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Relationships between conformational changes and antimicrobial activity of lysozyme upon reduction of its disulfide bonds

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Abstract

The structural changes and antimicrobial activity of hen lysozyme in which its disulfide bonds were reduced to varying degrees, were investigated. Lysozyme was reduced with 2 mM dithiothreitol at pH 8 and 30 °C from 0.5 to 4 h, and the released sulfhydryl groups were modified by iodoacetamide. The resulting reduced lysozymes were then tested for their antimicrobial activity against *Salmonella enteritidis*, *Escherichia coli*, *Proteus mirabilis* and *Serratia marcescens*. The treatment was found to induce lysozyme to expose its surface hydrophobicity and intrinsic fluorescence, change its CD behaviour and increase its binding affinity to the bacterial membranes, thereby enhancing its antimicrobial activity against Gram-negative bacteria, the extent of which depended on the degree of reduction and bacterial strains used. The reduced lysozymes showed greater bactericidal activity toward *S. enteritidis* than other strains tested and the partially reduced form was more powerful than the fully reduced one.

Keywords: Lysozyme; Conformational changes; Antimicrobial activity; Reduction; Intramolecular disulfide bonds; Dithiothreitol; Reduced lysozyme

1. Introduction

Lysozyme (Lz), because of its muramidase activity, has long been known to exert its antimicrobial action by specifically hydrolyzing the 1,4 β-D-linkage between N-acetylhexosamines in the peptidoglycan layer of the bacterial cell wall (Proctor & Cunningham, 1988). In this effect, Lz is predominantly sensitive to Gram-positive bacteria while being slightly or even inertly reactive to Gram-negative bacteria, the discrepancy being due to the presence of the outer membrane (i.e. lipopolysaccharides—LPS) of the latter, which can prevent the enzymatic activity of Lz (Ohno & Morrison, 1989) and/or prevent the diffusion of the molecule to the target site (Hughey & Johnson, 1987).

Three main approaches can be developed to improve the antimicrobial spectrum of action of Lz against resistant bacteria so that it would gain more useful applications. These include (1) disruption of the bacterial outer membrane to allow Lz to permeate to its target site, achieved either by chemical or physical treatments; (2) modification of the lysozyme molecule by chemical, physical, enzymatic and genetic engineering means, and (3) combined effects with other antimicrobials, resulting in a synergistic and/or simultaneous attack on different targets in the bacterial cell. The most promising effect, however, has been reportedly accomplished by increasing the surface hydrophobicity of Lz via genetic fusion of hydrophobic peptides (Ibrahim, Yamada, Matsushita, Kobayashi, & Kato, 1994; Ito, Kwon, Ueda, Tanaka, & Imanishi, 1997) or covalent attachment of hydrophobic ligands such as fatty acids (Liu, Sugimoto, Azakami, & Kato, 2000), cinnamic acid and caffeic acid (Bernkop-Schnüch, Krist, Vehabovic, & Valenta, 1998), to enable it to interact with the bacterial membranes and diffuse to the killing site.

The Lz structure is clearly characterized as a compactly folded molecule, the rigidity of which is stabilized by the four-disulfide bonds (^{6}Cys–^{127}Cys, ^{30}Cys–^{115}Cys, ^{64}Cys–^{80}Cys, and ^{76}Cys–^{94}Cys) (Canfield & Liu, 1965). These disulfide bonds are well known to be stable to denaturing agents and heat treatment, but easily disrupted with reducing agents, and reduction of these S–S bridges is conducive to a greater molecular flexibility and dramatic increase in exposed hydrophobic regions

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(Hayakawa & Nakamura, 1986; Li-Chan & Nakai, 1989; Joseph & Nagaraj, 1992; Volkin & Klibanov, 1987). Improvement of gelling, emulsifying and foaming properties of Lz by reduction of its disulfide bonds has been reported from our laboratory (Hayakawa & Nakamura, 1986) and by other investigators (Li-Chan & Nakai, 1989; Kato, Tanimoto, Muraki, Oda, Inoue, & Kobayashi, 1994). On the other hand, reduction of the S–S bridges of Lz results in loss of its enzymatic activity, which in turn leads to a loss of its ability to kill bacteria (Proctor & Cunningham, 1988). Yet, few reports have shown that reduced lysozyme (RLz), induced by reduction of the S–S bonds with dithiothreitol (DTT), still retains the same bactericidal potency toward *Streptococcus sanguis* and *Streptococcus faecalis* (Laible & Germaine, 1985) and *E. coli* (Pellegrini, Thomas, von Fellenberg, & Wild, 1992) as the native protein (NLz). In contrast, other investigators recently found that the antimicrobial activity of Lz, reduced with β-mercapto-ethanol, was poorer than that of NLz (Masschalck, van Houdt, van Haver, & Michiels, 2001). Although this controversy can be attributed to difference in the experimental conditions, it is questionable whether RLz could be renatured during the assay period (Pellegrini, Thomas, Bramaz, Klauser, Hunziker, & von Fellenberg, 1997), since unfolded lysozyme, without being blocked, is fully reversible (Anfinsen & Haber, 1961) and, particularly, addition of water induces a rapid reconstitution of the native secondary and tertiary structures of Lz (Galat, 1985) by reducing the probability of intermolecular interactions between its polypeptide chains (Roux, Delepierre, Goldberg, & Chaffotte, 1997). On the other hand, degrees of reduction/residual disulfide bonds, which may affect the antimicrobial activity of Lz, were not the focus of the previous studies.

This study was designed to investigate the correlations between the conformational changes and antimicrobial activity of Lz after reduction of its disulfide bonds to varying degrees to optimize structural requirements for its bactericidal action. Unlike RLz prepared in the previous studies, RLz samples in this study were alkylated, to ensure their stabilities in water and solution (Nishiyama & Maeda, 1992; Yamada et al., 1994) before testing their antimicrobial activity against several Gram-negative bacteria.

2. Materials and methods

2.1. Materials

Hen egg white lysozyme, crystallized six times, and Micrococcus luteus cells were supplied by Seikagaku Kogyo Co. Ltd. (Tokyo, Japan). DTT and iodoacetamide (IAM) were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Peroxidase-conjugated goat anti-rabbit IgG and rabbit anti-lysozyme IgG were purchased from Alpha Diagnostic Intl. Inc. (San Antonio, USA) and Polysciences, Inc. (Warrington, USA), respectively. Unless otherwise specified, all other chemicals used were reagent grade.

2.2. Protein determination

Protein concentration was spectrophotometrically determined on the basis of extinction coefficient at 280 nm of 2.635 cm^2 mg^{-1} for NLz (Sophianopoulos, Rhodes, Holcomb, & van Holde, 1962). Insoluble proteins were removed by centrifugation.

2.3. Preparation of reduced lysozymes

Hen egg lysozyme was reduced with DTT at given times to achieve different levels of reduction. Lysozyme solution in Tris–HCl buffer (10 mM, pH 8), at a final protein concentration of 1 mg/ml, was incubated with 2 mM DTT at 30 °C. Aliquots (10 ml) were withdrawn at intervals of 0, 0.5, 1, 1.5, 2, 3, and 4 h, and immediately allowed to react with three molar equivalents of IAM with respect to DTT at 30 °C for 1 h in the dark. The samples, after being extensively dialysed against distilled water at 4 °C, were freeze-dried for further use.

2.4. Mass spectrometric analysis

Mass spectra of NLz and its derivatives were determined by a matrix-assisted laser desorption ionization time of flight (MALDI–TOF) mass spectrometer (Autoflex, Brüker Daltonics, 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 analysed 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 well-characterized protein samples—namely insulin (5734.56 Da), ubiquitin (8565.89 Da), cytochrome c (12361.09 Da), and myoglobin (16952.55 Da).

2.5. 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, an aliquot (120 μl) of each lysozyme solution

(0.01 mg/ml) was added, followed by repeated inversions for 20 s. The enzymatic activity, expressed as per cent of the residual activities with respect to that of the native protein, was estimated as the initial velocity of the decrease in turbidity of the cells monitored at 700 nm with a UV-1200 spectrophotometer (Shimadzu, Japan) at 37 °C within 2 min.

2.6. Measurement of surface hydrophobicity (S_o)

The method described by Hayakawa and Nakai (1985) was employed to determine S_o of Lz and its derivatives, using 1-anilino-naphthalene-8-sulfonate (ANS) as a hydrophobic probe.

2.7. Fluorescence measurement

Fluorescence spectra of lysozyme samples (0.1%) were measured in Tris–HCl buffer (50 mM, pH 8) at an excitation wavelength of 280 nm with a spectrofluorometer (Shimadzu RF-150, Japan).

2.8. Circular dichroism (CD)

CD spectra of lysozyme samples at a concentration of 0.2 mg/ml in Tris–HCl buffer (50 mM, pH 8) were recorded at 200–240 nm with a spectropolarimeter (Jasco, J-720, Japan) using a 0.1-cm cuvette, maintained at 25 °C with a circulating water bath. The data were expressed as mean residue ellipticity.

2.9. Bacterial cultures

The bacteria used in this study were *Salmonella enteritidis* (IFO3313), *Escherichia coli* K2, *Proteus mirabilis* (IFO3849) and *Serratia marcescens* (IFO3046), which were obtained from the Institute for Fermentation (Osaka, Japan). The cultures were grown overnight in a medium containing 1% polypeptone, 0.5% leaven extract, 0.3% glucose, 1% sodium chloride, 0.1% $MgSO_4$–$7H_2O$, and 1.5% agar at pH 7±0.1 and 37 °C. The optical density of the growth was estimated at 600 nm with a UV-1200 spectrophotometer (Shimadzu, Japan). The cells were harvested by being washed with sodium phosphate buffer (PB, 10 mM, pH 7.2), and then resuspended in the same buffer to achieve an optical density of 1×10^5colony forming units (CFU)/ml.

2.10. Determination of antimicrobial activity

The above bacterial suspensions were incubated with an equal volume of each lysozyme solution at a final concentration of 0.2 mg/ml in PB (10 mM, pH 7.2) on a rotary shaker at 30 °C for 1 h. Having been diluted to a countable number of the bacteria in 0.15 M saline, a 100-μl aliquot of each mixture was streaked onto desoxycholate-hydrogen sulfide lactose agar (DHL) or tryptic soy agar (TSA) plates and incubated at 37 °C for at least 24 h before characteristic colonies were counted. Controls consisted of only water instead of the protein solutions. The antimicrobial activity was expressed as log (CFU of control/CFU of sample).

2.11. Enzyme-linked immunosorbent assay (ELISA)

Binding of Lz and its derivatives to *Salmonella enteritidis* (SE) treated with 0.2% formalin was determined using solid phase ELISA. Used as a solid support, microtitre plates (Greiner Co. Laboratory, USA) were coated with 100 μl/well of SE (10 μg/ml) in 0.1 M sodium carbonate buffer (pH 9.6) at 4 °C overnight. Controls containing only buffer were also carried out. After being washed three times with 0.1 M sodium phosphate buffered saline (PBS, pH 7.3) containing 0.05% (v/v) Tween 20 (PBS-T), the plates were incubated with 200 μl/well of 0.2% (w/v) bovine serum albumin (BSA) in PBS-T. The BSA solution was subsequently discarded and the plates were washed three times with PBS-T prior to addition of 100 μl/well of lysozyme solution serially diluted from 10 μg to 5 ng/ml. Following incubation at 37 °C for 1 h and three washes with PBS-T, 100 μl/well of rabbit anti-lysozyme IgG (1:500) were added and allowed to interact at 37 °C for 1 h, and then substituted by 100 μl/well of peroxidase-conjugated goat anti-rabbit IgG (1:10 000). Subsequent to incubation at 37 °C for 1 h, the plates were washed three times with PBS-T again, followed by addition of 50 μl/well of freshly prepared substrate solution (ABTS Peroxidase Substrate System, Kirkegaard and Perry Laboratory Inc., Gaithersburg, MD) to develop the colour reaction, the intensity of which was measured at 405 nm in a microplate reader (BIO-RAD, model 550, Tokyo, Japan).

2.12. Statistical analysis

The effects of reduction of the S–S bonds on the enzymatic activity, surface hydrophobicity, antimicrobial activity, and ELISA binding were assessed using the *t*-test. All analyses were carried out three times and the samples were treated alike. Significant differences were defined at the 5% level.

3. Results

NLz had a mass molecule of 14301 Da (Fig. 1), which agrees well with the theoretical molecular mass of 14300 Da measured with the four disulfide bonds intact (Loo, Edmonds, Udseth, & Smith, 1990). Upon 1.5 h of reduction, the molecular mass of Lz increased by 116 Da, indicating that one of the disulfide bonds was

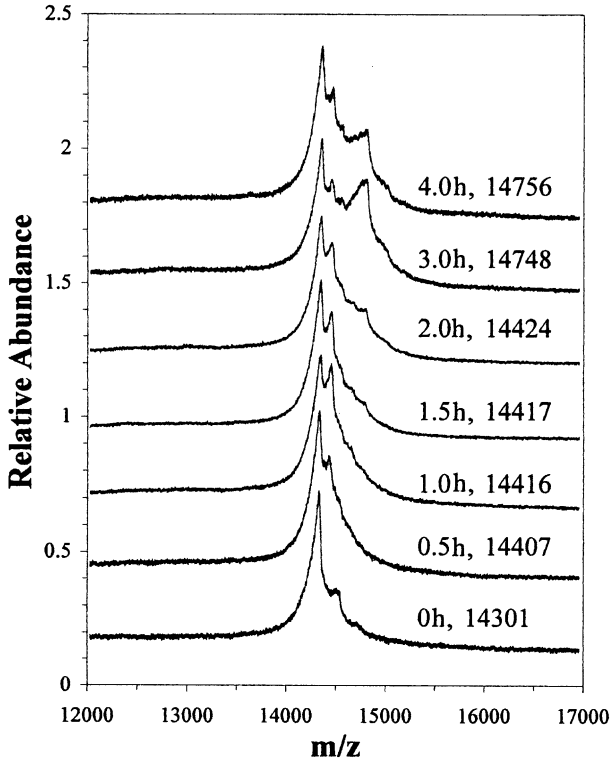

Fig. 1. Typical mass spectra of lysozyme in the native state and after reduction of the disulfide bonds at varying degrees. Lysozyme was reduced and carboxymethylated as described under Section 2. The average masses (expressed as Dalton) corresponding to each peak are shown with reducing times.

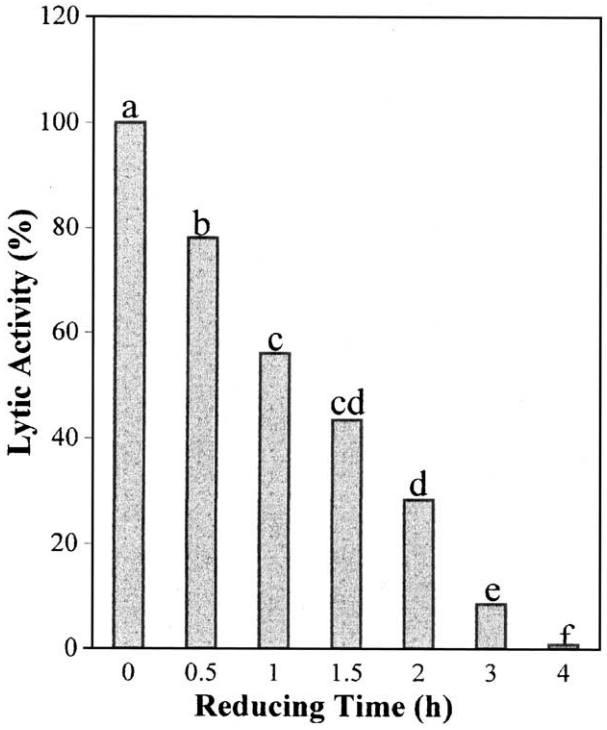

Fig. 2. Loss of the lytic activity of lysozyme as a function of reducing time. Substrate: *M. luteus*; lysozyme concentration: 0.01 mg/ml (120 μl). Means with different letters are significantly different ($P < 0.05$).

reduced by DTT; it was assumed that each reacted free SH group with IAM could increase its mass molecule by a fixed amount of 57 Da (Loo et al., 1990). It appeared that after one of the S-S bonds was released, other remaining S–S bonds were abruptly disrupted and the four S–S bonds were observed to be extensively, if not completely, reduced when the reaction times were extended to 3 and 4 h. It is noteworthy that the remaining appearance of the native peak was still discernible even when Lz was freely reduced with DTT in the presence of 6 M guanidine chloride (data not presented). This was attributable to the strongly charged molecular ions of Lz (Loo et al., 1990) and could be a reason why the peak of NLz was higher than that of RLz samples.

The enzymatic activity of Lz gradually decreased as a function of reducing time or residual disulfide bonds (Fig. 2), as a result of the disruption of the conformational structure forming the active site, due to unfolding of its molecule (Gilquin, Guilbert, & Perahia, 2000).

The S_o value of NLz was noticeably low (Fig. 3), because the accessibility of ANS to the hydrophobic residues, most of which are buried in the interior of the compact globular region (Hayakawa & Nakamura, 1986), was inhibited. It was, however, remarkable that after reduction of its S–S bonds with DTT, the S_o value

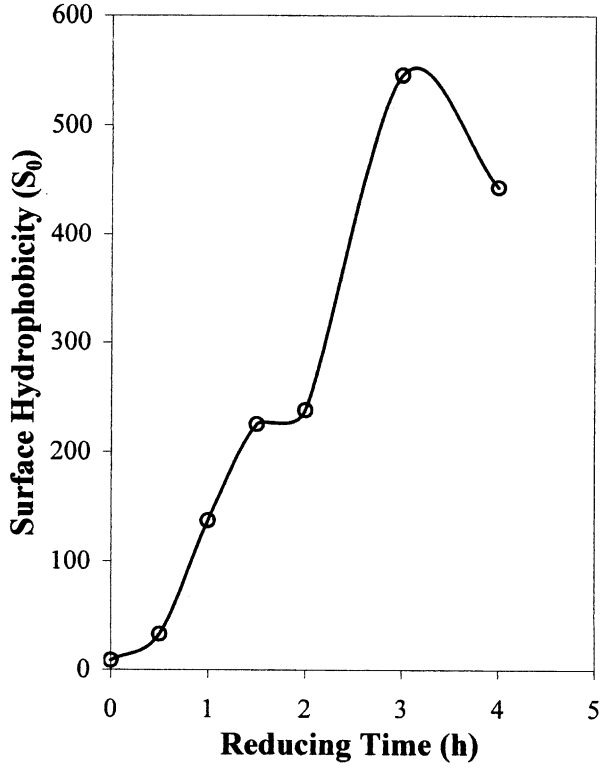

Fig. 3. Increased surface hydrophobicity of lysozyme upon reduction of the disulfide bonds with DTT. The hydrophobicity, measured by ANS assay, was calculated from the plot of fluorescence intensity against the protein concentrations (0.01–0.04%).

was found to considerably increase, reflective of exposure of hydrophobic amino acid residues buried in the folded structure (Gilquin et al., 2000). As observed, such an increase appeared to rely mainly on the levels of reduction, with RLz at 3 h having a substantially higher S_o value than that of other samples. The fact that RLz at 4 h showed a decreased S_o value suggests that upon extensive reduction of the S–S bonds, the hydrophobic sites were occluded, possibly as a consequence of an increased protein–protein interaction via hydrophobic association, the so-called hydrophobic collapse (Eyles, Radford, Robinson, & Dobson, 1994). This observation was reminiscent of previous data obtained for soy glycinin (Kim & Kinsella, 1986).

As shown in Fig. 4, all RLz samples exhibited increase of fluorescence intensity, along with an increase in the degree of reduction, with maximum wavelength shifting toward longer wavelength of 346 nm. The results were indicative of the conformational changes in the lysozyme molecule, presumably resulting from changes in the environment of the aromatic side chain chromophores; that is, some of the previously buried Trp residues, such as Trp-63,-111, and-123 could be exposed to a more polar environment, because of the dissociation of the native structure following reduction of the S–S bonds (Formoso & Forster, 1975).

Fig. 5 shows representative CD spectra of Lz before and after reduction of the S–S bond. While the CD spectra of RLz at 3 and 4 h were observed to deviate from that of NLz, the CD characteristics of NLz and RLz at 0.5, 1, 1.5, and 2 h were qualitatively similar. This implies that partially reduced lysozyme had the same backbone secondary structure as the native protein with a global loss of tertiary structure (data not shown), a feature characteristic of the molten-globule state (Ewbank & Creighton, 1991).

The effect of reduction of the S–S bonds of Lz on its antimicrobial activity is depicted in Fig. 6A–D. When tested against SE, all RLz samples showed greater sensitivity toward the organism than NLz, with the maximum activity being found after 1.5 h of reduction (Fig. 6A). The antimicrobial activity of RLz at 3 and 4 h, although still significantly higher ($P<0.05$) than that of NLz, was noticeably lower than that of partially reduced lysozyme (RLz at 1.5 h), suggesting that the partially reduced form was more bactericidal than its native and fully reduced ones. A comparison of the susceptibility of RLz toward SE and other bacterial strains, including *E. coli, P. mirabilis* and *S. marcescens*, showed that SE appeared to be more susceptible to RLz than other strains tested (Fig. 6B). The different responses, which could be ascribed to differences in the composition of the bacterial membranes (Braun, Rehn, & Wolff, 1970) that may influence the bactericidal action of RLz, imply that the bactericidal activity of RLz was strain-dependent. Also, RLz killed the sensitive bacteria

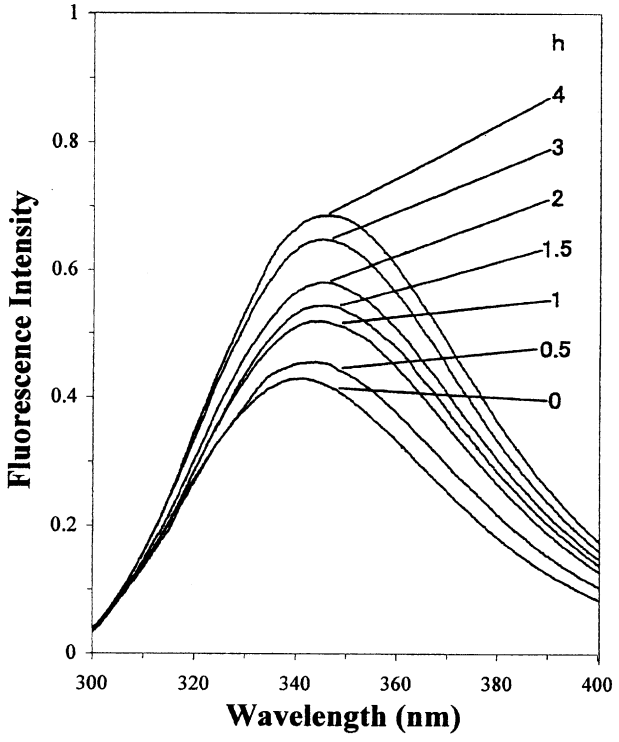

Fig. 4. Changes in fluorescence spectra of lysozyme upon reduction of the disulfide bonds with DTT. Lysozyme concentration: 0.1%. The fluorescence intensity was excited at 280 nm.

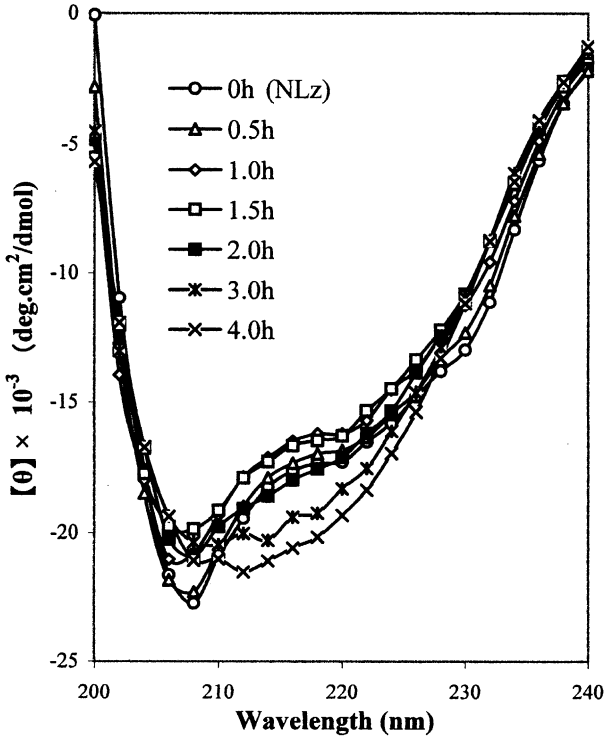

Fig. 5. Changes in CD spectra of lysozyme after reduction of its disulfide bonds with DTT. Lysozyme concentration, 0.2 mg/ml; pH 8.0; temperature, 25 °C.

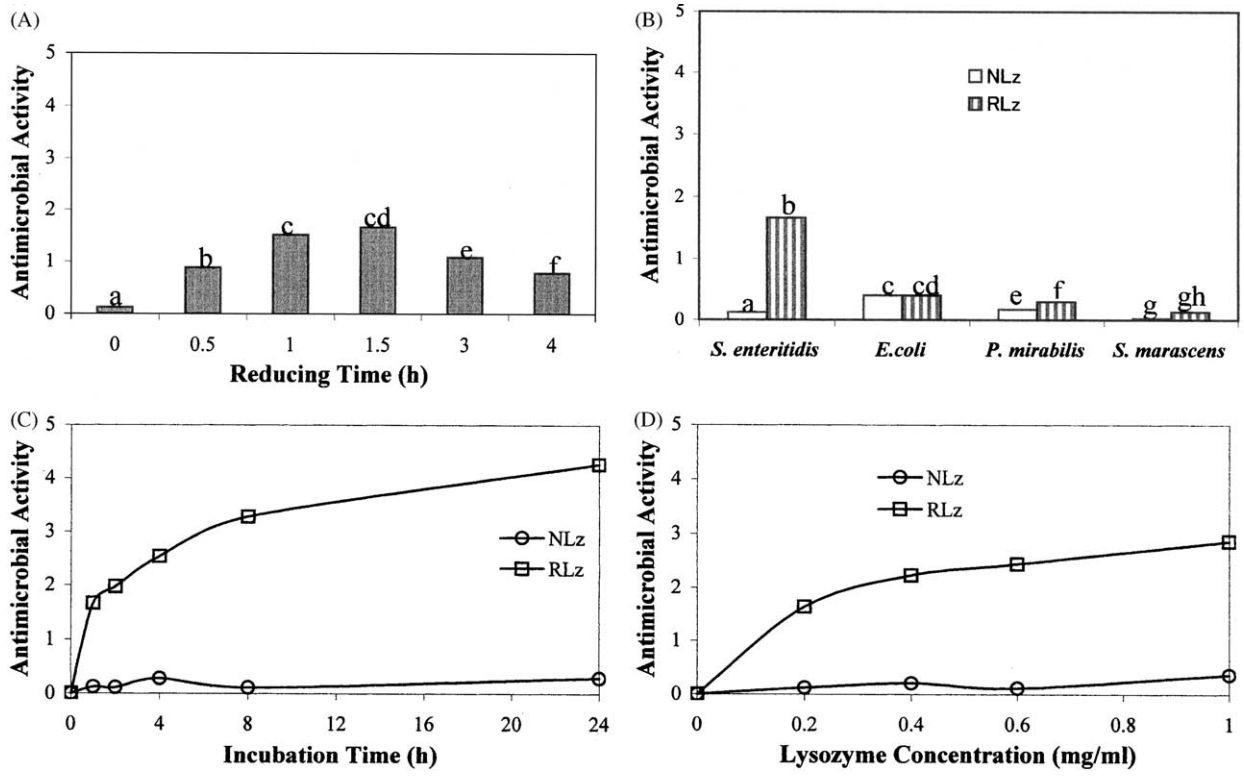

Fig. 6. Antimicrobial activity of lysozyme upon reduction of the S–S bonds against Gram-negative bacteria. (A) Antimicrobial activity against SE as a function of reducing time. (B) A comparison of the susceptibility of lysozyme and its reduced form against SE and other bacterial strains. Effects of incubation time (C) and protein concentration (D) on the antimicrobial activity of lysozyme and its reduced form against SE. The experimental conditions: the bacterial suspensions, 1×10^5 cfu/ml in PB (10 mM, pH 7.2); lysozyme concentration, 0.2 mg/ml; temperature, 30 °C; incubation time, 1 h; NLz, native lysozyme; RLz, reduced lysozyme at 1.5 h. Antimicrobial activity = log (cfu of control/cfu of sample). Means with different letters are significantly different at 5% level.

in a time-and dose-dependent manner; further prolonging the incubation time (Fig. 6C) and increasing the protein concentration (Fig. 6D) dramatically promoted its antimicrobial activity, while the activity of NLz was only slightly affected by these parameters.

Being highly sensitive to SE, RLz samples were tested for their interactions with the bacterial cells treated with formalin. The results, given in Fig. 7, demonstrate that all RLz samples had higher binding affinity to the SE surface than that of NLz. Since reduction of the S–S bonds of Lz was assumed to increase its surface hydrophobicity rather than other properties, this led to the hypothesis that the increased binding affinity of RLz to the bacterial cell surface was primarily assisted by the exposed hydrophobic regions derived from reduction of the S–S bonds.

4. Discussion

The data presented herein showed that reduction of the S–S bonds of Lz with DTT, despite causing a great reduction in its lytic activity (Fig. 2), not only retained but further promoted its antimicrobial activity toward Gram-negative bacteria, the extent of which was dependent upon the degrees of reduction and bacterial strains used (Fig. 6A and B). Our data thus tend to advocate another possible non-lytic mechanism of Lz, which has been challenged by some investigators (Laible & Germaine, 1985; Pellegrini et al., 1992; Ibrahim et al., 1996; Masschalck, Deckers, & Michiels, 2002)

Considering that the cationic and hydrophobic properties are critical for the antimicrobial action of Lz (Pellegrini et al., 1997), one would have expected an increase, to a certain extent, in its antimicrobial activity upon reduction of the S–S bonds, as the treatment could expose many of the hydrophobic groups (Fig. 3) and Trp residues (Fig. 4), previously buried in the compactly folded structure in its native state, to enable the molecule to interact with the bacterial membranes (Fig. 7). Nonetheless, the exposed surface hydrophobicity alone could not account for the promoted antimicrobial activity of RLz, because extensively or fully reduced lysozyme, notwithstanding having higher S_o values (Fig. 3) and binding affinity to the bacterial surface (Fig. 7), showed lower antimicrobial activity against SE than that of the partially reduced form (Fig. 6A). In other words, the conformational changes after reduction of the S–S bonds were also important for the bactericidal action of Lz. In this context, partially reduced

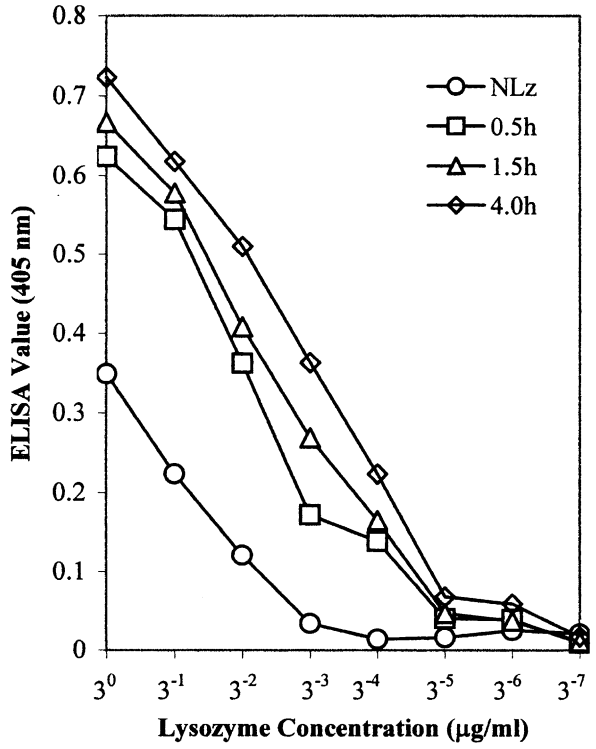

Fig. 7. Binding of lysozyme and its reduced forms to the SE surface. Lysozyme sample, 10 μg/ml (1/3 diluted in PBS-T); primary antibody, rabbit anti-lysozyme IgG (1:500); second antibody, peroxidase conjugated goat anti-rabbit IgG (1:10 000). Data are the mean of triplicate measurements with SD < 0.05.

lysozyme, which had unique properties distinct from native and fully reduced forms (Figs. 3 and 5), could serve as the best pathway for minimizing the energetic cost of making a compact conformation that was capable of inserting into the inner membrane (cytoplasmic membrane), where it could interact with negatively charged phosphate groups, because of its cationic nature (pI = 10.5). These findings were consistent with previously reported data for other proteins (Maher & Singer, 1986; Van der Goot, González-Mañas, Lakey, & Pattus, 1991).

Previously, Ibrahim et al. (1996) demonstrated that partially unfolded lysozyme (HLz), induced by heat treatment at 80 °C, was much more potentially bactericidal to a wide range of Gram-positive and negative bacteria than NLz, by virtue of the more hydrophobic dimeric form of the protein, whereas the bactericidal activity of RLz, reported herein, was strain-dependent (Fig. 6B). The discrepancy may be due to distinctions in the conformational changes of Lz prompted by the two treatments; heat-treatment causes the accumulation of chemical reactions in the Lz molecule, including isomerization of the Asp–Gly sequence, deamidation of the Asn residue, racemization of Asp and Asn residues, and cleavage of the Asp–X peptide bond (Tomizawa, Yamada, & Imoto, 1994), rather than influencing the stability of the disulfide bonds (Hayakawa & Nakamura, 1986; Li-Chan & Nakai, 1989; Volkin & Klibanov, 1987). Nevertheless, Masschalck et al. (2001) also found that the bactericidal action of denatured lysozymes, including HLz, was strain-specific.

The high sensitivity of RLz against SE would be advantageous for the food industry because the organism, to which antibiotics have been found to be resistant, due primarily to the widespread use of antibiotics in human and veterinary medicine (Threlfall, 2002), has recently been reported to be a serious concern as an emerging pathogen in a variety of food products, particularly poultry products (Murase et al., 2001). As such, RLz could be used as a potential antimicrobial agent against this strain, but applications in appropriate foods need to be validated, presumably on account of the toxicological properties of DTT used as the reducing agent. Hence, wider application is expected of RLz, using procedures that do not interfere with the organoleptic properties of foods. Understanding these minimal structural requirements, we are now investigating the possibility of using food additives as reducing agents so that RLz can be used for food applications.

5. Conclusion

Lysozyme molecular flexibility is restricted by the four intramolecular disulfide bonds which, when reduced by DTT and treated with IAM, resulted in loosened tertiary structure, thus exposing its hydrophobic regions. The exposed hydrophobic groups might favourably interact with and insert into the bacterial membranes. Partially reduced lysozyme, due to its unique structural properties, was more bactericidal than its fully reduced form toward certain bacteria.

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References

Anfinsen, C. B., & Haber, E. (1961). Studies on the reduction and reformation of protein disulfide bonds. *Journal of Biological Chemistry*, *236*, 1361–1363.

Bernkop-Schnüch, A., Krist, S., Vehabovic, M., & Valenta, C. (1998). Synthesis and evaluation of lysozyme derivatives exhibiting an enhanced antimicrobial action. *European Journal of Pharmaceutical Science*, *6*, 301–306.

Braun, V., Rehn, K., & Wolff, H. (1970). Supramolecular structure of the rigid layer of the cell wall of *Salmonella*, *Serratia*, *Proteus*, and *Pseudomonas fluorescens*. Number of lipoprotein molecules in a membrane layer. *Biochemistry*, *9*, 5041–5049.

Canfield, R. E., & Liu, A. K. (1965). The disulfide bonds of egg white lysozyme (muramidase). *Journal of Biological Chemistry*, *240*, 1997–2002.

Ewbank, J. J., & Creighton, T. E. (1991). The molten globule protein conformation probed by disulfide bonds. *Nature*, *350*, 518–520.

Eyles, S. J., Radford, S. E., Robinson, C. V., & Dobson, C. M. (1994). Kinetic consequences of the removal of a disulfide bridge on the folding of hen lysozyme. *Biochemistry*, *33*, 13038–13048.

Formoso, C., & Forster, L. S. (1975). Tryptophan fluorescence lifetimes in lysozyme. *Journal of Biological Chemistry*, *250*, 3738–3745.

Galat, A. (1985). Early stages in the trifluoroethanol-induced unfolding of hen egg-white lysozyme and its complex with $(GlcNAc)_3$. *Biochimica et Biophysica Acta*, *827*, 221–227.

Gilquin, B., Guilbert, C., & Perahia, D. (2000). Unfolding of hen egg lysozyme by molecular dynamics simulations at 300K: insight into the role of the interdomain interface. *Proteins*, *41*, 58–74.

Hayakawa, S., & Nakai, S. (1985). Relationships of hydrophobicity and net charge to the solubility of milk and soy proteins. *Journal of Food Science*, *50*, 486–491.

Hayakawa, S., & Nakamura, R. (1986). Optimization approaches to thermally induced egg white lysozyme gel. *Agricultural and Biological Chemistry*, *50*, 2039–2046.

Hughey, V. L., & Johnson, E. A. (1987). Antimicrobial activity of lysozyme against bacteria involved in food spoilage and foodborne disease. *Applied & Environmental Microbiology*, *53*, 2165–2170.

Ibrahim, H. R., Yamada, M., Matsushita, K., Kobayashi, K., & Kato, A. (1994). Enhanced bactericidal action of lysozyme to *Escherichia coli* by inserting a hydrophobic pentapeptide into its C-terminus. *Journal of Biological Chemistry*, *269*, 5059–5063.

Ibrahim, R. H., Higashiguchi, S., Koketsu, M., Juneja, R. L., Kim, M., Yamamoto, T., Sugimoto, Y., & Aoki, T. (1996). Partially unfolded lysozyme at neutral pH agglutinates and kills Gram-negative and Gram-positive bacteria through membrane damage mechanism. *Journal of Agricultural & Food Chemistry*, *44*, 3799–3806.

Ito, Y., Kwon, O. H., Ueda, M., Tanaka, A., & Imanishi, Y. (1997). Bactericidal activity of human lysozymes carrying various lengths of polyproline chain at the C-terminus. *FEBS Letters*, *415*, 285–288.

Joseph, M., & Nagaraj, R. (1992). Unfolding of lysozyme by breaking its disulfide bridges results in exposure of hydrophobic sites. *Biochemistry International*, *26*, 973–978.

Kato, A., Tanimoto, S., Muraki, Y., Oda, Y., Inoue, Y., & Kobayashi, K. (1994). Relationships between conformational stabilities and surface functional properties of mutant hen egg white lysozymes constructed by genetic engineering. *Journal of Agricultural & Food Chemistry*, *42*, 227–230.

Kim, S. H., & Kinsella, J. E. (1986). Effects of reduction with dithiotheitol on some molecular properties of soy glycinin. *Journal of Agricultural & Food Chemistry*, *34*, 623–627.

Laible, N. J., & Germaine, G. R. (1985). Bactericidal activity of human lysozyme, muramidase-inactive lysozyme and cationic polypeptides against *Streptococcus sanguis* and *Streptococcus faecalis*: inhibition by chitin oligosaccharides. *Infection & Immunity*, *48*, 720–728.

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

Liu, S. T., Sugimoto, T., Azakami, H., & Kato, A. (2000). Lipophilization of lysozyme by short and middle chain fatty acids. *Journal of Agricultural & Food Chemistry*, *48*, 265–269.

Loo, J. A., Edmonds, C. G., Udseth, H. R., & Smith, R. D. (1990). Effect of reducing disulfide-containing proteins on electrospray ionization mass spectra. *Analytical Chemistry*, *62*, 693–698.

Maher, P. A., & Singer, S. J. (1986). Disulfide bonds and the translocation of proteins across membranes. *Proceedings of the National Academy of Sciences of the USA*, *83*, 9001–9005.

Masschalck, B., van Houdt, R., van Haver, E. G. R., & Michiels, C. W. (2001). Inactivation of Gram-negative bacteria by lysozyme, denatured lysozyme, and lysozyme-derived peptides under high hydrostatic pressure. *Applied Environmental Microbiology*, *67*, 339–344.

Masschalck, B., Deckers, D., & Michiels, C. W. (2002). Lytic and nonlytic mechanism of inactivation of Gram-positive bacteria by lysozyme under atmospheric and high hydrostatic pressure. *Journal of Food Protection*, *65*, 1916–1923.

Murase, T., Senjyu, K., Maeda, T., Tanaka, M., Sakae, H., Matsumoto, Y., Kaneda, Y., Ito, T., & Otsuki, K. (2001). Monitoring of chicken houses and an attached egg-processing facility in a laying farm for *Salmonella* contamination between 1994 and 1998. *Journal of Food Protection*, *64*, 1912–1916.

Nishiyama, H., & Maeda, H. (1992). Reduced lysozyme in solution and its interaction with non-ionic surfactants. *Biophysical Chemistry*, *44*, 199–208.

Ohno, N., & Morrison, D. C. (1989). Lipopolysaccharides interaction with lysozyme. *Journal of Biological Chemistry*, *264*, 4434–4441.

Pellegrini, A., Thomas, U., von Fellenberg, R. F., & Wild, P. (1992). Bactericidal activities of lysozyme and aprotinin against Gram-positive and Gram-negative bacteria related to their basic character. *Journal of Applied Bacteriology*, *72*, 180–187.

Pellegrini, A., Thomas, U., Bramaz, N., Klauser, S., Hunziker, P., & von Fellenberg, R. (1997). Identification and isolation of a bactericidal domain in chicken egg white lysozyme. *Journal of Applied Microbiology*, *82*, 372–378.

Proctor, V. A., & Cunningham, F. E. (1988). The chemistry of lysozyme and its use as a food preservative and a pharmaceutical. *Critical Reviews in Food Science & Nutrition*, *26*, 359–395.

Roux, P., Delepierre, M., Goldberg, M. E., & Chaffotte, A. F. (1997). Kinetics of secondary structure recovery during refolding of reduced hen egg white lysozyme. *Journal of Biological Chemistry*, *272*, 24843–24849.

Sophianopoulos, A. J., Rhodes, C. K., Holcomb, D. N., & van Holde, K. E. (1962). Physical studies of lysozyme I. Characterization. *Journal of Biological Chemistry*, *237*, 1107–1112.

Threlfall, E. J. (2002). Antimicrobial drug resistance in Salmonella: problems and perspectives in food-and water-borne infections. *FEMS Microbial Reviews*, *26*, 141–148.

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

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

Volkin, D. B., & Klibanov, A. M. (1987). Thermal destruction processes in proteins involving cystine residues. *Journal of Biological Chemistry*, *262*, 2945–2950.

Yamada, H., Seno, M., Kobayashi, A., Moriyama, T., Kosaka, M., Ito, Y., & Imoto, T. (1994). An S-alkylating reagent with positive charges as an efficient solubilizer of denatured disulfide-containing proteins. *Journal of Biochemistry*, *116*, 852–857.