Effect of pH on NMR Relaxometry and Chicken Myosin Gel Properties

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ABSTRACT—*The effect of pH on low-field nuclear magnetic resonance (NMR) relaxometry datas, water holding capacity (WHC), gel strength, and microstructure of chicken myosin gel was investigated. There was a positive and significant correlation between WHC and gel strength. The WHC of the myosin gel significantly increased as the pH deviated from the myosin isoelectric point. T_{22} increased to 35.10ms at pH 6.5, indicating that the free water transformed to mobile water, which led to better WHC of chicken myosin gels. Maximum gel strength (30.78g) was obtained at pH 6.5. Scanning electron micrographs (SEM) showed that the mobility of water in the protein gel network correlated to the gel microstructure. A compact and uniform chicken myosin gel was acquired at pH 6.5.*

Keywords—chicken myosin, gel property, pH, NMR

1. INTRODUCTION

Chicken has been known as a "source of nutrition" and has become the second most consumed meat in the world. Gelation is one of the most important functional properties, which affects the properties of chicken products. Meat production involves gel formation involving denaturation and aggregation of protein (Hermansson1,1986), which induces a three dimensional network (Laier et al., 2004). Myosin is vast in chicken muscle protein and has a key role in chicken products. Change in pH significantly influences myosin conformation (Lin and Park, 1998) and other functional properties (Liu et al., 2007; Yongsawatdigul and Sinsuwan, 2007). Breakdown in protein structure with some reactive groups may cause alteration of pH on meat products and desired gel strength (Westphalen and Lonergan, 2005). Therefore, alteration of pH influences the myosin gel properties. A lot of information is available about the influences of pH on fish or porcine gel property (Liu et al., 2008; Liu et al., 2010). Nevertheless, there is dearth of knowledge on the relationship between the water mobility and chicken myosin gel quality is little reported.

NMR is widely used for its non-destructive nature. It has the capacity to determine the water distribution and mobility in meat product (Hills, 1989). Most studies have used NMR to determine the WHC and other properties of pork products (Bertram et al., 2001; Brown et al., 2000; Han et al, 2009). The spin-lattice relaxation time (T_1) and spin–spin relaxation time (T_2) are usually used to characterize the general features of proton relaxation (Han et al., 2009). T1 often measure the interaction of a spin with its surroundings, and T_2 determine the mobility of a spin. The greater changes of T_2 are often used for the determining WHC of meat products (Trout, 1988), which is often more sensitive under several surroundings than T_1 (Hinrichs et al., 2004). Without interaction with the dissolved molecules, the free water can be

distinguished from the immobile water, which include crystallization water or other chemically or physically bound water. NMR relaxation is useful in studying the effects of pH and other factors on myofibrillar proteins; therefore, the method may also be useful for discussing the effect of pH on chicken myosin gel properties.

In this research, myosin was prepared from chicken breast muscle. Myosin gel properties were determined by WHC assessment, gel strength measurement, and SEM. NMR relaxometry was used to monitor the water mobility of chicken myosin gel. Subsequently, the relationship between chicken myosin gels and water mobility at different pH were discussed. This study aims to investigate the effect of pH on the NMR Relaxometry and Chicken Myosin Gel Properties, providing more basis and data for chicken product designation and promote the development of meat industry.

2. MATERIALS AND METHODS

2.1 Materials

Live Yellow Hair Chickens were purchased from Huanghe Food City, zhengzhou, China. The chickens were immediately slaughtered, and the breast muscle was removed manually from the skin and bone before the muscles become stiff. The samples were placed in a plastic bag and taken to the laboratory within 30 min for myosin preparation. All chemicals used were analytical grade and purchased from Sigma (St. Louis, Mo, USA) or Huafeng Chemical Reagent Co., Ltd. (Zhengzhou, China).

2.2 Myosin preparation

Myosin was extracted according to previously described methods (Cao et al., 2012; Hermansson et al., 1986; Nauss et al., 1969; and Wang and Smith, 1994a, 1994c), with some modifications. Chicken breast muscle myosin was extracted immediately after sacrifice. Muscles were cut into 2×3cm pieces, homogenized for 3s in a meat grinder (250PP01B, Meideshenghuo Electrical Appliance Manufacturing Co., Ltd.), and the minced meat was then dispersed with three volumes of Guba-Straub (GS) solution (0.3mol/l KCL, 0.1mol/l KH2PO4, 0.05mol/l K2HPO4, 1mmol/l EDTA, 4mmol/l sodium pyrophosphate, pH 6.5) for 10min with vigorous stirring, but avoiding foaming. three volumes of distilled water were added to the suspension, and the muscle residue was centrifuged at 3000×g for 20min at 4℃ (Beckman ultracentrifuge, Model ALLLEGRA-64A, Beckman Coulter, Inc., Guangzhou, China). Filtration of the supernatant was carried by four layers of cotton cloth and 6.5 volumes of 1mmol/L EDTA (pH 7.0)was added to the filtrate with rapid stirring, and then allowed to precipitate for 2h or overnight. The supernatant was seperated by siphoning, and precipitated protein was collected by centrifugation at 10000×g for 10min at 4℃.

The precipitate was dissolved in 3mol/l KCL, stirred gently, allowed to solute for 2h or overnight, and then diluted with distilled water to 0.6mol/l KCL. Magnesium chloride (1mol/l) and sodium pyrophosphate (0.1mol/l) were added to a final concentration of 5mmol/l, respectively. The solution was stirred vigorously for 10min without foaming, diluted with distilled water to 0.3mol/l KCL, adjusted the pH to 6.6, and centrifuged at 10000×g for 15min at 4℃. The supernatant was diluted with distilled water to 0.03mol/l KCL, and allowed to precipitate for 2h or overnight. Afterward, the supernatant was seperated by siphoning, and precipitated protein was separated by centrifugation at 10000×g for 10 min at 4℃. The process was repeated.

The precipitate was resolved in 3mol/l KCL/25mmol/l PIPES (pH7.0), stirred gently, allowed to dissolve for 2h or overnight, and then diluted to 0.6mol/l KCL. Magnesium chloride and sodium pyrophosphate were added to a final concentration 5mmol/l. After the vigorous stirring for 10min, Solid $(NH_4)_2SO_4$ was added slowly at 35% saturation with constant stirring within 2 mins, and the solution was centrifuged at 10000×g for 25min at 4℃. Solid $(NH_4)_2SO_4$ was added again to the supernatant at 48% saturation. Myosin was collected by centrifugation at 10000×g for 15min at 4℃, suspended in 0.6mmol/l KCL, 20mmol/LKH_2PO_4- K_2HPO_4 (PBS, pH 6.0), and dialyzed for 24h with the same buffer. The extracted myosin solution was stored at 4℃and used within 7 days.

The myosin content was determined by the Gornall method, with bovine serum albumin (BSA) as standard. The protein concentrations were adjusted to 20mg/ml for thermal gel preparation (Gornall, 1949).

2.3. Electrophoresis

Myosin molecules comprise 2 heavy chains (205 KD) and 4 light chains (16, 18, 22, and 30KD, respectively) (Starr and Offer, 1971). The sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to verify the myosin purity(Laemmli, 1970). Figure 1 illustrates the electrophoretic pattern of chicken myosin. The purity of extracted myosin samples was greater than 90% as detected by densitometry(EC3 Imaging System, Ultra-Violet Products Ltd., UK), although small amount of actin and troponin remained.

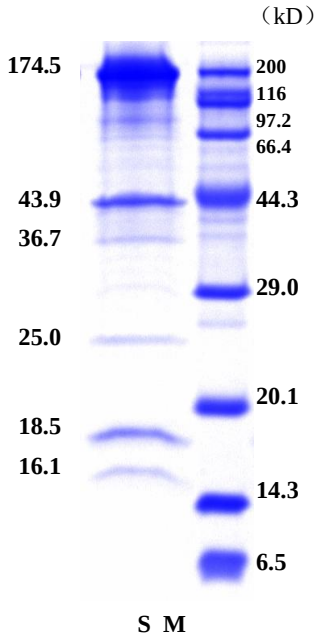

Figure 1: SDS-PAGE of extracted myosin (Lane 1) from chicken breast muscle. Lane 2 designates molecular weight markers. All lanes were loaded with 20ug of protein.

2.4. Thermal gel preparation

Myosin solutions of 20mg/ml at pH levels 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5) were prepared by adding 1M Tris, quickly mixed, and the pH value was determined using a pH meter (model 216, Jingke Instrument Co., Ltd., Shanghai, China). Aliquots sample were placed in Eppendorf tube for WHC measurement; 0.5ml in a chromatographic glass bottle for NMR measurement; and 3ml in a diameter of 18mm, height of 30mm glass bottle for gel strength measurement. A deal with three repeat was carried. The samples were heated in a water bath from 20℃ to 65℃ with heating rate of 1℃/min, followed by 20min at 65℃. Samples were immediately cooled using an ice bath, and then stored overnight at 4℃ for all the determinations.

2.5. Water-holding capacity (WHC)

The thermal myosin gels were centrifuged at 1000 g for 10min at 4℃. All the weights (g) of centrifuge tubes, protein samples, and water loss were all recorded. The formula used to calculate WHC (%):

$$\text{WHC } (\%) = \frac{CG - ML}{CG} \times 100 \quad (1)$$

where ML is the amount (g) of the gel water loss during the centrifugation and CG is the weight (g) of the thermal gel. All tests were carried with triplicates (Kocher and Foegeding 1993).

2.6. Gel Strength Determination

Texture analysis was carried out using a TA-XT plus Texture Analyzer (Stable Micro System Ltd., UK) at room temperature (Herrero, 2008). Compression was performed at 1.00 mm/sec test speed, and of 1.00 mm/sec post-test speed, using a 5mm diameter cylindrical stainless steel plunger (Part Code P/5). The probe returned to start position after penetration of 10mm. Force-deformation curves were derived at 1.00mm/sec crosshead speed. Gel strength (N) was defined as the force required to the ruptured gel. Results are the mean of the three repeated trials.

2.7. NMR spin–spin relaxation (T_2) measurements

NMR spin–spin relaxation measurements were carried on a Niumag Benchtop Pulsed NMR Analyzer PQ001 (Niumag Electric Corporation, Shanghai, China), operating at a resonance frequency of 23MHz. Approximately 0.5g of sample was placed in a 18mm glass tube and inserted in the NMR probe. Spin–spin relaxation time, T_2 was measured

using the Carr–Purcell–Meiboom–Gill sequence. T_2 measurements were carried out with a τ-value (time between 90° and 180° pulses) of 500μs. Data from 8000 echoes were acquired as 8 scan repetitions, with repetition time between subsequent scans of 8s(Zhang, 2013). Each measurement was carried with triplicates.

2.8 Post-processing of NMR T_2 data

Post-processing of NMR T_2 data was performed according to the method described by Han et al (2009). From the 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.

2.9. Scanning electron microscopy (SEM)

Microstructure of myosin gel was examined by scanning electron microscopy (SEM). Samples were processed according to the methods described by Liu et al. (2008). The samples were firstly fixed in 0.1 M glutaraldehyde in phosphate buffer (pH 7.3) for 2h, and then washed three times using 0.1M Na-phosphate (pH 7.3). The post-fixation stage involved exposure to 1% osmium tetroxide for 2h. Washed samples were dehydrated in 10% dimethyl sulfoxide (DMSO) for 6h, and then freeze-dried. Samples were coated with gold to a thickness of 10nm in an Ion SPUTTER (E-1010, Hitachi, Tokyo, Japan). Samples were observed at an accelerating voltage of 10kV and a magnification of 2000 using a scanning electron microscope (S-3400N II, Hitachi, Tokyo, Japan).

2.10. Statistical analysis

Correlation and variance analysis of all data were processed using the Statistical Package for Social Science (SPSS 19.0 for Windows). Least significant difference ($P<0.05$) was used to determine difference among treatments.

3. RESULTS AND DISCUSSION

3.1. Water-holding capacity

Figure 2 shows the WHC of chicken myosin gel at various pH values. From the result, WHC increased significantly ($P < 0.05$) from 26.03% to 40.66% as the pH increased from 5.5 to 8.5; the maximum WHC (39.51 to 40.66%) was achieved at pH 6.5 to pH 7.5. Literature suggested shows chicken-breast muscle gels retains water better at higher pH values (pH 7.0 to pH 7.4) than at lower pH values (pH 6.4 to pH 6.8) (Kristinsson and Hultin, 2003): Liu et al. (2008) and Bertram (2004) similarly Bertram et al. (2004) and Liu et al. (2008) reported the WHC of porcine protein gel had the same trends with increasing pH from 5.5 to 7.0. WHC was determined by the impact degree of pH on the inter-protein filaments electrostatic charge and electrostatic repulsion. During the denaturation of protein, some positive and negative polarisation centres appeared at polypeptide chains. These centers, –CO– and –NH, can form multilayer water system (Damodaran,1996). Above the pI, the increasing native charge lead to an increase in electrostatic repulsion between the protein molecules, more hydrogen binding sites for water molecules, and bigger surface of hydration (Liu et al., 2008; Westphalen et al., 2005). When the pH shifted close to the pI, proteins tended to coagulate with the increased protein–protein interactions (Surel and Famelart, 2003). At the same time, the changes of the hydration states of charged amino acids made the protein hydration gradually weak, which caused WHC of myosin gel increase with increasing pH.

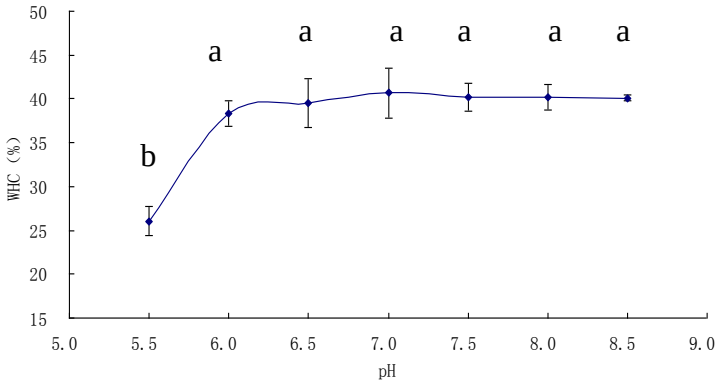

Figure 2: Effects of pH on Chicken breast myosin gel water-holding capacity. *a-b Means in the same curve with different letters are significantly different ($P<0.05$).

3.2. NMR proton relaxation

Figure 3 shows the spin–spin relaxation times (T_2) of chicken myosin gels at different pH values. The signal decay can fit into a distributed exponent including some separate peaks. NMR T_2 relaxation measurement was used to characterize the water properties in chicken gel systems. Generally, water was classified into three kind of water, including free water, mobile water, and bound water. Bound water is associated mainly with ionic chains. The other two types of water condensed into the clefts and crevices of the protein molecules, or in the capillaries of insoluble protein systems (Damodaran, 1996). Four peaks (the first two at below 10ms, one at 10ms to 100ms, and the other at about 1000ms) appeared in the spin–spin relaxation figure (Fig. 3). T_2 was characterized by two population, a minor of a few ms and a major population with a relaxation time around 1000 ms. According to the weighted geometric mean calculation method, the first two peaks were calculated and accounted as T_{21}, followed by T_{22}, and T_{23}, which characterized the bound water, mobile water, and free water, respectively.

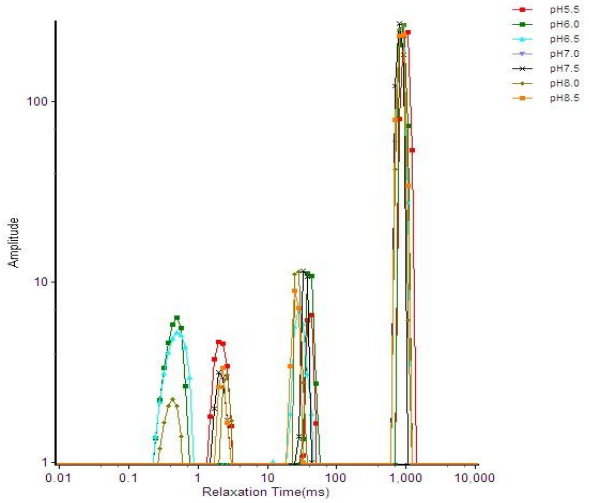

Figure 3: Spin–spin relaxation times (T_2) of chicken moysin gels at different pH values.

Table 1 shows the effects of pH on Spin–spin relaxation times (T_2) of chicken myosin gels. T_{21} had no significant change with the increase in pH *(P<0.05)*, and the maximum T_{21} (5.52ms) was achieved at pH 6.5, which characterized the water combined with protein molecules in the gel system. T_{22} decreased from 40.47ms to 31.72ms, and then increased to 36.92ms at pH 6.5 to pH 7.0; however, no further changes were observed with the increase in pH *(P<0.05)*. T_{23} decreased significantly at the beginning of the increase in pH *(P<0.05)*, with no further changes observed *(P>0.05)*. Therefore, mobility of free water decreased, which may transform to mobile water with the increase of pH.

Table 1: Effect of pH on Spin–spin relaxation times (T_2) of chicken moysin gels

pH	T_{21}(ms)	T_{22}(ms)	T_{23}(ms)
5.5	1.88±0.19	40.47±3.99	2063.07±1411.20[a]
6.0	1.04±0.57	31.72±6.50	932.60±0.00[b]
6.5	5.52±5.11	35.10±14.17	932.60±0.00[b]
7.0	1.25±1.08	36.92±6.67	811.13±0.00[b]
7.5	3.54±2.26	32.96±4.59	811.13±0.00[b]
8.0	2.09±1.69	32.96±4.59	932.60±0.00[b]
8.5	1.98±0.30	30.09±4.60	932.60±0.00[b]

All relaxation times values are mean ± standard deviation of three replicates (n = 3); *a-b Means in the same curve with different letters are significantly different ($P<0.05$).

Table 2 shows the effect of pH on peak areas of T_{21}, T_{22}, and T_{23} of myosin gel. PK_1 decreased with the increase in pH, indicating that bound water contents were reduced. PK_2 increased significantly, and the mobile water content increased ($P<0.05$). PK_3, however, had a small change with the increase in pH. Therefore, above pI, mobility of water increased, and the content of mobile water increased, which led to better WHC of chicken myosin gels.

Table 2: Effect of pH on peak areas of T_{21}、T_{22} and T_{23} of myosin gel

pH	PK_1	PK_2	PK_3
5.5	0.04 ± 0.01^{ab}	0.03 ± 0.00^{b}	0.93 ± 0.01^{ab}
6.0	0.05 ± 0.00^{a}	0.04 ± 0.00^{a}	0.91 ± 0.01^{b}
6.5	0.02 ± 0.03^{ab}	0.05 ± 0.00^{a}	0.93 ± 0.02^{ab}
7.0	0.04 ± 0.02^{ab}	0.04 ± 0.00^{a}	0.93 ± 0.02^{ab}
7.5	0.02 ± 0.02^{ab}	0.04 ± 0.00^{a}	0.94 ± 0.02^{ab}
8.0	0.02 ± 0.01^{ab}	0.04 ± 0.00^{a}	0.94 ± 0.01^{ab}
8.5	0.02 ± 0.01^{b}	0.04 ± 0.00^{a}	0.95 ± 0.01^{a}

All Peak areas values are mean ± standard deviation of three replicates (n = 3); *a-b Means in the same curve with different letters are significantly different ($P<0.05$).

3.3. Texture analysis

Table 3 shows the gel strength of chicken myosin gel at different pH values. Gel strength increased significantly (P<0.05) from 7.43g to 30.78g with increase in pH from 5.5 to 8.5, and the maximum gel strength 30.78g was achieved at pH 6.0 to pH 6.5. Shifting pH close to pI, the increased protein–protein interactions (Surel and Famelart, 2003) and the higher protein-protein polymerization rate caused soft and easy seepage gel (Saguer et al., 2008). The smaller electrostatic repulsion of pH 6.0 to pH 6.5 than pH 7.0 increased hydrophobic interactions and disulfide.

Table 3: Effects of pH on myosin gel strength

pH	Gel Strength (g)
5.5	7.43 ± 0.54^{b}
6.0	30.78 ± 9.72^{a}
6.5	10.48 ± 1.78^{b}
7.0	6.89 ± 1.29^{b}
7.5	9.79 ± 0.75^{b}
8.0	9.97 ± 0.52^{b}
8.5	11.01 ± 0.40^{b}

All Gel strength values are mean ± standard deviation of three replicates (n = 3); *a-b Means in the same curve with different letters are significantly different ($P<0.05$).

3.4. Microstructure of chicken myosin gel

Microstructure of chicken myosin gel was detected by Scanning electron micrographs as a function of pH. Chicken myosin solutions at low pH from 5.5 to 6.0 formed coarse and disordered gel networks with some blocky or spherical aggregates and big pores (Fig. 4a,b). The least charged groups of myosin at low pH may have made the aggregation rate faster than denaturation, which caused the coarse gel network and a low WHC (38.3%). Ordered and uniform gel was gained at pH 6.5 (Fig. 4c), coarse with some bigger pores myosin gel at pH 7.0 (Fig. 4d), myosin gel was compact, which included of some particles with various sizes at pH 7.5 (Fig. 4e), and myosin gel was uniform with some bigger pores at pH 8.0 (Fig. 4f). Hermansson (1979) suggested that proteins gel properties depend on the relative speed of unfolding and aggregation. Thus, the relative speed of unfolding and aggregation of chicken myosin at pH 6.5 was beneficial for the formation of a perfect gel network. Similarly, porcine myosin formed the perfect gel network at pH 6.5 (Liu et al., 2008) and pH 7.0 for fish myosin (Liu et al., 2010).

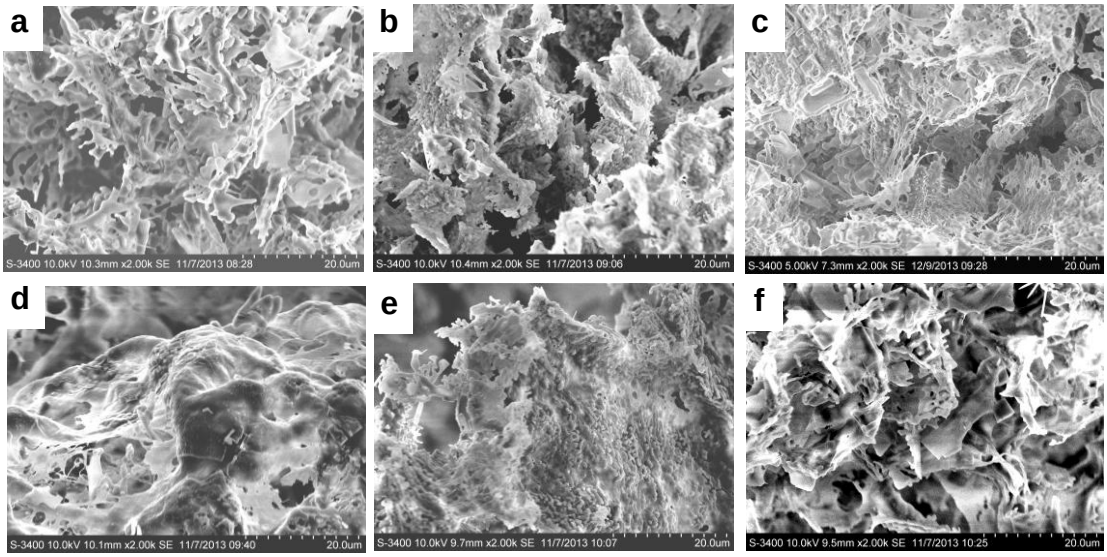

Figure 4: Scanning electron microscopys of myosin gel at various pH values. (a) pH5.5; (b) pH6.0; (c) pH6.5; (d) pH7.0; (e) pH7.5; (f) pH8.0.

3.5. Correlation Analysis

Table 4 shows the Pearson correlation coefficients among myosin gel properties viz. WHC, Gel strength, T_{21}, T_{22}, T_{23}, PK_1, PK_2 and PK_3. The results established extremely significantly negative($P < 0.05$) correlation between WHC and gel strength with the Pearson correlation coefficient of -0.706, and it was significant negative between T_{21} and PK_2 with the Pearson correlation coefficient of -0.548. The relationship was significant positive between T_{22} and T_{23} with the Pearson correlation coefficient of 0.524. Therefore, above pI, mobility of water increased, and the free water transformed to mobile water, with the content of mobile water increased, which led to better WHC and soft chicken myosin gels.

Table 4: Pearson correlation coefficients among the results of myosin gel properties

	WHC（%）	**Gel strength（g）**	**T_{21}（ms）**	**T_{22}（ms）**	**T_{23}（ms）**	**PK_1**	**PK_2**	**PK_3**
WHC（%）	1	-0.706**	-0.313	-0.012	0.213	0.124	0.100	-0.143
Gel strength（g）		1	-0.049	0.166	-0.220	0.098	-0.016	0.194
T_{21}（ms）			1	0.332	0.419	0.119	-0.548*	-0.295
T_{22}（ms）				1	0.524*	0.396	-0.206	0.243
T_{23}（ms）					1	0.213	-0.349	-0.188
PK_1						1	-0.206	0.293
PK_2							1	0.440
PK_3								1

Note: ** Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).

4. CONCLUSIONS

With the pH deviating the pI, T_{22} substantially increases, and the content of mobile water and WHC of myosin gels are improved significantly. Therefore, the increase in gel retention water only increased mobile water. The increase in the

pore size of myosin gel caused more water retention, and a compact and uniform chicken myosin gel was acquired at pH 6.5.

5. ACKNOWLEDGEMENTS

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