Prev. Nutr. Food Sci. 2018;23(1):60-69
https://doi.org/10.3746/pnf.2018.23.1.60
pISSN 2287-1098 · eISSN 2287-8602

Dextran-Conjugated Lysozymes Inhibit the Growth of *Shigella sonnei* and Viral Hemorrhagic Septicemia Virus.

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ABSTRACT: Lysozyme is well known as a natural antimicrobial agent, but its function is limited in that it only combats Gram-positive bacteria. We investigated the inhibitory effects of dextran-conjugated lysozymes (DLs) against some strains of Gram-negative bacteria and viral hemorrhagic septicemia virus (VHSV). The Maillard reactions of the DL were performed at various pHs (3.0, 7.0, and 8.5) in the presence of saturated KBr solution for 1, 3, 5, 7, and 9 days. The growth inhibition effects against Gram-negative bacterial strains, such as *Escherichia coli*, *Vibrio parahaemolyticus*, *Pseudomonas aeruginosa*, *Shigella sonnei*, and *Shigella flexneri* were found only in some DLs. DLs incubated at pH 7.0 for 9 days strongly inhibited growth of *S. sonnei* (92.9%). Fathead minnow (FHM) cells were infected with VHSV. DLs treated at all pHs for 1 day resulted in more than 80% viability of VHSV-infected FHM cells. The results of this study suggest that our DLs can be useful in food preservatives, pharmaceuticals, or aquatic feed due to their inhibitory effects against pathogenic bacteria and viral infections.

Keywords: denatured lysozyme, dextran, *Shigella sonnei*, viral hemorrhagic septicemia virus

INTRODUCTION

Lysozymes are well recognized as natural food preservatives. Lysozymes catalyze the breakage of the glycosidic linkage between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan in bacterial cell walls. Egg white lysozyme (EWL) is a stable protein with a molecular weight of 14.3 kDa consisting of 129 amino acid residues and four S-S bonds (1). EWL is the most abundant and easily obtainable source of lysozymes. It is a colorless, odorless, and tasteless preservative that does not affect food quality and is harmless to humans. Because of such properties, it has been used to preserve diverse types of foods, such as seafood, meat, fresh food, sausage, and hard cheese, and it is also used in some medicines (2-4). However, its antibacterial activity is limited to some Gram-positive bacteria, and it is not effective against Gram-negative bacteria (5). In order to improve its limited antimicrobial spectrum, attempts have been made to enhance its enzymatic activity by chemical reactions of lysozymes with nisin, sodium lactate, and ethylenediaminetetraactic acid. Studies have reported that structural modifications of lysozymes in a heating reaction improved the antimicrobial activity and functionality such as emulsification and solubility of lysozymes.

Bacterial dysplasia caused by *Shigella* has been reported to sicken more than 80～165 million people annually worldwide, causing more than 600,000 deaths (6). In Korea, the rate of infection decreased owing to the introduction of antibiotics and improvements in environmental sanitation in the 1950s. For the last two decades, however, the frequency of infection has increased rapidly, and there has been an increasing trend of mass outbreaks (7). Recently, the overuse of antibiotics has led to the emergence of antibiotic-resistant bacterial strains worldwide (8,9). The development of vaccines against *Shigella* has long been the goal of the World Health Organization (WHO), but there are no vaccines yet developed and various vaccines are in the study phase (10).

Viral hemorrhagic septicemia virus (VHSV) started in a trout farm in Europe in the 1980s and has been causing massive deaths in flounder farms in Japan and Korea since the 2000s. Especially in Korea, since 2006, the number of deadly incidents caused by viral diseases has

Received 15 November 2017; Accepted 11 January 2018; Published online 31 March 2018
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soared; the impact on the fishing industry reached 6,923 tons of fish in 2013, which represents a serious impact on aquatic resources (11). Although some vaccines are used to control VHSV-derived diseases, there is a problem with safety, and continuous research is required (12, 13). Studies on dextran-conjugated lysozymes (DLs) have not yet been conducted against pathogenic bacteria other than *Escherichia coli* and *Staphylococcus aureus*. Therefore, the purpose of this study was to characterize the chemical properties of DLs and expand their antimicrobial spectrum to Gram-negative bacteria and viruses.

MATERIALS AND METHODS

Preparation of DLs

Lysozyme and dextran from *Leuconostoc mesenteroides* (MW 9,000~11,000) were dissolved in 50 mM sodium acetate buffer (pH 3.0) or 50 mM sodium phosphate buffer (pH 7.0 and 8.5) at a ratio of 1:5, followed by drying using a freeze dryer. The lyophilized product was incubated in a 10 L volumetric desiccator containing 200 mL of saturated KBr solution (67.8%, w/v) at a relative humidity of 79% and 60°C for 1~9 days. After the reaction was completed, the product was lyophilized, pulverized, and used as DL. Lysozyme was heated under the same reaction condition without dextran for heat-treated lysozymes (HLs), and all samples were stored at −20°C.

Determination of binding rate

The number of free amino groups in the dextran-lysozyme conjugate was determined by modifying the trinitrobenzene sulfonic acid method (14). The sample was dissolved in 0.1 M sodium bicarbonate buffer (pH 8.6) at a concentration of 100 μg/mL, and 0.25 mL of 0.01% (w/v) 2,4,6-trinitrobenzene sulfonic acid (TNBSA) was added to 0.5 mL of the sample solution. After reacting at 37°C for 2 h, 0.25 mL of 10% sodium dodecyl sulfate (SDS) and 0.125 mL of 1 N HCl were added successively, and the absorbance was measured at 335 nm. The binding rate (%) was determined by the following equation:

$$\text{Binding rate (\%)} = \frac{\text{Control } A_{335} - \text{Sample } A_{335}}{\text{Control } A_{335}} \times 100$$

Lysozyme activity

The activity of the conjugates was determined by modifying the lysozyme assay as described in the Worthington Enzyme Manual (15). A pre-incubated sample solution (0.05 mL of 1 mg/mL) and 1.45 mL of 0.3 mg/mL *Micrococcus lysodeikticus* cells (Sigma-Aldrich Co., St. Louis, MO, USA) as a substrate at 25°C were mixed, and its absorbance was measured at 450 nm for 10 min using a spectrophotometer (Cary 60 UV-Vis, Agilent Technologies Inc., Santa Clara, CA, USA). The unit calculation (Unit/mg) of the enzyme is as follows.

$$\text{Units/mg} = \frac{\Delta A_{450}/\text{min} \times 1{,}000}{\text{mg enzyme in mixture}}$$

Measurement of solubility

The solubility of DL was determined using a method modified from that published by Abtahi and Aminlari (16). One mg of DL was completely dissolved in a different buffer using 0.1 M sodium acetate buffer (pH 3.0 and 5.0) and 0.1 M sodium phosphate buffer (pH 7.0 and 9.0) and centrifuged at 27,000 *g* for 15 min. The level of protein in the supernatant was quantified by the method of Bradford (5). Briefly, 10 μL of the supernatant was reacted with 200 μL of Coomassie Brilliant Blue G-250 for 10 min, and its absorbance was measured at 595 nm. Bovine serum albumin was used as a standard. The solubility of denatured lysozymes was determined by the following equation:

$$\text{Protein solubility (\%)} = \frac{\text{Protein in supernatant}}{\text{Total protein}} \times 100$$

Measurement of emulsion property

The emulsifying properties of samples were determined by a modified method put forth by Song et al. (17). One milliliter of purified corn oil (Sajo Co., Ltd., Seoul, Korea) was mixed with 3 mL of 0.1 M sodium phosphate buffer (pH 7.4) in a 15 mL conical tube, and the sample was added such that the protein concentration was 0.1%. After homogenization for 2 min at 25°C and 12,000 rpm in a homogenizer (HG-15D, Daihan Scientific Co., Ltd., Wonju, Korea), 50 μL of the emulsion in the tube bottom was immediately added to 5 mL of 0.1% SDS solution for 0~15 min, and then diluted to 1/1,000. The diluted emulsion was used to measure the absorbance at 500 nm using a spectrophotometer. The absorbance value of the first diluted emulsion (0 min) was expressed as emulsion activity (EA, A_{500} nm), and the time when the absorbance value of the first emulsion was halved was expressed as emulsion stability (ES, min).

Measurement of surface hydrophobicity

The surface hydrophobicity of samples was determined by modifying the method of Touch et al. (18). Samples were dissolved in 0.01 M sodium phosphate buffer (pH 7.2) and diluted to 0.01~0.04%. The diluted solution (3 mL) was added to 15 μL of 8 mM 1-anilino-8-naphthalene sulfonic acid (ANS). The fluorescence intensity was measured at an excitation wavelength of 390 nm and an absorption wavelength of 470 nm using a fluorescence spectrophotometer (Spectra max M2, Molecular Devices,

Sunnyvale, CA, USA). The fluorescence intensity was calculated by subtracting the fluorescence intensity of proteins without ANS, and the slope of the pure fluorescence intensity for each protein concentration was obtained and used as an index of surface hydrophobicity.

Measurement of antibacterial effects

Antibacterial activity was measured by modifying the method of Brandt et al. (19). *E. coli* (KCTC 1682), *Vibrio paraheamolyticus* (KCTC 2729), *Pseudomonas aeruginosa* (KCTC 1750), *Shigella sonnei* (KCTC 2518), and *Shigella flexneri* (KCTC 22192) were purchased from the Korean Collection for Type Cultures (Daejeon, Korea). All samples (10 mg/mL) were dissolved in 50 mM potassium phosphate buffer (pH 7.0) and filtered through a sterilized 0.2 μm syringe filter (Millipore, Temecula, CA, USA). The bacterial cells were incubated in brain heart infusion broth (Oxoid Ltd., Basingstoke, England) for *E. coli*, *P. aeruginosa*, *S. sonnei*, and *S. flexneri*, and tryptic soy broth (17 g/L of pancreatic digest of casein, 3 g/L of papaic digest of soybean, 2.5 g/L of dextrose, 5 g/L of sodium chloride, and 2.5 g/L of dipotassium phosphate) with 3% NaCl for *V. parahaemolyticus* at 37°C for 16 h, diluted with 200 μL of a liquid medium inoculated at about 10^6 CFU/mL, and 100 μL of the samples were mixed with the bacteria in 96-well plates. Sterilized liquid medium (100 μL) was used as a control, and samples were cultured at 37°C for 24 h. Using a microplate reader, the absorbance values were compared at 600 nm during the incubation period, and data is expressed as growth inhibition rate (%).

$$\text{Growth inhibition rate (\%)} = \left(1 - \frac{\text{Rxn. mix. with antimicrobial agent } \Delta A_{600}/\text{h}}{\text{Rxn. mix. without antimicrobial agent } \Delta A_{600}/\text{h}}\right) \times 100$$

Measurement of cell survival rate for VHSV infection of dextran conjugated lysozymes

Fathead minnow (FHM) cells, a type of fish cell, and viral hemorrhagic septicemia virus (VHSV, Genogroup IVa), a virus found in Japanese flounder fish, were obtained from the laboratory of Professor Kim of Chonnam National University. FHM cells were cultured in Leibovitz's L-15 (L-15_{10}) media containing 10% fetal bovine serum (FBS, Welgene Inc., Gyeongsan, Korea) and penicillin-streptomycin (100 U/mL) at 20°C. Virus at $10^{8.8}$ $TCID_{50}$/mL was stored at −80°C and used after dilution in Leibovitz's L-15 media (L-15_2) supplemented with 2% FBS.

Cytotoxicity and cell viability were measured using the method of Chavez et al. (20). FHM cells (100 μL, 1.0×10^5 cells/mL) were inoculated in sterile 96-well plates and cultured at 20°C for 24 h. After the medium was removed, DL was diluted in L-15_{10} at concentrations of 1 and 10 mg/mL, filtered through a sterilized 0.2 μm syringe filter, and cells were treated with 100 μL of each for 24 h. After that, the treatment solution was removed and 50 μL of L-15_2 medium and 50 μL of L-15_{10} medium were mixed together, and the cells were cultured for another 41 h. To measure cell viability, VHSV diluted in 100 μL of L-15_2 medium and L-15_{10} medium (1:1) was added to the cells for 41 h. The reactants for cytotoxicity and viability measurements were then reacted with 10 μL of a cell counting kit-8 reagent (Dojjindo Molecular Technologies Inc., Rockville, MD, USA) at 20°C for 8 h. Absorbance was measured at 450 nm using a microplate reader.

Statistical analysis

The results are expressed as the mean±standard deviation of at least three independent experiments. All results were statistically analyzed using the one-way ANOVA function in Minitab 16.0 software (Minitab Inc., State College, PA, USA), and significant differences between means were tested using Tukey's test ($P<0.05$).

RESULTS AND DISCUSSION

Formation of DLs

The aldehyde-type carbonyl group of the reducing sugar and the amino group of the amino acid or protein cause a Maillard reaction at temperatures above 50°C and moderate humidity. Most of the covalent bond formation occurs during the initial stage of the Maillard reaction (21). In this study, a covalent bond was formed by reacting the carbonyl group of dextran and the amino group of a lysozyme at different pHs and reaction times at 60°C and 79% relative humidity. When we measured the free amino group binding ratio of DLs, we observed that the binding rate increased as the reaction time became longer (Fig. 1). All DLs reacted during the first day and showed a binding rate of 40% or more. This indicates that the initial stage of the Maillard reaction, in which a covalent bond in DLs forms, occurred within one day. The free amino groups of the conjugates reacted at pH 3.0 were significantly greater than those reacted at other pHs. It can be inferred that the DL exhibits a higher binding rate under acidic conditions as the reaction time becomes longer. The Maillard reaction is known to differ in its reaction mechanisms under acidic, neutral, and alkaline conditions, and the reaction is generally increased in proportion to an increase in pH (22). However, the optimum pH range of the reaction depends on the reactants, and no definite study on the topic has yet been conducted (23). Most of the studies in which lysozymes and polysaccharides were coupled using the Maillard reaction revealed that one lysozyme molecule binds to two polysac-

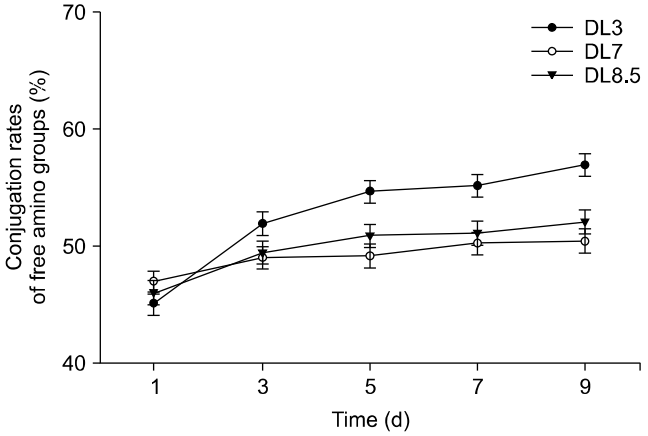

Fig. 1. Conjugation rates of amino groups of the dextran-conjugated lysozyme. DL, dextran-conjugated lysozyme at pH 3.0, 7.0, and 8.5.

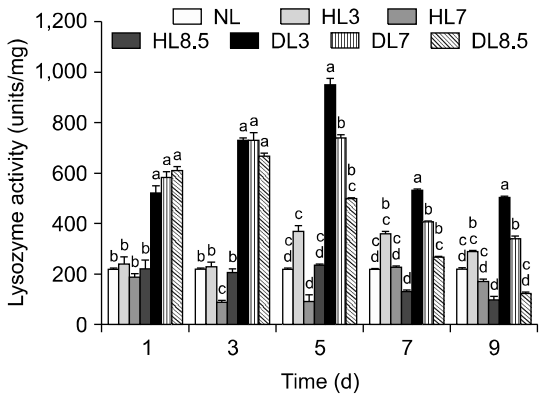

Fig. 2. Lysozyme activity of the heat denatured and the dextran-conjugated lysozyme. NL, native lysozyme; HL, heat denatured lysozyme without dextran at pH 3.0, 7.0, and 8.5; DL, dextran-conjugated lysozyme at pH 3.0, 7.0, and 8.5. The different letters (a-d) are significantly different at $P<0.05$.

charide molecules (24-26). However, our findings were similar to the findings of Scaman et al. (27) that 1～3 dextran molecules bind to one lysozyme molecule. Yeboah et al. (28) found that a lysozyme binds to glucose or fructose at the ε-amino groups of Lys-33 in addition to Lys-1 and Lys-98. Likewise, our DLs are expected to bind at Lys-1, Lys-98, and Lys-33.

Lysozyme activity of DLs

Most dextran-conjugation conditions increased lysozyme activity (Fig. 2). In the heat treatment groups, only HL3 was higher in enzyme activity than native lysozymes. Lysozyme activity was highest in DL reacted at pH 3.0 for 5 days, and lowest in DL reacted at pH 8.5 for 9 days. During the heat treatment of lysozymes, the folded state changes to an unfolded state. Unfolded polypeptides have a strong tendency to re-fold into the existing α-helix structure. During this process, an intermediate is formed that is accompanied by the formation of the protein's secondary structure during refolding (29-31). Under neutral conditions, refolding is a three-step process (unfolded structure－intermediate－refolding), whereas in acidic conditions, the reaction is explosive and takes part in a two-step process that does not involve an intermediate, resulting in increased enzymatic activity (32). These results support the results showing increases in enzymatic activity of DL and HL reacted at pH 3.0. Similarly, Lesnierowski et al. (33) reported that the activity of lysozymes heated under acidic conditions was higher than that of lysozymes reacted under neutral or alkaline conditions. The reason for the increase in enzymatic activity of DL is believed to be that the hydrophobicity caused by the heating reaction improves substrate adhesion and then catalyzes the enzymatic reaction, and that the peptide produced by the Maillard reaction may be synergistic with the DL. Studies have been conducted on the synergistic effect that occurs when the constituent peptides, which are altered according to Maillard reaction time, are mixed with lysozymes to improve enzymatic activity (28, 29).

Solubility of DLs

The solubility of HL and DL is shown in Table 1. After the heating reaction, both DL and HL showed a tendency to decrease in solubility compared to the natural lysozyme (NL). This may have resulted from an increased hydrophobicity and a strong positive charge owing to the formation of succinimidyl residues (34). In particular, HL exhibited a significant decrease in solubility of 2.36～4.87% in acidic conditions (pH 3.0～5.0), while DL showed a relatively high solubility of 13.27～57.66%. These results show that the Maillard reaction produces substances with high solubility under acidic conditions. In addition, binding of dextran improves solubility by increasing the capacity of the water molecules in the lysozyme (35). Increased solubility, caused by the combination of a lysozyme and polysaccharides, has been reported by many researchers (36-41).

Emulsifying property of DLs

The EA and ES of DL are shown in Table 2. NLs and dextran showed low EA and ES. HL did not show increased EA compared to NLs, but DL showed significantly increased EA and ES. Especially, the EA of DL7 reacted for 9 days was the highest (2.41), and the ES of DL7 reacted for 5 days was the highest (13.18 min). It has been shown that hydrophobic residues of DL are immobilized on oil droplets during emulsion formation, while polysaccharides promote the formation of thick steric stabilization layers around the emulsion that inhibit the adhesion of oil droplets and improve EA (42). In similar studies, Nakamura et al. (24) increased EA to 1.19 by binding of ovalbumin-galactomannan and Song et al. (17) improved EA to about 1.6 by combining chitosan and

Table 1. Solubility of the heat denatured and the dextran-conjugated lysozyme

pH value	Sample	Incubated time (d)				
		1	3	5	7	9
3	NL	49.34±1.03^{A}	49.34±1.03^{B}	49.34±1.03^{A}	49.34±1.03^{A}	49.34±1.03^{A}
	HL3	4.24±0.45^{Da}	3.21±0.44^{Db}	2.54±0.22^{Ec}	2.36±0.19^{Cc}	3.14±0.47^{Cb}
	HL7	3.31±0.15^{Dc}	4.32±0.63^{Db}	2.64±0.08^{Ed}	3.28±0.23^{Cc}	4.87±0.25^{Ca}
	HL8.5	3.75±0.31^{Db}	4.13±0.20^{Da}	2.84±0.13^{Ec}	2.37±0.31^{Cd}	2.93±0.06^{Cc}
	DL3	15.65±0.18^{Cc}	55.53±2.70^{ABa}	14.67±0.21^{Dc}	24.84±0.22^{Bb}	18.95±0.01^{Bc}
	DL7	19.51±0.34^{BCb}	57.66±0.02^{Aa}	25.04±0.63^{Cb}	20.67±0.52^{Bb}	24.74±0.30^{Bb}
	DL8.5	24.06±0.07^{Babc}	16.62±0.27^{Cc}	30.90±0.36^{Ba}	17.60±0.17^{Bbc}	25.58±0.07^{Bab}
5	NL	46.83±0.85^{A}	46.83±0.85^{B}	46.83±0.85^{A}	46.83±0.85^{A}	46.83±0.85^{A}
	HL3	4.24±0.46^{Da}	3.21±0.45^{Db}	2.54±0.23^{Eb}	2.36±0.19^{Db}	2.83±0.37^{Cb}
	HL7	3.31±0.15^{Db}	4.32±0.65^{Da}	2.64±0.08^{Ec}	3.28±0.23^{Db}	4.37±0.20^{Ca}
	HL8.5	3.75±0.32^{Da}	4.13±0.21^{Da}	2.84±0.14^{Eb}	2.37±0.32^{Db}	2.64±0.05^{Cb}
	DL3	14.14±0.14^{Cd}	49.61±2.15^{ABa}	13.27±0.17^{Dd}	22.74±0.18^{BCc}	28.69±0.01^{Bb}
	DL7	17.57±0.27^{BCc}	53.47±0.02^{Aa}	22.49±0.50^{Cc}	19.13±0.42^{Cc}	33.71±0.35^{Bb}
	DL8.5	21.62±0.06^{Bbc}	15.00±0.22^{Cc}	27.71±0.29^{Bab}	27.52±0.23^{Bab}	34.43±0.08^{Ba}
7	NL	78.18±0.75^{A}	78.18±0.75^{A}	78.18±0.75^{A}	78.18±0.75^{A}	78.18±0.75^{A}
	HL3	41.87±2.12^{Ca}	41.25±1.18^{BCa}	24.35±0.17^{Fb}	25.81±0.29^{Eb}	0.90±0.02^{Ec}
	HL7	41.73±1.07^{Cb}	45.26±0.98^{Ba}	41.92±1.10^{Cb}	34.83±0.90^{Cd}	40.04±1.36^{Bc}
	HL8.5	40.91±0.99^{Cb}	44.36±0.38^{Ba}	34.55±0.86^{Ec}	29.94±1.13^{Dd}	32.03±0.37^{Cd}
	DL3	76.58±0.27^{Aa}	23.79±0.06^{Eb}	−5.55±0.01^{Ge}	16.28±0.06^{Fd}	21.00±0.06^{Dc}
	DL7	63.06±0.74^{Ba}	37.13±0.26^{CDc}	37.50±0.17^{Dc}	38.87±0.18^{Bc}	42.57±0.13^{Bb}
	DL8.5	63.63±0.30^{Ba}	34.57±0.20^{Dc}	45.15±0.61^{Bb}	32.59±0.11^{CDc}	36.91±0.21^{BCc}
9	NL	99.75±2.12^{A}	99.75±2.12^{A}	99.75±2.12^{A}	99.75±2.12^{A}	99.75±2.12^{A}
	HL3	43.52±0.74^{Db}	45.73±0.58^{Ba}	19.27±0.29^{Ec}	18.81±0.44^{Ec}	0.00±0.00^{Fd}
	HL7	46.48±0.48^{Da}	47.88±1.13^{Ba}	43.81±0.77^{Cb}	36.42±1.48^{CDc}	43.37±0.72^{CDb}
	HL8.5	47.09±1.19^{Da}	46.03±1.10^{Ba}	40.36±0.34^{Cb}	33.71±1.09^{Dc}	34.86±1.13^{DEc}
	DL3	85.74±0.53^{Ba}	22.87±0.04^{Cc}	−6.30±−0.09^{Fe}	15.07±0.02^{Ed}	28.39±0.02^{Eb}
	DL7	72.96±0.60^{Ca}	47.66±0.17^{Bb}	32.10±0.23^{De}	43.12±0.20^{Bd}	45.58±0.20^{BCc}
	DL8.5	71.73±0.11^{Ca}	47.35±0.66^{Bcd}	57.15±0.40^{Bb}	40.26±0.45^{BCd}	53.33±0.47^{Bbc}

Means with the different capital letters (A-G) in the same column and small letters (a-e) in the same row are significantly different (n>3, P<0.05).
NL, native lysozyme; HL, heat denatured lysozyme at pH 3.0, 7.0, and 8.5; DL, dextran-conjugated lysozyme at pH 3.0, 7.0, and 8.5.

Table 2. Emulsifying properties of the heat denatured and the dextran-conjugated lysozyme

Properties	Sample	Incubated time (d)				
		1	3	5	7	9
Emulsifying ability (500 nm)	NL	0.07±0.31^{B}	0.07±0.31^{B}	0.07±0.31^{B}	0.07±0.31^{C}	0.07±0.31^{C}
	DE	0.27±0.02^{B}	0.27±0.02^{B}	0.27±0.02^{B}	0.27±0.02^{C}	0.27±0.02^{C}
	HL3	0.07±0.04^{B}	0.06±0.03^{B}	0.08±0.03^{B}	0.06±0.01^{C}	0.07±0.03^{C}
	HL7	0.07±0.04^{B}	0.06±0.01^{B}	0.08±0.04^{B}	0.06±0.00^{C}	0.06±0.04^{C}
	HL8.5	0.06±0.04^{B}	0.10±0.01^{B}	0.10±0.02^{B}	0.07±0.01^{C}	0.10±0.01^{C}
	DL3	1.53±0.09^{A}	1.19±0.23^{A}	1.48±0.03^{A}	1.20±0.14^{B}	1.44±0.57^{B}
	DL7	1.54±0.16^{Ab}	1.25±0.08^{Ab}	1.65±0.42^{Ab}	1.85±0.28^{Aab}	2.41±0.08^{Aa}
	DL8.5	1.59±0.26^{A}	1.29±0.12^{A}	1.75±0.34^{A}	1.62±0.15^{AB}	1.63±0.33^{B}
Emulsifying stability (min)	NL	0.06±0.02^{C}	0.06±0.02^{C}	0.06±0.02^{C}	0.06±0.02^{B}	0.06±0.02^{C}
	DE	0.18±0.27^{C}	0.18±0.27^{C}	0.18±0.27^{C}	0.18±0.27^{B}	0.18±0.27^{C}
	HL3	0.51±0.07^{C}	0.49±0.01^{C}	0.53±0.01^{C}	0.49±0.01^{B}	0.52±0.06^{C}
	HL7	0.51±0.06^{C}	0.50±0.02^{C}	0.54±0.02^{C}	0.49±0.02^{B}	0.49±0.02^{C}
	HL8.5	0.50±0.05^{C}	0.56±0.04^{C}	0.56±0.04^{C}	0.52±0.04^{B}	0.56±0.10^{C}
	DL3	6.79±0.62^{Bb}	9.46±1.22^{Bab}	9.86±1.22^{Bab}	13.01±2.94^{Aa}	9.37±0.43^{Aab}
	DL7	11.49±1.69^{Aab}	11.11±1.04^{Aab}	13.18±1.04^{Aa}	7.95±0.53^{Abc}	6.15±0.82^{Bc}
	DL8.5	12.37±3.39^{A}	12.61±0.27^{A}	12.39±0.27^{A}	12.01±3.92^{A}	11.69±2.20^{AB}

Means with the different capital letters (A-C) in the same column and small letters (a-c) in the same row are significantly different (n>3, P< 0.05).
NL, native lysozyme; DE, native dextran; HL, heat denatured lysozyme without dextran; DL, dextran-conjugated lysozyme at pH 3.0, 7.0, and 8.5.

lysozymes. Scaman et al. (27) improved EA to 0.4 by reacting dextran and lysozymes at pH 8.5 for 48 h. These emulsion properties are greater than those of commercially available emulsifiers such as soybean lecithin (0.35) and monoglyceride (0.36), and so these substances can be used as food emulsifiers. In addition, it is thought that they can be used as food additives.

Surface hydrophobicity of DLs

Increased surface hydrophobicity increases the binding affinity of a protein for a bacterial cell membrane and disrupts membrane permeability, thus increasing the degree of interaction between molecules on the inside and the outside of the membrane and increasing antimicrobial activity (43,44). The surface hydrophobicity of NLs is relatively low; the NL molecule is hydrophobic but exhibits low surface hydrophobicity because all polar groups appear on the surface and most hydrophobic groups are embedded within the molecule (18). The surface hydrophobicity of DL was significantly higher than that of NLs, but was significantly lower than that of HLs (Fig. 3). When heat is applied to lysozymes, the disulfide bond is broken, and a structural change occurs which increases the surface hydrophobicity via exposure to amino acid molecules with a hydrophobic group (45).

Both, heat denaturation and dextran conjugation of lysozyme significantly increased surface hydrophobicity, but dextran conjugation showed lower surface hydrophobicity than heat denaturation at the same conditions. The lower surface hydrophobicity of DL is probably because reduction of the amino group in the Maillard reaction during formation of DL, and the reaction between the hydrophobic amino acid residue and the surface hydrophobic luminescent material, ANS, was less than that which occurred during formation of HL. In addition, DL and HL

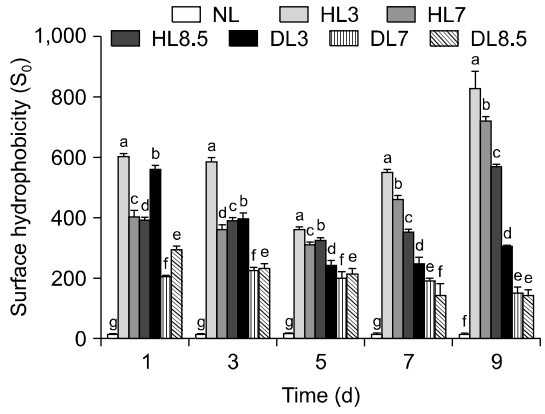

Fig. 3. Surface hydrophobicity of the heat denatured and the dextran-conjugated lysozyme. HL, heat denatured lysozyme without dextran at pH 3.0, 7.0, and 8.5 incubated for 1, 3, 5, 7, and 9 days; DL, dextran-conjugated lysozyme at pH 3.0, 7.0, and 8.5 incubated for 1, 3, 5, 7, and 9 days. The different letters (a-g) are significantly different at $P<0.05$.

at pH 3.0 exhibited significantly higher surface hydrophobicity values. This is consistent with the results presented by Lesnierowski et al. (33). Touch et al. (18) reported increased surface hydrophobicity to over 800 by heating lysozymes with cysteine and glutathione.

Antibacterial effects of DLs

This antibacterial effect is because the lysozyme hydrolyzes the glycosidic bond of N-acetylhexosamines, which constitutes the peptidoglycan layer of bacteria, but that bond is only found in some types of Gram-positive bacteria. Gram-negative bacteria have a complex outer membrane structure that is composed of lipoproteins, lipopolysaccharides, and certain hydrophobic peptides (46, 47). The growth inhibitory activity of DL was determined against five Gram-negative pathogenic bacteria (Table 3). There was no antibacterial activity against *P. aeruginosa*, *S. flexneri*, and *S. Sonnei* in the presence of native and heat denatured lysozymes. Growth inhibition of Gram-negative bacterial strains such as *P. aeruginosa*, *S. flexneri*, and *S. sonnei* was seen only after treatment with DL. Remarkably, DL7 showed 37.2~92.9% antibacterial activity against *S. sonnei*. The antibacterial activity of DL increased according to the reaction time, and about 90% of the antibacterial activity was observed in DL reacted for 9 days. The modified lysozyme, which has a high molecular weight, is composed mainly of β-sheets. It contains two dimeric forms of the lysozyme molecule. In this form, antibacterial activity against both Gram-positive and -negative bacteria was observed (45). Ibrahim et al. (45) suggested that the dimeric form of the lysozyme inhibits cell membrane function by inhibiting the transport of macromolecular precursors required for cell growth and cleavage, thereby exhibiting antibacterial effects.

Cell survival rate given VHSV infection and DL treatment

Cell viability was measured after pretreatment with DL at concentrations of 1 and 10 mg/mL for 24 h to determine the concentration of DL that displayed no cytotoxicity but had good antiviral activity. At a concentration of 1 mg/mL, NL, HL, and DL treatment were associated with a survival rate of 75% or more compared to the control group, and showed no serious toxicity (Fig. 4). However, at a 10 mg/mL, the survival rate was 70% or less when cells were treated with NLs, HL8.5 for 7 days, DL3 for 1~3 days, and DL8.5 for 9 days.

To confirm the antiviral activity of DL against VHSV for the purpose of preventing infection during the invasion stage of the VHSV life cycle, DL was added to cells at a concentration of 1 and 10 mg/mL for 24 h. After treatment, cells were infected with VHSV and cell viability was measured (Fig. 5). In line with the results of the cytotoxicity assays, pretreatment with lysozymes increased the survival rate by 5% compared to the control

Table 3. Antibacterial effects of the heat denatured and the dextran-conjugated lysozyme

Sample	Incubated time (d)				
	1	3	5	7	9
Escherichia coli					
NL	4.7±3.3^{E}	4.7±3.3^{C}	4.7±3.3^{D}	4.7±3.3^{C}	4.7±3.3^{C}
HL3	13.6±5.4^{CDEb}	11.1±3.6^{BCb}	25.1±5.8^{Aa}	28.7±0.6^{Aa}	30.9±0.6^{Aa}
HL7	11.7±4.9^{Deb}	11.8±2.7^{Bb}	8.5±4.2^{CDb}	20.5±0.2^{Aba}	13.2±0.5^{BCab}
HL8.5	14.5±4.4^{BCDbc}	13.8±2.6^{Bc}	20.8±0.7^{ABb}	29.7±0.6^{Aa}	28.9±0.9^{Aa}
DL3	22.2±0.9^{ABCa}	26.1±0.6^{Aa}	21.0±0.2^{Aba}	15.1±9.0^{BCa}	17.0±6.6^{Ba}
DL7	23.4±1.3^{Aba}	21.8±0.8^{Aa}	21.8±0.1^{Aba}	17.0±6.9^{ABCa}	17.0±7.0^{Ba}
DL8.5	25.7±0.2^{Aa}	24.3±0.5^{Aa}	17.5±1.6^{BCb}	17.5±7.5^{BCb}	17.6±6.5^{BCb}
Vibrio parahaemolyticus					
NL	3.4±3.1^{C}	3.4±3.1^{D}	3.4±3.1^{CD}	3.4±3.1^{BC}	3.4±3.1^{D}
HL3	0^{Cb}	15.3±1.4^{Aa}	16.5±5.3^{Aa}	17.3±0.6^{Aa}	14.3±4.2^{ABCa}
HL7	16.6±0.6^{Aa}	19.1±4.8^{Aa}	0^{Db}	17.1±5.6^{Aa}	17.6±3.5^{Aba}
HL8.5	20.0±3.9^{Aa}	20.6±3.0^{Aa}	0^{Db}	22.1±0.4^{Aa}	20.4±0.6^{Aa}
DL3	0^{Cc}	6.1±0.9^{CDb}	12.5±2.5^{ABa}	0^{Cc}	8.8±3.7^{BCDa}
DL7	0^{Cc}	13.4±0.2^{BCa}	8.1±0.8^{BCb}	7.1±1.3^{BCb}	8.1±3.5^{Db}
DL8.5	11.2±0.6^{Bab}	14.0±0.6^{ABa}	0^{Dd}	7.9±2.3^{Bbc}	6.9±3.0^{CDc}
Pseudomonas aeruginosa					
NL	0	0^{B}	0	0^{B}	0^{C}
HL3	0	0^{B}	0	0^{B}	0^{C}
HL7	0	0^{B}	0	0^{B}	0^{C}
HL8.5	0	0^{B}	0	0^{B}	0^{C}
DL3	0^{b}	0^{Bb}	3.40±5.5^{b}	0^{Bb}	28.3±1.4^{Aa}
DL7	0^{b}	0^{Bb}	0^{b}	19.2±14.7^{Aa}	14.8±5.1^{Bab}
DL8.5	0^{b}	13.8±1.1^{Aa}	0^{b}	13.4±1.6^{ABa}	13.8±1.1^{Ba}
Shigella sonnei					
NL	0^{B}	0^{C}	0^{D}	0^{D}	0^{B}
HL3	0^{B}	0^{C}	0^{D}	0^{D}	0^{B}
HL7	0^{B}	0^{C}	0^{D}	0^{D}	0^{B}
HL8.5	0^{B}	0^{C}	0^{D}	0^{D}	0^{B}
DL3	0^{Bd}	55.7±0.6^{Bc}	63.2±4.9^{Cb}	67.6±0.8^{Cb}	88.3±3.0^{Aa}
DL7	37.2±6.0^{Ac}	82.2±1.7^{Ab}	89.9±1.8^{Aa}	92.2±2.1^{Aa}	92.9±4.5^{Aa}
DL8.5	43.8±9.7^{Ab}	81.9±0.3^{Aa}	79.5±1.6^{Ba}	87.0±2.9^{Ba}	82.8±8.7^{Aa}
Shigella flexneri					
NL	0	0	0^{B}	0	0
HL3	0	0	0^{B}	0	0
HL7	0	0	0^{B}	0	0
HL8.5	0	0	0^{B}	0	0
DL3	0	0	14.3±2.4^{A}	0	2.3±12.6
DL7	0	0	16.8±1.5^{A}	2.9±7.5	3.7±16.4
DL8.5	0	0	0^{B}	0	0

Means with the different capital letters (A-D) in the same column and small letters (a-c) in the same row are significantly different (n>3, $P<0.05$).
NL, native lysozyme; HL, heat denatured lysozyme without dextran at pH 3.0, 7.0, and 8.5 for 1, 3, 5, 7, and 9 days; DL, dextran-conjugated lysozyme at pH 3.0, 7.0, and 8.5 for 1, 3, 5, 7, and 9 days.

group at 1 mg/mL, but cytotoxicity at 10 mg/mL decreased the survival rate. Pretreatment with most DL preparations at 1 mg/mL concentrations increased the cell survival rate (52.1~114.0%). Particularly, 1-d pretreatment with DL3, 1-d pretreatment with DL7, and 9-d pretreatment with DL8.5 produced a cell survival rate of 90% or more. In previous virus studies, lysozymes have been reported to possess antiviral activity against HIV (human immunodeficiency virus) and WSSV (white spot syndrome virus) (48,49). Lee-Huang et al. (50) reported that EWL exhibits antiviral activity due to the digestion of viral polysaccharides in HIV. However, Steinrauf et al. (51) suggested that lysozymes can bind DNA molecules through an electrostatic interaction, suggesting that the lysozyme may act on the transcription and replication of DNA and RNA, resulting in antiviral effects. Guanidyl-conjugated lysozymes produced a significant inhibition of the cytopathic effect of herpes simplex virus (HSV) and showed antiviral activity against HSV (52). Unlike these viruses, inhibitors of protein expression in *Rhabdoviridae*,

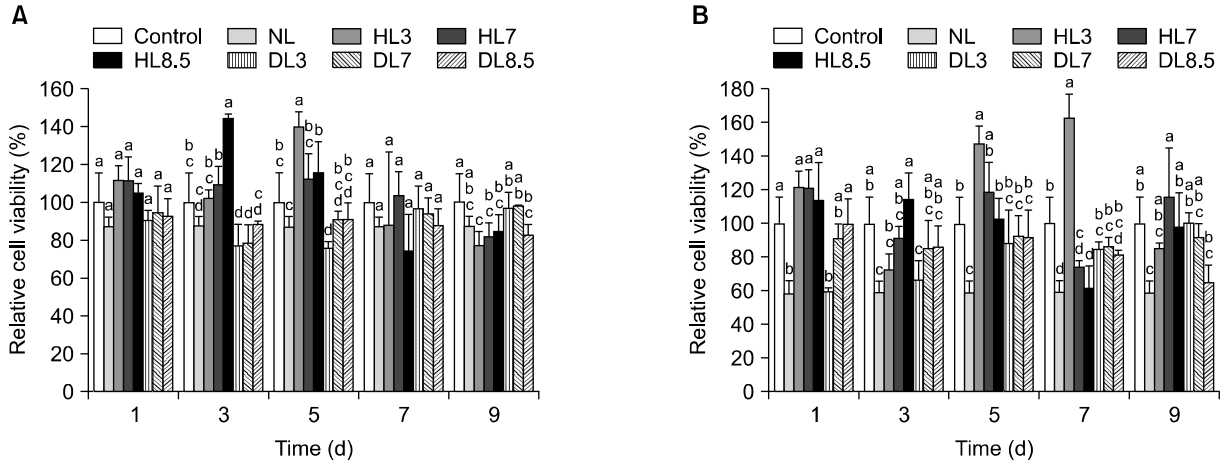

Fig. 4. Evaluation of cell cytotoxicity of the heat denatured and the dextran-conjugated lysozyme using CCK-8 assay. FHM cells were treated with 1 mg/mL (A) and 10 mg/mL (B) concentrations of dextran-conjugated lysozyme for 24 h. Cell viability was determined by CCK-8 assay. Control, not treated; NL, native lysozyme; HL, heat denatured lysozyme without dextran at pH 3.0, 7.0, and 8.5 for 1, 3, 5, 7, and 9 days; DL, dextran-conjugated lysozyme at pH 3.0, 7.0, and 8.5 for 1, 3, 5, 7, and 9 days. The different letters (a-d) are significantly different at $P<0.05$.

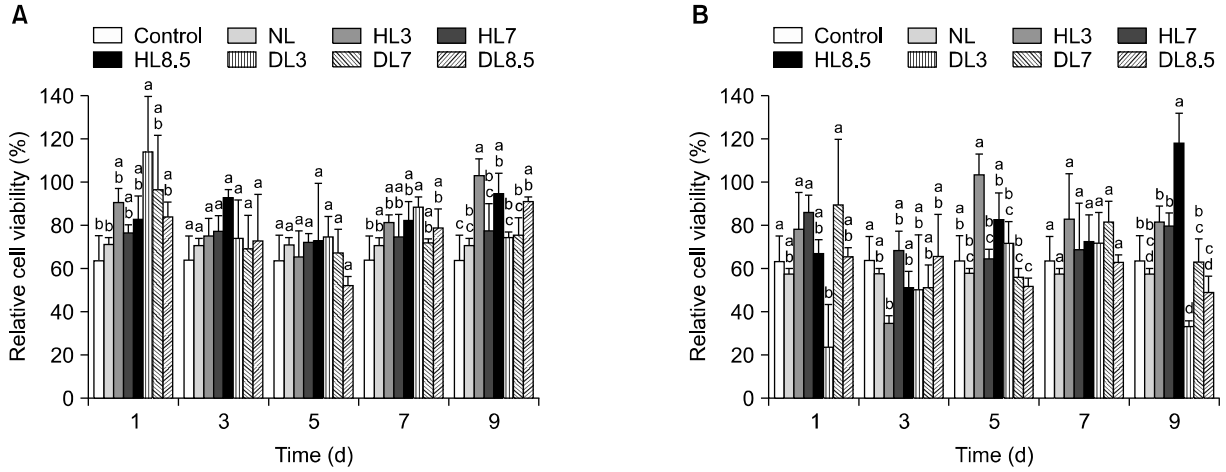

Fig. 5. Evaluation of antiviral activity of the heat denatured and the dextran-conjugated lysozyme using CCK-8 assay. Protective effects of dextran-conjugated lysozyme against viral hemorrhagic septicemia virus infected FHM cells. Cell viability of FHM cells treated 1 mg/mL (A) and 10 mg/mL (B) concentrations of dextran-conjugated lysozyme for 24 h. Cell viability was determined by CCK-8 assay. Control, not treated; NL, native lysozyme; HL, heat denatured lysozyme without dextran at pH 3.0, 7.0, and 8.5 for 1, 3, 5, 7, and 9 days; DL, dextran-conjugated lysozyme at pH 3.0, 7.0, and 8.5 for 1, 3, 5, 7, and 9 days. The different letters (a-d) are significantly different at $P<0.05$.

to which VHSV belongs, have not been investigated because of the complexity of the mechanisms involved in infection and fusion of such viruses (53). Jeong et al. (54) showed that inhibition of HSC71, one of the protein factors that increases in expression in VHSV infection, induces antiviral activity. Based on these studies, we speculate that DL also inhibited protein factors expressed during VHSV infection, such as the surface glycoprotein of VHSV, thereby improving the cell survival rate.

Although lysozyme is known to be effective only against Gram-positive bacteria, conjugation of lysozyme and dextran provided extended antimicrobial activity to include *E. coli*, *V. parahaemolyticus*, *P. aeruginosa*, *S. sonnei*, *S. flexneri*, and VHSV as well as enhanced lysozyme activity, EA and ES. It seems promising as antimicrobial agents, although further studies should be performed controlling the growth of pathogenic bacteria and viruses in various food and feed matrices.

ACKNOWLEDGEMENTS

This work was supported by the research program funded by the Small and Medium Business Administration (SMBA-S2230049) and the National Research Foundation (NRF-2016R1D1A1B03934137) of Korea.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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