CHAPTER 3

THE EFFECT OF METAL IONS ON LIPID OXIDATION, COLOR AND CHEMICAL PROPERTIES OF CUTTLEFISH (*SEPIA PHARAONIS*) SUBJECTED TO MULTIPLE FREEZE-THAW CYCLES

3.1 Abstract

The effect of different metal ions (Fe, Cu and Cd) at various concentrations (0, 5 and 25 ppm) on the lipid oxidation, discoloration and physicochemical properties of muscle protein in cuttlefish (*Sepia pharoanis*) subjected to multiple freeze-thaw cycles, were investigated. Lipid oxidation of all treatments increased as the freeze-thaw cycle increased. However, the rate of TBARS increases varied, depending on concentration, type and valency of metal ion. Fe(II) induced the lipid oxidation mostly effectively and its prooxidative effect was in a concentration dependent manner. The increased lipid oxidation of cuttlefish added with iron was coincidental with the increase in b*-value (yellowness), especially with increasing freeze-thaw cycles. Cu(I) and Cu(II) altered cuttlefish protein sulfhydryl content and the protein solubility decreased with the concomitant increased disulfide bond content. The oxidative changes of protein were observed only when a concentration of metal ions of 25 ppm was used. Those changes were more intense with increasing freeze-thaw cycles. Ca^{2+}-, Mg^{2+}- and Mg^{2+}-Ca^{2+}-ATPase activity of cuttlefish natural actomyosin decreased markedly in the presence of copper, whereas Mg^{2+}-EGTA-ATPase activity was increased. SDS-PAGE revealed that Cu(I) and Cu(II) induced the polymerization of muscle proteins stabilized 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 the lipid oxidation and the formation of yellow color from cuttlefish muscle, particularly with multiple freeze-thaw cycles.

3.2 Introduction

Cephalopods, including cuttlefish, squid and octopus, are an important marine resource for human consumption (Sikorski and Kolodziejska, 1986). Lipid oxidation in muscle foods including cephalopods is one of the major deteriorative reactions causing a loss in quality during frozen storage. The oxidation leads to the formation of free radicals and hydroperoxides. Such intermediary compounds are unstable and cause oxidation of pigments, flavors, and vitamins. Other compounds such as ketones, aldehydes, alcohols, hydrocarbons, acids and epoxides are formed during the oxidation of unsaturated fatty acids, produce off-flavor and can react with protein to produce off-color (Khayat and Schwall, 1983). Oxidized unsaturated fatty acids bind to protein and form insoluble lipid-protein complexes. Thus lipid oxidation processes lead to discoloration, drip losses, and off-flavor development (Decker and Hultin, 1992) and production of potentially toxic compounds (Xiong, 2000). Protein and lipid oxidation can therefore account for the toughened texture, poor flavor and/or unappealing odor of poorly stored frozen seafood (Khayat and Schwall, 1983).

Transition metals and heme protein are the major prooxidants in muscle foods (Decker *et al*., 1992). Both iron (Fe) and copper (Cu) are an essential trace element in cephalopods, since they are required as a cofactor for a number of enzymes and other cellular activities. For instance, cephalopods use Cu-containing protein, haemocyanin, as a respiratory pigment (Decleir *et al*., 1978). Both Fe and Cu are known to promote oxidative reaction through the pathways such as Fenton reaction (Walling, 1975).

$$H_2O_2 + Fe(II)/Cu(I) \longrightarrow {}^{0}OH + OH + Fe(III)/Cu(II)$$

The highly reactive hydroxyl radical causes oxidative damage to lipid membrances (Decker *et al*., 1989; Lauritzsen and Martinsen, 1999; Lauridsen *et al*., 2000). Proteins are also major targets of reactive oxygen species in cells, either through oxidation of their amino acid side chains to hydroxyl or carbonyl derivatives, or by a splitting of their peptide bonds (Srinivasan and Hultin, 1997). Degradation and polymerization of myofibrillar proteins

occurs in different model oxidation systems that closely resembling meat or processed meat conditions (Decker *et al*., 1993; Martinard *et al*., 1997; Srinivasan and Hultin, 1997; Liu *et al*., 2000; Liu and Xing, 2000). Liu and Xiong (2000) found marked changes in the electrophoretic pattern change of myosin treated with $FeCl_3/H_2O_2$/ascorbate system. Oxidation caused a disulfide cross-linkage of myosin to form a polymer. Decker *et al*. (1993) reported that hydroxyl radicals promoted the deterioration in functional properties of myofibrillar proteins (solubility, water holding capacity and gel strength). Liu *et al.* (2000) found that myofibrils oxidized by $FeCl_3/H_2O_2$/ascorbate showed a decrease in gel-forming ability and alteration in the functional groups of amino acids in the myofibrillar proteins. Site-specific metal-catalyzed protein oxidation occurs via hydroxyl free radicals, which are produced from H_2O_2 at specific iron-biding site on proteins.

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

3.3 Materials and Methods

Chemicals

Ammonium molybdate, 5-5'-dithio-bis (2-nitrobenzoic acid) (DTNB), adenosine 5'-triphosphate (ATP), 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. Louise, MO, USA).

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 with the

size of 8-10 cuttlefish/kg was 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 was kept in ice during preparation.

Sample preparation

Minced cuttlefish were frozen in liquid nitrogen and then powdered in a blender. The cuttlefish powder was mixed with 8 ml of water or stock solution containing different ions including iron, copper and cadmium to obtain the final concentration of 5 or 25 ppm. The cuttlefish paste was frozen at -18 °C 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).

Preparation of natural actomyosin

Natural actomyosin (NAM) was prepared according to the methods of Benjakul *et al*. (1997). Cuttlefish paste was homogenized (IKA Labortechnik, Salangor, Malaysia) in 10 volumes of 0.6 M KCl (4 °C) for a total of 4 min on ice. Homogenization was performed in 20 sec bursts, followed by 20 sec rest periods to avoid over heating. The homogenate was centrifuged at 5000 xg for 30 min at 4 °C and the supernatant, to which 3 volumes of chilled water were added, was collected. The diluted supernatant was centrifuged at 5000 xg for 30 min at 4 °C to precipitate the NAM. NAM was solubilized in 0.6 M KCl (1:1) and then recentrifuged at 5000 xg 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.

Thiobarbituric acid reactive substances (TBARS)

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 boiling water for 10 min, followed by cooling in running tap water. The mixture was centrifuged at 3600 xg for 20 min at room temperature. The absorbance of the supernatant was measured at 532 nm. The standard curve was prepared using malonaldehyde, and TBARS were expressed as mg malonaldehyde/kg sample.

Color

The color of the cuttlefish paste was measured using a colorimeter (Hunter Lab, Model Color Flex, Virginia, USA) and reported in the CIE color profile system as L*, a* and b*-values.

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 8mM 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.

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 13500 M^{-1} cm^{-1}.

Disulfide bond content

The disulfide bond content was determined using the 2-nitro-5-thiosulfobenzoate (NTSB) assay according to the method of Thannhauser *et al*. (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 13900 M^{-1} cm^{-1}.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to monitor the polymerization of the modified proteins. The cuttlefish paste was solubilized 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).

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. Diluted protein

solution (1 mL) was added with one of the following solutions 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; 0.1 mM EGTA for Mg^{2+}-EGTA-ATPase, 10 mM $CaCl_2$ and 2 mM $MgCl_2$ for Ca^{2+}-Mg^{2+}-ATPase. To each assay solution, 0.5 mL of 20 mM ATP 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 3500 xg for 5 min and the inorganic phosphate liberated in the supernatant was measured by the method of Fiske and Subbarow (1925). Specific activity was expressed as μmoles inorganic phosphate released/mg protein/min. A blank solution was prepared by adding chilled trichoroacetic acid prior to addition of ATP. Ca^{2+}-sensitivity was calculated according to Benjakul *et al*. (1997) as follows:

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

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 homogenized for 1 min at a speed of 12000 rpm, using an IKA homogenizer (Selangor, Malaysia). The homogenate was stirred at 4 °C for 4 h, followed by centrifuging at 8500 xg 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 solubilized in 0.5 M NaOH. The cuttlefish paste was also directly solubilized by 0.5 M NaOH to determine total protein. Protein content was determined using the Biuret method (Robinson and Hodgen, 1940).

Statistical analysis

A factorial design (5 metal ions × 2 concentrations × 6 storage times) was used. Data were subjected to analysis of variance (ANOVA) and mean comparison were carried out using Duncan's multiple rang test (DMRT) (Steel and Torrie, 1980). Statistical analyses were

performed using the Statistical Package for Social Science (SPSS 10.0 for windows, SPSS Inc., Chicago, IL).

3.3 Results and Discussion

Lipid oxidation

Lipid oxidation in muscle foods is one of the major deteriorative reactions causing losses in quality during frozen storage (Sahoo and 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 9. 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 *et al*. (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 and 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 organization of membrane lipids which could impact lipid oxidation pathways.

Discoloration

L*, a*, and b*-values of cuttlefish paste, with and without metal ions, as affected by multiple freeze-thaw cycles are shown in Figure 10. In samples with added metals, only those with Fe(II) and Fe(III) had an increase in a*-value and b*-value 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). No changes in L*-value or b*-value 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 color (b*-value) 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 flavor substances resembling fishy odor were produced by interaction of hydroperoxide decomposition products with primary and secondary amino groups of protein (Pokorny *et al*., 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 discolored because the lipid oxidation induced the formation of yellow fluorescent pigments in the fillet (Hamre *et al*., 2003).

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 Figure 11. 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 *et al*. (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 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 (Uchida and Kawakishi, 1990; Decker *et al*., 1992; Uchida *et al*., 1992).

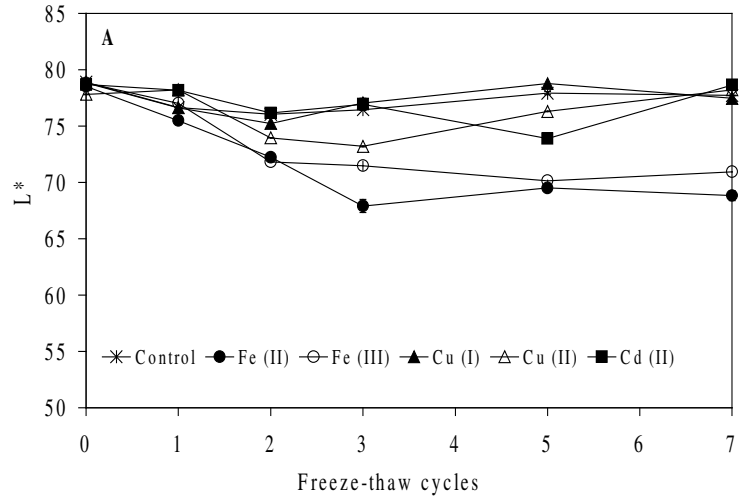
Table 9. Changes in TBARS of cuttlefish paste with different metal ions at concentration 5 ppm (*) and 25 ppm (**) during multiple freeze-thaw cycles

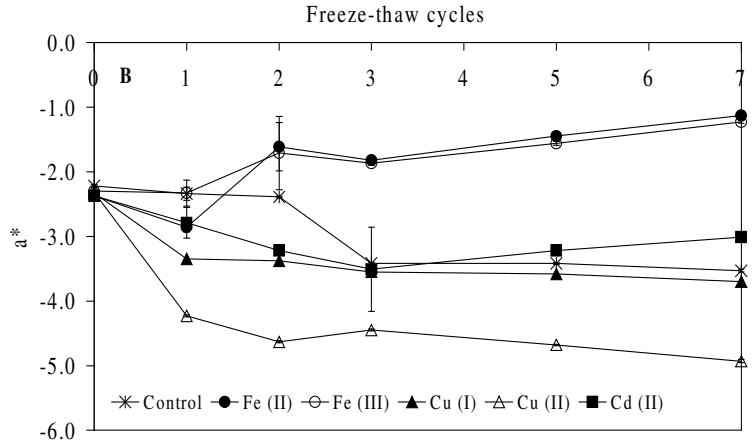
Freeze-thaw Cycles	TBARS values (mg malonaldehyde/kg sample) *					
	Control	Fe (II)	Fe (III)	Cu (I)	Cu (II)	Cd (II)
0	5.30±1.74^{c}	7.53±0.32^{f}	4.05±0.35^{e}	3.85±0.19^{c}	3.30±2.86^{c}	4.16±0.17^{d}
1	5.23±0.78^{c}	17.73±0.23^{e}	9.41±0.71^{e}	4.66±0.14b^{c}	4.67±0.67^{c}	4.60±0.40^{cd}
2	4.99±0.18^{c}	27.91±3.71^{d}	23.02±1.11^{d}	6.60±1.83^{b}	4.79±1.83^{c}	5.04±0.10^{cd}
3	6.03±0.33^{c}	60.85±2.88^{c}	44.26±2.20^{c}	4.92±0.90b^{c}	5.24±0.10^{c}	5.66±0.13^{cd}
5	8.37±1.93^{b}	85.47±1.10^{b}	71.58±5.67^{b}	5.66±0.66b^{c}	6.27±1.21^{b}	7.24±1.25^{b}

7	10.44±0.42[a]	119.52±0.44[a]	107.81±4.90[a]	5.67±0.45[a]	13.05±1.36[a]	11.75±0.09[a]

Freeze-thaw Cycles	TBARS values (mg malonaldehyde /kg sample) **					
	Control	Fe (II)	Fe (III)	Cu (I)	Cu (II)	Cd (II)
0	5.30±1.74[c]	21.77±1.23[d]	15.83±0.55[d]	5.28±0.23[bc]	4.49±0.08[d]	4.66±0.08[c]
1	5.23±0.78[c]	87.40±5.81[c]	63.56±4.46[c]	4.72±0.10[d]	4.85±0.36[d]	4.50±0.39[c]
2	4.99±0.18[c]	145.44±0.17[b]	111.15±9.88[b]	4.95±0.23[cd]	5.29±0.12[bc]	5.30±0.64[bc]
3	6.03±0.33[c]	141.77±7.03[b]	112.67±13.40[b]	4.76±0.26[d]	5.07±0.62[bc]	5.32±0.71[bc]
5	8.37±1.93[b]	136.83±16.73[b]	100.41±11.40[b]	5.67±0.45[b]	5.07±0.62[b]	6.54±0.69[b]
7	10.44±0.42[a]	146.19±3.95[a]	145.03±3.94[a]	11.83±0.17[a]	13.57±1.10[a]	9.52±1.05[a]

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


A
L*
Freeze-thaw cycles
Control
Fe (II)
Fe (III)
Cu (I)
Cu (II)
Cd (II)


Freeze-thaw cycles
B
a*
Control
Fe (II)
Fe (III)
Cu (I)
Cu (II)
Cd (II)

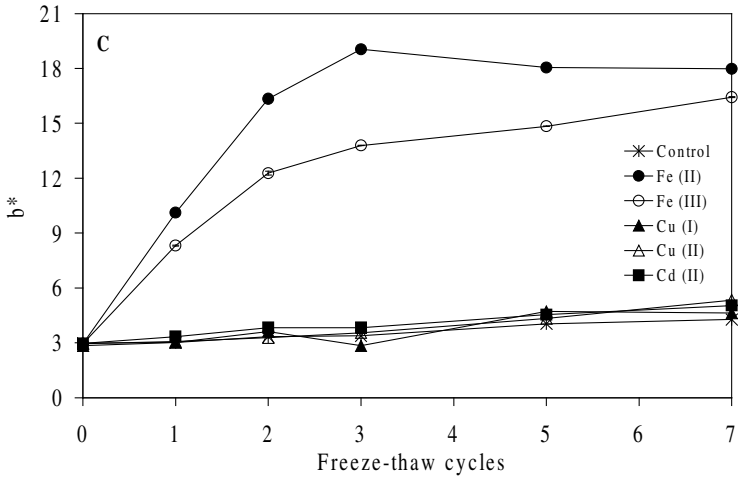

C
b*
Freeze-thaw cycles
Control
Fe (II)
Fe (III)
Cu (I)
Cu (II)
Cd (II)

Figure 10. Changes in L* (A), a* (B) and *b (C)-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 mean of triplicate determinations.

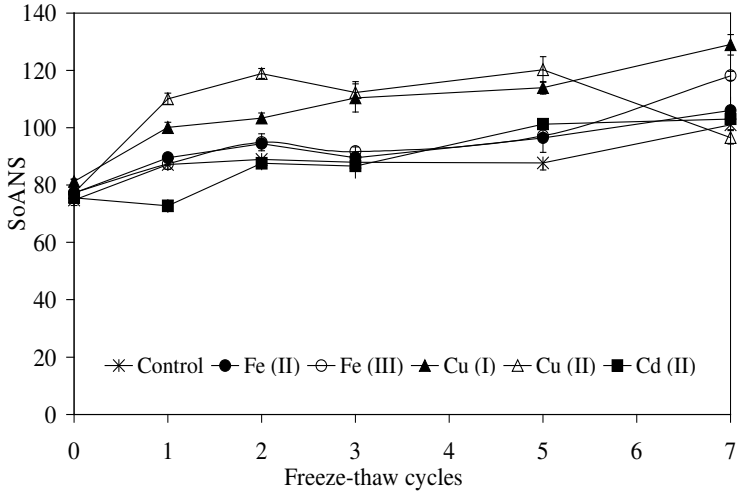


Figure 11. Changes in surface hydrophobicity (S_0ANS) of cuttlefish proteins exposed to different metal ions (25 ppm) and subjected to multiple freeze-thaw cycles. Error bars indicate the standard deviations from the mean of triplicate determinations.

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 Figure 12. 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 (Figure 12A). 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 (Figure 12B). With loss in free sulfhydryls, the formation of disulfides was greatest in the presence of copper. Conversion of sulfhydryl groups into disulfides and other oxidized species can be due to radical-mediated oxidation of protein (Dean *et al*., 1997). During refrigerated or frozen storage of beef heart surimi, one-third of the sulfhydryl groups are lost (Srinivasan and Xiong, 1996; Wang *et al*., 1997). Tunhun *et al*. (2002) reported that fish protein washed with $CuCl_2$ showed polymerization of myosin heavy chain through disulfide bonding.

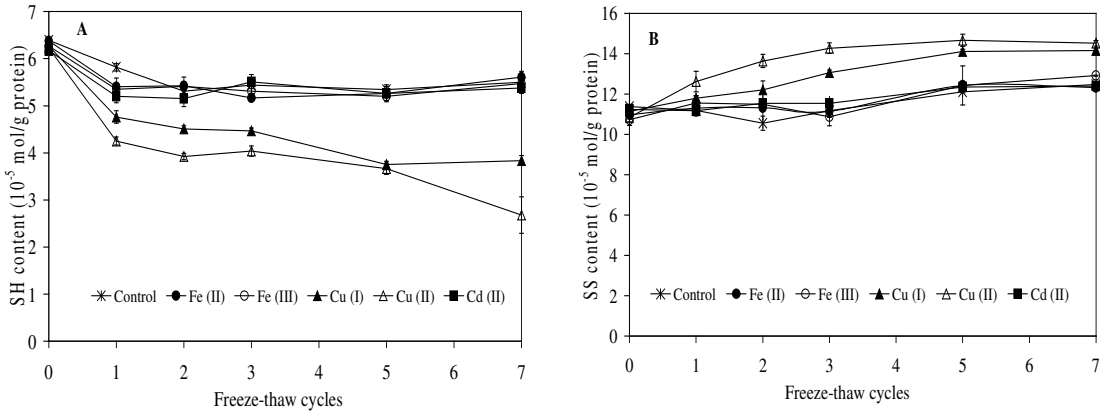


Figure 12. Changes in sulfhydryl content (A) and disulfide bond content (B) of cuttlefish proteins exposed to different metal ions (25 ppm) and subjected to multiple freeze-thaw cycles. Error bars indicate the standard deviations from the mean of triplicate determinations.

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 Figure 13. Cu(I) and Cu(II) catalyzed 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) molecular weight polymers with concomitant decreases in myosin, actin and troponin T band intensities (Figure 13A). 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 (Figure 13B). Similar changes in electrophoretic patterns were also reported (Srinivasan and Hultin, 1997) in oxidized cod muscle. No fragmentation could be observed in any of the cuttlefish protein samples. Srinivasan and Hultin (1997) also did not observe fragmentation in oxidized cod protein.

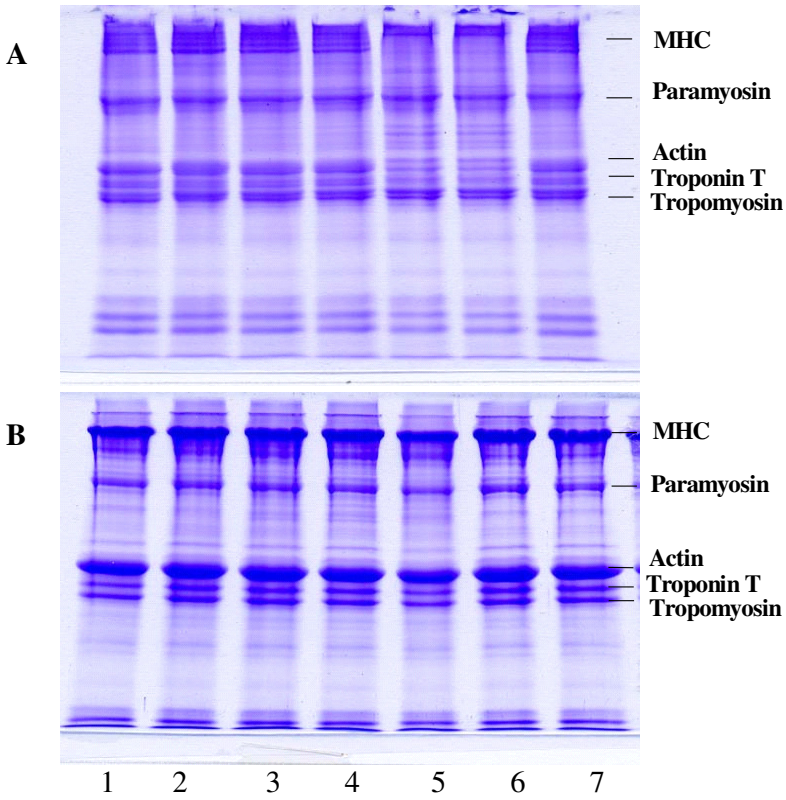


Figure 13. SDS-PAGE pattern of cuttlefish mince with subjected to different metal-ions (25 ppm) and subjected three freeze-thaw cycles, lane 1: non frozen cuttlefish, 2: Control (non added metal ion), lane 3: Fe(II), lane 4: Fe(III), 5: Cu(I), 6: Cu(II) and 7: Cd(II); A= Non-reducing condition, B= Reducing condition.

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 and Konno, 1998; Benjakul *et al*., 1997; Roura and Crupin, 1995). A decrease in ATPase activity can be due to conformational changes in the myofibrillar proteins (Okada *et al*., 1986) as well as protein-protein polymerization (Benjakul and Bauer, 2000). ATPase activity in cuttlefish proteins subjected to freeze-thaw cycling, in the presence and absence of metal ion, are depicted in Figure 14. 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 (Figure 13) 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 *et al*., 1997; Paredi and Crupkin, 1997). Igochi *et al*. (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 (Ebashi *et al*., 1968; Benjakul *et al*., 1997). The Ca^{2+}-sensitivity of all samples decreased with increasing numbers of freeze-thaw cycles (Figure 15). Cu-treated samples showed 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 (Figure 13).

Solubility

Decrease in protein solubility is often used as a marker of oxidative deterioration of muscle protein quality (Decker *et al*., 1993; Xiong and Decker, 1995; Srinivasan and Hultin, 1997). Solubilities of cuttlefish proteins in 0.6 M KCl in the presence of different metal ions

during multiple freeze-thaw cycles are depicted in Figure 16. 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 (Figure 11), disulfide bond formation (Figure 12B) and protein polymerization (Figure 13). Moral *et al.* (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 *et al*., 2002).

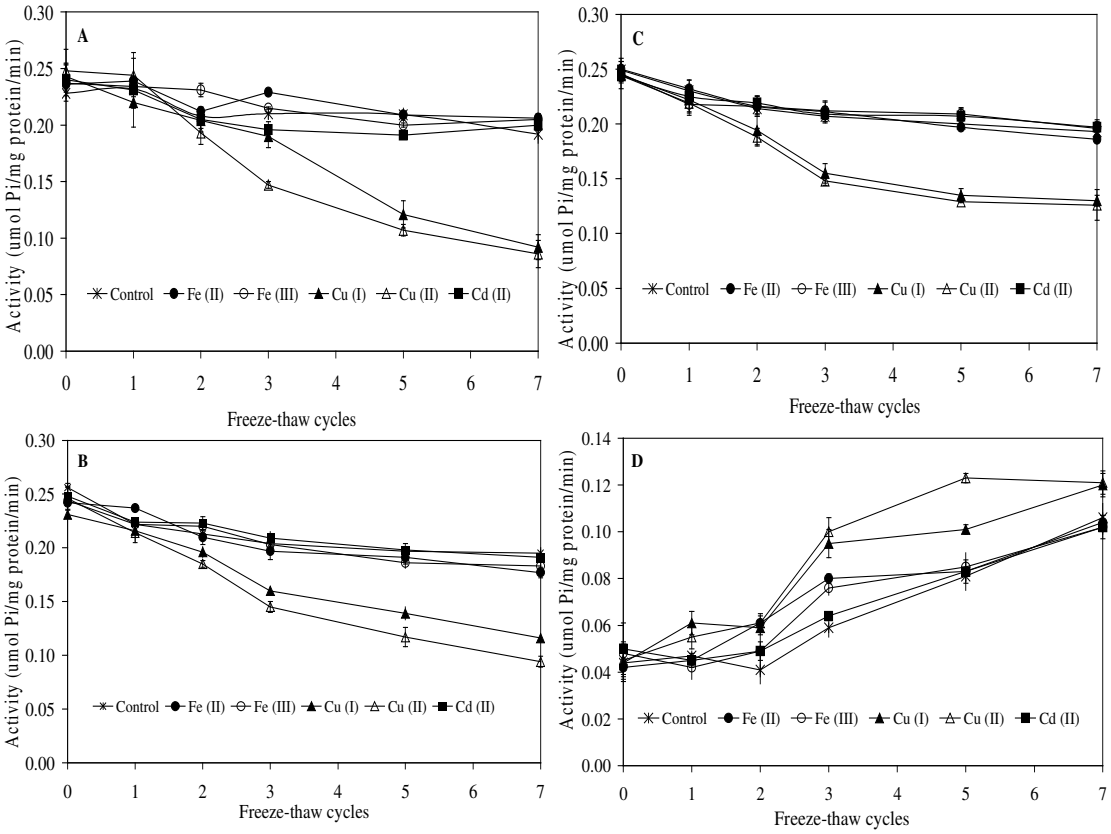


Figure 14. Changes in ATPase activity of cuttlefish proteins exposed to different metal ions (25 ppm) and subjected to multiple freeze-thaw cycles, A: Ca^{2+}-ATPase Activity, B: Mg^{2+}-ATPase Activity, C: Mg^{2+}-Ca^{2+}-ATPase Activity, D: Mg^{2+}-EGTA-ATPase Activity. Error bars indicate the standard deviations from the mean of triplicate determinations.

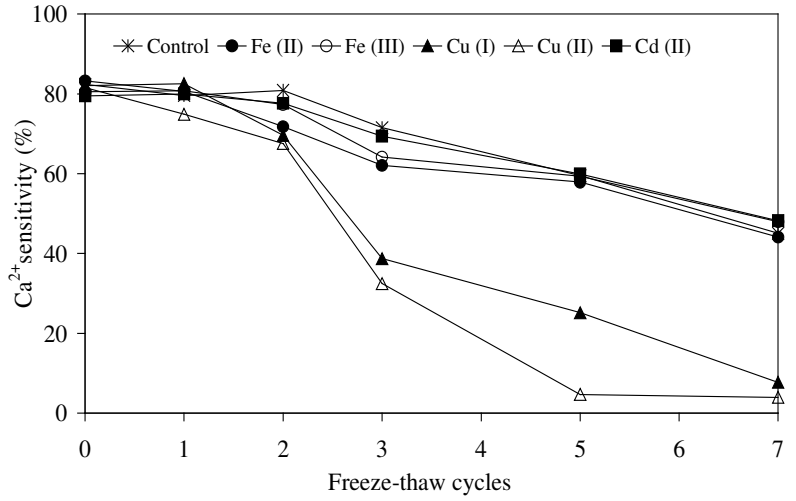


Figure 15. Changes in Ca^{2+}-sensitivity of cuttlefish proteins exposed to different metal ions (25 ppm) and subjected to multiple freeze-thaw cycles. Error bars indicate the standard deviations from the mean of triplicate determinations.

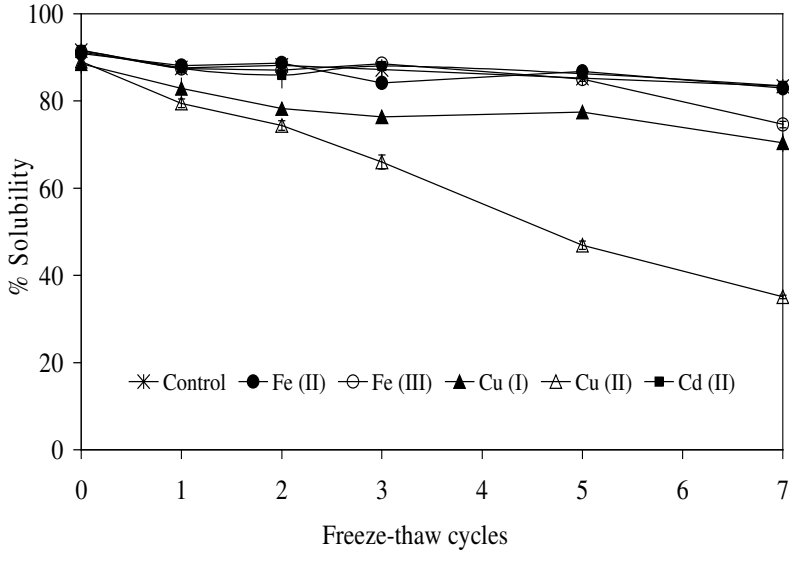


Figure 16. Changes in protein solubility of cuttlefish paste exposed to different metal ions (25 ppm) and subjected to multiple freeze-thaw cycles. Error bars indicate the standard deviations from the mean of triplicate determinations.

3.5 Conclusion

Cuttlefish are susceptible to lipid oxidation, discoloration and loss of protein functionality during frozen storage. The addition of metal ions to cuttlefish paste accelerated lipid oxidation, discoloration and loss of protein functionality during freeze-thaw cycling, although the components in the cuttlefish that were oxidized were highly dependent on the metal ion type. Iron showed the highest prooxidant effect on lipid oxidation and discoloration, 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*-value) in cuttlefish paste, suggesting that lipid oxidation is more closely related to discoloration 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.