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Effect of oxidation on the structural and functional properties of myofibrillar in *Coregonus peled*

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Abstract

The effects of the degree of oxidation (0.01 mmol/L $FeCl_3$, 0.1 mmol/L ascorbic acid, 1 - 20 mmol/L H_2O_2) principally induced by Fenton systems on the structural and functional properties of *Coregonus peled* myofibrillar proteins (MP) was investigated. When the oxidation levels of MP increased, their carbonyl groups increased but sulfhydryl groups decreased ($p < 0.05$). SDS-PAGE analysis revealed more cross-linking and protein polymerisation in oxidised MP. FTIR properties suggested that oxidation increased conformational changes of MP. The functional results indicated that moderate oxidation (5 - 10 mmol/L H_2O_2) of MP improved their foaming capacity thus providing better functional properties; while excessive oxidation (≥ 15 mmol/L H_2O_2) led to the deterioration of their functional properties due to the formation of large aggregates, and therefore, should be avoided in their application in the food processing industry.

Introduction

Protein oxidation, which occurs naturally during post-mortem storage, has been considered as an important cause for the deterioration of meat quality (Papuc *et al.,* 2017). The decrease in the number of sulfhydryl groups and the formation of carbonyl groups lead to further damage of the protein structure and its functional properties (Lund *et al.,* 2011). Muscle proteins, especially myofibrillar proteins (MP), are susceptible to destruction by reactive oxygen species (ROS) (Wang *et al.,* 2017). The well-known and most potent ROS that causes oxidative damage to meat proteins is the hydroxyl radical, which is generated from hydrogen peroxide (H_2O_2) and cellular reductants, catalysed by iron ions through the Fenton reaction. Protein oxidation by the hydroxyl radical-generating system (HRGS; Fe^{3+}, ascorbic acid, and H_2O_2 under different concentrations) has been widely studied in food systems for many years (Zhou *et al.,* 2013; Lu *et al.,* 2017).

Oxidative modifications of proteins always lead to a change in their physicochemical properties, thus resulting in the alteration of their functionality. Due to such modifications, not only the quality of fresh meat but also the processing properties of meat products are also affected (Cao *et al.,* 2018). However, such chemical processes are not always associated with deleterious effects on the protein functionality. Generally, excessive oxidation is deleterious, and can result in lower digestibility, partly due to protein aggregation. However, proteins that are oxidised moderately are easily digested by relevant proteases (Smuder *et al.,* 2010). In addition, Lu *et al.* (2017) reported that mild protein oxidation caused by meat storage or hydroxyl radicals could be an interesting strategy to promote gel texture and water-holding capacity in bighead carps (*Aristichthys nobilis*). Similarly, a recent study also revealed that both mild (0.1 - 1 mM H_2O_2) and strong (5 -1 0 mM H_2O_2) oxidative modifications could change the myofibril structural integrity by accelerating the formation of carbonyl compounds and disulphide bonds, especially under strong oxidation conditions (Zhang *et al.,* 2018).

Coregonus peled (family Salmonidae), also known as peled, is mainly freeze-stored for a longer shelf-life as the cold-water fish is prone to deterioration during storage and distribution. *C. peled* proteins are susceptible to damage by free radicals which results in the loss of product quality during their processing and storage (Deng *et al.,* 2019), and also changes the functional properties of proteins. Therefore, a deeper understanding of the mechanisms governing the changes in the functional properties by oxidation treatment is necessary to aid in designing reasonable processes for the development of *C. peled*

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muscle foods. Therefore, the present work was focused on the investigation on the effects of different oxidation levels on MP, and their relationship with the oxidation-induced changes in their functional properties.

Materials and methods

Materials

The *C. peled* samples were obtained from Xinjiang Serimu Lake Fishery Sci- and Tech-Development Co., Ltd. (Xinjiang, China). The dorsal skeletal muscle was collected, kept at -20°C, and used within seven days. Foetal bovine serum albumin standard was purchased from Sigma-Aldrich (Shanghai, China), and all other reagents were of analytical grade.

Preparation of MP

The MP were prepared following the procedures described by Hashimoto *et al.* (1979) and Chin *et al.* (2009), and the extracted proteins were kept at -80°C and used within three months. Briefly, the muscle samples were homogenised in 10 volumes of phosphate buffer A (50 mmol/L Na_2HPO_4, 50 mmol/L NaH_2PO_4, pH 7.5) for 1 min, and centrifuged at 8,000 *g* for 15 min at 4°C, and the aforementioned steps were repeated again. To the precipitate, 10 volumes of phosphate buffer B (50 mmol/L Na_2HPO_4, 50 mmol/L NaH_2PO_4, 0.6 mol/L NaCl, pH 7.5) were added, and the mixture was homogenised for 1 min, and centrifuged at 8,000 *g* for 15 min at 4°C. The supernatant was collected, and the precipitate was used for the second extraction; all the supernatants were pooled to yield the final MP.

The protein concentrations of MP were determined using the Biuret method, with bovine serum albumin protein as standard.

Protein oxidation

Protein oxidation by HRGS was performed following the procedures described by Park *et al.* (2006). Briefly, the protein isolates were dissolved in 50 mmol/L phosphate buffers (pH 6.0) containing the HRGS (0.01 mmol/L $FeCl_3$, 0.1 mmol/L ascorbic acid, 1 - 20 mmol/L H_2O_2), and oxidised at 4°C for 1, 3, and 5 h. The same phosphate buffers were used as blank; 1 mmol/L EDTA was added to terminate the oxidation. The degrees of oxidation of MP was evaluated based on the carbonyl and total sulfhydryl groups, following the procedures described by Oliver *et al.* (1987) and Di Simplicio *et al.* (1991), respectively.

Determination of nitrogen solubility index

The MP samples were homogenised with an equal volume of distilled water for 30 min. The pH was adjusted to 7.0 with 1 mol/L NaOH or HCl, and the solution was centrifuged at 8,000 *g* for 20 min (4°C). The nitrogen solubility index of each sample was calculated as the ratio of the supernatant protein concentration to the total protein concentration (Lowry *et al.,* 1951).

Determination of turbidity

Turbidity was measured following the procedures described by Xia *et al.* (2010). The solution of the MP samples (1 mg/mL protein) were heated from 30 to 80°C for 30 min in a water bath. The absorbance values of the samples at 600 nm were measured after 1 h.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE was performed following the procedures described by Liu *et al.* (2016) with slight modification. Briefly, the SDS-PAGE was carried out in a 12% polyacrylamide gels, and 10 μL of the samples (1 mg/mL protein) were loaded in each lane. The gels were run at a voltage of 70 and 110 V for the stacking gels and the separating gels, respectively.

Fourier transformed infrared spectroscopy (FTIR)

The sample pre-treatment was performed following the procedures described by Kang *et al.* (2016). Briefly, 1 mg of freeze-dried protein sample was mixed with 100 mg KBr, and pressed into tablet. The FTIR spectra of samples were determined using a FTIR spectrometer by scanning the whole band of (400 - 4,000 cm^{-1}) at room temperature. A curve-fitting procedure was performed to determine the position and percentage of the absorption peaks (1,700 - 1,600 cm^{-1}), using PeakFit Version 4.12 software (SPSS Inc., Chicago, IL, USA) at 1,700 and 1,600 cm^{-1}, with full width at half maximum height (FWHM) of 12.71 cm^{-1} and a maximum resolution factor (Wang *et al.,* 2016).

Foaming properties

The foaming capacity (Fc) and foaming stability (Fs) of the samples were determined following the procedures described by Motoi *et al.* (2004). Briefly, 10 mL of the sample (V_0) was introduced into a 50 mL plastic measuring cylinder, and mixed using a vortex mixer at 1,800 rpm for 1 min at 25°C. The volumes of the foam (V_1 and V_t) in the measuring cylinder were measured immediately

after 1 h, respectively. The Fc (%) and Fs (%) were determined using Eqs. 1 and 2, respectively:

$$Fc(\%) = \frac{V_1 - V_0}{V_0} \times 100\% \quad \text{(Eq. 1)}$$

$$Fs(\%) = \frac{V_t}{V_1} \times 100\% \quad \text{(Eq. 2)}$$

Emulsifying properties

The emulsifying properties of MP were determined following the procedures described by Pearce and Kinsella (1978). Briefly, pure soybean oil and protein isolate solution (v/v, 1:4) were added to a 50 mL plastic tube, and homogenised using a homogeniser (Scientz, Ningbo, China). Aliquots of the prepared emulsion (50 µL each) were removed from the bottom of the tube at 0 and 10 min, and were dispersed into 5 mL of 0.1% sodium dodecyl sulphate (SDS) solution. The absorbance values were immediately measured at 500 nm using a SP-752PC spectrophotometer (Shanghai Spectrum Instruments Co. Ltd, Shanghai, China), and 0.1% SDS was used as blank. The emulsifying activity [EAI (m^2/g)] and emulsifying stability [ESI (%)] were determined using Eqs. 3 and 4, respectively:

$$EAI = \frac{2 \times 2.303}{C \times (1-\varphi) \times 10^4} \times A_1 \times L \quad \text{(Eq. 3)}$$

$$ESI = \frac{A_2}{A_1} \times 100 \quad \text{(Eq. 4)}$$

where, A_1 and A_2 = absorbance values at 500 nm at 0 min and after 10 min, respectively; C = protein concentration (g/mL); and φ = oil volume fraction of the emulsion (φ = 0.2).

Statistical analysis

Origin 8.5.1 and SPSS 17.0 software were used for data processing and significant analysis, respectively, and all experiments were carried out in triplicate. Significant differences ($p < 0.05$) were measured using the Tukey's range test.

Results and discussion

Protein oxidation

Protein oxidation induced by ROS can decrease the number of sulfhydryl groups by forming disulphide bonds, which can lead to the conformation instability of proteins. ROS also attack the peptide bonds or side chain groups of proteins, thus forming carbonyl derivatives (Jiang and Xiong, 2016). As shown in Figure 1A, the carbonyl content of MP obviously increased with increasing degree of oxidation when compared with that of control. Meanwhile, the total sulfhydryl content of MP significantly decreased with increasing degree of oxidation ($p < 0.05$) (Figure 1B). The significant loss of the sulfhydryl content in MP may be attributed to either the disulphide formation or the damage of the side chains of sulphur-containing amino acid residues inside MP (Xiong *et al.,* 2010; Papuc *et al.,* 2017). In the presence of radical, the sulfhydryl groups are converted into intra- and inter-molecular cross-links, which might be greatly associated with the modification in the functional properties (Jongberg *et al.,* 2014). It has been shown that the increase in the oxidation time in the presence of hydroxyl radicals is accompanied by the increase in the carbonyl content as well as the increase in the protein susceptibility to thermal aggregation, which induces deterioration in the structural properties of porcine MP (Kong *et al.,* 2016).

Protein aggregation

Nitrogen solubility index

Protein solubility rarely serves as a functional property; however, it is an essential factor since it can influence the functional properties of most proteins (Higuera-Barraza *et al.,* 2016). As shown in Figure 2A, the nitrogen solubility index of MP significantly decreased as the degree of oxidation increased, with the maximum decrease being 67%. The decrease in nitrogen solubility of MP is in agreement with the results of other studies on protein modification due to oxidation (Utrera and Estévez, 2012). The decrease may be due to the high degrees of oxidation which result in protein denaturation and precipitation, which in turn is associated with protein solubility loss (Kramer *et al.,* 2012). However, there was almost no change in the nitrogen solubility index in MP oxidised by 1 mmol/L H_2O_2 for 1 and 3 h. The results indicated that mild oxidative modifications prevented a change in the nitrogen solubility.

Turbidity

Turbidity levels were used to monitor the MP aggregation. The formation of aggregates led to a steady increase in the optical density (Li *et al.,* 2013). The turbidity of MP increased significantly with the increase in the H_2O_2 concentration and at constant temperature; in addition, the turbidity of MP also increased with the increase in the temperature and at constant H_2O_2 concentration (Figure 2B). In fact, oxidation treatment of MP for the longest duration (3 h) exhibited an increase in turbidity with the increase

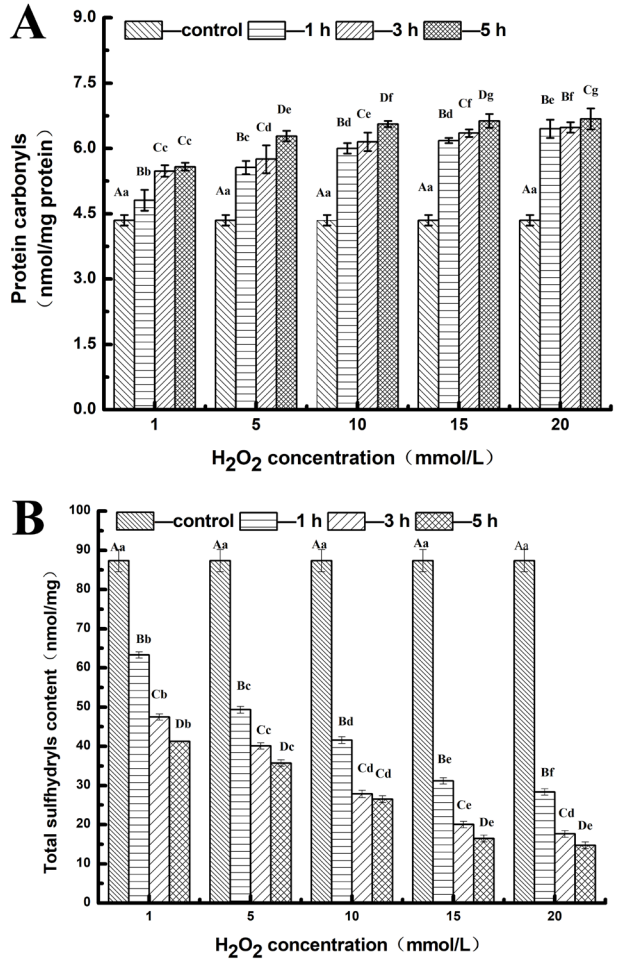

Figure 1. Protein carbonyl (A), and sulfhydryl contents (B) of *C. peled* myofibrillar proteins samples. Different uppercase letters indicate significant differences ($p < 0.05$) at the same oxidant concentration with different times; different lowercase letters indicate significant differences ($p < 0.05$) at the same oxidation time with different concentrations.

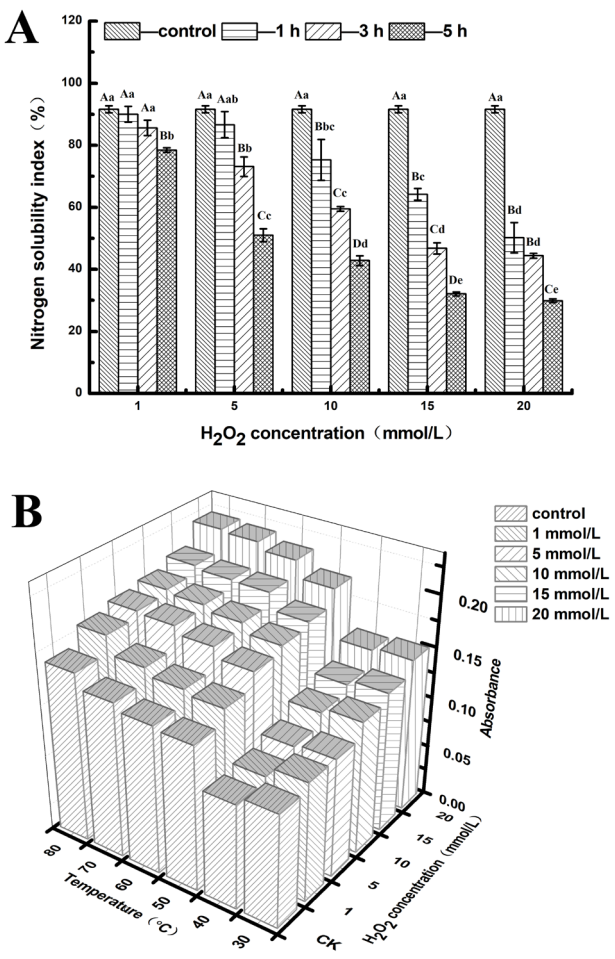

Figure 2. Effect of the Fe/H_2O_2/Asc system on the nitrogen solubility index (A), and turbidity (B) of *C. peled* myofibrillar proteins. Different uppercase letters indicate significant differences ($p < 0.05$) at the same oxidant concentration with different times; different lowercase letters indicate significant differences ($p < 0.05$) at the same oxidation time with different concentrations.

in the degree of oxidation and temperature. The results indicated that higher oxidation concentration may increase protein aggregation, which is comparable to the results of porcine longissimus dorsi muscle proteins oxidised by three different oxidation systems (Utrera and Estévez, 2012). Similar results were also reported in surimi protein solutions with increasing protein oxidation carbonyls (Parkington *et al.,* 2000). Many of the functional properties need to take account the association among individual proteins; hence, excessive oxidation leading to polymerisation and aggregation may exert deleterious influences in muscle foods (Reeg and Grune, 2015).

Protein conformation

SDS-PAGE

Non-reduced (-βME) and reduced (+βME) SDS-PAGE were performed to monitor the effect of oxidation on MP. As shown in Figure 3A, the most abundant MP bands were myosin heavy chain (MHC, 220 kDa) and actin (42 kDa). The reduced SDS-PAGE showed no difference in the intensity of all bands between samples and control after 3 h of oxidation. While based on the non-reduced SDS-PAGE profiles, the band intensity of MHC became thinner with increasing degree of oxidation (1 - 10 mM H_2O_2), and there was some protein aggregation on the top of the gel. β-ME is a strong reducing agent that can cleave inter and intra-molecular disulphide covalent bonds of proteins. The results indicated that oxidation induced the protein aggregation, which can be associated with disulphide cross-linkages (Li *et al.,* 2018). At 20 mM H_2O_2, the

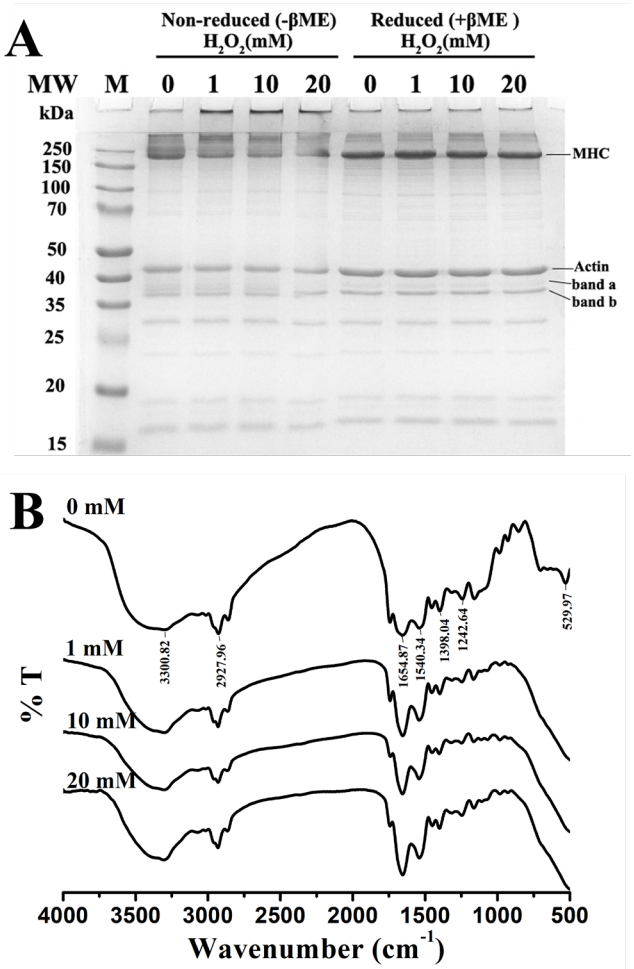

Figure 3. Effect of the Fe/H_2O_2/Asc system on the SDS-PAGE (A), and the FTIR spectra (B) of *C. peled* myofibrillar proteins.

band intensities of MHC decreased. Further addition of H_2O_2 likely promoted protein degradation. In addition, the reduced MP exhibited two adhesive bands (band a and band b) at approximately 35 - 40 kDa, while the non-reduced MP (oxidised with 20 mM H_2O_2) exhibited a single band. These results suggested that excessive oxidation can affect the intra- or inter- disulphide bonds of proteins (band a and b) and leads to aggregation (Cui *et al.,* 2012). These results also indicated that MP insignificantly degraded under H_2O_2 oxidation, and protein oxidation generated cross-linked protein derivatives including disulphides and carbonyls, thus resulting in the alteration of the functional properties of the proteins (Xiong *et al.,* 2009).

FTIR spectroscopy analysis

The FTIR spectra for MP and oxidised-MP incubated in different H_2O_2 doses are shown in Figure 3B. Amide A (3300.82 cm^{-1}) is attributed to NH stretching, and the peak slightly shifted from 3300.82 to 3302.60 cm^{-1} after H_2O_2 treatment, suggesting the changes of intermolecular forces in MP induced by oxidation (Li *et al.,* 2004). The weak peak at 2927.96 cm^{-1} was attributed to the C-H stretching vibration. Typical protein bands at 1654.87 and 1540.34 cm^{-1} are related to Amide I (C=O stretching and C-N stretching) and Amide II (N-H bending, C-N stretching, C-C stretching), respectively (Fan *et al.,* 2017), which increased and shifted to lower wavenumbers when the dose of H_2O_2 increased. This is because functional groups including NH2 may be lost as reported by Farhat *et al.* (1998). In addition, it was found that the peak (529.97 cm^{-1}) for amide VI disappeared with the oxidation progress, which is related to amide VI band (out of plane C=O bending) (Kong and Yu, 2007).

The amide I band (1,700 - 1,600 cm^{-1}) represents the C=O stretching vibration which provides information on the secondary structures of proteins (Kong and Yu, 2007). As shown in Table 1, the secondary structures of non-oxidised- and oxidised-MP were sensitive to oxidisation. Upon 10 mM H_2O_2 treatment, the content of α-helix significantly decreased by 64.14%, whereas the β-turn and random coils increased by 50.13 and 37.13% ($p < 0.05$), respectively, and the content of β-sheets in MP did not change significantly. In general, α-helices are stabilised by internal hydrogen bonds among peptide chains, and remain buried in the interior of the protein. The β-turn is caused by its highly ordered protein structure, and random coils

Table 1. Secondary structure in non-oxidised and oxidised treated myofibrillar proteins.

Structure distribution (%)	H_2O_2 concentration (mM)			
	0	1	10	20
α-helix	30.65 ± 0.54^{a}	21.10 ± 1.20^{b}	10.99 ± 0.14^{d}	18.12 ± 0.75^{bc}
β-sheet	24.22 ± 1.72^{ab}	24.93 ± 0.09^{ab}	23.21 ± 0.81^{bc}	26.91 ± 2.36^{a}
β-turn	30.20 ± 0.57^{c}	33.70 ± 2.08^{bc}	45.34 ± 3.76^{a}	37.43 ± 2.35^{b}
Random coil	14.92 ± 1.24^{bc}	20.27 ± 0.55^{a}	20.46 ± 1.07^{a}	17.55 ± 0.30^{ab}

For MP samples, means ($n = 3$) with different lowercase superscripts within the same row are significantly different ($p < 0.05$).

can originate from the unfolding of any higher-order structures, and the coils are related to protein flexibility (Chen *et al.,* 2017). Oxidation can induce partial unfolding of the proteins, thereby exposing certain sites that are normally hidden within proteins (Estévez, 2011). Analysis of the hydrophobicity of the protein surface from our previous studies confirmed this (Deng *et al.,* 2019). As the H_2O_2 treatment dose extended, protein molecule chains unfolded and partial hydrogen bonds ruptured, which disrupted some of the ordered structure where the α-helixes transitioned to β-turns and random coils. Sun *et al.* (2013) showed an increase in β-sheets accompanied by a decrease in the number of α-helixes, β-turns, and random coils as the concentration of oxidising agent increased. These differing findings may be related to the differences in the individual protein studied.

However, when the concentration of H_2O_2 increased to 20 mM, there was a readily visible increase in α-helixes from 10.99 to 18.12%, an increase in β-sheets from 23.22 to 26.91%, a decrease in the proportion of the overall protein made up of β-turns from 45.35 to 37.43%, and a decrease in random coils from 20.46 to 17.55% ($p < 0.05$). It was likely that the decrease in the random coils and β-turns contributed to an increase in α-helixes and β-sheets at 20 mM. These results are similar to those

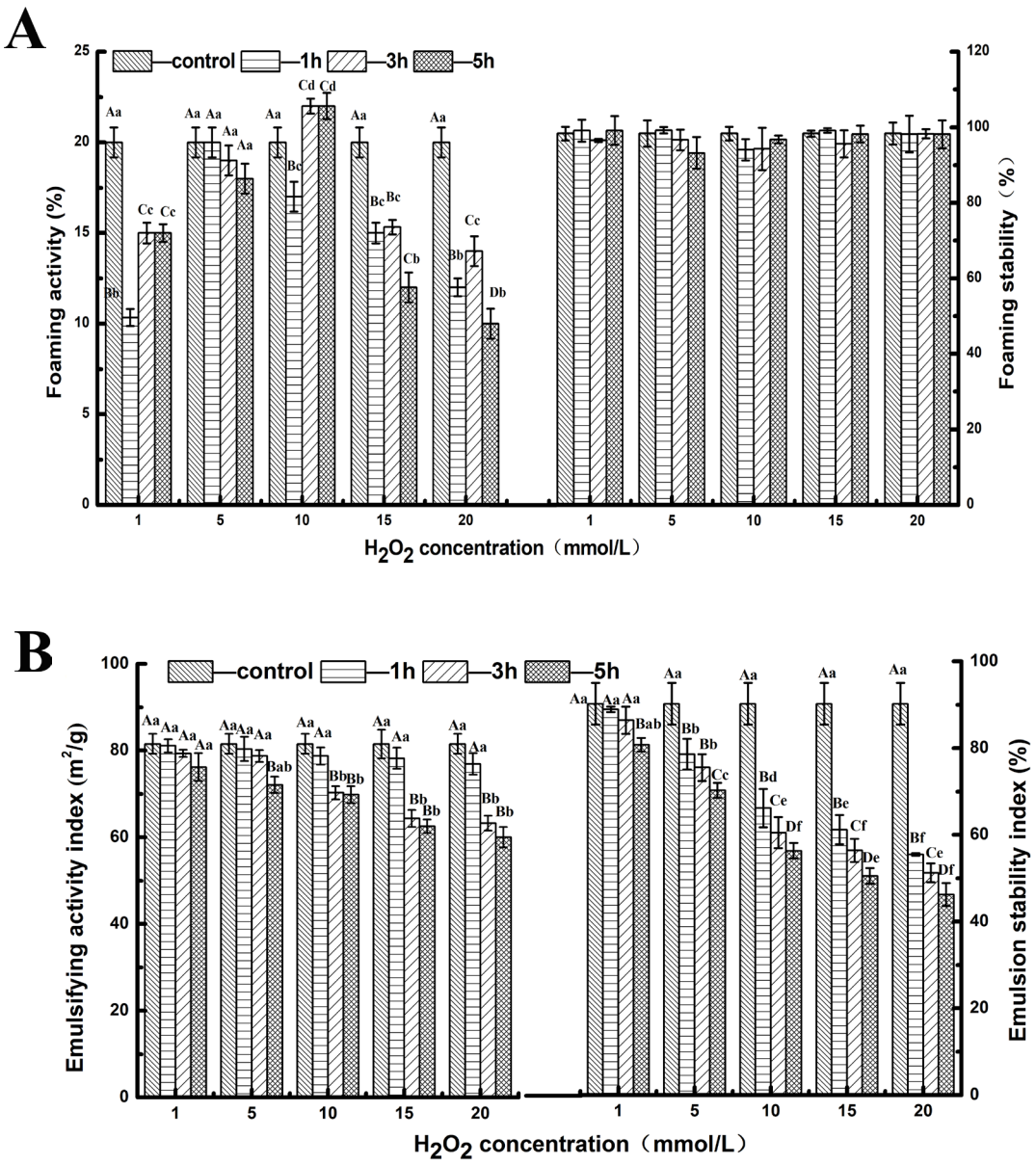

Figure 4. Effect of the Fe/H_2O_2/Asc system on the foaming properties (A), and the emulsifying properties (B) of *C. peled* myofibrillar proteins. Different uppercase letters indicate significant differences ($p < 0.05$) at the same oxidant concentration with different times; different lowercase letters indicate significant differences ($p < 0.05$) at the same oxidation time with different concentrations.

reported in soy protein isolate (Liu *et al.,* 2015).

Protein function

Foaming properties

A more intense loss in the foaming capacity of MP than other degrees of oxidation was induced by 1 mmol/L H_2O_2, while moderate oxidation (5 and 10 mmol/L H_2O_2) had lesser impact, and the foaming capacity was even strengthened by longer oxidation duration (Figure 4A). With further increase in H_2O_2 concentration, the foaming capacity significantly decreased as compared to that of control. The foaming stability of MP, however, exhibited no significant changes. Similar results were also observed for the foaming activities of whey protein isolates, which exhibited significant improvements ($p < 0.05$) with H_2O_2 concentrations up to 5 mmol/L and oxidation time up to 5 h (Kong *et al.,* 2013). Li *et al.* (2019) also reported that mild oxidation can increase the foaming capacities of the soy protein isolates (SPI) by an appropriate cleavage of the disulphide bond; however, excessive oxidation could decrease the foaming capacities but had no significant effects on the foaming stabilities. These results indicated that moderate oxidation could improve the foaming capacities of MP, while excessive oxidation, which induced the formation of large aggregates, may lead to a decrease in the foaming capacities of MP.

Emulsifying properties

The effects of oxidation on the emulsifying properties of MP are shown in Figure 4B. The emulsifying activity did not significantly change after oxidation with 1.0 and 5.0 mmol/L H_2O_2. With the increase in the H_2O_2 concentration (10 - 20 mmol/L), the emulsifying activity of MP significantly decreased ($p < 0.05$), except at oxidation for 1 h. The emulsifying stabilities of MP decreased rapidly with increasing H_2O_2 concentration and oxidation time, except when oxidised with 1 mmol/L for 1 and 3 h, which indicated that the emulsifying stability was more sensitive to oxidation. Heavy protein oxidation resulted in protein aggregation, which is one of the main causes of oxidation damages occurring in muscle proteins; excessive oxidation environment can lead to the formation of protein cross linkages, which further leads to protein polymerisation and aggregation (Tolstorebrov *et al.,* 2016). In this sense, > 5 mmol/L H_2O_2 oxidation may contribute to more aggregation in MP (Figure 2), which can potentially exert conformational changes in the protein, also influencing its water state and emulsifying properties. In contrast to the present findings, Liu *et al.* (2015) reported that moderate oxidation-induced structural modification can promote the emulsifying properties in the soy protein isolate. This may be interpreted by the fact that, in unfrozen samples, the oxidation of proteins by HRGS increased the emulsifying ability, while following the freeze/thaw process, oxidising the protein decreased the emulsifying ability (Srinivasan and Hultin, 1997).

Conclusion

The degree of oxidation of MP in *C. peled* muscle were effectively modulated by HRGS. Through SDS-PAGE and FTIR spectra analyses, we demonstrated that H_2O_2 addition to MP promoted the cross-linking, protein polymerisation, as well as increased conformational changes of MP. The addition of H_2O_2 had a significant influence on the functional properties of MP. Moderate oxidation (5 - 10 mmol/L H_2O_2) substantially increased the foaming capacity of MP, but had no significant influence on their emulsifying activity and emulsifying stability. However, excessive oxidation ($\geq$ 15 mmol/L H_2O_2) decreased the foaming capacity, emulsifying activity, and emulsifying stability. The results indicated that moderate oxidation could provide better functional properties to *C. peled* muscle proteins. Therefore, moderate oxidation (*i.e.,* treated by hydroxyl radicals or frozen-stored for a certain period) could be applied in *C. peled* muscle food production to achieve better functional properties.

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