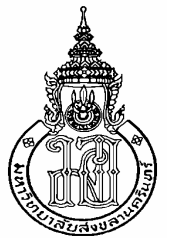


Development of Pink Color of Squid and the Effect of Chemical Treatment on Physico-Chemical Changes of Squid during Frozen Storage

Rattana Sungsri-in

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Food Science and Technology

Prince of Songkla University

2010

Thesis Title	Development of Pink Color of Squid and the Effect of Chemical Treatment on Physico-Chemical Changes of Squid during Frozen Storage
Author	Miss Rattana Sungsri-in
Major Program	Food Science and Technology

Major Advisor :

…….....
(Prof. Dr.Soottawat Benjakul)

Co-Advisor :

…….....
(Asst. Prof. Dr.Kongkarn Kijroongrojana)

Examining Committee :

……Chairperson
(Asst. Prof. Dr.Chakree Thongraung)

……
(Prof. Dr.Soottawat Benjakul)

……
(Asst. Prof. Dr.Kongkarn Kijroongrojana)

……
(Dr.Manat Chaijan)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirement for the Master of Science Degree in Food Science and Technology.

……
(Assoc. Prof. Dr.Krerkchai Thongnoo)
Dean of Graduate School

ชื่อวิทยานิพนธ์	การเกิดสีชมพูของหมึกกล้วยและผลของการใช้สารเคมีต่อการเปลี่ยนแปลงทางเคมี-กายภาพของหมึกกล้วยระหว่างการเก็บรักษาในสภาวะแช่แข็ง
ผู้เขียน	นางสาวรัตนา สังข์ศรีอินทร์
สาขาวิชา	วิทยาศาสตร์และเทคโนโลยีอาหาร
ปีการศึกษา	2552

บทคัดย่อ

การศึกษาการเกิดสีชมพูและการเปลี่ยนแปลงคุณภาพของหมึกกล้วยที่ผ่านการลอกและไม่ลอกหนังขนาด 6-10 ตัว/กก. (ขนาดใหญ่) และ 21-30 ตัว/กก. (ขนาดเล็ก) ระหว่างการเก็บรักษาในน้ำแข็งโดยใช้อัตราส่วนหมึกต่อน้ำแข็งที่แตกต่างกัน (1:1 และ 1:2 ; น.น./น.น.) เป็นเวลา 16 วัน พบว่าค่า a* และ b* ของหมึกกล้วยที่ไม่ลอกหนังเพิ่มขึ้นตามระยะเวลาเก็บรักษาที่เพิ่มขึ้น ($p<0.05$) แสดงให้เห็นการเกิดสีชมพูของหมึกกล้วย หมึกกล้วยที่ไม่ลอกหนังและเก็บรักษาในน้ำแข็งโดยใช้อัตราส่วนหมึกต่อน้ำแข็งเท่ากับ 1:1 มีค่า a* มากกว่าตัวอย่างที่เก็บรักษาโดยใช้อัตราส่วนหมึกต่อน้ำแข็งเท่ากับ 1:2 ($p<0.05$) หมึกกล้วยขนาดใหญ่มีค่า a* มากกว่าหมึกกล้วยขนาดเล็ก อย่างไรก็ตามไม่มีการเปลี่ยนแปลงค่า a* ในระหว่างการเก็บรักษาหมึกกล้วยที่ผ่านการลอกหนังทั้งสองขนาด อย่างไรก็ตามค่า b* ของหมึกกล้วยที่ลอกหนังเพิ่มขึ้นในระหว่างการเก็บรักษา ($p<0.05$) ปริมาณจุลินทรีย์ของหมึกกล้วยทั้งสองขนาดเพิ่มขึ้นอย่างต่อเนื่องในระหว่างการเก็บรักษาในน้ำแข็งสอดคล้องกับการเพิ่มขึ้นของพีเอช ปริมาณด่างที่ระเหยได้ทั้งหมด ปริมาณไตรเมทิลเอมีน และ ปริมาณแอมโมเนีย หมึกกล้วยทั้งสองขนาดที่เก็บรักษาโดยใช้อัตราส่วนหมึกต่อน้ำแข็งเท่ากับ 1:1 มีพีเอช ปริมาณด่างที่ระเหยได้ทั้งหมด ปริมาณไตรเมทิลเอมีน และ ปริมาณแอมโมเนีย มากกว่าหมึกที่เก็บรักษาโดยใช้อัตราส่วนหมึกต่อน้ำแข็งเท่ากับ 1:2 ($p<0.05$) ปริมาณฟอร์มัลดีไฮด์ และ TBARS ของหมึกกล้วยทั้งสองขนาดเพิ่มขึ้นตามระยะเวลาการเก็บรักษา การลอกหนังและการเก็บรักษาหมึกกล้วยโดยใช้อัตราส่วนน้ำแข็งที่แตกต่างไม่มีผลต่อปริมาณฟอร์มัลดีไฮด์ของหมึกกล้วยทั้งสองขนาดในระหว่างการเก็บรักษาในน้ำแข็ง หมึกกล้วยที่เก็บรักษาโดยใช้อัตราส่วนหมึกต่อน้ำแข็งเท่ากับ 1:1 มีค่า TBARS มากกว่าหมึกที่เก็บรักษาโดยใช้อัตราส่วนหมึกต่อน้ำแข็งเท่ากับ 1:2 อย่างไรก็ตามการลอกหนังไม่มีผลต่อค่า TBARS ดังนั้นการลอกหนังและใช้น้ำแข็งที่เพียงพอสามารถช่วยลดการเกิดสีชมพูและการสูญเสียคุณภาพของหมึกกล้วยในระหว่างเก็บรักษาในน้ำแข็ง

จากการศึกษาบทบาทของจุลินทรีย์ต่อการเกิดสีชมพู และการเปลี่ยนแปลงคุณภาพของหมึกกล้วยในระหว่างเก็บรักษาในน้ำแข็งโดยใช้อัตราส่วนหมึกต่อน้ำแข็งเท่ากับ 1:1 พบว่าหมึกกล้วยที่ไม่ผ่านการลอกหนังและแช่ในสารละลายโซเดียมเอไซด์ (NaN_3) ร้อยละ 0.1 ก่อนการเก็บรักษาในน้ำแข็ง มีค่า a* และ b* ปริมาณจุลินทรีย์ ปริมาณด่างที่ระเหยได้ทั้งหมด และปริมาณไตรเมทิลเอมีนน้อยกว่าตัวอย่างที่แช่ในสารละลาย NaN_3 ร้อยละ 0.01 และตัวอย่างที่ไม่แช่ NaN_3 (ชุดควบคุม) ตามลำดับ ($p<0.05$) ดังนั้นการแช่หมึกกล้วยในสารละลาย NaN_3 โดยเฉพาะที่ระดับความเข้มข้นสูง (ร้อยละ 0.1) ก่อนการเก็บรักษาในน้ำแข็งสามารถยับยั้งการเกิดสีชมพูและการสูญเสียคุณภาพของหมึกกล้วยได้ การซ้อนทับหมึกกล้วยที่ไม่ผ่านการลอกหนังและแช่ในสารละลาย NaN_3 ร้อยละ 0.1 ด้วยน้ำหนักมาตรฐาน (หนึ่งเท่าและสองเท่าของน้ำหนักหมึกกล้วย) และเก็บรักษาในน้ำแข็งโดยใช้อัตราส่วนหมึกต่อน้ำแข็งเท่ากับ 1:2 ให้ค่า a* และ b* มากกว่าหมึกกล้วยที่ผ่านการแช่สารละลาย NaN_3 และเก็บแบบไม่มีการซ้อนทับด้วยน้ำหนักมาตรฐาน อย่างไรก็ตามการซ้อนทับไม่มีผลต่อปริมาณด่างที่ระเหยได้ทั้งหมด ปริมาณไตรเมทิลเอมีน ปริมาณแอมโมเนีย ปริมาณฟอร์มัลดีไฮด์ ปริมาณจุลินทรีย์ พีเอช และ TBARS ($p>0.05$) ดังนั้นการใช้สารยับยั้งการเจริญของจุลินทรีย์และหลีกเลี่ยงการเก็บแบบซ้อนทับสามารถลดการเกิดสีชมพูของหมึกกล้วยในระหว่างการเก็บรักษาเป็นเวลานานในน้ำแข็ง

จากการศึกษาการแช่หมึกกล้วยที่เกิดสีชมพูด้วยสารละลายโซเดียมคลอไรด์ร้อยละ 3 และสารออกซิไดซิ่ง (ไฮโดรเจนเปอร์ออกไซด์, H_2O_2) ร้อยละ 0.05-0.5 และ โซเดียมไฮเปอร์คลอไรด์ (NaOCl ; 0-10 ppm) พบว่าหมึกกล้วยที่แช่สารละลาย H_2O_2ร้อยละ 0.5 ให้ค่า a* และ b* ต่ำสุด ($p<0.05$) สารละลาย NaOCl ไม่มีผลต่อการเปลี่ยนแปลงค่า a* และ b* ดังนั้นจึงเลือกสารละลายโซเดียมคลอไรด์ร้อยละ 3 และ H_2O_2 ร้อยละ 0.5 สำหรับแช่หมึกกล้วยที่เกิดสีชมพูก่อนการเก็บรักษาในสภาวะแช่แข็ง ในระหว่างเก็บรักษาที่อุณหภูมิ -18 องศาเซลเซียสเป็นเวลา 10 สัปดาห์ พบว่า การแช่หมึกกล้วยที่เกิดสีชมพูในสารละลาย H_2O_2 ร้อยละ 0.5 และ โซเดียมคลอไรด์ร้อยละ 3 ไม่มีผลต่อการเปลี่ยนแปลงค่า L* a* และ b* อย่างไรก็ตาม TBARS ปริมาณพันธะไดซัลไฟด์ การสูญเสียน้ำหนักหลังการทำละลาย และค่าแรงเฉือน ของหมึกกล้วยที่เกิดสีชมพูที่ผ่านการแช่ในสารละลาย H_2O_2 ร้อยละ 0.5 และโซเดียมคลอไรด์ร้อยละ 3 มีค่ามากกว่าหมึกกล้วยสีชมพูที่ไม่ผ่านการแช่สารละลายดังกล่าว (ชุดควบคุม) และหมึกกล้วยสด ในระหว่างเก็บรักษาแบบแช่แข็ง ($p<0.05$) การเพิ่มของพันธะไดซัลไฟด์สอดคล้องกับการลดลงของปริมาณหมู่ซัลฟ์ไฮดริล ซึ่งลดลงอย่างชัดเจนในตัวอย่างที่ผ่านการแช่สารละลาย H_2O_2 ตลอดระยะเวลาการเก็บรักษา การสูญเสียสภาพธรรมชาติและการจับตัวของโปรตีนกล้ามเนื้อระหว่างการเก็บรักษาในสภาวะแช่แข็งเกิดมากที่สุดในกล้ามเนื้อหมึกกล้วยสีชมพูที่ผ่านการแช่ในสารละลาย H_2O_2 ร้อยละ 0.5 และ

โซเดียมคลอไรด์ร้อยละ 3 เมื่อเปรียบเทียบกับชุดควบคุมและหมึกกล้วยสด ตามลำดับ ถึงแม้ว่าการแช่หมึกกล้วยที่เกิดสีชมพูด้วยสารออกซิไดซิ่งสามารถปรับปรุงสีของหมึกกล้วยที่เกิดสีชมพู แต่มีผลเหนี่ยวนำให้เกิดการจับตัวและเสียสภาพธรรมชาติของโปรตีนกล้ามเนื้อระหว่างเก็บรักษาในสภาวะแช่แข็ง ดังนั้นควรใช้หมึกกล้วยสดที่ไม่ผ่านการแช่สารออกซิไดซิ่งสำหรับการผลิตหมึกกล้วยแช่แข็ง เนื่องจากเกิดการสูญเสียคุณภาพเพียงเล็กน้อยในระหว่างเก็บรักษาในสภาวะแช่แข็งเป็นเวลานาน

Thesis Title	Development of Pink Color of Squid and the Effect of Chemical Treatment on Physico-Chemical Changes of Squid during Frozen Storage
Author	Miss. Rattana Sungsri-in
Major Program	Food Science and Technology
Academic Year	2009

ABSTRACT

Development of pink color and quality changes of squid with 6-10 squids/kg (large size) and 21-30 squids/kg (small size) with and without deskinning during iced storage at different squid/ice ratios (1:1 and 1:2, w/w) for 16 days were investigated. The increases in *a** and *b**-values of squid mantle were observed with increasing storage time ($p<0.05$), indicating the formation of pink color on squid mantle. The mantle from large squid without deskinning showed the higher *a**-value, compared with those of the small counterpart ($p<0.05$). The increases in *a** and *b**-values were more pronounced in squid without deskinning with a squid/ice ratio of 1:1 ($p<0.05$), regardless of size. No changes in *a**-value were observed in deskinned squid throughout the storage, regardless of squid/ice ratio and size ($p>0.05$). However, the slight increase in *b**-value was found in the squid with deskinning during the storage. Psychrophilic bacteria counts of squid increased continuously as the storage time increased, irrespective of size. Coincidental increases in pH, total volatile base (TVB), trimethylamine (TMA) and ammonia contents were observed during the storage. The rates of increase were greater in the samples with a squid/ice ratio of 1:1 than those found in the samples kept in ice with a squid/ice ratio of 1:2, regardless of size. Formaldehyde content and TBARS value of squid with both sizes increased with increasing storage time. For both squids, deskinning and squid/ice ratio had no impact on formaldehyde content of squid during iced storage ($p>0.05$). Squids stored in ice with a sample/ice ratio of 1:1 tended to have a higher TBARS value than those with a ratio of 1:2 during the storage. However, deskinning had no influence on TBARS value. Therefore, deskinning together with icing using a sufficient amount of ice could be a means to lower the pink discoloration and retard the losses in quality of squid stored in ice.

Role of microorganisms in pink discoloration and quality changes of squid stored in ice using a squid/ice ratio of 1:1 was investigated. During iced storage of 14 days, *a** and *b**-values, psychrophilic bacteria count, TVB and TMA contents of squid without deskinning treated with 0.1% sodium azide (NaN_3) prior to storage were lower than those treated with 0.01% NaN_3 and the control (without treatment), respectively ($p<0.05$). Therefore, NaN_3 treatment of squid especially at a higher level (0.1%) prior to the iced storage could retard the pink discoloration and the losses in quality of squid. Squids without deskinning treated with 0.1% NaN_3, stacked with standard weight (1X or 2X) and stored in ice with a squid/ice ratio of 1:2 showed the higher *a** and *b**-values than those without stacking. Nevertheless, stacking had no effect on TVB, TMA ammonia and formaldehyde contents, psychrophilic bacteria count, pH and TBARS value ($p>0.05$). Therefore, antimicrobials should be used and stacking should be avoided to retard pink discoloration of squid during the extended iced storage.

Effects of treatment of pink squid using 3% NaCl containing oxidizing agents (0.05-0.5% H_2O_2 or 0-10 ppm NaOCl) on color and physicochemical changes of squid during frozen storage were studied. The lowest decreases in *a** and *b**-values of squid were obtained with the treatment using 3% NaCl containing 0.5% H_2O_2. NaOCl (0-10 ppm) had no impact on *a** and *b**-values. Therefore, 3% NaCl containing 0.5% H_2O_2 was used to treat pink squid prior to frozen storage. During frozen storage at -18^{o}C for 10 weeks, the selected treatment had no effect on changes in *a** and *b**-values of squid during frozen storage. TBARS value, disulfide bond content, thaw drip and shear force of treated pink squid during frozen storage were more pronounced in treated squid, compared with pink squid without treatment (control) and fresh squid, respectively ($p<0.05$). The increase in disulfide bond with the coincidental decrease in sulfhydryl content was greater in the treated sample throughout the storage. The denaturation and aggregation of muscle proteins during frozen storage was more pronounced in treated pink squid than the control (without treatment) and fresh squid, respectively. Therefore, pink squid treated with oxidizing agent was prone to protein aggregation and denaturation during the extended frozen storage, though it could lower the pink color. Therefore, fresh squid without treatment using oxidizind agent is recommended for production of frozen squid with negligible loss in quality during extended storage.

ACKNOWLEDGEMENT

I would like to express my sincere appreciation to my advisor, Prof. Dr. Soottawat Benjakul of the Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, for his guidance and assistance in planning, writing, correcting the thesis and manuscript.

I also would like to express my thanks to my co-advisor, Asst. Prof. Dr. Kongkarn Kijroongrojana for her kindness and supporting.

I am also grateful to my examination committee for their comments.

My gratitude is also due to all of my friends who guided and gave me their helps during my research.

I would like to express my appreciation to my parent for their encouragement and support.

Finally, I would like to thank the financial support from Agro-Industry Practice School (AP), Faculty of Agro-Industry, Prince of Songkla University and National Center for Genetic Engineering and Biotechnology.

Rattana Sungsri-in

CONTENTS

CONTENTS (Continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

CHAPTER 1

INTRODUCTION

Cephalopods are marine animals that live in all oceans of the world. There are two subclasses: Nautiloidea and Coleoidea. Coleoideas comprise approximately 700 species, which include the major groups known as squid, cuttlefish and octopus (Sugiyama et al., 1989). Total world landing increased from 1.5 million tons in 1980 to approximatesly 3.3 million tons in 2001 (FAO, 2000). Squids are the main marine animals in the world cephalopod fishery production for about 67% of the total catch (FAO, 2003). Northwest Pacific is the biggest source of squid, followed by Southwest Atlantic and Western Central Pacific. Frozen cephalopods are the important product of Thailand, with the second export value and the sixth export quantity in 2001 (FAO, 2000). During January-September 2006, Thailand exported frozen cuttlefish and squid with the amount of 65,074 tons that decreased about 7% with a value of 10,613 million, compared with the same period in 2005. Europe is the biggest market and Japan is the second market. However, frozen cuttlefish and squid export values in USA, Canada and China increased about 39%, 31% and 31%, respectively, when compared with year 2005 (International Fish Trade Analysis Group of Department of Fisheries, 2006).

During handling or storage in ice, cephalopods undergo the changes in texture, flavor as well as discoloration. Quality changes of squids are generally dependent on storage conditions (Yamanaka et al., 1987; Ohashi et al., 1991; Lapa-Guimarães et al., 2005). During iced storage, psychrophilic bacteria counts, pH, total volatile base (TVB) and polyamine increased with increasing storage temperature (Yamanaka et al., 1987; Ohashi et al., 1991). The increases of agmatine in squid, used as a spoilage index, were observed as the storage time at 10^{o}C increased (Ohashi et al., 1991). The increases in free amino acids, especially arginine and ornithine, can be used as early freshness indicators (Ohashi et al., 1991). Generally, pH and TVB values are the suitable indicators for the initial decomposition of squid (Yamanaka et al., 1987). The acceptable limits of trimethylamine (TMA) and TVB are dependent on

the species and storage condition (Paarup et al., 2002). The formation of TVB and TMA in squid correlated well with the sensory changes (Vaz-Pires et al., 2008). Additionally, ammonia has been shown to be an excellent indicator of squid quality (Leblance and Gill, 1984; Paarup et al., 2002).

The changes in external appearance and color can be another index for quality changes. A decrease in the reddish brown color of skin has been used for the primary quality evaluation (Ke et al., 1984; Lakshmanan et al, 1993). The intensification and spreading of the pink color over the skin occurred with increasing storage time (Lapa-Guimarães et al., 2002). This might be associated with the disruption of chromatophores in the skin. Pink discoloration in squid mantle during handling or storage in ice is the problem associated with the losses in quality and acceptability. It is hypothesized that pigment released from the disrupted chromatophores localized in the skin most likely stains the mantle during the handling or storage. In general, large squids are more prone to the development of pink color, in comparison with the small squids and have the higher intensity of pink color developed. Additionally, the pink color development is commonly found in squid stored with the insufficient ice as well as under the stacking condition, particularly with the extended handling or post-harvested storage. Bleaching agents are commonly used to solve pink discoloration. However, this could induce the changes of proteins as well as lipids in squid during the subsequent frozen storage. Those alterations would result in the lowered quality. Therefore, the prevention of pink discoloration of squid and the development of the method to maintain the quality of squid with the extended storage should be of great benefit for fishermen and processors to minimize the economical loss caused by inappropriate handling or storage.

Reviews of Literature

1. Squid

Squid belongs to class Cephalopod, which forms part of phylum Mollusca and subclass Coleoideas. All species of squid have adapted to different environments, from coastal waters to oceanic regions, from surface waters to deep-sea zones, and from tropical seas to polar regions (Okuzumi and Fujii, 2000). External parts of squid are illustrated in Figure 1. The part generally termed the body is the mantle. The arms (leg or feet) are at the front while the fins are at the rear. The surface with the shell (inner shell) is the dorsal surface. The surface with the funnel is the ventral surface. The mantle dorsal surface has more chromatophores than the ventral surface. There are 10 arms with the suckers on. The dorsal arms are termed left arm I and right arm I, followed ventrally by left-right arm II, arm III and the tentacle (the longest arm) (Sugiyama et al., 1989).

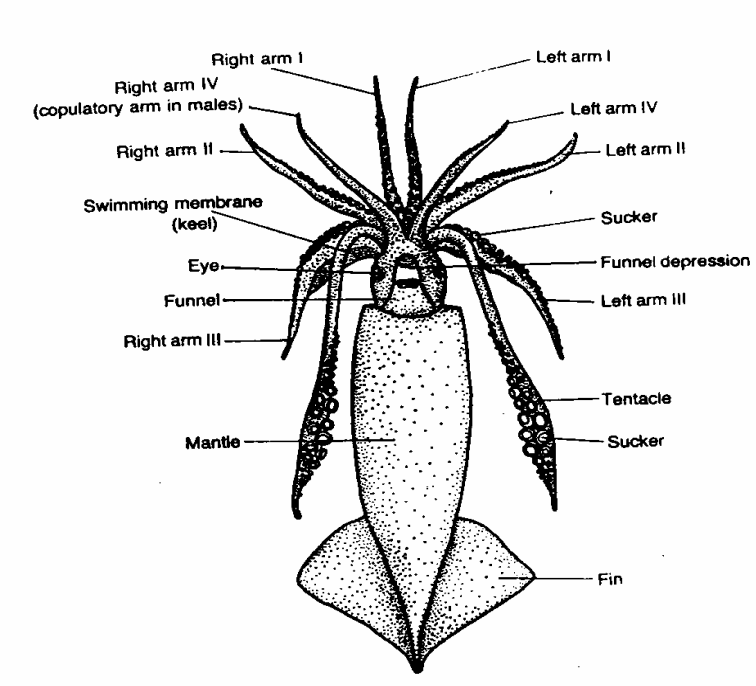


Figure 1. External parts of squid
Source : Sugiyama et al. (1989)

Loligo formosana (*Loligo chinensis*) (Figure 2) is the economically important squid in the Southeast Asia. This squid is caught along Western Pacific, southern Japan to northern and northeastern Australia. Fin length is about 70% of mantle length; arm sucker rings with 10-15 sharp and largest tentacular suckers with 20-30 sharp (Sweeney et al., 1992). In Thailand, *Loligo formosana* has been caught along the Gulf of Thailand and Andaman sea. It is very important for fishery of Thailand. Its common name is "Splendid squid" (Department of Fisheries, 2006). Among 10 kinds of squids caught in Thailand, *Loligo formosana* is caught for about 12.65% of the total squid catching (Department of Fisheries, 2006).

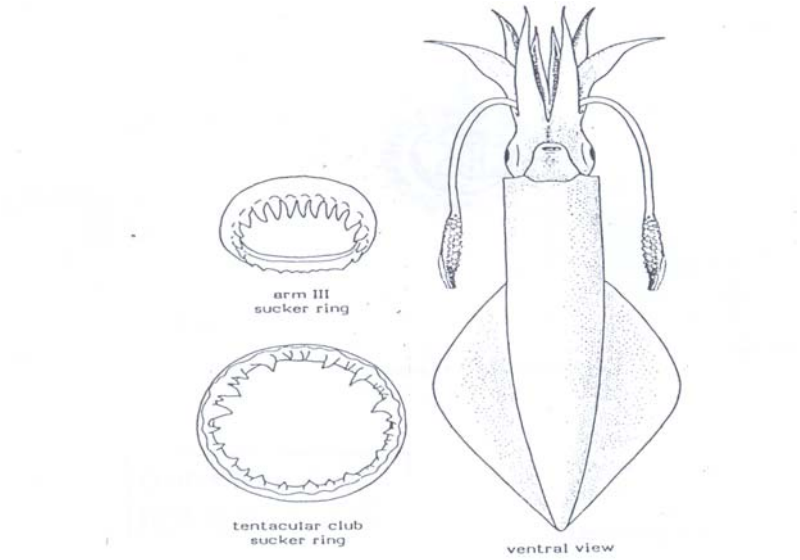


Figure 2. *Loligo formosana* (*Loligo chinensis*)
Source : FAO (1984) cited by Roongratri (1989)

2. Squid tissue

2.1 Skin tissue

The skin (epidermis) on the surface of *Todarodes pacificus* is composed of four layers (Figure 3). The first and second layers have nuclei and black chromatopores. The third layer is composed of many nuclei. The fourth layer is typical muscle fiber connective tissue. The thickness of each layer varies. The first layer is 60-100 μm; the second layer is 150-270 μm; the third layer is 50-60 μm and the fourth layer is 30-40 μm (Sugiyama et al., 1989).

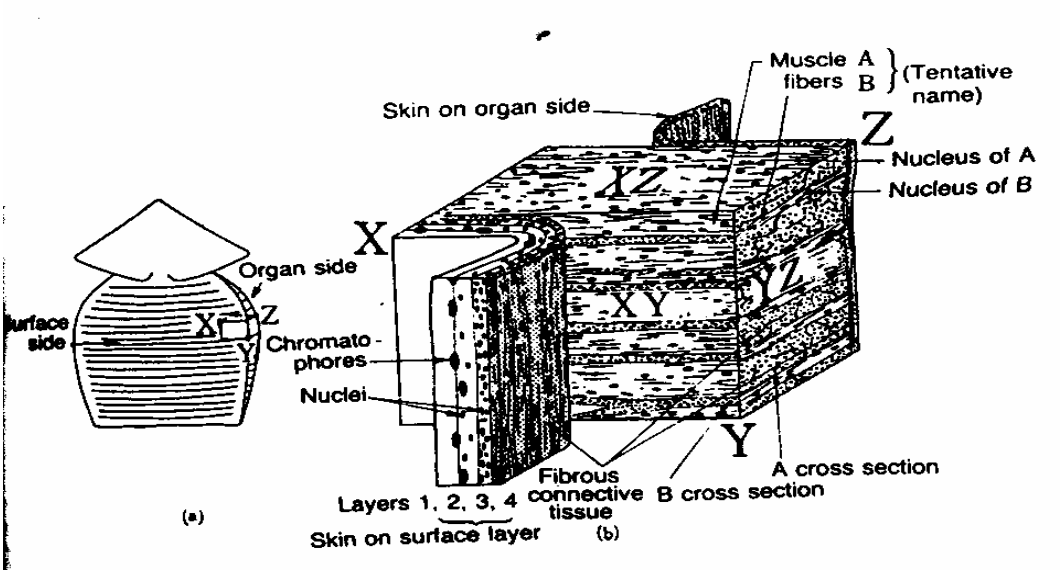


Figure 3. Tissue structure of mantle muscles of *T. pacificus*
Source : Sugiyama et al. (1989)

A chromatophore is a container for the color particles. It consists of a cell with an expandable membrane as a wall (Figure 4). Several muscle fibers are radially attached to the membrane and each fiber is connected with a nerve. If the animal is on a light surface, the muscle attached to the cell wall are relaxed, the chromatophores appear small and round. The skin of the animal appears light. When the animal moves into a dark background, the shade of the requied color are controlled by a nerve system. Instantly, the muscles contract and expand the walls of the chromatophores in all directions. Each chromatophore cell can now cover up to 60 times the surface than the small, contracted cell. The pigment flows to the periphery of the expanded cell covering the whole area. The skin now becomes dark (Kreuzer, 1984).

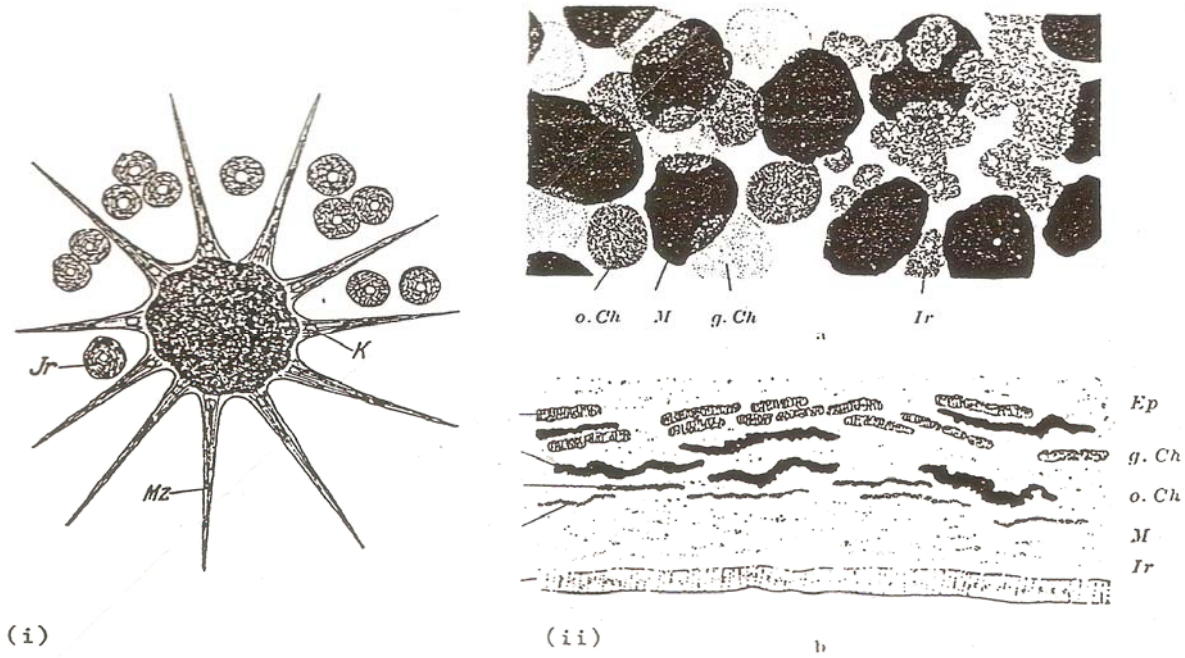
Ommochrome is the principal pigment of the epidermis. It is composed of yellow, orange and violet-purple chromatophores (Sugiyama et al., 1989). It has been extracted using hydrochloric acid and methanol, following fat removal. The absorption spectrum of the principal pigment separated through Sephadex G-75 gel filtration exhibits peaks at 280 and 526 nm. Ommochrome is a pigment produced using tryptophan as the starting material. The body surface pigment of *Loligo subulata*, *Sepia officinalis* and *T. pacificus* are bound with protein *in vivo* (Sugiyama et al., 1989).

2.2 Muscle tissue

The mantle of the squid *Loligo peali* is composed of five layers of tissue (Figure 5). The middle layer contains the muscle tissue with the muscle fibres group (Kreuzer, 1984). The muscle layer makes up about 98 percent of the total thickness of the mantle. The elongated cells of myofibrils are surrounding a central core which contains sarcoplasmic proteins. The muscle layer is covered on each side by a layer of connective tissue called the outer and the inner tunic. The outer side of each tunic is covered by a thin layer of tissue, the outer lining which connects the outer tonic with the skin. It consists of randomly oriented fibres. The inner surface of the mantle is visceral lining. It contains non-fibrous tissue (Kreuzer, 1984).

2.3 Proximate composition

The composition and general properties of squid vary depending on the species, the season, the growth stage and other factors. According to the 4th Amended Japanese Standard Food Content, the edible parts of squid contained 81.8% water, 15.6 % protein, 1.0% fat, and 1.5% ash. The species known as hotaru-ika has 78.9% water, 14.2% protein, 5.2% fat and 1.5% ash (whole body including internal organs) (Okuzumi and Fujii, 2000). Pavlos et al. (1995) reported that protein and lipid of marine depended on species and the season. Squid contained 13-15% protein and 0.2-1.0% lipid. Proximate composition of mantle of squid and cuttlefish is shown in Table 1. Lapa-Guimarães et al. (2005) reported that squid (*Loligo plei*) contained 74.2% water, 14.4% protein, 2.0% fat and 1.7% ash. Cuttlefish (*Seapia pharaonis*) contained 82.78% water, 14.91% protein, 0.47% fat, 1.20% ash and 0.64% collagen (Thanonkaew et al., 2006).



(i) Single chromatophore of *Sepiola* surrounded by muscle cells
Mz = muscle cell
Jr = iridocyte
K = nucleus of muscle
a = top view
b = side view

(ii) Skin of *Sepia officinalis*
M = black chromatophor
g Ch = yellow chromatophor
o Ch = orange chromatophor
Ir = iridocytes
Ep = epidermis

Figure 4. Chromatophores of cephalopod skin

Source : Kreuzer (1984)

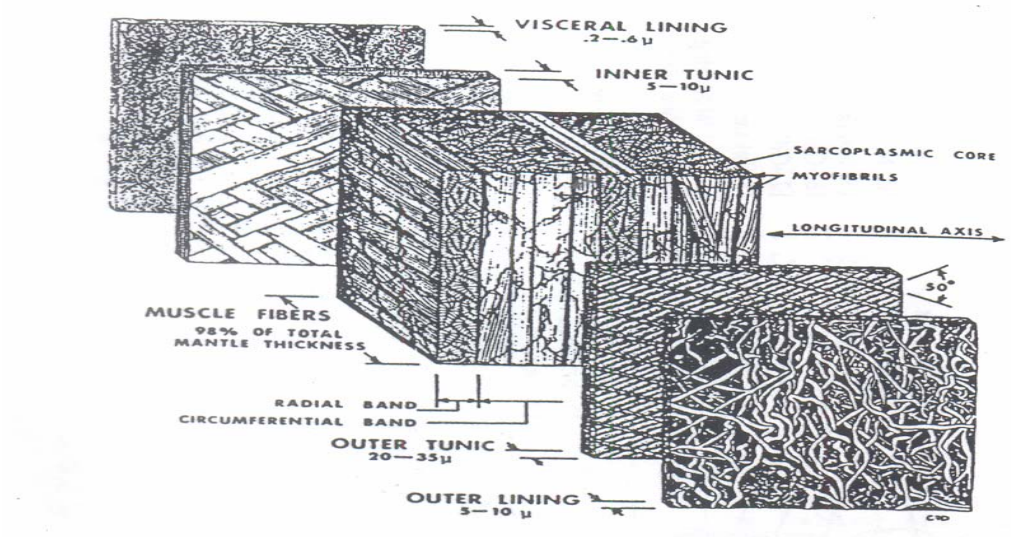


Figure 5. Tissue composition in the mantle of squid (*Loligo peali*)

Source : Otwell and Giddings (1980) cited by Kreuzer (1984)

Table 1. Proximate composition of mantle muscle of squid and cuttlefish (% on wet basis)

Species	Moisture	Protein	Lipid	Ash
Todarodes pacificus (1)	76.6	20.6	1.88	1.59
Todarodes pacificus (2)	78.0	19.8	1.95	1.57
Ommastrephesbartrami (1)	77.1	20.9	1.33	1.71
Ommastrephesbartrami (2)	74.8	23.0	1.56	1.92
Ommastrephesbartrami (3)	77.7	20.6	1.40	1.60
Nototodarus sloani gouldi	78.4	18.7	1.66	1.78
Nototodarus sloani sloani	77.1	20.2	1.67	1.74
Illex argentinus	78.8	18.2	2.03	1.71
Loligo opalescens	77.0	19.6	2.74	1.52
Sepia esculenta	81.5	15.6	1.28	1.56
Sepia pharaonis	76.4	20.2	1.36	1.86

Source : Suyama and Kobayashi (1980) cited by Kreuzer (1984)

2.3.1 Protein

Protein can be divided into three types based on their solubility. Myofibrillar protein is soluble in neutral saline solution but not in water. Sarcoplasmic protein is soluble in water and stroma protein is not soluble in neutral saline solution and water (Okuzumi and Fujii, 2000).

2.3.1.1 Myofibrillar protein

Squid contains 80% myofibrillar protein which is greater than other seafoods (Okuzumi and Fujii, 2000). There are thick filaments and fine filaments in the longitudinal axial direction as illustrated in Figure 6 (a). The thick filaments contain myosin and the fine filaments contain F-actin and troponin. Myosin of squid acts in the same way as vertebrate skeletal muscles. However the thick filaments of squid have a core of paramyosin, the unique protein for invertebrates (Sugiyama et al., 1989). Additionally, the filaments are thicker and longer than those found in vertebrates. The myofibrillar protein of the mantle muscle of *T. pacificus* is composed

of 54% myosin, 17% actin, 14% paramyosin, 8% tropomyosin and 7% miscellaneous (Table 2) (Sugiyama et al., 1989). Myofibrillar proteins of cuttlefish are composed of myosin heavy chain, actin and paramyosin as the major constituents (Thanonkaew et al., 2006; Dublán-García et al., 2006). Actomyosin from squid showed a lower relative percentage of myosin and a higher percentage of paramyosin and actin, compared with that of striated muscle of scallop (Mignino and Paredi, 2006). The actomyosin of squid is composed of 49.7% myosin, 30.0% actin and 12.5% paramyosin (Mignino and Paredi, 2006).

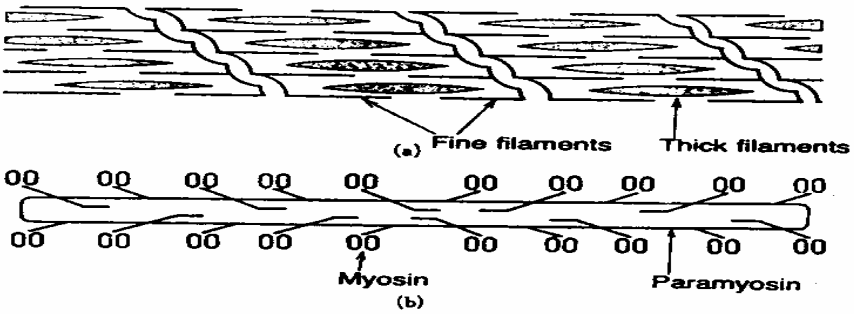


Figure 6. Schematic diagram of oblique muscle myofibril (a) and thick filament (b)
Source : Sugiyama et al. (1989)

Table 2. Composition of myofibrillar protein of some invertebrates (%)

Muscle	Myosin	Actin	Paramyosin	Troponin-myosin	Miscellaneous
Todarodes pacificus Oblique muscle	54	17	14	8	7
Patinopecten yessoensis Striated muscle	66	25	3	6	0
Oyster striated muscle	39	23	19	-	19
Oyster smooth muscle	29	9	38	-	24
Rabbit skeletal muscle	60	20	0	4.5	15.5

Source: Sugiyama et al. (1989)

2.3.1.2 Sarcoplasmic protein

Sarcoplasmic protein is water soluble. It constitutes 12 to 20% of total protein in mantle muscle of *T. pacificus* (Sugiyama et al., 1989). Sarcoplasmic protein is composed of proteinases that have the highest activity at weak alkaline, as well as TMAO demethylase, that causes the formation of dimethylamine and formaldehyde in frozen squid. Sarcoplasmic proteins in frozen squid showed 20 protein bands when determined by SDS-PAGE (Sikorski and Kolodziejska, 1986).

2.3.1.3 Stroma protein

Squid contains 2-3% collagen and octopus consists of the higher collagen content, which maybe associated with a tougher texture of octopus. Squid skin has about twice as much as collagen as squid meat (Okuzumi and Fujii, 2000). Sadowska and Sikorski (2007) reported that collagen contents in the muscle of squid *Illex argentinus* and *Loligo patagonica* were 1.0 and 0.3%, respectively. Cuttlefish contained 0.64% collagen (Thanonkaew et al., 2006).

2.3.2 Non-protein nitrogenous compounds

Squid muscle contains non-protein nitrogenous compounds at approximately 37% of total nitrogen content (Sugiyama et al., 1989). Those include free amino acids, betaines**,** trimethylamine oxide**,** nucleotides**,** octopine and trimethylamine (Okuzumi and Fujii, 2000). Amino acids, especially essential amino acids, determine primarily the nutritive food value. However, the amino acids can be used by invading bacteria, producing ammonia and putritive smell (Kreuzer, 1984).

Muscle of *T. pacificus* is composed of trimethylamineoxide (TMAO) (1280 mg/100 g), octopine (954 mg/100 g), proline (1050 mg/100 g), arginine (160 mg/100 g), taurine (97 mg/100 g) and betaine (671 mg/100 g). Octopine is produced through dehydration condensation of arginine and pyruvic acid in the presence of NADH. The reaction is catalyzed by octopine dehydrogenase, which can be formed in the muscles of mollusks such as squid, octopus and bivalves (Sugiyama et al., 1989).

Table 3. Non-protein nitrogenous compound in different fish and shellfish (%)

Class of Compound	Teleosts (Mackerel)	Elasmobranchs (Shark)	Crustacea (Shrimp)	Molluscs (Squid)
Amino acids	25	5	65	50
Peptides	5	5	15	15
Nucleotides	15	5	5	5
Creatine and	50	10	-	-
TMAO	15	20	5	15
Urea	-	55	-	-
Betaines	-	-	10	56
Ammonia and	5	-	-	-
Octopine	-	-	-	10

Source : Finne (1992)

2.3.3 Lipid

The lipid content of raw squid is about 1.0-2.0%. Squid lipid tends to be low in triglycerides but high in phospholipids. Okuzumi and Fujii (2000) studied the lipid composition of mantle from 5 species of squid as shown in Table 4. The phospholipids account for about 62-84%. The triglyceride content is extremely low at 0.8-3.2% and sterols account for 15-20% of total lipids. Thanonkaew et al. (2006) reported that phospholipid was the major component of lipids in head and mantle of cuttlefish , followed by diglyceride, triglyceride and free fatty acid, respectively.

Phospholipids in squids have lecithin and phosphatidyl-ethanolamine as the major compounds (Table 5). EPA (eicosapentaenoic acid ($C_{20:5}$)), DHA (docosahexaenoic acid ($C_{22:6}$)), and palmitic ($C_{16:0}$) are the dominant fatty acids in squid. The amount is in the descending order : DHA ($C_{22:6}$) > pamitic ($C_{16:0}$) > EPA ($C_{20:5}$). The total amounts of fatty acids in squid are in the order of total PUFA > total SFA > total MUFA. In general, total PUFA account for 46-56% of total fatty acid (Okuzumi and Fujii, 2000). PUFAs were found as the major fatty acids with the range of 50.3-54.9% in the mantle of cuttlefish. DHA and EPA were the most abundant

PUFAs in mantle of cuttlefish. For the saturated fatty acids, $C_{16:0}$ and $C_{18:0}$ were the most abundant fatty acids in the mantle of cuttlefish (Thanonkaew et al., 2006). DHA and EPA constituted 20-35% and 7-21% of total fatty acid in lipids of cuttlefish, squid and octopus. Arachidonic acid ($C_{20:4, n-6}$) ranged from 1.1 to1.9% (Passi et al., 2002).

Table 4. Lipid content of squid mantle meat (%)

	Phospholipids	Sterol	Free fatty acid	Triglycerides	Unknown substances
Japanese common	71.1	19.9	6.5	0.8	1.1
Spear squid	83.8	14.8	1.1	0.3	Not
Neon flying squid	61.8	15.8	21.6	0.1	0.7
Argentine shortfin	73.1	15.4	6.8	3.2	1.5
Mako gonate squid	84.0	3.6	3.6	trace	trace

Source : Okuzumi and Fujii (2000)

Table 5. Phospholipid content of squid mantle meat (%)

	LPC	SMP	PC	PS	PE	PI
Rubust clubhook squid	-	2.2	38.3	24.9	31.0	3.6
Boreo-Pacific gonate squid	1.3	2.1	56.6	14.7	23.2	2.1
Boreal clubhook squid	1.2	5.4	63.0	11.1	16.2	3.1
Neon flying squid	2.4	8.4	59.5	10.6	16.3	2.8
Argentine shortfin squid	1.4	4.4	68.6	7.2	17.2	1.2
Japanese common squid	11.4	3.1	65.8	2.7	11.9	5.1

PC= lysolecithin , SPM= sphingomyelin , PC= lecithin , PS= phosphatidylserine, PE= phosphatidylethanolamine , PI= phosphapidylinositol

Source : Okuzumi and Fujii (2000)

3. The quality changes of seafoods during chilled and frozen storage

3.1 The quality changes of chilled seafoods

Chilled storage is generally referred to storage at temperatures above freezing point, ranging from about 16^{o}C to -2^{o}C. The preservation effect of chilled storage is based on the fact that reducing the temperature decreases the rate of chemical reactions and growth of microorganisms (Doyle et al., 1997). The temperature of proper chilled storage is generally less than the minimal growth temperature of most foodborne microorganisms and the generation time of psychrotrophic microorganisms is also considerably increased (Doyle et al., 1997). The minimal growth temperature of most foodborne microorganisms is about 5^{o}C-15^{o}C (Adams and Moss, 2000).

Microorganisms such as *Shewanella putrefaciens* and *Pseudomonas* spp. are the specific spoilage bacteria of chilled seafoods (Herbert and Shewan, 1976; Gram and Huss, 1996). Fish spoilage depends on specific spoilage organisms. Spoilage is also dependent on the climatic and storage conditions, the type of fish and the even of place in which the fish was harvested (Drosinos and Nychas, 1996; Gram and Huss, 1996). Marine fish from temperate waters are spoiled by *Photobacterium phosphoreum* whereas gram-positive bacteria likely cause the spoilage of CO_2 packed fish from fresh or tropical waters (Gram and Huss, 1996). *Shewanella putrefaciens*, *Pseudoalteromonas* sp. and *Pseudomonas* sp. are spoilage bacteria of gutted squid stored in ice (Paarup et al., 2002). The major spoilage flora in cuttlefish (*Sepia officinalis*) and short fin (*Illex coindetii*) storage in ice was *Pseudomonas* (Vaz-Pires et al., 2008). Specific spoilage organisms of chilled seafood are shown in Table 6.

Table 6. Specific spoilage microorganisms of chilled seafood

Atmosphere	Temperate waters		Tropical waters	
	Marine	Fresh	Marine	Fresh
Aerobic	*S. putrefaclens*	*Pseudomonas* spp.	*S.putrefaclens*	*Pseudomonas* spp.
	Pseudomonas spp.		*Pseudomonas* spp.	
Vacuum	*S. putrefaciens*	Gram- positive bacteria	Lactic acid bacteria	Lactic acid bacteria
	P. phosphoreum	Lactic acid bacteria	others	
CO_2	*P. phosphoreum*	Lactic acid bacteria	Lactic acid bacteria	Lactic acid bacteria
			TMAO reducing bacteria	TMAO reducing bacteria

Source : Gram and Huss (1996)

TMAO reducing bacteria are capable of reducing TMAO to TMA (Gram and Huss, 1996). The most ammonia can be formed from the deamination of amino acids. Ammonia in chilled elasmobranches is associated with the decomposition caused by microorganisms. Many volatile compounds such as TMA, sulfur compounds, aldehydes, ketone, hypoxanthine and other low molecule weight compounds are produced by spoilage bacteria. The important precursors of volatile compounds are TMAO, cysteine, methionine, other amino acid, IMP, inosine and carbohydrates (Gram and Huss, 1996).

Icing has been used for a common chilling method to maintain the quality of squids. However, icing methods used have the impact on shelf-life differently. Squid (*Loligo plei*) quality markedly decreased after 7 days of storage in both contact and non-contact ice. However, the storage methods did not affect the

development of bacterial counts, in which both treatments were below 10^6 cfu/g of muscle after 16 days of storage. (Lapa-Guimaraes et al., 2002). Chemical and microbial changes in squid muscle (*Loligo plei*) during storage in ice by contact ice and non-contact ice were compared. Trimethylamine (TMA) and volatile basic nitrogen (VBN) contents increased during the first day of storage in the non-contact ice treatment, related with increasing psychrotrophic bacterial count. Free tryptophan and urea production by microorganisms markedly increased during the whole storage. Sample stored by contact ice had the increase in TMA and VBN after day 9 and no increase was observed in free tryptophan and urea contents (Lapa-Guimaraes et al., 2005). In general, the non protein nitrogen (NPN) continuously decreased in both squid and cuttlefish during storage due to the leaching process. The leaching of soluble compounds can affect of chemical test for freshness evaluation because increases of a soluble compound in the muscle will only be detected when the rate of its formation overcomes the loss rates (Vaz-Pires et al., 2008). Evisceration of squid is another means to extend the shelf-life along with iced storage. The gutting of ***T. eblanae*** extended shelf-life by approximately two days and reduced the production of ammonia and TMA. Agmatine production occurs during the early storage stages. This amine seems to be an excellent freshness indicator (Paarup et al., 2002). *Photobacterium phosphoreum* may contribute to spoilage through its activity in the digestive gland, followed by diffusion of volatile compounds and amines to the mantle (Moral et al., 2002). The diffusion of autolytic enzymes from gland to mantle was reported by Moral et al. (1998). The autolytic activity of squid (*Loligo vulgaris*) was found on day 4 of chilled storage and a drop was seen at day 7. These fluctuations were attributed to variations in the endogenous muscle proteases content and their activities, which are affected by the post-rigor condition and softening of the muscle. Autolysis increased sharply at day 10 of chilled storage. In addition, autolysis may be enhanced by the presence of proteases of microbial origin. The microbial protease activity may be significant after 10 day of iced storage, when muscle decomposition becomes apparent (Gomez-Guillen et al., 2003). The microbial action is less evident than autolysis effects in cephalopods until sensory rejection (Vaz-Pires et al., 2008). The autolysis or activity of endogenous enzymes was the main responsible for the change in sensory attributes (Vaz-Pires et al., 2008).

3.2 The quality changes in frozen seafoods

Freezing food is the method of food preservation, which slows both food decay by turning water to ice, makes it unavailable for bacterial growth and slows down most chemical rections. The temperature of proper frozen storage is generally about -18^{o}C or less (Ang et al., 1989).

Nevertheless, frozen storage can cause many physical-chemical changes such as rancid odor from oxidation reaction, decomposition of TMAO to dimethylamine (DMA) and formaldehyde (FA), losses in protein solubility and losses in water retention properties. Those changes lower the functional properties of muscle proteins, mainly due to denaturation and aggregation of myofibrillar proteins (Chang et al., 1997). High molecular weight protein aggregate stabilized by hydrophobic interaction as well as by disulfide bonds and other covalent cross- links are generally formed during frozen storage (Haard, 1992). Surface hydrophobicity and disulfide bond content of natural actomyosin from cuttlefish increased during frozen storage up to 12 weeks (Thanonkaew et al., 2007). Ruiz-Capillus et al. (2002) studied the functional properties of voladur (*Illex coindentii*) muscle proteins during frozen storage at -20^{o}C for 16 months. Solubility of protein in 5% NaCl showed a significant increase in the initial months followed by decrease. This solubility was generally greater in the mantle than in the arms. Soluble collagen in an acidic medium decreased in both mantle and arm throughout frozen storage (Moral et al., 2002). The viscosity of actomyosin from squid (*Illex argentinus*) decreased up to month 3 of frozen storage (Mignino et al., 2008). Viscosity is one of the most sensitive functional properties for measuring changes in myofibrillar protein during frozen storage (Barroso et al., 1998).

Factors influencing deterioration during frozen fish include rate of freezing, temperature and time of frozen storage, fish species, post-harvest history of fish prior to frozen storage and cryoprotective compounds (Haard, 1992). The squid (*Illex aryentinus*) proteins underwent aggregation, independent of the sex of specimens during frozen storage. Relative percentages of free fatty acid and diacylglycerides increased and triacylglycerides and phospholipid decreased after 8 months of frozen storage (Paredi et al., 2005). The catching method influences the

rates of autolysis and the functional properties of myofibrillar protein from frozen-stored squid mantle (Mignino et al., 2008).

3.2.1 Changes in proteins

Catalytic effect of ice and inorganic salts, the binding of fatty acids and lipid oxidation products, cross-linking induced by formaldehyde in the muscles of some fish, and other reactions lead to the formation of covalent bonds in denaturated proteins (Sikorski and Kolakowsha, 1990). In frozen fish, trimethylamine oxide (TMAO) undergoes demethylation to trimethylamine (TMA) and formaldehyde (FA) (Sikorski and Kolakowha,1990). TMAO can be broken down through a nonenzymic pathway during frozen storage, TMAO is degraded to FA and DMA or TMA by this pathway (Kimura et al., 2002). FA is hypothesized to be a cross-linking agent in muscle proteins. The aldehyde accumulated during frozen storage may bind to some groups in protein side chains and form intra and intermolecular methylene bridges as follows :

$RNH_2 + CH_2O \longrightarrow RNHCH_2OH$

$RCONH_2 + CH_2O \longrightarrow RCOHCH_2OH$

$RCONH_2 + RCOHCH_2OH \longrightarrow RCOHCH_2NHCOR + H_2O$

$ROH + CH_2O \longrightarrow ROCH_2OH$

$2ROH + CH_2O \longrightarrow ROCH_2OH + H_2O$

$2RSH + CH_2O \longrightarrow RSCH_2SH + H_2O$

These reactions may be involved in the formation of protein aggregates and may decrease the protein solubility (Sikorski and Kolakowsha, 1990). In addition, trimethylamine oxide demethylase (TMAOase) also leads to a losing and aggregation in protein solubility, which is associated with increases cross linking and aggregation of protein (Benjakul, et al., 2004). Leelapongwattana et al. (2008) reported that TMAOase from the kidney of lizardfish induced the formation of FA and caused the protein-crosslinking. Ueng and Chow (1998) found the increases in toughness of three species of cephalopods (*Loligo edulis*, *Sepia pharaonis* and *Illex argentinus*) during frozen storage at -20^{o}C for 4 months. There was no significant difference in drip and SDS gel electrophoresis patterns after 4 months of frozen storage among all species.

Stanley and Hultin (1982) reported that the toughening of frozen squid mantle might be caused by protein cross-linking because of the existence of high levels of DMA and formaldehyde. The formaldehyde was generally higher in viscera than in muscle of four species of frozen squid (Li et al., 2007).

3.2.2 Oxidation of lipid

Lipid oxidation in muscle is one of the major deteriorative reaction causing the losses in quality during processing and storage. The oxidation of unsaturated fatty acids leads to the formation of free radicals and hydroperoxides. Such intermediary compounds are unstable and cause the oxidation of pigments, flavors, and vitamins (Sahoo and Verma, 1999). The malonaldehyde formed as oxidation product of lipids was related with the change of proteins in frozen Baltic herring (Kussi et al., 1975). The reaction of malonaldehyde with the free amino groups of the proteins during frozen storage might cause the decrease in water binding capacity and solubility. The amount of free amino acid groups in these proteins also decreased (Kussi et al., 1975).

3.2.2.1 Factors affecting lipid oxidation

1. Fatty acid composition : Type of fatty acid and degree of unsaturation are the important factors affecting lipid oxidation. Relative rates of oxidation for arachidonic ($C_{20:4,n-6}$), linolenic ($C_{18:3,n-3}$), linoleic ($C_{18:2,n-6}$) and oleic ($C_{18:1}$) acids are approximately 40:20:10:1. The geometry of double bonds affect the rate of oxidation. Cis acids are oxidized more readily than their trans isomers (Nawar, 1985).

2. Metal : Transition metal ions are remarkable good promoters of free radical reaction because of singlet electron transfer during their change in oxidation states. A direct reaction between a metal catalyst and lipid molecule is envisaged in the chain initiation step (1). Non-enzymatic lipid oxidation is enhanced by metal ions such as iron, cobalt and copper as well as heme compounds (2), (3) (Jadhav et al., 1996). Thanonkaew et al. (2006) found that Fe^{2+} effectively induced lipid oxidation, while Cu^{+}, Cu^{2+} and Cd^{2+} showed negligible effects on lipid oxidation in cuttlefish muscle, particularly with multiple freeze-thaw cycles.

$$Mn^{2+} + R\text{-}H \longrightarrow Mn^{+} + H^{+} + R^{\bullet} \quad (1)$$

$$ROOH + Fe^{2+}\text{or } (Cu^{+}) \xrightarrow{\text{Fast}} RO^{\bullet} + Fe^{3+}\text{or } (Cu^{2+}) + OH^{-} \quad (2)$$

$$ROOH + Fe^{3+}\text{or } (Cu^{2+}) \xrightarrow{\text{Slow}} ROO^{\bullet} + Fe^{2+}\text{or } (Cu^{+}) + H^{+} \quad (3)$$

3. Temperature : The rate of oxidation increases with increasing temperature (Nawar, 1985). Saeed and Howell (2002) studied lipid oxidation of Atlantic mackerel during frozen storage at -20^{o}C and -3^{o}C for 24 months. An increase in lipid oxidation products was obtained with increasing storage time and storage temperature.

4. Oxygen : The rate of oxidation increases in direct proportion to the surface area of the lipid exposed to air (Nawar, 1985). Johnsen and Bjerheng (1995) studied the effect of rainbow trout packed by oxygen diffusion control at high, medium and low levels. The rate of oxidation as monitored by TBARS increased quickly in high level of oxygen diffusion and decreased in medium and low level of oxygen diffusion during frozen storage at -18^{o}C for 37 weeks.

5. Water activity : The rate of oxidation depends on water activity. It is very rapidly increased from a_w about 0.1 to about 0.3. At high water activities (a_w = 0.55-0.85), the rate of oxidation increases again, presumably as a result of increased mobilization of catalysts and oxygen (Nawar, 1985).

3.2.3 Effect of lipid oxidation on physicochemical changes and discoloration of squid during frozen storage

The changes in frozen stored squid mantle (*Illex argentinus*) involved denaturation and aggregation of protein components. These changes were caused by free fatty acids and lipid oxidation products, which can bind myofibrillar proteins and induce the aggregation of muscle proteins. This resulted in the decreased protein solubility and reduced enzymatic activities of actomyosin (Paredi et al., 2006). Thanonkaew et al. (2008) found that the increases in lipid oxidation of cuttlefish (*Sepia pharaonis*) muscle during frozen storage were correlated with the increases of hydrophobicity and decreases of protein solubility. Hydrophobic interaction might take place between the exposed hydrophobic residues. Frozen storage directly

affected conformational changes of protein molecules (Thanonkaew et al., 2008). Hydrophobicity of myofibrillar proteins of mantle squid (*Illex argentinus*) showed a trend to increase between the first and the fifth month of frozen storage (Mignino et al., 2008). Lipid oxidation and aldehydic lipid oxidation products were able to produce yellow pigmentation. Reaction between aldehydic lipid oxidation products and the amine groups of phospholipid, where the carbonyl group of the surface active aldehyde would be in close to the amine in the phospholipid headgroup caused the yellow discoloration in the squid (Thanonkaew et al., 2006).

4. Chemicals used for seafood processing

4.1 Phosphates

The addition of phosphates to seafood products generally increases water retention during processing, inhibits oxidation, contributes to better color, reduces drip losses and provides more tender texture (Dziezak, 1990). Denaturation of muscle proteins during frozen storage led to the lower water-holding capacity of proteins. Ice crystals resulted in the tissue damage and leakage from various organelles. As a result, water could be released from muscle more easily when the frozen storage time increased (Benjakul et al., 2003). The phosphates commonly used to treat seafood are sodium acid pyrophosphate (SAPP), sodiumtripolyphosphate (STPP) and sodium hexametaphosphate (SHMP) (Dziezak, 1990). Many phosphate blends have been applied in seafood which will be sold fresh, frozen uncooked or frozen precooked. There are four methods for applying phosphates to fish : immersion, spraying, injection or tumbling (Dziezak, 1990). Woyewoda and Bligh (1984) found that treatment of Atlantic cod fillets with 12% tripolyphosphate prior to frozen storage at -12 or -30^{o}C for 26 weeks could decrease thaw drip and cooked drip. Treatment of cod fillets with 3% phosphate and 5% salt effectively increased the yield of thawed fillet (Thorarinsdottir et al., 2004). Goncalves and Ribeiro (2009) reported that dipping shrimp in 5% phosphate blend solution (STPP + sodium tetra pyrophosphate + NaCl) prevented cooking yield losses, improved sensory quality and increased consumer's preference. The increase in sodium tripolyphosphate solution (STP) concentration (2-4%) for treatment reduced yield losses of peeled white and

brown shrimps (Erdogdu et al., 2004). Soaking cuttlefish with 0.5 % TPP could retard the decreases in solubility and increases in thaw drip of frozen cuttlefish. Phosphate could maintain the protein-water interaction and prevented the protein-protein interaction (Thanonkaew et al., 2008).

4.2 Hypochlorite (NaOCl)

Sodium hypochlorite (NaOCl) has a wide range of industrial, scientific and biomedical application (US EPA, 1994). Use of sodium hypochlorite has been approved in washing or assisting in lye peeling of fruits and vegetables (US FDA, 2004). Kosak and Toledo (1981) used chlorine pretreatment in combination with various packing techniques as a mean of delaying microbial growth. The fresh mullet was dipped in 1000 µg/ml free chlorine solution in combination with either a vacuum pack or polyethylene bag. The decrease in microbial enzymatic activity was found in sample treated with chlorine, yielding 12 day-extension of fresh fish quality at 2^{o}C.

NaOCl is an oxidizing agent, which can induce the physicochemical changes of muscle proteins. Washing bigeye snapper mince with 20 ppm NaOCl resulted in the highest increase in both the breaking force and the deformation of surimi gel from fish stored in ice. Actomyosin extracted from NaOCl-washed mince had higher surface hydrophobicity and disulfide bond content than of water- washed mince (Phatcharat et al., 2005).

4.3 Hydrogen peroxide (H_2O_2)

Hydrogen peroxide (H_2O_2) has a wide range of food applications. It can kill yeast, fungi and bacteria (Lueck, 1980). The maximum concentration of hydrogen peroxide used as an antimicrobial agent on meat carcasses, parts, trim and organs is 75 ppm (US FDA, 2004). Hydrogen peroxide degrades rapidly to oxygen and water (US EPA, 1989). Therefore residues in or on treated post-harvest food commodities are negligible. For food use, 1% hydrogen peroxide has no apparent acute toxicity (US EPA, 1989). Surumi produced from bigeye snapper by washing with hydrogen peroxide had the decreases in total volatile basic nitrogen (TVB-N) and trimethylamine (TMA) during ice storage for up to 14 days. This result indicated the

decrease of microbial enzymatic activity. The breaking force of surumi with H_2O_2 washing was higher than that of surumi with water washing (Phatcharat, 2006). Bhoite-Solomon et al., (1992) reported that H_2O_2 could cause myosin to form disulfide-cross-linked aggregates but not induce fragmentation of myosin. Anderson et al., (1975) reported that H_2O_2 showed bleaching effect on herring and whiter marinated herring product can be obtained. Shelf-life study of marinated herring indicated that H_2O_2 acted as a preservative, yielding a higher quality product with an extended shelf-life about 1 month of storage (Anderson et al., 1975). The cephalopod needs to be bleached because the flesh of cephalopod could be stained by ink, viscera and color pigment during handling and processing (Perkins, 1996). Soaking cuttlefish in 5% NaCl and 0.3% H_2O_2 for 15 min could improve the color of cuttlefish by increasing the L^*-value and decreasing the a^*-value (Thanonkaew et al., 2008).

Objectives

1. To investigate the effect of size, deskinning and squid/ice ratio on pink discoloration and quality changes of squid during iced storage.
2. To study the role of microbial growth and stacking load in pink discoloration and quality changes of squid during iced storage.
3. To elucidate the effect of chemical treatment on lowering pink discoloration and physico-chemical changes of squid mantle during frozen storage.

CHAPTER 2

MATERIALS AND METHODS

1. Materials

Squids (*Loligo formosana*) with the sizes of 21-30 and 6-10 squids/kg, caught by cast net from Songkhla coast along the Gulf of Thailand and off loaded after 24 h of capture, were purchased from a dock in Songkhla province. The squids were placed in the insulated boxes containing ice with a squid/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Had Yai, within 1 h.

2. Chemicals

Cabonate-free MgO, potassium carbonate and urea were obtained from Fluka (Buchs, Switzerland). Plate count agar, malondialdehyde, thiobarbituric acid, hydrochloric acid, 5-5′-dithio-bis (2-nitrobenzoic acid) (DTNB), sodium sulfite, ethylene diamine tetraacetic acid (EDTA), sodium azide and methyl red were procured from Sigma (St. Louise, MO, USA). Trichloro acetic acid, boric acid, hydrogen peroxide, sodium dodecyl sulfate (SDS), ethanol, formaldehyde, potassium chloride and acetic acid were purchased from Merck (Darmstadt, Germany). Sodium hypochlorite and sodium hydroxide were purchased from Ajax Finechem (Auckland, New Zealand).

3. Instruments

Instruments	Model	Company/Country
pH meter	Docu-pH Meter	Sartorius AG, Germany
Magnetic stirrer	BIG SQUID	IKA labortechnik, Germany
Homogenizer	T25 basic	IKA labortechnik, Malaysia
Oil bath	B-490	Buchi, Flawil, Switzerland
Water bath	W350	Memmert, Germany
Microcentrifuge	MIKRO20	Hettich Zentrifugan, Germany
Refrigerated centrifuge	RC-5B plus	Sorvall, USA
Spectrophotometer	UV-16001	Shimadzu, Japan
Colorimeter	ColorFlex	HunterLab Reston, USA
Texture analyzer	TA-XT2	Stable Micro Systems, England
Scaning electron microscope	JSM-5800LV	JEOL, Japan
Vortex mixer	G-560E	Scientific Industries Inc., USA
Light microscope	IX-70	Olympus Optical Company., Ltd, Japan
Auto clave	SS-325	Tomy Seiko co., Ltd, Japan
Hot air oven	Schutzart Din 40050-IP20	Memmert Gmbh and CO. KG, Germany

4. Methods

4.1 Effect of size, deskinning and squid/ice ratio on pink discoloration and quality changes of squid during iced storage

4.1.1 Preparation and storage of squids

Whole squids with the size of 6-10 squids/kg and 21-30 squids/kg with and without deskinning were placed in ice with the squid/ice ratio of 1:1 and 1:2 (w/w). The insulated boxes containing samples and ice were kept at room temperature (28-30^{o}C). During storage**,** the molten ice was replaced with the same amount of ice

every day. During storage of 16 days, the samples were randomly taken for analyses every 2 days. Before analyses, the samples with skin were subjected to deskinning and washing. The samples were kept in ice until analyzed.

4.1.2 Measurement of color

Color of squid mantle after skin removal was measured using a colorimeter (HunterLab, Model ColorFlex) and reported in CIE system color profile of L^* (lightness), a^* (redness/greenness) and b^* (yellowness/blueness). For each sample, the color was measured at six positions of the mantle.

4.1.3 Measurement of pH

pH of sample was determined according to the method of Benjakul et al., (1997). Prior to analysis, the squid was powdered in liquid nitrogen using a blender. Sample powder (5 g) was homogenized with 5 volumes of distilled water (w/v) and the pH was measured using a pH meter (Docu-pH_{Meter}, Sartorius AG, Germany).

4.1.4 Determination of total volatile base (TVB) and trimethylamine (TMA) contents

TVB and TMA contents were determined following the method of Conway and Byrne (1936). Sample powder (5 g) was homogenized with 4% trichloroacetic acid at a ratio of 1: 2 (w/v). The homogenate was filtered through Whatman No 1 paper. The filtrate (1 ml) was placed in the outer ring. The inner ring solution (1% boric acid containing the Conway indicator) was pipetted into the inner ring. To initiate the reaction, K_2CO_3 (1 ml) was mixed with the filtrate. The Conway unit was closed and incubated at 37^{o}C for 60 min. The inner ring solution was then titrated with 0.02 M HCl until the green color turned to pink. TMA was determined in the same manner as TVB, but 10% formaldehyde was added to the filtrate to tie up ammonia prior to the addition of K_2CO_3.

4.1.5 Determination of ammonia content

Ammonia content was determined as described by Parris and Foglia (1983). Sample powder (10 g) was placed in a 600 ml round bottom flask containing 200 ml of distilled water, 10 g carbonate - free MgO and a few drops of antifoam. The mixture was distilled and the distillate (100 ml) was collected in an erlenmeyer flask

containing 20 ml of 0.1 N HCl. Collected distillate was titrated using 0.05 N NaOH and methyl red was used as an indicator. Ammonia content was calculated and expressed as mg/g sample.

4.1.6 Determination of formaldehyde content

Formaldehyde content was determined according to the method of Nash (1953). Sample powder (5 g) was added with 20 ml of 5% trichloroacetic acid. The mixture was homogenized for 2 min and filtered using Whatman paper No.41. The filtrate was neutralized to pH 6.0-6.5. The final volume was made up to 50 ml using distilled water. The neutralized filtrate was determined using acetylacetone reagent and absorbance was measured at 412 nm. Formaldehyde content was calculated based on the standard curve with the amount range of 0-24 µg using the following formular :

$$\text{Formaldehyde}_{(\mu g/g)} = \frac{A/\text{volume of extract used} \times \text{Total volume of extract}}{W}$$

where: A = quantity of formaldehyde (µg) from the standard curve; W = weight of sample (g)

4.1.7 Determination of thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) were determined as described by Buege and Aust (1978). Sample powder (0.5 g) was dispersed in 2.5 ml of thiobarbituric acid reactive substances 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 water. The mixture was centrifuged at 3,600 xg for 20 min at room temperature. The absorbance of supernatant was measured at 532 nm. Standard curve was prepared using malondialdehyde (MDA)(0-2 µg/ml) and TBARS was expressed as mg MDA/kg sample.

4.1.8 Determination of psychrophilic bacterial count

Psychrophilic bacterial count was determined as described by Speck (1976). The sample (25 g) was taken aseptically and homogenized with 225 ml of normal saline solution (0.85 g NaCl in 100 ml water). Decimal dilutions were made using saline solution. Psychrophilic bacteria count was determined by plate count agar with the incubation at 4^{o}C for 7 days. The count was expressed as log cfu/g.

4.2 Effects of sizes and squid/ice ratio on disruption and distribution of chromatophores on squid mantle during iced storage

Fresh whole squid with the size of 6-10 squids/kg and 21-30 squids/kg (without deskinning) were placed in ice with squid/ice ratio of 1:1 and 1:2 (w/w). During storage**,** the molten ice was replaced with the same amount of ice everyday. During storage of 16 days, the chromatophores in the skin were visualized under the light microscope (Model SZH10, Olympus, Tokyo, Japan) and the magnified images were photographed (Model DP12, Olympus, Tokyo, Japan).

4.3 Role of microorganisms in pink discoloration and quality changes of squid stored in ice

4.3.1 Preparation of sodium azide treated squids

Whole squids (without deskinning) were soaked in 0.01 or 0.1% sodium azide (NaN_3) used as the antimicrobial agent at a squid/solution ratio of 1:5 (w/v) for 5 min. The samples were drained on the screens for 3 min at 4^oC and placed in ice with a squid/ice ratio of 1:1 (w/w). During storage**,** the molten ice was replaced with the same amount of ice every day. The samples were randomly taken for analyses every 2 days for up to 16 days as mentioned previously.

4.3.2 Color (as described in section 4.1.2)

4.3.3 TVB and TMA contents (as described in section 4.1.4)

4.3.4 Psychrophilic bacterial counts (as described in section 4.1.8)

4.3.5 Distribution of chromatophore (as described in section 4.2)

4.4 Effect of stacking on pink discoloration, chemical and quality changes of squid during iced storage

Whole squids with the sizes of 6-10 squids/kg were placed between the layers of ice. The squid/ice ratio was 1:2 (w/w). The squids and ice were placed in the insulated box. Different standard weights (4 and 8 kg) were placed on the top of squid/ice mixture containing 4 kg of squid. The samples were stored at room temperature (28 - 30^oC). The ice was changed every day. The samples were taken for analyses as mentioned in section 4.1 every 2 days up to 14 days.

4.5 Effect of chemical treatment on pink discoloration and physico-chemical changes of squid during frozen storage

4.5.1 Preparation of pink squid

Whole squids (6-10 squids/kg) were stored in ice with squid/ice ratio of 1:1 (w/w) yielding the highest pink discoloration (section 4.4) for 14 days. The molten ice was replaced with the same amount of ice every day.

4.5.2 Effect of bleaching agent on pink squids

Pink squids were deskinned and eviscerated. The samples were soaked in 3% NaCl containing oxidizing agents (H_2O_2 or NaOCl) at different levels (Table 7) using squid/solution ratio of 1:5 (w/v) for 3 h at 4^{o}C. Soaked samples were washed with cleaned water and drained on the screen for 5 min. The color was measured and reported as *L**, *a** and *b**-values. The treatment yielding the squid with the lowest pink color was chosen for further study.

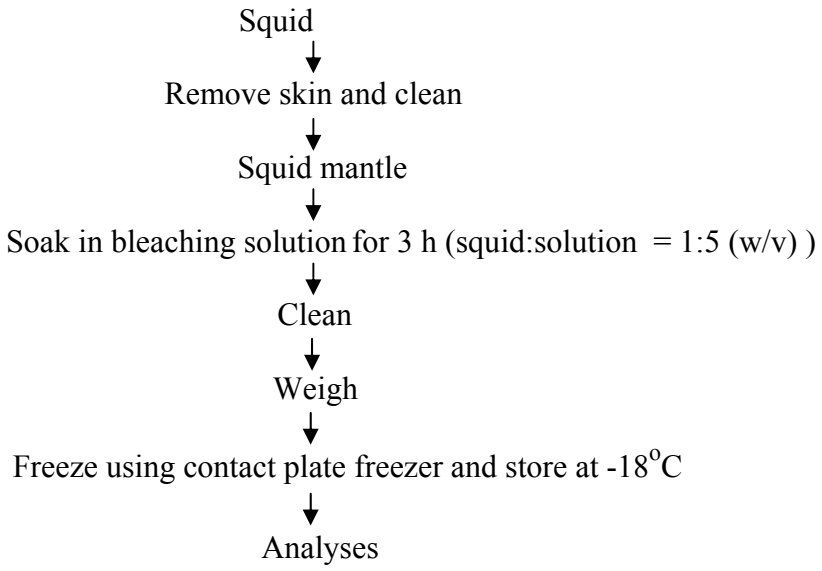


Figure 7. Scheme for the preparation of frozen bleached pink squid

Table 7. Bleaching solutions for pink squid

Treatments	H_2O_2 (%)	NaCl (%)	NaOCl (ppm)
1	0.05	3	0
2	0.25	3	0
3	0.50	3	0
4	0.05	3	5
5	0.25	3	5
6	0.50	3	5
7	0.05	3	10
8	0.25	3	10
9	0.50	3	10

The samples with and without bleaching (1 kg) were packed in metal tray (18x29x5 cm^3) and subjected to contact plate freezing (-18^oC) for 24 h. Samples were taken at week 0, 2, 4, 6, 8 and 10 for analyses. The samples were thawed using running tap water (25^oC-27^oC) until the core temperature reached 0-2^oC. Thawed samples were subjected to following analyses :

4.5.3 Analyses

4.5.3.1 Color

Color was dertermined as described in section 4.1.2

4.5.3.2 Thaw drip

The weight of squid was recorded before freezing and after frozen storage. To measure thaw drip, frozen squids were thawed using running tap water. Thawed samples were weighed and the amount of thaw drip was calculated according to the following formula (Santos and Regenstein, 1990) :

% Thaw drip = [(A-B)/A] x 100

where: A = Initial weight of sample ; B = Final weight of sample (after frozen storage and thawing)

4.5.3.3 Total sulfhydryl content

- Preparation of natural actomyosin

Natural actomyosin (NAM) was prepared according to the method of Benjakul et al. (1997). Squid powder was homogenized in 10 volumes of 0.6 M KCl ($4^{o}C$) for totally 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 $4^{o}C$ using a RC-5B Plus centrifuge. Supernatant was mixed with 3 volumes of chilled water. The diluted supernatant was centrifuged at 5000 xg for 30 min at $4^{o}C$ to precipitate the NAM. NAM was solubilized in 0.6 M KCl (1:1) and then recentrifuged at 5000 xg for 30 min at $4^{o}C$ 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 total sulfhydryl and disulfide bond contents.

- Total sulfhydryl content

Total sulfhydryl content was determined using 5, 5'-dithio-bis (2-nitrobenzoic acid) 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 was mixed with 0.4 ml of 0.1% DTNB and incubated at $40^{o}C$ for 25 min. The absorbance at 412 nm was measured using 0.6 M KCl solution as a blank. The sulfhydryl content was calculated using the extinction coefficient of 13600 $M^{-1}cm^{-1}$.

4.5.3.4 Disulfide bond content

Disulfide bond content was determined using the 2-nitro-5-thiosulfobenzoate 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 was added. The mixture was mixed thoroughly and incubated in 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 $M^{-1}cm^{-1}$.

4.5.3.5 TBARS (section 4.1.7).

4.5.3.6 Texture

Mantle was cut into the size of 1.0x2.0x0.5 cm and subjected to shear analysis using a texture analyzer (Texture analyzer, Model TA-XT2, Stable Micro Systems, Surrey, England) with a Warner-Bratzer shear apparatus according to the method of Dawson et al. (1991). The operating parameters consist of a cross head speed of 2 mm/s and a 25-kg load cell. The shear force perpendicular to the axis of muscle fiber was measured in ten replications for each sample. The peak of shear force profile was regarded as the shear force value.

4.5.3.7 Scanning Electron Microscopy (SEM)

Microstructure of squid mantle were analyzed as described by Palka and Daun (1999). The squid mantle was excised to obtain a piece of 0.5x1.0 cm^2. The samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4^oC. The solution was discarded and the dehydration was performed using 25, 50, 70, 95 and 100% (v/v) ethanol, respectively. For each concentration used, the dehydration was carried out for 1 h. The dried specimens were then cut in liquid nitrogen and the critical point drying was done by using liquid carbon dioxide. The fragments of dried tissue were mounted on holders with silver cement and coated twice with gold sputter coater (SPI-Module, SPI Supplies, West Chester, PA, USA). The microstructure was visualized using a scanning electron microscopy (JEOL, JSM-5800 LV, Tokyo, Japan). The micrographs were taken at magnification of 5000X.

5. Statistical analysis

Completely randomized design (CRD) was used. Experiments were run in triplicate. Data was subjected to analysis of variance (ANOVA) and mean composition was carried out using Duncan's Multiple range test (DMRT) (Steel and Torrie,1980). For pair comparison, T-test was used. Data analysis was performed using the Statistical Package for Social Science (SPSS 10.0 for window, SPSS Inc., Chicaco, IL).

CHAPTER 3

RESULTS AND DISCUSSION

1. Effect of size, deskinning and squid/ice ratio on pink discoloration and quality changes of squid during iced storage

1.1 Changes in color

The *a**-value of mantle from the squid without deskinning for both sizes, 6-10 squids/kg (large size) and 21-30 squids/kg (small size), increased continuously with increasing storage time ($p<0.05$), indicating the formation of pink color on squid mantle (Figure 8a and 8b). The increase in *a**-value was more pronounced in the samples stored in ice with a squid/ice ratio of 1:1, compared with those kept in ice at a ratio of 1:2, regardless of size ($p<0.05$). Therefore, the sufficient amount of ice could retard the increase in *a**-value of the mantle from squid without prior deskinning during the storage in ice. Lapa-Guimaraes et al. (2002) reported that both *a** and *b**-values in the squid skin and muscle of *Loligo plei* tended to increase during the storage. No changes in *a**-value were observed in the mantle of deskinned squid throughout the storage, irrespective of squid/ice ratio and size ($p>0.05$). Skin containing a number of chromatophores was most likely a major source of red or pink pigments, which were able to stain the mantle, especially as the storage time increased. The mantles from the large squid without deskinning showed the higher *a**-value, compared with those of the small size ($p<0.05$). The squids with the large size has the large chromatophore with a larger number than those having small size. As a result, the staining of chromatophore to mantle of squid was more pronounced in the former.

For *b**-value representing the yellowness of mantle, no changes were found in all samples within the first 2 days of storage ($p>0.05$) (Figure 9a and b). Thereafter, all samples had the increases in *b**-value up to 16 days ($p<0.05$). The mantle from the large squid without deskinning showed the higher *b**-value, compared with those of the small size ($p<0.05$). Thanonkaew et al. (2006) reported that the formation of yellow pigment in squid muscle could be due to nonenzymatic

browning reaction occurring between aldehydic lipid oxidation products and the amines on phospholipids head groups. Squid muscle contained phospholipids as the major lipid (Thanonkaew et al., 2006). It was noted that the mantle of squid without deskinning exhibited the higher increases in *b**-value**,** compared with the deskinned counterpart ($p<0.05$). Squid/ice ratio had no marked effect on *b**-value of the mantle of squid without deskinning throughout the storage of 16 days, regardless of size. For squids with both sizes, the higher *b**-value was noticeable in the mantle of deskinned squid stored in ice at a ratio of 1:2, compared with those kept with the squid/ice ratio of 1:1 ($p<0.05$). During the storage, the samples were immersed in molten ice, especially when the lower amount of ice was used (squid/ice ratio of 1:1). As a result, the exposures of samples to atmospheric oxygen should be lowered with the subsequent retarded lipid oxidation. This might lead to the higher *b**-value of sample kept in ice at squid/ice ratio of 1:2.

1.2 Changes in nitrogenous compounds

TVB and TMA contents of small and large squids with and without deskinning stored in ice with squid/ice ratios of 1:1 and 1:2 during storage are shown in Figure 10a and 10b. The initial TVB contents of squids with large and small size were 20.10 and 18.88 mg-N/100 g muscle, respectively. Which was lower than those of other cephalopods obtained 24-36 h after capture (Lapa-Guimarãaes et al., 2005; Vaz-Pires et al., 2008). TVB content of squid mantle with both sizes decreased gradually within the first 8-10 days of storage, suggesting the losses in volatile base compounds formed by endogenous or microbial enzymatic systems. This was probably due to the leaching effect of molten ice. After day 10, TVB content increased continuously up to 16 days of storage ($p<0.05$). This suggested that volatile compounds produced by microorganisms could be accumulated in the mantle, though some compounds might be washed out by molten ice. TVB content of squid with the large size was higher than those with the small size, especially after 10 days storage, indicating the higher spoilage of the former associated with spoilage microorganisms with increasing storage time.

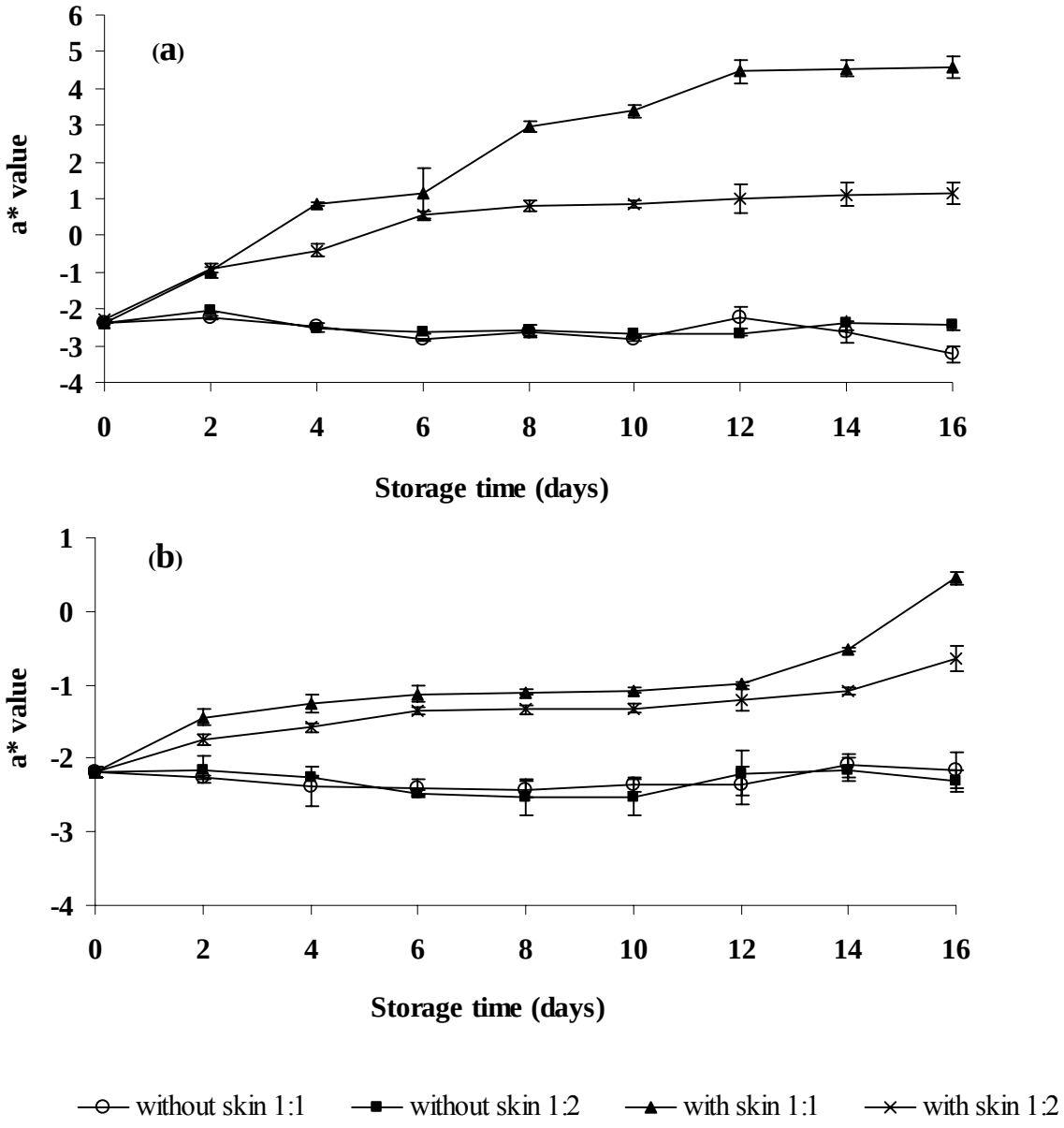


Figure 8. The *a**-value of the mantle of large (a) and small (b) squids with and without deskinning during iced storage using the squid/ice ratios of 1:1 and 1:2. Bars represent the standard deviation (n=3).

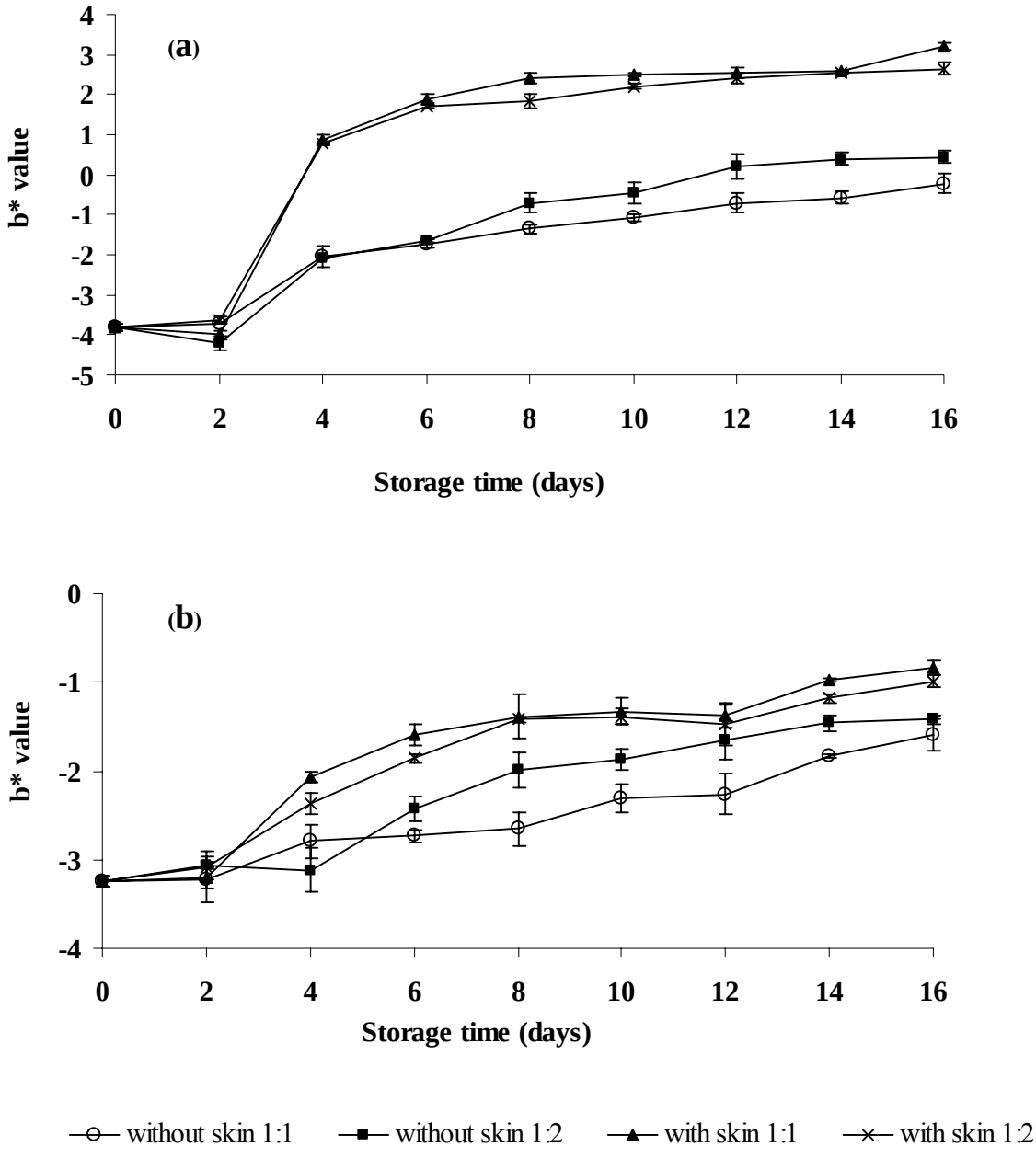


Figure 9. The *b**-value of the mantle of large (a) and small (b) squids with and without deskinning during iced storage using the squid/ice ratios of 1:1 and 1:2. Bars represent the standard deviation (n=3).

TVB has been considered useful as a spoilage indicator (Yamanaka et al., 1987; Ohashi et al., 1991). At the same time of storage, especially during 10-16 days, the samples kept in ice with a squid/ice ratio of 1:1 had the higher TVB content, compared with those kept in ice with squid/ice ratio of 1:2 ($p<0.05$). Rates of increases in TVB content were greater in the samples kept at 15^{o}C than those found in squid kept at 0^{o}C and 3.5^{o}C (Yamanaka et al., 1987). Ohashi et al. (1991) reported that the increases of TVB content were greater in squid kept at 10^{o}C than those found in squid stored at 0^{o}C and 5^{o}C. TVB include trimethylamine (TMA), dimethylamine (DMA), monomethylamine (MMA) and ammonia (Seibel and Walsh, 2002). At the same squid/ice ratio, the samples without deskinning had the higher TVB content than the deskinned counterpart ($p<0.05$). The skin could be a source of contaminated microorganisms. As a consequence, the microbial invasion into the mantle could be more pronounced in the samples without deskinning.

TMA content of squid with large size increased markedly during 4-6 days of storage ($p<0.05$) (Figure 11a). Thereafter, the slower rates of increases were obtained. It was noted that slower rates of increases were found in sample kept in ice with a squid/ice ratio of 1:2. After 16 days of storage, TMA contents of 8.30-11.23 and 6.51-7.43 mg-N/100 g sample were found in the samples kept in ice with the squid/ice ratio of 1:1 and 1:2, respectively. Squid with the small size had an increase in TMA content within the first 2 days. No changes in TMA were found during 2-10 days of storage. Subsequent increases in TMA content were found after 10 days of storage ($p<0.05$) (Figure 11b). In general, the results of TMA changes in squids were similar to those of TVB changes throughout the storage. TMA is produced by the reduction of trimethylamine oxide (TMAO) by TMAO reductase-producing organisms (Adam and Moss, 2000). TMA content is an indicator of bacterial deterioration in marine products (Seibel and Walsh, 2002). TMA has been found to be related with a loss of freshness of marine fishery products (Alur et al, 1995; Gram and Huss, 1996). The higher TVB and TMA contents were observed in squids kept in ice with a squid/ice ratio of 1:1, compared with those stored in ice with a squid/ice ratio of 1:2, regardless of size. Furthermore, deskinning tended to lower the rate of

increase in both TVB and TMA contents of squid mantle with both sizes during the extended storage.

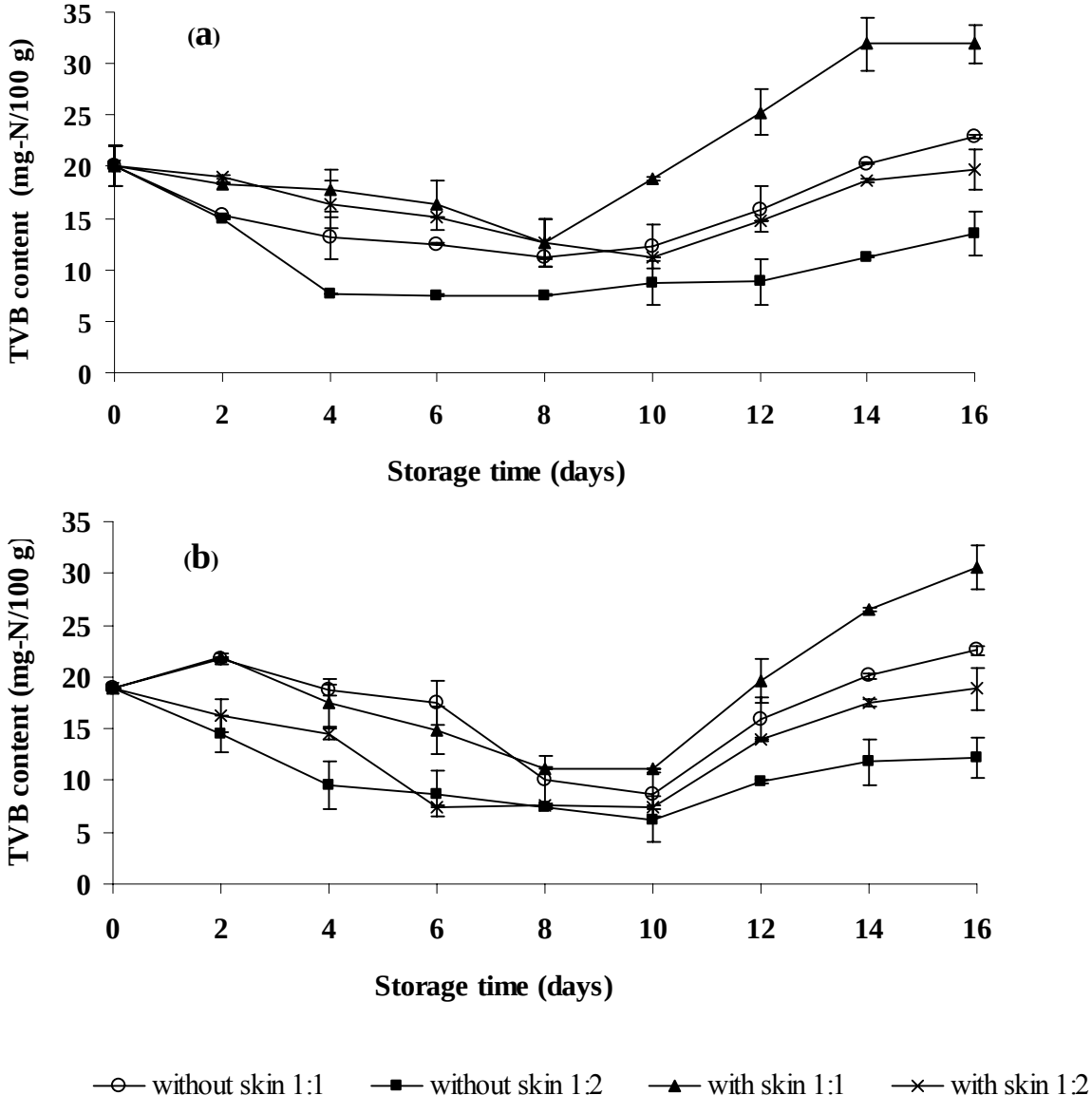


Figure 10. TVB content of large (a) and small (b) squids with and without deskinning during iced storage using the squid/ice ratios of 1:1 and 1:2. Bars represent the standard deviation (n=3).

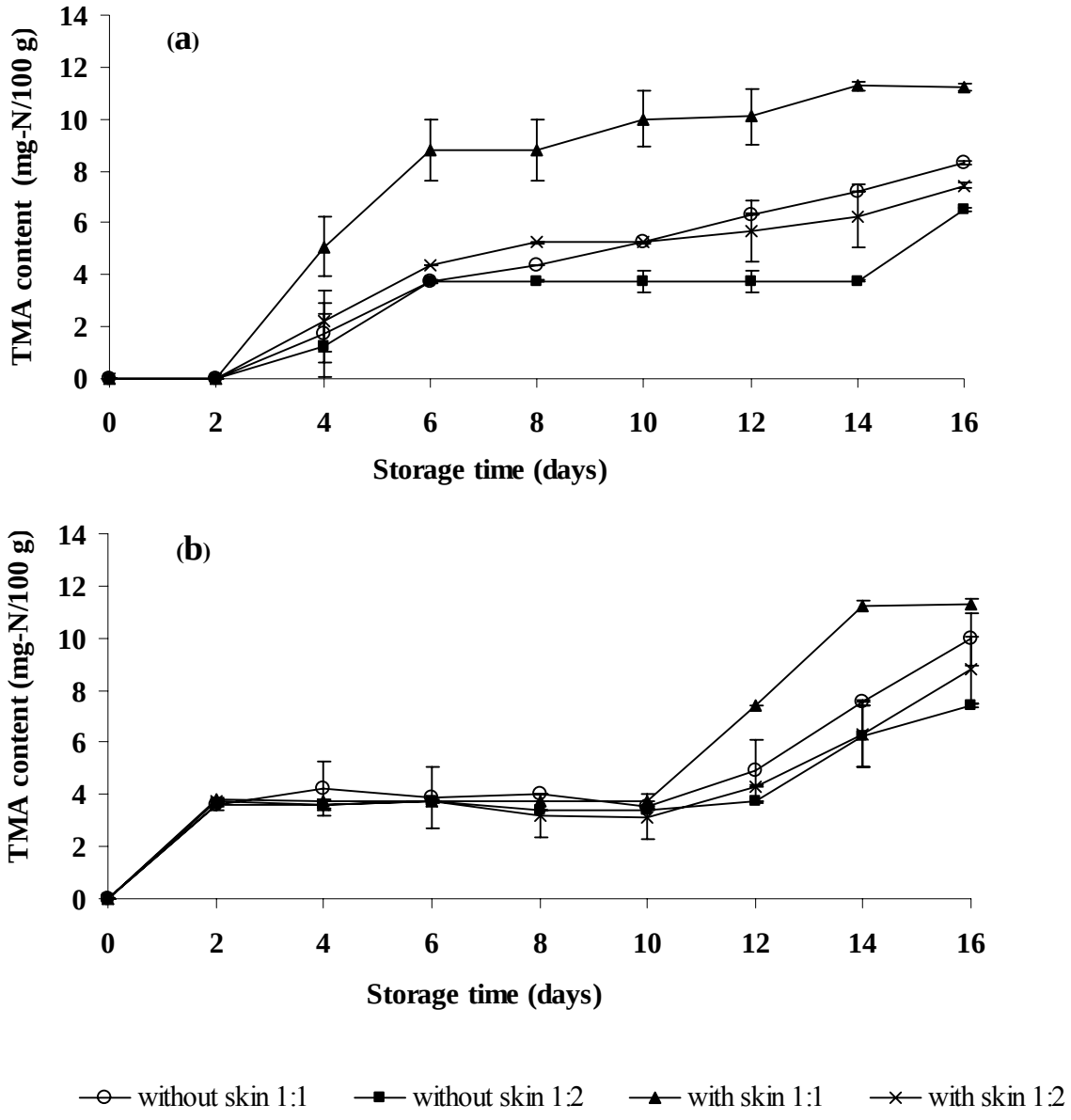


Figure 11. TMA content of large (a) and small (b) squids with and without deskinning during iced storage using the squid/ice ratios of 1:1 and 1:2. Bars represent the standard deviation (n=3).

Ammonia content of large and small squids with and without deskinning during iced storage with different squid/ice ratios are depicted in Figure 12a and 12b. No marked changes in ammonia content were found in the squid with large size kept in ice with a squid/ice ratio of 1:2 within the first 10 days of storages ($p>0.05$) (Figure 12a). The increases were noticeable during 12-16 days of storage ($p<0.05$). For the sample kept in ice with a squid/ice ratio of 1:1, the marked increases were found after 2 days of storage and remained constant up to day 12-14. The increases were also found at day 16 of storage ($p<0.05$). Squid with the small size had the increase in ammonia content within the first 4 days ($p<0.05$). The decreases were obtained during 4-8 days of storage ($p<0.05$). This was probably due to the loss caused by leaching effect. Thereafter, the increases were observed during 10-16 days of storage (Figure 12b). The rapid onset of ammonia production in squid at low bacterial cell densities indicates the role of autolysis, while the rapid ammonia production at high bacterial cell densities indicates a bacterial contribution (Paarup et al., 2002). Ammonia is derived from enzymatic deamination of free amino acids or from decomposition of nucleic bases (Huang et al, 1992). Ammonia content of squid kept in ice with a squid/ice ratio of 1:1 was higher than that found in squid stored in ice with a squid/ice ratio of 1:2, regardless of size ($p<0.05$), indicating that the former underwent the decomposition at a higher rate than the latter. Deskinning was also found to lower the rate of increase in ammonia of squid with both sizes during iced storage. Squids with the large size had the higher ammonia content, compared with those of the small size ($p<0.05$). It was noted that the decomposition of large squid was more intense than those with the small size.

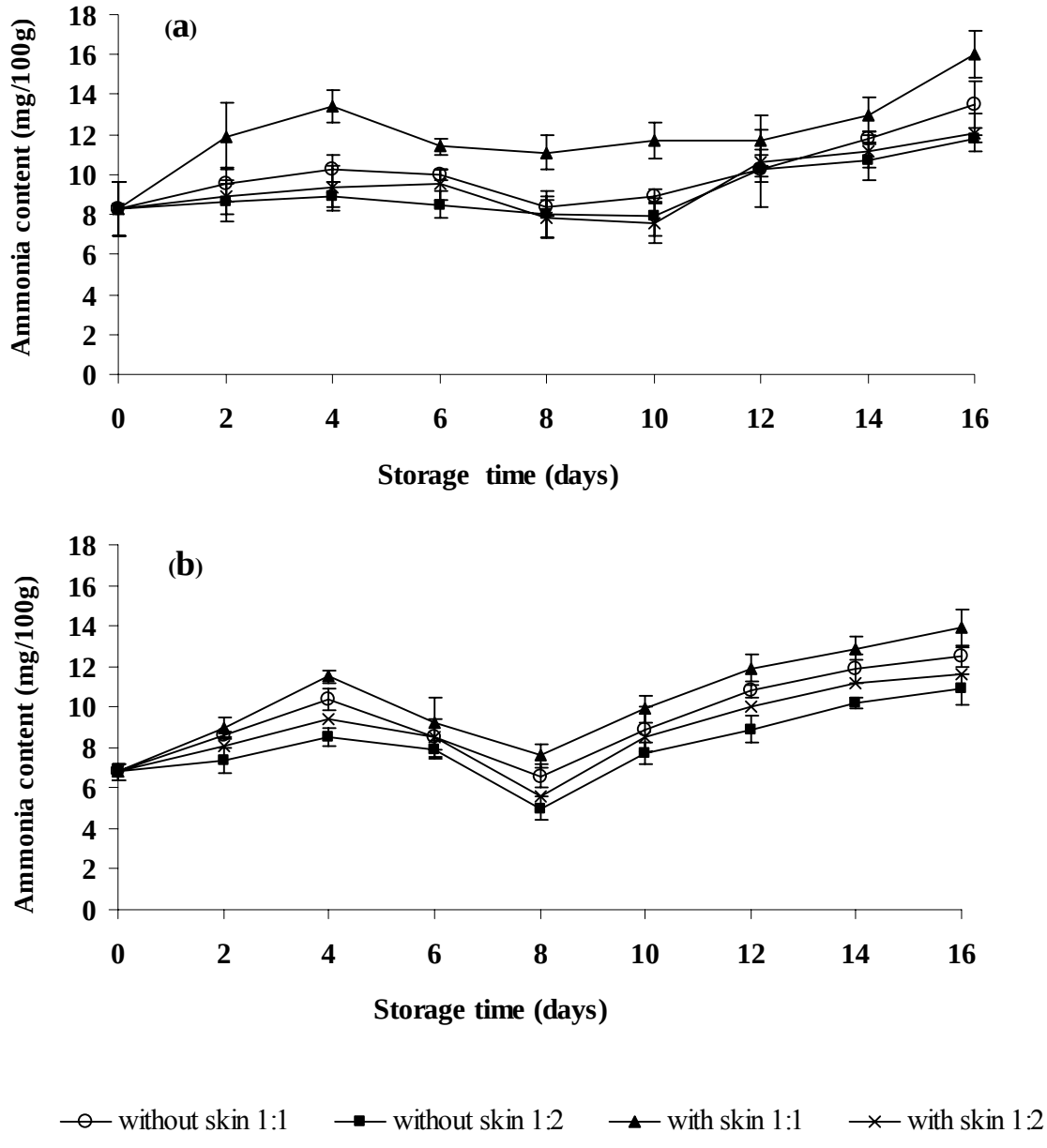


Figure 12. Ammonia content of large (a) and small (b) squids with and without deskinning during iced storage using the squid/ice ratios of 1:1 and 1:2. Bars represent the standard deviation (n=3).

1.3 Changes in pH

pH of large and small squids with and without deskinning stored in ice at different squid/ice ratios are shown in Figure 13a and 13b. The initial pH of squids with large and small size were 6.58 and 6.40, respectively. Marquiz-Rios et al. (2007) reported that an initial pH of *Dosidicus gigas* was 6.6, while Ohashi et al. (1991) reported that an initial pH of *Todarodes pacificus* was 6.0. For the large squid, pH of squid increased slightly during the storage ($p<0.05$). pH of squid with a small size increased continuously within the first 8 days. Thereafter, no marked changes were observed up to 14 days ($p>0.05$), followed by the increase at day 16 of storage ($p<0.05$). The rates of increases were greater in both samples with a squid/ice ratio of 1:1 than those found in samples kept in ice with a squid/ice ratio of 1:2. The increase in pH is generally associated with the formation of volatile bases (Ohashi et al.,1991). Yamanaka et al. (1987) reported that the rapid increase in pH in common squid (*Todarodes pacificus*) was noticeable when stored at 15^{o}C, compared with at 0^{o}C and 3.5^{o}C.

1.4 Changes in psychrophilic bacteria count (PBC)

PBC of large and small squids with and without deskinning increased with increasing storage time ($p<0.05$) (Figure 14a and 14b). No marked differences in PBC were found between squid with and without deskinning during the storage of 16 days when the same squid/ice ratio was used, regardless of size. For squids stored with the squid/ice ratio of 1:1, the higher PBC was noticeable in sample without deskinning, especially when the storage time was greater than 8 days ($p<0.05$). For the large squid, PBC increased from 2.9 x 10^2 to 1.5-5.6 x 10^6 cfu/g after 16 days of storage (Figure 14a). PBC of squid with a small size increased from 2.7 x 10^2 to 1.1-3.7 x 10^6 cfu/g after 16 days of storage (Figure 14b). Lapa-Guimarãaes et al. (2005) found that psychrotrophic counts in squids (*Loligo plei*) increased from 8 x 10^2 to 5 x 10^6 cfu/g after 12 days of iced storage. Generally, PBC in squid stored in ice with a squid/ice ratio of 1:1 (0-8^{o}C)was higher than that of squid stored in ice with a squid/ice ratio of 1:2 (0^{o}C) throughout the storage, regardless of size ($p<0.05$).

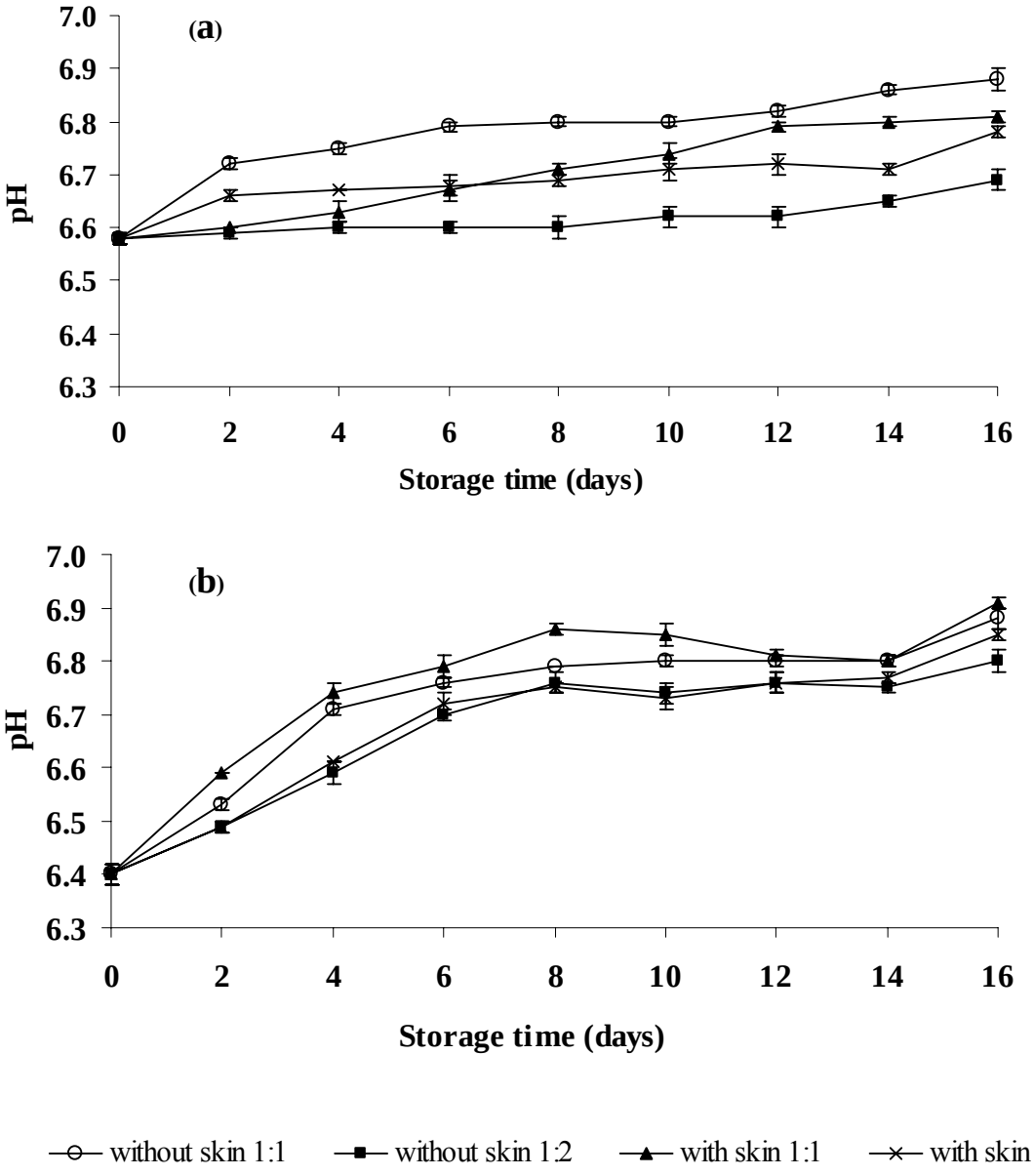


Figure 13. pH of large (a) and small (b) squids with and without deskinning during iced storage using the squid/ice ratios of 1:1 and 1:2. Bars represent the standard deviation (n=3).

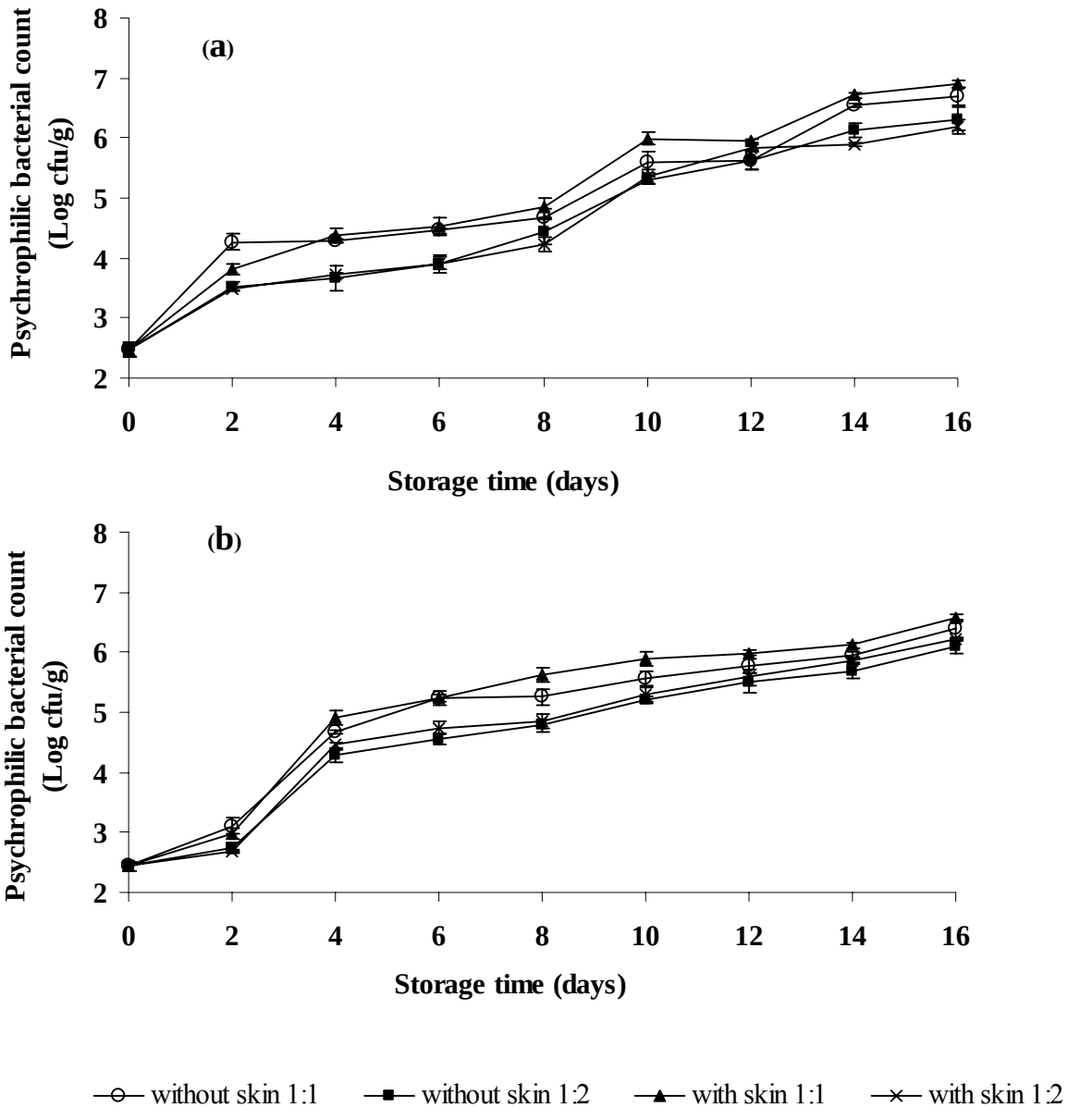


Figure 14. Psychrophilic bacterial count of large (a) and small (b) squids with and without deskinning during iced storage using the squid/ice ratios of 1:1 and 1:2. Bars represent the standard deviation (n=3).

The result indicated that a squid/ice ratio of 1:1 had the lower efficiency in retardation of the growth of psychrophilic bacteria, compared with the ratio of 1:2. Ohashi et al. (1991) reported that the increases in psychrophiles in common squid stored at 10^{o}C were higher than of squid kept at 0^{o}C and 5^{o}C. The increases in PBC were in agreement with the increases in TVB, TMA and ammonia contents (Figures 10, 11 and 12)

1.5 Changes in TBARS

TBARS value of large and small squids with and without deskining increased continuously during iced storage up to 16 days (Figure 15a and 15b). For squids with the large size, TBARS value increased from 0.30 to 0.79-1.14 mg/kg after 16 days of storage (Figure 15a). TBARS value of squid with a small size increased from 0.04 to 0.59-0.89 mg/kg after 16 days of storage (Figure 15b). No marked differences in TBARS value were found between squid with and without deskinning during the storage of 16 days when the same squid/ice ratio was used, regardless of size. Squid with a squid/ice ratio of 1:1 tended to have a higher TBARS than those with a squid/ice ratio of 1:2 during the storage, irrespective of size ($p<0.05$). The higher temperature of samples with squid/ice ratio 1:1 (0-8^{o}C) probably induced lipid oxidation in squid muscle. The rate of oxidation increased with increasing temperature (Jadhav et al., 1996). The lipid in cephalopod muscle is found mostly in the cellular membranes. The amount of phospholipids in cephalopods ranges from 61.8 to 84.0% of the total lipids (Southgate and Lou, 1995). Phospholipids contain unsaturated fatty acid that can be oxidized and decomposition to aldehydes (Hidalgo et al., 2004). The increases in TBARS was in accordance with the increase in *b**-value (Figure 9). The secondary product, particularly aldehydes might undergo Maillard reaction by reacting with amino group in squid muscle during the extended iced storage.

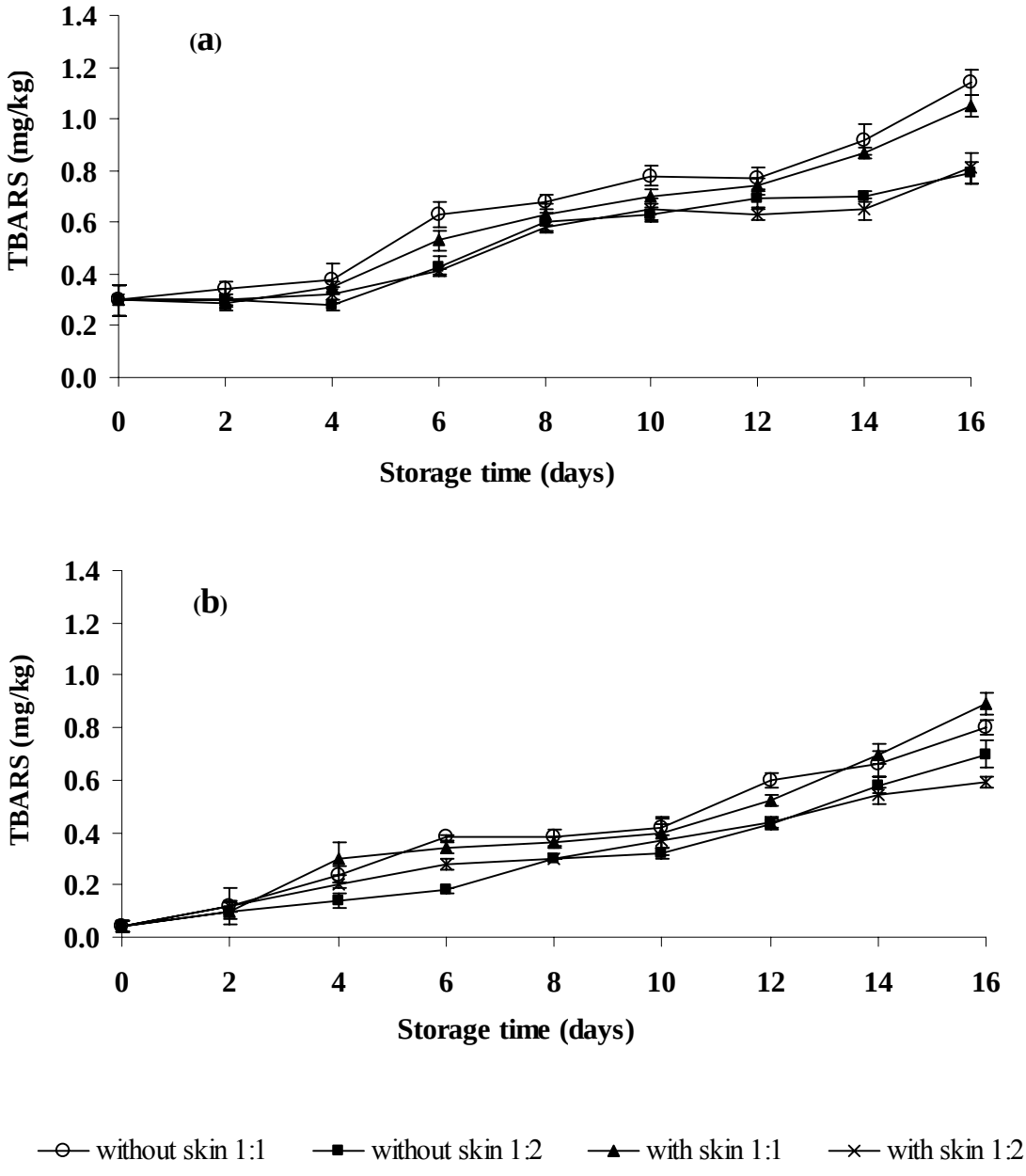


Figure 15. TBARS of large (a) and small (b) squids with and without deskinning during iced storage using the squid/ice ratios of 1:1 and 1:2. Bars represent the standard deviation (n=3).

1.6 Changes in formaldehyde content

Formaldehyde content of squid with both sizes increased with increasing storage time (Figure 16a and 16b). No differences in formaldehyde between all samples were noticeable during storage ($p>0.05$). No differences in formaldehyde content between squids with the large and small size were observed during the storage ($p>0.05$). Squid/ice ratio also had no pronounced effect on formaldehyde content. Formaldehyde is an enzymatic product from trimethylamine oxide. It can be formed in fish muscle during frozen storage, causing protein denaturation (Ang and Hultin, 1989). The formation of formaldehyde in seabass slice kept at 4^{o}C had led to the changes in texture of fish muscle (Masniyom et al., 2005). Trimethylamine oxide in the muscle of squid (*Illex argentinus*) reduced by chemical reactions to dimethylamine and formaldehyde (Kolodziejska et al., 1994). Li et al. (2007) found that four species of squid had variable formaldehyde levels, which was generally higher in viscera than in muscle of squid. Stanley and Hultin (1982) report that formaldehyde in squid muscle might cause protein cross-linking, leading to toughening of frozen squid.

2. Effect of size and squid/ice ratio on disruption and distribution of chromatophores in squid skin during iced storage

The morphologies of chromatophore in squid skin during iced storage with the squid/ice ratios of 1:1 and 1:2 are illustrated in Figure 17. The disruption of chromatophores in squid skin with both sizes increased with increasing storage time in ice. The skin of large squid had the larger chromatophore with a larger number than those with small size. The increased disruption of chromatophores was related with the increase in a^*-value of the mantle of squid without deskinning during iced storage (Figure 8). During the extended storage, the pigments in the chromatophore could be released and stained with the mantle, causing the pink color formation. Lakshmanan et al. (1993) used a red-brown color of chromatophores in the skin for excellent/very good or good to fair squid and moderate to intense pink spots for unacceptable squid for the sensory evaluation of squid skin color. The disruption of chromatophores in the skin was more pronounced in the squid stored in ice with a

squid/ice ratio of 1:1, compared with that using the ratio of 1:2. This was probably associated with the higher decomposition of squid skin as well as chromatophore membrane in the former. Those changes might be related with the higher PBC in the squid stored in ice with a squid/ice ratio of 1:1. Those microorganisms might produce the enzymes capable of hydrolyzing chromatophore membrane and skin, leading to the release of pigments through the soften skin to the mantle, where the staining took place.

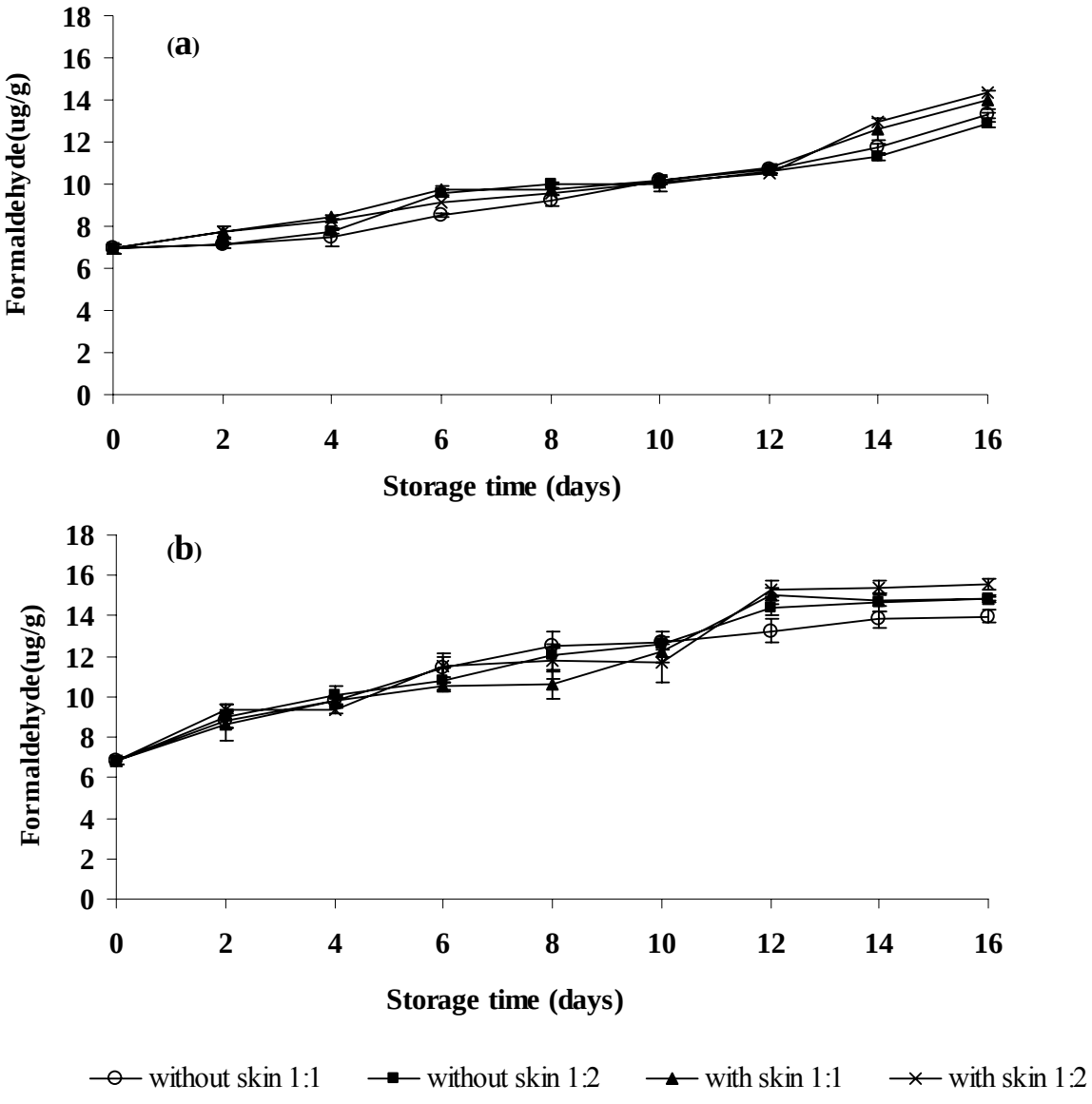


Figure 16. Formaldehyde content of large (a) and small (b) squids with and without deskinning during iced storage using the squid/ice ratios of 1:1 and 1:2. Bars represent the standard deviation (n=3).

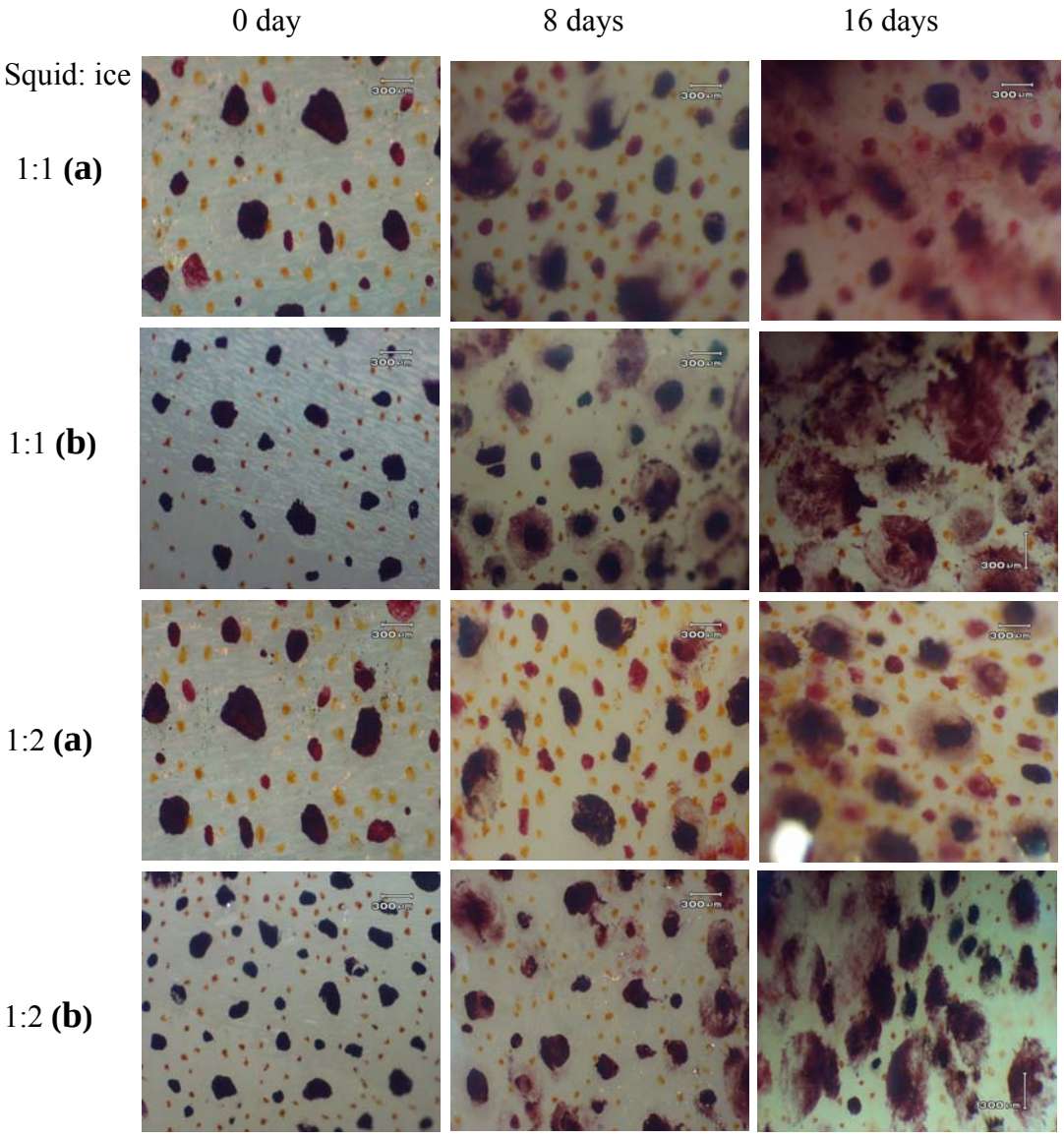


Figure 17. The distribution and disruption of chromatophores on the mantle of large (a) and small (b) squids stored in ice with squid/ice ratios of 1:1 and 1:2. Magnification : 30X.

3. Role of microorganisms in pink discoloration and quality changes of squid stored in ice

3.1 Changes in color

Large squid without deskinning was treated with NaN_3 at different levels as antimicrobial agent to retard the growth of microorganisms in squid during iced storage. During the storage, the *a** and *b**-values of squid treated with 0.01 or 0.1% NaN_3 and stored in ice with a squid/ice ratio of 1:1 increased up to 8 days of storage. Nevertheless, the rates of increases were lower than that found in the control (without NaN_3 treatment) ($p<0.05$) (Figure 18). After 10 days, a slight increase in *a**-value was found in the sample treated with 0.01% NaN_3 ($p<0.05$), while no changes in *a**-value were noticeable in those treated with 0.1% NaN_3 ($p>0.05$). The *a**-value of control squid (without treatment) increased sharply from day 10 to day 16 of storage ($p<0.05$). For *b**-value, the control samples tended to have the higher increase in *b**-value, followed by those treated with 0.01 and 0.1% NaN_3, respectively. Therefore, NaN_3 treatment of squid without deskinning, especially at higher level (0.1%) prior to the iced storage could retard the pink discoloration effectively. Additionally, the yellowish development of squid mantle was also retarded to some degrees with NaN_3 treatment.

Pink discoloration of mantle was more pronounced in squid without deskinning stored in ice with squid/ice ratio of 1:1, when the storage time increased (Figure 19). However, the pink discoloration was much lowered when NaN_3 was used to treat the squid prior to iced storage. Therefore, the growth of microorganisms was most likely associated with the pink discoloration. The retardation of the growth of psychrophilic bacteria using the safe antimicrobials or keeping the squid at low temperature, could lower pink discoloration of whole squid without prior deskinning. Microorganisms might involve in the enhancement of chromatophore disruption by weakening the chromatophore membrane. Also microorganisms could degrade the connective tissue of the skin, in which the pigments from the disrupted chromatophores could be released and stained the mantle to a higher extent as evidenced by the increased pink coloration.

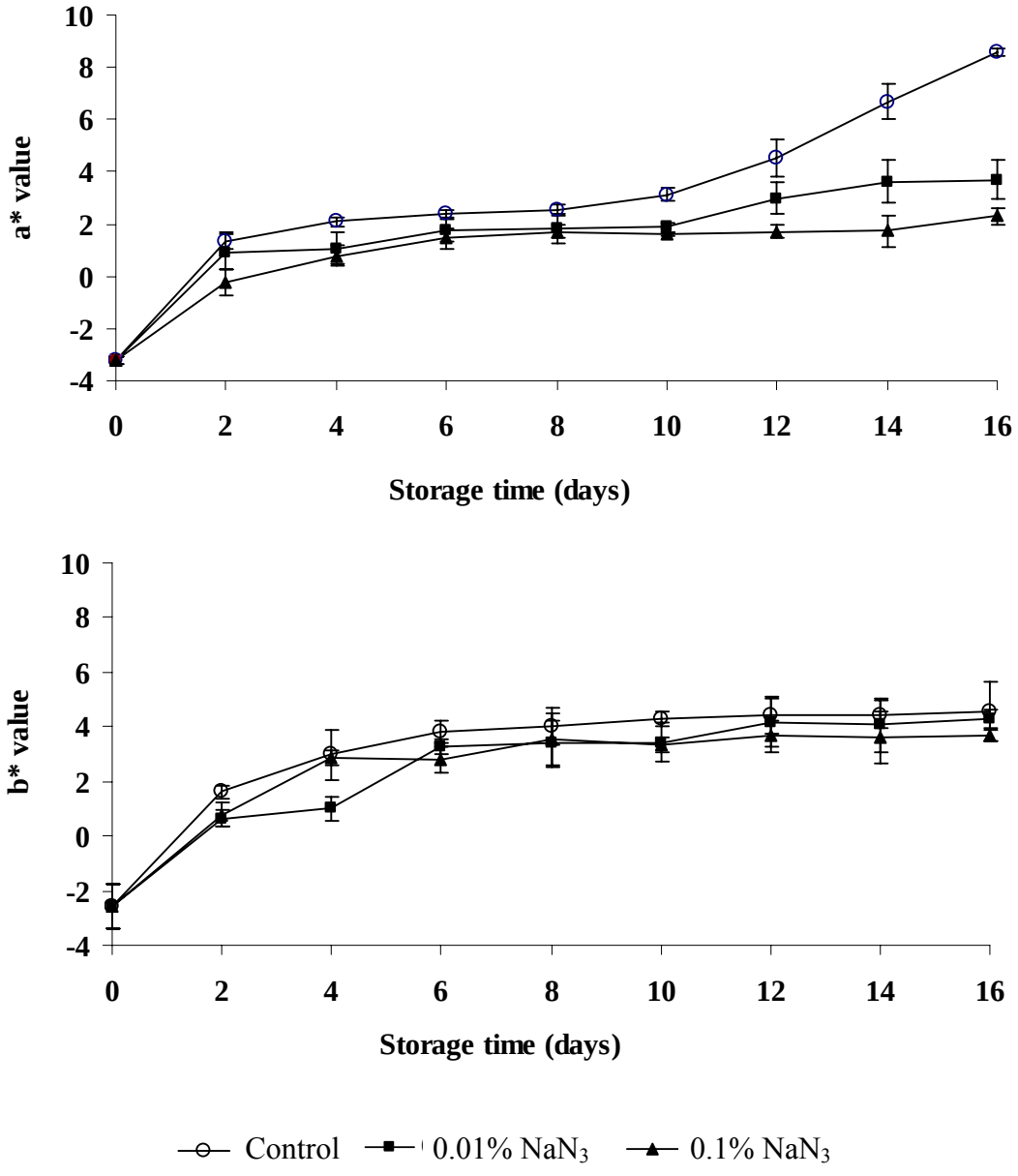


Figure 18. The *a** and *b**-values of the mantle of large squids without deskinning treated without and with 0.01 or 0.1% NaN_3 during iced storage using a squid/ice ratio of 1:1. Bars represent the standard deviation (n=3).

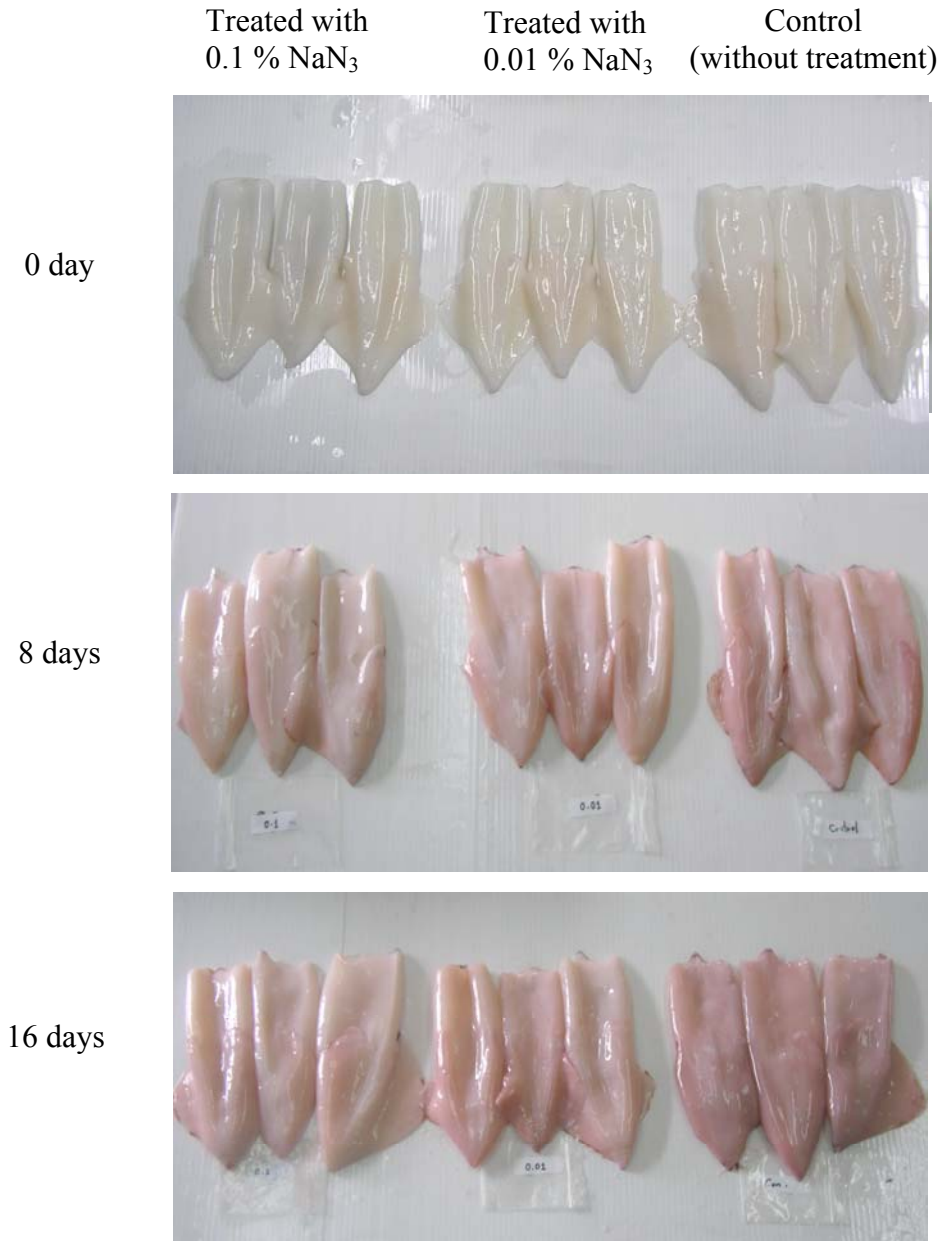


Figure 19. Pink discoloration of the mantle of large squids without deskinning treated without and with 0.01 or 0.1% NaN_3 at different storage times.

3.2 Changes in nitrogenous compounds

TVB and TMA contents of squid treated without and with NaN_3 solution during iced storage are depicted in Figure 20. TVB content of the control squid increased slightly up to 10 days of storage ($p<0.05$). After 10 days of storage, the sharp increases in TVB content were noticeable up to 16 days ($p<0.05$). These results were similar to those found for other squid species, in which the slow increases in TVB content were observed at the early storage (Ohashi et al., 1991; Lapa-Guimarães et al., 2005; Vaz-Pires et al., 2008). The increases in TVB contents of squid were lowered after being treated with NaN_3, particularly with the higher concentration. After 16 days of storage, the highest TVB content was observed in the control squid (94.30 mg-N/100 g), followed by squid treated with 0.01% NaN_3 (35.57 mg-N/100 g) and with 0.1% NaN_3 (29.06 mg-N/100 g), respectively. TMA content of the control squid was negligible within the first 2 days, followed by slight increase up to 8 days. Thereafter a sharp increase in TMA content was obtained ($p<0.05$). TMA content of squid treated with 0.01% NaN_3 was 3.80 mg-N/100 g muscle during 8-16 days of storage. TMA content of squid treated with 0.1% NaN_3 was slightly lower than those found in squid treated with 0.01% NaN_3. In general, the increases in TMA content were in accordance with the increase in TVB content. The result indicated that NaN_3, especially at 0.1%, could retard the spoilage of squid stored in ice as evidenced by the lowering of the TVB and TMA formation. This confirmed that microbial growth involved in part with the pink color development of whole squid during the extended storage in ice.

3.3 Changes in psychrophilic bacteria count (PBC)

During ice storage, the continuous increase in PBC was found in the control squid up to 14 days ($p<0.05$) (Figure 21). No further increase was noticeable at day 16 of storage ($p>0.05$). For the sample treated with NaN_3 at level of 0.01 and 0.1%, no changes in PBC were found during the first 8 days of storage ($p>0.05$). Thereafter, the marked increases in PBC were observed during 8-12 days of squid treated with 0.01% NaN_3, followed by the constant PBC up to 16 days. For those treated with 0.1% NaN_3, only slight increase in PBC was found up to 16 days of storage. After 16 days of storage, the highest PBC was observed in control squid 1.65 x 10^7 cfu/g), followed by squid treated with 0.01% NaN_3 (7.33 x 10^3 cfu/g) and with

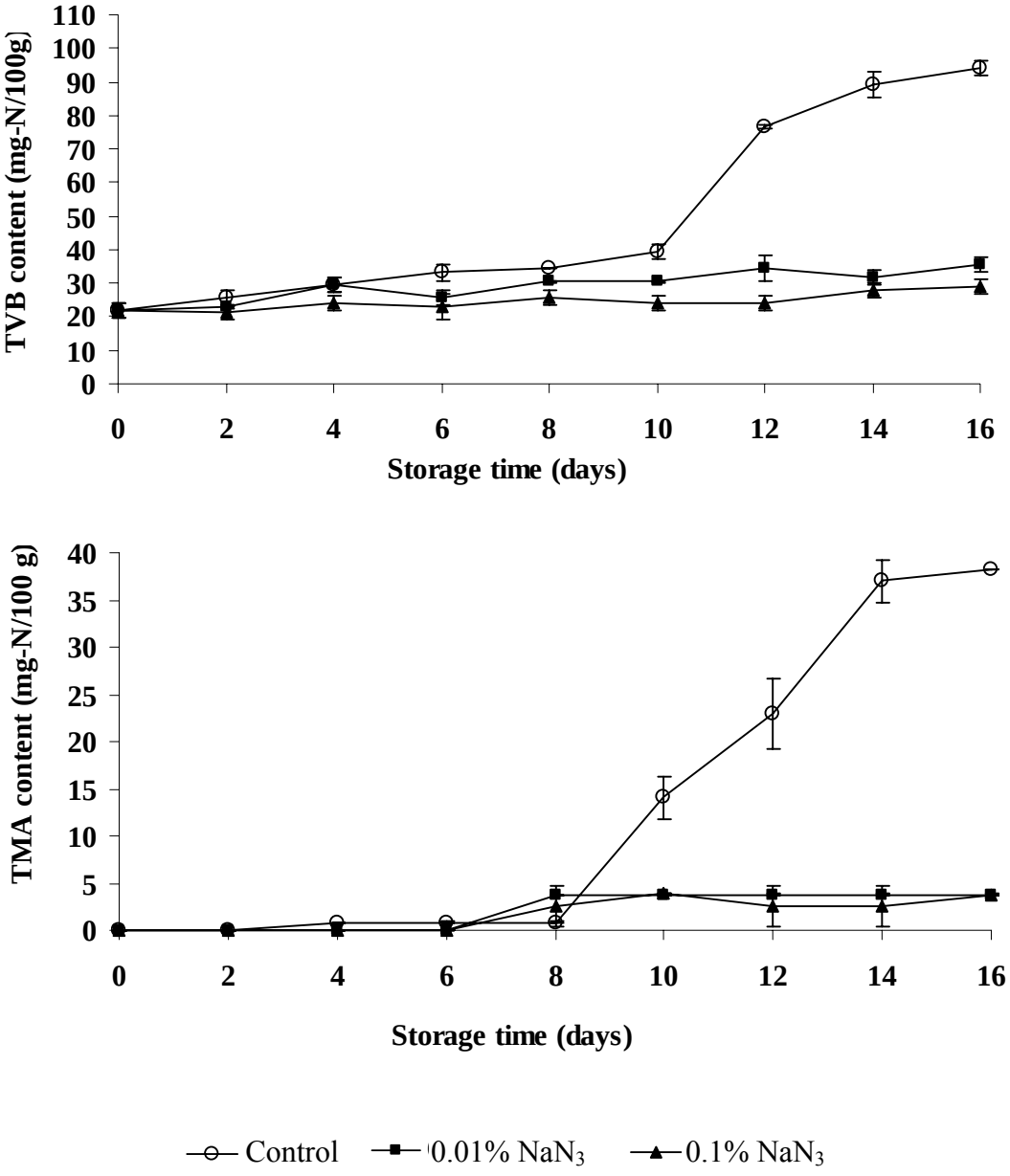


Figure 20. TVB and TMA contents of large squids without deskinning treated without and with 0.01 or 0.1% NaN_3 during iced storage using a squid/ice ratio 1:1. Bars represent the standard deviation (n=3).

0.1% NaN_3 (5.67 x 10^1 cfu/g), respectively. The result revealed that NaN_3 solution had an inhibition effect on the growth of psychrophilic bacteria. Lichstein and Soule (1944a) reported that NaN_3 at the concentration of 0.01-0.03% exhibited the growth of gram-negative and gram-positive spore-forming aerobic bacteria. The result indicated that the retarded growth of PBC was associated with the lowered pink discoloration of squid without deskinning during the iced storage. NaN_3 was added to the test solution either to prevent microbial growth or to inhibit existing microbial activity (Goel et al., 2003). Furthermore, NaN_3 exhibited the inhibitory activity toward the catalase activity of bacteria (Lichstein and Soule, 1944b). Similar increase in a^*-value of all samples was noticeable within the first 8 days of storages though PBC varied. Thus, microbial load partially contributed to pink discoloration in the initial period of storage. Nevertheless, microbial growth had the pronounced effect on the increase in redness of squid mantle after 8 days of storage. The result indicated that the retarded growth of PBC was associated with the lowered pink discoloration of squid without deskinning during the iced storage.

3.4 Changes in chromatophores

The disruption of chromatophores in the skin of squid treated without and with 0.01 or 0.1% NaN_3 are illustrated in Figure 22. After storage in ice for 8 or 16 days, the disruption of chromatophores in squid skin was most pronounced in the control squid, followed by squid treated with 0.01 and 0.1% NaN_3, respectively. The disruption was generally enhanced as the storage time increased. The disruption of chromatophores was related with pink discoloration, in which squid treated with 0.01 or 0.1% NaN_3 had the lower a^*-value during iced storage (Figure 18 and 19). The growth of microorganisms mainly contributed to the weaking or disruption of chromatophores in the skin causing the leakage and staining of pigment into the mantle.

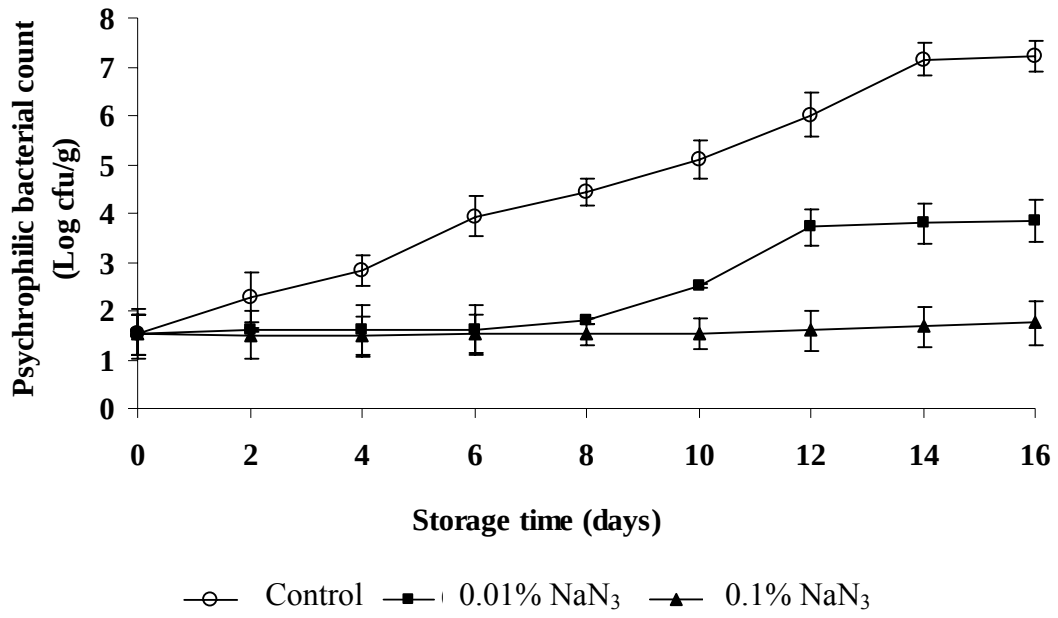


Figure 21. Psychrophilic bacterial count of large squids without deskinning treated without and with 0.01 or 0.1% NaN_3 during iced storage using a squid/ice ratio of 1:1.

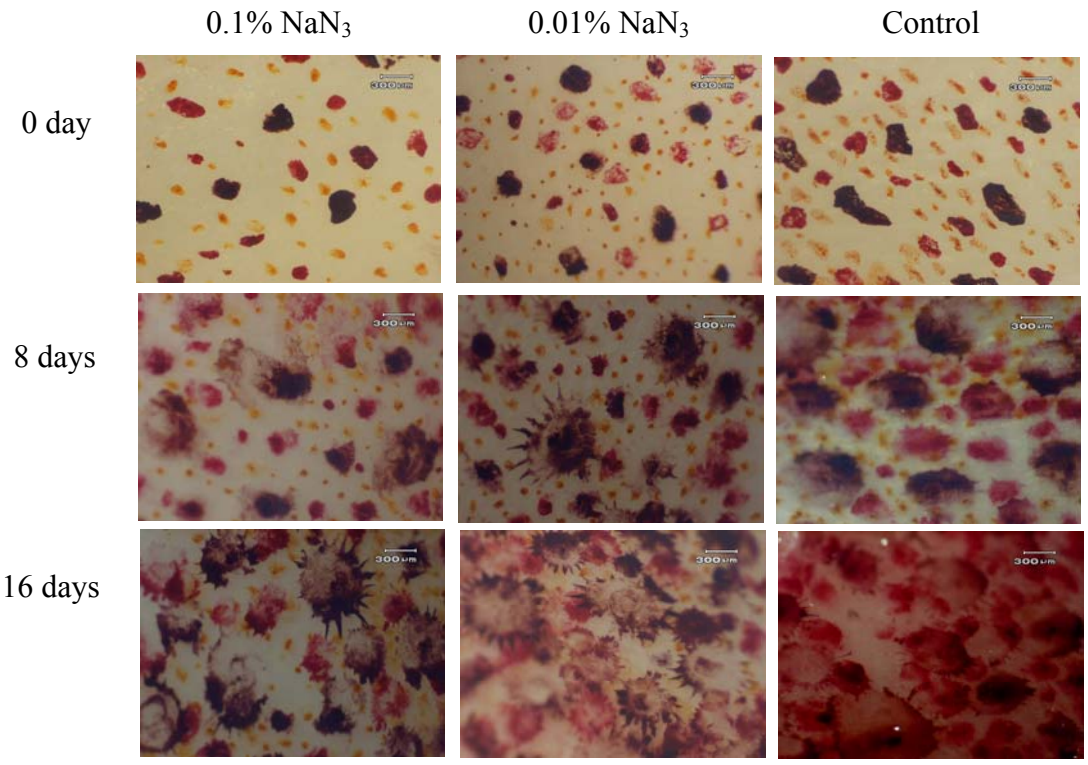


Figure 22. Morphology of chromatophores in the skin of large squids treated without and with 0.01 or 0.1% NaN_3 at different storage times. Magnification : 30X.

4. Effect of stacking on pink discoloration and quality changes of squid during iced storage

4.1 Changes in color

Squids without deskinning treated without and with 0.1% NaN_3 were stored in ice with a squid/ice ratio of 1:2 and different standard weights (1X SW and 2X SW) were placed on the top. During the storage, the *a**-value of all squid samples increased up to 10 days of storage in ice (Figure 23). After 10 days, no changes in *a**-value were found in the sample stacked with 2X SW ($p>0.05$), while the slight increases in *a**-value were found in those stacked with 1X SW and without stacking. The *b**-value of squid treated with 0.1% NaN_3 stacked with 1X SW and without stacking increased up to 14 days of storage (Figure 16). For squid treated with 0.1% NaN_3 stacked with 2X SW, the *b**-value increased up to 8 days of storage. After 8 days, a slight increase in *b**-value was found up to 14 days of storage ($p<0.05$). However, the rates of increase in *a** and *b**-values were lower than that found in squid without 0.1% NaN_3 treatment ($p<0.05$). In general, *a** and *b**-values of squids without 0.1% NaN_3 treatment stacked with 1X SW or 2X SW increased continuously up to 8 days of storage ($p<0.05$). After 8 days, the slight increases in *a** and *b**-values were found in the sample stacked with 1X SW ($p<0.05$), while no changes in *a** and *b**-values were found in those stacked with 2X SW ($p>0.05$). From the result, *a** and *b**-values of squids stacked with 2X SW showed the highest increase within the first 8-10 days of storage, followed by those stacked with 1X SW and the control, respectively, regardless of NaN_3 treatment ($p<0.05$). The result suggested that the compression force of standard weight most likely caused the disruption of chromatophores in the skin, causing the leakage and staining of pigment into the mantle of squid. The higher standard weight (2X SW) resulted in the disruption of chromatophores more effectively than the lower standard weight (1X SW). After 8-10 days, the *a** and *b**-values of sample stacked with 1X SW showed the highest increase up to 14 days of storage, followed by those stacked with 2X SW and the control, respectively, regardless of 0.1% NaN_3 treatment ($p<0.05$). This was probably because the stacking with a higher weight (2X SW) might break down

chromatophores, in which pigments might be leached out with molten ice at the early stage of iced storage. With the extended storage time, 2X SW did not have pronounced effect on staining. Therefore, lowering the stacking could retard the pink discoloration and yellowish development of squid mantle. Additionally, the retardation of microbial growth was reconfirmed for lowering pink discoloration.

4.2 Changes in nitrogenous compounds

TVB and TMA contents of squid without and with 0.1% NaN_3 treatment and stacked without and with 1X SW or 2X SW are shown in Figure 24. The initial TVB content of squid without and with 0.1% NaN_3 treatment was 15.29 mg-N/100 g sample. TVB content of squid without 0.1% NaN_3 treatment slightly increased up to 4 days of storage. After 4 days, the increases in TVB content were found up to 14 days of storage, regardless of standard weight used ($p<0.05$). No changes in TVB content of squid treated with 0.1% NaN_3 were found up to 10 days of storage ($p>0.05$). After 14 days of storage, TVB contents of 31.03-31.77 and 18.79-19.67 mg-N/100 g sample were found in the sample without and with 0.1% NaN_3 treatment, respectively. The slight increases in TVB content of squid treated with 0.1% NaN_3 were found, regardless of stacking. TMA content of squid without 0.1% NaN_3 treatment continuously increased after day 2 up to day 12 of storage. At day 14, the slower rates of increases were obtained in the sample without and with stacking ($p<0.05$). For squid with 0.1% NaN_3 treatment, TMA content of all samples increased after day 6 of storage ($p<0.05$). Squid without 0.1% NaN_3 treatment showed the higher TVB and TMA contents, compared with those with 0.1% NaN_3 treatment ($p<0.05$). The result indicated that NaN_3 could inhibit the growth of microorganisms in squid during iced storage. No differences in TVB and TMA contents were noticeable between squid without and with stacking during iced storage. Therefore, the stacking had no effect on TVB and TMA contents of squids during iced storage.

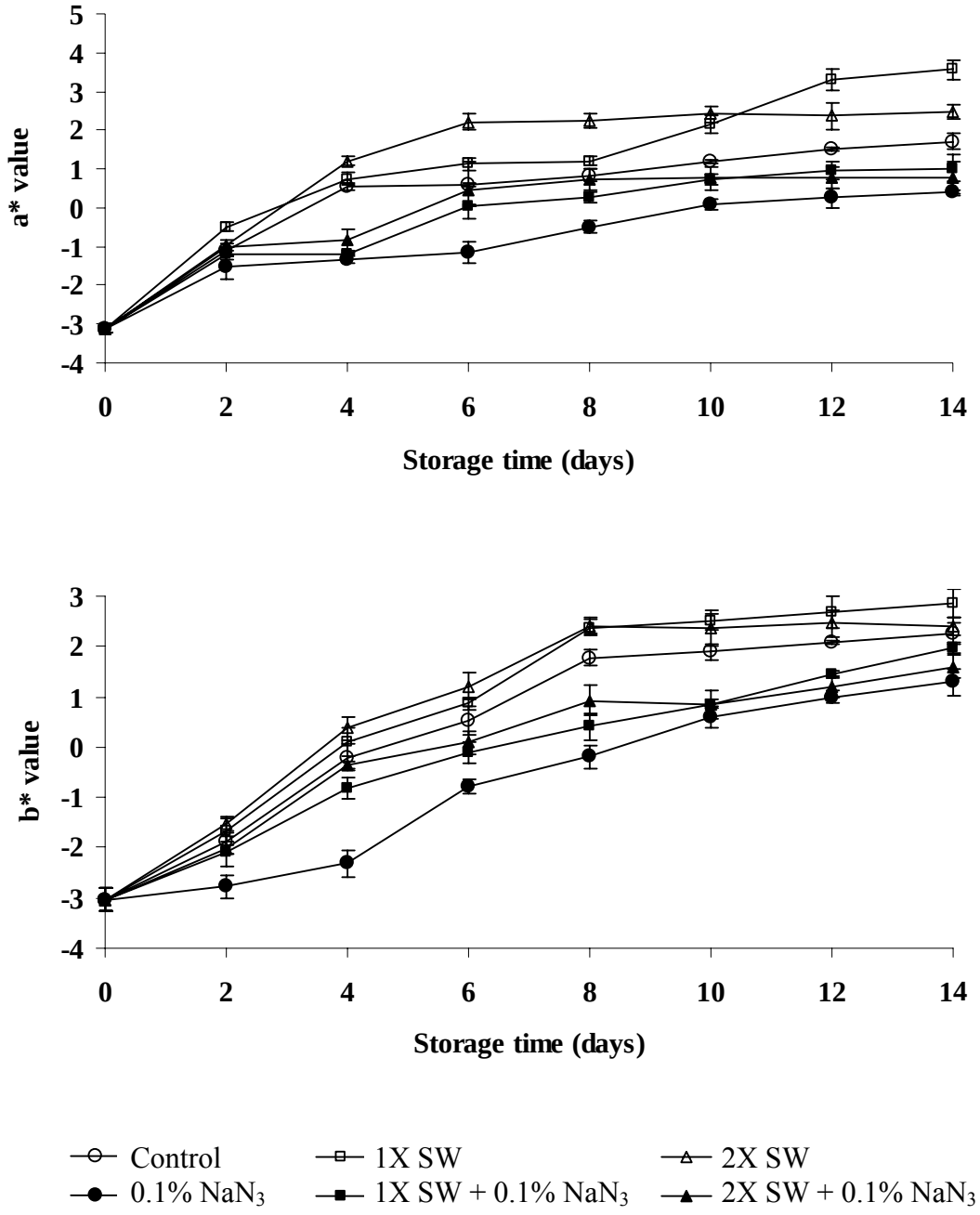


Figure 23. The *a** and *b**-values of the mantle of squids without and with 0.1% NaN_3 treatment stacked without and with standard weights (1X SW or 2X SW) during iced storage using a squid/ice ratio of 1:2. Bars represent the standard deviation (n=3).

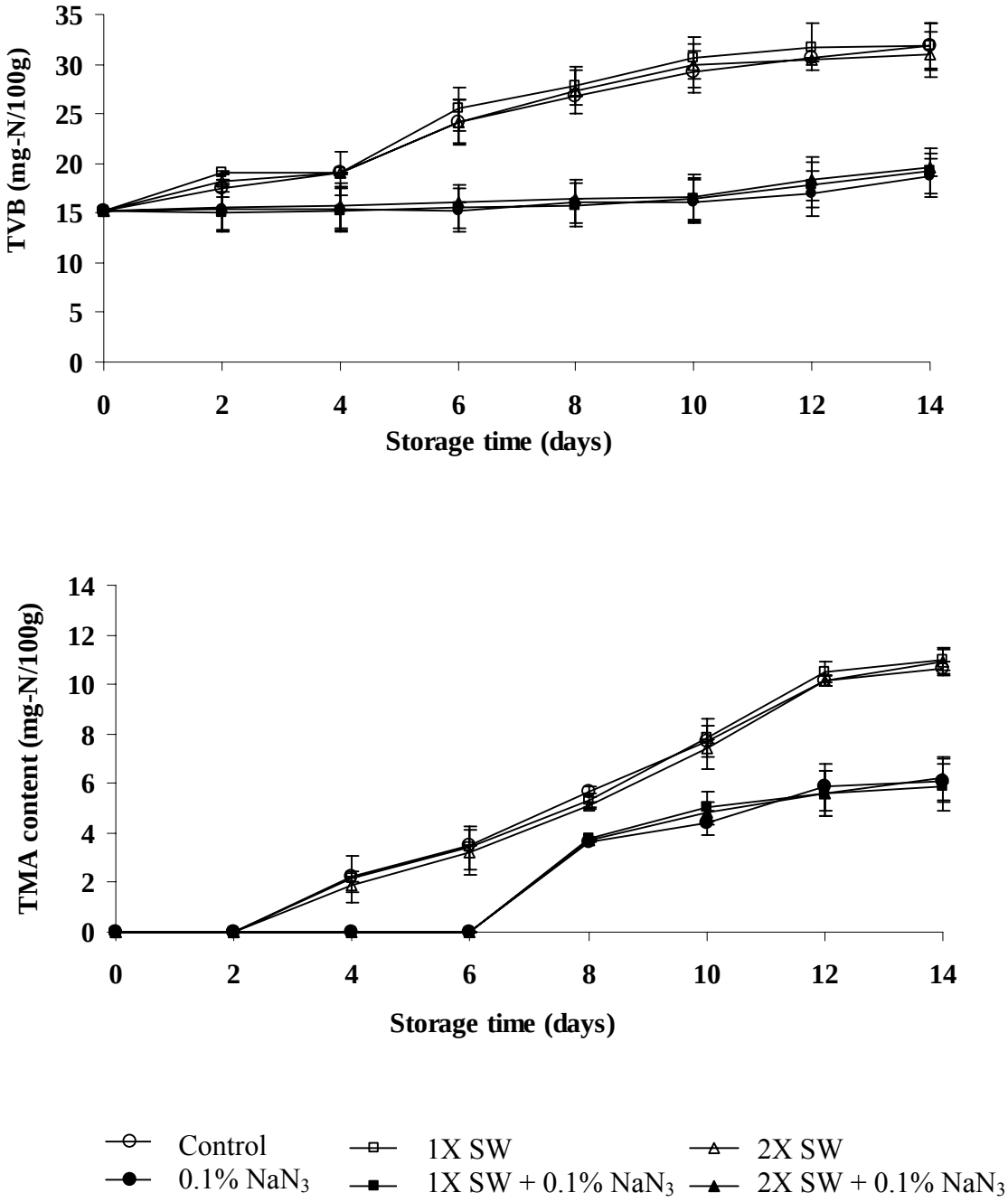


Figure 24. TVB and TMA contents of squids without and with 0.1% NaN_3 treatment stacked without and with standard weights (1X SW or 2X SW) during iced storage using a squid/ice ratio of 1:2. Bars represent the standard deviation (n=3).

Ammonia content of squid without and with 0.1% NaN_3 treatment and stacked with 1X SW or 2X SW during iced storage is shown in Figure 25. Ammonia content of squid without 0.1% NaN_3 treatment increased with increasing time, regardless of stacking ($p<0.05$). No changes in ammonia content were found in the squid with 0.1% NaN_3 treatment within the first 10 days of storage, irrespective of stacking ($p<0.05$). Thereafter, the increases were found up to 14 day of storage. Ammonia contents of 10.50-10.90 and 8.20-8.40 mg/100 g sample were found at day 14 in the sample without and with 0.1% NaN_3 treatment, respectively. Squids without 0.1% NaN_3 treatment had the higher ammonia content during iced storage, compared with those treated with 0.1% NaN_3. It was noted that NaN_3 could retard the decomposition of squid stored in ice. Nevertheless, stacking and weight of standard had no impact on ammonia content of squids stored in ice. Deamination was also inhibited by NaN_3 as evidenced by the lowered ammonia content.

4.3 Changes in pH

pHs of squid without and with 0.1% NaN_3 treatment stored in ice stacked without and with standard weights are shown in Figure 25. For squid without 0.1% NaN_3, pH of squids increased slightly within the first 2 days of storage. Thereafter, the increases in pH were found with increasing time ($p<0.05$). No changes were observed in pH of squid with 0.1% NaN_3 treatment within the first 2 days of storage but slightly increased up to 10 days of storage ($p<0.05$). During 10-12 days, the marked increases were obtained. At day 14, no change in pH was found ($p>0.05$). The rates of increases were greater in the sample without 0.1% NaN_3 treatment than those with 0.1% NaN_3 treatment, regardless of stacking ($p<0.05$). Stacking therefore had no effect on pH of squids during iced storage.

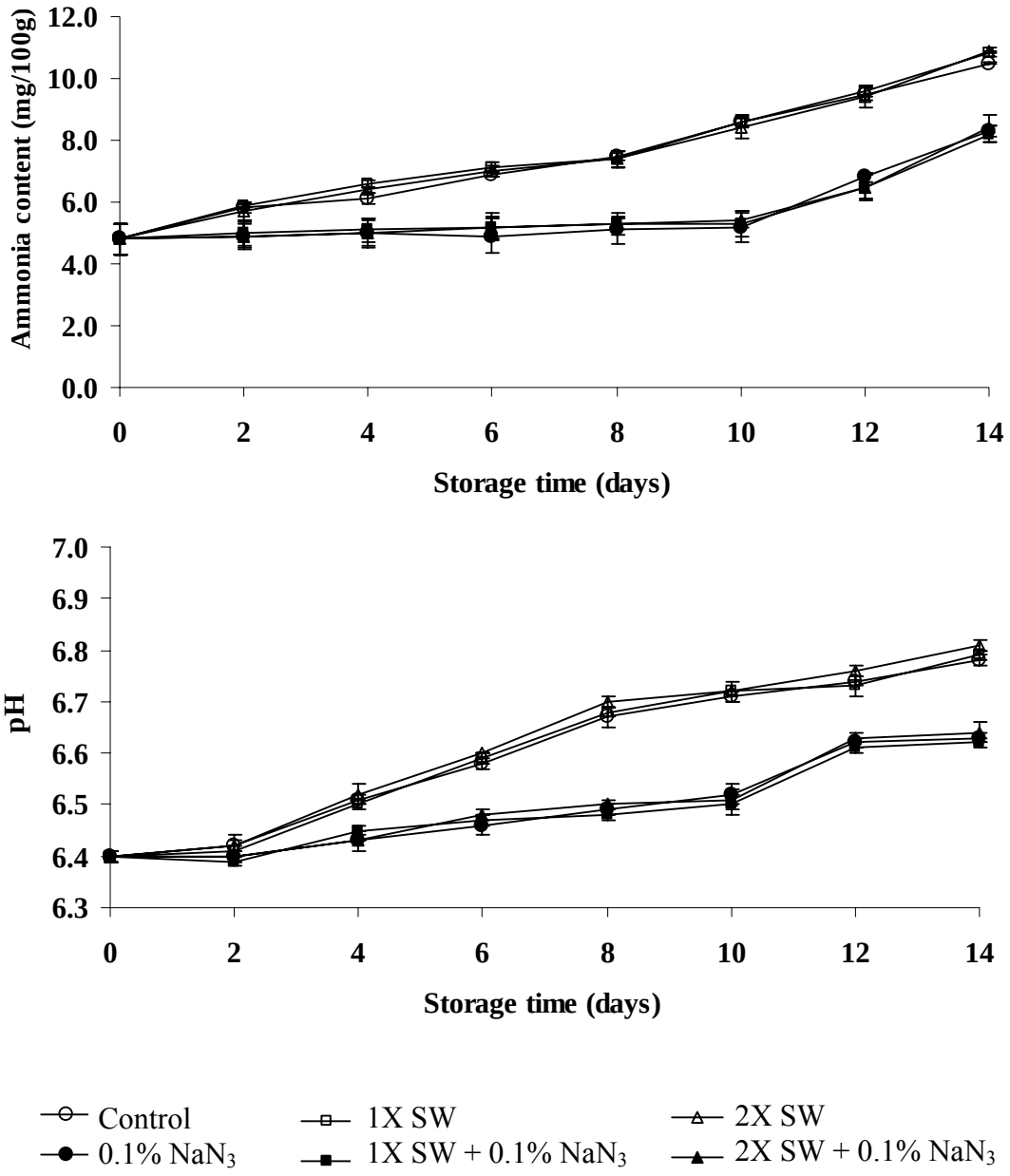


Figure 25. Ammonia content and pH of squids without and with 0.1% NaN_3 treatment stacked without and with standard weights (1X SW or 2X SW) during iced storage using a squid/ice ratio of 1:2. Bars represent the standard deviation (n=3).

4.4 Changes in psychrophilic bacterial count (PBC)

PBC of squid without and with 0.1% NaN_3 treatment stacked without and with standard weights during iced storage of 14 days are shown in Figure 26. No differences in PBC were found between squid without and with stacking, both 1X SW and 2X SW, during the storage of 14 days. The result revealed that the stacking had no effect on the growth of PBC. PBC of squid without 0.1% NaN_3 treatment increased with increasing time of iced storage, regardless of stacking ($p<0.05$). For the sample treated with 0.1% NaN_3, no changes in PBC were found within the first 10 days of storage ($p>0.05$). After 10 days, the slight increases in PBC were observed up to 14 days of storage ($p<0.05$). The higher PBC was found in squid without 0.1% NaN_3 treatment, compared with that found in those with 0.1% NaN_3 treatment throughout the storage, regardless of stacking ($p<0.05$). The result reconfirmed that NaN_3 could retard the growth of PBC in squids during iced storage.

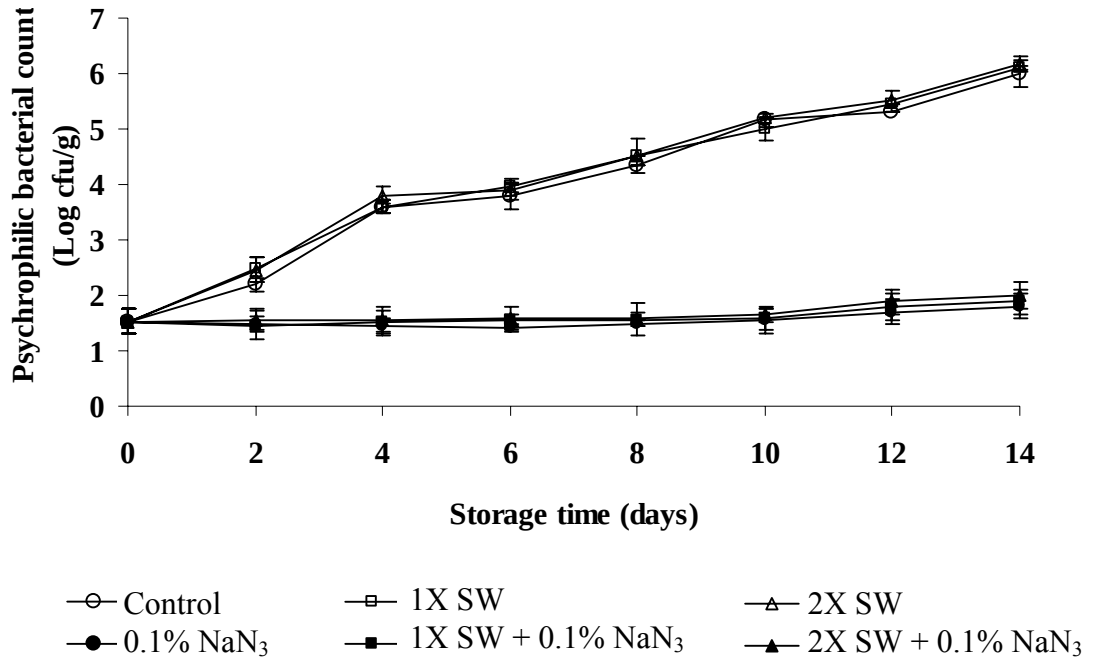


Figure 26. Psychrophilic bacterial count of squids without and with 0.1% NaN_3 stacked without and with standard weights (1X SW or 2X SW) during iced storage using a squid/ice ratio of 1:2. Bars represent the standard deviation (n=3).

4.5 Changes in TBARS

TBARS value of all samples increased continuously throughout the storage ($p<0.05$) (Figure 27). No differences in TBARS values between all samples were observed during iced storage with a squid/ice ratio of 1:2. The result indicated that NaN_3 treatment and stacking had no effect on lipid oxidation in muscle of squid during iced storage. Squids contained the high content of phospholipids, resulting in the high susceptibility toward autoxidation, especially during the extended storage.

4.6 Changes in formaldehyde content

Formaldehyde content of all samples increased up to 10 days of storage, followed by the marked increases up to 14 days of storage ($p<0.05$) (Figure 27). No differences in formaldehyde content between all samples were found during iced storage with a squid/ice ratios of 1:2 ($p>0.05$). Thus, NaN_3 treatment and stacking had no impact on formaldehyde formation in squid during iced storage.

4.7 Changes in chomatophores

The disruption of chromatophores in the skin of squid treated without and with 0.1% NaN_3 kept in ice without and with stacking with 1X SW or 2X SW is shown in Figure 28. The disruption of chromatophores in all samples increased with increasing time. However, after storage in ice for 7 or 14 days, the disruption of chromatophores in squid skin was more pronounced in squid without 0.1% NaN_3 treatment, compared with those treated with 0.1% NaN_3. The disruption of chromatophores in squid skin was most pronounced in squid stacked with 2X SW, followed by those stacked with 1X SW and without stacking, respectively. After storage in ice for 14 days, the dispersion of pigments in the skin of squid stacked with 2X SW was most pronounced, followed by those stacked with 1X SW. It was probably because the higher compression force of 2X SW to the skin of squid, leading to the disruption of chromatophores, which had the weaker sac caused by microbial action. The disruption of chromatophores were related with changes in color of squid during iced storage (Figure 23).

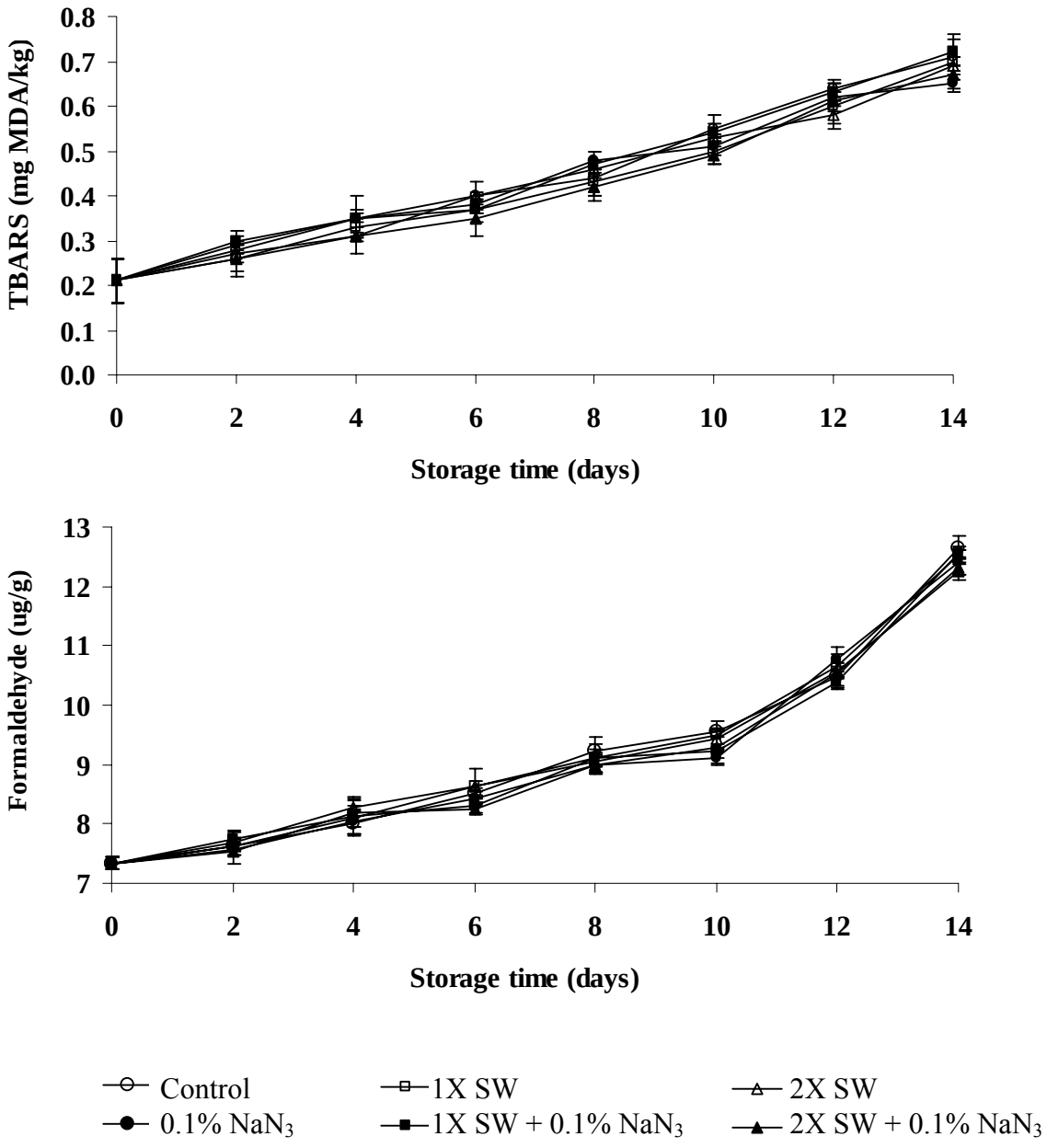


Figure 27. TBARS and formaldehyde content of squids without and with 0.1% NaN_3 treatment stacked without and with standard weights (1X SW or 2X SW) during iced storage using a squid/ice ratio of 1:2. Bars represent the standard deviation (n=3).

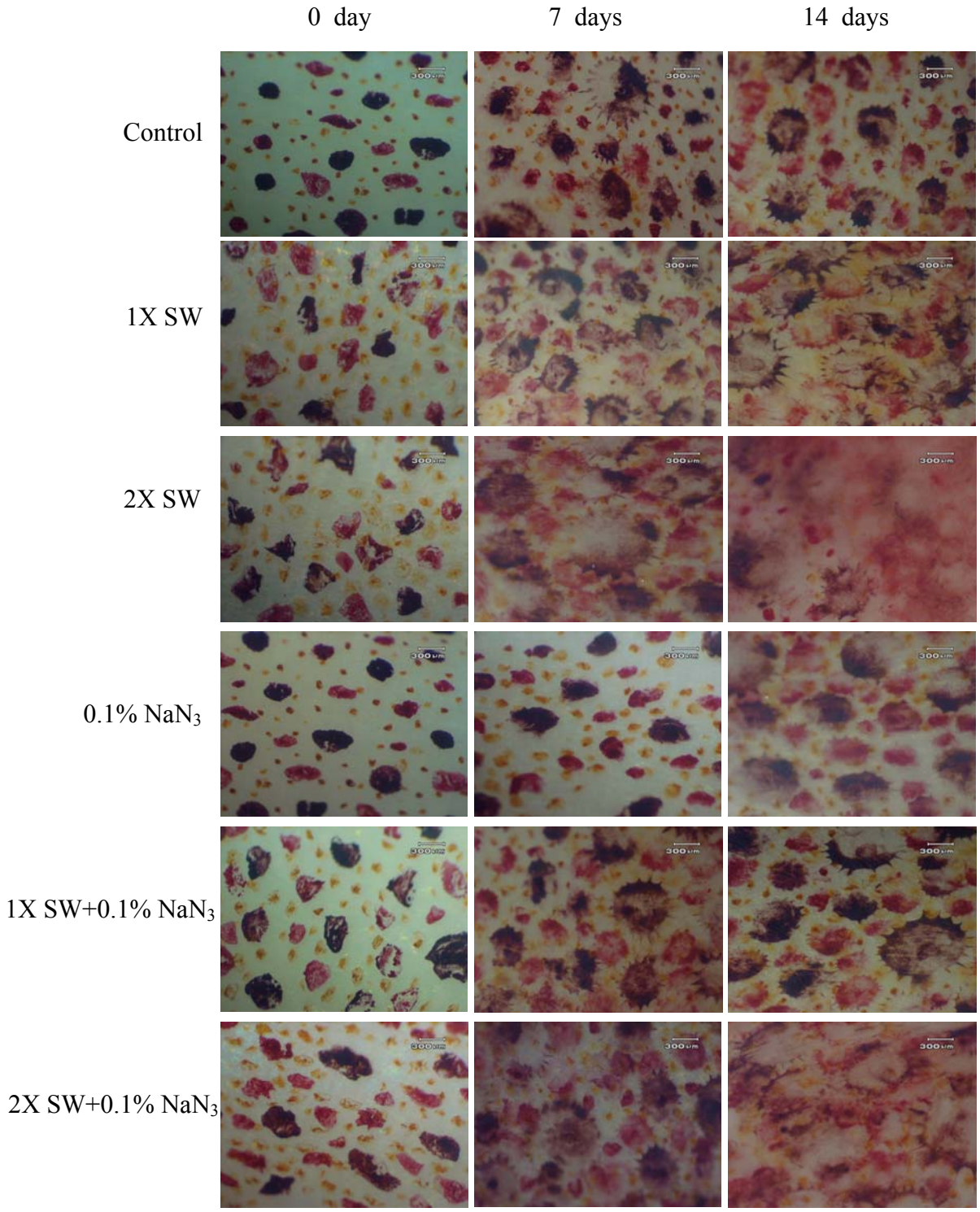


Figure 28. Morphology of chromatophores in the skin of squids with and without 0.1% NaN_3 treatment stacked without and with standard weights (1X SW or 2X SW) during iced storage using a squid/ice ratio of 1:2. Magnification : 30X.

5. Effect of chemical treatment on pink discoloration and physico-chemical changes of squid during frozen storage

5.1 Effect of bleaching agent on color of pink squids

Pink squids were treated with 3% NaCl containing oxidizing agents (H_2O_2 and NaOCl) at different levels for 3 h at 4^{o}C. The *a** and *b**-values of pink squid treated with oxidizing solutions were lower than those of the control squid (without bleaching) ($p<0.05$). No changes in *L**-value were observed in all samples ($p>0.05$). The result indicated that oxidizing agents had no effect on the lightness (*L**-value) of treated squids. The *a** and *b**-values of treated squid decreased with increasing H_2O_2 concentration ($p<0.05$). The lowest *a** and *b**-values of squid were obtained in all samples treated with bleaching solution containing 0.5% H_2O_2. Oxidation of pigment on squid mantle was more pronounced with increasing H_2O_2 concentration. The decomposition of H_2O_2 gives the highly reactive products, which can oxidize chromophores to substance that either does not contain a chromophore, or contains a chromophore that does not absorb visible light (Perkins, 1996). Aewsiri et al. (2009) found that the *a**-value of gelatin gel from cuttlefish skin decreased with increasing H_2O_2 concentration. At the same level of H_2O_2, NaOCl had no impact on *a**- and *b**-values of squid mantle, even at an increasing levels. Therefore, NaOCl had no effect on the changes of *a** and *b**-values. NaOCl is an oxidizing agent commonly used in water for food processing to reduce the microbial load (Rossoni and Gaylarde, 2000). In the present study, NaOCl concentration might not be high enough to function as an oxidizing agent, which was able to bleach the pink mantle. Therefore, the bleaching solution containing 0.5% H_2O_2, 3% NaCl (without NaOCl) was chosen to treat pink squid for bleaching purpose. After bleaching with solution containing 0.5% H_2O_2 and 3% NaCl, pink color was lowered (Figure 29). Therefore, bleaching solution could improve the color of pink squid. However, the mantle of fresh squid was whiter than that of bleached pink squid.

Table 8. *L**, *a** and *b**-values of pink squid treated with 3% NaCl containing oxidizing agents (H_2O_2 and NaOCl) at different levels for 3 h at 4°C

Treatments	*L**-value✧	*a**-value✧	*b**-value✧
Control	75.71±0.81^{a}✧✧	8.04±0.53^{a}	4.07±0.28^{a}
0.05% H_2O_2+3% NaCl+0 ppm NaOCl	74.17±0.89^{a}	3.49±0.21^{b}	2.13±0.07^{bc}
0.25% H_2O_2+3% NaCl+0 ppm NaOCl	76.08±1.57^{a}	1.60±0.45^{d}	1.57±0.70^{cd}
0.50% H_2O_2+3% NaCl+0 ppm NaOCl	74.69±1.00^{a}	-1.24±0.17^{e}	-0.34±0.21^{e}
0.05% H_2O_2+3% NaCl+5 ppm NaOCl	73.77±0.43^{a}	3.37±0.41^{b}	2.52±0.60^{b}
0.25% H_2O_2+3% NaCl+5 ppm NaOCl	74.47±1.17^{a}	1.72± 0.24^{d}	1.47±0.59^{cd}
0.50% H_2O_2+3% NaCl+5 ppm NaOCl	75.56±0.50^{a}	-1.18±0.08^{e}	-0.28±0.06^{e}
0.05% H_2O_2+3% NaCl+10 ppm NaOCl	75.21±1.48^{a}	2.65±0.50^{c}	2.00±0.11^{bc}
0.25% H_2O_2+3% NaCl+10 ppm NaOCl	75.39±1.04^{a}	1.76±0.20^{d}	1.17±0.34^{d}
0.50% H_2O_2+3% NaCl+10 ppm NaOCl	75.66±0.89^{a}	-1.26±0.06^{e}	-0.26±0.07^{e}

✧ Mean ± SD from triplicate determinations

✧✧ Different letters within the same column indicate significant differences ($p<0.05$).

Figure 29. Fresh squid, pink squid and pink squid treated with bleaching solution containing 0.5% H_2O_2 and 3% NaCl for 3 h at 4°C.

5.2 Changes in lipid oxidation of treated squid during frozen storage

Changes in TBARS values of pink squid treated with 3% NaCl containing 0.5% H_2O_2 during frozen storage at -18^{o}C for 10 weeks are shown in Table 9. TBARS in all samples increased with increasing frozen storage time ($p<0.05$). Pink squid treated with 3% NaCl containing 0.5% H_2O_2 had the higher TBARS value, compared with pink squid (without treatment) and fresh squid ($p<0.05$). However, as the storage time increased, no marked differences in TBARS between the control and fresh squid were observed ($p>0.05$). Treated pink squids showed the higher TBARS than pink squid (without treatment) after 4 weeks of storage ($p<0.05$). The result suggested that the formation of TBARS could be due to acceleration of lipid oxidation by oxygen radicals of hydrogen peroxide. The decomposition of H_2O_2 gives the highly reactive products: hydroperoxyl anion (HO_2^-) and hydroxyl ($OH^{\bullet}$) and hydroperoxyl ($HO_2^{\bullet}$) radicals, which can react with many substances (Perkins, 1996). Lipid oxidation in muscle foods occurs primarily in cell membrane lipids (Khayat and Schwall, 1983; Huang et al., 1993). Phospholipids are the major lipid in cuttlefish and contain a high content of unsaturated fatty acid (Thanonkeaw et al., 2006). TBARS in cuttlefish increased with increasing time during frozen storage at -18^{o}C for 16 weeks (Thanonkeaw et al., 2008). Paredi et al. (2006) found that free fatty acids (FFA) and diacylglycerols arised mainly from phospholipids and triacylglycerols during frozen storage of squid mantle ($p<0.05$). These FFA might undergo oxidation to a higher extent, especially in the presence of hydroxyl radical generated from H_2O_2 used.

Table 9. TBARS in fresh squid and pink squid treated without and with 3% NaCl containing 0.5% H_2O_2 during frozen storage at -18^{o}C for 10 weeks

Storage time (weeks)	TBARS✧ (mg malondialdehyde/kg sample)		
	Fresh squid	Pink squid	Treated pink squid
0	0.60 ± 0.16^{aB}✧✧	0.67 ± 0.06^{dB}	0.80 ± 0.05^{bA}
2	0.67 ± 0.07^{cB}	0.85 ± 0.04^{cdAB}	0.91 ± 0.15^{bA}
4	0.86 ± 0.08^{cA}	0.88 ± 0.10^{cA}	1.00 ± 0.13^{bA}
6	2.07 ± 1.13^{bB}	2.23 ± 0.37^{bB}	3.28 ± 0.69^{aA}
8	2.24 ± 0.04^{abB}	2.29 ± 0.18^{bB}	2.41 ± 0.14^{aA}
10	3.05 ± 0.62^{aB}	3.14 ± 0.05^{aAB}	3.53 ± 0.18^{aA}

✧ Mean ± SD from triplicate determinations

✧✧ Different letters within the same column indicate significant differences ($p<0.05$) and different capital letters within the same row indicate significant differences ($p<0.05$).

5.3 Changes in color

*L**, *a** and *b**-values of frozen pink squid treated without and with 3% NaCl containing 0.5% H_2O_2 in comparison with fresh squid are shown in Table 10. No changes in *L**, *a** and *b**-values were noticeable during frozen storage ($p>0.05$). The result suggested that soaking solution (3% NaCl containing 0.5% H_2O_2) had no effect on *L**, *a** and *b**-values of squid during frozen storage. No marked differences in *L**-value between all samples were observed during frozen storage ($p>0.05$). For *a** and *b**-values, fresh squid had the lowest in *a** and *b**-values, compared with pink squid treated with and without 3% NaCl containing 0.5% H_2O_2, respectively. Therefore, bleaching solution could improve color of pink squid and had no effect on color of treated squid during extended frozen storage. H_2O_2 can oxidize chromophores, leading to the decrease in redness and yellowness. Thanonkaew et al. (2008) reported that soaking the cuttlefish in 3% NaCl and 0.5% H_2O_2 for 15 min could improve the color of cuttlefish by increasing the *L**-value and decreasing the

*a**-value. Therefore, bleaching pink squid prior to frozen storage had no negative effect on color of frozen squids.

5.4 Changes in physicochemical properties of natural actomyosin (NAM)

The sulfhydryl and disulfide bond contents of NAM from pink squid treated with 3% NaCl containing 0.5% H_2O_2 are shown in Table 11 and 12, respectively. Sulfhydryl content of NAM from squid decreased with increasing time during frozen storage ($p<0.05$). Disulfide bond contents of NAM from squid coincidentally increased with increasing time of storage ($p<0.05$). The increases in disulfide bond content were more pronounced in the squid treated with 3% NaCl containing 0.5% H_2O_2, compared with untreated pink squid and fresh squid, respectively ($p<0.05$). The result suggested that treatment of pink squid with H_2O_2 resulted in the formation of disulfide bonds. The radicals generated by the decomposition of H_2O_2 may induce free radicals, causing the oxidation of sulfhydryl groups of proteins. Aewsiri et al. (2009) found that the oxidation of protein in gelatin increased with increasing concentration of H_2O_2 used for bleaching. The decreases in

Table 10. *L**, *a** and *b**-value in fresh squid and pink squid treated with 0.5% H_2O_2 and 3% NaCl (for 3 h) during frozen storage at -18^{o}C for 10 weeks

Color	Storage time (weeks)	Samples		
		Fresh squid	Pink squid	Treated pink squid
*L**-value✧				
	0	77.71 ± 1.28^{aA}✧✧	76.37 ± 0.45^{aA}	78.15 ± 1.38^{abA}
	2	76.20 ± 0.76^{aA}	73.95 ± 1.15^{bB}	77.75 ± 1.02^{abA}
	4	76.40± 0.64^{aB}	75.89 ± 0.60^{abB}	78.43 ± 0.86^{abA}
	6	76.32 ± 0.84^{aB}	75.78 ± 0.64^{abB}	79.60 ± 0.95^{aA}
	8	77.06 ± 0.53^{aA}	75.91 ± 0.72^{abA}	76.72 ± 1.42^{bA}
	10	76.97 ± 1.55^{aA}	77.35 ± 1.21^{aA}	79.16 ± 0.15^{aA}
*a**-value✧				
	0	-2.84 ± 0.06^{aC}	7.79 ± 0.58^{aA}	-1.24 ± 0.04^{aB}
	2	-2.68 ± 0.17^{aC}	7.65 ± 0.26^{aA}	-1.10 ± 0.11^{aB}
	4	-2.90 ± 0.13^{aC}	7.70 ± 0.30^{aA}	-1.31 ± 0.36^{aB}
	6	-2.78 ± 0.18^{aC}	7.68 ± 0.22^{aA}	-1.26 ± 0.13^{aB}
	8	-2.54 ± 0.19^{aC}	7.20 ± 0.09^{aA}	-1.25 ± 0.14^{aB}
	10	-2.74 ± 0.28^{aC}	7.25 ± 0.47^{aA}	-1.19 ± 0.15^{aB}
*b**-value✧				
	0	-2.13 ± 0.16^{abC}	4.03 ± 0.30^{aA}	-0.30 ± 0.06^{aB}
	2	-2.06 ± 0.06^{abC}	3.63 ± 1.26^{aA}	-0.21 ± 0.39^{aB}
	4	-2.21 ± 0.04^{bC}	4.00 ± 0.57^{aA}	-0.32 ± 0.11^{aB}
	6	-2.12 ± 0.18^{abC}	3.94 ± 0.38^{aA}	-0.47 ± 0.10^{aB}
	8	-2.06 ± 1.00^{abC}	3.81 ± 0.33^{aA}	-0.35 ± 0.19^{aB}
	10	-2.01 ± 0.07^{aC}	4.07 ± 0.14^{aA}	-0.25 ± 0.02^{aB}

✧ Mean ± SD from triplicate determinations

✧✧ Different letters within the same column under the same color parameter indicate significant differences ($p<0.05$) and different capital letters within the same row indicate significant differences ($p<0.05$).

sulfhydryl content were reported to be due to the formation of disulfide bonds via oxidation of sulfhydryl groups or disulfide interchanges (Hayakawa and Nakai, 1985). Myosin is susceptible to oxidizing agent during processing and storage. The disappearance of both heavy and light chains of myosin is much more pronounced than in actin and tropomyosin (Xiong, 2000). The amino acid most susceptible to oxidation are cysteine, histidine, and lysine. These amino acid are prone to oxidation where a hydrogen atom is abstracted from OH-, S- or N-containing groups (Doorn and Petersen, 2002).

Table 11. Sulfhydryl content of natural actomyosin extracted from fresh squid and pink squid treated without and with 3% NaCl containing 0.5% H_2O_2 during frozen storage at -18^{o}C for 10 weeks

Storage time (weeks)	Sulfhydryl content$^{\diamond}$ (10^{-5} mol/g protein)		
	Fresh squid	Pink squid	Treated pink squid
0	$8.86 \pm 0.87^{aA\diamond\diamond}$	7.37 ± 0.10^{aB}	7.08 ± 0.04^{aB}
2	7.52 ± 0.15^{bA}	6.44 ± 0.19^{bB}	6.18 ± 0.05^{bB}
4	7.33 ± 0.07^{bA}	6.15 ± 0.22^{bB}	5.06 ± 0.05^{cC}
6	6.43 ± 0.05^{cA}	5.78 ± 0.05^{cB}	4.51 ± 0.08^{dC}
8	5.60 ± 0.20^{dA}	5.50 ± 0.31^{cA}	3.77 ± 0.18^{eB}
10	5.20 ± 0.07^{dA}	5.17 ± 0.08^{dA}	3.30 ± 0.11^{fB}

$^{\diamond}$ Mean $\pm$ SD from triplicate determinations

$^{\diamond\diamond}$ Different letters within the same column indicate significant differences ($p<0.05$) and different capital letters within the same row indicate significant differences ($p<0.05$).

Table 12. Disulfide bond content of natural actomyosin extracted from fresh squid and pink squid treated without and with 3% NaCl containing 0.5% H_2O_2 during frozen storage at -18°C for 10 weeks

Storage time (weeks)	Disulfide bond content✧ (10^{-5} mol/g protein)		
	Fresh squid	Pink squid	Treated pink squid
0	$3.67 \pm 0.35^{cA✧✧}$	3.90 ± 0.14^{dA}	4.03 ± 0.19^{dA}
2	3.88 ± 0.06^{cC}	4.14 ± 0.19^{dB}	4.38 ± 0.08^{dA}
4	4.28 ± 0.81^{bB}	4.41 ± 0.67^{cB}	5.11 ± 0.09^{cA}
6	4.37 ± 0.19^{bB}	4.69 ± 0.05^{bB}	5.20 ± 0.45^{cA}
8	4.60 ± 0.19^{bB}	4.97 ± 0.28^{aB}	6.42 ± 0.25^{bA}
10	4.95 ± 0.06^{aB}	4.98 ± 0.07^{aB}	7.18 ± 0.29^{aA}

✧ Mean ± SD from triplicate determinations

✧✧ Different letters within the same column indicate significant differences ($p<0.05$) and different capital letters within the same row indicate significant differences ($p<0.05$).

5.5 Changes in thaw drip

Thaw drip of squid without and with bleaching during frozen storage in comparison with the fresh squid is shown in Table 13. Thaw drip of all samples increased with increasing time up to 10 weeks of frozen storage ($p<0.05$). The rates of increases were more greater in pink squid treated with 3% NaCl containing 0.5% H_2O_2, followed by the pink squid and fresh squid, respectively ($p<0.05$). It was probably because the oxidation of protein and lipid by the effect of oxidizing agent (H_2O_2), leading to aggregation of protein. As a result, water-holding capacity of proteins might be lowered. For the pink squid, the softening of muscle might occur by autolysis or microbial proteolysis. This might enhance the cell disruption induced by ice crystal during freezing and frozen storage. Aewsiri et al. (2009) found that the oxidation of protein from cuttlefish skin induced by H_2O_2 resulted in aggregation of protein. The oxidation products of lipid, specific secondary lipid oxidation products (aldehydes) appear to interact with protein to enhance the oxidation of protein (Chan et al., 1997). The loss of water holding capacity of muscle protein might be induced

by the growth of ice crystals during frozen storage, leading to the injured and aggregated muscle fibers. Continuous denaturation of muscle proteins during frozen storage led to the lower water-holding capacity of proteins. Ice crystal formed resulted in the tissue damage. As a result, water could be released from muscle more easily, particularly when the frozen storage time increased (Benjakul et al., 2003). Thanonkaew et al. (2008) reported that thaw drip of cuttlefish increased as the storage time increased up to 8 weeks of frozen storage. Thereafter, no change was observed throughout 16 weeks of frozen storage ($p<0.05$). Therefore, freshness and bleaching had the pronounced effect on the formation of exudates produced after freeze-thawing of squids.

5.6 Changes in texture

Shear force of all samples increased with increasing time up to 10 weeks of storage ($p<0.05$) (Table 14). The increases in toughness of squid mantles during frozen storage caused by protein aggregation because of injured muscle fibers (Ueng and Chow, 1998). At the same time of storage, The highest shear force was observed in pink squid treated with 3% NaCl containing 0.5% H_2O_2, followed by pink squid without treatment and fresh squid, respectively ($p<0.05$). Effect of H_2O_2 on lipid and protein oxidation probably led to the denaturation and aggregation of proteins and a loss of textural property of squid meat. Phatcharat et al. (2006) reported that the breaking force of surimi gel with H_2O_2 washing was higher than that of surumi gel with water washing. Bleaching of cuttlefish with H_2O_2 could improve bloom strength of gelatin gels (Aewsiri et al., 2009). Radicals produced during lipid oxidation of unsaturated fatty acids might attack protein molecules, leading to the formation of protein-free radicals. These protein-free radicals may cross-link with other proteins to form protein-protein aggregates (Karel et al., 1975). Several pathways of protein denaturation have been suggested in frozen marine meat. These include dehydration of proteins from ice crystal formation and increasing salt concentration (Ota and Tanaka, 1978). Therefore, bleaching of squid prior to frozen storage had the negative effect on toughening, especially during the extended storage.

Table 13. Thaw drip of fresh squid and pink squid treated without and with 3% NaCl containing 0.5% H_2O_2 during frozen storage at -18^{o}C for 10 weeks

Storage time (weeks)	% Thaw drip✧		
	Fresh squid	Pink squid	Treated pink squid
0	$0.15 \pm 0.01^{dC✧✧}$	0.47 ± 0.03^{dA}	0.34 ± 0.03^{dB}
2	1.54 ± 0.14^{cC}	1.97 ± 0.09^{cB}	2.30 ± 0.08^{cA}
4	2.30 ± 0.23^{bB}	2.61 ± 0.26^{bA}	2.68 ± 0.28^{bA}
6	2.38 ± 0.12^{bB}	2.64 ± 0.06^{aAB}	2.80 ± 0.15^{bA}
8	2.76 ± 0.04^{aC}	3.10 ± 0.13^{aB}	3.46 ± 0.15^{aA}
10	2.79 ± 0.15^{aB}	3.18 ± 0.10^{aA}	3.47 ± 0.10^{aA}

✧ Mean ± SD from triplicate determinations

✧✧ Different letters within the same column indicate significant differences ($p<0.05$) and different capital letters within the same row indicate significant differences ($p<0.05$).

Table 14. Shear force of fresh squid and pink squid treated without and with 3% NaCl containing 0.5% H_2O_2 during frozen storage at -18^{o}C for 10 weeks

Storage time (weeks)	Shear force✧ (g)		
	Fresh squid	Pink squid	Treated pink squid
0	$3442.55 \pm 478.95^{dB✧✧}$	3763.59 ± 461.08^{dAB}	4008.37 ± 262.96^{dA}
2	3638.73 ± 582.71^{cB}	4084.76 ± 645.83^{cAB}	4453.76 ± 570.74^{cdA}
4	3658.66 ± 734.02^{cB}	4229.34 ± 776.47^{cB}	4959.18 ± 740.58^{cA}
6	4137.07 ± 766.74^{bcB}	5908.50 ± 445.53^{bA}	6592.65 ± 220.14^{bA}
8	4722.28 ± 845.76^{bB}	7074.39 ± 200.19^{aA}	7809.43 ± 750.27^{aA}
10	5699.59 ± 768.71^{aC}	7219.08 ± 439.42^{aB}	8073.82 ± 273.22^{aA}

✧ Mean ± SD from triplicate determinations

✧✧ Different letters within the same column indicate significant differences ($p<0.05$) and different capital letters within the same row indicate significant differences ($p<0.05$).

5.7 Changes in microstructure

The changes in microstructure of squid muscle treated without and with 3% NaCl containing 0.5% H_2O_2 after 10 weeks of frozen storage, in comparison with fresh squid are shown in Figure 30 and 31. After frozen storage for 10 weeks, pink squid without and with treatment showed the gaps formed between the muscle bundles (Figure 30). The larger gaps between the muscle bundles were observed in bleached pink squid muscle, compared with the pink squid (without bleaching). It was probably because the aggregation of protein was induced by oxidizing agent (H_2O_2), leading to the formation aggregate between muscle fibers. Frozen storage for long periods are responsible for dehydration and compression of fibers due to the growth of the ice crystals (Otwell and Gidding, 1980; Llorca et al., 2007). Frozen storage caused partial dehydration of the intermyofibrillar spaces, permitting the myofilaments to compact (Llorca et al., 2007). No differences in arrangement of muscle fibers of fresh squid muscle and sample stored for 10 weeks were observed. At day 0 of storage, no marked differences in arrangement of muscle fibers between fresh squid, pinked squid without and with treatment were found. Sarcoplasmic network surrounding each fibers were more pronounced in fresh squid muscle, compared with pink squid (Otwell and Gidding, 1980). It was probably due to proteolysis by endogenous or microbial enzymatic systems of sarcoplasmic network of squid during iced storage before freezing and frozen. As a result, the quantity of sarcoplasmic network of pink squid should be lowered, regardless of treatment. Dublán-García et al. (2005) reported that the loosen muscle structure of squid during iced storage was caused by proteolysis, promoting a relaxed fiber structure, empty spaces between fibers and connective tissue fibrils forming amorphous areas.

In Figure 31, the outer lining of connective tissue is composed of primary fibrils (0.1 μ diameter) which compact into larger fibers (0.1-4.0 μ) (Otwell and gidding, 1980). The aggregation of collagen fibers of outer lining layer can be seen in treated pink squid muscle at 0 week of frozen storage. No aggregation of collagen fibers of outer lining layer in fresh squid muscle and the pink squid (without treatment) at 0 week of frozen storage were observed. After frozen storage for 10 weeks, the aggregation of collagen fibers of outer lining were more pronounced in treated pink squid muscle, compared with fresh squid muscle and pink squid without

treatment after frozen storage of 10 weeks. Oxidizing agent (H_2O_2) might result in the oxidation of protein and aggregation of collagen fibers of squid muscle. This in part contributed to the changes in color of squid.

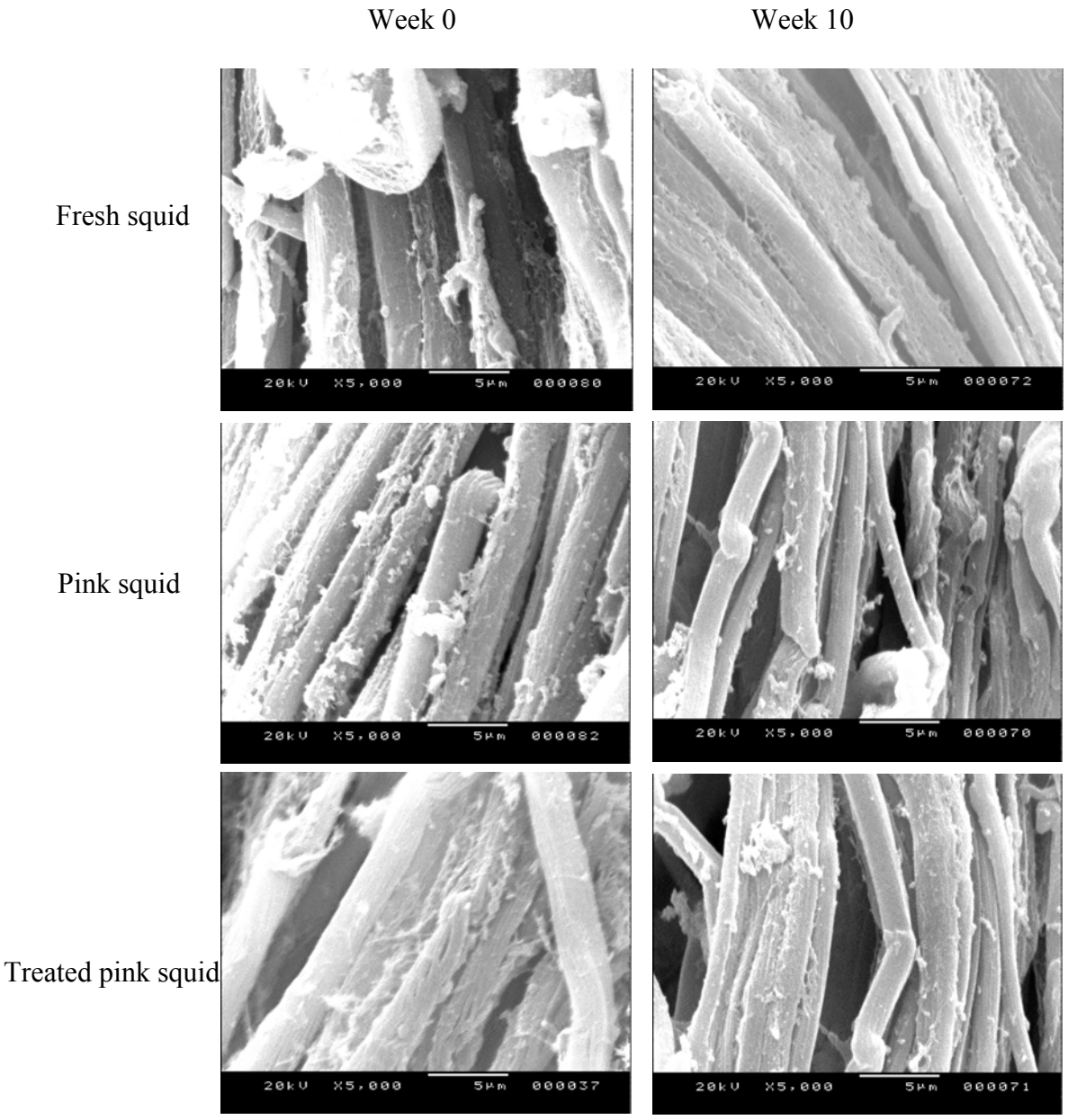


Figure 30. Scaning electron micrographs of longitudinal-section of fresh squid and squid stored at -18^{o}C for 10 weeks. Pink squids were treated with 3% NaCl containing 0.5% H_2O_2 (Magnification : 5000X).

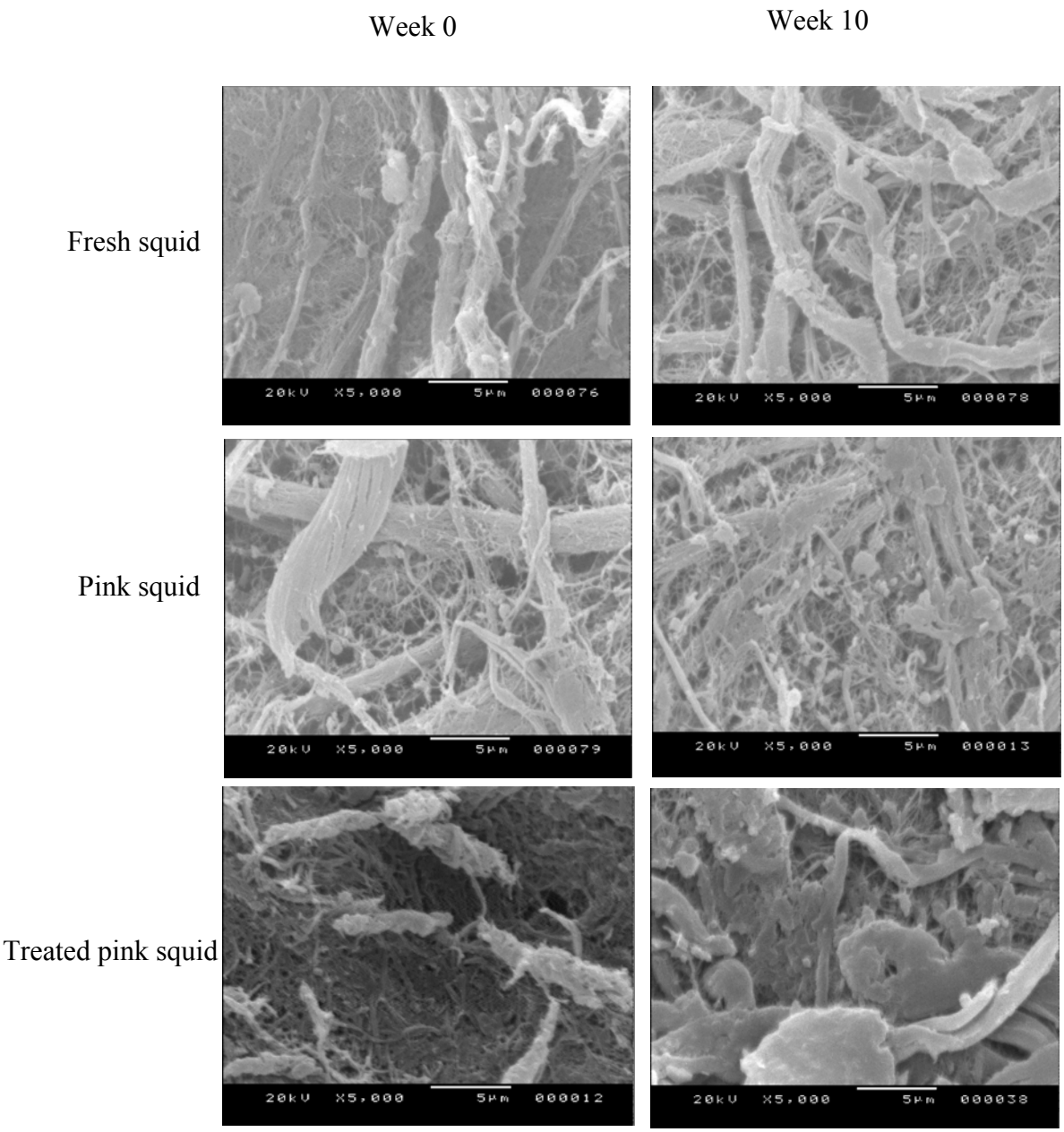


Figure 31. Scaning electron micrographs of surface layer of fresh squid and squid stored at -18^{o}C for 10 weeks. Pink squids were treated with 3% NaCl containing 0.5% H_2O_2 (Magnification : 5000X).

CHAPTER 4

CONCLUSION AND SUGGESTION

Conclusion

1. Pink discoloration on squid mantle without prior deskinning was associated with the decomposition of chromatophore membrane, leading to the release of pigments to the mantle of squid. The mantle of squid with the large size was more prone to the development of pink color than those with the small size and had the higher intensity of pink color developed. The use of sufficient amount of ice could retard pink discoloration and maintain the quality of squid during iced storage. Additionally, deskinning was an effective means to prevent pink discoloration.

2. The growth of microorganisms was most likely associated with the pink discoloration. The retardation of the growth of psychrophilic bacteria using the safe antimicrobials could lower pink discoloration and loss in quality of whole squid without deskinning.

3. Stacking exhibited the causative effect on the development of pink color. The use of antimicrobial agent and reduction of stacking during iced storage could retard development of pink color of squid without deskinning.

4. Treatment of pink squid with 3% NaCl containing 0.5% H_2O_2 could improve the color of pink squid. However, oxidizing agent (H_2O_2) induced the oxidation of lipid and protein, leading to the enhanced aggregation and denaturation of muscle protein during frozen storage. This was associated with the increased toughening of frozen squid muscle.

Suggestion

1. Effect of phosphates or non-phosphate treatment on quality changes of treated pink squid during frozen storage should be studied.

2. Effect of safe antimicrobial agents on quality changes and pink discoloration of squid during iced storage should be further investigated.

REFERENCES

Adams, M.R. and Moss, M.O. 2000. Food Microbiology 2nd Ed. p. 48-49. Royal Society of Chemistry. Philadelphia.

Aderson, W. E., Gosham, G.E. and Sim, G. G. 1975. Hydrogen peroxide bleaching of marinated herring. J. Food Technol. 10: 497-505.

Aewsiri, T., Benjakul, S. and Visessanguan, W. 2009. Functional properties of gelatin from cuttlefish (*Seapia pharaonis*) skin as affected by bleaching using hydrogen peroxide. Food Chem. 115: 243-249.

Alur, M. D., Doke, S. N., Warrier, S.B. and Nair, P. M. 1995. Biochemical methods for determination of spoilage of foods of animal origin: A critical evaluation. J. Food Sci. Technol. 32: 181-188.

Ang, J. F. and Hultin, H. O. 1989. Denaturation of cod myosin freezing after modification with formaldehyde. J. Food Sci. 54: 824-818.

Barroso, M., Careche, M. and Borderias, A. J. 1998. Qulaity control of frozen fish using rheological techniques. Trends Food Sci. Technol. 9: 223-229.

Benjakul, S., Seymour, T. A., Morrissey, M. T. and An, H. 1997. Physicochemical changes in Pacific whiting muscle proteins during iced storage. J. Food Sci. 62: 729-733.

Benjakul, S., Visessanguan, W., Thongkaew, C. and Tanaka, M. 2003. Comparative study on physicochemical changes of muscle proteins from some tropical fish during frozen storage. J. Food Res. Int. 36: 787-795.

Benjakul, S., Visessanguan, W. and Tanaka, M. 2004. Induced formation of dimethylamine and formaldehyde by lizardfish (*Saurida micropectoralis*) kidney trimethylamine-*N*-oxide demethylase. Food Chem. 84: 297-305.

Bhoite-Solomon, V., Kessler-Icekson, G., and Shaklai, N. 1992. Peroxidative cross-linking of myosin. Biochem. Int. 26: 181-189.

Buege, J. A. and Aust, S. D. 1978. Microsomal lipid peroxidation. Methods. Enzymol. 52: 32-34.

Chan, W. K. M., Faustman, C. and Decker, E. A. 1997. Oxymyoglobin oxidation as affected by oxidation products of phosphatidylcholine liposomes. J. Food Sci. 62: 709-712.

Change, C. C. and Regenstein, J. M. 1997. Textural change and functional properties of cod mince protein as affected by kidney tissue and cryoprotectants. J. Food Sci. 62: 299–304.

Conway, E. J. and Byrne, A. 1936. An absorption apparatus for the micro-determination of certain volatile substances. I. The micro-determination of ammonia. Biochem. J. 27: 419-429.

Dawson, P. L., Sheldon, B. W. and Miles, J. J. 1991. Effect of aseptic processing on the texture of chicken meat. Poultry Sci. 70: 2359–2367.

Department of Fisheries. 2006. Splendid squid. (Online). Available http : //www.ku.ac.th2/AgrInfo/thaifish/aquatic/aq272.html

Doorn, J. A. and Petersen, D. R. 2002. Covalent modification of amino acid nucleophiles by the lipid peroxidation products 4-hydroxy-2-nonenal and 4-oxo-2-nonenal. Chem. Res. Toxicol. 15: 1445-1450.

Doyle, M.P., Beuchat, L.R. and Montville, T.J. 1997. Food Microbiology. p. 501. ASM Press, Washington, D.C.

Drosinos, E. H. and Nychas, G. J. E. 1996. *Brochothrix thermosphacta*, a dominant microorganism in Mediterranean fresh fish (*Sparus aurata*) stored under modified atmosphere. Ital. J. Food Sci. 4: 323–329.

Dublán-García, O., Cruz-Camarillo, R., Guerrero-Legarreta, I. and Ponce-Alquicira, E. 2006. Effect of refrigerated storage on proteolytic activity and

physicochemical and microstructural properties of giant squid (*Gosidicus gigas*) mantle muscle. J. Muscle Foods. 17: 291-310.

Dziezak, J. D. 1990. Phosphates improve many foods. Food Technol. 44 (4): 80-92.

Ellman, G. L. 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82: 70-77.

Erdogdu, F., Balaban, M. O., Otwell, W. S. and Garrido, L. 2004. Cooked-related yield loss for Pacific white shrimp (*Penaeus vannamei*) previously treated with phosphates: effect of shrimp size and internal temperature distribution. J. Food Eng. 64: 297-300.

FAO. 2000. FAO cephalopod of the world, FAO species catalogue for fishery purpose. FAO. Rome.

FAO. 2003. FAO globefish cephalopods commodity update. FAO. Rome.

Finne, G. 1992. Non-protein nitrogen compounds in fish and shellfish. *In* Advances in Seafood Biochemistry. (Flick. G and Martin, R., eds.). p. 393–401. Technomic Publishing Company, Inc. Lancaster.

Goel, R. K., Cooper, A. T. and Flora, J. R. V. 2003. Sodium azide interference in chemical and biological testing. J. Environ. Eng. Sci. 2: 407-411.

Gomez-Guillen., M. C., Martinez-Alvarez., O. and Montero, P. 2003. Functional and thermal gelation properties of squid mantle proteins affected by chilled and frozen storage. Food Chem. Toxicol. 68: 1962-1967.

Goncalves, A. A. and Ribeiro, J. L. D. 2009. Effect of phosphate treatment on quality of red shrimp (*Pleoticus muelleri*) processed with cryomechanical freezing. LWT-Food Sci. Technol. 42: 1435-1438.

Gram, L. and Huss, H. H., 1996. Microbial spoilage of fish and fish products. Int. J. Food Microbiol. 33: 121-138.

Haard, N. F. 1992. Biochemical reaction in fish muscle during frozen storage. *In* Seafood Science and Technogy (Bligh, E.G., ed.) p. 176-209. Fishery News Books. Halifax.

Hayakawa, S. and Nakai, S. 1985. Contribution of hydrophobicity, net charge and sulfhydryl groups to thermal properties of ovalbumin. Can. Inst. Food Sci. Technol. J. 18: 290-295.

Herbert, R. A. and Shewan, J. M. 1976. Roles played by bacterial and autolytic enzymes in the production of volatile sulphides in spoiling North Sea cod (*Gadus morhua*). J. Sci. Food Agric. 27: 89-94.

Hidalgo, F. J., Nogales, F., and Zamora., R. 2004. Determination of pyrrolized phospholipid in oxidized phospholipids vesicles and lipoprotein. Anal. Biochem. 334: 155-163.

Huang, Y., Kouadio, K. and Gates, K. W. 1993. Effect of packaging on chemical changes and quality of refrigerated spot (*Leiostomus xanthurus*). J. Aqua. Food Prod. Technol. 1 (2): 111-127.

International Fish Trade Analysis Group of Department of Fisheries. 2006. Exporting of fishery products. (Online). Available http : //www.fisheries.go.th/foreign/ mainexport.html.

Jadhav, S. J., Nimbalkar. S. S., Kulkarni, A.D. and Madhavi, D. L. 1996. Lipid oxidation in biological and food systems. *In* Food Antioxidants. (Madhavi, D.L., Deshpande, S.S. and Salunkhe, D.K., eds.) p.5-63. Marcel Dekker, Inc. New York.

Johnsen, G. and Bjerheng, B. 1995. Frozen storage quality of rainbow trout (*Oncorhynchus mykiss*) as affected by oxygen, illumination and fillet pigment. J. Food Sci. 60: 284 – 288.

Karel, M., Schaich, K. and Roy, R. B. 1975. Interaction of peroxidizing methyl linoleate with some proteins and amino acids. J. Agric. Food Chem. 23: 159-163.

Ke, P. J., Burns, B. G., and Woyewoda, A. D. 1984. Recommended procedures and guidelines for quality evaluation of Atlantic short-fin squid (*Illex illecebrosus*). Int. J. Food Sci. Technol. 17: 276-281.

Khayat, A. and Schwall, D. 1983. Lipid oxidation in seafood. Food Technol. 37 (7): 130-140.

Kimura, S. 1972. Studies on marine invertebrate collagens. V. The neutral sugar composition and glucosylated hydroxylysine contents of several collagens. Bull. Jpn. Soc. Sci. Fish. 38: 1153-1161.

Kolodziejska, I., Niecikowska, C. and Sikorski, Z. E. 1994. Dimethylamine and formaldehyde in cooked squid (*Illex argentinus*) muscle extract and mantle. Food Chem. 50: 281-283.

Kosak, P. H. and Toledo, R. T. 1981. Effect of microbiological decontamination on the storage stability of fresh fish. J. Food Sci. 46: 1012-1014.

Kreuzer, R. 1984. Cephalopods: handling, processing and products. FAO Fish. Technol. Pap. 254: 1-108.

Kussi, T., Nikkila, D. E. and Sarolainen, K., 1975. Formation of malonaldehyde in frozen Baltic herring and its influence on changes in protein. Z. Lebensm Unters. Forsch. 159: 285-290.

Lakshmanan, P. T., Varme, P .R. G. and Iyer, T. S. G. 1993. Quality of commercially frozen cephalopod products from India. Food Control. 4: 159-164.

Lapa-Guimarãaes, J., Aparecida Azevedo da Silva, M., Eduardo de Felicio., P. and Guzman, E.C. 2002. Sensory, color and phychrotrophic bacterial analyses of squid (*Loligo plei*) during storage in ice. LWT-Food Sci. Technol. 35: 21-29.

Lapa-Guimarães, J., Felicio, P. E. de and Guzman, E. S. C. 2005. Chemical and microbial analyses of squid muscle (*Loligo plei*) during storage in ice. Food Chem. 91: 477-483.

Leblanc, R. J., and Gill, T. A. 1984i. Ammonia as an objective quality index in squid. Can. Food Sci. Technol. J. 17: 195-201.

Leelapongwattana, K., Benjakul, S., Visessanguan, W. and Howell, N. K. 2008. Effect of some additives on the inhibition of lizardfish trimethylamine-*N*-oxide demethylase and frozen storage stability of minced flesh. Int. J. Food Sci. Technol. 43: 448-455.

Li, J., Zhu, J. and Ye, L. 2007. Determination of formaldehyde in squid by high-performance liquid chromatography. Asia. Pac. J. Clin. Nutr. 16: 127-130.

Lichstein, H. C. and Soule, M. H. 1944a. Studies of the effect of sodium azide on microbic growth and respiration. The action of sodium azide on microbic growth. J. Bacteriol. 47: 221-230.

Lichstein, H. C. and Soule, M. H. 1944b. Studies of the effect of sodium azide on microbic growth and respiration. The action of sodium azide on bacterial catalase. J. Bacteriol. 47: 231-238.

Llorca, E., Hernando, I., Pérez-Munuera, I., Quiles, A., Larrea, V. and Lluch, M. A. 2007. Protein breakdown during the preparation of frozen batter-coated squid rings. Eur. Food Res. Technol. 225: 807-813.

Lueck, E. 1980. Antimicrobial food additives: characteristics, uses, effects. p. 35-197. Springer-Verlag. New York.

Marquiz-Rios., E., Moran- Palacio, E. F., Lugo-Sanchez, M. E., Ocano-Higuera, V. M. and Pacheco-Aguilar, R. 2007. Postmortem biochemical behavior of giant squid (*Dosidicus gigas*) mantle muscle stored in ice and its relation with quality parameters. J. Food Sci. 72: 356-362.

Masniyom, P., Benjakul, S. and Visessanguan, W. 2005. Combination effect of phosphate and modified atmosphere on quality and shelf-life extension of refrigerated seabass slices. LWT- Food Sci. Technol. 38: 745-756.

Mignino, L. A., Crupkin, M. and Paredi, M. E. 2008. Surface hydrophobicity and functional properties of myofibrillar proteins of mantle from frozen-stored squid (*Illex argentinus*) caught either jigging machine or trawling. LWT- Food Sci. Technol. 41: 678-685.

Mignino, L. A. and Paredi, M. E. 2006. Physico-chemical and functional properties of myofibrillar proteins from different species of mollusks. LWT-Food Sci. Technol. 39: 35-42.

Moral, A., Morales, J., Ruiz-Capillas, C. and Montero, P. 2002. Muscle protein solubility of some cephalopod (pota and octopus) during frozen storage. J. Sci. Food Agric. 83: 663-668.

Narwar, W. W. 1985. Lipid. *In* Food Chemistry (Fennema,O.R., ed) p.175-198. Marcel Dekker. New York.

Ohashi, E., Okamoto, M., Ozawa, A. and Fujita, T. 1991. Characterization of commom squid using several freshness indicators. J. Food Sci. 56: 161-163.

Okuzumi, M and Fujii, T. 2000. Nutriional and functional properties of squid and cuttlefish. p. 22-59. National Cooperative Association of Squid Processors. Tokyo.

Ota, F. and Tanaka., T. 1978. Some properties of the lipid portion in the frozen fish muscle fluid. Bull. Jpn. Soc. Sci. Fish. 44: 59-62.

Otwell, W. S. and Gidding, G. G. 1980. Scanning electron microscopy of squid, *Loligo pealei* : raw, cooked and frozen mantle. Mar. Fish. Rev. 42: 67-73.

Paarup, T., Sanchez, J. A.., Moral, A., Christensen, H., Bisgaard. and Gram, L. 2002. Sensory, chemical and bacteriological changes during storage of iced squid (*Todaropsis eblanae*). J. Appl. Microbiol. 92: 941-950.

Palka, K. and Daun, H. 1999. Changes in texture cooking losses and myofibrillar structure of bovine *M. semitendinosus* during heating. Meat Sci. 51: 237-243.

Paredi, M. E., Roldán, H. A., and Crupkin, M. 2005. Effect of frozen storage on the biochemical properties of actomyosin and lipids composition of male and female squid (*Illex argentinus*) mantle. Proceedings of Second Mercosur Congress in Chemical Engineering. Fourth Congress on Process Systems Engineering, Rio de Janeiro. Brasil.

Paredi, M. E., Roldan, H. A. and Crupkin, M. 2006. Changes in myofibrillar proteins and lipids of squid (*Illex argentinus*) during frozen storage. Food Chem. 30: 604-621.

Parris, N. and Foglia, T. A. 1983. Simplified alcoholic extraction procedure for ammonia in meat tissue. J. Agric. Food Chem. 31: 887-889.

Passi, S., Cataudella, S., Marco, P. D., De-Simone, F. and Rastrelli, L. 2002. Fatty acid composition and antioxidant levels in muscle tissue of different Meriterranean marine species of fish and shellfish. J. Agric. Food Chem. 50: 7314-7322.

Pavlos, A. K. 1995. Composition of the commercially important mediterranean finfish, crustaceans and molluscs. J. Food Comp. Anal. 8: 258-273.

Perkins, W. S. 1996. Advances made in bleaching practice. Alexander Technique International. 4: 92-94.

Phatcharat, S., Benjakul, S. and Visessanguan, W. 2006. Effect of washing with oxidizing agents on the gel-forming ability and physicochemical properties of

surimi produced from bigeye snapper (*Priacanthus tayenus*). Food Chem. 98: 431-439.

Roongratri, M. 1989. Study on spawning seasons and some biology aspects of *Loligo duvauceli* Orbigny and *Loligo chinensis* Gray along the eastern coast of the gulf of Thailand. Technical paper No. 20.

Rossoni, E. M. M. and Gaylarde, C. C. 2000. Comparison of sodium hypochlorite and peracetic acid as sanitizing agents for stainless steel food processing surfaces using epifluorescence microscopy. Int. J. Food Microbiol. 61: 81-85.

Ruiz-Capillas, C., Moral, A., Morales, J. and Montero, P. 2002. The effect of frozen storage on the functional properties of the muscle of volador (*Illex ciondetii*). Food Chem. 78: 149-156.

Sadowska, M. and Sikorski, Z. E. 2007. Collagen in the tissues of squid *Illex argentinus* and *Loligo patagonica* - contents and solubility. J. Food Biochem. 11: 109-120.

Saeed, S. and Howell, N. K. 2002. Effect of lipid oxidation and frozen storage on muscle proteins of Atlantic mackerel (*Scomber scombrus*). J. Sci. Food Agric. 82: 579-586.

Sahoo, J. and Verma, S. P. 1999. Oxidative problems in meat and meat products and use of antioxidant. Int. J. Food Sci. Technol. 36: 48-499.

Santos, E. E. M. and Regenstein, J. M. 1990. Effects of vacuum packaging, glazing and erythorbic acid on the shelf-life of frozen white hake and mackerel. J. Food Sci. 55: 64-70.

Seibel, B. A., and Walsh, P. J. 2002. Trimethylamine oxide accumulation in marine animals : relationship to acylglycerol storage. J. Exp. Biol. 205: 297-306.

Sikorski, Z. E. and Kolodiejska, I. 1986. The composition and properties of squid meat. Food Chem. 20: 213-224.

Sikorski, Z. E., Kolakowska, A. and Burt, J. R. 1990. Postharvest biochemistry and microbial change. *In* Seafood : resources, nutritional, composition and preservation. (Sikorski, Z.E., ed.). p. 55-57. CRC Press. Boca Raton.

Southgate, P. C. and Lou, D. C. 1995. Improving the *n*-3 PUFA composition of *Artemia* using microcapsules containing marine oils. Aquacuture. 134: 91-99.

Speck, M. L. 1976. Compendium of methods for the microbiological examination of foods. p.417-423. American Puplic Health Association. Washington, DC.

Stanley, D.W. and Hultin, H.O.1982. Quality factors in cooked North Atlantic squid. Can. Inst. Food Sci. Technol. J. 15: 277-282.

Steel, R G. D. and Torrie, J. H. 1980. Principle and procedures of statistics: a biometrical approach. McGraw-Hill. New York.

Sugiyama, M., Kousu, S., Hanabe, M. and Okuda, Y. 1989. Utilization of squid. A. A. Balkem/Rotterdam. Tokyo.

Sweeney, M. J., Roper, C. F. E., Mangold, K. M., Clarke, M. R. and Boleletzky S. U. 1992. “Larval” and Juvenile cephalopods : A manual for their identification. p. 117. Smithsonian Institution Press. Washington, D.C.

Thannhauser, T. W., Konisshi, Y. and Scheraga, H. A. 1987. Analysis for disulfide bonds in peptides and proteins. Methods Enzymol. 143: 115-118.

Thanonkaew, A., Benjakul, S.,Visessangvan, W. and Decker, E. A. 2006a. The effect of metal ions on lipid oxidation, colour and physicochemical properties of cuttlefish (*Sepia pharaonis*) subjected to multiple freeze-thaw cycles. Food Chem. 95: 591-599.

Thanonkaew, A., Benjakul, S., Visessangvan, W. and Decker, E. A. 2006b. Development of yellow pigmentation in squid (*Loligo peali*) as a result of lipid oxidation. J. Agric. Food Chem. 54: 956-962.

Thanonkaew, A., Benjakul, S. and Visessangvan, W. 2006c. Chemical composition and thermal property of cuttlefish (*Sepia pharaonis*) muscle. J. Food Comp. Anal. 19: 127-133.

Thanonkaew, A., Benjakul, S., Visessangvan, W. and Decker, E. A. 2007. Yellow discoloration of the liposome system of cuttlefish (*Sepia pharaonis*) as influenced by lipid oxidation. Food Chem. 102: 219-224.

Thanonkaew, A., Benjakul, S., Visessangvan, W. and Decker, E. A. 2008. The effect of antioxidants on the quality changes of cuttlefish (*Sepia pharaonis*) muscle during frozen storage. LWT- Food Sci. Technol. 41: 161-169.

Thorarinsdottir, K. A., Gudmundsdottir, S., Arason, S., Thorkelsson, and Kristbergsson, K. 2004. Effect of added salt, phosphates and protein on the chemical and physicochemical characteristics of frozen cod (*Gadus morhua*) fillets. J. Food Sci. 69: 144-152.

Ueng, Y. and Chow, C. 1998. Textural and histological changes of different squid mantle muscle during frozen storage. J. Agric. Food Chem. 46: 4728-4733.

United States Environment Protection Agency (US EPA). 1989. Drinking water criteria document for chlorine, hypochlorous acid and hypochlorite ion. Environmental Criteria and Assessment Office. U.S. EPA. Cincinnati.

United States Environment Protection Agency (US EPA). 1994. Chemical summary for chlorine. Office of Pollution Prevention and Toxics. US EPA. Cincinnati.

United States Food and Drug Administration (US FDA). 2004. Code of Federal Regulations. Title 21, Food and drugs. Chapter I. Food and Drug Administration. Part 173-secondary direct food additive permitted in food for human consumption. Section 173.315 Chemicals used in washing or to assist in the peeling of fruits and vegetables. US FDA. Silver Spring.

Vaz-Pires, P. Seixas, P. Mota, M. Lapa-Guimarãaes, J. Pickova, J. Lindo, A. and Silva, T. 2008. Sensory, microbiological, physical and chemical properties of

cuttlefish (*Sepia officinalis*) and broadtail shortfin squid (*Illex coindetii*) stored in ice. LWT- Food Sci. Technol. 41: 1655-1664.

Yamanaka, H., Shiomi., K. and Kikuchi., T. 1987. Agmatine as a potential index for freshness of common squid (*Todarodes pacificus*). J. Food Sci. 52: 936-938.

APPENDIX

Chemical composition of squid muscle

Table 15. Chemical compositions of squid muscle

Composition	g/100 g (wet basis)
Moisture	81.37 ± 0.07*
Protein	13.74 ± 0.09
Ash	0.92 ± 0.01
Lipid	1.35 ± 0.05
Hydroxyproline	0.05 ± 0.01

* Mean ± SD from triplicate determinations

Table 16. Lipid composition in squid muscle

Composition	% of total lipid
Phospholipid	61.46 ± 0.37*
Free fatty acid	20.12 ± 0.29
Triglycerides	18.42 ± 1.01

* Mean ± SD from triplicate determinations

Analytical methods

1. Determination of moisture content (AOAC, 2000)

1.1 Method

1. Dry the empty dish and lid in the oven at 105^{o}C for 3 h and transfer to desiccator to cool. Weigh the empty and lid.
2. Weigh about 3 g of sample to the dish. Spread the sample to the uniformity.
3. Place the dish with sample in the oven. Dry for 3 h at 105^{o}C.
4. After drying, transfer the dish with partially covered lid to the desiccator to cool. Reweigh the dish and its dried sample.

1.2 Calculation

$$\text{Moisture content (\%)} = \frac{(W1-W2)}{W1} \times 100$$

Where: W1 = weight (g) of sample before drying

W2 = weight (g) of sample after drying

2. Determination of protein content (AOAC, 2000)

2.1 Reagents

1. Kjedahl catalyst: Mix 9 part of potassium sulphate (K_2SO_4) with 1 part of copper sulphate ($CuSO_4$)
2. Sulfuric acid (H_2SO_4)
3. 40% NaOH solution (w/v)
4. 0.2 N HCl solution
5. 4% H_3BO_3 solution (w/v)
6. Indicator solution: Mix 100 ml of 0.1% methyl red (in 95% ethanol) with 200 ml of 0.2% bromocresol green (in 95% ethanol)

2.2 Method

1. Place sample (0.5-1.0 g) in digestion flask.
2. Add 5 g Kjedahl catalyst and 20 ml of conc. H_2SO_4.

3. Prepare a tube containing the above chemical except sample as blank. Place flasks in inclined position and heat gently until frothing ceases. Boil briskly until solution clears.
4. Cool and add 60 ml distilled water cautiously.
5. Immediately connect flask to digestion bulb on condenser and with tip of condenser immersed in standard acid and 5-7 indicator in receiver. Rotate flask to mix content thoroughly; then heat until all NH_3 is distilled.
6. Removed receiver, wash tip of condenser and titrate excess standard acid distilled with standard NaOH solution.

2.3 Calculation

$$\text{Protein content (\%)} = \frac{(A-B) \times N \times 1.4007 \times 6.25}{W1}$$

Where: A = volumn (ml) of 0.2 N HCl used sample titration
B = volumn (ml) of 0.2 N HCl used in blank titration
N = normality of HCl
W = weight (g) of sample
14.007 = atomic weight of nitrogen
6.25 = the protein-nitrogen conversion factor for fish and its by-products

3. Determination of ash content (AOAC, 2000)

3.1 Method

1. Place the crucible and lid in the furnace at 550^{o}C overnight to ensure that impurities on the surface of crucible are burned off.
2. Cool the crucible in the desiccator (30 min).
3. Weigh the crucible and lid to 3 decimal places.
4. Weigh about 5 g sample into the crucible. Heat over low Bunsen flame with lid half covered. When fumes are no longer produced, place crucible and lid in furnace.

5. Heat at 550^{o}C overnight. During heating, do not cover the lid. Place the lid after complete heating to prevent loss of fluffy ash. Cool down in the desiccator.
6. Weigh the ash with crucible and lid when the sample turns to gray. If not, return the crucible and lid to the furnace for the further ashing.

3.3 Calculation

$$\text{Ash content (\%)} = \frac{\text{Weight of ash} \times 100}{\text{Weight of sample}}$$

4. Determination of fat content (AOAC, 2000)

4.1 Reagent

1. Petroleum ether

4.2 Method

1. Place the bottle and lid in the incubator at 105^{o}C overnight to ensure that weight of bottle is stable.
2. Weigh about 3-5 g of sample to paper filter and wrap.
3. Take the sample into extraction thimble and transfer into soxhlet.
4. Fill petroleum ether about 250 ml into the bottle and take it on the heating mantle.
5. Connect the soxhlet apparatus and turn on the water to cool them and then switch on the heating mantle.
6. Heat the sample about 14 h (heat rate of 150 drop/min).
7. Evaporate the solvent by using the vacuum condenser.
8. Incubate the bottle at 80-90^{o}C until solvent is completely evaporated and bottle is completely dried.
9. After drying, transfer the bottle with partially covered lid to the desiccator to cool. Reweigh the bottle and its dried content.

4.3 Calculation

$$\text{Fat content (\%)} = \frac{\text{Weight of fat} \times 100}{\text{Weight of sample}}$$

5. Hydroxyproline content (Bergman and Loxley, 1963)

5.1 Reagent

1. 6 N HCl
2. Oxidant solution (the mixture of 7% (w/v)) chlororamine T and acetate/citrate buffer, pH 6 at ratio of 1:4 (v/v))
3. Ehlich's reagent solution (the mixture of solution A (2 g of *p*-dimethylamino benzaldehyde in 3 ml of 60% (v/v) perchloride acid (w/v))
4. Isopropanol
5. Hydroxyproline standard solution (400 ppm)

5.2 Method

5.2.1 Sample preparation

1. Weigh about 0.1-2.0 g sample (depending on type of sample) into screw cap tube.
2. Add 6 N HCl into the sample at the ratio of 1:10 (solid/acid, w/v).
3. Heat at 110^{o}C for 24 h in oil bath.
4. Clarify hydrolysate with activated carbon and filter through Whatman No. 4 filter paper.
5. Neutralize the filtrate with 10 M NaOH and 1 M NaOH to obtain the pH 6.0-6.5

5.2.2 Hydroxyproline determination

1. Transfer 0.1 ml of the neutralized sample into a test tube and add 0.2 ml of isopropanol then mix well.
2. Add 0.1 ml of oxidant solution and mix well.
3. Add 1.3 ml of Ehrlich's reagent solution.
4. Heat the mixture at 60^{o}C for 25 min in the water bath and then cool for 2-3 min in running water.
5. Add isopropanol at ratio of 3:13 (mixture/isopropanol,v/v) and mix well.
6. Read absorbance at 558 nm.

7. Plot the standard curves and calculate the unknown.

Table: Experimental set up for the hydroxyproline's assay

Tube number	Water (µL)	400 ppm Hydroxyproline (µL)	Effective hydroxyproline concentration (ppm)
1	100.0	0.0	0
2	97.5	2.5	10
3	95.0	5.0	20
4	92.5	7.5	30
5	90.0	10.0	40
6	87.5	12.5	50
7	85.0	15.0	60
8	0.0	0.0	unknown

6. Determination of trimethylamine (TMA-N) and total volatile basic nitrogen (TVB-N) by Conway's method (Conway and Byrne, 1936)

6.1 Reagents

1. Inner ring solution (1% boric acid solution containing indicator): Dissolve 10 g of boric in 1 liter flask, add 200 ml of ethanol and make up volume to 1 liter with distilled water.
2. Mixed indicator solution: Dissolve bromocresol green (BCG) 0.01 g and methyl red (MR) 0.02 g in 10 ml of ethanol.
3. 0.02 N HCI
4. Saturated $K_2 CO_3$ solution: Weigh 60 g of potassium carbonate, and add 50 ml of distilled water. Boil gently for 10 min. After cooling down, filter the mixture through filter paper.
5. 4% trichloroacetic acid ($CC1_3 COOH$), TCA, solution: Dissolve 40 g of TCA in 960 ml of distilled water.
6. Sealing agent: Weigh 3 g of Trangacanth gum, add 30 ml of distilled water, 15 ml of glycerine and 15 ml of 50% saturated K_2CO_3 solution and mix well.

7. Neutralized 10% formaldehyde solution: Add 10 g of $MgCO_3$ to 100 ml of formaline (35% formaldehyde solution) and shake in order to neutralize the acidity of formalion. Filter and dilute the filtrate with 3 volume of distilled water.

6.2 Method

6.2.1 Sample extraction:

1. Weigh 2 g of fish meat and place in a mortar and grind well.
2. Add 8 ml of 4% TCA solution and grind well.
3. Stand for 30 min at ambient temperature with occasional grinding.
4. Filter through filter paper (Whatman No. 41) or centrifuge at 3,000 rpm, for 10 min.
5. Keep the filtrate in -20 °C freezing if necessary.

6.2.2 Determination of TVB-N

1. Apply sealing agent to Conway's unit.
2. Pipette 1 ml of inner ring solution into inner ring.
3. Pipette 1 ml of sample extract into outer ring.
4. Slant the Conway's unit with cover.
5. Pipette 1 ml of saturated K_2CO_3 solution into outer ring.
6. Close the unit.
7. Mix gently.
8. Stand for 60 min at 37 °C in incubator.
9. Titrate the inner ring solution with 0.02 N HCI using a micro-burette until green color turns pink.
10. Prepare the blank test using 1 ml of 4% TCA instead of sample extract.

6.2.3 Determination of TMA-N

1. Apply sealing agent to Conway's unit.
2. Pipette 1 ml of inner ring solution into inner ring.
3. Pipette 1 ml of sample extract into outer ring.
4. Pipette 1 ml of neutralized 10% formaldehyde into outer ring.

5. Slant the Conway's unit with cover.
6. Pipette 1 ml of saturated K_2CO_3 solution into outer ring.
7. Close the unit.
8. Mix gently.
9. Stand for 60 min at 37 °C in incubator.
10. Titrate the inner ring solution with 0.02 N HCI using a micro-burette until green color turns pink.
11. Prepare the blank using 1 ml of 4% TCA instead of sample extract.

6.3 Calculation

$$\text{TMA-N or TVB-N (mg N/ 100g)} = \frac{(V_S - V_B) \times (N_{HCl} \times A_N) \times V_E \times 100}{W_S}$$

where: V_S = Titration volume of 0.02 N HCI for sample extract (ml)

V_B = Titration volume

N_{HCl} = Normality of HCI (0.02 N×f, factor of HCI)

A_N = Atomic weight of nitrogen (× 14)

W_S = Weight of muscle sample (g)

V_E = Volume of 4 % TCA used in extraction

7. pH determination (Benjakul et al., 1997)

7.1 Method

1. Weigh 5 g of sample. Add 5 volumes of distilled (w/v).
2. Homogenize for 2 min.
3. Measure pH using pH meter.

8. Triobarbituric acid-reactive substance (TBARS) (Buege and Aust, 1978)

8.1 Reagents

TBARS solution: Mix 0.375 g of thiobarbituric acid, 15 g of trichloroacetic acid and 0.875 ml of hydrochloric thoroughly in 100 ml of distilled water.

8.2 Method

1. Disperse ground sample (0.5 g) in 10 ml of TBA solution.
2. Heat the mixture in boiling water for 10 min, followed by cooling in running tap water.
3. Centrifuge the mixture at 3600xg for 20 min.
4. Measure the absorbance at 532 nm.
5. Prepare a standard curve with malonaldehyde bis (dimethyl acetal) (MDA) at concentration ranging from 0 to 10 ppm.
6. Calculate and express TBARS as mg malondialdehyde/kg.

9. Determination of total sulfhydryl content (Benjakul., 1997)

9.1 Reagents

1. 0.1% 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB)
2. 0.2 M Tris-HCl buffer, pH 6.8 (containing 8 M urea, 2% SDS and 10 mM EDTA)

9.2 Method

1. Mix actomyosin (1 ml, 4 mg/ml) with 9 ml of 0.2 M Tris-HCl.
2. Take 4 ml-aliquot of the mixture and add with 0.4 ml of 0.1% DTNB solution. Incubate the mixture at 40^{o}C for 25 min.
3. Measure the absorbance at 412 nm with spectrophotometer.
4. Prepare a blank by replacing the sample with 0.6 M KCl, pH 7.0.
5. Calculate SH content from the absorbance using the molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$ and express as mole/10^5 g protein.

9.3 Calculation

$$C \text{ (mole}/10^5 \text{ g protein)} = \frac{A}{\varepsilon b}$$

where: C = concentration

A = absorbance at 412 nm

ε = extinction coefficient of 13,600 M^{-1} cm^{-1}

b = path length 1 cm

10. Determination of disulfide bond (Thannhauser et al., 1987)

10.1 Reagents

1. 5,5'- dithiobis-2-nitrobenzoic acid (Ellman's reagent)
2. Na_2SO_3
3. glycine
4. sodium sulfite
5. EDTA
6. NTSB assay solution (Thannhauser et al., 1987)

10.2 Method

1. Add 10-200 µl of protein solution into 3 ml of the NTSB assay solution.
2. Incubate the reaction mixture in the dark for 25 min.
3. Measure the absorbance at 412 nm against a blank.
4. Calculate disulfide bond concentration from the absorbance using the molar extinction coefficient of 13,900 M^{-1} cm^{-1} and express as mole/10^5 g protein.

10.3 Calculation

$$C \text{ (mole/}10^5 \text{ g protein)} = \frac{A}{\varepsilon\, b}$$

where: C = concentration

A = absorbance at 412 nm

ε = extinction coefficient of 13,900 M^{-1} cm^{-1}

b = path length 1 cm

VITAE

Name Miss Rattana Sungsri-in

Student ID 5011020023

Educational Attainment

Degree	Name of Institution	Year of graduation
Bachelor of Science (Food Technology)	Walailak University	2002

Scholarship Awards during Enrolment

Agro-Industry Practice School (AP) from faculty of Agro-Industry, Prince of Songkla University and National Center for Genetic Engineering and Biotechnology.

List of Publication and Proceeding

Sungsri-in, R., Benjakul, S. and Kijroongrojana, K. 2009. Effects of deskinning and squid/ice ratio on pink discoloration and quality changes of squid (*Loligo formosana*) during iced storage. The 10th Annual Conference of Thai Society of Agricultural Engineering. Suranaree University of Technology, Nakhon Ratchasima, Thailand, 1-3 April 2009.

Sungsri-in, R., Benjakul, S. and Kijroongrojana, K. 2009. Pink discoloration and quality changes of squid (*Loligo formosana*) during iced storage. LWT- Food Sci. Technol. (Submitted).