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Production and properties of tragacanthin-conjugated lysozyme as a new multifunctional biopolymer

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ABSTRACT

In this communication we describe preparation and characterization of an enzyme-biopolymer conjugate composed of water-soluble part of gum tragacanth and chicken egg white lysozyme (LZM) under mild Maillard reaction conditions. SDS-PAGE together with FT-IR spectroscopy revealed that Maillard reactions occurred between LZM and tragacanthin (TRG). Under optimum conditions (pH = 8.5, 60 °C, RH = 79%, 8 days), approximately 2 TRG molecules were attached to one LZM molecule. DSC analysis showed that conjugation with TRG increased denaturation temperature by 6.35 °C. The resulting conjugates were characterized using scanning electron microscopy. The modified enzyme activity retained 77% of the original enzymatic activity after 8 days. TRG-conjugated LZM exhibited improved solubility and emulsion properties as compared with the native LZM. In addition, a significant increase in foam capacity and stability of LZM-TRG conjugate was detected. Conjugate with TRG significantly improved the inhibitory effect of LZM on the growth of *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* and *Salmonella typhi* in a dose dependent manner such that at 4000 μg/ml, LZM-TRG conjugate inhibited *S. aureus*, *B. cereus*, *E. coli* and *S. typhi* by 90%, 80%, 50% and 40%, respectively. Taken together these results suggest that the functional properties and antimicrobial activities of LZM can be improved by conjugation with TRG. The conjugation might expand the applications of LZM as a multifunctional ingredient in food and pharmaceutical industries.

1. Introduction

In recent years, a great deal of attention has been directed towards the application of biocatalytic functions of enzymes in food and pharmaceutical industries by manipulating their activity and stability. Enzymes are naturally present in the cytoplasm or organelle of living cells (Paustian, 2012). Enzymes have been recognized as efficient and environmentally friendly catalysts because of their high specificity and catalytic activity under mild conditions (Jia, Narasimhan, & Mallapragada, 2013; Patel, 2012; Wandrey, Liese, & Kihumbu, 2000).

Lysozyme (LZM) is an important natural bactericidal enzyme (EC 3.2.1.17) that is widely distributed in nature and helps protect against microbial infections. LZM lyses the cell wall of various Gram-positive bacteria by splitting β (1–4) linkages between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan in bacterial cell walls (Johnson & Larson, 2005). It is used in food preservation protocols and in pharmacology, and has various therapeutic applications (Johnson & Larson, 2005). LZM is a small protein (14.6 kDa) but it has lytic activity directed toward a very large substrate—the bacterial cell wall peptidoglycan (Fleming, 1974).

Polysaccharides widely exist in plants, microorganisms (fungi and bacteria), algae, and animals. In the past decades, some bioactive polysaccharides isolated from natural sources have attracted attention in the field of biochemistry and pharmacology. Tragacanth, a dried exudate obtained from the stems and branches of Asiatic species of Astragalus, is a very complex heterogenous anionic polysaccharide of high molecular weight (Weiping & Branwell, 2000). The primary source of gum tragacanth is the desert highlands of northern and western Iran, particularly the

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Zagros Mountains region. In Iran, the gum is harvested seasonally. Tragacanth consists of two 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 two polysaccharides are in a physical mixture and not chemically bonded (Balaghi, Mohammadifar, & Zargaraan, 2010).

The trend in the utilization of Maillard reaction to production and improvement of the functional biopolymers and nutroceuticals has increased and further increase in the consumption of these products is forecasted (Liu, Ru, & Ding, 2012; Liu, Zhao, Zhao, Ren, & Yang, 2012; Oliver, Melton, & Stanley, 2006). The Maillard reaction refers to a complex set of reactions initiated by reaction between amines and carbonyl compounds (Nursten, 2005). Covalent linking of enzymes with water-soluble biocompatible polymers is the most commonly used strategy to stabilize their structure and function. This phenomenon can pose a considerable improvement of enzymes functionalities, such as heat stability, solubility, emulsifying and foaming properties, antioxidant and antimicrobial effects (Li et al., 2010; Medrano, Abirached, Panizzolo, Moyna, & Añón, 2009; Nakamura, Kato, & Kobayashi, 1992; Nodake & Yamasaki, 2000; Song, Babiker, Usui, Saito, & Kato, 2002; Zalipsky, Mullah, Engbers, Hutchins, & Kiwan, 2007). For instance, dextran-conjugated LZM exhibited improved heat stability, increased solubility at different pH's and temperatures and better emulsifying property as compared with the unmodified LZM (Amiri, Ramezani, & Aminlari, 2007). Preparation of bioconjugates of synthetic polymers with LZM to be applied as functional coatings has been reported (Muszanska, Busscher, Herrmann, van der Mei, & Norde, 2011). The brush coatings consisting of pluronic-LZM conjugates were applied which exert bi-functionality, i.e. an anti-adhesive activity due to the polymer brush together with the antibacterial activity of the LZM.

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 (Nakamura et al., 1992). Conjugation with polysaccharides can extend the inhibitory effect of LYZ towards the Gram-negative bacteria (Alahdad, Ramezani, Aminlari, & Majzoobi, 2009; Amiri et al., 2007; Mohammadi Hashemi, Aminlari, & Moosavinasab, 2014; Muszanska et al., 2011; Nakamura et al., 1992; Song et al., 2002).

Based on the discussions outlined above and potential applications of conjugated enzymes, the objective of the present study was to fabricate novel LZM-TRG multifunctional biopolymer conjugate and to investigate its structural and functional properties.

2. Experimental procedure

2.1. Materials and chemicals

Iranian gum tragacanth used in this study was collected from plants growing in Southern mountainous areas of Iran. The raw gum was ground and sieved. Powdered gum with mesh size of 80–400 μm was used in this study. The powder composition was (g/100 g): 11.14 ± 0.3 moisture, 0.41 ± 0.05 nitrogen, 2.8 ± 0.01 ash and 85.65 carbohydrates. The ratio of the soluble to the insoluble fraction in the exudate was 0.39. Chicken egg white LZM was from Inovatech, Inc., Abbotsford, BC, Canada and *Micrococcus lysodeikticus* cell wall was from Sigma, St Louis, MO, USA. All other reagents were reagent grade and were commercially available.

2.2. Preparation of TRG by spray drying

One gram crude gum tragacanth was wetted with 1 ml ethanol. Two hundred ml water was added and mixture was rotated in a sealed bottle overnight to ensure good hydration of the biopolymers. Centrifugation at 3600 × *g* for 180 min allowed the separation of the soluble and insoluble parts (Mohammadifar, Musavi, Kiumarsi, & Williams, 2006). The supernatant was spray dried using a pilot plant spray dryer (Maham Industry Company, Neyshabur, Iran). The feed rate of the spray dryer was 24–28 ml/min, maintained by a peristaltic pump and the TRG solution was subjected to a two-fluid nozzle atomizer. An electric heater heated the inlet air to a temperature of 120–150 °C. The outlet air temperature was 75–85 °C.

2.3. Preparation of LZM-TRG conjugates

One hundred mg LZM and 500 mg TRG were dissolved in 10 ml of 0.05 M sodium phosphate buffer, pH = 7.0 and 8.5 ± 0.1. After thorough mixing, solutions were frozen and then lyophilized. The powder mixtures were incubated in sealed glass desiccators at 60 °C under the relative humidity of 79% provided by saturated KBr. Samples were removed at time intervals up to 10 days and analyzed as follows. For each experiment a control sample was included which contained no TRG.

2.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

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, 0.02 mg/ml 2-mercaptoethanol, and 0.004% bromophenol blue. Due to the gel forming potential of the TRG-LZM samples, one centrifugation step (4000 rpm, 5 min) was necessary after heating with SDS solution and prior to sample injecting in the wells. 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. Determination of free amino groups and extent of conjugation

The number of available $-NH_2$ groups were determined using the o-phthaldialdehyde (OPA) assay (Church, Porter, Catignani, & Swaisgood, 1985). An OPA reagent was prepared by dissolving 40 mg o-phthaldialdehyde in 1 ml absolute ethanol, then mixed with 2.5 ml 10% SDS, 0.1 ml 2-mercaptoethanol and made up to 50 ml with 0.1 M borate buffer (pH 9.85). The assay consisted of mixing 2 ml OPA reagent with 0.1 ml protein solution and measuring the absorbance of the mixture at 340 nm after 3 min incubation. The number of available $-NH_2$ groups was determined using leucine as the amino acid standard. The number of $-NH_2$ groups available per mole LZM was then calculated based on the solution protein concentration determined using the Lowry method (1951) and the average M_W of LZM (14,600 Da) (Scaman, Nakai, & Aminlari, 2006). To calculate the extent of conjugation, the available $-NH_2$ groups were determined before and after Maillard reaction and the differences in the available $-NH_2$ groups