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Yellow discoloration of the liposome system of cuttlefish (*Sepia pharaonis*) as influenced by lipid oxidation

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Abstract

Lipid oxidation, discoloration, loss of amine groups and pyrrolization of the liposome systems of cuttlefish (*Sepia pharaonis*) in the presence of $FeCl_3$ and ascorbic acid were studied. Thiobarbituric acid-reactive substances (TBARS) and the b^*-value of cuttlefish liposomes increased with a coincidental decrease in amine groups when the incubation temperatures (0, 4, 25, and 37 °C) and incubation times (0–24 h) were increased ($p < 0.05$). As lipid oxidation and yellow pigment formation in the cuttlefish liposome proceeded, a loss of amine groups and pyrrolization were also detected. The effects of $FeCl_3$ and ascorbic acid, at different concentrations, on TBARS production, b^*-value, loss of amine groups and pyrrolization of cuttlefish liposome were also investigated. Both $FeCl_3$ and ascorbic acid showed prooxidative effects in cuttlefish liposome in a concentration-dependent manner. Sodium chloride (0–2%) reduced TBARS, b^*-values and pyrrole compounds. These results suggest a positive correlation between lipid oxidation and the development of yellow pigments in cuttlefish phospholipids.

Keywords: Cuttlefish; Phospholipids; Lipid oxidation; Non-enzymatic browning; Yellow discoloration; Liposome

1. Introduction

Lipid oxidation is the main cause of rancidity in foods and different lipid oxidation products are able to modify different food components. Chemical reactions of oxidized lipids with amines, amino acids, and proteins have received considerable attention because they are associated with changes in functional properties, nutritive value, flavour, and colour of food (Nawar, 1996; Pokorny & Kolakowska, 2002; Xiong, 2000). Carbonylic lipid oxidation products, particularly aldehydes, can react with amine groups of proteins through the formation of Schiff bases. Those intermediates can further react with another aldehyde molecule, forming aldolization products (Korczak, Hes, Garmza, & Jedrusek-Golinska, 2004). Conjugated double bond formation, induced by aldolization, causes the production of pigments (Hidalgo & Zamora, 1993). Pyrroles formed in the reaction between oxidized lipid and proteins are important precursors of both colour and fluorescence compounds (Hidalgo, Nogales, & Zamora, 2005). The resulting products of reaction between oxidized lipid and protein are yellow, red or brown (Kikugawa, Ido, & Mikami, 1984). Our previous work demonstrated that the yellow colour intensity increased rapidly with increasing production of TBARS in the phospholipids of squid and egg yolk lecithin and yellow pigment formation correlated with a loss of primary amine groups (Thanonkaew, Benjakul, Visessanguan, & Decker, 2006c).

Frozen cuttlefish production and consumption is increasing worldwide. Though the microbiological deterioration is effectively arrested by frozen storage, various chemical reactions still take place. Yellow pigment

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formation sometimes occurs during frozen storage of cuttlefish, accompanied by the development of rancid odours (Thanonkaew, Benjakul, Visessanguan, & Decker, 2006b). Lauritzsen and Martinsen (1999) reported that the yellow/brown colour correlated with lipid oxidation of cod fillet during salting. During frozen storage of Norwegian spring-spawning herring for up to 9 weeks, there was an increase in TBARS and peroxide values, along with an increase in b^* value (Hamre, Lie, & Sandnes, 2003).

Cuttlefish muscle has a very high phospholipid content (Thanonkaew, Benjakul, & Visessanguan, 2006a). Those membrane phospholipids have a higher content of highly polyunsaturated fatty acids than do the neutral lipids. Additionally, the membrane phospholipids exist primarily in the form of a bilayer with a large surface area (Huang, Hultin, & Jafar, 1993). As a consequence, cuttlefish phospholipids are susceptible to oxidation. Since phospholipids contain amine groups, their oxidation can lead to aldehyde–amine interactions that produce colour. The objective of this study was to investigate the effect of lipid oxidation on the formation of yellow pigments in the phospholipid liposome system of cuttlefish (*Sepia pharaonis*).

2. Materials and methods

2.1. Chemicals

Butylated hydroxytoluene (BHT), L-ascorbic acid, *p*-dimethylamino-benzaldehyde and Triton X-100 were purchased from Sigma Chemical Co., (St. Louis, MO, USA). Ferric (III) chloride, and 2,4,6-trinitrobenzenesulfonic acid were obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Hydrochloric acid, methanol, chloroform, sodium dodecyl sulfate (SDS) monopotassium dihydrogen phosphate and dipotassium hydrogenphosphate were purchased from Merck (Darmstadt, Germany). Thiobarbituric acid (TBA) was procured from Fluka (Buchs, Switzerland).

2.2. Cuttlefish collection and preparation

Cuttlefish (*Sepia pharaonis*) of the size of 6–10 cuttlefish/kg, caught by cast net from the Songkhla coast, along the Gulf of Thailand, and off-loaded 12–24 h after capture, were purchased from a dock in Songkhla. The cuttlefish were placed in ice with a sample/ice ratio of 1:2 (w/w) with the temperature range of 0–1 °C and transported to the Department of Food Technology, Prince of Songkla University within 1 h. The cuttlefish were deskinned and eviscerated. The cuttlefish were then washed with tap water and kept in ice during preparation.

2.3. Preparation of cuttlefish liposome

Cuttlefish lipids were extracted as described by Bligh and Dyer (1959). Liposomes were prepared from the cuttlefish lipid according to the method of Decker and Hultin (1990). The cuttlefish lipids (5 mg/ml) were dispersed in 25 mM potassium phosphate buffer containing 0.12 M KCl, pH 7.2 (buffer A) with a homogenizer (Model RW 20, IKA Laboratechnik, Selangor, Malaysia) at a speed of 300 rpm for 5 min, followed by sonication (35% amplitude with a 5 s repeating cycle) using a high intensity ultrasonic apparatus (Digital Sonifier 450, Branson, Denberg, CT, USA) in an ice bath for 30 min. The suspension was referred to as "cuttlefish liposome system; CLS".

2.4. Lipid oxidation of cuttlefish liposome system

Lipid oxidation in CLS was accelerated with a non-enzymatic iron redox cycling system. The reaction medium contained NaCl (0–2%), ascorbic acid (0–200 μM), $FeCl_3$ (0–200 μM) and 5 mg of lipid in buffer A. All reagents were prepared fresh and added to the assay systems within 30 min. CLS was incubated at different temperatures [0 °C: an ice bath, 4 °C: cold room, 25 °C: ambient temperature and 37 °C: a temperature-controlled shaker (Unimax 1010 DT, Heidolph, Germany)]. All CLS samples were analyzed for TBARS and colour. CLS, incubated at 37 °C, was also analyzed for free amine groups and pyrrole compounds.

2.5. Determination of lipid oxidation

Lipid oxidation was monitored by measuring thiobarbituric acid-reactive substances (TBARS) according to the procedure of McDonald and Hultin (1987) with a slight modification. Thiobarbituric acid (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% butylated hydroxytoluene (BHT) in ethanol were added. CLS (1.0 ml) was added to 2 ml of the TBA/BHT mixture, vortexed, heated in a boiling water bath for 15 min, cooled to room temperature and centrifuged at 1600*g* for 20 min. The absorbance of supernatant was measured at 532 nm and the results were recorded as μmol thiobarbituric acid-reactive substances/mg liposome lipid. TBARS concentrations were determined from a malonaldehyde standard curve produced from 1,1,3,3-tetraethoxypropane.

2.6. Determination of free amine groups

Free amine groups were determined, using a modified spectroscopic method (Kubo, Sekine, & Saito, 2005). CLS was diluted (1:4) with 5% Triton X-100 and incubated at room temperature for 30 min. Diluted sample (1.5 ml) was then added to 30 μl of 100 mM 2,4,6-trinitrobenzenesulfonic acid (TNBS). The sample was incubated at room temperature for 1 h and the formation of the resulting trinitrophenyl derivatives was detected spectrophotometrically at 420 nm with UV–visible spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan). A blank was prepared in the same manner, except that buffer A was used instead of

CLS. Concentrations were calculated from a standard curve prepared with glycine.

2.7. Measurement of phospholipid pyrrolization

Phospholipid pyrrolization was used as an index of non-enzymatic browning, as described by Hidalgo, Nogales, and Zamora (2004). CLS was diluted (1:1) with 25 mM phosphate buffer containing 3% SDS. The diluted sample (1 ml) was reacted with 160 μl of 0.134 M Ehrlich reagent. The reagent was prepared by suspending 200 mg of *p*-dimethylamino benzaldehyde 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 the absorbance was measured at 570 nm. A blank was prepared in the same manner but the phosphate buffer A was used instead of CLS.

2.8. Colour measurement

Colour was measured, using a colorimeter (HunterLab, Model ColorFlex, Virginia, USA), and recorded by using the CIE colour system profile of L^*, a^* and b^*.

2.9. Statistical analysis

All experiments were run in triplicate. Statistic analysis was performed using one-way analysis of variance (ANOVA). Mean comparison was carried out using Duncan's multiple range test (Steel & Torrie, 1980).

3. Results and discussion

3.1. Effects of temperature and time on the changes of cuttlefish liposome system

In the presence of 200 μM ascorbate and 50 μM iron, the TBARS values of CLS increased with increasing temperatures and times ($p < 0.05$) (Fig. 1). Fig. 2 shows the discoloration of CLS incubated at different temperatures and times in the presence of 200 μM ascorbate and 50 μM iron. CLS had an increased b^*-value when the temperature and incubation time increased ($p < 0.05$). No marked changes in a^*-values were observed within the first 10 h of incubation at any temperatures tested. Thereafter, a^*-values decreased, particularly for CLS incubated at 37 °C for 24 h. L^*-values decreased slightly within 2–5 h, with no changes thereafter. Not surprisingly, both lipid oxidation and yellow pigment formation were more pronounced at

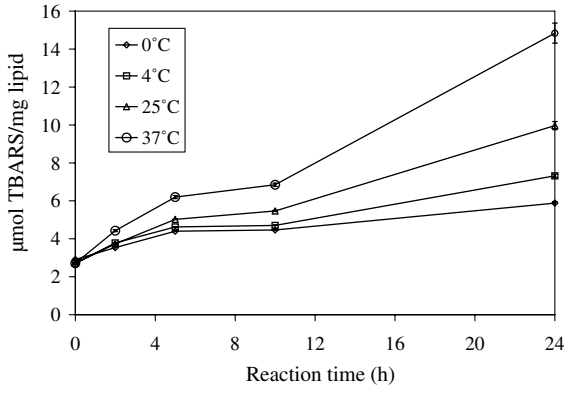

Fig. 1. Formation of thiobarbituric-acid reactive substances (TBARS) in liposomes made form cuttlefish lipids (5 mg lipid/ml) in the presence of 50 μM $FeCl_3$ and 200 μM ascorbic acid during incubation at different temperatures for 24 h. Error bars indicate standard deviations from triplicate determinations.

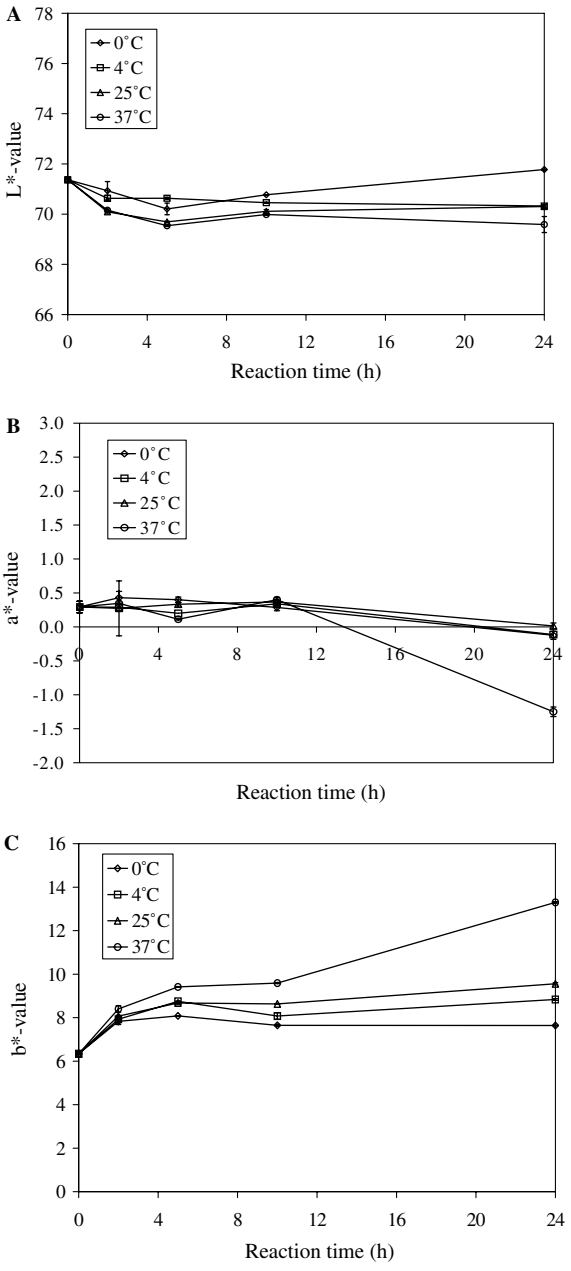

Fig. 2. Changes in L^* (A), a^* (B) and b^* (C)-value in liposomes made from cuttlefish lipids (5 mg lipid/ml) in the presence of 50 μM $FeCl_3$ and 200 μM ascorbic acid during incubation at different temperatures for 24 h. Error bars indicate standard deviations from triplicate determinations.

higher temperature, since temperature is an important factor in lipid oxidation and browning of foods.

Fig. 3 shows that TBARS formation and changes in b^*-values occurred over a similar time period in the presence of 200 μM ascorbate and 50 μM iron, suggesting that lipid oxidation products could be involved in the formation of yellow pigments. Interactions between phospholipids and lipid oxidation products can be monitored by the loss of free amines (Hidalgo et al., 2004; Kubo et al., 2005). Pyrroles formed in the reaction of oxidized lipids with protein are important precursors of both brown and fluorescing compounds (Zamora, Alaiz, & Hidalgo, 2000). Therefore, they were also measured to determine interactions between lipid oxidation products and phospholipid amines. Simultaneously with TBARS and yellow pigment formation, free amines decreased and pyrrole compounds were formed in the presence of 200 μM ascorbate and 50 μM iron, suggesting that lipid oxidation products were reacting with phospholipid amines to produce yellow pigments. These results were in agreement with our previous work on squid microsomes, squid liposomes and egg yolk lecithin liposomes, where yellow pigment formation correlated with the loss of the amine groups (mainly from phospholipids) and the formation of pyrrole compounds (Thanonkaew, Benjakul, Visessanguan, & Decker, 2005, 2006c).

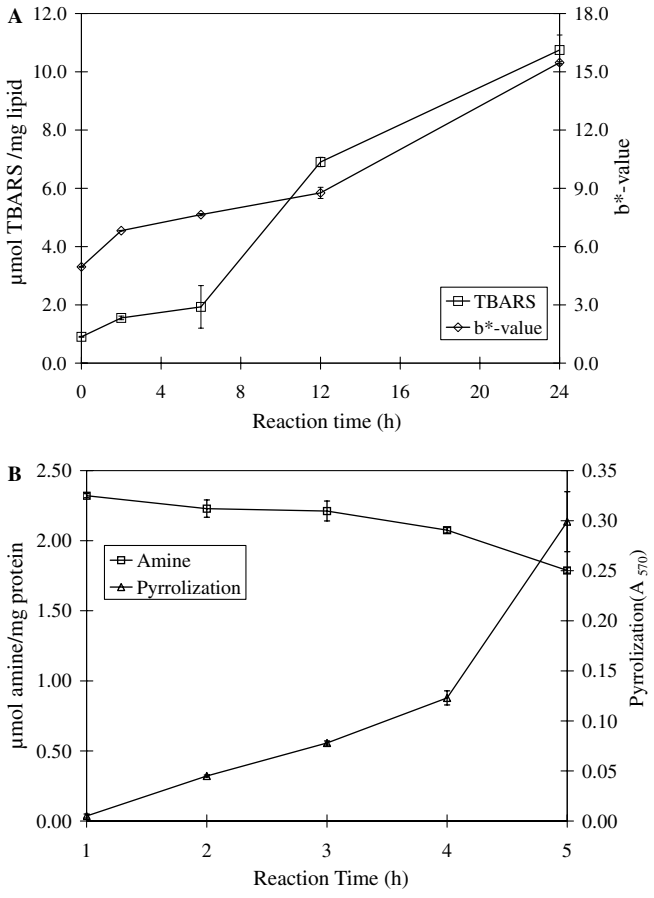

Fig. 3. Thiobarbituric acid-reactive substances (TBARS) formation and yellow pigment formation (b^*-value; A) as well as the loss of amine groups and the formation of pyrrole compounds (B) in liposomes made from cuttlefish lipids (5 mg lipid/ml) in the presence of 50 μM $FeCl_3$ and 200 μM ascorbic acid during incubation at different temperatures for 24 h. Error bars indicate standard deviations from triplicate determinations.

During oxidation of lipids, lipid hydroperoxides are degraded to carbonyl compounds, such as aldehydes and ketones (Khayat & Schwall, 1983). Among these carbonyls, aldehydes possess a pronounced ability to form Schiff-base adducts with amine groups (Korczak et al., 2004). Upon rearrangement of the Schiff base products, polymerization can occur to produce yellow-brown pigments. The amines in phospholipids, such as phosphatidylethanolamine, have been shown to participate in non-enzymatic browning reactions *in vitro* (Zamora, Nogales, & Hidalgo, 2005). Kikugawa et al. (1984) reported that the interaction between primary amines and malonaldehyde or/and monofunctional aldehydes produced yellow pigments. 4-Hydroxnonenal covalently attaches to aminophospholipids to form Schiff base adducts and cyclic pyrrolization products (Guichadant, Taibi-Tronch, Fay, & Lagarde, 1998).

3.2. Effects of $FeCl_3$ and ascorbic acid on the changes of cuttlefish liposome system

The ferric state (Fe^{3+}) can be reduced by ascorbic acid to produce the very prooxidative ferrous state (Fe^{2+}) of iron. The ferrous state of iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals via a Fenton type reaction (Dunford, 1987). TBARS formation in CLS was determined as a function of different $FeCl_3$ and/or ascorbic acid concentrations (0, 50, 100, 200 μM) after incubation at 37 °C for 24 h (Fig. 4A). TBARS formation increased in CLS with increasing $FeCl_3$ ($p < 0.05$) when ascorbate was held constant at 200 μM. TBARS value of CLS in the presence of 200 μM $FeCl_3$ was approximately eight-fold greater than that of CLS without $FeCl_3$. When different concentrations of ascorbic acid were used in the presence of 50 μM $FeCl_3$, a marked increase in TBARS was observed from 0 to 50 μM ascorbate with little further increase in TBARS from 100 to 200 μM ascorbate. Yellow pigment (b^*-value, Fig. 4B), amines (Fig. 4C) and pyrrole compounds (Fig. 4D) also changed when $FeCl_3$ and ascorbate were increased from 0 to 50 μM. However, few further changes in b^*-values, amines and pyrrole compounds were observed when iron or ascorbate were increased from 50 to 200 μM with the exception of amines, which continued to decrease with increasing iron concentrations (Fig. 4C).

3.3. Effect of NaCl on the changes of cuttlefish liposome system

NaCl is one of the most important additives used for improving texture of cephalopods, including cuttlefish. Table 1 shows the effect of NaCl on TBARS formation, b^*-value and pyrrolization in CLS in the presence of

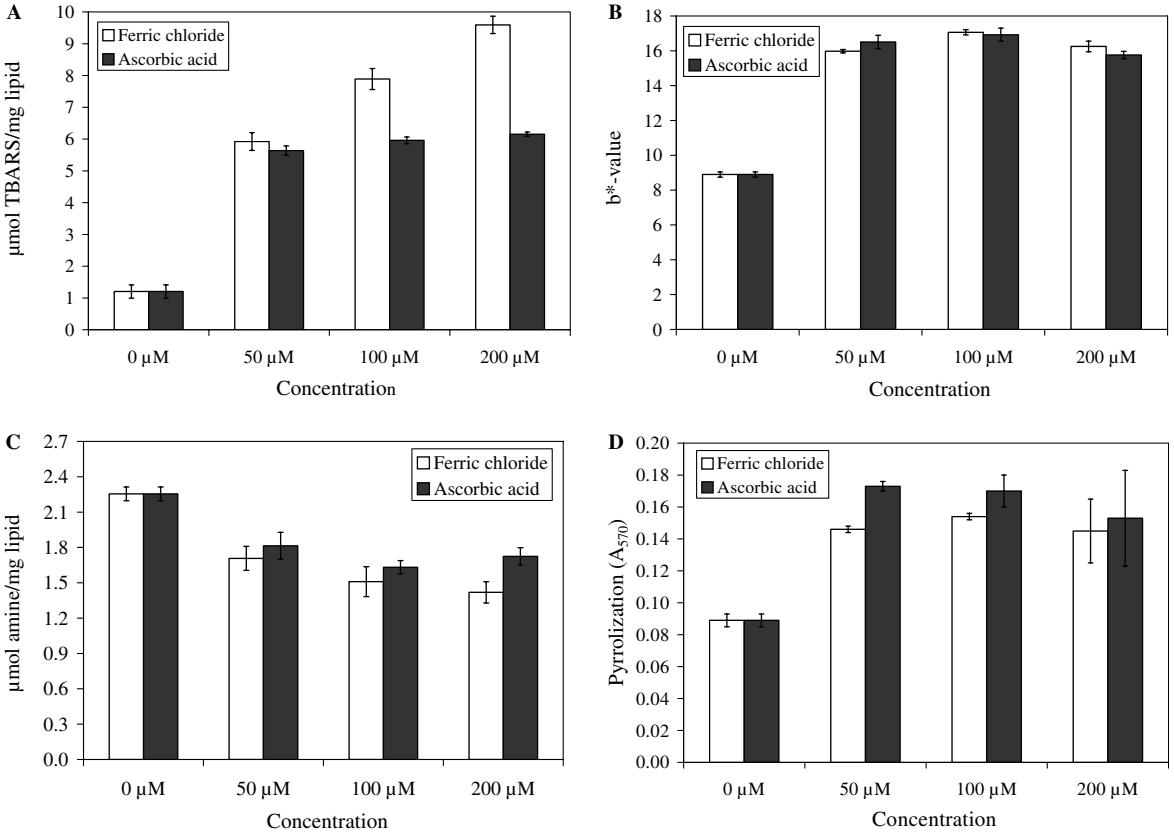

Fig. 4. Effect of $FeCl_3$ or ascorbic acid concentrations on the formation of thiobarbituric acid-reactive substances (TBARS; A), yellow pigments formation (b^*-value; B), loss of amine groups (C) and pyrrolization (D) in liposomes made from cuttlefish lipids (5 mg lipid/ml) during incubation at different temperatures for 24 h. To study the effect of $FeCl_3$ concentrations, ascorbic acid was fixed at 200 μM. For another study on the effect of ascorbic acid concentrations, $FeCl_3$ was fixed at 50 μM. Error bars indicate standard deviations from triplicate determinations.

Table 1
Effect of sodium chloride on thiobarbituric acid-reactive substances (TBARS) formation, b^*-value and pyrrole compounds of cuttlefish liposome systems

Samples	TBARS (μmol TBARS/mg lipid)	b^*-value	Pyrrole (Abs_{570})
1. Control	0.952 ± 0.010e*	6.791 ± 0.035d	0.100 ± 0.001e
2. 1% NaCl	0.951 ± 0.056e	2.733 ± 0.021g	0.093 ± 0.001f
3. 50 μM ASC + 50 μM $FeCl_3$	6.46 ± 0.073a	15.443 ± 0.055a	0.150 ± 0.003a
4. 50 μM ASC + 50 μM $FeCl_3$ + 0.2% NaCl	3.26 ± 0.075b	11.317 ± 0.020b	0.128 ± 0.001b
5. 50 μM ASC + 50 μM $FeCl_3$ + 0.5% NaCl	2.34 ± 0.077c	8.337 ± 0.006c	0.105 ± 0.001c
6. 50 μM ASC + 50 μM $FeCl_3$ + 1.0% NaCl	1.61 ± 0.099d	5.020 ± 0.066e	0.106 ± 0.014d
7. 50 μM ASC + 50 μM $FeCl_3$ + 2.0% NaCl	1.45 ± 0.057e	4.070 ± 0.035f	0.104 ± 0.001de

ASC, ascorbic acid.
Values are given as means ± SD from triplicate determinations.
Values in the same column with different letters are significantly different ($p < 0.05$).

50 μM ascorbate and 50 μM $FeCl_3$ after 24 h of incubation at 37 °C. NaCl by itself (1.0%) had no pronounced effect on the formation of TBARS and pyrrole compounds but decreased b^*-value in CLS. NaCl (0.2–2.0%), in the presence of iron and ascorbate, reduced TBARS, b^*-value and pyrrolization, with inhibition increasing with increasing NaCl concentrations, compared to iron and ascorbate alone. The result indicated that NaCl was inhibiting iron-promoted oxidation in CLS. Sodium chloride has been reported to inhibit lipid oxidation in emulsion (Mei, McClements, Wu, & Decker, 1997) and liposomes (Arnold, Bascetta, & Gunstone, 1991). Reduction of iron-catalyzed oxidation by sodium chloride could be due to the ability of sodium to displace iron from the surface of the negatively charged phospholipid lipid bilayer (Mei et al., 1997). Iron displacement would decrease iron-lipid interactions and thus decrease oxidation rates. In addition, chloride is able to form complexes with iron, resulting in decreased iron prooxidative activity (Osinchak, Hultin, Zajicek, Kelleher, & Huang, 1992). These results suggest that NaCl could be used to control lipid oxidation and discoloration in cuttlefish products.

4. Conclusion

Cuttlefish lipids dispersed into a liposome system were susceptible to lipid oxidation in the presence of the prooxidants, iron and ascorbic acid, with the susceptibility increasing with increasing temperature. Lipid oxidation products derived from the cuttlefish lipids in the liposomes could react with the amine groups of phospholipids to form complexes with yellow pigmentation. NaCl was able to reduce lipid oxidation, as well as yellow discoloration, in the liposome system, suggesting that NaCl might be a useful preservative for inhibiting discoloration of cuttlefish products.

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