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ORIGINAL PAPER

Effect of microbial transglutaminase on NMR relaxometry and microstructure of pork myofibrillar protein gel

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Abstract The effect of microbial transglutaminase (MTG) on nuclear magnetic resonance relaxation (NMR) behaviour, water holding capacity (WHC) and microstructure of pork myofibrillar protein (PMP) gel was studied. The enzymatic protein preparation had significantly lower values of spin–spin relaxation time (T_2), higher WHC and more porous microstructure in comparison with the control system. T_2 was reduced from 226 ms (peak value) of the PMP gel containing no MTG to 188 ms of the PMP gel containing 2 U/g protein. However, no further decrease was shown when the concentration of MTG increased. The reduction was attributed to reduced mobility of water protons in the system. Scanning electron micrographs (SEM) showed the mobility of water in the proteins gel network was related to gel microstructure.

Keywords PMP gel · MTG · NMR proton mobility · WHC · Microstructure

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Introduction

Myofibrillar proteins play an important role during meat processing, and are responsible for the formation of cohesive structures and for a firm texture following thermal treatment [1]. One of the most important properties of proteins in food systems is their ability to form gels after heating. Heat gelation contributes to forming fine texture, shaping the product, and retaining water in the product. Gelling and other functional properties of proteins are influenced by the physicochemical properties of proteins which change as a function of process variables, such as protein concentration, heating temperature and time, ionic strength, and pH [2]. And myofibrillar proteins are of great importance for functional properties of meat and meat products, in particular water holding capacity (WHC) [3]. In order to improve the functional properties of meat proteins, producers are currently using a wide range of vegetable additives, especially soy or whey protein derivates. However, consumers often reject these additives as being chemically modified or as being obtained from genetically modified plants. Another important way for improving their functional properties is the use of enzymatically catalyzed reactions to modify protein structure [4, 5].

In the meat industry, using transglutaminase (TG) to cross-link proteins has been found feasible. TG is an enzyme that catalyzes an acyl-transfer reaction between the γ-carboxyamide group of peptide or protein-bound glutaminyl residues, and primary amines. TG action over protein molecules, causes a cross-linking and polymerizing effect of protein molecules, through ε-(γ-glutamyl) lysine bonds. This TG-mediated protein cross-linking creates drastic physical changes in protein-rich foods [6]. Upon the discovery of an extracellular microbial transglutaminase (MTG), produced by a variant of *Streptoverticillium mobaraense*, utilization of

MTG in food industries was developed [7]. MTG has been used to catalyse the cross-linking of numbers of proteins, such as whey proteins, soy proteins, gluten, myosin and actomyosin [4, 6, 8]. Great improvements in the physical properties of meat product have been reported, such as elasticity and firmness [6]. However, effect of MTG on the pork myofibrillar proteins (PMP) gel nuclear magnetic resonance (NMR) relaxometry has not been reported.

NMR relaxometry is a non-destructive method that enables a characterization of water protons mobility and distribution, and several reports exist on the use of NMR to determine WHC and other technological properties in fresh pork [9–15], and see the review by Bertram et al. [16] for a detail. The general features of proton relaxation are characterized by spin-lattice relaxation time (T_1) and spin–spin relaxation time (T_2) [17]. T_1 and T_2 are measures of the interaction of a spin with its surroundings and the mobility of a spin, respectively. T_2 values are used most often for measuring WHC of meat and meat products, because they show greater changes in relaxation times [18], and the T_2 are usually more sensitive to the existence of several phases than the T_1. And, it is possible to distinguish between free water that does not interact with the solid particles or dissolved molecules and immobile water, e.g. crystallization water or other chemically or physically bound/immobilized water [19]. Bertram et al. [20] demonstrated that NMR relaxation is a useful tool for studying myofibrillar proteins as function of pH, ionic strength and heat treatment, and therefore the authors also expect that the method is useful for studying the effect of MTG on the PMP gel.

Up to now, the relationship between MTG-induced PMP gel structure changes and the changes in water mobility in the gel is still an almost unexplored area. The aim of the present work was therefore to study the effect of MTG-induced cross-linking on microstructure of PMP gels and its relationship with water proton relaxation behaviour.

Materials and methods

Materials

The meat used in the present study was porcine muscle lions from a single animal. The meat was frozen 24 h post-mortem and stored at −20 °C until the extraction. Commercial MTG (TG-B), provided by Yiming Biological Products Co., Ltd. (Taixing, China), was used. The enzymatic product is made up of 99% maltodextrine and 1% MTG with a declared enzyme activity of 100 U/g. This enzyme with optimum temperature and pH around 50 °C and 6.0, respectively, is active over large ranges of temperature (45–55 °C) and pH (5.0–8.0). EGTA was obtained from Sigma Chemical Co (Louis, USA). Triton X 100 was purchased from Amersco (San Francisco, USA). All other chemicals used in this work were also from commercial sources and of analytical grade.

Extraction of myofibrillar protein and preparation of gel system

Extraction of myofibrillar proteins was carried out essentially as described by Doerscher et al. [21] with some modifications according to the protocol of Goll et al. [22]. Briefly, Trimmed, ground muscle (about 100 g) was homogenized and washed using four volumes of a post-rigour extraction buffer (100 mmol L^{-1} Tris, 10 mmol L^{-1} EDTA, pH 8.3) in a Waring Blender, Model No. 8010ES (Waring Commercial, New Hartford, CT). Samples were then centrifuged 1,000×g for 20 min (Beckman Avanti J-E centrifuge, Beckman Coulter, Fullerton, CA, USA). The myofibrillar pellet was then re-suspended and washed three times in four volumes of a standard salt solution (SSS) (100 mmol L^{-1} KCl, 20 mmol L^{-1} K_2HPO_4/KH_2PO_4, 2 mmol L^{-1} $MgCl_2$, 1 mmol L^{-1} EGTA, 1 mmol L^{-1} NaN_3, pH 7.0) and was homogenized using a Ultra Turrax T25 BASIS (IKA Labortechnik, Germany). Centrifugation (1,000×g for 10 min) was used to collect the pellet between washes. The pellet was re-suspended and washed twice in four volumes of SSS + 1% Triton X-100 and centrifuged (1,500×g for 10 min). The pellet was then re-suspended and washed two additional times in four volumes of SSS and centrifuged (1,500×g for 10 min). Four volumes of 0.1 mmol L^{-1} KCl was added to re-suspend and wash the pellet and centrifuge (1,500×g for 10 min) was used to collect the pellet two additional times. The pellet was then re-suspended and washed in four volumes of 0.1 mmol L^{-1} NaCl and centrifuged (1,500×g for 10 min). The protein content of the final pellet was determined by the Biuret method [23] using bovine serum albumin as standard and was used in 24 h.

A 0.5% (w/v) MTG in 0.6 mol L^{-1} NaCl 50 mmol L^{-1} Na_2HPO_4/NaH_2PO_4 was used as stock solution. In order to study the effect of MTG concentration on the T_2 relaxation time and gel microstructure of PMP (40 mg mL^{-1} in 0.6 mol L^{-1} NaCl 50 mmol L^{-1} Na_2HPO_4/NaH_2PO_4 solution, pH 6.0), five tests were performed using different enzyme dosage (0, 2, 4, 6 and 8 U/g protein). After the enzyme added, the samples were stirred and maintained in a water bath at 50 °C for 60 min, afterwards they were cooled at 0–4 °C in a refrigerator or in an ice bath and aged overnight.

NMR spin–spin relaxation (T_2) measurements

NMR relaxation measurements were performed on a Niumag Benchtop Pulsed NMR Analyzer PQ001 (Niumag

Electric Corporation, Shanghai, China) operating at a resonance frequency for protons of 22.6 MHz. Approximately 2 g of sample was placed in a 15 mm glass tube and inserted in the NMR probe. Spin–spin relaxation time, T_2, was measured using the Carr-Purcell-Meiboom-Gill sequence [24, 25]. The T_2 measurements were made with a τ-value (time between 90° and 180° pulse) of 200 μs. Data from 12,000 echoes were acquired as 32 scan repetitions. The repetition time between subsequent scans was 6.5 s. Each measurement was performed in triplicate.

Post-processing of NMR T_2 data

Distributed exponential fitting of CPMG decay curves were performed in MultiExp Inv Analysis software (Niumag Electric Corporation, Shanghai, China). For a better fit, multi-exponential fitting analysis has been performed on the relaxation data in the software algorithm. This analysis resulted in a plot of relaxation amplitude for individual relaxation processes versus relaxation time. From such analyses, time constants for each process were calculated from the peak position, and the area under each peak (corresponding to the proportion of water molecules exhibiting that relaxation time) was determined by cumulative integration. Additionally, the width of the relaxation population was calculated as the standard deviation of the observed relaxation times for the given peak.

Water holding capacity

Water holding capacity (WHC, %) was determined according to the method developed by Kocher and Foegeding [26]. PMP gels were centrifuged at 10,000×g for 10 min at 4 °C. The weights (g) of the centrifuge tubes, protein samples and moisture loss were all recorded. The following formula was used to determine WHC (%):

$$\mathrm{WHC}(\%) = \frac{\mathrm{CG} - \mathrm{ML}}{\mathrm{CG}} \times 100$$

where ML is the amount (g) of moisture lost from the gel during centrifugation and CG is the weight (g) of the heated gel. Each measurement was performed in triplicate.

Scanning electron microscopy (SEM)

Gel samples were fixed in 2.5% glutaraldehyde in 0.1 mol L^{-1} phosphate buffer solution at pH 7.0 for at least 2 h. Ethanol dehydration was then performed using a series of solutions of increasing ethanol concentration. Each sample was freeze-dried, sputter-coated with 10 nm of gold. Sample observation and photomicrography were performed in a Hitachi S-3000 N SEM (Tokyo, Japan) operating at a voltage of 20 kV. Four fields were observed for each sample.

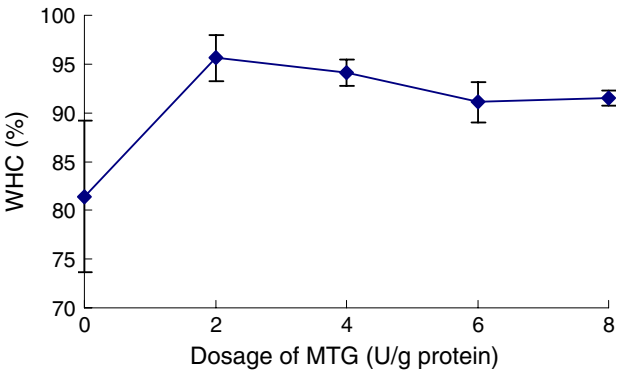

Fig. 1 Water holding capacity of PMP gel at various MTG concentrations. *Error bars* indicate mean values ± standard deviations of three replicates

Statistical analysis

Statistical analysis of results was performed using Statistical Analysis System (SAS 8.12, SAS Institute Inc., Cary, NC). Analysis of variance (ANOVA) was employed to determine the significance of main effects. Significant differences ($P < 0.05$) between means were identified using Duncan's multiple range test.

Results and discussion

WHC of PMP gel

The gelling process together with the cross-link of MTG entail the association of PMP chains which produce a continuous three dimensional network in which water is trapped. The WHC can indicate a protein's ability to bind water and is generally used to objectively evaluate the quality and yield of meat and meat products [18, 27]. As shown in Fig. 1, the WHC of PMP gel increased significantly ($P < 0.05$) from 81.4 to 95.6% as MTG increased from 0 to 2 U/g protein, however no further increase was observed with the increasing of MTG concentration from 2 to 8 U/g protein ($P > 0.05$).

Our observations agree with Motoki et al. [28], Ionescu et al. [29] and Kuraishi et al. [30] who found that MTG improves the WHC of proteins either by increasing the ability to swell and bind water, or by improving the ability to form a gel lattice.

NMR proton relaxation

Typically, the signal decay could be fitted into a distributed exponential consisting of one or two separate peaks. Figure 2 showed distributed T_2 relaxation times in samples

with various MTG concentrations. The T_2 was characterized by a minor population with a relaxation time of a few ms and a major population with a relaxation time around 170–230 ms. The position of the major component clearly shifted towards lower relaxation times with increasing MTG concentration. The peak of T_2 became wider after 2 U/g protein was added ($P < 0.05$), however no further changes were observed with the increasing of MTG dosage from 2 to 8 U/g protein at incubation temperature of 50 °C ($P > 0.05$).

Statistically significant effect was found on the T_2 ($P < 0.05$) with the adding of MTG to PMP (Fig. 3). A remarkable shift in the absolute peak value was observed after the enzyme was added, which led to a somewhat faster relaxation, the T_2 reduced from 226 ms of control to 188 ms of 2 U/g protein. However no further decrease was observed with the increasing of MTG concentration from 2 to 8 U/g proteins ($P > 0.05$).

The NMR data showing slower relaxation for samples with enzyme suggested less limited mobility of protein protons due to network formation. The observed relaxation times in PMP gel is reduced from the relaxation time of bulk water because the populations of fast relaxing protein exchangeable protons and slowly relaxing water protons are in chemical exchange.

The water properties in these gel systems were characterized using NMR T_2 relaxation measurement. These revealed a distribution of relaxation times with a major peak centred around 170–230 ms (Fig. 2). The enzymatic protein preparation had significantly lower values of spin–spin relaxation time T_2 in comparison with the control system. T_2 was reduced from 226 ms (peak value) of controlled the PMP gel without MTG to 188 ms of the 2 U/g protein MTG-induced PMP gel. The decrease in nuclear relaxation times indicates an overall decrease of water

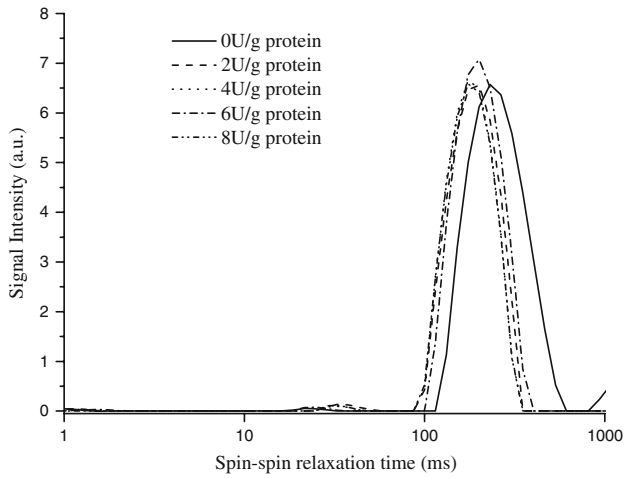

Fig. 2 Spin–spin relaxation times (T_2) of PMP with different MTG concentration

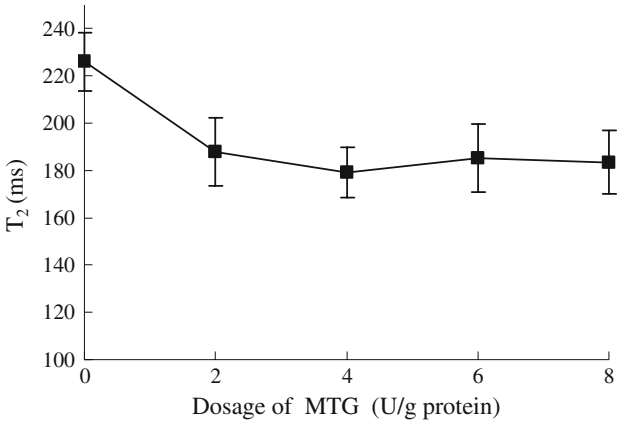

Fig. 3 Effect of adding MTG on the T_2 relaxation time. *Error bars* indicate mean values ± standard deviations of three replicates

mobility with the addition of MTG. The addition of MTG also broadened the T_2 population, which is similar to effect of the heating, and the NMR data revealed a gradual ongoing change in the characteristics of the myofibrillar water with increasing temperature [16, 31]. That change in the myofibrillar water was displayed as a broadening of the T_2 population, which resulted in this population expanding from the region 25–100 ms at 25 °C to 10–120 ms at 75 °C [32]. With addition of MTG and incubation at 50 °C, the myofibrillar proteins denatured and aggregated, finally a three dimensional gel network and cross links between proteins formed, Hills et al. [33] showed that protein aggregation reduces the water proton relaxation time. They also showed protein aggregation and gel formation resulted in increased relaxation rates and less dispersion in rates. Other authors also observed denaturation and gelling of extracted protein lower T_2 values [34]. A typical T_2 relaxation shows that distributed exponential fitting analysis identified three components, a minor component between 1 and 10 ms, probably reflecting bound water, a major component between 30 and 100 ms, and finally a component between 100 and 1,000 ms [35]. However, a distribution of relaxation times with a major peak centred around 170–230 ms was observed in PMP gel (Fig. 2). The differences observed between meat and protein are probably due to different magnetic equipment, differences in NMR parameters employed, including repetition time between succeeding scans, the τ-value (time between 90 and 180 pulse), differences in measuring temperature and, finally, intrinsic differences between meat and MTG-induced PMP gel.

Microstructure of MTG-induced PMP gel

As shown in Fig. 4, SEM revealed significant variations in shape and size of PMP gel with addition of MTG, which

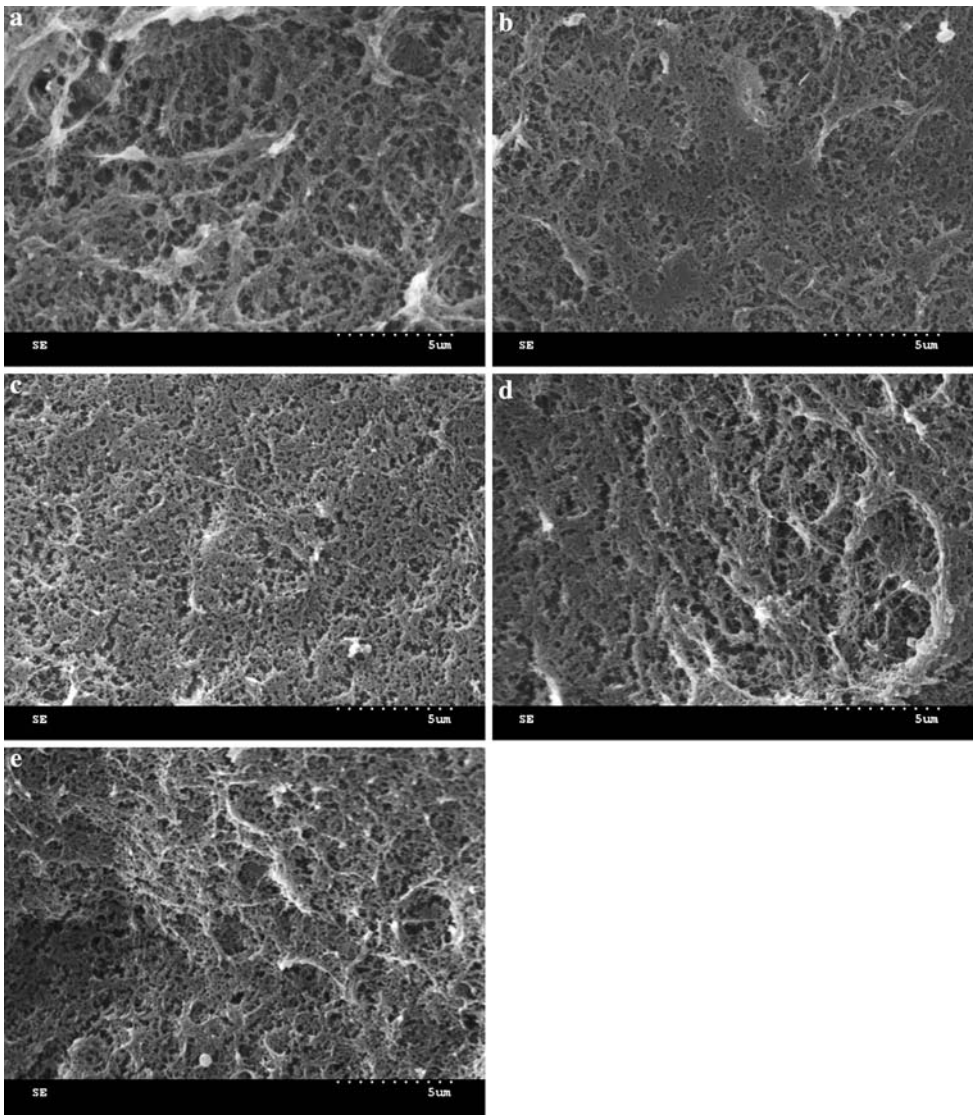

Fig. 4 Scanning electron micrographs of PMP gel with 0 U/g protein (**a**), 2 U/g protein (**b**), 4 U/g protein (**c**), 6 U/g protein (**d**), 8 U/g protein (**e**) MTG added (20 kV ×6,000)

had a granular without enzyme (Fig. 4a) and more porous microstructure with 2–8 U/g protein MTG added (Fig. 4b–e). The addition of the MTG resulted in gels with quite homogenous microstructure and decreasing the pore size of the gel as already shown in the study of Partanen et al. [36]. A few bridges between adjacent macromolecules are evident with the addition of MTG (Fig. 4b–e), which may lead to excellent WHC. As far as the dosage concerned, the amount of MTG added seems to be too high for the current study. It can be a reason of a lack of enzyme influence on protein preparations with addition of enzyme above 2 U/g of protein.

For the WHC of highly comminuted and heated meat products, the ability of the meat proteins to form different types of gels and colloidal systems which stabilize finely distributed fat particles and water are crucial factors [37]. And WHC is related to the microstructure of meat products [18]. The water is held or trapped within the muscle or muscle product by capillary action which is generated by small pores or capillaries. The magnitude of the force immobilizing the water is inversely related to the pore size according to the following relationship [38]:

$$h = \frac{\gamma^2 \cos\theta}{gdr}$$

[where h = capillary pressure (in cm of water), θ = wetting angle, r = capillary radius, g = acceleration due to gravity, d = density of the liquid, γ = surface tension]. In processed meat products, the combination of processing and heating causes the muscle proteins to rearrange and aggregate which results in the formation of an ordered three-dimensional protein lattice. It is the pores within this lattice that produce the capillary suction force. Typically, these pores are from 0.1 to 1.0 μm in diameter [39]. If water is immobilized in pores, it has a shorter distance to diffuse to the water/protein interface than non-immobilized

water and, as a result, a larger percentage of the immobilized water molecules will diffuse to the protein/water interface. Consequently, water immobilized in pores has lower T_2 values than free water and the T_2 values decrease progressively as the pore size decreases and approach that of bound water [18]. Therefore with the addition of MTG, the PMP gel formed more porous microstructure, which was related to the reduction of T_2 and may lead to higher WHC.

Conclusion

With the addition of MTG, changes in the gel network of PMP gels were introduced. The shorter relaxation time of gels with MTG observed with NMR suggested lower water proton mobility in the gel. The enzymatic gel formed higher WHC and more porous microstructure observed using SEM, which was related to the reduction of T_2.

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