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Glycation of lysozyme with galactose, galactooligosaccharides and potato galactan through the Maillard reaction and optimization of the production of prebiotic glycoproteins

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ABSTRACT

The production of glycated lysozyme (LZM), with galactose, galactooligosaccharides (GOSs) and potato galactan through the Maillard reaction, was investigated. The percent blocked lysine, estimated from the furosine content, reached a maximum value of 11.2% for LZM:galactan conjugates after 1 day incubation at a a_w of 0.65. A maximum percent blocked lysine of 7.0 and 13.5% were obtained for LZM:galactose/GOS conjugates at a lower a_w of 0.45 after 3 and 7 days, respectively. However, the low percent blocked lysine and the high protein aggregation index of LZM:galactose/GOS conjugates at a_w 0.79 and 0.65 revealed the prevalence of the degradation of the Amadori compounds and the protein cross-linking. Mass spectrometry of LZM conjugates revealed the formation of different glycoforms. Glycated LZMs containing up to seven galactose moieties were formed; while only mono- and diglycated LZMs with GOSs were detected. 2–3 mol of galactan were conjugated to 1 mol of LZM. Response surface methodology, based on a 5-level and 3-factor central composite design, revealed that molar ratio and temperature were the most significant variables for the glycation of LZM with GOSs. The optimal conditions leading to a high percent blocked lysine (16.11%) with a low protein aggregation index (0.11) were identified: temperature of 49.5 °C, LZM:GOS molar ratio of 1:9 and a_w of 0.65. To the best of our knowledge, this is the first study on the optimization of LZM glycation with GOSs.

1. Introduction

In order to improve the functional properties of proteins and their technological applications, several methods, based on genetic [1], physical [2], chemical [3], and enzymatic [4] modifications were reported. Among these modifications, the glycation of proteins via naturally occurring Maillard reaction, under controlled conditions, led to a significant improvement of protein functionalities, such as emulsifying properties, protein solubility and heat stability [5,6]. The protein glycation was also found to be a very efficient method to decrease, *in vivo*, the allergenicity of protein [7]. Moreover, the conjugation of prebiotic oligosaccharides with a protein could potentially increase their colonic persistence, allowing them to reach the distal colonic region, where most of chronic gut disorders originate [8]. Maillard reaction is, therefore, a promising approach to generate glycoproteins having improved functional and biological properties.

In the early stage of the Maillard reaction, the carbonyl group of a reducing carbohydrate interacts with the nucleophilic amino group in peptides/proteins, resulting in the reversible formation of N-substituted glycosylamine (Schiff base), which is labile and may undergo irreversible rearrangements [9]. The intermediate stage begins with the degradation of the Amadori/Heyn's products, which can undergo numerous transformations under various divergent pathways [10]. Further reactions lead to the formation of advanced glycation end products (AGEs) that are assumed to be responsible for a number of pathophysiological syndromes *in vivo* [11]. In order to produce protein–carbohydrate conjugates with improved functional and biological properties with minimal cross-linking and color or flavor development, the Maillard reaction should be well controlled and limited to its early stages [12]. Different conditions, such as the molar ratio of substrates, temperature, pH, incubation time, and water activity (a_w), have been reported to affect the rate of several steps involved in the Maillard reaction [13].

Although many studies have been reported on the glycation of proteins with small carbohydrates (i.e. galactose, glucose, fructose) [14,15], and polysaccharides (i.e. dextran, galactomannan, chitosan) [16,17], to our knowledge, only few studies on the glycation of proteins with oligosaccharides have been carried out to date

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[18,19]. Conflicting results have been reported regarding the effects of the carbohydrate length on the protein glycation [20–22]. Moreover, no optimization of the Maillard reaction conditions leading to the maximum formation of protein–oligosaccharide conjugates has been carried out.

The present study was aimed at investigating the glycation of a selected protein with mono-, oligo- and polysaccharides through the Maillard reaction. Lysozyme (LZM), derived from hen egg white protein, was selected as the model protein because it is well characterized and has many desirable properties including antimicrobial activity [23] and heat stability [24]. In these regards, the specific objectives were (a) to investigate the effect of a_w on the time course of the degree of LZM glycation with selected carbohydrates, including galactose, prebiotic galactooligosaccharides (GOSs) and potato galactan, (b) to characterize the structural properties of the resulting glycoproteins by MS in order to elucidate the reactivity of the selected carbohydrates and their actions and (c) to study and optimize the effects of selected glycation parameters on the conjugation of LZM with prebiotic GOSs by response surface methodology (RSM) using a 5-level 3-factor central composite design (CCD). These findings will bring more insight into the effect of carbohydrate length on the LZM glycation and more understanding of the interactions between the glycation parameters for the production of LZM conjugated with prebiotic oligosaccharides with higher glycation level. Such oligosaccharide/LZM conjugates may conceivably possess added functional properties and prebiotic activity as compared to LZM conjugated with mono and polysaccharides.

2. Materials and methods

2.1. Materials

LZM from chicken egg white (>96% purity), triolein and D-(+)-galactose were purchased from Sigma Chemical Co. (St.-Louis, MO). Potato galactan (~100 kDa) and endo-1-4-β-D-galactanase from *Aspergillus niger* were purchased from Megazyme (Wicklow, Ir). TNBSA (2,4,6-trinitrobezene sulfonic acid) and salts were purchased from Sigma Chemical Co. Furosine standard was purchased from Neosystem Lab. (Strasbourg, Fr).

2.2. Preparation of galactooligosaccharides

GOSs were prepared via the enzymatic hydrolysis of potato galactan. 5.0 enzymatic units of endo-1-4-β-D-galactanase were added to 200 ml of galactan solution (1 g l^{-1}) in 0.1 M sodium acetate buffer (pH 4.5). The mixture was incubated at 40 °C for 23 h. To remove the unhydrolyzed galactan, the hydrolysate was ultrafiltered using a stirred ultrafiltration unit (Amicon system, Millipore, Bedford, MA) fitted with a 3 kDa molecular mass cut-off membrane (Waters Corp., Milford, MA) and the filtrate containing the GOSs was recovered. The monosaccharides were removed from the GOS fraction by size exclusion chromatography on Biogel P2 column (Bio-Rad, Philadelphia, PA) previously conditioned with 20 mM ammonium carbonate buffer pH 7.0. The elution was carried out with 40 ml of the same buffer at a flow rate of 0.3 ml min^{-1}. Fractions of 1 ml were collected using a fraction collector (LKB FRAC-100, Pharmacia) and subjected to analysis by thin layer chromatography (TLC). TLC on silica-gel 60 F_{254} plates (EMD Chemicals, Gibbstown, NJ) was performed with butanol/acetic acid/water (4:5:2, v/v/v) as the developing mobile phase. For carbohydrate detection, the TLC plates were sprayed with 2% (v/v) H_2SO_4 in methanol and heated at 90 °C for the development of orange spots. The fractions containing GOSs were pooled and lyophilized. The lyophilized powder was desalted by elution with water using the same Biogel P2 column. The molar mass of GOSs was estimated from the average of its saccharidic distribution determined by electrospray ionization mass spectrometry (ESI-MS) (Section 2.5). The estimated molar mass of GOSs is 1990.9 g/mol.

2.3. Preparation of conjugates

LZM and each of galactose or GOSs or galactan at the molar ratio of 1:7 were dissolved in 0.1% (w/v) of 0.05 M sodium phosphate buffer (pH 7.0). The protein–carbohydrate mixtures were lyophilized at −25 °C. The powder mixtures were incubated in sealed glass desiccators at 60 °C. The samples were incubated for selected times (1–11 days) under controlled a_w values of 0.79 (KBr), 0.65 (KI), and 0.45 ($Mg(NO_3)_2$) and thereafter analyzed for their degree of glycation.

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

2.4.1. Measurement of proportion of free amino groups

The proportion of free amino groups of LZM was determined using TNBSA assay, according to the modified method of Goodwin and Choi [25]. To 0.5 ml of LZM conjugate solutions (10 mg ml^{-1}) in 0.1 M sodium bicarbonate buffer (pH 8.5), 0.25 ml of 0.01% TNBSA was added. The mixtures were incubated at 37 °C for 2 h. To solubilize the protein and avoid their precipitation, 0.25 ml of 10% sodium dodecyl sulfate (SDS) and 0.125 ml of 1 N HCl were added to the mixtures. 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-lysine. All assays were run in triplicates. The amounts of free amino groups obtained from unmodified LZM/carbohydrate mixtures, before incubation, were used as reference for the calculation of the percentage of blocked lysine.

2.4.2. Furosine analysis

To estimate the degree of glycation, ε-N-2-(furoylmethyl)-L-lysine (furosine) analysis was performed using a modified method of Moreno et al. [26]. 400 μl of LZM conjugates (2–4 mg of protein) was added to 1.1 ml of 8 N HCl. The mixtures were incubated at 110 °C for 23 h under nitrogen. The recovered hydrolysates were centrifuged at 14,000 × *g* for 10 min and 1 ml was applied to a previously activated Sep-pak C18 cartridge (Waters Corp., Milford, MA). Furosine was eluted with 3 ml of 3 N HCl, and the eluate was evaporated until dryness under nitrogen and resolubilized in 200 μl of water:acetonitrile:formic acid (95:4.5:0.5, v/v/v). Furosine concentration was determined using high-pressure-liquid chromatography (HPLC) according to the method described by Resmini et al. [27]. 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. The separation was performed on a Thermo Hypersil-Keystone Prism RPN (250 mm × 3 mm) column using an isocratic elution of 0.06 M sodium acetate buffer (pH 4.3) for 30 min at a flow rate of 0.4 ml min^{-1}. Injected sample volume was 20 μl and the detection of furosine was performed at 280 nm. The calibration curve was constructed using furosine standard. All assays were run in duplicates with quadruplicate injections (half of them containing internal standard of furosine).

2.4.3. Determination of percentage of blocked lysine

The concentration of blocked lysine was indirectly estimated from the furosine content, considering that tagatosyl-lysine Amadori compounds, formed during the Maillard reaction between lysine residues and galactose moieties upon 8 N acid hydrolysis, generates about 42% furosine [28]. The amount of initial free lysine before glycation was determined through the TNBSA assay. Since only six out of seven free amino groups per molecule of LZM were detected by the TNBSA assay, a conversion factor was used. The percentage of blocked lysine was estimated according to Eq. (b).

$$\text{Blocked lysine}\frac{1}{0.42} = (\text{furosine}) \times \left(\frac{\text{MW of furosine}}{\text{MW of lysine}}\right) \quad \text{(a)}$$

$$\text{Blocked lysine\%} = \frac{\text{blocked lysine} \times 100}{\text{initial free lysine} \times 1.167} \quad \text{(b)}$$

2.4.4. Measurement of the protein aggregation index and of the browning

To estimate the extent of protein cross-linking, favored during the advanced stages of Maillard reaction, the turbidity of LZM conjugates (10 mg ml^{-1}) was estimated spectrophotometrically at 500 nm. The browning of the LZM conjugates was measured in parallel at 420 nm. All assays were run in triplicates.

2.5. Structural characterization of lysozyme conjugates

The mass spectra of GOSs, LZM:galactose and LZM:GOS conjugates were analyzed by electrospray ionization mass spectrometry (ESI-MS) using a triple–quadrupole mass spectrometer equipped with a Surveyor LC pump, an LCQ advantage mass spectrometer (ion trap) and with Xcalibur® software to control the system acquisition and data processing. Samples were infused into the spectrospray ion source (fused silica capillary of 100 μm i.d.) at a rate of 1 μl min^{-1} from a low-pressure infusion pump (model 22, Harvard Apparatus, South Natick, MA). The peaks in each spectrum were identified by their observed m/z values using the following equation where M is the molecular weight of LZM (~14.3 kDa), N is the number of attached galactose molecules and W is the number of dehydrated water molecules:

$$\frac{m}{z} = \frac{M + N \times 180 - N \times 18 - W \times 18 + 12}{12}$$

The molecular weight of the LZM:galactan conjugates was estimated using a 25 ml Superose 12 gel filtration column (GE Healthcare). 20 μl of the aqueous solution of purified LZM:galactan conjugates (5 mg ml^{-1}) was applied to the column using a FPLC system (GE Healthcare). The elution was carried out with a 0.05 M sodium phosphate buffer at pH 7.0, containing 0.15 M of NaCl at a flow rate of