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Reduction of NaCl in oxidized myofibrillar protein by substitution with chloride salts: underlining the gel changes and gastric digestive properties

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ABSTRACT

The changes and gastric digestive properties of gel were investigated for oxidized myofibrillar protein (MP) (0–1 mmol/L H_2O_2) treated by 30% substitution of NaCl with KCl, $MgCl_2$, and KCl/$MgCl_2$. Mild oxidation level (0.3 mmol/L H_2O_2) facilitated the protein unfolding, and $MgCl_2$ obviously intervened the protein self-assembly by forming the salt bridge and more disulfide bonds. At the same oxidation concentration, the sodium-reduced MP substituted with KCl formed dense network structure with better gel strength (increased by 6%–18%) and water retention in comparision to the gel without sodium reduction. The KCl-substituted gel chewing work was also relatively higher among the low-sodium samples at the first chewing cycle. Reversely, the relatively soft structure and lower gel viscosity in simulated oral chewing were found in $MgCl_2$-substituted gel. These changes decreased the contact limit between pepsin and protein for better gastric disintegration or pepsin diffusivity, and increased the proportion of small molecular weight peptides (25.83%, < 5 kDa). Therefore, partial substitution of NaCl with other salt replacers could influence the physicochemical properties of low-sodium MP suspension or gel under different oxidation degree and the subsequent gastric digestion characteristics.

1. Introduction

Salt is an essential ingredient in the processing of meat products, and it is also the main source of sodium intake in human diets. Acceptable salt amount is conducive to maintaining human health through the regulation of blood pressure and physiological functions[1], and gaining the premium of meat products with better sensory experience and longer shelf life[2]. However, excessive sodium intake increases the risk of developing high blood pressure, cardiovascular diseases, and other related diseases[3]. Currently, the increased health awareness in sodium reduction promotes the development of low-sodium meat products. Three mainstream strategies for sodium reduction are proposed, including substitution with other chloride salts, modification of salt morphology, and alternative processing techniques for effective immersion of salt[4-5]. Among them, partial substitution of NaCl with other chloride salts (e.g., KCl and $MgCl_2$) is economical and easy-operated for consumers. More importantly, people should attach importance to regulating the substituted degree and types of these non-sodium salts for effectively avoiding the appearance of technological and sensory alterations[6].

Myofibrillar proteins (MP) are primarily responsible for functional traits, such as gelation, emulsification, and water-holding capacity (WHC) in cooked meat[7]. The reactive oxygen species are easily produced by the oxidation precursors in meat or pro-oxidation factors during meat

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processing and storage[8]. The functionality of proteins is thus changed due to the oxidation-induced peptide breakage, molecular cross-linking and aggregation, and amino acid side chain modification[9]. Moreover, numerous stimulated oxidative models have indicated that the physicochemical properties of oxidized MP and gel are dose-dependent with the substances used. Mild protein oxidation can change the aggregation mode of myosin in MP, enhance the non-covalent interaction between proteins, and form an ordered structure to improve the gel characteristics[10-12]. Currently, using other chloride salts to partially replace NaCl is an effective strategy to improve the gel properties of oxidized MP. These results reveal that KCl and $MgCl_2$ are promising in forming a dense gel network structure, while excessive substitution (> 40%, single or mixed) is prone to induce serious lipid or protein oxidation[4,13]. The differences of these chloride salts in gel properties are mainly related to ionic strength and the exposed charged groups, manifesting in the water-binding ability of protein particles, and the protein assembling induced by the uneven surface charge[1,14]. However, limited reports focus on the characteristics of oxidized protein based on partial substitution of NaCl using chloride salts under different oxidation levels.

It is widely recognized that the mechanical decomposition of food during oral processing is conducive to improving the accessibility of food bolus in subsequent digestion processes[15]. For solid-based MP gel, there is substance exchange between the gastric juice and gel matrix, and the diffusivity of pepsin is intervened by the structure, texture, and composition of gel[16-17]. Fluorescence recovery after photobleaching (FRAP), as a powerful tool to analyze the diffusion characteristics of the labeled molecules, has been successfully applied to the diffusion coefficients of fluorescein isothiocyanate (FITC)-dextrans in cheese and pepsin in soybean protein isolate gel[18-19]. Previous studies mainly focused on the characteristics of MP gel digestion products, but ignored the digestive behavior, especially the pepsin diffusion into gel matrix. In this work, MP was exposed to the most commonly free-radical system (hydroxyl-radical-generating system (HRGS)) to (i) investigate the structural changes of MP treated by different chloride salts (KCl, $MgCl_2$, and KCl/$MgCl_2$), and oxidation levels (0–1 mmol/L H_2O_2) by evaluating the carbonyl content, sulfhydryl groups, and surface hydrophobicity; (ii) investigate how these treatments intervened the gel traits (gel strength, WHC, water mobility, and microstructure), oral processing and digestive properties (pepsin diffusion); and (iii) evaluate the correlation between these indexes by Pearson's correlation analysis.

2. Materials and methods

2.1 Materials

Pork *longissimus dorsi* (48 h post-mortem) was obtained from the logistics department in South China University of Technology (Guangzhou, China), and the pigs were slaughtered at about 6 months of age following standard industrial procedures. The visible connective tissue and fat were trimmed away, and muscle was cut into cubes and frozen at –80 ℃ until use (within 7 days). Pepsin (3 000 U/mg, CAS NO. 9001-75-6) and chloride salts (NaCl, KCl, and $MgCl_2$) were purchased from Aladdin (Shanghai, China). FITC and *o*-phthaldialdehyde (OPA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All the other chemicals were of analytical or better grade.

2.2 Extraction of MP

The extraction of MP was performed following our previous study with slight modifications[20]. Ground raw muscle was blended with 4 volumes of pre-chilled buffer (0.1 mol/L NaCl, 2 mmol/L $MgCl_2$, 1 mmol/L EGTA, 6.1 mmol/L Na_2HPO_4, 3.9 mmol/L $NaH_2PO_4 \cdot H_2O$, pH 7.0), and homogenized using a high-speed disperser (FJ200-SH, Shanghai Specimen Model Factory, China) at 10 000 r/min for 60 s, followed by centrifugation (GL-21M; Xiangyi Laboratory Instrument Development Co., Ltd., China) at 8 000 r/min for 15 min. The precipitate was then extracted twice as above methods. The pellets were then washed three times with 0.1 mol/L NaCl solution (pH 6.25) and centrifuged. Before the last centrifugation, the homogenate should be filtered to remove connective tissue. The final obtained MP was suspended in 15 mmol/L piperazine-*N*,*N*′-bis(2-ethanesulfonics acid) (PIPES) buffer (pH 6.25), and the protein concentration was determined by the Biuret method using bovine serum albumin (BSA) as standard.

2.3 Sample treatment

MP suspensions (30 mg/mL final concentration) were prepared with 15 mmol/L PIPES buffer (pH 6.25) and different chloride salts. In current study, chloride salts (KCl, $MgCl_2$, and KCl/$MgCl_2$, 30% substitution of NaCl) were dissolved in MP suspensions following the formula depicted in Table 1, and all the samples were kept at a unified ionic strength (0.6 mol/L). To oxidize them, MP suspensions were incubated in dark at 4 °C for 24 h with HRGS (0–1 mmol/L H_2O_2, 10 μmol/L $FeCl_3$/100 μmol/L ascorbic acid). Oxidation reaction was terminated by adding termination agents (propyl gallate/Trolox C/EDTA, 1 mmol/L each)[21-22]. The non-oxidized MP suspension (0.6 mol/L NaCl, without HRGS) containing the same concentration of termination reagents was used as the Control group. The equation for calculating the ionic strength (I) is as follows:

$$I = \frac{1}{2} \sum_{i=1}^{n} c_i z_i^2 \quad (1)$$

Where c_i is the molar concentration of i, z_i is the number of charges (valence state of i) carried by ions.

Table 1
Different formulations that replace NaCl treatments in MP suspension.

Salt substitution	NaCl		KCl		$MgCl_2$	
	IS	C	IS	C	IS	C
Control	0.6	0.6	-	-	-	-
Na	0.6	0.6	-	-	-	-
30%-K	0.42	0.42	0.18	0.18	-	-
30%-Mg	0.42	0.42	-	-	0.18	0.06
30%-K+Mg	0.42	0.42	0.09	0.09	0.09	0.03

Note: IS and C represent ionic strength and concentration (mol/L), respectively. All the samples were kept at a unified ionic strength (0.6 mol/L).

2.4 Evaluation of oxidative changes of MP

2.4.1 Carbonyl content

The protein-bound carbonyl content of MP was detected by reactivity with 2,4-dinitrophenylhydrazine (DNPH) using a UV

spectrophotometer (UV-1800, Shimadzu, Japan), as described by the reference[14]. Carbonyl content was measured at 370 nm and expressed as nmol of DNPH equivalents/mg of protein using an absorption coefficient of 21 mmol/(L·cm) for protein hydrazones. Due to the protein loss during the repeated washing procedures, the protein concentration in the supernatant was calculated at 280 nm in the HCl control using BSA in 6 mol/L guanidine as standard.

2.4.2 SH and S-S groups content

The SH and S-S group levels were determined by Ellman's method using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and detailedly depicted in Fig. S1. The contents of SH and S-S groups are calculated by Eqs. (2) and (3), respectively:

$$\text{SH (μmol/g protein)} = \frac{10^6}{1.36 \times 10^4} \times A_{412} \times D_1/C \quad (2)$$

$$\text{S-S (μmol/g protein)} = \frac{10^6}{1.36 \times 10^4} \times A_{412} \times D_2/C - \text{SH} \quad (3)$$

Where 1.36×10^4 is the molar extinction coefficient, D is the dilution ratio ($D_1 = 6.04$, $D_2 = 15$), and C is protein concentration (5 mg/mL).

2.4.3 Surface hydrophobicity

The surface hydrophobicity of MP suspension (5 mg/mL) was determined using bromophenol blue (BPB) according to the reference[2], and the result was expressed as bound BPB (μg).

2.5 Preparation of heat-induced MP gels

Briefly, an aliquot of 12 mL MP suspension (30 mg/mL) was placed into a glass cylinder (d = 25 mm), and then heated in a temperature-controlled water bath (SY-358EB, Jichuang, Guangzhou, China). The heating procedures were conducted as follows: (i) equilibration at 25 ℃ for 5 min; (ii) 1 ℃/min (25–53 ℃ and kept at 53 ℃ for 5 min); and (iii) 1 °C/min (53–73 °C and kept at 73 °C for 5 min). After heating, the gels were immediately cooled to 4 ℃ for further study.

2.6 WHC and strength of MP gels

The gel samples (5 g) were centrifuged at 6 000 × g for 15 min at 4 °C, and WHC (%) was expressed as the final gel weight after centrifugation relative to the percentage of the weight before centrifuging. For gel strength, gels were penetrated with a cylindrical probe (P/0.5) attached to a texture analyzer (TA-XT plus, Stable Micro Systems Ltd., Surrey, U.K.) at a crosshead speed of 0.5 mm/s accompanied by 1.5 mm/s of pre-test or post-test speed, trigger force of 5 g, and deformation of 45%. The maximum sustained compression force was the gel strength.

2.7 Low-field nuclear magnetic resonance (LF-NMR)

Prior to the measurement, all the gel samples were equilibrated at 32 °C for 0.5 h. The gels were placed in an NMR tube with a resonant frequency of 21 MHz, and the relaxation measurements were then determined by an LF-NMR analyzer (NMI 20-040H-I, Newsmy, Suzhou, China). The operating parameters were set as follows: TR = 6 500 ms, SW = 200 kHz, NS = 8, and NECH = 9 500. The transverse relaxation time (T_2) involved in the exponential fitting of CPMG decay curves was implemented by using Multi-Exp Inv Analysis software (Niumag Electric Corp, Suzhou, China).

2.8 Microstructure

The gel slices (3 mm × 3 mm × 3 mm) were prepared according to the reference[2], and then observed at 250 × magnification through a scanning electron microscope (EM-30 plus, COXEM, Korea) at a voltage of 15 kV.

2.9 Simulated oral chewing

Gel samples were used to simulate oral chewing by a texture analyzer equipped with a MEC device (shown in Fig. S2). The MEC probe was kept at 36 °C using circulating water, and both the test and post-test speeds were 5 mm/s. The extrusion distance and number of chewing were set as 95 mm and 30 times, respectively[23]. The curve fitting of data was performed according to the procedure instruction of MEC Single Decay and calculated as follows:

$$W(n) = W_{\text{inf}} + w_1 \times \exp(-n/n_1) \quad (4)$$

Where W_{inf} represents the remained work after repeated stretches (used for measurement of sample viscosity), n reflects the cycle number, w_1 and n_1 reflect the contribution of energy loss per extrusion cycle and the speed of structural damage, respectively.

2.10 In vitro gastric digestion

Simulated gastric digestion was conducted following the INFOGEST method[24]. The MP gel after oral chewing was mixed with an equivalent weight of simulated gastric fluid (SGF), and pH was adjusted to 3.0 followed by addition of pepsin (2 000 U/mL in final gastric solution). The mixtures were then incubated with continuous vibration at 37 °C for 2 h using a water bath oscillator (SHZ-B, Boxun, Shanghai, China), and the digestion process was finally terminated by adjusting the pH to 7.0 with 1 mol/L NaOH.

2.11 Degree of hydrolysis (DH)

Simply, 400 μL of diluted digestion supernatant was mixed with 3 mL of OPA reagents, blended for 5 s, and incubated for exactly 2 min before being recorded at 340 nm (OD_{sample}) by a UV-1800 spectrophotometer. The blank and standard groups were measured by substituting the digestive solution with deionized water and serine standard solution, respectively. DH is calculated with the following equations:

$$\text{Serine } NH_2 = \frac{OD_{\text{sample}} - OD_{\text{blank}}}{OD_{\text{standard}} - OD_{\text{blank}}} \times 0.951\,6 \times \frac{1}{C_{\text{sample}}} \quad (5)$$

$$h = (\text{Serine } HN_2 - \beta)/\alpha \quad (6)$$

$$\text{DH (\%)} = \frac{h}{h_{\text{tot}}} \times 100 \quad (7)$$

Where β equals 0.4 mequv/g, α equals 1.0 mequv/g, h_{tot} equals 7.6 mequv/g for myofibrillar protein, and C_{sample} is the concentration of diluted solution.

2.12 Molecular weight (MW) distribution

The MW distribution of MP gel digests was determined by the method of size exclusion chromatography. In this work, the used Agilent HPLC system, gel chromatographic column, and mobile phase (including flow rate) were in accordance with the reference[19]. Aliquots of 15 μL samples were injected, and the eluted peptides were detected at 220 nm. The MW calibration curve was obtained from the following standards: BSA (68 000 Da), ovalbumin (44 500 Da), cytochrome c (12 384 Da), aprotinin (6 511 Da), and alcohol dehydrogenase (141 000 Da).

2.13 Fluorescence recovery after photobleaching (FRAP)

Pepsin was labeled with FITC (i.e., FITC-pepsin) according to the manufacturer's instructions, and MP gels were cut perpendicularly into cylindrical thin slices (d = 10 mm and h = 2 mm). A 5 μL amount of FITC-pepsin solution was pipetted onto the surface of the sliced MP gel, and the sample was then kept at 4 °C for approximately 24 h to allow adequate migration of fluorescent molecules toward the whole gel. An inverted CSLM (TiE-A1, Nikon, Japan) was used to determine the diffusion coefficients (D) of the FITC-pepsin within the water and oxidized MP gel. The FRAP protocol is simply schemed in Fig. S3. In this study, the ultrapure water (18.2 MΩ·cm) was mixed with FITC-pepsin (2:1, *V/V*) for the blank group. All the samples were set in a confocal dish (d = 20 mm, NEST, China), and observed using a 40 × objective lens (oil immersion) with a numerical aperture of 1.25 at 25 °C. A 488 nm argon laser was set to 0.4% of the maximum laser intensity to collect 6 pre-bleach images (fixed to 128 × 128 pixels, 1.52 s) as well as 100% for bleaching (2 bleaching images, 1.94 s) at a small circular region of interest (ROI) with a radius of 40 μm followed by fluorescence recovery. A series of 600 post-bleach images was acquired until full recovery was reached (2.32 min). Fifteen repetitions of the FRAP experiments were done for each sample.

Data were analyzed according to the theory using the following relation[25]:

$$D = \frac{r_e^2}{4\tau_D} \quad (8)$$

Where τ_D is the characteristic time of diffusion, obtained by analyzing the recorded images using the LAS-AF-Lite version 2.6.0 software, and r_e is the effective radius of the bleach spot determined by fitting the fluorescence intensity profiles of the first acquired post-bleach image to the Gaussian amplitude function.

2.14 Statistical analysis

All the experiments were conducted at least triplicate determinations, and values were expressed as mean ± standard deviation (SD). The obtained data were analyzed using one-way analysis of variance (ANOVA) (SPSS 22.0, Chicago, USA) to evaluate the effects of sodium-substituted chloride salts on the physicochemical changes and gastric digestive properties of MP under the HRGS-induced oxidative stress. The significant difference was determined at the $P < 0.05$ level for Duncan's multiple comparison.

3. Results and discussion

3.1 Physicochemical changes of MP in oxidized system

The changes of carbonyl groups and sulfhydryl groups are shown in Table 2. Compared with the Control group, the carbonyl contents of all oxidized MP without H_2O_2 were increased. This result might be correlated with the oxidation of residual fat in MP induced by the presence of pro-oxidant ($FeCl_3$ and VC)[26]. Under the incubation of 0 mmol/L H_2O_2, different salt ions facilitated the lipid oxidation in different degrees, and the carbonyl content in $MgCl_2$-substituted MP significantly increased ($P < 0.05$). Dichloride salts ($CaCl_2$ and $MgCl_2$) have been found to exhibit higher pro-oxidation effect than monovalent chloride salts (NaCl and KCl) in meat and seafood products[27-28]. Meanwhile, the oxidized MP containing KCl displayed relatively lower carbonyl content among all the oxidized groups. This phenomenon might be attributed that low dose of KCl reduced the pro-oxidant effect of NaCl[29]. Moreover, the carbonyl content in all samples had an evident increase as the H_2O_2 concentration increased from 0 to 1 mmol/L, which was consistent with the reference[30]. This result demonstrated that the Fenton system (Fe^{3+}, VC, and H_2O_2) had a distinct effect on the formation of carbonyls in the MP. Similarly, the species of chloride salts had great influence on the carbonyl content of oxidized MP, and the highest carbonyl content was obtained in the MP subjected to $MgCl_2$-substituted treatment.

Sulfhydryl group, as a vital parameter characterizing protein oxidation, is easily oxidized in the presence of H_2O_2, and prone to being attacked by ·OH to form disulfide bonds[31-32]. As depicted in Table 2, the MP suspensions undergoing oxidation manifested higher sulfhydryl contents and decreasing disulfide bonds than those of the non-oxidized MP. Both these two indexes were remarkable at the oxidation level of 1 mmol/L H_2O_2 for the groups treated with the same salt ions. The result indicated that partial sulfhydryl groups in MP were converted to disulfide bonds after oxidation, which was similar to the study reported by Shen et al.[21]. The decreased sulfhydryl content may be correlated with the denaturation of cysteine existing in the catalytic region of myosin, which is more susceptible to oxidative stress[33]. This change might induce the formation of inter- or intra-molecular disulfide bonds, and subsequently influence the network and the structural properties of MP gel. In the current study, the sulfhydryl contents in $MgCl_2$-substituted MP suspensions (30%-Mg and 30%-K + Mg) were always lower than those in MP suspensions only treated by 30%-K under the same oxidation level. Partial NaCl substitution with dichloride salts ($MgCl_2$ or $CaCl_2$) could promote sulfhydryl oxidation in the oxidized MP *via* protein denaturation or unfolding, which increased the accessibility of amino acids comprising hydrocarbons side chain groups in the hydrophobic core[14]. The above results indicated that the oxidized MP with compound salts (especially NaCl and $MgCl_2$) can better facilitate protein oxidation compared to the single NaCl marination at the same ionic strength.

Surface hydrophobicity can effectively reflect the conformation and structure of myofibrillar protein, and is usually characterized by the binding capacity between BPB molecules and MP[34]. In comparison to non-oxidized MP, the surface hydrophobicity in all oxidized MP significantly increased ($P < 0.05$). For the MP samples oxidized with 0 mmol/L H_2O_2, the residual fat oxidation

Table 2
Physicochemical changes of MP affected by chloride salts under different oxidation levels.

H_2O_2 concentration (mmol/L)	Samples	Carbonyl (nmol/mg prot)	SH (nmol/mg prot)	S-S (nmol/mg prot)	Surface hydrophobicity (μg bound BPB)
0	Control	1.60 ± 0.18^{a}	80.20 ± 3.02^{c}	12.20 ± 1.25^{a}	84.87 ± 5.37^{a}
	Na	2.18 ± 0.32^{ab}	78.75 ± 3.45^{bc}	23.39 ± 0.47^{bcde}	98.51 ± 3.52^{b}
	30%-K	2.17 ± 0.11^{ab}	78.25 ± 1.79^{bc}	21.55 ± 1.15^{bc}	98.63 ± 4.25^{b}
	30%-Mg	2.70 ± 0.36^{bcd}	74.43 ± 3.93^{abc}	27.54 ± 0.15^{f}gh	119.72 ± 1.02^{e}
	30%-K + Mg	2.30 ± 0.03^{b}	75.47 ± 4.49^{abc}	26.11 ± 0.47^{def}g	106.27 ± 8.15^{bc}
0.3	Na	2.38 ± 0.33^{bc}	79.82 ± 4.58^{c}	22.65 ± 0.63^{bcd}	103.45 ± 3.52^{bc}
	30%-K	2.27 ± 0.11^{b}	80.65 ± 4.30^{c}	20.56 ± 0.09^{b}	101.47 ± 4.02^{b}
	30%-Mg	2.97 ± 0.03^{cde}	74.91 ± 3.53^{abc}	26.65 ± 0.23^{efgh}	122.20 ± 4.59^{e}
	30%-K + Mg	2.61 ± 0.20^{bcd}	76.43 ± 4.42^{abc}	23.50 ± 0.81^{bcde}	114.21 ± 6.98^{cde}
1	Na	3.15 ± 0.10^{def}	76.30 ± 1.41^{abc}	29.25 ± 3.66^{gh}	99.44 ± 5.01^{b}
	30%-K	2.99 ± 0.39^{cde}	77.42 ± 1.39^{abc}	24.37 ± 2.58^{cdef}	97.86 ± 2.58^{b}
	30%-Mg	3.71 ± 0.32^{f}	71.18 ± 5.40^{a}	35.29 ± 1.23i	117.97 ± 5.50^{de}
	30%-K + Mg	3.36 ± 0.28^{ef}	72.18 ± 3.85^{ab}	30.19 ± 1.29^{hi}	107.90 ± 3.97^{bcd}

Note: All data are presented as mean ± standard deviation (SD). Different lower letters in the columns indicate significant differences ($P < 0.05$), the same below.

products induced by Fe^{3+} and VC addition could intervene the micro-environment of MP trytophan. In this study, oxidation treatment contributed to the exposure of hydrophobic amino acids initially blocked in the protein structure onto the polar surface, and thus enhanced their binding capacity with the hydrophobic molecules[35]. Regardless of salt species, the surface hydrophobicity of MP within oxidation conditions ranging from 0 to 1 mmol/L H_2O_2 first increased and then decreased. In other words, the slight oxidation induced the protein refolding, accompanied by the reduction of exposed hydrophobic amino acid groups[12]. Similar to the above-mentioned indexes, the addition of dichloride salt significantly increased the surface hydrophobicity of oxidized MP ($P < 0.05$) at the same oxidative concentration. Protein oxidation could promote intermolecular aggregation behaviors between protein molecules, and the exposure of hydrophobic patches was closely related with it[36]. Mg^{2+} ions that were destabilized ions in Hofmeister series would enhance the denaturation and unfolding of the protein in the oxidative environment[37]. Dichloride salts have been reported that they could facilitate the protein unfolding and the formation of "salt bridge" between Mg^{2+} and the -COOH terminal of protein side chain, making the hydrophobic groups exposed onto the protein surface[38].

3.2 Gel properties of oxidized MP

3.2.1 Gel strength and WHC

Gel-forming ability is an important functional characteristic of muscle protein during heating process, and gel strength is usually used to evaluate the gel properties[39]. Fig. 1A shows the gel strength of MP after partial substitution of NaCl with other chloride salts under different oxidation levels. In this study, the gel strength of oxidized MP with 0.6 mol/L NaCl in the range of 0–1 mmol/L H_2O_2 was always higher than that of the non-oxidized MP. Mild oxidation could decrease the loss of functional and structural characteristics of MP by improving the cross-linking and inter-molecular interactions, such as hydrophobic and ionic interactions[39]. Mild oxidation could induce partial unfolding of MP structure, and increase the MP solubility for enhanced gel strength[21]. In current study, the oxidized MP decreased in gel strength with the increasing oxidation degree, and the contribution of chloride salts was KCl > KCl/$MgCl_2$ > $MgCl_2$. Similarly, 25% substitution of NaCl with KCl, rather than $MgCl_2$ and $CaCl_2$, obviously improved the gel strength of the oxidized MP incubated by 1 mmol/L H_2O_2[13]. Both the NaCl and KCl were univalent salt, and they had similar physicochemical properties. The presence of K^+ ion changed the accessibility of amino acids comprising hydrocarbons side chain group in the hydrophobic core in protein, and prevented the excessive aggregation compared with Mg^{2+} ion[13]. Therefore, KCl could promote the MP unfolding and the formation of three-dimensional gel network for better gel quality[40-41].

WHC is commonly used to evaluate the quality of meat products, and it can greatly affect the sensory characteristics and economic value[2]. As shown in Fig. 1B, the WHC of oxidized MP gel with 0.6 mol/L NaCl increased only at the H_2O_2 concentration of 0–0.3 mmol/L, in comparison to that of the non-oxidized MP gel ($P < 0.05$). This result was attributed that mild oxidation could generate smaller and uniform-distributed pore size of MP gel[34]. Compared with 0 mmol/L H_2O_2 group, the WHC of MP gel decreased continuously regardless of the species of salt ions with the increase of oxidation degree. In current study, although the MP incubated with 1 mmol/L H_2O_2 exhibited no decrease in the gel strength compared with the non-oxidized MP, it had a significant negative effect on WHC ($P < 0.05$). Normally, serious oxidation will disrupt the protein structure, and induce the formation of voids in MP gel, causing the decrease of capillary action and protein hydration accompanied by lower WHC[42]. In this work, the 30%-KCl-substituted gel had the highest WHC among the sodium-reduced groups under the same oxidation level, which might be correlated with the enhanced gel strength for better retaining water. Mg^{2+} had higher charge intensity compared to that of K^+, which could tightly combine with protein groups and strengthen the interaction between protein molecules[43]. The presence of Cl^- can enhance the electrostatic repulsion between MP myofibrils, resulting in the swelling of the protein lattice[44]. Therefore, the presence of Mg^{2+} would prevent partial Cl^- from penetrating into the protein molecules, accompanied by negative effects on gel WHC[40,45].

3.2.2 Water distribution and mobility

The information concerning the water distribution and mobility in meat products is usually provided by LF-NMR, particularly the proton transverse relaxation time (T_2)[2]. The T_2 relaxation curve and corresponding peak proportion of MP gels treated with different chloride salts are shown in Fig. 1C and Table 3, respectively. Under the oxidation conditions of 0.3 and 1 mmol/L H_2O_2, two peaks appeared in the range of 0–10 ms (T_{2b}) in the samples containing $MgCl_2$. The existence of $MgCl_2$, to great extent, might facilitate the oxidation of protein accompanied by MP degradation and the covalent cross-linking between amino acid side chains[21]. This structural change caused the stronger mobility of the partial bound water in the protein molecular structure, and thus induced the appearance of second peak. As observed in Fig. 1C, the T_2 relaxation time (T_{21}, T_{22}, and T_{23}) of oxidized protein gel was higher than that of non-oxidized protein (Control group), especially the immobilized water (T_{21}). Generally, the peak point migrating to higher relaxation time is correlated

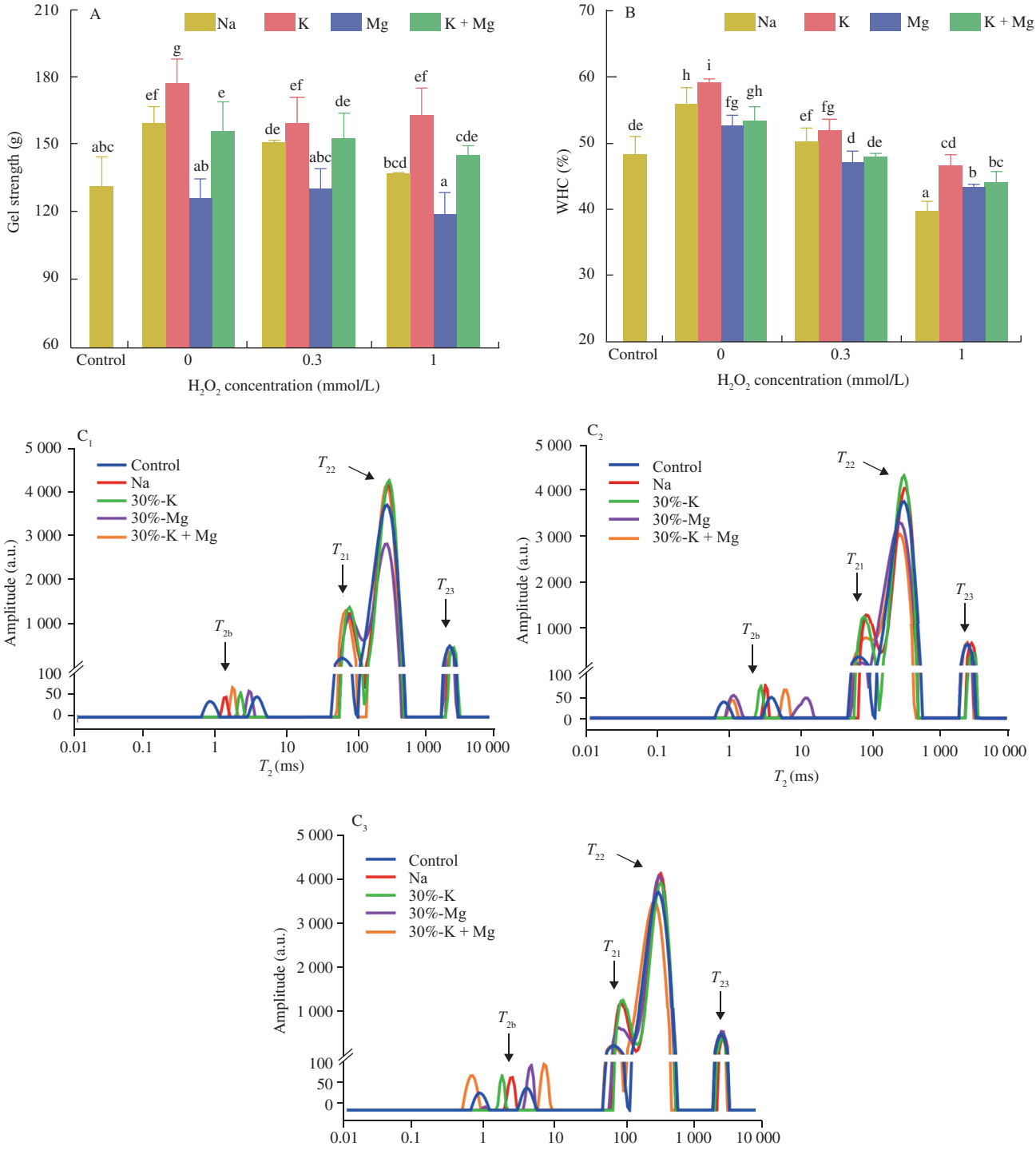

Fig. 1 The (A) gel strength, (B) WHC, and (C) T_2 relaxation times of sodium-reduced MP gel treated by chloride salts under different oxidation. C_1, C_2 and C_3 represent H_2O_2 concentrations of 0, 0.3, and 1.0 mmol/L, respectively. Different lower letters indicate significant differences ($P < 0.05$), the same as below.

Table 3
Water distribution of MP gels based on LF-NMR spectroscopy.

H_2O_2 concentration (mmol/L)	Samples	P_{2b} (%)	P_{21} (%)	P_{22} (%)	P_{23} (%)
0	Control	0.987 ± 0.277^{cd}	3.738 ± 2.456^{a}	90.299 ± 2.345^{c}	5.066 ± 0.735^{c}
	Na	0.387 ± 0.189^{ab}	12.739 ± 2.432^{cd}	83.808 ± 2.347^{b}	3.067 ± 0.528^{a}
	30%-K	0.283 ± 0.124^{ab}	16.838 ± 2.180^{e}	79.252 ± 2.246^{a}	3.627 ± 0.460^{abc}
	30%-Mg	0.251 ± 0.158^{a}	18.159 ± 4.722^{e}	77.007 ± 4.680^{a}	4.583 ± 0.957^{cde}
	30%-K + Mg	0.292 ± 0.151^{ab}	18.281 ± 2.390^{e}	77.207 ± 3.046^{a}	4.219 ± 1.178^{bcd}
0.3	Na	0.247 ± 0.161^{a}	17.412 ± 2.812^{e}	79.108 ± 2.837^{a}	3.234 ± 0.583^{ab}
	30%-K	0.310 ± 0.127^{ab}	16.401 ± 3.159^{e}	80.027 ± 3.478^{a}	3.261 ± 0.981^{ab}
	30%-Mg	1.282 ± 0.292^{e}	3.238 ± 0.958^{a}	89.869 ± 2.543^{c}	5.611 ± 1.891^{f}
	30%-K + Mg	0.857 ± 0.153^{c}	8.049 ± 3.508^{b}	85.655 ± 3.347^{b}	5.439 ± 1.139^{ef}
1	Na	0.500 ± 0.112^{b}	16.122 ± 2.290^{e}	80.099 ± 2.516^{a}	3.279 ± 0.463^{ab}
	30%-K	0.501 ± 0.131^{b}	14.959 ± 2.275^{de}	80.372 ± 1.934^{a}	4.168 ± 0.617^{bcd}
	30%-Mg	0.766 ± 0.332^{c}	10.360 ± 6.014^{bc}	84.758 ± 5.908^{b}	5.597 ± 1.038^{f}
	30%-K + Mg	1.192 ± 0.332^{de}	4.469 ± 0.535^{a}	89.955 ± 0.809^{c}	4.383 ± 0.803^{cd}

with the stronger water mobility[2]. In our study, oxidation treatment made some hydrophilic groups on the amino acid side chain of MP to be attacked, and intervened the network structure of MP gel[21]. Therefore, the oxidized MP gel manifested the decreased water-binding capacity, accompanied by enhanced water mobility.

Table 3 shows the corresponding proportion of T_2 peak area for the MP gel. Under the incubation condition of 0 mmol/L H_2O_2, all the samples treated with sodium replacement significantly decreased in P_{2b} value, manifesting lower connection between water molecules and protein macro-molecules[2]. As the addition of H_2O_2, the existence of $MgCl_2$ increased the P_{2b} value, especially in the 0.3 mmol/L H_2O_2 group, which might be related to the relatively high surface hydrophobicity of the oxidized MP. Protein could form more aggregates through hydrophobic interaction, and expose more hydrogen bonds in the gel network during the gelation process[19], exhibiting stronger combination of water molecules and protein macro-molecules. The free water is the most easily lost water in protein gel structure. At the oxidation levels with 0.3 mmol/L and 1 mmol/L H_2O_2, the relatively low proportion of free water (P_{23}) of MP gel in NaCl and 30%-KCl-substituted groups indicated that more water was restricted to gel network. With the increase of oxidation concentration, as a whole, the proportion of free water (P_{23}) in all groups exhibited an increasing trend. Therefore, the MP gel with higher oxidation degree and partial NaCl substitution with $MgCl_2$, manifested the relatively higher proportion of free water accompanied by lower WHC.

3.2.3 Microstructure

As presented in Fig. 2, the MP gel network under the incubation of 0 mmol/L H_2O_2 was relatively dense compared with that of the Control group. Especially, the 30%-KCl-substituted MP gel network was compact and orderly (red dotted box), which was attributed to the higher gel strength and WHC. The reference also reported that partial replacement (25%) of NaCl with monovalent chloride salt (KCl), rather than dichloride salt, was conducive to forming a more compact and uniform gel structure under the slight oxidation[13]. Taken together, the gel network structure tended to be rough and loose as the oxidation levels increased. Under the condition of 1 mmol/L H_2O_2, the uniformity of pore size in the gel network structure (yellow arrow) was obviously worse, and the uneven network reduced the water trapped in MP gel. To some extent, this result could explain the phenomenon that the gel strength of 30%-KCl-substituted MP was increased, but accompanied by decreased gel WHC under the oxidation degree of 1 mmol/L H_2O_2.

3.3 Chewing properties

The stimulated oral chewing of MP gel was evaluated by texture analyzer with MEC probe, and the work fitting curve and detailed fitting parameters of chewing process are shown in Table 4 and Fig. 3, respectively. It can be observed that the chewing work of 30%-KCl-substituted gel was relatively higher among the sodium-substituted groups at the first chewing cycle (especially at 0.3 and 1.0 mmol/L H_2O_2 of oxidation levels), which was consistent with the higher gel strength. The texture perception of first bite for semi-solid food gel depends on the fracture characteristics[46]. As exhibited in Table 4, the W_{inf} of almost all oxidized MP gel decreased, indicating that oxidation might decrease the viscosity of gel samples. Meanwhile, the increased w_1 values of oxidized MP gels were also correlated with higher destruction rate and better chewing properties[23]. The value of n_1 is negatively related to the structural fracture rate. At the oxidation levels of 0.3 and 1 mmol/L H_2O_2, the n_1 values (12.151 ± 1.740, and 10.997 ± 1.994, respectively) in oxidized gels treated with 30%-$MgCl_2$ were increased, which might be correlated with the soft network structure for slower structural fracture rate.

3.4 Gastric digestion

3.4.1 DH

The DH of MP gel marinated with chloride salts under different oxidative levels after simulated oral chewing and gastric digestion *in vitro* is shown in Fig. 4A. Compared with the non-oxidized MP gel (16.20%), the DH of oxidized MP gels without H_2O_2 in 0.6 mol/L NaCl and 30%-KCl-substituted group (15.49%) obviously decreased. On contrary, the increased DH of MP gel was found in 30%-$MgCl_2$-substituted and 30%-K + Mg-substituted

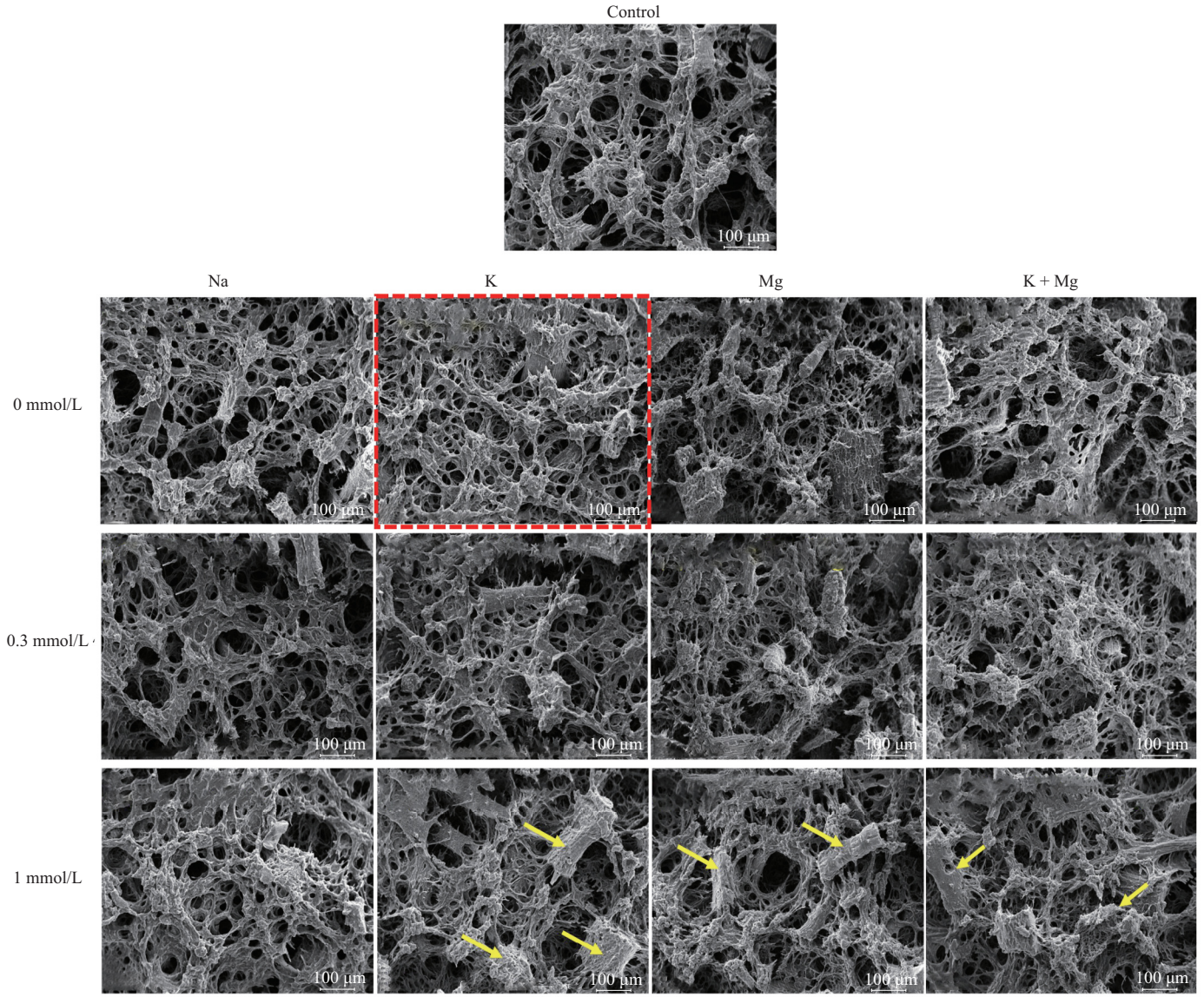

Fig. 2 Scanning electron microscope micrographs of MP gels with different oxidation levels. Scale bar = 100 μm.

Table 4
Parameters of the MP gel based on simulated chewing work fitting curve equation.

H_2O_2 concentration (mmol/L)	Samples	W_{inf}	w_1	n_1
0	Control	0.513 ± 0.004^{e}	0.094 ± 0.021^{a}	6.820 ± 2.370^{a}
	Na	0.501 ± 0.005^{e}	0.156 ± 0.030^{b}	6.221 ± 1.775^{a}
	30%-K	0.456 ± 0.005^{b}	0.142 ± 0.014^{b}	10.337 ± 1.923^{cd}
	30%-Mg	0.323 ± 0.003^{a}	0.210 ± 0.015^{c}	6.551 ± 0.713^{a}
	30%-K + Mg	0.346 ± 0.004^{a}	0.202 ± 0.016^{c}	7.645 ± 0.966^{b}
0.3	Na	0.349 ± 0.003^{a}	0.149 ± 0.015^{b}	6.634 ± 1.033^{a}
	30%-K	0.485 ± 0.004^{bc}	0.165 ± 0.013^{b}	9.791 ± 1.388^{c}
	30%-Mg	0.348 ± 0.004^{a}	0.159 ± 0.011^{b}	12.151 ± 1.740^{d}
	30%-K + Mg	0.337 ± 0.004^{a}	0.195 ± 0.015^{c}	8.384 ± 1.057^{bc}
1	Na	0.352 ± 0.003^{a}	0.149 ± 0.012^{b}	9.244 ± 1.273^{c}
	30%-K	0.522 ± 0.005^{c}	0.168 ± 0.017^{bc}	9.111 ± 1.616^{c}
	30%-Mg	0.451 ± 0.005^{b}	0.150 ± 0.014^{b}	10.997 ± 1.994^{cd}
	30%-K + Mg	0.343 ± 0.003^{a}	0.154 ± 0.011^{b}	9.369 ± 1.167^{c}

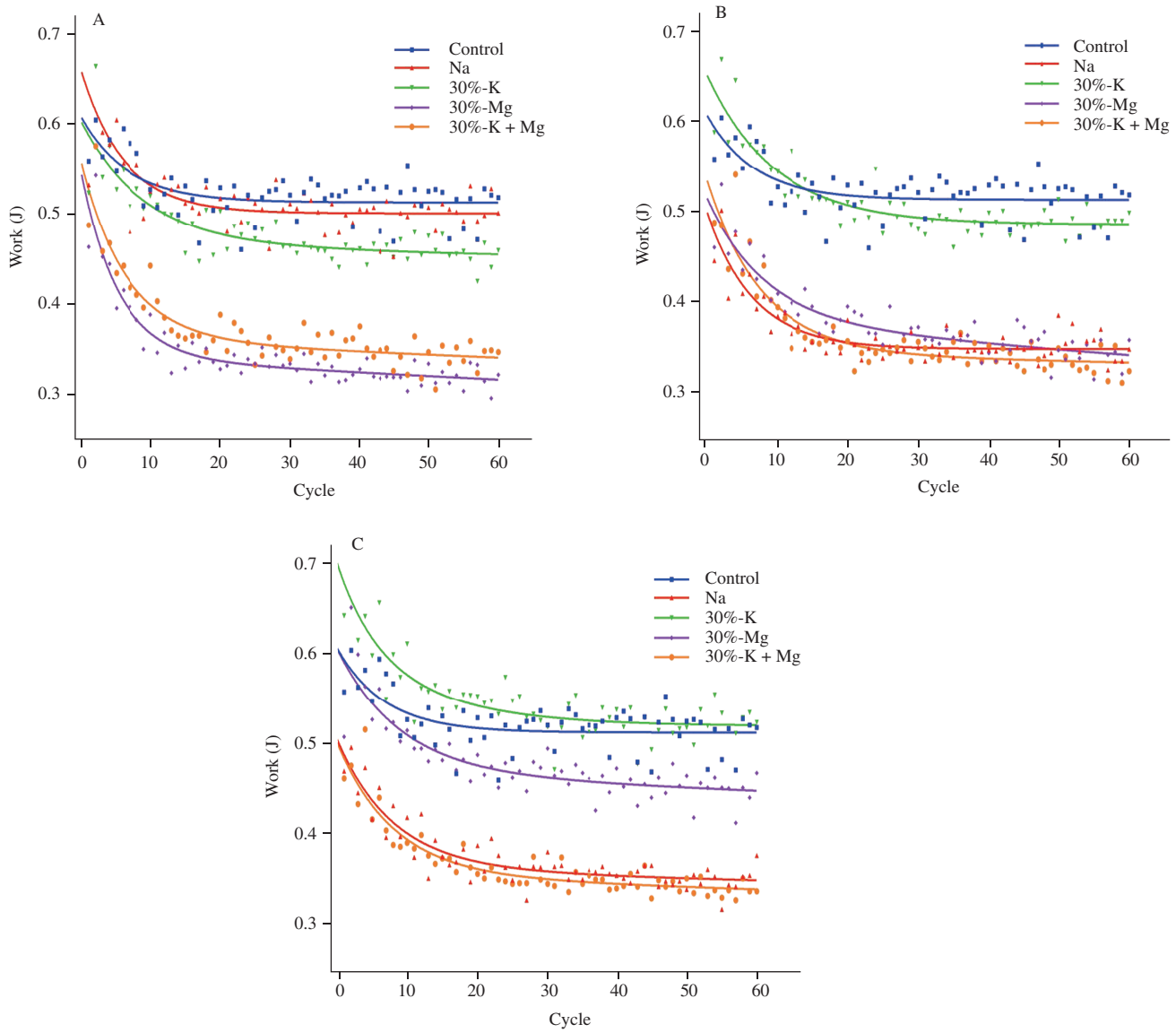

Fig. 3 Work fitting diagram of different MP gel samples under the simulated oral chewing. (A) H_2O_2 concentration 0 mmol/L; (B) H_2O_2 concentration 0.3 mmol/L; (C) H_2O_2 concentration 1 mmol/L.

groups. The reason for this phenomenon might be attributed to the relatively dense network structure of MP gel in 0 mmol/L-K and 0 mmol/L-Na groups, which hindered the diffusion of gastric juice. For the MP gels in 0 mmol/L-Mg and 0 mmol/L-K + Mg groups, the looser network and the larger pore size promoted the contact between gastric juice (especially the pepsin) and recognition sites in gel, manifesting higher DH. In other words, the microscopic network structure of gel had a vital influence on the gastric digestion. With the increase of oxidative levels, the DH of all sodium-reduced groups reached the highest value under the oxidation condition of 0.3 mmol/L H_2O_2. Mild oxidation of MP enhanced the sensitivity to proteolysis, but serious protein oxidation accompanied by the formation of aggregates and the degradation of specific amino acid side chains would change the recognition sites with weak proteolysis sensitivity[31]. In addition, the higher surface hydrophobicity of all oxidized MP marinated in 0.3 mmol/L H_2O_2, to some degree, contributed to the DH. The reference reported that protein molecule with more hydrophobic amino acids is the preferred substrate of proteasome[47], thus the partial unfolding of protein under mild oxidative stress is crucial for increasing the proteolysis.

3.4.2 *MW distribution*

Fig. 4B shows the MW distribution of digestive juice from MP gel treated with different chloride salts and oxidation levels. The proportion of MW peptides in the non-oxidized MP was 16.08% (> 50 kDa), 3.04% (30–50 kDa), 11.62% (10–30 kDa), 9.53% (7–10 kDa), 18.21% (5–7 kDa), 17.35% (3–5 kDa) and 24.16% (< 3 kDa), respectively. Under the condition of 0 mmol/L H_2O_2, the proportion of peptides with large molecular weight (> 50 kDa) in 0 mmol/L-Na and 0 mmol/L-K groups increased to 19.61% and

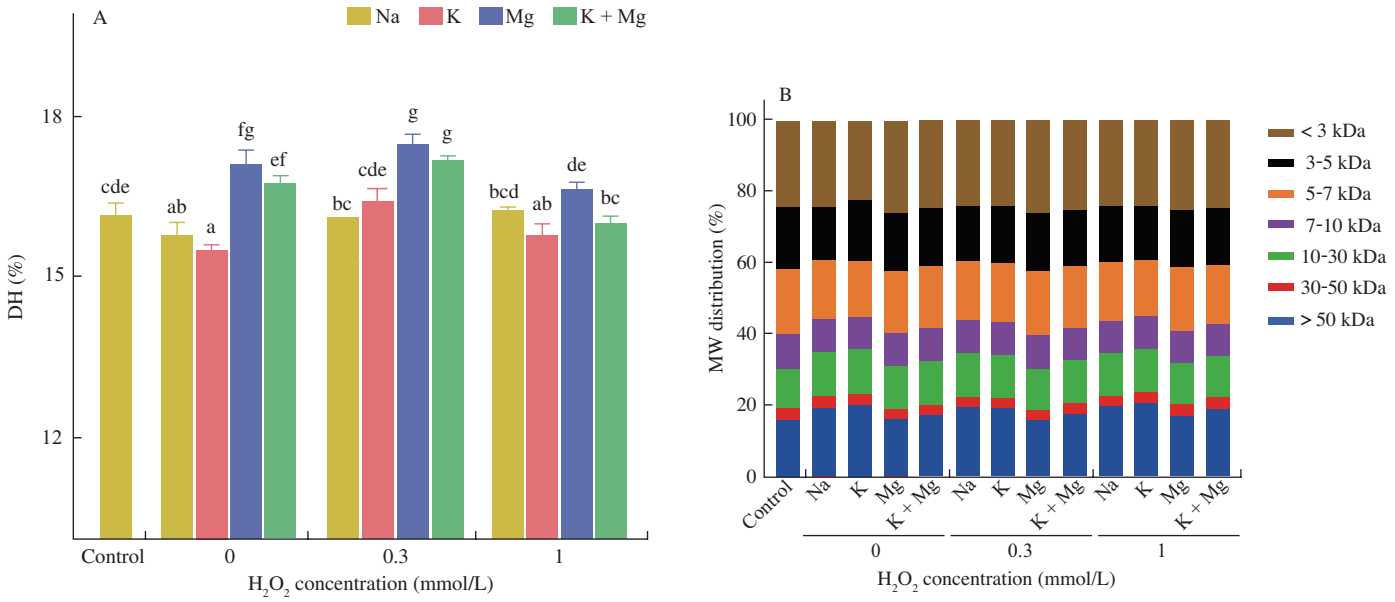

Fig. 4 The (A) DH and (B) MW distribution of MP gels after 2 h gastric digestion *in vitro*.

20.51%, respectively. Especially, the proportion of peptides with smaller MW (< 3 kDa) in 0 mmol/L-K group decreased to 22.29%. In other words, these 2 treatments, to some degree, were adverse to the gastric digestion of MP. Meanwhile, this phenomenon (higher proportion of large MW peptides and lower proportion of low MW peptides) still existed with the increase of oxidation levels. However, the samples added with $MgCl_2$ displayed the opposite phenomenon, especially in the 0.3 mmol/L-Mg group accompanied by higher peptides (15.83%) and 25.83% of smaller peptides (< 5 kDa). As far as the specific proportion value was concerned, the oxidation level of 0.3 mmol/L H_2O_2 was conducive to enhancing the gastric digestion degree of oxidized MP gel.

3.4.3 *Pepsin diffusion*

Based on the above-mentioned analysis of DH and MW distribution of oxidized MP gel after gastric digestion, we could speculate that the sodium-reduced MP gels with KCl and $MgCl_2$ were different in digestive characteristics under the same oxidation conditions. Therefore, pepsin diffusion (principle in Fig. S3) was conducted to better comprehend the difference in gastric digestive characteristics between KCl and $MgCl_2$-substituted MP gels. Fig. 5 shows the representative series of images of FITC-pepsin diffusivity in water and MP gel (Fig. 5A), the effective bleach radius (r_e) of ROI (Fig. 5B) and recovery curve of fluorescence intensity in ROI (Fig. 5C) after image and data processing. Fig. 5D further shows the effective diffusivity of FITC-pepsin in H_2O and MP gels determined by FRAP. It can be observed that the diffusion rate of FITC-pepsin in MP gels was significantly lower than that in water (99.85 $\mu m^2/s$), which was correlated with the restricted pepsin diffusion in the gel network structure. In addition, similar tendency between the diffusion rate of FITC-pepsin in the gel and the DH of oxidized gel after *in vitro* digestion was found, and the MP gels with mild oxidation (0.3 mmol/L H_2O_2) were beneficial to the diffusion of pepsin. This result was attributed that the enlarged pore size of gel network increased the aggregation distance between protein particles, and thus weakened the steric effects of gel network structure for FITC-pepsin diffusion[48]. At the same oxidation level, the diffusion rate of FITC-pepsin in $MgCl_2$-substituted groups was significantly higher than that in KCl-substituted group ($P < 0.05$). On the one hand, slight oxidation facilitated the unfolding of protein structure, and the existence of $MgCl_2$ further made more binding sites of pepsin in protein being exposed. On the other hand, $MgCl_2$-substituted MP gel had a larger pore size (Fig. 2), and the smaller steric hindrance was beneficial to pepsin diffusion. Moreover, the gel strength in $MgCl_2$-substituted MP was relatively soft, which was further conducive to the faster digestion and disintegration *in vitro*[49].

3.5 *Correlation analysis*

The correlation analysis between the oxidation characteristics, gel traits, and oral-gastric digestion characteristics of MP was evaluated (Fig. 6). A significant positive correlation (correlation coefficient of 0.89) between the diffusivity of pepsin and the DH ($P < 0.01$), which indicated that the diffusion of pepsin played an important role in regulating the gastric digestion of MP gel. The gastric digestion of protein is affected by many factors. The reference reported that compared with natural protein gel, oxidation will affect the digestion of SPI gel through the combined effects of pepsin diffusion, gel network structure, and hydrolysis degree[19]. In this study, it was found that the DH was positively correlated with the surface hydrophobicity (H_0) of MP ($P < 0.05$), and negatively correlated with gel strength and chewing work (W_{inf}). Based on the above discussion and data analysis, mild oxidation and the partial replacement of NaCl with $MgCl_2$ promoted the extension of protein structure, exposed more cleavage sites (hydrophobic amino acids) with pepsin, and

improved the surface hydrophobicity of protein[50]. Moreover, the network structure of heat-set gel in this condition was relatively loose accompanied by lower gel strength, which was conducive to the disintegration of MP gel structure. In the simulated chewing process, the relatively lower viscosity of MP gel decreased the contact limit between pepsin and protein and thus promoted the progress of gastric digestion of gel with higher DH. The high-viscosity food can hinder the diffusion of pepsin[51], which also support the speculation in this study to some extent. To sum up, the oral processing and digestive characteristics of oxidized MP gel were regulated by the gel traits (three-dimensional network, texture, and viscosity).

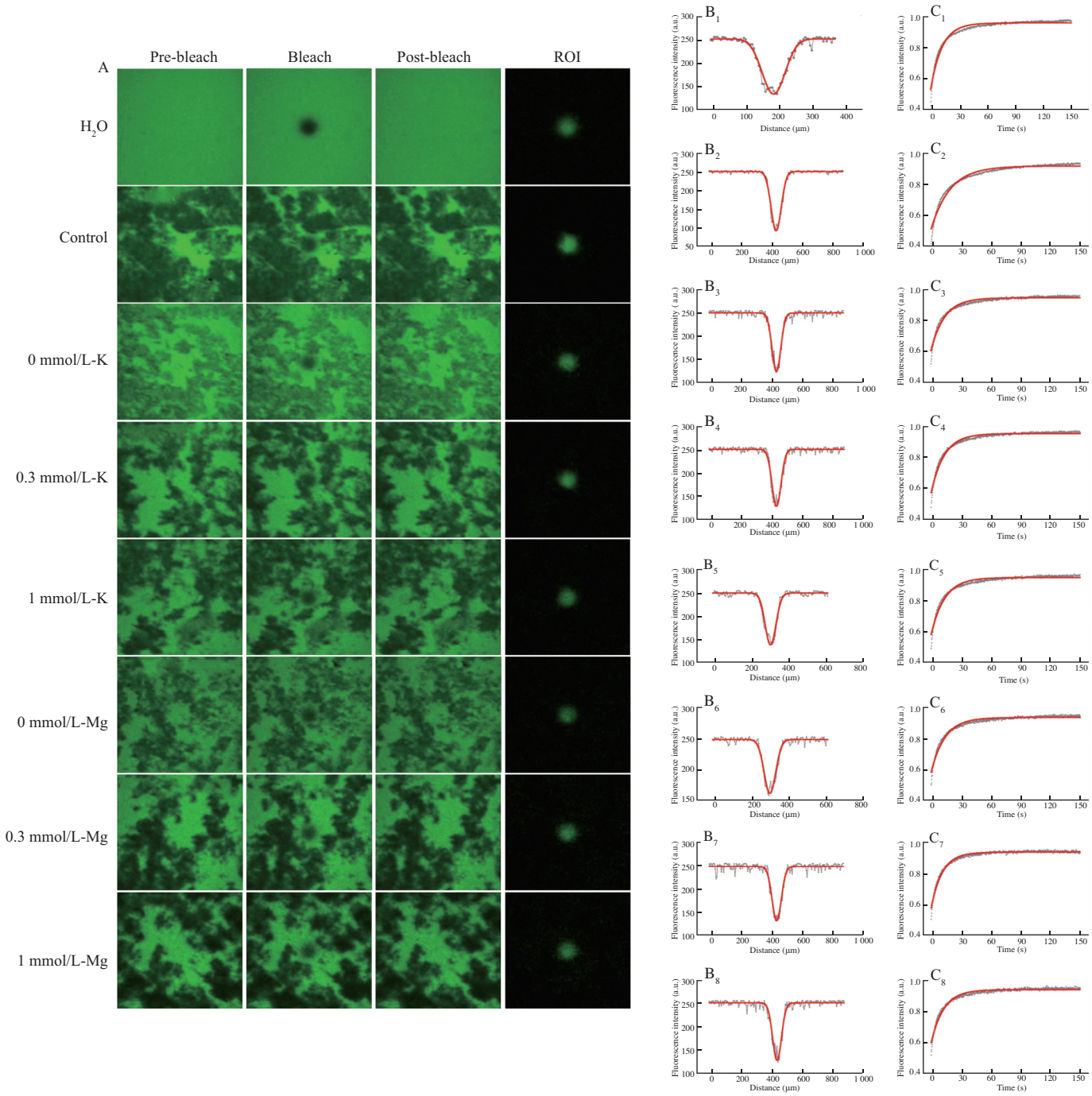

Fig. 5 Time series of confocal images of water and MP gel during the diffusion of (A) FITC-pepsin, (B) the effective bleach radius (r_e) of ROI, (C) the recovery curve of fluorescence intensity with rime in ROI , and (D) the effective diffusivity of FITC-pepsin in H_2O and MP gels determined by FRAP. The horizontal line in the box chart represents the average value, and the upper or lower error lines represent the largest or smallest data points in the upper or lower 1.5 quartile range. Subscripts 1-8 represent H_2O, Control, 0 mmol/L-K, 0.3 mmol/L-K, 1 mmol/L-K, 0 mmol/L-Mg, 0.3 mmol/L-Mg, 1 mmol/L-Mg.

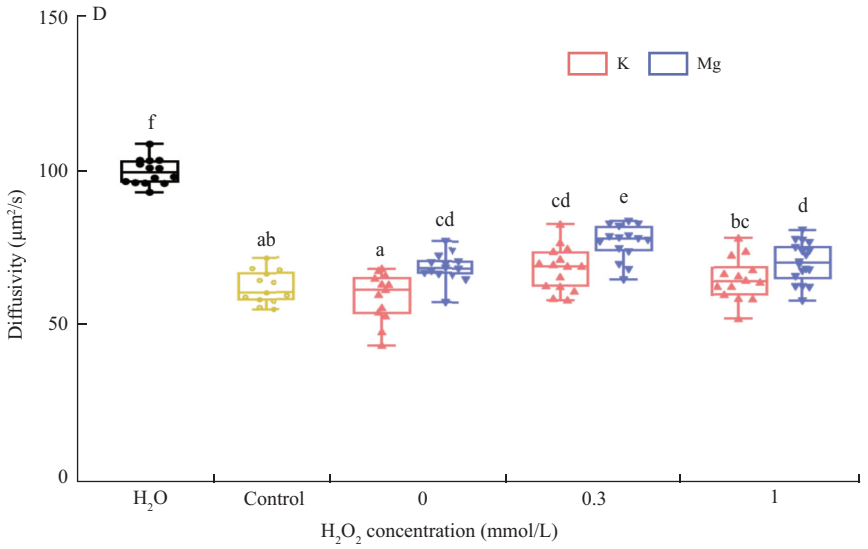

Fig. 5 (*Continued*)

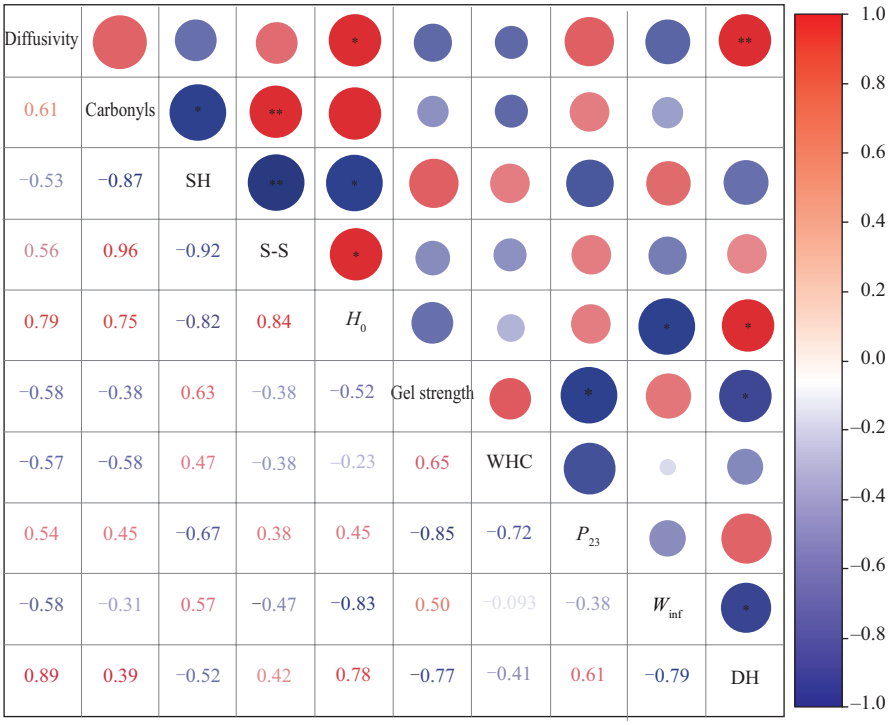

Fig. 6 Corrlation analysis between MP oxidation characteristics, gel properties, and *in vitro* gastric digestion characteristics performed by Pearson's correlation analysis. The correlation coefficient is proportional to the color depth. A correlation coefficient closer to –1 (blue) indicated a negative correlation, and that closer to +1 indicated a positive correlation (red). $^{*}P < 0.05$, $^{**}P < 0.01$.

4. Conclusions

Chloride salts (KCl, $MgCl_2$, and KCl/$MgCl_2$) were used to replace NaCl (30%) in oxidized MP. In the used HRGS model, the increased carbonyl content and surface hydrophobicity of MP were observed after oxidation. Among them, partial replacement of NaCl with $MgCl_2$, to great degree, promoted protein oxidation, and more sulfhydryl groups were oxidized to the disulfide bonds. Partial NaCl substitution with $MgCl_2$ promoted the sulfhydryl oxidation in the oxidized MP *via* protein denaturation or unfolding, which increased the accessibility of amino acids comprising hydrocarbons side chain group in the hydrophobic core. Within the mild oxidation levels (0.3 mmol/L H_2O_2), the sodium-reduced treatment with 30%-KCl facilitated the formation of dense network of gel, accompanied by enhanced gel strength, rendering more water to be restricted to gel matrix. However, this result made it hard for gel to be chewed, and the limited exposure of hydrophobic sites was averse to gastric digestion. Differently, the network structure of MP gel treated with mild oxidation and $MgCl_2$ was loose due to the enhanced hydrophobic interaction between protein molecules, and the relatively lower gel strength was beneficial to the disintegration of oral chewing and gastric digestion. As a result, the pepsin diffusivity,

degree of hydrolysis, and proportion of small molecular peptides were increased. However, the protein refolded as the oxidation degree increased to 1 mmol/L H_2O_2, and the gel network structure became rough and dispersed, resulting in hard diffusion of pepsin. In general, the digestive characteristics of oxidized gels were greatly attributed to the original protein structure and mechanical properties, and NaCl reduction substituted with $MgCl_2$ contributed to the MP gels with easier chewing and higher digestibility.

Declaration of competing interest

The authors declare that they have no conflict of interest in this work.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2024.9250331.

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