Role of Divalent Cations in the Novel Bactericidal Activity of the Partially Unfolded Lysozyme

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It was recently found that partial denaturation of hen egg white lysozyme at 80 °C for 20 min at pH 6.0 produces a strong bactericidal lysozyme (HLz) not only against Gram-positive bacteria but also against the Gram-negative ones. The novel antimicrobial action of the mildly denatured lysozyme was found to operate through a membrane-disrupting mechanism independent of its muramidase activity. To evaluate the role of Ca^{2+} and Mg^{2+}, which are known to stabilize the structure of bacterial membranes, in the antimicrobial activity of HLz, the antimicrobial activity in the presence of divalent cations and chelator EDTA was tested. The antimicrobial activity of HLz against test microorganisms *Staphylococcus aureus* and *Escherichia coli* K12 progressively decreased with an increase in Ca^{2+} or Mg^{2+} concentration, whereas 1 mM cation produced nonbactericidal lysozyme, either the native (NLz) or the heated enzyme (HLz). However, the inhibitory effect of divalent cations on the antimicrobial activity was more pronounced on HLz than on NLz. The antimicrobial activity of HLz against Gram-negative *E. coli*, which has been abolished in the presence of free Ca^{2+} (1 mM), was recovered by the addition of 1 mM EDTA, indicating the competition between free and membrane-bound Ca^{2+} on the part of HLz molecule. Addition of increasing concentration of NaCl up to 10 mM had no inhibitory effect on the strong antimicrobial activity of HLz. Thus, the inhibitory effect of divalent cations on the activity of HLz is unlikely to be simply due to charge suppression. Intrinsic fluorescence analysis revealed that Ca^{2+} induces conformational change of the HLz molecules, thus providing evidence that HLz exerts its antimicrobial action against Gram-negative bacteria by disrupting the normal electrostatic interactions between divalent cations and components of the outer membrane. Structural changes relevant to divalent cations sensitization of HLz are discussed.

Keywords: *Lysozyme; conformational changes; cyclic imide; antimicrobial action; calcium binding; tryptic peptides*

INTRODUCTION

Divalent cations such as Ca^{2+} and Mg^{2+} play a specific role in stabilizing the structure of bacterial membranes, as they form metal ion bridges between phosphate groups of phospholipids or lipopolysaccharides (LPS) and the carboxyl groups of membrane proteins (Asbell and Eagon, 1966). Their removal leads to the disintegration of certain functional membrane proteins, LPS from the outer membrane of Gram-negative bacteria, for example, and the collapse of membrane functions. It has been shown that EDTA damages outer membrane structure and releases 30−50% of the surface LPS by complexing Ca^{2+} and Mg^{2+}, which are crucial for the outer membrane of Gram-negative bacteria (Leive, 1974).

Lysozyme is an antimicrobial enzyme, hydrolyzing the β-1−4 linkage of the peptidoglycan backbone in the cell wall, that is highly active against Gram-positive bacteria but much less active against Gram-negative species. These differences in sensitivity are attributed to the unique membrane structure of Gram-negative bacteria, which includes the outer membrane, a permeability barrier that hinders the access of lysozyme to its site of action, the peptidoglycan layer. We recently reported that heat-denatured lysozyme at neutral pH is a potent bactericidal protein against various strains of both Gram-positive and -negative bacteria, regardless of its muramidase activity (Ibrahim et al., 1996a). Irreversibly denatured lysozyme at pH 6.0 (HLz) was found to be the most potent bactericidal derivative against Gram-positive and -negative bacteria. HLz retained about 60% of the native catalytic activity and exhibited 14-fold increase in surface hydrophobicity. Compared with the native lysozyme (NLz), HLz caused severe membrane damage and agglutinated the susceptible bacteria and showed enhanced binding capacity to the bacterial membrane fractions of *Escherichia coli*. Furthermore, HLz permeabilized the liposomal membrane made from *E. coli* phospholipids in a dose-dependent manner.

Since HLz is a polycationic molecule with characteristic amphiphilic nature, its disruptive effect on bacterial membranes may be dependent on its ability to displace divalent cations, which stabilize membrane integrity. After cation displacement, HLz, with exposed hydrophobic region to the surface of the molecule, inserts into the core of the membrane, thus distorting the normal package between phospholipids and functional membrane proteins. The best known of this kind of membrane disrupting action is that proposed for

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membrane active antibiotic polypeptides EM49 (Rosenthal et al., 1976). Here, we report specific protection of *Staphylococcus aureus* and *E. coli* from the novel antimicrobial action of HLz by addition of Ca^{2+} and Mg^{2+}, establishing that HLz may compete with Ca^{2+} and Mg^{2+} for binding sites in the outer membrane of *E. coli* K12. Furthermore, Ca^{2+} is shown to induce conformational changes in the HLz molecule without altering its residual enzyme activity.

MATERIALS AND METHODS

Materials. Hen egg white lysozyme recrystallized five times was a product of Taiyo Kagaku Inc. (Mie, Japan). *Micrococcus lysodeikticus*, a substrate of lysozyme, was purchased from Sigma (St. Louis, MO). Brain heart infusion (BHI), nutrient agar, and Bacto-peptone were from Difco (Detroit, MI). As test micro-organisms for the antimicrobial assay, *E. coli* IFO 3301 and *S. aureus* IFO 14462 were obtained from the Institute of Fermentation Osaka (Japan). Unless otherwise stated, all other chemicals were of the highest grade commercially available.

Thermal Denaturation of Lysozyme. Heat-induced denaturation of the lysozyme was performed by incubating 2 mL of 1 mg of lysozyme/mL of 10 mM sodium phosphate buffer (pH 6.0) in a screw-capped tube at 80 °C for 20 min. Immediately samples were quenched in an ice−water bath for 10 min. Insoluble aggregates of lysozyme formed during heating were removed by centrifugation (3000*g* for 15 min). The supernatants thus obtained were dialyzed in a Spectra/por dialysis tube (MWCO 6000−8000, Spectrum Medical Inc., Los Angeles, CA) against distilled water and lyophilized. Freeze-dried lysozyme derivatives were then dissolved in the appropriate buffer and used in the following experiments.

Lysozyme Activity. The lysis of *M. lysodeikticus* cells was determined according to a turbidometric method based on the decrease in turbidity of a 1.9 mL cell suspension (170 μg of dry cells/mL) in 100 mM sodium acetate buffer (pH 6.0), containing various concentrations of $CaCl_2$ (0, 62, 125, 250, and 500 μM), followed by the addition of 100 μL of lysozyme solution (50 μg/mL of the same respective buffer) after equilibration to achieve constant absorbance (0.75−0.8). The final concentration of lysozyme was kept at 3.5 μM. The decrease in absorbance at 450 nm (25 °C) was monitored using a Shimadzu MPS-2000 recording spectrophotometer (Shimadzu, Kyoto, Japan). The data are represented as the rate of decrease in absorbance per minute of the initial velocity of reaction.

Assay of Antimicrobial Activity. The bacteria were cultivated in BHI broth at 37 °C for 16 h with shaking and then diluted to 1:100 in BHI broth. The diluted culture was further incubated for 3−4 h until midlogarithmic phase was reached, on the basis of absorbance measurements at 675 nm. Bacterial pellets (3000*g* for 7 min at 4 °C) were washed two times and resuspended in 0.65% peptone broth (pH 7.3) to give absorbance of 0.002 cm^{-1} at 675 nm. $CaCl_2$, $MgCl_2$, and EDTA were added from a stock solution of 0.5 M to give a final concentration as indicated. One milliliter of the bacterial suspension was mixed with an equal volume of lysozyme in the same medium containing the respective cation and EDTA, to give a final lysozyme concentration of 200 μg/mL (0.014 mM). The mixture was incubated at 30 °C for 1−2 h. A 30-μL portion or dilutions (in 0.85% NaCl) were spotted in triplicate onto nutrient agar plates. The colony forming units (CFU) were obtained after the plates were incubated at 37 °C for 24 h. All assays were performed in triplicate, and the results, unless otherwise notified, are presented as CFU/mL (± SE). Controls consisted of bacteria incubated alone.

Fluorescence Measurements. Intrinsic fluorescence of 2-mL lysozyme samples (3.5 μM) in 100 mM sodium acetate buffer (pH 6.0) was performed with a Hitachi F-3000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan), using 1-cm path length cuvettes thermostated at 25 °C. The excitation and emission wavelengths used for tryptophan measurements were 280 and 300−400 nm, respectively. Effects of $CaCl_2$ concentration (final of 0, 62, 125, 250, and 500 μM) on fluorescence were examined by addition from a stock solution. It took several minutes for the intensity of the fluorescence to maximize after addition of $CaCl_2$.

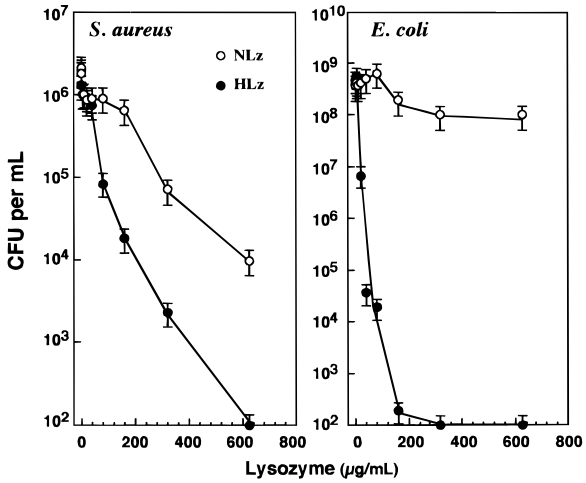

Figure 1. Effect of HLz concentration on bacterial viability. Bacteria, *S. aureus* (left) and *E. coli* K12 (right), were exposed to native (○) and HLz (●) lysozymes. The lysozyme concentration varied from 2.5 to 625 μg/mL, and incubation was for 2 h at 30 °C in Bacto-peptone broth (pH 7.3). CFU values were obtained on nutrient agar plates after incubation for 24 h. The vertical bars indicate the means of triplicate counts.

Reversed-Phase HPLC of Reduced and Aminoethylated Lysozyme. The general procedure for trypsin digestion involved suspension of reduced S-2-aminoethylated native or heated lysozyme at 1% concentration in distilled water as described by Okazaki et al. (1985). The enzymatic digestion was allowed to proceed for 120 min. A Wakosil-II 5C18-200 column, 4 × 250 mm (Wako, Osaka, Japan), was attached to a Shimadsu CBM-10A, LC work station class LC10 (Shimadzu, Kyoto, Japan), connected to PC-98-21 Bp NEC computer. The column was equilibrated with 1% acetonitrile containing 0.1% HCl. Twenty microliters of tryptic hydrolysates containing 50 μg of peptides was injected, and the column was eluted with a gradient of 40 mL of 1% acetonitrile and 40 mL of 40% acetonitrile, both containing 0.1% HCl at a flow rate of 0.4 mL/min for 200 min. Peptide elution was monitored by absorbance of effluent at 215 nm.

RESULTS

Effect of HLz Preparation on Viability of *S. aureus* and *E. coli* K12. We reported earlier (Ibrahim et al., 1996b) that heat-denatured lysozyme at pH 6.0 is a more potent bactericidal protein than the NLz against different species of Gram-positive and -negative bacteria, despite its reduced enzymatic activity (≈60% of the native activity). Figure 1 shows the antimicrobial potency of HLz against two representative strains of Gram-positive (*S. aureus*) and Gram-negative (*E. coli* K12) bacteria as a function of protein concentration. HLz killed both strains of bacteria within 2 h of incubation at 30 °C in a dose-dependent fashion. NLz was almost inactive against *E. coli* and showed less bactericidal activity against *S. aureus* in comparison with HLz. It should be noted that NLz exhibits an antimicrobial potency similar to that of HLz with an incubation time longer than 4 h. The sharp reduction in CFU of *E. coli* with increasing concentration of HLz may reflect its rapid and strong affinity to the bacterial membrane previously reported (Ibrahim et al., 1996b).

Effects of Ca^{2+} and Mg^{2+} on the Bactericidal Activity of HLz. The antistaphylococcal activity of HLz (0.014 mM) was gradually decreased with addition

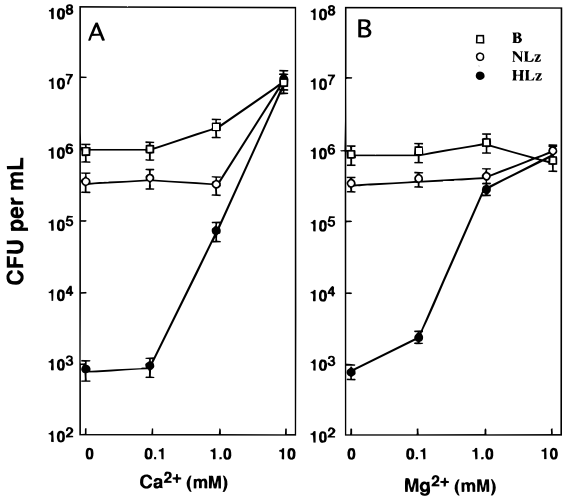

Figure 2. Effect of Ca^{2+} (A) and Mg^{2+} (B) on the antimicrobial action of HLz against *S. aureus*. Bacteria were incubated in peptone broth containing the indicated concentration of divalent cations with 200 μg/mL (0.014 mM) of NLz (○) or HLz (●) for 1 h at 30 °C. Controls (□) consisted of bacteria alone. CFU were obtained as in Figure 1.

of either Ca^{2+} (Figure 2A) or Mg^{2+} (Figure 2B) in a dose-dependent manner, whereas it was almost abolished with addition of 1 mM or more of either cation. The inhibition of antistaphylococcal activity of HLz was increased with the addition of 0.1−10 mM divalent cations. The effects of divalent cations in concentrations up to 1 mM on the antistaphylococcal activity of NLz were small or negligible. However, the antistaphylococcal activity of HLz was much more sensitive to addition of Mg^{2+} than of Ca^{2+}. Although the reason for this is unknown, differences in sensitivity appear to reflect the low Ca^{2+} content of the medium (BHI) used for bacterial growth. Addition of 10 mM Ca^{2+} increased the CFU of untreated (B) or NLz-treated cells relative to addition of 1 mM Ca^{2+}, and such effect was more pronounced in the presence of HLz (Figure 2A). This was not observed with addition of Mg^{2+} (Figure 2B). This phenomenon may be attributed to growth stimulatory activity of Ca^{2+} on this bacteria, most probably through activation of peptidoglycan synthesizing enzymes which are involved in cell division. The results of Figure 1 clearly indicate the involvement of membrane-bound divalent cations in the promoted antimicrobial activity of HLz, as is obvious from competition between free and bound cations on the part of HLz molecule.

Gram-negative *E. coli* K12 bacteria were also protected from the bactericidal effect of HLz by addition of Ca^{2+} and Mg^{2+} (Figure 3) in a manner similar to the Gram-positive *S. aureus*. The inhibition of the bactericidal effect of HLz against *E. coli* was observed with an increase in Ca^{2+} or Mg^{2+} concentration, and almost complete inhibition was detected with addition of 1 mM of either cation. In contrast, addition of Ca^{2+} had no effect on the viable cell count (CFU) of NLz-treated *E. coli* up to the maximum concentration used (10 mM) (Figure 3A, open circles), while an increase in Mg^{2+} concentration over 0.1 mM caused a slight increase in CFU of NLz-treated *E. coli*.

It has been shown that various Gram-negative strains including *E. coli*, which are normally insensitive to antibiotics, such as lysozyme, become completely sensitive after a brief EDTA treatment, a chelating agent (Bayer and Leive, 1977). Figure 4 shows the effect of EDTA on the antimicrobial activity against *E. coli* of NLz and HLz in the absence (Figure 4A) and presence

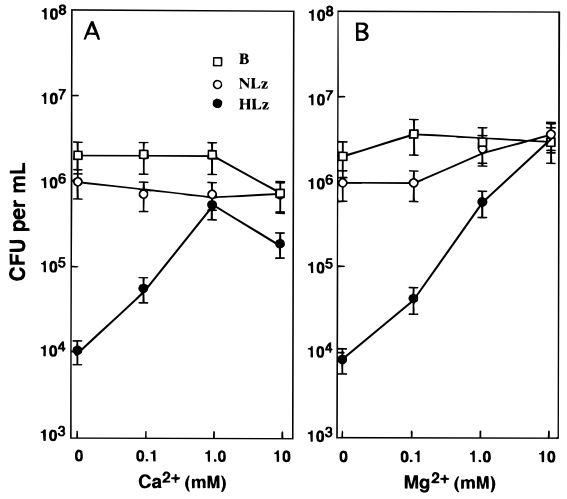

Figure 3. Effect of Ca^{2+} (A) and Mg^{2+} (B) on the antimicrobial action of HLz against *E. coli* K12. Assay conditions and symbols are the same as in Figure 2.

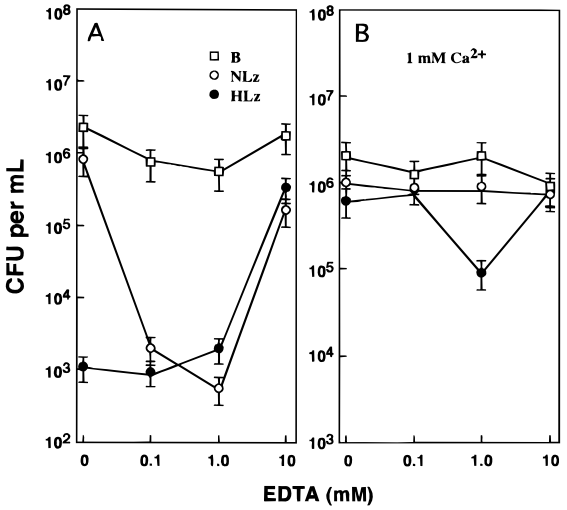

Figure 4. Effect of EDTA on the antimicrobial action of HLz against *E. coli* K12 in the absence (A) and presence of 1 mM Ca^{2+} (B). Assay conditions and symbols are the same as in Figure 2.

of 1 mM Ca^{2+} (Figure 4B). In the absence of free Ca^{2+}, addition of EDTA progressively increased antimicrobial activity of NLz against *E. coli* up to 1 mM and then was depressed with increasing concentration of EDTA to 10 mM (Figure 4A, open circles). On the other hand, addition of EDTA up to 1 mM had little or negligible effect on the antimicrobial activity of HLz, but the activity was decreased with increasing concentration of EDTA to 10 mM (Figure 4A, solid circles) in a similar manner for NLz. When 1 mM Ca^{2+} was incorporated in the assay medium (Figure 4B, solid circles), the antimicrobial activity of HLz was abrogated but partially recovered only in the presence of an equimolar concentration of EDTA (1 mM). Under the same assay condition (with 1 mM Ca^{2+}) EDTA had no or negligible effect on CFUs of blank- or NLz-treated *E. coli* (Figure 4B, open squares and circles, respectively). It should also be noted that removal of membrane-bound Ca^{2+} by the addition of 10 mM EDTA alone had negligible effect on the CFU of *E. coli* of blank-treated cells (Figure 4A, open squares) while depressing the activity of HLz (solid circles) as well as NLz (open circles). When free Ca^{2+} was complexed by the addition of an equimolar concentration of EDTA (1 mM), the antimicrobial action of HLz returned (Figure 4B, solid circles), indicating possible

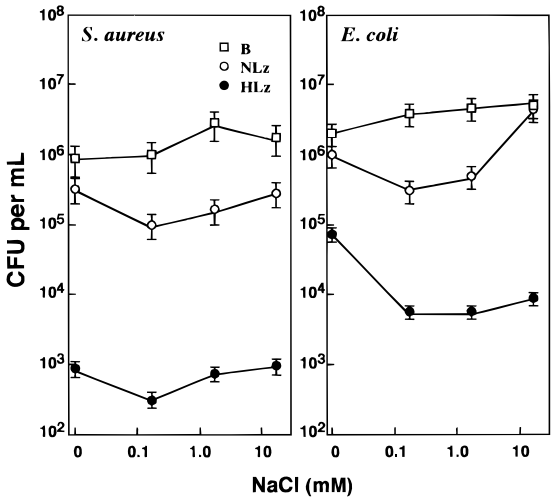

Figure 5. Effect of NaCl on the antimicrobial action of HLz against *S. aureus* (left) and *E. coli* K12 (right). Assay conditions and symbols are the same as in Figure 2.

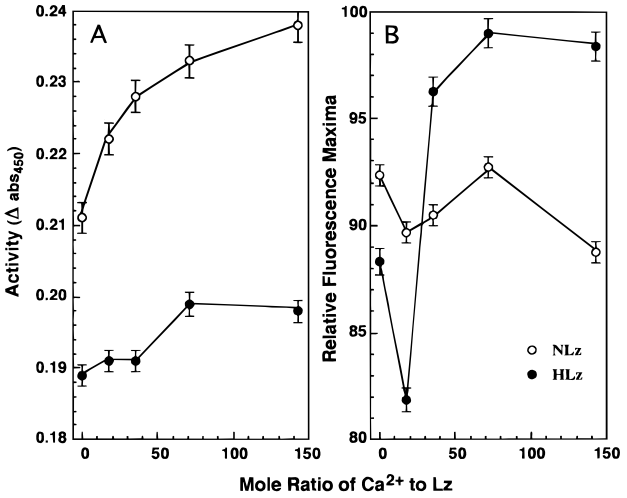

Figure 6. Effect of Ca^{2+} on the enzyme activity (A) and intrinsic fluorescence (B) of HLz. Solution of native (○) or HLz (●) lysozyme was 50 μg/mL (3.5 μM) in 100 mM sodium acetate buffer (pH 6.0), containing various concentrations of Ca^{2+}. Aliquots were used in enzyme assay against *M. lysodeikticus* dissolved in the same medium. Fluorescence intensity is expressed as maximum value of the relative fluorescnece intensity of a 2-mL solutuion after stable intensity was attained. Other assay conditions are described under Materials and Methods.

competition between free and membrane-bound Ca^{2+} on specific site(s) of HLz molecules.

It is unlikely that the inhibition of the antimicrobial action due to the addition of $CaCl_2$ to HLz is the result of salt action on the charge of HLz molecules, because addition of NaCl even up to 10 mM had no effect on the antimicrobial action of HLz against either *S. aureus* (Figure 5, left) or *E. coli* (Figure 5, right). Bactericidal action of HLz against *E. coli* was further promoted by increasing NaCl concentration (Figure 5, right, solid circles). Therefore, the results in Figure 4 suggest a direct involvement of the membrane-bound Ca^{2+} in exertion of the promoted bactericidal activity of HLz against Gram-negative *E. coli*, possibly by inducing certain conformational changes in HLz which predisposed the molecules of HLz to insertion into the core of the membrane, thus distorting its normal packing.

Effect of Ca^{2+} on the Intrinsic Fluorescence of HLz. To verify the possibility that Ca^{2+} induced conformational changes in HLz molecules, the effect of Ca^{2+} on intrinsic fluorescence and enzyme activity of HLz was examined, and the results are shown in Figure 6. The experiments were performed at a molar ratio of Ca^{2+} to lysozyme similar to that employed in the antimicrobial assays. Increasing the concentration of Ca^{2+} caused a progressive increase in the muramidase activity of NLz, but this effect was much less pronounced on HLz (Figure 6A). The effect of $CaCl_2$ on enzyme activity is probably a simple balancing action between the positively charged lysozyme molecules and the negatively charged cell wall substrate, as previously emphasized (Muraki et al., 1988). Interestingly, free Ca^{2+} markedly increased the enzyme activity of NLz (Figure 6A) while depressing its antistaphylococcal activity (Figure 2A), demonstrating that the antimicrobial action of native lysozyme is independent of its muramidase activity. With addition of Ca^{2+} the maximum fluorescence intensity of HLz was changed (Figure 6B, solid circles). At a low molar ratio of Ca^{2+} to lysozyme the intensity was decreased and then reversed at higher ratios, where it reached a steady state by addition of 1 mM Ca^{2+} (70 mole ratio). Compared with HLz, the effect of Ca^{2+} addition on the fluorescence intensity of NLz was small (open circles). These fluorescence changes in maximum intensity were accompanied with a consistent shift in maximum wavelength, whereas the decrease in intensity generally was associated with a red shift while an increase was accompanied by a blue shift, by about 2 nm, in HLz only but not NLz (data not shown). The data in Figure 6B illustrate a dramatic conformational change in HLz induced by Ca^{2+}. Conformational changes (Figure 6B) and inhibition of antimicrobial action (Figures 2 and 3) of HLz by free Ca^{2+} are in good agreement.

The deamidation is a common chemical reaction occurring *in vivo* (Kato et al., 1988) and has also been reported to be occurring during heat treatment of lysozyme (Tomizawa et al., 1995). Deamidation is the most likely candidate for providing a Ca^{2+} binding site on the HLz molecule. To investigate the possible chemical reactions that accompany heating, the native and heat-treated lysozymes were reduced, aminoethylated, and digested with TPCK-trypsin at pH 8.0 as described by Okazaki et al. (1985). The resulting tryptic peptides from HLz were compared with those from NLz on reversed-phase HPLC (Figure 7). The elution pattern of tryptic peptides of HLz (Figure 7B) was similar to that of NLz (Figure 7A) with the exception that three peptides (T8, T9, and T13) disappeared, while four new peaks (Figure 7B, solid peaks) are evident. A common sequence in the three peptides T8 (Asn46−Arg61), T9 (Tyr62−Arg68), and T13 (Leu98−Arg112) was the aspartylglycyl sequence Asp48−Gly49, Asp65−Gly66, and Asp101−Gly102, respectively. In addition, each peptide of them contains deamidatable amino acid residues (Asn or Gln) such as T8 possessed Asn46, Asn59, and Gln57, T9 possessed Asn65, and T13 possessed Asn103 and Asn106. The results suggest that the three peptides of HLz containing aspartylglycyl sequences were converted into β conformation, most probably via cyclic imide formation as has recently been reported (Tomizawa et al., 1994). Deamidation cannot be judged from the tryptic peptides pattern. However, when intact HLz was analyzed on a cation-exchange HPLC (TSK-gel CM-5PW column), mono- and dideamidated peaks (fast eluting peaks) were observed (data not shown).

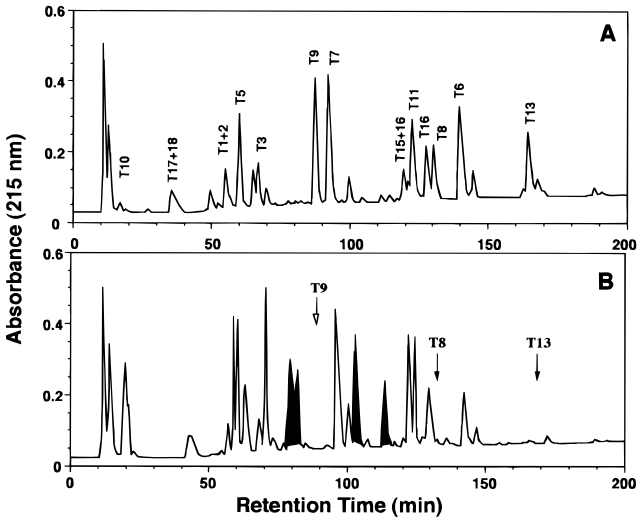

Figure 7. Reversed-phase HPLC elution patterns of tryptic peptides of reduced and aminoethylated NLz (A) and HLz (B) on a Wakosil-II 5C18-200 column (4 × 250 mm). The column was eluted with a gradient of 40 mL of 1% acetonitrile and 40 mL of 40% acetonitrile, both containing 0.1% HCl at a flow rate of 0.4 mL/min for 200 min.

DISCUSSION

A recent study showed that hen egg white lysozyme interacts with phospholipid vesicles and induces fusion and release of their aqueous content (Posse et al., 1994). In addition, interaction between lipopolysaccharide on the outer surface of Gram-negative bacteria and lysozyme has been demonstrated in earlier studies (Ohno and Morrison, 1989). Despite this finding that lysozyme interacts with bacterial membrane components, it does not kill Gram-negative bacteria. Our studies have demonstrated that the antimicrobial activity of lysozyme can be altered to include Gram-negative bacteria, without detrimental effect on its activity against Gram-positive bacteria due to heat denaturation at neutral pH (Ibrahim et al., 1996a,b). For instance, HLz showed stronger binding affinity to membrane fractions of *E. coli* K12, including the LPS and lipid bilayer, compared to untreated lysozyme. HLz caused fusion of liposomal membranes, made from *E. coli* phospholipids, and released its aqueous contents in a dose-dependent manner. In addition, a fluorescence study revealed that detectable structural changes occurred in HLz when incubated with *E. coli* phospholipid vesicles (Ibrahim et al., 1996b). The data suggested that HLz, with enhanced surface hydrophobicity, interacted with the hydrophobic core of the lipid bilayer and that a conformational change in the HLz molecule is induced. Such an interaction was demonstrated to position the positively charged region of HLz at the membrane aqueous interface, allowing polar interactions between the molecule and polar head groups of the phospholipids or LPS, as deduced from binding experiments using ELISA assay.

In the present study, free Ca^{2+} and Mg^{2+} (1 mM) protected *E. coli* K12 from the strong bactericidal action of HLz, whereas no such inhibitory effect occurred with addition of 1 mM EDTA (Figure 4B). This suggests that HLz may compete with these cations for negatively charged phosphate groups of phospholipids or LPS. Addition of NaCl at a molar ratio similar to that used for Ca^{2+} to HLz did not affect the antimicrobial activity of HLz (Figure 5), suggesting the specificity of Ca^{2+} effect on the antimicrobial action of HLz. The good agreement between changes in intrinsic fluorescence of HLz and the inhibitory effect on its antimicrobial action with increasing concentration of free Ca^{2+} supports the suggestion that specific interaction of Ca^{2+} with HLz is involved in the membrane-disrupting mechanism of HLz. It has been proposed that Ca^{2+} and Mg^{2+} play a specific structural role in membranes, particularly the outer membrane, as metal ion bridges between phosphate groups of phospholipids and LPS or membrane proteins (Leive, 1974). The polycationic nature of lysozyme and the inhibition of the antimicrobial activity of HLz by Ca^{2+} and Mg^{2+} (Figures 2 and 3) suggest that the bactericidal HLz might interfere with the normal electrostatic interactions between components of the bacterial membranes. Indeed, our earlier scanning electron microscopy study indicated that HLz caused intensive damage in the cellular morphology with the appearance of large holes on the outer surface of *E. coli* K12 (Ibrahim et al., 1996b). In addition, HLz was shown to interact strongly with isolated *E. coli* phospholipids and outer membrane preparations. Therefore, the data in Figure 3 together with that from the earlier study illustrate that HLz kills Gram-negative or even Gram-positive bacteria by disrupting metal ion bridges between the phosphates of lipopolysaccharide and phospholipid molecules.

The structural basis for the antimicrobial action of HLz against Gram-negative bacteria is yet to be clearly established, despite the fact that the membrane-damaging mechanism was clearly demonstrated (Ibrahim et al., 1996a,b). This is because of the complexity of structural changes and chemical reactions that occur upon heat-induced denaturation of a protein. The condition of heat-induced denaturation employed in the present study showed no dramatic changes in the secondary structures of HLz as revealed by CD spectra (data not shown), while it greatly promoted the surface hydrophobicity of HLz (Ibrahim et al., 1996b). Enhanced surface hydrophobicity of HLz can be attributed to its membrane-binding capability and subsequent damage to membrane integrity, but is unlikely to be accounted for by the observed Ca^{2+}-dependent antimicrobial action of HLz (Figure 4). The available data suggest that exposing the hydrophobic region facilitates membrane binding, acting with a cluster of basic residues of HLz that bind to phosphate groups of phospholipids or LPS through electrostatic attraction. For HLz to exert the effect on membrane integrity that has been observed in SEM images of HLz-treated *E. coli*, other factors must be important in determining its polymerization and the subsequent membrane pore formation previously reported (Ibrahim et al., 1996b). Calcium ions at the membrane interface may provide an electrostatic switch (McLaughlin and Aderem, 1995) that induces conformational changes in the HLz that result in both more exposure of the hydrophobic pocket and extrusion of the electrostatic attraction, thus leading to the fragmentation of bacterial membrane. Conversion of peptides T8, T9, and T13 to β conformation may alter the global conformation of the HLz molecule to accommodate the Ca^{2+} ion, particularly the three aspartylglycyl sequences that are located in loop structure, a characteristic Ca^{2+}-binding motif. Deamidation has also been detected in HLz and was revealed by NMR analysis (chemical shift) to occur at Asn44 and Asn46 (data not shown). Deamidation of these two asparagine residues together with the formation of β peptide containing the sequence (Asp48−Gly49) would result in

the formation of three consecutive Asp residues (Asp44, Asp46, and β Asp48) flanked with Arg45 and Thr47 and then followed by the native sequence Ser50−Thr51−Asp52. This sequence may provide a Ca^{2+}-sensitive region on the HLz molecule. However, this speculation is invalid until detailed molecular characterization and the precise relationship between deamidation, Ca^{2+}-binding, and antimicrobial action of lysozyme are established. An approach to detail these relationships by using site-directed mutagenesis is now in progress in our laboratory.

The bactericidal effect in the presence of a low concentration of EDTA (up to 1 mM) and its inhibition at higher concentration (complete removal of membrane-bound divalent cations) indicate a role for these divalent cations in the action of HLz (Figure 4). EDTA alone up to 1 mM did not reduce the viability of control *E. coli* at the concentration used but only in the presence of NLz. The action of HLz is taken as a combination between EDTA effect and muramidase catalysis, so it is likely that HLz displaces the divalent cations and thus inserts its exposed hydrophobic region into the lipid or LPS bilayer. The Ca^{2+} and EDTA experiments of this study do not exclude the possibility that HLz releases divalent cations from the membrane, which results in stimulation of autolysins, a class of endogenous muramidases that participate in hydrolysis and synthesis of peptidoglycan during cell growth. The autolysins are acidic proteins and could be inhibited and fixed to lipoteichoic acid and/or cardiolipin by ionic interactions that involve divalent cations (Hughes et al., 1973). Autolysis has been reported to be activated by the addition of chelating agents, e.g. EDTA or citrate, in Gram-positive (Ogata and Hongo, 1974) and -negative (Leduc and Van Heijenoort, 1980) bacteria. Loss of divalent cations associated with membranes could lead to liberation of part of autolysin, which would no longer be inhibited. In the presence of monovalent cations, e.g. Na^{+}, this phenomenon could be amplified as has previously been described (Ogata and Hongo, 1974). This was evident from the results of Figure 5 of the present work. Currently, biochemical experiments are in progress to elucidate the autolysin profile of Ca^{2+} in the presence of HLz and different divalent cations in the culture medium. Furthermore, it is worth emphasizing that the changes which occurred in the tertiary structure of lysozyme by heat treatment in neutral pH are biologically relevant to the sensitization to divalent cations and interaction with membrane components. The present study also indicates that care should be taken when HLz is considered for application in formulated food or drug systems, since the presence of a high concentration of divalent cations may abolish the novel antimicrobial action of such heat-activated lysozyme.

ABBREVIATIONS USED

NLz, native hen egg white lysozyme; HLz, lysozyme derivative that is heat-denatured at 80 °C (20 min) and pH 6.0; LPS, lipopolysaccharides; EDTA, ethylenediaminetetraacetic acid (disodium); ELISA, enzyme-linked immunosorbent assay; CFU, colony forming unit; SEM, scanning electron microscopy; TPCK, L-(tosylamino)-2-phenylethyl chloromethyl ketone.

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