JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 6449–6454 6449
DOI:10.1021/jf900246b

Preparation and Properties of Dextran Sulfate−Lysozyme Conjugate

Zahra Alahdad,[†] Roqhayeh Ramezani,[†] Mahmoud Aminlari,*[,†,§] and Mahsa Majzoobi[†]

[†]Department of Food Science and Technology, School of Agriculture, and [§]Department of Biochemistry, School of Veterinary, Shiraz University, Shiraz 71345, Iran

The purpose of this research was to conjugate lysozyme with dextran sulfate (DS) under mild Maillard reaction conditions and to investigate the functional properties of the resulting conjugate. The covalent attachment of DS to lysozyme was confirmed by SDS-PAGE and cation-exchange chromatography. Lytic activity of the conjugated and uncongugated lysozyme toward *Micrococcus lysodeikticus* was 40 and 71% that of native lysozyme, respectively. The lysozyme−DS conjugate was highly soluble at alkaline pH values and different temperatures (25, 40, and 60 °C). Moreover, the modified lysozyme exhibited increased heat stability, with better emulsion activity and emulsion stability than those of unmodified proteins. Lysozyme−DS conjugate showed antibacterial activity and significantly inhibited growth of *Staphylococcus aureus* and *Escherichia coli* in a dose-dependent manner. Taken together, the result of this study demonstrated that conjugation might increase the applicability of lysozyme in different food products for improving functional properties.

KEYWORDS: Lysozyme; dextran sulfate; functional properties; Maillard reaction

INTRODUCTION

Among different food components, proteins and polysaccharides play key roles in food systems by exerting effects such as gelling, thickening, surface, biological, and others properties. However, in recent years, food materials with new functionality have become strongly desired for the increasing variety of demands. For example, much attention has been paid to the preparation of proteins with new functions and, in particular, protein modification has captured wide interest. A variety of physical (*1*), enzymatic (*2*), and chemical methods such as covalent attachment of carbohydrates, amino acids, and fatty acids (*3*) and conjugation with glucosamine in the presence of a water-soluble carbodiimide (*4*) have been used to change the functional properties of food proteins. Among the various modifications applied to food proteins, the interaction between proteins and reducing sugars through mild Maillard reaction has recently been shown to produce marked changes in the functional properties of proteins, for example, solubility, water-binding capacity, foam and emulsion stability, heat stability, whipping ability, and gel forming capacity as well as antimicrobial functions (*5*). Moreover, it is well-known that Maillard reaction plays a very important role in food systems to provide flavor components. Conjugation of protein with polysaccharides is thought to improve protein functions more effectively. In particular, improvements in protein functions can be expected by complexation with branched and charged polysaccharides due to the difference in molecular weight or charge of the chemical species conjugated (*6*−*9*).

*Corresponding author (e-mail aminlari@shirazu.ac.ir; telephone 0098 711 2286950; fax 0098 711 2286940).

Lysozyme (EC 3.2.1.17, mucopeptide *N*-acetylmuramic hydrolase) has the ability to hydrolyze the β-1,4-glycoside linkage of the peptidoglycan in the bacterial cell wall. It exhibits more effective antibacterial activity toward Gram-positive than Gram-negative bacteria due to the differences in the structures of their cell walls. In the case of Gram-negative bacteria an additional barrier for lysozyme is the outer membrane composed of protein, phospholipids, and lipopolysaccharides (*10*). It has been reported that the range of lysozyme activity may be extended by using modifications leading to changes in the conformation of enzyme molecules. One of the methods for increasing the efficacy of enzyme activity is to create lysozyme conjugates with substances active toward Gram-negative bacteria (*3*, *5*, *10*).

Dextran sulfate (DS) is a polyanionic derivative of dextran and is prepared by sulfating a selected fraction of dextran. DS is a drug that was developed as an anticoagulant to delay clotting of blood in people who had heart disease or strokes (*11*). Several recent publications have reported on the preparation of non-covalently attached lysozyme−DS conjugate and have suggested application of protein−DS conjugates for improving the protein functional properties of caseins (*6*, *7*) and β-lactoglobulin (*8*, *9*).

The purpose of this investigation was to covalently attach DS to lysozyme and to study the effect of this modification on some functional properties and antibacterial characteristics of the conjugate.

MATERIALS AND METHODS

Materials. Hen egg white lysozyme (M_r 14600) was obtained from Inovatech, Inc. (Abbotsford, BC, Canada). DS (M_r 5000), carboxymethylcellulose (CM-52), and *Micrococcus lysodeikticus* cells were provided from Sigma (St. Louis, MO). Plate count agar and nutrient broth were obtained from Merck (Darmstadt, Germany). *Escherichia coli* IFO 1399 and

Published on Web 06/22/2009

Staphylococcus aureus IFO 1112 were purchased from the Persian Type Culture Collection (Tehran, Iran). All other chemicals were of reagent grade and were commercially available.

Preparation of Lysozyme−DS Conjugate. The lysozyme−DS conjugate was prepared as described for dextran with some modification (*12*). The DS used in this research contained approximately 17% sulfur, which is equivalent to approximately 2.3 sulfate groups per glucosyl residue. Briefly, 100 mg of lysozyme and various amounts of DS (100, 200, 300, and 500 mg) were mixed in 10 mL of 0.1 M sodium phosphate buffer (pH 7.0), and the mixture was homogenized and lyophilized. The powder was incubated at 60 °C for 1 week in a container containing a saturated KBr solution to provide a relative humidity (RH) of 79%. A control sample (without DS) was treated under the same conditions.

Color Evaluation of the Conjugate Samples. Color of samples was evaluated using the method described by Yam and Papadakis (*13*). Powder samples with similar thicknesses were put in a closed box, under a fluorescent light source with a radiation angle of 45°. Pictures were taken with a Sony digital camera (5 megapixel) and analyzed quantitatively using Adobe Photoshop 8 to determine colorimetric parameters.

Purification of Lysozyme−DS Conjugate. The lysozyme−DS was separated from the unreacted lysozyme by cation-exchange chromatography, using a CM-52 column. A 1×90 cm column was equilibrated with 10 mM ammonium carbonate buffer (pH 7.4). Six hundred milligrams of the powder was dissolved in 5 mL of the same buffer, and the solution was gently mixed and centrifuged at 2500*g* for 10 min to remove undissolved materials. The supernatant was added to a 5 × 20 cm CM-25 cation-exchange column. After the absorbance at 280 nm of the elutent was <0.01, the column was eluted with buffer containing 1.0 M NaCl . All fractions containing the lysozyme−DS conjugate were pooled and concentrated by ultrafilteration. To remove the excess untreated DS that might have been eluted together with the conjugate fractions, the solutions were brought to 60% saturated ammonium sulfate and left overnight at 4 °C; the precipitate was dialyzed against distilled water. Five hundred milligrams of unconjugated lysozyme (no DS) was treated similarly.

Electrophoresis. Sodium dodecyl sulfate (SDS) slab gel electrophoresis was conducted according to the method of Laemmli (*14*) using a 10% acrylamide separating gel. Protein samples were added to the loading buffer to give a final concentration of 1 mg/mL protein, 0.1 M Tris-HCl (pH 6.8), 0.4% SDS, 10% glycerol, and 0.004% bromophenol blue. The running gel (140 × 140 × 1 mm) was a 10% polyacrylamide gel in 1.2 M Tris-HCl (pH 8.8) and 0.3% SDS. The stacking gel contained 3% acrylamide in 0.25 M Tris-HCl (pH 6.8) and 0.2% SDS. The electrode buffer contained 0.025 M Tris-HCl, 0.192 M glycine, and 0.15% SDS at pH 8.16. Electrophoresis was performed at a constant 15 mA current, and gels were stained with 0.25% Coomassie Brilliant Blue R-250 in 50% methanol and destained with 10% acetic acid/7% methanol.

Determination of the Degree of Conjugation. Total sugar content of the conjugated proteins was determined according to the phenol−sulfuric acid method using glucose as standard (*15*). The number of moles of DS attached to 1 mol of lysozyme was then calculated by taking into account the molecular masses of DS (M_r 5000) and lysozyme (M_r 14600).

Lysozyme Activity. Lysozyme activity was measured by the lysis of *M. lysodeikticus* cell walls according to the method of Imoto and Yagishita (*16*). Nine milligrams of dried *M. lysodeikticus* cell walls was dissolved in 25 mL of 0.1 M potassium phosphate buffer (pH 7.0) and diluted to a final volume of 30 mL with the same buffer. Lysozyme or modified lysozyme at a concentration of 1 mg of protein/mL was dissolved in cold distilled water. The cell wall suspension (2.9 mL) was poured into a cuvette and incubated at 25 °C for 4−5 min to achieve temperature equilibration. The enzyme solution (0.1 mL) was added to the cuvette, and the change in absorbance at 450 nm was then recorded. One unit of activity of lysozyme is defined as the decrease in the absorbance at 450 nm of 0.001/min at pH 7.0 and 25 °C using*M. lysodeikticus* cells as a substrate.

Measurement of Solubility at Different pH Values and Temperatures. The effect of pH and temperature on protein solubility was assessed according to the method of Abtahi and Aminlari (*17*). Briefly, 30 mg of powdered protein samples was dissolved in 1 mL of appropriate buffer (0.1 M sodium acetate, pH 3.0 and 5.0, 0.1 M sodium phosphate, pH 7.0 and 9.0), mixed thoroughly at 25 °C for 1 h, and divided into two equal parts. One part was used for total protein determination according to the microkjedahl method (AOAC) (*18*), and the other part was centrifuged at 27000*g* for 15 min and the protein content of the supernatant determined (*19*). Solubility was expressed as the percent of protein in the supernatant with respect to the total protein content. The effect of temperature on solubility was determined as described above except that the lysozyme samples were dissolved in phosphate buffer, pH 9.0 (the maximum pH value for solubility of modified lysozyme, see Results and Discussion) and heated in water baths maintained at 25, 40, or 60 °C for 1 h. Samples were stored at ambient temperature for 2 h to achieve temperature equilibration, and solubility was measured as above.

Evaluation of the Emulsifying Properties of the Lysozyme−DS Conjugate. The emulsifying properties were determined according to the method of Pearce and Kinsella (*20*). To prepare emulsion, 1.0 mL of corn oil and 3.0 mL of protein solution in 0.1 M phosphate buffer (pH 7.4) was shaken together and homogenized at 1200 rpm for 2 min at 25 °C, using a Silverstone homogenizer. One hundred microliters of emulsion was taken from the bottom of the test tube after different time intervals and diluted with 5.0 mL of 0.1% SDS solution. The absorbance of diluted emulsion was then determined at 500 nm. The emulsifying activity is the absorbance at zero time. The time required to obtain a 50% reduction in absorbance is a measure of emulsion stability. Protein was determined spectrophotometrically at 280 nm (by a model 6405 spectrophotometer, Bibby Scientific Ltd., Essex, U.K.), using 26.3 as the extinction coefficient for a 1% solution of lysozyme (*21*).

Evaluation of Heat Stability. Heat stability of the unconjugated and DS-conjugated lysozyme was determined by measuring the turbidity (absorbance at 500 nm) of a 0.075% protein solutions (7.5 mg/10 mL of 0.1 M sodium phosphate, pH 7.4, held at 50−95 °C) using a model 6405 spectrophotometer (Bibby Scientific Ltd.). Starting at 50 °C, the temperature was increased at 1 °C/min and the absorbance recorded each 5 min (*20*).

Detection of Antimicrobial Activity. The antimicrobial assay for lysozyme derivatives and DS used *E. coli* (as representative Gram-negative bacteria) and *S. aureus* (as Gram-positive). Microorganisms were incubated in nutrient broth at 37 °C for 16 h and then decimally diluted to give a concentration of 10^6 CFU/mL. To 4.5 mL of the bacterial suspension in 50 mM phosphate buffer (pH 7.0) was added 0.5 mL of lysozyme, modified lysozyme, or DS solution in 50 mM phosphate buffer to give final lysozyme concentrations of 100, 200, and 400 μg/mL. Mixtures were incubated at 37 °C for 5 h. Decimal dilutions were carried out in sterile physiological saline solution adjusted to pH 7.2. A 100 μL aliquot was spread on plate count agar for both *E. coli* and *S. aureus* and to determine viable counts (*5*).

Statistical Analysis. All data were analyzed by ANOVA procedure of SPSS 13.0, and mean comparison was performed using Duncan's multiple-range test (DMRT, $P < 0.05$).

RESULTS AND DISCUSSION

Formation of Lysozyme−DS Conjugate. When lysozyme−DS mixtures were dry-heated at 60 °C, a light brown color developed after about 1 week of incubation. As shown in **Table 1**, when lysozyme−DS mixtures and lysozyme alone were dry-heated at 60 °C, there was an overall increase in redness and yellowness for the DS−lysozyme samples indicating Maillard-type reaction between the ε-amino group of lysine and the reducing end carbonyl group of DS. The formation of covalently bound lysozyme−DS conjugates through the Maillard reaction was confirmed by SDS-PAGE (**Figure 1**). The SDS-PAGE patterns are presented for various weight ratios of DS to lysozyme. Conjugation resulted in the appearance of diffused bands, indicating a wide distribution of molecular weights of the products of the reaction. Similar electrophoretic patterns were observed by other workers for other protein−polysaccharide mixtures (*5*, *11*, *22*−*25*). As shown in **Figure 1**, the diffusion of the bands increased when the weight ratio of DS to protein increased from 1:1 to 1:5. **Figure 2** shows the elution profiles of CM-52 column chromatography of lysozyme and lysozyme−DS

Table 1. Effect of Heating at 60 °C for 1 Week on Colorimetric Parameters *L*, *a*, and *b*[a]

sample	*L*	*a*	*B*
native lysozyme	71.8 a ± 0.8	−8.0 b ± 0.2	11.4 c ± 0.5
dextran sulfate	71.0 b ± 0.1	−9.0 c ± 0.2	13.2 b ± 0.4
heated lysozyme[b]	68.8 c ± 0.4	−9.8 d ± 0.4	12.0 c ± 0.7
glycosylated lysozyme[c]	62.6 d ± 0.5	−7.4 a ± 0.5	28.0 a ± 0.7

[a] In each column different letters indicate significant difference ($P < 0.05$). [b] No DS, dry heated at 60 °C for 1 week. [c] Lysozyme/DS (1.2 mol of DS/mol of lysozyme), dry heated at 60 °C for 1 week.

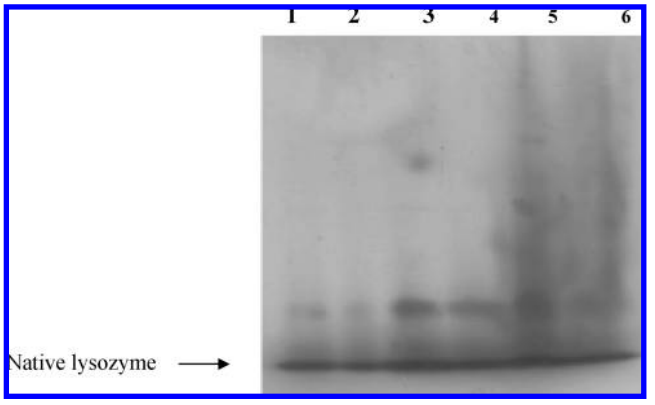

Figure 1. SDS-PAGE of DS-conjugated lysozyme (10% gel, 20 μg of protein per well). Lanes: 1, native lysozyme; 2, heated lysozyme without dextran sulfate; 3−6, modified lysozyme with weight ratios of lysozyme/DS of 1:1, 1:2, 1:3, and 1:5, respectively, heated at 60 °C for 1 week and at 79% RH.

conjugate. Whereas unmodified lysozyme was eluted from the column when 1.0 M NaCl was added to the buffer, the glycated lysozyme was eluted without salt, suggesting that DS was covalently attached to lysozyme, thereby decreasing the number of positively charged amino groups of protein. Furthermore, the presence of the negatively charged DS facilitated the elution of conjugate from the cationic resin. As indicated in **Figure 2B−E**, with increasing weight ratios of DS to lysozyme, the peak height corresponding to glycated lysozyme was increased and, simultaneously, that corresponding to unmodified lysozyme decreased. These results were further confirmed by the measurement of the total sugar content of conjugates. The fractions containing pure conjugate were pooled and used for subsequent studies. The results indicated that increasing the weight ratio of DS to lysozyme resulted in a significantly higher content of the polysaccharide of the lysozyme. At weight ratios of 1:1, 1:2, 1:3, and 1:5 averages of 0.6 ± 0.02, 0.8 ± 0.07, 1.0 ± 0.01, and 1.2 ± 0.06 mol of DS were attached per mole of lysozyme, respectively. These multiple forms probably originate from the formation of molecules with different numbers of polysaccharides attached to each molecule of proteins. Lysozyme has seven free amino groups (*22*). It is, therefore, conceivable that these multiple derivatives belong to lysozyme with one to seven DS molecules covalently attachment to amino groups. The 0.6−1.2 mol of DS/mol of lysozyme is therefore an average number. However, on the basis of the results of other studies, it is unlikely that all seven amino groups are available for conjugation, and some may be left unconjugated (*3*, *10*, *22*, *24*, *25*). In all subsequent experiments the conjugate containing the highest level of DS (i.e., 1.2 mol/mol lysozyme) was used.

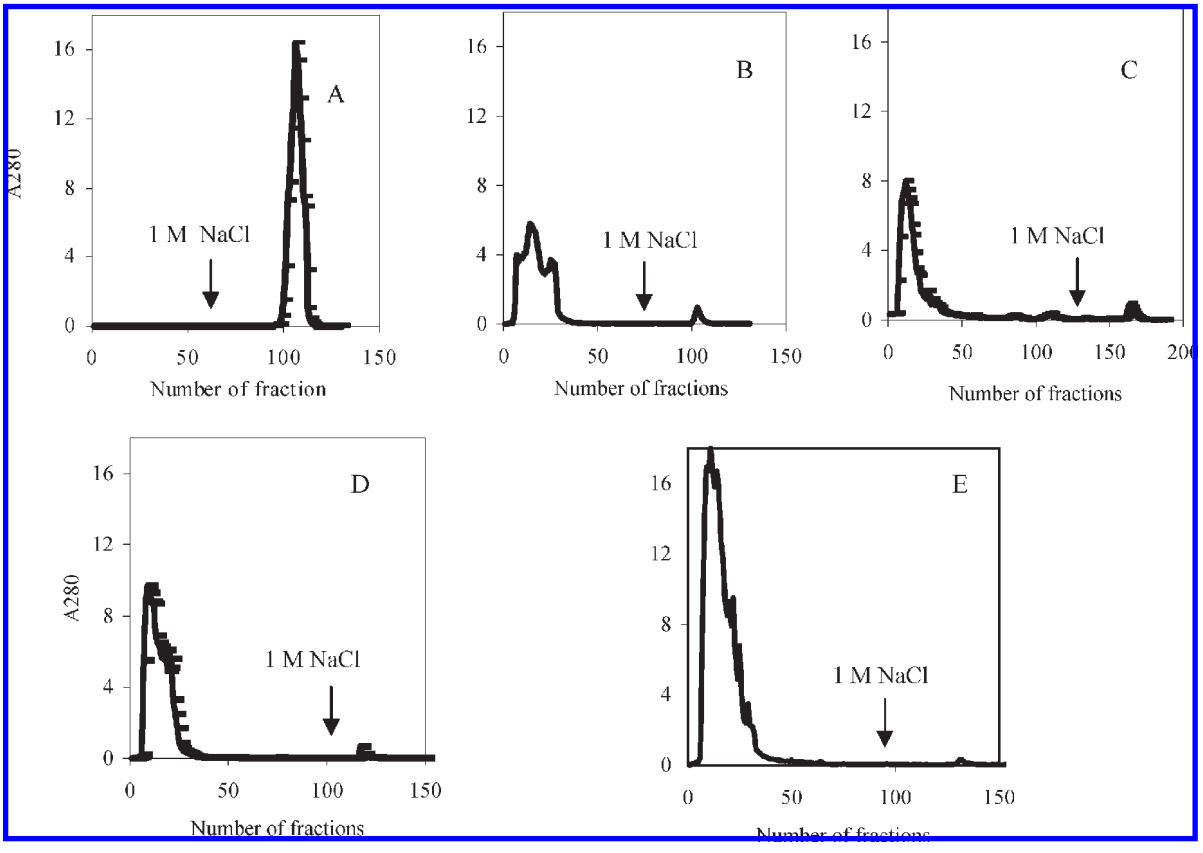

Figure 2. CM-52 chromatography of lysozyme and DS−lysozyme conjugates: unmodified lysozme (**A**); modified lysozyme with weight ratios of lysozyme/DS of 1:1 (**B**), 1:2 (**C**), 1:3 (**D**), and 1:5 (**E**). A 5 × 20 cm column was equilibrated with 10 mM ammonium carbonate buffer (pH 7.4). A 5 mL sample containing 600 mg of protein dissolved in the same buffer was added to the column, and after the absorbance at 280 nm of the elutent was <0.01; the column was eluted with buffer containing 1.0 M NaCl.

It has been reported that the interaction of different proteins with DS is of electrostatic nature and is characteristically affected by salt concentration (*6*, *8*, *9*) Our data, however, confirm the presence of a covalent bond between lysozyme and DS via Maillard reaction as it was not affected by pH or other conditions that might cause dissociation of DS from lysozyme. Similar results have been reported by other investigators (*3*, *5*, *10*, *21*, *22*, *26*, *27*). This novel approach might make this conjugate more useful in food systems undergoing pH changes or high salt concentration without the possibility of decoupling of polymers.

Enzymatic Activity. The lysozyme activity of the conjugate was measured by the lytic activity against *M. lysodeikticus*. **Table 2** indicates that heating of lysozyme at 60 °C for 1 week resulted in a 30% loss of enzymatic activity as compared with the native lysozyme. On the other hand, glycosylation led to a 60% loss of enzymatic activity of lysozyme. Both steric hindrance and blocking of the positive charges might be responsible for the decrease in the lytic activity of the conjugated enzyme. This result is in agreement with those of Ibrahim et al. (*21*, *26*), who reported 44 and 27% reductions in the enzyme activity of lysozyme derivatives incorporating four perillaldehyde and four palmitic acid residues with respect to unmodified lysozyme, respectively. The effect of surface charge modification has been found to play a critical role in the enzymatic activity of lysozyme. It has been reported that the net positive charge of lysozyme plays an important role in the initiation and efficiency of its lytic action against the negatively charged cell wall of *M. lysodeikticus* (*27*). Results of the current study indicate that modification of lysozyme possibly resulted in a decrease in the number of surface positive charge together with an increase in the surface negative charge and concomitant decrease in the activity of lysozyme.

Effect of pH and Temperature on Solubility. The solubility of glycated lysozyme at different pH values and temperatures is shown in **Table 2**. The native lysozyme was soluble in the whole pH range studied. After 1 week of heating at 60 °C, a decrease in solubility was observed in the whole pH range with a maximum solubility at pH 5.0. According to the data of **Table 2**, an interesting property of the complexation of lysozyme with the polysaccharide is the increase in the protein solubility at a pH close to its isoelectric point. After the lysozyme had been heated in the presence of DS, the resulting glycated lysozyme exhibited lower solubility than native lysozyme. In conjugated sample increasing the pH toward alkaline values resulted in a significant increase in solubility, with a maximum at pH 9.0. High solubility of glycated lysozyme at pH >7.0 is probably due to low pK_a of sulfate groups and shifting the pI of lysozyme to acidic pH values. In this research, native, heated lysozyme and glycated lysozyme were heated at different temperatures and at optimum pH (9.0). As indicated in **Table 2**, three samples presented a significant decrease in solubility as a function of the temperature of heating. Nevertheless, in the whole temperature range, the DS−lysozyme conjugate exhibited higher solubility than when lysozyme alone was heated. The effect of conjugation of lysozyme and other proteins with different polysaccharides on solubility has been reported by other investigators (*4*, *5*, *22*, *29*−*32*). Variation in the degree of solubility is probably due to difference in the types of polysaccharides used and the degree of conjugation. The significant increase in solubility of the glycated proteins is probably due to the hydration capacity of the polysaccharides (*27*) and increased negative charge of the conjugated lysozyme. As described earlier, Vardhanabhuti and Foegeding (*8*) have reported that interaction between β-lactoglobulin and DS is electrostatically driven and that DS at low concentration decreased aggregation of β-lactoglobulin, resulting in increased solubility of the mixture. At higher DS concentrations solubility decreased due to aggregation. Data gathered in this study indicate higher solubility of

Table 2. Effect of Conjugation with Dextran Sulfate on the Properties of Lysozyme[a,b]

sample	units of lysozyme activity/mg of lysozyme	% solubility at different pH values					% solubility at different temperatures[e]			emulsion activity (A_{500nm})	emulsion stability (min)
		3	5	7	9		25 °C	40 °C	60 °C		
native lysozyme	427.5 a ± 8.8	99.9 a,A ± 0.5	99.7a,A ± 0.5	99.8 a,A ± 0.2	98.7 a,B ± 0.4		98.7 a,A ± 0.4	96.8 a,A ± 0.8	90.6 a,A ± 1.1	0.1 b ± 0.00	2.0 b ± 0.0
heated lysozyme[c]	303.8 b ± 7.7	75.6 b,B ± 1.2	89.5 b,A ± 2.0	83.7 b,A ± 7.0	70.6 c,B ± 0.9		70.6 c,A ± 0.9	58.5 c,B ± 1.9	36.4 c,C ± 3.1	0.1 b ± 0.01	2.7 b ± 0.3
conjugated lysozyme[d]	171.9 c ± 2.7	28.3 c,D ± 5.9	49.7 c,C ± 8.6	65.1 c,B ± 5.3	82.9 b,A ± 3.8		82.8 b,A ± 2.1	72.8 b,B ± 6.8	62.0 b,C ± 3.3	1.3 a ± 0.21	4.5 a ± 0.7

[a] In each column different lowercase letters indicate significant difference ($P < 0.05$). [b] In each row different uppercase letters indicate significant difference ($P < 0.05$). [c] No DS, dry heated at 60 °C for 1 week and at 79% RH. [d] Lysozyme/DS (1.2 mol of DS/mol of lysozyme), dry heated at 60 °C for 1 week and at 79% RH. [e] Solubility was measured at pH 9.0

DS−lysozyme as compared with heated lysozyme alone. Such behavior might render DS−lysozyme a more attractive candidate as a food ingredient for improving functionality.

Emulsifying Properties. Table 2 indicates that glycation of lysozyme with DS resulted in an increase in emulsion activity and emulsion stability. Whereas no significant difference between native lysozyme and heated lysozyme was observed, the emulsifying activity and emulsion stability of lysozyme−DS conjugte were 14.2 and 2.25 times higher than those of native lysozyme, respectively. Several studies have shown that heated proteins exhibit increased emulsifying properties. The difference in the heating conditions might be the reason for the differences observed in the emulsifying properties of heated lysozyme obtained in the present study and those reported by others (*33*, *34*). Kato et al. (*31*) have noted that the branched macromolecule structure may be efficient in wrapping around the oil droplets, resulting in the excellent emulsifying properties. The role of polysaccharide in the stabilization of emulsion by a polysaccharide−protein conjugate is probably related to the anchorage of the hydrophobic residues of protein molecule in the oil droplet during emulsion formation and the orientation of the polysaccharide moiety to the aqueous phase of emulsion preparation, thereby accelerating the formation of the thick steric stabilizing layer around the emulsion and inhibiting the coalescence of oil droplets (*35*). In addition to this factor, DS used in the present study has a high net charge; therefore, the conjugates covering the oil droplets might inhibit their coalescence by electrostatic repulsion. Increased emulsion stability in the case of β-lactoglobulin−alginate conjugates compared to the emulsion of β-lactoglobulin alone has also been reported (*32*). The β-lactoglobulin−alginate conjugates showed good emulsion stability because both conjugates were high in net charge, which displayed a strong protective effect of shielding in the emulsifier. Overall, in the emulsion systems containing both proteins and polysaccharides, protein typically forms an adsorbed primary layer at the oil−water interface, whereas hydrophilic polysaccharides possibly form a thick secondary layer that enhances the steric stabilizing properties on the outside of protein-coated droplets (*36*). Therefore, emulsion stability seems to be the contribution of the electrostatic protection along with steric stabilization of droplets covered by the mixed protein−polysaccharide absorbed layers.

Heat Stability. To study the heat stability of lysozyme modified with DS, 0.075% solutions of lysozyme and lysozyme modified with DS were heated at various temperatures in the range of 60−95 °C for 10 min, and solution turbidity was measured at 500 nm. As shown in **Figure 3**, the turbidity of native and heated lysozyme increased significantly as heating temperature increased beyond 60 °C, whereas lysozyme glycated with DS resisted heat-induced aggregation up to 70 °C, after which its turbidity increased slightly. Similar results were reported for lysozyme−galactomanan and lysozyme−dextran conjugates (*20*, *21*).

Antimicrobial Activity of Modified Lysozyme. Antibacterial actions of the native and DS-modified lysozyme and DS alone were assessed by employing *E. coli* (as a Gram-negative bacterium) and *S. aureus* (as a Gram-positive bacterium). **Tables 3** and **4** show the antimicrobial effects of the samples against *S. aureus* and *E. coli* at different concentrations. The results for *E. coli* indicate that DS alone significantly decreased the number of bacteria. However, higher antimicrobial activity was observed when the lysozyme−DS conjugate was used, and the antimicrobial activity was greatly intensified with increasing concentration of the glycated enzyme. These antimicrobial effects were not observed with lysozyme alone (**Table 3**). The antimicrobial effect of the conjugate against Gram-positive bacteria was also investigated using one typical strain of *S. aureus*. As indicated in **Table 4**, lysozyme showed antimicrobial activity against *S. aureus* at 37 °C, and DS was more effective than lysozyme in causing decrease in bacterial growth. The conjugation of lysozyme with DS led to the loss of antimicrobial activity of lysozyme and DS. One of the major problems in the application of lysozyme as a natural preservative in the food and drug industry is that the antimicrobial action is limited to Gram-positive bacteria with little action on Gram-negative bacteria, including foodborne pathogens (*22*, *23*). The data of the present study clearly indicate that conjugating lysozyme with DS resulted in the creation of an bactericidal agent against Gram-negative bacteria. According to the results of other researchers the outer membrane of the Gram-negative bacterial

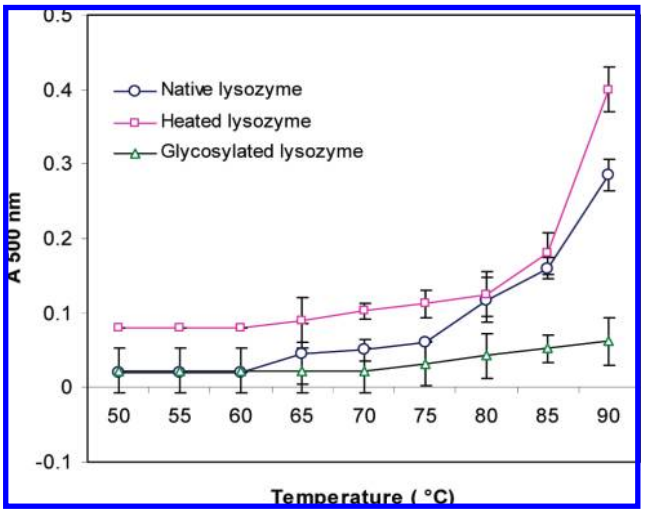

Figure 3. Heat stability of native lysozyme, heated lysozyme, and glycosylated lysozyme. Absorbance at 500 nm of a 0.075% protein in 0.1 M sodium phosphate, pH 7.4, was recorded as function of heating, starting at 50−90 °C. The temperature was increased 1 °C/min, and absorbance was recorded after holding at each temperature for 5 min.

Table 3. Antimicrobial Effect of Lysozyme, Dextran Sulfate (DS), and DS-Conjugated Lysozyme against *E. coli*[a,b]

	log CFU		
sample	100 μg/mL	200 μg/mL	400 μg/mL
no DS, no lysozyme		7.4 a,A ± 0.0	
heated lysozyme[c]	7.1 a,A ± 0.3	7.3 a,A ± 0.6	7.2 a,A ± 0.3
DS	6.6 d,B ± 0.05	6.1 b,C ± 0.07	5.8 c,C ± 0.01
conjugated lysozyme[d]	6.9 c,AB ± 0.1	6.6 b,BC ± 0.2	6.3 b,C ± 0.3

[a] In each column different lowercase letters indicate significant difference ($P < 0.05$). [b] In each row different uppercase letters indicate significant difference ($P < 0.05$). [c] No DS, dry heated at 60 °C for 1 week and at 79% RH. [d] Lysozyme/DS (1.2 mol of DS/mol of lysozyme), dry heated at 60 °C for 1 week and at 79% RH.

Table 4. Antimicrobial Effect of Native Lysozyme, Dextran Sulfate (DS), and Glycosylated Lysozyme against *S. aureus*[a,b]

	log CFU		
sample	100 μg/mL	200 μg/mL	400 μg/mL
no DS, no lysozyme		8.0 a,A ± 0.03	
heated lysozyme[c]	7.4 c,B ± 0.09	7.1 c,B ± 0.04	6.9 c,C ± 0.1
DS	7.1 d,AB ± 0.4	6.8 d,BC ± 0.2	6.6 d,C ± 0.2
conjugated lysozyme[d]	7.7 b,B ± 0.1	7.6 b,C ± 0.1	7.4 b,C ± 0.2

[a] In each column different lowercase letters superscripts indicate significant difference ($P < 0.05$). [b] In each row different uppercase letters superscript indicate significant difference ($P < 0.05$). [c] No DS, dry heated at 60 °C for one week and at 79% RH. [d] Lysozyme: DS (1.2 mol DS/ mole lysozyme), dry heated at 60 °C for one week and at 79% RH.

cell wall is very sensitive to surface active agents (*10*). Therefore, the antimicrobial effect against *E. coli* is probably related to the surfactant properties of DS−lysozyme conjugate. The decrease of antimicrobial activity of lysozyme against *S. aureus* is probably due to the blocking of positive charge on the surface of lysozyme with DS. As shown in **Table 2** a 70% loss in lytic activity of lysozyme occurred due to modification with DS.

Taken together, the results of this research show that DS conjugation to lysozyme is effective in improving the solubility at alkaline pH values and different temperatures and in increasing heat stability and emulsifying properties. In addition, although glycosylation of lysozyme resulted in decreased antimicrobial activity against *S. aureus*, it did extend the antimicrobial spectrum of lysozyme toward *E. coli* as representative of Gram-negative bacteria. Thus, lysozyme−DS conjugate might provide a novel class of ingredient in formulated food systems or as therapeutic agent. However, before this product can be recommended for these applications, its safety issue needs to be investigated.

LITERATURE CITED

(1) Maforimbo, E.; Skurray, G.; Uthayakumaran, S.; Wrigley, C. W. Improved functional properties for soy−wheat doughs due to modification of the size distribution of polymeric proteins. *J. Cereal Sci.* **2006**, *43*, 223−229.

(2) Ramezani, R.; Aminlari, M.; Fallahi, H. Effect of chemically modified soy protein and ficin-tendrized meat on the quality attributes of sausage. *J. Food Sci.* **2003**, *68*, 85−88.

(3) Takahashi, K.; Lou, X. F.; Ishii, Y.; Hattori, M. Lysozyme−glucose stearic acid monoester conjugate formed through Maillard reaction as an antimicrobial emulsifier. *J. Agric. Food Chem.* **2000**, *48*, 2044−2049.

(4) Ramezani, R.; Esmailpour, M.; Aminlari, M. Effect of conjugation with glucosamine on the functional properties of lysozyme and casein. *J. Sci. Food Agric.* **2008**, *88*, 2730−2737.

(5) Amiri, S.; Ramezani, R.; Aminlari, M. Antibacterial activity of dextran-conjugated lysozyme against *Escherichia coli* and *Staphylococcus aureus* in cheese curd. *J. Food Prot.* **2008**, *71*, 411−415.

(6) Jourdain, L.; Leser, M. E.; Schmitt, C.; Michel, M.; Dickinson, E. Stability of emulsions containing sodium caseinate and dextran sulfate: relationship to complexation in solution. *Food Hydrocolloids* **2008**, *22*, 647−659.

(7) Semenova, M. G.; Belyakova, L. E.; Polikarpov, Y. N.; Antipova, A. S.; Dickinson, E. Light scattering study of sodium caseinate dextran sulfate in aqueous solution: relationship to emulsion stability. *Food Hydrocolloids* **2009**, *23*, 629−639.

(8) Vardhanabhuti, B.; Foegeding, E. A. Effects of dextran sulfate, NaCl, and initial protein concentration on thermal stability of β-lactoglobulin and α-lactalbumin at neutral pH. *Food Hydrocolloids* **2008**, *22*, 752−762.

(9) Vardhanabhuti, B.; Yucel, U.; Coupland, J. N.; Foegeding, E. A. Interactions between β-lactoglobulin and dextran sulfate at near neutral pH and their effect on thermal stability. *Food Hydrocolloids* **2008**, in press, doi: 10.1016/j.foodhyd. 2008.09.006.

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

(11) Mahner, C.; Lechner, M. D.; Nordmeier, E. Synthesis and characterization of dextran and pullulan sulphate. *Carbohydr. Res.* **2001**, *331*, 203−208.

(12) Scaman, C.; Nakai, S.; Aminlari, M. Effect of pH, temperature and sodium bisulfite or cysteine on the level of Maillard-based conjugation of lysozyme with dextran, galactomanan and mannan. *Food Chem.* **2006**, *99*, 368−380.

(13) Yam, K. L.; Papadakis, S. E. A simple digital imaging method for measuring and analyzing color of food surfaces. *J. Food Eng.* **2004**, *61*, 137−142.

(14) Laemmli, U. K. Cleavage of structural proteins during the assembly of head of bacteriophage T_4. *Nature* **1970**, *227*, 680−685.

(15) Dubois, M.; Gilles, K. A.; Hamilton, K.; Rebers, A. P.; Smith, F. Colorimetric method for determination of sugars and related substances. *J. Anal. Chem.* **1959**, *28*, 350−356.

(16) Imoto, T.; Yagishita, K. A simple activity measurement of lysozyme. *Agric. Biol. Chem.* **1971**, *35*, 1154−1156.

(17) Abtahi, S.; Aminlari, M. Effect of sodium sulfite, sodium bisulfite, cysteine, and pH on protein solubility and sodium dodecyle sulfate−polyacrylamide gel electrophoresis of soybean milk base. *J. Agric. Food Chem.* **1997**, *45*, 4768−4772.

(18) AOAC. *Official Methods of Analysis of AOAC International*; Horwitz, W., Ed.; Washington, DC, 2000.

(19) Lowry, P. H.; Rosebrough, N. J.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1952**, *193*, 265−275.

(20) Pearce, K. M.; Kinsella, J. E. Emulsifying properties of proteins: Evaluation of a turbidimeric technique. *J. Agric. Food Chem.* **1978**, *26*, 716−723.

(21) Ibrahim, H. R.; Hatta, H.; Fujiki, M.; Kim, M.; Yamomoto, T. Enhanced antimicrobial action of lysozyme against Gram-negative and Gram-positive bacteria due to modification with perillaldehyde. *J. Agric. Food Chem.* **1994**, *42*, 1813−1817.

(22) Aminlari, M.; Ramezani, R.; Jadidi, F. Effect of Maillard-based conjugation with dextran on the functional properties of lysozyme and casein. *J. Sci. Food Agric.* **2005**, *85*, 2617−2624.

(23) Song, Y.; Babiker, E. E.; Usui, M.; Saito, A.; Kato, A. Emulsifying properties and bactericidal action of chitosan−lysozyme conjugates. *Food Res. Int.* **2002**, *35*, 459−466.

(24) Nakamura, S.; Gohya, Y.; Losso, J. N.; Nakai, S.; Kato, K. Protective effect of lysozyme−galactomannan or lysozyme−palmitic acid conjugates against *Edwardsiella tarda* infection in carp, *Cyprinus carpio* L. *FEBS Lett.* **1996**, *383*, 251−254.

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

(26) Ibrahim, H. R.; Kato, A.; Kobayashi, K. Antimicrobial effects of lysozyme against Gram-negative bacteria due to covalent binding of palmitic acid. *J. Agric. Food Chem.* **1991**, *33*, 2077−2082.

(27) Nakamura, S.; Kobayashi, K.; Kato A. Role of positive charge of lysozyme in the excellent emulsifying properties of Maillard-type lysozyme−polysaccharide conjugate. *J. Agric. Food Chem.* **1994**, *42*, 2688−2691.

(28) Muraki, M.; Morikawa, M.; Jigami, Y.; Tanaka, H. Engineering of human lysozyme as a polyelectrolyte by the alteration of molecular surface charge. *Protein Eng.* **1988**, *2*, 49−54.

(29) Babiker, E.; Hiroyuki, A.; Matsudomi, N.; Iwata, H.; Ogawa, T;, Bando, N.; Kato, A. Effect of polysaccharide conjugation or trans glutaminase treatment on the allergenicity and functional properties of soy protein. *J. Agric. Food Chem.* **1998**, *48*, 866−871.

(30) Jimenez-Castano, L.; Villamiel, M.; Lopez-Fandino, R. Glycosylation of individual whey proteins by Maillard reaction using dextran of different molecular mass. *Food Hydrocolloids* **2007**, *21*, 433−443.

(31) Kato, A.; Murata, K.; Kobayashi, K. Preparation and characterization of ovalbumin−dextran conjugate having excellent emulsifying properties. *J. Agric. Food Chem.* **1988**, *36*, 421−425.

(32) Hattori, M.; Aiba, Y.; Nagasawa, K.; Takahashi, K. Functional improvements of alginic acid by conjugating with β-lactoglobulin. *J. Food Sci.* **1996**, *61*, 1171−1176.

(33) Kato, A.; Osako, Y.; Matsudomi, N. Changes in the emulsifying and foaming properties of proteins during heat denaturation. *Agric. Biol. Chem.* **1983**, *47*, 33−37.

(34) Voutsinas, L. P., Cheung, E., Nakai, S. Relationships of hydrophobicity to emulsifying properties of heat denatured proteins. *J. Food Sci.* **2006**, *48*, 26−32.

(35) Kato, A.; Shimokawa, K.; Kobayashi, K. Improvement of the functional properties of insoluble gluten by Pronase digestion followed by dextran conjugation. *J. Agric. Food Chem.* **1991**, *39*, 1053−1056

(36) Dickinson, E. Interfacial structure and stability of food emulsions as affected by protein polysaccharide interaction. *Soft Matter* **2008**, *22*, 59−74.

Received January 21, 2009. Revised manuscript received March 27, 2009. Accepted May 26, 2009. This research was financially supported by Grants 87-GR-VT-11 and 87-GR-AGR-29 from Shiraz University Research Council.