CHAPTER 6

DEVELOPMENT OF YELLOW PIGMENTATION IN SQUID (*LOLIGO PEALI*) AS A RESULT OF LIPID OXIDATION

6.1 Abstract

The impact of lipid oxidation on yellow pigment formation in squid lipids and proteins was studied. When the squid microsomes were oxidized with iron and ascorbate, thiobarbituric acid reactive substances were observed to increase simultaneously with b*-value (yellowness) and pyrrole compounds concomitantly with a decrease in free amines. Oxidized microsomes were not able to change the solubility, sulfhydryl content, or color of salt-soluble squid myofibrillar proteins. Aldehydic lipid oxidation products were able to decrease solubility and sulfhydryl content of salt-soluble squid myofibrillar proteins but had no impact on color. Aldehydic lipid oxidation products increased b*-value (yellowness) and pyrrole compounds and decreased free amines in both squid phospholipid and egg yolk lecithin liposomes. The ability of aldehydic lipid oxidation products to change the physical and chemical properties of egg yolk lecithin liposomes increased with increasing level of unsaturation and when the carbon number was increased from 6 to 7. These data suggest that off-color formation in squid muscle could be due to nonenzymatic browning reactions occurring between aldehydic lipid oxidation products and the amines on phospholipids head groups.

6.2 Introduction

Lipid oxidation in muscle foods is one of the major deteriorative reactions causing losses in quality during processing and storage. The lipid oxidation process leads to discoloration, protein denaturation, off-flavor development and production of potentially toxic compounds (Xiong, 2000). During the oxidation of lipids, carbonyl compounds such as aldehydes and ketones are formed through the degradation of lipid hydroperoxides. Among the secondary

products, aldehydes have received the most attention because of their off-flavors and reactivity with amine groups such as amino acids (Gardner, 1979). Nonenzymatic browning has long been recognized as a consequence of oxidizing lipids in the presence of protein (Gardner, 1979). Most investigators theorize that nonenzymatic browning in muscle foods during lipid oxidation starts with the condensation of aldehydes with amines via Schiff base reaction pathways (Pokorny, 1981; Kikugawa *et al*., 1984; Husain *et al*., 1986). Frozen cephalopods undergo quality deterioration during storage primarily due to discoloration (yellowness), which is sometimes accompanied by the development of a rancid odor. Lipid is a minor component of cephalopods. The squid mantle contains only 1.0-2.0% lipid (Okuzumi and Fujii, 2000). Lipid oxidation in muscle foods is not strongly dependent on the amount of total lipid present because oxidation occurs primarily in cell membrane lipids, which have a high degree of unsaturation and large surface area (Khayat and Schwall, 1983; Huang *et al*., 1993). The lipid in cephalopod muscle is found mostly in the cellular membranes. The amount of phospholipids in cephalopods varies from species to species, ranging from 61.8 to 84.0% of the total lipids (Southgate and Lou, 1995). Phospholipids could contribute to quality deterioration during storage because they contain unsaturated fatty acid that can oxidize to produce aldehydes that cause off-flavors and can react with the amine groups in the polar head groups of the phospholipids such as phosphatidylethanolamine to form nonenzymatic browning products (Pokorny and Kolakowska, 2002; Hidalgo *et al*., 2004). Alternately, aldehydes produced by lipid oxidation could react with the amines of proteins and peptides, causing alterations in color.

Because quality deterioration due to discoloration and development of off-flavors in frozen squid causes the rejection of the product and thus economic loss, this project focused on gaining a better understanding on how these processes occur. Yellow pigment formation was studied in both squid protein and lipid fractions. Because most lipids in squid are cellular membrane lipids, squid microsomes and liposomes were used as models to study the factors effecting lipid oxidation and discoloration.

6.3 Materials and Methods

Materials

Ferric(III) chloride, butylated hydroxytoluene (BHT), L-ascorbic acid, thiobarbituric acid (TBA), monopotassium dihydrogen phosphate, dipotassium hydrogen phosphate, *p*-(dimethylamino) benzaldehyde, 2,4,6-trinitrobenzenesulfonic acid (TNBS), phosphatidylcholine (egg yolk lecithin; 60%), Triton X-100 and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hydrochloric acid, ethanol, methanol, chloroform, potassium chloride, hexanal, *trans*-2-hexenal, *trans*-2,4-hexadienal, *trans*-2-heptenal, *trans*-2-octenal and *trans*-2-nonenal were purchased from Fisher Chemicals (Fair Lawn, NJ, USA). Propanal and trichloroacetic acid were purchased from Acros Organics (Fari Lawn, NJ, USA).

Squid (*Loligo peali*), caught in the Northwest Atlantic and off-loaded within 24 h of capture, were purchased from Point Judith Fishermen's Co., Narragansett, RI, USA. The squid (12-18 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 squid were immediately cleaned and deskinned. The squid mantles were cut into 1 cm^2 pieces, packed in polyethylene bags (300 g/bag), and stored at -80 °C.

Preparation of squid microsomal fraction, liposomes and salt-soluble myofibrillar proteins

Squid muscle microsomes were isolated according to the method of Brannan and Decker (2001). Frozen squid was thawed under running tap water until the core temperature was 4 °C. Squid muscle was chopped in a stainless steel blender for 1 min, and the resulting paste (25 g) was homogenized in 100 mL of 0.12 M KCl/25 mM phosphate buffer, pH 7.2, in a Tissuemizer (20000 rpm; Tekmar, Cincinnati, OH, USA) 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 was determined by using the method of Lowry *et al*. (1951). Isolated microsomes were standardized to 30 mg of protein/mL of 0.12 M KCl/25 mM phosphate buffer, pH 7.2, and stored at -80 °C until use.

The lipids from the squid microsomes were extracted by homogenizing 1 part microsome with 5 parts solvent (chloroform/methanol, 2:1) for 2 min. The solvent phase was then collected and evaporated under nitrogen. Liposomes were prepared from the isolated squid phospholipids or egg yolk lecithin according to the method of Decker and Hultin (1990a). The squid microsome lipid or egg yolk lecithin (5 mg/mL) was dispersed in 0.12 M KCl/25 mM potassium phosphate buffer, pH 7.2, with a Potter-Elvehjem homogenizer follow by sonication (35% amplitude with 5 sec repeating cycle; Sonicator, Sonic Dismembrator, model 500, Fisher Scientific, Pittsburg, PA, USA) in an ice bath for 30 min.

Isolation of salt-soluble myofibrillar proteins (SSP) was accomplished according to the method of Benjakul *et al*. (1997). Squid was homogenized (Tissuemizer, 20,000 rpm; Tekmar) in 10 volumes of 0.6 M KCl (4 °C) for a total of 2 min on ice. Homogenization was performed in 20 sec bursts followed by 20 sec rest periods to avoid overheating. The homogenate was centrifuged at 5000 xg for 30 min at 4 °C, and the supernatant was diluted with 3 volumes of 4 °C water. The diluted supernatant was centrifuged at 5000 xg, for 30 min at 4 °C to precipitate the SSP. The pellet was mixed thoroughly with glycerol to a final concentration of 30% (v/v) glycerol and was stored at -80 °C. Prior to analysis, the frozen SSP was thawed with cool running tap water. The glycerol was removed by mixing the thawed SSP with 10 volumes of chilled water followed by gentle stirring at 4 °C for 30 min. To isolate the SSP, the mixture was centrifuged at 7500 xg at 4 °C for 30 min. The resulting SSP was kept in ice until use. Protein concentration in the SSP samples was determined according to the Biuret method as described by Layne (1957).

Lipid oxidation of liposomes or the microsomal fraction of squid muscle

Lipid oxidation in liposomes or the microsomal systems was accelerated with a nonenzymatic iron redox cycling system. The reaction medium contained a final concentration of 200 μM ascorbate, 50 μM $FeCl_3$, and 5 mg of squid microsomal protein or 5 mg of lipid (for liposomes)/mL of 25 mM potassium phosphate/0.12 M KCl buffer, pH 7.2. Initiation of the reaction was done by addition of the microsomes or liposomes to the other reactants. To directly assess the impact of lipid oxidation products on the chemical characteristics of phospholipids and salt-soluble proteins, stock solutions of lipid oxidization products in ethanol (100 mM) were added directly to the liposomes or proteins at a final concentration of 0.05-10 mM. Controls contained an equivalent amount of ethanol. All samples were incubated in a shaking incubator (INOVA™ 4080, New Brunswick Scientific, Edison, NJ, USA) at 37 °C under atmospheric conditions. The assay medium was sampled at various times and analyzed for thiobarbituric acid reactive substances (TBARS), color, free amines, solubility, sulfhydryls, and pyrrolization as described below.

To determine if lipid oxidation products from microsomes could affect the properties of salt-soluble proteins (SSP), the microsomal fraction of squid muscle (5 mg of squid microsomal protein/mL of 25 mM potassium phosphate/0.12 M KCl buffer, pH 7.2) was oxidized with 200 μM ascorbate/50 μM $FeCl_3$ for 6 h at 37 °C. The oxidized squid microsomes (10 mL) were placed inside in a dialysis bag with a molecular weight cut-off of 500, and the dialysis bags were placed in 100 mL of the SSP solution (5 mg of protein/mL, in 25 mM phosphate buffer, pH 7.2, containing 0.6 M KCl). The reaction was carried out at 25 °C for 8 h.

Measurement of chemical and physical alterations in the microsomes, liposomes and salt-soluble myofibrillar proteins

Lipid oxidation was monitored by measuring TBA reactive substances (TBARS) as 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 was added 3 mL of 2% BHT in ethanol. Microsomes (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 micromoles of TBARS per milligram of microsomal lipid. TBARS concentrations were determined from a malonaldehyde standard curve produced from 1,1,3,3-tetraethoxypropane.

The color of microsomes, liposomes, and proteins was measured using a UV-visible spectrophotometer equipped with an integrating sphere (UV-2101PC, Shimadzu Scientific Instruments, Columbia, MD, USA) according to the method of Chantrapornchai *et al*. (2001). Lipids from the microsomes or liposomes were dissolved in hexane/isopropanal (3:2; 3 mL of microsomes or liposomes to 5 mL of solvent), vortexed for 30 sec, and centrifuged at 2000 xg for 15 min. The solvent phase was collected and used for determination of b*-value. Spectral reflectance measurements were made over the wavelength range of 380-780 nm using a scanning speed of 700 nm/min. The data were reported as b*-value according to the method of Chantrapornchai *et al*. (2001).

Free amine groups were determined using a modified spectroscopic method according to that found in Kubo *et al.* (2005). Samples were diluted (1:4) with 5% Triton X-100 and incubated at room temperature for 30 min. Then, 1.5 mL of diluted solution was added to 30 μL of 100 mM TNBS. Samples were incubated at room temperature for 1 h, and the formation of the resulting trinitrophenyl derivatives was detected spectrophotometrically at 420 nm with a UV-visible spectrophotometer (UV-210PC, Shimadzu Scientific Instruments). A blank was prepared under the same conditions using buffer instead of the microsome or liposomes. Concentrations were calculated from a standard curve prepared with glycine.

Phospholipid pyrrolization was employed as an index of nonenzymatic browning according to a modified method of Hidalgo *et al.* (2005). Samples were diluted (1:1) with 25 mM phosphate buffer containing 3% SDS. The diluted solution (1 mL) was reacted with 160 μL of 0.134 M Ehrlich reagent (the reagent was prepared by suspending 200 mg of *p*-dimethylaminobenzaldehyde in 2 mL of ethanol and adding 8 mL of 1.25 N HCl). The resulting solution was incubated at 45 °C for 30 min, and absorbance was measured at 570 nm. After color development, microsome samples were centrifuged at 1600 x*g* for 30 min to remove protein. A blank was prepared under the same conditions using buffer instead of the microsome or liposomes.

Total sulfhydryl content of the SSP was determined using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) according to the method of Ellman (1959) as modified by Benjakul *et al*. (1997). One mL of SSP solution (5 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 was 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. Sulfhydryl content was calculated using the extinction coefficient of 13500 M^{-1} cm^{-1}. Protein solubility was determined after centrifugation of the protein solutions at 5000 x*g* for 30 min using the Biuret method as described by Layne (1957). Percent solubility was calculated as follows: (soluble protein concentration after treatment/protein concentration of untreated sample) × 100.

Statistics

Completely randomized design (CRD) was used in this study. All experiments were run in triplicate. Statistical analysis was performed using one-way analysis of variance (ANOVA). Mean separations were achieved using Duncan's multiple-range test (Steel and Torrie, 1980).

6.4 Results and Discussion

Yellow discoloration in squid muscle is a major cause of quality deterioration. Previous studies have shown that yellow pigments can form during the oxidation of squid muscle microsomes as well as liposomes prepared from phospholipids isolated from the squid microsomes (Thanonkaew *et al*., 2005). This research suggested that the amines in squid phospholipids were interacting with aldehydes produced by lipid oxidation to form yellow pigments via nonenzymatic browning-type reactions. Nonenzymatic browning occurs when carbonyls such as aldehydes react with amines to form Schiff base products. Upon rearrangement of the Schiff base products, polymerization can occur to produce yellow-brown pigments. These reactions can be monitored by measuring the loss of free amino groups and the formation of pyrrole compounds. Figure 27A shows that lipid oxidation (as determined by TBARS) in squid

microsomes increases at the same time as formation of yellow compounds (as determined by b*-value). Simultaneous with lipid oxidation and yellow pigment formation was a decrease in free amine groups (Figure 27B) and the formation of pyrrole compounds (Figure 27C) typical of nonenzymatic browning reactions (Zamora *et al*., 2000). The decreases in TBARS after 15 h of the incubation time suggested that TBARS might further react with the proteins or phospholipids in squid microsome. Furthermore, the losses in TBARS via volatilization might occur.

The above results suggest that yellow pigment formation in pigment formation in squid could be due to nonenzymatic browning reactions between fatty acid decomposition products and phospholipid head groups. However, browning could also occur as a result of interactions between fatty acid decomposition products and the amines in proteins. Chan *et al.* (1997) developed a model system to study interactions between lipid oxidation products and myoglobin by placing oxidized liposomes in a dialysis bag. This model prevents direct interactions between the liposomes and myoglobin, while allowing low molecular weight lipid oxidation products to pass through the dialysis membrane to modify myoglobin. A similar model was used in this study to determine if fatty acid decomposition products could interact with muscle proteins to form yellow pigments. Squid muscle microsomes (5 mg of protein/mL) were oxidized with iron (50 μM) and ascorbate (200 μM) for 6 h to produce a TBARS value of 26.2 μmol/mg of lipid. The oxidized microsomes were then placed in dialysis bags with a molecular weight cut-off of 500, and the dialysis bags were placed in a solution containing salt-soluble myofibrillar proteins (5 mg/mL). The ratio of lipid to myofibrillar protein in squid muscle would be on the order of 1 part phospholipid to 8 parts myofibrillar protein (Layne*,* 1957; Southgate and Lou, 1995). In this experiment, the microsome to SSP ratios tested were 1:10 and 1:5. Modification of the SSP was determined by changes in free sulfhydryls, protein solubility, and b*-value. Addition of phosphate buffer or phosphate buffer plus the iron (50 μM) and ascorbate (200 μM) to the dialysis bags had no impact on the sulfhydryl content (Figure 28A) or solubility (Figure 28B) of the salt-soluble myofibrillar proteins. Similarly, addition of oxidized microsomes to the dialysis bag at a microsome to salt-soluble myofibrillar proteins ratio of 1:10 or 1:5 had no impact on the sulfhydryl content (Figure 28A) or protein solubility (Figure 28B). No changes in b*-value were observed for any of the treatments (data not shown).

To verify that aldehydic lipid oxidation products could alter the properties of the squid SSP, *trans*-2-hexenal was added directly to the protein at concentrations ranging from 0.05 to 10 mM during incubation at 25 °C for 15 h. Reactions between *trans*-2-hexenal and salt-soluble myofibrillar proteins were complete after 8 h of incubation (data not shown). In the presence of *trans*-2-hexenal at concentrations ≥0.5 mM, protein sulfhydryls decreased (Table 11) with 10 mM *trans*-2-hexenal, causing a >59% decrease in free sulfhydryls after 8 h of incubation. Protein solubility decreased at *trans*-2-hexenal concentrations ≥5 mM, with 10 mM *trans*-2-hexenal decreasing solubility by >27% (Table 11). Addition of *trans*-2-hexenal to the salt-soluble myofibrillar proteins had no significant ($p>0.05$) impact on b*-value (Table 11). These data indicated that *trans*-2-hexenal was capable of modifying the salt-soluble myofibrillar proteins without causing yellow pigment formation.

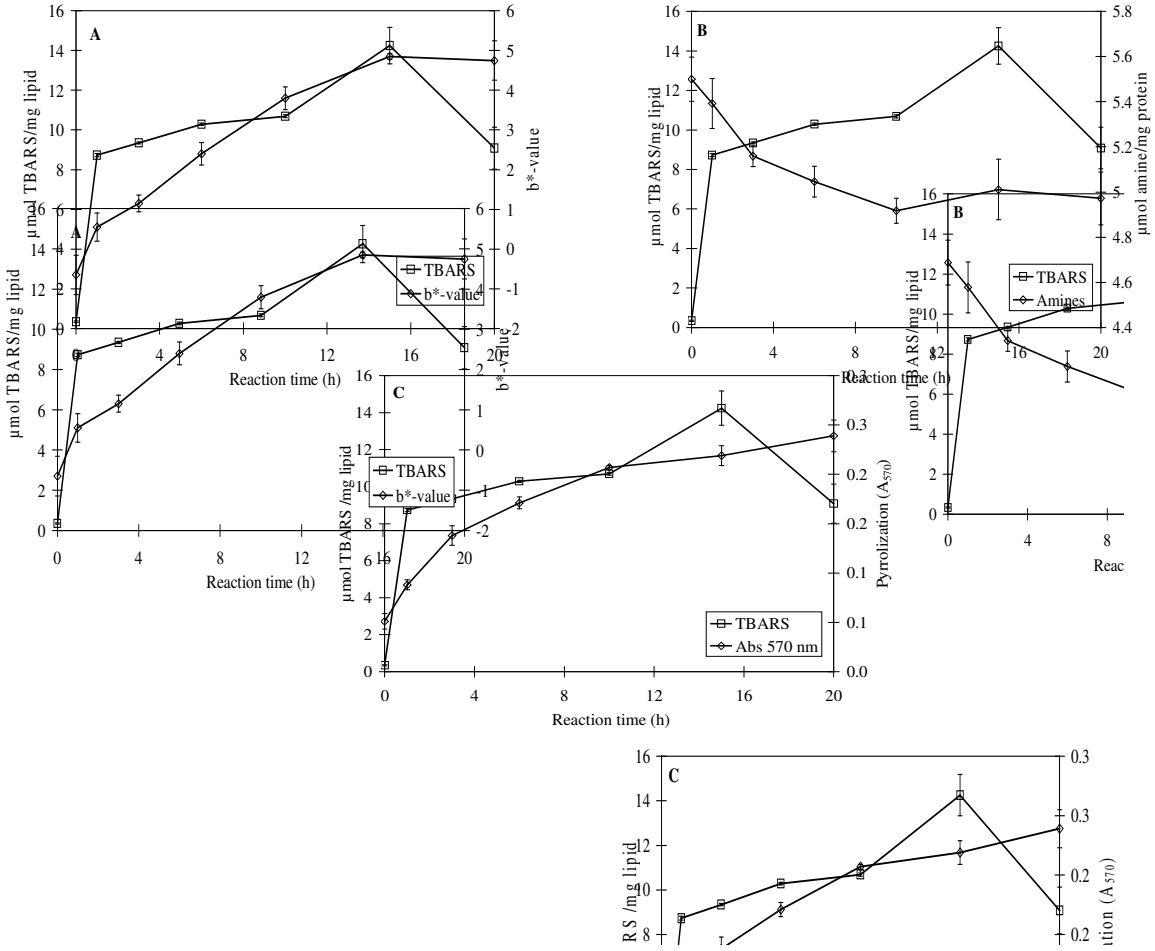

Figure 27. Relationship between development of lipid oxidation (measured as TBARS) with yellow pigment formation (b*-value; A), loss of amines (B) and formation of pyrrole compounds (C) in squid muscle microsomes. Oxidation was promoted with 200 μM ascorbic acid and 50 μM $FeCl_3$ in the presence of 5 mg of microsomal protein/mL of 0.12 M KCl/25 mM potassium phosphate buffer, pH 7.2, at 37 °C. Error bars represent standard deviations of triplicate determinations.

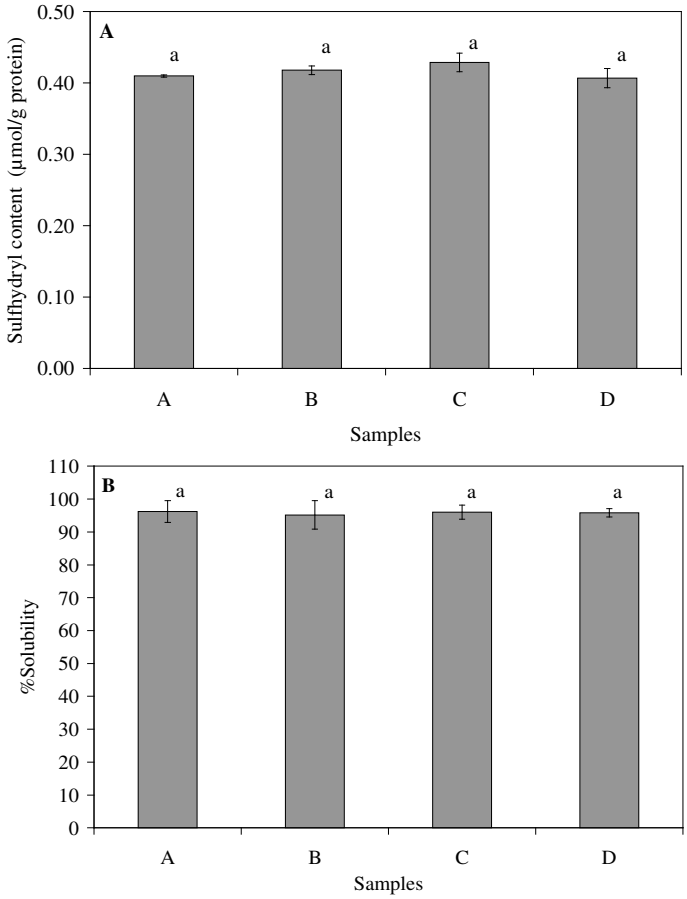

Figure 28. Changes in sufhydryls and solubility of 10 parts of squid muscle salt-soluble proteins (5 mg/mL 0.6 M KCl/25 mM potassium phosphate buffer, pH 7.2) after 8 h of exposure at 25 °C to (A) 1 part of 0.12 M KCl/25 mM potassium phosphate buffer, pH 7.2, (B) 1 part of 200 μM ascorbic acid and 50 μM $FeCl_3$, (C) 1 part of

squid muscle microsomes (5 mg of microsomal protein/mL of 0.12 M KCl/25 mM potassium phosphate buffer, pH 7.2) oxidized by 200 μM ascorbic acid and 50 μM $FeCl_3$, and (D) 2 parts of squid muscle microsomes (5 mg of microsomal protein/mL of 0.12 M KCl/25 mM potassium phosphate buffer, pH 7.2) oxidized by 200 μM ascorbic acid and 50 μM $FeCl_3$. Error bars represent standard deviations of triplicate determinations. Different letters indicate significant differences ($p<0.05$).

Table 11. Interaction between *trans*-2-hexenal and salt-soluble myofibrillar proteins

trans-2-Hexenal (mM)	SH content (μmol/g protein)	%Solubility	b*-value
0.00	0.526±0.003[a]	98.27±2.11[ab]	0.10±0.20[a]
0.05	0.528±0.010[a]	99.14±1.45[a]	0.03±0.20[a]
0.50	0.384±0.003[b]	94.18±2.04[b]	-0.03±0.15[a]
5.00	0.239±0.003[c]	85.43±1.13[c]	-0.03±0.15[a]
10.00	0.215±0.001[d]	72.47±4.29[d]	-0.13±0.32[a]

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

To determine if nonenzymatic browning reactions could occur in phospholipids in the absence of protein, changes in TBARS, b*-value, free amines, and pyrroles were determined in liposomes produced from isolated squid phospholipids and egg yolk lecithin. Iron (50 μM) and ascorbate (200 μM) were able to accelerate TBARS formation in both the squid and egg yolk liposomes (Figure 29A). The higher TBARS in the squid liposomes could be due to

the higher concentrations of long chain polyunsaturated fatty acids, which would be more susceptible to lipid oxidation and upon oxidation would produce a higher yield of TBARS. As with the squid microsomes, increases in b*-value (Figure 29B), decreases in free amines (Figure 29C), and increases in pyrroles (Figure 29D) were observed in both the squid and egg phospholipid liposomes. The simultaneous formation of yellow pigments and loss of amines suggest that the amines in the phospholipids were able to act as the substrates for nonenzymatic browning reactions.

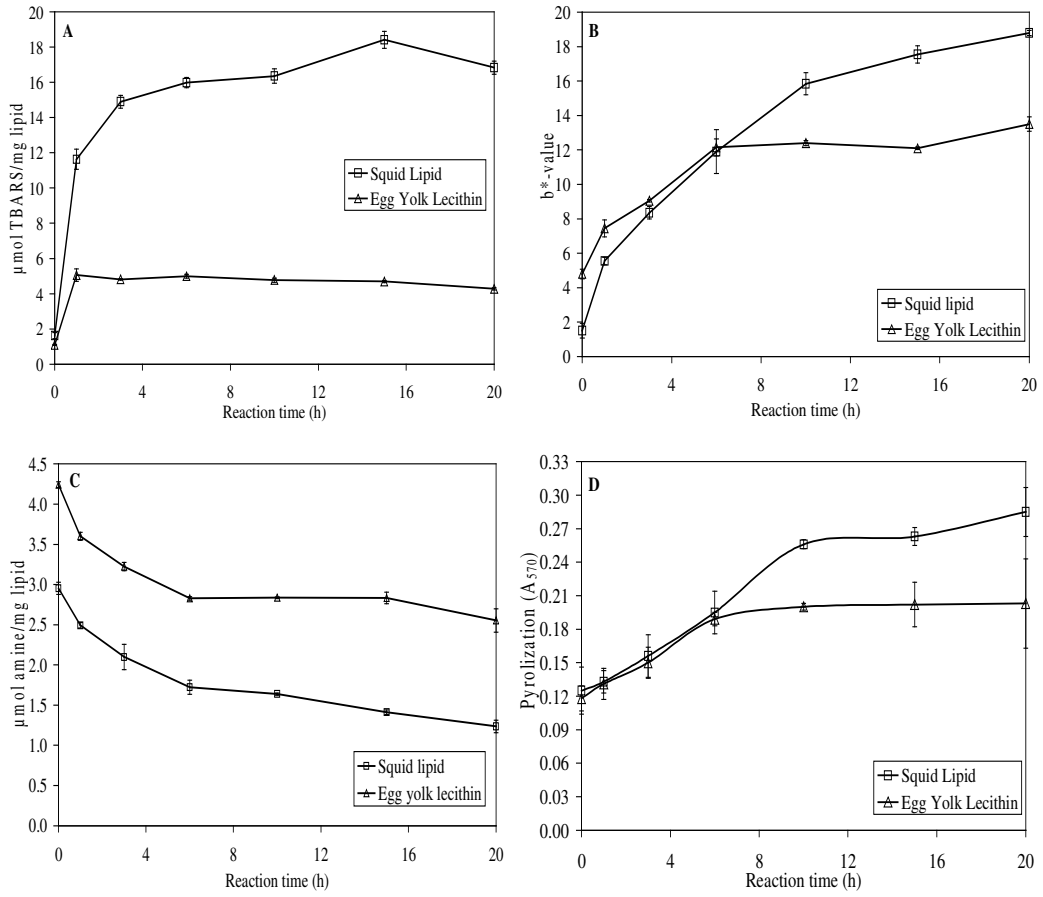

Figure 29. Formation of thiobarbituric acid reactive substances (TBARS; A) and yellow pigments (b*-value; B), loss of amines (C), and formation of pyrrole compounds (D) in liposomes (5 mg of lipid/mL of 0.12 M KCl/25 mM potassium phosphate buffer at pH 7.2) prepared from squid microsomal lipids and egg yolk lecithin during oxidation by 200 μM ascorbic acid and 50 μM $FeCl_3$ at 37 °C. Error bars represent standard deviations of triplicate determinations.

To determine if unsaturated aldehydes that can arise from lipid oxidation could produce yellow pigment formation in phospholipid liposomes, various concentrations of *trans*-2-hexenal and *trans*-2-nonenal (0-10 mM) were added to egg yolk liposomes and changes in b*-value were monitored (Figure 30). After 15 h of incubation at 37 °C, both *trans*-2- hexenal and *trans*-2-nonenal increased b*-value at concentrations 0.5 mM, with *trans*-2-nonenal causing a greater change than *trans*-2-hexenal. The impact of a variety of saturated and unsaturated aldehydes (5 mM) on the b*-value, free amines, and pyrroles of the egg yolk liposomes was also determined (Figure 31). The saturated aldehydes, propanal and hexanal, had the least impact on b*-value and the chemical properties of the liposomes. All of the monounsaturated aldehydes changed b*-value, free amines, and pyrroles ($p \leq 0.05$). As the monounsaturated aldehydes increased from six carbons to seven carbons, a 1.5-fold increase in b*-value was observed (Figure 31A). Further increasing the chain length to nine carbons had little impact on b*-value. As with b*-value, increasing carbon chain length from six to seven caused a dramatic decrease in free amines (Figure 31B). However, when carbon chain length was increased to nine, fewer free amines were lost. Similar trends were observed for increases in pyrroles (Figure 31C). The six-carbon dialdehyde, *trans*-2,4-hexadienal, increased b*-value and free amines more ($p \leq 0.05$) than *trans*-2-hexenal but changed pyrroles in a similar manner to *trans*-2-hexenal.

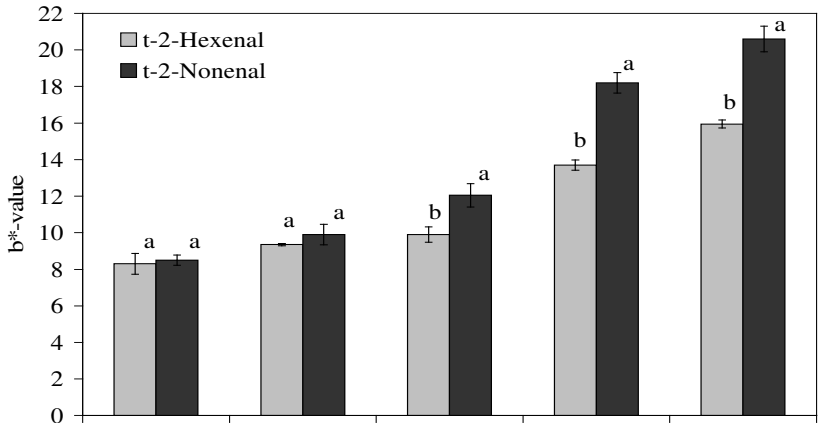

Figure 30. Changes in b*-value in egg yolk lecithin liposomes (5 mg of lipid/mL of 0.12 M KCl/25 mM potassium phosphate buffer at pH 7.2) upon exposure to various concentrations of *trans*-2-hexenal or *trans*-2-nonenal at 37 °C for 15 h. Error bars represent standard deviations of triplicate determinations. Different letters indicate significant differences ($p<0.05$).

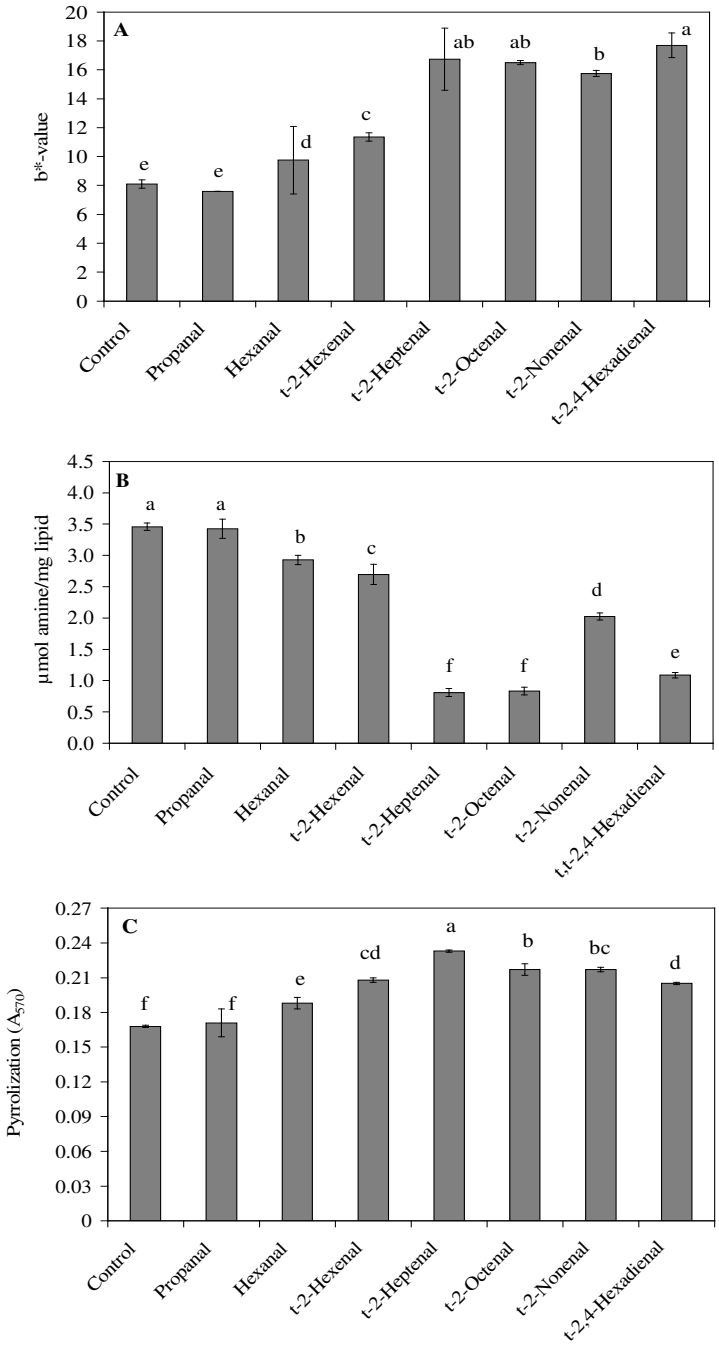

Figure 31. Changes in b*-value (A), amines (B), and pyrrole compounds (C) in egg yolk lecithin liposomes (5 mg of lipid/mL of 0.12 M KCl/25 mM potassium phosphate buffer at pH 7.2) exposed to different aldehydic lipid oxidation products (5.0 mM) at 37 °C for 15 h. Error bars represent standard deviations of triplicate determinations. Different letters indicate significant differences ($p<0.05$).

Yellow color is associated with lipid oxidation in muscle foods such as fish (Lauritzsen and Martinsen, 1999; Hamre *et al*., 2003), cephalopods (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 in cod fillets during the salting process, with increases in lipid oxidation and yellow pigment formation. During frozen storage of the Norwegian spring-spawning herring for up to 9 weeks, there was an increase in lipid oxidation products found simultaneously with yellow color deterioration (Hamre *et al*., 2003). An increase in lipid oxidation in cuttlefish during freeze-thaw cycling was coincidental with an increase in b*-value (yellowness; Thanonkaew *et al*., 2006). Yu *et al*. (2002) also reported that lipid oxidation correlated with an increase in b*-value in cooked turkey products during refrigerated storage.

Several researchers have proposed that the correlation between lipid oxidation and yellow pigment formation in muscle foods was due to nonenzymatic browning reactions occurring between lipid oxidation products and amino groups in proteins. However, in this study salt-soluble myofibrillar proteins were not found to produce yellow pigments in the presence of oxidized muscle microsomes or up to 10 mM concentrations of *trans*-2-hexenal and *trans*-2-nonenal, compounds commonly found in oxidized lipids that readily react with proteins (Chan *et al*., 1997). Instead, yellow pigment formation was observed directly in muscle microsomes (Figure 27). Previous work showed that formation of yellow pigments in muscle microsomes was primarily due to the phospholipid fractions because yellow pigment formation was observed in both microsomes and liposomes produced from isolated squid microsome phospholipids (no proteins; Thanonkaew *et al*., 2005). The ability of phospholipid bilayer to participate in nonenzymatic browning reactions with lipid oxidation products was confirmed using liposomes produced by egg yolk lecithin (Figure 30). The amines in phospholipids such as phosphatidylethanolamine have been shown to participate in nonenzymatic browning reactions *in vitro* (Zamora *et al*., 2005). Introduction of oxygen into fatty acid decomposition products to form

aldehydes would produce lipid oxidation products with polar head groups that would be surface active. Migration of the aldehyde group to the cell membrane interface could place the carbonyl in close proximity to the phospholipid amine, thus facilitating nonenzymatic browning (Figure 32). In contrast, it would be more difficult for the aldehydic lipid oxidation products to participate in nonenzymatic browning reactions with salt-soluble myofibrillar proteins because they would have to partition out of the membrane into the aqueous phase. This would in effect dilute the concentration of the aldehydes at the site of the amines in the proteins compared to the amines in the phospholipids. The reactivity of the aldehydes increased with increasing unsaturation (Figure 31) because double bonds will make the aldehydes more electrophilic (Chan *et al*., 1997). The aldehydic products produced by lipid oxidation could partition into the water, water-phospholipid interface, or bilayer lipid phase (A, B, and C in Figure 32, respectively). Reactivity of the aldehydes in the liposomes increased with increasing carbon number (seven or more carbons), which could be due to the larger, less water soluble aldehydes being more highly retained in the phospholipids bilayer or at the water-phospholipid interface (Figure 32B), where they could react with the amines, compared to *trans*-2- hexenal, which could partition into the aqueous phase (Figure 32A).

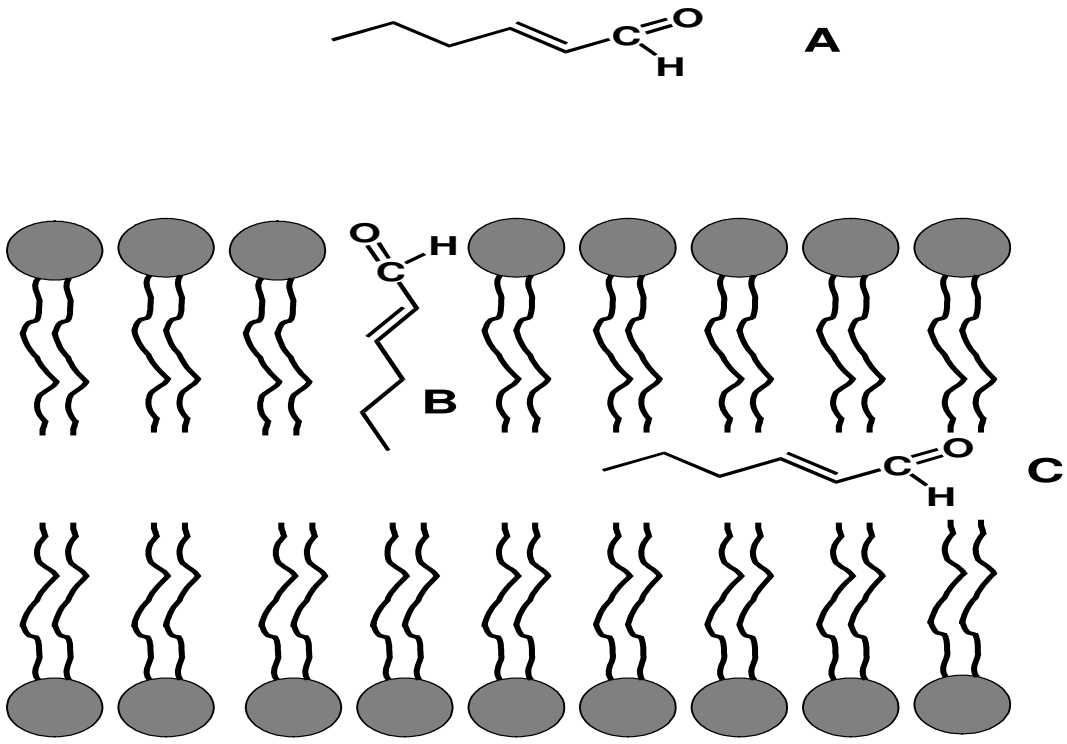

Figure 32. Proposed model for the impact of physical location of aldehydic lipid oxidation products on their reactivity with phospholipid amines. Physical locations include aqueous phase (A), interfacial region (B), and lipid phase (C).

6.5 Conclusion

Lipid oxidation and aldehydic lipid oxidation products were able to produce yellow pigment formation in squid microsomes, squid phospholipid liposomes, and egg yolk lecithin liposomes but not in squid salt-soluble myofibrillar proteins. Nonenzymatic browning reactions in squid muscle would occur primarily between aldehydic lipid oxidation products and the amine groups of phospholipids. Reactions between aldehydic lipid oxidation products and the amine groups of phospholipids could be occurring rapidly because of the physical orientation of the aldehydes in phospholipid bilayer, where the carbonyl group of the surface-active aldehyde would be in close proximity to the amine in the phospholipids head group.