Partially Unfolded Lysozyme at Neutral pH Agglutinates and Kills Gram-Negative and Gram-Positive Bacteria through Membrane Damage Mechanism

Hisham R. Ibrahim,*,† Shinji Higashiguchi,‡ Mamoru Koketsu,‡ Lekh R. Juneja,‡ Mujo Kim,‡ Takehiko Yamamoto,‡ Yasushi Sugimoto,† and Takayoshi Aoki†

Department of Biochemical Science and Technology, Faculty of Agriculture, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890, Japan, and Central Research Laboratories, Taiyo Kagaku Company, Ltd., Yokkaichi, Mie 510, Japan

The antimicrobial mechanism and structural changes of hen egg white lysozyme irreversibly inactivated at 80 °C and at different pHs were investigated. We found that heat denaturation of lysozyme at increasing temperatures for 20 min at pH 6.0 results in progressive loss of enzyme activity while greatly promotes its antimicrobial action to Gram-negative bacteria. Interestingly, lysozyme devoid of enzyme activity (heated at 80 °C and pH 7.0 or at pH 6.0 over 90 °C) exhibited strong bactericidal activity against Gram-negative and -positive bacteria, suggesting action independent of catalytic function. The most potent antimicrobial lysozyme to either Gram-negative or -positive bacteria was that heated at 80 °C and pH 6.0 (HLz80/6), retaining 50% of the native enzymatic activity, which exhibited a 14-fold increase in surface hydrophobicity, with two exposed thiol groups. HLz80/6-induced agglutination coincided with severe reduction in colony-forming ability of the susceptible bacteria in a dose-dependent manner. Denatured lysozyme HLz80/6 showed promoted binding capacity to peptidoglycan of *Staphylococcus aureus* and lipopolysaccharide of *Escherichia coli* as assessed by ELISA. Addition of HLz80/6 to *E. coli* phospholipid vesicles resulted in a blue shift in the intrinsic tryptophan fluorescence accompanied by an increase in the size of the vesicles, indicating enhanced protein−membrane binding and subsequent fusion of liposomes. Direct membrane damage of *E. coli* membrane by HLz80/6 was revealed by electron microscopy observation. Thus, the results introduce an interesting finding that partial unfolding of lysozyme with the proper acquisition of the hydrophobic pocket to the surface can switch its antimicrobial activity to include Gram-negative bacteria without a detrimental effect on the inherent bactericidal effect against Gram-positive ones. The data suggest that the unique antimicrobial action of unfolded lysozyme attributes to membrane binding and subsequent perturbation of its functions.

Keywords: *Lysozyme; conformational changes; antimicrobial action; agglutination; membrane interaction and fusion*

INTRODUCTION

Among the antimicrobial proteins, the mechanism by which lysozyme kills the sensitive bacteria is known to be the degradation of the glycosidic β-linkage between *N*-acetylhexosamines of the peptidoglycan layer in the bacterial cell wall (Fleming, 1922; Jolles and Jolles, 1984). Its antimicrobial function is limited to certain Gram-positive bacteria owing to the differences found in composition as well as the accessibility of the peptidoglycan to the enzyme action (Salton and Pavlik, 1960; Salton et al., 1968). Aside from its bacteriolytic action, it has been demonstrated that lysozymes have many other functions, including inactivation of certain viruses by forming insoluble complexes (Hasselberger, 1978), important roles in surveillance of membranes of mammalian cells (Osserman et al., 1974), enhancement of phagocytic activity of polymorphonuclear leukocytes (Kokoshis et al., 1978) and macrophages (Thacore and Willet, 1966), and stimulation of proliferation and antitumor functions of monocytes (Lemarbre et al., 1981). Recently, studies have shown that lysozyme interacts with and induces fusion of phospholipid vesicles (Posse et al., 1994). In parallel, immunochemical *in vivo* studies using confocal microscopy have demonstrated that lysozyme is synthesized and secreted with surfactant apoprotein A by rat alveolar type II epithelial cells and alveolar macrophages, suggesting its role in the extracellular remodeling of surfactant phospholipids in the air spaces of lung (Gibson and Phadke, 1994). The latter studies have also shown that lysozyme was distributed peripherally within the lamellar body in its native molecular weight, 14 kDa, and a considerable amount of a dimer of 2 times the molecular weight.

Lysozyme is known to be specifically bactericidal to certain Gram-positive bacteria but less effective against Gram-negative ones, despite its strong interaction with and disruptive effect on lipopolysaccharides (LPS) of the later species (Ohno and Morrison, 1989) and the positive correlation between the increased level of lysozyme secretion in many tissues and bacterial infections (Brouwer et al., 1984). Though in small amount, an irreversible dimeric form of lysozyme was detected in avian egg whites and within the lumen of the cystlike aggregates of alveolar macrophages (Back, 1984; Gibson and Phadke, 1994), presumably through intermolecular disulfide exchange. The tendency of lysozyme to associate into dimers and higher polymers as a function of pH, concentration, and temperature has also been

* Author to whom correspondence should be addressed (telephone, +81 (99) 285-8658; fax, +81 (99) 285-8525; e-mail, hishamri@chem.agri.kagoshima-u.ac.jp).
† Kagoshima University.
‡ Taiyo Kagaku Co., Ltd.

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reported (Sophianopoulos and Holde, 1964). The biological function(s) of such irreversible dimeric forms of lysozyme have so far not been studied. Although conclusive evidence is lacking, the literature abounds with evidence pertaining to the functional role of lysozyme in various tissues as a defense mechanism suggesting that it may primarily be related to structural properties and secondarily to its direct bacteriolytic action (Laible and Germaine, 1985; Cisani et al., 1989). Studies on structure−antimicrobial properties relationships of lysozyme are essential to identify the mechanism of lysozyme's role in the defense from pathogens which remains to be unraveled.

Recently, we suggested that antimicrobial action of lysozyme can be switched to include Gram-negative bacteria by altering its surface hydrophobicity through genetic (Ibrahim et al., 1994b, 1992) and chemical (Ibrahim et al., 1993) modifications or interaction with phenolic aldehyde (Ibrahim et al., 1994a) with the molecule. To more directly test the hypothesis that antimicrobial action as well as specificity of lysozyme may be related to conformational changes and can be independent of enzyme activity, we studied the antimicrobial action against Gram-negative and -positive bacteria of irreversibly heat-denatured lysozyme at different pHs. We detail here an intriguing finding that unfolded lysozyme is a potent bactericidal agent against both Gram-negative and -positive bacteria regardless of its enzyme activity. The most potent bactericidal lysozyme retains 50% of its enzyme activity and possesses two free thiol groups. The mechanism of antimicrobial action of denatured lysozyme was found to be enhanced agglutination activity leading to the subsequent killing of the exposed bacteria, through membrane interaction and direct damage to its functions.

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). *cis*-Parinaric acid (CPA) was purchased from Wako Pure Chemicals (Osaka, Japan). Alkaline phosphatase coupled to goat anti-mouse IgG was obtained from Zymed (San Francisco, CA). Phospholipids were isolated from *Escherichia coli* K12 according to the method of Viitanen et al. (1986). LPS was prepared from *E. coli* K12 according to the modified phenol−water extraction procedures of Morrison and Leive (1975), and peptidoglycans were purified as described earlier (Rotta, 1974). Brain heart infusion (BHI), nutrient agar, and peptone were obtained from Difco (Detroit, MI). *E. coli* IFO 3301 (*E.c.*), *Salmonella enteritidis* IFO 3313 (*S.e.*), *Pseudomonas aeruginosa* IFO 3080 (*P.a.*), *Staphylococcus aureus* IFO 14462 (*S.a.*), *Bacillus subtilis* IFO 3007 (*B.s.*), and *Bacillus cereus* IFO 13690 (*B.c.*) were obtained from the Institute of Fermentation Osaka (Japan) as test microorganisms for antimicrobial assays. Unless otherwise stated, all other chemicals were of the highest grade commercially available.

Thermal Denaturation of Lysozyme. Heat-induced inactivation of the lysozyme was performed by incubating 2 mL of 1 mg of lysozyme/mL of 10 mM sodium acetate buffer (pH 5.0) or sodium phosphate buffer (pH 6.0−8.0) 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 (MW CO: 6−8000; Spectrum Medical Inc., Los Angeles, CA) against distilled water and lyophilized. The temperature dependency of heat inactivation was carried out only at pH 6.0 because of the intensive aggregation that occurs upon heating over 80 °C at pH 7.0−8.0 or rapid refolding at pH 5.0 of lysozyme. Freeze-dried lysozyme derivatives were then dissolved in the appropriate buffer and used in the following experiments after the determination of the protein content by the Lowry method modified by Miller (Miller, 1959).

Acid−Polyacrylamide Gel Electrophoresis. The effect of heating temperature at pH 6.0 on the polymerization of lysozyme was assessed by using discontinuous acid−PAGE according to the method of Lewis (Lewis et al., 1968). Protein samples were electrophoresed at a constant current of 6 mA/plate for 4 h on nondenaturing 15% polyacrylamide gels at pH 4.5. Protein bands were visualized by Coomassie brilliant blue R-250.

SDS−Polyacrylamide Gel Electrophoresis. Protein samples were incubated for 2 h in 0.125 M Tris-HCl buffer (pH 6.8), containing 2% SDS and 15% glycerol, in the presence or absence of 5% β-mercaptoethanol. Electrophoresis was performed according to the method of Laemmli (Laemmli, 1970); then the gels were stained as in acid−PAGE.

Surface Hydrophobicity. The surface hydrophobicity was determined according to the method of Kato and Nakai (1980) using fluorescence probe (CPA). Ten microliters of an ethanolic solution of *cis*-parinaric acid was added to 2 mL of various protein concentrations in 10 mM phosphate buffer (pH 7.2). The mixture was excited at 325 nm, and the relative fluorescence intensity was recorded at 420 nm in a Hitachi F-3000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The hydrophobicity is presented as initial slope (S_0) which was calculated from the plot of fluorescence intensity versus protein concentration.

Estimation of Free Sulfhydryl Groups. The free thiol groups were determined by using DTNB, 5,5′-dithiobis(2-nitrobenzoic acid), according to the procedure of Ellman (Ellman, 1959).

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 50 mM sodium phosphate buffer (pH 6.2) following the addition of 100 μL of lysozyme solution (20 μg/mL) after equilibration to achieve constant absorbance (0.75−0.8). The decrease in absorbance at 600 nm (25 °C) was monitored using a Shimadzu MPS-2000 recording spectrophotometer (Shimadzu, Kyoto, Japan). The activity is estimated as the rate of decrease in absorbance/min of the initial velocity of reaction. The data of heated lysozyme derivatives are represented as percent of the native protein.

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, based on 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 7.4 (Difco), to give absorbance at 675 nm of 0.002 cm^{-1} (2×10^5 cells/mL). One milliliter of the bacterial suspension was mixed with an equal volume of various concentrations of lysozyme in the same medium. The mixture was incubated at 37 °C for 1 h, unless otherwise indicated. A 100 μL portion or dilutions (in 0.85% NaCl) were plated onto nutrient agar plates. The colony-forming unit (cfu) was obtained after incubating the plates at 37 °C for 24 h. All assays were performed in triplicate and the results, unless otherwise notified, are presented as percent survival ± SE to the controls consisting of bacteria incubated alone.

Preparation of Liposomes. Liposomes made from *E. coli* phospholipids were prepared as follows. A 100 μL aliquot (50 mg dry weight of lipids/mL of argon-saturated 2 mM β-mercaptoethanol) was added to 900 μL of 50 mM potassium phosphate buffer, pH 7.2, in a microfuge tube. The mixture was sonicated for 2 s at maximum output followed by a 3 s pause, and the cycle was repeated until the turbid suspension was clarified. The suspension was centrifuged at 356000*g* for 1 h at 4 °C. The liposomes pellet was gently resuspended in 50 mM potassium phosphate buffer, pH 7.2, and centrifugation was repeated. Finally, the liposomes were resuspended in the

same buffer and centrifuged for 3 min at 10000*g* to remove any multilamellar vesicles.

Fluorescence Measurements. Steady state fluorescence measurements were performed with a Hitachi F-3000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan), using 1 cm path length cuvettes thermostated at 25 °C. Excitation and emission band pass was set at 5 nm each. The excitation and emission wavelengths used for tryptophan measurements were 280 and 300−400 nm, respectively.

Binding of Lysozyme to Liposomal Membrane. The binding of lysozyme to lipid vesicles was assessed by monitoring the changes in the tryptophan spectra of lysozyme upon addition of small unilamellar vesicles of *E. coli* phospholipids. Increasing aliquots of vesicle suspension were added to a solution of 1 μM lysozyme in 10 mM sodium phosphate buffer (pH 7.2), and the fluorescence of suspension was recorded after each addition of vesicles. Fluorescence intensities were corrected for light scattering caused by the lipid vesicles.

Fusion of Liposomal Membrane. Lysozyme-induced fusion of lipid membranes was determined by measuring the increase of a vesicle's diameter by dynamic light scattering, NICOMP submicron particle sizer, Model 370 (Santa Barbara, CA), on addition of protein (final concentration of lipid was 71.4 μM and that of lysozyme was 2.8 μM, 40 μg/mL), in 50 mM sodium phosphate buffer (pH 7.2). A parallel experiment was conducted with liposomes with no protein. Volume weighting of the Gaussian analysis was set at vesicles mode. The results are represented as mean diameter (nm) of the vesicles.

Agglutination of Bacteria. To observe the agglutination of the bacterial cells, the absorbance of the suspension was measured at 600 nm. A 1.0 mL aliquot of the cell suspension (2×10^6 cells/mL) in 1% peptone, pH 7.4, was supplemented with an equal volume of the same medium containing different concentrations of lysozyme. The absorbance was measured after 30 min at 30 °C. Controls contained no protein. Simultaneously, 100 μL or dilutions in saline were plated onto nutrient agar to determine the cfu. Agglutination assays were done three times, and the average value was obtained.

Binding to LPS and Peptidoglycan by ELISA. Microtiter plates (96-well) were coated with LPS or peptidoglycan by incubating 100 μL/well (10 μg/mL in 0.1 M Na_2CO_3, pH 9.6) at 37 °C for 3 h. After rinsing and blocking with bovine serum albumin, lysozyme sample was added, allowed to interact for 1 h at 37 °C, and then flicked out. Mouse anti-lysozyme IgG monoclonal antibody was incubated with the plates for 1 h and then substituted with the alkaline phosphatase-conjugated goat anti-mouse IgG for 1 h. Finally, a 100 μL aliquot of 0.1% *p*-nitrophenyl phosphate-Na_2 in diethanolamine buffer (pH 9.8) was added and incubated at 25 °C until sufficient color had developed (ca. 20 min). The reaction was stopped by addition of 50 μL/well 2 N NaOH, and absorbance at 405 nm was measured in a microtiter plate reader. Mean absorbance and standard errors of the means were calculated, after subtracting the values of the controls from four replications of each well.

Electron Microscopy. Suspensions of *E. coli* K12 at midlog phase (10^8 cells/mL) made in 0.65% peptone, pH 7.4, were incubated with different doses of lysozyme at 30 °C for 30 min. After fixing by the addition of glutaraldehyde (final concentration: 2.5%), cell pellets obtained by centrifugation were washed in 20 mM phosphate buffer, pH 7.2, containing 2.5% glutaraldehyde. The fixed cells were carefully spotted onto the slide, then dehydrated in ethanol gradients, and embedded in Spurr resin. Dried cells were coated with gold and examined by a scanning electron microscope, JEOL, JSM-T330 (JEOL-Technics Co., Ltd., Tokyo, Japan).

RESULTS

Antimicrobial Activity of Denatured Lysozyme at Different pHs. In preliminary experiments on the irreversible denaturation of lysozyme, we found a pH dependence of aggregation and refolding commitment of the molecule. Stable irreversibly denatured lysozyme was obtained by heating at 80 °C for 20 min at pHs higher than 5.0. Figure 1A shows the progressive loss of enzymatic activity of lysozyme with rising the heating pH, whereas a complete loss of activity was reached by heating at pH 7.0. Denatured lysozymes at any pH tested were bactericidal to the Gram-positive *S. aureus* as well as the native lysozyme, although the denatured lysozyme at pH 6.0 exhibited stronger bactericidal activity against this strain (Figure 1A). Denatured lysozyme at pH 7.0 or 8.0 showed slightly reduced activity but was still potent against *S. aureus* compared with the native enzyme, although they were devoid of enzymatic activity. These results clearly indicate that the antimicrobial action of lysozyme can be uncoupled from its muramidase activity. Strikingly, denatured lysozymes at pH 6.0 and 7.0, whose residual lytic activity is 50% and less than 1%, respectively, exhibited very potent bactericidal activity against the Gram-negative *E. coli* K12 (Figure 1B), which is known as a resistant microorganism to the action of lysozyme. Heated lysozymes at pH 5.0 showed no advantage in the antimicrobial action over the native lysozyme against *E. coli*, most probably as the denaturation was reversible under this pH. Heating at pH 8.0 produced inactive lysozyme with slightly reduced antimicrobial activity to *E. coli* compared to those heated at pH 6.0 and 7.0. These results demonstrate the dual antimicrobial activity of denatured lysozyme at pH 6.0 (HLz80/6) against both Gram-positive and -negative bacteria. In particular, HLz80/6 retains about 50% of the native enzyme activity and was found readily soluble in water, while that heated at pH 7.0 showed only 50% solubility (data not shown).

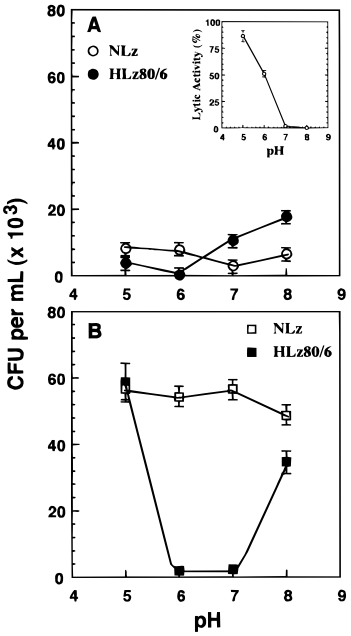

Figure 1. Effect of heated lysozyme at different pHs on viability of *S. aureus* IFO 14462 (A) and *E. coli* K12 (B). Unheated NLz (open symbols) and heated HLz (solid symbols) were incubated at 100 μg/mL with the test bacteria (10^5 cells/mL) in 0.65% peptone broth, pH 7.4, at 37 °C for 1 h before determining CFU on nutrient agar plates. Loss of enzymatic activity (*M. lysodeikticus* as substrate) as a function of heating pH of lysozyme is also given (A, inset).

Next, we investigated the effect of heating temperature at pH 6.0 (as it produces denatured lysozyme with better solubility) on the muramidase and antimicrobial activities of lysozyme (Figure 2). Increasing heating temperature over 50 °C associated with progressive inactivation, where enzyme activity was completely

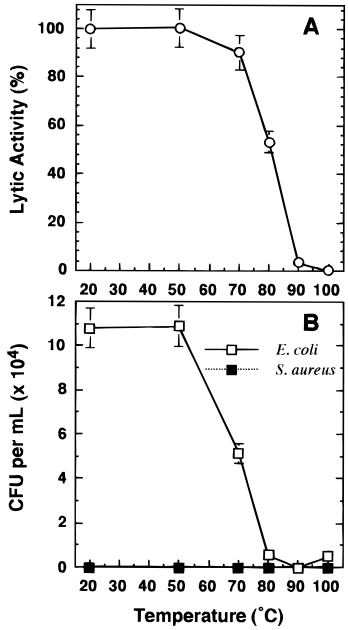

Figure 2. Effect of heating temperature at pH 6.0 on enzymatic activity (A) and bactericidal action (B) of lysozyme against *S. aureus* IFO 14462 and *E. coli* K12. Antimicrobial assay was essentially performed as described in Figure 1.

abolished at 100 °C (Figure 2A). All lysozyme derivatives, including the native enzyme (20 °C), were potent bactericidals against *S. aureus* regardless of the residual muramidase activity (Figure 2B). The antimicrobial activity against the Gram-negative *E. coli* K12 was observed at a temperature where irreversible inactivation (70 °C) occurred. An increase in denaturation temperature over 70 °C was accompanied with concomitant loss in enzymatic activity and proportional promotion in the antimicrobial action against *E. coli* K12 (Figure 2B). A very good correlation between loss in enzymatic activity and promotion of the antimicrobial action to *E. coli* can be drawn. Denatured lysozymes at 80, 90, and 100 °C (HLz80/6, HLz90/6, and HLz100/6, respectively) exhibited almost complete loss in CFU of *E. coli* K12. It should be noted that HLz80/6 and HLz90/6 possessed 53% and less than 1% residual enzymatic activity, respectively (Figure 2A), again suggesting that the antimicrobial action of denatured lysozyme is not dependent upon muramidase activity and the mechanism of action seems to be novel to structural factors.

We further tested the antimicrobial effects of HLz80/6 against three strains of Gram-negative (*E. coli* K12, *S. enteritidis*, and *P. aeruginosa*) and three strains of Gram-positive (*S. aureus*, *B. subtilis*, and *B. cereus*) bacteria, and the results are shown in Figure 3. The CFU of all bacteria tested was severely reduced within 1 h (Figure 3A) and completely lost within 24 h (Figure 3B) by HLz80/6. Native lysozyme did not show either bacteriostatic or bactericidal activity against both *E. coli* K12 and *S. enteritidis* but was efficiently bactericidal against the other bacteria tested. Similar results were obtained with HLz90/6 and HLz100/6 (data not shown). These results clearly indicate that the novel bactericidal activity against Gram-negative bacteria of the partially denatured lysozyme HLz80/6 is not limited to *E. coli* and may be general to a wide spectrum of bacteria including the resistant *Salmonella*. However, screening for the antimicrobial spectrum of HLz80/6 against different bacteria should await further investigation now in progress.

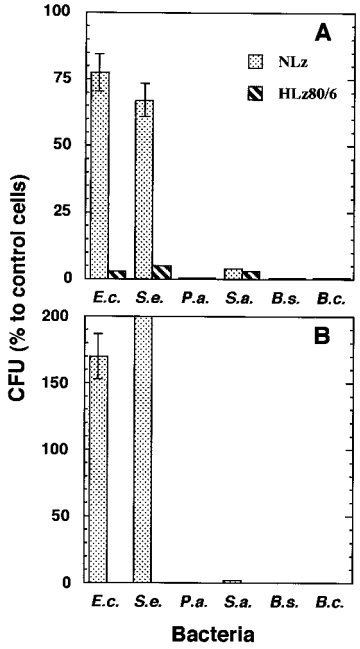

Figure 3. Antimicrobial effects of the denatured lysozyme at 80 °C (20 min) and pH 6.0, HLz80/6, against different Gram-negative (*E. coli* K12, *S. enteritidis*, and *P. aeruginosa*) and Gram-positive (*S. aureus*, *B. subtilis*, and *B. cereus*) bacteria. Bacteria (10^5 cells/mL) were incubated with 100 μg/mL native (NLz) or HLz80/6 in 0.65% peptone broth, pH 7.4, at 37 °C for 1 (A) or 24 (B) h before determining the CFU on nutrient agar plates. The results are represented as percent of the control cells incubated with no protein.

Table 1. Changes of Surface Hydrophobicity and Thiol Groups of Lysozyme as a Function of Heating Temperature at pH 6.0

heating temp (°C)	hydrophobicity[a] (S_0)	thiol groups[b] (mol/mol)
N	230 ± 1.90	0 ± 0.000
70	234 ± 2.60	0 ± 0.000
80	2629 ± 54.0	1.90 ± 0.080
90	3200 ± 47.0	4.73 ± 0.370
100	3500 ± 50.0	3.20 ± 0.270

[a] Surface hydrophobicity was determined by CPA assay. [b] Free sulfhydryl groups were estimated according to Ellman procedure using DTNB as described under Materials and Methods.

Structural Properties of Denatured Lysozyme. To determine the conformational changes associated with the promotion of the antimicrobial action of denatured lysozyme at pH 6.0, changes in surface hydrophobicity and possible cleavage of disulfide bonds of lysozyme were monitored as a function of heating temperature (Table 1). Consistent with the data of enzymatic activity (Figure 2A), the surface hydrophobicity has been increased with an increase of heating temperature, indicating unfolding of the lysozyme molecule. HLz80/6 appears to contain a single disulfide bond cleaved or probably intermolecular disulfide isomerization, where two thiol groups/molecule were freed. Approximately two disulfide bonds were cleaved in HLz90/6, and this did not cause proportional increase in surface hydrophobicity compared with the great changes that occurred in hydrophobicity of HLz80/6. This may suggest that cleavage or isomerization of a single S−S bond/molecule, by heating at pH 6.0 and 80 °C for 20 min, is sufficient to expose most of the hydrophobic region and thus produce the novel functional conformation of lysozyme.

Electrophoretic patterns indicate that more than 60% of HLz80/6 exists as oligomeric forms (Figure 4). Heat-

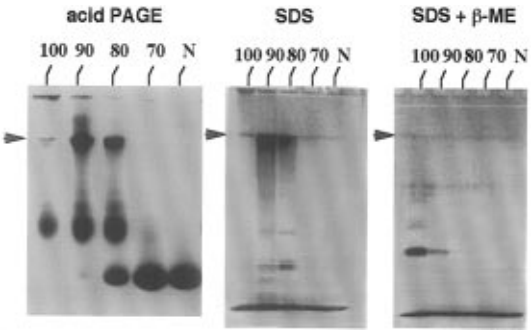

Figure 4. Electrophoretic patterns of heat-denatured lysozyme at different temperatures and pH 6.0. Samples of heated lysozyme were applied to 15% polyacrylamide nondenaturing acid−PAGE (left) or gradient (4/20) SDS−PAGE in the absence (middle) and presence of β-mercaptoethanol (right). Progressive disappearance of the native band (fast migration) with coincident formation of the dimeric denatured form (slow migration) of lysozyme is shown. All gels were stained with Coomassie brilliant blue R-250. Arrow heads indicate the start of separating gel.

ing at 90 °C resulted in the appearance of polymers with a much higher molecular weight than those of HLz80/6, with the presence of a considerable amount of dimeric form while monomers almost disappeared. At 100 °C the majority of lysozyme molecules were converted into high molecular weight polymers with a little amount of dimers. The chemical forces holding the dimers and oligomers of denatured lysozymes seemed to be mainly intermolecular disulfide linkage with little contribution of the noncovalent interactions, as revealed by denaturing gel electrophoresis in the presence (Figure 4, right) and absence (Figure 4, middle) of reducing agent. It can be suggested, therefore, that the free thiols on the denatured lysozyme emerged as a result of interchain disulfide exchange. Furthermore, it can be concluded that the enhanced antimicrobial activity of denatured lysozyme against *E. coli* K12 (Figure 2B) may correlate to the extent of oligomerization.

Binding to Bacterial Surface. To verify the ability of HLz80/6 to bind the surface of the susceptible bacteria, interaction of native and HLz80/6 lysozymes with the purified peptidoglycan (PG) and LPS, the major constituents of the outermost surface of Gram-positive and -negative bacteria, respectively, was determined by ELISA assay. The bound lysozyme was detected by anti-lysozyme monoclonal antibody. Lysozyme type was allowed to interact with microtiter plates coated with the purified peptidoglycan of either *S. aureus* (triangles) or *E. coli* (squares) and *E. coli* LPS (circles), and the results are shown in Figure 5. As can be seen the binding capacity of HLz80/6 to PG of *S. aureus* (Figure 5A, solid triangles) and *E. coli* LPS (Figure 5B, solid circles) was highly promoted compared with the native lysozyme (open respective symbols). Binding of HLz80/6 to *E. coli* peptidoglycan (Figure 5A, solid squares) was almost the same as the native lysozyme (open squares). The results demonstrate that the promoted antimicrobial action of HLz80/6 may be due to enhanced binding affinity to the bacterial surface.

Binding and Fusion to Lipid Vesicles. The association of HLz80/6 with the lipid bilayer was examined by monitoring the changes in its tryptophan fluorescence intensity (RFI) and emission maxima (λ_{max}) on addition of increasing amounts of *E. coli* phospholipid vesicles. Since the λ_{max} of Trp of a molecule is sensitive to the polarity of the microenvironment, where λ_{max} of 350 nm is indicative of exposure of Trp to aqueous

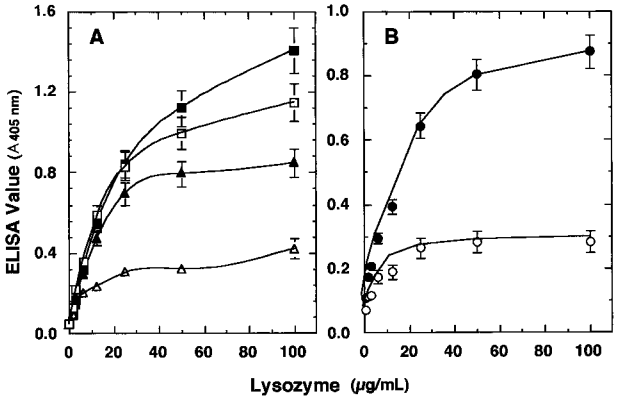

Figure 5. Binding of HLz80/6 to the isolated peptidoglycan (A) from *S. aureus* (triangles) or *E. coli* K12 (squares) and purified LPS (B) from *E. coli* K12 (circles). The indicated concentrations of native (open symbols) or HLz80/6 (closed symbols) were allowed to interact with microtiter plates coated with the respective fraction for 1 h at 37 °C. The binding was measured by ELISA using mouse anti-lysozyme monoclonal IgG and goat anti-mouse serum polyclonal IgG coupled to alkaline phosphatase; *y* axis scales refer to color developed at 405 nm.

Table 2. Emission Maximum (λ_{max}) and Relative Fluorescence Intensity (RFI) of Heat-Denatured Lysozymes at 80 and 90 °C (pH 6.0), in the Presence of Liposomal Membrane Prepared from *E. coli* K12 Phospholipids, when Excited at 280 nm[a]

lipid/protein ratio (mol/mol)	80 °C				90 °C			
	NLz		HLz		NLz		HLz	
	λ_{max}	RFI	λ_{max}	RFI	λ_{max}	RFI	λ_{max}	RFI
0	342	80	344	65	342	69	346	50
10	342	77	342	64	342	65	342	51
20	342	75	340	64	342	64	342	52
40	342	74	340	64	342	63	341	52
80	342	72	340	63	341	62	340	50
160	342	70	340	62	342	61	342	50

[a] Valeus represent the average of three independent observations. Variation was <5%.

environment, and a value of 340 nm indicates shielding from aqueous environment (Joseph and Nagaraj, 1995), we can suppose that the association of HLz80/6 with the liposomal membrane induces a blue shift (shorter wavelength) in the emission maximum. As can be seen in Table 2, denatured lysozyme at either 80 °C (HLz80/6) or 90 °C (HLz90/6) showed quenching in RFI and a red shift in λ_{max} compared with the native lysozyme in the absence of vesicles, indicating more exposure of Trp upon unfolding of the molecule. However, λ_{max} values of denatured lysozymes were significantly shifted to shorter wavelengths by addition of vesicles in a dose-dependent fashion. These results clearly demonstrate the interaction of denatured lysozyme with bilayer lipids, whereas the exposed Trp residues of the denatured lysozyme apparently became located in an apolar environment in the presence of vesicles. It can also be noted that in the presence of vesicles, native lysozyme showed a decrease in RFI values, suggesting conformational changes induced more exposure of Trp residues to the solvent. On the other hand, denatured lysozymes showed much less pronounced quenching in RFI values in the presence of vesicles, indicating that the hydrophobic Trp residues are involved in protein−lipid interaction.

The association of proteins to the lipid bilayer frequently alters the membrane integrity leading to fusion. Kinetic fusion of *E. coli* phospholipid vesicles on addition

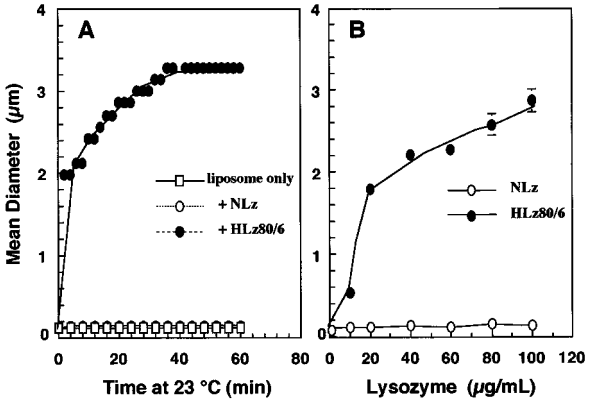

Figure 6. Kinetics of HLz80/6-induced fusion of *E. coli* phospholipid vesicles. Vesicles (71.4 μM) were incubated in 50 mM phosphate buffer, pH 7.2, with 2.8 μM lysozyme for various lengths of time (A) or different doses of lysozyme for 10 min at 23 °C. Changes in vesicles diameter were determined by a particle sizer as described under Materials and Methods.

of HLz80/6 compared with the native lysozyme is shown in Figure 6. The results clearly indicate that binding of HLz80/6 to vesicles leads to lipid fusion in time-dependent (Figure 6A) and dose-dependent (Figure 6B) manners. Taken all together, ELISA, fluorescence, and light scattering data clearly indicate that the potentially bactericidal HLz80/6 strongly associates with the bacterial membrane, and as a consequence, it may kill the bacteria through agglutination leading to disintegration of the membrane functions. To confirm this hypothesis, we examined the effect of HLz80/6 on the agglutination and killing of *S. aureus* (Gram-positive) and *E. coli* (Gram-negative) cells. As expected, the turbidity of the cell suspensions of *S. aureus* and *E. coli* increased linearly within 30 min on the addition of HLz80/6 in a dose-dependent fashion but not with the native lysozyme (Figure 7). Agglutination of bacteria by HLz80/6 was accompanied with marked reduction in CFU of both bacteria. The native lysozyme killed *S. aureus* with almost the same potency as HLz80/6 did, without increasing the turbidity of the suspension (Figure 7A). It can be speculated that HLz80/6 interacts with *S. aureus* resulting in aggregation of the cells, and the subsequent killing may be due to perturbation of membrane integrity and in part to the 53% residual muramidase activity (Figure 2A). It can also be noted that the dependency of the bactericidal action to *S. aureus* of HLz80/6 was similar to that of cell agglutination, whereas it began at 40 μg/mL HLz80/6 (Figure 2A). On the other hand, the dramatic reduction of CFU of *E. coli* began with a HLz80/6 concentration greater than 100 μg/mL, while the increase of turbidity of the suspension was observed at a much lower protein concentration (Figure 7B). The data of agglutination and killing of *E. coli* by HLz80/6 support the hypothesis that the novel bactericidal effect of HLz80/6 against Gram-negative bacteria, or even the Gram-positive ones, may be related to its strong interaction with and the disruption of the bacterial membrane and clearly indicate that HLz80/6 directly interacts with the bacterial surface.

Further evidence to support the hypothesis that HLz80/6 disrupted membrane integrity was found by SEM observation of morphological changes associated with the promoted susceptibility of Gram-negative *E. coli* (Figure 8). SEM micrography did not show any morphological changes between untreated cells and those treated with 200 μg/mL native lysozyme for 30

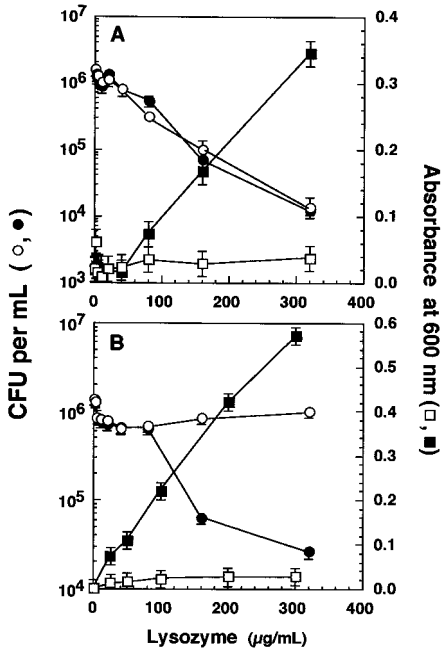

Figure 7. Effect of HLz80/6 on the turbidity of cell suspensions and viability of *S. aureus* (A) and *E. coli* K12 (B). Incubation of bacteria with lysozyme sample lasted for 30 min in 1% peptone, pH 7.4. Absorbance at 600 nm of cell suspension was recorded (squares), and CFU values were determined (circles) on nutrient agar plates as a function of lysozyme concentration. Open symbols, native lysozyme; solid symbols, HLz80/6.

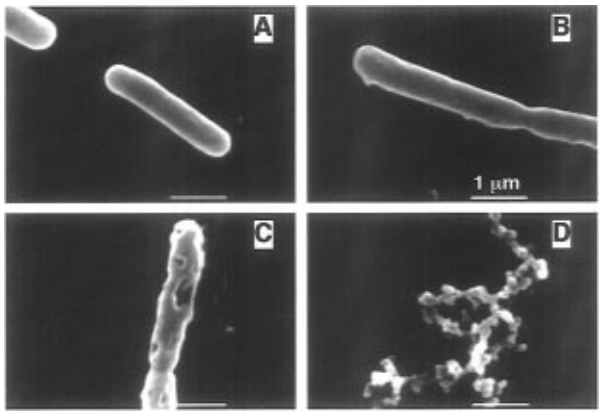

Figure 8. Scanning electron micrographs of *E. coli* K12 (10^8 cells/mL) incubated without (A) and with 200 μg/mL native lysozyme (B) or 100 μg/mL (C) or 200 μg/mL HLz80/6 at 30 °C for 30 min in 0.65% peptone, pH 7.4. Magnification, 20000× (figure reproduced at 50% of original size).

min, except the chains formed in the presence of native lysozyme (Figure 8A,B). At 100 μg/mL HLz80/6, cells exhibited chain formation associated with the appearance of large holes in the membrane, indicating direct damage of the bacterial membrane (Figure 8C). Addition of 200 μg/mL HLz80/6 produced completely collapsed morphology of bacteria with the appearance of aggregates of membrane debris (Figure 8D). When native lysozyme or HLz80/6 was added to *S. aureus* cultures, the cells were unstable and readily lysed during preparation for SEM examination (data not shown).

DISCUSSION

Laible and Germaine (1985) have reported that muramidase-inactive human lysozyme, prepared by reduc-

tion of essential disulfide bonds with dithiothreitol (DTT), was equal in bactericidal potency to native lysozyme against Gram-positive *Streptococcus* species. Antiviral activity of heat-inactivated hen egg white lysozyme against herpes simplex virus was reported by other workers (Cisani et al., 1989). Myeloperoxidase-dependent oxidative inactivation of microbicidal enzymes of human neutrophils was found to result in more than 50% inactivation of lysozyme (Vissers and Winterbourn, 1987). Despite these lines of evidence that the antimicrobial function of lysozyme may be operationally independent of its enzymatic activity, there was no study so far undertaken to examine the antimicrobial mechanism(s) of inactivated lysozyme against Gram-negative bacteria. The study presented in this paper shows that the enhanced antimicrobial action against Gram-negative bacteria (*E. coli* K12) was proportional to the degree of inactivation (by rising temperature at pH 6.0) of lysozyme, without detrimental effect on its inherent microbicidal activity to Gram-positive bacteria (Figure 2). Among the different denatured lysozymes, the denatured lysozyme that possessed about 50% residual enzymatic activity, HLz80/6, was the most potent bactericidal compound against different bacteria, including *S. enteritidis* (Figure 3). HLz80/6 showed a 10-fold increase in hydrophobicity over the native lysozyme with two exposed thiol groups (Table 1). Further evidence for acquisition of hydrophobic properties is the decrease in the intrinsic fluorescence intensity and the red shift observed, indicating that its tryptophan residues have become exposed to the surface (Table 2). More than 60% of HLz80/6 exists as oligomeric forms, and the major chemical forces of the interchain interactions were disulfide linkage with a little contribution from noncovalent cross-links (Figure 4). The observation that vesicle-bound HLz80/6 exhibits a blue shift in its tryptophan fluorescence maxima (Table 2) indicates that tryptophan residues are located in the lipid bilayer and reflects acquisition of a hydrophobic pocket. However, HLz80/6 shows enhanced binding capacity to peptidoglycan and LPS (Figure 5), the major components of the outer surface of *S. aureus* (Gram-positive) and *E. coli* (Gram-negative), respectively. The promoted lipid bilayer fusion properties of HLz80/6 has also been demonstrated (Figure 6). The agglutination and the consequent loss of viability (Figure 7) clearly indicate that HLz80/6 disintegrates the bacterial membrane upon binding. Taken all together, these findings support our proposal that the plasticity of HLz80/6 may allow for interference with biosynthetic processes occurring at the membrane important for bacterial viability, such as inhibition of the transport of nutrients and macromolecular precursors. Relevant to this finding is the report that antibiotics likewise disrupt the cell membrane, inhibiting the transport and incorporation of precursor molecules into peptidoglycan and lipids (Alborn et al., 1991). The bactericidal effects as a result of membrane disintegration caused by HLz80/6 have been evident from the SEM observations of *E. coli* (Figure 8).

The amphiphilic nature of HLz80/6 suggests a possible way in which the denatured molecule of lysozyme might interact and disrupt the membranes of bacteria. A cluster of hydrophobic side chains on the surface of HLz80/6 (Table 1) provides a hydrophobic patch of solvent accessible surface area that is surrounded with a set of the positively charged Arg and Lys side chains of lysozyme (Blake et al., 1965). Molecules of HLz80/6 disrupt the membrane by distorting lipid−lipid interactions (Figure 6). The denatured lysozyme does this by burying its hydrophobic surface into the lipid bilayer, while the positively charged residues interact with lipid phosphate groups (Table 2). This is in agreement with the enhanced interaction of HLz80/6 with the negatively charged PG and LPS and the restored epitope accessibility of the membrane-bound HLz80/6 to the anti-lysozyme IgG (Figure 5).

Although, the data presented in this study clearly demonstrate the direct effects of HLz80/6 on the membrane integrity of the susceptible bacteria which are sufficiently lethal, such activity could well provoke responses like autolytic enzymes that are secondary to the direct action of the denatured lysozyme on the cell membrane. The irregularity in the appearance of the collapsed structure of *E. coli* induced by HLz80/6 at higher concentration may further reflect the efficiency with which HLz80/6 triggers the enzymatic activities of the enzymes involved in the autolysis of either Gram-negative or -positive bacteria (Ledoc et al., 1982). The bactericidal effects of denatured lysozyme against Gram-positive bacteria appear to be related to its ability to interact with the bacterial membrane and subsequent stimulation of endogenous autolytic muramidases, as has previously been proposed (Laible and Germaine, 1985). The present study introduces an interesting finding that antimicrobial action of lysozyme can be switched to include Gram-negative bacteria by partial, or even complete, denaturation of the enzyme molecule. The denaturation temperature as well as the pH necessary to achieve the maximum antimicrobial activity of lysozyme were also emphasized. Our results may also imply that the antimicrobial action of lysozyme *in vivo* under varying redox potentials of infection-induced neutrophil stimulation (Vissers and Winterbourn, 1987) should be re-evaluated. Further studies verifying the possible role of autolysins in the enhanced bactericidal effects would provide better insight into the precise mechanism of action of the novel bactericidal activity of denatured lysozyme.

ABBREVIATIONS USED

CPA, *cis*-parinaric acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ELISA, enzyme-linked immunosorbent assay; HLz80/6, lysozyme derivative that is heat-denatured at 80 °C (20 min) and pH 6.0; NLz, native lysozyme; LPS, lipopolysaccharide; PAGE, polyacrylamide gel electrophoresis; PG, peptidoglycan; SEM, scanning electron microscopy.

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LITERATURE CITED

Alborn, W. E.; Allen, N. E.; Preston, D. A. Dapomycin Disrupts Membrane Potential in Growing *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **1991**, *35*, 2282−2287.

Back, J. F. Changes in the proteins of the vitelline membrane of hen's eggs during storage. *Biochim. Biophys. Acta* **1984**, *799*, 319−321.

Blake, C. C.; Koeing, D. F.; Mair, G. A.; North, A. C. T.; Phillips, D. C.; Sarma, V. R. Structure of Hen Egg-white Lysozyme. *Nature* **1965**, *206*, 757−761.

Brouwer, J.; van Leeuwen-Herverts, T.; Otting van de Ruit, M. Determination of lysozyme in serum, urine, cerebrospinal

fluid and feces by enzyme immunoassay. *Clin. Chim. Acta* **1984**, *142*, 21−30.

Cisani, G.; Varaldo, P. E.; Pompei, R.; Valisena, S.; Satta, G. Cell Fusion Induced by Herpes Simplex is Inhibited by Hen Egg-white Lysozyme. *Microbios* **1989**, *59*, 73−83.

Ellman, G. L. Tissue Sulfhydryl Groups. *Arch. Biochem. Biophys.* **1959**, *82*, 70−77.

Fleming, A. On a remarkable bacteriolytic element found in tissues and secretions. *Proc. R. Soc. London Ser. B* **1922**, *93*, 306−317.

Gibson, K. F.; Phadke, S. Intracellular Distribution of Lysozyme in Rat Alveolar Type II Epithelial Cells. *Exp. Lung Res.* **1994**, *20*, 595−611.

Hasselberger, F. X. *Uses of Enzymes and Immobilized Enzymes*; Nelson-Hall: Chicago, IL, 1978; p 128.

Ibrahim, H. R.; Yamada, M.; Kobayashi, K.; Kato, A. Bactericidal Action of Lysozyme against Gram-negative Bacteria Due to Insertion of A Hydrophobic Pentapeptide into Its C-terminus. *Biosci., Biotechnol., Biochem.* **1992**, *56*, 1361−1363.

Ibrahim, H. R.; Kobayashi, K.; Kato, A. Length of Hydrocarbon Chain and Antimicrobial Action to Gram-negative Bacteria of Fatty Acylated Lysozyme. *J. Agric. Food Chem.* **1993**, *41*, 1164−1168.

Ibrahim, H. R.; Hatta, H.; Fujiki, M.; Kim, M.; Yamamoto, T. Enhanced Antimicrobial Action of Lysozyme against Gram-Negative and Gram-Positive Bacteria Due to Modification with Perillaldehyde. *J. Agric. Food Chem.* **1994a**, *42*, 1813−1817.

Ibrahim, H. R.; Yamada, M.; Matsushita, K.; Kobayashi, K.; Kato, A. Enhanced Bactericidal Action of Lysozyme to *Escherichia coli* by Inserting A Hydrophobic Pentapeptide into Its C-terminus. *J. Biol. Chem.* **1994b**, *269*, 5059−5063.

Jolles, J.; Jolles, P. What's new in lysozyme research? *Mol. Cell. Biochem.* **1984**, *63*, 165−189.

Joseph, M.; Nagaraj, R. Interaction of Peptides Corresponding to Fatty Acylation Sites in Proteins with Model Membranes. *J. Biol. Chem.* **1995**, *270*, 16749−16755.

Kato, A.; Nakai, S. Hydrophobicity Determination by a Fluorescence Probe Method and Its Correlation with Surface Properties of Proteins. *Biochim. Biophys. Acta* **1980**, *624*, 13−20.

Kokoshis, P. L.; Williams, D. L.; Cook, J. A.; Di-Luzio, N. R. Increased resistance to *Staphylococcus aureus* infection and enhancement in serum lysozyme activity by glucan. *Science* **1978**, *199*, 1340−1342.

Laemmli, U. K. Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4. *Nature* **1970**, *227*, 680−685.

Laible, N. J.; Germaine, G. R. Bactericidal Activity of Human Lysozyme, Muramidase-inactive Lysozyme, and Cationic Polypeptides against *Streptococcus sanguis* and *Streptococcus faecalis*: inhibition by chitin oligosaccharides. *Infect. Immunol.* **1985**, *48*, 720−728.

Ledoc, M.; Kasra, R.; Heijenoort, J. Induction and Control of The Autolytic System of *Escherichia coli*. *J. Bacteriol.* **1982**, *152*, 26−34.

Lemarbre, P.; Rinehart, J. J.; Kay, N. E.; Vesella, R.; Jacobs, H. S. Lysozyme enhances monocyte-mediated tumoricidal activity: a potential amplifying mechanism of tumor killing. *Blood* **1981**, *58*, 994−999.

Lewis, U. J.; Cheever, E. V.; Seavey, B. K. Purification of Bovine Growth Hormone and Prolactin by Preparative Electrophoresis. *Anal. Biochem.* **1968**, *24*, 162−175.

Miller, G. L. Protein Determination for Large Numbers of Samples. *Anal. Chem.* **1959**, *31*, 964.

Morrison, D. C.; Leive, L. Fractions of Lipopolysaccharide from *Escherichia coli* 0111:B4 Prepared by Two Extraction Procedures. *J. Biol. Chem.* **1975**, *250*, 2911−2919.

Ohno, N.; Morrison, D. C. Lipopolysaccharide Interaction with Lysozyme. *J. Biol. Chem.* **1989**, *264*, 4434−4441.

Osserman, E. F.; Klockars, M.; Halper, J.; Fischer, R. E. Studies of the effects of lysozyme on mammalian cells. In *Lysozyme*; Osserman, E. F., Canfield, R. E., Beychok, S., Eds.; Academic Press: New York and London, 1974.

Posse, E.; De Arcuri, B. F.; Morero, R. D. Lysozyme interactions with phospholipid vesicles: relationships with fusion and release of aqueous content. *Biochim. Biophys. Acta* **1994**, *1193*, 101−106.

Rotta, J. Biologically Active Components of The Cells of Gram-positive Bacteria. *J. Hyg. Epidemiol. Microbiol. Immunol.* **1974**, *18*, 353−358.

Salton, M. R. J.; Pavlik, J. G. Studies of the bacterial cell wall. VI. Wall composition and sensitivity to lysozyme. *Biochim. Biophys. Acta* **1960**, *39*, 398−407.

Salton, M. R.; Freer, J. H.; Ellar, D. J. Electron transport components localized in a lipid-depleted sheet isolated from *Micrococcus lysodeikticus* membranes by deoxycholate extraction. *Biochem. Biophys. Res. Commun.* **1968**, *33*, 909−9015.

Sophianopoulos, A. J.; Holde, K. E. v. Physical studies of muramidase (lysozyme). II. pH-dependent dimerization. *J. Biol. Chem.* **1964**, *239*, 2516−2524.

Thacore, H.; Willet, H. P. The formation of spheroplasts of *Mycobacterium tuberculosis* in tissue culture cells. *Am. Rev. Respir. Dis.* **1966**, *93*, 786−790.

Viitanen, P.; Newman, M. J.; Foster, D. L.; Wilson, T. H.; Kaback, R. Purification, Reconstitution, and Characterization of the *lac* Pemease of *Escherichia coli*. *Methods Enzymol.* **1986**, *125*, 429−452.

Vissers, M. C.; Winterbourn, C. C. Myeloperoxidase-dependent Oxidative Inactivation of Neutrophil Neutral Proteinase and Microbicidal Enzymes. *Biochem. J.* **1987**, *245*, 277−280.

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