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Transglutaminase-catalyzed glycosylation of natural actomyosin (NAM) using glucosamine as amine donor: Functionality and gel microstructure

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ABSTRACT

Functionality of muscle proteins is closely related to the qualitative characteristics of the final products. Enzymatic glycosylation was explored as a way to upgrade the functionality of muscle proteins, thus increasing their successful utilization by the food industry. After actomyosin was extracted from chicken muscle and reacted with glucosamine using transglutaminase, the changes in functional properties and gel microstructure of glycoconjugates were investigated. The reaction was confirmed by sequential information obtained from an Orbitrap-LC–MS and relative quantification of glucosamine-adducts in conjugated actomyosin was assessed by matrix-assisted laser desorption/ionization mass spectrometry. The highest conjugation efficiency was obtained at 37 °C and a 1:1 protein/sugar ratio in the reaction mixture. For this treatment, solubility at the isoelectric point (pI) increased from 8.7 to 34% as compared to non-treated actomyosin, probably due to the hydrophilicity conferred by glucosamine attachment. Particle size and distribution analyses revealed that enzymatic conjugation of NAM-glucosamine at 37 °C improves emulsifying activity and stability of NAM, particularly at the protein's pI. Glycosylation exhibited a protective effect on actomyosin during heat-induced gelation. Cryo-electron scanning microscopy of actomyosin glycoconjugates revealed a strong effect of the level of glycosylation of the gels' microstructure. In particular, a highly dense network with a more closed nature was found for the glycoconjugates produced at 37 °C. Due to their improved solubility and emulsifying properties, actomyosin glycoconjugates represents a promising new additive for the food industry.

1. Introduction

The recovery of proteins from poultry and fish by-products for their utilization as food ingredients is of increasing interest in the food industry. One of the methods for protein extraction is using a pH-shifting process, based on acid/alkaline solubilization followed by precipitation at the pI (Hrynets, Omana, Xu, & Betti, 2010). Even though the method results in the production of high-protein ingredients, partial denaturation during the extraction process affects their functionality, solubility in particular. As a result, the functional properties of these isolated proteins have to be further improved. For this purpose, a wide spectrum of modifications, such as deamidation, succinylation, acetylation, and others could be used (Saberi, Kadivar, & Keramat, 2008). However, not all of the currently existing methods have been extensively used or explored at industrial-scale processing. Recent, significant attention has been given to protein modification by covalent attachment of carbohydrates during the Maillard reaction (glycation), usually performed in dry-state conditions (Zhu, Damodaran, & Lucey, 2008). Despite substantial functional improvement using this dry-state process, its application in food technology is limited due to the difficulty of controlling the reaction, long reaction time, and formation of mutagenic compounds, such as advanced glycation end products (AGEs) (Brands, Alink, van Boekel, & Jongen, 2000; Zhu et al., 2008). Conducting the reaction in a liquid state would be preferable to overcome these limitations and make it viable for industrial scale up. For instance, the Maillard reaction may be limited to its very initial stage of Schiff base formation, obtaining a light coloured product (Zhu et al., 2008). In this regard, Hrynets, Ndagijimana, and Betti (2013) proposed a new method to take advantage of the higher reactivity of the amino sugar glucosamine as compared to glucose. A fast glycation rate at a moderate temperature in a liquid aqueous environment was demonstrated possible, with subsequent positive effects on protein functionality. However, in order to

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further increase glycoconjugation efficiency at lower temperatures, a further reduction of browning, and the minimization of AGEs formation, glycosylation methods involving enzymes are proposed as a promising alternative.

Enzyme-mediated conjugation (glycosylation) may be very useful for site-specific conjugation due to its strict substrate specificity and amenability to mild reaction conditions. In this respect, glycosyltransferases and glycosidases might be considered as glycosylation catalysts (Colas, Caer, & Fournier, 1993). A glycosyltransferase (EC 2.4) catalyzed reaction uses a complex sugar nucleotide as the donor and has a very stringent substrate specificity (Ban et al., 2012). In comparison, glucosidase (EC 3.2) uses readily available donor substrates and has less specificity for acceptors. However, the low yield of transglycosylation is a major limiting factor to its usage in glycosynthesis (Milosavic, 2012). Transglutaminases (TGase; EC 2.3) belong to the group of acyltransferases, which are widely distributed in animal tissues, plants, and microorganisms. They are capable of catalysing acyl transfer reactions between the γ-carboxyamide of protein/peptide glutaminyl residues (acyl donors) and various primary amines (acyl acceptors). When the ε-amino group of lysine residues act as acyl acceptors, ε-(γ-glutamyl)-lysine isopeptide bond is formed. In the absence of primary amines in the reaction system, water acts as acyl acceptor resulting in the deamidation of the glutaminyl residue (Hu, Ren, Zhao, Cui, & He, 2011). Since the enzyme recognizes a wide variety of primary amines (Folk, 1983), a variety of conjugates can be produced using TGase-mediated acyl-transfer reactions.

Glucosamine (GlcN) is a naturally occurring amino sugar that is a building block of glycosaminoglycans found in cartilage (Black et al., 2009). It is produced commercially by the hydrolysis of crustacean shells and exoskeletons, a by-product of the marine food production (Arbia, Arbia, Adour, & Amrane, 2013). As an important constituent of joints, it is also known as a nutraceutical proposed to reduce the incidence of osteoarthritis. Taking into consideration that GlcN possesses amino and hydrophilic hydroxyl groups (Chung, Tsai, & Li, 2006), its incorporation into actomyosin via TGase would be useful for improvement of muscle protein functionality. Therefore, this study aimed: (1) to develop an enzymatic process for actomyosin glycosylation using TGase at two protein to glucosamine ratios (1:1 and 1:3) and two reaction temperatures (25 and 37 °C) and (2) to investigate its contribution on functionality. Actomyosin complex extracted from *Pectoralis major* of broiler chickens was used as a model protein. Sugar moieties covalently bound to actomyosin were evaluated using mass spectrometry techniques, while subsequent effects on functionality were evaluated by determining protein solubility, emulsifying properties, gelation behaviour and gel microstructure.

2. Material and methods

2.1. Materials

Fresh chicken breast was obtained from a local store. D-(+)-Glucosamine hydrochloride, sodium phosphate monobasic, sodium phosphate dibasic, sodium azide, guinea pig liver transglutaminase (EC 2.3.2.13), proteomics grade trypsin and ProteoMass peptides MALDI-MS calibration kit were purchased from Sigma–Aldrich (St. Louis, MO, USA). All the reagents and chemicals used in the study were of analytical grade.

2.2. Methods

2.2.1. Experimental design

Broiler chicken breasts were kept at 4 °C before treatments. Natural actomyosin (NAM) was extracted according to the procedure indicated in Section 2.2.2. Then buffered solutions of NAM:GlcN at 1:1 or 1:3 protein to sugar ratios were incubated at 25 and 37 °C for 6 h with (+) or without (−) TGase (glycosylation and glycation, respectively). This duration was chosen to minimize the extent of glycation (Hrynets et al., 2013) while providing enough time for glycosylation to occur. Non-treated and incubated NAM at 25 and 37 °C without GlcN and TGase were also included as controls to ensure that changes in protein functionality are due to sugar incorporation. The entire experiment from NAM extraction through final glycosylated/glycated products was replicated 3 times. Functionality measurements (solubility, emulsion properties, gels microstructure, rheological behaviour) were also conducted in triplicate.

2.2.2. Extraction of natural actomyosin (NAM)

Actomyosin from chicken *Pectoralis major* was extracted following the method described by Benjakul, Visessanguan, Ishizaki, and Tanaka (2001) with slight modifications. Briefly, fresh chicken breast muscle (100 g) was homogenized (Power Gen 1000 S1, Fisher Scientific, Schwerte, Germany) in chilled 0.6 M KCl at 1:10 (w/v) for 2 min. The obtained homogenate was centrifuged at 5000 × *g* for 40 min at 4 °C using Avanti® J-E refrigerated centrifuge (Beckman Coulter Inc., Palo Alto, CA, USA). NAM was precipitated by addition of three volumes of chilled deionized water and centrifuging at 5000 × *g* for 30 min at 4 °C. NAM 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 5000 × *g* for 10 min at 4 °C. The pellet from the final centrifugation step is hereafter called “natural actomyosin” or NAM.

2.2.3. Enzymatic glycosylation of natural actomyosin (NAM)

Glycosylation was conducted based on the optimization study of Hrynets et al. (2013). Mixtures of NAM and GlcN in ratios of 1:1 and 1:3 (w/w) were dissolved in 50 mM PBS (pH 7.5) in the presence of 0.55 M KCl. The pH of the solutions was adjusted to 7.5, when needed. The reaction was initiated by addition of 10 U of TGase/g of protein and 5 mM of $CaCl_2$. One enzymatic unit is defined as the amount needed to catalyse the formation of 1.0 μmol of hydroxamate per min from Z-Gln-Gly and hydroxylamine at pH 6.0 at 37 °C. Obtained aliquots were incubated in an Innova 44 (New Brunswick Scientific, Edison, NJ, USA) shaker under constant agitation for 6 h at 25 or 37 °C. To remove the enzyme and unreacted sugar, collected aliquots were subjected to an ultrafiltration membrane system with molecular weight cut-offs of 100 and 3 kDa (Amicon Ultra, Millipore Corporation, Bedford, MA, USA), respectively. The post-ultrafiltration ionic strength was measured for glycosylated samples using a conductivity meter (Oakton Acorn CON 6, Vernon Hills, IL, USA) calibrated prior to the measurements. Thereafter, the ionic strength of the sample set was adjusted to 0.55 M KCl.

2.2.4. Evaluation of protein solubility

The solubility of glycosylated and control samples was determined as described by Montero, Jimenez-Colmenero, and Borderias (1991). One ml of each sample and control solutions was adjusted to pHs ranging from 2.0 to 12.0, at 1 pH intervals. The final volume was then made up to 2 ml with distilled water having the same pH as non-diluted sample solution. After centrifugation (9000 × *g* for 15 min, 4 °C) the protein content of the clear supernatant and the original solution was determined according to the Biuret assay (Gornall, Bardawill, & David, 1949) using bovine serum albumin (BSA) as a reference protein. The solubilities of glycosylated and control NAM were expressed as the percentage of the protein in the supernatant to that of the protein solution before centrifugation.