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Characterization of glycated lysozyme with galactose, galactooligosaccharides and galactan: Effect of glycation on structural and functional properties of conjugates

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ABSTRACT

Glycated lysozyme (LZM) with galactose, galactooligosaccharides (GOSs) and galactan, through the Maillard reaction, were produced and characterized structurally and functionally. The extent of glycation of LZM was evaluated by the measurement of the blocked lysine and the formed furosine. The results indicated a high initial reactivity of galactose as compared to GOSs and galactan. In the presence of GOSs, the oxidative and cross-linking side reactions competed with the initial formation of Amadori product. The longer chain carbohydrate galactan exhibited the lowest initial rate of glycation with no significant cross-linking side reaction. Electrospray ionization mass spectrometry revealed the formation of glycated LZM containing up to eight galactose moieties; while only mono- to tetra-glycated LZM with GOSs were detected. 2–3 mol of galactan were conjugated to 1 mol of LZM. Galactan:LZM conjugates exhibited higher solubility, thermal stability and emulsion stability as compared to the unmodified LZM and LZM:galactose conjugates. LZM:GOS conjugates demonstrated the most improvement in the emulsion stability than the other conjugates. Furthermore, the glycation of LZM with galactose/GOSs/galactan decreased its immunoreactivity.

1. Introduction

The Maillard reaction comprises of complex series of naturally-occurring reactions with the protein glycation reactions being the early and intermediates ones (Nursten, 2005, chap. 1). These glycation reactions include the initial condensation reaction between amino groups of proteins and the carbonyl group of a reducing carbohydrate to form Amadori/Heyns intermediate products, followed by dehydration of the sugar moieties, cross-linking and degradation of proteins (Davidek, Clety, Aubin, & Blank, 2002; Nursten, 2005, chap. 1). To control and to limit the Maillard reaction to its early stages, the effects of selected parameters, including protein:carbohydrate ratio, temperature, pH, incubation time, and water activity (a_w), on the reaction rate have been studied by our group (Seo, Karboune, Yaylayan, & L'Hocine, 2012) and by others (Jakas, Katić, Bionda, & Horvat, 2008; Jiménez-Castaño, Villamiel, Martín-Álvarez, Olano, & López-Fandiño, 2005).

Maillard-type glycation under controlled conditions has been reported to improve emulsifying properties (Dickinson & Galazka, 1991), solubility (Niu, Jiang, Pan, & Zhai, 2011), heat stability (Broersen, Voragen, Hamer, & de Jongh, 2004), water/oil holding capacity (Matemu, Kayahara, Murasawa, & Nakamura, 2009), and foam-forming properties (Fechner, Knoth, Scherze, & Muschiolik, 2007) of proteins. In addition, a decrease in the allergenicity of proteins, *in vivo*, upon glycation has also been reported (Arita, Babiker, Azakami, & Kato, 2001). Moreover, the glycation of proteins with prebiotic oligosaccharides could lead to novel prebiotic products with an enhanced colonic persistence (Gibson, Probert, Loo, Rastall, & Roberfroid, 2004). The structural modifications of proteins via the Maillard reaction are, therefore, expected to expand the use of proteins in many applications and to fulfill the current needs of high-quality multi-functional food ingredients.

In order to produce glycoproteins with improved properties via the Maillard reaction, proper understanding of the effects of carbohydrate moieties on the protein glycation extent and on the functional properties of resulting protein–carbohydrate conjugates are of high interest. As an overall, it has been found that the carbohydrate reactivity in the Maillard reaction decreased as the chain length increased (Corzo-Martínez, Moreno, Villamiel, & Harte, 2010; Li et al., 2009; Niu et al., 2011). However, conflicting results (Corzo-Martínez et al., 2010; Li et al., 2009; Matemu et al., 2009; Shu, Sahara, Nakamura, & Kato, 1996) have been reported on the effects of the carbohydrate moieties (mono, oligo and polysaccharides) on the functional properties of conjugates. For

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instance, the emulsifying properties were reported to be unchanged for whey protein–chitosan oligosaccharide conjugates (Matemu et al., 2009), lower for wheat germ protein–glucose/lactose/maltodextrin conjugates (Niu et al., 2011) or higher for rice protein–glucose conjugates (Li et al., 2009) as compared to native proteins and/or to proteins conjugated with higher molecular weight carbohydrates. Water and oil-binding capacities, which are dependent on the length/charge of carbohydrate moieties and on the glycation extent, have been identified as important factors for the emulsifying properties of conjugates (Matemu et al., 2009). The extent of the improvement of the solubility of proteins upon Maillard-type glycation was also dependent on the molecular weight of carbohydrates (Li et al., 2009; Niu et al., 2011). Low molecular weight carbohydrates (glucose, lactose) imparted better solubility to rice proteins upon glycation than higher molecular ones (dextrin, maltodextrin) (Li et al., 2009). In contrast, the glycation of wheat germ protein with high-molecular weight dextran led to a more significant improvement in the protein solubility as compared to the glycation with smaller carbohydrates (glucose and xylose) (Niu et al., 2011). Considering these conflicting results, there is a need to further elucidate the effect of carbohydrate chain length and binding numbers on the functional properties of the glycoproteins.

As part of ongoing work (Seo et al., 2012), the present study was aimed at the investigation of the effects of glycation of lysozyme (LZM), through the Maillard reaction, with mono- (galactose), prebiotic oligo- (potato galactooligosacharides, GOSs) and polysaccharides (potato galactan), on the structures and the functional properties of glycated LZMs. LZM has many desirable properties as a food protein including antimicrobial activity (Hughey & Johnson, 1987) and heat stability (Tomizawa, Yamada, & Imoto, 1994); however, LZM has poor functional emulsifying properties and it is known to be one of the most important allergenic food ingredients (Frémont, Kanny, Nicolas, & Moneret-Vautrin, 1997). The specific objectives of this study were (a) to evaluate the glycation of LZM with galactose, GOSs, and galactan, (b) to characterize the structures of glycated LZMs at different glycation times by mass spectrometry, and (c) to investigate the functional properties of the purified LZM conjugates such as protein solubility, heat stability and emulsifying capacity. The allergenicity of the glycated LZM was also evaluated indirectly by measuring their immunoreactivity.

2. Materials and methods

2.1. Materials

LZM from chicken egg white (>96% purity), triolein, D-(+)-galactose, TNBS (2,4,6-trinitrobenzene sulfonic acid) and salts were purchased from Sigma Chemical Co. (St-Louis, MO). Biogel P2 extra fine, Bradford reagent and low range SDS-PAGE standards were purchased from Bio-Rad (Philadelphia, PA). Furosine standard was purchased from Neosystem Lab. (Strasbourg, France). Potato galactan (~100 kDa) and endo-*1→4-β*-D-galactanase from *Aspergillus niger* were purchased from Megazyme (Wicklow, Ireland).

2.2. Preparation of galactooligosaccharides

Potato galactan was enzymatically hydrolyzed to produce GOSs according to the method developed by Seo et al. (2012). Endo-*1→4-β*-D-galactanase (25 U/L) was added to potato galactan solution (1 g/L) in 0.1 mol/L sodium acetate buffer (pH 4.5). After 23 h incubation at 40 °C, the reaction mixture was ultrafiltered using a stirred ultrafiltration unit (Amicon system, Millipore, Billerica, MA) fitted with a 3 kDa molecular mass cut-off membrane. The recovered filtrate was fractionated by size exclusion chromatography on Biogel P2 column using 20 mmol/L ammonium carbonate buffer (pH 7.0) as a mobile phase at a flow rate of 0.3 ml/min. The fractions containing GOSs were pooled and lyophilized. The desalting of GOSs was performed using the same Biogel P2 column and water as mobile phase. The average of molar mass distribution of GOSs from ESI-MS analysis (data not shown) was 1990.9 g/mol.

2.3. Preparation of conjugates

LZM and carbohydrates (galactose or GOSs or galactan) were dissolved (10 mg/ml) in 0.05 mol/L sodium phosphate buffer (pH 7.0) at the molar ratio of 1:7, and were lyophilized at −25 °C. The powder mixtures were incubated in sealed glass desiccators at 60 °C for various periods (1, 3, 5, 7 days) under controlled a_w values of 0.65 (saturated KI) for LZM:galactan mixtures and 0.45 (saturated $Mg(NO_3)_2$) for LZM:galactose/GOSs mixtures. The mixtures were stored at −20 °C after incubation until further analyses.

2.4. Determination of the extent of lysozyme glycation with selected carbohydrates

2.4.1. Measurement of proportion of free amino groups

The free amino groups of LZM after glycation with selected carbohydrates were determined using trinitrobenzene sulfonic acid (TNBS) assay (Goodwin & Choi, 1970). First, 0.25 ml of TNBS solution (0.01g/100 ml) was added to 0.5 ml of LZM conjugate solutions (10 mg/ml) in 0.1 mol/L sodium bicarbonate buffer (pH 8.5) then incubated at 37 °C for 2 h. Then, 0.25 ml of 10 g sodium dodecyl sulfate (SDS) solubilized in 100 ml of distilled water and 0.125 ml of 1 mol equi/L HCl were added to solubilize the protein and to avoid their precipitation. The absorbance of the reaction mixtures was measured spectrophotometrically (DU 800, Beckman Coulter, Fuellerton, CA) at 335 nm against a buffer blank. The standard curve was constructed using L-leucine. The percentage of blocked lysine was calculated as the concentration of the initial free amino acids of LZM minus the concentration of free amino acids of LZM upon glycation, divided by the initial value. All assays were run in triplicates.

2.4.2. Furosine analysis

To estimate the degree of glycation, the ε-N-2-(furoylmethyl)-L-lysine (furosine) was determined using a modified method of Moreno, López-Fandiño, and Olano (2002). LZM conjugates (2–4 mg of protein) were added to 1.1 ml of 8 mol equi/L HCl, and were incubated at 110 °C for 23 h under nitrogen. The recovered hydrolysates were centrifuged at 14,000 × *g* for 10 min before applying 1 ml to a previously activated Sep-pak C18 cartridge (Waters Corp., Milford, MA). For the elution of furosine, 3 ml of 3 mol equi/L HCl was used. The eluate was evaporated until dryness under nitrogen. Furosine concentration was determined using high-pressure-liquid chromatography (HPLC) according to the method described by Resmini, Pellegrino, and Batelli (1990). A Beckman HPLC System equipped with a programmable solvent module (model 126), a photodiode array detector and a system Gold software for data collection, was used. Thermo Hypersil-Keystone Prism RPN (250 × 3 mm, 5 μm, Thermo Scientific, Waltham, MA, US) column was used for the separation. Sodium acetate buffer (0.06 mol/L, pH 4.3) was used as the mobile phase at an isocratic mode for 30 min at a flow rate of 0.4 ml/min. Injected sample volume was 20 μl and the detection of furosine was performed at 280 nm. The calibration curve was constructed using a furosine standard. All assays were run in duplicates with quadruplicate injections with or without internal standard.