Non-enzymatic glycation of natural actomyosin (NAM) with glucosamine in a liquid system at moderate temperatures

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Muscle protein functionality plays an important role in routine applications in the food industry. Glycation by the Maillard reaction is a naturally occurring process, which can be used to develop new ingredients with improved functionality using a food grade approach. Actomyosin was conjugated with glucose or glucosamine in a liquid system at moderate temperatures (40°C). Sugar to protein conjugation was evident by UV-Vis spectral changes, with the glycation level determined by matrix assisted laser desorption/ionisation mass spectrometry. Parameters for glycation of muscle protein were optimised using the bidimensional hierarchical clustering analyses. The best glycation conditions were 40°C for 8 h at 1:3 protein:sugar ratio. Solubility and emulsifying properties of glycoconjugates were significantly improved as compared to non-glycated proteins. At pH 7 glycated actomyosin was on average 31% more soluble compared to non-treated protein. Glucosamine was found to be more effective for glycation and provided higher protein functionality as compared to glucose.

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1. Introduction

The functional behaviour of proteins is one of the most important aspects which determines their technological usability, described in terms of solubility and emulsification. As this functionality is vital in food processing, many efforts have been made to develop new food ingredients with improved properties. Among the number of studies on functionality improvement, non-enzymatic glycation, the so-called Maillard reaction, has received much attention as a natural way to produce glycoconjugates with upgraded functional properties. The Maillard reaction is initiated by the interaction between the free amino groups in protein and carbonyl groups of a reducing sugar to produce an imine (Schiff base) (Dutta, Cohenford, & Dain, 2006). This adduct further rearranges into the Amadori product which, upon dehydration and rearrangements, forms highly reactive deoxyosones – potent precursors of protein cross-links (Elgawish, Glomb, Friedlander, & Monnier, 1996). Advanced glycation end products (AGEs), generated at the late stages of the Maillard reaction, are affiliated with diabetic complications and other diseases (Hsu & Zimmer, 2010).

The Maillard reaction, as a tool for glycation, was reported to be superior to other systems, since no chemical reagents are required for the reaction to proceed (Saeki, 2012). For instance, the solubility of scallop (Katayama, Shima, & Saeki, 2002) and chicken myofibrillar proteins (Nishimura, Murakoshi, Katayama, & Saeki, 2011) were highly improved with the progress of the Maillard reaction. In addition, emulsifying properties (Shu, Nakamura, & Kato, 1998) and thermal stability (Fujisawa, Oosawa, & Saeki, 1998) of different proteins have also been upgraded via glycation.

The usual preparation of protein-carbohydrate complexes by the Maillard reaction involves dry heating and storage of the lyophilised mixtures from 1 to 3 weeks (Zhu, Damodaran, & Lucey, 2008). Despite substantial evidence of functionality improvement by glycation in dry conditions, its application in food technology is limited. The limitations are attributed to difficulties in controlling the extent of glycation, uneven distribution of reactants (Kato, 2002), long reaction time (Zhu et al., 2008), possible protein denaturation (French, Harper, Kleinholz, & Green-Church, 2002), undesirable colour changes (Tanaka, Kunisaki, & Ishizaki, 1999) and formation of antinutritional compounds (Brands, Alink, van Boekel, & Jongen, 2000). In view of the drawbacks of current glycation techniques, no feasible industrial scale up method is available, thus no commercially produced conjugate ingredients have been obtained (Zhuo et al., 2012).

Water activity (a_w) of the reaction mixture plays an important role in the rate of numerous Maillard reaction pathways, as it is responsible for molecular mobility, protein conformation, surface area and accessibility of reactive amino groups in protein (Oliver, 2011). In a recent study (Qi, Liao, Yin, Zhu, & Yang, 2010) attempting to overcome drawbacks of glycation in dry conditions, acid precipitated soya protein–dextran conjugates were prepared in an 80% or 95% ethanol-reacting system at 50°C for 6 h or 60°C for 24 h, respectively. Zhu et al. (2008) also reported the formation of whey protein–dextran complexes in a liquid medium at 60°C for 24 h.
The main myofibrillar proteins, myosin and actin are the most important muscle proteins for technological purposes. Actomyosin, the main state of actin and myosin in post-mortem muscle, is accepted to be an unstable heat sensitive molecule (Jacobson & Henderson, 1973). Correspondingly, moderate temperature of reaction is an essential factor for actomyosin glycation. According to previous reports, if elevated temperatures and advanced stages of reaction are used during protein glycation in solution, protein denaturation and polymerisation are likely to occur (Igaki et al., 1990). Therefore, there is a strong need to establish a method suitable for glycation of muscle proteins in wet conditions.

Concurrently with water activity, the nature of reducing sugars considerably influences the reaction rate and, consequently, protein functionality (Oliver, 2011). The reactivity of reducing sugars in the Maillard reaction has been reported to increase with decreasing molecular weight (Jouppila, 2006).

Glucosamine (GlcN) is an amino sugar that is important for the biosynthesis of glycated proteins and lipids. It is a major constituent of glycosaminoglycans and glycolipids, and is important for the structure and function of cartilage in the joints of the human body (Henrotin, Mobasheri, & Marty, 2012). Kraehenbuehl, Davidek, Devaud, and Mauroux (2008) reported higher reactivity of GlcN as compared to fructose, galactose, glucose or xylose when reacting with cysteine at 125 °C for 25 min. If such a fast reactivity occurs during glycation it would be expected to increase the rate of the reaction and avoid usage of elevated reaction temperatures. Taking into account that GlcN possesses amino and hydrophilic hydroxyl groups (Chung, Tsai, & Li, 2006), its incorporation into actomyosin could be a practical strategy to improve muscle protein functionality. Therefore, the aim of this study is to demonstrate the possibility of actomyosin glycation with GlcN by the Maillard reaction in a liquid medium at moderate temperatures, with faster reaction rates, and to investigate its contribution to functionality mediated by glycoproteins.

2. Materials and methods

2.1. Materials

Fresh chicken breast was obtained from the local store. Analytical grade D(+)-glucosamine hydrochloride, D(+)-glucose (Glc), L-lysine, sodium phosphate monobasic, and sodium phosphate dibasic were purchased from Sigma–Aldrich (St. Louis, MO, USA). Other chemicals and reagents were of analytical grade. All buffers were prepared with Milli-Q purified distilled water (Millipore, Bedford, MA, USA).

2.2. Extraction of natural actomyosin (NAM)

Actomyosin from chicken Pectoralis major was extracted according to the method described by Benjakul, Visessanguan, Ishizaki, and Tanaka (2001) with slight modifications. Briefly, chicken breast muscle (fresh or frozen) was homogenised in chilled 0.6 M KCl at 1:10 (w/v) for 2 min in a homogeniser (Fisher Scientific, Power Gen 1000 S1, Schwerte, Germany). Homogenate was centrifuged at 5000g for 40 min at 4 °C using a Avanti® J–E refrigerated centrifuge (Beckman Coulter Inc., Palo Alto, CA, USA). Three volumes of chilled denionised water were added to precipitate NAM. After centrifugation at 5000g for 30 min at 4 °C the pellet was re-suspended in chilled 50 mM potassium phosphate buffer solution (PBS) (pH 7.5) containing 0.55 M KCl. The precipitate was collected by centrifugation at 5000g for 10 min at 4 °C. The pellet from the final centrifugation step is hereafter called “natural actomyosin” or NAM.

2.3. Glycation of NAM

Experimental design for NAM glycation is presented in Fig. 1. As follows, mixtures of NAM and sugars (Glc or GlcN) in ratios of 1:1, 1:3 and 1:6 (w/w) were dissolved in 50 mM PBS (pH 7.5) in the presence of 0.55 M KCl. The pH values of the solutions were adjusted by careful addition of 0.1 M HCl or 0.1 M NAOH. The aliquots of the solutions were incubated in an Innova 44 (New Brunswick Scientific, USA) shaker under constant agitation. After incubation, unreacted carbohydrate moieties were removed by ultrafiltration employing a Amicon Ultra 3K membrane (molecular weight cut-off of 3.000 NMWL; Millipore Corporation, Bedford, MA, USA). To study the effect of different reaction temperatures, NAM/Glc and NAM/GlcN solutions at different ratios were heated at 25, 37 and 40 °C from 0 to 12 h. To elucidate the effect of different protein to sugar ratios, NAM/Glc and NAM/GlcN solutions were incubated at different temperatures with samples taken every 2 h of reaction. At least three individual reaction conditions were performed.

2.4. Assessment of the Maillard reaction evolution by ultraviolet–visible (UV–Vis) spectroscopy

To confirm glycation occurrence and assess the extent of the Maillard reaction, UV–Vis spectroscopy analyses were performed. Glycated proteins in 50 mM PBS (pH 7.5) in presence of 0.55 M KCl were analysed using a spectrophotometer (Beckman Instruments, Fullerton, CA, USA). The samples were diluted 11 times, loaded into a 96-well microplate (200 μL) and scanned from 280 to 500 nm. Glc or GlcN solutions were analysed under the same conditions.

2.5. Protein oxidation measurement

Determination of carbonyl level in proteins is used as an index of the extent of protein oxidative damage. Content of carbonyl groups in NAM extracted from fresh or frozen chicken breast were detected by reactivity with 2,4-dinitrophenylhydrazine (DNPH) to form protein hydrazones according to Chan, Omana, and Betti (2011). NAM solutions were diluted to a protein concentration ranging from 0.7 to 1.0 mg/ml and precipitated with 10% TCA (w/v). After centrifugation (2000g, 10 min, 4 °C) the blank was treated with 4 ml of 2 M HCl and the test samples (pellet) with 4 ml of 0.2% DNPH (w/v) in 2 M HCl. The tubes were incubated for 1 h at 25 °C in the dark with agitation every 10 min. After precipitation with 10% TCA, the solutions were further centrifuged to collect the protein precipitates. Next, the pellet was washed 2 times with 1 ml of ethanol-ethyl acetate (1:1, v/v), precipitated with 10% TCA and centrifuged. The final precipitate was dissolved in 2 ml 6 M
2.6. Evaluation of protein solubility

The solubility of glycated and control samples was determined according to the method of Montero, Jimenez-Colmenero, and Borderias (1991). Briefly, one ml of each sample or control solution was added to glass test tubes and the pH adjusted to end points ranging from 2.0 to 12.0. The final volume was then attuned to 2 ml with distilled water having the same pH as the sample solution. After centrifugation (9000g for 15 min at 4 °C) protein content of the clear supernatant and original suspension was determined according to the Biuret assay (Gornall, Bardawill, & David, 1949) using BSA as a reference protein. The solubility of glycated and control NAM was expressed as the percent of the protein in the supernatant to that of the protein solution before centrifugation. The ionic strength post-ultrafiltration was calculated for glycated samples using a conductivity metre (Oakton Acorn CON 6, Vernon Hills, IL, USA) calibrated prior to the measurements. Thereafter, the ionic strength of the samples set was adjusted to 0.55 M KCl.

2.7. Study in a model lysine–glucosamine system

For the purpose of the study in the model system, mixture of GlcN and Lysine (Lys) at 1:3 (w/w) ratio in a 50 mM PBS was incubated at 40 °C under constant agitation. After predetermined heating time (0, 2 and 8 h) the obtained samples were immersed in ice for rapid cooling. Aliquots were further analysed by Orbitrap-LC-MS/MS. Solutions of GlcN were separately prepared, incubated and analysed at the same conditions as described above.

2.7.1. LC–MS/MS analyses

Mixture of Lys–GlcN obtained from different incubation times were dissolved in aqueous 25% (v/v) acetonitrile and 0.2% (v/v) formic acid. Nanoflow HPLC (Easy-nLC II, Thermo Scientific) coupled to the LTQ XL-Orbitrap hybrid mass spectrometer (Thermo Scientific, Bremen, Germany) was used to resolve and analyse the samples. Nanoflow chromatography and electrospray ionisation were accomplished by using a PicoFrit fused silica capillary column (ProteoPepII, C18) with 100 μm inner diameter (300 Å, 5 μm, New Objective). Sample solutions were injected onto the column at a flow rate of 3000 nl/min and resolved at 500 nl/min using 30 min linear ACN gradients from 5 to 50% v/v aqueous ACN in 0.2% v/v formic acid. The mass spectrometer was operated in data-dependent acquisition mode, recording high-accuracy and high-resolution survey Orbitrap spectra using external mass calibration, with a resolution of 60,000 and m/z range of 100–2000. Ten most intense multiple charged ions were sequentially fragmented by using collision induced dissociation, and spectra of their fragments were recorded in the linear ion trap; after two fragmentations all precursors selected for dissociation were dynamically excluded for 60 s.

2.7.2. Detection of ammonia (NH₃)

To verify the release of NH₃ in the course of Lys glycation with GlcN, the mixture of GlcN and Lys at 1:3 (w/w) ratio in a 50 mM PBS was incubated at 40 °C for 0, 2 and 8 h under constant agitation. The NH₃ concentration was measured immediately with the commercial ammonia quantitative determination kit (Sigma, St. Louis, MO, USA), according to the manufacturer’s instructions. The assay is based on the following reaction: NH₃ reacts with α-ketoglutaric acid (KGA) and reduces nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of the L-glutamate dehydrogenase (GDH) to form L-glutamate and oxidised nicotinamide adenine dinucleotide phosphate (NADP⁺):

\[
\text{KGA} + \text{NH}_3^+ + \text{NADPH} \rightarrow \text{L-Glutamate} + \text{NADP}^+ + \text{H}_2\text{O}
\]

The oxidation of NADPH to NADP⁺ results in a decrease in the absorbance at 340 nm that is proportional to the concentration of NH₃.

2.8. Sample pre-treatment and digestion prior to matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/TOF-MS) analyses

Control and glycated samples were diluted in a ratio of 1:1 with sample buffer containing 5% β-mercaptoethanol. After heating for 5 min (Thermomixer R, Eppendorf North America, Westbury, NY), 20 μl of the sample was loaded on 4–20% Mini-PROTEAN TGX pre-cast gel (Bio-Rad Laboratories, Inc., Hercules, CA) and run in a Mini-PROTEAN tetra cell attached to a PowerPac Basic electrophoresis apparatus (Bio-Rad Laboratories Inc., 1000 Alfred Nobel Drive, Hercules, CA, USA) samples were stained with Coomassie Brilliant Blue. Afterward, excised gels were reduced with 10 mM β-mercaptoethanol, alkylated with 50 mM iodoacetamide, washed with 100 mM ammonium bicarbonate/acetonitrile (1:1, v/v) solution and dried in a SpeedVac (AES 2000, Savant Instruments Inc., Farmingdale, NY, USA). Dried gel bands were subjected to overnight proteolytic digestion at 37 °C with 0.2 μg of trypsin. After incubation, peptides were extracted with 30 μl of 100 mM ammonium bicarbonate followed by extraction with 30 μl solution containing 5% formic acid and 50% acetonitrile in water twice and dried to ~15 μl in a SpeedVac.

2.9. Evaluation of glycoconjugation by MALDI-TOF/TOF-MS

With the aim of knowing the relative amount of carbohydrates linked covalently to NAM, MALDI-TOF/TOF-MS analyses were carried out. For this purpose, the samples (obtained as described in the section above) were diluted tenfold in 50% acetonitrile/water + 0.1% trifluoroacetic acid. Afterwards, 1 μl of each sample was mixed with 1 μl of α-cyano-4-hydroxycinnamic acid (4-HCCA, 10 mg/ml in 50% acetonitrile/water +0.1% trifluoroacetic acid). One microlitre of the sample/matrix solution was then spotted onto a stainless steel target plate and allowed to dry in air. All mass spectra were obtained using a Bruker UltraFlex MALDI-TOF/TOF-MS (Bruker Daltonic GmbH, Bremen, Germany). Ions were analysed in positive mode after acceleration from the ion source by 25 kV. External calibration was performed by use of a standard peptide mixture.

2.10. Emulsifying activity index (EAI) and emulsion stability index (ESI)

The measurements of emulsifying activity and stability indexes were conducted according to the method described by Mouré, Domínguez, Zuniga, Soto, and Chamy (2002) with slight modifications. Briefly, 3 ml of protein solution (2 mg/ml in 50 mM PBS (pH 7.5), containing 0.55 M KCl) was added to 1.0 ml of corn oil. The mixture was homogenised by using Power Gen 1000 51 homogeniser (Fisher Scientific, Schwerte, Germany) operated for 1 min at setting 3. Immediately after homogenisation, 0.05 ml of emulsion was diluted to 5 ml with 0.1% sodium dodecyl sulphate (SDS) solution and the absorbance of the sample was measured at 500 nm against 0.1% (w/w) SDS solution blank using a
spectrophotometer (V-530, Jasco Corporation, Tokyo, Japan). The effect of pH on EAI was determined by careful pH adjustment to 3.0 or 10.0 with a set of NaOH and HCl of different concentrations.

The EAI was calculated from the following equation:

$$EAI = 2.33 \times A_0$$

where $A_0$ is the absorbance estimated just after emulsion preparation. The emulsion stability index (ESI) was determined by measuring the absorbance of these emulsions after 10 min of standing and was calculated as follows:

$$ESI = 10 \times \frac{A_0}{(A_0 - A_{10})},$$

where $A_{10}$ is the absorbance determined after 10 min.

### 2.11. Data analyses

The experiment was replicated at least three times. All the data were expressed as means ± standard deviation. Analysis of variance (ANOVA) was conducted to test for significant differences among treatments using the statistical analysis system computer software (SAS, Version 9.0, NC, USA). Means were compared using Tukey’s adjustment at the 95% confidence interval.

The results from UV–Vis spectra were analysed by using bidimensional hierarchical clustering analyses (heat map) performed within the programming and visualisation environment R version 2.10.1 (Ihaka & Gentleman, 1996). The heat map was developed based on 126 samples (average from three repetitions) × 40 wavelength values for fresh and frozen NAM separately. In the graph, each column represented a wavelength, each row represented a different glycation treatment and the colour of each square corresponded to the magnitude of absorbance values, with green representing higher values and red representing lower values. The raw data underwent a Z-score transformation with the cells coloured accordingly:

$$Z = \frac{\text{Observed value} – \text{Average}}{\text{Standard deviation}}$$

### 3. Results and discussion

#### 3.1. UV–Vis spectrum

In the current study Maillard-type carbohydrate–protein conjugates were produced by the reaction between free amino groups of NAM and carbohydrate groups of Glc or GlcN. The extent of protein glycation is an important factor in the beneficial and detrimental effects on protein functionality (Oliver, Melton, & Stanley, 2006). Hence, this study focused on determining the best conditions to achieve high yields of glycation, along with avoiding protein polymerisation and minimizing the formation of undesirable reaction side products. To achieve this objective the study was designed as depicted in Fig. 1. Buffered solutions of NAM/Glc or NAM/GlcN at different protein to sugar ratios (1:1; 1:3 or 1:6) were incubated at 25, 37 or 40 °C with aliquots collected at different time intervals. The progress of reaction with time was monitored with UV–Vis spectroscopy. Typical spectra obtained for NAM–Glc and NAM–GlcN conjugates are shown in Fig. 2A and B, respectively. Due to the large number of variables, not all spectra are presented, however the results from all spectra are summarised in a heat map (described in the following part of this manuscript).

UV–Vis absorption spectra profiles demonstrated that the rate of Maillard reaction products formation was dependent on the type of sugar, its concentration, reaction time and temperature. The absorbance values at 280–320 nm. A continuous rise of absorbance values was observed as heating time increased. The highest absorbance readings were obtained when a 1:6 protein to carbohydrate ratio was used and increased mainly after 6 h of incubation. The rate of product formation was faster when GlcN was used as compared to Glc. NAM–Glc or NAM–GlcN mixture at the beginning of reaction corresponded to the absorbance maximum in the range from 287 to 290 nm, which might be dominated by contributions from the Tyr, Phe and Trp side-chains (Jamin & Lacapere, 2007). Upon glycation the bathochromic shift was observed in the region of 305–307 nm depending on the reaction variables. This shift was achieved faster and was more evident for the samples glycated with GlcN compared to Glc. Previously, Zhu et al. (2008) reported that conjugation between whey protein and dextran resulted in the peak shift from 304 to 307 or 310 nm after 48 or 72 h of reaction. The authors assigned $\lambda_{max}$ of 304 to the Schiff base formation, while 307–310 nm were attributed to the formation of Amadori compounds or intermediate stages of the glycation reaction. In the model reaction of 2,4-dihydroxybenzaldehyde (DNBA-P) with aminocaproic acid, Schiff base formation was observed at $\lambda_{max}$ of 296 nm (Bionski, De Moissac, Perie, & Sygusch, 1997), although the reaction between DHBA-P with lysine residues of aldolase resulted in Schiff base formation at 311 nm. Zhu et al. (2008) proposed that the different values for shift $\lambda_{max}$ reported in previous studies were related to the different environments of the Schiff bases. As presented in Fig. 2A and B, besides the absorption at the corresponding $\lambda_{max}$ there was a tailing absorption which extended to the visible wavelength region. This may indicate the formation of some chromophores, acquired from the rearrangement products of the Maillard reaction (Zhu et al., 2008).

Higher reactivity of GlcN compared to other sugars (i.e. fructose, galactose, glucose, etc.) when reacted with cysteine at a 4:1 M ratio was observed by Kraehenbuehl et al. (2008). Greater acceleration in glycation rate by GlcN might be related to the difference in the molecular structure as compared to Glc (Fig. 3). It is speculated here that GlcN might convey higher catalytic activity for non-enzymatic browning by a specific mechanism related to the presence of a primary amine at the C-2 position in the pyranose ring. The close proximity of this amino group to the reactive anomerical centre apparently might be responsible for GlcN higher reactivity.

Because of GlcN amino group, the condensation between GlcN molecules or GlcN molecules with their products in solution may occur (Zhang et al., 2004). GlcN has been reported to undergo spontaneous dimerisation and rearrangement, forming deoxyfructosazone and 2,5-bis-(tetrahydroxybutyl)pyrazine and other products (Jun, Shao, Ho, Koetter, & Lech, 2003). In view of this, it is expected that the reaction mixture contained not only glycoconjugates and unreacted protein/sugar, but also the products derived from GlcN autocondensation and/or rearrangement. In the current study GlcN solutions were also separately incubated as a carbohydrate control (data not shown). The slight increase in absorbance values over time was observed only at 1:6 protein to sugar ratios using 40 °C for incubation.

The results from all UV–Vis spectra for NAM extracted from fresh meat are summarised in a heat map (Fig. 4). Described here for the first time for glycation analyses, this method allows for visualisation of large-scale experimental data in a single graph, helping to explore and interpret results more efficiently. From the heat map it is evident that samples glycated with Glc formed clusters tightly together, coloured in bright red on the left side of the heat map. The central part of the heat map consisted of clusters with treatments displaying the highest absorbance, which corresponded to GlcN glycated samples (bright green). Accordingly, the following treatments were selected as optimal for glycation reactions: 1:3 NAM–GlcN incubated from 8 to 12 h and 1:6 NAM–GlcN from 6 to 10 h of reaction at 40 °C.
Fig. 2. UV–Vis absorbance spectra of NAM–GlcN (A) and NAM–Glc (B) conjugates over time. The reaction was performed at 40 °C at 1:3 protein–carbohydrate ratio from 0 (control) to 12 h.

Fig. 3. Difference in the molecular structure between glucosamine and glucose.

Fig. 4. Hierarchal clustering of protein–sugar UV–Vis absorbance data for actomyosin extracted from fresh chicken Pectoralis major. The vertical axis represent individual wavelength (280–320 nm). The horizontal axis represents individual glycation treatments. The colours in each cell indicate the absorbance of a particular sample relative to the mean level from all samples for the specific wavelength. The colour scale extends from bright green (maximum absorbance) to bright red (minimum absorbance). The heat map was developed based on 126 samples (average from three repetitions) × 40 wavelength values.
3.2. Effect of frozen storage on glycation parameters

Frozen storage is an important practise used for preservation of muscle-based food. Allowing for longer storage time and better production control, it is also necessary for the purpose of transportation and export. Even though frozen storage is applied to slow down undesirable biochemical reactions in meat, cell disruption due to formation of ice crystals might have negative effects on meat physicochemical properties and subsequently its functionality (Soyer, Ozalp, Dalmis, & Bilgin, 2010). Due to the changes during frozen storage, conditions for glycation for this type of meat might significantly differ from those which are optimal for fresh meat.

In this respect, determination of the optimal conditions to produce NAM glycoconjugates from frozen material was included into the experimental design (Fig. 1). NAM was extracted from frozen (3 weeks; −20 °C) chicken breast, glycated and analysed by UV–Vis spectroscopy in the same way as described above for fresh NAM. Collected UV–Vis spectra were organised into the heat map (Fig. 5). For the UV–Vis absorbance there was an evident shift to the longer wavelength (318–320 nm) as compared to the fresh, where clustering was observed at broader range of wavelength. This shift corresponded to the samples incubated at 25, 37 and 40 °C at different NAM/GlcN ratios. Moreover, the glycation proceeded slightly faster even at lower temperatures, as compared to the fresh meat system. This suggests that freezing of NAM prior to glycation might be responsible for the acceleration of the reaction rate. It was also observed that after 8 h of incubation, frozen NAM–GlcN sample solutions developed a slightly yellow colour. Even though a faster time of glycation seems to be a positive aspect here, there is a chance that glycation may not to be limited to the initial stages of the Maillard reaction, proceeding further to Amadori or the AGEs. This might negatively impact protein functionality by causing higher occurrence of protein cross-linking and faster accumulation of unwanted reaction products. At the same time, considering the complexity of the Maillard reaction, it would be beneficial to gain further insight into the parameters allowing for better control of the reaction at these particular conditions.

3.2.1. Protein oxidation during frozen storage

There are reports in the literature indicating that frozen storage promotes oxidative deterioration of proteins, causing production of carbonyl compounds. For example, Soyer et al. (2010) found that freezing of chicken leg meat over 3 months at −7 °C led to significant increases in carbonyl content. To further elucidate the effect of frozen storage, the amount of carbonyl groups in fresh and frozen NAM was measured. The amount of carbonyls significantly increased ($p = 0.0059$) from 2.14 to 2.92 nmoles carbonyls/mg protein. This result was in agreement with the report of Chan et al. (2011) who found that the amount of carbonyls significantly increased from 1.72 to 2.0 nmol carbonyls/mg protein during frozen storage of turkey breast meat. It has been reported that oxidation subjects proteins to unfolding, enhancing the exposure of certain protein segments and reactive amino acid residues (Li, Xiong, & Chen, 2012). In this case, higher availability of Lys exposed for reaction with Glc or GlcN might be the reason for the faster reaction rate in frozen NAM systems. However, the increase in absorbance values at earlier incubation times and shifts in wavelength were predominant for GlcN glycoconjugates, as compared to Glc.

Fig. 5. Hierarchical clustering of protein–sugar UV–Vis absorbance data for actomyosin extracted from frozen chicken Pectoralis major. The vertical axis represent individual wavelength (280–320 nm). The horizontal axis represents individual glycation treatments. The colours in each cell indicate the absorbance of a particular sample relative to the mean level from all samples for the specific wavelength. The colour scale extends from bright green (maximum absorbance) to bright red (minimum absorbance). The heat map was developed based on 126 samples (average from three repetitions) × 40 wavelength values.
This suggests that carbonyls produced by NAM oxidation can be attacked by nucleophiles, such as –NH$_2$ of GlcN. Thus, one possible explanation for the glycation rate differences between fresh and frozen NAM with GlcN, is the simultaneous involvement of two functional groups from sugar molecules (aldehyde and primary amine) and protein (protein-bound –NH$_2$ and carbonyl groups).

3.3. Protein solubility profiles as a function of pH

On the basis of the optimum reaction variables selected from the heat map for fresh NAM, the following reaction conditions were designed: 40°C, 1:3 and 1:6 protein to carbohydrate ratio during 4, 8 and 12 h of incubation. In order to determine the optimum time of incubation and conclude the best protein to carbohydrate ratio, protein solubility was tested at pH values of 3, 5, 7, 8 and 10 (data not shown). It was found that at a 1:3 protein to carbohydrate ratio, higher solubility was obtained for the samples incubated for 8 h as compared to those incubated at 4 and 12 h. In contrast, NAM incubated at a 1:6 protein to carbohydrate ratio was more soluble after 12 h of incubation. Even though the optimum incubation time was found to be the same for Glc and GlcN, solubility of NAM treated with Glc was significantly lower ($p < 0.05$). Based on this, the complete solubility profile of the Glc/GlcN glycated NAM was built as a function of pH for samples treated for 8 h with 1:3 NAM–Glc/GlcN ratio and 1:6 protein:sugar ratio for 12 h (Fig. 6). The solubility of native NAM gradually decreased from pH 3 to 5, where it became the least soluble, corresponding to its isoelectric point (pI). In contrast, the solubility of NAM glycated either with Glc or GlcN was improved, increasing from 6.6% to 28% on average for glycoconjugates. The solubility of NAM glycated with GlcN was higher than those glycated with Glc, particularly in the pH range from 8 to 12. Chung et al. (2006) also reported that solubility of chitosan–glucose, derivatives was higher compared to chitosan–glucose, reaching the highest values after 3 days of incubation at 70°C. Moreover, the same authors reported the formation of precipitates during dialysis for Glc glycated chitosan.

There was only a slight difference in solubility between samples treated at 1:3 and 1:6 protein to sugar ratio for both Glc and GlcN. This possibly indicates that all available –NH$_2$ groups in protein reacted when 3-fold parts of sugar were in excess over protein. A further increase in the amount of sugar in the reaction mixture did not significantly influence the solubility. To render carp myofibrillar proteins soluble in low ionic strength media, Saeki (1997) used a 9-fold weight of glucose over protein. In comparison, Jimenez-Castano, Lopez-Fandino, Olano, and Villamiel (2005) found that conjugate formed at a 2:1 weight ratio of dextran to β-lactoglobulin exhibited higher solubility in the pH range from 3 to 9 compared to the native protein. Such a difference in the amount of sugar required for successive grafting might be dictated by the size and reactivity of the reactants, as well as amphipathicity and molecular flexibility of protein (Jung, Choi, Kim, & Moon, 2006).

The average increase in solubility at pH 7–12 as a result of glycation with GlcN was 23% compared to native protein. In addition, the shift in isoelectric point to acidic values was observed for the samples glycated with Glc at 1:6 protein to sugar ratio and for GlcN at 1:3 and 1:6 ratios. This shift was expected, as protein becomes more acidic upon glycation since sugar attachment leads to the neutralisation of positive charges on protein (Luthra & Balasubramanian, 1993). However, this explanation mainly applies to Glc. GlcN, which carries its own –NH$_2$ group, was not expected to induce the pI shift. Judging from the difference in molecular structure between Glc and GlcN (Fig. 3), the explanation of pI shift may possibly involve the –NH$_2$ group of GlcN in the progress of glycation.

3.4. Model study in lysine–glucosamine system

A plausible reason for the isoelectric point shift to more acidic values for GlcN glycoconjugates might be the liberation of ammonia from NAM–Glc or GlcN conjugate during reaction. To clarify this, the model study in amino acid–sugar system was designed. Lysine is a very reactive amino acid towards glycation. Histidine, tryptophan, and arginine residues also react, but to a lesser extent (Ames, 1992). The mass spectra for the study in the model system are presented in Fig. 7. At time 0 (control, Fig. 7A), two prominent peaks with $m/z$ 147.11 and 180.08 Da corresponding to protonated molecular ions [M+H]$^+$ of Lys and GlcN, respectively, were observed. After 2 h of incubation (Fig. 7B) the early glycation product (Schiff base) formation was detected, corresponding to the protonated ion with molecular weight of 308.18 Da, calculated based on the mass shift of +161 Da. This mass shift was calculated based on water release upon GlcN to protein (Lys) attachment. In the samples that had been heated for 8 h (Fig. 7C), a decrease in peak intensity was found for Lys and GlcN. Ions with $m/z$ of 308.18 were not detected after 8 h of glycation, but a new ion with $m/z$ 291.97 was formed possibly due to ammonia liberation [[(Lysine + GlcN) – (H$_2$O + NH$_3$)]. Due to the presence of the –NH$_2$ group on GlcN it may undergo variable reactions, including autocondensation and rearrangements. The

![Fig. 6. Solubility of actomyosin as dependent on pH, type of sugar and protein to sugar ratio. NAM–Glc/GlcN mixtures in weight ratios of 1:3 and 1:6 were incubated at 40°C for 8 or 12 h. Results are means of three independent experiments ± standard deviations.](image-url)
products of this reaction were not found at 0 and 2 h of glycation, but were identified after 8 h. For instance, an ion with m/z 321.00 indicated the presence of GlcN autocondensation product with further rearrangement to give 2,5-bis(tetrahydroxybutyl)pyrazine (Candiano, Ghiggeri, Gusmano, Benfenati, & Icardi, 1988). The same authors also reported a peak at m/z 185.07. However the structure of the product associated with that molecular mass was not indicated. Further evidence of GlcN autocondensation adducts is a peak of m/z 341.30, which derives from GlcN dimerisation (Kerwin, Whitney, & Sheikh, 1999). Ion peaks of m/z 321.00 and 185.07 with higher intensities were also identified in the sample, where GlcN was incubated without Lys (data not shown). This further confirms the assignments of those peaks to GlcN rearrangement products.

Therefore, the proposed pathway of ammonia release during Lys glycation with GlcN appears to be possible and the obtained results could be extended to the NAM–GlcN system. This model gives a preliminary indication of the involvement of ammonia release during GlcN–Lys conjugation and its influence on protein isoelectric point. It is also possible that the pl shift is induced by blocking the −NH₂ group of covalently attached GlcN by other free GlcN molecules or compounds deriving from rearrangements products of GlcN alone or NAM–GlcN conjugates. Therefore, more detailed investigation is necessary to figure out the mechanism and kinetics behind the course of glycation with GlcN.

3.4.1. Verification of ammonia (NH₃) release in the model

To further confirm the results obtained from mass spectrometry about ammonia release in the model, an enzymatic assay based on GDH was performed. It was revealed that 0.91 ± 0.17 μg/ml of NH₃ was released after 8 h of glycation, proving the data attained from mass spectrometry. This finding also clarifies the mechanism about the higher reactivity of GlcN compared to Glc; the presence of −NH₂ group adjacent to the reactive aldehyde group accelerates the release of NH₃ during initial phase of glycation. In comparison, the acetylated form of GlcN (N-acetyl-glucosamine) normally found in the connective tissue of human body has lower reactivity due to the presence of an amide bond (Greene & Wuts, 1999, Chap. 7). It appears that in a biological system acetylation of amino sugars is a strategy to control their reactivity.

The proposed mechanism also suggests that the aldehyde group of GlcN is an acceptor of nucleophile −NH₂ groups from amino acids. On the other hand, when proteins are oxidised with the formation of carbonyls (i.e. freezing) glucosamine can also act as a nucleophile donor through the −NH₂ group.

3.5. Protein glycation by MALDI-TOF/TOF-MS

To determine the relative levels of the glycated proteins, MALDI-TOF/TOF-MS analyses were performed. Previous reports indicated the usefulness of this approach to study the glycation in vivo and in vitro, since the number of Glc molecules that condense onto the protein can be evaluated by the mass increase (Lapolla, Fedele, & Traldi, 2000). This task is also simplified since the majority of ionised peptides from MALDI are singly charged, thus observed mass/charge (m/z) ratios of peaks denote the mass of the peptide. In this study, solutions of NAM, separately or glycated with Glc or GlcN were incubated at 40 ºC for 8 or 12 h, at different protein to sugar ratios. Thereafter, native and glycated NAM were digested with endoprotease trypsin to produce a set of peptides, which were further analysed by MALDI-TOF/TOF-MS. Hydrolysis was carried out prior to mass spectrometry in order to yield a better mass resolution. The difference of +162 Da between the control (peptides from non-treated NAM) and glycated peptides was attributed to attachment of Glc residue within one particular treatment and the loss of a water molecule (Meltretter & Pischetsrieder, 2008). As this study is the first focusing on MALDI-TOF/TOF-MS analyses of muscle protein glycation with GlcN, no information is available on the possible mechanism of this reaction and thus identification of GlcN–protein glycation products. In addition, as shown in the model Lysine–GlcN study, possible ammonia liberation during NAM reaction with GlcN might have an impact on the calculation of molecular weight of the resulting glycation product. In this respect, calculation of glycation for GlcN–NAM conjugates was performed in two ways. First, each detected mass shift of +161 Da represented the successive increase of one GlcN residue as a result of Schiff base formation at the initial stages of Maillard reaction and further dehydration. Second, a +144 Da shift in molecular mass indicated a single glycation due to the loss of both water and ammonia. It is worth mentioning that the mass shift (Δm = 161 Da) was confirmed by the model study (Lysine–GlcN) as being suitable for subsequent calculation of Schiff base formation between NAM and GlcN.

The profiles from various treatments (Figs. 8 and 9) showed that the relative number of the glycated peptides varied among treatments. The highest percentage of glycated peptides was found for NAM–GlcN (1:3) conjugates, reaching 32% of the total peptides. In contrast, 14% of glycopeptides were produced by conjugation with Glc. Only a slight increase (on average about 1%) in glycation level was observed with an increase in the sugar amount to 6-fold over protein and prolonged reaction time (12 h) for both Glc and

Fig. 7. Orbitrap LC–MS/MS spectrum of Lys–GlcN conjugates at 0 h (A), 2 h (B) and 8 h (C) of incubation.
GlcN. These results are supported by the findings of the protein solubility study, where the highest values were obtained for samples glycated with GlcN, as compared to those with Glc. It is also in agreement with observations that increase in the amount of sugar does not considerably influence the level of glycation, as well as protein solubility.

With increase in incubation time up to 12 h (Fig. 9), the number of peptides calculated by the second method (considering MALDI-TOF/TOF mass spectra of non-treated actomyosin (A), and actomyosin conjugated with glucosamine (B) or glucose (C) at 40 °C for 8 h at a 1:3 protein to sugar ratio. The data were obtained after total digestion with trypsin. The filled circle refers to $M_w$ obtained from a mass shift of 161 or 162 resulting from a release of 1 mol of H$_2$O during glycation with GlcN or Glc, respectively. The stars indicated $M_w$ obtained from a mass shift of 144 corresponding to a release of 1 mol of H$_2$O and 1 mol of NH$_3$ during glycation with GlcN.

Fig. 9. MALDI-TOF/TOF mass spectra of non-treated actomyosin (A), and actomyosin conjugated with glucosamine (B) or glucose (C) at 40 °C for 12 h at 1:6 protein to sugar ratio. The data were obtained after total digestion with trypsin. The filled circle refers to $M_w$ obtained from a mass shift of 161 or 162 resulting from a release of 1 mol of H$_2$O during glycation with GlcN or Glc, respectively. The stars indicated $M_w$ obtained from a mass shift of 144 corresponding to a release of 1 mol of H$_2$O and 1 mol of NH$_3$ during glycation with GlcN.
ammonia release) doubled, supporting the proposed explanation of ammonia liberation as a reason for a shift in isoelectric point for NAM–GlcN conjugates.

3.6. Emulsifying properties

Among the dominant properties for protein's favourable industrial application is the ability to emulsify fat and form a stable emulsion. Emulsifying properties of non-treated NAM and NAM glycated with Glc or GlcN at different pH values were determined and the results are presented in Table 1. Emulsion activity index at all pH values was significantly higher for NAM–GlcN conjugates compared to non-heated and incubated NAM. In contrast, the EAI of NAM–Glc conjugates was significantly higher from control NAM only at strongly acidic pH. At pH 3, significantly higher EAI was found for GlcN conjugates in comparison to those of Glc. Such a strong difference, particularly at this pH, was probably caused by the pI shift into acidic values for GlcN glycoconjugates, providing higher solubility and thus higher ability to emulsify fat particles. The results from ESI had the same trend as EAI, with significant improvement of emulsion stability for GlcN conjugates compared to that of control NAM. Although incorporation of Glc into NAM enhanced EAI, no significant improvement was brought to emulsion stability. Improvement in emulsifying properties of glycoconjugates, and in particular NAM–GlcN, was possibly caused by changes in the amphiphilic properties of protein. It seems reasonable to propose that if proteins absorb at the oil–water interface and forming viscoelastic layer, while hydrophilic sugar residues orient towards water, prevention of oil droplets from coalescence occurred (Fujiwara et al., 1998). Moreover, as solubility is a determining factor in a protein's ability to form an emulsion its improvement (Fig. 6) upon glycation provides beneficial effects on emulsifying properties.

Morgan, Leonil, Molle, and Bouhallab (1999) showed that lactosylation of β-lactoglobulin in dry conditions (50 °C, 48 h) did not significantly modify the native protein structure, while the treatment in solution (60 °C, 130 h) resulted in important structural changes. Thus, it is speculated here that protein structural modifications induced by glycation in liquid conditions might have a strong influence on its emulsifying activity and stability. This is especially related to NAM–GlcN conjugates, since a shift in protein pI confirms a higher degree of modification and hence changes in three-dimensional protein structure.

In previous studies by Fujiwara et al. (1998) it was found that myofibrillar proteins from carp developed excellent emulsifying properties during glycation with dextran. Saeki (1997) who incorporated Glc into carp myofibrillar protein also found that emulsifying properties of glycoconjugates were superior to that of unglycated protein.

4. Conclusions

This study offers important information about the glycation of muscle protein in a liquid environment achieved at moderate temperature and faster reaction rate. It also gives an insight into glycation by using protein, which undergoes oxidative changes as a result of frozen storage. The optimised method for muscle protein glycation proposed here leads to significant improvement in protein solubility, which is of primary importance in muscle food processing. The present work showed that use of GlcN for non-enzymatic glycation accelerates the speed of reaction, which allows shorter time and lower temperatures for sugar incorporation into protein, avoiding its heat denaturation. Further research is required to determine the mechanism of glycation with GlcN related to kinetics of the reaction. In addition, as GlcN is known to have medical effects on joint health, it would be beneficial to evaluate the biological activity of those proteins to which GlcN was covalently attached.

Acknowledgments

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References


Table 1

<table>
<thead>
<tr>
<th>Treatments</th>
<th>pH 3.0</th>
<th>pH 7.5</th>
<th>pH 10.0</th>
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<tr>
<td>NAM non-incubated</td>
<td>2.6±0.1</td>
<td>4.3±0.7</td>
<td>6.0±0.3</td>
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<tr>
<td>NAM incubated (4°C, 8 h)</td>
<td>2.1±0.2</td>
<td>4.0±0.6</td>
<td>5.8±0.6</td>
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<tr>
<td>NAM–Glc (1:3, 40°C, 8 h)</td>
<td>3.9±0.5</td>
<td>5.2±0.7</td>
<td>7.6±0.9</td>
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<tr>
<td>NAM–GlcN (1:3, 40°C, 8 h)</td>
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<td>6.5±1.3</td>
<td>8.3±1.1</td>
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<td>Level of significance</td>
<td>0.0012</td>
<td>0.0225</td>
<td>0.0152</td>
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< 0.05) difference between mean.

< 0.05) difference between mean.


