CHAPTER 5

LIPID OXIDATION IN MICROSOMAL FRACTION OF SQUID MUSCLE (*LOLIGO PEALI*)

5.1 Abstract

Frozen squid is susceptible to both lipid oxidation and yellow/brown discoloration during frozen storage. The involvement of lipid oxidation in the microsomal fraction of squid muscle on oxidative rancidity and discoloration was investigated using iron and either enzymatic or nonenzymatic redox cycling pathways. Lipid oxidation was measured by thiobarbituric acid reactive substances (TBARS), and color changes were measured spectrophotometrically using an integrating sphere. The lipid oxidation was not observed in the squid microsomes in the presence of Fe(III) and β-nicotinamide adenine dinucleotide disodium salt (NADH) or β-nicotinamide adenine dinucleotide phosphate, reduced (NADPH), suggesting that the enzymatic redox cycling pathway was not active. Iron-promoted TBARS formation was observed in the nonenzymatic pathway when ascorbic acid was used as a reducing compound. Nonenzymatic lipid oxidation increased with increasing temperature (4 °C to 37 °C), iron (0 μM to 100 μM) and ascorbic acid (0 to 200 μM) concentrations. As lipid oxidation in the microsomes or isolated microsomal lipids increased, color changes were observed as could be seen by an increase in b*-value (yellowness) and a decrease in a*-value (redness). The ability of iron and ascorbate to promote both lipid oxidation and pigment formation in the microsomal fraction suggests that this pathway could be responsible for quality deterioration of squid muscle during storage.

5.2 Introduction

Lipid oxidation in muscle foods during processing and storage causes quality deterioration including changes in flavor, color, texture and nutritive value. In addition, the

presence of lipid oxidation products such as lipid hydroperoxides, aldehydes and oxidized cholesterol derivatives in muscle foods can cause a health risk because these compounds are potentially toxic (Chan and Decker, 1994). Skeletal muscle contains high concentrations of unsaturated fatty acids, especially in cellular membranes such as mitochondria, sarcoplasmic reticulum and microsomes. Because the muscle membrane phospholipids exist primarily in the form of a bilayer, they have a much larger surface area than the neutral lipids, which exist primarily as intracellular fat droplets (Huang *et al*., 1993).

In the muscle tissues of squid, almost all lipids reside in the cellular membranes. Thus, studies to evaluate lipid oxidation in the membranes from squid muscle are important in understanding how these reactions may affect quality. Iron can promote lipid oxidation in muscle foods by enzymatic or nonenzymatic pathways (Decker and Hultin, 1990). Enzymatic metal promoted lipid oxidation occurs in the presence of iron, which is reduced by either β-nicotinamide adenine dinucleotide disodium salt (NADH) or β-nicotinamide adenine dinucleotide phosphate, reduced (NADPH), and membrane-bound enzymes located in the sarcoplasmic reticulum and mitochondria (Lin and Hultin, 1976; Borhan *et al*., 1984; Rhee, 1988). Nonenzymatic metal promoted lipid oxidation occurs in the presence of reducing agents such as superoxide, ascorbate or cysteine (Kanner *et al*., 1987). The microsomal or sarcoplasmic reticulum enzymatic lipid oxidation systems have been reported in chicken, beef cattle and many marine fish (Lin and Hultin, 1976; Player and Hultin, 1977; McDonald *et al*., 1979; Rhee *et al*., 1984; McDonald and Hultin, 1987; Decker *et al*., 1988; Huang *et al*., 1998; Soyer and Hultin, 2000; Lin *et al*., 2003). However, there are no published reports describing whether this enzymatic lipid oxidation system is active in squid muscle. Therefore, the objective of this work was to study both enzymatic and nonenzymatic pathways of lipid oxidation in microsomal fraction of squid muscle.

5.3 Materials and Methods

Chemicals

Adenosine-5-diphosphate (ADP), NADH, trichloroacetic acid, were procured from Acros Organics (Morris Plains, NJ, USA). Ferric(III) chloride, butylated hydroxytoluene (BHT), L-ascorbic acid, NADPH, thiobarbituric acid (TBA), malonaldehyde, monopotassium dihydrogen phosphate, and dipotassium hydrogen phosphate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hydrochloric acid, ethanol, methanol, chloroform, and potassium chloride were obtained from Fisher Chemicals (Morris Plains, NJ, USA).

Materials

Squids (*Loligo peali*), caught in Northwest Atlantic and offloaded within 24 h of capture, were purchased from Point Judith Fishermen's Co. (Narragansett, RI, USA). The squids (12 to 18 squid/kg) were placed in ice with a squid/ice ratio of approximately 1:2 (w/w) and transported to the Department of Food Science, University of Massachusetts within 4 h. The squids were immediately cleaned and deskinned. The squid mantles were cut into approximately 1 cm^2 pieces, packed in polyethylene bags (300 g/bag), and stored at -80 °C.

Preparation of squid microsomal fraction

Squid muscle microsomes were isolated according to the method of Brannan and Decker (2001). Frozen squids were thawed under running tap water until the core temperature was approximately 4 °C. Squid muscle was chopped in a stainless-steel blender for 1 min, and the resulting paste (25 g) was then homogenized in 100 mL of 0.12 M KCl/25 mM phosphate buffer, pH 7.2, in a Tissuemizer (20000 rpm; Tekmar, Cincinnati, Ohio, U.S.A.) for 2 min, followed by centrifugation for 30 min at 10000 xg at 4 °C (Sorvall Superspeed RC2-B, Newton, CT, USA). The resulting supernatant was ultracentrifuged for 60 min at 100000 xg (Sorvall Ultra 80, DuPont, Wilmington, DE, USA) to pellet-insoluble muscle components including the microsomes. Myofibrillar proteins were then solubilized from the pellet in 0.6 M KCl, 25 mM phosphate buffer, pH 7.2, and a microsome containing pellet was isolated by ultracentrifugation for 60 min at 100000 xg. Protein in the microsomal fraction pellet was determined by the method of Lowry *et al*. (1951). Lipid content of the microsomes (44%) was calculated from the

differences between the total weight of dried microsomes and the protein content. Isolated microsomes were standardized to 30 mg of protein per mL of 0.12 M KCl, 25 mM phosphate buffer, pH 7.2, and stored at -80 °C until use.

Lipid oxidation in microsomal fraction of squid muscle

Lipid oxidation in the microsomal fraction of squid muscle was studied by both enzymatic and nonenzymatic pathways. The reaction medium of the enzymatic system contained a final concentration of 200 μM ADP, 50 μM $FeCl_3$, 200 μM NADH or NADPH, and 2 mg squid microsomal protein/mL of 25 mM potassium phosphate buffer (pH 7.2)/0.12 M KCl. The nonenzymatic system had a similar composition, except that ascorbic acid was substituted for NADH or NADPH. Initiation of the reaction was done by addition of the microsomes to the other reactants. Incubation was performed in a shaking incubator (INOVA™ 4080, New Brunswick Scientific, Edison, NJ, USA) at 4 °C or 37 °C. All incubations were conducted under exposure to the atmosphere in Erlenmeyer flasks. All reagents were prepared fresh and added to the assays within 30 min. The assay medium was sampled at various times and analyzed as described subsequently.

Preparation of liposomes from squid microsomal lipids

The lipids from the squid microsomes were extracted by homogenizing 1 part microsome with 5 parts solvent (chloroform: methanol; 2:1) for 2 min. Solvent was then removed by evaporation under nitrogen. Liposomes were prepared from the isolated squid phospholipids according to the method of Decker and Hultin (1990a). The squid microsome lipid (0.2 mg/mL) was dispersed in 0.12 M KCl, 25 mM potassium phosphate buffer, pH 7.2, with a Potter-Elvehjem homogenizer (Wheaton Science Products, Millville, NK, USA), followed by sonication (35% amplitude with 5 sec repeating cycle; Sonicator, Sonic Dismembrator, Model 500, Fisher Scientific, Pittsburgh, PA, USA) in an ice bath for 30 min. Squid liposomes were oxidized at 37 °C in the presence of 200 μM ADP, 50 μM $FeCl_3$, and 200 μM ascorbic acid. The assay medium was sampled at various times and analyzed as described subsequently.

Measurement of lipid oxidation

Thiobarbituric acid reactive substances (TBARS) were determined by a modification of the procedure of McDonald and Hultin (1987). TBA stock solution consisted of 15% trichloroacetic acid (w/v) and 0.375% TBA (w/v) in 0.25 M HCl. To 100 mL of TBA stock solution, 3 mL of 2% BHT in ethanol were added. Microsome or liposome solutions (1.0 mL) were added to 2 mL of the TBA solution, vortexed, heated in a boiling water bath for 15 min, cooled to room temperature, and centrifuged at 1600 xg for 20 min. The absorbance of supernatant was measured at 532 nm, and the results were reported as μmol TBARS/mg microsomal protein in experiments using microsomes. In the experiment comparing microsomes and liposomes, oxidation is expressed as μmol TBARS/mg microsomal lipid assuming a lipid concentration in the microsomes of 44%. TBARS concentrations were determined from a malonaldehyde standard curve (derived from 1,1,3,3-tetraethoxypropane).

Color measurement

The color of oxidized microsomes and liposomes were measured using a UV-visible spectrophotometer equipped with an integrating sphere (UV-210PC, Shimadzu Scientific Instruments, Columbia, MD, USA) according to the method of Chantrapornchai *et al*. (2001). Spectral reflectance measurements were made over the wavelength range of 380 to 780 nm using scanning speed of 700 nm/min. The data were reported as L*, a*, b*-values according to the method of Chantrapornchai *et al*. (2001).

Statistics

Completely randomized design (CRD) was used in this study. All experiments were run in triplicate. Statistic analysis was performed using one way analysis of variance

(ANOVA). Mean separations were achieved using Duncan's multiple range test (Steel and Torrie, 1980).

5.4 Results and Discussion

Enzymatic and nonenzymatic lipid oxidation in microsomal fraction of squid muscle

Promotion of lipid oxidation via iron reduction by enzymes in the microsomal fraction of skeletal muscle is dependent on NADH or NADPH as a cofactor that provides reducing equivalents. ADP plays a role in the microsomal enzyme system by chelating iron to maintain its solubility (McDonald and Hultin, 1987). In general, mammalian and avian species prefer NADPH, whereas fish species prefer NADH (Lin and Hultin, 1976; Player and Hultin, 1977; Rhee *et al*., 1984; Lin *et al*., 2003). In the squid microsomes, TBARS were observed to increase in the absence of all cofactors after 8 h at 37 °C and 120 h at 4 °C (Figure 21). Addition of iron and ADP promoted lipid oxidation at both 4 °C and 37 °C compared with the control. Oxidation generally increased with increasing incubation temperature and time. However, NADH and NADPH in combination with iron and ADP increased TBARS formation in microsome less effectively, compared with ADP and iron at either incubation temperature. At 4 °C, NADH had little effect on oxidation rates, whereas NADPH inhibited lipid oxidation with TBARS concentrations being less than the control (without iron, and ADP). At 37 °C, TBARS of samples with ADP, iron, and NADH or NADPH samples were similar but were lower than that of samples with ADP and iron. The lack of an increase in lipid oxidation in the presence of NADH or NADPH suggests that the enzymatic iron-promoted lipid oxidation by squid muscle microsomes was not active. The microsomes used in this study were obtained from frozen squid and then stored at -80 °C after isolation until thawing immediately before each experiment. It is possible that these freeze-thaw steps could have inactivated the microsomal enzyme system. However, previous research has shown that the microsomal enzyme system from flounder was stable to frozen temperatures (Apgar and Hultin, 1982). In addition, enzyme promoted lipid oxidation was

observed in over 13 species of marine fish after similar freeze-thaw procedures used in these experiments (Decker *et al*., 1988). These data suggest that the squid microsomes used in this study did not contain an active enzyme system that was able to promote lipid oxidation in the presence of iron, ADP, and NADH or NADPH. Because the enzyme system might be inactivated by freeze-thawing, the oxidation pathway would not be an important prooxidant factor in frozen squid.

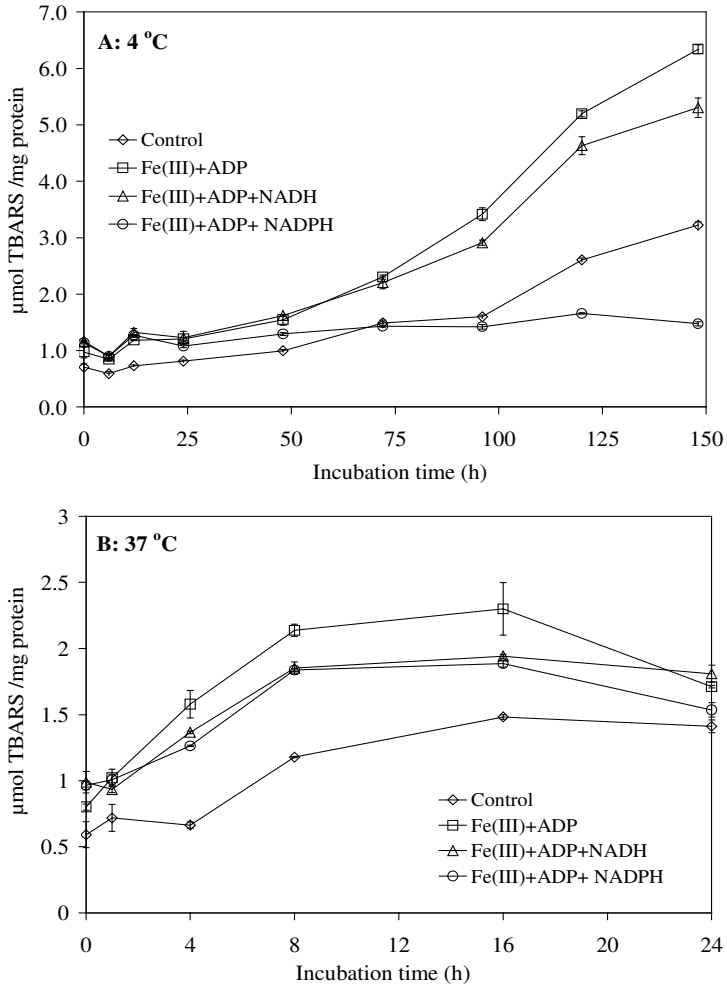

Figure 21. Formation of thiobarbituric acid reactive substances (TBARS) in squid muscle microsomes (2 mg microsomal protein/mL) in the presence or absence of 200 μM ADP, 50 μM $FeCl_3$, 200 μM NADH, 200 μM NADPH during incubation at

4 °C (A) and 37 °C (B). Error bars indicate the standard deviations from the mean of triplicate determinations.

Promotion of lipid oxidation in the squid microsomes was observed in the presence of a nonenzymatic system consisting of iron, ADP and ascorbic acid (Figure 22). Nonenzymatic lipid oxidation in the squid microsomal fraction increased with increasing temperature from 4 °C to 37 °C. Figure 23 shows that TBARS production in the presence of 200 μM ascorbic acid increased with increasing iron concentrations up to 100 μM. During the 4 h of incubation, TBARS formation could be observed with as little as 1.0 μM iron. Increasing ascorbic acid concentrations in the presence of 50 μM $FeCl_3$ also increased TBARS formation in the squid muscle microsomes (Figure 24). TBARS production in the presence of 200 μM ascorbic acid was more than 9-fold higher than 0 μM, 4-fold higher than 50 μM, and 2-fold higher than 100 μM ascorbic acid after 4 h of incubation. Ascorbate can act as both a prooxidant and antioxidant. However, at concentrations of ascorbate and iron found in mackerel muscle (approximately 100 μM), ascorbate concentration is most likely in the prooxidant range (Decker and Hultin, 1992). For example, removal of ascorbate from the soluble fraction of mackerel ordinary muscle decrease $FeCl_3$-stimulated lipid oxidation suggesting that ascorbate was capable of reducing metals in mackerel muscle (Decker and Hultin, 1990b).

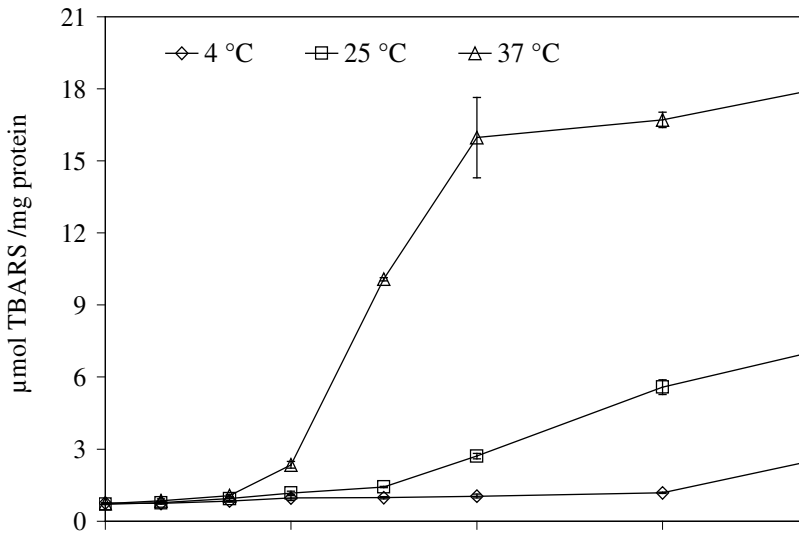

Figure 22. Formation of thiobarbituric acid reactive substances (TBARS) in squid muscle microsomes (2 mg microsomal protein/mL) in the presence of 200 μM ADP, 50 μM $FeCl_3$ and 200 μM ascorbic acid during incubation at 4, 25 and 37 °C. Error bars indicate the standard deviations from the mean of triplicate determinations.

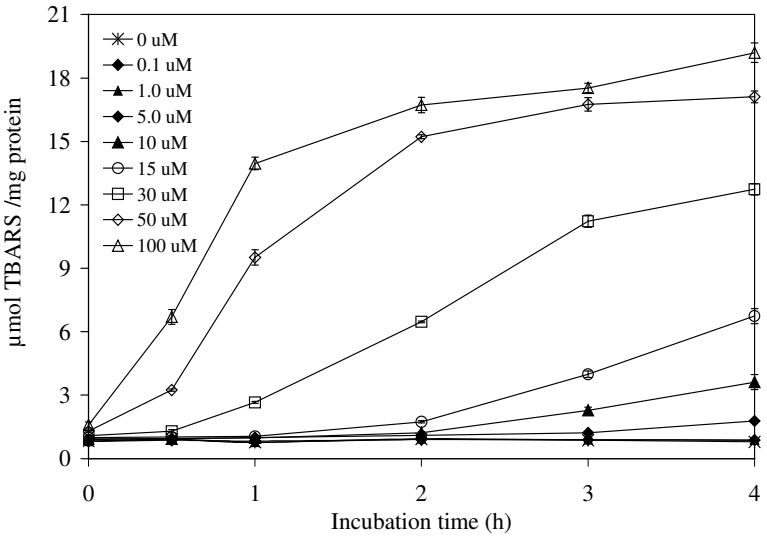

Figure 23. Effect of iron concentration on thiobarbituric acid reactive substances (TBARS) formation in squid muscle microsomes in the presence of 200 μM ADP and 200 μM ascorbic acid during incubation at 37 °C. Error bars indicate the standard deviations from the mean of triplicate determinations.

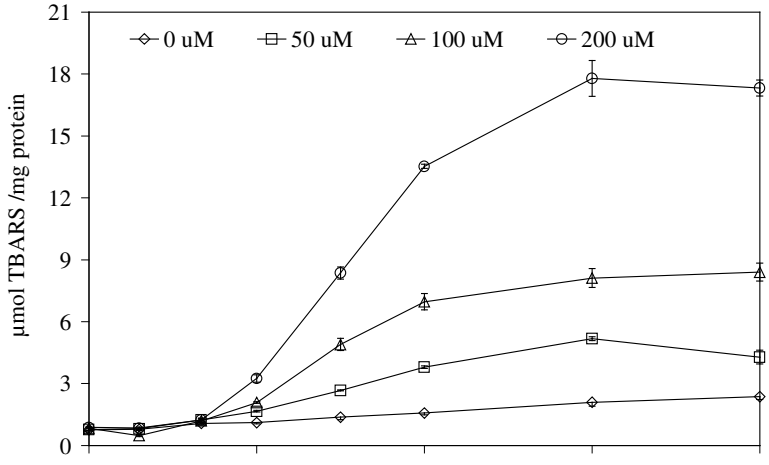

Figure 24. Effect of ascorbic acid concentration on thiobarbituric acid reactive substances (TBARS) formation in squid muscle microsomes in the presence of 200 μM ADP and 5 0 μM $FeCl_3$ during incubation at 37 °C. Error bars indicate the standard deviations from the mean of triplicate determinations.

The effect of nonenzymatic lipid oxidation on discoloration of squid muscle microsomes and microsomal lipids

The effect of iron (50 μM) and ascorbic acid (200 μM) on TBARS formation and discoloration in squid muscle microsomes (microsome system) and squid muscle microsomal lipids (liposome system) is shown in Figures 25 and 26. TBARS formation was observed to increase in the micosome system after 2 h and in the liposome systems after 1 h of incubation (Figure 25). The microsomes were added to the model system at 2 mg microsomal protein/mL. Muscle microsomes contain 44% lipid meaning that approximately 0.9 mg microsomal lipid/mL was present in the microsome system compared with 0.2 mg lipid/mL in the liposome systems. Despite the lower lipid concentrations in the liposomes, TBARS formation occurred more rapidly and to a greater extent, suggesting that the microsomal protein might decrease lipid oxidation rates. In the liposome system, a decrease in a*-value (red color) and an increase in b*-value (yellow color) were observed (Figure 26B, 26C) with the concomitant increase in TBARS formation. In the microsome system, a*-value also decreased after 1 h, whereas b*-value did not increase until 2 h of incubation. The slower change in b*-value in the microsomes could be due to slower lipid oxidation rates. L*-value did not change during the 6 h of incubation (Figure 26A).

Yellow color formation is associated with lipid oxidation in muscle foods such as fish (Lauritzen and Martinsen, 1999; Hamre *et al*., 2003), cuttlefish (Thanonkaew *et al*., 2006), and turkey (Yu *et al*., 2002). Lauritzen and Martinsen (1999) reported that the yellow/brown color was correlated with lipid oxidation of the muscle cod fillet during the salting. During frozen storage of Norwegian spring-spawning herring for up to 9 weeks, there was an increase in TBARS and peroxide values found simultaneously with a loss of a*-value and increase in b*-value (Hamre *et al*., 2003). In our previous work, increased lipid oxidation in cuttlefish during

freeze-thaw cycling was coincidental with an increase in b*-value and a decrease of L*-value and a*-value (Thanonkaew *et al*., 2006a). Yu *et al*. (2002) also found that the increase of lipid oxidation in cooked turkey muscle was correlated with the decrease of redness (a*-value) and the increase of yellowness (b*-value). Lipid oxidation and discoloration in turkey muscle were inhibited by rosemary extract.

Pokorny (1981) described the formation of brown pigments from interactions between lipid oxidation products and proteins. The pigments are proposed to be formed by the condensation of amino groups in proteins with aldehydes produced by the decomposition of lipid hydroperoxides. Lipid oxidation-protein reaction products not only cause color change but also cause a destruction of essential amino acids and formation of antinutritional and toxic compounds (Friedman and Gumbmann, 1986). Our results show that brown pigmentation can also be produced during the oxidation of isolated microsomal lipids (liposome system) extracted from squid muscle microsomes. These results suggest that lipid oxidation-induced brown pigment formation might not only occur from interactions between oxidation products and amines in proteins but also amines in phospholipids.

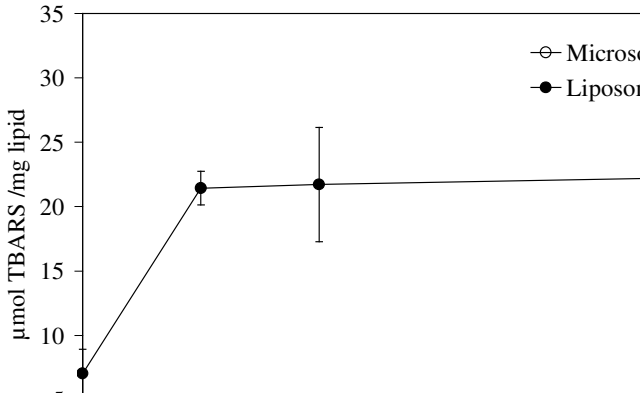

Figure 25. Formation of thiobarbituric acid reactive substances (TBARS) in squid muscle microsomes (approximately 0.9 mg microsomal lipid/mL; microsomal system) or lipids isolated from muscle microsomes (0.2 mg lipid/mL; liposome system) in the presence of 200 μM ADP, 50 μM $FeCl_3$ and 200 μM ascorbic acid during incubation at 37°C. Error bars indicate the standard deviations from the mean of triplicate determinations.

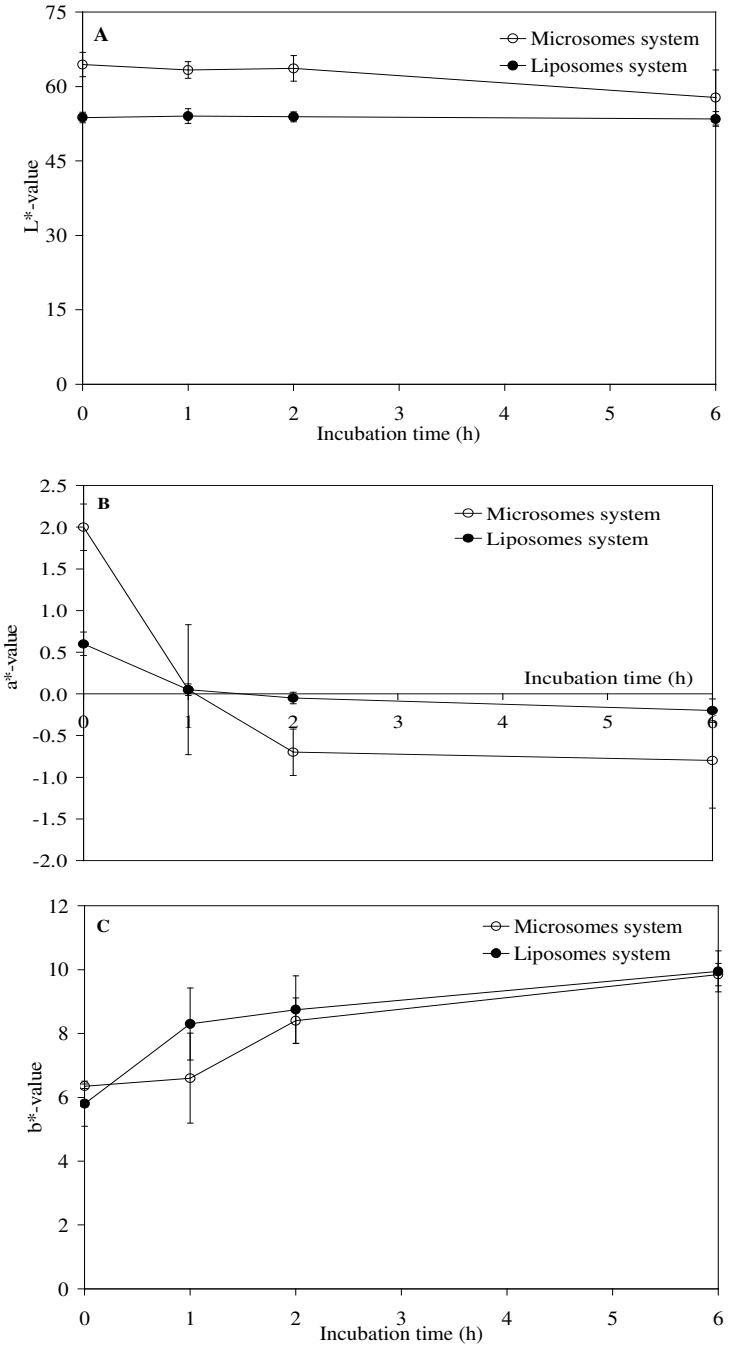

Figure 26. Changes in L* (A), a* (B) and b* (C)-values in squid muscle microsomes (2 mg microsomal protein/mL; microsomal system) or lipids isolated from muscle microsomes (0.2 mg lipid/mL; liposome system) in the presence of 200 μM ADP, 50 μM $FeCl_3$ and 200 μM ascorbic acid during incubation at 37 °C. Error bars indicate the standard deviations from the mean of triplicate determinations.

5.5 Conclusion

Lipid oxidation and simultaneous brown pigment formation is a quality deterioration problem in frozen squid. Lipid oxidation was not observed in microsomes in the presence of ADP, Fe (III), and NADH or NADPH, suggesting that the enzymatic redox cycling pathway was not active in squid muscle. Iron-promoted TBARS formation was observed in the nonenzymatic pathway when ascorbic acid was used as a reducing compound. As lipid oxidation in the microsomes or isolated microsomal lipids increased, color changes were observed as could be seen by an increase in b*-value and a decrease in a*-value. The ability of iron and ascorbate to promote both lipid oxidation and pigment formation in the microsomal fraction suggests that this pathway could be responsible for quality deterioration of squid muscle during storage.