Properties of Gelatin from Seabass (*Lates calcarifer*) Skin and Swim Bladder as Influenced by Extraction Conditions and Hydrocolloids

Sittichoke Sinthusamran

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

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

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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 ผลของสภาวะการสกัดและไฮโดรคอลลอยด์ต่อสมบัติของเจลาตินจากหนังและถุงลมปลากะพงขาว (*Lates calcarifer*)
ผู้เขียน นายสิทธิโชค สินธุสำราญ
สาขาวิชา วิทยาศาสตร์และเทคโนโลยีอาหาร
ปีการศึกษา 2559

บทคัดย่อ

เจลาตินได้จากการสูญเสียสภาพธรรมชาติด้วยความร้อน หรือการย่อยสลายบางส่วนของคอลลาเจน จากการศึกษาเปรียบเทียบคุณลักษณะของคอลลาเจนที่ละลายด้วยกรด จากหนังและถุงลมของปลากะพงขาว (*Lates calcarifer*) พบว่าคอลลาเจนที่สกัดได้ สามารถจำแนกเป็นชนิด type I โดยมีสายโซ่แอลฟาและสายโซ่บีตา เป็นองค์ประกอบหลัก มีน้ำหนักโมเลกุลเท่ากับ 112-122 กิโลดาลตัน และ 187 กิโลดาลตัน ตามลำดับ คอลลาเจนที่ละลายด้วยกรดจากทั้งสองแหล่งมีปริมาณกรดอิมิโน (โพรลีนและไฮดรอกซีโพรลีน) เท่ากับ 194-195 หน่วยต่อ 1000 หน่วย อุณหภูมิการสูญเสียสภาพธรรมชาติของคอลลาเจนจากถุงลม (35.02 องศาเซลเซียส) มีค่าสูงกว่าคอลลาเจนจากหนัง (33.33 องศาเซลเซียส) จากการวิเคราะห์ศักย์ซีตา พบว่าคอลลาเจนจากหนังและถุงลมมีค่า pI เท่ากับ 6.46 และ 6.64 ตามลำดับ

จากการสกัดเจลาตินจากหนังปลากะพงขาวที่สภาวะอุณหภูมิ (45, 55, 65 และ 75 องศาเซลเซียส) และเวลา (3, 6 และ 12 ชั่วโมง) การสกัดต่างๆ พบว่าผลผลิตของเจลาตินเพิ่มขึ้นเมื่ออุณหภูมิและเวลาการสกัดเพิ่มขึ้น ($P<0.05$) เจลาตินทั้งหมดประกอบด้วยสายโซ่บีตา และสายโซ่แอลฟาเป็นองค์ประกอบหลัก และมีปริมาณกรดอิมิโนที่สูง (198-202 หน่วยต่อ 1000 หน่วย) โดยทั่วไป ค่าความแข็งแรงเจลของเจลาตินลดลงเมื่ออุณหภูมิและระยะเวลาการสกัดเพิ่มขึ้น ($P<0.05$) ที่ระยะเวลาการสกัดเดียวกัน เจลาตินสกัดที่อุณหภูมิ 45 องศาเซลเซียส มีค่าความแข็งแรงเจล (303.4-369.2 กรัม) สูงกว่าเจลาตินสกัดที่ 55 องศาเซลเซียส (282.0-322.6 กรัม) ($P<0.05$) เจลาตินจากหนังปลากะพงขาวสามารถเกิดเจลได้ภายใน 30 นาที ที่อุณหภูมิ 25 องศาเซลเซียส การสกัดเจลาตินจากปลาขนาดต่างๆ (2, 4 และ 6 กิโลกรัมต่อตัว) คือ G2, G4 และ G6 ตามลำดับ ได้ผลผลิตร้อยละ 38.33, 40.50 และ 43.48 ของน้ำหนักแห้ง ตามลำดับ G2 มีสายโซ่แอลฟาเป็นองค์ประกอบหลัก ส่วน G4 และ G6 ประกอบด้วยสายโซ่แอลฟา สายโซ่บีตา และสายโซ่แกมมา รวมทั้งมีสายโซ่เชื่อมประสานกันที่มีขนาดโมเลกุลใหญ่ในปริมาณสูง ค่าประจุสุทธิของ G2 G4 และ G6 มีค่าเท่ากับศูนย์เมื่อพีเอชเท่ากับ 6.73 6.41 และ 7.12 ตามลำดับ เมื่อเปรียบเทียบตัวอย่างเจลาตินทั้งหมด พบว่า G6 ให้ความแข็งแรงของเจลสูงสุด (321.5 กรัม) ($P<0.05$) อุณหภูมิในการเกิด

เจลและอุณหภูมิที่เจลหลอมละลายของเจลาตินทุกตัวอย่าง มีค่าอยู่ในช่วง 17.09-19.01 องศาเซลเซียส และ 26.92-28.85 องศาเซลเซียส ตามลำดับ นอกจากนี้ เจลาตินทั้งหมดสามารถเกิดเจลที่อุณหภูมิห้องได้โดยไม่ขึ้นกับขนาดของปลา อย่างไรก็ตาม G6 มีระยะเวลาการเซ็ตตัวที่อุณหภูมิห้องน้อยกว่า G2 และ G4

การสกัดเจลาตินจากถุงลมปลากะพงขาวที่อุณภูมิต่างๆ (45, 55, 65 และ 75 องศาเซลเซียส) เป็นเวลา 6 ชั่วโมง พบว่าผลผลิตเจลาติน (ร้อยละ 44.83-71.95 ของน้ำหนักแห้ง) เพิ่มขึ้นเมื่ออุณหภูมิการสกัดสูงขึ้น เจลาตินทั้งหมดมีสายโซ่แอลฟาเป็นองค์ประกอบหลัก และมีสายโซ่บีตาในปริมาณรองลงมา เจลาตินจากถุงลมปลามีปริมาณกรดอิมิโนที่สูง (195 หน่วยต่อ 1000 หน่วย) การศึกษาสเปคตราฟูเรียร์ทรานฟอร์มอินฟาเรด (FTIR) และสเปคตราเซอร์คูลาร์ไดโครอิสซึม (CD) แสดงให้เห็นการสูญเสียสภาพโครงสร้างของเกลียวแอลฟาสามสาย โดยทั่วไปค่าความแข็งแรงเจลของเจลาตินเพิ่มขึ้น เมื่อเพิ่มอุณหภูมิการสกัดถึง 65 องศาเซลเซียส ($P<0.05$) เจลาตินสกัดที่อุณหภูมิ 65 องศาเซลเซียส เป็นระยะเวลา 6 ชั่วโมง ให้ความแข็งแรงของเจล (280.9 กรัม) สูงกว่าเจลาตินจากกระดูกวัวทางการค้า (246.3 กรัม) ($P<0.05$)

การศึกษาผลของการเติมวุ้นสกัดจากสาหร่ายผมนาง (*Gracilaria tenuistipitata*) (GA) และวุ้นทางการค้า (CA) ที่ระดับต่างๆ (ร้อยละ 0, 5, 10, 15 และ 20 ทดแทนปริมาณเจลาติน) ต่อสมบัติของเจลาตินทางการค้าจากหนังปลา (FG) พบว่าค่า critical linear stress และค่า failure stress ของเจลผสม เพิ่มขึ้น เมื่อความเข้มข้นของวุ้นเพิ่มขึ้น ($P<0.05$) โดยไม่ขึ้นกับชนิดของวุ้น การเติมวุ้นร้อยละ 15 และ 20 ทำให้เจลผสม FG/CA มีค่า failure stress สูงกว่าเจลผสม FG/GA ($P<0.05$) อย่างไรก็ตาม การเติมวุ้นทั้งสองชนิดลดค่าคะแนนความชอบของเจลเจลาติน เมื่อเติมแคปปา-คาราจีแนน (KC) ที่ระดับต่างๆ (ร้อยละ 0, 25, 50, 75 และ 100 ทดแทนปริมาณเจลาติน) ลงไปใน FG พบว่า ค่าความแข็งแรงเจล และค่าความแข็งของเจลผสมเพิ่มขึ้น ($P<0.05$) เมื่อปริมาณ KC เพิ่มขึ้น ในขณะที่ค่าความยืดหยุ่นของเจลผสมลดลง ($P<0.05$) เมื่อระดับของ KC เพิ่มสูงกว่าร้อยละ 25 ค่าโมดูลัสสะสม (Elastic modulus; G') ของเจลผสม FG/KC ลดลง เมื่อปริมาณ KC เพิ่มสูงขึ้น ซึ่งอาจเกี่ยวข้องกับการแยกเฟสระหว่างพอลิเมอร์ชีวภาพสองชนิดในเจลผสม นอกจากนี้ อุณหภูมิของการเกิดเจลและอุณหภูมิการหลอมละลายของเจลเพิ่มขึ้นเมื่อระดับของ KC เพิ่มขึ้น ($P<0.05$) สเปคตรา FTIR บ่งชี้ว่าเกิดอันตรกิริยาระหว่าง FG และหมู่ซัลเฟต ของ KC แสดงว่าการเติม KC สามารถปรับปรุงสมบัติเจลของ FG

การศึกษาผลของการเติม GA และ KC ที่ร้อยละ 10 และ 20 ทดแทนปริมาณเจลาติน ต่อสมบัติและคุณลักษณะทางประสาทสัมผัสของเจลาตินจากหนัง (SK) และถุงลม (SW) ปลากะพงขาว พบว่า ค่าความแข็งของเจลจาก SK และ SW ที่ผสม GA เพิ่มขึ้นเมื่อปริมาณ GA

เพิ่มขึ้น อย่างไรก็ตาม การเติม KC มีผลลดค่าความแข็งของเจล ส่วนค่าความยืดหยุ่นและค่าความแข็งแรงของพันธะภายในตัวอย่างของทั้งเจลจาก SK และ SW ลดลงเมื่อระดับของGA และ KC เพิ่มสูงขึ้น อุณหภูมิของการเกิดเจลและอุณหภูมิที่เจลเกิดการหลอมละลายโดยทั่วไปเพิ่มสูงขึ้นเมื่อระดับสารไฮโดรคอลลอยด์เพิ่มขึ้น ($P<0.05$) ดังนั้น GA ที่ร้อยละ 10 สามารถเพิ่มคุณลักษณะประสาทสัมผัสของเจลาตินเจลทั้งสองชนิด อย่างไรก็ตาม การเติมสารไฮโดรคอลลอยด์ทั้งสองชนิดที่ร้อยละ 10 มีผลทำให้ความสามารถในการย่อยในระบบจำลองทางเดินอาหารลดลง

ดังนั้น คอลลาเจนและเจลาตินสามารถสกัดจากหนังและถุงลมของปลากะพงขาว (*Lates calcarifer*) การเติมไฮโดรคอลลอยด์ เช่น วุ้น และ แคปปา-คาราจีแนน ในระดับที่เหมาะสมสามารถเพิ่มค่าความแข็งแรงเจล และเพิ่มอุณหภูมิของการเกิดเจลและอุณหภูมิที่เจลเกิดการหลอมละลาย การค้นพบพื้นฐานจากวิทยานิพนธ์นี้อาจช่วยเพิ่มการใช้ประโยชน์ของเจลาตินจากปลาโดยเฉพาะสำหรับอุตสาหกรรมอาหาร

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Major Program Food Science and Technology

Academic Year 2016

ABSTRACT

Gelatin is obtained by thermal denaturation or partial hydrolysis of collagen. Acid soluble collagens (ASCs) from skin and swim bladder of seabass (*Lates calcarifer*) were isolated and comparatively characterised. ASCs from both skin and swim bladder were identified to be type I. Both α- and β-chains constituted were the major components with MW of 112-122 kDa and 187 kDa, respectively. ASC from both sources contained imino acids (proline and hydroxyproline) of 194–195 residues/1000 residues). Thermal transition temperature of swim bladder ASC (35.0 °C) was slightly higher than the skin counterpart (33.3 °C). Based on zeta potential analysis, ASCs from skin and swim bladder had a net charge of zero at pH 6.46 and 6.64, respectively.

Gelatin from seabass skin was extracted under various extraction conditions. The yield of gelatin increased when the extraction temperature and time increased ($P<0.05$). All gelatins contained β-chain and α–chains as the predominant components and showed a high imino acid content (198-202 residues/1000 residues). Generally, gel strength of gelatins decreased as extraction temperature and time increased. At the same extraction time, gel strength of gelatin extracted at 45 °C (303.4-369.2 g) was higher than that of gelatin extracted at 55 °C (282.0-322.6 g) ($P<0.05$). Gelatin from the seabass skin could be set at 25 °C within 30 min. When gelatin was extracted from the skin of seabass with different average sizes (2, 4 and 6 kg/fish), termed G2, G4 and G6, respectively, yields were 38.22, 40.50 and 43.48% (based on dry weight), respectively. G2 contained α-chains as dominant component, while G4 and G6 comprised α-, β- and γ-chains with a larger content of high MW cross-links. Net charge of G2, G4 and G6 became zero at pHs of 6.73, 6.41 and 7.12, respectively. Among all gelatin samples, G6 exhibited the highest gel strength (321.5 g) ($P<0.05$). Gelling and melting temperatures of all gelatins were 17.09-19.01 and

26.92-28.85 °C, respectively. Furthermore, all gelatins were able to set at room temperature, regardless of size of seabass used. However, G6 had the shorter setting time than others.

Gelatin from swim bladder of seabass was also extracted at different temperatures (45, 55, 65 and 75 °C). The yield of gelatin (44.83-71.95%, based on dry weight) increased with increasing extraction temperatures. All gelatins contained α-chains as the predominant components, followed by β-chain. Gelatin from seabass swim bladder showed a high imino acid content (195 residues/1000 residues). FTIR and CD spectra revealed the loss of triple helix during heating. Gel strength generally increased as the extraction temperature increased up to 65 °C ($P<0.05$). Gelatin extracted at 65 °C for 6 h showed a higher gel strength (280.9 g), compared to bovine gelatin (246.3 g) ($P<0.05$).

The impact of agar extracted from *Gracilaria tenuistipitata* (GA) and commercial agars (CA) at various levels (0, 5, 10, 15 and 20% gelatin substitution) on properties of commercial fish gelatin (FG) gel was studied. The critical linear stress and failure stress of FG/agar mixed gels increased with increasing agar levels ($P<0.05$), regardless of agar types. At 15 and 20% of agar used, the FG/CA mixed gels exhibited higher failure stress than FG/GA mixed gel ($P<0.05$). Nevertheless, the incorporation of both agars lowered the likeness score of gelatin gel. When κ-Carrageenan (KC) at different levels (0, 25, 50, 75 and 100% gelatin substitution) was incorporated into FG, gel strength and hardness of FG/KC mixed gel increased with increasing KC content, while springiness of mixed gel decreased when KC was higher than 25% ($P<0.05$). Elastic modulus G′ of FG/KC mixed gel decreased as KC content increased. This could be associated with the phase separation between the two biopolymers present in the mixed gels. Moreover, gelling and melting temperatures also increased as the levels of KC increased ($P<0.05$). Based on Fourier transform infrared (FTIR) spectra, interaction between FG and sulphate groups of KC occurred. Therefore, the addition of KC into FG improved the gelling property of FG.

Physical and sensory properties of gelatin from skin and swim bladder of seabass (SK and SW, respectively) as affected by the incorporation of GA or KC at 10 and 20% substitution were investigated. Hardness of both SK and SW gels containing GA increased with increasing level of GA. However, the addition of KC

lowered hardness of mixed gels. Springiness and cohesiveness of either SK or SW gels decreased as the level of both GA or KC increased. Gelling and melting temperature generally increased when the level of hydrocolloids was increased. The addition of 10% GA could therefore increase sensory properties of both gelatin gels. However, the addition of both hydrocolloids at 10% decreased digestibility of mixed gelatin gel in gastrointestinal tract model.

Overall, collagen and gelatin were successfully extracted from the skin and swim bladder of seabass (*Lates calcarifer*). To enhance gel strength and increase gelling and melting temperatures of gelatin, the hydrocolloids such as agar and κ-carrageenan at an appropriate level was incorporated. The fundamental findings of this thesis might be used to widen the application of fish gelatin, particularly for food industry.

ACKNOWLEDGEMENT

I would like to express my deepest appreciation and sincere gratitude to my advisor, Prof. Dr. Soottawat Benjakul of the Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, for his kindness, mentorship and assistance during my study. I also would like to thank for his invaluable suggestion and lessons for my academic knowledge and personal life. His determination and perseverance to train me to be a good researcher with responsibility, vigilance and honesty are always acknowledged.

I would like to express my profound gratitude to my co-advisor, Assoc. Prof. Dr. Yacine Hemar of the School of Chemical Sciences, Faculty of Science, University of Auckland, New Zealand for his warm welcome, kindness, generous contribution and unconditional help, particularly during my 8 month-stay in New Zealand.

I am grateful to my examining committee, Asst. Prof. Dr. Manee Vittayanont and, Asst. Prof. Dr. Kongkarn Kijroongrojana of the Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University and Assoc. Prof. Dr. Jirawat Yongsawatdigul of the School of Food Technology, Institute of Agricultural Technology, Suranaree University of Technology for the valuable time devoted to my thesis and their kindness, comments and helpful suggestion.

My deep gratitude is also dedicated to my dear friends and colleagues of Fish Chemistry and Biochemistry lab (2205) members who shared the hard time with me during my study and gave me their help. Among all the friends that have companied me during my journey, I would like to express my deep thanks to Dr. Phanat Kittipattanabawon, Dr. Rossawan Intarasirisawat, Dr. Samart Sai-Ut, Dr. Suthasinee Yarnpakdee, Dr. Muralidharan Nagarajan, Dr. Sirima Takeungwongtrakul, Dr. Theerapol Senphan, Dr. Phakawat Tongnuanchan, Dr. Thanasak Sae-leaw, Dr. Supatra Karnjanapratum, Ms. Phanngam Kaewruang, Mr. Naveen Kumar Vate and other new lab members. Thank also to Mr. Zhao Li, Ms. Pasongsin Khamchan and Mr. Praveen Warnakulasooriya for their valuable help and friendship during my research at School of Chemical Sciences, Faculty of Science, University of Auckland,

New Zealand. Finally, I would like to express my deepest appreciation to my beloved family for the great encouragement and support.

This study could not be succeeded without the financial support from Thailand Research Fund under the Royal Golden Jubilee Ph.D. Program and the Graduate School of Prince of Songkla University. I gratefully acknowledge this financial support.

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CHAPTER 1
INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Seabass (*Lates calcarifer*) is a warm-water species, which is one of economically important fish species in Thailand and other countries in Southeast Asia. It is cultured in the south of Thailand, especially in Songkhla lake area (Masniyom *et al.*, 2002). Seabass are cultured over 7,752 metric tons and production and value of seabass farm increased to 753 million bahts in year 2000 (Fisheries Statistical and Information Technology Sub-Division, 2000). Seabass has been commonly used for fillet production for local consumption and for export. During processing, byproducts consisting of skin, bone and scales are generated. Skin and bone are the rich sources of collagen (Gómez-Guillén *et al.*, 2002). Additionally, the skin from seabass has been used for making the crispy fried fish skin for domestic consumption. However, the market value is still low. The utilization of fish skin for gelatin and collagen production can increase the value of those processing wastes and eliminate harmful environmental problems caused by inappropriate disposal/treatment (Binsi *et al.*, 2009). Furthermore, swim bladder is another byproduct, which has not been fully exploited. Swim bladder is well known to contain collagen as the major component and has been processed as fish maw. Therefore, both skin and swim bladder can serve as the potential sources for collagen and gelatin productions with a high market value.

Collagen is the major structural protein in the connective tissue of vertebrates and constitutes about 30% of the total animal proteins (Jongjareonrak *et al.*, 2005b). Gelatin is a fibrous protein obtained by the thermal denaturation of collagen from animal skin and bone. Collagen and gelatin have a wide range of applications in food manufacturing, leather and film industries, pharmaceutical, cosmetic and biomedical materials (Benjakul *et al.*, 2009). Generally, bovine and porcine skin and bone are the main sources for gelatin production. However, the

religious restrictions have led to increasing interest for alternative sources of collagen and gelatin. By-products from fish processing have became an important alternative source for collagen and gelatin productions (Jongjareonrak *et al.*, 2005b). However, the use of collagen and gelatin from aquatic sources still is limited, due to poorer property, when compared to those from land animals (Karim and Bhat, 2009). As a consequence, it shows the inferior quality to bovine or porcine gelatin. Additionally, the property of collagen and gelatin varies, depending upon species or habitats. This is associated with their chemical compositions (Foegeding *et al.*, 1996). Collagens from cold-water fish have lower thermal stability than do mammalian collagens because the formers contain a lower imino acid content than do the latters (Foegeding *et al.*, 1996). Moreover, cold-water fish gelatins have the limited applications, mainly due to the lower gel strength and lower stability of gels, compared with mammalian counterparts (Kittiphattanabawon *et al.*, 2010b).

Seabass is a warm-water fish, and its gelatin may have the better gelling property than those from cold water fish. Boran *et al.* (2010) reported that the gel strength and rheological properties (gelling and melting temperatures) of gelatin from silver carp skin (warm water fish) were similar to those of chicken bone and porcine gelatin. Since extraction condition, including temperature, time and the protease inhibitors have been known to affect the properties of gelatin from fish skin, the better quality gelatin can be produced under the optimal condition (Kaewruang *et al.*, 2013a; Nagarajan *et al.*, 2013). To widen the application of fish gelatin, the improvement of gel properties of fish gelatin has been carried out via several means. Chemical or natural cross-linkers have been used to strengthen fish gelatin gels (Kosaraju *et al.*, 2010). For instance, Gómez-Guillén *et al.* (2001) reported that the addition of transglutaminase to gelatin can increase melting temperature and gel strength of gelatin gel. Some hydrocolloids with gelling properties, such as κ-carrageenan, pectin and gellan, were also added to gelatin to improve its gelling properties (Haug *et al.*, 2004b; Lau *et al.*, 2000; Liu *et al.*, 2007). As a consequence, property of gelatin from aquatic sources can be improved and resulting gelatin can be marketable and fully employed.

However, no information regarding characteristics and properties of collagen and gelatin from skin and swim bladder of seabass has been reported. The

optimal extraction condition of gelatin can be a promising procedure to gain the high quality of gelatin from seabass skin and swim bladder. Furthermore, there is little information regarding the use of polysaccharides e.g. agar and κ-carrageenan, etc. to improve textural and rheological properties as well as sensory evaluation of fish gelatin. The information gained can provide an alternative and effective approach for better utilization of fish skin and swim bladder from seabass. As a consequence, applications of seabass gelatin can be broadened and farmer or processers can get the increasing revenue from those byproducts.

1.2 Review of Literature

1.2.1 Seabass

Seabass (*Lates calcarifer*) also called Giant sea perch and Asian seabass belong to the class *Pisces* in the family *Centropomidae* (Figure 1). It is one of economically important fish species in the tropical and subtropical areas of the western Pacific and Indian Ocean countries, including India, Burma, Sri Lanka, Bangladesh, Malay Peninsula, Java, Borneo, Celebes, Philippines, Papua New Guinea, Northern Australia, Southern China and Taiwan. It is euryhaline, which can live in both fresh and salt water (Boonyaratpalin *et al.*, 1994). In Thailand, seabass have been cultured in several provinces such as Samut Prakan, Prachuab Kiri Khan, Songkhla, Pattani and others. Seabass have been commercially cultivated in brackishwater ponds and marine cages in Thailand. A number of seabass farms are located in the south of Thailand, especially in the lake of Songkhla. The seabass is a relatively highly-priced and widely-accepted species, leading to a very attractive commodity for both large and small-scale fish culture enterprises. In general, seabass has been mainly used in fillet production, in which both skin and swim bladder are generated as byproducts. Skin has been used for making crispy skin for domestic consumption, whilst swim bladder has not been fully utilized. Swim bladder has been used for making a fish maw. Alternatively, skin and swim bladder from seabass can be used as raw material for collagen and gelatin extraction with an increase market value.

Figure 1. Seabass fish
Source: FAO (2005)

1.2.2 Fish collagen

1.2.2.1 Molecular characteristics of fish collagen

Collagen is the major structural protein in the connective tissue of vertebrates and constitutes about 30% of total animal protein (Ogawa *et al.*, 2004). Collagen is the fibrous protein, contributing to unique physiological functions of tissues in skins, tendons, bones, cartilages, etc. (Jongjareonrak *et al.*, 2005b). The structural unit of collagen is tropocollagen, a long cylindrical protein about 2800 Å in diameter. Collagen is a rod-shaped protein consisting of three polypeptide chains (called α-chains) intertwined to form a triple-helical structure. Each α-chain has a molecular mass of about 100,000 dalton, yielding a total molecular mass of about 300,000 dalton for collagen (Figure 2). The three chains are twisted right-handed to form the triple helix by hydrogen bonding between adjacent –CO and –NH group (Foegeding *et al.*, 1996). The triple helix of collagen is assembled from specific polypeptide chain (α-chains) with the Gly-X-Y repeat and contains proline and hydroxyproline in the X and Y position, respectively (Xu *et al.*, 2002).

At present, at least 29 variants of collagen have been identified, and each differs considerably in amino acid sequence, structure and function, more likely associated with specific genetic variants (Liu *et al.*, 2012). The most common collagen is type I collagen. It contains two identical polypeptide chains, designated α-1 (I), and a third chain α-2, which has a different amino acid sequence (Foegeding *et al.*, 1996). Some types of collagen are observed among different organs and connective tissue layers (Table 1).

Table 1. Collagens and their distribution.

Type	Triple helix	Distribution
I	Two identical α1(I) chains + one α2 chain	skin, tendon, bone
II	three α1(II) chains	Intervertebral disc, cartilage
III	three α1(III) chains	Cardiovascular vessel, uterus
IV	three α1(IV) chains	basement membrane, kidney

Source: Wong (1989)

Each collagen molecule contains about 33% glycine, 12% proline, 11% hydroxyproline and alanine and 1% hydroxyproline and is devoid of tryptophan and cysteine (Benjakul *et al.*, 2012b; Pearson and Young, 1989). Glycine represents nearly one-third of the total residues and it is distributed uniformly at every third position throughout most of collagen molecule. The repetitive occurrence of glycine is absent in the first 14 amino acid residues from N-terminus and the first ten residues from the C-terminus, in which these end portions are termed "telopeptides" (Figure. 2) (Benjakul *et al.*, 2012b; Foegeding *et al.*, 1996).

Lower content of imino acids (proline and hydroxyproline) is generally found in collagen of cold-water fish, compared with warm-water fish. Imino acids contribute to the stability of helix structure of collagen (Jongjareonrak *et al.*, 2005a). The presence of proline stabilizes the helix structure by preventing rotation of the N-C bond. Hydroxyproline also stabilizes the collagen molecule (Nalinanon *et al.*, 2010). Collagen with small contents of both imino acids denatures at lower temperatures than do those with larger contents (Benjakul *et al.*, 2012b; Foegeding *et al.*, 1996). Collagen from calf skin had a higher imino acid content (about 215 residues per 1000 total residues) than did collagen from bighead carp (156–175 residues per 1000 total residues) (Duan *et al.*, 2009). The amino acid compositions of collagen varied with raw material (Table 2).

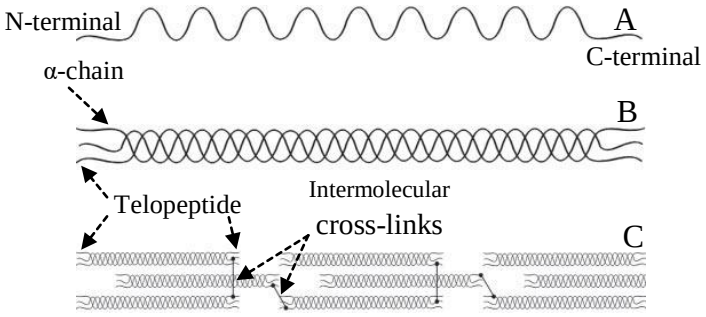

Figure 2. Arrangement of collagen fibril in collagen fiber. Collagen polypeptide (A) Tropocollagen (B) and Collagen fibril (C)

Source: Benjakul *et al.* (2012b)

Commonly, fish collagen has been isolated from fish processing byproducts including skin, bone or scale (Jongjareonrak *et al.*, 2005a). Swim bladder can be used for collagen extraction (Liu *et al.*, 2012). Skin, scales or fins collagen from several fish species have been isolated and characterized as type I collagen.

Thermal stability of collagen from different sources is correlated with the contents of imino acids, their habitat, seasons and age (Foegeding *et al.*, 1996; Singh *et al.*, 2011). Transition temperature of collagen from tropical fish is generally higher than that from cold-water fish (Muyonga *et al.*, 2004a; Nalinanon *et al.*, 2010). The maximal transition temperatures (T_{max}) or denaturation temperature (T_d) of collagen from cold-water fish including dusky spinefoot (28.7 °C) (Bae *et al.*, 2008), tigger puffer (28.4 °C) (Bae *et al.*, 2008), arabesque greenling (15.4 °C) (Nalinanon *et al.*, 2010), Pacific saury (24-25 °C) (Mori *et al.*, 2013) and Nothern pink (28.5 °C) (Kozlowska *et al.*, 2015) are generally lower than those of collagens from tropical fish. T_{max} above 30 °C have been reported for collagen from subtropical and tropical fish such as Rohu (35.5 °C) (Pati *et al.*, 2010), brownbanded bamboo shark (34.45 °C) (Kittiphattanabawon *et al.*, 2010a), striped catfish (35.3 °C) (Singh *et al.*, 2011), bighead carp (35.2 -37.3 °C) (Liu *et al.*, 2012) and grass carp (36.4 °C) (Liu *et al.*, 2015b). A high content of imino acid contributes to stabilization of collagen triple helix (Xu *et al.*, 2002). Thermal stability of collagen is considered by the hydrogen-bonded networks, which connect the hydroxyl group of hydroxyproline in one strand to the amide carboxyl of another chain (Benjakul *et al.*, 2012b). Therefore, the imino acid content of fish collagens is associated with their thermal stability and correlates with the water temperature of their normal habitat (Foegeding *et al.*, 1996).

Extraction process can affect thermal stability of collagen from fish skin to some degree. Pepsin soluble collagen (PSC), in which pepsin has been used as the aid of extracting, showed the slightly lower T_{max} and enthalpy, compared with acid soluble collagen (Nalinanon *et al.*, 2007). The partial removal of non-helical telopeptides by pepsin might be associated with lower stability of resulting PSC (Nalinanon *et al.*, 2007).

Table 2. Amino acid compositions of fish collagens as compared with calf skin (residues per 1000 residues).

Amino acid	Source of collagen			
	Calf skin[1]	Brownbanded bamboo skin[2]	Bighead carp bladder[3]	Grass carp skin[4]
Alanine	119	105	123	119
Arginine	50	51	53	53
Aspartic acid/asparagine	45	42	47	49
Cysteine	-	1	1	-
Glutamine/glutamic acid	75	77	78	78
Glycine	330	318	331	334
Histidine	5	7	3	5
Isoleucine	11	18	11	12
Leucine	23	24	21	22
Lysine	26	29	27	29
Hydroxylysine	-	6	5	2
Methionine	6	12	15	14
Phenylalanine	3	14	15	15
Hydroxyproline	94	93	80	68
Proline	121	111	95	113
Serine	33	41	30	37
Threonine	18	23	28	25
Tyrosine	3	3	3	3
Valine	21	25	34	22
Total	1000	1000	1000	1000
Imino acid	215	204	175	181

Sources: [1]Giraud-Guille *et al.* (2000), [2]Kittiphattanabawon *et al.* (2010a), [3]Liu *et al.* (2012) and [4]Liu *et al.* (2015b)

1.2.2.2 Extraction of fish collagen

Generally, collagen can be produced from bovine and porcine hides and bones, and fish byproducts such as skin, bone, scale, and so on (Kittiphattanabawon *et al.*, 2010c). Extraction of collagen is generally separated into three main steps, including preparation, extraction and recovery. All procedures are performed at low temperature (4 °C) to avoid thermal denaturation. The extraction of collagen was prepared by elimination of noncollagenous protein and decalcification before acid solubilization (Benjakul *et al.*, 2012b). Pretreatments are used to remove the contaminants including noncollagenous proteins, lipids and pigments and increase the purity of extracted collagen (Benjakul *et al.*, 2012b). To remove noncollagenous proteins and pigment, alkaline pretreatment is widely used (Jongjareonrak *et al.*, 2005b; Kittiphattanabawon *et al.*, 2010c; Liu *et al.*, 2012; Nagai *et al.*, 2008; Singh *et al.*, 2011; Zhang *et al.*, 2009). However, NaCl and H_2O_2 have also been used to remove noncollagenous proteins and pigments, respectively, in raw material prior to extraction of collagen (Wang *et al.*, 2007; Zhang *et al.*, 2009).

Collagen is generally dissolved in the acidic pH ranges and the ionic strength affects the collagen solubility. Collagen has been extracted using acid solubilization process and referred to as “acid-soluble collagen, ASC”. Extraction is conducted using acidic condition, in which the positive charge of collagen polypeptides becomes dominant. As a consequence, collagen undergoes repulsion, leading to increased solubilization (Benjakul *et al.*, 2012b). Organic acids such as acetic, citric and lactic acids, are directly used to extract collagen from animal tissues (Skierka and Sadowska, 2007). Acetic acid is the most popular organic solvent used for collagen solubilization due to its high extractability (Wang *et al.*, 2008). Sadowska *et al.* (2003) reported that the extractability of collagen depends both on the concentration of acetic acid and on the ratio of raw material to acid. pH value can regulate the charge density of protein, thereby affecting the electrostatic interaction of proteins (Wang *et al.*, 2008). Skierka and Sadowska (2007) reported the influence of different acids (citric, lactic, acetic and hydrochloric acid; HCl) on the extraction of collagen from Baltic cod skin. Collagen extraction with 0.5 M acetic or lactic acid gave the highest yield of 90%, but 0.15 M HCl gave the lowest yield (18%).

Collagens extracted with HCl and citric acids with pepsin treatment were partially denatured. However, collagens extracted with acetic acid and lactic acid were present in their native form. Thus, the type and concentration of organic acid directly affects the characteristic and properties of collagen.

However, the yield of acid-soluble collagen is lower. Inter-chain crosslinks at the telopeptide region of the collagen result in low solubility in acid (Zhang *et al.,* 2007). Pepsin has been used for increasing the yield of collagen since it can specifically cleave peptides at the telopeptide region, thereby facilitating the extraction of collagen from fibrils or skin matrix (Nalinanon *et al.*, 2007). Nalinanon *et al.* (2007) reported that collagen extracted from skin of bigeye snapper using both bigeye snapper pepsin (BSP) or porcine pepsin (PP) showed higher extraction yield than that of the collagen extracted without pepsin addition. However, higher protein molecular weight (MW) components, including γ-chains were found at a greater extent in ASC extracted from the skin of bigeye snapper than in PSC. Ogawa *et al.* (2003) reported that band intensity of β and γ components were higher in ASC than in PSC for collagens from skin of black drum and sheepshead sea bream.

After extraction, the collagen solution is generally precipitated by adding salt prior to dialysis and freeze-drying (Benjakul *et al.*, 2012b). Collagen can be precipitated with sodium chloride or with polysaccharides (Sadowska *et al.*, 2003). The concentrations of NaCl used for collagen precipitation varies from 0.9 to 2.6 M. This can be adjusted to maximize the collagen recovery and removal of impurities (Benjakul *et al.*, 2012b). Sadowska and Kołodziejska (2005) reported the effect of pH and ionic strength on the precipitation of collagen fibrils with k-carrageenan. Addition of NaCl to collagen dissolved in HCl at concentrations corresponding to an ionic strength of 0.72 and 0.90 resulted in collagen precipitation with the yields of 22 and 84%, respectively. Collagen precipitated with an ionic strength of 0.9 at various pHs of 2.2, 3, 4 and 5 showed collagen yield of 11, 40, 53 and 62%, respectively. After collagen precipitation, the collagen pellet is then collected using centrifugation, following by dialysis against 0.1 M acetic acid and distilled water. The dialysate is finally freeze-dried and the powder obtain is used as collagen (Benjakul *et al.*, 2012b). The conditions for the extraction of collagen from different sources are summarized in Table 3.

Table 3. Yields and molecular compositions of collagens from different fish

Raw materials	Pretreatment[a]	Extraction[a]	%Yield (based on wet weight)	Collagen type	Molecular compositions	References
Tilapia skin/scale (*Oreochromis niloticus*)	0.1 M $NaHCO_3$ for 6 h	0.5 M acetic acid for 24 h	27.2 (skin) 3.2 (scale)	type I	$(\alpha1)_2\alpha2$-heterotrimer	Chen *et al.* (2016)
Squid outer skin (*Doryteuthis singhalensis*)	0.1 M NaOH for 72 h following 10% butanol for 24 h	0.5 acetic acidfor 72 h and re-extract with 05 M acetic acid containg 10% (w/v) pepsin	56.8[b] (ASC) 24.6[b] (PSC)	type I	$(\alpha1)_2\alpha2$-heterotrimer	Veeruraj *et al.* (2015)
Northern pink scale (*Esox lucius*)	0.1 M NaOH for 24 h, followed by 0.5M EDTA for 96 h	0.5 M acetic acid for 48 h and re-extract with 0.5 M acetic acid containing 1% (w/w) pepsin for 48 h	[C]	type I	$(\alpha1)_2\alpha2$-heterotrimer	Kozlowska *et al.* (2015)
Grass carp swim bladder (*Ctenopharyngodon idella*)	0.1 M NaOH for 36 h, followed by 10% (v/v) butanol for 24 h	0.5 M acetic acid for 72 h	46[b]	type I	$(\alpha1)_2\alpha2$-heterotrimer	Liu *et al.* (2015a)
Horse mackerel scale (*Trachurus japonicus*)	0.1 M NaOH for 6 h, followed by 0.5 M Na_2EDTA for 24 h	0.5 M acetic acid for 96 h	1.51[b]	type I	$(\alpha1)_2\alpha2$-heterotrimer	Minh Thuy *et al.* (2014)

[a]All procedures were performed at 4 °C.;[b]based on dry weight; [c]NM:not mentioned

Table 3. Yields and molecular compositions of collagens from different fish (Continued)

Raw materials	Pretreatment[a]	Extraction[a]	%Yield (based on wet weight)	Collagen type	Molecular compositions	References
panish mackerel skin (*Scomberomorous niphonius*)	0.1 M NaOH for 48 h, followed by 10% (v/v) butanol for 48 h	0.5 M acetic acid for 48 h and re-extract with 0.5 M acetic acid containing porcine pepsin for 48 h	13.68 (ASC) 3.49 (PSC)	type I	$(\alpha1)_2\alpha2$-heterotrimer	Li *et al.* (2013)
Spanish mackerel bone (*Scomberomorous niphonius*)	0.1 M NaOH for 48 h, followed by 0.5 M Na_2EDTA for 5 day and 10% butanol for 48 h	0.5 M acetic acid for 72 h and re-extract with 0.5 M acetic acid for 72 h	12.54 (ASC-1) 14.27 (ASC-2)	type I	$(\alpha1)_2\alpha2$-heterotrimer	Li *et al.* (2013)
Bighead carps (*Hypophthalmicthth ys nobilis*) skin (s) and swimbladder (w)	0.1 M NaOH for 36 h, followed by 10% (v/v) butanol for 36 h	0.5 M acetic acid containing porcine pepsin for 72 h	17.5 (PSC-s) 14.6 (PSC-w)	type I type I	$(\alpha1)_2\alpha2$-heterotrimer	Liu *et al.* (2012)
Ballon fish skin (*Diodon holocanthus*)	0.1 M NaOH for 72 h, followed by 10% (v/v) butanol for 24 h	0.5 M acetic acid for 36 h and re-extract with 0.5 M acetic acid containing porcine pepsin for 30 h	4[b] (ASC) 19.5[b] (PSC)	type I	$(\alpha1)_2\alpha2$-heterotrimer	Huang *et al.* (2011)

[a]All procedures were performed at 4 °C.;[b]based on dry weight; [c]NM:not mentioned

Table 3. Yields and molecular compositions of collagens from different fish (Continued)

Raw materials	Pretreatment[a]	Extraction[a]	%Yield (based on wet weight)	Collagen type	Molecular compositions	References
Trash fish, leather jacket skin (*Odonus niger*)	0.8 M NaCl for 30 min, followed by 0.1 M NaOH for 72 h	I: 0.5 M acetic acid for 72 h II: 0.5 M acetic acid for 72 h and re- extract with 0.5 M acetic acid containing porcine pepsin for 72 h III: 0.5 M acetic acid containing porcine pepsin for 72 h	50.24[b] (I) 55.48[b] (II) 70.94[b] (III)	type I	$(\alpha1)_2\alpha2$-heterotrimer	Nagarajan *et al.* (2011)
Bigeye snapper skin (*Priacanthus tayenus*, T and *Priacanthus macracanthus*, M)	0.1 M NaOH for 6 h, followed by 10% (v/v) butanol for 18 h	0.5 M acetic acid containing pepsin from tongol tuna stomach extract (TP) or porcine (PP) for 48 h	7.74 (T-PP) 8.73 (T-TP) 7.06 (M-PP) 7.29 (M-TP)	type I	$(\alpha1)_2\alpha2$-heterotrimer	Benjakul *et al.* (2010)
Arabesque greenling skin (*Pleurogrammus azonus*)	0.1 M NaOH for 6 h, followed by 10% (v/v) butanol for 18 h	0.5 M acetic acid for 24 h and re-extract with 0.5 M acetic acid containing albacore tuna pepsin for 24 h	30.31[b] (ASC) 14.03[b] (PSC)	type I	$(\alpha1)_2$ $\alpha2$- or-$\alpha1$ $\alpha2$ $\alpha3$-heterotrimer	Nalinanon *et al.* (2010)
Brownbanded bamboo shark skin (*Chiloscyllium punctatum*)	0.1 M NaOH for 6 h	0.5 M acetic acid for 48 h and re-extract with 0.5 M acetic acid containing porcine pepsin for 48 h	9.38 (ASC) 8.36 (PSC)	type I	$(\alpha1)_2$ $\alpha2$heterotrimer	Kittiphattanabawon *et al.* (2010a)

[a]All procedures were performed at 4 °C.;[b]based on dry weight; [c]NM:not mentioned

Table 3. Yields and molecular compositions of collagens from different fish (Continued)

Raw materials	Pretreatment[a]	Extraction[a]	%Yield (based on wet weight)	Collagen type	Molecular compositions	References
Carp skin (*Cyprinus carpio*)	0.1 M NaOH for 6 h, followed by 1.0% (v/v) dish drops (detergent) to remove fat	0.5 M acetic acid for 72 h	41.3[b] (ASC)	type I	$(\alpha1)_2$ α2heterotrimer	Duan *et al.* (2009)
Carp bone (*Cyprinus carpio*)	0.1 M NaOH for 24 h, followed by 0.5 M EDTA-2Na for 5 day and then 0.1% (v/v) dish drops (detergent) to remove fat	0.5 M acetic acid for 72 h and re-extract with 0.5 M acetic acid for 48 h	1.06[b] (ASC)	type I	$(\alpha1)_2$ α2heterotrimer	Duan *et al.* (2009)
Largefin longbarbel catfish skin (*Mystus macropterus*)	0.1 M NaOH containing 0.5% non-ionic detergent for 24 h, followed by 15% (v/v) butanol for 24 h and 3% H_2O_2 for 24 h	0.5 M acetic acid for 24 h and re-extract with 0.5 M acetic acid for 12 h (ASC) 0.5 M acetic acid containing porcine pepsin for 30 h (PSC)	16.8[b] (ASC) 28.0[b] (PSC)	type I	α1α2α3-herterotrimer	Zhang *et al.* (2009)
Nile tilapia skin (*Oreochromis niloticus*)	0.1 M NaOH for 48 h, followed by 10 % (v/v) butanol for 24 h	0.5 M acetic acid for 3 days and re-extract with same solution for 2 days (ASC)	39.4[b] (ASC)	type I	α1α2α3-herterotrimer	Zeng *et al.* (2009)

[a]All procedures were performed at 4 °C.;[b]based on dry weight; [c]NM:not mentioned

1.2.3 Fish gelatin

Gelatin is a fibrous protein produced by thermal denaturation or partial degradation of collagen from animal skin and bone (Benjakul *et al.*, 2012b; Foegeding *et al.*, 1996). Extraction process of gelatin involves the disruption of non-covalent bonds, mainly induced by heating at temperature higher than the denaturation temperature (Figure 3) (Bigi *et al.*, 2002). Gelatin has been widely used in food, material, pharmacy and photography industries. It has been used for improving the elasticity, consistency and stability of foods (Benjakul *et al.*, 2009; Tabarestani *et al.*, 2010). At a critical temperature, known as the shrinkage temperature, collagen fibrils shrink to less than one-third of their original length (Benjakul *et al.*, 2012b). This shrinkage involves a disassembly of fibers and a collapse of the triple-helical arrangement of polypeptide subunits in the collagen molecule. During the conversion of collagen to gelatin, many non-covalent bonds are broken along with some covalent inter- and intra-molecular bonds (Schiff base and also condensation bonds) and a few peptide bonds. This results in conversion of the helical collagen structure to a more amorphous form, known as gelatin (Figure 3). These changes constitute the denaturation of the collagen molecule but not to the point of a completely unstructured product. If the latter happens, glue instead of gelatin is produced (Foegeding *et al.*, 1996).

Normally, gelatin is manufactured on a large scale from byproducts generated during animal slaughter and processing, such as skin and bone (Jongjareonrak *et al.*, 2006b). There are two types of gelatin obtained from different methods including different characteristics, gelatin type-A (an acid process with isoelectric point at pH 6-9) and gelatin type-B (an alkaline process with isoelectric points at pH 5) (Benjakul *et al.*, 2009). Extraction condition such as temperature and time as well as chemicals used for pretreatment can influence the length of polypeptide chains and the functional properties of gelatin (Kołodziejska *et al.*, 2004).

Fish gelatins have been extracted from skin or bone of different fish species including rainbow trout skin (Tabarestani *et al.*, 2010), tiger-toothed croaker and pink perch skin and bone (Koli *et al.*, 2012), splendid squid skin (Nagarajan *et al.*,

2012b), unicorn leatherjacket skin (Kaewruang *et al.*, 2013b), cobia skin (Silva *et al.*, 2014), African catfish skin (Alfaro *et al.*, 2014), *Catla catla* swim bladder (Chandra and Shamasundar, 2015), clown featherback skin (Kittiphattanabawon *et al.*, 2016), Nile tilapia skin and channel catfish (Zhang *et al.*, 2016). Acid process is the most suitable process for less fully cross-linked collagen raw materials and it has been widely used as a process for fish gelatin extraction. On the other hand, alkaline process is appropriate for the more complex collagens found in bovine hides (Benjakul *et al.*, 2009).

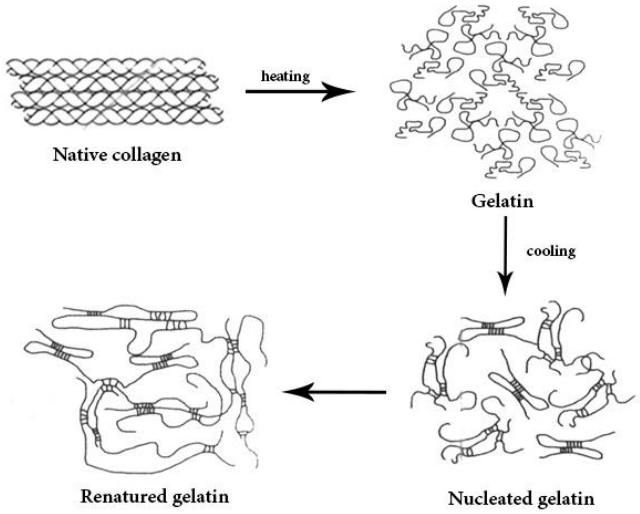

Figure 3. Collagen conversion into gelatin
Source: Modified from Harrington and Rao (1970)

1.2.3.1 Production of gelatin

Gelatin production consists of three main steps: 1), pretreatment processes by the removal of non-collagenous components from the raw materials (skin and bones), 2) the conversion of collagen to gelatin by heating the collagen in the presence of water, and 3) recovery of gelatin in its final form (Foegeding *et al.*, 1996).

1.2.3.1.1 Pretreatment processes

Pretreatment is an important step in preparing collagen before gelatin extraction. Pretreatment aims to remove other constituents in raw material to lower

the contaminants in the gelatin. Fat removal is an essential step, especially for raw material containing high fat. However, some fish skin with low fat content was not defatted prior to gelatin extraction (Benjakul *et al.*, 2009; Kittiphattanabawon *et al.*, 2010b; Koli *et al.*, 2012; Taheri *et al.*, 2009). The degree of conversion of collagen into gelatin is related to both the pretreatment and the extraction process, in which pH, temperature and time are three major factors (Montero and Gómez-Guillén, 2000).

- Removal of non-collagenous protein

This step is implemented to increase the purity of the extracted gelatin. To remove non-collagenous proteins and pigments, alkaline solutions at a proper concentration has been used (Benjakul *et al.*, 2012b). Alkaline pretreatment is used to break some crosslinks and to inactivate the proteases involved in the degradation of collagen (Zhou and Regenstein, 2005). NaOH solution has been widely used for gelatin extraction (Arnesen and Gildberg, 2007; Kittiphattanabawon *et al.*, 2012; Tabarestani *et al.*, 2010). Zhou and Regenstein (2005) reported that the type of alkali did not make a significant difference, but the concentration of alkali was critical. Alkaline pretreatment time and alkaline concentration affected yield of gelatin from channel catfish skin (Yang *et al.*, 2008). The yield of gelatin increased with increasing alkaline concentrations in the range of 0-1 M. However, the longer time of alkaline pretreatment decreased the yield of gelatin. Higher concentrations of alkali or longer pretreatment times decreased gel strength of gelatin gel (Yang *et al.*, 2007). The concentration of alkali, time and temperature used for pretreatment varied with raw materials (Table 4).

- Swelling process

Prior to gelatin extraction, swelling-process is commonly required to enhance the extraction efficiency. Swelling induces protein unfolding by disruption of non-covalent bonding and predispose the collagen to subsequent extraction and solubilization (Stainby, 1987). Two processes associated with different swelling approaches are as follows:

1. Acid process

Acid hydrolysis is a milder treatment that effectively solubilizes collagens of animals slaughtered at a young age such as pigs (Foegeding *et al.*, 1996). The pretreatment is designed to convert the collagen into a form suitable for extraction. A sufficient number of the covalent crosslinks in the collagen must be broken in order to enable the release of free α-chains (Johnston-Banks, 1990). The process is also designed to remove other organic substances, such as proteoglycan, blood, mucins, sugars, etc., that also occur naturally in the raw material (Johnston-Banks, 1990). It is optimized by each manufacturer to give the required physical and chemical properties (Johnston-Banks, 1990). Normally, soaking the raw material in dilute acid for 18-24 h is sufficient to bring about the conversion. Additionally, the acid pretreatment can partly prevent the degradation of collagen caused by endogenous proteases and minimize the enzymatic breakage of intra-chain peptide bonds of collagen during extraction (Zhou and Regenstein, 2005). Indigenous proteases associated with skin of some fish species have been reported such as unicorn leatherjacket (Kaewruang *et al.*, 2013a) and bigeye snapper (*Priacanthus tayenus*) (Benjakul *et al.*, 2009). Those proteases directly contributed to degradation of gelatin, especially during gelatin extraction process at optimal temperature (Kaewruang *et al.*, 2013a).

Moreover, type of acid and concentration affected the yield and properties of gelatin. Gómez-guillœn and Montero (2001) reported that the type of acid used in processing of gelatin from megrim skins affected the yield and functional properties (viscoelastic and gelling properties). Different swelling capacity and pH of extraction are governed by the acid used. The concentration of H^+ also influenced yield and quality of gelatin (Gudmundsson and Hafsteinsson, 1997). Pretreatment of skin from unicorn leatherjacket skin using phosphoric acid yielded a gelatin with an improved gel strength (Ahmad and Benjakul, 2011).

2. Alkaline process

Type B gelatins are produced by alkaline hydrolysis of beef materials and results in deamidation and a greater range of molecular weight species

(Foegeding *et al.*, 1996). Alkaline pretreatments are normally applied to bovine hide and ossein. Liming process is particularly designed for gelatin extraction from mammalian skins and bones, which normally takes a few days to four months, depending on the concentration and temperature used. Lime [$Ca(OH)_2$] is normally preferred due to its ability to regulate the desired alkalinity and does not cause the collagen to swell (Jamilah *et al.*, 2011). Gelatins from the skins of red tilapia (*Oreochromis nilotica*), walking catfish (*Clarias batrachus*) and striped catfish (*Pangasius sutchi fowler*) obtained through a liming process [$Ca(OH)_2$] for 14 days had the yields of 12.92, 13.06 and 11.17% (wet basis), respectively (Jamilah *et al.*, 2011). Gel strength of gelatin was affected by liming process used. Gelatin extracted from red tilapia skin by liming process had higher gel strength than that of gelatin extracted using acid conditioning process (Jamilah *et al.*, 2011). Kaewdang *et al.* (2016) extracted gelatin from swim bladder of yellowfin tuna (*Thunnus albacores*) using various alkaline pretreatment. Pretreatment of swim bladder using the alkaline mixture of Na_2CO_3:NaOH at a ratio of 7:3 (4%, w/v) rendered the gelatin with the highest extraction yield (35.96%). Yield and gel strength of gelatin from yellowfin tuna swim bladder increased with increasing proportion of NaOH in alkaline mixture. The different gel strength from the same source by varying the gelatin extraction method has been associated with the compositions and characteristics of the resulting gelatin (Gudmundsson and Hafsteinsson, 1997).

1.2.3.1.2 Extraction of gelatin

The second step of gelatin production is the extraction of the pretreated raw material. The extraction process is designed to maximize the yield in combination with the most desirable physical properties. 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). Conversion of collagen into soluble gelatin can be achieved by heating the collagen. The intra- and intermolecular covalent crosslink of collagen can be cleaved by thermal process, leading to gelatin solubilization in the medium (Karim and Bhat, 2009). The extraction process can influence the length of the polypeptide chains and the functional properties of the gelatin. This depends on the processing parameters (temperature, time, and pH)

(Karim and Bhat, 2009). Gelatin from African catfish skin extracted at higher temperature had the lower gelatin viscosity and gelling properties (gel strength, and melting temperature), which was caused by increased degradation of protein chains (Alfaro *et al.*, 2014). Kittiphattanabawon *et al.* (2016) also reported that gelatin from the skins of clown featherback showed the lower gel strength and longer setting time as extraction temperature increased from 45 to 85 °C and time increased from 6 to 12 h. In addition, the higher extraction temperature and longer extraction time lowered gelling temperature of gelatin from clown featherback skin.

Additionally, the presence of the endogenous protease, serine protease, in the skin of some fish species such as bigeye snapper and unicorn leatherjacket has been reported (Jongjareonrak *et al.*, 2006b; Kaewruang *et al.*, 2013a; Nalinanon *et al.*, 2008). The indigenous protease had impact on the properties of gelatin. Ahmad *et al.* (2011) reported that endogenous serine protease found in the skin of unicorn leatherjacket had maximal activity at 50 °C, which is in the temperature range used for gelatin extraction. The use of protease inhibitors during extraction could be a mean to lower degradation. Gelatin extracted in the presence of protease inhibitor showed higher gel strength than the gelatin extracted by the typical process in absence of protease inhibitor. Nevertheless, Kaewruang *et al.* (2013a) reported that the addition of protease inhibitor during gelatin extraction resulted in a lower yield, regardless of extraction temperature.

1.2.3.1.3 Filtration, bleaching and drying

After extraction, the gelatins are filtered to remove suspended or insoluble matters such as fat or un-extracted collagen fibres. This process is usually performed as quickly as possible to minimize loss of properties. Materials such as diatomaceous earth have been used to render gelatin solutions of high clarity (Johnston-Banks, 1990). Activated carbon has also been used to reduce the color and turbidity of gelatin solution (Cho *et al.*, 2006). Color and clarity of a gelatin gel are important properties, depending on the application for which the gelatin is intended (Zarai *et al.*, 2012). The turbidity and color of gelatin solution is commonly caused by inorganic, protein and muco-substance contaminants. Turbidity is largely dependent on type of raw material, gelatin extraction process and clarification process (Muyonga

et al., 2004b; Zarai *et al.*, 2012). However, the color did not affect functional properties of gelatin (Jellouli *et al.*, 2011).

Moreover, the pigments in skin of some species, especially cuttlefish and squid, may pose a color problem and bleaching can be performed prior to gelatin extraction (Aewsiri *et al.*, 2009; Hoque *et al.*, 2011a; Nagarajan *et al.*, 2013). Hydrogen peroxide (H_2O_2) is an oxidizing agent that is widely used as bleaching agent in seafood processing and it can affect the properties of food proteins including gelatin (Aewsiri *et al.*, 2009; Hoque *et al.*, 2011a). The decomposition of H_2O_2 in aqueous solution occurs by dissociation and hemolytic cleavage of O–H or O–O bonds, with the formation of highly reactive products: hydroperoxyl anion, and hydroperoxyl and hydroxyl radicals, which can react with many substances, including chromatophores (Aewsiri *et al.*, 2009). Aewsiri *et al.* (2009) reported that soaking cuttlefish skin in 5% H_2O_2 for 48 h at 4 °C could improve the color of the resulting gelatin, while bloom strength of gelatin gel increased with increasing H_2O_2 concentration and bleaching time. On the other hand, Nagarajan *et al.* (2013) reported that the gel strength of gelatin from splendid squid skin decreased as H_2O_2 concentrations increased.

The final stage of gelatin extraction is evaporation, sterilization and drying (Johnston-Banks, 1990). Drying processes directly affected the functional properties of gelatin because the heat used in these procedures can denature the protein (Cepeda *et al.*, 1998). Kwak *et al.* (2009) studied the effect of three drying processes, freeze-drying, hot-air drying and spray drying, on the functional properties of gelatin extracted from shark cartilage. The highest gel strength and foam formation ability were found in gelatin obtained using freeze-drying, due to lower degradation of gelatin at low drying temperature. The spray drying gelatin could not form a gel network. However, spray-dried gelatin exhibited the best emulsion capacities. Lower drying temperatures with less protein denaturation are desirable for making gelatins with acceptable gel strength (Kwak *et al.*, 2009). Sae-Leaw *et al.* (2016) reported that gelatin from seabass skin obtained from freeze-drying had higher gel strength than that from spray-drying. Gel strength of spray-dried gelatin decreased with increasing temperature of spray-drying. However, freeze-dried gelatin had lower solubility when compared with spray-dried counterpart.

Table 4. Extraction conditions, yield and gel strength of gelatin extracted from different fish

Raw materials	Pretreatments		Extraction	Yield (based on wet weight)	Gel strength	References
	Non-collagenous matter removal	Swelling				
Clown featherback skin (*Chitala ornata*)	0.1 M NaOH at 15-20 °C for 2 h	0.05 M acetic acid at 26-28 °C for 30 min	Water at 45 °C for 6 h	74%[b]	284 g	Kittiphattanabawon *et al.* (2016)
Nile tilapia skin (T) (*Oreochromis niloticus*) and channel catfish skin (C) (*Icatalurus punctatus*)	0.2 M NaOH at 2 °C for 40 min	0.2% H_2SO_4 at 2 °C for 40 min followed by 0.2% citric acid 2 °C for 40 min	Water at 45 °C for 12 h	94.1%[a] (T) 80.6%[a] (C)	Not mention	Zhang *et al.* (2016)
Yellowfin tuna skin (*Thunnus albacares*)	0.2 M NaOH at 20 °C for 30 min	0.1 M acetic acid at 20 °C for 1 h	Water at 55 °C for 1 h	6.68%	289.8g	Karayannakidis and Zotos (2015)
Zebra blenny skin (*Salaria basilisca*)	0.05 M NaOH at 4 °C for 1 h	100 mM glycine-HCl buffer pH 2 containing crude acid protease from zebra blenny at 4 °C for 18 C	Water at 50 °C for 4 h	14.65%	151.3g	Ktari *et al.* (2014)

[a]Based on dry weight basis. [b]Based on protein content.

Table 4. Extraction conditions yield and gel strength of gelatin extracted from different fish (Continued)

Raw materials	Pretreatments		Extraction	Yield (based on wet weight)	Gel strength	References
	Non-collagenous matter removal	Swelling				
Splendid squid skin (*Loligo formosana*)	0.05 M NaOH at 26-28 °C for 6 h	0.05 M phosphoric acid at 4 °C for 12 h	Water at 60 °C for 12 h	21.8%	122 g	Nagarajan *et al.* (2012b)
Marine snail (*Hexaplex trunculus*)	0.02 M NaOH at 26-28 °C for 1 h	Not mention	3% (v/v) acetic acid at 60 °C for 9 h	3%	103 g	Zarai *et al.* (2012)
Red tilapia (*Oreochromis nilotica*) (R), Walking catfish (*Clarias batrachus*) (W) and Striped catfish (*Pangasius sutchi fowler*) (S) skin	-	2.7% (w/v) $[Ca(OH)_2]$ at 20 °C for 14 days	Water at 48 °C for 12 h	12.92% (R) 13.06% (W) 11.17 (S)	384.9 g (R) 147.4 g (W) 238.9 (S)	Jamilah *et al.* (2011)
Grey triggerfish skin (*Balistes capriscus*)	0.2 M NaOH at 4 °C for 1.5 h	0.05 M acetic acid at 4 °C for 6 h	Water at 50 °C for 18 h	5.67%	168.3 g	Jellouli *et al.* (2011)

[a]Based on dry weight basis.

Table 4. Extraction conditions yield and gel strength of gelatin extracted from different fish (Continued)

Raw materials	Pretreatments		Extraction	Yield (based on wet weight)	Gel strength	References
	Non-collagenous matter removal	Swelling				
Giant catfish (*Pangasianodon gigas*)	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	20.1 %	153 g	Jongjareonrak *et al.* (2010)
Brownbanded bamboo shark (Chiloscyllium punctatum) (BBS) skin and blacktip shark (Carcharhinus limbatus) (BTS)	0.1 M NAOH at 15-20 °C for 2 h, followed by 1 M HCl for 1 h	0.2 M acetic acid at 25-26 °C for 15 h	Water at 45 °C for 6 h	19.06% (BBS) 21.17% (BTS)	214 g (BBS) 206 g (BTS)	Kittiphattanabawon *et al.* (2010b)
Cuttlefish skin (*Sepia pharaonis*)	0.05 M NaOH at 26-28 °C for 6 h, followed by 5% (v/v) H_2O_2 at 4 °C for 16 h	None	Water at 60 °C for 12 h	36.82 %[a] (dorsal skin) and 59.69 %[a] (ventral skin)	126 g (dorsal skin) and 137 g (ventral skin)	Aewsiri *et al.* (2009)
Giant catfish (*Pangasianodon gigas*)	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	20.1 %	153 g	Jongjareonrak *et al.* (2010)

[a]Based on dry weight basis.

1.2.3.2 Chemical compositions

The proximate compositions of fish gelatin have been reported to vary among the fish species, mainly due to the differences in collagen content and the compositions of raw material (Benjakul *et al.*, 2012a; Muyonga *et al.*, 2004b). Generally, gelatins contain protein as the major component but low moisture, fat and ash contents (Jongjareonrak *et al.*, 2006b). The purity of gelatin depends on extraction condition, especially efficiency in removal of water, fat, ash and other constituents (Jongjareonrak *et al.*, 2010; Zarai *et al.*, 2012). Protein content of skin gelatins of different fishes ranges from 78.4 to 91.4 g/100 g, (Table 5). In general, the recommended moisture and ash content of edible gelatin is less than 15 and 2%, respectively (Nagarajan *et al.*, 2012b). The proximate compositions of different fish gelatins are summarized in Table 5.

Table 5. Proximate compositions of gelatin from different fish species

Raw material	Contents (g/ 100 g wet weight)				References
	Protein	Moisture	Fat	Ash	
Cobia skin	88.6	9.4	1.6	1.0	Silva *et al.* (2014)
Croaker skin	88.2	10.2	0.6	0.9	Silva *et al.* (2014)
Cuttlefish skin	91.4	6.48	0.28	0	Balti *et al.* (2011)
Fesh water (*Catla catla*) fish swim bladder	92.8	5.5	Not mention	1.5	Chandra and Shamasundar (2015)
Giant catfish skin	89.1	9.3	0.7	0.3	Jongjareonrak *et al.* (2010)
Grey triggerfish skin	89.9	7.4	0.1	0.9	Jellouli *et al.* (2011)
Grouper bone	82.4	4.0	3.9	6.6	Jeya Shakila *et al.* (2012)
Red snapper bone	78.5	6.2	5.2	10.3	Jeya Shakila *et al.* (2012)
Splendid squid	90.0	10.5	0.2	0.2	Nagarajan *et al.* (2012b)
Tilapia	90.76	7.32	0.02	0.09	Weng *et al.* (2014)
Zebra blenny	90.6	7.45	0.6	3.1	Ktari *et al.* (2014)

The amino acid composition particularly imino acid (proline and hydroxyproline) of gelatin is one of the most important factors affecting the functional properties of gelatin, especially gel strength (Aewsiri *et al.*, 2008; Benjakul *et al.*, 2009). Taheri *et al.* (2009) reported that the limited application of gelatins from both cold- and warm-water fish was due to lower gel strength and lower stability of gels. Based on amino acid composition of gelatin, glycine, alanine, proline and hydroxyproline are the most abundant amino acids in fish gelatin (Kittiphattanabawon *et al.*, 2010b). However, fish gelatins have lower imino acid contents (proline+hydroxyproline) than mammalian gelatin, and this may be the reason for their denaturation at lower temperature (Karim and Bhat, 2009). Imino acid content correlates with gel strength of gelatin. It played a role in gel formation. Hydroxyl groups of hydroxyproline are thought to be responsible for the stability of the helix by inter-chain hydrogen bonding via a bridging water molecule as well as direct hydrogen bonding to the carbonyl group (Wong, 1989). Jellouli *et al.* (2011) characterized the chemical and functional properties of gelatin from grey triggerfish skin (GSG), compared with halal bovine gelatin (HBG). GSG contained a lower number of imino acid (176 residues/ 1000 residues) than HBG (219 residues/ 1000 residues). The gel strength of the GSG (168.3 g) was lower than that of HBG (259 g) ($p < 0.05$). Balti *et al.* (2011) reported that the low gel strength of gelatin extracted from cuttlefish skin, compared with bovine gelatin may be due to the possible low content of proline and hydroxyproline, which could result in less organized triple helical structures. The amino acid compositions of different fish gelatins are shown in Table 6.

1.2.3.3 Functional and physical properties of gelatin

Gelatin has many extraordinary properties, especially gelling property, which is the most important property of gelatin (Karim and Bhat, 2009). The gelatin has been used as ingredients to improve the elasticity, consistency and stability of foods. It is one of the hydrocolloid used to enhances the viscosity of aqueous systems and to form aqueous gels (Choi and Regenstein, 2000). Other functional properties, including emulsifying properties, foam forming properties and film formation are also important properties for food applications (Montero and Gómez-Guillén, 2000). The

functional properties of gelatin are generally related to their chemical characteristics (Muyonga *et al.*, 2004b).

Table 6. Amino acid compositions of fish gelatin as compared to bovine gelatin (residues per 1000 residues)

Amino acids	Source of gelatin				
	Bovine gelatin[1]	Grey triggerfish skin[2]	Tilapia scale[3]	Octopus skin[4]	Clown featherback skin[5]
Alanine	115	113	166	52	123
Arginine	48	78	56	20	52
Aspartic acid/asparagine	44	59	38	35	42
Cysteine	0	0	0	0	1
Glutamine/glutamic acid	74	60	60	54	71
Glycine	341	289	310	310	334
Histidine	5	11	5	165	5
Isoleucine	11	17	7	68	10
Leucine	25	25	10	31	19
Lysine	26	32	22	14	16
Hydroxylysine	7	9	-	-	16
Methionine	5	8	2	7	12
Phenylalanine	12	19	24	31	14
Hydroxyproline	96	74	60	45	85
Proline	123	102	159	56	122
Serine	29	40	20	33	34
Threonine	17	29	10	34	24
Tyrosine	1	7	11	6	4
Valine	21	28	31	39	17
Total	1000	1000	1000	1000	1000
Imino acid	219	176	219	101	207

Sources: [1]Balti *et al.* (2011), [2]Jellouli *et al.* (2011), [3]Weng *et al.* (2014), [4]Jridi *et al.* (2015) and [5]Kittiphattanabawon *et al.* (2016)

1.2.3.3.1 Gelation

Gelation is one of the most important functional properties of gelatin, which is influenced by many factors such as sources of raw material, the presence of endogenous protease in raw material and the conditions of gelatin extraction, especially the temperature using for extraction of gelatin (Kittiphattanabawon *et al.*,

2010b). An aqueous solution of a few percent gelatin forms a low viscous solutions at temperature above 40 °C. On cooling, the gelatin solution starts to form transparent elastic thermo-reversible gels when the temperature is below the setting temperature (Babin and Dickinson, 2001; Normand *et al.*, 2000). Gelling or setting temperature of fish gelatin varies with fish species. In general, gelling temperature of fish gelatin are lower than that of mammalian counterpart. Gelling and melting temperatures of fish gelatin are shown in Table 7. The interaction initiates via 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 held together, predominantly by hydrogen bonded junction zones (Babin and Dickinson, 2001). The amino acid composition and amount of β- and γ-components were considered as factors governing gelation of gelatin (Tabarestani *et al.*, 2010). Imino acids, especially hydroxyproline, are associated with the gel formation of gelatin by hydrogen bonding through hydroxyl group (Benjakul *et al.*, 2012a). In addition, Kittiphattanabawon *et al.* (2016) reported that gelatin with large amount of α-, β- and γ-chains yielded gel network with strong strands.

Table 7. Gelling and melting temperatures (°C) of gelatin extracted from different fish

Raw materials	Gelling temperature	Melting temperature	References
Bovine skin	23.8	33.8	Cho *et al.* (2005)
Porcine skin	25.6	36.5	Cho *et al.* (2005)
Bigeye snapper (*Priacanthus hamrur*) skin	10.0	16.8	Binsi *et al.* (2009)
Chennel catfih (*Ictalurus punctaus*) skin	18	24	Liu *et al.* (2008)
New Zealand hoki skin	-	21.4	Mohtar *et al.* (2013)
Skipjack tuna (*Katsuwonus pelamis*) skin	18.7	24.2	Shyni *et al.* (2014)
Dog shark (*Scoliodon sorrakowah*) skin	20.8	25.8	Shyni *et al.* (2014)
Squid skin	5	16	Abdelmalek *et al.* (2016)
Silver carp skin	18.7	27.1	Boran *et al.* (2010)

Extraction conditions have been known to determine gelling property of gelatin. Nagarajan *et al.* (2012b) extracted the gelatin from splendid squid skin at different temperatures (50, 60, 70 and 80 °C). Gelatin extracted with extraction temperature of 50 °C exhibited the highest gel strength. The higher extraction temperature caused protein degradation, producing protein fragments and lowering gelling ability. Kittiphattanabawon *et al.* (2016) reported that increasing extraction temperature and time decreased gel strength of gelatin from clown featherback skin. In addition, gelling temperature also decreased when extraction temperature and time were increased. Kaewruang *et al.* (2013a) reported that gelatin from skin of unicorn leatherjacket extracted at 45 and 55 °C had the lower gel strength than gelatin extracted at 65 °C, mainly caused by endogenous heat stable proteases. Moreover, the imino content had correlation with gel strength by hydrogen bonding between free water molecules and the hydroxyl group of the hydroxyproline in gelatin (Fernández-Díaz *et al.*, 2001). Also, type and concentration of chemical used for pretreatment of raw material affected gelation properties of gelatin (Gómez-guillœn and Montero, 2001; Zhou and Regenstein, 2005). Gelatins from different sources show varying gel strengths (Table 4). Silva *et al.* (2014) studied the characteristics and functional properties of gelatin from different sources (cobia skin and croaker skin). It was found that gelatin from cobia skin had higher gel strength, gelling and melting temperatures than that of gelatin from croaker skin. This was associated with the different intrinsic characteristics (molecular weight distribution and amino acid composition). Several organic acids (formic, acetic, propionic, lactic, malic, tartaric and citric acids) had varying influences on functional properties of gelatin extracted from skin of megrim (Gómez-guillœn and Montero, 2001). Gelatins with formic acid or lactic acid pretreatment showed the higher gel strength than those pretreated with another acid. Ahmad and Benjakul (2011) reported that the gel strength of gelatin from unicorn leatherjacket skin pretreated with phosphoric acid was higher than that of gelatin from skin pretreated with acetic acid.

1.2.3.3.2 Film formation

Most of food protein exhibit film forming ability. Among protein, gelatin has been used as the biomaterial for film formation, due to its fibrous structure, which favors the chain to chain interaction. Edible film and coating materials have been widely used as tools in food packaging to extend the shelf-life and improve the quality of food systems by acting as a barrier to moisture and oxygen (Avena-Bustillos *et al.*, 2011; Benjakul *et al.*, 2008).

Films from different gelatins generally exhibit different properties as affected by raw material source, extraction method, molecular weight and structure, amino acid compositions, film preparation method and degree of hydration or presence of plasticizer (Chiou *et al.*, 2008; Hoque *et al.*, 2011b; Jongjareonrak *et al.*, 2006a). However, fish gelatin film has poor water vapor barrier property (WVP), leading to the limited application of gelatin-based film as food packaging (Tongnuanchan *et al.*, 2013).

Chain length of gelatin is a factor determining the property of film. Hoque *et al.* (2011b) prepared the films from gelatin of cuttlefish skin with different degree of hydrolysis (DHs) containing glycerol at varying levels. All films showed different properties and molecular characteristics. Gelatin molecules with the shorter chain had low film-forming properties, owing to the lower junction zones mainly via hydrogen bond. This led to lower mechanical properties and thermal stability of their film. Moreover, the mechanical properties of gelatin-based film were also affected by glycerol content. The increases in glycerol would lower the interaction between chains by preventing protein-protein interaction. Jongjareonrak *et al.* (2006a) reported that the decreased tensile strength (TS) and increased elongation at break (EAB) were obtained for gelatin film from bigeye snapper skin and brownstripe red snapper skin with increasing glycerol content (25-75%, based on protein). TS and EAB of films from splendid squid (*Loligo formosana*) decreased as the extraction temperature increased (Nagarajan *et al.*, 2012a). Higher content of low molecular weight in gelatin extracted at higher temperature might impair the formation of junction zones. WVP value of films from splendid squid (*Loligo formosana*) also increased with increasing extraction temperatures (Nagarajan *et al.*, 2012a).

The increase in protein content of film forming solution from 2 to 3% increased thickness, TS and EAB of film, but did not affect WVP of film (Jongjareonrak *et al.*, 2006a). Films incorporated with essentials oils showed the lower TS but higher EAB with increasing essential oils, compared with the control film (without oil incorporated). The incorporation of essential oils at the higher lever (100% based on protein) reduced WVP of film from 3.11 x 10^{-11} gm^{-1} Pa^{-1} to 1.88 x 10^{-11} gm^{-1} Pa^{-1} (Tongnuanchan *et al.*, 2013).

1.2.4 Quality improvement of fish gelatin

Gelatin obtained from fish processing byproducts has the limited applications, due to its low gel strength, gelling and melting temperatures, when compared to gelatin from land animals (Karim and Bhat, 2009). Therefore, the improvement of gel properties of fish gelatin has been carried out to widen their applications. Chemical or natural cross-linkers have been used to strengthen fish gelatin gels (Kosaraju *et al.*, 2010). Gómez-Guillén *et al.* (2001) reported that the addition of microbial transglutaminase to gelatin can increase melting temperature and gel strength of gelatin gel. Sarabia *et al.* (2000) reported that the addition of salts at high concentration (0.5 M) to megrim gelatin affected the rheological properties of gelatin, especially increasing the melting temperature. Some hydrocolloids with high gelling properties, such as κ-carrageenan, pectin and gelatin, were used to increase the gel strength and gelation behaviors of gelatin gel (Haug *et al.*, 2004a; Lau *et al.*, 2000; Liu *et al.*, 2007).

1.2.4.1 Enzyme crosslinking

Transglutaminase has been widely used in the cross-linking of several proteins. Casein, ovomucin and soy bean glycine were modified by the incorporation of a transglutaminase (Lee *et al.*, 1997). The enzyme acts by catalyzing an acyl transfer reaction between the γ-carboxamind group of glutamine residues and the amino group of lysine residues of peptide chain (Folk, 2006). Gómez-Guillén *et al.* (2001) reported that the addition of microbial transglutaminase to a fish skin gelatin considerably enhanced gel strength, melting point and viscosity at 60 °C. The efficacy

depended on the concentration of the enzyme and the incubation time. Elasticity and cohesiveness of the gels increased with increasing concentration of transglutaminase. However, at higher concentration of transglutaminase, gel strength and hardness decreased, which was associated with excessively rapid gel network formation. Partial inactivation of the enzyme by heating showed no negative impact on properties of the gelatin. Kołodziejska *et al.* (2004) also reported that the enzymatically crosslinked gels did not melt during 30 min of heating in a boiling water bath. Furthermore, fish gelatin solution formed gels at room temperature when transglutaminase was added. Jongjareonrak *et al.* (2006b) noted that the addition of microbial transglutaminase increased the bloom strength of skin from bigeye snapper and brownstripe red snapper gelatins. However, bloom strength decreased when the concentration of enzyme was higher than 0.01% (w/w). This result was in accordance with Gómez-Guillén *et al.* (2001). Moreover, Mohtar *et al.* (2013) showed that the addition of transglutaminase at optimum conditions increased the gel strength and melting point of hoki gelatin gel, which was increased by approximately 41% and 4.5 °C, respectively.

1.2.4.2 Natural/chemical crosslinking

Gel properties of gelatin can also be improved by introducing covalent chemical crosslinks between single strand and chain segments (Chiou *et al.*, 2006). Chemical crosslinking reactions has been used in industrial process such as production of hardened gelatin gels for photographic emulsions and the manufacture of hardened gelatin-acacia coacervates for ink encapsulation in pressure-sensitive paper (Strauss and Gibson, 2004). Various cross-linkers, including glutaraldehyde, genipin and carbodiimides have been widely used to cross-link gelatin (Chiou *et al.*, 2006). Glutaraldehyde is one of the most widely used protein cross-linkers because it reacts rapidly with amine groups in the gelatin and is also relatively inexpensive. However, there have been concerns about the toxicity of the glutaraldehyde. Genipin (isolate from the fruits of *Gardenia jasminoides*) has increased interest as an alternative cross-linker to glutaraldehyde because of its lower toxicity (Karim and Bhat, 2009). In another study, Chiou *et al.* (2006) studied the rheological and physical properties of gelatin from Alaska pollock skin and Alaska pink salmon skin using genipin and glutaraldehyde as crosslinking agents. Both fish gelatin containing

genipin exhibited faster cross-linking rates, particularly at higher pH values. However, Alsaka pink salmon gelatin showed the greater dependence on pH. In addition, gelatin samples added with genipin had higher gel strength than those containing glutaraldehyde. Chemical modifications of gelatin from New Zealand hoki skin using three different cross-linking agents (genipin, glutaraldehyde and caffeic acid) were reported by Mohtar *et al.* (2014). Gelatin cross-linked with glutaraldehyde had higher gel strength and melting temperature than those cross-linked with caffeic acid and genipin. Intra- and inter-molecular cross-linking of gelatin mediated by those three compounds are shown in Figure 4.

Use of plant phenolics as cross-linkers of gelatin gels and gelatin based coacervates for future use as food ingredients was reported by Strauss and Gibson (2004). Polyphenols are known to react under oxidizing conditions with side chain amino groups of peptides, leading to formation of cross-links in proteins. Several phenolic acids (caffeic, chlorogenic and ferulic), instant coffee and commercial white grape juice are an examples of plant polyphenols or sources of polyphenols. Gelatin gels crosslinked using these materials had higher mechanical strength with reduced swelling and fewer free amino groups (Strauss and Gibson, 2004). Gel strength of gelatin from swim bladder of yellowfin tuna increased when ethanolic extract from coconut husk (rich in tannic acid) was incorporated (Kaewdang and Benjakul, 2015). Yan *et al.* (2011) reported that the gel strength of gelatin increased with increasing the level of gallic acid and rutin. Both cross-linking agents could enhance the storage and loss modulus of hydrogel. The result suggested that gallic acid and rutin molecules mainly interacted with skeletal C-N-C group and carboxyl group of gelatin molecules in the formation of gels. However, both cross-linking agents had no effect on the gelling and melting temperature of gel.

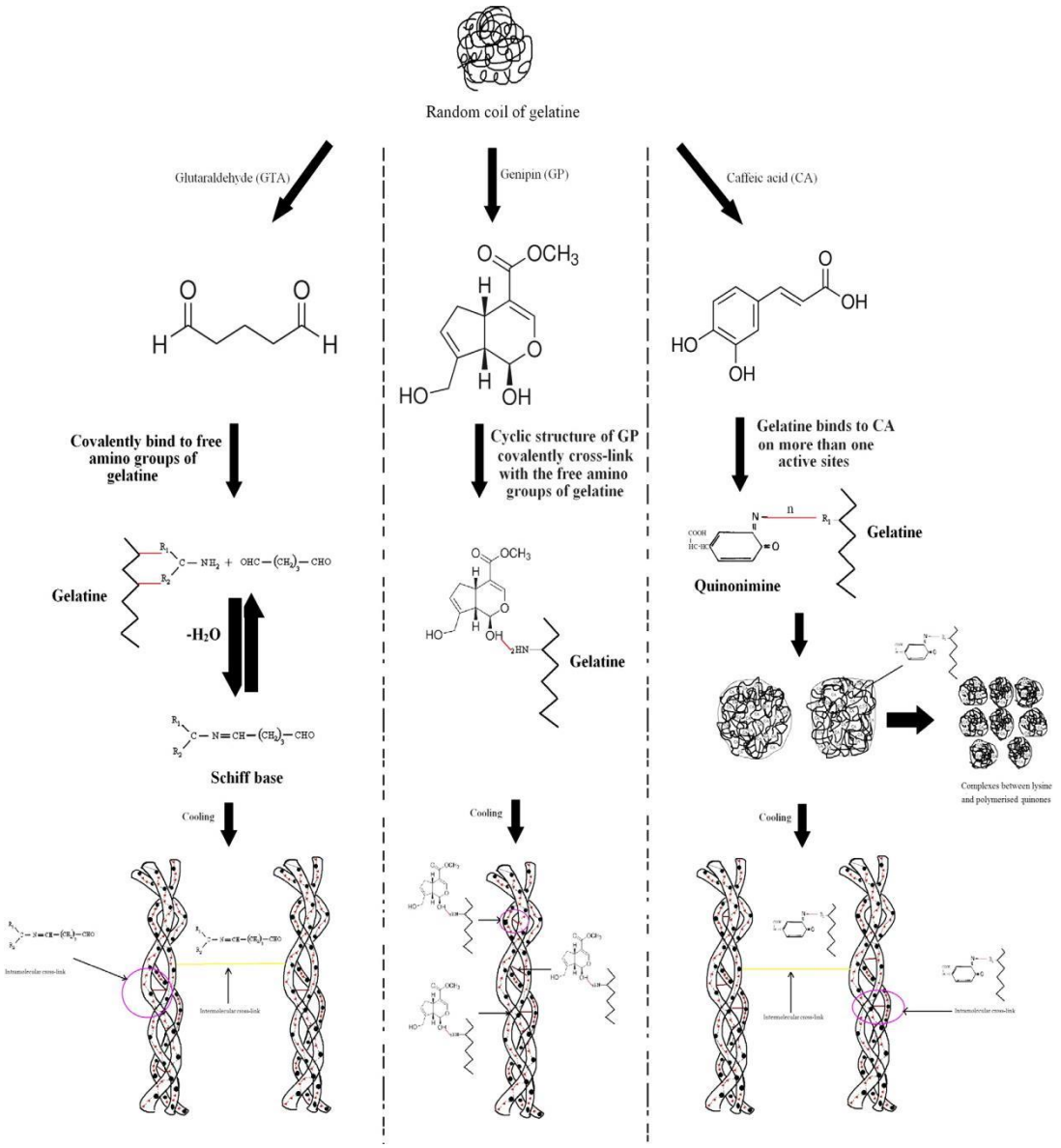

Figure 4. Schematic representation of the intra-molecular and inter-molecular cross-linking structures of gelatin with glutaraldehyde (GTA), genipin (GP) and caffeic acid (CA)

Source: Mohtar *et al.* (2014)

1.2.4.3 Protein-polysaccharide mixing

A mixed system consisting of fish gelatin combined with other high Bloom fish gelatins or other hydrocolloids could enhance gel strength, gelling and melting temperature of fish gelatin (Karim and Bhat, 2009; Zhou *et al.*, 2006).

Pollock gelatin mixed with tilapia gelatin or pork gelatin had increased gel strength with the two-step melting process, which might be useful in food product development to control the texture (Choi and Regenstein, 2000). Badii and Howell (2006) noted that fish gelatin combined with egg albumen proteins showed synergistic interactions and compatible gel structures, leading to a higher gel strength. The modification of gelatin with egg proteins brings about a potential means to widen the use of porcine and bovine gelatins in desserts and bakery products.

Polysaccharides and protein are natural polymers that are widely used as functional ingredients for various food colloids or emulsion formulations. Their interaction in formulation generally changes the rheological properties of food colloids and affects the food product texture and colloidal stability. The formation of polysaccharides-protein complexes and their solubility depend on various factors such as charge and nature of biopolymers, pH, ionic strength and temperature of the medium (Ghosh and Bandyopadhyay, 2012). Proteins contribute to the structural and textural properties of food by changing rheology of food emulsion through their gelation (Benichou *et al.*, 2007). The stability of food colloids is electrostatic interactions, hydrophobic interactions, H-bonding and Van der Waals interactions (Ghosh and Bandyopadhyay, 2012).

1.2.4.3.1 Distribution of polysaccharide/gelatin

Two bio-polymers can exist either in a single phase systems or in a phase separated systems, depending on the nature of bio-polymers, their concentration, and solution conditions (Figure 5). When two bio-polymers have opposite charge, both biopolymer agglomerates to form soluble complexes (single phase) or insoluble precipitates (Two phase system) (Vladimir, 2006). When two non-interacting bio-polymers are mixed together, these exist either in a single phase system (where the biopolymers while separated are still uniformly distributed throughout the medium) or they exist in two distinct phases (each phases comprises one of the bio-polymer with a very high concentration compared to the other). Therefore, in the protein-polysaccharide system, phase separation occurs through two different mechanisms which are associative phase separation and segregative phase separation (Vladimir, 2006). Associative phase separation is the aggregation between

two oppositely charged bio-polymers (electrostatic attraction driven). This leads to the phase separation, with one phase enriched with two different bio-polymers (coacervation or precipitation) (Figure 5). Segregative phase separation occurs either due to strong electrostatic repulsion (between two similarly charged bio-polymers). In this case, at low concentration, two biopolymers can co-exist in a single phase whereas at higher concentration, phase separation occurs. (Figure 5) (Ghosh and Bandyopadhyay, 2012).

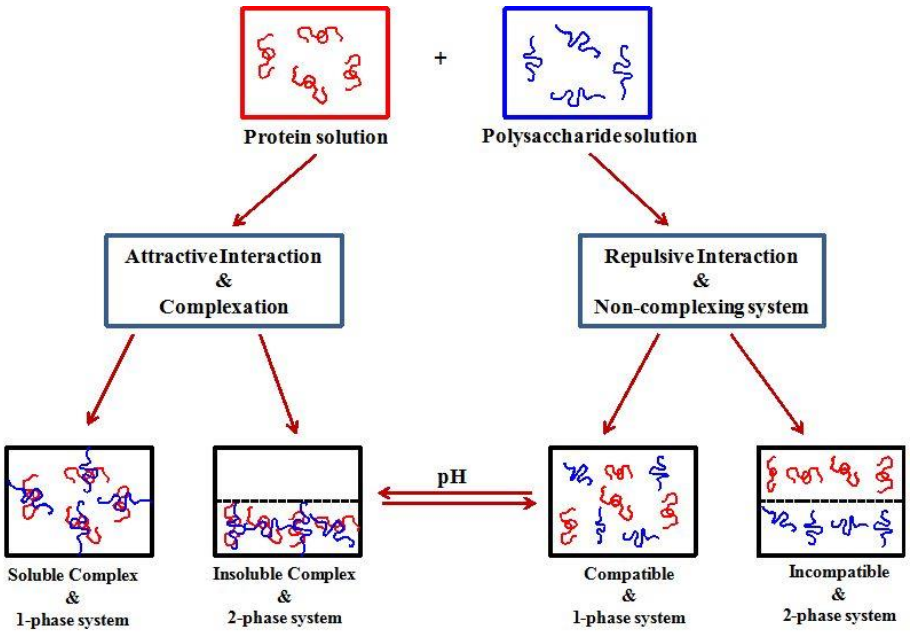

Figure 5. Schematic representation of the possible mode of interaction between polysaccharides and proteins

Source: Ghosh and Bandyopadhyay (2012)

Polysaccharides are important factors governing the functional properties of food systems and the quality of many foods (Fonkwe *et al.*, 2003). Increasing attention in gelation and interaction of mixed solution and gel of protein and polysaccharides for improvement of foods has been gained (Fonkwe *et al.*, 2003).When two biopolymers (proteins and/or polysaccharide) are mixed together, different behaviors can occur (Panouillé and Larreta-Garde, 2009). In most cases, mixing two or more biopolymers result in phase separation. This can be associative (first phase being enriched in both polymers, and the second one is mainly the

solvent) or segregative (each phase being enriched with one of the two biopolymer) (Panouillé and Larreta-Garde, 2009; Tolstoguzov, 1995). Segregative phase separation, also called thermodynamic incompatibility, is mainly due to repulsion between the two biopolymers (Doublier *et al.*, 2000). Critical parameters are the limit of co-solubility of the biopolymers and their critical gelation concentration. When the concentrations of each polymer are higher than the critical concentration, mixed gels undergo independent three-dimensional networks (Tolstoguzov, 1995). Synergistic effect is generally related to excluded volume effects and water distribution between the two phases (Panouillé and Larreta-Garde, 2009).

1.2.4.3.2 Impact of polysaccharides on properties of gelatin

The addition of polysaccharides into fish gelatin could influence the properties of fish gelatin gel, especially texture and rheological properties.

- Textural properties

Texture is the sensory property of the structural, mechanical and surface properties of food (Yuan *et al.*, 2016). In general, texture of commercial gelatin gel is evaluated by measuring the "gel strength", which in the gelatin industry refers to non-fracture rigidity (Zhou and Regenstein, 2007). However, this parameter cannot represent all the texture properties encountered during human consumption (Zhou and Regenstein, 2007). Texture profile analysis (TPA) is the easiest and quantifiable analytical technique that has been widely used in the food industry (Yang *et al.*, 2013). Hardness, cohesiveness and springiness have been widely used to investigate the structural characteristics of gelatin gel. Lau *et al.* (2000) studied the effect of gellan/gelatin ratios and calcium ion levels on the textural properties of mixed gels. Hardness of mixed gel increased with increasing levels of gellan and ions. However, hardness decreased when ions were increased at concentration higher than 5 mM. Springiness and cohesiveness decreased when the calcium ions concentration was increased. Cohesiveness of gellan/gelatin mixed gels without ions was higher than that of gellan/gelatin mixed gel containing ions. Incorporation of gellan into gelatin showed a synergistic increase in gel strength, the increases in hardness and

springiness and improvement in gel firmness (Fonkwe *et al.*, 2003). Presence of cations could induce the gelation of gellan gum with the formation between the aggregated helices (Chandrasekaran *et al.*, 1988). Nevertheless, hardness decreased as the ions increased. This was associated with the increased repulsive forces in junction zones, leading to reducing the formation of linkages between aggregated helics (Tang *et al.*, 1996). Gelation behavior and texture of gelatin dessert from gelatin mixed with different polysaccharides (dextran, gellan and guar gum) were reported by Fonkwe *et al.* (2003). In general, gel strength of gelatin mixed with all polysaccharides was higher than that of gelatin gel without polysaccharides. Gelatin mixed with different polysaccharides showed the different gel strength. The highest gel strength was found in gelatin mixed with gellan at 0.0064% (w/v). Somboon *et al.* (2014) also studied the properties of mixed gels between fish gelatin and agar. It was found that gelatin/agar mixed gel showed the higher hardness, compared to gelatin gel alone, regardless of the total solid concentration. Derkach *et al.* (2015) studied the effect of κ-carrageenan as a co-gelator on the rheological properties of gelatin. Increasing carrageenan levels increased the gel strength of the mixed gel. The increased strength of gel was associated with the interaction between the positively charged of gelatin and negatively charged of carrageenan as confirmed by the shift of amide peak and sulphate peak in the FTIR spectra (Derkach *et al.*, 2015).

- Syneresis

Syneresis is the loss of water during ageing of gels, and has been used to indicate the instability of gel network (Stanley, 2006). This is an undesirable phenomenon which can be reduced by selection of appropriate hydrocolloids at suitable concentration (Banerjee and Bhattacharya, 2011). The different hydrocolloid mixed gel showed the different syneresis of gel samples. Syneresis of gel from gellan mixed with agar decreased as hydrocolloids concentration increased (Banerjee and Bhattacharya, 2011), indicating that a high concentration of hydrocolloids in mixed gel might reduce syneresis of food gels (Banerjee and Bhattacharya, 2011). Nevertheless, syneresis of gelatin gel was lower than that of gelatin /agar mixed gels (Somboon *et al.*, 2014).

- Turbidity

Turbidity is another quality attribute of gels, determining the preference of consumers. Haug *et al.* (2004b) reported that the turbidity of mixture containing fish gelatin and κ-carrageenan was influenced by the concentration of hydrocolloids, pH and ionic strength. The turbidity is most likely a result of phase separation of the system. The effects of gelatin ratio and calcium concentration on turbidity were reported by Lau *et al.* (2000). In general, turbidity of gel increased with increasing concentration of calcium. Increasing turbidity might be associated with the formation of light scattering aggregates in the gel network (Lau *et al.*, 2000). Moreover, the increase amount of gelatin in mixed gel increased the turbidity of gel, which was more likely related with phase separated polymers due to incompatibility between the protein and polysaccharides. Lee *et al.* (2003) also reported that turbidity of gel from gellan mixed gelatin was affected by NaCl concentration and the ratio of gellan to gelatin. Turbidity dramatically increased with increasing NaCl and gelatin concentration.

- Rheological properties

Rheological properties of gelatin determined by G^* (complex modulus), G' (storage modulus) and G'' (loss modulus) are correlated with gel strength, gelling and melting temperatures. Gel strength, gelling and melting temperatures are one of the most important functional properties of gelatin (Binsi *et al.*, 2009). The rheological behavior of mixed protein/hydrocolloids was affected by the polymer ratio and ions in mixed systems (Derkach *et al.*, 2015; Haug *et al.*, 2004b). Lee *et al.* (2003) studied the effect of gellan/gelatin and concentration of NaCl on the dynamic viscoelasticity of mixed solutions. In general, G' decreased with increasing gelatin proportion. However, the highest G' was found in solution at gellan/gelatin ratio of 60:40. The incorporation of gelatin to gellan exhibited a synergistic effect, which was related to the strong interaction between the two polymers in the mixed solutions. For all gellan/gelatin proportion, G' increased as NaCl concentration increased. Furthermore, mixed system of fish gelatin with κ-carrageenan or gellan gum has been reported by Pranoto *et al.* (2007). The melting temperature of fish gelatin gels

increased as the levels of κ-carrageenan or gellan gum increased. Derkach *et al.* (2015) studied the effect of carrageenan on rheological and gelation properties of carrageenan/gelatin gel. Gelatin mixed with carrageenan gel (modified gel) had higher *G′* than gels from gelatin or carrageenan alone. The addition of κ-carrageenan in mixed gel exhibited the synergetic effect ($G'_{gelatin\ +\ carrageenan} > G'_{gelatin} + G'_{carrageenan}$) which was associated with electrolyte complexes during structure formation. The incorporation of carrageenan strongly affected the thermo-stability of κ-carrageenan/gelatin mixed system. Haug *et al.* (2004b) studied the mechanical properties and the gelling kinetics of fish gelatin mixed with κ-carrageenan gel as monitored by rheological measurements. Gelling temperatures of mixed gelatin/carrageenan gel was higher than that of gel from fish gelatin or carrageenan alone. However, those gels showed no differences in melting temperatures. At 4 °C, fish gelatin gel had higher *G′* value than carrageenan gel. When fish gelatin was mixed with carrageenan, *G′* value was approximately 20% lower, compared to fish gelatin alone. Thus, hydrocolloids and their levels used directly affect the rheological property of gelatin.

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1.4 Objectives

1.4.1 To isolate and characterize acid soluble collagen from skin and swim bladder of seabass (*Lates calcarifer*).

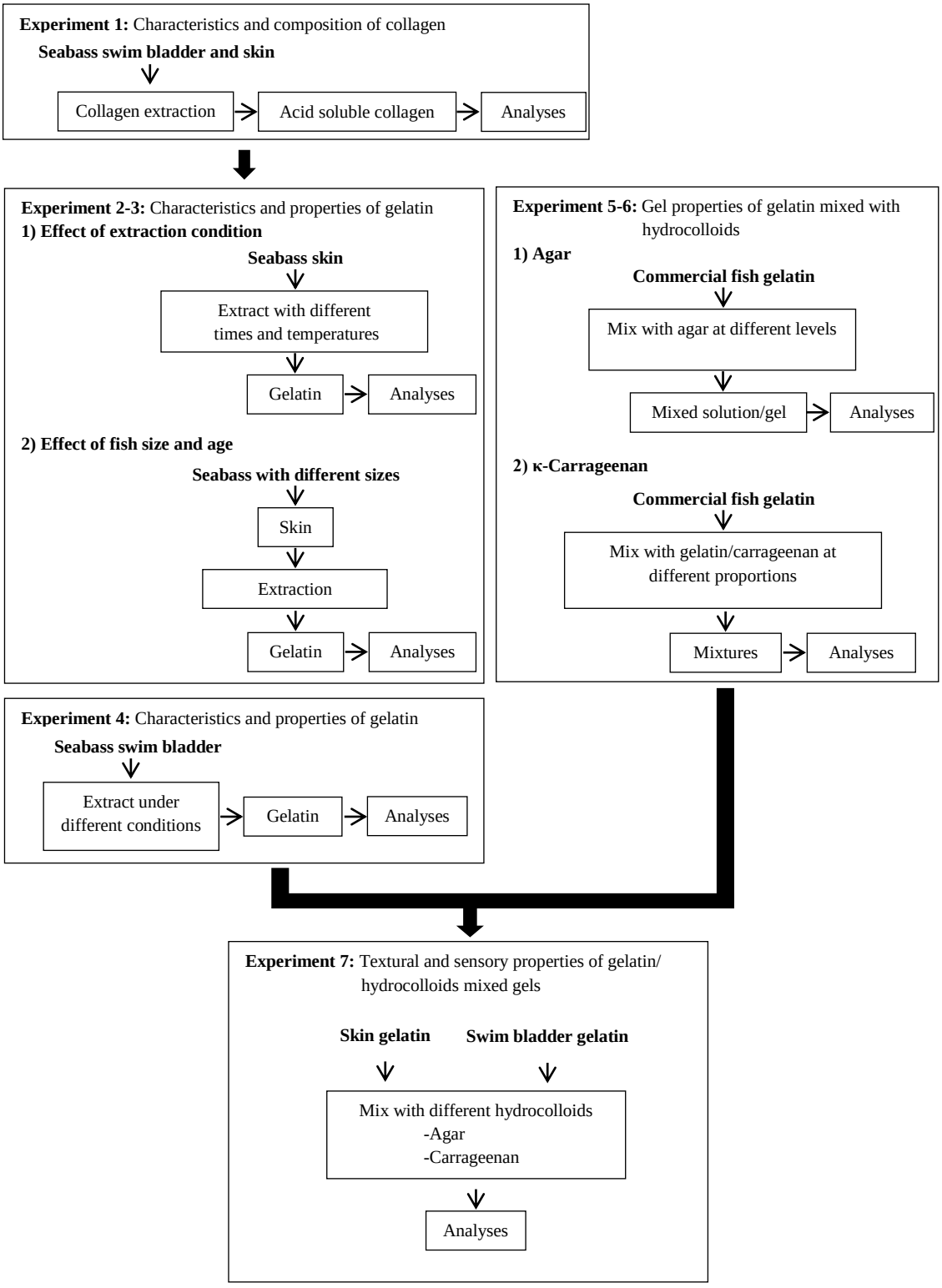
1.4.2 To investigate the impact of extracting conditions on characteristics and properties of gelatin from the skin and swim bladder of seabass.

1.4.3 To study physical and gelling properties of fish gelatin as influenced by agar.

1.4.4 To investigate the textural and rheological properties of fish gelatin as influenced by κ-carrageenan.

1.4.5 To comparatively examine the physical and sensory properties of gelatin from the skin and swim bladder of seabass mixed with different polysaccharides (agar and κ-carrageenan).

1.5 Flow chart of experiments



CHAPTER 2

EXTRACTION AND CHARACTERISTICS OF ACID SOLUBLE COLLAGENS FROM SKIN AND SWIM BLADDER OF SEABASS (*LATES CALCARIFER*)

2.1 Abstract

Acid soluble collagens (ASCs) from skin and swim bladder of seabass (*Lates calcarifer*) were isolated and comparatively characterised. Higher yield (28.5%) was obtained for ASC from swim bladder, compared with that from skin (15.8%). ASCs from both skin and swim bladder had the similar protein patterns and were identified to be type I. Both α- and β-chains constituted as major components with MW of 112-122 kDa and187 kDa. Fourier transform infrared (FTIR) spectra revealed that both ASCs were triple helix in structure. ASC from both sources contained glycine as the major amino acid with imino acids (proline and hydroxyproline) of 194–195 residues/1000 residues). Peptide maps of both ASCs digested by chymotrypsin and trypsin showed slight differences, suggesting some differences in their primary structure. The thermal transition temperature of swim bladder ASC (35.0 °C) was slightly higher than its skin counterpart (33.3 °C). Based on zeta potential analysis, ASCs from skin and swim bladder had a net charge of zero at pH 6.46 and 6.64, respectively. Therefore, both the skin and swim bladder of seabass could be used potentially for collagen extraction.

2.2 Introduction

Collagen is the major structural protein in the connective tissue of vertebrates and constitutes about 30% of total animal protein. Collagen is the fibrous protein, contributing to unique physiological functions of tissues in skins, tendons, bones, cartilages, etc. (Jongjareonrak *et al.,* 2005b). At present, at least 29 variants of collagen have been identified, and each differs considerably in amino acid sequence, structure and function, more likely associated with specific genetic variants (Liu *et al.,* 2012). Collagen has been widely used in food, cosmetic, biomedical and

pharmaceutical industries (Ogawa *et al.*, 2004). The original sources of collagen are bovine and porcine skin and bones. However, collagens obtained from porcine skin or bone are prohibited for some religious and ethnic groups, such as Jews and Muslims (Ahmad and Benjakul, 2010). Additionally, the outbreaks of bovine spongiform encephalopathy (BSE), transmissible spongiform encephalophathy (TSE) and foot-and-mouth disease (FMD) have brought the anxiety among consumers of collagen and collagen-derived products from these land-based animals (Jongjareonrak *et al.*, 2005a). As a consequence, the demand for collagen from alternative sources, particularly from aquatic origin has been increasing over the year. Fish skin, a by-product from fish processing plant, has been widely used for gelatin production, e.g. skins of bigeye snapper and brownstripe red snapper (Jongjareonrak *et al.*, 2006), unicorn leatherjacket (Ahmad and Benjakul, 2011) and Nile perch (Muyonga *et al.*, 2004b), etc.

Swim bladder, also called air bladder, is an internal gas-filled organ found in most fish. It has been widely used as raw material for making fish maw. The fish species used for fish maw production include mainly brown croaker (*Protonibea* diacanthus), seabass (*Lates calcarifer*), croaker (*Otolithes spp.*), giant catfish (*Arius thalassinus*), bigeye snapper (*Priacanthus tayenus*) and solder croaker (*Boesmania microlepis*) (Yellowdawn, 2011). Recently, pepsin-soluble collagens with the yields of 40 and 59% were extracted from swim bladder of catfish and bighead carp, respectively (Bama *et al.*, 2010; Liu *et al.*, 2012).

Seabass (*Lates calcarifer*) is an economically important species in the south of Thailand. Its skin has been used for making crispy skin for domestic consumption, whilst swim bladder has not been utilized. Both skin and swim bladder can be the potential sources for production of collagen, a high market value product. Nevertheless, no information regarding characteristics and properties of collagen from both skin and swim bladder of seabass has been reported.

2.3 Objective

To investigate the isolation and comparative characteristic of the acid soluble collagen (ASC) from the skin and swim bladder of seabass, cultured in Thailand.

2.4 Materials and methods

2.4.1 Chemicals

Folin-Ciocalteu's phenol reagent and *p*-dimethylamino-benzaldehyde were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS), Coomassie Blue R-250 and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). Acetic acid, hydrochloric acid, sodium hydroxide, sodium chloride, butanol, methanol, isopopanal and tris(hydroxymethyl) aminomethane were purchased from Lab-Scan (Bangkok, Thailand). Type I collagen from calf skin and trypsin from bovine pancreas (EC 3.4.21.4, 10,000 units/mg protein) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). α-Chymotrypsin from bovine pancreas (EC 3.4.21.1, 1,000 USP chymotrypsin units/mg) was procured from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). High-molecular-weight markers were purchased from GE Healthcare UK Limited (Buckinghamshire, UK).

2.4.2 Collection and prpeparation of skin and swim bladder from seabass

Seabass (*Lates calcarifer*) with average weight of 6-8 kg were purchased from a farm in Koyo Island, Songkhla, Thailand. The fish were kept in ice with a fish/ice ratio 1:3 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h after capture. Fish were washed using cold tap water. Skins were then removed, descaled, and cut into small pieces (0.5 x 0.5 cm^2). Swim bladders were also removed, washed with cold tap water and cut into small pieces (0.5 x 0.5 cm^2) using a scissor. The average thickness of skin and swim bladder was 0.5 and 0.1 mm, respectively. The skin and swim bladder were placed in polyethylene bags and stored at -20 °C until used, but not longer than 3 months. Before the use for collagen extraction, the frozen materials were thawed with running water until the core temperature reaches 8-10 °C. Then both tissues were cut into small pieces (0.5 x 0.5 cm^2) using a scissor.

2.4.3 Extraction of acid soluble collagens (ASCs) from skin and swim bladder of seabass

ASCs were extracted from the prepared skin and swim bladder of seabass following the method of Kittiphattanabawon *et al.* (2005) and Nalinanon *et al.* (2007) with a slight modification. Prior to extraction, the frozen skin and swim bladder were thawed using running water for 30 min. The extraction process of collagen from both tissues started from non-collagenous protein removal using alkaline solution, followed by defatting using butanol. Thereafter, the pretreated tissues were subjected to extraction using acetic acid. The soluble collagens were precipitated by "salting-out", dialysed and freeze-dried. All extraction procedures were carried out at 4 °C.

2.4.3.1 Pretreatment of skin and swim bladder

To remove non-collagenous proteins, the thawed skin and swim bladder were mixed with 0.1 M NaOH at a solid to alkali solution ratio of 1:10 (w/v). The mixture was continuously stirred for 6 h. The alkali solution was changed every 2 h. Thereafter, the residues were washed with cold water until a neutral or weak basic pH of wash water was obtained.

2.4.3.2 Extraction of ASCs

The pretreated skin and swim bladder were soaked in 0.5 M acetic acid with a sample to solvent ratio of 1:50 (w/v) for 48 h with continuous stirring using an overhead stirrer model W20.n (IKA®-Werke GmbH & CO.KG, Staufen, Germany). The mixtures were filtered with two layers of cheesecloth. The collagen in the supernatant was precipitated by adding NaCl to a final concentration of 2.6 M in the presence of 0.05 M tris(hydroxymethyl) aminomethane at pH 7.5. The resultant precipitate was collected by centrifugation at 20,000 g for 60 min using a refrigerated centrifuge model Avanti® J-E (Beckman Coulter, Inc., Palo Alto, CA, USA). The pellet was dissolved in a minimum volume of 0.5 M acetic acid, dialysed against 10 volumes of 0.1 M acetic acid for 12 h. Thereafter, it was dialysed against 10 volumes

of distilled water with changes of water for five times. The resulting dialysate was freeze-dried and referred to as ‘‘acid soluble collagen, ASC”. ASCs from both skin and swim bladder were subjected to analyses.

2.4.4 Analyses

2.4.4.1 Yield

The yield of ASC was calculated based on dry weight of starting material.

$$\text{Yield (\%)} = \frac{\text{Weight of freeze-dried collagen}}{\text{Weight of initial dry skin or swim bladder}} \times 100$$

2.4.4.2 Amino acid analysis

Amino acid composition of ASC samples was analysed using an amino acid analyser. The samples were hydrolysed under reduced pressure in 4 M methanesulphonic acid containing 0.2% (v/v) 3-2(2-aminoethyl) indole at 115 °C 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).

2.4.4.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS–PAGE was performed by the method of Laemmli (1970). ASC samples were dissolved in 5% SDS solution. The mixtures were then heated at 85 °C for 1 h using a temperature controlled water bath model W350 (Memmert, Schwabach, Germany), followed by centrifugation at 8500 g for 5 min using a microcentrifuge (MIKRO20, Hettich Zentrifugan, Germany) to remove undissolved debris. Solubilised samples were mixed at 1:1 (v/v) ratio with sample buffer (0.5 M Tris–HCl, pH 6.8 containing 5% SDS and 20% glycerol in the presence or absence of 10% (v/v) βME). 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 20 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 were used to estimate the molecular weight of proteins. Type I from calf skin was used as standard.

2.4.4.4 Peptide mapping

ASC samples (5 mg) were suspended in 0.5 ml of 50 mM Tris-HCl, pH 8.0, containing 10 mM $CaCl_2$. The mixture was then incubated at 50 °C for 30 min. To initiate the digestion, 50 µl of chymotrypsin solution (0.1 µg/µl) were added to the mixture and the reaction was performed for 5 min. The reaction was terminated by adding 650 µl of 5% (w/v) SDS (85 °C) to the reaction mixture and boiling 10 min. Peptides generated by the chymotrypsin digestion were separated by SDS–PAGE using 7.5% separating gel and 4% stacking gel.

For peptide mapping with trypsin, the samples (5 mg) were suspended in 0.5 ml of 10 mM Tris-HCl, pH 8.0, containing 1 mM $CaCl_2$. The mixtures were preheated at 30 °C for 1 h. To initiate the digestion, 100 µl of trypsin solution (0.1µg/µl) was added to the mixture and incubated at 30 °C for 45 min. The reaction was terminated and peptide map was determined as previously described.

2.4.4.5 Fourier transform infrared (FTIR) spectroscopic analysis

FTIR spectra of both ASCs were obtained by using a FTIR spectrometer (EQUINOX 55, Bruker, Ettlingen, Germany) equipped with a deuterated l-alanine tri-glycine sulphate (DLATGS) detector. The horizontal attenuated total reflectance accessory (HATR) was mounted into the sample compartment. The internal reflection crystal (Pike Technologies, Madison, WI, USA), made of zinc selenide, had a 45° angle of incidence to the IR beam. Spectra were acquired at a resolution of 4 cm^{-1} and the measurement range was 4000–650 cm^{-1} (mid-IR region) at room temperature. Automatic signals were collected in 32 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, Ettlingen, Germany).

2.4.4.6 Differential scanning calorimetry (DSC)

ASC samples were rehydrated by adding deionised water at a solid to solution ratio of 1:40 (w/v) (Rochdi *et al.*, 2000). The mixtures were allowed to stand for 2 days at 4 °C prior to analysis. Differential scanning calorimetry (DSC) was performed using a differential scanning calorimeter model DSC 7 (Perkin Elmer, Norwalk, CT, USA). Calibration was run using Indium thermogram. The samples (5–10 mg) were accurately weighed into aluminium pans and sealed. The samples were scanned at 1 °C/min over the range of 20–50 °C using iced water as the cooling medium. An empty pan was used as the reference. The maximum transition temperature (T_{max}) was estimated from the thermogram. Total denaturation enthalpy (ΔH) was estimated by measuring the area of DSC thermogram.

2.4.4.7 ζ- potential analysis

ASC samples were dissolved in 0.5 M acetic acid to obtain a final concentration of 0.05% (w/v). The mixtures were continuously stirred at 4 °C using a magnetic stirrer model BIG SQUID (IKA®-Werke GmbH & CO.KG, Staufen, Germany) until the samples were completely solubilised. Zeta (ζ) potential of ASC solutions was measured by Zeta potential analyzer, model ZetaPALs (Brookhaven Instruments Co., Holtsville, NY, USA). Solutions (20 ml) were transferred to autotitrator model BI-ZTU (Brookhaven Instruments Co., Holtsville, NY, USA), in which the pH of solutions was adjusted to 2–11 using either 1.0 M nitric acid or 1.0 M KOH. The zeta potential of solution at all pHs determined was recorded. The isoelectric point (pI) was estimated from pH rendering zero zeta-potential.

2.4.5 Statistical analysis

The experiments were carried out in triplicate using three different lots of samples. The data were presented as means ± standard deviation.

2.5 Results and discussion

2.5.1 Extraction yield

ASCs were isolated from skin and swim bladder of seabass with yields of 15.8 and 28.5% (wet weight basis), corresponding to 58.1 and 85.3% (dry weight basic), respectively. The lower yield of ASC from skin might be attributed to the higher covalent cross linking at the telopeptide region of collagen molecules through the condensation of aldehyde groups, as well as intermolecular cross-linkage of molecules in the skin (Jongjareonrak *et al.*, 2005a). The yields of ASCs from skin of young and adult of Nile perch (*Lates niloticus*) were 63.1 and 58.7% (on dry weight basis), respectively. Stronger skin matrix is required to maintain a structure and to protect body from environments they reside, whilst swim bladder containing less complexed structure with lower collagen cross-links is located inside (Le Guellec *et al.*, 2004). Long *et al.* (1996) reported that the structure of skin has a complex and strong fibrous connective tissue and the thickness of skin varies between species and with age, time of year and anatomical location. In the present study, collagen could be extracted with ease from the swim bladder when acetic acid was used as an extraction medium. Fish collagen is soluble in acidic solution at pH lower than 5.0 (Benjakul *et al.*, 2010). It was noted that ASC from seabass swim bladder was higher than pepsin soluble collagen (PSC) from bighead carp (59.0%, dry weight basis) (Liu *et al.*, 2012). The result suggested that swim bladder of seabass might have less cross-linked collagen than that from bighead carp. Pepsin has been used for increasing the yield of collagen since it can specifically cleave peptides at telopeptide region, thereby facilitating the extraction of collagen from fibrils or skin matrix (Nalinanon *et al.*, 2007).

2.5.2 Amino acid composition

Amino acid composition of ASCs from seabass skin and swim bladder, expressed as residues per 1000 total residues, is shown in Table 8. Both ASCs showed similar amino acid compositions, in which glycine (326-331 residues/1000 residues), constituted as the major amino acid, followed by alanine (134 residues/1000

residues). Low contents of hydroxylysine (5-8 residues/1000 residues), histidine (5-6 residues/1000 residues), tyrosine (4-5 residues/1000 residues) and cysteine (1 residues/1000 residues) were found in both ASCs. Generally, glycine in collagen represents nearly one third of the total residues and occurs as every third residue in collagen except for the first 14 amino acid residues from the N-terminus and the first 10 residues from the C-terminus (Jongjareonrak *et al.*, 2005a; Kittiphattanabawon *et al.*, 2010b). The imino acid content (proline + hydroxyproline) of ASC from skin and swim bladder were 195 and 194 residues /1000 residues, respectively. The imino acid contents of ASCs from skin and swim bladder of seabass were slightly lower than those of calf skin collagen (215 residues/1000 residues) (Herbage *et al.*, 1977) and pig skin collagen (about 220 residues/ 1000 residues) (Zhang *et al.*, 2007), but were much higher than those of PSC from skin (165 residues/1000 residues) and swim bladder (175 residues/1000 residues) of bighead carp (Liu *et al.*, 2012). Differences in the living environments and habitat temperature amongst animals were associated with varying imino acid contents (Rigby, 1968). In addition, the imino acid content has been known to determine thermal stability of collagen and the formation of junction zones via hydrogen bonding. Hydroxyproline plays a role in stabilising the triple helical structure by the formation of interchain hydrogen bond through the hydroxyl group (Kittiphattanabawon *et al.*, 2005). The results suggested that ASC from the same species more likely contained similar amino acid composition, regardless of tissues used.

2.5.3 Protein patterns

Protein patterns of ASC from skin and swim bladder of seabass determined under non-reducing and reducing conditions are illustrated in Figure 6. No differences in protein patterns between both ASCs determined under both conditions were observed. Thus, both ASCs from skin and swim bladder had similar protein components and contained no disulphide bond (Kittiphattanabawon *et al.*, 2010b). The result was in accordance with the negligible cysteine content in both ASCs (Table 8). Collagen from other fish skin including carp (Duan *et al.*, 2009), largefin longbarbel catfish (Zhang *et al.*, 2009), unicorn leatherjacket (Ahmad and Benjakul, 2010) and striped catfish (Singh *et al.*, 2011) had no disulphide bond. ASCs from both

Table 8. Amino acid compositions of ASC from skin (SK) and swim bladder (SW) of seabass (residues/1000 residues).

Amino acids	**SK**	**SW**
Alanine	134	134
Arginine	53	53
Aspartic acid/asparagine	46	46
Cysteine	1	1
Glutamine/glutamic acid	70	71
Glycine	331	326
Histidine	6	5
Isoleucine	10	9
Leucine	20	23
Lysine	27	25
Hydroxylysine	5	8
Methionine	14	14
Phenylalanine	13	13
Hydroxyproline	79	83
Proline	117	111
Serine	28	27
Threonine	22	24
Tyrosine	4	5
Valine	21	22
Total	1000	1000
Imino acid	195	194

skin and swim bladder comprised α-chains and β-chains (dimers), as the dominants constituents with MW of 112-122 and 187 kDa, respectively. Both ASCs contained α_1-chains and α_2-chain at a ratio of approximately 2:1, suggesting that they belonged to type I collagen. In addition, high MW components, including γ-chains as well as the cross-linked proteins were also observed in both ASCs from skin and swim bladder. The result indicated that covalent cross-links, both inter- and intra-crosslink, could be extracted from both skin and swim bladder with the aid of acetic acid. Due to

the similarity with collagen type I, collagen from both tissues can be used in foods, especially as the supplement in health drink. Additionally, it can be used for production of collagen hydrolysate with bioactivities, which can be further used as the functional food (Cúneo *et al.*, 2010; Ding *et al.*, 2011).

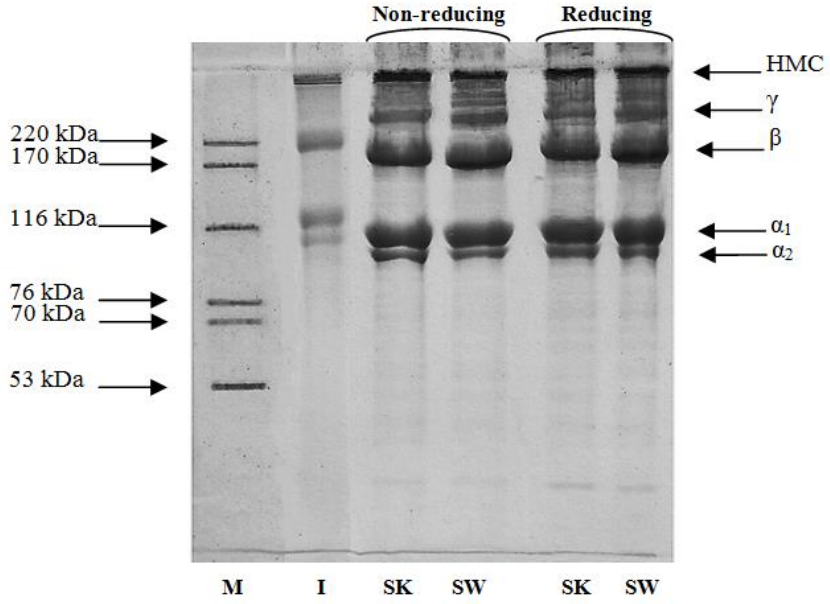


Figure 6. SDS–PAGE patterns of ASC from skin (SK) and swim bladder (SW) of seabass under non-reducing and reducing conditions. M, I and HMC denote high molecular-weight protein markers, type I collagen from calf skin and high MW cross-linked components, respectively.

2.5.4 Peptide mapping

Peptide maps of ASCs from skin and swim bladder of seabass digested by chymotrypsin and trypsin are shown in Figure 7. After the digestion by chymotrypsin, band intensity of α_1 and α_2-chains as well as high MW cross-link, γ- and β-components of both ASCs, entirely disappeared. Additionally, the generated peptides from both ASCs had MWs below 157 kDa and the peptide with MW of 97 kDa was predominant. When comparing peptide map between ASC from skin and swim bladder, some differences were noticeable. Peptide fragments with MW of 89 and 58 kDa were found in ASC from skin, whilst those bands were negligible in ASC from swim bladder. The chymotrypsin shows a high specific hydrolysis of peptide

bonds on the carboxyl side of hydrophobic amino acid residues, such as phenylalanine, tyrosine, tryptophan and leucine (Simpson, 2000). Thus, the slight difference in peptide map between ASC from two sources indicated the slight difference in their primary structures, especially in terms of sequence and composition of amino acids.

For peptide maps of both ASCs digested by trypsin, band intensity of α- and β- and γ-chains decreased to some degrees. The peptide maps between both ASCs digested by trypsin showed some slight differences, particularly for peptide having MW of 55 kDa which was noticeable in skin ASC sample. It was noted that peptide with MW of 27 kDa was found only in swim bladder ASC. Trypsin has been known to cleave peptides on the carboxyl group side of lysine and arginine residues (Simpson, 2000). Due to the low contents of lysine (25-27 residues/1000 residues) and arginine residues (52-53 residues/ 1000 residues), both ASCs were not preferably cleaved by trypsin as evidenced by more resistance to hydrolysis of both ASCs. Thus, ASC skin and swim bladder might have the slight differences in their sequence and composition of amino acids.

2.5.5 Fourier transform infrared (FTIR) spectra

FTIR spectra in the range of 4000-650 cm^{-1} of ASCs from skin and swim bladder of seabass are presented in Figure 8. The amide A band of both ASCs was found at the wavenumber of 3292 cm^{-1}. This band is related to a free N-H stretching vibration and shows the existence of hydrogen bonds, which commonly occurs in the range of 3400-3440 cm^{-1} (Doyle *et al.*, 1975). However, when the NH group of a peptide is involved in hydrogen bond, the position is shifted to lower wavenumber. Amide B band of both ASCs was observed at the wavenumber of 2925 and 2921 cm^{-1}, associated with asymmetrical stretch of CH_2 (Muyonga *et al.*, 2004a). This result was in accordance with those of collagen from other fish skin (Kittiphattanabawon *et al.*, 2010a; Liu *et al.*, 2012).

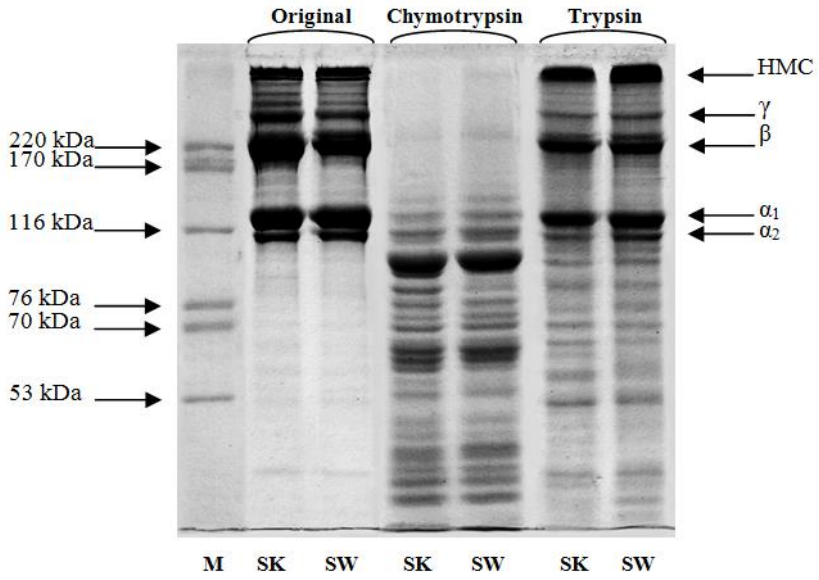


Figure 7. Peptide maps of ASC from skin (SK) and swim bladder (SW) of seabass digested by chymotrypsin and trypsin. M and HMC denote high molecular-weight protein markers and high MW cross-linked components, respectively.

Amide I bands of ASC from skin and swim bladder were found at wavenumbers of 1631 and 1632 cm^{-1}, respectively. Payne and Veis (1988) reported that amide I band with the characteristic wavenumber in the range of 1600-1700 cm^{-1} was mainly associated with backbone C=O stretching vibration or hydrogen bond coupled with COO^-. The decrease in molecular order was reflected by a shift of amide I peak to lower frequency. Additionally, both ASCs exhibited the amide II band at wavenumbers of 1532 – 1533 cm^{-1}, which generally appeared at 1550-1600 cm^{-1} and resulted from N-H bending vibration coupled with a C-N stretching vibration (Krimm and Bandekar, 1986). A shift of amide II peak to lower wavenumber is associated with existence of hydrogen bonds in each collagen (Duan *et al.*, 2009). Furthermore, the amide III bands were observed at wavenumbers of 1233-1234 cm^{-1}, more likely associated with N–H deformation and C–N stretching vibration (Muyonga *et al.*, 2004a). The ratio of peak amplitude between amide III and 1454 cm^{-1} band was 0.98 and 1.00 for skin and swim bladder ASCs, respectively. The absorption ratio of approximately 1.0 indicates the presence of triple helical structure of both ASCs

(Benjakul *et al.*, 2010; Heu *et al.*, 2010). Therefore, both ASCs from skin and swim bladder of seabass showed a slight difference in the secondary structure, but they remained in triple-helix.

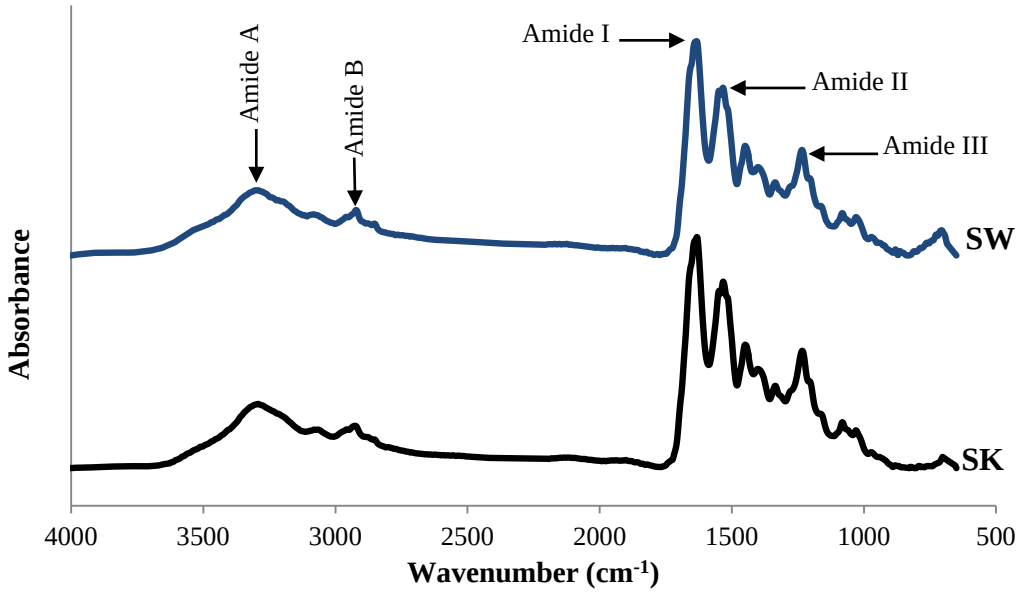


Figure 8. FTIR spectra of ASC from skin (SK) and swim bladder (SW) of seabass

2.5.6 Thermal transition

The maximum transition temperature (T_{max}) and enthalpy (ΔH) of ASCs from skin and swim bladder in deionized water are shown in Figure 9. T_{max} values of ASCs from skin and swim bladder were observed at 33.3 and 35.0 °C, respectively, in which the latter was higher. ΔH of ASC from swim bladder (0.918 J/g) was slightly higher than that of ASC from the skin (0.860 J/g). The higher T_{max} and ΔH indicated higher thermal stability. The T_{max} values of ASC from skin of Nile perch was 36 °C (Muyonga *et al.*, 2004a). The result suggested that ASC from swim bladder exhibited higher thermal stability than skin counterpart, Liu *et al.* (2012) also reported that collagen from the internal tissues (swim bladder and bone) of bighead carp was slightly higher than that of collagen from the external tissues (fin, scale and skin). T_{max} values of ASC from both skin and swim bladder were slightly lower than those of mammalian collagen such as porcine skin collagen (37 °C) (Nagai *et al.*, 2008) and calf skin collagen (40.8 °C) (Duan *et al.*, 2009). Nevertheless, T_{max} of both

ASCs were higher than those of collagens from the skins of dusky spinefoot (28.7 °C) (Bae *et al.*, 2008), Japanese seabass (26.5 °C), cub mackerel (25.6 °C), bullhead shark (25.0 °C) (Takeshi and Suzuki, 2000) and arabesque greenling (15.4 °C) (Nalinanon *et al.*, 2010). Higher denaturation temperature for collagen of warm water fish species, compared with collagen from cold water fish species, was reported by Muyonga *et al.* (2004a). The difference in T_{max} amongst collagen from different species correlated with the different imino acid contents (proline and hydroxyproline) (Kittiphattanabawon *et al.*, 2005). The higher imino acid content is associated with increasing thermal denaturation of collagens, governed by the pyrrolidine rings of proline and hydroxyproline and hydrogen bonding through the hydroxyl group of hydroxyproline (Benjakul *et al.*, 2010). However, T_{max} value might be also determined by the conformation and amino acid sequence of collagen. The differences in T_{max} and ΔH between both ASCs were coincidental with the slight difference in amino acid compositions (Table 8) and peptide map (Figure 7). Thus, thermal properties of ASC were affected by the tissues used for collagen extraction.

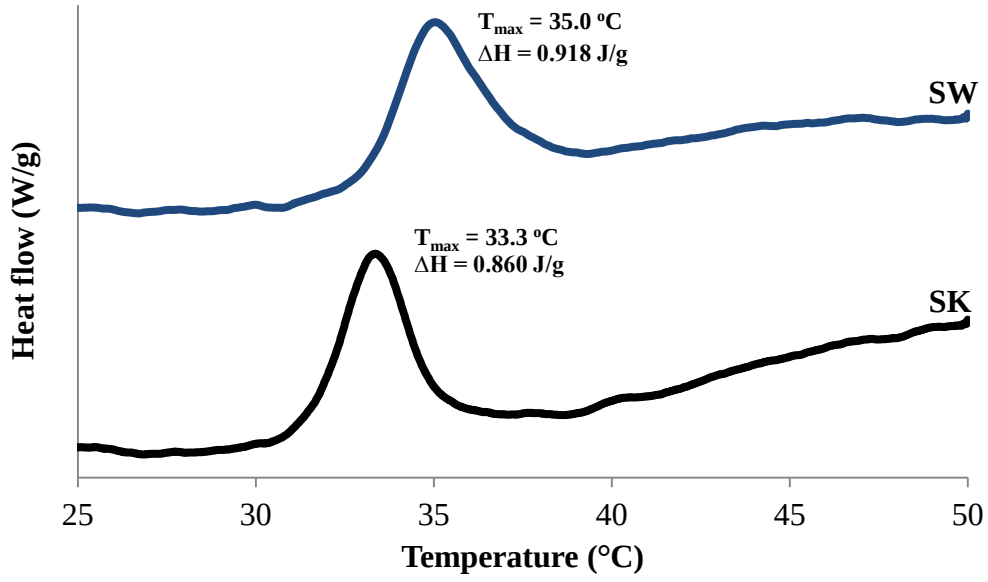


Figure 9. DSC thermogram of ASC from skin (SK) and swim bladder (SW) of seabass

2.5.7 ζ- potential

ζ- potential of ASCs from skin and swim bladder of seabass at different pHs is shown in Figure 10. When pHs increased from 2 to 6, the positive surface charge of both ASCs continuously decreased. Zero surface net charge of skin and swim bladder ASCs was observed at pHs of 6.46 and 6.64, respectively, which were estimated to be their isoelectric points (pI). When the positive charges are balanced out by the negative charges, the proteins show the least repulsion between molecules (Kittiphattanabawon *et al.*, 2010a). At pHs above their pIs, both ASCs had a negative charge and became more negatively charged as pH was far from pI. Collagens from skin of different fish showed varying pIs, e.g. 4.72 for striped catfish (Singh *et al.*, 2011), 6.40 for ornate threadfin bream (Nalinanon *et al.*, 2011) and 6.21 for bamboo shark (Kittiphattanabawon *et al.*, 2010a). Additionally, the slight differences in pI between collagens might be caused by the slight difference in their amino acid compositions and distribution of amino acid residues, particularly on the surface domains.

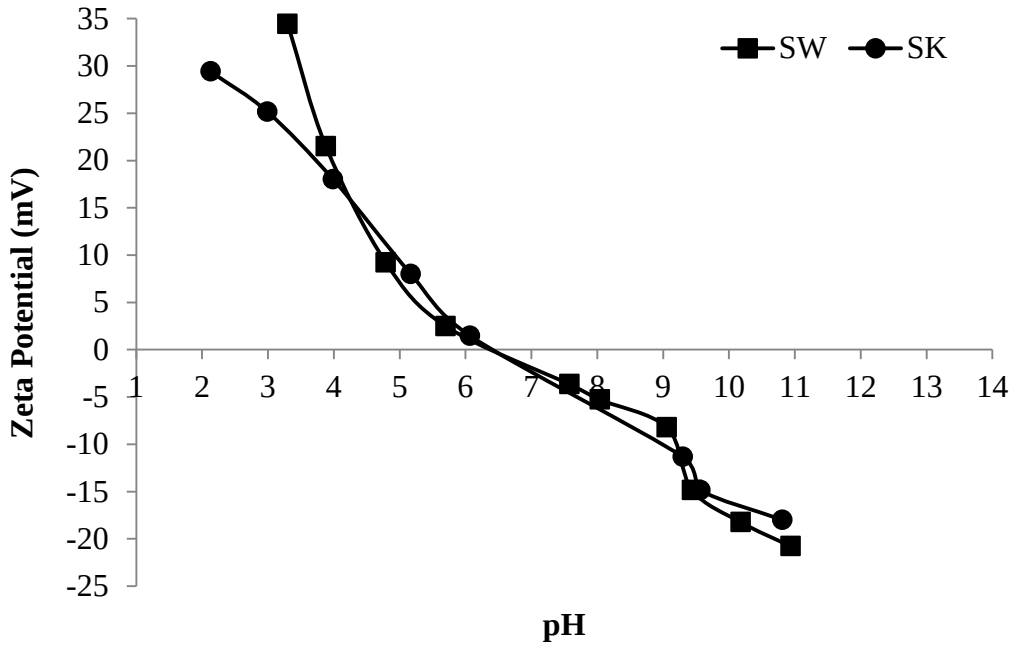


Figure 10. ζ- potential of ASC from skin (SK) and swim bladder (SW) of seabass

2.6 Conclusion

ASC from different tissues of seabass, skin and swim bladder, could be extracted. Swim bladder rendered the higher yield, compared with skin. Both ASCs were type I collagens with similar amino acid compositions. Both ASCs were in triple-helix structure and showed slight differences in thermal stability. T_{max} values of ASC from skin and swim bladder were 33.3 and 35.0 °C, respectively. ASC from skin and swim bladder had pI of 6.46 and 6.64, respectively. Thus, collagenous sources could be a factor determining properties of ASC.

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CHAPTER 3

CHARACTERISTICS AND FUNCTIONAL PROPERTIES OF GELATIN FROM SKIN OF SEABASS (*LATES CALCARIFER)*

3.1 Abstract

Characteristics and gel properties of gelatin from seabass skin, as influenced by extraction conditions (Temperature: 45, 55, 65 and 75 °C; Time: 3, 6, 9 and 12 h), were studied. The yield of gelatin increased when the extraction temperature and time increased (P<0.05). Yields of gelatin extracted at 45 and 55 °C for various times were 51.6-57.3% and 62.0-66.4% (dry weight basis), respectively. All gelatins contained β-chain and α–chains as the predominant components and showed a high imino acid content (198-202 residues/1000 residues). Fourier transform infrared (FTIR) spectra of obtained gelatins indicated a loss of molecular order of triple helix. Generally, gel strength of gelatins decreased as extraction temperature and time increased. At the same extraction time, gel strength of gelatin extracted at 45 °C (303.4-369.2 g) was higher than that of gelatin extracted at 55 °C (282.0-322.6 g) (P<0.05). Gel strength of all gelatins from the seabass skin was higher than that of commercial bovine gelatin (208.0 g) (P<0.05). Gelatin from the seabass skin could be set at 25 °C within 30 min, however gelatins extracted at 45 °C had the shorter setting time than those extracted at 55 °C (P<0.05). Gels of gelatin extracted at higher temperature with longer time had the lower L^*- and a^*- values but higher b^*- and ΔE^*- value, compared with those extracted at lower temperature with shorter time. Gelling and melting temperatures were 19.5-20.0 °C and 26.3-27.0 °C, respectively. All gelatins could be set at 25 ºC within 30 min, however gelatins extracted at 45 ºC had a shorter setting time than those extracted at 55 ºC. Gelatin from seabass skin showed higher gel strength than bovine gelatin and could be used as a potential replacement for land animal gelatins.

3.2 Introduction

Seabass (*Lates calcarifer*) is one of economically important species of Thailand and other countries in the Southeast Asia. It has been widely used for fillet production, in which a large amount of byproducts e.g. skin, bone, scales, etc. is generated. Fish processing byproducts can account 75% of the total catch weight. About 30% of byproducts consist of skin and bone, which can serve as the source of collagen and gelatin. The utilisation of fish skin and bone for gelatin production can add value to processing byproducts and to eliminate harmful environmental aspects (Binsi *et al.*, 2009; Gómez-Guillén *et al.*, 2002).

Gelatin is a fibrous protein produced by thermal denaturation or partial degradation of collagen from animal skin and bone. It has been widely used in food, material, pharmacy and photography industries (Benjakul *et al.*, 2009; Tabarestani *et al.*, 2010). Generally, the typical sources of gelatin are skins and bones of pig and cow. However, outbreaks of mad cow disease (bovine spongiform encephalopathy, BSE) have caused an anxiety for customers. In addition, porcine gelatin cannot be used for halal and kosher food markets (Kittiphattanabawon *et al.*, 2010). Therefore, an increasing interest in production of fish gelatin from fish skins and bones as alternative sources has been gained (Jongjareonrak *et al.*, 2006).

In general, gelatins from the skins of cold-water fish have the limited applications, mainly due to the lower gel strength and lower stability of gels, compared with mammalian counterparts. Additionally, fish gelatin has the lower gelling and melting temperatures than mammalian gelatin (Kittiphattanabawon *et al.*, 2010). This is governed by the lower imino acid (proline and hydroxyproline) content (Kittiphattanabawon *et al.*, 2010). Skin gelatins from several fish species, e.g. yellowfin tuna (Cho *et al.*, 2005), grass carp (Kasankala *et al.*, 2007), brownbanded bamboo shark and blacktip shark (Kittiphattanabawon *et al.*, 2010), splendid squid (Nagarajan *et al.*, 2012) and unicorn leatherjacket (Kaewruang *et al.*, 2013) have been produced.

Seabass skin has been used for production of snack, etc. to increase its market value. Recently, collagen from seabass skin was extracted and identified as type I collagen (Sinthusamran *et al.*, 2013). To widen its utility, the production of

gelatin can be another approach, which can increase the revenue for processor on farmer. Seabass is a warm-water species, and its gelatin might have the better gelling property than gelatin from cold water fish. However, extraction condition, including temperature, time and the protease inhibitors have been known to affect the properties of gelatin from skin of some fish species (Kaewruang *et al.*, 2013; Nagarajan *et al.*, 2013). Nevertheless, no information regarding gelatin extraction from seabass skin under varying conditions has been reported.

3.3 Objective

To study the extraction conditions including extraction temperatures and times on characteristics and functional properties of gelatin from the skin of seabass.

3.4 Materials and methods

3.4.1 Chemicals

All chemical were of analytical grade. 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). Food grade bovine bone gelatin with the bloom strength of 150-250 g was obtained from Halagel (Thailand) Co., Ltd. (Bangkok, Thailand). Fish gelatin produced from tilapia skin (~240 bloom) was obtained from Lapi Gelatine S.p.A (Empoli, Italy).

3.4.2 Collection and preparation of skins from seabass

Descaled skin of seabass (*Lates calcarifer*) was obtained from the Kingfisher Holdings Co., Ltd., Songkhla, Thailand. Descaled skin was kept in ice with a skin/ice ratio 1:3 (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 and the skin was washed using cold tap water. The skin was placed in polyethylene bags and stored at -20 °C until used, but not longer than 3 months. Prior to gelatin extraction, the frozen skin was thawed with

running water until the core temperature reached 8-10 °C. Then skin was cut into small pieces (1.0 x 1.0 cm^2) using a scissor.

3.4.3 Extraction of gelatin from the skin of seabass

Gelatin was extracted from seabass skin according to the method of Jongjareonrak *et al.* (2006) with a slight modification as described in the following scheme (Figure 11).

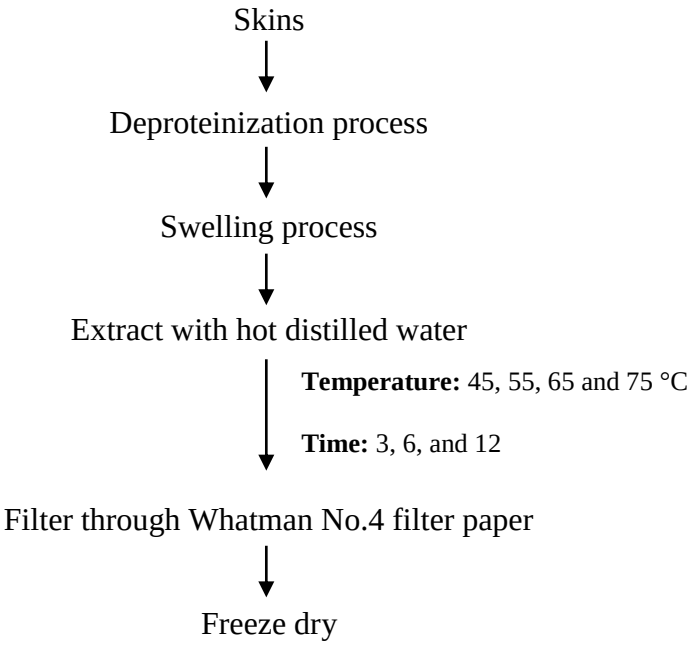

Figure 11. Scheme for extraction of gelatin from skin

3.4.3.1 Preatreatment of skin

Before gelatin extraction, skin was soaked in 0.1 M NaOH with a skin/solution ratio of 1:10 (w/v) to remove non-collagenous proteins. The mixture was stirred for 3 h at room temperature (28-30 °C) using an overhead stirrer model W20.n (IKA®-Werke GmbH & CO.KG, Staufen, Germany). The alkaline solution was changed every 1 h for totally 3 times. The residues were washed with tap water until neutral or faintly basic pH was obtained. Then, the residues were mixed with 0.05 M acetic acid at a skin/solution ratio of 1:10 (w/v) to swollen the collagenous material in fish skin matrix. The mixture was stirred at room temperature for 2 h. The skin was washed using tap water until neutral or faintly acidic pH of wash water was obtained.

3.4.3.2 Extraction of gelatin

The swollen skins were extracted with distilled water at a ratio of 1:10 (w/v) at different temperatures (45, 55, 65 or 75 °C) for 12 h. The mixtures were filtered using a Buchner funnel with Whatman No.4 filter paper (Schleicher & Schuell, Maidstone, England). Then, the filtrates were freeze-dried using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). Gelatin samples were subsequently subjected to analyses for Yield, protein pattern and gel strength.

The extraction temperature, which gives the gelatin with high yield and/or gel strength, was selected for further step. The swollen skin was mixed with distilled water at a ratio of 1:10 (w/v) at the selected temperatures. The extraction was performed for various times (3, 6, and 12 h) with continuous stirring. At the designated time, the mixtures were filtered and freeze-dried as previously described in the method. Gelatin samples were subsequently subjected to analyses.

3.4.4 Analyses

3.4.4.1 Yield

The yield of gelatin was calculated based on dry weight of starting material.

$$\text{Yield (\%)} = \frac{\text{Weight of freeze-dried gelatin (g)}}{\text{Weight of initial dry skin (g)}} \times 100$$

3.4.4.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS–PAGE was performed by the method of Laemmli (1970). Gelatin samples were dissolved in 5% SDS solution. The mixtures were then heated at 85 °C 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 20 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 were used to estimate the molecular weight of proteins.

3.4.4.3 Free amino group content

Free amino group content was determined following the method of Benjakul and Morrissey (1997). Properly diluted samples (125 µl) were mixed thoroughly with 2.0 mL of 0.2 M phosphate buffer, pH 8.2, followed by the addition of 1.0 mL of 0.01% 2,4,6- trinitrobenzenesulfonic acid (TNBS) solution. The mixtures were then placed in a temperature-controlled 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 using a double beam spectrophotometer and the free amino group content was expressed in terms of *L*-leucine.

3.4.4.4 Amino acid analysis

Amino acid composition of gelatin samples was analysed using an amino acid analyser. Gelatin samples were hydrolysed under reduced pressure in 4 M methane sulphonic acid containing 0.2% 3-2(2-aminoethyl)indole at 115 °C 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).

3.4.4.5 Fourier transform infrared (FTIR) spectroscopic analysis

FTIR spectra of gelatin samples were obtained by using a FTIR spectrometer (EQUINOX 55, Bruker, Ettlingen, Germany) equipped with a deuterated l-alanine tri-glycine sulphate (DLATGS) detector. The horizontal attenuated total reflectance accessory (HATR) was mounted into the sample compartment. The

internal reflection crystal (Pike Technologies, Madison, WI, USA), made of zinc selenide, had a 45° angle of incidence to the IR beam. Spectra were acquired at a resolution of 4 cm^{-1} and the measurement range was 4000–650 cm^{-1} (mid-IR region) at room temperature. Automatic signals were collected in 32 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, Ettlingen, Germany).

3.4.4.6 Determination of gel strength

Gelatin gel was prepared by the method of Kittiphattanabawon *et al.* (2010). Gelatin was dissolved in distilled water (60 °C) to obtain a final concentration of 6.67% (w/v). The solution was stirred until gelatin was solubilised completely and transferred to a cylindrical mold with 3 cm diameter and 2.5 cm height. The solution was incubated at the refrigerated temperature (4 °C) for 18 h prior to analysis.

Gel strength was determined at 8–10 °C using a texture analyser (Stable Micro System, Surrey, UK) with a load cell of 5 kg, cross-head speed of 1 mm/s, equipped with a 1.27 cm diameter flat-faced cylindrical Teflon® plunger. The maximum force (grams), taken when the plunger had penetrated 4 mm into the gelatin gels, was recorded.

3.4.4.7 Determination of gelling and melting temperatures

Gelling and melting temperatures of gelatin samples were measured following the method of Boran *et al.* (2010) using a controlled stress rheometer (RheoStress RS 75, HAAKE, Karlsruhe, Germany). Gelatin solution (6.67%, w/v) was prepared in the same manner as described previously. The solution was preheated at 35 °C for 30 min. The measuring geometry used was 3.5 cm parallel plate and the gap was set at 1.0 mm. The measurement was performed at a scan rate of 0.5 °C/min, frequency of 1 Hz, oscillating applied stress of 3 Pa during cooling from 35 to 5 °C and heating from 5 to 35 °C. The gelling and melting temperatures were calculated, where tan δ became 1 or δ was 45°.

3.4.4.8 Measurement of setting time

Setting time of gelatin solution was determined at 4 °C and 25 °C according to the method of Kittiphattanabawon *et al.* (2010). Gelatin solution (6.67%, w/v) was prepared in the same manner as described previously. The solution (2 mL) was transferred to thin wall (diameter of 12 mm and length of 75 mm) test tubes (PYREX®, Corning, NY, USA) and preheated at 60 °C for 10 min, followed by incubation in an ice bath (4 °C) or at room temperature. An aluminum needle with the diameter and length of 0.1 and 25 cm, respectively, was inserted manually in the gelatin solution and raised every 10 s. The time at which the needle could not detach from the gelatin sample was recorded as the setting time. The setting time was expressed as min.

3.4.4.9 Determination of gel color

The color of gelatin gels (6.67% w/v) was measured by a Hunter lab colourimeter (Color Flex, Hunter Lab Inc., Reston, VA, USA). L^*, a^* and b^* values indicating lightness/brightness, redness/greenness and yellowness/blueness, respectively, were recorded. The colourimeter was warmed up for 10 min and calibrated with a white standard. Total difference in color (ΔE^*) was calculated according to the following equation (Gennadios *et al.*, 1996):

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

where ΔL^* , Δa^* and Δb^* are the differences between the corresponding color parameter of the sample and that of white standard (L^* = 93.63, a^* = -0.94 and b^* = 0.40).

3.4.4.10 Microstructure analysis of gelatin gel

Microstructure of gelatin gel was visualised using scanning electron microscopy (SEM). 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 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 acceleration voltage of 20 kV.

3.4.6 Statistical analysis

All experiments were run in triplicate using three different lots of skin samples. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out by using Duncan's multiple range test (Steel and Torrie, 1980). For pair comparison, T-test was used. Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for windows: SPSS Inc., Chicago, IL, USA).

3.5 Results and discussion

3.5.1 Effect of extraction temperature

3.5.1.1 Extraction yield

Yield of gelatin extracted at different temperatures from the skin of seabass is shown in Table 9. Extraction yield increased when extraction temperature was increased up to 55 °C ($P<0.05$). However, no differences in yields of gelatin between extracted at temperature higher than 55 °C were observed ($P>0.05$). Yield of 58.16, 70.82, 73.05 and 74.51% (based on dry weight) was found for gelatin extracted at 45, 55, 65 and 75 °C, respectively. Kaewruang *et al.* (2013) reported that the increasing extraction temperatures resulted in the increased yield of gelatin from unicorn leatherjacket skin. The increasing temperature directly provided more energy to disrupt bondings stabilizing the collagen structure, leading to larger amount of gelatin (Nagarajan *et al.*, 2012). The gelatin extractability also was influenced by the pretreatment conditions and the properties and the preservation method of the starting raw material (Karim and Bhat, 2009). Therefore, the optimal gelatin in terms of quantity and quality could be obtained by optimizing the extraction temperature.

Table 9. Yield and gel strength of gelatin from the skin of seabass extracted at different temperatures

Extraction temperature	Yield (%, based on dry weight)	Gel strength (g)
45	58.16±0.74a	312.9±7.04a
55	70.82±4.81b	286.5±.88b
65	72.05±6.67b	236.0±3.12c
75	74.51±3.19b	200.2±3.02d

Values are presented as mean±SD (n = 3).
Different lowercase letters within the same column indicate significant difference (P< 0.05).

3.5.1.2 Protein pattern

Protein pattern of gelatin from the skin of seabass extracted with different extraction temperatures is shown in Figure 12. For all extraction temperature, all gelatin samples consist of β- and α-components as the major constituents. β- and α-chains had the MW of 190 and 125-110 kDa, respectively. In general, the band intensity of β-, γ- and α-chains decreased with increasing extraction temperature. When extraction temperature higher than 55 °C, γ-chains were more degraded, leading to decrease of γ-chains band. The decreasing α- and β-cahins band intensity was observed in gelatin extracted at 65 and 75 °C. The result was in accordance the reported of Kaewruang *et al.* (2013) and Nagarajan *et al.* (2012). Extraction temperature played an important role in protein components of resulting gelatin (Nagarajan *et al.*, 2012). Muyonga *et al.* (2004) reported that peptides with an MW less than α-chain of gelatin from Nile prch skin and bone decreased with increasing extraction temperature. The result noted that high exraction temperature used in gelatin extraction more likely caused the hydrolysis of α- and β-chains. Gelatins with higher content of α-chains possessed better functional properties including foaming properties, gel strength, gelling and melting temperautres (Gómez-Guillén *et al.*, 2002). Therefore, the result revealed that extraction temperature strongly affected the molecular weight distributiom, which contributed to their function properties.

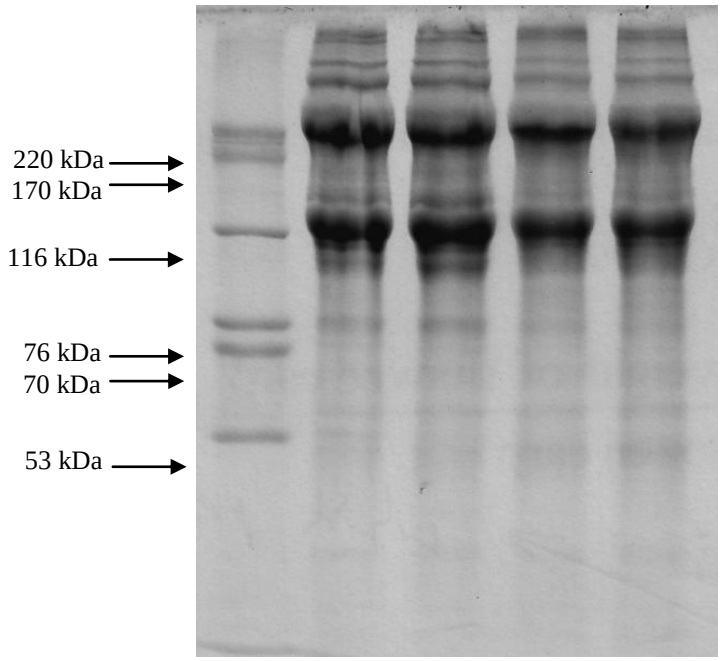

Figure 12. SDS-PAGE pattern of gelatin from skin of seabass extracted at different temperatures for 12 h. M denotes high molecular weight markers. Numbers denote the extraction temperature (°C).

3.5.1.3 Gel strength

Gel strength of gelatin extracted at different temperatures from the skin of seabass is shown in Table 9. Gel strength of gelatin gels decreased as the extraction temperature increased ($P<0.05$). Gelatin extracted at 45 °C for 12 h exhibited the higher gel strength (312.9 g). Gelatin obtained from lower extraction temperature, more likely had the longer chain. As a result, the interaction via inter-junction zone could take place as indicated by the higher gel strength (Kaewruang *et al.*, 2013). The result was in agreement with Kaewruang *et al.* (2013) and Nagarajan *et al.* (2012) who reported that gel strength of gelatin decreased when higher extraction temperatures was used. Moreover, gelatin extracted at 75 °C had the lowest gel strength, compared to other gel samples ($P<0.05$). Decreases in gel strength was related to decreasing of band intensity of α- and β-cahins in protein pattern (Figure 12). The high extraction temperature might have more the degradation of protein. As a result, protein degradation fragments might reduce the ability of α-chains to form a

stronger gel network (Nagarajan *et al.*, 2012). The different in gel strength between might be governed by molecular weight distribution and amino acid composition (Kittiphattanabawon *et al.*, 2010). Therefore, gelatin samples obtained from extraction temperature of 45 and 55 °C with higher yield and gel strength were selected for further study.

3.5.2 Effect of extraction time

3.5.2.1 Extraction yield

The yields of gelatin extracted under different extraction conditions are shown in Table 10. Gelatin extracted at 45 or 55 °C had the increasing yield as the extraction times increased up to 6 h ($P<0.05$). In general, the higher yields were obtained when extracted at 55 °C, in comparison with 45 °C for all extraction times used. Yields of 51.55-57.27% and 62.04-66.36% (on dry weight basis) were found for gelatin extracted at 45 and 55 °C, respectively. The highest yield from seabass skin (66.36%) was obtained when extraction was carried out at 55 °C for 12 h ($P<0.05$). With extraction time of 12 h, no differences in yield were noticeable when extraction temperatures were higher than 55 °C ($P>0.05$) (Table 9). The result suggested that higher heat applied likely destabilised the bondings between α-chains in native mother collagen more effectively. As a consequence, the triple helix structure turned to be amorphous and could be extracted into the medium with ease, leading to the higher yield. Increasing extraction time also provided more energy to destroy those bondings, in which free α-chains or β-chain were more released from skin complex. It was noted that the extraction time longer than 6 h did not increase the yield ($P>0.05$). The cross-linked proteins stabilised by covalent bonds in skin network might not be destroyed even with higher extraction temperature and time. Kittiphattanabawon *et al.* (2010) reported that the extraction yield of gelatin from skin of brownbanded bamboo shark and blacktip shark increased when extraction temperature and time increased. The different yields of fish skin gelatin have been reported for Nile perch (64.3%) (Muyonga *et al.*, 2004), greater lizardfish (35.1%) (Taheri *et al.*, 2009) and tiger-toothed croaker (36.8%) and pink perch (27.3%) (Koli *et al.*, 2012). The yield and characteristics of gelatin are associated with the kind of raw material and gelatin

extraction process including pretreatment process (Kittiphattanabawon *et al.*, 2010; Montero and Gómez-Guillén, 2000; Nagarajan *et al.*, 2012). Seabass is a warm-water fish and its skin had complex structure and strong fibrous, which was resistant to high temperature environmental (Athauda *et al.*, 2012). As a result, the harsher extraction conditions were required to obtain the higher yield.

3.5.2.2 Protein Pattern

Protein patterns of gelatin from skin of sea ass bextracted with different extraction temperatures and times are shown in Figure 13. All gelatin samples contained β- and α–chains with MW of 193 and 125-113 kDa, respectively, as the major constituents. It was noted that gelatin samples had both α_1- and α_2-chains. Protein patterns of gelatin were similar to those found in collagen of seabass skin reported by Sinthusamran *et al.* (2013). This indicated that α- chains and β-chains of mother collagen were retained with negligible degradation. On the other hand, Kaewruang *et al.* (2013) reported that no α–chains and β-chain were remained in gelatin from skin of unicorn leatherjacket extracted at 45 and 55 °C, caused by endogenous heat stable proteases. Thus, it was suggested that seabass skin might contain no proteases.

3.5.2.3 Free amino group content

Free amino group content of gelatin from seabass skin extracted under different extraction conditions is depicted in Table 10. Free amino group content increased as extraction temperature and time increased ($P<0.05$). This was in agreement with Nagarajan *et al.* (2012) who reported that free amino group content of the gelatin extracted from splendid squid skin increased with increasing extraction temperature. At higher temperature with extended time, peptide chains could be broken to higher extent as evidenced by higher free α-amino group content. This result reconfirmed that gelatin extracted at high temperature, especially with the longer extraction times, showed slightly lower band intensity of β- and α–chains (Figure 12). This was mainly due to thermal degradation, leading to the increasing free amino group.

However, the band intensity of β- and α-chains in gelatin slightly decreased with increasing extraction temperature and time. This might be caused by some degradation induced by the thermal process. Thermal degradation of gelatin from skin of brownbanded bamboo shark and blacktip shark was more pronounced with increasing temperatures (Kittiphattanabawon *et al.*, 2010). However, at the same extraction conditions, α-chains and cross-linked components of gelatin from seabass skin were more tolerant to thermal hydrolysis than those of brownbanded bamboo shark and blacktip shark skin. Gelatins from splendid squid skin with higher extraction temperatures contained lower band intensity of α–chains than those obtained with lower extraction temperature (Nagarajan *et al.*, 2012). Generally, gelatins with higher content of α-chains showed the better functional properties including gelling, emulsifying and foaming properties (Gómez-Guillén *et al.*, 2002). Thus, the components of gelatin from seabass skin were quite thermo-tolerant, compared with other species previously reported (Kaewruang *et al.*, 2013; Kittiphattanabawon *et al.*, 2010).

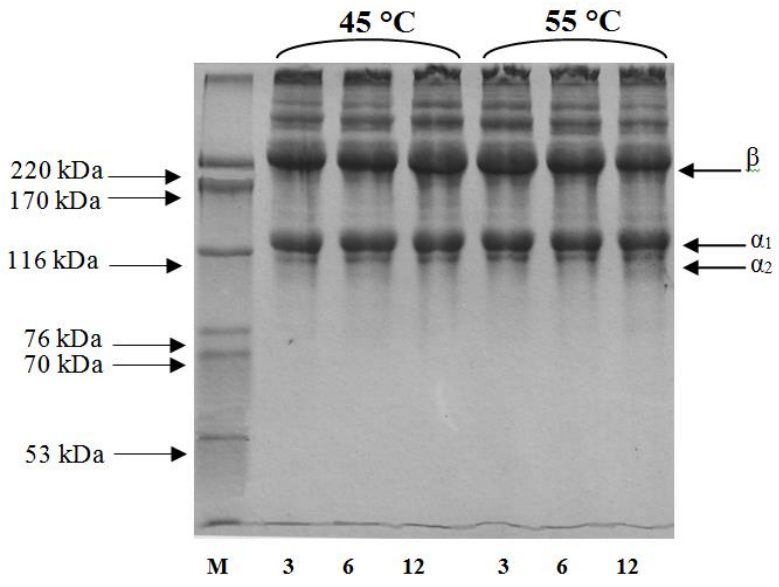

Figure 13. SDS-PAGE pattern of gelatin from skin of seabass extracted at different temperatures for various times. M denotes high molecular weight markers. Numbers denote the extraction times (h).

Table 10. Extraction yield (%, dry weight basis) and gel color of gelatin from skin of seabass extracted at different temperatures for various times.

Temperature (°C)	Time (h)	Yield (%)	Color value				Free amino group content (mmol/g)
			L^*	a^*	b^*	ΔE^*	
45	3	51.55±2.28Aa	22.10±0.15Aa	-1.28±0.17Aa	-1.94±0.11Ac	71.57±0.15Aa	0.032±0.002Aa
	6	55.72±1.21Bb	20.95±0.05Bb	-1.37±0.15Aab	-1.91±0.14Ac	72.72±0.05Bb	0.046±0.002Aa
	12	57.27±0.87Bb	18.64±0.14Cc	-1.57±0.28Abc	-1.80±0.37Abc	75.03±0.15Cc	0.109±0.002Bb
55	3	62.04±1.54Ac	17.84±0.79Ad	-1.79±0.14Ac	-1.66±0.22Aabc	75.83±0.80Ad	0.062±0.002Aa
	6	65.31±1.28Bd	17.41±0.08ABd	-1.75±0.15Ac	-1.54±0.29Aab	76.25±0.08ABd	0.072±0.004Aa
	12	66.36±1.40Bd	16.88±0.29Be	-1.74±0.21Ac	-1.37±0.26Aa	76.77±0.29Be	0.115±0.004Bc

Values are presented as mean±SD (n = 3).
Different uppercase letters within the same column under the same temperature indicate significant difference ($P< 0.05$). Different lowercase letters within the same column indicate significant differences ($P< 0.05$).

3.5.2.4 Fourier transform infrared (FTIR) spectra

FTIR spectra of gelatin from the skins of seabass extracted at 45 and 55 °C with different extraction times are shown in Figure 14, respectively. Generally, all gelatins showed similar spectra. Amide I band of gelatin extracted at 45 or 55 °C for various times appeared at 1642.8-1644.8 cm^{-1} and 1643.5-1644.6 cm^{-1}, respectively. The amide I vibration mode is primarily a C=O stretching vibration coupled to contributions from the CN stretch, CCN deformation and in-plane NH bending modes (Bandekar, 1992). The absorption in the amide I region is probably the most useful for infrared spectroscopic analysis of the secondary structure of proteins (Surewicz and Mantsch, 1988). The absorption peak at amide I (1634.7 cm^{-1}) was characteristic for the coil structure of gelatin (Nagarajan *et al.*, 2012). Kittiphattanabawon *et al.* (2010) reported that higher extraction temperature of gelatin increased the amount of low molecular weight components, in which the reactive group (C=O) could be more exposed. Amide I band of gelatin extracted at higher temperature and longer time was shifted to the higher wavenumber (Kittiphattanabawon *et al.*, 2010). Additionally, gelatin extracted at 45 and 55 °C for all extraction times exhibited the amide II bands at the wavenumber of 1539.7-1542.7 cm^{-1} and 1539.5-1543.2 cm^{-1}, respectively. Amide II band resulted from an out-of-phase combination of CN stretch and in-plane NH deformation modes of the peptide group (Bandekar, 1992; Lavialle *et al.*, 1982). It was noted that the amide II band of gelatins extracted at both temperatures showed similar spectra. Furthermore, the amide III bands of all gelatin samples were observed at wavenumbers of 1233-1234 cm^{-1}, which indicated the disorder in gelatin molecules and were more likely associated with loss of triple helix state (Friess and Lee, 1996). The amide III band represents the combination peaks between CN stretching vibrations and NH deformation from amide linkages as well as absorptions arising from wagging vibrations from CH_2 groups of glycine backbone and proline side-chains (Jackson *et al.*, 1995).

The amide A band was found at 3288.8-3304.3 cm^{-1}, associated with stretching vibrations of NH group coupled with hydrogen bonding. Normally, a free NH stretching vibration is found in the range of 3400-3440 cm^{-1}. The position of this

band shifts to lower frequencies because the NH group of a peptide is involved in a hydrogen bond. (Doyle *et al.*, 1975). Gelatin extracted at 45 °C for 3 h showed the lowest amplitude than other gelatin samples. This result indicated the lower free amino groups, associated with the lower degradation. The amide B band was observed at 3069.8-3078.6 and 3070.7-3078.6 cm^{-1} for gelatin extracted at 45 and 55 °C, respectively, corresponding to the asymmetric stretching vibration of =C-H as well as NH_3^+. Amongst all samples, gelatin extracted for longer time had the lowest wavenumber of amide-B peak, suggesting the interaction of $-NH_3$ group between peptide chains (Nagarajan *et al.*, 2012). Thus, the secondary structure and functional group of gelatins obtained from skin of seabass were affected by extraction temperatures and times.

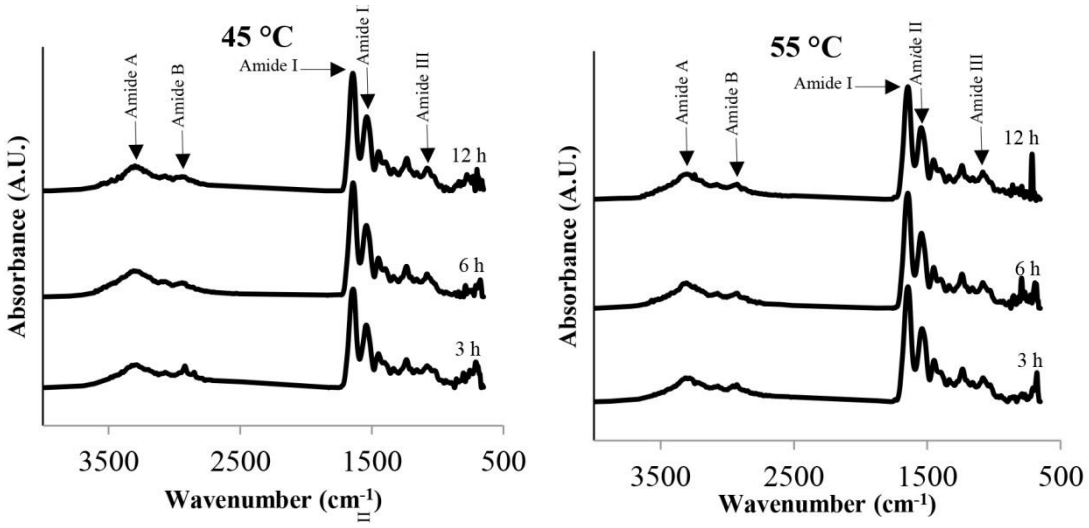

Figure 14. FTIR spectra of gelatin from skin of seabass extracted at different temperatures for various times.

3.5.2.5 Amino acid composition

Amino acid compositions of gelatin extracted under different extraction conditions are shown in Table 11. All gelatin samples showed similar amino acid compositions, in which glycine was the major amino acid (331-334 residues/1000 residues), followed by alanine (134-137 residues/1000 residues). Low contents of cysteine (1 residues/1000 residues), tyrosine (3-4 residues/1000 residues),

histidine (5-6 residues/1000 residues) and hydroxylysine (5 residues/1000 residues) were observed in all gelatin samples. Generally, glycine occurs every third position of α-chain and represents nearly one third of total residues (Benjakul *et al.*, 2009). For imino acids, all gelatins contained proline and hydroxyproline of 116-118 and 82-84 residues/1000 residues, respectively. The imino acid content of gelatin from seabass skin (198-202 residues/1000 residues) was higher than that reported in 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 (186.29-187.42 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, it was lower than that found in bovine gelatin (219 residues/1000 residues) (Jellouli *et al.*, 2011). Gelatin with higher content of hydroxyproline affected viscoelastic properties of gelatin and ability to develop the strong gel structure (Benjakul *et al.*, 2009). Kittiphattanabawon *et al.* (2010) reported that gelatin from brownbanded bamboo shark skin with higher content of imino acids had higher bloom strength than gelatin from blacktip containing lower imino acids. OH groups of hydroxyproline might be involved in hydrogen bondings with adjacent chains. This could strengthen gel network. These results indicated that the extraction conditions did not affect amino acid compositions of gelatin from seabass skin.

Table 11. Amino acid compositions of gelatins from skin of seabass extracted at different temperatures for various times

Amino acids (residues/1000 residues)	45 °C			55 °C		
	3 h	6 h	12 h	3 h	6 h	12 h
Alanine	134	135	135	137	135	135
Arginine	55	54	54	54	54	54
Aspartic acid/asparagine	43	43	43	43	43	43
Cysteine	1	1	1	1	1	1
Glutamine/glutamic acid	71	71	71	72	72	72
Glycine	334	334	334	331	332	331
Histidine	5	6	6	5	6	5
Isoleucine	9	9	9	9	9	9
Leucine	18	18	18	18	18	18
Lysine	28	28	28	28	28	28
Hydroxylysine	5	5	5	5	5	5
Methionine	14	14	14	13	13	13
Phenylalanine	13	13	13	13	13	13
Hydroxyproline	82	82	82	83	84	84
Proline	116	116	116	116	117	118
Serine	26	26	25	26	27	26
Threonine	22	22	22	22	22	22
Tyrosine	4	4	4	4	4	3
Valine	20	20	20	20	20	20
Total	1000	1000	1000	1000	1000	1000
Imino acid	198	199	199	199	201	202

3.5.2.6 Gel Strength

Gel strength of gelatin gels from seabass skin extracted with distilled water under varying conditions is shown in Figure 15. Gel strength is one of the most important functional properties of gelatins. The highest gel strength (369.2 g) was found in gelatin extracted at 45 °C for 3 h ($P<0.05$). Gel strength of gelatin gels decreased as the extraction temperature increased ($P<0.05$). At the same extraction temperature, gel strength of gelatin decreased when extraction time increased ($P<0.05$). This result was in agreement with the reports of Muyonga *et al.* (2004), Kittiphattanabawon *et al.* (2010) and Nagarajan *et al.* (2012). The decrease in gel strength was in accordance with the slight decreases in β- and γ-chains band intensity (Figure 12). Amounts 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). According to the amino acid composition (Table 11), all gelatins had high imino acid content (198-202 residues/1000 residues). In the present study, seabass skin gelatin had the higher gel strength (282.0-369.2 g) than bovine gelatin (208.0 g) ($P<0.05$). Different gel strength was reported for gelatin from skin of different species including splendid aquid (85-132 g) (Nagarajan *et al.*, 2012), rainbow trout (459 g) (Tabarestani *et al.*, 2010), brownbanded bamboo shark and blacktip shark (206-214 g) (Kittiphattanabawon *et al.*, 2010), salmon (195 g) (Arnesen and Gildberg, 2007) and yellowfin tuna (426 g) (Cho *et al.*, 2005). Gel strength is a function of complex interactions determined by molecular weight distribution (Gómez-Guillén *et al.*, 2002). In this study, solution of gelatin extracted for longer times showed higher turbidity. The result suggested that α- or β-chains might undergo conformation changes, which favoured the aggregation to some extent, especially via the exposed reactive group. The larger bundles or strands might not form the fine and ordered gel structure, as evidenced by the poorer gel strength.

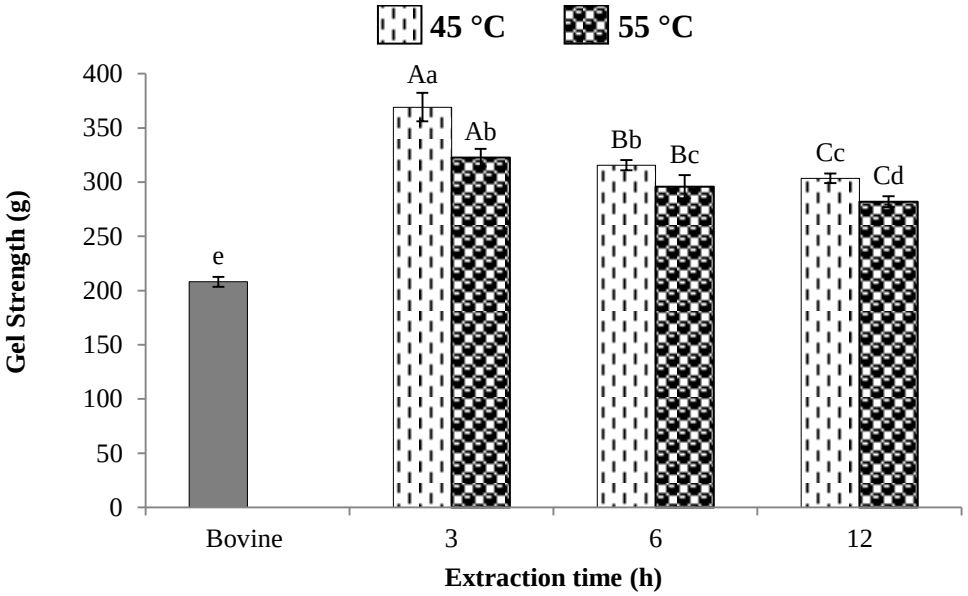

Figure 15. Gel strength of gelatin from skin of seabass extracted at different temperatures for various times. Bars represent the standard deviation (n=3). Different uppercase letters on the bars within the same extraction temperature indicate significant differences (P< 0.05). Different lowercase letters on the bars indicate significant differences (P< 0.05).

3.5.2.7 Gelling and melting temperatures

Thermal transitions monitored by changes in the phase angle (δ) of dissolved gelatins during cooling (35 to 5 °C) and subsequent heating (5 to 35 °C), are depicted in Figure 16A and 16B, respectively. Gelling temperatures of all gelatin samples with different extraction conditions were in the range of 19.5-20.0 °C. This was considered as the sharp decrease in phase angle during cooling. Changes in phase angle indicated the rapid transition, the formation of junction zones to three-dimensional network with subsequent development of strong gel matrix. No marked differences in the gelling temperatures were observed amongst different gelatins obtained from varying extraction conditions. Nagarajan *et al.* (2012) reported that gelatin extraction conditions affected physico-chemical properties of gelatin such as molecular weight distribution, amount of β- and γ-components and free amino group content. Therefore, gelling temperature was not much affected by extraction conditions used in the present study. The gelling temperature in this study was higher

than those of gelatins from skin of yellowfin tuna (18.7 °C) (Cho *et al.*, 2005), bigeye snapper (10.0 °C) (Binsi *et al.*, 2009) and silver carp (18.7) (Boran *et al.*, 2010). Setting temperature was affected by molecular weight and relative content of γ-, β- and α-chain componets (Muyonga *et al.*, 2004).

Melting temperatures of gelatin gel from seabass skin were in the range of 26.3-27.0 °C. Varying melting temperatures were reported for gelatin from bigeye snapper skin (16.8 °C) (Binsi *et al.*, 2009), yellowfin tuna skin (24.3 °C) (Cho *et al.*, 2005), grass carp skin (26.8 °C) (Kasankala *et al.*, 2007) and silver skin (27.1 °C) (Boran *et al.*, 2010). Pro-rich regions in gelatin molecules of cold water fish were lower than those of warm blooded animals, which were directly correlated with thermal stability of gelatin gel (Gómez-Guillén *et al.*, 2002). With higher melting temperature, gel could be maintained for a longer time, thereby providing the better mouth feel when consumed. The gelling and melting temperatures depend on fish species used as raw material, which may have different living environments and habitat temperatures (Gómez-Guillén *et al.*, 2002).

3.5.2.8 Setting time for gel formation

Setting time required for the gel formation of gelatin from seabass skin at 4 °C and 25 °C is presented in Figure 17A and 17B, respectively. Setting time at 4 °C of gelatin extracted with both temperatures increased as extraction time increased ($P<0.05$). Gelatin extracted at 45 °C had lower setting time at 4 °C than that of commercial bovine gelatin ($P<0.05$). Setting time of gelatin extracted at 45 and 55 °C was in the ranges of 0.83-1.03 and 0.97-1.23 min, respectively. This result was in accordance with Kittiphattanabawon *et al.* (2010) who reported that the gelatin extracted from blacktip shark skin at higher temperature (75 °C) required the longer setting time.

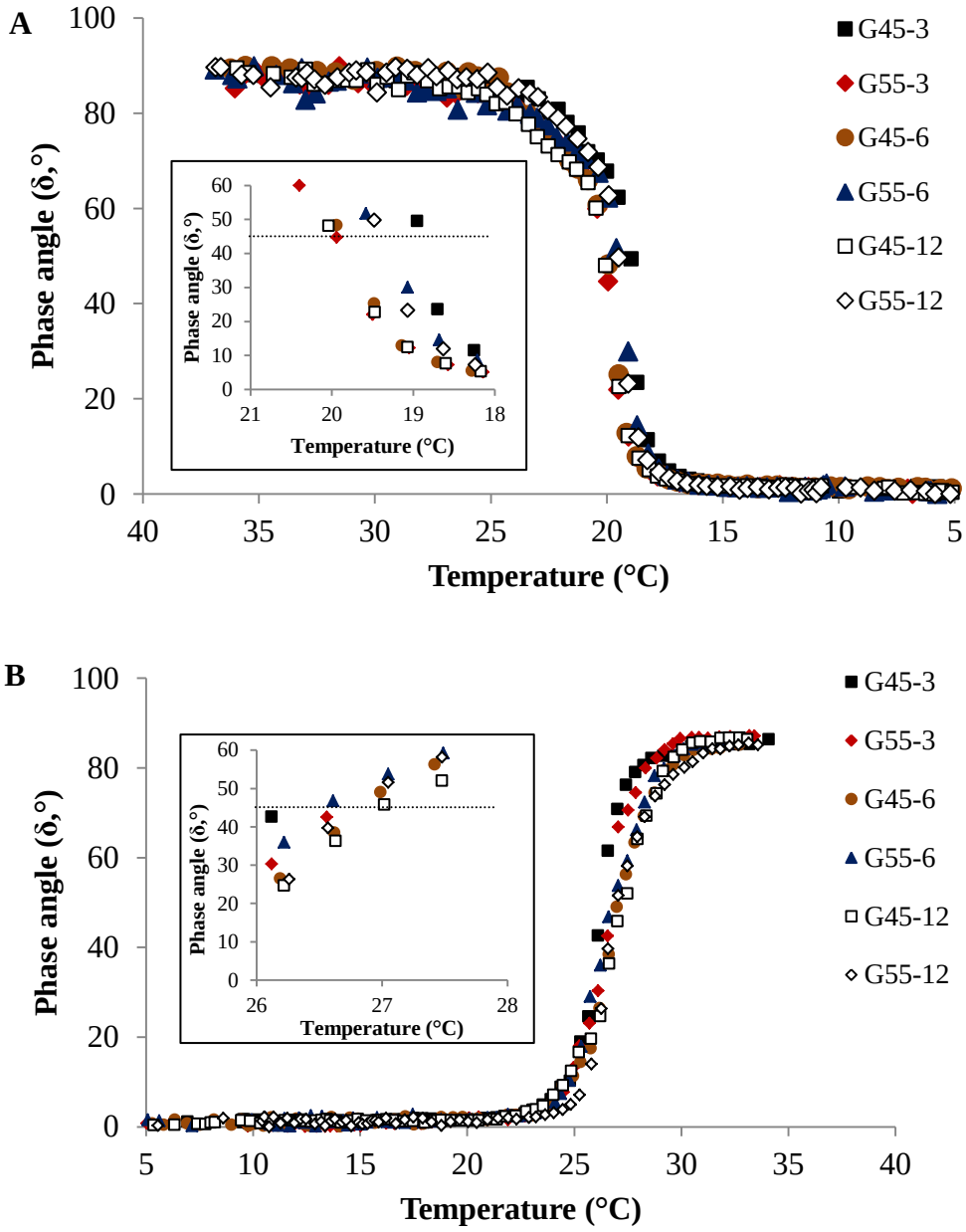

Figure 16. Changes in phase angle (δ, °) of gelatin solution (6.67%, w/v) from the skin of seabass extracted at different temperatures for various times during cooling (A) and subsequent heating (B).

Setting times at 25 °C of gelatin extracted at 45 and 55 °C for various times were 19.2-21.6 and 23.4-29.2 min, respectively. Similar result was observed, in comparison with setting at 4 °C. However, the longer setting time was required at 25 °C. Amongst all gelatins, that extracted at 45 °C for 3 h had the lowest setting times (19.2 min) ($P<0.05$). The low molecular weight peptides are associated with long setting time of gelatin (Kittiphattanabawon *et al.,* 2010). The gelatin extracted at higher temperature for longer time more likely had higher degradation caused by thermal degradation, compared with those extracted at lower temperature for shorter time. Consequently, the formation of gel network of gelatin extracted at higher temperature needed the longer time for alignment and connection between chains to form gel network. As a result, the lower temperature and longer time were needed for setting. Thus, different extraction conditions affected the setting time of gelatin from seabass skin. It was noted that gelatin from seabass skin was able to set at 25 °C, which was different from gelatin of other fish species, which could not set at room temperature.

3.5.2.9 Color

Color of gelatin gel from seabass skin with different extraction temperatures and times expressed as L^*, a^* and b^* are shown in Table 10. Higher lightness (L^*- values) was observed for gels of gelatin extracted at 45 °C, compared with those of gelatin extracted at 55 °C ($P<0.05$). At extraction temperature of 45 °C, L^*-values of gelatin gels slightly decreased with increasing extraction times ($P<0.05$). It was found that the decreases in redness (a^*- value) were observed when the extraction temperatures and times increased ($P<0.05$). Nevertheless, no differences in a^*- value were found between gelatins extracted at different temperatures ($P>0.05$). For yellowness (b^*- value), the increases were observed in all gelatin gels when the extraction temperatures increased ($P<0.05$). This might be due to non-enzymatic browning reaction taken place at higher temperature, especially for a longer time (Ajandouz and Puigserver, 1999). Amongst all gelatin samples, that extracted at lower temperature (45 °C) for the shortest time (3 h) showed the lowest total difference in color value (ΔE^*) (71.57) with the highest lightness (L^*- values). Benjakul *et al.* (2009) reported that pigments could be removed during pretreatment prior to gelatin

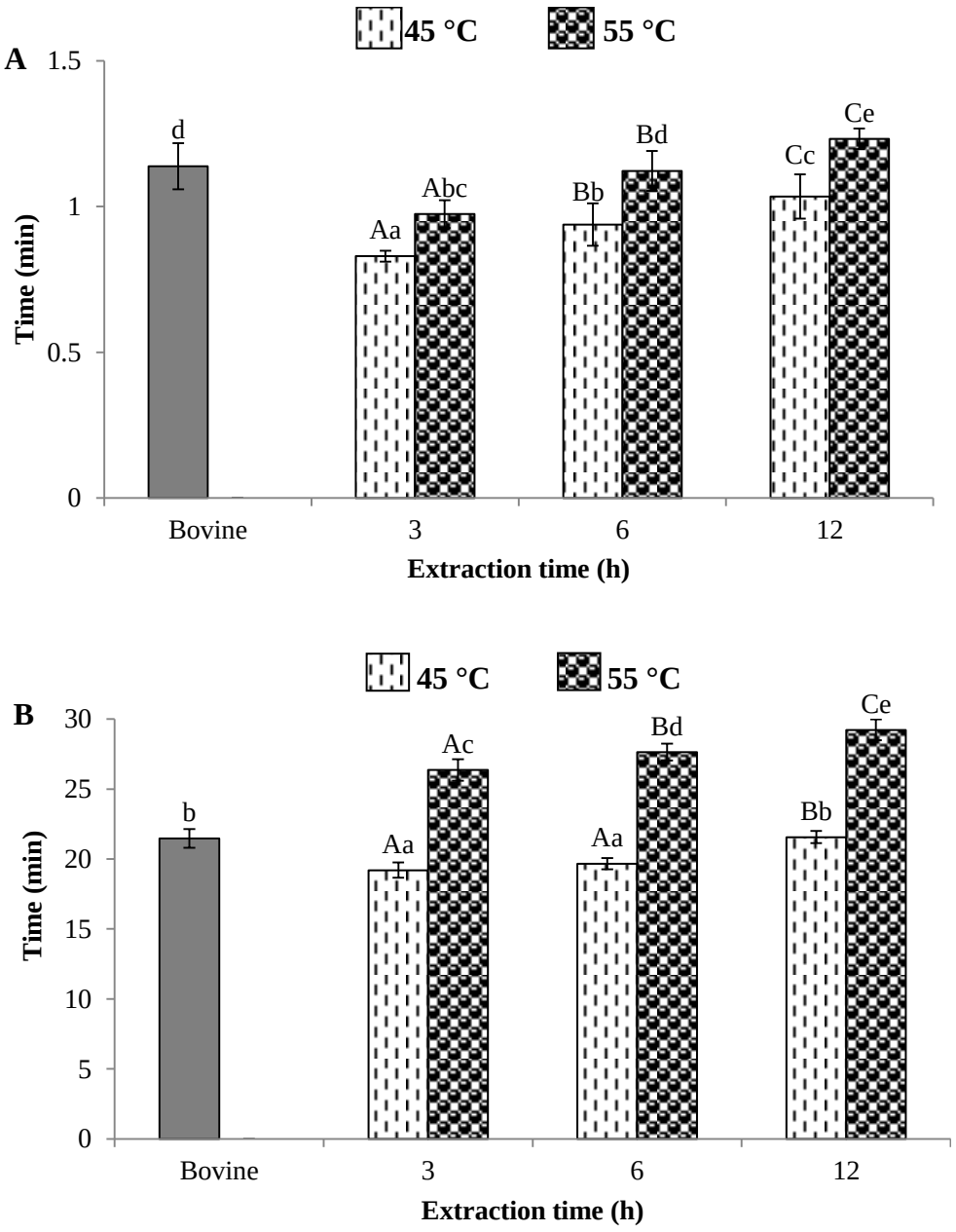

Figure 17. Setting time at 4 °C (A) and 25 °C (B) of gelatin from the skin of seabass extracted at different temperatures for various times. Bars represent the standard deviation (n=3). Different uppercase letters on the bars within the same extraction temperature indicate significant differences ($P<0.05$). Different lowercase letters on the bars indicate significant differences ($P<0.05$).

extraction. These results showed that the extraction conditions had the impact on color of gelatin extracted from the skin of seabass.

3.5.2.10 Microstructures of gelatin gel

The microstructures of gelatin gels from seabass skin with different extraction conditions are illustrated in Figure 18. In general, the conformation and chain length of proteins in gel matrix directly contributed to the gel strength of gelatin (Benjakul *et al.*, 2009). Gelatin extracted at 45 °C for 3 h showed the finest gel network with very small voids. Conversely, the larger voids were found in gel of gelatin extracted at higher temperature for longer time. The fine gel structure of gelatin extracted at lower temperature for shorter time was in accordance with the higher gel strength (Figure 15). Larger strands of network were formed in gelatin gel with longer extraction times. As a consequence, this type of gel could not be resistant to force applied, leading to lower gel strength. It is well known that the distribution of α-, β- and γ-chains is important factor affecting property of gelatin. Extraction condition might have the impact on the molecular weight distribution and peptides of gelatin, which also affected the arrangement of peptides in network during gelation.

3.6 Conclusion

The skin of seabass could be a promising source of gelatin. Gelatin extracted at higher temperature and longer time had higher extraction yield, but showed the poorer gel property. Gelatin obtained from seabass skin with different extraction conditions contained β- and α-chains as the major components. All gelatins from different extraction temperatures and times showed higher gel strength than that of commercial bovine gelatin. They were able to set at 25 °C within 30 min with high gelling (19.5-20.0 °C) and melting (26.3-27.0 °C) temperatures. Therefore, gelatin from seabass skin could be used as a replacer for bovine or porcine gelatin.

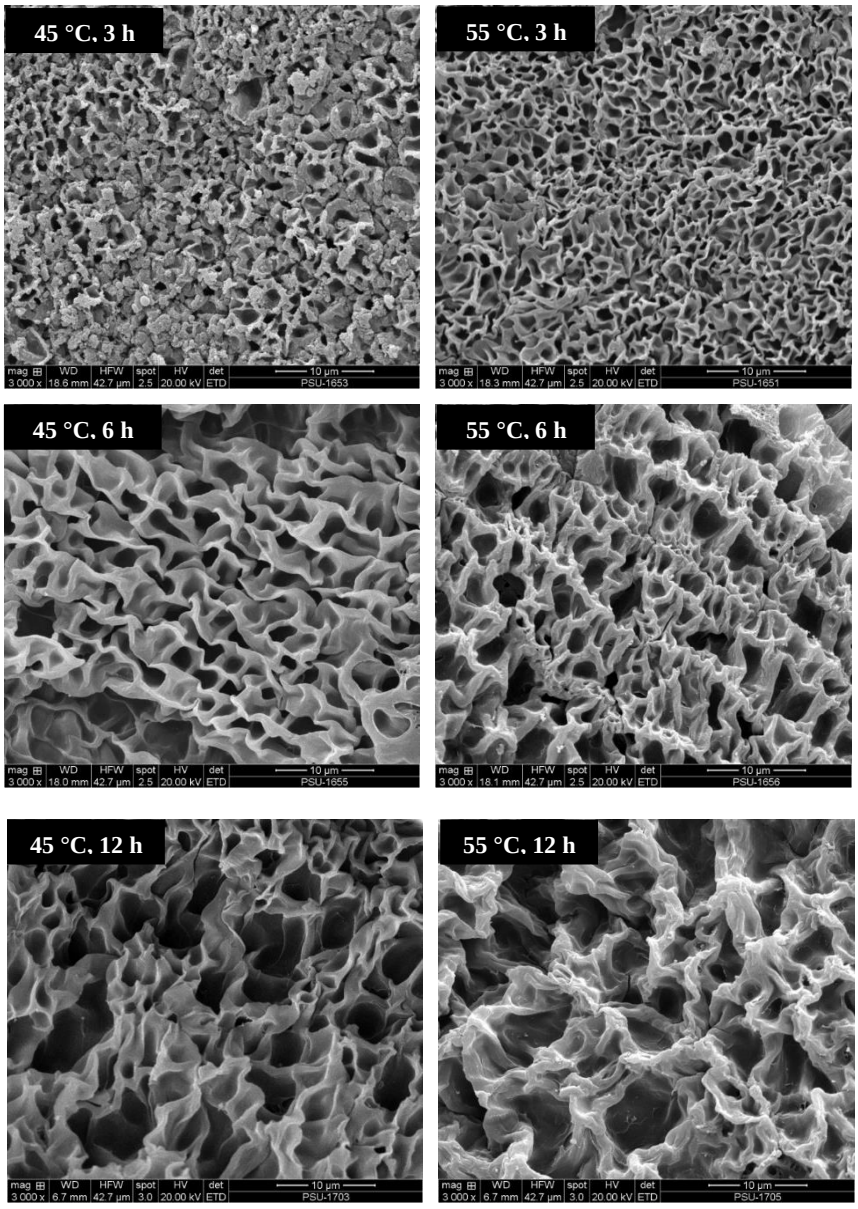

Figure 18. Microstructures of gelatin gel from skin of seabass extracted at different temperatures for various times. Magnification: 3000 times

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CHAPTER 4

MOLECULAR CHARACTERISTICS AND PROPERTIES OF GELATIN FROM SKIN OF SEABASS WITH DIFFERENT SIZES

4.1 Abstract

Gelatin was extracted from the skin of seabass (*Lates calcarifer*) with different average sizes (2, 4 and 6 kg/fish), termed G2, G4 and G6, respectively and their characteristics and functional properties were determined. Yields of G2, G4 and G6 were 38.22, 40.50 and 43.48% (based on dry weight), respectively. G2 contained α-chains as dominant component, whilst G4 and G6 comprised α-, β- and γ-chains with a larger content of high MW cross-links. All gelatins had the similar imino acid (hydroxyproline and proline) content. Net charge of G2, G4 and G6 became zero at pHs of 6.73, 6.41 and 7.12, respectively. Amongst all gelatin samples, G6 exhibited the highest gel strength (321.5 g) ($P<0.05$), but had the lowest turbidity ($P<0.05$). Gels of G6 sample had the lower L^*-value but higher a^*-, b^*- and ΔE^*-value, compared with others. Gelling and melting temperatures of all gelatins were 17.09-19.01 and 26.92-28.85 °C, respectively. Furthermore, all gelatins were able to set at room temperature, regardless of size of seabass used. G6 had the shorter setting time at room temperature than others. Therefore, size of seabass, in which skin was used for gelatin extraction, had the impact on yield, composition and properties of resulting gelatin.

4.2 Introduction

Gelatin is the denatured or partially hydrolysed form of collagen (Chen *et al.*, 2014). It represents a major biopolymer with several applications in food, materials, pharmacy and photography industries (Jellouli *et al.*, 2011). Due to its gelling property and surface behaviour (e.g., formation and stabilisations of foams and emulsions), gelatin has been widely used to enhance the elasticity, consistency and

stability of food products (Benjakul *et al.*, 2012; Benjakul *et al.*, 2009; Nur Azira *et al.*, 2014). In general, the main sources of gelatin are skins and bones of pig and cow obtained from processing by-products. Gelatin from those sources can be a problem for certain consumers, e.g., Muslims and Jews, in which porcine gelatin is prohibited. Occurrence of bovine spongiform encephalopathy (BSE) has led to awareness for consumption of bovine gelatin (Nur Azira *et al.*, 2014). Nowadays, an increasing interest has been paid to alternative sources of gelatin, especially from the skins and bones from fish processing by-products (Kittiphattanabawon *et al.*, 2010; Sinthusamran *et al.*, 2014). Fish gelatin can be extracted from skin of several fish species including skipjack tuna, dog shark (Shyni *et al.*, 2014), cobia (Silva *et al.*, 2014), farmed Amur sturgeon (Nikoo *et al.*, 2014), seabass (Sinthusamran *et al.*, 2014), and brownbanded bamboo and blacktip shark (Kittiphattanabawon *et al.*, 2010). It has been known that the extraction conditions including temperature, time as well as pretreatment affect the functional properties of gelatin from fish processing by-products (Kaewruang *et al.*, 2013a; Nagarajan *et al.*, 2012; Sinthusamran *et al.*, 2014). Additionally, characteristics and properties of gelatin vary with species and age of raw material (Karim and Bhat, 2009; Singh *et al.*, 2011). Muyonga *et al.* (2004b) reported that gelatin from adult Nile perch skin exhibited the better gel properties than young Nile perch skin when the same extraction condition was used.

Seabass is one of economically important fish in Thailand. A number of seabass farms are located in the south of Thailand, especially in the lake of Songkhla. Due to its delicacy, a large amount of seabass has been exported as well as domestically consumed. During processing, particularly fillet production, skin is generated as byproduct. Skins from seabass has been used as raw material for collagen and gelatin extraction with higher yield (Sinthusamran *et al.*, 2013). Gelatin from seabass skin had higher gel strength than bovine gelatin and could be set at room temperature within 30 min (Sinthusamran *et al.*, 2014). Size or age of seabass, in which skin is used for gelatin extraction, can have the impact on composition and properties of gelatin. Nevertheless, no information regarding gelatin extracted from skin of seabass with different sizes has been reported.

4.3 Objective

To investigat the chemical characteristics and functional properties of gelatin from seabass as influenced by sizes of fish.

4.4 Materials and methods

4.4.1 Chemicals

All chemicals were of analytical grade. 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). Fish gelatin produced from tilapia skin (~240 bloom) was obtained from Lapi Gelatine S.p.A (Empoli, Italy).

4.4.2 Collection and preparation of skins from seabass

Fresh seabass (*Lates calcarifer*) with different sizes of 1.7-2.3, 3.7-4.3 and 5.7-6.2 kg/fish, equivalent to average size of 2, 4 and 6 kg/fish, respectively, were obtained from a farm in Koyo Island, Songkhla, Thailand. The fish were kept in ice with a fish/ice ratio 1:3 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h after capture. Fish were washed using cold tap water. Skins were then removed, descaled, and cut into small pieces (1.0 x 1.0 cm^2) using a scissor. The skin was placed in polyethylene bags and stored at -20 °C until used, but not longer than 2 months.

4.4.3 Extraction of gelatin from the skin of seabass

Gelatin was extracted from seabass skin according to the method of Jongjareonrak *et al.* (2006). Before gelatin extraction, skin was soaked in 0.1 M NaOH with a skin/solution ratio of 1:10 (w/v) to remove non-collagenous proteins. The mixture was stirred for 3 h at room temperature (28-30 °C) using an overhead stirrer model W20.n (IKA®-Werke GmbH & CO.KG, Staufen, Germany). The alkaline solution was changed every 1 h for totally 3 times. The pretreated skin was

washed with tap water until neutral or faintly basic pH was obtained. Then, the washed skin was mixed with 0.05 M acetic acid at a skin/solution ratio of 1:10 (w/v) to swell collagenous material in the fish skin matrix. The mixture was stirred at room temperature for 2 h. The skin was washed using tap water until neutral or faintly acidic pH of wash water was obtained. Finally, the swollen skin was mixed with distilled water at a ratio of 1:10 (w/v) at 45 °C for 3 h with continuous stirring. The mixtures were filtered using a Buchner funnel with Whatman No.4 filter paper (Whatman International, Ltd., Maidstone, England). Then, the filtrates were freeze-dried using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The freeze-dried gelatins extracted from seabass skin with an average size of 2, 4 and 6 kg/fish were referred to as ‘G2’, ‘G4’ and ‘G6’, respectively. Gelatin samples were subsequently subjected to analyses.

4.4.4 Analyses

4.4.4.1 Yield

The yield of gelatin was calculated based on dry weight of starting material.

$$\text{Yield (\%)} = \frac{\text{Weight of freeze-dried gelatin (g)}}{\text{Weight of initial dry skin (g)}} \times 100$$

4.4.4.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS–PAGE was performed by the method of Laemmli (1970). Gelatin samples were dissolved in 5% SDS solution. The mixtures were then heated at 85 °C 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 20 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 were used to estimate the molecular weight of proteins.

4.4.4.3 Amino acid analysis

Amino acid composition of gelatin samples was analysed using an amino acid analyser. The samples were hydrolysed under reduced pressure in 4 M methanesulphonic acid containing 0.2% (v/v) 3-2(2-aminoethyl) indole at 115 °C 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).

4.4.4.4 Fourier transform infrared (FTIR) spectroscopic analysis

FTIR spectra of gelatin samples were obtained by using a FTIR spectrometer (EQUINOX 55, Bruker, Ettlingen, Germany) equipped with a deuterated l-alanine tri-glycine sulphate (DLATGS) detector. The horizontal attenuated total reflectance accessory (HATR) was mounted into the sample compartment. The internal reflection crystal (Pike Technologies, Madison, WI, USA), made of zinc selenide, had a 45° angle of incidence to the IR beam. Spectra were acquired at a resolution of 4 cm^{-1} and the measurement range was 4000–650 cm^{-1} (mid-IR region) at room temperature. Automatic signals were collected in 32 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, Ettlingen, Germany).

4.4.4.5 Measurement of ζ -potential

Gelatin samples were dissolved in distilled water at a concentration of 0.5 mg/ml. The mixture was stirred at room temperature for 6 h. The Zeta (ζ) potential of each sample (20 ml) was measured using a zeta potential analyser (ZetaPALS, Brookhaven Instruments Co., Holtsville, NY, USA). ζ -Potential of samples adjusted

to different pHs with 1.0 M nitric acid or 1.0 M KOH using an autotitrator (BIZTU, Brookhaven Instruments Co., Holtsville, New York, USA) was determined. The pI was estimated from pH rendering ζ-potential of zero.

4.4.4.6 Determination of gel strength

Gelatin gel was prepared by the method of Kittiphattanabawon *et al.* (2010). Gelatin was dissolved in distilled water (60 °C) to obtain a final concentration of 6.67% (w/v). The solution was stirred until gelatin was solubilised completely and transferred to a cylindrical mold with 3 cm diameter and 2.5 cm height. The solution was incubated at the refrigerated temperature (4 °C) for 18 h prior to analysis.

Gel strength was determined at 8–10 °C using a texture analyser (Stable Micro System, Surrey, UK) with a load cell of 5 kg, cross-head speed of 1 mm/s, equipped with a 1.27 cm diameter flat-faced cylindrical Teflon® plunger. The maximum force (grams), taken when the plunger had penetrated 4 mm into the gelatin gels, was recorded.

4.4.4.7 Determination of gel color

The color of gelatin gels (6.67% w/v) was measured by a Hunter lab colourimeter (Color Flex, Hunter Lab Inc., Reston, VA, USA). L^*, a^* and b^* values indicating lightness/brightness, redness/greenness and yellowness/blueness, respectively, were recorded. The colourimeter was warmed up for 10 min and calibrated with a white standard. Total difference in color (ΔE^*) was calculated according to the following equation (Gennadios *et al.*, 1996):

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

where ΔL^* , Δa^* and Δb^* are the differences between the corresponding color parameter of the sample and that of white standard (L^* = 93.63, a^* = -0.94 and b^* = 0.40).

4.4.4.8 Measurement of turbidity

Turbidity of gelatin solution (6.67 %, w/v) was determined as per the method of Fernández-Díaz *et al.* (2001) with a slight modification. Gelatin solution was preheated at 40 °C for 15 min. The turbidity of gelatin solutions was measured by reading the absorbance at 360 nm using a double-beam spectrophotometer (model UV-1601, Shimadzu, Kyoto, Japan).

4.4.4.9 Measurement of setting time

Setting time of gelatin solution was determined at 4 °C and room temperature according to the method of Kittiphattanabawon *et al.* (2010). Gelatin solution (6.67%, w/v) was prepared in the same manner as described previously. The solution (2 ml) was transferred to thin wall (12 x 75 mm^2) test tubes (PYREX®, Corning, NY, USA) and preheated at 60 °C for 10 min, followed by incubation in an ice bath (4 °C) or at room temperature. An aluminium needle with the diameter and length of 0.1 and 25 cm, respectively, was inserted manually in the gelatin solution and raised every 10 s. The time at which the needle could not detach from the gelatin sample was recorded as the setting time. The setting time was expressed as min.

4.4.4.10 Gelling and melting temperatures

Gelling and melting temperatures of gelatin samples were measured following the method of Boran *et al.* (2010) using a controlled stress rheometer (RheoStress RS 75, HAAKE, Karlsruhe, Germany). Gelatin solution (6.67%, w/v) was prepared in the same manner as described previously. The solution was preheated at 35 °C for 30 min. The measuring geometry used was 3.5 cm parallel plate and the gap was set to 1.0 mm. The measurement was performed at a scan rate of 0.5 °C/min, a frequency of 1 Hz, oscillating applied stress of 3 Pa during cooling from 35 to 5 °C and heating from 5 to 35 °C. The gelling and melting temperatures were calculated, where tan δ became 1 or δ was 45°.

4.4.4.11 Microstructure analysis of gelatin gel

Microstructure of gelatin gel was visualised using scanning electron microscopy (SEM). 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 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 acceleration voltage of 20 kV.

4.4.5 Statistical analysis

All experiments were run in triplicate using three different lots of skin samples. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out by using Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for windows: SPSS Inc., Chicago, IL, USA).

4.5 Results and discussion

4.5.1 Extraction yield

The yields of gelatin extracted from the skin of seabass with different sizes are shown in Table 12. Extraction yields of G2, G4 and G6 were 38.22, 40.50 and 43.48% (based on dry weight), respectively. The highest yield was found for G6 sample extracted from the skin of largest size, in comparison with those of smaller sizes ($P<0.05$). Difference in yield can be associated with varying extraction processes as well as the species or tissue used (Karim and Bhat, 2009). The result was in accordance with Muyonga *et al.* (2004b) who reported that the lower yield of gelatin was obtained from young Nile perch skins, compared to adult Nile perch skin. The age of animal used as raw material for gelatin extraction has the influences on the connective tissues and protein compositions (Foegeding *et al.*, 1996). The number of cross-links in collagen increased with increasing age of the animal (Muyonga *et al.*, 2004a). The result suggested that the lower yield of gelatin from seabass with smaller

size might be associated with lower cross-linking in collagen molecule. Leaching of collagen might take place to a higher extent during the swelling process. As a result, the lower yield was gained when skin of seabass with smaller size was used. On the other hand, the cross-links might be attached tightly in the skin matrix. Thus, the loss during swelling could be lower. The yields of fish skin gelatin varied among species, e.g. dog shark (62.3%), tuna (48.1%), rohu (39.55%) (Shyni *et al.*, 2014), cobia (24.1%), croaker (30.3%) (Silva *et al.*, 2014), tiger-toothed croaker (36.8%) and pink perch (27.3%) (Koli *et al.*, 2012). Therefore, size or age of seabass directly affected the extraction yield of gelatin.

4.5.2 Protein patterns

The protein patterns of gelatin extracted from the skin of seabass with different sizes are illustrated in Figure 19. Gelatins from seabass skin had the typical molecular distribution of collagen type I, in which the ratio of α_1-chain and α_2-chain was approximately 2:1 Apart from α-chain, β- and α-chains with MW of 195 and 125-110 kDa, respectively, were obtained. It was noted that G2 sample had α-chains as the major constituents. Commercial fish gelatin also contained α-chains as major components with some degraded proteins. The band intensity of β-chains, γ-chain and high molecular weight cross-link increased when skin from larger size seabass was used for extraction. The increase in band intensity of proteins larger than α–chains was in agreement with the lower degradation. Generally, G2 samples exhibited more degraded peptides with the range of 100-25 kDa. Cross-links with higher MW found in skin of seabass with the average size of 4 and 6 kg/fish might be more resistant to degradation during extraction at 45 °C, compared with small MW components present in skin of smallest size seabass (2 kg/fish). It was noted that the ratio of α_2/α_1 band intensity of G4 and G6 became lower than that of G2. It was presumed that α_2 might under cross-linking to form a larger MW component such as β-, γ-chains as well as high MW cross-links. The protein components of gelatin as well as degradation of proteins contribute to gelling behaviour of gelatin, especially gel strength, setting time and gelling temperature (Kittiphattanabawon *et al.*, 2010). Therefore, the components of gelatin from seabass skin were affected by size of seabass used.

Table 12. Extraction yield, gel color, gelling and melting temperatures of gelatin extracted from seabass skin with different sizes

Gelatin samples	Yield (%, dry wt basis)	L^*	a^*	b^*	ΔE^*	Gelling temperatures (°C)	Melting temperatures (°C)
CF	NM	4.17±0.37a	-0.18±0.36b	-3.35±0.40a	91.27±0.25d	15.84±0.28a	25.10±0.57a
G2	38.22±0.21c	17.07±0.49d	-0.47±0.11a	-1.31±0.14b	73.09±0.68a	17.09±0.42b	26.92±0.35b
G4	40.50±0.31b	16.33±0.34c	-0.43±0.07ab	-0.91±0.11c	74.25±0.44b	18.43±0.29c	27.62±0.63bc
G6	43.48±1.27a	14.46±0.04b	-0.33±0.14ab	-1.19±0.16bc	76.86±0.08c	19.01±0.63c	28.85±0.62c

Mean±SD (n = 3).

Different lowercase letters in the same column indicate significant differences (P< 0.05). NM: Not mentioned

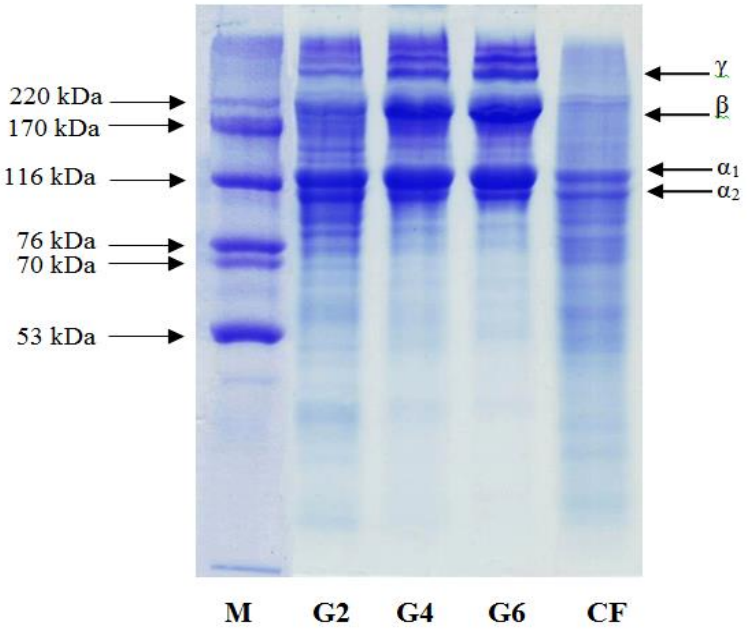

Figure 19. SDS-PAGE patterns of gelatin extracted from the skin of seabass with different sizes. M and CF denote high molecular weight markers and commercial fish gelatin.

4.5.3 Amino acid compositions

Amino acid compositions of gelatin extracted from the skin of seabass with different sizes are presented in Table 13. All gelatins had glycine as the major amino acid (327-337 residues/1000 residues) and had a relatively high content of alanine (123-138 residues/1000 residues). The amino acid composition of all gelatins showed very low contents of cysteine (1 residues/1000 residues), tyrosine (3-4 residues/1000 residues), histidine (5 residues/1000 residues) and hydroxylysine (5 residues/1000 residues). In general, cysteine and tryptophan are not found in collagen and gelatin (Benjakul *et al.*, 2009; Kittiphattanabawon *et al.*, 2010; Sinthusamran *et al.*, 2014). For imino acids, all gelatins contained proline and hydroxyproline contents of 113-128 and 79-82 residues/1000 residues, respectively. The imino acid content of G2 (209 residues/1000 residues) and G4 (207 residues/1000 residues) was higher than that of G6 (192 residues/1000 residues). Commercial fish gelatin had imino acid of 204 residues/1000 residues. Gelatin of skin from young Nile perch (192 residures/1000 residues) showed similar imino acid content to that of skin from adult

Nile perch (193 residues/1000 residues) (Muyonga *et al.*, 2004b). The imino acid content of seabass skin gelatin was higher than that of farmed Amur sturgeon skin (171 residues/1000 residues) (Nikoo *et al.*, 2014), dog shark skin (165 residues/1000 residues), rohu skin (154 residues/1000 residues), tuna skin (160 residues/1000 residues) (Shyni *et al.*, 2014) and grass carp skin (201 residues/1000 residues) (Kasankala *et al.*, 2007). Mammalian gelatins generally contain 30% imino acids, which was higher than fish gelatin (Shyni *et al.*, 2014). The species, environment living habitat and body temperature of fish are the main factors governing hydroxyproline and proline content (Zhang *et al.*, 2012). Moreover, hydroxyproline content of gelatin was also affected by the extraction conditions (Nikoo *et al.*, 2014). The stability of triple-helix in collagen molecule correlated with imino acid content (Sinthusamran *et al.*, 2013). The difference in imino acid content, especially hydroxyproline, was considered as the important factor influencing viscoelastic properties and gel formation of gelatin (Benjakul *et al.*, 2009; Shyni *et al.*, 2014). In the present study, there was similar hydroxyproline content amongst all samples, but G6 showed the lowest proline content. The difference in amino acid composition could be due to the differences in cross-links in the starting skin matrix. With increasing fish age, the cross-linking of collagen in skin can be enhanced (Muyonga *et al.*, 2004b). Those imino acids might contribute to cross-links. As a consequence, the chains rich in proline were not leached out during extraction as indicated by the lower proline in the extracted gelatin. This result suggested that size or age of fish raw materials might determine the amino acid composition of the resulting gelatin.

4.5.4 Fourier transform infrared (FTIR) spectra

FTIR spectra of gelatin extracted from the skin of seabass with various sizes are depicted in Figure 20. FTIR spectroscopy has been used to study the changes in functional groups and the secondary structure of gelatin (Nikoo *et al.*, 2014). All gelatin samples showed similar spectra, which had the major peaks in amide region. G2, G4 and G6 samples exhibited the amide I bands at the wavenumber of 1637, 1638 and 1637 cm^{-1}, respectively. Normally, amide I band of gelatin appeared at 1700-1600 cm^{-1}, which was associated primarily with a C=O stretching vibration/hydrogen bonding coupled with COO (Bandekar, 1992). The absorption in the amide I is

probably the most useful for infrared spectroscopic analysis of the secondary structure of protein (Kittiphattanabawon *et al.*, 2010). In the present study, the amide I peak (1637-1638 cm^{-1}) of all samples was in agreement with Yakimets *et al.* (2005) who reported that the absorption peak at 1633 cm^{-1} was the characteristic of coiled structure of gelatin. The amide I bands of all gelatin samples were shifted to the higher wavenumber, compared with seabass skin collagen (Sinthusamran *et al.*, 2013). Loss of triple helix occurred during heating via breaking down hydrogen-bonds between α-chains (Kaewruang *et al.*, 2013b; Muyonga *et al.*, 2004b). The Amide II band of G2, G4 and G6 appeared at 1540, 1536 and 1536 cm^{-1}, respectively. The amide II vibration modes are related to an out-of-phase combination of a CN stretch and in-plane NH deformation modes of the peptide group (Bandekar, 1992; Lavialle *et al.*, 1982). In addition, amide III was detected around the wavenumber of 1236, 1235 and 1235 cm^{-1} for G2, G4 and G6, respectively, more likely associated with N–H deformation and C–N stretching vibration as well as the absorptions arising from wagging vibrations of CH_2 groups in the glycine backbone and proline side-chains (Jackson *et al.*, 1995; Muyonga *et al.*, 2004a). It was noted that the amide III band of gelatins extracted from skin of seabass with different sizes showed similar spectra.

The amide A band of G2, G4 and G6 samples was found at 3294, 3299 and 3293cm^{-1}, respectively. A free NH stretching vibration is found in the range of 3400-3440 cm^{-1}. However, when NH group of peptide is involved in hydrogen bond, its position is shifted to lower frequencies, usually 3300 cm^{-1} (Doyle *et al.*, 1975). The amide B band was observed at 2918, 2922 and 2923 cm^{-1} for G2, G4 and G6, respectively. It represents CH stretching vibrations of the -CH_2 groups (Nagarajan *et al.*, 2012). Amplitude of Amide B peak was lower in G6 sample and the peak was shifted to the lower wavenumber, compared with other samples. This suggested that CH_2 groups were more likely interacted via cross-linking. This was in accordance with the higher MW cross-links in G6 sample. Therefore, gelatins from seabass skin showed slight difference in the secondary structure, as affected by size of raw material used.

Table 13. Amino acid compositions of gelatins extracted from seabass skin with different sizes

Amino acids	(residues/1000 residues)			
	CF	**G2**	**G4**	**G6**
Alanine	123	136	136	138
Arginine	54	52	51	53
Aspartic acid/asparagine	43	43	44	44
Cysteine	1	1	1	1
Glutamine/glutamic acid	72	70	70	71
Glycine	331	327	328	337
Histidine	5	5	5	5
Isoleucine	11	9	9	9
Leucine	21	18	18	18
Lysine	28	26	26	27
Hydroxylysine	5	5	5	5
Methionine	10	13	14	14
Phenylalanine	12	13	13	13
Hydroxyproline	82	81	79	80
Proline	122	128	128	113
Serine	35	26	27	27
Threonine	23	22	22	2.2
Tyrosine	3	3	4	4
Valine	21	20	20	20
Imino acid	204	209	207	192

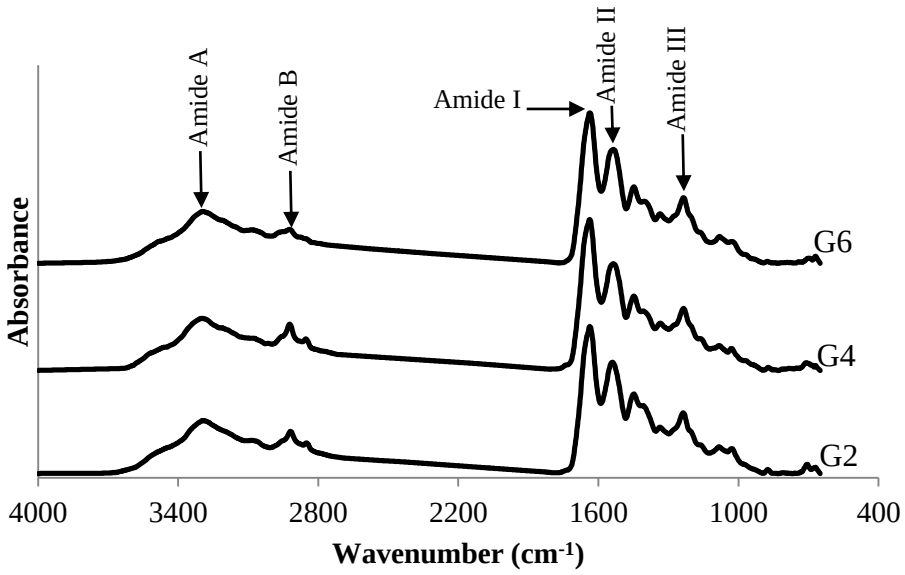

Figure 20. FTIR spectra of gelatin extracted from seabass skinwith different sizes.

4.5.5 Zeta-potential

The ζ-potential of gelatins extracted from the skin of seabass having different sizes tested at different pHs is shown in Figure 21. Generally, gelatin samples showed positive charge at acidic pH ranges and became negatively charged under alkaline conditions. Zero surface net charge was obtained at pH 6.73, 6.41 and 7.12 for G2, G4 and G6, respectively, which were estimate to be their isoelectric points (pI). At pI, the positive charges are balanced out by the negative charges (Bonner, 2007). Gelatin from G6 had higher pI (7.12) than other gelatins extracted from skin of seabass with smaller sizes (G2 and G4). Gelatin from skin of different fish showed varying pIs, e.g 6.65-7.15 for unicorn leatherjacket (Ahmad and Benjakul, 2011), 8.8 for young Nile perch and 9.4 for adult Nile perch (Muyonga *et al.*, 2004b). Thus, the differences in pI of all gelatin samples might be caused by the difference in their amino acid compositions and distribution of amino acid residues, which were more likely influenced by size or age of raw material used.

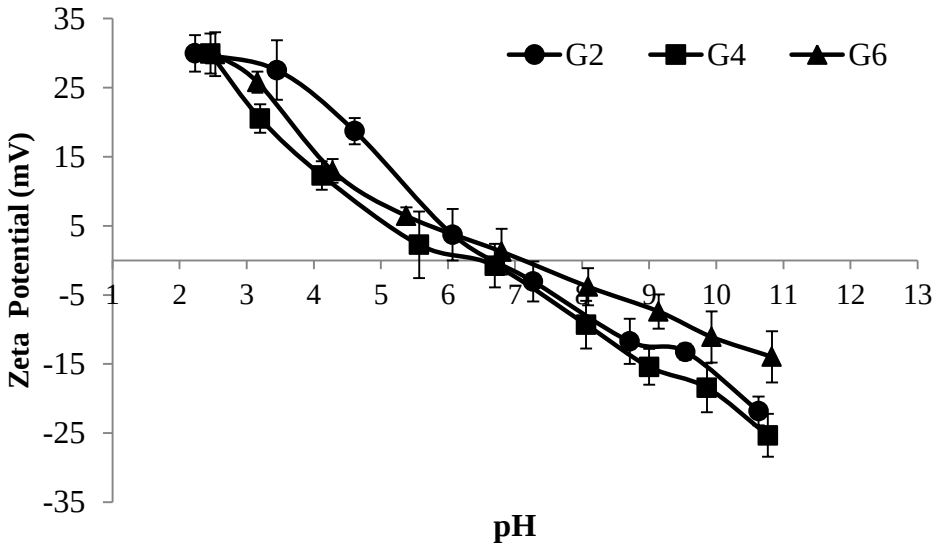

Figure 21. Zeta-potential of gelatins extracted from seabass skin with different sizes. Bars represent the standard deviation (n=3).

4.5.6 Gel strength of gelatin

Gel strength of gelatin from the skin of seabass with different sizes is shown in Figure 22A. Gel strength of gelatin increased as the size of fish increased ($P<0.05$). G6 had the highest gel strength, compared with others ($P<0.05$). All gelatin gels from seabass skin exhibited higher bloom strength (223-322 g) than that of commercial fish gelatin from tilapia skin (212 g) ($P<0.05$). Muyonga *et al.* (2004b) reported that gelatin from young Nile perch skins (222 g) had slightly lower gel strength than adult Nile perch skin gelatin (229 g). The result suggested that gelatin with greater ratio of high-molecular weight components showed the better gelling properties via higher stabilising interactions (Tabarestani *et al.*, 2010). G4 and G6 showed higher band intensity of β-chain, γ-chains and high MW cross-links than G2 (Figure 18). Those large components might serve as the strong strands in gel network. Gelatins with different gel strength were reported for farmed Amur sturgeon skin (141 g) (Nikoo *et al.*, 2014), cobia skin (232 g), croaker skin (212 g) (Silva *et al.*, 2014), dog shark skin (206 g), tuna skin (177 g) (Shyni *et al.*, 2014), tiger-toothed croaker (170 g), pink perch (140 g) (Koli *et al.*, 2012) and grey triggerfish (168 g) (Jellouli *et al.*, 2011). The difference in gel strength amongst species was mainly due to the

different amounts of the β- and α-components and the amino acid composition of gelatin (Sinthusamran *et al.*, 2014). Furthermore, imino acids, especially hydroxyproline, are involved in gel formation of gelatin via hydrogen bonding through hydroxyl group (Benjakul *et al.*, 2012). However, similar hydroxyproline contents were observed amongst all gelatin samples as shown in Table 13. The result suggested that components of gelatin, particularly cross-links, most likely played an essential role in gel formation and determined gel strength of gelatin.

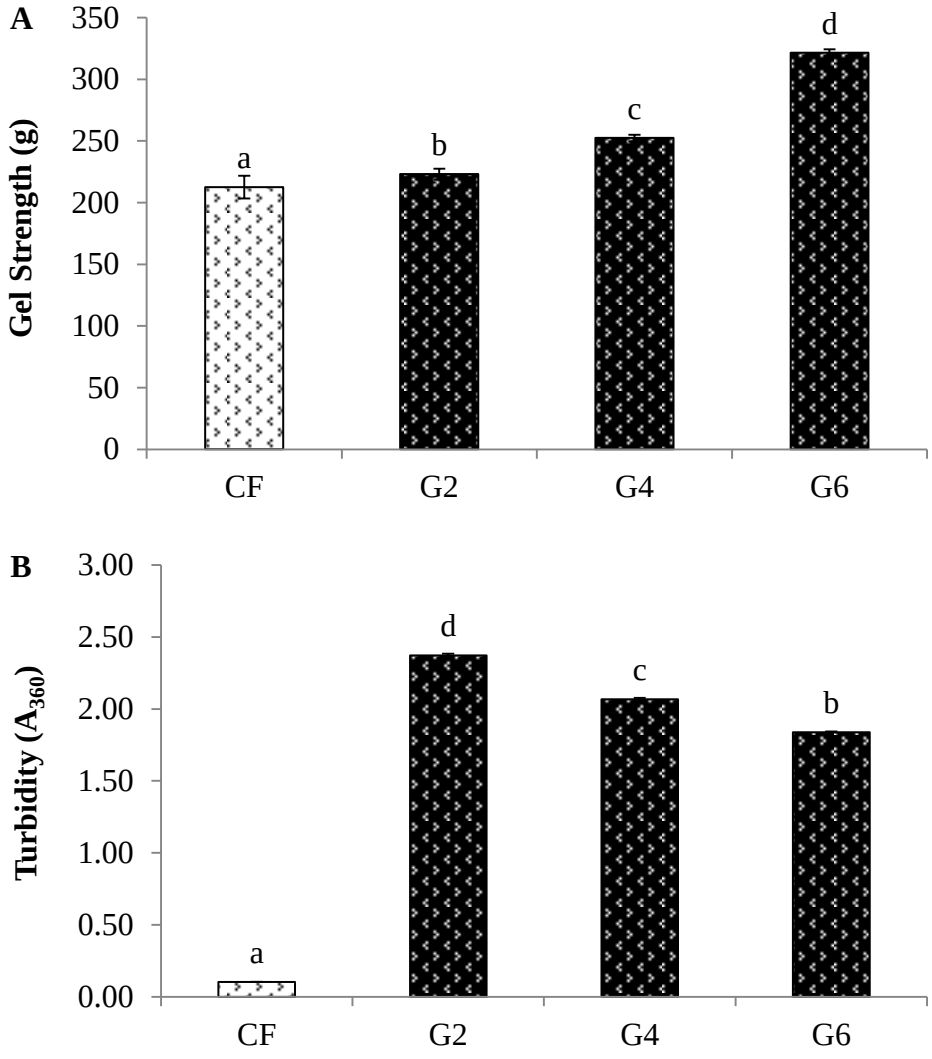

Figure 22. Gel strength (A) and turbidity (B) of gelatin extracted from seabass skin with different sizes. CF denotes commercial fish gelatin. Bars represent the standard deviation (n=3). Different lowercase letters on the bars indicate significant differences ($P< 0.05$).

4.5.7 Color and turbidity of gelatin gel

Colors of different gelatin gels from skin of seabass and commercial fish gelatin expressed as L^*, a^*, b^* and ΔE^* are shown in Table 12. Gelatin gel from G2 showed the higher L^*-value (lightness) than others (G4 and G6) ($P<0.05$). The L^*-value of gelatin gels slightly decreased when skin from seabass with larger sizes was used ($P<0.05$). G2 sample had the lower redness value (a^*-value), compared to commercial fish gelatin ($P<0.05$). No differences in a^*-value were found amongst gelatins from seabass with different sizes ($P>0.05$). Additionally, gelatin gels from seabass had higher b^*-value than that from commercial fish gelatin ($P<0.05$). Slight differences in b^*-value were found amongst gelatin gels from seabass skin with different sizes ($P<0.05$). For total difference in the color value (ΔE^*), commercial fish gelatin showed the highest ΔE^*-value (91.27), compared with gelatin gels from seabass skin. ΔE^*-value increased as size of seabass used for gelatin extraction increased. This trend was in agreement with the decrease in L^*-value as the size of fish used increased. Thus, size or age of seabass as raw material had the impact on color of gelatin and their gels.

Turbidity of solutions of gelatin extracted from skin with different sizes expressed as A_{360} is shown in Figure 22B. Gelatin solution exhibited the lower turbidity when size of seabass used for extraction increased ($P<0.05$). The highest turbidity was found in solution of G2 sample ($P<0.05$). Turbidity of gelatin solution from commercial fish gelatin was much lower, compared with all gelatins from seabass skin ($P<0.05$). The turbidity of gelatin solution is dependent on species of raw material, gelatin extraction process and clarification/filtration process (Koli *et al.*, 2012; Muyonga *et al.*, 2004b). In this study, only filtration process was implemented, whilst the clarification process has been used for commercial gelatin to remove both light- and heavy-impurities (Muyonga *et al.*, 2004b). Koli *et al.* (2012) reported that inorganic, protein and muco-substance contaminants mainly affected turbidity and dark color of gelatin solution. Both G4 and G6 samples contained high MW components at higher level than G2 (Figure 18). Solution of G2 sample with smaller peptides or proteins might prevent transmission of light more effectively, thereby increasing the light scattering. This might contribute to higher turbidity of gelatin

solution obtained from skin of fish with smaller sizes. These results showed that the size or age of raw materials had the impact on turbidity of gelatin solution.

4.5.8 Setting time for gel formation

The setting times required for the gel formation of gelatin extracted from the skin of seabass having different sizes at 4 °C and room temperature (26±2 °C) are presented in Figure 23A and 23B, respectively. The setting times at 4 °C of G2, G4 and G6 were 2.13, 2.80 and 3.60 min, respectively. The setting time at 4 °C of gelatin increased as seabass size used for gelatin extraction increased ($P<0.05$). G2 and G4 samples had a shorter setting time at 4 °C than the commercial fish gelatin ($P<0.05$). Due to the higher proportion of high MW peptide chains of G6, the arrangement of those chains formation of gel network plausibly took a longer time as indicated by longer setting time. The longer setting time at 4 °C of G6 might be also governed by the slower rate of interaction between those chains, particularly via hydrophobic interaction. Hydrophobic-hydrophobic interaction was lowered at low temperature (Dias *et al.*, 2010). In addition, the nucleation zone during gel formation required hydroxyproline (Ledward, 1989). With high proportion of cross-links or β- and γ-chains, hydroxyproline residues could be localized inside the molecules. This could lower the accessibility or exposure of hydroxyproline to form H-bond. Thermal degradation of peptides in gelatin during heating extraction affected the setting time of gelatin gel (Sinthusamran *et al.*, 2014).

When setting was carried out a room temperature, setting times of 32.0, 28.83 and 19.13 min were found for G2, G4 and G6, respectively. Gelatin samples from seabass skin had the longer setting time when small size of seabass was used ($P<0.05$). All gelatins from seabass were able to undergo setting at room temperature. This result was in agreement with Sinthusamran *et al.* (2014) who reported that gelatin from seabass skin was able to set at room temperature within 30 min. Conversely, commercial fish gelatin was not able to set within 3 h at room temperature. Setting phenomenon of gelatin samples at room temperature was different from that occurred at lower temperature (4 °C). At higher setting temperatures, higher entropy of gelatin molecules was presumed. Those γ- and β-components found in G4 or G6 samples might promote the formation of gel network

with rapid gelation (Fernández *et al.*, 2003). When those high MW cross-links aligned themselves under the high entropy conditions, they could form gel easily as evidenced by lower setting time. Different extraction condition, especially extraction temperature and time also influenced the setting time of gelatin (Kittiphattanabawon *et al.*, 2010; Muyonga *et al.*, 2004b). Furthermore, difference in setting time of fish gelatin might be due to different age or size of fish used as raw material (Shyni *et al.*, 2014). Thus, setting time could be affected by size of seabass used as raw material. Additionally, setting temperature was another factor affecting time employed for gel formation.

4.5.9 Gelling and melting temperatures

Gelling and melting temperatures of gelatin extracted from the skin of seabass with different sizes are presented in Table 12. Thermal transition of gelatin solution were monitored by changes in phase angle (δ) during cooling (35-5 °C, 0.5 °C/min) and subsequent heating (5-35 °C, 0.5 °C/min). The formation of junction zones in the three-dimensional network of gelatin gel can be monitored in term of changes of phase angle (Sinthusamran *et al.*, 2014). The gelling temperatures were 17.09, 18.43 and 19.01 °C for G2, G4 and G6, respectively, which were higher than commercial fish gelatin (15.84 °C) ($P<0.05$). The higher gelling temperatures were observed in G4 and G6 samples extracted from the skin of larger size, in comparison with that of smaller size (G2) ($P<0.05$). The gelling temperature of gelatin tended to increase with increasing amount of cross-links. This result indicated that the molecular weight distribution in gelatin was associated with gelation. Muyonga *et al.* (2004b) also reported that chemical compositions, especially the content of γ-, β-, and α-chain components influenced gelling point of gelatin gel. Additionally, different gelling temperature was related to amino acid composition, especially proline and hydroxyproline, which is governed by temperatures of habitat, where fish live (Jeya Shakila *et al.*, 2012; Nikoo *et al.*, 2014). The imino acid content is normally available to form hydrogen bonds with water (Karim and Bhat, 2009). In general, warm water fish or mammalian gelatin has higher gelling and melting temperatures than the cold water fish gelatin (Nikoo *et al.*, 2014). Most of fish gelatin had lower gelling

temperature than mammalian gelatin (Jeya Shakila *et al.*, 2012). Varying gelling temperatures were reported for gelatin from different fish, e.g. cobia skin (19.9 °C), croaker skin (17.8 °C) (Silva *et al.*, 2014), dog shark skin (20.8 °C), tuna skin (18.7 °C) (Shyni *et al.*, 2014), farmed Amur sturgeon skin (14.2 °C) (Nikoo *et al.*, 2014), red snapper bone (16.0 °C) and grouper bone (16.0 °C) (Jeya Shakila *et al.*, 2012).

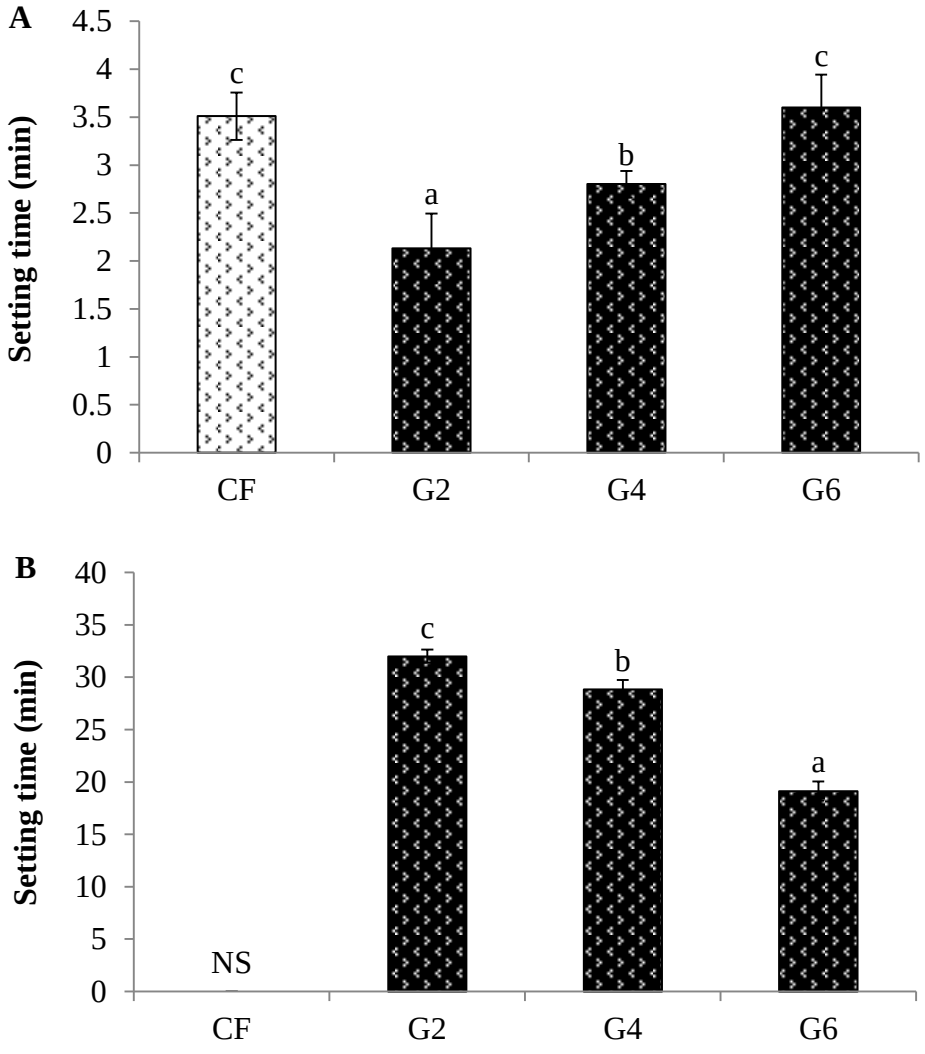

Figure 23. Setting time of gelatin extracted from the skin of seabass with different sizes at 4 °C (A) and room temperature (26±2 °C) (B). NS: Gel was not set within 3 h. Bars represent the standard deviation (n=3). Different lowercase letters on the bars indicate significant differences ($P<0.05$).

Melting temperatures of G2, G4 and G6 were 26.92, 27.62 and 28.85 °C, respectively. The melting temperatures of gelatin from seabass skin were higher than that of commercial fish gelatin (25.10 °C) ($P<0.05$). Nevertheless, the melting temperature of gelatins from seabass skin were lower than those of bovine bone (29.7 °C) and porcine skin (32.3 °C) (Gudmundsson, 2002). It was noted that the melting temperatures of all gelatins from seabass skin were higher than those of gelatins from the dog shark skin (25.8 °C), tuna skin (24.2 °C) (Shyni *et al.*, 2014), farmed Amur sturgeon skin (22 °C) (Nikoo *et al.*, 2014), tiger-toothed croaker skin (20.36 °C), pink perch skin (19.23 °C) (Koli *et al.*, 2012) and tilapia skin (22.4 °C) (Zeng *et al.*, 2010). Since seabass used in the present study was tropical fish (25-32 °C), gelatin gel might be more stable than those from temperate zone (15-25 °C) (Haugen and Sanger, 2011). Thermal stability of gelatin gel is associated with the temperature of habitat of the animals (Karim and Bhat, 2009). G4 and G6 samples had higher melting temperature than G2 ($P<0.05$). High MW components might contribute to the stability and stronger network of gel.

In the present study, G6 showed the higher gelling as well as melting temperatures, although it showed the lower imino acid content than G2 and G4. It was noted that G6 had the higher band intensity of β-chains, γ-chain and high molecular weight cross-link, compared with G2 and G4 (Figure 19). Due to the higher percentage of β- and γ-components, a shorter maturation time might be required since the entanglement of chain took place with ease. Also, those large components might serve as the strong strands in gel network, leading to the increased thermal stability of the gelatin gel (Wangtueai *et al.*, 2010) as indicated by the higher melting temperature. As a consequence, G6 had higher gelling and melting temperatures. Gelatin extracted from tropical fish has higher gelling and melting temperature than that gelatin extracted from temperate fish. Thus, the differences in gelling and melting temperature of seabass gelatin might be governed by the differences in components, especially those with high MW.

4.5.10 Microstructures of gelatin gels

Microstructures of the gel from commercial fish gelatin and gels from gelatin extracted from skin of seabass with different sizes are shown in Figure 24. Generally, all gelatin gels from seabass skin were sponge or coral-like in structure. The gel from G2 had a looser network with larger voids and thinner strands, compared with gels from other gelatin samples (G4 and G6). G6 showed thicker strands in gel network, compared with others. The gelation process was governed by cross-linked polypeptide network stabilized mainly by hydrogen-bonded junction zones (Benjakul *et al.*, 2012). Gelatin with high molecular weight distribution could form the junction zones with ordered gel structure, leading to higher gel strength. Sinthusamran *et al.* (2014) reported that gelatin extracted from the seabass skin at milder condition (45 °C for 3 h) had a higher gel strength with the finest structure and smaller voids in gel matrix, compared with gelatin extracted under the harsher conditions. Zhang *et al.* (2012) reported that the denser strands of gel structure were governed by greater content of high molecular weight peptides (γ- and β-chains) in gelatin, whilst looser strands in gel matrix were found in gelatin sample containing smaller and shorter peptides. Based on protein pattern (Figure 1), it was found that gelatin extracted from skin of seabass with the larger sizes contained higher content of longer or larger peptides chains. Those chains could build up the strong strands, which could strengthen the network as evidenced by the stronger gel strength.

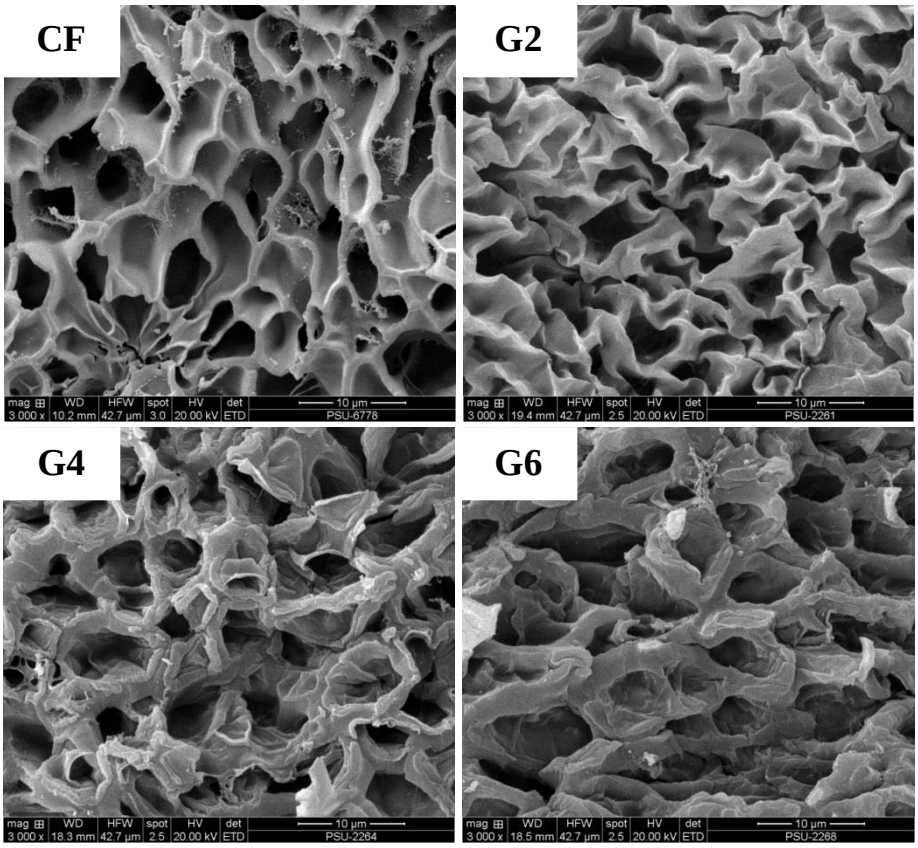

Figure 24. Microstructures of gel from commercial fish gelatin (CF), gelatin extracted from seabass skin with the average size of 2 kg/fish (G2), 4 kg/fish (G4) and 6 kg/fish (G6). Magnification: 3000 times.

4.6 Conclusion

Yield and gelling properties of gelatin were influenced by the size of seabass. Gelatin obtained from the skin of seabass with 6 kg/fish had the highest yield and better gelling properties. This was associated with the higher content of high MW components or cross-links. Nevertheless, gels of all gelatins were able to set at room temperature. The gelling and melting temperatures were in the range of 16.49-18.64 °C and 26.92-27.69 °C, respectively, which were higher than that of commercial fish gelatin. Thus, skin from seabass with different size used as raw material for gelatin extraction had the influence on chemical compositions and properties of gelatin.

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CHAPTER 5

CHARACTERISTICS AND FUNCTIONAL PROPERTIES OF GELATIN FROM SEABASS (*LATES CALCARIFER*) SWIM BLADDER

5.1 Abstract

Characteristics and properties of gelatin from seabass swim bladder extracted at different temperatures (45, 55, 65 and 75 °C) were studied. The yield and recovery of gelatin from swim bladder (44.83-71.95% and 49.08-74.83%, based on dry weight) increased with increasing extraction temperatures. All gelatins contained α-chains as the predominant components, followed by β-chain. Gelatin from seabass swim bladder showed a high imino acid content (195 residues/1000 residues). FTIR and CD spectra revealed the loss of triple helix during heating via breaking down hydrogen bonds between α-chains. Gel strength increased as the extraction temperature increased up to 65 °C ($P<0.05$). Gelatin extracted at 65 °C for 6 h showed a higher gel strength, compared to commercial bovine gelatin ($P<0.05$). Gelling and melting temperatures were 10.4-19.7 and 19.3-28.4 °C, respectively, depending on extraction temperature. Therefore, seabass swim bladder could serve as an alternative collagenous material for gelatin production.

5.2 Introduction

Gelatin is a fibrous protein produced by thermal denaturation of collagen. It represents a major biopolymer with wide range of applications in food, pharmaceutical, cosmetic and photographic industries (Regenstein and Zhou, 2007). In general, the main sources of gelatin production are porcine and bovine skin and bones (Kittiphattanabawon *et al.*, 2010). Nevertheless, religious restriction has led to an increasing interest in alternative sources for gelatin (Sinthusamran *et al.*, 2014). Fish processing by-products have become important for gelatin production. Fish gelatin can be extracted from skin of several aquatic animals including clown featherback (Kittiphattanabawon *et al.*, 2016), Nile tilapia and channel catfish

(Zhang *et al.*, 2016), octopus (Jridi *et al.*, 2015), cobia (Silva *et al.*, 2014) and unicorn leatherjacket (Kaewruang *et al.*, 2013b). Nevertheless, the utilization of fish gelatin is limited, due to its poor gelling property (Muyonga *et al.*, 2004). Moreover, it has been known that extraction conditions, especially extraction temperature and time, determine the properties of gelatin (Sinthusamran *et al.*, 2014). Increasing extraction temperature and time (harsh conditions) generally render the gelatin with the higher yield, but decreased gelling properties (Kittiphattanabawon *et al.*, 2016). Thus, extraction condition, including temperature and time, should be optimized.

Seabass (*Lates calcarifer*) is an economically important fish in Thailand. A large amount of seabass has been exported as well as domestically consumed (Sinthusamran *et al.*, 2015). In general, by-products including skins, bones, scales and swim bladder, constituting around 30%, are generated during dressing (Alfaro *et al.*, 2014; Sinthusamran *et al.*, 2013). Skin, bone and scales have been widely used for gelatin production to increase the value. However, seabass swim bladder rich in collagen has been rarely used for gelatin extraction. Swim bladders from a few marine fish species were used for the production of isinglass (fining agents) (Chandra and Shamasundar, 2015). Swim bladder of fresh water carp was used for gelatin extraction with high yield (13.5%) and gel strength (264.6 g) (Chandra and Shamasundar, 2015). Thus, the seabass swim bladder can be the potential source for production of gelatin with prime quality. Nevertheless, no information regarding the characteristics and properties of gelatin from seabass swim bladder has been reported.

5.3 Objective

To investigate the extraction of gelatin from swim bladder of seabass (*Lates calcarifer*) at various temperatures and to determine properties of resulting gelatins.

5.4 Materials and methods

5.4.1 Chemicals

All chemicals were of analytical grade. 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). Fish gelatin (FG) produced from tilapia skin (~240 bloom) was obtained from Lapi Gelatine S.p.A (Empoli, Italy). Food grade bovine bone gelatin (BG) with the gel strength of 150–250 g was purchased from Halagel (Thailand) Co., Ltd. (Bangkok, Thailand).

5.4.2 Preparation of seabass swim bladder

Swim bladders of seabass (*L. calcarifer*) with a length of 20-25 cm were obtained from Sirikhun Seafood Co., Ltd, Samutsakhon, Thailand. The frozen swim bladders packed in a polyethylene bag, were kept in ice with a skin/ice ratio of 1:3 (w/w) using a polystyrene box as a container and transported to the Department of Food Technology, Prince of Songkla University, within 10 h. Upon arrival, frozen swim bladders were thawed with running water until the core temperature reached 8-10 °C. The samples were washed with tap water and cut into small pieces (1×1 cm^2) using scissors. Prepared swim bladder samples were placed in polyethylene bags and stored at -20 °C until used, but not longer than 2 months. Swim bladder had moisture, protein, fat and ash content of 69.74, 29.67, 0.34 and 0.23%, as determined by AOAC methods with the analytical numbers of 927.05, 984.13, 920.39 B and 942.05, respectively (AOAC, 2000). The conversion factor of 5.4 was used for calculation of protein content. It was rich in hydroxyproline (24.48 mg/g) as measured by the method of Bergman and Loxley (1963).

5.4.3 Extraction of gelatin from swim bladder

Gelatin was extracted from swim bladder according to the method of Jongjareonrak *et al.* (2006) with a slight modification. Before gelatin extraction, swim

bladder was soaked in 0.1 M NaOH, with a sample/solution ratio of 1:10 (w/v), to remove non-collagenous proteins. The mixture was stirred continuously for 3 h at room temperature (28–30 °C) using an overhead stirrer with a propeller (W20.n IKA®-Werke GmbH & CO.KG, Staufen, Germany). The alkaline solution was changed every 1 h for totally 3 times. The residues were then washed with tap water until a neutral or faintly basic pH was obtained. The deproteinized matters were then mixed with 0.05 M acetic acid at a sample/solution ratio of 1:10 (w/v) to swell collagenous material. The mixture was stirred at room temperature for 2 h. The swollen swim bladders were washed using tap water until wash water became neutral or faintly acidic in pH.

To extract the gelatin, the swollen swim bladders were mixed with distilled water at a ratio of 1:10 (w/v) at 45, 55, 65 and 75 °C for 6 h with continuous stirring. The mixtures were filtered with two layers of cheesecloth. Then, the filtrates were mixed with 1 % (w/v) activated carbon for 1 h with continuous stirring. The mixtures were centrifuged at 17,500x*g* for 15 min at 25 °C using a Beckman model Avanti J-E centrifuge (Beckman Coulter, Inc., Palo Alto, CA, USA) to remove insoluble material. The supernatants were filtered using a Buchner funnel with Whatman No.4 filter paper (Whatman International, Ltd., Maidstone, England). Finally, the filtrates were freeze-dried using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark) at -50 °C for 72 h. The dry gelatins extracted from swim bladder at 45, 55, 65 and 75 °C were referred to as ‘G45’, ‘G55’, ‘G65’ and ‘G75’, respectively. The gelatin samples were subsequently subjected to analyses.

5.4.4 Analyses

5.4.4.1 Determination of yield and recovery

The yield of gelatin was calculated based on dry weight of starting material.

$$\text{Yield (\%)} = \frac{\text{Weight of freeze-dried gelatin (g)}}{\text{Weight of initial dry swim bladder (g)}} \times 100$$

Recovery was also determined using the following equation:

$$\text{Recovery (\%)} = \frac{[\text{HYP of gelatin (g/g)} \times \text{weight of gelatin (g)}]}{[\text{HYP of initial swim bladder (g/g)} \times \text{weight of initial swim bladder (g)}} \times 100$$

Hydroxyproline (HYP) content in both gelatin and initial swim bladder was determined according to the method of Bergman and Loxley (1963).

5.4.4.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS–PAGE was performed by the method of Laemmli (1970). Gelatin samples were dissolved in 5% SDS solution. The obtained mixtures were then heated at 85 °C for 1 h using a temperature controlled water bath model W350 (Memmert, Chwabach, Germany). Solubilized 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 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-Red Laboratories,Hercules, CA, USA) with Molecular Analyst Software version 1.4 (image analysis systems).

5.4.4.3 Fourier transform infrared (FTIR) spectroscopic analysis

FTIR spectra of gelatin samples were obtained using a FTIR spectrometer (EQUINOX 55, Bruker, Ettlingen, Germany) equipped with a deuterated l-alanine tri-glycine sulphate (DLATGS) detector. A horizontal attenuated total reflectance accessory (HATR) was mounted into the sample compartment. The internal reflection crystal (Pike Technologies, Madison, WI, USA), made of zinc selenide, had a 45° angle of incidence to the IR beam. Spectra were acquired at room temperature, at a resolution of 4 cm^{-1} and the measurement range was 4000–400 cm^{-1}

(mid-IR region). Automatic signals were collected in 32 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, Ettlingen, Germany).

5.4.4.4 Circular dichroism (CD) spectroscopic analysis

Gelatin samples (0.05 mg protein/mL) were dissolved in 10 mM sodium phosphate buffer (pH 7.0) according to the method of Wierenga *et al.* (2003). CD spectra were measured at 20 °C, with a scan speed of 100 nm/min from 190 to 250 nm, a data interval of 0.2 nm, a bandwidth of 1 nm and a response time 0.125 s using a JASCO J-801 spectrometer (Jasco Corp, Tokyo, Japan). The secondary structure was estimated using a CDPro software with the reference spectra as described by Johnson (1999).

5.4.4.5 Surface hydrophobicity

Surface hydrophobicity of gelatin samples was determined by the method of Benjakul, Seymour, Morrissey and An (1997b). Gelatin was dissolved in 10 mM phosphate buffer, pH 6.0, containing 0.6 M NaCl to obtain a final protein concentration of 5 g/L. The gelatin solution was diluted to 0.125, 0.25, 0.5, and 1 g/L using the same buffer. The diluted gelatin solutions (2 mL) were well mixed with 10 μL of 8 mM 1-anilinonaphthalene-8-sulfonic acid (ANS) in 0.1 M phosphate buffer, pH 7.0. The relative fluorescence intensity of ANS-protein conjugates was measured using a spectrofluorometer (RF-15001, Shimadzu, Kyoto, Japan) at the excitation wavelength of 374 nm and the emission wavelength of 485 nm. Surface hydrophobicity was calculated from initial slopes of plots of relative fluorescence intensity versus protein concentration (g/L) using linear regression analysis. The initial slope was referred to as SoANS.

5.4.4.6 Determination of gel strength

Gelatin gel was prepared by the method of Kittiphattanabawon et al. (2010). Gelatin was mixed with distilled water (60 °C) to obtain the concentration of

6.67% (w/v). The solution was stirred until gelatin was solubilized completely and transferred to a cylindrical mold with 3 cm diameter and 2.5 cm height. The solution was incubated at the refrigerated temperature for 18 h prior to analysis.

Gel strength was determined at 8-10 °C using a texture analyzer (Stable Micro System, Surrey, UK) with a load cell of 5 kg, cross-head speed of 1 mm/s, equipped with a 1.27 cm diameter flat-faced cylindrical Teflon® plunger. The maximum force (gram) was recorded, when the plunger had penetrated 4 mm into the gel samples.

5.4.4.7 Determination of gel color

The color of gelatin gels (6.67% w/v) was measured by a Hunter lab colorimeter (Color Flex, Hunter Lab Inc., Reston, VA, USA). L^*, a^* and b^* values indicating lightness/brightness, redness/greenness and yellowness/blueness, respectively, were recorded. The colorimeter was warmed up for 10 min and calibrated with a white standard. Total difference in color (ΔE^*) was calculated according to the following equation (Gennadios *et al.*, 1996):

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

where ΔL^*, Δa^* and Δb^* are the differences between the corresponding color parameter of the sample and that of white standard (L^* = 90.77, a^* = -1.27 and b^* = 0.50).

5.4.4.8 Determination of gelling and melting temperatures

Gelling and melting temperatures of gelatin samples were measured following the method of Boran *et al.* (2010). The measurement was performed using a RheoStress RS 1 rheometer (HAAKE, Karlsruhe, Germany) in the oscillatory mode. The measuring geometry used was a stainless steel 60-mm-diameter parallel plate and the gap was set at 1.0 mm. Gelatin solution (6.67%, w/v) was prepared in the same manner as described in the methods. The solution was preheated at 60 °C for 30 min.

Then the solution (2.9 mL) was loaded on the Peltier plate and equilibrated at 60 °C for 10 min before measurements. The measurements were conduct at a constant frequency of 1 Hz, and a constant applied stress of 3 Pa. The samples were cooled from 60 to 5 °C and subsequently heated to 60 °C at a constant rate of 1.0 °C/min. The gelling and melting temperatures were calculated, where tan δ became 1 or δ was 45°.

5.4.4.9 Microstructure analysis of gelatin gel

Microstructure of gelatin gel (6.67%, w/v) prepared as previously described was visualized using a scanning electron microscopy (SEM). 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 with serial concentrations 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 acceleration voltage of 20 kV.

5.4.5 Characterization of selected gelatin

5.4.5.1 Proximate analysis

Proximate compositions were determined as per AOAC method (AOAC, 2000).

5.4.5.2 Amino acid analysis

Amino acid composition of gelatin samples was analyzed using an amino acid analyzer. Gelatin samples were hydrolyzed under reduced pressure in 4 M methane sulphonic acid containing 0.2% 3-2(2-aminoethyl)indole at 115 °C for 24 h. The hydrolysates were neutralized 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 analyzer (MLC-703; Atto Co., Tokyo, Japan).

5.4.6 Statistical analysis

All experiments were run in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA) and mean omparisons were carried out using the Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for windows: SPSS Inc., Chicago, IL, USA).

5.5 Results and discussion

5.5.1 Yield and recovery of gelatin

Yield and recovery of gelatin from seabass swim bladder extracted at different temperatures varied as shown in Table 14. Yields of 44.83-71.95% and recovery of 49.08-74.83% were obtained. The yield was the amount of solid released from pretreated swim bladder matrix, while the recovery represented the percentage of collagen converted to gelatin by heat (Kaewruang *et al.*, 2013a). Both extraction yield and recovery increased as the extraction temperature increased ($P<0.05$). The highest yield (71.95%) was found in gelatin extracted at 75 °C (G75). However, there was no difference in the recovery between G65 and G75 ($P>0.05$). Similar results were reported for gelatin from clown featherback skin (Kittiphattanabawon *et al.*, 2016) and seabass skin (Sinthusamran *et al.*, 2014), in which the yield of gelatin increased as extraction temperatures increased. The higher temperature used for extraction more likely provided higher energy, thereby destroying hydrogen bonds stabilizing the triple helix in collagen structure to higher extent. As a consequence, α- and β-chains were more released into medium, resulting in the higher amount of gelatin obtained (Kaewruang *et al.*, 2013a; Sinthusamran *et al.*, 2014). Collagen from seabass swim bladder showed a T_{max} of 35.02 °C (Sinthusamran *et al.*, 2013). In the present study, extraction temperatures were above T_{max} and could induce the denaturation of collagen to gelatin. By raising the temperature of extraction, higher conversion of collagen to gelatin could be achieved as shown by the increased recovery.

Table 14. Extraction yield and recovery of gelatin from swim bladder of seabass extracted at different temperatures

Samples	Yield (%)	Recovery (%)
G45	44.83±1.33a	49.08±1.45a
G55	55.35±1.33b	63.77±1.49b
G65	64.69±1.67c	73.27±1.91c
G75	71.95±2.31d	74.83±2.40c

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

Different lowercase letters within the same column indicate significant differences (P<0.05).

5.5.2 Protein pattern

Protein patterns of gelatin from seabass swim bladder extracted at different temperatures are illustrated in Figure 25. All gelatin samples consisted of α_1- and α_2-chains with MW of 130 and 115 kDa, respectively, as the major constituents. FG and BG also contained α-chains as major components. MW of α_1-chain (150 kDa) and α_2-chain (133 kDa) of BG was higher than that from other gelatin samples. Among gelatin from swim bladders, that extracted at 45 °C had the lowest band intensity of α-chains as well as β- and γ -chains. With lower heat, energy was not sufficient to destroy the bondings stabilizing triple helix of mother collagen. As a consequence, all components were released to lower degree as evidenced by lower yield and recovery (Table 14). Additionally, some heat labile proteins might be co-extracted at 45 °C as shown by the higher band intensity of protein with MW lower than α-chains. However, the band intensity of protein with MW lower than α-chain decreased with increasing the extraction temperature. This might be associated with precipitation and aggregation of those low MW contaminating proteins at high temperature. During centrifugation or filtration, those precipitated proteins could be removed. Nevertheless, slight decrease in band intensity of both γ- and β-chains was found in G75. This might be caused by degradation induced by the thermal process (Sinthusamran *et al.*, 2014). Kittiphattanabawon *et al.* (2016) reported that bands of α-, β-, and γ-chains were more degraded with increasing extraction temperature and time. Sinthusamran *et al.* (2014) also found that the band intensity of protein components in gelatin from seabass skin slightly decreased with increasing extraction

temperatures. The result reconfirmed that extraction temperatures played a paramount role in components of gelatin extracted from collagenous matrix of seabass swim bladder.

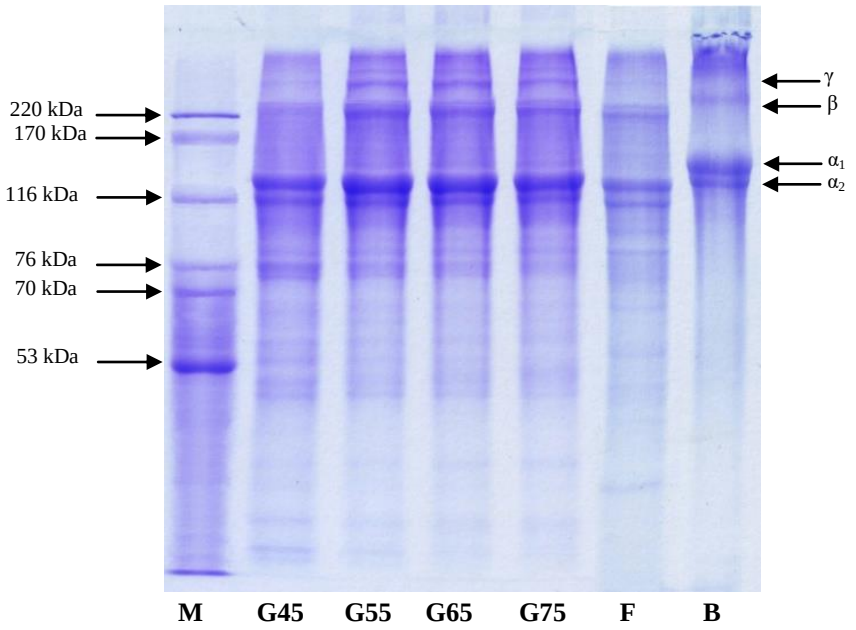

Figure 25. SDS-PAGE patterns of gelatin from seabss swim bladder extracted at different temperatures. G45, G55, G65 and G75 denote gelatin from seabass swim bladder extracted at 45, 55, 65 and 75 °C, respectively, for 6 h. M, F and B denote high molecular weight markers, commercial fish gelatin and bovine gelatin, respectively.

5.5.3 Fourier transform infrared (FTIR) spectra

FTIR spectra of gelatin from the swim bladder of seabass extracted at various temperatures are presented in Figure 26. Generally, all gelatin samples showed similar spectra, in which amide A, amide B, amide I, amide II and amide III bands were detected. The amide I band of G45, G55, G65 and G75 was found at wavenumbers of 1636, 1632, 1632 and 1639 cm^{-1}, respectively. This band is related to a C=O stretching vibration coupled to contributions from the CN stretch (Payne and Veis, 1988). Amide I band of all gelatin samples, especially G75 was shifted to higher wavenumber. The result was in agreement with the slight decrease in α- and β-chain of G75. Kittiphattanabawon *et al.* (2010) reported that gelatin extracted from shark

skins at the higher temperature with longer time generally contained a higher amount of low molecular weight components, in which the C=O group were more exposed. Amide II band of G45, G55, G65 and G75 was observed at wavenumber ranging from 1529 to 1546 cm^{-1}. Amide II band represents the combination between bending vibration of N-H groups and stretching vibrations of C-N groups (Nagarajan *et al.*, 2012). Furthermore, the amide III bands of all gelatin samples were observed at wavenumbers of 1233-1238 cm^{-1}, demonstrating disorder from an α-helical to a random coil structure, leading to the loss of triple helix state. The amide III was more likely associated with C-N stretching vibrations and N-H deformation from the amide linkages as well as the absorptions arising from wagging vibration of CH_2 groups in the glycine backbone and proline side-chains (Muyonga *et al.*, 2004). In the present study, amide III peak of G75 had the lower amplitude when compared with that of other gelatin samples. This suggested that G75 plausibly had more disordered structure due to higher heat applied for extraction.

Amide A band of G45, G55, G65 and G75 samples was located at 3294, 3298, 3296 and 3294 cm^{-1}, respectively. Amide A arises from the stretching vibrations of the NH group coupled with hydrogen bonding (Nagarajan *et al.*, 2012). Normally, a free NH stretching vibration is found in the range of 3400-3440 cm^{-1}. When the NH group of a peptide is involved in a H-bond, the position is shifted to lower frequency, usually 3300 cm^{-1} (Doyle *et al.*, 1975). Additionally, the higher amplitude of amide A was found in G75, which was associated with the degradation of gelatin, leading to higher free amino groups. However, lower wavenumber of G75 also implied that NH groups of this sample more likely interacted with adjacent chains via H-bond, resulting in the decreased wavenumber. Amide B band was observed at 2932, 2920, 2922 and 2924 cm^{-1} for G45, G55, G65 and G75, respectively. It represents CH stretching vibrations of the $–CH_2$ groups (Nagarajan *et al.*, 2012). Amide B peaks of G55 and G65 were shifted to the lower wavenumber, compared with other samples. The result suggested that CH_2 groups were plausibly interacted with adjacent molecules. This was in accordance with the higher MW cross-links in both G55 and G65. Therefore, the extraction temperature affected the secondary and functional group of gelatins obtained from swim bladder of seabass.

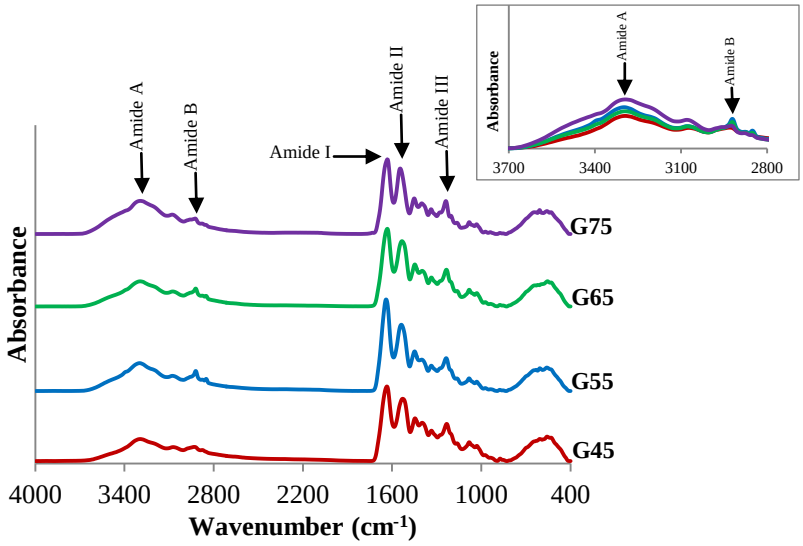

Figure 26. FTIR spectra of gelatins from seabass swim bladder extracted at different extraction temperatures. G45, G55, G65 and G75 denote gelatin from seabass swim bladder extracted at 45, 55, 65 and 75 °C, respectively, for 6 h. Bars represent the standard deviation (n=3).

5.5.4 CD-spectra

CD-spectra of gelatin from swim bladder extracted at different temperatures are depicted in Figure 27. Circular dichroic (CD) spectroscopy has been used to characterize the secondary structure of protein (Li *et al.*, 2008). It can also be used to analyze the conformation of peptides (Greenfield, 2006). Native collagen generally exhibited a CD spectra with a positive peak at around 220-230 nm and a negative peak at around 200 nm (Aewsiri *et al.*, 2011). After complete denaturation of collagen, the positive peak at 220-230 nm, characteristic of the triple-helix, disappears completely and only the negative peak at 200 nm of gelatin remains (Aewsiri *et al.*, 2011). The negative peak at 200 nm was related to the random conformation of gelatin (Wu *et al.*, 2007). In the present study, CD spectra of all samples from seabass swim bladder showed the maximum negative peak at 197-200 nm, but the positive peak in the CD spectra almost disappeared, suggesting that the triple helical structure was almost converted to the random coil. The result was in agreement with that reported by Nikoo *et al.* (2014) on the farmed Amur sturgeon skin gelatin. However, small positive peak at 218-220 nm was still found in G55 and G65 samples,

suggesting the presence of some α-helix. This was coincidental with higher content of longer chain length (β- and γ-chain) of protein (Figure 25). In addition, the positive peak as observed in G55 and G65 samples might be related with the partial refolding of protein chains. α-, β- and γ-chains of G55 and G65 at high content could enhance refolding inter- or intra-molecularly to some degree during measurement at 20 °C. G75 had more random coil in structure as evidenced by the decrease in positive ellipticity at 220 nm (Aewsiri *et al.*, 2011). This indicated that higher extraction temperature more likely caused unfolding and degradation of collagen, leading to higher random structure. Thus, extraction conditions mainly caused the changes in the secondary structure of resulting gelatin.

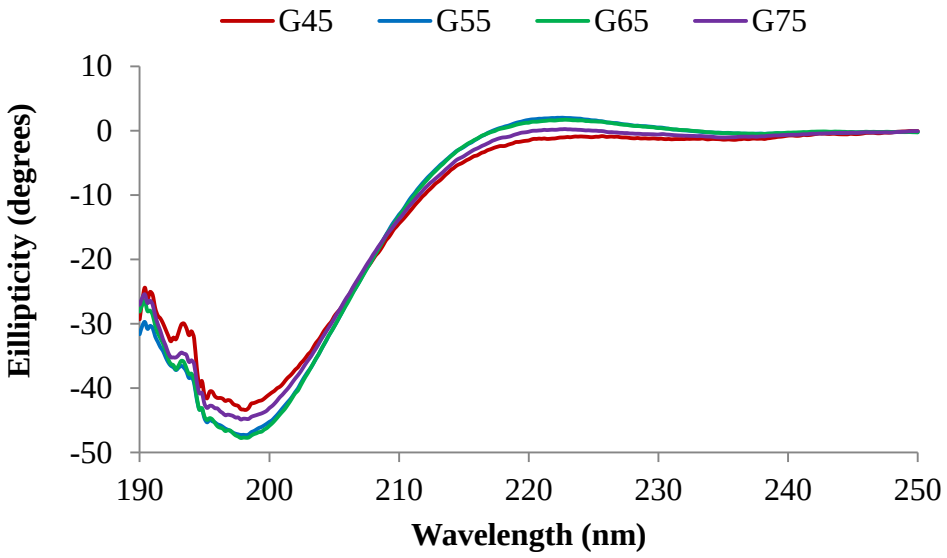

Figure 27. CD spectra of gelatins from seabass swim bladder extracted at different extraction temperatures. G45, G55, G65 and G75 denote gelatin from seabass swim bladder extracted at 45, 55, 65 and 75 °C, respectively, for 6 h. Bars represent the standard deviation (n=3).

5.5.5 Surface hydrophobicity

The surface hydrophobicity (S_oANS) of gelatin from the swim bladder extracted at different temperatures is shown in Figure 28. ANS probe can bind to hydrophobic amino acids (tyrosine, phenylalanine and tryptophan) and has been used

to indicate the surface hydrophobicity of proteins (Benjakul *et al.*, 1997). G45 showed higher S_oANS than G55 and G65 ($P<0.05$). However, no difference in S_oANS between G55 and G65 was observed ($P>0.05$). As extraction temperature increased, drastic increase in S_oANS was noticeable, particularly for G75. An increase in S_oANS of G75 was more likely associated with an exposure of the interior of molecule (Sae-leaw *et al.*, 2016). Kittiphattanabawon *et al.* (2012) also reported that S_oANS of gelatin extracted from shark skin increased as the extraction temperature increased. The hydrophobic domains might be more exposed or released, when the peptide molecules were cleaved by heat (Kittiphattanabawon *et al.*, 2012). The difference in S_oANS from gelatin extracted at different temperatures might be governed by the variation in protein conformation, related with protein degradation and precipitation induced by heat.

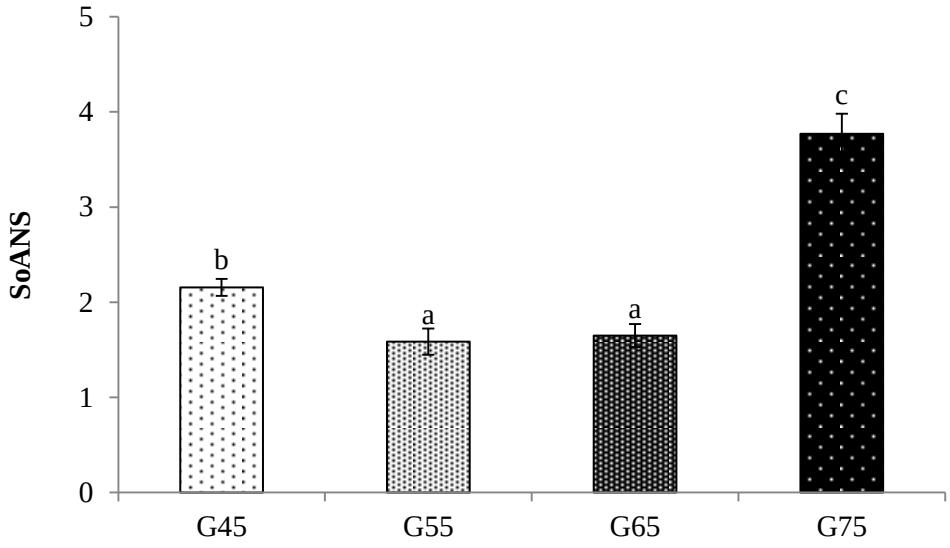

Figure 28. Surface hydrophobicity of gelatins from the seabass swim bladder extracted at different extraction temperatures. G45, G55, G65 and G75 denote gelatin from seabass swim bladder extracted at 45, 55, 65 and 75 °C, respectively, for 6 h. Bars represent the standard deviation (n=3).

5.5.6 Gel strength

Gel strength of gelatin from seabass swim bladder extracted at different temperatures in comparison with FG and BG is shown in Table 15. Gelatin gel is a thermally reversible gel network stabilized mainly by hydrogen bond (Benjakul *et al.*, 2012). Gel strength of gelatin increased as extraction temperature increased up to 65 °C ($P<0.05$). The highest gel strength (280.9 g) was found in G65 sample ($P<0.05$). The increase in gel strength was coincidental with higher proportion of α-, β- and γ-chains (Figure 24). However, when gelatin was extracted at 75 °C, gel strength of gelatin decreased ($P<0.05$). This was related with the slight decreases in those aforementioned components. Sinthusamran *et al.* (2014) also reported that gelatin from seabass skin extracted at lower temperature had higher gel strength than those extracted at higher temperature. Among all samples, the lowest gel strength (188.3 g) was observed for gelatin extracted at 45 °C ($P<0.05$). The low gel strength of G45 was related to the lower proportion of α-, β- and γ-chains (Figure 25). FG and BG had the gel strength of 201.6 and 246.3 g, respectively. G55 and G65 showed higher gel strength than BG and FG ($P<0.05$). The results suggested that long chain components (α-, β- and γ-chains) were the major factor affecting the development of strong gel (Sinthusamran *et al.*, 2014). Furthermore, the ways those chains interacted each other or the junction zones were formed were also crucial for gel formation. In addition, gelatin contained high hydroxyproline content (Table 16). The OH groups of hydroxyproline might be involved in gel formation by hydrogen bonding with adjacent chains (Kittiphattanabawon *et al.*, 2010). Gelatin with different gel strength was reported for clown featherback skin (225-284 g) (Kittiphattanabawon *et al.*, 2016), *Catla catla* swim bladder (264.6 g) (Chandra and Shamasundar, 2015), seabass skin (223-322 g) (Sinthusamran *et al.*, 2015), farmed Amur sturgeon skin (141 g) (Nikoo *et al.*, 2014), cobia skin (232 g) and croaker skin (212 g) (Silva *et al.*, 2014). In the present study, G65 with higher amount of α-, β- and γ-chains showed the highest gel strength and 65 °C was consider as the optimal temperature for extraction of gelatin from seabass swim bladder.

Table 15. Gel strength gelling and melting temperatures as well as gel color of gelatin from seabass swim bladder extracted at different temperatures

Sample	Gel strength (g)	Gelling temperature (°C)	Melting temperature (°C)	L^*	a^*	b^*	ΔE^*
FG	201.6±5.0b	ND	ND	71.16±0.14f	-0.97±1.42a	8.09±0.31e	26.48±0.39a
BG	246.3±10.8c	ND	ND	62.21±0.79e	0.05±0.02b	28.76±0.61f	40.06±0.55b
G45	188.3±8.3a	10.4±3.1a	19.3±3.2a	52.59±0.55d	-1.59±0.07a	-2.63±0.05a	43.79±0.43c
G55	263.5±8.3d	19.6±1.4b	28.3±1.4b	49.77±0.20c	-1.55±0.04a	1.52±0.26b	47.54±0.18d
G65	280.9±4.6e	19.7±0.6b	28.4±0.7b	48.14±0.18b	-1.58±0.08a	7.38±0.22d	50.10±0.16e
G75	204.3±8.19b	18.9±0.5b	27.4±0.4b	42.52±0.23a	-1.41±0.02a	4.46±0.17c	55.10±0.20f

Values are presented as mean ± SD (n = 3). ND: Not determined

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

5.5.7 Color of gel

The color of gelatin gel from seabass swim bladder extracted at different temperatures expressed as L^*, a^*, b^* and ΔE^* is shown in Table 15. FG and BG had the higher lightness (L^*-value), compared with all gelatin from seabass swim bladder, regardless of extraction temperatures ($P<0.05$). The L^*-value of gel from swim bladder gelatin decreased with increasing extraction temperatures ($P<0.05$). BG showed the higher a^*-value ($P<0.05$). No differences in a^*-value were found among gelatins from swim bladder with different extraction temperatures ($P>0.05$). Furthermore, yellowness (b^*-value) increased as extraction temperature increased up to 65 °C ($P<0.05$). However, b^*-value of gelatin gel decreased when extraction temperature was higher than 65 °C ($P<0.05$). The increase in b^*-value might be associated with non-enzymatic browning reaction during extraction at higher temperature, leading to higher yellowness (Sinthusamran *et al.*, 2014). The decrease in b^*-value found in G75 might be due to the denaturation of indigenous pigments. The higher b^*-value (28.76) was found in gel from BG ($P<0.05$). Kittiphattanabawon *et al.* (2016) reported that the higher yellowness in gelatin gel from bovine bone might be affected by the harsher extraction process as required for bone with complex structure, leading to formation of coloring components. For total difference in color value (ΔE^*-value), FG exhibited the lowest ΔE^*-value (26.48) ($P<0.05$). ΔE^*-value of gel from swim bladder gelatin increased with increasing extraction temperatures ($P<0.05$). This coincided with the decrease in L^*-value. The result indicated that extraction temperature had the impact on color of gel from gelatin extracted from seabass swim bladder.

5.5.8 Gelling and melting temperatures

Gelling and melting temperatures of gelatin from seabass swim bladder with different extraction temperatures are presented in Table 15. Changes in the phase angle have been used to monitor the thermal transitions of gelatin solution (Sinthusamran *et al.*, 2014). The gelling temperatures of G45, G55, G65 and G75 were 10.4, 19.6, 19.7 and 18.9 °C, respectively. It was noted that G45 had the lowest gelling temperature ($P<0.05$). There were no differences in gelling temperature among

G55, G65 and G75 ($P>0.05$). Low gelling temperature observed in G45 might be associated with lower amount of α-, β- and γ-chains and high content of low MW peptides (Figure 24). Short chains could not undergo network formation effectively (Sinthusamran *et al.*, 2015). Bovine gelatin had the gelling temperature of ~24.7 °C as reported by Kittiphattanabawon *et al.* (2016). The gelling temperature of gelatin from seabass swim bladder was lower than that of bovine gelatin. Gelation process is related to formation of junction zones in the three-dimensional network of gelatin gel, which was governed by amino acid compositions as well as molecular weight distribution (Muyonga *et al.*, 2004; Sinthusamran *et al.*, 2014). The imino acid plays the importance role in formation of nucleation zones via hydrogen bonds with water (Karim and Bhat, 2009; Kittiphattanabawon *et al.*, 2010). The result indicated that extraction temperature affected the gelling temperature of gelatin from seabass swim bladder.

The melting temperatures of gelatin from seabass swim bladder with different extraction temperatures were in the range of 19.3-28.4 °C. G45 showed the lowest melting temperature (~19.3 °C) ($P<0.05$), indicating that gel could not be maintained at room temperature for a longer time. Higher melting temperatures were found in gelatin extracted at 55-75 °C ($P<0.05$). Nevertheless, there were no difference in melting temperatures among those samples ($P>0.05$). Gelatin gel with higher melting temperature provides a better mouth feel when consumed, compared to gelatin with lower melting temperature (Sinthusamran *et al.*, 2014). Varying melting temperatures were reported for gelatin from yellowfin tuna skin (26.9-33.9 °C) (Gurunathan *et al.*, 2015), *Catla catla* swim bladder (23.3 °C) (Chandra and Shamasundar, 2015), cobia skin (26.8 °C), croaker skin (25.7 °C) (Silva *et al.*, 2014), seabass skin (26.3-27.0 °C) (Sinthusamran *et al.*, 2014) and New Zealand hoki skin (21.4 °C) (Mohtar *et al.*, 2013). The present study demonstrated that the high MW components of gelatin more likely contributed to the enhanced junction zone formation, in which strong gel network was developed. Such a gel required higher temperature to disrupt the network formed.

5.5.9 Microstructure of gelatin gels

Microstructures of gel from gelatin with different extraction temperatures are illustrated in Figure 29. The microstructure of gel is directly related to the gel strength, which is governed by the conformation and chain length of gelatin (Benjakul *et al.*, 2009). In general, all gelatin gels from seabass swim bladder showed a sponge or coral-like in structure. G45 gel with lower gel strength showed the gel network with the thinner strands and larger voids, compared with G55 and G65 gels. The finer structure and denser strands in gel matrix observed in gels from G55 and G65 samples were in accordance with higher gel strength (Table 15). Zhang *et al.* (2012) reported that the higher amount of high MW peptides (γ- and β-chains) contributed to denser strands and smaller voids of gelatin gel from silver carp skin. However, gel from G75 had the looser strands and very large voids. Such a gel network had the less resistance to the force applied, leading to a lower gel strength. Basically, the gelatin gel matrix was developed via the formation of hydrogen-bonded junction zones (Sinthusamran *et al.*, 2015). The results revealed that G55 and G65 yielded the gel network, which was stronger than G45 and G75. Thus, components and their chain length directly affected the arrangement of proteins in the gel network.

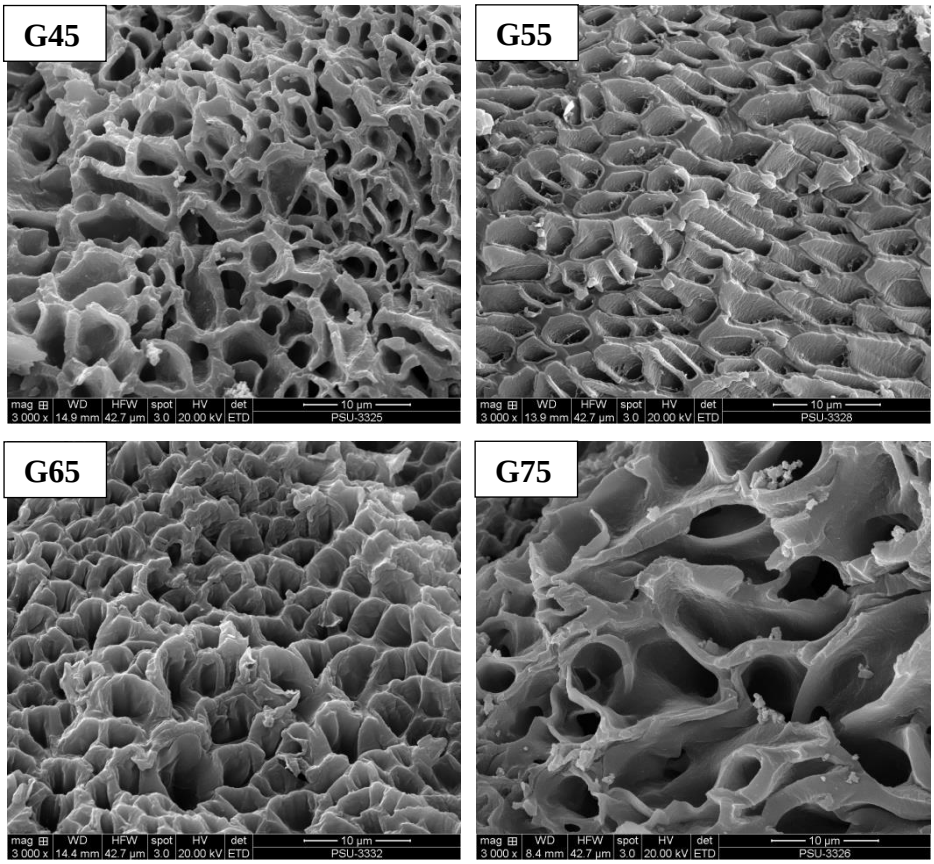

Figure 29. Microstructures of gelatin gel from the swim bladder of seabass extracted at different temperatures. G45, G55, G65 and G75 denote gelatin from seabass swim bladder extracted at 45, 55, 65 and 75 °C, respectively, for 6 h. Magnification: 3000 times.

5.5.10 Characteristics of selected gelatin

5.5.10.1 Proximate composition

Gelatin (G65) from swim bladder, possessing the highest gel strength, had high protein content (91.98%) with low fat (2.06%) and ash (1.55%) contents. The moisture content was 3.92%. Similar compositions of gelatins from *Catla catla* swim bladder (5.5% moisture, 92.8% protein and 1.5% ash) (Chandra and Shamasundar, 2015), cobia skin (9.4% moisture, 88.6% protein, 1.6% fat and 1.0% ash) and croaker skin (10.2% moisture, 88.2% protein, 0.6% fat and 0.9% ash) (Silva *et al.*, 2014) have been reported. In general, the recommended moisture and ash

content of edible gelatin are less than 15 and 2%, respectively (Nagarajan *et al.*, 2012). Therefore, the composition of obtained gelatin was complied with the standard.

5.5.10.2 Amino acid profile

Amino acid composition of G65 is shown in Table 16. Glycine is a major amino acid in G65 (334 residues/1000 residues), followed by alanine (136 residues/1000 residues), proline (108 residues/1000 residues) and hydroxyproline (87 residues/1000 residues). Glycine is located at every third position of the triple helix of collagen (Balti *et al.*, 2011). No cysteine was found in G65. Cysteine is not generally present in the structure of type I collagen (Nagarajan *et al.*, 2012). The contents of tyrosine (3 residues/1000 residues), histidine (5 residues/1000 residues) and isoleucine (7 residues/1000 residues) were low. The imino acid content (195 residues/1000 residues) of G65 was higher than that of Amur sturgeon skin (~171 residues/1000 residues) (Nikoo *et al.*, 2014), skipjack tuna skin(~149 residues/1000 residues), dog shark skin (~165 residues/1000 residues), rohu skin (~154 residues/1000 residues) (Shyni *et al.*, 2014) and croaker skin (188 residues/1000 residues) (Silva *et al.*, 2014). Nevertheless, it was slightly lower than that found in clown featherback skin (207 residues/1000 residues) (Kittiphattanabawon *et al.*, 2016), cobia skin (~205 residues/1000 residues) (Silva *et al.*, 2014), seabass skin (~198-202 residues/1000 residues) (Sinthusamran *et al.*, 2014) and grass carp skin (~201 residues/1000 residues) (Kasankala *et al.*, 2007). The differences in the species, environment living habitat, body temperature of fish and age of fish are the main factor affecting the contents of proline and hydroxyproline, which contributed to the stability of triple helical structure in collagen molecule (Sinthusamran *et al.*, 2013; Zhang *et al.*, 2012). High content of hydroxyproline enhanced the viscoelastic and gelling properties of gelatin (Benjakul *et al.*, 2009). Hydroxyproline might be involved in hydrogen bonding between molecules during gelation of gelatin.

Table 16. Amino acid compositions of gelatins from seabass swim bladder extracted at 65 °C for 6 h (residues/1000 residues)

Amino acid	**Content (residues/1000 residues)**
Alanine	136
Arginine	53
Aspartic acid/asparagine	44
Cysteine	0
Glutamine/glutamic acid	71
Glycine	334
Histidine	5
Isoleucine	7
Leucine	21
Lysine	25
Hydroxylysine	8
Methionine	14
Phenylalanine	14
Hydroxyproline	87
Proline	108
Serine	25
Threonine	24
Tyrosine	3
Valine	20
Total	1000
Imino acid	195

5.6 Conclusion

Characteristics and properties of gel were affected by extraction temperatures. Extraction yield and recovery increased with increasing extraction temperatures. Gelatin extracted at 65 °C, having α- and β-chains as major components, exhibited the highest gel strength and also showed higher gel strength than bovine gelatin. Nevertheless, G65 had similar gelling and melting temperatures, compared with those extracted at 55 and 75 °C. Therefore, gelatin with high gel properties and extraction yield could be extracted from seabass swim bladder at 65 °C for 6 h.

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CHAPTER 6

RHEOLOGICAL AND SENSORY PROPERTIES OF FISH GELATIN GELS AS INFLUENCED BY AGAR FROM *GRACILARIA TENUISTIPITATA*

6.1 Abstract

Agar extracted from *Gracilaria tenuistipitata* and commercial agars were incorporated into fish gelatin at various levels (0, 5, 10, 15 and 20% gelatin substitution). *G. tenuistipitata* agar (GA) had lower failure stress (~16 kPa) than commercial agar (CA) (~20 kPa). However, the former showed higher failure strain (~30%) with lower melting temperature (65.9 °C). The critical linear stress and failure stress of agar/gelatin mixed gels increased with increasing agar levels ($P<0.05$). At 15 and 20% of agar used, the mixed gels containing CA exhibited higher failure stress than those with added GA ($P<0.05$). Two melting points of agar/gelatin mixed gels were observed, corresponding to the melting temperatures of gelatin and agar gels. Nevertheless, the incorporation of agar lowered the likeness score of gelatin gel. Thus, both GA and CA had the impact on rheological property and the selected sensory characteristics of fish gelatin, depending on the level of substitution.

6.2 Introduction

Gelatin is a protein derived from collagen by thermal denaturation or partial degradation. It has a wide range of applications in food and pharmaceutical industries (Kittiphattanabawon *et al.*, 2010). The quality of gelatin depends mainly on its chemical and structural characteristics (Benjakul *et al.*, 2009). Rheological and gelling properties, setting behavior and melting temperature are the major properties of gelatin, which are associated with molecular weight, amino acid composition and the α/β ratio of chains present in the gelatin (Karim and Bhat, 2009). Gelatins extracted from various raw materials have different setting and melting temperatures as well as gel strengths (Sinthusamran *et al.*, 2014).

In recent years, fish gelatin has become an important alternative to bovine and porcine gelatin in food industry owing to the bovine spongiform encephalopathy (BSE) crisis and to religious reasons (Kaewruang *et al.*, 2014). In addition, fish skin and bones, which constitute approximately 30% of total byproducts, are usually discarded during fish processing (Sai-Ut *et al.*, 2012). As a consequence, several attempts have been paid to exploit those underutilized resources. However, fish gelatin still has limited applications, due to its low gel strength, gelling and melting temperatures, when compared to gelatin from land animals (Karim and Bhat, 2009). Therefore, the improvement of gel properties of fish gelatin has been carried out to widen their applications. Chemical or natural cross-linkers have been used to strengthen fish gelatin gels (Kosaraju *et al.*, 2010). Gómez-Guillén *et al.* (2001) reported that the addition of transglutaminase to gelatin can increase melting temperature and gel strength of gelatin gel. Some hydrocolloids with gelling properties, such as κ-carrageenan, pectin and gellan, were also used in order to improve its gel properties (Haug *et al.*, 2004; Lau *et al.*, 2000; Liu *et al.*, 2007).

Agar, a hydrophilic hydrocolloid, is extracted from red algae (Rhodophyta) (Romero *et al.*, 2008). Agar has an excellent gelling ability and it has been widely used in medical and pharmaceutical industries, and for laboratory experiments (Romero *et al.*, 2008). The characteristic and properties of agar depend on their chemical compositions, which are influenced by the extraction processes (Kumar and Fotedar, 2009). Furthermore, the type, pattern, degree of substitution, and molecular weight have been known to determine the gelling properties of agar (Villanueva *et al.*, 2010). *Gracilaria tenuistipitata* has grown rapidly and became the dominant red algae in the Lake of Songkhla in Thailand. It has been used for agar extraction (Yarnpakdee *et al.*, 2015). The agar could be employed as a gelling agent to modify the gel property of fish gelatin.

6.3 Objective

To compare the physical and chemical properties of agar extracted from *Gracilaria tenuistipitata* to those of a commercially available agar, and to investigate the effect of these two agars on textural and sensory characteristics of fish gelatin gel.

6.4 Materials and methods

6.4.1 Chemicals

All chemicals were of analytical grade. Resorcinol and acetal (Kosher) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fish gelatin from tilapia skin (~240 bloom) was obtained from Lapi Gelatine S.p.A (Empoli, Italy). Commercial agar powder was obtained from New Zealand Manuka Bioactives Ltd. (Whakatane, New Zealand).

6.4.2 Preparation of agar from *Gracilaria tenuistipitata*

G. tenuistipitata were harvested from the Lake of Songkhla, Koyo Island, Songkhla, Thailand. Semi-sun dried algae were purchased and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand within 1 h. Algae were washed with tap water. The clean algae were dried using a tray dryer at 60 °C for 12 h. The samples were cut into small pieces and kept in polyethylene bag at 4 °C until used for agar extraction.

6.4.3 Agar extraction

The agar was extracted from the prepared algae following the method of Yarnpakdee *et al.* (2015). Prior to extraction, dried algae were soaked in 5% NaOH, with a dried algae/solution ratio of 1:50 (w/v) for 24 h at room temperature. The mixture was heated at 90 °C for 3 h with continuous stirring using an overhead stirrer (W20.n, IKA®-Werke GmbH & CO.KG, Stanfen, Germany). After alkaline treatment, the algae were washed in tap water until the neutral pH was obtained. Finally, the pretreated algae were mixed with distilled water at a ratio of 1:50 (w/v) at 95 °C for 2 h. The mixture was filtered through a cheesecloth and filtered under pressure using a Buchner funnel, with a Whatman No. 4 filter paper. The filtrate was transferred into plastic container. Filtrate was allowed to gel at room temperature (25-26 °C), frozen for 24 h and thawed at room temperature for approximately 4 h. Thereafter, the thawed gel was frozen for another 24 h prior to freeze-drying using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark) at -50 °C for 72 h.

6.4.4 Analyses of agar

Agar extracted from *Gracilaria tenuistipitata* (GA) was subjected to analyses in comparison with commercial agar (CA).

6.4.4.1 Determination of sulphate content

The sulphate content of agar samples was measured turbidimetrically using a $BaCl_2$-gelatin method after hydrolysis in 0.5 M HCl as described by Yarnpakdee *et al.* (2015). Agar powder (50 mg) was hydrolyzed in 6 mL of 0.5 N HCl at 105-110 °C for 12 h. After cooling, the solutions were filtered with a Whatman No. 1 filter paper. A portion of filtrate (0.2 mL) was transferred to a 10-mL tube containing 3.8 mL of 3% (w/v) TCA and 1 mL of $BaCl_2$-gelatin reagent. The solution was mixed and incubated at room temperature for 20 min. The absorbance at 360 nm was measured using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Blank was prepared in a same manner with distilled water replacing the agar sample. A standard curve was prepared using K_2SO_4 at concentration ranging from 0.1 to 1 mg/mL (~0.0533-0.533 mg SO_4^{2-}/mL). The sulphate content was calculated and expressed as the percentage of total solid.

6.4.4.2 Determination of 3,6-anhydrogalactose content

The 3,6-anhydrogalactose (3,6-AG) content was determined colorimetrically using the resorcinol-acetal method as described by Yarnpakdee *et al.* (2015). Agar solution (0.02 mg/mL, 2 mL) was transferred into 20 mm×150 mm screw cap tube containing 10 mL of cold resorcinol-acetal reagent (a mixture of 100 mL of 37% HCl, 9 mL of 13.6 mM resorcinol solution and 1 mL of 0.0695 mM acetal solution). The mixtures were mixed thoroughly and allowed to cool in ice bath for 3 min. The tubes were placed in 25 ºC water bath for 4 min and then heated at 80 °C for 10 min in the dark. After cooling, the absorbance at 555 nm was read within 15 min. A standard curve was prepared using D-fructose at concentration ranging from 0.018 to 0.09 mM. The 3,6-AG content was calculated and expressed as the percentage of total solid.

6.4.4.3 Compression measurements

Compression behavior was investigated following the method of Kreger and Voytik-Harbin (2009). Compression testing was performed using a Paar Physica MCR 302 stress-controlled rheometer (Anton-Paar, Austria) using a stainless steel 25 mm diameter parallel plate geometry. Agars were dissolved in distilled water at 95 °C to obtain a final concentration of 1.5% (w/v). The agar solutions were transferred to a 25 mm diameter and 10 mm height mould plate. The solutions were incubated at a refrigerated temperature (4-6 °C) for 18 h. Then an agar gel slab obtained from moulds was loaded onto the Peltier plate set at 8 °C.

Normal force was measured in response to compressive strain generated by depressing the top plate at a rate of 0.05 mm/s. Stress-strain plots were generated for each sample. Compressive strain was calculated as $(L\text{-}L_o)/L_o$, where L is the actual height during compression and L_o=initial height. Stress was calculated as normal force applied by the rheometer divided by plate area. Compressive moduli were calculated in two regions of the stress-strain curves. A critical linear stress was calculated by linear regression as the slope of the curve from approximately 10 to 30% strain, which was in a straight line region. The failure stress was the point when the compressive stress became greater than the gel ability, which then caused the matrix collapse. Failure stress and failure strain values were taken at this point. Compression measurements were performed on 5 independent replicates for each sample (n=5).

6.4.4.4 Determination of melting points

Melting point was measured using a Paar Physica MCR 302 stress-controlled rheometer (Anton-Paar, Austria) with a stainless steel 25 mm diameter parallel plate geometry and 2 mm height gap. Agar gel was loaded into the rheometer geometry and soya oil was added at the periphery of the samples to minimize evaporation. Melting point measurement was conducted at constant frequency of 1 Hz and a constant applied strain of 1% and the samples were heated from 5 to 95 °C at a constant rate of 1 °C/min.

6.4.4.5 Determination of syneresis

Gel syneresis was determined as described by Banerjee and Bhattacharya (2011). Hot agar solutions (1.5% w/v; 90 °C, 30 mL) were poured into 50 mL graduated centrifuge tubes and their masses (*m1*) were recorded. The samples were allowed to cool at room temperature for gel formation. Gels were matured at 4 °C for 12 h. Before measurement, gels were equilibrated at room temperature for 2 h. Then, these samples were centrifuged at 2,150×g at 25 °C for 10 min using a thermostated centrifuge (Beckman Coulter, Palo Alto, CA, USA). After centrifugation, gels along with the tubes were weighed (*m2*) again after discarding the supernatant water. The percentage of syneresis was calculated as:

$$\text{Syneresis (\%)} = \frac{(m1-m2)}{m1} \times 100$$

6.4.5 Effect of agar addition on properties of gelatin gel

6.4.5.1 Preparation of agar/gelatin mixed gel with different agar substitutions

Fish gelatin and agar samples were separately solubilized in distilled water at 60 °C and 95 °C, respectively. Solutions of GA or CA were mixed with gelatin solution to obtain 0, 5, 10, 15 and 20% gelatin substitution. Total solid content of all solutions was 5% (w/v). The mixtures were transferred to a mould plate with 25 mm diameter and 10 mm height for compression test. A mould plate with 25 mm diameter and 2 mm height was used for the melting tests. The resulting agar/gelatin mixed gels were incubated at refrigerated temperature for 18 h prior to analysis.

6.4.5.2 Analyses of agar/gelatin mixed gel

Gels were determined for compression properties and melting point as described previously.

For sensory evaluation, fifty non-trained panelists (aged between 25-40 years) were selected. They were the students and staffs from the Department of Food Technology, who were acquainted with gelatin products. Gel samples were cut into a bite-size (1 cm thickness and 2 cm diameter) and coded with 3-digit random numbers. Gel samples (8-10 °C) were served on white paper dishes at room temperature under the fluorescent daylight-type illumination. The samples stored at 4 °C were removed from the refrigerator and tempered for 2 min at room temperature prior to sensory testing. The panelists were asked to evaluate for appearance, color, firmness and springiness liking of gel samples using 9-point hedonic scale (1, extremely dislike; 2, very much dislike; 3, moderately dislike; 4, slightly dislike; 5, neither like nor dislike; 6, slightly like; 7, moderately like; 8, very much like; 9, extremely like) (Meilgaard *et al.*, 2006). Between samples, the panelists were asked to rinse their mouth with distilled water.

6.4.6 Statistical analysis

All experiments were run in triplicate using three different lots of samples and a completely randomized design (CRD) was used. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out using Duncan's multiple range test (Steel and Torrie, 1980). Randomized complete block design (RCBD) was used for analysis of acceptance test. For two-sample comparison, T-test was used. Correlation coefficient between the physical properties and the sensory data was also analyzed. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for windows: SPSS Inc., Chicago, IL, USA).

6.5 Results and discussion

6.5.1 Chemical compositions and properties of GA

6.5.1.1 Sulphate content

Sulphate contents in GA and CA were 2.65±0.37 and 1.86±0.45%, respectively (Table 17). CA sample had lower sulphate content than GA. The sulphate

content has been known to govern the gel strength, gelling and melting temperatures of agar (Sousa *et al.*, 2010). Sulphate content of agar from *G. cliftonii* treated with alkali was in a range of 2.16-3.01% (Kumar and Fotedar, 2009) and native agar (without alkaline pretreatment) showed higher sulphate content than alkali-pretreated agar (Villanueva *et al.*, 2010). Yarnpakdee *et al.* (2015) also reported that lower sulphate content was found in agar from *G. tenuistipitata* algae pretreated with alkali, compared with agar without alkaline pretreatment. The alkaline pretreatment is widely applied to remove the sulphate group at C-6 of the 4-linked galactopyranose unit in polysaccharide chains. This process is able to cleave sulphate ester at C-6 of the *L*-galactose (Murano, 1995). Extraction conditions, especially alkaline concentration, pretreatment time, etc. were found to affect the sulphate content of agar (Villanueva *et al.*, 2010). Sulphate content $\leq$ 4% is required for agar by the international food industry (Armisen, 1995), and the agar from *G. tenuistipitata* in the present study had the sulphate content below this limit.

6.5.1.2 3,6-Anhydro-L-galactose content (3,6-AG)

3,6-AG contents of GA and CA are shown in Table 17. The 3,6-AG content of CA (63.38$\pm$3.77%) was higher than that of GA (50.56$\pm$1.92%) ($P<0.05$). 3,6-AG contents of both agars were higher than those of agar extracted from *G. vermiculophylla* (42.5-44.2%) (Villanueva *et al.*, 2010) and *G. eucheumatoides* (31.34%) (Romero *et al.*, 2008). 3,6-AG content was affected by extraction conditions such as alkaline concentration and pretreatment time. The alkaline pretreatment is able to eliminate the unstable sulphate unit when the hydroxyl group at C-3 of the L-galactose unit is free, giving rise to the formation of more stable 3,6-anhydro-L-galactose (Rebello *et al.*, 1997). However, the pretreatment at high alkaline concentration resulted in chain breakage rather than the formation of 3,6-AG in polysaccharide chains (Yarnpakdee *et al.*, 2015). Generally, the increase in 3,6-AG content is related to the decrease in sulphate content (Villanueva *et al.*, 2010). Gel properties of agar, especially gel strength, gelling and melting temperatures were also influenced by 3,6-AG content (Sousa *et al.*, 2010).

Table 17. Chemical composition and properties of agar extracted from *G. tenuistipitata* and commercial agar

Properties	**GA**	**CA**
Sulfate content (%)	2.65±0.37b	1.86±0.45a
3,6-Anhydrogalactose content (%)	50.56±1.92a	63.38±3.77b
Critical linear stress (kPa)	34.74±9.59a	65.89+6.44b
Failure stress (kPa)	16.46±0.97a	20.45±1.82b
Failure strain (%)	29.78±2.64b	23.44±1.43a
Melting temperature (°C)	65.90±2.01a	80.00±1.73b
Syneresis (%)	7.17±0.40a	6.93±0.44a

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

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

6.5.1.3 Compression properties of gel

Agar is a neutral polysaccharide with fewer anionic sites, which require no specific counterions or other additives to induce gelation (Stanley, 2006). Prior to gelation, heating is required to dissolve agar, before the hot solution is cold-set (Armisen and Galatas, 1987). Left-handed dual helices of agar can form gel network capable of immobilizing the water, mainly by hydrogen bond formation as the primary mechanism (Braudo *et al.*, 1991; Stanley, 2006). When comparing compression properties of both agars, CA had a higher critical linear stress than GA ($P<0.05$) (Table 17), indicating that CA gels was more resistance to the applied force and was more deformable. The critical linear stress represented the highest stress at which the material did not lose the stress-strain proportionality (Kreger and Voytik-Harbin, 2009). Moreover, the failure stress of CA (~20 kPa) was higher than that of GA (~16 kPa). The higher failure stress of CA can be related to the higher amount of 3,6-AG and lower sulphate content, in comparison with GA (Table 17). The presence of sulphate content is responsible for the poor gelling ability of agar (Freile-Pelegrín and Murano, 2005). Due to the kink in the helical structure of agar during gel formation (Rees, 1972), agar rich in sulphate exhibited weak gel with low gel strength. On the other hand, the failure strain of GA (~30%) was higher than that of

CA (~23%) ($P<0.05$), indicating that CA gel was more brittle with less springiness than GA gel.

6.5.1.4 Melting temperature

Melting points of both agar gels are presented in Table 17. GA and CA had melting points of 65.9 and 80.0 °C, respectively. In general, melting point of GA was lower than those of agar from *G. cervicornis* (67 °C), *G. blodgettii* (88 °C), *G. crassissima* (93 °C) (Freile-Pelegrín and Murano, 2005) and *G. vermiculophylla* (73.6-80.4 °C) (Villanueva *et al.*, 2010). Agar gels are known for their "gelation hysteresis" due to the large difference between their gelling and melting temperatures (Armisen and Galatas, 1987). During cooling, agar gels are formed at temperature between 32 and 43 °C, depending on species of algae used. When the gel is heated to 85 °C or higher, the gel becomes a sol (Armisen and Galatas, 1987). The molecular weight distribution in agar plays an important role in the gelling behavior (Kumar and Fotedar, 2009). Sousa *et al.* (2010) reported that agar with high gel strength generally possessed higher gelling and melting points. In the present study, CA with higher critical linear stress and failure stress (Table 17) had the higher melting point, compared to GA.

6.5.1.5 Syneresis

Syneresis is the loss of water during ageing of agar gels, and indicates the instability of gel network (Stanley, 2006). Syneresis of gels from GA and CA ranged from 6.93 to 7.17% as shown in Table 17. There was no difference in syneresis between gels from GA and CA ($P>0.05$). Mao *et al.* (2001) reported that agar gel with low strength generally had high syneresis. Agar gels with higher concentrations generally showed a lower syneresis than those having lower concentrations (Banerjee and Bhattacharya, 2011). Stanley (2006) suggested that syneresis is attributed to the aggregation of double helices, leading to the contraction of the polymer network, which in turn results in the decrease in the space available for holding water.

6.5.2 Effect of agar addition on gel properties

6.5.2.1 Compression properties of agar/gelatin mixed gel

Compression properties of gelatin gels mixed with GA or CA at different substitution levels are presented in Table 18. Gelatin gel had a critical linear stress and failure stress of ~20 and ~32 kPa, respectively. When agars were added into gelatin, the critical linear stress of the mixed gels was increased ($P<0.05$). However, no difference in critical linear stress was observed between CA gel added with 15 and 20% agar ($P>0.05$). Failure stress of agar/gelatin mixed gels increased when GA at levels higher than 5% was incorporated ($P<0.05$). However, no difference in failure stress amongst the samples containing 10-20% GA was observed ($P>0.05$). The similar result was noticeable with CA/gelatin mixed gel. This result suggested that mixed gel containing higher agar levels (10-20%) became more brittle with decreased degree of deformation. Lau *et al.* (2000) reported that hardness of hydrocolloids gel, which refers to the strength of the gel structure, increased as the content of gel forming material was increased. Additionally, at 15 and 20% agar levels, agar/gelatin mixed gels containing CA exhibited higher failure stress than those containing GA. The difference in critical linear stress and failure stress values between gelatin gels mixed with GA and CA was more likely associated with different chemical compositions and compressive properties between two agars (Table 17).

The failure strain of agar/gelatin mixed gel with different agar levels is shown in Table 18. In general, the failure strain of gel decreased when agar content was increased. However, gelatin gel mixed with 5% GA had the higher failure strain (~75%), compared with gelatin gel ($P<0.05$). Agar gel is brittle and crunchy in texture (Mao *et al.*, 2001), and it might lower the springiness of gelatin gel (Banerjee and Bhattacharya, 2011). Both agars were generally able to increase the hardness (failure stress) but decreased the springiness (failure strain) of gel. Thus, the addition of both agars, GA and CA, affected the texture properties of gelatin gel, depending on the level used.

Table 18. Critical linear stress (kPa), failure stress (kPa) and failure strain (%) of gel from gelatin mixed with agar extracted from *G. tenuistipitata* or commercial agar at different levels

Gelatin substitution (%)	Critical linear stress (kPa)		Failure stress (kPa)		Failure strain (%)	
	GA	CA	GA	CA	GA	CA
0	20.48±3.79a		32.65±0.58a		71.68±1.32b	
5	21.23±2.36aA	28.04±5.32abA	39.48±5.98abA	38.71±5.35aA	74.89±3.30aA	63.71±4.48cB
10	23.25±3.54aA	32.67±5.16bB	42.74±7.80bcA	57.04±11.68bA	62.57±0.73cA	64.83±2.92cA
15	36.54±2.84bA	47.07±3.80cB	48.13±7.14cA	62.75±2.91bB	48.79±1.77dA	55.81±3.78dB
20	48.11±8.40cA	51.52±9.59cA	43.56±3.94bcA	51.95±11.90bB	45.04±2.24eA	45.99±6.29eA

Values are presented as mean ± SD (n = 3). Different lowercase letters within the same column including gelatin gel (without agar) indicate significant differences ($P<0.05$). Different uppercase letters within the same row under the same testing parameter indicate significant differences ($P<0.05$).

6.5.2.2 Syneresis

Syneresis of agar/gelatin mixed gels containing GA and CA at different levels after storage for 12 h at 4 °C is shown Table 19. Amongst all gel samples, fish gelatin gel had the lowest syneresis, which was 0.10%. In contrast, the highest syneresis (0.25%) was found in the mixed gel containing 20% CA. Syneresis of mixed gel increased with increasing agar levels, regardless of agar type. However, gels of gelatin mixed with CA at 5-10% or GA at 5-15% showed no differences in syneresis, compared with gel from fish gelatin alone ($P>0.05$). The interaction between gelatin and agar, especially in the presence of agar at high level, might provide more compact structure, thereby holding a limited amount of water. Syneresis is governed by the type of hydrocolloid and Banerjee and Bhattacharya (2011) reported that an increase in hydrocolloids (gellan and agar) concentration decreased syneresis of mixed gel. Agar gel is known to be prone to extensive syneresis, which has an effect on gel strength (Mao *et al.*, 2001). Therefore, the incorporation of agar in gelatin gel slightly increased the amount of unbound water released from the gel matrix.

Table 19. Syneresis (%) and melting points (°C) of gel from gelatin mixed with agar extracted from *G. tenuistipitata* or commercial agar at different levels

Gelatin substitution	Syneresis		1st melting point		2nd melting point	
(%)	GA	CA	GA	CA	GA	CA
0	0.10±0.01a		24.83±0.29a		ND	
5	0.09±0.04aA	0.12±0.02aA	22.47±1.10bA	21.25±1.06bA	66.20±2.12aB	80.75±0.35aA
10	0.10±0.02aA	0.14±0.04abA	22.10±1.56bA	25.00±2.50aA	70.25±3.61aB	81.83±0.14abA
15	0.13±0.03aA	0.18±0.03bA	22.00±1.00bA	22.50±2.29aA	70.27±4.65aB	82.00±0.87abA
20	0.19±0.04bA	0.25±0.03cA	21.67±0.58bA	20.57±2.29bA	70.57±2.14aB	82.42±0.95bA

Values are presented as mean ± SD (n = 3). Different lowercase letters within the same column including gelatin gel (without agar) indicate significant differences (P< 0.05). Different uppercase letters within the same row under the same parameter indicate significant differences (P< 0.05).

ND: Not Detected

6.5.2.3 Melting temperature

Melting points of agar/gelatin mixed gels with different agar levels are shown in Table 19. Melting point of gelatin gel was 24.8 °C. The melting points of agar/gelatin mixed gels were found in the temperature range of 21.3-25.0 °C and 66.2-82.4 °C, corresponding to melting points of gelatin and agar, respectively. For the first melting point, the mixed gel had varying values, depending on the level of agar present in the system. It was more likely that some interaction between both hydrocolloids occurred. The second melting points were found to be similar when the same agar was added, regardless of substitution levels. However, gel containing 20% CA showed the higher melting point than that added with 5% CA ($P<0.05$). At the same agar level, the second melting points of gelatin mixed with CA was higher than those of gelatin mixed with GA ($P<0.05$). Higher melting points were in agreement with the higher failure stress of agar/gelatin mixed gels, especially those containing CA (see Table 18). Overall, both agars had an influence on the melting behavior of fish gelatin. The obviously different melting points in agar/gelatin mixed system can be due to phase separation between two different hydrocolloids, gelatin and agar. In general, phase separation due to thermodynamic incompatibility occurs when two different biopolymers in solution are mixed (Tolstoguzov, 1997). Phase separation occurs with protein in the presence of a neutral polysaccharide. The gel network of such a mixed system, undergoing phase separation, is stabilized by electrostatic interactions or hydrogen bonding or hydrophobic interactions (Doublier *et al.*, 2000). Tolstoguzov (1997) reported that unfolded proteins such as gelatin were prone to phase-separation through thermodynamic incompatibility.

6.5.2.4 Sensory characteristics

Appearance, color, firmness, springiness and overall likeness scores of various gel samples are shown in Table 20. In general, gelatin gel had the higher likeness scores for all attributes tested, compared with gelatin/agar mixed gels ($P<0.05$). With the same agar type, the increasing levels of agar resulted in the decreases in likeness score ($P<0.05$). Nevertheless, gelatin gel mixed with 5% CA had similar appearance, color and springiness likeness score to gelatin gel ($P>0.05$).

Decreases in appearance and color likeness scores might be associated with increasing gel turbidity (less transparent) caused by the distribution of agar in the gel matrix. Firmness and springiness of mixed gel decreased when agar content was increased. At the same agar level, firmness and springiness likeness scores of gel mixed with CA were higher than those of gel containing GA ($P<0.05$). For overall likeness, gelatin gel showed the highest likeness score ($P<0.05$). Incorporation of agar, both GA and CA, lowered the likeness score of mixed gel, particularly when the level of agar increased. In the present study, the addition of both agars into gelatin gel affected the gel texture, especially the failure stress and strain (Table 18). Gel with higher brittleness and lower elasticity in texture might be undesirable for gelatin gel product. As a result, the likeness score of all attributes for agar/gelatin mixed gel was decreased. Generally, the unique characteristic of gelatin gel is its melt-in-the-mouth property during ingestion (Zhou and Regenstein, 2007). Agar gel also has thermo-reversible property but it melts at higher temperatures (Table 19). The presence of agar increased the melting point of gelatin gel (Table 19), resulting in unmolten residues in the mouth. Furthermore, agar more likely contributed to the lowering the deformability of the mixed gel. Therefore, the addition of both agars from GA and CA showed negative effects on the sensory properties of gelatin-agar gels.

Table 20. Likeness scores of appearance, color, firmness, and springiness from gelatin mixed with agar extracted from *G. tenuistipitata* or commercial agar at different levels

Gelatin substitution (%)	Appearance		Color		Firmness		Springiness		Overall	
	GA	CA	GA	CA	GA	CA	GA	CA	GA	CA
0	8.17±0.83a		8.00±0.83a		7.80±0.92a		7.70±1.18a		7.80±0.96a	
5	6.70±1.09bA	7.80±0.85aA	6.77±1.14bA	7.73±0.87abB	6.60±1.22bA	7.30±0.84bB	6.93±0.98bA	7.33±0.99bB	6.67±1.24bA	7.33±1.12abA
10	6.43±0.82bA	7.23±0.90bB	6.57±1.19bA	7.43±0.86bcB	6.23±1.10bcA	6.83±0.95cB	6.43±1.14bA	7.13±0.90bB	6.50±1.11bA	7.10±1.06bB
15	5.93±1.05cA	6.90±0.88bcB	5.80±1.35cA	7.10±0.92cB	5.77±1.28cA	6.23±1.01dA	6.53±1.14bA	6.57±1.01cA	6.23±1.14bA	6.20±1.42cA
20	5.67±1.35cA	6.70±1.24cB	5.73±1.36cA	6.73±1.05dB	5.90±1.27cA	6.23±1.28dA	6.63±1.19bA	6.53±1.17cA	6.37±1.16bA	6.40±1.25cA

Values are presented as mean ± SD (n = 3). Different lowercase letters within the same column including gelatin gel (without agar) indicate significant differences ($P<0.05$). Different uppercase letters within the same row under the same quality attribute indicate significant differences ($P<0.05$).

6.6 Conclusion

G. tenuistipitata could be a promising source of agar as it exhibited similar chemical composition (sulphate and 3,6-AG content), when compared to commercial agar. The textural properties of agar/gelatin mixed gels were strongly affected by the agar levels. Both low strain modulus and failure stress of agar/gelatin mixed gel increased with increasing levels of agar. However, commercial agar showed a higher gel strengthening effect than *G. tenuistipitata* agar. For both agars, their incorporation to gelatin decreased the failure strain (springiness) of agar/gelatin mixed gels. However, the incorporation of agar, especially at higher levels, showed a negative impact on sensory property of agar/gelatin mixed gel. This was more likely due to the changes in mouth feel associated with the increased brittleness and lower melting of resulting gel.

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CHAPTER 7

PHYSICAL AND RHEOLOGICAL PROPERTIES OF FISH GELATIN GEL AS INFLUENCED BY K-CARRAGEENAN

7.1 Abstract

Physical and rheological properties of commercial fish gelatin (FG) (5%, w/v) gel containing κ-carrageenan (KC) at different levels (0, 25, 50, 75 and 100% of gelatin substitution) were studied. Gel strength and hardness of FG/KC mixed gel increased with increasing KC content, while springiness of mixed gel decreased when KC was higher than 25% ($P<0.05$). Rheological measurements revealed that the value of elastic modulus G′ of FG/KC mixed gel decreased as KC content increased. This could be associated with the phase separation between the two biopolymers present in the mixed gels. Moreover, gelling and melting temperatures also increased as the levels of KC increased ($P<0.05$). Based on fourier transform infrared (FTIR) spectra, interaction between FG and sulphate groups of KC occurred. This study shows that the addition of KC into FG improves the gelling property of FG.

7.2 Introduction

Gelatin is a fibrous protein obtained by thermal denaturation of collagen. It has a wide range of applications in food, pharmaceutical, cosmetic and photographic industries (Regenstein and Zhou, 2007). Generally, bovine and porcine skin and bone have been utilised for gelatin production. However, religious restrictions have led to increasing interest in alternative sources of gelatin (Kittiphattanabawon *et al.*, 2012). Fish processing by-products have became an important alternative source for gelatin production (Sinthusamran *et al.*, 2015). Unfortunately, fish gelatin has a poorer gelling property, compared to those from land animals (Haug *et al.*, 2004). To improve the gelling properties of fish gelatin, several approaches such as the use of cross-linkers, the addition of salts, etc. have been considered (Kosaraju *et al.*, 2010; Sarabia *et al.*, 2000).

Kappa (κ)-carrageenan is a sulphated polysaccharide extracted from red seaweeds. It consists of a repeating disaccharide unit of α(1-4)-3,6-anhydro-D-galactose and β(1-3)-D-galactose (Şen and Erboz, 2010). Salt and temperature govern the conformational transition of carrageenan. During cooling, carrageenan molecules form gels via the binding of monovalent cations in junction zones, resulting in a hard brittle gel (Haug *et al.*, 2004; Şen and Erboz, 2010). The gel properties (such as gelling temperature and gel strength) of κ-carrageenan are greatly dependent on the concentration and type of carrageenan as well as the concentration of monovalent cations (Şen and Erboz, 2010). Carrageenan has a wide range of applications such as making dessert jelly, improving texture and water holding in processed meat products, etc. (Derkach *et al.*, 2015; Nishinari *et al.*, 1996; Şen and Erboz, 2010). Carrageenan was also used to improve gel strength and to increase the gelling and melting temperatures of gelatin (Venugopal, 2011).

When biopolymers are used in combination, the mixed system may undergo phase separation. This phenomenon is the result of the limited compatibility of these biopolymers, especially in the case of aqueous protein-polysaccharide systems (Antonov and Gonçalves, 1999). When two or more biopolymers are mixed, phase separation can result in one phase being enriched in both polymers or each phase being enriched with one of the two biopolymers (Panouillé and Larreta-Garde, 2009). However, Derkach *et al.* (2015) reported that the polyelectrolyte complexes between carrageenan and gelatin are formed via the interaction of positively charged groups on gelatin and the polysaccharide sulphate ions. They reported that increasing carrageenan concentration accelerated the gelation and increased the strength of the gelatin gel. Furthermore, films obtained from fish gelatin mixed with κ-carrageenan had an increased tensile strength when the levels of κ-carrageenan or gellan gum increased (Pranoto *et al.*, 2007). Although there is some literature regarding the impact of carrageenan on the gelling properties of gelatin, the effect of κ-carrageenan at various levels on the physical and rheological properties of fish gelatin in the absence of cations has not yet been well elucidated.

7.3 Objective

To investigate the effect of κ-carrageenan levels (without cations) on the properties and microstructure of commercial fish gelatin gels. Further characterisation of gel structure, gelation rate and melting behaviours was conducted by texture profile analysis and rheological measurements.

7.4 Materials and methods

7.4.1 Chemicals

All chemicals were of analytical grade. Fluorescein isothiocyanate isomer I (FITC) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fish gelatin from tilapia skin (~240 bloom) was obtained from Lapi Gelatine S.p.A (Empoli, Italy). κ-Carrageenan powder with gel strength of 508 g (1.5% w/v, at 20 °C) was obtained from High Science Co, Ltd. (Songkhla, Thailand).

7.4.2 Preparation of gelatin/carrageenan mixed solutions

Fish gelatin (FG) or κ-carrageenan (KC) were mixed with distilled water to obtain a concentration of 5 g/100 mL each, followed by heating at 60 °C for FG and 90 °C for KC. The mixtures were stirred until the samples were fully solubilised. FG and KC solutions were mixed to obtain FG:KC ratios of 100:0, 75:25, 50:50, 25:75 and 0:100, w/w, referred to as “FG”, “FG-25KC”, “FG-50KC”, “FG-75KC” and “KC”, respectively. The mixtures were transferred to a cylindrical mould with 3 cm diameter and 2.5 cm height. Those mixtures were incubated at refrigerated temperature for 18 h prior to analysis.

7.4.3 Analyses

7.4.3.1 Measurement of gel strength

Gel strength was determined at 8-10 °C using a texture analyser (Stable Micro System, Surrey, UK) with a load cell of 5 kg, cross-head speed of 1 mm/s, equipped with a 1.27 cm diameter flat-faced cylindrical Teflon® plunger. The

maximum force (grams) was recorded, when the plunger had penetrated 4 mm into the gel samples.

7.4.3.2 Texture profile analysis (TPA)

TPA was performed using a TA-XT2 texture analyser (Stable Micro Systems, Surrey, UK) with a load cell of 50 kg, equipped with a 5.0 cm diameter flat-faced cylindrical aluminium probe. FG/KC mixed gels were placed on the instrument's base, and the tests consisted of two compression cycles with a time interval of 8 sec. TPA textural parameters were measured at 8-10 °C using a crosshead speed of 0.5 mm/s and 50% compression of the original sample height. Hardness and springiness were calculated from the force–time curves generated for each sample. Hardness is defined as the maximum force peak on the first compression cycle, whilst springiness is defined as the height recovered during the time between the end of the first compression and the start of the second compression (Lau *et al.*, 2000).

7.4.3.3 Determination of gel color

The color of gel samples was measured by a Hunter lab colorimeter (Color Flex, Hunter Lab Inc., Reston, VA, USA). L^*, a^*, and b^* values indicating lightness/brightness, redness/greenness, and yellowness/blueness, respectively, were recorded. The colorimeter was warmed up for 10 min and calibrated with a white standard (L^* = 90.77, a^* = -1.27 and b^* = 0.50). Total difference in color (ΔE^*) was calculated according to the following equation (Gennadios *et al.*, 1996):

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad (1)$$

where ΔL^*, Δa^* and Δb^* are the differences between the corresponding color parameter of the sample and that of white standard.

7.4.3.4 Rheological measurements

The rheological properties of mixed FG/KC samples were determined using a Paar Physica MCR 301 stress-controlled rheometer (Anton-Paar, Graz,

Austria) fitted with a cup and bob (Couette geometry). The inner diameter of the cup was 27.5 mm and the diameter of the bob was 26.5 mm, giving a gap between the cup and the bob of 1.0 mm.

A sample volume of 17 ml was transferred to the rheometer. The surface of the sample in the Couette geometry was covered with a thin layer of mineral oil (one drop of oil) to prevent evaporation. The sample was initially maintained at a temperature of 60 °C for 15 min for equilibration. Then the sample was cooled from 60 to 5 °C. The sample was left for 12 h at 5 °C to ensure that the gel was fully developed. Frequency sweeps were performed at a constant strain of 1% and a constant temperature of 5 °C at frequencies ranging from 0.01 to 100 Hz. The elastic modulus G' and the loss (viscous) modulus G'' were recorded.

Finally, gelling and melting temperature measurements were conducted at a constant frequency of 1 Hz and a constant applied strain of 1%. The samples were heated from 5 to 60 °C and subsequently cooled to 5 °C at a constant rate of 1.0 °C/min. The gelling and melting temperatures were defined as where tan δ (G'' / G') = 1 or δ = 45°.

7.4.3.5 Fourier transform infrared (FTIR) spectroscopy

Prior to FTIR analysis, FG/KC mixed gels were freeze-dried. FTIR spectra of samples were obtained using a FTIR spectrometer (EQUINOX 55, Bruker, Ettlingen, Germany) equipped with a deuterated l-alanine tri-glycine sulphate (DLATGS) detector. The horizontal attenuated total reflectance accessory (HATR) was mounted into the sample compartment. The internal reflection crystal (Pike Technologies, Madison, WI, USA), made of zinc selenide, had a 45° angle of incidence to the IR beam. Spectra were acquired at room temperature and at a resolution of 4 cm^{-1} and the measurement range was 4000–400 cm^{-1} (mid-IR region). Spectra were averaged over 32 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from a clean empty cell at 25 °C. Analysis of spectral data was carried out using the OPUS 3.0 data collection software programme (Bruker, Ettlingen, Germany).

7.4.3.6 Confocal laser scanning microscopy

Microstructures of FG/KC mixed gel samples were examined with a confocal laser scanning microscope (CLSM; Olympus, FV300, Tokyo, Japan). Carrageenan was firstly stained using FITC according to the method of Hans Tromp *et al.* (2001) with a slight modification. KC (1 g) and pyridine (100 µL) were dissolved in dimethyl sulfoxide (DMSO) (20 mL) and stirred at room temperature for 30 min. FITC (50 mg) were added in the mixture and heated at 95 °C for 3 h using a temperature controlled water bath model W350 (Memmert, Schwabach, Germany). Finally, the solution was cooled down to room temperature and washed with ethanol until the solvent became colorless. The pellet was stored overnight at room temperature and dried at 60 °C for 24 h. The stained carrageenan powder was then stored at room temperature.

FG/stained-KC mixed solutions were prepared as described in Section 2.2. A few drops of mixed solution were placed onto glass cavity slides and coverslips were placed on top. The samples were incubated at 4 °C overnight to form gels prior to analysis. The samples were observed under Olympus 20x objective (Olympus, Tokyo, Japan) at room temperature using the fluorescence mode at an excitation wavelength of 492 nm and an emission wavelength of 520 nm. Digital images were acquired in tif format and in 1024 x 1024 pixel resolution.

7.4.4 Statistical analysis

All experiments were run in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out using Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for windows: SPSS Inc., Chicago, IL, USA).

7.5 Results and discussion

7.5.1 Gel strength

Gel strength data for the FG/KC mixtures are compared with FG and KC in Table 21. In general, FG exhibited the lowest gel strength (~211 g), whilst the highest gel strength was found for KC sample (~430 g) ($P<0.05$). In general, the gel strength of FG/KC mixed gels increased as KC content increased ($P<0.05$), although there was no difference in gel strength between FG (~211 g) and FG-25KC (~222 g) ($P>0.05$). The increase in gel strength of FG containing KC might be governed by the interaction between the negatively charged sulphate ester groups of KC and the positively charged chains of FG (Pranoto *et al.*, 2007). When KC was incorporated, FG gel became more resistant to applied force as shown by the increased gel strength. Typically κ-carrageenan is considered to undergo coil to helix transitions upon cooling and the helices aggregate in the presence of cations (Thrimawithana *et al.*, 2010). In the present study where cations were omitted the increased gel strength of FG containing KC is most likely due to the interaction between those two hydrocolloids via ionic interactions. The KC sulphate groups are likely to undergo ionic interactions with positively charged domains of the FG. The role of cations in the induction of KC gel can be considered negligible in these systems. Therefore, the incorporation of KC could increase the strength of FG gel in a concentration-dependent manner. In fact, the gel strength of the FG/KC mixtures (G) can be fitted using a simple "blending law" (Kasapis, 2008):

$$\frac{1}{G} = \frac{\emptyset_F}{G_F} + \frac{\emptyset_C}{G_C} \qquad (2)$$

Where G_F (=211 g) and G_C (=430 g) are the gel strength of the fish gelatin and the carrageenan alone, and ϕ_F and ϕ_c (=1-ϕ_F) are the volume fractions of the gelatin and the carrageenan in the mixture. As seen in Figure 30, the results yielded by equation (2) are in very good agreement with the experimental gel strength values reported in Table 21.

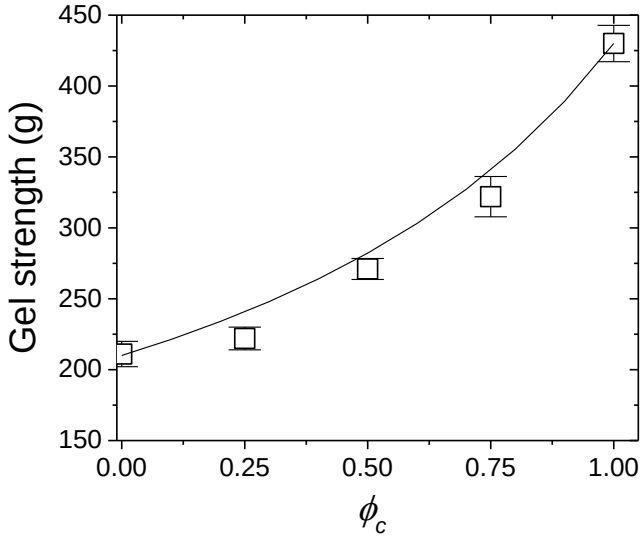

Figure 30. Gel strength of the FG/KC mixtures as a function of κ-carrageenan volume fraction. Solid line is obtained using Equation (2).

7.5.2 Texture profile analysis (TPA) measurements

Texture profile analysis (TPA) is an easily quantifiable analytical technique that has been widely used in the food industry (Yang *et al.*, 2013). Hardness of gels from FG, KC as well as FG/KC mixed gels are presented in Table 21. The hardness of the FG gel was ~1395 g, the lowest value compared to other FG gel containing KC or KC alone ($P<0.05$), except FG-25KC gel. The hardness of the FG gels generally increased as the level of KC increased above 25% ($P<0.05$) and KC had the highest hardness (~6235 g). This result was in agreement with the increase in gel strength with the increase in the amount of incorporated KC. Lau *et al.* (2000) reported that hardness of gellan/gelatin mixed gels increased when the ratio of gellan to gelatin was increased where gellan, like carrageenan, is an anionic polysaccharide. In the present study, carrageenan molecules could be expected to disperse in the gelatin solution, exerting a filler effect, in addition to exhibiting electrostatic attractive interactions with gelatin molecules. These phenomena resulted in the observed increase in the hardness of FG/KC mixed gels.

Springiness of FG, KC and FG/KC mixed gels is shown in Table 21. Springiness is a measure of the rate at which a deformed material returns to its undeformed condition after the deforming force is removed (Yuan *et al.*, 2016). In other words, springiness indicates the elastic recovery of a material. FG generally had the highest springiness (96.60%), whereas KC showed the lowest value (74.05%) ($P<0.05$). In general, the springiness of the mixed gels decreased with increasing levels of KC ($P<0.05$) although there were no differences in the springiness of the FG-50KC : FG-75KC pair and the FG : FG-25KC pair ($P>0.05$). It was noted that FG gel became more brittle (lower springiness) when KC was added, especially at higher levels. It is known that KC gels are typically less springy and are broken easily during compressive testing (Huang *et al.*, 2007). This rigidity of the KC gel causes the FG to become more rigid or brittle with increasing KC levels. Thus, the addition of KC at a high level negatively affected the textural characteristic of mixed FG/KC gels, particularly springiness.

Table 21. Gel strength, hardness, springiness and gelling and melting temperatures of fish gelatin, carrageenan and mixed gels

Samples	Gel strength (g)	Hardness (g)	Springiness (%)	Gelling temperature (°C)	Melting temperature (°C)
FG	211±8.93d	1395±72d	96.60±0.47a	17.7±0.6e	25.8±0.7c
FG-25KC	222±8.01d	1545±56d	93.03±1.66a	39.6±1.1d	28.5±1.0bc
FG-50KC	271±7.43c	1928±59c	88.48±2.78b	43.6±0.6c	31.6±2.4b
FG-75KC	322±14.23b	4249±171b	85.23±3.31b	45.7±0.4b	53.4±4.2a
KC	430±12.80a	6235±196a	74.05±4.03c	57.3±0.8a	52.7±2.2a

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

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

7.5.3 Color

The color of FG/KC mixed gels and FG and KC gels are expressed as L^*, a^*, b^* and ΔE^* in Table 22. Amongst all gel samples, FG gel had the highest lightness (L^*-value), compared with other gels (P<0.05). The L^*-values of FG/KC mixed gels decreased with increasing levels of KC (P<0.05). However, no difference in L^*-value between FG-25KC and FG-50KC gels was observed (P>0.05). The lowest L^*-value was found in KC gel (~43.12). Furthermore, addition of KC increased the redness (a^*-value) of FG gel although FG-25KC had the highest a^*-value of all the gels (P<0.05). The yellowness (b^*-value) of the FG-50KC gel was the lowest among all gels tested (P<0.05), while the FG gel had the highest b^*-value (~8.63) (P<0.05). This might be associated with non-enzymatic browning reaction of gelatin occurring during gelatin production (Sinthusamran *et al.*, 2014). Amongst all gel samples, FG gel showed the lowest total difference in the color value (ΔE^*) (22.94) with the highest lightless (L^*-value). The ΔE^*-value of FG/KC mixed gel was increased as KC content increased (P<0.05). However, no difference in ΔE^*-value was observed between FG-25KC and FG-50KC (P>0.05). The result also suggested that the color of mixed gels might be influenced by the color of individual hydrocolloid as well as the way the hydrocolloids are dispersed and interact with each other. For instance, phase separation is expected to influence color measurements. Therefore, the addition of carrageenan had an impact on the color of FG/KC mixed gels.

Table 22. Color values of fish gelatin, carrageenan and mixed gels

Samples	Color values			
	L^*	a^*	b^*	ΔE^*
FG	71.39±0.87a	-1.64±0.05a	8.63±0.24a	22.94±0.78a
FG-25KC	49.85±0.68b	0.29±0.10d	5.49±0.44b	43.30±0.65b
FG-50KC	49.05±0.47b	-1.10±0.16b	0.16±0.23e	43.78±0.47b
FG-75KC	48.03±0.25d	-1.11±0.09b	1.87±0.23d	44.82±0.26c
KC	43.12±0.14e	-0.49±0.13c	3.77±0.20c	49.82±0.13d

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

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

7.5.4 FTIR spectra

FTIR spectra were used to probe the interaction between FG and KC (Derkach *et al.*, 2015). As shown in Figure 31, the major bands in FTIR spectra of FG gel were amide I, amide II, amide III, amide A and amide B. Amide I, II and III correspond to wavenumbers of 1646 cm^{-1} (vibration of C=O stretching), 1554 cm^{-1} (bending vibration of N-H group and C-N group) and 1240 cm^{-1} (C-N stretching vibration and N-H deformation), respectively (Nagarajan *et al.*, 2012; Payne and Veis, 1988). Amide A at 3416 cm^{-1}, is associated with the N-H stretching vibration (Doyle *et al.*, 1975). Amide B at 2958 cm^{-1}, representing CH stretching vibrations of $–CH_2$ group (Nagarajan *et al.*, 2012). For KC spectra, the main bands were observed at 1250, 928 and 847 cm^{-1}, corresponding to ester sulphate group, 3,6-anhydride galactose group and D-galactose group, respectively (Derkach *et al.*, 2015). The amplitudes of those peaks increased as the proportion of KC increased in the mixture.

When KC was incorporated into FG, a slight shift of amide I band (1646 cm^{-1}) to higher wavenumbers, 1654, 1662 and 1656 cm^{-1}, for FG-25KC, FG-50KC and FG-75KC, respectively. The shift of amide I band to higher wavenumber indicated a decrease of ordered structures as the result of complex formation (Derkach *et al.*, 2015). Amide B of FG-25KC, FG-50KC and FG-75KC shifted to lower wavenumbers at 2937, 2935 and 2929 cm^{-1}, respectively, compared with that of FG, indicating that CH_2 groups more likely interacted with other side chains. Moreover, the sulphate groups in FG-25KC, FG-50KC and FG-75KC shifted to lower wavenumbers, 1241, 1242 and 1245 cm^{-1}, respectively. The shift of the absorption bands of the sulphate groups to lower frequency, compared to the band of KC gel (1250 cm^{-1}), indicates the interaction of sulphate group of KC via amide groups of FG. The results suggested that the interaction between amide groups of gelatin and sulphate groups of KC occurred, leading to the formation of polyelectrolyte complexes.

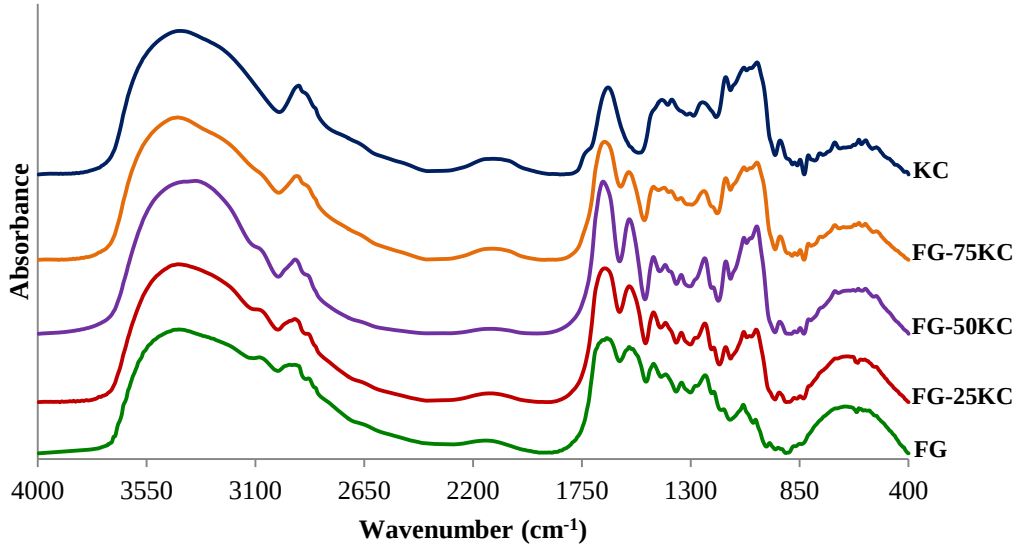

Figure 31. FTIR spectra of fish gelatin, carrageenan and mixed gels. Gels were freeze-dried prior to analysis.

7.5.5 Rheological properties

7.5.5.1 Time sweep

Time sweep measurements at a constant strain of 1% and a constant frequency of 1Hz were performed to determine the gelation course of FG, KC and FG/KC mixtures at 5 °C for 12 h. The elastic modulus (*G′*) and loss modulus (*G″*) as a function of time for all gels are represented in Figure 32. The gelation process is indicated by the a sharp increases of both *G′* and *G″* (Sinthusamran *et al.*, 2014). When the solution was cooled to 5 °C, the *G′* and *G″* of all FG/KC mixtures increased rapidly at ~30 mins. The result indicated the rapid formation of junction zones for gel network, leading to a transition from sol to gel (Mohtar *et al.*, 2014). During cooling, gelatin molecules undergo a random coil to helix conformation transition and water is entrapped inside the gel structure (Derkach *et al.*, 2015). The rate of increase in *G′* is generally higher than that of the *G″*. After ~30 min of cooling, *G′* was higher than *G″* value for KC and for all the mixed gels, indicating that all solutions were gelled (Mohtar *et al.*, 2013). FG (without KC incorporation) took a longer time (~40 min) for the gelation to occur, compared with those containing KC at all levels which

gelified in a shorter time (≤30 min). This suggested that the addition of KC could enhance the gelation rate of FG, especially the on-set time, that is the time at which gelation occurs. This result is in agreement with Derkach *et al.* (2015) who reported that an increase in κ-carrageenan content accelerated the formation of network structures in fish gelatin. This was consistent with the increases in hardness and gel strength (Table 1). *G′* and *G″* of KC markedly increased within ~20 min indicating a shorter time for gelation of KC, compared with other FG/KC mixtures. However, after ~30 mins of cooling, *G′* and *G″* of KC slightly decreased. *G′* and *G″* of FG and FG/KC mixed samples remained constant after 60 min of cooling. This might be related to the enhanced formation of junction zones or chain interactions between FG and KC when KC was present (Derkach *et al.*, 2015; Pranoto *et al.*, 2007).

7.5.5.2 Frequency sweep

Figure 33 shows the frequency sweeps of FG, KC and FG/KC mixtures performed to investigate the viscoelastic behaviour of these systems. At all frequencies, the *G′* was higher than *G″* by more than 10 fold, while *G′* and *G″* did not vary markedly with the frequency. This indicated that FG and FG/KC mixtures can be considered as strong gels (Mohtar *et al.*, 2014). The *G′* values obtained from FG and FG-25KC showed nearly a constant value in the range of frequency from 0.01 to 100 Hz, suggesting that the stable cross-linked gel network was formed (solid-like behaviour) (Mohtar *et al.*, 2014). For FG gel the *G″* decreased when the frequency increased up to 1 Hz, which suggests that FG gel might not be able to form strong junction zones of gel network. However, at higher frequency, FG-50KC and FG-75KC gel samples showed an increased *G′* and *G″*, suggesting that a higher gel strength for each sample was obtained (Mohtar *et al.*, 2014). The result is in agreement with the gel strength and hardness of the resulting mixed gels (Table 1). Carrageenan without cations had lower *G′* values than carrageenan mixed with cations (Thrimawithana *et al.*, 2010). Electrostatic repulsions between polymer chains and coil formation decreased as the counter-ions concentration increased. This might increase the cross-linking, which provided the gel with a higher *G′* value (Haug *et al.*, 2004; Núñez-Santiago *et al.*, 2011). In the present study, no cations were added. The

elastic modulus *G′* is affected by the interactions between biopolymers as well as phase separation in the system. Therefore, the levels of KC directly affected the gel structure, especially via the interactions amongst polymers.

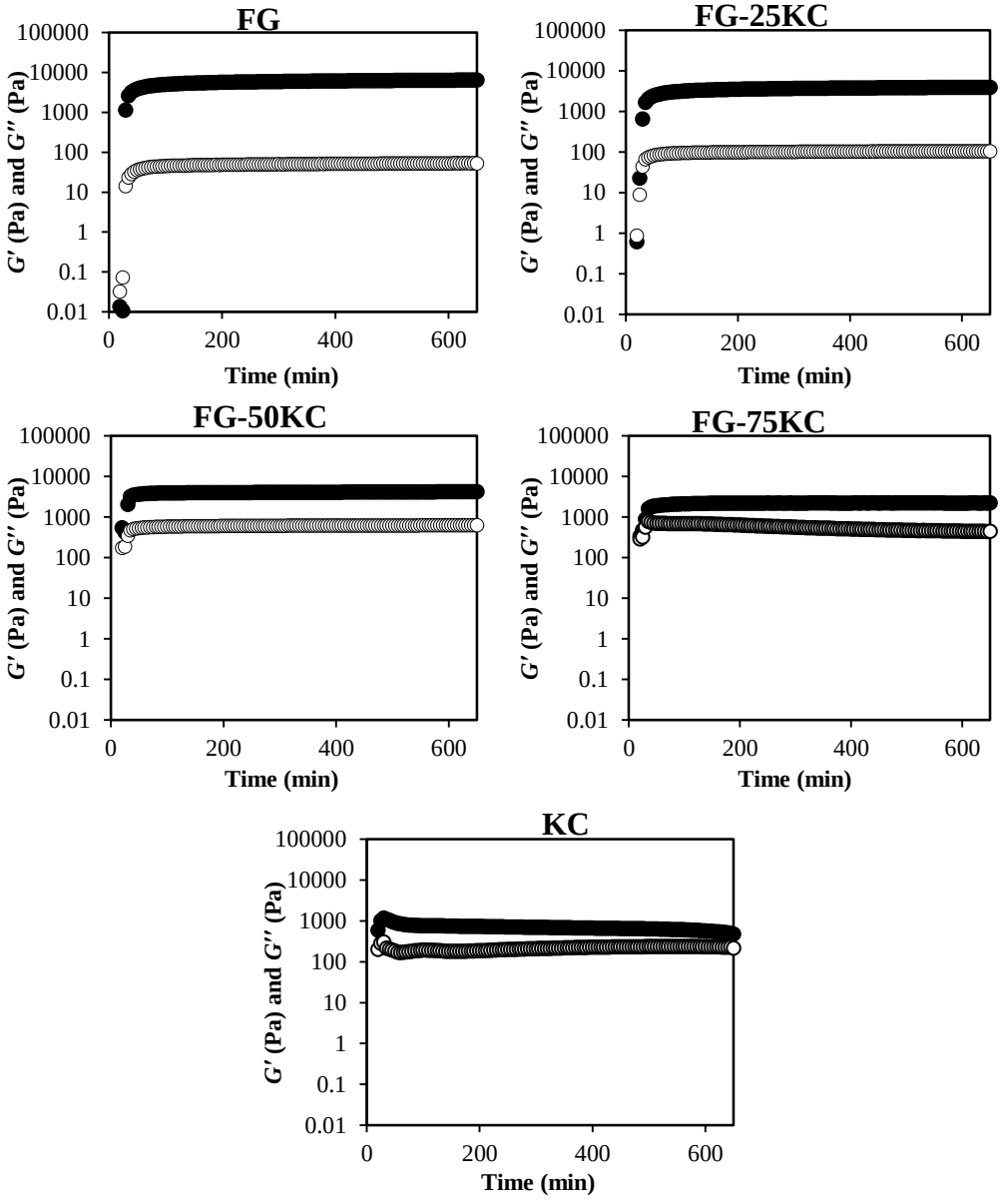

Figure 32. Dynamic viscoelastic storage (*G′*: solid symbols) and loss modulus (*G″*: open symbols) as function of time of fish gelatin (FG), carrageenan (KC) and mixed gels (FG-25KC, FG-50KC and FG-75KC). All measurements were taken at 5 °C.

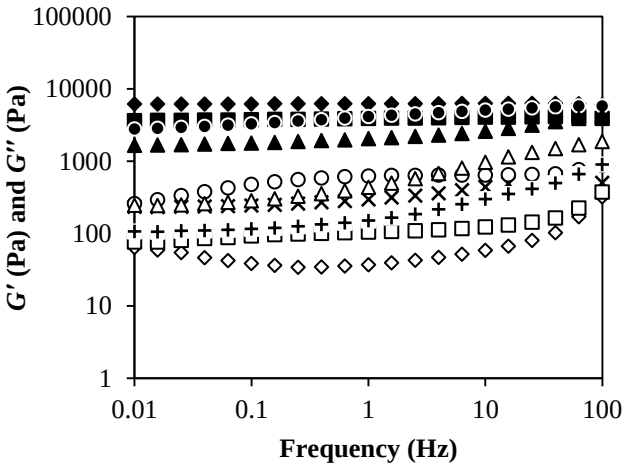

Figure 33. Dynamic viscoelastic storage (*G′*: solid symbols) and loss modulus (*G″*: open symbols) as function of frequency of fish gelatin (FG; ♦), carrageenan (KC; G:′ ×; G″: +) and mixed gels (FG-25KC; ■, FG-50KC; ●, and FG-75KC; ▲). All measurements were taken at 5 °C.

7.5.6 Gelling and melting temperatures

Gelling and melting temperatures of FG, KC and FG/KC mixtures are shown in Table 21. Gelling temperatures of FG and KC were ~18 and ~57 °C, respectively. FG did not set at room temperature (25-28 °C). Gelling temperature of FG/KC mixed gel increased as KC levels increased ($P<0.05$). The gelling temperatures of the mixtures were different, depending on the levels of KC present, meaning that it is more likely that interactions between both hydrocolloids occurred. Sulphate groups of KC presumably have an electrostatic interaction with the FG (Derkach *et al.,* 2015). This is supported by the shift of sulphate peaks to lower frequency due to the interaction between sulphate group and amide groups (Figure 30). However, the presence of KC in FG gel can result in thermodynamic incompatibility in mixed systems (Haug *et al.,* 2004). During gelation of the FG/KC mixed gel, KC probably underwent ordering and formed a network, whilst gelatin was still in a random coil conformation. Thus, the mixed system might have a bi-continuous phase (Haug *et al.,* 2004). Melting temperature of FG/KC mixed gel samples was increased with increasing KC content ($P<0.05$). However, the addition of 25% KC had no impact on the melting temperature, ($P>0.05$). Haug *et al.* (2004)

reported that a gel of 1% carrageenan alone and a gel of 10% fish gelatin with 1% carrageenan containing 20 mM KCl had the same melting temperature. Increasing melting temperature at higher KC concentrations (>25%) was in accordance with increasing gelling temperature of FG/KC mixtures. Amongst all mixtures, the highest melting temperature was found in FG-75KC (~53.4 °C) ($P<0.05$), which was similar to that of KC alone (~52.7 °C). This suggests that KC directly contributes to the melting behaviour of a mixed gel to a greater extent than the gelatin. In this study, the presence of KC might have increased the stability of the gel network of FG, leading to the increased melting temperature. Thus, the addition of KC strongly affected the thermo-stability of FG/KC mixed gel.

7.5.7 CLSM

The microstructures of FG/KC mixed gels were visualised by CLSM, as shown in Figure 34. KC appeared as bright green domains since it was stained with FITC, while the dark regions represent FG. The microstructure of gel networks is considered as an important factor influencing the physical properties of gel (Sinthusamran *et al.*, 2014). For all gels, KC was localised in specific regions of the matrix. At 25% KC (Figure 33A), KC (small green spot) was rarely observed in the gel network. FG gel containing 25% KC had less KC-rich regions, compared with those containing 50 and 75% KC. When KC levels were increased up to 50%, there were larger KC aggregates observed (Figure 33B). The largest KC aggregates were found in FG-75KC (Figure 33C), and were dispersed in FG continuous phase. KC gel could provide reinforcement for the gelatin network. As a consequence, the gel could be more resistant to the force, leading to a higher gel strength and hardness (Table 21). Thus, KC levels in FG had an influence on the gel network associated with the textural and rheological properties of gel.

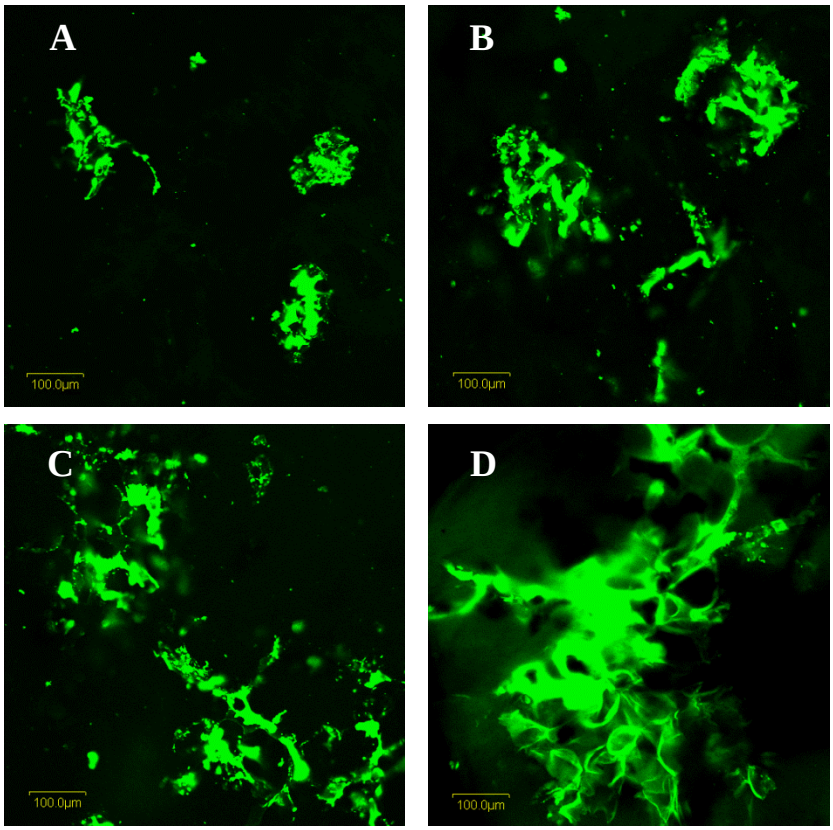

Figure 34. CSLM micrograph of fish gelatin/carrageenan mixed gels (FG-25KC (A), FG-50KC (B), FG-75KC (C) and KC (D)). Magnification: 20X

7.6 Conclusion

Fish gelatin is known to show lower gel strength as well as lower thermal stability, compared to bovine and porcine gelatins. The addition of KC to FG (without cations) can result in an increase in the gel strength, gelation rate, and thermal stability of the mixed gels.

KC had a significant effect on the textural and rheological properties of FG. Textural properties (gel strength, hardness and springiness) of FG/KC mixed gels were affected by KC levels. Gel strength and hardness increased as KC levels increased. FG-75KC showed the highest gel strength and hardness. However, the springiness of the mixed gels decreased with increasing levels of KC. The addition of KC caused the formation of gel networks between FG and KC as elucidated by FTIR spectra. Moreover, KC also affected the gelation rate of FG as measured by a time sweep. Gelling and melting temperatures of FG/KC mixed gels increased when KC

levels were increased. CLSM indicated that KC was dispersed in gel matrix of FG. The present study demonstrates the prospective use of KC to improve the gelling properties of FG gels. Thus, the addition of KC could improve the texture and rheological properties, particularly increasing gelling and melting temperature of FG.

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CHAPTER 8

PHYSICAL AND SENSORY PROPERTIES OF GELATIN FROM SEABASS AS AFFECTED BY AGAR AND K-CARRAGEENAN

8.1 Abstract

Physical and sensory properties of gelatin from skin and swim bladder of seabass (SK and SW, respectively) as affected by *Gracilaria tenuistipitata* agar (GA) or κ-carrageenan (KC) at 10 and 20% substitution were investigated. Hardness of both SK and SW gels containing GA increased with increasing level of agar. However, the addition of KC lowered hardness of mixed gels. Springiness and cohesiveness of either SK or SW gels decreased as the level of both GA or KC increased. Gelling and melting temperatures generally increased when the level of hydrocolloids was increased. The highest gelling (36.27 °C) and melting temperatures (43.44 °C) were obtained for SK added with 20% GA and 20% KC, respectively. However, the addition of both hydrocolloids at 10% affected gel microstructure differently and decreased digestibility of mixed gelatin gel in gastrointestinal tract model. Furthermore, the addition of GA at 10% could increase likeness score of sensory properties of gelatin gel. Therefore, the addition of hydrocolloids with appropriate level could improve the texture and sensory properties of gelatin from seabass.

8.2 Introduction

Gelatin is a fibrous protein extracted from collagenous material by thermal denaturation. It is one of the most versatile biopolymers and has a wide range of applications in food, pharmaceutical, cosmetic and photographic industries (Regenstein and Zhou, 2007). The gelatin primarily has been produced from skin and bone of pig and cow. However, the outbreaks of bovine spongiform encephalopathy (BSE), foot-and-mouth disease (FMD) as well as bird flu have resulted in anxiety

among users of gelatin from land animal origin. Additionally, the gelatin obtained from pig skin or bone cannot be consumed for Muslim and Jewish, due to the religious restrictions (Benjakul *et al.*, 2012a; Sadowska *et al.*, 2003). Currently, an increasing interest has been paid to alternative sources of gelatin, particularly from the by-products of fish processing (Kittiphattanabawon *et al.*, 2010; Sinthusamran *et al.*, 2014). However, the application of fish gelatin is limited, due to its poor gelling property, compared with mammalian counterpart (Muyonga *et al.*, 2004). Gelatin from different fish species have varying thermal and rheological properties such as gel strength, gelling and melting temperatures (Sinthusamran *et al.*, 2014). The textural and sensory properties of gelatin gel are greatly influenced by the rheological properties, which were governed by their chemical compositions and chain length (Benjakul *et al.*, 2012b; Kołodziejska *et al.*, 2004).

To improve the property of fish gelatin, some polysaccharides can be used as co-gelators. However, the effectiveness of individual polysaccharide on improvement of gelatin can be varied. Their compatibility is an important factor. Interaction of charged gelatin and polysaccharide macroions leads to the formation of polyelectrolyte complexes (Derkach *et al.*, 2015). In addition, polysaccharides possess several functional properties, especially gelation (Lau *et al.*, 2000). The mixtures between gelatin and various polysaccharides, such as maltodextrin, pectin, alginate, κ-carrageenan and agar have been characterized (Haug *et al.*, 2004; Panouillé and Larreta-Garde, 2009; Sinthusamran *et al.*, 2016). When two biopolymers (protein and polysaccharides) are mixed together, different behaviors can occur. In most cases, the mixtures of two or more biopolymers may have a phase separation, which can be associative (first phase being enriched in both polymers, the second one in solvent) or segregative (each phase being enriched with one of the two biopolymers) (Panouillé and Larreta-Garde, 2009). The addition of κ-carrageenan into gelatin could improve the gelation rate, viscoelasticity, gel strength and melting temperature of resulting mixture (Derkach *et al.*, 2015). Sinthusamran *et al.* (2016) reported that gelatin gel become more brittle when agar was incorporated, especially at high levels. Failure stress of agar/gelatin mixed gels increased as levels of agar increased (Sinthusamran *et al.*, 2016). Lau *et al.* (2000) reported that hardness of gellan/gelatin mixed gels

increased as proportion of gellan increased. The incorporation of ions increased the brittleness but decreased springiness and cohesiveness of gellan/gelatin mixed gel.

Seabass (*Lates calcarifer*) is one of an economically important fish in Thailand. Skin and swim bladder can be used for gelatin production, in which their market values can be increased (Binsi *et al.*, 2009). To widen the uses of gelatin from seabass, the addition of some selected polysaccharides might improve the rheological, textural and sensory properties of mixed gels. Nevertheless, no information regarding the textural and sensory properties of gelatin from skin and swim bladder of seabass as influenced by the addition of hydrocolloids exists. Therefore, the aims of the study were to investigate the impact of incorporation of agar or κ-carrageenan at various levels on physical and sensory properties of gelatin from skin and swim bladder of seabass.

8.3 Objective

To investigate the impact of incorporation of agar or κ-carrageenan at various levels on physical and sensory properties of gelatin from skin and swim bladder of seabass.

8.4 Materials and methods

8.4.1 Gelatin and κ-carrageenan

All chemicals were of analytical grade. Fish gelatin produced from tilapia skin (~240 bloom) was obtained from Lapi Gelatine S.p.A (Empoli, Italy). Food grade bovine bone gelatin with the gel strength of 150–250 g was purchased from Halagel (Thailand) Co., Ltd. (Bangkok, Thailand). κ-Carrageenan powder with gel strength of 508 g (1.5% w/v, at 20 °C) was procured from High Science Co, Ltd. (Songkhla, Thailand).

8.4.2 Preparation of gelatin from seabass skin and swim bladder

Gelatins were extracted from skin and swim bladder of seabass according to the method of Jongjareonrak *et al.* (2006) with a slight modification. Before gelatin extraction, skin and swim bladder were soaked in 0.1 M NaOH with a

sample/solution ratio of 1:10 (w/v) in order to remove non-collagenous proteins. The mixture was stirred continuously for 3 h at room temperature (28–30 °C) using an overhead stirrer with a propeller (W20.n IKA®-Werke GmbH & CO.KG, Staufen, Germany). The alkaline solution was changed every 1 h for totally 3 times. The residues were then washed with tap water until a neutral or faintly basic pH of wash water was obtained. The deproteinized matters were then mixed with 0.05 M acetic acid at a sample/solution ratio of 1:10 (w/v) to swell collagenous material. The mixture was stirred at room temperature for 2 h. The swollen skin and swim bladders were washed using tap water until wash water became neutral or faintly acidic in pH.

To extract the gelatin, the swollen skin and swim bladders were mixed with distilled water at a ratio of 1:10 (w/v) with continuous stirring at 55 and 65 °C, respectively, for 6 h. The mixtures were filtered with two layers of cheesecloth. Then, the filtrates were mixed with 1 % (w/v) activated carbon for 1 h with continuous stirring. The mixtures were centrifuged at 17,500xg for 15 min at 25 °C using a Beckman model Avanti J-E centrifuge (Beckman Coulter, Inc., Palo Alto, CA, USA) to remove insoluble material. The supernatants were filtered using a Buchner funnel with a Whatman No.4 filter paper (Whatman International, Ltd., Maidstone, England). Finally, the filtrates were freeze-dried using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark) at -50 °C for 72 h. Dry gelatins extracted from skin and swim bladder were referred to as ‘SK’ and ‘SW’, respectively.

8.4.3 Preparation of agar from *Gracilaria tenuistipitata*

The agar was extracted from the algae, Gracilaria tenuistipitata, following the method of Yarnpakdee *et al.* (2015). Prior to extraction, dried algae were soaked in 5% NaOH, with the algae/solution ratio of 1:50 (w/v) for 24 h at room temperature. The mixture was heated at 90 °C for 3 h with continuous stirring using an overhead stirrer. After alkaline treatment, the algae were washed in tap water until the neutral pH was obtained. Finally, the pretreated algae were mixed with distilled water at a ratio of 1:50 (w/v) at 95 °C for 2 h. The mixture was firstly filtered through a cheesecloth. The filtrate was further filtered under pressure using a Buchner funnel with a Whatman No. 4 filter paper. The filtrate was transferred into plastic container and allowed to gel at room temperature (25-26 °C). Gel was subsequently frozen for

24 h and thawed at room temperature for approximately 4 h. Thereafter, the thawed gel was frozen for another 24 h prior to freeze-drying. The dried matter was blended and sieved (20 mesh). The powder was referred to as “agar”.

8.4.4 Preparation of gelatin/hydrocolloids mixed gels

Gelatins (SK or SW) and hydrocolloids (agar or κ-carrageenan) were separately solubilized in distilled water at 60 °C and 95 °C, respectively. Solutions of agar (GA) or κ-carrageenan (KC) were mixed with gelatin solution to obtain the mixed solution with 10 and 20% gelatin substitution. Those samples were named as “SK-10GA or SK-10KC or SW-10GA or SW-10KC” for SK and SW gelatin mixed with A or C to obtain 10% substitution. “SK-20GA or SK-20KC or SW-20GA or SW-20KC” represented SK and SW gel containing GA or KC with 20% substitution. Total solid content of all solutions was 6.67% (w/v). The mixtures were transferred to a cylindrical mould with 3 cm diameter and 2.5 cm height. Those mixtures were incubated at refrigerated temperature for 18 h. Gelatin (SK and SW) and gelatin-hydrocolloids mixed gels were then subjected to analyses in comparison with commercial bovine gelatin (BG) and commercial fish gelatin (FG).

8.4.5 Analyses

8.4.5.1 Texture profile analysis (TPA)

TPA was performed using a TA-XT2 texture analyzer (Stable Micro Systems, Surrey, UK) with a load cell of 50 kg, equipped with a 5.0 cm diameter flat-faced cylindrical aluminum probe. Gel samples were placed on the instrument's base, and the tests consisted of two compression cycles with a time interval of 5 sec. TPA textural parameters were measured at 8-10 °C using a crosshead speed of 1.0 mm/s and 50% compression of the original sample height. Hardness, springiness, cohesiveness, gumminess and chewiness were calculated from the force-time-curves generated for each sample. Hardness (the maximum force peak on the first compression cycle), springiness (the height recovered during the time between the end of the first compression and the start of the second compression), cohesiveness (the ratio of the area under the first and second compression), gumminess (the hardness

multiplied by cohesiveness) and chewiness (the hardness multiplied by cohesiveness and springiness) were calculated from the force–time curves generated for each sample (Lau *et al.*, 2000; Yang *et al.*, 2007).

8.4.5.2 Determination of gel color

The color of gel samples (6.67%, w/v) was measured by a Hunter lab colorimeter (Color Flex, Hunter Lab Inc., Reston, VA, USA). L^*, a^*, and b^* values indicating lightness/brightness, redness/greenness, and yellowness/blueness, respectively, were recorded. The colorimeter was warmed up for 10 min and calibrated with a white standard (L^* = 90.77, a^* = -1.27 and b^* = 0.50). Total difference in color (ΔE^*) was calculated according to the following equation (1) (Gennadios *et al.*, 1996):

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

where ΔL^* , Δa^* and Δb^* are the differences between the corresponding color parameter of the sample and that of white standard.

8.4.5.3 Determination of syneresis

Gel syneresis was determined as described by Banerjee and Bhattacharya (2011). All gelatin or gelatin-hydrocolloid solutions (30 mL) were poured into 50 mL graduated centrifuge tubes and their masses (*m1*) were recorded. The samples were allowed to cool at room temperature for 15 min. Solutions were subsequently incubated at 4 °C for 12 h. Before measurement, gels were equilibrated at room temperature for 3 h. Then, these samples were centrifuged at 2,150×*g* at 25 °C for 10 min using a centrifuge (Beckman Coulter, Palo Alto, CA, USA). After centrifugation, gels along with the tubes were weighed (*m2*) again after discarding the supernatant. The percentage of syneresis was calculated as follows:

$$\text{Syneresis (\%)} = \frac{(m1\text{-}m2)}{m1} \times 100$$

8.4.5.4 Determination of gelling and melting temperatures

Gelling and melting temperatures of gelatin and gelatin-hydrocolloid mixtures were measured following the method of Boran *et al.* (2010). The measurement was performed using a RheoStress RS1 rheometer (HAAKE, Karlsruhe, Germany) in the oscillatory mode. The measuring geometry used was a stainless steel 60-mm-diameter parallel plate and the gap was set at 1.0 mm. All samples (6.67%, w/v) were incubated at 60 °C for 30 min. Then the solution (2.9 mL) was loaded on the Peltier plate and equilibrated at 60 °C for 5 min before measurements. The measurements were conducted at a constant frequency of 1 Hz, and a constant applied stress of 3 Pa. The samples were cooled from 60 to 5 °C and subsequently heated to 90 °C at a constant rate of 1.0 °C/min. The gelling and melting temperatures were calculated, where tan δ became 1 or δ was 45°.

8.4.5.5 Sensory evauation

Fifty non-trained panelists (aged between 25-40 years) were selected. They were the students and staffs from the Department of Food Technology, who were acquainted with gelatin products. Gel samples were cut into a bite-size (1 cm thickness and 2 cm diameter) and coded with 3-digit random numbers. Gel samples (8-10 °C) were served on dishes at room temperature under the fluorescent daylight-type illumination. The samples stored at 4 °C were removed from the refrigerator and tempered for 2 min at room temperature prior to sensory testing.The panelists were asked to evaluate for appearance, color, odor, texture, melting characteristic and overall liking of gel samples using 9-point hedonic scale (1, extremely dislike; 2, very much dislike; 3, moderately dislike; 4, slightly dislike; 5, neither like nor dislike; 6, slightly like; 7, moderately like; 8, very much like; 9, extremely like) (Meilgaard *et al.*, 2006). For texture attribute, panelists were asked to evaluate for firmness and elasticity of gel during ingestion. Melting characteristic was evaluated from the melting rate of gel during ingestion. Between samples, the panelists were asked to rinse their mouth with distilled water.

8.4.5.6 Microstructure analysis of gelatin gel

Microstructure of gels from SK and SW without and with GA and KC at 10% substitution, prepared as previously described, was visualized using a scanning electron microscope (SEM). 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 with serial concentrations 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 acceleration voltage of 20 kV.

8.4.5.7 Gastrointestinal tract model system

Gastrointestinal tract model system was prepared according to the method of Lo *et al.* (2006) with a slight modification. Gels of SK and SW without and with GA and KC at 10% substitution were added with distilled water to obtain a concentration of 0.25 g gel sample/ml. Gels of BG and FG were also used. The mixture was homogenized at 8,000 rpm for 2 min using a homogenizer (IKA Labortechnik homogenizer, Selangor, Malaysia). The solution was adjusted to pH 2.0 with 1 M HCl and pepsin dissolved in 0.1 M HCl was added to obtain the final concentration of 40 mg pepsin/g sample. The mixture was incubated at 37 °C for 1 h with a continuous shaking (Memmert Model SV 1422, Schwabach, Germany). After sampling, the sample was immediately placed in a boiling water bath for 10 min to terminate enzyme activity. Thereafter, the pH of the reaction mixture was raised to 5.3 with 1 M NaOH before the addition of 20 mg pancreatin/g sample. Subsequently, the pH of mixture was adjusted to 7.5 with 1 M NaOH. The mixture was incubated at 37 °C for 3 h with a continuous shaking. The sample was immediately placed in a boiling water bath for 10 min to terminated enzyme activity. During digestion, the mixture was taken at 0, 1 and 4 h for determination of α-amino group content and DH was calculated.

Free amino group content was determined following the method of Benjakul and Morrissey (1997). Properly diluted samples (125 µl) were mixed

thoroughly with 2.0 ml of 0.2 M phosphate buffer, pH 8.2, followed by the addition of 1.0 ml of 0.01% 2,4,6- trinitrobenzenesulfonic acid (TNBS) solution. The mixtures were then placed in a temperature-controlled 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 using a double beam spectrophotometer (Model UV-1800, Shimadzu, Kyoto, Japan) and the free amino group content was expressed in terms of L-glycine (the dominant amino acid). DH was determined following the method of Benjakul and Morrissey (1997) and defined as follows:

$$DH = \left[\frac{L_t - L_0}{L_{max} - L_0}\right] \times 100$$

where L_t corresponded to the amount of free amino acid released at time t. L_0 was the amount of free amino acid in original gel samples. L_{max} was the maximum amount of free amino acid in gel samples obtained after acid hydrolysis using of 6 N HCl. The hydrolysis was run at 105 °C for 24 h in heating bath (BUCHI Labortechnik AG, Tokyo, Japan). The acid-hydrolyzed sample was filtered through Whatman paper no. 1 to remove the unhydrolyzed debris. The supernatant was neutralized with 6 N NaOH before determination of free amino acid content.

8.4.6 Statistical analysis

All experiments were run in triplicate using three different lots of samples and a completely randomized design (CRD) was used. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out using the Duncan's multiple range test (Steel and Torrie, 1980). Randomized complete block design (RCBD) was used for analysis of acceptance test. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for windows: SPSS Inc., Chicago, IL, USA).

8.5 Results and discussion

8.5.1 Textural properties of gelatin gel as affected by agar and κ-carrageenan

8.5.1.1 Hardness

Hardness of gels from various gelatins and gelatins mixed with agar or κ-carrageenan at different levels is shown in Table 23. Hardness of SK and SW samples was ~4488 and ~4311 g, respectively. Their hardness was higher than that of BG (3545 g) and FG (3467 g) gels ($P<0.05$). On other hand, Boran *et al.* (2010) reported that porcine and bovine gelatin gel had higher hardness than fish gelatin. Hardness is related to the strength of gel structure under compression (Boran *et al.*, 2010; Lau *et al.*, 2000). Hardness of both SK and SW gels increased as the level of agar added increased ($P<0.05$). The highest hardness was observed in SK-20GA ($P<0.05$). In contrast, when κ-carrageenan was incorporated into SK or SW gels, the resulting mixed gel showed the decreases in hardness, regardless of gelatin types ($P<0.05$). Nevertheless, there was no difference in hardness between gelatin gels containing 10 and 20% κ-carrageenan ($P>0.05$). The result indicated that SK or SW gels containing agar had the higher hardness than those mixed with κ-carrageenan ($P<0.05$). The lowered hardness of mixed gel containing κ-carrageenan might be associated with the phase separation between the two biopolymers presented in the mixed gel. It was reported that less amount of carrageenan induced phase separation in the mixed gelatin/carrageenan gel (Doublier *et al.*, 2000). In the present study, the agar was more efficient in increasing the hardness of gelatin gel, compared to carrageenan. Therefore, the types and levels of hydrocolloids directly affected gel strength of gelatin.

8.5.1.2 Springiness

Various gelatin gel and gelatin mixed with agar or κ-carrageenan at various levels had different springiness as presented in Table 23. Springiness is a perception of gel “rubberiness” in the mouth, and indicates how much the gel structure is broken by the initial compression (Lau *et al.*, 2000). Springiness of gelatin

(BG, FG, SK and SW) gel alone was in the range of 97.4-98.1%. Springiness of SK gel decreased with increasing level of agar ($P<0.05$). When κ-carrageenan was higher than 10%, springiness of mixed gel decreased ($P<0.05$). However, there was no difference in springiness among all gels ($P<0.05$), except for SK and SW containing agar at 20%, which showed the lower value ($P<0.05$). Polysaccharides with less springiness such as low-methoxyl pectin, carrageenan and agar gels might break down more easily during compression than gelatin gel (Lau *et al.*, 2000). The incorporation of polysaccharides into gelatin gel could therefore reduce the springiness of mixed gel. The incorporation of agar (10%) or carrageenan (10 and 20%) into either SK or SW gels had no impact on springiness of resulting mixed gels ($P>0.05$). Therefore, the addition of agar at level higher than 10% lowered the springiness of gelatin gels from seabass skin and swim bladder.

8.5.1.3 Cohesiveness

Cohesiveness of different gelatin gels without and with agar or κ-carrageenan at different levels is presented in Table 23. Cohesiveness of FG, BG, SK and SW gel sample was 90.1, 88.8, 88.7 and 88.2%, respectively, which were higher than those of SK or SW gels added with agar or κ-carrageenan ($P<0.05$). Cohesiveness is a measure of the degree of difficulty in breaking down the internal structure of gel (Lau *et al.*, 2000). For the same gelatin, cohesiveness of gels decreased with increasing level of agar or carrageenan ($P<0.05$). Furthermore, at the same level of hydrocolloids used, no differences in cohesiveness were observed for both SK and SW gels. On the other hand, Lau *et al.* (2000) reported that the incorporation of gellan into gelatin resulted in the increased cohesiveness of gel structure. The internal structure of gellan/gelatin gel might be more difficult to break during the first compression than gelatin gel alone. For the present study, the internal structure of gelatin gel alone could be more difficult to break gel during ingestion, compared with gelatin gel containing hydrocolloids, especially at higher level (20%). Generally, most of gel matrix was not broken during the first compression. More energy was required to break the remaining gel matrix during the second compression, leading to the increased toughness (Lau *et al.*, 2000). Agar and κ-

carrageenan addition thus had the impact on cohesiveness of gelatin gels from seabass skin and swim bladder.

8.5.1.4 Gumminess

Gumminess of gelatin gels in the absence and presence of agar or κ-carrageenan at different levels is shown in Table 23. For both SK and SW gels, gumminess increased as the levels of agar increased ($P<0.05$). Gumminess is the energy required to break down a semi-solid food ready for swallowing (Yarnpakdee *et al.*, 2015). However, when κ-carrageenan was added into gelatin gel, gumminess of mixed gels decreased ($P<0.05$). Decrease in gumminess of gelatin/carrageenan mixed gel was related to the decreased hardness of gels (Table 23). The highest gumminess was observed in SW gels containing 20% agar, compared to other gel samples ($P<0.05$). The result suggested that the incorporation of agar increased the gumminess of gelatin gels.

8.5.1.5 Chewiness

Chewiness is related to the energy required to masticate a solid food to a state ready for swallowing (Yang *et al.*, 2007). Chewiness of different gelatin and gelatin-hydrocolloid gels is shown in Table 23. Chewiness of both SK and SW gels increased with increasing levels of agar ($P<0.05$). Nevertheless, chewiness of gel decreased when carrageenan content was increased ($P<0.05$). SK and SW containing agar gel had higher chewiness than BG and FG samples ($P<0.05$). The lowest chewiness was found in both SK and SW gels containing 20% κ-carrageenan ($P<0.05$). The highest chewiness was found for SW gel added with 20% agar ($P<0.05$). The result suggested that the incorporation of agar into gelatin gel more likely affected the chewiness of mixed gel. Gel from channel catfish skin gelatin with the higher chewiness was considered as the better quality gel (Yang *et al.*, 2007). Thus, the addition of agar into SK and SW gelatins could improve the chewiness of resulting gels.

Table 23. Hardness, springiness, cohesiveness, gumminess and chewiness of gel from seabass skin and swim bladder gelatins mixed with agar or carrageenan at different levels

Gelatin sample	Level/Hydrocolloid	Hardness (g)	Springiness (%)	Cohesiveness (%)	Gumminess (g)	Chewiness (g)
BG		3545±18E	98.1±0.15A	88.8±0.42A	3149±12E	3088±12E
FG		3467±23E	97.4±0.90A	90.1±0.81A	3122±25E	3041±46E
SK-	0	4488±41cD	98.0±0.17aA	88.7±0.87aA	3982±75cD	3905±81bCD
	10GA	5116±80bC	96.6±0.06bAB	85.6±0.35bB	4381±50bC	4231±45aB
	20GA	6419±118aA	94.9±0.06cBC	72.3±1.10dE	4638±154aB	4400±147aB
	10KC	3571±119dE	97.4±0.69aA	83.6±0.58bB	2985±120dEF	2908±137cEF
	20KC	3546±76dE	96.5±0.40bAB	75.1±2.37cD	2665±140eG	2571±146dG
SW-	0	4311±49cD	98.1±0.0aA	88.2±0.92aA	3802±82cD	3728±80bD
	10GA	4988±205bC	96.6±0.52abAB	85.4±0.29bB	4262±188bC	4116±161bBC
	20GA	6119±426aB	94.1±3.12bC	81.2±0.0cC	4970±347aA	4683±473aA
	10KC	3571±119dE	97.8±0.29aA	84.9±1.44bB	3120±55dE	3052±62cE
	20KC	3678±120dE	96.5±0.29abAB	79.8±0.58cC	2825±42dFG	2727±50cFG

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

Different lowercase letters within the same gelatin sample indicate significant differences (P<0.05). Different uppercase letters within the same column indicate significant differences (P<0.05).

BG: bovine gelatin, FG: fish gelatin; SK:seabass skin gelatin, SW: seabass swim bladder gelatin, GA: agar, KC: κ-carrageenan

Numbers (10, 20) in front of GA or KC represent concentration (%) of hydrocolloids added.

8.5.2 Color

The color of gels from various gelatins without and with agar or κ-carrageenan at different levels expressed as L^*, a^*, b^* and ΔE^* is shown in Table 24. In general, FG and BG gels had the higher lightness (L^*-value), compared with SK and SW gels as well as all gelatin gels mixed with hydrocolloids ($P<0.05$). The L^*-value of gelatin-hydrocolloid gels decreased when content of both agar and κ-carrageenan increased ($P<0.05$). However, no differences in L^*-value between SW gels containing 10 and 20% agar were observed ($P>0.05$). Among all gel samples, SW gels added with 10 or 20% agar and 20% κ-carrageenan showed the lowest L^*-value, compared with others ($P<0.05$). The lowest redness (a^*-value) was found in FG gel (-1.81), followed by BG gel (0.73) ($P<0.05$). SK gel had higher a^*-value than SW gel ($P<0.05$). The incorporation of agar or carrageenan into gelatin gel increased a^*-value of SK and SW gel ($P<0.05$). The yellowness (b^*-value) of mixed gel decreased as levels of agar or carrageenan increased ($P<0.05$). However, the addition of agar at 10% had no impact on b^*-value of SK gel ($P>0.05$). The highest b^*-value (31.00) was found in gel from BG gel ($P<0.05$). The higher yellowness in gelatin gel from bovine bone might be affected by the harsher extraction process required for bone with complex structure, leading to the formation of coloring components (Kittiphattanabawon *et al.*, 2016). For total difference in color value (ΔE^*-value), FG gel exhibited the lowest ΔE^*-value (20.51), compared to other gel samples ($P<0.05$). ΔE^*-value of gel increased when agar or carrageenan contents were increased ($P<0.05$). SK gel generally had higher ΔE^*-value than SW gel. The result suggested that color of individual hydrocolloids as well as the interaction between biopolymer affected the color of mixed gels. It can be inferred that the addition of either agar or κ-carrageenan had the impact on the color of gelatin gel.

Table 24. Color (L^*, a^*, b^* and ΔE^*) of gel from seabass skin gelatin and swim bladder gelatins mixed with agar or carrageenan at different levels

Gelatin sample	Level/ Hydrocolloid	L^*	a^*	b^*	ΔE^*
BG		64.41±1.71B	0.73±0.11H	31.00±1.13A	41.74±0.34G
FG		74.47±2.29A	-1.81±0.07I	9.64±0.35I	20.51±1.90H
SK-	0	55.40±0.72aC	1.66±0.08dG	25.44±0.21aC	44.82±0.49dF
	10GA	52.22±0.69bD	3.36±0.56bC	24.77±1.21aC	47.52±0.10bcD
	20GA	48.69±0.01cE	3.61±0.03bB	19.47±0.11cE	48.26±0.04bD
	10KC	52.60±0.60bD	3.78±0.03cB	23.85±0.17bD	46.64±0.40cE
	20KC	41.50±0.32dG	4.30±0.16aA	19.43±0.13cE	57.60±0.25aA
SW-	0	53.09±0.39aD	2.02±0.03dF	26.29±0.3aB	47.53±0.30dD
	10GA	39.38±0.51cH	2.72±0.16cE	15.50±0.11dG	55.61±0.53bB
	20GA	38.10±0.28cH	3.13±0.18bCD	11.52±0.18eH	55.97±0.23bB
	10KC	45.32±1.18bF	3.21±0.12bCD	23.40±0.68bD	53.41±0.17cC
	20KC	38.15±0.34cH	3.80±0.10aB	18.08±0.19cF	57.80±0.17aA

Values are presented as mean ± SD (n = 3). ND: Not determined

Different lowercase letters within the same gelatin sample indicate significant differences (P<0.05). Different uppercase letters within the same column indicate significant differences (P<0.05). BG: bovine gelatin, FG: fish gelatin; SK:seabass skin gelatin, SW: seabass swim bladder gelatin, GA: agar, KC: κ-carrageenan. Numbers (10, 20) in front of GA or KC represent concentration (%) of hydrocolloids added.

8.5.3 Syneresis

Syneresis of of gels from different gelatins in the absence and presence of agar or κ-carrageenan at various levels after storage for 12 h at room temperature (25 °C) is shown in Figure 35. Syneresis is the phenomenon of liquid being exuded from a gel and this is basically undesirable for gel product (Haug and Draget, 2011). In general, gelatin gel alone (BG, FG, SK and SW) showed the lower syneresis than those mixed with agar or κ-carrageenan ($P<0.05$). Syneresis of mixed gels increased when agar or carrageenan at level higher than 10% were added ($P<0.05$). The incorporation of agar or carrageenan at 10% had no impact on syneresis of mixed gels ($P>0.05$). The highest syneresis were found in SK and SW gels containing 20% κ-carrageenan ($P<0.05$). The interaction between gelatin and carrageenan, especially the presence of carrageenan at higher level (20%), might decrease interaction between molecules. This resulted in the non-ordered network, which had poorer capacity of holding water as indicated by the increased syneresis. Banerjee and Bhattacharya (2011) reported that an increase in the levels of hydrocolloids (gellan and agar) decreased syneresis of mixed gel. Therefore, the incorporation of carrageenan and agar in gelatin gel increased the amount of unbound water, which was released from the gel matrix easily.

8.5.4 Gelling and melting temperatures

Gelling and melting temperatures of solution containing various gelatins without and with agar or κ-carrageenan at different levels are shown in Table 25. Gelling and melting temperatures of various gelatins were in the range of 16.74-21.99 °C and 25.21-31.67 °C, respectively. Among all gelatin (without hydrocolloids), BG exhibited higher gelling and melting temperatures, compared with SK, SW and FG samples ($P<0.05$). The lowest gelling (16.74 °C) and melting (25.21 °C) temperatures were found in FG ($P<0.05$). No difference in gelling and melting temperatures was observed between SK and SW gels ($P>0.05$). In general, the differences in setting and melting temperature of gelatin were governed by molecular weight distribution (Muyonga *et al.*, 2004; Sinthusamran *et al.*, 2014). Gelling and

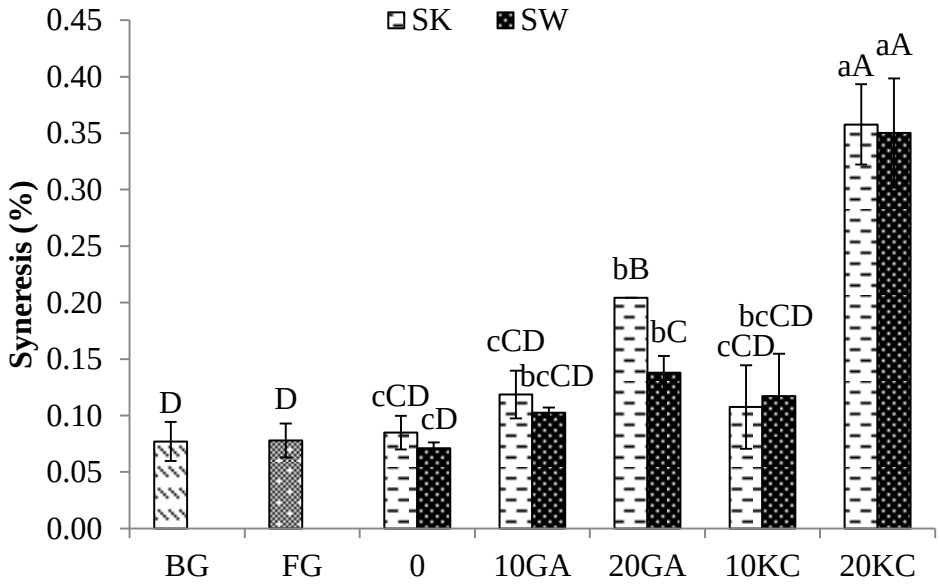

Figure 35. Syneresis of gel from seabass skin and seabass swim bladder gelatins mixed with agar or carrageenan at different levels. BG: bovine gelatin, FG: fish gelatin, SK: seabass skin gelatin, SW: seabass swim bladder gelatin, GA: agar, KC: κ-carrageenan. Bars represent the standard deviation (n=3). Different lowercase letters on the bars within the same gelatin sample indicate significant differences ($P<0.05$). Different uppercase letters on the bars indicate significant differences ($P<0.05$).

melting temperatures of SK or SW mixed with agar increased as the level of agar increased ($P<0.05$), except for gelatin mixed with 10% agar. Gelling temperature of gelatin gel containing κ-carrageenan increased as the level of κ-carrageenan increased ($P<0.05$). The results suggested that the interaction between gelatin and hydrocolloids (agar and κ-carrageenan) could be enhanced in the presence of κ-carrageenan at higher level. Derkach *et al.* (2015) reported that the gelation rate of gelatin increased with increasing concentration of κ-carrageenan. This might be associated with formation of polyelectrolyte complexes. The increase in the melting temperature with increasing κ-carrageenan was also reported by Pranoto *et al.* (2007). Nevertheless, SW gels containing κ-carrageenan (10 and 20%) had no impact on melting temperatures ($P>0.05$). Among all gel samples, the highest gelling temperature was found in SK gel containing 20% κ-carrageenan, while SK or SW gels containing 20%

agar showed the highest melting temperature (P<0.05). Mixed gels were more thermally stable, indicating the stronger network of gel, particularly when the appropriate level of hydrocolloids was used. The result suggested that both agar and κ-carrageenan strongly affected the thermo-stability of gelatin and the level of both hydrocolloids played a role in complexation of mixed system.

Table 25. Gelling and melting temperatures of seabass skin and swim bladder gelatins mixed with agar or carrageenan at different levels

Gelatin sample	Level/Hydrocolloid	Gelling temperature	Melting temperature
BG		21.99±0.58E	31.67±0.66C
FG		16.74±0.97H	25.21±0.71E
SK-	0	19.87±1.10dFG	28.98±0.58cD
	10GA	20.87±1.04dEF	31.58±0.81bC
	20GA	28.92±0.85bC	43.40±1.89aA
	10KC	25.26+0.42cD	31.30+0.83bC
	20KC	36.27±0.41aA	33.97±0.56bB
SW-	0	18.62±0.46eG	28.55±0.57cD
	10GA	20.76±0.65dEF	30.82±1.00bC
	20GA	27.71±0.57bC	42.66±0.87aA
	10KC	24.38±1.01cD	28.97±0.56cD
	20KC	33.27±0.84aB	28.45±0.45cD

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

Different lowercase letters within the same gelatin sample indicate significant differences (P<0.05). Different uppercase letters within the same column indicate significant differences (P<0.05).

BG: bovine gelatin, FG: fish gelatin; SK:seabass skin gelatin, SW: seabass swim bladder gelatin, GA: agar, KC: κ-carrageenan

Numbers (10, 20) in front of GA or KC represent concentration (%) of hydrocolloids added.

8.5.5 Sensory properties

Scores of appearance, color, odor, texture, melting characteristic and overall likeness of gels from BG, FG, SK, SW and SK or SW mixed with agar or κ-carrageenan at different levels are presented in Table 26. In general, BG and FG gel samples showed no differences in likeness scores for all attributes ($P<0.05$). Appearance, color, odor, texture and melting characteristic likeness scores of SK and SW gel alone showed the similar result to that of BG and FG gel samples ($P>0.05$). Moreover, no differences in appearance, color, odor and texture likeness between FG and SK samples were observed ($P>0.05$). Melting characteristic likeness scores of SK gel containing agar or κ-carrageenan at 10% was also increased ($P<0.05$). Moreover, there was no difference in appearance, odor and texture likeness scores between SK gel and SK gel added with agar or κ-carrageenan at 10% ($P>0.05$). Nevertheless, the addition of either agar or κ-carrageenan at 10% could improve the thermal stability of gelatin gel as evidenced by the increase in melting characteristic likeness score. Decreases in appearance and color likeness score of mixed gels were more likely caused by the increased gel turbidity (less transparent) with coincidental decreased lightness value (Table 24). Texture likeness score of mixed SW gels increased when agar or κ-carrageenan at a level of 10% was incorporated ($P<0.05$), except SW gel containing 20% carrageenan. The addition of both hydrocolloids into gelatin gel plausibly affected the gel texture, especially the hardness (Table 23). Nevertheless, the addition of both hydrocolloids at 10% had no impact on texture likeness score of SK gel. Drastic increasing hardness of mixed gel negatively affected the textural properties of gelatin gel as shown by the decrease likeness score of texture of gel containing 20% hydrocolloids. The presence of both hydrocolloids at 10% increased the melting characteristic likeness score of mixed gel, regardless of gelatin type. This was plausibly associated with increased melting temperatures of mixed gel (Table 25). However, the addition of both hydrocolloids at 20% had no effect on the likeness score of melting characteristic of both gelatins (SK and SW gels) ($P>0.05$). The addition of hydrocolloids at optimum level could improve the melt-in-mouth property during ingestion of gelatin gel, which is the unique characteristic of gelatin gel (Zhou and Regenstein, 2007). SK gel added with GA or KC at 10% had the comparable

Table 26. Likeness score of appearance, color, odor, texture, melting characteristic and overall of gel from seabass skin and swim bladder gelatins mixed with agar or carrageenan at different levels

Gelatin sample	Level/ Hydrocolloid	Attributes					
		Appearance	Color	Odor	Texture	Melting characteristic	Overall
BG		7.30±1.45ABC	7.40±1.14ABC	7.30±1.38ABC	7.30±0.92ABC	6.70±1.22ABCD	7.43+0.87AB
FG		7.80±1.11A	7.40±2.06ABC	7.50±1.40A	7.70±1.22A	7.50±1.15A	7.80+1.11A
SK-	0	7.40±0.82abAB	7.00±1.21bcBCD	6.80±1.39abABC	7.30±0.66abABC	6.58±1.29bcBCD	7.15±1.17bB
	10GA	8.00±0.79aA	8.10±0.85aA	7.40±1.31aAB	7.50±1.47aAB	7.30±1.22aABC	7.90±0.85aA
	20GA	6.05±1.85dEFG	6.25±1.83cDE	6.00±1.84bD	6.75±1.07bcBCD	6.60±1.27bcBCD	6.45±1.28cCDE
	10KC	6.80±1.70bcBCD	6.90±1.41bcBCD	6.70±1.59abABCD	7.60±0.94aA	7.50±0.69aA	7.30±1.03abAB
	20KC	6.60±0.50cdCDE	7.10±1.07bBC	6.00±1.84bD	6.60±1.39cCD	6.50±1.79cCD	6.40±1.05cDE
SW-	0	7.50±0.95aAB	7.70±0.92aAB	7.10±1.25aABC	6.70±0.85bBCD	6.40±1.85bD	7.00±1.03aBCD
	10GA	6.00±1.17bcEFG	5.90±1.41cE	6.50±1.32aCD	7.45±0.93aAB	7.35±0.8aAB	6.85±0.88a BCD
	20GA	5.80±1.20bcFG	5.60±1.31cE	5.10±1.33bE	6.40±1.73bD	6.40±1.85bD	5.70±1.30bF
	10KC	6.45±1.47bDEF	6.75±0.97bCD	6.60±0.97aBCD	7.25±1.02aABC	6.80±1.24abABCD	6.85±1.09aBCD
	20KC	5.60±1.23cG	5.80±1.28cE	5.20±1.82bE	6.60±1.31bCD	6.10±1.48bD	5.90±1.25bEF

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

Different lowercase letters within the same gelatin sample indicate significant differences (P<0.05). Different uppercase letters within the same column indicate significant differences (P<0.05).

BG: bovine gelatin, FG: fish gelatin; SK:seabass skin gelatin, SW: seabass swim bladder gelatin, GA: agar, KC: κ-carrageenan

Numbers (10, 20) in front of GA or KC represent concentration (%) of hydrocolloids added.

overall likeness, compared to FG gel ($P>0.05$). Therefore, the addition of hydrocolloid at optimum level was able to improve the sensory properties of gelatin gel from seabass skin and swim bladder.

8.5.6 Microstructure of gel

Microstructures of BG and FG as well as SK and SW gels without and with agar or κ-carrageenan at 10% are illustrated in Figure 36. The microstructure of gel is directly related to the strength of gel, which is governed by the conformation and chain length of gelatin (Benjakul *et al.*, 2009). Generally, all gelatin gels were sponge or coral-like in gel structure. SK and SW gel exhibited similar gel network. SK and SW showed the finer structure and denser strands in gel matrix, compared with BG and FG gels. This was related with the higher hardness of SK and SW, in comparison with BG and FG (Table 23). The thinner strands were observed in BG and FG gels. The ordered structure with larger strands of gel matrix were in accordance with higher strength of gel (Yang *et al.*, 2008). The differences in gel microstructure of gelatin were strongly affected by the distribution of α-, β- and γ-chains in gelatin (Sinthusamran *et al.*, 2014). When agar (10%) was incorporated into gelatin gel, the gel network of SK or SW gel had more compact and denser structure with less voids in network, compared to SK or SW gels alone. This result was correlated with higher hardness of SK or SW gel containing 10% agar, compared with SK or SW gel alone (Table 23). Agar and gelatin might form inter-penetrated network by intermolecular interactions via hydrogen bonds between biopolymers (Tian *et al.*, 2011). This could build up the denser gel structure, which increased the strength of network. Conversely, phase separation between proteins aggregates (larger strands) and κ-carrageenan (very fine strands) took place in SK or SW gels containing 10% κ-carrageenan. Formation of κ-carrageenan aggregate occurred separately and those aggregates were localized between gelatin networks. The polysaccharide contributed to the thermodynamic activity of gelatin solution. This phenomenon could disturb the continuous gel network of gelatin, leading to the decrease of hardness in the mixed gels (Table 23). The result revealed that the incorporation of agar or κ-carrageenan determined the formation and arrangements of polysaccharides and gelatins in the network during gelation.

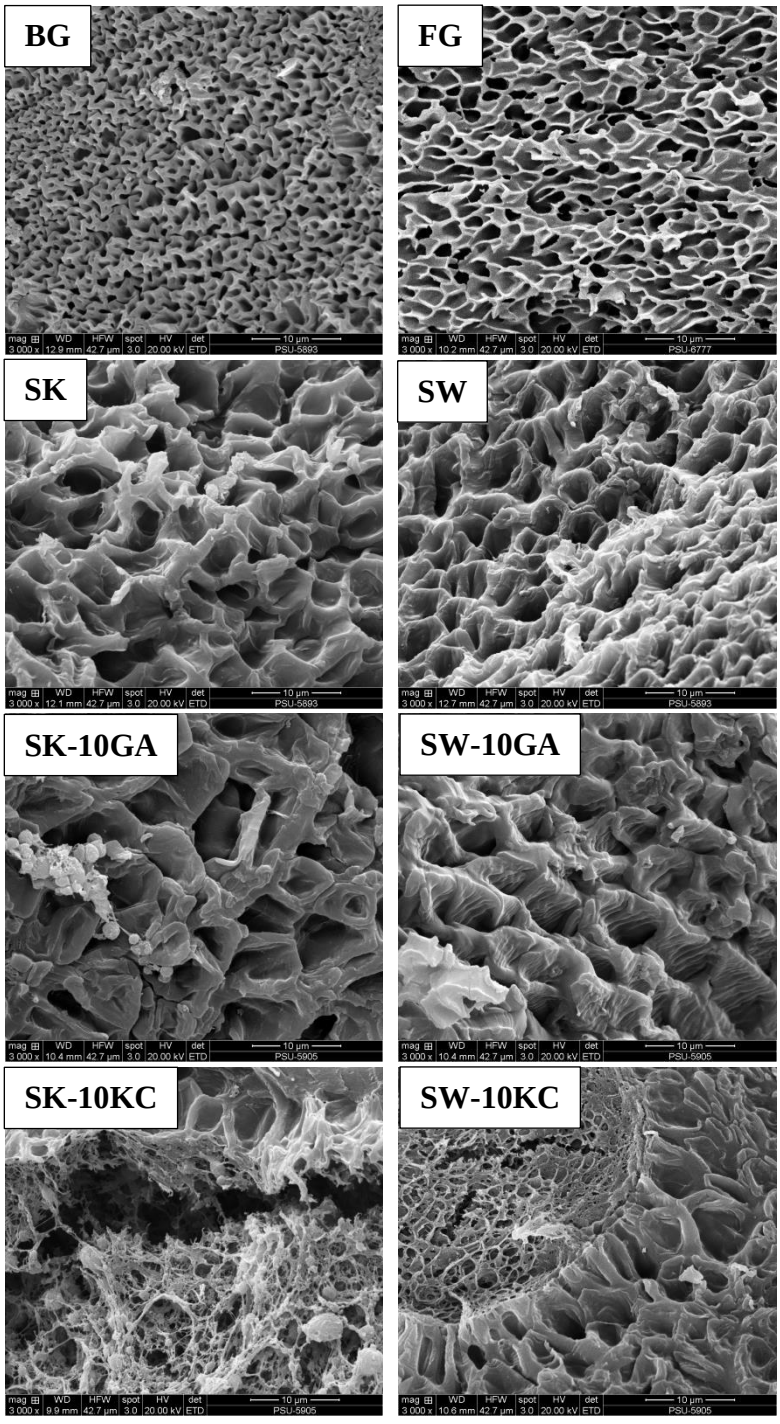

Figure 36. Microstructures of gel from seabass skin and swim bladder gelatins mixed with agar or carrageenan at 10%. BG: bovine gelatin, FG: fish gelatin, SK: seabass skin gelatin, SW: seabass swim bladder gelatin, GA: agar, KC: κ-carrageenan. Number of 10 in front of GA or KC represents concentration (%) of hydrocolloids added. Magnification: 3000 times

8.5.7 DH of different gels in gastrointestinal tract model system

Gastrointestinal tract model system was used to simulate the ingestion system of human body for gels from different gelatins in the absence and presence of agar or κ-carrageenan at 10%, as illustrated in Figure 37. In general, during pepsin digestion, DH of gel from gelatin without agar or κ-carrageenan was in the range of 6.40-11.64%. SK and SW gels showed the highest DH, compared with other gel samples ($P<0.05$). When agar or κ-carrageenan at 10% was incorporated into SK or SW gel, DH of mixed gel decreased ($P<0.05$). However, no differences in DH were observed between SW gel containing 10% agar and 10% κ-carrageenan ($P>0.05$). For SK gel, that added with 10% agar showed the lower DH than that containing 10% κ-carrageenan ($P<0.05$). The results indicated that the interaction between gelatin and polysaccharides via electrostatic interactions or hydrogen bonding or hydrophobic interaction more likely took place to the higher extent in the former (Doublier *et al.*, 2000). The interaction between gelatin and hydrocolloids could yield more compact structure. When the intermolecular interaction ensured, peptide bonds were embedded. This could impede the hydrolysis by enzyme. Pepsin has been known to cleave peptides specifically at the telopeptid-region in cross-linked molecules of collagen structure (Nalinanon *et al.*, 2007). For intestinal simulated system, all gel samples had the increased DH, compared to those digested with pepsin ($P<0.05$). The result suggested that pancreatin further cleaved the peptides, leading to the increased DH of samples. Pancreatin normally contains many enzymes, including amylase, lipase and proteases, which gave more hydrolysis activity (You *et al.*, 2010). SK or SW gel containing 10% agar showed the lowest DH, compared with other gel samples after pancreatin digestion ($P<0.05$). There was no difference in DH between SK and SW gels containing 10% agar ($P>0.05$). The highest DH was found in SK and SW gel ($P<0.05$) and both gels showed no differences in DH values ($P>0.05$). The present study suggested that gel gelatin mixed with hydrocolloids, especially agar, might be inter-connected, resulting in the compact structure. As a consequence, decreased DH of mixed gel in gastrointestinal tract model system was observed. This was coincidental with the larger strands, which had less surface area exposed for hydrolysis (Fig. 36). The gel network of gelatin is generally stabilized by junction

zone via hydrogen bonding through hydroxyl group, depending on imino acids content (Benjakul *et al.*, 2012a). Therefore, the addition of agar or κ-carrageenan could lower digestibility as examined by gastrointestinal tract model system.

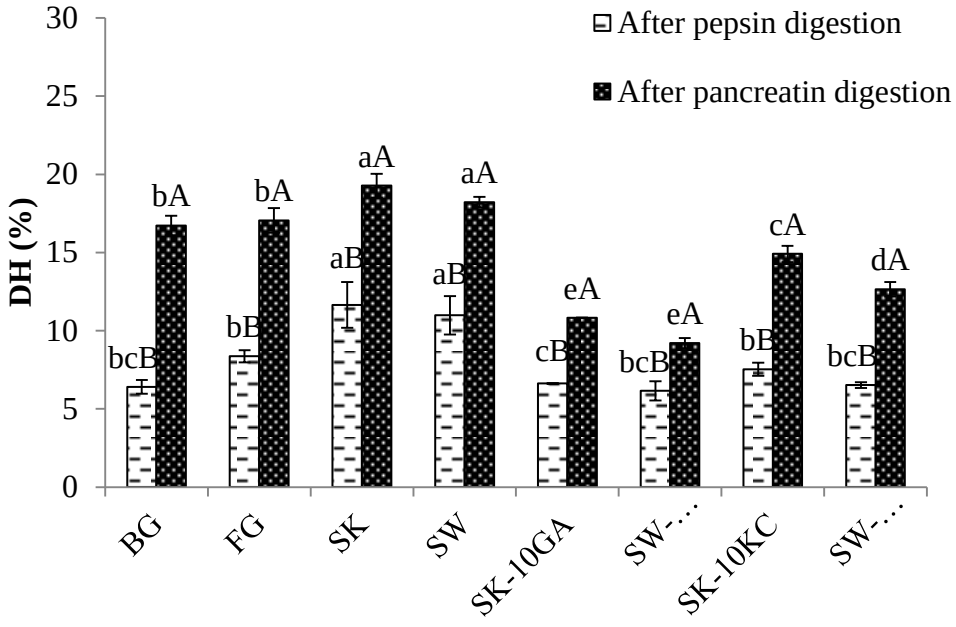

Figure 37. Degree of hydrolysis (%) of gel from seabass skin and seabass swim bladder gelatin mixed with agar or carrageenan at 10% in gastrointestinal tract model system. BG: bovine gelatin, FG: fish gelatin, SK: seabass skin gelatin, SW: seabass swim bladder gelatin, GA: agar, KC: κ-carrageenan. Number of 10 in front of GA or KC represents concentration (%) of hydrocolloids added. Bars represent the standard deviation (n=3). Different lowercase letters on the bars within the same digestion time indicate significant differences ($P<0.05$). Different uppercase letters on the bars within the same gel sample indicate significant differences ($P<0.05$).

8.6 Conclusion

Physical and sensory properties of gelatin from skin and swim bladder of seabass were strongly influenced by the agar or κ-carrageenan with the range of 10-20%. Both agar and κ-carrageenan affected the textural properties of gelatin gels. The addition of agar could increase hardness of gelatin gel, but springiness of mixed gel was decreased. However, hardness decreased when κ-carrageenan was added into SK or SW gels. Gelling and melting temperatures of gelatin gel increased as the level of hydrocolloids increased. The addition of both agar and κ-carrageenan lowered digestion of gelatin gel as determined by gastrointestinal tract model system. The addition of agar or carrageenan also affected the sensory properties of gel from gelatin from seabass. Therefore, the addition of 10% agar in gelatin was recommended to improve the textural and gelling properties as well as sensory properties of gelatin from both seabass skin and swim bladder.

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CHAPTER 9

CONCLUSION AND SUGGESTION

9.1 Conclusions

1. Collagens from the skin and swim bladder of seabass were characterized as type I and contained α- and β-chains as major components. Both collagens had a triple-helix structure and showed slight differences in thermal stability.

2. Gelatin extracted from seabass skin at higher temperature for longer time showed the higher yield, but had poorer gel properties. Gelatin extracted at 45 °C for 3 h exhibited the highest gel strength. All gelatins from seabass skin had higher gel strength than commercial bovine gelatin. They were able to set at 25 °C within 30 min.

3. Yield and gelling properties of gelatin were influenced by the size of seabass. Gelatin obtained from the skin of larger seabass (6 kg/fish) had the highest yield and better gelling properties. This was associated with the higher content of high MW components or cross-links.

4. Gelatin from seabass swim bladder extracted at 65 °C for 6 h, having α- and β-chains as major components, exhibited the highest gel strength. It showed higher gel strength than commercial bovine gelatin.

5. Textural properties of commercial fish gelatin gels (FG) were strongly affected by both *Gracilaria tenuistipitata* agar (GA) and commercial agar (CA). Both low strain modulus and failure stress of agar/gelatin mixed gel increased with increasing agar content. Incorporation of both agars to gelatin decreased the failure strain (springiness) of resulting gels. However, the addition of both agars, especially at higher levels, had a negative impact on sensory properties.

6. κ-Carrageenan (KC) had a significant effect on the textural and rheological properties of FG. Gel strength and hardness increased as KC level increased. FG gel containing 75% KC showed the highest gel strength and hardness. However, the springiness of the mixed gels decreased with increasing levels of KC. Gelling and melting temperatures of fish FG/KC mixed gels increased when KC levels were increased.

7. Physical and sensory properties of gelatin from skin (SK) and swim bladder (SW) of seabass were strongly influenced by GA or KC with the range of 10-20%. Both GA and KC affected the textural properties of both gelatin gels. The addition of both GA and KC lowered digestion of gelatin gel. The addition of GA at 10% increased sensory properties of gel from seabass gelatin.

9.2 Suggestions

1. Improvement of turbidity, color and odor of gelatin from seabass skin and swim bladder should be further studied.

2. The behaviors and interaction between seabass gelatin and other hydrocolloids during gelation should be examined.

3. Applications of gelatin from seabass as food ingredients e.g. the stabilizer, gelling and clarifying agent, in some food products should be further investigated.

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Scholarship Awards during Enrolment

- Scholarship of Academic Distinction, Prince of Songkla University
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List of Publication and Proceedings

Publications

Sinthusamran, S., Benjakul, S. and Kishimura, H. 2013. Comparative study on molecular characteristics of acid soluble collagens from skin and swim bladder of seabass (*Lates calcarifer*). Food Chemistry. 138(4): 2435-2441.

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