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Dependence of microbial transglutaminase on meat type in myofibrillar proteins cross-linking

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ABSTRACT

The objectives of this study were to determine the factors that cause differences in the improvements of gel strength and ε(γ-glutamyl)lysine (G-L) content in chicken and beef (Japanese black cattle) myofibrillar proteins after adding microbial transglutaminase (MTG). As the amount of MTG added increased, the breaking strength increased progressively ($p < 0.01$) in chicken and beef samples, with the exception of chicken samples treated at 40 °C. The values of elasticity in the chicken samples were lower than those of the beef samples ($p < 0.01$). Surprisingly, the elasticity level, ε(γ-glutamyl)lysine contents and myosin heavy chain (MHC) band sizes of chicken and beef at all levels of MTG were significantly different ($p < 0.01$). The results of this study suggest that MTG activity was affected by MTG inhibitors; that MTG develops the texture of myofibrils differently in different species. However, the activity is limited and inconstant among meat proteins, as suggested by the data collected from the chicken samples. As a result, when the transferable amino acid residues are depleted (cross-linked) by MTG activity, the function of MTG will be insignificant. The correlation between MTG and different sources of meat protein is quite unstable but it is strong, which was observed when chicken and beef responded differently to MTG because their chemical and physiological properties were different. The remarkable rate of formation of cross-linked proteins and the discrepancy between the expected and observed amount of dipeptide raises the possibility that there are enzymes capable of reversing the reaction induced by transglutaminase in chicken and beef myofibrils. In summary, our results suggest that access of MTG to chicken and beef myofibrils is different because it depends on physiological (muscles and their fibre types), biological (substrates) and biochemical (inhibitors and amino acids) variables.

1. Introduction

Treatment with microbial transglutaminase (MTG) enhances the texture and gel strength of meat and meat proteins in many products by forming a bond between glutamine and lysine, which improves the rigidity and gel elasticity of meat products, avoiding some undesirable attributes such as stickiness, high viscosity and excessive meat adhesiveness.

Muguruma and his associates conducted many studies on the functionality of MTG and its effects on the gelation properties of meat products. The results of a study on the improvement in chicken sausage texture induced by transglutaminase at a low level of phosphate suggested that the texture was improved by the formation of a network structure, which contributed to the hardness of meat gels with added biopolymers (Muguruma et al., 2003). There have been many studies of the gelation of chicken myofibrillar proteins: Lesiow and Xing (2001) reported that under dynamic condition aggregation has a major role in producing differences of gel elasticity between myofibrillar proteins in both white and red meat. A study of the inconsistency in the improvement of gel strength in chicken and pork sausage induced by MTG was conducted by Kawahara, Ahhmed, Ohta, Nakade, and Muguruma (2007), who suggested that the binding between myofibrillar proteins and MTG is strong. Furthermore, MTG achieves different levels of improvement in chicken and pork products that are treated mechanically, such as sausages.

It is necessary to understand the protein reactions induced by MTG binding and the effect on ATPase activity in meat proteins because of the important economic benefits of using MTG to improve the textural quality of meat products. MTG catalyses the formation

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of an ε-(γ-glutamyl)lysine bond, and therefore depends on the availability of glutamic acid and lysine residues, as well as other factors that are discussed below. El Alaoui, Legastelois, Roch, Chantepie, and Quash (2006) reported that the amount of N isopeptides follows transglutaminase activity closely during the lag phase of growth of both chicken embryo cells and human carcinoma of the larynx cells. Some details of the mechanism of MTG activity are not known and the substrates of tissue transglutaminase have not been identified in cells or tissues (Fesus & Tracsa, 1989).

MTG aggregates proteins by catalysing the formation of bonds between amino acid residues by the transfer of the active radicals of certain amino acids (free or aggregated) of isopeptides to other amino acids. It is well-known that each amino acid has at least one carboxyl (COOH) group, which is acidic, and one amino (NH_2) group, which is basic. Preferably, amino acids join together in long or short chains, the amino group of one amino acid linking with the carboxyl group of another. The linkage is known as a peptide bond, and a chain of amino acids is known as a polypeptide. However, peptide bonds sometimes occur by the action of enzymes, such as MTG, which was used in this study. It plays a functional role in coupling glutamine with lysine, which eventually forms a polymer with ε(γ-glutamyl)lysine structure. The potential catalytic functions of MTG as well as other residues cause the side chains of glutamine (C-terminal) to interact with the side chains of lysine (N-terminal). So MTG catalyses the interconnections of myofibrils and giant polymers can be created. This final stage of protein aggregation and accumulation improves the gel elasticity of meat protein and forms a protein–protein network (PPN). The gelation that is induced by adding MTG to meat products has been observed by many researchers, and gel strength is further enhanced by heat treatment subsequent to the action of MTG.

We have conducted studies on the effects of MTG on cross-linking proteins in different minced meats (Ahhmed et al., 2007a; Kawahara et al., 2007), and we have observed that MTG catalyses protein–protein interaction differently depending on the temperature. This study was designed to compare the effects of MTG on the gel properties of different myofibrillar proteins. We investigated the action of MTG on chicken and beef myofibrillar proteins at two different temperatures. Ramirez and Xiong (2003) studied the effects of transglutaminase on the gelation of a myofibrillar and soy protein mixture, and reported that transglutaminase is an excellent agent for producing an adhesive mixed protein gel structure with a reduced requirement for myofibrillar proteins. Consumers in many countries have become more demanding about food quality (Ahhmed et al., 2007a), and consumer acceptance of processed meat is determined by the product quality, particularly flavour, texture, and storage stability (Ramirez & Xiong, 2003). Earlier studies showed that the proteins in chicken and beef respond differently to the activity of MTG. There is a need to determine the reason why MTG improves the texture of meat differently and the mechanisms underlying its actions with myofibrillar proteins of different species. The objectives of this study were to determine reactive MTG levels for chicken and beef myofibrillar proteins as substrates and to focus specifically on the factors affecting the reaction of MTG with meat proteins.

2. Materials and methods

2.1. Materials

The thighs of 8-week-old chickens were sourced from a butcher in Miyazaki, Japan, and stored for 1 day at 4 °C; the pH upon arrival in the laboratory was 5.5. The *biceps femoris* muscles of 5–6-year-old post-breeding Japanese black cattle were obtained from Minami Kyushu Chikusan Kogyo Ltd., Kasgoshima, Japan. The beef was vacuum-packed and stored for 4–5 days at 4 °C; the pH was 5.6 and the meat was grade A-3. The visible fatty tissue was removed from both types of meat.

2.2. Methods

The samples were divided into two groups: group 1 contained the control and positive samples and was incubated at 40 °C for 30 min in a water-bath (Thermo-minder, Sm-05, Taitec, Tokyo, Japan); group 2 contained negative and positive samples and was heated at 78 °C for 30 min in a shaking water-bath (Personal-11, Taitec, Tokyo, Japan). Group 1 samples were incubated at 40 °C to avoid heat-denaturation of proteins. The functional properties of myofibrillar proteins are related to their thermal stability and interactions (Foegeding & Liu, 1995). The samples were subjected to a test of textural properties with a creep metre, and other measurements were made, such as protein extractability and evaluation of the degree of protein cross-linking by SDS-PAGE. Protein concentrations were determined by the biuret method (Gornall, Baradawill, & David, 1949). The ε(γ-glutamyl)lysine (G-L) content was determined by HPLC after enzymatic digestion as described (Ahhmed, Kawahara, Soeda, & Muguruma, 2005; Ahhmed et al., 2007a; Kawahara et al., 2007).

2.3. Preparation of MTG solution

Ando et al. (1989) reported the purification of MTG from the culture filtrate of strain S8112, which was assumed to belong to the genus *Streptoverticillium*. The transglutaminase secreted by *S. mobaraense* is used in the food industry (Kikuchi, Date, Yokoyama, Umezawa, & Matsui, 2003). In this study, MTG was obtained from Ajinomoto Co., Japan, and dissolved in 20 mM NaCl as described previously (Ahhmed et al., 2007a; Erwanto et al., 2005; Kawahara et al., 2007). The concentration of MTG used in this study was 3.4 mg/ml.

2.4. Preparation of myofibril samples

The chicken and beef were minced separately in a meat grinder (MK-GL 20-W National), placed into a borate/KCl solution, homogenised in a laboratory knife mill (Grindomix GM 100, Retsch Kurt Retsch GmbH & Co. KG, Germany), and centrifuged (Himac CR 20E, Hitachi, Tokyo, Japan) at 12,000 rpm for 20 min at 3 °C. The homogenates were centrifuged three times; after each centrifugation, the homogenates were mixed gently with borate/KCl buffer solution and centrifuged again. The supernatant, which contained water-soluble proteins, mitochondrial enzymes, haemoglobin, myoglobin, and inorganic substances, was discarded. The upper layer of the precipitant was removed and used as a myofibrillar protein pellet as described (Ahhmed et al., 2007b). The final samples were made from 50 g of myofibrils, 30 ml of distilled water, 1.4 g of NaCl, and 0.21 g of sodium pyrophosphate; MTG (3.4 mg/ml) was added in 0.1 ml portions to different levels; zero, 0.1 ml, 0.2 ml, 0.3 ml up to1.0 ml (we studied the action of MTG at 10 levels).

2.5. Textural properties test

The texture of the chicken and beef myofibrillar protein preparations was measured to determine the effect of adding MTG to meat products. Shear force was evaluated with a knife fixed on a creep metre (Rheoner II, Yamaden Co. Ltd., Tokyo, Japan). The samples were subjected to a puncture test as described previously (Ahhmed et al., 2007a). The samples were prepared as a 1 cm × 1 cm × 1 cm cube and the knife speed was 1 mm/s. Five

samples from each treatment were used; each sample was sliced into five pieces and each individual piece was measured 2 or 3 times and the six measurements closest to the mean were accepted.

2.6. Extraction of proteins

Protein extractability is a very important indicator of how the proteins were engaged in reactions with MTG in chicken and beef muscle fibre. Samples (2 g) from each treatment of chicken and beef myofibrils were dissolved separately in 28 ml of two different solutions: (1) the water-soluble protein solution (WSPS) was a low-ionic strength solution (pH 6) containing 50 mM imidazole-HCl and 2 mM EDTA; (2) Guba Starub-adenosine triphosphate solution (GS-ATP), a high-ionic strength solution (pH 6.5) containing 0.09 M KH_2PO_4, 0.06 M K_2HPO_4, 0.3 M KCl, and 1 mM ATP. The contents were homogenised in a Polytron homogeniser (Kinematica Co., Littau, Switzerland) at setting 4, then centrifuged at 12,000 rpm for 30 min at 4 °C (Himac CR 20E). The supernatants were recovered and passed through filter paper (No. 5A, Advantec Toyo K. Ltd., Tokyo, Japan). The filtrate was used as the extracted protein solution, and the concentration of protein was determined using the biuret method (Gornall et al., 1949), measuring absorption at 540 nm with bovine serum albumin as a standard.

2.7. Electrophoreses

SDS-PAGE at ~20 mA/gel as described (Laemmli, 1970) was used to determine the diversity of protein molecular weight using a gradient slab gel (7.5–17.5% acrylamide) containing 2-mercaptoethanol; the gels were stained with Coomassie Brilliant Blue.

2.8. Evaluation of MHC band intensity

The best chosen electrophoresis gels were examined in an intensity metre (Bio-Rad, Segrate, S.N. 76S, Milan, Italy) connected to a computer running Quantity One (version 4.62) 1-D analysis software for viewing the band pattern.

2.9. Determination of ε(γ-glutamyl)lysine content

2.9.1. Sample preparation for HPLC

The determination of ε(γ-glutamyl)lysine content (G-L) was done as previously described (Dondero, Figueroa, Morales, & Curotto, 2006; Kumazawa, Seguro, Takamura, & Motoki, 1993). Briefly, 50 g sample was added to 100 ml of distilled water. The mixture was chopped at a standard speed for 2 min in a food processor (8551 Mini Electric Chop, SEB, France). Then the mixture was homogenised 3 times for 1 min at 30 s intervals in a Polytron homogeniser (Kinematica Co., Littau, Switzerland) on setting 5, the fastest speed. The concentration of protein was checked and then a volume containing 25 mg of protein was taken from each sample. These were called original samples (OSs), and had different volumes because the concentration of protein differed among them, due to the action of MTG.

Digestive enzymes pronase, leucine aminopeptidase, prolidase and carboxypeptidase obtained from Sigma-Aldrich, Inc. (St. Louis, MO) were added consecutively to OSs.

2.9.2. HPLC assay

The ε(γ-G)L content in the hydrolysate of chicken and beef samples was determined by reverse-phase high-performance liquid chromatography (RP-HPLC) using an Intersil ODS C_{18} column (4.6 mm × 250 mm, 5 μm film thickness, GL Sciences, Inc., Tokyo, Japan). A 40 μl sample of each hydrolysate was added to 160 μl of OPA, to give final collections (FC), which were mixed gently for 2 min. Then 10 μl of FC was injected in the HPLC pump using a 25 μl microsyringe. The HPLC pumps were connected to a fluorescence monitor (RF-535; Shimadzu Corporation, Tokyo, Japan). FC hydrolysates were fractionated by a gradient elution of 3 l of **A**, a filtered solution of 20 mM potassium acetate (pH 5.5) with 1% tetrahydrofuran (THF) purchased from Wako, Osaka, Japan, and **B**, 1 l of degassed methanol with 1% THF. The flow rate was 1.2 ml/min and the starting solution was 80% **A**, and 20% **B**. Excitation was at 334 nm and absorbance at 440 nm was measured; the run time was 55 min.

2.10. Statistical analysis

Data for breaking strength and concentration of extracted proteins were obtained from five independent experiments and expressed as the mean ± SEM. Data for the G-L content were sourced from three independent experiments and expressed as mean ± SEM. All significant differences mentioned in the text are indicated in the figures with superscript letters on the bars. Two-factorial analysis of meat type (chicken and beef) with or without treatment with MTG was carried out using ANOVA followed by Tukey's method to indicate statistically significant differences.

3. Results and discussion

3.1. Textural properties

Fig. 1a shows how MTG improves the texture in chicken and beef myofibrillar proteins incubated at 40 °C. MTG-induced cross-links between proteins, and the hardness of the samples with 0.2–1.0 ml of added MTG solution increased significantly compared to the untreated samples ($p < 0.01$). It is clear that MTG catalysed the interconnections of myofibrils and giant polymers were created. Therefore, when the mechanical knife was plunged into the samples, most likely it encountered some hard polymers and strong networks within the samples, which might have affected the behaviour of the knife. The knife may have met less resistance in samples with the addition of 0.8 ml, 0.9 ml and 1.0 ml of MTG solution than that found for the addition of 0.6 ml or 0.7 ml of MTG solution. Catalysing bond formation between chicken myofibrillar proteins by MTG makes the proteins bind together more tightly. The more substrate is added, the more the reaction rate increases, because more active sites (cross-bridge) become occupied. This can continue until most of the enzyme becomes saturated with substrate (at levels of 0.7–1.0 ml) and the rate reaches a maximum. However, the gel strength of the samples that were incubated at 78 °C showed a gradual and systematic increase (Fig. 1b), which was much more stable than the increase of samples incubated at 40 °C. The values showed a significant ($p < 0.01$) increase with the addition of 0.2–1.0 ml of MTG solution. Heat treatment may have a role in improving the elasticity of chicken myofibrillar samples. The breaking strength values increased, which might be associated with the addition of MTG with the rise in temperature (Ahhmed et al., 2007a).

Samples of beef myofibrillar proteins that had been treated with MTG and incubated at 40 °C were improved in terms of gel elasticity (Fig. 1a). Transglutaminase may serve as an excellent agent for producing an adhesive mixed protein gel structure with a reduced concentration requirement for extracted myofibrillar proteins (Ramirez & Xiong, 2003). The impact of the first addition of MTG (0.1 ml) was large compared to values for non-treated samples. The increase was significant ($p < 0.01$) compared to what was seen with the chicken samples incubated at 40 °C. The enhancement of the gel consistency of beef myofibrils was stepwise at all levels. The results suggest that the values were increased, and the incre-

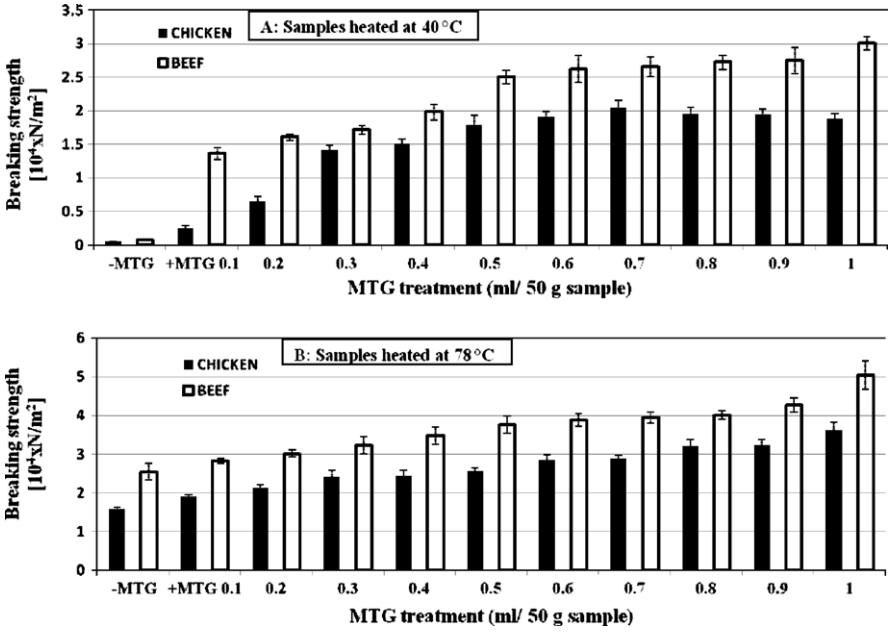

Fig. 1. Changes in gel breaking strength of chicken and beef myofibrils as functions of MTG and temperature.

ment was significant ($p < 0.01$) at all levels (0.1–1.0 ml of MTG), when compared to the values of the non-treated samples. Samples of beef treated with MTG and heated at 78 °C for 30 min showed the same improvement in elasticity and gel consistency (Fig. 1b). Undoubtedly, MTG improves textural properties with less retention of water. Variability in the means was observed; values of samples treated with 0.5–10 ml of MTG solution were significantly different ($p < 0.01$) from the values of non-treated samples. The values of samples treated with 0.1–0.4 ml of MTG solution were not significantly different from those of the control samples. The breaking strength increased progressively with increased addition of MTG, showing that it is possible to improve the mechanical properties of beef gels by adding MTG (Dondero et al., 2006). In this study, beef samples generally showed a greater ($p < 0.01$) improvement than the chicken samples. MTG improves gel formation between myofibrils of both species but not to the same extent. This variability depended on some factors that are addressed below. Regardless of the treated samples, values for the non-heat-treated samples of chicken and beef had almost the same gel consistency as the samples heated at 40 °C; $0.047 \times 10\ N/m^2$ and $0.067 \times 10\ N/m^2$, respectively.

3.2. Measurements of extracted proteins

Fig. 2 shows values of protein concentration that were extracted from samples dissolved in GS-ATP. As an overview, the values of the control samples in beef are significantly higher than those for the chicken samples, which may be related to storage time, as the longer meat is stored, the greater the amount of amino acids released. Nishimura, Okitani, Rhyu, and Kato (1990) showed that aminopeptidases B and C were present in beef, pork and chicken muscle. The impact of MTG on the myofibrils of both meat types at the first level (0.1 ml of MTG solution) in this study showed a significant reduction of protein concentration ($p < 0.01$). The concentration of protein for chicken and beef decreased gradually with added MTG, and the values in both chicken and beef with 1.0 ml of MTG solution were reduced drastically, compared to the control values. Fig. 3 shows values of protein concentration in chicken and beef myofibrils that were extracted in WSPS. All values for beef were slightly higher than those for chicken. A gradual decrease in protein concentration was observed for both chicken and beef but the values were not significantly different from those of the controls. This implies that partial reaction of MTG occurred with

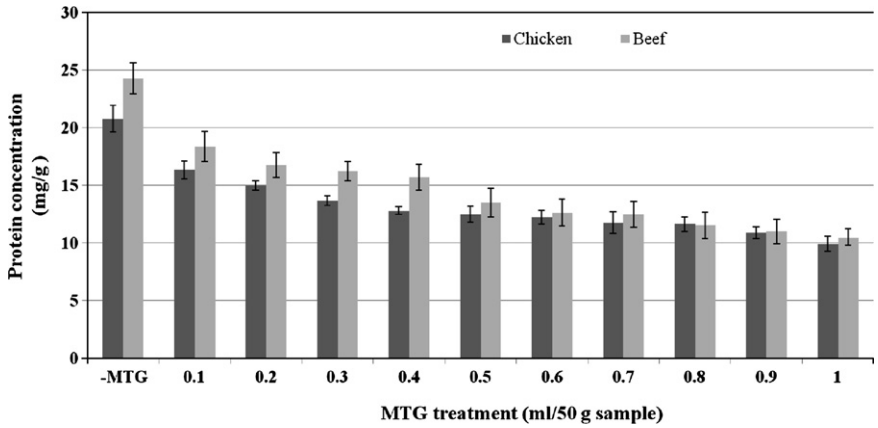

Fig. 2. Protein concentrations of chicken and beef myofibrils as affected by MTG. The samples were incubated at 40 °C for 30 min and extracted in GS-ATP solution.

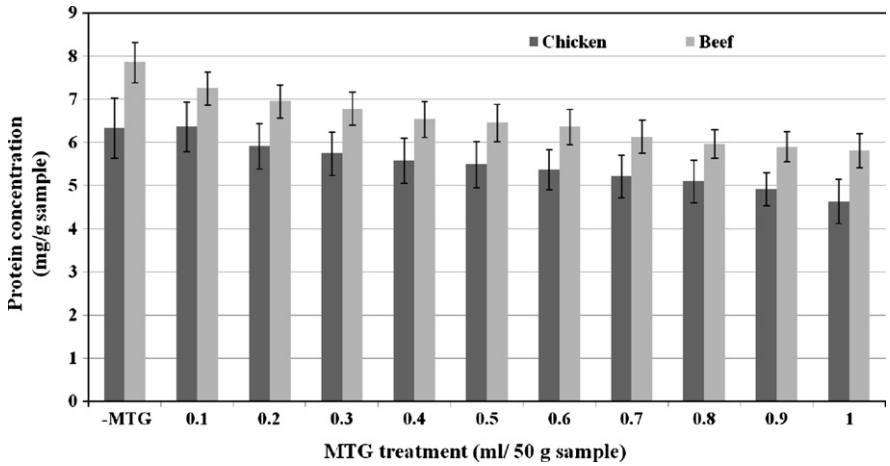

Fig. 3. Protein concentrations of chicken and beef myofibrils as affected by MTG. The samples were incubated at 40 °C for 30 min and extracted in WSP solution.

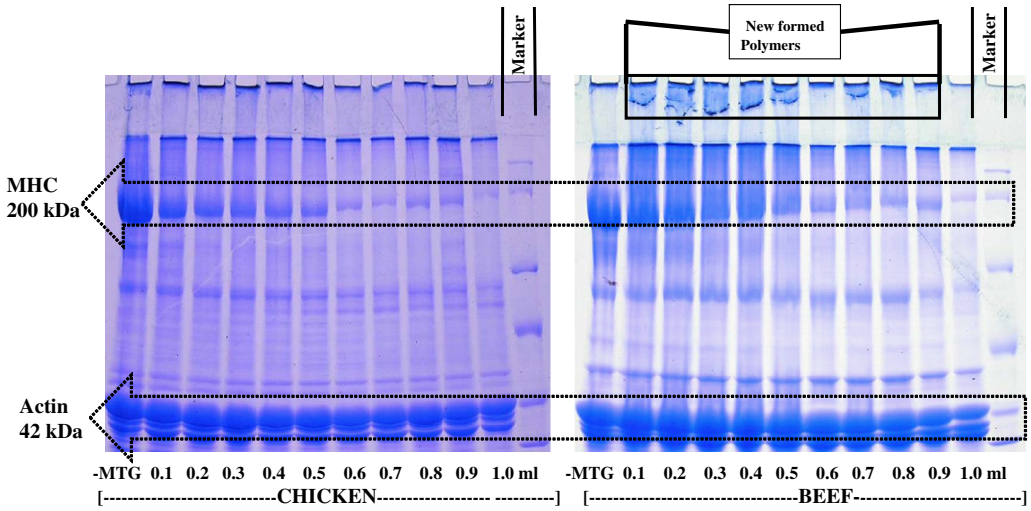

Fig. 4. SDS-PAGE pattern illustrating how MTG influences MHC in chicken and beef myofibrils. The samples were incubated at 40 °C for 30 min, samples were extracted in a GS-ATP solution.

WSPS, whereas the major reaction was implemented in the myosin heavy chain (MHC) proteins (Fig. 4). Therefore, the profound reduction of proteins extracted in GS-ATP observed with 1.0 ml of MTG solution in both meat types is due to the protein–protein cross-links in MHC proteins. It was difficult to extract much protein, as most were aggregated by the action of MTG (Fig. 2). This reveals that ATP had a role as a refractive element; it cleared away or released the tight bond in actomyosin so that myosin was available to MTG. As a possible explanation of the contrast in protein extractability between the two species, it is known that chicken myofibrils contain aminopeptidase H, which has been thought to affect the MTG substrate. Simply, a reaction between the substrate and MTG was slowed by the efficacy of aminopeptidase H. Nishimura et al. (1990) showed that hydrolase H was present in pork and chicken. Aminopeptidases C and H have a high level of activity against almost all substrates of MTG.

3.3. SDS-PAGE

The samples used for electrophoresis were those incubated at 40 °C for 30 min, and proteins extracted in GS-ATP (Fig. 4). These samples were heat-treated but without protein denaturation, allowing us to observe the change in MHC bands induced by adding MTG to myofibrils. MTG reacted positively with myofibrillar proteins at the MHC, which was the main reaction, as shown by significant ($p < 0.01$) reduction of the concentrations of protein for chicken and beef. The two major structural domains of MHC are the globular head (subfragment 1 or S1), and the helical tail or rod (Miller, Teal, & Stockdale, 1989).

Clearly, the intensity of MHC bands was reduced gradually as the amount of added MTG increased (Fig. 5A and B). This provides evidence that MTG reacts with proteins of the MHC in both meats. However, we could not detect any band (data not shown), upon SDS-PAGE of samples extracted in the low-ionic strength solution, which indicates that the majority of the reaction took place with other proteins. Earlier, we reported (Ahhmed et al., 2007a, 2007b; Kawahara et al., 2007) that the SDS-PAGE patterns of chicken and beef myofibrils showed reductions in band intensity for the MHC, in agreement with the data for samples in SG-ATP in this study. Differences in MHC recovery with the addition of TGase inhibitors were presumed to be due to the differences in TGase activity, and to different susceptibilities to inhibitors between species (Benjakul, Vissanguan, & Pecharat, 2004). Moreover, some newly formed polymers were found in beef samples. The differ-

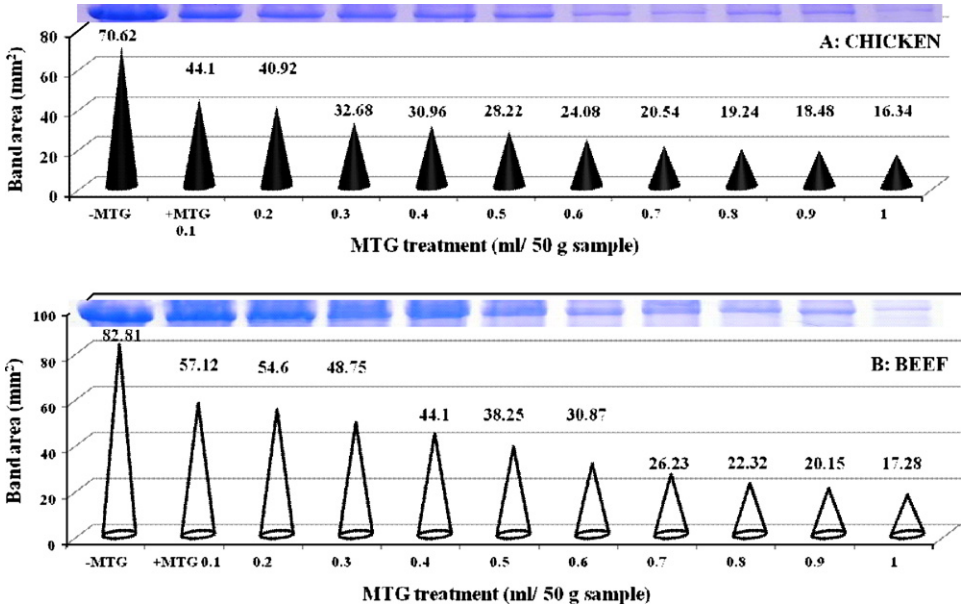

Fig. 5. Changes in the intensity of the MHC bands in chicken (A) and beef (B) myofibrils as the function of MTG treatments. The samples were incubated at 40 °C for 30 min.

ences of all bands, which appeared in the SDS-PAGE pattern between the chicken and beef samples were small, so we used an intensity metre to evaluate the band differences.

3.4. Evaluation of MHC band intensity

Fig. 5A shows values (mm^2/band) for the MHC band intensity after SDS-PAGE of the chicken samples. Fig. 5B shows values of the MHC band intensity for the beef samples. The intensity of the bands was reduced drastically at the first addition of MTG solution (0.1 ml); the intensity was reduced by 37% for chicken and 26% for beef when compared to their control values. The values for the other bands (0.2–1.0 ml of MTG) in both chicken and beef samples decreased gradually as the amount of MTG increased. The band intensity in the control samples for beef was higher than that in the control samples for chicken. The relative front (R_F, the distance of a band from the top of its lane, divided by the total length of the lane) of all samples was evaluated with the intensity metre. The R_F values of the MHC bands in the treated samples of beef and chicken were slightly higher than those in the control samples (data not shown). As a result, the increased R_F values indicate that the molecular weights of the MHC bands were reduced in all treated samples. On the other hand, the extractability of MHC was hindered after the samples were treated with MTG, implying that the proteins were aggregated and had formed a PPN.

3.5. ε(γ-Glutamyl)lysine contents

The G-L content was determined by biological assays. Pronase, leucine aminopeptidase, prolidase, and carboxypeptidase were used to degrade all peptides to amino acids, with the exception of the dipeptide G-L. The G-L contents in the myofibrillar proteins of both chicken and beef increased significantly ($p < 0.01$) in samples treated with two levels of MTG (Fig. 6). MTG acts as an intermediate factor in coupling glutamyl and lysine residues in one strong complex by the affinity relation through covalent bonds between 3-glutamyl and 5-lysine. The G-L contents of the chicken and beef samples were increased by 25% and 46%, respectively with the addition of 1.0 ml of MTG solution, compared to those of non-treated samples.

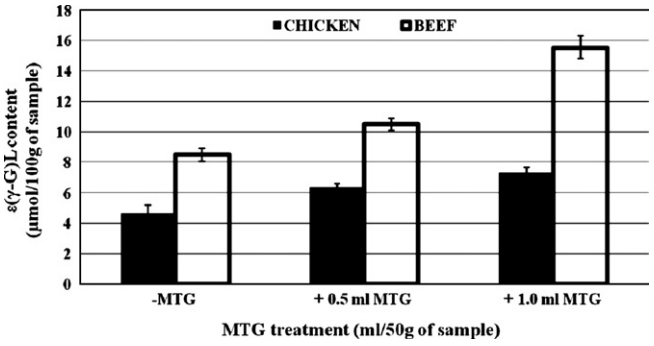

Fig. 6. Changes in ε-(γ-glutamyl)lysine content (μmol/100 g of sample). The samples were treated at 40 °C for 30 min and then digested by series of proteases (Pronase, leucine amino peptidase, prolidase and carboxypeptidase).

The results of this study suggest that there are remarkable differences in the myofibrillar protein within the same muscle type in different species; the leg muscle fibres of chicken and beef are different in biochemical as well as functional characteristics. The findings of this study are in agreement with other reports that the myosin in red and in white muscles from well-nourished broiler chickens has characteristic differences in certain physicochemical properties (Asghar, Morita, Samejima, & Yasui, 1984). The discrepancy between our findings in an earlier study and those described here in terms of gel strength, is due to differences in sample type and origin. In previous studies, we used whole meat, which contains components such as mitochondrial enzymes, haemoglobin, myoglobin, elastin and collagen. We suggest that fibronectin could be important for gel formation, in addition to the roles of collagen and actin in protein cross-linking (Ahhmed et al., 2007a). We emphasise that the ability of MTG to produce PPN varies, and depends on many factors. Skill et al. (2004) reported that transglutaminase-mediated cross-linking of collagen is able to increase both the rate and level of its deposition as well as reduce its rate of degradation, thus tipping the balance toward collagen accumulation. In the current study, fibronectin probably contributed to increasing gel strength and accumulated protein matrices of sausages made

from whole meat (chicken and beef). Fibronectin is made in the liver by hepatocytes and is mainly found in a soluble form in blood plasma. Obviously, it was present in the whole meat samples of chicken and beef, and was capable of causing some differences in gel strength and of contributing to the creation of the PPN.

The question arises of why MTG reacts differently with meat proteins from different species. In order of importance, we will consider some factors, such as muscle physiology and morphogenesis that have the potential to affect the results of treating myofibrils with MTG. The age of the muscles could be related to differences in morphology between chicken (8 weeks) and beef (5–6 years). The sarcolemma composition of the filaments was also considered as a possible contributor. Through the lifespan of an animal, the number of cross-links between collagen molecules and fibrils increases, which in turn increase collagen heat stability (Lepetit, 2007). In this study, differences in the gelation properties induced by MTG in chicken and beef myofibrillar protein are thought to be dependent, at least in part, on muscle morphogenesis.

Lesiow and Xing (2001) suggested that the gelation properties of myofibrillar proteins are influenced by the distribution of specific fibre types in the muscle samples from which the myofibrillar proteins are extracted. Chicken muscles are much smaller than beef muscles, and this difference interfered with the myofibril extraction process, which could have a vital role in the shape of the sarcolemma as well as differences in the development of the connective tissues in beef and chicken.

Another factor that might alter the function of MTG and make differences in the gel improvements of myofibrils is the identity of amino acids. In particular, there may have been significant amounts of free amino acids that caused some side-reaction with MTG rather than being bonded with each other by amine bonds that generally form the peptides. Rhyu, Nishimura, Kato, Okitani, and Kato (1992) reported that aminopeptidases degrade the peptides produced from proteins through the action of endopeptidases to free amino acids; the degradation to free amino acids in chicken skeletal muscle was due mainly to the action of neutral aminopeptidases (Okitani, Otsuka, Sugitani, & Fujimaki, 1974). Here, there might have been more free amino acids in the chicken samples than there were in the beef samples, as chicken retained more aminopeptides than beef. Nishimura et al. (1990) showed that aminopeptidases C and H contributed to the increase of glutamic acid during storage of pork and chicken. Most probably, this enzyme had released amino acids from the protein complex in chicken so that we found myofibrils of chicken are less rigid than myofibrils in beef; i.e., the myofibrils of beef are much coagulated. This is related to the water-holding capacity, which is higher in chicken than in beef, as chicken contains considerable amounts of glutamic acid. Thus, glutamic acid has a potential role in retaining water among connective tissue, so that chicken myofibrils seemed to contain more water when compared to those in beef, as beef proteins contained less liquid and were over-coagulated. Amount of water may depend on the available space within the myofibrillar structure, whereas the volume of myofibrils qualifies the water-holding capacity of the muscle. Some variations exist between muscles of different species, highly related to the lifespans of the animals and their muscle fibres.

Rhyu et al. (1992) reported that aminopeptidase in chicken muscle had relative activities of 51.3% against glutamic acid and 91.8% against lysine, as well as 79% against dipeptides, specifically Lys-Glu. The relative activity of aminopeptidase was about 0.64 against glutamic acid and 5.78 against lysine in chicken, whereas it was 0.18 against glutamic acid and 3.18 against lysine in beef (Nishimura et al., 1990). We suggest that chicken had a considerable amount of inhibitors of MTG, so that the formation of G-L was reduced in chicken when compared to beef.

Thirdly, the amount and the distance between amino acid residues in the protein chain probably contributed to the differences found in chicken and beef myofibrils. The localisation of amino acid residues in chicken is different from that in beef, showing strongly that the distance between amino acid residues within the protein is not the same in all species. The action of MTG is inconstant and dependent on many variables (Ahhmed et al., 2007b). The cross-linking reaction is specific, and requires that the substrates are located appropriately for cross-linking to occur (Miller & Johnson, 1995). It may be that the transferable amino acids in beef had a greater chance to make PPN than in chicken because the distance between them was less. MTG must have the ability to aggregate the distributed amino acids in chicken myofibrils; however, Maita, Hayashida, Tanioka, Komine, and Matsuda (1987) found 24 residues from the heavy chain of myosin S1 in chicken after digestion with *Staphylococcus aureus* V8 protease. From their study we found 7 Gly and 5 Lys residues in chicken, exactly half the number of amino acid residues thought to be responsible for the formation of ε(γ-glutamyl)lysine in myosin S1. It is possible that different amounts of amino acids with the ability to react with MTG, such as glutamic acid and lysine, occur in different species.

It is likely that some inhibitor(s) had an effect on the reaction of MTG within the myofibrils of chicken and beef. Conversely, the addition of transglutaminase inhibitors, including *N*-methylmaleimide, ammonium chloride and EDTA, resulted in a marked decrease in protein hydrolysis, especially with increasing concentration (Benjakul et al., 2004). Undoubtedly, chicken samples had inhibitors that linked with the muscle fibres so that MTG could not react properly with myofibrils. *Vibrio* B-30 collagenase, which cleaves gelatin type I and collagen types IV, VII, and X, appeared to be efficacious for the tenderisation of restructured beef products (Miller, Strange, & Whiting, 1989). Therefore, inhibitors such as collagenase must have had an impact on the chicken myofibrils. Inhibitors that restrain the activity of MTG in chicken, such as aminopeptidase, are abundant in chicken. Furthermore, cystamine, which is formed when cystine is heated, has a role as an MTG inhibitor. Together, these may have affected MTG activity in the chicken samples, because the beef samples showed much greater improvements in elasticity, especially in the samples incubated at 40 °C, and the gel strength of the control samples was almost the same in both chicken and beef. However, the inconsistency of improvements to the gel strength appeared after treatment with MTG. As the temperature increased, more cystamine was formed, and MTG activity was reduced in chicken; that is why elasticity in the chicken samples was much less than in beef (Fig. 1b). It has been reported that reducing agents such as 1 mM cystine inhibited the activity of glutamyl aminopeptidase (Maehashi et al., 2003). Nishimura, Kato, Okitani, and Kato (1991) and Rhyu et al. (1992) reported that chicken contains aminopeptidases C and H. Glutamyl aminopeptidase was also purified from chicken and it hydrolyses Glu and Asp, but not Leu. It is believed that this enzyme has a relative activity against Glu of 100% (Maehashi et al., 2003). Considering all the evidence, it is clear that cystamine does inhibit MTG. So, as the amount of cystine increases, the amount of glutamyl aminopeptidase increases, and the more cystamine is formed by heating, which means less MTG activity. It has been reported that the mechanism of cystamine inhibition could not be ascertained with certainty, but it was mentioned in the same article that cystamine inhibited transglutaminase activity, that the ε(γ-glutamyl)lysine content in the cell was decreased, and that several proliferation markers were enhanced (Birckbichler, Orr, Patterson, Conway, & Carter, 1981).

The microbial content of meat increases during storage. We should state that there is a speculative correlation between the content of microorganisms and the breakdown of amino acids, which is accompanied by an estimable amount of putrescines,

which are organic compounds produced by the breakdown of amino acids in living and dead organisms, and are thought to be inhibitory to MTG function. Kenneth and Dolynchuk (1996) showed that cross-link formation by tissue transglutaminase activity was inhibited during treatment of hypertrophic scar by putrescine. Additionally, they suggested that putrescines might prevent cross-linking of isopeptides by tissue transglutaminase. In the presence of TGase inhibitors, MHC was more retained, especially as the concentration of TGase inhibitors increased (Benjakul et al., 2004). It is possible, of course, that the differences in improvement of gel strength were not caused solely by the inhibitors discussed above; there may be unidentified inhibitors that have an important role. Also, we have to note that there might be co-enzymes in the beef samples that could enhance the reaction of MTG with the beef myofibrils.

4. Conclusion

This study is the first report of the range of access of MTG to myofibrils of different species. The correlation between MTG activity and protein source is unstable but it is strong. In these studies, we observed that chicken and beef responded to MTG differently. Although it is difficult to establish a clear cause and effect relation between MTG and meat proteins, we believe that the increase in disordered gel elasticity, cross-linking and G-L content between chicken and beef myofibrils that were observed even when these samples were treated using the same method is due to certain factors that had a pronounced effect. This study has focused on these factors, some of which are physiological and some are biochemical. The fact that MTG reacts differently with myofibrils of different species may be because of: (a) variation in muscle physiology and morphogenesis; (b) the identity of free amino acids, especially those with the ability to react with MTG; (c) the amount and distance between transferable amino acids; and (d) the amount of MTG inhibitors.

Consequently, as the textural characteristics developed differently in beef and chicken via the action of MTG, the protein cross-linking performances were different in chicken and beef. In conclusion, we suggest that using kinetic studies to determine whether MTG is competitive with some inhibitors is necessary for final confirmation. Studies are underway on myosin B, as the reaction of MTG most likely took place within this protein type.

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