Preparation of Antimicrobial Reduced Lysozyme Compatible in Food Applications

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The structural and antimicrobial functions of lysozyme reduced with food-compatible reducing agents—cysteine (Cys) and glutathione (GSH)—were investigated. The disulfide bonds were partially reduced by thiol–disulfide exchange reactions under heat-induced denaturing conditions from 55 to 90 °C. The results showed that treatment of lysozyme with Cys and GSH resulted in the introduction of new half-cystine residues (2–3 residues/mol of protein). The released SH groups, in turn, rendered the lysozyme molecule more flexible, being accompanied by a dramatic increase in the surface hydrophobicity and exposure of tryptophan residues. As a consequence, the resulting reduced lysozymes were more capable of binding to lipopolysaccharides (LPS) and permeabilizing the bacterial outer membrane, as evidenced by the liposome leakage experiment, than were native or heated lysozyme. Both reduced lysozymes displayed significantly higher antimicrobial activity than native or heated lysozyme against *Salmonella enteritidis* (SE) in sodium phosphate buffer (10 mM, pH 7.2) at 30 °C for 1 h. Their minimal inhibitory concentrations (MICs) against the tested bacteria were about 150- and 25-fold lower than their respective MICs of native or heated lysozyme. The results suggest that partially reduced lysozyme could be used as a potential antimicrobial agent for prevention of SE attack.

KEYWORDS: Antimicrobial activity; reduced lysozyme; cysteine; glutathione; thiol–disulfide exchange reactions

INTRODUCTION

Lysozyme, a naturally occurring antimicrobial protein, has been attractively used as a preservative in various food applications (*1–3*) as well as in cosmetics and medical applications (*4*), as a consequence of its safety and stable activity under a variety of conditions (*5–7*). Nevertheless, the utilization of lysozyme, to some extent, is still limited because of its poor sensitivity toward Gram-negative bacteria, most of which are known as foodborne and spoilage agents (*8*).

Many strategies have been and are being developed to broaden the antimicrobial spectrum of action of lysozyme against Gram-negative bacteria so that it would gain more useful applications. These strategies include conjugation of lysozyme with dextran or galactomannan (*9, 10*), lipolization of lysozyme with fatty acids (*11, 12*), equipping lysozyme with a hydrophobic compound (*13*), genetic fusion of lysozyme with hydrophobic peptides (*14, 15*), heating the lysozyme molecule (*16, 17*), and hydrolysis of its polypeptide to yield peptides with hydrophobic and cationic properties (*17, 18*) or with a helix–loop–helix (HLH) motif (*19*).

More recently, we (*20*) have found that the lysozyme molecular flexibility is restricted by the four disulfide bonds

(^{6}Cys–^{127}Cys, ^{30}Cys–^{115}Cys, ^{64}Cys–^{80}Cys, and ^{76}Cys–^{94}Cys), which when reduced by dithiothreitol (DTT) and treated with iodoacetamide (IAM) resulted in loosened structure, thus exposing its hydrophobic regions. The exposed hydrophobic groups favorably interact with bacterial surfaces, thereby interfering with the bacterial membrane biosynthesis. Noteworthy is the finding that partial reduction of the disulfide bonds converted native lysozyme to partially unfolded species with the physicochemical characteristics of the molten-globule state. The molten-globule state is more potentially bactericidal to Gram-negative bacteria, particularly *Salmonella enteritidis* (SE), than the fully reduced form. However, the resulting reduced lysozyme may not be readily accepted in food applications owing to the toxicological properties of DTT used as the reducing agent. For reduced lysozyme to be widely accepted in food applications, reduction procedures that do not interfere with the organoleptic properties of foods should be employed. For this purpose, the present study was directed into investigating the structural and antimicrobial functions of lysozyme partially reduced by thiol–disulfide exchange reactions between the S–S bonds of lysozyme and food-compatible reducing agents—cysteine (Cys) and glutathione (GSH).

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MATERIALS AND METHODS

Materials. The materials used in this study were supplied by the indicated companies: hen egg white lysozyme and *Micrococcus luteus* cells, Seikagaku Kogyo Co. Ltd. (Tokyo, Japan); l-cysteine and IAM, Nacalai Tesque Inc. (Kyoto, Japan); GSH and lipopolysaccharides isolated from *S. enteritidis*, Sigma Chemical Co. (St. Louis, MO); 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB or Ellman's reagent) and peroxidase, Wako (Tokyo, Japan); rabbit anti-lysozyme IgG, Polysciences, Inc. (Warrington, PA); alkaline phosphatase conjugated goat anti-rabbit IgG, Chemicon International (Temecula, CA); egg yolk phosphatidylcholine, Avanti Polar Lipids, Inc. (Birmingham, AL); glucose oxidase (348 units/mg), Oriental Yeast Co., Ltd. (Tokyo, Japan). Unless otherwise specified, all other chemicals used were of reagent grade.

Preparation of Reduced Lysozyme. Lysozyme at a final protein concentration of 10 mg/mL was incubated with freshly prepared Cys and GSH at a final concentration of 40 mM in 10 mM Tris-HCl buffer at pH 8.0 for 15 min, on the basis of the results from the initial investigations on pH, concentration, and time dependency, under heating conditions from 55 to 90 °C. Having reached optimum conditions for the reactions, the resulting reduced lysozymes were allowed to react with 2 molar equiv of IAM with respect to the thiol concentration at 30 °C for 1 h in the dark, the condition under which the released SH groups were blocked by alkylation reaction. As a control, lysozyme heated without Cys and GSH was also subjected to the same treatment as the samples; hence, any free SH groups released by heat treatment would be trapped by IAM. The samples, after being exhaustively dialyzed against distilled water at 4 °C to remove salts and excess reagents, were freeze-dried for the following experiments.

Determination of Total Sulfhydryl Group Content. Prior to being blocked with IAM, the released SH groups of lysozyme were determined using the method of Beveridge et al. (*21*) with a slight modification. Aliquots (10 mL) were withdrawn at given conditions and allowed to incubate with 10 mL of 12% trichloroacetic acid (TCA) for 1 h and then centrifuged at 5000*g* for 10 min. After repeated precipitation and centrifugation, duplicate samples (10 mg) were suspended in 1 mL of glycine buffer containing 2% sodium dodecyl sulfate (SDS) and 6 M urea. The suspensions were routinely diluted in the same buffer to completely dissolve the denatured protein, after which 0.05 mL of Ellman's reagent (4 mg/mL) was added to develop the reaction color. Total SH content was calculated according to the equation

$$\mu\text{M SH/g of protein} = (73.53 A_{412\text{nm}} D)/C$$

where $A_{412\text{nm}}$ = the absorbance at 412 nm, C = sample concentration determined on the basis of the extinction coefficient at 280 nm of 2.635 cm^2 mg^{-1} for native lysozyme (*22*), and D = dilution factor.

Mass Spectrometric Analysis. Mass spectra of native lysozyme and its derivatives were determined by a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Autoflex, Bruker Daltonik, Bremen, Germany) equipped with a nitrogen laser and a delayed extraction ion source. Each lysozyme solution (1 μL) at a final concentration of 2−3 pmol/μL was directly spotted onto a target plate, which had been overlaid with 1 μL of matrix (saturated sinapinic acid in ethanol). The target plate was allowed to dry and then introduced to the ion source. Positively charged ions were analyzed in linear mode. Spectra were acquired as the sum of ions generated by irradiation of the samples with at least 50 laser shots. Peak abundances were collected at integer *m/z* values. Calibration was periodically checked with a low molecular weight standard (Protein Calibration Standard I, Bruker Daltonik).

Lytic Activity Assay. Dried cells of *Micrococcus luteus* (41.1 mg) were suspended in 100 mL of sodium phosphate buffer (55 mM, pH 6.2) under stirring conditions at 4 °C overnight. To 2.88 mL of the *M. luteus* suspension placed in a plastic cuvette was added an aliquot (120 μL) of each lysozyme solution (0.01 mg/mL), followed by repeated inversions for 20 s. The activity was estimated as the initial velocity of the decrease in turbidity of the cells monitored at 700 nm with a UV-1200 spectrophotometer (Shimadzu, Kyoto, Japan) at 37 °C within 2 min.

Determination of Surface Hydrophobicity (S_0). The surface hydrophobicity of lysozyme and its derivatives was determined according to the method described by Hayakawa and Nakai (*23*), using 1-anilinonaphthalene-8-sulfonate (ANS) as a hydrophobic probe. To measure the fluorescence intensity, 3 mL of each lysozyme solution serially diluted in sodium phosphate buffer (10 mM, pH 7.2) to obtain protein concentrations from 0.01 to 0.04% was mixed with 15 μL of 8 mM ANS. The fluorescence intensity was excited at 360 nm and recorded at 430 nm with a spectrofluorometer (Shimadzu RF-150). Expressed as initial slopes, the hydrophobicity was calculated from the plot of fluorescence intensity against the protein concentrations employed.

Measurement of Tryptophan Fluorescence. Tryptophan fluorescence of native "lysozyme" and its derivatives was measured in sodium phosphate buffer (10 mM, pH 7.2) with a spectrofluorometer (Shimadzu RF-150). The excitation wavelength (λ_{ex}) was 280 nm, and the emission wavelength (λ_{em}) was from 300 to 400 nm.

Antimicrobial Activity Assay. *S. enteritidis* IFO3313, obtained from the Institute for Fermentation (Osaka, Japan), was chosen as a model for Gram-negative bacteria to be tested. SE was precultured in a medium containing 1% polypeptone, 0.5% leaven extract, 0.3% glucose, 1% sodium chloride, 0.1% MgSO$_4$·7H$_2$O, and 1.5% agar at 37 °C for 24 h. Cells were harvested by washing with sodium phosphate buffer (10 mM, pH 7.2) and then resuspended in the same buffer to measure the optical density of the growth. The bacterial density, expressed as colony-forming units (CFU/mL), was estimated from an established standard curve previously prepared from CFU/mL versus optical density at 600 nm using a UV-1200 spectrophotometer (Shimadzu). The resuspended cultures were further diluted to achieve a density of 1×10^6 CFU/mL. An aliquot (20 μL) of the bacterial suspension was incubated with an equal volume of each lysozyme solution to obtain a final concentration of 0.2 mg/mL in sodium phosphate buffer (10 mM, pH 7.2) on a rotary shaker at 30 °C for 1 h. Controls were subjected to the same treatment as the samples but with water instead of the protein solution. Having been diluted to a countable number of the bacteria in saline solution (0.15 M), a 100 μL aliquot of each mixture was streaked onto desoxycholate−hydrogen sulfide lactose agar (DHL) plates. The plates were examined for characteristic colonies after incubation at 37 °C for at least 24 h. The antimicrobial activity is expressed as log(CFU of control/CFU of sample). The experiment was done in triplicate, and the samples were treated alike.

Minimal inhibitory concentrations (MICs) were determined according to the method described by Zimmermann and Rosselet (*24*) with a minor modification. The above bacterial suspension, diluted to a density of 10^3 CFU/mL, was allowed to interact with each lysozyme solution with increasing protein concentrations for 2 h at 30 °C. A 100 μL aliquot of each mixture was directly streaked onto DHL plates and incubated at 37 °C for 24 h. Viable cell counts were made in triplicate at the beginning and after 2 h of incubation. The concentration at which no significant decrease in viable cell count occurs during 2 h of incubation is defined as the MIC. The experiment was carried out three times, and the samples were treated alike.

Binding to LPS by Enzyme-Linked Immunosorbent Assay (ELISA). Microtiter plates (Greiner Co. Laboratory) were coated with 100 μL/well of lipopolysaccharides (LPS) isolated from SE (10 μg/mL) in 0.1 M sodium carbonate buffer (pH 9.6) at 4 °C overnight, after which they were washed three times with PBS containing 0.05% (v/v) Tween 20 (PBS-T). After blocking with 200 μL/well of 0.2% (w/v) bovine serum albumin (BSA) in PBS-T, the plates were washed with PBS-T again. Thereafter, 100 μL/well of lysozyme solution serially diluted from 10 μg/mL to 5 ng/mL was added to the plates. Following incubation at 37 °C for 1 h and three washings with PBS-T, 100 μL/well of rabbit anti-lysozyme IgG (1:500) was added and allowed to interact at 37 °C for 1 h and then substituted by 100 μL/well of alkaline phosphatase conjugated goat anti-rabbit IgG (1:10000). After incubation at 37 °C for 1 h, the plates were washed three times with PBS-T again, followed by the addition of 50 μL/well (1 mg/mL) of *p*-nitrophenyl phosphate solution dissolved in Tris-HCl buffer (100 mM, pH 9.8). The developed reaction color was then read on a microplate reader at 405 nm.

Liposome Leakage Measurement. Lysozyme-induced leakage of liposome contents was examined using glucose release. A mixture of egg yolk phosphatidylcholine and LPS of SE (1:1) (*25*) was dissolved in chloroform and then dried in a round-bottom flask by rotary evaporation under reduced pressure. The lipid film was kept under high vacuum for at least 2 h, after which 0.2 mL of 0.3 M glucose (*26*) was added. The flask was then washed twice with 5 mL of Tris-HCl buffer (10 mM, pH 7.5). The lipid suspension was extensively extruded through a polycarbonate membrane filter with a pore diameter of 0.1 *μ*m, using a filter holder (Advantec Inc., Tokyo, Japan) by applying a pressure gradient. Untrapped glucose was removed by centrifugal filter, using a 10K NMWL membrane (Millipore Corp.). Lipid concentration was estimated using a Wako phospholipid test kit (Wako, Osaka, Japan). The derived liposomes (0.5 mL, 0.5 mg/mL) were then allowed to interact with an equal volume of each lysozyme solution at final concentrations (0−100 *μ*g/mL) for 1.5 h. The mixtures were then centrifuged in a microcentrifuge for 5 min, and the supernatants were removed for glucose release determination, performed using the modified enzymatic method of Fox and Robyt (*27*). A 50 *μ*L aliquot from the supernatant was added to microtiter plates (Greiner Co. Laboratory), followed by the addition of 50 *μ*L of the stock assay reagent containing 22 mg of glucose oxidase and 3 mg of peroxidase in 200 mL of Tris-HCl buffer (10 mM, pH 7.5) and 50 *μ*L of the substrate (ABTS, Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD). After incubation (35 min), the reaction was stopped by the addition of 50 *μ*L of 2 N HCl prior to reading of the color intensity on a microplate reader at 405 nm. One hundred percent leakage was achieved after solubilization of the liposomes with 100 *μ*L of a 10% Triton X-100 prepared in 0.1 M Tris (pH 8.0). Liposome leakage was calculated as

$$\% \text{ liposome leakage} = (A_s - A_0)/(A_t - A_0) \times 100$$

where A_s is the absorbance achieved by lysozyme and its derivatives and A_0 and A_t are the absorbance without lysozyme solution (liposome only) and with Triton X-100, respectively.

Statistical Analysis. Data obtained were assessed by ANOVA (SPSS version 8.0 for Microsoft Windows; SPSS, Chicago, IL), and the mean pairs were compared using Student's *t* test. Significant differences were defined at the 5% level.

RESULTS

It is apparent from **Figure 1** that without the addition of the reducing agents, only trace quantities of free SH groups were detected after lysozyme solutions were heated up to 90 °C for 15 min. Likewise, neither Cys nor GSH treatment could release free SH groups of lysozyme at temperatures <55 °C, indicating that the added thiols had not participated in the thiol−disulfide exchange reactions, due presumably to the inaccessibility of the added thiols to the disulfide bonds. As such, burial of the disulfide bonds of lysozyme may prevent their contact and block the reactions, as thiol−disulfide exchange reactions could initiate only when a thiol and disulfide bonds come into contact (*28*). It, however, appeared that a significant increase in the SH content was observed with increasing temperature, with the maximum increase being found at 85 °C. Under this condition, treatment of lysozyme with Cys and GSH resulted in the introduction of free SH content up to 166.8 and 206.3 *μ*M/g of protein, corresponding to 2.3 and 3.0 mol/mol of protein, respectively. It should be pointed out that, although no attempt was made to completely reduce the S−S bonds, increasing temperature, thiol concentration, and incubation time did not significantly increase the SH content (data not shown), because the ability of heat treatment to unfold/denature the lysozyme molecule is limited; that is, it may not reach the value expected for the fully unfolded polypeptide chains (*29*). An attempt to increase the heating temperatures to >90 °C was found to decrease the reaction rate, by virtue of the formation of
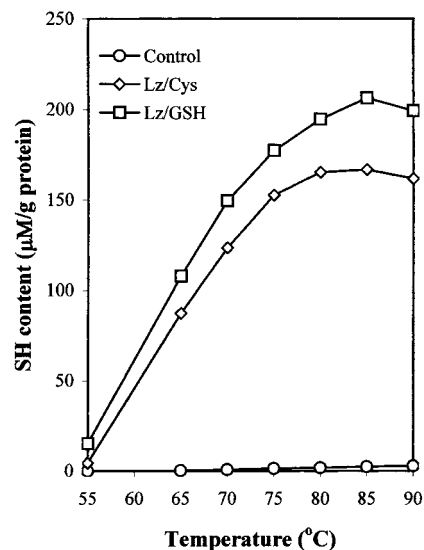

Figure 1. Release of sulfhydryl content of lysozyme by thiol–disulfide exchange reactions between lysozyme and cysteine and glutathione as a function of heating temperature. Lysozyme (10 mg/mL) was incubated with Cys and GSH (40 mM) in Tris-HCl buffer (10 mM, pH 8.0) for 15 min. Control, lysozyme heated in the absence of reducing agents; Lz/Cys, lysozyme heated in the presence of Cys; Lz/GSH, lysozyme heated in the presence of GSH.
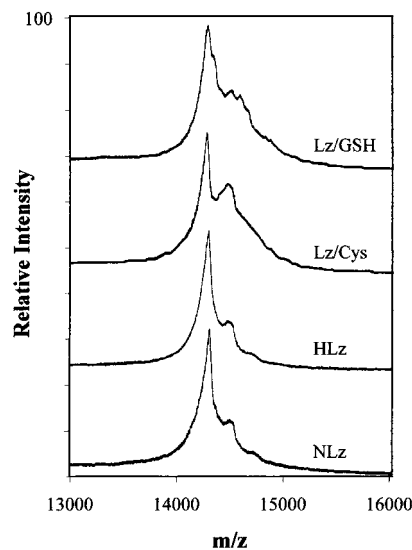

Figure 2. Typical mass spectra of lysozyme in the native state and after heating and reduction of the disulfide bonds. Lysozyme was reduced and alkylated as described under Materials and Methods. The average masses corresponding to each peak were 14301 ± 3.2 Da (NLz), 14301 ± 4.1 Da (HLz), 14468 ± 2.8 (Lz/Cys), and 14576 ± 5.3 (Lz/GSH). NLz, native lysozyme; HLz, lysozyme heated without reducing agents; Lz/Cys, lysozyme reduced with Cys; Lz/GSH, lysozyme reduced with GSH.

precipitates of the lysozyme molecule at elevated temperatures. This observation is reminiscent of the previous paper by Volkin and Klibanov (*30*).

To qualitatively verify the extent to which the disulfide bridges of lysozyme were reduced by heating and the thiol−disulfide exchange reactions, the increments of the lysozyme mass molecule after alkylation of lysozyme with IAM were detected by mass spectrometry. As shown in **Figure 2**, the molecular mass of lysozyme dramatically increased from 14301 Da in the native state to 14468 and 14576 Da, respectively, after treatment with Cys (lysozyme reduced with Cys, Lz/Cys)
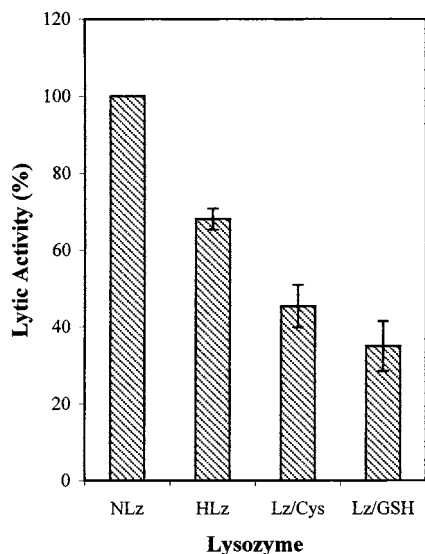
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Figure 3. Loss of the lytic activity of lysozyme after heating and reduction of the disulfide bonds. The lytic activity, assessed against *M. luteus* substrate, was expressed as percent of the residual activities of heated and/or reduced lysozyme with respect to that of the native protein. NLz, native lysozyme; HLz, lysozyme heated without reducing agents; Lz/Cys, lysozyme reduced with Cys; Lz/GSH, lysozyme reduced with GSH.

Figure 4. Changes in surface hydrophobicity of lysozyme heated with and without addition of cysteine and glutathione. The hydrophobicity, measured by ANS assay, was calculated from the plot of fluorescence intensity against the protein concentrations (0.01–0.04%). NLz, native lysozyme; HLz, lysozyme heated without reducing agents; Lz/Cys, lysozyme reduced with Cys; Lz/GSH, lysozyme reduced with GSH.

and GSH (lysozyme reduced with GSH, Lz/GSH). No increase in the mass molecule of heated lysozyme (HLz) was found, implying that none of the SH groups were released and blocked by IAM. The results confirm that the four disulfide bonds were partially reduced only in the presence of Cys and GSH as a result of the thiol−disulfide exchange reactions but not by the heat treatment.

There were great losses of the enzymatic activity of HLz, Lz/Cys, and Lz/GSH compared to native lysozyme (NLz) (**Figure 3**). Such considerable reductions were ascribed to the irreversible heat denaturation of the lysozyme molecule at high temperature (85 °C) with prolonged incubation (15 min), which led to secondary temperature-induced chemical changes of protein groups—namely, isomerization of Asp-Gly, deamidation of Asn, racemization of Asp and Asn, and cleavage of the Asp−X peptide bond (*31*). The effect of reduction of the disulfide bonds could further lower the enzymatic activity of Lz/Cys and Lz/GSH compared with HLz, possibly due to more intensive disruption of the conformational structure forming the active site, leading to a loss in their abilities to interact with its substrate (*M. luteus*).

The surface hydrophobicity of NLz was found to be remarkably low (**Figure 4**) because many of the amino acids bearing hydrophobic site groups are huddled together, forming a hydrophobic box inside the compactly folded structure. Even though there was a slight increase in the surface hydrophobicity of lysozyme upon heating, it is remarkable that the increased exposure of the hydrophobic surface was much more pronounced upon partial reduction of the disulfide bridges of lysozyme with the reducing agents. On the basis of the results, it is concluded that partial reduction of the disulfide bonds is required for lysozyme to expose its hydrophobic regions, as has been suggested by previous investigators (*32−34*).

Presented in **Figure 5** are the changes in tryptophan fluorescence of lysozyme upon heating and reduction of its disulfide bonds. The quantum yield of NLz and HLz was found to be the same, but the latter was red-shifted. The red shift of the fluorescence spectrum of HLz is indicative of a change in the
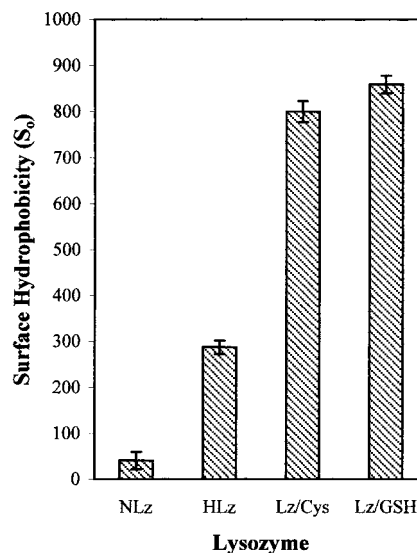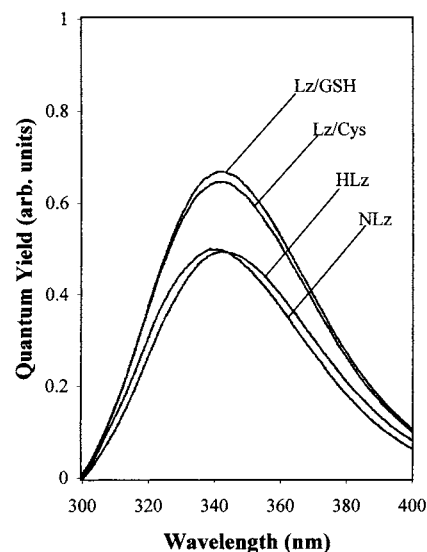
Figure 5. Changes in tryptophan fluorescence of lysozyme upon heating and reduction of the disulfide bonds with cysteine and glutathione. All samples at a concentration of 0.1% were measured in phosphate buffer (10 mM, pH 7.2). The quantum yield was excited at 280 nm, and emission from 300 to 400 nm was recorded. NLz, native lysozyme; HLz, lysozyme heated without reducing agents; Lz/Cys, lysozyme reduced with Cys; Lz/GSH, lysozyme reduced with GSH.

local environment due to unfolding, whereas its low quantum yield is reflective of the remnants of structure maintained by the four disulfide bonds (*35*). By comparison, the tryptophan fluorescence intensity of Lz/Cys and Lz/GSH dramatically increased with maximum wavelength shifting toward longer wavelength, indicating the disappearance of tertiary interactions that quench the fluorescence in the native state, and a change of the Trp residues to a more polar environment (*36*).

The antimicrobial activities of lysozyme and its modified forms are depicted in **Figure 6**. As shown, native lysozyme displayed weak antimicrobial activity against *S. enteritidis*. Note that there was a slight but significant increase ($P < 0.05$) in
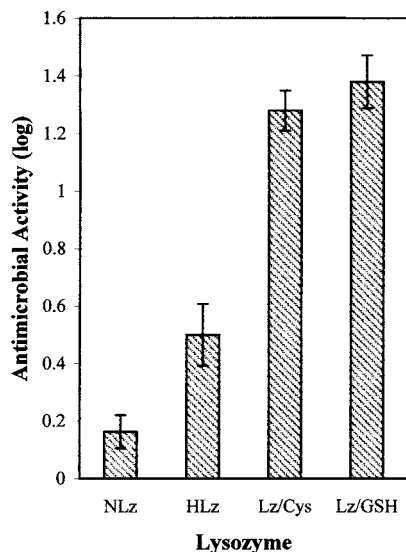
Figure 6. Antimicrobial activity of native, heated, and reduced lysozymes against *S. enteritidis* IFO3313 (SE). The antimicrobial activity is expressed as log(CFU of control/CFU of sample). Bacterial suspension, 1×10^6 CFU/mL; protein concentration, 0.2 mg/mL; temperature, 30 °C; incubation time, 1 h. NLz, native lysozyme; HLz, heated lysozyme; Lz/Cys, lysozyme reduced with Cys; Lz/GSH, lysozyme reduced with GSH.

Table 1. Minimal Inhibitory Concentrations (MICs)[a] of Lysozyme and Its Modified Forms against *S. enteritidis*

sample	MIC (μg/mL)
native lysozyme (NLz)	≤150
heated lysozyme (HLz)	≤25
lysozyme reduced with Cys (Lz/Cys)	≤1.25
lysozyme reduced with GSH (Lz/GSH)	≤1.0

[a] MIC is defined as the concentration at which there is no significant decrease ($P \geq 0.05$) in means of viable cell count from three sets of experiments each at the beginning and after 2 h of incubation at 30 °C.

the antimicrobial activity of HLz over NLz. This increase, nevertheless, was remarkably less pronounced than that of Lz/Cys and Lz/GSH, whereas there was no significant difference ($P > 0.05$) in the antimicrobial activity between both reduced lysozymes. It is of interest to note that the bactericidal potencies of lysozyme reduced with Cys or GSH were almost comparable to that of lysozyme reduced with DTT in our previous study (*20*), suggesting that Cys and GSH could be employed as alternative candidates to DTT for the preparation of antimicrobial reduced lysozyme while retaining comparable potency. This may also reflect that the structural requirements rather than the reducing agent affect the antimicrobial activity of lysozyme. Also noteworthy is the fact that the MICs of Lz/Cys and Lz/GSH were found to be about 150- and 25-fold lower than the respective MICs of NLz and HLz (**Table 1**) and are comparable to the MICs of some of the antibiotics against *Salmonella* spp. reported by Gutmann et al. (*37*). These clinically achievable concentrations may offer the potential use of reduced lysozyme as an effective antimicrobial agent not only in foods but also in medical and veterinary applications to inactivate and/or kill SE, to which antibiotics have been found to be resistant due primarily to the widespread use of antibiotics in human and veterinary medicine (*38*).

The ELISA binding experiment reveals that Lz/Cys and Lz/GSH had significantly ($P < 0.05$) higher binding affinity to lipopolysaccharides (LPS) than did NLz and HLz (**Figure 7**).
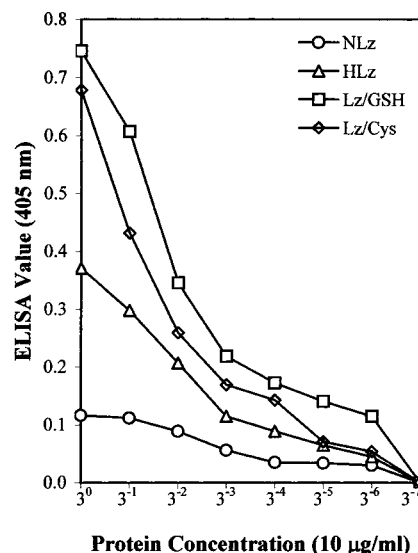

Figure 7. Binding of lysozyme and its derivatives to lipopolysaccharides of *S. enteritidis*. Lysozyme samples at the same concentration (10 μg/mL) were 1/3 diluted in PBS-T. Data are the mean of triplicate measurements with SD < 0.05. NLz, native lysozyme; HLz, heated lysozyme; Lz/Cys, lysozyme reduced with Cys; Lz/GSH, lysozyme reduced with GSH.

The facts that native lysozyme failed to bind to LPS and that there was a good relationship between the increased surface hydrophobicity and binding affinity suggest that binding of lysozyme to LPS is dominantly hydrophobic with little effect contributed by ionic binding, which agrees with previously reported data of Ohno and Morrison (*39*).

To test the abilities of lysozyme and its derivatives to permeabilize the outer membrane of bacteria, leakage of glucose trapped in the LPS-containing liposomes was measured; glucose, trapped in the liposome interior, cannot be released from the liposome interior unless lipid membranes are perturbed. The results, shown in **Figure 8**, demonstrate that Lz/Cys and Lz/GSH were far better than NLz and HLz in spanning the lipid bilayer, presumably through interactions of their exposed hydrophobic segments with the lipid hydrocarbon acyl chains (*40*). In this effect, the surface hydrophobicity was assumed to be aligned parallel to the surface of the vesicle membrane, and some nonpolar residues shallowly penetrated into the bilayer. Judging from these results, it is more likely that reduced lysozyme is lethal to bacteria by membrane interference and/or perturbation.

DISCUSSION

The ability to break a specific disulfide bond is largely contingent upon (1) the origin, size, amino acid composition, and sequence of the protein (*30*) and (2) the nature of the reductant, the disulfide stability, the kinetics of the forward and reverse reactions, and the nature and redox state of the environment in which the reaction occurs (*28*). In the case of lysozyme, its disulfide bonds in the native state, embedded in the compact globular molecule, are highly protected from reduction (*32*). In our previous study (*20*), partial and extensive reduction of the disulfide bonds of lysozyme was achieved using DTT as the reducing agent at pH 8.0 and 30 °C. The efficiency of DTT results from the thermodynamically favored formation of an intramolecular disulfide ring structure within the reagent (*41*–*43*), which competently shifts the equilibrium of the reaction toward complete reduction of the protein even under

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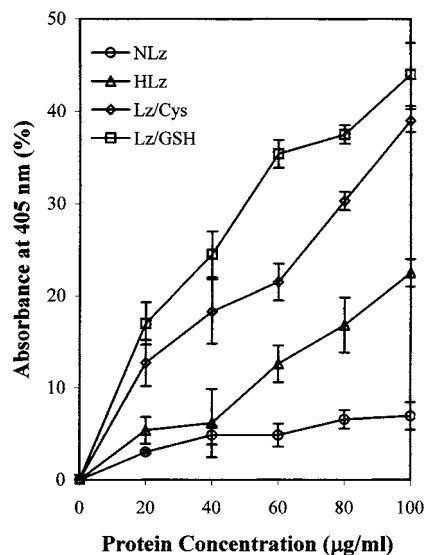*J. Agric. Food Chem.,* Vol. 51, No. 17, 2003   **5159**

Figure 8. Liposome leakage induced by lysozyme and its derivatives. Release of glucose trapped in the interior of LPS-containing liposomes was monitored by measuring the color intensity developed by glucose oxidase in the presence of peroxidase with ABTS. The maximum intensity, corresponding to 100%, was determined by addition of Triton X-100 to the vesicle suspension. NLz, native lysozyme; HLz, heated lysozyme; Lz/Cys, lysozyme reduced with Cys; Lz/GSH, lysozyme reduced with GSH.

non-denaturing condition. As monothiols, known to be weaker reducing agents than the corresponding dithiols (*43, 44*), Cys and GSH were unable to break the disulfide bonds of lysozyme under the condition that could be achieved by DTT. Nonetheless, upon heat denaturation, the susceptibility of the disulfide bonds of lysozyme to reduction increased because of the enhanced accessibility of the disulfide bonds in the denatured protein compared with the native conformation of the protein, meaning that some, but not all, of the disulfide bonds of lysozyme were reshuffled and/or exposed on the surface by heating, thus initiating the thiol−disulfide exchange reactions (**Figure 1**). Yet, the effect of heat treatment alone did not lead to disruption of the disulfide bonds of lysozyme (**Figures 1** and **2**), which appears to be contradictory to previous findings showing a dramatic increase in SH groups of lysozyme upon heating at pH 6 and 80 °C for 20 min (*16*). Consistent with our results, Li-Chan and Nakai (*33*) found only trace quantities of SH groups after heating a lysozyme solution at pH 7.2 and 80 °C for 12 min. Reportedly, destruction of the disulfide bonds of lysozyme to release free SH groups, even if in small quantities (<15 μM), could be accomplished at 100 °C via β-elimination reaction when some chemicals, for example, guanidine hydrochloride, are used to overcome aggregations (*30, 45*). Nevertheless, the rate of reduction of these S−S bonds was found to be much slower at pH 6 than at pH 8 (half-lives of 9.2 h at pH 6 and only 0.63 h at pH 8) (*30*).

The intact disulfide bonds tend to shield the hydrophobic regions and suppress the conformation of lysozyme even when the protein is denatured/unfolded (*32−34, 46*). Therefore, partial reduction of the disulfide bonds could increase the conformational flexibility of the lysozyme molecule, as evidenced by the dramatic increase in the surface hydrophobicity (**Figure 4**) and the tryptophan fluorescence intensity (**Figure 5**).

Changes in the conformation of lysozyme had an effect on its antimicrobial activity. As can be observed in **Figure 6**, native lysozyme produced little inactivation effect on the tested bacteria, regardless of concentration and time tested (*20*).

However, when subjected to heat treatment under the condition employed, the lysozyme molecule altered its conformation (**Figure 5**) with a slight increase in the surface hydrophobicity (**Figure 4**), thereby resulting in higher antimicrobial activity than that of the native protein (**Figure 6**). On the other hand, the reduced lysozymes, notwithstanding the substantial losses of the enzymatic activity (**Figure 3**), exhibited remarkably enhanced antimicrobial activity against SE when compared with NLz and HLz. Presumably, there are two salient factors contributing to such predominantly enhanced activity. First, and perhaps more importantly, the reduced lysozymes had substantially higher surface hydrophobicity than did NLz and HLz (**Figure 4**), thereby greatly promoting their binding affinity to the components of the bacterial outer membrane (**Figure 7**), which is viewed as being critical for the protein to increase the permeability of the outer membrane of bacteria (*39*) and for successive translocation/insertion into the inner membrane (*47*). Second, as far as the mechanism of protein translocation is concerned, it is generally accepted that a protein lacking the tertiary conformation can be more effectively translocated into a membrane than that crossed-linked by disulfide bridges (*34, 48−50*) because the protein is believed to be folded into its amphipathic structure upon successive translocation, although it is still obscure at which point this process is prevalent (i.e., during transit across the outer membrane or during insertion into the cytoplasmic membrane) (*50*). In this context, partially unfolding species of the reduced lysozymes could serve as an intermediate in the protein folding upon interactions with the transport machinery and as the best pathway for translocation across the outer membranes, as evidenced by the liposome leakage experiment (**Figure 8**). Having transited the outer membrane, the reduced lysozymes with the cationic nature (p*I* 10.5) may interfere/interact with the negatively charged surface of the cytoplasmic membrane, formed by the headgroups of phosphatidylglycerol and cardiolipin (*50*). Ultimately, once the functional integrity of the cytoplasmic membrane has been disrupted, cell damage or death could result.

In conclusion, this study provides further evidence that hydrophobic and cationic properties but not muramidase activity are implicated in the bactericidal action of lysozyme. Partial reduction of the disulfide bonds is necessary for lysozyme to expose its hydrophobic regions to maximally perform the bactericidal action. Reduced lysozyme kills microorganisms by interference and/or disruption of the bacterial membranes. Produced by using food additives as the reducing agents, reduced lysozyme could be employed as an effective method for reducing or eliminating the likelihood of foodborne outbreaks induced by SE, thus minimizing economic losses to the food industry due to product recalls. Experiments to test its potential applications in food systems are under way in our laboratory.

LITERATURE CITED

(1) Hughey, V. L.; Wilger, P. A.; Johnson, E. A. Antibacterial activity of hen egg white lysozyme against *Listeria monocytogenes* Scott A in foods. *Appl. Environ. Microbiol.* **1989**, *55*, 631−638.

(2) Gill, A. O.; Holley, R. A. Surface application of lysozyme, nisin, and EDTA to inhibit spoilage and pathogenic bacteria on ham and bologna. *J. Food Prot.* **2000**, *63*, 1338−1346.

(3) Marchal, R.; Chaboche, D.; Marchal-Delahau, L.; Gerland, C.; Gandon, J. P.; Jeandet, P. Detection and quantification of lysozyme in champagne wines. *J. Agric. Food Chem.* **2000**, *48*, 3225−3231.

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Touch et al.

(4) Proctor, V. A.; Cunningham, F. E. The chemistry of lysozyme and its use as a food preservative and a pharmaceutical. *Crit. Rev. Food Sci. Nutr.* **1988**, *26*, 359−395.

(5) Yang, T. S.; Cunningham, F. E. Stability of egg white lysozyme in combination with other antimicrobial substances. *J. Food Prot.* **1993**, *56*, 153−156.

(6) Chang, H. M.; Yang, C. C.; Chang, Y. C. Rapid separation of lysozyme from chicken egg white by reductants and thermal treatment. *J. Agric. Food Chem.* **2000**, *48*, 161−164.

(7) Johansen, C.; Gram, L.; Meyer, A. S. The combined inhibitory effect of lysozyme and low pH on growth of *Listeria monocytogenes. J. Food Prot.* **1994**, *57*, 561−566.

(8) Hughey, V. L.; Johnson, E. A. Antimicrobial activity of lysozyme against bacteria involved in food spoilage and food-borne disease. *Appl. Environ. Microbiol.* **1987**, *53*, 2165−2170.

(9) Nakamura, S.; Kato, A.; Kobayashi, K. New antimicrobial characteristics of lysozyme−dextran conjugate. *J. Agric. Food Chem.* **1991**, *39*, 647−650.

(10) Nakamura, S.; Kato, A.; Kobayashi, K. Bifunctional lysozyme galactomannan conjugate having excellent emulsifying properties and bactericidal effect. *J. Agric. Food Chem.* **1992**, *40*, 735−739.

(11) Ibrahim, H. R.; Kobayashi, K.; Kato, A. Length of hydrocarbon chain and antimicrobial action to gram-negative bacteria of fatty acetylated lysozyme. *J. Agric. Food Chem.* **1993**, *41*, 1164−1168.

(12) Liu, S. T.; Sugimoto, T.; Azakami, H.; Kato, A. Lipophilization of lysozyme by short and middle chain fatty acids. *J. Agric. Food Chem.* **2000**, *48*, 265−269.

(13) 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.* **1994**, *42*, 1813−1817.

(14) 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.* **1994**, *269*, 5059−5063.

(15) Arima, H.; Ibrahim, H. R.; Kinoshita, T.; Kato, A. Bactericidal action of lysozymes attached with various sizes of hydrophobic peptides to the C-terminus using genetic modification. *FEBS Lett.* **1997**, *415*, 114−118.

(16) Ibrahim, R. H.; Higashiguchi, S.; Koketsu, M.; Juneja, R. L.; Kim, M.; Yamamoto, T.; Sugimoto, Y.; Aoki, T. Partially unfolded lysozyme at neutral pH agglutinates and kills gram-negative and gram-positive bacteria through membrane damage mechanism. *J. Agric. Food Chem.* **1996**, *44*, 3799−3806.

(17) Düring, K.; Porsch, P.; Mahn, A.; Brinkmann, O.; Gieffers, W. The non-enzymatic microbicidal activity of lysozymes. *FEBS Lett.* **1999**, *449*, 93−100.

(18) Pellegrini, A.; Thomas, U.; Bramaz, N.; Klauser, S.; Hunziker, P.; von Fellenberg, R. Identification and isolation of a bactericidal domain in chicken egg white lysozyme. *J. Appl. Microbiol.* **1997**, *82*, 372−378.

(19) Ibrahim, R. H.; Thomas, U.; Pellegrini, A. A helix-loop peptide at the upper lip of the active site cleft of lysozyme confers potent antimicrobial activity with membrane permeabilization action. *J. Biol. Chem.* **2001**, *276*, 43767−43774.

(20) Touch, V.; Hayakawa, S.; Saitoh, K. Unpublished data.

(21) Beveridge, T.; Toma, S. J.; Nakai, S. Determination of SH− and S−S groups in some food proteins using Ellman's reagent. *J. Food Sci.* **1974**, *39*, 49−51.

(22) Sophianopoulos, A. J.; Rhodes, C. K.; Holcomb, D. N.; Van Holde, K. E. Physical studies of lysozyme. I. Characterization. *J. Biol. Chem.* **1962**, *237*, 1107−1112.

(23) Hayakawa, S.; Nakai, S. Relationships of hydrophobicity and net charge to the solubility of milk and soy proteins. *J. Food Sci.* **1985**, *50*, 486-491.

(24) Zimmermann, W.; Rosselet, A. Function of the outer membrane of *Escherichia coli* as a permeability barrier to *β*-lactam antibiotics. *Antimicrob. Agents Chemother.* **1977**, *12*, 368−372.

(25) Matsuzaki, K.; Sugishita, K.; Miyajima, K. Interactions of an antimicrobial peptide, magainin 2, with lipopolysaccharide-containing liposomes as a model for outer membranes of Gram-negative bacteria. *FEBS Lett.* **1999**, *449*, 221−224.

(26) Kinsky, S. C.; Haxby, J. A.; Zopf, D. A.; Alving, C. R.; Kinsky, C. B. Complement-dependent damage to liposomes prepared from pure lipids and Forssman hapten. *Biochemistry* **1969**, *8*, 4149−4159.

(27) Fox, J. D.; Robyt, J. F. Miniaturization of three carbohydrate analyses using a microsample plate reader. *Anal. Biochem.* **1991**, *195*, 93−96.

(28) Gilbert, H. F. Thiol/disulfide exchange equilibria and disulfide bond stability. *Methods Enzymol.* **1995**, *251*, 8−28.

(29) Tanford, C.; Aune, K. C. Thermodynamics of the denaturation of lysozyme by guanidine hydrochloride. III. Dependence on temperature. *Biochemistry* **1970**, *9*, 206−211.

(30) Volkin, D. B.; Klibanov, A. M. Thermal destruction processes in proteins involving cystine residues. *J. Biol. Chem.* **1987**, *262*, 2945−2950.

(31) Tomizawa, H.; Yamada, H.; Imoto, T. The mechanism of irreversible inactivation of lysozyme at pH 4 and 100 °C. *Biochemistry* **1994**, *33*, 13032−13037.

(32) Hayakawa, S.; Nakamura, R. Optimization approaches to thermally induced egg white lysozyme gel. *Agric. Biol. Chem.* **1986**, *50*, 2039−2046.

(33) Li-Chan, E.; Nakai, S. Effect of molecular changes (SH groups and hydrophobicity) of food proteins on their functionality. In *Food Proteins*; Kinsella, J. E., Soucie, W. G., Eds.; AOCS: Champaign, IL, 1989; pp 232−251.

(34) Joseph, M.; Nagaraj, R. Unfolding of lysozyme by breaking its disulfide bridges results in exposure of hydrophobic sites. *Biochem. Int.* **1992**, *26*, 973−978.

(35) Formoso, C.; Forster, L. S. Tryptophan fluorescence lifetimes in lysozyme. *J. Biol. Chem.* **1975**, *250*, 3738−3745.

(36) Imoto, T.; Forster, L. S.; Rupley, J. A.; Tanaka, F. Fluorescence of lysozyme: Emissions from tryptophan residues 62 and 108 and energy migration. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 1151−1155.

(37) Gutmann, L.; Billot-Klein, D.; Williamson, R.; Goldstein, F. W.; Mounier, J.; Acar, J. F.; Collatz, E. Mutation of *Salmonella paratyphi* A conferring cross-resistance to several groups of antibiotics by decreased permeability and loss of invasiveness. *Antimicrob. Agents Chemother.* **1988**, *32*, 195−201.

(38) Chang, Y. H. Prevalence of *Salmonella* spp. in poultry broiler and shell eggs in Korea. *J. Food Prot.* **2000**, *63*, 655−658.

(39) Ohno, N.; Morrison, D. C. Lipopolysaccharide interaction with lysozyme. *J. Biol. Chem.* **1989**, *264*, 4434−4441.

(40) Kuboi, R.; Mawatari, T.; Yoshimoto, M. Oxidative refolding of lysozyme assisted by negatively charged liposomes: Relationship with lysozyme-mediated fusion of liposomes. *J. Biosci. Bioeng.* **2000**, *90*, 14−19.

(41) Cleland, W. W. Dithiothreitol, a new protective reagent for SH groups. *Biochemistry* **1964**, *3*, 480−482.

(42) Lapanje, S.; Rupley, J. A. Enthalpy of reduction of disulfide cross-links in denatured lysozyme. *Biochemistry* **1973**, *12*, 2370−2372.

(43) Szajewski, R. P.; Whitesides, G. M. Rate constants and equilibrium constants for thiol−disulfide interchange reactions involving oxidized glutathione. *J. Am. Chem. Soc.* **1980**, *102*, 2011−2025.

(44) Rothwarf, D. M.; Scheraga, H. A. Equilibrium and kinetic constants for the thiol−disulfide interchange reaction between glutathione and dithiothreitol. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 7944−7948.

(45) Ahern, T. J.; Klibanov, A. M. The mechanism of irreversible enzyme inactivation at 100 °C. *Science* **1985**, *228*, 1280−1284.

(46) Kuroki, R.; Inaka, K.; Taniyama, Y.; Kidokoro, S.; Matsushima, M.; Kikuchi, M.; Yutani, K. Enthalpic destabilization of a mutant human lysozyme lacking a disulfide bridge between cysteine-77 and cysteine-95. *Biochemistry* **1992**, *31*, 8323−8328.

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J. Agric. Food Chem., Vol. 51, No. 17, 2003 **5161**

(47) Eilers, M.; Schatz, G. Binding of a specific ligand inhibits import of a purified precursor protein into mitochondria. *Nature* **1986**, *322*, 228−232.

(48) Maher, P. A.; Singer, S. J. Disulfide bonds and the translocation of proteins across membrane (secretion/mitochondrial protein import/cysteine residues). *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 9001−9005.

(49) Van der Goot, F. G.; González-Mañas, J. M.; Lakey, J. H.; Pattus, F. A "molten-globule" membrane insertion intermediate of the pore-forming domain of colicin A. *Nature* **1991**, *354*, 408−410.

(50) Hancock, R. E. W.; Chapple, D. S. Peptide antibiotics. *Antimicrob. Agents Chemother.* **1999**, *43*, 1317−1323.