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Microbial transglutaminase-induced structural and rheological changes of cationic and anionic myofibrillar proteins ☆

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ABSTRACT

This study investigated the effects of microbial transglutaminase (TG) on structural changes in porcine myofibrillar protein (MP) under varying pH (2.0–6.0) and two ionic strength conditions (0.1 M versus 0.6 M NaCl). Lowering the pH below the isoelectric point (pI) of myosin induced protein unfolding as revealed by surface hydrophobicity and differential scanning calorimetry. Although the MP solubility at the low ionic strength (0.1 M NaCl) was maximal at pH 3.0, both SDS-PAGE profiles and dynamic rheology indicated TG could not cross-link MP under this condition. Based on the carboxyl group content, the TG-catalyzed deamidation was dominant at a pH lower than the pI of myosin (pH 5.0) while cross-linking occurred at higher pH. Moreover, deamidation had no effect on rheological properties of MP. The results indicate that the TG reaction was governed by the pH of substrate protein, and the reaction intensity was related to the solubility of protein.

1. Introduction

Microbial transglutaminase (TG) is a group of glutamine-γ-glutamyl transferases produced from the actinomycete *Streptoverticillum mobaraense* that catalyzes the acyl transfer between the γ-carboxyamide group of peptide-bound glutamine and ε-amino group of peptide-bound lysine resulting in cross-linking among proteins by the formation of ε-(γ-glutamyl)lysine isopeptide bonds (Belitz, Grosch, & Schieberle, 2009). For TG reactions in the absence of free amines, water acts as an acyl-acceptor (Orrù, Caputo, D'Amato, Ruoppolo, & Esposito, 2003). The TG catalyzes either inter- or intra-molecular cross-linking of proteins or peptides, therefore, homologous or heterologous protein polymers can be formed by a TG reaction (Han & Damodaran, 1996). It has been reported that the impact of a TG reaction depends on protein sources; casein, myofibrillar protein (MP), gelatin, α-lactalbumin, and bovine serum albumin were shown to be good substrates individually or in combination with each other (Han & Damodaran, 1996; Sakamoto, Kumazawa, & Motoki, 1994).

The reason why TG shows a high substrate specificity and often varies in catalytic efficacy in different food systems is not completely understood. From a macroscopic point of view, the isoelectric point (pI) of TG is about pH 9, and the optimum reaction of TG ranges from pH 5 to 9 (Sakamoto et al., 1994). Kashiwagi et al. (2002) hypothesized that TG contains 2 major reactive sites, i.e., cysteine (Cys^{64}) and aspartic acid (Asp^{255}) residues within the primary structure of the TG polypeptide. The catalytic reaction was thought to be initiated by transferring the thiolated ion of Cys^{64} to Asp^{255} via glutamine residues of a protein followed by reversing to Cys^{64} via lysine residues (Kashiwagi et al., 2002). To identify factors involved in TG catalyzed cross-linking of food proteins, the TG reaction has been conducted at different pH levels and ionic strengths (Hong & Chin, 2010; Xiong, Agyare, & Addo, 2008). However, there is no report regarding TG reaction at a pH lower than pI of substrates.

MP is a group of key proteins largely responsible for textural and functional properties of meat products. The TG catalyzed cross-linking of MP is strongly influenced by protein solubility, thus, a TG-catalyzed formation of cold-set MP gels can occur at higher than 0.3 M NaCl concentration (Hong & Chin, 2010). On the other hand, Ramírez-Suárez and Xiong (2003) noted deamidation before TG catalyzed cross-linking of MP. Xiong et al. (2008) investigated the carboxyl group content as a function of reaction time. The authors reported that the carboxyl group of MP at low ionic strengths increased significantly at the initial incubation time and then decreased, but at high ionic strengths it increased slightly throughout 18 h of incubation. These reports indicated that deamidation and cross-linking reactions were not necessarily related to each other although both were time-dependent processes.

Although it is difficult to precisely identify the factor(s) regulating deamidation or cross-linking processes of MP owing to the complexity of the TG reaction, ionic strength (and related protein solubility) seems to have a major role in TG catalysis. From the solubility point of view, myosin is soluble at a pH lower than its pI at low ionic

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strengths (Lin & Park, 1998); however, the TG reaction under this condition has not been evaluated. Furthermore, MP molecules under acidic conditions have different structural characteristics than those at high pH and high ionic strength, i.e., they are positively charged at a pH lower than the pI and unfold at extremely acidic conditions (Kristinsson & Hultin, 2003; Lin & Park, 1998; Xiong, 2004). Although there have been studies reporting that TG has a high affinity for unfolded proteins (Seki, Nozawa, & Ni, 1998; Wang, Zhao, Yang, Jiang, & Chun, 2007), no information regarding the effects of protein charge on a TG reaction is available.

Furthermore, because the optimum pH for TG catalysis is in the 5–9 range where TG is positively charged and the substrate MP is negatively charged, one would expect the reaction of enzyme with MP being initiated by electrostatic attractions. The opposite charges, combined with increased exposures of reactive groups (glutamine, lysine) in MP, would promote the TG reaction. On the other hand, TG might have a reduced affinity for MP under conditions where the substrate proteins are converted to cations (positively charged) even if the substrate is fully soluble. Hence, the present study was undertaken to test the above hypothesis, specifically, to investigate the influence of MP structural changes induced by varying pH on the efficacy of TG for cross-linking MP in different ionic strength media and the resulting rheological behavior of the enzyme-treated protein.

2. Materials and methods

2.1. Materials

A total of 15 crossbred pigs were raised at the University of Kentucky Swine Farm on a typical commercial corn-soybean meal diet (14.0% protein, 3.34 Mcal/kg metabilisable energy). *Musculus longissimus dorsi* with normal post-rigor pH (ca. 5.4) was randomly selected from 24-h postmortem pork carcasses. The meat was trimmed of external visible fat and connective tissue, and cut into 1 cm cubes. The meat cubes were divided into approximately 200-g portions, vacuum packaged (−0.9 bar) in polynylon pouches (52 cm^3/m^2 of oxygen transmission rate and negligible moisture vapor permeability provided by manufacturer), and frozen at −30 °C prior to use (within 3 months). The TG (Activa-TI, 1% enzyme and 99% maltodextrin) with 1 U/g activity of powdered preparation was denoted by Ajinomoto (Ajinomoto Inc., Teaneck, NJ, USA). All chemicals used in this study were reagent grade.

2.2. MP extraction

Before MP isolation, the frozen meat cubes were thawed at 4 °C overnight. MP was extracted by removing the sarcoplasmic fraction and connective tissue according to the method of Xiong (1993). In brief, minced meat was washed three times using 4 vol. (v/w) of 0.1 M NaCl in 50 mM sodium phosphate buffer (pH 6.25) followed by washing with 8 vol. (v/w) of 0.1 M NaCl (pH 6.0). The final protein suspension was filtered through two layers of cheese cloth before centrifugation. The protein concentration of the final pellet (MP) was determined by the Biuret method (Gornall, Bardawill, & David, 1949) with bovine serum albumin as a standard. The extracted MP was stored on ice prior to use (within 4 days).

2.3. Enzyme reaction

Cold MP suspensions were made by the dilution of MP in a 25 mM sodium citrate buffer containing targeted NaCl concentrations (0.1 M versus 0.6 M) and then adjusted to pH 2.0, 3.0, 4.0, 5.0 or 6.0 using 1 M HCl. The final MP concentration was 10 mg/mL in all sample formulations except for thermal and dynamic rheology measurements in which a 40 mg/mL MP concentration was used and for electrophoresis (4 mg/mL MP). The TG powder was pre-dissolved in distilled and deionized water before being added to the MP suspension to obtain a final enzyme preparation concentration of 0.5% (w/w) except for electrophoresis (0.2%). The mixture was well mixed by homogenization for 30 s at 4 °C with a PT 10/35 Polytron homogenizer equipped with a low-form PTA 20TG generator (Brinkman Instruments Inc., Westbury, NY, USA) at a speed setting of 2 before incubation at 4 °C for 24 h unless otherwise specified.

2.4. Protein solubility

Aliquots (10 g) of control and TG-reacted (4 °C, 24 h) MP samples (10 mg/mL) were centrifuged at 1000×*g* for 15 min at 4 °C, and the protein concentration of the supernatants was measured by the Biuret methods (Gornall et al., 1949). Solubility was defined as the percent protein concentration of the supernatants over that of the original MP suspension.

2.5. Protein surface hydrophobicity

The surface hydrophobicity of control and TG-reacted MP samples (10 mg/mL) was measured according to Hayakawa and Nakai (1985). The samples after 24 h of incubation at 4 °C were centrifuged at 10,000×*g* for 15 min to remove particulates. The supernatants were diluted with 25 mM sodium citrate buffer (0.1 or 0.6 M NaCl with targeted pH levels) to obtain a range of protein concentrations (0.05–0.2 mg/mL). An aliquot of the supernatants (4 mL) was reacted with 20 μL of 8 mM 1-anilino-8-naphthalenesulfonate magnesium salt (ANS) in 10 mM phosphate buffer (pH 7.0) for 15 min at ambient temperature in dark. The relative fluorescent intensity (RFI) was recorded using a FluoroMax-3 spectrofluorometer (Horiba Jovin Yvon Inc., Edison, NJ, USA). The excitation and emission wavelengths were 390 and 470 nm, respectively, and the splits were set at 5 nm. The slope (S_o) of the RFI as a function of protein concentration was determined from the linear regression plot.

2.6. Differential scanning calorimetry (DSC)

The thermal stability of control and TG-reacted (4 °C, 24 h) MP samples (40 mg/mL) was measured using a Model 2920 Modulated DSC apparatus (TA Instruments, Inc., New Castle, DE, USA). Indium was used for instrument calibration. Approximately 20 mg of samples were hermetically sealed in alodined aluminum pans and heated from 20 °C to 90 °C at a rate of 10 °C/min. An empty pan was used as reference.

2.7. Carboxyl group content

The free carboxyl group content in control and TG-reacted MP samples (10 mg/mL) was determined by the method of Kobayashi and Chiba (1994). Samples incubated at 4 °C for 24 h were centrifuged at 10,000×*g* for 15 min, and the supernatants were diluted to 0.2 mg/mL protein using 25 mM sodium citrate buffer with targeted NaCl and pH levels. An aliquot of the supernatant was mixed with an equal volume of 4% (w/v) *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC)-HCl in 100 mM *N*,*N*,*N*′,*N*′-tetramethylenediamine (TEMED)-HCl buffer (pH 4.75) and incubated at 30 °C for 30 min. After a 10-fold dilution with water, a 0.1 mL aliquot was mixed with 0.1 mL *o*-phathalaldehyde (OPA) reagent and 8 mL of water (Kobayashi & Ichishima, 1990). The fluorescence intensity (kilo photon count per second, kpc/s) was measured using a FluoroMax-3 spectrofluorometer (Horiba Jovin Yvon Inc., Edison, NJ, USA) with excitation (340 nm) and emission (455 nm) slits set at 5 nm.

2.8. Gel electrophoresis

MP suspensions (4 mg/mL) in a 25 mM sodium citrate buffer at pH 3.0 or 6.0 containing targeted NaCl concentrations (0.1 M versus