Production of Protein Hydrolysate Possessing Antioxidant Activities with Negligible Muddy- and Fishy- Odour/Flavour from Freshwater and Marine Fish

Suthasinee Yarnpakdee

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Author	Ms. Suthasinee Yarnpakdee
Major Program	Food Science and Technology

Major Advisor:

…….....
(Prof. Dr. Soottawat Benjakul)

Co-advisor:

…….....
(Assoc. Prof. Dr. Hordur G. Kristinsson)

Examining Committee:

…….Chairperson
(Asst. Prof. Dr. Manee Vittayanont)

…….…….....……
(Prof. Dr. Soottawat Benjakul)

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

…….…….....……
(Assoc. Prof. Dr. Nurul Huda)

The Graduate School, Prince of Songkla University, has approved this thesis as fulfillment of the requirements for the Doctor of Philosophy Degree in Food Science and Technology.

…….……
(Assoc. Prof. Dr. Teerapol Srichana)
Dean of Graduate School

This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

……..……..….Signature
(Prof. Dr. Soottawat Benjakul)
Major Advisor

……..……..….Signature
(Ms. Suthasinee Yarnpakdee)
Candidate

I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

……...Signature
(Ms. Suthasinee Yarnpakdee)
Candidate

ชื่อวิทยานิพนธ์ การผลิตโปรตีนไฮโดรไลเสตที่มีกิจกรรมการออกฤทธิ์ต้านออกซิเดชันและปราศจากกลิ่นรสโคลนและกลิ่นคาวจากปลาน้ำจืดและปลาทะเล

ผู้เขียน นางสาวสุธาสินี ญาณภักดี

สาขาวิชา วิทยาศาสตร์และเทคโนโลยีอาหาร

ปีการศึกษา 2556

บทคัดย่อ

จากการศึกษาปฏิกิริยาออกซิเดชันของลิปิดและการเกิดกลิ่นคาวในโปรตีนไฮโดรไลเสตจากปลานิลสดและที่ผ่านการเก็บรักษาในน้ำแข็ง พบว่าในระหว่างการเก็บในน้ำแข็งเป็นเวลา 18 วัน ปริมาณเหล็กที่เป็นองค์ประกอบของฮีมมีค่าลดลง สอดคล้องกับการเพิ่มขึ้นของปริมาณเหล็กที่ไม่ใช่องค์ประกอบของฮีม ค่าเพอร์ออกไซด์ (PV) และ thiobarituric acid reactive substances (TBARS) ($P < 0.05$) ปริมาณฟอสโฟลิปิดมีค่าลดลงสอดคล้องกับการเพิ่มขึ้นของปริมาณกรดไขมันอิสระ และเมื่อผลิตโปรตีนไฮโดรไลเสตจากปลานิลสดและที่ผ่านการเก็บรักษาในน้ำแข็ง พบว่าผลิตภัณฑ์จากปฏิกิริยาออกซิเดชัน การเกิดกลิ่นคาว รวมถึงปริมาณสารประกอบที่ระเหยได้มีค่าสูงขึ้นในโปรตีนไฮโดรไลเสตที่ผลิตจากปลาไม่สด ($P < 0.05$) อย่างไรก็ตาม เมื่อเติมสารต้านออกซิเดชันผสมในระหว่างกระบวนการย่อยสลาย ส่งผลให้ผลิตภัณฑ์จากปฏิกิริยาออกซิเดชัน ค่า b* ΔC* และ ΔE* กลิ่นคาว/กลิ่นรสคาว และปริมาณสารประกอบที่ระเหยได้ลดลงอย่างเด่นชัดในโปรตีนไฮโดรไลเสตที่เตรียมทั้งจากปลาสดและที่ผ่านการเก็บรักษา

เมื่อศึกษาผลของการปฏิบัติเบื้องต้นด้วยวิธีต่าง ๆ ต่อองค์ประกอบทางเคมีของเนื้อปลานิล พบว่าเนื้อปลาที่ผ่านกระบวนการล้าง/ การกำจัดลิปิดที่เป็นองค์ประกอบของเยื่อหุ้มเซลล์และการละลายด้วยสารละลายด่าง (โปรตีนไอโซเลต: PI) มีค่าปริมาณไมโอโกลบินและปริมาณเหล็กที่เป็นองค์ประกอบของฮีมต่ำสุด รวมทั้งมีปริมาณลิปิดทั้งหมดและฟอสโฟลิปิดต่ำสุด เมื่อย่อยสลายเนื้อปลาบดและโปรตีนไอโซเลต เป็นเวลา 120 นาที พบว่าโปรตีนไอโซเลตมีระดับการย่อยสลาย (DH) สูงกว่าเนื้อปลาบด นอกจากนี้โปรตีนไฮโดรไลเสตที่เตรียมจากโปรตีนไอโซเลตยังมีค่า PV TBARS และปริมาณเหล็กที่ไม่ใช่องค์ประกอบของฮีมต่ำกว่าตลอดช่วงการย่อยสลาย ($P < 0.05$) และเมื่อเติมโปรตีนไฮโดรไลเสตจากเนื้อปลาบดและโปรตีนไอโซเลต (ร้อยละ 0.3-0.5 โดยน้ำหนักต่อปริมาตร) ในนม พบว่านมที่ผ่านการเติมโปรตีนไฮโดรไลเสตจากเนื้อปลาบด มีคะแนนความชอบลดลงในทุกระดับความเข้มข้น ($P < 0.05$) ในทางตรงกันข้ามการเติมโปรตีนไฮโดรไลเสตที่เตรียมจากโปรตีนไอโซเลตที่ระดับร้อยละ 0.5 ไม่มีผลต่อคะแนนความชอบสำหรับทุกคุณลักษณะ เมื่อเปรียบเทียบกับนมที่ไม่ผ่านการเติมไฮโดรไลเสต

เมื่อศึกษาองค์ประกอบทางเคมีของเนื้อปลาทูลังที่ผ่านการปฏิบัติเบื้องต้นด้วยวิธีต่าง ๆ พบว่า โปรตีนไอโซเลตมีปริมาณโปรออกซิแดนซ์ ฟอสโฟลิปิด และผลิตภัณฑ์จากปฏิกิริยาออกซิเดชันของลิปิดต่ำที่สุด และจากการติดตามระดับการย่อยสลายและการเปลี่ยนแปลงทางเคมีในระหว่างกระบวนการไฮโดรไลซิสเนื้อปลาบดและโปรตีนไอโซเลตด้วยเอนไซม์อัลคาเลส เป็นเวลา 2 ชั่วโมง พบว่าโปรตีนไฮโดรไลเสตจากโปรตีนไอโซเลตมีระดับการย่อยสลายสูงกว่า ในขณะที่ปริมาณเหล็กที่ไม่ใช่องค์ประกอบของฮีม ค่า PV และ TBARS มีค่าต่ำกว่า เมื่อเติมโปรตีนไฮโดรไลเสตจากเนื้อปลาบดและโปรตีนไอโซเลต (ร้อยละ 0.1-0.3 โดยน้ำหนักต่อปริมาตร) ในนม พบว่าโปรตีนไฮโดรไลเสตจากเนื้อปลาบดทุกระดับมีผลลดคะแนนความชอบของนม ($P < 0.05$) ส่วนโปรตีนไฮโดรไลเสตจากโปรตีนไอโซเลตร้อยละ 0.2 ไม่ส่งผลต่อคะแนนความชอบในทุกคุณลักษณะ เมื่อเปรียบเทียบกับนมชุดควบคุม (ที่ไม่ผ่านการเติมไฮโดรไลเสต)

จากศึกษาเปรียบเทียบผลของออกซีฮีโมโกลบิน (oxy-Hb) และเมทฮีโมโกลบิน (met-Hb) ต่อปฏิกิริยาออกซิเดชันของลิปิดที่สัมพันธ์กับการเกิดกลิ่นคาวของโปรตีนไฮโดรไลเสตจากปลานิล พบว่าในระหว่างการย่อยสลายโปรตีนไอโซเลตเป็นเวลา 120 นาที พบว่าปริมาณเหล็กที่ไม่ใช่องค์ประกอบของฮีม ค่า PV และ TBARS ของไฮโดรไลเสตเพิ่มขึ้นเล็กน้อย ($P < 0.05$) ทั้งนี้ทุกพารามิเตอร์มีค่าเพิ่มขึ้นอย่างเด่นชัดเมื่อเติม oxy-Hb หรือ met-Hb โดยเฉพาะอย่างยิ่งภายใน 60 นาทีแรกของการย่อยสลาย โดยชุดทดลองที่เติม met-Hb ให้ค่าที่สูงกว่า บ่งชี้ว่า met-Hb เป็นตัวเร่งปฏิกิริยาออกซิเดชันที่มีประสิทธิภาพกว่า oxy-Hb นอกจากนี้ ค่า b*, ΔE^* และ ΔC^* รวมทั้งกลิ่นคาว/กลิ่นรสคาว และปริมาณสารประกอบที่ระเหยได้มีค่าสูงขึ้นอย่างเด่นชัดในโปรตีนไฮโดรไลเสตที่เติม oxy-Hb หรือ met-Hb

การศึกษาองค์ประกอบทางเคมีและสารประกอบกลิ่นโคลนของกล้ามเนื้อส่วนบนและส่วนล่างของปลานิลและปลาดุกอุย พบว่าปลานิลประกอบด้วยโปรตีนสูง (ร้อยละ 93.1-93.8) ในขณะที่ปลาดุกอุยมีปริมาณโปรตีน (ร้อยละ 55.2-59.5) และไขมัน (ร้อยละ 36.6-42.2) เป็นองค์ประกอบหลักเมื่อคิดบนฐานน้ำหนักแห้ง โดยกล้ามเนื้อส่วนล่างมีปริมาณของลิปิดและฟอสโฟลิปิดสูงกว่า สอดคล้องกับปริมาณของจีออสมิน และ 2-methylisoborneol (2-MIB) ที่สูงกว่า โดยในเนื้อปลานิลและปลาดุกอุยพบปริมาณจีออสมิน เท่ากับ 1.5 และ 3.2 ไมโครกรัมต่อกิโลกรัมตามลำดับ สำหรับปริมาณ 2-MIB ที่พบในเนื้อปลาดุกอุยเท่ากับ 0.8 ไมโครกรัมต่อกิโลกรัม แต่ไม่พบในเนื้อปลานิล ทั้งนี้เมื่อเตรียมโปรตีนไอโซเลต ปริมาณของลิปิดและฟอสโฟลิปิดมีค่าลดลง พร้อมกับมีการลดลงของปริมาณจีออสมินและ 2-MIB นอกจากนี้โปรตีนไฮโดรไลเสตที่

เตรียมจากโปรตีนไอโซเลตให้สีที่อ่อนกว่า และมีการลดลงของปริมาณสารประกอบกลิ่นโคลน เมื่อเปรียบเทียบกับโปรตีนไฮโดรไลเสตที่เตรียมจากเนื้อปลาบด

จากการศึกษาสมบัติการออกฤทธิ์ต้านออกซิเดชันและสมบัติด้านประสาทสัมผัสของโปรตีนไฮโดรไลเสตที่เตรียมจากโปรตีนไอโซเลตของปลานิลโดยกระบวนการย่อยสลายขั้นตอนเดียวและสองขั้นตอน พบว่าโปรตีนไฮโดรไลเสตที่เตรียมโดยใช้เอนไซม์โปรติเอสเดี่ยว ๆ ได้แก่ อัลคาเลส (HA) ฟลาโวไซม์ (HF) โปรตาเม็ก (HPr) และปาเปน (HPa) มีกิจกรรมการออกฤทธิ์ต้านออกซิเดชันเพิ่มขึ้น เมื่อ DH เพิ่มขึ้นถึงร้อยละ 40 ($P < 0.05$) เมื่อเปรียบเทียบไฮโดรไลเสตทั้งหมด HA ที่ DH ร้อยละ 40 มีกิจกรรมการออกฤทธิ์ต้านออกซิเดชันสูงสุด และเมื่อ HA ถูกย่อยสลายต่อด้วยปาเปน ส่งผลให้ไฮโดรไลเสตที่ได้ (HAPa) มีกิจกรรมการออกฤทธิ์ต้านออกซิเดชันสูงสุดสำหรับทุกวิธีการทดสอบที่ใช้ ($P < 0.05$) อีกทั้งยังพบว่ากิจกรรมการออกฤทธิ์กำจัดอนุมูล ABTS และการกำจัดโลหะของ HAPa มีความคงตัวในช่วงพีเอชกว้าง (1-11) และในระหว่างการให้ความร้อน (30-100 °ซ) โดยกิจกรรมการออกฤทธิ์ทั้งสองมีค่าเพิ่มขึ้นในระบบทางเดินอาหารจำลอง โดยเฉพาะอย่างยิ่งในสภาวะจำลองของลำไส้ นอกจากนี้ HAPa (100-1,000 มิลลิกรัมต่อลิตร) ยังสามารถยับยั้งปฏิกิริยาออกซิเดชันของลิปิดในระบบเบต้า-แคโรทีน-ลิโนเลเอต และเลซิติน-ลิโพโซมโดยแปรผันตามความเข้มข้นที่ใช้ เมื่อทำการแยกเพปไทด์จาก HA และ HAPa พบว่า เพปไทด์ที่มีน้ำหนักโมเลกุล เท่ากับ 513 และ 1,484 ดาลตัน แสดงกิจกรรมการออกฤทธิ์กำจัดอนุมูล ABTS และกำจัดโลหะสูงสุดตามลำดับ และจากศึกษารูปแบบของกรดอะมิโน พบว่า ทั้ง HA และ HAPa มีกรดอะมิโนชนิดไฮโดรโฟบิกในปริมาณสูง (ร้อยละ 38.26-38.85) และพบกรดกลูตามิก/กลูตามีน ไลซีน และ กรดแอสพาร์ติก/แอสพาราจีน เป็นกรดอะมิโนหลัก อย่างไรก็ตาม HAPa มีการยอมรับที่สูงกว่า HA เนื่องจากมีความขมที่น้อยกว่า

เมื่อศึกษากิจกรรมการออกฤทธิ์ต้านออกซิเดชันของโปรตีนไฮโดรไลเสตจากปลานิล อันได้แก่ HA HAPa และแฟรกชั่นที่แยกจากคอลัมน์ Sephadex G-25 ของทั้งสองตัวอย่าง (FHA และ FHAPa) ในระบบจำลองทางเคมีและเซลล์ พบว่า FHAPa แสดงกิจกรรมการออกฤทธิ์ต้านออกซิเดชันในระบบจำลองทางเคมีสูงสุดแต่ไม่พบกิจกรรมการกำจัดโลหะ เมื่อตรวจสอบความสามารถการออกฤทธิ์ต้านออกซิเดชันในเซลล์ของ HA HAPa และ แฟรกชั่นของทั้งสองตัวอย่าง โดยศึกษาการยับยั้งความเสียหายของเซลล์มะเร็งตับและดีเอ็นเออันเกิดจากปฏิกิริยาออกซิดชันที่เหนี่ยวนำด้วยไฮโดรเจนเปอร์ออกไซด์ (H_2O_2) และ AAPH พบว่า เซลล์ที่ผ่านการเติมไฮโดรไลเสตและแฟรกชั่นของทั้งสองตัวอย่างก่อนการเร่งให้เกิดปฏิกิริยาออกซิเดชันมีการรอดชีวิตเพิ่มขึ้น ส่วนการเพิ่มจำนวนของสารประกอบความว่องไวสูงที่มีออกซิเจน (ROS) ในเซลล์ที่

เร่งด้วย H_2O_2 และ AAPH มีค่าลดลงเมื่อมีการเติมไฮโดรไลเสตหรือแฟรกชั่น โดยเฉพาะอย่างยิ่งเมื่อใช้ร่วมกับ Trolox เข้มข้น 50 ไมโครโมลาร์ นอกจากนี้ FHAPa ยังมีความสามารถในการยับยั้งการตัดของสายดีเอ็นเอที่เหนี่ยวนำด้วยไฮโดรเจนเปอร์ออกไซด์ และอนุมูลเพอร์รอกซีโดยแปรผันตามความเข้มข้นที่ใช้

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ABSTRACT

Lipid oxidation and fishy odour development in protein hydrolysate from fresh and ice-stored Nile tilapia were investigated. During iced storage of 18 days, haem iron content decreased with a concomitant increase in non-haem iron content, peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) values ($P < 0.05$). Phospholipid content decreased with a coincidental increase in free fatty acid content. When protein hydrolysates were produced from fresh and 18 days ice-stored Nile tilapia muscle, higher lipid oxidation and fishy odour/flavour along with higher amount volatile compounds were obtained in hydrolysate for unfresh sample ($P < 0.05$). However, the addition of mixed antioxidants during hydrolysis process markedly lowered lipid oxidation, b*, ΔC^*, ΔE^* values, fishy odour/flavour as well as the formation of volatile compounds in the resulting hydrolysates prepared from both fresh and unfresh samples.

Impact of different pretreatments on chemical compositions of Nile tilapia were elucidated. Mince prepared using washing/membrane lipid removal/alkaline solubilisation process (protein isolate: PI) contained the lowest remaining myoglobin and haem iron content and also showed the lowest total lipid and phospholipid contents. When mince and PI were hydrolysed using Alcalase for up to 120 min, a higher degree of hydrolysis (DH) was found in PI. Furthermore, hydrolysate from PI had lower PV, TBARS and non-haem iron content throughout hydrolysis ($P < 0.05$). When hydrolysate powder produced from mince and PI (0.3-0.5%, w/v) were fortified in milk, that from mince yielded the milk with the lower likeness score at all levels used ($P < 0.05$). On the other hand, the fortification of hydrolysate from PI up to

0.5%, had no effect on likeness of all attributes, compared with milk without fortification ($P > 0.05$).

When chemical compositions of Indian mackerel mince with different pretreatments were studied, PI had the lowest pro-oxidant, phospholipid and lipid oxidation product contents ($P < 0.05$). During hydrolysis of mince and PI using Alcalase for 2 h, DH and chemical changes were monitored. A higher DH was found in hydrolysate from PI, whilst lower non-haem iron content, PV and TBARS were also observed ($P < 0.05$). When hydrolysate powder from mince and PI (0.1-0.3% w/v) were fortified in milk, the former resulted in a lower likeness score of milk at all levels used ($P < 0.05$). The addition of the latter up to 0.2% had no effect on likeness of all attributes, compared with control milk (without fortification).

Lipid oxidation associated with fishy odour development in Nile tilapia protein hydrolysate as affected by oxyhaemoglobin (oxy-Hb) and methaemoglobin (met-Hb) was comparatively studied. During hydrolysis of PI up to 120 min, non-haem iron content, PV and TBARS of resulting hydrolysate slightly increased ($P < 0.05$). When oxy-Hb or met-Hb was incorporated, the marked increases in all parameters were observed, especially within the first 60 min of hydrolysis. The higher increases were obtained with the latter, suggesting that met-Hb was more pro-oxidative than oxy-Hb. The marked increases in the b*, ΔE^*, ΔC^* values, fishy odour/flavour and volatile compounds were also found in the resulting hydrolysate containing either oxy-Hb or met-Hb.

Chemical compositions and muddy compounds in dorsal and ventral muscles of Nile tilapia and broadhead catfish were investigated. On a dry weight basis, Nile tilapia was rich in protein (93.1–93.8%), whilst broadhead catfish contained protein (55.2–59.5%) and lipid (36.6–42.4%) as the major constituents. Ventral portion had higher lipid or phospholipid contents with coincidentally higher geosmin and/or 2-methylisoborneol (2-MIB) contents. Geosmin was found in mince of Nile tilapia and broadhead catfish at levels of 1.5 and 3.2 μg/kg, respectively. Broadhead catfish mince had 2-MIB at a level of 0.8 μg/kg, but no 2-MIB was detected in Nile tilapia counterpart. When PI was preprared, lipid and phospholipid contents were lowered with concomitant decrease in geosmin and 2-MIB contents. Protein hydrolysate produced

from PI had a lighter colour and a lower amount of muddy compounds, compared with that prepared from mince.

The antioxidant and sensory properties of protein hydrolysate from Nile tilapia PI prepared using one- and two- step hydrolysis were tested. Hydrolysates prepared using single protease including Alcalase (HA), Flavourzyme (HF), Protamex (HPr) and papain (HPa) had increases in antioxidant activities as the DH increased up to 40% ($P < 0.05$). Amongst all hydrolysates, HA having 40% DH showed the highest antioxidant activities. When HA was further hydrolysed by papain, the resulting hydrolysate (HAPa) exhibited the highest antioxidant activities for all assays tested ($P < 0.05$). ABTS radical scavenging activity and metal chelating of HAPa generally remained constant in a wide pH range (1–11) and during heating at 30–100 °C. Both activities increased in the simulated gastrointestinal tract model system, especially in intestine condition. HAPa (100–1,000 mg/l) could retard lipid oxidation in β-carotene-linoleate and lecithin-liposome model systems in a dose dependent manner. Peptides in both HA and HAPa with molecular weight of 513 Da and 1,484 Da possessed the strongest ABTS radical scavenging activity and metal chelating activity, respectively. Based on the amino acid profile, both HA and HAPa contained a high amount of hydrophobic amino acids (38.26–38.85%) and had glutamic acid/glutamine, lysine and aspartic acid/asparagine as the dominant amino acids. However, HAPa showed a higher acceptability than did HA, owing to the lower bitterness.

When the antioxidant activities of Nile tilpapia protein hydrolysates, HA, HAPa and their Sephadex G-25 fractions (FHA and FHAPa), were determined in both chemical and cellular based models. Amongst all samples, FHAPa showed the highest chemical antioxidant activities, however it had no metal chelation activity. Cellular antioxidant ability of HA, HAPa and their fractions agianst H_2O_2 and AAPH induced oxidative damage of HepG2 cell and DNA were tested. When cell was pretreated with all hydrolysates or fractions, cell viabiliy increased. Cell reactive oxygen species (ROS) generation mediated by H_2O_2 and AAPH decreased with treatment of hydrolysates or their fractions, especially in combination with 50 µM Trolox. FHAPa effectively inhibited H_2O_2 and peroxyl radical induced DNA scission in a dose dependent manner.

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CHAPTER 1
INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Fish have been of increasing demand among the consumers due to the high nutritive value. Fish can be processed into different finished products with varying market values. Apart from fish flesh or edible portions, the left-over such as head, skin, viscera, etc. can be used as raw material for production of value-added products (Šližytė *et al.*, 2009). Hydrolytic processes have been developed to convert fish proteins into more marketable and acceptable forms (Halldorsdottir *et al.*, 2014a; Klompong *et al.*, 2008). Enzymatic hydrolysis of fish proteins has been known to be an efficient way to produce potent bioactive peptides (Šližytė *et al.*, 2009; Thiansilakul *et al.*, 2007a). The numerous peptides derived from hydrolysed food protein have potential for nutritional or pharmaceutical applications (Grienke *et al.*, 2014; Je *et al.*, 2013; Khiari *et al.*, 2014). Some peptides have been recognised for bioactivities and have gained increasing attention as functional foods.

A major problem associated with hydrolysate preparation from fish is the presence of pro-oxidants such as haem proteins and unstable lipid substrates, which are susceptible to oxidation. The oxidation products could decrease the stability of protein hydrolysates (Raghavan *et al.*, 2008). Additionally, lipid oxidation generally contributes to the development of undesirable odour and flavour, especially fishy odour (Maqsood and Benjakul, 2011a). The offensive odour/smell is a crucial factor limiting the use of hydrolysates, particularly in foods or drinks which have a light odour. Even though lean fish are traditionally used for fish processing with a consistent high quality owing to their negligible lipid and haem pigment contents, lipid oxidation is still a chemical reaction related with the quality loss of lean fish and their products. Phospholipid membranes are believed to be the key substrate for lipid oxidation (Liang and Hultin, 2005b). It is well known that unsaturated fatty acids are presented in membrane and also contain a large surface area to contact with pro-oxidants and oxygen (Liang and Hultin, 2005a). Moreover, haem proteins, myoglobin (Mb) and hemoglobin (Hb), have a close relationship with lipid oxidation, which could influence the

deterioration in lipid-based food (Maqsood *et al.*, 2012; Richards and Li, 2004; Thiansilakul *et al.*, 2010). Apart from fishy odour, muddy flavour is a severe problem found in freshwater fish, mainly causing the rejection by consumers. The common problems are due to the absorption of odourous compounds, particularly geosmin and 2-methyl-iso-borneol (2-MIB) produced by microorganisms in water, where fish inhabit. Fish flesh with those offensive odour/flavour more likely yields the resulting protein hydrolysate with undesirable attributes. Therefore, the appropriate technology which can tackle these problems and yield hydrolysate without off-odour or off-flavour should be taken into consideration. Additionally, the hydrolysate containing bioactive peptides can be obtained, which can be of health benefit. As a consequence, the use of aquatic resource can be maximised and new marketable products from fish flesh can be gained.

1.2 Review of Literature

1.2.1 Production and quality aspects of some economically important fish

1.2.1.1 Nile tilapia

Nile tilapia (*Oreochromis niloticus*) becomes an important economic fish species for freshwater aquaculture. It is popular in Southeast Asia or other countries including China, Bangladesh, India etc., owing to its white flesh and delicacy. Nowadays, it is rapidly expanding in Asia with annual production exceeding 3.3 million tons in 2012 out of a total global production of 5.15 million tons (FAO, 2012). Thailand is one of the main producers of Nile tilapia in the world. Its export has increased from 8,240 tons in 2000 to 204,700 tons in 2010 (Department of Fisheries, 2012). Traditionally, Nile tilapia is sold as whole frozen fish or as fillets frozen products (Grundy-Warr *et al*., 2011). During an extended storage, tilapia became more rancid, associated with lipid oxidation (Dergal *et al*., 2013). The freshness of fish is reduced by the oxidative rancidity and the organoleptic properties are rapidly deteriorated (Azhar and Nisa, 2006). Shakhtour *et al.* (2014) reported that the increases in FFA and TBA were correlated with the stronger off-odour/flavour of red tilapia during of ice storage. In addition, muddy flavour is a major problem associated with freshwater fish,

including Nile tilapia (Yamprayoon and Noomhorm, 2000). Those off-flavours lower the acceptability of Nile tilapia (Dergal *et al.*, 2013; El-Hanafy *et al.*, 2011).

1.2.1.2 Catfish

Farm raised catfish (*Clarias spp.*) has been one of the most popularly commodities in Thailand's domestic freshwater fish market. Two native species, *Clarias batrachus* and *Clarias macrocephalus* have been commercially cultured. However, consumers have always favoured *C. macrocephalus* because of its better flesh quality (taste and firmness) (Senanan *et al*., 2004). The annual production in Thailand is estimated to be 113,800 tons with a value of 5,329.8 million baht in 2011 (Department of Fisheries, 2012). Majority of cultured catfish is sold in raw and some are exported in chilled and frozen fillets. Based on aquaculture, catfish are mainly cultured intensively and fed with trash fish, chicken offal or pelleted feed, which generally cause poor water quality and heavy phytoplankton blooms throughout most of the grow-out period (Yi *et al*., 2003). This led to the presence of the offensive odour associated with fish flesh and become a serious problem (Yi *et al*., 2003). Due to high fat content, catfish meat is also prone to oxidation related with rancidity (Pourashouri, *et al*. 2009). Pourashouri *et al.* (2009) reported that rancidity development in Wels catfish (*Silurus glanis*) fillets during frozen storage at -18 ºC for 6 months was associated with lipid deterioration and PUFA oxidation. However, soaking its fillet with ascorbic acid and citric acid could improve rancid stability during storage. Additionally, catfish has muddy flavour, especially those raised in the earth pond (Lovell, 1983).

1.2.1.3 Indian mackerel

Indian mackerel (*Rastrelliger kanagurta*) is one of the most economically import pelagic fish species due to it is abundance. Due to the limited of marine white muscle fish caused by the overexploitation, dark muscle fish has gained more attention as a potential alternative raw material. In 2011, the catch of Indian mackerel in the Gulf of Thailand was approximately 54,200 tons with a value of 1,867 million bath (Department of Fisheries, 2012). In general, Indian mackerel is introduced to industry to develop the various kinds of products (Chaijan *et al*., 2006; Panpipat *et*

al., 2010). This species has been used for domestic consumption. To maximise the utility, hydrolysis process might be one way to transform underutilised fish protein resources into value added product with nutritive value and bioactivity (Klompong *et al*., 2008). Nevertheless, large quantity of lipids and myoglobin associated the muscle tissue from those fatty dark-fleshed fish affected the stability of their products. During hydrolysis, lipid oxidation along with the development of fishy- or rancid odour took place and contributed to quality deterioration of hydrolysate produced from mackerel (Hou *et al*., 2011a; Wu *et al*., 2003).

1.2.2 Off-odour/flavour in fish and fish products

Off-odour or off-flavour in fish and fish products generally causes a major reduction in acceptability for consumers or makes them unsuitable for sale, thereby lowering market value (Robin *et al.*, 2006). The compounds associated with off-odour/flavour are generated by enzymatic reaction, lipid autoxidation, microbial action and environmentally or thermally derived reaction (Šližytė *et al.*, 2009). Amongst these reactions, lipid oxidation is closely related with the alteration of odour and flavour in muscle based food. Oxidation of unsaturated lipids not only produces offensive odours/flavours but also decreases the nutritional quality and safety. The typical offensive fishy- and rancid-odour/flavour have contributed to the major sensory defects. Additionally, another unpleasant odour/flavour, a muddy flavour, has been considered to be an undesirable contaminant in freshwater fish. The problems with such odour/flavour taints are well documented in lowering overall quality and marketability of fish and their products.

1.2.2.1 Fishy odour/flavour

Fishy odour/flavour is the typical off-odour/flavour associated with fish flesh. Generally, trimethylamine (TMA) is the main component responsible for an unpleasant ‘fishy’ odour, primarily found in marine fish. The compound is generated by the reduction of TMAO caused by some microorganisms with TMAO reductase (Gram and Huss, 1996). When oxygen levels are depleted, TMAO serves as a terminal electron acceptor for anaerobic respiration of those microorganisms (Gram and

Dalgaard, 2002). TMA at low concentrations can contribute to the strong fishy odour. Rochat *et al.* (2009) used olfactometric analysis and found that trimethylamine was a key fishy odourant in shrimps. Sallam *et al.* (2007) reported that fishy odour formation in marinated Pacific saury during vacuumed package storage was correlated with the development of total volatile base (TVB) and TMA. Moreover, lipid oxidation plays an important role in fishy odour development (Maqsood and Benjakul, 2011a). Some oxidation products, aldehyde, ketone or alcohol, were identified as fishy volatiles. Varlet *et al.* (2006) reported that carbonyl compounds, such as heptanal or (*E,Z*)-2,6-nonaldienal, contributed to typical fishy odour in salmon (*Salmo salar*) stored in cold room (3 °C) for 18 h. The fishy volatiles identified in the boiled sardine were dimethyl sulfide, acetaldehyde, propionaldehyde, butyraldehyde, 2-ethylfuran, valeraldehyde, 2,3-pentanedione, hexanal and 1-penten-3-ol (Kasahara and Osawa, 1998). Thiansilakul *et al.* (2011) reported that the major fishy volatile compounds found in washed Asian seabass mince were hexanal, 1-octen-3-ol and 2-pentyl furan, when Mb was incorporated. The volatile compounds associated with fishy odour in washed Asain seabass added with Hb from tilapia included heptanal, hexanal, octanal, nonanal and 2-octenal as dominant compounds (Maqsood and Benjakul, 2011a). Tao *et al.* (2014) reported that hexanal, (Z)-4-heptenal, (E)-2-nonenal, 1-penten-3-ol, 1-octen-3-ol, 2-pentylfuran and TMA were identified as the key fishy odourants in the cooked meat of farmed obscure puffer.

1.2.2.2 Rancid odour/flavour

Rancid odour is often related to a significant number of volatile compounds that can be produced from the oxidation of polyunsaturated fatty acids (PUFA). PUFAs are capable of reacting with molecular oxygen via a free radical chain mechanism, forming fatty acyl hydroperoxides and non-volatile as well as volatile hydroperoxide breakdown products. Many of the products, especially the volatile fraction, create undesirable off-flavours known as rancidity. This results in a reduction of the commercial shelf-life of food stuffs. Aldehydes are the main volatile secondary oxidation products responsible for off-flavours/odours during processing and storage. Several volatiles are associated with the characteristic odours and flavours of oxidised fish, described as rancid, painty, fishy and cod-liver like (Pearson *et al.*, 1977).

Oxidation of unsaturated fatty acids in fish is related to the formation of *E*-2-pentenal, *E*-2-hexenal, *Z*-4-heptenal, (*E*,*E*)-2,4-heptadienal and 2,4,7-decatrienal (Frankel, 1998). Other volatile compounds formed during oxidation of fish lipids are 1-penten-3-ol, 1-octen-3-ol, 1,5-octadien-3-one and 2,6-nonadienal and some of them had high odour impact (Milo and Grosch, 1993). Halldorsdottir *et al.* (2014a) reported that a stronger rancid odour of cod protein hydrolysate was related with higher TBARS development, especially when proxidants (Hb, iron and fish oil) were present.

1.2.2.3 Muddy flavour

A serious problem in fish aquaculture is related with the objectionable flavouring compounds from the culture environment (Percival *et al.*, 2008). Generally, fish readily absorb the organic and inorganic compounds through their gills and skin as well as from digestive tract. Off-flavour brings about the loss in market value and unacceptability of consumers. This flavour is described as ‘muddy or earthy/musty’ flavours, which are positively caused by geosmin and 2-MIB. The former compound renders an earthy pond-bottom taste, whilst the latter one is associated with a musty taste. These are mainly metabolites produced by cyanobacteria, actinomycetes, and certain fungi (Jensen *et al.*, 1994; Lovell and Sackey, 1973). Actinomycetes are more numerous in the sediments of pond which have been identified as producers of geosmin and 2-MIB. Cyanobacteria are dominant phytoplankton found in water, which can produce geosmin-related off-flavour compounds. Typically, fish readily absorbs these compounds through their gills, then transfer to digestive tract and finally accumulated in lipid rich fish tissues. In aquaculture systems, earthy and musty off-flavours have been detected in Nile tilapia (*Oreochromis niloticus*) (Yamprayoon and Noomhorm, 2000), shrimp (Whitfield *et al.*, 1988) Atlantic salmon (*Salmo salar*) (Farmer *et al.*, 1995), rainbow trout (*Salmo gairdneri*) (From and Hørlyck, 1984), catfish (Lovell *et al.*, 1986; Martinez *et al.*, 2003), cultured largemouth bass (*Micropterus salmoides*) and white sturgeon (*Acipenser transmontanus*) (Schrader *et al.*, 2005). Each compound is easily detectable in fish by humans. The sensory threshold concentration differs between species of fish. For geosmin, the sensory threshold in rainbow trout was estimated to be 0.9 µg/kg (Rohrlack *et al.*, 2005). This was slightly higher than the

value of 0.7 μg/kg reported in the channel catfish for geosmin (Dionigi *et al.*, 2000; Johnsen and Kelly, 1990) and 0.6 μg/kg for MIB in rainbow trout (Persson, 1988).

Generally, the muddy flavour could be reduced from live fish during holding in clean flowing water for 10–15 days, depending on the amount of contamination and temperature, however the fish can easily lose 10–15% of its live weight in this process (Tapiador, 1974). Burr *et al.* (2012) also found that the depuration of salmon in odour-free water for 10-15 days decreased geosmin and 2-MIB in fish flesh. After 20 days, fish lost significantly more weight (5.8%), compared to day 5 (3.8%). Boonanuntanasarn *et al.* (2014) reported that the use of activated carbon at 30 mg/kg as feed supplement in Nile tilapia for 4 weeks could reduce geosmin in fish fillet. Typically, fish can be purged of taint compounds if transferred to geosmin/MIB-free water but the process is much slower than the rate of uptake (Percival *et al.*, 2008). Moreover, off odours can be minimised during processing of fish by soaking, dipping and washing in tamarind pulp, lemon juice, lemon grass, banana leaf ash, salt solution or a mixture (Bakar and Hamzah, 1997; Mohsin *et al.*, 1999). Rohani and Yunus (1994) reported that soaking of gutted and cleaned tilapia in salt solution (5% for 30 min) before deboning and preparation of surimi also removed the muddy odour and flavour to some degree. Soaking tilapia flesh in lambanong (distilled alcoholic beverage made from coconut sap) for 1 h prior to cooking also reduced off-flavour (Mabesa *et al.*, 1989).

1.2.3 Lipid oxidation

Oxidation of lipids is a major cause of deterioration of food and food products, especially those having high content of unsaturated fatty acids. Lipid oxidation is one of the main factors limiting the quality and acceptability of muscle foods (Maqsood *et al.*, 2012). Oxidation of lipids is accentuated in the immediate post-slaughter period, during handling, processing, storage and cooking. This process leads to discolouration, drip losses, off-odour/flavour development, texture defects and the production of potentially toxic compounds (Morrissey *et al.*, 1998; Richards and Hultin, 2002).

1.2.3.1 Lipid autoxidation

The oxidation of lipids occurs by a free radical chain reaction involving three processes: (1) initiation – the formation of free radicals; (2) propagation – the free radical chain reactions; and (3) termination – the formation of non-radical products (Frankel, 1998; Nawar, 1996) (Figure 1).

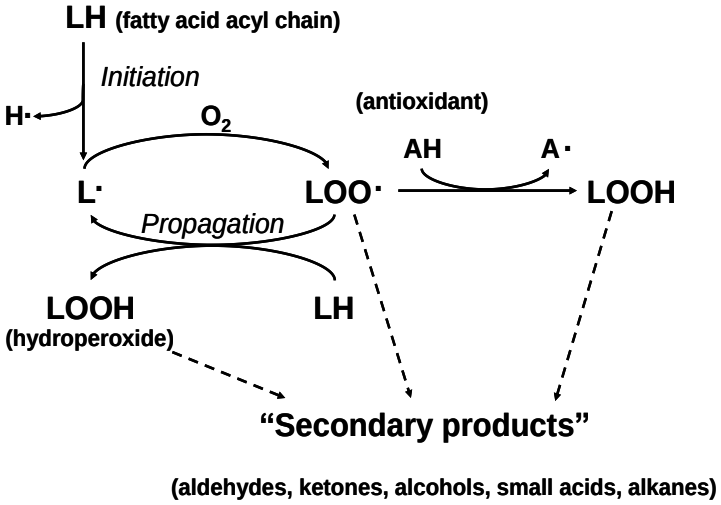


Figure 1. Autoxidation of polyunsaturated lipids
Source: Huss (1995)

Initiation: The autoxidation of lipid proceeds via typical free radical mechanisms. The process is initiated by abstraction of a hydrogen atom from the central carbon of the pentadiene structure found in most fatty acid acyl chains (LH) containing more than one double bond:

$$\text{-CH=CH-CH}_2\text{-CH=CH-} \longrightarrow \text{-CH=CH-CH-CH=CH-} + H^{\bullet}$$

The formation of lipid radical ($L^{\bullet}$) is usually mediated by trace metals, irradiation, light or heat. Also, the initiation of oxidation may take place by hydroperoxide (LOOH) decomposition, generating a highly reactive alkoxyl lipid radical ($LO^{\bullet}$) and a hydroxyl radical ($HO^{\bullet}$). Lipid hydroperoxides which exist in trace quantities prior to the oxidation can be formed by the reaction of an unsaturated fatty acid such as linoleic acid with oxygen in the singlet excited state or enzymatically by the action of lipoxygenase (Nawar, 1996; Sae-leaw *et al.*, 2013). Sae-leaw *et al.* (2013)

found that the quality changes of Nile tilapia skin during ice storage was associated with the lipid oxidation, lipid hydrolysis along with the release or activation of lipoxygenase. Due to resonance stabilization of lipid radical ($L^{\bullet}$) species, the reaction sequence is usually accompanied by a shift in position of the double bonds, resulting in the formation of isomeric hydroperoxides that often contain conjugated diene groups (-CH=CH-CH=CH-) (Nawar, 1996). Conjugated diene shows a characteristic UV absorption at 232-234 nm (Nakayama *et al.*, 1994).

Propagation*:* In propagation reaction, free radicals are converted into other radicals. Propagation of free-radical oxidation processes occurs by chain reactions that consume oxygen and yield new free-radical species (peroxy radicals, $LOO^{\bullet}$). Lipid peroxy radicals initiate a chain reaction with other molecules (LH), resulting in the formation of lipid hydropeoxides (LOOH) and lipid free radicals ($L^{\bullet}$). This reaction, when repeated many times, produces an accumulation of hydroperoxides. The propagation reaction becomes a continuous process as long as unsaturated lipid or fatty acid molecules are available. Lipid hydroperoxide, the primary products of autoxidation, are odourless and tasteless (Jadhav *et al.*, 1996).

Termination: A free radical is any atom with unpaired electron in the outermost shell. Owing to the bonding-deficiency and structural unstability, the radicals therefore tend to react with others to restore normal bonding. When there is a reduction in the amount of unsaturated lipids (or fatty acids) present, radicals bond to one another, forming a stable non-radical compounds. The radicals can also be removed by reaction with an antioxidant (AH) whose resulting radical ($A^{\bullet}$) is much less reactive (Huss, 1995; Jadhav *et al.*, 1996).

Although fish lipids are responsible for health benefit, lipid oxidation is inevitably associated with undesirable odour/flavours development of marine-based food products. Hydroperoxide, a primary oxidation product during fish storage, is readily decomposed to a variety of volatile compounds including aldehydes, ketones and alcohols. The off-odour problems give rise to a difficulty in increase of human consumption of fish and their products. Many reports have been demonstrated that the development of off-odour upon heating of fish meat was related to oxidation of lipids,

which gradually increases intensity during storage (Koizumi *et al.*, 1987; Thiansilakul *et al.*, 2010). Domínguez *et al.* (2014) reported that the increases of cooking temperature increased lipid oxidation and total volatile compounds of foal meat. During fish processing and storage, quality may decline as a result of several reactions affecting both protein and lipid fractions and decreasing the nutritional and sensory properties of the product. Lipid oxidation in fish starts immediately after the harvest and during the processing, making the fish smell rancid. The removal of subcutaneous fat and dark muscle during hand-mincing can lower lipid oxidation (Eymard *et al.*, 2009). Total lipid hydroperoxide content and TBARS of the yellowtail (*Seriola quinqueradiata*) dark muscle were higher than those of the ordinary muscle during 2 days of iced storage. Those changes were accompanied with the increasing intensity of fishy, spoiled and rancid off-odour as well as increasing metMb formations. However, no correlation was found between the content of total lipid hydroperoxide and the odour intensities in ordinary muscle (Sohn *et al.*, 2005). Rancidity in herring fillets could be detected sensorially after 2.5 days on ice (Undeland *et al.*, 1999). Additionally, the formation of tertiary lipid oxidation-fluorescense compounds and hydrolysis products were strongly correlated to rancidity (Karlsdottir *et al*., 2014). The development of lipid oxidation during storage seemed to depend on the composition of the mince matrix and its initial oxidative status (Eymard *et al.*, 2009). Lipid oxidation products can modify proteins by inducing cross linking, resulting in modifications of amino acids of nutritional interest and a decrease in protein functionality due to protein denaturation (HidalgoI and Zamora, 1993). Although frozen storage has been known to terminate microbial spoilage, the deterioration reaction including lipid oxidation still takes place. Storage temperature is crucial in controlling lipid oxidation in fish. Karlsdottir *et al.* (2014) reported that lower frozen storage temperature (-30 ° C) showed more significantly preventive effect on lipid deterioration of saithe and hoki muscles than higher temperature (-20 °C). The impact of lipid oxidation on the quality of aquatic food products are schematically illustrated in Figure 2.

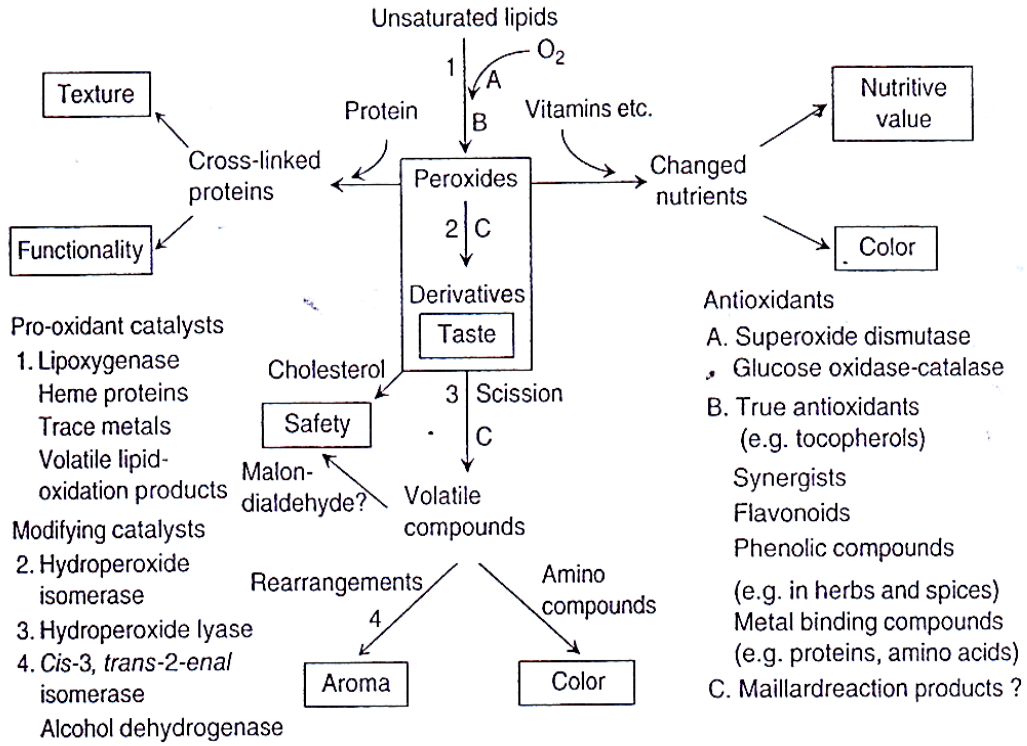


Figure 2. The impact of lipid oxidation on the quality of aquatic food products
Source: Bao and Ohshima (2014)

1.2.3.2 Lipid oxidation mediated by haem proteins (HP)

The most abundant HP presented *in vivo* are haemoglobin (Hb) and myoglobin (Mb), which are potent catalysts of lipid oxidation in muscle tissue after death. Lipid oxidation processes negatively affect food quality primarily by promoting off-odours and off-flavours (Maqsood and Benjakul, 2011a). HP also provides the colour of muscle, depending upon their redox state. Reduced, oxygenated HP, renders meat with a bright red colour, whilst oxidised pigments are brown in colour (Thiansilakul *et al.*, 2012b). Fully reduced, deoxygenated pigments appear purple at high concentrations (Richards *et al.*, 2005). The conversion of haem proteins from the reduced- to met-form is a process called HP autoxidation. The reaction is also responsible for acceleration of lipid oxidation in muscle based food (Richards *et al.*, 2005).

A) HP structures

Mb and Hb are monomeric and tetrameric, respectively, as shown in Figure 3. Hb, which transports oxygen and other gases, is the main protein in blood and highly concentrated in the erythrocytes (Jensen, 2004). Mb, the oxygen-storage protein retained by the intracellular structure is a major pigment in the dark muscle of fish (Chaijan *et al.*, 2005). Mb consists of a globin portion plus a porphyrin haem, containing an iron atom coordinated inside the haem ring. Typically, Hb tetramer consists of two α-chains and two β-chains, in which each chain contains one haem group. The four chains are held together by covalent attractions. The HP from different fish species varies with respect to the difference in the amino acid sequences (Grunwald and Richards, 2006; Richards *et al.*, 2005). Differences in the numbers and identity of amino acids have been reported in HP from different sources (Jensen *et al.*, 1998).

(A) (B)

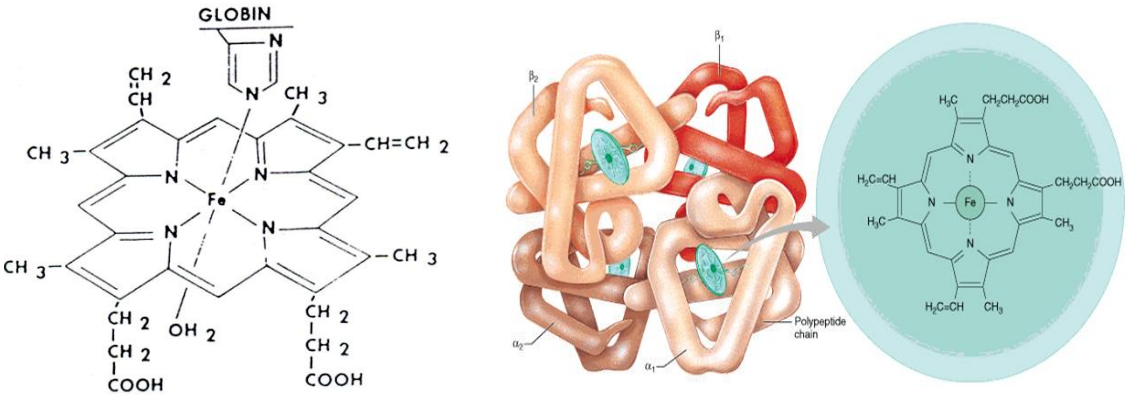


Figure 3. Chemical structures of Mb (A) and Hb (B)

Source: Pearson and Young (1989)

In its reduced state, the iron can be bound to O_2 (oxyHP-Fe(II)O_2), which is stabilised via hydrogen bonding by the nearby distal histidine, or it can be without the oxygen (deoxyHP-Fe(II)), such as at low pH (Hargrove *et al.*, 1997) or at low oxygen tension (Stryer, 1988). A continuous oxidation to metHP-Fe(III)-state take place *in vivo*, though subsequent enzymatic reduction to the deoxy-forms occurs (Baron and Andersen, 2002). Both oxidised and reduced forms can be pro-oxidative (Grunwald and Richards, 2006). Thiansilakul *et al*. (2012b) reported that lipid oxidation in washed

bighead carp mince initiated by Mb and Hb, especially met-form, brought about the increased fishy and rancid off-odour. However, Hb promoted the larger amounts of peroxides, thiobarbituric acid-reactive substances and hexanal than did Mb. Pro-oxidative activity of HP depended on their molecular properties. Several different mechanisms of the pro-oxidative power of HP have been proposed (Everse and Hsia, 1997). Richards *et al.* (2005) reported autoxidation and haemin loss of trout Mb and Hb associated with lipid oxidation in washed trout muscle at pH 6.3. Haem is anchored more loosely in met-form, compared to reduced-form. HP mutants with low haem affinity promoted lipid oxidation in washed cod more effectively, compared to mutants with high haem affinity (Grunwald and Richards, 2006). Alternatively, displaced haemin or released iron can stimulate lipid oxidation (Tokur and Korkmaz, 2007).

B) Autoxidation of HP

Different forms of iron have been proposed to initiate lipid oxidation, causing pathological conditions in different tissue and resulting in oxidative deterioration in muscle food. The process by which ferrous state is converted to ferric state is called 'autoxidation'. Superoxide anion radical ($O_2^{\bullet-}$) or $^{\bullet}OOH$ is liberated in this process, depending on whether deoxy- or oxy-HP undergoes autoxidation (Brantley *et al.*, 1993). $O_2^{\bullet-}$ and $^{\bullet}OOH$ can readily be converted to hydrogen peroxide (H_2O_2), which enhances the ability of HP to promote lipid oxidation (Grunwald and Richards, 2006). Additionally, H_2O_2 can react with metHP to generate ferryl HP radicals ($^{\bullet}$HPFe(IV)), which can abstract hydrogen from polyunsaturated fatty acids and hence initiate lipid oxidation (Maqsood and Benjakul, 2011a). The schematic diagram of Hb autoxidation and its role in lipid oxidation as shown in Figure 4. Many factors have been known to be associated with autoxidation of HP as follows:

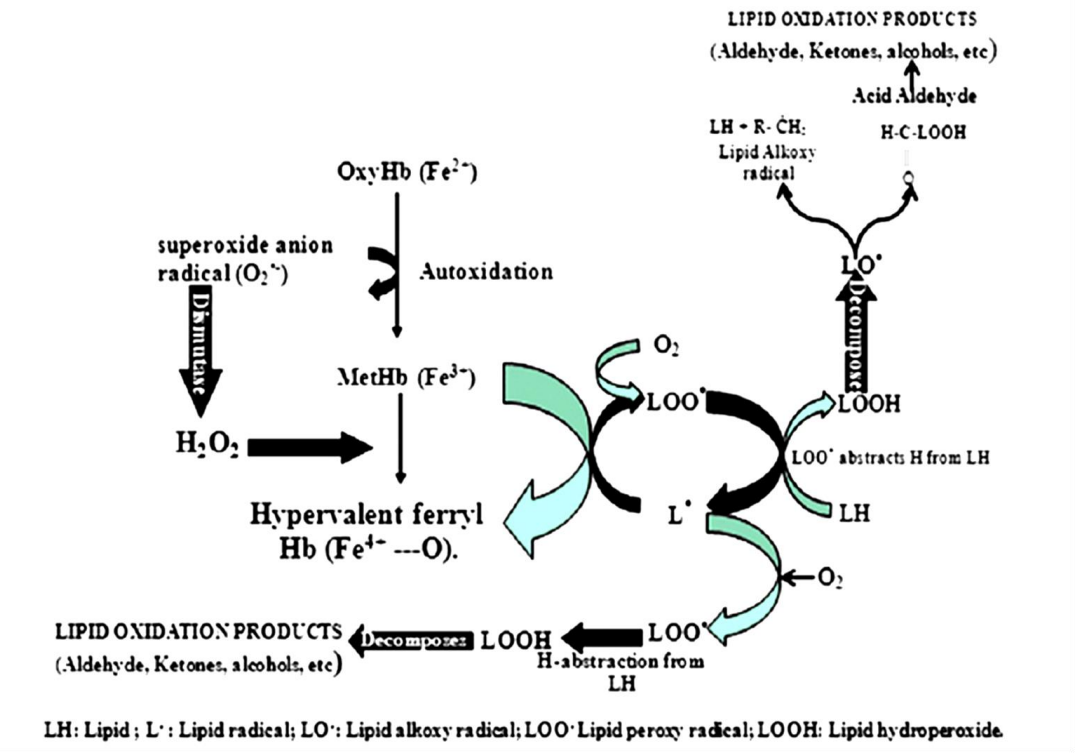


Figure 4. Schematic diagram of Hb autoxidation and its role in lipid oxidation
Source: Maqsood *et al.* (2012)

(1) pH: The autoxidation reaction is enhanced by a low pH (Richards and Hultin, 2000). According to the Bohr's effect, increasing proton generally decreases the affinity of HP for oxygen molecule, leading to a lowering of oxy-HP (Stryer, 1988). Additionally, low pH favours the protonation of the ferryl species, which exhibit great instability and can be considered as possessing a radical-like nature (Reeder and Wilson, 2001). Livingston and Brown (1981) reported that autoxidation of Mb became greater and faster as pH decreased. The increasing autoxidation rate constant was also found in milkfish (*Chanos chanos*) Mb with decreasing pH in a range of 5.5-7.0 (Chen and Chow, 2007). Autoxidation of Hbs was found to occur around pH 7.0 and below but much less at pH 8.0. Hb-subunits were found to autoxidise more rapidly than Hb-tetramers (Griffon *et al.*, 1998). The decrease in oxygen affinity coincided with increased pro-oxidative activity of Hb in a washed cod system (Richards and Hultin, 2000).

(2) Temperature: Autoxidation of HP is enhanced as temperature increases (Livingston and Brown, 1981). Rising the temperature of fresh red meat tended to promote the autoxidation of HP (Giddings and Hultin, 1974). Heating was able to rapidly convert ferrous-HP to met-HP based on the brown appearance of cooked meat. Chaijan *et al.* (2007) reported that the higher temperature, particularly above 40 ºC, and the longer incubation time induced the metMb formation as well as the conformational changes of Mb. However, no metMb formation was noticeable at temperature lower than 20 ºC. Thiansilakul *et al.* (2011) reported that the oxyMb and metMb from Eastern little tuna were precipitated when the temperature reached 60 ºC. Heating also unfolds globin of haem proteins, resulting in denaturation of globin. Thus, heating should facilitate the release of haemin from globin, followed by iron released from haemin. These changes are responsible for promoting lipid oxidation in fish muscle (Grunwald and Richards, 2006; Thiansilakul *et al.*, 2012a).

(3) Oxygen and oxygen consumption: Oxygen and oxygen consumption play the important role on autoxidation of deoxy- and oxyHP. Lowering the oxygenation of HP was found to enhance the autoxidation. Brantley *et al.* (1993) denoted that in the presence of oxygen, deoxy-HP is susceptible to rapid autoxidation, whereas fully oxygenated HP should be more resistant to autoxidation. Tissue oxygen consumption decreased with post-mortem time. Tang *et al.* (2004) found that bovine mitochondrial respiration in a closed system resulted in decreased oxygen partial pressure (pO_2) and enhanced the conversion of oxyHP to deoxyHP or metHP. Poorly oxygenated Hb autoxidises faster than highly oxygenated Hb (Richards and Dettmann, 2003). The phenomenon is governed by the spin state of the iron atom inside the haem ring (Livingston and Brown, 1981). The ferrous iron atom of deoxy-HP (Fe^{2+}) is a 5-coordinated complex, where the iron has 4 bonds to the porphyrin haem ring and 1 bond to a histidine residue of the globin. This causes the iron to be in a high spin state and hence highly susceptible to oxidation to ferric met-HP (Fe^{3+}). Lee *et al.* (2003a) reported that the grinding process for tuna (*Thunnus albacares*) patties is likely a cause of greater surface metMb formation in patties than in steaks because of the higher surface for air exposure. To minimise metMb formation in fresh beef, oxygen must either be totally excluded from the packaging environment or present at saturating levels (Faustman and Cassens, 1990).

(4) Lipid oxidation product: Lipid oxidation results in a variety of secondary products, which are predominantly *n*-alkanals, *trans*-2-alkenals, 4-hydroxy-*trans*-2-alkenals and malondialdehyde (Lynch and Faustman, 2000). The aldehyde products are more water-soluble than their parent compounds and could potentially interact with HP (Chan *et al.*, 1997). Hexanal, hexenal and 4-hydroxynonenal have been reported to enhance tuna oxyMb oxidation (Lee *et al.*, 2003a). Porcine metMb formation was greater in the presence of 4-hydroxynonenal (Lee *et al.*, 2003b). Faustman *et al.* (1999) explained that 4-hydroxynonenal covalently attached to oxyMb caused structural alterations, which would make the protein more susceptible to oxidation. The addition of known oxidation products of oleic and linoleic acid (2-octene, propanal, decanal, nonanal, hexanal, 2-nonenal and 2-heptenal) could accelerate the oxidation of oxy HP (Chan *et al.*, 1997). However, there was no difference in oxyHP oxidation between the oxidised or unoxidised bovine muscle extracts containing lipid oxidation product during 3 days of storage at 4 ºC. The final products of lipid oxidation, as opposed to free-radical intermediates or lipid hydroperoxides, are not directly responsible for oxyHP oxidation (O'Grady *et al.*, 2001).

C) Impact of HP on lipid oxidation

Lipid oxidation is a principal cause of quality deterioration and shelf-life shortening in fish muscle based food. The chemical reaction is influenced by various factors. Generally, Mb with high concentration is retained in muscle tissue, whist Hb is mainly located in red blood cells. In seafood processing, blood may not be fully removed and can react with the muscle lipids (Richards *et al.*, 1998). Under fluctuating oxygen supply and pH decrease of post-mortem system, the haem pigments like Hb and Mb become catalytic in lipid peroxidation by various mechanisms (Maqsood and Benjakul, 2011a; Thiansilakul *et al.*, 2012b). The detachment of ferric haemin from the globin can follow metMb/Hb formation to promote lipid oxidation reactions (Everse and Hsia, 1997). When the critical levels of peroxides are present, iron can be released from haemin to participate in lipid oxidation processes (Richards *et al.*, 2002). The HP destruction, ferryl radical formation, haem dissociation or free

iron release have been related with lipid oxidation in muscle food. The mechanism of HP involving both one- and two-electron transfer processes is shown in Figure 5.

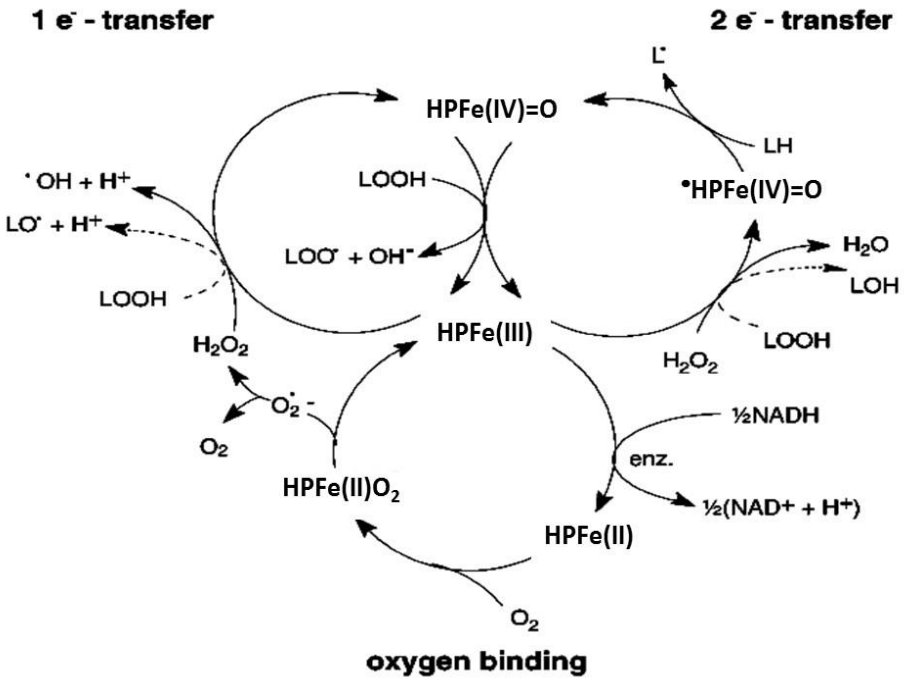


Figure 5. HP involvement in one- and two-electron transfer processes in lipid oxidation

Source: Adapted from Carlsen *et al.* (2005)

Haem-initiated lipid oxidation, especially Mb and Hb, have been intensively reported. Trout Hb promoted lipid oxidation more effectively as compared to bovine Hb, whilst avian Hbs had intermediate activity (Richards and Hultin, 2002). HP has been known as a promoter of lipid oxidation in muscle tissue. There was no significant difference in Hb levels estimated in whole muscle from bled and unbled sockeye salmon (Porter *et al.*, 1992). Lipid oxidation in washed Asian sea bass mince initiated by Mb brought about the increased fishy and rancid off-odour, especially at low pH, in which metMb was the dominant form (Thiansilakul *et al.*, 2012b). Pro-oxidative activity of Hb from different fish species, including Asian sea bass, tilapia and grouper varied, depending on the molecular properties of Hb (Maqsood and Benjakul, 2011a). Richards *et al.* (2005) reported autoxidation and haemin loss of trout Mb and Hb associated with lipid oxidation in washed fish muscle at pH 6.3. Haemin

loss rate was considered crucial in promoting lipid oxidation, compared to autooxidation rate. Trout Hb was prone to haemin loss and became a stronger promoter of lipid oxidation than trout Mb, whilst trout Mb had a more rapid autoxidation rate than trout Hb (Richards *et al.*, 2005). Released haemin readily converts pre-formed lipid hydroperoxides to radicals that facilitate lipid oxidation (Van Der Zee *et al.*, 1996).

It is well known that HP has a close relationship with lipid oxidation, which negatively affects the quality of muscle food. To tackle the deterioration mediated by HP, several strategies have been proposed. Bleeding was also shown to retard lipid oxidation of minced trout muscle during storage at 2 °C (Richards and Hultin, 2002). Maqsood and Benjakul (2011b) demonstrated that the removal of blood from the Asian seabass muscle was effective in lowering lipid oxidation as reflected by lower formation of peroxide and TBARS values. Thiansilakul *et al.* (2012a) reported that the uses of phenolic compound, i.e cafeic acid and gallic acid, were able to retard haem release and lipid oxidation in washed bighead carp mince as induced by haem proteins. Grunwald and Richards (2012) found that haemopexin extracted from porcine serum could inhibit Hb, but not haemin induced lipid oxidation in washed cod muscle. Nevertheless, Cai *et al.* (2013) noted that the recombinant streptococcal protein apoShp strongly inhibited lipid oxidation of trout muscle since it specifically bound the haemin released from Hb.

1.2.4 Fish protein hydrolysate

Protein hydrolysate is derived from fish proteins, in which peptides are broken down to varying sizes by chemical or enzymatic process. The hydrolysis affects the properties of resulting hydrolysate. In general, short chain peptides seem to be more advantageous than both intact protein and free amino acids (Clemente, 2000). Nevertheless, the modification in structure, chain length and amino acid sequence depend on treatment, hydrolytic conditions, type of enzyme, temperature, protein concentration, etc. (Foh *et al.*, 2011). Additionally, characteristics and properties of fish protein hydrolysate are determined by raw material used. Different proteinaceous substances yield the hydrolysate with varying nutritive value as well as functional properties (Najafian and Babji, 2012). Generally, protein hydrolysate can be produced

from fish flesh, by-products such as skin, bone, etc. as well as fish flesh or mince (Kim and Wijesekara, 2010).

1.2.4.1 Raw materials for protein hydrolysate production

A) Fish muscle

Lean fish are traditionally used for protein hydrolysate preparation. Hydrolysate from lean fish exhibits higher sensory property than those from dark fleshed fish. Due to a high diversity in protein structure, amino acid compositions and sequence, a large range of fish protein substrate can yield novel peptides with specific or multi-functional bioactivity via hydrolysis (Harnedy and FitzGerald, 2012). Protein hydrolysates based on whole fish, fish fillet or pretreated muscle have been produced (Table 1). To increase the value of dark fleshed fish or to maximise the use of those species, production of protein hydrolysates is of choice (Khaled *et al.*, 2014; Klompong *et al.*, 2007; Thiansilakul *et al.*, 2007b). Owing to the high amount of lipids and pro-oxidants in dark muscle, the additional pretreatment is recommended prior to production of hydrolysate. The use of washed mince or surimi generally gives the high quality hydrolysate (Kristinsson and Rasco, 2000a). Myofibrillar protein isolate was used for enzymatic hydrolysis (Theodore *et al.*, 2008). The protein isolate is more preferable for the protease used. As a consequence, the hydrolysis could take place at the higher degree, compared with that found in the intact muscle (Theodore *et al.*, 2008). Protein isolate from herring was also used for protein hydrolysate production (Taheri *et al.*, 2014).

B) Fish processing by-products

Industrial fish processing for human consumption yields more than 60% by-products, however the amount of by-product depends on raw materials, process used or product required (Ockerman and Hansen, 2000; Raa *et al.*, 1982). Large amounts of protein-rich by-products from the seafood industry are discarded or processed into fish meal (Sathivel *et al.*, 2003). Those by-products are prone to microbial spoilage as well as chemical deterioration. Thus, handling and storage time

Table 1. Fish protein hydrolysate produced from fish muscle with different pretreatments

Type of samples	Treatments	Sources	References
Lean fish	mince	capelin (*Mullotus vilhus*)	Pacheco-Aguilar *et al.* (2008)
		Zebra blenny (*Salaria basilisca)*	Khiari *et al.* (2014)
		Northern whiting fish (*Sillago sihama*)	Venkatesan and Nazeer (2014)
	washed mince/surimi	tilapia (*Oreochromi niloticus*)	Foh *et al.* (2010)
		cod (*Gadus morhua*)	Halldorsdottir *et al.* (2013)
	protein isolate	bluewhiting (*Micromesistius australis*)	Yoshie-Stark *et al.* (2009)
		ornate threadfin bream (*Nemipterus hexodon*)	Nalinanon *et al.* (2011)
		brownstripe red snapper (*Lutjanus vita*)	Khantaphant *et al.* (2011a)
		saithe (*Pollachius virens*)	Halldórsdóttir *et al.* (2011)
Fatty fish	mince	mackerel (*Scomber austriasicus*)	Wu *et al.* (2003)
		yellow stripe trevally (*Selaroides leptolepis leptolepis*)	Klompong *et al.* (2009)
		monkfish *(Lophius litulon)*	Chi *et al.* (2014)
		sallmon (*Salmo salar*)	Girgih *et al.* (2013)
		round scad (*Decapterus maruadsi*)	Jiang *et al.* (2014)
		sardinelle *(Sardinella aurita)*	Khaled *et al.* (2014)
	protein isolate	channel catfish (*Ictalurus punctatus*)	Theodore *et al.* (2008)
		herring (*Clupea harengus*)	Taheri *et al.* (2014)

should be minimised and further processing should be rapidly implemented. Enzymatic hydrolysis has been developed to convert by-products into more profitable forms. Frame, bone, head, skin, viscera or etc. can potentially be used to produce the protein hydrolysate. By-products (heads, viscera, frames, skin, trimmings) of black scabbardfish (*Aphanopus carbo*) (Batista *et al.*, 2010) and those (head and viscera) from sardinella (*Sardinella aurita*) (Bougatef *et al.*, 2010) as well as surimi waste (frame, bone, skin and refiner discharge) from threadfin bream (*Nemipterus spp*.) (Wiriyaphan *et al.*, 2012) and silver carp (*H. molitrix*) (Liu *et al.*, 2014) were used for protein hydrolysate production. Frame from yellowfin sole (*Limanda aspera*) (Jun *et al.*, 2004), Alaska pollack (*Theragra chalcogramma*) (Je *et al.*, 2005), hoki (*Johnius belengerii*) (Kim *et al.*, 2007), backbones from tuna (Je *et al.*, 2007) and Atlantic cod (*Gadus morhua*) (Šližytė *et al.*, 2005a) were also used for preparation of protein hydrolysate. Halldorsdottir *et al.* (2014b) reported that protein hydrolysate with antioxidant activity was produced from cod bone (*Gadus morhua*). Apart from solid by-products, liquid effluent such as cooking juice from tuna was used as the proteinaceous source to produce active peptides (Hsu *et al.*, 2009; Jao and Ko, 2002).

Recently, increasing attention has been paid for hydrolysate derived from fish collagen and gelatin. In general, skin, bone and scale are the excellent sources of collagen and gelatin (Venugopal, 2010). Collagen and gelatin are rich in imino acids, both proline and hydroxyproline, and contain glycine around one-third of total amino acids (Vercruysse *et al.*, 2005). Numerous studies indicate that collagen or gelatin derived hydrolysates possess bioactivities. Hydrophobic amino acids such as proline play a role in inhibiting lipid peroxidation and contribute to ACE inhibitory activity (Byun and Kim, 2001; Mendis *et al.*, 2005b). Peptides with proline or hydroxyproline are generally resistant to degradation by digestive enzymes, thereby having a better chance of reaching their target site in an intact form (Sarmadi and Ismail, 2010). Gelatin hydrolysates with antioxidant activity have been produced from skin gelatin of unicorn leatherjacket (Sai-Ut *et al.*, 2014), Amur sturgeon (Nikoo *et al.*, 2014a), seabass (Senphan and Benjakul, 2014) and zebra blenny (Ktari *et al.*, 2014). Furthermore, gelatin hydrolysates with antioxidant and ACE inhibitory properties were also prepared from other aquatic sources such as bullfrog (Qian *et al.*, 2008), squid (Giménez *et al.*, 2009), jellyfish (Zhuang *et al.*, 2010), sea cucumber (Pérez-Vega *et al.*, 2013) and

oyster (Wang *et al.*, 2014). Most hydrolysates were mainly prepared with the aid of proteolytic enzymes. Since the gelatin can be hydrolysed at high temperature, thermal hydrolysis was also applied to produce the gelatin hydrolysate with bioactivity (Gómez-Guillén *et al.*, 2011).

Viscera containing a high level of proteolytic enzymes can be used for protein hydrolysate production, in which the additional protease is less required due to the presence of indigenous proteases. Bougatef *et al.* (2010) prepared protein hydrolysate for sardinelle viscera via autolysis process at 40-50 °C and pH 8. Nevertheless, the addition of external enzyme can expedite and increase the degree of hydrolysis. Motamedzadegan *et al.* (2010) hydrolysed yellowfin tuna visceral waste protein using Neutrase. Enzyme activity of 39.61 AU/kg protein, temperature of 53°C, and hydrolysis time of 141 min were found to be the optimal conditions, in which more than 35% DH was obtained. The tuna visceral protein hydrolysates had high protein (74.56%), but low lipid (1.86%) contents. Chotikachinda *et al.* (2013) produced protein hydrolysate from skipjack tuna viscera as feeding attractants for Asian seabass. In addition, protein hydrolysate from defatted skipjack tuna roe (*Katsuwonous pelamis*) was reported to have antioxidant and functional properties (Intarasirisawat *et al.*, 2012).

1.2.4.2 Production of protein hydrolysate

A) Enzymatic hydrolysis

Biological processes using added enzymes are employed more frequently due to the ease of control and mild condition. Enzymatic hydrolysis holds the most promise for the future because it renders the products of high functionality and nutritive value (Kristinsson and Rasco, 2000a). Plant, animal and microbial enzymes can be used for the production of protein hydrolysate. Amongst microbial enzymes, bacterial and fungal proteases are most frequently used. Fungal proteases, which often contain a mixture of several enzymes, display a broader substrate specificity. As a consequence, a more pronounced hydrolysis of protein can be attained (Guérard *et al.*, 2001; Kristinsson and Rasco, 2000a). Enzymatic hydrolysis can be performed with the aid of proteolytic enzymes, both endopeptidases, which cleave the peptide bonds within protein molecules and exopeptidases, which hydrolyse peptide bonds from either N- or

C- terminal (Clemente, 2000; Raksakulthai and Haard, 2003). Proteases have been used widely to obtain a more selective hydrolysis since they are specific for peptide bonds adjacent to certain amino acid residues (Wu *et al.*, 2003). The selection of enzymes is usually based on a combination of efficacy and economics (Lahl and Braun, 1994). In comparison to animal- or plant-derived enzymes, microbial enzymes offer several advantages including a wide variety of available catalytic activities, greater pH and temperature stabilities (Guerard *et al.*, 2002). The scheme for the production of fish protein hydrolysate using enzymes is given in Figure 6.

(1) Plant enzymes

Plant proteases can be produced from some plants and those extracted from edible fruits are considered as acceptable. In terms of value or volume, papain is the major enzyme, though bromelain and ficin also appear in commerce (Caygill, 1979; Stepek *et al.*, 2004). Proteases from plant can be used for hydrolysis of fish protein. Crude proteolytic enzyme from papaya latex was used to prepare gelatin hydrolysate from shark skin (Kittiphattanabawon *et al.*, 2012). It yielded gelatin hydrolysate with higher degree of hydrolysis (DH), in comparison with Neutrase. Karnjanapratum and Benjakul (2014) also reported that glycyl endopeptidase extracted from papaya latex yielded tilapia gelatin hydrolysate with higher antioxidant activity and less undesirable odour, especially when the enzyme was partially purified. Papain has been used to produce hydrolysate from the muscle of blue mussel (Wang *et al.*, 2013), grass carp (Li *et al.*, 2013) and spotless smoothhound (Wang *et al.*, 2014). Gelatin hydrolysate of from cobia skin (Yang *et al.*, 2008), chum salmon skin (Fu and Zhao, 2013) and Indian mackerel backbone (Sheriff *et al.*, 2014) have been also produced using papain. Moreover, Salampessy *et al.* (2010) noted that protein hydrolysate from insoluble leatherjacket proteins prepared using bromelain contained antimicrobial peptide against *S. aureus*, *B. cereus*, *E. coli* and *C. albicans.* Plant proteases can be more effective in hydrolysis of some fish proteins in comparison with microbial or animal protease, depending on their specificity toward substrates used and degree of hydrolysis required (Kittiphattanabawon *et al.*, 2012).

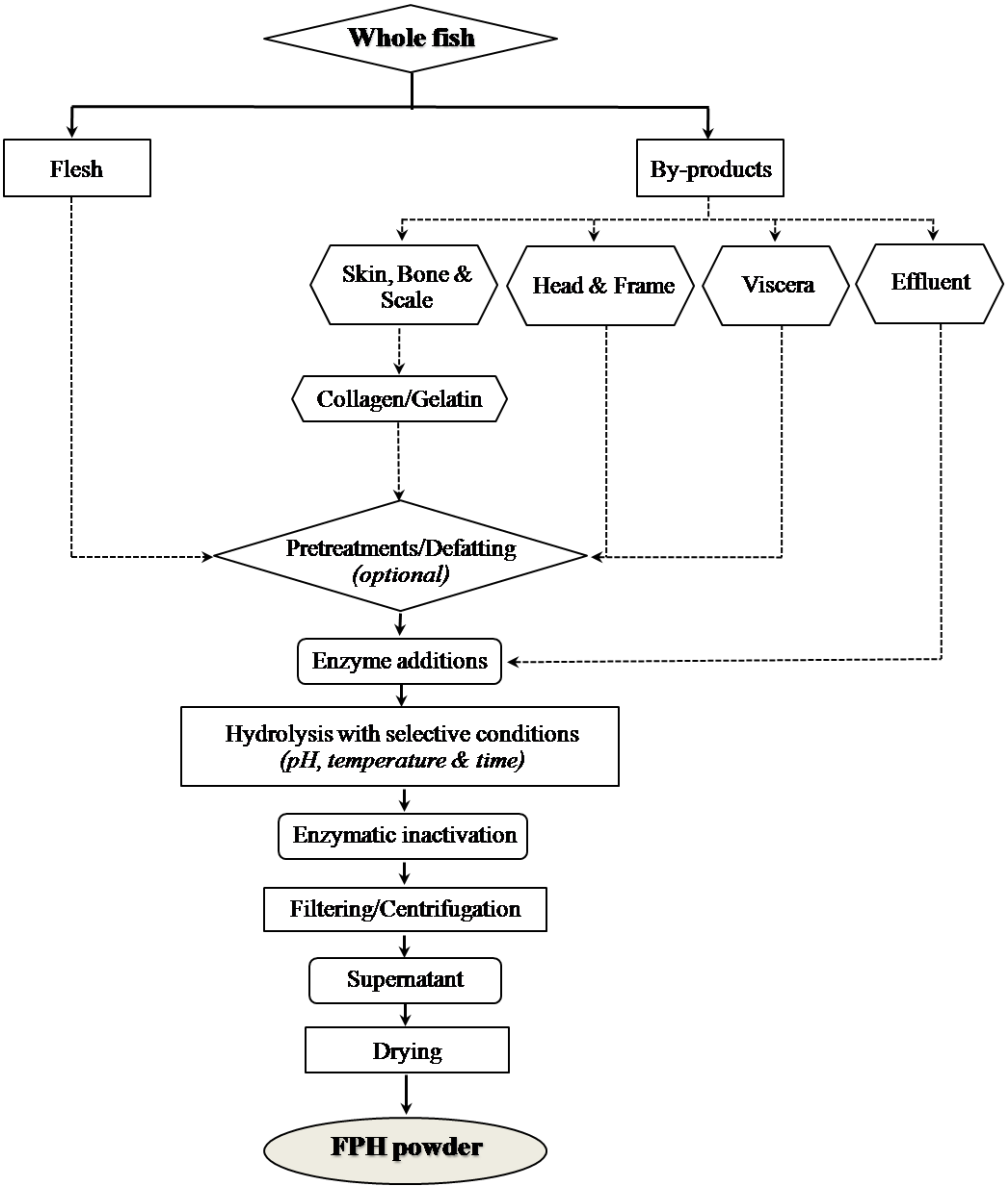


Figure 6. Scheme of fish protein hydrolysate production

Source: Adapted from Kristinsson and Rasco (2000a)

(2) Fish enzymes

Fish enzymes, especially from viscera, have been proven as a potential tool for preparing hydrolysate. Fish digestive proteolytic enzymes include pepsin, trypsin, chymotrypsin, gastricsin and elastase (Vecchi and Coppes, 1996). Apart from commercial protease, fish endogenous enzymes have been used for protein hydrolysate production (Table 2). Protein hydrolysate from ornate threadfin bream

Table 2. Fish proteases used for production of fish protein hydrolysate

Proteases	Raw material	References
Pepsin, mackerel intestine proteases	Yellowfin sole (*Limanda aspera*) frame	Jun *et al.* (2004)
Mackerel intestine proteases	Alaska pollack (*Theragra chalcogramma*) frame	Je *et al.* (2005)
Smooth hound intestine protease	Smooth hound (*Mustelus mustelus*) meat	Bougatef *et al.* (2009)
Cuttlefish hepatopancreas protease	Cuttlefish (*Sepia officinalis*) muscle	Balti *et al.* (2010)
Sardine viscera protease	Sardinelle (*Sardinella aurita*) heads and viscera	Bougatef *et al.* (2010)
Skipjack tuna pepsin	Ornate threadfin bream (*Nemipterus hexodon*) muscle	Nalinanon *et al.* (2011)
Pyloric caeca of brownstripe red snapper protease	Brownstripe red snapper (*Lutjanus vitta*) muscle	Khantaphant *et al.* (2011b)
Cuttlefish and sardinelle gastrointestinal protease	Cuttlefish (*Sepia officinalis)* by products	Ktari *et al.* (2013)
Hybrid catfish viscera protease	toothed ponyfish (*Gazza minuta*)	Klomklao *et al.* (2013)
Hepatopancreas of Pacific white shrimp	Seabass (*Lates calcarifer*) skin	Senphan and Benjakul (2014)

muscle was prepared using skipjack tuna pepsin and could serve as a promising source of functional peptides with antioxidant properties (Nalinanon *et al.*, 2011). Proteases from pyloric caeca of brownstripe red snapper were used to produce hydrolysate from preteated mince from brownstripe red snapper with antioxidant activity with the lowered amount of lipids and pro-oxidants (Khantaphant *et al.*, 2011a). Pepsin and mackerel intestinal crude enzymes were used for preparing yellowfin sole frame protein hydrolysates containing antioxidative peptides (Jun *et al.*, 2004). Antioxidant peptides were also obtained in Alaska pollack frame protein hydrolysate prepared using crude proteinase from mackerel intestine (Je *et al.*, 2005). Cod frame protein hydrolysates with antioxidative activity were prepared with crude proteinase extracted from tuna

pyloric caeca (Jeon *et al.*, 1999). Additionally, smooth hound muscle hydrolysate prepared using its gastrointestinal proteases possessed the higher antioxidative activities, compared with that prepared using bovine trypsin (Bougatef *et al.*, 2009). Sardinelle by-products protein hydrolysates obtained by various proteases were found to have antioxidant activity. Hydrolysis with crude extract from sardine viscera yielded the hydrolysate with the highest antioxidant activity, compared with other proteases (Bougatef *et al.*, 2010). Senphan and Benjakul (2014) reported that the application of protease from hepatopancreas of Pacific white shrimp yielded gelatin hydrolysate from seabass skin with higher antioxidant activity than those prepared using Alcalase.

(3) Microbial enzymes

Commercial microbial proteases have been successfully and widely used to produce protein hydrolysates with bioactivities (Kristinsson and Rasco, 2000b). Proteolytic enzymes from microorganisms such as Alcalase (Yu-lian Dong *et al.*, 2005; Gbogouri *et al.*, 2004), Flavourzyme (Klompong *et al.*, 2007; Thiansilakul *et al.*, 2007a), Neutrase (Phanturat *et al.*, 2010; Šližytė *et al.*, 2005b), Protamex (Liaset *et al.*, 2003) and Protease N (Liaset *et al.*, 2002; Wu *et al.*, 2003) are effective for hydrolysate preparation since they are able to cleave fish substrates broadly and potentially. A wide variety of commercial enzymes from microbial have been used to hydrolyse fish proteins (Table 3).

(3.1) Alcalase

Alcalase (Subtilisin carlberg: EC 3.4.21.14) is an alkaline bacterial protease produced from *Bacillus licheniformis*. It has been proven to be one of the best enzyme used in the preparation of protein hydrolysate (Guérard *et al.*, 2001). Alcalase is endopeptidase, which is able to hydrolyse proteins with broad specificity for peptide bonds and prefers a large uncharged residue. Benjakul and Morrissey (1997) reported that Alcalase was more active at alkaline pH and remained active to pH 6.0. The activity was high in pH range of 6.5-8.5 but showed considerable loss of activity at pH 10.5. Alcalase showed a high activity toward Pacific whiting waste in the high temperature

Table 3. Commercial proteases used for production of fish protein hydrolysate

Proteases	Sources	References
Alcalase, Flavourzyme	Round scad (*Decapterus maruadsi*) mince	Thiansilakul *et al.* (2007a)
Alcalase, Flavourzyme	Yellow stripe trevally (*Selaroides leptolepis*) mince	Klompong *et al.* (2007)
Alcalase, Flavourzyme	Silver carp (*Hypophthalmichthys molitrix*) mince	Dong *et al.* (2008)
Papain	Grass carps (*Ctenopharyngodon idellus*) muscle	Ren *et al.* (2008)
Protamex	Atlantic mackerel (*Scomber scombrus*)	Beaulieu *et al.* (2009)
Orientase	Tuna (*Thunnus tonggol*) cooking juice	Hsu *et al.* (2009)
Papain, Protamex	Loach (*Misgurnus anguillicaudatus*) meat	You *et al.* (2009)
Papain	Loach (*Misgurnus anguillicaudatus*) meat	You *et al.* (2010)
Orientase, Protease, XXIII	Tuna (*Thunnus tonggol*) dark muscle	Hsu (2010)
Protamex	Black scabbardfish (*Aphanopus carbo*) heads, viscera, frames, skin, trimmings	Batista *et al.* (2010)
Alcalase, Papain	Meriga (*Cirrhinus mrigala*) fish egg	Chalamaiah *et al.* (2010)
Alcalase, Flavourzyme	Bluewing Searobin (*Prionotus punctatus*) meat	Santos *et al.* (2011)
Alcalase, Trypsin, Protamex	Alaska pollock frame muscle	Hou *et al.* (2011)
Alcalase	Nile tilapia (*Oreochromis niloticus*) meat	Foh *et al.* (2011)
Alcalase	Yellowfin Tuna (*Thunnus albacares*) viscera	Ovissipour *et al.* (2012)
Alcalase	Threadfin bream (*Nemipterus spp*.) bone, skin	Wiriyaphan *et al.* (2012)
Papain	Scallop (*Patinopecten yessoensis*) meat	Zhou *et al.* (2012)
Protease A, Protease N, Protamex, Prozyme 6	Lanternfish (*B. pterotum*) mince	Cai *et al.* (2013)
Papain	Small yellow croaker (*psendosciaena polyactis***)** mince	Ji *et al.* (2013)
Protease P	Cod bone mince (*Gadus Morhua*)	Halldorsdottir *et al.* (2014b)
Flavourzyme	Whitemouth croaker (*Micropogonias furnieri*)	Zavareze *et al.* (2014)

range (55-70 °C) with an optimum at 60 °C (Benjakul and Morrissey, 1997). Normah *et al.* (2005) found that the hydrolysis of threadfin bream (*Nemipterus japonicus*) meat by Alcalase was optimum at 60 ºC and pH 8.5 for 120 min using an enzyme/substrate ratio of 1:3. Under this hydrolysis condition, 20% hydrolysis was achieved and 70% nitrogen was recovered. Bhaskar *et al.* (2008) reported that the degree of hydrolysis close to 50% was obtained for visceral wastes of freshwater carp, when Alcalase was used with the following conditions: an enzyme to substrate level of 1.5% (v/w), pH 8.5, 50 ºC and a hydrolysis time of 135 min. Bigeye tuna head protein hydrolysate prepared using Alcalase exhibited antioxidant activities (Yang *et al.*, 2011). Ghassem *et al.* (2014) also reported that Alcalse hydrolysed sarcoplasmic protein from snakehead fish yielding hydrolysate possessing ACE inhibitory property. Peptides isolated from skate gelatin hydrolysate derived by Alcalase could protect human endothelial cells from H2O2 induced oxidative damage (Ngo *et al*., 2014). Alcalase has been documented to be a promising candidate for hydrolysing fish proteins based on enzyme cost per activity (Kristinsson and Rasco, 2000b).

(3.2) Neutrase

Neutrase is a neutral protease derived from *Bacillus amyloliquefaciens*, with a broad specificity. It exhibits the optimal activity at 45-55°C and pH 5.5-7.5. Complete loss in activity is noticeable at 80 °C (Rao *et al.*, 1998). Benjakul and Morrissey (1997) found that Neutrase showed the optimum activity against Pacific whiting solid wastes at pH 7.0 and 55 °C. Protein hydrolysate from tilapia and *Arca Subcrenata* meat prepared using Neutrase exhibited the antioxidant activities (Foh *et al.*, 2011; Song *et al.*, 2008). Ahn *et al*. (2010) reported that tuna liver protein by-products hydrolysed by Neutrase showed excellent DPPH, hydrogen peroxide and hydroxyl radical scavenging, and reducing power. Protein hydrolysates from tilapia meat prepared using Neutrase showed significant ability to scavenge ROS and reduce ferric ions (Raghavan *et al.*, 2008). Je *et al*. (2007, 2008) performed hydrolysis using Neutrase to produce antioxidant peptides from bigeye tuna backbone and dark muscle protein. Peptides from hydrolysates showed both free radical scavenging effects and lipid peroxidation inhibitory activity. Lee *et al.* (2010) produced ACE-inhibitory peptides from tuna frame protein using Neutrase with hydrolysis time

of 8 h. Gelatin hydrolysates prepared from bigeye snapper skin using Neutrase in combination with Alcalase exhibited antioxidant activity in the linoleic acid oxidation and the lecithin liposome systems (Phanturat *et al.*, 2010).

(3.3) Flavourzyme

Flavourzyme is a fungal protease/peptidase complex produced by submerged fermentation of a selected strain of *Aspergillus oryzae*, which has not been genetically modified and is used for the hydrolysis of proteins under neutral or slightly acidic conditions. Flavourzyme is the endo- and exopeptidase enzyme mixture, which can minimise the bitterness in protein hydrolysate (Rossini *et al.*, 2009; Thiansilakul *et al.*, 2007a). The optimal working conditions for Flavourzyme are reported to be at pHs 5.0-7.0 with an optimal temperature around 50 °C (Šližyte *et al.*, 2005). Silver carp muscle was defatted and hydrolysed using Flavourzyme at pH 7 and 50ºC (Dong *et al.*, 2008). Nilsang *et al.* (2005) reported that the spray-dried hydrolysate produced from fish soluble concentrate using Flavourzyme contained high protein content (66%) and had the bitterness less than that of 1 ppm caffeine solution. Protein hydrolysate from round scad and yellow stripe trevally prepared using Flavourzyme showed the antioxidant activities (Klompong *et al.*, 2007; Thiansilakul *et al.*, 2007a).

(3.4) Other enzymes

Several microbial proteases have been used for hydrolysate production. Protease N was used for preparing hydrolysate from mackerel with antioxidant activity (Wu *et al.*, 2003). Additionally, freshwater clam muscle hydrolysate with ACE inhibitory activity was prepared using Protomex, followed by Flavourzyme (Tsai *et al.*, 2006). Kim *et al.* (2007) employed pepsin and trypsin for enzymatic hydrolysis to produce antioxidant peptides from hoki frame protein. The hydrolysate from lantern fish protein prepared by Protease N was reported to show a preventive effect against oxidative stress of HepG 2 cell as induced by H_2O_2 (Chai *et al.*, 2013).The combined proteases including Alcalase, chymotrypsin, pancreatin and pepsin were also used to increase the bioactivity of hydrolysates (Korhonen and Pihlanto, 2003). For some fish species, the hydrolysis can be maximised by

optimization of autolysis mediated by endogenous proteases. Autolysis of fish promotes the hydrolysis process and lowers the cost of enzymes. Pacific hake was hydrolysed by its endogenous proteases from infected *K. paniformis*, which produced cathepsin L-like enzyme. Hence, the production of protein hydrolysate from Pacific hake could be achieved without adding any commercial enzymes (Samaranayaka and Li-Chan, 2008). Nevertheless, hydrolysates produced autolytically showed considerably lower protein recovery, compared with that produced by commercial enzymes (Shahidi *et al.*, 1995).

B) Factors affecting fish protein hydrolysate production

(1) Type of enzyme and substrates

A wide variety of commercial enzymes has been used successfully to hydrolyse fish and other food proteins. The choice of substrate and protease employed as well as the degree to which the protein is hydrolysed generally affects the physicochemical properties of the resulting hydrolysates (Kristinsson and Rasco, 2000b). Proteolytic enzymes from microorganisms such as Alcalase, Neutrase, Protease N and Protamex have been found to be more suitable to produce hydrolysate because of their high productivity (Benjakul and Morrissey, 1997; Liaset *et al.*, 2003; Wu *et al.*, 2003). Enzyme from plants and animals such as papain, bromelain, ficin and pepsin are still used for hydrolysis (Aristotelis *et al.*, 2011). Even though acidic condition is better for microbial growth prevention, acid proteases generally show lower hydrolytic activity. Thus, milder enzymes at neutral and slightly alkaline condition have been used more frequently (Kristinsson and Rasco, 2000b). Due to high proteolytic activity of microbial proteases, those enzymes have been used intensively and widely for protein hydrolysate production (Nalinanon *et al.*, 2011; Ovissipour *et al.*, 2013; Wiriyaphan *et al.*, 2012).

(2) Enzyme concentration

With increasing enzyme concentration upon the hydrolytic reaction, the substrate must be present in an excess amount. The amount of product formed over a specified period of time is dependent upon the level of enzyme (Klompong *et al.*, 2007). The relationship between enzyme activity and concentration

is affected by many factors such as temperature, pH, etc. (Linder *et al.*, 1995). Cheftel *et al*. (1971) reported that an increase in enzyme concentration has a positive effect on overall proteolysis with subsequent increases in solubilisation of fish protein concentrate. Benjakul and Morrissey (1997) found that when the enzyme concentration was increased, DH of Pacific whiting solid wastes treated with both Alcalase and Neutrase increased. Guerard *et al.* (2002) suggested that the concentration of hydrolysable bonds was one of the main variables controlling the hydrolysis rate. Protein hydrolysate from yellow stripe trevally showed the higher DH with increasing Alcalase concentration (Klompong *et al.*, 2007). Increase in DH of protein hydrolysate from round scad was obtained when the higher enzyme/substrate ratio was used (Thiansilakul *et al.*, 2007a). Intarasirisawat *et al.* (2012) also found a linear relationship when $\log_{10}$ of enzyme concentration and DH were plotted. Sheriff *et al.* (2014) reported that protein hydrolytsate from Indian mackerel prepared using papain showed a significant higher rate of DH when the enzyme/substrate ratio increased to 4:100 (w/w), especially at the initial stage of hydrolysis. It is more likely that proteases are able to localise close to substrate, thereby cleaving peptides to a higher degree. However, DH is not always proportional to the level of proteases added. This is governed by cleavage sites available in the substrates (Linder *et al.*, 1995).

(3) Hydrolysis time

The enzymatic hydrolysis of protein is characterised by an initial rapid phase, during which a large of peptide bonds are hydrolysed. Thereafter, the rate of enzymatic hydrolysis decreases and reaches a stationary phase where no apparent hydrolysis takes place (Shahidi *et al.*, 1995). Normah *et al.* (2005) reported an increase in DH in the first 30 min of reaction when Alcalase was used to hydrolyse threadfin bream muscle. Subsequently, the rate of hydrolysis remained constant. Hydrolysis curves of shrimp waste using Alcalase and pancreatin showed high initial reaction rates, followed by decreases in the reaction rate up to the stationary phase (De Holanda and Netto, 2006). With increasing hydrolysis time, DH of sardinella heads and viscera hydrolysed by Alcalase increased rapidly in the first 3 h, followed by the decreasing rate (Souissi *et al.*, 2007). Dong *et al.* (2008) reported that the hydrolysis of silver carp protein with Alcalase or Flavourzyme proceeded at a high rate during the initial 15 min

and then slowed down thereafter, indicating that maximum cleavage of peptides occurred within the first 15 min of hydrolysis. The similar result was also reported by Giménez *et al.* (2009) for the hydrolysis of skin from sole and squid with Alcalase, where the maximum cleavage of peptides occurred at the beginning of hydrolytic reaction.

1.2.4.3 Pretreatment processes

Although fish can serve as a potential raw material for protein hydrolysate production, the presence of pro-oxidants such as HP and unstable lipid substrates is a drawback (Raghavan and Kristinsson, 2008). These constituents are involved in undesirable characteristic and instability of hydrolysates (Khantaphant *et al.*, 2011a; Raghavan *et al.*, 2008). Furthermore, HP can also become oxidised during the hydrolysis process, thereby promoting lipid oxidation and developing of unpleasant odour (Khantaphant *et al.*, 2011a; Theodore *et al.*, 2008). To overcome such problems, the pretreatment of protein substrate including washing, defatting or centrifugation in order to remove the excess fat and pigments are strongly recommended. Recently, a new method yielding the protein isolate with low amount of undesirable compounds has been developed (Khantaphant *et al.*, 2011a). The resulting hydrolysate showed the superior property to that prepared directly from fish mince. One advantage was that hydrolysate had the negligible fishy odour/smell. Therefore, appropriate pretreatment is required prior to hydrolysis process.

A) Defatting

Lean fish or their derived material is preferable as the protein substrate for enzymatic hydrolysis to avoid an extensive lipid oxidation. Fatty fish are highly prone to oxidative rancidity. This may be due to the highly unsaturated fatty acids present in fish lipids. Due to the abundance of underutilized pelagic fatty fish, they can serve as the proteinaceous substrates for hydrolysate production (Klompong *et al.*, 2007; Thiansilakul *et al.*, 2007b). Fatty fish species such as mackerel, herring, round scad and yellow stripe trevally would require additional treatment such as centrifugation and solvent extraction to remove the fat (Klompong *et al.*, 2007;

Thiansilakul *et al.*, 2007a). Fish protein hydrolysate with high lipid content turned to be darken caused by lipid oxidation (Hoyle and Merritt, 1994). Furthermore, the oxidative deterioration was associated with the development of offensive odour, particularly fishy odour, in resulting hydrolysate (Raghavan and Hultin, 2009). Many types of solvents have been used to remove fat of fish meat prior to hydrolysis. Holy and Merit (1994) used an ethanol extraction to remove fat from herring mince with a fish/solvent ratio of 1:2 at 70 °C. Lipid content in final product was reduced to 0.9% from 4.0% of raw material. Klompong *et al*. (2007) reported that yellow tripe trevally mince subjected to defatting with isopropanol had a much lower fat content (0.67%) when compared to whole mince (3.23%). Luo *et al.* (2013) noted that the pretreatment of *S. lewin* muscle by defatting using isopropanol with a fish/solvent ratio of 1:4 (w/v) yielded hydrolysate with high antioxidant property. Jiang *et al*. (2014) found that protein hydrolysate prepared from isopropanol defatted silver carp mince using papain contained peptide possessing antibacterial-zinc complexes. Before being hydrolysed, shrimp by-product was defatted with petroleum ether, in which fat content at a level of < 0.1% was obtained (Huang *et al.*, 2011). However, the protein substrates were less susceptible toward hydrolysis. The lower DH was obtained in defatted mince, when compared to mince (Thiansilakul *et al.*, 2007a). Hoyle and Merritt (1994) found that denatured fish protein possessed poor wettability, thereby lowering the dispersibility and accessibility of enzyme to the substrate. Moreover, endogenous proteases in fish muscle might undergo denaturation during defatting process, especially at high temperature (Klompong *et al.*, 2007). In the presence of solvent and higher temperature, proteins undergo denaturation and aggregation. Therefore, the type of solvent and condition used for defatting were closely related with hydrolysis efficiency as well as properties of resulting hydrolysate.

B) Phospholipid membrane removal

Lean fish species, particulary ordinary portion, are preferable raw material for protein hydrolysate preparation with a consistent high quality owing to their negligible lipid contents and haem pigments. Nevertheless, oxidative deterioration still takes place in resulting lean fish hydrolysate as evidenced by the development of off-odour (Halldorsdottir *et al.*, 2014a). The unpleasant odour/flavour lowers the

consumer acceptance and limits their use. Even though washing is the effective means used for the removal of sarcoplasmic proteins, lipids and pro-oxidative aqueous components, etc. phospholipid membranes are still retained in washed mince. Phospholipid membranes are believed to be the key substrate for lipid oxidation (Liang and Hultin, 2005b). Phospholipids consist of highly polyunsaturated fatty acids and have largely surface area. Therefore, they are prone to oxidation, which cause the adverse effects on quality and acceptability of protein hydrolysates. The application of $CaCl_2$ and citric acid in combination with washing process has been known to show the great impact on phospholipid membrane separation. Ca^{2+} and citric acid are able to disconnect the linkages between cytoskeletal proteins and membrane lipid, linked together via electrostatic interaction. Citric acid plays a role as a binding agent for the basic amino acid residues of cytoskeletal proteins, thereby competing with the acidic phospholipids of membranes (Hrynets *et al.*, 2011). The scheme of phospholipid membrane removal from fish muscle protein is shown in Figure 7. Hrynets *et al.* (2011) reported that the use of 6 or 8 mM of citric acid resulted in substaintial removal of lipid and pigments from mechanically separated turkey meat. The addition of 10 mM $CaCl_2$ and 5 mM citric acid during acid or alkaline aided protein isolation significantly improved lipid reduction in mussel protein (Vareltzis and Undeland, 2008). Additionally, Ca^{2+} could interact with the polar head of phospholipid to form a calcium-phospholipid complex. The membranes released from the cytoskeletal proteins might aggregate to a large particle as induced by Ca^{2+} addition and sediment by centrifugation (Liang and Hultin, 2005a). After membrane lipids are removed, the hydrolysis can be enhanced, more likely due to the higher accessibility of protein substrances. Khantaphant *et al.* (2011a) reported that protein hydrolysate produced from brownstripe red snapper mince pretreated by membrane separation/washing had the highest antioxidant properties with lesser amount of pro-oxidants and phospholipid content. Moreover, the chitosan and chitin have been applied to aid membrane separation. Liang *et al.* (2007) reported that the addition of chitosan with a molecular weight of 310-375 kDa prior to either acidification or alkalization of cod homogenate resulted in the 55 and 80% of membrane removal, respectively, whilst the control treatment showed 31% membrane removal. The efficiency of chitosan was ascribed to its high positive

charge/molecule, thereby interacting with negatively charged membrane (Liang *et al.*, 2007).

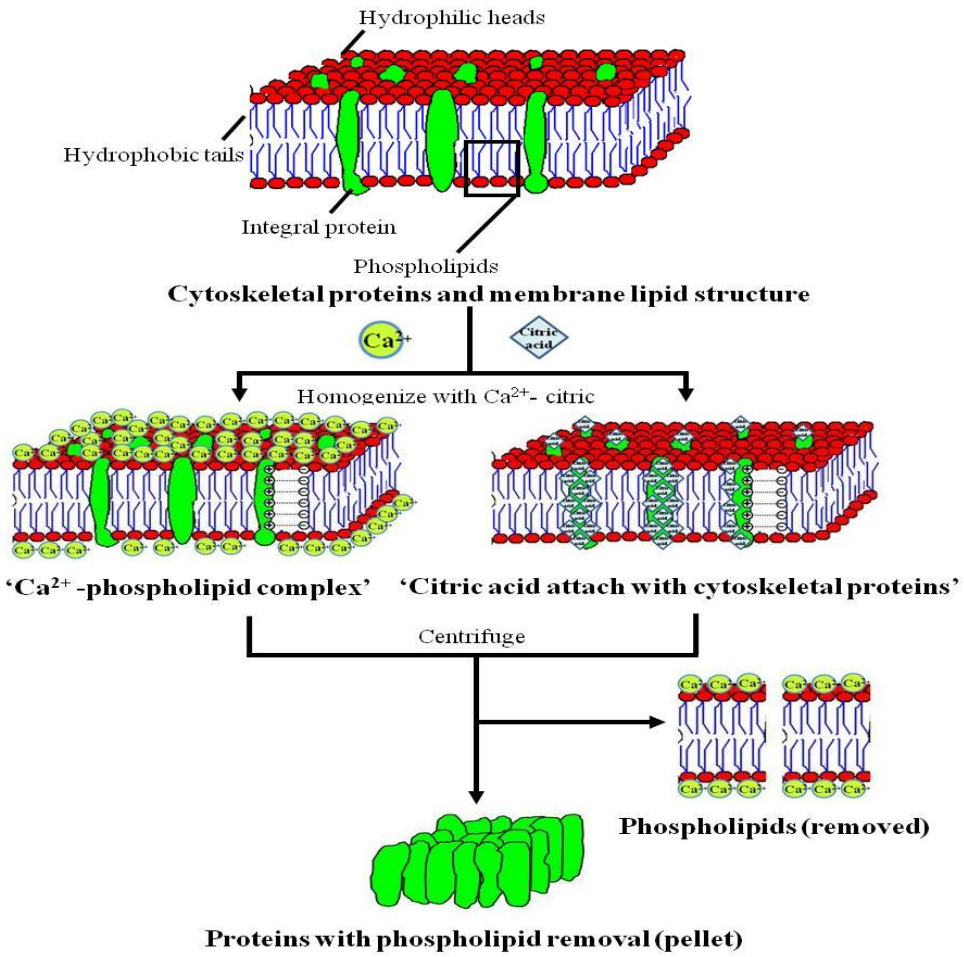


Figure 7. Phospholipid membrane removal from fish muscle protein

Source: Benjakul *et al.* (2014).

C) Preparation of protein isolate

Many protein-rich fish processing by-products or underutilized fish species are not successfully used to maximise their benefit. A new technology, acid/alkaline pH-shift method, were developed for recovery of proteins via the isolation of protein from complex structures (Hultin and Kelleher, 2000). The technique involves solubilisation of muscle at acidic or alkaline pH, followed by centrifugation to remove insoluble materials. The upper layer containing neutral lipids and the bottom sediments

layer containing connective tissue and insoluble lipids membrane were discarded, whilst the middle layer containing proteins were subsequently subjected to precipitation by adjusting pH to their isoelectric pH (~5.5) (Nalinanon *et al.*, 2011). The protein isolates were then recovered by centrifugation. This process not only removes undesirable materials, especially lipids or sarcoplasmic proteins, but also concentrates the myofibrillar proteins (Baxter and Skonberg, 2008). Vareltzis and Undeland (2008) compared the impact of acid and alkaline processes on a blue mussel mince and found that the acid process yielded the isolate with a lower lipid content than did alkaline process. Undeland *et al.* (2002) reported that protein isolates prepared from herring using alkaline solubilisation technique had lower total lipid and phospholipid contents than acid solubilisation technique. The highest total pigment removal was also achieved for tilapia mince after alkaline aided process (Rawdkuen *et al.*, 2009). The protein isolate could yield a much purer protein substrate for enzymatic hydrolysis. By removing the contaminant materials, the stability of the final product is greatly enhanced as these are the main substrates for oxidative reactions in fish muscle (Hultin, 1994). Hydrolysate produced from alkaline treated protein isolate show the superior functional properties and bioactive activities. Those included hydrolysates produced from by-product of Cape hake (Pires *et al.*, 2012), or the meat of channel catfish (Theodore *et al.*, 2008), brownstripe red snapper (Khantaphant *et al.*, 2011a) and saithe (Halldórsdóttir *et al.*, 2011). Although acid aided process is not popular for protein hydrolysate production, due to the destruction of HP associated with the promotion of lipid oxidation, its isolate can be served as a potential source for acid protease hydrolysis. Nalinanon *et al.* (2011) prepared ornated threadfin bream hydrolysate with antioxidant activity from protein isolate using acid solubilised process. Skipjack tuna pepsin was used to hydrolyse protein isolate at acid pH. To lower sarcoplasmic protein as well as HPs, pre-washing is recommended prior to solubilisation. As a result, those pro-oxidative proteins are less co-precipitated during precipitation process at pI (Theodore *et al.*, 2008).

1.2.5 Antioxidant properties of protein hydroysate

1.2.5.1 *In vitro* chemical based antioxidant activity

Antioxidant compounds in food play an important role as health protecting factors and they are used to preserve food products to retard discolouration and deterioration caused by oxidation (Decker *et al.*, 2005). Lipid oxidation is of great concern to the food industry and consumers, since it leads to the development of undesirable off-flavours and potentially toxic reaction products (Arvanitoyannis *et al.*, 2006). Protein hydrolysates formed by the enzymatic digestion of aquatic products and by-products have been proven to be good sources of antioxidant peptides (Bougatef *et al.*, 2010; Kudo *et al.*, 2009; Ren *et al.*, 2008). During hydrolysis, peptide bond cleavage allows the release of active peptides capable of sequestering oxygen radicals, chelating pro-oxidant metal ions and inhibiting lipid peroxidation in food systems (You *et al.*, 2010). Protein substrates and types of proteases have the influence on the peptides produced and their activities. Jun *et al*. (2004) reported that hydrolysate prepared from yellowfin sole frame using pepsin showed a stronger antioxidant activity in a linoleic acid model system than those produced using Alcalase, α-chymotrypsin, papain, Pronase E, Neutrase, and trypsin. Protein hydrolysate from Alaska pollack frame prepared using mackerel intestine crude enzyme also exhibited antioxidant activity in a linoleic acid oxidation system (Je *et al.*, 2005). Tuna cooking juice was hydrolysed using Protease XXIII from *Aspergillus oryzae* and the hydrolysate displayed the DPPH radical scavenging ability (Jao and Ko, 2002). Kim *et al.* (2007) prepared antioxidant peptide from hoki frame using different six proteases (pepsin, trypsin, papain, α-chymotrypsin, Alcalase and Neutrase). Peptic hydrolysate showed the highest inhibition against linoleic acid oxidation and it had radical scavenging activities. When Alaska pollack skin gelatin was hydrolysed with Alcalase, Pronase E, and collagenase, the hydrolysate prepared using Pronase E exhibited the highest antioxidant activity (Kim *et al.*, 2001). Furthermore, Batista *et al.* (2010) produced protein hydrolysate from black scabbardfish by-products using Protamex. The resulting hydrolysate possessed DPPH and hydroxyl radical scavenging activities and reducing power. Different proteases exhibited the varying specificity and the hydrolysis rate, resulting in the formation of different peptides with various bioactivities. Phanturat *et al.* (2010) used

the pyloric caeca extract from bigeye snapper (*Priacanthus macracanthus*) for preparation of gelatin hydrolysate with antioxidant activity. The antioxidant peptide of gelatin hydrolysate had MW of 1.7 kDa. The amino acid composition and sequence as well as the chain length are the factors governing the antioxidant activity of peptides. With the same raw materials, the different proteases used render the peptides with different characteristics, especially bioactivity. To enhance the bioactivity of peptides produced, several approaches have been implemented such as the use of multi-step of hydrolysis with different proteases (Phanturat *et al.*, 2010). Sequence of proteases used for hydrolysis also showed the pronounced impact on the bioactivity of peptides formed (Klompong *et al.*, 2007; Phanturat *et al.*, 2010). Different radical scavenging activity is not determined by only amino acid composition but also special amino acid sequences (Mendis *et al.*, 2005b). Antioxidant peptides derived from different marine animals showed varying potencies to scavenge free radicals. Different antioxidant peptides were identified from protein hydrolysate from different aquatic animals (Table 4). The antioxidant activity may not be attributed to a single antioxidant mechanism (Rajapakse *et al.*, 2005). Some peptides are capable of chelating metal, which acts as the pro-oxidant (Klompong *et al.*, 2007). Jao and Ko (2002) reported that hydrolysate of tuna cooking juice contained several amino acids, such as tyrosine, methionine, histidine, tryptophan, lysine and proline, which function as antioxidants. Quenching of free radicals by natural antioxidants has been reported through donation of hydrogen (Byun and Kim, 2001). Davalos *et al.* (2004) indicated that tryptophan, tyrosine and methionine showed the highest antioxidant activity, followed by cysteine, histidine and phenylalanine. Histidine and hydrophobic amino acids were suggested by Peña-Ramos *et al.* (2004) as the key factor in the delay of lipid oxidation. Peptides, which exhibited good antioxidant activity, usually contain certain amino acids such as histidine, proline, tyrosine and lysine (Wang *et al.*, 2008). Mendis *et al.* (2005a) reported that the presence of non-aromatic amino acids such as proline, alanine, valine and leucine in jumbo squid skin hydrolysate contributed to the higher antioxidant activities. Phenylalanine and leucine residues at N- and C-terminal of peptide also contributed to the activity. Peptides containing tyrosine residues at the C-terminus, lysine or phenylalanine residues at the N-terminus and tyrosine residues in their sequences had strong free radical scavenging activity (Guo *et al.*, 2009). Bougatef *et al.* (2010) indicated that

peptides containing histidine, tryptophan and tyrosine residues possessed antioxidant activity. Moreover, Suetsuna *et al*. (2000) indicated that some other amino acids such as proline, alanine and leucine contribute to free radical scavenging activity. Leucine and proline could favour antioxidant activity when it is located in the C-terminal of the sequence (Suetsuna *et al.*, 2000). Gu *et al.* (2012) reported that Tyr residue at the N-terminus of synthesis tetrapeptide (Tyr-Glu-Cys-Gly) contributed to ABTS radical scavenging ability. In addition, the carboxyl of Glu located next to Tyr could induce the release of hydrogen atom of phenolic hydroxyl in Tyr, enhancing the antioxidant activity. Wiriyaphan *et al.* (2013) reported that the high ABTS radical scavenging activity of FLGSFLYEYSR peptide was attributed to two Tyr residues in the sequence. Furthermore, the antioxidant activity of protein hydrolysate has been linked to the high content of hydrophobic amino acids, which could increase their solubility in lipids and therefore enhance their antioxidant activity. Octapeptide isolated from salmon by-product contained five amino acid residues (two Phe, two Leu and one His) responsible for antioxidant activity (Ahn *et al.*, 2014). Moreover, histidine- and methionine-containing peptides were reported to have antioxidant activity due to the imidazole group, which could donate proton to free radical. Wang *et al.* (2014) isolated two antioxidant peptides including PVMGD and QHGV from oyster protein hydrolysate, both of which containing methionine and histidine in their sequence.

1.2.5.2 Cellular based antioxidant activity

Even though chemically based antioxidant activity has been widely reported, all assays conducted under non-physiological conditions. The result displayed was probably not correlate with biological response. Biological systems are much more complex than the simple chemical mixtures employed, and antioxidant compounds may operate via multiple mechanisms (Liu and Finley, 2005). However, human and animal models are expensive and time-consuming, whereas a cell culture model allows for rapid and inexpensive screenings. Various cell culture models are therefore an invaluable tool to assess these potential health benefits of food antioxidants. Human adenocarcinoma colon cancer (Caco-2) cell monolayers have been the most commonly reported in the literature for studying intestinal permeability of

Table 4. Antioxidative peptides from fish protein hydrolysate

Sources	Enzymes	MW (Da)	Sequences	References
Tuna cooking juice	Orientase	1305	Pro-Val-Ser-His-Asp-His-Ala-Pro-Glu-Tyr	Hsu *et al.* (2009)
		938	Pro-Ser-Asp-His-Asp-His-Glu	
		584	Val-His-Asp-Tyr	
Tuna dark muscle by-product	Protease XXIII	756	Pro-Met-Asp-Tyr-Met-Val-Thr	Kuo-Chiang (2010)
		978	Leu-Pro-Thr-Ser-Glu-Ala-Ala-Lys-Tyr	
Loach muscle	Papain	464	Pro-Ser-Tyr-Val	You *et al.* (2010)
Horse mackerel skin	Pepsin, Trypsin, α-Chymotrypsin	856	Asn-His-Arg-Tyr-Asp-Arg	Sampath Kumar *et al.* (2012)
Nile tilapia frame	Trypsin	456.1	Asp-Cys-Gly-Tyr	Fan *et al.* (2012)
		702.3	Asn-Tyr-Asp-Glu-Tyr	
Sphyrna lewini muscle	Alcalase	475.5	Trp-Asp-Arg	Wang *et al.* (2012)
		677.7	Pro-Tyr-Phe-Asn-Lys	
Nile tilapia skin gelatin	Pronase E and multifect neutral	317.3	Glu-Gly-Leu (Da)	Zhang *et al.* (2012)
		645.2	Tyr-Gly-Asp-Glu-Tyr	
Flounder fish muscle	α-Chymotrypsin	406.1	Val-Cys-Ser-Val	Ko *et al.* (2013)
		360.1	Cys-Ala-Ala-Pro	
Monkfish muscle	Trypsin	629.7	Glu-Trp-Pro-Ala-Gln	Chi *et al.* (2014)
		668.8	Phe-Leu-His-Arg-Pro	
		633.8	Leu-Met-Gly-Gln-Trp	
Round scad muscle	Alcalase	706.8	His-Asp-His-Pro-Val-Cys	Jiang *et al.* (2014)
		614.7	His-Glu-Lys-Val-Cys	
Ethanol soluble protein from spotless smooth	Papain	217.3	Gly-Ala-Ala	Wang *et al.* (2014)
		378.5	Gly-Phe-Val-Gly	
		682.0	Gly-Ile-Ile-Ser-His-Arg	
		486.7	Glu-Leu-Leu-Ile	
		519.6	Lys-Phe-Pro-Glu	

bioactive compounds due to their similarity to the intestinal endothelium cells (Liu and Finley, 2005; Vermeirssen *et al.*, 2005). Upon culturing as a monolayer, Caco-2 cells differentiate to form tight junctions between cells to serve as a model of paracellular movement of compounds across the monolayer. Caco-2 cells express transporter proteins, efflux proteins, and Phase II conjugation enzymes to model a variety of transcellular pathways as well as metabolic transformation of test substances (Van Breemen and Li, 2005). Small di- and tri-peptides may be absorbed intact across the brush border membrane using H^+-coupled PepT1 transporter system (Vermeirssen *et al.*, 2002). Larger water- soluble peptides can cross the intestinal barrier paracellularly via the tight junction between cells, whilst highly lipid-soluble peptides may diffuse via the transcellular route (Miguel *et al*., 2008). Peptides may also enter the enterocytes via endocytosis, which entails membrane binding and vesiculisation of the material (Ziv and Bendayan, 2000). The intestinal basolateral membrane also possesses a peptide transporter, which facilitates the exit of hydrolysis-resistant small peptides from the enterocyte into the portal circulation (Gardner, 1984).The contribution of each route and the ability of individual peptides to transport across the membrane, depend upon the molecular size, and other structural characteristics such as hydrophobicity, as well as their resistance to brush-border peptidases (Satake *et al.*, 2002; Shimizu *et al.*, 1997).

In addition, the liver cells have been noted to be the main target for antioxidant compounds once they have been absorbed from the gastrointestinal tract. The use of normal human hepatocytes, which closely represent the parent cell types, is restricted due to limited growth potential, expensive, legal and ethical considerations. Under proper culture, the human hepatocarcinoma (HepG2) cell line can display highly morphological and functional differentiation, resembling normal human hepatocytes (Narayanan *et al.*, 2001). Wiriyaphan *et al.* (2012) reported that hydrolysate prepared from surimi by-product (frame, bone and skin) using Alcalase showed non-cytotoxic on HepG2 cell. The low molecular weight peptide associated with a good antioxidant property could efficiently penetrate into the cell (Wiriyaphan *et al.*, 2012). Ahn *et al.* (2014) reported that octapeptide purified from salmon by-protduct protein hydrolysate showed a protection effect against H_2O_2 induced hepatic damage in Chang liver cells. Moreover, Halldorsdottir *et al.* (2014b) also found that the addition of hydrolysate from cod bone mince containing brown algae extract in HepG2 cell yielding a lower cell

reactive oxygen species generation as induced by AAPH, compared to those without brown algae extract. Apart from Caco2 or HepG2 cells, several other human cell lines have been applied in cellular antioxidant activity studies including fibro sarcoma cell (HT1080) (Kim and Kim, 2010), colon rectal adenocarcinoma (HT-29)(Bellion *et al.*, 2009), human Jurkat T cells (Lahart *et al.*, 2011) or etc.

1.2.6 Application of protein hydrolysate

1.2.6.1 Food Application

Fish protein hydrolysates have been extensively applied in food products. Several hydrolysates have been found to have excellent interfacial properties and thus may have potential use as emulsifying ingredients in a variety of products, e.g. dressing, margarine and meat batter. They can be used as the aid in the formation and stabilisation of foam based product, e.g. whipped cream meringues and mousse (Kristinsson, 2007). In addition, protein hydrolysates from fish have shown to display cryoprotective ability in frozen product. Nikoo *et al.* (2014b) also found that tetrapeptide (Pro-Ala-Gly-Tyr) isolated from Amur sturgeon skin gelatin showed cryoprotective effect in Japanese seabass mince. Damodaran (2007) demonstrated the ability of gelatin hydrolysate to inhibit ice crystal growth in ice cream mix. Khan *et al.* (2003) used fish-scrap protein hydrolysate as the cryoprotectant to prevent protein denaturation of lizardfish surimi during frozen storage at -25 °C. Cheung *et al.* (2009) found that the increases in expressible moisture content from 22 to 33% and cooking loss from 3 to 16% were found in control cod sample after 6 cycle of freeze-thaw. The poor water retention properties were improved in samples containing 8% of protein hydrolysate from Pacific hake prepared using Alcalase and Flavourzyme. In some countries, fish protein hydrolysates are used as a milk substitute and as flavouring compounds (James *et al.*, 1996). During hydrolysis, the oligopeptides released have been known to serve as potential flavourants. Protein hydrolysate from headed-gutted or frame red hake mince prepared using Flavourzyme under natutral pH (6.8) and water/fish ratio of 2:5 was used as for natural flavour stock (Imm and Lee, 1999). Hydrolysate with the highest acceptability contained higher percentages of free amino acids, especially Glu, giving both umami and sweet tastes. Protein hydrolysates derived

from bonito muscle protein using thermolysin were used as salt replacement substances in a food or beverage formula (LeBlanc, 2005). Due to the detrimental effect of sodium chloride, in particular elevating blood pressure in human, the alternative hydrolysates with salty flavour were applied (Imm and Lee, 1999). Based on bioactivities, fish protein hydrolysates are increasingly used to extend shelf-life and to improve the stability of lipid and lipid-containing foods. The incorporation of capelin hydrolysate at a level of 3% in ground pork could effectively reduce lipid oxidation by 60.4%, when compared with untreated sample (Shahidi *et al.*, 1995). The application of Nile tilapia protein hydrolysate with 13% DH in dip solution could improve oxidative stability of mahi mahi red muscle during storage at 4°C (Dekkers *et al.*, 2011). Intarasirisawat *et al.* (2014) reported that the addition of skipjack roe protein hydrolysate not only improved textural properties of broadhead catfish emulsion sausage but also retarded lipid oxidation during the refrigerated storage. Additonally, peptide *CgPep33*, from the Pacific oyster with high inhibitory activity against *Botrytis cinerea* growth could reduce gray mold disease incidence of strawberry fruit after harvest. Therefore, *CgPep33* can be used as a substitute for fungicidal to control postharvest diseases and to extend the shelf-life of fruit and vegetable (Liu *et al.*, 2007).

1.2.6.2 Cell and microbial cultivations

Protein hydrolysates provide a readily available source of nitrogen for microorganisms. The supplementation of cell culture media with hydrolysates can yield a number of benefits such as cell viability, cell proliferation and target protein production. Protein hydrolysate contains soluble amino acids, peptides, minerals and essential elements required for microbial growth (Essien *et al.*, 2005; Kurbanoğlu and Algur, 2004). Klompong *et al.* (2009) produced protein hydrolysates from yellow stripe trevally and used as culture media. Hydrolysate prepared using Flavourzyme (HF_{25}) with 25% DH showed the higher bacterial productivity ratio than did Bacto Peptone. When HF_{25} and commercial Bacto Peptone were used as microbial media to determine microbial load of environmental and food samples and pathogenic bacteria, HF_{25} generally exhibited similar potential in culturing those microorganisms. The peptides with the MW of 6,500 Da, di-peptides and amino acids from tuna treated with Alcalase served as a suitable nitrogenous source in microbial media (Guérard *et al.*, 2001). For

bacteria, *Staphylococcus aureus* and *Escherichia coli*, HF with 25% DH (HF_{25}) yielded the highest cell density and specific growth rate (μ_{max}) and the lowest generation time (t_d) (Klompong *et al.*, 2012). Protein hydrolysate from hake filleting waste was shown to support growth of bacteria and archaea (Martone *et al.*, 2005). Fish peptones from tuna, cod, salmon, and unspecified fish were compared to one made with a casein using a new method based on Gompertz modeling of microbial growth. Cumulative results obtained from 6 species of bacteria, yeasts, and fungi revealed that fish peptones were very effective as microbial media (Dufossé *et al.*, 2001). Ghorbel *et al*. (2005) reported that defatted *Sardinella* meat protein hydrolysate may be an excellent nitrogen source for growth of *Rhizopus oryzae* and the production of lipase. In comparison with commercial peptone, a slight improvement in lipase production was obtained when fish media were used. Due to high lipase activity obtained with cheap fish meal, clearly indicated that these substrates could be used in industrial fermentation processes. Hydrolysates generated from yellowfin tuna (*Thunnus albacares*) head waste showed higher effectiveness in promoting the growth of lactic acid bacteria better than the commercial MRS media (Safari *et al.*, 2012).

1.2.6.3 Pharmaceutical and nutraceutical applications

Bioactive peptides from fish origin have been defined as specific protein fragments that may exert regulative activities on body functions, in particular, reducing the risk of disease or enhancing a certain physiological function (Hartmann and Meisel, 2007). Fish protein hydrolysates have been known as a source of biologically active peptides with promising health benefits, in terms of nutritional or pharmaceutical properties

The possible roles of marine-derived bioactive peptides in reducing the risk of cardiovascular diseases by lowering plasma cholesterol level and anti-cancer activity by reducing cell proliferation on human breast cancer cell lines, have been demonstrated (Picot *et al.*, 2006). Hydrolysates obtained from three blue whiting, three cod, three plaice and one salmon showing significant inhibition on two human breast cancer cell lines, MCF-7/6 and MDA-MB-231 (Picot *et al.*, 2006). Hosomi *et al.* (2010) reported the Alaska pollock (*Theragra chalcogramma*) fillet protein hydrolysates decreased the serum and liver cholesterol contents of experimental rats through the

enhancement of faecal acidic and neutral excretions. In addition, marine-derived peptides from *Crassostrea gigas* and *Ruditapes philippinarum*have showed anti-inflammatory effects by inhibiting nitric oxide production in lipopolysaccharide-stimulated RAW264.7 macrophages (Hwang *et al.*, 2012; Lee *et al.*, 2012). Moreover, calcium-binding bioactive peptides derived from pepsin hydrolysates of Alaska pollack (*Theragra chalcogramma*) and hoki (*Johnius belengerii*) frame can be introduced to oriental people with lactose indigestion or intolerance and calcium fortified fruit juices or calcium rich foods as alternatives to dairy products (Jung *et al.*, 2006; Jung *et al.*, 2005). Kim *et al.* (2006) and Jung *et al.* (2005) reported that peptides from fish protein hydrolysate are capable of accelerating calcium absorption.

Hydrolysed gelatin and collagen products have been used in pharmaceuticals and foods. Wu *et al.* (2004) demonstrated the safety of oral ingestion of a high dose (1.66 g/kg of body weight) of collagen hydrolysate in an animal model. Clinical studies suggest that the ingestion of 10 g collagen hydrolysate daily reduces pain in patients with osteoarthritis of knee or hip. The uses of collagen hydrolysate in combination with calcitonin rich diet had the greater effect on inhibiting bone collagen breakdown than calcitonin alone (Moskowitz, 2000). Bello and Oesser (2006) postulated that collagen hydrolysate ingestion stimulates an increase in synthesis of extracellular matrix macromolecules by chondrocytes, compared with untreated controls. In addition, fish skin collagen hydrolysates from salmon and trout have been reported to affect lipid absorption and metabolism in rats (Saito *et al.*, 2009). Besides the lipid-lowering effect, chicken bone collagen hydrolysates have been shown to reduce pro-inflammatory cytokine production in mice (Zhang *et al.*, 2010). Kittiphattanabawon *et al.* (2013) also reported that gelatin hydrolysate from blacktip shark skin prepared using papaya latex enzyme with DH of 40% effectively inhibited DNA oxidation by scavenging hydroxyl and peroxyl radicals. Even though fish protein hydrolysate exhibited many potential benefits, very few commercial products containing its hydrolysate are available as human food. The products with various health promotions from aquatic resources, especially from fish protein hydrolysate, have the good trend in market.

1.3 Objectives

1.3.1 To investigate the impact of freshness and antioxidant incorporation on lipid oxidation and fishy odour development during hydrolysis process of Nile tilapia muscle.

1.3.2 To elucidate the effect of pretreatments on the removal of pro-oxidants and phospholipid membranes from Nile tilapia mince and to monitor lipid oxidation and fishy odour development during hydrolysis process.

1.3.3 To investigate the impact of pretreatments on pro-oxidants and phospholipid membranes removal of Indian mackerel mince and to study lipid oxidation and fishy odour formation during hydrolysis process.

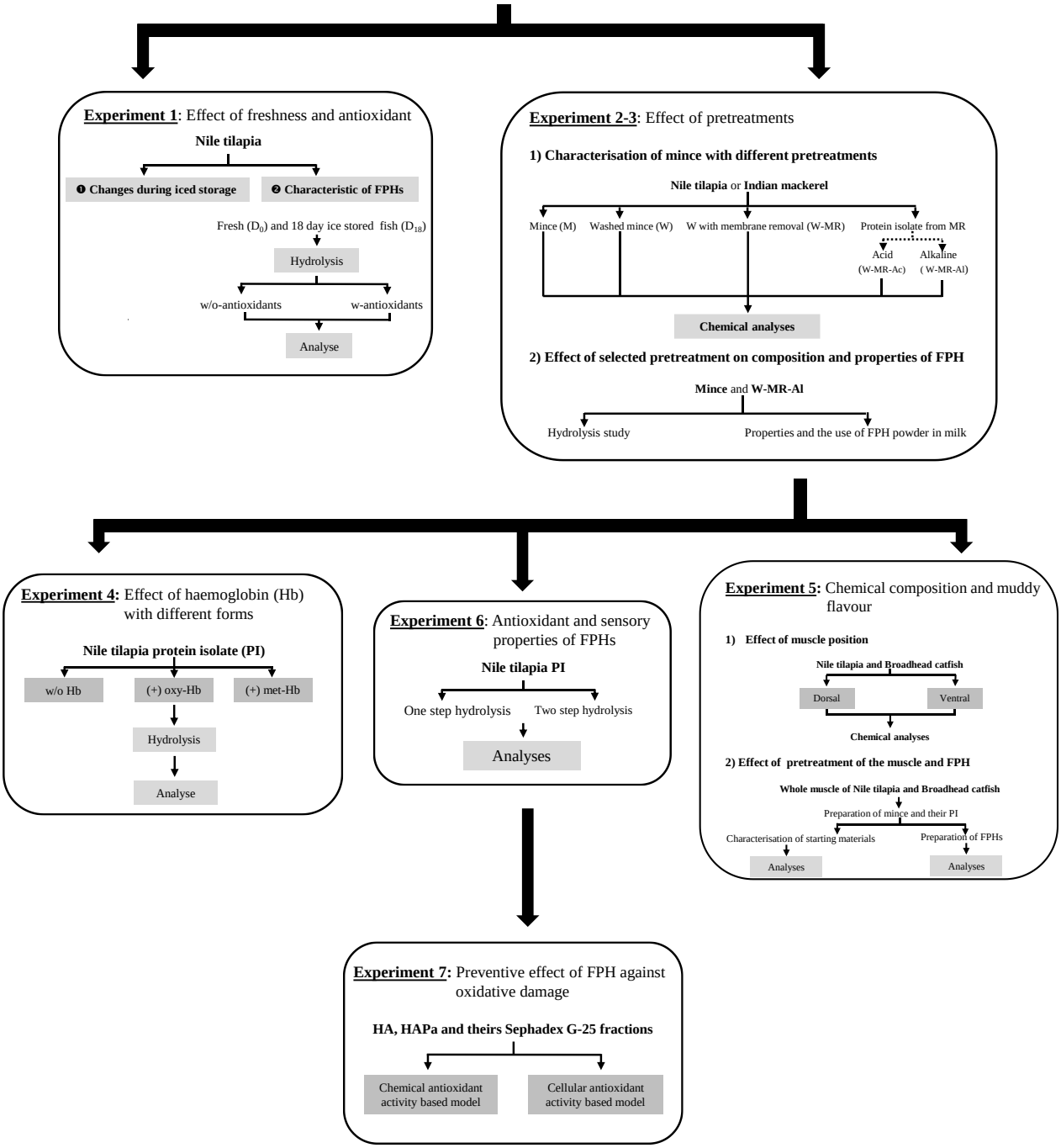
1.3.4 To study the impact of Hb with different forms, oxy-Hb and met-Hb, on lipid oxidation and fishy odour development during hydrolysis of Nile tilapia PI.

1.3.5 To investigate the effect of muscle position and pretreatment on muddy flavour/odour associated with the muscle and protein hydrolysate produced from Nile tilapia and broadhead catfish.

1.3.6 To produce protein hydrolysate from Nile tilapia PI prepared by one-step and two-step hydrolysis using various types of commercial proteases and to investigate their antioxidant activities and sensory characteristic.

1.3.7 To investigate antioxidant properties of Nile tilapia protein hydrolysates and their fractions in both chemical and cellular antioxidant based models.

1.4 Flow chart of experiments



CHAPTER 2

LIPID OXIDATION AND FISHY ODOUR DEVELOPMENT IN PROTEIN HYDROLYSATE FROM NILE TILAPIA (*OREOCHROMIS NILOTICUS*) MUSCLE AS AFFECTED BY FRESHNESS AND ANTIOXIDANTS

2.1 Abstract

Lipid oxidation and fishy odour development in protein hydrolysate from fresh and ice-stored Nile tilapia (*Oreochromis niloticus*) were investigated. During iced storage of 18 days, haem iron content decreased with a concomitant increase in non-haem iron content ($P < 0.05$). Peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) values increased. Phospholipid content decreased with a corresponding increase in free fatty acid content. The results suggested that lipid hydrolysis and oxidation took place during storage. When protein hydrolysates were produced from fresh and 18 days ice-stored Nile tilapia muscle, higher lipid oxidation and fishy odour/flavour along with higher amount volatile compounds were obtained in hydrolysate from unfresh sample ($P < 0.05$). However, the addition of mixed antioxidants during hydrolysis process markedly lowered lipid oxidation, b*, ΔC*, ΔE* values, fishy odour/flavour as well as the formation of volatile compounds in the resulting hydrolysates prepared from both fresh and unfresh samples. Therefore, hydrolysate from Nile tilapia muscle with reduced fishy odour and lighter colour could be prepared by using fresh fish and incorporation of mixed antioxidants during hydrolysis.

2.2 Introduction

Off-odour or off-flavour in fish and fish products generally causes a major reduction in acceptability for consumers or makes them unsuitable for sale (Robin *et al.*, 2006). The compounds associated with off-odour or off-flavour are generated by enzymatic reactions, lipid autoxidation, microbial action and

environmentally or thermally derived reaction (Selli *et al.*, 2009). Amongst these reactions, lipid oxidation is closely related with the alteration of odour and flavour in fish (Maqsood and Benjakul, 2011a). Lipids of fish muscle are prone to oxidation due to the higher content of unsaturated fatty acids than those of mammals and birds (Sohn *et al.*, 2006). A main substrate for lipid oxidation is membrane phospholipids, which are more susceptible to oxidation than the neutral triacylglycerols. This phenomenon can be catalysed by haem protein (Maqsood and Benjakul, 2011a; Thiansilakul *et al.*, 2010), iron (Tokur and Korkmaz, 2007) as well as lipoxygenase (Richards and Hultin, 2002). Recently, fish protein hydrolysates (FPH) have gained increasing attention as a nutritious fish product (Khantaphant *et al.*, 2011a). They can serve as food ingredients and also provide desirable characteristics to food product such as athletic drinks, emulsified meat products, cereal based foods, etc. They can be used for patients with gastrointestinal tract complications (Khantaphant *et al.*, 2011a).

However, during the hydrolysis process, oxidation may take place due to the presence of pro-oxidants such as haem, etc. Additionally, the post-mortem changes of fish are related with deterioration and development of off-flavour in fish muscle (Thiansilakul *et al.*, 2010). Thus, the freshness of fish used for protein hydrolysate production may be another crucial factor affecting the rate of lipid oxidation as well as fishy odour formation in resulting hydrolysates. However, no information regarding the effect of fish quality and the oxidation taking place during hydrolysis associated with off-odour has been reported. The objectives of this study were to investigate the chemical changes in Nile tilapia muscle during iced storage and to determine the role of antioxidants on lipid oxidation and fishy odour development occurring the hydrolysis process.

2.3 Materials and Methods

2.3.1 Chemicals

Alcalase 2.4L (E.C. 3.4.21.62) was obtained from Novozyme (Bagsvaerd, Denmark). Palmitic acid, cupric acetate, 2, 4, 6-trinitrobenzenesulphonic acid (TNBS), bathophenanthroline disulphonic acid, 1,1,3,3-tetramethoxypropane and Trolox were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA).

Trichloroacetic acid, anhydrous sodium sulphate, sodium nitrite, isooctane, ferrous chloride and iron standard solution were obtained from Merck (Darmstadt, Germany). Disodium hydrogen phosphate, sodium dihydrogen phosphate and 2-thiobarbituric acid and cumene hydroperoxide were procured from Fluka (Buchs, Switzerland). Methanol, acetone, chloroform and ammonium thiocyanate were obtained from Lab-Scan (Bangkok, Thailand). All chemicals were of analytical grade.

2.3.2 Fish samples

Live Nile tilapia (*Oreochromis niloticus*) with a weight of 0.8–1.0 kg/fish were purchased from a local market in Hat Yai, Songkhla, Thailand. Fish were stored in ice and transported to the Department of Food Technology, Prince of Songkla University within 30 min. Upon arrival, fish were killed by ice-shocking and stored in ice until use.

2.3.3 Changes of Nile tilapia during iced storage

Whole fish (without evisceration) were washed and kept in ice with a fish/ice ratio of 1:2 (w/w). The fish were placed and distributed uniformly between the layers of ice in insulated boxes at room temperature (28–30 ºC). To maintain the fish/ice ratio, ice was replaced every 2 day. The temperature of fish was 0–2 ºC as measured by the thermocouple throughout the storage of 18 days. Five stored fish were randomly taken every 6 days, pooled and used as the composite sample. Fish samples were washed, eviscerated and filleted. The flesh from dorsal portion was chopped with a knife to uniformity and used for analyses.

2.3.3.1 Measurement of haem and non-haem iron contents

Haem iron content of fish muscle was determined as described by Cheng and Ockerman (2004) with slight modifications. The ground sample (2 g) was mixed with 9 ml of acid acetone (90% acetone, 8% deionised water and 2% HCl, v/v/w). The mixture was macerated with a glass rod and allowed to stand for 1 h at room temperature. The extract was filtered with aWhatman No. 42 filter paper (Whatman International Ltd., Maidstone, UK) and the absorbance was read at 640 nm against an

acid acetone blank using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). Haem iron content was calculated with the factor of 0.00882 μg/μg haematin using the following formula:

$$\text{Haem iron (mg/100 g)} = \text{total pigment (ppm)} \times 0.00882$$

where total pigment (ppm) = $A_{640} \times 680$.

Non-haem iron content of fish muscle was determined according to the method of Schricker *et al.* (1982). The ground sample (1.0 g) was weighed and transferred into a screw cap test tube and 50 μl of 0.39% (w/v) sodium nitrite were added. Four millilitres of 40% trichloroacetic acid and 6 M HCl (ratio of 1:1 (v/v), prepared freshly) were added. The tightly capped tubes were placed in an incubator shaker (W350, Memmert, Schwabach, Germany) at 65 ºC for 22 h and then cooled at room temperature for 2 h. The supernatant (400 μl) was mixed with 2 ml of the non-haem iron colour reagent (prepared freshly). After vortexing using a Vortex-Genie2 mixer (Scientific Industries, Bohemia, NY, USA) and standing for 10 min, the absorbance was measured at 540 nm. The colour reagent was prepared by mixing a 1:20:20 ratio (w/v/v) of: (1) bathophenantroline (0.162 g dissolved in 100 ml of double deionised water with 2 ml thioglycolic acid [96–99%]); (2) double deionized water; (3) saturated sodium acetate solution.

The non-haem iron content was calculated from an iron standard curve. Iron standard solutions (Fe $(NO_3)_2$ in HNO_3), with concentrations ranging from 0 to 5 ppm, were used. The concentration of non-haem iron was expressed as mg/100 g sample.

2.3.3.2. Measurement of peroxide value

Peroxide value (PV) was determined according to the method of Richards and Hultin (2002) with slight modifications. A ground sample (1 g) was homogenised at a speed of 13,500 rpm for 2 min in 11 ml of chloroform/methanol (2:1, v/v) using an IKA Labortechnik homogeniser (Selangor, Malaysia). Homogenate was then filtered using Whatman No. 1 filter paper. Two millilitres of 0.5% NaCl were then added to 7 ml of the filtrate. The mixture was vortexed at a moderate speed for 30 s and

then centrifuged at 3000×g for 3 min at 4 ºC using a refrigerated centrifuge (Beckman Coulter, Palo Alto, CA, USA) to separate the sample into two phases. Twenty-five microlitres of ammonium thiocyanate and 25 µl of iron (II) chloride were added to the mixture. The reaction mixture was allowed to stand for 20 min at room temperature prior to reading the absorbance at 500 nm. The blank was prepared in the same manner, except the distilled water was used instead of ferrous chloride. A standard curve was prepared using cumene hydroperoxide at a concentration range of 0.5–2 ppm. PV was expressed as mg of cumene hydroperoxide/kg sample.

2.3.3.3 Measurement of thiobarbituric acid reactive substances

Thiobarbituric acid-reactive substances (TBARS) were determined as described by Buege and Aust (1978). The ground sample (0.5 g) was homogenised with 2.5 ml of a solution containing 0.375% thiobarbituric acid (w/v), 15% trichloroacetic acid (w/v) and 0.25 M HCl. The mixture was heated in a boiling water bath (95–100 ºC) for 10 min to develop a pink colour, cooled with running tap water and centrifuged at 3600×g at 25 ºC for 20 min. The absorbance of the supernatant was measured at 532 nm. A standard curve was prepared using 1,1,3,3-tetramethoxypropane at the concentrations ranging from 0 to 6 ppm. TBARS value was calculated and expressed as mg malonaldehyde/kg sample.

2.3.3.4 Measurement of free fatty acid (FFA) content

Prior to analysis, lipid was extracted by the Bligh and Dyer method (Bligh and Dyer, 1959). The sample (25 g) was homogenised with 200 ml of a chloroform: methanol: distilled water mixture (50:100:50, v/v/v) at a speed of 9,500 rpm for 2 min at 4 ºC. The homogenate was added with 50 ml of chloroform and homogenised at 9,500 rpm for 1 min. Thereafter, 25 ml of distilled water were added and homogenised at the same speed for 30 s. The homogenate was centrifuged at 3000 ×g at 4 ºC for 15 min and transferred into a separating flask. The chloroform phase was drained off into a 125 ml Erlenmeyer flask containing about 2–5 g of anhydrous sodium sulphate, shaken well, and decanted into a roundbottom flask through Whatman No. 4 filter paper. The solvent was evaporated at 25 ºC using an EYELA rotary evaporator

N-1000 (Tokyo, Japan) and the residual solvent was removed by flushing nitrogen. Free fatty acid content was determined according to the method of Lowry and Tinsley (1976). The lipid sample (0.1 g) was added to 5 ml of isooctane and swirled vigorously to dissolve the sample. The mixture was then treated with 1 ml of 5% (w/v) cupric acetate-pyridine reagent, prepared by dissolving 5 g of the reagent grade cupric acetate in 100 ml of water, filtering and adjusting the pH to 6.0–6.2 using pyridine. The mixture was shaken vigorously for 90 s using a vortex mixer and allowed to stand for 10–20 s. The upper layer was subjected to absorbance measurement at 715 nm. A standard curve was prepared using palmitic acid in isooctane at concentrations ranging from 0 to 10 μmol/ml. FFA content was expressed as g FFA/100 g lipid.

2.3.3.5 Measurement of phospholipid content

Phospholipid content was measured based on the direct spectrophotometric measurement of complex formation between phospholipids and ammonium ferrothiocyanate as described by Stewart (1980). Lipids extracted by the Bligh and Dyer method (20 μl) were dissolved in chloroform to obtain a final volume of 2 ml. One millilitre of thiocyanate reagent (a mixture of 0.10 M ferric chloride hexahydrate and 0.40 M ammoniumthiocyanate) was added. After thorough mixing for 1 min, the lower layer was removed and the absorbance at 488 nm was measured. A standard curve was prepared using phosphatidylcholine (0-50 ppm). The phospholipid content was expressed as mg/100 g lipid.

2.3.4 Lipid oxidation and characteristics of fish protein hydrolysate as influenced by fish freshness and incorporated antioxidants

2.3.4.1 Preparation of Nile tilapia protein hydrolysates

Fresh mince or mince from 18 days ice-stored Nile tilapia (100 g) was mixed with distilled water with a mince/water ratio of 1:4 (w/v). The mixtures were adjusted to pH 8.0 using 2 N NaOH. One half of the protein mixtures (2% w/v) had no added antioxidant, whilst the other had the antioxidants, EDTA and Trolox, added to obtain a final concentration of 2 mM and 100 μM, respectively. The mixtures were homogenised at a speed of 11,000 rpm for 1 min and the homogenates were pre-

incubated at 50 °C for 10 min. The hydrolysis reaction was initiated by adding Alcalase at the amount required (0.91-0.92% w/w protein) to obtain a DH of 30% following the method of Benjakul and Morrissey (1997).

After 2 h of hydrolysis, the reactions were terminated by heating the mixture in boiling water for 10 min. The mixture was then centrifuged at 2,000 ×g at 4 °C for 10 min and the supernatant was collected. The protein hydrolysate was lyophilised using a Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lynge, Denmark). The lyophilised fish protein hydrolysate produced from fresh and 18 days ice-stored Nile tilapia were referred to as 'FPHD$_0$' and 'FPHD$_{18}$', respectively. The obtained hydrolysates were subjected to determination of TBARS and PV as mentioned above. Additional analyses were also performed as outlined below.

2.3.4.2 Colour measurement

The colour of FPHD$_0$ and FPHD$_{18}$ were measured by a colourimeter (ColourFlex, Hunter Lab Reston, VA, USA) and reported in the CIE system. L^*, a^*, b^* and ΔE^* and ΔC^* to indicate lightness, redness/greenness, yellowness/blueness, total difference of colour and the difference in chroma, respectively. The ΔE^* and ΔC^* were calculated as follows:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where ΔL*, Δa* and Δb* are the differentials between colour parameter of the samples and the colour parameter of the white standard (L^*= 93.55, a^*= -0.84, b^*= 0.37)

$$\Delta C^* = C^*_{sample} - C^*_{standard}$$

where $C^* = \sqrt{(a^*)^2 + (b^*)^2}$

2.3.4.3 Sensory evaluation

Sensory evaluation for fishy-odour/flavour in fish protein hydrolysate was conducted using Generic descriptive analysis (Meilgaard *et al*., 2007). The sensory panel (10-15 panellists) was firstly screened for perception of fishy odour/flavour,

familiarity with fish consumption and ability to determine differences between fish protein hydrolysate solution (International Standard ISO 8586-1, 1993; ASTM Special Technical Publication 758, 1981; Meilgaard, 2007).

Eight trained panellists (5 female and 3 male) between the ages of 25-32 were selected. Prior to the evaluation, the panellists were trained three hours a week for totally one month. Panellists were trained with standards for two sessions using a scale of 0 to 5, which anchored 'none' to 'extremely strong' for fishy odour/flavour as per the method of Thiansilakul *et al.* (2010) and Shaviklo *et al.* (2012) with a slight modification. Fish protein hydrolysate produced from Nile tilapia stored in ice for 18 days was prepared as used as a source of samples with fishy odours/flavours. The working standard was prepared by dissolving fish protein hydrolysate in water to obtain concentration of 0, 0.5 and 1% (w/v) representing the score of 0, 2.5 and 5, respectively.

To test the samples, all fish protein hydrolysates (0.75%) were placed in a sealable plastic cup and heated at 60 °C in a temperature controlled water bath for 5 min before serving. The samples were coded with 3-digit random numbers and presented to panellists on a tray in individual booths. Serving orders were completely randomised. The panellists were asked to open the sealable cup and sniff the headspace above the samples for determining fishy odour. To evaluate the fishy flavour, panelists were asked to taste the sample and rinsed their mouth between different samples.

2.3.4.4 Measurement of volatile compound

The volatile compounds in $FPHD_0$ and $FPHD_{18}$, with and without the addition of antioxidants, were determined using solid-phase microextraction gas chromatography mass spectrometry (SPME-GCMS) following the method of Iglesias and Medina (2008) with a slight modification.

2.3.4.4.1 Extraction of volatile compounds by SPME fiber

To extract volatile compounds, 1 g of sample was mixed with 4 ml of deionised water and stirred continuously to dissolve sample. The mixture was heated at 60 °C in 20 ml headspace vial with equilibrium time of 10 h. The SPME fiber (50/30 μm DVB/Carboxen^{TM}/PDMS StableFlex^{TM}) (Supelco, Bellefonte, PA, USA)

was conditioned at 270 °C for 15 min before use and then exposed to the headspace. The 20 ml-vial (Agilent Technologies, Palo Alto, CA, USA) containing the sample extracted and the volatile compounds were allowed to absorb into the SPME fiber at 60 °C for 1 h. The volatile compounds were then desorbed in the GC injector port for 15 min at 270 °C.

2.3.4.4.2 GC-MS analysis

GC-MS analysis was performed in a HP 5890 series II gas chromatography (GC) coupled with HP 5972 mass-selective detector equipped with a splitless injector and coupled with a quadrupole mass detector (Hewlett Packard, Atlanta, GA, USA). Compounds were separated on a HP-Innowax capillary column (Hewlett Packard, Atlanta, GA, USA) (30 m × 0.25 mm ID, with film thickness of 0.25 µm). The GC oven temperature program was: 35 ºC for 3 min, followed by an increase of 3 ºC/min to 70 ºC, then an increase of 10 ºC/min to 200 ºC, and finally an increase of 15 ºC/min to a final temperature of 250 ºC and holding for 10 min. Helium was employed as a carrier gas, with a constant flow of 1 ml/min. The injector was operated in the splitless mode and its temperature was set at 270 ºC. Transfer line temperature was maintained at 260 ºC. The quadrupole mass spectrometer was operated in the electron ionisation (EI) mode and source temperature was set at 250 ºC. Initially, full-scan-mode data were acquired to determine appropriate masses for the later acquisition in scan mode under the following conditions: mass range: 25-500 amu and scan rate: 0.220 s/scan. All the analyses were performed with ionisation energy of 70 eV, filament emission current at 150 µA, and the electron multiplier voltage at 500 V.

2.3.4.4.3 Analyses of volatile compounds

Identification of volatile compounds in the samples was done by consulting ChemStation Library Search (Wiley 275 L). Identification of compound was performed, based on the retention time and mass spectra in comparison with those of standards from Chemstation Library Search (Wiley 275 L). Quantification limits calculated to a signal to noise (S/N) ratio of 10. The identified volatile compounds,

related with lipid oxidation, including aldehydes, alcohols, ketones, etc., were presented in the term of abundance of each identified compound.

2.3.5 Statistical analysis

Experiments were run in triplicate using three lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Ducan's multiple range tests. T-test was used for pair comparison (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

2.4 Results and Discussion

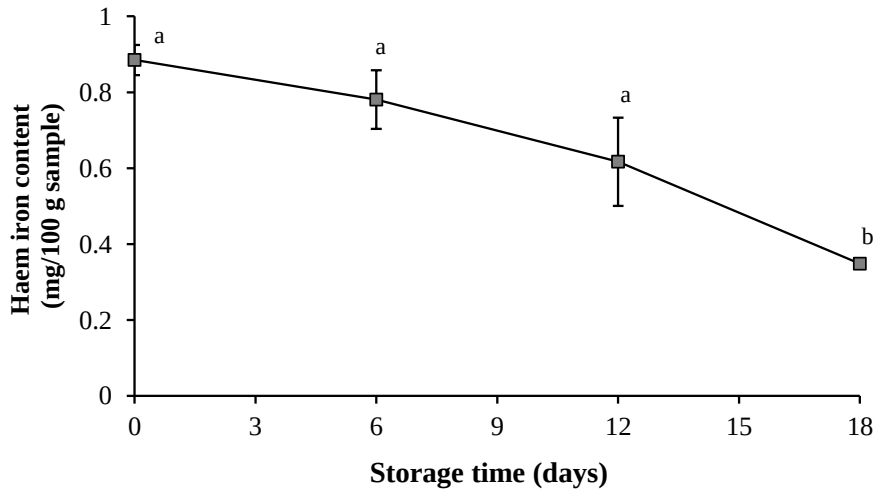
2.4.1 Changes of Nile tilapia muscle during iced storage

2.4.1.1 Haem iron and non-haem iron contents

Changes of haem and non-haem iron contents of Nile tilapia muscle during iced storage are depicted in Figure 8A and 8B, respectively. Haem iron content of Nile tilapia muscle decreased with increasing storage time ($P < 0.05$). The haem iron content decreased from 0.88 to 0.34 mg/100 g sample after 18 days of storage. During storage, the denaturation of haem proteins might occur. In general, haem iron constituted 25–44% of the total iron in fish (Benjakul and Bauer, 2001). Haem iron content is governed by fish species, processing involved as well as post-mortem handling or storage time (Thiansilakul *et al.*, 2010). Benjakul and Bauer (2001) reported that haem iron content in catfish (*Silurus glanis Linne*) fillet was 0.72 mg/100 g and decreased to 0.5–0.6 mg/100 g after 3 days of frozen storage. Decker and Hultin (1990) reported that the deterioration of subcellular organelles, e.g. mitochondria and the release cytochrome c, could be responsible for the increase in soluble haematin.

Non-haem iron contents in Nile tilapia muscle during iced storage are shown in Figure 8B. No changes in non-haem iron content were observed within the first 12 days of iced storage ($P > 0.05$). The increase in non-haem iron content with extended storage time was in agreement with the decrease in haem iron content (Figure

(A)



(B)

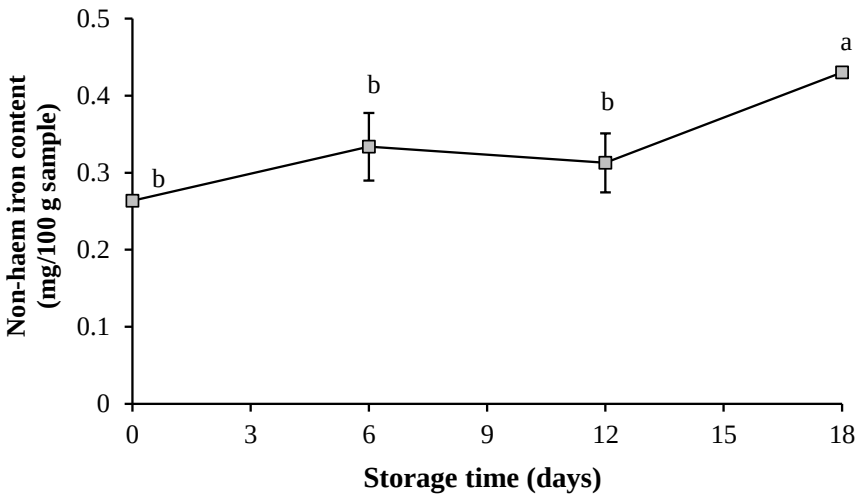


Figure 8. Changes in haem iron (A) and non-haem iron (B) contents of Nile tilapia muscle during iced storage. Different letters indicate significant differences ($P < 0.05$). Bars represent the standard deviations ($n = 3$).

8A). This result suggested that the disruption of porphyrin ring more likely occurred during extended storage. As a result, the release of free iron became more pronounced. Haem pigment or other iron-containing proteins were possibly denatured with increasing storage time, resulting in the release of iron (Decker and Hultin, 1990).

Additionally, a greater release of free iron was related with the degradation of fish muscle (Chaijan *et al.*, 2005). The damage of the globin molecule was suggested as the cause of the instability of haem molecule with the subsequent release of iron. Non-haem iron has been reported to act as pro-oxidant in the fish muscle (Thiansilakul *et al.*, 2010). Metal ions, especially Fe^{3+} and Fe^{2+}, exhibit their pro-oxidative activity in the muscle system and contribute to the accelerated lipid oxidation (Chaijan *et al.*, 2005). Thus, fish stored for an extended time contained a higher content of non-haem iron, which more likely plays a role in acceleration of lipid oxidation in muscle.

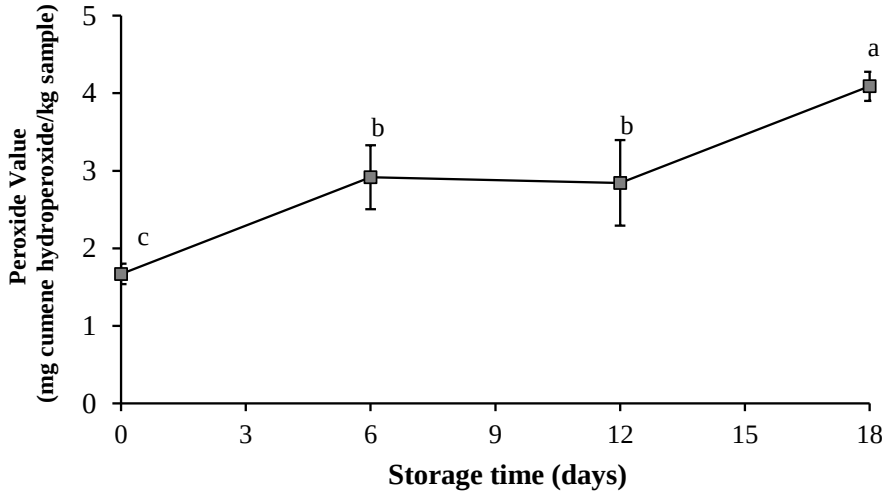
2.4.1.2 PV and TBARS

Lipid oxidation of Nile tilapia muscle during iced storage was monitored by measuring PV and TBARS values (Figure 9). Primary oxidation was monitored by the changes in PV as shown in Figure 9A. PV of fresh sample was 1.67 mg hydroperoxide/kg, suggesting that lipid oxidation occurred during postmortem. handling to some extent. Continuous increase in PV was observed throughout iced storage of 18 days ($P < 0.05$). Oxidation of unsaturated fatty acids takes place in the presence of pro-oxidants like haem proteins during storage. In fish, especially in lean fish, the phospholipid membrane is believed to be the key substrate for lipid oxidation due to its highly unsaturated fatty acid composition (Undeland *et al.*, 1998). Pacheco-Aguilar *et al.* (2000) reported that peroxide formation of oily Monterey sardine increased up to 15 days of storage at 0 ºC. Nevertheless, the slight decreases in PV were found in seabass and red tilapia at the end of iced storage (day 15), plausibly due to the decomposition of hydroperoxide to other compounds. The increase in PV correlated with the increased amount of non-haem iron (Figure 8B), which more likely acted as a pro-oxidant in fish muscle. Haem dissociation, haem destruction and iron release play a role in the acceleration of lipid oxidation.

The changes in TBARS values of Nile tilapia muscle during iced storage are shown in Figure 9B. TBARS values of Nile tilapia increased throughout storage. The marked increase in TBARS was probably due to the decomposition of hydroperoxides into secondary oxidation products, especially aldehydes, in the later, stages of lipid oxidation. Increases in TBARS formation in sardine anchovies, Atlantic bonito and bluefish were caused by iron catalysed oxidation (Tokur and Korkmaz,

2007). Undeland *et al.* (1998) reported that the abundance of haemoproteins and free metals in the dark muscle and under the skin layer favoured and promoted lipid oxidation in the herring minced fillets. Thus, the lipid oxidation became more pronounced in Nile tilapia when storage time increased. This contributed to the deterioration and unacceptability of Nile tilapia meat.

(A)



(B)

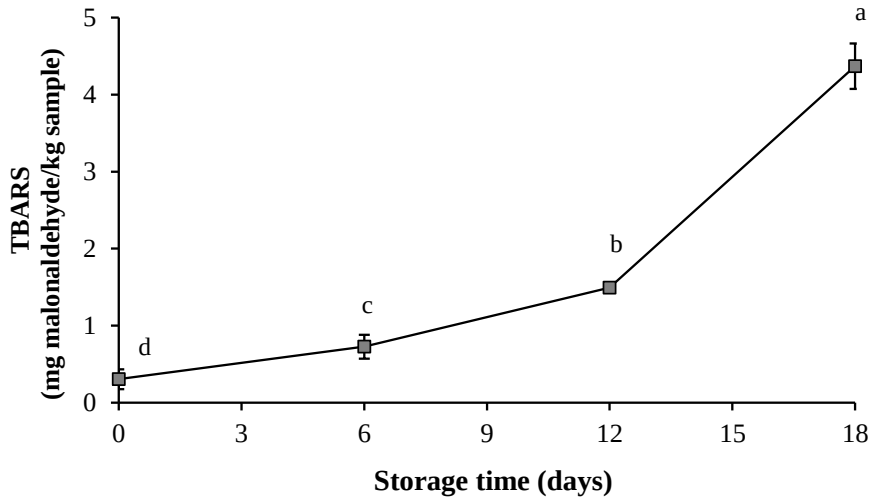


Figure 9. Changes in peroxide value (A) and TBARS value (B) of Nile tilapia muscle during iced storage. Different letters indicate significant differences ($P < 0.05$). Bars represent the standard deviations ($n = 3$).

2.4.1.3 Phospholipid and free fatty acid (FFA) contents

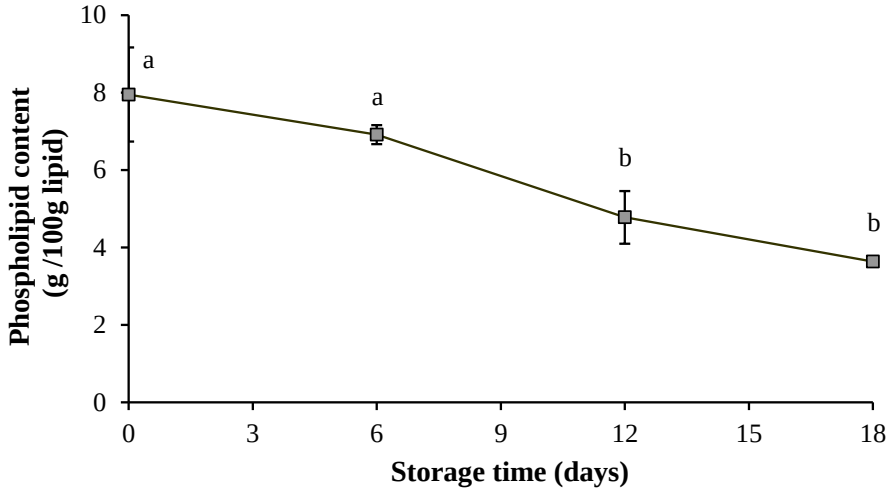
Changes in phospholipid and FFA contents in Nile tilapia muscle during iced storage are depicted in Figure 10. No changes in phospholipid content were observed within the first 6 days of iced storage ($P > 0.05$). Subsequently, a gradual decrease was found up to day 18 of iced storage ($P < 0.05$). At the end of the storage (day 18), phospholipid content decreased by 54.6%, when compared with fresh Nile tilapia muscle (day 0). On the other hand, FFA increased with increasing storage time ($P < 0.05$). The result indicated that hydrolysis of lipids occurred to a great extent at the end of the storage. Hydrolysis of glycerol-fatty acid esters is one important change that occurs in fish muscle lipids during post-mortem storage. This is catalysed by lipase and phospholipase (Pacheco-Aguilar *et al.*, 2000). Since the whole fish were stored ungutted in ice, lipases or phospholipase from internal organs might be released into muscle, where lipids were localised. Kolakowska (2002) also found active phospholipase in fish pyloric caeca. Phospholipids in sardine muscle were decreased to a higher extent, compared with triglyceride. This was possibly due to the higher phospholipase activity than lipase activity in sardine muscle (Chaijan *et al.*, 2006). Released free fatty acids are more prone to oxidation, compared to those esterified with a glycerol backbone. This coincided with the increased lipid oxidation as evidenced by the increases in PV and TBARS value in Nile tilapia stored in ice for extended time (Figure 9).

2.4.2 Lipid oxidation and characteristic of fish protein hydrolysate as influenced by fish freshness and antioxidants incorporated

2.4.2.1 Lipid oxidation in fish protein hydrolysate

PV and TBARS values of fish protein hydrolysate powder produced from fresh and 18 days ice-stored Nile tilapia in the presence and absence of mixed antioxidants (100 μM Trolox and 2 mM EDTA) are shown in Table 5. PV and TBARS values of fish protein hydrolysate powders from fresh muscle ($FPHD_0$) were 33.93 mg hydroperoxide/kg dry sample and 80.21 mg MDA/kg dry sample, respectively. Higher

(A)



(B)

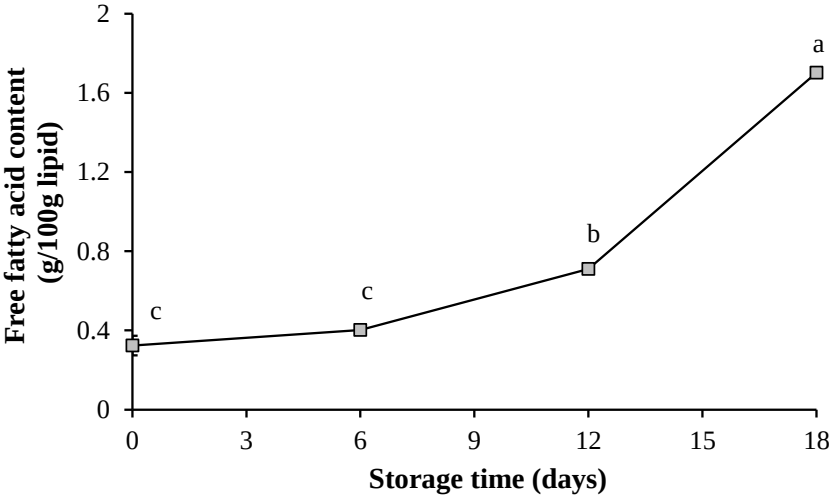


Figure 10. Changes in phospholipid (A) and free fatty acid contents (B) of Nile tilapia muscle during iced storage. Different letters indicate significant differences ($P < 0.05$). Bars represent the standard deviations ($n = 3$).

PV and TBARS values were found in fish protein hydrolysate powder ($FPHD_{18}$) when 18 day old fish was used as the raw material ($P < 0.05$). During extended storage, lipid hydrolysis along with the oxidation of haem protein and the release of free iron or other pro-oxidants might favour lipid oxidation during hydrolysis. Lipids and free fatty acids in fish mince likely underwent oxidation during hydrolysis at 50 ºC. Higher temperature is known to accelerate lipid oxidation. Pretreatment of mince, especially phospholipid

membrane separation, could decrease pro-oxidants and phospholipids in the hydrolysate from brownstripe red snapper (Khantaphant *et al.*, 2011a).

When antioxidants (Trolox and EDTA) were incorporated during hydrolysis, PV was decreased by 83.9% and 70.4% when fresh and 18 days in ice-stored fish were used, respectively. TBARS values were also decreased by 99.2% and 98.5%, respectively. This suggested that both Trolox and EDTA effectively prevented lipid oxidation of fish muscle during hydrolysis. Trolox and EDTA are well known as effective antioxidants for preventing rancidity of many lipid systems, in particular fish oils and fish muscle or surimi (Chaijan *et al.*, 2005). Trolox, a water soluble tocopherol, behaves like a chain-breaking electron donor by competing with the substrate for the chain-carrying peroxyl radicals (Georgantelis *et al.*, 2007) and is able to retard the decomposition of hydroperoxides (Jeong-Ho Sohn *et al.*, 2005). Strong antioxidant effects of Trolox was found in yellowtail dark muscle during iced storage (Jeong-Ho Sohn *et al.*, 2005). Verma and Sahoo (2000) reported that the addition of 10 ppm a-tocopherol acetate could inhibit lipid oxidation in ground chevon meat during refrigerated storage. Haem and non-haem irons have been reported to accelerate lipid oxidation of fish, pig and chicken meat (Jittrepotch *et al.*, 2006). EDTA, a metal chelator, has been known to suppress the non-haem iron catalysed lipid oxidation (Wang and Regenstein, 2009). EDTA is able to chelate metal ions to form 1:1 metal: EDTA complexes (Wang and Regenstein, 2009). Igene *et al.* (1979) reported that the addition of 2% EDTA effectively chelated the non- haem iron and significantly reduced lipid oxidation in cooked beef and chicken meat. The incorporation of both Trolox and EDTA in the fish homogenate could therefore prevent oxidation of lipids during hydrolysis via radical scavenging and metal chelation, respectively. As a consequence, reduced lipid oxidation was achieved.

2.4.2.2 Colour of fish protein hydrolysate

L^*, a^*, b^*, $\Delta E^*$and $\Delta C^*$of fish protein hydrolysate powder from fresh and 18 days ice-stored Nile tilapia in the absence and presence of mixed antioxidants are presented in Table 5. All fish protein hydrolysate powders were light yellow in colour. Myoglobin and haemoglobin, which are responsible for colour characteristics of fish flesh, remained in the fish protein hydrolysate and the oxidation of these proteins might

Table 5. TBARS, PV and colour of fish protein hydrolysate powders produced from fresh ($FPHD_0$) and 18 days ice-stored ($FPHD_{18}$) Nile tilapia muscle in the absence and presence of mixed antioxidants

Parameters	$FPHD_0$		$FPHD_{18}$	
	Without antioxidants	With antioxidants	Without antioxidants	With antioxidants
Peroxide value (mg hydroperoxide /kg dry sample)	33.93±15.10aA[*,**]	5.45±2.90bA	114.02±45.76aB	33.71±13.18bB
TBARS value (mg MDA/kg dry sample)	80.21±9.80aA	0.67±0.13bA	121.35±5.87aB	1.84±0.35bB
L*	94.57±0.17aA	94.20±0.54aA	93.29±0.21aB	92.40±0.22bB
a*	-1.04±0.09bA	-1.18±0.05aA	-1.16±0.11aA	-1.20±0.08aA
b*	7.80±0.56aA	5.62±0.30bA	6.60±0.24aB	5.53±0.77bA
ΔE*	7.59±0.53aA	5.32±0.41bA	6.12±0.24aB	5.05±0.77bA
ΔC*	6.61±0.50aA	4.40±0.29bA	5.36±0.24aB	4.32±0.77bA

Values are given as mean ± SD (n=3)

[*] Different letters within the same row under the same freshness of raw material indicate significant differences ($P<0.05$).

[**] Different capital letters within the same row under same condition of antioxidants incorporation indicate significant differences ($P < 0.05$).

cause a yellow-brownish colour in resulting hydrolysate (Venugopal and Shahidi, 1996). Generally, protein hydrolysate powders from fresh fish had higher L* (lightness), ΔE* (total colour difference) and ΔC* (colour intensity difference) than those produced from 18 days ice-stored fish. A high b* value was found in the powder produced from fresh fish, but no difference between powders from fresh and ice stored fish was found when the mixed antioxidants was incorporated ($P > 0.05$). However, no differences in a* (redness) value of all powders were observed ($P > 0.05$). During extended storage, the dark brown colour was developed in herring hydorolysates as indicated by a* decrease in lightness with coincidental increase in b*-value (Hoyle and Merritt, 1994). For the protein hydrolysate prepared from the same raw material, the decreases in all parameters, except for a* value, were obtained when mixed antioxidants were added during hydrolysis ($P < 0.05$). Lipid oxidation products, especially aldehyde compounds, were more likely involved in a yellowish discolouration, via the Maillard reaction. The result was in accordance with the lower PV and TBARS formation in hydrolysates prepared in the presence of mixed antioxidants. Lipid oxidation products played a role in yellow discolouration of fish muscle product, mainly by providing carbonyl groups involved in the Maillard reaction (Khantaphant *et al.*, 2011a). The result indicated that the incorporation of both Trolox and EDTA during hydrolysis process had a synergistic effect on both lowering lipid oxidation and improving colour of the protein hydrolysates.

2.4.2.3 Fishy odour and flavour of fish protein hydrolysate

Although numerous fish protein hydrolysates have been shown to have bioactivity, such as antioxidative activity (Khantaphant *et al.*, 2011a; Thiansilakul *et al.*, 2007b), one of the limiting factors for introducing fish protein hydrolysates or derivatives into foodstuffs is their fishy odour and flavour. Stronger fishy odour and flavour were found in fish protein hydrolysate powder produced from 18 days ice-stored Nile tilapia ($FPHD_{18}$), compared with that prepared from fresh sample (Table 6). Stored fish therefore gave hydrolysates with a stronger fishy odour/flavour, which was correlated with higher lipid oxidation (Table 5). The formation of secondary lipid oxidation products is one of the main causes of the development of undesirable odours in fish muscle, especially fishy odour (Thiansilakul *et al.*, 2010). Khantaphant (2010)

reported that the fortification of soy bean milk with brownstripe red snapper protein hydrolysate resulted in fishy flavour in the product.

When antioxidants were incorporated, fishy odour and flavour intensities were markedly decreased for both $FPHD_0$ and $FPHD_{18}$ ($P < 0.05$) (Table 6). This was in accordance with the lower TBARS and PV in hydrolysate (Table 5). It was noted that there was no significant difference in fishy odour/flavour intensities between both fresh and unfresh samples when the mixed antioxidants were incorporated. This was postulated that the mixed antioxidant was effective to inhibit lipid oxidation associated with fishy odour development, even when unfresh fish was used. Nam and Ahn (2003) reported that the addition of mixed antioxidants including sesamol, trolox and gallate in pork homogenates could reduce the production of rancid volatiles induced by irradiation. Wiener sausages containing antioxidant, particularly whey powder or rosemary extracts, had improved sensory scores, compared to the sample without antioxidants (Coronado *et al.*, 2002). The result revealed that the addition of Trolox and EDTA during hydrolysis was a promising means to minimise fishy odour/flavour in resulting hydrolysate.

Table 6. Fishy odour and flavour of fish protein hydrolysate powder produced from fresh and 18 days ice-stored Nile tilapia muscle

Samples	Treatments	Fishy	
		Odour	Flavour
$FPHD_0$	Without antioxidants	3.05±0.09aB[*,**]	3.10±0.10aB
	With antioxidants	2.40±0.20bA	2.26±0.46bA
$FPHD_{18}$	Without antioxidants	4.00±0.71aA	4.16±0.80aA
	With antioxidants	2.50±0.35bA	2.75±0.25bA

Values are given as mean ± SD, evaluated by 8 trained panellists.

Score are based on a 6-point scale (0 = none and 5 = extremely strong).

[*]Different letters within the same column under the same freshness of raw material indicate significant differences ($P < 0.05$).

[**]Different capital letters within the same column under the same condition of antioxidants incorporated indicate significant differences ($P < 0.05$).

2.4.2.4 Volatile compounds in fish protein hydrolysate

Selected volatile compounds in fish protein hydrolysate powder produced from fresh and 18-days ice-stored Nile tilapia without and with antioxidant addition are presented in Table 7. Lipid oxidation of fish muscle is known to produce volatile aldehydic compounds including hexanal, heptanal, octanal, nonanal, etc. (Maqsood and Benjakul, 2011a). In the absence of mixed antioxidants, $FPHD_0$ contained several volatile compounds including furan (2-ethylfuran, 2-amylfuran and cis-2-(1-pentenyl)furan), aldehyde (hexanal and nonanal), ketone (2,6-di(*t*-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one), alcohol (1-octen-3-ol, 2-octen-1-ol and E,E-2,4-octadien-1-ol) and other volatile substances (2,3-octanediene). It was noted that $FPHD_{18}$ contained higher abundance of all volatile compounds. Furthermore, a new volatile substance (1,3-cyclooctadiene) was generated when fish stored in ice for 18 day was used for protein hydrolysate production. It was postulated that higher lipid oxidation and greater decomposition of hydroperoxide occurred in hydrolysate prepared from ice stored fish, compared with that produced from fresh sample. Among aldehydes, which are known to be the most predominant volatiles produced during lipid oxidation, heptanal has been recognised as a reliable indicator of flavour deterioration for fish products, whilst hexanal contributes to the rancidity in meats (Maqsood and Benjakul, 2011). Carbonyl compounds involving 4-heptenal, octanal, decanal and 2,4-decadienal were responsible for fishy odour in salmon fish (Varlet *et al.*, 2006). Fu *et al.* (2009) reported that 2,4-heptadienal contributed to fishy odour as affected by lipoxygenase, whilst hexane and nonanal were responsible for oxidised oil odour as catalyzed by haemoglobin in a silver carp mince model system. Alcohols and ketones are the secondary products produced by the decomposition of hydroperoxide (Maqsood and Benjakul, 2011a). Furans are formed by the decomposition of 12-hydroperoxide of linolenate (18:3n-3), the 14-hydroperoxide of eicosapentaenoate (20:5n-3) and the 16-hydroperoxide of docosahexaenoate (22:6n-3), which can undergo β-cleavage to produce a conjugated diene radical, which can react with oxygen to produce a vinyl hydroperoxide. Cleavage of the vinyl hydroperoxide by loss of a hydroxyl radical leads to the formation of alkoxyl radical, that undergoes cyclisation and produces furan (Maqsood and Benjakul, 2011a). However, the development of volatiles was

significantly reduced as the mixed antioxidants were incorporated during hydrolysis. The result confirmed that both of Trolox and EDTA were effective in retarding the lipid oxidation, thereby preventing the formation of volatile lipid oxidation compounds, which contributed to fishy odour and flavour in Nile tilapia protein hydrolysate.

Table 7. Volatile compounds in fish protein hydrolysate powder produced from fresh and 18 days ice-stored Nile tilapia muscle in the absence and presence of mixed antioxidants

Compounds	Abundance			
	$FPHD_0$		$FPHD_{18}$	
	Without antioxidants	With antioxidants	Without antioxidants	With antioxidants
2-Ethylfuran	580,000	ND	720,000	ND
Hexanal	500,000	ND	500,000	ND
2-amylfuran	1,200,000	520,000	1,220,000	ND
Cis-2-(1-pentenyl)furan	920,000	580,000	1, 020,0000	580,000
2,3 -octanediene	70,000	ND	75,000	62,000
Nonanal	1,002,000	68,000	1,020,000	90,000
1-octen-3-ol	1,800,000	ND	1,700,000	ND
2-octen-1-ol	85,000	ND	95,000	60,000
E,E-2,4-octadien-1-ol	65,000	75,000	75,000	62,000
1,3-cyclooctadiene	ND	ND	70,000	100,000
2,6-di (t-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one	60,000	50,000	ND	ND

ND: Not detectable

2.5 Conclusion

Freshness of Nile tilapia used as raw material for protein hydrolysate had an influence on lipid oxidation and fishy odour or flavour of resulting hydrolysate. The results show that fresh fish was the most appropriate for production of hydrolysate with reduced fishy odour/flavour. Incorporation of the antioxidants, Trolox and EDTA in combination effectively prevented lipid oxidation and the development of fishy odour/flavour in hydrolysate. Therefore, use of fresh fish along with antioxidant incorporation is recommended for production of fish protein hydrolysate with high acceptability.

CHAPTER 3

EFFECT OF PRETREATMENTS ON CHEMICAL COMPOSITIONS OF MINCE FROM NILE TILAPIA (*OREOCHROMIS NILOTICUS*) AND FISHY ODOUR DEVELOPMENT IN PROTEIN HYDROLYSATE

3.1 Abstract

Fish protein hydrolysates (FPH) have gained increasing attention as nutritious fish products. Lipid oxidation associated with fishy odour in FPH limits its utility. Thus, an appropriate pretreatment of fish mince prior to hydrolysis by lowering pro-oxidants and lipid substrates could tackle such a problem. Different pretreatments of Nile tilapia minces including (1) washing (W), (2) washing and membrane removal (W-MR), and (3) washing/membrane removal followed by acid or alkaline solubilisation (W-MR-Ac or W-MR-Al) were conducted prior to hydrolysis. During the hydrolysis process, degree of hydrolysis (DH) and chemical changes were monitored. Colour and sensory properties of milk fortified with hydrolysates prepared from mince without and with pretreatment were also determined. Amongst the pretreated mince samples, W-MR-Al contained the lowest remaining myoglobin and haem iron contents and also showed the lowest total lipid and phospholipid contents ($P < 0.05$). When mince and W-MR-Al were hydrolysed using Alcalase for up to 120 min, higher DH were found in W-MR-Al. Furthermore, lower peroxide values, thiobarbituric acid reactive substances, and non-haem iron contents of hydrolysates from W-MR-Al were also observed ($P < 0.05$). When FPH powder from mince and W-MR-Al (0.3% to 0.5%) were fortified in milk, the former yielded a lower likeness score ($P < 0.05$) at all levels used. The addition of the latter up to 0.5% had no effect on likeness of all attributes, compared with control (without FPH). An appropriate pretreatment of mince was a promising approach to lower fishy odour problem, caused by lipid oxidation in FPH.

3.2 Introduction

Lipid oxidation is a great concern to the food industry because it can cause an adverse effect on flavour, odour, texture, and nutritional value of muscle-based

foods (Liang and Hultin, 2005a). Even though lean fish are traditionally used for fish processing with a consistent high quality owing to their negligible lipid contents and haem pigments, lipid oxidation still takes place in lean fish and their products. Phospholipid membranes with a high content of unsaturated fatty acids are believed to be the key substrate for lipid oxidation (Liang and Hultin, 2005b) In addition, membranes also have a large surface area and thus to contact with pro-oxidants in the aqueous phase around membranes (Liang and Hultin, 2005a). Moreover, haem proteins in the raw material can also become oxidised, thereby promoting lipid oxidation and development of unpleasant odour/flavour (Raghavan *et al.*, 2008)

Nile tilapia is popular in Southeast Asia and other counties like China, India, etc., owing to its white flesh and delicacy. In general, it is sold as whole fish or as fillets. To add more value, hydrolysis processes have been developed to convert fish protein into the potent bioactive peptides. Nevertheless, one problem connected to protein hydrolysate from fish flesh is the presence of pro-oxidants such as haem proteins and unstable lipid substrates (Raghavan and Kristinsson, 2008). Oxidation of lipids is associated with fishy odour development which can make hydrolysates unsuitable for use as food supplements. From a previous work (chapter 2), fishy smell in protein hydrolysate from Nile tilapia mince was mainly caused by lipid oxidation, especially when fish which were not fresh were used. To tackle such a problem, pretreatments of mince prior to enzymatic hydrolysis, in which both pro-oxidants and lipids are removed or reduced, are required Protein isolate with a low amount of undesirable compounds has been successfully prepared (Halldórsdóttir *et al.*, 2011; Raghavan *et al.*, 2008). In brief, fish muscle proteins (myofibrillar and sarcoplasmic proteins) are solubilised at acidic (pH 2.0 to 3.5) or alkaline (pH 10.5 to 11.5) and undesirable contaminants are separated from soluble proteins by centrifugation. Subsequently, soluble proteins are precipitated at their isoelectric points (pH approximately 5.5) (Hultin and Kelleher, 2000; Kristinsson *et al.*, 2005b; Liang and Hultin, 2005a). Hydrolysates from brownstripe red snapper protein isolate showed negligible fishy odour, compared with that prepared directly from fish mince (Khantaphant *et al.*, 2011a). As a consequence, a wider range of applications for FPH can be achieved. Since the protein hydrolysate from Nile tilapia mince had the low acceptability associated with fishy odour development, the appropriate pretreatment,

which was able to remove lipids effectively prior to hydrolysate preparation, could be a promising means to tackle such a problem. However, no information regarding the pretreatments of mince from Nile tilapia and their effect on fishy odour in the resulting protein hydrolysate has been reported. The objectives of this study were to elucidate the effect of pretreatments on the removal of pro-oxidants and phospholipid membranes from Nile tilapia mince and to monitor lipid oxidation and fishy odour development during a hydrolysis process.

3.3 Materials and methods

3.3.1 Chemicals

Alcalase 2.4 L (E.C. 3.4.21.62) was obtained from Novozyme (Bagsvaerd, Denmark). 2,4,6-trinitrobenzenesulfonic acid, bathophenanthroline disulphonic acid, sodium dodecyl sulphate (SDS) and 1,1,3,3-tetramethoxypropane were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid, sodium nitrite, ferrous chloride, and iron standard solution were obtained from Merck (Darmstadt, Germany). Disodium hydrogen phosphate, sodium dihydrogen phosphate and 2-thiobarbituric acid and cumene hydroperoxide were procured from Fluka (Buchs, Switzerland). Methanol, acetone, chloroform, and ammonium thiocyanate were obtained from Lab-Scan (Bangkok, Thailand). All chemicals were of analytical grade.

3.3.2 Fish samples

Fresh Nile tilapia (*Oreochromis niloticus)* with a weight of 0.5 to 0.8 kg/fish were purchased from a local market in Hat Yai, Songkhla province, Thailand. Fish were transported in ice with a fish/ice ratio of 1:2 (w/w) to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand within 30 min.

3.3.3 Preparation of mince with different pretreatments

3.3.3.1 Preparation of mince

Whole fish were washed and flesh was separated manually. Flesh was minced to uniformity using Moulinex AY46 blender (Group SEB, Lyon, France) in a walk-in-cold room (4 °C). The mince obtained was placed in polyethylene bags and kept in ice not longer than 2 h before use.

3.3.3.2 Preparation of washed mince

Mince was homogenised with five volumes of cold distilled water (2 to 4 °C) using an IKA Labortechnik homogeniser (Selangor, Malaysia) at a speed of 11,000 rpm for 2 min. The homogenate (4 °C) was stirred for 15 min, followed by centrifugation at 9,600 ×g for 10 min at 4 °C using a Beckman Coulter centrifuge Model Avant J-E (Beckman Coulter, Inc., Fullerton, CA, USA). The washing process was repeated twice. The sample obtained was referred to as ‘washed mince, (W)’.

3.3.3.3 Preparation of mince with membrane removal

Prior to membrane separation, the mince was subjected to washing as previously described. Membranes were then removed from washed mince by treatment with Ca^{2+} and citric acid according to the method of Liang and Hultin (2005a) with a slight modification. Washed mince sample was homogenised with nine volumes of cold solution including 8 mM $CaCl_2$ and 5 mM citric acid, using a homogeniser at a speed of 11,000 rpm for 2 min. After continuous stirring for 60 min (4 °C), the sample was centrifuged at 4,000 × g for 15 min at 4 °C, and the mince obtained was referred to as ‘washed mince with membrane removal (W-MR)’.

3.3.3.4 Preparation of protein isolate with membrane removal

To prepare the protein isolate, acid and alkaline solubilisation processes were used as described by Rahavan and Hultin (2009) with a slight modification. The W-MR sample was homogenised with nine volumes of cold distilled water (2 to 4 °C) at a speed of 11,000 rpm for 1 min. The homogenate was adjusted to pH either 3.0 or

11.0. The mixtures were kept on ice for 60 min. Homogenates were then centrifuged at 5,000 ×g for 10 min at 4 °C. Acid and alkaline soluble fractions were collected and adjusted to pH 5.5 to precipitate the myofibrillar proteins. The mixture was then centrifuged at 10,000 ×g for 20 min. The pellet obtained was reerred to as ‘acid and alkaline solubilised protein isolate with membrane removal followed by acid or alkaline solubilisation (W-MR-Ac and W-MR-Al), respectively.

3.3.4 Analyse

All prepared mince samples were subjected to following analyses:

3.3.4.1 Determination of pH

All samples were subjected to pH measurement as described by Benjakul and Morrissey (1997). The samples were homogenised with ten volumes of deionised water (w/v) at a speed of 11,000 rpm for 1 min. pH of homogenate was measured using a Model Docu-pH Meter (Sartorius AG, Gottingen, Germany).

3.3.4.2 Determination of myoglobin content

Myoglobin content was determined by a direct spectrophotometric measurement (Chaijan *et al.*, 2005). Sample (2.0 g) was mixed with 20 ml of cold 40 mM phosphate buffer (pH 6.8), and the mixture was homogenised at 13,500 rpm for 10 s. The mixture was centrifuged at 3,000 ×g for 30 min at 4 °C, and the supernatant was filtered through a Whatman filter paper No.1 (Whatman International Ltd., Maidstone, England). The absorbance of the supernatant was read at 525 nm using a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). Myoglobin content was calculated from the molar extinction coefficient of 7.6×10^{-3} and a molecular weight of 16,110 dalton (Gomez-Basauri and Regenstein, 1992). The myoglobin content was expressed as mg/100 g dry sample.

3.3.4.3 Determination of haem and non-haem iron content

Haem iron content was determined as per the method of Cheng and Ockerman (2004) with a slight modification. To a ground sample (2 g), 9 ml of acid

acetone (90% acetone, 8% deionised water, and 2% HCl, v/v/w), were added. The mixture was macerated with a glass rod and allowed to stand for 1 h at room temperature. The extract was filtered with a Whatman No. 42 filter paper (Whatman International Ltd., Maidstone, UK), and the absorbance was read at 640 nm against an acid acetone using a spectrophotometer. Haem iron content was calculated with the factor of 0.00882 μg/μg hematin using the following formula:

Haem iron (mg/100 g dry sample) = total pigment (ppm) × 0.00882

where total pigment (ppm) $=A_{640} \times 680$.

Non-haem iron content was determined according to the method of Schricker et al. (1982). The ground sample (1.0 g) was transferred into a screw cap test tube and 50 μl of 0.39% (w/v) sodium nitrite were added. Thereafter, 4 ml of 40% trichloroacetic acid and 6 M HCl (ratio of 1:1 (v/v), prepared freshly) were added. The tightly capped tubes were placed in an incubator shaker (W350, Memmert, Schwabach, Germany) at 65 °C for 22 h and then cooled at room temperature for 2 h. The supernatant (400 μl) was mixed with 2 ml of the non-haem iron colour reagent (prepared freshly). After vortexing using a Vortex-Genie2 mixer (Scientific Industries, Bohemia, NY, USA) and standing for 10 min, the absorbance was read at 540 nm. The colour reagent was prepared by mixing a 1:20:20 ratio (w/v/v) of: (1) bathophenantroline (0.162 g dissolved in 100 ml of double deionised water with 2 ml thioglycolic acid); (2) double deionised water; (3) saturated sodium acetate solution.

The non-haem iron content was calculated from an iron standard curve. The iron standard solutions ($Fe(NO_3)_2$ in HNO_3) with concentrations ranging from 0 to 5 ppm were used. The concentration of non-haem iron was expressed as mg/100 g dry sample.

3.3.4.4 Determination of lipid content

Lipid content was determined by a Soxhlet apparatus according to the method 920.39B of AOAC (2000). Lipid content was expressed as g/100 g dry sample.

3.3.4.5 Determination of phospholipid content

The phospholipid content was determined by measuring phosphorus according to the method of Suzuki and Suyama (1985) with a slight modification. To the samples (0.6 to 0.8 g), 20 ml of 4 M NaOH were added and mixed vigorously. The samples were heated in a boiling water bath (90 to 95°C) for 30 min and then cooled at room temperature for 1 h. The mixture was mixed with 20 ml of 4 M HCl for neutralization. The supernatant (0.2 ml) was mixed with 2 ml of phosphate reagent (ammonium molybdate solution: malachite green, 1:3 v/v). The mixture was then incubated at room temperature for 30 min. The absorbance was measured at 620 nm. Disodium hydrogen phosphate solutions with concentrations of 0 to 15 µg/ml were used for a standard curve preparation. A factor of 25 was used for converting phosphorus content to phospholipid based on an average molecular weight of phosphatidyl choline divided by atomic weight of phosphorus (Sigfusson and Hultin, 2002). The phospholipid content was expressed as mg/100 g dry sample.

3.3.4.6 Determination of peroxide value

The peroxide value (PV) was determined according to the method of Richards and Hultin (2000) with a slight modification. Ground sample (4.5 to 5.5 g) was homogenised at a speed of 13,500 rpm for 2 min in 11 ml of chloroform/methanol (2:1, v/v). The homogenate was then filtered using a Whatman No.1 filter paper. To 7 ml of filtrate, 2 ml of 0.5% NaCl were added. The mixture was vortexed at a moderate speed for 30 s, followed by centrifugation at 3,000 ×g for 3 min at 4 °C using a refrigerated centrifuge to separate the sample into two phases. To the lower phase (3 ml), 2 ml of cold chloroform/methanol (2:1) mixture, 25 µl of 30% (w/v) ammonium thiocyanate and 25 µl of 20 mM iron (II) chloride were added. The reaction mixture was allowed to stand for 20 min at room temperature prior to reading the absorbance at 500 nm. The blank was prepared in the same manner, except distilled water was used instead of ferrous chloride. A standard curve was prepared using cumene hydroperoxide at concentrations ranging from 0.5 to 2 ppm. PV was expressed as mg cumene hydroperoxide/kg dry sample.

3.3.4.7 Determination of thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) were determined as described by Buege and Aust (1978). A ground sample (0.5 g) was homogenised with 2.5 ml of a solution containing 0.375% (w/v) thiobarbituric acid, 15% (w/v) trichloroacetic acid, and 0.25 M HCl. The mixture was heated in a boiling water bath (95 to 100 °C) for 10 min to develop a pink colour, cooled with running tap water, and centrifuged at 3,600 × g at 25 °C for 20 min. The absorbance of the supernatant was measured at 532 nm. A standard curve was prepared using 1,1,3,3-tetramethoxypropane at concentrations ranging from 0 to 10 ppm. TBARS was calculated and expressed as mg malonaldehyde/kg dry sample.

3.3.4.8 Determination of trimethylamine content

Trimethylamine (TMA) content was determined according to the method of Conway and Byrne (1933). A ground sample (5 g) was mixed with 20 ml of 4% (w/v) trichloroacetic acid and homogenised at a speed of 11,000 rpm for 1 min. The homogenate was filtered using a Whatman No. 4 filter paper. In the outer ring, formaldehyde (10% v/v) (1 ml) was added to the filtrate (1 ml) to fix ammonia present in the sample. To initiate the reaction, saturated K_2CO_3 (1 ml) was mixed with the prepared sample to release TMA. TMA was trapped in 1 ml of the inner ring solution (1% boric acid (w/v) containing the Conway indicator). The Conway unit was incubated at 37 °C for 60 min. The titration of the inner ring solution was performed using 0.02 M HCl, and the amount of TMA was calculated. TMA content was expressed as mg N/100 g dry sample.

3.3.4.9 SDS-polyacrylamide gel electrophoresis

Protein patterns were determined by SDS-polyacrylamide gel electrophoresis using a 4% stacking gel and a 10% running gel according to the method of Laemmli (1970). Samples (3 g) were solubilised in 27 ml of 5% SDS. The mixture was homogenised for 1 min at a speed of 13,000 rpm and incubated at 85 °C for 1 h to solubilise all proteins. Proteins (15 μg), determined by the Biuret method (Robinson and Hogden, 1940), 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 (Sigma, St Louis, MO, USA) in 50% (v/v) methanol and 7.5% (v/v) acetic acid and destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid for 12 min, followed by 5% (v/v) methanol and 7.5% (v/v) acetic acid for 3 h. A wide range molecular weight marker was used to estimate the molecular weight.

Pretreated mince with the lowest pro-oxidant and lipid contents was selected for the hydrolysis study.

3.3.5 Effect of selected pretreatment on composition and properties of protein hydrolysate

3.3.5.1 Changes during hydrolysis

To study the impact of pretreatment on changes in protein hydrolysates, mince and W-MR-Al (100 g) were mixed with distilled water (2 to 4 °C) using a sample/water ratio of 1:4 (w/v) to obtain a final protein concentration of 2% (w/v). The mixtures were adjusted to pH 8.0 and were then pre-incubated at 50 °C for 20 min prior to enzymatic hydrolysis using Alcalase. The hydrolysis reaction at 50 °C was initiated by adding Alcalase at a level of 1% (w/w). Samples were taken at different times during hydrolysis (0, 10, 20, 30, 60, 90, and 120 min), and the reactions were terminated by heating the sample in boiling water for 10 min. The obtained protein hydrolysates were then subjected to analyses of non haem iron content, PV, and TBARS as previously described. Degree of hydrolysis (DH) of hydrolysates was also determined as per the method of Benjakul and Morrissey (1997).

3.3.5.2 Properties and the use of protein hydrolysates

After 2 h of hydrolysis, the reaction mixtures were heated for 10 min in boiling water to terminate the hydrolytic reaction. The mixture was then centrifuged at 2,000 ×g at 4°C for 10 min. The supernatant obtained was lyophilised using a Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lynge, Denmark). The lyophilised fish protein hydrolysates produced from mince and W-MR-Al were referred to as 'FPH_{mince}'

and '$FPH_{W\text{-}MR\text{-}Al}$', respectively. The resulting hydrolysates were subjected to colour measurement and were fortified in low-fat milk.

3.3.5.3 Colour measurement

The colour of both hydrolysate powders was measured by a colourimeter (ColourFlex, Hunter Lab Reston, VA, USA) and reported in the CIE system. L*, a*, b*, ΔE*, and ΔC* representing lightness, redness/greenness, yellowness/blueness, total difference of colour, and the difference in chroma, respectively, were reported. ΔE* and ΔC*were calculated as follows:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where ΔL*, Δa*, and Δb* are the differentials between colour parameter of the samples and the colour parameter of the white standard (L*=92.82, a* = −1.24, b*=0.50)

$$\Delta C^* = C^*\text{sample} - C^*\text{standard}$$

where $C^* = \sqrt{(a^*)^2 + (b^*)^2}$

3.3.5.4 Preparation of low fat milk fortified with protein hydrolysates

Low fat milk, containing 1% milk fat (Foremost, Frieslandfoods Co., Ltd., Samutprakan, Thailand), was purchased from a local supermarket, Hat Yai, Thailand. FPH_{mince} and $FPH_{W\text{-}MR\text{-}Al}$ were added to the milk at different levels (0.3, 0.4, and 0.5%) and mixed well. The resulting milks (25 to 26 °C) were subjected to sensory evaluation.

3.3.5.5 Sensory evaluation

A likeness evaluation of low fat milk fortified with and without protein hydrolysates was performed by 30 untrained panellists at the ages of 22 to 30, who were regular milk consumers. The samples were coded with 3-digit random numbers and presented to panellists on a tray in individual booths. Serving orders were completely randomised. The assessment was conducted for colour, odour, flavour, and overall

likeness using a 9-point hedonic scale: 1, dislike extremely; 5, neither like nor dislike; 9 like extremely (Meilgaard *et al.*, 2007).

3.3.6 Statistical analysis

Experiments were run in triplicate using three different batches of samples. Data were subjected to analysis of variance. Comparison of means was carried out by Duncan's multiple range tests. The t-test was used for pair comparison (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

3.4 Results and discussion

3.4.1 Characteristics of mince with different pretreatments

3.4.1.1 Chemical compositions

Mince from Nile tilapia subjected to various pretreatments showed varying compositions as presented in Table 8. Pretreatments included washing the mince, washing the mince with W-MR and preparing acid- and alkaline-aided protein isolate from the washed mince with W-MR-Ac and W-MR-Al. Different samples had the varying pH (5.41 to 6.57), depending on pretreatments used. Mince and washed mince showed similar pH (6.35 to 6.57). The lower pH was found in W-MR sample (5.92). Membrane removal process using citric acid more likely resulted in the decrease in pH. For both W-MR-Ac and W-MR-Al samples, the lowest pHs (5.41 to 5.55) were found and were similar to pH used for protein precipitation (5.5).

No TMA was detected in Nile tilapia mince and all pretreated mince samples. This result indicated that Nile tilapia, which was cultured in a freshwater farm, did not contain trimethylamine-N-oxide (TMAO), a compound found in marine fish for osmoregulation. TMAO can be reduced to TMA, a fishy odour compound (Gram and Huss, 1996). As a result, it could be presumed that the fishy odour developed in Nile tilapia mince was not mainly due to the formation of TMA. The washing process reduced myoglobin by 51.7%, compared with that found in mince. However, more myoglobin was removed when the membrane removal process was implemented ($P <$

Table 8. Chemical compositions of Nile tilapia mince after different pretreatments

Parameters	M	W	W-MR	W-MR-Ac	W-MR-Al
pH	6.35±0.02b[*,**]	6.57±0.10a	5.92±0.18c	5.41±0.02d	5.55±0.01e
Myoglobin content (mg/g dry sample)	5.34±0.50a	2.58±0.36b	0.95±0.11c	0.41±0.16d	0.11±0.01d
Haem iron content (mg /100 g dry sample)	4.68±0.25a	2.94±0.07b	2.57±0.23c	0.84±0.06d	0.74±0.34d
Non-haem iron content (mg /100 g dry sample)	2.25±0.25a	1.68±0.16b	1.57±0.36b	0.89±0.16c	0.59±0.03c
Lipid content (g/100 g dry sample)	1.66±0.60a	1.49±0.26a	0.70±0.15b	0.27±0.05bc	0.10±0.05c
Phospholipid content (mg/100 g dry sample)	13.52±0.23a	7.00±0.20b	5.30±0.27c	2.69±0.16d	2.35±0.05d
PV (mg hydroperoxide/kg dry sample)	28.89±2.02a	22.43±0.36b	15.32±1.86c	8.09±0.12d	4.42±0.64e
TBARS (mg MDA/kg dry sample)	10.58±0.07a	8.42±0.06b	5.63±0.04c	4.52±0.12c	1.79±0.07d
TMA content (mg N/100 g sample)	----------ND[†]----------				

*Mean ± SD (n=3).

** Different letters within the same row indicate the significant differences ($P < 0.05$).

[†]ND: not detectable

M: mince; W: washed mince; W-MR: washed mince with membrane removal; W-MR-Ac: W-MR with acid solubilisation and W-MR-Al: W-MR with alkaline solubilisation.

0.05). The lowest myoglobin contents were found in W-MR-Ac and W-MR-Al samples ($P < 0.05$). Pretreatment of washed mince with $CaCl_2$ and citric acid might enhance the removal of myoglobin by increasing the polarity of myoglobin, in which it could be leached out more easily. When the solubilisation process via acid or alkaline pH adjustment was used, the dissociation of protein complexes might occur. This could enhance the liberation of myoglobin from the muscle. The reduction in haem proteins, myoglobin and haem iron contents, not only improved the colour of mince, but also increased its oxidative stability. Total lipids and phospholipids in the washed mince were also significantly lowered by 10.2% and 48.2%, respectively. Similar results were also observed by Tongnuanchan *et al.* (2011) who noted that lipid content of washed red tilapia mince was deceased by 14.4%, in comparison with that found in mince. Washing is a process, which can remove lipids and undesirable materials such as blood, pigment and odourous substances (Rawdkuen *et al.*, 2009). Nevertheless, lipoproteins or membrane phospholipids associated with other muscle proteins might not be leached out easily. Additionally, some lipids were solidified at low temperatures during the washing process. This might lead to less removal of those lipids.

It was found that W-MR had marked decrease in lipids and phospholipids, compared with mince. $CaCl_2$ and citric acid used for membrane removal might disconnect the linkage between membranes and cytoskeleton protein, which linked together via electrostatic interaction. Ca^{2+} as well as citric acid might interact with those components, especially the phospholipids membrane, thereby providing the charge or polarity to those components. As a consequence, they were more likely soluble in the aqueous phase. As a result, the releases of the phospholipid membranes from attached cytoskeletal were facilitated due to the loosened structure. In addition, Ca^{2+} could interact with the polar head of phospholipid to form a calcium-phospholipid complex. In addition, citric acid might play a role as a binding agent for the basic amino acid residues of cytoskeletal proteins, thereby competing with the acidic phospholipids of membranes (Hrynets *et al.*, 2011). Hrynets *et al.* (2011) reported that addition of 6 or 8 mM of citric acid resulted in the substantial removal of lipid and pigments from mechanically separated turkey meat. Vareltzis *et al.* (2008) reported that the presence of 10 mM $CaCl_2$ and 5 mM citric acid during acid or alkaline aided protein isolation significantly improved lipid reduction in mussel proteins.

When the pH-shift methods were applied in mince with membrane removal, much more lipids and phospholipids were removed. The dissociation of protein complexes might provide a larger surface, in which both citric acid and $CaCl_2$ could help in removing phospholipids more effectively. Total lipid in W-MR-Ac and W-MR-Al decreased by 83.7% and 94.0%, compared with that found in mince, whilst phospholipids decreased by 80.1% and 82.6%, respectively. During pretreatment, not only undesirable materials, especially lipids and haem proteins, were removed but also lipid oxidation products were eliminated as indicated by the lower PV and TBARS values. The lowest PV and TBARS were found in the W-MR-Al sample. Higher PV and TBARS values were found in W-MR-Ac compared with W-MR-Al ($P < 0.05$). The result suggested the greater susceptibility of acid treated protein isolate toward oxidation. Raghavan and Hultin (2009) reported that cod protein isolate prepared using the acid process was significantly more susceptible to lipid oxidation than using the alkaline process. Amongst various pro-oxidative constituents of muscle tissue, phospholipid membrane, and haem proteins are mainly involved in oxidative deterioration (Liang and Hultin, 2005a; Raghavan and Hultin, 2009; Thiansilakul *et al.*, 2011). Thus, the alkaline solubilisation process was more appropriate than the acid solubilisation process since lipid oxidation could be minimised under alkaline condition.

3.4.1.2 Protein patterns

Protein patterns of different pretreated minces are depicted in Figure 11. Myosin heavy chain (MHC), actin, tropomyosin, and troponin were found in fish mince. After washing or membrane separation, some sarcoplasmic proteins or some cytoskeletal proteins which interact with the membrane phospholipids might be removed. As a result, the myofibrillar proteins became concentrated as indicated by the increased band intensity of MHC, actin, tropomyosin, and troponin, whilst some protein bands with molecular weight of 49.8, 39.3, and 37.0 kDa disappeared. No marked differences in protein patterns were observed in W-MR compared to those found in isolates. However, a slight degradation of MHC band was obtained in W-MR-Ac. It was also found that band intensity of all myofibrillar proteins increased in W-MR-Ac and W-MR-Al, when compared with other treatments. When proteins became charged

at alkaline or acidic conditions, protein repulsion occurred, thereby solubilising the protein. When pH is adjusted to 5.5, the myofibrillar proteins are precipitated, whilst several sarcoplasmic proteins or cytoskeleton proteins remained in the supernatant. As a consequence, myofibrillar proteins were more concentrated as evidenced by the increase in band intensity. Since W-MR-Al sample had the highest myofibrillar protein proportion with the lowest lipids, pro-oxidants, myoglobin, haem iron and non-haem iron contents as well as lipid oxidation products, it was chosen for preparation of protein hydrolysate.

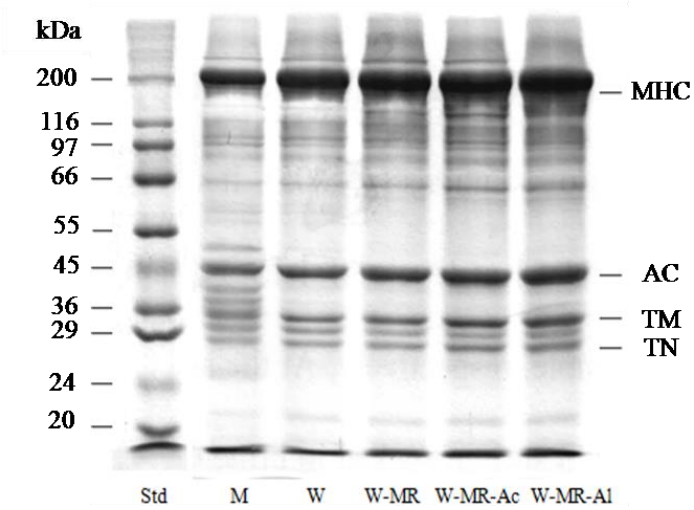


Figure 11. Protein patterns of Nile tilapia mince before and after different pretreatments. M; mince; W: washed mince; W-MR: washed mince with membrane removal; W-MR-Ac: W-MR with acid solubilisation; W-MR-Al: W-MR with alkaline solubilisation. MHC: myosin heavy chain, AC: actin; TM: tropomyosin; TN: troponin.

3.4.2 Changes in DH and compositions of protein hydrolysate during hydrolysis

3.4.2.1 Changes in DH

Hydrolysis of mince and W-MR-Al by Alcalase as a function of time is shown in Figure 12. Degree of hydrolysis (DH) of protein hydrolysates increased as hydrolysis time increased ($P < 0.05$). DH is known to have a great impact on several

properties of protein hydrolysates (Abdalla and Roozen, 1999; Adler-Nissen, 1979). Rapid hydrolysis was observed within the first 60 min, indicating that a large number of peptide bonds were hydrolysed (Shahidi *et al.*, 1995). Thereafter, the hydrolysis rate was decreased, mainly due to a decrease in available hydrolysis sites in substrate, enzyme autodigestion, and/or product inhibition (Kristinsson and Rasco, 2000b). At the same time of hydrolysis, the hydrolysates obtained from W-MR-Al possessed a higher DH than did those derived from mince ($P < 0.05$). The result suggested that the W-MR-Al sample was more susceptible to cleavage by Alcalase. This was possibly due to more exposure of peptide bonds of the protein isolates, where Alcalase could cleave the peptides more effectively. Alcalase has several advantages toward hydrolysis including (1) broad specificity (2) availability (3) inexpensive price and (4) resistance to gastrointestinal hydrolysis (Ovissipour *et al.*, 2009; Tiengo *et al.*, 2009; Zhu *et al.*, 2010). During alkaline solubilisation, the repulsion between protein molecules results in the dissociation of actomyosin complex. The liberated peptide chains and looser protein structures were more prone to hydrolysis by Alcalase. Thus, the configuration of protein was another important factor governing the rate of hydrolysis, thereby affecting the properties of hydrolysate obtained.

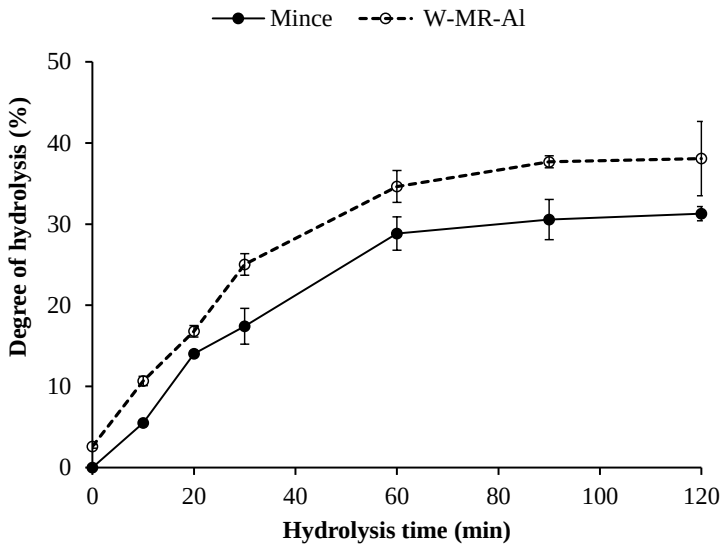


Figure 12. Changes in DH of Nile tilapia mince and W-MR-Al during hydrolysis with Alcalase at a level of 1% (w/w). The reaction was performed at 50 °C and pH 8. Bars represent the standard deviation (n=3).

3.4.2.2 Changes in non-haem iron content

Changes in non-haem iron content in hydrolysates from mince and W-MR-Al during hydrolysis were monitored as depicted in Figure 13A. An increase in non-haem iron content was observed with increasing hydrolysis time for both samples ($P < 0.05$). It was suggested that the disruption of the porphyrin ring of haem protein remaining in both samples more likely occurred during the hydrolysis process at 50 °C. Chaijan *et al.* (2005) noted that the destabilization of the haem-globin complex occurs under harsh conditions such as very acidic pH and high temperature, leading to the release of free iron called 'non-haem iron'. Thiansilakul *et al.* (2011) found that heating could weaken the porphyrin ring with the subsequent iron released. Released iron might act as a pro-oxidant, which is able to enhance lipid oxidation. Furthermore, Kristensen and Andersen (1997) revealed that heated haem iron had a more significant effect on pro-oxidative activity than did heated free iron. Regardless of hydrolysis time, less non-haem iron was found in the hydrolysate from W-MR-Al than in hydrolysate from mince ($P < 0.05$). Since water soluble components, including haem proteins, were largely removed by washing in combination with membrane removal and alkaline solubilisation process, non-haem iron content was also reduced. The result was in agreement with the low amount of myoglobin and haem iron retained in W-MR-Al (Table 8). Therefore, the pro-oxidant present during hydrolysis can be lowered by combined pretreatments of protein substrate via washing, membrane removal, followed by alkaline solubilisation.

3.4.2.3 Changes in PV and TBARS

Lipid oxidation of hydrolysates produced from mince and W-MR-Al expressed as PV and TBARS values is shown in Figure 13B and 13C, respectively. For hydrolysates produced from mince, a continuous increases in PV was found up to 60 min of hydrolysis process, followed by a gradual decrease up to the end of hydrolysis ($P < 0.05$), suggesting that the hydroperoxides formed were decomposed to other compounds. However, no changes in PV were observed in hydrolysate from W-MR-Al

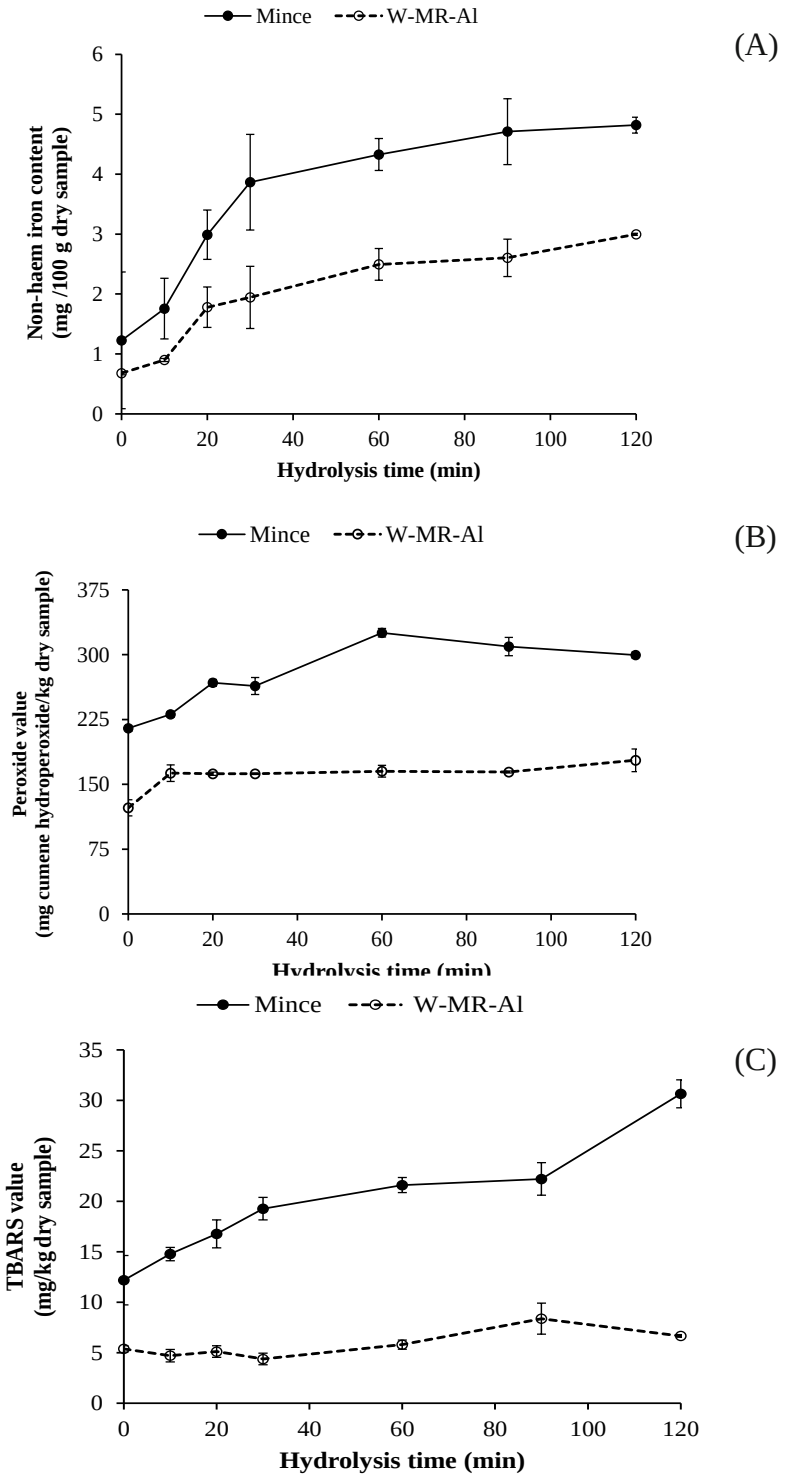


Figure 13. Changes in non-haem iron content (A), PV (B) and TBARS (C) values of Nile tilapia mince and W-MR-Al during hydrolysis with Alcalase at a level of 1% (w/w). The reaction was performed at 50 °C and pH 8. Bars represent the standard deviation (n=3).

during 10 to 120 min of hydrolysis ($P > 0.05$). This was more likely due to presence of more lipids and pro-oxidants in mince, compared with W-MR-Al. Apart from myoglobin, hemoglobin in fish muscle also serves as an effective catalyst of lipid oxidation (Apte and Morrissey, 1987). Haem dissociation, haem destruction and iron released play a role in lipid oxidation of fish muscle (Gandemer, 1999). Generally, washing/membrane removal in combination with the alkaline solubilisation process could yield hydrolysate with negligible lipid oxidation.

TBARS values of both hydrolysates increased as hydrolysis time increased ($P < 0.05$) (Figure 13C). The result reconfirmed that lipid oxidation took place during hydrolysis, particularly when mince was used as the substrate. A much higher increase in TBARS of hydrolysates produced from mince was observed, and this was more likely related to the increase in non-haem iron content during extended hydrolysis (Figure 13A). For hydrolysates produced from W-MR-Al, a slight increase in TBARS was observed after 60 min of hydrolysis ($P < 0.05$). TBARS tended to decrease at 120 min, possibly caused by a loss of low-molecular-weight decomposition products. Adduction of aldehyde with other compounds, especially proteins, might also contribute to the lowered TBARS. Even though a large amount of lipid as well as pro-oxidant could be removed, the lipid oxidation of hydrolysate produced from W-MR-Al still occurred to some extent. Some lipoproteins might be co-precipitated with myofibrillar proteins during isoelectric precipitation (Kristinsson *et al.*, 2005). Thus, W-MR-Al could yield hydrolysate with lower lipid oxidation products.

3.4.3 Colour and fortification of protein hydrolysate powders in low fat milk

Colour of freeze-dried hydrolysates produced from mince and W-MR-Al is presented in Table 9. All hydrolysates exhibited a slight creamy yellowish colour. There were differences in L* (lightness), b* (yellowness), ΔE* (total colour difference), and ΔC* (colour intensity difference) between $FPH_{W\text{-}MR\text{-}Al}$ and FPH_{mince}. Higher L* and lower b* values were observed in $FPH_{W\text{-}MR\text{-}Al}$, compared with those of FPH_{mince} ($P < 0.05$). This was in accordance with the lower ΔE* and ΔC* in the former. Generally, myoglobin and haemoglobin have been known to be responsible for colour characteristics of fish flesh or their product. The pretreatment of mince via

washing/membrane removal in combination with alkaline solubilisation could remove or significantly reduce those pigments, leading to lower amounts of coloured compounds retained in the resulting mince. Moreover, lipid oxidation causes poor visual appearance and a yellowish discolouration via the Maillard reaction. It was noted that yellow discolouration of hydrolysate produced from an extended storage Nile tilapia muscle was mainly caused by lipid oxidation and could be inhibited by antioxidant (Trolox and EDTA) incorporation (chapter 2). In the present study, the lower lipid oxidation occurred in hydrolysate from W-MR-Al. Thus, discolouration caused by browning reaction might be retarded.

Table 9. Colour parameters of protein hydrolysates prepared from Nile tilapia mince and W-MR-Al

Parameters	FPH_{mince}	$FPH_{W\text{-}MR\text{-}Al}$
L*	91.80±0.26b[†]	93.40±0.30a
a*	-1.12±0.09a	-0.85±0.06a
b*	5.37±0.58a	2.98±0.25b
ΔE*	4. 98±0.51a	2.59±0.17b
ΔC*	4.15±0.57a	1.77±0.26b

Values are given as mean ± SD (n=3).

[†]Different letters within the same row indicate the significant differences ($P < 0.05$).

When low-fat milk was fortified with both hydrolysates at different levels (0.3% to 0.5%, w/v), colour, odour, flavour, and overall likeness scores were evaluated as shown in Table 10. For colour likeness, there was no difference amongst all milk samples tested ($P > 0.05$). The milk fortified with $FPH_{W\text{-}MR\text{-}Al}$ had no changes in odour, flavour, and overall likeness scores when the levels were added up to 0.5% ($P > 0.05$). This indicated that $FPH_{W\text{-}MR\text{-}Al}$ could be fortified in milk at a high level without detrimental effect on sensory property. For the sample fortified with FPH_{mince}, no difference in odour likeness score was observed when added at up to 0.4% ($P > 0.05$). However, FPH_{mince} at a level of 0.4% resulted in a decrease in flavour and overall likeness score ($P < 0.05$), indicating the presence of offensive flavourant in milk. The

Table 10. Likeness score of low fat milk fortified with different levels of protein hydrolysate prepared from Nile tilapia mince and W-MR-Al

Attributes	Control[†] (w/o hydrolysate)	FPH_{mince} (%)			$FPH_{W\text{-}MR\text{-}Al}$ (%)		
		0.3	0.4	0.5	0.3	0.4	0.5
Color	8.25±0.75a[*,†]	8.17±0.85a	7.83±1.00a	8.03±0.98a	8.21±0.77a	8.00±0.85a	8.07±0.86a
Odour	7.87±0.82a	7.68±0.80a	7.45±1.06a	6.44±1.93b	7.81±0.83a	7.62±1.17a	7.50±1.38a
Flavour	7.70±0.75a	7.46±0.90ab	6.84±1.51b	6.18±1.89c	7.67±0.96a	7.52±1.28a	7.33±1.14ab
Overall	7.57±0.69a	7.17±1.42a	6.46±1.72b	6.22±1.90b	7.43±1.19a	7.24±1.30a	7.28±1.65a

Values are given as mean ± SD, evaluated by 30 untrained panellists.

[†]Score are based on a 9-point hedonic scale (1: Dislike extremely, 5: Neither like nor dislike, 9: Like extremely).

[*] Different letters within the same row indicate the significant differences ($P < 0.05$).

decrease in likeness was more likely related with fishy odour/flavour in FPH. Sohn *et al.* (2005) reported that the offensive odour detected in fish flesh was directly related with the formation of secondary lipid oxidation products. Additionally, a stronger fishy odour/flavour of FPH produced from stored Nile tilapia was correlated with a higher lipid oxidation products (chapter 2). Therefore, $FPH_{W\text{-}MR\text{-}Al}$ could serve as a nutritive ingredient with some bioactive activities and could be supplemented without the adverse effect on sensory properties.

3.5 Conclusion

Pretreatment of Nile tilapia mince played a significant role in the reduction of prooxidants and lipids, especially neutral lipids and membrane phospholipids. Washing, along with a process to remove membranes prior to alkaline solubilisation, was very effective in preparing a substrate for protein hydrolysate production, in which fishy odour and flavour could be significantly lowered. FPH prepared from the appropriate pretreatment could be fortified in low fat milk at a level up to 0.5%.

CHAPTER 4

EFFECT OF PRETREATMENT ON LIPID OXIDATION AND FISHY ODOUR DEVELOPMENT IN PROTEIN HYDROLYSATES FROM THE MUSCLE OF INDIAN MACKEREL

4.1 Abstract

Impact of different pretreatments on chemical compositions of Indian mackerel mince was studied. Mince prepared using washing/membrane removal/alkaline solubilisation process (W–MR–Al) contained the lowest remaining myoglobin and haem iron content and also showed the lowest total lipid and phospholipid contents. When mince and W–MR–Al were hydrolysed using Alcalase for up to 120 min, a higher degree of hydrolysis (DH) was found in W–MR–Al after 30 min of hydrolysis. Furthermore, hydrolysate from W–MR–Al had lower peroxide value (PV), thiobarbituric acid reactive substances (TBARS) and non-haem iron content throughout hydrolysis period ($P < 0.05$). When hydrolysate powder produced from mince and W–MR–Al (0–0.3% w/v) were fortified in milk, the former resulted in the lower likeness score ($P < 0.05$) at all levels used. The addition of the latter, for up to 0.2%, had no effect on likeness of all attributes, compared with milk without fortification ($P > 0.05$). Therefore, the appropriate pretreatment of mince yielded hydrolysate with lower fishy odour.

4.2 Introduction

Proteins from seafood resources are well balanced with regard to their amino acid composition. Enzymatic hydrolysis is a process that has been developed some time ago to convert proteins into more marketable and acceptable forms. Numerous fish protein hydrolysates have been reported to possess antioxidative activities and various other bioactive properties such as those from brownstripe red snapper (Khantaphant *et al.*, 2011a), ornate threadfin bream (Nalinanon *et al.*, 2011), round scad (Thiansilakul *et al.*, 2007b) and mackerel (Wu *et al.*, 2003). Due to their functional properties, fish protein hydrolysates have been used in foods. With their excellent interfacial properties, they may have the potential use as emulsifying

ingredients in a variety of products, e.g. dressing, margarine and meat batter (Kristinsson, 2007). In addition, Khan *et al.* (2003) used fish scrap protein hydrolysate as the cryoprotectant to prevent protein denaturation of lizardfish surimi during frozen storage at -25 °C. However, a major problem connected to hydrolysate preparation from fish flesh is the presence of pro-oxidants such as haem proteins and unstable lipid substrates (Raghavan *et al.*, 2008). Lipid oxidation generally contributes to the development of undesirable odour, especially fishy odour (Maqsood and Benjakul, 2011a). This offensive odour limits the use of fish protein hydrolysates, particularly in foods or drinks which have a light odour or smell.

Fatty fish have been known to contain high levels of myoglobin, other haem proteins, low molecular weight (LMW) transition metal complexs and lipoxygenases in their dark muscle (Chaijan *et al.*, 2005; Thiansilakul *et al.*, 2012b). Furthermore, phospholipid membranes are believed to be the key substrate for lipid oxidation due to their highly unsaturated fatty acid composition (Liang and Hultin, 2005a). Contamination of pro-oxidants and oxidised lipids in mince can have a great impact on functionality and antioxidant properties of protein hydrolysates (Raghavan and Kristinsson, 2008). Haem proteins in the raw material can also become oxidised during the hydrolysis process, thereby promoting lipid oxidation and development of unpleasant odours/flavours (Raghavan *et al.*, 2008). Therefore, the removal of pro-oxidants and lipids could be a promising means to alleviate such problems occurring in hydrolysates from fatty fish flesh. Khantaphant *et al.* (2011a) reported that pretreatment of fish mince also affected antioxidant activity of the resulting fish protein hydrolysate.

One recent successful method to recover fish proteins is the pH shift method, where the fish proteins are separated and recovered from other undesirable components (i.e. lipids, connective tissue, skin and bones) by their pH-dependent solubility properties (Kristinsson *et al.*, 2005b). In brief, fish muscle proteins (myofibrillar and sarcoplasmic proteins) are solubilised at acidic or alkaline pH (pH ~2.0 to 3.5 or pH ~ 10.5 to 11.5, respectively) and undesirable contaminants are separated from soluble proteins by centrifugation (Hultin and Kelleher, 2000; Kristinsson *et al.*, 2005b; Liang and Hultin, 2005a). Subsequently, protein isolates are prepared by precipitating the myofibrillar and sarcoplasmic proteins at their isoelectric points (pH ~5.5) (Kristinsson *et al.*, 2005b). Protein isolate prepared from blue mussel

meat pretreated with the solution containing 10 mM calcium chloride and 5 mM citric acid had much lower amount of phospholipids (Vareltzis *et al*., 2008). Due to the high fat content and high amount of pigments in dark fleshed fish, the appropriate pretreatment of muscle before hydrolysis, in which pro-oxidant and membrane lipids can be removed, should be taken into consideration. Fish protein hydrolysate with negligible fishy odour can have the wider applications in food systems. As a consequence, seafood industry can have the promising procedure for production of high quality fish protein hydrolysate, a value-added product. Additionally, fish protein hydrolysate with less fishy odour can be supplemented as the source of nutritive bioactive peptides in various beverages and drinks such as fruit juices, milk, etc. without the negative effect on sensorial property. The objectives of this study were to elucidate the effect of pretreatments on the removal of pro-oxidants and phospholipid membranes and to study lipid oxidation and fishy odour development during the hydrolysis process of Indian mackerel. The impact of fish protein hydrolysates on the sensory property of milk was also evaluated.

4.3 Materials and methods

4.3.1 Chemicals

Alcalase 2.4L (E.C. 3.4.21.62) was obtained from Novozyme (Bagsvaerd, Denmark). 2,4,6-trinitrobenzenesulphonic acid (TNBS), bathophenanthroline disulphonic acid, sodium dodecyl sulphate (SDS), β-mercaptoethanol (β-ME) and 1,1,3,3-tetramethoxypropane were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid, sodium nitrite, ferrous chloride and iron standard solution were obtained from Merck (Darmstadt, Germany). Disodium hydrogen phosphate, sodium dihydrogen phosphate and 2-thiobarbituric acid and cumene hydroperoxide were procured from Fluka (Buchs, Switzerland). Methanol, acetone, chloroform and ammonium thiocyanate were obtained from Lab Scan (Bangkok, Thailand). All chemicals were of analytical grade.

4.3.2 Fish samples

Indian mackerel (*Rastrelliger kanagurta*) with a weight of 0.4–0.5 kg/fish were purchased from a local market in Songkhla province, Thailand. The fish, off-loaded approximately 24–36 h after capture, were transported in ice with a fish/ice ratio of 1:2 (w/w) to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand within 1 h.

4.3.3 Preparation of mince with different pretreatments

4.3.3.1 Preparation of mince

Whole fish were washed and the flesh was separated manually. Flesh was minced to uniformity using Moulinex AY46 blender (Group SEB, Lyon, France) in a walk-in-cold room (4 °C) .The mince obtained was placed in polyethylene bags and kept in ice not longer than 2 h before use.

4.3.3.2 Preparation of washed mince

Mince was homogenised with five volumes of cold distilled water (2–4 °C) using an IKA Labortechnik homogeniser (Selangor, Malaysia) at a speed of 11,000 rpm for 2 min. The homogenate (4 °C) was stirred for 15 min prior to centrifugation at 9,600 ×g for 10 min at 4 °C using a Beckman Coulter centrifuge Model Avant J-E (Beckman Coulter, Inc., Fullerton, CA, USA). The washing process was repeated twice. The sample obtained was referred to as ‘washed mince; W’.

4.3.3.3 Preparation of mince with membrane removal

Prior to membrane separation, the mince was subjected to washing as previously described. Membranes were then removed from the W sample by treatment with Ca^{2+} and citric acid according to the method of Liang and Hultin (2005a) with a slight modification. The W sample was homogenised with nine volumes of cold 8 mmol/l $CaCl_2$ solution in the presence of 5 mmol/l citric acid at a speed of 11,000 rpm for 2 min. After continuous stirring for 60 min at 4 °C, the sample was centrifuged at

4,000 ×g for 15 min at 4 °C and the mince obtained was referred to as ‘washed mince with membrane removal; W–MR’.

4.3.3.4 Preparation of protein isolate with membrane removal

To prepare the protein isolate, acid and alkaline solubilisation processes were used as described by Raghavan and Hultin (2009) with a slight modification. The W–MR sample was homogenized with nine volumes of cold distilled water (2–4 °C) at a speed of 11,000 rpm for 1 min. The homogenate was adjusted to pH either 3.0 or 11.0. The mixtures were placed on ice for 60 min. Homogenates were then centrifuged at 5,000 ×g for 10 min at 4 °C. Acid and alkaline soluble fractions were collected and adjusted to pH 5.5 to precipitate the myofibrillar proteins. The homogenate was then centrifuged at 10,000 × g for 20 min. The pellet obtained was referred to as ‘acid and alkaline solubilised protein isolate with membrane removal; W–MR–Ac and W–MR–Al.

4.3.4 Analyse

All prepared samples were subjected to following analyses.

4.3.4.1 Determination of pH

pH of all samples was measured as described by (Benjakul *et al.*, 1997). The samples were homogenized with 10 volumes of deionised water (w/v) at a speed of 11,000 rpm for 1 min. Homogenate obtained was subjected to pH measurement using a Model Docu-pH Meter (Sartorius AG, Germany).

4.3.4.2 Determination of myoglobin content

Myoglobin content was determined by direct spectrophotometric measurement (Chaijan *et al.*, 2005). Sample (2.0 g) was mixed with 20 ml of cold 40 mmol/l phosphate buffer (pH 6.8), followed by homogenisation at 13,500 rpm for 10 s. The mixture was centrifuged at 3,000 × g for 30 min at 4 °C and the supernatant was filtered through a Whatman filter paper No.1 (Whatman International Ltd., Maidstone, England). The absorbance of the supernatant was read at 525 nm using a

spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). Myoglobin content was calculated from the molar extinction coefficient of 7.6×10^{-3} and a molecular weight of 16,110 dalton (Gomez-Basauri and Regenstein, 1992). The myoglobin content was expressed as mg/100 g dry sample.

4.3.4.3 Determination of haem and non-haem iron content

Haem iron content was determined as described by Cheng and Ockerman (2004) with a slight modification. The ground sample (2 g) was mixed with 9 ml of acid acetone (90% acetone, 8% deionized water and 2% HCl, v/v/w). The mixture was macerated with a glass rod and allowed to stand for 1 h at room temperature. The extract was filtered with a Whatman No. 42 filter paper (Whatman International Ltd., Maidstone, UK) and the absorbance was read at 640 nm against an acid acetone using a spectrophotometer. Haem iron content was calculated with the factor of 0.00882 µg/ µg haematin using the following formula:

$$\text{Haem iron (mg/100 g dry sample)} = \text{total pigment (ppm)} \times 0.00882$$

where total pigment (ppm) = $A_{640} \times 680$.

Non-haem iron content was determined according to the method of Schricker et al. (1982). The ground sample (1.0 g) was transferred into a screw cap test tube and 50 µl of 0.39% (w/v) sodium nitrite were added. Four millilitres of 40% trichloroacetic acid and 6 mol/l HCl (ratio of 1:1 (v/v), prepared freshly) were added. The tightly capped tubes were placed in an incubator shaker (W350, Memmert, Schwabach, Germany) at 65 °C for 22 h and then cooled at room temperature for 2 h. The supernatant (400 µl) was mixed with 2 ml of the non-haem iron colour reagent (prepared freshly). After vortexing, using a Vortex- Genie2 mixer (Scientific Industries, Bohemia, NY, USA) and standing for 10 min, the absorbance was measured at 540 nm. The colour reagent was prepared by mixing a 1:20:20 ratio (w/v/v) of: (1) bathophenantroline (0.162 g dissolved in 100 ml of double deionised water with 2 ml thioglycolic acid [96–99%]); (2) double deionised water; (3) saturated sodium acetate solution. The non-haem iron content was calculated from an iron standard curve. The iron standard solutions ($Fe(NO_3)_2$ in HNO_3) with concentrations ranging from 0 to 5

ppm were used. The concentration of non-haem iron was expressed as mg/100 g dry sample.

4.3.4.4 Determination of lipid content

Lipid content was determined by a Soxhlet apparatus according to the method 920.39B of AOAC (2000). Lipid content was expressed as g/100 g dry sample.

4.3.4.5 Determination of phospholipid content

The phospholipid content was determined as phosphorus content according to the method of Suzuki and Suyama (1985) with a slight modification. To the samples (0.6–0.8 g), 20 ml of 4 mol/l NaOH was added and mixed vigorously. The samples were heated in a boiling water bath (90–95 °C) for 30 min and then cooled at room temperature for 1 h. The mixture was mixed with 20 ml of 4 mol/l HCl for neutralisation. The supernatant (0.2 ml) was mixed with 2 ml of phosphate reagent (ammonium molybdate solution: malachite green, 1:3 v/v). The mixture was then incubated at room temperature for 30 min. The absorbance was measured at 620 nm. The phosphorus content was calculated from a phosphate standard curve. Disodium hydrogen phosphate solutions with concentrations of 0 to 15 µg/ml were used as a standard. A factor of 25 was used for converting phosphorus content to phospholipid based on an average molecular weight of phosphatidyl choline divided by atomic weight of phosphorus (Sigfusson and Hultin, 2002). The phospholipid content was expressed as mg/100 dry sample.

4.3.4.6 Determination of peroxide value

The peroxide value (PV) was determined according to the method of Richards and Hultin (2000) with a slight modification. Ground sample (4.5–5.5 g) was homogenised at a speed of 13,500 rpm for 2 min in 11 ml of chloroform/methanol (2:1, v/v). The homogenate was then filtered using a Whatman No.1 filter paper. Two millilitres of 0.5% NaCl was then added to 7 ml of the filtrate. The mixture was vortexed at a moderate speed for 30 s and then centrifuged at 3,000 × g for 3 min at 4 °C using a

refrigerated centrifuge (Avanti J-E centrifuge, Beckman Coulter, Palo Alto, CA, USA) to separate the sample into two phases. Twenty-five microliters of 30% (w/v) ammonium thiocyanate and 25 µl of 20 mmol/l iron (II) chloride were added to the mixture. The reaction mixture was allowed to stand for 20 min at room temperature prior to reading the absorbance at 500 nm. The blank was prepared in the same manner, except distilled water was used instead of ferrous chloride. A standard curve was prepared using cumene hydroperoxide at concentrations ranging from 0.5 to 2 ppm. PV was expressed as mg cumene hydroperoxide/kg dry sample.

4.3.4.7 Determination of thiobarbituric acid reactive substances

Thiobarbituric acid-reactive substances (TBARS) were determined as described by Buege and Aust (1978). A ground sample (0.5 g) was homogenised with 2.5 ml of a solution containing 0.375% (w/v) thiobarbituric acid, 15% (w/v) trichloroacetic acid and 0.25 mol/l HCl. The mixture was heated in a boiling water bath (95–100 °C) for 10 min to develop a pink colour, cooled with running tap water and centrifuged at 3,600 × g at 25 °C for 20 min. The absorbance of the supernatant was measured at 532 nm. A standard curve was prepared using 1,1,3,3-tetramethoxypropane at concentrations ranging from 0 to 10 ppm. TBARS was calculated and expressed as mg malonaldehyde/kg dry sample.

4.3.4.8 Determination of trimethylamine content

TMA contents were determined according to the method of Conway and Byrne (1933). A ground sample (5 g) was mixed with 20 ml of 4% trichloroacetic acid (w/v) and homogenised at a speed of 11,000 rpm for 1 min. The homogenate was filtered using a Whatman No. 4 filter paper and the filtrate was used for analysis. In the outer ring, formaldehyde (10%, w/v) (1 ml) was added to the filtrate (1 ml) to fix ammonia present in the sample. To initiate the reaction, saturated K_2CO_3 (1 ml) was mixed with the prepared sample to release TMA. TMA was trapped in 1 ml of the inner ring solution (1% boric acid (w/v) containing the Conway indicator). The Conway unit was incubated at 37 °C for 60 min. The titration of the inner ring solution was performed

using 0.02 mol/l HCl and the amount of TMA was calculated. TMA content was expressed as mg N/100 g dry sample.

4.3.4.9 SDS-polyacrylamide gel electrophoresis

Protein patterns were determined by SDS–PAGE using a 4% stacking gel and a 10% running gel according to the method of Laemmli (1970). Samples (3 g) were solubilised in 27 ml of 5% SDS. The mixture was homogenised for 1 min at a speed of 13,000 rpm and incubated at 85 °C for 1 h to solubilise all proteins. Proteins (15 µg), determined by the Biuret method (Robinson and Hogden, 1940) 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 and destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid for 12 min, followed by 5% (v/v) methanol and 7.5% (v/v) acetic acid for 3 h. A wide range molecular weight marker was used to estimate the molecular weight.

4.3.5 Effect of selected pretreatment on composition and properties of protein hydrolysate

Prepared mince with the lowest pro-oxidant and lipid contents was selected for hydrolysis study.

4.3.5.1 Changes during hydrolysis

To study the impact of pretreatment on changes in the protein hydrolysates, mince or W–MR–Al (100 g) was mixed with distilled water (2–4 °C) using a sample/water ratio of 1:4 (w/v) to obtain a final protein concentration of 2% (w/v). The mixtures were adjusted to pH 8.0 using 2 mol/l NaOH or 2 mol/l HCl, respectively. The mixtures were then pre-incubated at 50 °C for 20 min prior to enzymatic hydrolysis using Alcalase. The hydrolysis reaction at 50 °C was initiated by adding Alcalase at a level of 1% (w/w). Samples were taken at different time points during hydrolysis (0, 10, 20, 30, 60, 90 and 120 min) and the reactions were terminated

by heating the sample in boiling water for 10 min. The obtained hydrolysates were then subjected to determination of degree of hydrolysis (DH) according to the method of Benjakul and Morrissey (1997), non-haem iron content, PV and TBARS as mentioned above.

4.3.5.2 Properties and the use of protein hydrolysates

After 2 h of hydrolysis, the remaining mixtures were heat treated as described above to terminate the reaction. The mixture was then centrifuged at 2,000 × g at 4 °C for 10 min. The supernatant obtained was lyophilised using a Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lynge, Denmark). The lyophilised fish protein hydrolysates produced from mince and W–MR–Al were referred to as 'FPHmince' and '$FPH_{W–MR– Al}$', respectively. The resulting hydrolysates were subjected to colour measurement and to fortification of low-fat milk.

4.3.5.3 Colour measurement

The colour of both hydrolysate powders was measured by a colourimeter (ColourFlex, Hunter Lab Reston, VA, USA) and reported in the CIE system. L*, a*, b*, ΔE* and ΔC* representing lightness, redness/greenness, yellowness/blueness, total difference of colour and the difference in chroma, respectively, were reported. DE* and DC* was calculated as follows:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where ΔL*, Δa*and Δb* are the differentials between colour parameter of the samples and the colour parameter of the white standard (L* = 92.82, a*= -1.24, b*= 0.50).

$$\Delta C^* = C^*\text{sample} - C^*\text{standard}$$

where $C^* = \sqrt{(a^*)^2 + (b^*)^2}$

4.3.5.3 Preparation of low fat milk fortified with protein hydrolysates

Low fat milk, containing 1% milk fat (Foremost, Frieslandfoods Co., Ltd., Samutprakan, Thailand), was purchased from a local supermarket, Hat Yai, Thailand. FPH_{mince} and $FPH_{W\text{-}MR\text{-}Al}$ were added to the milk at different levels (0.1, 0.2, and 0.3%) and mixed well. The resulting milks (25 to 26 °C) were subjected to sensory evaluation.

4.3.5.4 Sensory evaluation

A likeness evaluation of low fat milk fortified with and without protein hydrolysates was performed by 30 untrained panelists at the ages of 22 to 30, who were regular milk consumers. The samples were coded with 3-digit random numbers and presented to panellists on a tray in individual booths. Serving orders were completely randomised. The assessment was conducted for colour, odour, flavour, and overall likeness using a 9-point hedonic scale: 1, dislike extremely; 5, neither like nor dislike; 9 like extremely (Meilgaard *et al.*, 2007).

4.3.6 Statistical analysis

Experiments were run in triplicate using three different batches of samples. Data were subjected to analysis of variance. Comparison of means was carried out by Duncan's multiple range tests. The t-test was used for pair comparison (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

4.4 Results and discussion

4.4.1. Characteristics of mince with different pretreatments

4.4.1.1 Chemical compositions

Mince from Indian mackerel subjected to different pretreatments had different compositions as shown in Table 11. Pretreatments included washing the mince (W), washing the mince with membrane removal (W–MR) and making acid- and

alkaline-aided protein isolate from washed mince with membrane removal (W–MR–Ac and W–MR–Al). Different samples varied in pH, ranging from pH 5.30 to 6.95, depending on pretreatment processes used. It was noted that all pretreatments resulted in a decrease in myoglobin and prooxidative haem and non-haem iron content ($P < 0.05$). Total lipid and phospholipid contents were decreased by the pretreatments. Lipid oxidation products in pretreated samples also decreased as indicated by the decrease in PV and TBARS values. TMA content was lower in pretreated mince, compared with that of untreated mince.

The washing process reduced myoglobin by 50.2%, compared with that found in mince. It also significantly lowered total lipid and phospholipids in the mince by 72.5 and 69.3%, respectively. Washing has been reported to be important to remove lipids and undesirable materials such as blood, pigment and odourous substances (Kristinsson *et al.*, 2005b). Neutral lipids were more easily removed during washing than the membrane phospholipids (Raghavan *et al*., 2008a). Tongnuanchan *et al.* (2011) reported that lipid content of washed mince was decreased by 14.4%, in comparison with that found in mince. Nevertheless, lipoproteins or membrane phospholipids associated with other muscle proteins might not be leached out easily. A higher reduction in neutral lipids is expected, since they are not as tightly associated with other structural components of the muscle like phospholipids. In tropical fish like Indian mackerel a significant portion of the lipids will likely solidify at the low temperatures used during the washing process, thus aiding in their removal. Haem proteins have been known to be a prooxidant in muscle foods (Thiansilakul *et al.*, 2011). Chaijan *et al.* (2010) reported that myoglobin in ordinary muscle of sardine and mackerel mince were reduced by 23 and 75% by washing. The reduction in haem proteins seen here for Indian mackerel not only is positive for the colour of products produced from the mince but can also lead to increased oxidative stability.

The membrane removal process markedly decreased the amount of myoglobin and phospholipids by 95.5 and 77.9%, compared with that found in washed mince. The decreases by 97.7 and 93.2% were obtained, compared with those found in mince (without any pretreatment). This process also contributed to a reduction in other components, for example resulting in lower PV and TBARS values than both unwashed and washed mince. Membrane bound phospholipids are believed to be a key substrate

Table 11. Chemical compositions of untreated Indian mackerel mince compared to mince after different pretreatments

Parameters	M	W	W-MR	W-MR-Ac	W-MR-Al
pH	6.69±0.31b[†]	6.95±0.08a	5.55±0.14c	5.39±0.07d	5.60±0.03c
Myoglobin content (mg/ g dry sample)	16.42±0.17a	8.18±0.20b	0.37±0.09c	0.21±0.02d	0.28±0.03cd
Heam iron content (mg/100 g dry sample)	9.05±1.08a	5.08±0.89b	2.64±1.23c	1.96±0.69c	1.75±0.71c
Non-heam iron content (mg/100 g dry sample)	7.79±0.06a	7.08±0.09b	6.31±0.03c	6.11±0.14d	4.97±0.10e
Lipid content (g/100 g dry sample)	10.93±0.73a	3.01±0.42b	2.18±0.64c	0.19±0.03d	0.34±0.02d
Phospholipid content (mg/100g dry sample)	123.10±5.10a	37.76±2.07b	8.36±1.28c	8.02±0.39c	6.66±0.15c
PV (mg hydroperoxide/kg dry sample)	48.52±0.75a	38.79±0.21b	33.48±1.25c	26.88±0.06d	11.01±0.57e
TBARS (mg MDA/kg dry sample)	61.51±0.43a	40.77±0.51b	31.02±2.57c	24.31±3.71d	11.05±3.24e
TMA content (mg N/100 g sample)	0.79±0.11a	0.58±0.02b	0.39±0.00c	0.32±0.00d	0.31±0.00d

Values are given as mean ± SD (n=3).

M: mince; W: washed mince; W-MR: washed mince with membrane removal; W-MR-Ac: W-MR with acid solubilisation and W-MR-Al: W-MR with alkaline solubilisation.

[†] Different letters within the same row indicate the significant differences ($P < 0.05$).

for lipid oxidation due to their highly unsaturated fatty acid composition. In addition, membranes have a large surface area to contact pro-oxidants in the aqueous phase of the cell, and their lipids are located near the site of electrons leaked from the electron transport process (Liang and Hultin, 2005). Ca^{2+} as well as citric acid used for membrane removal might interact with those components, especially membrane lipids, thereby providing the charge or polarity to those components. As a consequence, they were more likely soluble in water phase. When Ca^{2+} and citric acid were applied for washing, Ca^{2+} and citric acid were also able to disconnect the linkages between cytoskeletal proteins and membrane lipid, linked together via electrostatic interaction. Citric acid might play a role as a binding agent for the basic amino acid residues of cytoskeletal proteins, thereby competing with the acidic phospholipids of membranes (Hrynets *et al.*, 2011). As a result, the release of the membrane phospholipids from attached cytoskeletal proteins occurred. Additionally, Ca^{2+} could interact with the polar head of phospholipid to form a calciumphospholipid complex. The membranes released from the cytoskeletal proteins might aggregate to a large particle as induced by Ca^{2+} addition and sediment by centrifugation (Liang and Hultin, 2005a). Hrynets *et al.* (2011) reported that addition of 6 or 8 mmol/l of citric acid resulted in substaintial removal of lipid and pigments from mechanically separated turkey meat. The present result was in accordance with Khantaphant *et al.* (2011a) who reported that the membrane separation process resulted in the lowering of myoglobin, haem and non-haem iron contents in brownstripe red snapper mince. In the present study, the phospholipid content was estimated from total phosphorus content in the sample. Therefore, other phosphorus containing compounds including, phosphoproteins, ATP, inorganinc phosphate or etc., might partially contribute to the estimated phospholipid content.

The use of another pretreatment process, the acid or alkaline solubilisation and isoelectric precipitation processes, also resulted in a reduction of all components tested. Nevertheless, haem iron and phospholipid contents were not affected by these processes ($P > 0.05$). At acidic or alkaline pH used for solubilisation, proteins undergo dissociation via the repulsion between charged protein chains. As a result, the components associated with myofibrillar proteins can be more liberated into water phase. Thus, most constituents including remaining lipids, haem proteins, etc.

can be removed or separated with relative ease. When comparing the components between W–MR–Ac and W–MR–Al samples, it was found that the former had higher PV and TBARS values than the latter ($P < 0.05$). Additionally, a lower phospholipid content was found in the latter. Thus, the alkaline solubilisation process was more effective than the acid solubilisation process in removing some components from the Indian mackerel muscle. Kristinsson and Hultin (2003) suggested that higher lipid removal by the alkaline process may be due to the greater emulsification ability of the proteins at alkaline pH. The first centrifugation step caused a portion of the membrane phospholipids to sediment in the bottom layer of the centrifuge tube, and also cause significant separation of neutral lipids to the top (Nalinanon *et al.*, 2011). Kristinsson *et al.* (2005b) reported that the acid- and alkaline-aided processes of channel catfish led to more reduction in lipids than did the conventional washing process. The reduced level of phospholipids may in part explain the significantly lower PV and TBARS levels. It is also possible that the high pH treatment provided protection towards lipid oxidation, as has been seen in other studies (Kristinsson and Hultin, 2004).

4.4.1.2 Protein patterns

Protein patterns of mince samples with different pretreatments are shown in Figure 14. The mince contained several protein bands, in which the myosin heavy chain (MHC) was the dominant protein, followed by actin, tropomyosin, troponin, respectively. During washing or membrane separation, some sarcoplasmic proteins or some cytoskeletal proteins which interact with the membrane phospholipids might have been removed. As a consequence, myofibrillar proteins became concentrated as evidenced by the increased band intensity of MHC obtained with coincidental removal of some proteins, especially those having molecular weights between actin and tropomyosin. For the protein isolate prepared from washed mince with membrane removal, the W–MR–Al sample had similar protein patterns to those of W–MR. However, the decrease in band intensity of MHC and other proteins including actin, troponin and tropomyosin of the W–MR–Ac sample were noticeable. The result indicated that the acid solubilisation process resulted in enhanced degradation of muscle protein. This was more likely mediated by endogenous acidic

proteases which were active under acidic condition. Choi and Kim (2005) reported that degradation of MHC in croaker and jack mackerel under acidic conditions was higher than that obtained under alkaline conditions. For W–MR–Al, the dissociation of actomyosin complex more likely took place after alkaline solubilisation. When the solubilised proteins were recovered by isoelectric precipitation, undesirable materials might have been co-precipitated to a small extent. As a result, myofibrillar proteins could become more concentrated with reduced levels of pro-oxidants and lipid substrates. Therefore, the W–MR–Al sample, which had a higher proportion of myofibrillar proteins, lowest phospholipid content and the least oxidation product was selected as a substrate for protein hydrolysate production and compared to using mince as a substrate.

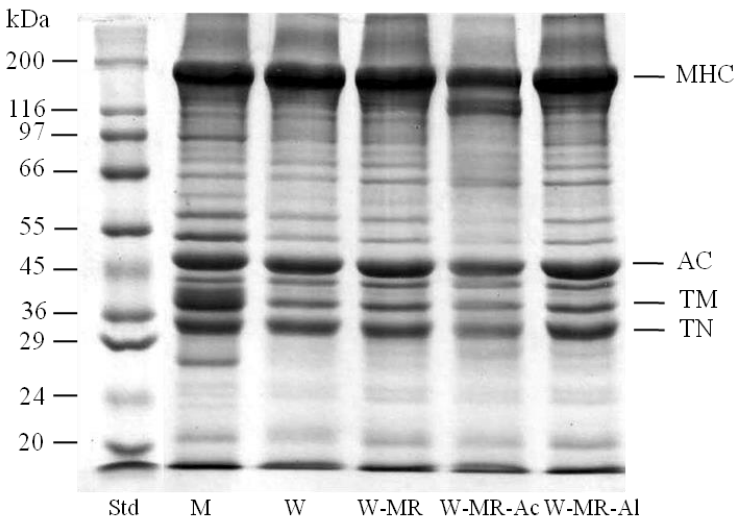


Figure 14. Protein patterns of Indian mackerel mince before and after different pretreatments as determined by SDS-PAGE electrophoresis. Std: standard protein marker; M: mince; W: washed mince; W-MR: washed mince with membrane removal; W-MR-Ac: W-MR with acid solubilisation; W-MR-Al: W-MR with alkaline solubilisation. MHC: myosin heavy chain, AC: actin; TM: tropomyosin; TN: troponin.

4.4.2 Changes in DH and compositions in protein hydrolysate during hydrolysis

4.4.2.1 Changes in DH

Hydrolysis of mince and W–MR–Al using Alcalase is shown in Figure 15. Degree of hydrolysis (DH) of protein hydrolysates increased as hydrolysis time increased ($P < 0.05$). DH is known to have the influence on several properties of protein hydrolysates (Adler-Nissen, 1979). Rapid hydrolysis was observed within the first 30 and 60 min, respectively, when mince and W–MR–Al were used. The rapid hydrolysis in the initial phase indicated that a large number of peptide bonds were hydrolysed. Thereafter, the hydrolysis rate was decreased, mainly due to a decrease in available hydrolysis sites, enzyme autodigestion and/or product inhibition (Kristinsson and Rasco, 2000a). Typical hydrolysis curves, like that seen here for Indian mackerel, were reported for Pacific whiting solid wastes (Benjakul and Morrissey, 1997), round scad (Thiansilakul *et al.*, 2007b) and brownstripe red snapper (Khantaphant *et al.*, 2011a). Within the first 30 min, mince was hydrolysed more rapidly whilst W–MR–Al demonstrated higher DH up to 120 min of hydrolysis ($P < 0.05$). The result suggested that overall W–MR–Al was more susceptible to cleavage by Alcalase. This was possibly due to more exposure of peptide bonds of the protein isolates, where Alcalase could cleave the peptides more effectively. During alkaline solubilisation, the repulsion between protein molecules resulted in the dissociation of actomyosin complex. The looser protein structures could have been more prone to hydrolysis by Alcalase. Thus, the configuration of protein was another important factor governing the rate of hydrolysis. As a consequence, different DH at the same time point could be obtained by using different proteinaceous starting materials.

4.4.2.2 Changes in non-haem iron content

Non-haem iron content in hydrolysates from mince and W– MR–Al during hydrolysis was monitored as shown in Figure 16A. The increase in non-haem iron content was observed with increasing hydrolysis time for both samples ($P < 0.05$). During hydrolysis, enzymatic breakdown of haem proteins, like any other protein in the

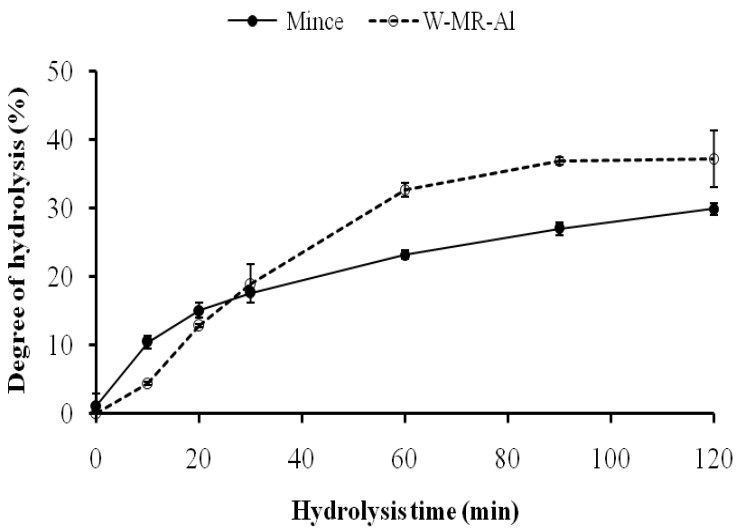
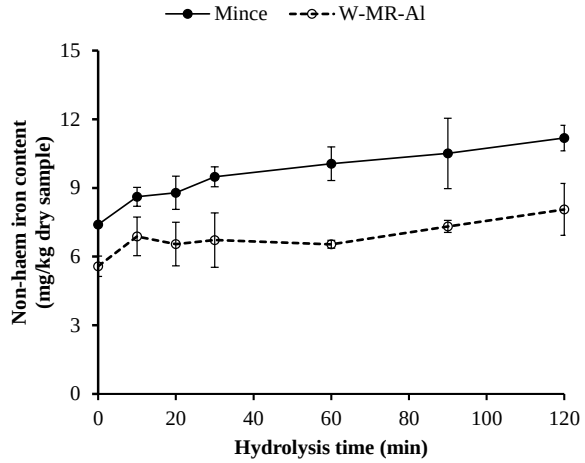


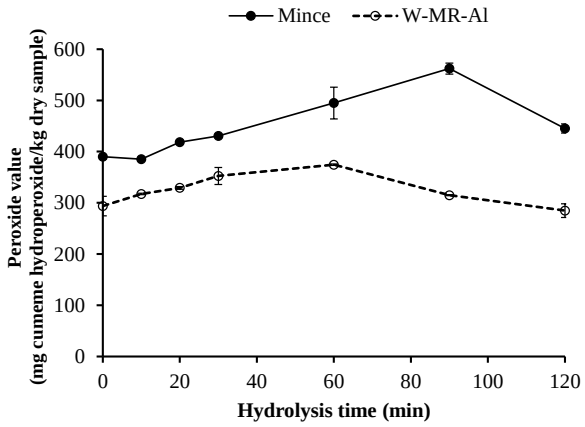
Figure 15. Changes in DH of Indian mackerel mince and W-MR-Al during hydrolysis with Alcalase at a level of 1% w/w. The reaction was performed at 50 °C, pH 8 and was terminated at 100°C for 10 min. The bars represent the standard deviation (n=3).

sample, will lead to substantial changes in their conformation and eventually the destabilisation and/or release of haem which normally is bound to the protein. This result suggested that the disruption of porphyrin ring of haem protein remaining in both samples more likely occurred during the hydrolysis process. The heating step used to arrest the reaction could also lead to an increase in iron content since unhydrolysed or partially intact haem proteins would be denatured, thereby destabilising the haem group. Kristensen and Andersen (1997) reported a slight increase in free iron (0.46 to 0.50 ppm) from horse heart metMb after heating with temperature of 25 to 90 °C. Thiansilakul *et al.* (2011) found that conformational changes of apo-myoglobin by heating might weaken porphyrin ring with the subsequent iron released. Released iron might act as a pro-oxidant, which was able to enhance lipid oxidation. Furthermore, Kristensen and Andersen (1997) revealed that heated haem iron had a more significant effect on pro-oxidative activity than did heated free iron. At the same hydrolysis time point, a lower non-haem iron content was found in the hydrolysate from W–MR–Al than what was found in mince ($P < 0.05$). The removal of water soluble components, including haem proteins, by washing resulted in fewer porphyrin rings which could be

(A)



(B)



(C)

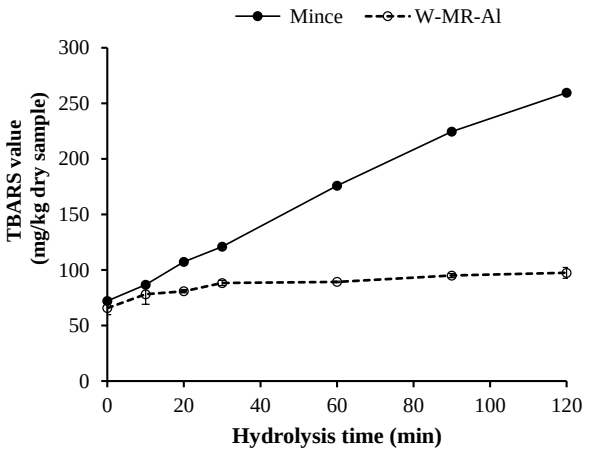


Figure 16. Changes in (A) non-haem iron content, (B) PV and (C) TBARS value of Indian mackerel mince and W-MR-Al during hydrolysis with Alcalase at a level of 1% w/w. The reaction was performed at 50°C, pH 8 and was terminated at 100°C for 10 min. The bars represent the standard deviation (n=3).

disrupted during hydrolysis. The result was in agreement with the low amount of myoglobin and low haem iron content retained in W–MR–Al (Table 11). Therefore, the pro-oxidant present during hydrolysis can be minimised by pretreatment of the protein substrate via washing, membrane removal, followed by alkaline solubilisation.

4.4.2.3 Changes in PV and TBARS

Lipid oxidation of hydrolysates produced from mince and W–MR–Al during hydrolysis of 120 min expressed as PV and TBARS values is shown in Figure 16B and 16C, respectively. A marked increase in PV was observed during hydrolysis of mince and W–MR–Al at 50 °C which peaked after 90 and 60 min, respectively. Thereafter, a decrease in PV was seen ($P < 0.05$) which suggested that the hydroperoxides formed were decomposed to other compounds. The result clearly demonstrated that lipid oxidation took place during hydrolysis. When comparing PV values of the hydrolysates from mince and W–MR–Al, higher overall values were observed for the former. The result suggested that washing/membrane removal in combination with alkaline solubilisation process more likely removed some pro-oxidants as well as lipid substrates from the muscle. Apart from myoglobin, haemoglobin in fish muscle also serves as an effective catalyst of lipid oxidation (Richards and Hultin, 2000). Haem dissociation, haem destruction and iron released play a role in lipid oxidation of fish muscle (Gandemer, 1999). Thiansilakul *et al.* (2011) reported that negligible PV was found in washed Asian seabass mince but increased dramatically when myoglobin was incorporated into the mince. The removal of those compound was associated with the enhanced stability of the hydrolysates during hydrolysis.

Changes in TBARS during hydrolysis of both substrates are shown in Figure 16C. The TBARS value of both hydrolysates increased as hydrolysis time increased ($P < 0.05$). The result reconfirmed that lipid oxidation took place during hydrolysis, particularly when mince was used as the substrate. A much larger increase in TBARS of hydrolysates produced from mince was observed and this was most likely in part related to the increase in non-haem iron content during extended hydrolysis (Figure 16A). The higher neutral and phospholipid contents also to some extent were expected to account for this much higher level of oxidation of the hydrolysed mince.

Haem proteins and haem-iron, both found in substantially higher levels in mince compared to W–MR–Al, are very potent pro-oxidants and are known to cleave preformed hydroperoxides into secondary oxidation products like those measured in the TBARS assay (Undeland *et al.*, 2002). The higher initial level of both can also account for this increase in TBARS seen during hydrolysis of the mince. For hydrolysate produced from W–MR–Al, only a slight increase in TBARS was observed within the first 30 min of hydrolysis ($P < 0.05$). Thereafter, TBARS values remained steady until the end of hydrolysis ($P > 0.05$). Even though a large amount of lipid as well as pro-oxidant could be removed, the lipid oxidation of hydrolysate produced from W–MR–Al still occurred to some extent. This was probably because some membrane lipids were still retained in W–MR–Al along with some active prooxidants. Some lipids might be co-precipitated with the proteins during isoelectric precipitation (Kristinsson *et al.*, 2005b). The result however showed that W–MR–Al yielded hydrolysate with significantly reduced levels of lipid oxidation products compared to untreated mince.

4.4.3 Colour and fortification of protein hydrolysate powders in low fat milk

L*, a*, b*, ΔE* and ΔC*values of protein hydrolysate powder from Indian mackerel mince and W–MR–Al are shown in Table 12. Freeze-dried hydrolysates were creamy brownish in colour. Batista *et al.* (2010) reported that hydrolysate produced from black scabbardfish exhibited the dark greynish due to a black skin pigment of this species. $FPH_{W–MR–Al}$ showed the lower a* (redness), b* (yellowness), ΔE* (total colour difference) and ΔC* (colour intensity difference) than FPH_{mince}. Washing, membrane removal in combination with alkaline solubilisation could remove pigments such as haemoglobin or myoglobin, leading to lower amounts of coloured compounds retained in the resulting mince (Table 11). Additionally, during the hydrolysis process, FPH_{mince} underewent lipid oxidation to a higher extent, thereby producing carbonyl compounds (e.g. aldehydes and ketones). Those compounds could react with amino groups of free amino acids or peptides via non-enzymatic browning reaction. This can result in a product with a more yellow colour than $FPH_{W–MR–Al}$.

Table 12. Colour of protein hydrolysates prepared from Indian mackerel mince and W-MR-Al

Parameters	FPH_{mince}	$FPH_{W\text{-}MR\text{-}Al}$
L^*	92.21±0.21a†	92.37±0.32a
a^*	-0.55±0.05a	-0.96±0.03b
b^*	11.85±0.17a	9.54±0.63b
ΔE^*	11.39±0.15a	9.05±0.64b
ΔC^*	10.5±30.17a	8.25±0.63b

Values are given as mean ± SD (n=3)

FPH: lyophilised fish protein hydrolysate

† Different letters within the same row indicate the significant differences ($P < 0.05$)

When low-fat milk was fortified with both hydrolysates at different levels (0.1–0.3%), the likeness scores of sensory panelists were found to be different (Table 13). Regarding the colour likeness, there was no difference in scores among all milk samples tested ($P > 0.05$). The milk fortified with $FPH_{W–MR–Al}$ had no change in likeness scores for odour when the levels were added up to 0.3% ($P > 0.05$). However, $FPH_{W–MR–Al}$ at a level of 0.3% resulted in the decrease in flavour and overall likeness score ($P < 0.05$). For the sample fortified with 0.1% FPH_{mince}, no changes in odour likeness score were observed ($P > 0.05$). However, the fortification of FPH_{mince} at all levels resulted in a decrease in other attributes tested ($P < 0.05$). Therefore, FPH_{mince} contributed to a decrease in sensory properties. This was most likely due to the fishy odour caused by lipid oxidation in hydrolysate. Lipid oxidation in fish muscle is well known to be associated with fishy odours (Maqsood and Benjakul, 2011a). Sohn *et al.* (2005) reported that the offensive odour detected in fish flesh was directly related with the formation of secondary lipid oxidation. Therefore, it was concluded that the strongfishy smell in the resulting hydrolysates from fish mince is the major problem, whilst the use of W–MR–Al as raw material significantly reduces this problem. Thus, the hydrolysate produced from Indian mackerel with the W–MR–Al pretreatment is expected to be fortified into milk at a level of 0.2% without detrimental effect on sensory property.

Table 13. Likeness score of low fat milk fortified with protein hydrolysates prepared from Indian mackerel mince and W-MR-Al at different levels

Attributes	Control† (w/o hydrolysate)	FPH_{mince}			$FPH_{W\text{-}MR\text{-}Al}$		
		0.1%	0.2%	0.3%	0.1%	0.2%	0.3%
Colour	8.13±0.80a*,†	7.93±0.96a	7.90±0.98a	8.00±0.89a	7.90±0.94a	8.07±0.75a	8.07±0.75a
Odour	7.66±0.77a	7.40±1.19a	6.74±1.39bc	6.21±1.22c	7.65±1.09a	7.46±1.17a	7.13±1.29ab
Flavour	7.38±0.78a	6.39±1.03bc	5.86±1.25cd	5.35±1.29d	7.14±1.09a	6.79±1.26ab	6.41±1.42bc
Overall	7.59±0.84a	6.69±1.23bc	6.16±1.31c	6.17±1.13c	7.14±1.24ab	7.08±1.08ab	6.92±1.20b

Values are given as mean ± SD, evaluated by 30 untrained panellists.

†Score are based on a 9-point hedonic scale (1: dislike extremely, 5: Neither like nor dislike, 9: Like extremely).

* Different letters within the same row indicate the significant differences ($P < 0.05$).

4.5 Conclusion

Pretreatment of mince played a significant role in lowering prooxidants and lipids, especially neutral lipids and membrane phospholipid in the resulting Indian mackerel mince. Washing along with a process to remove membranes prior to alkaline solubilisation was very effective in preparing a substrate for protein hydrolysate production, where lipid oxidation was significantly reduced and fishy odour and taste issues were minimised. Milk was successfully fortified with fish protein hydrolysates prepared with the pretreatment at a level of 0.2%.

CHAPTER 5

LIPID OXIDATION AND FISHY ODOUR IN PROTEIN HYDROLYSATE DERIVED FROM NILE TILAPIA (*OREOCHROMIS NILOTICUS*) PROTEIN ISOLATE AS INFLUENCED BY HAEMOGLOBIN

5.1 Abstract

Lipid oxidation associated with fishy odour development in Nile tilapia protein hydrolysate as affected by oxyhaemoglobin (oxy-Hb) and methaemoglobin (met-Hb) was comparatively studied. During hydrolysis of protein isolate (PI) up to 120 min, non-haem iron content, peroxide value and thiobarbituric acid reactive substances slightly increased ($P < 0.05$). When oxy-Hb or met-Hb was incorporated, the marked increases in all parameters were observed, especially within the first 60 min of hydrolysis. The higher increases were obtained with the latter, suggesting that met-Hb was more pro-oxidative than oxy-Hb. However, no differences in degree of hydrolysis of all samples were observed ($P > 0.05$). The marked increases in the b*, ΔE*, ΔC* values, fishy odour/flavour and volatile compounds were also found in the resulting hydrolysate containing either oxy-Hb or met-Hb. Thus, Hb, particularly met-Hb, induced lipid oxidation and the development of a fishy odour/flavour in fish protein hydrolysate.

5.2 Introduction

Lipid oxidation is one of the key problems associated with the loss in quality of muscle foods (Maqsood *et al.*, 2012). This undesirable reaction lowers the acceptability of muscle and its products through discolouration, loss of nutritive value and development of off-odour. Haem proteins, such as myoglobin (Mb) and haemoglobin (Hb), have been shown to promote lipid oxidation in various foods model systems (Maqsood and Benjakul, 2011a; Thiansilakul *et al.*, 2012b) Hb is a major contributor to lipid oxidation in fish and fish product, since the blood is not practically removed prior to processing (Richards *et al.*, 2005). Thiansilakul *et al*. (2012b) reported

that Hb induced lipid oxidation in washed bighead carp (*Hypophthalmichthys nobilis*) mince more effectively than Mb.

Hb has a haem group, containing an iron (Fe) ion held in a heterocyclic ring. States of iron ion, either Fe^{2+} (ferrous) or Fe^{3+} (ferric), known as deoxy/oxy and met form, respectively, can be changed via reduction/oxidation mechanisms. Conversion of the ferrous form into the ferric form is called autoxidation, which is responsible for discolouration and acceleration of lipid oxidation. Superoxide anion radical is liberated in this process and is readily converted to hydrogen peroxide (H_2O_2) by dismutation. H_2O_2 formed can further generate ferrylHb, a strong pro-oxidant, that can facilitate lipid oxidation (Kanner and Harel, 1985). Alternatively, displacement of haem or released iron can also participate in lipid oxidation. Several mechanisms of the pro-oxidative power of Hb have been proposed (Everse and Hsia, 1997; Thiansilakul *et al.*, 2012b). Richards *et al*. (2005) reported that autoxidation and haemin loss of trout Mb and Hb were associated with lipid oxidation in washed fish muscle at pH 6.3. Grunwald and Richards (2006) revealed that rate of haem loss in haem protein was crucial for the induction of lipid oxidation.

Lean fish species, especially Nile tilapia, have been known to be a preferable raw material for protein hydrolysate production owing to their low contents of lipid and haem pigment. However, oxidative deterioration still took place in resulting hydrolysate and was associated with off-odour development (chapter 3). This was more likely due to the pro-oxidative effect of haem proteins in the muscle, which could act as pro-oxidant during hydrolysis. Recently, lipid oxidation and fishy odour could be found in hydrolysate from fish protein isolate (PI) (chapter 3). The remaining lipid, haem protein and non-haem iron released during hydrolysis could contribute to lipid oxidation of protein hydrolysate. Fishy odour mediated by lipid oxidation is undesirable and limits the application of hydrolysate, especially for supplementation. Nevertheless, little information regarding the effect of Hb on lipid oxidation and off-odour development during hydrolysis has been reported. Thus, the objective of this study was to elucidate the impact of Hb with different forms, oxy-Hb and met-Hb, on lipid oxidation and fishy odour development during hydrolysis of PI.

5.3 Materials and Methods

5.3.1 Chemicals

Alcalase 2.4L (E.C. 3.4.21.62) with the activity of 2.4 unit/g was obtained from Novozyme (Bagsvaerd, Denmark). 2,4,6-Trinitrobenzenesulfonic acid (TNBS), bathophenanthroline disulfonic acid, sodium dodecyl sulfate (SDS) and 1,1,3,3 -tetramethoxypropane were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid, sodium nitrite, ferrous chloride and iron standard solution were obtained from Merck (Darmstadt, Germany). Disodium hydrogen phosphate, sodium dihydrogen phosphate, 2-thiobarbituric acid and cumene hydroperoxide were procured from Fluka (Buchs, Switzerland). Methanol, acetone, chloroform and ammonium thiocyanate were obtained from Lab-Scan (Bangkok, Thailand). All chemicals were of analytical grade.

5.3.2 Fish samples

Live Nile tilapia (*Oreochromis niloticus*) with a weight of 0.8–1.0 kg/fish were purchased from a local market in Hat Yai, Songkhla province, Thailand. Fish were transported in a tank to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand within 30 min. Upon arrival, four fish were bled from the caudal vein by severing cut off. Blood was collected in a 50 ml polypropylene tube rinsed with 150 mM NaCl solution containing sodium heparin (30 U/ml) with a ratio of 1:4.The collected blood was stirred continuously in order to avoid coagulation.

After blood collection, whole fish were washed and the flesh was separated manually. Flesh was minced to uniformity using a Moulinex AY46 blender (Group SEB, Lyon, France) in a walk-in cold room (4 °C). The mince (pH 6.73) obtained was placed in polyethylene bags and kept in ice not longer than 2 h before use.

5.3.3 Preparation of different forms of haemoglobin

5.3.3.1 Preparation and quantification of haemoglobin

Hb was prepared according to the method of Fyhn *et al*. (1973). Heparinised blood was washed with four volumes of cold 1.7% NaCl in 1 mM Tris

buffer, pH 8.0. Centrifugation was done at 700 × g for 10 min at 4 °C using an Avianti J-E centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA). After the plasma was removed, the red blood cells were washed for three times in 10 volumes of the same buffer, followed by centrifugation at 700 ×g. Red blood cells were lysed in three volumes of 1 mM Tris buffer (pH 8.0) for 1 h. One-tenth volume of 1 M NaCl was then added to aid in stromal removal before centrifugation at 10,000 ×g for 15 min at 4 °C. Prepared Hb was stored at −40 °C until used.

Hb content was determined following the method described by Brown (1961). The prepared Hb was mixed with 50 volumes of cold 50 mM Tris, pH 8.0 to obtain the absorbance at 415 nm of 0.5–0.6. Approximately 1 mg of sodium dithionite was added to 1.5 ml of prepared Hb and mixed thoroughly. The solution was scanned from 400 to 440 nm with a scanning rate of 100 nm/min using a UV 1800 spectrophotometer (Shimadzu, Kyoto, Japan) and 50 mM Tris buffer (pH 8.0) was used as blank. A standard curve was generated from bovine Hb. Calculation of the Hb content was performed using the absorbance at the peak (~415 nm) minus the absorbance at the valley.

5.3.3.2 Preparation of oxy- and met-haemoglobin

Oxy- and met-Hb were prepared as per the method of Tang *et al*. (2004) with some modifications. To obtain the oxy form, 1.5 mg of sodium dithionite was added to an aliquot of Hb solutions (3 ml; 2 mg protein/ml). Met-Hb was prepared by adding 1.5 mg of potassium ferricyanide to 3 ml of Hb solutions. To remove sodium dithionite and potassium ferricyanide, the samples were placed in a dialysis bag and dialysed against 10 volumes of cold 50 mM Tris-buffer, pH 8.0 with four changes of dialysis buffer. The dialysates obtained, representing of oxy-Hb and met-Hb, respectively, were subjected to absorption spectra analysis. The spectra were recorded from 350 to 750 nm at the scanning rate of 100 nm/min using 50 mM Tris-buffer, pH 8.0 as blank. To determine the proportions of three Hb forms, deoxy-Hb, oxy-Hb and met-Hb were calculated by a modified Krzywicki's equations (Tang *et al.*, 2004) as follows:

$$[\text{Deoxy-Hb}] = -0.543R_1 + 1.594R_2 + 0.552R_3 - 1.329$$

$$[\text{Oxy-Hb}] = 0.722R_1 - 1.432R_2 - 1.659R_3 + 2.599$$

$$[\text{Met-Hb}] = -0.159R_1 - 0.085R_2 + 1.262R_3 - 0.520$$

where $R_1 = A_{582}/A_{525}$, $R_2 = A_{557}/A_{525}$ and $R_3 = A_{503}/A_{525}$.

5.3.4 Effect of different forms of haemoglobin on lipid oxidation in protein hydrolysate

5.3.4.1 Preparation of protein isolate

PI was prepared using alkaline solubilising process from washed mince following the method as described in chapter 3. Washed mince was homogenised with five volumes of cold distilled water (2–4 °C) using an IKA Labortechnik homogeniser (Selangor, Malaysia) at a speed of 11,000 rpm for 1 min. The homogenate was adjusted to pH 11 and placed on ice for 60 min with a continuous stirring. The mixture was then centrifuged at 5,000 ×g for 10 min at 4 °C. The alkaline soluble fraction obtained, referred to as 'PI solution', is used as substrate for hydrolysis study.

5.3.4.2 Preparation of protein hydrolysate containing different forms of haemoglobin

PI solution was mixed with distilled water to obtain a final protein concentration of 2% (w/v) as determined by the Biuret method (Robinson and Hogden, 1940). The solution was then adjusted to pH 8.0 using 2 M NaOH. Oxy-Hb and met-Hb solutions were added in the prepared PI solution to obtain a final Hb concentration of 13 μmol/kg. For the control sample, distilled water was added instead of Hb solution. All mixtures were pre-incubated at 50 °C for 10 min prior to enzymatic hydrolysis using Alcalase at a level of 1% (w/w). The hydrolysis was conducted at 50 °C up to 120 min. The samples were taken at the designated time (0, 10, 20, 30, 60, 90 and 120 min) and placed into the temperature-controlled water bath (Memmert, Schwabach, Germany) at 90 °C for 10 min to terminate the enzymatic reaction. The obtained hydrolysates with different hydrolysis times were lyophilised using a Scanvac Model Coolsafe 55 freeze

dryer (Coolsafe, Lynge, Denmark). All dried hydrolysate samples were then subjected to analyses.

5.3.4.3 Analyses

A) Determination of the degree of hydrolysis

The degree of hydrolysis (DH) of protein hydrolysate was determined according to the method of Benjakul and Morrissey (1997). Protein hydrolysate with appropriate dilution (125 µl) was added with 2 ml of 0.2 M phosphate buffer (pH 8.2) and 1 ml of 0.01% TNBS solution. The solutions were mixed thoroughly and placed in a temperature-controlled water bath at 50 °C for 30 min in the dark. The reaction was terminated by adding 2 ml of 0.1 M sodium sulfite. The mixtures were cooled at room temperature for 15 min. The absorbance was read at 420 nm and the α-amino group was expressed in terms of L-leucine. The DH was defined as follows:

$$DH = [(L_t - L_0)/(L_{max} - L_0)]\times 100$$

where L_t is the amount of α-amino acid released at time t, L_0 is the amount of free amino group in original PI solution, and L_{max} is the total amino group content in the initial PI solution obtained after acid hydrolysis (6 M HCl at 100 °C for 24 h).

B) Determination of non-haem iron content

Non-haem iron content was determined according to the method of Schricker *et al.* (1982). The hydrolysate samples (100 mg) were weighed and transferred into a screw-cap test tube and 50 µl of 0.39% sodium nitrite were added. Four millilitres of a mixture of 40% trichloroacetic acid and 6 M HCl (ratio of 1:1 (v/v), prepared freshly) were added. The tightly capped tubes were placed in an incubator shaker (W350, Memmert, Schwabach, Germany) at 65 °C for 22 h and then cooled at room temperature for 2 h. The supernatants (400 µl) were mixed with 2 ml of the batho colour reagent, a freshly prepared mixture of bathophenanthroline disulfonic acid, double-deionised water and saturated sodium acetate solution at a ratio of 1:20:20

(w/v/v). After vortexing using a Vortex-Genie2 mixer (Scientific Industries, Bohemia, NY, USA) and standing for 10 min, the absorbance was measured at 540 nm.

The non-heme iron content was calculated from the standard curve, in which Fe $(NO_3)_2$ dissolved in HNO_3 with concentrations ranging from 0 to 5 ppm was used. The concentration of non-haem iron content was calculated and expressed as mg/100 g dry sample.

C) Determination of peroxide value (PV)

PV was determined according to the method of Richards and Hultin (2000) with slight modifications. The hydrolysate samples (100 mg) were homogenised at a speed of 13,500 rpm for 2 min in 11 ml of chloroform/methanol (2:1). Homogenate was then filtered using a Whatman No.1 filter paper. Two millilitres of 0.5% NaCl were then added to 7 ml of the filtrate. The mixtures were vortexed at a moderate speed for 30 s and then centrifuged at 3,000 ×g for 3 min at 4 °C to separate the sample into two phases. To the lower phase (3 ml), 2 ml of cold chloroform/methanol (2:1) mixture, 25 µl of 30% ammonium thiocyanate and 25 µl of 20 mM iron (II) chloride were added. The reaction mixture was allowed to stand for 20 min at room temperature prior to reading the absorbance at 500 nm. The blank was prepared in the same manner, except distilled water was used instead of ferrous chloride. A standard curve was prepared using cumene hydroperoxide at concentrations ranging from 0.5 to 2 ppm. PV was expressed as mg of cumene hydroperoxide/kg dry sample.

D) Determination of thiobarbituric acid reactive substances (TBARS)

TBARS were determined as described by Buege and Aust (1978). The hydrolysate samples (100 mg) was homogenised with 2.5 ml of a solution containing 0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 M HCl. The mixture was heated in a boiling water bath (95-100 ºC) for 10 min to develop a pink colour, cooled with running tap water and centrifuged at 3,600 ×g at 25 ºC for 20 min. The absorbance of the supernatant was measured at 532 nm. A standard curve was

prepared using 1,1,3,3-tetramethoxypropane with concentrations ranging from 0 to 10 ppm. TBARS were calculated and expressed as mg malonaldehyde/kg dry sample.

5.3.5 Preparation and characterisation of hydrolysate powder

Hydrolysates derived from PI in the absence and presence of oxy-Hb and met-Hb were prepared with the hydrolysis time of 120 min as mentioned previously. The obtained hydrolysate mixtures were centrifuged at 2,000 ×g for 10 min at 4 °C. The supernatant were collected and then lyophilised. The resulting powders were subjected to analyses as follows:

5.3.5.1 Colour measurement

The colour of hydrolysate powder was measured by a colourimeter (ColourFlex, Hunter Lab Reston, VA, USA) and reported in the CIE system. L^*, a^*, b^*, ΔE^* and ΔC^* indicating lightness, redness/greenness, yellowness/blueness, total difference of colour and the difference in chroma, respectively, were determined. The ΔE^* and ΔC^* were calculated as follows:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where ΔL*, Δa* and Δb* are the differentials between colour parameter of the samples and the colour parameter of the white standard (L^*= 92.82, a^*= -1.24 b^*= 0.50)

$$\Delta C^* = C^*_{sample} - C^*_{standard}$$

where $C^* = \sqrt{(a^*)^2 + (b^*)^2}$

5.3.5.2 Evaluation of fishy odour/flavour

Sensory evaluation for fishy-odour/flavour in fish protein hydrolysate was conducted using Generic descriptive analysis (Meilgaard *et al*., 2007). The sensory panel (10-15 panellists) was screened for perception of fishy odour/flavour, familiarity with fish consumption and ability to determine differences between fish protein

hydrolysate solution (International Standard ISO 8586-1, 1993; ASTM Special Technical Publication 758, 1981; Meilgaard, 2007).

Fishy odour and flavour in hydrolysate samples were evaluated by 10 trained panellists (7 female and 3 male) with the ages of 25-32. Prior to the evaluation, the panellists were trained three hours a week for totally one month. Panellists were trained with standards for two sessions using a 15-cm unstructured line scale anchored from 'none' to 'extremely strong' for fishy odour/flavour as per the method of Thiansilakul *et al.* (2010) and Shaviklo *et al.* (2012) with a slight modification. Fish protein hydrolysate produced from Nile tilapia stored in ice for 18 days was prepared and used as a standard with fishy odour/flavour. The working standard was prepared by dissolving fish protein hydrolysate in water to obtain concentration of 0, 0.25, 0.5 and 1.0% (w/v) representing the score of 0, 5, 10 and 15, respectively.

To test the samples, hydrolysate samples (1%) were placed in a sealable plastic cup and heated at 60 °C in a temperature controlled water bath for 5 min before serving. The samples were coded with 3-digit random numbers and presented to panellists on a tray in individual booths. Serving orders were completely randomised. The panellists were asked to open the cup and sniff the headspace above the samples for determining fish odour. To evaluate the fishy flavour, panelists were asked to taste the sample and rinsed their mouth between different samples.

5.3.5.3 Determination of volatile compounds

The volatile compounds in hydrolysate samples were determined using a solid-phase microextraction gas chromatography mass spectrometry (SPME-GCMS) following the method of Iglesias and Medina (2008) with a slight modification.

A) Extraction of volatile compounds by SPME fibre

To extract volatile compounds, 1 g of sample was mixed with 4 ml of deionised water and stirred continuously to dissolve sample. The mixture was heated at 60 °C in 20 ml headspace vial (Agilent Technologies, Palo Alto, CA, USA) with equilibrium time of 10 h. The SPME fibre (50/30 µm DVB/Carboxen^{TM}/PDMS StableFlex^{TM}) (Supelco, Bellefonte, PA, USA) was conditioned at 270 °C for 15 min

before use and then exposed to the headspace. The volatile compounds were allowed to absorb into the SPME fibre at 60 °C for 1 h. The volatile compounds were then desorbed in the GC injector port for 15 min at 270 °C.

B) GC-MS analysis

GC-MS analysis was performed in a HP 5890 series II gas chromatography coupled with HP 5972 mass-selective detector equipped with a splitless injector and coupled with a quadrupole mass detector (Hewlett Packard, Atlanta, GA, USA). Compounds were separated on a HP-Innowax capillary column (Hewlett Packard, Atlanta, GA, USA) (30 m × 0.25 mm ID, with film thickness of 0.25 µm). The GC oven temperature program was: 35 ºC for 3 min, followed by an increase of 3 ºC/min to 70 ºC, then an increase of 10 ºC/min to 200 ºC, and finally an increase of 15 ºC/min to a final temperature of 250 ºC and holding for 10 min. Helium was employed as a carrier gas, with a constant flow of 1 ml/min. The injector was operated in the splitless mode and its temperature was set at 270 ºC. Transfer line temperature was maintained at 260 ºC. The quadrupole mass spectrometer was operated in the electron ionisation (EI) mode and source temperature was set at 250 ºC. Initially, full-scan-mode data were acquired to determine appropriate masses for the later acquisition in scan mode under the following conditions: mass range: 25-500 amu and scan rate: 0.220 s/scan. All analyses were performed with ionisation energy of 70 eV, filament emission current at 150 µA, and the electron multiplier voltage at 500 V.

C) Analysis of volatile compounds

Identification of volatile compounds in the samples was done by consulting ChemStation Library Search (Wiley 275 L). Identification of compound was performed, based on the retention time and mass spectra in comparison with those of standards from Chemstation Library Search (Wiley 275 L). Quantification limits calculated to a signal to noise (S/N) ratio of 10. The identified volatile compounds, related with lipid oxidation, including aldehydes, alcohols, ketones, etc., were presented in the term of abundance of each identified compound.

5.3.6 Statistical analysis

Experiments were run in triplicate using three lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests. T-test was used for pair comparison (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

5.4 Results and Discussion

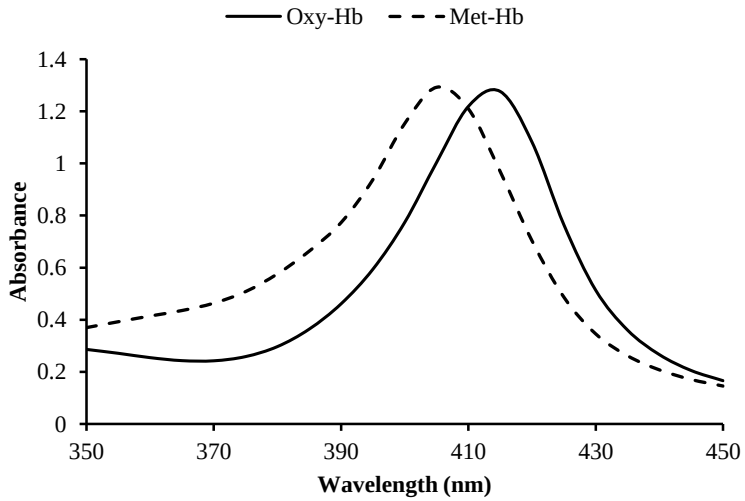
5.4.1 Absorption spectra of oxy-Hb and met-Hb

The absorption spectra of oxy-Hb and met-Hb are depicted in Figure 17. The intense peaks in a blue region (350–450 nm) corresponding to the Soret bands were found at 414 and 406 nm for oxy-Hb and met-Hb solutions, respectively (Figure 17A). The typical spectrum of oxy-haem protein shows a higher wavelength than that of met-haem protein (Tang *et al*., 2004). Thiansilakul *et al*. (2012b) reported that Soret bands of oxy-Hb and met-Hb isolated from bighead carp blood (pH 7) were found at 412 and 406 nm, respectively. The Soret band results mainly from the interaction of the haem moiety with apoHb, hence it can be used to monitor the integrity of haemoproteins (Chen and Chow, 2007). The result suggested that met-Hb had less affinity for the haem–globin complex than did oxy-Hb. This result was in accordance with Grunwald and Richards (2006) who reported that haemin is more strongly bound to globin in the oxy form as compared to the met form.

In a region of 450–750 nm, representing the redox state, the peaks at wavelengths of 541 and 576 nm were found for oxy-Hb solution, whilst met-Hb solution exhibited the peaks at wavelengths of 500, 536, 573 and 630 nm (Figure 17B). Similar spectra patterns were reported for Hb from tilapia (Kristinsson *et al.*, 2005a) flounder and cod (Undeland *et al.*, 2004). Kristinsson *et al*. (2005a) reported that the shift of wavelength and the decrease in peak height of oxy-Hb were caused by autoxidation, followed by met-Hb formation during storage at 4 °C. In the present study, oxy-Hb solution had the highest proportions of oxy-Hb (61.63%), followed by met-Hb (26.17%) and deoxy-Hb (12.20%), respectively. This indicated that the

oxidation of oxy-Hb to met-Hb occurred to some extent during the preparation of oxy-Hb solution. For met-Hb solution, met-Hb (91.09%) was dominant and deoxy-Hb (4.41%) and oxy-Hb (4.50%) were found at the lower levels. Oxy-Hb and met-Hb were reported to have different pro-oxidative activity in several food model systems (Richards *et al.*, 2005).

(A)



(B)

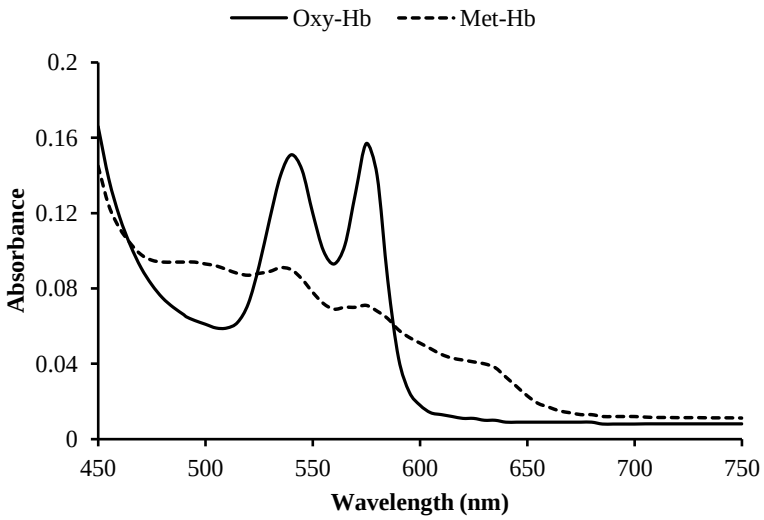


Figure 17. Absorption spectra in the regions of 350-450 (A) and 450-750 nm (B) of oxy-Hb and met-Hb (2 mg/ml, pH 8.0) from Nile tilapia blood.

5.4.2 Effect of oxy-Hb and met-Hb on DH and lipid oxidation of PI during hydrolysis

5.4.2.1 Changes in DH

Hydrolysis of PI in the absence and presence of oxy-Hb or met-Hb as a function of time is shown in Figure 18. Rapid hydrolysis was observed within the first 30 min as evidenced by the sharp increase in DH, followed by a slower rate of hydrolysis. At the initial stage of hydrolysis, a large number of peptides bonds were hydrolysed (Shahidi *et al.*, 1995).Thereafter, a reduction of hydrolysis rate was mainly due to the lower peptide bonds available for hydrolysis, enzyme inhibition and deactivation (Dong *et al.*, 2008). Similar patterns of hydrolysis curve were reported for different fish protein, including defatted skipjack roe (Intarasirisawat *et al.*, 2012), tuna dark muscle (Hsu, 2010) and smooth hound (Bougatef *et al.*, 2009). Nevertheless, the hydrolysis rates varied when different substrates were used. When oxy-Hb or met-Hb was incorporated, the similar DH was noticeable, compared with the control (without haem protein) at all hydrolysis times ($P > 0.05$). A small amount of Hb with both forms therefore had no influence on enzymatic hydrolysis of PI mediated by Alcalase.

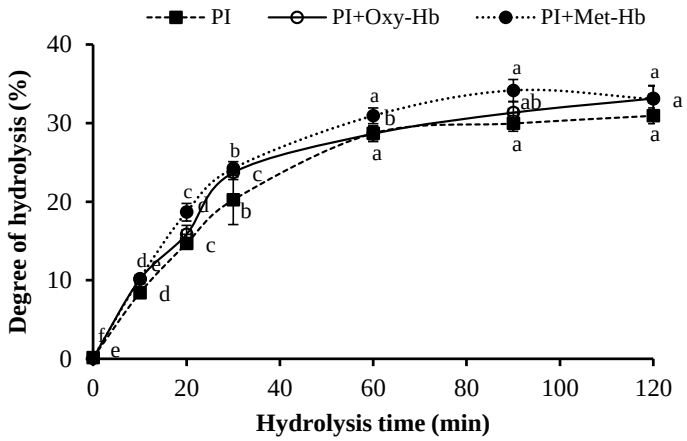
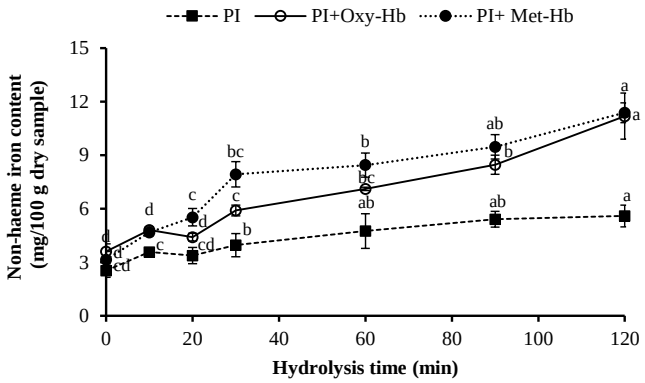


Figure 18. Changes in DH of Nile tilapia PI in the absence and presence of oxy-Hb or met-Hb (13 µmol/kg) during hydrolysis. The reaction was performed at 50 °C, pH 8 and was terminated at 90 °C for 10 min. Different letters within the same hydrolysate indicate significant differences ($P < 0.05$). The bars represent the standard deviation (n =3).

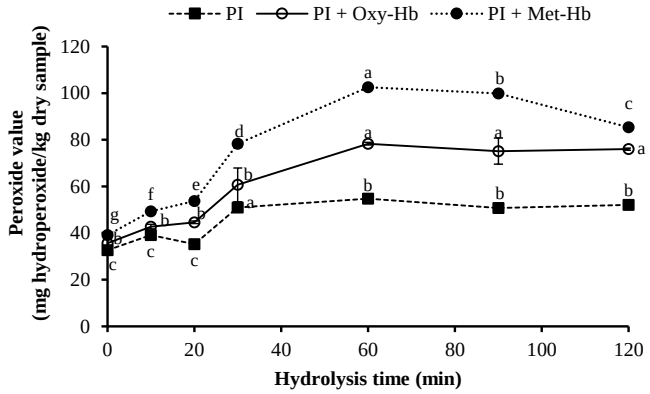
5.4.2.2 Changes in non-haem iron content

Changes in non-haem iron content of hydrolysate from Nile tilapia PI as affected by oxy-Hb or met-Hb during hydrolysis are shown in Figure 19A. The increases in non-haem iron content of all samples were observed with increasing hydrolysis time ($P < 0.05$). The result suggested that the disruption of porphyrin ring of haem protein occurred during hydrolysis, especially at high temperature (50 °C) used for enzymatic hydrolysis. Additionally, the cleavage of proteins, especially globin, by enzymatic hydrolysis into a shorter chain led to the destabilisation of haem group with subsequent release of free iron. Normally, haem group is surrounded in a hydrophobic pocket-like structure of globin, in which iron is occupied four sites with nitrogen of porphyrin ring and the other one site with histidine residue of globin. Grunwald and Richard (2006) noted that the cooking process loosened the haemin-globin linkage, allowing haemin mediated lipid oxidation in cooked washed fish mince to proceed. Increasing temperature might be associated with protein unfolding. Due to the interruption of covalent bonding in haem structure, iron atom could undergo changes in redox form and was easily kicked out (Grunwald and Richards, 2006). When oxy-Hb or met-Hb was present, the higher increase in non-haem iron content was noticeable with increasing hydrolysis time, compared with the control (without haem protein). Hbs, both reduced and oxidised forms, have been known as the potential pro-oxidative substance in various food systems (Maqsood and Benjakul, 2011a; Richards and Hultin, 2000; Vareltzis *et al.*, 2008). Higher non-haem iron content was found in the sample containing met-Hb, compared with that comprising oxy-Hb during 30 and 90 min of hydrolysis ($P < 0.05$). The result suggested that met-Hb with lower affinity of haem–globin complex was more prone to destruction, in which free iron from haem moiety could be more released. Free iron released could be involved actively in lipid oxidation. The haemin loss from oxy-Hb could occur, following the formation of met-Hb which was loosely bound. The release of iron from oxy-Hb was more likely based on 2 steps, oxidation and destruction. Thus, the degree of haem loss was lower in oxy-Hb containing treatment. When the free radicals are presented at a critical level, intrinsic haem autoxidation could be more pronounced (Richards and Hultin, 2000). Thus, the

(A)



(B)



(C)

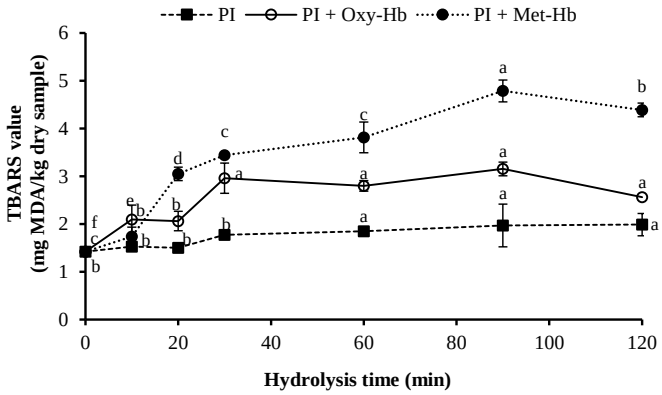


Figure 19. Changes in (A) non haem iron content, (B) peroxide and (C) TBARS values of Nile tilapia PI in the absence and presence of oxy-Hb or met-Hb (13 µmol/kg) during hydrolysis. Different letters within the same hydrolysate indicate significant differences ($P < 0.05$). The bars represent the standard deviation (n =3).

weakening of haem-globin complex at high temperature with pronounced hydrolysis might contribute to the release of free iron. Therefore, haem protein, particularly met-form, might be a potential pro-oxidant by providing free iron for acceleration of lipid oxidation during the hydrolysis of PI.

5.4.2.3 Changes in PV

PV of hydrolysate derived from PI without and with oxy-Hb or met-Hb during hydrolysis is shown in Figure 19B. PV of all samples tended to increase as hydrolysis time increased up to 60 min, indicating that lipid oxidation occurred during hydrolysis. Thereafter, no changes in PV were obtained in the control sample and hydrolysate containing oxy-Hb ($P > 0.05$). On the other hand, the gradual decreases in PV were observed for hydrolysate with met-Hb, which might be due to the decomposition of hydroperoxide to other compounds. At the same hydrolysis time, the lowest PV was noticeable in the control sample and the highest PV was found in hydrolysate containing met-Hb. The result indicated that met-Hb could enhance lipid oxidation more effectively than oxy-Hb. The potential mechanisms of met-Hb mediated lipid oxidation were widely proposed (Richards *et al.*, 2005; Thiansilakul *et al*., 2012b). MetHb formed can release its haem group 60 times greater than oxy- and deoxyHb (Hargrove *et al.*, 1997). In the present study, PI was used for hydrolysate preparation and potent pro-oxidants and lipid substrate, particularly triacylglyceride and phospholipid membrane, could be removed to high extent. Total lipid content in PI was 0.10 mg/100 g dry sample. Pretreatment of Nile tilapia muscle by alkaline solubilisation was an effective means to eliminate pro-oxidants and phospholipid membrane than other pretreatments (chapter 3). However, PV was still detectable in hydrolysate from PI, suggesting that some lipid substrates were still retained and underwent oxidation, particularly in the presence of effective pro-oxidant such as met-Hb. Apart from pro-oxidative role of haem proteins in lipid oxidation, superoxide radicals generated during autoxidation can promote oxidation (Maqsood and Benjakul, 2012). Superoxide radicals can be converted to H_2O_2, which facilitates ferrylHb catalyst formation (Kanner and Harel, 1985) and increases the reactivity of dissociated haemin (Robinson *et al.*, 2009). Thus, haem protein, especially met-Hb, played a role in acceleration of

lipid oxidation during hydrolysis. As a consequence, a higher content of hydroperoxide, a primary oxidation product, could be formed to a higher extent.

5.4.2.4 Changes in TBARS

Changes in TBARS of hydrolysate from Nile tilapia PI in the presence and absence of oxy-Hb and met-Hb during hydrolysis are shown in Figure 19C. The slight increases in TBARS were observed in the control hydrolysate, reconfirming that lipid oxidation still occurred during hydrolysis. TBARS values have been used to measure the concentration of relative polar secondary reaction products, especially aldehydes (Nawar, 1996). A progressive increase in TBARS formation was found when Hb was incorporated. Higher TBARS value was found in the samples with met-Hb, in comparison with that observed in those containing oxy-Hb during 20 and 120 min of hydrolysis. The similar trend was noticeable in comparison with PV and non-haem iron content (Figure 19A and 19B). Hb autoxidation, Hb subunit formation, ferryl radical formation, haem dissociation, haem destruction or iron release might rapidly occur and stimulate lipid oxidation. For hydrolysate from PI having oxy-Hb or met-Hb, TBARS slightly decreased after 90 min of hydrolysis. Those secondary products, especially those with carbonyl groups, plausibly reacted with free amino, peptide or protein presented in the hydrolysate, to form shiff's base (Dillard and Tappel, 1973). The amine-carbonyl or Maillard reaction products formed might have the antioxidative properties (Yu *et al.*, 2013), thereby retarding lipid oxidation. In addition, fish protein hydrolysates containing active peptides have been known to possess antioxidative activity. Klompong *et al.* (2008) reported that hydrolysate from yellow stripe trevally muscle prepared using Alcalase and Flavourzyme showed DPPH and ABTS radical scavenging activities as well as ferric reducing antioxidant power. The peptides with antioxidative activity generated during hydrolysis might partially contribute to the retardation of lipid oxidation. Furthermore, the volatile oxidation products might be lost during hydrolysis process. Higher release of free iron from met-Hb could be considered as a major contributor for acceleration of lipid oxidation of hydrolysate. Haemin release could break down lipid hydroperoxide to generate many radical species (alkoxy, peroxy or haem radical). Those radicals had the ability to abstract hydrogen from polyunsaturated fatty acid, thereby inducing lipid oxidation (Richards and Li, 2004).

Those secondary volatile products formed might be associated with off-odour development in the resulting protein hydrolysate.

5.4.3 Colour and sensory properties of protein hydrolysate

Colours of freeze-dried hydrolysate powders derived from PI in the absence and presence of oxy-Hb or met-Hb are presented in Table 14. The hydrolysates had a slight creamy yellowish in colour. Hb and Mb have been known to be responsible for colour of fish flesh and their product (Faustman *et al.*, 2010). Difference in redox state of haem proteins resulted in the discolouration of fish muscle. The lowest b*, ΔE* and ΔC* were obtained in hydrolysate without Hb addition ($P < 0.05$). Pre-washing and alkaline solubilisation of fish muscle yielded the resulting PI with lower pigments and haem proteins (chapter 3). The ligher colour of hydrolysate from Nile tilapia was obtained when PI was used as the starting material, compared with mince (chapter 3). When Hb was incorporated, the more yellowish colour were noticeable as indicated by the increases in b*, ΔE* and ΔC*-values. Additionally, the enhanced lipid oxidation might be associated with the higher yellowish colour of hydrolysates, especially those containing haem proteins. However, no changes in L* and a*-values of all samples were found ($P > 0.05$). Lipid oxidation has been known to play a role in yellow discolouration, mainly by providing the carbonyl groups involved in Maillard reaction (Thanonkaew *et al.*, 2007). Those carbonyls could react with amino group of peptide or free amino acids after hydrolysis via glycation. Greater concentrations of iron and haem proteins are associated with greater rates of lipid oxidation (Faustman *et al.*, 2006). The result was coincidental with the increase in PV and TBARS observed during hydrolysis of samples containing haem proteins, especially met-Hb (Figure 19B and 19C).

Fishy odour and flavour identified as typical off-odour/flavour of different samples are shown in Table 14. A negligible fishy odour/flavour was found in hydrolysate without Hb addition. The profound development of fishy odour and flavour took place in hydrolysate when Hbs were incorporated ($P < 0.05$). This indicated the role of Hb in development of fishy odour/flavour during hydrolysis process, mainly via the induction of lipid oxidation. The formation of secondary lipid oxidation products is

Table 14. Colour and sensory property of protein hydrolysate from Nile tilapia PI in the absence and presence of oxy-Hb or met-Hb

Parameters	Hydrolysates		
	without Hb	**with oxy-Hb**	**with met-Hb**
Colour			
L^*	93.20±0.34^{a†}	92.91±0.51^{a}	93.22±0.17^{a}
a^*	-0.61±0.02^{a}	-0.52±0.15^{a}	-0.57±0.03^{a}
b^*	4.20±0.03^{c}	4.48±0.13^{b}	4.87±0.10^{a}
ΔE^*	3.70±0.48^{c}	3.98±0.37^{b}	4.37±0.18^{a}
ΔC^*	2.90±0.02^{c}	3.17±0.16^{b}	3.57±0.19^{a}
Sensory property††			
Fishy odour	0.65±0.46^{b}	2.92±0.86^{b}	3.97±0.80^{a}
Fishy flavour	0.90±0.68^{c}	3.98±0.61^{b}	6.08±1.16^{a}

Values are given as mean ± SD (n=3).

† Different superscripts in the same row indicate significant differences ($P < 0.05$).

††Score are based on 15 cm unstructured line scales (0: none and 15: extremely strong fishy odour/flavour), evaluated by 10 trained panellists.

one of the main causes of the development of undesirable odours in fish muscle (Thiansilakul *et al.*, 2010). Sohn *et al*. (2005) reported that the changes in total lipid hydroperoxide content and TBARS of yellowtail (*Seriola quinqueradiata*) dark muscle were accompanied with the increasing intensity of fishy- and rancid off-odour. Fu *et al.* (2009) demonstrated that lipid oxidation associated with off-odour in silver carp mince were mediated by lipoxygenase and Hb known as fishy- and rancid-off odour. Amongst all samples tested, the highest fishy odour and flavour intensity were obtained in the hydrolysate added with met-Hb. Deoxy-Hb and/or met-Hb have previously been reported to be more active than other forms of haem proteins in initiating lipid oxidation (Richards and Hultin, 2000). The result suggested that a greater lipid oxidation generally correlated well with the development of undesirable odour in resulting hydrolysate. Therefore, the presence of Hb in fish muscle, not only promoted lipid oxidation, but was also associated with off-odour and flavour as well as increased discolouration of resulting hydrolysate. However, a low fishy odour score of hydrolysate from PI containing of Hb was obtained, when compared with that of hydrolysate from mince or unfresh mince (chapter 2). This was plausibly due to the fact

that haemoproteins and lipids were largely removed during pre-washing and alkaline solubilisation for PI preparation. This might lead to the lowered lipid oxidation. Although Hb is proven as stronger pro-oxidants, fishy odour derived from lipid oxidation was not developed obviously in the appropriately pretreated substrate with a small content of lipids.

5.4.4 Volatile compounds in fish protein hydrolysates

Volatile compounds developed in protein hydrolysate in the absence and presence of oxy-Hb or met-Hb is displayed in Table 15. Alcohols, aldehydes and ketones have been known to be associated with lipid oxidation (Maqsood and Benjakul, 2011a). In the absence of Hb, hydrolysate contained nonanal, 1-octen-3-ol, 1-hexanol and benzaldehyde, which were more likely due to the oxidation of lipids remaining in PI. Although most of lipids, both neutral and phospholipids, were removed effectively by alkaline solubilisation process, lipids were still retained to some level (chapter 3). When oxy-Hb or met-Hb was incorporated, 2-pentylfuran, 1-hexanol and benzaldehyde were found as dominant volatile compounds. Furthermore, other volatile aldehydes including heptanal were also detected when either oxy- or met-Hb was present. This was more likely contributed to fishy odour development of protein hydrolysate. Volatile alcohols (cis-p-methane-9-ol and 1-decanol) and ketones (3-hexen-2-one and 6-methyl-5-hepten-2-one) were also found in the hydrolysate containing oxy- or met-Hb. This indicated that lipid oxidation took place to a higher extent in the sample containing Hb. Amongst all sample tested, the highest amount of volatile compounds were found in hydrolysate added with met-Hb. Higher formation of volatile lipid oxidation products in met-Hb added sample was coincidental with higher TBARS formation (Figure 19C) and lower sensory score (Table 14). The result suggested that residual lipids in PI were more prone to oxidation in the presence of Hb, a strong pro-oxidant. Also, it was found that met-Hb was more pro-oxidative than oxy-Hb. Varlet *et al*. (2006) reported that carbonyl compounds involving 4-heptenal, octanal, decanal and 2,4-decadienal were responsible for fishy odour in salmon flesh (*Salmo salar*). The fishy volatiles identified in washed grouper, tilapia and sea bass mince accelerated by Hb were 2-ethylfuran, pentenylfuran and 2-pentyl furan (Maqsood and Benjakul, 2011a). During Maillard reaction, the intermediate products, such as α-dicarbonyl compounds or α-

aminoketones, undergo further reaction, in which furan is formed. Limacher *et al.* (2008) demonstrated that 2-methylfuran was largely formed by recombination of C_2 and C_3 fragments from aldo type sugars and/or amino acids. The furan was associated with almond like odour. Hexanal and nonanal were major compounds associated with rancid odour in silver carp mince mediated by Hb (Fu *et al.*, 2009). The result suggested that lipid oxidation of protein hydrolysate was more enhanced when Hb was present, especially in oxidized form. Therefore, the offensive odour associated with hydrolysate, caused by oxidative deterioration induced by Hb could be suppressed by removal of Hb from PI as much as possible prior to hydrolysis.

Table 15. Volatile compounds in protein hydrolysate from Nile tilapia PI in the absence and presence of oxy-Hb or met-Hb

Compounds/ Abundance	**Hydrolysates ($\times 10^9$)**		
	without Hb	**with oxy-Hb**	**with met-Hb**
3-hexen-2-one	ND*	0.47	0.45
2-pentyl furan	ND	0.87	1.11
6-methyl-5-hepten -2-one	ND	0.29	0.31
Heptanal	ND	0.34	0.43
Nonanal	0.74	0.59	0.67
1-octen-3-ol	0.20	0.15	0.32
1-hexanol	1.38	1.50	3.10
Benzaldehyde	1.38	1.78	2.40
Cis –p-methane-9-ol	ND	ND	0.70
1-decanol	ND	0.90	0.98

*ND: Not detectable

5.5 Conclusion

Hb was proven to be an effective pro-oxidant in hydrolysate derived from Nile tilapia-PI. Both oxy-Hb and met-Hb could accelerate lipid oxidation, in which the primary and secondary oxidation products were formed. However, met-Hb showed the greater impact on fishy off-odour development and discolouration of resulting hydrolysate in comparison with oxyHb.

CHAPTER 6

CHEMICAL COMPOSITIONS AND MUDDY FLAVOUR/ODOUR OF PROTEIN HYDROLYSATE FROM NILE TILAPIA AND BROADHEAD CATFISH MINCE AND PROTEIN ISOLATE

6.1 Abstract

Chemical compositions and muddy compounds in dorsal and ventral muscles of Nile tilapia and broadhead catfish were comparatively studied. On a dry weight basis, Nile tilapia was rich in protein (93.1–93.8%), whilst broadhead catfish contained protein (55.2–59.5%) and lipid (36.6–42.4%) as the major constituents. Ventral portion had higher lipid or phospholipid contents with coincidentally higher geosmin and/or 2-methylisoborneol (2-MIB) contents. Geosmin was found in mince of Nile tilapia and broadhead catfish at levels of 1.5 and 3.2 μg/kg, respectively. Broadhead catfish mince had 2-MIB at level of 0.8 μg/kg, but no 2-MIB was detected in Nile tilapia counterpart. When pre-washing and alkaline solubilisation were applied for preparing protein isolate (PI), lipid and phospholipid contents were lowered with concomitant decrease in geosmin and 2-MIB contents. Protein hydrolysate produced from PI had a lighter colour and a lower amount of muddy compounds, compared with that prepared from mince. Therefore, PI from both Nile tilapia and broadhead catfish could serve as the promising proteinaceous material, yielding protein hydrolysate with the negligible muddy odour and flavour.

6.2 Introduction

Nowadays, aquaculture provides approximately one-third of the world's fishery products. Muddy flavour and odour are amongst the most severe problems encountered in aquaculture and cause unacceptability by consumer as well as the reduction of market value of product. Two chemical compounds associated with muddy taints are known as geosmin and 2-methylisoborneol (2-MIB). The former compound renders an earthy pond-bottom taste, whilst the latter one is associated with a musty

taste (Robin *et al.*, 2006). These metabolites produced by cyanobacteria, actinomycetes and certain fungi, are excreted into the environment (Jensen *et al.*, 1994; Lovell and Sackey, 1973). Typically, fish readily absorbs these compounds through the gills, then transfer through the digestive tract, and finally accumulate in lipid rich tissue. Although both compounds are present at an extremely low level in fish tissue, they can cause the muddy flavour due to their low threshold values. Sensory threshold of geosmin in rainbow trout was estimated to be 0.9 μg/kg (Rohrlack *et al.*, 2005). This is slightly higher than that of channel catfish (0.7 μg/kg) (Dionigi *et al.*, 2000; Johnsen and Kelly, 1990). Threshold value of 2-MIB in rainbow trout was 0.6 μg/kg (Persson, 1980).

To reduce muddy flavour, several strategies have been proposed. Rohani and Yunus (1994) reported that soaking of gutted tilapia in salt solution (5% w/v) for 30 min prior to deboning was able to lower muddy off-flavour of surimi to some degree as reflected by the higher hedonic score. Additionally, soaking, dipping or washing the farm-fish with tamarind pulp, lemon juice, lemon grass and banana leaf ash can also minimise muddy taint associated with fish tissue (Bakar and Hamzah, 1997; Mohsin *et al.*, 1999). Recently, acid- and alkaline-aided solubilisation has shown significant potential to reduce lipids and phospholipids in fish muscle. DeWitt *et al.* (2007) reported that 2-MIB and gesosmin spiked in channel catfish (*Ictalurus punctatus*) were effectively removed by acid and alkaline solubilisation. Due to their lipophilic nature, these muddy compounds might be localised in the lipid rich portion in fish muscle. However, little information regarding the distribution of muddy compounds in Nile tilapia and broadhead catfish muscle has been reported. Additionally, the development of muddy flavour and odour associated with hydrolysate and its reduction has not been reported. The objective of this study was to investigate the effect of muscle position and pretreatment on muddy flavour and odour associated with the muscle and protein hydrolysate produced from Nile tilapia and broadhead catfish.

6.3 Materials and Methods

6.3.1 Chemicals and reagents

Geosmin, 2-methylisoborneol (2-MIB) and isobornyl acetate with purity more than 95% were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO,

USA). Methanol, hexane and water were HPLC grade, and obtained from Lab-Scan (Bangkok, Thailand). Alcalase 2.4L (E.C. 3.4.21.62) with the activity of 2.4 unit/g was obtained from Novozyme (Bagsvaerd, Denmark).

6.3.2 Collection and preparation of fish muscle

Fresh Nile tilapia (*Oreochromis niloticus*) and broadhead catfish (*Clarias macrocephalus*) with the age of 4-months and the body weight of 0.5–0.8 kg/fish were collected from a farm with earth ponds in Ranote, Songkhla province. Fish were transported in ice with a fish/ice ratio of 1:2 (w/w) to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand within 1 h. Upon arrival, fish were washed with tap water and the flesh was separated manually into dorsal and ventral portions. The dark meat located at the lateral line was discarded. The portions were then minced to uniformity using a Moulinex AY46 blender (Group SEB, Lyon, France) in a walk-in-cold room (4 °C). The mince samples from both portions were placed in polyethylene bags and kept in ice not longer than 2 h before analyses.

6.3.3 Analyses

6.3.3.1 Proximate analysis

All samples were subjected to proximate analysis, including moisture, protein, lipid and ash contents following the AOAC (2000) methods with analytical No. of 950.46, 928.08, 960.39 and 920.153, respectively.

6.3.3.2 Determination of phospholipid content

Prior to analysis, lipid was extracted according to the method of Lee *et al.* (1996) with a slight modification. One gram of sample was homogenised at a speed of 13,500 rpm for 2 min in 11 ml of chloroform/methanol (2:1, v/v) mixture using an IKA Labortechnik homogeniser (Selangor, Malaysia). Homogenate was then filtered using a Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, UK). Two millilitres of 0.5% NaCl were added to the filtrate, vortexed and then centrifuged at 3,000 ×g for 3 min to separate into two phases. The chloroform phase was collected and mixed with 0.5 g of anhydrous sodium sulphate. The solvent was then liberated by

nitrogen flushing until dryness. Phospholipid content was measured based on the direct spectrophotometric measurement of complex formation between phospholipids and ammonium ferrothiocyanate as described by Stewart (1980). A standard curve was prepared using phosphatidylcholine (0–50 ppm). The phospholipid content was expressed as mg/100 g sample.

6.3.3.3 Determination of geosmin and 2-MIB

Geosmin and 2-MIB were extracted using vacuum distillation/liquid–liquid extraction following the method of Tanchotikul and Hsieh (1990) with a slight modification.

Vacuum distillation: Ground samples (50 g) were weighed in a round bottom flask and mixed with 12.5 ml of HPLC grade water. To the mixture, 2.5 ml of methanol containing 50 ppb isobornyl acetate were added. Flask was then equipped with vacuum distillation apparatus. The flask containing sample was heated in an EYELA water bath OSB-2000 (Tokyo, Japan) by increasing the temperature from 50 to 90 °C with heating rate of 2 °C/min. Vacuum distillation was conducted at 0.073 MPa for 45 min. Two collection tubes submerged in ice bath (0 °C) were used to trap the condensed distillate. The distillate obtained (~20 ml) was then subjected to liquid–liquid extraction.

Liquid–liquid extraction: Liquid–liquid extraction was performed to separate the tested compounds. The distillate (20 ml) was extracted with 3 ml of hexane in a separatory funnel. The extraction process was repeated for three times. The hexane layer was transferred to a 25 ml Erlenmeyer flask containing 2–3 g of anhydrous sodium sulphate and shaken well. The mixture was then filtered using a Whatman No.4 filter paper to remove sodium sulphate. The solvent in the filtrate was removed by flushing nitrogen until dryness. Volume of analytes was made up to 200 µl using hexane prior to GC–MS analysis.

Gas chromatography–mass spectrometry: Compounds were separated on a TRACE TR-WAXMS capillary column (Thermo Scientific, San Jose, CA, USA) (30 m × 0.25 mm ID, with film thickness of 0.25 µm) equipped with gas chromatography (GC) (Trace GC Ultra/ ISQMS, Thermo Scientific Inc., San Jose, CA, USA). The GC conditions were operated as per the method as mentioned in chapter 2.

The mass spectrometers were run in the selected ion monitoring mode. Ions at m/z 95, 135 and 168 were monitored for 2-MIB, whilst 112, 126 and 182 were monitored for geosmin. The selected ion at m/z 93, 95, 121 and 136 were used to monitor for isobornyl acetate.

For semi-quantification, the calibration curve was prepared using a mixed solution of standard geosmin and 2-MIB at concentrations ranging from 25 to 400 ppb, in which isobornyl acetate (50 ppb) was used as an internal standard. A linear correlation was observed between the peak area and concentrations. The linear equation obtained was used to convert peak areas to concentrations of target compounds in the samples. The limit detection was 5 ppb for each compound.

6.3.4 Effect of pretreatment on chemical compositions and sensory properties of protein hydrolysate derived from the muscle of Nile tilapia and broadhead catfish

6.3.4.1 Preparation of fish mince and protein isolate (PI)

Flesh of Nile tilapia and broadhead catfish without lateral line was minced to uniformity using a blender. To prepare PI, the prepared mince was pre-washed and subjected to membrane separation as previously described in chapter 3. Fish mince was homogenised with five volumes of cold distilled water (2–4 °C) at a speed of 11,000 rpm for 1 min. The homogenate was adjusted to pH 11 using 2 M NaOH and placed on ice for 60 min with a continuous stirring. The mixture was then centrifuged at 5,000 ×g for 10 min at 4 °C. The alkaline soluble fraction was collected and adjusted to pH 5.5 to precipitate the proteins. The mixture was then centrifuged at 10,000 ×g for 20 min. The pellet obtained was referred to as ‘PI’. The mince and PI were subjected to determination of total lipid, phospholipid, geosmin and 2-MIB contents as described above.

6.3.4.2 Production of protein hydrolysate

Mince and PI from Nile tilapia and broadhead catfish were mixed with distilled water to obtain a final protein concentration of 2% (w/v). The mixtures were then adjusted to pH 8 using 2 M NaOH. All mixtures were pre-incubated at 50 °C for

10 min prior to enzymatic hydrolysis using Alcalase at 1.1–1.3% (w/w) for Nile tilapia, and 3.8–4.3% (w/w) for broadhead catfish to obtain DH of 30%, following the method of Benjakul and Morrissey (1997). After 2 h of hydrolysis, the reactions were terminated by heating the mixture in boiling water for 10 min. The mixture was then centrifuged at 2000 ×g at 4 °C for 10 min using an Avianti J-E centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA). The supernatant was collected and then lyophilised using a Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lynge, Denmark). The dry matters referred to as 'hydrolysates' prepared from mince and PI of both Nile tilapia and broadhead catfish were subjected to determination of geosmin and 2-MIB as mentioned previously. Colour and muddy odour/flavour of hydrolysate samples were also determined.

6.3.4.3 Colour measurement

The colour of hydrolysate powder was measured by a colourimeter (ColourFlex, Hunter Lab, Reston, VA, USA) and reported in the CIE system. L*, a*, b* and ΔE* and ΔC* indicating lightness, redness/greenness, yellowness/blueness, total difference in colour and the difference in chroma, respectively, were determined as per the method described in chapter 3.

6.3.4.4 Evaluation of muddy odour/flavour

Sensory evaluation for muddy odour/flavour in fish protein hydrolysate was conducted using Generic descriptive analysis (Meilgaard *et al*., 2007). The sensory panel (10-15 panellists) was screened for perception of muddy odour/flavour, familiarity with fish consumption and ability to determine differences between fish protein hydrolysate solution (International Standard ISO 8586-1, 1993; ASTM Special Technical Publication 758, 1981; Meilgaard, 2007).

Muddy odour and flavour in hydrolysate samples were evaluated by 10 trained panelists (7 female and 3 male) with the ages of 25–32. Prior to the evaluation, the panelists were trained three hours a week for totally one month. Panelists were trained with standards of mixed solution containing of geosmin and 2 MIB with a ratio of 1:1 (w/w) using a 15 cm unstructured line scale anchored from 'none' to 'extremely

strong' for muddy odour/flavour as per the method of Percival *et al.* (2008) with a slight modification. The working standards at final concentrations of 0, 1, 2 and 5 ppb represented the score of 0, 5, 10 and 15, respectively.

To test the samples, hydrolysate samples (1%, w/v) were placed in a sealable plastic cup and heated at 60 °C in a temperature controlled water bath for 5 min before serving. The samples were coded with 3-digit random numbers and presented to panellists on a tray in individual booths. Serving orders were completely randomised. The panelists were asked to open the sealable cup and sniff the headspace above the samples for determining muddy odour. To evaluate the muddy flavour, panelists were asked to test the samples and rinse their mouth between different samples.

6.3.5 Statistical analysis

Experiments were run in triplicate using three lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

6.4 Results and Discussion

6.4.1 Chemical compositions, geosmin and 2-MIB of dorsal and ventral muscles of Nile tilapia and broadhead catfish

Chemical compositions of dorsal and ventral muscle from Nile tilpia and broadhead catfish are shown in Table 16. Both portions of Nile tilapia and broadhead catfish had high moisture content, accounting for 82.1 – 83.7 and 70.5 – 72.7%, respectively. Puwastien *et al.* (1999) also reported that the protein content of raw freshwater fish muscle ranged from 17 to 20 g/100 g. Proteins constituted as the major component in Nile tilapia dorsal and ventral muscle, representing 93.1 – 93.8% (dry weight basis). Lipid content in Nile tilapia muscle was low (1.3 – 1.7% dry weight basis). For broadhead catfish muscle, proteins were found at lower level (55.2–59.5%, dry weight basis), compared with those present in Nile tilapia. This was in accordance

with high content of lipid (36.6 – 42.4%, dry weight basis) in broadhead catfish muscle. It was noted that ventral portion had the higher lipid content than dorsal counterpart. This was obviously observed in broadhead catfish sample. When considering phospholipid content in both muscle portions of both species, it was noted that higher phospholipid content was found in ventral portion, compared with dorsal counterpart ($P < 0.05$). Also, it was found that broadhead catfish muscle contained higher phospholipid than Nile tilapia ($P < 0.05$). This was in agreement with the higher lipid content in the former. Phospholipids in muscle are mainly membrane lipids, which are important in the extracellular transport of lipids in the blood (Tocher *et al.*, 2008). Based on high protein and low lipid contents in muscle, Nile tilapia could be classified as lean fish. Conversely, broadhead catfish was named as fatty fish. Similar ash contents were found in all samples, ranging from 0.9% to 1.0% (wet weight basis). The differences in composition in fish muscle depend on the species and variety, the state of nutrition as well as the parts of the organisms (Sikorski, 1994). The results revealed that the dorsal portion was rich in protein with low contents of ash, lipid and phospholipid ($P < 0.05$). Nakamura *et al.* (2006) reported that the lipid content of dorsal was significantly lower than that of the ventral part. The lipid content of rainbow trout fillet tends to decrease, following the cranio caudal-direction, where the muscles are used for swimming. (Akhan *et al.*, 2010). Chaijan *et al.* (2010) noted that meat from lateral line of farm raised giant catfish (*Pangasianodon gigas*) had 2.0 and 15.9-fold fat content, when compared to those from ventral and dorsal, respectively. Therefore, chemical compositions varied, depending on species and muscle portions. This might affect quality, oxidation stability as well as acceptability.

Geosmin and 2-MIB contents of dorsal and ventral muscles of Nile tilapia and broadhead catfish are shown in Table 16. 2-MIB and geosmin were separated at the retention times of 8.5 and 12.8 min, respectively (Figure 20). Nile tilapia used in the present study had low geosmin content (0.7–1.1 μg/kg) (Figure 20B). Ventral portion contained higher geosmin than dorsal part. This was more likely due to the fact that geosmin is lipophilic and localised in lipid rich portion like ventral part. However, 2-MIB was not detectable in Nile tilapia dorsal and ventral portions. Therefore, geosmin was more likely the major contributor to muddy flavour and odour in Nile

Table 16. Chemical compositions and muddy compounds in dorsal and ventral muscles of Nile tilapia and broadhead catfish

Compositions	Nile tilapia		Broadhead catfish	
	Dorsal	Ventral	Dorsal	Ventral
Moisture (%)	82.1±0.1a[*,†]	83.7±0.1a	70.5±3.9c	72.7±1.3b
Ash (%)	1.0±0.0a (5.6±0.0B)[**]	1.03±0.0a (6.3±0.2A)	1.02±0.1a (5.2±0.5B)	0.87±0.1b (3.1±0.4C)
Protein (%)	16.6±0.1ab (93.1±0.4A)	15.3±0.1c (93.8±0.7A)	17.5±0.1a (59.5±0.5B)	15.7±1.2bc (55.2±1.8C)
Lipid (%)	0.2±0.0c (1.3±0.2C)	0.3±0.0c (1.7±0.0C)	11.4±0.2b (36.6±0.6B)	12.5±0.1a (42.4±0.5A)
Phospholipid (mg/100 g sample)	66.2±6.9d (305.6±31.9D)	90.0 ±5.4c (370.0±22.0C)	155.9±9.1b (528.6±30.9B)	204.3±6.08a (749.4±22.3A)
Geosmin (µg/kg)	0.7±0.0c (3.9±0.3C)	1.1±0.1b (6.4±0.7B)	1.1±0.0b (3.7±0.1C)	5.70±0.4a (20.9±1.1A)
2-MIB (µg/kg)	ND[††]	ND	0.9±0.0b (3.1±0.1)	1.4±0.4a (5.1±1.5A)

[*] Values are given mean ± SD (n = 3), expressed as wet weight basis.

[**]Values in the parenthesis were expressed as dry weight basis.

[†]Different lowercase or uppercase within the same row indicate the significant differences ($P < 0.05$).

[††]ND: Non-detectable.

tilapia. For broadhead catfish muscle, geosmin and 2-MIB were detected and varied with portions (Figure 20C). Ventral portion had higher geosmin (5.7 µg/kg) than dorsal part (1.1 µg/kg). Furthermore, higher 2-MIB content was observed in ventral part (1.4 µg/kg), compared with dorsal part (0.9 µg/kg). This was in accordance with higher lipid content in ventral portion, in which both geosmin and 2-MIB could be accumulated. Yamprayoon and Noomhorm (2000) reported that geosmin distribution of Nile tilapia, held in water containing 50 µg/L geosmin, was in the decreasing order of intestine, abdominal tissue, skin and flesh, respectively. Percival *et al.* (2008) reported that the high muddy flavour was dominant in barramundi fillets from belly cut, whilst the low level was detected in fillets from tail and shoulder cut. Thaysen (1936) reported that

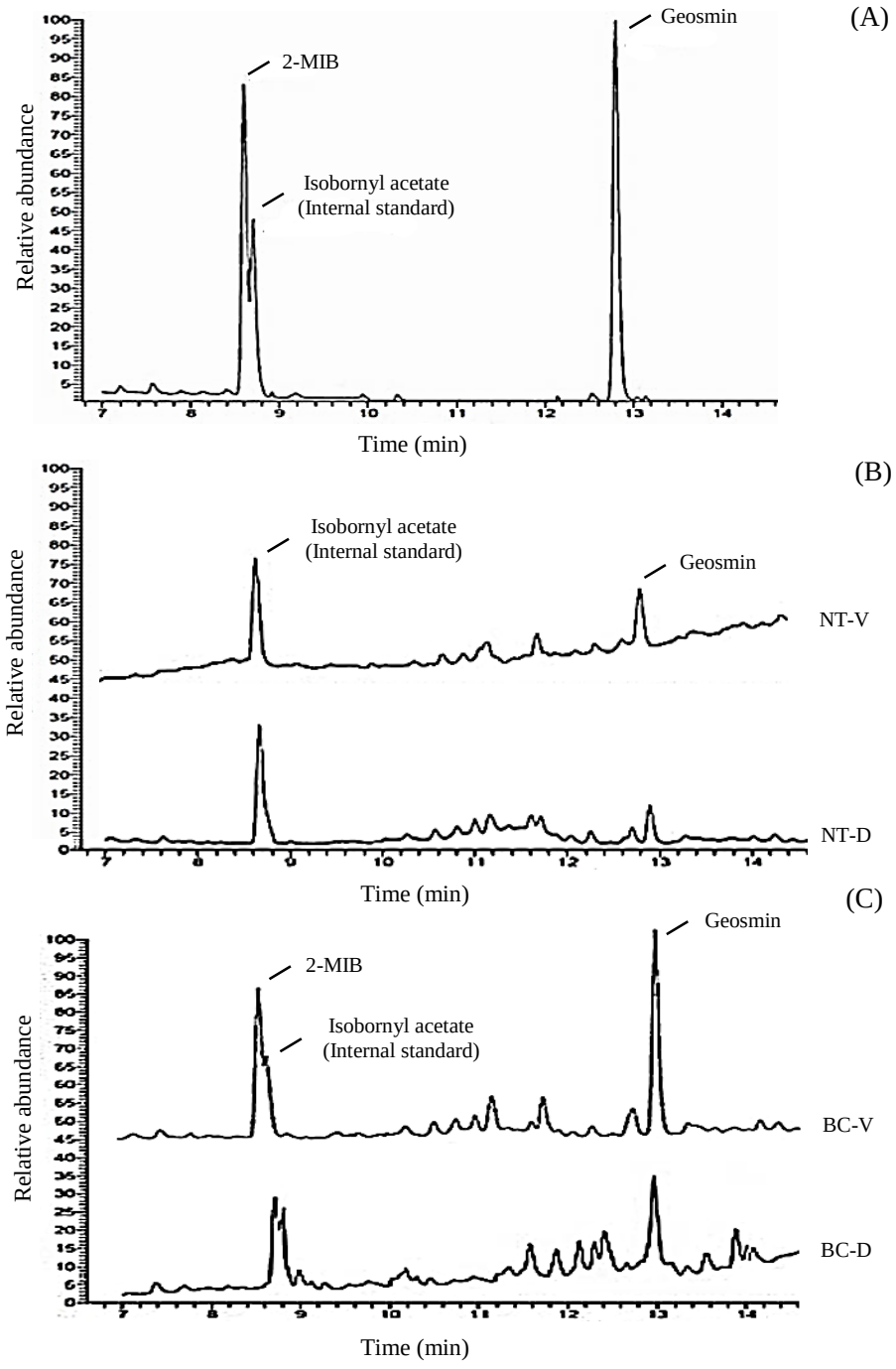


Figure 20. GC–MS chromatogram of geosmin, 2-MIB and isobornylacetate in standard mixture (A) dorsal and ventral ordinary muscle of Nile tilapia (B) and broadhead catfish (C). NT-D: Nile tilapia dorsal muscle; NT-V Nile tilapia ventral muscle; BC-D: broadhead catfish dorsal muscle; BC-V: broadhead catfish ventral muscle.

muddy compounds enter in fish, principally through the gills and transported to the muscle, especially lipid containing tissues, by the blood stream. Geosmin was previously reported to be the major taste and odour compound in various freshwater fish (Robertson and Lawton, 2003; Robin *et al.*, 2006; Yamprayoon and Noomhorm, 2000). Generally, 2-MIB and geosmin were identified as musty- and earthy-odour and flavour, respectively. Fish acquire these compounds from water, which are further bioconcentrated in lipid predominant tissues such as intestine, subcutaneous muscle or stomach tissue (From and Hørlyck, 1984). Different fish may have varying geosmin and 2-MIB contents, which can be related with different muddy flavour. Schrader *et al.* (2005) reported that geosmin in sturgeon fillet was 675 ng/kg, whilst the concentration at 23 ng/kg was present in largemouth bass. Howgate (2004) suggested that the uptake of geosmin or 2-MIB in fish tissue were related with fat content. Additionally, muddy compounds presented in fish flesh are influenced by water temperature, season of harvest, fish portion, size, or habitat (Howgate, 2004). Johnsen and Lloyd (1992) demonstrated that lean channel catfish with fat content less than 2.5% contained less MIB than channel catfish with a greater fat (> 2.5%) during 24 h exposure in water containing taint compounds.

6.4.2 Chemical compositions, geosmin and 2-MIB of mince and PI from Nile tilapia and broadhead catfish

Lipid and phospholipid contents of mince and PI derived from Nile tilapia and broadhead catfish are presented in Table 17. Lipid contents were 0.4% and 12.1% (wet weight basis) for Nile tilapia and broadhead catfish mince, respectively. *Sardinella maderensis* and *Sardinella aurita* are fatty fish containing more than 5% (wet weight) of lipids, whereas *Cephalopholis taeniops* is a lean fish with approximately 1% lipids (Njinkoué *et al.*, 2002). Generally, lipid rich storage tissue, contains triglyceride as the principal lipid, whilst leaner tissue comprises mainly phospholipids. Phospholipids were generally found in white muscle (Ackman and Ratnayake, 1992). Phospholipid content was generally in accordance with lipid content, in which the higher phospholipid content was obtained in broadhead catfish mince. Phospholipid content in mince of broadhead catfish (244.3 mg/100 g sample) was much higher than that of Nile tilapia (74.3 mg/100 g sample).

Table 17. Chemical compositions and muddy compounds in mince and PI of Nile tilapia and broadhead catfish

Compositions	Nile tilapia		Broadhead catfish	
	Mince	PI	Mince	PI
Lipid (%)	0.4±0.0c[*,†] (2.1±0.1B)[**]	0.03±0.0d (0.1±0.1C)	12.1±0.1a (42.3±0.1A)	0.5±0.3b (1.6±0.7B)
Phospholipid (mg/100 g sample)	74.3±5.7b (331.5±3.2B)	19.6±2.8d (81.5±13.5C)	244.3±6.3a (901.2±70.2A)	40.1±1.6c (281.0±8.8B)
Geosmin (µg/kg)	1.5±0.5b (7.2±0.5B)	ND	3.2±0.0a (13.0±0.1A)	ND
2-MIB (µg/kg)	ND[††]	ND	0.8±0.0 (2.9±0.1)	ND

[*] Values are given mean ± SD (n = 3), expressed as wet weight basis.

[**]Values in the parenthesis were expressed as dry weight basis.

[†]Different lowercase or uppercase within the same row indicate the significant differences ($P < 0.05$).

[††]ND: Non-detectable.

PI had the marked differences in lipid and phospholipid, compared with mince of both species. Based on dry weight basis, lipid and phospholipid in PI were decreased by 93.7% and 75.4% for Nile tilapia and 95.6% and 83.6% for broadhead catfish, respectively, compared with those found in mince. Since the alkaline solubilisation process was applied, proteins were more likely dissociated at alkaline condition, mainly due to the enhanced repulsion. As a result, lipids or phospholipids could be liberated to a higher extent. It was noted that neutral lipid was more likely removed than phospholipids during PI preparation. The higher lipid removal by the alkaline solubilisation process might be due to the higher emulsification ability of the proteins at alkaline pH (Kristinsson *et al.*, 2006). Phospholipids are strongly bound to proteins and are hardly removed (Nylander, 2004). In muscle tissue, phospholipid membranes are connected with cytoskeletal proteins through electrostatic attraction between the acidic phospholipids of membranes and the basic amino acid residues of the cytoskeleton protein (Haleva *et al.*, 2004). Moayedi *et al.* (2010) reported that

neutral lipids were removed from chicken dark meat PI by 61.5%, whilst phospholipid remained unchanged. Lipid oxidation in mackerel protein hydrolysate derived from PI was more likely due to the residual phospholipid membrane (chapter 4). The result showed that alkaline solubilisation yielded proteinaceous substrate with significantly reduced levels of lipid, compared to untreated mince.

The levels of geosmin and 2-MIB of Nile tilapia and broadhead catfish mince and PI are displayed in Table 17. Geosmin in broadhead catfish mince (3.2 μg/kg) was higher than that of Nile tilapia mince (1.5 μg/kg). Based on dry weight basis, geosmin content in broadhead catfish mince was 1.8-fold-higher than that found in Nile tilapia mince. 2-MIB at a level of 0.8 μg/kg was only detected in broadhead catfish mince. Both geosmin and 2-MIB have been reported as the major causes of muddy flavour in fresh water fish such as channel catfish flesh (Grimm *et al.*, 2004) and rainbow trout (Lovell and Broce, 1985), etc. When alkaline solubilisation process was implemented for PI preparation, both compounds were not detectable in the resulting PI. Their levels in PI might be lower than the detection limit by GC–MS. Since most lipids were removed, geosmin and 2-MIB accumulated in lipids were also simultaneously eliminated. As a result, negligible levels of both compounds were retained in resulting PI. This result was in accordance with DeWitt *et al.* (2007) who reported that pretreatments of channel catfish via acid and alkaline solubilisation effectively reduced 2-MIB by 86.1%. When the centrifugation (10,000 ×g) was combined with acid solubilisation using either phosphoric acid or acetic acid, geosmin was significantly removed (DeWitt *et al.*, 2007). Forrester *et al.* (2002) reported that 2-MIB in catfish fillets treated with 2% citric acid together with vacuum tumbling decreased from 4.4 to 2.8 μg/kg. It can be inferred that alkaline solubilisation was able to remove geosmin and 2-MIB from mince of both species potentially.

6.4.3 Colour, geosmin, 2-MIB and sensory property of protein hydrolysate from mince and PI of Nile tilapia and broadhead catfish

In general, hydrolysate was hygroscopic and was brownish yellow in colour. Different colours were observed for all hydrolysates prepared using Alcalase as shown in Table 18. Hydrolysate derived from broadhead catfish mince showed higher

a*(redness), b* (yellowness), ΔE*(total difference in colour) and ΔC* (difference in chroma) than that derived from Nile tilapia mince ($P < 0.05$). A higher lightness was found in Nile tilapia hydrolysate, as indicated by the higher L*. Generally, fatty fish contained a high amount of lipids, especially those with unsaturated fatty acids. Lipid oxidation during hydrolysis of muscle proteins was associated with yellowish discolouration of hydrolysate via Maillard reaction (chapter 4). The carbonyl groups of oxidation product, especially aldehydes and ketone, could react with amino groups of free amino acids or peptides generated during hydrolysis, leading to yellow colour development. Additionally, the dark colour of some fish protein hydrolysates was possibly from the colour of fish itself. Protein hydrolysate from the muscle of round scad, a dark fleshed fish, showed a dark colour (L*= 58.0, a*= 8.4 and b* = 28.3) (Thiansilakul *et al*., 2007b). Pre-washing prior to alkaline solubilisation of fish mince yielded the resulting PI with a lower water soluble pigment, such as haem proteins (chapter 3 and 4). Furthermore, PI showed a lower content of lipid and phospholipid than mince (Table 18). The lighter colour of hydrolysates produced from PI of both species was noticeable as shown by the decreases in all parameters observed, except for L* value ($P < 0.05$). Therefore, the varying colours of fish protein hydrolysate depended on the composition of the raw material, especially pigment and lipid contents. Prewashing of mince followed by alkaline solubilsation used for PI preparation could improve the colour of the corresponding hydrolysate.

Hydrolysate derived from broadhead catfish mince contained a higher geosmin content than that of Nile tilapia ($P < 0.05$). This is in agreement with the higher levels found in the former mince. When comparing with mince, geosmin content of hydrolysate decreased from 7.2 to 5.4 μg/kg and 13.0 to 11.2 μg/kg (dry weight basis) for Nile tilapia and broadhead catfish, respectively. When PI was used for hydrolysate production, the lower geosmin content was observed in the hydrolysate from Nile tilapia, whilst no geosmin was detectable in hydrolysate from PI of broadhead catfish. Geosmin was detected in hydrolysate from PI of Nile tilapia, but it was not detected in PI (Table 17). Geosmin in hydrolysate could be better released and recovered by distillation, in comparison with that associated with PI. Additionally, no 2-MIB was detected in hydrolysate derived from PI of both species. The result reconfirmed that

pretreatment of mince using alkaline solubilisation together with pre-washing effectively lowered the muddy compounds in the corresponding hydrolysates.

Muddy odour and flavour associated with hydrolysate derived from mince and PI of both species are shown in Table 18. Lower muddy odour and flavour scores were found in hydrolysates derived from Nile tilapia muscle than those produced from broadhead catfish ($P < 0.05$). This indicated that the stronger muddy odour and flavour associated with hydrolysates was directly related to muddy compounds, mainly geosmin. Earthy flavour caused by geosmin in white sturgen was more intense than that of the largemouth bass (Schrader *et al.*, 2005). Robertson and Lawton (2003) postulated that the range of geosmin at concentration near 1 μg/kg represented ‘‘untainted’’, whereas the level above 2 yielded ‘‘off-flavour’’ in trout flesh. When PI was used as the starting material, the negligible muddy flavour was noticeable in the resulting hydrolysate. This was plausibly due to the fact that lipids were largely removed during pre-washing and alkaline solubilisation for PI preparation. As a result, the muddy compounds associated with lipids were more leached out. Even though no muddy compounds, both geosmin and 2-MIB were detected in hydrolysate from broadhead catfish PI (Table 18), the muddy odour/flavour was perceived by the panellists. This was possibly because the residues were below the detection limit of GC-MS. It has been known that both compounds have extremely low threshold. As a result, the muddy odour/flavour could be detected to some degree by panelists. Mohsin *et al.* (1999) demonstrated that washing the Nile tilapia fillet with the 5% banana leaf ash could remove its muddy flavour. Rohani and Yunus (1994) reported that soaking of gutted and cleaned tilapia in salt solution (5% for 30 min) before deboning and preparation of surimi also removed the muddy odour and flavour. Yamprayoon and Noomhorm (2000) reported that marinating tilapia in acetic acid solution resulted in decreased earthy–musty odour, partly because the acid enhanced the desorbing of 2-MIB and geosmin from the muscle proteins. Therefore, PI could be used as the promising proteinaceous substrate for production of protein hydrolysate with negligible muddy flavour and odour.

Table 18. Colour, muddy compounds and sensory property of protein hydrolysate from mince and PI of Nile tilapia and broadhead catfish

Parameters	Nile tilapia		Broadhead catfish	
	Mince	PI	Mince	PI
L^*	93.2±1.5a[§,†]	94.5±0.9a	86.3±1.6b	93.2±0.2a
a^*	-0.7±0.1c	-0.8±0.1c	0.1±0.2a	-0.3±0.1b
b^*	8.2±1.4c	3.3±0.7d	14.1±1.1a	9.5±0.5b
ΔE^*	7.9±1.4b	3.4±0.7c	15.2±1.5a	9.1±0.6b
ΔC^*	6.9±1.4c	2.0±0.7d	12.7±1.1a	8.2±0.5b
Geosmin (µg/kg dry sample)	5.4±0.3b	1.0±0.1c	11.2±1.3a	ND
2-MIB (µg/kg dry sample)	ND[††]	ND	2.6±0.1	ND
Muddy odour[**]	3.3±1.1b	0.5±0.5b	6.6±1.3a	1.1±0.8b
Muddy flavour[**]	5.8±1.2b	0.5±0.6c	7.9±1.5a	1.0±0.9bc

[§]Values are given as mean ± SD (n = 3), expressed as dry weight basis.

[**] Score are based on 15 cm unstructured line scales (0: none and 15: extremely strong), evaluated by 10 trained panelists.

[†] Different lowercase within the same row indicate the significant differences ($P < 0.05$).

[††]ND: Non-detectable.

6.5 Conclusions

Freshwater fish, both lean and fatty fish, had high nutritional values. However, muddy flavour and odour associated with muscle and their products were the critical problem. Geosmin was found as a main muddy compound associated with Nile tilapia, whilst both geosmin and 2-MIB contributed to muddy flavour and odour in broadhead catfish. Both compounds were more concentrated in ventral portion, where higher lipid and phospholipid were located. Prewashing in combination with alkaline solubilisation used for PI preparation had the marked influence on lowering muddy flavour and odour in resulting hydrolysate. Therefore, protein hydroysate with negligible muddy flavour and odour could be prepared from PI of both Nile tilapia and broadhead catfish.

CHAPTER 7

ANTIOXIDANT AND SENSORY PROPERTIES OF PROTEIN HYDROLYSATE DERIVED FROM NILE TILAPIA (*OREOCHROMIS NILOTICUS*) BY ONE- AND TWO-STEP HYDROLYSIS

7.1 Abstract

Antioxidant and sensory properties of Nile tilapia protein hydrolysates prepared by one- and two-step hydrolysis using commercial proteases were investigated. Hydrolysates prepared using single protease including Alcalase (HA), Flavourzyme (HF), Protamex (HPr) and papain (HPa) had increases in antioxidant activities as the degree of hydrolysis (DH) increased up to 40 % ($P < 0.05$). Amongst all hydrolysates, HA having 40 % DH showed the highest antioxidant activities. When HA was further hydrolysed by papain, the resulting hydrolysate (HAPa) exhibited the highest antioxidant activities for all assays tested ($P < 0.05$). ABTS radical scavenging activity and metal chelating of HAPa generally remained constant in a wide pH range (1–11) and during heating at 30–100 °C. Both activities increased in the simulated gastrointestinal tract model system, especially in intestine condition. HAPa (100–1,000 ppm) could retard lipid oxidation in β-carotene-linoleate and lecithin-liposomemodel systems in a dose dependent manner. Peptides in both HA and HAPa with molecular weight of 513 Da and 1,484 Da possessed the strongest ABTS radical scavenging activity and metal chelating activity, respectively. The amino acid profile of both HA and HAPa contained a high amount of hydrophobic amino acids (38.26–38.85 %) and had glutamic acid/glutamine, lysine and aspartic acid/asparagine as the dominant amino acids. However, HAPa showed a higher acceptability than did HA, owing to the lower bitterness. Therefore, the use of Alcalase in combination with papain for hydrolysis of protein isolate rendered the hydrolysate with antioxidant properties and reduced bitterness, which could serve as the functional supplement.

7.2 Introduction

Antioxidants play an important role as health protecting factors and are used to preserve food products to retard off-odour/flavour and discolour caused by lipid oxidation (Decker *et al.*, 2005). Lipid oxidation is of great concern for food industry and consumers, since it is associated with the development of undesirable off-flavours and potentially toxic products (Decker *et al.*, 2005). Protein hydrolysates produced by the enzymatic digestion of aquatic processing byproducts have been proven to be a promising source of antioxidant peptides (Bougatef *et al.*, 2010; Shahidi *et al.*, 1995). Under the controlled enzymatic hydrolysis, the functional peptides could be released from the starting proteins, yielding the hydrolysate with varying properties. The substrate and protease employed as well as the degree of hydrolysis greatly affect physicochemical properties of the resulting hydrolysate. The type of enzyme generally dictates the cleavage patterns of the peptide bonds. Jun *et al.* (2004) reported that protein hydrolysates from yellowfin sole (*Limanda aspera*) frame prepared by Alcalase, Neutrase, papain, trypsin, pepsin, α-chymotrypsin, and Pronase E had different antioxidant activities.

Lean fish, such as Nile tilapia etc., are traditionally used for protein hydrolysate preparation. With an appropriate hydrolytic process, the hydrolysate might contain novel peptides with specific or multi-functional bioactivity (Khantaphant *et al.*, 2011a). However, the presence of pro-oxidants, such as haem proteins and unstable lipid substrates associated with the muscle, is a drawback (Raghavan *et al.*, 2008). These constituents contribute to undesirable characteristic and instability of hydrolysate (Raghavan *et al.*, 2008). Recently, protein isolate (PI) from the pretreated muscle using pH-shifted method has been proven to be a potential substrate, yielding the hydrolysate with a negligible fishy odour (chapter 3). Khantaphant *et al.* (2011a) reported that hydrolysate property from brownstripe red snapper (*Lutjanus vita*) PI was superior to that prepared directly from fish mince. However, the bitterness associated with hydrolysates is a critical problem for further applications, especially as the supplement for health or functional foods or drinks. To conquer the drawback, the use of several proteases in the step-wise process could remove the domain causing the bitterness in peptides. Nevertheless, there was no information regarding the step-wise hydrolysis of

Nile tilapia muscle, especially from PI. Khantaphant *et al.* (2011b) reported that protein hydrolysates prepared from the muscle of brownstripe red snapper using Alcalase or Flavourzyme in the first step hydrolysis together with protease from its pyloric caeca for the second step contained the peptides with the highest antioxidant and ACE inhibitory activities. Thus, this work aimed to produce protein hydrolysate from Nile tilapia PI prepared by one-step and two-step hydrolysis using various types of commercial proteases and to investigate their antioxidant activities and sensory characteristic.

7.3 Materials and Methods

7.3.1 Chemical/enzymes

Alcalase 2.4 L (E.C. 3.4.21.62) (2.4 AU/g), Flavourzyme 500 L (E.C. 3.4.21.77) (500 LAPU/g) and ProtamexTM (EC. 3.4.21.14/3.4.24.28) (1.5 AU/g) were provided by Novozymes (Bagsvaerd, Denmark). Papain (E.C. 3.4.22.2) (≥3 AU/mg), 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6- trinitrobenzenesulfonic acid (TNBS), 1,1,3,3-tetramethoxypropane, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt (ferrozine), 2,4,6-tripyridyl-triazine (TPTZ), l-α –phosphatidylcholine (lecithin) and linoleic acid were purchased from Sigma (St. Louis, MO, USA). Thiobarbituric acid (TBA), potassium persulphate, β-carotene and Tween 40 were obtained from Fluka (Buchs, Switzerland). Sodium sulphite was obtained from Riedel-deHaën (Seelze, Germany). All chemicals were of analytical grade.

7.3.2 Preparation of PI from Nile tilapia mince

PI was prepared from pre-washed Nile tilapia mince using alkaline solubilisation process as per the method as described in chapter 3. Washed mince was homogenized with five volumes of cold distilled water (2–4 °C) using an IKA Labortechnik homogeniser (Selangor, Malaysia) at a speed of 11,000 rpm for 1 min. The homogenate was adjusted to pH 11 and placed on ice for 60 min with a continuous stirring. The mixture was then centrifuged at 5,000 ×g for 10 min at 4 °C using an Avanti J-E centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA). The alkaline

soluble fraction obtained, referred to as ‘PI solution’, was used as substrate for hydrolysis.

7.3.3 Production of Nile tilapia protein hydrolysate with antioxidant activities

7.3.3.1 One-step hydrolysis using various single proteases

PI solution was mixed with distilled water to obtain a final protein concentration of 2% (w/v) as determined by the Biuret method (Robinson and Hogden, 1940). The hydrolysis was conducted for 1 h using 0.08–5.54 % (w/w) Alcalase (pH 8.0, 50 °C), 0.07–11.69 % (w/w) Flavourzyme (pH 7.0, 50 °C), 0.58–8.67 % (w/w) Protamex (pH 6.0, 40 °C) and 0.83–4.69 % (w/w) papain (pH 7.0, 40 °C) to obtain the desirable degree of hydrolysis (DH) (10, 20, 30 and 40 %) as described by Benjakul and Morrissey (1997). After 1 h of hydrolysis, the reactions were terminated by placing the mixtures in boiling water for 10 min. Thereafter, the mixture was centrifuged at 2,000×g at 4 °C for 10 min. The supernatants were collected and then lyophilised using a Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lynge, Denmark).

The hydrolysates prepared using Alcalase, Flavourzyme, Protamex and papain were referred to as HA, HF, HPr and HPa, respectively. Those samples were subjected to analyses.

7.3.3.2 Two-step hydrolysis using different proteases

After 1 h of the first hydrolysis, the selected hydrolysate (HA) was adjusted to the desirable pH using 2 M NaOH or HCl for proteases used for the second step. To initiate the second step of hydrolysis, different proteases at the same amount (5.54 % w/w) used in the first step were added into the pre-incubated mixture with optimal temperature of the corresponding proteases (50 °C for Flavourzyme and 40 °C for Protamex and papain). Reaction was conducted for 1 h and the mixture was submerged in boiling water for 10 min to inactivate the enzymes. Thereafter, the mixture was centrifuged at 2,000 ×g at 4 °C for 10 min and the supernatant was collected, followed by lyophilisation to obtain hydrolysate powder. HA with further

hydrolysis using Flavourzyme, Protamex and papain were referred to as HAF, HAPr and HAPa, respectively. All hydrolysates were subjected to analyses.

7.3.4 Analyses

7.3.4.1 Determination of degree of hydrolysis (DH)

DH of protein hydrolysate was determined according to the method of Benjakul and Morrissey (1997). Protein hydrolysate with an appropriate dilution (125 µl) was added with 2 ml of 0.2 M phosphate buffer (pH 8.2) and 1 ml of 0.01% TNBS solution. The solutions were mixed thoroughly and placed in a temperature-controlled water bath at 50 °C for 30 min in the dark. The reaction was terminated by adding 2 ml of 0.1 M sodium sulphite. The mixtures were cooled at room temperature for 15 min. The absorbance was read at 420 nm and α-amino group was expressed in terms of *L*-leucine. The DH was defined as follows:

$$DH = [(L_t - L_0)/(L_{max} - L_0)] \times 100$$

where L_t is the amount of α-amino acid released at time t, L_0 is the amount of free amino group in original PI solution, and L_{max} is the total amino group content in the initial PI solution obtained after acid hydrolysis (6 M HCl at 100 °C for 24 h).

7.3.4.2 Determination of antioxidant activities

A) DPPH radical scavenging activity

DPPH radical scavenging activity was determined according to the method of Binsan *et al.* (2008). Sample solution (1.5 ml) was added with 1.5 ml of 0.1 mM DPPH in 95 % ethanol. The mixture was incubated at room temperature for 30 min in dark. The resulting solution was measured at 517 nm. The control was prepared in the same manner except that distilled water was used instead of the sample. The DPPH radical scavenging activity was calculated from Trolox standard curve (0–60 µM) and expressed as µmol Trolox equivalents (TE)/g solid.

B) ABTS radical scavenging activity

ABTS radical scavenging activity was determined as described by Binsan *et al.* (2008). ABTS radical ($ABTS^{\bullet+}$) was produced by reacting 7.4 mM ABTS stock solution with 2.6 mM potassium persulphate at a ratio of 1:1 (v/v). The mixture was allowed to react in dark for 12 h at roomtemperature. Prior to assay, $ABTS^{\bullet+}$ solution was diluted with methanol to obtain an absorbance of 1.1 (± 0.02) at 734 nm. To initiate the reaction, 150 µl of sample was mixed with 2.85 ml of $ABTS^{\bullet+}$ solution. The mixture was incubated at room temperature for 2 h in dark. The absorbance was then read at 734 nm. Trolox standard curve (0 – 600 µM) was prepared. Distilled water was used instead of the sample and prepared in the same manner to obtain the control. ABTS radical scavenging activity was expressed as µmol TE/g solid.

C) Ferric reducing antioxidant power (FRAP)

The ability of samples to reduce ferric ion (Fe^{3+}) was evaluated as per the method of Benzie and Strain (1996). FRAP reagent (a freshly prepared mixture of 10 mM TPTZ solution in 40 mM HCl, 20 mM $FeCl_3.6H_2O$ solution and 300 mM acetate buffer, pH 3.6 (1:1:10 v/v/v) (2.85 ml) was incubated at 37 °C for 30 min prior to mixing with 150 µl of sample. The reaction mixture was allowed to stand in dark for 30 min at room temperature. Absorbance at 593 nm was read and FRAP was calculated from the Trolox standard curve (0 – 600 µM) and expressed as µmol TE/g solid. The control was prepared in the same manner except that distilled water was used instead of the sample.

D) Metal chelating activity

Chelating activity of samples towards ferrous ion (Fe^{2+}) was measured by the method of Binsan *et al.* (2008) with a slight modification. Sample (200 µl) was mixed with 800 µL of distilled water. Thereafter, 0.1 ml of 2 mM $FeCl_2$ and 0.2 ml of 5 mM ferrozine were added. The mixture was allowed to react for 20 min at room temperature. The absorbance was then read at 562 nm. The standard curve of EDTA (0 – 30 µM) was prepared. The control was prepared in the same manner except that distilled water was used instead of the sample. Ferrous chelating activity was expressed

as μmol EDTA equivalents (EE)/g solid. The hydrolysate prepared using a two-step hydrolysis, showing the highest antioxidant activity, was used for further studies.

7.3.5 Stability of the selected protein hydrolysate

7.3.5.1 pH and thermal stability

The selected protein hydrolysate powder (25 mg) was dispersed in 8 ml of distilled water previously adjusted to different pHs (1, 3, 5, 7, 9 and 11) using 1 M HCl or NaOH. The final volume was made up to 10 ml with the water having the corresponding pH and allowed to stand at room temperature for 30 min. Thereafter, the pH of the mixtures was adjusted to 7.0 and their volume was made up to 25 ml withdistilled water. The residual antioxidant activities were determined for ABTS radical scavenging activity and metal chelating activity. Activity (%) relative to that obtained without pH adjustment was reported.

To determine thermal stability, protein hydrolysate solution (1 mg hydrolysate/ml; 10 ml) was placed in a temperature controlled water bath at different temperatures (30, 40, 50, 60, 70, 80, 90 and 100 °C) for 30 min. Thereafter, the solutions were suddenly cooled in iced water. The residual antioxidant activities were determined for ABTS radical scavenging activity and metal chelating activity and expressed as the activity (%) relative to those without heat treatment.

7.3.5.2 Stability in gastrointestinal tract model system (GIMs)

GIMs using an in vitro pepsin-pancreatin hydrolysis was carried out according to the method of You *et al.* (2010) with a slight modification. The pH of protein hydrolysate solution (1 mg hydrolysate/ml; 25 ml) was adjusted to pH 2.0 with 1 M HCl. Pepsin solution (E/S 1:35 w/w) was then added and the mixture was incubated with continuous shaking at 100 rpm for 1 h at 37 °C (stomach condition). The pH was then adjusted to 5.3 with 0.9 M $NaHCO_3$ solution and further to pH 7.5 with 6 M NaOH. Pancreatin was added (E/S 1:35 w/w), and the mixture was further incubated with continuous shaking for 3 h at 37 °C (intestine condition). To terminate the digestion, the solution was submerged in boiling water for 10 min. During digestion, the mixture was sampled at 0, 20, 40, 60, 80, 100, 120, 150, 180, 210 and 240 min for measurement

of ABTS radical scavenging activity and metal chelating activity. The residual activities were expressed as the activity (%) relative to that without digestion.

7.3.6 Antioxidant activity of the selected protein hydrolysate in different model systems

7.3.6.1 β-carotene linoleic acid emulsion model system

The antioxidant activity of the protein hydrolysate in the β-carotene linoleic acid emulsion model system was determined as described by Binsan *et al.* (2008). β-carotene (1 mg) was dissolved in 10 ml of chloroform. Thereafter, the solution (3 ml) was added to 20 mg linoleic acid and 200 mg Tween 40. Chloroform was then removed by purging with nitrogen. Fifty millilitres of oxygenated distilled water were added to the β-carotene emulsion and mixed well. Hydrolysate (200 µl) was then mixed with 3 ml of oxygenated β-carotene emulsion to obtain the final concentrations of 100, 500 and 1,000 ppm. The oxidation of β-carotene emulsion was monitored spectrophotometrically at 470 nm after 0, 10, 20, 30 40, 60, 90 and 120 min of incubation at 50 °C in dark. Trolox at a level of 100 ppm was also used as positive control. The control was prepared by using distilled water instead of protein hydrolysate in the assay system.

7.3.6.2 Lecithin liposome model system

The antioxidant activity of hydrolysates in a lecithin liposome system was determined according to the method of Frankel *et al.* (1997) as modified by Thiansilakul *et al.* (2007b). Lecithin liposome system was prepared by suspending lecithin in deionised water at a concentration of 8 mg/ml. The mixture was stirred with a glass rod, followed by sonication at room temperature (25–28ºC) for 30 min in a sonicating bath (ElmaModel S30H, Singen, Germany). Hydrolysate solution (3 ml) was added to the lecithin liposome system (15 ml) to obtain final concentrations of 100, 500 and 1,000 ppm. The mixture was sonicated for 2 min. To initiate the reaction, 20 µL of 0.15 M cupric acetate were added. The mixture was shaken in dark at 120 rpm using a shaker (Heidolph Model Unimax 1010, Schwabach, Germany) at 37 °C. The system containing 100 ppm Trolox was also prepared. The control was prepared in the same

manner, except that distilled water was used instead of hydrolysate. Oxidation in lecithin liposome systems was monitored at 6 h intervals for 48 h by determining the formation of TBARS (Buege and Aust, 1978) and conjugated diene (Frankel *et al.*, 1997).

7.3.7 Fractionation of antioxidant peptides from the selected protein hydrolysate

HA and HAPa were fractionated using a Sephadex G-25 column (1.6 × 63.5 cm). Sample (100 mg) was dissolved in distilled water (2 ml). The mixture was loaded onto a column and the elution was performed using distilled water at a flow rate of 0.5 ml/min. The 3 ml-fractions were collected and their absorbance was monitored at 220 and 280 nm. The standards, including insulin chain B (3,496 Da), vitamin B_{12} (1,356 Da), Gly-Tyr (238 Da) and tyrosine (181 Da) were used. Blue dextran (2,000 kDa) was used to measure the void volume of the column. The fractions were determined for their ABTS radical scavenging activity and chelating activity. The molecular weight of fraction exhibiting antioxidant activity was estimated from the plot between the available partition coefficient (K_{av}) against the logarithm of the molecular weight of the protein standards.

7.3.8 Amino acid analysis

HA and HAPa were hydrolysed with 4.0 M methanesulphonic acid under reduced pressure at 110 °C for 22 h to prevent the oxidation of tryptophan. The hydrolysates were neutralised with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot of 100 µl was applied to an amino acid analyser (MLC-703; Atto Co., Tokyo, Japan).

7.3.9 Sensory properties of the selected protein hydrolysate

Sensory evaluation for fishy-, muddy- odour/flavour and bitter taste in fish protein hydrolysate was conducted using Generic descriptive analysis (Meilgaard *et al.*, 2007). The sensory panel (10-15 panellists) was screened for perception of muddy odour/flavour, familiarity with fish consumption and ability to determine

differences between fish protein hydrolysate solution (International Standard ISO 8586-1, 1993; ASTM Special Technical Publication 758, 1981; Meilgaard, 2007).

Prior to the evaluation, the 10 panellists (7 female and 3 male) with the ages of 25–32 were trained three hours a week for totally one month as per the method mentioned in chapter 5 and 6. Panellists were trained with standards for two sessions using a 15 cm line scale anchored from 'none' to 'extremely strong' for fishy-, muddy-odour/flavour and bitterness. The working standards included the stored fish protein hydrolysate (0–1 %), mixed solution of geosmin and 2-MIB with a ratio of 1:1 w/w (0–5 ppb) and caffeine solution (0–0.5 mg/ml) for fishy-, muddy-odour/flavour and bitterness, respectively. To evaluate the samples, hydrolysate solutions (1 % w/w) were placed in a sealable plastic cup and heated at 60 °C in a temperature controlled water bath for 5 min prior to serving. The samples were coded with 3-digit random numbers and presented to panellists on a tray in individual booths. Serving orders were completely randomised. The panellists were asked to open the cup and sniff the headspace above the samples for determining odour. To evaluate the flavour, panellists were asked to taste the sample and rinse their mouth between different samples. The evaluation of acceptance was performed by 30 untrained panellists who were familiar with fish consumption. The assessment was conducted for the odour, colour and overall likeness using a 9-point hedonic scale: 1, dislike extremely; 2, dislike very much; 3, dislike moderately; 4, dislike slightly; 5, neither like nor dislike; 6, like slightly; 7, like moderately; 8, like very much; 9, like extremely.

7.3.10 Statistical analysis

Experiments were run in triplicate using three lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests. The T-test was used for pair comparison (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

7.4 Results and Discussion

7.4.1 Antioxidant activities of protein hydrolysate prepared using various single proteases with different DHs

7.4.1.1 DPPH radical scavenging activity

DPPH radical scavenging activities of Nile tilapia protein hydrolysates including HA, HF, HPr and HPa with different DHs are depicted in Figure 21A. DPPH is a stable free radical that shows the maximal absorbance at 517 nm. When DPPH encounters a proton-donating substance, such as an antioxidant, the radical is scavenged. The colour changes from purple to yellow and the absorbance is reduced (Binsan *et al.*, 2008).The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen-donating ability, thereby terminating the radical chain reaction. The differences in activities were observed amongst hydrolysates and the activity varied with DHs. As the DH increased, DPPH radical scavenging activities increased ($P < 0.05$). However, DPPH radical scavenging activity of HPa decreased when DH was above 30 %. The result was in agreement with Thiansilakul *et al.* (2007a) who reported that DPPH radical scavenging activity of protein hydrolysate from round scad muscle prepared using Flavourzyme and Alcalase increased as DH increased. Nevertheless, DPPH radical scavenging activity of protein hydrolysates prepared from alkaline-aided channel catfish protein isolates using Protamex decreased with increasing DH (Theodore *et al.*, 2008). At all DHs, HA showed the highest activity, compared to other hydrolysates ($P < 0.05$). The highest activity was observed in HA with 40 % DH ($P < 0.05$). Rajapakse *et al.* (2005) reported that high DPPH or other radical scavenging activities for the protein hydrolysates or peptides derived from giant squid muscle are usually associated with high hydrophobic amino acid or hydrophobicity. The results revealed that the efficiency in hydrogen donation of peptides in hydrolysate was governed by types of proteases used and DH.

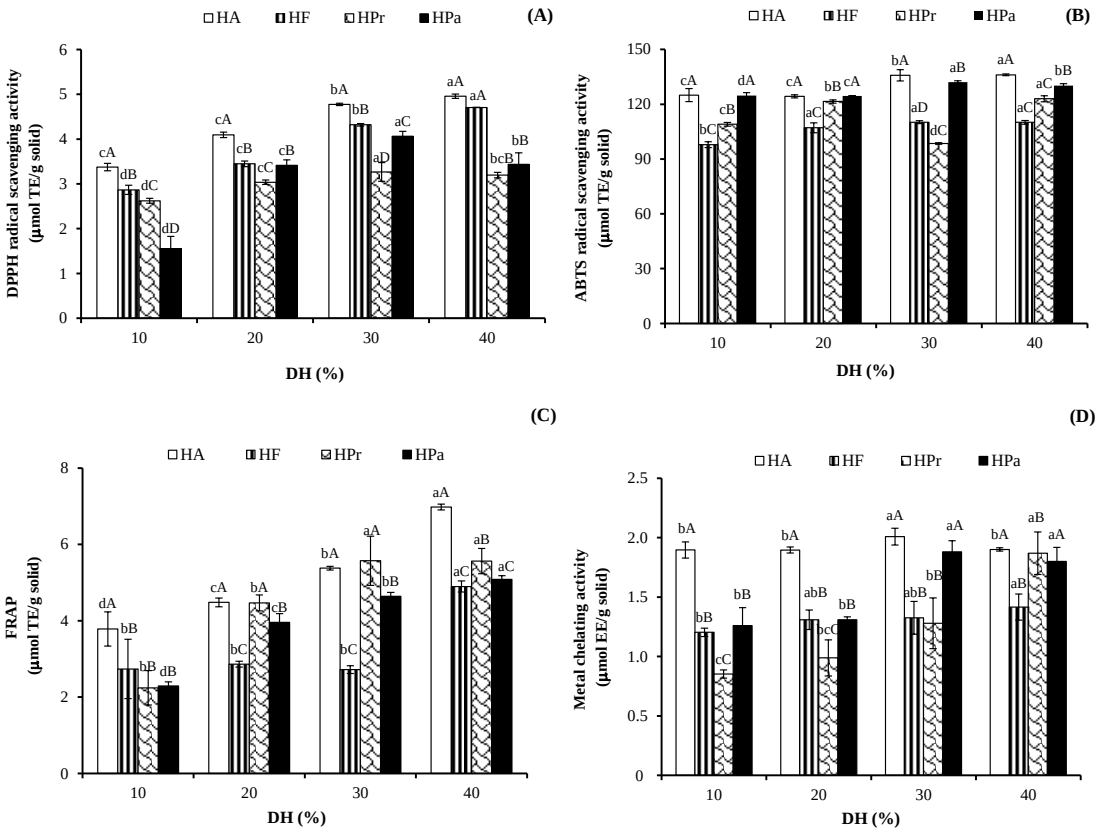


Figure 21. Antioxidant activities of Nile tilapia protein hydrolysate prepared using various single proteaseses with different DHs determined by DPPH radical scavenging activity (A), ABTS radical scavenging activity (B), FRAP (C) and metal chelating activity (D). HA, HF, HPr and HPa: hydrolysate prepared using Alcalase, Flavourzyme, Protamex and papain, respectively. Bar represent stand deviations (n=3). Different lowercase letters within the same type of enzyme and different uppercase letters within the same DH indicate the significant differences ($P < 0.05$).

7.4.1.2 ABTS radical scavenging activity

Protein hydrolysates prepared using various proteases showed varying ABTS radical scavenging activities (Figure 21B). The ABTS assay is a colourimetric assay that evaluates the potential of an antioxidant to inhibit the formation of a coloured radical cation $ABTS^{\bullet+}$, a blue-green chromophore with the characteristic absorption at 734 nm. The $ABTS^{\bullet+}$ is generated by the oxidation of ABTS with potassium persulphate and reduced in the presence of electron-donating antioxidants or of chain breaking

anitioxidant (Raghavan *et al.*, 2008). The highest activity was observed in all hydrolysates with 40 % DH ($P < 0.05$), regardless of type of enzymes used. There were no differences in ABTS activity for HF with DH of 20 – 40 % ($P > 0.05$). However, the lowest activity was observed in HPr with 30 % DH, compared with HPr having other DHs ($P < 0.05$). Peptides produced might be different in terms of amino acid composition, sequence and chain length. The result suggested that the hydrolysates contained peptides or proteins, which served as hydrogen or electron donors to radicals by converting them to more stable products. Li *et al.* (2012) reported that grass carp hydrolysate prepared using Alcalase with DH ranging from 10 to 20 % had increased ABTS scavenging activity with increasing DH.

7.4.1.3 Ferric reducing antioxidant power (FRAP)

FRAP of Nile tilapia protein hydrolysates as affected by DH and types of proteases is shown in Figure 21C. FRAP is generally used for measurement of the reducing ability (TPTZ-Fe^{3+} to TPTZ-Fe^{2+}). All protein hydrolysates prepared using different proteases had the increases in FRAP when DH increased ($P < 0.05$). The result was in agreement with DPPH radical scavenging activity (Figure 21B). However, FRAP of HF was not different as DH was in the range of 10 – 30 % ($P > 0.05$). At 40 % DH, HA showed the highest activity (6.98 μmol TE/g solid), followed by HPr (5.56 μmol TE/g solid), HPa (5.05 μmol TE/g solid) and HF (4.90 μmol TE/g solid), respectively. The reducing ability of hydrolysates was possibly due to the presence of peptides, which donated electrons to free radicals, leading to the prevention or retardation of propagation. Protein hydrolysates from yellow stripe trevally (Klompong *et al.*, 2008) and alkaline-solubilised tilapia (Raghavan *et al.*, 2008) have been reported to possess FRAP.

7.4.1.4 Metal chelating activity

The ability of protein hydrolysates prepared using various proteases with different DHs in metal chelating is depicted in Figure 21D. Generally, the metal chelating activity of hydrolysates increased as the DHs increased ($P < 0.05$), except for HA and HF, in which no difference in activity was observed with increasing DHs ($P >$

0.05). At the same DH tested, HA exhibited the higher metal chelating activity than others ($P < 0.05$). Ferrous ion (Fe^{2+}) is a pro-oxidant and can interact with hydrogen peroxide in a Fenton reaction to produce reactive oxygen species and hydroxyl (OH•) radicals, leading to the initiation and/or acceleration of lipid oxidation (Binsan *et al.* 2008). For the assay, ferrozine produces a violet complex with Fe^{2+}. The formation of this complex is interrupted in the presence of a chelating agent, resulting in the decreased violet colour. Thus, protein hydrolysate containing peptides had the ability in chelation of metals. As a consequence, prooxidative metals could be sequestered, leading to the lowered oxidation.

Based on the results, enzymatic hydrolysis most likely increased the antioxidant activity of Nile tilapia PI to different degrees. The DH greatly influenced the peptide chain length as well as the exposure of terminal amino groups of products (Thiansilakul *et al.*, 2007a). Wu *et al.* (2003) found that changes in size, level and composition of free amino acids of peptides affected the antioxidant activity. Furthermore, Shahidi *et al.* (1995) reported that the composition of protein hydrolysates from capelin depended on the type of enzyme used. Proteases from various types most likely cleaved the peptide bonds in protein structure at the different positions, leading to the different products with various antioxidant activities. Alcalase and Protamex are endopeptidase capable of hydrolysing proteins with broad specificity for peptide bonds and prefers the uncharged residue, whereas Flavourzyme is a mixture of endo-and exopeptidase, which can produce both free amino acids and peptides (Je *et al.*, 2009). Papain has fairly broad specificity; it has endopeptidase, amidase, and esterase activities. It exhibits specific substrate preferences, primarily for bulky hydrophobic or aromatic residues (Tavano, 2013). Amongst all hydrolysates, HA with 40 % DH showed the highest antioxidant activity and it was selected for further hydrolysis using other proteases.

7.4.2 Antioxidant activities of protein hydrolysate prepared using two-step process

Antioxidant activities of hydrolysates prepared by a two-step hydrolysis process using combined proteases including HAF, HAPr and HAPa with DHs of 48.9,

50.6 and 53.8 %, respectively (data not shown), in comparison with HA are depicted in Figure 22.

7.4.2.1 DPPH radical scavenging activity

DPPH radical scavenging activity of protein hydrolysates from Nile tilapia PI using a two-step hydrolysis with two different proteases is shown in Figure 22A. The increases in DPPH radical scavenging activity were observed for all hydrolysates, when a two-step hydrolysis was employed ($P < 0.05$). Je *et al.* (2009) reported that the DPPH scavenging activity of hydrolysates from a two-step hydrolysis process was more potent than that of one step hydrolysis. The present result indicated that the two-step hydrolysis plausibly enhanced the liberation of bioactive peptides from the hydrolysate prepared at the first step. The peptides generated more likely showed a greater ability to scavenge DPPH radicals or to donate hydrogen. However, the scavenging activity against DPPH radical was not different between HAF and HAPr ($P > 0.05$), whilst the highest activity was observed for HAPa ($P < 0.05$). Thus, DPPH radical scavenging activity increased when papain was used for the second hydrolysis.

7.4.2.2 ABTS radical scavenging activity

ABTS radical scavenging activity of protein hydrolysates prepared by a two-step hydrolysis in comparison with HA is depicted in Figure 22B. The hydrolysate prepared by a two-step process showed higher ABTS radical scavenging activity, compared with HA ($P < 0.05$). However, the ability to scavenge the ABTS radicals varied with enzymes used. Amongst all hydrolysates, HAPa had the highest ABTS radical scavenging activity ($P < 0.05$). This was in accordance with DPPH radical scavenging activity, in which the highest activity was found in HAPa (Figure 22A). HAPa with the highest DH had the active peptides, which could react with free radicals to form more stable products. Khantaphant *et al.* (2011b) reported that the increases in ABTS radical scavenging activity of brownstripe red snapper muscle hydroysate varied, depending on the hydrolysis time of the second hydrolysis step.

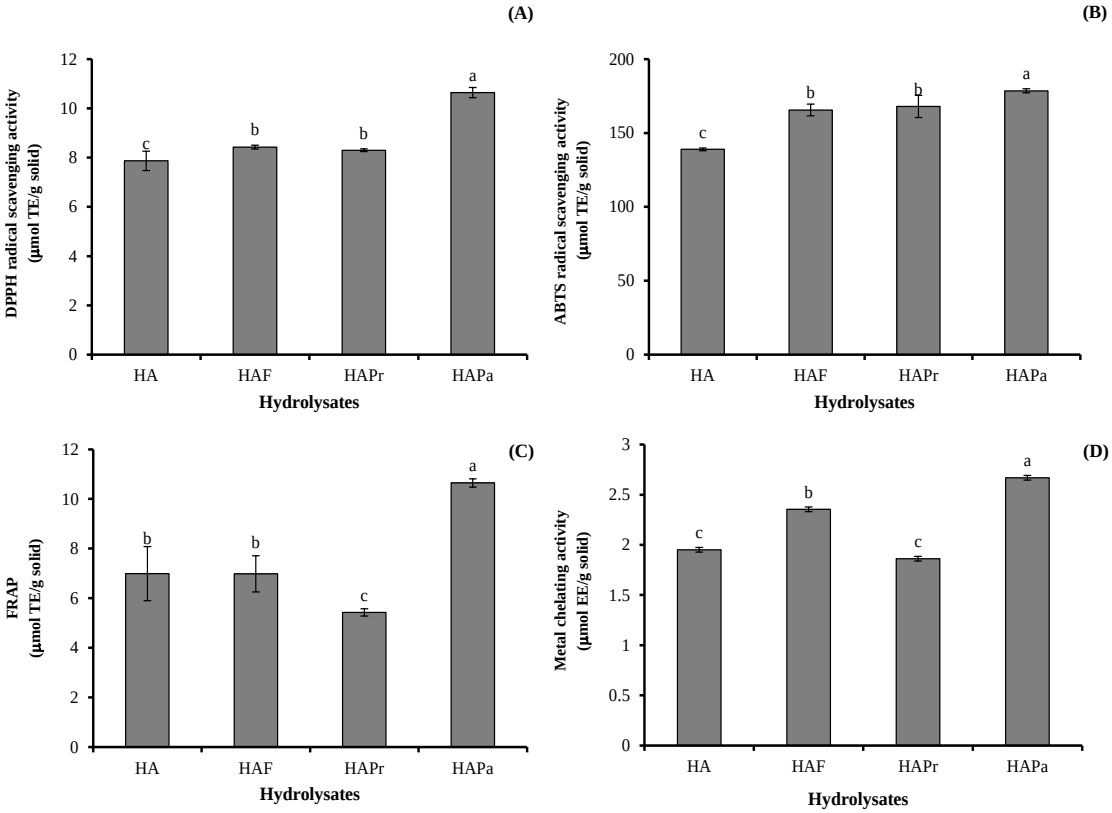


Figure 22. Antioxidant activities of Nile tilapia protein hydrolysate prepared by a two-step hydrolysis using various proteases as determined by DPPH radical scavenging activity (A), ABTS radical scavenging activity (B), FRAP (C) and metal chelating activity (D). HA: hydrolysate prepared using Alcalase. HAF, HAPr and HAPa: HA further hydrolysed with Flavourzyme, Protamex and papain, respectively. Bar represent stand deviations (n=3). Different lowercase letters in the same parameter indicate the significant differences ($P < 0.05$).

7.4.2.3 FRAP

Figure 22C shows FRAP of Nile tilapia protein hydrolysates prepared using a two-step hydrolysis process. The highest FRAP was noticeable in HAPa ($P < 0.05$) and its FRAP was much higher than that of HA ($P < 0.05$). On the other hand, HAPr had a decrease in FRAP by 22.4%, compared with that of HA. There was no difference in FRAP between HAF and HA ($P > 0.05$). The differences in FRAP might

be governed by peptides in the hydrolysates. Protamex used in the second step might generate peptides with the lower FRAP. A number of studies have shown that the antioxidant activity of hydrolysates was dependent on their molecular weight distribution (Wu *et al.*, 2003). The highest reducing power of protein hydrolysate from whole anchovy sprat prepared using bromelain and Promod® was observed, whilst the lowest activity was found in those prepared by autolysis and Flavourzyme (Ovissipour *et al.*, 2013).

7.4.2.4 Metal chelating activity

Metal chelating activity of Nile tilapia protein hydrolysate as affected by a two-step hydrolysis process is shown in Figure 22D. In general, the increases in metal chelating ability of all hydrolysates prepared by two-step process were observed, except for HAPr. This might be attributed to more exposure of effective sites capable of chelating ferrous ion. HAPa showed the strongest metal chelating activity and the activity increased by 36.4 %, compared with that found in HA ($P < 0.05$). Thus, metal chelating activity of hydrolysate was affected by types of protease used in the second step of hydrolysis.

According to antioxidant activities tested by different assays, HAPa exhibited the highest activity and it was used for stability study.

7.4.3 Stability of the selected protein hydrolysate (HAPa)

7.4.3.1 pH and thermal stabilities

The influence of pH on antioxidant activity of HAPa as monitored by ABTS radical scavenging and metal chelating activities is shown in Figure 23A. ABTS radical scavenging activity of HAPa was quite stable over the pH range of 1–11 ($P > 0.05$). Nevertheless, metal chelating activity of HAPa decreased by 15.3–25.9 % after pH adjustment to acidic and alkaline pH. This was possibly due to the changes of charge in peptides, particularly at N- and C-terminal, mediated by pH adjustment. Nalinanon *et al.* (2011) reported that the antioxidant peptides derived from ornate threadfin bream muscle possessing ABTS radical-scavenging activity lost its activity to some extent at

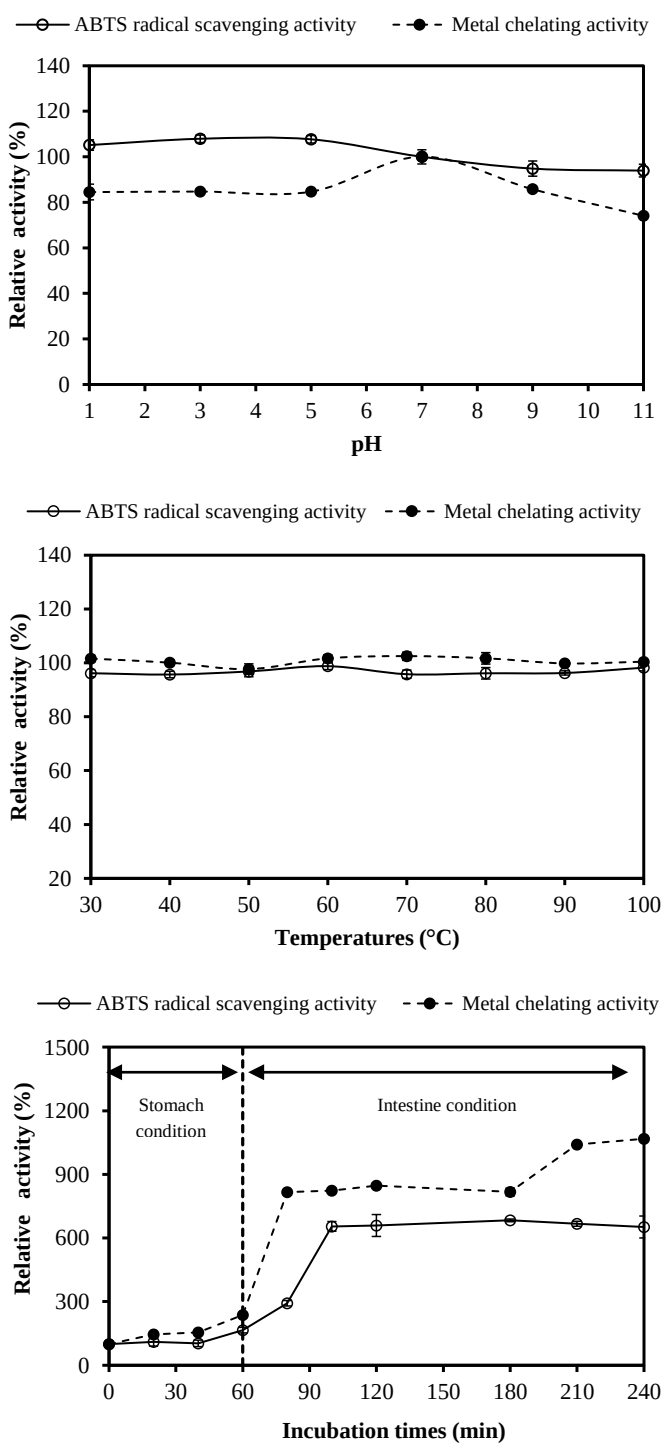


Figure 23. Antioxidant stability of the selected Nile tilapia protein hydrolysate prepared using two-step hydrolysis process (HAPa) as affected by pH (A), heating (B) and GIMs (C) as monitored by ABTS radical scavenging activity and metal chelating activity. Bars represent standard deviation (n=3).

high pH. Due to the stability over a wide pH range, antioxidant peptides of protein hydrolysate from Nile tilapia PI had the potential for applications in any food system in wide pH ranges.

Thermal stability of HAPa is depicted in Figure 23B. Both ABTS radical scavenging and metal chelating activities of HAPa remained constant when subjected to the heating at 30 – 100 °C for 30 min. An activity of more than 98%was retained after heat treatment. A slight decrease in ABTS radical scavenging activity might be due to either degradation or aggregation of some antioxidant peptides, caused by heat treatment. Peptides with smaller sizes were more stable to aggregation at high temperature (Nalinanon *et al.*, 2011). In general, proteins were vulnerable to heat treatment, leading to the aggregation of protein and the exposure of hydrophobic domain. Peptides derived from many protein sources with increased hydrophobicity have been reported to correlate with antioxidant properties (Klompong *et al.*, 2008). The result was in accordance with Binsan *et al.* (2008) who reported that antioxidant in the water extract from Mungoong showed high stability when temperature increased up to 100 °C, in which an activity of more than 80 % was retained. Thus, peptides in HAPa were stable to heating process. As a consequence, HAPa could be used or supplemented as a source of natural antioxidants in thermally processed foods.

7.4.3.2 Stability in gastrointestinal tract model system (GIMs)

Antioxidant activities of HAPa in GIMs were monitored (Figure 23C). GIMs have been used to simulate the ingestion system of human body. HAPa showed the slight increase in ABTS radical scavenging activity and metal chelating activity during pepsin digestion ($P < 0.05$). With further hydrolysis in intestinal simulated system, the sharp increases in ABTS radical scavenging were obtained within the first 40 min under the intestine condition ($P < 0.05$). Thereafter, no difference in activity was found up to 240 min ($P > 0.05$). For metal chelating activity, the marked increase was found in the first 20 min under intestine condition and remained constant up to 180 min, followed by the increase at the end of digestion (240 min). Nalinanon *et al.* (2011) also found the increase in antioxidant activity of protein hydrolysate from ornate threadfin bream muscle after being ingested in the simulated model system. The result suggested that pancreatin might cleave the peptides to some degrees, leading to the

release of new antioxidant peptides. This could enhance the antioxidant activities of hydrolysates in gastrointestinal tract after ingestion.

7.4.4 Antioxidant activities of the selected hydrolysate (HAPa) in different model systems

7.4.4.1 β-carotene linoleate model system

The antioxidant activity of HAPa at different concentrations in a β-carotene-linoleate model system in comparison with Trolox (100 ppm) is shown in Figure 24A. The decrease in A_{470} indicates the oxidation of β-carotene in the system caused by free radical from oxidation of linoleic acid (Chandrasekara and Shahidi, 2011). Free radicals formed are able to attack the highly unsaturated β-carotene molecules, leading to the losses in chromophore and characteristic orange colour of β-carotene (Binsan *et al.*, 2008). A sharp decrease in A_{470} was noticeable in system without antioxidant (control), whilst systems containing HAPa retarded the decrease in A_{470} in a dose dependent manner. Nevertheless, no difference in A_{470} between the systems with HAPa at levels of 500 and 1,000 ppm was observed up to 120 min ($P > 0.05$). The antioxidant activity of HAPa was generally lower than Trolox. This indicated that β-carotene bleaching was retarded mainly due to the elimination of free radicals by HAPa. The difference in antioxidant activity between HAPa and Trolox might be caused by the difference in their polarity. The hydrophobic antioxidants have higher efficiency than hydrophilic antioxidants in preventing oxidation in oil-in-water emulsion systems by preferably orienting at the oil-water interface (Chandrasekara and Shahidi, 2011). Khantaphant *et al.* (2011b) reported that the ability of hydrolysates to prevent the bleaching of β-carotene was governed by the amphiphilic properties of amino acid compositions. The use of HAPa was thus able to retard lipid oxidation in oil-in-water emulsions.

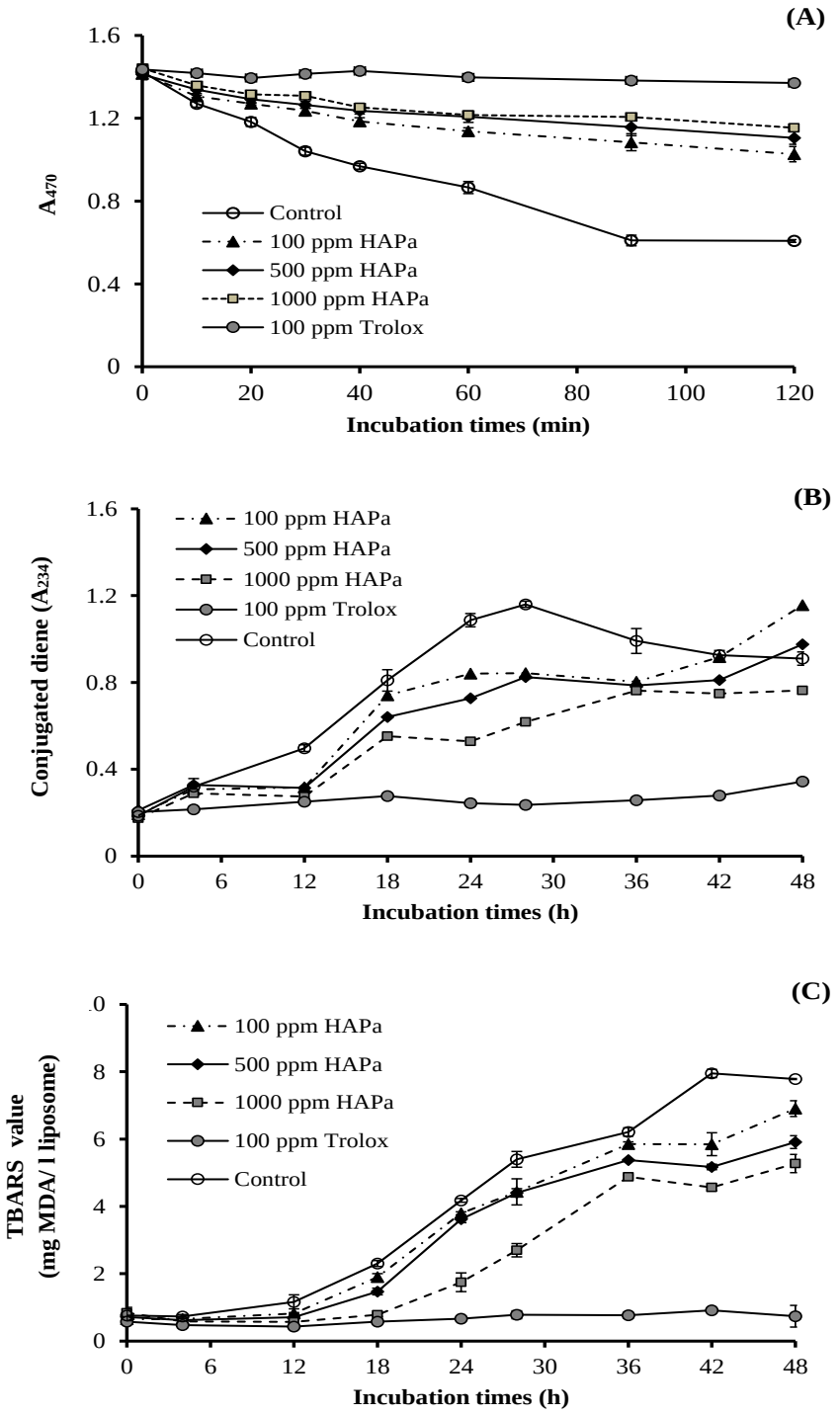


Figure 24. Changes in A_{470} of β-carotene linoleic acid system (A) and the formation of CD (B) and TBARS (C) in lecithin liposome system containing the selected Nile tilapia protein hydrolysate (HAPa) at different levels. Bars represent standard deviation (n=3).

7.4.4.2 Lecithin liposome model system

HAPa at different levels affected the oxidation of the lecithin liposome system differently as indicated by different conjugated dienes and TBARS values during the incubation at 37 °C for 48 h (Figure 24B and 24C). The oxidation of all systems generally increased throughout 48 h of incubation. However, a slight decrease was observed in the control after 30 h of incubation ($P < 0.05$). The formation of CD occurs during the early stages of lipid oxidation (Frankel *et al.*, 1997). The decrease in CD was probably due to the transformation of CD into hydroperoxide or the secondary products. The system containing HAPa had lower increases in CD, compared with the control. However, the rate of changes varied with the concentration used. System containing HAPa at a level of 1,000 ppm showed the lowest CD formation, compared with other concentrations ($P < 0.05$). The result indicated that HAPa containing antioxidant peptide could retard the formation of CD. However, no marked changes in CD of system containing 100 ppmTrolox were observed during incubation of 48 h. The result indicated that HAPa had a lower efficacy in prevention of oxidation at the early stage than did Trolox.

Changes in TBARS of lecithin-liposome system containing HAPa at various concentrations during incubation of 48 h are shown in Figure 24C. TBARS values increased as the incubation time increased ($P < 0.05$). The increase in TBARS indicated the formation of the secondary lipid oxidation products. TBARS in the control (without any additive) increased markedly after incubation for 4 h, whilst those containing HAPa showed the increases in TBARS after 12 h ($P < 0.05$). The longer induction period indicates a stronger antioxidant activity (Wu *et al.*, 2003). The antioxidant effect of HAPa toward TBARS formation was dose-dependent. The system added with 1,000 ppm HAPa showed the lower TBARS formation than those added with other levels ($P < 0.05$). The similar changes in TBARS value were observed within the first 28 h of incubation when the HAPa at levels of 100 and 500 ppm were added to liposome system ($P > 0.05$). Nevertheless, Trolox exhibited higher antioxidant activity than HAPa. This was evidenced by no changes in TBARS during incubation of 48 h ($P>0.05$). Therefore, HAPa, especially at 1,000 ppm, could retard lipid oxidation in lecithinliposome system, however the efficiency was lower than Trolox.

7.4.5 Molecular weight distribution of the selected protein hydrolysates

Nile tilapia protein hydrolysates, HA and HAPa, prepared using one-step and two-step hydrolysis were fractionated using a Sephadex G-25 gel filtration chromatography as shown in Figure 25. A_{220} was used to monitor peptide bonds, whereas A_{280} was the parameter representing peptides, proteins or amino acids with aromatic rings. The different peaks of A_{280} were observed between HA and HAPa, indicating the presence of proteins or peptides containing aromatic amino acids with varying MW. However, A_{220} was greater than A_{280}, indicating the prevalence of peptide bonds in hydrolysate (Klompong *et al.*, 2009). Both hydrolysates showed a distinct peak of A_{280} with the fraction no. 44. However, several small A_{280} peaks were found in both samples. HAPa contained a larger proportion of the low MW peptides than did HA. The result was in accordance with a higher DH obtained in HAPa. Pre-hydrolysis of cod protein using Alcalase with subsequent hydrolysis by Kojizyme yielded the hydrolysate with a higher proportion of peptide below 455 Da (Liaset *et al.*, 2000). Wu *et al.* (2003) reported that three major antioxidant peptides with MW of 1,400, 900 and 200 Da were fractionated from mackerel protein hydrolysate derived by autolysis, whilst the higher proportion of all peaks were observed when Pronase E was used in combination with autolysis.

When the obtained fractions were measured for ABTS radical scavenging activity and metal chelating activity (Figure 25B and 25D), different fractions exhibited varying antioxidant activities. The fraction containing peptides with MW of 1,484 Da exhibited the highest metal chelating activity, whilst peptide with MW of 513 Da showed remarkable ABTS radical scavenging activity for both HA and HAPa. However, stronger activities were noticeable in HAPa than those in HA. The proteases used for hydrolysis showed a pronounced impact on the size and antioxidant activity of peptides formed. Low MW peptides possessing metal chelating activity and ABTS radical scavenging activity were generated by enzymatic hydrolysis. The enhanced exposure of the functional groups of peptides could favour their antioxidant activities. Several reports suggested that phenolic hydroxyl group present in aromatic amino acids contributed substantially to scavenging of radicals via acting as electron donors (Wu *et al.*, 2003). Peptides containing tyrosine residues at the C-terminus, lysine

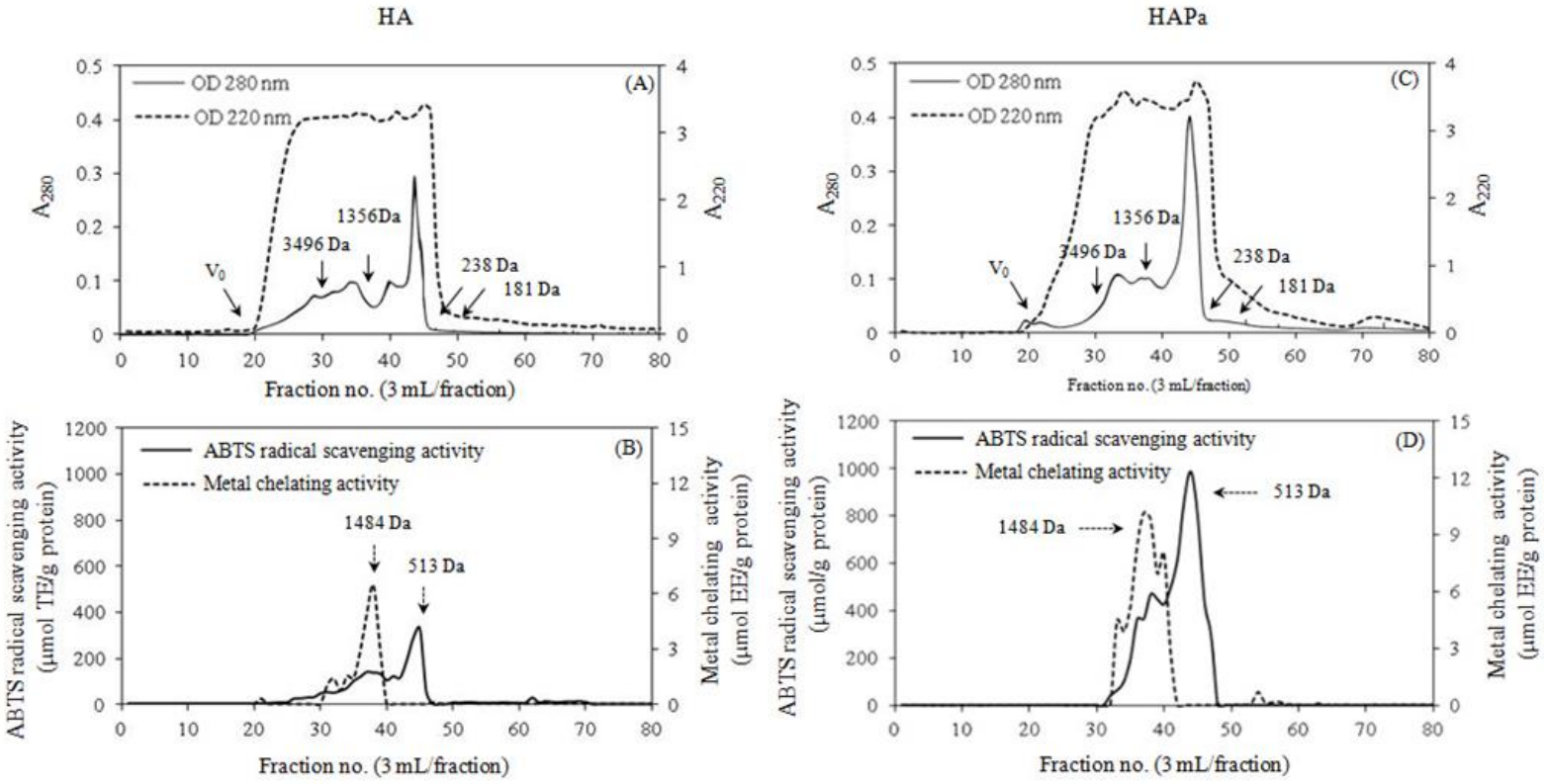


Figure 25. Elution profiles of antioxidant peptides from the selected Nile tilapia protein hydrolysates, HA and HAPa, by a Sephadex G-25 column as monitored by A_{220} and A_{280} (A and C). Elution was performed using distilled water with a flow rate of 0.5 ml/min. Fractions (3 ml) were determined for ABTS radical scavenging activity and metal chelating activity (B and D). Solid arrows in (A) and (C) indicate the standard peaks. Dash arrows in (B) and (D) indicate the peaks with antioxidant activities.

or phenylalanine residues at the N-terminus and tyrosine in their sequence had strong free radical scavenging activity (Wang *et al.*, 2008). Hydrolysates from yellow stripe trevally muscle with MW of 2.4 kDa exhibited higher antioxidant properties than those with MW of 35, 0.47 and 57 kDa, respectively (Klompong *et al.*, 2009). The result suggested that Nile tilapia hydrolysate mostly contained certain peptides with antioxidant activities. The use of two-step hydrolysis process particularly using Alcalase, followed by papain, could enhance the antioxidant activity of resulting hydrolysate.

7.4.6 Amino acids composition of the selected protein hydrolysates

Amino acid compositions of HA and HAPa are presented in Table 19. The major amino acids of both samples were glutamic acid/glutamine, lysine and aspartic acid/asparagines, which accounted for 17.87–18.35 %, 10.68–10.90 % and 10.49–10.64 % of the total amino acids, respectively. The result was in accordance with Foh *et al.* (2010) who reported that the hydrolysates from tilapia mince using Alcalase, Flavourzyme and Neutrase were rich in glutamic acid, aspartic acid and lysine. Khantaphant *et al.* (2011b) also found glutamic acid/glutamine, aspartic acid/asparagines, alanine, lysine and leucine as the most abundant amino acids in hydrolysates from brownstripe red snapper using Alcalase or Flavourzyme in conjunction with pyloric caeca protease. However, negligible contents of cysteine (0.02–0.03 %), hydroxylysine (0.03 %) and tryptophan (0.64–0.72 %) were observed in both HA and HAPa. Shahidi *et al.* (1995) reported that the sensitive amino acids, such as methionine and tryptophan were presented at smaller amount after hydrolysis of capelin proteins. Based on total amino acids, essential amino acids constituted 43.30 and 43.01 % of total amino acids for HA and HAPa, respectively. Therefore, they could be a dietary protein supplement to poorly balanced dietary proteins. Although both HA and HAPa showed the similar amino acid composition, HAPa had the higher antioxidant activity. The difference was plausibly determined by several factors including the sequence of amino acid, chain length, etc. The higher antioxidant activity of HAPa might be due to a higher content of peptides with a shorter chain than did HA. The result was evidenced by a higher %DH obtained in HAPa. In addition, the

Table 19. Amino acid composition of selected protein hydrolysate derived from Nile tilapia PI (HA and HAPa)

Amino acids (%)	HA	HAPa
Alanine†	6.26	6.10
Arginine	6.52	6.49
Aspartic Acid/Aspargine	10.49	10.64
Cysteine†	0.02	0.03
Glutamic Acid/Glutamine	18.35	17.87
Glycine	3.90	3.90
Histidine*	2.67	2.65
Hydroxylysine	0.03	0.03
Isoleucine*,†	4.58	4.63
Leucine*,†	8.63	8.49
Lysine*	10.90	10.68
Methionine*,†	3.29	3.25
Phenylalanine*,†	3.63	3.74
Proline†	2.96	3.32
Serine	4.10	4.19
Threonine*	4.77	4.71
Tryptophan†	0.64	0.72
Tyrosine†	3.42	3.71
Valine*,†	4.83	4.86
Hydrophobic amino acids	38.26	38.85
Essential amino acids	43.30	43.01
Non-essential amino acids	56.60	57.00

* Essential amino acids

†Hydrophobic amino acids

hydrophobic amino acid of HA and HAPa were 38.26 and 38.85 %, respectively. The slight differences in amino acid composition between HA and HAPa depended on the existing differences in enzyme specificity and hydrolysis conditions (Klompong *et al.*, 2009). Peptides derived from many materials with increased hydrophobicity were reported to relate with antioxidant activity (Rajapakse *et al.*, 2005). Kim *et al.* (2001) indicated that some amino acids including histidine, proline, alanine, and leucine can scavenge free radicals. Suetsuna *et al.* (2000) suggested that phenolic hydroxyl groups present in aromatic amino acids contribute substantially to radical scavenging by acting

as potent electron donors. As a result, the different antioxidant activity between hydrolysates (Figure 22) was possibly associated with differences in hydrophobic amino acids in the peptides. Nevertheless, the hydrophobicity has been reported to play a major role in bitter taste (FitzGerald and O'cuinn, 2006). Bitter taste intensity of a peptide might be governed by several factors, such as degree of hydrolysis, concentration and location of bitter taste residues and number of carbons on the R group of branched chain amino acids (Leksrisompong *et al.*, 2012). The results suggested that both HA and HAPa could serve as an excellent source of useful nutrient, based on their amino acid profiles.

7.4.7 Sensory properties of the selected protein hydrolysates

The intensity of fishy-, muddy-odour/flavour and bitterness of HA and HAPa are presented in Table 20. Fishy- and muddy odour/flavour were negligible in both samples. However, no differences in those attributes were observed between HA and HAPa ($P > 0.05$). Fishy odour/flavour development associated with Nile tilapia protein hydrolysate was mainly caused by lipid oxidation, whereas muddy taint was related to muddy compounds known as geosmin (chapter 6). During the preparation of PI, the undesirable materials including lipid or phospholipid were removed. HA showed a higher bitterness score than did HAPa ($P < 0.05$). This might be associated with the formation of peptides containing bulky hydrophobic groups toward their C-terminal such as valine, isoleucine, phenylalanine, tryptophan, leucine and tyrosine (Wu *et al.*, 2003). Hydrolysis process with Alcalase might induce the exposure of the buried hydrophobic peptides, resulting in detection of bitter taste by human taste buds. Peptides containing hydrophobicity (Q) values > 1,400 cal/mole and molecular masses < 6 kDa are bitter in taste (Ney, 1979). Additionally, the presence of internally sited Pro residues was also shown to be a major and distinct contributor to peptide bitterness (Ney, 1979). Further hydrolysis of HA by papain was probably produced peptide with less bitterness by the removal of hydrophobic amino acid side chain to some extent. Due to the similar content of hydrophobic amino acids (Table 19), the differences in bitterness between HA and HAPa were plausibly governed by amino acid sequences of peptides in both hydrolysates. The result was in accordance with Wróblewska and Troszyñska (2005) who reported that the application of two-step hydrolysis with

Alcalase, followed by papain improved the sensory quality of whey protein hydolysate via lowering the bitterness as compared to those produced using Alcalase, Alcalase+Pepsin or Alcalase+Alcalase. Liaset *et al.* (2000) found that the combination of Alcalase and Kojizyme effectively yielded a non-bitter cod byproduct hydrolysate.

Table 20. Sensory properties of the selected protein hydrolysates derived from Nile tilapia PI (HA and HAPa)

Tests	Attributes	HA	HAPa
Intensity †	Fishy - odour	1.00±0.82[a]	1.45±1.03[a,§]
	Fishy - flavour	2.19±1.35[a]	2.96±2.01[a]
	Muddy - odour	0.60±0.41[a]	0.96±0.80[a]
	Muddy - flavour	1.20±0.72[a]	1.46±1.24[a]
	Bitterness	10.14±1.03[a]	6.24±1.87[b]
Acceptance ††	Colour	8.00±0.55[a]	7.83±0.72[a]
	Odour	6.92±0.90[a]	6.73±1.42[a]
	Taste	6.25±1.14[b]	7.08±0.73[a]
	Overall	6.14±0.79[b]	6.75±0.75[a]

Values are given as mean ± SD.

[§]Different superscripts in the same row indicate significant differences ($P < 0.05$).

[†]Score are based on 15 cm unstructured line scales (0: none and 15: extremely strong odour/flavour), evaluated by 10 trained panellists.

[††]Score are based on 9-point hedonic scales (1: dislike extremely, 5: Neither like nor dislike, 9: Like extremely), evaluated by 30 untrained panellists.

Colour, odour, taste and overall likeness scores of HA and HAPa are shown in Table 20. There was no difference in likeness scores of colour and odour between HA and HAPa ($P > 0.05$). According to the criterion for acceptability limit, the score greater than 5 indicates acceptability (Meilgaard *et al.*, 2007). Pre-washing prior to alkaline solubilisation of fish mince yielded the resulting PI with a lower water soluble pigment, such as haem proteins (chapter 3 and 4). As a result, the lighter colour was obtained with both hydrolysates, leading to high acceptability. A high likeness score of odour was in agreement with a low intensity of fishy- or muddy odour.

However, a slightly lower score in odour likeness was probably governed by the offensive smell associated with enzymes used. For taste and overall likeness, HA exhibited the lower likeness scores, compared with HAPa ($P < 0.05$). This was more likely due to the lower bitterness in HAPa. Saha and Hayashi (2001) noted that the formation of bitter peptide is the most serious problem in the practical use of food protein hydrolysates. Thus, the application of a two-step hydrolysis process using Alcalase followed by papain could be a promising mean for production of muscle protein hydrolysate with improved sensory quality via the reduction of bitterness.

7.5 Conclusion

Protein hydrolysate from Nile tilapia PI prepared using different single proteases exhibited various antioxidant activities, depending on DH and types of enzyme used. Hydrolysate prepared using Alcalase with 40 % DH possessed the highest antioxidant activities. The application of a two-step hydrolysis process using Alcalase and papain for the first and the second steps, respectively yielded the hydrolysate with antioxidant activities, both in vitro and oxidation model systems. The activities were stable in a wide range of pH (1–11) and during heating (30–100 °C). The increase in activity was found after digestion in GIMs. The antioxidant peptides in both HA and HAPa were characterised to have MW of 513 and 1,484 Da, capable of scavenging free radicals and metal chelation, respectively. Additionally, both hydrolysates were rich in essential amino acids. However, HAPa showed a stronger antioxidant activity together with a higher sensorial acceptability than did HA. Therefore, hydrolysate from Nile tilapia PI prepared using two-step hydrolysis could serve as the natural antioxidant for food preservation or as functional foods.

CHAPTER 8

PREVENTIVE EFFECT OF NILE TILAPIA PROTEIN HYDROLYSATE AGAINST OXIDATIVE DAMAGE OF HEPG2 CELLS AND DNA MEDIATED BY H_2O_2 AND AAPH

8.1 Abstract

Antioxidant activities of protein hydrolysate prepared from Nile tilapia protein isolate using Alcalase (HA), Alcalase followed by papain (HAPa) and their Sephadex G-25 fractions (FHA and FHAPa) were investigated in both chemical and cellular based models. Amongst all samples, FHAPa showed the highest chemical antioxidant activities, however it had no metal chelation activity. Cellular antioxidant ability of HA, HAPa and their fractions agianst H_2O_2 and AAPH induced oxidative damage of HepG2 cell and DNA were tested. When cells were pretreated with all hydrolysates or fractions, cell viabiliy increased. Cell reactive oxygen species (ROS) generation, mediated by H_2O_2 and AAPH, decreased with treatment of hydrolysates or their fractions, especially in combination with 50 µM Trolox. FHAPa effectively inhibited H_2O_2 and peroxyl radical induced DNA scission in a dose dependent manner. Therefore, Nile tilapia protein hydrolysates could serve as a functional food ingredient.

8.2 Introduction

Generally, oxygen consumption inherent in cell growth leads to the generation of a series of reactive oxygen species (ROS). The formations of ROS including superoxide radical ($O_2^{-\bullet}$), hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), etc. are inevitably consequence in aerobic organisms during respiration. Some of them are essential for biological functions involved in cell mechanism, proliferation, apoptosis and signal transduction (Martindale and Holbrook, 2002; Owuor and Kong, 2002). Nevertheless, the excessive production of ROS can attack biological macromolecules such as protein, lipid and DNA, etc, leading to cell or tissue injury. Under normal condition, ROS are effectively eliminated by the antioxidant defense system, such as antioxidant enzymatic and non-enzymatic factors in human body (Kim *et al.*, 2007). However, under pathological conditions, the imbalance between the

generation and the elimination of ROS was contributed to oxidative stress on cell, resulting in many disorders. Oxidative damage has been associated with numerous chronic diseases including diabetes, cancer, neurodegenerative and coronary heart disease (Chai *et al.*, 2013).

In recent years, a large number of studies has been conducted to find potent radical scavengers to protect against oxidative stress (García-Nebot *et al.*, 2014; Himaya *et al.*, 2012). Numerous antioxidant compounds, particularly fish peptide, have been widely studied. Potent antioxidant peptides have been isolated from protein hydrolysates from the muscle of yellow stripe trevally (*Selaroides leptolepis*) (Klompong *et al.*, 2009), cod (*Gadus morhua*) (Halldorsdottir *et al.*, 2014), spotless smoothhound (*Mustelus griseus*) (Wang *et al.*, 2014) and monkfish (*Lophius litulon*) (Chi *et al.*, 2014), etc. Protein, peptides and free amino acids released during enzymatic hydrolysis are capable of modulating specific biological function. Different modes of actions, namely the donation of electrons/hydrogen atom, direct scavenging of free radicals and sequestration of pro-oxidative metal-ions, have been observed in various protein hydrolysates (Samaranayaka and Li-Chan, 2011). The activity is closely related to the amino acid composition and sequence, size and configuration of peptides (Alemán *et al.*, 2011). Additionally, protein hydrolysates possessing antioxidant activity have been governed by the type of proteases used for step-wise hydrolysis (Phanturat *et al.*, 2010). Recently, the application of Alcalase in combination with papain for hydrolysis of Nile tilapia protein isolate (PI) gave a hydrolysate with antioxidant properties and reduced bitterness (chapter 7). Although protein hydrolysates derived from PI have been widely reported to exhibit *in vitro* antioxidant activity, a little information regarding the antioxidant ability on cell-based models, especially those induced by different types of free radicals, have been reported. Thus, the objective of this study was to investigate antioxidant properties of Nile tilapia protein hydrolysates prepared using Alcalase (HA), Alcalase/papain (HAPa) and their Sephadex G-25 fractions in both chemical and cellular antioxidant based models.

8.3 Materials and Methods

8.3.1 Chemical/enzymes

Alcalase 2.4 L (E.C. 3.4.21.62) (2.4 AU/g) was provided by Novozymes (Bagsvaerd, Denmark). Papain (E.C. 3.4.22.2) (≥3AU/mg), 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,4,6-trinitrobenzenesulfonic acid (TNBS), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), 2,2′-azobis (2-methylpropionamidine) (AAPH) and hydrogen peroxide (H_2O_2) were purchased from Sigma (St. Louis, MO, USA). Sephadex G-25 was procured from GE-Healthcare (Uppsala, Sweden). The HepG-2 human hepatoma cell line was obtained from American Type Culture Collection (ATCC 8065, Rockville, MD, USA). Cell culture medium and all the other materials required for culturing were obtained from Invitrogen (Carlsbad, CA, USA). Plasmid DNA (pUC 18) was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). All chemicals were of analytical grade.

8.3.2 Fish sample collection

Fresh Nile tilapia (*Oreochromis niloticus*) with a weight of 0.8-1.0 kg/fish were purchased from a local market in Hat Yai, Songkhla, Thailand. Fish were stored in ice and transported to the Department of Food Technology, Prince of Songkla University within 30 min. Upon arrival, fish flesh was separated manually and minced using a Moulinex AY46 blender (Group SEB, Lyon, France) in a walk-in-cold room (4 °C). The mince was placed in polyethylene bags and stored in ice until used.

8.3.3 Preparation of Nile tilapia protein isolate (PI)

PI from pre-washed mince was prepared following the method as mentioned in chapter 3. Washed mince was homogenised with five volumes of cold distilled water (2-4 °C) using an IKA Labortechnik homogeniser (Selangor, Malaysia) at a speed of 11,000 rpm for 1 min. The homogenate was adjusted to pH 11 and placed on ice for 60 min with a continuous stirring. The mixture was then centrifuged at 5,000 ×g for 10 min at 4 °C using an Avianti J-E centrifuge (Beckman Coulter, Inc., Fullerton,

CA, USA). The alkaline soluble fraction obtained, referred to as 'PI solution', was used as substrate for hydrolysis.

8.3.4 Production of Nile tilapia protein hydrolysates

PI solution was mixed with distilled water to obtain a final protein concentration of 2% (w/v) as determined by the Biuret method (Robinson and Hogden, 1940). The hydrolysis was conducted for 1 h using Alcalase at a level of 5.54% (w/w) (pH 8.0, 50 °C) to obtain the degree of hydrolysis (DH) of 40% as described by Benjakul and Morrissey (1997). After 1 h of hydrolysis, the reaction was terminated by placing the mixture in boiling water for 10 min. One half of the resulting hydrolysate was subjected to centrifugation at 2,000 ×g at 4 °C for 10 min. The supernatant was lyophilised using a Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lynge, Denmark). Another portion was further hydrolysed using papain at the same amount used in the first step. Reaction was conducted for 1 h at pH 7.0 and 40 ºC and the mixture was submerged in boiling water for 10 min to terminate the enzyme. A DH of 53% was obtained. Thereafter, the mixture was subjected to centrifugation and lyophilisation as mentioned previously. The resulting hydrolysates using Alcalase and Alcalase/papain were named as 'HA' and 'HAPa', respectively.

To fractionate both hydrolysates, HA and HAPa were further separated using Sephadex G-25 gel filtration chromatography as described by Yarnpakdee *et al.* (2014). Fractions with the highest ABTS radical scavenging activity were pooled. Pooled fractions obtained from HA and HAPa were referred to as 'FHA' and 'FHAPa', respectively. All samples were kept in -20 ºC until further analysis.

8.3.5 Chemical antioxidant activities

8.3.5.1 DPPH radical scavenging activity

DPPH radical scavenging activity was determined as described by Binsan *et al.* (2008). Sample solution (10 mg/ml; 1.5 ml) was added with 1.5 ml of 0.1 mM DPPH in 95% ethanol. The mixture was allowed to stand for 30 min in dark at room temperature. The resulting solution was measured at 517 nm using a UV-160 spectrophotometer (Shimadzu, Kyoto, Japan). The blank was prepared in the same

manner except that distilled water was used instead of the sample. A standard curve was prepared using Trolox in the range of 0–60 M. The activity was expressed as µmol Trolox equivalents (TE)/g solid.

8.3.5.2 ABTS radical scavenging activity

ABTS radical scavenging activity was measured as per the method of Binsan *et al.* (2008). The stock solutions included 14.8 mM ABTS solution and 5.2 mM potassium persulphate solution. The working solution was prepared by mixing two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in dark. The solution was then diluted by mixing 1 ml of ABTS solution with 50 ml of methanol in order to obtain an absorbance of 1.1 ± 0.02 at 734 nm. Sample (10 mg/ml; 150 µl) was mixed with 2,850 µl of ABTS solution and the mixture was left at room temperature for 2 h in dark. The absorbance was then measured at 734 nm. The blank was prepared in the same manner except that distilled water was used instead of the sample. A standard curve of Trolox ranging from 0 to 600 µM was prepared. The activity was expressed as µmol TE/g solid.

8.3.5.3 Oxygen radical absorbance capacity (ORAC)

The oxygen radical absorbance capacity (ORAC) assay was performed according to Ganske and Dell (2006), Trolox (0–50 µM) and samples (0.3-0.7 mg/ml) were prepared in phosphate buffer (10 mM, pH 7.4). The reaction was conducted in a black opaque microplate (200 µl, 96 wells, MJ Research, Waltham, MA, USA). For each well, the following solutions were added: (1) 60 µl of 10 nM Fluorescein solution; (2) 10 µl of Trolox dilutions for standard or 10 µl of sample solution or 10 µl of phosphate buffer for blank. The microplate was incubated for 15 min at 37 ºC without shaking in the POLARstar Optima microplate reader (BMG labtech, Offenburg, Germany). After incubation, 30 µl of 120 mM AAPH solution was quickly added manually using a multi-channel pipette. The fluorescence (excitation at 485 nm and emission at 520 nm) was recorded every minute for 100 minutes. The area under the fluorescence decay curve (AUC) was calculated by the normalised curves with the following equation:

$$AUC = (f_0/f_0 + f_{99}/f_0) \times 0.5 + (f_1/f_0 + ... + f_{98}/f_0)$$

where f_0 was the fluorescence reading at the initiation of the reaction and f_{99} was the last measurement. The net AUC was obtained by subtracting the AUC of the blank from was measured by reading the absorbance at 593 nm. The blank was prepared in the same manner except that distilled water was used instead of the sample. The standard curve was prepared using Trolox ranging from 0 to 600 µM. The activity was expressed as µmol TE/g solid.

8.3.5.4 Ferric reducing antioxidant power (FRAP)

FRAP assay was performed according to Benzie and Strain (1996). FRAP reagent was prepared by mixing 25 ml of 300 mM acetate buffer (pH 3.6), 2.5 ml of 10 mM TPTZ solution in 40 mM HCl, and 2.5 ml of 20 mM $FeCl_3.6H_2O$ solution. The mixed solution was incubated at 37 °C for 30 min in a water bath (Memmert, D-91126, Schwabach, Germany) and was referred to as FRAP solution. A sample (10 mg/ml; 150 µl) was mixed with 2,850 µl of FRAP solution and kept for 30 min in dark at room temperature. The ferrous tripyridyltriazine complex (coloured product) was measured by reading the absorbance at 593 nm. The blank was prepared in the same manner except that distilled water was used instead of the sample. The standard curve was prepared using Trolox ranging from 0 to 600 µM. The activity was expressed as µmol TE/g solid.

8.3.5.5 Metal chelating activity

Metal chelating activity was investigated as described by Binsan *et al.* (2008) with a slight modification. Sample (10 mg/ml; 940 µl) was mixed with 20 µl of 2 mM $FeCl_2$ and 40 µl of 5 mM ferrozine. The mixture was allowed to stand at room temperature for 20 min. Absorbance at 562 nm was read. The blank was prepared in the same manner except that distilled water was used instead of the sample. EDTA with the concentrations range of 0–30 µM was used as standard. Metal chelating activity was expressed as µmol EDTA equivalent (EE)/g solid.

8.3.5.6 Hydrogen peroxide (H_2O_2) scavenging activity

Hydrogen peroxide scavenging activity was assayed according to the method of Wettasinghe and Shahidi (2000). Sample (10 mg/ml, 200 µl) was mixed with 300 µl of 40 mM H_2O_2. Total reaction volume was brought up to 1 ml with 45 mM sodium phosphate buffer (pH 7.4). After 40 min of incubation at room temperature (25-28 ºC), the absorbance was measured at 230 nm. For sample blank, hydrogen peroxide was omitted and replaced by sodium phosphate buffer (pH 7.4). Trolox (0–5 mM) was used as standard. The hydrogen peroxide scavenging activity was expressed as µmol TE/g solid.

8.3.5.7 Singlet oxygen scavenging activity

Singlet oxygen scavenging activity was determined as described by Kittiphattanabawon *et al.* (2012) with a slight modification. The chemical solutions and sample were prepared in 45 mM sodium phosphate buffer (pH 7.4). The reaction mixture consisted of 0.4 ml of sample, 0.5 ml of 200 µM *N*,*N*-dimethyl para-nitrosoaniline (DPN), 0.2 ml of 100 mM histidine, 0.2 ml of 200 mM sodium hypochlorite, and 0.2 ml of 200 mM H_2O_2, and the total volume was made up to 2 ml with 45 mM sodium phosphate buffer (pH 7.4). The absorbance of the reaction mixture was measured at 440 nm after incubation at room temperature (25 °C) for 40 min. Sample blanks were run in the same manner, except DPN, histidine, and NaOCl solutions were replaced by sodium phosphate buffer. A standard curve of Trolox (0–10 mM) was prepared. Singlet oxygen scavenging activity was expressed as µmol TE/g solid.

8.3.6 Cellular antioxidant activities

8.3.6.1 Cell culture

HepG2 cells (ATCC 8065, American Type Culture Collection, Rockville, MD, USA) were cultured as per the method of Halldórsdóttir *et al.* (2014a). The cells were maintained in Minimum Essential α (MEMα) and supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), penicillin (50 units/ml) and

streptomycin (50 µg/ml). Cells were incubated at 37 ºC in a fully humidified environment under 5% CO_2 and HepG2 cells at passage 80–100 were used for the experiments. Cell culture medium was replaced every other day, and cells were subcultured at 3–5 days intervals before reaching 90% confluence.

8.3.6.2 Cytotoxicity effect of H_2O_2 or AAPH on HepG2 cells

Cytotoxicity effect of H_2O_2 and AAPH was determined as described by Wiriyaphan *et al.* (2012) with a slight modification. Cells were seeded at 6.0×10^4 cells per well on black 96-well plates (BD Falcon™, Franklin Lakes, NJ, USA) and incubated in a humidified incubator containing 5% CO_2 at 37 ºC, for 24 h. Then, the cells were treated with H_2O_2 or AAPH at various concentrations (200–1000 µM; 100 µl) and incubated for another 24 h. After incubation, the culture medium was removed and the cell viability was evaluated by exposure to 100 µl of 10% PrestoBlue® (Invitrogen) solution in MEMα at 37 ºC in dark for 1 h. Thereafter, the fluorescence was measured at excitation wavelength (λ_{ex}) of 570 nm and emission wavelength (λ_{em}) of 610 nm using a POLARstar OPTIMA microplate reader. Control cells were prepared in the same manner without oxidative stressors addition. The result was expressed as percentage of viable cells compared to the control culture as follows:

$$\text{Cell viability (\%)} = \left(\frac{\text{Mean fluorescence of treated cells}}{\text{Mean fluorescence of control cells}}\right) \times 100$$

The oxidative stressors at a level rendered a ~ 50% cell viability were selected for further studies

8.3.6.3 Protective effect of HA, HAPa and their Sephadex G-25 fractions against oxidative stress on HepG2 cell

A) Cell viability determination

Cells were placed in a 96-well plate (6.0×10^4 cells/well) and incubated in a humidified incubator containing 5% CO_2 and at 37 ºC, for 24 h. Then, cells were pretreated with various hydrolysates and fractions (HA, FHA, HAPa and FHAPa) at different concentrations (0.5, 1 and 2 mg/ml; 100 µl) in the presence and

absence of 50 µM Trolox and further incubated for 24 h. In the first set, the cells were subjected to determination of the viability using Prestoblue® assay as described above. For the second set, the culture medium was removed and replaced with a fresh medium containing 100 µl of 800 µM H_2O_2 or 800 µM AAPH to give the oxidative stress. Subsequently, the cells were further incubated at 37 ºC in dark for another 24 h. The control cells were cultured without any treatment. A commercial antioxidant, 50 µM of Trolox, was used as positive control. After incubation, cell viability was assessed and the results were expressed as a percentage of viable cells, compared to the control culture (without any treatment).

B) Cell ROS determination by DCFH-DA

Intracellular formation of ROS was assessed using an oxidation sensitive dye DCFH-DA as the substrate according to the method of Wolfe and Liu (2007) with some modification. HepG2 cells (6×10^4 cells/well) seeded in black 96-well plates were loaded with 100 µL DCFH-DA (1 µM in Hank's Balanced Salt Solution, HBSS) and incubated in the dark for 30 min. Cells were then treated with various hydrolysates and fractions at different concentrations (0.5, 1 and 2 mg/ml; 100 µl) in the presence and absence 50 µM Trolox and incubated for another 1 h. After removal of the test compounds, 100 µL of 800 µM H_2O_2 or 800 µM AAPH in HBSS were added. The formation of 2′,7′-dichlorofluorescin (DCF) due to oxidation of DCFH in the presence of ROS was read every 10 min for 90 min at λ_{ex} of 485 nm and the λ_{em} of 535 nm using a POLARstar OPTIMA microplate reader. Negative control wells consisted of cells connect to the DCFH-DA probe and oxidative stressors (H_2O_2 or AAPH). The % cellular ROS was expressed as the percentage of relative fluorescence intensity of negative control cell.

8.3.7 Protective effect on DNA oxidation induced by H_2O_2 and AAPH

The ability of HA, HAPa and their fractions to protect DNA damage from ROS was assessed as per the method Chandrasekara and Shahidi (2011) with a minor modification. A reaction was conducted in an Eppendorf tube at a total volume of 10 µL. Supercoiled plasmid DNA (pUC 18) (0.125 µg/µL, 4 µL) dissolved in 10

mM Tris-HCl containing 1 mM EDTA (pH 8.0) was mixed with 2 µL of different hydroysates or fractions to obtain a final concentration of 0.5, 1.0 and 2.0 mg/ml. To initiate the oxidation reaction, 4 ml of 30 mM AAPH or 30 mM H_2O_2 were added. The mixture was incubated at 37 ºC for 1 h in the dark. The controls were prepared in the same manner by using distilled water instead of oxidants. After incubation, 2 ml of the loading dye (0.25% bromophenol blue, 50% glycerol) were added to the reaction mixture. The mixture (12 ml) was loaded onto 1% agarose gel, and the DNA bands were stained with ethidium bromide. Electrophoresis was conducted at 100 V for 50 min using a horizontal gel electrophoresis system (Sub cell® model 192 cell, Biorad, Hercules, CA, USA) equipped with PowerPac™ basic power supply (Biorad, Hercules, CA, USA). The DNA bands were visualised under transillumination of UV light using Gel Doc™ 2000 Gel Documentation System (Biorad, Hercules, CA, USA). The retention of supercoiled DNA strand (%) was calculated using following equation:

$$\text{Retention (\%)} = \left(\frac{\text{Intensity of supercoil DNA of sample}}{\text{Intensity of supercoil DNA of control}}\right) \times 100$$

8.3.8 Statistical analysis

Experiments were run in triplicate using three lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

8.4 Results and Discussion

8.4.1 Chemical antioxidant activity of HA, HAPa and their Sephadex G-25 fraction

Chemically based antioxidant activities of different hydrolysates and their fractions (HA, FHA, HAPa and FHAPa) are shown in Table 21.

8.4.1.1 DPPH and ABTS radical scavenging activities

DPPH scavenging activities ranged from 4.59 to 15.89 µmol TE/g solid, whilst ABTS radical scavenging activity varied from 106.33 to 322.79 µmol TE/g solid. DPPH and ABTS radical scavenging activities are based on the ability of antioxidants to donate a hydrogen atom or an electron to stabilise radicals, by converting them to the non-radical species (Binsan *et al.*, 2008; Chandrasekara and Shahidi, 2011). When the radicals are scavenged, the absorbance is decreased. The radical scavenging activity is visually noticeable as the changes from purple to yellow for DPPH and the changes from green to colourless for ABTS assays. In general, crude protein hydrolysates (HA or HAPa) had lower DPPH and ABTS radical scavenging activities in comparison with their corresponding fractions (FHA or FHAPa). The differences in scavenging capacity for DPPH and ABTS radicals were likely related with the different peptides in each sample. This might be governed by the specificity of the enzyme hydrolysis conditions and degree of hydrolysis when different processes were used. Peptides containing more hydrophobic side chain were found to be more accessible by $DPPH^{\bullet}$ (Zhu *et al.*, 2008). The shorter peptides, including di- or tri- peptides, that are more hydrophilic were found to readily react with water soluble $ABTS^{\bullet+}$ but not with lipid soluble $DPPH^{\bullet}$ (Zhu *et al.*, 2008). The activities of both HAPa and FHAPa were much higher than that those of HA and FHA for both assay tested. The result suggested that the application of two-step hydrolysis using Alcalase and papain might liberate peptides with higher antioxidant activity. Amongst all samples, FHAPa showed the highest DPPH and ABTS radical scavenging activities ($P < 0.05$). After gel filtration, peptides with high radical scavenging activity were fractionated. Simultaneously, the peptides without radical scavenging capacity were removed.

8.4.1.2 ORAC

All hydrolysates and fractions showed ORAC in the ranges of 369.17 to 378.16 µmol TE/ g solid. After fractionation, higher ORAC was obtained. It was noted that FHA had higher ORAC than FHAPa ($P < 0.05$). FHA might contain a larger proportion of peptides possessing a higher capacity in scavenging peroxyl radical than

Table 21. Antioxidant activity of Nile tilapia protein hydrolysates and Sephadex G-25 fractions prepared using different enzymes

Chemical antioxidant assays†	HA	FHA	HAPa	FHAPa
DPPH radical scavenging (µmol TE/ g solid)	4.59±0.42$^{*, d}$	9.05±0.39^{c}	11.46±0.41^{b}	15.89 ± 0.17^{a}
ABTS radical scavenging (µmol TE/ g solid)	106.33±3.35^{d}	268.93±7.41^{b}	147.51±8.29^{c}	322.79 ± 4.44^{a}
ORAC (µmol TE/ g solid)	369.17± 26.51^{c}	949.07 ± 38.98^{a}	378.16±17. 64^{c}	789.20±24.35^{b}
FRAP (µmol TE/ g solid)	10.13± 0.41^{c}	10.78±2.70^{c}	13.56±0.02^{b}	16.84 ± 0.10^{a}
Metal chelating activity (µmol EE/ g solid)	1.36± 0.29^{b}	ND**	5.70±0.53^{a}	ND
Singlet oxygen scavenging activity (µmol TE/ g solid)	686.02±1.50^{c}	1413.88±42.48^{b}	779.34±86.86^{c}	1642.72±61.59^{a}
H_2O_2 scavenging activity (µmol TE/ g solid)	174.60±17.57^{d}	350.10 ± 2.29^{c}	507.93±10.27^{b}	618.48±5.14^{a}

† Values are given as mean ± SD (n=2).

*Different superscripts in the same row indicate the significant difference.

**Not detectable.

HA: hydrolysate prepared using Alcalase

FHA: Sephadex G-25 fraction of HA

HAPa: hydrolysate prepared using Alcalase, followed by papain

FHAPa: Sephadex G-25 fraction of HAPa

FHAPa. Peroxyl radicals, which are formed by direct reaction of triplet oxygen with alkyl radicals during the propagation step in fatty acid oxidation, produce hydroperoxides by abstracting hydrogen from other molecules (Chandrasekara and Shahidi, 2011). ORAC is the assay, which based on the inhibition of the peroxyl radical induced oxidation initiated by thermal decomposition of the azo compound, AAPH

(Glazer, 1990). Fluorescein, which has been used as a fluorescence probe, reacts with peroxyl radical and hence causing fluorescence decay. The fluorescence decay was inhibited in the presence of antioxidants (Zhu *et al.*, 2008). Samaranayaka *et al*. (2010) reported that ORAC of both crude and fractionated Pacific hake (*Merluccius productus*) protein hydrolysate was found in the range of 225 - 330 µmol TE/ g sample. Theodore *et al.* (2008) also reported that ORAC of protein hydrolysate prepared from catfish isolates ranged from 2 to 4 µmol TE/g protein. Peptides in Nile tilapia protein hydrolysate could therefore serve as the powerful antioxidant for donating a hydrogen atom to peroxyl radical.

8.4.1.3 FRAP

Table 21 shows FRAP of various hydrolysates and their fractions. A higher FRAP was noticeable in HAPa (13.56 µmol TE/g solid), compared with that of HA (10.13 µmol TE/g solid) ($P < 0.05$). FHAPa (16.84 µmol TE/g solid) also showed higher FRAP than FHA (10.78 µmol TE/g solid). Further cleavage of peptides in two-step hydrolysis process by papain led to an enhanced reducing ability, plausibly due to the increases in the exposure of some amino group, such as leucine, lysine, methionine or iso-leucine at terminal of released peptides. FRAP is a method based on electron transfer and is used frequently to measure antioxidant activity of peptides in reducing the Fe^{3+} - Fe^{2+} transformation. You *et al.* (2011) reported that loach peptides having a stronger reducing power contained histidine, methionine, tryptophan, lysine, and tyrosine in theirs sequences. Rapeseed fraction with the strongest reducing power had abundant hydrophobic amino acids, which were considered to contribute to enhancing the reducing power of peptides (Zhang *et al.*, 2008). After fractionation, FRAP of HAPa sample increased as evidenced by higher FRAP for FHAPa. However, there was no difference in FRAP between HA and FHA ($P > 0.05$).

8.4.1.4 Metal chelating activity

The metal chelating activity of HA and HAPa was 1.36 and 5.70 µmol TE/g solid, respectively, whilst FHA and FHAPa had no metal chelating activity. The result indicated that the further hydrolysis using papain resulted in enhanced metal

chelating capacity. This might be attributed to more exposure of effective sites capable of chelating ferrous ion. Nevertheless, some peptides possessing metal chelating activity were possibly removed during fractionation. Ferrous ion (Fe^{2+}) is a pro-oxidant and can interact with hydrogen peroxide in a Fenton reaction to produce reactive oxygen species and hydroxyl ($OH^{\bullet}$) free radicals, leading to the initiation and/or acceleration of lipid oxidation (Stohs and Bagchi, 1995). Yarnpakdee *et al.* (2014) demonstrated that the peptide from Nile tilapia protein hydrolysates with MW of 513 Da exhibited the strongest ABTS radical scavenging activity, whereas those with MW 1484 Da possessed the highest metal chelating activity. Thus, varying peptides in Nile tilapia protein hydrolysates, especially in preparation using different enzymes, showed different metal chelating activity.

8.4.1.5 Singlet oxygen scavenging activity

The greatest singlet oxygen radical scavenging activity was observed for FHAPa, followed by FHA, HAPa and HA, respectively. The result demonstrated that HAPa and its fraction were stronger in scavenging singlet oxygen than did HA. Active peptides possessing singlet oxygen scavenging activity might be more generated during the second hydrolysis mediated by papain. Singlet oxygen, which is a highly reactive, electrophilic and non-radical molecule, can be formed by the reaction between photosensitizers and triplet oxygen in the presence of light (Min and Boff, 2002). Singlet oxygen can directly react with electron-rich double bonds of unsaturated fatty acids without the formation of free-radical intermediates (Choe and Min, 2005). This was correlated well with Kittiphattanabawon *et al*. (2012) who reported that peptides with the shorter chain length from blacktip shark skin were able to trap or bind singlet oxygen to a higher extent. In general, the higher activity was noticeable in Sephadex G-25 fractions, compared with that found in hydrolysates. The result suggested that peptides with singlet oxygen scavenging activity were more concentrated in the fraction obtained.

8.4.1.6 H_2O_2 scavenging activity

H_2O_2 scavenging activity of all hydrolysates and their fractions is presented in Table 21. H_2O_2 is a reactive non radical, which can permeate biological membranes and be converted to more reactive species such as hydroxyl radical and singlet oxygen (Choe and Min, 2005). Hydrogen peroxide is the precursor for the generation of hydroxyl radical, which is a strong initiator of lipid oxidation (Choe and Min, 2005). HAPa exhibited higher ability for scavenging H_2O_2 than HA ($P < 0.05$). This might be governed by the differences in peptide produced by enzyme used. Guo *et al.* (2009) reported that dipeptide derived from royal jelly protein by Protease N containing tyrosine residue at its C-terminus was associated with strong hydrogen peroxide scavenging activity. Both fractions, FHA and FHAPa, had a higher H_2O_2 scavenging activity than the corresponding hydrolysates ($P < 0.05$). Similar results were observed to those of DPPH and ABTS radical scavenging activities.

From the above measurements, HAPa showed higher antioxidant activities than those of HA. This result indicates that the second step hydrolysis could release bioactive peptides from the hydrolysates obtained from the first step. Changes in size, amount, exposure of the terminal amino groups of the products obtained, and composition of free amino acids or small peptides determine antioxidative activity (Thiansilakul *et al.*, 2007b; Wu *et al.*, 2003). The small peptides generally showed a higher antioxidant activity (Qian *et al.*, 2008). In addition, their antioxidant activities, except for metal chelating activity, were enhanced after fractionation. Therefore, HA and HAPa contained various antioxidant peptides with various modes of action. The partial purification using gel filtration could be an effective means to concentrate antioxidant peptides.

8.4.2 Cellular antioxidant activity of hydrolysates and Sephadex G-25 fractions of HepG2 cell

8.4.2.1 Effect of oxidative stressors on cytotoxicity of HepG2 cell

Effect of H_2O_2 and AAPH on HepG2 cell viability is depicted in Figure 26. When the cells were exposed to oxidative stressors, the viability of cells was

decreased ($P < 0.05$). With increasing levels of H_2O_2 or AAPH, the cell viability was significantly lowered. Cell viability was 49.1% and 14.2% after exposure to 1000 μM H_2O_2 and 1000 μM AAPH, respectively. The result suggested that HepG2 cells were sensitive to H_2O_2 and peroxyl radical, especially when the level was higher than 200 μM ($P < 0.05$). In general, ROS can cause oxidative stress and damage of biomolecules in the cell, leading to cell death and serious chronic diseases (Suh *et al.*, 2011). Zhang *et al.* (2012) reported that the use of H_2O_2 (50-200 μM) exhibited a dose dependent decrease in PC12 cell viability. Wiriyaphan *et al.* (2012) found that HepG2 cell had a 44% of cell death when exposed to tert-butyl hydroperoxide at a concentration of 500 μM. Elisia and Kitts (2008) noted that the exposure of Caco2 cell to 15 mM AAPH led to cell apoptosis. However, no difference in viability between cells exposed to H_2O_2 and AAPH at levels below 600 μM was noticeable ($P > 0.05$). A damaging effect was found when HepG2 cells were challenged with APPH than H_2O_2 at levels of 800-1000 μM ($P < 0.05$). The result indicated that peroxyl radical acted as a stronger oxidative stressor in HepG2 cell, compared with H_2O_2. It is well known that H_2O_2 itself is not highly reactive, however it forms a highly reactive hydroxyl radical ($OH^{\bullet}$) in the presence of transition metal ions, such as Fe^{2+} and Cu^{+} (Halliwell and Gutteridge, 1992). Wijeratne *et al.* (2005) reported that the loss in viability of Caco2 cells exposed to 10 mM H_2O_2 was caused by the changes in cell membrane permeability. This could lead to the increased entry of toxins to cells. Although hydroxyl radicals have been proven as highly damaging free radical in cells, they have short half-life (10^{-9} s) (Kim *et al.*, 2011). Since peroxyl radicals have a long half-life (10^{-2} s), they show a greater affinity to diffuse into cells. This leads to be more macromolecular damage. Poli *et al.* (2004) reported that peroxyl radical induced lipid peroxidation contributed to the adverse changes of biomembrane composition. Thus, it was postulated that HepG2 cells were more highly sensitive to peroxyl radical than H_2O_2. Based on cell viability test, the level of 800 μM H_2O_2 or AAPH, causing ~ 50% cell viability, was selected for study on the role of hydrolysates and fractions in prevention of cells toward oxidative stress.

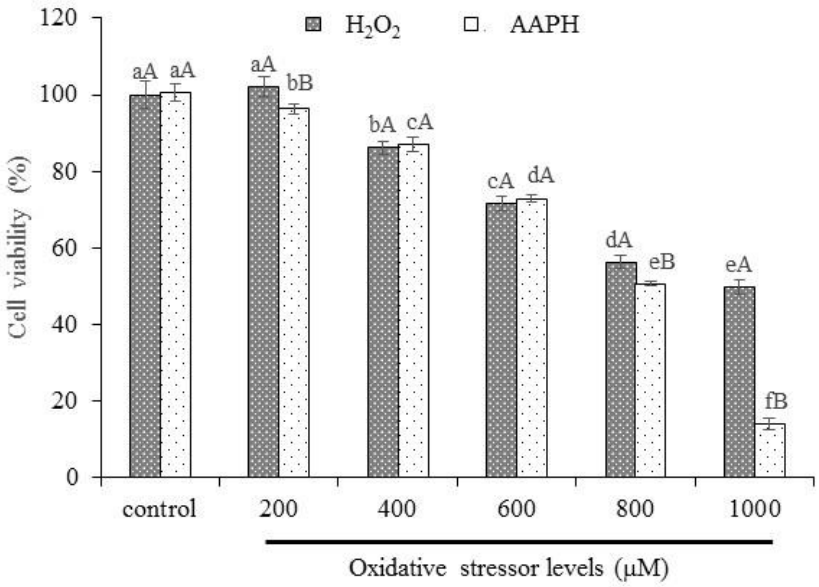


Figure 26. Dose-dependent toxic effects of H_2O_2 and AAPH on HepG2 cell viability. HepG2 cells were exposed to oxidative stressors with different concentrations (200-1000 µM) for 24 h. Bars represent the standard deviation (n = 2). Different letters within the same oxidative stressors indicate the significant differences ($P < 0.05$). Different capital letters within the same concentration indicate the significant difference ($P < 0.05$).

8.4.2.2 Protective effect of hydrolysates and fractions on oxidative damage of HepG2 cell

A) Cell viability

The effect of different hydrolysates and fractions in the absence and presence of Trolox on viability of HepG2 cells was assessed as shown in Table 22. The viability of cells treated with various hydrolysates and fractions was slightly higher than that observed for non-treated control cells, regardless of Trolox incorporation. However, no difference was observed for the concentration range of 0.5-2.0 mg/ml. All samples with concentrations selected for this study were non-cytotoxic on HepG 2 cells. The slightly higher cell viability in the presence of hydrolysates or fractions might be attributed to nutrient balance for cell survival. Chun *et al.* (2007) found that the addition of soy protein hydrolysate on Chinese hamster ovary cells resulted in the increased cell

Table 22. The viability of HepG2 cell treated with Nile tilapia protein hydrolysates and Sephadex G-25 fractions in the absence and presence of oxidative stressors

Samples	Concentrations (mg/ml)	Cell viability (%)		
		w/o oxidative stressors	H_2O_2	AAPH
HA	0.5	105.94±0.33^{*,†,defghij}	98.02±3.24^{cde}	93.05±2.17^{bcd}
	1	103.38±3.55^{defghi}	94.80±2.82^{def}	92.68±0.77^{bcdefgh}
	2	102.31± 4.91^{fghjkl}	92.61±5.00^{ef}	89.63±2.95^{gh}
FHA	0.5	107.42±1.34^{defghij}	93.06±1.31^{ef}	89.49±2.83^{h}
	1	111.18±0.99^{bcde}	96.93±5.22^{def}	93.59±2.25^{bcd}
	2	111.04±2.70^{bcde}	97.36±4.15^{cdef}	94.34±1.39^{bc}
T+ HA	0.5	100.09±6.05^{def}	95.65±1.04^{def}	93.42±1.38^{bcd}
	1	102.65±3.34^{efghij}	95.55±3.46^{def}	94.77±1.69^{b}
	2	102.24±3.65^{hijkl}	95.38±3.29^{def}	89.88±3.21^{fgh}
T+ FHA	0.5	105.44±2.85^{fghijk}	94.74±5.35^{def}	90.86±3.57^{defgh}
	1	111.58±2.70^{bcd}	95.94±4.94^{def}	93.15±1.56^{bcd}
	2	108.21±6.67^{dfgh}	95.18±3.42^{def}	93.63±3.06^{b}
HAPa	0.5	107.42±1.27^{ijk}	95.84±1.27^{def}	89.59±0.89^{gh}
	1	107.79±1.61^{ghijkl}	97.14±2.86^{def}	90.73±2.51^{efgh}
	2	104.77±2.40^{efghijk}	91.58±3.48^{f}	91.10±1.83^{cdefgh}
FHAPa	0.5	108.81±1.73^{defg}	99.98±4.17^{cde}	91.49±1.29^{bcdefgh}
	1	116.03±0.67^{ac}	107.64±1.98^{ab}	91.74±.0.92^{bcdefgh}
	2	117.08±1.11^{a}	111.40±1.96^{a}	93.03±1.12^{bcd}
T+HAPa	0.5	103.09±0.78^{jkl}	102.97±4.13^{bc}	89.91±2.86^{cd}
	1	105.98±0.11^{ijkl}	96.70±3.39^{def}	94.19±1.43^{bcd}
	2	108.96±0.20^{kl}	96.92±5.21^{def}	92.86±1.19^{bd}
T+FHAPa	0.5	109.36±0.27^{de}	98.06±4.99^{cd}	93.00±1.56^{bcd}
	1	116.34±1.74^{ab}	106.63±2.77^{ab}	94.34±1.31^{bc}
	2	117.98±0.05^{a}	109.64±1.97^{a}	94.29±1.31^{bc}
T		105.43±4.39^{fghijk}	93.05±2.00^{ef}	91.57±1.28^{bcdefgh}
Control	-	100.00±0.00^{l}	100.00±1.97^{cd}	100.00±0.95^{a}
Control damage	-	-	58.08±1.65^{g}	48.14±0.52^{i}

†Value are given as mean ± SD

* Different superscripts in the same column indicate the significant different.

HA: hydrolysate prepared using Alcalase

FHA: Sephadex G-25 fraction of HA

HAPa: hydrolysate prepared using Alcalase, followed by papain

FHAPa: Sephadex G-25 fraction of HAPa

T: Trolox

Control: without antioxidant, hydrolysates or fractions

Control damage: including oxidative stressor (without antioxidant, hydrolysates or fractions)

intensity and cell growth promotion. Short chain proteins, peptides or amino acids generated during hydrolysis might be required for cell metabolism. Zhang *et al*. (2012) reported that the protective ability of WPH on cell death was increased with increasing peptide concentration (50-200 μg/ml). Samaranayaka *et al.* (2010) also reported that fish protein hydrolysate derived from Pacific white hake showed no toxicity to human hepatocellular liver carcinoma cells when treated at concentrations up to 1 mg/ml.

When the cells were subsequently exposed to H_2O_2 and AAPH, the protective ability of hydrolysates and fractions in the absence and presence of Trolox is presented in Table 22. The lowest cell viability (58.1 and 46.2%) was observed when cells were only treated with H_2O_2 and AAPH, respectively. Pretreated cells in the presence of different hydrolysates or fractions significantly elevated the cell viability to a range of 92.6-111.4% and 89.6-94.8% for H_2O_2 and AAPH induced oxidative stress cells, respectively. The result was similar to cells incorporated with Trolox (91.6-93.0%). This indicated that Nile tilapia protein hydrolysate exerted a protective effect against free-radical induced cytotoxicity of HepG2 cell. The difference in protection mechanism might be related to peptides, which reacted with free radicals in different manners. It was noted that the concentrations tested did not have an impact on cell viability. The concentration used was probably excessive to overcome H_2O_2 or AAPH induced cell death. Furthermore, there was no difference in cell viability, irrespective of Trolox incorporation. Kim *et al.* (2007) reported that MRC-5 cell viability increased with increasing concentrations of antioxidant peptide from hoki frame protein hydrolysate. The viability of cell exposed to *t*-BHP-induced cytotoxicity increased up to 91.1% when hydrolysate at a concentration of 55.5 μM was used. Mendis *et al.* (2005a) reported that the cell viability of antioxidant peptide purified from jumbo squid skin treated *t*-BHP induced oxidative stress human lung fibroblast cells was increased in a dose dependent manner (25-100 μM). Chai *et al.* (2013) reported that the survival rate of neuroblastoma cells increased (67.2-82.3%) when the cells were treated with lanternfish hydrolysate (0.10 to 1.44 mg/ml) and H_2O_2 (400 μM) for 24 h. Although there was no significant correlation between results obtained from chemical (Table 21) and cell antioxidant based assays, the results confirmed the antioxidant activity of Nile tilapia protein hydrolysate.

B) Cell ROS generation

The cellular ROS scavenging activities of different hydrolysates and their fractions in the absence and presence of Trolox against H_2O_2 and AAPH induced intracellular ROS generation is shown in Figure 27. In general, the cells were labeled with DCFH-DA fluorescence probe for 30 min. When DCFH-DA diffuses through cell membrane, it is esterified into DCFH inside the cytosol and then is oxidised by intracellular ROS to form fluorescing DCF (Wolfe and Liu, 2007). As shown in Figure 27A (a,b) and 27B (a,b), the fluorescence gradually increased as the time increased up to 90 min. The much higher fluorescence intensity was noticeable in AAPH challenged system than did H_2O_2 containing counterpart, indicating that peroxyl radical was more potent oxidising agent than H_2O_2. Peroxyl radical generally has a higher stability, leading to a higher effectiveness toward cells. García-Nebot *et al.* (2014) reported that H_2O_2 had a poor activity on DCFH oxidation in such a short induction time. When pre-treated cells were incorporated with hydrolysates and fractions, the reduction of fluorescence intensity by either H_2O_2 or AAPH challenged systems was evoked, regardless of Trolox incorporation.The lower florescence intensity indicated the suppression of DCFH-DA oxidation, which corresponded to the reduction in intracellular ROS production.

For H_2O_2 induced system, the ROS generation in cell treated with various hydrolysates or their fractions was observed in the range of 31.7-78.4%, compared to control damage cell (Figure 27A). It was suggested that Nile tilapia hydrolysate had a cellular radical scavenging effect. The ROS reduction was observed in a dose-dependent manner ($P < 0.05$). In the absence of Trolox, the intracellular ROS production (53.0 – 78.3%) was higher than that with Trolox (alone) (48.5%) at a low dosages used (0.5 mg/ml). Both HAPa and FHAPa exhibited a lower ROS production, compared with HA and FHA. The difference in protective ability between HA and HAPa might be caused by the difference in the peptides. Lima *et al.* (2006) noted that the antioxidant efficacy on cell depends on both abilities to penetrate the cell membrane and the antioxidant capacity in solution. Due to a higher DH obtained in HAPa, low MW peptides plausibly penetrated into lipid bilayer of HepG2 cell more effectively and reacted with free radical inside cells. Wiriyaphan *et al.* (2012) reported that the higher

protective ability of refiner discharge threadfin bream hydrolysate derived by Alcalase against cytotoxicity was associated with lower MW peptides, resulting in a higher cell permeability. The further hydrolysis of HA by papain did not only produce shorter peptides but also enhance the exposure of the functional groups, which favoured antioxidant activity. The result was in accordance with a higher chemical antioxidant activity observed in HAPa comparing with HA (Table 21). Several studies have also documented that protein hydrolysates from fish are a good source of free radical scavenging peptides (Halldórsdóttir *et al.*, 2011; Raghavan and Kristinsson, 2008; Thiansilakul *et al.*, 2007a). Peptides with higher ratios of hydrophobic amino acids (e.g. tyrosine, tryptophan, phenylalanine, histidine, methionine or cysteine) are considered more effective (Davalos *et al.*, 2004; Ren *et al.*, 2008). Aromatic residues, such as tryptophan (indolic group) or tyrosine (phenolic group), may stabilise ROS by means of direct electron transfer and resonance (Ajibola *et al.*, 2011; Qian *et al.*, 2008). Je *et al.* (2008) reported that the purified peptide isolated from bigeye tuna had a considerable radical scavenging effect in HT1080 cell at a concentration of 50 µg/ml. Amongst all samples, FHAPa exhibited the strongest cytoprotective ability as evidenced by the lowest cell ROS generation. It was postulated that the antioxidant peptides might be more concentrated in their fractions. When the samples (hydrolysates or fractions) and Trolox were combined, ROS generation was sharply decreased (29.2-45.2%) and the percentage of cell ROS generation was lower than that found in a system incorporated with Trolox (alone) at all dosages tested, suggesting a synergistic effect on cytoprotection. A similar pattern was observed in the system simulated with AAPH (Figure 27B). Nile tilapia protein hydrolysates were more effective in scavenging peroxyl radical than did H_2O_2, as evidenced by the lower percentage of cell ROS generation obtained. FHAPa most effectively reduced ROS generation induced by H_2O_2 and AAPH, especially when used in combination with Trolox.

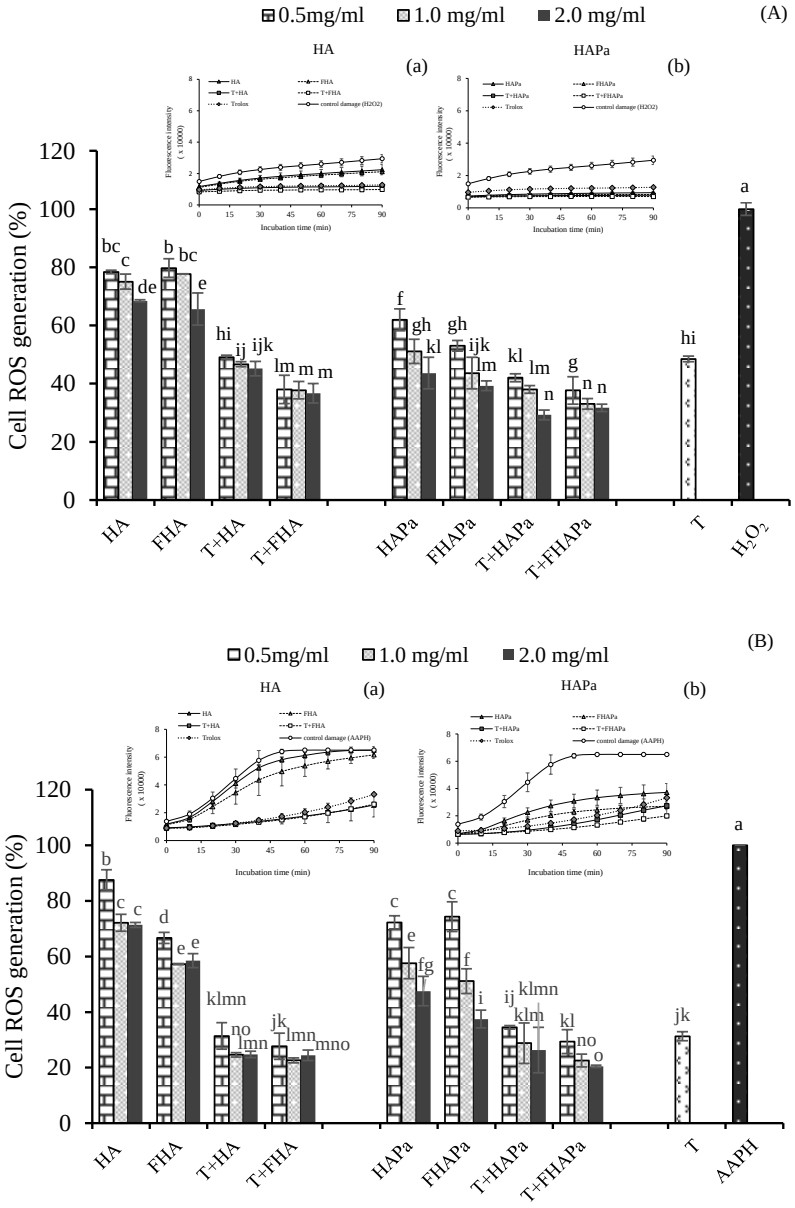


Figure 27. The cellular radical scavenging activities of HA, HAPa and their fractions on HepG2 cell. The cells were labeled with 100 μM fluorescence dye (DCFH-DA) and treated with various concentrations of different samples (0.5, 1.0 and 2.0 mg/ml) for 1 h prior exposure to 800 μM H_2O_2 (A) or 800 μM AAPH (B). Fluorescence intensity (FI) of the oxidation of DCFH to DCF was monitored at E_x = 493 nm and E_m = 527 nm. The changes of FI as a function of time were tested using different samples at level of 2 mg/ml. The results were calculated as the percentage of relative FI of H_2O_2 or AAPH. Values are expressed as the mean ± S.D. (n = 2). Different letters on the bar indicate the significant differences ($P < 0.05$).

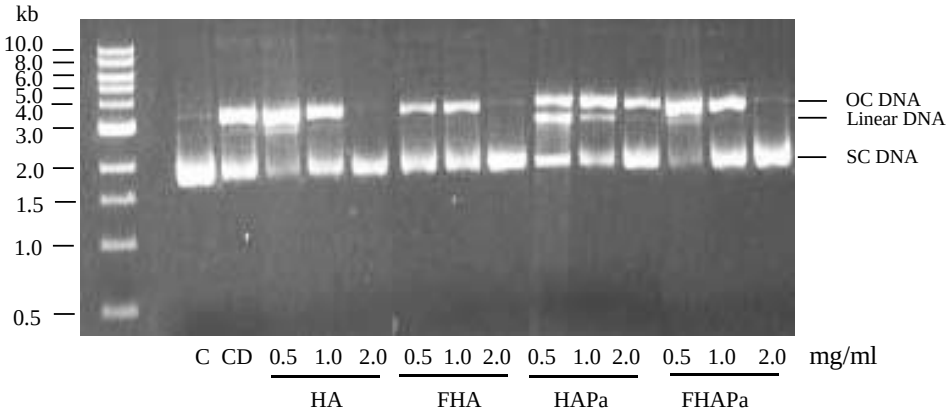
8.4.3 Protective ability of hydrolysates and their fractions against DNA damage

DNA is another sensitive biotarget for ROS-mediated oxidative damage, leading to mutagenicity (Martinez *et al.*, 2003). Protective effect of different hydrolysates and fractions against H_2O_2 and AAPH induced DNA damage is depicted in Figure 28. The antioxidant ability of hydrolysates or fractions was assessed, based on their protection of supercoiled DNA strand from scission by oxidative stressor into the open circular or linear form. As shown in Figure 28A and 28B, the supercoiled DNA was converted into the open circular DNA when exposed to H_2O_2 or AAPH. The supercoiled DNA band of sample treated with H_2O_2 was retained by 50.7%, whilst those treated with AAPH was not detectable (lane CD). Peroxyl radical induced DNA breakage was more pronounced than that induced by H_2O_2, suggesting that peroxyl radical resulted in a higher damage in biological molecules. This was in accordance with the decrease cell viability of HepG2 cell treated with AAPH (Figure 26). Oxidative stress in cells caused by ROS, particularly H_2O_2 and peroxyl radicals, generally leads to DNA damage. There are a wide variety of reports for DNA modifications caused by ROS, including strand scission, sister chromatid exchange, DNA-DNA and DNA-protein cross-links as well as base modification (Davies, 1995). H_2O_2 has been known to be a non-reactive radical, however it becomes highly reactive when reacts with transition metal, yielding the hydroxyl radical ($HO^{\bullet}$). Klompong *et al.* (2009) reported that $OH^{\bullet}$ induced oxidative stress resulted in pUC18 DNA relaxation, whilst H_2O_2 (alone) treated DNA had no damage. Kim *et al*. (2007) reported that the supercoiled pBR322 DNA was completely changed to open circular form when exposed to Fe^{2+}-H_2O_2 solution, whereas it decreased to 50% when treated with H_2O_2 alone. However, hydroxyl radicals have a shorter half-life as compared to peroxyl radicals (Kittiphattanabawon *et al.*, 2013). Therefore, the major oxidative damages in the cells were caused by peroxyl radical to a higher extent.

When DNA was incorporated with all hydrolysates and fractions at levels of 0.5-2 mg/ml, the retention of supercoiled DNA increased in a dose dependent manner for both H_2O_2 and AAPH induced systems. For DNA induced with AAPH, supercoiled DNA band intensity was retained by 24.2%, 76.7%, 22.9% and 92.6%

when HA, FHA, HAPa and FHAPa at a level of 2 mg/ml were present, respectively. It was noted that the use of all hydrolysates or fractions at 2 mg/ml could effectively protect DNA scission from H_2O_2 as evidenced by 100% supercoiled DNA observed, except for HAPa, in which 89.5% retention were found. The result indicated that Nile tilapia protein hydrolysates had a protective ability against DNA scission induced by H_2O_2 and peroxyl radical to different degrees. This was possibly due to the differences in ability to scavenge free radicals and H_2O_2 as reported in Table 21. According to a result, HAPa showed higher protective effect, compared to HA, especially after fractionation. The difference in their capacity might be related to peptides in various hydrolysates. Surguladze *et al.* (2004) reported the scission of supercoiled DNA strand to nicked circular form by free radicals. The rate of nicking correlated with the iron content and was strongly inhibited by radical scavengers and chelators. Je *et al.* (2009) reported that the second step hydrolysates from tuna liver showed more active protection toward supercoiled DNA conversion than did the hydrolysates from the first step. Oxidative stress caused by ROS, such as H_2O_2 and peroxyl radical, resulted in damaged DNA, which may possibly be implicated in mutagenesis and carcinogenesis. Therefore, hydrolysates, especially those prepared using Alcalase and papain contributed to inhibitory effect against DNA oxidation induced by H_2O_2 or peroxyl radical.

(A)



(B)

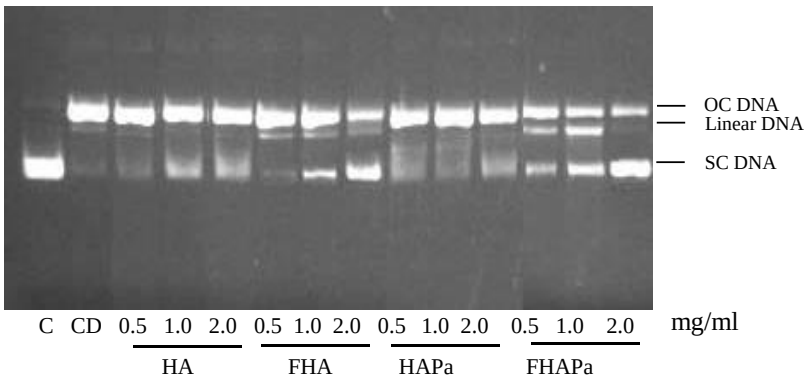


Figure 28. Agarose gel electrophoresis of DNA treated with H_2O_2 (A) and AAPH (B) in the absence and presence of HA and HAPa and theirs fractions at different concentrations (0.5, 1.0 and 2.0 mg/ml). C denote control; CD denote control damage (DNA + oxidative stressors); HA, FHA, HAPa and FHAPa denote oxidative DNA damage pre-treated with various hydrolysates or fractions (DNA + oxidative stressors + hydrolysates or fractions).

8.5 Conclusion

Protein hydrolysate from Nile tilapia PI prepared using Alcalase and Alcalase together with papain exhibited a good antioxidant potential, in both chemical and cellular based assays. Hydrolysates and their fractions exerted a protection ability against H_2O_2 and peroxyl radical induced oxidative damage on HepG2 cell and DNA

via scavenging free radical and H_2O_2. Generally, HAPa, especially after fractionation together with the combination of 50 μM Trolox, showed a stronger antioxidant activities than did HA. Therefore, protein hydrolysate from Nile tilapia prepared by two step hydrolysis using Alcalase and papain could yield peptides with high antioxidant activity and could serve as functional food.

CHAPTER 9
CONCLUSION AND SUGGESTION

9.1 Conclusions

1. Freshness of Nile tilapia used as raw material for protein hydrolysate had an influence on lipid oxidation and fishy odour/flavour of resulting hydrolysate. Fresh fish was the most appropriate for production of hydrolysate with reduced fishy odour/flavour. Incorporation of the antioxidants effectively prevented lipid oxidation and the development of fishy odour/flavour in hydrolysate.

2. Pretreatment of Nile tilapia minces played a significant role in the reduction of prooxidants and lipids, especially neutral lipids. Washing, along with a process to remove membranes prior to alkaline solubilisation, yielded protein isolate (PI), which was the promising substrate for protein hydrolysate production, in which fishy odour and taste could be significantly reduced.

3. PI was the promising raw material for protein hydrolysate preparation from Indian mackerel by lowering prooxidant and membrane phospholipids. Fishy odour/flavour development of hydrolysate from PI was significantly lowered.

4. Hb was proven to be an effective pro-oxidant in hydrolysate derived from Nile tilapia PI. Both oxy-Hb and met-Hb could accelerate lipid oxidation. However, met-Hb showed the greater impact on fishy off-odour development and discolouration of resulting hydrolysate in comparison with oxy-Hb. In order to improve the quality of hydrolysate by lowering fishy odour/flavour, the contamination of Hb should be alleviated.

5. Geosmin and/or 2-MIB mainly contributed to muddy flavour and odour in freshwater fish. Both compounds were more concentrated in ventral portion, where higher lipid and phospholipid were located. Prewashing, followed by membrane removal, in combination with alkaline solubilisation used for PI preparation had the marked influence on lowering muddy flavour and odour in resulting hydrolysate.

6. Protein hydrolysate from Nile tilapia PI prepared using different single proteases exhibited various antioxidant activities, depending on DH and types of enzyme used. Hydrolysate prepared using Alcalase (HA) with 40 % DH possessed the highest antioxidant activities. The application of a two-step hydrolysis process using Alcalase and papain (HAPa), respectively, yielded the hydrolysate with increased antioxidant activities. The antioxidant peptides in both HA and HAPa had MW of 513 and 1,484 Da, respectively.

7. HA, HAPa and their fractions from Nile tilapia exerted a protection ability against H_2O_2 and peroxyl radical induced oxidative damage on HepG2 cell and DNA. Therefore, protein hydrolysate could play a role as antioxidant in cellular system.

9.2 Suggestions

1. Impact of drying methods on odourous compound and antioxidant activity of Nile tilapia protein hydrolysate should be further studied.

2. The antioxidative peptides in Nile tilapia hydrolysate should be purified and identified. The synthesised peptides should be tested for *in vitro* and cellular antioxidant activities.

3. Bioactivity and immunomodulating ability of Nile tilapia protein hydrolysate should be focused.

4. The fortification of Nile tilapia protein hydrolysate prepared from the proper pretreatment in other food systems should be further employed.

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VITAE

Name Ms. Suthasinee Yarnpakdee

Student ID 5311030028

Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Agro-Industry, First Class Honor)	Prince of Songkla University	2006
Master of Science (Food Science and Technology)	Prince of Songkla University	2008

Scholarship Awards during Enrolment

1. Scholarship for Academic Distinction, Prince of Songkla University
2. Ph.D. Student Research Scholarship by Thailand Research Fund under the Royal Golden Jubilee Ph.D. Program (PHD/0226/2552) collaborated with Prince of Songkla University

List of Publication and Proceedings

Publications

1. Yarnpakdee, S., Benjakul, S., Nalinanon, S. and Kristinsson, H. G. 2012. Lipid oxidation and fishy odour development in protein hydrolysate from Nile tilapia (*Oreochromis niloticus*) muscle as affected by freshness and antioxidants. Food Chem. 132: 1781-1788.

2. Yarnpakdee, S., Benjakul, S. and Kristinsson, H. G. 2012. Effect of pretreatments on chemical compositions of mince from Nile tilapia (*Oreochromis niloticus*) and fishy odor development in protein hydrolysate. Int Aquat Res. 4: 7.

3. Yarnpakdee, S., Benjakul, S., Kristinsson, H. G. and Maqsood, S. 2012. Effect of pretreatment on lipid oxidation and fishy odour development in protein hydrolysates from the muscle of Indian mackerel. Food Chem. 135: 2474-2482.

4. Yarnpakdee, S., Benjakul, S. and Kristinsson, H. G. 2014. Lipid oxidation and fishy odour in protein hydrolysate derived from Nile tilapia (*Oreochromis niloticus*) protein isolate as influenced by haemoglobin. J. Sci Food Agric. 94: 219-226.

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Book Chapter

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