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Maillard induced glycation behaviour of individual milk proteins

Hugo B. Cardoso, Peter A. Wierenga, Harry Gruppen, Henk A. Schols*

Laboratory of Food Chemistry, Wageningen University, Bornse Weilanden 9, 6708 WG Wageningen, The Netherlands

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ABSTRACT

This paper set out to differentiate the Maillard induced glycation reactivity of individual milk proteins using different saccharides under well-defined reaction conditions. α-Lactalbumin, β-lactoglobulin and β-casein were incubated with mono-, di- and trisaccharides in the dry state under standardised buffered conditions and glycation was expressed relative to the available reactive groups per protein (DG). Protein reactivity, described by the DG_{max} and initial speed of glycation (v), followed the same order for each protein-saccharide incubation: α-lactalbumin > β-lactoglobulin ≫ β-casein. Glycation of whey proteins by different monosaccharides was double that of β-casein. Differences in DG between whey proteins and β-casein decreased with increased saccharide size. A two-fold difference was found for glycation in the presence of the dimers lactose and maltose for β-casein but not for the whey proteins. The percentage of glycated lysines increased with increased lysine to protein size ratio.

1. Introduction

The Maillard reaction stands for a cascade of reactions also known as non-enzymatic browning of foods. The initial step of this cascade consists of the reaction between a reducing end of a saccharide and a free amino group of a protein, leading to protein glycation (Fennema, Damodaran, & Parkin, 2008; Hodge, 1953; Maillard, 1912; Sikorski, Pokorny, & Damodaran, 2008). Depending on conditions used, this protein glycation can result in protein aggregation (Broersen et al., 2007; Pellegrino, van Boekel, Gruppen, Resmini, & Pagani, 1999). This reaction can take place during the heat processing of foods with a low relative humidity (e.g. spray drying of milk) as well as during the processing of liquid foods (e.g. sterilisation/pasteurisation of beverages/liquid foods). The Maillard reaction proceeds at a higher rate in dry products than in liquid products (Schroeder, Iacobellis, & Smith, 1955). Protein reactivity in the Maillard induced glycation remain poorly understood due to discrepancies in literature. For milk proteins, α-lactalbumin and β-lactoglobulin were shown to have identical reactivity in one study (Czerwenka, Maier, Pittner, & Lindner, 2006), whilst in another study they were shown to have vastly different reactivities (Nacka, Chobert, Burova, Leonil, & Haertle, 1998).

The Maillard induced glycation can be influenced by several parameters such as the type of protein, saccharide, temperature and pH. Protein glycation depends on the reactive groups in the protein primary structure. The lysine residue has been found to be the most reactive group, followed by the arginine residue and the *N*-terminal α-amino group (Munch et al., 1999). The reactivity of the lysine residue itself has been shown to vary depending on neighbouring amino acids, either by changes in lysine pKa by neighbouring amino acids or through the catalysis of the Amadori compound (Bunn et al., 1979; Iberg & Fluckiger, 1986; Shilton, Campbell, & Walton, 1993). The saccharide represents another parameter influencing the Maillard reaction. Research has shown that for the reaction performed in the dry state in a non-buffered system, the level of glycation of α-lactalbumin decreases with an increase in saccharide size (ter Haar, Schols, & Gruppen, 2011). Incubation with different saccharide isomers can also lead to differences in glycation (Ledesma-Osuna, Ramos-Clamont, & Vazquez-Moreno, 2008), since different structures might differently influence the initial nucleophilic attack due to differences in reducing end reactivity. Temperature can have two distinct effects on Maillard reaction. An increase in temperature can lead to increased glycation in the early stage of the Maillard reaction (Cheison, Josten, & Kulozik, 2013; Chen, Liang, Liu, Labuza, & Zhou, 2012; Naranjo, Malec, & Vigo, 1998). In later stages, increased temperature can influence the reaction mechanism and consequently lead to the formation of various intermediary and end products such as melanoidin structures (Benzingpurdie, Ripmeester, & Ratcliffe, 1985). A widely accepted temperature used for the glycation of milk proteins is 60 °C (Czerwenka et al., 2006; Fenaille, Morgan, Parisod, Tabet, & Guy, 2003; Nacka et al., 1998; Naranjo et al., 1998; Pinto et al., 2012; ter Haar et al.,

Abbreviations: DG, degree of glycation; PB, 10 mM phosphate buffer pH 8.0; PBS, 10 mM phosphate buffer pH 8.0 with 150 mM NaCl; AmOH, 5% ammonium hydroxide solution; NaOH, 0.5 M sodium hydroxide solution

* Corresponding author.
E-mail addresses: hugo.cardoso@wur.nl (H.B. Cardoso), peter.wierenga@wur.nl (P.A. Wierenga), harry.gruppen@wur.nl (H. Gruppen), henk.schols@wur.nl (H.A. Schols).

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2011). The pH is another parameter shown to influence the Maillard reaction (Schroeder et al., 1955). An acidic pH can protonate both the lysine amino group and the saccharide aldehyde group and by doing so, preventing the formation of the protein-saccharide conjugate. A pH of 8.0 as been already widely used for the study of glycation of whey proteins (Ajlouni & Pan, 2014; Deng, Wierenga, Schols, Sforza, & Gruppen, 2017; Enomoto et al., 2009; ter Haar et al., 2011) and of caseins (Lee, Sen, Clifford, Whitaker, & Feeney, 1979). Ammonia has been previously used to set the pH at 8.0 prior to freeze-drying (ter Haar et al., 2011), but this system leads to a poorly controlled starting pH which in turn might be easily influenced by acidic compounds known to form throughout the reaction (Andrews, 1975; Davidek, Clety, Aubin, & Blank, 2002; Martins, Marcelis, & van Boekel, 2003). Phosphate buffers on the other hand, were shown to catalyse the Amadori rearrangement (Davidek et al., 2002; Yamaguchi et al., 2009). Consequently, a standardized method is key to compare the reactivity of different proteins and to identify key parameters in this reactivity.

In the present study, protein reactivity was studied using three milk proteins (α-lactalbumin, β-lactoglobulin and β-casein) individually reacted with saccharides of different size and structure in the dry state under standardized conditions.

2. Materials and methods

2.1. Materials

Bovine apo-α-lactalbumin was obtained as a commercial powder (BioPURE, Davisco Foods International, Le Sueur, MN, USA) containing 95% (w/w) protein of which 90% (w/w) was α-lactalbumin (Heijnis, Wierenga, Berkel, & Gruppen, 2010). Bovine β-lactoglobulin (Lot #SLBM8373, ≥90% purity measured by PAGE; 14–16% nitrogen content), β-casein (BioUltra, Lot #SLBK9882, ≥98% purity measured by PAGE; protein by biuret > 75%), rhamnose, galactose, glucose and maltotriose were obtained from Sigma-Aldrich (St Louis, MO, USA). Lactose and maltose were obtained from Merck (Kenitworth, NJ, USA). Mannose was acquired from Acros Organics (Thermo Fischer Scientific, Waltham, MA, USA). UHPLC grade water and acetonitrile containing 0.1% (v/v) formic acid were acquired from Biosolve (Valkenswaard, NB, The Netherlands). Ultrapure water was obtained using the Milli-Q system from Millipore (Billerica, MA, USA). No glycation was present in α-lactalbumin, β-lactoglobulin or β-casein acquired, as measured with the protocol described below.

2.2. Preparation of the Maillard induced protein-saccharide conjugates

In order to have a stable buffer system for the Maillard reaction and to be able to compare the effect of the reaction conditions with results previously described (ter Haar et al., 2011), different sample preparation conditions were initially tested using α-lactalbumin and glucose. Samples were either prepared in phosphate buffers or in aqueous solutions. The phosphate buffers were a 10 mM sodium phosphate buffered system pH 8.0 without or with 150 mM sodium chloride (PB and PBS respectively). The pH of aqueous solutions was adjusted to pH 8.0 by either 5% (w/v) ammonium hydroxide (AmOH) or by 0.5 M sodium hydroxide (NaOH). The pH of PB and PBS samples was readjusted to 8.0 prior to freeze-drying, using NaOH when needed. α-Lactalbumin, β-lactoglobulin and β-casein were individually mixed with each individual mono, di and trisaccharide in the PBS solution. A 1:2 M ratio lysine residue:saccharide reducing-end was used at a final protein concentration of 0.5% (w/v). Of each protein:saccharide solution, 1 mL was placed in a 1.5 mL vial and freeze-dried. The resulting powder was incubated up to 48 h at 60 °C and 65% relative humidity (RH), using a humidity chamber model HCP 108 (Memmert, Schwabach, Germany). Samples were incubated for 0, 2, 4, 8, 12, 24 or 48 h and subsequently stored at −20 °C. All samples were prepared in triplicate and incubated. The pH of the samples was measured after incubation and solubilisation in Milli-Q water.

2.3. Analysis of protein-saccharide conjugates by UPLC-MS

Incubated samples were solubilised in PBS to a final protein concentration of 5 mg/mL stirred at 4 °C overnight and centrifuged (10 min; 22,000*g*, room temperature). An aliquot of the supernatant was diluted 50 times using Milli-Q water. The diluted samples were analysed by a protocol adapted from ter Haar et al. (2011), using a Waters (Milford, MA, USA) UPLC H-Class system coupled to a Waters Synapt G2Si mass spectrometer. Samples were desalted online using a MassPREP Micro desalting UPLC column (Waters), using a column temperature of 80 °C. Ten µL of the diluted sample were injected in the UPLC system. The separation was performed using water containing 0.1% (v/v) formic acid (solvent A) and acetonitrile containing 0.1% (v/v) formic acid (solvent B) at a constant flow of 0.5 mL/min. The following elution profile was used: 95% A from 0 to 0.5 min; 95% A to 5% A from 0.5 to 9 min; 5% A to 95% A from 9 to 10 min and 95% A from 10 to 11 min for reconditioning. From 0.5 min to 5 min the flow was diverted to the MS detector, the remainder of the time it was diverted to the waste. UV detection was done at 214 nm using the Waters UPLC H-Class system UV detector. Samples were ionised using the positive and the resolution mode with ionisation energy of 3.00 kV, source temperature of 150 °C and cone gas flow of 200 L/h. The chromatographic and spectrometric data was acquired using Waters MassLynx V4.1 and was processed using Waters BiopharmaLynx v1.3.2 by the following process: 1) MS data was selected from 4.00 to 5.00 min from each chromatogram; 2) the baseline of the resulting MS spectrum was subtracted and the MS spectrum was then smoothed using the software default conditions; 3) the region of 800 to 2200 *m/z* of the spectrum of each sample was then deconvoluted to a resulting mass range from 10 to 30 kDa. The weighted average molecular weight of the protein-saccharide conjugate was subsequently calculated by integrating the *m/z* signals in the mass spectrum data to the molecular weight range of the protein-saccharide conjugate (dependent on protein).

2.4. Degree of glycation (DG)

The DG is the number of saccharides attached measured expressed as percentage of possible modification sites per protein (consisting of lysines, arginines and the *N*-terminal α-amino group). The parameters used to calculate the DG as described in Eq. (1) were as follows: native protein molecular weight ($Mw_{protein}$); average molecular weight of the modified protein ($Mw_{mod\text{-}protein}$); number of available NH_2 groups per protein ($n_{reactive\text{-}groups}$); molecular weight of the saccharide minus a water molecule ($Mw_{anhydro\text{-}saccharide}$).

$$DG\ (\%) = \frac{Mw_{mod-protein} - Mw_{protein}}{\mathrm{Mw}_{anhydro-saccharide} \times \mathrm{n}_{\mathrm{reactive-groups}}} \times 100\% \quad (1)$$

In order to derive the reaction rate constant and the theoretical DG_{max}, the calculated DG was best fitted to a first order reaction equation (Eq. (2)).

$$\mathrm{DG}\ (\%) = DG_{max}(1 - e^{(-k \times t)}) + DG_0 \quad (2)$$

DG_{max} stands for the DG plateau value (%) obtained from curve fitting, DG_0 is the fitted initial DG at time zero (%), t stands for time (h) and *k* is the reaction rate constant (h^{-1}).

In order to calculate the initial reaction speed, the measured DG values of the first 4 h were fitted to a zero order reaction equation (Eq. (3)).

$$\mathrm{DG}(\%) = v * t + DG_0 \quad (3)$$

v stands for zero order reaction speed ($\%.h^{-1}$). The *v* was calculated in order to differentiate the speed at which saccharides were attaching to the proteins in the first phase of the reaction. The DG_0 (%) in Eqs. (2) and (3) correspond to a fitted value and may differ slightly for a specific protein-saccharide combination.