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Title:
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Date:
2017-01-15

Citation:
Wang, L., Zhang, M., Fang, Z. & Bhandari, B. (2017). Gelation properties of myofibrillar protein under malondialdehyde-induced oxidative stress. Journal of the Science of Food and Agriculture, 97 (1), pp.50-57. https://doi.org/10.1002/jsfa.7680.

Persistent Link:
https://hdl.handle.net/11343/291165

Gelation Properties of Myofibrillar Protein under Malondialdehyde Induced Oxidative Stress

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/jsfa.7680

ABSTRACT

BACKGROUND: Structure of myofibrillar protein (MP) can be readily altered by oxidation, leading to the unfolding of MP structure, which further promote protein-protein interactions, and thus influence the MP gelling properties. The objective of the study was to investigate the effect of malondialdehyde induced oxidative stress on the gelation properties of myofibrillar protein (MP). Structural changes of the oxidized myofibrillar proteins were evaluated by the contents of carbonyl and total sulfhydryls, surface hydrophobicity, SDS-PAGE and Fourier transform infrared spectroscopy. The oxidative stability of the MP gels as indicated by lipid hydroperoxide was also determined.

RESULTS: With the addition of MDA concentration less than 10 mM, the MP gels showed an improved elasticity, gel strength, water holding capacity, and oxidative stability. Nevertheless, higher MDA concentration (25-50 mM) significantly reduced the gel quality, probably due to the formation of excessive covalent bonds in the system.

CONCLUSION: Results suggested that protein aggregation was occurred in the oxidized system. Myosin was involved in gel formation through non-disulfide covalent bond.

KEYWORDS: Gelation; myofibrillar protein; malondialdehyde; protein oxidation

INTRODUCTION

Silver carp (*Hypophthalmichthys molitrix*) is a popular species of freshwater fish in China with an annual harvest of 3.7 million metric tons.[1] Its muscle is a common protein resource used for manufacturing surimi products - an intermediate material for producing various texturized food products. The formation of protein gels in surimi products is one the most important functional properties, which determine the unique product quality such as texture, juiciness, fat content, and sensory characteristics.[2,3] Myofibrillar proteins (MP), contributing 55-65 % of the total silver carp muscle protein, are the main gelling substances in surimi-based foods. The gelation behavior of MP has been well documented in terms of factors influencing gelation properties as well as rheological properties.[4] Most of the previous research focused on how myosin and actin react in different animal muscles, and how the interactions of MP with other food additives influence the texture and rheological properties of the protein gels.[5]

Recently, protein oxidation has attracted great interests in the research of the mechanisms of gel formation.[5,6] Studies have shown that the secondary and tertiary structure of MP can be readily altered by oxidation, leading to the unfolding of MP structure, which further promote effective protein-protein interactions, and thus influence the MP gelling properties.[7,8] Because of the coexistence of protein and lipid in meat system, it is unlikely that the oxidation of lipids and proteins takes place independently.[9] Reports also have confirmed that the onset of lipid oxidation could take place faster, and the lipid-derived radicals and hydroperoxides further promote the protein oxidation.[10] Malondialdehyde (MDA) is an abundant individual aldehyde

resulting from lipid peroxidation, which could have promoted the MP gelation in the presence of certain ionic strength.[11] Xiong et al.[8] suggested that MDA could change MP conformation through modifying its side chains and polypeptide backbone. Meanwhile, MDA can react with the amino groups of MP, producing strong intermolecular cross-links of the Schiff base type that also promote the gel formation.[12] Therefore, we hypothesized that the direct incubation of MDA with MP could modify the structure of MP and thus lead to the formation of MP gels. The objective of this study was to test this hypothesis by determination of the physicochemical changes of MP and gel properties after the MDA modification.

EXPERIMENTAL

Materials and chemicals

Fresh silver carp was purchased at a local market in Wuxi city, China. Chemicals of 1, 1, 3, 3 - tetramethoxypropane were purchased from Sigma-Aldrich (St Louis, MO, USA). All other chemicals were of analytical reagent grade.

Extraction of MP

MP was extracted from the dorsal muscle of silver carp by homogenizing the fish mince with 4 volumes of cold distilled water (d4 ^{o}C) according the method of Park et al.[13] After addition of 0.1 M NaCl, the pH of the MP suspension was adjusted to 6.25 before centrifugation. The pellet was finally suspended in 20 mM sodium phosphate

buffer (pH 6.0), and the protein concentration was determined according to the Biuret method using BSA as standard.[14]

Preparation of MDA solution

MDA solution was freshly prepared by hydrolyzing 1, 1, 3, 3 - tetramethoxypropane according to the method described by [15] with minor modifications. Firstly, 8.4 mL (50 mM) 1, 1, 3, 3 - tetramethoxypropane was mixed with 10.0 mL 5.0 M HCl and 31.6 mL distilled water and incubated at 40 °C in the dark for 30 min. After acidic hydrolysis, the resulting mixtures were adjusted with NaOH (6 M) solution to pH 6.0 to obtain the MDA solution. Finally, the MDA solution was diluted to 100 mL using 50 mM phosphate buffer (pH 6.0). The concentration of MDA was determined by spectrophotometric measurements of the dilution 10^{-5} at 267 nm and calculated using the $\mu = 31{,}500$.

MDA modified myofibrillar protein

The MP suspension (40 mg/mL, in 50mM PB, pH 6.0) were mixed with different concentrations of MDA (0, 0.5, 2.5, 5, 10, 25, 50 mM). The mixtures were immediately transferred into tightly sealed glass vials and incubated at 25 °C in the dark for 24 h. After incubation, a series of MDA treated MP gel samples was obtained.

Analytical methods

Determination of carbonyl content

Carbonyl groups of the samples were measured following the procedure described by Uzun et al.[16] Diluted MP samples were incubated with 2, 4 dinitrophenylhydrazine (DNPH) reagent for 30 min at room temperature to form protein hydrazones. Samples were homogenized and diluted in 7 volumes (w/v) of distilled water. The DNPH reacted samples were then recovered by centrifugation after 10 % trichloroacetic acid (TCA) precipitation, and washed with ethanol: ethyl acetate (1:1, v:v) solution three times. The protein content in the final pellets were dissolved in 6 M guanidine hydrochloride and determined by reading the absorbance at 370 nm. A standard curve was prepared by measuring the absorbance of blank and HCl treated protein samples. Results were expressed as nmol of DNPH equivalents/mg of protein using an absorption coefficient of 21 mM^{-1} cm^{-1}.

Total sulfhydryls (SH)

The SH group contents of MP were determined by a modification of Ellman's method using 5, 5'-dithio-bis (2-nitropyridine acid) (DTNB) according to Shimada and Cheftel [17] A molar extinction coefficient of 1.36 × l04 M^{-1} cm^{-1} was used. Results were expressed in moles of SH per miligram of protein.

Protein surface hydrophobicity

Protein surface hydrophobicity of MP was determined using bromophenol blue (BPB) according to the description of Chelh et al.[18] Results were expressed as bound BPB in mg.

Tryptophan fluorescence

Tryptophan fluorescence of MP was determined on a FluoroMax-3 spectrofluorometer (Hitachi HighTechnologies Co., Tokyo, Japan) with MP suspension. The excitation wavelength was set at 283 nm and the emission spectra were recorded from 300 to 400 nm. Background spectra under the same conditions were recorded and subtracted from the treatment samples.

Rheological characterization

The rheological properties of the MP gels at different MDA levels were characterized using a AR-G2 Rheometer (Malvern Instruments, Westborough, MA, USA) with a parallel plate (diameter=35 mm), at 25 °C. The gap between two plates was set to 1.0 mm. For determination of steady shear viscosities, shear rate was ramped from 1 to 200 s^{-1}. Shear stress, shear rate, and steady shear (apparent) viscosity (·) were recorded by a RheoWin 4 Data Manager. Dynamic viscoelastic properties were characterized using small-amplitude oscillatory frequency sweep mode. The frequency was oscillated from 0.1 to 100 rad/s, and all measurements were performed within the identified linear viscoelastic region and made at 0.5 % strain. The elastic modulus (G2), loss modulus (G3), and loss tangent (tan ´) were recorded.

Gel strength

The gel strength of the MP gels was measured using a cylinder measuring probe (P/0.5) attached to a TA.TX2 texture analyzer (TA-XT plus, Stable Micro Systems, Ltd., Surrey, United Kingdom) at a constant probe speed of 1.0 mm/min at room temperature (25 ± 1 °C). The gel strength is defined as the initial force required to disrupt the gels. All samples were tested in triplicate.

Water holding capacity (WHC)

The WHC values of the MP gels were determined according to the method of Xia et al.[19] with slight modifications. The MP gels (3 g) were centrifuged at 8,000g for 30 min at 4 °C and the WHC (%) was expressed as the final weight as a percentage of the weight before centrifugation.

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

SDS-PAGE was performed on the MP gels according to the method described by Flores et al.[20] The diluted MP sol was mixed with certain volumes of sample buffer (with and without 5% ²-mercaptoethanol (²-ME)) to obtain a theoretical concentration of 1 mg mL^{-1} protein. Samples were vortexed and incubated at room temperature overnight. The ones with ²-ME were boiled for 3 min before centrifugation (10,000g, 10 min). Then, 15 μL of protein patterns was loaded onto the polyacrylamide gel made of 12% running gel and 5% stacking gel. Electrophoresis was run on an SE 250 Mighty Small II vertical slab gel electrohoresis unit (Hoefer Scientific Instruments, San Francisco, CA, USA)

Fourier transform infrared spectroscopy (FT-IR)

The FTIR spectra of the MP samples were recorded using a Nicolet 8210E FTIR spectrometer equipped with a deuterated triglycine sulfate detector (Nicolet, Madison, WI, USA) according to Sun et al.[21] The spectrometer was continuously purged with dry air from a Balston dryer (Balston, Cleveland, OH, USA). One mg of sample was mixed with 100 mg KBr and ground gently with an agate pestle and mortar under an infrared lamp (Tianjin Tianguang Optical Instrument Co. Ltd., Tianjin, China) and

afterwards was pressed into a 13 mm diameter disk by applying 15 tons pressure for 1 min using a tablet device (Tianjin Tianguang Optical Instrument Co. Ltd., Tianjin, China). The FTIR spectra were obtained in the wave number range from 400 to 4000 cm^{-1} during 128 scans, with 2 cm^{-1} resolution (Omnic 6.0 software, Nicolet, Madison, WI, USA). A straight baseline passing through the ordinates at 1,700 and 1,600 cm^{-1} was adjusted as an additional parameter to obtain a best fit using the OPUS 6.5 (Bruker Optics, Ettlingen, Germany). The curve-fitting procedure was performed to determine the position and percentage of the absorption peaks using PeakFit Version 4.12 software (SPSS Inc., Chicago, IL, USA), with full width at half maximum height (FWHM) of 14.2 cm^{-1} and a maximum resolution factor.

Data processing

Statistical calculations were performed using the statistical package SPSS 11.5 (SPSS Inc., Chicago, IL, USA) for one-way ANOVA. all of the experiments were repeated with three different batches of MP. Data were expressed as means plus standard deviations (SD) of triplicate determinations.

RESULTS AND DISCUSSION

Carbonyl and sulfhydryl contents

Many amino acid side chain groups are readily modified by oxidants, and have been commonly applied to the assessment of the extent of oxidative modification of proteins.[22] As shown in Fig.1, carbonyl content of the non-oxidized MP (control) was 1.48 nmol/mg proteins, a similar value to those reported for myofibrillar proteins.[23,24]

Compared to the control, the protein carbonyl content began to increase significantly ($p < 0.05$) in the presence of 0.5 mM MDA, indicating the occurrence of protein oxidation. The content of carbonyl increased with the increasing MDA concentration from 0.2 to 50 mM ($p > 0.05$), and a maximum value of 9.26 nmol carbonyls/mg protein was observed at 50 mM MDA. The results were in agreement with previous reports on linoleic acid-induced oxidaizing system, hydroxyl radical-generating system, metmyoglobin oxidizing system and MDA-induced oxidazing system on pork muscle myofibrillar proteins.[13,25]

The contents of SH of the control and oxidized MP are also shown in Fig. 1. The SH content of the control was 72.74 nmol/mg protein, similar to that in porcine MP.[26] Compared to the control, about 30 % of SH was lost after 10 mM MDA addition, which was quite similar to that obtained by Zhou et al.[27] who found a 30 % loss of SH in a MetMb/H_2O_2 system after 1 h incubation. Further addition of MDA resulted in heavier loss of SH.

Protein surface hydrophobicity

The amount of protein bound bromophenol blue (BPB) is a parameter estimating the protein hydrophobicity.[28] Reports have confirmed that oxidation of protein could expose the originally occluded hydrophobic amino acids to the polar surface to allow enhanced binding with the hydrophobic probe of BPB.[29] Fig. 2 shows the surface hydrophobicity of the control and oxidized MP. Compared to the control (139.67 mg bound BPB), the surface hydrophobicity was not significantly ($p>0.05$) altered in the range of 0.5 to 10 mM oxidizing agent. Nevertheless, further addition of MDA

significantly increased ($p<0.05$) the the protein surface hydrophobicity. The oxidative stress with addition of MDA might result in the unfolding of myofibrillar proteins, and thus the exposure of non-polar amino acids to the protein surface, which resulting in the increased surface hydrophobicity. This result was in agreement with the reports of Li et al.[30] who observed that oxidation enhanced protein surface hydrophobicity of porcine MP.

Intrinsic tryptophan fluorescence

Intrinsic tryptophan fluorescence properties are particularly sensitive to the polarity of the tryptophan microenvironment. Generally, in the folded state, tryptophan residues are located within the core (a hydrophobic environment) of the protein, having a high quantum yield and therefore high fluorescence intensity. While in an unfolded state, they tend to expose to solvent (a hydrophilic environment), leading to reduced fluorescence intensity. As shown in Fig.3, the tryptophan fluorescence intensity of oxidized MP decreased remarkably compared with the non-oxidized MP, suggesting protein unfolding. The addition of MDA led to further decreased fluorescence intensity, especially at the high concentration of MDA, indicating further unfolding and possible interactions between MDA and tryptophan residues.

Gel strength and WHC

Gel strength and WHC of the MP gel samples are depicted in Fig. 4. The gel strength is one of the most important functional properties of proteins gels, and this was increased ($P<0.05$) in the MP gels with the increasing addition of MDA in the range of 0.5-10 mM (Fig.4A). The maximum gel strength reached 1.90 N, indicating an

evident increase compared with that of control. This could be mainly due to the interaction of proteins under MDA induced oxidative stress. This was further supported by the continuous loss of myosin in the SDS-PAGE analysis (Fig.6) and discussed later. The MDA induced myosin cross-links could increase protein-protein interactions and thus contribute to the enhancement of the gel network, which was related to the increase in gel strength.[19] Nevertheless, with further addition of MDA (25−50 mM), a drastic decrease in gel strength was observed (Fig.4A), probably due to excessive carbonylation during oxidation that decreased the gelling capacities.

The parameter of WHC can be recognized as an indication of the amount of water retained within the protein gel network structure. The WHC of MP gels showed a very similar changing tendency with that of gel strength (Fig. 4B), suggesting the gel strength play an important role in the water holding capacity. Specifically, myosin is of paramount importance for protein gelation.[31] The influence of myosin cross-links on gelation was intensified with the increasing concentration of MDA, thus resulted in a stronger gel network formation and a higher WHC in the gel matrix.

Dynamic rheological testing of myofibrillar protein

The effects of MDA induced oxidation on the rheological properties of myofibrillar protein are shown in Fig.5. Variations of G2and tan ′ with frequency were recorded using small strain measurement. As shown in Figure 5A, G2of MP in the test linear viscoelastic range increased with increasing MDA concentration from 0.5 to 10 mM. The maximum G2of the MP treated with 10 mM MDA reached 1,186 Pa, approximate 5.0 times higher than that of the non-oxidative MP, suggesting the elastic property of

the MP was markedly enhanced. This might be due to the formation of more protein-protein cross-links under MDA induced oxidative stress that resulted in an entanglement gel network. Nevertheless, further addition of MDA (25-50 mM) sharply decreased the G2, suggesting a weakened gel structure, which was corresponded well to the decrease of gel strength as shown in Figure 4A.

The loss factor, tan ´, indicative of whether elastic or viscous properties were predominant in a gel, was further measured. As shown in Fig.5B, the tan ´ was independent of the frequency throughout the tested range and the tan ´ values were <1, indicating all the MP exhibited a predominant elastic behavior with frequency independency. In addition, with increasing MDA concentrations from 0.5 to 10 mM, an evident decrease in the tan ´ values was observed, suggesting enhanced elastic properties. However, when MDA concentration was further increased (25-50 mM), the tan ´ values of the gels were also increased (Fig.5B), suggesting a weaker elastic properties.

SDS-PAGE

Protein patterns of the MDA induced MP in the absence and presence of ²-ME are shown in Fig 6. In the absence of ²-ME and with the increasing concentration of MDA, a continuous decrease in band intensity of myosin heavy chain (MHC) was observed, accompanied by the accumulation of high molecular weight compound at the top of the gel (Fig.6A). This indicated the increased protein cross-links under MDA oxidation and MHC played a prominent role in this process. The results were in agreement with a previous report on linoleic acid-induced oxidative modifications of

porcine MP.[32] In the presence of ² -ME (Fig. 6B), essentially the loss MHC could all been recovered, which indicated that the increasing formation of disulfide bond or aggregates formed in this system.

FTIR

The amide I band between 1600 and 1700 cm^{-1} was investigated to gain insight in changes in secondary structure of the MP.[33] As shown in Table 1, nine bands were determined in the amide I region. These bands were located at the following positions: 1685, 1675, 1664, 1654, 1646, 1639, 1629, 1617and 1612cm^{-1}, which were similar to those reported for MP structures under heat induced oxidation.[34] The amounts of each secondary structural element given in percentage of the control and oxidized MPs are shown in Table 1. With the increasing addition of MDA, a continuous decrease ($p<0.05$) of amino acid side chain was observed, resulting in a significant decrease in arginine (Arg) at 1612 cm^{-1}. Further more, μ-NH2 groups of Arg are believed to be readily react with MDA. Buttkus[35] reported that the reaction of myosin with MDA modified 50-60% of the μ-NH2 in the protein. The intensity of band at 1639 cm^{-1}, a potential indicator for tightly bound water, was increased with the MDA addition ($P < 0.05$), suggesting an increase in WHC. With more addition of MDA (Fig.7), an increment in the percentage of ±-helical (central band at 1653 cm^{-1}) was observed ($P<0.05$), indicating a more compact structure of the protein gels. Ju and Kilara [36] also found a positive correlation between ±-helical content and gel hardness. An increase ($P<0.05$) in turn structure (from 11.85% to 14.65%) with the increase of MDA was found, indicating a more rigid state. Further more, a partial transition from ² -Sheet to

random structure was also observed for the protein gels with the addition of MDA (Table 1).This could be the formation of aggregates due to the enhanced protein-protein interaction under high oxidative stress (Fig. 3), which again were in good agreement with the surface hydrophobicity analysis (Fig. 2).

CONCLUSIONS

Results from this study demonstrated that exposure of myofibrillar protein to increased concentrations of MDA resulted in significant changes of protein structure, which further affected the rheological and gelling properties of myofibrillar protein. Increases in ² -sheets structure accompanied by decreases in turns, ±-helix, and random structures were observed after treatment with the oxidizing agent. Moderate oxidative modification (10 mM addition to the protein) improved the gel strength, WHC and network of the MP gel whereas the excessive oxidation led to a significant reduction in these qualities. This research could have provided new information for better understanding the effect of oxidation on meat protein.

Acknowledgments

We acknowledge the financial support by Jiangsu Province(China)“Collaborative Innovation Center for Food Safety and Quality Control” Industry Development Program, and Jiangsu Province (China) Infrastructure Project (Contract No. BM2014051) which have enabled us to carry out this study.

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Table 1 Structure assignment, wavenumber and percentage of secondary structure of the control and oxidized MP (mean values ± SD).

Structure	Wavenumber(cm^{-1})	Percentage(%)						
		control	0.5mM	2.5mM	5mM	10mM	25mM	50mM
AA side chains	1612	2.27±0.09^{f}	2.02±0.02^{e}	1.83±0.03^{d}	1.62±0.07^{c}	1.51±0.02^{b}	1.44±0.08^{ab}	1.36±0.06^{a}
² - sheet	1617	5.90±0.21^{e}	5.29±0.33^{d}	4.89±25^{c}	4.62±0.23^{bc}	4.52±0.17^{bc}	4.32±0.10^{ab}	4.1±0.21^{a}
² - sheet	1629	10.72±0.38^{a}	9.27±0.71^{b}	8.48±0.39^{b}	7.62±0.29^{a}	7.58±0.20^{a}	7.24±0.24^{a}	7.05±0.12^{a}
² - sheet	1639	13.90±0.68^{c}	13.55±0.5^{c}	13.2±0.17^{bc}	12.95±0.40^{abc}	12.86±0.11^{abc}	12.53±0.12^{ab}	12.36±0.15^{a}
Random	1646	12.93±0.17^{a}	12.8±0.27^{ab}	12.74±0.20^{ab}	12.67±0.30^{ab}	12.58±0.37^{ab}	12.46±0.17^{a}	12.33±0.09^{a}
± - Helix	1654	15.13±0.27^{a}	15.52±0.12^{b}	16.38±0.3^{bc}	17.12±0.17^{c}	17.65±0.17^{cd}	17.99±0.34^{d}	18.23±0.24^{d}
Random	1664	13.87±0.1^{a}	14.04±0.31^{ab}	14.26±0.28^{b}	14.47±0.06^{bc}	14.77±0.25^{bcd}	14.92±0.32^{cd}	15±0.12^{cd}
² - sheet	1675	13.46±0.11^{a}	13.88±0.26^{b}	14.31±0.2^{bc}	14.58±0.42^{c}	14.59±0.12^{c}	15.29±0.12^{d}	15.46±0.23^{d}
Turn	1685	11.84±0.2^{a}	12.22±0.21^{a}	13.83±0.64^{b}	14.36±0.23^{b}	14.46±0.2^{b}	14.72±0.14^{b}	14.65±0.53^{b}
Total ± - Helix		15.13±0.27^{a}	15.52±0.12^{b}	16.38±0.3^{bc}	17.12±0.17^{c}	17.65±0.17^{cd}	17.99±0.34^{d}	18.23±0.24^{d}
Total ² - sheet		43.98±0.42^{e}	41.99±0.29^{d}	40.88±0.32^{c}	39.77±0.36^{b}	39.55±0.20^{ab}	39.38±0.34^{ab}	38.97±0.45^{a}
Total Random		13.87±0.1^{a}	14.04±0.31^{ab}	14.26±0.28^{b}	14.47±0.06^{bc}	14.77±0.25^{bcd}	14.92±0.32^{cd}	15±0.12^{cd}
Total Turn		11.84±0.2^{a}	12.22±0.21^{a}	13.83±0.64^{b}	14.36±0.23^{b}	14.46±0.2^{b}	14.72±0.14^{b}	14.65±0.53^{b}
Total AA side chains		2.27±0.09^{f}	2.02±0.02^{e}	1.83±0.03^{d}	1.62±0.07^{c}	1.51±0.02^{b}	1.44±0.08^{ab}	1.36±0.06^{a}

Different superscripts in the same row indicate significant different (P d 0.05)

Figure captions

Fig. 1 Carbonyl and SH content of MP treated with different concentrations of MDA

Fig. 2 Surface hydrophobicity of MP at different oxidizing levels

Fig. 3 Tryptophan fluorescence of MP at different oxidizing levels

Fig. 4 Gel strength (A) and WHC（B）of MP at different oxidizing levels

Fig.5 Typical dependence profiles of storage modulus (G2) (A) and tan ′ (B) on frequency for MP gels at various MDA concentrations

Fig. 6 Representative SDS-PAGE patterns of the MDA-induced MP gels in the absence (A) or presence (B) of 5% ² -ME

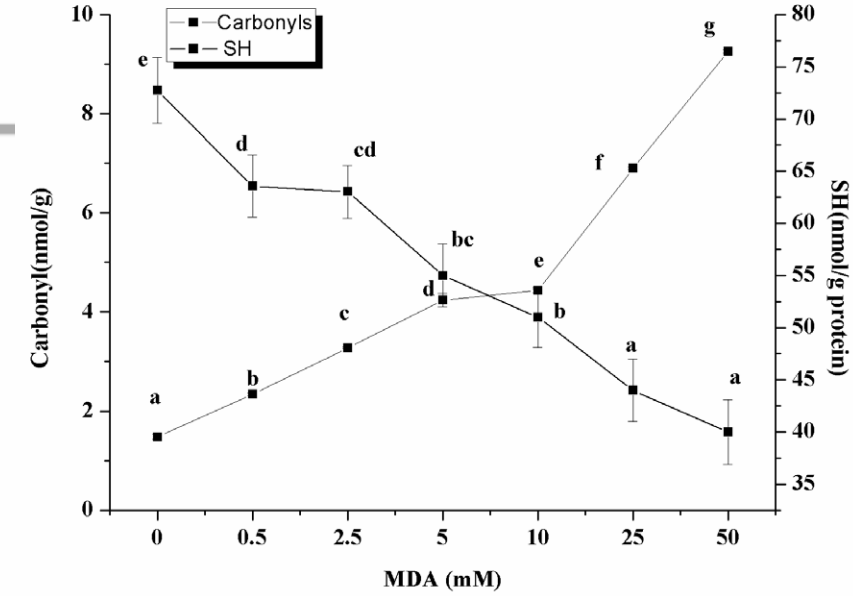


Fig.1 Carbonyl and SH content of MP treated with different concentrations of MDA

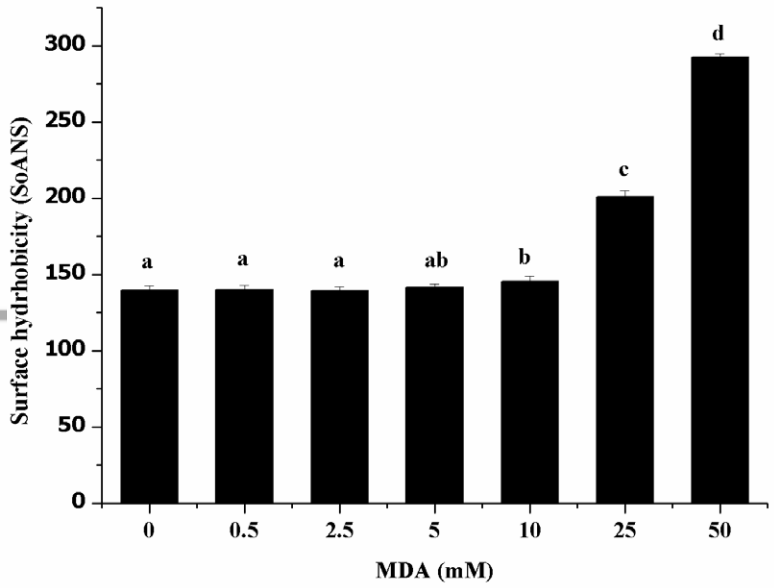


Fig.2 Surface hydrophobicity of MP at different MDA levels

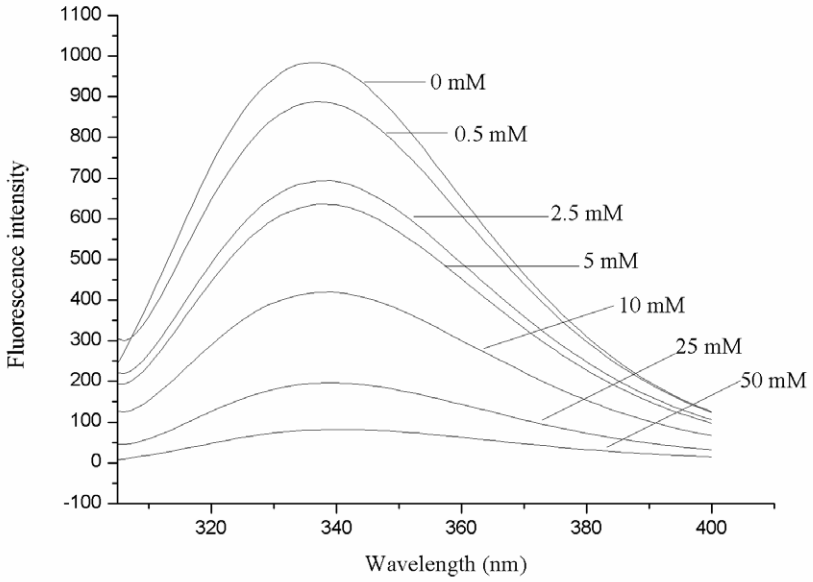
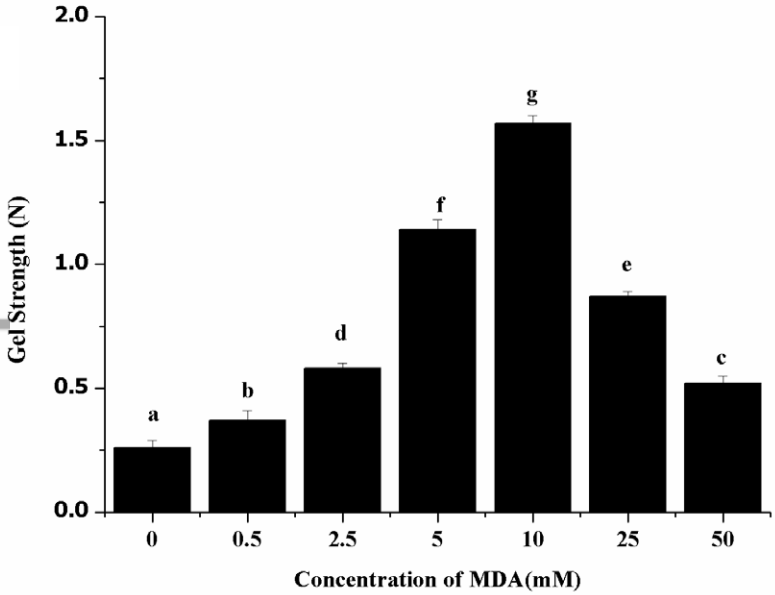


Fig.3 Tryptophan fluorescence of MP at different MDA levels

A



B

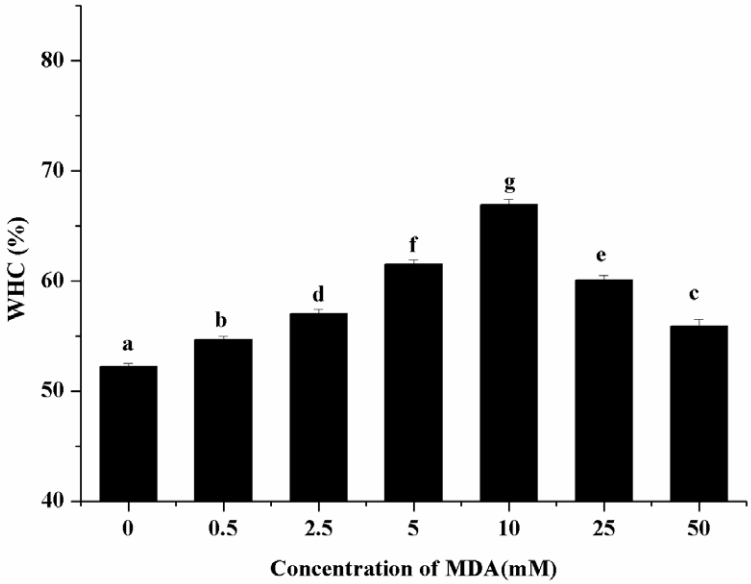


Fig 4 Gel strength (A) and WHC（B）of MP at different MDA levels

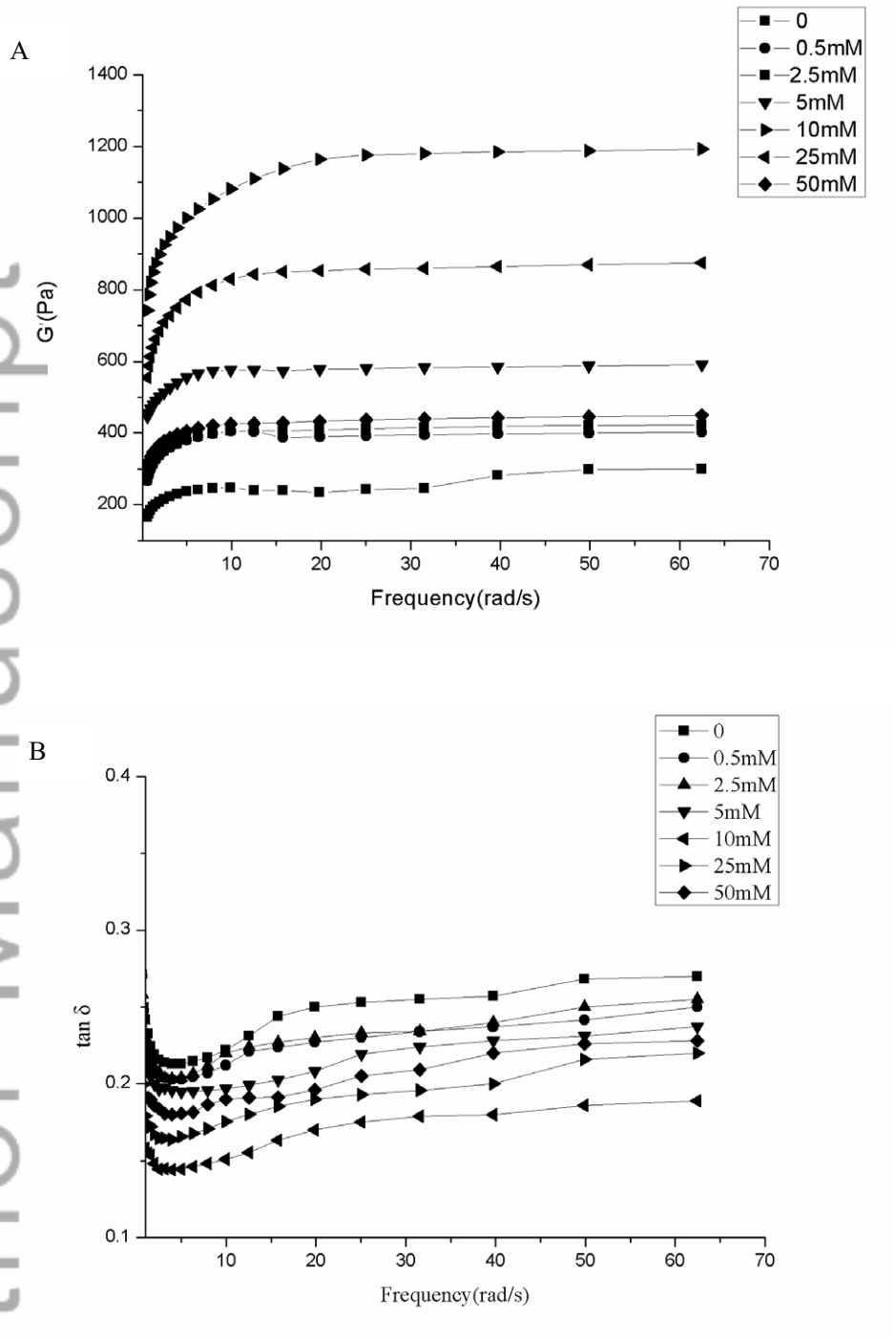


Fig.5 Typical dependence profiles of storage modulus (G2) (A) and tan′ (B) on frequency for MP gels at various MDA concentrations

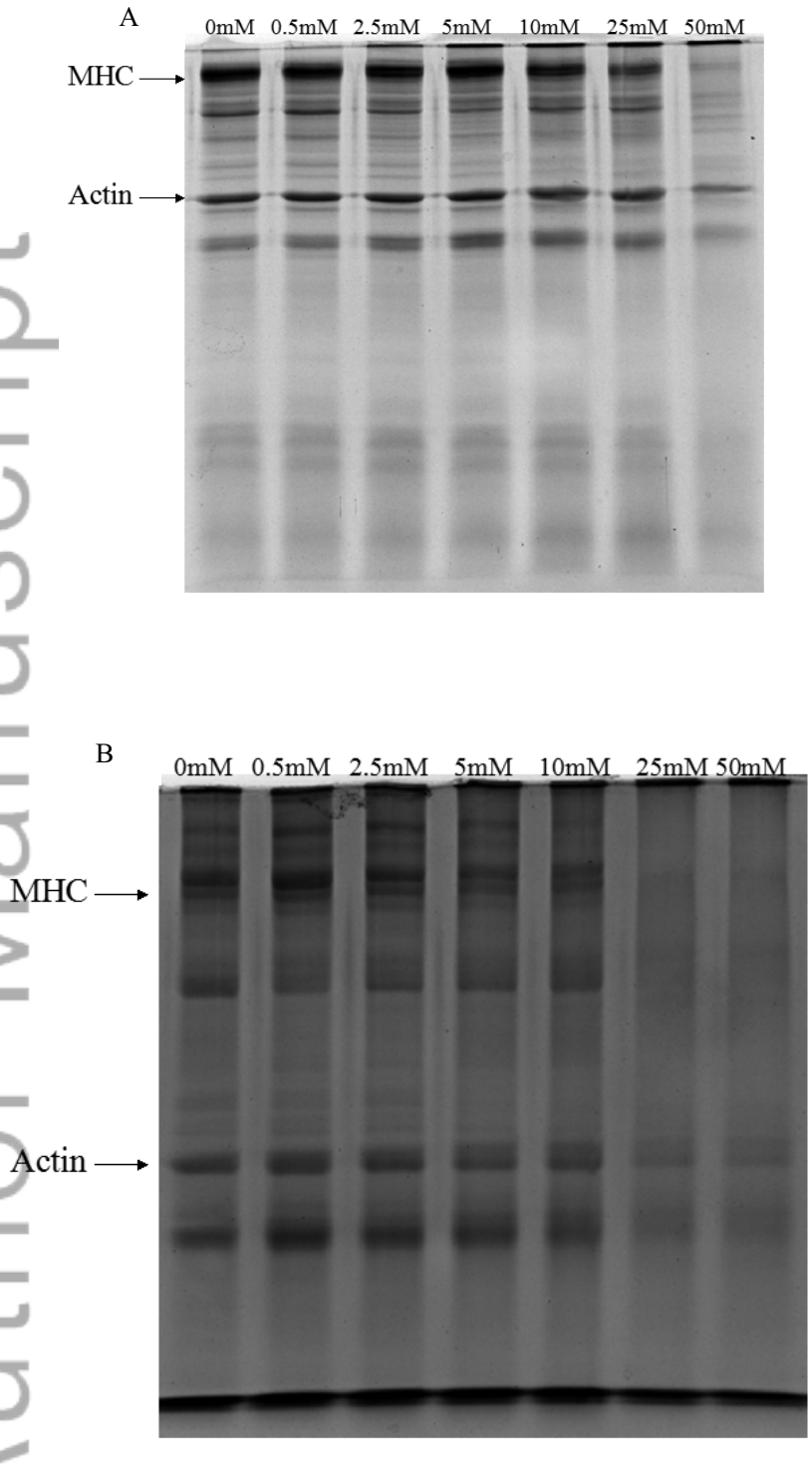


Fig.6 Representative SDS-PAGE patterns of the MDA induced MP gels in the

absence (A) or presence (B) of 5% ² -ME