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Effect of pH, temperature and sodium bisulfite or cysteine on the level of Maillard-based conjugation of lysozyme with dextran, galactomannan and mannan

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Abstract

The antimicrobial activity of lysozyme against Gram positive bacteria is well known. Application of this enzyme as a natural antimicrobial or preservative agent in food and pharmaceutical industry is under consideration in many laboratories. The antimicrobial effect of lysozyme can be extended towards Gram negative bacteria by chemical modification including conjugation with carbohydrates. The purpose of this investigation was to find the optimum experimental conditions for glycation of lysozyme with polysaccharides dextran, galactomannan and mannan and to evaluate some functional properties of the modified enzyme. Lysozyme was allowed to react with dextran under Maillard reaction condition at different pH and temperatures and in the presence of 50 mmol/L sodium bisulfite or cysteine and the extent of glycation was determined by ion exchange chromatography and SDS-PAGE. The optimum condition for glycation was pH 8.5 and 60 °C with a protein to derxtran molar ratio of 1:5. Same results were obtained for galactomannan but mannan did not react. Under these condition three moles dextran was attached to one mole lysozyme. Sodium bisulfite inhibited glycation at pH 8.5 and temperatures above 40 °C while cysteine prevented glycation at all pH and temperatures. Dextran-conjugated lysozyme exhibited improved heat stability, pH and heat solubility and better emulsifying property as compared with the unmodified lysozyme. These results suggest that it is possible to improve the properties of this enzyme in order to make it more suitable for application in foods and pharmaceuticals.

Keywords: Lysozyme; Dextran; Galactomannan; Mannan; Glycation; Antimicrobial; Functional properties

1. Introduction

Chicken egg-white lysozyme is well-known as an enzyme that has the ability to cause lysis of bacterial cells (Imoto, Johnson, North, Phillips, & Rupley, 1972). The protein has a molecular weight of 14,600 (129 amino acid residues) and a high isoelectric point (*p*I 11.0) (Liu, Sugimoto, Azakami, & Kato, 2000). Because of the presence of four disulfide bonds, the flexibility of the main chain is very restricted, even in the solution, resulting in a considerably high structural stability (Takahashi, Lou, Ishii, & Hattori, 2000). Due to its antibacterial activity, attempts have been made to use it as a natural preservative in foods. However, due to limited lytic spectrum only to Gram positive bacteria, it is not suitable to use it as a food preservative (Hayashi, Kasumi, Kubo, Harguchi, & Tsumura, 1989; Nakamura, Kato, & Kobayashi, 1991). Expanded antimicrobial activity as well as improved functional properties will make lysozyme a very desirable food additive which can be applied in a vast variety of food systems under different conditions.

Both antibacterial activity and functional properties can be improved by chemical and enzymatic modifications. Recently much attention has been directed towards the

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preparation of new functional proteins. A number of studies to improve protein functionality have involved chemical modification, such as alkylation, esterification, amidination, deamidination, covalent attachment of carbohydrates and fatty acids, thiol-disulfide exchange, and enzymatic modification (Abtahi & Aminlari, 1997; Babiker, 2002; Bloksona, 1975; Diftis & Kiosseoglou, 2003; Kato, Murata, & Kobayashi, 1988). These modifications generally improve solubility, emulsion stability, foam stability, heat stability, water binding capacity, gel forming property and change in isoelectric point (Abtahi & Aminlari, 1997; Babiker et al., 1998; Diftis & Kiosseoglou, 2003; Hattori, Okada, & Takahashi, 2000b; Kato, Ibrahim, Watanabe, Honma, & Kobayashi, 1990a; Nagasava, Ohgata, Takahashi, & Hattori, 1996; Ramezani, Aminlari, & Fallahi, 2003). Among the various modifications applied to food proteins, covalent coupling of carbohydrates through mild Maillard-type reaction appears to produce marked changes in the functional properties, particularly water solubility and heat stability (Nakamura et al., 1991, Nakamura, Kato, & Kobayashi, 1992a, Nakamura, Kato, & Kobayashi, 1992b, 1996; Nagasava et al., 1996; Shu, Sahara, & Kato, 1996; Takahashi et al., 2000). These properties make the modified proteins more attractive in biotechnological processes, where increased solubility and heat stability of proteins is required to ensure efficient transformation processes (Caer, Baniel, Subriade, Guegguen, & Colas, 1990; Rhichardson, 1977). In addition, it is well established that bisulfite and sulfhydryl containing compounds inhibit Maillard reaction (Freidman, 1996; Freidman & Molnar-Perl, 1990), thereby providing a means to control the degree of progress of Maillard reaction in food systems.

Because of the significance of the role of Maillard reaction in food stability, flavor development, nutrition and health, it is very important to develop rational approaches to minimize the adverse consequences of this reaction and optimize the beneficial effects while attempting to establish a condition in which the highest yield of polysaccharide-conjugated protein can be produced. The purpose of this investigation was to study the effect of pH, temperature and presence sodium bisulfite and cysteine on the glycation of lysozyme with polysaccharides dextran, galactomannan and mannan and to measure some functional properties of the modified lysozyme.

2. Material and methods

2.1. Materials

Chicken egg-white lysozyme was from Inovatech, Inc., Abbotsford, BC, Canada. Dextran (MW ~ 10,500), *Micrococcus lysodeicticus* cells and mannan (from *Sacharomyces cerevisiae*, MW 40,000) were from Sigma, St. Louis, MO, USA. Carboxy methyl cellulose resins (CM-52) was from Merck (Darmstadt, Germany) and protein molecular weight markers were supplied by Fermentas (Molndal, Sweden). Mannase hydrolyzed guar gum galactomannan (average molecular weight of 15 kDa) was gift from Dr. Soichiro Nakamura, Ube College, Yamaguchi, Japan. All other chemicals were reagent grade and were commercially available.

2.2. Preparation of lysozyme-dextran conjugate

One hundred mg lysozyme and 500 mg dextran were added to 2.0 ml 0.05 mol/L sodium phosphate buffer pH 8.5 and 0.05 mol/L sodium acetate pH 3.5. Other sets of samples were prepared in the same manner except that the solution contained 50 mmol/L sodium bisulfite or 50 mmol/L cysteine. pH of solutions was adjusted if necessary and after thorough mixing and incubating at room temperature for 1 h, solutions were frozen at –35 °C and then lyophilized. For each experiment a control sample was included which contained no dextran. Lyophilized powder was incubated at 40 or 60 °C for one week and at 80 °C for one day under the relative humidity of 79% provided by saturated KBr.

2.3. FPLC

Conjugation was followed by FPLC using a HiTrap SP column (Pharmacia, Upsala, Sweden) equilibrated with 0.03 mmol/L ammonium bicarbonate. Elution of proteins was carried out with 0–1.0 mol/L NaCl gradient in the same buffer.

2.4. Electrophoresis

Slab SDS-PAGE was performed according to the discontinuous buffer system of Laemmli (1970). Protein samples were added to the loading buffer to give final concentration of 1 mg/ml protein, 0.01 mol/L Tris–HCl, pH 6.8, 0.4% SDS, 10% glycerol, and 0.004% bromophenol blue. The running gel was made of 15% (w/v) acrylamide in 1.2 mol/L Tris–HCl, pH 8.8 and 0.3% SDS. The stacking gel contained 3.0% acrylamide in 0.25 mol/L Tris–HCl, pH 6.8 and 0.2% SDS. The electrode buffer compromised 0.025 mol/L Tris–HCl, 0.192 mol/L glycine, and 0.15% SDS at pH 8.16. Electrophoresis was performed at constant 25 mA and gels were stained with 0.25% Coommassie Brilliant blue R-250 in 50% acetic acid/25% methanol and destained with a 10% acetic acid/7.0% methanol.

2.5. Kinetics of glycation of lysozyme with dextran and galactomannan

Lysozyme (600 mg) was dissolved in 10 ml 0.05 mol/L acetate buffer, pH was adjusted to 3.5 and 3.0 g dextran or galactomannan was added. In order to study the effect of bisulfite, similar solution was prepared except that mixture contained 50 mmol/L sodium bisulfite. Samples were frozen and lyophilized. Powder was divided into several parts and each part was incubated at 60 °C under the

relative humidity of 79% in the presence of saturated potassium bromide. Samples were removed at time intervals and analyzed for degree of glycation by FPLC and SDS-PAGE. Similar experiment was repeated in phosphate buffer pH 8.5 instead of acetate buffer.

2.6. Glycation of lysozyme with mannan

Glycation was performed as described for dextran with slight modifications. Briefly, 60 mg lysozyme was dissolved in 1 ml 0.05 mmol/L sodium phosphate, pH 8.5, 300 mg mannan was added, pH adjusted, and the mixture was frozen and lyophilized. Powder was incubated at 60 °C for one or two weeks.

2.7. Glycation of urea-denatured lysozyme with dextran

Reaction of lysozyme with dextran was carried out at pH 8.5 except that the solution contained 8 mol/L urea. After freezing and lyophilization, powder was incubated at 60 °C for one or two weeks. The dried powder was dissolved in water (50 mg/ml) and the solution was dialyzed in a 25,000 MW cut off dialysis bag for two days at 4 °C. Samples were analyzed by SDS-PAGE and FPLC.

2.8. CM-52 cation exchange column chromatography of dextran-conjugated lysozyme

In order to obtain sufficient amounts of pure product for functional studies, glycated lysozyme was prepared under optimum condition (i.e. 60 °C, pH 8.5 for 48 h, see results, Table 1) and was subjected to CM-52 cation-exchange chromatography. One hundred mg dextran-conjugated lysozyme was dissolved in 1 ml 20 mmol/L ammonium carbonate buffer, pH 7.7. The solution was gently mixed and centrifuged at 2500*g* for 10 min to remove undissolved materials. The supernatant was applied to a 5 × 10 cm CM-52 cation exchange column previously equilibrated with 20 mmol/L ammonium carbonate buffer, pH 7.7. One hundred mg unconjugated lysozyme was treated similarly. Proteins were then eluted with the same buffer followed by buffer containing 1 and 2 mol/L NaCl. Samples were collected, dialyzed against distilled water overnight at 4 °C, lyophilized and used for studying functional properties.

Table 1
Factors affecting glycation of lysozyme with dextran[a]

% Glycation			
Temperature	No treatment	Sodium bisulfite (50 mM)	Cysteine (50 mM)
40 °C			
pH 3.5	33	28	5
pH 8.5	57	53	15
60 °C			
pH 3.5	62	63	6
pH 8.5	77	27	1
80 °C			
pH 3.5	67	2	21
pH 8.5	4	4	6

[a] Samples were held at 40 °C and 60 °C for one week and at 80 °C for one day.

2.9. Analytical studies

Total sugar content of proteins was determined by the phenol sulfuric acid method using glucose as standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Number of moles of dextran attached to one mole protein was then calculated taking into account the molecular weight of dextran (10,500, Sigma) and lysozyme (14,600; Liu et al., 2000). Lysozyme activity was determined as described by Imoto and Yagishita (1971). One unit activity of lysozyme is defined as decrease in the absorbance at 450 nm of 0.001 per min at pH 7.0 and 25 °C using *Micrococcus lysodeikticus* cells as substrate. Protein solubility was assessed by measuring protein concentration after solutions of protein were centrifuged according to the method described earlier (Abtahi & Aminlari, 1997). Briefly, 30 mg powdered protein samples were dissolved in 1 ml of appropriate buffer (0.1 mol/L sodium acetate, pH 3.0, 0.1 mol/L sodium phosphate, pH 7.0 or 9.0), mixed thoroughly at 25 °C for 1 h and divided into two equal parts. One part was used for total protein determination by microkjeldahl method (AOAC, 1975) and the other part was centrifuged at 2700*g* for 15 min and the protein content of the supernatant was determined by Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). Solubility was expressed as percent of protein concentration in the supernatant with respect to the total protein content. Effect of temperature on solubility was determined as described above except that the lysozyme samples were dissolved in acetate buffer, pH 3.0 (the optimum pH for solubility, see results section) and heated in water baths maintained at 25, 40, or 60 °C for 48 h. Solubility was measured as above.

The emulsion activity and stability was measure by the method of Pearce and Kinsella (1978). One ml corn oil was added to 3 ml of 1 mg/ml protein in 0.1 mol/L sodium phosphate, pH 7.4 and the mixture was homogenized at fixed speed at 25 °C for 1 min. At 1 min intervals (0 to 10 min), 0.1 ml of the emulsion was removed, added to 5 ml 0.1% SDS and absorbance at 500 nm was recorded immediately and plots of absorbance against time was prepared. Emulsion activity is the absorbance at zero time. The time required to obtain a 50% reduction in absorbance is a measure of emulsion stability.

Heat stability of unconjugated and dextran conjugated lysozyme was determined by measuring the turbidity (absorbance at 500 nm) of protein solutions (7.5 mg per 10 ml 0.1 mol/L sodium phosphate, pH 7.4) held at 50 °C to 95 °C (Nakamura et al., 1991). Starting at 50 °C the temperature was increased 1° per min and absorbance recorded each 5 min.

Duplicate analysis was performed in all analytical studies.

3. Results

Effect of different conditions on the degree of glycation is shown in the chromatograms and SDS –PAGE patterns (Figures 1 to 7). Figs. 1–3 show the effect of pH, temperature and sodium bisulfite or cysteine on the properties of and degree of conjugation of lysozyme with dextran. Results are summarized in Table 1. Samples held at 40 °C for different length of time were white while those kept at 60 °C were yellow and at 80 °C were brown. The color of samples held at pH 8.5 was darker than those held at pH 3.5. In the absence of bisulfite or cysteine and at pH 3.5, the levels of glycation increased with temperature (33%, 62% and 67% at 40, 60 and 80 °C, respectively. At pH 8.5 maximum glycation occurred at 60 °C (77%) as compared with 57% at 40 °C and 4% at 80 °C. High pH and high temperature converted lysozyme into a form which had less affinity for HiTrap cation exchange resin. At pH 8.5, increasing temperature to 80 °C decreased % glycation (Fig. 3, Table 1). Sodium bisulfite decreased glycation at high pH and temperatures above 40 °C. Cysteine essentially prevented glycation at all pH and temperatures (Table 1).

Figs. 4 and 5 show the SDS-PAGE pattern of lysozyme treated under different conditions. These data further confirm the effect of pH, temperature and presence of cysteine and bisulfite on the lysozyme molecule and on the process of glycation, as described for FPLC results.

Fig. 6 shows the kinetics of glycation of lysozyme with dextran at different pH's. An increase in the degree of glycation is observed as the incubation time increased at pH 3.5 or 8.5 (Fig. 6A and B). At pH 3.5, glycation starts at

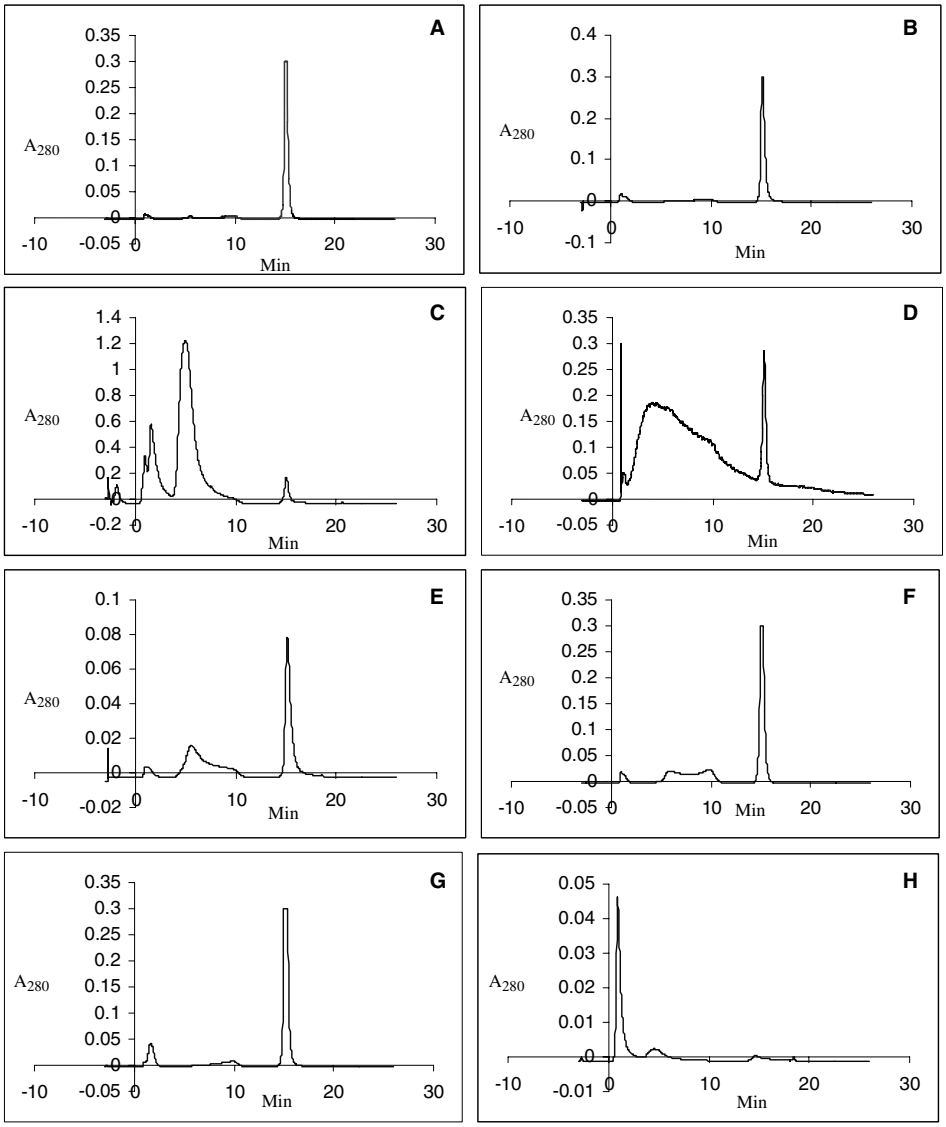


Fig. 1. FPLC results showing the effect of pH and sodium bisulfite and cysteine on the glycation of lysozyme with dextran at 40 °C for one week: (A) lysozyme, pH 3.5, (B) lysozyme, pH 8.5 (C) lysozyme and dextran at pH 3.5, no sodium bisulfite (D) lysozyme and dextran at pH 8.5, no cysteine (E) lysozyme and dextran at pH 3.5, 50 mM sodium bisulfite, (F) lysozyme and dextran at pH 8.5, 50 mM sodium bisulfite, (G) lysozyme and dextran at pH 3.5, 50 mM cysteine, and (H) lysozyme and dextran at pH 8.5, 50 mM cysteine.

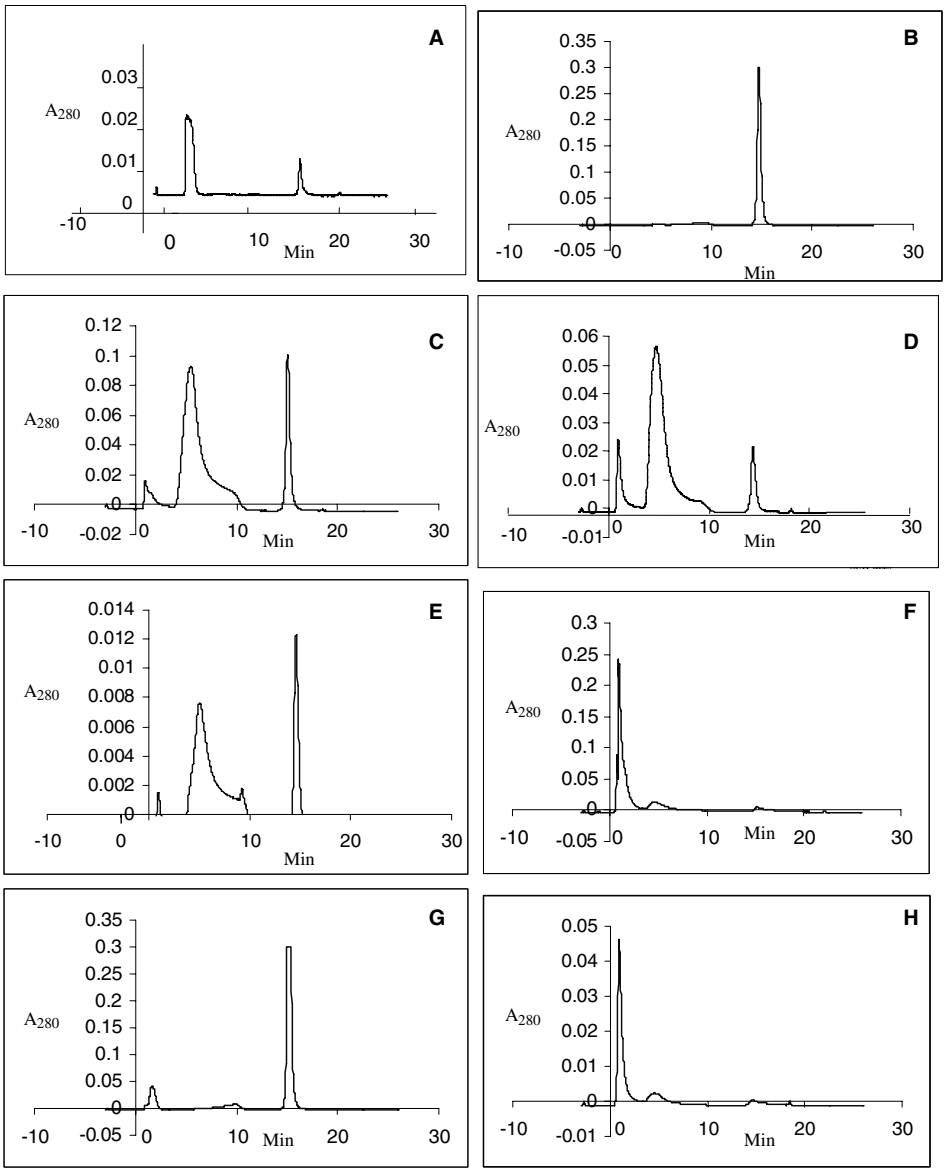


Fig. 2. FPLC results showing the effect of pH and sodium bisulfite on the glycation of lysozyme with dextran at 60 °C for one week: (A) lysozyme, pH 3.5, (B) lysozyme at pH 8.5 (C) lysozyme and dextran at pH 3.5, no sodium bisulfite, (D) lysozyme and dextran at pH 8.5, no sodium bisulfite, (E) lysozyme and dextran at pH 3.5, 50 mM sodium bisulfite, (F) lysozyme and dextran at pH 8.5, 50 mM sodium bisulfite, (G) lysozyme and dextran at pH 3.5, 50 mM cysteine and (H) lysozyme and dextran at pH 8.5, 50 mM cysteine.

24 h and is reached the highest value after one week. Sodium bisulfite did not significantly change the pattern of glycation kinetic at pH 3.5 as evidenced by SDS-PAGE (Fig. 6C). At pH 8.5, glycation started 2 h after incubation and reached the highest value after 48 h.

Urea denaturation prevented glycation of lysozyme with dextran. Denaturation resulted in insolubilization of dried lysozyme, even in the presence of dextran while the undenatured glycated lysozyme was soluble.

The FPLC result of glycation of lysozyme with galactomannan is shown in Fig. 7. At pH 3.5, the level of lysozyme decreased and an increase in the glycation product and a form of modified lysozyme with low affinity for HiTrap cation exchange resin is observed as the time of incubation increased (Fig. 7-A, Table 2). The highest value (88%) was obtained at 48 h. After one week, more than 70% of lysozyme was converted to a product that did not bind to the resin. After 2 weeks essentially all of lysozyme was converted to this form. The same results are observed when the reaction was carried out at pH 8.5 and the highest degree of glycation (92%) occurred after 16 h (Fig. 7-B). Sodium bisulfite did not change the pattern of glycation kinetic as evidenced by SDS-PAGE (Fig. 7-C). No significant reaction occurred between lysozyme and mannan at pH 8.5 and 60 °C for one or two week. Similar results was obtained at pH 3.5.

Fig. 8 shows the results of CM-52 cation exchange chromatography of lysozyme and glycated lysozyme. While

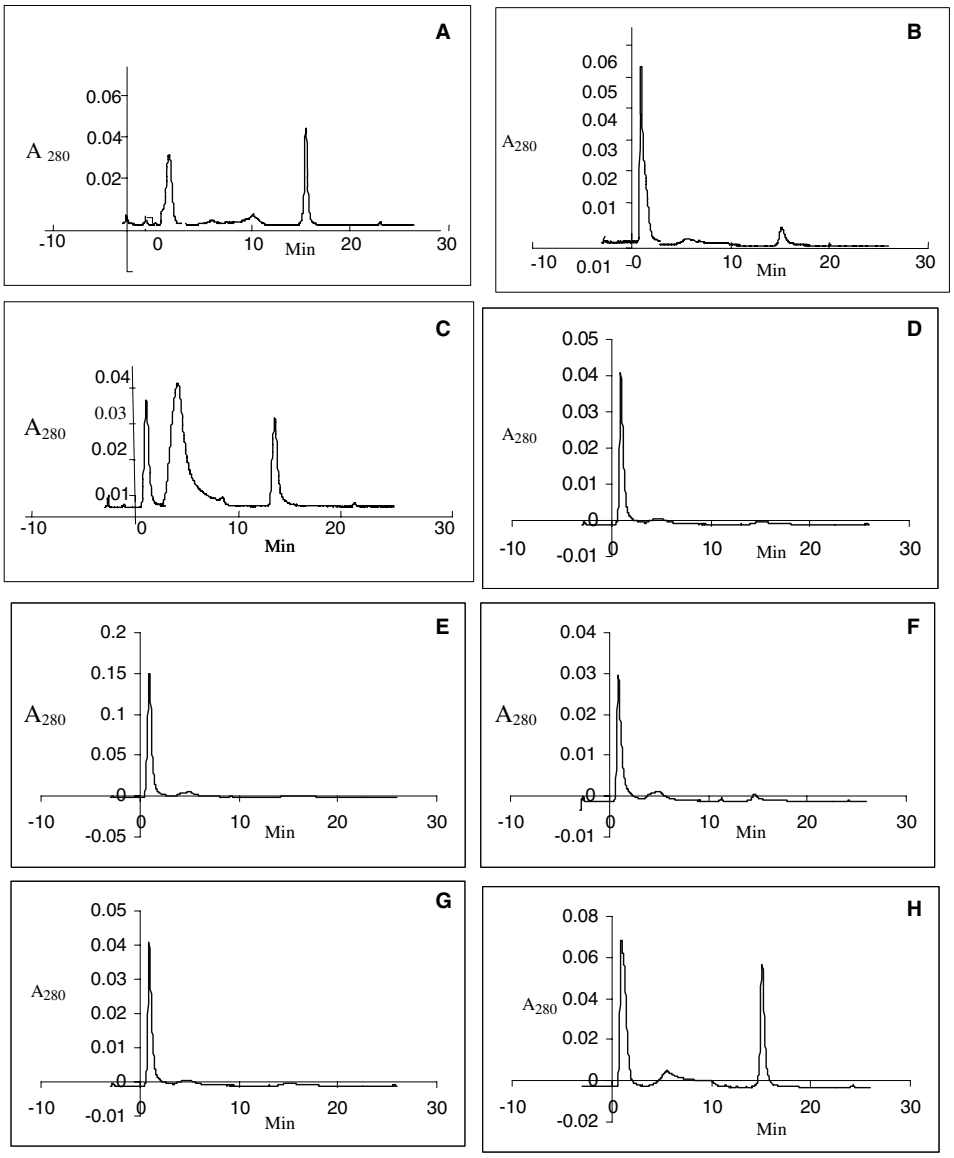


Fig. 3. FPLC results showing the effect of pH and sodium bisulfite on the glycation of lysozyme with dextran at 80 °C for one day: (A) lysozyme, pH 3.5, (B) lysozyme, pH 8.5, (C) lysozyme and dextran at pH 3.5, no sodium bisulfite, (D) lysozyme and dextran at pH 8.5, no sodium bisulfite, (E) lysozyme and dextran at pH 3.5, 50 mM sodium bisulfite, (F) lysozyme and dextran at pH 8.5, 50 mM sodium bisulfite, (G) lysozyme and dextran at pH 3.5, 50 mM cysteine and (H) lysozyme and dextran at pH 8.5, 50 mM cysteine.

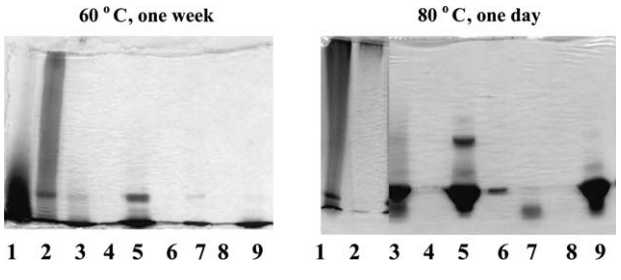


Fig. 4. SDS-PAGE analysis of dextran-conjugated lysozyme in the presence of cysteine 60 °C, one weak 80 °C **one day**: (1) lysozyme + dextran, pH 3.5, (2) lysozyme + dextran, pH 8.5, (3) lysozyme + dextran, pH 3.5, 50 mM cysteine, (4) lysozyme + dextran, pH 8.5, 50 mM cysteine, (5) lysozyme, pH 3.5, (6) lysozyme, pH 8.5, (7) lysozyme, pH 3.5, 50 mM cysteine, (8) lysozyme, pH 8.5, 50 mM cysteine and (9) untreated lysozyme.

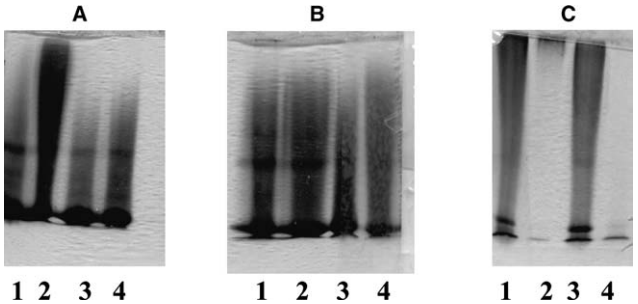
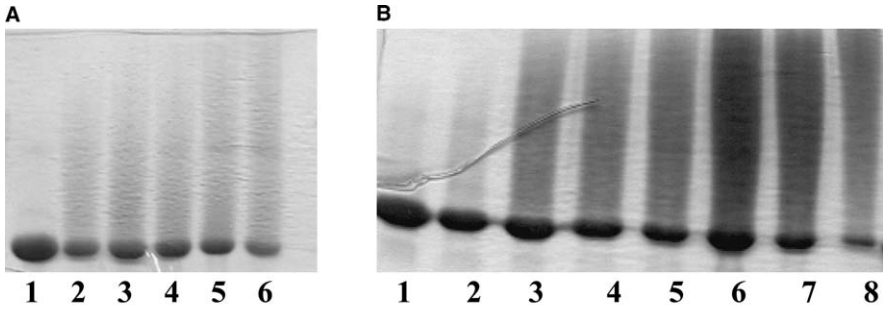


Fig. 5. SDS-PAGE analysis of dextran-conjugated lysozyme at different temperatures in the presence of sodium bisulfite: (A) 40 °C for 1 week (B) 60 °C for 1 week (C) 80 °C for 1 day. (1) Lysozyme + dextran, pH 3.5, (2) lysozyme + dextran, pH 8.5, (3) lysozyme + dextran, pH 3.5, 50 mM sodium bisulfite and (4) lysozyme + dextran, pH 8.5, 50 mM sodium bisulfite.



Panel A		Panel B		Panel C	
1	0 hours	1	0 hour	1	0 hours
2	24 hours	2	2 hours	2	24 hours
3	48 Hours	3	4 Hours	3	48 Hours
4	72 Hours	4	8 Hours	4	72 Hours
5	One week	5	16 Hours	5	One week
6	Two weeks	6	24 Hours	6	Two weeks
		7	48 Hours		
		8	One week		

Fig. 6. Time course of glycation of lysozyme with dextran at pH 3.5 (A) pH 8.5, (B) and pH 3.5 in the presence of 50 mM sodium bisulfite at 60 °C (C). (15% acrylamide gel, 20μg protein per well).

unmodified lysozyme is eluted from the column at 2 mol/L NaCl, the glycated lysozyme was eluted at 1 mol/L NaCl. The fraction shown by horizontal arrow in Fig. 8 (chromatogram B) was essentially free from unmodified lysozyme and was used for analytical studies and determination of functional properties.

Table 3 shows the effect of glycation on several properties of lysozyme. Under best condition glycation resulted in attachment of about 3 mol dextran per mole lysozyme. Glycation at 60 °C for 48 h at pH 8.5 resulted in 20% loss of specific activity (76.5–60.6). Glycation improved the solubility of heated lysozyme at all pH's studied. The best result was obtained when modified lysozyme was held at pH 3.5. Likewise, glycated lysozyme was more soluble than unglycated at different temperatures. Higher temperature slightly decreased solubility of both types of lysozymes. The effect of modification on the heat stability of lysozyme is shown in Fig. 9. Significantly higher turbidity of protein samples was observed at higher temperatures. At all temperatures above 60 °C the absorbances of unmodified lysozyme was significantly higher than those of conjugated lysozyme, indicating resistance of latter to precipitation due to heat. Table 3 further show that the glycation of lysozyme produced a better and a more stable emulsion.

4. Discussion

Carbohydrate–protein conjugates can be produced by Maillard-type reaction by allowing the ε-amino group of lysine residues or N-terminal amino group of proteins to react with the carbonyl group of carbohydrates under controlled temperature, pH and relative humidity (Nakamura et al., 1992a, 1992b). The effect of experimental conditions (pH, temperature, concentration of reactants, etc) on the Maillard reaction has been extensively studied using mainly model systems containing mono- or disaccharides and either small amino group containing molecules such as lysine or proteins such as gluten, casein, soy proteins or ovalbumin (Ajanduz, Tchiakpe, Dalleore, Benajiba, & Puigserver, 2001; Ashoor & Zent, 1984; Bell, 1997; Branss & Van Boekel, 2003; Ellingson, Muller, & Kemmerere, 1954; Feather & Mossine, 1998; Freidman, 1996; Hodge, 1953; Wedzicha & Leong, 1998). The present communication describes conjugation of selected polysaccharides to lysozyme under different conditions by Maillard-type reac-

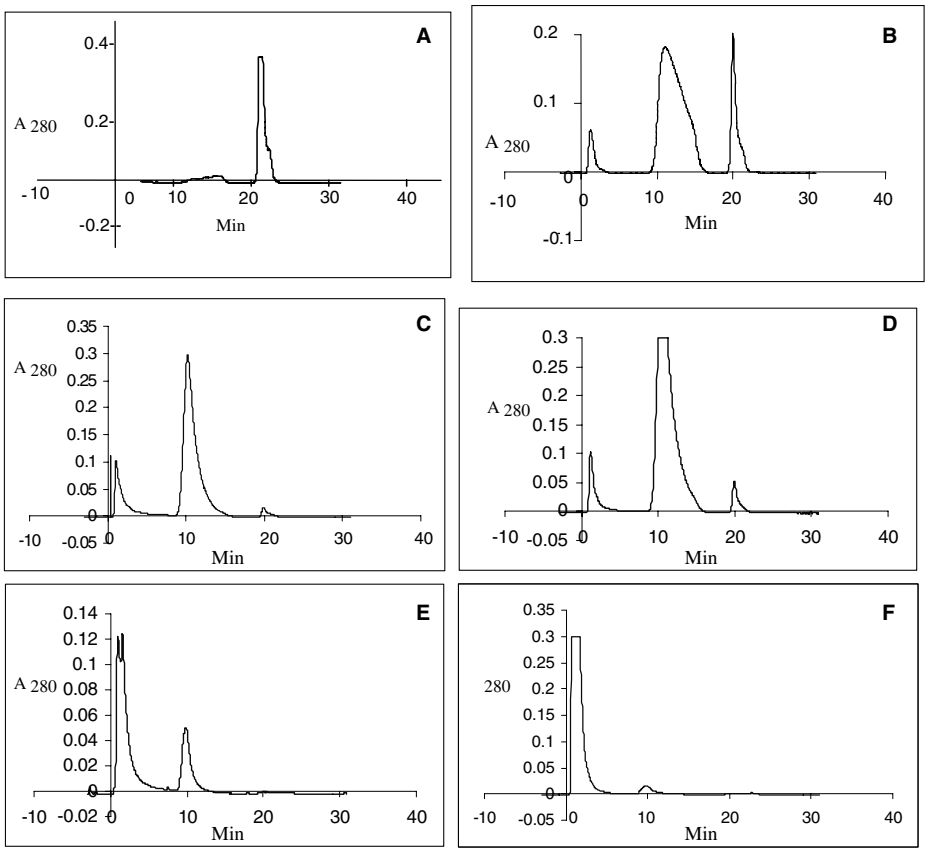


Fig. 7-A. FPLC results of glycation of lysozyme with galactomannan at pH 3.5 and 60 °C: (A) 0 h (B) 24 h (E) one week (C) 48 h (D) 72 h and (F) two weeks.

tion. Degree of covalent attachment was followed by FPLC and SDS-PAGE and molar ratio of polysaccharide attached to lysozyme was calculated considering the molecular weight of polysaccharides and lysozyme. As with low molecular weight sugars, the reaction is strongly affected by pH, temperature and presence of reducing agents. In the absence of sodium bisulfite or cysteine, 77% of lysozyme molecules were glycated at pH 8.5 and 60 °C. While at 80 °C the corresponding value is 67% at pH 3.5, it is reduced to 4% at pH 8.5. These data suggest that a combination of high pH's and temperatures are not suitable for glycation and these conditions should be avoided in foods in which protection of protein by conjugation with polysaccharides is desired. As shown in Figs. 2(B), 3(B), 5 (samples 6), in the absence of dextran, lysozyme treated at pH 8.5 and temperatures above 60 °C is converted to a form which does not bind to the cation exchange resin used in this study. It has been reported that lysozyme heated at 80 °C and pH 7 experiences extensive unfolding and exposure of hydrophobic groups on the surface of the molecule (Ibrahim et al., 1996a, Ibrahim, Higashiguchi, Juneja, Kim, & Yamamoto, 1996b). The lysozyme prepared in this study under above mentioned condition was very insoluble. Although the samples used for FPLC and SDS-PAGE were centrifuged, the lysozyme molecules were probably denatured and aggregated. When dextran was present in the reaction mixture, some degree of protection was achieved against this phenomena at 60 °C (Fig. 2, compare B with D) but not at 80 °C (Fig. 3, compare B with D). These data are further confirmed by the reaction of urea-denatured lysozyme.

Presence of sodium bisulfite or cysteine inhibited glycation under all conditions. It is well established that sodium bisulfite and sulfhydryl containing compounds inhibit Maillard reaction in model systems (Freidman, 1996; Freidman & Molnar-Perl, 1990) and in protein and carbohydrate containing foods (Molnar-Perl & Freidman, 1990; Freidman, Molnar-Perl, & Knighton, 1992). The inhibitory effect of these compounds is due to a multiplicity of the mechanisms, including, among many mechanisms, their ability to act as reducing agents in vivo and in vitro and strong nucleophilicity that can trap electrophilic compounds and intermediates (Finot, 1990; Freidman, 1996; Voldrich, Dupont, Dobios, & Philipson, 1995). Bisulfite was less effective in preventing glycation at low pH as compared with high pH. Bisulfite is in equilibrium with other derivatives of sulfur dioxide, including sulfurous acid and metabisulfte, the proportion of each derivative depends on the pH of the medium, ionic strength of the sulfur compound, and the concentration of neutral salts. Metabisulfite ($S_2O_5^{2-}$) and SO_2 are the predominant derivatives at high and low pH's, respectively (Wedzicha, 1984). This

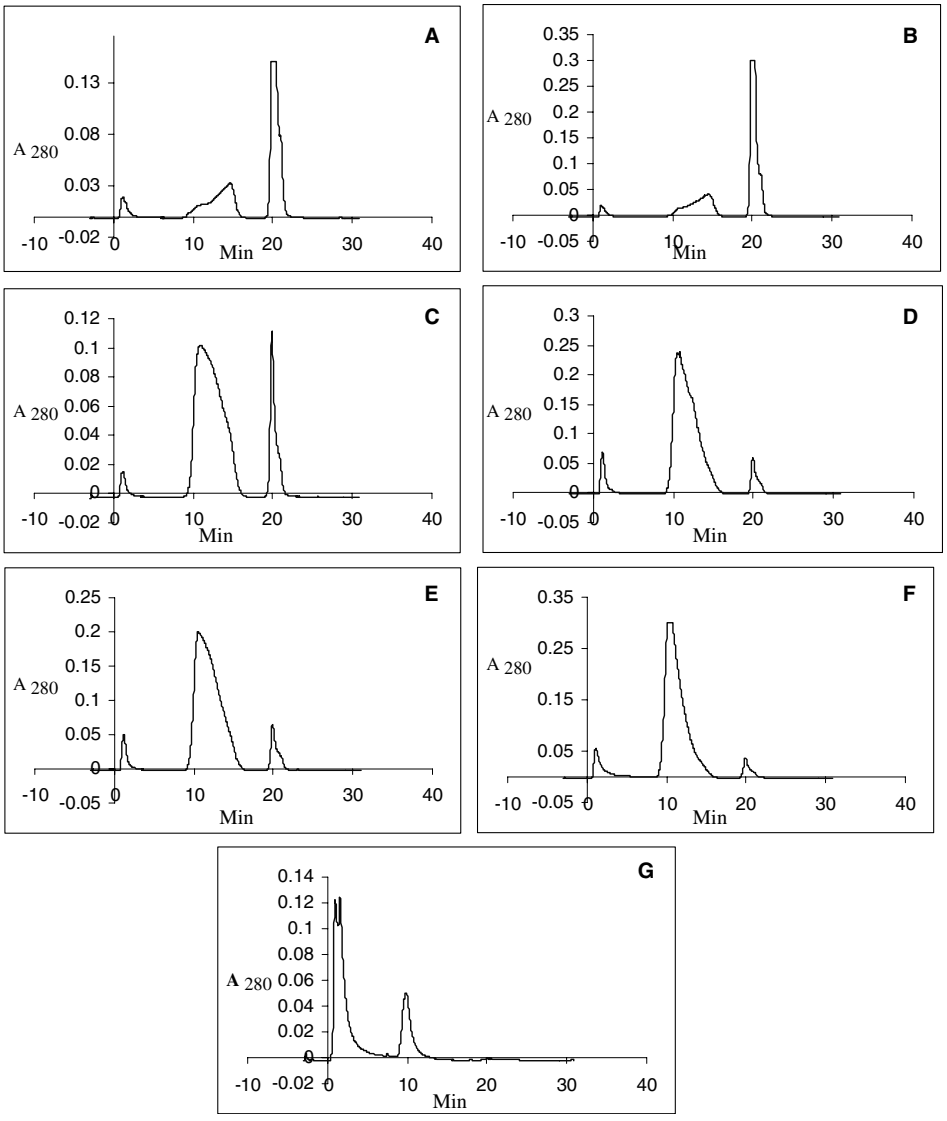


Fig. 7-B. FPLC results of glycation of lysozyme with galactomannan at pH 8.5 and 60 °C: (A) 0 h (B) 2 h (C) 8 h (D) 16 h (E) 24 h (F)48 h and (G) one week.

phenomena might explain the inability of bisulfite to inhibit glycation at low pH, i.e. unavailability of bisulfite due to its conversion to SO_2 at low pH's.

The shape and the retention time of the glycated peaks in FPLC and the extensively diffused bands in SDS-PAGE is an indication of the multiplicity of conjugated derivatives obtained during the course of reaction of lysozyme with dextran. These multiple forms probably originate from molecules with different number of polysaccharides attached to each molecule of lysozyme, extensive variability in conformation of different molecules, disulfide-sulfhydryl exchange reactions, protein–protein interaction, isopeptide bond formation, or other unknown mechanisms, that might affect the retention time in FPLC and the electrophoretic mobility (Diftis & Kiosseoglou, 2003). Lysozyme has seven free amino groups, two of which have been shown to be available to participate in Maillard reaction (Nakamura et al., 1991, Nakamura, Gohya, & Kato, 1996; Takahashi et al., 2000). The amino terminal Lys-1 and Lys-98 are the two binding sites for polysaccharides (Nakamura et al., 1996). However, our results, based on the calculation of the molar ratio of dextran attached to lysozyme at optimum condition, indicate modification of three amino groups. The higher yield obtained might be due to the higher pH (8.5) used in this study. As the calculated number of attached molecules is in fact an average, one might expect presence of multiple molecular forms, containing 0–3 moles dextran, as evidenced by FPLC and SDS-PAGE results. The position of the third amino groups reacting with dextran remains to be determined. Yeboah et al. (2004) have shown three sites for glycation of lysozyme with fructose and glucose. These sites include the ε-amino group of Lys-1, Lys-33 and Ly-98. By use of ESI-MSMS peptide mapping, molecular modeling and trypsin digestion these authors showed that glycation results in global conformation change in lysozyme molecule.

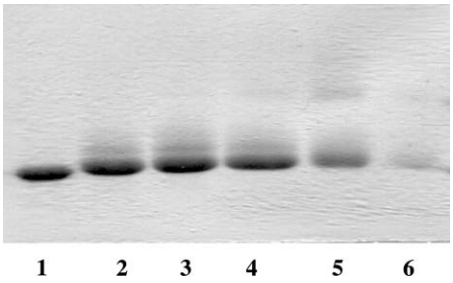


Fig. 7-C. Kinetics of glycation of lysozyme with galactomannan at pH 3.5 and 60 °C. (15% acryl amide gel, 20 μg protein per well): (1) 0 h, (2) 24 h (3) 48 h (4) 72 h (5) one week, and (6) two weeks.

The data on time course of reaction of lysozyme with dextran (Fig. 6) emphasizes the effect of pH and temperature on the rate of Maillard reaction involving polysaccharides and suggest that different rates of glycation is expected in foods containing different polysaccharides at different pH and temperatures. Again, sodium bisulfite can decrease the rate of the reaction. The results further show that different polysaccharides have different reactivity toward lysozyme under similar conditions (Fig. 7 and results on mannan). Galactomannan readily react with lysozyme to give the highest yield of glycated product under the optimum condition of pH and temperature (i.e. pH 8.5 at 60 °C for 48 h) (Table 2). Similar to the reaction with dextran, higher temperatures for longer period of time was unfavorable for glycation and an increase in the proportion of the form with no affinity for binding to the cation exchange resin was resulted (peak I, Table 2 and Fig. 7). FPLC results indicate that the peak corresponding to the galactomannan conjugated lysozyme (Fig. 7 and peak II in Table 2) are sharper with a longer retention time than the similar peak obtained with dextran. Similarly SDS-PAGE showed the bands related to the galactomannan conjugated lysozyme is less diffused than that obtained with dextran conjugated lysozyme. These results suggest that the glycated product of lysozyme with guar gum galactomannan, a linear heteropolysaccharide made of β-(1,4)-linked D-mannose with small side chain consisting of 1, 6-linked α-galactose units attached to every other mannose residues (McCleary, Clark, Dea, & Rees, 1985), is less heterogeneous than the product obtained with dextran. No attempt was made to determine the number of galactomannan molecules attached to one molecule of lysozyme. Nakamura et al., 1996 reported 2 moles of galactomannan attached to one mole lysozyme when the reaction was carried out in water at 60 °C. Mannan, a homopolysaccharide made of α-(1-2)- and of α-(1-6)-linked D-mannose residues (Kobayashi et al., 1994) did not react with lysozyme under the conditions used in this study. The differential reactivity of different polysaccharide observed in this study might be explained in terms of differences in conformations, in sizes, or in solubility of these polymers (Bemiller & Whistler, 1996).

Both steric hindrance and blocking of the positive charges might be responsible for the decrease in lytic activity of the enzyme observed in this study (Table 3). Similar

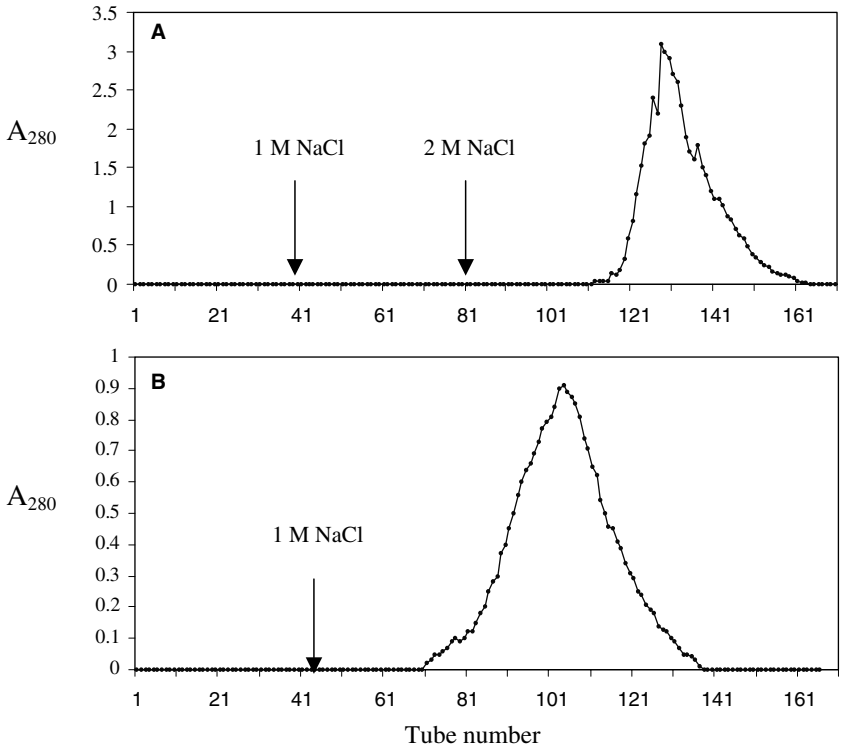


Fig. 8. CM-52 chromatography of unmodified lysozyme (A) and dextran-congugated lysozyme (B) (Glycated lysozyme was prepared under optimum condition (i.e. 60 °C, pH 8.5 for 48 h, see results, Table 1) and contained 3 moles dextran per mole lysozyme (Table 3)).

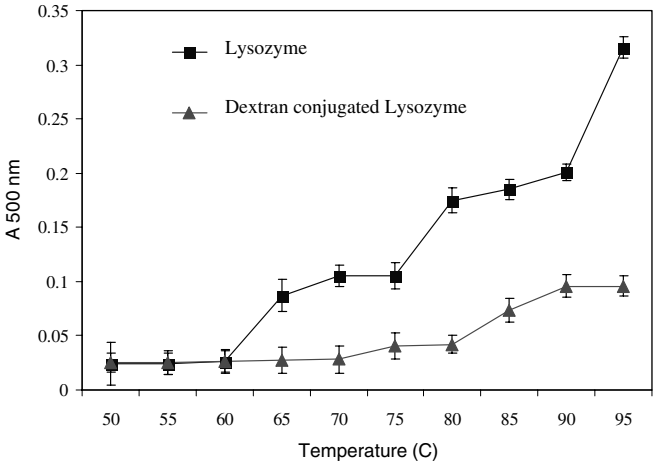


Fig. 9. Heat stability of lysozyme and dextran conjugated lysozyme.

Table 2
Glycation of lysozyme with galactomannan at 60 °C

Incubation Time	%		
	Peak I	Peak II	Peak III
pH 3.5			
0 h	2	10	88
24 h	5	76	20
48 h	8	88	4
72 h	14	84	2
One week	65	35	0
Two weeks	97	3	0
pH 8.5			
0 h	2	8	90
2 h	4	6	90
8 h	2	81	17
16 h	5	89	6
24 h	5	88	7
48 h	4	92	4
One week	53	47	0

Peak I: fraction with no affinity for Hitrap cation exchange resin, Peak II: glycated lysozyme, Peak III: unmodified lysozyme.

results have been reported by other investigators using different molecule binding to lysozyme, including fatty acid (Ibrahim, Kato, & Kobayashi, 1991; Liu et al., 2000), perillaldehyde (Ibrahim, Hatta, Fujiki, Kim, & Yamamoto, 1994), glucose–stearic monoester (Takahashi et al., 2000) and dextran (Nakamura et al., 1991). Most of these investigations have shown that the modified enzymes retain their antimicrobial properties in spite of loss of some lytic activity. The antimicrobial effect of the highly conjugated lysozyme in different foods is under current study in this laboratory.

Marked improvement in solubility at low and high pH's and temperatures and in emulsion activity and stability of the dextran-conjugated lysozyme was obtained in agreement with results of other investigators. Improved antimicrobial and functional properties of dextran- and galactomannan-lysozyme conjugates was reported (Nakamura et al., 1991,

Table 3
Mean (and range) of enzymatic activity pH stability, heat stability and emulsion activity and stability of lysozyme and modified lysozyme[a]

Lysozyme samples	Mg dextran per Mg protein	Mole dextran per mole lysozyme	Units per mg protein	% Solubility at different pH's[a,b]			% Solubility at different temperatures (°C)[a,b]			Emulsion activity (A_{500} nm)	Emulsion stability (Min)
				3	7	9	25	40	60		
Lysozyme[c]	0[a]	0[a]	76.5[a] (±2.0)	$81^{a,A}$(±1)	$72^{a,B}$(±1)	$62^{a,C}$(±2)	$81^{a,A}$(±1)	$78^{a,B}$(±2)	$68^{a,C}$(±3)	$0.11^{a,A}$(±0.01)	$0.1^{a,B}$(±0.01)
Dextran-conjugated lysozyme[d]	0.598[b](±0.087)	2.99[b](±0.17)	60.6[b](±2.8)	$99^{b,A}$(±1)	$78^{b,B}$(±1)	$83^{b,C}$(±1)	$99^{b,A}$(±0.5)	$92^{b,B}$(±1)	$89^{b,C}$(±1)	$0.40^{b,A}$(±0.02)	$2.02^{b,B}$(±0.07)

[a] In each column different superscript letters indicate significant difference ($P < 0.05$).
[b] In each row different superscript uppercase letters indicate significant difference ($P < 0.05$).
[c] Sample from Fig. 8A.
[d] Sample from Fig. 8B.

1992b, Nakamura, Kobayashi, & Kato, 1994). Polymannosylated lysozyme was produced by site-directed mutagenesis and was shown to have improved emulsifying properties (Shu et al., 1998). Some other proteins which have been shown to attain improved functional properties due to glycation include β-lactoglobulin (Hattori, Numamoto, Kobayashi, & Takahashi, 2000a, 2000b; Kitabatake, Cuq, & Cheftel, 1985), ovalbumin (Kato, Takayoshi, Kato, Nakamura, & Matsuda, 1995, 1981, 1988, 1990b), soy bean proteins (Babiker et al., 1998; Diftis & Kiosseoglou, 2003) and gluten (Kato, Shimokawa, & Kobayashi, 1991). These effects are probably due to the high hydration capacity of attached carbohydrates (Nakamura et al., 1991, 1992b, 1994). Majority of the studies on lysozyme were performed in an aqueous solution of the protein and carbohydrate followed by a prolonged (two weeks) heating of the lyophilized and dried powder at 60 °C which resulted in attachment of 2 moles polysaccharide per mole lysozyme (Nakamura et al., 1991, 1992b, 1994). A more efficient glycation was achieved in our study by performing the experiment at pH 8.5, thereby reducing the incubation time at 60 °C to 48 h. This treatment resulted in heat, pH and emulsion stability at least comparable with those reported by other investigators.

Taken together, the results of this study shows that a more efficient and rapid glycation of lysozyme with excellent functional properties can be achieved by controlling pH, temperature and time of reaction while sodium bisulfite can be used to reduce degree of glycation. On the other hand, cysteine at 50 mmol/L can be used to prevent or stop this reaction. Furthermore, due to their improved pH and heat stability, the modified lysozyme might be used in a vast variety of foods processed at different pH and temperatures, without adverse effects on their beneficial properties.

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