รายงานวิจัยฉบับสมบูรณ์

การเปลี่ยนแปลงสีของเนื้อปลาสวาย (**Pangasius hypophthalmus**) แล่ระหว่างแช่เยือกแข็ง และการป้องกัน

โดย ผู้ช่วยศาสตราจารย์ ดร.โชษณ ศรีเกตุ

มิถุนายน 2563

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ผศ.ดร.โชษณ ศรีเกตุ มหาวิทยาลัยราชภัฏอุบลราชธานี

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและ
มหาวิทยาลัยราชภัฏอุบลราชธานี

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ชื่อนักวิจัย: นายโชษณ ศรีเกตุ สาขาวิชาวิทยาศาสตร์และเทคโนโลยีอาหาร คณะเกษตรศาสตร์ มหาวิทยาลัยราชภัฏอุบลราชธานี

E-mail Address: psriket@hotmail.com

ระยะเวลาโครงการ: 1 กรกฎาคม 2558 ถึง 30 มิถุนายน 2560

งานวิจัยนี้เกี่ยวข้องกับการจำแนกคุณลักษณะทางเคมีกายภาพ ผลของเหล็กต่อการเกิดปฏิกิริยาออกซิเดชันของไขมัน การเปลี่ยนแปลงสี การเกิดสีเหลืองและการป้องกันการเปลี่ยนแปลงคุณภาพของเนื้อปลาสวาย (**Pangasius hypophthalmus**) แล่ระหว่างการเก็บรักษาแบบแช่เยือกแข็ง องค์ประกอบทางเคมีของเนื้อปลาสวายที่ศึกษาได้แก่ องค์ประกอบโดยประมาณ ปริมาณคอลาเจน สารประกอบไนโตรเจนที่เป็นโปรตีนและไม่ใช่โปรตีน องค์ประกอบของไขมัน รูปแบบกรดไขมัน และปริมาณแร่ธาตุ ค่าสีและคุณสมบัติทางความร้อน ผลของกระบวนการแช่เยือกแข็งทำละลายของเนื้อปลาสวายต่อการเกิดการเปลี่ยนแปลงคุณภาพโดยติดตามค่า TBARS ค่าสี พื้นผิวไม่ชอบน้ำ หมู่ซัลฟ์ไฮดริล พันธะไดซัลไฟด์ รูปแบบโปรตีน กิจกรรม ATPase และการละลายของโปรตีน ศึกษาการเกิดออกซิเดชันของไขมัน การเปลี่ยนแปลงสี หมู่เอมีน สารประกอบไพโรล และหมู่ซัลฟ์ไฮดริลของระบบไลโพโซม และไมโครโซมที่เติม $FeCl_3$ และกรดแอสคอร์บิก ศึกษาผลของกรดแทนนิกต่อการป้องกันการเปลี่ยนแปลงคุณภาพของเนื้อปลาสวายแล่ระหว่างการเก็บรักษาแบบแช่แข็ง ดังนั้นวัตถุประสงค์ของงานวิจัยนี้คือเพื่อจำแนกคุณลักษณะทางเคมีกายภาพ ผลของเหล็กต่อการเปลี่ยนแปลงสี และผลของกรดแทนนิกต่อการป้องกันการเปลี่ยนแปลงสี และคุณภาพของเนื้อปลาสวายแล่ระหว่างการเก็บรักษาแบบแช่เยือกแข็ง

เนื้อปลาสวายมีโปรตีนร้อยละ 55.36 และไขมันร้อยละ 40.96 การจำแนกสารประกอบไนโตรเจนพบว่าโปรตีนไมโอไฟบริลเป็นองค์ประกอบหลัก (ร้อยละ 71.81) โดยมีไมโอซิน และแอคตินเป็นโปรตีนหลัก ไตรกลีเซอไรด์เป็นไขมันหลัก (ร้อยละ 80.79) ในเนื้อปลาสวายรองลงมาคือฟอสโฟลิปิด เนื้อปลาสวายมีกรดไขมันชนิดไม่อิ่มตัวเชิงเดี่ยวเป็นกรดไขมันหลัก และมีกรดโอเลอิกและกรดปาล์มิติกในปริมาณสูง พบว่ามี DHA (22:6), EPA (20:5) และ ARA (20:4) ในขันมันจากปลาสวาย แมกนีเซียมพบเป็นแร่ธาตุหลักในเนื้อปลาสวายรองลงมาคือ แคลเซียม สังกะสี และเหล็ก อุณหภูมิสูงสุดในการสูญเสียสภาพธรรมชาติ (T_{max}) ของไมโอซินและแอคตินเท่ากับ 54.33 and 78.00 องศาเซลเซียส ตามลำดับ

การศึกษาผลของเหล็กที่ความเข้มข้นแตกต่างกัน (0-25 ppm) ต่อการเกิดปฏิกิริยาออกซิเดชันของไขมัน ค่าสี และการเปลี่ยนแปลงของโปรตีนปลาสวายระหว่างการแช่แข็งทำละลาย พบว่าค่า TBARS ของทุกตัวอย่างเพิ่มขึ้นเมื่อจำนวนรอบการแช่แข็งทำละลายเพิ่มขึ้น ($p<0.05$) การเพิ่มขึ้นของค่า TBARS ที่เหนี่ยวนำโดยเหล็กขึ้นอยู่กับความเข้มข้นที่ใช้ การเพิ่มขึ้นของการเกิดออกซิเดชันของไขมันในตัวอย่างที่เติมเหล็กสอดคล้องการเพิ่มขึ้นของค่า b* (สีเหลือง) การเพิ่มขึ้นของการเกิด

ออกซิเดชันของโปรตีนและการละลายที่ลดลงเด่นชัดขึ้นเมื่อจำนวนรอบของการแช่แข็งทำละลายเพิ่มขึ้น อย่างไรก็ตามการเติมเหล็กมีผลเพียงเล็กน้อยต่อการเปลี่ยนแปลงดังกล่าว การลดงลงของกิจกรรม Ca^{2+}-ATPase ของแอคโตไมโอซินธรรมชาติพบทุกตัวอย่างเมื่อการแช่แข็งทำละลายเพิ่มขึ้น รูปแบบโปรตีน (SDS-PAGE) พบว่ามีโปรตีนที่มีโมเลกุลใหญ่ในตัวอย่างที่เติมเหล็ก ดังนั้นเหล็กสามารถเหนี่ยวนำให้เกิดสีเหลืองในกล้ามเนื้อปลาสวายร่วมกับการเกิดออกซิเดชันของไขมันโดยเฉพาะเมื่อแช่แข็งทำละลายเป็นจำนวนหลายรอบ

เมื่อระบบไลโพโซมและไมโครโซมถูกออกซิไดซ์ด้วยเหล็กและแอสคอร์เบท ค่า TBARS ค่า b* และสารประกอบไพโรลเพิ่มขึ้นซึ่งสอดคล้องกับหมู่เอมีนที่ลดลง โดยเฉพาะเมื่อปริมาณเหล็ก (25-100 μM) และเวลาในการทำปฏิกิริยาเพิ่มขึ้น (0-20 h) ผลของออกซิไดซ์ไลโพโซมที่ปริมาณต่างกัน (ร้อยละ 1 2 และ 5) ต่อโปรตีนไมโอไฟบริลที่ละลายในเกลือ พบว่าผลิตภัณฑ์จากปฏิกิริยาออกซิเดชันของไขมันส่งผลให้หมู่ซัลฟ์ไฮดริลลดลง และพื้นผิวไม่ชอบน้ำ และปริมาณคาร์บอนิลเพิ่มขึ้น ซึ่งผลการวิจัยนี้ชี้ให้เห็นถึงความสัมพันธ์ของปฏิกิริยาออกซิเดชันกับการเกิดสีเหลืองในกล้ามเนื้อปลาสวาย

การศึกษาผลของกรดแทนนิก (ร้อยละ 0.02-0.08) ต่อการเกิดปฏิกิริยาออกซิเดชันของไขมันและค่าสีของของไลโพโซมปลาสวายระหว่างทำปฏิกิริยาที่อุณหภูมิ 37 องศาเซลเซียส เป็นเวลา 9 ชั่วโมง พบว่าเมื่อไลโพโซมถูกออกซิไดซ์ด้วยเหล็กและแอสคอร์เบท ค่า TBARS ค่า b* และค่าสารประกอบไพโรลของตัวอย่างควบคุมเพิ่มขึ้นซึ่งสอดคล้องกับการลดลงของหมู่เอมีนโดยเฉพาะเมื่อเวลาในการทำปฏิกิริยา (0-9 h) เพิ่มขึ้น กรดแทนนิกสามารถใช้ป้องกันการเกิดปฏิกิริยาออกซิเดชันของไขมันในไลโพโซมโดยลดค่า TBARS และทำให้ค่า L* เพิ่มขึ้น ดังนันกรดแทนนิกโดยเฉพาะที่ความเข้มข้นสูงสามารถใช้ลดการเกิดปฏิกิริยาออกซิเดชันของไขมันและการเปลี่ยนแปลงสีของไลโพโซมปลาสวายระหว่างการทำปฏิกิริยาที่อุณหภูมิ 37 องศาเซลเซียส เป็นเวลา 9 ชั่วโมง

การศึกษาการเปลี่ยนแปลงคุณภาพของเนื้อปลาสวายแล่ที่แช่และไม่แช่กรดแทนนิกที่ความเข้มข้นแตกต่างกัน (ร้อยละ 0-1.0) ระหว่างการเก็บรักษาที่อุณหภูมิ -20 องศาเซลเซียสเป็นเวลา 16 สัปดาห์ พบว่าค่า TBARS ของทุกตัวอย่างเพิ่มขึ้นเมื่อเวลาในการเก็บรักษาเพิ่มขึ้น ($p<0.05$) กรดแทนนิกแสดงความสามารถในการเป็นสารต้านอนุมูลอิสระในปลาสวายแล่แช่แข็งโดยทำให้ค่า TBARS ต่ำลงเมื่อเปรียบเทียบกับตัวอย่างควบคุม การแช่ในสารละลายกรดแทนนิกปรับปรุงสีของเนื้อปลาแล่ให้ดีขึ้นโดยเพิ่มค่าความสว่าง และลดค่าสีเหลืองของปลาแล่ระหว่างเก็บรักษาแบบแช่แข็ง ค่าพื้นผิวไม่ชอบน้ำของแอคโตไมโอซินธรรมชาติของปลาสวายเพิ่มขึ้นเมื่อเวลาในการเก็บรักษาแบบแช่แข็งเพิ่มขึ้นถึง 16 สัปดาห์ การเพิ่มขึ้นของพันธะไดซัลไฟด์สอดคล้องกับการลดลงของปริมาณซัลฟ์ไฮดริล การละลายของโปรตีนค่อยๆ ลดลงเมื่อเก็บรักษานานขึ้น การแช่เนื้อปลาแล่ในสารละลายกรดแทนนิกสามารถช่วยการละลายของโปรตีนและลดการสูญเสียน้ำหนักของเนื้อปลาสวายแล่แช่แข็ง ดังนั้นการแช่ปลาแล่ในสารละลายกรดแทนนิกก่อนการแช่เยือกแข็งสามารถใช้เป็นวิธีป้องกันการเกิดออกซิเดชันของไขมัน และป้องกันการเปลี่ยนแปลงคุณภาพของเนื้อปลาสวายแล่ระหว่างการเก็บรักษาแบบแช่แข็ง

คำหลัก: ปลาสวาย เนื้อปลาแล่ การเปลี่ยนแปลงสี สารต้านอนุมูลอิสระ

Abstract

Project Code: TRG5880010

Project Title: Discoloration of Sawai (**Pangasius hypophthalmus**) fillet during frozen storage and its prevention

Investigator: Mr. Chodsana Sriket, Program in Food Science and Technology, Faculty of Agriculture, Ubon Ratchathani Rajabhat University

E-mail Address: psriket@hotmail.com

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This research covers the physicochemical characteristics, effect of iron on lipid oxidation and discoloration, development of yellow discoloration and prevention of quality changes of Sawai (**Pangasius hypophthalmus**) meat during frozen storage. Chemical composition of Sawai meat including proximate compositions, collagen content, protein and non protein nitrogenous compounds, lipid compositions, fatty acid profiles and mineral content were studied. Color and thermal property were studied. Thiobarbituric acid reactive substances (TBARS), color, surface hydrophobicity, total sulfhydryl content, disulfide bond content, protein pattern, ATPase activity and protein solubility as affected by multiple freeze-thaw of Sawai meat were monitored. Lipid oxidation, discoloration, loss of amine groups, pyrrolization and total sulfhydryl content of Sawai liposome and microsomal systems in the presence of $FeCl_3$ and ascorbic acid were studied. During frozen storage of Sawai fillet, the quality changes as affected by tannic acid were investigated. Therefore, the objectives of this research were to study physicochemical, effects of iron on lipid oxidation, discoloration and physicochemical changes of Sawai muscle as well as to investigate the effect of tannic acid on discoloration and quality changes of Sawai fillet during frozen storage.

Chemical compositions and thermal property of Sawai meat were studied. High protein (55.36% dry basis) and lipid (40.96% dry basis) contents were found in Sawai meat. Fractionation of nitrogenous constituents revealed that myofibrillar protein (71.81%) was the major component in the muscles; myosin heavy chain (MHC) and actin were the predominant proteins. Triglyceride was the main lipid (80.79%) in Sawai meat, followed by phospholipid. Sawai meat had monounsaturated fatty acids as the major component and was rich in oleic acid and palmitic acid. Docosahexaenoic acid (DHA; 22:6), eicosapentaenoic acid (EPA; 20:5) and arachidonic acid (ARA; 20:4) were also found in Sawai lipid. Magnesium (Mg) was the dominant mineral in Sawai meat, followed by calcium (Ca). Zinc (Zn) and iron (Fe) were also found at high concentrations.

Sawai meat exhibited thermal transition temperatures (Tmax) of 54.33 and 78.00ºC for myosin and actin, respectively.

The effects of different iron concentrations (0, 5, 10, 15, 20 and 25 ppm) on the lipid oxidation, color, and protein changes of Sawai pastes subjected to multiple freeze-thaw cycles, were investigated. The thiobarbituric acid reactive substance (TBARS) values of all samples increased as the number of freeze-thaw cycle increased ($p<0.05$). The increase in TBARS value of Sawai pastes induced by the presence of iron was dose dependent. Increases in lipid oxidation of the samples containing iron were concomitant with the increase in b*-value (yellowness). The increase in protein oxidation and decrease in protein solubility were more pronounced when the number of freeze-thaw cycles increased. However, iron at all concentrations exhibited negligible effects on those changes. Decreases in Ca^{2+}-ATPase activity of fish natural actomyosin were found in all samples with increased freeze-thawing processes. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed that proteins with high molecular weight (MW) were observed in the sample added with iron. Therefore, iron induced the yellow discoloration in Sawai muscle, associated with lipid oxidation, particularly with multiple freeze-thaw cycles.

The impact of lipid oxidation on yellow discoloration in Sawai lipids and proteins was studied. When the Sawai liposomes and microsomes were oxidized with iron and ascorbate, thiobarbituric acid reactive substance (TBARS) were observed to increase simultaneously with b* values (yellowness) and pyrrole compounds concomitantly with a decrease in free amines, especially when iron content (25-100 μM) and incubation times (0-20 h) increased. Effect of oxidized liposomes at different contents (1, 2 and 5%) on salt-soluble Sawai myofibrillar proteins was also studied. Lipid oxidation products were able to decrease sulfhydryl content and increase surface hydrophobicity and carbonyl content of salt-soluble Sawai myofibrillar proteins. These results suggest a positive correlation between lipid oxidation and the development of yellow color in Sawai muscle.

The impact of tannic acid (0.02-0.08%) on lipid oxidation and color in Sawai liposomes during incubation at 37 °C for 9 h was studied. When the Sawai liposomes were oxidized with iron and ascorbate, thiobarbituric acid reactive substance (TBARS) in the control samples were observed to increase simultaneously with b* values (yellowness) and pyrrole compounds concomitantly with a decrease in free amines, especially when incubation times (0-9 h) increased. Tannic acid could prevent lipid oxidation and color changes of fish liposomes by decreasing the TBARS value and increasing the L*-value. Therefore, tannic acids, especially high concentration

were able to decrease the lipid oxidation and color changes of the fish liposomes during incubation at 37 °C for 9 h.

Quality changes of Sawai fillet soaked with and without different concentrations of tannic acid (0-1.0%) during frozen storage at -20 °C for 16 weeks were investigated. TBARS in all Sawai samples increased when the storage time increased ($p<0.05$). Tannic acid showed antioxidative effect in frozen fish fillet as indicated by lower TBARS content compared with control sample. Soaking the fish fillet in tannic acid solutions could improve the color of fish fillet by increasing L*-value and decreasing b*-value during frozen storage. Surface hydrophobicity (SoANS) of Sawai natural actomyosin (NAM) increased when the frozen storage period increased up to 16 weeks. The increase in disulfide bond content was generally coincidental with the decrease in sulfhydryl content. Protein solubility decreased slightly during prolonged storage. Soaking Sawai fillet with tannic acid could retard the decreases in solubility and increase in thaw drip of frozen Sawai fillet. Therefore, soaking in tannic acid solution before freezing could be a means to prevent lipid oxidation and quality changes of Sawai fillet during frozen storage.

Keywords: Sawai, fillet, discoloration, antioxidant

EXECUTIVE SUMMARY

Catfish such as Sawai (**Pangasius hypophthalmus**) has been increasingly produced and consumed due to its delicacy and useful nutrients. Viet Nam is largest producer followed by Thailand, Cambodia, Lao People's Democratic Republic, Myanmar, Bangladesh and China (POSMA, 2009). Thailand is an important source of catfish production including Swai, Basa fish and giant catfish. Thailand exported frozen Sawai fillet with a value of 5.47 million baht in year 2011 (AFTA, 2011). Although, the microbiological deterioration is effectively prohibited by frozen storage, various chemical reactions still take place. During frozen storage, fish protein undergoes denaturation and aggregation, leading to decrease in protein functionality such as solubility and water binding ability (Moral **et al.**, 2002; Ruiz-Capillas **et al.**, 2002). Frozen catfish fillet undergo quality deterioration during storage primarily due to discoloration (yellowness), which is sometimes accompanied by the development of a rancid odor. Texture also turns to be toughen and rubbery. Quality deterioration due to discoloration and development of off-flavors in frozen fish causes the rejection of the product and economic loss. It is hypothesized that the contamination of metal ion in fish during living, catching, post harvest handling or processing, may contribute to the acceleration of lipid oxidation of fish muscle. As a consequence, lipid oxidation products such as peroxide, aldehyde, ketone, etc. are formed. Interaction of peroxides or carbonylic peroxide decomposition product with active groups of protein can lead to protein polymerization or aggregation as well as discoloration in fish. Our preliminary study showed that Sawai meat contained high PUFA and it is very susceptible to oxidation. Lipid oxidation is of great concern to the food industry and consumers since it contributes to the development of poorer flavor, color and texture, reduces nutritive value and produces potentially toxic reaction products (Namiki, 1990).

Textural changes and muscle discoloration of some fish species including giant catfish (**Pangasianodon gigas**) (Rawdkuen **et al.**, 2008), herring fillets (Hamre **et al.**, 2003) and Indian catfish fillet (Kunnath **et al.**, 2013) during storage have been reported. Those changes have been reported as a result from lipid oxidation (Kunnath **et al.**, 2013; Rawdkuen **et al.**, 2008; Thanonkaew **et al.**, 2007). To alleviate such a muscle discoloration mediated by lipid oxidation problem, the use of antioxidant has been reported (Bao **et al.**, 2009; Nicolalde **et al.**, 2006; Park **et al.**, 2013). Phenolic compounds have been used to prevent the quality changes of stripe catfish minced, fillet and sausage during storage (Maqsood **et al.**, 2012). However, information regarding lipid oxidation, discoloration and physicochemical changes in muscle proteins as well as the prevention of quality changes by using natural antioxidant in Sawai, especially cultured in Thailand, during

the extended frozen storage is scarce. The knowledge gained can be supportive for a development on the appropriate method to maintain the prime quality of frozen products. As a consequence, frozen fish take a less risk in a rejection from the consumer or importer. Also, Thai products can be competitive in the world market.

Chemical compositions and thermal property of Sawai meat were studied. High protein (55.36% dry basis) and lipid (40.96% dry basis) contents were found in Sawai meat. Fractionation of nitrogenous constituents revealed that myofibrillar protein (71.81%) was the major component in the muscles; myosin heavy chain (MHC) and actin were the predominant proteins. Triglyceride was the main lipid (80.79%) in Sawai meat, followed by phospholipid. Sawai meat had monounsaturated fatty acids as the major component and was rich in oleic acid and palmitic acid. Docosahexaenoic acid (DHA; 22:6), eicosapentaenoic acid (EPA; 20:5) and arachidonic acid (ARA; 20:4) were also found in Sawai lipid. Magnesium (Mg) was the dominant mineral in Sawai meat, followed by calcium (Ca). Zinc (Zn) and iron (Fe) were also found at high concentrations. Sawai meat exhibited thermal transition temperatures (Tmax) of 54.33 and 78.00ºC for myosin and actin, respectively. These data could be useful for consumers and food industry.

The effects of different iron concentrations (0, 5, 10, 15, 20 and 25 ppm) on the lipid oxidation, color, and protein changes of Sawai (**Pangasianodon hypophthalmus**) pastes subjected to multiple freeze-thaw cycles, were investigated. The thiobarbituric acid reactive substance (TBARS) values of all samples increased as the number of freeze-thaw cycle increased ($p<0.05$). The increase in TBARS value of Sawai pastes induced by the presence of iron was dose dependent. Increases in lipid oxidation of the samples containing iron were concomitant with the increase in b*-value (yellowness). The increase in protein oxidation and decrease in protein solubility were more pronounced when the number of freeze-thaw cycles increased. However, iron at all concentrations exhibited negligible effects on those changes. Decreases in Ca^{2+}-ATPase activity of fish natural actomyosin were found in all samples with increased freeze-thawing processes. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed that proteins with high molecular weight (MW) were observed in the sample added with iron. Therefore, iron induced the yellow discoloration in Sawai muscle, associated with lipid oxidation, particularly with multiple freeze-thaw cycles.

The impact of lipid oxidation on yellow discoloration in Sawai (**Pangasianodon hypophthalmus**) lipids and proteins was studied. When the Sawai liposomes and microsomes were oxidized with iron and ascorbate, thiobarbituric acid reactive substance (TBARS) were observed to increase simultaneously with b* values (yellowness) and pyrrole compounds concomitantly with a decrease in free amines, especially when iron content (25-100 μM) and

incubation times (0-20 h) increased. Effect of oxidized liposomes at different contents (1, 2 and 5%) on salt-soluble Sawai myofibrillar proteins was also studied. Lipid oxidation products were able to decrease sulfhydryl content and increase surface hydrophobicity and carbonyl content of salt-soluble Sawai myofibrillar proteins. These results suggest a positive correlation between lipid oxidation and the development of yellow color in Sawai muscle.

The impact of tannic acid (0.02-0.08%) on lipid oxidation and color in Sawai (**Pangasianodon hypophthalmus**) liposomes during incubation at 37 °C for 9 h was studied. When the Sawai liposomes were oxidized with iron and ascorbate, thiobarbituric acid reactive substance (TBARS) in the control samples were observed to increase simultaneously with b* values (yellowness) and pyrrole compounds concomitantly with a decrease in free amines, especially when incubation times (0-9 h) increased. Tannic acid could prevent lipid oxidation and color changes of fish liposomes by decreasing the TBARS value and increasing the L* - value. Therefore, tannic acids, especially high concentration were able to decrease the lipid oxidation and color changes of the fish liposomes during incubation at 37 °C for 9 h.

Quality changes of Sawai (**Pangasianodon hypophthalmus**) fillet soaked with and without different concentrations of tannic acid (0-1.0%) during frozen storage at -20 °C for 16 weeks were investigated. TBARS in all Sawai samples increased when the storage time increased ($p<0.05$). Tannic acid showed antioxidative effect in frozen fish fillet as indicated by lower TBARS content compared with control sample. Soaking the fish fillet in tannic acid solutions could improve the color of fish fillet by increasing L*-value and decreasing b*-value during frozen storage. Surface hydrophobicity (SoANS) of Sawai natural actomyosin (NAM) increased when the frozen storage period increased up to 16 weeks. The increase in disulfide bond content was generally coincidental with the decrease in sulfhydryl content. Protein solubility decreased slightly during prolonged storage. Soaking Sawai fillet with tannic acid could retard the decreases in solubility and increase in thaw drip of frozen Sawai fillet. Therefore, soaking in tannic acid solution before freezing could be a means to prevent lipid oxidation and quality changes of Sawai fillet during frozen storage.

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Chapter 1

Chemical compositions and characteristic of Sawai (**Pangasianodon hypophthalmus**) meat

Abstract

Chemical compositions and thermal property of Sawai meat were studied. High protein (55.36% dry basis) and lipid (40.96% dry basis) contents were found in Sawai meat. Fractionation of nitrogenous constituents revealed that myofibrillar protein (71.81%) was the major component in the muscles; myosin heavy chain (MHC) and actin were the predominant proteins. Triglyceride was the predominant lipid (80.79%) in Sawai meat, followed by phospholipid. Sawai meat had monounsaturated fatty acids as the major component and was rich in oleic acid and palmitic acid. Docosahexaenoic acid (DHA; 22:6), Eicosapentaenoic acid (EPA; 20:5) and Arachidonic acid (ARA; 20:4) were also found in Sawai lipid. Magnesium (Mg) was the dominant mineral in Sawai meat, followed by calcium (Ca). Zinc (Zn) and iron (Fe) were also found at high concentrations. Sawai meat exhibited thermal transition temperatures (T_{max}) of 54.33 and 78.00°C. Sawai meat quality is plausibly dependent upon its compositional and thermal properties.

Keywords: lipid; mineral; **Pangasianodon hypophthalmus**; Sawai; unsaturated fatty acids

1.1 Introduction

Catfish such as Sawai (**Pangasianodon hypophthalmus**) has been increasingly produced and consumed due to its taste and useful nutrients. Viet Nam is largest producer followed by Thailand, Cambodia, Lao People's Democratic Republic, Myanmar, Bangladesh and China (Orban et al., 2008). Sawai is a fast growing species which reached market sizes within 8 months. So, it has become an economically important cultured freshwater fish in Thailand. This fish is mainly exported as frozen fillet. Recently, frozen catfish fillet market has been increasing due to its meat qualities (Wang & Hsieh, 2016). Catfish meat has been also reported as a good source of lipid and unsaturated fatty acids (Wang et al., 2012). However, the chemical composition of fish meat is influenced by various factors such species, size, feeding fish habits, environmental conditions and season (Balçik Misir et al., 2014; Suárez et al., 2015). Variation of chemical compositions and characteristics of fish muscle would affect the nutritional value and the organoleptic properties of fish. For example, marine fish contains high amount of n-3 polyunsaturated fatty acids (n-3 PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) but monounsaturated fatty acid (MUFA) was dominant in freshwater fish (Di Lena et al., 2016; Trbović et al., 2013).

Generally, fish meat quality can be varied with species, more likely governed by compositions and thermal property of tissue. There are many reports on the chemical compositions and muscle characteristics of marine fish species (Balçik Misir et al., 2014; Suárez et al., 2015). However, information on that content of freshwater fish species such as catfish is limited. The understanding of the chemical and thermal properties including lipid class, fatty acid profile and mineral content of Sawai meat could provide the nutritive values of this species. Nevertheless, information regarding chemical compositions and thermal property of Sawai meat, especially cultured in Thailand, is scarce. Therefore, the objective of this research was to study the chemical composition and thermal property of meat from Sawai cultured in Thailand.

1.2 Materials and methods

1.2.1 Chemicals

Chloroform, methanol and sulfuric acid were purchased from Merck (Darmstadt, Germany). Bovine serum albumin (BSA), acrylamide,**N,N,N,N**-tetramethyl ethylene diamine (TEMED) and bis-acrylamide were obtained from Fluka (Buchs, Switzerland). ß-mercaptoethanol (BME) was procured from Sigma (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS), coomassie Blue R-250 and protein molecular weight markers were obtained from Bio-Rad Laboratories (Hercules, CA, USA). All chemicals used were of analytical grade.

1.2.2. Sample preparation

Sawai (**P. hypophthalmus**) with an average weight of 2 kg were obtained from fish farm in Ubon Ratchathani province, Thailand. Three different lots (10 fish each) were used for the study. The live samples were placed in plastic box added oxygen and transported to the Food Science and Technology Program, Ubon Ratchathani Rajabhat University, Ubon Ratchathani province, Thailand. Upon the arrival, fish were immediately washed and filleted. The fillets were placed in polyethylene bags and kept in ice at a meat/ice ratio of 1:3 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla province, Thailand within 9 h. The fillets were ground to obtain uniformity using a blender (Type AY46, Moulinex, Group SEB Thailand Ltd., Bangkok, Thailand). The minced samples were placed in polyethylene bags and kept in ice (0-2°C) until the analyses. The storage time was not longer than 5 h.

1.2.3 Proximate Analysis

Fish meat was analyzed for moisture, protein, fat, and ash contents according to AOAC methods (AOAC, 1999) described in the analytical numbers of 950.46, 920.153, 960.39 and 928.08, respectively. The values were expressed as % of wet weight basis.

1.2.4 Determination of hydroxyproline content

The hydroxyproline content of fish muscle was analyzed according to the method of Bergman and Loxley (1963) with a slight modification. The sample was hydrolyzed with 6 M HCl in a screw cap tube at 110°C for 24 h in an oil bath (model B-490, BUCHI, Flawil, Switzerland). The hydrolyzed sample was clarified with activated carbon and filtered through Whatman No. 4 filter paper. The filtrate was then neutralized with 10 M NaOH to obtain a pH of 6.0–6.5. The neutralized sample (0.1 mL) was transferred into a test tube and isopropanol (0.2 mL) was added and mixed well; 0.1 mL of oxidant solution (mixture of 7% chloramine T and 0.1 M acetate/citrate buffer, pH 6, at a ratio of 1:4 (v/v)) was added and mixed thoroughly. A 1.3 mL of Ehrlich's reagent solution (mixture of solution A; 2 g of **p**-dimethylamino-benzaldehyde in 3 mL of 60% perchloric acid) and isopropanol at a ratio of 3:13 (v/v) were added and mixed. The mixture was heated at 60°C for 25 min in a water bath (Memmert, Schwabach, Germany) and then cooled for 2-3 min using running water. The solution was diluted to 5 mL with isopropanol. Absorbance of the mixture was measured at the wavelength of 558 nm. Hydroxyproline standard solution, with concentration ranging from 0 to 60 ppm, was also included. Hydroxyproline content was calculated and expressed as mg/g of sample.

1.2.5 Determination of protein and non-protein nitrogenous compounds

Non-protein nitrogenous constituents, sarcoplasmic protein, myofibrillar protein, alkali-soluble protein, and stromal protein in fish meat were fractionated according to the method of Hashimoto et al. (1979) Nitrogen content in each fraction was measured by the Kjeldahl method (AOAC, 1999). Protein patterns of different fractions were determined using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) with 10% running gel and 4% stacking gel, as described by Laemmli (1970). Samples (10 μg protein) determined by the Biuret test (Robinson & Hogden, 1940) were loaded. After separation, proteins were stained and destained.

1.2.6 Determination of lipid composition and fatty acid profile

Lipid in the fish was extracted by the Bligh and Dyer method (Bligh & Dyer, 1959). The lipid compositions were determined by thin-layer chromatography/flame ionization detector (TLC-FID). Scanned quartz rods (silica gel powder-coated Chromarod S III) were dipped in 3% boric acid solution for 5 min, dried and rescanned with the TLC-FID analyzer. The sample solution (1 μL) was spotted on the rod and the separation was carried out with the mixture of benzene: chloroform: acetic acid (52:20:0.7) for approximately 35 min. Then, the rods were dried in an oven (105°C) for 5 min before analyzing with the flame ionization detector. The analytical conditions were H_2 flow rate of 160 mL/min, air flow rate of 2000 mL/min and scanning speed of 30 s/scan. Retention time of lipid composition standards was used to identify chromatographic peaks. Peak area was quantitated and expressed as % of total lipid.

The fatty acid compositions were determined as fatty acid methyl esters (FAME) using a gas chromatography, GC-14A (Shimadzu, Kyoto, Japan) equipped with fused silica capillary column Carbowax-30 M (30 m, 0.25 mm ID) and flame ionization detector (FID). Helium was used as the carrier gas at a flow rate of 30 cm/s. The initial temperature of the column was set at 170°C and was increased to 225°C with a rate of 1°C/min and then held at 220°C for an additional 20 min. The detector temperature was set at 270°C, while the temperature at the injection port was maintained at 250°C. Retention time of FAME standards was used to identify chromatographic peaks. Peak area was quantitated and expressed as % of total lipid (AOAC, 1999).

1.2.7 Determination of mineral content

Copper (Cu), manganese (Mn), zinc (Zn), nickel (Ni), iron (Fe), calcium (Ca), magnesium (Mg) and lead (Pb) contents were determined by the inductively coupled plasma optical emission

spectrophotometer (ICP-OES) (Perkin–Elmer, Model 4300 DV, Norwalk, CT, USA) according to the method of AOAC (AOAC, 1999). Ground fish meat (4 g) was mixed well with 4 mL of nitric acid. The mixture was heated on the hot plate until digestion was completed. The digested samples were transferred to a volumetric flask and the volume was made up to 10 mL with deionised water. The solution was subjected to (ICP-OES) analysis. Flow rates of argon to plasma, auxiliary and nebulizer were kept at 15, 0.2, and 0.8 L/min, respectively. Sample flow rate was set at 1.5 mL/min. The wavelengths for analysis of Fe, Cu, Mn, Ni, Zn, Ca, Mg and Pb were 238.2, 327.4, 257.6, 231.6, 206.2, 317.9, 285.2 and 220.4 nm, respectively. The concentration of mineral was calculated and expressed as mg/kg sample.

1.2.8 Differential scanning calorimetry (DSC)

Thermal transitions of Sawai meat were measured using a differential scanning calorimeter (DSC; Perkin–Elmer, Model DSCM, Norwalk, CT, USA). The samples (15-20 mg) were placed in the DSC hermetic pans, assuring a good contact between the sample and the pan bottom. An empty hermetic pan was used as a reference. Calibration was made using Indium thermogram. The samples were scanned at 1°C/min over the range of 20-100°C. Thermal transition temperature (T_{max}) was measured, and the denaturation enthalpies (ΔH) were estimated by measuring the area under the DSC transition curve.

1.2.9 Statistical analysis

For each experiment, fish minces from 10 randomly selected fish were used as the composite sample. The composite sample was divided into 3 replications, each of which were considered experimental units (n = 3). Data were subjected to analysis of variance (ANOVA) and mean comparison was carried out using Duncan's multiple range test (DMRT) (Steel & Torrie, 1980). Statistical analyses were performed using the Statistical Package for Social Science (SPSS 13.0 for windows, SPSS Inc., Chicago, IL).

1.3 Results and discussions

1.3.1 Proximate composition and hydroxyproline content in Sawai meat

Sawai meat contained 70.34% moisture, 16.42% protein, 12.15% fat, 0.86% ash and 0.20 mg/g sample hydroxyproline. High protein (55.36%, dry weight basis) and lipid (40.96%, dry weight basis) contents were found in Sawai meat. These results indicated that Sawai meat could be served as a good source of proteins and lipid. The slight difference in chemical composition was observed between Sawai and that reported for other fish species. Edible parts (dorsal, ventral

and lateral line muscle) of giant catfish (**Pangasianodon gigas**) contained 75.51-81.67% moisture, 14.36-19.00% protein, 0.54-8.60% fat, 1.11-1.47% ash and 0.73-0.83 mg/g hydroxyproline (Chaijan et al., 2010). Trboviḉ et. al. (2013) also reported that proximate composition of common carp (**Cyprinus carpio**) consisted of 71.44-77.47% moisture, 15.14-18.28% protein, 3.02-11.57% fat, and 1.05-1.06% ash. The different chemical content between Sawai and other fish species might be due to the different fish species and feeding conditions. Haard (1992) reported that fish are classified into 4 groups according to their lipid content including lean fish (<2%); low-fat fish (2-4%); medium-fat fish (4-8%) and high-fat fish (>8%). Base on lipid content, Sawai in the present study is classified to high-fat fish. Differences in chemical compositions might be associated with the differences in sensory properties, nutritional value, and shelf-life of different fish.

1.3.2 Proteins and non-protein nitrogenous compounds

The contents of different proteins and non-protein nitrogenous components in Sawai meat are shown in Table 1.

Table 1. Nitrogenous constituents of Sawai meat

Components	Nitrogen content (mg N/g meat)
Non-protein nitrogen	2.36±0.05*
Sarcoplasmic protein	6.05±0.25 (23.36) **
Myofibrillar protein	18.60±0.10 (71.81)
Alkali-soluble protein	0.35±0.07 (1.36)
Stromal protein	0.88±0.02 (3.41)

*Means ± SD (n=3).

**Numbers in parenthesis represent percentage distribution.

Myofibrillar protein (71.81% of total protein) was the major protein in Sawai meat, followed by sarcoplasmic protein (23.36% of total protein). Fish sarcoplasmic proteins mainly consist of myoglobin and enzymes (Hui Y. H., 2012). This finding was in agreement with Chaijan et al. (2010) and Sriket et al. (2013) who reported that myofibrillar and sarcoplasmic proteins were the major proteins in fish and shellfish muscle. Alkali-soluble protein and stromal protein were found as the minor components in Sawai meat. Alkali-soluble protein was reported as a cross-linked myofibrillar protein (Kristinsson et al., 2005), and collagen was presented as the major protein in stroma (Gökoğlu & Yerlikaya, 2015). Sawai meat contained 2.36 mg N/g meat of non-protein

nitrogenous compound. Non-protein nitrogenous constituents including free amino acids, nucleotide, peptides and betaine play a crucial role in fish and shellfish flavor (Sikorski et al., 1990; Sriket et al., 2013). Additionally, the changes in non-protein nitrogenous compounds could be used as a quality index for monitoring the shelf-life of fish. Protein patterns of whole fish meat and different protein fractions from Sawai meat are shown in Fig. 1.

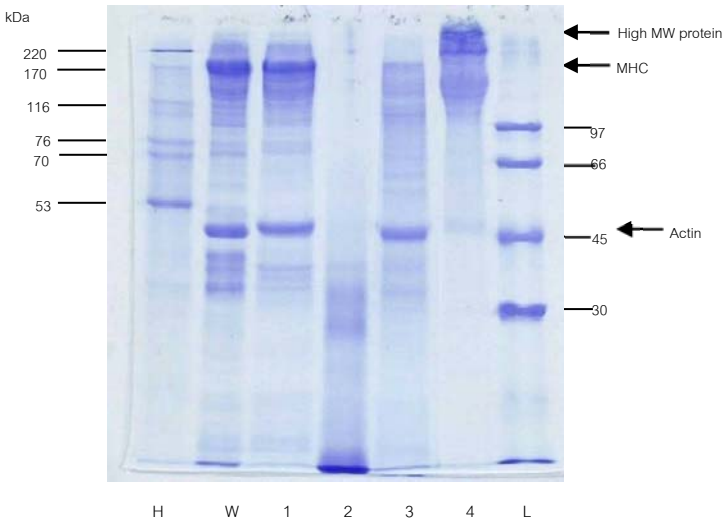

Figure 1 Protein patterns of Sawai meat and various protein fractions (H: high molecular weight protein markers; W: whole meat; 1, 2, 3, 4: myofibrillar, sarcoplasmic, alkali-soluble and stromal protein fractions, respectively; L: low molecular weight protein markers; MHC: myosin heavy chain).

Myosin heavy chain (MHC) was the dominant protein component in Sawai meat, followed by actin. This result was in agreement with previously reported in other species such as Asian hard clam, **Meretrix lusoria**, (Karnjanapratum et al., 2013) and freshwater prawn, **Macrobrachium rosenbergii** (Sriket et al., 2013). MHC and actin are the major proteins in myofibrillar protein and constitute around 40-60% of the total protein in fish and shellfish (Hui, 2012). Two major protein bands, corresponding to MHC and actin, were observed in myofibrillar protein fraction (Fig. 1). Generally, sarcoplasmic protein fraction expressed mainly low-molecular-weight proteins (Fig. 1). For the alkali soluble protein fraction, the protein with MW of 45 kDa was present as the major proteins. Stromal protein fraction contained proteins with MW of 116 and 220 kDa. It was noted that there were some cross-links with high MW protein in this fraction as evidenced by the presence of the protein band close to the stacking gel.

1.3.3 Lipid composition and fatty acid profile of Sawai meat

Table 2. Lipid compositions of Sawai meat

Composition	Content (% of total lipid)
Phospholipid	17.07±0.55*
Triglyceride	80.79±0.28
Diglyceride	1.58±0.06
Monoglyceride	0.45±0.03
Free fatty acid	0.11±0.01

*Means ± SD (n=3).

In general, triglyceride, phospholipids, diglyceride, monoglyceride and free fatty acid were observed in Sawai lipid (Table 2). Triglyceride was the major component (80.79%) of lipid in Sawai meat, followed by phospholipid (17.07%) and traces amounts of diglyceride, monoglyceride and free fatty acid (Table 2). The result was similar to Senphan and Benjakul (2015) who reported that triglyceride was the main component in striped catfish (**P. hypophthalmus**) lipid. It was noted that free fatty acid content was very low in Sawai lipid. This might be due to very low lipase activity in Sawai meat during postmortem storage. However, it was reported that free fatty acid tends to be high in low lipid tissue (Oliveira & Bechtel, 2006). The fatty acid profile of lipid extracted from Sawai meat is shown in Table 3. Oleic acid (18:1) is the most abundant fatty acid (35 g/100 g) in Sawai meat, followed by palmitic acid (16:0). Fish meat also contains many n-3 polyunsaturated fatty acids (n-3 PUFA) and n-6 polyunsaturated fatty acids (n-6 PUFA) such as eicosapentaenoic acid (EPA: C20:5n-3), docosahexaenoic acid (DHA: C22:6n-3) linoleic acid (C18:2n-6) and arachidonic acid (ARA: 20:4n-6). The consumptions of fish oil rich in DHA and EPA have been reported to reduce risk of cardiovascular and neurological diseases (Khawaja, et al., 2014) EPA, DHA and ARA were also reported to maintain cell membrane structure integrity and proved as the precursors of eicosanoids hormones in the human body (Ateş et al., 2013; Inhamuns & Franco, 2008).

Table 3. Fatty acid compositions (g/100g) of lipid from Sawai meat

Fatty acids	Content
Caproic acid C6:0	0.31
Caprylic acid C8:0	0.06
Capric acid C10:0	0.07
Lauric acid C12:0	1.01
Myristic acid C14:0	1.48
Myristoleic acid C14:1	0.03
Pentadecanoic acid C15:0	0.17
Palmitic acid C16:0	23.87
Palmitoleic acid C16:1 n-7	3.85
Heptadcanoic acid C17:0	0.25
Stearic acid C18:0	6.72
Cis-9-Octadecenoic acid C18:1 n-9	35.67
Cis-Vaccenic acid C18:1 n-7	2.32
Cis-9,12-Octadecadienoic acid C18:2 n-6	12.37
Cis-9,12,15-Octadecatrienoic acid C18:3 n-3	0.65
Cis-6,9,12-Octadecadienoic acid C18:3 n-6	0.17
Moroctic acid C18:4 n-3	0.08
Arachidic acid C20:0	0.11
Cis-11-Eicosenoic acid C20:1 n-9	0.67
Cis-11,14-Eicosadienoic acid C20:2 n-6	0.64
Cis-8,11,14-Eicosatrienoic acid C20:3 n-6	0.79

Cis-11,14,17-Eicosatrienoic acid C20:3 n-3	0.10
Cis-5,8,11,14-Eicosatetraenoic acid C20:4 n-6 (ARA)	1.63
Eicosatetraenoic acid C20:4 n-3	0.10
Cis-5,8,11,14,17-Eicosapentaenoic acid C20:5 n-3 (EPA)	0.37
Behenic acid C22:0	0.05
Cis-13-Docosenoic acid C22:1 n-9	0.05
Cis-13,16-Docosenoic acid C22:2	0.03
Cis-4,7,10,13,16,19-docosahexaenoic acid C22-6 n-3 (DHA)	2.51
Tricosanoic acid C23:0	0.04
Lignoceric acid C24:0	0.51
Nervonic acid C24:1	0.04
MUFA	42.58
PUFA	19.44
UFA	62.02
SFA	34.60

MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; UFA: unsaturated fatty acids; SFA: saturated fatty acids.

In addition, as shown in Table 3, MUFAs (42.58 g/100g) were found as the major fatty acids in Sawai meat followed by SFAs (34.60 g/100g) and PUFAs (19.44 g/100g). Caldironi and Manes (2006) revealed that to reduce the risk of cardiovascular disease, a diet should provide a 1.5:1:1 relation among, MUFA, PUFA and SFA. Generally, the fatty acid composition of fish muscle is influenced by fish species, season, diet and wild or cultured types. However, high unsaturated fatty acids (62.02 g/100g, Table 3) found in lipid extracted from Sawai meat might be implied that Sawai meat is susceptible to lipid oxidation during processing and storage. Sawai

fillet exported to European countries is not successful because the yellow discoloration of the fillets due to lipid oxidation has been reported (Khawaja et al., 2014).

1.3.4 Mineral content in Sawai meat

The content of different minerals in the Sawai meats is shown in Table 4.

Table 4 Mineral contents in Sawai meat

Minerals	Content (mg/kg)
Cu	4.36±0.06*
Mn	ND
Zn	50.35±0.03
Ni	ND
Fe	27.41±0.05
Pb	ND
Ca	439.21±6.55
Mg	1,662±11.84

*Means ± SD (n=3).

ND: Non-detectable.

From the results, it was found that Mg (1,662 mg/kg) was the dominant mineral in Sawai meats, followed by Ca (439.21 mg/kg). Thanonkaew et al. (2006) reported that Ca is an essential for muscle contraction, hard tissue structure and as a cofactor for enzymatic activity. Zn (50.35 mg/kg) and Fe (27.41 mg/kg) were also found at high amounts (Table 4). Zn is a metal required by a wide variety of metal-dependent enzymes. Iron is recommended for pregnant woman (Bogard et al., 2015). High content of Ca (1023.71-1225.55 mg/kg), followed by Zn (10.79-17.29 mg/kg) and Fe (6.81-9.34 mg/kg) in plaice (**Pleuronectes platessa**) and garfish **(Belone belone)** was also reported (Staszowska et al., 2013). Low levels of Ca (40.1 mg/kg), Zn (3.48 mg/kg) and Fe (12.0 mg/kg) in African catfish (**Clarias gariepinus**) were found. Calcium ranging from 8.6 to 1,900 mg/100 g, zinc from 0.6 to 4.7 mg/100 g and iron from 0.34 to 19 mg/100 g were reported for fish and shellfish (Bogard et al., 2015). Mineral contents in marine and freshwater fish can vary due to the difference in species, sex, feeding and living eviroment. Major sources of minerals for fish are water and feed.

From the result, Sawai meat would be recommended as good source of minerals due to high content of calcium, zinc and iron (Table 4). However, on the other hand, Fe and Cu have been known as the major catalysts for lipid oxidation (Chaiyasit et al., 2007; Thanonkaew et al.,

2006). Those minerals might contribute to the oxidation of fish meat during processing and storage. High content of Fe found in Sawai meat might be implied that it is susceptible to lipid oxidation. Additionally, high contents of unsaturated fatty acids in Sawai meat (Table 3) might cause the meat to be more prone to oxidation. No toxic mineral such as lead (Pb) was detectable in Sawai meat.

1.3.5 Thermal transition of Sawai meat

T_{max} and ΔH of Sawai meat are shown in Table 5.

Table 5. T_{max} and enthalpy of whole meat from Sawai

Sample	T_{max} I (ºC)	ΔH (J/g)	T_{max} II (ºC)	ΔH (J/g)
Sawai meat	54.33±1.02*	1.17±0.01	78.00±1.40	0.32±0.01

*Means ± SD (n=3).

DSC analysis was used to determine the thermal transition or unfolding temperature of protein and also to quantify the enthalpy of conformational transition (John & Shastri, 1998). Sawai meat revealed two major transition peaks, corresponding to myosin and actin peaks. T_{max} of the first (54.33°C) and second (78.00°C) peaks, with ΔH of 1.17 and 0.32 J/g, respectively, were observed. The result was slightly difference from Techarang and Apichartsrangkoon (2015) who reported two major endothermic transitions, with T_{max} of 56.09 (myosin) and 68.09°C (actin) in **P. hypophthalmus**. Aussanasuwannakul et al. (2012) reported that T_{max} of the first and second peaks of rainbow trout (**Oncorhynchus mykiss**) fillets were 38.89-44.27 and 77.27-78.19°C, respectively. The differences in thermal properties between Sawai meat and other species may be due to the differences in species, season, habitat, temperature, feed, etc. (Aussanasuwannakul et al., 2012; Sriket et al., 2013).

1.4 Conclusion

Sawai meat can serve as a good source of nutrients, especially protein, lipid and minerals for human consumption. Triglyceride was the main component in Sawai lipid. MUFA and oleic acid were the major fatty acids in Sawai lipid. Sawai meat was also rich in unsaturated fatty acids and essential elements. These data would be benefit for consumers and food industry.

Chapter 2

Effect of Iron on Physicochemical Changes of Sawai (**Pangasianodon hypophthalmus**) Pastes During Multiple Freeze-Thaw Cycles

Abstract

Effects of different iron concentrations (0, 5, 10, 15, 20 and 25 ppm) on the lipid oxidation, color and protein changes of Sawai (**Pangasianodon hypophthalmus**) pastes subjected to multiple freeze-thaw cycles, were investigated. TBARS value of all samples increased as the number of freeze-thaw cycle increased ($p<0.05$). The increase in TBARS values of Sawai pastes induced by iron was in a dose dependent manner. Increasing in lipid oxidation of the samples containing iron was concomitant with the increase in b*-value (yellowness). The changes in protein oxidations and protein solubility were more pronounced when freeze-thaw cycles increased. However, iron at all concentrations showed negligible effects on those changes. Decreasing in Ca^{2+}-ATPase activity of fish natural actomyosin was found in all samples with increasing freeze-thawing process. SDS-PAGE showed that proteins with high MW were observed in the sample with added iron. Therefore, iron induced the yellow discoloration in Sawai muscle, associated with lipid oxidation, particularly with multiple freeze-thaw cycles.

Keywords: freeze-thaw cycles; **Pangasianodon hypophthalmus**; iron; Sawai, actomyosin

2.1 Introduction

Catfish, such as Sawai (**Pangasianodon hypophthalmus**), have been increasingly produced and consumed due to its taste and nutritional values. Vietnam is the largest producer followed by Thailand, Cambodia, Lao People's Democratic Republic, Myanmar, Bangladesh and China (Orban et al., 2008). Thailand is an important source of catfish production including Sawai, Basa (**Pangasius bocourti**) and giant catfish (**Pangasianodon gigas**). Normally, catfish have been exported as frozen fillet. Although, frozen storage can effectively prevent the microbiological deterioration, various chemical reactions still take place. During storage and freeze-thaw process, fish fillet undergo quality deterioration (muscle discoloration or yellowness), which is sometimes accompanied by the development of a rancid odor. Texture also tends to become tougher and rubbery. Quality deterioration in frozen fish causes the rejection of the product and economic loss. Textural changes and muscle discoloration of some fish species including giant catfish (**P. gigas**) (Rawdkuen, Jongjareonrak, Benjakul & Chaijan, 2008), herring fillets (Hamre, Lie & Sandnes, 2003) and Indian catfish fillet (Kunnath, Lekshmi, Chouksey, Kannuchamy & Gudipati, 2015) during storage have been reported. Those changes have been reported to result from lipid oxidation (Kunnath et al., 2015; Rawdkuen et al., 2008).

Several hypotheses from different studies showed that the contamination of iron in fish during handling and processing (Thanonkaew, Benjakul, Visessanguan & Decker, 2006) or endogenous iron (Chaijan, 2008) and the released iron from heme protein as affected by storage conditions (Maqsood, Benjakul & Kamal-Eldin, 2012) may contribute to the acceleration of lipid oxidation of fish muscle. Iron and heme proteins have also been reported to have the capacity to promote lipid oxidation in muscle foods (Kathirvel & Richards, 2012; Min & Ahn, 2005). As a consequence, lipid oxidation products such as peroxides, aldehydes, and ketones are formed. Interaction of peroxides or carbonylic peroxide decomposition products with active groups of protein can lead to protein polymerization or aggregation as well as discoloration in fish. A preliminary study showed that Sawai meat contained high amounts of unsaturated fatty acids and it is very susceptible to oxidation. Lipid oxidation is of great concern to the food industries and consumers since it contributes to the development of poor color, odor and texture as well as reduces the nutritive values (Logan, Nienaber & Pan, 2013; Undeland, 2016). Muscle discoloration related with lipid oxidation of some fish species has been reported. However, the information regarding lipid oxidation, discoloration and physicochemical changes in muscle proteins influenced by iron in Sawai during extended frozen storage is limited. Therefore, the objective of this research was to study the effects of iron on lipid oxidation, discoloration and physicochemical changes of Sawai muscle during multiple freeze-thaw cycles.

2.2 Material and methods

2.2.1 Chemicals

Ammonium molybdate, 5-5´-dithio-bis (2-nitrobenzoic acid) (DTNB), adenosine 5´-triphosphate (ATP), **p**-nitrophenyl-α-glucopyranoside, **p**-nitrophenyl-**N**-acetyl-β-D-glucose amide, 1-anilinonaphthalene-8-sulphonic acid (ANS) and iron (II) chloride, thiobarbituric acid (TBA) and malondialdehyde (MDA) were purchased from Sigma (St. Louis, MO, USA). Potassium chloride (KCl), sodium dodecyl sulphate (SDS) and urea were obtained from Ajax Finechem (Wellington, New Zealand).

2.2.2 Sample preparation

Sawai (**P. hypophthalmus**) weighing 2-3 kg, killed by ice-shocking, were purchased from a fish farm in Ubon Ratchathani, Thailand. The fish were kept in ice using a fish/ice ratio of 1:2 (w/w) during transportation to the Program in Food Science and Technology, Faculty of Agriculture, Ubon Ratchathani Rajabhat University. Upon arrival, fish were washed with tap water, filleted, deskinned and cut into slices with a thickness of 1-2 cm. The slices were ground using a blender (Model MX-898N, Panasonic, Panasonic Sdn. Bhd., Kuala Lumpur, Malaysia) to obtain uniform material. Minced fish were frozen in liquid nitrogen and then powdered in a blender. The fish powders were mixed with 8 mL of sterilized deionized water (GenPure^{TM}, Thermo Fisher Scientific, Waltham, MA, USA) (control) or stock solution containing iron ($FeCl_2$) to obtain the final concentrations of 5, 10, 15, 20 and 25 ppm. The fish pastes were packaged in polyethylene bags and frozen at -20 °C using an air blast-freezer for 48 h, followed by thawing using running tap water (25-27 °C) until the core temperature reached 0-2 °C. The mixtures were subjected to different freeze-thaw cycles (0, 1, 2, 3, 5 and 7 cycles).

2.2.3 Determination of thiobarbituric acid reactive substances (TBARS)

TBARS were determined as described by Buege and Aust (1978). Fish paste (2 g), containing various concentrations of iron, was dispersed in 10 mL of thiobarbituric acid solution (0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 N HCl). The mixture was heated in boiling water for 10 min, followed by cooling in running tap water. The mixture was centrifuged at 3600 ×g for 20 min using a refrigerated centrifuge (Model CR22N, Hitachi, Hitachi Koki Co., Ltd., Tokyo, Japan) at 25 °C. The absorbance of the supernatant was measured at 532 nm using a spectrophotometer (Model UV 1800, Shimadzu, Kyoto, Japan). The standard curve was prepared using malondialdehyde (Sigma), and TBARS were expressed as mg malonaldehyde/kg sample.

2.2.4 Determination of color

The fish paste was spread in the sample cup and color was measured through the bottom of the sample cup using a colorimeter (Color Flex, Hunter Lab Inc., Reston, VA, USA) and reported in the CIE color profile system as L*, a* and b*-values.

2.2.5 Determination of surface hydrophobicity

Surface hydrophobicity (SoANS) was determined as described by Benjakul, Seymour, Morrissey and An (1997) using ANS as a probe. Natural actomyosin (NAM) was prepared as described by Benjakul et al. (1997). Fish paste was homogenized in chilled 0.6 M KCl, pH 7.0 at a ratio of 1:10 (w/v) using a homogenizer (IKA, Labortechnik, Selangor, Malaysia). To avoid overheating, the sample was placed in ice and homogenized for 20 s, followed by a 20 s rest interval for a total extraction time of 4 min. The homogenate was centrifuged at 5000 ×g for 30 min at 4 ºC using a refrigerated centrifuge. To the supernatant, three volumes of chilled deionized water (4 ºC) were added to precipitate NAM. The NAM was collected by centrifuging at 5000 ×g for 20 min at 4 ºC. The NAM pellet was dissolved in chilled 0.6 M KCl, pH 7.0 for 30 min at 4 ºC and then centrifuged at 5000 ×g for 20 min at 4 ºC. The supernatant was collected and used as NAM. NAM solution (4 mg/mL) was diluted in 10 mM sodium phosphate buffer, pH 6.0, containing 0.6 M NaCl to produce protein concentrations of 0.125, 0.25, 0.5 and 1 mg/mL, followed by incubation at room temperature (25-28 ºC) for 10 min. The diluted protein solution (2 mL) was mixed with 20 μL of 8 mM ANS in 0.1 M phosphate buffer, pH 7.0 and the fluorescence intensity of ANS-conjugates was immediately measured using a spectrofluorometer (RF-15001, Shimadzu, Kyoto, Japan) at an excitation wavelength of 374 nm and an emission wavelength of 485 nm. The initial slope of the plot of fluorescence intensity versus protein concentration was referred to as SoANS.

2.2.6 Determination of total sulfhydryl content

The total sulfhydryl content was determined using DTNB according to the method of Ellman (1959) as modified by Benjakul et al. (1997). One mL of NAM solution (4 mg protein/mL determined using the Biuret method (Robinson & Hogden, 1940) with bovine serum albumin (Sigma) as the standard assuming 100% purity) was mixed with 9 mL of 0.2 M Tris-HCl buffer, pH 6.8, containing 8 M urea, 2% SDS and 10 mM EDTA. Four mL of the mixture were mixed with 0.4 mL of 0.1% DTNB and incubated at 40 °C for 25 min. The absorbance at 412 nm was measured using the spectrophotometer and a 0.6 M KCl solution was used as a blank. The sulfhydryl content was calculated using the extinction coefficient of 13500 M^{-1} cm^{-1}(Ellman, 1959).

2.2.7 Determination of disulfide bond content

The disulfide bond content was determined using the NTSB assay according to the method of Thannhauser, Konishi and Scheraga (1987). To 0.5 mL of NAM solution (4 mg/mL), 3.0 mL of freshly prepared NTSB assay solution were added. The mixture was mixed thoroughly and incubated in the dark at room temperature for 25 min. The absorbance at 412 nm was measured using the spectrophotometer. The disulfide bond content was calculated using the extinction coefficient of 13900 M^{-1} cm^{-1}(Ellman, 1959).

2.2.8 Determination of Ca^{2+}-ATPase activity

ATPase activity was determined using the method of Benjakul et al. (1997). NAM was diluted to 3–5 mg/mL with 0.6 M KCl, pH 7.0. Diluted NAM solution (0.5 mL) was added to 0.3 mL of 0.5 M Tris–maleate, pH 7.0. Then, 0.5 mL of 10 mM $CaCl_2$ and 3.45 mL of distilled water were added. To initiate the reaction, 0.25 mL of 20 mM ATP were added. The reaction was done for 10 min at 25 ºC and terminated by adding 2.5 mL of chilled (4 ºC) 15% (w/v) trichloroacetic acid. The reaction mixture was centrifuged at 3500 ×g for 5 min and the inorganic phosphate liberated in the supernatant was measured using the method of Fiske and Subbarow (1925). Briefly, the supernatant (250 µL) was mixed with deionized water (625 µL), Elon reagent (1,250 µL) and ammonium molybdate (250 µL). The mixture was incubated at 25 ºC for 45 min. Inorganic phosphate was measured by reading the absorbance at 640 nm. KH_2PO_4 was used as phosphate standard. Specific activity was expressed as µmol inorganic phosphate (Pi) released/mg protein/min. A blank solution was prepared by adding chilled trichloroacetic acid prior to addition of ATP.

2.2.9 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to monitor the polymerization of the modified proteins. The fish paste was solubilized in 5% SDS (1:9, w/v) and dissolved in sample buffer with and without β-mercaptoethanol. SDS-PAGE was done using 4% stacking gels and 10% running gels (Laemmli, 1970). Proteins (15 µg) determined using the Biuret method were loaded onto the gel and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-Protean II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.02% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid for 3 hr and destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid for 15 min, followed by 5% (v/v) methanol and 7.5% (v/v) acetic acid for 3 hr. High molecular weight markers (GE healthcare UK Limited, Buckinghamshire, UK) were used for estimation of MW of protein bands.

2.2.10 Determination of protein solubility

Solubility was determined according to Benjakul and Bauer (2000). To 1 g fish paste, 20 mL of 0.6 M KCl were added and the mixture was homogenized for 1 min at a speed of 12000 rpm, using an IKA homogenizer. The homogenate was stirred at 4 °C for 4 h, followed by centrifuging at 8500 ×g for 30 min at 4 °C. To 10 mL of supernatant, cold (4 °C) 50% (w/v) trichloroacetic acid was added to obtain a final concentration of 10%. The precipitate was washed with 10% trichloroacetic acid and solubilized in 0.5 M NaOH. The fish paste was also directly solubilized using 0.5 M NaOH to determine total protein. Protein content was determined and expressed as the percentage of total protein in the sample.

2.2.11 Statistical analysis

Experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA) and mean comparison was carried out using Duncan's multiple range test (DMRT) (Steel & Torrie, 1980). Statistical analyses were done using the Statistical Package for Social Science (SPSS 13.0 for Windows, SPSS Inc., Chicago, IL, USA). The significance level was set at $p<0.05$.

2.3 Results and discussion

2.3.1 Changes in TBARS values

Changes in TBARS in Sawai paste samples with and without iron are shown in Fig 2. The differences in TBARS values found in Sawai pastes with and without iron addition suggested that iron addition increased TBARS after sample mixing. The catalytic effect of iron and heme protein on lipid oxidation has been reported (Maqsood et al., 2012).

The increase in TBARS for all samples with increasing freeze-thaw cycles was significant ($p<0.05$), with the control sample's TBARS increasing sharply after 2 cycles ($p<0.05$). The repeated ice crystal formation during multiple freeze-thaw cycles could disrupt the muscle structure of the samples (Benjakul et al., 2000). Disruption of muscle food structure induced by multiple freeze-thaw cycles has been reported (Ali, Rajput, Li, Zhang & Zhou, 2016). The freeze–thawing process could affect the physical organization of membrane lipids, which could impact lipid oxidation pathways. As a consequence, the formation of some compounds such as aldehydes and hydrocarbons caused by the lipid oxidation could interact with protein to produce off-colors (Wasowicz, Gramza, Hes, Jeleñ, Korczak & Malecka, 2004). For each freeze-thaw cycle, samples with iron had higher TBARS values compared to control ($p<0.05$). In addition, the rate of lipid oxidation induced by iron was in a dose dependent manner.

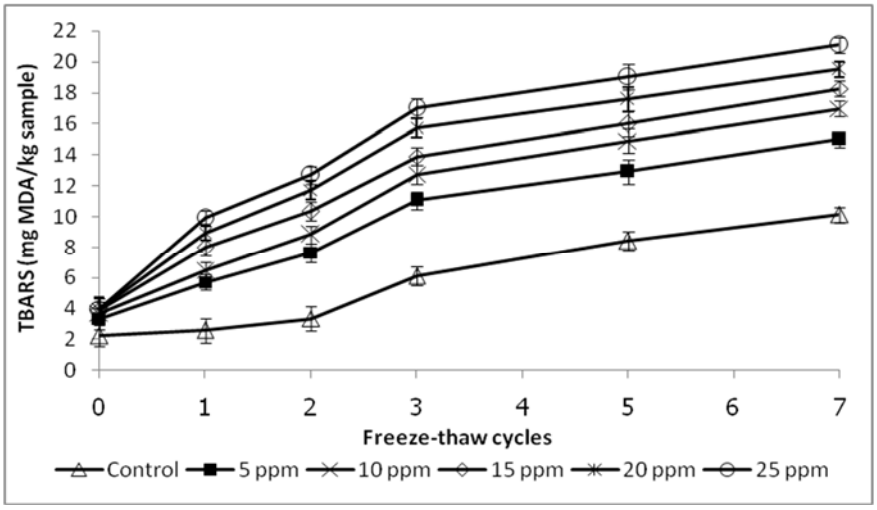

Figure 2 Changes in TBARS values of Sawai paste with different concentrations of iron during multiple freeze-thaw cycles. Bars represent the standard deviation from triplicate determinations.

2.3.2 Changes in colors

Changes in L*, a* and b* values are shown in Fig. 3. After iron addition, there was no difference in L* (lightness), a* (redness) and b* (yellowness) values of the Sawai pastes, compared to the control ($p \geq 0.05$). However, the decrease in L* values of all samples was observed when the number of freeze-thaw cycles increased (Fig. 2a). It was noted that initially, iron added samples had lower L* value than control ($p<0.05$).

The decrease in a* value in all samples was observed when the number of freeze-thawing cycles increased ($p<0.05$) (Fig. 3b). For each freeze-thaw cycle, there was no difference in a* values between samples with iron and controls (Fig. 3b). The decreased a* value in all samples might result from a water bleaching effect. Jeong et al. (2011) reported that a* value and heme pigment content of beef muscle decreased as the numbers of freeze-thawing increased. Pigment degradation can also lead to changes in muscle color (Alonso, Muela, Tenas, Calanche, Roncales & Beltran, 2016). Freeze-thawing effect on drip loss and a* value of muscle has been reported (Alonso et al., 2016; Cheng, Sun & Pu, 2016).

The increase in b* values of all samples increased with freeze-thawing up to 5 cycles ($p<0.05$). However, higher b* values for each cycle with iron compared to the controls were found (Fig. 3c). A positive correlation ($R^2 = 0.97$) between lipid oxidation (TBARS) and yellow color (b*) formation was found in the samples containing iron. In addition, at the first two freeze-thaw cycles, the gradual increase in b* value of controls was correlated ($R^2 = 0.97$) with the slow increase in TBARS value (Fig. 2). Aldehydes, ketones and carbonyl compound products from the oxidation of unsaturated fatty acids can react with free amino groups in protein. These reactions lead to

the formation of discoloration and production of unpleasant odors (Pokorny, 1981; Wsowicz, Gramza, Hes, Jeleñ, Korczak & Malecka, 2004). Fish quality deterioration such as lipid oxidation and discoloration induced by freeze-thawing has been reported (Mousakhani-Ganjeh, Hamdami & Soltanizadeh, 2015; Mousakhani-Ganjeh, Hamdami & Soltanizadeh, 2016). These results indicated that iron addition can facilitate the yellow color formation of Sawai pastes, particularly with increasing numbers of freeze-thaw cycles.

2.3.3 Changes in surface hydrophobicity

Changes in surface hydrophobicity (SoANS) of natural actomyosin extracted from Sawai pastes are shown in Fig. 4a. SoANS gradually increased as the number of freeze-thaw cycles increased, regardless of iron concentration (Fig. 4a). However, after 5 freeze-thaw cycles, SoANS of all samples remained unchanged ($p \geq 0.05$). The hydrophilic residues are generally exposed to water, while the hydrophobic groups are localized in the molecule. Sriket et al. (2007) reported an increase in SoANS of NAM extracted from black tiger and white shrimps during 5 freeze-thaw cycles. The increase in surface hydrophobicity of freeze-thawed Sawai pastes was in agreement with other studies that increased surface hydrophobicity was observed as frozen storage time increased (Kobayashi & Park, 2017; Li, Kong, Xia, Liu & Li, 2013). In addition, some studies also showed that the increased protein oxidation leading to increased hydrophobicity correlated with increased lipid oxidation (Kong, Guo, Xia, Liu, Li & Chen, 2013; Xia, Kong, Liu & Liu, 2009), suggesting that protein structural changes might result from the reaction between functional groups of proteins and oxidation products of polyunsaturated fatty acids (Tokur & Korkmaz, 2007). However, in these experiments, an increase in SoANS was more pronounced when the number of freeze-thaw cycles increased, while the addition of iron had a negligible effect on the SoANS.

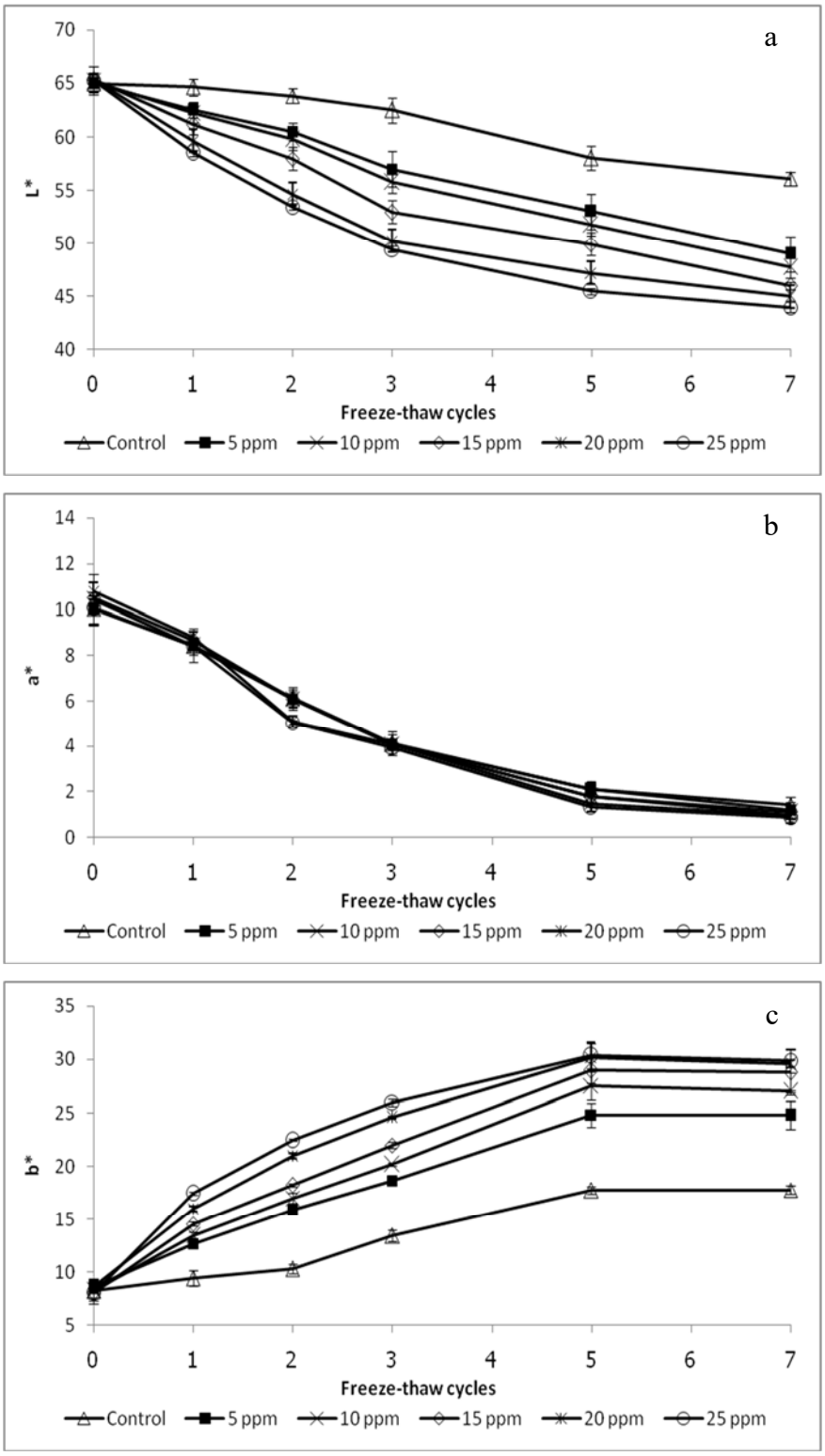

Figure 3 Changes in L* value (a) a* value (b) and b* value (c) of Sawai paste with different concentrations of iron during multiple freeze-thaw cycles. Bars represent the standard deviation from triplicate determinations.

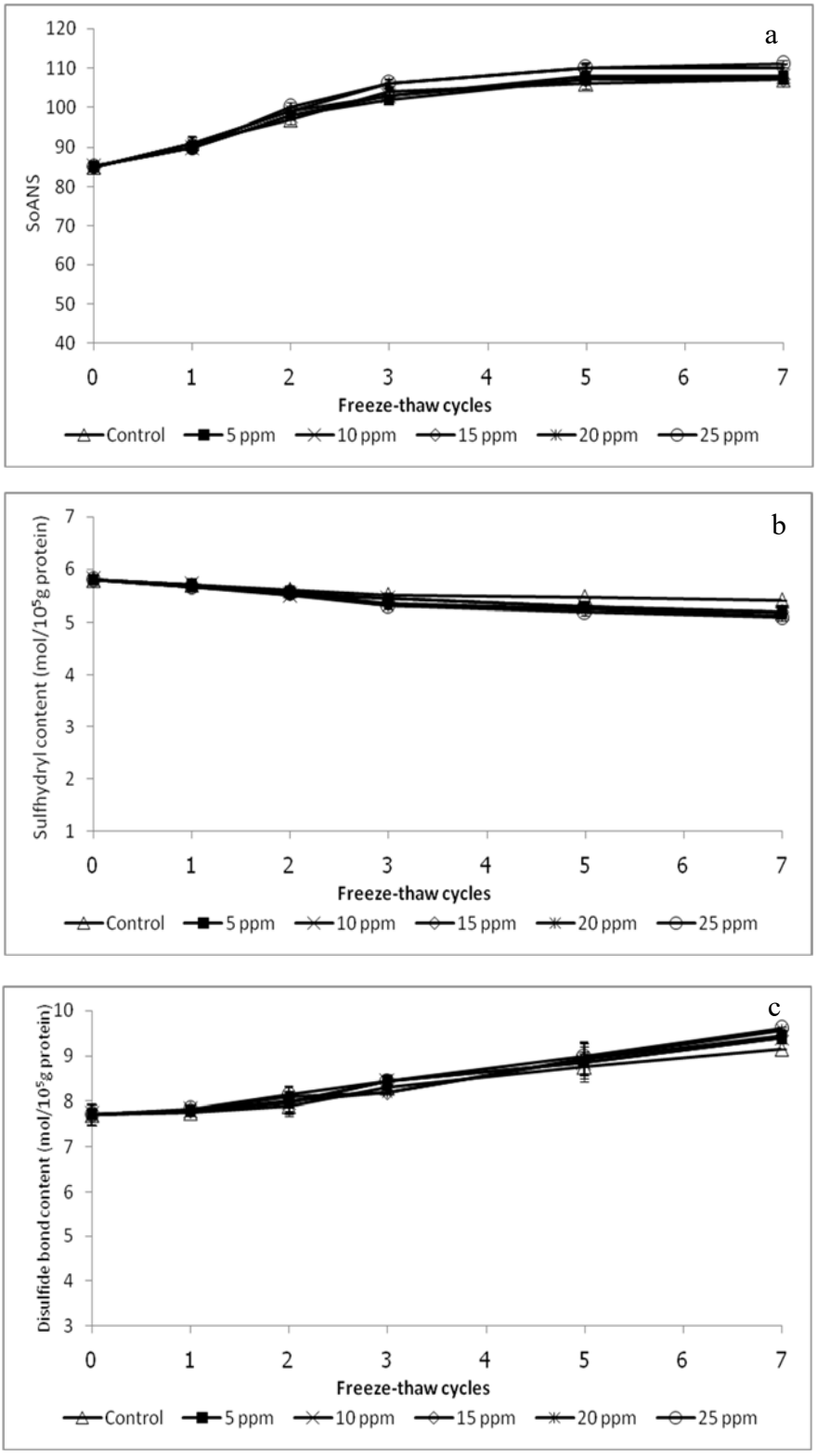

Figure 4 Changes in surface hydrophobicity (SoANS) (a) sulfhydryl content (b) and disulfide bond content (c) of natural actomyosin extracted from Sawai paste with different concentrations of iron during multiple freeze-thaw cycles. Bars represent the standard deviation from triplicate determinations.

2.3.4 Changes in sulfhydryl and disulfide bond contents

Changes in sulfhydryl content and disulfide bond content of Sawai NAM are shown in Fig. 4. Decreasing sulfhydryl content for all samples was observed with increasing freeze-thaw cycles ($p<0.05$) (Fig. 4b). For the first three freeze-thaw cycles, different sulfhydryl contents for all samples were not observed ($p\geq0.05$). However, after three freeze-thaw cycles, samples with iron (5-25 ppm) had slightly lower sulfhydryl content, compared to the control. These findings were slightly different from Thanonkaew et al. (2006) who reported that there is no difference in sulfhydryl content between samples with iron and controls. This might be due to the different specificity of iron to catalyze protein changes with different fish (Tokur et al., 2007).

Generally, disulfide bonds increased in all samples, regardless of iron concentration (Fig. 4c). The increase in disulfide bond content was correlated (R^2 = 0.92) with the decrease in sulfhydryl content (Fig. 4b). However, with the 7th cycle, a higher disulfide bond content was found with iron (5-25 ppm) compared to controls ($p<0.05$). Conversion of sulfhydryl groups into disulfides and other oxidized species can be due to radical-mediated oxidation of protein (Dean, 1997). Decreasing sulfhydryl group content of common carp (**Cyprinus carpio**) surimi as influenced by frozen storage and multiple freeze-thaw cycles has been reported (Kong et al., 2013; Li et al., 2013). Polymerization of high molecular weight protein via disulfide bonding in fish muscle induced by iron was also reported (Tokur et al., 2007).

2.3.5 Changes in Ca^{2+}-ATPase activity

Ca^{2+}-ATPase activity is shown in Fig. 5. A decrease in Ca^{2+}-ATPase activity of all samples with increasing freeze-thaw cycles was observed. Decreasing Ca^{2+}-ATPase activity might be due to the conformational changes and protein polymerization. A decline in Ca^{2+}-ATPase activity of fish sample with increasing frozen storage time indicated myosin denaturation, especially in the head region (Benjakul et al., 2000). Kobayashi and Park (2017) reported that Ca^{2+}-ATPase activity of NAM extracted from frozen tilapia (**Oreochromis niloticus**) decreased when frozen storage time increased. Decreasing Ca^{2+}-ATPase activity of NAM extracted from carp surimi induced by freeze-thawing has also been reported (Kong et al., 2013). The results correlated with the increase in surface hydrophobicity (R^2 = 0.95) (Fig. 4a) and disulfide bond content (R^2 = 0.94) (Fig. 4c). However, there were no differences for Ca^{2+}-ATPase activity between iron and control samples during the first two cycles ($p\geq0.05$). Thereafter, slightly lower Ca^{2+}-ATPase activity with iron, compared to controls was found (Fig. 5). These results suggested that iron affected Ca^{2+}-ATPase activity of Sawai pastes to some degree, specifically as freeze-thaw cycles increased.

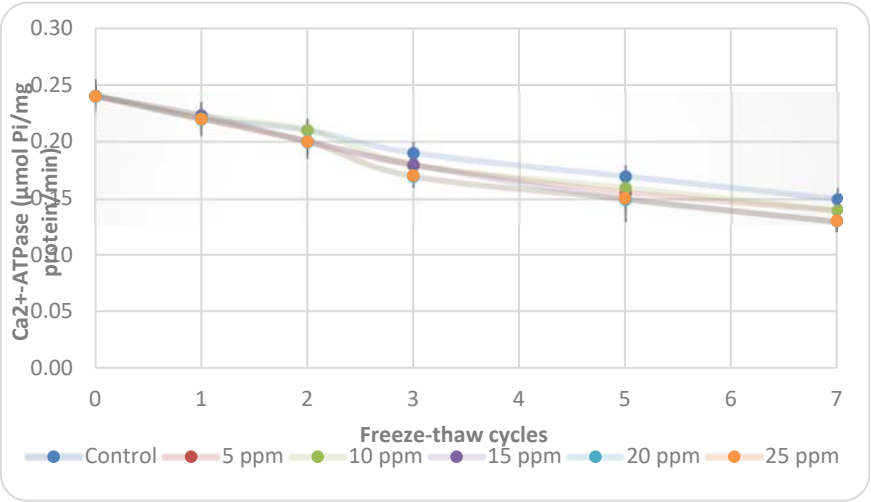

Figure 5 Changes in Ca^{2+}-ATPase activity of natural actomyosin extracted from Sawai paste with different concentrations of iron during multiple freeze-thaw cycles. Bars represent the standard deviation from triplicate determinations.

2.3.6 Changes in protein solubility

Decreases in protein solubility were observed (Fig. 6). The decreased solubility of Sawai pate samples during freeze-thawing correlated (R^2 = 0.92) with the decreased sulfhydryl group content (Fig. 4b) and Ca^{2+}-ATPase activity (Fig. 5) as well as the increased SoANS (Fig. 4a) and disulfide bond content (Fig. 4c). The decrease in protein solubility is used as an indicator of protein denaturation. This may be caused by the formation of hydrophobic and disulphide bonds (Campo-Deano, Tovar & Borderias, 2010). After two freeze-thaw cycles, the samples containing iron showed lower protein solubility ($p<0.05$) than control which correlated (R^2 = 0.99) with lower Ca^{2+}-ATPase activity (Fig. 5). Apart from the effect of freeze-thaw cycles, iron addition might directly affect protein solubility by binding to carboxyl groups. The decrease in protein solubility of common carp surimi during extended frozen storage has been reported (Li et al., 2013). Changes in protein structure and functional properties such as protein polymerization and loss of solubility, caused by oxidation, have also been reported (Ooizumi & Xiong, 2004). Protein oxidation of some fish species (sardine, Atlantic bonito and bluefish) induced by iron addition has also been reported (Tokur et al., 2007).

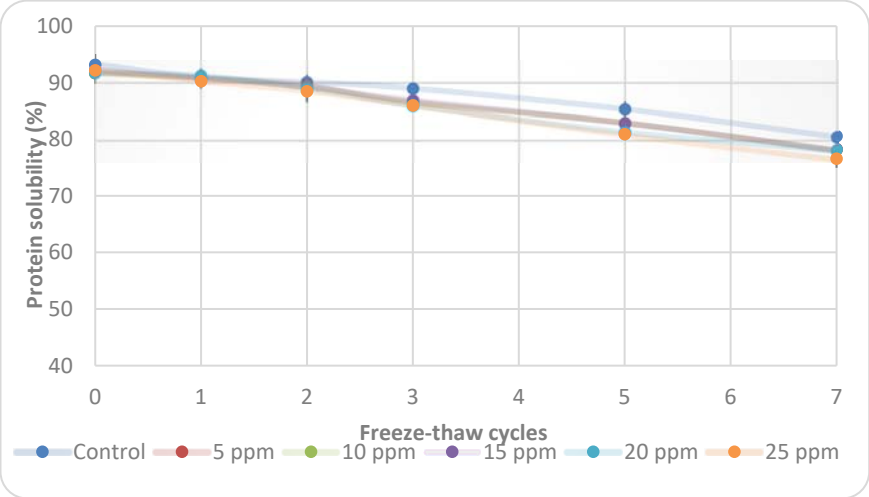

Figure 6 Changes in protein solubility of Sawai paste with different concentrations of iron during multiple freeze-thaw cycles. Bars represent the standard deviation from triplicate determinations.

2.3.7 Changes observed with SDS-PAGE

Protein patterns using SDS-PAGE are shown in Figure 7. The aggregation of Sawai protein induced by iron addition (5-25 ppm) can be seen as evidenced by the formation of high (higher than the myosin heavy chain, MHC) molecular weight polymers with concomitant decreases in myosin and actin intensities (Fig. 6b). Protein aggregation was explained by the loss of sulfhydryls and the formation of disulfide bonds between or within polypeptides (Xia, Kong, Xiong & Ren, 2010).

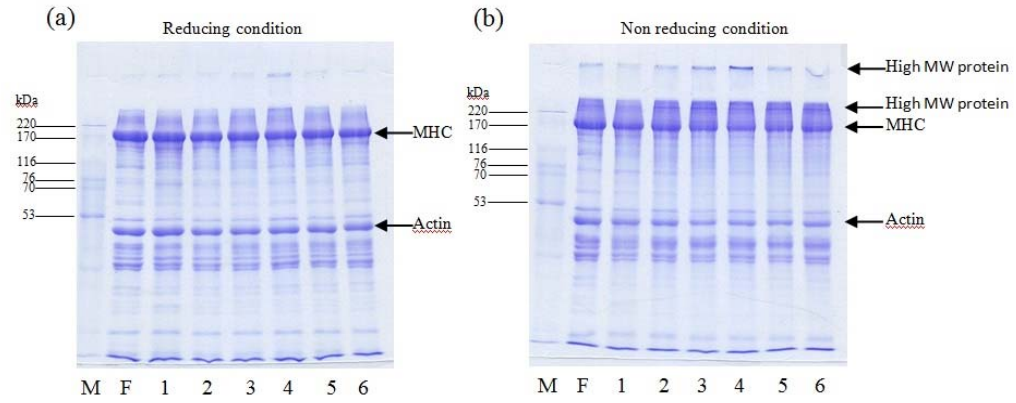

Figure 7 SDS-PAGE pattern of Sawai paste with different concentrations of iron (0-25 ppm) and subjected to multiple freeze-thaw cycles. F: fresh sample, 1: control sample (no iron addition), 2: sample with 5 ppm iron, 3: sample with 10 ppm iron, 4: sample with 15 ppm iron, 5: sample with 20 ppm iron, 6: sample with 25 ppm iron.

The disappearance of polymers and the reappearance of myosin and actin in the presence of reducing agents suggested that the polymers were formed via disulfide linkages between proteins (Fig. 6a). These results confirmed that iron accelerated protein oxidation in Sawai pastes, especially with 7 freeze-thaw cycles. Similar changes in electrophoretic patterns have also been reported (Thanonkaew et al., 2006) in oxidized cuttlefish muscle. No fragmentation could be observed in any of the fish protein samples. Thanonkaew et al. (2006) also did not observe any fragmentation in oxidized cuttlefish protein.

2.4 Conclusion

The addition of iron to Sawai pastes increased lipid oxidation, yellowness (b* value) and the loss of protein functionality during freeze-thawing. Increased lipid oxidation and yellow color of Sawai pastes with iron addition was observed in a dose dependent manner. However, iron had only negligible effects on protein oxidation even with increasing freeze-thaw cycles. The formation of a yellow color (increasing b* values) was due to iron addition, suggesting that lipid oxidation is closely related to yellow discoloration. Therefore, to maintain fish quality, the prevention of the yellow discoloration induced by lipid oxidation should continue to be studied.

Chapter 3

Development of yellow discoloration in Sawai (**Pangasianodon hypophthalmus**) muscle as affected by lipid oxidation

Abstract

The impact of lipid oxidation on yellow discoloration in Sawai (**Pangasianodon hypophthalmus**) lipids and proteins was studied. When the Sawai liposomes and microsomes were oxidized with iron and ascorbate, thiobarbituric acid reactive substance (TBARS) were observed to increase simultaneously with b* values (yellowness) and pyrrole compounds concomitantly with a decrease in free amines, especially when iron content (25-100 μM) and incubation times (0-20 h) increased. Effect of oxidized liposomes at different contents (1, 2 and 5%) on salt-soluble Sawai myofibrillar proteins was also studied. Lipid oxidation products were able to decrease sulfhydryl content and increase surface hydrophobicity and carbonyl content of salt-soluble Sawai myofibrillar proteins. These results suggest a positive correlation between lipid oxidation and the development of yellow color in Sawai muscle.

Keywords: Sawai; yellow discoloration; microsomes; liposome; lipid oxidation

3.1 Introduction

Lipid oxidation in muscle foods is one of the major deteriorative reactions causing losses in quality during processing and storage. The lipid oxidation process leads to discoloration, protein denaturation and off-flavor (Utrera, Parra, & Estévez, 2014). During the oxidation of lipids, carbonyl compounds such as aldehydes and ketones are formed through the degradation of lipid hydroperoxides. Among the secondary products, aldehydes have received the most attention because of their off-flavors and reactivity with groups such as amino acids (Vieira, Zhang, & Decker, 2017). Nonenzymatic browning has long been recognized as a consequence of oxidizing lipids in the presence of protein (Potes, Lim, & Roos, 2017). Most investigators theorize that nonenzymatic browning in muscle foods during lipid oxidation starts with the condensation of aldehydes with amines via Schiff base reaction pathways (Muhasinath, Mehta, Prasadnaik, Chouksey, & Nayak, 2019; Thanonkaew, Benjakul, Visessanguan, & Decker, 2006)

Frozen catfish such as Sawai (**Pangasianodon hypophthalmus**) have been increasingly produced and consumed worldwide. Although the microbiological deterioration is effectively retarded by frozen storage, various chemical reactions still take place (C. Sriket, Benjakul, & Senphan, 2019). Yellow pigment formation sometimes occurs during frozen storage of fish, accompanied by the development of rancid odours (Thanonkaew, Benjakul, Visessanguan, & Decker, 2006; Yerlikaya & Gökoğlu, 2010). Textural changes and muscle discoloration of some fish species including Basa (**Pangasius bocourti**) fillet (P. Sriket & La-ongnual, 2018), rainbow trout (**Oncorhynchus mykiss**) muscle (Burgaard & Jørgensen, 2011), Cod (**Gadus morhua**) muscle salmon muscle (Pahila, Kaneda, Nagasaka, Koyama, & Ohshima, 2017) and common carp (Cyprinus carpio) fillets (Li, Qin, Zhang, Li, Prinyawiwatkul, & Luo, 2019) during storage have been reported. Pahila, Kaneda, Nagasaka, Koyama, and Ohshima (2017) and Mi, Guo, and Li (2016) report that these changes occurred as a result of lipid oxidation. Sawai muscle has very high lipid content (C. Sriket, Benjakul, & Senphan, 2017). Those lipids have a high content of unsaturated fatty acids. As a consequence, Sawai lipids are susceptible to oxidation and their oxidation can lead to the muscle discoloration. The quality deterioration due to discoloration in frozen fish causes the rejection of the product has been reported (C. Sriket, Benjakul, & Senphan, 2019). So, this research focused on gaining a better understanding on how these processes occur. Yellow pigment formation was studied in both Sawai protein and lipid fractions. Therefore, objective of this study was to investigate the effect of lipid oxidation on the formation of yellow pigments in the microsomes and liposomes systems of Sawai cultured in Thailand.

3.2 Material and Methods

3.2.1 Chemicals

Ferric (III) chloride, butylated hydroxytoluene (BHT), L-ascorbic acid, thiobarbituric acid (TBA), monopotassium dihydrogen phosphate, dipotassium hydrogenphosphate, **p**-dimethylamino benzaldehyde, 2,4,6-trinitrobenzenesulfonic acid (TNBS), Triton X-100, and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co. (St. Louis, MO). Hydrochloric acid, methanol and chloroform, were obtained from Merck (Darmstadt, Germany). All chemicals were of analytical grade.

3.2.2 Sample preparation

Sawai (**P. hypophthalmus**) weighing 2-3 kg, killed by ice-shocking, were purchased from a fish farm in Ubon Ratchathani, Thailand. The fish were kept in ice using a fish/ice ratio of 1:2 (w/w) and transported to the Program in Food Science and Technology, Faculty of Agriculture, Ubon Ratchathani Rajabhat University, Ubon Ratchathani province, Thailand within 1 hr. Upon arrival, the fish were washed with tap water, filleted, deskinned and cut into slices with a thickness of 1-2 cm. The slices were minced to obtain a uniform mixture.

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

Sawai muscle microsomes were isolated according to the method of Brannan (2011). Fish minced (25 g) was homogenized in 100 mL of 0.12 M KCl/25 mM phosphate buffer, pH 7.2, in a Tissuemizer (20000 rpm; Tekmar, Cincinnati, OH, USA) for 2 min, followed by centrifugation for 30 min at 10000 xg at 4 °C (Sorvall Superspeed RC2-B, Newton, CT, USA). The resulting supernatant was ultracentrifuged for 60 min at 100000 xg (Sorvall Ultra 80, DuPont, Wilmington, DE, USA) to pellet insoluble muscle components including the microsomes. Myofibrillar proteins were then solubilized from the pellet in 0.6 M KCl/25 mM phosphate buffer, pH 7.2, and a microsome-containing pellet was isolated by ultracentrifugation for 60 min at 100000 xg. Protein in the microsomal fraction was determined by using the method of Lowry, Rosebrough, Farr, and Randall (1951). Isolated microsomes were standardized to 30 mg of protein/mL of 0.12 M KCl/25 mM phosphate buffer, pH 7.2, and stored at -80 °C until use.

The lipids from the Sawai microsomes were extracted by homogenizing 1 part microsome with 5 parts solvent (chloroform/methanol, 2:1) for 2 min. The solvent phase was then collected and evaporated under nitrogen. Liposomes were prepared from the isolated Sawai phospholipids according to the method of Decker and Hultin (1990). The fish microsome lipid (5 mg/mL) was

dispersed in 0.12 M KCl/25 mM potassium phosphate buffer, pH 7.2, with a Potter-Elvehjem homogenizer follow by sonication (35% amplitude with 5 sec repeating cycle; Sonicator, Sonic Dismembrator, model 500, Fisher Scientific, Pittsburg, PA, USA) in an ice bath for 30 min.

Isolation of salt-soluble myofibrillar proteins (SSP) was accomplished according to the method of Benjakul et al. (1997). NAM pellet was mixed thoroughly with glycerol to a final concentration of 30% (v/v) glycerol and was stored at -80 °C. Prior to analysis, the frozen NAM was thawed with cool running tap water. The glycerol was removed by mixing the thawed NAM with 10 volumes of chilled water followed by gentle stirring at 4 °C for 30 min. The mixture was centrifuged at 7500 xg at 4 °C for 30 min. The resulting NAM was kept in ice until use. Protein concentration in the NAM was determined according to the Biuret method as described by Robinson and Hogden (1940).

3.2.4 Lipid oxidation of liposomes or the microsomal fraction of Sawai muscle

Lipid oxidation in liposomes or the microsomal systems was accelerated with a nonenzymatic iron redox cycling system according to the method of Thanonkaew, Benjakul, Visessanguan, and Decker (2006) with slight modification. The reaction medium contains a final concentration of 200 μM ascorbate and 5 mg of fish microsomal protein or 5 mg of lipid (for liposomes)/mL of 25 mM potassium phosphate/0.12 M KCl buffer, pH 7.2 in the presence of $FeCl_3$ at various levels (25, 50 and 100 μM). The assay medium was sampled at various times and analyzed for thiobarbituric acid reactive substances (TBARS), color, free amine, and pyrrolization as described below.

To directly assess the impact of lipid oxidation products on the chemical characteristics of SSP or NAM, the solutions containing the highest level lipid oxidization products were added directly to SSP at different levels (1, 2 and 5%). Control contains an equivalent amount of water. All samples were incubated in a shaking incubator (INOVATM 4080, New Brunswick Scientific, Edison, NJ, USA) at 37 °C under atmospheric conditions for 9 h. The samples were determined for sulfhydryl content, surface hydrophobicity and carbonyl content as described below.

3.2.5 Measurement of chemical and physical alterations in the microsomes, liposomes and salt-soluble myofibrillar proteins (SSP).

3.2.5.1 Lipid oxidation was monitored by measuring TBA reactive substances (TBARS) as determined by a modification of the procedure of MCDONALD and HULTIN (1987). TBA stock solution consisted of 15% trichloroacetic acid (w/v) and 0.375% TBA (w/v) in 0.25 M HCl. To 100 mL of TBA stock solution was added 3 mL of 2% BHT in ethanol. Microsomes (1.0 mL) were

added to 2 mL of the TBA solution, vortexed, heated in a boiling water bath for 15 min, cooled to room temperature, and centrifuged at 1600**g** for 20 min. The absorbance of supernatant was measured at 532 nm, and the results were reported as micromoles of TBARS per milligram of microsomal protein. TBARS concentrations were determined from a malonaldehyde standard curve produced from 1,1,3,3-tetraethoxypropane.

3.2.5.2 The color of microsomes and liposomes was measured, using a colorimeter (HunterLab, Model ColorFlex, Virginia, USA), and recorded by using the CIE color system profile of L*, a* and b*.

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

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

3.2.5.5 Total sulfhydryl content of the SSP was determined using 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) according to the method of Ellman (1959) as modified by Nikoo, Benjakul, Gavlighi, Xu, and Regenstein (2019). One milliliter of SSP solution (5 mg/mL) was mixed with 9 mL of 0.2 M Tris-HCl buffer, pH 6.8, containing 8 M urea, 2% SDS, and 10 mM EDTA. Four milliliters of the mixture was mixed with 0.4 mL of 0.1% DTNB and incubated at 40 °C for 25 min. The absorbance at 412 nm was measured using a 0.6 M KCl solution as a blank. Sulfhydryl content was calculated using the extinction coefficient of 13500 M^{-1} cm^{-1}.

3.2.5.6 Surface hydrophobicity (SoANS) of the SPP was determined using the method of Kaewmanee, Benjakul, and Visessanguan (2011) using ANS as a probe. SSP solution (4 mg/mL)

was diluted in 10 mM sodium phosphate buffer, pH 6.0, containing 0.6 M NaCl to produce protein concentrations of 0.125, 0.25, 0.5 and 1 mg/mL, followed by incubation at room temperature for 10 min. The diluted protein solution (2 mL) was mixed with 20 μL of 8 mM ANS in 0.1 M phosphate buffer, pH 7.0 and the fluorescence intensity of ANS-conjugates was immediately measured using a spectrofluorometer (RF-15001, Shimadzu, Kyoto, Japan) at an excitation wavelength of 374 nm and an emission wavelength of 485 nm. The initial slope of the plot of fluorescence intensity versus protein concentration was referred to as SoANS.

3.2.5.7 Carbonyl content of SSP was determined by derivatization with DNPH (2,4-dinitrophenyl hydrazine) according to Larsson and Undeland (2010) with some modifications as described by Larsson and Undeland (2010). SSP (0.5 g) was homogenized in the Ultra Turax in 10 mL of buffer (50 mM Tris, 1 mM EDTA, pH 7.4) containing 0.01% BHT. Then 300 μL of the homogenate was precipitated with 0.5 mL of TCA (30%). The mixture was centrifuged at 13,400 xg for 3 min at 4 ºC and the pellet was incubated with 10 mM 2,4-dinitrophenylhydrazine (DNPH) dissolved in 2 M HCl for 60 min. A blank was made by adding an equal volume of 2 M HCl instead of DNPH solution. The samples were precipitated with 0.5 mL of TCA (30%) and the excess DNPH was removed by washing with 1.0 ml of ethanol–ethyl acetate (1:1) with 10 mM HCl. The protein pellets were finally dissolved in 1 mL of 6 M guanidine chloride in 20 mM KH_2PO_4 (pH 2.3) and left overnight at 4 ºC. The carbonyl concentration (nmol/mg protein) was calculated from the absorbance at 280 nm and 370 nm of the samples by using an absorption coefficient at 370 nm of 22,000 M^{-1} cm^{-1} for the formed hydrazones.

3.2.6 Statistical analysis

Experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out using Duncan's multiple range test (DMRT) Steel and Torrie (1980). Statistical analyses were done using the Statistical Package for Social Science (SPSS 13.0 for Windows, SPSS Inc., Chicago, IL, USA).

3.3 Results and discussion

3.3.1 Changes in TBARS of Sawai liposome and microsome

Lipid oxidation and yellow discoloration in fish muscle is a major cause of quality deterioration. Previous studies have shown that yellow pigments can form during the oxidation of fish muscle. The changes in TBARS of Sawai liposome and microsome during incubation at 37 ºC for 20 h in the presence of 200 μM ascorbic acid and various concentrations of $FeCl_3$ (0-100 μM) are shown in figure 8a and b. The increases in lipid oxidation (as determined by TBARS) in

both Sawai liposome and microsomes as concentration of iron and reaction time increased were observed (Fig. 8). The ferric state (Fe^{3+}) can be reduced by ascorbic acid to produce the very prooxidative ferrous state (Fe^{2+}) of iron. The ferrous state of iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals via a Fenton type reaction(Bhawya & Anilakumar, 2010). Lipid oxidation of Sawai liposomes and microsomes in the presence of 100 μM $FeCl_3$ was approximately six-fold greater than that of control samples.

The increases in TBARS of squid liposome and microsome as temperatures and reaction times increased were also reported (Thanonkaew, Benjakul, Visessanguan, & Decker, 2006; Thanonkaew, Benjakul, Visessanguan, & Decker, 2007). Peroxidation of liposomal lipids is affected by many factors such as inducing of peroxidation by the composition and physical properties of the liposomes, with respect to both oxidizable and non-oxidizable lipids, and the antioxidants, when present in the system (Schnitzer, Pinchuk, & Lichtenberg, 2007).

3.3.2 Changes in colors of Sawai liposome and microsome

The changes in colors of Sawai liposome and microsome during incubation at 37 ºC for 20 h in the presence of 200 μM ascorbic acid and various concentrations of $FeCl_3$ (0-100 μM) are depicted in figure 9 and 10. Following iron and ascorbate addition, there was no difference in L*, a* and b* values in Sawai liposomes compared to the control sample ($p \geq 0.05$). However, a decrease in the L* values of all samples was observed as the reaction times increased (Fig. 9a). No changes in the a* values in all samples were observed during the incubation ($p > 0.05$) (Fig. 9b). The b* values of all samples increased when the reaction times increased ($p < 0.05$). However, higher b* values were found in the liposome with added iron compared to the control sample (Fig.9c). A positive correlation between lipid oxidation (TBARS) (Fig. 8a) and yellow color (b*) was found in the liposomes containing iron. The same trend in colors changes of Sawai microsomes with Sawai liposomes during incubation at 37 ºC for 20 h in the presence of 200 μM ascorbic acid and various concentrations of $FeCl_3$ (0-100 μM) was found (Fig. 10).

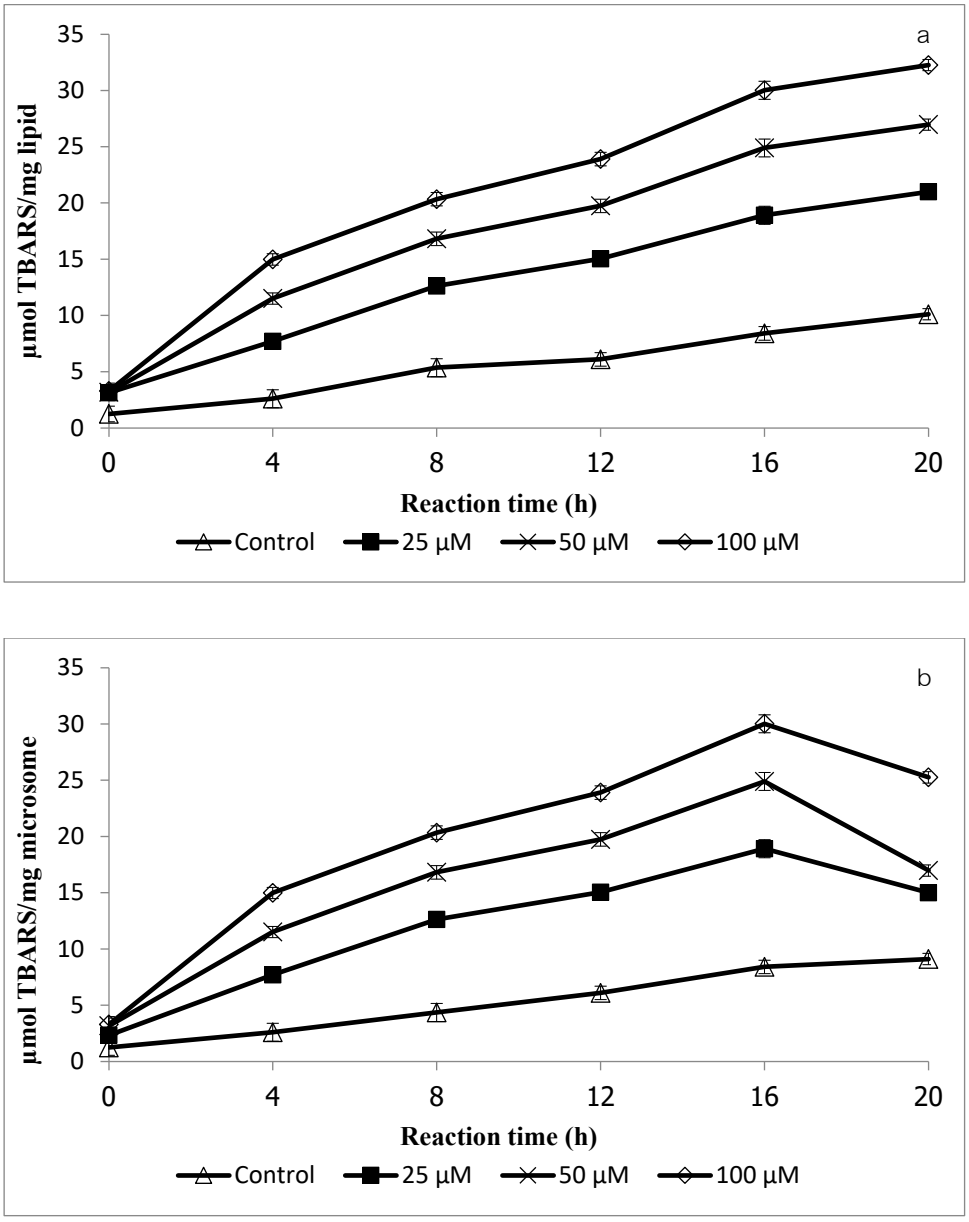

Figure 8 Formation of thiobarbituric acid-reactive substances (TBARS) in Sawai muscle microsomes (5 mg microsomal protein/mL) (a) and liposome made from Sawai lipids (5 mg lipid/mL) (b) in the presence of 200 µM ascorbic acid and various concentrations of $FeCl_3$ (0-100 µM) during incubation at 37 ºC for 20 h. Error bars indicate standard deviations from triplicate determinations.

The above results suggest that yellow pigment formation in pigment formation in Sawai could be due to nonenzymatic browning reactions between fatty acid decomposition products and phospholipid head groups. However, browning could also occur as a result of interactions between fatty acid decomposition products and the amines in proteins (Benjakul & Visessanguan,

2010). Aldehydes and carbonyl compounds, i.e. heptanal, hexanal, octanal etc., products from the oxidation of unsaturated fatty acids, can react with free amino groups in protein. These reactions lead to discoloration and the production of unpleasant odor (Ross & Smith, 2006). Lipid oxidation in squid liposomes and microsomes related to yellow discoloration has been reported (Thanonkaew, Benjakul, Visessanguan, & Decker, 2006; Thanonkaew, Benjakul, Visessanguan, & Decker, 2007). Not surprisingly, lipid oxidation and yellow discoloration in both Sawai liposomes and microsomes were more pronounced at higher concentration of iron and reaction time, since iron is an important catalyst for lipid oxidation and browning of muscle food.

3.3.3 Changes in amine groups and pyrrole compounds of Sawai liposome and microsome

The changes in amine groups and pyrrole compounds of Sawai liposome and microsome during incubation at 37 ºC for 20 h in the presence of 200 μM ascorbic acid and various concentrations of $FeCl_3$ (0-100 μM) are shown in figure 11 and 12. Changes in free amines was used to be an indicator for the interactions between phospholipids and lipid oxidation products (Hidalgo et al., 2004; Kubo et al., 2005). In the presence of ascorbic acid and various concentrations of iron, the loss of amine ($p<0.05$) was observed in both Sawai liposomes (fig. 11a) and microsomes (fig. 12a). The loss of free amines in both Sawai liposomes and microsomes occurred over a similar time period in the presence of ascorbate and iron, suggesting that lipid oxidation products could be involved in the formation of yellow pigments in Sawai muscle.

In the presence of ascorbic acid and various concentrations of iron, the increase of pyrroles ($p<0.05$) was also observed in both Sawai liposomes (fig. 11b) and microsomes (fig. 12b). It was noted that the slow increase in pyrroles in the control sample of both liposomes and microsomes systems was observed, especially after 8 h of reaction time. This is presumably that temperature and time also are the important factors for lipid oxidation. Pyrroles formed in the reaction between oxidized lipids and protein are important precursors of browning (Zamora & Hidalgo, 2011). Simultaneously with TBARS and yellow pigment formation, free amines decreased and pyrrole compounds were formed in the presence of ascorbate and iron, suggesting that lipid oxidation products were reacting with amines to produce yellow pigments (Thanonkaew, Benjakul, Visessanguan, & Decker, 2007).

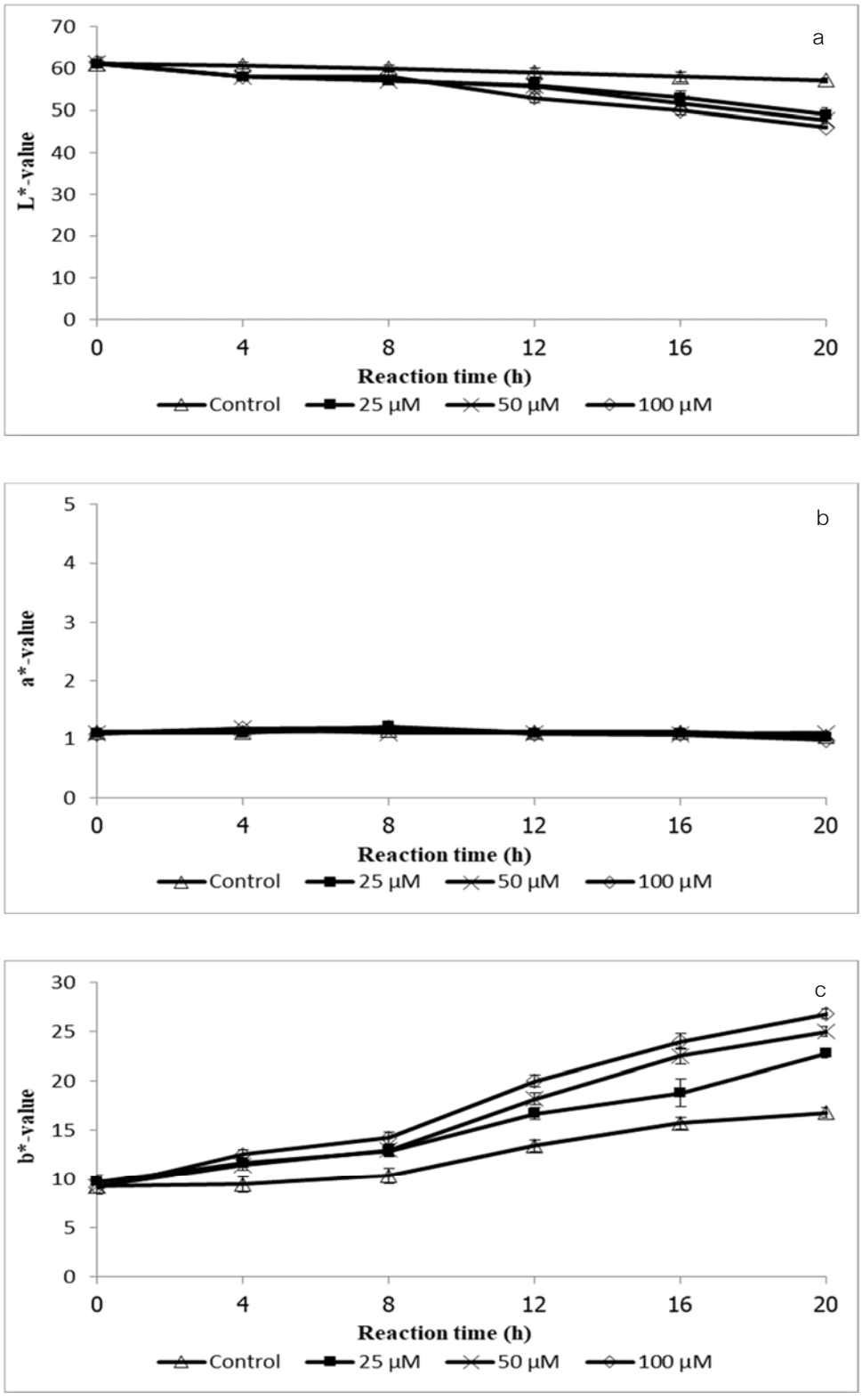

Figure 9 Changes in L* (a), a* (b) and b* (c)-value in liposomes made from Sawai lipids (5 mg lipid/mL) in the presence of 200 μM ascorbic acid and various concentrations of $FeCl_3$ (0-100 μM) during incubation at 37 ºC for 20 h. Error bars indicate standard deviations from triplicate determinations.

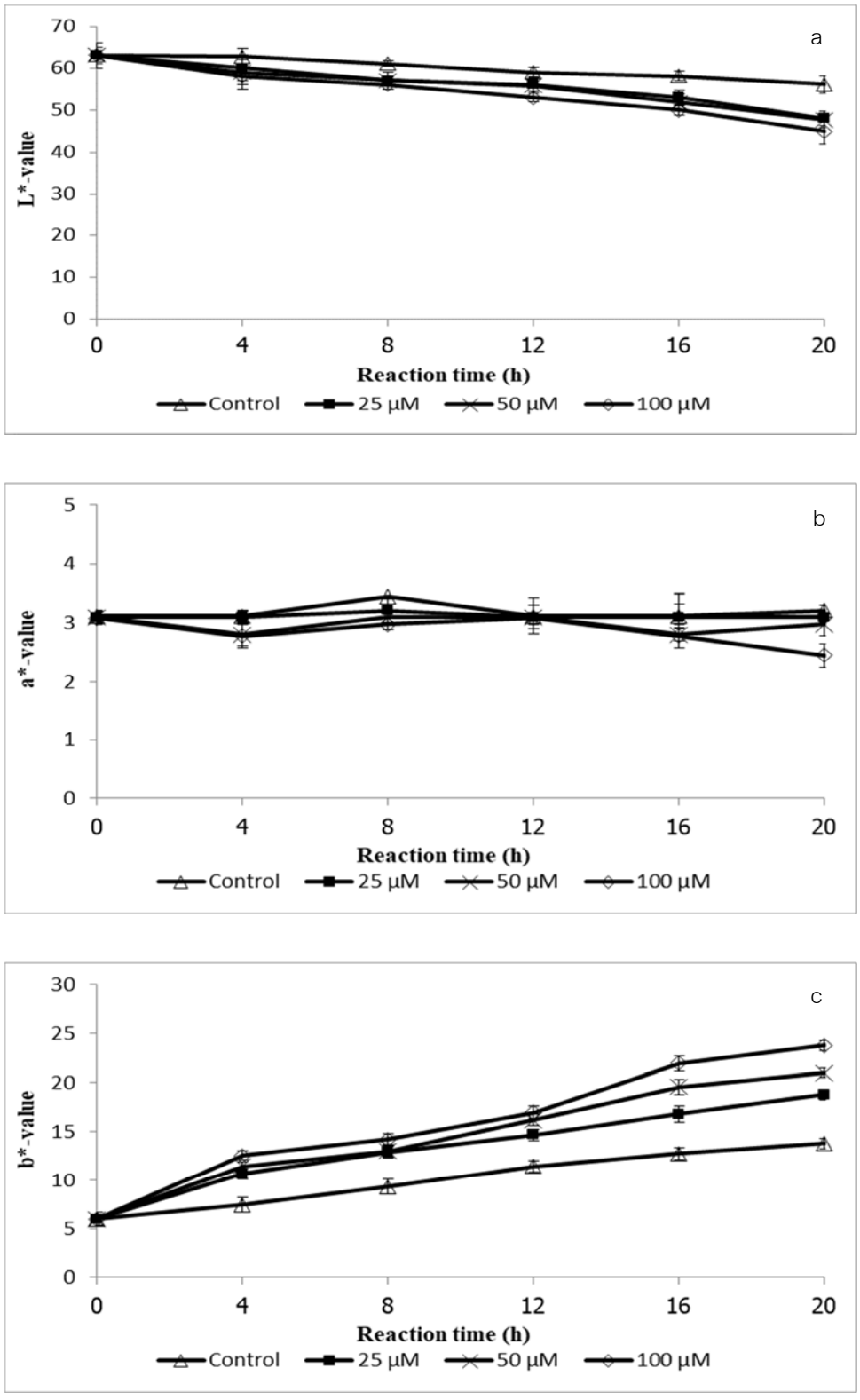

Figure 10 Changes in L* (a), a* (b) and b* (c)-value in Sawai muscle microsomes (5 mg microsomal protein/mL) in the presence of 200 μM ascorbic acid and various concentrations of $FeCl_3$ (0-100 μM) during incubation at 37 ºC for 20 h. Error bars indicate standard deviations from triplicate determinations.

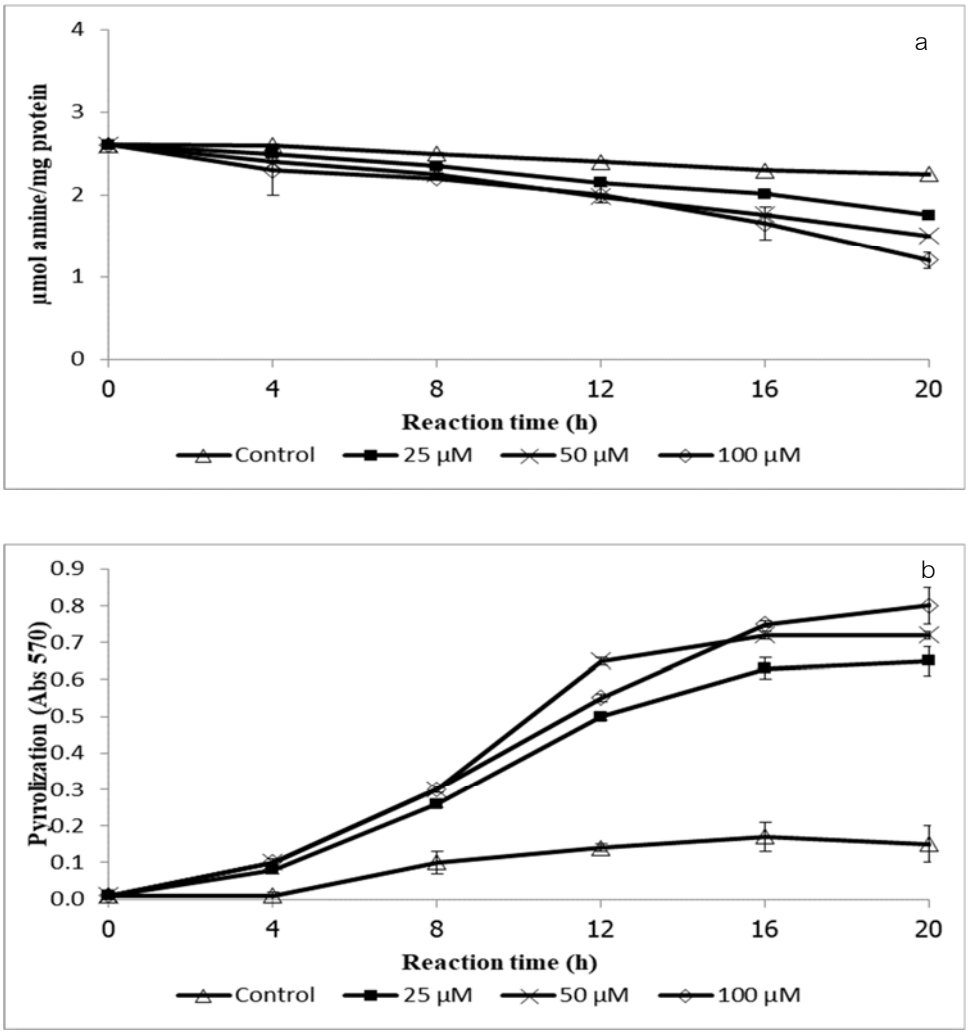

Figure 11 Changes in amine groups (a) and the formation of pyrrole compounds (b) in liposomes made from Sawai lipids (5 mg lipid/mL) in the presence of 200 µM ascorbic acid and various concentrations of $FeCl_3$ (0-100 µM) during incubation at 37 ºC for 20 h. Error bars indicate standard deviations from triplicate determinations.

3.3.4 Changes in SSP of Sawai as affected by oxidized Sawai liposomes

To verify that lipid oxidation products from Sawai liposomes could alter the properties of the Sawai SSP, oxidized Sawai liposomes was added directly to the SSP at content ranging from 1, 2 and 5% during incubation at 37 °C for 9 h. In the presence of oxidized Sawai liposomes at higher content (5%), sulfhydryl content of SPP decreased (Fig. 13a) with increasing time of incubation. SoANS of SPP increased at oxidized Sawai liposomes increased, especially after 9 h of incubation (Fig. 13b). Addition of oxidized Sawai liposomes to the SPP had significant ($p<0.05$) impact on carbonyl content (Fig. 13c). It was noted that the increase in carbonyl content of SPP was in a dose dependent. The formation of carbonyls was used to indicate the proteins

oxidation (Soyer, Özalp, Dalmış, & Bilgin, 2010). Nikoo, Benjakul, and Xu (2015) revealed that malondialdehyde, the secondary lipid oxidation product, can covalently bind to proteins; therefore some of the formed carbonyls could be due to lipid oxidation. Carbonyl groups, product from lipid oxidation involved in yellow discoloration of Basa (**Pangasius bocourti**) fillet during frozen storage was also reported (P. Sriket & La-ongnual, 2018). These data indicated that oxidized Sawai liposome was capable of modifying the SSP of Sawai.

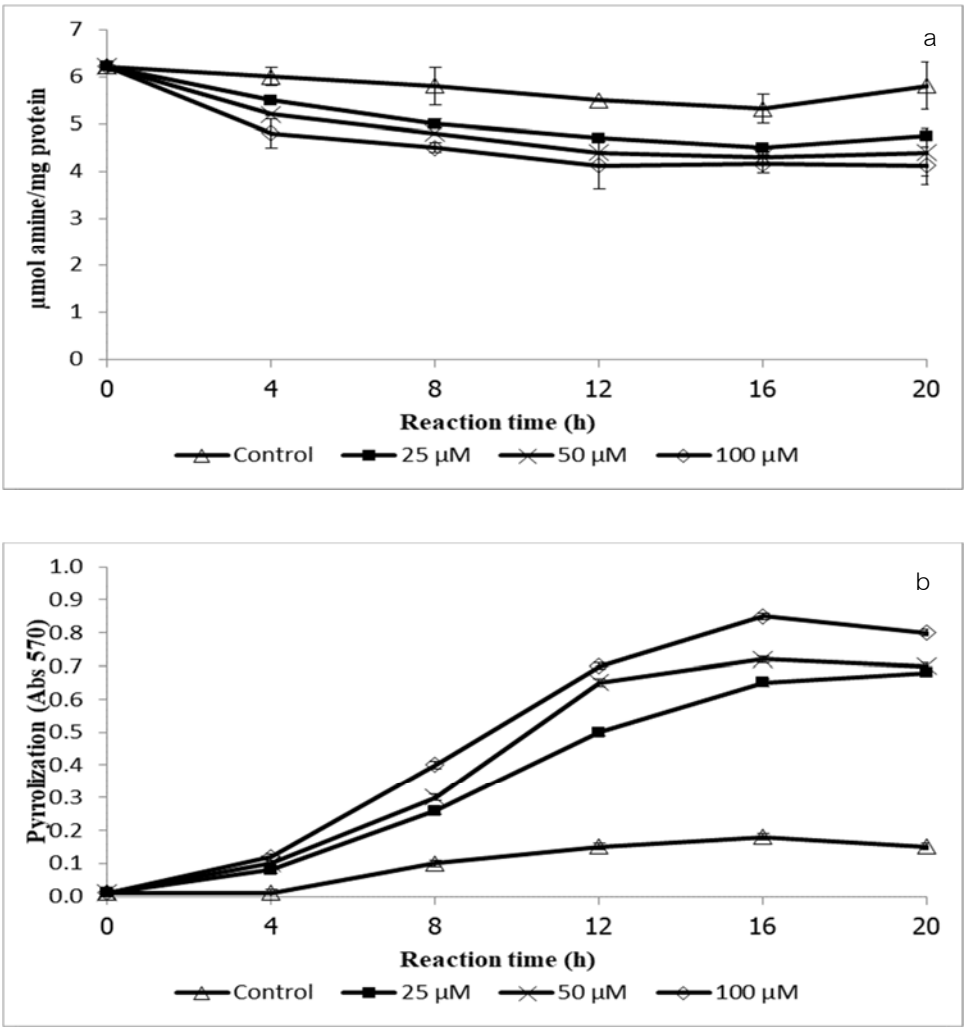

Figure 12 Changes in amine groups (a) and the formation of pyrrole compounds (b) in Sawai muscle microsomes (5 mg microsomal protein/mL) in the presence of 200 µM ascorbic acid and various concentrations of $FeCl_3$ (0-100 µM) during incubation at 37 ºC for 20 h. Error bars indicate standard deviations from triplicate determinations.

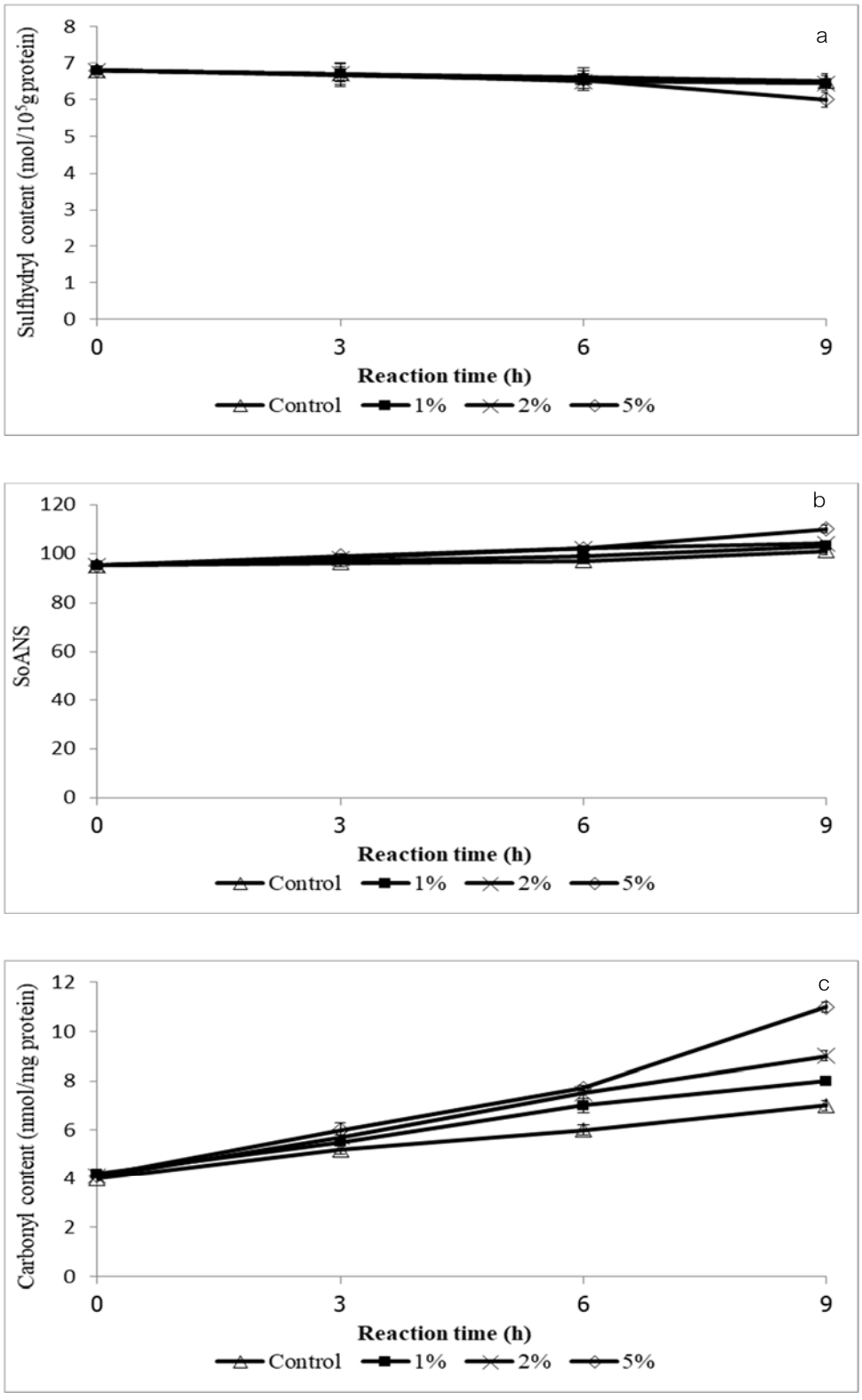

Figure 13 Changes in total sulfhydryl content (a), surface hydrophobicity (b) and carbonyl content (c) of NAM extracted from Sawai muscle after exposure at 37 ºC for 9 h with various contents (0, 1, 2 and 5%) of Sawai liposome (5 mg lipid/mL) oxidized by 200 μM ascorbic acid and 100 μM $FeCl_3$. Error bars indicate standard deviations from triplicate determinations.

3.4 Conclusion

Sawai lipids in liposomes and microsomes systems were susceptible to lipid oxidation in the presence of the iron and ascorbic acid, with the susceptibility increasing with increasing iron concentration and reaction time. Lipid oxidation products were able to produce yellow pigment formation in Sawai liposomes and microsomes. Oxidized Sawai liposome could increase protein oxidation in SPP. These results suggest a positive correlation between lipid oxidation and the development of yellow pigments in Sawai meat.

Chapter 4

Effect of tannic acid on Sawai (**Pangasianodon hypophthalmus**) liposome lipid oxidation

Abstract

The impact of tannic acid (0.02-0.08%) on lipid oxidation and color in Sawai (**Pangasianodon hypophthalmus**) liposomes during incubation at 37 °C for 9 h was studied. When the Sawai liposomes were oxidized with iron and ascorbate, thiobarbituric acid reactive substance (TBARS) in the control samples were observed to increase simultaneously with b* values (yellowness) and pyrrole compounds concomitantly with a decrease in free amines, especially when incubation times (0-9 h) increased. Tannic acid could prevent lipid oxidation and color changes of fish liposomes by decreasing the TBARS value and increasing the L* - value. Therefore, tannic acids, especially high concentration (0.08%) were able to decrease the lipid oxidation and color changes of the fish liposomes during incubation at 37 °C for 9 h.

Keywords: Sawai; yellow discoloration; antioxidant; liposome; lipid oxidation

4.1 Introduction

Fish are highly perishable products and their deterioration is mainly from the biological reactions such as lipids oxidation. The lipid oxidation process leads to discoloration, protein denaturation and off-flavor (Utrera, Parra, & Estévez, 2014). During the oxidation of lipids, carbonyl compounds such as aldehydes and ketones are formed through the degradation of lipid hydroperoxides. Among the secondary products, aldehydes have received the most attention because of their off-flavors and reactivity with groups such as amino acids (Vieira, Zhang, & Decker, 2017). Nonenzymatic browning has long been recognized as a consequence of oxidizing lipids in the presence of protein (Potes, Lim, & Roos, 2017). Most investigators theorize that nonenzymatic browning in muscle foods during lipid oxidation starts with the condensation of aldehydes with amines via Schiff base reaction pathways (Muhasinath, Mehta, Prasadnaik, Chouksey, & Nayak, 2019; Thanonkaew, Benjakul, Visessanguan, & Decker, 2006)

Frozen catfish such as Sawai (**Pangasianodon hypophthalmus**) have been increasingly produced and consumed worldwide. Although the microbiological deterioration is effectively retarded by frozen storage, various chemical reactions still take place (C. Sriket, Benjakul, & Senphan, 2019). Yellow pigment formation sometimes occurs during frozen storage of fish, accompanied by the development of rancid odours (Thanonkaew, Benjakul, Visessanguan, & Decker, 2006; Yerlikaya & Gökoğlu, 2010). Textural changes and muscle discoloration of some fish species including Basa (**Pangasius bocourti**) fillet (P. Sriket & La-ongnual, 2018), rainbow trout (**Oncorhynchus mykiss**) muscle (Burgaard & Jørgensen, 2011), Cod (**Gadus morhua**) muscle salmon muscle (Pahila, Kaneda, Nagasaka, Koyama, & Ohshima, 2017) and common carp (**Cyprinus carpio**) fillets (Li, Qin, Zhang, Li, Prinyawiwatkul, & Luo, 2019) during storage have been reported. Pahila, Kaneda, Nagasaka, Koyama, and Ohshima (2017) and Mi, Guo, and Li (2016) report that these changes occurred as a result of lipid oxidation. Sawai muscle has very high lipid content (C. Sriket, Benjakul, & Senphan, 2017). Those lipids have a high content of unsaturated fatty acids. As a consequence, Sawai lipids are susceptible to oxidation and their oxidation can lead to the muscle discoloration. The quality deterioration due to discoloration in frozen fish causes the rejection of the product has been reported (C. Sriket, Benjakul, & Senphan, 2019). To lower the lipid oxidation, the treatment of fish with potential antioxidants is required. Phenolic compounds have been used as the natural antioxidant to retard lipid oxidation in foods. Recently, Maqsood and Benjakul (2010) found that tannic acid exhibited the highest antioxidative activity by different in vitro assays as well as in fish oil-in-water emulsion and fish mince. Tannic acid is affirmed as Generally Recognised as Safe (GRAS) by the Food and Drug Administration (FDA) for the use as a direct additive in some food products such as meat products (21 CFR184.

1097, US Code of Federal Regulation, 2006). Therefore, the use of tannic acid could be a promising means to lower lipid oxidation of Sawai containing a high amount of fat, which has become an economically important species in Thailand. To our knowledge, no information on effect of tannic acid on lipid oxidation of Sawai liposome system has been reported. Therefore, objective of this study was to investigate the effect of tannic acid on lipid oxidation on the formation of yellow pigments in the liposomes system of Sawai cultured in Thailand.

4.2 Material and Methods

4.2.1 Chemicals

Tannic acid, Ferric (III) chloride, L-ascorbic acid, thiobarbituric acid (TBA), monopotassium dihydrogen phosphate, dipotassium hydrogenphosphate, **p**-dimethylamino benzaldehyde, 2,4,6-trinitrobenzenesulfonic acid (TNBS), Triton X-100, and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co. (St. Louis, MO). Hydrochloric acid, methanol and chloroform, were obtained from Merck (Darmstadt, Germany). All chemicals were of analytical grade.

4.2.2 Sample preparation

Sawai (**P. hypophthalmus**) weighing 2-3 kg, killed by ice-shocking, were purchased from a fish farm in Ubon Ratchathani, Thailand. The fish were kept in ice using a fish/ice ratio of 1:2 (w/w) and transported to the Program in Food Science and Technology, Faculty of Agriculture, Ubon Ratchathani Rajabhat University, Ubon Ratchathani province, Thailand within 1 hr. Upon arrival, the fish were washed with tap water, filleted, deskinned and cut into slices with a thickness of 1-2 cm. The slices were minced to obtain a uniform mixture.

4.2.3 Preparation of Sawai liposomes

Fish lipids were extracted as described by Bligh and Dyer (1959). Liposomes were prepared from the Sawai lipids according to the method of Decker and Hultin (1990). The fish lipid (5 mg/mL) was dispersed in 0.12 M KCl/25 mM potassium phosphate buffer, pH 7.2, with a Potter-Elvehjem homogenizer follow by sonication (35% amplitude with 5 sec repeating cycle; Sonicator, Sonic Dismembrator, model 500, Fisher Scientific, Pittsburg, PA, USA) in an ice bath for 30 min. The suspension was referred to as ‘‘fish liposome system”.

4.2.4 Lipid oxidation of liposomes

Lipid oxidation in liposomes systems was accelerated with a nonenzymatic iron redox cycling system according to the method of Thanonkaew, Benjakul, Visessanguan, and Decker

(2006) with slight modification. The reaction medium contains a final concentration of 200 μM ascorbate and 5 mg of lipid/mL of 25 mM potassium phosphate/0.12 M KCl buffer, pH 7.2 in the presence of 100 μM $FeCl_3$. To study the effect of tannic acid on fish liposome lipid oxidation, tannic acid at different levels (0.02, 0.04, 0.06 and 0.08%) was added. The control (without tannic acid) was prepared as the same manner. The systems were incubated at 37 ºC for 9 h. The assay medium was sampled at various times and analyzed for thiobarbituric acid reactive substances (TBARS), color, free amine, and pyrrolization as described below.

4.2.5 Determination of lipid oxidation

Lipid oxidation was monitored by measuring TBA reactive substances (TBARS) as determined by a modification of the procedure of MCDONALD and HULTIN (1987). TBA stock solution consisted of 15% trichloroacetic acid (w/v) and 0.375% TBA (w/v) in 0.25 M HCl. To 100 mL of TBA stock solution was added 3 mL of 2% BHT in ethanol. Liposomes (1.0 mL) were added to 2 mL of the TBA solution, vortexed, heated in a boiling water bath for 15 min, cooled to room temperature, and centrifuged at 1600**g** for 20 min. The absorbance of supernatant was measured at 532 nm, and the results were reported as micromoles of TBARS per milligram of liposomes lipid. TBARS concentrations were determined from a malonaldehyde standard curve produced from 1,1,3,3-tetraethoxypropane.

4.2.6 Determination of color

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

4.2.7 Determination of free amine groups

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

4.2.8 Determination of phospholipid pyrrolization

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

4.2.9 Statistical analysis

Experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out using Duncan's multiple range test (DMRT) Steel and Torrie (1980). Statistical analyses were done using the Statistical Package for Social Science (SPSS 13.0 for Windows, SPSS Inc., Chicago, IL, USA).

4.3 Results and discussion

4.3.1 Changes in TBARS of Sawai liposome

Lipid oxidation and yellow discoloration in fish muscle is a major cause of quality deterioration. Previous studies have shown that yellow pigments can form during the oxidation of fish muscle. The changes in TBARS of Sawai liposome with and without tannic acids (0.02-0.08%) during incubation at 37 ºC for 9 h in the presence of 200 µM ascorbic acid and µM 100 $FeCl_3$ are shown in Figure 14. The increases in lipid oxidation (as determined by TBARS) in Sawai liposome as reaction time increased were observed (Fig. 14). The ferric state (Fe^{3+}) can be reduced by ascorbic acid to produce the very prooxidative ferrous state (Fe^{2+}) of iron. The ferrous state of iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals via a Fenton type reaction (Bhawya & Anilakumar, 2010). The increases in TBARS of squid liposome as temperatures and reaction times increased were also reported (Thanonkaew, Benjakul, Visessanguan, & Decker, 2006; Thanonkaew, Benjakul, Visessanguan, & Decker, 2007). Peroxidation of liposomal lipids is affected by many factors such as inducing of peroxidation by the composition and physical properties of the liposomes, with respect to both oxidizable and non-oxidizable lipids, and the antioxidants, when present in the system (Schnitzer, Pinchuk, & Lichtenberg, 2007). Control sample showed the higher formation of TBARS throughout the incubation time of 9 h, compared with other samples ($P < 0.05$). When tannic acid (0.02% -

0.08%) was added in the fish liposomes, the formation of TBARS was retarded effectively. Tannic acid especially at higher level (0.08%) was therefore very effective in retarding the lipid oxidation. Apart from acting as a radical scavenger, tannic acid, especially at a level of 0.08%, could chelate iron. Tannic acid has the ability to chelate iron, particularly in the free form (Lopes, Schulman, & Hermes-Lima, 1999). Tannic acid chelates iron due to its ten galloyl groups and might also be able to inhibit iron-mediated oxyradical formation like other iron chelators, such as desferrioxamine (DFO), 1,10-phenanthroline and pyridoxal isonicotinoyl hydrazone (PIH) (Lopes et al., 1999). Recently, tannic acid was demonstrated to show the higher ferric reducing antioxidant power (FRAP) than other phenolic compounds (catechin, ferulic acid and caffeic acid) (Maqsood & Benjakul, 2010). Lopes et al. (1999) and Andrade, Ginani, Lopes, Dutra, and Hermes-Lima (2006) also reported that tannic acid was able to reduce Fe (III) to Fe (II). Thus, tannic acid (0.02-0.08%) was effective in retarding lipid oxidation in fish liposomes during incubation at 37 ºC for 9 h.

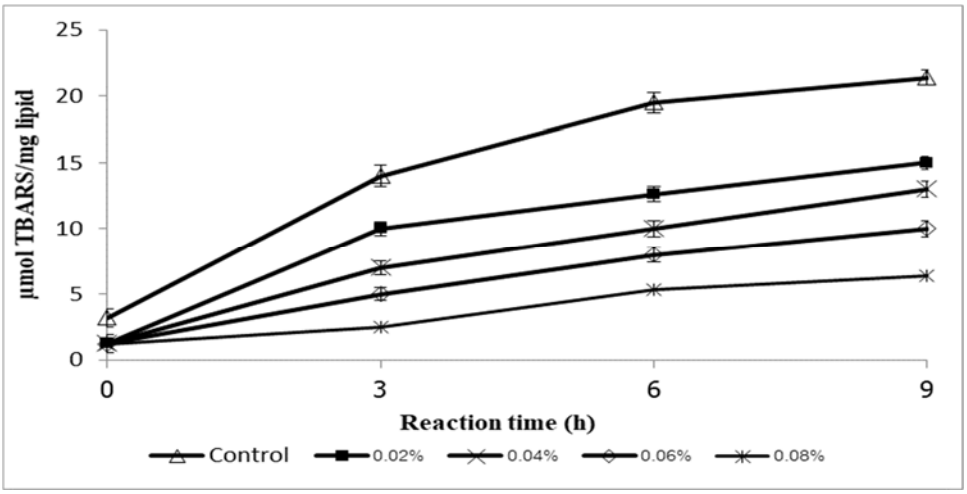

Figure 14 Formation of thiobarbituric acid-reactive substances (TBARS) in Sawai liposome (with and without different concentrations of tannic acids) made from Sawai lipids (5 mg lipid/mL) in the presence of 200 μM ascorbic acid and 100 μM $FeCl_3$ during incubation at 37 ºC for 9 h. Error bars indicate standard deviations from triplicate determinations.

4.3.2 Changes in colors of Sawai liposome

The changes in colors of Sawai liposome with and without tannic acids (0.02-0.08%) during incubation at 37 ºC for 9 h in the presence of 200 μM ascorbic acid and 100 μM $FeCl_3$ are depicted in Figure 15. Following iron and ascorbate addition, there was no difference in L*, a* and b* values in Sawai liposomes compared to the control sample ($p \geq 0.05$). However, a

decrease in the L* values of all samples was observed as the reaction times increased (Fig. 19a). No changes in the a* values in all samples were observed during the incubation ($p>0.05$) (Fig. 15b). The b* values of all samples increased when the reaction times increased ($p<0.05$). However, higher b* values were found in the control sample compared to the other samples (Fig. 15c). A positive correlation between lipid oxidation (TBARS) (Fig. 14) and yellow color (b*) was found.

The above results suggest that yellow pigment formation in pigment formation in Sawai could be due to nonenzymatic browning reactions between fatty acid decomposition products and phospholipid head groups. However, browning could also occur as a result of interactions between fatty acid decomposition products and the amines in proteins (Benjakul & Visessanguan, 2010). Aldehydes and carbonyl compounds, i.e. heptanal, hexanal, octanal etc., products from the oxidation of unsaturated fatty acids, can react with free amino groups in protein. These reactions lead to discoloration and the production of unpleasant odor (Ross & Smith, 2006). Lipid oxidation in squid liposomes related to yellow discoloration has been reported (Thanonkaew, Benjakul, Visessanguan, & Decker, 2006; Thanonkaew, Benjakul, Visessanguan, & Decker, 2007).

4.3.3 Changes in amine groups and pyrrole compounds of Sawai liposome

The changes in amine groups and pyrrole compounds of Sawai liposome without and with tannic acids (0.02-0.08%) during incubation at 37 ºC for 9 h in the presence of 200 µM ascorbic acid and 100 µM $FeCl_3$ are shown in figure 16 and 17. Changes in free amines was used to be an indicator for the interactions between phospholipids and lipid oxidation products (Hidalgo et al., 2004; Kubo et al., 2005). The loss of amine ($p<0.05$) was observed in all liposome samples, especially when incubation time increased (Fig. 16). The loss of free amines in Sawai liposomes occurred over a similar time period in the presence of ascorbate and iron, suggesting that lipid oxidation products could be involved in the formation of yellow pigments in Sawai muscle. However, samples containing tannic acid had higher amine content, compared with control sample. Therefore, tannic acid could prevent the oxidation of fish liposome to some degree.

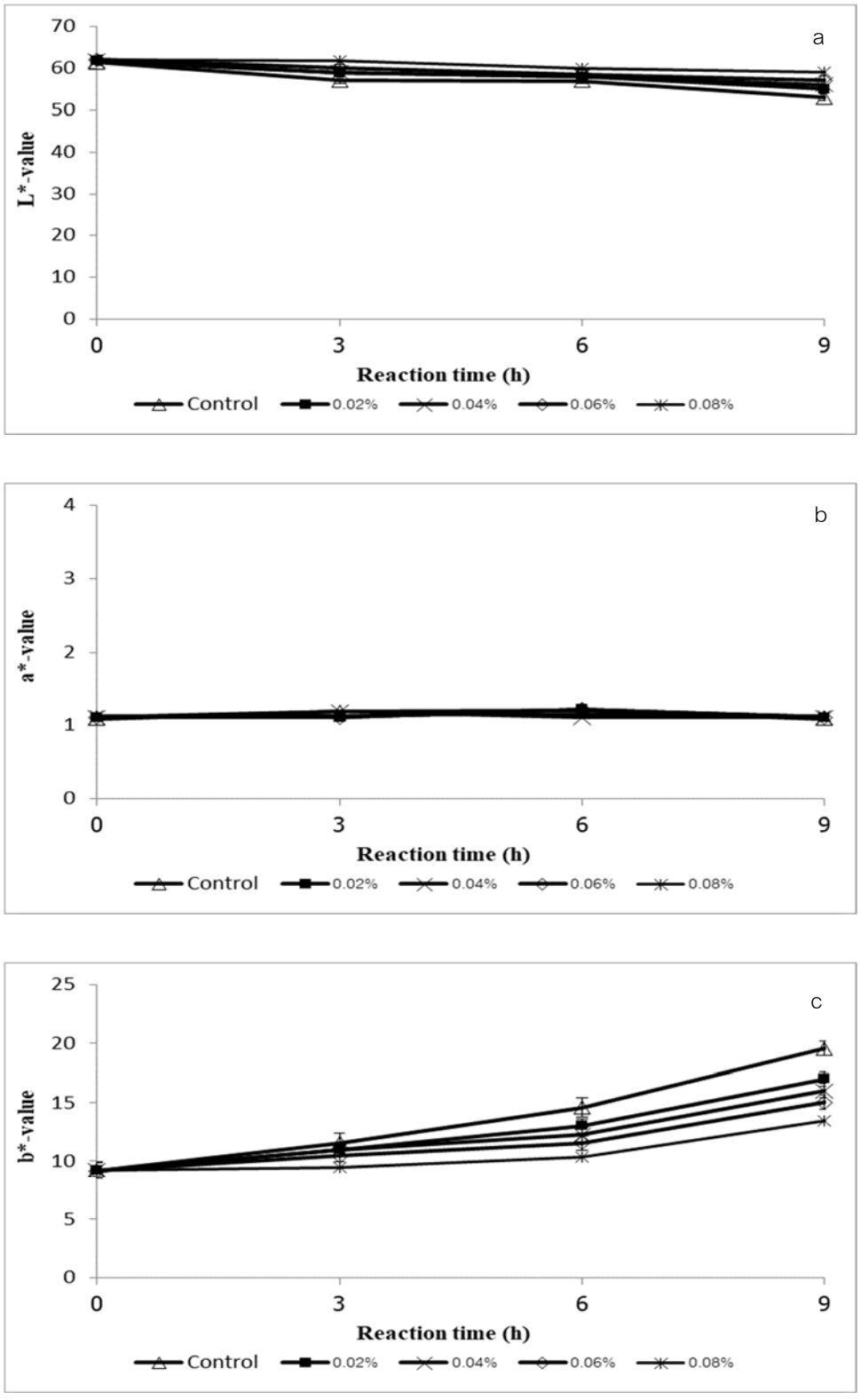

Figure 15 Changes in L* (a), a* (b) and b* (c)-value in liposomes (with and without different concentrations of tannic acids) made from Sawai lipids (5 mg lipid/mL) in the presence of 200 μM ascorbic acid and 100 μM $FeCl_3$ during incubation at 37 ºC for 9 h. Error bars indicate standard deviations from triplicate determinations.

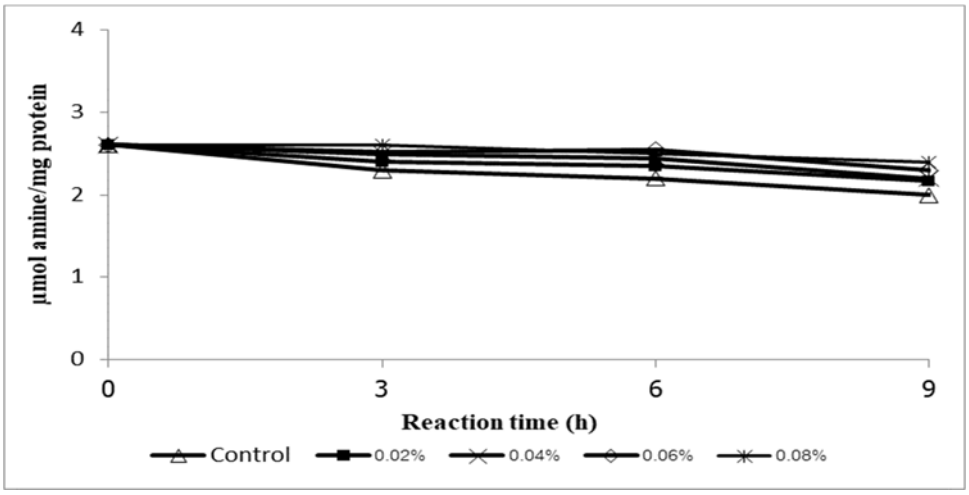

Figure 16 Changes in amine in liposomes (with and without different concentrations of tannic acids) made from Sawai lipids (5 mg lipid/mL) in the presence of 200 µM ascorbic acid and 100 µM $FeCl_3$ during incubation at 37 ºC for 9 h. Error bars indicate standard deviations from triplicate determinations.

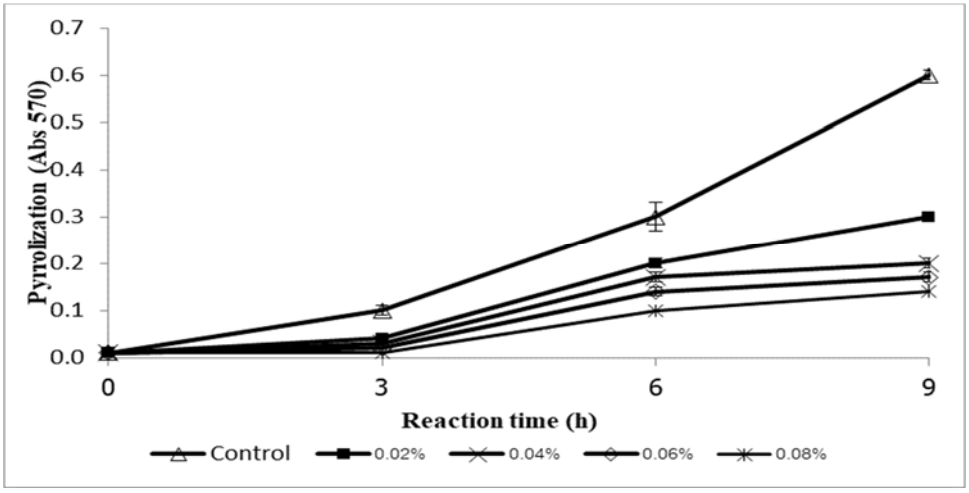

Figure 17 Changes in the formation of pyrrole compounds in liposomes (with and without different concentrations of tannic acids) made from Sawai lipids (5 mg lipid/mL) in the presence of 200 µM ascorbic acid and 100 µM $FeCl_3$ during incubation at 37 ºC for 9 h. Error bars indicate standard deviations from triplicate determinations.

In the presence of ascorbic acid and iron, the increase of pyrroles ($p<0.05$) was also observed in all Sawai liposomes (Fig. 17). It was noted that the slow increase in pyrroles in the tannic acid containing samples of liposomes, compared with control sample was observed, especially after 3 h of reaction time. This is presumably that tannic acid could prevent lipid

oxidation in fish liposome to some degree. Pyrroles formed in the reaction between oxidized lipids and protein are important precursors of browning (Zamora & Hidalgo, 2011). Simultaneously with TBARS and yellow pigment formation, free amines decreased and pyrrole compounds were formed in the presence of ascorbate and iron, suggesting that lipid oxidation products were reacting with amines to produce yellow pigments (Thanonkaew, Benjakul, Visessanguan, & Decker, 2007).

4.4 Conclusion

Tannic acids (0.02-0.08%) were effective in retarding the lipid oxidation in Sawai liposomes as indicated by lower TBARS formation as well as lower development of yellow color (b* value). Addition of tannic acid at a level of 0.02-0.08% could prevent the loss amine groups and reduce the pyrrole formation after reaction for 9 h at 37 ºC. Thus, tannic acid, especially at the higher level (0.08%) can be used as an effective natural antioxidant in the fish liposome system.

Chapter 5

Effect of tannic acid on muscle discoloration and quality changes of Sawai (**Pangasianodon hypophthalmus**) fillet during frozen storage

Abstract

Quality changes of Sawai (**Pangasianodon hypophthalmus**) fillet soaked with and without different concentrations of tannic acid (0-1.0%) during frozen storage at -20 °C for 16 weeks were investigated. TBARS in all Sawai samples increased when the storage time increased ($p<0.05$). Tannic acid showed antioxidative effect in frozen fish fillet as indicated by lower TBARS content compared with control sample. Soaking the fish fillet in tannic acid solutions could improve the color of fish fillet by increasing L*-value and decreasing b*-value during frozen storage. Surface hydrophobicity (SoANS) of Sawai natural actomyosin (NAM) increased when the frozen storage period increased up to 16 weeks. The increase in disulfide bond content was generally coincidental with the decrease in sulfhydryl content. Protein solubility decreased slightly during prolonged storage. Soaking Sawai fillet with tannic acid could retard the decreases in solubility and increase in thaw drip of frozen Sawai fillet. Therefore, soaking in tannic acid solution before freezing could be a means to prevent lipid oxidation and quality changes of Sawai fillet during frozen storage.

Keywords: Sawai; yellow discoloration; lipid oxidation; tannic acid; antioxidant

5.1 Introduction

Catfish, such as Sawai (**Pangasianodon hypophthalmus**) and Basa (**Pangasius bocourti**), have been increasingly produced and consumed due to its taste and nutritional values. Thailand is an important source of catfish production including Sawai, Basa and giant catfish (**Pangasianodon gigas**). Normally, catfish have been exported as frozen fillet. Although, frozen storage can effectively prevent the microbiological deterioration, various chemical reactions still take place. Frozen fish products are in general characterized by having a lower quality than fresh ones due to the osmotic removal of water, denaturation of protein and mechanical damage (P. Sriket & La-ongnual, 2018; Thyholt & Isaksson, 1997). Lipid oxidation occurring during frozen storage leads to the formation of free radicals and hydroperoxides, which cause oxidation of pigments and flavors. Yellow pigment formation sometimes occurs during frozen storage of catfish, accompanied by the development of rancid odors (C. Sriket, Benjakul, & Senphan, 2019; P. Sriket & La-ongnual, 2018). Lipid oxidation products such as peroxides, aldehydes, and ketones are formed. Interaction of peroxides or carbonylic peroxide decomposition products with active groups of protein can lead to protein polymerization or aggregation as well as discoloration in fish. Lipid oxidation processes lead to discoloration, drip losses, and off-flavor development (Decker & Hultin, 1990) and production of potentially toxic compounds (Xiong, 2000). Furthermore, lipid oxidation in fish muscle during frozen storage showed a detrimental effect on protein structure and functionality (Saeed & Howell, 2002). Protein and lipid oxidation can account for the toughened texture, poor flavor and/or unappealing odor of poorly stored frozen seafood. Basa fish (**Pangasius bocourti**) fillet turned yellow when frozen (P. Sriket & La-ongnual, 2018). The changes of color are correlated with an increase in TBARS and peroxide values (Rawdkuen, Jongjareonrak, Benjakul, & Chaijan, 2008; Wsowicz, Gramza, Hes, Jeleñ, Korczak, & Malecka, 2004). Recently, lipid oxidation was reported to promote formation of yellow pigment in Sawai paste with repeated freeze-thawing (C. Sriket, Benjakul, & Senphan, 2019). Prevention of oxidative deterioration of muscle seafood during processing or storage can be achieved by the incorporation of antioxidants (Olatunde & Benjakul, 2018). Tannic acid is a natural antioxidant and has been successfully used as an antioxidant in many fish and fish products. Quality deterioration due to discoloration, poor texture, development of off-flavors and yellow discoloration in frozen Sawai causes the rejection of the product. Those changes may be associated with lipid oxidation of Sawai meat, whose lipids contained high levels of unsaturated fatty acids (C. Sriket, Benjakul, & Senphan, 2017). Therefore, the use of natural antioxidant could retard the quality losses of frozen fish. This

study aimed to investigate the stabilizing effect of tannic acid on lipid oxidation, discoloration, and protein denaturation of Sawai (**P. hypophthalmus**) muscle during frozen storage.

5.2 Material and Methods

5.2.1 Chemicals

Tannic acid, 1-anilinonaphthalene-8-sulphonic acid (ANS), 5-5´- dithio-bis (2-nitrobenzoic acid) (DTNB), β-mercaptoethanol (β-ME), cysteine, sodium bisulfite, sodium ascorbate, sodium D-isoascorbate (sodium erythorbate), thiobarbituric acid and malondialdehyde (MDA) were obtained from Sigma (St. Louis, MO, USA). Acrylamide, **N,N,N´,N´**-tetramethylethylenediamine (TEMED) and bis-acrylamide were procured from Fluka (Buchs, Switzerland). All chemicals were of analytical grade.

5.2.2 Sample preparation

Sawai (**P. hypophthalmus**) weighing 2-3 kg, killed by ice-shocking, were purchased from a fish farm in Ubon Ratchathani, Thailand. The fish were kept in ice using a fish/ice ratio of 1:2 (w/w) and transported to the Program in Food Science and Technology, Faculty of Agriculture, Ubon Ratchathani Rajabhat University, Ubon Ratchathani province, Thailand within 1 hr. Upon arrival, the fish were washed with tap water and filleted. Tannic acid with different concentrations (0.2, 0.4, 0.6, 0.8 and 1.0%) was prepared. Fish fillet were soaked in tannic acid at various concentrations (0-1.0%) (4 °C) at a ratio of 1:1 (w/v) for 30 min. After soaking, the samples were drained at 4 °C for 5 min. Each treatment was placed in polyethylene bags, frozen and stored at -20 °C. Samples without treatment (soaked in the same volume of sterilized water) were used as the control. Analyses of the frozen fish were carried out at 0, 2, 4, 8, 12 and 16 weeks except for volatile compound was analyzed at the end of storage.

Before analysis, frozen fish fillet was thawed using running tap water (25-27 °C) until the core temperature reached 0-2 °C. Thawed fish was measured for thaw drip and color. Thawed fish fillet was cut, finely chopped and kept in ice before analysis. The thawed samples were subjected to analysis for chemical composition and physicochemical properties as follows:

5.2.3 Determination of thaw drip

The weight of fish was recorded before freezing and frozen storage. After the thawing process, the thawed samples were weighed and the amount of thaw drip was calculated according to the following formula (Santos & Regenstein, 1990): % Thaw drip [(A-B)/A]×100
where A = initial weight of sample; B = final weight of sample.

5.2.4 Determination of color

The fish fillet was spread in the sample cup and color was measured through the bottom of the sample cup using a colorimeter (Color Flex, Hunter Lab Inc., Reston, VA, USA) and reported in the CIE color profile system as L*, a* and b*-values.

5.2.5 Determination of Shear Force

Shear force of thawed fish fillet was measured using the TA-XT2i texture analyzer (Stable Micro Systems, Surrey, England) equipped with a Warner-Bratzler shear apparatus (Brauer, Leyva, Alvarado, & Sandez, 2003). The cylindrical longitudinal muscle samples of the fillet were cut out with a borer of 11 mm in diameter. The blade was pressed down at a constant speed of 2 mm/s through the sample, cutting the muscle fiber transversely. Shear force (N) was recorded from six measurements.

5.2.6 Determination of thiobarbituric acid reactive substances (TBARS)

TBARS were determined as described by Buege and Aust (1978). Fish (2 g), containing various concentrations of iron, was dispersed in 10 mL of thiobarbituric acid solution (0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 N HCl). The mixture was heated in boiling water for 10 min, followed by cooling in running tap water. The mixture was centrifuged at 3600 ×g for 20 min using a refrigerated centrifuge (Model CR22N, Hitachi, Hitachi Koki Co., Ltd., Tokyo, Japan) at 25 °C. The absorbance of the supernatant was measured at 532 nm using a spectrophotometer (Model UV 1800, Shimadzu, Kyoto, Japan). The standard curve was prepared using malondialdehyde (Sigma), and TBARS were expressed as mg malonaldehyde/kg sample.

5.2.7 Determination of surface hydrophobicity

Surface hydrophobicity (SoANS) was determined as described by Benjakul, Seymour, Morrissey and An (1997) using ANS as a probe. Natural actomyosin (NAM) was prepared as described by Benjakul et al. (1997). Fish meat was homogenized in chilled 0.6 M KCl, pH 7.0 at a

ratio of 1:10 (w/v) using a homogenizer (IKA, Labortechnik, Selangor, Malaysia). To avoid overheating, the sample was placed in ice and homogenized for 20 s, followed by a 20 s rest interval for a total extraction time of 4 min. The homogenate was centrifuged at 5000 ×g for 30 min at 4 ºC using a refrigerated centrifuge. To the supernatant, three volumes of chilled deionized water (4 ºC) were added to precipitate NAM. The NAM was collected by centrifuging at 5000 ×g for 20 min at 4 ºC. The NAM pellet was dissolved in chilled 0.6 M KCl, pH 7.0 for 30 min at 4 ºC and then centrifuged at 5000 ×g for 20 min at 4 ºC. The supernatant was collected and used as NAM. NAM solution (4 mg/mL) was diluted in 10 mM sodium phosphate buffer, pH 6.0, containing 0.6 M NaCl to produce protein concentrations of 0.125, 0.25, 0.5 and 1 mg/mL, followed by incubation at room temperature (25-28 ºC) for 10 min. The diluted protein solution (2 mL) was mixed with 20 µL of 8 mM ANS in 0.1 M phosphate buffer, pH 7.0 and the fluorescence intensity of ANS-conjugates was immediately measured using a spectrofluorometer (RF-15001, Shimadzu, Kyoto, Japan) at an excitation wavelength of 374 nm and an emission wavelength of 485 nm. The initial slope of the plot of fluorescence intensity versus protein concentration was referred to as SoANS.

5.2.8 Determination of total sulfhydryl content

The total sulfhydryl content was determined using DTNB according to the method of Ellman (1959) as modified by Benjakul et al. (1997). One mL of NAM solution (4 mg protein/mL determined using the Biuret method (Robinson & Hogden, 1940) with bovine serum albumin (Sigma) as the standard assuming 100% purity) was mixed with 9 mL of 0.2 M Tris-HCl buffer, pH 6.8, containing 8 M urea, 2% SDS and 10 mM EDTA. Four mL of the mixture were mixed with 0.4 mL of 0.1% DTNB and incubated at 40 °C for 25 min. The absorbance at 412 nm was measured using the spectrophotometer and a 0.6 M KCl solution was used as a blank. The sulfhydryl content was calculated using the extinction coefficient of 13500 M^{-1} cm^{-1}(Ellman, 1959).

5.2.9 Determination of disulfide bond content

The disulfide bond content was determined using the NTSB assay according to the method of Thannhauser, Konishi and Scheraga (1987). To 0.5 mL of NAM solution (4 mg/mL), 3.0 mL of freshly prepared NTSB assay solution were added. The mixture was mixed thoroughly and incubated in the dark at room temperature for 25 min. The absorbance at 412 nm was measured using the spectrophotometer. The disulfide bond content was calculated using the extinction coefficient of 13900 M^{-1} cm^{-1}(Ellman, 1959).

5.2.10 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to monitor the polymerization of the modified proteins. The fish fillet was solubilized in 5% SDS (1:9, w/v) and dissolved in sample buffer with and without β-mercaptoethanol. SDS-PAGE was done using 4% stacking gels and 10% running gels (Laemmli, 1970). Proteins (15 μg) determined using the Biuret method were loaded onto the gel and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-Protean II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.02% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid for 3 hr and destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid for 15 min, followed by 5% (v/v) methanol and 7.5% (v/v) acetic acid for 3 hr. High molecular weight markers (GE healthcare UK Limited, Buckinghamshire, UK) were used for estimation of MW of protein bands.

5.2.11 Determination of protein solubility

Solubility was determined according to Benjakul and Bauer (2000). To 1 g fish meat, 20 mL of 0.6 M KCl were added and the mixture was homogenized for 1 min at a speed of 12000 rpm, using an IKA homogenizer. The homogenate was stirred at 4 °C for 4 h, followed by centrifuging at 8500 ×g for 30 min at 4 °C. To 10 mL of supernatant, cold (4 °C) 50% (w/v) trichloroacetic acid was added to obtain a final concentration of 10%. The precipitate was washed with 10% trichloroacetic acid and solubilized in 0.5 M NaOH. The fish meat was also directly solubilized using 0.5 M NaOH to determine total protein. Protein content was determined and expressed as the percentage of total protein in the sample.

5.2.12 Determination of volatile compounds

The volatile lipid oxidation compounds in thaw fish fillet were determined by solid phase micro-extraction coupled with gas chromatography mass spectrometry (SPME-GCMS) (Iglesias & Medina, 2008).

5.2.13 Statistical analysis

Experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out using Duncan’s multiple range test (DMRT) (Steel & Torrie,

1980). Statistical analyses were done using the Statistical Package for Social Science (SPSS 13.0 for Windows, SPSS Inc., Chicago, IL, USA).

5.3 Results and discussion

5.3.1 Changes in TBARS of Sawai fillet

Lipid oxidation and yellow discoloration in fish muscle is a major cause of quality deterioration. Previous studies have shown that yellow pigments can form during the oxidation of Sawai muscle (C. Sriket, Benjakul, & Senphan, 2019). Changes in the TBARS values of Sawai fillet treated with and without tannic acids (0-1.0%) during frozen storage at -20 ºC for 16 weeks are shown in Figure 18. The formation of TBARS in Sawai fillet increased when the frozen storage time increased ($p<0.05$), especially at 16 weeks of storage. Among all samples, those treated with various concentrations of tannic acids, tended to have lower value with extended storage, compared with control sample. TBARS values of control sample increased more rapidly than other samples, particularly at week 8 and 12 of frozen storage ($p<0.05$). It was presumed that prooxidative metal ions in the intact muscle might cause the oxidation of lipid in Sawai fillet. As a result, lipid oxidation still took place as evidenced by the formation of TBARS. Tannic acid could effectively prevent the lipid oxidation of Sawai fillet during frozen storage. Maqsood & Benjakul (2010) reported that tannic acid showed high efficacy in retardation of lipid oxidation in minced fish as evidenced by the lower peroxide value (PV), conjugated diene (CD) and thiobarbituric acid reactive substances (TBARS) values.

5.3.2 Changes in colors of Sawai fillet

Color plays an important part in the appearance, presentation and acceptability of frozen fish. Generally, color changes can occur during frozen storage due to lipid oxidation and pigment degradation processes (Alonso, Muela, Tenas, Calanche, Roncales, & Beltran, 2016; Jeong, Kim, Yang, & Joo, 2011). L*, a*, and b* -values of frozen Sawai fillet soaked with and without tannic acids (0-1.0%) are shown in Figure 19a, b and c. L* -value of all samples decreased slightly at 8 weeks of storage (Fig. 19a). Thereafter, L* -values decreased up to 16 weeks of frozen storage. Control sample had lower L* -values but higher b* -values (Fig.19b). than tannic acids treated samples ($p<0.05$). A decrease in the a* -value in the fish fillet was observed when the samples were stored for 8 weeks ($p<0.05$) (Figure 19c). Pigment degradation can also lead to the changes in muscle color

(P. Sriket & La-ongnual, 2018). The frozen storage's influence on the a*- value of muscle has been reported (P. Sriket & La-ongnual, 2018). The increase in b* values of the fish fillet was observed after 8 weeks of storage ($p<0.05$). A positive correlation between lipid oxidation (TBARS) (Fig. 18) and yellow color (b*) (Fig. 19b) formation was found in the fish samples. The increase in lipid oxidation in Sawai pastes during freeze-thaw cycling was coincidental with an increase in b* - value (C. Sriket, Benjakul, & Senphan, 2019). Sriket & La-ongnual (2018) also found that the increase of lipid oxidation in Basa fillet during frozen storage was correlated with the decrease in redness and the increase in yellowness.

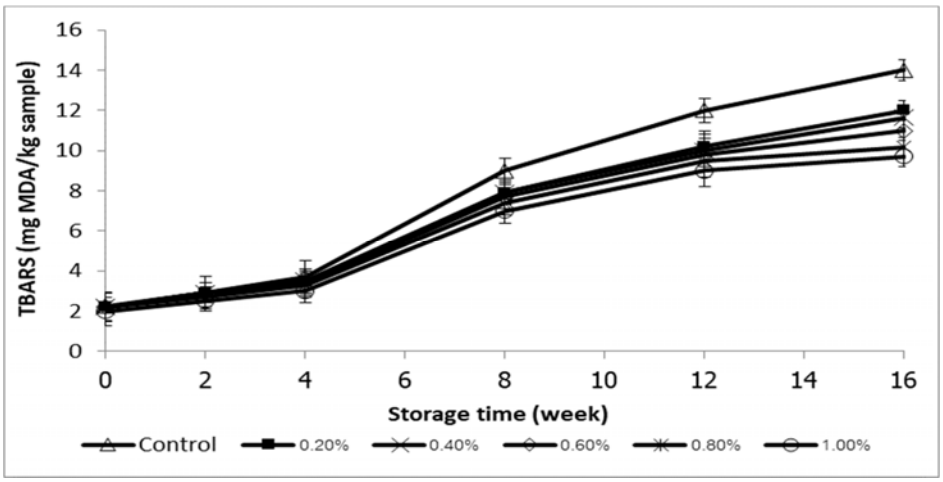

Figure 18 Changes in the TBARS values of Sawai fillet treated with and without tannic acids (0-1.0%) during frozen storage at -20 ºC for 16 weeks. Error bars indicate standard deviations from triplicate determinations.

The above results suggest that yellow pigment formation in pigment formation in Sawai could be due to nonenzymatic browning reactions between fatty acid decomposition products and phospholipid head groups. However, browning could also occur as a result of interactions between fatty acid decomposition products and the amines in proteins (Thanonkaew, Benjakul, Visessanguan, & Decker, 2005; Thanonkaew, Benjakul, Visessanguan, & Decker, 2006). Aldehydes and carbonyl compounds, i.e. heptanal, hexanal, octanal etc., products from the oxidation of unsaturated fatty acids, can react with free amino groups in protein. These reactions lead to discoloration and the production of unpleasant odors (Pokorny, 1981; Wsowicz, Gramza, Hes, Jeleñ, Korczak, & Malecka, 2004). Lipid from Sawai meat contained high unsaturated fatty acids (C. Sriket, Benjakul, & Senphan,

2017). However, tannic acid, especially high concentration (1.0%) could effectively retard the color changes of Sawai fillet during frozen storage.

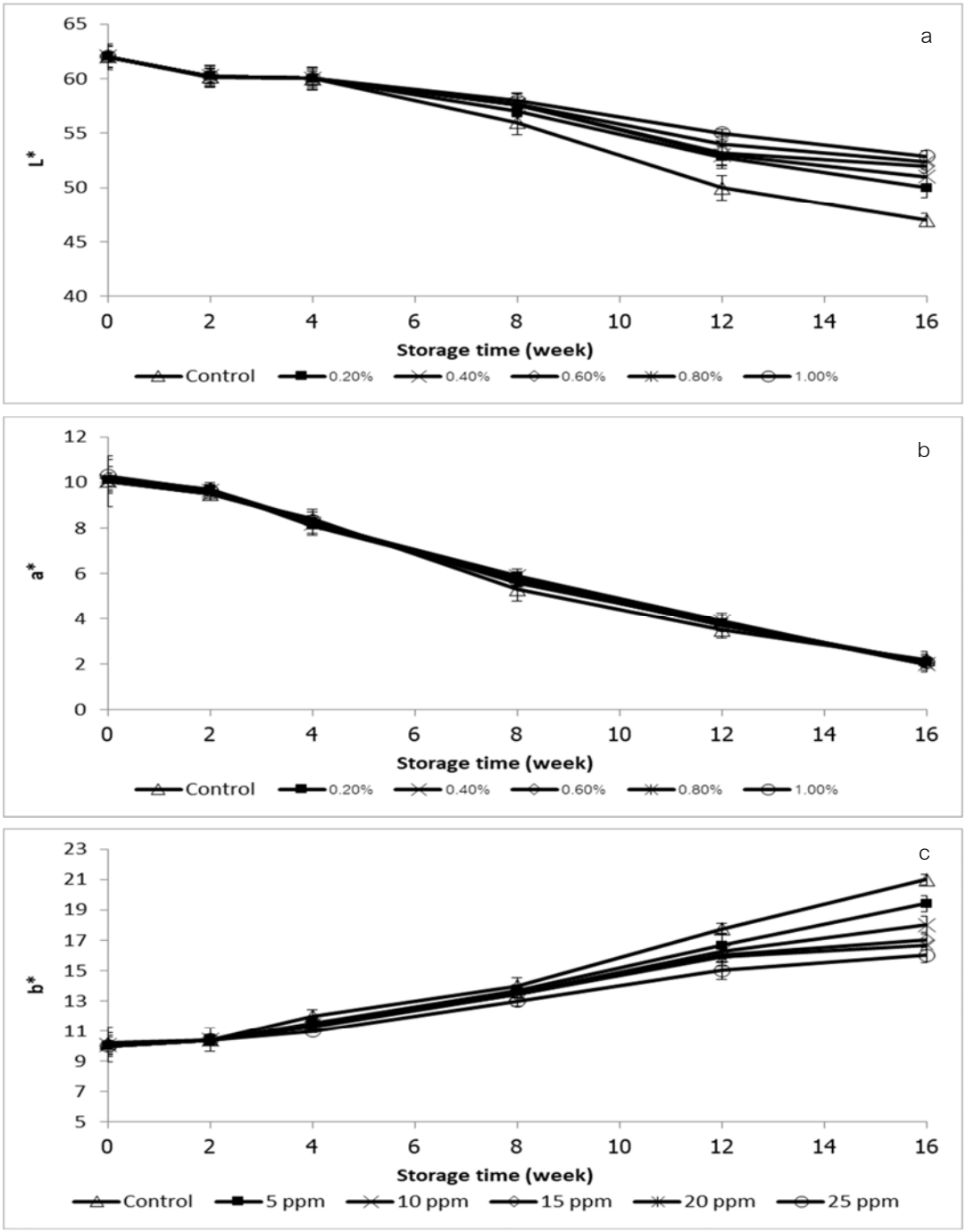

Figure 19 Changes in the L*, a* and b*- values of Sawai fillet treated with and without tannic acids (0-1.0%) during frozen storage at -20 ºC for 16 weeks. Error bars indicate standard deviations from triplicate determinations.

5.3.3 Changes in surface hydrophobicity of NAM extracted from Sawai fillet

Changes in SoANS indicate conformational changes in protein structure. The changes in SoANS of NAM extracted from Sawai fillet with and without tannic acids (0.2-1.0%) are depicted in Figure 20. SoANS of NAM increased as the frozen storage period increased up to 16 weeks. P. Sriket & La-ongnual (2018) also reported an increase in the SoANS of NAM extract from Basa muscle during 20 weeks of frozen storage. However, tannic acids did not affect SoANS of NAM from frozen Sawai fillet ($p>0.05$). An increase in surface hydrophobicity possibly resulted from structural alterations of proteins induced by frozen storage. The enhanced hydrophobicity was coincidental with the increased oxidation of lipids, suggesting that protein structural changes might result from the modifications of amino acid residue side chains by lipid free radicals or fatty acid decomposition products. Hydrophobic interaction might take place between the exposed hydrophobic residues, leading to the aggregation of protein. Thus, frozen storage directly affected conformational changes in protein molecules.

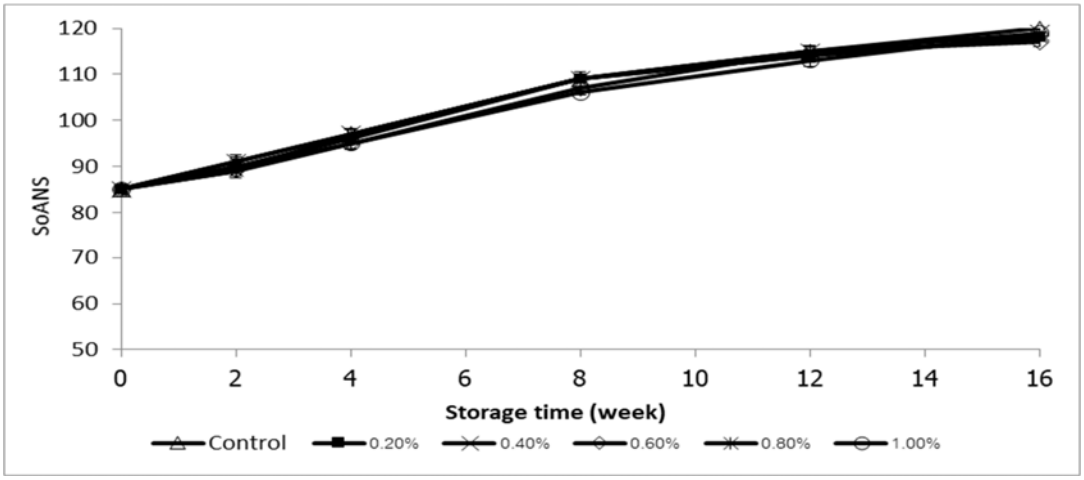

Figure 20 Changes in surface hydrophobicity of NAM extracted from Sawai fillet treated with and without tannic acids (0-1.0%) during frozen storage at -20 ºC for 16 weeks. Error bars indicate standard deviations from triplicate determinations.

5.3.4 Changes in sulfhydryl and disulfide bond content of NAM extracted from Sawai fillet

The sulfhydryl and disulfide bond contents of NAM from Sawai fillet with different treatments are shown in Figure 21a and b. Sulfhydryl content of NAM from Sawai fillet decreased with the increase in disulfide bond content during frozen storage up to 16 weeks ($p<0.05$). During frozen

storage, the conformational changes of protein molecules might be associated with the exposure of reactive sulfhydryl groups, which were prone to oxidation or disulfide interchange. Under normal frozen storage conditions, muscle proteins also undergo destabilization (Benjakul & Bauer, 2000). Myosin is susceptible to oxidizing agents during processing and storage. The disappearance of both heavy and light chains of myosin is much more pronounced in myosin than in actin and tropomyosin (Xiong, 2000). In general, the denaturation and aggregation of protein starts with the formation of disulfide bonds, followed by a rearrangement of hydrophobic and hydrogen-bonded regions on an intra- and inter-molecular basis (Buttkus, 1970). From the results, tannic acids exhibited no marked effect on sulfhydryl and disulfide bond contents of Sawai fillet, when compared with non-treated sample. Conversion of sulfhydryl groups into disulfides and other oxidized species is one of the radical-mediated oxidation of protein (Dean, Fu, Stocker, & Davies, 1997).

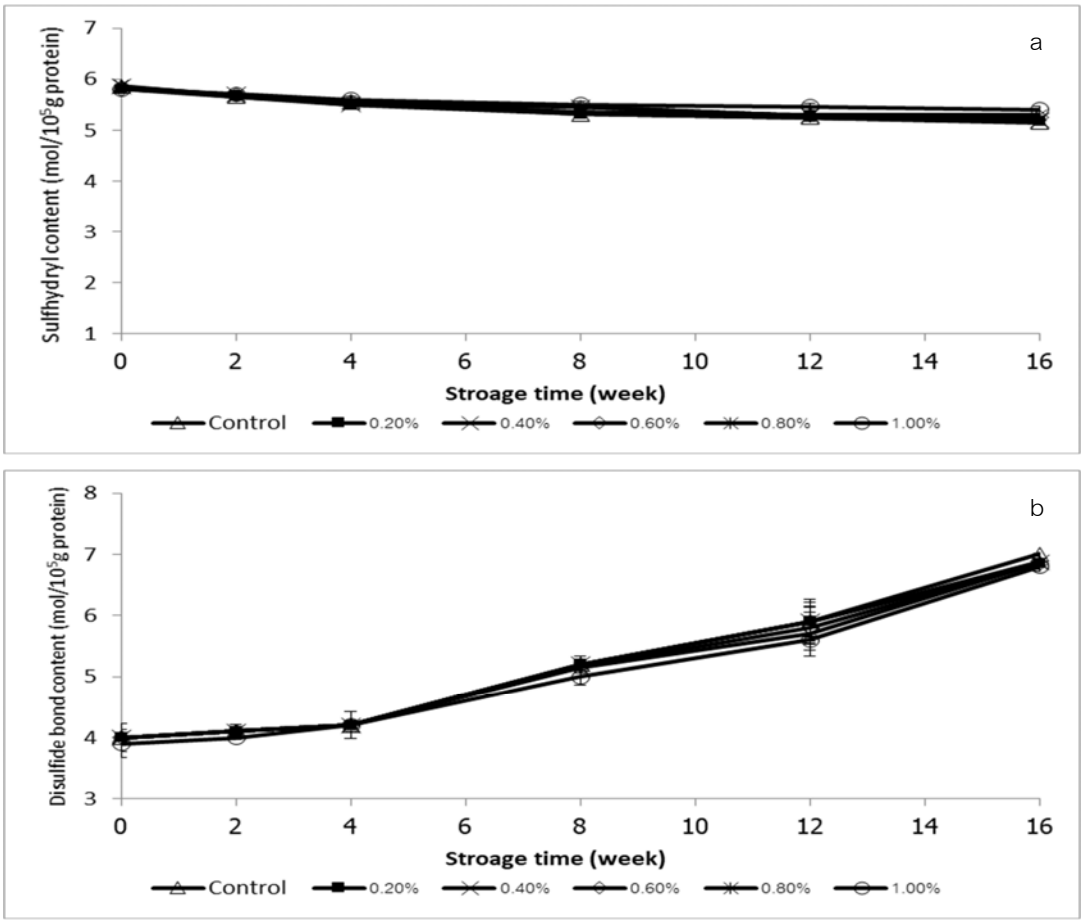

Figure 21 Changes in sulfhydryl (a) and disulfide bond (b) contents of NAM extracted from Sawai fillet treated with and without tannic acids (0-1.0%) during frozen storage at -20 ºC for 16 weeks. Error bars indicate standard deviations from triplicate determinations.

5.3.5 Changes in protein pattern (SDS-PAGE) of Sawai fillet

Under both non-reducing (without β-mercaptoethanol) and reducing conditions, similar SDS-PAGE patterns of Sawai fillet treated with different concentrations of tannic acids (0.2-1.0%) were obtained (Fig. 22). Two major protein bands were observed in Sawai muscle, corresponding to myosin heavy chain (MHC) and actin. The result was in accordance with that reported by Sriket et al. (2017). However, the band intensity of all proteins was higher under non-reducing condition. Disulfide bonds could be formed in Sawai muscle during 16 weeks of frozen storage (Fig. 21b). Recently, Sriket et al. (2019) reported that iron effectively catalyzed protein denaturation and caused dramatic changes in Sawai muscle proteins by the formation of disulfide bond. However, tannic acids showed no profound on protein pattern of Sawai fillet during frozen storage.

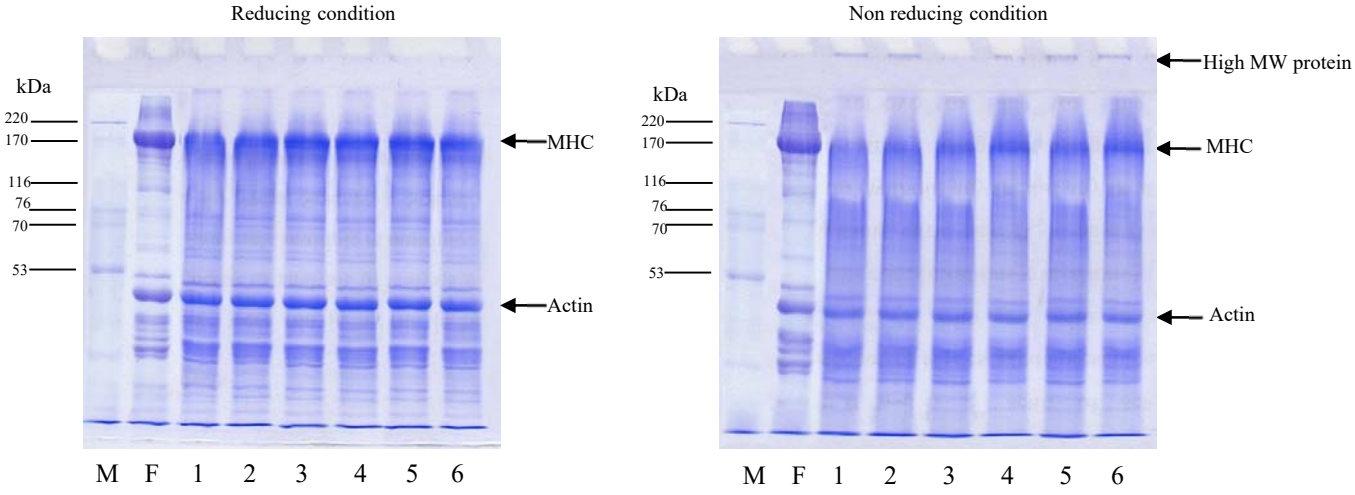

Figure 22 SDS-PAGE pattern of Sawai fillet with different concentrations of tannic acids (0-1.0%) during frozen storage for 16 weeks. M: high molecular weight marker, F: fresh sample, 1: control sample (no tannic acid), 2: sample with 0.2% tannic acid, 3: sample with 0.4% tannic acid, 4: sample with 0.6% tannic acid, 5: sample with 0.8% tannic acid, 6: sample with 1.0% tannic acid.

5.3.6 Volatile compounds in Sawai fillet

Selected volatile compounds in Sawai fillet without and with various concentration of tannic acids (0.2-1.0%) after stored for16-weeks are presented in Table 6. Lipid oxidation of fish muscle is known to produce volatile aldehydic compounds including hexanal, heptanal, octanal, nonanal, etc. (Maqsood & Benjakul, 2011). In the absence of tannic acids, fish fillet contained several volatile

compounds including hexanal, nonanal, 1-octanol, 1-nonanol and decanal. It was noted that control sample contained higher abundance of all volatile compounds (Table 6). It was postulated that higher lipid oxidation and greater decomposition of hydroperoxide occurred in control sample, compared with tannic acid treated samples. Among aldehydes, which are known to be the most predominant volatiles produced during lipid oxidation, hexanal contributes to the rancidity in meats (Maqsood & Benjakul, 2011). Carbonyl compounds involving 4-heptenal, octanal, decanal and 2,4-decadienal were responsible for fishy odor in salmon fish (Varlet, Knockaert, Prost, & Serot, 2006). Fu, Xu, and Wang (2009) reported that hexane and nonanal were responsible for oxidized oil odor as catalyzed by hemoglobin in a silver carp mince model system. Alcohols and ketones are the secondary products produced by the decomposition of hydroperoxide (Maqsood & Benjakul, 2011). However, the development of volatiles was significantly reduced as the tannic acid were incorporated before freezing of fish fillet. The result confirmed that tannic acids, especially 0.6-1.0% were effective in retarding the lipid oxidation, thereby preventing the formation of volatile lipid oxidation compounds of frozen Sawai fillet.

Table 6 Volatile compounds of Sawai fillet with and without various concentrations (0.2-1.0%) of tannic acid during frozen storage for 16 weeks.

	Samples					
Compounds	Control	0.2%	0.4%	0.6%	0.8%	1.0%
Hexanal	7.0*	3.6	2.6	2.2	1.6	1.4
Nonanal	9.1	8.6	7.9	6.3	5.7	5.5
Hexane	6.8	5.6	5.0	3.4	2.8	2.1
1-Octanol	1.8	1.8	ND	ND	ND	ND
1-Nonanol	1.1	1.0	ND	ND	ND	ND
Decanal	1.4	1.0	0.9	ND	ND	ND

ND: not detectable

*Value are expressed as abundance ($\times 10^5$)

5.3.7 Changes in protein solubility

Protein solubility of Sawai fillet treated with and without tannic acids (0.2-1.0%) during frozen storage is shown in Figure 23. Protein solubility decreased slightly during the first 8 weeks of storage. However, the solubility of control sample decreased rapidly after 8 weeks of frozen storage ($p<0.05$).

During frozen storage, Sawai fillet treated with tannic acids could imbibe more water and the migration of water to form ice crystals was retarded. As a result, less aggregation of protein occurred. The decrease in salt-soluble protein concentrations of Sawai fillet during frozen storage was coincidental with the increase in SoANS (Fig. 20), loss of sulfhydryl content and disulfide bond formation (Fig. 21a and b). A gradual decrease in protein extractability during frozen storage of Basa (**P. bocourti**) fillet was also reported (P. Sriket & La-ongnual, 2018). The decrease in solubility of fish fillet might be caused by protein denaturation and protein aggregation induced by freezing and frozen storage. Thermodynamically, a decrease in protein solubility is the result of a shift from a tendency of proteins to interact with water towards a situation where proteins interact with each other (Vojdani, 1996). Freezing reduces the solubility of proteins, especially the myofibrillar proteins, which are generally extracted with 0.6 M NaCl. The solubility of Sawai fillet treated with tannic acids (0-1.0%) was maintained throughout 16 weeks of storage. In the presence of tannic acids, protein-water interaction could be maintained and protein-protein interaction was more likely prevented.

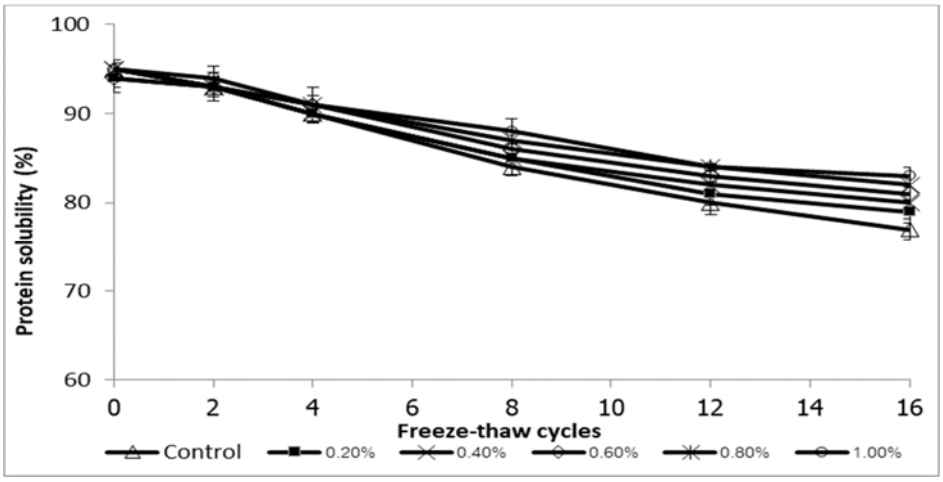

Figure 23 Changes in protein solubility of Sawai fillet treated with and without tannic acids (0-1.0%) during frozen storage at -20 ºC for 16 weeks. Error bars indicate standard deviations from triplicate determinations.

5.3.8 Changes in thaw drip

The lost moisture, known as thaw drip, normally occurs in fish and carries with it soluble proteins, minerals and other nutrients (Gillies, 1975). Thaw drip in frozen Sawai fillet with different concentrations of tannic acids (0.2-1.0%) is shown in Figure 24. No changes in thaw drip of fish fillets

during the first 8 weeks of storage were observed ($p>0.05$). Thereafter, thaw drip of fish fillet increased markedly ($p<0.05$). At 16 weeks of storage, the highest thaw drip was found with control sample (25%), whereas fish fillet treated with tannic acids tended to have the lower thaw drip (17%). Thaw drip of samples soaked with 0.2, 0.4, 0.6, 0.8 and 1.0% tannic acid solutions were 23, 21, 19, 18 and 17%, respectively.

Therefore, tannic acid treatment significantly decreased the thaw drip of frozen fish fillet. During frozen storage, Sawai fillet treated with tannic acids could imbibe more water and the migration of water to form ice crystals was retarded. Continuous denaturation of proteins in fish muscle 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, particularly when the frozen storage time increased. Soaking the fish fillet with tannic acids, especially 0.6-1.0% resulted in reduced thaw drip of frozen fish fillet.

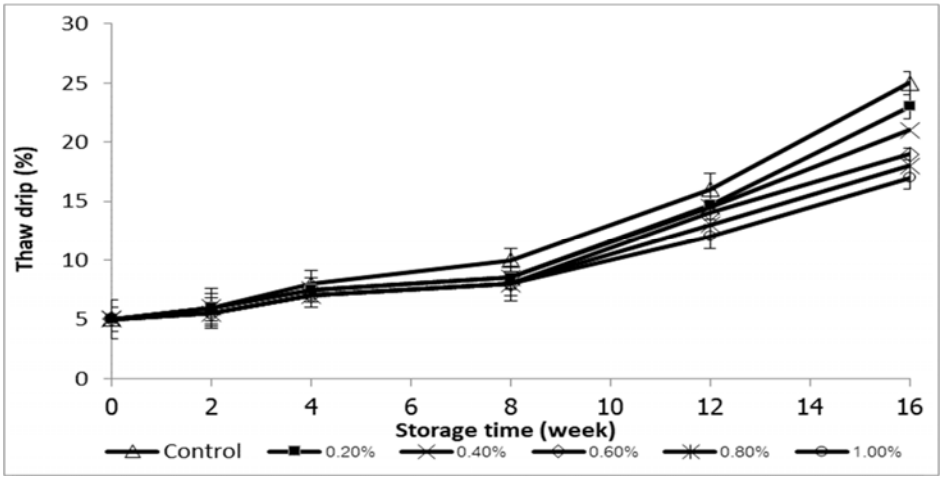

Figure 24 Changes in thaw drip of Sawai fillet treated with and without tannic acids (0-1.0%) during frozen storage at -20 ºC for 16 weeks. Error bars indicate standard deviations from triplicate determinations.

5.3.9 Changes in shear force

The effects of tannic acids (0.2-1.0%) on the shear value of Sawai fillet during frozen storage are shown in Fig. 25. Generally, no changes in shear force of fish fillets during the first 8 weeks of storage were observed. Thereafter, shear force of all samples sharply decreased ($p<0.05$). However, shear force of control sample was generally lower than those with treatments ($p < 0.05$). The

decrease in shear force suggested the loss in integrity of muscle fibers, leading to the weakening of muscle. Fish fillet treated with tannic acids had the higher shear force than control sample during frozen storage ($p<0.05$). The result suggested that tannic acids, especially 1.0% more likely retarded the loss in integrity of muscle fibers via maintaining the water in the muscle and lowering the thaw drip (Fig. 24).

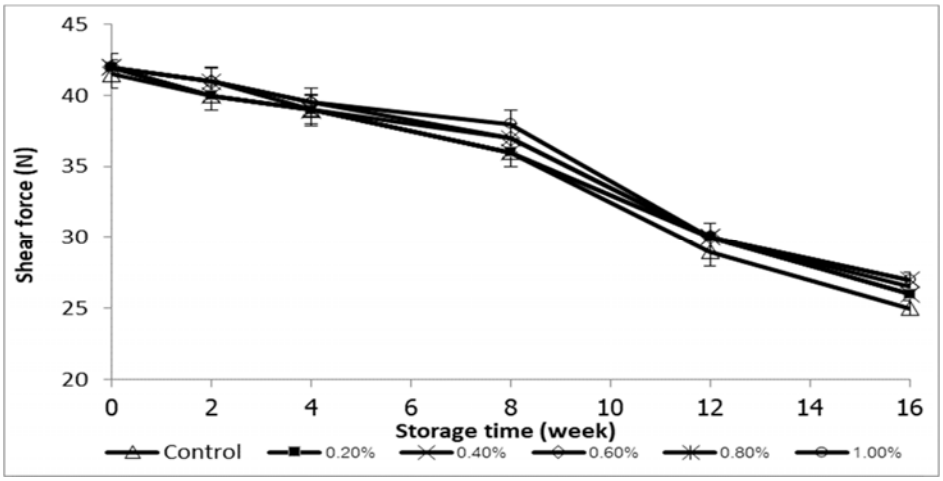

Figure 25 Changes in shear force of Sawai fillet treated with and without tannic acids (0-1.0%) during frozen storage at -20 ºC for 16 weeks. Error bars indicate standard deviations from triplicate determinations.

5.4 Conclusion

Tannic acid (0.2-1.0%) was effective in retarding the lipid oxidation as indicated by lower TBARS formation, yellow discoloration as well as lower development of volatile compounds. Additionally, tannic acid at a level of 0.6-1.0% could maintain the quality of fish fillet to the highest extent after storage for 16 weeks at -20 °C. Therefore, soaking before freezing of Sawai fillet with tannic acids solution, especially at concentration of 0.6-1.0% was an important process to prevent the lipid oxidation, color and quality changes of Sawai fillet during frozen storage.

OUTPUTS

Sriket, C., Benjakul, S. and Senphan, T. 2019. Effect of iron on physicochemical changes of Sawai (**Pangasianodon hypophthalmus**) pastes during multiple freeze-thaw cycles. Turkish Journal of Fisheries and Aquatic Sciences. 19(11), 947-956.

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APPENDIX