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The effect of metal ions on lipid oxidation, colour and physicochemical properties of cuttlefish (*Sepia pharaonis*) subjected to multiple freeze–thaw cycles

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Abstract

The effects of different metal ions at various concentrations (0, 5, 25 ppm) on lipid oxidation, discolouration and physicochemical properties of muscle protein in cuttlefish (*Sepia pharaonis*) subjected to multiple freeze–thaw cycles, were investigated. Lipid oxidation of all treatments increased as the freeze–thaw cycle increased. However, the rate of the TBARS increases varied, depending on concentration, type and valency of the metal ion. Fe(II) induced lipid oxidation most effectively and its prooxidative effect was in a concentration-dependent manner. Cu(I), Cu(II) and Cd(II) showed negligible effects on lipid oxidation. The increased lipid oxidation of cuttlefish with added iron was coincidental with the increase in b^* values (yellowness), especially with increasing freeze–thaw cycles. Cu(I) and Cu(II) altered cuttlefish protein sulfhydryl content and the protein solubility decreased with a concomitant increase in the disulfide bond content. The oxidative changes of proteins were observed only when a concentration of metal ions of 25 ppm was used. Those changes were more intense with increasing freeze–thaw cycles. The Ca^{2+}, Mg^{2+}, and Mg^{2+}–Ca^{2+}–ATPase activities of cuttlefish natural actomyosin decreased markedly in the presence of copper, whereas the Mg^{2+}–EGTA–ATPase was increased. SDS-PAGE revealed that Cu(I) and Cu(II) induced the polymerization of muscle proteins stabilised by disulfide bond formation. However, Fe(II), Fe(III) and Cd(II) exhibited no pronounced effect on the oxidation of cuttlefish muscle proteins. Therefore, copper mainly caused the oxidation of protein, while iron induced lipid oxidation and the formation of a yellow colour in cuttlefish muscle, particularly with multiple freeze–thaw cycles.
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Keywords: Lipid oxidation; Freeze–thaw cycles; discolouration; Metal ions; Protein denaturation

1. Introduction

Cephalopods, including cuttlefish, squid and octopus, are an important marine resource for human consumption (Sikorski & Kolodziejska, 1986). Lipid oxidation in muscle foods including cephalopods is one of the major deteriorative reactions causing a loss in quality during frozen storage. Lipid oxidation leads to the formation of free radicals and hydroperoxides. Such intermediary compounds are unstable and cause oxidation of pigments, flavours, and vitamins. Other compounds, such as ketones, aldehydes, alcohols, hydrocarbons, acids, and epoxides, formed during the oxidation of unsaturated fatty acids, produce off-flavours and can interact

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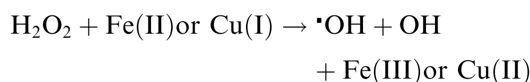
with proteins to produce off-colours (Khayat & Schwall, 1983). Oxidised unsaturated fatty acids bind to protein and form insoluble lipid–protein complexes. Thus lipid oxidation processes lead to discolouration, drip losses, texture changes and off-flavour development (Decker & Hultin, 1992) and production of potentially toxic compounds (Xiong, 2000). Protein and lipid oxidation can therefore account for the toughened texture, poor flavour and/or unappealing odour of poorly frozen stored seafood (Khayat & Schwall, 1983).

Transition metals and heme proteins are the major prooxidants in muscle foods (Decker & Hultin, 1992). Both iron (Fe) and copper (Cu) are essential trace elements in cephalopods, since they are required as cofactors for a number of enzymes and other cellular activities. For instance, cephalopods use a Cu-containing protein, haemocyanin, as a respiratory pigment (Decleir, Vlaemink, Geladi, & Grieken, 1978). Both Fe and Cu are known to promote oxidative reactions through pathways such as Fenton-type reactions (Walling, 1975).



$$H_2O_2 + Fe(II)\text{or } Cu(I) \rightarrow {}^{\bullet}OH + OH + Fe(III)\text{or } Cu(II)$$

The highly reactive hydroxyl radical causes oxidative damage to lipid membranes (Decker, Huang, Osinchak, & Hultin, 1989; Lauridsen, Jensen, Skibsted, & Bretelsen, 2000; Lauritzsen, Martinsen, & Olsen, 1999). Proteins are also major targets, either through oxidation of their amino acid side chains to hydroxyl and carbonyl derivatives, or by a splitting of their peptide bonds (Srinivasan & Hultin, 1997). Degradation and polymerisation of myofibrillar proteins occurs in different model oxidation systems that closely resemble meat or processed meat conditions (Decker, Xiong, Calvert, Crum, & Blanchard, 1993; Liu & Xiong, 2000; Liu, Xiong, & Butterfield, 2000; Srinivasan & Hultin, 1997). Liu and Xiong (2000) found marked changes in the electrophoretic pattern of myosin treated with $FeCl_3/H_2O_2$/ascorbate system, where oxidation caused a disulfide cross-linkage of myosin to form a polymer. Decker et al. (1993) reported that hydroxyl radicals promoted a deterioration in functional properties of myofibrillar proteins (solubility, water holding capacity and gel strength). Liu et al. (2000) found that myofibrils oxidised by $FeCl_3/H_2O_2$/ascorbate showed a decrease in gel-forming ability and an alteration of the functional groups of amino acids in the myofibrillar proteins. Site-specific metal-catalysed oxidation of amino acids in proteins occurs via hydroxyl free radicals, which are produced from H_2O_2 at specific iron-binding sites on proteins.

Although metal ions in cephalopods have been intensively studied, the influence of metal ions on quality changes during processing or storage is poorly understood. The objective of this work was to study the effects of metal ions on lipid oxidation, discolouration and physicochemical changes of cuttlefish muscle during multiple freeze–thaw cycles.

2. Materials and methods

2.1. Chemicals

Ammonium molybdate, 5-5′-dithio-bis(2-nitrobenzoic acid) (DTNB), adenosine 5′-triphosphate (ATP), ρ-nitrophenyl-α-glucopyranoside, ρ-nitrophynyl-*N*-acetyl-β-D-glucose amide, β-mercaptoethanol (βME), cysteine, sodium bisulfite, iron (II) chloride, iron (III) chloride, copper (I) chloride, copper (II) chloride and cadmium (II) chloride were obtained from Sigma (St. Louis, MO, USA).

2.2. Materials

Cuttlefish (*Sepia pharaonis*), caught by cast net from Songkhla coast along the Gulf of Thailand and offloaded about 24 h after capture, were purchased. The cuttlefish, of a size of 8–10 cuttlefishes/kg, were placed in ice with a cuttlefish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 1 h. The cuttlefish were cleaned and deskinned, eviscerated and the eyes were removed. The cuttlefish were kept in ice during preparation.

2.3. Sample preparation

Minced cuttlefish muscles were frozen in liquid nitrogen and then powdered in a blender. The cuttlefish powder (312 g) was mixed with 8 ml of water or stock solutions containing different ions (e.g., iron or copper) to obtain final concentrations of 5 or 25 ppm. The cuttlefish paste was frozen at −18 °C in a polyethylene bag using an air blast-freezer for 48 h, followed by thawing using running tap water (25–27 °C) until the core temperature reached 0–2 °C. The mixtures were subjected to different freeze–thaw cycles (0, 1, 2, 3, 5 and 7 cycles).

2.4. Preparation of natural actomyosin

Natural actomyosin (NAM) was prepared according to the methods of Benjakul, Seymour, Morrissey, and An (1997). Cuttlefish paste was homogenised (IKA Labortechnik, Salangor, Malaysa) in 10 vol. of 0.6 M KCl (4 °C) for a total of 4 min on ice. Homogenisation was preformed in 20 s bursts, followed by 20 s rest periods to avoid over heating. The homogenate was centrifuged at 5000*g* for 30 min at 4 °C and the supernatant, to which 3 vol. of chilled water were added, was collected.

The diluted supernatant was centrifuged at 5000*g* for 30 min at 4 °C to precipitate the NAM. NAM was solubilised in 0.6 M KCl (1:1) and then recentrifuged at 5000*g* for 30 min at 4 °C to precipitate insoluble protein. The supernatant was collected and the protein content was adjusted to 4 mg/ml. The NAM solution was used for analysis of surface hydrophobicity, sulfhydryl contents, disulfide bond contents and enzyme activity.

2.5. Thiobarbituric acid-reactive substances

Thiobarbituric acid reactive substances (TBARS) were determined as described by Buege and Aust (1978). Cuttlefish paste, containing different types and concentrations of ions (2 g), was dispersed in 10 ml of thiobarbituric acid solution (0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 N HCl). The mixture was heated in a boiling water for 10 min, followed by cooling in running tap water. The mixture was centrifuged at 3600*g* for 20 min at room temperature. The absorbance of the supernatant was measured at 532 nm. The standard curve was prepared using malondialdehyde (MDA) and TBARS were expressed as mg MDA/kg sample.

2.6. Colour

The colour of the cuttlefish paste was measured using a colourimeter (HunterLab, Model colourFlex, Virginia, USA) and reported in the CIE colour profile system as L^*, a^* and b^*.

2.7. Surface hydrophobicity

Surface hydrophobicity (S_0ANS) was determined as described by Benjakul et al. (1997) using 1-anilinonaphthalene-8-sulphonic acid (ANS) as a probe. NAM solution (4 mg/ml) was diluted in 10 mM phosphate buffer, pH 6.0, containing 0.6 M NaCl to produce protein concentrations of 0.1%, 0.2%, 0.3% and 0.5%, followed by incubation at room temperature for 10 min. The diluted protein solution (2 ml) was mixed with 20 μl of 8 mM ANS in 0.1 M phosphate buffer, pH 7.0 and the fluorescence intensity of ANS-conjugates was immediately measured at an excitation wavelength of 374 nm and an emission wavelength of 485 nm. The initial slope of the plot of fluorescence intensity versus protein concentration was referred to as S_0ANS.

2.8. Total sulfhydryl content

The total sulfhydryl content was determined using 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) according to the method of Ellman (1959) as modified by Benjakul et al. (1997). One ml of NAM solution (4 mg/ml) was mixed with 9 ml of 0.2 M Tris–HCl buffer, pH 6.8, containing 8 M urea, 2% SDS and 10 mM EDTA. Four ml of the mixture were mixed with 0.4 ml of 0.1% DTNB and incubated at 40 °C for 25 min. The absorbance at 412 nm was measured using a 0.6-M KCl solution as a blank. The sulfhydryl content was calculated using the extinction coefficient of 13,500 M^{-1} cm^{-1}.

2.9. Disulfide bond content

The disulfide bond content was determined using the 2-nitro-5-thiosulfobenzoate (NTSB) assay according to the method of Thannhauser, Konishi, and Scheraga (1987). To 0.5 ml of NAM solution (4 mg/ml), 3.0 ml of freshly prepared NTSB assay solution were added. The mixture was mixed thoroughly and incubated in the dark at room temperature for 25 min. The absorbance at 412 nm was measured. The disulfide bond content was calculated using the extinction coefficient of 13,900 M^{-1} cm^{-1}.

2.10. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to monitor the polymerisation of the modified proteins. The cuttlefish paste was solubilised in 5% SDS (1:9, w/v) and dissolved in sample buffer with and without β-mercaptoethanol. SDS-PAGE was performed using 4% stacking gels and 10% running gels (Laemmli, 1970).

2.11. ATPase activity

ATPase activity was determined according to the method of Benjakul et al. (1997). NAM solution was diluted to 3.0 mg/ml with 0.6 M KCl, pH 7.0. To the diluted protein solution (1 ml), one of the following solutions was added for each ATPase activity assay to a total volume of 9.5 ml: 10 mM $CaCl_2$ for Ca^{2+}–ATPase; 2 mM $MgCl_2$ for Mg^{2+}–ATPase; 2.0 mM $MgCl_2$ and 0.5 mM EGTA for Mg^{2+}–EGTA–ATPase, 0.1 mM $CaCl_2$ and 2.0 mM $MgCl_2$ for Mg^{2+}–Ca^{2+}–ATPase. To each assay solution, 0.5 ml of 20 mM ATP was added to initiate the reaction. The reaction was conducted for 10 min at room temperature and terminated by adding 5 ml of chilled 15% (w/v) trichloroacetic acid. The reaction mixture was centrifuged at 3500*g* for 5 min and the inorganic phosphate liberated into the supernatant was measured by the method of Fiske and Subbarow (1925). The specific activity was expressed as μmoles of inorganic phosphate released/mg protein/min. A blank solution was prepared by adding chilled trichloroacetic acid to the diluted NAM solution prior to the addition of ATP. Ca^{2+}-sensitivity was calculated according to Benjakul et al. (1997) as follows:

$$\text{Ca}^{2+}\text{-sensitivity} = 1 - \frac{\text{Mg}^{2+}\text{–EGTA–ATPase activity}}{\text{Mg}^{2+}\text{–Ca}^{2+}\text{–ATPase activity}} \times 100$$

2.12. Protein solubility

Solubility was determined according to Benjakul and Bauer (2000). To 1 g cuttlefish paste, 20 ml of 0.6 M KCl were added and the mixture was homogenised for 1 min at a speed of 12,000 rpm, using an IKA homogeniser (Selangor, Malaysia). The homogenate was stirred at 4 °C for 4 h, followed by centrifuging at 8500*g* for 30 min at 4 °C. To 10 ml of supernatant, cold 50% (w/v) trichloroacetic acid was added to obtain a final concentration of 10%. The precipitate was washed with 10% trichloroacetic acid and solubilised in 0.5 M NaOH. The cuttlefish paste was also directly solubilised by 0.5 M NaOH to determine total protein. Protein content was determined using the Biuret method (Robinson & Hodgen, 1940).

2.13. Statistical analyses

Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out using Duncan's multiple range test (DMRT) (Steel & Torrie, 1980). Statistical analyses were performed using the Statistical Package for Social Science (SPSS 10.0 for windows, SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Lipid oxidation

Lipid oxidation in muscle foods is one of the major deteriorative reactions causing losses in quality during frozen storage (Sahoo & Verma, 1999). The levels of TBARS in cuttlefish paste with added metal-ions at 5 and 25 ppm during different freeze–thaw cycles are presented in Table 1. TBARS, in all treatments, increased as the number of freeze–thaw cycles increased ($p < 0.05$). The rate of lipid oxidation varied, depending on concentration and type of metal ion. TBARS formation increased with increasing metal ion concentrations. The TBARS values of Fe(II) and Fe(III)-treated samples increased more rapidly, than did other samples. Of the two iron-containing samples, Fe(II) induced lipid oxidation in cuttlefish paste most effectively, as evidenced by the greatest TBARS values after 3–5 and 1–5 freeze–thaw cycles for 5 and 25 ppm iron, respectively. Samples containing Cu or Cd had TBARS levels similar to the control. Mizushima, Takama, and Zama (1977) reported the catalytic effect of copper, iron, and hemin on lipid oxidation of Alaska pollack and mackerel oil at 40 °C in the order: Fe(II) > hemin > Cu(II) > Fe(III). Freeze–thaw cycling produces the repeated ice crystal formation that could disrupt cellular structure (Benjakul & Bauer, 2000). Lipid oxidation in muscle foods is thought to occur at the cellular membrane level and thus oxidative deterioration is a problem in both lean and fatty fish muscle. Therefore, freeze–thawing could result in changes in the physical organisation of membrane lipids which could impact lipid oxidation pathways.

3.2. Discolouration

L^*, a^* and b^* values of cuttlefish paste, with and without metal ions, as affected by multiple freeze–thaw cycles are shown in Fig. 1. In samples with added metals, only those with Fe(II) and Fe(III) had an increase in a^* and b^* values as the number of freeze–thaw cycles increased. In addition, a decrease in L^* value was only observed in the samples containing Fe(II) and Fe(III).

Table 1
Changes in thiobarbituric acid-reactive substances (TBARS) in cuttlefish paste with different metal ions at 5 ppm (*) or 25 ppm (**) during multiple freeze–thaw cycles

Freeze–thaw cycles	Control	Fe(II)	Fe(III)	Cu(I)	Cu(II)	Cd(II)
*TBARS values (mg MDA/kg sample)**						
0	5.30 ± 1.74^{c}	7.53 ± 0.32^{f}	4.05 ± 0.35^{e}	3.85 ± 0.19^{c}	3.30 ± 2.86^{c}	4.16 ± 0.17^{d}
1	5.23 ± 0.78^{c}	17.7 ± 0.23^{e}	9.41 ± 0.71^{e}	4.66 ± 0.14^{bc}	4.67 ± 0.67^{c}	4.60 ± 0.40^{cd}
2	4.99 ± 0.18^{c}	27.9 ± 3.71^{d}	23.0 ± 1.11^{d}	6.60 ± 1.83^{b}	4.79 ± 1.83^{c}	5.04 ± 0.10^{cd}
3	6.03 ± 0.33^{c}	60.9 ± 2.88^{c}	44.3 ± 2.20^{c}	4.92 ± 0.90^{bc}	5.24 ± 0.10^{c}	5.66 ± 0.13^{cd}
5	8.37 ± 1.93^{b}	85.5 ± 1.10^{b}	71.6 ± 5.67^{b}	5.66 ± 0.66^{bc}	6.27 ± 1.21^{b}	7.24 ± 1.25^{b}
7	10.4 ± 0.42^{a}	120 ± 0.44^{a}	108 ± 4.90^{a}	5.67 ± 0.45^{a}	13.1 ± 1.36^{a}	11.8 ± 0.09^{a}
*TBARS values (mg MDA/kg sample)***						
0	5.30 ± 1.74^{c}	21.8 ± 1.23^{d}	15.8 ± 0.55^{d}	5.28 ± 0.23^{bc}	4.49 ± 0.08^{d}	4.66 ± 0.08^{c}
1	5.23 ± 0.78^{c}	87.4 ± 5.81^{c}	63.6 ± 4.46^{c}	4.72 ± 0.10^{d}	4.85 ± 0.36^{d}	4.50 ± 0.39^{c}
2	4.99 ± 0.18^{c}	145 ± 0.17^{b}	111 ± 9.88^{b}	4.95 ± 0.23^{cd}	5.29 ± 0.12^{bc}	5.30 ± 0.64^{bc}
3	6.03 ± 0.33^{c}	142 ± 7.03^{b}	113 ± 13.40^{b}	4.76 ± 0.26^{d}	5.07 ± 0.62^{bc}	5.32 ± 0.71^{bc}
5	8.37 ± 1.93^{b}	137 ± 16.73^{b}	100 ± 11.40^{b}	5.67 ± 0.45^{b}	5.07 ± 0.62^{b}	6.54 ± 0.69^{b}
7	10.4 ± 0.42^{a}	146 ± 3.95^{a}	145 ± 3.94^{a}	11.8 ± 0.17^{a}	13.6 ± 1.10^{a}	9.52 ± 1.05^{a}

Values are given as ±SD from three replications. Values in the same column with different superscripts are significantly different ($p < 0.05$).

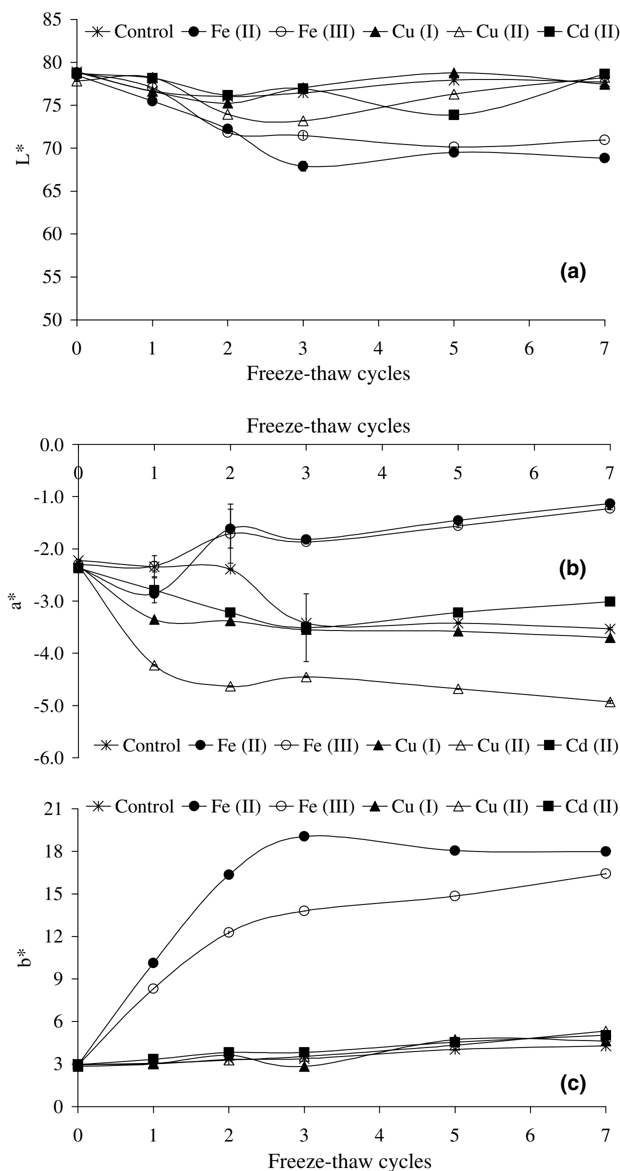


Fig. 1. Changes in L^*, a^* and b^* values of cuttlefish paste containing different metal ions (25 ppm) and subjected to multiple freeze–thaw cycles. Error bars indicate the standard deviations from the means of triplicate determinations.

No changes in L^* or b^* values were observed in samples containing Cu or Cd. Cu(II) caused a decrease in a^* value during the freeze–thawing process. A positive correlation between lipid oxidation (TBARS) and yellow colour (b^*) formation was seen in the samples containing Fe. Free radicals and carbonyl compounds, produced from oxidation of the highly unsaturated fatty acids in the cell membranes, can react with free amino groups in proteins with a subsequent condensation to polymeric brown pigments (Pokorny, 1981). Both soluble and insoluble brown pigments and flavour substances resembling fishy odour were produced by interaction of hydroperoxide decomposition products with primary and secondary amino groups of protein (Pokorny, El–Zeany, & Janicek, 1974). The pigments are probably formed by ionic condensation of primary amino groups of protein with conjugated unsaturated aldehydes or similar reactive lipid-oxidation products that are produced by cleavage of unsaturated hydroperoxides. Frozen herring fillets became discoloured because the lipid oxidation induced the formation of yellow fluorescent pigments in the fillet (Hamre, Lie, & Sandnes, 2003).

3.3. Surface hydrophobicity

Changes in surface hydrophobicity (S_0ANS) indicate conformational changes in protein structure. The changes in S_0ANS in cuttlefish muscle protein, with and without different metal ions (25 ppm), as influenced by freeze–thaw cycles are shown in Fig. 2. Samples with 5 ppm of the metal ions showed no differences in S_0ANS from the control (data not shown). After 1 cycle of freeze–thawing, S_0ANS of all cuttlefish samples increased ($p < 0.05$), except for the sample treated with Cd. The S_0ANS gradually increased as the number of freeze–thaw cycles increased. Cu was the only metal ion that was able to increase S_0ANS more than the control. S_0ANS in samples containing Cu(II) decreased from 5 to 7 freeze–thaw cycles. The decrease in S_0ANS of sample with Cu(II) after seven freeze–thaw cycles suggested that the protein molecules might aggregate with each other via hydrophobic interaction, leading to fewer hydrophobic groups being available to react with ANS. Wang, Xiong, and Srinivasan (1997) reported an increase in S_0ANS of beef heart surimi protein stored at either −15 or −29 °C for more than 3 months. The enhanced hydrophobicity was coincidental with the increased oxidation of lipids, suggesting that protein structural changes might result from modifications of amino acid residue side chains by lipid free radicals or fatty acid decomposition products. However in these

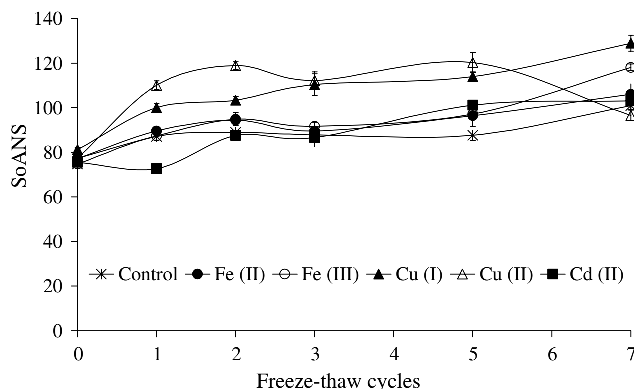


Fig. 2. Changes in surface hydrophobicity (S_0ANS) of cuttlefish proteins exposed to different metal ions (25 ppm) and subjected to multiple freeze–thaw cycles. Error bars indicate the standard deviations from the mean of triplicate determinations.

experiments, an increase in S_0ANS was only seen in the Cu-containing samples, where lipid oxidation was not different from the control. In general, proteins can bind copper ions more effectively than iron, a factor that can induce site-specific oxidation of proteins in the presence of hydroperoxides (Decker, Crum, & Calvert, 1992; Uchida & Kawakishi, 1990; Uchida, Kato, & Kawakishi, 1992).

3.4. Sulfhydryl and disulfide bond content

The impacts of metal ions and freeze–thaw cycling on free sulfhydryl and disulfide bond contents in cuttlefish protein are shown in Fig. 3. Neither free sulfhydryl nor disulfide bond concentrations in samples containing 5 ppm of the metal ions were different from the control (data not shown). In the presence of 25 ppm metals, the free sulfhydryl content of cuttlefish muscle protein decreased after one freeze–thaw cycle (Fig. 3(a)). The extent of decrease was dependent on the metal ion and copper caused the greatest loss of free sulfhydryls. Free sulfhydryl contents in the control, Fe(II)-, Fe(III)- and Cd(II)-containing samples were constant after one cycle of freeze–thawing. After seven freeze–thaw cycles, Cu(I)- and Cu(II)-treated samples had 3.84 and 2.68×10^{-5} moles free sulfhydryls/g protein, respectively. The free sulfhydryl content of the control cuttlefish protein decreased to 5.50×10^{-5} moles/g protein after seven freeze–thaw cycles. The observed decreased in free sulfhydryl content was accompanied by an increase in disulfide bond content (Fig. 3(b)). With loss in free sulfhydryls, the formation of disulfides was greatest in the presence of copper. Conversion of sulfhydryl groups into disulfides and other oxidised species can be due to radical-mediated oxidation of protein (Dean, Fu, Stocker, & Davies, 1997). During refrigerated or frozen storage of beef heart surimi, one-third of the sulfhydryl groups are lost (Srinivasan & Xiong, 1996; Wang et al., 1997). Tunhun, Itoh, Morioka, Kubota, and Obetake (2002) reported that fish protein washed with $CuCl_2$ showed polymerisation of myosin heavy chain through disulfide bonding.

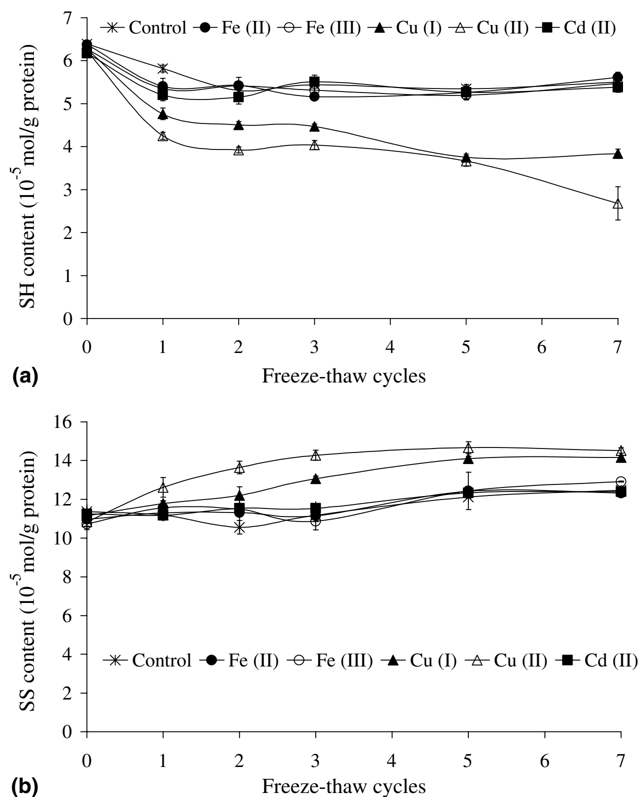


Fig. 3. Changes in sulfhydryl content (a) and disulfide bond content (b) of cuttlefish proteins exposed to different metal ions (25 ppm) and subjected to multiple freeze–thaw cycles. Error bars indicate standard deviations from the means of triplicate determinations.

3.5. SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) patterns of cuttlefish muscle, in the presence and absence of 25 ppm metal ions and subjected to multiple freeze–thaw cycles, are shown in Fig. 4. Cu(I) and Cu(II) catalysed the aggregation of protein in cuttlefish muscle protein, as evidenced by the formation of high (greater than myosin heavy chain, MHC) and intermediate (between actin and paramyosin)

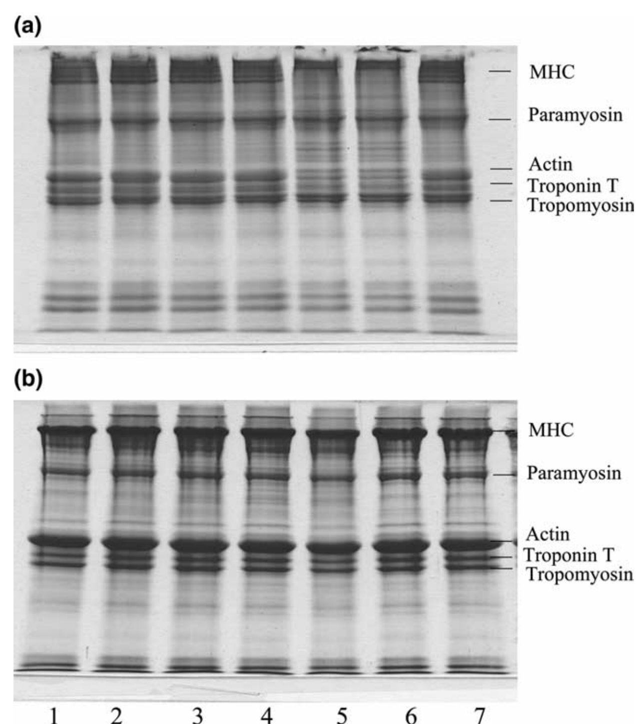


Fig. 4. SDS-PAGE pattern of cuttlefish proteins exposed to different metal-ions (25 ppm) and subjected three freeze–thaw cycles; 1: non-frozen cuttlefish, 2: Control (no added metal ion); 3: Fe(II); 4: Fe(III); 5: Cu(I); 6: Cu(II); 7: Cd(II).

molecular weight polymers with concomitant decreases in myosin, actin and troponin T band intensities (Fig. 4a). The disappearance of polymers and the reappearance of myosin, actin and troponin T in the presence of reducing agents suggested that the polymers were formed via disulfide linkages between these proteins (Fig. 4b). Similar changes in electrophoretic patterns were also reported (Srinivasan & Hultin, 1997) in oxidised cod muscle. No fragmentation could be observed in any of the cuttlefish protein samples. Srinivasan and Hultin (1997) also did not observe fragmentation in oxidised cod protein.

3.6. ATPase activity

Ca^{2+}–ATPase, Mg^{2+}–ATPase, Mg^{2+}–Ca^{2+}–ATPase, and Mg^{2+}–EGTA–ATPase activities can be used as indicators for chemical or structural changes in myosin, actin, actin–myosin and troponin–tropomyosin, respectively (Azuma & Konno, 1998; Benjakul et al., 1997; Roura & Crupin, 1995). A decrease in ATPase activity can be due to conformational changes in the myofibrillar proteins (Okada, Inoue, & Akiba, 1986) as well as protein–protein polymerisation (Benjakul & Bauer, 2000). ATPase activity in cuttlefish proteins subjected to freeze–thaw cycling, in the presence and absence of metal ion, are depicted in Fig. 5. As seen with the other measurements of protein oxidation, Cu(II) and Cu(I) caused the greatest loss of activity in all the different measurements of ATPase activities. Copper was able to decrease all the ATPase activities measured, suggesting that myosin, actin, actin–myosin and troponin–tropomyosin were all susceptible to oxidation. The oxidation of these myofibrillar proteins, as measured by loss of ATPase activity, was in good agreement with the SDS-electrophoresis data (Fig. 4) which also showed that actin, myosin and troponin T were susceptible to copper-induced oxidation. Of all the ATPase activities, copper was able to decrease Ca^{2+}–ATPase activity most effectively, suggesting that myosin was the most susceptible to Cu-induced oxidation. Mg^{2+}–ATPase activity, in actomyosin extracted from mantles of squid, decreased during 3 months of frozen storage (Joseph, Varmar, & Venketaraman, 1997; Paredi & Crupkin, 1997). Igochi, Tsuchiya, and Mustsumoste (1981) reported a slight decrease in the Ca^{2+}–ATPase of squid actomyosin caused by freezing.

A decrease in Ca^{2+}-sensitivity indicates the loss in Ca^{2+} regulation of tropomyosin (Benjakul et al., 1997; Ebashi, Kodama, & Ebashi, 1968). The Ca^{2+}-sensitivity of all samples decreased with increasing numbers of freeze–thaw cycles (Fig. 6). Cu-treated samples showed

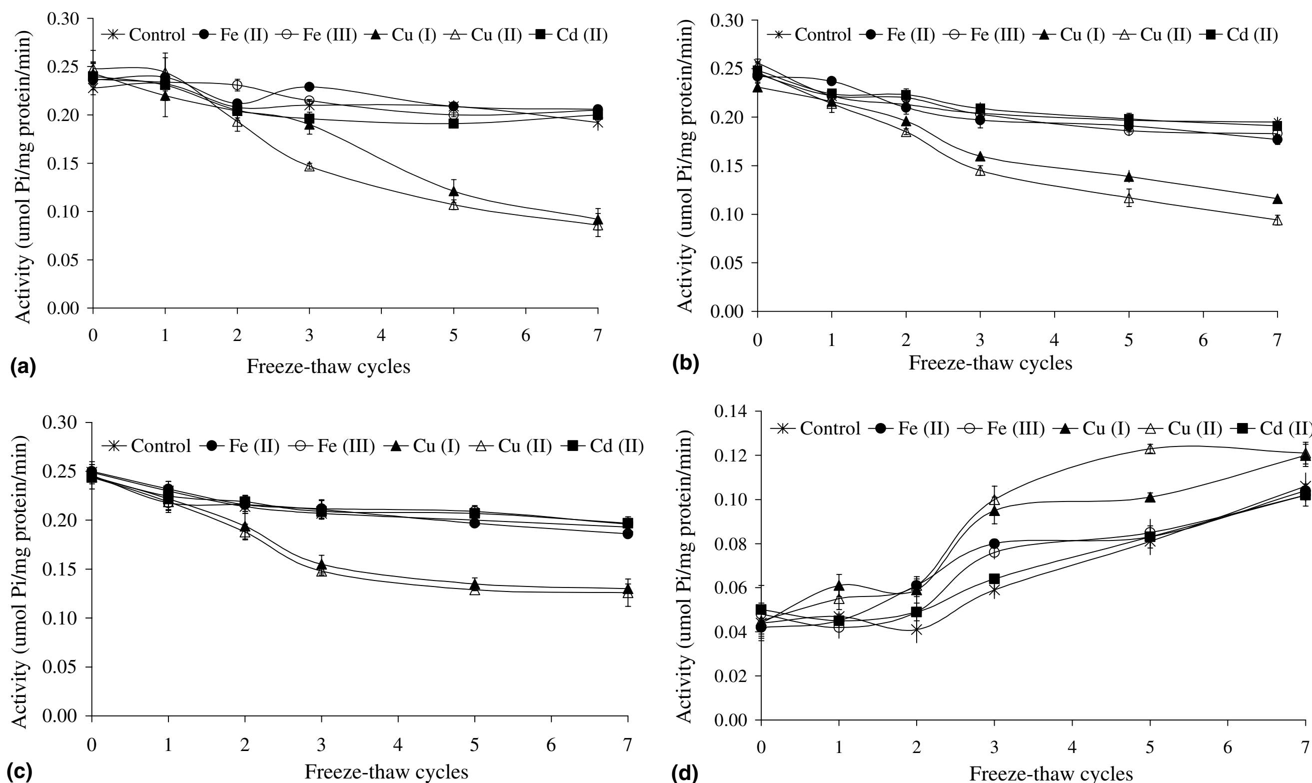


Fig. 5. Changes in ATPase activity of cuttlefish proteins exposed to different metal ions (25 ppm) and subjected to multiple freeze–thaw cycles; a: Ca^{2+}–ATPase activity, b: Mg^{2+}-activity, c: Mg^{2+}–Ca^{2+}–ATPase activity, d: Mg^{2+}–EGTA–ATPase-activity. Error bars indicate standard deviations from the means of triplicate determinations.

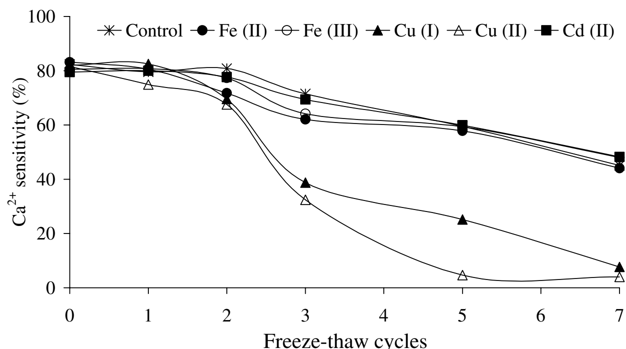


Fig. 6. Changes in Ca^{2+} sensitivity of cuttlefish proteins exposed to different metal ions (25 ppm) and subjected to multiple freeze–thaw cycles. Error bars indicate standard deviations from the means of triplicate determinations.

the most dramatic decrease in Ca^{2+}-sensitivity, especially, with repeated freeze–thaw cycling. These results suggested that copper was able to alter the biological activity of tropomyosin, even though no changes in its molecular weight could be observed by electrophoresis (Fig. 4).

3.7. Solubility

Decrease in protein solubility is often used as a marker of oxidative deterioration of muscle protein quality (Decker et al., 1993; Srinivasan & Hultin, 1997; Xiong & Decker, 1995). Solubilities of cuttlefish proteins in 0.6 M KCl in the presence of different metal ions during multiple freeze thaw cycles are depicted in Fig. 7. Protein solubility decreased only slightly during freeze–thaw cycling with the exception of samples treated with copper. Unlike other measurements of protein oxidation, a large difference between the activities of Cu(I) and Cu(II) was observed with Cu(II) causing a larger decrease in protein solubility. Thermodynamically, a decrease in protein solubility is the result of a shift from a tendency of proteins to interact with water towards a situation where proteins interact with each other (Vojdani, 1996). The decrease in salt-soluble protein concentrations in copper-treated samples was in agreement with other protein oxidation data, which showed that copper induced an increase in surface hydrophobicity (Fig. 2), disulfide bond formation (Fig. 3b) and protein polymerisation (Fig. 4). Moral, Tejada, and Borderias (1983) reported a 60% or greater decrease in protein solubility in gutted muscle of squid (*Loligo valgaris*) after 13.5 months of frozen storage. A gradual decrease in protein extractability during frozen storage of squid (*Loligo duvauceli*) and voladur (*Illex coindentii)* was also reported (Joseph et al., 1997; Ruiz-Capillas, Moral, Morales, & Montero, 2002).

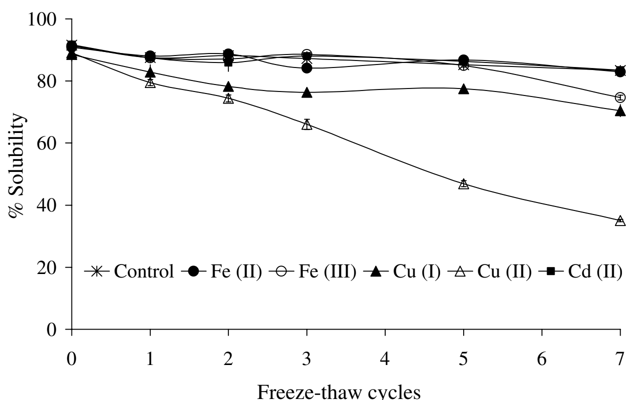


Fig. 7. Changes in protein solubility of cuttlefish mince exposed to different metal ions (25 ppm) and subjected to multiple freeze–thaw cycles. Error bars indicate standard deviations from the means of triplicate determinations.

4. Conclusions

Cuttlefish are susceptible to lipid oxidation, discolouration and loss of protein functionality during frozen storage. The addition of metal ions to cuttlefish paste accelerated lipid oxidation, discolouration and loss of protein functionality during freeze–thaw cycling, although the components in the cuttlefish that were oxidised were highly dependent on the metal ion type. Iron showed the highest prooxidant effect on lipid oxidation and discolouration, while copper mainly caused alterations in the physical and chemical properties of cuttlefish muscle proteins. Binding of copper to proteins could explain why copper promoted protein oxidation while subsequently being unable to promote lipid oxidation since the protein binding could prevent copper–lipid interactions. Only iron caused the formation of yellow pigments (increase in b^*) in cuttlefish paste, suggesting that lipid oxidation is more closely related to discolouration than is protein oxidation. Since cuttlefish are susceptible to oxidation of both its lipids and proteins, this suggests that both metals could be active prooxidants during storage.

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