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Influence of combined salt concentration and low-frequency ultrasound on physicochemical and antioxidant properties of silver carp myofibrillar protein

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Abstract

The present work aimed to evaluate the combined influences of salt sodium chloride (NaCl) concentration and low-frequency ultrasound on the functionality of silver carp myofibrillar protein (MP). Silver carp MP was set using different salt concentrations (0.2, 0.4, 0.6, and 0.8 M NaCl) and low-frequency ultrasound treatment (12 min, 350 W). The samples showed a high solubility (85.30 - 93.80%) across various treatments. The turbidity was lowered significantly by increasing the salt concentration with no sonication, while the turbidity changes were insignificant with the combination treatment (NaCl + U12). Ultrasonic treatment of combined 0.4 M salt (NaCl 0.4 M + U12) achieved the highest water holding capacity (WHC) making up 6.46 g water/g MP. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) inhibition ranged from 16.33 to 43% and 13.27 to 25.69%, respectively, and the highest values were shown in the sample containing 0.4 M salt with sonication (NaCl 0.4 M + U12). It could be concluded that the best treatment was ultrasonic with 0.4 M salt (NaCl 0.4 M + U12). The treatment provided the optimal antioxidant activity with good protein solubility and WHC. The integrated results indicate that employing NaCl with ultrasonic treatments (NaCl + U12) could improve the functionality of silver carp MP. These results are very promising for increasing the potential advantage of this fish species as the sources of proteins for several applications.

Introduction

Silver carp fish (*Hypophthalmichthys molitrix*) are a major and inexpensive freshwater fish species in China due to their ease of cultivation, rapid growth rate, high nutrition efficiency, and nutritional content (Wang *et al.,* 2013). Farmed silver carp are a freshwater fish that correspond to marine fish in polyunsaturated fatty acid *n*-3 (PUFA) content, with about 36% total fatty acids (Li *et al.,* 2011). In past years, several products were developed from this fish such as surimi (Luo *et al.,* 2008; Fu *et al.,* 2012; Liu *et al.,* 2014), fermented sausages (Xu *et al.,* 2010), and restructured fish (Wang *et al.,* 2013).

As the modern food industry develops, one ongoing challenge is finding innovative technologies to improve processing effectiveness and diminish energy loss. In this regard, ultrasound as an innovative technique has been extensively considered in recent years (Xiong *et al.,* 2016). A number of researchers have noted the positive impact of high-intensity low-frequency ultrasound (up to 10 kW and frequency range of 20 - 100 kHz) on the properties of meat protein as a result of protein modification (Bekhit *et al.,* 2014). Liu *et al.* (2017) noticed the enhancement of solubility and dispersion of silver carp myosin by ultrasound at 100 - 250 W and 20 kHz for 3 - 12 min. Saleem and Ahmad (2016) noted better properties of chicken actomyosin with regard to water holding capacity (WHC), regular three-dimensional networks, and gelling properties as the effect of sonication at the low frequency of 20 kHz. Amiri *et al.* (2018) reported that high-intensity ultrasound (100 or 300 W, and 20 kHz for 10, 20, or 30 min) could enhance the pH, WHC, solubility, particle size (reduced), foaming, emulsifying properties, and gel strength of beef myofibrillar proteins. Finally, Higuera-Barraza *et al.* (2017) confirmed the enhancement characteristics of

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squid (*Dosidicus gigas*) mantle protein upon emulsification by ultrasound (20 or 40% amplitude, 20 kHz, and applied for 30, 60, or 90 s).

Myofibrillar proteins (MP) are important proteins principally in charge of the texture and functional characteristics of emulsion on gel-type meat products. MP influences the properties of emulsion to different degrees depending on the solubility where salt-soluble myofibrillar proteins are involved in gel network formation (Hong and Xiong, 2012; Liu *et al.,* 2013; Wong, 2018). The functional characteristics of MP are influenced by factors such as pH, ionic strength, heat or hydrostatic pressure treatment, protein oxidation, and protein or non-protein additives. The determining factor among these is ionic strength due to the salt-soluble characteristics of MP (Wu *et al.,* 2016).

Sodium chloride (NaCl) is one of the principal factors that affect the character of protein gel, and play an influential role in gel formation and flavour release. The intensity of flavour in meat processing products was dependent on the concentration of salt namely the Cl^- anion and Na^+ cation which generated the saltiness sensation (Feng *et al.,* 2018a). An amount of 2 - 3% (0.47 - 0.68 M) NaCl was commonly needed to solubilise the MP to get the desired functional attributes of MP (Wu *et al.,* 2016). The salt concentration had a notable effect on gel formation and structural change of acid-induced silver carp myofibrils. It was necessary to add the right amount of salt to allow the silver carp myofibrils to further solubilise and induce gel formation under acidic conditions. NaCl 1.7 - 2% might be needed by silver carp myofibrils to form an acid-induced gel with premium gel properties (Xu *et al.,* 2013).

To the best of our knowledge, this is the first work that deals with the preparation of silver carp myofibrillar protein using combined salt (NaCl) concentration and low-frequency ultrasound. The present work intended to examine the influence of salt concentration and low-frequency ultrasound on the physicochemical properties, and subsequently analyse the antioxidant activities of the obtained myofibrillar protein.

Materials and methods

Raw material

Live silver carp (51 - 58 cm length, 2 - 3 kg weight) were purchased from a local supermarket (Wuxi, Jiangsu, China). The fish samples were transported directly to the laboratory. There, they were scaled, eviscerated, beheaded, skinned, filleted, and then washed under running water. The obtained dorsal muscle was frozen at -60°C prior to myofibril preparation.

Chemicals and reagents

Bovine serum albumin (BSA) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were purchased from Sigma Chemical Co. (Shanghai, China). Other chemicals and reagents were purchased from Sinopharm Chemical Reagent, Shanghai, China. All chemicals and reagents used in the present work were of high purity and analytical grade.

Myofibril protein preparation

Myofibril protein (MP) was prepared corresponding to the method represented by Qiu *et al.* (2013) with some modifications. Frozen slices were kept overnight at 4°C, and then cut into small parts. Two grams of trimmed meat were added with a 15 mL phosphate buffer solution (4°C, 50 mM, and pH 7.5), and then homogenised for 30 s (T 10 basic Ultra Turrax, IKA, Staufen, Germany). The homogenates were centrifuged for 15 min at 10,000 rpm and 4°C (Sigma Laboratory Centrifuges 4K15, Germany). The supernatants were poured, and the precipitates were washed again using the same method. Thereafter, 20 mL of phosphate buffer (4°C, 50 mM, and pH 7.5) containing 0.6 M NaCl was added to the precipitate followed by homogenisation for 30 s using a homogeniser. The homogenates were then centrifuged at 10,000 rpm and 4°C for 15 min. The precipitates were washed again using the same method. The supernatants resulting from the washing process were collected twice and manually pooled. The supernatants were separated from connective tissue using three layers of cheesecloth, and used as myofibrillar proteins (MP). The concentration of protein was determined by the biuret assay (Gornall *et al.,* 1949) with BSA as standard. This MP solution was kept at 4°C, and used within 48 h.

Myofibril treatment by different salt concentrations and sonication

Sonication of the sample was carried out using an ultrasonic probe system (JY88-II Ultrasonic Cell Disruptor, 500 W, and 20 kHz; Scientz Biotechnology Co. Ltd., Ningbo, China) provided with a 6 mm diameter horn. The intensity of ultrasound was examined calorimetrically in accordance with the method described by Jambrak *et al.* (2009). The ultrasound intensity was 58.59 W/cm^2 to produce an energy of 350 W.

NaCl concentration treatment was performed in line with the previous method with modification (Xu *et al.,* 2013). The MP solutions were adjusted to a concentration of 3 mg/mL with deionised water containing 0.0, 0.2, 0.4, 0.6, and 0.8 mol/L NaCl. After 12 h of incubation at 4°C, the ultrasound was emitted toward a 20 mL protein dispersion in a beaker (50 mL) which was placed in an ice bath to maintain the solution temperature below 12°C (to avoid overheating). The sonication process of samples was carried out at 350 W for 12 min (pulse duration of 4 s turned on, 1 s turned off). During ultrasonic treatment, the probe was submerged into the MP dispersion to a depth of 1 cm from the bottom (half the depth of the solution).

The samples treated with 12 min sonication in combination with NaCl were labelled as NaCl 0.2 M + U12, NaCl 0.4 M + U12, NaCl 0.6 M + U12, or NaCl 0.8 M + U12. The ones treated with only NaCl or ultrasound were denoted as NaCl 0.2 M, NaCl 0.4 M, NaCl 0.6 M, NaCl 0.8 M, and U12, respectively. The sample with no treatment served as control. All measurements were taken in triplicate. All the samples were kept at 4°C before analyses.

Protein solubility measurement

The solubility of the MP solution was measured following the method of Añon *et al.* (2012) with slight modifications. The MP solutions (4 mL) were centrifuged (Sigma Laboratory Centrifuges 4K15, Germany) for 20 min at 10,000 rpm and 4°C. The supernatant protein content was determined using the biuret method (Gornall *et al.,* 1949). Protein solubility was stated as a percentage of supernatant protein concentration towards protein concentration before centrifugation. Three replicates were performed for every treatment group.

Turbidity determination

The MP solution turbidity was examined using a modified version of the previously reported procedure (Benjakul *et al.,* 2001). Sample absorptions were read at 660 nm using a UV-VIS spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) to capture the turbidity values. The measurements were taken in triplicate.

WHC measurement

The WHC of MP gel was measured with a few modifications to the centrifugation technique described by Zhang *et al.* (2017). Briefly, 4 mL of MP solution were transferred to a 4 mL centrifuge tube. The tube was placed in a water bath, and heated for 30 min at 40°C, and then 20 min at 90°C. The protein gel was cooled in ice-cold water for 1 h, and thereafter stored at 4°C for 20 h. The tube was centrifuged at 10,000 rpm for 20 min (TGL-16C, Anke Instrument Ltd., Shanghai, China). The supernatant was poured off, and the remaining water was carefully disposed of using Whatman filter paper as described by Wen *et al.* (2017). The WHC was calculated using Eq. 1:

$$\text{WHC} = \frac{\text{Protein gel (g)} - \text{Protein content (g)}}{\text{Protein content (g)}} \quad \text{Eq. 1}$$

DPPH radical scavenging assay

The antioxidant activity of samples obtained by DPPH was analysed in accordance with Noman *et al.* (2019). Half millilitres of the sample were added to 3 mL DPPH in methanol (0.1 mM). The mixture was then homogenised by vortex for 10 s, and stored at room temperature in the dark for 30 min. Then, using a UV spectrophotometer (UV-1800PC, Mapada) at 517 nm, the absorbance was read, and the antioxidant activity was determined using Eq. 2:

$$\text{DPPH Inhibition (\%)} = \frac{\text{Abs. (blank)} - \text{Abs. (sample)}}{\text{Abs. (blank)}} \times 100 \quad \text{Eq. 2}$$

ABTS radical scavenging activity

Antioxidant activities in the samples were determined using ABTS assay following Najafian *et al.* (2013) with slight modifications. Briefly, 2.6 mL of potassium sulphate was blended with 7.4 mL of ABTS solvent to get a supply solution of ABTS radicals. The working solution was then formulated by mixture of equal amounts of the supply solutions, and incubated at room temperature in the dark for 16 h. Thereafter, it was diluted with methanol (98%) to get an absorbance of 0.70 ± 0.02 at 734 nm. Then the sample (20 µL) was blended with 3.5 mL of prepared $ABTS^+$ solution, and thereafter the mixture was incubated at room temperature in the dark for 30 min. The absorbance was read at 734 nm by UV spectrophotometer (UV-1800PC, Mapada). Distilled water was used as a blank. The antioxidant activity of $ABTS^+$ was determined using Eq. 3:

$$\text{ABTS Inhibition (\%)} = [1 - (\frac{Abs.\ (sample)}{Abs.\ (blank)})] \times 100 \quad \text{Eq. 3}$$

Statistical analysis

The obtained data were expressed as mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) was performed to establish the statistical differences. Significant differences

between means were identified using Duncan's multiple range test ($p < 0.05$). Statistical analyses were performed using IBM SPSS Statistics 20.0 (IBM SPSS software, USA).

Results and discussion

The gel properties of meat products generally depend on the chemical composition of the myofibrils, which are mainly composed of myosin (Feng *et al.,* 2018c). Chen *et al.* (2020) demonstrated an ultrasonication-induced "stress response" through the action of cavitation on myosin which led to an increase in the stability of the emulsion. Additionally, the texture and expressible moisture content of the gel were improved after treatment. The nanostructure of the myofibril was shown as an intact strip-like structure before hydrolysis when examined with atomic force microscopy (Feng *et al.,* 2016; 2017a). The nanostructure of the myofibril changed to a circular shape after enzymatic hydrolysis, which led to a change in the properties of the protein (Feng *et al.,* 2017b). Shi *et al.* (2019) reported that the ultrasound greatly affects the physical and chemical properties of muscle proteins. The ultrasonication treatment led to notable changes in the nanostructure of myosin and a notable change of myosin aggregates into smaller ones with a more consistent distribution and evident enhancement in the solubility of myosin from silver carp (Liu *et al.,* 2017).

Due to the multiple effects of ultrasound on gel properties, the effects of sonication on protein solubility, turbidity, water holding capacity, and antioxidant activity were studied in the present work.

Protein solubility

The solubility of proteins varies greatly depending on several factors including the amount of salt-soluble protein, surface reactive groups, and pH shift (Zhou and Yang, 2019). In our previous study, it was noted that the pH of the MP solution was 7.3 (Nasyiruddin *et al.,* 2019).

The results obtained in the present work indicated that the ultrasound treatment improved myofibrillar protein solubility with or without salt (Figure 1). The solubility increased in control from 86.72 to 87.68% when treated with ultrasound. Moreover, it was observed that the ultrasound treatment also improved myofibrillar protein solubility at different levels of salt; where the solubility increased from 85.29 to 90.95%, 85.30 to 91.85%, 86.32 to 92.84%, and 87.77 to 93.80% at 0.2, 0.4, 0.6, and 0.8 M, respectively.

Gao *et al.* (2020) indicated that the solubility of silver carp surimi gels ranged from 85.02 to 93.63% at salt levels of 0 - 2%, and they confirmed that the ultrasound treatment improved this. Li *et al.* (2020) confirmed that treating the protein with ultrasound led to the breakdown of large protein molecules into smaller molecules, thus increasing their surface area and exposure of polar molecules, which in turn led to high solubility.

Ultrasonic treatment has a positive influence on protein solubility. Ultrasound produces high-shear-energy waves and turbulence from the cavitation phenomenon. It would violently agitate and then break the particles (reduction in diameter). As the sonication reduces the particle size of the protein, it can then provide more surface area and greater charge,

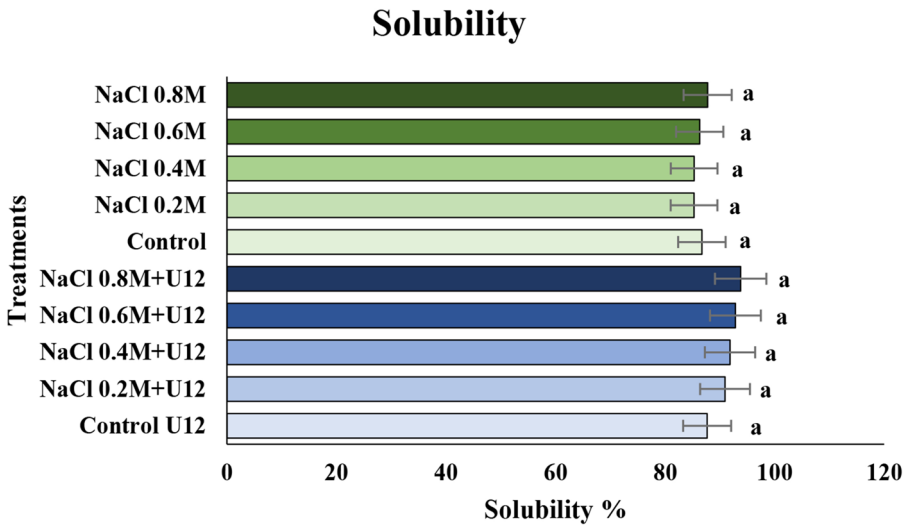

Figure 1. Effect of treatment by different levels of salt and ultrasonication on the solubility of the myofibrillar protein of silver carp. Duncan's multiple range test ($p < 0.05$, and $n = 3$) showed no significant differences between samples.

which present a stronger protein-water interaction (Zhang *et al.,* 2017). Many functional characteristics of proteins relate to their solubility, and the excellent solubility of proteins makes them a potential source of application in the food and pharmaceutical industries (Noman *et al.,* 2019).

Turbidity

The turbidity of samples ranged from 0.031 to 0.242 turbidity (A_{660}). The treatment of samples with different levels of salt resulted in reduced turbidity, especially at higher concentrations (Figure 2). In contrast, the treatment of samples by ultrasound had a greater impact on the turbidity of the samples, generating a significant reduction in turbidity.

The turbidity of protein pellets was involved in the content of protein aggregate (Benjakul *et al.,* 2001). The possible reason for these results is that since MP is considered a salt-soluble protein (Wu *et al.,* 2016), MP solution tends to have better solubility with increasing salt concentration. It would therefore impact the smaller size of protein aggregates which then increases specific surface area available for light scattering.

In addition, ultrasound produced high shear energy waves and turbulence from the cavitation phenomenon. Sonication causes particles to be agitated violently, broken, and decreased in diameter, further increasing the specific surface area for light scattering (Zhang et al., 2017).

Water holding capacity (WHC)

The WHC of the myofibril proteins obtained under different salt concentrations are shown in Figure 3. In the samples treated by brines and sonication, the addition of NaCl concentration from

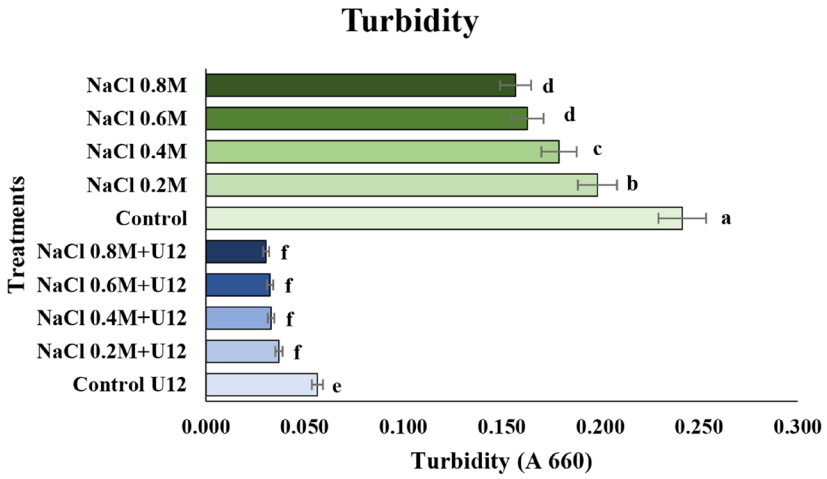

Figure 2. Effects of treatment by different levels of salt and ultrasonication on the turbidity of the myofibrillar protein of silver carp. Different letters (a - f) represent significant differences found by Duncan's multiple range test ($p < 0.05$, and $n = 3$).

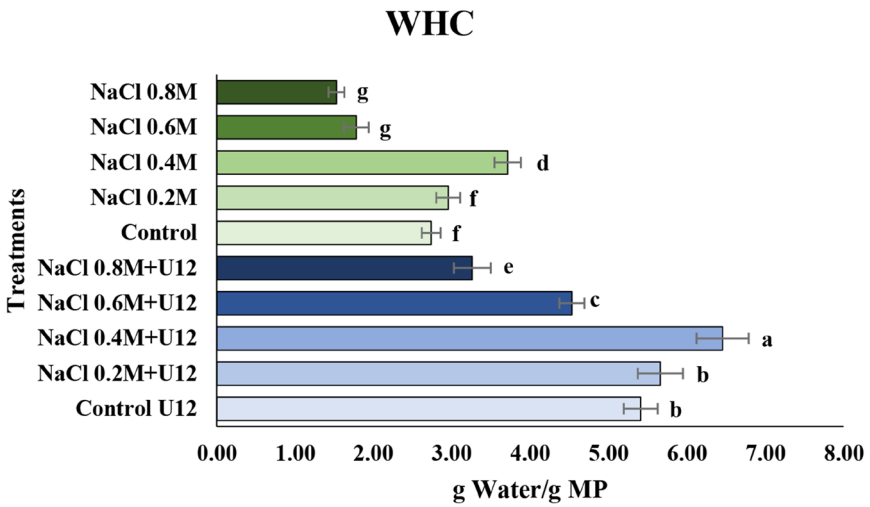

Figure 3. Effects of treatment by different levels of salt and ultrasonication on the WHC of the myofibrillar protein of silver carp. Different letters (a - g) represent significant differences found by Duncan's multiple range test ($p < 0.05$, and $n = 3$).

0.2 into 0.4 M resulted in an increase in WHC from 5.66 to 6.46 g water/g MP. Furthermore, as salt concentration increased, the WHC significantly decreased as compared to control. In the samples treated by brines only, the increasing concentration of saline had the same effect on the WHC of the samples but at significantly lower values than the samples treated with the combination treatment. Differences in the value of WHC were probably due to the variance in the effect of the salt concentrations, which may have affected the polar groups such as –COOH and $–NH_2$ (Balti *et al.,* 2010). These results are in line with Feng *et al.* (2018b) who reported that the WHC of porcine MP gels were enhanced significantly as the NaCl concentration was increased from 0.2 to 0.5 M.

The strengthened effect on WHC values by ultrasonication was associated with a reduction in the size of protein particles which led to the networks of protein gel becoming denser and more uniform, thus promoting the water binding in the gel. The protein gel with a homogenous and fine structure has a higher value of WHC as compared to one with a non-homo geneous structure, since the small pores of the homogenous structure may entrap water molecules firmly (Saleem and Ahmad, 2016; Zhang *et al.,* 2017; Wen *et al.,* 2017; Amiri *et al.,* 2018).

Antioxidant activities

DPPH radical scavenging activity

Free radical scavenging is an essential mechanism by which antioxidants inhibit the oxidative process. The absorbance decreases gradually as DPPH$^{\bullet}$ encounters a proton-donating substrate like antioxidant compounds. The antioxidant activity of the samples against DPPH is shown in Figure 4a. The addition of brine concentration resulted in a significant enhancement of antioxidant activity in both treatments as compared to control, with the combination treatment yielding a higher increase in antioxidant activity.

The highest inhibition (43.00%) was observed at 0.4 M salt concentration with sonication treatment (NaCl 0.4 M + U12), followed by treatment of 0.2 M salt concentration and ultrasound (NaCl 0.2

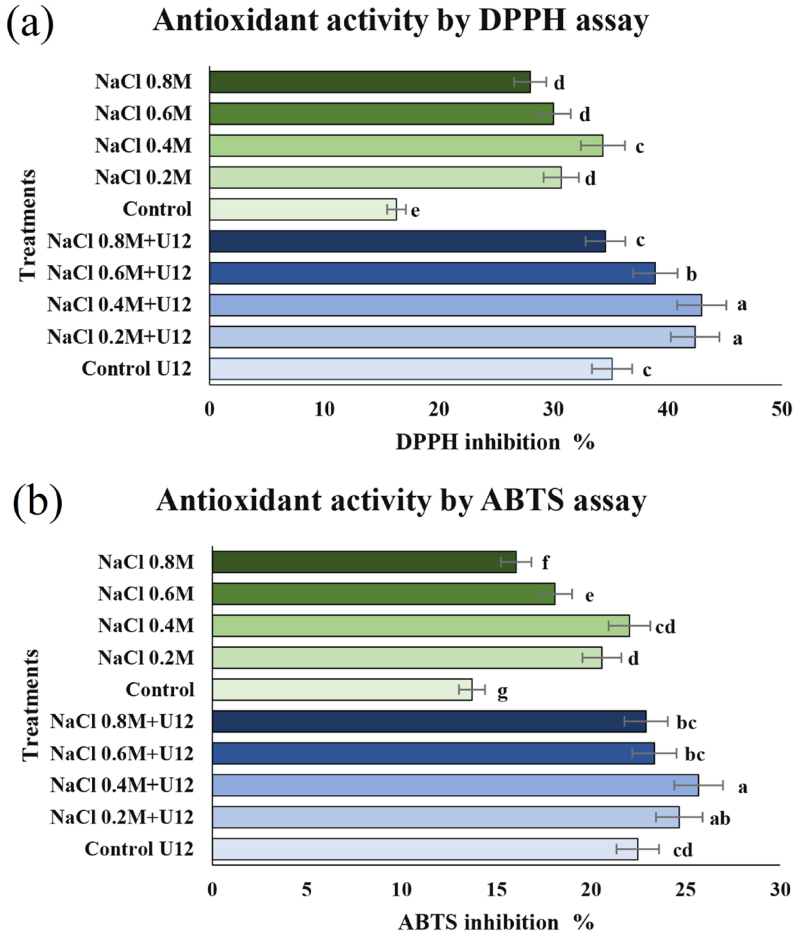

Figure 4. Effects of treatment by different levels of salt and ultrasonication on the antioxidant activity of the myofibrillar protein of silver carp against DPPH (a) and ABTS (b). Different letters (a - g) represent significant differences found by Duncan's multiple range test ($p < 0.05$, and $n = 3$).

M + U12) at 42.42%, both of which had insignificant difference. Chi *et al.* (2014) reported that antioxidant activity was related to the progress of reductones which have a role as terminators of free radical chain reactions. In addition, the increasing value of DPPH inhibition by sonication is correlated with the unfolding of protein structures as well as the destruction of hydrophobic interactions of protein molecules (Yu and Tan, 2017).

ABTS radical scavenging activity

Figure 4b shows the change of ABTS radical scavenging activity for every myofibril protein obtained from different treatments using four saline concentrations and ultrasonic techniques. The highest inhibition of ABTS (25.69%) was observed in the treatment with saline concentration of 0.4 M with sonication (NaCl 0.4 M + U12). From these results, it is clear that the enhancement of salt concentration from 0.2 to 0.4 M generated improved antioxidant activity in both treatments, while increasing concentration to higher levels caused a decrease in this activity. The differences in radical scavenging activity between myofibril proteins obtained using different treatments could probably be due to changes in their peptide sizes and composition of amino acids resulting from the sample preparation by brines (Pires *et al.,* 2013). The ultrasonication effect which showed a beneficial impact on ABTS inhibition of protein solutions might be caused by an enhancement of hydrophobic proteins or amino acid materials following sonication (Zou *et al.,* 2018).

Conclusion

The utilisation of saline solutions at different concentrations improved the functionality of myofibrillar protein obtained from silver carp. The application of salt concentration from 0.2 to 0.4 M generated the increase of DPPH and ABTS inhibition in both treatments as compared to control, especially in samples treated with brine and ultrasound combined. Further studies are needed to investigate the application of other ultrasound conditions on myofibrillar protein in order to add potential applications of protein in the food systems.

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