Physicochemical changes of myosin and gelling properties of washed tilapia mince as influenced by oxidative stress and microbial transglutaminase

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Revised: 4 June 2014 / Accepted: 10 June 2014 / Published online: 21 June 2014
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Abstract Physicochemical properties of myosin from tilapia subjected to oxidation via Fenton's reaction using H_2O_2 (0, 0.05, 0.1, 1 and 5 mM) were determined. With increasing H_2O_2 concentrations and times (from 0 to 12 h), sulfhydryl group content and Ca^{2+} -ATPase activity decreased, while carbonyl content and surface hydrophobicity increased to a higher extent. After being subjected to oxidation, cross-linking via disulfide bond along with increased storage modulus (G') was observed. Microbial transglutaminase (MTGase) induced polymerization of myosin in both non-oxidized and oxidized forms and increased gel G' . Gel properties of washed mince and oxidized washed mince were determined in the presence and absence of MTGase. A stronger gel was observed when 0.3 unit MTGase/g was added, regardless of oxidation process. Nevertheless, the gel strengthening effect of MTGase was hampered when mince was subjected to severe oxidation. Excessive protein aggregation of oxidized samples prior to gelation resulted in the reduction of gel strength and water-holding capacity. Negative effect of protein oxidation on gelation could therefore be alleviated to some degree by MTGase addition.

Keywords Fish · Myosin · Protein oxidation · Gelation

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Introduction

Oxidation processes generally affect the quality of foods via inducing a number of changes in proteins such as modification of amino acid side chains, protein fragmentation, polymerization, and structural alteration. Several chemicals have been known to induce the oxidation. Fenton's reaction is one of the mechanisms to generate hydroxyl radicals ($\text{OH}^\cdot$) from H_2O_2 in the presence of Fe^{2+} . Hydroxyl radicals are reactive species, which can undergo side reaction with amino acid residues, react with another carbon-centered radical to form a protein-protein cross-links, etc. Those reactions lead to the changes in composition and configuration (Liu and Xiong 2000a; Berlett and Stadtman 1997).

Myofibrillar proteins, especially myosin, are well known to be primarily responsible for gelation therefore textural properties of comminuted muscle foods (Sun and Holley 2011). The heat induced gelation of myosin results in the formation of three-dimensional networks that hold water in a less mobile state. However, myosin is susceptible to oxidation, causing cross-linking between individual protein molecules. Cross-linked heterogeneous myosin can be formed in oxidized myofibrillar proteins (Liu and Xiong 2000a). The increase in carbonyl contents (Srinivasan and Hultin 1997; Rowe et al. 2004) was associated with the reductions in thermal stability of myosin (Liu and Xiong 2000b). Gelation of comminuted muscle foods is generally influenced by several physicochemical processes (Ooizumi and Xiong 2006). Oxidized proteins have varying functional properties, particularly gelation. Decker et al. (1993) reported that oxidation of turkey white muscle myofibrillar proteins by iron or copper and ascorbate caused a decreased gel strength. Srinivasan and Hultin (1997) also reported that frozen cod surimi treated with free-radical generating system had a poorer gel quality.

Transglutaminase (EC 2.3.2.13) is a transferase capable of catalyzing cross-linking, resulting in the formation of ϵ -(γ -

glutamyl) lysine cross-link in target proteins via acyl transfer between the ϵ -amino groups of a lysine residue and γ -amide group of a glutamine residue (DeJong and Koppelman 2002). Microbial transglutaminase (MTGase) has been widely used in processed fish and meat in order to strengthen protein-based gels. MTGase has been reported to increase gel strength of surimi from different fish such as lizardfish (Benjakul et al. 2008), threadfin bream (Benjakul et al. 2004; Jiang et al. 2000), and sardine (Kudre and Benjakul 2013; Karayannakidis et al. 2008). Efficiency of MTGase in improving gel properties of proteins depends on many factors, e.g., the amount of MTGase as well as protein substrates (Asagami et al. 1995; DeJong and Koppelman 2002).

Structural unfolding and aggregation are two common changes induced by oxidants, which result in, respectively, the exposure and masking of amino side chain groups recognized by enzymes. These changes could either favor or suppress the MTGase induced protein gelation (Li et al. 2012). However, there is no published report on the impact of MTGase on gelling properties of oxidized myosin in minced fish products despite the critical importance of gel formation and the susceptibility to oxidation (Decker et al. 1993; Srinivasan and Hultin 1997; Ooizumi and Xiong 2006). Thus, the objectives of this study were to determine the reactivity of MTGase toward both oxidized and non-oxidized myosin from tilapia, a major aquacultural species, and to study the effect of MTGase on gelling properties of washed mince as influenced by oxidation process.

Materials and methods

Chemicals/enzyme

All chemicals were of analytical grade. Ascorbic acid, sodium dodecyl sulphate (SDS), β -mercaptoethanol (β ME), Trolox, propyl gallate and glutaraldehyde were purchased from Sigma (St. Louis, MO, USA). Sodium hydroxide, hydrochloric acid, *N, N, N', N'*-tetramethyl ethylene diamine (TEMED), acrylamide, and bisacrylamide were procured from Fisher Scientific (Fair Lawn, NJ, USA). Microbial transglutaminase (MTGase; Activa-TI with the activity of 100 unit/g powder) from *Streptoverticillium mobaraense* containing 1 % pure enzyme blended with 99 % maltodextrin was donated by Ajinomoto Food Ingredients (Chicago, IL, USA).

Physicochemical changes in tilapia myosin as affected by oxidation

Fish samples

Live tilapia (*Oreochromis niloticus*) with the average size of 1–1.2 kg were obtained from a local market in Kentucky, U.S.A.

Fish were transported to the Department of Animal and Food Science, University of Kentucky within 1 h. Fish was then washed humanely killed, and filleted manually. The fillets were subjected to mincing using a mincer with the hole diameter of 5 mm and kept on ice during preparation.

Extraction of myosin

Myosin was isolated from fillet as described by Wang and Smith (1994) with a slight modification. All steps were performed below 10 °C to minimize proteolysis and protein denaturation. Fish mince was homogenized with 3 volumes of Guba-Straub solution (0.3 M KCl containing 0.1 M KH_2PO_4 , 50 mM K_2HPO_4 , 1 mM EDTA, and 4 mM sodium pyrophosphate) for 1 min using a Polytron model PT 10/35 (Brinkmann Instruments, Westbury, NY, USA) at a speed of 11,000 rpm. Thereafter, 3 volumes of distilled water were added to the mixture. The homogenate was filtered through 2 layers of cheesecloth and diluted with 6.5 volumes of 1 mM EDTA with rapid stirring. The mixture was kept at 4 °C overnight before centrifugation at 1000 $\times$ g for 1 h using a refrigerated centrifuge (Sorvell RC-5B, Newtown, CT, USA). The pellet was collected and resuspended with 2 volumes of 25 mM PIPES buffer, pH 7 containing 3 M KCl. The mixture was stirred for 30 min on ice and subsequently diluted with 5 volumes of distilled water. Magnesium chloride and sodium pyrophosphate were added to obtain the final concentrations of 5 mM and 3 mM, respectively. The mixture was centrifuged at 40,000 $\times$ g for 2 h at 4 °C. Myosin in the supernatant was isolated by fractional precipitation with ammonium sulfate (35–48 % saturation). The precipitate was collected and redissolved in 20 mM Tris-HCl (pH 6.8) containing 0.6 M KCl. After 24 h of dialysis against 100 volumes of the same buffer with two changes. The dialyzed solution was collected and measured for protein concentration according to the Biuret method (Robinson and Hogden 1940) using bovine serum albumin as standard. Densitometric data showed that the myosin sample had approximately 90 % purity.

Oxidation of myosin

Myosin was firstly diluted to a final protein concentration of 20 mg/ml in 20 mM Tris buffer containing 0.6 M NaCl (pH 6.8). This solution was then subjected to oxidation by hydroxyl radical generated by the Fenton's reaction. Hydroxyl radicals were produced by mixing 10 μM FeCl_3 /100 μM ascorbic acid with H_2O_2 at different concentrations (0.05, 0.1, 1 and 5 mM) for various times (2, 6, and 12 h) at 4 °C. The oxidation reaction was terminated by propyl gallate/Trolox C/EDTA to obtain the final concentration of 1 mM each. The non-oxidized myosin solution containing propyl

gallate/Trolox C/EDTA was used as the control. All samples were subjected to analyses:

Analyses

Total sulfhydryl group content Total sulfhydryl group content was determined using 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) according to the method of Ellman (1959) with a slight modification. To 1 ml of myosin solution (4 mg/ml), 9 ml of 0.2 M Tris-HCl buffer, pH 8, containing 8 M urea, 2 % SDS and 10 mM EDTA, were added. To 3 ml of the mixture, 0.3 ml of 0.1 % DTNB, dissolved in 0.2 M Tris-HCl (pH 8.0) was added and the mixture was incubated at 40 °C for 25 min. The absorbance at 412 nm was measured using a double beam spectrophotometer (model UV-1800, Shimadzu, Kyoto, Japan). A blank was conducted by replacing the sample with 0.6 M KCl. Sulfhydryl group content was calculated, using the extinction coefficient of $13,600 \text{ M}^{-1} \text{ cm}^{-1}$ and was expressed as $\text{mol}/10^5 \text{ g protein}$.

Carbonyl content Protein carbonyl content, an index of protein oxidation, was measured according to the method of Levine et al. (1990) with slight modifications. A 100- μl aliquot of protein solution (20 mg protein/ml) was reacted with 1 ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 N HCl for 1 h at room temperature; another 100 μl of sample was added with 1 ml 2 N HCl (control). After incubation, 1 ml of 20 % trichloroacetic acid was added to precipitate the protein. The mixture was centrifuged at 10,000 $\times g$ for 10 min using a microcentrifuge (Eppendorf-5415D, Eppendorf, Hamburg, Germany). The precipitate was washed twice with 1.5 ml of an ethanol:ethyl acetate (1:1; v/v) mixture to remove unreacted DNPH, blow-dried, and dissolved in 1 ml of 20 mM potassium phosphate (pH 2.3) containing 6 M guanidine hydrochloride. The absorbance was read at 370 nm for carbonyl content and 280 nm for protein content. The carbonyl concentration was calculated using a molar extinction coefficient of $22,000 \text{ M}^{-1} \text{ cm}^{-1}$.

Ca^{2+} -ATPase activity Ca^{2+} -ATPase activity of myosin with and without oxidation was determined according to the method of Benjakul et al. (1997). Diluted myosin solution (1 ml) was mixed with 0.6 ml of 0.5 M Tris-maleate (pH 7.0) and 1 ml of 0.1 M CaCl_2 . Deionized water was added to make up a total volume of 9.5 ml. To the mixture, 0.5 ml of 20 mM adenosine 5-triphosphate (ATP) solution was added to initiate the reaction. The reaction was conducted for 8 min at 25 °C and terminated by adding 5 ml of chilled 15 % (w/v) trichloroacetic acid. The reaction mixture was centrifuged at $3,500 \times g$ for 5 min and the inorganic phosphate liberated in the supernatant was measured by the method of Fiske and Subbarow (1925). The Ca^{2+} -ATPase activity was expressed as micromoles inorganic phosphate released/mg protein/min.

A blank was prepared by adding chilled trichloroacetic acid prior to addition of ATP.

Surface hydrophobicity Surface hydrophobicity was determined by assessing fluorescence intensity using 8-anilino-1-naphthalene sulfonate (ANS), as a fluorescence probe. A series of protein solutions (0.2–3 mg/ml, 4 ml) were thoroughly mixed with 20 μl of 8.0 mM ANS, and the fluorescence intensity was measured after 20 min using a FluoroMax-3 spectrofluorometer (Horiba JobinYvon Inc., Edison, NJ, USA) at the excitation wavelength of 374 nm and the emission wavelength of 485 nm. Protein surface hydrophobicity was calculated from initial slopes of plots of relative fluorescence intensity versus protein concentration (mg/ml) using a linear regression analysis. The initial slope was referred to as “SoANS”.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) Protein patterns were determined using SDS-PAGE under reducing and non-reducing conditions according to the method of Laemmli (1970). The samples were mixed with an equal volume of sample buffer with and without 10% β -mercaptoethanol (β ME), representing the reducing and non-reducing condition, respectively. The mixtures were then boiled for 3 min. The samples (20 μg protein) were loaded onto the polyacrylamide gel made of 10 % running gel and 4 % stacking gel and subjected to electrophoresis at a constant current of 15 mA per gel, using a Mini Protein III unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.02 % (w/v) Coomassie Brilliant Blue R-250 in 50 % (v/v) methanol and 7.5 % (v/v) acetic acid and destained with 50 % methanol (v/v) and 7.5 % (v/v) acetic acid, followed by 5 % methanol (v/v) and 7.5 % (v/v) acetic acid.

MTGase cross-linking of oxidatively stressed myosin

Oxidized myosin was prepared using 10 μM FeCl_3 /100 μM ascorbic acid in the presence of H_2O_2 at different concentrations (0.05, 0.1, 1 and 5 mM) for various times (2, 6, and 12 h) at 4 °C. Myosin (non-oxidized) and oxidized myosin samples in 20 mM Tris-HCl containing 0.6 M NaCl (pH 6.8) were incubated with MTGase at 25 unit/g protein at 4 °C for 2 h. All samples were subjected to analyses as below:

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were subjected to SDS-PAGE as mentioned above.

Rheological test

For oscillatory shear analysis, a Bohlin VOR rheometer (Bohlin Instruments, Inc., Cranbury, NJ, USA) was used to

examine the dynamic formation of a protein network during thermal process. Samples were heated from 20 to 80 °C at a 1 °C/min between two parallel plates (1 mm gap) in an oscillatory mode at a fixed frequency of 0.1 Hz with a maximum strain of 0.02. Changes in the storage modulus, G' (i.e., rigidity due to elastic response), were recorded.

MTGase effect on gel properties of washed mince as affected by oxidation process

Preparation of washed mince with and without oxidation

Tilapia fillets were subjected to mincing using a mincer with the hole diameter of 5 mm. Mince obtained were placed in polyethylene bag and imbedded in ice until use.

To prepare washed mince, the conventional washing process was implemented. Mince was washed with cold water (4 °C) using a water/mince ratio of 3:1 (w/w). The mixture was stirred gently for 10 min in a cold room (4 °C) and the washed mince was filtered with a layer of nylon screen. Washing was performed three times. Finally, the washed mince was centrifuged at $700\times g$ for 15 min using a basket centrifuge (Model CE 21 K, Grandiumpiant, Belluno, Italy). To prepare the oxidized mince, the washed mince was mixed with 10 μM FeCl_3 , 100 μM ascorbic acid and 0.1 mM H_2O_2 using a mince/solution ratio of 1:3 (w/v). The mixture was stirred gently for 10 min in a cold room (4 °C), followed by filtration using a layer of nylon screen. The sample was then washed with cold water again before centrifugation at $700\times g$ for 15 min using a basket centrifuge. The obtained mince samples were used for gel preparation.

Gel preparation

To prepare the gels, mince samples were ground for 2 min using a MoulinexMasterchef 350 mixer (Paris, France). Moisture content was adjusted to 82 % and NaCl was added to the samples to obtain the concentration of 2.5 % (w/w). After grinding for 2 min, the paste was added with various amounts of MTGase (0, 0.3 and 0.6 units/g sample). The mixture was chopped for another 2 min at 4 °C to obtain the homogenous paste. The paste was then stuffed into polyvinylidene casing with a diameter of 2.5 cm and both ends of casing were sealed tightly. Two-step heated gels were prepared by setting the paste at 40 °C for 30 min, followed by heating at 90 °C for 20 min in a temperature controlled water bath (Memmert, Schwabach, Germany). The gels were then cooled in iced water and stored for 24 h at 4 °C prior to analyses.

Analyses

Texture analysis Texture analysis of gels was performed using a Model TA-XT2 texture analyzer (Stable Micro

System, Surrey, UK). Gels were equilibrated at room temperature for 2 h before analysis. Five cylindrical samples (2.5 cm in length) were prepared and tested. Breaking force (gel strength) and deformation (elasticity/deformability) were measured by the texture analyzer equipped with a spherical plunger (5-mm diameter; depression speed 60 mm/min).

Expressible moisture content Expressible moisture content (%) was measured according to the method of Benjakul et al. (2008). Cylindrical gel samples were cut into a thickness of 5 mm, weighed (X), and placed between 2 pieces of Whatman paper no. 1 at the bottom and 1 piece of paper on the top. A standard weight (5 kg) was placed on the top of the sample for 2 min, and then the sample was removed from the papers and weighed again (Y). Expressible moisture content was calculated and expressed as percentage of sample weight as follows:

$$\text{Expressible moisture content (\%)} = [(X-Y)/X] \times 100$$

Whiteness Gel samples were subjected to whiteness measurement using a colorFlex (HunterLab, Reston, Va., USA). Illuminant C was used as the light source of measurement. CIE L^* , a^* , and b^* values were measured. Whiteness was calculated using the following equation (NFI 1991):

$$\text{Whiteness} = 100 - [100 - (L^{*2} + a^{*2} + b^{*2})]^{1/2}$$

Microstructure Microstructure of gels was determined using a scanning electron microscope (SEM). Gels were cut into small pieces ($0.25\times 0.25\times 0.25\text{ cm}^3$) and fixed with 2.5 % glutaraldehyde in 0.2 M phosphate buffer, pH 7.2 for 2 h at room temperature. The fixed samples were rinsed with distilled water twice. Fixed specimens were dehydrated in graded ethanol solution with serial concentrations of 50, 70, 80, 90, and 100 %. Samples were subjected to critical point dried (Balzers mod. CPD 030, Liechtenstein, Switzerland) using CO_2 as transition fluid. The prepared samples were mounted on copper specimen holders, sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA) and examined on a FEI Quanta 400 scanning electron microscope (FEI Company, Hillsboro, OR, USA) at an acceleration voltage of 20 kV.

Statistical analysis

The experiments were run in triplicate with three different lots (replicates) of samples. Data were subjected to analysis of variance (ANOVA) and mean comparison was

carried out using Duncan's multiple range tests. *T*-test was used for pair comparison (Steel and Torrie 1980). Analysis was performed using the Statistical Package for Social Science package (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

Results and discussion

Physicochemical properties of myosin as affected by oxidation process

Total sulfhydryl group content

Total sulfhydryl (SH) group contents of myosins oxidized with H_2O_2 (0–5 mM) in the presence of FeCl_3 and ascorbic acid for 2–12 h are shown in Table 1. SH group content of oxidized myosin decreased as the concentration of H_2O_2 and incubation time increased ($P<0.05$), suggesting the increasing formation of disulfide bonds. When H_2O_2 at a concentration of 5 mM was used, the decrease in SH group by 50.2–54.8 % was obtained, compared with that observed in the control. SH groups, which are abundant in myofibrillar protein, undergo inter- and intramolecular disulfide cross-linking, which occurs during radical-mediated oxidation of proteins (Dean et al. 1997). Hydroxyl radical formed by the Fenton's reaction caused the reduction of SH group by 7–55 %, compared to that found in the control myosin. Cysteine residues are particularly sensitive to oxidation and converted to disulfides by all forms of reactive oxygen species even under mild condition (Berlett and Stadtman 1997). Frederiksen et al. (2008) found that SH groups of myosin are the main target for oxidative modification induced by hypervalent myoglobin species. (Liu

et al. 2000) also reported the reduction in SH group content of myofibrillar protein from pectoralis muscle after the protein was oxidized for 1 h and continued to decrease after 24 h with the concomitant increase in disulfide bonds. Thus, the oxidation process resulted in the formation of disulfide bond in myosin.

Carbonyl content

Carbonyl content is one of the most reliable measures of protein oxidation (Levine et al. 1990). Carbonyl contents of myosin under different oxidation condition are shown in Table 1. Carbonyl contents of oxidized myosin increased as the higher levels of H_2O_2 and longer incubation time were used ($P<0.05$). Myosin oxidized using 5 mM H_2O_2 for 12 h showed approximately 5-fold increase in carbonyl contents, compared to the control (non-oxidized). Li et al. (2012) reported that the exposures of myofibrillar protein extracted from longissimus muscle to 5 mM H_2O_2 in the presence of FeCl_3 for 24 h significantly increased carbonyl content (2.76 $\mu\text{mol/g}$ protein) of resulting protein. The increase in protein carbonyls is one of the key biochemical changes that occur during protein oxidation. Carbonyl groups (aldehydes and ketones) are produced on protein side chains (especially of Pro, Arg, Lys, and Thr) when they are oxidized (Estévez 2011). Protein-bound carbonyls can also be derived from direct oxidative attack on amino acid side chains, fragmentation of the peptide backbone via α -amidation pathway, or cleavage associated with the oxidation of glutamyl residues (Estévez 2011). The cleavage of peptide backbones and covalent attachments of secondary lipid oxidation products, such as malondialdehyde and 4-hexyl-2 nonenal, also contributed to carbonyl formation in myofibrillar proteins (Estévez 2011;

Table 1 Physicochemical changes of myosin from tilapia as affected by oxidation via Fenton's reaction using H_2O_2 at various concentrations for different times

H_2O_2 (mM)	Time (h)	Total sulfhydryl content (mol/ 10^5 g protein)	Carbonyl content ($\mu\text{mol/g}$ protein)	Ca^{2+} -ATPase activity ($\mu\text{mol Pi/mg}$ protein/10 min)	Surface hydrophobicity
0	0	2.21 $\pm$ 0.12 ^c	0.41 $\pm$ 0.07 ^a	1.58 $\pm$ 0.05 ^j	85.42 $\pm$ 1.88 ^a
0.05	2	2.05 $\pm$ 0.20 ^c	0.60 $\pm$ 0.05 ^{ab}	1.54 $\pm$ 0.02 ^{ij}	89.46 $\pm$ 3.60 ^b
	6	2.02 $\pm$ 0.03 ^{bc}	0.75 $\pm$ 0.26 ^{abc}	1.50 $\pm$ 0.03 ^{hi}	93.93 $\pm$ 2.43 ^c
	12	1.94 $\pm$ 0.04 ^{bc}	0.93 $\pm$ 0.10 ^{abcd}	1.48 $\pm$ 0.05 ^{gh}	102.05 $\pm$ 0.03 ^e
0.1	2	2.06 $\pm$ 0.19 ^c	0.88 $\pm$ 0.08 ^{abcd}	1.50 $\pm$ 0.03 ^{hi}	98.72 $\pm$ 0.06 ^d
	6	1.94 $\pm$ 0.02 ^{bc}	0.94 $\pm$ 0.06 ^{abcd}	1.48 $\pm$ 0.03 ^{gh}	101.19 $\pm$ 1.02 ^e
	12	1.95 $\pm$ 0.01 ^{bc}	1.25 $\pm$ 0.34 ^{cde}	1.45 $\pm$ 0.03 ^{fg}	109.44 $\pm$ 0.29 ^f
1	2	1.71 $\pm$ 0.03 ^{bc}	1.12 $\pm$ 0.34 ^{bcde}	1.41 $\pm$ 0.02 ^f	108.28 $\pm$ 0.07 ^f
	6	1.69 $\pm$ 0.02 ^{bc}	1.24 $\pm$ 0.18 ^{cde}	1.29 $\pm$ 0.01 ^e	119.83 $\pm$ 0.21 ^g
	12	1.61 $\pm$ 0.18 ^{bc}	1.45 $\pm$ 0.15 ^{cfig}	1.11 $\pm$ 0.02 ^d	136.29 $\pm$ 0.17 ⁱ
5	2	1.10 $\pm$ 0.17 ^a	1.42 $\pm$ 0.43 ^{cfig}	1.06 $\pm$ 0.02 ^c	133.58 $\pm$ 0.52 ^h
	6	1.03 $\pm$ 0.02 ^a	1.61 $\pm$ 0.12 ^{fg}	0.78 $\pm$ 0.01 ^b	144.71 $\pm$ 0.20 ^j
	12	1.00 $\pm$ 0.01 ^a	1.95 $\pm$ 0.23 ^g	0.42 $\pm$ 0.02 ^a	153.98 $\pm$ 0.52 ^k

Values are given as mean $\pm$ SD ($n=3$)

*Different lowercase superscripts in the same column indicate the significant differences ($P<0.05$)

Xiong 2000). In addition, Estévez et al. (2009) reported that specific carbonyl compounds in oxidized myofibrillar protein namely, α -aminoadipic and γ -glutamic semialdehydes, were formed from oxidized lysine, proline and/or arginine in the presence of Fe^{3+} and H_2O_2 . These semialdehydes contributed to 70 % carbonyls found in oxidized proteins. Therefore, tilapia myosin was susceptible to oxidation when exposed to hydroxyl radicals generated by Fenton's reaction.

Ca^{2+} -ATPase activity

Ca^{2+} -ATPase activity of non-oxidized and oxidized myosin as a function of H_2O_2 levels and incubation time is shown in Table 1. Ca^{2+} -ATPase of oxidized myosin decreased with time and H_2O_2 concentrations increased. The result suggested that denaturation of myosin was pronounced by the oxidation. With increasing level of H_2O_2 , hydroxyl radicals were generated to a higher extent. As a consequence, those radicals could induce the oxidation of protein, leading to the loss of ATPase activity found at the head of myosin heavy chain. Ca^{2+} -ATPase activity is considered to be a good indicator of integrity of myosin molecule (Roura and Crupkin 1995).

The decrease in Ca^{2+} -ATPase activity was in agreement with the lowered SH group content and increased carbonyl group content (Table 1). Enhanced protein oxidation and reduced SH group content were suggested to be related with the inactivation of Ca^{2+} -ATPase (Klebl et al. 1998). Reactive SH groups located at myosin head are involved in ATPase activity (Wells et al. 1979). The oxidation of sulfhydryl groups, especially in the head region caused the decrease in Ca^{2+} -ATPase activity (Sompongse et al. 1996; Benjakul et al. 2003). Moreover, the reduced Ca^{2+} -ATPase activity might be governed by structural changes of myosin heavy chain, especially at head domain induced by hydroxyl radicals (Ishibashi et al. 1996; Xu et al. 1997). The result suggested that hydroxyl radicals more likely induced the denaturation of myosin and the rate of denaturation was determined by H_2O_2 concentration in Fenton's reaction as well as reaction time.

Surface hydrophobicity

Changes in surface hydrophobicity of tilapia myosin were observed upon the oxidation by Fenton's reaction as influenced by H_2O_2 concentrations and incubation time (Table 1). Surface hydrophobicity of myosin increased as the concentration of H_2O_2 and time increased ($P < 0.05$). After incubation for 12 h with 5 mM H_2O_2 in the presence of FeCl_3 and ascorbic acid, surface hydrophobicity increased by 80.2 %, compared with the control (non-oxidized). During the oxidation, the proteins underwent the conformational changes, in which the hydrophobic portions which buried inside the protein molecules were exposed. As a consequence,

conformational changes of the peptide chain occurred and reformed in a manner different from those in the native structure (Morawetz 1972). Chao et al. (1997) reported that the exposure of liver proteins to a metal-catalyzed oxidation system or peroxy radical generating system led to the increases in surface hydrophobicity. Li et al. (2012) also found that hydrophobicity of myofibrillar protein under mild oxidative condition increased. Oxidation process therefore induced the configuration changes of myosin, reflecting the denaturation of myosin.

SDS-PAGE

Protein patterns of non-oxidized myosin and those oxidized by H_2O_2 at different levels in the presence of FeCl_3 and ascorbic acid as the function of time are depicted in Fig. 1a. In the absence of βME , the oxidized myosin had the decrease in band intensity of myosin. The decrease was more pronounced when H_2O_2 concentration and incubation time increased. At the high concentration of H_2O_2 (5 mM), the myosin band intensity completely disappeared, especially for samples incubated for 6 h and 12 h. Compared to control (non-oxidized), the changes in band intensity of sample oxidized with H_2O_2 up to 0.1 mM H_2O_2 was negligible, when incubation times of 2 and 6 h were used. The disappearance of myosin band was more likely due to the formation of large aggregates localized on the stacking gel.

Under the reducing condition, myosin band was almost recovered, indicating that the cross-linking of myosin took place mainly via disulfide bonds. The result was in accordance with the loss of SH group, particularly when the higher concentration of H_2O_2 was used (Table 1). A slight decrease in band intensity of myosin was found as the high concentration of H_2O_2 and longer time were used. Liu and Xiong (2000a) reported that the $\text{FeCl}_3/\text{H}_2\text{O}_2$ /ascorbate-induced oxidation caused fragmentation and polymerization of myosin from chicken breast and the cross-linking was mediated mainly by disulfide bonds. As indicated by Stadtman and Berlett (1997), oxidation may induce formation of protein aggregates through Schiff base adducts or through formation of carbon-carbon covalent bonds by the interaction of carbon-centered radicals in protein molecules or due to the formation of other covalent bonds, such as Tyr–Tyr and active carbonyl– NH_2 interactions (Baron et al. 2007; Li et al. 2012; Xiong et al. 2010). Thus, the non-disulfide covalent cross-links could be formed in myosin, when hydroxyl radicals were generated at higher extent.

MTGase cross-linking of myosin as affected by oxidation process

SDS-PAGE

When MTGase was incorporated in myosin and those subjected to oxidation under various conditions, it was

Fig. 1 Protein patterns of myosin and those subjected to oxidation via Fenton's reaction using H_2O_2 at various concentrations for different times in the absence (a) and presence (b) of MTGase at 25 unit/g protein. SDS-

PAGE was conducted under non-reducing and reducing conditions. Numbers designate the H_2O_2 concentration (mM) and incubation time (h)

noted that band intensity of myosin in all samples were decreased (Fig. 1b), compared with that found in the corresponding sample without MTGase (Fig. 1a). It was postulated that MTGase could induce the cross-linking in both non- and oxidized myosin. It was noted that similar protein patterns were found between those without and with MTGase addition (Fig. 1a and b). Under reducing condition, most of MHC band was regained. However, the band intensity was still lower than those found in sample without MTGase addition. The result indicated that MTGase could induce cross-linking of myosin via non-disulfide covalent bond to some degree. Those bonds were not destroyed by β ME used. Li et al. (2012) found that more loss of MHC band intensity from oxidized myofibrillar protein from pork was noticed, compared to non-oxidized one when MTGase was incorporated. MTGase was therefore capable of cross-linking of myosin, regardless of oxidation.

Rheology

Non-oxidized and oxidized myosin under different oxidizing conditions showed varying rheological property. Storage modulus (G') of different myosins during heating was monitored as shown in Fig. 2. Generally, G' value is a measure of deformation energy stored in the sample during shear process, representing the elastic behavior of a sample (Tabilo-Munizaga and Barbosa-Cánovas 2005). The increase in G' of all myosins was observed at 40 °C, indicating the onset of gelation or the formation of an elastic protein network. In control sample (without oxidation), the G' reached the maximum of the first peak at around 42–43 °C, followed by a slight decrease. The initial increase of G' could be related to interactions that occurred between protein molecules at low temperatures. The subsequent decrease was possibly due to disentanglement and the increased mobility of myosin molecules as a result of

Fig. 2 Rheogram of myosin and myosin subjected to oxidation via Fenton's reaction using H_2O_2 at various concentrations for different times without (a) or with MTGase at 25 unit/g protein (b)

breaking of protein–protein bonds (Chen et al. 1999; Lanier et al. 2004). Denaturation (unfolding) of heavy meromyosin and cross-linking of myosin filaments were responsible for the initial G' increase at $<50^\circ\text{C}$ (Egelandsdal et al. 1986). Denaturation of light meromyosin and subfragment-1 (S-1), which leads to increased filamental “fluidity”, caused G' to temporarily decrease (Brenner et al. 2009; Choi and Kim 2009; Liu et al. 2014). Subsequently, G' of myosin solution increased. This second increase in G' was more likely related to the formation of more permanent, irreversible myosin filaments or complexes. The phase angle, a ratio of G''/G' , decreased when the samples were heated from 20 to 37°C . It increased and reached the maximum at 40°C . Thereafter, it decreased gradually and remained constant during heating at 50 – 80°C (data not shown). Changes in

the phase angle reflected a transition of the viscous myosin sol to elastic myosin gel, which was in accordance with the increase of G' . Yongsawatdigul and Park (2003) reported that the unfolding of actomyosin helical structure, hydrophobic interaction and disulfide formation took place and became greater at high temperature ($>50^\circ\text{C}$).

When myosin was oxidized, a higher G' with the lower phase angle was found, compared with that of the control (data not shown). This indicated that the larger aggregation induced by oxidation condition resulted in enhanced entanglement, associated with increased viscosity. At final temperature, oxidized myosin using 1 mM H_2O_2 for 12 h exhibited the highest G' , while the control sample showed the lowest G' . Further oxidation using higher H_2O_2 concentration slightly lowered the G' . Thus, degree of protein cross-linking directly affected the network formation as evidenced by different G' .

After oxidation, most myosin underwent cross-linking, mainly via disulfide bond as evidenced by the loss of SH group and reduction of myosin band in SDS-PAGE (Fig. 1b). Therefore, less viscosity with more solid-like was found due to strong protein aggregation. When oxidation took place under the strong condition (5 mM H_2O_2), the excessive cross-linking of myosin before heating could cause premature aggregation, thereby limiting ordered interactions of reactive functional groups. This resulted in the inhibition of fine gel network formation (Liu et al. 2000). Xiong et al. (2010) investigated the oxidation in pectoralis myofibrillar protein from chicken. Myosin tail (light meromyosin or rod) was likely susceptible to hydroxyl radicals attack and the subsequent aggregation of myosin monomers via tail–tail interaction occurred. However, aggregation of non-oxidized myosin occurred by head–head interaction (Ooizumi and Xiong 2006).

When MTGase (25 unit/g) was added, both non-oxidized and oxidized samples had the higher final G' at 80 °C than those without MTGase addition. MTGase catalyzes the cross-linking of polypeptides through the formation of isopeptides between lysine and glutamine residues (Folk 1970). For the oxidized samples, the final G' decreased when oxidation took place under strong condition (using 5 mM H_2O_2). Due to high reactivity of carbonyls with free amines, particularly $\epsilon\text{-NH}_2$ of lysine, oxidation-induced carbonyl production was probably an important cause for the reduced MTGase cross-linking in myosin exposed to higher concentrations of H_2O_2 . Both the production and the consumption of carbonyls appeared to be at the expense of lysine and affected MTGase catalysis (Li et al. 2012). Moreover, disulfide bond formed during oxidation might lower the accessibility of reactive Glu and Lys or yielded the steric hindrance for MTGase cross-linking. Therefore, the degree of protein oxidation affected the rheological property of tilapia myosin.

Effect of MTGase on gelling properties of washed mince as affected by oxidation process

Breaking force and deformation

Breaking force and deformation of gels from washed mince (control) and oxidized washed mince added without and with MTGase at different levels (0–0.6 unit/g) are depicted in Fig. 3. Generally, breaking force of the gel was positively correlated with gel strength, while the deformation represented the elasticity of the gels. Without MTGase addition, the control gels had higher breaking force and deformation, compared to those from oxidized washed mince ($P<0.05$). Breaking force and deformation of gel from oxidized washed mince decreased by 24 % and 32 %, respectively, compared with those of control gel. Oxidation was associated with lowered solubility, caused by cross-linking. Protein conformation and cross-linking were induced by the oxidation as evidenced by the increases in

Fig. 3 Breaking force and deformation of gels from washed mince and oxidized washed mince as affected by addition of MTGase at different levels. Bars represent the standard deviation ($n=3$). Different letters within same oxidative condition indicate significant differences ($P<0.05$). Different capital letters within the same level of MTGase indicate significant differences ($P<0.05$). Numbers designate the level of MTGase added (unit/g)

surface hydrophobicity and carbonyl content, with the loss of SH group content and Ca^{2+} -ATPase activity (Table 1). Li et al. (2012) also reported that changes in protein induced by radicals were manifested by carbonylation of amino acid side chains, cleavage of peptide backbones and formation of disulfide cross-linking. Those changes might be associated with poorer gelation. Xiong et al. (1993) reported that the inhibition of oxidation by using propyl gallate, ascorbate and tripolyphosphate during washing resulted in a strong gel of restructured meat. When protein was oxidized, cross-linking via disulfide bonds or protein-protein interaction via Schiff-based occurred, leading to lower solubility of protein. During setting at 40 °C, the formation of isopeptide catalyzed by endogenous transglutaminase has been reported to play a crucial role in

gel strengthening of processed fish product (Kamath et al. 1992). Oxidation mediated by hydroxyl radicals could induce the loss of activity of endogenous transglutaminase. As a result, the setting phenomenon could be impeded. Thus, oxidation taken place before gel setting resulted in the lower breaking force and deformation of gels. In addition, not only protein was oxidized but some lipids which were still retained after washing process could also be oxidized. The compounds generated from lipid oxidation can modify proteins by inducing cross-linking, resulting in modifications of amino acids and a decrease in protein functionality including gelation (Eymard et al. 2009).

Breaking force of control gels increased as MTGase at the higher levels was incorporated. For oxidized samples, MTGase at 0.3 unit/g increased breaking force ($P<0.05$). Nevertheless, the addition of MTGase at 0.6 unit/g did not increase breaking force of the gel ($P>0.05$). Higher amount of MTGase might induce the formation of non-disulphide covalent bond to a greater extent. As a result, the strength of gel matrix was enhanced. MTGase catalyses an acyl-transfer between lysine and glutamine residues of proteins. There was no difference in breaking force and deformation between oxidized and non-oxidized sample when MTGase 0.3 unit/g was added. However, with the addition of 0.6 unit/g MTGase, gel from the oxidized washed mince had the decreases in breaking force and deformation by 19.7 % and 9.8 %, respectively, compared to those of non-oxidized samples. Disulfide bond regulated by hydroxyl radicals might cause the cross-linking, in the way which lowered the accessibility of glutamine and lysine for MTGase reaction. Oxidation induced carbonyl production was probably one of the important causes for reduced MTGase cross-linking (Li et al. 2012). The addition of MTGase at 0.3 unit/g was able to induce protein cross-linking and improve property of gel from oxidized mince to be equivalent to the control gel. Visessanguan et al. (2003) also reported that the iron-catalyzed oxidation decreased the gel-forming ability of big-eye snapper (*Priacanthustayenus*) and the addition of MTGase could partially recovered the gel strength and setting response to some degree. Therefore, oxidation of muscle protein mainly reduced breaking force and deformation of gel. With the excessive amount of MTGase, the cross-linking of previously oxidized proteins with the large aggregates might occur, in which gel with coagulated network was formed. This led to poor gel network. However, the addition of MTGase at an appropriate level was able to improve gel strength of oxidized mince to some extent.

Expressible moisture

Expressible moisture content of gels from washed mince (control) and oxidized washed mince added with MTGase at different levels (0–0.6 unit/g) is shown in Table 2. In the absence of MTGase, oxidized samples

Table 2 Expressible moisture content and whiteness of gels from washed mince and oxidized washed mince added with MTGase at different levels

MTGase levels	samples	Expressible moisture (%)	Whiteness
0	Control	3.88±0.41 ^{ba}	86.09±0.27 ^{ba}
	Oxidized	4.03±0.26 ^{ba}	86.62±0.38 ^{ba}
0.3	Control	3.72±0.18 ^{abA}	85.32±0.51 ^{ba}
	Oxidized	3.76±0.19 ^{aA}	85.30±0.59 ^{aA}
0.6	Control	3.17±0.25 ^{ab}	85.04±0.55 ^{aA}
	Oxidized	3.70±0.15 ^{aA}	85.24±0.47 ^{aA}

Values are given as mean±SD ($n=3$)

*Different lowercase superscripts in the same column indicate the significant differences ($P<0.05$)

** Different uppercase superscripts in the same column under the same MTGase levels indicate the significant differences ($P<0.05$)

showed the non-significantly higher expressible moisture content, compared to control gel. The lower expressible moisture content of gels suggested more water retained in the gel network (Niwa 1992). When MTGase was added, the decrease in expressible moisture content was observed. For the control gel, the expressible moisture content decreased as MTGase level increased. However, no significant differences in expressible moisture content were obtained between gels from oxidized washed mince added with MTGase at 0.3 and 0.6 unit/g ($P>0.05$). Bertram et al. (2007) reported that reduced WHC of purified myofibrils upon oxidation with Hb and H_2O_2 was found together with an increase in formation of the cross-linked oxidation product. When MTGase at 0.6 unit/g was added, expressible moisture content of control and oxidized sample was reduced by 18.3 % and 8.2 %, respectively compared to those without MTGase addition. It was noted that the addition of MTGase could increase the ability of gel in water holding as evidenced by the lowered expressible moisture content ($P<0.05$). MTGase induced protein cross-linking via covalent cross-linking, causing the water to be bound or retained in the gel network with more inter-connection. Moreno et al. (2008) reported that the addition of 1 % MTGase increased water holding capacity of restructured fish muscle. The result suggested that water holding capacity of gel network was determined by protein substrates and level of MTGase incorporated.

Whiteness

Whiteness of gels made from washed mince and oxidized washed mince added with MTGase at different levels is shown in Table 2. No differences in whiteness

were found between gels from washed mince and oxidized washed mince. At the same level of MTGase, no differences in whiteness were found between the control and oxidized sample ($P>0.05$). However, the addition of MTGase slightly lowered the whiteness of gel. The higher gel strength induced by MTGase may cause gel network of samples to become denser. This might be associated with the higher light absorption. Kang et al. (2007) reported that gels from post-rigor pork had small pockets with denser myofibrillar gel matrix. This might cause more light to be absorbed in the gel matrix, resulting in the darker color of gel.

Microstructure

Microstructures of gel from washed mince and oxidized washed mince added with different levels of MTGase (0–0.6 unit/g sample) are illustrated in Fig. 4. Gel of oxidized washed mince displayed a coarse gel matrix with a slightly larger void, while the control gels (without oxidation) had a finer three-dimensional filamentous protein network with smaller void. The finer and more ordered structure of gel was in accordance with higher breaking force (Fig. 3) along with higher water holding capacity (Table 2). Less continuous network with slightly larger strands were observed in gels prepared from

oxidized washed mince. This might be caused by the large aggregate of proteins induced by oxidation process. Those large bundles as indicated by the increased G' (Fig. 2) could not form the fine network. Therefore, those large aggregates induced by oxidation could not be completely dissociated prior to thermal aggregation. This led to the coarser network with poor water holding capacity.

When MTGase was incorporated, protein could undergo the cross-linking more effectively. In control samples, gel structure became more compact and denser with smaller voids as higher amount of MTGase was added. Gel network became more rigid. The result was in agreement with Kudre and Benjakul (2013) who reported that MTGase addition was able to improve the gel matrix of sardine surimi, which became more compact and filamentous. For oxidized samples, gel became denser with the addition of MTGase up to 0.3 unit/g. However, an excessive amount of MTGase (0.6 unit/g) resulted in the discontinuous network, leading to the lower gel strength in comparison with the gel of control sample added with the same MTGase level (Fig. 1). Thus, oxidation process and MTGase addition affected gel network formation of tilapia washed mince.

Fig. 4 Electron microscopic image of gels from washed mince and oxidized washed mince as affected by addition of MTGase at different levels (Magnification: 10,000x)

Conclusion

The oxidation induced the physicochemical and conformation changes of myosin from tilapia. Those changes determined the susceptibility of protein to MTGase cross-linking, in which protein cross-linking induced by MTGase was impeded when the severe oxidation took place. The oxidation also lowered the gel-forming ability of washed mince but MTGase at appropriate level could strengthen the gel. The gel formability and setting response of oxidized samples were partially recovered by the addition of MTGase. Therefore, MTGase could be an effective means to improve gel properties from mince when oxidation occurred at low degree.

Acknowledgments The authors would like to express their sincere thanks to Thailand Research Fund under the Royal Golden Jubilee Ph.D. Program to Sochaya Chanarat (PHD/0138/2551) and the Grant-in-Aid for dissertation from Graduate School, Prince of Songkla University, Thailand for financial support. The TRF senior research scholar program was also acknowledged.

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