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Article

Effect of Malondialdehyde-induced Oxidation Modification on Physicochemical Changes and Gel Characteristics of Duck Myofibrillar Proteins

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Abstract: This paper focuses on the effect of malondialdehyde-induced oxidation modification (MiOM) on the gel properties of duck myofibrillar proteins (DMPs). The DMPs were firstly prepared and treated with oxidation modification at various concentrations of malondialdehyde (0, 0.5, 2.5, 5.0 and 10.0 mmol/L). Physicochemical changes (carbonyl group and free thiol group content) and gel properties (gel whiteness, gel strength, water-holding capacity, rheological properties and micro-structure properties) were then investigated. Results showed that, with the increase of MDA oxidation, the content of protein carbonyl group increased ($p<0.05$), whereas, the content of free thiol group decreased significantly ($p<0.05$). Meanwhile, there is a clearly decreasing trend of gel whiteness; Hardness and water holding capacity of protein gels increased significantly under the oxidation of low concentrations of MDA (0 to 5 mmol/L), while the hardness of gels decreased under the oxidation of high concentrations (10 mM). The storage modulus and loss modulus of the oxidized DMPs also increased with increasing concentration; Furthermore, microstructure analysis confirmed that the gels oxidised at lower concentrations were more compact and homogeneous in pore size compared with high concentration or blank group. To sum, moderate oxidation of malondialdehyde is beneficial to the improvement of the gel properties of duck meat, However, excessive oxidation is not conducive to the formation of dense structured gels.

Keywords: malondialdehyde; duck meat; myofibrillar proteins; physicochemical changes; gel properties

1. Introduction

In general, myofibrillar proteins played an important biological function protein. Therein, the content of myosin and actin is more than 2/3. Myofibrillar proteins has better functionality, and changes in its structure would lead to changes in producing the formation of good textural characteristics, and their gel characteristics are the basis for the processing of minced meat products and directly affect the sensory properties of the final meat product[1]. In particularly, oxidation modification showed the most significant influence. The oxidation of proteins could weak the interaction between proteins, thus altering their gel formation and thus having an impact on the quality of cleavage, intermolecular interactions causing second/third structural changes in the protein[2]. The side chains contain amino acids such as arginine, tyrosine, leucine, cysteine, phenylalanine, histidine, tryptophan, proline, lysine, and methionine, which are very sensitive to the action of ROS[3]. Among them, cysteine and methionine can oxidize histidine to oxidized histidine at lower ROS concentrations due to their high sensitivity to sulfide centers, while leucine and valine are converted to hydroxyl derivatives. In addition, lysine, arginine and

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proline are more prone to form carbonyl residues under metal catalysis[4].Sulfur-containing amino acids such as cysteine are most susceptible to oxidation by free radicals, which is reversible, and it has certain cyclic oxidation and reduction effects[5].

As known, malondialdehyde (MDA), which is naturally generated under meat processing conditions, is the most abundant individual aldehyde resulting from lipid peroxidation. MDA could have further promoted the myofibrillar proteins gelation in the presence of certain ionic strength[6]. Studies by Zhou et al showed that gels are formed by the dissolution of myofibrillar proteins at a certain ionic strength and form covalent bonds with non-disulfide bonds with the secondary fat oxidation product malondialdehyde (MDA) [7]. Previous studies also confirmed that the secondary and tertiary structure of myofibrillar proteins can be readily altered by oxidation, leading to the unfolding of myofibrillar proteins structure, which further promotes effective protein–protein interactions[8]. As a consequence, the oxidation of the protein structure causes changes in its physical properties, which are related to the degree of protein oxidation, and generally, the longer the oxidation time, the worse the functional properties of the protein[9]. It is still need to mention according to finding of wang et al., mild oxidation facilitated the formation of elastic gel lattice structure, while excessive oxidation decreased the gel cohesion and elasticity[10]. MDA could also bind to proteins and modified the interaction among proteins, and further lead to shifts in the functional properties of myofibrillar proteins in processed muscle foods. Xiong et al. suggested that MDA could change myofibrillar proteins conformation through modifying its side chains and polypeptide backbone[11]. Meanwhile, MDA can react with the amino groups of myofibrillar proteins, producing strong intermolecular cross-links of the schiff base type that also promote the gel formation[12]. However, there are few studies on the mechanism of its action on DMPs by MDA, chosen as an effective secondary oxidation product in fat peroxidation process. Hence, we hypothesised that the direct incubation of MDA with duck myofibrillar proteins (DMPs) could modify the structure of DMPs and thus lead to influence the formation of compact and rigid gels. The objective of this study was investigate effect of malondialdehyde oxidation modification on physicochemical changes and gel characteristics of duck myofibrillar proteins, aiming to provides a basis for controlling the degree of meat oxidation and the rational use of oxidants.

2. Results and Discussion

2.1. Morphological observation

The state of the samples formed after the protein was oxidized by different oxidation efforts of MDA is shown in Figure 1. The samples were all kept upside down for 15 min after the water bath heating and then photographed. Under the ionic strength of 0.6M NaCl, the state of MP samples changed from solution to gel with the increase of MDA concentration, and the samples treated with 0.5mM and higher concentrations became gel-like and did not collapse easily, while the samples treated in absent of MDA still became sol-like and flowed easily.

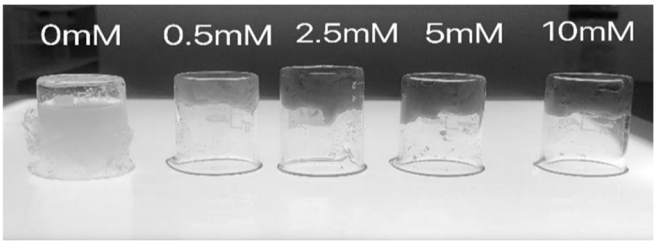

Figure 1. Morphology of DMPs treated with different MiOM (0, 0.5, 2.5, 5 and 10 mM).

2.2. Carbonyl and free thiol content changes

Protein carbonylation is an important oxidation reaction of proteins due to its -NH- or -NH2 groups on the side chains of amino acids, which leads to oxidation to generate

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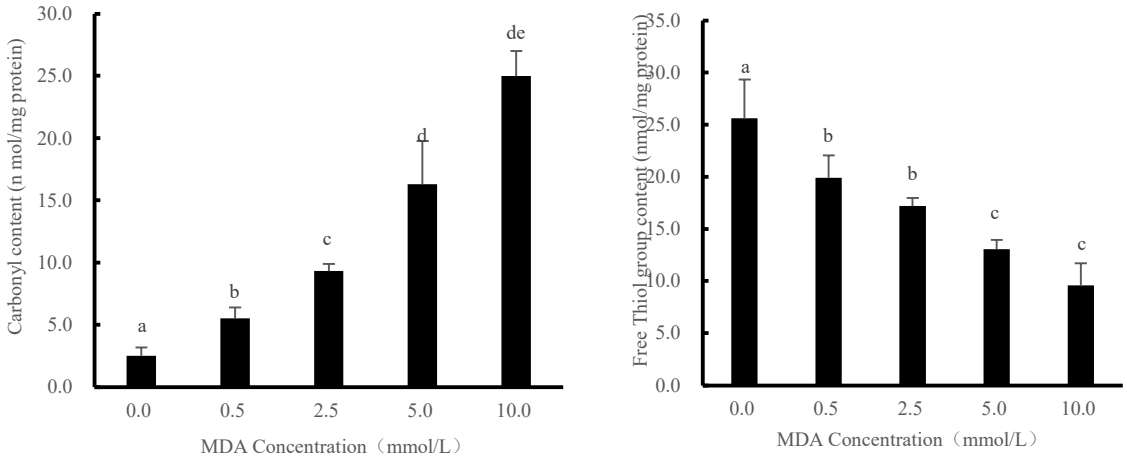
new carbonyl groups[4]. Figure 2a showed the variation of carbonyl content of the samples after oxidation treatment with different concentrations of MDA. The protein carbonyl content of the sample treated in absent of MDA was 2.52 nmol/mg protein. The carbonyl content of DMPs increased significantly with the increase of MDA concentration from 0 to 10 mM ($p<0.05$), as MDA could add to primary amines in the protein molecule in a certain ratio to produce an enamine adduct, resulting in a new carbonyl group. In addition, the molecular structure of MDA is composed of two carbonyl groups combined, and the oxidation of one of the MDA molecules with the protein can introduce another carbonyl group at the same time. Generally, MDA could act on the nucleophilic side chain groups of cysteine, histidine and lysine residues, and then react to form schiff base[13].

In proteins, the sulfhydryl group is the most reactive and responsive one, so amino acid residues containing sulfur are easily interacted to generate disulfide bonds, so the earliest oxidation of sulfhydryl group occurs in proteins, which will lead to the change of protein structure and then cause some effects on protein functions[14]. As shown in Figure 2b, the total sulfhydryl content of DMPs gradually decreased with the increase of MDA concentration, from 25.62 nmol/mg steadily dropping to 9.58 nmol/mg, which is due to the endogenous sulfhydryl groups are exposed when the protein structure is decomposed, exposured to pro-oxidant compounds, thus the total of sulfhydryl groups is continuously reduced, which were confirmed by recent finding of soglia et al[15]. Previous reports also indicated that the loss of sulfhydryl in plasma proteins is due to the interaction between protein sulfhydryl and α,β- Michael addition of unsaturated aldehyde occurred[16]. Moreover, MDA are electrophilic and could preferentially withdraw hydrogen atom from the sulfhydryl group of cysteine, resulting in a decrease in the content of sulfhydryl groups[17]



(**a**) (**b**)

Figure 2. Changes of carbonyl (a) and free thiol (b) content of DMPs treated with different MiOM (0, 0.5, 2.5, 5 and 10 mM).

2.3. SDS-PAGE profile analysis

As Figure 3 showed, samples under non-reducing conditions (-DTT), the intensity of band of myosin heavy chain (MHC) basically dimished after 2.5 mM, 5 mM, and 10 mM MDA treatment, and the other bands of the myofibrillar proteins disappeared when the MDA concentration exceeded 2.5 mM (actin bands were slightly blurred), a phenomenon that indicates excessive cross-linking of proteins with increasing oxidation concentrations. Under reducing conditions (+DDT), simlar results were found as under non-reducing conditions, the disappeared myosin heavy chain components were not recovered, large polymers remained on the loading part of gel, suggesting that the cross-linking was largely not through disulfide bonds or similar mechanisms, and that the covalent bonds of such cross-linking are strong and difficult to break. It is need to mention here less high

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molecular polymer is formed under non-reducing conditions compared with reducing conditions. We supposed that it could be due to the excessive cross-linking of proteins induced by high concentration of MDA, forming polymers with extremely high molecular weight that cannot enter the separation gel at all[18]. Louise previously reported that malondialdehyde can react with lens proteins to form non-disulfide bonded covalent crosslinks [19]. The oxidative stimulation with 0.6 M NaCl and different concentrations of MDA led to the unfolding of myosin, increasing the site of action of both, while the formation of non-disulfide bonds in this gel was not conducive to the speration using SDS-PAGE between myosin and other proteins. The protein cross-linking induced by non disulfide bond under the action of MDA could be more likely related to the formation of Schiff base aforementioned. Recent finding also indicated protein aggregation occurred in the MDA-induced oxidised system. Myosin was involved in gel formation through non-disulfide covalent bond.[20] . Disulfide bonds were responsible for most of the cross-linking, and malonaldehyde appeared to contribute to the cross-linking as well. [11]

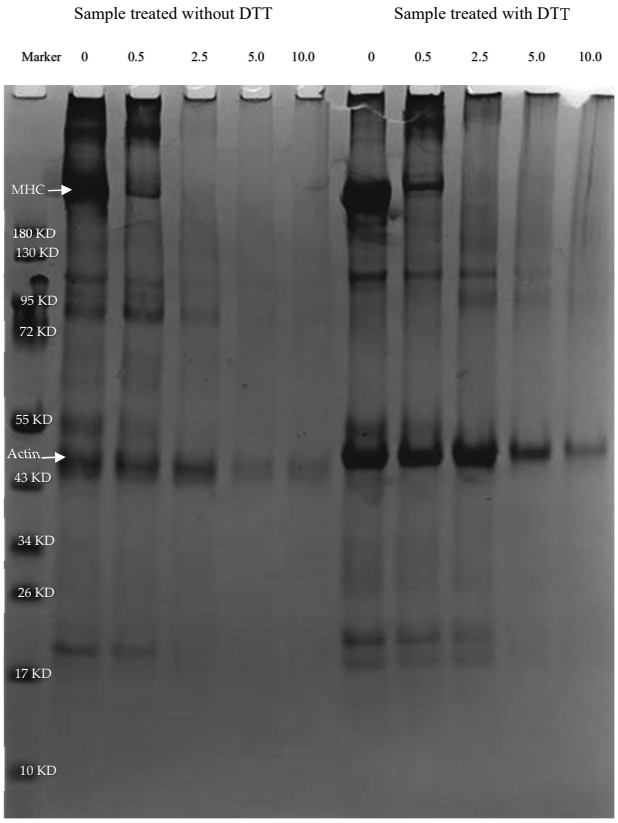

Figure 3. SDS-PAGE pattern changes of DMPs treated with different MiOM (0, 0.5, 2.5, 5 and 10 mM).

2.4. Gel strength and water holding capacity (WHC) analysis

The gel strength is an important quality characteristic of protein gels. The MDA-induced oxidation modification plays an important role in affecting the gel strength as shown in Figure 4a. It clearly indicated that gel strength of the samples increased significantly up to 0.23 N from 0.02 N, with the increase of MDA concentration. This is due to the disulfide bond-induced cross-linking between the proteins and other non-disulfide bonds during the heat treatment of the gels, while the formation of these covalent bonds facilitates the reinforcement of the gel structure, thus The gel strength of the samples was improved[21]. However, the gel strength of the samples decreased significantly with the further increase of MDA concentration when combining 5mM groups with 10 mM groups, which was due to the formation of excessive covalent bonds caused by the high concentration of oxidation, which in turn possibly led to the breakage of protein bonds and the decrease of gel strength[22]. Previous study showed that disulfide and other covalent

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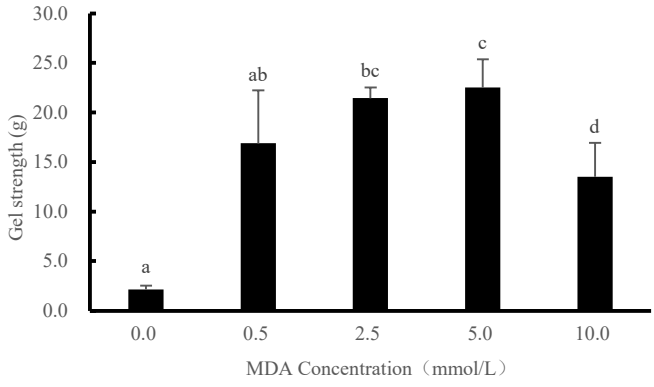
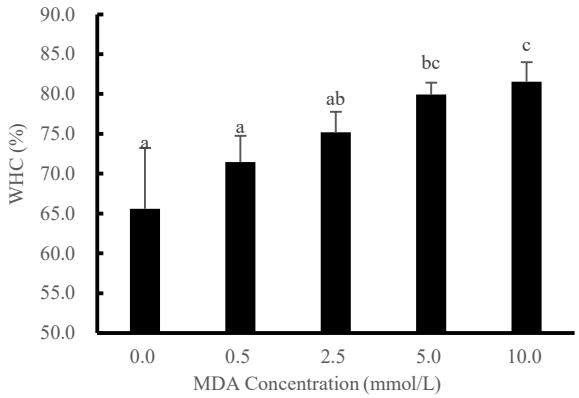
bonds were enhanced and caused protein cross-linking and aggregation, thus affecting gel strength, whereas hydrophobic interactions were weakened upon high extent of oxidation[23].

Similar to gel strength, analysis of the water holding capacity of gels can also reflect the quality of different treated myofibrillar proteins gels. The gel water holding capacity is related to the spatial structure of the gel and is a response to the ability of the gel to retain water molecules[24]. As shown in Figure 4b, the WHC of the gel samples increased continuously with the increase of MDA concentration, from 65.6% initially to 85.55%, and the trend of its water holding capacity was basically the same as the trend of carbonyl changes. It is speculated MDA could promote protein carbonylation and was responsible for the formation of protein cross-linking throughout incubation treatment, which could change the gel mesh structure and facilitate the improvement of the gel water holding capacity[25].

As can be seen in Figure 4c, the whiteness values of the samples decreased significantly with increasing MDA content, Results showed that the brightness, redness values and yellowness values of the colloids were affected significantly, which was corresponding with aforementioned morphological observation results. Xia et al. also found that repeated thawing-freezing treatment of meat caused non-enzymatic oxidation reactions between fat oxidation products and amino acids in proteins, resulting in a decrease in their whiteness[26].



(**a**)



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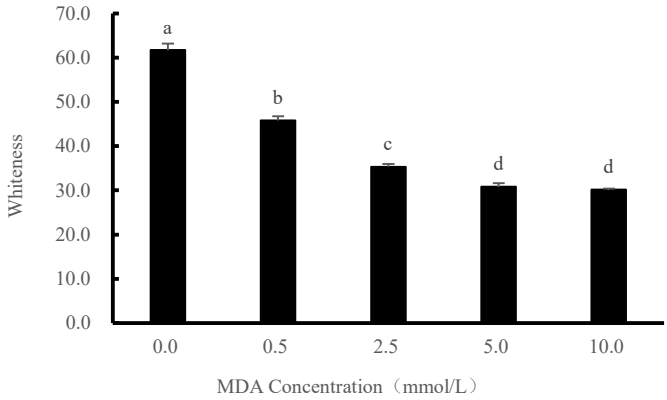

(c)

Figure 4. Changes of gel strength (a), WHC (b) and whiteness (c) of DMPs treated with different MiOM (0, 0.5, 2.5, 5 and 10 mmol/L).

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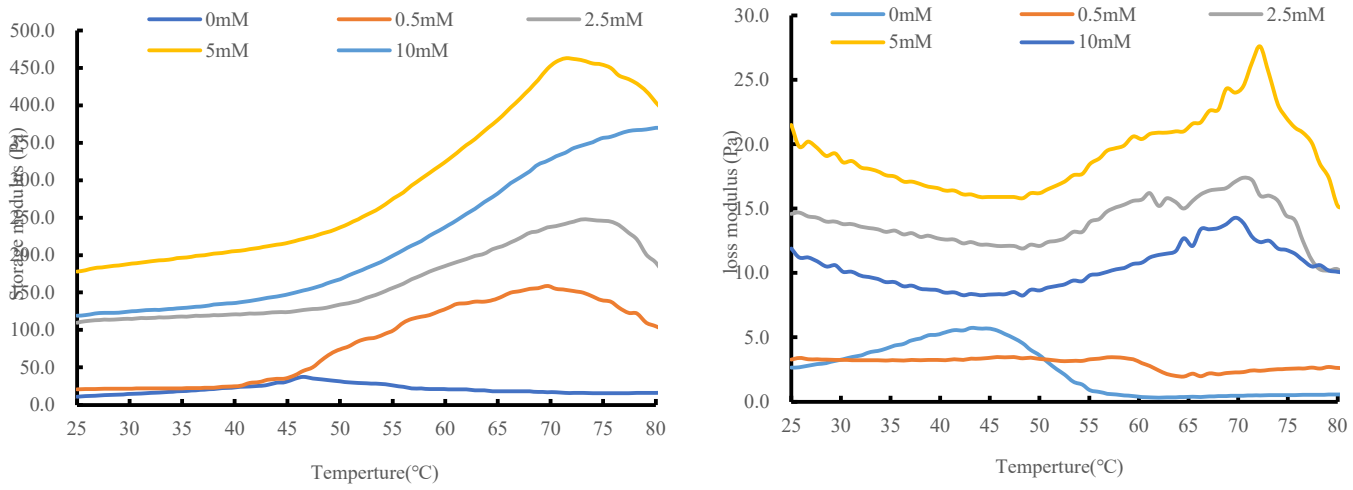
2.5. Rheological characterization

The storage modulus is an important parameter in the process of dynamic rheological property determination, and the experimental results are shown in Figure 5a. Firstly, when the MDA concentration was 0, the curve peaked from 25 ℃ to 46.5 ℃, and the curve gradually decreased until 70.1 ℃, and the curve continued to increase until the test was completed, indicating that 46.5 ℃ is the gel point of myofibrillar proteins gel. In the temperature interval from 25 to 46.5℃, myofibrillar proteins underwent high-temperature denaturation, which exposed its amino acids, resulting in changes in the gel structure, thus forming a gel with high elasticity; in the temperature interval from 46.5 to 70.1℃, the original gel matrix was destroyed, and in the interval from 70.1 to 80 ℃, the The ordered cross-linking of disulfide bonds continued and a stable, uniform, dense, three-dimensional elastic network with high energy density was formed. The storage modulus trend was similar among various MiOM groups, however, the final storage modulus also increased significantly with the increase of oxidation concentration at low MDA concentration (from 0 - 5 mmol/L), It should be emphasized that G' of 10 mmol/L groups were lower compared with 5 mmol/L groups. It confirmed the fact that at low oxidation concentrations MDA (0.5, 2.5, 5 mmol/L) oxidation protein-protein interactions are enhanced to produce good binding in proteins, while excess high oxidation concentrations of MDA (10 mmol/L) oxidation cause protein aggregation and gel contraction, which were adverse to gel-forming capacity of DMPs, as described in previous results of gel strength.

The trend of changes of loss modulus G'' (Figure5b) is roughly similar to that of storage modulus G'. The G'' curve of blank group (0 mmol/L) firstly peaked at 43.1℃, and then decreases, plateaused at 56℃. Results also showed that, with the MDA concentration increases, the loss modulus gradually increases until around 70℃, which was possibly due to the fact that, as the temperature increases, the previously formed gel matrix is destroyed at low concentration oxidation gradient[27]. Meanwhile, the loss modulus curves of G'' under 10 mM MDA oxidation were overall lower than those under 2.5 mM and 5 mM oxidation, which was coincide with the aforementioned results of gel strength and storage modulus.



(**a**) (**b**)

Figure 5. Storage modulus (G') and Loss modulus (G'') changes of DMPs treated with different MiOM (0, 0.5, 2.5, 5.0 and 10.0 mmol/L).

2.6. Nuclear magnetic characterization

Figure 6 showed the effect of MDA concentration on the relaxation time (T_2) changes of DMPs gel. As seen, three typical peaks were found, therein a peak appeared in 20 - 96 ms, a larger peak emerged at 131-446 ms, and a peak existed after 800 ms. In our previous paper, DMPs gel generally showed three peaks in the fitted NMR relaxation curve, which

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corresponded to moderate immobilized water, immobilized water, and free water[28]. That is T_{21} represented moderate immobilized water with peaks from 20 to 96 ms, T_{22} represented immobilized water with peak from 131 to 446 ms, and T_{23} represented free water with the peak after 800 ms. Generally, It is understandable that DMPs cross-link with each other, forming a three-dimensional structure that locks in a large amount of water molecules, resulting in the formation of immobilized water. Our results also showed that the area of T_{22} (immobilized water) increased with the increase of MDA concentration (from 0 to 5.0 mmol/L). Moreover, it could be found that there is a decreasing trend of peak area at 10 mmol/L concentration compared with 2.5 and 5 mmol/L groups. Furthermore, with the increase of MDA concentration, there is a decreasing tread of T_{23} (free water), which indicates that especially low concentration of MDA oxidant helps to convert free water into immobilized water and reduce the content of free water. These results explained well the increase of WHC mentioned above, and also was corresponding with the finding of Wang et al, the fraction of free water declined from 7.66% to 0.15% as the MDA addition increased from 0 to 50 mM. Moreover, the relaxation components T_{2b} disappeared with the addition of MDA mainly due to enhanced protein flexibility and surface hydrophobicity[29]. Xia also reported that WHC had a significant positive correlation with percentage of immobile water, while having a negative correlation with carbonyl group content and T_{23} [30].

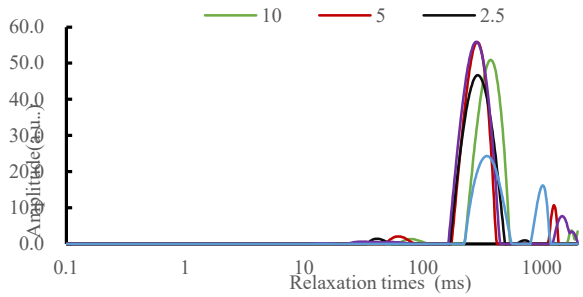

Figure 6. Storage modulus (G') and Loss modulus (G'') changes of DMPs treated with different MiOM (0, 0.5, 2.5, 5 and 10 mM).

2.7. Gel microstructure analysis

The results of gel microstructure analysis was shown in Figure 7. Samples under 2.5mM and 5mM MDA treatment, the gel mesh structure gradually tightened and formed an orderly gel mesh structure with porous and uniform pore size, which corresponds to the enhanced gel strength, as mentioned before. In contrast, the gel structure after 10 mM treatment was still firm, but the collapse of the gel surface structure could be seen in the figure, which was consistent with the decrease in gel strength in our previous results. It clearly shows that the gel mesh structure can be improved under low concentration of MDA oxidation[31]. Zhou et al also reported similar result, the gel mesh structure can be improved under low concentration of MDA oxidation. while the higher MDA could cause gel collapse, which was believed to be due to the excessive covalent bond existed.[32]

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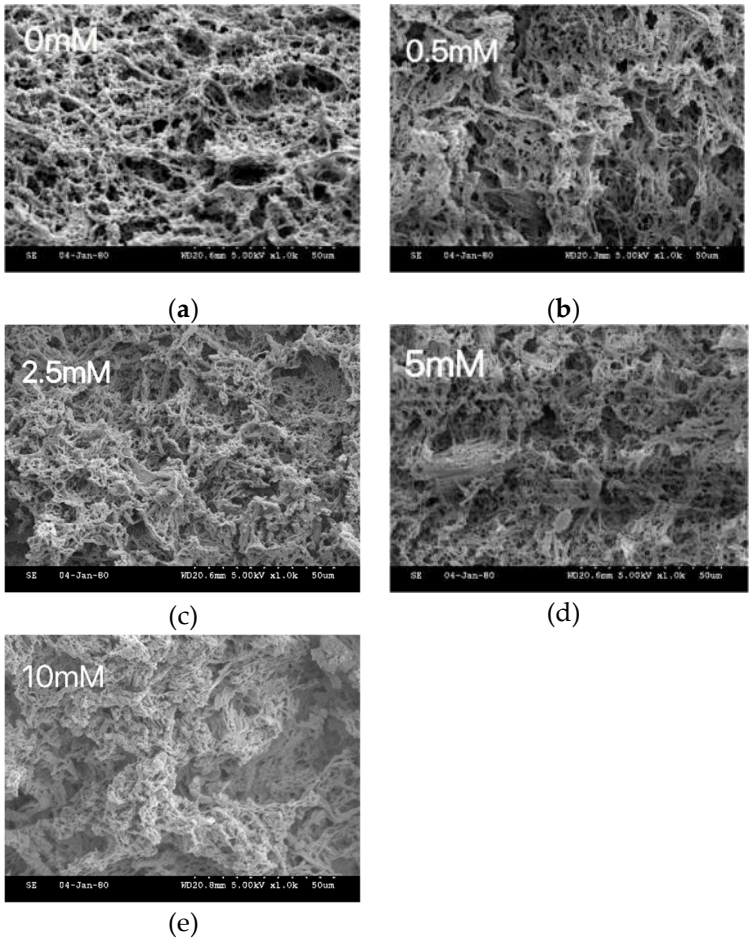

Figure 7. Microstructure analysisof DMPs treated with different MiOM (0, 0.5, 2.5, 5 and 10 mM).

3. Materials and methods

3.1. samples and DMPs preparation

The duck breast meat used in this experiment was obtained from the Nanjing Lishui local market, and the duck breasts were removed after conventional slaughter, and all the muscle and fat were peeled off and placed in a self-sealing bag in a refrigerator at -80°C.

To extract DMPs, 30 g of duck breast meat was added to the extract (5 times the volume), homogenized at high speed for 20 s and then placed in a high-speed refrigerated centrifuge with the following conditions: 4°C, centrifuged at 4000 g for 10 min, repeated three times, filtered through gauze, 1% TritonX-100 (5 times the volume) to wash the above precipitate, repeated three times, with the same centrifugation conditions, and finished with 0.1M NaCl (5 times volume) to dissolve, homogenize, centrifuge again, discard the supernatant, repeat three times, and finally filter through white gauze to collect the precipitated DMPs [9]。

3.2. MDA oxidation-modified myofibrillar protein treatment

The MDA stock solution was mainly prepared through acid treatment of 1,1,3,3-tetramethoxypropane obtained from Sigma–Aldrich Chemical Co. (St Louis, MO, USA) as described by Wu, et al.[33] with minor modifications.

The MDA-induced oxidation systerm of DMPs was referred to Zhou Fei Bai et al [7] with appropriate modifications. At First, adjusted concentration of DMPs to 40 mg/mL, then mixed with MDA stock solution at different ratio (MDA concentration: 0 mM, 0.5 mM, 2.5 mM, 5 mM, 10 mM respectively) . The mixture was then placed in a 10 mL centrifuge tube at 25°C for 24 h. The protein samples were heated directly in a water bath after the above treatment. The heating conditions were: linear heating at a starting

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temperature of 25°C for 1°C/min to 85°C, ice bath cooling after 5 min, and storage of the samples in a refrigerator at 4°C。

3.3. Carbonyl content determination

The protein carbonyl content was determined according to soglia et al with modification[34]. The DMPs sample obtained was adjusted to a concentration of 20 mg/mL. During the determination, 5% SDS was using to resolve the precipitate,. DNPH reagent were used to marker to lab carbonyl. At last, the solution was measured A280 and A370. The carbonyl content equation (1) was calculaated as follow:

$$\text{Carbonyl content} = \frac{\left[A_{370} - A_{370(\text{blank})}\right] \times 10^6}{22000 \times \left[A_{280} - \left(A_{370} - A_{370(\text{blank})}\right) \times 0.43\right]} \quad (1)$$

3.4. free thiols content determination

The protein sulfhydryl content was appropriately carried out according to the method of Bao et al [35]. The concentration of the oxidized DMPs obtained was adjusted to 2 mg/mL, and 300 μL of this protein concentration solution was used. Particularly, 0.5 mL of 10 mM DTNB was added to the solution, mixed well, and A412 (after) was measured at room temperature and protected from light for 30 min, and zeroed with 0.1 M Tris-HCl. The molar extinction coefficient was 13600L/(mol-cm), and the sulfhydryl content was calculated as follow:

$$\text{Thiol groups[n mol/mg protein]} = \frac{\left[A_{412(\text{after})} - A_{412\ (\text{before})}\right]}{A_{280} \times 14150} \quad (2)$$

3.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (SDS-PAGE)

DMPs with different MDA-induced oxidation treatments were adjusted to 2 mg/mL. The DMPs were pretreated using LDS sample buffer (invitrogen, thermal fisher, USA) with or without DTT, and then heated in metal bath at 99℃ for 5-10min water bath for 5 min . Electrophoresis were then run at 220 V for 45 min .The Coomassie Brilliant Blue R-250 solution was stained for 30 min and then decolorized and photoed for analysis.

3.6. Gel hardness and water holding capacity (WHC)

The gel strength of DPMs samples was performed according Zhu et al[36]. using a Texture Analyzer (TA-XT plus Plaser, Stable Micro Systems UK). Measurement conditions: P/0.5 R probe with a pre-measurement speed of 1 mm/s, a measurement speed of 0.5 mm/s, a post-measurement speed of 10 mm/s, compression mode, and a depth distance of 5 mm. Each treated sample is repeated 3 times.

The WHC of the gel was determined using centrifugation methods. Gel was centrifuged at 4°C (6000r/min, 15min), after which the tube was placed upside down on absorbent paper for 30min, and the mass of the tube m and the total mass before centrifugation was recorded as m1, and the total mass after being placed for 30min was m2. Then the water holding capacity was calculated as follow:

$$WHC(\%) = \frac{m2 - m}{m1 - m} \times 100 \quad (3)$$

3.7. Gel whiteness determination

The gel samples were determined using a CR400 colorimeter (Minolta Camera, Japan). Brightness (L*), red (a*), and yellow (b*) were measured three times before use, and the gel whiteness was calculated by as:

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$$Whiteness = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}} \quad (4)$$

3.8. Rheological properties test

The freshly oxidized protein solution was measured using a rheometer (MCR-301, Anton Paar, Austria) in oscillatory mode as described by Zhuang [37]. Parameters: 50 mm plate material was selected with a gap of 1 mm between the upper and lower plates, frequency 0.1 Hz, strain 2%,25°C/min, heating temperature 2°C/min and cooling rate 5°C/min. Before the test, paraffin oil had to be dripped into the edge of the plate to isolate the sample from the outside air in order to avoid the sample being heated. The storage modulus G′ and loss modulus G′′ was then recorded.

3.9. Low field NMR measurements

T2 relaxation times were measured according Han et al.[38] with a NMR analyzer (MesoMR23-060H-1, Niumag elctric Co., China). A standard oil sample was first calibrated, and then a centrifuge tube of about 2 g was placed in the tester, and the spin-spin relaxation time was selected as the CPMG sequence. The proton resonance frequency was set at 22.6 MHz and the measurement was performed at a temperature of 32°C. The relevant parameters: the number of repetition sampling (NS) was 4 times, the repetition interval (time wait, TW) was 2000 ms, the number of echoes (NECH) was 9000, each test was performed 3 times, and the obtained curve was an exponential decay sample curve. A large number of data inversions were achieved through the data query function in the software menu.

3.10. Gel microstructure analysis

The gel samples were cut into squares (3mm × 3mm × 3mm) and fixed with 4% malondialdehyde, Gels were then analyzed with a Hitachi S-3000N scanning electron microscope (Tokyo, Japan) at an accelerating voltage of 20 kV .

3.11. Statistical analysis

Data were processed with SPSS 20.0 software and subjected to one-way ANOVA with Duncan's multiple range test for statistical analysis

4. Conclusions

MDA-induced oxidation could alter the physicochemical sructure of DMPs, as the carbonyl content increasing and sulfhydryl content decreasing significantly; furthmore, the gel whiteness and WHC showed a decreasing trend. It is needed to mention, under proper oxidation condition, the protein gel hardness increased significantly under the oxidation of low concentration of MDA (from 0 to 5 mM), however, decreased under the oxidation of high concentration (10 mM). The enhanced covalent bonding facilitates the consolidation of the gel structure. These results suggested that the disulfide bonding induced by the heating process under mild low concentration of MDA oxidation might improve the gel structure and thus the gel quality.

Author Contributions: Conceptualization, project administration, writing-review and editing, Xueshen Zhu; investigation, data curation, writing-original draft,Zhenghao Ma; methodology, , Xinyu Zhang; funding acquisition, visualization, Xuefang Huang， Junya Liu; software, supervision,Xinbo Zhang; All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The datasets generated for this study are available on request to the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

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