Lipophilization of Lysozyme by Short and Middle Chain Fatty Acids

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Hen egg white lysozyme was lipophilized with short and middle chain saturated fatty acids (caproic, capric, or myristic acid). The yield, bactericidal properties, and structural properties of lipophilized lysozymes were investigated. The yield of lipophilization of lysozyme greatly increased with the decrease in the chain length of fatty acid. Lipophilization broadened the bactericidal action of lysozyme to Gram-negative bacteria with little loss of enzymatic activity. The bactericidal activity increased in proportion to the number of bound short chain fatty acids. The thermal stability of lipophilized lysozyme decreased in proportion to the chain length and number of bound fatty acids.

Keywords: *Lysozyme; lipophilization; bactericidal action; enzymatic activity; thermal stability*

INTRODUCTION

Lysozyme is a well-known protein with bactericidal activity, but its activity is unfavorably limited to Gram-positive bacteria. To broaden its bactericidal action to Gram-negative bacteria, many of them food-borne pathogens, some promising attempts have been made (Nakamura et al., 1991, 1992; Ibrahim et al., 1991, 1994, 1996). Among them, the lipophilization of lysozyme is most promising. The lipophilization of lysozyme would facilitate its penetration through the protective barrier (outer membrane), thereby decomposing the peptidoglycan layer of Gram-negative bacteria (Ibrahim et al., 1991). Ibrahim et al. (1993) have investigated the effect of lipophilization with long chain fatty acids on the bactericidal action of lysozyme. However, the yield of this lipophilization is considerably low, probably due to the insolubilization of the products. This problem may be solved by lipophilization with shorter chain fatty acids. Therefore, the characterization of lysozyme lipophilized with shorter chain fatty acids should be investigated to optimize the efficiency of lipophilization to increase potential for industrial applications of lysozyme. Thus, in the present paper, lysozyme was lipophilized with caproic acid, capric acid, and myristic acid, respectively. The bactericidal action and the structural properties of lipophilized lysozymes were investigated.

MATERIALS AND METHODS

Materials. Lysozyme was purified from fresh egg white by recrystallizing at pH 9.5 in the presence of 5% NaCl. *o*-Phthalaldehyde was purchased from Wako Co., Japan; MacConkey medium was obtained from Nissui Seiyaku Co., Japan; *Micrococcus lysodeikticus* cells were purchased from Sigma (St Louis, MO); *Escherichia coli* K-12 was from the Institute for Fermentation, Osaka, Japan. Other chemicals were of analytical grade.

Lipophilization of Lysozymes. Three kinds of fatty acids (caproic acid, C6:0; capric acid, C10:0; myristic acid, C14:0) were covalently attached to the lysine residue of lysozyme according to the method of Haque et al. (1982), with some modification. Five milliliters of dimethyl sulfoxide containing *N*-hydroxysuccinimide ester of each kind of fatty acid (final concentration 0.7 mM) was added drop-by-drop to 25 mL of 1% $NaHCO_3$ solution containing lysozyme (final concentration 0.2 mM) with agitation at the same time. The reaction mixture was incubated with gentle agitation at 30 °C for 6 h. The reaction was terminated by the addition of 25 mL of 100 mM glycine solution and incubation at 30 °C for 10 min. The reaction mixture was dialyzed against distilled water at room temperature and finally against 20 mM phosphate buffer, pH 7.0 at 4 °C, for 1 day. To remove the unreacted fatty acid, the dialysates were centrifuged at 12 000 rpm for 10 min at 5 °C. Supernatant was collected for the following steps.

Polyacrylamide Gel Electrophoresis (PAGE). PAGE was performed using 7% acrylamide gel. Twenty microliters of sample (0.05%) containing 0.05% brilliant green was applied. Electrophoresis was performed at a constant current of 5 mA for 4 h with an electrophoretic buffer of 0.35 M β-alanine (pH 4.3). Gel sheets were stained with Coomassie brilliant blue R-250.

Separation of Lipophilized Lysozymes. Lipophilized lysozymes were separated by cation-exchange chromatography on a CM-Toyopearl 650 M (Tosoh, Co., Tokyo, Japan) column (1.6 × 70 mm). The modified lysozymes were applied onto the column equilibrated with starting buffer, 20 mM phosphate buffer (pH 7.0). The adsorbed proteins were eluted with a stepwise gradient elution with starting buffer containing 0.1, 0.2, and 0.3 M NaCl, at 4 °C with a flow rate of 0.6 mL/min. The effluents were detected at 280 nm. The fraction of each peak was collected, dialyzed against distilled water, and lyophilized.

Measurement of Degree of Lipophilization. The number of fatty acids attached to lysozyme molecules was determined by measuring the free amino groups by a spectrophotometric method using *o*-phthalaldehyde (Church et al., 1983).

Measurement of Bactericidal Activity to *E. coli*. *E. coli* K-12 cultured for 10 h in Luria-Bertani medium was diluted 10^3 times with 20 mM phosphate buffer (pH 7.0), and then 4.5 mL of this solution was mixed with 0.5 mL of lysozyme solution (final concentration 0.05% w/v). The mixture was incubated at 20 °C for 30 min, and then a 100-μL portion was surface plated onto MacConkey agar plate. Colonies were counted after incubation at 37 °C overnight. The lysozyme-free solution was used as a control.

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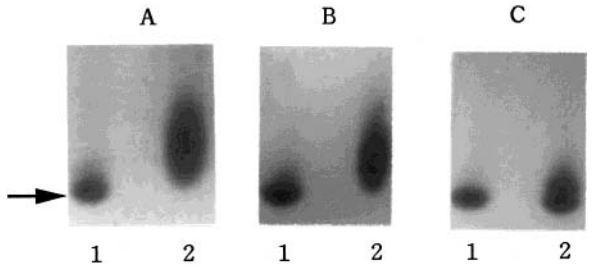

Figure 1. PAGE pattern of the supernatant of the products of lipophilization. Panels A, B, and C represent the pattern of lysozymes lipophilized by caproic, capric, and myristic acids, respectively: lane 1, native lysozyme; lane 2, supernatant of the product of lipophilization; arrow indicates the position of native lysozyme.

Table 1. Number of Fatty Acids Attached to Fractions of Lipophilized Lysozymes

fatty acid attached	monoacylated	diacylated	triacylated
caproic acid	–	1.8	2.8
capric acid	0.8	1.8	2.4
myristic acid	0.8	1.9	–

Enzymatic Action against Ethylene Glycol Chitin and *M. lysodeikticus*. Hydrolytic action against ethylene glycol chitin was measured according to the method of Imoto and Yagishita (1971). Lytic action against *M. lysodeikticus* was determined by a turbidimetric method (Muraki et al., 1988). The lyophilized cells of *M. lysodeikticus* were suspended in 50 mM sodium acetate or sodium phosphate buffer (pH 4.0−9.0, $OD_{450} = 0.7$). The initial decrease in OD_{450} of the suspensions caused by the lysis of *M. lysodeikticus* was measured at 20 °C for 1 min using a Hitachi U-2000 spectrophotometer.

Circular Dichroism (CD) Analysis. The far-ultraviolet (200−250 nm) CD spectra were measured to estimate the conformational change in lipophilized lysozymes according to the method of Kato and Takagi (1988). Lysozyme solutions were adjusted to 0.05 mg/mL in 40 mM glycine-HCl buffer (pH 5.5). CD spectra were recorded at 25 °C on a J-600 spectropolarimeter (Jas Co., Tokyo, Japan) with a 1.0-cm cuvette. The thermal stability of lipophilized lysozymes was estimated from the denaturation curves, which were obtained from the change in the ellipticity of CD spectra at 222 nm, during heating in the range of 50−83 °C at a rate of 1 °C/min. Melting temperature (T_m) was determined from the transition point of the denaturation curve.

RESULTS

Lipophilization of Lysozymes. The supernatant of lipophilized lysozyme product had a PAGE pattern shown in Figure 1. Since lipophilization decreased positive charge of lysine residues in the lysozyme, the lipophilized lysozyme was less mobile than the native lysozyme. The electrophoretic pattern indicated that the most lipophilized lysozymes were obtained with caproic acid and the least yield was for the myristoylated lysozyme. The yield of lipophilization of lysozyme could be estimated to be about 40%, 80%, and 90% for myristic, capric, and caproic acids, respectively, suggesting lipophilization yield improved in inverse proportion to the fatty acid chain length.

Lipophilized lysozyme separation into mono-, di- and triacylated proteins by cation-exchange chromatography was based on the differences in the free amino group of each fraction. The number of fatty acids bound to lysozyme in each fraction is shown in Table 1. Triacylated lysozyme was easily obtained from caproylation, but not from myristoylation. On the other hand, monoacylated lysozyme was obtained from myristoylation, but not from caproylation. This suggests the reaction efficiency of lipophilization is better for the shorter chain fatty acids.

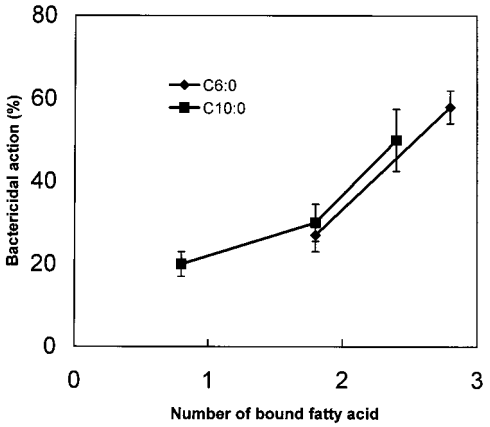

Figure 2. Bactericidal activity of lysozymes attached with caproic or capric acid against *E. coli* K-12 as a function of the number of bound fatty acids. C6:0 and C10:0 represente caproylated and caprylated lysozyme, respectively. Each point represents the mean of three trials, and standard deviations are shown as bars.

Bactericidal Activity of Lipophilized Lysozymes to *E. coli*. Bactericidal activity against *E. coli* K-12, a typical Gram-negative bacterium, was measured for each fraction of caproylated and caprylated lysozyme. The bactericidal action was represented as a function of the number of bound fatty acids (Figure 2). The bactericidal action increased with increasing bound fatty acid in the cases of caproic and capric acids. This suggests that the bactericidal action of these fatty acids-attached lysozyme can be strengthened with the number of bound fatty acids. On the other hand, it was not the case for myristic, palmitic, and stearic acids, in which monoacylated lysozyme revealed enough bactericidal action (Ibrahim et al., 1993).

Enzymatic Action of Lipophilized Lysozymes. Figure 3 shows the hydrolytic activity of lipophilized lysozymes against ethylene glycol chitin, a synthetic substrate used in lysozyme assays. In all cases, dramatic changes of enzymatic activity with lipophilization were not observed, albeit a slight decrease in action occurred at acidic pH. This result suggests that lipophilization of free amino groups has little effect on the active site of lysozyme. It is consistent with the result of acetylation of lysozyme (Yamasaki et al., 1968a).

The lytic activity of lipophilized lysozymes against *M. lysodeikticus* is shown in Figure 4. Lytic activity of lipophilized lysozymes was almost the same as that of native protein when the pH value was lower than 6.0, though it decreased greatly in the vicinity of pH 8.0, where it is optimal for native lysozyme. This result indicates the potential for lipophilization in improving the bactericidal action of lysozyme, because it not only broadens the bactericidal action to Gram-negative bacteria but also almost completely reserves its hydrolytic activity and bactericidal action to *M. lysodeikticus*, a typical Gram-positive bacterium, at acidic pH.

The shift of optimal pH of lytic activity may be due to the lowering of the isoelectric point of lysozyme through lipophilization, resulting in changes in the optimal pH of the enzyme−substrate interaction. The importance of positive charge of lysozyme in the lysis

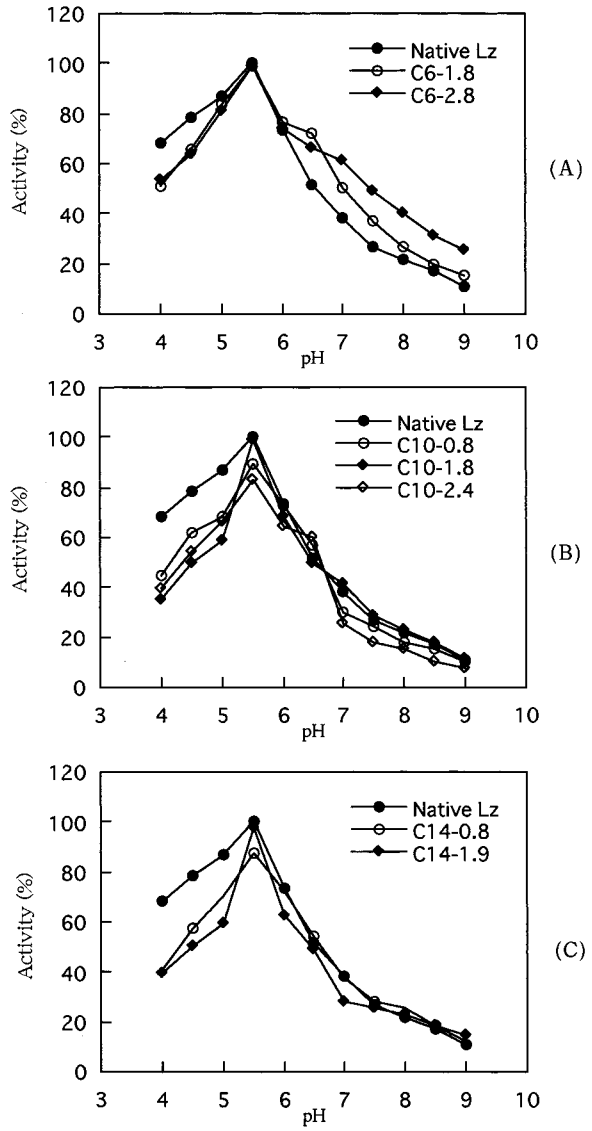

Figure 3. Hydrolytic activity of native and lipophilized lysozymes as a function of pH. Panels A, B, and C show the hydrolytic activity of caproylated, caprylated, and myristoylated lysozyme, respectively. Native Lz represents native lysozyme. Other symbols are consistent with Table 1. The activity is expressed as a percentage of that of native lysozyme at pH 5.5.

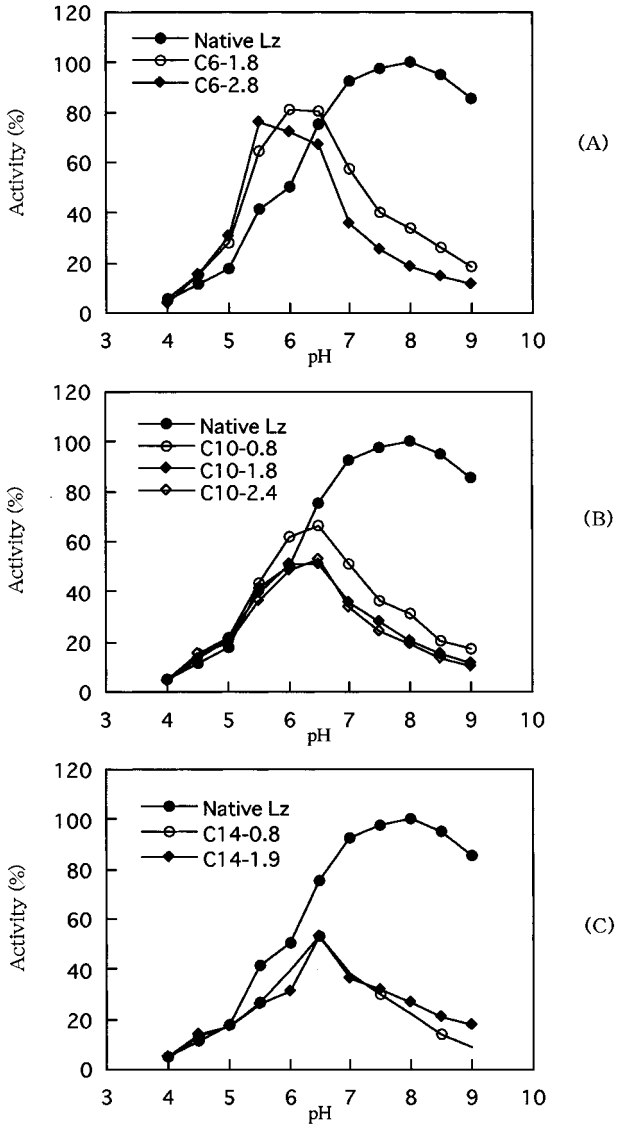

Figure 4. Lytic activity of native and lipophilized lysozymes against *M. lysodeikticus* as a function of pH. Symbols are the same as those in Figure 3. The activity is expressed as a percentage of that of native lysozyme at pH 8.0.

of *M. lysodeikticus* is consistent with the observation obtained by Yamasaki et al. (1968b) and Kato et al. (1994).

Effect of Lipophilization on the Thermal Stability of Lysozymes. Figure 5 shows the CD spectra of native and lipophilized lysozymes. Minimum value of ellipticity of lipophilized lysozymes slightly shifted to 210 from 208 nm, and a slight decrease of ellipticity was found, suggesting that some changes in the secondary structure of lysozyme occurred with lipophilization.

Table 2 shows the ratio of α-helix structure in native and lipophilized lysozymes, estimated from the curve-fitting analysis of the CD pattern (Kato and Takagi, 1988). These data indicated that the α-helix content decreased with lipophilization. It has been reported that lysozyme is lipophilized at 33 and 97 lysine residues (Ibrahim et al., 1993) and that both of these residues were involved in the formation of α-helix (Wilson et al., 1992). Therefore, the decrease in the content of α-helix may be due to the partial destruction of α-helix containing the lysine residue at positions 33 and 97 by lipophilization.

The thermal stability was estimated from the decreases in the ellipticity at 222 nm during heating from 50 to 83 °C. Changes in the melting temperature (T_m) were represented as a function of the number of fatty acids attached to lysozyme (Figure 6). The T_m value decreased with the increase in the binding number and length of the hydrocarbon chain of the fatty acid. This result indicates that thermal stability of lysozyme decreases in proportion to the number or chain length of bound fatty acid. Therefore, denaturation becomes more feasible for lipophilized lysozymes with increasing length of attached fatty acid, thereby bringing about poor solubility. This is the reason the yield of lipophilization of lysozyme decreased with length of fatty acid.

DISCUSSION

As described above, lipophilization of lysozyme with short chain fatty acids effectively broadened bactericidal action of lysozyme to Gram-negative bacteria without

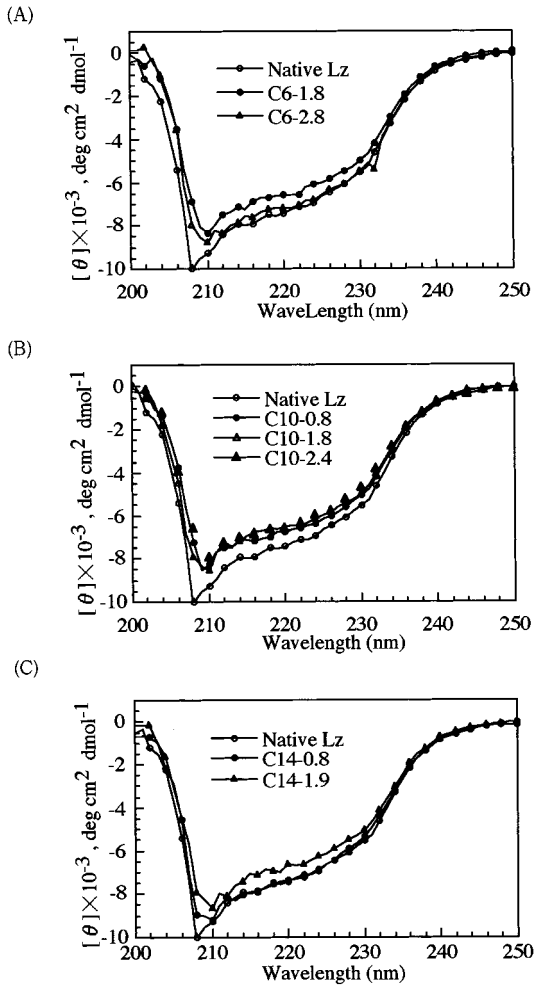

Figure 5. Far-UV CD spectra of native and lipophilized lysozymes. Symbols are the same as those in Figure 3.

Table 2. Ratio of α-Helix Structure of Native and Lipophilized Lysozymes

sample	α-helix (%)
native lysozyme	37.4
C6−1.8	35.1
C6−2.8	33.2
C10−0.8	33.7
C10−1.8	32.8
C10−2.4	30.9
C14−0.8	31.2
C14−1.9	31.0

lowering its action to Gram-positive bacteria at acidic pH. The advantage of the lipophilization with short chain fatty acids is the remarkable increase in the yield of 80−90%, for the yield of myristoylation was about 40% and the yield of palmitoylation and stearoylation was less than 10% (data not shown).

To elucidate further the relationship between the lipophilization and bactericidal action to Gram-negative bacteria, our data and those of Ibrahim et al. (1993) were combined in Figure 7. Bactericidal activity increased with the number of bound fatty acids in the cases of caproylated and caprylated lysozyme, but it tended to decrease in the cases of myristoylated, palmitoylated, and stearoylated protein. Although bactericidal activity of monoacylated lysozyme increased with the fatty acid chain length, di- and triacylated lysozyme activity was not affected by fatty acid chain length. These results suggest that optimal bactericidal action of lipophilized lysozyme results from appropriate lipophilization. Ibrahim et al. (1991) described previously that lipophilized lysozyme was liable to penetrate the outer membrane and degrade the peptidoglycan of Gram-negative bacteria. Although penetration would be improved by increasing the hydrophobicity of the fatty acid, strong hydrophobic interaction between lipophilized lysozyme and the outer membrane may bind lysozyme in the outer membrane, preventing lytic activity to the peptidoglycan. Therefore, the balance between tendency of penetration into the outer membrane and translocation to the peptidoglycan layer of Gram-negative bacteria may have an important role in the bactericidal action of lysozyme.

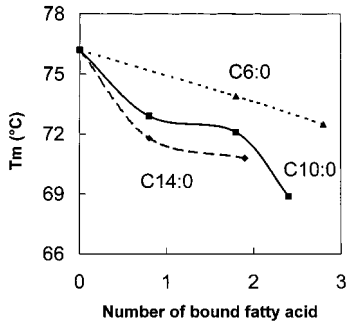

Figure 6. Variation of T_m of lipophilized lysozymes as a function of the number of bound fatty acids. C14:0, C10:0, and C6:0 represent myristoylated, caprylated, and caproylated lysozyme, respectively.

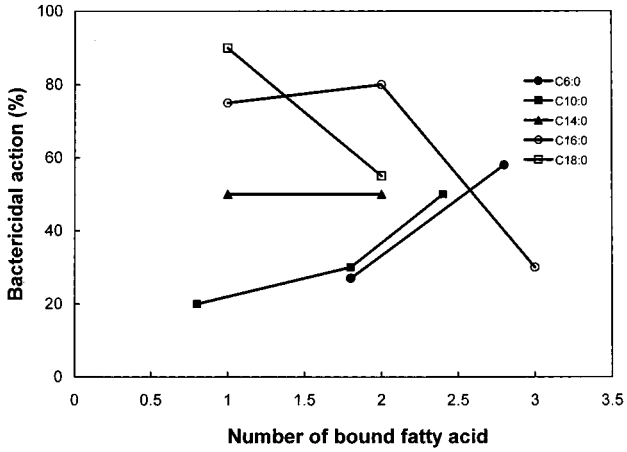

Figure 7. Bactericidal action of lipophilized lysozyme against *E. coli* K-12 as a function of the number of bound fatty acids. C6:0, C10:0, C14:0, C16:0, and C18:0 represent caproylated, caprylated, myristoylated, palmitoylated, and stearoylated lysozyme, respectively.

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