IUFoST 2006
DOI: 10.1051/IUFoST:20060308

Antimicrobial Activity of Duck Egg Lysozyme against *Salmonella enteritidis*

Naknukool, S., Hayakawa, S., Uno, T., Ogawa, M.
Department of Biochemistry and Food Science, Faculty of Agriculture, Kagawa University,
Ikenobe, Miki, Kagawa, Japan 761-0795
hayakawa@ag.kagawa-u.ac.jp

Abstract

The objective of the study was to investigate antimicrobial activity of native and reduced forms of duck lysozyme (dLz) against *Salmonella enteritidis*. Moreover, antimicrobial activity of dLz was compared with chicken lysozyme (cLz). The purified dLz was reduced with dithiothreitol. Free SH groups of reduced dLz were trapped with iodoacetamide. Lytic activity against *Micrococcus luteus* of reduced dLz decreased according to a period of reduction time. Whereas, reduction of dLz enhanced antimicrobial activity against *S. enteritidis* IFO3313 and other *S. enteritidis* found in contaminated food. The antimicrobial activity of reduced dLz depended on the time of reduction as 1.5 h reduction showed highest antimicrobial activity. Incubation of lysozyme with *S. enteritidis* at various temperatures showed that the optimum temperature for antimicrobial activity of reduced Lz is 45-49 ˚C. The presence of polyphosphate (0.05-0.10%) enhanced the antimicrobial effect of reduced dLz. On the other hand, addition of NaCl (0.05-0.10 M), glucose (10%), sucrose (10%) and bovine serum albumin (0.5-1.0%) decreased the antimicrobial activity of reduced dLz. Meanwhile, glycine (0-0.5%) did not show any effect on reduced dLz inhibitory activity. Combination of lactoferrin (0.1 mg/ml) and reduced dLz (0.1 mg/ml) showed a synergistic effect. In all conditions, dLz showed higher activity than cLz in both native and reduced forms. The result of the study indicated that reduced dLz trends to be a more efficient antimicrobial agent against *S. enteritidis*. To achieve the highest sufficiency, the essential roles of food components and incubation temperature should be concerned in reduced dLz application.

Keywords: *Duck lysozyme, Antimicrobial activity, Reduced lysozyme*

Introduction

A trend in non-chemical food preservation is alerted according to the demand of consumers who are concerned about health caring. Natural food antimicrobial agents such as lysozyme, nisin, lactoferrin and lactoperoxidase are widely used in the food industry to inhibit growth of microorganism including foodborne pathogens. Lysozyme (Lz) has been known as a basic protein in egg white, containing muramidase activity. It specifically hydrolyses the 1,4-β-linkages between N-acetyl muramic acid and N-acetylglucosamine in the glycan, which stabilizes cell walls of gram-positive bacteria. According to this effect, Lz strongly affects gram-positive bacteria but not much to gram-negative bacteria because of the composition of outer membrane. Many researchers attempt to enhance the antimicrobial activity of Lz against

gram-negative bacteria by using chemical or physical treatment to disrupt the bacteria membrane (Vannini et al., 2004), combining with other antimicrobial or/and chemical substances (Facon, 1996; Boland et al., 2003; Branen et al., 2004) and modifying the structure of Lz (Ibrahim et al., 1992; Liu et al., 2000; Mine et al., 2004; Hunter et al., 2005). Recently, reduction of Lz's disulfide bonds, exposing the hydrophobic surface of Lz, was introduced to improve the antimicrobial action of Lz against *S. enteritidis* (Touch et al., 2004)

Most of the reports on antimicrobial activity are about chicken Lz (cLz). Duck Lz (dLz) shows some differences in amino acid sequence. The molecule of dLz is stabilized with four disulfide bonds in the same position as cLz. dLz reveals enzymatic activity 1.34-1.53 time that of chicken Lz (cLZ). It contains 3 isoforms (DL-1, DL-2 and DL-3) which are different according to the displacement of Ser-37 to Gly, Gly-71 to Arg (DL-2 and DL-3) and Pro-79 to Arg (DL-3). The last two displacements induce the conformational change of dLz from β-turn to random coil (Prager et al., 1971; Hermann et al., 1973; Kondo et al., 1982).

In the present paper, we investigated the antimicrobial activity of dLz against *S. enteritidis* to create an alternative agent for food preservation. Furthermore, dLz was studied in various conditions for understanding in the effect of food components on antimicrobial activity of dLz.

Materials and Methods

Duck eggs were purchase from Agricultural, Food and Environmental Sciences Research Centre of Osaka Prefecture (Osaka, Japan). Hen egg white lysozyme, crystallized six times, and *Micrococcus luteus* cell were supplied by Seikagaku Kogyo Co. Ltd. (Tokyo, Japan). SP-Sepharose^{TM} Fast Flow, used for dLz purification, was purchased from Amersham Bioscience (New Jersey, USA). Dithiolthreiol (DTT) and iodoacetamide (IAM) were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Unless specified, all other chemicals used were reagent grade.

Duck lysozyme purification

Duck egg white was mixed gently with 3-fold volumes of sodium acetate buffer (ionic strength 0.05, pH 5.0). After centrifuged at 6000 × *g* for 15 min, the supernatant was applied to SP-Sepharose column and washed with sodium acetate buffer before eluted stepwise with 0.1, 0.3 and 0.5 M NaCl in sodium phosphate buffer (ionic strength 0.05, pH 9.0). The dLz containing fractions were collected and dialysed against Mili Q water for 3 days before freeze-drying for further experiment. The purity of dLz containing fractions was determined by SDS-PAGE in a 15% gel (Laemmli, 1970).

Reduction of Lysozyme

The 1 mg/ml Lz in 10 mM Tris-HCl buffer, pH 8.0 were reduced by 2 mM DTT at 30 ˚C for 0.5-4 h and treated with 5 mM IAM at 30 ˚C for 1 h in the dark. Reduced Lz were dialysed against Mili Q water for 3 days to remove salts and excess reagents before freeze-drying for further use.

IUFoST 2006
DOI: 10.1051/IUFoST:20060308

Lytic Activity Assay

Lytic activity of *Micrococcus luteus* was determined according to turbidometic method. Dried cells of *M. luteus* were suspended in 55 mM sodium phosphate buffer, pH 6.2 to the concentration of 0.4 mg/ml and stirred at 4 ˚C overnight. The decrease of absorbance at 700 nm, 37 ˚C was reported after the addition of bacteria suspension 2.88 ml and 0.12 ml of each reduce/non-reduced dLz solution (1 mg/ml) for 2 min. One unit of lytic activity is the amount of Lz that produce 0.001 decrease in absorbance per min. The activity is expressed in U/mg of protein.

Determination of Antimicrobial Activity

The bacteria that were used in this study were *S. enteritidis* IFO3313, obtained from NITE Biological Resource Centre (Chiba, Japan) and other strains from contaminated food, E991011, E990241, E990925, E990253 and E990579, obtained from National Institute of Infectious Diseases (Tokyo, Japan). They were cultured overnight at 37 ˚C in a medium containing 1% polypeptone, 0.5% yeast extract, 0.3% glucose, 1% NaCl, 0.1% $MgSO_4$-$7H_2O$, and 1.5% agar at pH 7.0. The cells were resuspended in 10 mM phosphate buffer pH 7.2, containing 0.15 mM NaCl before diluted with the same buffer to achieve the concentration of 10^5 CFU/ml as measured by the absorbance at 600 nm.

10^5 CFU/ml bacteria solution was incubated with reduced/non-reduced Lz at the final concentration of 0.1 mg/ml in 10 mM phosphate buffer, pH 7.2 at 30 ˚C for 1 h. Countable dilution of bacteria solution, diluted with saline water (0.15 mM NaCl), 100 µl was streaked onto desoxycholate-hydrogen sulfide lactose agar (DHL) and incubated at 37 ˚C for 24 h before examined for characteristic colonies. Controls were subject to the same treatment by using water instead of the protein solutions. The antimicrobial activity was expressed as log A_0/A_1 (A_0 = CFU/ml of control; A_1 = CFU/ml of sample). The experiment was done in triplicate.

The Effect of Incubation Temperature

0.1 mg/ml of reduced Lz was incubated with 10^5 CFU/ml *S. enteritidis* IFO 3313 at various incubation temperatures (30, 45, 47, and 49 ˚C) for 1 h. Then the antimicrobial activity of reduced Lz was determined as mentioned above.

The Effect of Food Component and Combination with Lactoferrin to Antimicrobial Activity of Duck and Chicken Lysozyme

The combination effects of 0.1 mg/ml reduced Lz with different kinds of food components were investigated. The food components used in this study are as follows: 0.05 and 0.1 M NaCl, 5 and 10% Glucose (Glu), 5 and 10% Sucrose (Suc), 0.1 and 0.5% Glycine (Gly), 0.5 and 1.0% BSA, 0.05 and 0.10% polyphosphate and 0.1 mg/ml lactoferrin. The mixtures of Lz and food components were incubated with 10^5 CFU/ml *S. enteritidis* IFO 3313 at 30 ˚C, for 1 h before determined the antimicrobial activity as described above. The effect of 0.50% glycine, 0.10% polyphosphate and 0.2 mg/ml lactoferrin were also studied by incubated in the same condition above.

Result and Discussion

The dLz was obtained by single-step purification, using ion exchange chromatography. After eluted with sodium phosphate buffer (ionic strength 0.05, pH 9), containing 0.1, 0.3 and 0.5 M NaCl, the result was as shown in Fig. 1. Peak 3 (P3) and Peak 4 (P4) presented on the same position (~14 kDa) on SDS-PAGE that related to the molecular mass of Lz. To identify these two bands, the N-terminal amino acid sequence was analysed and resulted in four amino acid sequences (KVYS) that completely same as reported. Thus, these two bands were confirmed as dLz. The difference of these two peaks may be due to the multiple forms of dLz that have been mentioned in previous works (Prager et al., 1971; Hermann et al., 1973; Kondo et al., 1982).

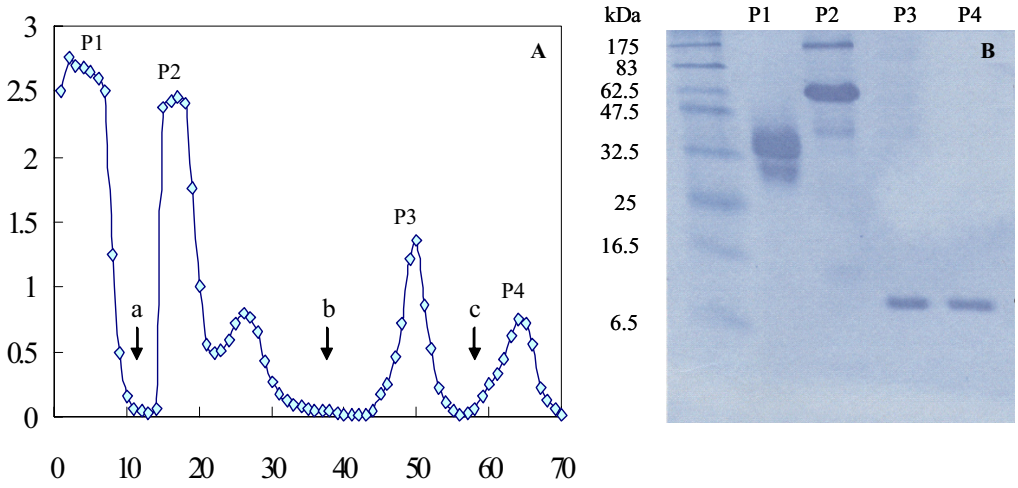

Figure 1. Chromatogram (A) and SDS-PAGE pattern (B) of duck lysozyme from SP-Sepharose column eluted with sodium carbonate buffer (ionic strength 0.05, pH 9.0), containing 0.1 (a), 0.3 (b) and 0.5 (c) M NaCl.

After being reduced by 2 mM DTT and trapped free sulfhydryl group with IAM, the lytic activity of dLz against *M. luteus* decreased as a function of reduction time (Fig. 2A). The reduction of SH groups results in the disruption of the conformation forming the active site, due to unfolding of its molecule which affect muramidase activity of Lz (Gilquin et al., 2000). On the other hand, the antimicrobial activity of dLz against *S. enteritidis* IFO3313 increased depending on reduction time (Fig. 2B). The reduction time of 1.5 h showed the highest antimicrobial activity against *S. enteritidis* IFO3313. In cLz, the exposure of hydrophobic region which is buried in the interior of the compact lysozyme molecule was reported after it was reduced by DTT (Touch et al., 2004). Increasing in exposed hydrophobic region is an important factor of binding affinity which can expand the spectrum of Lz antimicrobial activity against gram-negative bacteria, containing of the lipopolysaccharide at the outer membrane. In the reduction time over 1.5 h, the antimicrobial activity enhancement of the reduced Lz was slightly decreased. These may result from the protein-protein interaction, occurring via the hydrophobic association. The reduction time of 1.5 h was selected to be the condition of reduced Lz in following experiments.

IUFoST 2006
DOI: 10.1051/IUFoST:20060308

In comparison of the antimicrobial activity of native and reduced Lz (Fig. 3), reduced Lz of both species demonstrated stronger antimicrobial activity against *S. enteritidis* IFO3313 than native counterpart. The efficiency of reduced Lz against *S. enteritidis* from contaminated food was also investigated (Fig. 4). The result presented that reduced Lz showed wide antimicrobial efficiency against food-contaminating *Salmonella*. Moreover, reduced dLz possessed higher antimicrobial action against all *S. enteritidis* than cLz.

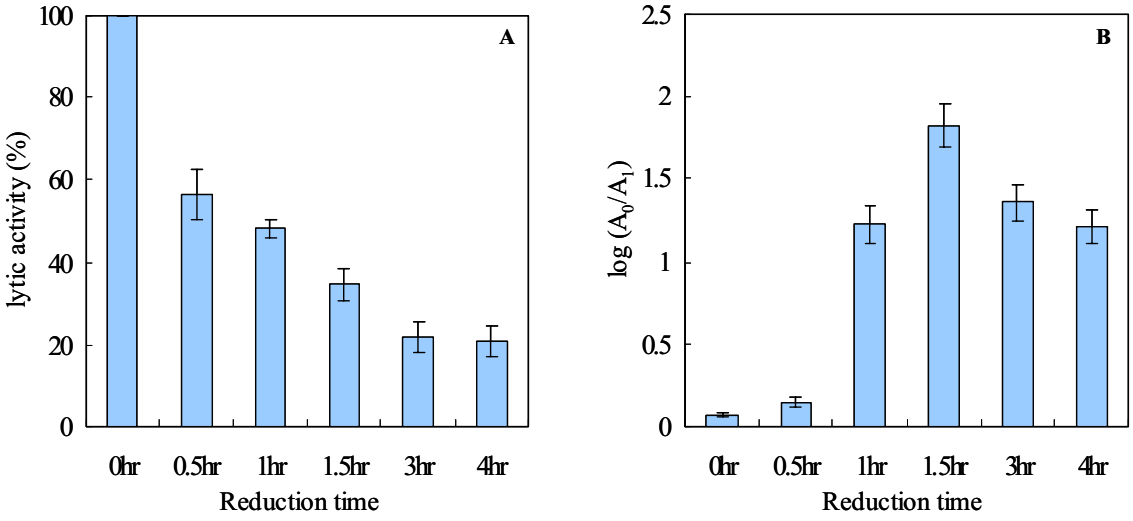

Figure 2. Effect of reduction time on (A) lytic activity against *M. luteus* and (B) antimicrobial activity against *S. enteritidis* IFO3313 of duck lysozyme. The experiment was done in triplicate. (A) Lysozyme 0.4 μ g/ml; *M. luteus* 0.4 mg/ml; temperature 37 °C; incubation time 2 min. (B) Lysozyme 0.1 mg/ml; *S. enteritidis* 10^5 CFU/ml; temperature 30 °C; incubation time 1 h.

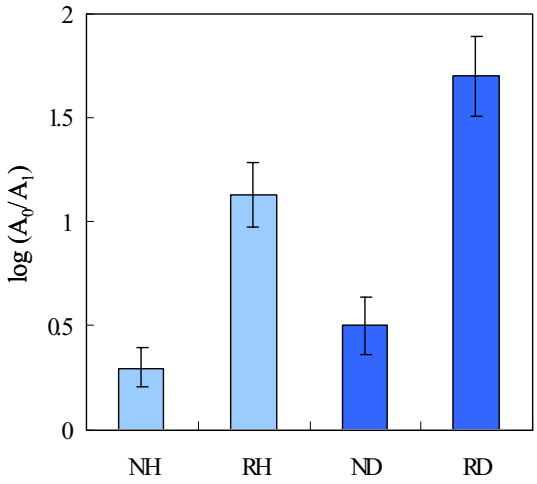

Figure 3. Antimicrobial activity of reduced / non-reduced lysozyme against *S. enteritidis* IFO3313. The experiment condition was reduced lysozyme 0.1 mg/ml; *S. enteritidis* IFO3313 10^5 CFU/ml; temperature 30 °C; incubation time 1 h. NH, native chicken lysozyme; RH, reduced chicken lysozyme; ND, native duck lysozyme; RD, reduced duck lysozyme.

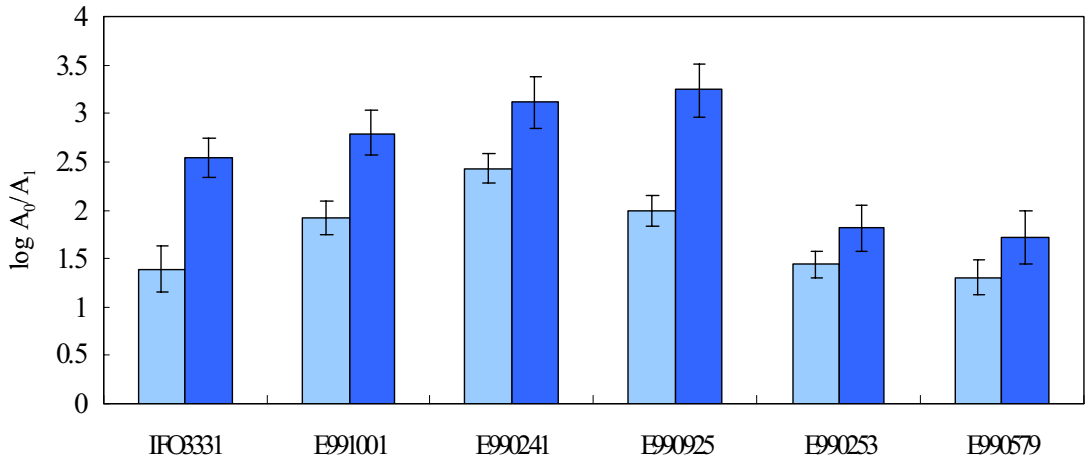

Figure 4. Antimicrobial activity of reduced lysozyme against several *S. enteritidis* contaminated in food. The antimicrobial activity was measured at reduced lysozyme concentration of 0.1 mg/ml; *S. enteritidis* 10^5 CFU/ml; temperature 30 °C; incubation time 1 h. Reduced chicken lysozyme (□); Reduced duck lysozyme (■)

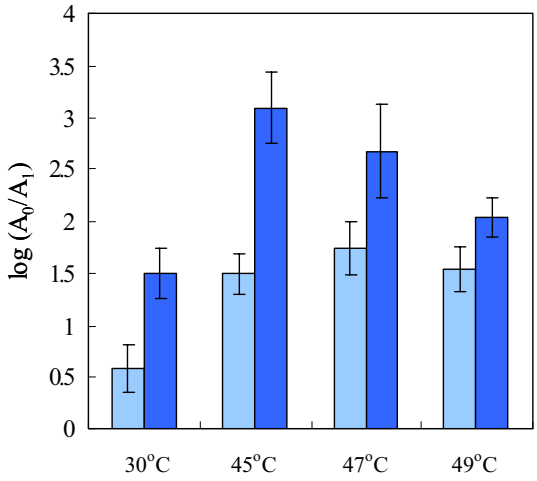

Figure 5. The effect of incubation temperature on antimicrobial activity of reduced lysozyme. The antimicrobial activity was measure at reduced lysozyme concentration of 0.1 mg/ml; *S. enteritidis* IFO 3313 10^5 CFU/ml; incubation time 1 h. Reduced chicken lysozyme (□), Reduced duck lysozyme (■)

Since there are many factors that can affect antimicrobial activity, the effect of incubation temperature and food components were studied. Studying in incubation time, at 45 ˚C reduced dLz showed the highest antimicrobial activity (Fig. 5). Meanwhile, reduced cLz was proposed the highest activity when incubated at 47 ˚C. It was assumed that the rising of incubation temperature induced the bacteria physiology to be less active and affected bacteria membrane to be more fluid, enhancing the diffusion of reduced Lz (Russell, 2002). Moreover, hydrophobic interaction is a temperature dependence reaction. Therefore, reduced Lz may penetrate into bacteria membrane easier at the high incubation temperature. Thus, 45-47 ˚C was appropriate incubation temperature for reduced Lz to be used as antimicrobial agent.

IUFoST 2006
DOI: 10.1051/IUFoST:20060308

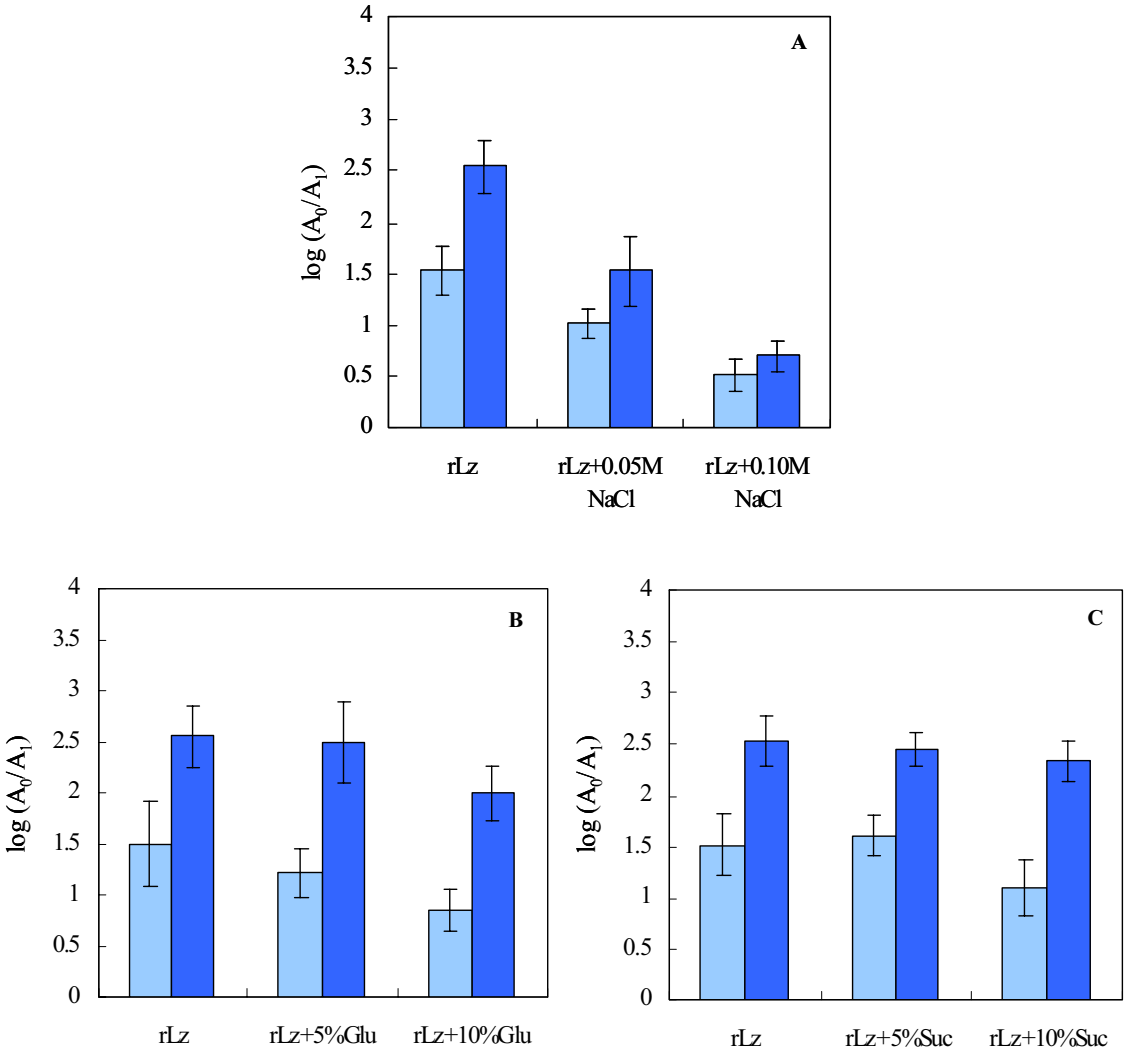

Figure 6. The effect of NaCl (A), glucose (B) and sucrose (C) on antimicrobial activity of reduced lysozyme. The measurement was performed at reduced lysozyme 0.1 mg/ml; *S. enteritidis* IFO3313 10^5 CFU/ml; temperature 30 °C; incubation time 1 h. rLz, reduced lysozyme; chicken lysozyme (□); duck lysozyme (■)

Salt or NaCl and sugar are widely used in the food industry as preservative agents, to impact sensory characteristics and to satisfy the human dairy requirement. According to the result of the effect of NaCl, it showed that addition of 0.05-0.1 M NaCl decreased the activity of reduced Lz to the growth of *S. enteritidis* IFO3313 (Fig. 6A). The bactericidal effect of nisin against *Listeria monocytogenes* is the highest at low (near 0%) or high NaCl concentration (near 6%), while between 2 and 4% NaCl, the activity of nisin is low (Bouttefroy et al., 2000). The bactericidal activity of heat-treated Lz at 80 ˚C for 20 min at pH 6.0, termed HL80/6, against both *E. coli* and *S. aureus* is abolished by 0.1% and 1% NaCl (Ibrahim et al., 1996). The difference in the effect of NaCl may be caused by the differences of antimicrobial agents and types of bacteria. The decrease in antimicrobial activity of reduced Lz may be due to the effect of monovalent cations which bind to the negatively charge head groups of the phospholipids in the cytoplasmic membrane and negative-charge of outer membrane as previously reported for divalent cations binding with cell membrane of

gram-positive bacteria (Abee, 1995). This interaction may prevent the attachment of reduced Lz to bacteria cell. Moreover, Na^+ ions may have stimulated the biological process at the membrane by increasing the rate of membrane synthesis to repair the damage caused by reduced Lz (Ibrahim et al., 1996).

At the concentration of 5 and 10% glucose and sucrose slightly decreased antimicrobial activity of reduced Lz (Fig.6B and C). In addition, sucrose was demonstrated an ability of decrease the effect of HL80/6 to *E.coli* when the concentration of sucrose reached to 1.5%, as reported in the previous study (Ibrahim et al., 1996). To be used as the foundational nutrition in bacteria growth, may explain the less effect of glucose and sucrose. However, small decrease in antimicrobial activity could be detected. The result may be from the increase in viscosity of the solution that influence to the attraction of reduced Lz and bacteria membrane.

Glycine and D-alanine are known as the essential components of bacteria cell that result in easier accessible approach into cells, making them to be interested as carrier molecules (Mishra et al., 2005). The result showed that glycine alone and its combination with reduced Lz possessed no effect on *S. enteritidis* (Fig. 7A). The effect of glycine up to 0.4% was not sufficient on the viability of either *S. aureaus* or *E. coli,* was reported (Ibrahim et al., 1996). However, the effect of combination between HL80/6 and glycine possessed the synergistic effect on both bacteria. This combination was sufficient in low-salt condition.

The addition of BSA affected the decrease in antimicrobial activity of reduced Lz as shown in Fig. 7B. As the incubation pH is 7.2, BSA (pI= 4.6) contains a negative charge that can bind to positive charge of Lz (pI=10.7). This electrostatic interaction may prevent reduced Lz to bind to bacteria membrane with negative charge, resulting in the reduction of antimicrobial sufficiency of reduced Lz. Furthermore BSA is the good source of nutrition that bacteria can use for recovery during the incubation time.

According to the experiment, polyphosphate alone possessed a small effect on *S. enteritidis* (Fig. 8A). Meanwhile, the combination with polyphosphate improved the antimicrobial activity of reduced Lz. Phosphate is known as chelating compound, widely used as food additive in meat product and processing. The chelation of structurally essential metal ions, Ca^{2+} and Mg^{2+}, in the cell walls, chelation of enzyme synthesis and inhibition of enzyme activity and changes in the water activity of the media increase the antimicrobial activity via improving the penetration property of antimicrobial compounds (Boland et al., 2003; Cutter et al., 1995; Vareltzis et al., 1997). Phosphate forms a metal-chelate ring by oxygen atoms founding the polyphosphate structure and metal ions. Thus, the disruption of polyphosphate to bacteria cell can increase the activity of reduced Lz against *S. enteritidis*.

IUFoST 2006
DOI: 10.1051/IUFoST:20060308

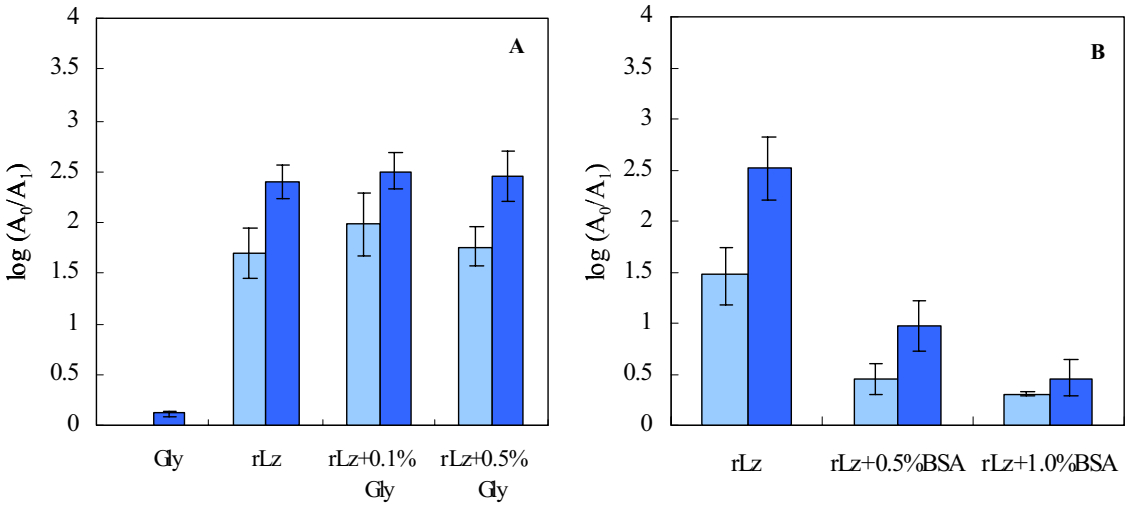

Figure 7. The antimicrobial effect of reduced lysozyme in the presence of glycine (A) and BSA (B). Reduced lysozyme 0.1 mg/ml; *S. enteritidis* IFO3313 10^5 CFU/ml; temperature 30 °C; incubation time 1 h. rLz, reduced lysozyme; chicken lysozyme (□); duck lysozyme (■)

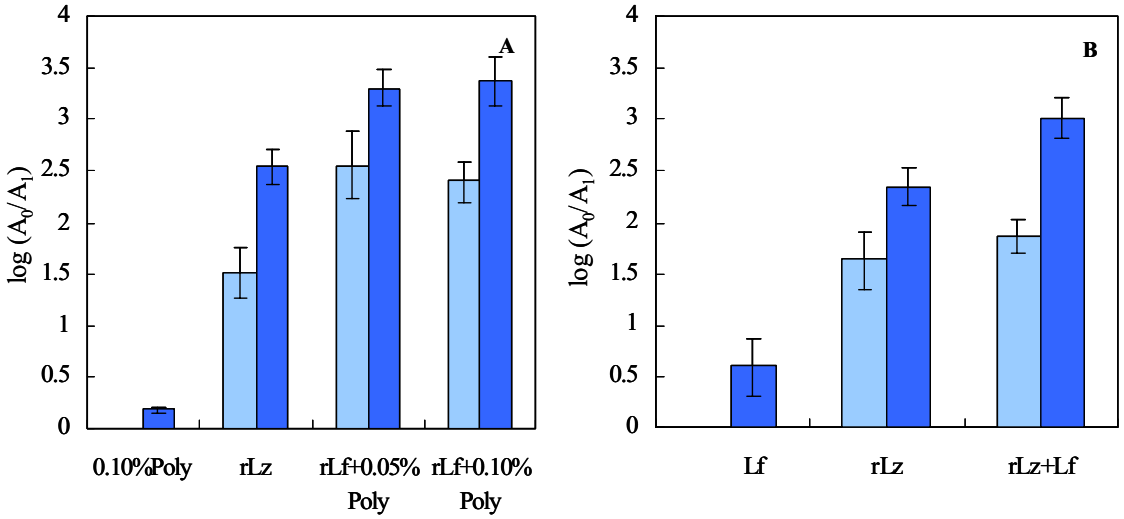

Figure 8. The effect of reduced lysozyme in the present of polyphosphate (A) and lactoferrin (B) on antimicrobial activity. Reduced lysozyme 0.1 mg/ml; *S. enteritidis* IFO3313 10^5 CFU/ml; temperature 30 °C; incubation time 1 h. rLz, reduced lysozyme; chicken lysozyme (□); duck lysozyme (■)

Lactoferrin, a glycoprotein, is found in milk and other exocrine secretions. It possesses an iron binding activity that can disrupt the structure of bacteria membrane. Not only that, the ability to release LPS from the outer membrane of gram-negative bacteria was reported recently (Ellison et al., 1991). The LPS-binding site is located on its N-terminal. Lactoferrin showed the synergistic effect in antimicrobial activity against *V. cholera*, *E. coli*, *S. typhimurium* and *S. enteritidis* when combined with native Lz, as reported (Elass-Rochard et al., 1995). It also showed the synergistic, when used connately with reduced Lz as showed in this study (Fig. 8B).

As observed, the antimicrobial activity of dLz in native and reduced form, in all conditions of experiment, showed higher activity against *S. enteritidis* than cLz. It may be due to the differences of amino acid composition between these two Lzs. Compared with cLz, the contents of arginine, valine and tyrosine residue dLz were higher by a few residues, while those of aspartic acid, alanine and phenylalanine residues were lower by two, one and two residues, respectively (Kondo, 1982). The difference of arginine content in both Lz was also presented in other report. They showed that in dLz contains 13-15 arginine residues, while only 11 residues consist in cLz (Prager et al., 1971; Hermann et al., 1973; Kondo et al., 1982). Arginine plays an important role in the antimicrobial agent. The cationic charges of arginine provide an effective means of attracting with negative charged surfaces such as LPS, teichoic acid, or phosphatidyl glycerol phospholipids head group. Moreover, arginine can form a complex with tryptophans via cation-π interaction which is favourable to penetrate into a lipid bilayer (Jing et al., 2003). The difference in arginine content may be responsible for high antimicrobial activity of dLz.

Conclusion

The high antimicrobial activity of reduced Lz against *S. enteritidis* was reported in this study. It is supposed that reduced dLz could be the alternative antimicrobial agent for food industry to supply the customers who are concerned about natural foods, as it proposed higher activity than reduced cLz. Also this study, investigated that food components of ingredients such as NaCl, sucrose, glucose and BSA have antagonism effects in antimicrobial activity of reduced Lz, while polyphosphate and lactoferrin promoted the efficiency of reduced lysozyme. In addition, Glycine did not have much effect. The effect of reduced duck lysozyme still needs more information about its activity in other bacteria and the effect of other food components.

Reference

Abee, T. Pore-forming bacteriocins of gram-positive bacteria and self protection mechanism of produces organisms. *FEMS Microbiol. Lett*. 1995, 129, 1-10

Boland, J. S.; Davidson, P. M.; Weiss, J. Enhanced inhibition of *Escherichia coli* 0157: H7 by Lysozyme and Chelators. *J. Food Prot*. 2003, 66(10), 1783-1789

Bouttefroy, A.; Mansour, M.; Linder, M.; Melliere, J. Inhibitory combinations of nisin, sodium chloride, and pH on *Listeria monocytogenes* ATCC 15313 in broth by an experimental design approach. *Int. J. Food Microbiol*. 2000, 54, 109-115

Branen, J. K.; Davidson, P. M. Enhancement of nisin, lysozyme and monolaurin antimicrobial activities by ethylenediamineteraacetic acid and lactoferrin. *Int. J. Food Microbiol*. 2004, 90, 63-74

IUFoST 2006
DOI: 10.1051/IUFoST:20060308

Cutter, C. N., Siragusa, G. R. Population reduction of gram negative pathogens following treatments with nisin and chelators under various conditions. *J. Food Prot*. 1995, 58, 977-983

Elass-Rochard, E.; Roseanu, A.; Legrand, D.; Trif, M.; Salmon, V.; Motas, C., Montreuil, J.; Spik, G. Lactoferrin-lipopolysaccharide interaction: involvement of the 28-34 loop region of human lactoferrin in the high affinity binding to *Escherichia coli* 055B5 lipopolysaccharide. *Biochem. J*. 1995, 312, 839-845

Ellison, R.T.; Giehl, T.J. Killing of gram-negative bacteria by lactoferrin and lysozyme. *J. Clin. Invest*. 1991, 88, 1080-1091

Facon, M. J.; Skura, B. Antibacterial activity of lactoferricin, lysozyme and EDTA against *Salmonella enteritidis*. *Int. Dairy J*. 1996, 6, 303-313

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

Hermann, J.; Jolles, J.; Jolles, P. The disulfide bridges of duck egg-white lysozyme II. *Arch. Biochem. Biophys*. 1973, 158, 355-358

Hunter, H. J.; Jing, W.; Shibli, D.; Trinh, T.; Park, I. Y.; Kim, S. C.; Vogel, H. J. The interactions of antimicrobial peptides derived from lysozyme with model membrane systems. *Biochim. Biophys. Acta*. 2005, 1668, 175-189

Ibrahim, H. R.; Kato, A. Design of amphipathic lysozyme using chemical and genetic modification to achieve optimal food functionality and diverse antimicrobial action In *Food proteins structure and functionality*; Schwenke, K. D.; Mothes, R., Eds; VCH Publishers, New York, 1992, 16-28

Ibrahim, H. R.; Higashiguchi, S.; Sugimoto, Y.; Aoki, T. Antimicrobial synergism of partially-denatured lysozyme with glycine: effect of sucrose and sodium chloride. *Food Res. Int*. 1997, 29(8), 771-777

Jing, W.; Demcoe, A. R.; Vogel, H. J. Conformation of a bactericidal domain of puroindoline : a structure and mechanism of action of a 13-residue antimicrobial peptide. *J. Bacteriol.* 2003, 185, 4938-4947

Kondo, K.; Fujio, H.; Amano, T. Chemical and immunological properties and amino acid sequences of three lysozyme from Peking-Duck egg white. *J. Biochem*. 1982, 91, 571-587

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 1970, 157, 105-132

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

Mine, Y.; Ma, F.; Lauriau, S. Antimicrobial peptides released by enzymatic hydrolysis of hen egg white lysozyme. *J. Agric. Food Chem*. 2004, 52, 1088-1094

Mishra, S.; Narain, U.; Mishra, R.; Misra, K. Design, development and synthesis of mixed bioconjugates of piperic acid-glycine, curcumin-glycine/alanine and curcumin-glycine-

piperic acid and their antibacterial and antifungal properties. *Bioorgan. Med. Chem*. 2005, 13 (5), 1477-1486

Prager, E. M.; Wilson, A. C. Multiple lysozymes of duck egg white. *J. Biol Chem*. 1971, 246(2), 523-530

Russell, N. J. Bacterial membranes: the effects of chill storage and food processing. An overview. *Int. J. Food Microbiol*. 1997, 79, 27-34

Touch, V.; Hayakawa, S.; Saitoh, K. Relationships between conformational changes and antimicrobial activity of lysozyme upon reduction of its disulfide bonds. *Food Chem*. 2004, 84, 421-428

Vannini, L.; Lanciotti, R.; Baldi, D.; Guerzoni, M.E. Interactions between high pressure homogenization and antimicrobial activity of lysozyme and lactoperoxidase. *Int. J. Food Microbiol*. 2004, 94, 123-135

Vareltzis, K.; Soultos, N.; Koidis, P.; Ambrosiadis, Genigeorgis, C. Antimicrobial effects of sodium tripolyphosphate against bacteria attached to the surface of chicken carcasses. *Lebensm.-Wiss. u. -Technol.* 1997, 30, 665-669