Modified Lysozymes as Novel Broad Spectrum Natural Antimicrobial Agents in Foods

Ladan Aminlari, Marjan Mohammadi Hashemi, and Mahmoud Aminlari

Abstract: In recent years much attention and interest have been directed toward application of natural antimicrobial agents in foods. Some naturally occurring proteins such as lactoperoxidase, lactoferrin, and lysozyme have received considerable attention and are being considered as potential antimicrobial agents in foods. Lysozyme kills bacteria by hydrolyzing the peptidoglycan layer of the cell wall of certain bacterial species, hence its application as a natural antimicrobial agent has been suggested. However, limitations in the action of lysozyme against only Gram-positive bacteria have prompted scientists to extend the antimicrobial effects of lysozyme by several types of chemical modifications. During the last 2 decades extensive research has been directed toward modification of lysozyme in order to improve its antimicrobial properties. This review will report on the latest information available on lysozyme modifications and examine the applicability of the modified lysozymes in controlling growth of Gram-positive and Gram-negative bacteria in foods. The results of modifications of lysozyme using its conjugation with different small molecule, polysaccharides, as well as modifications using proteolytic enzymes will be reviewed. These types of modifications have not only increased the functional properties of lysozyme (such as solubility and heat stability) but also extended the antimicrobial activity of lysozyme. Many examples will be given to show that modification can decrease the count of Gram-negative bacteria in bacterial culture and in foods by as much as 5 log CFU/mL and in some cases essentially eliminated *Escherichia coli*. In conclusion this review demonstrates that modified lysozymes are excellent natural food preservatives, which can be used in food industry.

Keywords: chemical-enzymatic modifications, conjugation, glycation, lysozyme, Maillard reaction, natural antimicrobial

Practical Application: The subject described in this review article can lead to the development of methods to produce new broad-spectrum natural antimicrobial agents, based on modification of chicken egg white lysozyme, which might potentially replace the currently used synthetic food preservatives.

Introduction

In recent years consumer demand for "natural" foods has driven development of products without additives. In order to meet this demand much attention and interest have been directed toward identification and application of naturally made compounds such as antimicrobial agents, in food and pharmaceuticals (Branen and Davidson 2004). Some naturally occurring proteins such as lactoperoxidase, lactoferrin, and lysozyme have received much attention and are being considered as potential antimicrobial agents to replace the currently used synthetic food preservatives. (Proctor and Cunningham 1988; Santos and others 1994; Brown and others 2008; Clardy and others 2009; Demain 2009). Lysozyme (muramidase EC: 3.2.1.17) has been found in many different sources, from viruses to vertebrates, and has been subjected to extensive scrutiny, both as a protein model and a natural antimicrobial and pharmaceutical agent. Chicken egg white has the highest content of lysozyme, from which this enzyme is purified and is commercially produced (Proctor and Cunningham 1988).

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Lysozyme can hydrolyze or dissolve bacterial cell wall, which is how it received its name. The cell wall of Gram-positive bacteria (that is, the peptidoglycan frame wrapping around the bacterial cells) is the main substrate for lysozyme, hence the susceptibility of this group of bacteria to lysozyme. Gram-negative bacteria are resistant because of the presence of the outer lipopolysaccharide (LPS) layer in their cell wall (Jolles and Jolles 1984; Masschalck and Michiels 2003; Seo and others 2013). Many attempts to broaden the antimicrobial spectrum of lysozyme have been made in the past decade or so. Several laboratories, including the authors', have shown that it is possible to broaden the antimicrobial effect of lysozyme by several types of modifications, which consequently make these lysozyme derivatives excellent food preservatives. The purpose of this communication is to review the production and application of several of these lysozyme derivatives.

Lysozyme molecule

In 1963, Canfield reported the chemical composition of the chicken egg white lysozyme and determined its amino acid sequence and showed that the molecular weight of this molecule was 14307 (Canfield 1963). Chicken lysozyme was the 1st enzyme of which the amino acids sequence was obtained allowing determination of the 3-dimensional structure by X-ray crystallography. From this information, a detailed mechanism of action of lysozyme was proposed (Jolles and others 1963; Blake and others 1965; Rezaei Behbehani and others 2011). The hen egg white lysozyme is a single polypeptide chain made of 129 amino acids

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and has mainly α helical conformation with 4 disulfide bonds which cross-link cysteines residues 64 to 80, 76 to 94, 6 to 127, and 30 to 115 (Vaney and others 1996; Wang and others 2009).

Lysozyme utilizes the mucopolysaccharide part of the peptidoglycan in the bacterial cell wall as substrate. The mucopolysaccharide structure consists of alternate N-acetylmuramicacid and N-acetylglucosamine units linked by a β, 1–4 glycosidic linkage. These polysaccharide molecules are cross-linked by short peptides. Lysozyme hydrolyzes the β (l-4) glycosidic bonds, thereby lysing the bacterial cell wall, resulting in the death of bacterial cells (Figure 1; Pellegrino and Tirelli 2000).

Hen egg white lysozyme belongs to O-glycosyl hydrolases (EC 3.2.1.x) which comprise more than 45 different families, varying in substrate specificity and the mode of action, mostly governed by the details of their 3-dimensional structures (Davies and Henrissat 1995). Glycoside hydrolases are a widespread group of enzymes, which hydrolyse the glycosidic bond between 2 or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety (glycoside hydrolase family classification: http://www.cazy.org/Glycoside-Hydrolases.html). According to CAZy database, chicken lysozyme is a member of eukaryotic glycoside hydrolases (Family 22, C-type), which have sequence homology with the serum milk protein α-lactalbumin. Hydrolysis of the glycosidic bond by the enzymes of this family requires 2 critical residues: a proton donor and a nucleophile/base. The mechanistic studies have established that the aspartic acid residue in the active site must be in its de-protonated state while glutamic acid must be protonated (Dickerson and Geis 1969; Proctor and Cunningham 1988). In hen egg white lysozyme, the enzymatic hydrolysis of the substrate C2 acetamido group also participates in catalysis. The hydrolysis occurs via a mechanism in which anomeric configuration is retained (McCarter and Withers 1994). This is opposed to the alternative mechanism in which a product with opposite stereochemistry is formed.

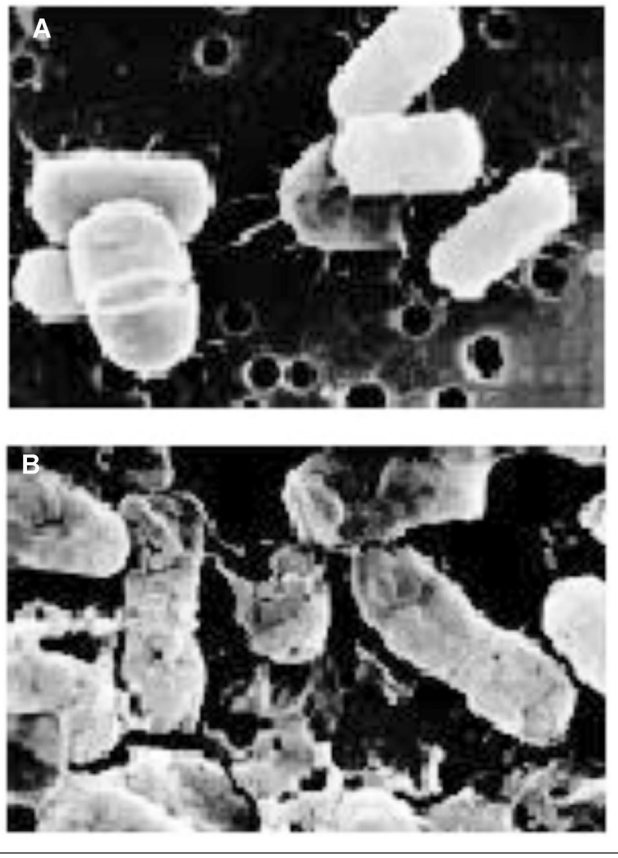


Figure 1–The lytic action of lysozyme on the cell wall of *C. tyrobutyricum*:. (A) no lysozyme, (B) in the presence of lysozyme (Reprinted with permission from Neova Technologies Inc. [1999], copyright Neova Technologies Inc.).

The sequence of events leading to the hydrolysis of glycosidic linkage in the oligosacharide substrate by hen egg white lysozyme is very well elucidated (Strynadka and James 1991). In the well-established model, the unionized proton of Glu-35 attacks the C1−O oxygen weakening the C−O glycosidic linkage. Breakage of the bond leads to the formation of an oxycarbonium ion, which is stabilized by nearby Asp-52. In the next step, the Glu-35 proton is replaced by another from ionizing water molecule and the resulting hydroxyl ion then attacks the carbonium ion and completes the reaction (Strynadka and James 1991).

By using electrospray ionization mass spectrometry, Vocadlo and others (2001) showed formation of a covalent glycosyl-enzyme intermediate during the catalytic cycle of hen egg white lysozyme. The mechanism proposed by these authors includes distortion of substrate, formation of a covalent intermediate, and the electrophilic migration of C1 along the reaction coordinate for all retaining beta-glycosidases.

Another chicken lysozyme is the family 23, G-type lysozyme, which corresponds to family 1 of the peptidoglycan lytic transglycosylases and cleaves peptidoglycan without intervention of a water molecule (Blackburn and Clarke 2001).

Lysozyme sources and its role as antimicrobial agent in body defense mechanism. Lysozyme is present in many biological sources and is produced by viruses, bacteria, fungi, plants, and various tissues and fluids of birds and mammals (such as avian eggs and animal secretions including tears, saliva, and respiratory and cervical secretions; Jolles and Jolles 1984; Tenovuo and others 1991; Oliver and Wells 2013). Lysozyme is one of the major components of the breast milk in terms of providing some defense mechanism against infection (Lönnerdal 2003). In the gastrointestinal tract, it can perform the function of protection (Tenovuo and others 1991). Lysozymes are defense mechanism provided by nature against bacterial infection in plants and animals. Lysozyme is considered as an important innate immune component of the most mammals including humans (Varahan and others 2013). Some bacteriophages produce lysozyme that can hydrolyse the peptidoglycans of the bacterial cell wall, thereby allowing penetration of the phage (Smith and others 2013).

Lysozymes are classified into 6 types: phage-type (T4 phage lysozyme), plant lysozyme, G-type (goose-type lysozyme, bacterial lysozyme), C-type (which includes chicken lysozyme, stomach lysozyme, and calcium-binding lysozyme), and i-type (invertebrate lysozyme) (Wang and others 2011, Wang and others 2013a, 2013b). The antimicrobial effects of C-type lysozyme does not depend on its enzymatic activity since partially denatured lysozyme shows bactericidal activity against Gram-negative bacteria while retaining its activity against Gram-positive bacteria (Ibrahim and others 1996; Masschalck and others 2001). Heating lysozyme at elevated temperature resulted in loss of enzymatic activity while its antimicrobial action against Gram-negative bacteria was enhanced, suggesting that this action is independent of catalytic function of lyzozyme, but might be related to a heat-induced change in conformation of the enzyme molecule, rendering bacterial membrane more permeable (Ibrahim 1998).

In general, lysozyme shows *in vitro* antimicrobial activity against some Gram-positive bacteria such as *Staphylococcus aureus*,

Micrococcus luteus, *Bacillus stearothermophilus*, *Clostridium tyrobutyricum* but little action against Gram-negative bacteria (Cunningham and others 1991). Other important functions of lysozyme include antiviral and antitumor properties, immune modulatory, and anti-inflammatory activities (Mo 1998; Ibrahim and Akoi 2003; Mao and others 2005; Pellegrini1 and Engels 2005). Invertebrates do not produce immunoglobulins. In these organisms, lysozyme may serve as a primary protective system (Wei and others 2012; Wang and others 2013b). High concentrations of lysozyme in the egg white of many birds provide protective vigilance for developing embryo until immunoglobulins are produced at hatching (Jolles and Jolles 1984).

Gram-negative bacteria, including foodborne pathogens, resist lysozyme due to steric hindrance posed by the outer LPS layer, hence lysozyme cannot access the peptidoglycan (Ibrahim and others 1991). Thus modification of lysozyme, which can broaden its antibacterial properties against both Gram-negative and Gram-positive bacteria, can also increase the usefulness of lysozyme (Seo and others 2013).

Lysozyme as a food preservative

Lysozyme has received considerable interest as a food preservative (Proctor and Cunningham 1988; Cegielska-Radziejewska and Szablewski 2013). Since lysozyme prevents growth of deleterious bacteria, it can be acceptable to apply it in food processing to prolong shelf life of foods, especially when used in accordance with good manufacturing practice (World Health Organization [WHO] 1993). Use of lysozyme in preventing late blowing in hard cheeses caused by *Clostridium tyrobacterium* (Pellegrino and Tirelli (2000)) is practiced in the dairy industry (Intl. Dairy Federation Bulletin No. 216/1987). In many countries, lysozyme is used as a preservative in many types of foods (including vegetables, sea foods, soy bean products, meat products, and semihard cheeses, and as a component of pharmaceutical products; Cunningham and others (1991; Losso and others 2000; Naidu 2000; Gill and Holley 2003). In the European Union, its use in specific products such as hard cheeses and in wine making to control infection (E number E 1105 as a food additive) is allowed (Losso and others 2000). Proctor and Cunningham (1988) and Cunningham and others (1991) have reviewed the use of lysozyme as a food preservative and the factors affecting lysozyme activity. Dubois-Prevost (1970) reviewed the use of lysozyme in formula milk for infant feeding to aid digestibility. Lysozyme has a pI of more than 10 and its positive charge at pH 7 is believed to be responsible for its antiviral activities (Cisani and others 1984). Lysozyme as an antimicrobial agent for treatment of processed ham and bologna was reported (Gill and Holley 2003).

Lysozyme modifications

Chemical modification is widely used in proteins chemistry as a means to produce derivatives with novel functional properties useful to food manufacturers (Kinsella 1976; Carne 1994; El-Adawy 2000; Niu and others 2011; Seo and others 2013). Some common chemical modifications include acetylation, amidation, esterification, succinylation, phosphorylation, thiolation, and glycosylation (Kester and Richardson 1984; Means and Feeney 1998). For example, glycosylation can change the hydrophilicity or net charge of protein surface. These alterations can affect protein–protein or protein–water interaction and bring about changes in isoelectric point and conformation of protein, leading to significant changes in solution and interfacial behavior of glycosylated proteins (Aminlari and others 2005; Alahdad and others 2009; Corzo-Martinez and others 2010; Seo and others 2013).

During the last 2 decades or so extensive research has been directed toward modification of lysozyme in order to improve its antimicrobial properties. Several chemical and enzymatic procedures have been developed to alter and/or improve these properties. In the following section, some of these developments are reviewed.

Modification using small molecules

Lipophilization is a method for altering the properties of proteins. Ibrahim and others (1991) covalently attached palmitic acid to chicken egg white lysozyme using N-hydroxysuccinimide ester of the palmitic acid. Under optimum conditions 2 moles palmitic acid were attached to 1 mole lysozyme with little effect on its enzymatic activity. Extensive conjugation resulted in loss of lytic activity. The decrease in lytic activity was proportional to the increase in the degree of modification with palmitic acid. The conjugated lysozyme showed a substantial antimicrobial activity against Gram-negative bacteria such as *Escherichia coli* and *Edwardsiella tarda*. Authors concluded that extended antimicrobial activity was due to insertion of lysozyme–palmitoyl conjugate in the LPS layer of cell wall membrane. In an attempt to improve the bacteriocidal action of lysozyme, short and middle chain saturated fatty acids (caproic, capric, or myristic acid) were attached to lysozyme (Liu and others 2000a). Lipophilization broadened the antimicrobial effect of lysozyme to Gram-negative bacteria without decreasing enzymatic activity. The bactericidal activity increased with increase in the number of short chain fatty acids bound to lysozyme. Lipophilization results in low recovery of enzyme activity. However, this may be overcome by glycosylation of lysozyme before lypophilization. Liu and others (2000b) used Maillard reaction to glycosylate hen egg white lysozyme followed by lipophilization using palmitic acid. This procedure increased the yield of the modified enzyme, and the derivatives demonstrated strong antimicrobial activity against *E. coli*. The authors concluded that combined glycosylation and lipophilization of lysozyme can be a promising method for potential industrial applications of the lysozyme (Liu and others 2000a, 2000b).

Takahashi and others (2000) prepared a lysozyme–glucose stearic acid monoester conjugate. In this type of modification, neutral saccharide and fatty acyl moieties are combined and attached to lysozyme molecule, which resulted in lowering the isoelectric pH of lysozyme to 6 to 7, much lower than that of native lysozyme, and increase in denaturation temperature to 74 °C. The modified lysozyme showed considerable resistance to proteolysis. Furthermore, the emulsifying activity and the emulsion stability of the conjugate were markedly improved. The conjugate maintained more than 50% of its enzymatic activity while retaining 70% of the bactericidal activity (Takahashi and others 2000).

Perillaldehyde, or perilla aldehyde, is a phenolic aldehyde found most abundantly in the annual herb "perilla," but also in a wide variety of other plants and essential oils. It is a monoterpenoid containing an aldehyde functional group. Perillaldehyde, or volatile oils from perilla that are rich in perillaldehyde, are used as "food additives," as flavoring and antimicrobial and in perfumery to add spiciness (Sonboli and others 2012). The aldehyde group can participate in Maillard reaction. This functional group was used to attach perillaldehyde to lysozyme by Ibrahim and others (1994a). The modification resulted in derivatives in which about 0.5 to 4 amino groups were blocked by perillaldehyde. Modification with the highest degree of conjugation retained more than 70% of the enzymatic activity as compared with that of native lysozyme and

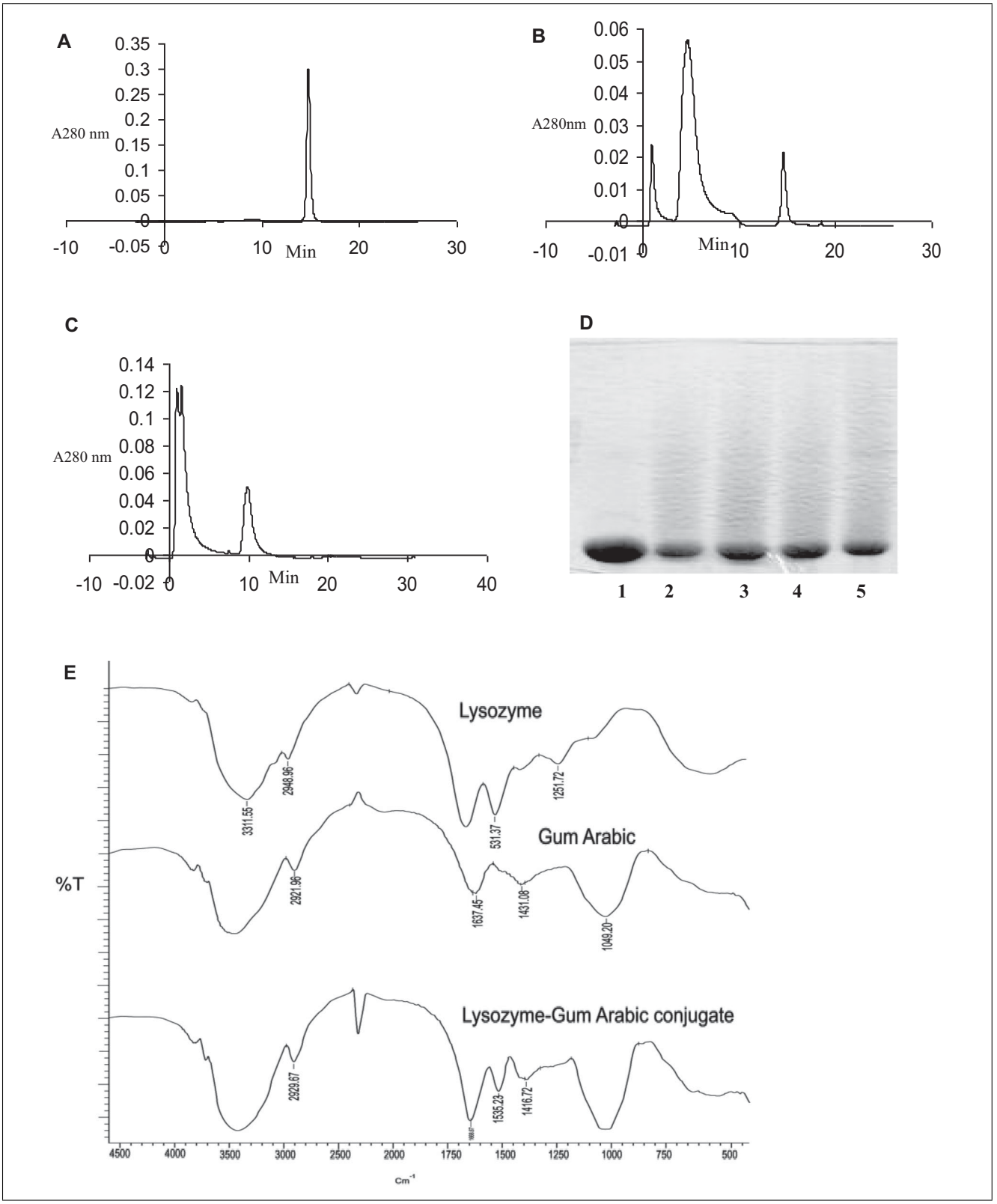


Figure 2–The effect of conjugation of lysozyme with dextran and galactomannan. (A) Lysozyme alone in buffer at pH 7.0 heated at 60 °C for 1 wk. (B) Lysozyme:dextran (1:5 weight ratio, heated at 60 °C for 1 wk; from Scaman and others 2006). (C) Lysozyme:guar gum galactomann (1:5 weight ratio, heated at 60 °C for 1 wk; from Scaman and others 2006). (D) Time course of conjugation of lysozyme with dextran heated at 60 °C (15% acryl amide gel, 20 μg protein per well) 1: 0 h, 2: 24 h, 3: 48 h, 4: 72 h, and 5: 1 wk (from Scaman and others 2006). (E) FT-IR spectra of gum Arabic, lysozyme, and lysozyme–gum Arabic conjugate (from Mohammadi Hashemi 2012).

Table 1–Free amino groups and the enzymatic activity of lysozyme–dextran conjugate.

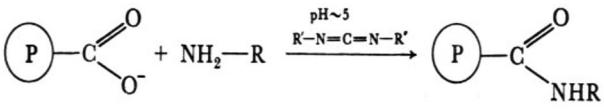
Lysozyme derivatives	Free amino group	Enzymatic activity (units/mg protein)
Lysozyme	7	539.5
Lysozyme–dextran	3.3	336.4
Heated-lysozyme	7	432.4

Source: From Amiri and others (2008).

exhibited antimicrobial activity against both Gram-negative bacteria (*E. coli K12*) and Gram-positive bacteria (*S. aureus*). The authors suggested that the perillaldehyde-conjugated lysozyme may be considered as a novel bactericidal agent in formulated foods (Ibrahim and others 1994a).

Cinnamaldehyde is the organic compound responsible for the flavor and odor of cinnamon. The most common application of cinnamaldehyde is as flavoring in chewing gum, ice cream, candy, and beverages. Antimicrobial activity of cinnamaldehyde against both Gram-positive and Gram-negative bacteria has been reported (Ooi and others 2006). Lysozyme was covalently modified with cinnamaldehyde by allowing the aldehyde group of cinnamaldehyde to react with the amino groups of lysozyme followed by reduction with sodium borohydride. Results showed enhanced antimicrobial activity against both the Gram-negative *E. coli* and the Gram-positive *S. aureus* compared with the unmodified lysozyme (Valenta and others 1997).

Glucosamine (2-amino-D-glucopyranose) is an amino sugar abundantly found in nature, such as in chitin, the polysaccharides making the exoskeletons of crustaceans and other arthropods, as the main precursor for the synthesis of glycosylated proteins and lipids, and in the cell walls of fungi and many higher organisms. It is used as a nonvitamin, nonmineral, dietary supplement by adults and as part of treatment for osteoarthritis (Sawitzke and others 2008). We tried to conjugate glucosamine to lysozyme (Ramezani and others 2008). In this research, we prepared the glucosamine–lysozyme derivative by a carbodiimide-promoted amide formation between the amino group of glucosamine and the carboxyl groups of lysozyme as shown in Scheme 1 (Means and Feeney 1998; Ramezani and others 2008):



Scheme 1: Carbodiimide promoted amide formation between glucosamine (NH_2-R) and lysozyme ($P-COO^-$).

The degree of glycation was followed by determination of sugar content of lysozyme and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). At a 1:1:1 weight ratio of protein:glucosamine:carbodiimide in phosphate buffer, glycation of lysozyme resulted in coupling of 0.11 mole glucosamine to 1 mole lysozyme when the mixture was held at 4 °C for 6 h. A 5% decrease in enzymatic activity of glycosylated lysozyme occurred but solubility of the modified lysozyme at different pHs and different temperatures was higher as compared with unmodified proteins. Also the conjugate had increased heat stability and better emulsion activity, emulsion stability, and foam capacity. These changes might be useful in the application of lysozyme as a natural antimicrobial at adverse conditions of temperatures and pHs (Ramezani and others 2008).

Under similar conditions, caffeic acid and cinnamic acid conjugated lysozyme were synthesized by formation of amide bonds between the carboxyl group of these organic acids and the amino groups lysozyme catalyzed by a water soluble carbodiimide (Bernkop-Schnürch and others 1998). The resulting conjugates showed antimicrobial activity against *E. coli* at a concentration of 0.05% for the lysozyme derivative having the highest activity. However, the efficacy of lysozyme derivatives against *S. aureus* was slightly reduced.

Modification with polysaccharides

Polysaccharides are extensively used in food industry as hydrocolloids, either in native or modified forms, for many different purposes such as thickening or gel-setting agents, stabilizers for emulsions and dispersions, film-forming, coating substances to protect sensitive food from undesired change, and inert fillers to increase the proportion of indigestible ballast substances in a diet (Belitz and others 2009). Different properties of different polysaccharide are a reflection of the conformation dictated by type and number of monosaccharides and the positions and types of linkages in the chain of a polysaccharide. Polysaccharide-conjugated protein can be used as new functional biopolymers that have great potentials in foods as novel natural antibacterial agents (Schmitt and others 1998; Seo and others 2013). As will be discussed, when these macromolecules are attached to lysozyme, their unique properties are incorporated into the produced bifunctional conjugates.

Kato (2002) reviewed the properties of protein–polysaccharide conjugates produced by Maillard-type reaction in order to develop new modified proteins for industrial applications. Protein–polysaccharide conjugates usually show superior emulsifying properties and increased heat stability and enhanced antimicrobial activity compared to the conventional chemically synthesized counterparts. Conjugation might also decrease the allergenicity of natural proteins (Kato 2002).

Starting year 2000, we began performing extensive studies to find the optimum conditions for conjugation of lysozyme with selected polysaccharides including dextran, dextran sulfate, galactomannan, mannan, gum Arabic, inulin, and tragacanth (a hydrocolloid produced only in Iran). We used the well-known Maillard reaction to attach polysaccharides to lysozyme. Maillard reaction compromises a series of naturally occurring chemical reactions between the free carbonyl groups of carbohydrates and the unprotonated amino groups of proteins (Nursten 2005; BeMiller and Huber 2008). Hen egg white lysozyme has 7 free amino groups (6 lysine residues plus N-terminal). However not all of them can participate in Maillard reaction. The amino terminal of Lys-1 and Lys-98 of lysozyme are reported to be the 2 binding sites for polysaccharides (Nakamura and others 1996). However, as will be shown, the differences in the number of modifying groups attached to lysozyme depends on size, conformation, and availability of reducing end aldehyde group or in the solubility of these polymers in water such that the number of reagents coupled might vary from 0 (yeast mannan) to approximately 4.0 (dextran).

We also attempted to evaluate the antimicrobial and functional properties of the modified enzymes. Lysozyme was allowed to react with polysaccharides at different pHs and temperatures, with different weight ratios of polysaccharides to lysozyme, and in the presence of sodium bisulfite or cysteine. The extent of conjugation was followed by different

techniques including fast protein liquid chromatography (FPLC), SDS-PAGE, determination of free amino groups of lysozyme, and-Fourier transform infrared spectroscopy (FT-IR) (Figure 2 A to D). Additionally, large-scale ion-exchange and gel exclusion chromatography were used to separate the unreacted materials from conjugated products and to prepare enough modified lysozyme for antimicrobial and functional studies. Some of the results will be presented later.

The results obtained so far indicate that polysaccharides have different reactivities toward lysozyme under similar condition, depending on their size, conformation, and availability of reducing end aldehyde group or in solubility of these polymers in water (Aminlari and others 2005; Amiri and others 2008; Mohammadi Hashemi 2012). Such differences are probably the source of variation observed in their activities against different bacteria.

Dextran is a branched homopolysaccharide in which glucose units are attached through α, 1–4 glycosidic bonds with branches of different lengths attached to the main chain through α, 1–6 bonds. The molecular weights of these polymers range from 3 to 2000 kDa. The results of FPLC (Figure 2B) and SDS-PAGE (Figure 2D) indicate that the optimum condition for conjugation was 60 °C with lysozyme to polysaccharide molar ratio of 1:5 incubated for 1 wk (Scaman and others 2006; Amiri and others 2008). FPLC chromatogram shows that the dextran-conjugated lysozyme elutes from the cation exchange resin prior to the lysozyme, an indication of the decrease in the positive charge of the amino

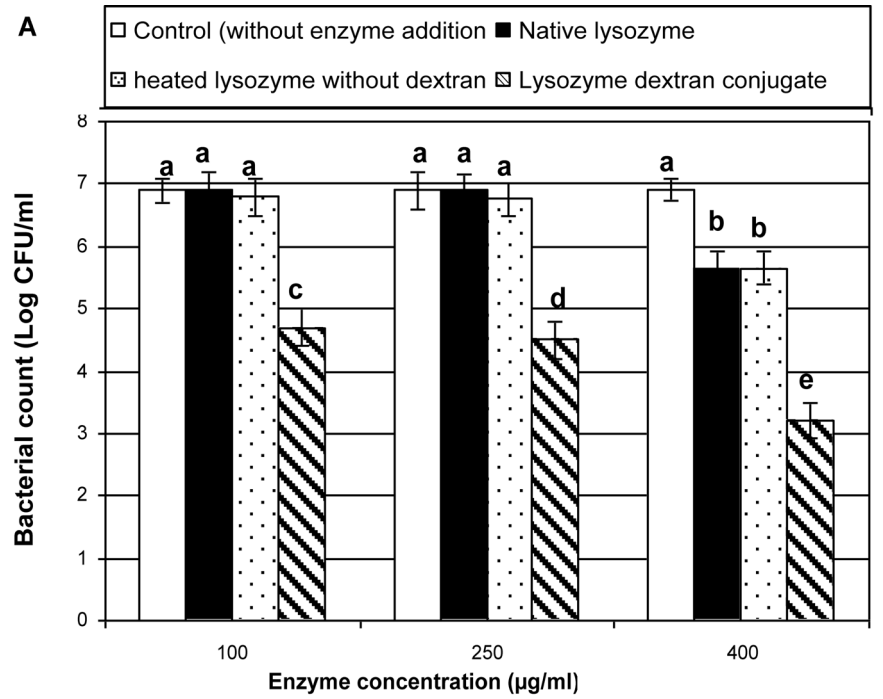


Figure 3–Antimicrobial activity of lysozyme and dextran conjugated lysozyme against: (A) *E. coli* and (B) *S. aureus*. The enzyme–bacterial suspensions were incubated at 37 °C for 16 h. Each point is the average of 3 replicates. Columns with different letters have significant differences ($P < 0.05$; from Amiri and others 2008).

groups of lysozyme, apparently reacted with dextran. The SDS-PAGE pattern further confirms conjugation (Figure 2D). As the incubation time increased the intensity of the diffused bands increased, indicating the multiplicity of the reaction products of lysozyme with dextran (Scaman and others 2006).

Under optimum conditions approximately 3.7 moles dextran were attached to 1 mole lysozyme. The same results were obtained for galactomannan (Figure 2C). Although conjugation of lysozyme with dextran resulted in approximately 50% loss of free amino groups and approximately 40% loss of enzymatic activity as compared with those of unmodified lysozyme (Table 1), Nakamura and others (1991) conjugated lysozyme with dextran. Their results showed attachment of 2 moles dextran to 1 mole lysozyme. The reason for the difference might be related to the different conditions used by different authors. Amiri and others (2008) used dextran with the MW of ~10 kDa, while Nakamura (1991) used dextran with the MW of 60 to 90 kDa. Also Amiri and others (2008) performed reaction in 0.1 M phosphate buffer at pH 7.0, followed by lyophilization and the powder was incubated at 60 °C for 1 wk. In the Nakamura experiment, the reaction was performed in water and this mixture was incubated at 60 °C for 0 to 3 wk.

The lysozyme dextran conjugate exhibited significant enhancement of *in vitro* antibacterial effect against *S. aureus* and *E. coli* in a dose-dependent manner (Figure 3; Amiri and others 2008). Dextran conjugated lysozyme also showed higher solubility at different temperatures (Figure 4), increased heat stability with better emulsion, and foaming properties (Scaman and others 2006; Amiri and others 2008).

We applied the dextran conjugated lysozyme as a food preservative in cheese and milk. Conjugated lysozyme reduced the *E. coli* count by more than 6 log CFU/mL in cheese curd after 40 d storage (Figure 5; Amiri and others 2008). *In vitro* studies indicated that the dextran conjugated lysozyme decreased the *E. coli* and *S. aureus* by 2.3 and 0.4 log cycle when added to milk (Safari 2013; Figure 6). These results indicate that by chemical modification it might be possible to increase the applicability of lysozyme as a natural antimicrobial ingredient in milk and other dairy products. The antimicrobial activity of the dextran conjugate lysozyme against Gram-negative bacteria might be attributed to the combined effects of the remaining enzymatic activity against the cell of bacteria as well as the excellent surface properties of the conjugate (Nakamura and others 1996).

Dextran conjugated lysozyme was applied as an antibiotic for treatment of bovine mastitis. Mastitis is the bacteria-induced inflammation of cattle mammary glands, which results in low quality and low yield of milk, and incurs substantial economic loss to dairy industry worldwide. Dextran conjugated lysozyme was more effective in reducing the count of bacteria isolated from mastitis-inflected cows, including Gram-positive bacteria (*L. monocytogenes*, *S. aureus*, *Bacillus cereus*) and Gram-negative bacteria (*E. coli* and *Kelebsiella*, spp) *in vitro* at 400 ppm, but had no effect on *Streptococcus agalacticus.* Thus, the dextran conjugated lysozyme might be a suitable candidate as an antimicrobial agent in pharmaceutical preparations for treatment of mastitis (Kuhi 2010).

A lysozyme–dextran nanogel containing lysozyme in the core and dextran as coating was prepared by Li and others (2008). Their method involved dry heating of lysozyme and dextran to produce the conjugated derivative followed by heat-denaturation of lysozyme to produce nanogels. The authors suggested that the stable gel produced this way might be useful for loading drugs. The applicability of this method for forming coating materials for foods needs further investigation (Li and others 2008).

Guar gum is a glactomannan composed of a main chain of β-D-mannopyranosyl units joined by (1–4) bonds with single-unit α-Dgalactopyranosyl branches attached to O-6 (BeMiller and Huber 2008). We covalently attached this polysaccharide to lyszyme under Maillard reaction conditions (Scaman and others 2006). Under optimal conditions (60 °C, pH 8.5, 1 wk), almost 100% of lysozyme molecules were modified with galactomannan, as evidenced by SDS-PAGE and FPLC (Figure 2 C). Nakamura and others (1992), Nakamura and others (1996), and Nakamura and Kato (2000) prepared lysozyme–galactomannan conjugates through the Maillard reaction and showed that the modified enzyme retained about 80% of its enzymatic activity and had activity against the Gram-negative pathogen *E. tarda.*

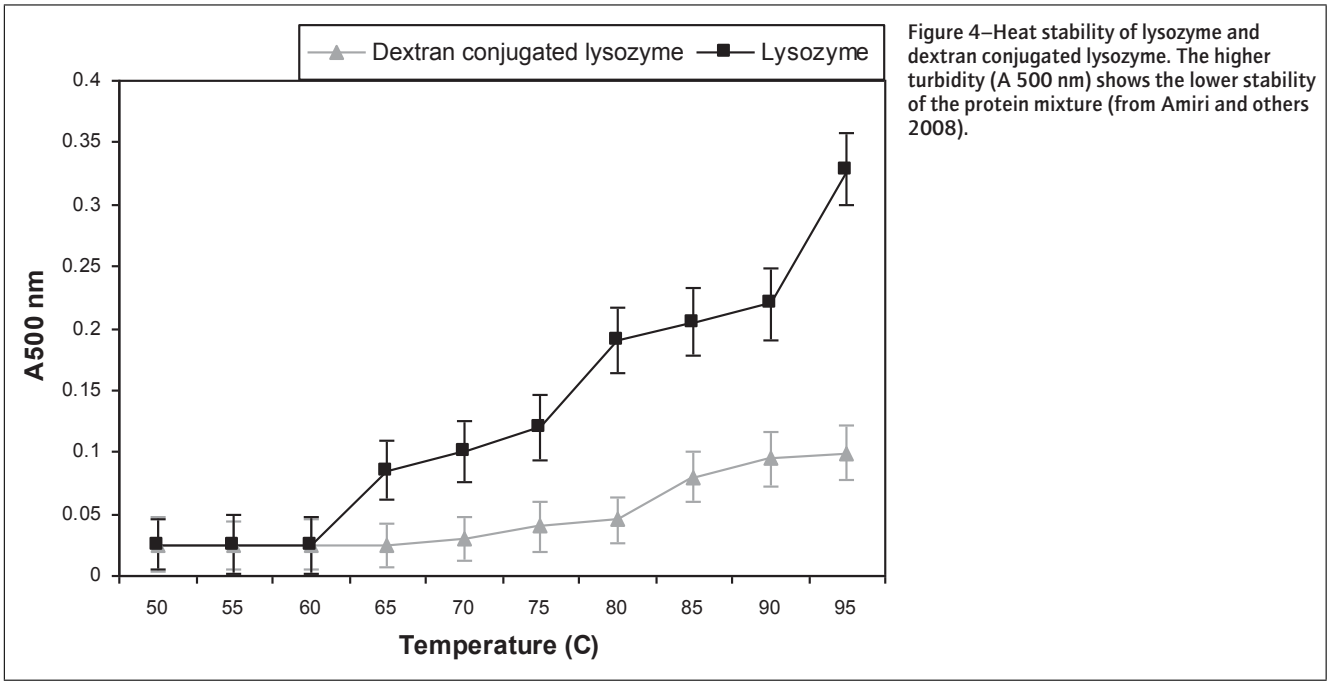


Figure 4–Heat stability of lysozyme and dextran conjugated lysozyme. The higher turbidity (A 500 nm) shows the lower stability of the protein mixture (from Amiri and others 2008).

When the conjugate was fed to the carp *Cyprinus carpio* L., it protected the fish against bacterial infection with this bacterium. The authors suggested that the modified lysozymes could be used in the formulated food systems as a novel antimicrobial agent.

The antimicrobial action of lysozyme was extended toward the Gram-negative bacterium *E. coli* K-12 through conjugation with chitosan (Song and others 2002). A lysozyme–chitosan composite film was produced, which exhibited antibacterial properties against *E. coli* and *Streptococcus faecalis* (Park and others 2004; Duan and others 2008).

Lysozyme was covalently attached to citrate cross-linked nonwoven cotton using a water-soluble carbodiimide-induced amide formation between the enzyme and cellulose molecules. Textiles with immobilized-lysozyme demonstrating antimicrobial properties provide potential barriers to microbial invasion

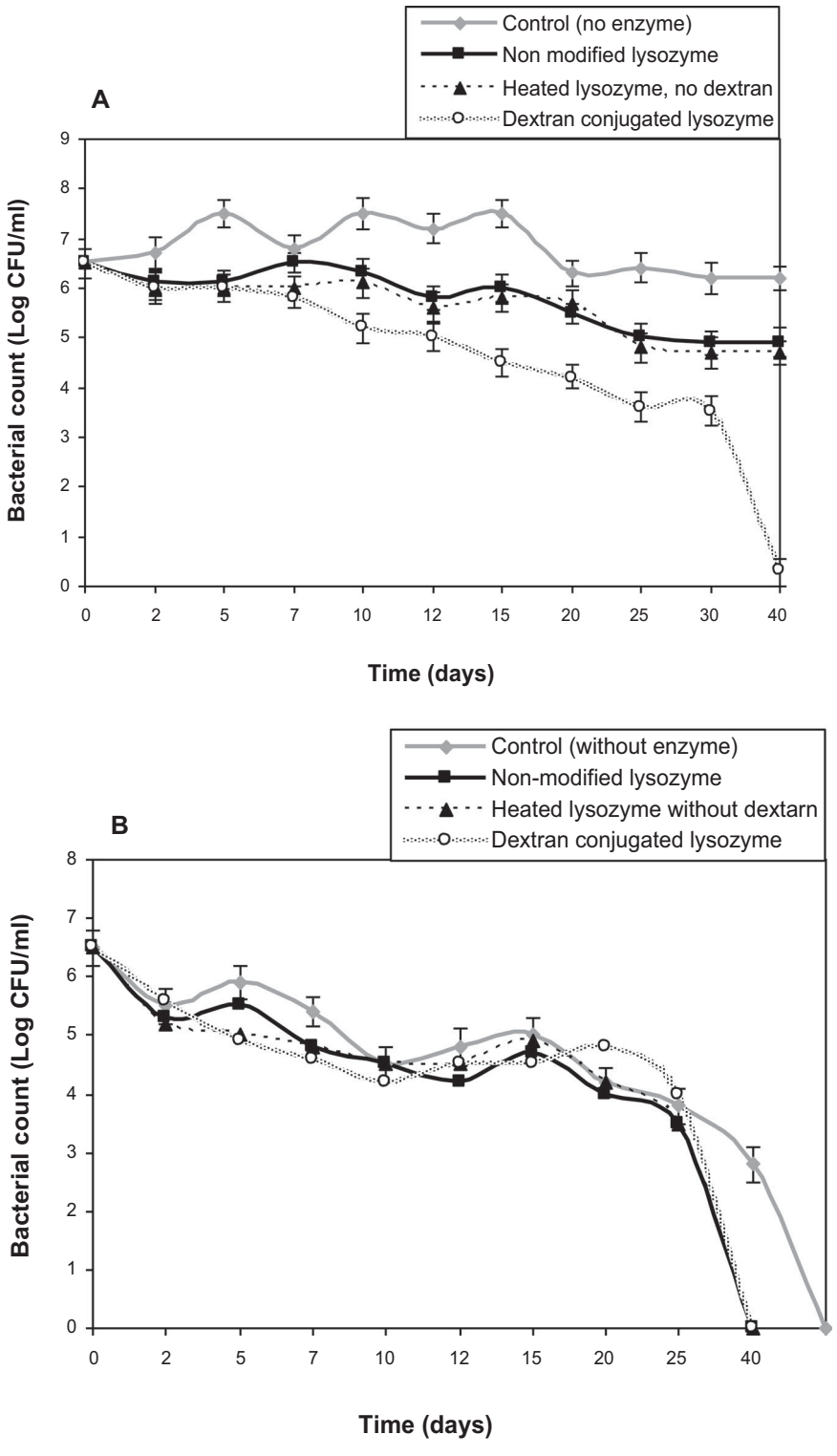


Figure 5–Antimicrobial activity of lysozyme and dextran conjugated lysozyme against: (A) *E. coli* and (B) *S. aureus* in cheese curd at 4 °C. Each point is the average of 3 replicates (from Amiri and others 2008).

through hydrolysis of the bacterial cell wall (Edwards and others 2011).

Mannan, a homopoly-saccharide made of α-(1–2)- and of α-(1–6)-linked D-mannose residues produced by the yeast *Saccharomyces cerevisiae* did not react with lysozyme at all (Scaman and others 2006). Inulin, a polysaccharide isolated from chicory and artichoke is a member of a class of carbohydrates called fructans. These are considered as dietary fibers and are believed to be of significant technological and nutritional importance (Flamm and others 2001). Fructans are linear or branched fructose polymers, mainly linked by β, 2 $\rightarrow$1 glycosidic bonds without free reducing end aldehyde group. As such, these polymers do not react with lysozyme. However, when we oxidized this polysaccharide with periodate, the newly formed carbonyl groups did react with lysozyme under mild Maillard reaction condition (Haghighimanesh and others 2012).

Gum Arabic, a hydrocolloid obtained from the Acacia tree is widely used as a hydrocolloid in the food industry. Gum Arabic is a complex polysaccharide with different ratios of arabinogalactan oligosaccharides and glycoproteins. Depending on the source, the proportion of L-arabinose relative to D-galactose in the glycan moiety might be different (Williams and Phillips 2009). Under optimal conditions (pH 8.5 and 60 °C for 10 d) with a lysozyme:gum Arabic weight ratio of 1:4, about 4.4 mmol gum Arabic was coupled to 1.0 mol Lysozyme. The FT-IR spectra of the conjugated product of lysozyme and gum Arabic showed disappearance of some of the amino groups of lysozyme and the carbonyl group of gum Arabic concomitant with an appearance of the bands related to amide linkage between lysozyme and the polysaccharide (Figure 2E; Mohammadi Hashemi 2012). The conjugate showed higher solubility at acidic pHs and at different temperatures, increased heat stability with better emulsion and foaming properties. Additionally, lysozyme–gum Arabic conjugate showed antioxidant properties and significantly inhibited growth of *S. aureus* and *E. coli in vitro* and in mayonnaise. The conjugate eliminated these species of bacteria within 3 to 5 d as compared to the control, which showed no bacterial growth after 10 d. The result of this study demonstrated that conjugation of lysozyme with gum Arabic can increase the spectrum of lysozyme application in food products and it can be a suitable candidate as a natural preservative in mayonnaise (Figure 7; Mohammadi Hashemi 2012).

Xanthan gum, secreted by *Xanthomonas campestris* is an anionic extracellular polysaccharide extensively used as a stabilizer, thickener, or emulsifier in food products. Xanthan gum is a linear (1–4) linked β–D glucose backbone (as in cellulose) with a trisaccharide side chain on every other glucose at C3, containing a glucuronic acid residue linked (1–4) to a terminal mannose unit and (1–2) to a 2nd mannose that connects to the backbone. Approximately 50% of the terminal mannose residues are pyruvated and the non-terminal residue usually carries an acetyl group at C6. (Stokke and others 1998; Sworn 2009). Lysozyme–xanthan gum conjugate was prepared according to the condition of Maillard reaction. Lysozyme and xanthan gum in a 1:1 weight ratio were mixed in phosphate buffer, pH 8.5, mixture lyophilized, and the powder was incubated at 60 °C for 10 d. Conjugation was confirmed by SDS-PAGE, enzyme activity, and determination of sugar content of the product. Results showed that under optimal conditions approximately 1.9 mmol xantham gum was attached to 1 mol lysozyme (Mohammadi Hashemi and others 2014). The conjugate showed higher solubility at acidic pHs and at different temperatures, increased heat stability with improved emulsion, and foaming properties. Additionally, the conjugate showed antioxidant properties and significantly inhibited the growth of *S. aureus* and *E. coli* in a dose-dependent manner. These findings may broaden food applications of lysozyme as a functional ingredient with high-quality emulsifier, foam producer, or a natural antibacterial agent in food or pharmaceutical industries (Mohammadi Hashemi and others 2014).

We also studied the conjugation of lysozyme with dextran sulfate and barley beta-glucan (Ansari 2008; Alahdad and others 2009). Dextran sulfate is a biocompatible polyanionic polymer. Each glucose unit in the dextran chain has approximately 2 sulfate groups, located normally at C2 and C4 of glucose units (Anitha and others 2011). Dextran sulfate is applied as an anticoagulant (Ricketts 1954), an antivirus (Nakashima and others 1989), and to accelerate hybridization rates of DNA fragments (Amasino 1985). Beta-glucan, a polysaccharide mainly found in the cell walls of barley and oats, is a linear polymer made of D-glucose units linked by β-1–3 and β-1–4 glycosidic bonds. It displays the physiological

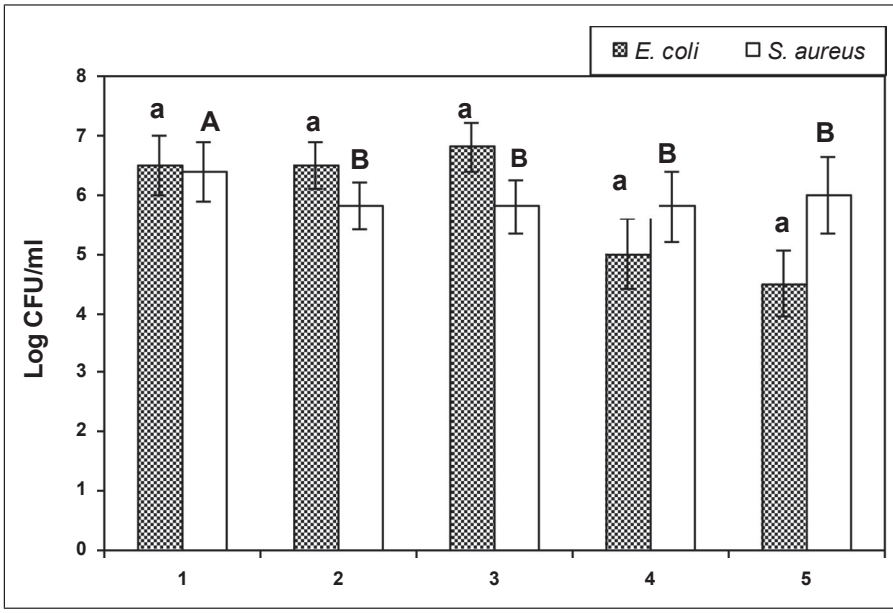


Figure 6–Antimicrobial activity of lysozyme and dextran conjugated lysozyme against *E. coli* and *S. aureus* in milk. The enzyme–milk–bacterial suspensions were incubated at 37 °C for 48 h. Each point is the average of 3 replicates. 1: no enzyme, 2: native lysozyme, 3: heated lysozyme, 4: fraction 1 cation exchange chromatography (Figure 2B), 5: fraction 2 from cation exchange chromatography (Figure 2B). Columns with different letters are significantly different ($P < 0.05$; from Safari and others 2013).

properties of dietary fibers such as cholesterol lowering, blood glucose regulation, immunostimulation, and antitumor activity (Lazaridou and Biliaderis 2007). Lysozyme modification with both polysaccharides resulted in improvement of solubility at high pH values due to shifting of pI to lower than that of native lysozyme (approximately 11), improvement of emulsion activity, and effectively inhibiting the growth of *E. coli* (Ansari 2008; Alahdad and others 2009). Results of studies of this sort indicate that one can simultaneously take advantage of both polymers (the polysaccharides and lysozyme) by attaching them together.

Tragacanth, dried exudates obtained from the stems and branches of Asiatic species of Astragalus, is a highly branched, complex heterogeneous anionic polysaccharide of high molecular weight (840 kDa), mainly composed of D-galacturonic acid, D-galactose, L-fucose (6-deoxy-L-galactose), D-xylose, L-arabinose, and L-rhamnose (LoÂpez-Franco and Higuera-Ciapara 2009). The exact proportion of each sugar varies between gums from different locations. The primary source of gum tragacanth is the desert highlands of northern and western Iran, particularly the Zagros Mountains region. In Iran, the gum is harvested seasonally (Balaghi and others 2010). Tragacanth consists of 2 main fractions: a water-insoluble component called bassorin, which has the capacity to swell and form a gel, and a water-soluble component called tragacanthin (TRG). The easy separation of TRG and bassorin suggests that the 2 polysaccharides are in a physical mixture and not chemically bonded (LoÂpez-Franco and Higuera-Ciapara 2009). TRG was allowed to react with lysozyme. Under optimum conditions (pH 8.5, 60 °C, RH = 79%, 8 d), approximately 2 TRG molecules were attached to 1 lysozyme molecule (Koshani

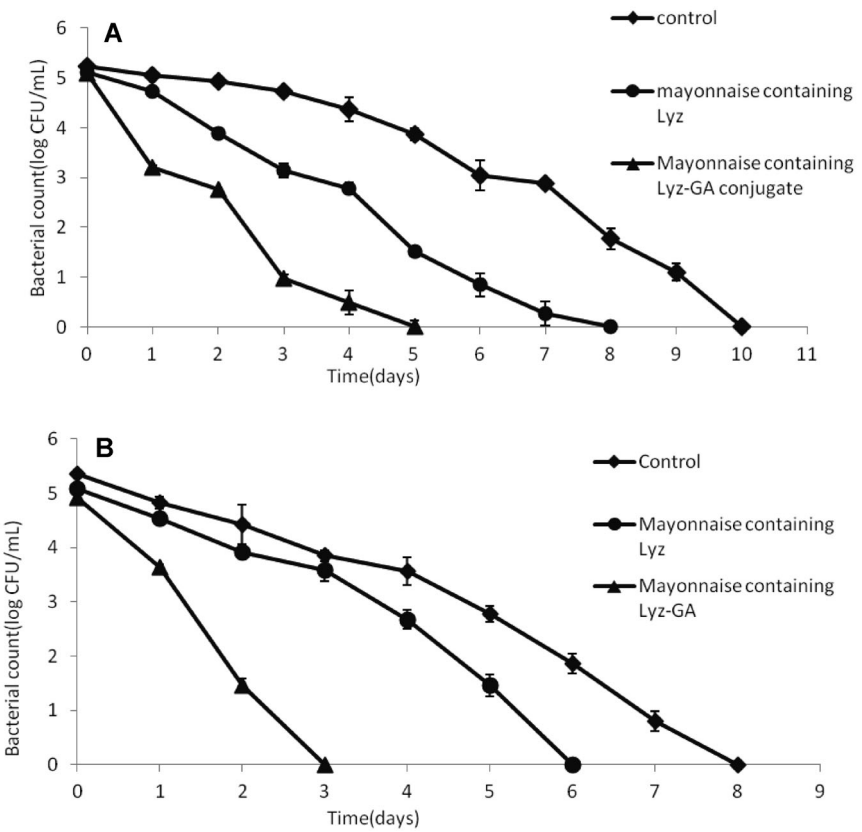


Figure 7–Antimicrobial activity of lysozyme (Lys) and lysozyme–gum Arabic (Lyz–GA) conjugate against *E. coli* (A) and *S. aureus* (B) in mayonnaise stored at 4 °C. Each point is the average of 3 replicates (from Mohammadi Hashemi 2012).

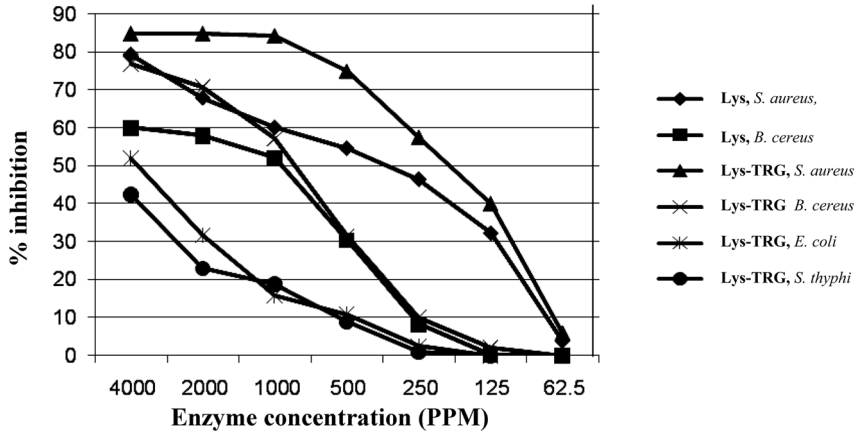


Figure 8–Antimicrobial activity of lysozyme (Lys) and Lysozyme–tragacanthin (TRG) conjugate against *S. aureus*, *B. cereus*, *E. coli*, and *S. thyphi*. TRG alone had no antimicrobial activity and unconjugated Lys did not have antimicrobial activity against *E. coli* and *S. thyphi* under similar conditions (from Koshani 2013).

Table 2–Modified lysozymes and their properties.

Modified lysozymes	Effect on functionality	Reference
Palmitic acid	Enhanced antimicrobial against *E. coli* (WT-3301)	Ibrahim and others (1991)
Short and middle chain saturated fatty acids	Enhanced antimicrobial against G-positive bacteria	Liu and others (2000a)
Glucose–stearic acid monoester	Enhanced activity against *E. coli* and *E. tarda* (NG8104)	Nakamura and others (1996)
Perillaldehyde	Enhanced activity against *E. coli* K12 and *S. aureus*	Ibrahim and others (1994a)
Cinnamaldehyde	Enhanced antimicrobial activity against *E. coli* and *S. aureus*	Valenta and others (1997)
Glucosamine	Improved solubility at different pHs and temperatures, increased heat stability, emulsion activity and stability, and foam capacity	Ramezani and others (2008)
Caffeic acid–cinnamic acid	Antimicrobial activity against *E. coli* (ATCC 8739), decreased activity against) *S. aureus* (ATCC 6538)	Bernkop-Schnürch and others (1998)
Dextran	Enhanced activity against *E. coli* and *S. aureus* in cheese	Amiri and others (2008)
Dextran	Enhanced activity against *E. coli* and *S. aureus* in milk	Safari (2013)
Dextran	Treatment of bacterial isolates from cows with mastitis	Kuhi (2010)
Dextran	Preparation of a lysozyme–dextran nanogel	Li and others (2008)
Dextran	Increased heat stability, higher emulsifying property	Nakamura and others (1991)
Galactomannan	Antibacterial emulsifier	Takahashi and others (2000)
Galactomannan	Emulsifier, antibacterial against G-negative pathogen *E. tarda*	Nakamura and others (1996, 1992) and Nakamura and Kato (2000)
Chitosan	Enhanced bactericidal action against *E. coli* K-12	Song and others (2002)
Chitosan	Lysozyme-composite film with activity against *E. coli*, *L. monocytogenes*, and *S. faecalis*	Duan and others (2008) and Park and others (2004)
Cellulose	Preparing a textile with potential barrier to microbial invasion	Edwards and others (2011)
Gum Arabic	Enhanced activity against *E. coli* and *S. aureus* in mayonnaise	Mohammadi Hashemi (2012)
Xanthan gum	Enhanced activity against *E. coli and S. aureus*	Mohammadi Hashemi and others (2013)
Dextran sulfate	Enhanced activity against *E. coli and S. aureus*	Alahdad and others (2009)
Barley beta-glucan	Enhanced activity against *E. coli* and *S. aureus*	Ansari (2008)
Tragacanth	Enhanced activity against *E. coli*, *S. typhimyrium*, *B. cereus*, and *S. aureus*	Koshani (2013)
Inulin	Improved functional properties	Haghighi-manesh and others (2012)
Trypsin and ficin digestion of dextran-conjugated lysozyme	Enhanced activity of peptides *E. coli* and *B. cereus*	Tavana Babnary (2013)

2013). Differential scanning calorimetric (DSC) analysis showed that conjugation increased the denaturation temperature by 6.35 °C. The antimicrobial evaluation of the conjugated products using minimum inhibitory concentration (MIC) method revealed that conjugation enhanced the antimicrobial effect of lysozyme against Gram-positive *S. aureus* and *B. cereus* and Gram-negative bacteria *E. coli* and *S.* typhimurium (Figure 8). Tragacnthin alone had no antimicrobial activity and unconjugated lysozyme did not have antimicrobial activity against *E. coli* and *S. thyphimirium* under similar conditions (Koshani 2013).

Enzymatic hydrolysis of glycated lysozymes

One method for modification of lysozyme is enzymatic hydrolysis, which results in formation of small peptides with enhanced antibacterial activity attributed to the positive charge, hydrophobic amino acids, and an amphiphilic helical conformation (Pellegrini and others 1997; Pellegrino and Tirelli 2000; Ibrahim and others 2002, 2005; Masschalck and others 2003; Mine and others 2004; Gonzalez and others 2010). Treatment of lysozyme with clostripapain resulted in production of a pentapeptide with antimicrobial activity but without the muramidinase activity (Pellegrini and others 1997). The proposed mechanism for bactericidal activity of the lysozyme fragment included inhibition of DNA and RNA synthesis and alteration of the membrane permeability (Pellegrino and Tirelli 2000). These findings might contribute to the notion that the peptides produced by hen egg white lysozyme hydrolysis might be applied in food and pharmaceutical industries as novel antimicrobial agents (Sompong and others 1970, Memarpoor-Yazdi and others 2012).

In an attempt to further improve the bacteriocidal effects of these bioactive peptides, we first glycated lysozyme with dextran (as described earlier) and then digested the glycated product with trypsin and ficin. Isolated peptides produced by trypsin digestion of glycated lysozyme had significantly higher antibacterial activity against *E. coli* than the respective unglycated peptides but not against *B. cereus*. However, ficin digestion of glycated lysozyme had action against both microorganisms (Tavana Babnary 2013).

Other modifications

In one approach hen egg white lysozyme was pegylated with methoxypolyethylene glycol-p-nitrophenyl carbonate, a derivative of polyethylene glycol (PEG). PEG derivatives are extensively used for modification of proteins to improve their physical, biochemical, and functional properties as well as thermal stabilities (Freitas and Abrahao-Neto 2010). PEGylation of lysozyme produced conjugates that retained full enzyme activity over a broad pH range and were active at 50 °C and resisted proteolytic degradation. This type of modified lysozymes could have application in food treatments involving temperature and pH fluctuations without losing lysozyme activity.

A pentadecapeptide encompassing amino acids 98–112, derived from hen egg white lysozyme (Ile-Val-Ser-Asp-Gly-Asn-Gly-Met-Asn-Ala-Trp-Val-Ala-Trp-Arg) showed antimicrobial activity against Gram- and Gram-negative (Ibrahim and others 1994b; Pellegrini and others 1997). The Ile98–Met105 segment was inactive while Asn106–Arg112 was weakly active and replacement of Asn in the C-terminal peptide with Arg showed improved antimicrobial activity. The mode of action of these peptides appear to involve interaction with the phospholipids of the bacterial

cell membrane and facilitating internalization of the peptides and perturbing intracellular functions (Hunter and others 2005).

A genetically polymannosylated lysozyme derivative was produced in the yeast expression system. The average molecular weight of polymannosyl lysozyme was 75 kDa (Kato and others 1994). These findings suggest that genetic engineering in addition to chemical modification might be applied to improve lysozyme.

Heat-induced change in the conformation of lysozyme is another method for improving its antimicrobial activity. Heating lysozyme at elevated temperatures resulted in loss of enzymatic activity while its antimicrobial action against Gram-negative bacteria was enhanced, suggesting that this action is independent of catalytic function of lyzozyme but might be related to a heat-induced change in conformation of the enzyme molecule, which can affect permeability of bacterial membrane (Ibrahim 1998).

Another type of modification involves sulfhydryl–disulfide exchange reactions. Treatment with cysteine and gluthation might reduce disulfides bonds and introduce new half-cystine residues. Touch and others (2003) used this approach to modify hen egg white lysozyme which has 4 disulfide linkages. Using the sulfhydryl compounds along with heat-induced denaturing conditions resulted in a reduced form of lysozymes which was more flexible and demonstrated increase in the surface hydrophobicity. This modified lysozyme bound more strongly to LPSs layer of *Salmonella enteritidis*, permeabilized the bacterial outer membrane, and demonstrated significantly higher antimicrobial activity against these bacteria than native or heated lysozyme. The authors suggested that reduced lysozymes could have potential as antimicrobial agents for prevention of *S. enteritidis* attack (Touch and others 2003).

Still another type of modification applies the thermo-chemical method to produce a oligomeric forms of lysozyme (Cegielska-Radziejewska and others 2010). The method involved heating lysozyme solutions at pH 4.0 and 70 °C for 15 min in the presence of 1% to 2% hydrogen peroxide. This procedure resulted in conversion of monomer to dimer and trimer of lyozyme, the level of which was proportional to H_2O_2 concentration and heat treatment. Under optimal condition, 70% of lysozyme molecules were converted to oligomers. These oligomers had significantly lower specific enzyme activity and higher surface hydrophobicity. The effect of this modification was studied by applying the oligomeric lysozyme against selected bacterial strains of Gram-positive *S. epidermidis* (PCM, 2118/ATCC 14990) and Gram-negative *Pseudomonas fluorescens* (NCTC 5887). The authors found that in case of lysozyme monomer, no inhibitory action was observed against *P. fluorescens* while the preparation containing higher proportion of oligomers was most effective against these bacteria and lowered the count by 3.3 logarithmic cycles as compared with control samples. The antibacterial action against *S. epidermidis* showed similar results except that a higher antibacterial activity was found for preparations with milder degrees of modification (Cegielska-Radziejewska and others 2010).

Conclusions and Prospects

Nature provides us with remarkable antimicrobial agents. However, inherent limitations of these natural antimicrobials might hinder their vast usage. It therefore becomes necessary to modify and expand the application of these agents. Lysozyme, an abundantly found protein, is well known for its antimicrobial property. In this communication, we reviewed different modifications of this interesting molecule. Both lysozyme and the carbohydrates used in its modification are naturally present in many sources commonly used as food and they might easily interact (Schmitt and others 1998). Table 2 summarizes different compounds used to modify lysozyme molecule. In spite of extensive research on preparation and characterization of these modified products, little work has been reported to demonstrate their safety for human consumption. Nevertheless, it is known that most heated food stuffs experience Maillard reaction and the products of this versatile reaction are present in the foods we eat. It is therefore conceivable that the modified lysozymes reported in this review are not expected to impose health threat. However, it is suggested that more studies be performed to approve the safety of the modified lysozyme if they are to be used in food systems. Entering this intriguing field, the curious mind will find extraordinary ways to overcome the limitations, thereby expanding the potentiality of lysozyme as a natural antibiotic.

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