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Preparation of and studies on the functional properties and bactericidal activity of the lysozyme–xanthan gum conjugate

Marjan Mohammadi Hashemi [a], Mahmoud Aminlari [a,b,*], Marzieh Moosavinasab [a]

[a] Department of Food Science and Technology, School of Agriculture, Shiraz University, Shiraz 71345, Iran
[b] Department of Biochemistry, School of Veterinary Medicine, Shiraz University, Shiraz 71345, Iran

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ABSTRACT

The purpose of this research was to produce a xanthan gum–lysozyme conjugate (Lyz–XM) and to investigate the effect of conjugation on functional properties and antimicrobial properties of the conjugated products. The antimicrobial activity of Lyz is limited to Gram positive bacteria, but its antibacterial spectrum can be extended towards Gram negative bacteria by conjugation with carbohydrate through the Maillard reaction. Lyz was allowed to react with XG under mild Maillard reaction condition (pH 8.5 and 60 °C for 10 days) and 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 XG was attached to one mol Lyz. The Lyz–XG conjugate showed higher solubility at acidic pHs and at different temperatures, increased heat stability with improved emulsion and foaming properties. Additionally, Lyz–XG conjugate showed antioxidant properties and significantly inhibited the growth of *Staphylococcus aureus* and *Escherichia coli* in a dose dependent manner. These findings may broaden food applications of Lyz as a functional ingredient with high quality emulsifier, foam producer or natural antibacterial agent in food or pharmaceutical industries.

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1. Introduction

Lysozyme (E.C.3.2.1.17, N-acetyl-muramic-hydrolase) is a well-known bacteriolytic enzyme that has the ability to lyse bacterial cells. Lysozyme is found abundantly in many secretes of biological cells. The largest concentration is in tears, but for commercial extraction, hen egg white has become the major source of this enzyme. The hen egg white lysozyme has a molecular weight of approx. 14.6 kDa. It is a single polypeptide chain consisting of 129 amino acids, in which lysine is the N-terminal amino acid and leucine is at the C-terminal. The lysozyme (Lyz) molecule is cross-linked in four different places by disulfide bridges which cause high thermal stability of the enzyme, together with six helix regions (Proctor, Cunningham, & Fung, 1988).

Lyz has received considerable attention as a natural preservative but because of the structural differences in the cell wall, Gram-positive bacteria are most susceptible to lysozyme, so its industrial application is limited to Gram positive bacteria but not on Gram negatives. It has been reported that chemical and enzymatic modifications can change Lyz to a potent bactericidal agent against Gram-negative bacteria like *Escherichia coli* (Amiri, Ramezani, & Aminlari, 2007; Nakamura, Kato, & Kobayashi, 1991; Scaman, Nakai, & Aminlari, 2006; Song, Babiker, Usui, Saito, & Kato, 2002). Covalent coupling of polysaccharides to Lyz through mild Maillard-type reaction leads to protein–polysaccharide conjugates by condensation between the carbonyl group of a reducing end of polysaccharide and the un-protonated amino group of Lyz (Yadav, Strahan, Mukhopadhyay, Hotchkiss, & Hicks, 2012). The conjugate's antibacterial activity and functional properties, including solubility, emulsion stability, foam stability, heat stability, water binding capacity, and antioxidant activity can be improved (Aminlari, Ramezani, & Jadidi, 2005; Dickinson, 2003).

Xanthan gum is an anionic extracellular polysaccharide secreted by the microorganism *Xanthomonas campestris*, and is extensively used as a stabilizer, thickener or emulsifier in food products. It is soluble in cold water and solutions exhibit highly pseudoplastic flow. Its viscosity has excellent stability over a wide pH and temperature range. The structure of xanthan gum consists of a linear (1 → 4) linked β-D-glucose backbone with a charged trisaccharide side chain on each second glucose residue of the trisaccharide (Stokke, Christensen, & Smidsrød, 1998; Williams & Phillips, 2000). The reaction between xanthan and some proteins like soy proteins

* Corresponding author. Department of Biochemistry, School of Veterinary Medicine, Shiraz University, P.O. Box 1731, Shiraz 71345, Iran. Tel.: +98 711 2286950; fax: +98 711 2286940.
E-mail address: Aminlari@shirazu.ac.ir (M. Aminlari).

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(Carp, Bartholomai, & Pilosof, 1999) and whey protein isolate (Benichou, Aserin, Lutz, & Garti, 2007; Laneuville, Paquin, & Turgeon, 2000) have been studied.

The aim of this study was to investigate on the production possibility of Lyz–XG conjugate through Maillard reaction and to survey the effect of this modification on the enzyme activity, the antimicrobial activity against test organisms *Staphylococcus aureus* and *E. coli* and the functional properties of the Lyz–XG conjugate.

2. Material and methods

2.1. Materials

Hen egg white Lyz (Mr 14600) was obtained from Inovatech, Inc. (Abbotsford, BC, Canada), xanthan gum and *Micrococcus lysodeikticus* cells and diphenyl picryl hydrazyl (DPPH) were from Sigma (St. Louis, MO) and protein molecular weight markers were from Fermentas (Molndal, Sweden). All other chemicals were of reagent grade and were commercially available.

2.1.1. Preparation of Lyz–XG conjugation in lyophilized powder

Conjugation of Lyz with XG was performed as described previously (Amiri et al., 2007) with some modification. 200 mg Lyz and 200 mg xanthan were added to 10 ml 0.1 mol/L sodium phosphate buffer pH 8.5. After thorough mixing and incubating at room temperature for 1 h, the solution was frozen at −70 °C and lyophilized. The powder was divided into several parts and each part was incubated at 60 °C under the relative humidity of 79% provided by saturated potassium bromide. The conjugation of Lyz with XG was studied by removing samples at different time intervals, proteins were precipitated by saturated ammonium sulfate and analyzed for degree of conjugation by sugar analysis, SDS-PAGE and enzyme activity. A control sample in which Lyz was treated as above except that no XG was present was also prepared.

2.1.2. Preparations of Lyz–XG conjugate in aqueous media

Conjugation of Lyz with XG was also studied without lyophilization, in a phosphate buffer to observe the effect of dilution and water activity on the degree of conjugation. A mixture of Lyz and XG in weight ratio of 1:1 (40 mg Lyz, 40 mg XG) was dissolved in 2.0 ml 0.1 mol/L sodium phosphate buffer pH 8.5 at room temperature, then incubated at 60 °C. Samples were examined as described above after 10 days.

2.2. 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, and 0.004% bromophenol blue. The running gel was made of 10% (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% Coomassie Brilliant blue R-250 in 50% acetic acid/25% methanol and destained with a 10% acetic acid/7.0% methanol.

2.3. Fourier transform infrared spectroscopy (FT-IR)

Structure of samples was studied by recording Fourier transform infrared 115 (FT-IR) spectra of XG, Lyz, Lyz–XG conjugate on a BRUCKER spectrophotometer (EQUINOX 55, Germany) by grinding the dry sample with 300 mg KBr and pressing the dry mixture into a pellet. This procedure for preparing KBr pellets does not alter the structure of proteins in the dry solid. All the spectra were recorded in the transmittance mode with a resolution of 2 cm^{-1} in the range of 4500–500 cm^{-1}.

2.4. Determination of the sugar content of the conjugated Lyz

Total sugar content of samples was determined by the phenol sulfuric acid method using glucose as standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1959). Number of moles of xanthan attached to one mole Lyz was calculated taking into account the molecular weight of ~ 10^6 for XG (Stokke et al., 1998) and 14,600 for Lyz (Proctor et al., 1988).

2.5. Color evaluation of the conjugated samples

Color of samples was evaluated using the method described by Yam and Papadakis (2004). Powder samples with similar thicknesses were put in a closed box under a fluorescent light source with a radiation angle of 45°. Pictures were taken with a Sony digital camera (5 megapixel) and analyzed quantitatively using Adobe Photoshop 8 to determine *L*, *a*, *b* colorimetric parameters. The *L*-value gives the lightness (difference to a white standard), the *a*-value gives the intensity of the red color (positive values) and green color (negative values) and the *b*-value the intensity of yellow and blue, respectively. The total color difference (ΔE) was calculated as follows (Hunter, 1975 , pp. 133–140):

$$\Delta E_{L\times a\times b} = \sqrt{\left[(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2 \cdots\right.}, \quad \Delta L = L - L_0,$$
$$\Delta a = a - a_0, \quad \Delta b = b - b_0$$

where L_0, a_0 and b_0 indicate color parameters of untreated (native) Lyz.

2.6. Lysozyme activity

Lyz activity was measured by the lysis of *M. lysodeikticus* cell walls according to the method of Imoto and Yagishita (1971). Nine milligrams of dried *M. lysodeikticus* cell wall was dissolved in 25 ml of 0.1 M potassium phosphate buffer (pH 7.0) and diluted to a final volume of 30 ml with the same buffer. Lyz or modified Lyz at a concentration of 1 mg of protein/mL was dissolved in cold distilled water. The cell wall suspension (2.9 ml) was poured into a cuvette and incubated at 25 °C for 4–5 min to achieve temperature equilibration. The enzyme solution (0.1 ml) was added to the cuvette, and the change in absorbance at 450 nm was then recorded. One unit of activity of Lyz is defined as the decrease in the absorbance at 450 nm of 0.001/min at pH 7.0 and 25 °C using *M. lysodeikticus* cells as a substrate (Imoto & Yagishita, 1971).

2.7. Functional properties

2.7.1. Measurement of solubility

Protein solubility was determined according to the method of Jimenes-Castano, Villiamiel, and Lopez-fandino (2007) with some modification Total nitrogen in each sample was determined by the standard micro-Kjeldahl procedure. Each sample was centrifuged using International Centrifuge Universal, Model UV, USA, at 2700 × *g* for ten minutes. The nitrogen content of the supernatant was measured and the solubility was calculated as:

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