Production and Properties of Reduced Fishy Odour Gelatin and Gelatin Hydrolysate from Seabass (*Lates calcarifer*) Skin

Thanasak Sae-leaw

A Thesis Submitted in Fulfillment of the Requirements for the Degree of
Doctor of Philosophy in Food Science and Technology
Prince of Songkla University
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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.

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ชื่อวิทยานิพนธ์ การผลิตและสมบัติของเจลาตินและเจลาตินไฮโดรไลเสตลดกลิ่นคาวจากหนังปลากะพงขาว
ผู้เขียน นายธนศักดิ์ แซ่เลี่ยว
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บทคัดย่อ

จากการศึกษาการเก็บรักษาหนังปลากะพงขาว (ซึ่งใช้เป็นวัตถุดิบในการสกัดเจลาติน) ในน้ำแข็งเป็นเวลา 18 วัน และทำการตรวจสอบการเปลี่ยนแปลงขององค์ประกอบของกรดไขมัน การไฮโดรไลซิสและออกซิเดชันของลิปิด การพัฒนากลิ่นคาวและสารประกอบที่ระเหยได้ พบว่าค่าเปอร์ออกไซด์ (PV) เพิ่มขึ้นจนถึงวันที่ 6 และลดลงจนถึงวันที่ 18 ($P < 0.05$) ค่า thiobarbituric acid reactive substances (TBARS) กรดไขมันอิสระ (FFA) และกิจกรรมของเอนไซม์ไลพอกซีจิเนส (LOX) มีค่าเพิ่มขึ้นตลอดระยะเวลาการเก็บรักษา ($P < 0.05$) โดย FFA และสารไฮโดรเปอร์ออกไซด์ที่เกิดขึ้นสามารถยืนยันได้จากการเปลี่ยนแปลงของสเปคตราการดูดกลืนแสงของ Fourier transform infrared (FTIR) ในช่วงคลื่น 3600-3200 cm^{-1} และ 1711 cm^{-1} ตามลำดับ นอกจากนี้กลิ่นคาวรวมถึงสารประกอบที่ระเหยได้มีปริมาณเพิ่มขึ้นตามระยะเวลาการเก็บรักษา

เมื่อนำหนังปลากะพงขาวที่เก็บรักษาในน้ำแข็งที่เวลาต่างกันมาทำการสกัดเจลาตินและศึกษาสมบัติต่าง ๆ พบว่าได้ผลผลิตเจลาตินไม่แตกต่างกัน ส่วนปริมาณหมู่แอลฟา-อะมิโนของเจลาตินมีค่าเพิ่มขึ้นตามระยะเวลาการเก็บรักษาที่เพิ่มขึ้น ในขณะที่ความเข้มของแถบสายโซ่แอลฟา ความขาวและความแข็งแรงของเจลมีค่าลดลง นอกจากนี้กลิ่นคาว การเกิดออกซิเดชันของลิปิด และสารประกอบที่ระเหยได้ในเจลาตินมีปริมาณเพิ่มขึ้นเมื่อใช้หนังปลากะพงขาวที่เก็บรักษาในน้ำแข็งเป็นเวลานานขึ้น

เมื่อศึกษาผลของการปฏิบัติเบื้องต้นและการกำจัดไขมันในหนังปลากะพงขาวด้วยวิธีต่าง ๆ ต่อกลิ่นคาวและสมบัติของเจลาติน พบว่าหนังปลาที่ปฏิบัติเบื้องต้นด้วยกรดซิตริกตามด้วยการกำจัดไขมันด้วยไอโซโพรพานอล (ร้อยละ 30 โดยปริมาตร) มีปริมาณฟอสโฟลิปิดต่ำสุด ($P < 0.05$) อย่างไรก็ตามเจลาตินที่สกัดได้ (G-Ci-Def) มีผลผลิตและความแข็งแรงของเจลต่ำกว่าเจลาตินจากหนังปลาที่ปฏิบัติเบื้องต้นด้วยกรดอะซิติก (G-Ac) หรือปฏิบัติเบื้องต้นด้วยกรดซิตริก (G-Ci) ($P < 0.05$) G-Ci-Def มีค่า L^* สูงสุด และมีค่า a^* b^* ΔE^* และ ΔC^* ต่ำสุด เมื่อเปรียบเทียบกับตัวอย่างอื่น ๆ ($P < 0.05$) ตัวอย่าง G-Ci-Def มีกลิ่นคาวและสารประกอบที่ระเหยได้ปริมาณต่ำสุด

จากการศึกษาคุณลักษณะและสมบัติเชิงหน้าที่ของเจลาตินที่สกัดจากหนังปลากะพงขาวที่ไม่ได้กำจัดไขมัน (G-Ac) และกำจัดไขมัน (G-Ci-Def) พบว่าเจลาตินทุกตัวอย่างสามารถละลายน้ำได้มากกว่าร้อยละ 90 ที่ความเป็นกรด-ด่างช่วงกว้าง (1–10) G-Ci-Def มีความสามารถในการเกิดโฟมและความคงตัวของโฟมมากกว่าตัวอย่าง G-Ac อิมัลชันที่เตรียมจาก G-Ci-Def มีขนาดหยดน้ำมัน (d_{32} และ d_{43}) เล็กกว่าอิมัลชันที่เตรียมจาก G-Ac เมื่อเก็บรักษาไว้เป็นเวลา 10 วันที่อุณหภูมิห้อง (28-30 องศาเซลเซียส) อิมัลชันที่เตรียมจาก G-Ci-Def มีความคงตัวมากกว่าอิมัลชันที่เตรียมจาก G-Ac ซึ่งบ่งชี้ด้วยการเพิ่มขึ้นของ d_{32} และ d_{43} ที่ต่ำกว่า และมีแฟคเตอร์การเกาะกลุ่มและดัชนีการรวมตัวที่ต่ำกว่า นอกจากนี้อิมัลชันที่เตรียมจากเจลาตินที่สกัดจาก G-Ci-Def มีค่าศักย์ซีตามากกว่าอิมัลชันที่เตรียมจาก G-Ac

จากการศึกษาผลของการทำแห้งแบบพ่นฝอยต่อสมบัติและกลิ่นและกลิ่นรสคาวของ G-Ac และ G-Ci-Def พบว่าเจลาตินที่ทำแห้งแบบระเหิดมีค่าความแข็งแรงของเจลสูงกว่าเจลาตินที่ทำแห้งแบบพ่นฝอย ($P < 0.05$) เมื่ออุณหภูมิที่ใช้ในการทำแห้งแบบพ่นฝอยเพิ่มขึ้นส่งผลให้ความแข็งแรงของเจลมีค่าลดลง ($P < 0.05$) กลิ่นคาวที่ลดลงในเจลาตินที่ทำแห้งแบบพ่นฝอยสอดคล้องกับปริมาณของสารประกอบที่ระเหยได้ที่ลดลงเมื่อเปรียบเทียบกับเจลาตินที่ทำแห้งแบบระเหิด นอกจากนี้กลิ่นคาวที่ลดลงยังมีความสัมพันธ์กับค่า PV และ TBARS ที่ลดลง เมื่อนำ G-Ac และ G-Ci-Def ที่สกัดพร้อมกับการเติมกรดแทนนิก (0-0.4 กรัม/กิโลกรัมหนังแห้ง) มาทำแห้งแบบถาด พบว่าการเติมกรดแทนนิกในระหว่างการสกัดเจลาตินสามารถลดปฏิกิริยาออกซิเดชันของลิปิด รวมถึงกลิ่นคาว และสารประกอบที่ระเหยได้ในเจลาติน

เมื่อนำหนังปลากะพงขาวที่ปฏิบัติเบื้องต้นด้วยกรดซิตริกตามด้วยการกำจัดไขมันและทำการย่อยสลายด้วยเอนไซม์อัลคาเลสด้วยวิธีต่าง ๆ ได้แก่ การไฮโดรไลซิสในระหว่างกระบวนการสกัดและหลังจากกระบวนการสกัดเจลาติน พบว่าเจลาตินไฮโดรไลเสตที่ไฮโดรไลซ์ในระหว่างกระบวนการสกัดเจลาตินมีระดับการไฮโดรไลซิส (DH) และผลผลิตสูงกว่าเจลาตินไฮโดรไลเสตที่ไฮโดรไลซ์หลังจากกระบวนการสกัดเจลาติน เจลาตินไฮโดรไลเสตที่ไฮโดรไลซ์ในระหว่างกระบวนการสกัดเจลาตินมีปริมาณสารประกอบที่ระเหยได้ต่ำกว่าเจลาตินไฮโดรไลเสตที่ไฮโดรไลซ์หลังจากกระบวนการสกัดเจลาติน เจลาตินไฮโดรไลเสตที่ไฮโดรไลซ์ในระหว่างกระบวนการสกัดเจลาตินมีกิจกรรมการออกฤทธิ์ต้านอนุมูล DPPH FRAP และการกำจัดโลหะสูงกว่าเจลาตินไฮโดรไลเสตที่ไฮโดรไลซ์หลังจากกระบวนการสกัดเจลาติน ($P < 0.05$) เมื่อศึกษากิจกรรมการออกฤทธิ์ต้าน

ออกซิเดชันในระบบทางเดินอาหารจำลอง พบว่ากิจกรรมการออกฤทธิ์ต้านอนุมูล DPPH และ FRAP ของเจลาตินไฮโดรไลเสตมีค่าคงที่ ส่วนการกำจัดโลหะมีค่าเพิ่มขึ้น

เมื่อศึกษากิจกรรมการออกฤทธิ์ต้านออกซิเดชัน ฤทธิ์ในการควบคุมการตอบสนองของระบบภูมิคุ้มกัน และฤทธิ์ต้านการเพิ่มจำนวนของเซลล์มะเร็งในระบบจำลองทางเซลล์ ของเจลาตินไฮโดรไลเสตที่เตรียมจากหนังปลากะพงขาว พบว่าเจลาตินไฮโดรไลเสตมีกิจกรรมการออกฤทธิ์ยับยั้งความเสียหายของดีเอ็นเอในเซลล์ U937 อันเกิดจากปฏิกิริยาออกซิเดชันที่เหนี่ยวนำด้วยไฮโดรเจนเปอร์ออกไซด์ (H_2O_2) เจลาตินไฮโดรไลเสตแสดงกิจกรรมการออกฤทธิ์ควบคุมการตอบสนองของระบบภูมิคุ้มกันโดยการลดการผลิต interleukin-6 (IL-6) และ IL-1β ในเซลล์ RAW264.7 ที่ถูกกระตุ้นด้วยลิโปพอลิแซคคาไรด์ (LPS) นอกจากนี้เจลาตินไฮโดรไลเสตยังแสดงกิจกรรมการออกฤทธิ์ต้านการเพิ่มจำนวนของเซลล์มะเร็งลำไส้ (Caco-2) และเซลล์มะเร็งตับ (HepG2) โดยมีฤทธิ์แปรผันตามความเข้มข้น (1-25 มิลลิกรัมต่อมิลลิลิตร)

เมื่อเติมเจลาตินไฮโดรไลเสตในน้ำแอปเปิ้ลที่ระดับต่าง ๆ (ร้อยละ 0.1 0.2 และ 0.3 โดยน้ำหนักต่อปริมาตร) พบว่าน้ำแอปเปิ้ลที่เติมเจลาตินไฮโดรไลเสตมีค่า *L** ลดลง ในขณะที่มีค่า *a** และ *b** เพิ่มขึ้นเมื่อเติมในระดับที่มากขึ้น ($P < 0.05$) นอกจากนี้น้ำแอปเปิ้ลที่เติมเจลาตินไฮโดรไลเสตยังมีสีน้ำตาลและความขุ่นที่เพิ่มขึ้นตามระดับการเติมที่เพิ่มขึ้น ($P < 0.05$) น้ำแอปเปิ้ลที่เติมเจลาตินไฮโดรไลเสตมีกิจกรรมการออกฤทธิ์ต้านออกซิเดชัน ได้แก่ กิจกรรมการออกฤทธิ์ต้านอนุมูล DPPH FRAP และการกำจัดโลหะเพิ่มขึ้น ตามระดับการเติมที่เพิ่มขึ้น การเติมเจลาตินไฮโดรไลเสตที่ระดับร้อยละ 0.2 (โดยน้ำหนักต่อปริมาตร) ไม่มีผลต่อคะแนนความชอบในทุกคุณลักษณะของน้ำแอปเปิ้ล

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ABSTRACT

The skins of seabass (*Lates calcarifer*), used as the raw material for gelatin extraction, were stored in ice for 18 days. Changes in fatty acid profile, lipid hydrolysis and oxidation, development of fishy odour and volatile compounds were monitored. Peroxide value (PV) increased up to day 6 and subsequently decreased up to 18 days ($P < 0.05$). The continuous increases in thiobarbituric acid reactive substances (TBARS) values, free fatty acid (FFA) content and lipoxygenase (LOX) activity were noticeable with increasing storage time ($P < 0.05$). Formation of FFA and hydroperoxide was confirmed by the changes in amplitude of peak at 3600–3200 cm^{-1} and 1711 cm^{-1} in Fourier transform infrared spectra, respectively. With increasing storage time, the increase in fishy odour intensity was observed along with the formation of volatile compounds.

When gelatins from seabass skin stored in ice for different times were extracted and characterised, no differences in extraction yields of gelatins were obtained when skins stored for up to 18 days were used. With increasing storage time, the gelatin had the increased α-amino group content ($P < 0.05$) with coincidental decrease in α-chain band intensity and whiteness. Gel strength of gelatin decreased as the skin was stored in ice for a longer time ($P < 0.05$). Fishy odour intensity of gelatin increased with the increases in lipid oxidation and volatile compounds when skin stored in ice for a longer time was used.

The impact of different pretreatments and defatting of seabass skin on fishy odour and properties of gelatin was investigated. Skin pretreated with citric acid, followed by defatting using 30% (v/v) isopropanol had the lowest remaining phospholipid content ($P < 0.05$). Nevertheless, the resulting gelatin (G-Ci-Def) showed lower yield and gel strength, compared to those from skins pretreated with acetic acid

(G-Ac) or citric acid (G-Ci) ($P < 0.05$). G-Ci-Def exhibited the highest L^* value with the lowest a^*, b^*, ΔE^* and ΔC^* values, compared to other gelatins ($P < 0.05$). The lowest fishy odour with lower amount of volatile compounds was found in G-Ci-Def, compared to other gelatins.

Gelatins from non-defatted (G-Ac) and defatted seabass skin (G-Ci-Def) were characterised and evaluated for their functional properties in comparison with commercial fish skin gelatin. All gelatins had a relative solubility greater than 90% in the wide pH ranges (1−10). G-Ci-Def had higher foam expansion and stability than G-Ac sample. Emulsion containing G-Ci-Def had smaller oil droplet size (d_{32}, d_{43}), compared with that having G-Ac ($P < 0.05$). After 10 days of storage at room temperature (28−30 ºC), emulsion stabilised by G-Ci-Def showed the higher stability as indicated by the lower increases in d_{32}, d_{43}, lower flocculation factor, coalescence index and higher zeta potential than that containing G-Ac.

Effects of spray drying on properties and fishy odour/flavour of both G-Ac and G-Ci-Def were evaluated. Generally, a higher gel strength was found in the freeze-dried gelatin, compared with spray-dried counterpart ($P < 0.05$). Gel strength of gelatin decreased as the inlet temperature for spray drying increased ($P < 0.05$). The lower fishy odour/flavour with coincidentally lower abundance of volatile compounds was found in gelatin obtained by spray drying, in comparison with freeze-dried counterpart. The lower fishy odour/flavour in spray-dried gelatin was in accordance with the lower PV and TBARS. When G-Ac and G-Ci-Def were extracted in the absence and presence of tannic acid at various concentrations (0−0.4 g/kg), followed by tray drying, the addition of tannic acid during gelatin extraction could lower lipid oxidation, fishy odour as well as the formation of volatile compounds in the resulting gelatins.

Gelatin hydrolysates were prepared from seabass skin, pretreated with citric acid and followed by defatting, using Alcalase with different processes. Those included hydrolysis during or after gelatin extraction. Samples hydrolysed during gelatin extraction showed a higher degree of hydrolysis (DH) and yield, compared with those hydrolysed after gelatin extraction ($P < 0.05$). A lower abundance of volatile

compounds was found in the hydrolysates produced during gelatin extraction, in comparison with those obtained after gelatin extraction. Hydrolysates prepared during gelatin extraction had higher 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, ferric reducing antioxidative power (FRAP) and ferrous ion chelating activity ($P < 0.05$). Based on a simulated *in vitro* gastrointestinal digestion study, DPPH radical scavenging activity and FRAP of the hydrolysates were retained, whilst ferrous ion chelating activity increased.

Antioxidant, immunomodulatory and antiproliferative potentials of gelatin hydrolysates from seabass skin were studied in cell model systems. Gelatin hydrolysates had the ability to protect against H_2O_2-induced DNA damage in U937 cells. All samples showed immunomodulatory potential by significantly reducing interleukin-6 (IL-6) and IL-1β production in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells. Gelatin hydrolysate also showed antiproliferative activities in human colon cancer (Caco-2) and liver cancer (HepG2) cell lines.

Gelatin hydrolysate from seabass skin was fortified in apple juice at different levels (0.1, 0.2 and 0.3%, w/v). The decrease in L^*-value and the increases in a^*- and b^*-values, browning index and turbidity of apple juice were observed with increasing concentration of gelatin hydrolysate ($P < 0.05$). Apple juice added with gelatin hydrolysate had the increases in antioxidative activities, including DPPH radical scavenging activity, ferric reducing antioxidant power and ferrous ion chelating activity in a dose-dependent manner ($P < 0.05$). Fortification of gelatin hydrolysate up to 0.2% (w/v) into apple juice had no effect on likeness scores of all attributes.

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Seabass (*Lates calcarifer*) is an economically important fish species in Thailand and other countries, especially the tropical and subtropical regions of Asia and the Pacific. Thailand is the major producer of seabass, which is widely cultured in the south, especially in Songkhla lake area. During processing or dressing of seabass, the skin is removed and considered as a byproduct. Seabass skin could serve as a potential source for the production of high value-added products, e.g. collagen, gelatin as well as hydrolysates with bioactivities (Senphan and Benjakul, 2014; Sinthusamran *et al.*, 2013).

Generally, gelatin is produced from skins and bones of pig and cow. The outbreak of bovine spongiform encephalopathy (BSE) has resulted in anxiety among consumers of bovine gelatin. Additionally, gelatin obtained from pig and cow skin cannot be used due to the religious constraint (Al-Mazeedi *et al.*, 2013). As a consequence, more increasing interest and attempt have been paid to the alternative gelatin sources, especially fish skin from seafood processing. Nowadays, fish protein hydrolysates have gained increasing attention from consumers due to their various bioactivities. Fish protein hydrolysates have shown a wide range of biological functions, including antioxidant, anti-hypertensive, antimicrobial, immunomodulatory, mineral binding, anti-thrombotic and antiproliferative effects (Chalamaiah *et al.*, 2012). However, fish gelatin hydrolysate still has the strong fishy odour/flavour, thereby limiting the amount for fortification in foods, especially in drinks with mild odour/flavour (Yarnpakdee *et al.*, 2012b). To maximise the use of gelatin or gelatin hydrolysate from fish skin, the prevention of lipid oxidation to lower fishy odour/flavour development during storage of skin or during processing is required.

Fish skin contains lipids with high degree of unsaturation (Kołakowska *et al.*, 2002). Phospholipid membranes are believed to be the key substrate for lipid oxidation (Liang and Hultin, 2005a). Those lipids in skin can be oxidised during gelatin extraction and hydrolysis processes at high temperature, thereby promoting lipid oxidation and development of unpleasant odours/flavours, especially fishy odour. Spray drying can be an approach to remove undesirable odour from fish derived products. During the transformation of liquid feed into dry powder at high temperature, volatile odourous compounds could be eliminated to some degree (Sai-Ut *et al.*, 2014). Therefore, the appropriate pretreatment facilitating the removal of membrane lipids or other pro-oxidants of skin prior to gelatin extraction, as well as the use of antioxidant during gelatin extraction along with an effective drying method could be a promising means to lower fishy odour/flavour occurring in fish skin gelatin and derived products. Additionally, the gelatin hydrolysate containing bioactive peptides can be obtained, which can be of health benefit. Fish skin gelatin and hydrolysate with negligible fishy odour/flavour can be widely applied in foods without the negative effect on sensorial property. As a consequence, the use of aquatic by-product can be maximised and high value-added products from fish skin can be gained.

1.2 Review of literature

1.2.1 Fish skin

Fish skin is the envelope for the body that separates and protects the animal from its environment (osmotic pressure and physical force). The skin is a multifunctional organ and may serve important roles in chemical and physical protection, communication, sensory perception, movement, respiration, excretion and thermal regulation. Furthermore, it is an important first-line defense system against pathogens, as fish are continuously exposed to multiple microbial challenges in their aquatic habitat (Rakers *et al.*, 2010). Skin structure varies with fish species. In general, the layers of skin of adult teleosts are the cuticle or mucus layer, epidermis and dermis. The dermis is mainly composed of dense connective tissue with a large amount of collagen fibres (Hawkes, 1974).

Some fish skin contains more lipid than the muscle tissue. Makanjuola (2012) revealed that the lipid contents in the skin of catfish, croaker and mackerel were higher, compared with those of flesh. The lipid content in the skin of lean fish (e.g. cod, blue whiting) ranges from 0.2 to 3.9%, w/w (Kołakowska *et al.*, 2002). Lipid content in skins of fish from the Senegalese coast ranged from 2.4% (*Cephalopholis taeniops*) to 26.0% (*Sardinella maderensis*) (Njinkoué *et al.*, 2002). The lipid content of the skin of fatty fish is much higher and can be more than 50% (w/w) (Kołakowska *et al.*, 2002). The proximate compositions of skins from some fish species are shown in Table 1.

Table 1. Chemical composition of selected fish skins

Composition (%)	Giant catfish	Striped catfish	Catfish	Croacker	Mackerel
Protein	34.03	27.26	26.77	27.20	28.00
Moisture	64.86	51.85	68.57	67.40	62.72
Lipid	2.69	20.24	2.36	4.60	7.40
Carbohydrate	ND	ND	1.22	0.11	1.18
Ash	0.25	0.23	0.66	0.69	0.70

ND: Not determined

Source: Makanjuola (2012); Thitipramote and Rawkdkuen (2011)

Skins of three fish species caught in Senegalese, including *Sardinella maderensis*, *Sardinella aurita* and *Cephalopholis taeniops* had high concentrations of *n*-3 polyunsaturated fatty acids (PUFAs) (Table 2). Eicosapentaenoic acid (EPA) (C20:5 *n*-3) and docosahexaenoic acid (DHA) (C22:6 *n*-3) were the major fatty acids in the skins of three fish species. These two fatty acids together accounted for 24.7% and 12.9% of total fatty acids in the skin of *S. maderensis* and *S. aurita*, respectively. The percentages of fatty acids therefore differ amongst species. Palmitic acid (C16:0) was the main fatty acid in the skin of all fish species (20–33% of total fatty acids) (Njinkoué *et al.*, 2002).

Table 2. Fatty acid composition of lipids from three Senegalese fish species

Fatty acids (%)	*Sardinella maderensis*	*Sardinella aurita*	*Cephalopholis taeniops*
C12:0	ND	ND	ND
C14:0	8.1	7.8	5.0
C14:1	ND	ND	ND
C15:0	0.5	0.6	0.7
C15:1	ND	ND	ND
C16:0	20.5	20.5	28.4
C16:1 *n*-7	ND	ND	ND
C17:0	2.0	1.8	1.0
C17:1 *n*-9	1.4	1.2	0.3
C18:0	5.8	5.6	8.4
C18:1 *n*-9	8.2	15.5	12.5
C18.2 *n*-6	1.2	0.7	0.8
C18:2	0.7	0.8	0.3
C18:3 *n*-6	0.3	0.8	0.3
C18:3	0.8	0.3	0.3
C18:3 *n*-3	0.3	0.3	0.3
C18:4 *n*-3	2.0	1.3	0.3
C20:0	1.2	0.8	0.3
C20:1 *n*-12	1.9	0.3	ND
C20:1 *n*-9	ND	1.6	1.5
C20:1 *n*-7	0.3	0.3	0.3
C20:2 *n*-9	0.3	1.2	ND
C20:2 *n*-6	ND	ND	ND
C20:3 *n*-6	0.3	0.6	1.7
C20:3 *n*-3	ND	ND	ND
C20:4 *n*-6	1.0	ND	ND
C20:4 *n*-3	0.5	0.4	0.3
C20:5 *n*-3	20.5	10.4	3.1
C21:5 *n*-3	ND	2.1	0.6
C22:0	2.3	2.5	0.3
C22:1 *n*-11	ND	0.4	1.7
C22:5 *n*-6	ND	ND	ND
C22:5 *n*-3	1.5	0.8	2.6
C22:6 *n*-3	4.2	2.5	6.9
C24:1 *n*-9	ND	ND	ND
C24:0	0.5	0.3	1.6
Σ SFA	41.5	40.3	46.4
Σ MUFA	23.2	34.0	33.6
Σ PUFA	33.6	22.5	17.5

ND: Not-detectable.

Source: Njinkoué *et al.* (2002)

1.2.2 Lipid oxidation

Lipid oxidation is a general term which is used to describe a complex sequence of chemical changes that result from the interaction of lipids with oxygen (Kim and Min, 2008). Triacylglycerols and phospholipids have low volatility and thus do not directly contribute to the aroma of foods. During lipid oxidation reactions, the fatty acids esterified to triacylglycerols and phospholipids are decomposed to form small, volatile molecules that produce the off-aromas known as oxidative rancidity. Those volatile compounds are detrimental to food quality (McClements and Decker, 2008).

Oxidation of lipids is a major cause of deterioration of food and food products, especially those containing high content of unsaturated fatty acids. Lipid oxidation causes nutritional losses and produces undesirable odour/flavour, colour, and toxic compounds, which make foods less acceptable or unacceptable to consumers (Kim and Min, 2008).

1.2.2.1 Lipid autoxidation

Autoxidation is the direct reaction of molecular oxygen with organic compounds under mild conditions. 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, 2005) (Figure 1).

Initiation: The autoxidation of lipids proceeds by a free radical chain mechanism. In the presence of initiators, unsaturated lipids (LH) lose a hydrogen radical ($H^{\bullet}$) to form lipid radicals ($L^{\bullet}$). The formation of lipid radical 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 hydroperoxide, which exists in trace quantities prior to the oxidation, breaks down to yield radicals. Lipid hydroperoxides are formed by various pathways including the reaction of singlet oxygen with unsaturated lipids or the lipoxygenase-catalysed oxidation of

polyunsaturated fatty acids (Jadhav *et al.*, 1996). Due to resonance stabilisation of lipid radical (L•) 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-). Conjugated diene shows a characteristic UV absorption at 232-234 nm (Nakayama *et al.*, 1994).

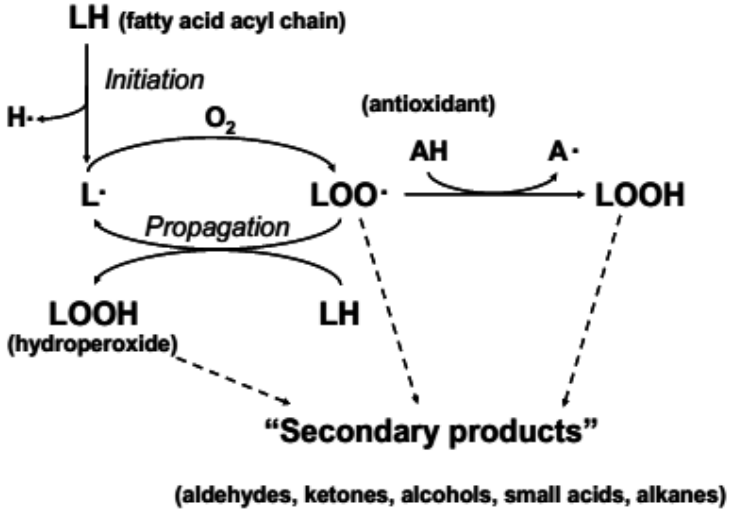

Figure 1. Autoxidation of polyunsaturated lipid

Source: Huss (1995)

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 (lipid peroxy radicals, LOO•) Lipid peroxy radicals initiate a chain reaction with other molecules, resulting in the formation of lipid hydroperoxides (LOOH) and lipid free radicals (L•). 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, radicals therefore tend to react whenever possible 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. Thus the termination reactions lead to interruption of the repeating sequence of propagating steps of the chain reaction (Jadhav *et al.*, 1996).

1.2.2.2 Enzymatic lipid oxidation

Several enzyme systems capable of initiating lipid oxidation have been identified in foods. There are two broad groups of enzymatic lipid oxidation such as lipoxygenase (LOX) and cyclooxygenase (COX) (Ashton, 2002).

LOX is a dioxygenase that oxygenates polyunsaturated fatty acids containing 1,4-cis,cis-pentadiene structure to pentadienyl, upon abstraction of H. This results in the formation of pentadienyl radical intermediate (Kanner and Rosenthal, 1992). The pentadienyl radical may react with oxygen to give peroxyl radical isomers, followed by fatty acid hydroperoxides as the final products. LOX exists in different tissues of many higher plants and animals. It has also been found in the gill, muscle and skin of fish and is capable of initiating the oxidation of PUFAs to produce unstable hydroperoxides (Hsieh *et al.*, 1988b). The most active LOX is in the gill and skin of fish (Hsieh *et al.*, 1988a). Fish gill tissue contains two LOX (12- and 15-LOX), each being active toward PUFA (AA, EPA, DHA) but exhibiting different hydroperoxide addition sites. LOX from sardine skin were more active on α-linolenic acid (18:3(*n*-3)) than linoleic acid (18:2(*n*-6)) (Mohri *et al.*, 1990). The skin contains active LOX, which significantly enhances lipid oxidation of fish (Mohri *et al.*, 1992). In general, skin lipids undergo more rapid oxidation than muscle lipids (Ke and Ackman, 1976).

Enzymatic oxidation induced by LOX is involved in the production of aroma compounds associated with rancidity as well as fishy odour in fish (Fu *et al.*, 2009; Josephson *et al.*, 1984; Josephson *et al.*, 1987). LOX activity is also found in several tissues of fish (Hsieh and Kinsella, 1989b). German *et al.* (1985) identified a 12-LOX from rainbow trout skin that produced 12-hydroxyeicosatetraenoic acid (12-HETE) as the major monohydroxy product. 12-LOX was also detected in trout gill

tissue and was related to the generation of oxidative volatile carbonyl compounds in a model system (Hsieh *et al.*, 1988a; Hsieh and Kinsella, 1989a). LOX activity found in Menhaden gill homogenate has been directly linked to the production of volatiles (Grun and Barbeau, 1994). 12-LOX activity was also detected in the skin and gill tissue of sixteen fish species. Relative activities in both tissues showed species variation (Hsieh *et al.*, 1988a). Trout gill 12-LOX was inhibited by flavonoids (Hsieh *et al.*, 1988b).

Moreover, LOX has also been detected in the tissues of pelagic species including sardine (Mohri *et al.*, 1990; Mohri *et al.*, 1992) and mackerel flesh (Harris and Tall, 1994). The activity of partially purified LOX from sardine skin readily oxidised the esterified fatty acids such as methyl linoleate and trilinolein (Mohri *et al.*, 1992). LOX in a crude homogenate from mackerel flesh oxidised linoleic acid and DHA more efficiently than EPA or linolenic acid (Harris and Tall, 1994). The 12-LOX from Atlantic mackerel muscle may also have a role in the onset of lipid oxidation although it is possible that the enzyme was derived from blood left in the capillaries and not the muscle itself (Saeed and Howell, 2001). Activities of these enzymes may be induced by tissue damage during capture, thus having the impact on post-mortem storage (Ashton, 2002).

1.2.3 Lipid oxidation in fish

Lipids in fish can be divided into two main groups: neutral and polar lipids. The neutral lipids consist of triglycerides (TGs), which are present primarily in adipose tissue and generally serve as an energy source. The total amount of neutral lipid fraction correlates positively to the total fat content of fish muscles and it varies widely due to species, season, diet, geographical origin, etc. On the contrary, polar lipids consist mainly of phospholipids (PLs) and glycolipids are found in membranes serving a structural role. The total polar lipid content is relatively constant among different fish and is also independent of environmental factors (Ackman and Ratnayake, 1992). The PLs of membranes are considered most susceptible to lipid oxidation. This has been explained by the fact that PLs have more PUFAs, compared with TGs, and a large surface area of membranes effectively increases the exposure of PLs to aqueous pro-oxidants (Huang *et al.*, 1993). Phospholipids represent around 1% of the total weight

of the flesh. Although present in much smaller amounts, the physical form of phospholipids in the membrane bilayer present a large surface area for oxidation reactions to occur, compared with 'droplets' of storage TG present in the muscle, associated with storage depots of fatty fish (Ashton, 2002; Hultin, 1994). Furthermore, the involvement of membranes in an electron transport processes gives a high possibility of electron leakage. The reaction of these electrons with molecular oxygen produces certain reactive oxygen species, such as superoxide and hydrogen peroxide (Hultin, 1994).

The levels of lipid in fish flesh vary, depending on species, ranging from lean fish (< 2% total lipid) such as cod, haddock and pollock, to high lipid species (8–20% total lipid) such as herring, mackerel and farmed salmon. A total lipid level of 5% has been suggested as a cut-off point between low and medium fat fish. In addition to species variability, lipid levels vary with sex, diet, seasonal fluctuation and tissue (Body and Vlieg, 1989). It is well known that fatty fish are susceptible to lipid oxidation and rancidity development because of the high content of PUFAs in their lipids, particularly the nutritionally important *n*-3 fatty acids, including EPA and DHA (Harris and Tall, 1994). The fatty acid profile of fish varies quite considerably between and within species and is also influenced by several factors (Body and Vlieg, 1989).

The onset of lipid oxidation in fish results in loss of quality. The losses in quality are usually evident in the later stages of lipid oxidation and are associated with the attributes of flavour, colour, and nutritional value. The impacts of lipid oxidation on the quality of fish are schematically illustrated in Figure 2.

1.2.3.1 Lipid oxidation in fish during storage

The oxidation of PUFAs requires an active form of oxygen because the reaction of PUFAs with ground state oxygen is spin restricted. The spin restriction is overcome by an activation reaction (initiation) involving a catalyst to initiate free radical chain reactions (propagation). The lipid hydroperoxides formed are unstable and subsequently break down to volatile compounds, which are associated with off-odour and off-flavour development (Kamal-Eldin *et al.*, 2003). The volatiles produced during

storage have been thoroughly characterised for a number of fish species including anchovy (Triqui and Reineccius, 1995), Atlantic salmon (Refsgaard *et al.*, 1998), Nile tilapia (Yarnpakdee *et al.*, 2012c) and mackerel (Refsgaard *et al.*, 1999). The mechanism of breakdown of lipid hydroperoxides to produce volatiles was reviewed (Hsieh and Kinsella, 1989b).

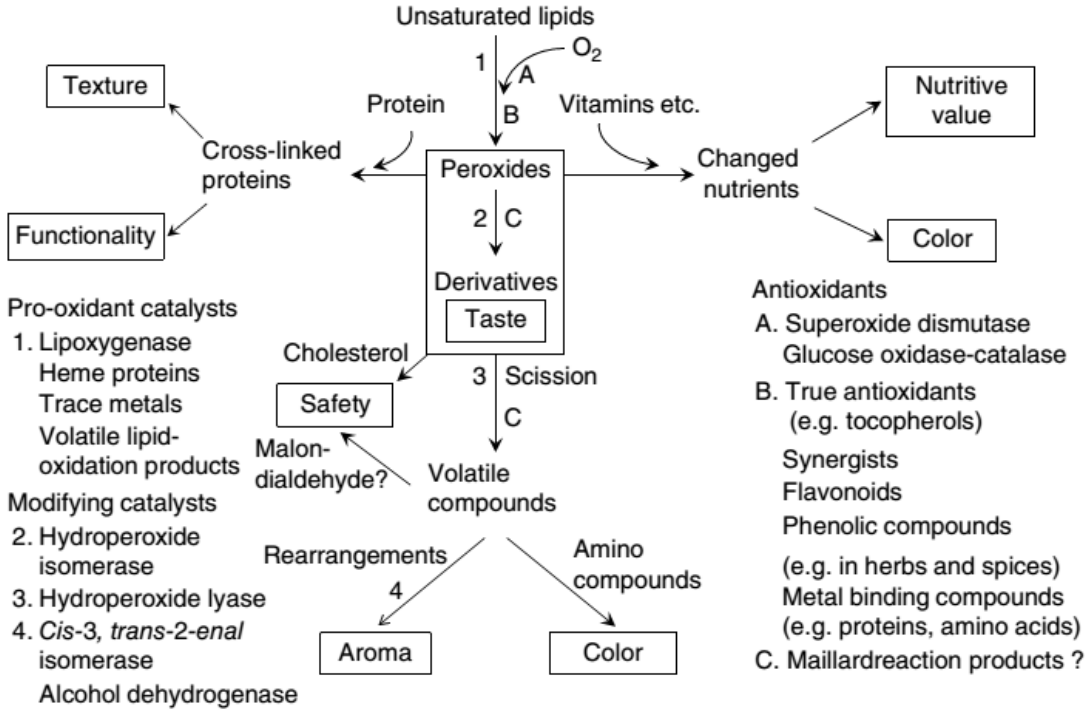

Figure 2. The impact of lipid oxidation on the quality of fish

Source: Bao and Ohshima (2014)

Storage condition has been shown to determine oxidative stability of fish. Aryee *et al.* (2012) studied the effect of storage temperatures at (25, 4, -18 and -80 °C) on the quality indices; free fatty acid (FFA) content, peroxide value (PV), thiobarbituric acid reactive substances (TBARS) of crude oil recovered from salmon fish skins. Higher temperatures resulted in higher PV, TBARS and FFA content in the salmon skin oil.

LOX enzymatic activity was monitored during chilling by testing its effect on the production of hydroxides, breakdown products from hydroperoxides (Medina *et al.*, 1999a). LOX was active for up to 48 h of chilled storage. Refsgaard *et*

al. (2000) indicated that enzymatic hydrolysis of neutral lipids followed by oxidative reactions of free fatty acids plays a major role in the sensory deterioration of salmon during frozen storage. FFA and volatile oxidation products were developed simultaneously in rainbow trout fillets stored at -20 °C for 13 months (Baron *et al.*, 2007). Lipid deterioration, lipolysis and lipid oxidation in farmed giant catfish muscle increased as refrigerated storage time increased (Rawdkuen et al., 2008). Aubourg *et al.* (2005) reported that hydrolytic and oxidative rancidity development were observed during 12 months of frozen storage at -20 °C. Comparison of whole fish and fillet of horse mackerel and mediterranean hake, PV and TBARS formation in fillets was greater than that found in whole fish (Simeonidou *et al.*, 1997). This could be due to a greater access of oxygen to fish tissue, more contact between white muscle tissue and blood contaminants as well as the greater activation of enzymatic system as a result of filleting (Richards *et al.*, 1998). Lipid oxidation proceeded most rapidly in fillet of herring under the skin during iced storage, probably explained by its high initial pro-oxidative activity (Undeland *et al.*, 1999). Yamaguchi and Toyomizu (1984) found that TBARS in the skin of lean fish during cold storage was caused by the dioxygenase, which is a prooxidant involved in lipid oxidation.

1.2.3.2 The use of antioxidants in fish and fish products

Fish have high content of PUFAs, leading to quality losses due to their oxidative sensitivity (Erickson, 2008). Lipid oxidation causes nutritional losses and produces undesirable flavour, colour, texture and toxic compounds, which make foods less acceptable or unacceptable to consumers (Kim and Min, 2008). Hence, fish and fish products should be protected from oxidative deterioration using antioxidants in order to prevent offensive odour/flavour, rancidity, loss of nutritional value, and consumer rejection (Chen *et al.*, 2008). Incorporation of antioxidants into fish muscle can effectively retard lipid oxidation. Although synthetic antioxidants have been widely used, nowadays, there is a growing interest to replace such compounds by naturally occurring antioxidants (Shi *et al.*, 2000). Several natural plant extracts from kiam wood, green tea, grape seed, rosemary and extra virgin olive oil have been proven to be effective in retarding lipid oxidation in many fish products (Gokoglu *et al.*, 2012; Maqsood and Benjakul, 2013; Medina *et al.*, 2003; Medina *et al.*, 1999b; Yarnpakdee

et al., 2012c) (Yerlikaya and Gokoglu, 2010). Ethanolic kiam wood extract (EKWE) could serve as a potential natural antioxidant in prevention of lipid oxidation and retardation of development of fishy odour and volatile lipid oxidation compounds in washed mince during iced storage (Maqsood and Benjakul, 2013). Yerlikaya and Gokoglu (2010) reported that green tea and grape seed extracts could retard lipid oxidation in bonito fillets treated during frozen storage of 5 months. Tomato and garlic extracts could delay TBARS, *p*-anisidine and conjugated-diene values in fish croquette during frozen storage (Gokoglu *et al.*, 2012). The addition of mixed antioxidants (EDTA and Trolox) during hydrolysis process markedly lowered lipid oxidation, fishy odour as well as the formation of volatile compounds in protein hydrolysate from Nile tilapia muscle (Yarnpakdee *et al.*, 2012c).

1.2.4 Fish gelatin

Gelatin is biopolymer obtained from partial denaturation or hydrolysis of collagen. It has a wide range of applications in food and non-food industries (photographic, cosmetic, and pharmaceutical) (Regenstein and Zhou, 2007). For medical and pharmaceutical purposes, it can be used for encapsulation, production of hard and soft capsules, wound dressing, adsorbent pads, and edible film formation. Furthermore, it can be applied for biomaterial based packaging and photographic industries (Regenstein and Zhou, 2007). Generally, gelatin is obtained from mammals, especially pig and cow skins and bones. Outbreaks of mad cow disease (bovine spongiform encephalopathy, BSE) have caused the anxiety for customers. Additionally, the gelatin obtained from pig skin and pig bone cannot be used in Kosher and Halal foods due to religious constraints. Furthermore, an increasing attention to health issues by consumers has also resulted in a high demand of fish gelatin (Kittiphattanabawon *et al.*, 2010). As a consequence, the alternative sources for gelatin production have been intensively increased, especially skin, bone, and scale from seafood processing by-products due to their abundance and low cost. However, fish gelatins have limited application, mainly owing to the lower gel strength and lower gel stability, compared with those from their mammalian counterparts (Benjakul *et al.*, 2012).

The process of gelatin production involves the disruption of non-covalent bonds and is partially reversible during gelation (Bigi *et al.*, 1998). Gelatin possesses several functional properties, including gelation, film formation, etc. (Wasswa *et al.*, 2007). Those properties are governed by their molecular properties and associated with type of raw material, processing parameters, etc. (Benjakul *et al.*, 2012). To enhance its interfacial property, e.g. emulsifying or foaming property, some modification has been implemented (Aewsiri *et al.*, 2011).

1.2.4.1 Production of gelatin

Production of gelatin can be divided into three main steps, (1) pretreatment, (2) extraction and (3) drying (Figure 3). All processes used for gelatin extraction have the direct impact on the yield and properties of gelatin obtained. The process has been optimised for different raw materials (Cho *et al.*, 2005; Cho *et al.*, 2004; Zhou and Regenstein, 2004).

1.2.4.1.1 Pretreatment of raw material

A) Removal of non-collagenous proteins

Prior to gelatin extraction from raw material, the pretreatment is practically implemented to increase purity of gelatin extracted. Alkaline solution under appropriate condition has been used to remove non-collagenous materials and break some interchain cross-links (Zhou and Regenstein, 2005). Also, the process is able to inactivate proteases involved in degradation of collagen (Regenstein and Zhou, 2007). During alkaline pretreatment, the type of alkali does not make a significant difference, but the concentration of alkali is critical (Zhou and Regenstein, 2005). Yoshimura *et al.* (2000) reported that alkali attacks predominantly the telopeptide region of the collagen molecule during pretreatment. Thus, some collagen can be solubilised by an alkaline solution. Long time and high concentration of alkaline pretreatment decreased the yield of gelatin from skin of channel catfish (Yang *et al.*, 2007).

Raw materials

↓

Non-collagenous protein removal

↓

Fat removal (optional)

↓

Demineralization (optional)

↓

Swelling

↓

Extraction with distilled water

↓

Filtration

↓

Clarification (optional)

↓

Drying

↓

Gelatin powder

Figure 3. Scheme for gelatin extraction

Source: Benjakul *et al.* (2012)

B) Removal of fat

Raw material with high fat content can be associated with the poor quality gelatin. Soaps may be formed during treatment with alkali prior to gelatin extraction. This soap can be contaminated into the resulting gelatin. Nevertheless, fat removal process was not included in the pretreatment process for gelatin extraction from fish skin with low fat content (Aewsiri *et al.*, 2008; Zhou and Regenstein, 2004). A simple defatting can be achieved by hot water (Waldner, 1977). Muyonga *et al.* (2004) removed fat in bone of Nile perch by tumbling in warm water (35 °C). Some solvents including isopropanol, butanol and ethanol have been used to remove fat from

fish skin used for gelatin extraction (Khantaphant and Benjakul, 2008). Khantaphant and Benjakul (2008) prepared defatted skin from brownstripe red snapper using 10% butanol prior to gelatin extraction.

C) Removal of minerals

Demineralisation of the raw material, especially bone or scale, aims to remove the calcium and other inorganic substances to facilitate the extraction of collagenous component (Waldner, 1977). The inorganic substances in raw material can be removed by treatment with dilute hydrochloric acid solution, whereby the calcium phosphate is dissolved as acid phosphates (Waldner, 1977). Fresh bone is commonly treated with hydrochloric acid solution, in which almost all minerals are completely removed. Depending on the nature of the material, temperature, and acid concentration, the demineralisation time can be varied. HCl concentration used is in the range of 2−6% (Waldner, 1977). Acid hydrolysis of protein should be minimised during demineralisation. High temperature should also be avoided since it can enhance the hydrolysis of protein. Bone from Nile perch was demineralised with 3% HCl at room temperature prior to extraction using warm water (60 °C) (Muyonga *et al.*, 2004). Mackerel and blue whiting bones were demineralised at room temperature for 18 h using 0.25 N HCl (Khiari *et al.*, 2013). The bone of *Pangasius* catfish was demineralised using 3 % HCl at room temperature for 21 h (Mahmoodani *et al.*, 2012). Demineralisation of tuna fin was carried out at room temperature using 0.6 N HCl (Aewsiri *et al.*, 2008).

D) Swelling of pretreated raw materials

Prior to gelatin extraction, pretreatment is generally required to enhance the extraction efficiency. Swelling is important because it can favour protein unfolding by disruption of non-covalent bonding and predispose the collagen to subsequent extraction and solubilisation (Stainsby, 1987). Pretreatments can be classified into two processes and are selectively used on the basis of the raw materials.

(1) Acid process

Acid hydrolysis is a milder treatment that effectively solubilises collagens of animals slaughtered at a young age such as pigs (Foegeding *et al.*, 1996). The pretreatment is aimed to convert the collagen into a form suitable for extraction. The covalent cross-links, particularly at the peptide region of the collagen must be disrupted to enable the release of free α-chains during the extraction (Johnston-Banks, 1990). The process is able to remove other organic substances. Sulfuric and hydrochloric acids are used, often with the addition of phosphoric acid to retard colour development (Johnston-Banks, 1990). Additionally, the acid pretreatment can partially inactivate endogenous proteases involved in degradation. As a result, the enzymatic breakage of intrachain peptide bonds of collagen during extraction can be lowered (Zhou and Regenstein, 2005).

Moreover, type of acid and concentration affected the yield and properties of gelatin. The concentration of H^+ used in processing of gelatin from cod skins affected yield and quality of resulting gelatin (Gudmundsson and Hafsteinsson, 1997). Megrim skin was treated with 0.05 M acetic acid or 0.05 M propionic acid prior to gelatin extraction using distilled water at 45°C for 30 minutes. The gelatin obtained had the highest elastic modulus, viscous modulus, melting temperature, and gel strength. On the other hand, gelatin obtained from skin swollen with citric acid exhibited the lowest turbidity of gelatin solution, whereas propionic acid led to the most turbid gelatin solution (Gómez-guillén and Montero, 2001). Giménez *et al.* (2005) reported that lactic acid (25 mM) could be an excellent substitute for acetic acid for the skin swelling process. The obtained gelatin showed similar properties to that prepared by using 50 mM acetic acid without the negative organoleptic properties. Gelatin obtained from the acid process is known as type A gelatin.

(2) Alkaline process

Type B gelatins are generally produced by alkali hydrolysis of bovine materials. This process results in deamidation as well as degradation, leading to the lowered chain length (Foegeding *et al.*, 1996). Alkaline pretreatments are normally

applied to bovine hide and ossein. Lime is most commonly used for this purpose as it is relatively mild and does not cause significant damage to the raw material by excessive hydrolysis. However, 8 weeks or more are required for complete treatment. Lime at concentrations of up to 3% is used in conjunction with small amounts of calcium chloride or caustic soda. Alkaline process using caustic soda takes 10-14 days for pretreatment (Johnston-Banks, 1990). Gelatin obtained from the alkaline process is known as type B gelatin. Cho *et al.* (2004) optimised the extraction condition for production of gelatin from shark cartilage with alkaline treatment. The maximum yield (79.9%) for gelatin production was obtained when alkali treatment using 1.6 N NaOH for 3.16 days and hot-water extraction at 65 °C for 3.4 h were implemented.

1.2.4.1.2 Extraction of gelatin

Thermal process is commonly implemented for gelatin extraction. When the heat is applied, collagen fibrils shrink to less than one-third of their original length at a critical temperature known as the shrinkage temperature (Foegeding *et al.*, 1996). This shrinkage involves a disassembly of fibres and a collapse of the triple-helical arrangement of polypeptide subunits in the collagen molecule. During the collagen to gelatin transition, many non-covalent bonds are broken along with some covalent inter- and intramolecular bonds and a few peptide bonds are cleaved. Heat applied at temperature higher than transition temperature (T_{max}) is able to disrupt the bonds, mainly H-bond, which stabilises collagen structure (Figure 4). This results in conversion of the helical collagen structure to a more amorphous form known as gelatin. Nevertheless, when the collagen molecule is completely destructured, glue is produced instead of gelatin. The conversion of collagen to gelatin yields molecules of varying mass, depending on the temperatures used, indigenous proteinases, etc. Generally, gelatin is a mixture of fractions with varying molecular weights from 15 to 400 kDa (Foegeding *et al.*, 1996).

The maximum yield together with the desirable physical properties is the main objective for extraction. The pH of extraction can be selected either for the maximum extraction rate (low pH) or for the maximum physical properties (neutral pH) (Johnston-Banks, 1990). Basically, the efficient pretreatment conditions allow the

manufacturer to use lower extraction temperatures, and still obtain gelatins with high gel strength (Johnston-Banks, 1990). Shorter time is generally associated with higher extraction temperatures if neutral pH levels are chosen. However, lower gel strength is obtained (Johnston-Banks, 1990). Muyonga *et al.* (2004) reported that Nile perch skin extracted at higher temperature yielded lower gel strength, melting point, setting temperature, and longer setting time. Gelatin from channel catfish skin showed lower gel strength as extraction temperature was increased from 60 °C to 75 °C (Yang *et al.*, 2007). Additionally, gelatin extracted from the skin of brownbanded bamboo shark and blacktip shark using higher temperature had decreasing band intensity of major components (α-, β- and γ -chains) as well as increasing low-molecular weight components. Such changes correlated well with lowered gel strength. Shorter chain fragments of gelatin could not form the junction zone, thereby limiting the strong network formation (Kittiphattanabawon *et al.*, 2010) (Figure 4).

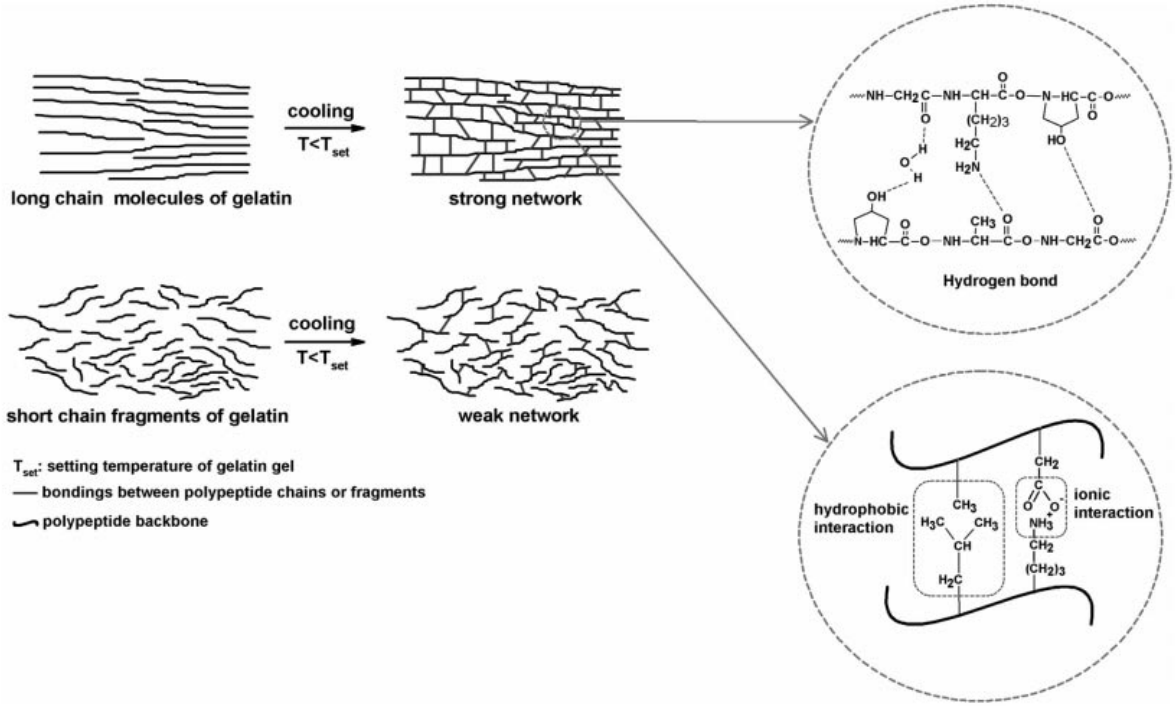

Figure 4. Gelatin network associated with hydrogen bond, hydrophobic interaction, and ionic interaction

Source: Benjakul *et al.* (2012)

1.2.4.1.3 Drying

After extraction, the gelatins are filtered to remove suspended or insoluble matters including fat, unextracted collagen fibers, and other residues. Diatomaceous earth or activated carbon can be used to clarify gelatin solution. The final stage is evaporation, sterilisation, and drying. These are performed as quickly as possible to minimise the loss of properties (Johnston-Banks, 1990). Kwak *et al.* (2009) extracted gelatin from shark cartilage using three drying methods, (1) freeze drying, (2) hot-air drying, and (3) spray drying. Freeze-dried gelatin showed the highest gel strength and foam formation ability, but its foam stability was the lowest. Nevertheless, spray-dried gelatin exhibited the best emulsion capacities. Gudmundsson and Hafsteinsson (1997) evaporated gelatin solution from cod skin under vacuum at 43–45 ºC until 85–90% of the water had been removed. The concentrate containing the gelatin was then air dried in a fume hood at 45 ºC to remove remaining water. One portion of gelatin liquid was freeze-dried. Freeze-dried gelatin had considerably higher bloom value than air-dried gelatin. Air drying more likely causes protein denaturation than lyophilisation, thus reducing gel forming properties (Gudmundsson and Hafsteinsson, 1997).

1.2.4.2 Functional properties of gelatin

Gelatin is a gelling protein, which has widely been applied in the food and pharmaceutical industries (Cho *et al.*, 2005). Gelatin gel has "melt-in-the-mouth" characteristic and shows an excellent release of flavour (Choi and Regenstein, 2000). Moreover, its functional properties, including gelation, emulsifying properties, foam-forming properties, and film formation, are important for the food industry as it enhances the elasticity, consistency, and stability of food products, and it is also used as an outer film to protect foods against light and oxygen (Montero and Gómez-Guillén, 2000).

1.2.4.2.1 Gelation

An aqueous solution of gelatin becomes slightly viscous at temperature above its melting temperature. On cooling, the gelatin solution starts to form transparent

elastic thermoreversible gels when the temperature is below the setting temperature (Babin and Dickinson, 2001). The interaction initiates a disorder-to-order transition, as the random coil gelatin molecules seek to return to the ordered triple helix conformation. Gelatin gel is a reversibly cross-linked biopolymer network stabilised mainly by hydrogen-bonded junction zones. Furthermore, hydrophobic and ionic interactions are also involved in the gelation of gelatin (Figure 4). The gelation of gelatin is dependent on many factors such as source of raw material for gelatin extraction, presence of endogenous protease in raw material, and the conditions for gelatin extraction, particularly temperature. High temperature generally renders high yield, but the gelatin obtained has shorter chains (Kittiphattanabawon *et al.*, 2010). These fragments cannot form the junction zone effectively. As a result, a poor gel is formed or these fragments cannot set, especially at room temperature (25 °C).

Pretreatment of skin using various acids also affects the gelling property of gelatin from fish skin. Gelatin extracted from the skin of megrim pretreated with 50 mM acetic acid and propionic acid exhibited the highest gel strength (Gómez-guillén and Montero, 2001). Furthermore, the skin from Dover sole swollen with 50 mM acetic acid yielded the gelatin with the highest gel strength, compared with that swollen in 25 and 50 mM lactic acid (Giménez *et al.*, 2005).

Extraction temperature is another important factor affecting the gelation of gelatin. Normand *et al.* (2000) reported that higher extraction temperature caused protein degradation, thus producing protein fragments and lowering gelling ability. Gómez-Guillén *et al.* (2002) found that squid gelatin extracted at 80 °C showed very weak gel. For gelatin from Nile perch skin and bone, the gelatin extracted at 50°C exhibited the higher gel strength than corresponding bone gelatin. Gelatin extracted from skins at the higher temperature exhibited lower gel strength but temperature had no effect on gel strength of bone gelatin (Muyonga *et al.*, 2004). Bloom strength of gelatin from brownbanded bamboo shark and blacktip shark decreased with increasing extraction temperature and time. The marked decrease in bloom strength was noticeable when extraction temperature of 75 °C was used for extraction of gelatin from the skin of both sharks (Kittiphattanabawon *et al.*, 2010). Gelatins from different fish species extracted under varying conditions had different gel strength (Table 3).

Gelatins from tuna or tilapia skin (warm-water fish) had a melting point of 25-27 °C (Choi and Regenstein, 2000) and had a bloom value of 200-250 g. These gelatins more closely resemble bovine or pig gelatin, which melts at 32-35 °C. Fish gelatin with lower gel melting temperatures had a better release of aroma and offered a stronger flavour (Choi and Regenstein, 2000). By increasing the concentration of gelatin or by using gelatin mixtures, desserts made from fish gelatins would be more similar to desserts made from high bloom pork skin gelatin (Zhou and Regenstein, 2007). Cheng *et al.* (2008) observed that combinations of fish gelatin with pectin have been used to make a low-fat spread. A decrease in the fish gelatin to pectin ratio (3:0, 2:1, 1:1, and 1:2) resulted in an increase in bulk density, firmness, compressibility, adhesiveness, elasticity, and meltability. On the other hand, use of gelatin/sodium alginate blends to form casings could lower water losses and lipid oxidation during chilled storage as compared to pectin casing (Liu *et al.*, 2007). Binsi *et al.* (2009) used gelatin from the skin of bigeye snapper to modify the texture of threadfin bream mince gel. The addition of fish gelatin (0.1-1%) to fish mince resulted in higher storage modulus (G′) values from the beginning of heating regime. A maximum G′ value of 443.7 kPa at 68.3 °C was obtained in the presence of 0.5% gelatin, which was 42% higher than that of fish mince without the added gelatin. Thus, gelatin can be used in combination with other gelling agents or hydrocolloids for the unique purpose or applications.

1.2.4.2.2 Emulsifying and foaming properties

Emulsions and foams are heterogeneous systems consisting of one phase dispersed in another. An emulsion is a dispersion or suspension of two immiscible liquids, while a foam is a gas phase dispersed in liquid (Hill, 1998). Proteins extracted from the different natural sources, for example, soy, milk, fish, meat, and plant, can be used as the emulsifier or foaming agents because of their ability to facilitate the formation and improve the stability of emulsion or foam (Surh *et al.*, 2006). Gelatin is surface-active and is capable of acting as an emulsifier in oil-in-water emulsions and foaming agent (Binsi *et al.*, 2009). The hydrophobic areas on the peptide chain are responsible for emulsifying and foaming properties of gelatin (Galazka *et al.*, 1999).

Table 3. Extraction conditions and gel strength of gelatin extracted from different fish species

Raw materials	Pretreatments		Extraction conditions	Gel strength	References
	Non-collageneous matter removal	Swelling			
Giant catfish (*Pangasianodon gigas*) skin	0.2 M NaOH at 4 °C for 90 min	0.05 M acetic acid at 24–26 °C for 3 h	Water at 45 °C for 12 h	153 g	Jongjareonrak *et al.* (2010)
Brownbanded bamboo shark (BBS, *Chiloscyllium punctatum*) and blacktip shark (BTS, *Carcharhinus limbatus*) skins	0.1 M NaOH at 15–20 °C for 2 h, followed by 1 M HCl at 15–20 °C for 1 h	0.2 M acetic acid at 25–26 °C for 15 min	Water at 45 °C, 60 °C, and 75 °C for 6 and 12 h	56.53-217.26g (BBS) 10.43-207.83g (BTS)	Kittiphattanabawon *et al.* (2010)
Bigeye snapper (*Priacanthus tayenus*) skin	0.025 M NaOH at 26–28 °C for 2 h	None	0.2 M acetic acid in the presence of pepsin (15 unit/g) at 4 °C for 48 h, followed by incubation at 45 °C for 12 h	138.6 g	Nalinanon *et al.* (2008)
Tuna (*Katsuwonus pelamis*) fin	0.025 N NaOH at 26–28 °C for 1 h, followed by 0.6 N HCl at 26–28 °C for 5 days	0.2 M acetic acid at 26–28 °C for 3 h	Water at 50 °C for 12 h	126 g	Aewsiri *et al.* (2008)
Channel catfish (*Ictalurus punctatus*) skin	0.2 M NaOH at 4 °C for 84 min	0.115 M acetic acid at 4 °C for 60 min	Water at 55 °C for 3 h	252 g	Yang *et al.* (2007)

Table 3. Extraction conditions and gel strength of gelatin extracted from different fish species (cont.)

Raw materials	Pretreatments		Extraction conditions	Gel strength	References
	Non-collageneous matter removal	Swelling			
Shortfin scad (*Decapterus macrosoma*) skin	0.2% (w/v) NaOH for 2 h	0.2% (w/v) H_2SO_4 for 2 h, followed by 1.0% (w/v) citric acid for 2 h	Water at 40–50 °C for 12 h	177 g	Cheow *et al.* (2007)
Shark (*Isurus oxyrinchus*) cartilage	1.6 N NaOH at 8 °C for 3.14 days	None	Water at 65 °C for 3.4 h	111.9 kPa	Cho *et al.* (2004)
Nile perch (adult) (*Lates niloticus*) bone	3% HCl at 20–25 °C for 9–12 h	None	Four-step extractions with water at 50 °C, 60 °C, 70 °C and 100 °C, respectively, for 5 h each step	134 g (50 °C) 151 g (60 °C) 160 g (70 °C)	Muyonga *et al.* (2004)
Nile perch (*Lates niloticus*) skin	0.01 M H_2SO_4 at 20–25 °C for 16 h	None	Four step-extractions with water at 50 °C, 60 °C, 70 °C and 100 °C, respectively, for 5 h each step	229 g (50 °C) 175 g (60 °C) 134 g (70 °C)	Muyonga *et al.* (2004)
Red tilapia (*Oreochromis nilotica*) skin	0.2% (w/v) NaOH for 40 min	0.2% (w/v) H_2SO_4, followed by 1.0% (w/v) citric acid	Water at 45 °C for 12 h	128 g	Jamilah and Harvinder (2002)
Black tilapia (*Oreochromis mossambicus*) skin	0.2% (w/v) NaOH for 40 min	0.2% (w/v) H_2SO_4, followed by 1.0% (w/v) citric acid	Water at 45 °C for 12 h	181 g	Jamilah and Harvinder (2002)

Gelatin concentration has an impact on interfacial properties, but these properties are also governed by the source of the raw material. Aewsiri *et al.* (2009) reported that the higher emulsifying and foaming properties were observed in gelatin from precooked tuna fin when the concentration of gelatin was increased. In contrast, the emulsifying capacity of gelatin from bigeye snapper decreased with increasing gelatin concentration (Binsi *et al.*, 2009). Additionally, Aewsiri *et al.* (2009) studied the emulsifying properties, emulsion activity index (EAI) and emulsion stability index (ESI), and foaming properties, foam expansion (FE) and foam stability (FS) of gelatin from cuttlefish skin with and without bleaching using hydrogen peroxide (H_2O_2). Emulsions containing gelatin from bleached dorsal and ventral skin were more stable than those of gelatin without bleaching. A longer bleaching time and higher H_2O_2 concentration led to a lower ESI of gelatin for all samples, except for gelatin from dorsal skin, in which the highest ESI was obtained when the skin was bleached with 5% H_2O_2 for 48 h ($P < 0.05$). Surh *et al.* (2006) found that the oil-in-water emulsion prepared with high-molecular weight fish gelatin (~120 kDa) was more stable than that prepared with low-molecular weight fish gelatin (~50 kDa). For foam-forming ability, gelatin from unbleached skin, both dorsal and ventral, had a slightly lower FE than gelatin extracted from bleached skin, while bleaching had no effect on the FS of gelatin from ventral skin, but gelatin from dorsal skin bleached with 5% H_2O_2 for 48 h showed the highest FS (Aewsiri *et al.*, 2009). Jongjareonrak *et al.* (2010) reported that foam capacity and foam stability of gelatin from farmed giant catfish were higher than those of gelatin from calf skin. Gelatin from shark cartilage and precooked tuna fin showed lower foam capacity and foam stability than gelatin from porcine skin (Aewsiri *et al.*, 2008; Cho *et al.*, 2004).

1.2.4.2.3 Film formation

Film and coating from biopolymers such as proteins and polysaccharides have been receiving increasing attention since synthetic packaging films have led to serious ecological problems because of their non-biodegradability. Proteins are important biopolymers possessing good film-forming ability (Benjakul *et al.*, 2008). Moreover, their mechanical and barrier properties are generally superior to

polysaccharide-based films (Cuq *et al.*, 1998). Amongst all proteins, gelatin has attracted the attention for the development of edible films due to its abundance, biodegradability, and excellent film-forming properties (Bigi *et al.*, 2002). Thus, it is one of the first materials applied to edible coatings and films. The main parameters affecting film-forming properties of gelatin are the source of raw material, extraction method, molecular weight, film preparation method, and degree of hydration or type and level of plasticizer used (Jongjareonrak *et al.*, 2006).

Giant squid gelatin obtained from pepsin aid process during the swelling step showed good film-forming properties (Giménez *et al.*, 2009). Its puncture force, puncture deformation, and water permeability were 4.94 N, 46%, and 1.89×10^{-8} g mm h^{-1} cm^{-2} Pa^{-1}, respectively. Zhang *et al.* (2007) characterised edible film from channel catfish gelatin extracted using different pretreatment methods. The pretreatment with 0.25 M NaOH and 0.09 M acetic acid, followed by extraction at 50°C for 3 h was selected as the optimum extraction method. The resultant film had tensile strength (TS), percentage elongation (%E), and water vapour permeability (WVP) comparable to the film obtained from commercial mammalian gelatin. Additionally, Nile perch skin gelatin films were also found to exhibit film strength (stress at break) and percentage of strain (elongation at break) similar to that of bovine bone gelatin (Muyonga *et al.*, 2004). TS and %E of catfish gelatin films significantly increased from 27.1 MPa to 61.7 MPa and from 105.1% to 115.1%, respectively, when the gelatin contents were increased from 0.5% to 1%. Due to the hydrophilicity in nature, gelatin film shows the poor water vapour barrier property. Water vapour permeability (WVP) of film from channel catfish increased with increasing gelatin contents from 0.5% to 2.5% (Zhang *et al.*, 2007).

1.2.5 Protein hydrolysates

Protein hydrolysates can be defined as proteins that are chemically or enzymatically broken down to peptides of varying sizes (Adler-Nissen, 1986). The chain length of peptides formed during the hydrolysis process is one of the parameters determining both functional and organoleptic properties of protein hydrolysate. The modification in structure, chain length and amino acid sequence depend on treatment,

hydrolytic conditions, type of enzyme, temperature, protein concentration, etc. (Najafian and Babji, 2012). Additionally, characteristics and properties of fish protein hydrolysate are determined by raw material used. Different proteinaceous substances yield the hydrolysate with varying nutritive value, functional properties as well as bioactivities (Najafian and Babji, 2012). Generally, protein hydrolysate can be produced from fish flesh, by-products such as head, skin, trimmings, frames and bones. Bioactive peptides generally contain 2–20 amino acid units. The amino acid composition and sequences can affect the activity of those peptides (Ryan *et al.*, 2011). Bioactivities of fish-derived peptides are governed by their structural properties and their amino acid composition and sequences. Bioactive peptides may be involved in various biological functions, including angiotensin-I-converting enzyme (ACE) inhibiting activity, antioxidant, immunomodulatory, antimicrobial and anticoagulant activities (Najafian and Babji, 2012).

However, the use of fish hydrolysates into food applications has been restricted by their bitterness (Dauksas *et al.*, 2004). Recently, much attention has been focused on the identification and characterisation of the structure, composition and sequence of bioactive peptides. Biologically active peptides play an important role in metabolic regulation and modulation. These peptides can be used as functional food ingredients, or nutraceuticals and pharmaceuticals to promote human health and prevent disease. The importance of fish as a source of novel bioactive substances is growing rapidly (Chalamaiah *et al.*, 2012).

1.2.5.1 Production of protein hydrolysates

Chemical and biological methods have been used for protein hydrolysis. The chemical hydrolysis process is used less frequently in food. This technique has several drawbacks such as considerable racemisation, destruction of amino acids and peptides, low selectivity, low extraction efficiency, solvent residue and environmental pollution (Wang *et al.*, 2010). Furthermore, hydrolysate obtained exhibit poor functional properties. In contrast, the biological processes using added enzymes are employed more frequently and enzyme hydrolysis holds the most promise because it

renders the products of high functionality and nutritive value (Kristinsson and Rasco, 2000b).

A) Preparation of substrate

Lean fish species or their derived material is preferred as the protein substrate for enzymatic hydrolysis to avoid extensive lipid oxidation. However, the abundant underutilised pelagic fish can be used. The production is started by evisceration of the whole fish. Thereafter a fish is ground, mixed with water and homogenised until a viscous homologous mixture is obtained. In some instances, a buffer solution such as phosphate buffer and boric acid-NaOH buffer is added to the minced fish (Baek and Cadwallader, 1995).

However, the presence of buffer salts may affect the final properties of the hydrolysates. Process for fatty and lean species is different. Fatty species such as mackerel, herring, menhaden, sprat and anchovy require additional treatments such as centrifugation or solvent extraction to remove excess fat (Klompong *et al.*, 2007). A fish protein hydrolysate with high lipid content turned to be darken as a result from lipid oxidation (Hoyle and Merritt, 1994). Fish protein hydrolysate suitable for human consumption has a lipid content not exceeding 0.5% by weight (Shahidi *et al.*, 1995). Hoyle and Merritt (1994) used an ethanol extraction to remove fat from minced herring at the fish/ethanol ratio of 1:2 at 70 °C for 30 min. Lipid content in final product was reduced to 0.9 from 4.0% of raw herring. Before placing the treated substrate in the reaction vessel, chemical reagent such as NaCl, sorbic acid or ethanol are occasionally added to the minced fish to minimise bacterial degradation (Sikorski *et al.*, 1981). Yellow stripe trevally mince was defatted using isopropanol at 75 ºC for 90 min (Klompong *et al.*, 2007). Defatted mince had a much lower fat content (0.67%) than whole mince (3.23%). Ultrasound-assisted solvent extraction with isopropanol was used for separating lipid from mackerel muscle, resulting in a degreasing rate of 95% (Hou *et al.*, 2011). Silver carp by-products including head, skin, fin, bone and residual meat were defatted using 90% ethanol at 70 ºC for 30 min, prior to enzymatic hydrolysis (Duan *et al.*, 2010).

B) Enzymatic hydrolysis

Enzymatic hydrolysis can be employed to produce fish protein hydrolysates with the aid of proteolytic enzymes, both endopeptidases, which cleave the peptide bonds within protein molecules, and exopeptidases, which hydrolyse peptide bonds from either the N- or the C-terminal (Clemente, 2000). This can be done by proteolytic enzymes already present in the fish viscera and muscle (endogenous protease), or by adding enzymes from other sources (Kristinsson and Rasco, 2000a). The autolytic process is commonly used for animal feed or fertilizer application but typically not for food use with the exception of fish sauce because it is difficult to control the rate of hydrolysis (Kristinsson and Rasco, 2000a). Each protease has a unique specificity for peptide bonds adjacent to certain amino acid residues. It is therefore possible to selectively hydrolyse protein to obtain the specific properties (Wu *et al.*, 2003). Plant, animal and microbial enzymes can be used for the production of protein hydrolysate. Amongst microbial enzymes, bacterial and fungal proteases are the 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 (Kristinsson and Rasco, 2000a). In comparison to animal- or plant-derived enzymes, microbial enzymes offer several advantages, including having more versatile catalytic activities and being more stable at a greater pH range and higher temperatures (Guérard *et al.*, 2001). The scheme for the production of FPH using enzymes is given in Figure 5.

The enzymatic hydrolysis of proteins with different proteases is an effective method of producing peptides with the improved nutritional properties and bioactivities (Kohen *et al.*, 1988). This process takes place under mild circumstances and racemisation reactions can be minimised (Kristinsson and Rasco, 2000a). Enzymes act on proteins at specific sites, depending upon the site of cleavage. The general principle of the process is simple (Figure 5) but there are many factors need to be carefully considered (Kristinsson, 2007). The important factors affecting the characteristics and properties of protein hydrolysates included temperature, time of hydrolysis, pH, degree of hydrolysis, the nature of the substrate, type of enzyme used and enzyme to substrate level (Kristinsson and Rasco, 2000b).

A wide variety of commercial enzymes has been used successfully to hydrolyse fish and other food proteins. Proteolytic enzymes from microorganisms such as Alcalase, Neutrase, Protease N and Protamex are most suitable to prepare fish protein hydrolysates due to their high productivity (Gildberg *et al.*, 1989; Guérard *et al.*, 2001; Liaset *et al.*, 2002; Rebeca *et al.*, 1991). Enzymes from plants and animals such as papain, bromelain, ficin and pepsin are still used for hydrolysis (Aspmo *et al.*, 2005; Liaset *et al.*, 2002). The selection of enzymes is usually based on a combination of efficacy and economics (Lahl and Braun, 1994).

C) Factors affecting fish protein hydrolysate production

(1) Types of enzymes 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 reactivity (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).

(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).

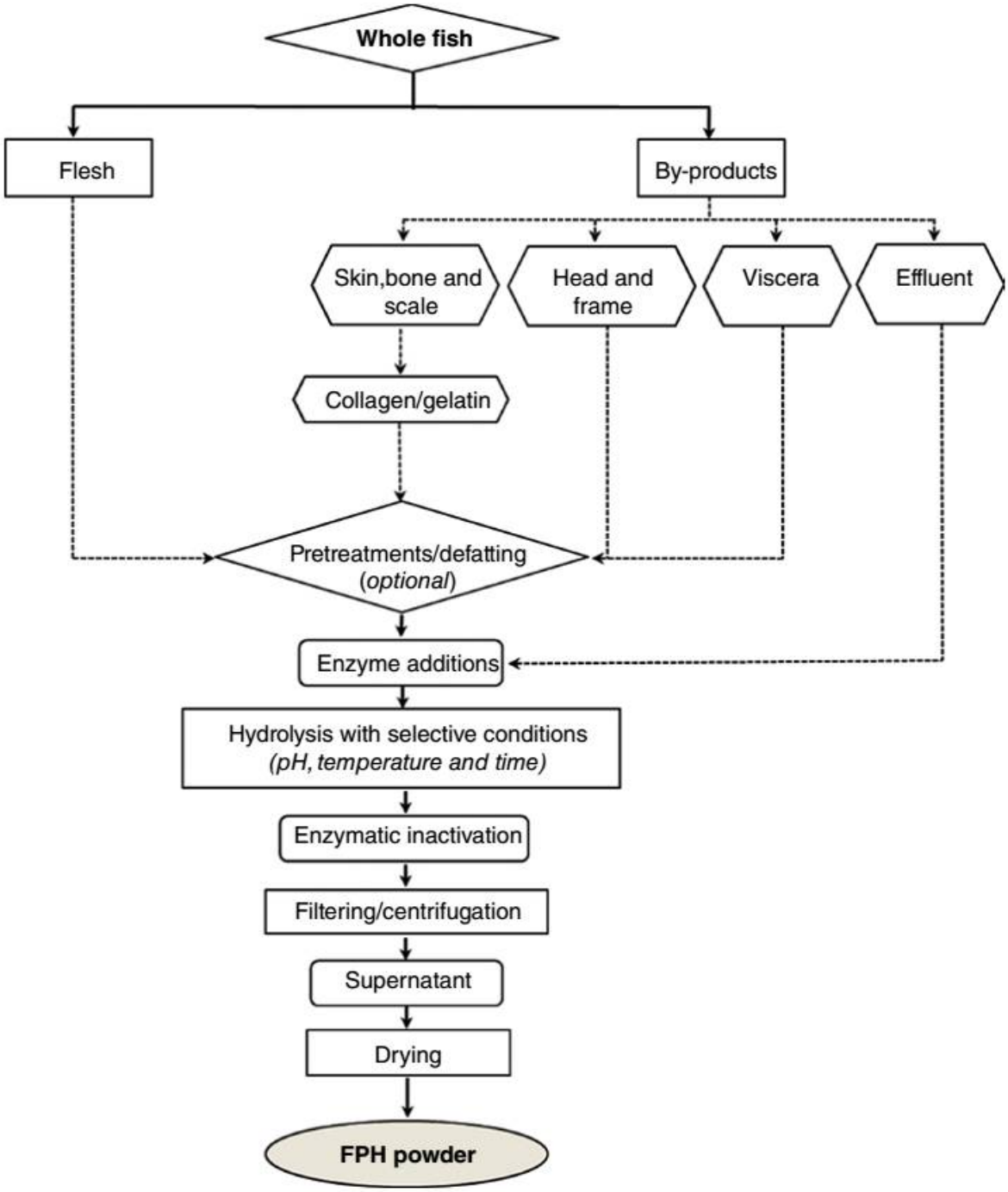

Figure 5. Scheme of fish protein hydrolysate production

Source: Benjakul *et al.* (2014)

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 log10 of enzyme concentration and DH were plotted. Sheriff *et al.* (2014) reported that protein hydrolysate 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.

1.2.5.2 Pretreatment processes

Although fish can serve as a potential raw material for protein hydrolysate production, the presence of pro-oxidants such as unstable lipid substrates is a drawback (Raghavan and Kristinsson, 2008). These constituents are involved in undesirable characteristic and instability of hydrolysates (Khantaphant *et al.*, 2011; Raghavan and Kristinsson, 2008). To overcome such problems, the pretreatment of protein substrate including defatting in order to remove the fat is strongly recommended.

A) Defatting

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 underutilised pelagic fatty fish, they can serve as the proteinaceous substrates for hydrolysate production (Klompong *et al.*, 2007). Fatty fish species such as mackerel, herring, round scad and yellow stripe trevally would require additional treatment such as solvent extraction to remove the fat (Klompong *et al.*, 2007; Thiansilakul *et al.*, 2007a). 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. Hoyle and Merritt (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 *Sphyrna 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.*, 2011a). 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.*, 2014). The unpleasant odour/flavour lowers the consumer acceptance and limits their use. 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 (Khantaphant *et al.*, 2011). 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). Hrynets *et al.* (2011) reported that the use of 6 or 8 mM of citric acid resulted in substantial 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 substances. Khantaphant *et al.* (2011) 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).

1.2.5.3 Termination of enzymatic reaction

When protein substrate is hydrolysed until the desired degree of hydrolysis (DH) is obtained, it is necessary to terminate the enzymatic reaction. Otherwise the enzymes would remain active in the substrate and further hydrolyse the protein and peptides. Deactivation of enzymes is achieved either by chemical or thermal means (Kristinsson and Rasco, 2000a). Chemical inactivation can be carried out by either lowering or raising the pH of the slurry to a point where the enzyme is inactivated. Some enzymes are more sensitive to pH changes rather than temperature changes (Kristinsson and Rasco, 2000b). Alcalase is a relatively thermostable enzyme, but it is very sensitive to acid pH. Complete inactivation of Alcalase therefore is obtained by lowering the pH to 4.0 (Adler-Nissen, 1986). After hydrolysis of capelin muscle protein using commercially available Alcalase, Neutrase and papain, the hydrolytic reaction

was stopped by lowering the pH to 3-4 in order to deactivate the enzyme (Shahidi *et al.*, 1995). Thermal inactivation is the easy method for termination of enzyme. The slurry of hydrolysate and enzymes are transferred to a heat bath, where the enzymes are deactivated by exposing them to temperatures ranging from 75 to 100 °C for 5 to 30 min, depending on the type of enzyme (Kristinsson and Rasco, 2000b). Papain is very heat tolerant, and has been reported to need at least 90 °C for 30 min to be fully inactivated (Hoyle and Merritt, 1994). Alcalase and Neutrase activities were stopped by heating at 90 °C for 5 min (Benjakul and Morrissey, 1997). However, terminating the reaction by heat is undesirable (Haque, 1993). Heat denaturation of protein can lead to the exposure of hydrophobic residues and subsequently protein aggregation.

1.2.5.4 Collection of protein hydrolysate

Hydrolysis of protein molecules brings about the peptides and free amino acids which are obtained in the soluble fraction (Mohr, 1980). Commonly, the slurry is desludged by centrifugation, in which four fractions are formed. Those include oil layer on the top, emulsion, fish protein hydrolysate and sludge on the bottom of the centrifugation vessels (Šližyte *et al.*, 2005). Since lipid in the final hydrolysate is a major concern for fish protein hydrolysate, it is important to remove it. Lipid residues in product must be lower than 0.5% to prevent alteration of the lipid during storage (Spinelli *et al.*, 1972). Fish protein hydrolysates can be filtrated using suction filtration of the sludge (Onodenalore and Shahidi, 1996) and or by passing it through a 2-mm mesh screen (Vieira *et al.*, 1995). The insoluble fraction or the sludge precipitated during centrifugation can be used as animal feed. The final product can be neutralised, in which a fairly high salt content is found in the final product. Alternatively, ultrafiltration membranes have been introduced into the production of protein hydrolysates, mainly to control the molecular size of the product (Kristinsson and Rasco, 2000a). The final soluble fraction is generally dried to convert the hydrolysate to a powder form, which can be incorporated into food formulations. Spray drying of the soluble fraction is one of the most energy consuming and expensive steps in the production of protein hydrolysates.

1.2.5.5 Characteristics and sensory property

The use of gelatin hydrolysates as food supplements, they have to be palatable. However, the use of those fish-derived products are limited due to fishy odour associated with lipid oxidation as well as the bitterness caused by certain peptides (Raghavan *et al.*, 2010). Those undesirable characteristics limit the use of fish gelatin hydrolysates, particularly in foods or drinks which have a light odour or smell. An appropriate pretreatment of raw material is a promising approach to lower unpleasant problems in the resulting products. Muyonga *et al.* (2004) reported that gelatins prepared from the skin and bone of Nile Perch and treated with activated carbon were found to be free of fishy odour. Yarnpakdee *et al.* (2012a) showed that washing of Nile tilapia mince, along with a process to remove membranes prior to alkaline solubilisation, was effective in preparing a substrate for protein hydrolysate production, in which fishy odour and taste could be significantly lowered. The resulting protein hydrolysate could be fortified in low fat milk at a level up to 0.5% or higher. Moreover, milk was successfully fortified with fish protein hydrolysates from Indian mackerel mince prepared with the pretreatment at a level of 0.2% (Yarnpakdee *et al.*, 2012b). As a consequence, a wider range of applications for fish derived hydrolysates can be achieved. The colour of gelatin depends on the raw materials used for extraction. However, the colour does not affect functional properties of gelatins (Ockerman and Hansen, 1988).

Taste is one of the most important sensory characteristics that determine the acceptance or rejection of protein hydrolysates. Tastes of hydrolysates include bitter, bland, fishy, glutamic acid-like, sour and umami (Hoyle and Merritt, 1994; Maehashi *et al.*, 1999; Shahidi *et al.*, 1994). The tastes are influenced by parameters used in the hydrolysis of the protein, including DH, type of enzyme and duration of hydrolysis (Hoyle and Merritt, 1994). Bitterness is a major hindrance in the application of hydrolysates for food use. It is related with the presence of bitter peptides containing hydrophobic amino acids such as phenylalanine, tyrosine, leucine and isoleucine (Ishibashi *et al.*, 1987; Raghavan *et al.*, 2010). Ishibashi *et al.* (1987) reported that the intensity of bitterness increased when the level of phenylalanine or tyrosine in the peptides increased, especially when they were located at the C-terminal position.

1.2.6 Gelatin hydrolysate

Gelatin hydrolysate can be prepared as the value-added products by enzymatic hydrolysis. Gelatin hydrolysates have gained increasing interest, especially for health concern (Je *et al.*, 2007). Enzymatically hydrolysed fish skin gelatin has shown better biological activities, compared to that of the peptides derived from fish muscle protein (Kim and Mendis, 2006). Gelatin peptides have unique Gly-Pro-Hyp sequence in their structure, and it is presumed that the observed antioxidative and antihypertensive properties of gelatin peptides can be associated with their unique amino acid compositions (Kim *et al.*, 2001). The different gelatin hydrolysates had different amino acid compositions and sequences, depending on gelatin sources and enzymes used. A number of commercial proteases have been used for the production of gelatin hydrolysates, including trypsin, chymotrypsin, pepsin, Alcalase, Properase E, Pronase, collagenase, bromelain and papain (Kim *et al.*, 2001; Lin and Li, 2006; Mendis *et al.*, 2005a; Yang *et al.*, 2008). Bioactive peptides can be released by enzymatic proteolysis of gelatin and may act as potential physiological modulators of metabolism during the intestinal digestion of the diet; these peptides could be the good candidates as nutraceuticals (Je *et al.*, 2007).

Mendis *et al.* (2005b) produced gelatin hydrolysates from jumbo squid skin with different proteolytic enzymes. The extent of hydrolysis was estimated by assessing the degree of hydrolysis (DH) and it was observed to be 97, 87 and 56% for trypsin-, α-chymotrypsin- and pepsin-derived hydrolysates, respectively. Thornback ray gelatin hydrolysates were prepared with proteases from *Bacillus subtilis* A26, *Raja clavata* crude alkaline protease extract, Alcalase and Neutrase (Lassoued *et al.*, 2015). At the same level of enzyme, crude proteases from *R. clavata* showed a higher DH (17%), followed by crude enzyme from *B. subtilis* A26 (11%), Alcalase (9%) and Neutrase (7%), respectively. Crude proteolytic enzyme from papaya latex yielded gelatin hydrolysate from shark skin with higher DH than that hydrolysed with Neutrase (Kittiphattanabawon *et al.*, 2012). Karnjanapratum and Benjakul (2015a) prepared gelatin hydrolysates from autolysed non-swollen and swollen unicorn leatherjacket skin using partially purified glycyl endopeptidase from papaya latex. Autolysed swollen skin was more hydrolysed by partially purified glycyl endopeptidase as indicated by

higher α-amino group content, compared with autolysed non-swollen skin. Six proteases, including Alcalase, bromelain, Flavourzyme, Neutrase, papain and trypsin, were used for hydrolysis of gelatin from Nile tilapia skin. The highest DH was observed in hydrolysate prepared with papain (60%), followed by those prepared using Flavourzyme (57%), bromelain (51%), trypsin (45%), Neutrase (16%) and Alcalase (16%), respectively (Choonpicharn *et al.*, 2015). Gelatin hydrolysate from unicorn leatherjacket skin prepared with protease from *Bacillus amyloliquefaciens* H11 yielded a higher α-amino acid content than did Alcalase. Gelatin hydrolysates from sole and squid skin were prepared using Alcalase with DH of ~35 and ~50%, respectively (Giménez *et al.*, 2009).

1.2.6.1 Radical scavenging activities

The beneficial effects of gelatin hydrolysate are well known in scavenging free radicals and reactive oxygen species or in preventing oxidative damage by interrupting the radical chain reaction of lipid peroxidation (Rajapakse *et al.*, 2005). Gelatin hydrolysates from autolysed non-swollen and swollen unicorn leatherjacket skin prepared using partially purified glycyl endopeptidase from papaya latex showed ABTS radical scavenging activity (Karnjanapratum and Benjakul, 2015a). Thornback ray gelatin hydrolysates prepared using proteases from *Bacillus subtilis* A26 exhibited the highest DPPH radical scavenging activity, compared with Alcalase and Neutrace (Lassoued *et al.*, 2015). Gelatin from Nile tilapia skin hydrolysed using Flavourzyme showed high ABTS radical scavenging activity (Choonpicharn *et al.*, 2015). Alemán *et al.* (2011a) prepared gelatin hydrolysates from squid and tuna skin with different enzymes (collagenase, trypsin and pepsin). Alcalase and pepsin gave the hydrolysates with the highest and lowest ABTS radical scavenging activity, respectively. Hydrolysate from Korean rockfish skin gelatin prepared using Alcalase and Flavourzyme exhibited free radical scavenging activities towards DPPH, hydroxyl, superoxide and alkyl radicals (Kim *et al.*, 2011). Pepsin hydrolysate from Japanese flounder skin gelatin had ability to scavenge hydroxyl and DPPH radicals (Himaya *et al.*, 2012b). The IC_{50} values of gelatin hydrolysates from blue shark skin to scavenge DPPH and hydroxyl radicals were 13.30 and 4.30 mg/mL, respectively (Weng *et al.*, 2014). The peptide isolated from Amur sturgeon skin gelatin showed scavenging

activity against DPPH, ABTS and hydroxyl radicals (Nikoo *et al.*, 2014). The oxygen radical absorbance capacity (ORAC) of gelatin hydrolysate from blacktip shark skin with different DHs ranged from 268.16 to 709.42 μmol TE/g sample (Kittiphattanabawon *et al.*, 2012).

Differences in free radical scavenging activity between gelatin hydrolysates from bigeye snapper skin prepared using different enzymes possibly resulted from existing differences in the enzyme specificity toward protein substrates, in which a wide variety of peptides with different modes of actions for inhibiting lipid oxidation were generated during hydrolysis. Changes in size, amount, the exposure of the terminal amino groups of the products obtained and the composition of free amino acids or small peptides affect the antioxidative activity (Thiansilakul *et al.*, 2007b; Wu *et al.*, 2003). Low molecular weight peptides generally showed higher antioxidative activity (Qian *et al.*, 2008).

1.2.6.2 Fe^{2+}Chelating activity

Transition metals, such as Fe, Cu, and Co, have been known to react very quickly with peroxides by acting as one-electron donors to form alkoxyl radicals. Therefore, chelation of transition metal ions would retard the oxidation process (Gordon, 2001). In comparison with other ions, ferrous ion is a key active species responsible for ROS formation in cells, leading to increased levels of lipid peroxidation (Huang *et al.*, 2002). The formation of a violet complex by ferrozine and Fe^{2+} is interrupted in the presence of a chelating agent (Decker and Welch, 1990). Peptides are well known as metal chelators and this is one of the antioxidant mechanisms for many active peptides. The activity is affected by size and amino acid sequence (Peng *et al.*, 2009). Karnjanapratum and Benjakul (2014) prepared gelatin hydrolysate from unicorn leatherjacket skin by autolysis-assisted process mediated by indigenous protease in combination with thermal hydrolysis and hydrolysis using papain. Hydrolysates possessed ferrous chelating activity in the range of 4.83–12.35 μmol EDTA equivalents/g solid. Bromelain hydrolysate from Nile tilapia skin gelatin showed high ferrous ion chelating activity (86.90%) (Choonpicharn *et al.*, 2015). Giménez *et al.* (2009) evaluated the ferrous ion chelating activity of gelatin hydrolysates obtained from

sole and squid skin gelatins by Alcalase. Both hydrolysates showed a high chelating ability, with values above 80% at 0.2 mg/ml assay concentration. Carboxyl and amino groups in the side chains of the acidic (Glx, Asx) and basic (Lys, His, Arg) amino acids are thought to play an important role in chelating metal ions (Saiga *et al.*, 2003). These amino acids are abundant in both gelatin hydrolysates, especially in the squid (~225 vs. 196 residues/1000 residues). His residues, which are present at low concentrations in both hydrolysates, have been also reported to contribute to the chelating effect of protein hydrolysates (Chen *et al.*, 1998).

1.2.6.3 Ferric reducing antioxidant power

The ferric reducing antioxidant power (FRAP) assay is based on the reduction of a ferroin analog, TPTZ-Fe (III), to the TPTZ-Fe (II) complex by antioxidants in acidic medium (Antolovich *et al.*, 2002). In this assay, the yellow colour of the test solution changes to various shades of green and blue, depending on the reducing power of each compound (Ferreira *et al.*, 2007). Since the reducing power of bioactive compounds is associated with their antioxidative activity, the reducing power assay provides a reliable method to study the antioxidative activity of various compounds (Antolovich *et al.*, 2002). Gelatin from Nile tilapia skin hydrolysed using Alcalase showed the highest FRAP, compared with those prepared using bromelain, Flavourzyme, trypsin, papain and Neutrase (Choonpicharn *et al.*, 2015). Gelatin hydrolysate from seabass skin prepared using ammonium sulphate precipitated fraction (ASPF) from Pacific white shrimp hepatopancreas and Alcalase had the increases in FRAP when DH increased ($P < 0.05$) (Senphan and Benjakul, 2014). Gelatin hydrolysates from unicorn leatherjacket skin prepared using extracellular protease from *Bacillus amyloliquefaciens* H11 exhibited higher FRAP than that prepared using Alcalase ($P < 0.05$). FRAP of gelatin hydrolysates from the skin of bigeye snapper prepared using different proteinases, pyloric caeca extract (PCE), Neutrase or Alcalase, increased as DH increased (Phanturat *et al.*, 2010). Giménez *et al.* (2009) reported that gelatin hydrolysates from skins of sole and squid hydrolysed using Alcalase exhibited FRAP. A significant higher FRAP was found in gelatin hydrolysate from squid skin in comparison with sole skin ($P < 0.05$).

1.2.6.4 Prevention of lipid peroxidation

In vitro lipid peroxidation inhibition activity of peptides was determined by assessing their ability to inhibit oxidation of linoleic acid in an emulsified model system, in which carbon-centred, peroxyl radicals and hydroperoxides are involved in the oxidation process (Burton and Ingold, 1986). Lipid peroxidation inhibition assay carried out using linoleic acid model system helped to identify the antioxidative activity, by retarding the formation of primary oxidation products in the oxidative sequence (Chen *et al.*, 1995).

The hydrolysate from seabass skin gelatin prepared using ammonium sulphate precipitated fraction (ASPF) from Pacific white shrimp hepatopancreas and Alcalase could inhibit lipid oxidation in a lecithin liposome system in a dose dependent manner (Senphan and Benjakul, 2014). Lipid peroxidation were inhibited more than 50% by gelatin hydrolysates from Nile tilapia skin prepared using Flavourzyme, trypsin and bromelain (Choonpicharn *et al.*, 2015). Gelatin hydrolysate from hoki fish skin hydrolysed by trypsin, α-chymotrypsin, and pepsin exhibited antioxidative activity against lipid peroxidation in a linoleic acid model system (Mendis *et al.*, 2005b). Trypsin-derived hydrolysate exhibited a significantly higher peroxidation inhibition, compared to that of natural antioxidant, α-tocopherol. The other two hydrolysates had a lower inhibition of lipid peroxidation, compared to tryptic hydrolysate. Tryptic gelatin hydrolysate was rich in Gly (37.5%), Pro (9.3%), Glu (8.8%), Ala (8.6%), Arg (7.1%), and Hyp (5.98%) (Mendis *et al.*, 2005b). Gelatin hydrolysates from bigeye snapper skin prepared using Alcalase + Neutrase or Alcalase + PCE also showed antioxidative activity in the linoleic oxidation system (Phanturat *et al.*, 2010). Additionally, peptides from Alaska pollack skin (Kim *et al.*, 2001), jumbo squid skin gelatin (Mendis *et al.*, 2005b) and bullfrog skin (Qian *et al.*, 2008) also showed inhibitory activity in the linoleic acid model system.

Antioxidative activity of gelatin hydrolysates was possibly attributed to the ability of peptides to interfere with the propagation cycle of lipid peroxidation, thereby slowing radical-mediated linoleic acid oxidation (Thiansilakul *et al.*, 2007b). Antioxidative peptides with 5-16 amino acid residues could inhibit autooxidation of

linoleic acid (Chen *et al.*, 1995). In the free radical-mediated lipid peroxidation system, antioxidative activity of peptides or proteins is dependent on molecular size and properties such as hydrophobicity and electron transferring ability of the amino acid residues in the sequence (Qian *et al.*, 2008). Many antioxidative peptides have hydrophobic amino acid residues such as Val, or Leu at the N-terminus of the peptides, which are more likely able to interact with the fatty acids (Chen *et al.*, 1995; Kim *et al.*, 2001).

1.2.7 Antioxidative peptides from gelatin hydrolysates

The levels and composition of free amino acids and peptides in hydrolysate have been found to contribute to antioxidative activities (Thiansilakul *et al.*, 2007a). Phenolic hydroxyl groups present in aromatic amino acids contribute substantially for scavenging of radicals, mostly via electron donation (Suetsuna *et al.*, 2000). Free radical scavenging has been reported to be the main antioxidative mechanism of these peptides due to the amino acids, especially His, Pro, Ala, Val and Leu (Kim *et al.*, 2001; Mendis *et al.*, 2005b). Peptides from gelatin hydrolysates of blue shark skin with MW ranging from 2,000 to 150 Da possessed strong antioxidative activity. The peptides mainly composed of four peptides of Glu-Gly-Arg, Gly-Pro-Arg, Gly-Tyr and Gly-Phe, as well as four amino acids of Arg, Leu, Tyr and Phe (Weng *et al.*, 2014). Potent scavenging activities of squid gelatin peptides on hydroxyl and carbon-centered radicals could be due to non-aromatic amino acids such as Pro, Ala, Val and Leu (Mendis *et al.*, 2005b). The peptide isolated from Nile tilapia scale gelatin was identified as Asp-Pro-Ala-Leu-Ala-Thr-Glu-Pro-Asp-ProMet-Pro-Phe (1382.57 Da). This peptide could scavenge hydroxyl, DPPH and superoxide radicals at the IC_{50} values of 7.56, 8.82 and 17.83 μM, respectively (Ngo *et al.*, 2010). Potent antioxidative peptides from Pacific cod skin gelatin hydrolysate contained amino acid sequences of Thr-Cys-Ser-Pro (388 Da) and Thr-Gly-Gly-Gly-Asn-Val (485.5 Da) (Ngo *et al.*, 2011). The dominant peptide isolated from Amur sturgeon skin gelatin hydrolysate was found to be Pro-Ala-Gly-Tyr with MW of 405.99 Da (Nikoo *et al.*, 2014). Mendis *et al.* (2005a) isolated two antioxidative peptides derived from jumbo squid gelatin hydrolysate, Phe–Asp–Ser–Gly–Pro–Ala–Gly–Val–Leu (880.18 Da) and Asn–Gly–Pro–Leu–Gln–Ala–Gly–Gln–Pro–Gly–Glu–Arg (1241.59 Da).

Antioxidative activity of protein hydrolysates is highly dependent on their sequence and the amino acid composition (Chen *et al.*, 1998). Many antioxidative peptides include hydrophobic amino acid residues Val or Leu at the N-terminus end and Pro, His or Tyr in the sequences (Chen *et al.*, 1996). In case of gelatin peptides, the abundance of hydrophobic amino acids in their sequences seems to be responsible for the higher antioxidative effects in comparison with other antioxidative peptides due to an increase of their solubility in lipids (Mendis *et al.*, 2005b).

Relationship between molecular weight (MW) distribution and antioxidative activity of protein hydrolysate was studied (Je *et al.*, 2005). Antioxidative peptides varied depending upon gelatin sources and enzyme used (Table 4). Peptide from unicorn leatherjacket skin gelatin hydrolysate having MW of 1,170 Da showed high ABTS radical scavenging activity (Karnjanapratum and Benjakul, 2015a). Gelatin hydrolysate from Pacific cod skin gelatin with MW of 1301 Da exhibited the highest antioxidative activity via hydroxyl radical scavenging activity (Himaya *et al.*, 2012a). The peptides with molecular mass of 797 Da in gelatin hydrolysates from hoki fish skin acted as strong superoxide, carbon-centred and DPPH radical scavenging activities (Mendis *et al.*, 2005b). Kim *et al.* (2001) reported that gelatin from Alaska pollack skin hydrolysed with Alcalase and Pronase E was composed of peptides ranging from 1.5 to 4.5 kDa and showed high antioxidative activity. Two different peptides contained 13 and 16 amino acid residues, respectively, and both peptides contained a Gly residue at the C-terminus and the repeating motif Gly-Pro-Hyp.

1.2.8 Bioactivities of protein hydrolysates in cell model systems

1.2.8.1 Cellular antioxidant

The antioxidant activity of foods and dietary supplements has been extensively studied with the development of many new antioxidant activity assays in recent years. However, these chemical-based assays do not reflect the cellular physiological conditions and do not consider the bioavailability and metabolism issues. Biological assays are much more complex than the simple chemical mixtures employed and antioxidant compounds may act through multiple mechanisms (Liu, 2004).

Table 4. Antioxidative peptides from gelatin hydrolysate

Sources	Enzymes	Active peptide sequences	MW (Da)	References
Hoki (*Johnius belengerii*) skin	Trypsin	His-Gly-Pro-Leu-Gly-Pro-Leu	797	Mendis *et al.* (2005b)
Nile tilapia (*Oreochromis niloticus*) scale	Alcalase	Asp-Pro-Ala-Leu-Ala-Thr-Glu-Pro-Asp-Pro-Met-Pro-Phe	1382.5	Ngo *et al.* (2010)
Pacific cod (*Gadus macrocephalus*) skin	Papain	Thr-Cys-Ser-Pro Thr-Gly-Gly-Gly-Asn-Val	388 485.5	Ngo *et al.* (2011)
Pacific cod (*Gadus macrocephalus*) skin	Pepsin, trypsin and α-chymotrypsin	Leu-Leu-Met-Leu-Asp-Asn-Asp-Leu-Pro-Pro	1301	Himaya *et al.* (2012a)
Japanese flounder (*Palatichtys olivaceus*) skin	Pepsin	Gly-Gly-Phe-Asp-Met-Gly	582	Himaya *et al.* (2012b)
Nile tilapia (*Oreochromis niloticus*) skin	Properase E and Multifect	Glu-Gly-Leu Tyr-Gly-Asp-Glu-Tyr	317.3 645.2	Zhang *et al.* (2012)
Nile tilapia (*Oreochromis niloticus*) skin	Properase E	Leu-Ser-Gly-Tyr-Gly-Pro	592.2	Sun *et al.* (2013)
Amur sturgeon (*Acipenser schrenckii*) skin	Alcalase	Pro-Ala-Gly-Tyr	405.9	Nikoo *et al.* (2014)

The best measures are from animal models and human studies. However, these are expensive, time-consuming, not suitable for initial antioxidant screening of foods and dietary supplements (Liu and Finley, 2005). Cell culture models provide an approach which is cost-effective, relatively fast, and address some issues of uptake, distribution, and metabolism. Therefore, there is a need for cell culture models to access the bioactivity of foods and dietary supplements (Liu and Finley, 2005).

Many studies have demonstrated the ability of hydrolysates from marine sources to protect against oxidant-induced DNA damage. The octapeptide from salmon byproduct protein hydrolysate showed protection ability against hydroxyl radical-induced DNA damage and H_2O_2-induced hepatic damage in Chang liver cells (Ahn *et al.*, 2014). Protein hydrolysates from Nile tilapia protein isolate prepared using Alcalase and Alcalase together with papain exhibited a good antioxidant potential in cellular based assays. Hydrolysates and their fractions exerted a protective ability against H_2O_2 and peroxyl radical induced oxidative damage on HepG2 cells and DNA via scavenging free radical and H_2O_2 (Yarnpakdee *et al.*, 2015). Peptide from tilapia gelatin exhibited protective effect against free radical-induced cellular and DNA damage in murine microglial cells (BV-2) (Vo *et al.*, 2011). Wiriyaphan *et al.* (2012) reported that protein hydrolysates from threadfin bream surimi byproducts protected HepG2 cells against *tert*-butyl hydroperoxide-induced oxidative damage. Peptide isolated from Nile tilapia scale gelatin could prevent oxidative DNA damage caused by hydroxyl radical in RAW 264.7 cells (Ngo *et al.*, 2010). Ryu *et al.* (2011) reported that peptides derived from seaweed pipefish suppressed H_2O_2-induced DNA damage in human dermal fibroblasts. Peptides from skate skin gelatin could be an effective intracellular radical scavenger by increasing the expression levels of antioxidant enzymes in endothelial EA.hy926 cells (Ngo *et al.*, 2014). Protein hydrolysate from threadfin bream surimi byproduct (frame, bone and skin) displayed cytoprotective effects against *tert*-butyl hydroperoxide (TBHP)-induced cytotoxicity of Caco-2 cells (Wiriyaphan *et al.*, 2015). TBHP-induced DNA damage may be triggered by iron metabolism, leading to free radical formation and further radical-mediated processes. Compounds with a metal ion chelating ability showed a protective mechanism against TBHP-induced DNA strand breaks in Caco-2 cells. Thus, high metal chelating activity of fish protein hydrolysate might partly

contribute to its high cytoprotection (Wiriyaphan *et al.*, 2015). The protective effect of tuna liver hydrolysates was related to their scavenging of H_2O_2, hydroxyl radical and chelating activity toward Fe^{2+}. Such activities lead to the inhibition of the Fenton reaction, and therefore, protected the DNA from oxidant-induced strand breaks (Je *et al.*, 2009).

1.2.8.2 Immunomodulatory activity

In order to develop a better understanding of immune-mediated disorders, it is necessary to measure cytokine production (Katial *et al.*, 1998). Cytokines are proteins which play an important role in the human immune response. The functions of these proteins are diverse and include roles in immunity, inflammatory response, cancer, autoimmunity and allergy (Borish and Rosenwasser, 1996). Cytokines are produced by a broad range of cells, including immune cells, e.g. macrophages, B lymphocytes, T lymphocytes and mast cells, as well as endothelial cells, fibroblasts, and various stromal cells. Lipopolysaccharide (LPS) is a strong immune activator that induces inflammation and antibody production during severe infection and septic shock. Macrophages play an important role in a host's defense against bacterial infection, and are major cellular targets for LPS action. The inflammatory response has been extensively studied in LPS-stimulated RAW 264.7 macrophage cells, which are very sensitive to LPS stimulation and respond by activation of the proinflammatory transcription factors. Those result in tumor necrosis factor (TNF), interleukin (IL)-1β, IL-6, IL-8 and nitric oxide production (Wang and Mazza, 2002). These cytokines activate a beneficial inflammatory response that promotes local coagulation to confine tissue damage. However, the excessive production of these proinflammatory cytokines can be even more dangerous than the original stimulus, overcoming the normal regulation of the immune response and producing pathological inflammatory disorders (Ulloa and Tracey, 2005).

Enzymatic hydrolysates of fish proteins have shown immunomodulatory activity. Sweetfish-derived protein hydrolysates prepared by trypsin and α-chymotrypsin exhibited immunomodulatory activity on LPS-induced RAW264.7 macrophage cells by inhibition the production of cytokine IL-6 (Sung *et*

al., 2012). A phosvitin-derived peptide from zebrafish markedly suppressed the expression of proinflammatory cytokine genes, including IL-1β, IL-6, TNF-α and IFN-γ (Ding *et al.*, 2012). Ahn *et al.* (2012) reported that salmon byproduct protein hydrolysates showed immunomodulatory activity by inhibiting nitric oxide (NO) production and proinflammatory cytokines, including TNF-α, IL-6 and IL-1β in RAW264.7 macrophage cells. Protein hydrolysate from blue mussel prepared using pepsin exhibited NO inhibitory activity in LPS-stimulated RAW264.7 macrophage cells (Park *et al.*, 2014). Peptide fraction from ark clam (*Arca subcrenata*) could suppress the production of NO in LPS-induced RAW264.7 macrophage cells as well as the secretion of proinflammatory cytokines IL-6 and TNF-α in human cervical cancer HeLa cells (Wu *et al.*, 2014).

1.2.8.3 Antiproliferative effects against cancer cells

Cancer is one of the leading causes of death in the world (Kaufmann and Earnshaw, 2000). Chemopreventive compounds offer a promising anticancer approach by reducing the morbidity and mortality of cancer through delaying the process of carcinogenesis (Sheih *et al.*, 2009). The mechanisms which suppress tumorgenesis often involve inhibition of tumor cell mediated protease activity, attenuation of tumor angiogenesis, promotion of cell cycle arrest, induction of apoptosis and immunostimulation, etc (Lee *et al.*, 2003; Sheih *et al.*, 2009; Tzianabos, 2000; Yang *et al.*, 2001).

Fish derived peptides have been shown to have potent *in vitro* antitumor activity against several human cancer cell lines. A pepsin hydrolysate from half-fin anchovy exhibited antiproliferative activity in human prostate (DU-145), human lung (1299) and human esophagus cancer cells (109) (Song *et al.*, 2011). Loach protein hydrolysates prepared by papain digestion showed antiproliferative activity for Caco-2 and HepG2 cancer cell lines at doses of 5–40 mg/ml in a dose-dependent manner (You *et al.*, 2011). Peptide fractions of Japanese threadfin bream and tropical two-wing flyingfish hydrolysates prepared using trypsin exerted significant antiproliferative effect on HepG2 with IC_{50} values of 48.5 mg/ml and 21.6 mg/ml, respectively (Naqash and Nazeer, 2010). Picot *et al.* (2006) reported that hydrolysates obtained from three

blue whiting, three cod, three plaice and one salmon showed significant inhibition on two human breast cancer cell lines, MCF-7/6 and MDA-MB-231. Peptides prepared from enzymatic hydrolysates of tuna dark muscle showed antiproliferative activity on human breast cancer cell line MCF-7 (Hsu *et al.*, 2011). Gelatin hydrolysate from giant squid hydrolysed with Esperase showed strong cytotoxic effect on cancer cells, MCF-7 (human breast carcinoma) and U87 (glioma) cells (Alemán *et al.*, 2011b). Lee *et al.* (2004) reported that peptide having antiproliferative activity was found to be a 440.9 Da hydrophobic peptide and was able to induce apoptosis in human U937 lymphoma cells through the increase of caspase-3 and caspase-8 activity. It has been reported that low molecular weight peptides have greater molecular mobility and diffusivity than the high molecular weight peptides, which appears to improve interactions with cancer cell components and enhances anticancer activity (Jumeri and Kim, 2011). Moreover, a study on the mechanism of action revealed that modulation of hydrophobicity of peptides plays a crucial role against cancer cells (Huang *et al.*, 2011b).

1.2.9 Applications of protein hydrolysates

1.2.9.1 Food applications

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 food 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. Gelatin hydrolysate from unicorn leatherjacket skin exhibited cryoprotective effect (Karnjanapratum and Benjakul, 2015b). Nikoo *et al.* (2014) 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 ºC. 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 cycles of freeze-thaw. The water retention properties were improved in samples containing 8% of protein hydrolysate from Pacific hake prepared using Alcalase and Flavourzyme. 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 ºC (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.

1.2.9.2 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 (Kurbanoglu and Algur, 2004). Klompong *et al.* (2009) produced protein hydrolysates from yellow stripe trevally and used as culture media. Hydrolysate prepared using Flavourzyme (HF25) with 25% DH showed the higher bacterial productivity ratio than did commercial Bacto Peptone. When HF25 and Bacto Peptone were used as microbial media to determine microbial load of environmental and food samples and pathogenic bacteria, HF25 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 44 bacteria, *Staphylococcus aureus* and *Escherichia coli*, HF with 25% DH (HF25) yielded the highest cell density and specific growth rate (μmax) and the lowest generation time (td) (Klompong *et al.*, 2009). 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 (Dufosse *et al.*, 2001). Ghorbel *et al.* (2005) reported that defatted *Sardinella* meat protein hydrolysate was 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. Additionally, hydrolysates generated from yellowfin tuna head waste showed higher effectiveness in promoting the growth of lactic acid bacteria better than the commercial MRS media (Safari *et al.*, 2012).

1.2.9.3 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 fillet protein hydrolysates decreased the serum and liver cholesterol contents of experimental rats through the 45 enhancement of faecal acidic and neutral excretions. In addition, marine-derived peptides from *Crassostrea gigas* and *Ruditapes philippinarum*have showed antiinflammatory 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 and hoki 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 and Mendis (2006) reported that peptides from fish protein hydrolysate are capable

of accelerating calcium absorption. Hydrolysed gelatin and collagen products have recently 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). 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

1.4.1 To evaluate the chemical changes in seabass skin during iced storage and to study the effects of fish freshness on lipid oxidation and fishy odour development in gelatin from skin.

1.4.2 To study the effect of pretreatments, defatting, drying methods and antioxidant incorporation on lipid oxidation, fishy odour and characteristics of gelatin from seabass skin.

1.4.3 To produce gelatin hydrolysate from seabass skin and to evaluate its bioactivities both in chemical and cellular systems.

1.4.4 To study the impact of fortification of gelatin hydrolysate from seabass skin on characteristics and bioactivities of apple juice.

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CHAPTER 2

FATTY ACID COMPOSITION, LIPID OXIDATION AND FISHY ODOUR DEVELOPMENT IN SEABASS (*LATES CALCARIFER*) SKIN DURING ICED STORAGE

2.1 Abstract

Changes in fatty acid profile, lipid hydrolysis and oxidation, development of fishy odour and volatile compounds in seabass (*Lates calcarifer*) skin during 18 days of iced storage were investigated. Peroxide value (PV) increased up to Day 6 and subsequently decreased up to 18 days ($P < 0.05$). The continuous increases in thiobarbituric acid reactive substances (TBARS) values, free fatty acid (FFA) content and lipoxygenase (LOX) activity were noticeable with increasing storage time ($P < 0.05$). Formation of FFA and hydroperoxide was confirmed by the changes in amplitude of peak at 3600–3200 cm^{-1} and 1711 cm^{-1} in Fourier transform IR spectra, respectively. With increasing storage time, the increase in fishy odour intensity was observed along with the formation of volatiles. Hexanal and nonanal constituted as the dominant volatile aldehydes in skin stored in ice for an extended time. Therefore, the delay of skin processing must be avoided to prevent the formation of undesirable fishy odour in skin and its products.

2.2 Introduction

Iced storage is an important preservation method to maintain the quality of fish during handling and storage. Icing has been used widely to reduce undesirable biochemical and chemical reactions and to retard the growth of spoilage microorganisms (Sikorski and Pan, 1994). However, lipid deterioration still easily takes place and limits the shelf-life of fish during storage. Both lipolysis and lipid oxidation in fish are associated with quality loss (Pacheco-Aguilar *et al.*, 2000). Hydrolysis, induced by lipases and phospholipases, produces free fatty acids that undergo further oxidation (Toyomizu *et al.*, 1981). Fish lipids are relatively more susceptible to oxidation due to high degree of unsaturation and low content of endogenous antioxidants, compared with other food lipids (Kolakowska *et al.*, 2002). Lipid

oxidation is associated with the development of undesirable odour, especially fishy odour, in fish stored for an extended time (Maqsood and Benjakul, 2011; Pacheco-Aguilar *et al.*, 2000). Development of fishy odour in seabass and red tilapia muscle was primarily associated with lipid oxidation (Thiansilakul *et al.*, 2010). In addition, lipid oxidation induced by lipoxygenase was responsible for a strong fishy odour in silver carp mince (Fu *et al.*, 2009). Moreover, fishy odour in protein hydrolysate caused by lipid oxidation was reported by Yarnpakdee, Benjakul, Nalinanon and Kristinsson (Yarnpakdee *et al.*, 2012).

Seabass (*Lates calcarifer*) is one of economically important species of Thailand and other countries in Southeast Asia owing to its white flesh and delicacy. During processing or dressing, skin is generated and considered as a byproduct. To fully exploit those skins, the conversion to value-added products such as collagen, gelatin as well as hydrolysate with bioactivities has been employed (Anand *et al.*, 2013; Nagai and Suzuki, 2000; Sinthusamran *et al.*, 2013). Hydrolysate from fish skin has gained increasing interest as the supplement for skin and health care products, e.g. beauty drink, etc. Owing to strong fishy odour/flavour of those products, the use of skin is limited. After deskinning, the skins are used as the starting material for manufacturing of collagen or its derived products. Before being further processed, those skins are generally stored in ice and they more likely undergo deterioration during the storage. Due to the greater surface area for chemical or microbial reactions, as well as the tissue damage during dressing, quality loss could be more pronounced than those intacted with whole fish. This might be associated with the rapid development of fishy odour in the stored skin.

However, no information regarding the alteration of lipids via chemical and enzymatic reactions and its development of fishy odour in seabass skin stored in ice has been reported. This study aimed to evaluate the changes in fatty acid composition, lipid hydrolysis and oxidation as well as fishy odour development and volatile compounds in seabass skin during iced storage of 18 days.

2.3 Materials and methods

2.3.1 Chemicals

Trichloroacetic acid, anhydrous sodium sulphate and ferrous chloride were obtained from Merck (Darmstadt, Germany). 1,1,3,3-tetramethoxypropane and linoleic acid were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Methanol, acetone, chloroform and ammonium thiocyanate were obtained from Lab-Scan (Bangkok, Thailand). All chemicals were of analytical grade. Disodium hydrogen phosphate, sodium dihydrogen phosphate, 2-thiobarbituric acid and cumenehydroperoxide were procured from Fluka (Buchs, Switzerland).

2.3.2 Fish collection and preparation

Fresh seabass (*Latescalcarifer*) having the average weight of 0.8-1.0 kg from a farm in KoYor, Songkhla were deskinned after capture. The skins were placed in a polystyrene box containing ice using a skin/ice ratio 1:2 (w/w). The samples were transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h.

Upon arrival, seabass skins were washed, placed in polyethylene bag and kept in ice with a skin/ice ratio of 1:2 (w/w). The packed samples were placed and distributed uniformly between the layers of ice in the insulated boxes, which were subsequently left at room temperature (28–30 °C). To maintain the skin/ice ratio, the molten ice was removed and replaced with new ice every 2 days. The temperature of skin was maintained at 0–2 °C throughout the storage of 18 days. Fish skins were randomly taken every 3 days, pooled and used as the composite sample. Fish skins were descaled manually. The skins were then washed with cold tap water, drained and cut into small pieces (0.5 × 0.5 cm^2) using the scissor. The skins were pulverised by blending in the presence of liquid nitrogen. The skin powder was subjected to analyses.

2.2.3 Changes in fatty acid compositions of lipids from seabass skin during iced storage

2.3.3.1 Extraction of lipid

Lipid of skin was extracted as per the method of Bligh and Dyer (Bligh and Dyer, 1959). Skin powder (25 g) was homogenised with 200 ml of a chloroform:methanol:distilled water mixture (50:100:50, v/v/v) at the speed of 9,500 rpm for 2 min at 4 °C using an IKA Labortechnik homogeniser (Selangor, Malaysia). The homogenate was added with 50 ml of chloroform and homogenised at 9,500 rpm for 1 min. Subsequently, 25 ml of distilled water were added and then homogenised again for 30 s. The homogenate was centrifuged at 3,000 ×*g* at 4 °C for 15 min using a RC-5B plus centrifuge (Sorvall, Norwalk, CT, USA). Aqueous phase was transferred into a separating flask. After being separated, the chloroform layer was drained off into a 125 ml Erlenmeyer flask containing about 2–5 g of anhydrous sodium sulphate, shaken very well, and decanted into a round-bottom flask through a Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, England). The solvent was evaporated at 25 °C using an EYELA rotary evaporator N-100 (Tokyo Rikakikai, Co., Ltd., Tokyo, Japan), and the residual solvent was removed by flushing with nitrogen.

2.3.3.2 Determination of fatty acid profile

Fatty acid profile was determined as fatty acid methyl esters (FAMEs). FAMEs were prepared according to the method of AOAC (AOAC, 2000). The prepared methyl ester was injected to the gas chromatography (Shimadzu, Kyoto, Japan) equipped with the flame ionisation detector (FID) at a split ratio of 1:20. A fused silica capillary column (30 m × 0.25 mm), coated with bonded polyglycol liquid phase, was used. The analytical conditions were: injection port temperature of 250 °C and detector temperature of 270 °C. The oven was programmed from 170 to 225 °C at a rate of 1 °C/min (no initial or final hold). Retention times of FAME standards were used to identify chromatographic peaks of the samples. Fatty acid content was calculated, based on the peak area ratio and expressed as g fatty acid/100 g lipid.

2.3.4 Oxidation and hydrolysis of lipids from seabass skin during iced storage

2.3.4.1 Measurement of peroxide value

Peroxide value (PV) was determined according to the method of Richards and Hultin (2002) with slight modifications. Skin powder (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 a homogeniser. Homogenate was then filtered using Whatman No. 1 filter paper. To 7 ml of the filtrate, 2 ml of 0.5% NaCl were added. 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 to separate the sample into two phases. Twenty-five µl of 30% (w/v) ammonium thiocyanate and 25 µl of 20 mM iron (II) chloride were added to 3 ml of lower phase. The reaction mixture was allowed to stand for 20 min at room temperature and the absorbance at 500 nm was read. The blank was prepared in the same manner, except the distilled water was used instead of ferrous chloride. A standard curve was prepared using cumenehydroperoxide at a concentration range of 0.5–2 ppm. PV was expressed as mg cumenehydroperoxide equivalents/kg skin after blank subtraction.

2.3.4.2 Measurement of thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) were determined as described by Buege and Aust (1978). Skin powder (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 mM HCl. The mixture was heated in a boiling water bath (95–100 °C) for 10 min. Thereafter, the mixture was 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 the concentrations ranging from 0 to 6 ppm. TBARS value was calculated and expressed as mg malonaldehyde equivalents/kg skin.

2.3.4.3 Fourier transform infrared spectra analysis

Fourier transform infrared **(**FTIR) analysis of lipid was performed in a horizontal ATR Trough plate crystal cell (45° ZnSe; 80 mm long, 10 mm wide and 4

mm thick) (PIKE Technology, Inc., Madison, WI, USA) equipped with a Bruker Model Vector 33 FTIR spectrometer (Bruker Co., Ettlingen, Germany). Prior to analysis, the crystal cell was cleaned with acetone, wiped dry with soft tissue and the background scan was run. For spectra analysis, lipid sample (200 µl) was applied directly onto the crystal cell and the cell was clamped into the mount of the FTIR spectrometer. The spectra, in the range of 4,000−400 cm^{-1} (mid-IR region) with automatic signal gain, were collected in 16 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean, empty cell at 25 °C. Analysis of spectral data was carried out using the OPUS 3.0 data collection software programme (Bruker Co., Ettlingen, Germany).

2.3.4.4 Measurement of free fatty acid

Free fatty acid (FFA) content was determined according to the method of Lowry and Tinsley (1976). Lipid sample (0.1 g) was mixed with 5 ml of isooctane and swirled vigorously to dissolve the sample. The mixture was then added 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-Genie2 mixer (Bohemia, NY, USA) and allowed to stand for 20 s. The absorbance at 715 nm of the upper layer was read. A standard curve was prepared using palmitic acid in isooctane at concentrations ranging from 0 to 10 mM. FFA content was expressed as g FFA/100 g lipid.

2.3.5 Changes in lipoxygenase (LOX) in the skin of seabass during iced storage

2.3.5.1 Extraction of LOX

Skin powder was suspended in 50 mM phosphate buffer, pH 7.0, containing 1 mM glutathione and 0.04% Tween-20, at a ratio of 1:9 (w/v). The mixture was homogenised at 11,000 rpm for 3 min. The suspension was centrifuged for 15 min at 4 °C at 15,000 ×*g* to remove the tissue debris. The supernatant was collected and referred to as “LOX extract”.

2.3.5.2 Assay for LOX

LOX activity was assayed spectrophotometrically by monitoring the formation of conjugated dienes at the absorbance of 234 nm as per the method of Hamberg and Samuelsson (1967) and Liu and Pan (2004) with some modifications. To 0.1 ml of LOX extract, 0.9 ml of 50 mM phosphate buffer, pH 7.0, containing 1 mM glutathione and 0.04% Tween-20 was added. Subsequently, 200 µl of 0.2 mM linoleic acid was added to the reaction mixture to initiate the reaction. After incubation at 25 °C for 3 min, the absorbance at 234 nm of the mixture was recorded at 0 and 3 min of reaction. One unit was defined as LOX causing an increase in absorbance at 234 nm of 0.001/min under the specified condition.

2.3.6 Development of fishy odour and volatile compounds in seabass skin during iced storage

2.3.6.1 Measurement of fishy odour

Seabass skins stored in ice at day 0, 3, 6, 9, 12, 15 and 18 were used for evaluation of fishy odour. Fishy odour was evaluated by 8 trained panellists with the ages of 25–32. Prior to the evaluation, the panellists were trained three times a week. Panellists were trained with standards for two sessions using a 15-cm line scale anchored from none (score = 0) to extremely strong fishy odour (score = 4). Before testing, 10 g of fish skins were placed in a sealable plastic cup. All samples were kept on ice until evaluation. The sealable plastic cups were placed at room temperature for 10 min before evaluation. The panellists were asked to open the sealable cup and sniff the headspace above the samples for determining the intensities of fishy odour.

2.3.6.2 Measurement of volatile compounds

Seabass skins stored in ice at day 0, 6, 12 and 18 were determined for volatile compounds using solid-phase microextraction gas chromatography mass spectrometry (SPME-GC-MS) following the method of Iglesias and Medina (2008) with a slight modification.

2.3.6.2.1 Extraction of volatile compounds

To extract volatile compounds, 3 g of sample was homogenised at a speed of 13,500 rpm for 2 min with 8 ml of deionised water. The mixture was centrifuged at 2,000 ×*g* for 10 min at 4 ºC. The supernatant (6 ml) was heated at 60 °C with equilibrium time of 10 h in a 20-ml headspace vial. The SPME fibre (50/30 lm DVB/Carboxen™/ PDMS StableFlex™) (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 extract and the volatile compounds were allowed to adsorb 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.

2.3.6.2.2 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 °C for 3 min, followed by an increase of 3 °C/min to 70 °C, then an increase of 10 °C/min to 200 °C, and finally an increase of 15 °C/min to a final temperature of 250 °C 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 °C. Transfer line temperature was maintained at 260 °C. The quadrupole mass spectrometer was operated in the electron ionisation (EI) mode and source temperature was set at 250 °C. 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.

2.3.6.2.3 Analysis of volatile compounds

Identification of volatile compounds in the samples was done by consulting ChemStation Library Search (Wiley 275.L). Identification of compounds 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 were calculated to a signal-to-noise (S/N) ratio of 10. The volatile compounds identified related to lipid oxidation, included aldehydes, alcohols, ketones, etc., and were expressed in the terms of relative abundance.

2.3.7 Statistical analysis

Experiments were run in triplicate using three different lots of samples. The data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test. 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 in fatty acid compositions of seabass skin during iced storage

Fatty acid compositions of lipids extracted from seabass skin stored in ice for 18 days are presented in Table 5. Skin lipids from fresh seabass comprised palmitic acid (C16:0) (23.40 g/100 g lipid) as the most abundant fatty acid, followed by oleic acid (C18:1(*n*−9)) (20.90 g/100 g lipid), docosahexaenoic acid (C22:6(*n*-3)) (9.82 g/100 g lipid) and linoleic acid (C18:2(*n*−6)) (9.29 g/100 g lipid), respectively. The result was in agreement with Njinkoué *et al.* (2002) who reported that palmitic acid was the major fatty acid in lipids from skin of three edible fish from the Senegalese coast. Palmitic acid was also found as the predominant saturated fatty acid in skin lipids of wild and cultivated gilthead seabream and European seabass, whilst oleic acid was

Table 5. Fatty acid profiles of lipids from seabass skin during iced storage

Fatty acids (g/100 g lipid)	Storage time (days)		
	0	6	18
C6:0	ND	ND	0.02 ± 0.00a
C8:0	ND	ND	0.02 ± 0.00a
C10:0	ND	0.02 ± 0.00a	0.02 ± 0.00a
C12:0	0.15 ± 0.00*b**	0.17 ± 0.01a	0.12 ± 0.00c
C13:0	0.03 ± 0.00a	0.03 ± 0.00a	ND
C14:0	2.97 ± 0.03b	3.08 ± 0.03a	2.94 ± 0.01b
C14:1	0.06 ± 0.00b	0.09 ± 0.00a	0.03 ± 0.00c
C15:0	0.55 ± 0.02a	0.54 ± 0.01a	0.55 ± 0.01a
C16:0	23.41 ± 0.11c	24.08 ± 0.10b	24.53 ± 0.13a
C16:1 *n*-7	4.71 ± 0.03a	4.56 ± 0.04b	4.50 ± 0.04b
C17:0	0.64 ± 0.01b	0.64 ± 0.01b	0.68 ± 0.01a
C17:1	0.65 ± 0.01a	0.64 ± 0.01a	0.60 ± 0.01b
C18:0	5.93 ± 0.07b	5.97 ± 0.08b	6.41 ± 0.04a
C18:1 *n*-9	20.90 ± 0.11a	20.81 ± 0.09a	20.74 ± 0.13a
C18:1 *n*-7	2.05 ± 0.03a	1.98 ± 0.02b	1.98 ± 0.01b
C18:2 *n*-6	9.29 ± 0.05b	9.64 ± 0.03a	7.81 ± 0.01c
C18:3 *n*-3	0.97 ± 0.02b	1.03 ± 0.02a	0.83 ± 0.00c
C18:3 *n*-6	0.36 ± 0.01a	0.36 ± 0.01a	0.26 ± 0.00b
C18:4 *n*-3	0.47 ± 0.00a	0.48 ± 0.01a	0.48 ± 0.00a
C20:0	0.34 ± 0.00c	0.40 ± 0.00b	0.42 ± 0.00a
C20:1 *n*-7	0.11 ± 0.00a	0.11 ± 0.00a	0.12 ± 0.01a
C20:1 *n*-9	0.42 ± 0.01b	0.43 ± 0.00b	0.69 ± 0.01a
C20:1 *n*-11	0.08 ± 0.00a	0.06 ± 0.00b	0.07 ± 0.00c
C20:2 *n*-6	0.19 ± 0.00b	0.19 ± 0.00b	0.20 ± 0.00a
C20:3 *n*-6	0.25 ± 0.00a	0.26 ± 0.01a	0.22 ± 0.00b
C20:3 *n*-3	0.07 ± 0.00a	0.07 ± 0.00a	0.07 ± 0.00a
C20:4 *n*-6 (AA)	1.88 ± 0.02a	1.70 ± 0.02b	1.89 ± 0.01a
C20:4 *n*-3	0.27 ± 0.01a	0.27 ± 0.01a	0.27 ± 0.00a
C20:5 *n*-3 (EPA)	3.07 ± 0.02a	2.99 ± 0.02b	2.89 ± 0.01c
C21:0	0.07 ± 0.00b	0.07 ± 0.00b	0.08 ± 0.00a
C22:1 *n*-9	0.08 ± 0.00a	0.08 ± 0.00a	0.08 ± 0.00a
C22:1 *n*-11, *n*-13	0.15 ± 0.00a	0.09 ± 0.00b	0.07 ± 0.00c
C22:2	ND	0.02 ± 0.00a	0.02 ± 0.00a
C22:4 *n*-6	0.35 ± 0.01a	0.34 ± 0.01a	0.35 ± 0.00a
C22:5 *n*-6	0.90 ± 0.01b	0.86 ± 0.01c	0.91 ± 0.01a
C22:6 *n*-3 (DHA)	9.82 ± 0.04a	9.53 ± 0.01b	9.59 ± 0.03b
C23:0	0.20 ± 0.00a	0.20 ± 0.00a	0.21 ± 0.00a
C24:0	1.70 ± 0.01a	1.59 ± 0.02b	1.57 ± 0.02b
C24:1	0.21 ± 0.00a	0.21 ± 0.00a	0.22 ± 0.00a
Unidentified peak	6.56 ± 0.45b	6.28 ± 0.45b	7.36 ± 0.49a
Saturated fatty acids (SFA)	35.98 ± 0.21c	36.78 ± 0.16b	37.58 ± 0.20a
Monounsaturated fatty acids (MUFA)	29.57 ± 0.21a	29.16 ± 0.17a	29.23 ± 0.22a
Polyunsaturated fatty acids (PUFA)	27.90 ± 0.19a	27.78 ± 0.14a	25.81 ± 0.06b

*Values are expressed as means ± standard deviation (n = 3).

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

ND: not-detectable.

the major monounsaturated fatty acid (MUFA) (Sağglık *et al.*, 2003). Lipids from fresh seabass skin contained 35.98% saturated fatty acids (SFA), 29.57% MUFA and 27.90% polyunsaturated fatty acids (PUFA). Amongst PUFA, docosahexaenoic acid C22:6(*n*−3) (DHA) was the dominant fatty acid, followed by eicosapentaenoic acid C20:5(*n*−3) (EPA). In the present study, DHA constituted approximately 2-fold higher than EPA. DHA contents of skin lipids from wild and cultivated gilthead seabream and European seabass were higher than EPA (Sağglık *et al.*, 2003). DHA is usually more abundant in fish lipids than EPA (Kolakowska *et al.*, 2002). DHA has been found as the main lipid in cell membrane, mainly phospholipids (Erickson, 2008).

During iced storage, SFAs including, palmitic acid, stearic acid and arachidonic acid increased as the storage time increased. This was coincidental with the decreases in MUFA and PUFA. Decreases in PUFAs, especially EPA and DHA, were observed with increasing storage time. EPA decreased by 2.28% and 5.86% at day 6 and day 18, respectively. For DHA, the decreases by 2.95% and 2.34% were found at day 6 and day 18, respectively. The decreases in DHA and EPA contents were due to their susceptibility to oxidation during the extended storage (Thiansilakul *et al.*, 2010). At day 18 of iced storage, MUFA and PUFA contents decreased by 1.15% and 7.49%, respectively, whereas SFA content increased by 4.28%, compared with that found at day 0. PUFAs were generally more prone to oxidation than MUFAs. Therefore, changes in fatty acids took place in seabass skin stored in ice, in which the content of unsaturated fatty acids decreased during the storage.

2.4.2 Lipid oxidation and hydrolysis of seabass skin during iced storage

2.4.2.1 Peroxide value (PV)

PV of seabass skin during iced storage of 18 days is depicted in Figure 6A. At day 0, PV of 5.49 mg hydroperoxide/kg skin was found in seabass skin. The result suggested that lipid oxidation occurred during postmortem handling to some extent. The increase in PV was noticeable within the first 6 days of iced storage ($P < 0.05$). Subsequently, a gradual decrease in PV was observed up to 18 days ($P < 0.05$). The increase in PV of seabass skin was more likely due to the formation of

hydroperoxide at the early stage of lipid oxidation. Lipid peroxidation takes place via several pathways including the reaction of singlet oxygen with unsaturated fatty acids or the lipoxygenase-catalysed oxidation of PUFA (Wang and Hammond, 2010). A decrease in PV with extended storage was more likely due to the decomposition of hydroperoxide formed to the secondary oxidation products during the advanced stages of oxidation (Boselli *et al.*, 2005). The result suggested that lipid oxidation took place in the skin of seabass during iced storage, probably owing to the high content of unsaturated fatty acids (Table 5). Furthermore, the skin with the large surface area might be more exposed to atmosphere, in which the oxidation could proceed with ease.

2.4.2.2 Thiobarbituric acid reactive substances (TBARS)

TBARS values of seabass skin during iced storage of 18 days are shown in Figure 6B. A continuous increase in TBARS value in seabass skin was found up to 12 days of iced storage ($P < 0.05$). Thereafter, no changes in TBARS value were noticeable ($P > 0.05$). TBARS have been used to measure the concentration of relatively polar secondary reaction products, especially aldehydes (Nawar, 1996). TBARS value of 4.62 mg MDA/kg skin found in the skin at day 0 reflected the presence of lipid oxidation products in the skin after capture or during handling. During the extended storage, lipid oxidation proceeded in the skin as evidenced by the increases in TBARS value. At the end of iced storage (day 18), TBARS value increased by 44.51% when compared with that found in fresh skin. The increase in TBARS value of seabass skin during iced storage indicated the destruction of hydroperoxides into the secondary oxidation products in the later stages of lipid oxidation (Jacobsen, 2010). Hydroperoxides break down in several steps, yielding a wide variety of decomposition products, including aldehydes, etc. Lipid oxidation generates a wide range of secondary aldehyde products, including n-alkanals, trans-2-alkenals, 4-hydroxy-trans-2-alkenals, and malonaldehyde (Jacobsen, 2010). Fish skin has been known to contain high content of phospholipids. Those phospholipids are susceptible to oxidation due to high PUFA content (Erickson, 2008). Additionally, seabass skin contained a high content of PUFA (Table 5). Those fatty acids underwent oxidation to a higher extent with increasing storage time as indicated by the decrease in those fatty acids (Table 5). Although PV decreased continuously during 12-18 days, TBARS values remained unchanged. This

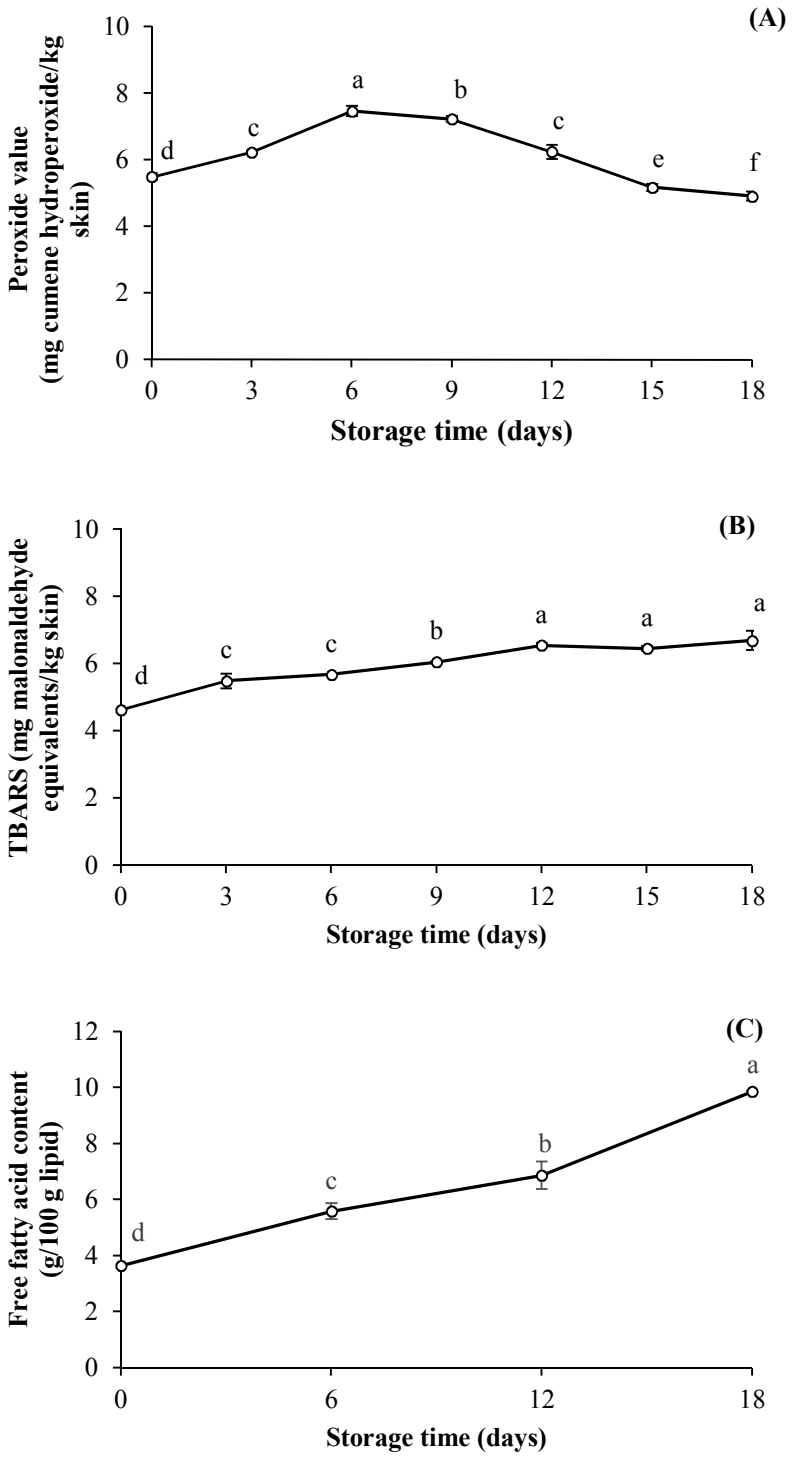

Figure 6. Changes in peroxide values (A), TBARS values (B) and free fatty acid contents (C) of seabass skin during iced storage. Different letters indicate significant difference ($P < 0.05$). Bars represent standard deviations ($n = 3$).

might be due to the balance between decomposition of hydroperoxide and the loss of some secondary products during that period. Thus, those lipid oxidation products were present in seabass skin and could lead to the quality loss, particularly the offensive fishy odour.

2.4.2.3 Free fatty acid (FFA) content

Changes in FFA contents in lipids from seabass skin during iced storage of 18 days are depicted in Figure 6C. FFA content of seabass skin increased as the storage time increased ($P < 0.05$). The result indicated that lipid hydrolysis occurred in seabass skin during the storage in ice. It was found that FFA content increased from 3.63 g/100 g lipid at day 0 to 9.85 g/100 g lipid after 18 days of storage. Lipases, phospholipase A and phospholipase B are important enzymes involved in hydrolysis of fish lipids (Hwang and Regenstein, 1993). Nayak *et al.* (2003) reported that extracellular lipase, produced by certain microorganisms, such as *Pseudomonas fragi*, contributed to the lipolytic breakdown of fish lipids. Lipase and phospholipase are also found in fish skin (Audley *et al.*, 1978). FFA released are more prone to oxidation, compared with those esterified with a glycerol backbone. This plausibly led to the increased lipid oxidation as evidenced by the increases in PV and TBARS value in seabass skin stored in ice for the extended time (Figure 6A & B).

2.4.2.4 FTIR spectra of lipids from skin during iced storage

FTIR spectra of lipid extracted from the skin of seabass stored in ice at day 0, 6, 12 and 18 are illustrated in Figure 7. Dominant peaks were found at a wavenumber range of 3050–2800 cm^{-1}, representing CH stretching vibrations, which overlap with –OH group in carboxylic acids (3100–2400 cm^{-1}). The peaks detected at 1163 and 1237 cm^{-1} were associated with the stretching vibration of the C−O ester groups and the bending vibration of CH_2 groups, and the peaks observed at 1117 and 1099 cm^{-1} represented the stretching vibration of the C−O ester groups (Guillén and Cabo, 1997b). Based on spectra, it indicated the presence of ester bonds between fatty acids and glycerol backbone of triglyceride or phospholipids. Absorbance peak between 3600 and 3400 cm^{-1} was mostly associated with the −OO−H stretching

vibrations of hydroperoxide moieties (Van de Voort *et al.*, 1994). The larger peak representing hydroperoxide was found in the lipids from seabass skin within the first 6 days of iced storage. On the other hand, the amplitude of peak became lower after 18 days of storage. The result was confirmed by the higher PV in formers (Figure 6A). During extended storage, the loss or decomposition of hydroperoxides formed in seabass skin might occur, yielding the secondary lipid oxidation products. A higher ratio of $A_{2854}/A_{3600-3100}$was observed in lipids from seabass skin after 18 days of storage, indicating the advanced oxidation process of lipid in skin when fish were stored for a longer time. This was concomitant with the increases in TBARS value at the end of storage (Figure 6A). Guillén and Cabo (2004) reported that the ratio between the absorbance band at 2854 cm^{-1}, due to the symmetrical stretching vibration of $–CH_2$ groups and the absorbance band between 3600 and 3100 cm^{-1} ($A_{2854}/A_{3600-3100}$), could be used to monitor oxidation process. After 18 days of iced storage, the higher amplitude of peak at the wavenumber of 2854 cm^{-1} was observed. The increased amplitude in this region more likely indicated the higher amounts of aldehyde formed in the skin at the end of storage.

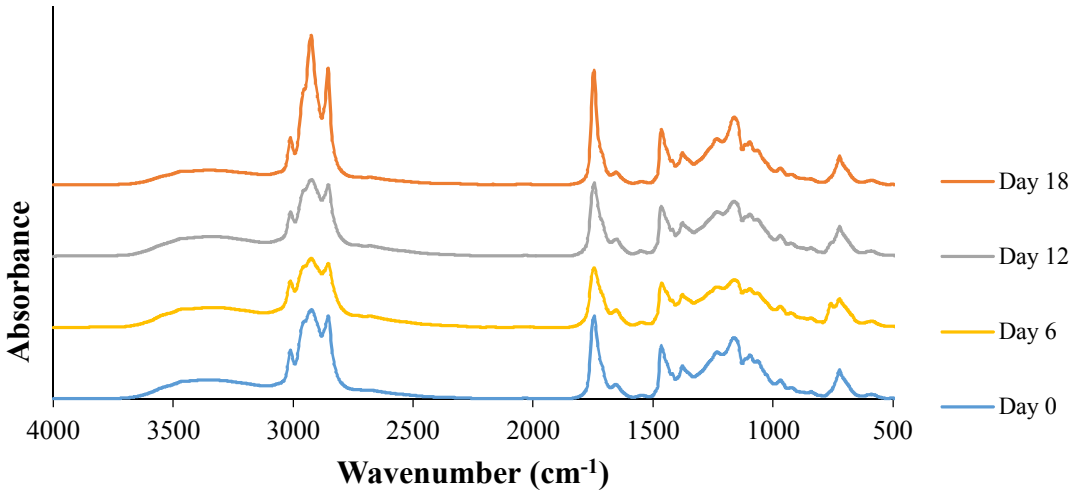

Figure 7. FTIR spectra of lipids from seabass skin during iced storage.

Peak at wavenumbers of 1745 cm^{-1} was considered to be a triglyceride peak for lipid from skin of seabass. Generally, the ester carbonyl functional group of triglycerides was observed at the wavenumber of 1741–1746 cm^{-1} (Guillén and Cabo,

1997b). After 18 days of storage, a shift to lower wavenumbers of this peak was found. These changes were in accordance with an increase in peak amplitude with a wavenumber of 1711 cm^{-1}, which represents the C=O carboxylic group of free fatty acids (Guillén and Cabo, 1997a). The increase in amplitude of peak at 1711 cm^{-1} suggested the enhanced formation of FFA mediated by hydrolysis. This observation was coincidental with the increased FFA content (Figure 6C). Therefore, lipid oxidation and hydrolysis became more pronounced in seabass skin when the iced storage time increased.

2.4.3 Lipoxygenase (LOX) activity in seabass skin during iced storage

The changes in LOX activities in skin of seabass during iced storage are depicted in Figure 8. No changes in LOX activity were observed within the first 6 days of iced storage ($P > 0.05$). After 9 days of storage, the marked increases in LOX activity were found up to 18 days of iced storage ($P < 0.05$). At day 18, LOX activity increased by 193%, compared with that found at day 0. The increase in LOX activities in skin during iced storage might be associated with the increase in extractable LOX or the activation of LOX in skin during the extended storage in ice. LOX is a dioxygenase that oxygenates PUFAs containing 1,4-cis,cis-pentadiene structure to pentadienyl, upon abstraction of H. As a result, pentadienyl radical intermediate is formed (Kanner and Rosenthal, 1992). The pentadienyl radical may react with oxygen to give peroxyl radical isomers, followed by fatty acid hydroperoxides as the final products. LOX exists in higher plants and gill, muscle and skin of fish and is capable of initiating the oxidation of PUFAs to produce unstable hydroperoxides (Hsieh *et al.*, 1988). LOX has been found in several fish skin, including trout (German *et al.*, 1985), Baltic herring (Stodolnik and Samson, 2000), sardine (Mohri *et al.*, 1990), herring (Medina *et al.*, 1999) and lake herring (Medina *et al.*, 1999). It was noted that the marked increase in LOX activity was observed after 9 days of storage. However, other parameters indicating lipid oxidation including PV and TBARS were not increased drastically. Lipid oxidation plausibly took place via auto-oxidation as well as via the LOX meditated reaction at the faster rate at the beginning of storage. With increasing storage time, the lipids or fatty acids available for LOX might be limited. As a result, the oxidation products might not be formed to a higher level, though LOX activity

increased. The result indicated that LOX might play a partial role in acceleration of lipid oxidation in the skins during the storage.

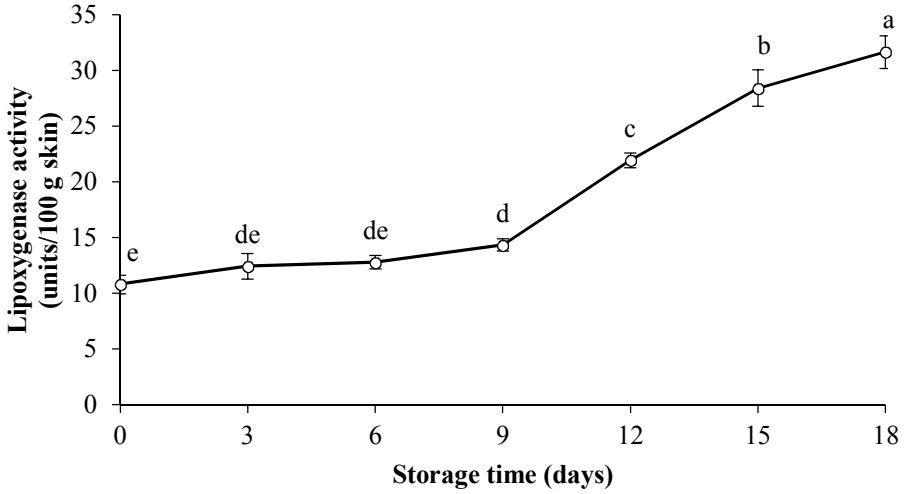

Figure 8. Changes in LOX activities in seabass skin during iced storage. Different letters indicate significant differences ($P < 0.05$). Bars represent the standard deviations (n = 3).

2.4.4 Fishy odour of seabass skin during iced storage

Development of fishy odour in seabass skin during 18 days of iced storage is shown in Figure 9. The continuous increase in fishy odour of seabass skin was noticeable throughout 18 days of iced storage ($P < 0.05$). Fishy odour intensity in seabass skin at day 0 was coincidental with the presence of lipid oxidation products as indicated by the detectable TBARS value (Figure 6B). No changes in fishy odour intensity were observed within the first 3 days of storage ($P > 0.05$). Thereafter, fishy odour was more intense after day 6 and dramatically increased up to 18 days of storage ($P < 0.05$). Thiansilakul *et al.* (2010) reported that fishy odour of seabass and red tilapia muscle were evident after 6 days of storage in ice.

In general, the development of fishy odour in seabass skin was in accordance with the marked increase in TBARS and LOX activity (Figure 6B & 8). Additionally, it was in accordance with the increased peak amplitude representing aldehyde found in fish skin after storage for 18 days (Figure 7). The formation of the

secondary lipid oxidation products is one of the main causes of the development of undesirable odour in fish flesh, especially fishy odour (Thiansilakul *et al.*, 2010). Those volatile compounds have low organoleptic thresholds (McGill *et al.*, 1977). The off-odour of fish muscle was mainly caused by lipid oxidation catalysed by the haem proteins and irons, as well as LOX (Fu *et al.*, 2009). LOX was affiliated with strong fishy odour and 2,4-heptadienal (*E,E*) mainly contributes to fishy odour (Fu *et al.*, 2009). Due to the continuous increases in LOX activity, it was presumed that LOX might be involved in the development of fishy odour in seabass skin during iced storage, which was accompanied with the formation of lipid oxidation products.

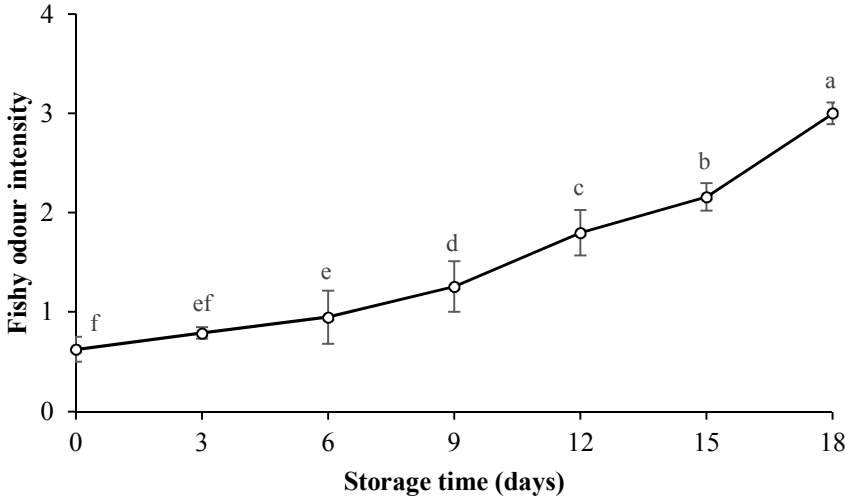

Figure 9. Changes in fishy odour intensity of seabass skin during iced storage. Score ranges from none (score = 0) to extremely strong (score = 4). Different letters indicate significant differences ($P < 0.05$). Bars represent the standard deviation (n = 3).

2.4.5 Volatile compounds of seabass skin during iced storage

Volatile compounds in seabass skin during iced storage are shown in Table 6. Aldehydes are the most prominent volatiles produced during lipid oxidation and have been used successfully to monitor lipid oxidation in a number of foods (Ross and Smith, 2006). Lipid oxidation of fish muscle is known to produce volatile aldehydic compounds including hexanal, heptanal, octanal, nonanal, etc. (Ross and Smith, 2006).

Table 6. Volatile compounds in seabass skin during iced storage

Compounds	Storage time (days)			
	0	6	12	18
Hexanal	0.97*	1.26	2.91	5.22
Heptanal	0.14	0.13	0.42	0.88
Octanal	ND	ND	0.31	1.20
Nonanal	0.25	0.27	0.89	2.15
2-Octenal	ND	ND	0.42	0.73
1-Octen-3-ol	0.72	0.86	0.94	1.15
1,5-Octadien-3-ol	0.11	0.13	0.18	0.26
2,6-Dimethyl-7-octen-2-ol	0.19	0.29	0.32	0.42
2-Ethylhexanol	0.30	0.41	0.44	0.60
Benzaldehyde	ND	ND	ND	1.68
2-Nonenal	ND	ND	ND	0.55
3,7-Dimethyl-1,6-octadien-3-ol	ND	ND	ND	0.43
1-Octanol	0.16	0.33	0.45	0.49
Pentadecane	0.65	0.25	0.15	0.31
Menthol	0.21	0.24	0.30	0.42
E-2-decenal	ND	ND	0.62	ND
3-Undecen-2-one	0.30	0.36	0.61	0.76
Heptadecane	0.31	ND	ND	ND
2,4-Decadienal	ND	ND	0.56	ND
1-Dodecanol	0.87	2.24	1.05	1.26
2,6-di(t-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one	0.18	0.25	0.26	0.26

* Values are expressed as abundance ($\times 10^9$) (n = 2).

ND: not detectable.

Amongst all aldehydic compounds, hexanal was found as the major aldehydes formed in seabass skin, followed by nonanal and octanal, respectively (Table 6). In addition, alcohol, ketone and other volatile substances were also formed in seabass skin during iced storage of 18 days. Fresh seabass skin contained the lower amounts of volatile compounds, when compared with the skins stored in ice for a longer time. Higher formation of volatile lipid oxidation products in seabass skin correlated well with the fishy odour intensities and LOX activities (Figure 8 & 9). Decomposition of hydroperoxides to the secondary volatile oxidation products were more pronounced

in seabass skin stored in ice. Propanal and heptanal can serve as a reliable indicator of flavour deterioration for fish products, whilst hexanal contributes to the rancidity in meats (Ross and Smith, 2006). Iglesias and Medina (2008) reported that propanal, 1-penten-3-ol, 2,3-pentanedione, hexanal and 1-octen-3-ol were the main volatile compounds formed during the storage of Atlantic horse mackerel muscle at 4 °C. Hexanal, heptanal and 1-octen-3-ol are generated from *n*–6 PUFA oxidation (Iglesias and Medina, 2008).

Alcohols such as 1-octen-3-ol, 1,5-octadien-3-ol, 2,6-dimethyl-7-octen-2-ol increased throughout 18 days of iced storage. Alcohols are known as the secondary products produced by the decomposition of hydroperoxides of fatty acids (Girard and Durance, 2000). 1-Octen-3-ol is an important contributor to off-flavour due to its low odour threshold and it is reported to be formed from oxidation of arachidonic acid by 12-LOX (Hsieh and Kinsella, 1989). Hexanal and 1-octen-3-ol, the major volatile compounds, contributed to the strong intensities of fishy and rancid off-odours in washed Asian seabass mince containing myoglobin (Thiansilakul *et al.*, 2011). 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 (*Salmosalar*). Carbonyl compounds, which are produced from oxidation of PUFA by LOX or by autoxidation, might contribute to fishy odour/flavour (Josephson *et al.*, 1984). Ketone, especially 3-undecen-2-one, increased with increasing storage time. Since seabass skin contains high levels of PUFA and LOX, carbonyl compounds could be easily generated via oxidation process. The fishy volatiles identified in boiled sardines were dimethyl sulfide, acetaldehyde, propionaldehyde, butyraldehyde, 2-ethylfuran, valeraldehyde, 2,3-pentanedione, hexanal, and 1-penten-3-ol (Kasahara and Osawa, 1998). The development of volatile compounds coincidentally occurred with the enhanced lipid oxidation. The subsequent decomposition of primary lipid oxidation products led to the formation of several volatile compounds, which more likely contributed to unacceptable offensive fishy odour in the skin of seabass prior to processing.

2.5 Conclusion

During iced storage, seabass skin underwent lipid oxidation with the coincidental release or activation of LOX. Development of fishy odour took place along with the formation of volatile compounds. Therefore, skin from fresh fish should be used and the delay of further processing should be avoided to prevent undesirable fishy odour in skin and its products.

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CHAPTER 3

PHYSICO-CHEMICAL PROPERTIES AND FISHY ODOUR OF GELATIN FROM SEABASS (*LATES CALCARIFER*) SKIN STORED IN ICE

3.1 Abstract

Properties and fishy odour of gelatins from seabass skin stored in ice for different days were investigated. No differences in extraction yields of gelatins were observed when skins stored for up to 18 days were used. With increasing storage time, the gelatin obtained had the increased α-amino group content ($P < 0.05$) with coincidental decrease in α-chain band intensity but had the decreased whiteness. Total viable count and psychrophilic bacterial count of skin increased continuously as the storage time increased. Gel strength of gelatin decreased as the skin was stored in ice for a longer time ($P < 0.05$). Lipid oxidation took place in skin during the storage as monitored by peroxide value and thiobarbituric acid reactive substances. Fishy odour intensity of gelatin increased with the increases in volatile aldehydes and alcohols when skin stored in ice for longer time was used. Therefore, the delay of skin processing must be avoided to prevent the formation of undesirable fishy odour and loss in properties of resulting gelatin.

3.2 Introduction

Gelatin is biopolymer obtained from collagen by partial denaturation or hydrolysis. It has a wide range of applications in food and non-food industries (Regenstein and Zhou, 2007). Generally, gelatin is obtained from mammals, especially pig and cow skins and bones. Outbreaks of mad cow disease (bovine spongiform encephalopathy, BSE) have caused the anxiety for customers. Additionally, porcine gelatin cannot be used in Kosher and Halal foods due to religious constraints (Kittiphattanabawon *et al.*, 2010). As a consequence, fish gelatin, particularly from fish processing byproducts, has gained increasing attention.

Seabass (*Lates calcarifer*) is an economically important fish species in Thailand and other countries, especially tropical and subtropical regions of Asia and

Pacific. In general, it is sold as whole fish or fillets. During processing or dressing of seabass, skin is generated and considered as a byproduct. Conversion to value-added products such as collagen, gelatin as well as hydrolysate with bioactivities has been considered as a means to fully exploit aquatic resources (Sinthusamran *et al.*, 2013). However, fishy odour associated with gelatin, particularly extracted from unfresh fish, can limit the application, especially as human food ingredients or supplement.

Lipid oxidation is associated with the development of undesirable odour, especially fishy odour, in fish stored for an extended time (Maqsood and Benjakul, 2011). Fishy odour in protein hydrolysate from Nile tilapia muscle caused by lipid oxidation was reported by Yarnpakdee *et al.* (2012). The delay in production of gelatin from fish skin might lead to the undesirable fishy odour of obtained gelatin. This might limit the utilisation of gelatin from fish skin. However, there is no information regarding the properties and fishy odour of gelatin extracted from skin stored in ice for an extended time. Thus, the aim of this study was to evaluate the changes in gelling property, colour, lipid oxidation, fishy odour and volatile compounds development in gelatin extracted from seabass skin stored in ice up to 18 days.

3.3 Materials and methods

3.3.1 Chemicals

L-leucine and 2,4,6-trinitrobenzenesulphonic acid (TNBS) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Sodium dodecyl sulphate (SDS), Coomassie Blue R-250, and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). High molecular weight markers were purchased from GE Healthcare UK Limited (Buckinghamshire, UK). Ferrous chloride were obtained from Merck (Darmstadt, Germany). 2-Thiobarbituric acid and cumene hydroperoxide were purchased from Fluka (Buchs, Switzerland). Food grade bovine bone gelatin with the bloom strength of 150-250 g was obtained from Halagel (Thailand) Co., Ltd. (Bangkok, Thailand). All chemicals were of analytical grade.

3.3.2 Collection and preparation of seabass skins

Fresh skins of seabass (*Lates calcarifer*) were obtained from Kingfisher Holdings Co., Ltd., Songkhla, Thailand. Skins were kept in a polystyrene box containing ice using a skin/ice ratio 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Upon arrival, the remaining meat was removed manually. The skins were washed with tap water, drained for 5 min on a screen, placed in polyethylene bag and kept in ice with a skin/ice ratio of 1:2 (w/w). The packed samples were placed and distributed uniformly between the layers of ice in the insulated boxes, which were subsequently left at room temperature (28–30 °C). To maintain the skin/ice ratio, the molten ice was removed and replaced with new ice every 2 days. The temperature of skin was maintained at 0–2 °C throughout the storage of 18 days. The skin samples were taken at day 0, 6, 12 and 18 for microbiological analysis.

3.3.3 Microbiological analysis

Total viable count (TVC) and psychrophilic bacteria count from fish skins stored in ice were monitored according to the method of Alfaro *et al.* (2013) with a slight modification. Each sample (10 g) was mixed with 90 ml of peptone water (0.1%, w/v) in a stomacher bag and homogenised for 2 min. Appropriate 1:10 dilutions of the resultant homogenate were prepared using 0.1% peptone water. From each dilution, 0.1 ml was spread onto Plate Count Agar (PCA) plates in triplicate, using a glass spreader. The plates were incubated at 37 ºC for 2 days and at 5 ºC for 7 days for determination of TVC and psychrophilic bacterial count, respectively. The plates containing 30-300 colonies were counted and the results were expressed as log of colony forming units per gram (log CFU/g).

3.3.4 Extraction of gelatin from seabass skins

Fresh skins and skins stored in ice for 6, 12 and 18 days were used for gelatin extraction as described by Benjakul *et al.* (2009) with some modifications. To remove non-collagenous proteins, skins were soaked in 0.05 M NaOH with a skin/solution ratio of 1:10 (w/v). The mixture was stirred for 2 h at room temperature

(about 26–28 °C). The alkaline solution was changed every hour. Alkaline-treated skins were then washed with tap water until neutral or faintly basic pH of wash water was obtained. Thereafter, the skins were rinsed with distilled water. Subsequently, the skins were then soaked in 0.05 M acetic acid with a skin/solution ratio of 1:10 (w/v) for 1 h with gentle stirring to swell the collagenous material in the skin matrix. Acid-treated skins were washed thoroughly as previously described. After swelling, the swollen skins were immersed in distilled water (55 °C) with a skin/water ratio of 1:10 (w/v) in a water bath (W350, Memmert, Schwabach, Germany) for 6 h with a continuous stirring using an overhead stirrer (RW 20.n, IKA labortechnik, Germany) at a speed of 150 rpm to extract the gelatin from skin matter. The mixture was then filtered using two layers of cheesecloth. The resultant filtrate was lyophilised using a freeze-drier (Model Duratop™ lP/Dura Dry™ lP, FTS® System, Inc., Stone Ridge, NY, USA). The lyophilised gelatins produced from fresh skins and skins stored in ice for 6, 12 and 18 days were referred to as GD0, GD6, GD12 and GD18, respectively. The obtained gelatins were subjected to analyses.

3.3.5 Analyses

3.3.5.1 Yield

Gelatin yield was calculated by the following equation:

$$\text{Yield (\%)} = \frac{\text{weight of lyophilised gelatin (g)}}{\text{weight of dry skin (g)}} \times 100$$

3.3.5.2 α-amino group content

The α-amino group content was determined according to the method of Benjakul and Morrissey (1997). Lyophilised gelatin samples were dissolved in distilled water. Properly diluted samples (125 µl) were mixed thoroughly with 2.0 ml of 0.20 M phosphate buffer, pH 8.2, followed by the addition of 1.0 ml of 0.01% TNBS solution. The mixtures were then placed in a water bath at 50 °C for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulphite. The mixtures were cooled down at room temperature for 15 min. The absorbance was measured at 420 nm and α-amino group content was expressed in terms of L-leucine.

3.3.5.3 Protein patterns

Protein patterns were determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). The gelatin samples were dissolved in 5% SDS and the mixtures were incubated at 85 ºC for 1 h using a temperature controlled water bath. Solubilised samples were mixed at a 1:1 (v/v) ratio with sample buffer (0.5 M Tris–HCl, pH 6.8 containing 5% SDS and 20% glycerol). Samples were loaded onto a polyacrylamide gel made of 7.5% separating gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA/gel. After electrophoresis, gels were stained with 0.05% (w/v) Coomassie blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid for 30 min. Finally, they were destained with the mixture of 50% (v/v) methanol and 7.5% (v/v) acetic acid for 30 min and destained again with the mixture of 5% (v/v) methanol and 7.5% (v/v) acetic acid for 1 h. High molecular weight protein markers (GE Healthcare UK Limited, Buckinghamshire, UK) including myosin (220 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa) and glutamic dehydrogenase (53 kDa) were used to estimate the molecular weight of proteins. Quantitative analysis of protein band intensity was performed using a Model GS-700 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA, USA) with Molecular Analyst Software version 1.4 (image analysis systems).

3.3.5.4 Gel strength

Gelatin gel was prepared as per the method of Fernández-Díaz *et al.* (2001) with a slight modification. Gelatin sample was dissolved in distilled water at 60 °C to obtain the final concentration of 6.67% (w/v). The gelatin solution was stirred until the gelatin was solubilised completely and cooled in a refrigerator at 10 °C for 16–18 h for gel maturation. The dimension of the sample was 3 cm in diameter and 2.5 cm in height. Gel strength of samples was determined by a Model TA-XT2 Texture Analyser (Stable Micro System, Surrey, UK) with a load cell of 5 kN and equipped with a 1.27 cm diameter flat-faced cylindrical Teflon plunger. The maximum force (in gram) was recorded when the penetration distance reached 4 mm. The speed of the plunger was 0.5 mm/s.

3.3.5.5 Fourier transform infrared (FTIR) spectroscopy

Attenuated total reflectance Fourier transform infrared spectrometer Model Equinox 55 (Bruker Co., Ettlingen, Germany) equipped with a horizontal ATR trough plate crystal cell (45° ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology, Inc., Madison, WI, USA) was used. For spectra analysis, the samples were placed onto the crystal cell and the cell was clamped into the mount of the FTIR spectrometer. The spectra, in the range of 4000-400 cm^{-1} (mid-IR region) with automatic signal gain, were collected in 32 scans at a resolution of 4 cm^{-1} and were rationed against a background spectrum recorded from the clean and empty cell at 25 °C. Analysis of spectral data was carried out using the OPUS 3.0 data collection software programme (Bruker Co., Ettlingen, Germany).

3.3.5.6 Colour

Colour of gelatin samples were measured by a Hunter Lab Colourimeter (Color Flex, Hunter Lab Inc., VA, USA). Before measurement, the samples (sponge-like), were cut into small pieces and spread over the plate. L^*, a^* and b^* indicating lightness/brightness, redness/greenness and yellowness/blueness, respectively, were recorded. Total difference in colour (ΔE^*) and the difference in chroma (ΔC^*) were calculated according to the following equation.

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

where ΔL^*, Δa^* and Δb^* are the differences between the corresponding colour parameter of the sample and that of white standard ($L^* = 93.55$, $a^* = -0.84$, $b^* = 0.37$).

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

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

3.3.5.7 Peroxide value (PV)

PV was determined according to the method of Richards and Hultin (2002) with slight modifications. 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 a homogeniser. 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 °C using a refrigerated centrifuge (Avanti® J-E, Beckman Coulter, Palo Alto, CA, USA) to separate the sample into two phases. To 3 ml of lower phase, 25 µl of 30% (w/v) ammonium thiocyanate and 25 µl of 20 mM 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 cumene hydroperoxide equivalents/100 g sample.

3.3.5.8 Thiobarbituric acid reactive substances (TBARS)

TBARS were determined as described by Buege and Aust (1978). 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 mM 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 3600 ×*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 the concentrations ranging from 0 to 6 ppm. TBARS value was calculated and expressed as mg malonaldehyde equivalents/100 g sample.

3.3.5.9 Fishy odour intensity

Fishy odour intensity was evaluated according to the method of Yarnpakdee *et al.* (2012) with a slight modification. Evaluation of fishy odour was conducted by 8 trained panelists with the ages of 25–32. Prior to the evaluation, the panelists were trained three times a week. Panelists were trained with standards for two sessions using a 15-cm line scale anchored from none (score = 0) to extremely strong fishy odour (score = 4). Gelatin produced from seabass skin stored in ice for 18 days was prepared and used as a source of fishy odour. The working standard was prepared

by dissolving gelatin in water to obtain concentration of 0, 0.5 and 1% (w/v) representing the score of 0, 2 and 4, respectively. To test the samples, all gelatins (0.75%) were placed in a sealable plastic cup and heated at 60 ºC in a temperature controlled water bath for 5 min before serving. The panelists were asked to open the sealable cup and sniff the headspace above the samples for determining the intensity of fishy odour.

3.3.5.10 Volatile compounds

Volatile compounds in gelatin powder samples were determined using a solid-phase microextraction gas chromatography mass spectrometry (SPME-GC-MS) following the method of Iglesias and Medina (2008) with a slight modification.

3.3.5.10.1 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 disperse the sample. The mixture was heated at 60 °C in 20 ml headspace vial with equilibrium time of 10 h. The SPME fibre (50/30 µm DVB/Carboxen™/ PDMS StableFlex™) (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 extract and 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.

3.3.5.10.2 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 °C for 3 min, followed by an increase of 3 °C/min to 70 °C, then an increase of 10 °C/min to 200 °C, and finally an increase

of 15 °C/min to a final temperature of 250 °C 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 °C. Transfer line temperature was maintained at 260 °C. The quadrupole mass spectrometer was operated in the electron ionisation (EI) mode and source temperature was set at 250 °C. Initially, full scan mode data was 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.

3.3.5.10.3 Analyses of volatile compounds

Identification of volatile compounds in the samples was done by consulting ChemStation Library Search (Wiley 275.L). Identification of compounds 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 were calculated to a signal-to-noise (S/N) ratio of 10. The identified volatile compounds related to lipid oxidation, including aldehydes, alcohols, ketones, etc., were presented in the term of abundance of each identified compound.

3.3.6 Statistical analysis

Experiments were run in triplicate using three different lots of samples. The data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test. 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 Microbiological changes in seabass skin during the extended iced storage

Both TVC and physchrophilic bacterial count in skin of seabass increased as the iced storage time increased up to 18 days. The initial TVC was 3.95

log CFU/g and reached 4.15, 5.30 and 5.38 log CFU/g at day 6, 12 and 18 days, respectively. Psychrophilic bacterial count of skin increased from less than 1 log CFU/g at day 0 to 3.0, 5.51 and 5.85 log CFU/g at day 6, 12 and 18, respectively. The results indicated that psychrophilic bacteria became dominant in seabass skin with increasing iced storage time. Psychrophilic microorganisms are known to produce the enzymes active at low temperatures. Those include proteases, lipases, amylases and phosphatase (Gerday *et al.*, 1997). Those microorganisms might induce the spoilage or degradation of skin, used for gelatin extraction. As a result, the property of resulting gelatin could be governed by microbial growth.

3.4.2 Yield and characteristics of gelatin extracted from seabass skin during the extended storage

3.4.2.1 Yield

Yield of gelatin extracted from the skin of seabass stored in ice for various days is shown in Table 7. No differences in extraction yield (63.7–67.2%) were observed for all samples ($P > 0.05$). The different yields of fish skin gelatin have been reported for Nile perch (64.3%) (Muyonga *et al.*, 2004a), tiger-toothed croaker (36.8%) and pink perch (27.3%) (Koli *et al.*, 2012). The yield of gelatin depends on the raw material, pretreatment conditions, the extracting parameters such as time, temperature and pH, as well as preservation method of the initial raw material (Kittiphattanabawon *et al.*, 2010). It was noted that seabass skin might undergo decomposition or degradation to a low extent during iced storage. As a result, skin matrix remained unchanged and the extraction condition used (55 ºC, 6 h) more likely exhibited similar efficacy in conversion of collagen to gelatin as evidenced by the similar yields.

3.4.2.2 α-Amino group content

The α-amino group content of gelatin from fresh and ice-stored seabass skin is presented in Table 7. The highest α-amino group content was observed in GD18 ($P < 0.05$). With increasing storage time, microbial proteases as well as indigenous proteases in skin might partially hydrolyse proteins in skin as indicated by increased α-amino group content. The increase in α-amino group content was generally in

agreement with the increasing TVC and psychrophilic bacterial count. Additionally, skin of some fish species e.g. unicorn leather jacket was reported to contain indigenous protease (Ahmad *et al.*, 2011). During extraction, those peptides produced in skin during iced storage could be extracted along with α-chains. Sinthusamran *et al.* (2014) found that higher temperature used for extraction of gelatin from seabass skin caused thermal degradation to some degree. Thus, the degradation of skin proteins was affected by storage time to some extent.

Table 7. Extraction yield, free amino group content, PV and TBARS of gelatin extracted from the skin of seabass stored in ice for different days

Parameters	GD0	GD6	GD12	GD18
Yield (%)	65.45 ± 4.82*a**	67.20 ± 2.21a	66.52 ± 5.80a	63.70 ± 4.82a
α-amino group content (mmol/g sample)	0.33 ± 0.01b	0.34 ± 0.01b	0.34 ± 0.02b	0.37 ± 0.01a
PV (mg cumene hydroperoxide equivalents/100 g sample)	10.99 ± 0.35a	11.10 ± 1.05a	11.97 ± 0.47a	7.75 ± 1.04b
TBARS (mg malonaldehyde equivalents/100 g sample)	1.20 ± 0.07a	2.77 ± 0.04b	3.07 ± 0.02b	6.97 ± 0.52c

* Mean ± SD (n = 3).

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

GD0, GD6, GD12 and GD18 represent gelatin extracted from skin stored in ice for 0, 6, 12 and 18 days, respectively.

3.4.2.3 Protein patterns

Protein patterns of gelatin extracted from the skin of seabass stored in ice for different days are shown in Figure 10. All gelatins contained α-chains as the major components. β-components (α-chain dimers) and γ-components (α-chain trimers) were also noticeable. Amongst all samples, GD0 showed higher band intensity of α_1- and α_2-chains than the others. With increasing storage time, all components showed the decreases in band intensity. The band intensity of α_1- and α_2-chains of GD18 decreased by 60 and 42 %, compared with that observed in GD0, respectively. The band intensity

of β-chain also decreased as the iced storage time increased. Gelatin extracted from skin stored for 18 days (GD18) had the decrease in β-chain band intensity by 59%, compared with that found in GD0. This was more likely related with protein degradation in skin during iced storage. The decrease in band intensity of major components in gelatin was coincidental with the increased α-amino group content (Table 7) as well as the microbial growth in skin (data not shown). During gelatin extraction, the conversion of collagen to gelatin with varying molecular mass took place, due to the cleavage of inter-chain cross-links (Zhou *et al.*, 2006). With the less compactness of skin stored for an extended time, more drastic degradation of gelatin plausibly occurred.

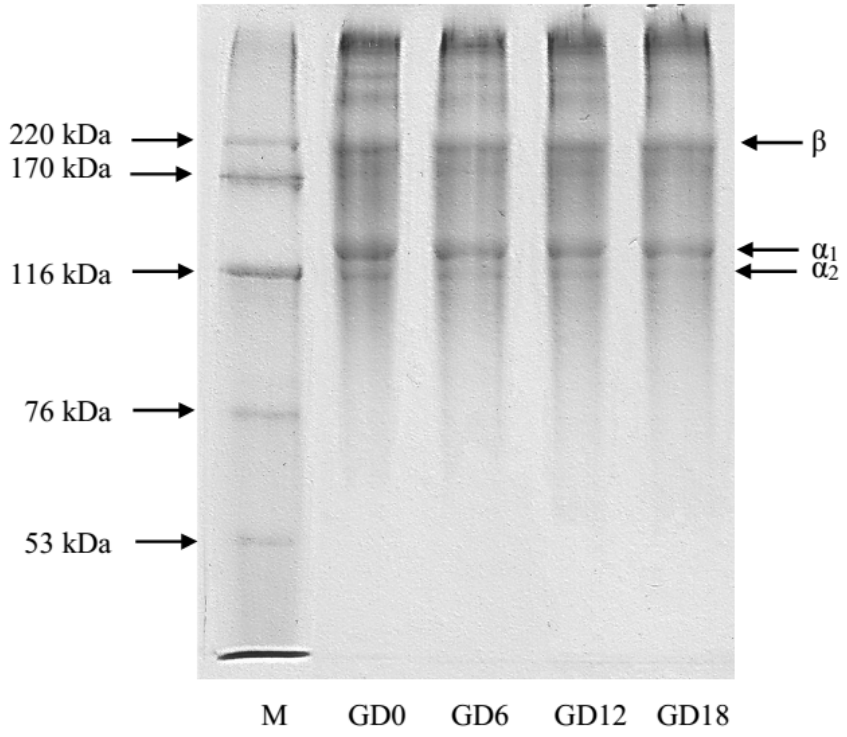

Figure 10. Protein pattern of gelatin extracted from the skin of seabass stored in ice for different days. M denote high molecular weight markers. GD0, GD6, GD12 and GD18 represent gelatin extracted from skin stored in ice for 0, 6, 12 and 18 days, respectively.

3.4.2.4 Gel strength

Gel strength of gelatin from seabass skin stored in ice for different days is shown in Figure 11. Gelatin prepared from seabass skin had higher gel strength (265.4-276.4 g), compared with commercial bovine bone gelatin (197.7 g) ($P < 0.05$). This might be due to the higher α- and β-chains in gelatin from seabass skin, in comparison with that of bovine gelatin as previously reported by Sinthusamran *et al.* (2014). Both α- and β-chains of gelatin from seabass skin played a role in development of gel network, in which the junction zone could be formed and the subsequent interaction between chains tool place via several bondings. As a consequence, the stronger gel could be formed. Gel strength of gelatin extracted from seabass skin decreased when skins used as raw material were stored for 12 and 18 days ($P < 0.05$). The difference in gel strength between the samples could be due to the differences in intrinsic characteristics, such as molecular weight distribution, amino acid composition as well as the chain length of the protein (Badii and Howell, 2006). The quality of gelatin is generally determined by the gel strength or bloom value, which can be classified as low (< 150 g), medium (150–220 g) and high bloom (220–300 g) (Johnston-Bank, 1983). Different gel strength was reported for gelatin from skin of different species including unicorn leatherjacket (170-178 g) (Kaewruang *et al.*, 2013a), brownbanded bamboo shark and blacktip shark (206-214 g) (Kittiphattanabawon *et al.*, 2010) and yellowfin tuna (426 g) (Cho *et al.*, 2005). The decrease in gel strength of gelatin was coincidental with the increase in α-amino group content as well as slight decrease in α- and β- chain band intensity, especially when the storage time increased. Gelatin with the shorter chain might not undergo aggregation effectively and the junction zones could not well developed, thereby leading to the poorer gel. Protein degradation may reduce the ability of α-chains to anneal correctly by hindering the growth of the existing nucleation sites (Ledward, 1986). Nevertheless, only slight decrease in gel strength was noticeable for gelatin extracted from seabass. This reconfirmed that α- or β-chains in seabass skin were quite stable and resistant to degradation either induced by thermal hydrolysis or protease induced degradation.

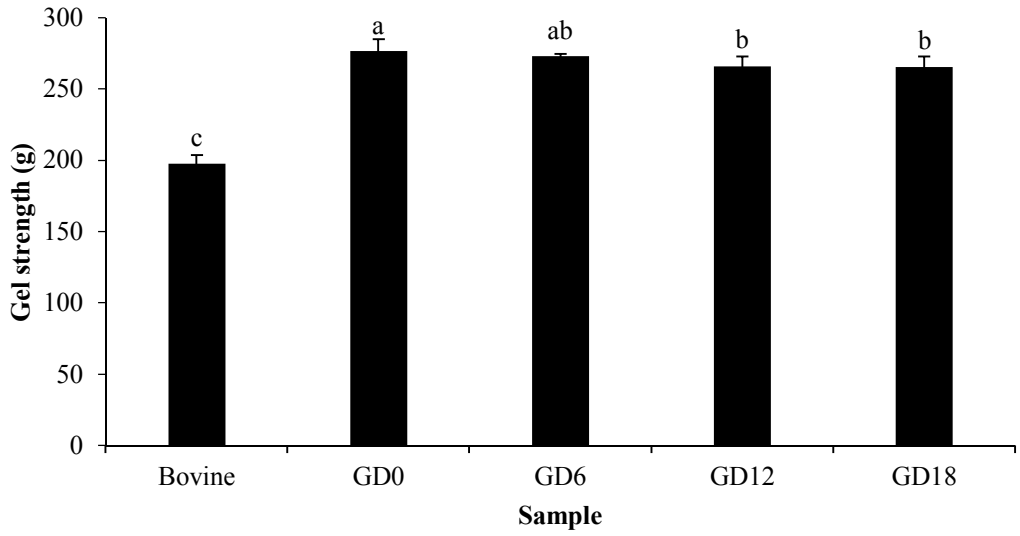

Figure 11. Gel strength of gelatin extracted from the skin of seabass stored in ice for different days. Different letters on the bars indicate significant difference ($P < 0.05$). Bars represent the standard deviations (n = 3). GD0, GD6, GD12 and GD18 represent gelatin extracted from skin stored in ice for 0, 6, 12 and 18 days, respectively.

3.4.2.5 FTIR spectra

FTIR spectra of gelatin extracted from seabass skin stored in ice for different days are depicted in Figure 12. Functional groups and secondary structure of gelatin were monitored by FTIR (Muyonga *et al.*, 2004b). All gelatin samples had the major peaks in amide region. FTIR spectra of seabass skin gelatin were similar to those found with other fish skin gelatins (Kittiphattanabawon *et al.*, 2010). The absorption in the amide I region, owing to C=O stretching vibration, can be used to determine the secondary structure of proteins (Bandekar, 1992). Its location depends on the hydrogen bonding and the conformation of protein structure (Uriarte-Montoya *et al.*, 2011). In the present study, the amide I peak was observed in the range of 1637–1641 cm^{-1}. Kaewruang *et al.* (2013b) reported that the characteristic absorption bands in the amide I region of gelatins from unicorn leatherjacket extracted at different extraction temperatures and times were noticeable at the wavenumbers of 1631–1641 cm^{-1}. Amide I band, between 1700 and 1600 cm^{-1}, was useful for infrared spectroscopic

analysis of the secondary structure of proteins (Muyonga *et al.*, 2004b). The absorption peak at amide I was characteristic for the coil structure of gelatin (Yakimets *et al.*, 2005). GD0, GD6, GD12 and GD 18 exhibited the amide I band at the wavenumber of 1637.27, 1639.20, 1639.20 and 1641.13 cm^{-1}, respectively. The amide I band of gelatin extracted from seabass skin was shifted to the higher wavenumber when the storage time increased. This indicated the greater loss of triple helix due to the pronounced destruction of triple helix via breaking down H-bonds between α-chains (Muyonga *et al.*, 2004b).

The characteristic absorption bands of all gelatins in the amide II region were noticeable at the wavenumbers of 1550.49–1548.56 cm^{-1}. The amide II vibration mode is attributed to an out-of-phase combination of C–N stretch and inplane N–H deformation modes of the peptide group (Bandekar, 1992). Gelatins from the skin of seabass extracted at different temperatures for various times exhibited the amide II bands at the wavenumbers of 1540–1543 cm^{-1} (Sinthusamran *et al.*, 2014). Amide II band of gelatins at 1560–1500 cm^{-1} was reported by Yakimets *et al.* (2005). In addition, the amide III was detected at the wavenumber of 1236 cm^{-1} for all gelatins. Nikoo *et al.* (2014) also reported that skin gelatin from Amur sturgeon showed Amide III at the wavenumber of 1236.2–1242.0 cm^{-1}. The amide III represents the combination peaks between C–N stretching vibrations and N–H deformation from amide linkages as well as absorptions arising from wagging vibrations from CH_2 groups from the glycine backbone and proline side-chains (Jackson *et al.*, 1995).

Amide A band, arising from the stretching vibrations of the N–H group, appeared at 3295.75, 3297.68, 3297.68 and 3301.53 cm^{-1} for GD0, GD6, GD12 and GD18, respectively. The result was similar to those found in the gelatin from seabass skin reported by Sinthusamran *et al.* (2014). The amide A band is associated with the N–H stretching vibration and shows the existence of hydrogen bonds. Normally, a free N–H stretching vibration occurs in the range of 3400–3440 cm^{-1}. When the N–H group of a peptide is involved in a H-bond, the position is shifted to lower frequencies (Doyle *et al.*, 1975). The higher wavenumber of GD18 indicated the higher content of N–H, which was more likely exposed when the hydrolysis of peptides took place. This was in accordance with the increase in α-amino group content (Table 7). The amide B band

was observed in the range of 3083.62–3093.26 cm^{-1}. GD18 showed the lowest wavenumber for the amide B peak, suggesting the interaction of $–CH_2$ groups between peptide chains. Therefore, the secondary structure and functional group of gelatins obtained from seabass skin was affected by iced storage time.

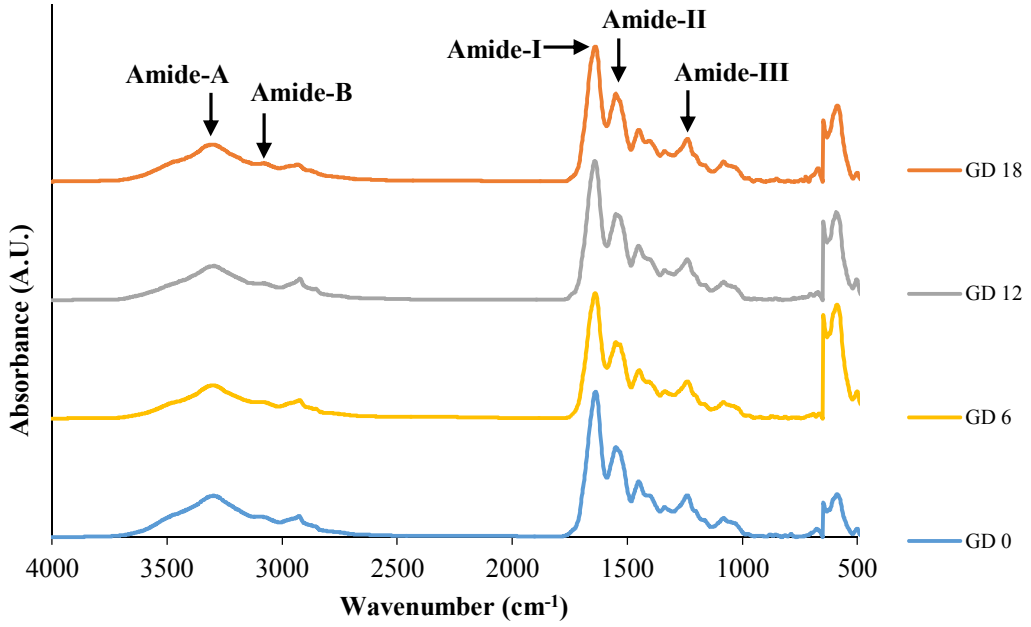

Figure 12. Fourier transform infrared spectra of gelatin extracted from the skin of seabass stored in ice for different days. GD0, GD6, GD12 and GD18 represent gelatin extracted from skin stored in ice for 0, 6, 12 and 18 days, respectively.

3.4.2.6 Colour

Differences in colour were observed between gelatins extracted from seabass skin stored in ice for different days ($P < 0.05$) (Table 8). GD0 and GD6 showed higher L^*- value (lightness) than others ($P < 0.05$). The a^*-value (redness) and b^*-value (yellowness) of gelatin increased when the storage time of seabass skin increased ($P < 0.05$). Amongst all gelatin samples, GD0 showed the lowest ΔE^* (13.64). This was concomitant with the highest lightness (L^*-value). During the storage, lipid oxidation products, especially aldehyde compounds, were formed, especially with increasing storage time. Lipid oxidation products played a role in yellow discolouration by

providing carbonyl groups involved in the Maillard reaction (Khantaphant *et al.*, 2011). During extraction at high temperature, the reaction between carbonyl group and amino group of gelatin, particularly released during the extended time (Table 7), was more favourable. This was evidenced by the marked increases in *a**- and *b**-value in gelatin extracted from seabass skin stored in ice for 18 days (GD18). The result indicated that the extended storage time of seabass skin had the negative effect on colour of extracted gelatin.

3.4.2.7 Lipid oxidation

Lipid oxidation of gelatin extracted from seabass skin stored in ice was monitored as a function of time by measuring PV and TBARS values (Table 7). PV of gelatin extracted from fresh seabass skin was 10.99 mg cumene hydroperoxide equivalents/100 g sample. Continuous increase in PV of gelatin was observed when skins were stored in ice for up to 12 days ($P < 0.05$). Nevertheless, a decrease in PV was noticeable for the gelatin extracted from skin stored for 18 days ($P < 0.05$). The increase in PV of gelatin was more likely due to the formation of hydroperoxide, a primary lipid oxidation products. The decrease in PV of gelatin from seabass skin kept in ice for a longer time was more likely due to the decomposition of hydroperoxide to the secondary oxidation products (Boselli *et al.*, 2005). TBARS value of gelatin produced from fresh skin of seabass was 1.20 mg MDA equivalents/100 g sample (Table 7). TBARS of gelatin increased as skins were kept in ice with increasing time ($P < 0.05$). The increase in TBARS was likely due to the decomposition of hydroperoxides into the secondary oxidation products, especially aldehydes. During extended storage of fish skin in ice, lipid hydrolysis along with the release or activation of lipoxygenase or other pro-oxidants occurred. This might favour lipid oxidation during gelatin extraction at high temperature (55 ºC).

3.4.2.8 Fishy odour intensity

Development of fishy odour in gelatin extracted from fresh seabass skin and skins stored in ice is shown in Table 8. The continuous increase in fishy odour of gelatin was noticeable when skins were stored in ice with increasing time ($P < 0.05$).

Stored fish skin therefore yielded the resulting gelatins with a strong fishy odour, which was coincidental with the higher lipid oxidation products as indicated by the increased TBARS value (Table 7). The formation of secondary lipid oxidation products is one of the main causes of the development of undesirable odours in fish, especially fishy odour (Yarnpakdee *et al.*, 2012). Those volatile compounds have low organoleptic thresholds (McGill *et al.*, 1977). The off-odour of fish muscle was mainly caused by lipid oxidation catalysed by the haem proteins and irons, as well as lipoxygenase (Fu *et al.*, 2009). Such an off-odour was governed by lipid autoxidation and lipoxygenase induced oxidation. Those odourous compounds might be co-extracted along with gelatin. Furthermore, the new compounds associated with the fishy odour might be formed during the extraction at high temperature, particularly for skin kept for a longer time. The result suggested that fishy odour was more pronounced when unfresh skins were used as raw material for gelatin extraction.

Table 8. Colour and fishy odour of gelatin extracted from the skin of seabass stored in ice for different days

Parameters	GD0	GD6	GD12	GD18
L^*	83.48 ± 0.29*a**	83.55 ± 0.37a	80.09 ± 0.29b	77.99 ± 0.40c
a^*	-1.18 ± 0.03d	-0.25 ± 0.03c	-0.37 ± 0.07b	0.25 ± 0.03a
b^*	9.57 ± 0.32d	11.02 ± 0.12c	11.79 ± 0.20b	13.78 ± 0.25a
ΔE^*	13.64 ± 0.42d	14.76 ± 0.28c	17.66 ± 0.33b	20.57 ± 0.44a
ΔC^*	8.72 ± 0.32d	10.10 ± 0.12c	10.87 ± 0.20b	12.86 ± 0.25a
Fishy odour	0.48 ± 0.21d	1.32 ± 0.26c	1.93 ± 0.35b	3.22 ± 0.56a

* Mean ± SD (n = 3).

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

GD0, GD6, GD12 and GD18 represent gelatin extracted from skin stored in ice for 0, 6, 12 and 18 days, respectively.

3.4.2.9 Volatile compounds

Selected volatile compounds in gelatin produced from iced stored seabass skin are shown in Table 9. Aldehydes are the most prominent volatiles found in gelatin from seabass skin stored in ice. Aldehydes have been used as the index of

lipid oxidation in a number of foods (Ross and Smith, 2006). Amongst all aldehydic compounds, hexanal was found as the major aldehydes formed in gelatin, followed by nonanal and 2-decenal, respectively (Table 9). In addition, alcohol, ketone and other volatile substances were also found in gelatin extracted and those compounds could be detectable in gelatin from skin stored in ice for 18 days. In general, gelatin from fresh seabass skin contained the lower amounts of volatile compounds, when compared with those extracted from the skins stored in ice for an extended time. Higher abundance of volatile lipid oxidation products in gelatin from seabass skin correlated well with the TBARS value and fishy odour intensities as shown in Table 7 and 8, respectively. Propanal and heptanal can serve as a reliable indicator of flavour deterioration for fish products, whilst hexanal contributes to the rancidity in meats (Ross and Smith, 2006). Iglesias and Medina (2008) reported that propanal, 1-penten-3-ol, 2,3-pentanedione, hexanal and 1-octen-3-ol were the main volatile compounds formed during the storage of Atlantic horse mackerel muscle at 4 °C. Hexanal, heptanal and 1-octen-3-ol are generated from *n*–6 PUFA oxidation (Iglesias and Medina, 2008). Linoleic acid was found at high level (9.29 g/100 g lipid) in seabass skin lipid (data not shown).

Alcohols such as 1-octanol, 1-dodecanol and 1-tridecanol of gelatin increased when skins used for gelatin extraction were stored in ice for longer time. Alcohols are known as the secondary products produced by the decomposition of hydroperoxides of fatty acids (Girard and Durance, 2000). Furthermore, new volatile alcohols (1-octen-3-ol and heptanol) were generated when fish skin stored in ice for 18 days was used for gelatin production. 1-Octen-3-ol is an important contributor to off-flavour due to its low odour threshold and it is reported to be formed from oxidation of arachidonic acid by 12-lipoxygenase (Hsieh and Kinsella, 1989). Seabass skin lipid contained arachidonic acid at a level of 1.88 g/100 g lipid (data not shown). Hexanal and 1-octen-3-ol, the major volatile compounds, contributed to the strong intensities of fishy and rancid off-odours in washed Asian seabass mince containing myoglobin (Thiansilakul *et al.*, 2011). 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. Carbonyl compounds, which are produced from oxidation of polyunsaturated fatty acids by lipoxygenase or by autoxidation, contributed to fishy

odour/flavour (Josephson *et al.*, 1984). The fishy volatiles identified in boiled sardines were dimethyl sulfide, acetaldehyde, propionaldehyde, butyraldehyde, 2-ethylfuran, valeraldehyde, 2,3-pentanedione, hexanal, and 1-penten-3-ol (Kasahara and Osawa, 1998). The development of volatile compounds was in accordance with the increased lipid oxidation (Table 7). Thus, the formation of several volatile compounds tended to unacceptable offensive fishy odour in gelatin extracted from unfresh skin.

Table 9. Volatile compounds in gelatin extracted from seabass skin stored in ice for different days

Compounds	GD0	GD6	GD12	GD18
Hexanal	2.45*	2.48	3.05	4.86
Heptanal	0.53	0.65	1.74	2.04
2,4-Nonadienal	ND	ND	ND	0.80
Cyclohexanone	ND	ND	ND	2.91
Octanal	0.73	2.47	2.97	4.29
2-Heptenal	ND	ND	0.31	0.95
Nonanal	1.51	4.71	5.11	6.10
Octenal	ND	ND	ND	2.94
1-Octen-3-ol	ND	ND	ND	0.41
Heptanol	ND	ND	ND	0.78
Nonenal	ND	0.45	2.14	2.42
1-Octanol	0.56	1.68	1.63	1.99
2,6-Nonadienal	ND	ND	ND	0.44
2-Undecanone	ND	ND	ND	1.00
2-Decenal	1.15	3.37	5.10	5.23
13-Octadecenal	ND	ND	ND	0.41
2,4-Nonadienal	ND	ND	ND	0.42
3-Undecen-2-one	ND	ND	ND	0.70
2-Undecenal	0.78	2.59	2.81	3.99
2,4-Decadienal	ND	ND	ND	0.84
1-Dodecanol	0.63	0.79	1.63	12.21
1-Tridecanol	0.20	0.22	0.41	2.82

* Values are expressed as abundance ($\times 10^8$). ND: not detectable. GD0, GD6, GD12 and GD18 represent gelatin extracted from skin stored in ice for 0, 6, 12 and 18 days, respectively.

3.5 Conclusion

Freshness of seabass skin used as raw material for gelatin production had a marked influence on fishy odour and colour of resulting gelatin but it did not drastically affect gelling property. Therefore, use of fresh skin is recommended to prevent undesirable fishy odour in resulting gelatin or its products and the delay in production of gelatin from skin must be avoided.

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CHAPTER 4

EFFECT OF PRETREATMENT AND DEFATTING OF SEABASS SKINS ON PROPERTIES AND FISHY ODOUR OF GELATIN

4.1 Abstract

The impact of different pretreatments and defatting processes of seabass skins on fishy odour and other properties of gelatin was investigated. Skin pretreated with 0.05 M citric acid, followed by defatting using 30% isopropanol (G-Ci-Def) had the lowest remaining phospholipid content ($P < 0.05$). Nevertheless, G-Ci-Def had a lower yield and gel strength, compared to those from skins pretreated with acetic acid (G-Ac) or citric acid (G-Ci) ($P < 0.05$). All gelatins contained α-chains as the predominant component as indicated by SDS-PAGE. G-Ci-Def showed the highest L^* value with the lowest a^*, b^*, ΔE^* and ΔC^* values, compared to the other gelatins ($P < 0.05$). All gelatins were sponge or coral-like in structure but varied in patterns as evaluated by SEM. The lowest fishy odour with lower amounts of volatile compounds was found in G-Ci-Def, compared to other gelatins. Therefore, gelatin with negligible fishy odour could be prepared from citric acid pretreated and isopropanol defatted skin.

4.2 Introduction

Gelatin is a biopolymer obtained from partial denaturation of collagen. It has a wide range of applications in food and non-food industries (Regenstein and Zhou, 2007). In general, gelatin is obtained from mammals, especially pig, and cow skins and bones (Gómez-Guillén *et al.*, 2011). However, outbreaks of bovine spongiform encephalopathy (mad cow disease) caused some concern for some consumers (Benjakul *et al.*, 2012). Additionally, porcine gelatin cannot be used in Kosher and Halal foods (Regenstein and Zhou, 2007). As a consequence, alternative sources for gelatin production have gained increasing attention. Amongst those potential materials, seafood processing byproducts including skin, scale, or bone have been used for gelatin production due to their abundance and low cost (Benjakul *et al.*, 2012).

Seabass (*Lates calcarifer*) is an economically important fish species in Thailand and other countries, especially tropical and subtropical regions of Asia and Pacific. It has been widely consumed domestically and also exported to many countries (Ravisankar and Thirunavukkarasu, 2010). During processing or dressing of seabass, skins are generated and considered as a byproduct. Recently, seabass skin had been used to produce collagen gelatin and hydrolysate with bioactivities (Senphan and Benjakul, 2014; Sinthusamran *et al.*, 2014). However, fishy odour associated with gelatin, particularly when extracted from fish skin stored in ice for a long time, can limit the application, especially as human supplements or food ingredients. Lipid oxidation in fish is associated with the development of undesirable fishy odour (Thiansilakul *et al.*, 2011). Fishy odour in protein hydrolysate from Nile tilapia muscle caused by lipid oxidation was also reported by Yarnpakdee *et al.* (2012b). Fish skin contains lipids with a high degree of unsaturation (Kołakowska *et al.*, 2002). Polyunsaturated fatty acid, especially, docosahexaenoic acid (C22:6 *n*−3, DHA) was found at high levels (9.82 g/100 g lipid) in seabass skin (data not shown). Furthermore, phospholipid membranes are believed to be the key substrate for lipid oxidation due to their highly unsaturated fatty acid composition (Liang and Hultin, 2005). Those lipids in skin can be oxidised during the extraction process at high temperature, thereby promoting lipid oxidation and developing unpleasant odours/flavours in the resulting gelatin (Hou *et al.*, 2011). This markedly limits the utilisation of gelatin from fish skin. Therefore, the appropriate pretreatment facilitating the removal of lipids or other pro-oxidants prior to gelatin extraction, could be a promising means to tackle such problems occurring in gelatin from fish skin. As a consequence, fish skin gelatin with negligible fishy odour could have wider applications in food systems. The aim of the present study was to evaluate the effect of pretreatments and defatting processes on fishy odour intensity, lipid oxidation and physico-chemical properties of gelatin from seabass skin.

4.3 Materials and methods

4.3.1 Chemicals

1,1,3,3-Tetramethoxypropane and L-leucine were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Sodium dodecyl sulphate (SDS),

Coomassie Blue R-250, and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). High molecular weight markers including myosin (220 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa) and glutamic dehydrogenase (53 kDa), were obtained from GE Healthcare UK Limited (Buckinghamshire, UK). Ferrous chloride was obtained from Merck (Darmstadt, Germany). Malachite oxalate green was purchased from Panreac Co. (Barcelona, Spain). Cumene hydroperoxide, disodium hydrogen phosphate, and 2-thiobarbituric acid were purchased from Fluka (Buchs, Switzerland). Ethanol and isopropanol were obtained from RCI Labscan (Bangkok, Thailand). All chemicals were of analytical grade.

4.3.2 Collection of seabass skins

Skins of fresh seabass (*L. calcarifer*) with a weight of 2.5–3 kg were obtained from the Kingfisher Holdings Co., Ltd., Songkhla, Thailand. Skins were kept in a polystyrene box containing ice using a skin/ice ratio 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, within 1 h. Upon arrival, the skins were descaled and the remaining meat was manually removed using knifes and the skins were washed with ice-cold tap water (1–3 ºC). The skins were pooled to make a composite sample, placed in polyethylene bags and stored at –20 ºC until used in less than 2 months. Prior to gelatin extraction, the frozen skins were thawed with running water (25–26 ºC) until the core temperature reached 8–10 ºC and cut into small pieces (1.0 × 1.0 cm^2) using scissors.

4.3.3 Preparation of skins with different pretreatments and defatting processes

4.3.3.1 Removal of non-collagenous proteins

The skins were soaked in 0.1 M NaOH with a skin/alkali solution ratio of 1:10 (w/v) to remove non-collagenous proteins. The mixture was stirred for 3 h at room temperature (28–30 ºC) using an overhead stirrer model RW20.n (IKA®-Werke GmbH & Co. KG, Staufen, Germany) at a speed of 300 rpm. The alkaline solution was

changed every 1 h for 3 h. Alkali-treated skin was washed with tap water until a neutral or slightly basic pH (7.0−7.5) of wash water was obtained.

4.3.3.2 Acid pretreatment

After being treated with alkaline solution, the skins were swollen using 0.05 M acetic acid or 0.05 M citric acid at a skin/solution ratio of 1:10 (w/v). The mixture was stirred at a speed of 300 rpm at room temperature for 2 h and the swollen skin was washed using tap water until the wash water became neutral or slightly acidic in pH (6.5−7.0). The samples obtained are referred to as 'swollen skin with acetic acid or citric acid; Ac and Ci', respectively.

4.3.3.3 Removal of phospholipids

To remove phospholipids, alkali-treated skins were mixed with 0.05 M citric acid containing 50 mM $CaCl_2$ using a skin/solution ratio of 1:10 (w/v). The mixture was stirred at a speed of 150 rpm for 1 h at 4 ºC and the skin was washed thoroughly with 10 volumes of tap water. The skin was finally rinsed with distilled water. The sample obtained is referred to as 'swollen skin with phospholipid removal; Ci-Pr'.

4.3.3.4 Defatting

Both Ci and Ci-Pr samples were defatted using ethanol or isopropanol at concentrations of 30, 50 and 70% (v/v) using a solid/solvent ratio of 1:10 (w/v) at room temperature with continuous shaking (200 rpm) using a shaking water bath (W350, Memmert, Schwabach, Germany) for 1 h. The solvent was removed and the defatted skin was washed with 10 volumes of tap water to remove the solvents. The defatted skins were then subjected to a final rinse with 10 volumes of distilled water.

All samples with acid pretreatment, phospholipid removal, and/or defatting were drained on the screen for 20 min at 4 ºC. The samples were analysed for phospholipid content.

4.3.4 Deterimanation of phospholipid content

Two ml of perchloric acid (70%, v/v) were added to 0.6 to 0.8 g of sample and mixed vigorously. The mixtures were heated in a boiling water bath for 1 h and then cooled at room temperature for 1 h. The mixtures were centrifuged at 9000 ×*g* for 10 min at 4 ºC using a refrigerated centrifuge model Avanti® J-E (Beckman Coulter, Inc., Palo Alto, CA, USA). The supernatant (0.2 ml) was mixed with 2 ml of malachite green stock solution prepared as described by Zhou and Arthur (1992). The mixture was then incubated at room temperature for 30 min and absorbance was measured at 660 nm using a spectrophotometer (Model UV-1800, Shimadzu, Kyoto, Japan). Disodium hydrogen phosphate solutions with concentrations of 0 to 15 µg/ml were used to prepare a standard curve for estimation of phospholipid content. A factor of 25 was used for converting phosphorus content to phospholipid based on an average molecular weight of phosphatidyl choline divided by the atomic weight of phosphorus (Sigfusson and Hultin, 2002). The removal of phospholipid was expressed as a percentage of phospholipid reduction compared to that found in fresh skin.

4.3.5 Extraction of gelatin from the skin of seabass

Amongst all, the specifically defatted skins were selected for gelatin extraction (skin pretreated with 0.05 M citric acid, followed by defatting using 30% isopropanol). Skins pretreated with 0.05 M acetic acid (typical process) or pretreated with 0.05 M citric acid were also subjected to gelatin extraction as controls. To extract gelatin, the prepared skins were mixed with distilled water at a ratio of 1:10 (w/v) at 55 ºC in a water bath (W350, Memmert, Schwabach, Germany) for 6 h with a continuous stirring using an overhead stirrer (RW 20.n, IKA®-Werke GmbH & Co. KG, Staufen, Germany) at a speed of 150 rpm. The mixtures were then filtered using a Buchner funnel with a Whatman No. 4 filter paper (Whatman International, Ltd., Maidstone, England). Thereafter, the filtrates were frozen at −40 ºC for 12 h and then lyophilised using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark) at −50 ºC for 72 h. Gelatins obtained from seabass skins pretreated with 1) acetic acid, 2) citric acid and 3) citric acid followed by defatting were referred to as ‘G-Ac’, ‘G-Ci’ and ‘G-Ci-Def’, respectively. Lyophilised gelatin samples were subsequently subjected to analyses.

4.3.6 Analyses

4.3.6.1 Yield

Gelatin yield was calculated based on the dry weight of the starting material using the following equation:

$$\text{Yield (\%)} = \frac{\text{weight of lyophilised gelatin (g)}}{\text{weight of dry skin (g)}} \times 100$$

4.3.6.2 SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

Protein patterns were determined using SDS-PAGE according to the method of Laemmli (1970). The gelatin samples (15 mg/ml protein) were dissolved in 5% SDS and the mixtures were incubated at 85 ºC for 1 h using the temperature-controlled water bath model W350 (Memmert GmbH + Co. KG, Schwabach, Germany). Solubilised samples were mixed at a 1:1 (v/v) ratio with sample buffer (0.5 M Tris–HCl, pH 6.8 containing 5% SDS and 20% glycerol). Samples were loaded onto a polyacrylamide gel made of 7.5% separating gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA/gel using a Mini Protein III unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After electrophoresis, gels were stained with 0.05% (w/v) Coomassie blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid for 3 h. Finally, they were destained with a mixture of 50% (v/v) methanol and 7.5% (v/v) acetic acid for 30 min and destained again with a mixture of 5% (v/v) methanol and 7.5% (v/v) acetic acid for 1 h. High molecular weight protein markers were used to estimate the molecular weight of proteins. Quantitative analysis of protein band intensity was done using an Imaging Densitometer model GS-700 (Bio-Rad Laboratories, Hercules, CA, USA) with Molecular Analyst Software version 1.4 (image analysis systems).

4.3.6.3 Gel strength

Gelatin gels were prepared according to the method of Fernández-Díaz *et al.* (2001) with a slight modification. Gelatin sample was dissolved in distilled water at 60 ºC to obtain a final concentration of 6.67% (w/v). The gelatin solution was stirred

until the gelatin was solubilised completely and cooled in a refrigerator at 4 ºC for 16–18 h for gel maturation. The dimension of the sample is 3 cm in diameter and 2.5 cm in height. Gel strength of samples was determined at 8–10 ºC using a texture analyser model TA-XT2 (Stable Micro System, Surrey, UK) with a load cell of 5 kN, cross-head speed of 1 mm/s and equipped with a 1.27 cm diameter cylindrical flat-faced Teflon plunger. The maximum force (g) was recorded when the penetration distance reached 4 mm.

4.3.6.4 Fourier transform infrared (FTIR) spectroscopy

Attenuated total reflectance Fourier transform infrared spectrometer model Equinox 55 (Bruker Co., Ettlingen, Germany) equipped with a horizontal ATR trough plate crystal cell (45° ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology, Inc., Madison, WI, USA) was used. For spectra analysis, the samples were placed onto the crystal cell and the cell clamped into the mount of the FTIR spectrometer. The spectra, in the range of 4000-400 cm^{-1} (mid-IR region) with automatic signal gain, were collected in 32 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean and empty cell at 25 ºC. Analysis of spectral data was carried out using the OPUS 3.0 data collection software programme (Bruker Co, Ettlingen, Germany.).

4.3.6.5 Colour

Colour of gelatin gels were measured using a Hunter Lab Colourimeter (Color Flex, Hunter Lab Inc., Reston, VA, USA). L^*, a^* and b^* indicating lightness/brightness, redness/greenness and yellowness/blueness, respectively, were recorded. The colourimeter was calibrated with a white standard. Total difference in colour (ΔE^*) and the difference in chroma (ΔC^*) were calculated as described by Wrolstad and Smith (2010).

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

where ΔL^*, Δa^* and Δb^* are the differences between the corresponding colour parameter of the sample and that of white standard ($L^* = 93.55$, $a^* = -0.84$, $b^* = 0.37$).

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

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

4.3.6.6 Microstructure

The microstructure of the gelatin gel was visualised using scanning electron microscopy (SEM). Gelatin gels were prepared in the same manner as those for gel strength measurement. Gelatin gels having a thickness of 2–3 mm were fixed with 2.5% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 12 h. The samples were then rinsed with distilled water for 1 h and dehydrated in ethanol using a serial volume fraction of 50−100% in 10% increments. The samples were subjected to critical point drying. Dried samples were mounted on a bronze stub and sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA). The specimens were observed with a scanning electron microscope (JEOL JSM-5800 LV, Tokyo, Japan) at an acceleration voltage of 20 kV.

4.3.6.7 Peroxide value (PV)

PV was determined according to the method of Richards and Hultin (2002). 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 a homogeniser. The homogenate was then filtered using Whatman No. 1 filter paper (Whatman International, Ltd., Maidstone, England). Two millilitres of 0.5% (w/v) NaCl were then added to 7 ml of the filtrate. The mixture was vortexed for 30 s and then centrifuged at 3000 ×*g* for 3 min at 4 ºC using a refrigerated centrifuge (Avanti® J-E, Beckman Coulter, Palo Alto, CA, USA) to separate the sample into two phases. 25 µl of Ammonium thiocyanate (30%, w/v) and 25 µl of 20 mM iron (II) chloride were added to 3 ml of lower phase and the reaction mixture was allowed to stand for 20 min at room temperature prior to reading the absorbance at 500 nm. A blank was prepared in the same manner, except that 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 cumene hydroperoxide equivalents/100 g dry gelatin.

4.3.6.8 Thiobarbituric acid reactive substances (TBARS)

TBARS were determined as described by Buege and Aust (1978). 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 mM 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 concentrations ranging from 0 to 6 ppm. TBARS value was calculated and expressed as mg malonaldehyde equivalents/100 g dry gelatin.

4.3.6.9 Fishy odour intensity

Sensory evaluation for fishy odour intensity was carried out according to the method of Yarnpakdee *et al.* (2012b) using 8 trained panelists 25-32 years of age. Prior to evaluation, panelists were trained with standards for two sessions to give them familiarity with the scale of fishy odour intensity. Training was carried out for totally 3 days. Gelatin produced from seabass skin stored in ice for 18 days was prepared and used as a source of fishy odour. The standard was prepared by dissolving fishy gelatin in water to obtain concentrations of 0, 0.5 and 1% (w/v) representing the score of 0, 2 and 4, respectively. To test the samples, 20 ml of all gelatin solutions (0.75%) were placed in a 40-ml plastic cup covered with lid and heated at 60 ºC in a temperature controlled water bath for 5 min before serving. The panelists were asked to open the sealable cup and sniff the headspace above the samples to determine the intensity of fishy odour, with the score from 0 (none) to 4 (extremely strong fishy odour).

4.3.6.10 Volatile compounds

Volatile compounds in gelatin powder samples were determined using a solid-phase micro-extraction gas chromatography mass spectrometry (SPME-GC-MS) following the method of Iglesias and Medina (2008) with a slight modification.

4.3.6.10.1 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 disperse the sample. The mixture was heated at 60 ºC in 20 ml headspace vial with equilibration time of 10 h. The SPME fibre (50/30 µm DVB/Carboxen™/ PDMS StableFlex™) (Supelco, Bellefonte, PA, USA) was conditioned at 270 ºC for 15 min before use and then exposed to the headspace. The vial containing the sample extract and the volatile compounds were allowed to absorb into the SPME fibre at 60 ºC for 1 h. The volatile compounds were then desorbed in the GC injector port for 15 min at 270 ºC.

4.3.6.10.2 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 programme was: 35 ºC for 3 min, followed by an increase to 70 ºC at 3C/min, 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 was 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.

4.3.6.10.3 Analyses of volatile compounds

Identification of volatile compounds in the samples was done by consulting ChemStation Library Search (Wiley 275.L). Identification of compounds

was performed, based on the retention time and mass spectra in comparison with those of standards from library. Quantification limits were calculated to a signal-to-noise (S/N) ratio of 10. The volatile compounds identified related to lipid oxidation, included aldehydes, alcohols, ketones, etc., and were expressed as abundance of each identified compound.

4.3.7 Statistical analysis

All experiments were run in triplicate using three different lots of samples. The data were subjected to analysis of variance (ANOVA). Comparison of means was carried out using Duncan's multiple range test. Statistical analysis was done using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA). Differences between means at the 5% ($P < 0.05$) level were considered significant.

4.4 Results and discussion

4.4.1 Phospholipid reduction of skin with different pretreatments and defatting processes

Phospholipid reduction of seabass skin subjected to various pretreatments and defatting processes is presented in Table 10. Acid pretreatment (Ac, Ci) and membrane removal (Ci-Pr) with and without subsequent defatting with isopropanol or ethanol at various concentrations yielded skin with varying phospholipid contents. Acid pretreatment using citric acid (Ci) reduced phospholipid content by 61.4%, compared to fresh skin (without any treatment). The removal of phospholipid was higher than typical pretreatment using acetic acid (Ac) with 50.6% reduction. However, phospholipid was removed to a higher extent with membrane removal process ($P < 0.05$) using citric acid in combination with $CaCl_2$ (Ci-Pr). Membrane bound phospholipids are believed to be the key substrates for lipid oxidation due to their highly unsaturated fatty acid composition (Ashton, 2002). Additionally, 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). When Ca^{2+} and citric acid were applied,

they were able to disconnect the electrostatic linkages between cytoskeletal proteins and membrane lipid. 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 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, 2005). Those complexes might be removed during washing. Yarnpakdee *et al.* (2012a) reported that the membrane removal process using Ca^{2+} and citric acid markedly decreased the amount of phospholipids in the muscle of Indian mackerel by 93.2%, compared to that found in mince.

The defatting process using ethanol or isopropanol at different concentrations (30, 50 and 70%) was applied for skin subjected to citric acid pretreatment (Ci) and membrane lipid removal (Ci-Pr). The efficacy of both isopropanol and ethanol in phospholipid removal from seabass skin decreased as the concentrations increased, regardless of prior membrane lipid removal (Table 10). With the same solvent used for defatting, skin pretreated with citric acid in combination with $CaCl_2$ showed lower phospholipid removal. Divalent cations, such as Ca^{2+} can form cross-links between protein molecules (Damodaran, 2008). Ca^{2+} might induce cross-linking with outer matrix of skin via salt bridge, thus limiting the extractability of solvents. The most phospholipid reduction was observed in the citric acid pretreated skin (Ci), followed by defatting with 30% isopropanol ($P < 0.05$), in which phospholipids were removed by 97.0%, compared to skin. The result was in agreement with that reported by Thiansilakul *et al.* (2007) who found that isopropanol could remove lipid in round scad muscle effectively. Removal of phospholipids varied with polarities of solvents used. Apart from removal of neutral lipids, the alcohols were able to remove membrane lipids to different degrees. Solvent mixture with higher polarity might extract a greater content of phospholipids, which are polar lipids (Kudre and Benjakul, 2013).

Table 10. Phospholipid reduction of seabass skin with different pretreatments and defatting processes

Pretreated skin	Defatting process	Phospholipid reduction (%)
Ac	-	50.6 ± 1.57^{h}
Ci	-	61.4 ± 3.58^{g}
Ci-Pr	-	79.1 ± 1.76^{f}
Ci	30% ethanol	91.0 ± 0.52^{bc}
Ci	50% ethanol	93.2 ± 0.36^{b}
Ci	70% ethanol	86.7 ± 0.59^{d}
Ci	30% isopropanol	97.0 ± 0.35^{a}
Ci	50% isopropanol	91.6 ± 0.78^{b}
Ci	70% isopropanol	91.0 ± 0.50^{bc}
Ci-Pr	30% ethanol	89.1 ± 1.04^{cd}
Ci-Pr	50% ethanol	88.4 ± 1.74^{d}
Ci-Pr	70% ethanol	82.9 ± 0.59^{e}
Ci-Pr	30% isopropanol	88.6 ± 0.76^{cd}
Ci-Pr	50% isopropanol	86.8 ± 1.34^{d}
Ci-Pr	70% isopropanol	83.2 ± 0.76^{e}

Values are expressed as mean ± SD (n = 3).
Different superscripts in the same column indicate significant differences ($P < 0.05$).
Ac, Ci and Ci-Pr represent skins pretreated with acetic acid, citric acid and skin with phospholipid removed, respectively.

4.4.2 Extraction yield

Gelatin extracted from different seabass skins showed different extraction yields (Table 11). The yields of G-Ac, G-Ci and G-Ci-Def were 62.0, 46.4 and 39.1%, respectively. G-Ac showed the highest yield, compared to others ($P < 0.05$). Acetic acid more likely provided a greater swelling capacity of destabilisation of acid labile cross-links at the telopeptide region and amide bonds of the triple helical structure of collagen as well as non-covalent intra- and inter-molecular bonds, compared to citric acid (Gómez-Guillén and Montero, 2001). Acetic acid with smaller size (60.05 g/mol)

might be able to penetrate into skin matrix more effectively than citric acid (192.12 g/mol). As a consequence, the higher ability to modify the charges associated with enhanced repulsive force could be obtained when acetic acid was implemented. This result was in accordance with Bougatef *et al.* (2012) who reported that yield of gelatin extracted from smooth hound skin pretreated with acetic acid was higher than that pretreated with citric acid. The type of acid affects pH of the extraction solution, which strongly influences swelling capacity and breaking of collagen crosslinks (Gómez-Guillén and Montero, 2001). The different yields of cod skin gelatin as affected by chemical pretreatments had been reported (Gudmundsson and Hafsteinsson, 1997).

Table 11. Extraction yield, gel strength, colour, PV, TBARS and fishy odour of gelatin from seabass skin with different pretreatments and defatting processes

Parameters	G-Ac	G-Ci	G-Ci-Def
Yield (%)	62.0 ± 0.82^{a}	46.4 ± 0.38^{b}	39.1 ± 0.72^{c}
Gel strength (g)	219 ± 7.54^{a}	222 ± 2.96^{a}	202 ± 3.19^{b}
L^*	22.6 ± 0.44^{c}	23.3 ± 0.23^{b}	30.2 ± 0.08^{a}
a^*	-0.33 ± 0.13^{a}	-0.46 ± 0.13^{b}	-1.01 ± 0.09^{c}
b^*	3.40 ± 0.28^{a}	2.10 ± 0.17^{b}	-2.03 ± 0.11^{c}
ΔE^*	71.1 ± 0.44^{a}	70.3 ± 0.23^{b}	63.4 ± 0.09^{c}
ΔC^*	2.50 ± 0.29^{a}	1.23 ± 0.16^{b}	1.35 ± 0.07^{b}
PV (mg cumene hydroperoxide equivalents/100 g dry gelatin)	14.1 ± 0.81^{a}	11.2 ± 0.56^{b}	9.20 ± 0.27^{b}
TBARS (mg malonaldehyde equivalents/100 g dry gelatin)	3.40 ± 0.28^{a}	2.64 ± 0.17^{b}	2.07 ± 0.13^{c}
Fishy odour intensity	3.49 ± 0.38^{a}	2.50 ± 1.21^{a}	0.89 ± 0.69^{b}

Values are expressed as mean ± SD (n = 3).

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

G-Ac, G-Ci and G-Ci-Def represent gelatin extracted from skin pretreated with acetic acid, citric acid, and citric acid followed by defatting using 30% isopropanol, respectively.

The yield of gelatin depends on the raw material, pretreatment conditions, the extracting parameters such as time, temperature and pH, as well as preservation method of the initial raw material (Kittiphattanabawon *et al.*, 2010). During gelatin extraction using hot water, hydrogen bonds stabilising triple helix of mother collagen were destroyed, leading to helix-to coil transition. This resulted in the conversion of collagen to soluble gelatin (Benjakul *et al.*, 2009). The skin matrix with appropriate swelling was likely more loose in structure and could facilitate the extraction or breaking down hydrogen bond stabilising triple helix of mother collagen, resulting in higher yields. Nevertheless, the decrease in yield was noticeable for G-Ci-Def ($P < 0.05$). Defatting process using isopropanol might cause aggregation via dehydration (Bull and Breese, 1978). The compact and denser structue of skin matrix as induced by defatting might not allow hot water to pass through and extract the gelatin effectively. Therefore, defatting of pretreated skin markedly showed the negative effect on extraction of gelatin from skin.

4.4.3 Protein patterns

Protein patterns of gelatin extracted from the skin of seabass subjected to different pretreatments and defatting are shown in Figure 13. All gelatin samples contained α-chain with a MW of 129–119 kDa as the major constituent. Gelatin samples also contained β-components (α-chain dimers) and γ-components (α-chain trimers). The protein patterns of gelatin were similar to those found in the gelatin of seabass skin reported by Sinthusamran *et al.* (2014). Amongst all samples, G-Ac showed the highest band intensities of α_1-, β- and γ-chains. The band intensity of α_1-, β- and γ-chains of G-Ci decreased by 6.0, 28.6 and 18.5%, respectively, compared to that observed in G-Ac. G-Ci-Def also showed decreased band intensities of α_1-, β- and γ-chains by 8.0, 11.4 and 3.7%, respectively, compared to G-Ac. G-Ci had the band with MW around 80 kDa with the low band intensity of β-chain but not G-Ci-Def. During defatting, the aggregation of protein components might be pronounced. This led to the less extraction of major components including protein with MW of 80 kDa. Protein with MW of 80 kDa could be the degradation products, especially when skin was pretreated with citric acid. During gelatin extraction, the cleavage of inter-chain cross-links might take place (Zhou *et al.*, 2006).

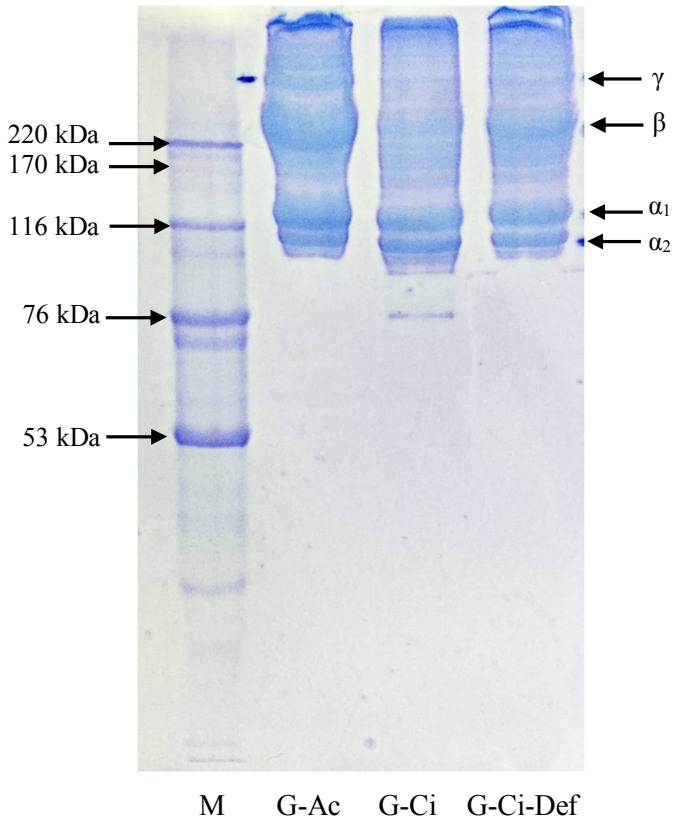

Figure 13. Protein pattern of gelatin extracted from the skin of seabass with different pretreatments and defatting processes. M denote high molecular weight markers. G-Ac, G-Ci and G-Ci-Def represent gelatin extracted from skin pretreated with acetic acid, citric acid, and citric acid followed by defatting using 30% isopropanol, respectively.

4.4.4 Gel strength

Gel strength of gelatin extracted from seabass skin with different pretreatments and defatting processes are shown in Table 11. Gel strength is one of the most important functional properties of gelatins (Karim and Bhat, 2009). Gel strength of G-Ci (222 g) and G-Ac (219 g) was higher than that of G-Ci-Def (202 g) ($P < 0.05$). During defatting with isopropanol, proteins might be vulnerable to solvent, leading to the exposure of the hydrophobic domain and aggregation of proteins (Sikorski *et al.*, 1981). Isopropanol and other alcohols have been known to compete with protein in water binding (Sikorski *et al.*, 1981). As a result, water was more removed from protein molecules in the presence of solvents (Sikorski *et al.*, 1981). This might lead to self-

aggregation of gelatin molecules via dehydration (Bull and Breese, 1978). Those large aggregated gelatin molecules could not form the fine and ordered gel network. The difference in gel strength between the samples could be due to the differences in intrinsic characteristics, such as molecular weight distribution, the chain length of protein as well as complex interactions determined by the amino acid composition and the ratio of α/β chains present in the gelatin (Badii and Howell, 2006). The gel strength of gelatin from the skin of dover sole varied considerably with acid pretreatments (Giménez *et al.*, 2005). Based on protein patterns (Figure 13), G-Ac and G-Ci showed different patterns though they had similar gel strength. Additionally, G-Ac and G-Ci-Def had similar protein pattern, but gel strength was different ($P < 0.05$). The configuration of protein and the way the inter-junction was developed to form the stronger network were therefore crucial for gel formation. Gel matrix with higher interconnection and finer strand shows higher gel strength (Benjakul et al. 2012). Location of imino acids in the peptide chain also contributed to gel formation (Ledward, 1986). Limited hydrolysis, in which the free α-chains and some oligomers could be released, might favour the aggregation or cross-links, mainly via hydrogen bond formation (Damodaran, 1997). This was evidenced in G-Ci sample, in which low MW chain (~80 kDa) was found. Thus, defatting generally showed the adverse effect on gel formation of gelatin.

4.4.5 Fourier transform infrared (FTIR) spectra

FTIR spectra of gelatin from seabass skin subjected to different pretreatments and defatting processes are illustrated in Figure 14. The FTIR spectroscopy together with attenuated total reflectance (ATR) has been used to determine chemical, physicochemical and morphological properties, gelation as well as intermolecular cross-linking of collagen and gelatin (Muyonga *et al.*, 2004). All gelatin samples had the major peaks in amide region (Kittiphattanabawon *et al.*, 2010; Sinthusamran *et al.*, 2014). FTIR spectra of seabass skin gelatin were similar to those found with other fish skin gelatins (Kittiphattanabawon *et al.*, 2010). The absorption in the amide-I region is due to C=O stretching/hydrogen bonding coupled with COO (Bandekar, 1992). Its location depends on the hydrogen bonding and the conformation of protein structure (Uriarte-Montoya *et al.*, 2011). In the present study, the amide-I

peak was observed in the range of 1630–1643 cm^{-1}. Amide I band, between 1700 and 1600 cm^{-1}, was useful for infrared spectroscopic analysis of the secondary structure of proteins (Muyonga *et al.*, 2004). The absorption peak at amide-I was characteristic for the coil structure of gelatin (Yakimets *et al.*, 2005). G-Ac, G-Ci and G-Ci-Def exhibited the amide-I band at the wavenumber of 1630, 1643 and 1637 cm^{-1}, respectively. The amide-I band of G-Ci and G-Ci-Def was shifted to the higher wavenumber, compred to G-Ac. The change in amide-I band of gelatin suggested that the pretreatment or defatting might affect the helix coil structure of gelatin, especially via exposure of hidden domains.

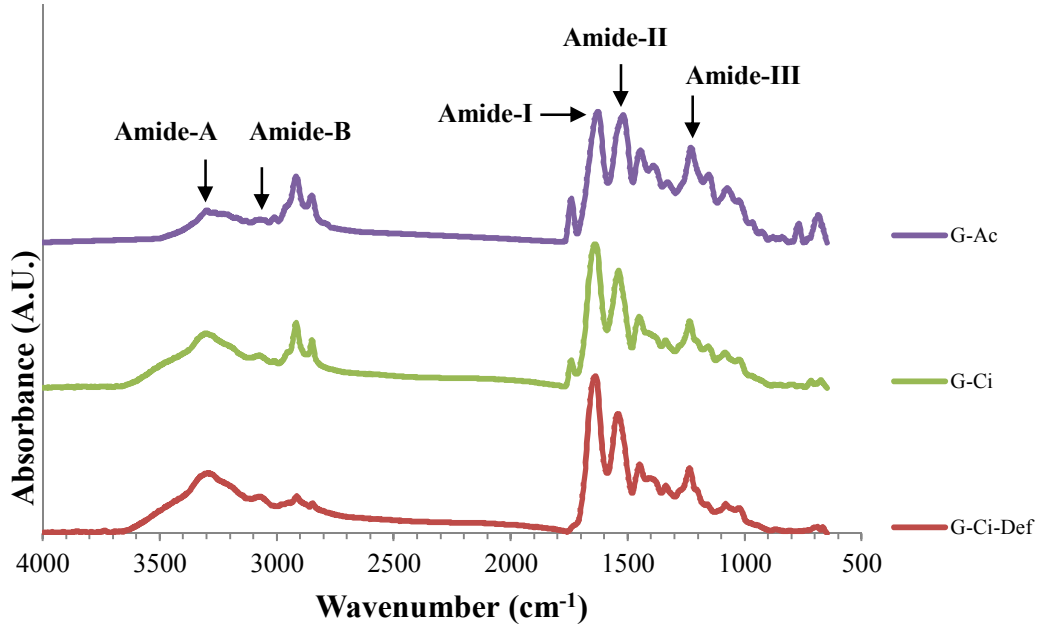

Figure 14. Fourier transform infrared spectra of gelatin extracted from the skin of seabass with different pretreatments and defatting processes. G-Ac, G-Ci and G-Ci-Def represent gelatin extracted from skin pretreated with acetic acid, citric acid, and citric acid followed by defatting using 30% isopropanol, respectively.

The characteristic absorption bands of G-Ac, G-Ci and G-Ci-Def in the amide-II region were noticeable at the wavenumbers of 1520, 1539 and 1541 cm^{-1}, respectively. The amide II vibration mode is attributed to an out-of-phase combination of C–N stretch and inplane N–H deformation modes of the peptide group (Bandekar,

1992). It was noted that the amide II band of G-Ci and G-Ci-Def showed similar spectra. Thus, citric acid pretreatment might favour the dissociation of α-chain, as indicated by the shift to higher wavenumber. Amide II band of gelatins at 1560–1500 cm^{-1} was reported by Yakimets *et al.* (2005). In addition, the amide-III bands of all gelatin samples were detected at the wavenumbers of 1230-1236 cm^{-1}. The amide-III represents the combination peaks between C–N stretching vibrations and N–H deformation from amide linkages as well as absorptions arising from wagging vibrations from CH_2 groups from the glycine backbone and proline side-chains (Jackson *et al.*, 1995). G-Ac had the highest amplitude, whereas G-Ci-Def exhibited the lowest amplitude at amide-III region. This indicated that the greater disorder of molecular structure of native collagen due to transformation of an α-helical to a random coil structure occurred (Muyonga *et al.*, 2004). However, defatting might induce the change, especially interaction of gelatin components as indicated by lower amplitude.

Amide-A band, arising from the stretching vibrations of the N–H group coupled with hydrogen bonding, appeared at 3298, 3303 and 3292 cm^{-1} for G-Ac, G-Ci and G-Ci-Def, respectively. The amide-A band is associated with the N–H stretching vibration and shows the existence of hydrogen bonds. Normally, a free N–H stretching vibration is found in the range of 3400–3440 cm^{-1} (Muyonga *et al.*, 2004). The position of amide-A band shifted to a lower frequency due to the NH group of a peptide being involved in hydrogen bonding (Nagarajan *et al.*, 2012). The higher wavenumber of G-Ci indicated the higher content of N–H, which was likely exposed when hydrolysis of peptides took place. This was coincidental with the formation of band with 80 kDa (Figure 13). Furthermore, the lowest wavenumber of G-Di-Def was probably related to the higher aggregation induced during defatting using isopropanol. The amide B band was observed at 3070, 3074 and 3074 cm^{-1} for G-Ac, G-Ci and G-Ci-Def, respectively, corresponding to the asymmetric stretching vibration of =C–H as well as $–NH_3^+$ (Nagarajan *et al.*, 2012). Amongst all samples, G-Ac showed the lowest wavenumber for the amide-B peak, suggesting the interaction of $–NH_3$ groups between peptide chains. Therefore, the secondary structure and functional group of gelatin obtained from seabass skin were affected by acid pretreatments and defatting processes.

A higher ratio of $A_{2854}/A_{3600–3100}$ was observed in G-Ac, indicating that higher oxidation took place in gelatin extracted from skin with typical pretreatment using acetic acid. Citric acid has been known as metal chelator, which could sequester prooxidant (Gordon, 1990). As a result, lower oxidation was found in gelatin when skin was pretreated with citric acid. Guillén and Cabo (2004) reported that the ratio between the absorbance band at 2854 cm^{-1}, due to the symmetrical stretching vibration of $–CH_2$ groups and the absorbance band between 3600 and 3100 cm^{-1} ($A_{2854}/A_{3600–3100}$), could be used to monitor oxidation process. The increased amplitude in this region may indicate higher amounts of aldehyde formed in the resulting gelatin (Guillén and Cabo, 2004).

4.4.6 Colour of gelatin gel

The colour of gelatin gel from seabass skin with various pretreatments and defatting processes is shown in Table 11. All samples exhibited a creamy whitish colour. Differences in colour were observed between gelatin gel samples ($P < 0.05$). G-Ci-Def showed the highest L^*- value (lightness), followed by G-Ci and G-Ac, respectively ($P < 0.05$). G-Ci-Def had the lowest a^*-value (redness) and b^*-value (yellowness) ($P < 0.05$). With citric acid pretreatment, pigments in the skin were more likely removed to a greater extent, compared to that pretreated with acetic acid. Additionally, defatting using isopropanol might facilitate removal of some particular pigments resuting in the increase in lightness. Fish skin contains chromatophores, which are cells that contain colour pigments (Fujii, 1993). Amongst all gelatin samples, G-Ci-Def showed the lowest ΔE^* (total colour difference). During gelatin extraction at high temperature (55 ºC), lipid oxidation products, especially aldehyde compounds, might be formed (Frankel, 2005). Lipid oxidation products played a role in yellow discolouration by providing carbonyl groups involved in the Maillard reaction (Khantaphant *et al.*, 2011). Therefore, skin with defatting, which showed the lowest phospholipid content (Table 10), was less susceptible to discolouration mediated by Maillard reaction. This was indicated by the lowest a^*- and b^*-value of G-Ci-Def ($P < 0.05$). Defatted fish skin was recommended as the starting material for production of gelatin with lighter colour.

4.4.7 Microstructures of gelatin gels

The microstructures of different gelatin gels as affected by pretreatments and defatting are illustrated in Figure 15. All gelatin gels were sponge or coral-like in structure. Gel network of G-Ac had the larger strand with uniform voids. For G-Ci and G-Ci-Def, similar structure was noticeable. However, G-Ci was finer with small strands than G-Ci-Def gel. The coarser gel network observed in G-Ci-Def gel was in accordance with the lower gel strength (Table 11), compared to G-Ac gel, which possessed a finer gel structure. In general, the conformation and association of protein molecules in gel matrix directly contributed to the gel strength of gelatin (Benjakul *et al.*, 2009). It has been known that the microstructure of gel network is related to the physical properties of the gelatin gel (Pang *et al.*, 2014). The coarser network had less inter-connected protein chains than the finer counterpart. As a result, the matrix was weaker. Sinthusamran *et al.* (2014) also found that gelatin from seabass skin with the finer gel network had the higher gel strength than gel possessing the coarser network. As a result, the gel with the coarser network was less resistant to the force applied, leading to a lower gel strength. The result revealed that the pretreatment with citric acid and defatting with isopropanol of raw material had a negative impact on the arrangement and association of gelatin molecules in gel matrix.

4.4.8 Lipid oxidation

Lipid oxidation of gelatin extracted from pretreated and defatted seabass skin was monitored by measuring PV and TBARS values (Table 11). PV of G-Ac was higher than G-Ci and G-Ci-Def ($P < 0.05$) and G-Ci-Def had the lowest PV ($P < 0.05$). The result suggested that pretreatment with citric acid, followed by defatting using 30% isopropanol may have removed lipid substrates as well as some pro-oxidants from the skin more effectively. When comparing TBARS of different gelatins, the highest value was observed in G-Ac ($P < 0.05$) and G-Ci-Def showed the lowest TBARS ($P < 0.05$). Although a large amount of phospholipid could be removed, lipid oxidation of gelatin produced from pretreated skin still occurred to some degree. Membrane lipids or some active pro-oxidants might still retain in G-Ci-Def to some extent. The detectable TBARS in gelatin was most likely due to decomposition of hydroperoxides into

secondary oxidation products in the later stages of lipid oxidation (Jacobsen, 2010). Hydroperoxides break down in several steps, yielding a wide variety of decomposition products, especially aldehydes, including n-alkanals, trans-2-alkenals, 4-hydroxy-trans-2-alkenals and malonaldehyde (Frankel, 2005; Jacobsen, 2010).

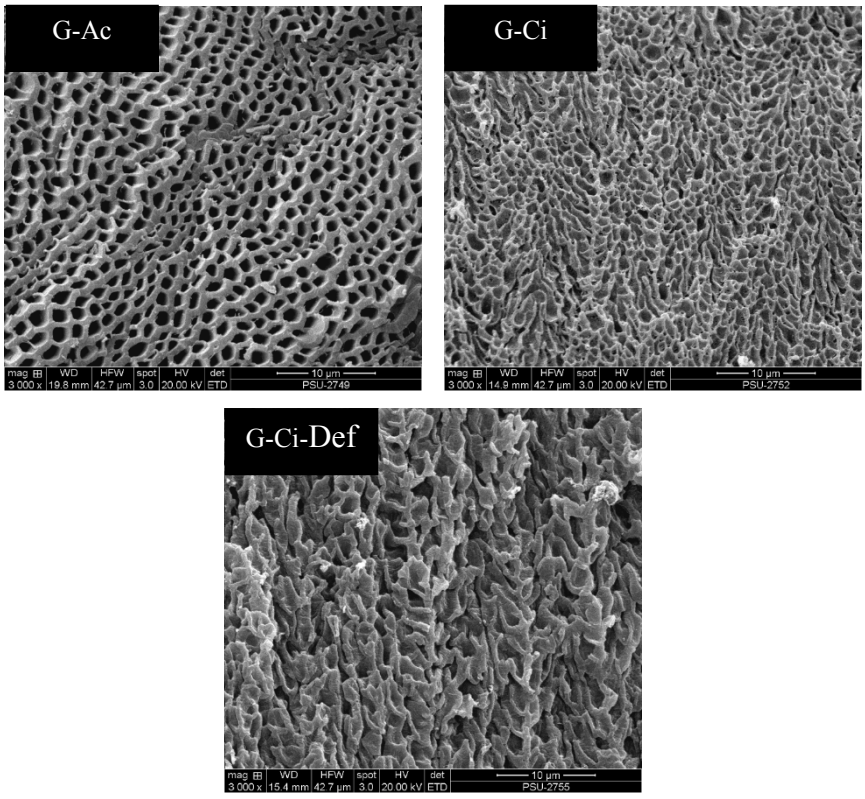

Figure 15. Microstructures of gelatin gel from the skin of seabass with different pretreatments and defatting processes. Magnification: 3000×. G-Ac, G-Ci and G-Ci-Def represent gelatin extracted from skin pretreated with acetic acid, citric acid, and citric acid followed by defatting using 30% isopropanol, respectively.

4.4.9 Fishy odour intensity

Fishy odour intensity of gelatin extracted from various pretreated and defatted seabass skins is shown in Table 11. It was found that G-Ci-Def had a marked decrease in fishy odour intensity, compared to G-Ac and G-Ci. Fishy odour intensity of G-Ci-Def was 3.9 fold lower than G-Ac. This was in accordance with the lower lipid

oxidation products monitored by PV and TBARS values in G-Ci-Def. Additionally, it was in accordance with the increased peak amplitude representing aldehydes in G-Ac (Figure 14). The formation of secondary lipid oxidation products, which was mediated by lipid autoxidation and lipoxygenase induced oxidation, is one of the main causes of the development of undesirable offensive fishy odour in fish skin. The result suggested that seabass skin pretreated with citric acid, followed by defatting prior to gelatin extraction was a promising means to minimise fishy odour in resulting gelatin via lowering lipid substrate for oxidation process.

4.4.10 Volatile compounds

Selected volatile compounds in gelatin produced from different pretreated and defatted seabass skin are shown in Table 12. Aldehydes are the most prominent volatiles found in gelatin from seabass skin. Aldehydes have been used as the index of lipid oxidation in a number of foods because they possess low odour threshold values and are the major contributors to development of off-odour and off-flavour (Ross and Smith, 2006). Numerous aldehydes produced during oxidation included octanal, nonanal, pentanal, hexanal, etc. (Ross and Smith, 2006). Amongst all aldehydic compounds, hexanal was the major aldehyde formed in gelatin, followed by nonanal and pentanal, respectively. It was noted that G-Ci-Def had hexanal 5.9 fold lower than G-Ac. No pentanal was detected in G-Ci-Def. For other aldehydes, G-Ci-Def also showed lower abundance than G-Ac and G-Ci. Propanal and heptanal can serve as a reliable indicator of flavour deterioration for fish products (Ross and Smith, 2006). Iglesias and Medina (2008) reported that propanal, 1-penten-3-ol, 2,3-pentanedione, hexanal and 1-octen-3-ol were the main volatile compounds formed during the storage of Atlantic horse mackerel muscle at 4 °C. The fishy volatiles identified in boiled sardines were dimethyl sulfide, acetaldehyde, propionaldehyde, butyraldehyde, 2-ethylfuran, valeraldehyde, 2,3-pentanedione, hexanal, and 1-penten-3-ol (Kasahara and Osawa, 1998). 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. Carbonyl compounds, which are produced from oxidation of polyunsaturated fatty acids by lipoxygenase or by autoxidation, contributed to fishy odour/flavour (Josephson *et al.*, 1984). Hexanal, heptanal and 1-

octen-3-ol are generated from *n*–6 polyunsaturated fatty acid oxidation (Iglesias and Medina, 2008).

Table 12. Volatile compounds in gelatin extracted from seabass skin with different pretreatments and defatting processes

Compounds	G-Ac	G-Ci	G-Ci-Def
Aldehydes			
Pentanal	2.23	1.98	ND
Hexanal	12.85	2.26	2.20
2-Pentenal	1.16	0.67	0.32
Heptanal	1.91	1.93	1.86
2-Hexenal	1.25	0.94	1.10
Octanal	1.81	1.90	1.66
Heptenal	1.01	0.70	0.66
Nonanal	3.60	3.04	2.77
2-Octenal	1.59	1.50	1.47
3-Cyclohexene-1-carboxaldehyde	0.30	ND	ND
Decanal	0.41	0.37	0.29
Nonenal	0.75	0.59	0.48
2,6-Nonadienal	0.70	0.59	0.55
2-Decenal	1.20	0.79	0.81
2,4-Nonadienal	0.31	0.45	0.29
2-Undecenal	0.34	0.33	0.37
Alcohols			
1-Penten-3-ol	1.18	0.73	0.55
1-Pentanol	0.94	0.75	0.74
1-Octen-3-ol	3.49	2.38	2.37
2-Ethylhexanol	1.37	0.93	0.85
1-Octanol	1.17	0.93	0.78
2-Octen-1-ol	0.69	0.28	0.31
1-Dodecanol	0.61	0.47	0.41
Ketones			
2,3-Octanedione	0.32	ND	ND
6-Methyl-5-hepten-2-one	0.34	ND	ND
3,5-Octadien-2-one	0.55	0.29	0.42
3-Undecen-2-one	0.55	0.58	0.43

Values are expressed as abundance ($\times 10^8$). ND: not detectable. G Ac, G-Ci and G-Ci-Def represent gelatin extracted from skin pretreated with acetic acid, citric acid, and citric acid followed by defatting using 30% isopropanol, respectively.

Alcohols including 1-penten-3-ol, 1-pentanol, 1-octen-3-ol and 1-octanol of G-Ci-Def were lower in abundance, compared to G-Ac and G-Ci (Table 12). 1-Octen-3-ol was the most abundant alcohol in all gelatins. Alcohols are known as secondary products of decomposition of hydroperoxides of fatty acids (Ross and Smith, 2006). 1-Octen-3-ol is an important contributor to off-flavour due to its low odour threshold and it was reportedly formed from oxidation of arachidonic acid by 12-lipoxygenase (Hsieh and Kinsella, 1989). Thiansilakul *et al.* (2011) reported that 1-octen-3-ol contributed to the strong intensities of fishy and rancid off-odours in washed Asian seabass mince containing myoglobin.

Other volatile compounds were also formed but generally decreased after pretreatment with citric acid, followed by defatting. Ketone is another secondary lipid oxidation product derived from decomposition of hydroperoxide (Iglesias and Medina, 2008). In the present study, 2,3-octanedione, 6-methyl-5-hepten-2-one, 3,5-octadien-2-one and 3-undecen-2-one were ketones found in gelatin samples. 2,3-Octanedione and 6-methyl-5-hepten-2-one were not present in G-Ci and G-Ci-Def. Lower levels of 3,5-octadien-2-one and 3-undecen-2-one were found in G-Ci and G-Ci-Def, compared to G-Ac.

In general, the lower amount of secondary oxidation products, including aldehydes, alcohols and ketones in G-Ci-Def was in accordance with the lower PV and TBARS values (Table 11). The results suggested that lipid oxidation and decomposition of hydroperoxides to secondary volatile lipid oxidation products were lower in G-Ci-Def, compared to G-Ac. Thus, the pretreatment of seabass skin with citric acid, followed by defatting was effective in retarding the formation of secondary lipid oxidation products, which contribute to the offensive fishy odour in gelatin.

4.5 Conclusion

Pretreatment and defatting of seabass skin played a significant role in the reduction of lipids, especially membrane phospholipids. Pretreatment of skin using citric acid, followed by defatting with 30% isopropanol prior to gelatin extraction, was very effective in preparing a substrate for gelatin production, in which volatile lipid oxidation products and fishy odour could be significantly lowered. Gelatin prepared

from the skin with appropriate pretreatments and defatting could be widely used in foods without any detrimental effect on sensory attributes.

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CHAPTER 5

CHARACTERISTICS AND FUNCTIONAL PROPERTIES OF GELATIN FROM SEABASS SKIN AS INFLUENCED BY DEFATTING

5.1 Abstract

Gelatins from non-defatted and defatted seabass skin were characterised and evaluated for their functional properties in comparison with commercial fish skin gelatin. All gelatins contained α_1- and α_2-chains as the predominant components and showed a high imino acid content (199–201 residues/1000 residues). All gelatins had a relative solubility greater than 90% in the wide pH ranges (1−10). Foaming properties of all gelatins increased with increasing concentrations (1−3%, w/v). Gelatin from defatted skin had higher foam expansion and stability than that extracted from non-defatted skin. Emulsion containing gelatin from defatted skin had smaller oil droplet size (d_{32}, d_{43}), compared with that having gelatin from non-defatted skin ($P < 0.05$). After 10 days of storage at room temperature (28−30 ºC), emulsion stabilised by gelatin from defatted skin showed the higher stability as indicated by the lower increases in d_{32}, d_{43}, and lower flocculation factor and coalescence index. Coincidentally, emulsion stabilised by gelatin from defatted skin had higher zeta potential than that containing gelatin from non-defatted skin. Thus, defatting of seabass skin directly affected characteristics and functional properties of resulting gelatin.

5.2 Introduction

Gelatin is a fibrous protein obtained from partial denaturation or hydrolysis of collagen. It is an important functional biopolymer with a wide range of applications in food and non-food industries (Regenstein and Zhou, 2007). Generally, gelatin is obtained from mammals, especially pig and cow skins and bones. However, outbreaks of mad cow disease (bovine spongiform encephalopathy, BSE) as well as the prohibition of porcine gelatin for Muslims and Jews, have led to the search for the alternative sources (Regenstein and Zhou, 2007). Amongst those potential materials, seafood processing byproducts (skin, scale, swim bladder or bone) have been used for gelatin production due to their abundance and low cost.

Seabass (*Lates calcarifer*) is an economically important fish species in Thailand and other countries, especially tropical and subtropical regions of Asia and Pacific (Boonyaratpalin and Williams, 2002). During dressing of seabass, skin is generated as a byproduct. Recently, seabass skin has been used to produce collagen, gelatin as well as hydrolysate with bioactivities (Senphan and Benjakul, 2014; Sinthusamran *et al.*, 2013).

Gelatin has been used as a foaming, emulsifying and wetting agent in food, pharmaceutical, medical and technical applications due to its surface-active properties (Balti *et al.*, 2011). The properties of gelatins are generally influenced by several factors, such as raw material and the intrinsic properties, including chemical composition, amino acid composition as well as molecular weight distribution of the protein components (Regenstein and Zhou, 2007). In addition, functional properties associated with the chain length of gelatin could be influenced by the chemicals used for pretreatment and the extraction conditions, such as temperature and time (Kołodziejska *et al.*, 2004). Gómez-Guillén and Montero (2001) reported that various organic acids (formic, acetic, propionic, lactic, malic, tartaric, and citric acids) influenced the viscoelastic and gelling properties of gelatin from megrim skin. Fish gelatin possessing fishy odour directly limits its uses, particularly in food industries. Offensive fishy odour was attributed to lipid oxidation of fish skin during extended storage or during gelatin extraction. To conquer this drawback, the pretreatment using citric acid along with defatting process have been implemented to remove both membrane and neutral lipids prior to gelatin extraction. Nevertheless, the removal of lipids from skin used as raw material may affect the physicochemical as well as functional properties of resulting gelatin. Thus, the objective of this study was to investigate the effect of pretreatments and defatting processes on characteristics and functional properties of gelatin from seabass skin.

5.3 Materials and methods

5.3.1 Chemicals

L-leucine was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Sodium dodecyl sulphate (SDS), Coomassie Blue R-250, and N,N,N',N'-

tetramethylethylenediamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). High molecular weight markers were obtained from GE Healthcare UK Limited (Buckinghamshire, UK). Fish gelatin produced from tilapia skin (~240 bloom) was obtained from Lapi Gelatine S.p.a. (Empoli, Italy). All chemicals were of analytical grade.

5.3.2 Collection of seabass skins

Skins of seabass (Lates calcarifer) with a weight of 2.5–3 kg were obtained from the Kingfisher Holdings Co., Ltd., Songkhla, Thailand. Skins were kept in a polystyrene box containing ice with a skin/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Upon arrival, the remaining meat was removed manually. The skins were washed with cold tap water (≤ 10 ºC), pooled and used as the composite sample. The samples were placed in polyethylene bags and stored at –20 ºC until used. However, the storage time did not exceed 2 months. Prior to gelatin extraction, the frozen skins were thawed with running tap water (25–26 ºC) to obtain the core temperature of 0–2 ºC. The skins were then cut into small pieces (1.0 × 1.0 cm^2) using scissors.

5.3.3 Preparation of seabass skin

5.3.3.1 Removal of non-collagenous proteins

The skins were soaked in 0.05 M NaOH with a skin/alkaline solution ratio of 1:10 (w/v) in order to remove non-collagenous proteins. The mixture was stirred for 3 h at room temperature (28–30 ºC) using an overhead stirrer model RW20.n (IKA®-Werke GmbH & CO.KG, Staufen, Germany) at a speed of 300 rpm. The alkaline solution was changed every 1 h for totally 3 times. Alkali-treated skins were washed with tap water until a neutral or slightly basic pH (7.0–7.5) of wash water was obtained.

5.3.3.2 Acid pretreatment and defatting

After non-collagenous proteins were removed, the obtained skins were separated into two portions. The first one was subjected to pretreatment using 0.05 M acetic acid at a skin/solution ratio of 1:10 (w/v). The mixture was stirred at room

temperature for 2 h. The swollen skins were washed using tap water until wash water became neutral or slightly acidic in pH (6.5-7.0). The typically swollen skins obtained were referred to as 'non-defatted skins'.

For the second portion, the skins were pretreated by stirring skin with 0.05 M citric acid using a skin/solution ratio of 1:10 (w/v) for 2 h to remove phospholipids. After stirring and washing, the pretreated skins were defatted using 30% (v/v) isopropanol with a skin/solvent ratio of 1:10 (w/v). The mixture was stirred continuously at 200 rpm at room temperature for 1 h. The solvent was then removed and the defatted skins were washed with 10 volumes of tap water. The defatted skins were then rinsed with distilled water. The obtained skins were referred to as 'defatted skins'.

5.3.4 Extraction of gelatin

Both non-defatted and defatted skins were subjected to gelatin extraction. Both skins were mixed with distilled water at a ratio of 1:10 (w/v). Extraction was carried out at 55 ºC in a water bath (W350, Memmert, Schwabach, Germany). The mixtures were stirred continuously for 6 h using an overhead stirrer (RW 20.n, IKA®-Werke GmbH & CO.KG, Staufen, Germany) at a speed of 150 rpm. The mixtures were then filtered using a Buchner funnel with a Whatman No. 4 filter paper (Whatman International, Ltd., Maidstone, England).

The gelatin solutions were lyophilised using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The lyophilised gelatins obtained from non-defatted and defatted skins were referred to as 'NDFG' and 'DFG', respectively. The obtained gelatins were subjected to analyses.

5.3.5 Analyses

5.3.5.1 Fat content

Fat content was determined following the methods of AOAC (2000) with the analytical No. of 960.39.

5.3.5.2 Protein patterns

Protein patterns were determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). High molecular weight protein markers (GE Healthcare UK Limited, Buckinghamshire, UK) were used to estimate the molecular weight of proteins.

5.3.5.3 Amino acid analysis

Amino acid composition of gelatin samples was analysed according to the method of Nagarajan et al. (2012) with a slight modification. The samples were hydrolysed under reduced pressure in 4 M methanesulphonic acid containing 0.2% (v/v) 3-2(2-aminoethyl) indole at 115 ºC for 24 h. The hydrolysates were neutralised with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot of 0.04 ml was applied to an amino acid analyser (MLC-703; Atto Co., Tokyo, Japan).

5.3.5.4 Surface hydrophobicity

Surface hydrophobicity (S_0ANS) was determined as described by Benjakul et al. (1997) using 1-anilinonaphthalene-8-sulfonic acid (ANS) as a probe. Gelatin solution (10 mg/ml) was diluted in 10 mM sodium phosphate buffer (pH 6.0) to obtain the protein concentrations of 0.1, 0.2, 0.3, and 0.5%. The diluted protein solution (2 ml) was mixed with 20 µl of 8 mM ANS in 0.1 M sodium phosphate buffer (pH 7.0). The fluorescence intensity was immediately measured at an excitation wavelength of 374 nm and an emission wavelength of 485 nm. The initial slope of the plot of fluorescence intensity versus protein concentration was referred to as S_0ANS.

5.3.5.5 Solubility

Solubility of gelatin at different pH values was determined following the method of Kittiphattanabawon et al. (2012). The gelatin sample was dissolved in distilled water at 60 ºC to obtain a final concentration of 1% (w/v) and the mixture was stirred at room temperature for 30 min. The gelatin solution was adjusted to different pHs (1−10) with either 6 M NaOH or 6 M HCl. The solution was centrifuged at 8500×*g* at room temperature for 10 min using a centrifuge (Microfuge 20, Beckman Coulter,

Inc., Krefeld, Germany). Protein content in the supernatant was determined by the Biuret method (Robinson and Hogden, 1940) using bovine serum albumin as a standard. Relative solubility was calculated and compared with total protein content of the sample. Total protein content was determined after complete solubilisation of the sample using 0.5 M NaOH.

5.3.5.6 Viscosity

Viscosity of gelatin solution (6.67%, w/v) was measured using a rheometer (HAAKE™ RheoStress™ 1, Thermo Scientific, 500, Anton Paar GmbH, Germany) equipped with a cone and plate geometry. The diameter and angle of cone was 35 mm and 1º, respectively and the gap was 0.01 mm. Approximately 1.3 ml of gelatin solution was applied to the plate and the excess sample was removed. The sample was covered with silicone oil to prevent evaporation during measurement. Measurement was performed over a shear rate up to 1,000 s^{-1} at 60 °C. Shear viscosity was expressed as mPas.

5.3.5.7 Foaming property

Foam expansion (FE) and foam stability (FS) of gelatin sample solutions were determined as described by Shahidi *et al.* (1995) with a slight modification. Gelatin solution (1, 2 or 3%) was transferred into 100 ml cylinders. The solution was homogenised at a speed of 13,400 rpm for 1 min at room temperature using an IKA Labortechnik homogeniser (Selangor, Malaysia). The sample was allowed to stand for 0 and 60 min. FE and FS were then calculated using the following equations:

$$\text{FE (\%)} = (V_T/V_0) \times 100$$

$$\text{FS (\%)} = (V_t/V_0) \times 100$$

where, V_T is total volume after whipping; V_0 is the original volume before whipping and V_t is total volume after leaving at room temperature for 60 min.

5.3.5.8 Emulsifying property

Oil-in-water emulsions were prepared as described by Aewsiri et al. (2013). Gelatins were dissolved in distilled water to obtain a final concentration of 2% (w/v). Emulsions were prepared by homogenising the mixture of sunflower oil and gelatin solution (1:9, v/v) at a speed of 10,000 rpm for 2 min using a homogeniser. These coarse emulsions were then passed through a two-stage high-pressure homogeniser (M-110P, Microfluidiser, Newton, MA, USA) at 3,000 psi for 3 passes. NaN_3 (0.02%, w/w) was added to the emulsions as an antimicrobial agent. All samples were then stored at room temperature (28–30 °C) for 10 days. The samples were taken at day 0, 5 and 10 for analyses.

5.3.5.8.1 Measurement of oil droplet size

Particle size distribution of oil droplets in emulsion was determined using a ZetaPlus zeta potential analyser (Brookhaven Instruments Corporation, Holtsville, NY, USA) at 25 °C. Prior to analysis, the samples were 100-fold diluted with 1% (w/v) sodium dodecyl sulphate (SDS) in order to dissociate flocculated droplets. The surface-weighted mean diameter (d_{32}) and the volume-weighted mean particle diameter (d_{43}) of the oil droplets were calculated using the following equations:

$$d_{32} = \frac{\sum n_i d_i^3}{\sum n_i d_i^2}$$

$$d_{43} = \frac{\sum n_i d_i^4}{\sum n_i d_i^3}$$

where n_i and d_i are the number of droplets of a determined size range and the droplet diameter, respectively.

5.3.5.8.2 Determination of flocculation and coalescence

To determine flocculation factor (F_f) and coalescence index (C_i), the emulsions were diluted with distilled water in the presence and absence of 1% (w/v) SDS. F_f and C_i were calculated using the following equations:

$$F_{\mathrm{f}} = \frac{d_{43-\mathrm{SDS}}}{d_{43+\mathrm{SDS}}}$$

$$C_{\mathrm{i}} = \frac{d_{43+\mathrm{SDS,t}} - d_{43+\mathrm{SDS,in}} \times 100}{d_{43+\mathrm{SDS,in}}}$$

where $d_{43+\mathrm{SDS}}$ and $d_{43-\mathrm{SDS}}$ are the volume-weight mean particle diameter of the emulsion droplets in the presence and absence of 1% (w/v) SDS, respectively. $d_{43+\mathrm{SDS,in}}$ is initial value of the volume-weight mean particle diameter of the emulsion droplets in the presence of 1% (w/v) SDS; $d_{43+\mathrm{SDS,t}}$ is the volume weight distribution of the emulsion droplets in the presence of 1% (w/v) SDS at the designated storage time.

5.3.5.8.3 ζ-Potential analysis

The electrical charge (ζ-potential) of oil droplets in the emulsions was determined using a ZetaPlus zeta potential analyser (Model ZetaPALS, Brookhaven Instruments, Co., Holtsville, NY, USA) at room temperature. The oil-in-water emulsions were diluted 250-fold prior to measurements. The diluted emulsions were mixed thoroughly and then injected into the measurement chamber of the instrument. The ζ-potential of each individual sample was calculated from the average of five measurements.

5.3.6 Statistical analysis

Experiments were run in triplicate using three different lots of samples. The data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test. 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 Chemical composition and physicochemical property of gelatin from non-defatted and defatted seabass skins

5.4.1.1 Fat content

Fat contents of gelatins extracted from non-defatted (NDFG) and defatted DFG) seabass skins are shown in Table 13. Fat content of NDFG was 1.65%, whilst DFG contained the lower fat content (0.53%). Fat in fish skin was rich in polyunsaturated fatty acids. As a result, skins used as raw material for gelatin extraction were susceptible to oxidation, causing the fishy odour in resulting gelatin. Therefore, it is necessary to remove fat from fish protein (Hou *et al.*, 2011). Thiansilakul et al. (2007) found that isopropanol could remove fat in fish muscle effectively. Varying fat contents of gelatins were reported when different fish skins were used, e.g. giant catfish (1.85%) (Sai-Ut *et al.*, 2012), Pangasius catfish (2.63%) (See *et al.*, 2010), Cobia (2.62−3.66%) (Amiza and Siti Aishah, 2011), and two species of bigeye sanpper *Priacanthus tayenus* (0.71%) and *Priacanthus macracanthus* (0.67%) (Benjakul *et al.*, 2009). The result indicated that fat content in gelatin was lowered by 68% when skin with prior defatting was used for extraction.

Table 13. Fat content, surface hydrophobicity and viscosity of gelatin extracted from non-defatted and defatted seabass skins

Samples	Fat (%, dry weight)	Surface hydrophobicity	Viscosity (mPas)
COM	0.37 ± 0.06b	1.36 ± 0.06c	9.28 ± 0.05b
NDFG	1.65 ± 0.12a	3.13 ± 0.06b	24.59 ± 1.67a
DFG	0.53 ± 0.08b	5.71 ± 0.14a	5.50 ± 0.01c

Values are presented as mean ± SD (n = 3).

Different lowercase letters in the same column indicate significant difference ($P < 0.05$). COM, NDFG and DFG represent commercial fish skin gelatin, gelatin from non-defatted seabass skin and gelatin from defatted seabass skin, respectively.

5.4.1.2 Protein patterns

Protein patterns of gelatin extracted from NDFG and DFG are shown in Figure 16. Both gelatin samples consisted of α-chain as the major constituent. Gelatin samples also contained β-components (α-chain dimers) and γ-components (α-chain trimers). NDFG showed higher band intensities of α-, β- and γ-chains than DFG. It was noted that peptides or proteins with the MW lower than α_2-chanis were noticeable in DFG. For commercial fish gelatin (COM), both α_1- and α_2-chains were dominant and it showed similar protein pattern with that found in DFG.

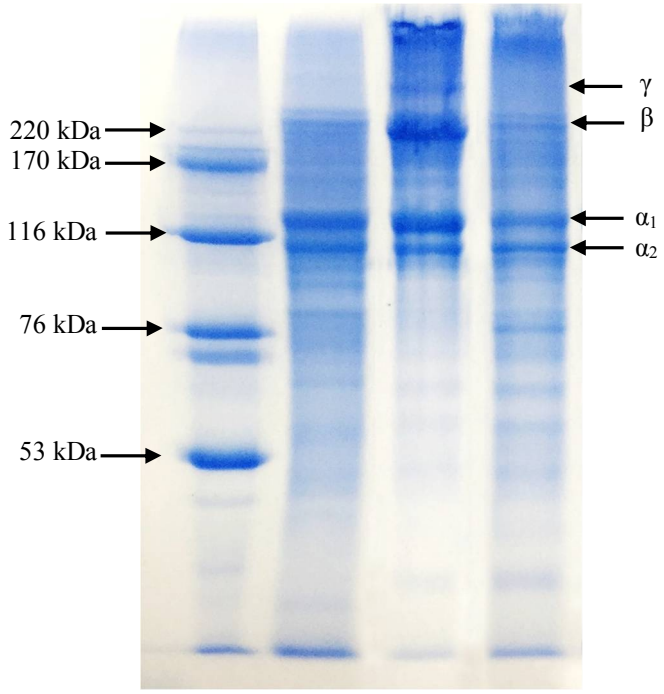

Figure 16. Protein pattern of gelatin extracted from non-defatted and defatted seabass skin. M denote high molecular weight markers. COM, NDFG and DFG represent commercial fish skin gelatin, gelatin from non-defatted seabass skin and gelatin from defatted seabass skin, respectively.

Citric acid was used to facilitate the removal of phospholipids (Yarnpakdee *et al.*, 2012). Basically, citric acid plays a role as a binding agent for basic amino acid residues of cytoskeletal proteins (Hrynets *et al.*, 2011). When isopropanol was used for further defatting, the aggregation of protein in skin matrix might be

induced. As a result, citric acid might be retained as the residue in the defatted skin. Residual citric acid plausibly induced the hydrolysis of α-, β- and γ-chains localised in the skin, especially during extraction at high temperature. The result suggested that pretreatment of seabass skins using citric acid and defatting using isopropanol affected protein components of the resulting gelatin.

5.4.1.3 Amino acid composition

Amino acid compositions of NDFG and DFG samples are shown in Table 14. Both gelatins showed similar amino acid compositions, in which glycine was the major amino acid (326–334 residues/1000 residues), followed by alanine (132–134 residues/1000 residues). Low contents of cysteine (1 residue/1000 residues), tyrosine (3–4 residues/1000 residues), histidine (5–6 residues/1000 residues) and hydroxylysine (5−6 residues/1000 residues). Generally, glycine occurs every third position in the α-chain and represents nearly one third of total residues (Benjakul *et al.*, 2009). For imino acids (proline and hydroxyproline), NDFG and DFG samples showed similar content (199 and 195 residues/1000 residues, respectively). The imino acid content of gelatin from seabass skin was higher than that reported in gelatins from splendid squid skin (184 residues/1000 residues) (Nagarajan *et al.*, 2012), grey triggerfish skin (176 residues/1000 residues) (Jellouli *et al.*, 2011), bigeye snapper skins (186–187 residues/1000 residues) (Benjakul *et al.*, 2009), salmon skin (166 residues/1000 residues) and cod skin (154 residues/1000 residues) (Arnesen and Gildberg, 2007). Nevertheless, imino content of both samples was lower than that found in COM sample (205 residues/1000 residues) and bovine gelatin (219 residues/1000 residues) reported by Jellouli et al. (2011).

5.4.1.4 Surface hydrophobicity

Surface hydrophobicity (S_0ANS) of NDFG and DFG samples is shown in Table 13. DFG showed a higher S_0ANS than NDFG ($P < 0.05$). When the skin was defatted using isopropanol, the hydrophobic domains might be more exposed and interacted with ANS probe. The increased S_0ANS indicates an exposure of the interior of the molecule (Mutilangi *et al.*, 1996). ANS, a fluorescence probe, has been found to bind to hydrophobic amino acids containing an aromatic ring, such as tyrosine,

phenylalanine and tryptophan, and can be used to indicate the surface hydrophobicity of proteins (Benjakul *et al.*, 1997). The difference in surface hydrophobicity between different gelatins might be caused by the differences in amino acid compositions and sequence, as well as chain length (Kittiphattanabawon *et al.*, 2012). Different surface hydrophobicity more likely determines interfacial properties of gelatin. Hydrophobic domains preferably migrate and localise at oil-water interphase (Townsend and Nakai, 1983).

Table 14. Amino acid compositions of gelatin extracted from non-defatted and defatted seabass skins

Amino acids (residues/1000 residues)	COM	NDFG	DFG
Alanine	121	134	132
Arginine	54	54	54
Aspartic acid/asparagine	43	44	47
Cysteine	0	1	1
Glutamic acid /glutamine	72	73	75
Glycine	333	334	326
Histidine	4	5	6
Isoleucine	11	9	10
Leucine	23	18	21
Lysine	26	27	27
Hydroxylysine	5	5	6
Methionine	10	14	14
Phenylalanine	12	13	13
Hydroxyproline	83	79	78
Proline	122	120	117
Serine	35	27	28
Threonine	23	22	24
Tyrosine	3	3	4
Valine	19	18	20
Total	1000	1000	1000
Imino acid	205	199	201

COM, NDFG and DFG represent commercial fish skin gelatin, gelatin from non-defatted seabass skin and gelatin from defatted seabass skin, respectively.

5.4.2 Functional properties of gelatin from non-defatted and defatted seabass skin

5.4.2.1 Solubility

The solubility of NDFG and DFG samples at different pH values is depicted in Figure 17. Solubility is a prerequisite for other functional properties of proteins. Homogeneous dispersibility of protein molecules in colloidal systems is also associated with the enhanced interfacial properties (Zayas, 1997). Relative solubility of both gelatins was greater than 90% at all pHs (1–10) tested. In general, solubility of NDFG was higher than that of DFG, particularly in the pH range of 3−8. It was suggested that NDFG had higher amount of hydrophilic domains. This was in accordance with the lower S_0ANS of NDFG in comparison with DFG (Table 13). The lowest solubility of seabass skin gelatin was observed at pH 7. COM sample had the lowest solubility at pH 6. The minimum solubility of proteins occurs at the isoelectric point due to the lack of electrostatic repulsion, which promotes aggregation and precipitation via hydrophobic interactions (Zayas, 1997). Generally, type-A gelatin or acid-processed gelatin has isoelectric points, varying from pH 6 to 9 (Stainsby, 1987). Kittiphattanabawon *et al.* (2012) found that the lowest solubility of gelatins from brownbanded bamboo and blacktip shark was observed at pH about 8–9. The difference in solubility of different gelatins might result from the differences in molecular weight and the content of polar and non-polar groups in amino acids (Zayas, 1997). For type B gelatin such as bovine gelatin, pI is in the acidic pH range (4−5). This was due to deamidation of glutamine and asparagine, in which glutamic acid and aspartic acid are produced (Stainsby, 1987).

5.4.2.2 Viscosity

Apart from gel strength, viscosity is the second most important commercial property of gelatin (Koli *et al.*, 2012). The viscosities of NDFG and DFG were 24.59 and 5.94 mPas, respectively, whilst COM sample had viscosity of 5.50 mPas (Table 13). The difference in viscosity of gelatins could be due to the differences in chain length or molecular size distribution. With lower average molecular weight of gelatin, the lower gel strength and viscosity of the solution were obtained (Cole, 2000).

Commercial gelatins have viscosity in the range of 2.0−7.0 mPas and viscosity may reach 13.0 mPas for gelatins with specific applications (Johnston-Banks, 1990). See et al. (2010) reported that viscosities of gelatins from snakehead, catfish, Pangasius catfish and red tilapia were 3.40, 5.24, 3.82 and 1.73 mPas, respectively. In the present study, the lower viscosity coincided well with the lower band intensity of all components including α-, β- and γ-chains found in DFG and COM samples.

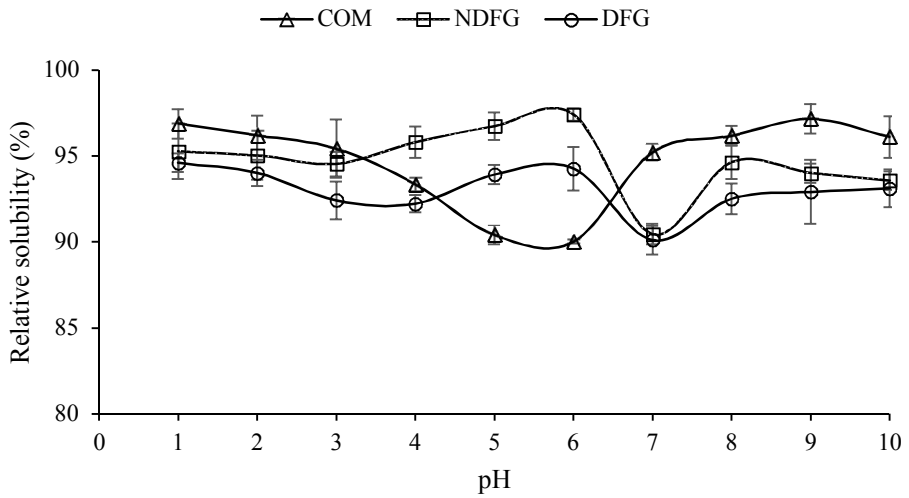

Figure 17. Relative solubility of gelatin extracted from non-defatted and defatted seabass skin. COM, NDFG and DFG represent commercial fish skin gelatin, gelatin from non-defatted seabass skin and gelatin from defatted seabass skin, respectively.

5.4.2.3 Foaming property

Foam expansion (FE) and foam stability (FS) of NDFG and DFG samples in comparison with COM sample at various concentrations (1−3%) are shown in Figure 18. FE of all gelatins increased as the concentration of gelatin increased ($P < 0.05$), except for COM sample, where no difference was found between 1 and 2% ($P > 0.05$) (Figure 18A). Protein foams with higher concentration have the denser and more stable films (Zayas, 1997). In general, the foaming ability of proteins is correlated with their film-forming ability at the air–water interface. When proteins rapidly adsorb at the newly created air–liquid interface during bubbling and undergo unfolding and molecular rearrangement at the interface, a better foaming ability can be obtained,

compared with proteins that adsorb slowly and resist unfolding at the interface (Damodaran, 1997).

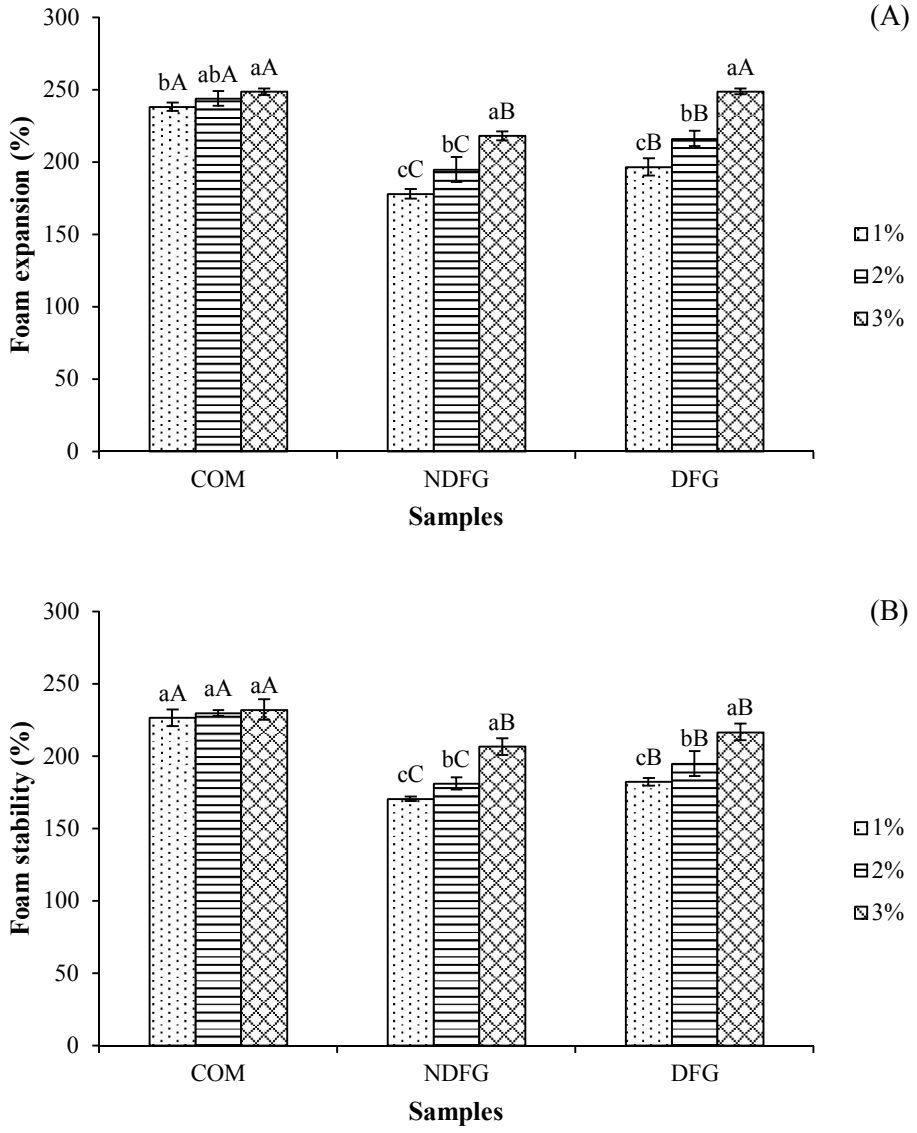

Figure 18. Foam expansion and foam stability of gelatin extracted from non-defatted and defatted seabass skin. COM, NDFG and DFG represent commercial fish skin gelatin, gelatin from non-defatted seabass skin and gelatin from defatted seabass skin, respectively. Different lowercase letters within the same samples indicate significant differences ($P < 0.05$). Different uppercase letters within the same level of gelatin indicate significant differences ($P < 0.05$). Bars represent the standard deviation (n=3).

At the same concentrations, DFG showed a higher FE than NDFG sample ($P < 0.05$). The lower foaming capacity of NDFG was in accordance with the lower S_0ANS (Table 13). Positive correlation between hydrophobicity of proteins and foaming characteristics was reported by Townsend and Nakai (1983). The foaming capacity of protein was improved by exposing more hydrophobic residues and by increasing its ability to decrease surface tension (Mutilangi *et al.*, 1996). Furthermore, DFG with higher proportion of smaller MW proteins or peptides could migrate or rearrange at the air–water interface more effectively. FS of all seabass skin gelatins increased with increasing protein concentrations ($P < 0.05$) (Figure 18B). FS of DFG was higher than NDFG sample when the same concentration was used ($P < 0.05$). FS is directly affected by protein concentration, which influences the thickness, mechanical strength and cohesiveness of film (Zayas, 1997). The stability of foams depends on various parameters, such as the rate of attaining equilibrium surface tension, bulk and surface viscosities, steric stabilisation, and electrical repulsion between the two sides of the foam lamella (Liu *et al.*, 2003). It was noted that COM sample showed higher FS than both NDFG and DFG samples at all concentrations tested ($P < 0.05$).

5.4.2.4 Emulsifying property

5.4.2.4.1 Oil droplet size distribution

Particle sizes of oil droplet in emulsions containing NDFG and DFG samples, in comparison with COM sample, expressed as the surface-weighted mean (d_{32}) and the volume-weighted mean particle diameter (d_{43}), are shown in Table 15. Generally, emulsions stabilised by NDFG had higher d_{32} and d_{43}, compared with those containing DFG and COM samples ($P < 0.05$). Similar d_{32} and d_{43} were found between emulsion containing DFG and COM samples at all storage time tested ($P < 0.05$). DFG more likely migrated and adsorbed at the surface of the newly formed oil droplets faster and more effectively than NDFG. The d_{32} is directly related to specific surface area. The smaller d_{32} indicates the higher specific surface area, which offers the increase in protein loads for adsorbing at interface of emulsions (Hebishy *et al.*, 2013). d_{43} can be used as the index of coalescence and flocculation. The increase in d_{43} reflects the association of individual droplets into larger flocs (Hebishy *et al.*, 2013).

During the storage up to 10 days, the increases in d_{32} and d_{43} were noticeable in all samples ($P < 0.05$). This indicated the instability of the emulsion, in which collapse of the emulsion by the coalescence mechanism as well as the Ostwald ripening phenomenon or assembly of individual droplets by flocculation occurred to a higher extent as the storage time increased. With extended storage time, oil droplets more likely aligned themselves closely, leading to flocculation and creaming. These phenomena could foster the coalescence of emulsion. At day 10 of storage, d_{32} and d_{43} of emulsion containing NDFG were higher than those containing DFG and COM samples ($P < 0.05$). Nevertheless, both d_{32} and d_{43} were not different between emulsions containing DFG and COM samples ($P > 0.05$). Thus, pretreatment and defatting process determined emulsifying property of gelatin from seabass skin.

5.4.2.4.2 Flocculation and coalescence

Flocculation factor (F_f) and coalescence index (C_i) of emulsions during 10 days of storage at room temperature are presented in Table 15. After emulsification, emulsion containing NDFG or DFG showed higher F_f and C_i than that containing COM sample ($P < 0.05$). During 10 day of storage, increases in F_f and C_i were observed for all emulsions. The results coincided with increases in d_{32} and d_{43} in these samples after storage. After 10 days, the highest F_f was found in the emulsion containing NDFG ($P < 0.05$). This was in agreement with the highest d_{32} and d_{43} of this sample. Emulsion underwent flocculation when the repulsive forces between the drops were not sufficiently strong. Furthermore, when adhesion energies were large enough, the adhesion could be promoted (Langevin *et al.*, 2004). Coalescence occurs when two or more oil droplets approach together and join together to form a larger one after the interfacial membrane is ruptured. The process is irreversible and results in the instability of emulsion (Long *et al.*, 2013). In the present study, the highest C_i was also found in emulsion containing NDFG after 10 days of storage ($P < 0.05$). Pretreatment and defatting thus affected the instability of emulsion differently.

5.4.2.4.3 ζ-Potential

ζ-Potential of emulsions containing different gelatins as a function of storage time is shown in Table 15. ζ-potential is the potential difference between the

Table 15. Particle size, flocculation, coalescence and ζ-potential of oil droplets in emulsions containing gelatin extracted from non-defatted and defatted seabass skins during storage

Samples	Storage time (day)	d_{32} (μm)	d_{43} (μm)	Flocculation factor (F_f)	Coalescence index (C_i)	ζ-Potential (mV)
COM	0	0.74 ± 0.03bB	0.74 ± 0.03cB	0.89 ± 0.04cB	-	20.85 ± 1.47aA
	5	0.77 ± 0.02bB	0.79 ± 0.01bB	1.18 ± 0.06bA	6.39 ± 1.74bA	19.07 ± 1.53bA
	10	0.83 ± 0.01aB	0.83 ± 0.01aB	1.52 ± 0.03aB	12.56 ± 1.83aB	17.48 ± 0.75cA
NDFG	0	1.07 ± 0.01cA	1.04 ± 0.06 bA	1.03 ± 0.02cA	-	17.87 ± 1.59aC
	5	1.12 ± 0.02bA	1.12 ± 0.02aA	1.19 ± 0.05bA	7.88 ± 2.02bA	16.90 ± 0.85abB
	10	1.19 ± 0.02aA	1.19 ± 0.02aA	1.59 ± 0.04aA	17.16 ± 1.42aA	15.56 ± 0.52bB
DFG	0	0.75 ± 0.03cB	0.75 ± 0.03cB	1.01 ± 0.01cA	-	19.40 ± 1.65aB
	5	0.80 ± 0.01bB	0.80 ± 0.01bB	1.19 ± 0.05bA	7.06 ± 1.99bA	18.24 ± 0.96bA
	10	0.86 ± 0.01aB	0.85 ± 0.01aB	1.49 ± 0.03aB	13.17 ± 1.64aB	16.91 ± 0.59cA

Values are presented as mean ± SD (n = 3).

Different lowercase letters in the same column within the same sample indicate significant difference ($P < 0.05$).

Different uppercase letters in the same column within the same storage time indicate significant difference ($P < 0.05$).

COM, NDFG and DFG represent commercial fish skin gelatin, gelatin from non-defatted seabass skin and gelatin from defatted seabass skin, respectively.

dispersion medium and the stationary layer of fluid attached to the dispersed droplet (El-sayed *et al.*, 2012). This value provides the useful information on the electrostatic repulsion between oil droplets, closely related to coalescence and stability phenomena (Wang *et al.*, 2010). A high ζ-potential implies the resistance to cohesion or coalescence of emulsion because of the electrostatic repulsion of adjacent emulsion droplets (Ishii and Nii, 2014). Oil droplets in all emulsions had the positive charge. At day 0, the lowest ζ-potential was noticeable for emulsion containing NDFG, followed by DFG and COM samples, respectively. With increasing storage time, ζ-potential of all samples decreased. The layer of protein surrounding droplets might undergo aggregation via ionic interaction during the extended storage as indicated by the decrease in ζ-potential. The insufficient electrostatic repulsion might lead to the development of flocculation and coalescence. After 10 days of storage, emulsion containing NDFG had the lowest positive charge ($P < 0.05$). The differences in charge directly determined the repulsion between oil droplets, thereby affecting emulsion stability in different fashions.

5.5. Conclusion

Pretreatment using citric acid and defatting using isopropanol of seabass skin had an impact on components and functional properties of resulting gelatin. Gelatin obtained from defatted skin had higher solubility, foaming properties as well as emulsion stability effect than that prepared from non-defatted skin. Functional properties of gelatin obtained from defatted skin were generally similar to commercial tilapia skin gelatin. Therefore, gelatin from defatted seabass skin could serve as an alternative source for production of gelatin with property equivalent to commercial gelatin.

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CHAPTER 6

EFFECT OF PRETREATMENTS AND DRYING METHODS ON PROPERTIES AND FISHY ODOUR/FLAVOUR OF GELATIN FROM SEABASS (*LATES CALCARIFER*) SKIN

6.1 Abstract

Effects of different pretreatments of seabass skin and various drying methods on properties and fishy odour/flavour of resulting gelatin were evaluated. All gelatins contained α- and β-chains as the predominant components. Generally, a higher gel strength was found in the freeze-dried gelatin, compared with spray-dried counterpart ($P < 0.05$). Gel strength of gelatin decreased as the inlet temperature for spray drying increased ($P < 0.05$). All gelatin samples had creamy whitish colour but became more yellow as the inlet temperature for spray drying increased. All gelatin gels were sponge or coral-like in structure. Gelatin from skin pretreated with citric acid had lower fishy odour/flavour than that from skin pretreated using acetic acid. The lower fishy odour/flavour with coincidentally lower abundance of volatile compounds, including aldehydes, ketones and alcohols, etc. was found in gelatin obtained by spray drying, in comparison with freeze-dried counterpart. The lower fishy odour/flavour in spray-dried gelatin was in accordance with the lower thiobarbituric acid reactive substances and peroxide values. Thus, spray drying in conjunction with an appropriated pretreatment could be an effective method for production of gelatin with negligible undesirable fishy odour and flavour.

6.2 Introduction

Gelatin is a protein obtained from partial denaturation or hydrolysis of collagen. It is widely used in food, pharmaceutical, cosmetic, and photographic applications because of its unique functional and technological properties (Regenstein and Zhou, 2007). Generally, gelatin is obtained from mammals, especially pig and cow skins and bones. However, outbreaks of mad cow disease (bovine spongiform encephalopathy, BSE) caused some concern for customers. Additionally, porcine

gelatin cannot be used in Kosher and Halal foods due to religious constraints (Kittiphattanabawon *et al.*, 2010). As a consequence, alternative sources for gelatin production have gained increasing attention. Fish processing byproducts including skin, scale or bone, etc. have been used for gelatin production due to their abundance and low cost (Benjakul *et al.*, 2012).

Seabass (*Lates calcarifer*) is an economically important fish species in Thailand and other countries, especially tropical and subtropical regions of Asia and Pacific. During processing or dressing of seabass, skin is generated and considered as a byproduct. Recently, seabass skin has been used to produce collagen, gelatin as well as bioactive hydrolysates (Senphan and Benjakul, 2014; Sinthusamran *et al.*, 2013). However, fishy odour associated with gelatin, particularly that extracted from unfresh fish skin, can limit its range of applications, especially as human food ingredients or supplements. Fishy flavour and odour in protein hydrolysate from Nile tilapia muscle caused by lipid oxidation were also reported by Yarnpakdee *et al.* (2012). Therefore, undesirable fishy flavour and odour can markedly limit the utilisation of gelatin from fish skin.

Drying is a process used for food preservation throughout the world. The most common methods applied in food industry, apart from conventional air drying, are spray drying and freeze drying (Tsami *et al.*, 1998). Conventional air drying is a slow process. The products obtained, when compared to the non-processed products, are generally characterised by low porosity, high apparent density and reduced quality (Ratti, 2001). Freeze drying is based on the removal of water by sublimation of the frozen product (Ratti, 2001). Powders obtained after freeze drying generally have low bulk density, high porosity as well as good aroma and taste retention. However, the process is time consuming and requires a large amount of energy. It has been reported that the freeze drying process is 4–5 times more expensive when compared with spray drying, and approximately nine times more expensive than single-stage evaporation process (Hammami and René, 1997). Spray drying is not a one-step processing. Redispersion, heat treatment, homogenization and evaporation are important processes prior to spray drying (Hayashi, 1989). This process is widely applied in food industry due to many advantages of the powders gained, such as good quality and low water

activity. Spray drying can be an approach to remove undesirable odour, especially fishy odour from fish derived products. Sai-Ut *et al.* (2014) prepared gelatin hydrolysate from the skin of unicorn leatherjacket with reduced off-odour using spray drying. During the transformation of liquid feed into dry powder at high temperature, volatile odourous compounds can be eliminated to some degree. Appropriate pretreatment such as the removal of membrane lipid and depot fat of skin before gelatin extraction along with effective drying method could be a promising means to lower fishy odour in fish skin gelatin. The objective of this study was to investigate the effect of different pretreatments and drying methods on the properties and fishy odour/flavour intensity of gelatin from seabass skin.

6.3 Materials and methods

6.3.1 Chemicals

L-leucine and 1,1,3,3-tetramethoxypropane were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Sodium dodecyl sulphate (SDS), Coomassie Blue R-250, and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). High molecular weight markers were obtained from GE Healthcare UK Limited (Buckinghamshire, UK). Ferrous chloride was obtained from Merck (Darmstadt, Germany). Disodium hydrogen phosphate, 2-thiobarbituric acid and cumene hydroperoxide were purchased from Fluka (Buchs, Switzerland). Food grade bovine bone gelatin was obtained from Halagel (Thailand) Co., Ltd., (Bangkok, Thailand).All chemicals were of analytical grade.

6.3.2 Collection of seabass skins

Descaled skins of fresh seabass (*L. calcarifer*) with a weight of 2.5–3 kg were obtained from the Kingfisher Holdings Co., Ltd., Songkhla, Thailand. Skins were kept in a polystyrene box containing ice using a skin/ice ratio 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h after deskinning process. Upon arrival, the remaining meat was removed manually. The skins were washed with cold tap water (≤ 10 ºC). The skins were pooled and used as the composite sample. The samples were placed in polyethylene bags and stored at

–20 ºC until used, but not longer than 2 months. Prior to gelatin extraction, the frozen skins were thawed with running water (25–26 ºC) until the core temperature reached 8–10ºC. The skins were then cut into small pieces (1.0 × 1.0 cm^2) using scissors.

6.3.3 Preparation of seabass skin

6.3.3.1 Removal of non-collagenous proteins

The skins were soaked in 0.1 M NaOH with a skin/alkaline solution ratio of 1:10 (w/v) in order to remove non-collagenous proteins. The mixture was stirred for 3 h at room temperature (28–30ºC) using an overhead stirrer model RW20.n (IKA®-Werke GmbH & CO.KG, Staufen, Germany) at a speed of 300 rpm. The alkaline solution was changed every 1 h for totally 3 times. Alkaline-treated skin was washed with tap water until a neutral or slightly basic pH (7.0–7.5) of wash water was obtained.

6.3.3.2 Acid pretreatment and defatting

After treatment with alkaline solution, the obtained skins were subjected to pretreatment using 0.05 M acetic acid or 0.05 M citric acid at a skin/solution ratio of 1:10 (w/v).The mixture was stirred at room temperature for 2 h. The swollen skins were washed using tap water until wash water became neutral or slightly acidic in pH (6.5-7.0).

Citric acid pretreated skins were defatted using 30% isopropanol with a solid/solvent ratio of 1:10 (w/v) at room temperature for 1 h. The mixture was shaken continuously at 200 rpm. The solvent was then removed and the defatted skins were washed with 10 volumes of tap water. The defatted skins were then rinsed with distilled water.

6.3.4 Extraction of gelatin

Skins pretreated with acetic acid or citric acid, followed by defatting were subjected to gelatin extraction. To extract gelatin, pretreated skins were mixed with distilled water at a ratio of 1:10 (w/v) at 55 ºC in a water bath (W350, Memmert, Schwabach, Germany) and stirred continuously for 6 h using an overhead stirrer (RW

20.n, IKA®-Werke GmbH & CO.KG, Staufen, Germany) at a speed of 150 rpm. The mixtures were then filtered using a Buchner funnel with a Whatman No. 4 filter paper (Whatman International, Ltd., Maidstone, England). The solution had 1.28% solid content (w/v).

6.3.5 Drying of gelatin

Gelatin solutions (1.27% solid) were dried by freeze drying or spray drying. For freeze drying, gelatin solution was frozen at -40 ºC for 6 h. The frozen samples were subjected to sublimation using a freeze dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark) at –50 ºC for 72 h. Freeze-dried gelatins obtained from seabass skin pretreated with acetic acid or citric acid, followed by defatting were referred to as 'FD-AC' and 'FD-CI', respectively.

The spray drying was performed using a spray dryer (LabPlant SD-06 Basic, North Yorkshire, England) equipped with a spry-drying chamber having the dimensions of 500 mm height and 210 mm diameter and a spray nozzle type of two-liquid nozzle (0.5 mm in size). A cyclone separator, a hot-air blower, and an exhaust blower were equipped. The gelatin solution was fed by a peristaltic pump at 485 ml/h into the chamber, atomised by hot air (air velocity of 2 m/s) from the blower in a downward current flow mode, using the following process conditions: inlet temperature of 160, 180 and 200 ºC, and an atomizing pressure of 2.8 bars. The samples obtained from skin pretreated with acetic acid or citric acid, followed by defatting and spray-dried with the inlet temperatures of 160, 180 and 200 ºC were referred to as 'SD-AC-160', 'SD-AC-180', 'SD-AC-200', 'SD-CI-160', 'SD-CI-180' and 'SD-CI-200', respectively. Moisture contents of gelatin powders were 6.79, 4.01, 4.00, 4.05, 6.04, 4.42, 4.28 and 4.10% for FD-AC, SD-AC-160, SD-AC-180, SD-AC-200, FD-CI, SD-CI-160, SD-CI-180 and SD-CI-200, respectively. The gelatin powders were transferred into the double zipped bag and kept in plastic vacuum box prior to storage at –40 ºC. The storage time was not longer than 1 month. Dried gelatin samples were subsequently subjected to analyses.

6.3.6 Analyses

6.3.6.1 Protein patterns

Protein patterns were determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) with slight modifications. The gelatin samples (15 mg/ml protein) were dissolved in 5% SDS and the mixtures were incubated at 85 ºC for 1 h using a temperature controlled water bath model W350 (Memmert, Schwabach, Germany). Solubilised samples were mixed at a 1:1 (v/v) ratio with sample buffer (0.5 M Tris–HCl, pH 6.8 containing 5% SDS and 20% glycerol). Samples were loaded onto a polyacrylamide gel made of 7.5% separating gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA/gel. After electrophoresis, gels were stained with 0.05% (w/v) Coomassie blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid for 30 min. Finally, they were destained with the mixture of 50% (v/v) methanol and 7.5% (v/v) acetic acid for 30 min and destained again with the mixture of 5% (v/v) methanol and 7.5% (v/v) acetic acid for 1 h. High molecular weight protein markers (GE Healthcare UK Limited, Buckinghamshire, UK) including myosin (220 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa) and glutamic dehydrogenase (53 kDa) were used to estimate the molecular weight of proteins.

6.3.6.2 Gel strength

Gelatin gel was prepared as per the method of Fernández-Díaz *et al.* (2001) with a slight modification. Gelatin (6.67%, w/v) was dissolved in distilled water at 60 °C and stirred until the gelatin was completely solubilised before cooling in a refrigerator at 4 °C for 16–18 h for gel maturation. Bovine gelatin gel was also prepared in the same manner. The dimension of the sample was 3 cm in diameter and 2.5 cm in height. Gel strength of samples was determined at 8–10 ºC using a texture analyser Model TA-XT2 (Stable Micro System, Surrey, UK) with a load cell of 5 kN, cross-head speed of 1 mm/s and equipped with a 1.27 cm diameter flat-faced cylindrical Teflon plunger. The maximum force (in gram) was recorded when the penetration distance reached 4 mm.

6.3.6.3 Fourier transform infrared (FTIR) spectra

Attenuated total reflectance Fourier transform infrared spectrometer model Equinox 55 (Bruker Co., Ettlingen, Germany) equipped with a horizontal ATR trough plate crystal cell (45° ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology, Inc., Madison, WI, USA) was used. For spectral analysis, samples were placed onto the crystal cell and the cell was clamped into the mount of FTIR spectrometer. The spectra, in the range of 4000-400 cm^{-1} (mid-IR region) with automatic signal gain, were collected over 32 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean and empty cell at 25 °C. Analysis of spectral data was carried out using the OPUS 3.0 data collection software programme (Bruker Co., Ettlingen, Germany).

6.3.6.4 Solubility

Solubility of gelatin was determined, following the method of Kittiphattanabawon *et al.* (2012) with a slight modification. The gelatin sample was dissolved in distilled water at 60 °C to obtain a final concentration of 1% (w/v) and the mixture was stirred at room temperature (28–30 °C) for 30 min. The mixture (8 ml) was transferred to a centrifuge tube and the pH was adjusted with either 2 N NaOH or 2 N HCl to obtain a neutral pH. The volume of solution was made up to 10 ml with distilled water. The solution was centrifuged at 8,500 ×*g* at room temperature for 10 min using a microcentrifuge (MIKRO20, Hettich Zentrifugan, Germany). Protein content in the supernatant was determined by the Biuret method(Robinson and Hogden, 1940) using bovine serum albumin as a standard. Protein solubility was calculated, compared with total protein content of the sample. Total protein content was determined after complete solubilisation of the sample using 0.5 M NaOH.

6.3.6.5 Colour

Colour of spray-dried gelatin powder was measured by a Hunter Lab Colourimeter (Color Flex, Hunter Lab Inc., Reston, VA, USA). For lyophilised gelatin, it was cut into small pieces prior to colour measurement. Samples were spread over the plate and L^*, a^* and b^* indicating lightness/brightness, redness/greenness and

yellowness/blueness, respectively, were recorded. The colourimeter was calibrated with a white standard. Total difference in colour (ΔE^*) was calculated according to the following equation:

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

where ΔL^*, Δa^* and Δb^* are the differences between the corresponding colour parameter of the sample and that of white standard ($L^* = 93.55$, $a^* = -0.84$, $b^* = 0.37$).

6.3.6.6 Microstructure

The microstructure of gelatin gel was visualised using a scanning electron microscopy (SEM). Gelatin gels (2–3 mm thick) were fixed with 2.5% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 12 h. The samples were then rinsed with distilled water for 1 h and dehydrated in ethanol with a serial concentration of 50, 70, 80, 90 and 100% (v/v). Samples were subjected to critical point drying. Dried samples were mounted on a bronze stub and sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA). The specimens were observed with a scanning electron microscope (JEOL JSM-5800 LV, Tokyo, Japan) at an accelerating voltage of 20 kV.

6.3.6.7 Peroxide value (PV)

PV was determined according to the method of Richards and Hultin (2002) with slight modifications. 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 a homogeniser. The homogenate was then filtered using Whatman No. 1 filter paper (Whatman International, Ltd., Maidstone, England). 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 °C using a refrigerated centrifuge (Avanti® J-E, Beckman Coulter, Palo Alto, CA, USA) to separate the sample into two phases. 25 µl of Ammonium thiocyanate (30%, w/v) and 25 µl of 20 mM iron (II) chloride were added to 3 ml of lower phase and the reaction mixture was allowed to stand for 20 min at room temperature prior to reading the absorbance at 500 nm. A blank was prepared

in the same manner, except that distilled water was used instead of ferrous chloride. A standard curve was prepared using cumene hydroperoxide over a concentration range of 0.5–2 ppm. PV was expressed as mg cumene hydroperoxide equivalents/100 g sample.

6.3.6.8 Thiobarbituric acid reactive substances (TBARS)

TBARS were determined as described by Buege and Aust (1978)with slight modifications. 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 mM 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 3600 ×*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 6 ppm. TBARS value was calculated and expressed as mg malonaldehyde equivalents/100 g sample.

6.3.6.9 Sensory evaluation

Sensory evaluation for fishy odour/flavour intensity was carried out according to the method of Yarnpakdee *et al.* (2012) with a slight modification. Evaluation of fishy odour and flavour was conducted by 8 trained panelists between the ages of 25–32. Prior to the evaluation, the panelists were trained three times a week. Panelists were trained with standards for two sessions with the score ranging from none (score = 0) to extremely strong fishy odour and flavour (score = 4). Gelatin produced from 18 day ice-stored fish was used as a source of fishy odour and flavour. Working standards were prepared by dissolving gelatin in water to obtain concentration of 0, 1 and 2% (w/v), representing scores of 0, 2 and 4, respectively. To test the samples, all gelatin samples (1.5%, w/v) were placed in a sealable plastic cup and heated at 60 ºC in a temperature controlled water bath for 5 min before serving. The panelists were asked to open the sealable cup and sniff the headspace above the samples in order to determine the intensity of fishy odour. To evaluate the fishy flavour, the panelists were asked to taste the sample and rinse their mouths between samples.

6.3.6.10 Volatile compounds

Volatile compounds in gelatin powder samples were determined using a solid-phase micro-extraction gas chromatography mass spectrometry (SPME-GC-MS) following the method of Iglesias and Medina (2008) with a slight modification.

6.3.6.10.1 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 disperse the sample. The mixture was heated at 60 °C in 20 ml headspace vial with equilibration time of 10 h. The SPME fibre (50/30 µm DVB/Carboxen™/ PDMS StableFlex™) (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 extract and 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.

6.3.6.10.2 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 programme was: 35 °C for 3 min, followed by an increase to 70 °C at 3 °C/min, then an increase of 10 °C/min to 200 °C, and finally an increase of 15 °C/min to a final temperature of 250 °C 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 °C. Transfer line temperature was maintained at 260 °C. The quadrupole mass spectrometer was operated in the electron ionisation (EI) mode and source temperature was set at 250 °C. Initially, full scan mode data was 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.

6.3.6.10.3 Analyses of volatile compounds

Identification of volatile compounds in the samples was done by consulting ChemStation Library Search (Wiley 275.L). Identification of compounds 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 were calculated to a signal-to-noise (S/N) ratio of 10. The volatile compounds identified related to lipid oxidation, included aldehydes, alcohols, ketones, etc., and were expressed in the terms of relative abundance.

6.3.7 Statistical analysis

Experiments were run in triplicate using three different lots of samples. The data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test. 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 Effects of pretreatments and drying methods on characteristics and properties of gelatin

6.4.1.1 Protein patterns

Protein patterns of gelatin extracted from the skin of seabass subjected to different pretreatments and drying methods are shown in Figure19. All gelatin samples contained α-chain with a MW of 130–120 kDa as the major constituent. Gelatin samples also consisted of β-component (α-chain dimer) and γ-component (α-chain trimer). The protein patterns of gelatin were similar to those found in the gelatin of seabass skin reported by Sinthusamran *et al.* (2014). When comparing the band intensity between gelatin extracted from skin pretreated with acetic acid and citric acid,

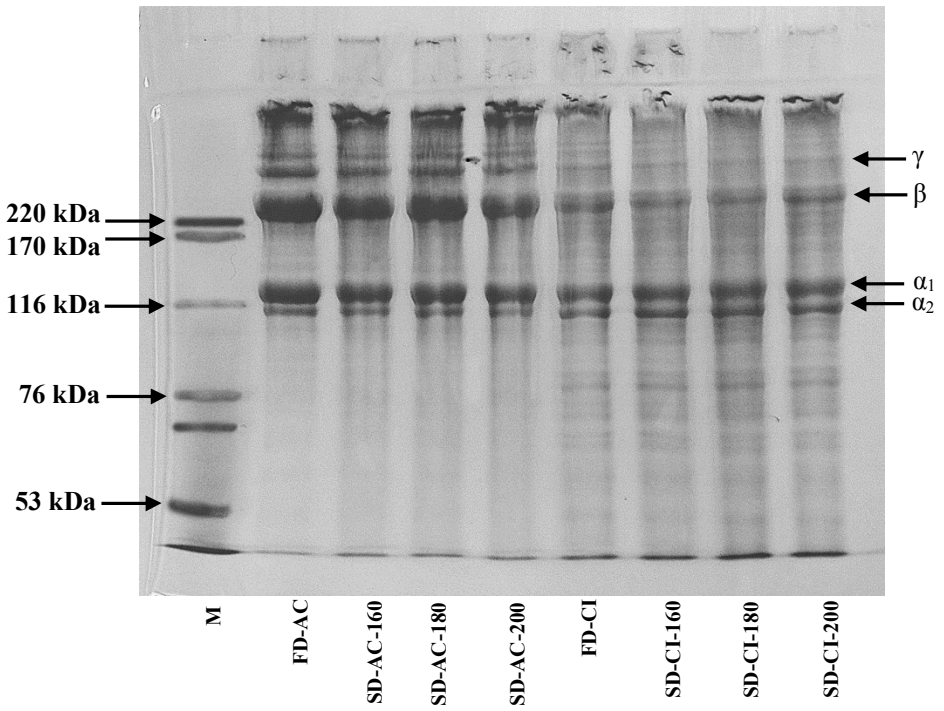

Figure 19. Protein pattern of gelatin extracted from the skin of seabass with different pretreatments and drying methods. M denote high molecular weight markers. FD-AC and FD-CI represent freeze dried gelatins from skin pretreated with acetic and citric acid, followed by defatting, respectively. SD-AC-160, SD-AC-180 and SD-AC-200 represent spray dried gelatins obtained from skin pretreated with acetic acid and spray dried at inlet temperature of 160, 180 and 200 ºC, respectively. SD-CI-160, SD-CI-180 and SD-CI-200 represent spray dried gelatins obtained from skin pretreated with citric acid, followed by defatting and spray dried at inlet temperature of 160, 180 and 200 ºC, respectively.

it was found that gelatins prepared from acetic acid had higher band intensity of all components, compared with those pretreated with citric acid. Citric acid plays a role as a binding agent for basic amino acid residues of cytoskeletal proteins (Benjakul *et al.*, 2012). The acid might be therefore retained in the skin to some extent. When defatting process was implemented, the aggregation of protein might be induced. As a result, those acids could be retained as the residue in the resulting gelatin. Citric acid retained plausibly induced the hydrolysis of α-, β- and α-chains of gelatin, especially during

extraction at high temperature. It was noted that the degradation peptides or proteins with the MW lower than α_2-chains were noticeable in gelatin from skin pretreated with citric acid, regardless of drying methods. During gelatin extraction, the cleavage of a number of intra- and intermolecular covalent crosslinks might take place to different degrees, depending on processing parameters (temperature, time, and pH), pretreatment, and properties and preservation method of the starting raw material used (Karim and Bhat, 2009). Kittiphattanabawon *et al.* (2010) also reported the difference in degradation between gelatin from skin of two shark species. No marked difference in protein patterns was observed for gelatin from skin pretreated with acetic acid, when spray drying at different inlet temperatures was used. Nevertheless, the spray-dried sample from skin pretreated with citric acid showed slight increase in degradation, compared with the corresponding freeze-dried sample. The result suggested that pretreatment and defatting of raw material played a role in protein components of the resulting gelatin.

6.4.1.2 Gel strength

Gel strength of gelatin extracted from seabass skin with different pretreatments and drying methods is depicted in Figure 20. Gel strength is one of the most important functional properties of gelatin. In the present study, gelatin from seabass skin had a higher gel strength (221–331 g) than bovine gelatin (207 g) ($P < 0.05$), irrespective of pretreatment and drying methods used. Generally freeze-dried gelatin had higher gel strength than spray-dried counterpart ($P < 0.05$). Amongst all samples, the highest gel strength (331 g) was found in FD-AC ($P < 0.05$). For the gelatin prepared with the same pretreatment, gel strength decreased as the spray drying temperatures increased ($P < 0.05$). However, no difference in gel strength between SD-CI-180 and SD-CI-200 was observed ($P > 0.05$). Higher drying temperature most likely caused protein degradation, thereby producing protein fragments and lowering gelling ability. A weak gelatin gel was associated with the formation of small fragments (Gómez-Guillén *et al.*, 2002). Freeze- dried gelatin from shark cartilage showed a higher gel strength than spray-dried gelatin (Kwak *et al.*, 2009). The decrease in gel strength was in accordance with the slight decreases in α-, β- and γ-chains band intensity of gelatin dried at higher temperatures (Figure19). The amount of β- and γ-

components and the amino acid composition of gelatin were reported as the factors governing gelation of gelatin (Taheri *et al.*, 2009). The presence of protein degradation fragments in gelatin might reduce the ability of α-chains to anneal correctly during stabilisation overnight and thus hindering the growth of the existing nucleation sites (Ledward, 1986). The difference in gel strength could be due to the differences in intrinsic characteristics, such as molecular weight distribution, the chain length of protein as well as complex interactions determined by the amino acid composition and the ratio of α/β chains present in the gelatin (Badii and Howell, 2006). The configuration of protein and the way the inter-junction was developed to form the stronger network were therefore crucial for gel formation. Location of imino acids in the peptide chain also contributed to gel formation (Ledward, 1986). Thus, pretreatments and drying methods generally affected the gel formation of gelatin from seabass skin.

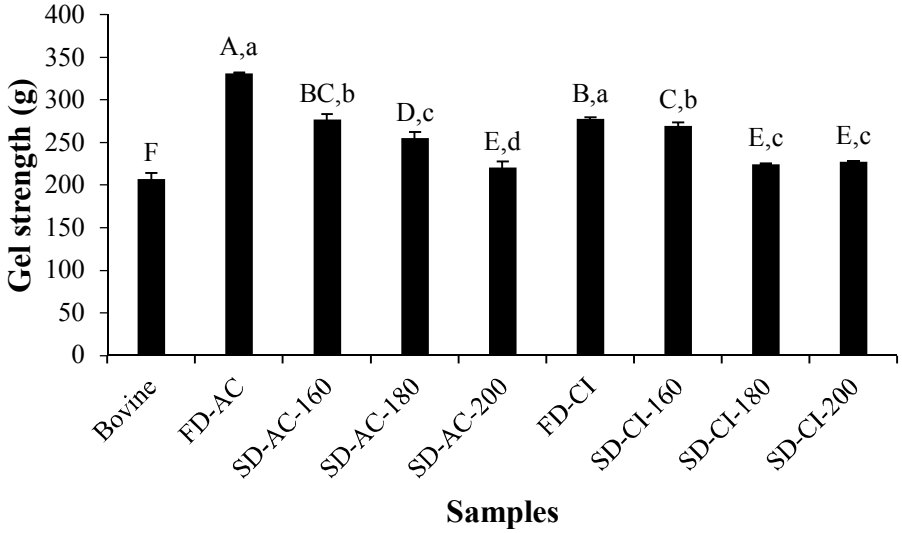

Figure 20. Gel strength of gelatin extracted from the skin of seabass with different pretreatments and drying methods. Bars represent the standard deviation (n= 3). Different uppercase letters on the bars indicate significant differences ($P < 0.05$). Different lowercase letters on the bars within the same pretreatment indicate significant differences ($P < 0.05$). Key: see the caption for Figure19.

6.4.1.3 Solubility

Solubility of gelatin powder as influenced by pretreatments and drying methods is presented in Table 16. Solubility is a prerequisite for other functional properties of proteins or peptides. Soluble proteins provide a homogeneous dispersibility of the molecules in colloidal systems and enhance the interfacial properties (Thiansilakul *et al.*, 2007). For the gelatin with the same pretreatment, solubility of gelatin obtained by spray drying was higher than that obtained from freeze drying ($P < 0.05$). The relative solubility of gelatin samples was greater than 90%, except for FD-AC (81.22%). The solubility of gelatin obtained by spray drying at higher inlet temperatures was generally higher than that of gelatin spray dried at lower temperatures. It was suggested that gelatin dried at higher inlet temperatures contained smaller peptides with higher amount of hydrophilic groups (Kittiphattanabawon *et al.*, 2012). During drying, proteins might be aligned and aggregated to some degree. This led to the decreased solubility of gelatin dried with either freeze- or spray-drying.

6.4.1.4 Colour of gelatin powder

The colour of gelatin powder from seabass skin obtained from various pretreatments and drying methods expressed as L^*, a^* and b^* is shown in Table 16. All samples had creamy whitish colour. SD-AC-160 exhibited the highest L^*- value (lightness), whilst FD-AC showed the lowest L^*- value ($P < 0.05$). The higher b^*-value (yellowness) was found for gelatins from skin pretreated with acetic acid, compared with those pretreated with citric acid, when the same drying condition was used. SD-AC-200 had the highest b^*-value ($P < 0.05$). With citric acid pretreatment, pigments in the skin were more likely removed to a higher extent, compared with that pretreated with acetic acid. Additionally, defatting using isopropanol might facilitate the removal of some particular pigments. Amongst all gelatin samples, FD-AC showed the highest ΔE^* (total colour difference). This was concomitant with the lowest lightness (L^*-value). It was noted that a^*- and b^*-values of gelatin increased with increasing inlet temperature for spray drying ($P < 0.05$). During spray drying, carbonyl compounds (e.g. aldehydes and ketones) mainly from lipid oxidation were formed. Those compounds could react with amino groups of free amino acids or peptides via non-enzymatic

Table 16. Solubility, colour, PV, TBARS and fishy odour/flavour intensities of gelatin from seabass skin with different pretreatments and drying methods

Samples	FD-AC	SD-AC-160	SD-AC-180	SD-AC-200	FD-CI	SD-CI-160	SD-CI-180	SD-CI-200
Solubility (%)	81.22 ± 2.62C,c	91.81 ± 1.34B,b	95.76 ± 1.00A,a	93.12 ± 1.35B,ab	92.62 ± 0.61B,c	95.52 ± 0.63A,b	95.59 ± 0.88A,b	97.83 ± 0.54A,a
*L**	69.06 ± 0.21G,d	96.63 ± 0.03A,a	95.85 ± 0.07C,b	95.35 ± 0.19D,c	81.09 ± 0.27F,d	96.21 ± 0.06B,a	95.69 ± 0.10C,b	93.99 ± 0.37E,c
*a**	-0.50 ± 0.07E,d	-0.44 ± 0.02D,c	-0.35 ± 0.01B,b	-0.30 ± 0.02A,a	-0.40 ± 0.03CD,c	-0.39 ± 0.01BC,ab	-0.38 ± 0.02BC,ab	-0.36 ± 0.04BC,a
*b**	6.09 ± 0.12C,c	5.80 ± 0.02D,d	6.82 ± 0.11B,b	7.40 ± 0.11A,a	1.94 ± 0.05G,c	4.37 ± 0.05F,b	5.01 ± 0.10E,a	5.14 ± 0.22E,a
Δ*E**	25.15 ± 0.23A,a	6.25 ± 0.02E,d	6.87 ± 0.09D,c	7.28 ± 0.08C,b	12.56 ± 0.26B,a	4.83 ± 0.02G,c	5.13 ± 0.07F,b	4.82 ± 0.21G,c
PV (mg cumene hydroperoxide equivalents/100 g sample)	32.08 ± 0.76A,a	19.90 ± 0.62E,b	20.23 ± 0.54E,b	20.74 ± 0.33DE,b	30.50 ± 0.22B,a	21.87 ± 0.75C,b	21.58 ± 0.66CD,b	21.55 ±0.30CD,b
TBARS (mg malonaldehyde equivalents/100 g sample)	4.41 ± 0.00A,a	3.72 ± 0.01C,b	3.37 ± 0.04D,c	2.57 ± 0.01E,d	4.16 ± 0.00B,a	2.58 ± 0.00E,b	2.43 ± 0.00F,c	2.02 ± 0.01G,d
Fishy odour	2.65 ± 0.32A,a	2.27 ± 0.39AB,a	2.23 ± 0.40AB,a	1.51 ± 0.57C,b	2.18 ± 0.41B,a	1.63 ± 0.48C,b	0.90 ± 0.27D,c	0.70 ± 0.40D,c
Fishy flavour	3.17 ± 0.24A,a	2.81 ± 0.34AB,ab	2.60 ± 0.54B,b	1.64 ± 0.56CD,c	2.42 ± 0.43B,a	1.89 ± 0.39C,b	1.28 ± 0.44DE,c	0.91 ± 0.35E,c

Values are expressed as mean ± SD (n = 3).
Different uppercase letters within the same row indicate significant differences ($P < 0.05$).
Different lowercase letters within the same row under the same pretreatment indicate significant difference ($P < 0.05$).
Key: see the caption for Figure19.

browning reaction. This could result in a product with more yellow colour in gelatin spray-dried at higher temperature as indicated by the increased *b**-value. Lipid oxidation products played a role in yellow discolouration by providing carbonyl groups involved in the Maillard reaction (Khantaphant *et al.*, 2011).

6.4.1.5 Lipid oxidation

Lipid oxidation of gelatin with different pretreatments and drying methods was monitored by measuring PV and TBARS values (Table 16). PV is an indicator of the initial stages of lipid oxidation. PV of gelatin obtained by freeze drying was higher than that obtained by spray drying ($P < 0.05$). The higher PV of freeze dried gelatin indicated the higher presence of hydroperoxide generated via lipid oxidation process. Lipid hydroperoxides are formed via several pathways including the reaction of singlet oxygen with unsaturated fatty acids or the lipoxygenase-catalysed oxidation of polyunsaturated fatty acids (Jacobsen, 2010). Hydroperoxide might be decomposed to small MW components when spray drying was implemented. No differences in PV were found when inlet temperatures of spray drying increased ($P > 0.05$). At high temperature, hydroperoxides are readily decomposed to alkoxy radicals and then form a wide variety of decomposition products, including aldehydes, ketones, acids esters, alcohols, and short-chain hydrocarbons (Kim and Min, 2008).

When comparing TBARS of gelatins prepared from the same pretreated skin, higher values were observed in freeze-dried samples, compared with spray-dried counterparts. TBARS of gelatin samples from spray drying process decreased with increasing inlet temperature ($P < 0.05$). The result suggested that the secondary volatile lipid oxidation products were more removed from the samples when inlet temperature of spray drying increased. The detectable TBARS in gelatin most likely indicated the decomposition of hydroperoxides into the secondary oxidation products in the later stages of lipid oxidation (Jacobsen, 2010). Hydroperoxides break down in several steps, yielding a wide variety of decomposition products including aldehydes, etc. Lipid oxidation generates a wide range of secondary aldehyde products, including n-alkanals, trans-2-alkenals, 4-hydroxy-trans-2-alkenals and malonaldehyde (Jacobsen, 2010). At the same inlet temperature used for spray drying, the lower TBARS were found for the

sample prepared from skin pretreated using citric acid. Citric acid was reported to help in removal of membrane lipids (Liang and Hultin, 2005). As a result, the oxidation in gelatin occurred to a lower extent.

6.4.1.6 Fishy odour/flavour intensity

Fishy odour/flavour intensity of gelatin extracted from various pretreated skins with different drying methods is shown in Table 16. Odour and flavour are one of the most important sensory characteristics that determine acceptance or rejection of food. Gelatin from seabass skin pretreated with citric acid, followed by defatting had lower fishy odour/flavour, compared with those extracted from skin pretreated using acetic acid ($P < 0.05$). This suggested the removal of membrane lipids using citric acid. Fishy odour/flavour of gelatin from spray drying decreased as the inlet temperature increased ($P < 0.05$). This was in accordance with the lower secondary lipid oxidation products as monitored by TBARS values. The formation of secondary lipid oxidation products is one of the main causes of the development of undesirable offensive fishy odour in fish protein hydrolysate (Yarnpakdee *et al.*, 2012). The result suggested that seabass skin pretreated with citric acid, followed by defatting prior to gelatin extraction in combination with spray drying was a promising means to minimise fishy odour/flavour in the resulting gelatin.

6.4.2 Characteristics of selected gelatins

Spray-dried gelatin from skin pretreated with citric acid spray dried at the lowest inlet temperature (SD-CI-180) and which exhibited the lowest fishy odour/flavour was selected for characterisation, in comparison with freeze-dried gelatin from skins pretreated with acetic acid (FD-AC) or citric acid (FD-CI).

6.4.2.1 Fourier transform infrared (FTIR) spectra

FTIR spectra of the selected gelatins from seabass skin subjected to different pretreatments and drying methods are illustrated in Figure 21. The FTIR spectroscopy together with attenuated total reflectance (ATR) has been used to determine the functional groups and conformation of collagen and gelatin (Muyonga *et*

al., 2004). All gelatin samples exhibited the major absorption bands in amide band region. The absorption in the amide I region is due to C=O stretching/hydrogen bonding coupled with COO (Bandekar, 1992). Its location depends on the hydrogen bonding and the conformation of protein structure (Uriarte-Montoya *et al.*, 2011). In the present study, the amide I peak was observed in the range of 1651–1655 cm^{-1}. The position of amide I band of SD-CI-180 shifted to a higher wavenumber. The result indicated that SD-CI-180 had a higher degree of molecular order, since the shift of this peak to a higher frequency was associated with an increase in the molecular order (Payne and Veis, 1988). Amide I band, between 1700 and 1600 cm^{-1}, was useful for infrared spectroscopic analysis of the secondary structure of proteins (Muyonga *et al.*, 2004).

The characteristic absorption bands of FD-AC, FD-CI and SD-CI-180 in the amide II region were noticeable at the wavenumbers of 1545, 1543 and 1541 cm^{-1}, respectively. The amide II vibration mode is attributed to an out-of-phase combination of C–N stretch and inplane N–H deformation modes of the peptide group (Bandekar, 1992). Amide II band of gelatins at 1560–1500 cm^{-1} was reported by Yakimets *et al.* (2005). In addition, the amide III bands of all gelatin samples were detected at the wavenumbers of 1240-1242 cm^{-1}, which indicated disorder in the gelatin molecules and were more likely associated with loss of triple helix state (Muyonga *et al.*, 2004). The amide III represents the combination peaks between C–N stretching vibrations and N–H deformation from amide linkages as well as absorptions arising from wagging vibrations from CH_2 groups from the glycine backbone and proline side-chains (Jackson *et al.*, 1995).

Amide A band, arising from the stretching vibrations of the N–H group coupled with hydrogen bonding, appeared at 3402, 3397 and 3310 cm^{-1} for FD-AC, FD-CI and SD-CI-180, respectively. The amide A band is associated with the N–H stretching vibration and shows the existence of hydrogen bonds. Normally, a free N–H stretching vibration is found in the range of 3400–3440 cm^{-1} (Muyonga *et al.*, 2004). The position of amide A band shifted to a lower frequency suggesting that the NH group of a peptide is involved in hydrogen bonding (Doyle *et al.*, 1975). The result indicated that drying at high temperature induced the aggregation of gelatin components. The amide B band was observed at 3082, 3082 and 3080 cm^{-1} for FD-AC, FD-CI and SD-

CI-180, respectively, corresponding to the asymmetric stretching vibration of =C–H as well as $–NH_3^+$ (Sinthusamran *et al.*, 2014). Amongst all samples, SD-CI-180 showed the lowest wavenumber for the amide B peak, suggesting the interaction of $–NH_3$ groups between peptide chains as induced by high temperature during drying. Therefore, the secondary structure and functional group of gelatin obtained from seabass skin were affected by acid pretreatments and drying processes.

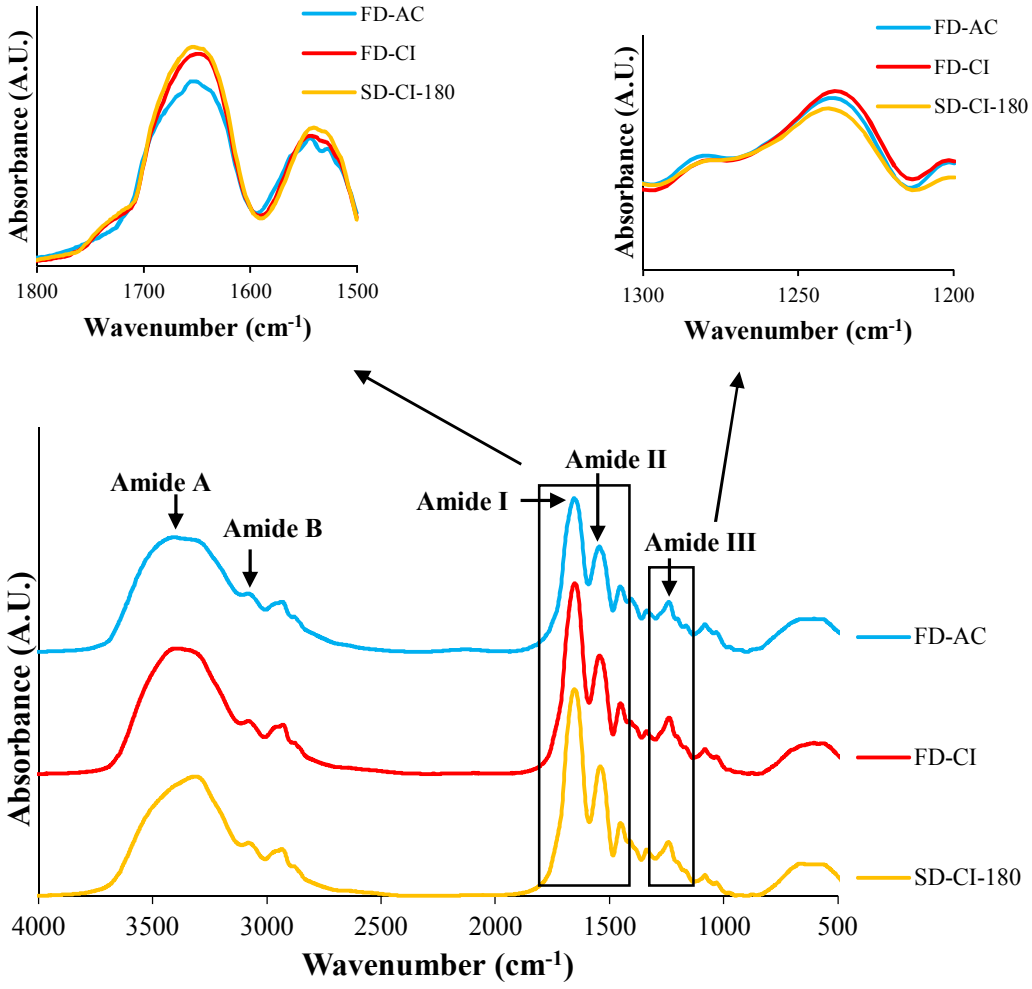

Figure 21. Fourier transform infrared spectra of gelatin extracted from the skin of seabass with different pretreatments and drying methods. Key: see the caption for Figure19.

6.4.2.2 Microstructures of gelatin gels

The microstructures of different gelatin gels as affected by pretreatments and drying methods are illustrated in Figure 22. All gelatin gels were sponge or coral-like in structure. FD-AC showed the finest gel network with small voids. It was found that FD-CI showed the thinner strand than FD-AC. As a consequence, the former could resist to the force applied to a lower extent as indicated by the lower gel strength (Figure 20). The coarser gel with largest voids was found in gel from gelatin obtained by spray drying (SD-CI-180). The coarser gel was in accordance with the poorer gel strength (Figure 20). The coarser network had less inter-connected protein chains than the finer counterpart. As a result, the weaker matrix was formed. Sinthusamran *et al.* (2014) also found that gelatin from seabass skin with the finer gel network had the higher gel strength than gel possessing the coarser network. In general, the conformation and association of the protein molecules in gel matrix directly contributed to the gel strength of gelatin (Benjakul *et al.*, 2009). The result suggested that the pretreatment and drying methods had an impact on the arrangement and association of gelatin molecules in the gel matrix. It has been known that the microstructure of gel network is related to the physical properties of the gelatin gel. The gelatin gel network was generally governed by the pretreatment conditions and gelatin concentration (Yang *et al.*, 2008).

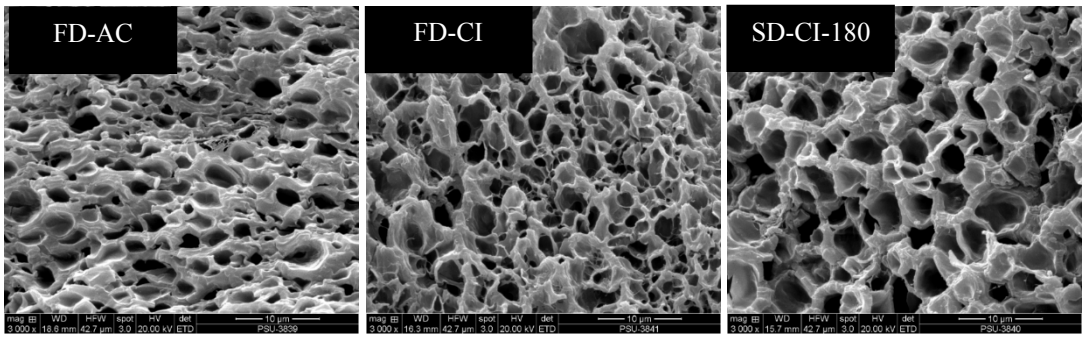

Figure 22. Microstructures of gelatin gel from the skin of seabass with different pretreatments and drying methods. Magnification: 3000×. Key: see the caption for Figure19.

6.4.2.3 Volatile compounds

Selected volatile compounds in gelatin produced from different pretreated skins and drying methods including FD-AC, FD-CI and SD-CI-180 are shown in Table 17. Aldehydes were the most prominent volatiles found in gelatin from seabass skin. Aldehydes have been used as the index of lipid oxidation in a number of foods because they possess low threshold values and are the major contributors to the development of off-odour and off-flavour (Ross and Smith, 2006). Numerous aldehydes produced during oxidation included octanal, nonanal, pentanal, hexanal, etc.(Ross and Smith, 2006). Amongst all aldehydic compounds, hexanal was found as the major aldehyde formed in gelatin, followed by pentanal and heptanal, respectively (Table 17). Hexanal showed very low threshold (0.15 ppm) (Frankel, 2012). This volatile more likely contributed to the off-odour in the gelatin powder. It was found that SD-CI-180 showed the lower abundance of those aldehydic compounds than FD-AC and FD-CI. No hexanal was found in SD-CI-180. Propanal and heptanal can serve as a reliable indicator of flavour deterioration for fish products, whilst hexanal contributes to the rancidity in meats. Iglesias and Medina (2008) reported that propanal, 1-penten-3-ol, 2,3-pentanedione, hexanal and 1-octen-3-ol were the main volatile compounds formed during the storage of Atlantic horse mackerel muscle at 4 °C. 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. Carbonyl compounds, which are produced from oxidation of polyunsaturated fatty acids by lipoxygenase or by autoxidation, contributed to fishy odour/flavour (Josephson *et al.*, 1984). Hexanal, heptanal and 1-octen-3-ol are generated from n–6 polyunsaturated fatty acid oxidation (Iglesias and Medina, 2008).

Alcohols including 1-penten-3-ol, 1-pentanol, 2-penten-1-ol, 1-hexanol, 1-octen-3-ol, 1-heptanol and 1-octanol of SD-CI-180 were lower in abundance, compared with FD-AC and FD-CI (Table 17). 1-Penten-3-ol and 1-pentanol were the most dominant alcohols in FD-AC. Alcohols are known as the secondary products produced by the decomposition of hydroperoxides of fatty acids (Ross and Smith, 2006). 1-Octen-3-ol is an important contributor to off-flavour due to its low odour threshold and it was reported to be formed from oxidation of arachidonic acid by 12-

Table 17. Volatile compounds in gelatin extracted from seabass skin with different pretreatments and drying methods

Compounds	FD-AC	FD-CI	SD-CI-180
Aldehydes			
Pentanal	36.4	13.7	3.3
Hexanal	451.0	267.0	ND
2-Pentenal	1.3	ND	ND
3-Methyl-2-butenal	17.6	8.1	ND
Heptanal	18.9	8.1	2.3
2-Hexenal	10.7	6.1	ND
Octanal	10.6	3.8	1.6
2-Heptenal	5.6	ND	ND
Nonanal	14.4	7.2	ND
2,4-Hexadienal	3.6	5.2	ND
2-Octenal	12.5	3.3	1.0
2,4-Heptadienal	2.2	ND	ND
2-Nonenal	6.8	4.3	ND
2,6-Nonadienal	3.9	2.0	ND
2-Decenal	5.0	2.8	ND
Ketones			
Cyclohexanone	ND	ND	1.6
3,5-Octadien-2-one	5.4	4.8	ND
3,5-Octadiene-2-one	2.2	ND	ND
2,6-Di(t-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one	2.5	2.2	1.6
Alcohols			
1-Penten-3-ol	25.4	13.8	1.9
1-Pentanol	19.4	9.8	2.4
2-Penten-1-ol	5.7	2.8	ND
1-Hexanol	3.9	ND	ND
1-Octen-3-ol	9.9	5.2	0.7
1-Heptanol	8.2	3.0	ND
1-Octanol	8.1	3.6	ND
Furans			
2-Ethylfuran	37.0	ND	ND
2-Amylfuran	11.1	ND	ND
Others			
1-(3-butenyl)cyclohexene	7.4	ND	ND
3-Ethyl-1,4-hexadiene	6.6	ND	ND
Oxime-, methoxy-phenyl	7.3	3.5	ND

Values are expressed as abundance ($\times 10^7$).

ND: not detectable. FD-AC and FD-CI represent freeze dried gelatins from skin pretreated with acetic and citric acid, followed by defatting, respectively. SD-CI-180 represent spray dried gelatins obtained from skin pretreated with citric acid, followed by defatting and spray dried at inlet temperature of 180 ºC.

lipoxygenase (Hsieh and Kinsella, 1989). 1-Octen-3-ol, the major alcohol, contributed to the strong intensity of fishy and rancid off-odours in washed Asian seabass mince containing myoglobin (Thiansilakul *et al.*, 2011).

Other volatile compounds were also formed but generally decreased after pretreatment with citric acid, followed by defatting and spray drying. Ketone is another secondary lipid oxidation product derived from the decomposition of hydroperoxide (Iglesias and Medina, 2008). In the present study, cyclohexanone 3,5-octadien-2-one, 3,5-octadiene-2-one and 2,6-di(t-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one were found in gelatin samples. Nevertheless, 3,5-octadien-2-one and 3,5-octadiene-2-one were not found in SD-CI-180. Furans including 2-ethylfuran and 2-amylfuran were noticeable in FD-AC but were not detectable in FD-CI and SD-CI-180. The fishy volatiles identified in boiled sardines were dimethyl sulfide, acetaldehyde, propionaldehyde, butyraldehyde, 2-ethylfuran, valeraldehyde, 2,3-pentanedione, hexanal, and 1-penten-3-ol (Kasahara and Osawa, 1998). Although the spray drying could remove the low MW volatiles from the gelation solution, drying at high temperature could induce lipid oxidation to some extent. However the rate of removal was most likely much higher than the rate of formation. This was evidenced by the very low amount of volatiles in the spray-dried sample.

In general, the lower abundance of secondary oxidation products, including aldehydes, alcohols and ketones in SD-CI-180 was in accordance with the lower PV and TBARS values (Table 16). Thus, the pretreatment of seabass skin with citric acid, followed by defatting could lower the lipids, which were prone to oxidation. Additionally, spray drying was effective in removal of the volatile secondary lipid oxidation products, which contribute to the offensive fishy odour in the resulting gelatin.

6.5 Conclusion

Pretreatment of seabass skin using citric acid, followed by defatting prior to gelatin extraction, was effective in preparing the skin for gelatin extraction. Spray drying, particularly with inlet temperature of 180 ºC could lower several

odourous compounds associated with fishy flavour and odour more effectively than freeze-drying. Therefore, gelatin prepared from the skin with appropriate pretreatment and drying method could be widely applied in foods without any detrimental effect on sensory properties.

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CHAPTER 7

EFFECTS OF DEFATTING AND TANNIC ACID INCORPORATION DURING EXTRACTION ON PROPERTIES AND FISHY ODOUR OF GELATIN FROM SEABASS SKIN

7.1 Abstract

Gelatins from seabass skin without and with defatting, extracted in the absence and presence of tannic acid at various concentrations (0−0.4 g/kg), were characterised. Gelatins from skin without defatting had higher gel strength ($P < 0.05$) with coincidentally higher band intensity of α- and β-chains, compared with those extracted from defatted skin. Gelatins from defatted skin had lower fishy odour with concomitantly lower thiobarbituric acid reactive substances and peroxide value. Lower abundance of volatile compounds, including aldehydes, ketones and alcohols was found in gelatin extracted from defatted skin. The addition of tannic acid during gelatin extraction could lower lipid oxidation, fishy odour as well as the formation of volatile compounds, especially hexanal and heptanal, in the resulting gelatins, regardless of defatting. Therefore, gelatin with reduced fishy odour could be prepared from seabass skin, in which 0.2 g/kg tannic acid was incorporated during extraction.

7.2 Introduction

Gelatin is a protein obtained from partial denaturation or hydrolysis of collagen. Due to its unique functional and technological properties, gelatin has been widely used in food, pharmaceutical, cosmetic and photographic applications (Regenstein and Zhou, 2007). Generally, gelatin is obtained from mammals, especially pig and cow skins and bones. However, porcine gelatin cannot be used in Kosher and Halal foods, whilst bovine counterpart is not consumed by Hindu because of religious constraints (Kittiphattanabawon *et al.*, 2012). As a consequence, alternative sources, particularly fish processing byproducts including skin, scale or bone, etc. have gained attention for gelatin production due to their availability and the need to increase their value (Benjakul *et al.*, 2012).

Seabass (*Lates calcarifer*) is an economically important fish species in Thailand and other countries, especially the tropical and subtropical regions of Asia and the Pacific. During processing or dressing of seabass, the skin is removed and considered as a byproduct. Recently, seabass skin has been used to produce collagen, gelatin as well as hydrolysates with bioactivities (Senphan and Benjakul, 2014; Sinthusamran *et al.*, 2013). However, fishy odour associated with gelatin, particularly that extracted from unfresh fish skin, can limit its applications, especially as human food ingredients or supplements. Lipid oxidation products mainly contributed to fishy odour in fish gelatin. Furthermore, fishy flavour and odour in protein hydrolysate from Nile tilapia muscle was also reported to be caused by lipid oxidation (Yarnpakdee *et al.*, 2012b).

Fish skin contains lipids with high degree of unsaturation. Those lipids can be oxidised during the extraction at high temperature, thereby promoting lipid oxidation and development of unpleasant odours/flavours in the resulting gelatin. The removal of lipids from skin by the appropriate pretreatment could be a means to lower the oxidation. Additionally, the incorporation of antioxidant during gelatin extraction might retard lipid oxidation and decrease the formation of lipid oxidation products and offensive fishy odour in the resulting gelatin. Nevertheless, there is no information regarding the influence of antioxidant incorporation during gelatin extraction on properties and fishy odour of gelatin from fish skin. Therefore, the objective of this study was to investigate the effect of tannic acid incorporation during gelatin extraction of non-defatted and defatted skins on the properties and fishy odour of gelatin from seabass skin.

7.3 Materials and methods

7.3.1 Chemicals

L-leucine and 1,1,3,3-tetramethoxypropane were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Sodium dodecyl sulphate (SDS), Coomassie Blue R-250, and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). High molecular weight

markers were obtained from GE Healthcare UK Limited (Buckinghamshire, UK). Ferrous chloride was obtained from Merck (Darmstadt, Germany). Cumene hydroperoxide and 2-thiobarbituric acid were purchased from Fluka (Buchs, Switzerland). All chemicals were of analytical grade.

7.3.2 Collection of seabass skins

Descaled skins of fresh seabass (*Lates calcarifer*) with a weight of 2.5–3 kg were obtained from the Kingfisher Holdings Co., Ltd., Songkhla, Thailand. Skins were kept in a polystyrene box containing ice using a skin/ice ratio 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Upon arrival, the remaining meat was removed manually. The skins were washed with cold tap water (≤ 10 ºC), pooled and used as the composite sample. The samples were placed in polyethylene bags and stored at –20 ºC until used, but not longer than 2 months. Prior to gelatin extraction, the frozen skins were thawed with running tap water (25–26 ºC) until the core temperature reached 8–10 ºC. The skins were then cut into small pieces (1.0 × 1.0 cm^2) using scissors.

7.3.3 Preparation of seabass skin

7.3.3.1 Removal of non-collagenous proteins

The skins were soaked in 0.1 M NaOH with a skin/alkaline solution ratio of 1:10 (w/v) in order to remove non-collagenous proteins. The mixture was stirred for 3 h at room temperature (28–30 ºC) using an overhead stirrer model RW20.n (IKA®-Werke GmbH & CO.KG, Staufen, Germany) at a speed of 300 rpm. The alkaline solution was changed every 1 h for totally 3 times. Alkali-treated skins were washed with tap water until a neutral or slightly basic pH (7.0–7.5) of wash water was obtained.

7.3.3.2 Acid pretreatment and defatting

Following the treatment with alkaline solution, the obtained skins were separated into two portions. The first one was subjected to pretreatment using 0.05 M acetic acid at a skin/solution ratio of 1:10 (w/v). The mixture was stirred at room temperature for 2 h. The swollen skin was washed using tap water until wash water

became neutral or slightly acidic in pH (6.5-7.0). The skins obtained were referred to as 'typically swollen skins'.

For the second portion, the skins were pretreated with 0.05 M citric acid using skin/solution ratio of 1:10 (w/v) for 2 h to remove phospholipids. After stirring and washing as previously described, the skins treated with citric acid were defatted using 30% (v/v) isopropanol with a skin/solvent ratio of 1:10 (w/v) at room temperature for 1 h. The mixture was stirred continuously at 200 rpm. The solvent was then removed and the defatted skins were washed with 10 volumes of tap water. The defatted skins were then rinsed with distilled water. The obtained skins were referred to as 'defatted skins'.

7.3.4 Extraction of gelatin

Both typically swollen skins and defatted skins were subjected to gelatin extraction. Skins were mixed with distilled water containing tannic acid at various concentrations (0, 0.2 and 0.4 g/kg, based on skin dry matter) at a ratio of 1:3 (w/v). Extraction was carried out at 55 ºC in a water bath (W350, Memmert, Schwabach, Germany) and the mixtures were stirred continuously for 6 h using an overhead stirrer (IKA®-Werke GmbH & CO.KG, Staufen, Germany) at a speed of 150 rpm. The mixtures were then filtered using a Buchner funnel with a Whatman No. 4 filter paper (Whatman International, Ltd., Maidstone, England). The filtrates were further dried.

7.3.5 Drying of gelatin

Gelatin solution was transferred into stainless steel tray with the thickness of 0.5 cm. Air velocity was 1.4 m/s. Drying was carried out at 60 ºC for 24 h. All gelatin samples were analysed.

7.3.6 Analyses

7.3.6.1 Protein patterns

Protein patterns were determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) as modified

by Sinthusamran *et al.* (2014). High molecular weight protein markers (GE Healthcare UK Limited, Buckinghamshire, UK) were used to estimate the molecular weight of proteins.

7.3.6.2 Gel strength

Gelatin gel was prepared as per the method of Fernández-Díaz *et al.* (2001). The maximum force (in gram) was recorded when the penetration distance reached 4 mm.

7.3.6.3 Colour

Colour of gelatin gels was measured by a Hunter Lab Colourimeter (Color Flex, Hunter Lab Inc., Reston, VA, USA). *L**, *a** and *b** indicating lightness/brightness, redness/greenness and yellowness/blueness, respectively, were recorded. Total difference in colour (ΔE^*) was calculated as described by Yarnpakdee *et al.* (2012b)

7.3.6.4 Peroxide value (PV)

PV was determined according to the method of Richards and Hultin (2002). A standard curve was prepared using cumene hydroperoxide at concentrations ranging from 0 to 2 ppm. PV was expressed as mg cumene hydroperoxide equivalents/kg sample.

7.3.6.5 Thiobarbituric acid reactive substances (TBARS)

TBARS were determined as described by Buege and Aust (1978). A standard curve was prepared using 1,1,3,3-tetramethoxypropane at concentrations ranging from 0 to 6 ppm. TBARS value was calculated and expressed as mg malonaldehyde equivalents/kg sample.

7.3.6.6 Sensory evaluation

Sensory evaluation for fishy odour intensity was carried out according to the method of Yarnpakdee *et al.* (2012b) using 8 trained panelists with the ages of 25–32. Prior to the evaluation, the panelists were trained three times a week. Panelists

were trained with standards for two sessions. To test the samples, all gelatin samples (0.75%, w/v) were placed in a sealable plastic cup and heated at 60 ºC in a temperature controlled water bath for 5 min before serving. The panelists were asked to open the sealable cup and sniff the headspace above the samples in order to determine the intensity of fishy odour, with the score from 0 (none) to 4 (extremely strong fishy odour).

7.3.6.7 Volatile compounds

Volatile compounds in gelatin samples were determined using a solid-phase micro-extraction gas chromatography mass spectrometry (SPME-GC-MS) following the method of Iglesias and Medina (2008). The identified volatile compounds were expressed in the terms of relative abundance.

7.3.6.8 Microstructure

The microstructure of gelatin gel was visualised using a scanning electron microscopy (SEM). Gelatin gels (2–3 mm thick) were fixed with 2.5% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 12 h. The samples were then rinsed with distilled water for 1 h and dehydrated in ethanol with a serial concentration of 50, 70, 80, 90 and 100% (v/v). Dried samples were mounted on a bronze stub and sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA). The specimens were observed with a scanning electron microscope (JEOL JSM-5800 LV, Tokyo, Japan) at an accelerating voltage of 20 kV.

7.3.7 Statistical analysis

Experiments were run in triplicate using three different lots of samples. The data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test. 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 Effects of defatting and tannic acid incorporation during extraction on properties and fishy odour of gelatin

7.4.1.1 Protein patterns

Protein patterns of gelatin extracted from the skin of seabass without and with defatting in the absence and presence of tannic acid during extraction are shown in Figure 23. All gelatin samples contained α-chain with a MW of 122–115 kDa as the major constituent. Gelatin samples also consisted of β-component (α-chain dimer) and γ-component (α-chain trimer). Gelatins obtained from skin without defatting had higher band intensities of all components than those from skin with defatting, irrespective of tannic acid incorporation. No marked difference in protein patterns was observed for gelatin from the same skin, when tannic acid at different concentrations was incorporated during gelatin extraction. It was noted that peptides or proteins with the MW lower than α_2-chains, particularly with MW of 85 and 70 kDa, were noticeable in gelatin from defatted skin. Additionally, the proportion of β-chain in gelatin from defatted skin was lower than that observed in gelatin from non-defatted skin. Citric acid was used to facilitate the removal of phospholipids (Yarnpakdee *et al.*, 2012a). Basically, citric acid plays a role as a binding agent for basic amino acid residues of cytoskeletal proteins (Benjakul *et al.*, 2012). When isopropanol was used for further defatting, the aggregation of protein in skin matrix might be induced. As a result, citric acid might be retained as the residue in the defatted skin. Citric acid retained plausibly induced the hydrolysis of α-, β- and γ-chains localised in the skin, especially during extraction at high temperature. During gelatin extraction, intra- and intermolecular covalent crosslinks might be hydrolysed to different degrees, depending on processing parameters (temperature, time, and pH), pretreatment, and properties and preservation method of the starting raw material used (Karim and Bhat, 2009). The result suggested that defatting to remove both phospholipids and neutral lipids of skin played a major role in protein components of the resulting gelatin. On the other hand, tannic acid added during extraction had no profound impact on protein constituents of gelatin.

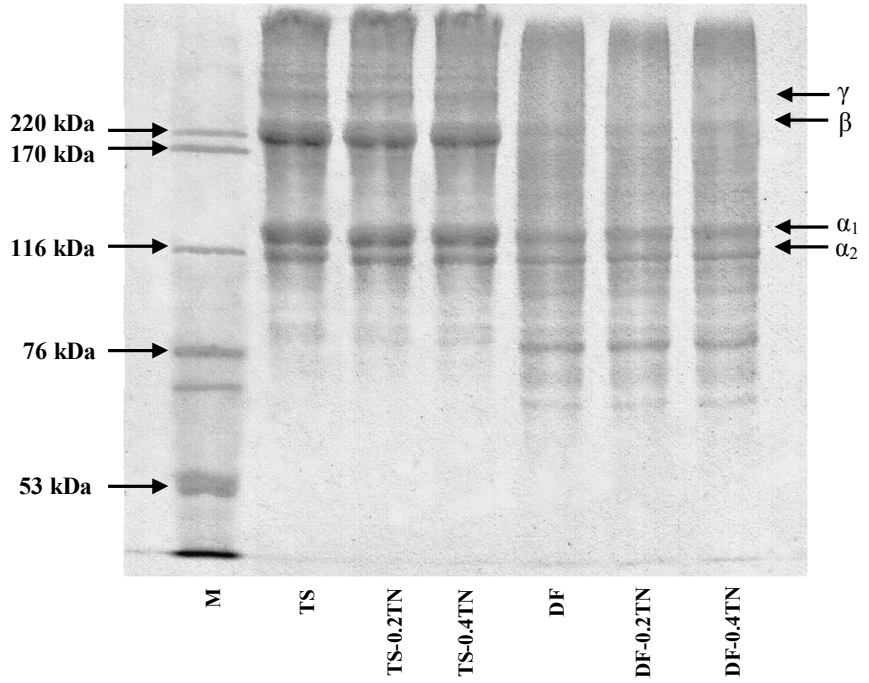

Figure 23. Protein patterns of gelatin extracted from seabass skin as affected by defatting and tannic acid incorporation during gelatin extraction. M denote high molecular weight markers. TS, TS-0.2TN and TS-0.4TN represent gelatins from typically swollen (non-defatted) seabass skin and extracted in the presence of tannic acid at concentrations of 0, 0.2 and 0.4 g/kg, respectively.

7.4.1.2 Gel strength

Gel strength of gelatin extracted from defatted and non-defatted seabass skin in the absence and presence of tannic acid at different levels during gelatin extraction is presented in Table 18. Gel strength is one of the most important functional properties of gelatin. In the present study, gelatin from seabass skin without defatting had similar gel strength (190-192 g) to bovine gelatin (193 g) ($P > 0.05$) (data not shown). Generally, gelatin from defatted skin had slightly lower gel strength than those from non-defatted skin. No differences in gel strength were found between gelatins extracted in the absence and presence of tannic acid when the same skin was used for gelatin extraction ($P > 0.05$). The lower gel strength of gelatin from defatted skin was in accordance with the lower α-, β- and γ-chains band intensities (Figure 23). The amount of β- and γ-components and the amino acid composition of gelatin were

reported as the factors governing gelation of gelatin (Taheri *et al.*, 2009). Although the protein patterns were different between the gelatin samples extracted from skin without and with defatting, there was no big difference in gel strength. It was noted that gelatin from defatted skin still had α-, and β-chains to some extent. Those chains could still form the gel in the similar manner, in which inter-junction was formed and the gel matrix could be developed. During defatting with isopropanol, proteins might be vulnerable to solvent, leading to the exposure of the hydrophobic domain and aggregation (Sikorski *et al.*, 1981). Isopropanol and other alcohols have been known to compete with proteins in water binding. As a result, water was more removed from protein molecules in the presence of solvents (Sikorski *et al.*, 1981), leading to protein aggregation of skin matrix. As a result, poor gel was developed. The difference in gel strength could be due to the differences in intrinsic characteristics, such as molecular weight distribution, the chain length of protein as well as complex interactions determined by the amino acid composition and the ratio of α/β chains present in the gelatin (Badii and Howell, 2006). The configuration of protein and the way the inter-junction was developed to form the stronger network were therefore crucial for gel formation. Location of imino acids in the peptide chain also contributed to gel formation (Ledward, 1986). Thus, defatting of skin prior to gelatin extraction affected the gel formation of gelatin. Nevertheless, tannic acid (0.2–0.4 g/kg) incorporated in extracting medium had no impact on gel strength of resulting gelatin.

7.4.1.3 Colour of gelatin gel

The colour of gelatin gel from seabass skin without and with defatting, extracted in the absence and presence of tannic acid, expressed as L^*, a^* and b^* is shown in Table 18. In general, gelatin from defatted skin had higher lightness (L^*-value) than those extracted from non-defatted skin ($P < 0.05$). Furthermore, the former showed the lower yellowness (b^*-value) than the latter ($P < 0.05$). ΔE^* (total colour difference) of gelatin extracted from defatted skin was lower than those from non-defatted skin ($P < 0.05$). The result suggested that defatting using isopropanol facilitated the removal of lipids as well as fat soluble pigments. Fats or lipids rich in PUFA were prone to oxidation. Lipid oxidation products formed, particularly aldehydes, further underwent glycation with amino groups in gelatin during extraction or drying. This lead

Table 18. Gel strength, PV, TBARS, fishy odour and colour of gelatin from seabass skin as affected by defatting and tannic acid incorporation during extraction

Parameters	TS	TS-0.2TN	TS-0.4TN	DF	DF-0.2TN	DF-0.4TN
Gel strength (g)	191.93 ± 3.81a	191.72 ± 1.63a	190.41 ± 2.10a	186.26 ± 2.20b	186.18 ± 0.99b	186.26 ± 0.45b
PV (mg cumene hydroperoxide equivalents/kg sample)	85.63 ± 0.61a	82.96 ± 0.30b	68.48 ± 0.47c	48.86 ± 0.88d	42.65 ± 0.94e	35.71 ± 0.31f
TBARS (mg MDA equivalents/kg sample)	50.47 ± 2.08a	24.80 ± 3.59b	14.30 ± 1.19c	14.78 ± 1.28c	8.57 ± 1.08d	6.33 ± 1.57d
Fishy odour	3.83 ± 0.22a	3.14 ± 0.48b	2.72 ± 0.17b	1.76 ± 0.27d	1.17 ± 0.20e	0.98 ± 0.24e
*L**	23.59 ± 0.22c	22.31 ± 0.17d	21.57 ± 0.26e	25.87 ± 0.37a	25.56 ± 0.62a	25.13 ± 0.48b
*a**	-0.42 ± 0.14d	-0.14 ± 0.04ab	-0.07 ± 0.02a	-0.43 ± 0.09d	-0.30 ± 0.09c	-0.22 ± 0.08bc
*b**	7.80 ± 0.45b	8.77 ± 0.33a	8.98 ± 0.46a	0.56 ± 0.32d	0.62 ± 0.12d	1.34 ± 0.17c
Δ*E**	70.36 ± 0.19c	71.73 ± 0.19b	72.50 ± 0.23a	67.68 ± 0.37e	67.89 ± 0.62e	68.43 ± 0.48d

Values are expressed as mean ± SD (n = 3).

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

TS, TS-0.2TN and TS-0.4TN represent gelatins from typically swollen (non-defatted) seabass skin, in which tannic acid at concentrations of 0, 0.2 and 0.4 g/kg was incorporated during gelatin extraction, respectively.

DF, DF-0.2TN and DF-0.4TN represent gelatins from defatted seabass skin, in which tannic acid at concentrations of 0, 0.2 and 0.4 g/kg was incorporated during gelatin extraction, respectively.

to the higher yellowness in gelatin. When tannic acid was incorporated during gelatin extraction, *L**-value was decreased, whilst *b**-value increased, especially with increasing tannic acid concentration. Tannic acid might be oxidised during gelatin extraction to some degree. Oxidised tannic acid with brown colour more likely contributed to darker colour of gelatin. Thus, defatting was able to improve the whiteness and lower the yellowness in gelatin from seabass skin. Conversely, incorporation of tannic acid during extraction at level of 0.4 g/kg resulted in slight increase in darkness and yellowness of gelatin.

7.4.1.4 Lipid oxidation

Lipid oxidation of gelatin from skin as influenced by defatting and tannic acid incorporation during extraction was monitored by PV and TBARS values (Table 18). PV of gelatin from non-defatted skin was higher than that obtained from defatted skin ($P < 0.05$). The result suggested that defatting with isopropanol more likely removed lipids, both phospholipids and neutral lipids as well as some pro-oxidants from the skin effectively. PV is an indicator of the initial stage of lipid oxidation. The higher PV of gelatin indicated the higher presence of hydroperoxide generated via lipid oxidation process. Lipid hydroperoxides are formed via several pathways including the reaction of singlet oxygen with unsaturated fatty acids or the lipoxygenase-catalysed oxidation of polyunsaturated fatty acids (Jacobsen, 2010). When tannic acid was incorporated during gelatin extraction, the lower PV in gelatin was observed with increasing tannic acid concentration ($P < 0.05$), regardless of skin used. The result showed that tannic acid was very effective in retarding the propagation stage of lipid oxidation. Tannic acid showed radical-scavenging activity via hydrogen donating and reducing power, thereby terminating the propagation (Maqsood and Benjakul, 2010).

TBARS of gelatin from non-defatted skin was higher than those of gelatin from defatted skin ($P < 0.05$) (Table 18). Citric acid was reported to help in removal of membrane lipids (Liang and Hultin, 2005) and could act as a metal chelator (Choe and Min, 2009), whilst isopropanol was able to remove neutral lipids. As a result,

the oxidation in gelatin from defatted skin occurred to a lower extent. Citric acid was able to disconnect the linkages between cytoskeletal proteins and phospholipids, linked together via electrostatic interaction (Liang and Hultin, 2005). Moreover, citric acid 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. The removal of phospholipids in fish muscle prior to protein hydrolysis has been reported to lower lipid oxidation products as well as fishy odour in the resulting hydrolysate (Yarnpakdee *et al.*, 2012a). When tannic acid was incorporated during gelatin extraction, the lower TBARS was observed in gelatin from non-defatted skin as tannic acid concentration increased ($P < 0.05$). The result suggested that tannic acid was able to prevent lipid oxidation of gelatin during extraction. However, no differences in TBARS value were found in gelatin extracted from defatted skin, which tannic acid at levels of 0.2 and 0.4 g/kg was used ($P > 0.05$). Tannic acid is well known as an effective natural antioxidant for preventing lipid oxidation of many food systems (Maqsood *et al.*, 2014). The detectable TBARS in gelatin most likely indicated the decomposition of hydroperoxides into the secondary oxidation products in the later stage of lipid oxidation (Jacobsen, 2010). Hydroperoxides break down in several steps, yielding a wide variety of decomposition products including various aldehydes e.g. n-alkanals, trans-2-alkenals, 4-hydroxy-trans-2-alkenals and malonaldehyde (Jacobsen, 2010).

7.4.1.5 Fishy odour intensity

Fishy odour intensity of gelatin extracted from non-defatted and defatted skins as affected by tannic acid incorporation during extraction is shown in Table 18. Gelatin from defatted skin had lower fishy odour, compared with those extracted from skin without defatting ($P < 0.05$). Fishy odour of gelatin also decreased when tannic acid was incorporated during gelatin extraction ($P < 0.05$). This was in accordance with the lower secondary lipid oxidation products as monitored by TBARS values. However, no difference in fishy odour was found between gelatin extracted in the presence of tannic acid at concentration of 0.2 and 0.4 g/kg ($P > 0.05$). The formation of secondary lipid oxidation products is the main contributor to undesirable offensive fishy odour in

fish skin. The result suggested that the use of defatted skin in combination with tannic acid incorporation during gelatin extraction was a promising means to minimise the formation of fishy odour in the resulting gelatin.

7.4.2 Characteristics of the selected gelatins

Gelatins extracted from non-defatted skin (TS), from defatted skin (DF) and from defatted skin in the presence of 0.2 g/kg tannic acid (DF-0.2TN) were compared.

7.4.2.1 Microstructures of gelatin gels

The microstructures of gels of different gelatins are illustrated in Figure 24. All gelatin gels were sponge or coral-like in structure. TS gel showed the finest gel network with smaller voids, compared with DF and DF-0.2TN samples. TS gel could resist to the force applied to a higher extent as indicated by the higher gel strength (Table 18). Sinthusamran *et al.* (2014) found that gelatin from seabass skin with the finer gel network had the higher gel strength than gel possessing the coarser network. The coarser gel with the larger voids was found in gel from gelatin from defatted skin. The coarser network had less inter-connected protein chains than the finer counterpart. As a result, the weaker matrix was formed. When tannic acid at a level of 0.2 g/kg was incorporated during extraction, the similar network was observed in both gels, compared with that from the corresponding skin. Similar microstructure was related with the similar gel strength of gelatin extracted from defatted skin without and with tannic acid incorporation (Table 18). In general, the conformation and association of the protein molecules in gel matrix directly contributed to the gel strength of gelatin (Benjakul *et al.*, 2009). Gelatin gel network was generally governed by the pretreatment conditions and gelatin concentration (Yang *et al.*, 2008). Thus, the defatting mainly had an impact on the arrangement and association of gelatin molecules in the gel matrix, whilst the incorporation of tannic acid at the level used showed the negligible effect on gel structure.

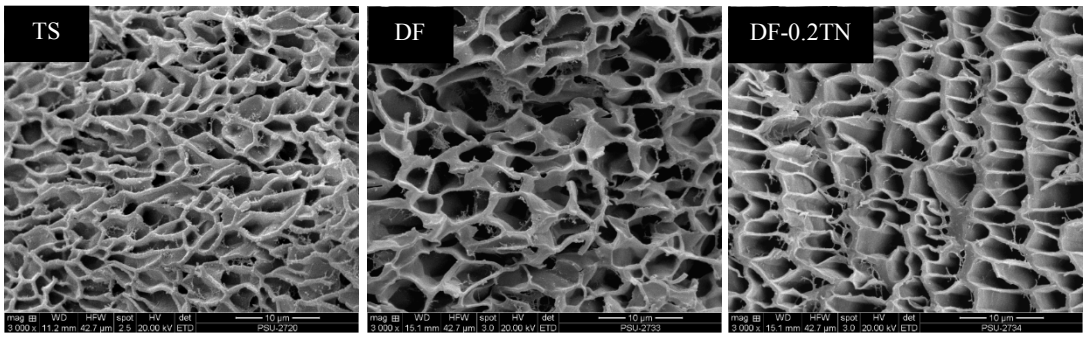

Figure 24. Microstructures of gelatin gel from the skin of seabass as affected by defatting and tannic acid incorporation during gelatin extraction. Magnification: 3000×. TS and DF represent gelatins obtained from typically swollen (non-defatted) and defatted seabass skin, respectively. DF-0.2TN represents gelatin obtained from defatted seabass skin and extracted in the presence of 0.2 g/kg tannic acid.

7.4.2.2 Volatile compounds

Selected volatile compounds in different gelatin samples are shown in Table 19. Aldehydes were the most prominent volatiles found in gelatin from seabass skin. Aldehydes have been used as the index of lipid oxidation in a number of foods because they possess low threshold values and are the major contributors to off-odour and off-flavour (Ross and Smith, 2006). Numerous aldehydes produced during oxidation included octanal, nonanal, pentanal, hexanal, etc. (Ross and Smith, 2006). Amongst all aldehydic compounds, hexanal and heptanal were found as the major aldehydes in gelatin, followed by octanal, 2-hexenal and 2,6-nonadienal, respectively (Table 19). It was noted that DF and DF-0.2TN samples showed the lower abundance of those aldehydic compounds than TS sample. Propanal and heptanal can serve as a reliable indicator of flavour deterioration for fish products, whilst hexanal contributes to the rancidity in meats (Iglesias and Medina, 2008). Ross and Smith (2006) reported that propanal, 1-penten-3-ol, 2,3-pentanedione, hexanal and 1-octen-3-ol were the main volatile compounds formed during the storage of Atlantic horse mackerel muscle at 4°C. 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. Carbonyl compounds, which are produced from oxidation of polyunsaturated fatty

acids by lipoxygenase or by autoxidation, contributed to fishy odour/flavour (Josephson *et al.*, 1984). Hexanal, heptanal and 1-octen-3-ol are generated from *n*–6 polyunsaturated fatty acid oxidation (Iglesias and Medina, 2008).

Table 19. Selected volatile compounds in different gelatins extracted from seabass skin

Compounds	TS	DF	DF-0.2TN
Aldehydes			
Hexanal	37.92	23.16	20.98
2-Pentenal	6.71	3.95	4.46
Heptanal	34.34	21.22	18.58
2-Hexenal	9.42	7.35	6.82
Octanal	12.44	9.19	8.19
2-Octenal	4.45	4.20	4.08
Nonenal	6.12	4.97	3.10
2,6-Nonadienal	9.25	8.16	3.04
2,4-Decadienal	2.28	3.03	2.85
Hexadecanal	1.21	0.78	0.86
Alcohols			
1-Penten-3-ol	23.93	10.63	10.15
1-Pentanol	5.38	4.80	4.82
2-Decanol	31.66	20.06	21.48
1-Octen-3-ol	13.78	11.47	10.39
1-Octanol	1.61	1.48	1.20
Ketones			
3,5-Octadien-2-one	8.92	7.25	7.43
2-Undecanone	8.46	4.10	5.22
3-Undecen-2-one	3.80	3.40	1.54
Furans			
2-Allylfuran	4.55	3.61	2.12
2-Ethylfuran	85.72	64.51	59.16
2-Amylfuran	15.16	14.78	14.66

Values are expressed as abundance ($\times 10^8$).

TS and DF represent gelatins obtained from typically swollen (non-defatted) and defatted seabass skin, respectively.

DF-0.2TN represents gelatin obtained from defatted seabass skin and extracted in the presence of 0.2 g/kg tannic acid.

Alcohols including 1-penten-3-ol, 1-pentanol, 2-decanol, 1-octen-3-ol and 1-octanol of DF and DF-0.2TN samples were lower in abundance, compared with TS sample (Table 19). 1-Penten-3-ol and 2-decanol were the most dominant alcohols in gelatin samples. Alcohols are known as the secondary products generated via the decomposition of hydroperoxides (Ross and Smith, 2006). 1-Octen-3-ol is an important contributor to off-flavour due to its low odour threshold and it was reported to be formed from oxidation of arachidonic acid by 12-lipoxygenase (Hsieh and Kinsella, 1989). Thiansilakul *et al.* (2011) reported that 1-octen-3-ol contributed to the strong intensity of fishy and rancid off-odours in washed Asian seabass mince containing myoglobin.

Ketones and furans were also found but generally decreased after defatting or when tannic acid was incorporated during gelatin extraction. Ketone is another secondary lipid oxidation product derived from the decomposition of hydroperoxide (Iglesias and Medina, 2008). In the present study, 3,5-octadien-2-one, 2-undecanone and 3-undecen-2-one were found in gelatin samples. Furans including 2-allylfuran, 2-ethylfuran and 2-amylfuran were noticeable in gelatin samples. The fishy volatiles identified in boiled sardines were dimethyl sulfide, acetaldehyde, propionaldehyde, butyraldehyde, 2-ethylfuran, valeraldehyde, 2,3-pentanedione, hexanal, and 1-penten-3-ol (Kasahara and Osawa, 1998).

In general, the lower abundance of secondary oxidation products, including aldehydes, alcohols and ketones in DF and DF-0.2TN was in accordance with the lower PV and TBARS values (Table 19). Thus, defatting of skin could lower the lipids, which were prone to oxidation. Additionally, the incorporation of tannic acid during gelatin extraction was effective in retarding lipid oxidation, thereby preventing the formation of lipid oxidation products, which contribute to the offensive fishy odour in the resulting gelatin.

7.5 Conclusion

Defatting of seabass skin using citric acid, followed by isopropanol and the incorporation of tannic acid during gelatin extraction effectively prevented lipid

oxidation, the development of volatile compounds and fishy odour in the resulting gelatin. Therefore, gelatin prepared from the skin with appropriate defatting along with antioxidant incorporation during extraction could be widely applied in foods without the detrimental impact on sensory properties.

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CHAPTER 8

ANTIOXIDANT ACTIVITIES AND SELECTED CHARACTERISTICS OF GELATIN HYDROLYSATES FROM SEABASS (*LATES CALCARIFER*) SKIN AS AFFECTED BY PRODUCTION PROCESSES

8.1 Abstract

Antioxidant activities and selected characteristics of gelatin hydrolysates from seabass skin as affected by production processes were investigated. Hydrolysates were prepared using different processes, including hydrolysis during and after gelatin extraction. Samples hydrolysed during gelatin extraction showed a higher degree of hydrolysis (DH) and yield compared with those hydrolysed after gelatin extraction ($P < 0.05$). All hydrolysates had a creamy yellowish colour. A lower abundance of volatile compounds was found in the hydrolysates produced during gelatin extraction, in comparison with those obtained after gelatin extraction. Hydrolysates prepared during gelatin extraction had higher 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, ferric reducing antioxidative power (FRAP) and ferrous ion chelating activity ($P < 0.05$). Following a simulated *in vitro* gastrointestinal digestion, the DPPH radical scavenging activity and FRAP of the hydrolysates was retained, whilst ferrous ion chelating activity increased. The most appropriate conditions for the generation of antioxidant hydrolysates from seabass skin were identified.

8.2 Introduction

Protein hydrolysates have attracted increasing interest as potential ingredients for various health-promoting functional foods due to their bioactivities (Gómez-Guillén *et al.*, 2011). Collagen and gelatin have been earmarked as a source of biologically active peptides with promising health benefits for nutritional or pharmaceutical applications (Gómez-Guillén *et al.*, 2011). Production of gelatin hydrolysates from fish skins may provide an alternative to meat that is acceptable for Kosher and Halal products and may also serve as a substitute for markets concerned

about bovine spongiform encephalopathy (BSE). A number of commercial proteases have been used for the production of gelatin derived hydrolysates and peptides. Alcalase, a commercial protease from a microbial source, has been used in numerous studies investigating gelatin hydrolysis because of its broad specificity as well as the high degree of hydrolysis that can be achieved in a relatively short time under moderate conditions (Benjakul and Morrissey, 1997). This enzyme demonstrated extensive proteolytic activity during the hydrolysis of skin gelatin from Alaska pollack, sole and giant squid, producing hydrolysates which exhibited high antioxidant activity (Giménez *et al.*, 2009; Kim *et al.*, 2001).

Seabass (*Lates calcarifer*) is an economically important fish species in Thailand and other countries, especially tropical and subtropical regions of Asia and the Pacific. During processing or dressing of seabass, skin is generated and considered as a byproduct. Recently, seabass skin has been used to produce collagen and gelatin, as well as hydrolysates with demonstrated bioactivities (Senphan and Benjakul, 2014; Sinthusamran *et al.*, 2013). It is well known that protein hydrolysates and peptides can act as free radical scavengers, as well as transition metal chelators and exert antioxidant activities against enzymatic (lipoxygenase-mediated) and non-enzymatic peroxidation of lipids and fats (Sarmadi and Ismail, 2010). Gelatin hydrolysates from bigeye snapper skin prepared using a protease derived from fish pyloric caeca exhibited antioxidative activity including DPPH and ABTS radical scavenging activity and ferric reducing antioxidant power (Phanturat *et al.*, 2010). Kittiphattanabawon *et al.* (2012) reported that gelatin hydrolysates from blacktip shark skin prepared using papaya latex enzyme also showed antioxidant activities in various model systems.

In vitro digestion models are widely used to study the structural changes, digestibility, and release of food components under simulated gastrointestinal conditions (Hur *et al.*, 2011). Stability of antioxidant peptides during gastrointestinal digestion is an important parameter governing bioactivity of a peptide *in vivo*. When peptides pass through the gastrointestinal tract, they are modified by gastrointestinal proteinases, which could consequently alter antioxidant properties. The effects of an *in vitro* gastrointestinal digestion on the antioxidant activity of peptides may be used as an indicator of the stability and hence the potential bioactivity of peptides *in vivo*.

The direct hydrolysis of pretreated skin, a major source of collagen and gelatin, without prior gelatin extraction, can shorten the processing time and production costs. The obtained peptides can serve as a functional supplement in foods or drinks. The present study aimed to investigate the impact of different processing techniques on the selected characteristics and antioxidant activities of hydrolysates from seabass skin. In addition, the impact of a simulated *in vitro* gastrointestinal digestion on the antioxidant activity of the hydrolysates was evaluated.

8.3 Materials and methods

8.3.1 Chemicals

Alcalase (EC 3.4.21.62) (food grade) from *Bacillus licheniformis* was obtained from Novozyme (Bagsvaerd, Denmark). 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was from Fluka Chemie (Buchs, Switzerland). 2,4,6-Trinitrobenzenesulphonic acid (TNBS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyltriazine (TPTZ), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4"-disulphonic acid sodium salt (ferrozine), ethylenediaminetetraacetic acid (EDTA), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), porcine pepsin (EC 3.4.23.1, 480 units/mg), glycodeoxycholate, Hank's balanced salts solution (HBSS), sodium taurocholate, sodium taurodeoxycholate and porcine pancreatin (EC 232-468-9) were procured from Sigma–Aldrich, Inc. (St. Louis, MO, USA). All other chemicals and reagents were from Sigma Chemical Co. (Dublin, Ireland). All solvents used were of HPLC grade.

8.3.2 Collection of seabass skins

Descaled skins of fresh seabass (*L. calcarifer*) with a weight of 2.5–3 kg were obtained from the Kingfisher Holdings Co., Ltd., Songkhla, Thailand. Skins were kept in a polystyrene box containing ice using a skin:ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Upon arrival, the remaining meat was removed manually. The skins were washed with cold tap water (1–3 ºC). The skins were pooled and used as the composite sample. The samples were placed in polyethylene bags and stored at –20 ºC

until used, but not longer than 2 months. Prior to gelatin extraction and hydrolysis, the frozen skins were thawed with running water (25–26 ºC) until the core temperature reached 8–10 ºC. The skins were then cut into small pieces (1.0 × 1.0 cm^2) using scissors.

8.3.3 Preparation of seabass skins

8.3.3.1 Removal of non-collagenous proteins

The skins were soaked in 0.05 M NaOH with a skin:alkali solution ratio of 1:10 (w/v) in order to remove non-collagenous proteins. The mixture was stirred for 3 h at room temperature (28–30 ºC) using an overhead stirrer model RW20.n (IKA®-Werke GmbH & CO.KG, Staufen, Germany) at a speed of 300 rpm. The alkaline solution was changed every 1 h for a total of 3 times. Alkali-treated skin was washed with tap water until a neutral or faintly basic pH of wash water was obtained.

8.3.3.2 Acid pretreatment and defatting

Following treatment with alkaline solution, the obtained skins were subjected to swelling process using 0.05 M citric acid at a skin:solution ratio of 1:10 (w/v). The mixture was stirred at room temperature for 2 h. The swollen skin was washed using tap water until wash water became neutral or faintly acidic in pH.

Acid pretreated skins were defatted using 30% isopropanol with a solid:solvent ratio of 1:10 (w/v) at room temperature for 1 h. The mixture was continuously shaken at 200 rpm. The solvent was then removed and the defatted skin was washed with 10 volumes of tap water to ensure the solvent was completely removed. The defatted skin was then rinsed with distilled water.

8.3.4 Preparation of gelatin hydrolysate

Gelatin hydrolysis was done either during gelatin extraction or after gelatin extraction according to the method of Senphan and Benjakul (2014) with a slight modification. Alcalase was used to hydrolyse gelatin and protease activity was determined as per the method of An *et al.* (1994). The prepared skins were mixed with

distilled water at a ratio of 1:10 (w/v). Prior to hydrolysis, the pH of the mixture was adjusted to 8.0 using 2 M NaOH. For samples hydrolysed during gelatin extraction, Alcalase was added into the mixture at concentrations of 1 or 2 units/g swollen skin dry matter. The mixtures were incubated for 6 h at 55 ºC in a water bath with continuous stirring using an overhead stirrer at a speed of 150 rpm. For remaining samples, the gelatins were extracted for 6 h at 55 ºC and then hydrolysed with Alcalase at concentrations of 1 or 2 units/g swollen skin dry matter for 3 h at 55 ºC. Following hydrolysis, all samples were heated in a boiling water bath for 10 min to inactivate the enzyme. The obtained hydrolysates were centrifuged at 8000 ×*g* for 10 min. The supernatants were lyophilised prior to analyses. Gelatin hydrolysates produced during gelatin extraction with enzyme concentrations of 1 and 2 units/g dry swollen skin were referred to as 'P1-1U' and 'P1-2U', respectively. Gelatin hydrolysates obtained after gelatin extraction with enzyme concentrations of 1 and 2 units/g dry swollen skin were referred to as 'P2-1U' and 'P2-2U', respectively.

8.3.5 *In vitro* gastrointestinal digestion

The simulated *in vitro* gastrointestinal digestion procedure was performed according to the method of Garrett *et al.* (1999) with minor modifications. Briefly, 0.4 g of each sample was dissolved in 8 ml Hank's balanced salts solution (HBSS) and the solution was transferred to an amber bottle. To mimic the gastric phase of human digestion, freshly prepared pepsin (0.04 g/ml in 0.1 N HCl) was added and the pH was adjusted to 2.0 using 0.1 N HCl. The samples were overlaid with nitrogen gas and incubated at 37 °C for 1 h in an orbital shaking water bath (Grant OLS200, Grant Instruments, Cambridge, UK) at 95 rpm. After gastric digestion, the pH was increased to 5.3 using 0.9 M sodium bicarbonate, followed by the addition of 200 µl of freshly prepared bile salts (0.04 g/ml glycodeoxycholate, 0.04 g/ml taurocholate and 0.025 g/ml taurodeoxycholate) and 100 µl of porcine pancreatin (0.08 g/ml). Subsequently, the pH was increased to 7.4 using 1 M NaOH. Samples were overlaid with a layer of nitrogen gas and incubated for a further 2 h at 37 °C in an orbital shaking water bath to mimic the duodenal phase of human digestion. Following intestinal digestion, the digested sample was centrifuged at 14,000 ×*g* using a centrifuge (Sigma 4K15, SIGMA Laborzentrifugen GmbH, Osterode, Germany) for 30 min at 4 ºC. The

supernatant was collected and filtered through a 0.45 μm membrane filter. Samples were stored at −18 °C until further analysis.

8.3.6 Analyses

8.3.6.1 Degree of hydrolysis (DH)

DH of the hydrolysates was determined as described by Benjakul and Morrissey (1997). Hydrolysate samples (125 μl) were mixed with 2.0 ml of 0.2 M phosphate buffer (pH 8.2) and 1.0 ml of 0.01% TNBS solution. The solution was mixed thoroughly and placed in a temperature controlled water bath at 50 ºC for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulphite. The mixture was cooled at room temperature for 15 min. The absorbance was read at 420 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) and α-amino group was expressed in terms of L-leucine. The DH was calculated as follows:

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

where L is the amount of α-amino groups of hydrolysate sample. L_0 is the amount of α-amino groups in the original swollen skin. L_{max} is the total α-amino groups in the swollen skin or the gelatin obtained after acid hydrolysis (6 M HCl at 100 ºC for 24 h).

8.3.6.2 Yield

The yield of gelatin hydrolysate was calculated based on the dry weight of the swollen skin dried matter by the following equation:

$$\text{Yield (\%)} = \frac{\text{Weight of lyophilised gelatin hydrolysate (g)}}{\text{Weight of initial swollen dried matter (g)}} \times 100$$

8.3.6.3 Colour

The colour of the gelatin hydrolysates was measured by a Hunter Lab Colourimeter (Color Flex, Hunter Lab Inc., Reston, VA, USA). L^*, a^* and b^* indicating lightness/brightness, redness/greenness and yellowness/blueness, respectively, were recorded. The colourimeter was calibrated with a white standard. Total difference in colour (ΔE^*) was calculated according to the following equation:

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

where ΔL^*, Δa^* and Δb^* are the differences between the corresponding colour parameter of the sample and that of white standard ($L^* = 93.55$, $a^* = -0.84$, $b^* = 0.37$).

8.3.6.4 Volatile compounds

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

To extract volatile compounds, 1 g of sample was mixed with 4 ml of deionised water and stirred continuously to disperse the sample. The mixture was heated at 60 °C in 20 ml headspace vial with an equilibrium time of 10 h. The SPME fibre (50/30 µm DVB/Carboxen™/ PDMS StableFlex™; 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 extract and the volatile compounds was 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.

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 °C for 3 min, followed by an increase of 3 °C/min to 70 °C, then an increase of 10 °C/min to 200 °C, and finally an increase of 15 °C/min to a final temperature of 250 °C 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 °C. Transfer line temperature was maintained at 260 °C. The quadrupole mass spectrometer was operated in the electron ionisation (EI) mode and source temperature was set at 250 °C. Initially, full scan mode data was 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.

Identification of volatile compounds in the samples was done by consulting ChemStation Library Search (Wiley 275.L). Identification of compounds 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 were calculated to a signal-to-noise (S/N) ratio of 10. The identified volatile compounds related to lipid oxidation, including aldehydes, alcohols, ketones, etc., were presented as abundance of each identified compound.

8.3.6.5 Antioxidant activities

8.3.6.5.1 DPPH radical scavenging activity

DPPH radical scavenging activity was determined as described by Brand-Williams *et al.* (1995) with a slight modification. Sample (100 µl) was mixed with 900 µL of 0.06 mM DPPH in methanol. The sample was mixed vigorously and allowed to stand at room temperature in the dark for 1 h. The absorbance of the resulting solution was measured at 515 nm using a microplate reader (Thermo Scientific Varioskan® Flash Multimode Reader, Fisher Scientific UK Ltd., Leicestershire, UK). The blank was prepared in the same manner except that distilled water was used instead of the sample. The scavenging effect was calculated as follows:

$$\text{Radical scavenging activity (\%)} = [(B - A)/B] \times 100$$

where A is A_{515} of sample and B is A_{515} of the blank.

8.3.6.52 Ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power (FRAP) assay was carried out as described by Benzie and Strain (1996). Briefly, 2 ml of working FRAP reagent (0.01 M TPTZ in 0.04 M HCl, 0.02 M $FeCl_3.6H_2O$ and 0.3 M acetate buffer), prepared fresh daily, was mixed with 1 ml sample. After a 2 h incubation in the dark, absorbance was

measured at 593 nm. A standard curve was prepared using Trolox in the range of 20–100 µM. Data were expressed as µmol Trolox equivalents (TE)/g sample.

8.3.6.5.3 Ferrous ion-chelating ability

The chelating ability of samples toward ferrous ion (Fe^{2+}) was determined according to the method of Thiansilakul *et al.* (2007) with a slight modification. Briefly, the samples (250 µl) were mixed with 1 ml of 0.1 M sodium acetate buffer pH 4.9 and 50 µl of 2 mM $FeCl_2$. The reaction was initiated by the addition of 5 mM ferrozine (100 µl). Following 20 min incubation at room temperature, the absorbance was measured at 562 nm using a microplate reader (Thermo Scientific Varioskan® Flash Multimode Reader, Fisher Scientific UK Ltd., Leicestershire, UK). The blank was prepared in the same manner except that distilled water was used instead of ferrozine. The ferrous ion-chelating ability was calculated by the equation:

$$\text{Ferrous ion chelating ability (\%)} = [(B - A)/B] \times 100$$

where A is A_{562} of sample and B is A_{562} of the blank.

8.3.6.5.4 Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay was performed as described by Kittiphattanabawon *et al.* (2012) with some modifications. The samples were dissolved in 75 mM phosphate buffer (pH 7.0) to obtain a final concentration of 0.1 mg/ml. The prepared sample (25 µl) was loaded onto a white polystyrene, nontreated 96-well microplate (Costar Corning Inc., Corning, NY, USA). Only the internal wells of the microplate were used. 50 µl of 0.04 µM fluorescein dissolved in 75 mM phosphate buffer (pH 7.0) was added to each sample. The loaded microplate was allowed to equilibrate at 37 ºC for 20 min in a microplate reader (Thermo Scientific Varioskan® Flash Multimode Reader, Fisher Scientific UK Ltd., Leicestershire, UK). The reaction was initiated by the addition of 100 µl of 221 mM AAPH. The reaction was performed at 37 ºC. The fluorescence intensity was measured every 5 min for 90 min with excitation and emission filters of 485 and 535 nm, respectively. The control was prepared in the same manner, except that 75 mM phosphate buffer (pH 7.0) was used instead of the sample. The area under

the fluorescence decay curve (AUC) of the samples was calculated by the normalised curves with the following equation:

$$AUC = AUC = 0.5 + (f_2/f_1) + (f_3/f_1) + (f_4/f_1) +...+ 0.5(f_n/f_1)$$

where f_1 is the fluorescence reading at the initiation of the reaction and f_n is the last measurement. The net AUC was obtained by subtracting the AUC of the blank from that of a sample or standard. Trolox (0–100 μM) was used as the standard. The ORAC was expressed as μmol Trolox equivalents (TE)/g sample.

8.3.7 Statistical analysis

Experiments were run in triplicate using three different lots of samples. The data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Tukey's test. 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 Degree of hydrolysis (DH)

In the present study, seabass skin was hydrolysed by Alcalase for production of antioxidant peptides. Enzymatic hydrolysis of fish proteins generates a mixture of free amino acids, di-, tri-, and oligopeptides, whilst also increasing the solubility of the hydrolysate and improving their functional properties and bioavailability (Bougatef *et al.*, 2012). The extent of protein hydrolysis was estimated by assessing the degree of hydrolysis (DH). DH of gelatin hydrolysates from seabass skin ranged from 36.19 to 38.85% (Table 20). When swollen seabass skin was hydrolysed using Alcalase at levels of 1 and 2 units/g swollen skin, different DH were obtained, depending on the process used. A higher DH was observed in P1-1U and P1-2U, which were the hydrolysates produced during gelatin extraction, than in the hydrolysates produced after gelatin extraction (P2-1U and P2-2U). With the same production process, concentration of enzyme (1 or 2 units/g swollen dry skin) did not significantly ($P > 0.05$) affect DH.

8.4.2 Yield of gelatin hydrolysate

The yields of gelatin hydrolysates prepared from different processes are presented in Table 20. Different yields were obtained for gelatin hydrolysates prepared by different processes ($P < 0.05$). In general, hydrolysate prepared by gelatin extraction in combination with hydrolysis showed higher yield than those prepared by hydrolysis after gelatin extraction when the same enzyme concentration was used ($P < 0.05$). The highest yield (67.68%) was found for P1-2U sample ($P < 0.05$) and was higher than that of P2-2U (61.76%). It was found that the yield increased with increasing enzyme levels ($P < 0.05$). During gelatin extraction at 55 ºC, the heat applied destroyed hydrogen bonds stabilizing the triple helix of collagen in the pretreated skin. As a result, the conversion of collagen to gelatin took place. Simultaneously, enzyme, with the optimum temperature of 55 ºC, was activated, thereby inducing hydrolysis of the released gelatin. The partially hydrolysed peptides or loosened skin matrix could favour the migration of enzyme to substrate. Also, the exposed substrates were readily available for hydrolysis by protease. During gelatin extraction simultaneous with hydrolysis, peptides released or loosened skin matrix could serve as the preferable substrate rather than the process with gelatin extraction, followed by hydrolysis. This was evidenced by the higher yield when hydrolysis was conducted during gelatin extraction. Thus, production processes affected the yield of gelatin hydrolysate from seabass skin.

8.4.3 Colour of gelatin hydrolysate

The colour of the gelatin hydrolysates obtained from seabass skin was expressed as L^*, a^* and b^* (Table 20). All samples exhibited a creamy yellowish colour. Differences in colour were observed between gelatin hydrolysates from various processes. Generally, hydrolysate produced after gelatin extraction had higher L^*-value (lightness) than those hydrolysed during gelatin extraction ($P < 0.05$). P2-1U exhibited the highest L^*- value, whilst P1-2U showed the lowest L^*- value ($P < 0.05$). P2-1U had the lowest a^*-value (redness). The lower b^*-value (yellowness) was found in sample hydrolysed with the lower level of enzyme. The process used for hydrolysate production did not affect b^*-value. Amongst all samples, P1-2U showed the highest

ΔE^* (total colour difference). This was concomitant with the lowest L^*-value. It is likely that at higher concentrations of enzyme the pigments in the skin were eluted to a greater extent. During hydrolysis, carbonyl compounds (e.g. aldehydes and ketones) produced from lipid oxidation are formed. Those compounds can react with amino groups of free amino acids or peptides via the Maillard reaction (Khantaphant *et al.*, 2011) resulting in a product with a more yellow colour such as that observed in the hydrolysate prepared after gelatin extraction, as indicated by the increased b^*-value.

Table 20. DH, yield and colour of gelatin hydrolysates from seabass skin with different processes

Parameters	P1-1U	P1-2U	P2-1U	P2-2U
DH (%)	38.85 ± 0.88^{a}	38.19 ± 0.11^{a}	36.19 ± 0.23^{b}	36.28 ± 1.11^{b}
Yield (%)	59.66 ± 2.59^{b}	67.68 ± 2.14^{a}	52.72 ± 0.80^{c}	61.76 ± 3.21^{b}
L^*	77.03 ± 0.04^{c}	76.31 ± 0.06^{d}	87.92 ± 0.05^{a}	85.75 ± 0.02^{b}
a^*	1.70 ± 0.05^{c}	2.08 ± 0.05^{a}	1.57 ± 0.04^{d}	2.03 ± 0.01^{b}
b^*	11.58 ± 0.03^{b}	20.55 ± 0.08^{a}	17.69 ± 0.11^{b}	20.58 ± 0.05^{a}
ΔE^*	20.12 ± 0.03^{c}	26.66 ± 0.07^{a}	18.30 ± 0.11^{d}	21.79 ± 0.05^{b}

Values are expressed as mean ± SD (n = 3).
Different superscript letters in the same row indicate significant differences ($P < 0.05$).
P1-1U and P1-2U represent gelatin hydrolysate produced during gelatin extraction with enzyme concentrations of 1 and 2 units/g dry swollen skin, respectively. P2-1U and P2-2U represent gelatin hydrolysate produced after gelatin extraction with enzyme concentrations of 1 and 2 units/g dry swollen skin, respectively.

8.4.4 Volatile compounds of gelatin hydrolysate

Selected volatile compounds in seabass skin hydrolysates produced from different processes are shown in Table 21. Aldehydes were found to be the most prolific volatile compounds in gelatin hydrolysates obtained from seabass skin. Aldehydes have been used as the index of lipid oxidation in a number of foods because they possess low threshold values and are the major contributors to the development of off-odour and off-flavour (Ross and Smith, 2006). The various aldehyde compounds formed during oxidation included octanal, nonanal, pentanal and hexanal. Hexanal was found as the major aldehyde formed in gelatin hydrolysates (Table 21). It was found

that P1-1U and P1-2U had a lower abundance of volatiles in comparison with P2-1U and P2-2U (Table 21). This result suggested that hydrolysis of gelatin during extraction could reduce the hydrolysis time at high temperature, leading to the lower abundance of volatile lipid oxidation products. Iglesias and Medina (2008) reported that propanal, 1-penten-3-ol, 2,3-pentanedione, hexanal and 1-octen-3-ol were the main volatile compounds formed during the storage of Atlantic horse mackerel muscle at 4 °C. Varlet *et al.* (2006) reported that carbonyl compounds including 4-heptenal, octanal, decanal, and 2,4-decadienal were responsible for the fishy odour of salmon flesh.

Table 21. Volatile compounds in gelatin hydrolysates from seabass skin with different processes

Compounds	P1-1U	P1-2U	P2-1U	P2-2U
Aldehydes				
Hexanal	17.40	17.57	26.94	35.04
2-Pentenal	0.14	0.49	0.52	1.28
Heptanal	0.26	0.68	0.54	0.74
2-Hexenal	ND	1.70	0.72	1.14
Octanal	ND	0.45	0.35	0.99
Nonanal	0.40	2.08	0.53	1.05
2-Octenal	ND	0.50	ND	0.50
2,6-Nonadienal	ND	0.43	ND	0.42
Alcohols				
1-Penten-3-ol	0.24	0.40	0.25	0.62
1-Octen-3-ol	0.16	0.54	0.23	0.48
Farnesol	0.30	0.85	0.35	0.57
2-Ethylhexanol	0.53	0.74	0.71	0.68
1-Hexadecanol	0.23	1.00	0.39	0.71
Others				
2-Amylfuran	0.69	2.40	0.30	1.81
Styrene	0.61	1.39	0.93	1.13
Tetradecane	0.50	0.52	ND	ND
5-Isopropenyl-3-isopropyl-2,2-dimethyl-2,5-dihydrofuran	0.20	0.20	0.49	1.08

Values are expressed as abundance ($\times 10^9$).

ND: Not detectable. Key: see the caption for Table 20.

Alcohols including 1-penten-3-ol, 1-octen-3-ol, 2-ethylhexanol and 1-hexadecanol were lower in P1-1U than the remaining samples (Table 21). Alcohols are the secondary products produced by the decomposition of hydroperoxides of fatty acids (Ross and Smith, 2006). 1-Octen-3-ol is an important contributor to off-flavour due to its low odour threshold and it was reported to be formed from oxidation of arachidonic acid by 12-lipoxygenase (Hsieh and Kinsella, 1989). 1-Octen-3-ol contributed to the strong intensity of fishy and rancid off-odours in washed Asian seabass mince containing myoglobin (Thiansilakul *et al.*, 2011). Other volatile compounds were also found in skin hydrolysates. Furans including 2-amylfuran and 5-isopropenyl-3-isopropyl-2,2-dimethyl-2,5-dihydrofuran were detected in all samples. .

Generally, secondary oxidation products such as aldehydes and alcohols were lower in P1-1U and P1-2U than in P2-1U and P2-2U indicating that hydrolysis of gelatin during extraction was effective in the reduction of volatile secondary lipid oxidation products, which contribute to the offensive fishy odour in gelatin hydrolysates.

8.4.5 Antioxidant activities of gelatin hydrolysates

The antioxidant activity of protein hydrolysates or peptides can occur via several mechanisms. Therefore, different assays (DPPH, FRAP, ferrous ion chelating ability and ORAC) were performed to assess the antioxidant properties of the gelatin hydrolysates.

8.4.5.1 DPPH radical scavenging activity

The DPPH radical scavenging assay has been widely used to evaluate antioxidant properties of compounds such as free radical scavengers or hydrogen donors (Klompong *et al.*, 2007). Gelatin hydrolysates from seabass skin showed a dose dependent DPPH scavenging effect ranging from 19 to 79 % (Figure 25A). At concentrations below 25 mg/ml no significant difference ($P > 0.05$) in DPPH radical scavenging activity between samples was found. At higher concentrations, P1-1U had the highest DPPH radical scavenging activity, whilst P2-2U showed the lowest activity. DPPH radical scavenging activity was previously reported in protein hydrolysates

prepared from the skin of seabass (Senphan and Benjakul, 2014), catfish (Alemán *et al.*, 2011), Alaska pollack (Jia *et al.*, 2010) and bigeye snapper (Phanturat *et al.*, 2010). Increasing quantities of active peptides react with free radicals to convert them to stable products (Batista *et al.*, 2010). At the highest concentration (125 mg/ml), the sample with the highest DPPH scavenging activity (79%) was P1-1U. The EC_{50} values (concentration required to inhibit 50% of DPPH radical) of gelatin hydrolysate were 60, 73, 76 and 87 mg/ml for P1-1U, P1-2U, P2-1U and P2-2U, respectively (data not shown). Samples prepared by hydrolysis after gelatin extraction, showed higher EC_{50} values and therefore had a lower scavenging activity, than P1-1U and P1-2U (prepared during gelatin extraction). The results indicated that gelatin hydrolysates from seabass skin contain peptides which may act as hydrogen donors to free radicals and thereby have the potential to prevent or retard lipid oxidation via a chain breaking reaction.

8.4.5.2 Ferric reducing antioxidant power (FRAP)

The ability of gelatin hydrolysates to reduce Fe^{3+} to Fe^{2+} was determined and expressed as FRAP using the well-known antioxidant Trolox as a comparison standard (Figure 25B). All samples showed FRAP values in the range of 0.35– 2.68 µmol TE/g sample. The highest FRAP was obtained in P1-1U at a concentration of 7.5 mg/ml ($P < 0.05$). No significant differences ($P > 0.05$) in the FRAP value of P1-1U were observed at different concentrations. Hydrolysates prepared from several fish skins such as bigeye snapper (Phanturat *et al.*, 2010), sole (Giménez *et al.*, 2009) and seabass (Senphan and Benjakul, 2014) also exhibited FRAP activity. Processing conditions can greatly influence peptide chain length as well as the exposure of terminal amino groups of products. Greater FRAP values indicate that hydrolysates could donate an electron to free radicals, leading to the prevention or retardation of propagation (Klompong *et al.*, 2007). The results of the present study suggest that gelatin hydrolysates hydrolysed during gelatin extraction possibly contain higher amount of peptides which can donate electrons to free radicals.

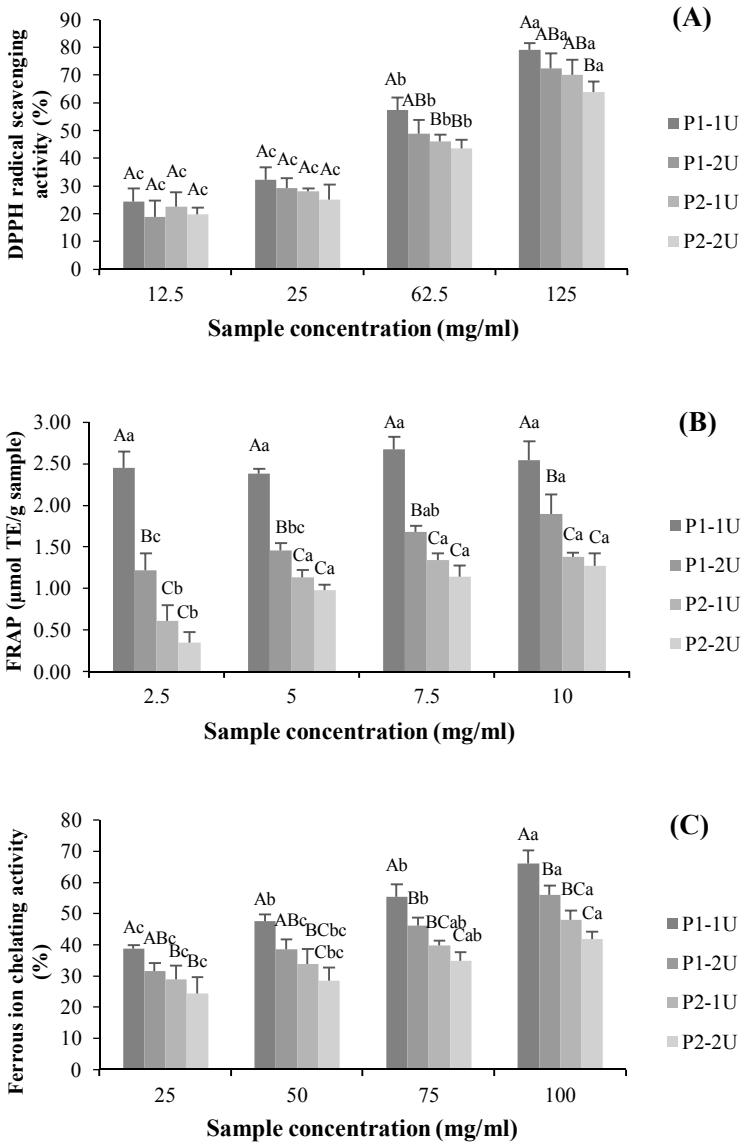

Figure 25. DPPH radical scavenging activity (A), FRAP (B) and ferrous ion chelating activity (C) of gelatin hydrolysates from seabass skin prepared using different processes. Bars represent the mean ± standard deviation (n = 3). Different uppercase letters on the bars within the same hydrolysate concentration indicate significant differences ($P < 0.05$). Different lowercase letters on the bars within the same hydrolysate sample indicate significant differences ($P < 0.05$). Key: see the caption for Table 20.

8.4.5.3 Ferrous ion chelating activity

The transition metal ions such as Fe^{2+} and Cu^{2+} participate in the formation of free radicals or reactive oxygen species that accelerate lipid oxidation (Sarac, 1999), therefore, chelation of transition metal ions could retard or interrupt the oxidation process (Gordon, 2001). In comparison with other ions, ferrous ion is a key active species responsible for ROS formation in cells, leading to the increase of lipid oxidation (Huang *et al.*, 2002).

The ferrous ion chelating ability of the gelatin hydrolysates are shown in Figure 25C. Gelatin hydrolysate samples demonstrated the ability to chelate ferrous ion in a dose dependent manner ($P < 0.05$). At the same concentration, P1-1U generally showed the highest ferrous ion chelating activity, whilst P2-2U exhibited the lowest activity ($P < 0.05$). The EC_{50} values of ferrous ion chelating activity were 57, 84, 112 and 137 mg/ml for P1-1U, P1-2U, P2-1U and P2-2U, respectively (data not shown). The peptides present in the hydrolysates most likely had different metal ion chelating capacity, depending on the amino acid sequences and chain length of peptide fragments. Samaranayaka and Li-Chan (2008) reported that ferrous ion chelating ability ranged from approximately 7 to 46% for hydrolysates derived from Pacific hake muscle prepared using different hydrolysis processes. Thiansilakul *et al.* (2007) found a chelating activity of 60% in round scad protein muscle hydrolysate. Carboxyl and amino groups in the side chains of the acidic (Glx, Asx) and basic (Lys, His, Arg) amino acids are thought to play an important role in chelating metal ions (Saiga *et al.*, 2003). Transition metals, such as Fe, Cu and Co in foods affect both the rate of autoxidation and breakdown of hydroperoxide to volatile compounds. Transition metal ions react very quickly with peroxides by acting as one-electron donors to form alkoxyl radical (Gordon, 2001). Skin hydrolysates could act as the secondary antioxidant which could scavenge pro-oxidative metal ions.

8.4.5.4 Oxygen radical absorbance capacity (ORAC)

ORAC is the only assay that combines both inhibition time and degree of inhibition into a single quantity (Prior *et al.*, 2005). The protective effect of an

antioxidant is calculated from the net integrated area under the fluorescence decay curve (AUC) and reported as Trolox equivalents. The fluorescence decay was highest for the control, whilst inhibition was observed in the presence of gelatin hydrolysates. P1-1U showed the highest inhibition toward fluorescence decay ($P > 0.05$) (Figure 26A). The ORAC values of the gelatin hydrolysates ranged from 550.25 to 623.88 µmol TE/g sample (Figure 26B). However, no statistical ($P > 0.05$) differences in ORAC values were determined between the samples. Kittiphattanabawon *et al.* (2012) reported that gelatin hydrolysates from blacktip shark skin prepared using papaya latex enzyme showed ORAC in the range of 268.16 to 709.42 µmol TE/g sample.

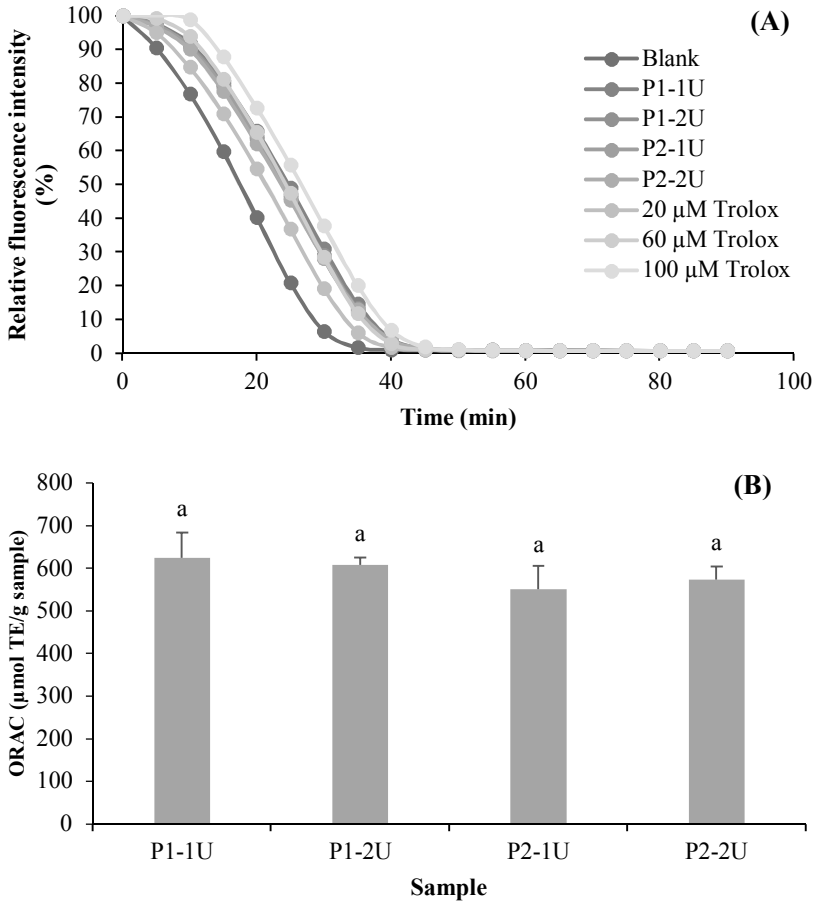

Figure 26. Fluorescence decay curves of fluorescein (A) and oxygen radical absorbance capacity (ORAC) of gelatin hydrolysates (B). Bars represent the mean ± standard deviation (n = 3). Different letters on the bars indicate significant differences ($P < 0.05$). Key: see the caption for Table 20.

Differences in DPPH radical scavenging activity, FRAP, ferrous ion chelating activity and ORAC between gelatin hydrolysates from seabass skin prepared using different processes possibly resulted from differences in the experimental conditions, by which a wide variety of peptides with different modes of actions for inhibiting lipid oxidation were generated during hydrolysis. Changes in size, amount, the exposure of the terminal amino groups of the products obtained and the composition of free amino acids or small peptides affect the antioxidative activity (Thiansilakul *et al.*, 2007). Low molecular weight peptides have generally shown higher antioxidant activity (Qian *et al.*, 2008).

8.4.6 Changes in antioxidant activities of gelatin hydrolysate upon *in vitro* gastrointestinal digestion

A simulated *in vitro* gastrointestinal digestion model was used to study the stability of gelatin hydrolysates following incubation with several digestive proteases. *In vitro* methods for simulating the human digestive tract are being extensively used since they are rapid, safe and do not have the same ethical restrictions as *in vivo* methods. The antioxidant activity of gelatin hydrolysates, as determined by DPPH scavenging activity, FRAP and ferrous ion chelating activity, following simulated *in vitro* gastrointestinal digestion are presented in Figure 27.

After simulated gastric digestion, the DPPH radical scavenging activity of all samples slightly decreased ($P < 0.05$), compared to those of non-digested samples (Figure 27A). Thereafter, a slight increase was noticeable after intestinal digestion, compared with corresponding gastric digests ($P < 0.05$). It was noted that no significant differences in DPPH radical scavenging activity of all samples were found between non-digested samples and those obtained after duodenal digestion ($P > 0.05$). A similar change in antioxidant activity during gastrointestinal digestion of peptides has also been reported recently (Sanjukta *et al.*, 2015). The decrease in activity after pepsin digestion may be due to the degradation of some original peptides and increase in activity was probably due to further formation of peptides with higher antioxidant activity (Sanjukta *et al.*, 2015). The conditions of the gastrointestinal tract, such as digestive enzymes and pH values in the stomach may influence the structures and functions of the peptides.

The loss of activity observed could be attributed to the acidic condition (pH 2.0) under gastric phase digestion.

FRAP activity demonstrated a similar trend to DPPH radical scavenging activity following simulated *in vitro* gastrointestinal digestion. No significant changes in FRAP of all samples were found as a result of the gastric and intestinal digestion, except P1-1U (Figure 27B). After 1 h of digestion by pepsin, the FRAP of P1-1U decreased slightly but increased following intestinal digestion ($P < 0.05$). A similar result was observed from collagen peptides from Alaska pollock skin (Guo *et al.*, 2015).

The changes in metal chelating activity of gelatin hydrolysates upon simulated in vitro gastrointestinal digestion are presented in Figure 27C. The ferrous ion chelating activity of the samples before gastrointestinal digestion ranged from 30.05 to 52.50% and were significantly decreased ($P < 0.05$) to 15.87 – 19.69% after gastric phase digestion. After duodenal phase digestion, ferrous ion chelating activity increased to 62.61 – 72.11% ($P < 0.05$). In the gastric phase, pepsin may disrupt the structure of peptides and reduce their abilities to bind Fe^{2+}. The decrease in metal chelating activity might also result from the degradation of peptides under high acidic pH in gastric phase. During duodenal phase digestion, pancreatin may cleave the peptides to a greater extent, leading to the release of new antioxidant peptides or modification of the antioxidant peptide sequences. High affinity metal binding groups may became more exposed or be newly formed. Guo *et al.* (2015) reported that during simulated gastrointestinal digestion of collagen hydrolysate from Alaska pollock skin, different peptides and free amino acids were released by the action of digestive enzymes, which changed the composition of the hydrolysate and the ability of the peptides to bind metal ions. Pepsin shows a high specificity towards the aromatic amino acids e.g. Phe, Tyr, and Trp, and Leu and Glu at the carboxyl side of a peptide bond (Simpson, 2000), whilst pancreatin contains many proteases, including endopeptidases (trypsin, α-chymotrypsin, and elastase) and the exopeptidases (carboxypeptidases A and B) with broad specificity (Young *et al.*, 2011). Nalinanon *et al.* (2011) also found increased antioxidant activity of protein hydrolysate from ornate threadfin bream after being digested in the simulated model.

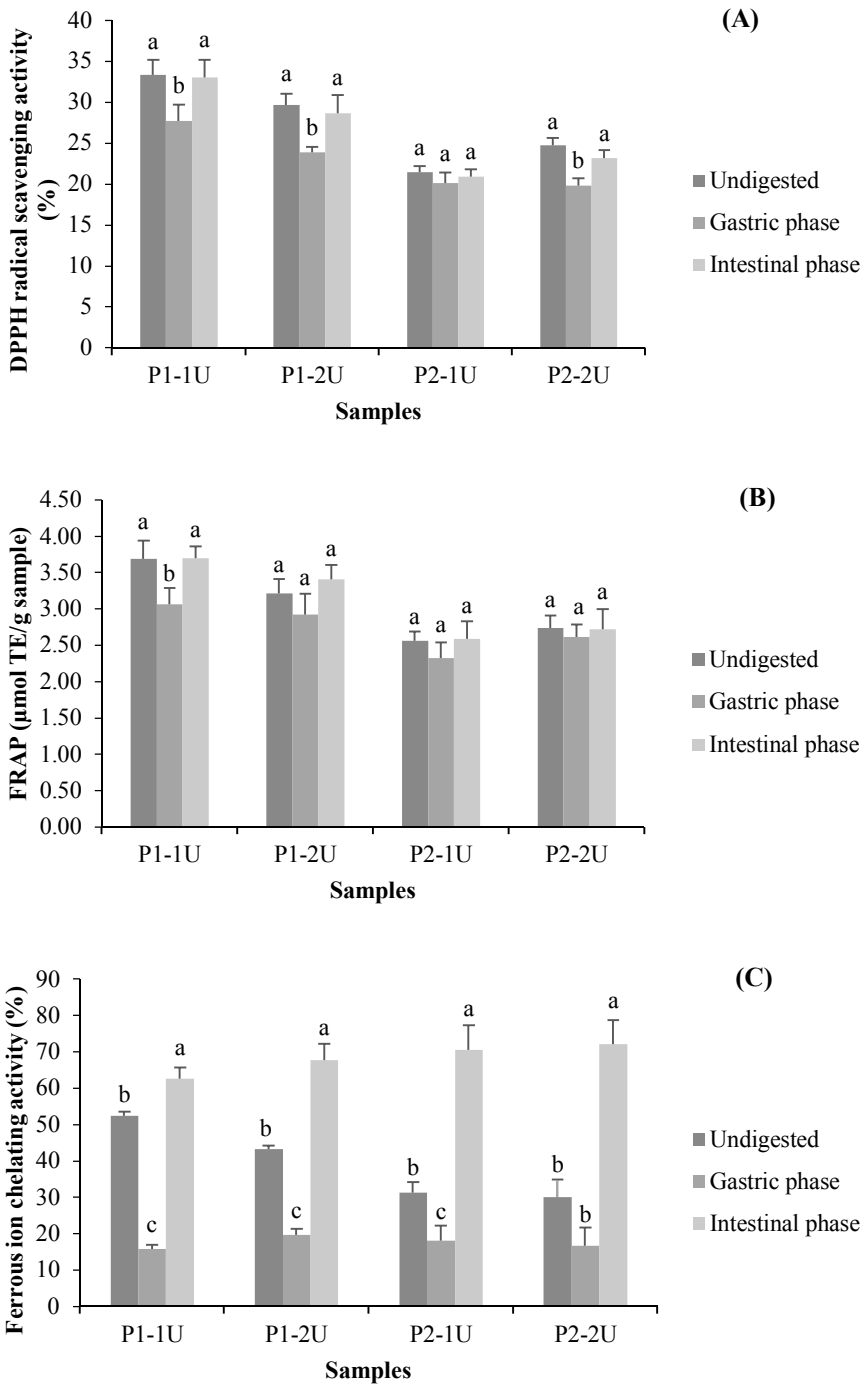

Figure 27. Changes in DPPH radical scavenging activity (A), FRAP (B) and ferrous ion chelating activity (C) of gelatin hydrolysates from seabass skin after the simulated *in vitro* gastrointestinal digestion. Bars represent the mean ± standard deviation (n = 3). Different letters within the same sample indicate significant differences ($P < 0.05$). Key: see the caption for Table 20.

8.5 Conclusion

Production processes affected the selected characteristics and antioxidant activities of seabass skin hydrolysates prepared using Alcalase. Hydrolysis of gelatin during extraction was effective in the reduction of volatile secondary lipid oxidation products. The hydrolysates produced during gelatin extraction demonstrated greater antioxidant activity than those hydrolysates prepared after gelatin had been extracted. DPPH radical scavenging activity and FRAP of seabass skin hydrolysates were stable after *in vitro* simulated gastrointestinal digestion, whilst metal chelating activity was improved. Therefore, hydrolysate from seabass skin could serve as a potential source of functional food and/or neutraceutical peptides.

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CHAPTER 9

ANTIOXIDANT, IMMUNOMODULATORY AND ANTIPROLIFERATIVE EFFECTS OF GELATIN HYDROLYSATE FROM SEABASS (*LATES CALCARIFER*) SKIN

9.1 Abstract

This study investigated the antioxidant, immunomodulatory and antiproliferative potentials of gelatin hydrolysates from seabass skin in cell model systems. Gelatin hydrolysates were prepared from seabass skins using different processes and enzyme concentrations. The ability of the hydrolysates to protect against H_2O_2-induced DNA damage was assessed in U937 cells by the Comet assay and one of the samples demonstrated DNA protective effects. All samples showed immunomodulatory potential by significantly ($P < 0.05$) reducing interleukin-6 (IL-6) and IL-1β production in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells. Antiproliferative activities of seabass skin hydrolysates were measured using human colon cancer (Caco-2) and liver cancer (HepG2) cell lines as the model cell cultures. The inhibition of cell proliferation of Caco-2 and HepG2 cancer cells occurred in a dose-dependent manner at concentrations of 1–25 mg/ml. Therefore, seabass skin hydrolysates could serve as a potential functional food ingredient.

9.2 Introduction

In recent years, several attempts have been made to valorize protein rich fish processing by-products through the production of value-added food ingredients for commercial markets. Fish protein hydrolysates have gained attention due to the availability of large quantities of raw materials and the presence of a high protein content with good amino acid profile and demonstrated bioactivities (Chalamaiah *et al.*, 2012). Fish bioactive peptides have shown a wide range of biological functions including antioxidant, antihypertensive, antimicrobial, antiproliferative, immunomodulatory, mineral binding and antimicrobial effects due to their structural properties and amino acid composition and sequences (Ngo *et al.*, 2014). Hence, it can

be suggested that fish-derived bioactive proteins may be used as an alternative to synthetic ingredients and may provide many health benefits, as ingredients in functional foods, pharmaceuticals and/or cosmetics (Kim and Wijesekara, 2013). A number of studies have demonstrated that hydrolysates derived from different fish skin such as cobia (Yang *et al.*, 2008), tilapia (Yang *et al.*, 2009), sole (Giménez *et al.*, 2009), brownstripe red snapper (Khantaphant and Benjakul, 2008), bigeye snapper (Phanturat *et al.*, 2010), and horse mackerel and croaker (Sampath Kumar *et al.*, 2012) have potential antioxidant effects.

Enzymatic hydrolysates of fish proteins have also demonstrated immunomodulatory activity. Protein hydrolysates derived from fish such as salmon and sweetfish have demonstrated anti-inflammatory potential by inhibiting the production of nitric oxide and pro-inflammatory cytokines in LPS-stimulated RAW264.7 mouse macrophage cells (Ahn *et al.*, 2012; Sung *et al.*, 2012).

Chemopreventive compounds offer a promising anticancer approach by reducing the morbidity and mortality of cancer through delaying the process of carcinogenesis (Sheih *et al.*, 2009). Hydrolysates from fish have shown potential chemopreventative activity. A pepsin hydrolysate from half-fin anchovy exhibited antiproliferative activity in human prostate (DU-145), human lung (1299) and human esophagus cancer cells (109) (Song *et al.*, 2011). A gelatin hydrolysate from giant squid showed a strong cytotoxic effect in human breast carcinoma (MCF-7) and glioma (U87) cells (Alemán *et al.*, 2011). Naqash and Nazeer (2010) also found that peptide fractions from Japanese threadfin bream and tropical two-wing flying fish hydrolysates exerted antiproliferative effects in HepG2 cells.

Seabass (*Lates calcarifer*) is an economically important fish species in Thailand and other countries, especially the tropical and subtropical regions of Asia and the Pacific. During processing or dressing of seabass, the skin is removed and considered a by-product. Recently, seabass skin has been used to produce collagen and gelatin, as well as hydrolysates with potential bioactivity (Senphan and Benjakul, 2014; Sinthusamran *et al.*, 2013). Although protein hydrolysates derived from fish have been widely reported to exhibit *in vitro* antioxidant activity, little information regarding the

antioxidant, immunomodulatory and antiproliferative activities in cell culture systems have been reported. Therefore, the objective of this study was to investigate the protective effects of seabass skin gelatin hydrolysates, prepared using Alcalase, against oxidative DNA damage in addition to their potential immunomodulatory and antiproliferative effects in cell model systems.

9.3 Materials and methods

9.3.1 Chemicals

Dulbecco's modified Eagle's medium (DMEM), Royal Park Memorial Institute (RPMI)-1640 medium, Hanks balanced salt solution (HBSS) and non-essential amino acids were purchased from Sigma-Aldrich Chemical Co. (Dublin, Ireland). Human histiocytic lymphoma cells (U937 cells), mouse leukaemic macrophages (RAW264.7 cells), human hepatoma cells (HepG2 cells) and human carcinoma cells (Caco-2 cells) were purchased from the European Collection of Animal Cell Cultures (Salisbury, UK). Foetal bovine serum was purchased from Invitrogen (Paisley, Scotland). Cell culture plastics were supplied by Cruinn Diagnostics (Greiner Bio-One, Frickenhausen, Germany). All other cell culture chemicals and reagents were from Sigma Chemical Co. (Dublin, Ireland). All solvents used were of HPLC grade.

9.3.2 Collection of seabass skins

Descaled skins of seabass (*L. calcarifer*) were obtained from the Kingfisher Holdings Co., Ltd., Songkhla, Thailand. Skins were kept in a polystyrene box containing ice using a skin/ice ratio 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Upon arrival, the remaining meat was manually removed. The skins were washed with cold tap water (1–3 ºC). The skins were pooled and used as the composite sample. The samples were placed in polyethylene bags and stored at –20º C until used, but not longer than 2 months. Prior to gelatin extraction, the frozen skins were thawed with running water (25–26 ºC) until the core temperature reached 8–10 ºC. The skins were then cut into small pieces (1.0 × 1.0 cm^2) using scissors.

9.3.3 Preparation of seabass skins

The skins were soaked in 0.05 M NaOH with a skin/alkali solution ratio of 1:10 (w/v) in order to remove non-collagenous proteins. The mixture was stirred for 3 h at room temperature (28–30 ºC) using an overhead stirrer (RW20.n, IKA®-Werke GmbH & CO.KG, Staufen, Germany) at a speed of 300 rpm. The alkaline solution was changed every 1 h for a total of 3 times. Alkali-treated skin was washed with tap water until a neutral or faintly basic pH of wash water was obtained.

Following treatment with alkaline solution, the obtained skins were subjected to a swelling process using 0.05 M citric acid at a skin/solution ratio of 1:10 (w/v). The mixture was stirred at room temperature for 2 h. The swollen skin was washed using tap water until the pH of the wash water became neutral or faintly acidic.

Acid pretreated skins were defatted using 30% isopropanol with a solid/solvent ratio of 1:10 (w/v) at room temperature for 1 h. The mixture was continuously shaken at 200 rpm. The solvent was then removed and the defatted skin was washed with 10 volumes of tap water to ensure the solvent was completely removed. The defatted skin was then rinsed with distilled water.

9.3.4 Preparation of gelatin hydrolysate

Gelatin hydrolysis was done either during gelatin extraction or after gelatin extraction according to the method of Senphan and Benjakul (2014) with a slight modification. The prepared skins were mixed with distilled water at a ratio of 1:10 (w/v) at 55 ºC in a water bath (model W350, Memmert, Schwabach, Germany) for 6 h with continuous stirring using an overhead stirrer at a speed of 150 rpm to extract the gelatin from skin matter. The mixtures were then filtered using a Buchner funnel with Whatman No. 4 filter paper (Whatman International, Ltd., Maidstone, England).

Prior to hydrolysis, the pH of the gelatin solution was adjusted to pH 8.0 using 2 M NaOH. Alcalase was added into the mixture at concentrations of 1 or 2 units/g swollen skin dry matter. For samples hydrolysed during gelatin extraction, Alcalase was added into the mixture at concentrations of 1 or 2 units/g swollen skin dry

matter. The mixtures were incubated for 6 h at 55 °C in a water bath with continuous stirring using an overhead stirrer at a speed of 150 rpm. For remaining samples, the gelatins were extracted for 6 h at 55 °C and then hydrolysed with Alcalase at concentrations of 1 or 2 units/g swollen skin dry matter for 3 h at 55 °C. Following hydrolysis, all samples were heated in a boiling water bath for 10 min to inactivate the enzyme. The obtained hydrolysates were centrifuged at 8000×*g* for 10 min. The supernatants were lyophilised prior to analyses. Gelatin hydrolysates produced during gelatin extraction with enzyme concentrations of 1 and 2 units/g dry swollen skin were referred to as 'sample 1' and 'sample 2', respectively. Gelatin hydrolysates obtained after gelatin extraction with enzyme concentrations of 1 and 2 units/g dry swollen skin were referred to as 'sample 3' and 'sample 4', respectively.

9.3.5 Determination of antioxidant activity in cell model systems

9.3.5.1 Cell culture

U937 cells were grown in RPMI-1640 medium supplemented with 10% (v/v) foetal bovine serum (FBS). RAW264.7 and HepG2 cells were maintained in DMEM supplemented with 10% (v/v) FBS. Caco-2 cells were cultured in DMEM supplemented with 10% (v/v) FBS and 1 % non-essential amino acids. Cells were cultured in the absence of antibiotics. The cells were grown at 37 °C in a 5 % (v/v) CO_2 atmosphere in a humidified incubator. Reduced serum media (2.5% FBS) was used for all experiments.

9.3.5.2 Determination of IC_{50} in RAW264.7 cells

RAW264.7 cells (2×10^4 cells/ml) were supplemented with increasing concentrations (0–15 mg/ml) of gelatin hydrolysates in 96-well flat-bottom plates with a final volume of 200 µl at 37 ºC for 24 h. Following incubation, cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (MTT I proliferation kit, Roche Diagnostics; Burgess Hill, West Sussex, UK). In this assay, MTT (a yellow tetrazolium salt) is converted to a formazan derivative (purple colour), and the absorbance of the converted dye is measured at 570 nm. Briefly, 10 µl MTT reagent 1 and 100 µl DMEM were added to cells and incubated

for 4 h at 37 ºC. Following incubation, 100 µl MTT reagent 2 was added and cells were incubated for further 24 h at 37 ºC. Absorbance was read at 570 nm using a microplate reader (Thermo Scientific Varioskan® Flash Multimode Reader, Fisher Scientific UK Ltd., Leicestershire, UK). The IC_{50} value (the concentration of sample that induced 50% decrease in viable cells) for each gelatin hydrolysate was calculated using the data obtained from the MTT assay and Prism software (version 4.0, GraphPad Inc., San Diego, CA, USA).

9.3.5.3 Determination of DNA damage (Comet assay)

U937 cells (1×10^5 cells/ml) were treated with gelatin hydrolysate (2 and 5 mg/ml) for 24 h in a 24-well plate with a final volume of 1 ml media, containing reduced FBS (2.5%, v/v) at 37 °C. Following incubation, cells was treated with 40 µM H_2O_2 for 30 min. Oxidative DNA damage in the U937 cells was assessed using the Comet assay as described by McCarthy *et al.* (2012). Briefly, slides were prepared by coating with 1% (w/v) normal gelling agarose (NGA). Cells (30 µl) were then mixed with 1% (w/v) low melting point (LMP) agarose, placed on a microscope slides, covered with a coverslip and the mini-gels were allowed to solidify on ice. Slides were then placed in cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM tri(hydroxymethyl)-aminomethane, fresh 1% Triton® X-100 and 10% dimethyl sulfoxide) for 1.5 h at 4 ºC. Slides were aligned in a horizontal gel electrophoresis tank (Horizon® 20·25, GIBCO BRL Life Technologies, Gaithersburg, MD, USA) which was filled with fresh electrophoresis solution (1 mM EDTA, 300 mM NaOH; pH 13). Slides were allowed to sit in this buffer for 30 min. Electrophoresis was conducted at 20V, 300 mA for 25 min at 4 ºC. After electrophoresis, the slides were neutralized using 0.4 M Tris for 5 min (×3) and rinsed with distilled water. Slides were stained with ethidium bromide (20 µg/ml) for 5 min and rinsed with distilled water. The Komet 5.5 image analysis software (Kinetic Imaging, Liverpool, UK) was used to score 50 cells for each slide using a fluorescence microscope (Optiphot-2, Nikon). DNA damage was expressed as percentage tail DNA.

9.3.6 Cytokine production (Immunomodulatory activity)

RAW264.7 cells, at a density of 2×10^5 cells/ml, were seeded in 96-well plates in the presence of lipopolysaccharide (LPS, 0.5 μg/ml) and treated with test samples (5 and 10 mg/ml) for 24 h at 37 ºC. Production of the cytokines interleukin-6 (IL-6) and interleukin-1β (IL-1β) was determined using ELISA kits (eBioscience mouse IL-6 and IL-1β ELISA Ready-SET-Go kits, Insight Biotechnology, Wembley, U.K.). Absorbance was read at 450 nm using a microplate reader. Data were expressed as a percentage of the LPS-stimulated RAW264.7 cell control.

9.3.7 Measurement of inhibition of HepG2 and Caco-2 cell proliferation

The antiproliferative activities of hydrolysates were assessed in Caco-2 and HepG2 human cancer cell lines. Caco-2 and HepG2 cells at a density of 2×10^4 cells/ml in growth media were seeded in each well of 96-well flat-bottom plates. Cells were incubated with gelatin hydrolysates (1-25 mg/ml) at 37 ºC for 24 h. Cell proliferation was determined using the MTT assay as previously described.

9.3.8 Statistical analysis

Experiments were carried out in triplicate using three different lots of samples. The data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test. Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

9.4 Results and discussion

9.4.1 Effect of gelatin hydrolysates on cell viability in RAW264.7 cells

Cytotoxicity of gelatin hydrolysates from seabass skin was tested in the RAW264.7 cell line to determine the non-cytotoxic concentration (Table 22). Cells were treated with or without increasing concentrations (0–15 mg/ml) of gelatin hydrolysates for 24 h and the cell viability was measured by the MTT assay. All samples reduced RAW264.7 cell proliferation in a dose-dependent manner. The MTT

Table 22. Effect of gelatin hydrolysates (0–15 mg/ml) on cell proliferation in the RAW264.7 cell line

Sample	Cell proliferation (% of control)									
	0 mg/ml	1 mg/ml	2 mg/ml	3 mg/ml	4 mg/ml	6 mg/ml	8 mg/ml	10 mg/ml	12 mg/ml	15 mg/ml
Sample 1	100.0 ± 0.0^{a}	87.63 ± 9.25^{b}	85.85 ± 3.48^{bc}	79.21 ± 5.78^{bc}	73.57 ± 4.28^{cd}	66.88 ± 2.29^{de}	57.57 ± 2.61^{ef}	51.64 ± 2.79^{fg}	47.28 ± 2.79^{fg}	40.38 ± 2.11^{g}
Sample 2	100.0 ± 0.0^{ab}	103.41 ± 9.72^{a}	100.88 ± 7.37^{ab}	97.80 ± 9.91^{ab}	91.36 ± 6.95^{abc}	84.21 ± 9.72^{bc}	73.00 ± 4.35^{cd}	62.03 ± 3.78^{de}	55.78 ± 3.12^{de}	46.80 ± 1.24^{e}
Sample 3	100.0 ± 0.0^{a}	100.19 ± 7.19^{a}	96.90 ± 6.82^{ab}	89.44 ± 5.32^{ab}	85.99 ± 9.40^{abc}	78.91 ± 7.74^{bc}	66.76 ± 1.06^{cd}	58.45 ± 3.40^{de}	55.21 ± 3.60^{de}	45.24 ± 4.06^{e}
Sample 4	100.0 ± 0.0^{a}	94.23 ± 9.95^{a}	92.74 ± 9.96^{ab}	90.28 ± 4.53^{ab}	85.21 ± 7.17^{ab}	74.75 ± 9.37^{bc}	59.96 ± 7.16^{cd}	52.21 ± 2.91^{d}	47.70 ± 2.35^{d}	42.83 ± 4.77^{d}

Values are mean ± SD of 3 independent experiments, expressed as percentage relative to untreated RAW264.7 cells.

($^{a-g}$): Different superscript lowercase letters within the same row indicate significant differences ($P < 0.05$).

Sample 1 and sample 2 represent gelatin hydrolysate produced during gelatin extraction with enzyme concentrations of 1 and 2 units/g dry swollen skin, respectively.

Sample 3 and sample 4 represent gelatin hydrolysate produced after gelatin extraction with enzyme concentrations of 1 and 2 units/g dry swollen skin, respectively.

colourimetric assay is an established method of determining viable cell number in proliferation and cytotoxicity studies.

At a concentration of 1 mg/ml, sample 1 significantly ($P < 0.05$) reduced cell viability by approximately 12%, compared with untreated cells, in RAW264.7 (Table 22). Samples 2, 3 and 4 caused a significant reduction in cell viability at concentrations from 6–15 mg/ml ($P < 0.05$). IC_{50} values of each sample were calculated and found to be in the range from 11.2 to 13.6 mg/ml (data not shown). It was found that sample 2 was the least cytotoxic with the highest IC_{50} value of 13.6 mg/ml. This finding indicates that the processing conditions of the gelatin hydrolysates influenced their effect on RAW264.7 cell viability.

9.4.2 Ability of gelatin hydrolysate to protect against oxidant-induced DNA damage

The protective effects of gelatin hydrolysates against oxidant-induced DNA damage were determined using the Comet assay. U937 cells were pretreated with the samples (2 and 5 mg/ml) for 24 h prior to exposing the cells to 40 μM H_2O_2. The addition of 40 μM H_2O_2 to U937 cells significantly ($P < 0.05$) increased DNA damage from a control level of 2.1% tail DNA in untreated cells to 75.5% tail DNA (Figure 28). Pre-incubation (24 h) of U937 cells with sample 1 at 2 and 5 mg/ml significantly ($P < 0.05$) reduced DNA damage to approximately 66.4 and 60.4% tail DNA, respectively. However, the remaining samples did not exert protective effects against H_2O_2-mediated DNA damage at the concentrations tested. ROS-induced DNA damage is associated with carcinogenesis, neurodegenerative diseases (e.g., Parkinson's and Alzheimer's) and aging processes (Mandavilli *et al.*, 2002).

Few studies have demonstrated the ability of hydrolysates from marine sources to protect against oxidant-induced DNA damage. Peptide from oyster protein at concentrations ranging from 5.7 to 45.6 μmol/l showed protective effects on plasmid pBRr 322 DNA damage caused by hydroxyl radicals (Qian *et al.*, 2008). Ryu *et al.* (2011) reported that peptides derived from seaweed pipefish suppressed H_2O_2-induced DNA damage in human dermal fibroblasts (HDF). The protective effect of tuna liver

hydrolysates was related to their scavenging of H_2O_2, hydroxyl radical and chelating activity toward Fe^{2+}. Such activities lead to the inhibition of the Fenton reaction, and therefore, protected the DNA from oxidant-induced strand breaks (Je *et al.*, 2009). From the findings of the present study, it may be postulated that sample 1 had a higher Fe^{2+} chelating ability than the remaining samples which could account for its greater protective effect against oxidant-induced DNA damage. Seabass skin hydrolysates prepared using different processes exhibited various protective effects against DNA damage which may be due to the differences in their chain length, amino acid compositions and sequences.

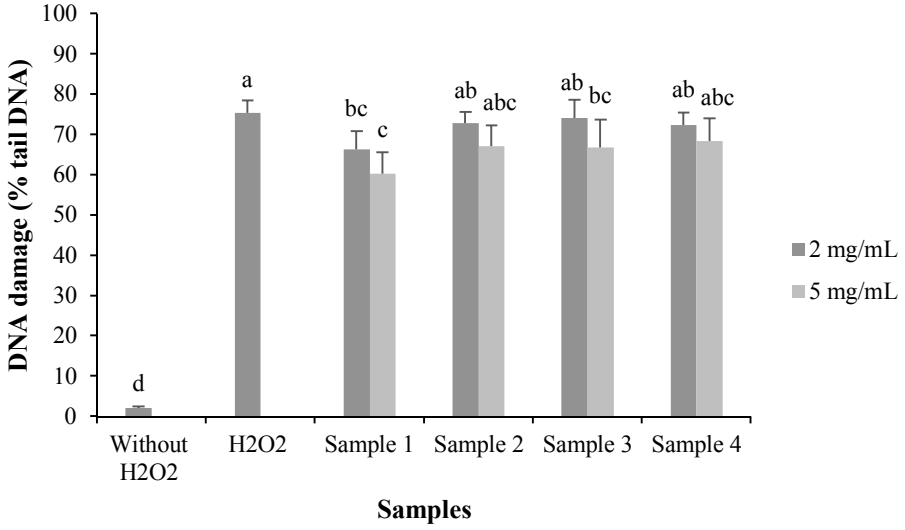

Figure 28. DNA damage induced by H_2O_2 in U937 cells following pretreatment with or without gelatin hydrolysates from seabass skin. Different lowercase letters on the bars indicate significant differences ($P < 0.05$). Bars represent the standard deviations ($n = 3$). Sample 1 and sample 2 represent gelatin hydrolysate produced during gelatin extraction with enzyme concentrations of 1 and 2 units/g dry swollen skin, respectively. Sample 3 and sample 4 represent gelatin hydrolysate produced after gelatin extraction with enzyme concentrations of 1 and 2 units/g dry swollen skin, respectively.

9.4.3 Immunomodulatory effects of gelatin hydrolysates

The immunomodulatory potential of gelatin hydrolysates from seabass skin was determined by measuring their effect on the production of cytokines IL-6 and IL-1β in lipopolysaccharide (LPS)-stimulated macrophage RAW264.7 cells (Figure 29A & B). All seabass skin hydrolysates, at the tested concentrations (5 and 10 mg/ml), significantly reduced ($P < 0.05$) interleukin-6 (IL-6) and IL-1β production in RAW264.7 cells. It was found that IL-6 production was reduced to the greatest extent by sample 1. Sample 1 at concentrations of 5 and 10 mg/ml significantly ($P < 0.05$) reduced the production of IL-6 by 47.0 and 42.8%, respectively, relative to the control cells.

The inflammatory response has been extensively studied in LPS-stimulated RAW 264.7 macrophage cells, which are very sensitive to LPS stimulation and respond by activation of the proinflammatory transcription factors resulting in tumor necrosis factor (TNF), IL-1β, IL-6, IL-8 and nitric oxide production (Wang and Mazza, 2002). These cytokines trigger a beneficial inflammatory response that promotes local coagulation to confine tissue damage. However, the excessive production of these proinflammatory cytokines can be more dangerous than the original stimulus, overwhelming the normal regulation of the immune response and producing pathological inflammatory disorders (Ulloa and Tracey, 2005).

Recently, peptides have gained much attention due to their health benefits and biological activities, especially their immunomodulatory effects. It has been reported that sweetfish-derived protein hydrolysates prepared by trypsin and α-chymotrypsin exhibited immunomodulatory activity in LPS-induced RAW264.7 macrophage cells by inhibiting the production of IL-6 (Sung *et al.*, 2012). A phosvitin-derived peptide from zebrafish markedly suppressed the expression of proinflammatory cytokine genes IL-1β, IL-6, TNF-α and IFN-γ (Ding *et al.*, 2012). Ahn *et al.* (2012) reported that protein hydrolysates derived from salmon by-product showed anti-inflammatory activity by inhibiting nitric oxide production and proinflammatory cytokines including TNF-α, IL-6 and IL-1β in RAW264.7 macrophage cells. The findings of the present study suggest that seabass skin hydrolysates could suppress the

production of proinflammatory cytokines IL-6 and IL-1β in macrophage cell lines, thereby protecting the cells from the detrimental effects of an excessive inflammatory response.

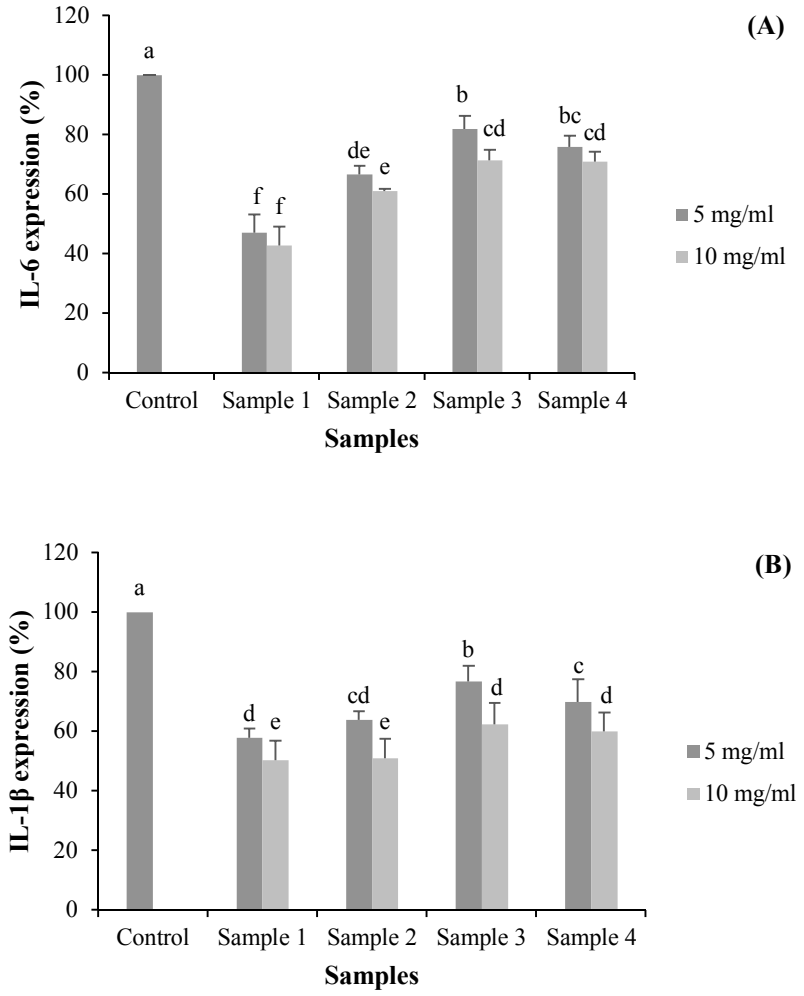

Figure 29. Effect of gelatin hydrolysates from seabass skin on cytokine IL-6 (A) and IL-1β (B) production in LPS-stimulated macrophage RAW264.7 cells. Different lowercase letters on the bars indicate significant differences ($P < 0.05$). Bars represent standard deviation ($n = 3$). Sample 1 and sample 2 represent gelatin hydrolysate produced during gelatin extraction with enzyme concentrations of 1 and 2 units/g dry swollen skin, respectively. Sample 3 and sample 4 represent gelatin hydrolysate produced after gelatin extraction with enzyme concentrations of 1 and 2 units/g dry swollen skin, respectively.

9.4.4 Antiproliferative effects against cancer cells

Human colon cancer (Caco-2) and liver cancer (HepG2) cell lines were used as the model cell culture system for investigating the antiproliferative activities of seabass skin gelatin hydrolysates prepared using different processes. The inhibition of cell proliferation in Caco-2 colon cancer cells occurred in a dose-dependent manner at concentrations of 1–25 mg/ml (Figure 30A). Sample 2 possessed greater antiproliferative effects than the other samples ($P < 0.05$). The proliferation of Caco-2 cells exposed to sample 2 was approximately 20% of the control, untreated cells at the highest concentration (25 mg/ml). The antiproliferative effect of gelatin hydrolysates from seabass skin in HepG2 cells is presented in Figure 30B. All samples inhibited HepG2 cell proliferation at doses of 1–25 mg/ml in a dose-dependent manner. Sample 4 showed the strongest antiproliferative activity in HepG2 cells ($P < 0.05$), reducing cell proliferation to 39% of the control, untreated cells at a concentration of 25 mg/ml. Hydrolysate samples exhibited differences in antiproliferative activity between the two cell types investigated possibly due to differences in cell membrane composition, fluidity and surface area (Leuschner and Hansel, 2004).

Protein hydrolysates from several marine sources such as half-fin anchovy (Song *et al.*, 2011), giant squid (Alemán *et al.*, 2011), blue whiting, cod, plaice and salmon (Picot *et al.*, 2006), tuna (Hsu *et al.*, 2011) and loach (You *et al.*, 2011), have shown antiproliferative activities against various cancer cells. Loach protein hydrolysates prepared by papain digestion showed dose-dependent antiproliferative activity in Caco-2 and HepG2 cells at concentrations ranging from 5–40 mg/ml (You *et al.*, 2011). Peptide fractions from Japanese threadfin bream and tropical two-wing flying fish hydrolysates prepared using trypsin exerted significant antiproliferative effects in HepG2 cells, with IC_{50} values of 48.5 mg/ml and 21.6 mg/ml, respectively (Naqash and Nazeer, 2010). Picot *et al.* (2006) reported that hydrolysates obtained from three blue whiting, three cod, three plaice and one salmon showed significant inhibition on two human breast cancer cell lines, MCF-7/6 and MDA-MB-231. Peptides prepared

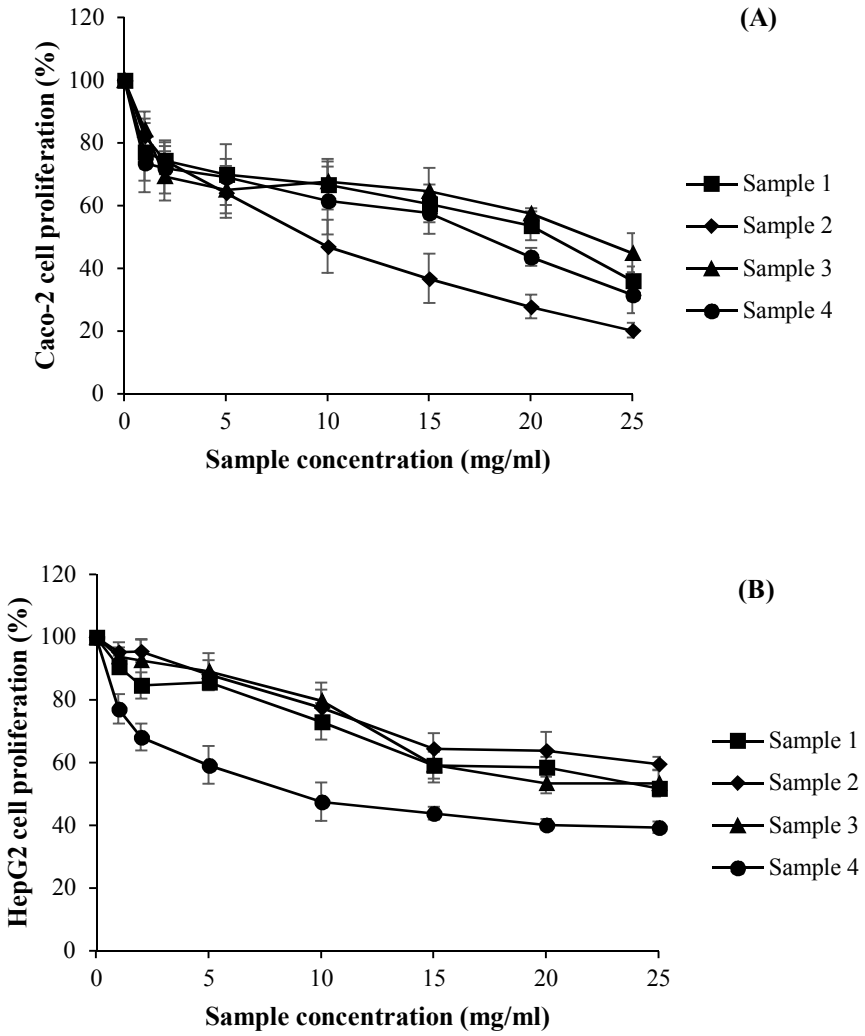

Figure 30. Percent inhibition of Caco-2 colon cancer (A) and HepG2 liver cancer cell (B) proliferation by gelatin hydrolysates from seabass skin. Bars represent standard deviation (n = 3). Sample 1 and sample 2 represent gelatin hydrolysate produced during gelatin extraction with enzyme concentrations of 1 and 2 units/g dry swollen skin, respectively. Sample 3 and sample 4 represent gelatin hydrolysate produced after gelatin extraction with enzyme concentrations of 1 and 2 units/g dry swollen skin, respectively.

from enzymatic hydrolysates of tuna dark muscle showed antiproliferative activity on human breast cancer cell line MCF-7 (Hsu *et al.*, 2011). Gelatin obtained from giant squid hydrolysed by Esperase showed a strong cytotoxic effect in MCF-7 (human breast carcinoma) and U87 (glioma) cells (Alemán *et al.*, 2011). Lee *et al.* (2004) reported

that a 440.9 Da hydrophobic peptide isolated from anchovy sauce demonstrated antiproliferative activity in human U937 lymphoma cells through the induction of apoptosis. It has been reported that low molecular weight peptides have greater molecular mobility and diffusivity than high molecular weight peptides, which appear to improve interactions with cancer cell components and enhance antiproliferative activity (Jumeri and Kim, 2011).

9.5 Conclusion

The findings of the present study demonstrated that the process used in the production of gelatin hydrolysates from seabass skin influences bioactivity, including antioxidant, immunomodulatory and antiproliferative effects in cell culture systems. Protein hydrolysates from seabass skin prepared using Alcalase could have potential applications as functional food ingredients with various health benefits.

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CHAPTER 10

CHARACTERISTICS, ANTIOXIDATIVE ACTIVITIES AND SENSORY PROPERTY OF APPLE JUICE FORTIFIED WITH GELATIN HYDROLYSATE FROM SEABASS SKIN

10.1 Abstract

Gelatin hydrolysate from seabass skin, prepared using Alcalase, was fortified in apple juice at different levels (0.1, 0.2 and 0.3%, w/v). The decrease in *L**-value and the increases in *a**- and *b**-values of apple juice were observed with increasing concentration of gelatin hydrolysate ($P < 0.05$). Browning index and turbidity of apple juice increased when the levels of gelatin hydrolysate increased ($P < 0.05$). Fortification of gelatin hydrolysate into apple juice could increase the antioxidative activities, including DPPH radical scavenging activity, ferric reducing antioxidant power and ferrous ion chelating activity in a dose-dependent manner ($P < 0.05$). Fortification of gelatin hydrolysate up to 0.2% into apple juice had no effect on likeness scores of all attributes. Therefore, gelatin hydrolysate from seabass skin at appropriate level could be used as a functional ingredient in apple juice without negative effect on sensory property.

10.2 Introduction

Protein hydrolysates have attracted an increasing interest as potential ingredients for various health-promoting functional foods due to their bioactivities (Gómez-Guillén *et al.*, 2011). Collagen and gelatin have been considered as a source of biologically active peptides with promising health benefits for nutritional or pharmaceutical applications (Gómez-Guillén *et al.*, 2011). Gelatin hydrolysates from fish skins serve as potential ingredient for Kosher and Halal products. As a consequence, they can be of high demand in the world market, regardless of religious constraint.

Seabass (*Lates calcarifer*) is an economically important fish species in Thailand and other countries, especially tropical and subtropical regions of Asia and the Pacific. During processing or dressing of seabass, skin is generated and becomes a

byproduct with low market value. Recently, seabass skin has been used to produce collagen and gelatin, as well as hydrolysates with antioxidative activities (Senphan and Benjakul, 2014; Sinthusamran *et al.*, 2013). Protein hydrolysates and peptides can act as free radical scavengers, as well as transition metal chelators. They also prevent enzymatic (lipoxygenase-mediated) and non-enzymatic peroxidation of lipids and fats (Sarmadi and Ismail, 2010). Gelatin hydrolysates from bigeye snapper skin prepared using a protease derived from fish pyloric caeca exhibited antioxidative activity including DPPH and ABTS radical scavenging activities and ferric reducing antioxidant power (Phanturat *et al.*, 2010). Kittiphattanabawon *et al.* (2012) reported that gelatin hydrolysates from blacktip shark skin prepared using papaya latex enzyme also exerted antioxidant activities in various model systems.

Recently, foods and beverages have been recognised as the key role in disease prevention and treatment. Thus, the production and consumption of functional foods or drinks has gained much importance as they provide a health benefit beyond the basic nutritional functions (Corbo *et al.*, 2014). Nowadays, beverages are by far the most active functional foods because of convenience and possibility to meet consumer demands. Size, shape, and appearance of packaging or container, as well as ease of distribution and storage expand the market of those shelf-stable products. Moreover, they are an excellent delivering means for nutrients and bioactive compounds including vitamins, minerals, antioxidants, ω-3 fatty acids, plant extracts, and fibre, prebiotics, and probiotics (Corbo *et al.*, 2014). Fruit juices are of the choices due to their health benefits. The fortification of active ingredients having functional properties is of interest for the development of novel functional beverages. The aim of this study was to investigate the characteristics, antioxidative and sensory properties of apple juice fortified with gelatin hydrolysate from seabass skin.

10.3 Materials and methods

10.3.1 Chemicals

Alcalase was obtained from Novozyme (Bagsvaerd, Denmark). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyltriazine (TPTZ), 3-(2-pyridyl)-5,6-

diphenyl-1,2,4-triazine-4',4''-disulphonic acid sodium salt (ferrozine), ethylenediaminetetraacetic acid (EDTA) and 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) were procured from Sigma–Aldrich, Inc. (St. Louis, MO, USA). All chemicals were of analytical grade.

10.3.2 Collection of seabass skins

Skins of fresh seabass (*L. calcarifer*) with a weight of 2.5–3 kg were obtained from the Kingfisher Holdings Co., Ltd., Songkhla, Thailand. Skins were kept in a polystyrene box containing ice using a skin:ice ratio 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, within 1 h. Upon arrival, the skins were descaled and the remaining meat was removed manually using a knife. The skins were washed with cold tap water (1–3 ºC). The skins were pooled and used as the composite sample. The samples were placed in polyethylene bags and stored at –20 ºC until used. The storage time was less than 2 months. Prior to gelatin extraction and hydrolysis, the frozen skins were thawed with running water (25–26 ºC) until the core temperature reached 8–10 ºC. The skins were then cut into small pieces (1.0 × 1.0 cm^2) using scissors.

10.3.3 Preparation of seabass skin

10.3.3.1 Removal of non-collagenous proteins

The skins were soaked in 0.05 M NaOH with a skin:alkali solution ratio of 1:10 (w/v) in order to remove non-collagenous proteins. The mixture was stirred for 3 h at room temperature (28–30 ºC) using an overhead stirrer model RW20.n (IKA®-Werke GmbH & CO.KG, Staufen, Germany) at a speed of 300 rpm. The alkaline solution was changed every 1 h for totally 3 times. Alkali-treated skins were washed with tap water until a neutral or faintly basic pH of wash water was obtained.

10.3.3.2 Acid pretreatment and defatting

After treatment with alkaline solution, the obtained skins were subjected to swelling together with membrane lipid removal using 0.05 M citric acid at a skin:solution ratio of 1:10 (w/v). The mixture was stirred at a speed of 300 rpm at room

temperature for 2 h. The swollen skins were washed using tap water until wash water became neutral or faintly acidic in pH.

Acid pretreated skins were defatted using 30% isopropanol with a solid:solvent ratio of 1:10 (w/v) at room temperature for 1 h. The mixture was continuously shaken at 200 rpm. The solvent was then removed and the defatted skin was washed with 10 volumes of tap water to ensure that the solvent was completely removed. The defatted skin was then rinsed with 10 volumes of distilled water.

10.3.4 Preparation of gelatin hydrolysate

Gelatin hydrolysis was performed according to the method of Senphan and Benjakul (2014) with a slight modification. The pretreated skins were mixed with distilled water at a ratio of 1:10 (w/v). Prior to hydrolysis, the pH of the mixture was adjusted to 8.0 using 2 M NaOH. Alcalase was added into the mixture at a concentration of 1 unit/g skin dry matter. The mixture was incubated for 6 h at 55 ºC in a water bath with continuous stirring using an overhead stirrer at a speed of 150 rpm. Following hydrolysis, the mixture was heated in a boiling water bath for 10 min to inactivate the enzyme. The obtained hydrolysate was filtered using a Buchner funnel with Whatman No.4 filter paper (Whatman International, Ltd., Maidstone, England). The supernatant was spray-dried using a spray dryer (LabPlant SD-06 Basic, North Yorkshire, England) equipped with a spry-drying chamber having the dimensions of 500 mm height and 210 mm diameter. A spray nozzle type of two-liquid nozzle (0.5 mm in size) was used. A cyclone separator, a hot-air blower, and an exhaust blower were equipped. The gelatin hydrolysate solution was fed by a peristaltic pump at 485 ml/h into the chamber, atomised by hot air (air velocity of 2 m/s) from the blower in a downward current flow mode, using an inlet temperature of 180 ºC and an atomising pressure of 2.8 bars.

10.3.5 Preparation of apple juice fortified with gelatin hydrolysate

Apple juice (Tipco F&B Co., Ltd., Ayutthaya, Thailand) was purchased from a local supermarket, Hat Yai, Thailand. Gelatin hydrolysate was added to apple juice to obtain different final levels (0.1, 0.2 and 0.3%, w/v) and mixed well. The resulting apple juices were subjected to analyses.

10.3.6 Analyses

10.3.6.1 Colour

The colour of the apple juice fortified with gelatin hydrolysates was measured by a Hunter Lab Colourimeter (Color Flex, Hunter Lab Inc., Reston, VA, USA). *L**, *a** and *b** indicating lightness/brightness, redness/greenness and yellowness/blueness, respectively, were recorded.

10.3.6.2 Browning intensity

Browning intensity of samples was measured according to the method of Ajandouz *et al.* (2001). The absorbance of samples was measured at 420 nm using a spectrophotometer (UV-1800, Shimadzu, Tokyo, Japan).

10.3.6.3 Turbidity

Turbidity of samples was determined by measuring the absorbance at 660 nm (Surajbhan *et al.*, 2012).

10.3.6.4 Antioxidative activities

10.3.6.4.1 DPPH radical scavenging activity

DPPH radical scavenging activity was determined as described by Brand-Williams *et al.* (1995) with a slight modification. Sample (30-fold diluted) (100 µl) was mixed with 900 µl of 0.06 mM DPPH in methanol. The sample was mixed vigorously and allowed to stand at room temperature in the dark for 1 h. The absorbance of the resulting solution was measured at 515 nm using a spectrophotometer (Model UV-1800, Shimadzu, Kyoto, Japan). The blank 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)/ml.

10.3.6.4.2 Ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power (FRAP) assay was carried out as described by Benzie and Strain (1996). Briefly, 2 ml of working FRAP reagent (0.01 M TPTZ in 0.04 M HCl, 0.02 M $FeCl_3.6H_2O$ and 0.3 M acetate buffer), prepared fresh daily, were mixed with 1 ml of sample (2-fold diluted). After a 2 h incubation in the dark, the absorbance was measured at 593 nm. A standard curve was prepared using Trolox in the range of 20–100 µM. Data were expressed as µmol Trolox equivalents (TE)/ml.

10.3.6.4.3 Ferrous ion chelating activity

The chelating ability of samples toward ferrous ion (Fe^{2+}) was determined according to the method of Thiansilakul *et al.* (2007) with a slight modification. Briefly, 250 µl of the samples (2-fold diluted) were mixed with 1 ml of 0.1 M sodium acetate buffer pH 4.9 and 50 µl of 2 mM $FeCl_2$. The reaction was initiated by the addition of 100 µl of 5 mM ferrozine. After 20 min of incubation at room temperature, the absorbance was measured at 562 nm. The blank was prepared in the same manner except that distilled water was used instead of ferrozine. The standard curve of EDTA (0-1.0 mM) was prepared. Ferrous ion chelating activity was expressed as µmol EDTA equivalents (EE)/ml.

10.3.6.5 Sensory evaluation

A likeness evaluation of apple juice with and without gelatin hydrolysate at different levels was performed by 30 untrained panellists at the ages of 22–30. The assessment was conducted for appearance, colour, odour, flavour, taste and overall likeness using a 9-point hedonic scale: 1, dislike extremely; 5, neither like nor dislike; 9 like extremely (Meilgaard *et al.*, 2007).

10.3.7 Statistical analysis

Experiments were run in triplicate using three different lots of samples. The data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by the Duncan's multiple range test. Statistical analysis was performed using

the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

10.4 Results and discussion

10.4.1 Physical properties

Physical properties (colour, browning intensity and turbidity) of apple juice fortified with gelatin hydrolysate from seabass skin at different levels are shown in Table 23. Generally, the addition of gelatin hydrolysate resulted in the decrease in *L**-value and the increases in *a**- and *b**-values, especially when the concentration of gelatin hydrolysate increased ($P < 0.05$). The increase in *b**-value ($P < 0.05$) indicated the increased yellowness of fortified apple juice. The increase in yellow colour of fortified apple juice was more likely caused by the colour of gelatin hydrolysate added.

Table 23. Colour, browning index and turbidity of apple juice fortified with seabass skin gelatin hydrolysate at different levels

Parameters	Control	Juice fortified with gelatin hydrolysate		
		0.1%	0.2%	0.3%
*L**	61.80 ± 0.35a	60.67 ± 0.38b	58.33 ± 0.03c	54.08 ± 0.03d
*a**	3.54 ± 0.13c	3.71 ± 0.04c	4.03 ± 0.06b	5.60 ± 0.09a
*b**	37.11 ± 0.13c	37.56 ± 0.16c	38.50 ± 0.23b	39.30 ± 1.11a
Browning index (A_{420})	0.56 ± 0.00d	0.60 ± 0.00c	0.76 ± 0.01b	0.84 ± 0.02a
Turbidity (A_{660})	0.03 ± 0.00d	0.08 ± 0.00c	0.12 ± 0.00b	0.17 ± 0.00a

Values are expressed as mean ± SD (n = 3).
Different letters within the same row indicate significant differences ($P < 0.05$).

The increase in browning intensity of fortified apple juice was observed when gelatin hydrolysate was added ($P < 0.05$). Browning intensity increased with increasing amount of gelatin hydrolysate ($P < 0.05$). This was in accordance with the increase in *b** value of fortified apple juice. Fortification of gelatin hydrolysate affected the turbidity of apple juice. When gelatin hydrolysate at higher level was added, the higher turbidity of apple juice was found ($P < 0.05$). Apple juice contains high amount of phenolic compounds (van der Sluis *et al.*, 2002). Those phenolics might interact with

gelatin hydrolysate, leading to the large aggregate as evidenced by the increased turbidity of apple juice. Undesirable haze and cloud may arise in apple juice by several mechanisms, including starch (dextrin) retrogradation, protein-phenol aggregation, phenol oxidation, crystallisation of carbohydrate macromolecules extracted from cell walls, etc. (Beveridge and Wrolstad, 1997).

10.4.2 Antioxidative activities of apple juice fortified with gelatin hydrolysate

Antioxidative activities of apple juice fortified with gelatin hydrolysate at different levels are depicted in Figure 31. DPPH radical scavenging activity, FRAP and ferrous ion chelating activity of apple juice fortified with gelatin hydrolysate (0.1−0.3%) were higher than that of the control apple juice ($P < 0.05$). The activities of apple juice tested by all assays increased with increasing amounts of gelatin hydrolysate added ($P < 0.05$). DPPH radical scavenging activity, FRAP and ferrous ion chelating activity increased by 16, 20 and 55%, respectively when apple juice was fortified with 0.3% gelatin hydrolysate. Phanturat (2008) reported that apple juice fortified with gelatin hydrolysate (0.3%) from bigeye snapper skin prepared using Alcalase and Neutrase showed the increase in DPPH radical scavenging activity by 22%, compared with the control. Soybean milk fortified with protein hydrolysate from the muscle of brownstripe red snapper (0.1−0.5%) showed the increase in DPPH radical scavenging activity and ferrous chelating activity ($P < 0.05$), whereas FRAP remained unchanged at all levels of hydrolysate added ($P < 0.05$). Apple juice has been reported as the good source of natural antioxidant such as flavonoids, etc. (van der Sluis *et al.*, 2002). Peptides in gelatin hydrolysates have been reported to possess antioxidative activities (Nikoo *et al.*, 2014; Weng *et al.*, 2014). Thus, fortification of gelatin hydrolysate could enhance the antioxidative activities of apple juice.

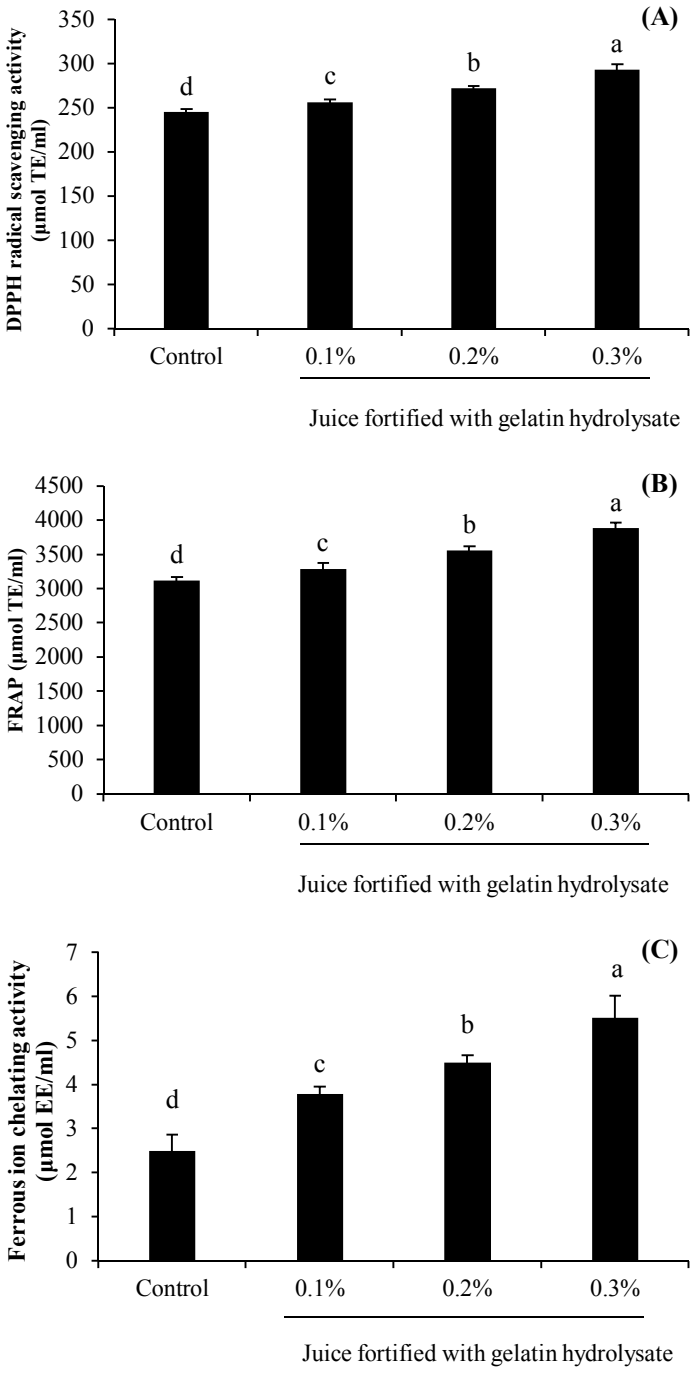

Figure 31. DPPH radical scavenging activity (A), FRAP (B) and ferrous ion chelating activity (C) of apple juice fortified with seabass skin gelatin hydrolysate at different levels. Bars represent the mean ± standard deviation (n = 3). Different letters on the bars indicate significant differences ($P < 0.05$).

10.4.3 Sensory properties

Sensory properties, including appearance, colour, odour, flavour, taste and overall likeness scores of the control apple juice and apple juice fortified with gelatin hydrolysate at levels of 0.1, 0.2 and 0.3% are shown in Table 24.

Table 24. Likeness scores of apple juice fortified with seabass skin gelatin hydrolysate at different levels

Attributes	Control	Apple juice fortified with gelatin hydrolysate		
		0.1%	0.2%	0.3%
Appearance	8.05 ± 0.67a	7.86 ± 0.85a	7.86 ± 0.91a	6.75 ± 0.74b
Colour	8.10 ± 0.70a	8.00 ± 0.89a	7.95 ± 0.80a	6.95 ± 0.86b
Odour	7.95 ± 0.89a	8.10 ± 0.89a	7.81 ± 0.87a	6.75 ± 0.73b
Flavour	8.15 ± 0.75a	8.05 ± 0.83a	7.88 ± 0.79a	6.70 ± 0.66b
Taste	7.90 ± 0.94a	7.76 ± 1.00a	7.70 ± 0.95a	6.80 ± 0.72b
Overall	7.95 ± 0.97a	7.81 ± 0.98a	7.60 ± 0.97a	6.69 ± 0.58b

Values are given as mean ± SD (n = 3).
Different letters within the same row indicate the significant differences ($P < 0.05$). Score are based on a 9-point hedonic scale (1: Dislike extremely, 5: Neither like nor dislike, 9: Like extremely).

The fortification of gelatin hydrolysate up to 0.2% had no impact on likeness scores of all attributes tested of apple juice ($P > 0.05$). However, the addition of gelatin hydrolysate at a level of 0.3% resulted in the decreases in likeness scores of all attributes ($P < 0.05$). Apple juice fortified with gelatin hydrolysate from bigeye snapper skin at a level of 0.3% had the similar sensory properties to the control (Phanturat, 2008). Yarnpakdee *et al.* (2012) reported that protein hydrolysate produced from Indian mackerel muscle with the proper pretreatment could be fortified into milk at a level of 0.2% without negative effect on sensory property. With higher level of seabass skin gelatin hydrolysate fortified, the odour and flavour likeness scores were decreased ($P < 0.05$). This was due to the stronger fishy odour/flavour of apple juice obtained. Additionally, the apple juice became more turbid and slightly darker when

0.3% gelatin hydrolysate was added. This was coincidental with the lower appearance and colour likeness scores ($P < 0.05$). The result suggested that gelatin hydrolysate from the skin of seabass could be added in apple juice to enhance antioxidative activities at a level of 0.2% without the detrimental effect on sensory properties.

10.5 Conclusion

The fortification of gelatin hydrolysate from seabass skin could enhance the antioxidative activities of fortified apple juice. Apple juice was successfully fortified with seabass skin gelatin hydrolysate at a level of 0.2%. Thus, seabass skin gelatin hydrolysate could be used as the functional ingredient in drink.

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CHAPTER 11

CONCLUSION AND SUGGESTION

11.1 Conclusions

1. Seabass skin underwent lipid oxidation with the coincidental release or activation of LOX during iced storage. Development of fishy odour took place along with the formation of volatile compounds in seabass skin during iced storage.

2. Freshness of seabass skin used as raw material for gelatin production had the marked influence on fishy odour and colour of resulting gelatin but it did not drastically affect gelling property.

3. Pretreatment and defatting of seabass skin played a significant role in the reduction of lipids, especially membrane phospholipids. Pretreatment of skin using citric acid, followed by defatting with 30% (v/v) isopropanol prior to gelatin extraction, was effective in prevention of volatile lipid oxidation product formation and fishy odour of resulting gelatin.

4. Gelatin obtained from defatted skin had higher solubility, foaming properties as well as emulsion stability than that prepared from non-defatted skin. Functional properties of gelatin obtained from defatted skin were generally similar to commercial tilapia skin gelatin.

5. Drying methods had an profound influence on properties and fishy odour and flavour of gelatin from seabass skin. Spray drying, particularly with inlet temperature of 180 ºC could lower several odourous compounds associated with fishy flavour and odour more effectively than freeze-drying.

6. The incorporation of tannic acid during gelatin extraction of pretreated/defatted skin effectively prevented lipid oxidation, the development of volatile compounds and fishy odour in the tray-dried gelatin.

7. Simultaneous hydrolysis of gelatin during extraction was effective in the reduction of volatile compounds and the resulting hydrolysate exerted the greater

antioxidant activity than those prepared via hydrolysis after gelatin extraction. DPPH radical scavenging activity and FRAP of seabass skin hydrolysates were stable after *in vitro* simulated gastrointestinal digestion, whilst metal chelating activity was improved.

8. Gelatin hydrolysates from seabass skin had various bioactivities, including antioxidant, immunomodulatory and antiproliferative effects in cell culture systems. Those bioactivities were governed by processes used.

9. The fortification of gelatin hydrolysate from seabass skin could enhance the antioxidative activities of fortified apple juice. Gelatin hydrolysate up to 0.2% (w/v) could be fortified into apple juice without the negative effect on sensory property.

11.2 Suggestions

1. Applications of gelatin as food ingredients e.g. the stabiliser and gelling agent, in some selected foods or beverages should be further studied.

2. The bioactive peptides in gelatin hydrolysate hydrolysate should be purified and identified. In addition, the identified peptides should be synthesised and tested for their bioactivities.

3. Bioactivities of gelatin hydrolysate in animal trial should be investigated.

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