Value-added Products from Yellowfin Tuna Swim Bladder: Collagen and Gelatin

Onouma Kaewdang

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

Prince of Songkla University

2015

Thesis Title Value-added products from yellowfin tuna swim bladder: collagen and gelatin

Author Miss Onouma Kaewdang

Major Program Food Science and Technology

Major Advisor :

…….……....

(Prof. Dr. Soottawat Benjakul)

Examining Committee :

….……...….....Chairperson

(Asst. Prof. Dr. Manee Vittayanont)

…….…...……Committee

(Prof. Dr. Soottawat Benjakul)

…….…….....Committee

(Dr. Tanong Aewsiri)

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

..…….....

(Assoc. Prof. Dr. Teerapol Srichana)

Dean of Graduate School

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

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

.....……..Signature
(Miss Onouma Kaewdang)
Candidate

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

……Signature
(Miss Onouma Kaewdang)
Candidate

ชื่อวิทยานิพนธ์ ผลิตภัณฑ์มูลค่าเพิ่มจากถุงลมปลาทูน่าพันธุ์ครีบเหลือง : คอลลาเจนและเจลาติน

ผู้เขียน นางสาวอรอุมา แก้วแดง

สาขาวิชา วิทยาศาสตร์และเทคโนโลยีอาหาร

ปีการศึกษา 2557

บทคัดย่อ

จากการสกัดคอลลาเจนที่ละลายในกรดและคอลลาเจนที่ละลายด้วยเปปซินจากถุงลมปลาทูน่าพันธุ์ครีบเหลืองพบว่าให้ผลผลิตโดยน้ำหนักแห้งร้อยละ1.07 และ 12.10 ตามลำดับ คอลลาเจนทั้งสองประกอบด้วย แอลฟา-1 และแอลฟา-2 และสามารถจำแนกเป็นคอลลาเจนชนิด type I คอลลาเจนที่ละลายในกรดและละลายด้วยเปปซินมีปริมาณกรดอิมิโนเท่ากับ 128 และ169 หน่วยต่อ 1000 หน่วยตามลำดับ สเปคตราของฟูเรียร์ทรานฟอร์มอินฟาเรด (FTIR) ของคอลลาเจนที่ละลายในกรดและละลายด้วยเปปซินบ่งชี้ว่าคอลลาเจนทั้งสองมีโครงสร้างเป็นฮีลิกซ์สามสาย (triple helix) คอลลาเจนทั้งสองชนิดมีความสามารถในการละลายในช่วงพีเอชเป็นกรด คอลลาเจนที่ละลายด้วยกรดและด้วยเปปซิน มีค่า pI เท่ากับ 6.05 และ 5.93 ตามลำดับ และมีค่า T_{max} เท่ากับ 32.97 และ 33.92 องศาเซลเซียส ตามลำดับ

เมื่อสกัดเจลาตินจากถุงลมปลาทูน่าที่ปฏิบัติเบื้องต้นโดยใช้ด่างที่ความเข้มข้นร้อยละ 4 (น้ำหนักต่อปริมาตร) ซึ่งเป็นสารผสมระหว่าง โซเดียมคาร์บอเนต (Na_2CO_3) และโซเดียมไฮดรอกไซด์ (NaOH) อัตราส่วน 9:1, 8:2, 7:3 และ 6:4 โดยเจลาตินที่ได้คือ G1, G2, G3 และ G4 ให้ผลผลิตร้อยละ 9.78, 14.91, 35.96 และ 13.60 ของน้ำหนักแห้ง ตามลำดับ เจลาตินทั้งหมดประกอบด้วยแถบโปรตีนแอลฟาเป็นองค์ประกอบหลัก สเปคตรา FTIR บ่งชี้การสูญเสียของโครงสร้างฮีลิกซ์สามสายของเจลาติน G3 ประกอบด้วยปริมาณกรดอิมิโนและค่าความแข็งแรงของเจลสูงสุด ($P < 0.05$) เมื่อเปรียบเทียบกับเจลาตินอื่นๆ เมื่อศึกษาโครงสร้างจุลภาคพบว่าโครงสร้างของเจล G3 มีลักษณะเป็นระเบียบและมีช่องว่างขนาดเล็กกว่าเมื่อเปรียบเทียบกับเจลาตินอื่น เมื่อสัดส่วนของโซเดียมไฮดรอกไซด์สำหรับการปฏิบัติเบื้องต้นเพิ่มขึ้นพบว่า ค่าความสว่างของเจลาตินมีค่าเพิ่มขึ้น ในขณะที่ค่าความแตกต่างของสีลดลง อุณหภูมิในการเกิดเจลและอุณหภูมิการหลอมละลายของเจลเจลาตินจากถุงลมอยู่ในช่วง 12.3-15.1 และ 21.3-22.3 องศาเซลเซียส ตามลำดับ เมื่อศึกษาผลของอุณหภูมิต่างๆ (60, 70 และ 80 องศาเซลเซียส) สำหรับการสกัดเจลาตินพบว่าผลผลิตเจลาตินมีค่าร้อยละ 35.6, 41.1 และ 47.3 โดยน้ำหนักแห้ง ตามลำดับ แถบโปรตีนแอลฟาของเจลาตินมีปริมาณลดลงเมื่ออุณหภูมิการสกัดสูงขึ้น แต่องค์ประกอบกรดอะมิโนของเจลาตินทั้งหมดไม่แตกต่างกัน กรดอิมิโนอยู่ในช่วง 169 ถึง 172 หน่วยต่อ 1000 หน่วย ความ

แข็งแรงของเจลาตินที่สกัดที่อุณหภูมิต่ำมีค่าสูงกว่าเจลาตินที่สกัดที่อุณหภูมิสูง อุณหภูมิการเกิดเจลและอุณหภูมิที่เจลเกิดการหลอมละลายของเจลาตินจากถุงลมมีค่าอยู่ในช่วง 11.0-15.24 และ 20.36-22.33 องศาเซลเซียส ตามลำดับ โดยอุณหภูมิการเกิดเจลและอุณหภูมิที่เจลเกิดการหลอมละลายของเจลาตินซึ่งสกัดที่อุณหภูมิต่ำมีค่าสูงกว่า โครงสร้างจุลภาคของเจลของเจลาตินที่สกัดที่อุณหภูมิ 60 องศาเซลเซียส มีช่องว่างขนาดเล็กกว่าเมื่อเทียบกับเจลาตินที่สกัดด้วยอุณหภูมิอื่น ดังนั้นสภาวะในการปฏิบัติเบื้องต้นและการสกัดส่งผลโดยตรงต่อสมบัติของเจลาตินจากถุงลม

สารสกัดเอทานอลจากกาบมะพร้าว (EECH) ซึ่งประกอบด้วยกรดแทนนิคปริมาณสูงสามารถใช้ในการปรับปรุงสมบัติของเจลาติน EECH ที่ระดับแตกต่างกัน (0.25, 0.5, 0.75, 1, 2, 3, และ 5 มิลลิกรัมต่อกรัมเจลาตินแห้ง) มีผลต่อสมบัติเจลของเจลาตินแตกต่างกัน ความแข็งแรงเจลของเจลาตินเพิ่มสูงขึ้นเมื่อความเข้มข้นของ EECH เพิ่มสูงขึ้นถึง 0.5 มิลลิกรัมต่อกรัม ($P < 0.05$) แล้วค่อยๆลดลงเมื่อปริมาณ EECH สูงขึ้นกว่า 0.5 มิลลิกรัมต่อกรัม การเติม EECH ที่ระดับต่างๆ ไม่เปลี่ยนแปลงแถบโปรตีนเมื่อวิเคราะห์ด้วยเทคนิคอิเล็กโตรโฟรีซีส (SDS-PAGE) ซึ่งแสดงให้เห็นว่าพันธะส่วนใหญ่ในเจลเป็นพันธะไฮโดรเจนและพันธะที่ไม่แข็งแรง โครงข่ายเจลมีรูปแบบที่สม่ำเสมอ และเจลมีเส้นใยโครงข่ายที่ใหญ่เมื่อเติม EECH 0.5 มิลลิกรัมต่อกรัม อุณหภูมิในการเกิดเจลและอุณหภูมิการหลอมละลายของเจลเพิ่มสูงขึ้นเมื่อเติม EECH ที่ระดับ 0.5 มิลลิกรัมต่อกรัม อย่างไรก็ตามค่าสีของเจลเจลาตินทั้งหมดเพิ่มขึ้นเล็กน้อยเมื่อความเข้มข้นของ EECH เพิ่มสูงขึ้น ดังนั้น EECH ที่ระดับที่เหมาะสมสามารถใช้เป็นสารจากธรรมชาติที่ช่วยเพิ่มความแข็งแรง เจลของเจลาติน

จากการเติมเอการ์ (Agar) ที่ระดับต่างๆ (ร้อยละการทดแทนเจลาตินเท่ากับ 5, 10, 15 และ 20) ลงไปในเจลาติน พบว่าเจลที่ได้มีสมบัติที่แตกต่างกัน ค่าความแข็งแรงเจล อุณหภูมิการเกิดเจลและอุณหภูมิที่เจลเกิดการหลอมละลายเพิ่มขึ้น เมื่อความเข้มข้นของเอการ์เพิ่มสูงขึ้นจากร้อยละ 5 ถึง 20 ($P < 0.05$) ความแข็งของเจลเพิ่มสูงขึ้นในขณะที่แรงยึดเกาะลดลง เมื่อระดับของเอการ์เพิ่มขึ้น ($P < 0.05$) แต่อย่างไรก็ตามไม่มีความแตกต่างของค่าความยืดหยุ่นระหว่างตัวอย่างที่ประกอบด้วยเอการ์ร้อยละ 5 ถึง 20 ($P > 0.05$) นอกจากนี้ไม่พบความแตกต่างของแถบโปรตีน ค่าความสว่าง (L^*) และค่าความเป็นสีแดง (a^*) เพิ่มขึ้น แต่ค่าความเป็นสีเหลือง (b^*) และค่าความแตกต่างของสี (ΔE^*) ลดลงเมื่อระดับของเอการ์เพิ่มขึ้น โครงข่ายเจลมีความสม่ำเสมอและเส้นใยโครงข่ายที่ใหญ่ขึ้น เมื่อมีการเติมเอการ์ลงไปเมื่อเทียบกับเจลเจลาติน (ไม่มีการเติมเอการ์) ดังนั้นการเติมเอการ์ในระดับที่เหมาะสมสามารถปรับปรุงสมบัติของเจลเจลาตินจากถุงลมปลาทูน่าพันธุ์ครีบเหลือง

Thesis Title Value-added Products from Yellowfin Tuna Swim Bladder: Collagen and Gelatin

Author Miss Onouma Kaewdang

Major Program Food Science and Technology

Academic Year 2014

ABSTRACT

Acid soluble (ASC) and pepsin soluble collagens (PSC) were extracted from swim bladder of yellowfin tuna (*Thunnus albacares*) with yields of 1.07 % and 12.10 % (dry weight basis), respectively. Both collagens consisted of α_1 and α_2 chains and were characterised to be type I collagen. Imino acid contents of ASC and PSC were 128 and 169 residues/1000 residues, respectively. Fourier transform infrared (FTIR) spectra of both ASC and PSC were almost similar and revealed the presence of triple helix. Both ASC and PSC had the highest solubility at acidic pH. ASC and PSC had pI of 6.05 and 5.93, respectively and possessed T_{max} of 32.97 and 33.92 °C, respectively.

When gelatins were extracted from the swim bladder of yellowfin tuna with various alkaline pretreatments using 4 % (w/v) alkaline mixtures (Na_2CO_3: NaOH) at different ratios (9:1, 8:2, 7:3 and 6:4), the corresponding gelatins termed "G1, G2, G3 and G4" had yields of 9.78, 14.91, 35.96, and 13.60 % (dry weight basis), respectively. All gelatins had α-chains as the major component. FTIR spectra of obtained gelatins revealed the significant loss of molecular order of the triple-helix. G3 having the highest imino acid content and exhibited the highest gel strength ($P < 0.05$), compared with others. Microstructure of G3 gel was finer with smaller voids, compared with others. With increasing proportion of NaOH, L^*- value of gelatin gel increased with coincidental decrease in ΔE^*- value. Gelling and melting temperatures of swim bladder gelatin were 12.3–15.1 and 21.3–22.3 °C, respectively. As gelatins were extracted at different temperatures (60, 70 and 80 °C), the extraction yields were 35.6 %, 41.1 % and 47.3 % (dry weight basis), respectively. The α-chains of gelatin decreased with increasing extraction temperatures. Similar amino acid compositions were noticeable among all gelatins, in which glycine constituted as the major amino acid. Imino acids ranged from 169 to 172 residues/1000 residues. The

gel strength of gelatin extracted at lower temperature was higher than that of gelatins extracted at higher temperatures. Gelling and melting temperatures for swim bladder gelatin were 11.07–15.24 and 20.36–22.33 °C, respectively. Higher gelling and melting points were observed for gelatin extracted at lower temperatures. Microstructure of gel of gelatin extracted at 60 °C was finer with smaller voids, compared with others. Thus, pretreatment and extraction conditions directly had the impact on properties of gelatin.

Ethanolic extract from coconut husk (EECH) rich in tannic acid was used to improve property of gelatin. EECH at different levels (0.25, 0.5, 0.75, 1, 2, 3 and 5 mg/g dry gelatin) showed varying impact on gel properties of gelatin. Gel strength of gelatin increased when EECH concentrations increased up to 0.5 mg/g ($P < 0.05$). Nevertheless, the gradual decrease in gel strength was found with increasing EECH levels. When EECH at different levels was incorporated, no marked changes in protein patterns determined by SDS-PAGE were observed, suggesting that most of bondings were hydrogen bond or other weak bonds. Gel matrix with uniformity and larger strands were observed with gels added with 0.5 EECH mg/g. Gelling and melting temperatures were also increased when EECH at a level of 0.5 mg/g was incorporated. However, color of all gelatin gels slightly increased with increasing concentrations of EECH. Therefore, EECH at an appropriate level could act as a natural gel enhancer of gelatin.

When agar at different levels (5, 10, 15 and 20 % of gelatin substitution) was incorporated into gelatin, varying properties of resulting gels were obtained. Gel strength, gelling and melting points continuously increased as agar concentration increased from 5 to 20 % of gelatine substitution ($P < 0.05$). Hardness increased, whereas cohesiveness decreased with increasing agar levels ($P < 0.05$). Nevertheless, there was no difference in springiness between sample containing 5-20 % agar ($P > 0.05$). No differences in protein patterns were observed, regardless of agar levels. L^* and a^* increased, but b^* and ΔE^* decreased as the level of agar increased. Gel matrix with uniformity and larger strands were observed with gels added with agar, compared with the control gelatin gel (without agar). Therefore, the addition of agar into gelatin at an appropriate level could improve the properties of gelatin gel from swim bladder of yellowfin tuna.

ACKNOWLEDGEMENT

I would like to express my special appreciation and sincere gratitude to my advisor Professor Dr. Soottawat Benjakul of the Department of Food Technology**,** Faculty of Agro-Industry, Prince of Songkla University, who has shown the attitude and the substance of a genius in regard to researching and teaching. I would like to thank for his kindness, comments and helpful suggestion. Without his supervision and constant help, this dissertation would not have been possible.

I am also grateful to my examining committee, Assist. Prof. Dr. Manee Vittayanont of the Department of Food Technology**,** Faculty of Agro-Industry, Prince of Songkla University and Dr. Tanong Aewsiri of the School of Agricultural Technology, Walailak University for their kindness, comments and helpful suggestion.

Furthermore, I would like to thank my parents for their endless love and support. Finally, I would also like to thank all of my dear friends for their most valuable help and friendship.

This study could not be succeeded without the financial support from Graduate School of Prince of Songkla University and PSU halal center, Pattani campus, for the financial support.

Onouma Kaewdang

CONTENTS

CONTENTS (Continued)

CONTENTS (Continued)

CONTENTS (Continued)

CONTENTS (Continued)

CONTENTS (Continued)

CONTENTS (Continued)

CONTENTS (Continued)

CONTENTS (Continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

CHAPTER 1

Introduction and review of literature

1.1 Introduction

Thailand is the world's largest producer and exporter of canned tuna. The most commercially important tuna species for the Thai tuna industry is skipjack tuna, followed by yellowfin tuna and tongol tuna, accounting for over 80 % of Thai frozen tuna imports (Nalinanon *et al*., 2010). During January and June, 2013, the amount of imported tuna to Thailand was 65,954.14 tons (Department of Fisheries, 2014). Large volumes of raw tuna go through the canning process, for which about two-thirds of the whole fish are utilized. Those byproducts including the internal organs can be used as animal feeds or pet foods. To better utilize those byproducts, severed kinds of value-added products have been produced, particularly collagen, gelatin and their derivatives. Gelatin from tuna processing byproduct can be extracted (Shyni *et al*., 2014; Aewsiri *et al*., 2008) and gelatin hydrolysate with bioactivity was also prepared with the aid of tuna pepsin (Nalinanon *et al*., 2011). Additionally, viscera can be the excellent sources of protease, such as trypsin (Klomklao *et al*., 2004) and pepsin (Nalinanon *et al*., 2011).

Fish collagen and gelatin have gained increasing attention. This is due to the outbreak of mad cow disease or Bovine Spongiform Encephalopathy in 1980 as well as the unacceptability of collagen produced from bovine and porcine sources for Muslims, Jews and Hindus. Collagen has been widely used in food, cosmetic, biomedical and pharmaceutical industries (Ogawa *et al*., 2004), due to its excellent biocompatibility and biodegradability (Liu *et al*., 2010). Apart from collagen, gelatin from fish processing byproduct, particularly skins, has become popular. Gelatin has been used as thickening agent, gelling agent, and film forming agent. However, the property of gelatin varies with species and extraction condition (Kaewruang *et al*., 2013; Nagarajan *et al*., 2012; Ahmad and Benjakul 2011). Bone and scale can be used for gelatin extraction but they more likely render the lower yield than skin (Matmaroh *et al*., 2011).

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 (Yellowdawn, 2011). Recently, pepsin-soluble collagens with the yields of 40 and 59% were extracted from the swim bladder of catfish and bighead carp, respectively (Bama *et al*., 2010; Liu *et al*., 2012). Swim bladder from tuna can be used as an alternative raw material for production of collagen and gelatin. Nevertheless, gelatins of fish origin have poorer gel strength, compared with mammalian counterpart, due to their lower imino acid content (Grossman and Bergman, 1992). Therefore, the improvement of fish gelatin properties has been conduced to conquer the problem. Phenolic compounds can interact with proteins through non-covalent and covalent interaction (Maqsood *et al*., 2013). The use of phenolic from cheap source such as coconut husk could be anther means to enhance the gel strength of gelatin from swim bladder. In addition, gelling agents have been incorporated to improve gel strength or increase gelling and melting points of gelatin gel (Somboon *et al*., 2014; Shrinivas *et al*., 2009). Thus, gelling agent, especially agarose could be blended with swim bladder gelatin to enhance gelling property. As a whole, properties of gelatin from tuna swim bladder could be improved, in which the wider application of swim bladder gelatin can be achieved. More importantly, halal food product, can be produced to serve the increasing demand, especially for marine collagen and gelatin.

1.2 Review of literature

1.2.1 Swim bladder

The swim bladder, gas bladder, fish maw or air bladder is an internal gas-filled that contributes to the ability of a fish to control its buoyancy, and thus to stay at the current water depth without wasting energy in swimming (Wikipedia, 1999). The swim bladder is also of use as a stabilizing organ because in the upright position the center of mass is below the center of volume due to the dorsal position of the swim bladder. Another function of the swim bladder is the use as a resonating chamber to produce or receive sound. The swim bladder is evolutionarily homologous to the lungs (Pelster, 2001)

The swim bladder normally consists of two gas-filled sacs located in the dorsal portion of the fish. Although in a few primitive species, there is only a single sac. It has flexible walls that contract or expand according to the ambient pressure. The walls of the bladder contain very few blood vessels and are lined with guanine crystals, which make them impermeable to gases (Pelster, 2001). By adjusting the gas pressure using the gas gland or oval window, the fish can obtain neutral buoyancy and ascend and descend to a large range of depths. Due to the dorsal position, it gives the fish lateral stability (Pelster, 2001). In physostomous swim bladders, a connection is retained between the swim bladder and the gut, the pneumatic duct, allowing the fish to fill up the swim bladder by "gulping" air and filling the swim bladder. Excess gas can be removed in a similar manner (Pelster, 2001).

In more derived varieties of fish, the physoclisti, the connection to the digestive tract is lost. In early life stages, fish have to rise to the surface to fill up their swim bladders, however, in later stages the connection disappears and the gas gland has to introduce gas (usually oxygen) to the bladder to increase its volume and thus increase buoyancy. In order to introduce gas into the bladder, the gas gland excretes lactic acid and produces carbon dioxide. The resulting acidity causes the hemoglobin of the blood to lose its oxygen (Root effect) which then diffuses partly into the swim bladder (Pelster, 2001). The blood flowing back to the body first enters a rete mirabile where virtually all the excess carbon dioxide and oxygen produced in the gas gland diffuses back to the arteries supplying the gas gland. Thus a very high gas pressure of oxygen can be obtained, which can even account for the presence of gas in the swim bladders of deep sea fish like the eel, requiring a pressure of hundreds of bars (Pelster, 2001).

In general, the combination of gases in the bladder varies. In shallow water fish, the ratios closely approximate that of the atmosphere, while deep sea fish tend to have higher percentages of oxygen. For instance, the eel *Synaphobranchus* has been observed to have 75.1 % oxygen, 20.5 % nitrogen, 3.1 % carbon dioxide, and 0.4 % argon in its swim bladder. In some fish, mainly freshwater species (e.g. common carp, wels catfish), the swim bladder is connected to the labyrinth of the

inner ear by the Weberian apparatus, a bony structure derived from the vertebrate, which provides a precise sense of water pressure (and thus depth), and improves hearing (Wittenberg *et al*., 1981). Swim bladders of bighead carp (*Hypophthalmichthys nobilis*) had 75.2 ±1.4 % moisture contents (wet weight basis) (Liu *et al*., 2012).

In some Asian cultures, the swim bladders of certain large fishes are considered a food delicacy. In China, they are known as fish maw and are served in soups or stews. Swim bladders are also used in the food industry as a source of collagen. They can be made into a strong, water-resistant glue, or used to make isinglass for the clarification of beer (Teresa, 2009). Recently, Sinthusamran *et al*. (2013) extracted collagen from swim bladder of seabass. Swim bladders of catfish and bighead carp were also used for collagen extraction (Bama *et al*., 2010; Liu et al., 2012).

1.2.2 Collagen

Collagen is the fibrous protein of animal connective tissue, contributing to the unique physiological functions of tissues in skins, tendons, bones, cartilages, (Foegeding *et al*., 1996; Ogawa *et al*. 2003; Muyonga *et al*. 2004; Yan *et al*., 2008; Kittiphattanabawon *et al*., 2010). The collagen fibers are essentially inextensible and, therefore provide mechanical strength and also allow flexibility between various organs of the body (Bailey *et al*., 1998).

The collagen monomer is a long cylindrical protein about 2,800 Å long and 14-15 Å in diameter (Foegeding *et al*., 1996). It consists of three polypeptide units (called α-chains). Each α-chain coils is a left-handed helix with three residues per turn, and three chains are twisted right-handed to form the triple helix held together by hydrogen bonding (Fig. 1) (Fan *et al*., 2012). Each α-chain contains ~1,000 amino acid residues and varies in amino acid compositions (Wong, 1989) and has a molecular mass of about 100,000 Da, yielding a total molecular mass of about 300,000 Da for collagen (Foegeding *et al*., 1996). Polypeptides of collagen are mostly helical but differ from the typical α-helix due to the abundance of hydroxyproline and proline, which interfere with α-helical structure (Foegeding *et al*., 1996).

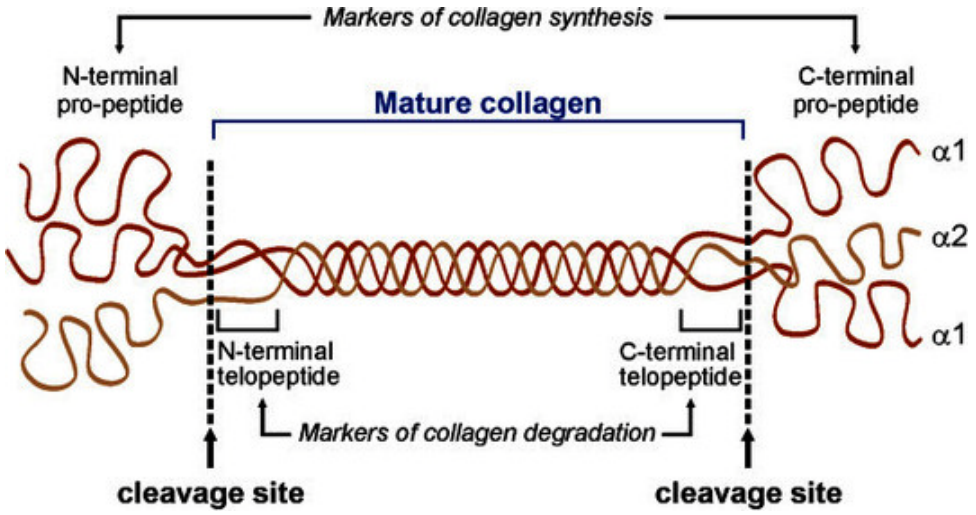

Figure 1. Structure of collagen molecule.
Source: Fan *et al*. (2012)

The prerequisite for the formation of homo- or heterotrimetric triple-helix is the repeating unique amino acid sequence (-Gly-X-Y-) in each α-chains to coil tightly around one another. The occurrence of proline and 4-hydroxyproline at X- and Y- positions, respectively, promotes the stability of the triple-helix by hydrogen bonding or by hydrogen water-bridges between the chains (Kovanen *et al*., 1998). Collagen has the high contents of glycine (33 %) and proline (12 %) with the occurrence of 4- hydroxyproline (12 %) and 5-hydroxylysine (1 %) (Burghagen, 1999). Glycine generally represents mainly one-third of the collagen molecule. The repetitive occurrence of glycine is absent in the first 14 amino acid residues from N-terminus and the first 10 from the C-terminus, with these end portions being termed “telopeptides” (Foegeding *et al*., 1996). Collagen is almost devoid of tryptophan (Jongjareonrak *et al*., 2006). Fish collagens have different amino acid composition, which can directly affect their physicochemical property and stability (Table 1). Collagen contents in fish muscles depend on the species as well as on the state of maturation and feeding of the fish (Regenstein and Regenstein, 1991). Additionally, the collagen in skin can be varied with species (Kittiphattanabawon *et al*., 2010).

Table 1. Amino acid composition of collagen from different fish

Amino acids (residues/1000 residues)	**Seabass swim bladder** [1]	**Amur sturgeon fish skin**[2]	**Spanish mackerel skin**[3]	**Grass skin**[4]	**Cobia skin**[5]
Alanine	134	109.96	135.4	125	134
Arginine	53	56.02	50.7	52.9	52
Aspartic acid/asparagines	46	39.17	50.1	49.3	46
Cysteine	1	-	3.1	0.3	-
Glutamine/glutamic acid	71	71.47	67.2	78.7	70
Glycine	326	327.52	341.6	326	329
Histidine	5	17.32	7.0	3.8	5
Isoleucine	9	11.15	13.4	11.8	11
Leucine	23	16.93	23.5	21	21
Lysine	25	23.35	20.8	27.5	25
Hydroxylysine	8	7.34	4.8	2.4	-
Methionine	14	5.84	10.7	13	14
Phenylalanine	13	13.44	15.3	13.6	14
Hydroxyproline	83	97.55	68.7	87.2	94
Proline	111	115.35	108.4	105	109
Serine	27	46.36	32.6	34.2	24
Threonine	24	22.71	19.5	24.7	23
Tyrosine	5	3.27	3.4	0	4
Valine	22	15.24	23.8	21.1	25
Imino acid	194	212.9	177.1	192	203

Source: [1]- Sinthusamran, *et al*. (2013), [2]- Wang, *et al*. (2014), [3]- Li, *et al*. (2013), [4]- Liu, *et al*. (2014), [5]- Zeng, *et al*. (2012)

Collagen is widely used in food, biomedical, pharmaceutical and cosmetics, and its consumption has been increasing along with the development of new industrial application (Nalinanon *et al*., 2007; Regenstein and Zhou 2007; Woo *et al*., 2008). Collagen exhibits weak antigenecity, and superior biodegradability to other natural polymers, such as albumin, etc. (Lee *et al*., 2001). Generally, commercial collagens are produced from bovine and porcine hides and bones. Currently, the increasing attention of fish collagen for replacement of mammalian collagen has been paid. Collagen can be extracted from fish skin, scale and bone. Collagen is also found in the body walls and cuticles of invertebrates (Meena *et al*., 1999). Fish collagen has been produced and characterized (Jongjareonrak *et al*., 2005; Nalinanon *et al*., 2007, 2008; Regenstein and Zhou 2007; Duan *et al*., 2009; Kittiphattanabawon *et al*., 2010). With the appropriate extraction technology, collagen from fish or aquatic animals can be used as the potential alternative for mammalian counterpart (Nalinanon *et al*., 2011).

1.2.2.1 Extraction of fish collagen

The acid solubilization process has been widely used for collagen extraction. The collagen can be extracted using acid solution and the collagen obtained is referred to as “acid-soluble collagen, ASC”. Extraction is conducted under acidic condition, in which the positive charge of collagen polypeptides becomes dominant (Benjakul *et al*., 2012). Collagen from total tissue can be isolated by direct extraction with organic acid (acetic, chloracetic, citric, lactic acids) or inorganic acid (hydrochloric acid) (Cheng *et al*., 2009). The yield of extracted collagen depends on the animal species, and parameters of extraction (Skierka and Sadowska, 2007). Collagen extraction is generally achievd by 0.5 M acetic acid treatment at 4 °C for 24-48 h. Compared to simply prolonging the extraction time, the use of several consecutive extraction may get better ASC yield (Sadowaka *et al*., 2003; Regenstein and Zhou, 2007; Skierka and Sadowska, 2007). Nalinanon *et al*. (2008) found that ASC yield from the skin of threadfin bream extracted using 0.5 M acetic acid was increased from 12.32 % to 34.90 % (based on hydroxyproline content) when the extraction time increase from 24 to 48 h.

To increase the yield of collagen, pepsin has been used to cleave the telopeptides, thereby increasing the extract able collagen. Pepsin is able to cleave telopeptides specifically, thus facilitating the extraction of collagen under the acidic condition (Benjakul *et al*., 2010). Nalinanon *et al*. (2011) used pepsin from porcine stomach mucosa to produce pepsin soluble collagen (PSC) from skin of ornate threadfin bream (*Nemipterus hexodon*). β-and α-chain of PSC was slightly lower in molecular weight, compared with those of ASC (Nalinanon *et al*., 2011). Nalinanon *et al*. (2011) used pepsin from the stomach of skipjack tuna (*Katsuwonus pelamis*) to increase the extraction yield of collagen from the skin of ornate threadfin bream (*Nemipterus hexodon*). There was no significant different in physicochemical property between PSC extracted with the aid of porcine pepsin and bigeye snapper pepsin (Nalinanon *et al*., 2007). The yield of PSC was generally higher than that of ASC.

Most of collagens extracted from fish skin were reported as type I collagen (Table 2). However, the alternative sources such as swim bladder can be used for extraction of collagen. Recently, PSCs with the yields of 40 and 59 % were extracted from the swim bladder of catfish and bighead carp, respectively (Bama *et al*., 2010; Liu *et al*., 2012). ASC from seabass swim bladder was extracted with the yield of 85.3 % (Sinthusamran *et al*., 2013).

1.2.2.2 The factors affecting collagen properties

There are many factors, which can affect the properties of collagen. Both intrinsic and extrinsic factors have been reported to the collagen properties as follows:

1.2.2.2.1 Age of animals

The age of the animal influences the connection tissue and its basic protein quantitatively. Modification of collagen with age via cross-linking increase the toughness to meat. The number of cross-links in collagen increased with increasing age of the animal and this may explain why meat from older animals is tougher than that from younger animals (Zayes, 1997). However most connective

tissue in fish is renewed annually and highly cross-linked protein is not generally found in fish (Foegeding *et al*., 1996). Collagenous tissue from older animals with more cross-linkages would be expected to be more resistant to swelling and have a lower water holding capacity (Zayes, 1997). Collagen from young animals is more easily solubilized but produces structures with low tensile strength. In contrast, collagen from old animals is difficult to solubilize and produces a structure with high tensile strength (Miller *et al*., 1983).

Table 2. ASC and PSC extracted from the skin of various fish

Collagen	Source	References
ASC	Grass carp (*Ctenopharyngodon idella*)	Liu *et al*. (2015)
ASC	seabass (*Lates calcarifer*)	Sinthusamran *et al*. (2013)
ASC	unicorn leatherjacket (*Aluterus monocerous*)	Ahmad *et al*. (2010)
ASC	Nile tilapia (*Oreochromis niloticus*)	Zeng *et al*. (2009)
PSC	Bigeye snapper (*Priacanthus tayenus* and *Priacanthus macracanthus*).	Benjakul *et al*. (2010)
ASC/PSC	Amur sturgeon (*Acipenser schrenckii*)	Wang *et al*. (2014)
ASC/PSC	Spanish mackerel (*Scomberomorous niphonius*)	Li *et al*. (2013)
ASC/PSC	sailfish (*Istiophorus platypterus*)	Tamilmozhi *et al*. (2013)
ASC/PSC	Cobia (*Rachycentron canadum*)	Zeng *et al*. (2012)
ASC/PSC	balloon fish (*Diodon holocanthus*)	Huang *et al*. (2011)
ASC/PSC	Striped catfish (*Pangasianodon hypophthalmus*)	Singh *et al*. (2011)
ASC/PSC	Brownbanded bamboo shark *(Chiloscyllium punctatum)*	Kittiphattanabawon *et al.* (2010)

1.2.2.2.2 Starvation

In some species of fish, the amount of collagen and the amount of cross-linking of the collagen increase with starvation. Thus the toughening process in fish seems to be much more reversible than it is in higher animals, where the amount of cross-linking increase with age (Regenstein and Regenstein, 1991). Foegeding *et al*. (1996) reported that starving fish have collagen with a greater degree of cross-linking than do fish that are well fed. Love *et al*. (1976) reported that myocommata are thickened with more intermolecular cross-links of collagen during starvation.

1.2.2.2.3 Habitat

Fish living in habitat with different temperatures have the collagen with different thermal stability. Collagen from tropical region shows the higher thermal stability, compared with that from cold water or temperate zone. T_{max} of collagen from cobia was 38.17 °C, which was higher than that of collagen from cod (15 °C) (Zeng *et al*., 2012; Duan *et al*. 2009). T_{max} of skin collagen from several cold- and temperate-water fish including deep-sea redfish (16.1 °C) (Wang *et al*., 2007), silver carp (35.9 °C) (Liu *et al*., 2014), grass carp (28.4 °C) (Zhang *et al*., 2007), striped catfish (39.3 °C) (Singh *et al*., 2011), balloon fish (29.64 °C) (Huang *et al*., 2011) have been reported.

1.2.2.2.4 Imino acid content

The thermal stability of collagen is related to the content of imino acids (proline and hydroxyproline). The higher the imino acid content, the more stable the helices are (Wong, 1989). Collagens that contain small concentrations of imino acid denature at lower temperatures than do those with large concentrations. The imino acid content of fish collagens correlates with the water temperature of their normal habitat and is associated with their thermal stability. Proteoglycan and glycoprotein contents can also affect collagen thermal stability (Foegeding *et al*., 1996).

1.2.2.2.5 pH and salt

Protein solubility in 0.5 M acetic acid, apparent viscosity and water-binding capacity of the collagenous of the collagen material from skin and muscle of hake (*Merluccius merluccius*) were influenced by pH and the ionic strength of the medium (Montero *et al*., 1999). Skin collagenous material showed higher functionality than muscle collagenous material. Maximum protein solubility, apparent viscosity and water-binding capacity were obtained at pH levels between 2 and 4 and with NaCl at concentrations of less than 0.25 M (Montero *et al*., 1999). Solubility of collagen from tiger puff skin reached the maximum at pH 3 and decreased instantly at pH 7 (Bae *et al*., 2008). Additionally, solubility of collagen from the skin of unicorn leatherjaket skin generally decreased with increasing NaCl concentration (Ahmad and Benjakul, 2010).

1.2.2.2.6 Processing/Preservation

Montero *et al*. (1995) compared 4 stabilizing methods: 1.) freezing, 2.) freeze-drying, 3.) partial solubilization with 0.05 M acetic acid then freezing and 4.) partial solubilization with 0.05 M acetic acid then freeze-drying. Only freeze-drying caused reduction solubility and emulsifying capacity. Viscosity was greatest when samples were pre-solubilized. Emulsifying capacity was not changed when samples were frozen and decreased when they were either freeze-dried or presolubilized. Optimum water-holding capacity was observed in samples which previously solubilized.

1.2.3. Gelatin

Gelatin is one of the commonly used food ingredients, obtained by the thermal denaturation of collagen (Bailey and Light, 1989; Hansen *et al*., 1994). Gelatin consists of random chains triple helix. Depending on the method in which collagens are pre-treated, two different types of gelatin with different characteristics including type-A, acid treated collagen, and type-B, alkaline treated counterpart, can be produced (Karim and Bhat, 2009). Acid treatment is most suitable for less fully cross-linked collagens commonly found in pig or fish skins, whereas alkaline

treatment is appropriate for the more complex collagens found in bovine hides (Foegeding *et al*., 1996).

The process involves the disruption of non-covalent bonds which directly affects gelling properties of gelatin (Bigi *et al*., 1998). Collagen fibrils shrink to less than one-thrid of their original length at a critical temperature, known as the shrinkage temperature (T_s), which varies with species (Belitz and Gwrosch, 1999). The shrinkage includes a disassembly of fibers and a collapse of the triple-helical arrangement of polypeptide subunits in the collagen molecule at critical temperature. The midpoint of the collagen-to-gelatin transition is defined as the melting temperature (Bremner, 1992). Generally, at heating temperature more than T_s, the triple-stranded helix of collagen is also destroyed to a great extent and exists as the random coils. During the collagen to gelatin transition, many non-covalent bonds are broken along with some covalent inter and intramolecular bonds and a few peptide bonds. This results in conversion of the helical collagen structure to a more amorphous form, known as “gelatin”. These changes constitute denaturation of the collagen molecule but not to the point of a completely unstructured product. If the latter event happens, glue instead of gelatin is produced (Foegeding *et al*., 1996). For fish collagen, T_s is 45 °C, while collagen from mammals has T_s of 60-65 °C (Belitz and Grosch, 1999).

The gelatin has been widely applied in the food industry as ingredients to improve the elasticity, consistency, and stability of foods. It can be used for medical and pharmaceutical purposes such as encapsulation, production of hard and soft capsules, wound dressing, adsorbent pads, etc. Furthermore, it can be applied for biomaterial-based packaging and photographic industries (Jongjareonrak *et al*., 2006). Generally, gelatin is obtained from mammals. However, mad cow disease (bovine spongiform encephalopathy, BSE) has caused the anxiety for consumers. Additionally, the gelatin obtained from pig skin and pig bone cannot be used in Kosher and Halal foods due to religious constraints. Gelatin production has been increased, especially from fish skin, and scale due to their abundance and low cost. However, fish gelatins have the limited application, mainly owing to low gel strength

and gel stability, compared with those from mammalian counterparts (Leuenberger, 1991; Benjakul *et al*., 2012).

Gelatin gel has “melt-in-the-mouth” characteristic and shows an excellent release of flavor (Choi and Regenstein, 2000). Moreover, its functional properties, including gelation, emulsifying properties, foam-forming properties, and film formation, are important for the food industry as it enhances the elasticity, consistency, and stability of food products. Gelatin film is used to protect foods against light and oxygen (Bonilla *et al*., 2012). Fiszman *et al*. (1999) studied the effect of the addition of gelatin on the microstructure and rheological properties of acid milk gels and yogurt. The addition of 1.5 % gelatin developed fairly firm and deformable gel with almost total absence of synersis. Kaewruang *et al*. (2013) added fish gelatin in surimi gel and found that fish gelatin at levels up to 3 % could be added to gel in conjunction with MTGase without any change in gel properties.

1.2.3.1 Gelatin structure

1.2.3.1.1 Primary structure

The primary structure and composition of gelatin resembles the parent collagen. This similarity has been substantiated for several tissues and species. Slight differences are due to the source of raw material in combination with the pretreatment and extraction procedures used (Mariod and Adam, 2013). Partial hydrolysis of the amide groups of asparagines and glutamine side chains of collagen results from the alkaline and to a lesser extent from acid pretreatments of the raw materials. This brings about an increase in the number of free carboxyl groups in the side chains of the amino acids in the gelatin molecule. The consequent increase in net negative charge on the gelatin molecule lowers its isoelectric point (Ofori, 1999). Prolonged exposure of collagen or gelatin to alkali during pretreatment procedures results in the conversion of the guanidine groups of arginine side chains to amino groups, being that of ornithines (Ofori, 1999). The reaction takes place by the removal of a urea group from the arginine side chain. The gelatins are slightly richer in the abundant amino acids, and trace amounts of amino acid such as cysteine. Tyrosine and isoleucine are

found to be the removal of some telopeptide during cross-link cleavage, which is lost in pretreatment solutions (Ofori, 1999).

1.2.3.1.2 Secondary structure and molecular weight

Various aspects of gelatin behavior in solution and gels have been explained in relation to its molecular weight. Gelatin is not polydispersed completely, but has a definite molecular weight distribution pattern, which corresponds to the α-chain and its oligomers (Johnston-Banks, 1990).

Oligomers of three α-chains will be exist mainly as intact triple helices whilst a certain proportion will exists as extended α-polymers bonded randomly by end-to-end or side-to-side bonds. The presence of oligomers with increasing numbers of α-chains becomes more complex and form self-aggregation (Giménez *et al*., 2005). Pretreatment also shows the profound impact on molecular weight of gelatin. Ahmad and Benjakul (2011) reported that gelatin from the skin of unicorn leatherjacket swollen with 0.2 M phosphoric acid had the decrease in major band intensity (α-, β-, and γ -chains) with the increasing low-molecular weight components, in comparison with that swollen using 0.2 M acetic acid.

1.2.3.2 Gelatin production

Production of gelatin involves three steps involving 1) pretreatment of the raw material, the removal of non-collagenous components from the stock (skin and bones), 2) extraction of the gelatin, the conversion of collagen to gelatin by heating in the presence of water and 3) recovery of gelatin in the final form (McMullen *et al*., 2005). All processes used for gelatin extraction have the direct impact on the yield and properties of gelatin obtained. The process has been optimized for different source of raw materials (Cho *et al*., 2005; Hou and Regenstein, 2004; Yang *et al*., 2007).

1.2.3.2.1 Pretreatment processes

1.2.3.2.1.1 Removal of non-collagenous protein

Prior to extraction from raw material, the pretreatment is practically implemented to increase purity of gelatin extracted. Alkaline solution has been used remove considerable amounts of non-collagenous materials (Zhou and Regenstein, 2005) and breaks some interchain cross-links. Also, the process is able to inactivate proteases involved in degradation of collagen (Regenstein and Zhou, 2007). During alkaline proteases, the type of alkali dose not make a significant difference, but the concentration of alkali is critical (Zhou and Rgenstein, 2005). Different alkaline solutions were able to leach out protein during the treatment differently. $Ca(OH)_2$ pretreatment solution had higher amounts of protein than the NaOH pretreatment solution (Hao *et al*., 2009). Liu *et al*. (2015) reported that NaOH could remove non-collagenous protein with the minimal loss of collagen, compared with $Ca(OH)_2$. Yoshimura *et al*. (2000) reported that alkali attacks predominantly the telopeptide region of the collagen molecule during pretreatment. Long time and high concentration of alkaline pretreatment decreased the yield of gelatin from skin of channel catfish (Yang *et al*., 2007). The concentration of alkali, time and temperature used for pretreatment varied with raw materials (Benjakul *et al*., 2012).

1.2.3.2.1.2 Swelling of raw materials

Swelling is important because it can favor protein unfolding by disruption of non-covalent bonding and predispose the collagen to subsequent extraction and solubilization (Stainby, 1987). Swelling process can be classified into two processes and are selectively used, based on the raw materials.

1. Acid process or type A gelatin

Acid hydrolysis is a milder treatment that effectively solubilizes collagens of animals slaughtered at a young age, such as pig (Foegeding *et al*., 1996). The swelling process is designed to convert the collagen into a form suitable for extraction. A sufficient number of the covalent cross-links in the collagen must be broken in order to enable the release of free α-chains. The process is also designed to

remove other organic substances, such as proteoglycan, blood, mucins, sugars, ets., that also occur naturally in the raw material (Zhou and Regenstein, 2005). It is optimized by each manufacturer to yield gelatin with the required physical and chemical sufficient to bring about the conversion. Sulphuric and hydrochloric acids are used, often with the addition of phosphoric acid to retard color development (Johnston-Banks, 1990). Different kinds of acids have been used for pretreatment of fish skin before extractions. Phosphoric acid and acetic acid have been used in fish skin pretreatment. Nevertheless, other acids including citric and sulfuric has been used for skin pretreatment before gelatin extraction (Gudmundsson and Hafasteinsson, 1997). After removal of non-collagenous protein, the materials are generally subjected to swelling using acids. The concentration of H^+ used in processing of gelatin from cod skin affected yield and quality of resulting gelatin (Gudmundsson and Hafsteinsson, 1997). Megrim skin was treated with 0.05 M acetic and 0.05 M propionic acid prior to gelatin extraction using distilled water at 45 °C for 30 minutes. The gelatin obtained had the highest elastic modulus, viscous modulus, melting temperature, and gel strength. On the other hand, gelatin obtained from skin swollen with citric acid exhibited the lowest turbidity of gelatin solution, whereas propionic acid led to the most turbid gelatin solution. Giménez *et al*. (2005) reported that lactic acid (25 mM) could be an excellent substitute for acetic acid for the skin swelling process. Gelatin obtained from the acid process is known as type A gelatin.

2. Alkaline process or type B gelatin

Type B gelatins are generally produced by alkali hydrolysis of bovine materials, which results in deamidation and a greater range of molecular weight species (Foegeding *et al*., 1996). Alkaline pretreatment is normally applied to bovine hide and ossein. Lime is most commonly used for this purpose; it is relatively mild and dose not cause significant damage to the raw material by excessive hydrolysis. Unfortunately, 8 weeks or more are required for complete treatment. Concentration up to 3 % lime are used in conjunction with small amounts of calcium chloride or caustic soda. Frequent renewal of the liquors of alkalinity present. If caustic soda is used, a 10-14 day pretreatment is possible (Johnston-Banks, 1990). Gelatin obtained from the alkaline process is named as type B gelatin. To increase the yield, alkaline process

was used for gelatin extraction from cartilage. Cho *et al*. (2004) optimized the extraction condition for production of gelatin from shark cartilage using response surface methodology. Gelatin production has two important step, including alkali treatment and hot-water extraction. The alkaline treatment removes non-collagenous protein. Hot water extraction causes thermal hydrolysis, leading to the solubilization of gelatin. The predicted maximum yield of 79.9 % for gelatin production was obtained when alkali treatment using 1.6 NaOH for 3.16 days and hot-water extraction at 65 °C for 3.4 h were implemented. Gelatin from tilapia skin could be extracted from alkaline pretreated skin with the yield of 21.55 % (Niu *et al*., 2013). Gelatin had mild to undetectable fishy odour with acceptable colour attributes. Its color was light yellowish to whitish. Additionally, gel strength of gelatin extracted by liming was high (300 g) (Jamilah *et al*., 2011).

1.2.3.2.2 Extraction of fish gelatin

After pretreatment, prepared skin or other raw materials are subjected to heat treatment in which hydrogen bond and other weak bonds, contributing to structural stabilization are destroyed (Fig. 2) (Hansan *et al*., 1994). Extraction temperature has been reported to determine functional property of gelatin, especially gelling property. Kittiphattanabawon *et al*. (2010) found that gelatin from shark skin had the decrease in gel strength with coincidentally increased degradation as the extraction temperature increased. However, Kaewruang *et al*. (2013) reported that gelatin from skin of unicorn leatherjacket had the higher gel strength when extracted at temperature higher than 55 °C. With extraction temperature of 45-55 °C, which was the optimal temperature for indigenous protease, the pronounced autolysis was obtained leading to the lower content of α-chains retained in the resulting gelatin (Kaewruang *et al*., 2013).

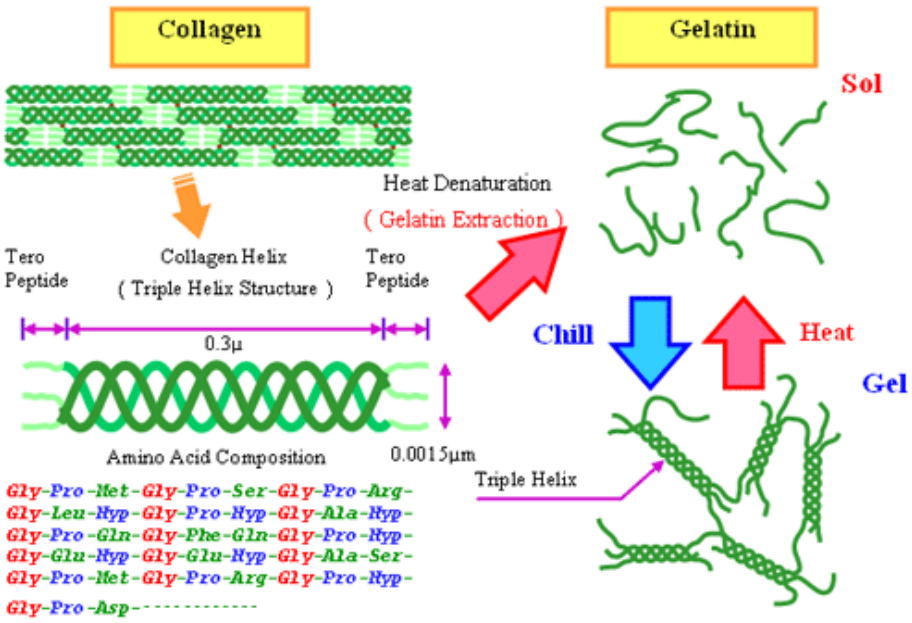

Figure 2. Transformation of collagen into gelatin.
Source: Nitta Gelatin Inc. Ltd. (2012)

Thermal treatment above 40 °C (above helix-to-coil transition temperatures for fish gelatins) destroys hydrogen bonding and cleaves a number of covalent bonds, which destabilizes the triple-helix via a helix-to-coil transition and results in conversion to gelatin (Djabourov, 1991). High-molecular weight polymers may occur in the resulting gelatin through the possible persistence of cross-links, depending on the nature and degree of solubilization. Gómez-Guillén *et al*. (2002) reported that gelatin was not extracted by heating the squid skin (*Dosidicus gigas*) in water at 45 °C. Therefore, heating the squid skin was preformed at 80 °C overnight. However, the yield of squid gelatin was only 2.6 % which was lower than that found in sole (8.3 %), megrim (7.4 %), cod (7.2 %) and hake (6.5 %) using a milder procedure. Therefore, invertebrate collagens present a really high cross-linking degree, compared to vertebrate collagens, mainly due to the high increase in lysine hydroxylation that participate in different types of cross-links via Schiff-base formation. Moreover, hydroxylysine is normally glycosylated in invertebrate collagens, and the sugar moieties contribute to the establishment of additional covalent cross-links (Gómez-Guillén *et al*., 2002).

The lower extraction yield of fish gelatin could be due to the loss of extracted collagen through leaching during the series of washing steps or due to incomplete hydrolysis of the collagen (Jamilah and Harvinder, 2002). Nalinanon *et al*. (2008) found that the addition of an appropriate protease inhibitor together with pepsin-aided process might be an effective means to obtain a higher yield with negligible hydrolysis of the peptides. Extraction of gelatin from the bigeye snapper using the pepsin-aided process in combination with a protease inhibitor (pepstatin A) markedly increased the yield from 22.2 to 40.3 %.

Table 3. Bloom strength of gelatin from various fish

Sample	**Extraction conditions**	**Bloom strength (g)**	**Yield (g/100 g)**	**References**
Seabass (*Lates calcarifer*)	Water (45°C for 3 h)	369	51.6	Sinthusamran *et al*. (2014)
Dog shark (*Scoliodon sorrakowah*)	Water (45°C for 12 h)	206	19.7	Shyni *et al*. (2014)
Leatherjacket (*Aluterus monoceros*)	Water (65°C for 9 h)	197	11.6	Kaewruang *et al*. (2013)
Tiger-toothed croaker (*Otolithes ruber*)	Water (45°C for 12 h)	170	7.56	Koli *et al*. (2012)
Rainbow trout (*Onchorhynchus mykiss*)	Water (50°C for 16 h)	459	9.36	Tabarestani *et al*. (2010)
Brownbanded bamboo shark (*Chiloscyllium punctatum*)	Water (45°C for 6 h)	214	19.06	Kittiphattanabawon *et al*. (2010)

Generally, the extraction yield of gelatin from skins ranged from about 5.5 to 21 % of the starting material (Giménez *et al*., 2005; Muyonga *et al*., 2004). The variation in yield depends on the differences in both proximate composition of the skins and the amount of soluble components in the skins (Muyonga *et al*., 2004), as these properties vary with the species and the age of the fish. In addition, the variation in the extraction method can also have an effect on yields and gel properties, especially gel strength (Table 3). Higher extraction temperature resulted in the higher

yield of gelatin, however the higher degradation took place (Kittiphattanabawon *et al*., 2010).

1.2.3.2.3 Recovery/drying

After extraction, the gelatin are filtered to remove suspended insolubles such as fat of unextracted collagen fibres. Gelaltin manufacture generally has a good process to clarify the impurities from the gelatin solution, such as chemical clarification and filtration processes (Ahmad and Benjakul, 2011). This is usually performed using materials such as diatomaceous earth to give solutions of high clarity. The final stage is evaporation, sterilization and drying. These are performed as quickly as possible to minimize loss of properties (Johnston-Banks, 1990). Kwak *et al*. (2009) prepared gelatin from shark cartilage using three drying methods, including freeze drying, hot-air drying and spay drying. Freeze-dried gelatin showed the highest gel strength and foam formation ability but had the lowest foam stability. Nevertheless, spray-dried gelatin exhibited the best emulsion capacities.

1.2.3.3 Composition and molecular properties of gelatin

Gelatin is a heterogeneous mixture of water-soluble proteins of high molecular weight (Budavari, 1996). On a dry weight basis, gelatin consists of 98 to 99 % protein. The molecular weight of these large proteins typically ranges between 20,000 and 250,000 Da (Kennan, 1994). Coils of amino acids are joined together by peptide bonds. The predominant amino acid sequence is Gly-Pro-Hyp (Poppe, 1997). Gelatin contains relatively high levels of these following amino acids: glycine (Gly) 26-34 %; proline (Pro) 10-18 %; and hydroxyproline (Hyp) 7-15 % (Veis, 1964; Poppe, 1997). Other significant amino acids include alanine (Ala) 8-11 %; arginine (Arg) 8-9 %; aspartic acid (Asp) 6-7 %; and glutamic acid (Glu) 10-12 %. (Hudson, 1994; Poppe, 1997). Gelatin is not a nutritionally complete protein. It contains no tryptophan and is deficient in isoleucine, threonine, and methionine (Potter and Hotchkiss, 1998). Sulfur-containing amino acids, cysteine and cystine, are also deficient in gelatin. Different amino acid compositions between fish gelatin from different sources are shown in Table 4. In general, gelatin contained the similar molecular properties to mother collagen, in which α- or β-chains could be present.

Table 4. Amino acid composition of gelatin from different fish

Amino acids (residues/1000 residues)	**Grey triggerfish skin**[1]	**Smooth hound skin**[2]	**Bigeye snapper skin**[3]	**Brownbanded bamboo shark skin**[4]	**Blacktip shark skin**[5]
Hydroxyproline	74	90	87.75	95	91
Aspartic acid	59	38	40	40	40
Threonine	29	22	25.03	22	20
Serine	40	36	29.80	41	29
Glutamic acid	60	78	92.94	76	76
Proline	102	112	98.54	113	110
Glycine	289	322	248.57	322	321
Alanine	113	113	49.04	106	120
Cysteine	0	1	-	1	1
Valine	28	25	17.82	24	25
Methionine	8	12	20.04	12	15
Isoleucine	17	19	10.30	17	18
Leucine	25	23	22.32	22	23
Tyrosine	7	2	6.07	2	2
Phenylalanine	19	13	20.01	13	13
Hydroxylysine	9	7	7.37	6	5
Histidine	11	7	4.46	7	7
Arginine	78	52	87.36	51	54
Lysine	32	28	53.02	28	27
Imino acids	176	202	186.29	208	201

Source: [1]- Jellouli *et al*. (2011), [2]- Bougatef *et al.* (2012), [3]- Benjakul *et al*. (2009), [4], [5]- Kittiphattanabawon *et al*. (2010)

However, the degradation of those chains induced by inappropriate extraction could lead to the differences in MW of chains (Nagarajan *et al*., 2012). Additionally, molecular distribution of gelatin was determined by size or age of fish (Sinthusamran *et al*., 2015). Gelatin from seabass with the larger size contained a larger proportion of α- and β-chains in comparison with gelatin from seabass with smaller size. Higher extraction temperature (75 °C) caused the drastic degradation of gelatin from blacktip and brownbanded bamboo shark (Kittiphattanabawon *et al*., 2010). Slightly higher degradation of α-chain was found in gelatin from seabass skin as the extraction temperature was increased (Sinthusamran *et al*., 2014).

1.2.3.4 Improvement of properties of fish gelatin gel

Gel strength is one of the most important functional properties of gelatin. Fish gelatin generally has lower concentrations of imino acid content (proline and hydroxyproline) than mammalian gelatin (Haug *et al*., 2004; Muyonga *et al*., 2004; Avena-Bustillos *et al*., 2006). Low gelling and melting temperatures and low gel strength of fish gelatin (Gómez-Guillén *et al*., 2002; Lui *et al*., 2008) limit its applications. Therefore, a number of chemical or enzymes as well as technologies have been used for improvement of gelatin properties (Chiou *et al*., 2006; Yi *et al*., 2006; Benjakul and Visessanguan, 2003).

1.2.3.4.1 Microbial transglutaminase

Microbial TGase (MTGase) have been successfully produced by fermentation process. TGase from *Streptoverticillium mobarense* is Ca^{2+} independent and also differs from mammalian TGase in molecular weight, thermal stability, isoelectric point, and substrate specificity (Ando *et al*., 1989; Tsai *et al*., 1996). MTGase can be obtained from *Streptoverticillium ladakanum* and *Streptoverticillium mobaraense* (Seki *et al*., 1998; Shann *et al*., 1998). MTGase has been known to induce the acyl transfer from glutamine (acyl donor) to lysine (acyl acceptor), in which ε-(γ-glutamyl) lysine isopeptides are formed (Fig. 3) (Cozzolino *et al*., 2010).

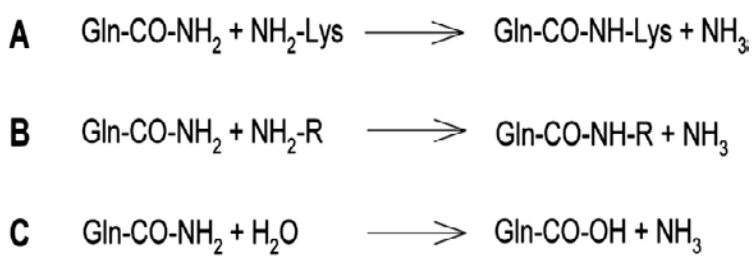

Figure 3. Reaction catalyzed by TGase.
Source: Ashie and Lanier (2000)

MTGase offers a means of upgrading the gelling quality of surimi (Ando *et al*., 1989). Lanier and Kang (2000) reported that the addition of MTGase has more pronounced effects on gel strengthening of Pacific whiting surimi, especially when combined with beef plasma (1 %), which inhibits heat stable protease. TGase from *Streptoverticillium ladakanum* and *Streptoverticillium mobaraense* catalyzed the crosslinking of fish myosin heavy chain (Seguro *et al*., 1995). MTGase was also applied for improvement of gelatin gel. Jongjareonrak *et al*. (2006) studied the effect of MTGase on the gel properties of gelatin from bigeye snapper skin and brownstrip red snapper skin. The addition of MTGase at concentrations up to 0.005 % and 0.01 % (w/v) increased the bloom strength of gelatin gel from bigeye snapper and brownstripe red snapper, respectively. Norziah *et al*. (2009) reported that the addition of MTGase with the concentration of 0.5 and 1.0 mg/g gelatin into fish gelatin extracted from the wastes of fish herring species (*Tenualosa ilisha*) increased gel strength. However, enzyme concentration above 1.0 mg/g gelatin resulted in a decreased gel strength.

1.2.3.4.2 Salt (organic salt)

The effect of different salts on the rigidity or melting temperature of warm blooded animal gelatins has been known for a long time (Harrington and Von Hippel, 1961). Sarabia *et al*. (1961) studied the effect of several salts on the viscoelastic properties of fish skin gelatins from megrim and tilapia. Although salts generally extended the setting time of gelatins, the melting temperatures were increased considerably, mainly by the addition of $MgSO_4$. Recently, Kaewruang *et al*.

(2014) demonstrated the impact of divalent salts ($CaCl_2$) on gel properties of gelatin from the skin of unicorn leatherjacket. Addition of $CaCl_2$ at 2.5-40 µmol/l increased the gel strength of gelatin from unicorn leatherjacket skin, compared with the control.

1.2.3.4.3 Ultraviolet (UV)

Ultraviolet (UV) irradiation as a physical, cost effective, non-thermal, and environmental friendly technology has received increasing attention during recent years. It has been successfully applied for preservation and decontamination of food products (Bintsis *et al*., 2000; Mertens and Knorr, 1992). UV irradiation has been used in medical and pharmaceutical research to crosslink collagen and gelatin films (Fujimori, 1965; Tomihata *et al*., 1992; Weadock *et al*., 1984). Bhat and Karim (2009) reported that fish gelatin was exposed to UV irraddition for 30 and 60 min to increase gel strength, mainly due to the enhanced crosslinking induced by irradiation treatments. Sung and Chen (2014) also reported that UV treatment can improve gel strength of gelatin and gel forming ability of gelatin processing products.

1.2.3.4.4 Phenolic compounds

Plant phenolic compounds, also denoted polyphenols, are defined as compounds possessing one or more aromatic rings bearing hydroxyl substituent(s), and which are derived from the secondary metabolism of plants (Parr and Bolwell, 2000; Robards *et al.,* 1999). In plants, phenolic compounds play a role in numerous processes, such as plant growth and reactions to stress and pathogen attack (Parr and Bolwell, 2000).

1. Sources

Phenolic compounds can be found in many foods and drinks from plant origin, e.g. fruits, vegetables, coffee (Clifford, 1999), tea (Lakenbrink *et al.,* 2000), beer, wine and chocolate (Arts *et al.,* 1999). Kiam (*Cotylelobium lanceotatum* craih) trees are very common in the southern part of Thailand. Pieces of wood from the kiam tree have been traditionally submerged in sugar palm sap to prevent or retard microbial fermentation (Chanthachum and Beuchat, 1997). The kiam wood extract containing phenolic compounds could be used for improvement of gel properties of

surimi (Balange, 2009). Temdee and Benjakul (2014) used the extracts from kiam wood and cashew bark rich in tannic acid as the gel enhancer for gelatin. Additionally, *Cocos nucifera* L. (family Arecaceae), commonly known as “coconut”, is considered as an important cash crop in tropical countries. A portion of this waste material is processed and used by the rope-making industry, but the majority remains unutilized (Dey *et al*., 2003) and various polyphenols are present in the husk (Sueli and Gustavo, 2007).

Apart from being naturally present in the raw materials used for foods, phenolic compounds are also added to some foods for their coloring properties and for their antioxidant effects (O'Connell and Fox, 2001; Richelle *et al.,* 2001). The presence of phenolic compounds may be easily observable due to the chromophoric groups that some phenolic compounds bear, e.g. the red-purple anthocyanins (Bakowska *et al.,* 2003), or by the brown and green reaction products of phenolic compounds with themselves or with proteins (Montavon *et al.,* 2003; Yabuta *et al.,* 2001). The presence of phenolic compounds can also affect the taste of food. Low concentrations of phenolic compounds may be responsible for desirable sweet, smoky or caramel flavours in foods e.g. dairy products (O'Connell and Fox, 2001). High concentrations of phenolic compounds in tea and wine provide the astringent sensation. The latter results from the precipitation of saliva proteins on the tongue by interactions with specific phenolic compounds (Baxter *et al.,* 1997; Charlton *et al.,* 2002). On the other hand, the interactions between phenolic compounds and proteins may lead to a decrease of protein digestibility, by blocking the substrate and/or inhibiting certain proteases (Kroll *et al.,* 2003).

2. Classification of phenolic compounds

Phenolic compounds represent a wide range of molecules with a molecular mass from about 100 to 3,000-4,000 Da (Haslam, 1996). They are produced by two principal pathways: the shikimate and the polyketide pathway (O'Connell and Fox, 2001). Different classifications of phenolic compounds have been proposed (O'Connell and Fox, 2001). For example, plant “tannins” are proposed to be broadly divisible into two majors groups: the proanthocyanidins and the

polyesters based on gallic and/or hexahydroxydiphenic acid (Haslam, 1989). However, this classification does not include simple phenols.

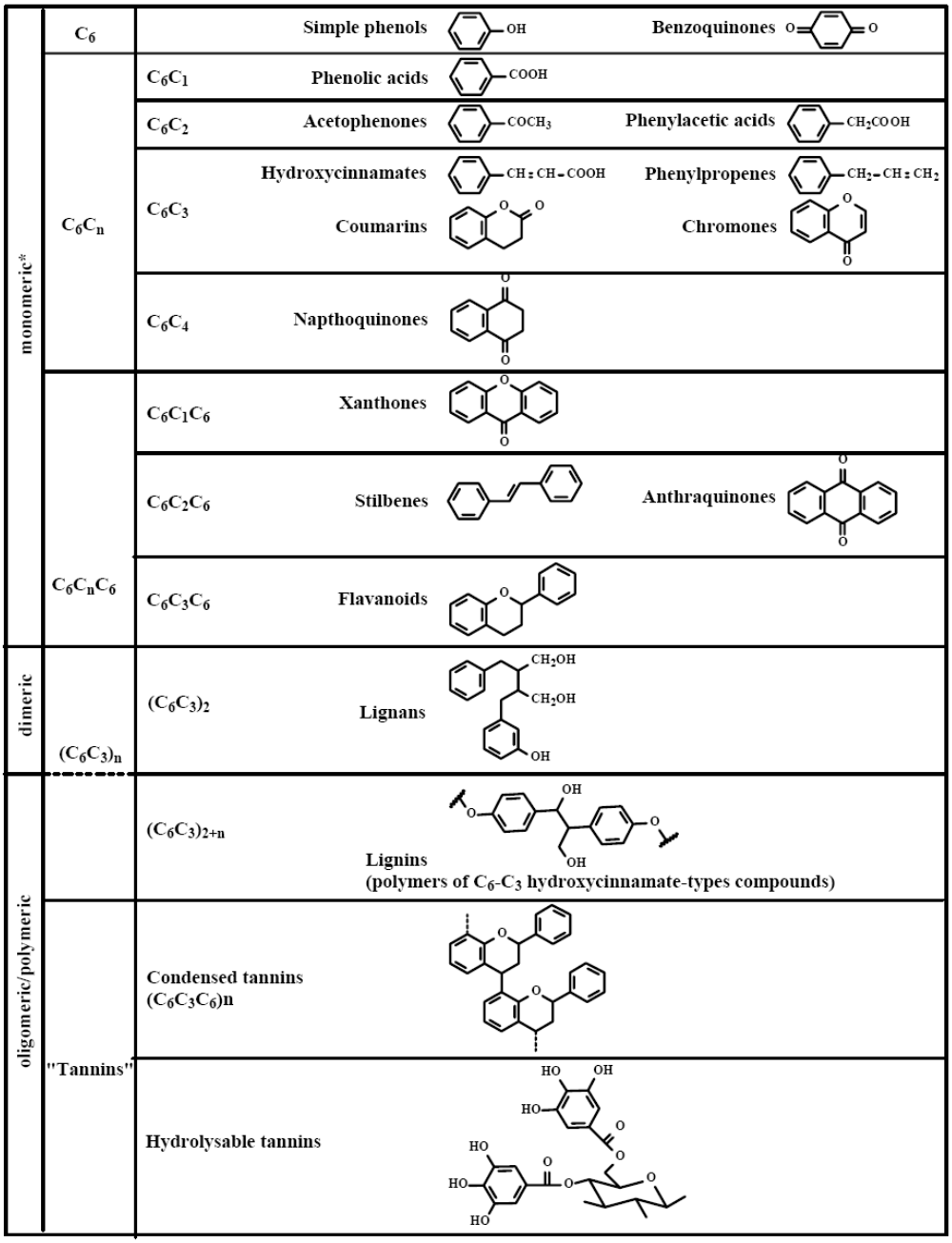

Figure 4. Classification of phenolic compounds. *Oligomers are occasionally formed in these groups.

Source: O'Connell and Fox (2001)

In Figure 4, a more complete classification according to the number of carbon atoms is given. It is adapted from the one given by O'Connell and Fox (2001) and divides the phenolic compounds into five groups: 1) the C_6 group, comprising simple phenols and benzoquinones; 2) the C_6C_n group, which includes phenolic acid derivatives and 3) hydroxycinnamic acid derivatives the C_6-C_n-C_6 group, which includes flavanoids (C_6-C_3-C_6); 4) the $(C_6$-$C_3)_n$, group consisting of lignans and lignins; and 5) the tannin group, which are divided into hydrolysable tannins and condensed tannins (O'Connell and Fox, 2001). The hydrolysable tannins are formed by gallic acid, 3-digallic acid or hexahydroxydiphenic acid, esterified to a polyol such as glucose or quinic acid (O'Connell and Fox, 2001).

3. Use as protein cross-linker

Generally, gelatins from fish skin have the poorer bloom strength, compared with mammalian gelatins, due to their lower imino acid content (Grossman and Bergman, 1992). The addition of phenolic compounds into gelatin solutions could increase the bloom strength of gelatin (Struss and Gibson, 2004). Phenolic compounds in the extracts could interact with proteins in two different ways: via non-covalent (reversible) interactions and via covalent interactions, which in most cases are irreversible (Prigent *et al*., 2003). Phenolic compound, especially tannic acid, could induce the cross-linking, mainly via hydrogen bond or hydrophobic interaction. Such cross-links formed contributed to the stronger gel network as evidenced by the increase in gel strength. Yan *et al*. (2011) reported that gelatin from walleye pollock skin had the increase in gel strength when cross-linked by gallic acid and rutin.

Additionally, phenolic–protein interaction concept has been introduced extensively for improving food properties (Kroll *et al*., 2003). Two types of complex formation mechanisms can be distinguished: a monodentate and a multidentate mechanism (Haslam, 1988). Both complex formation mechanisms lead to aggregation and precipitation of proteins (Haslam, 1988). Recently, Balange and Benjakul (2009) found that the incorporation of tannic acid into mackerel surimi increased its gel strength.

1.2.3.4.5 Agar/Agarose

1. Molecular structure

Agar consists of polysaccharides extracted from red (Rhodophyceae) seaweed, of *Gelidiaceae* and *Gracilariaceae* species. Agar is a mixture of two different polysaccharides, one a neutral agarose which consists of alternating repeat of (1-4) linked 3,6-anhydro-α-L-galactopyranose and (1-3) linked β-D-galactopyranose (Fig. 5) and the other a charged agaropectin (Singh, 1992). Agaropectin contains galactopyranose residues with sulfate and other charge groups present in varying degrees in the molecule (Araki, 1966). The molecular weight of agarose is about 120,000. Due to the substitutions in agaropectin, the molecular weight of agar is typically higher (up to 250,000), with a wide distribution (Labropoulos *et al*., 2002).

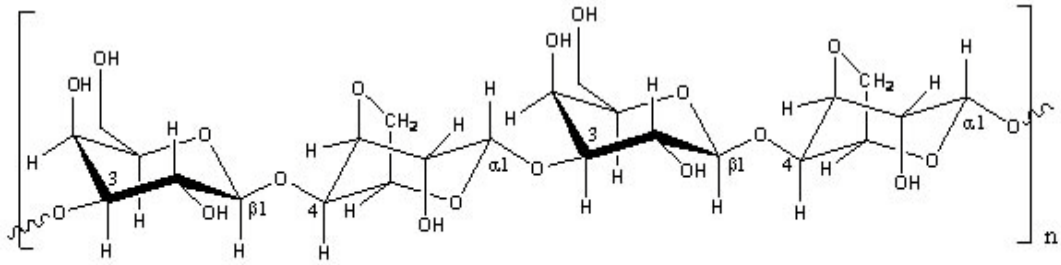

Figure 5. Chemical structure of agar.
Source: Singh (1992)

Agar has ability to form gel upon cooling of a hot solution at 30-40 °C and melt to sols upon heating at 90-95 °C. Gel formation mechanism is shown in Figure 6. At temperature above melting point of the gel, thermal agitation overcomes the tendency to form helices and polymer exists in solution as a random coil. Upon cooling, a three dimensional net work builds up in double helices form the junction points of the polymer chain. Furthermore, cooling leads to aggregation of these junctions. The presence of the sulfate at C-6, the 1,4-linked-L-galactose residues in the precursor of agarose, act as a “kink” to prevent the double helix forming (Rees,

1969). Closure of the ring to form the 3,6-anhydrode, and the elimination of the C-6 sulfate group make the chain straightened and lead to great regularity in the polymer. This results in enchancing gel strength due to increased capability of forming a double helix (Singh, 1992).

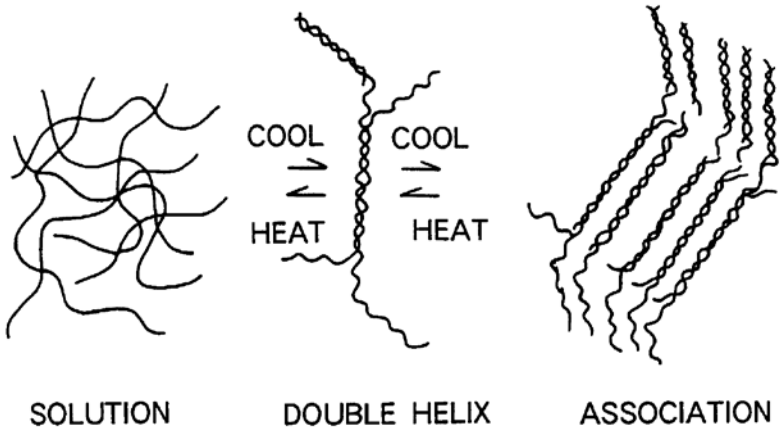

Figure 6. Gelling mechanism of agar polymer.
Source: Ito *et al*. 1998

2. Use of agar

Protein–polysaccharide interactions are the basis for many important biological processes. In foods, proteins and polysaccharides are the most important structure-forming ingredients (Tolstoguzov, 1991) and their use in mixed systems can improve or modify their functional behaviour (Dickinson and Izgi, 1996; Gurov and Nuss, 1986; Turgeon and Beaulieu, 2001). Somboon *et al*. (2014) studied properties of gels from mixed agar and fish gelatin. The agar concentration was fixed at 1 % (w/v), while the fish gelatin concentration was varied (5-25 %, w/v). The mixing ratio of agar with fish gelatin affects the property of mixed gel. The 1:15-1: 25 mixed gels had improved springiness and syneresis, compared to 1 % agar, but the gelling and melting temperatures were similar to those of fish gelatin. Additionally, Norziah *et al*. (2006) studied the property of mixtures of agar and k-carrageenan. Addition of k-carrageenan appeared to affect the rheological properties of agar. The effect was not of a synergistic type. In the presence of k-carrageenan, the gelation characteristics of agar gel were still clearly evident in the mixtures.

1.2.4 References

Aewsiri, T., Benjakul, S., Visessanguan, W. and Tanaka, M. 2008. Chemical compositions and functional properties of gelatin from pre-cooked tuna fin. Int. J. Food Sci. Technol. 43: 685-693.

Ahmad, M. and Benjakul, S. 2010. Extraction and characterisation of pepsin solubilised collagen from the skin of unicorn leatherjacket (*Aluterus monocerous*). Food Chem. 120: 817–824.

Ahmad, M., Benjakul, S. and Nalinanon, S. 2010. Compositional and physicochemical characteristics of acid solubilized collagen extracted from the skin of unicorn leatherjacket (*Aluterus monoceros*). Food Hydrocolloid. 24: 588-594.

Ahmad, M. and Benjakul, S. 2011. Characteristics of gelatin from the skin of unicorn leatherjacket (*Aluterus monoceros*) as influenced by acid pretreatment and extraction time. Food Hydrocolloid. 25: 381-388.

Ando, H., Adachi, M., Umeda, K., Matsuura, A., Nonaka, M., Uchio, R., Tanaka, H. and Motoki, M. 1989. Purification and characteristics of a novel transglutaminase derived from microorganisms. Agric. Biol. Chem. 53: 2613-2617.

Araki, C. 1966. Some recent studies on the polysaccharides of agarophytes. *In* Proceeding Fifth International Seaweed Symposium. (Young, E. G. and McLachlan J. L., eds.). p. 3-17. Pergammon Press. London.

Arts, I. C. W., Hollman, P. C. H. and Kromhout, D. 1999. Chocolate as a source of tea flavonoids. Lancet. 354: 488.

Ashie, L. N. A. and Lanier, T. C. 2000. Transglutaminases in seafood processing. *In* Seafood Enzymes. (Haard, N. F. and Simpson, B. K., eds.). p. 147-90. Marcel Dekker Inc. New York.

Avena-Bustillos, R. J., Olsen, C. W., Chiou, B., Yee, E., Bechtel, P. J. and McHugh, T. H. 2006. Water vapor permeability of mammalian and fish gelatin films. J. Food Sci. 71: 202–207.

Bae, I., Osatomi, K., Yoshida, A., Osako, K., Yamaguchi, A. and Hara, K. 2008. Biochemical properties of acid-soluble collagens extracted from the skins of underutilised fishes. Food Chem. 108: 49–54.

Bailey, A. J., Paul, R. G. and Knott, L. 1998. Mechanisms of maturation and ageing of collagen. Mech. Ageing Dev. 106: 1–56.

Bhat, R. and Karim, A. A. 2009. Ultraviolet irradiation improves gel strength of fish gelatin. Food Chem. 113: 1160–1164.

Balange, A. and Benjakul, S. 2009. Enhancement of gel strength of bigeye snapper (*Priacanthus tayenus*) surimi using oxidised phenolic compounds. Food Chem. 113: 61–70.

Bakowska, A., Kucharska, A. Z. and Oszmianski, J. 2003. The effects of heating, UV irradiation and storage on stability on the anthocyanin polyphenol copigment complex. Food Chem. 81: 349-355.

Bama, M., Vijayalakshimi, M., Jayasimman, R., Kalaichelvan, P. T., Deccaraman, M. and Sankaranarayanan, S. 2010. Extraction of collagen from cat fish (*Tachysurus maculatus*) by pepsin digestion and preparation and characterization of collage chitosan sheet. Inter. J. Pharm. Pharm. Sci. 2: 133–137.

Baxter, N. J., Lilley, T. H., Haslam, E. and Williamson, M. P. 1997. Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. Biochem. 36: 5566-5577.

Belitz, H. D. and Grosch, W. 1999. Meat. *In* Food Chemistry. (Belitz, H. D. and Grosch, W., eds.). p. 540-547. Springer. Heidelberg.

Benjakul, S. and Visessanguan, W. 2003. Transglutaminase-mediated setting in bigeye snapper surimi. Food Res. Int. 36: 253-266.

Benjakul, S., Oungbho, K., Visessanguan, W., Yaowapa Thiansilakul , Y. and Roytrakul , S. 2009. Characteristics of gelatin from the skins of bigeye snapper, *Priacanthus tayenus* and *Priacanthus macracanthus.* Food Chem. 116: 445-451.

Benjakul, S., Oungbho, K., Visessanguan, W., Thiansilakul, Y., and Roytrakul, S., Kishimura, H., Prodpran, T., Meesane, J. 2010. Extraction and characterisation of pepsin-solubilised collagens from the skin of bigeye snapper (*Priacanthus tayenus* and *Priacanthus macracanthus*). J. Sci. Food Agric. 90: 132-138.

Benjakul, S., Nalinanon, S. and Shahidi, F. 2012. Fish collagen. *In* Food Biochemistry and Food Processing. (Simpson, B. K., Nollet, L. M. L., Toldra, F., Benjakul, S., Paliyath, G. and Huieds, Y. H., eds.). p. 365-387. Wiley-Blackwell. Oxford.

Bigi, A., Panzavolta, S. and Roveri, N. 1998. Hydroxyapatite-gelatin films: a structural and mechanical characterization. Biomaterials. 19: 739-744.

Bintsis, T., Litopoulou-Tzanetaki, E. and Robinson, R. K. 2000. Existing and potential applications of ultraviolet light in the food industry–A critical review. J. Sci. Food Agric. 80: 637–645.

Bonilla, J., Atarés, L., Vargas, M. and Chiralt, A. 2012. Edible films and coatings to prevent the detrimental effect of oxygen on food quality: Possibilities and limitations. J. Food Eng. 110: 208-213.

Bougatef, A., Balti, R., Sila, A., Nasri, R., Graiaa, G. and Nasri, M. 2012. Recovery and physicochemical properties of smooth hound (*Mustelus mustelus*) skin gelatin. LWT - Food Sci. Technol. 48: 248-254.

Budavari, S. 1996. Merck Index. 12th ed. Whitehouse Station. Berlin.

Burghagen, M. 1999. Collagen. *In* Food Chemistry. (Belitz, H. D. and Grosch, W., eds.). p. 540-547. Springer-verlag. Berlin.

Bremner, H. A. 1992. Fish flesh structure and the role of collagen-Its postmortem aspects and implications for fish processing. *In* Quality Assurance in the Fish Industry. (Huss, H. H. *et al*., eds.). p. 39-62. Elsevier Amsterdam.

Chanthachum, S. and Beuchat, L. R. 1997. Inhibitory effect of kiam (*cotylelobium lanceotatum* craih). Wood extract on gram-positive food borne pathogens and spoilage micro-organisms. Food Microbiol. 14: 603-608.

Charlton, A. J., Baxter, N. J., Lokman, M. K., Moir, A. J. G., Haslam, E., Davies, A. P. and Williamson, M. P. 2002. Polyphenol/peptide binding and precipitation. J. Agric. Food Chem. 50: 1593-1601.

Cheng, F., Hsu, F., Chang, H., Lin, L. and Sakata, R. 2009. Effect of different acids on the extraction of pepsin-solubilised collagen containing melanin from silky fowl feet. Food Chem. 113: 563–567.

Chiou, B. S., Avena-Bustillos, R. J., Shey, J., Yee, E., Bechtel, P. J., Syed H. Imam, S. H., Glenn, G. M. and Orts, W. J. 2006. Rheological and mechanical properties of cross-linked fish gelatins. Polymer. 47: 6379-6386.

Cho, S. M., Kwak, K. S., Park, D. C., Gu, Y. S., Ji, C. I., Jang, D. H., Lee, Y. B. and Kim, S. B. 2004. Processing optimization and functional properties of gelatin from shark (*Isurus oxyrinchus*) cartilage. Food Hydrocolloid. 18: 573-579.

Cho, S. M., Gu, Y. S. and Kim, S. B. 2005. Extraction optimization and physical properties of gellowfin tuna (*Thunnus albacores*) skin gelatin compared to mammalian gelatins. Food Hydrocolloid. 19: 221-229.

Choi, S. S. and Regenstein, J. M. 2000. Physicochemical and sensory characteristics of fish gelatin. J. Food Sci. 65: 194–199.

Clifford, M. N. 1999. Chlorogenic acids and other cinnamates - nature, occurrence and dietary burden. J. Sci. Food Agric. 79: 362-372.

Cozzolino, A., Pierro, P. D., Mariniello, L., Sorrentino, A., Masi, P. and Porta, R. 2010. Incorporation of whey proteins into cheese curd by using transglutaminase. Biotechnol. Appl. Biochem. 38: 289–295.

Department of Fisheries, Thailand. 2014. Summary of the import-export of fish of Thailand-year. 2013. www.fisheries.go.th/it-database/dbit/images/import.xls.

Dey, G., Sachan, A., Ghosh, S. and Mitra, A. 2003. Detection of major phenolic acids from dried mesocarpic husk of mature coconut by thin layer chromatography. Ind. Crop. Prod. 18: 171-176.

Dickinson, E. and Izgi, E. 1996. Foam stabilization by protein–polysaccharide complexes. Colloids Surf. A: Physicochem. Eng. Aspects. 113: 191–201.

Djabourov, M., Pezron, I. and Leblond, J. 1991. Conformation of gelatin chains in aqueous solutions: 1. A light and small-angle neutron scattering study. Polymer. 32: 3201-320.

Duan, R., Zhang, J., Du, X., Yao, X. and Konno, K. 2009. Properties of collagen from skin, scale and bone of carp (*Cyprinus carpio*). Food Chem. 112: 702-706.

Fan, D., Takawale1, A., Lee1, J. and Kassiri, Z. 2012. Cardiac fibroblasts, fibrosis and extracellular matrix remodeling in heart disease. Fibrogenesis and Tissue Repair. 5: 1-15.

Fiszman, S. M., Lluch, M. A. and Salvador, A. 1999. Effect of addition of gelatin on microstructure of acidic milk gels and yoghurt and on their rheological properties. J. Inter. Dairy. 9: 895-901.

Foegeding, E. A., Lanier, T. C. and Hultin, H. O. 1996. Characteristics of edible muscle tissues. *In* Food Chemistry. (Fennema, O. R., ed.). p. 902–906. Marcel Dekker Inc. New York.

Fujimori, E. 1965. Ultraviolet light-induced change in collagen macromolecules. Biopolymer. 3: 115–169.

Gómez-Guillén, M. C., Turnay, J., Fema´ndez-Diaz, M. D., Ulmo, N., Lizarbe, M. A. and Montero. 2002. Structural and physical properties of gelatin extracted from different marine species: a comparative study. Food Hydrocolloid. 16: 25-34.

Giménez, B., Gómez-Guillén, M. C. and Montero, P. 2005. Storage of dried fish skins on quality characteristics of extracted gelatin. Food Hydrocolloid. 19: 958-963.

Gudmundsson, M. and Hafsteinsson, H. 1997. Gelatin from cod skins as affected by chemical treatments. J. Food Sci. 62: 37-47.

Gurov, A. N. and Nuss, P. V. 1986. Protein–polysaccharide complexes as surfactants. Nahrung-Food. 30: 349–353.

Grossman, S. and Bergman, M. 1992. Process for the production of gelatin from fish skin. United States Patent No. 5,093,474

Hao, S., Li, L., Yang, X., Cen, J., Shi, H., Bo, Q. and He, J., 2009. The characteristics of gelatin extracted from sturgeon (*Acipenser baeri*) skin using various pretreatments. Food Chem. 115: 124–128.

Haslam, E. and Lilley, T. H. 1988. Natural astringency in foodstuffs-A molecular interpretation. Crit. Rev. Food Sci. Nutri. 27: 1-40.

Hansen, F. and Sherief, P. M. 1994. Role and application of fish collagen. J. Seafood Export. 25: 19–24.

Harrington, W. F. and von Hippel, P. H. 1961. Arch. Biochem. Biopys. 92: 100.

Haug, I. J., Draget, K. I. and Smidsrod, O. 2004. Physical and rheological properties of fish gelatin compared to mammalian gelatin. Food Hydrocolloid. 18: 203-213.

Hou, P. Z. and Regenstein, J. M. 2004. Optimization of extraction conditions for Pollock skin gelatin. J. Food Sci. 69: C393-C398.

Huang, Y. R., Shiau, C. Y., Chen, H. H. and Huang, B. C. 2011. Isolation and characterization of acid and pepsin-solubilized collagens from the skin of balloon fish (*Diodon holocanthus*). Food Hydrocolloid. 25: 1507-1513.

Hudson, C. B. 1994. Gelatine-relating structure and chemistry of functionality. *In* Food Hydrocolloids: Structure, Properties, and Functions. (Nishihari, K. and Doi, E., eds.). Plenum. New York.

Jamilah, B. and Harvinder, K. G. 2002. Properties of gelatins from skins of fish-black tilapia (*Oreochromis mossambicus*) and red tilapia (*Oreochromis nilotica*). Food Chem. 77: 81-84.

Jamilah, B., Tan, K., hartina, M. U. and Azizah, A. 2011. Gelatins from three cultured freshwater fish skins obtained by liming process. Food Hydrocolloid. 25: 1256-1260.

Jellouli, K., Balti, R., Bougatef, A., Hmidet, N., Barkia, A. and Moncef Nasri, M. 2011. Chemical composition and characteristics of skin gelatin from grey triggerfish (*Balistes capriscus*). LWT - Food Sci. Technol. 44: 1965-1970.

Johnston-Banks, F. A. 1990. Gelatin. *In* Food Gels. (Harris, P., ed.). p. 233-289. Elsevier Applied Science. London.

Jongjareonrak, A., Benjakul, S., Visessanguan, W., Nagai, T. and Tanaka, M. 2005. Isolation and characterization of acid and pepsin-solubilised collagens from the skin of brownstripe red snapper (*Lutjanus vitta*). Food Chem. 93: 475–484.

Jongjareonrak, A., Benjakul, S., Visessanguan, W. and Tanaka, M. 2006. Skin gelatin from bigeye snapper and brownstripe red snapper: Chemical compositions and effect of microbial transglutaminase on gel properties. Food Hydrocolloid. 20: 1216-1222.

Kaewruang, P., Benjakul, S. and Prodpran, T. 2013. Molecular and functional properties of gelatin from the skin of unicorn leatherjacket as affected by extracting temperatures. Food Chem. 138: 1431–1437.

Kaewruang, P., Benjakul, S., Prodpran, T., Encarnacion, A. B. and Nalinanon, S. 2014. Impact of divalent salts and bovine gelatin on gel properties of phosphorylated gelatin from the skin of unicorn leatherjacket. LWT-Food Sci. Technol. 55: 477-482.

Karim, A. A. and Bhat, R. 2009. Fish gelatin: properties, challenges, and prospects as an alternative to mammalian gelatin. Food Hydrocolloid. 23: 563-576.

Keenan, T. R. 1994. Gelatin. *In* Kirk-Othmer Encyclopedia of Chemical Technology. (Kroschwitz, J., ed.). p. 406-416. Wiley. New York.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W., Kishimura, H. and Shahidi, F. 2010. Isolation and characterisation of collagen from the skin of brownbanded bamboo shark (*Chiloscyllium punctatum*). Food Chem. 119: 1519–1526.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W. and Shahidi, F. 2010. Isolation and characterization of collagen from the cartilages of brownbanded bamboo shark (*Chiloscyllium punctatum*) and blacktip shark (*Carcharhinus limb*atus). LWT - Food Sci. Technol. 43: 792–800.

Klomklao, S., Benjakul, S. and Visessanguan, W. 2004. Comparative studies on proteolytic activity of spleen extracts from three tuna species commonly used in Thailand. J. Food Biochem. 28: 355–372.

Koli, J. M., Basu, S., Nayak, B. B., Surendra B. Patange, S. B., Pagarkar, A.U. and Gudipati, V. 2012. Functional characteristics of gelatin extracted from skin and bone of Tiger-toothed croaker (*Otolithes ruber*) and Pink perch (*Nemipterus japonicus*). Food Biopro. Proc. 90: 555-562.

Kovanen, V., Ahtikoski, A. M., Koskinen, S. O. A., Takala, T. E. S. and Gosselin, L. E. 1998. Collagen synthesis activation after eccentric contraction-induced muscle damage in rats at different ages. Pathophysiol. 5: 126.

Kroll, J., Rawel, H. M. and Rohn, S. 2003. Reactions of plant phenolics with food proteins and enzymes under special consideration of covalent bonds. Food Sci. Technol. Res. 9: 205-218.

Kwak, K. S., Cho, S. M. Ji, C. I., Lee, Y. B. and Kim, S. B. 2009. Changes in functional properties of shark (*Isurus oxyrinchus*) cartilage gelatin produced by different drying methods. Int. J. Food Sci. Technol. 44: 1480-1484.

Labropoulos, K. C., Niesz, D. E., Danforth, S. C. and Kevrekidis, P. G. 2002. Dynamic rheology of agar gels: theory and experiments. Part I. Development of a rheological model. Carbohydr. Polym. 50: 393–406.

Landrault, N., Poucheret, P., Ravel, P., Gasc, F., Cros, G. and Teissedre, P. L. 2001. Antioxidant capacities and phenolics levels of French wines from different varieties and vintages. J. Agr. Food Chem. 49: 3341–3348.

Lanier, T. C. and Kang, I. S. 2000. Plasma as a source of functional food additives. Food System Functionality: Physicochemical and Biological Aspects. Vol II. New York. Technomics.

Lee, C. R., Grodzinsky, A. J. and Spector, M. 2001. The effects of cross-linking of collagen-glycosaminoglycan scaffolds on compressive stiffness, chondrocyte-mediated contraction, proliferation and biosynthesis. Biomaterials. 22: 3145-3154.

Leuenberger, B. H. 1991. Investigation of viscosity and gelation properties of different mammalian and fish gelatins. Food Hydrocolloid. 5: 353–361.

Li, B. Z., Wang, B., Chi, C. F., Zhang, Q. H., Gong, Y. D., Tang, J. J., Luo, H. and G. Ding. 2013. Isolation and characterization of acid soluble collagens and pepsin soluble collagens from the skin and bone of Spanish mackerel (*Scomberomorous niphonius*). Food Hydrocolloid. 31: 103-113.

Liu, H. Y., Li, D. and Guo, S. D. 2008. Extraction and properties of gelatin from channel catfish (*Letalurus punetaus*) skin. 2008. LWT- Food Sci. and Technol. 41: 414-419.

Liu, Z., Oliveira, A. C. M. and Su, Y. C. 2010. Purification and characterization of pepsin-solubilised collagen from skin and connective tissue of giant red sea cucumber (*Parastichopus californicus*). J. Agric. Food Chem. 58: 1270–1274.

Liu, D., Liang, L., Regenstein, J. M. and Zhou, P. 2012. Extraction and characterisation of pepsin-solubilised collagen from fins, scales, skins, bones and swim bladders of bighead carp (*Hypophthalmichthys nobilis*). Food Chem. 133: 1441-1448.

Liu, D., Zhou, P., Li, Y. and Regenstein, J. M. 2014. Comparison of acid-soluble collagens from the skins and scales of four carp species. Food Hydrocolloid. 41: 290-297.

Liu, D. Wei, G., Li, T., Hu, J., Lu, N., Regenstein, J. M. and Zhou, P. 2015. Effects of alkaline pretreatments and acid extraction conditions on the acid-soluble collagen from grass carp (*Ctenopharyngodon idella*) skin. Food Chem. 172: 836–843.

Love, R. M., Yamaguchi, Y., Creach, Y. and Lavety, J. 1976. The connective tissue and collagens of cod during starvation. Comp. Biochem. Physiol. 55: 487-492.

Matmaroh, K., Benjakul, S., Prodpran, T., Encarnacion, A. B. and Kishimura, H. 2011. Characteristics of acid soluble collagen and pepsin soluble collagen from scale of spotted golden goatfish (*Parupeneus heptacanthus*). Food Chem. 129: 1179-1186.

Maqsood, S., Benjakul, S. and Shahidi, F. 2013. Emerging role of phenolic compounds as natural food additives in fish and fish products. Crit. Rev. Food Sci. Nutri. 53: 162-179.

Mariod, A. A. and Adam, H. F. 2013. Review: gelatin, source, extraction and industrial applications. Acta Sci. Pol. Technol. Aliment. 12: 135-147.

Meena, C., Mengi, S. A. and Deshpande, S. G. 1999. Biomedical and industrial applications of collagen. Chem. Sci. 111: 319-329.

Mertens, B. and Knorr, D. 1992. Developments of non-thermal processes for food preservation. Food Technol. 46: 124–133.

McMullen, J., Fasina, O. O., Wood, C. W. and Feng, Y. 2005. Storage and handling properties of pellets from poultry litter. Appl. Eng. Agric. 21: 645-651.

Miller, A. T., Karmas, E. and Lu, M. F. 1983. Age-related changes in the collagen of bovine corium: stuies on extractability, solubility and molecular size distribution. J. Food. Science. 48: 681-686.

Montero, P., Jiménez-Colmenero, F. and Borderias, J. 1991. Effect of pH and the presence of NaCl on some hydration properties of collagenous material from trout (*Salmo irideus* Gibb) muscle and skin. J. Sci. Food Agric. 54: 137-146.

Montero, P., Álvarez, C., Marti, M. A. and Borderías, A. J. 1995. Plaice skin collagen extraction and function properties. J. Food Sci. 60: 1-3.

Montero, P., Gómez-Guillén, M. C. and Borderías, A. J. 1999. Functional characterisation of muscle and skin collagenous material from hake (*Merluccius merluccius*). Food Chem. 65: 55-59.

Montavon, P., Mauron, A. F. and Duruz, E. 2003. Changes in green coffee protein profiles during roasting. J. Agric. Food Chem. 51: 2335- 2343.

Muyonga, J. H., Cole, C. G. B. and Duodu, K. G. 2004. Characterisation of acid soluble collagen from skins of young and adult Nile perch (*Lates niloticus*). Food Chem. 85: 81–89.

Nagarajan, M., Benjakul, S., Prodpran, T., Songtipya, P. and Kishimura, H. 2012. Characteristics and functional properties of gelatin from splendid squid (*Loligo formosana*) skin as affected by extraction temperatures. Food Hydrocolloid. 29: 389-397.

Nalinanon, S., Benjakul, S., Visessanguan, W. and Kishimura, H. 2007. Use of pepsin for collagen extraction from the skin of bigeye snapper (*Priacanthus tayenus*). Food Chem. 104: 593–601.

Nalinanon, S., Benjakul, S., Visessanguan, W. and Kishimura, H. 2008. Improvement of gelatin extraction from bigeye snapper skin using pepsin-aided process in combination with protease inhibitor. Food Hydrocolloid. 22: 615–622.

Nalinanon, S., Benjakul, S. and Kishimura, H. 2010. Collagens from the skin of arabesque greenling (*Pleurogrammus azonus*) solubilized with the aid of acetic acid and pepsin from albacore tuna (*Thunnus alalunga*) stomach. J. Sci. Food Agr. 90: 1492–1500.

Nalinanon, S., Benjakul, S., Kishimura, H. and Osako, K. 2011. Type I collagen from the skin of ornate threadfin bream (*Nemipterus hexodon*): Characteristics and effect of pepsin hydrolysis. Food Chem. 125: 500–507.

Niu, L., Zhou, X., Yuan, C., Bai, Y., Lai, K., Yang, F. and Yiqun Huang, Y. 2013. Characterization of tilapia (*Oreochromis niloticus*) skin gelatin extracted with alkaline and different acid pretreatments. Food Hydrocolloid. 33: 336-341.

NGIL. 2012. Production and sale of gelatinizer for photograph, medical application and such as for food gelatin. Nitta Gelatin Inc. Japan.

Norziah, M. H., Foo, S. L. and Karim, A. A. 2006. Rheological studies on mixtures of agar (*Gracilaria changii*) and *κ*-carrageenan. Food Hydrocolloid. 20: 204-217.

Parr, A. J. and Bolwell, G. P. 2000. Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenol content or profile. J. Sci. Food Agric. 80: 985-1012.

Pelster, B. 2001. The generation of hyperbaric oxygen tensions in fish. News Physiol. Sci. 16: 287–91.

Poppe, J. 1997. Gelatin. *In* Thickening and Gelling Agents for Food. (Imeson, A., ed.). p.144-168. Blackie Academic and Professional. London.

Potter, N. N. and Hotchkiss, J. H. 1998. Food Science. 2nd Ed. Aspen. Gaithersburg.

Prigent, M., Dubois, T., Raposo, G., Derrien, V., Tenza, D. and Rosse, E. 2003. ARF6 controls postendocytic recycling through its downstream exocyst complex effector. J. Cell Biol. 163: 1111-1121.

O'connell, J. E. and Fox, P. F. 2001. Significance and applications of phenolic compounds in the production and quality of milk and dairy products. Int. Dairy J. 11: 103-120.

Ofori, R. A. 1999. Preparation of gelatin from fish skin by an enzyme aided process. Master of science thesis. Macdonald Campus of McGill University, Montreal, Canada.

Ogawa, M., Moody, M. W., Portier, R. J., Bell, J., Schexnayder, M. A. and Losso, J. N. 2003. Biochemical properties of black drum and sheepshead seabream skin collagen. J. Agric. Food Chem. 51: 8088–8092.

Ogawa, M., Portier, R. J., Moody, M. W., Bell, J., Schexnayder, M. A. and Losso, J. N. 2004. Biochemical properties of bone and scale collagens isolated from the subtropical fish black drum (*Pogonia cromis*) and sheepshead seabream (*Archosargus probatocephalus*). Food Chem. 88: 495–501.

Rees, D. A. 1972. Polysaccharide gels-a molecular review. Chemistry and Industry 16: 630–636.

Regenstein, J. M. and Regenstein, C. E. 1991. Introduction to Fish Technology. (Reinhold, V. N., ed.). Van Nostrand Reinhold. New York.

Regenstein, J. M. and Zhou, P. 2007. Collagen and gelatin from marine by-products. *In* Mazimising the Value of Marine By-products. (Shahidi, F., ed.). p. 279–303. Woodhead Publishing Limited. Cambridge.

Richelle, M., Tavazzi, I. and Offord, E. 2001. Comparison of the antioxidant activity of commonly consumed polyphenolic beverages (coffee, cocoa, and tea) prepared per cup serving. J. Agric. Food Chem. 49: 3438-3442.

Robards, K., Prenzler, P. D., Tucker, G., Swatsitang, P. and Glover, W. 1999. Phenolics compounds and their role in oxidative processes in fruits. Food Chem. 66: 401-436.

Sadowska, M., Kołodziejska, I. and Niecikowska, C. 2003. Isolation of collagen from the skins of Baltic cod (*Gadus morhua*). Food Chem. 81: 257–262.

Sarabia, A. I., Gómez-Guillén, M. C. and Montero, P. 2000. The effect of added salts on viscoelastic properties of fish skin gelatin. Food Chem. 70: 71-76.

Seki, N., Nakahara, C., Takeda, H., Maruyama, N. and Nozawa, H. 1998. Dimerization site of carp myosin heavy chains by the endogenous transglutaminase. Fish. Sci. 64: 314-319.

Seguro, K., Kymazawa, Y., Ohtsuka, T., Toiguchi, S. and Motoki, M. 1995. Microbial transglutaminase and ε-(γ-glutamyl) lysine cross-link effects on elastic properties of kamaboko gels. J. Food Sci. 60: 305-311.

Shann, T. J., Shou, Z. L. and Gou, J. T. 1998. Cross-linking of mackerel surimi actomyosin by microbial transglutaminase and ultraviolet irradiation. J. Agric. Food Chem. 46: 5278-5282.

Shrinivas, P., Kasapis, S. and Tongdang, T. 2009. Morphology and mechanical properties of bicontinuous gels of agarose and gelatin and the effect of added lipid phase. Langmuir. 25: 8763-8773.

Shyni, K., Hema, G. S., Ninan, G., Mathew, S., Joshy, C. G. and Lakshmanan, P. T. 2014. Isolation and characterization of gelatin from the skins of skipjack tuna (*Katsuwonus pelamis*), dog shark (*Scoliodon sorrakowah*), and rohu (*Labeo rohita*). Food Hydrocolloid. 39: 68-76.

Singh, T. 1992. Agar and Agar Production. Infofish, Kuala Lumpur.

Singh, P., Benjakul, S., Maqsood, S. and Kishimura, H. 2011. Isolation and characterisation of collagen extracted from the skin of striped catfish (*Pangasianodon hypophthalmus*). Food Chem. 124: 97-105.

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 Chem. 138: 2435-2441.

Sinthusamran, S., Benjakul, S. and Kishimura, H. 2014. Characteristics and gel properties of gelatin from skin of seabass (*Lates calcarifer*) as influenced by extraction conditions. Food Chem. 152: 276–284.

Sinthusamran, S., Benjakul, S. and Kishimura, H. 2015. Molecular characteristics and properties of gelatin from skin of seabass with different sizes. doi:10.1016/j.ijbiomac.2014.11.024.

Skierka, E. and Sadowska, M. 2007. The influence of different acids and pepsin on the extractability of collagen from the skin of Baltic cod (*Gadus morhua*). Food Chem. 105: 1302-1306.

Somboon, N., Karrila, T. T., Kaewmanee, T. and Karrila, S. J. 2014. Properties of gels from mixed agar and fish gelatin. Int. Food Res. J. 21: 485-492.

Stainby, G. 1987. Gelatin gels. *In* Advances in Meat Research. Vol. 4. (Pearson, A. M. *et al*., eds.). p. 209-222. Van Nostrand Reinhold Company Inc. New York.

Strauss, G. and Gibson, S. M. 2004. Plant phenolics as cross-linkers of gelatin gels and gelatin-based coacervates for use as food ingredients. Food Hydrocolloid. 18: 81–89.

Sueli, R. and Gustavo, A. S. P. 2007. Ultrasound extraction of phenolic compounds from coconut (*Cocos nucifere*) shell powder. J. Food Eng. 80: 869–872.

Sung, W. C. and Chen, Z. Y. 2014. UV treatment and γ irradiation processing on improving porcine and fish gelatin and qualities of their premix mousse. Radiat. Phys. Chem. 97: 208–211.

Tabarestani, H. S., Maghsoudlou, Y., Motamedzadegan, A. and Mahoonak, A. R. S. 2010. Optimization of physico-chemical properties of gelatin extracted from fish skin of rainbow trout (*Onchorhynchus mykiss*). Biores. Technol. 101: 6207–6214.

Tamilmozhi, S., Veeruraj, A., and Arumugam, M. 2013. Isolation and characterization of acid and pepsin-solubilized collagen from the skin of sailfish (*Istiophorus platypterus*). Food Res. Int. 54: 1499–1505.

Temdee, W. and Benjakul, S. 2014. Effect of oxidized kiam wood and cashew bark extracts on gel properties of gelatin from cuttlefish skins. Food Biosci. 7: 95–104.

Teresa, M. 2009. A Tradition of Soup: Flavors from China's Pearl River Delta. North Atlantic Books.

Tolstoguzov, V. B. 1991. Functional properties of food proteins and role of protein-polysaccharide interaction. Food Hydrocolloid. 4: 429–468.

Tomihata, K., Burczak, K., Shiraki, K. and Ikada, Y. 1992. Crosslinking and biodegradation of native and denatured collagen. Polymer Preprint. 33: 534–535.

Tsai, G. J., Lin, S. M. and Jiang, S. T. 1996. Transglutaminase from *Streptoverticillium ladakanum* and application to minced fish product. J. Food Sci. 61: 1234-1238.

Turgeon, S. L. and Beaulieu, M. 2001. Improvement and modification of whey protein gel texture using polysaccharides. Food Hydrocolloid. 15: 583–591.

Wang, L., An, X., Xin, Z., Zhao, L. and Hu, Q. 2007. Isolation and characterization of collagen from the skin of deep-sea redfish (*Sebastes mentella*). J. Food Sci. 8: E450-E455.

Wang, L., Liang, Q., Chen, T., Wang, Z., Xu, J. and Ma, H. 2014. Characterization of collagen from the skin of Amur sturgeon (*Acipenser schrenckii*). Food Hydrocollois. 38: 104-109.

Wikipedia. 1999. Swim bladder (Online). Available https://en.wikipedia.org/wiki/Swim_bladder (17 May 2013).

Wong, D. W. S. 1989. Mechanism and Theory in Food Chemistry. Van Nostrand Reinhold. New York.

Woo, J. W., Yu, S. J., Cho, S. M., Lee, Y. B. and Kim, S. B. 2008. Extraction optimization and properties of collagen from yellowfin tuna (*Thunnus albacores*) dorsal skin. Food Hydrocolloid. 22: 879–887.

Yabuta, G., Koizumi, Y., Namiki, K., Hida, M. and Namiki, M. 2001. Structure of green pigment formed by the reaction of caffeic acid esters (or chlorogenic acid) with a primary amino compound. Biosci. Biotechnol. Biochem. 65: 2121-2130.

Yan, M., Li, B., Zhao, X., Ren, G., Zhuang, Y. and Hou, H. 2008. Characterization of acid soluble collagen from the skin of walleye pollock (*Theragra chalcogramma*). Food Chem. 107: 1581–1586.

Yan, M., Li, B., Zhao, X. and Yi, J. 2011. Physicochemical properties of gelatin gels from walleye pollock (*Theragra chalcogramma*) skin cross-linked by gallic acid and rutin. Food Hydrocolloid. 25: 907-914.

Yang, H., Wang, Y., Jiang, M., Oh, J. H., Herring, J. and Zhou, P. 2007. 2-Step optimization of the extraction and subsequent physical properties of Channal catsish (*Ictalurus punctatus*) skin gelatin. J. Food Sci. 72: C188-C195.

Yellowdawn, T. H. 2011. The sun, human and food: A self-treatment and practice with natural food (2nd ed.). AuthorHouse. Bloomington.

Yi, J. B., Kim, Y. T., Bae, H. J., Whiteside, W. S. and Park, H. J. 2006. Influence of transglutaminase-induced cross-linking on properties of fish gelatin films. J. Food Sci. 71: E376-E383.

Yoshimura, K., Terashima, M., Hozan, D., Ebato, T., Nomura, Y. and Ishii, Y. 2000. Physical properties of shark gelatin compared with pig gelatin. J. Agric. Food Chem. 48: 2023–2027.

Zayas, J. F. 1997. Functionality of Proteins in Food. Springer. Berlin.

Zeng, S., Zhang, C., Lin, H., Yang, P., Hong, P. and Jiang, Z. 2009. Isolation and characterisation of acid-solubilised collagen from the skin of Nile tilapia (*Oreochromis niloticus*). Food Chem. 116: 879-883.

Zeng, S., Yin, J., Yang, S., Zhang, C., Yang, P. and Wu, W. 2012. Structure and characteristics of acid and pepsin-solubilized collagens from the skin of cobia (*Rachycentron canadum*). Food Chem. 135: 1975-1984.

Zhang, Y., Liu, W., Li, G., Shi, B., Miao, Y. and Wu, X. 2007. Isolation and partial characterization of pepsin-soluble collagen from the skin of grass carp (*Ctenopharyngodon idella*). Food Chem. 103: 906–912.

Zhou, P. and Regenstein, J. M. 2005. Effects of alkaline and acid pretreatments on Alaska pollock skin gelatin extraction. J. Food Sci. 70: 392–396.

1.3 Objectives

1.3.1 To extract and characterize collagen from swim bladder of yellowfin tuna.

1.3.2 To characterize gelatin from yellowfin tuna swim bladder prepared with different pretreatment and extraction conditions.

1.3.3 To investigate the effect of phenolic on the gel properties of gelatin extracted from swim bladder of yellowfin tuna.

1.3.4 To study the impact of agarose on the gel properties of gelatin extracted from swim bladder of yellowfin tuna.

CHAPTER 2

Characteristics of collagens from swim bladder of yellowfin tuna (*Thunnus albacares*)

2.1 Abstract

Acid soluble collagen (ASC) and pepsin soluble collagen (PSC) were extracted from swim bladder of yellowfin tuna (*Thunnus albacares*) with yields of 1.07 % and 12.10 %, respectively. Based on electrophoretic patterns, both ASC and PSC consisted of two α-chains (α_1 and α_2) and were characterised to be type I collagen. ASC had higher β-chain than PSC. Imino acid contents of ASC and PSC were 128 and 169 residues/1000 residues, respectively. Fourier transform infrared (FTIR) spectra of both ASC and PSC were almost similar and revealed the presence of triple helix. Both ASC and PSC had the highest solubility at acidic pH. From zeta potential analysis, net charge of zero was found at pH 6.05 and 5.93 for ASC and PSC, respectively. T_{max} of ASC and PSC were 32.97 and 33.92 °C, respectively. Swim bladder of yellowfin tuna could therefore serve as the alternative source of collagen for future applications.

2.2 Introduction

Thailand is the world's largest producer and exporter of canned tuna. The most commercially important tuna species for tuna industry include skipjack tuna, followed by yellowfin tuna and tongol tuna, accounting for over 80 % of Thai frozen tuna imports (Nalinanon *et al*., 2010). During January and June, 2013, the amount of imported tuna to Thailand was 65,954.14 tons (Department of Fisheries, 2014). A large amount of raw tuna go through the canning process, by which about two-thirds of whole fish is utilized (Klomklao *et al*., 2005). Thus, high amount of processing byproducts such as viscera, head, bone and swim bladder are discarded during canned tuna manufacturing. Swim bladder, also called air bladder, is an internal gas-filled organ found in most fish. Swim bladder can be the potential sources for the production of collagen. Sinthusamran *et al*. (2013) reported that swim bladder

of seabass was the excellent source of collagen. ASC was isolated from swim bladder of seabass with a yield of 85.3 % (dry weight basis).

Collagen has been typically isolated from the skins of land-based animals, such as cow and pig, and widely used in food, cosmetic, biomedical and pharmaceutical industries (Ogawa *et al.,* 2004). However, fish collagen has gained increasing interest to avoid bovine sponge encephalopathy (BSE), transmissible spongiform encephalopathy (TSE), foot-and-mouth disease (FMD) and avian influenza associated with collagen and collagen-derived products from land-based animals (Jongjareonrak *et al*., 2005). Additionally, porcine collagen cannot be consumed by Muslim and Judaism (Sadowska *et al*., 2003). Fish collagen has been extracted mainly from fish skin such as bigeye snapper (Nalinanon *et al*., 2007), striped catfish (Singh *et al*., 2011), brownbanded bamboo shark (Kittiphattanabawon *et al*., 2010), unicorn leatherjacket (Ahmad and Benjakul, 2010) and seabass (Sinthusamran *et al*., 2013). Collagen from skin of various species had the different properties, particularly denaturation temperature, which has been known to be influenced by habitat and environment the fish live (Singh *et al*., 2011).

During butchering of raw tuna, swim bladder was discarded and can be used the raw material for collagen extraction. Although skipjack tuna are the most abundant, they do not have swim bladder. On the other hand, swim bladder can be found in yellowfin tuna. However, no information on collagen from swim bladder from this species has been reported. Thus, the aim of this study was to extract and characterise ASC and PSC from swim bladder of yellowfin tuna.

2.3 Materials and methods

2.3.1 Chemicals

All chemicals were of analytical grade. Bovine haemoglobin, β-mercaptoethanol (β-ME), *L*-tyrosine, and Type I collagen from calf skin were purchased from Sigma Chemical Co. (St. Louis, MO). High molecular weight markers were obtained from GE Healthcare UK (Aylesbury, UK). Sodium dodecyl sulphate (SDS), Coomassie Blue R-250, and *N,N,N',N'*-tetramethylethylenediamine

(TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). Trichloroacetic acid, Folin–Ciocalteu's phenol reagent, acetic acid and tris (hydroxylmethyl) aminomethane were obtained from Merck (Darmstadt, Germany).

2.3.2 Collection and preparation of swim bladder and stomach

Swim bladders and stomach of yellowfin tuna (*Thunnus albacares*) were obtained from Songkla Canning Public Co., Ltd., Songkhla, Thailand. Swim bladders were 8-12 cm in length and stomach were 10-12 g in weight. Swim bladders and stomach were placed in polyethylene bags, inserted in ice using a sample/ice ratio of 1:2 (w/w) and transported to Prince of Songkla University, Hat Yai. Upon arrival, swim bladders and stomach were washed with distilled water, cut into pieces with the length of approximately 2 cm. The prepared samples were then placed in polyethylene bag and frozen at -20 °C. The samples were stored at -20 °C before use. The storage time was not longer than 3 months.

2.3.3 Proximate analysis

Swim bladders were subjected to proximate analysis. Moisture, protein, ash and fat contents were determined following the methods of AOAC (2000) with the analytical Nos. of 950.46, 928.08, 920.153 and 960.39, respectively. Hydroxyproline content was measured according to the method of Bergman and Loxley (1963) as described by Nagarajan *et al*. (2012).

2.3.4 Preparation of stomach extract

Frozen stomach was thawed using a running water (26–28 °C) until the core temperature reached -2 to 0 °C. Samples were finely ground in liquid nitrogen using a National Model MX-T2GN blender (Taipei, Taiwan). The powder was referred to as "stomach powder". To prepare the crude extract, stomach powder was suspended in 20 mM sodium phosphate buffer (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) at a ratio of 1:5 (w/v) and stirred continuously at 4 °C for 3 h. The suspension was centrifuged for 20 min at 4 °C at 20,000 g (Hitachi, CR 22 G III,Tokyo, Japan) to remove the tissue debris. The supernatant was collected and referred to as ''crude stomach extract".

2.3.5 Assay of proteolytic activity

Prior to assay, the crude stomach extract was adjusted to pH 2 with 1 M HCl for pepsin activation and the mixture was allowed to stand at 4 °C for 10 min, as described by Nalinanon *et al*. (2007).

Proteolytic activity of crude stomach extract was determined using haemoglobin as a substrate as per the method of Nalinanon *et al*. (2007) with a slight modification. To initiate the reaction, 200 µl of the extract were added into the assay mixture containing 200 µl of 2 % haemoglobin, 200 µl of distilled water and 625 µl of McIlvaine's buffer (0.2 M Na-phosphate and 0.1 M Na citrate, pH 2.0) pre-incubated at 50 °C. Appropriate dilution of the extract was made to ensure that the amount of enzyme was not excessive for the available substrate in the assay system. The reaction was conducted at 50 °C for 15 min. To terminate the enzymatic reaction, 200 µl of 50 % (w/v) trichloroacetic acid (TCA) were added. Unhydrolysed protein substrate was allowed to precipitate for 15 min at 4 °C, followed by centrifuging at 8,500 g for 10 min at room temperature (26–28 °C) using a MIKRO 20 centrifuge (Hettich Zentrifugen Tuttlingen, Germany). The oligopeptide content in the supernatant was measured by the Lowry method (Lowry *et al*., 1951) using tyrosine as a standard. One unit of activity was defined as the amount releasing 1 µmol of tyrosine per min under the specified condition. A blank was performed in the same manner, except that the acidified stomach extract was added into the reaction mixture after the addition of 50 % TCA.

2.3.6 Extraction of acid soluble collagen (ASC)

Firstly, non-collagenous proteins were removed from the thawed swim bladder by mixing the sample with 0.15 M NaOH using a sample to alkaline solution ratio of 1:10 (w/v) at 4 °C. The mixture was continuously stirred for 2 h. The alkaline solution was changed every 1 h. Thereafter, the residue was washed with cold distilled water until a neutral or weak basic pH of wash water was obtained. Washed sample was then defatted with 10 % butyl alcohol with a sample/solvent ratio of 1:10 (w/v) for 12 h and the solvent was changed every 3 h. Defatted sample was washed with 10 volumes of cold distilled water for three times.

To extract ASC, the pretreated sample was soaked in 0.5 M acetic acid with a sample to solvent ratio of 1:10 (w/v) for 48 h at 4 °C with continuous stirring using an overhead stirrer model W20.n (IKA®-Werke GmbH & CO.KG, Stanfen, Germany). The mixture was 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 and dialysed against 10 volumes of 0.1 M acetic acid for 12 h. Thereafter, it was dialysed against 10 volumes of distilled water. Dialysis water was changed for five times. The resulting dialysate was lyophilized using a SCANVAC CoolSafe™ freeze-dryer (Cool-Safe 55, ScanLaf A/S, Lynge, Denmark) and referred to as “acid soluble collagen, ASC”.

2.3.7 Extraction of pepsin soluble collagen (PSC)

Undissolved matter from acid soluble collagen extraction was further extracted in 0.5 M acetic acid containing crude stomach extract (20 units/g swim bladder) using a solid/solution ratio of 1:10 (w/v) for 48 h at 4 °C. After extraction, the supernatant was obtained in the same manner for ASC preparation. The precipitation, dialysis as well as lyophilisation were carried out in the same manner with ASC. The collagen obtained was referred to as “pepsin soluble collagen, PSC”.

2.3.8 Analyses

Both ASC and PSC were subjected to the following analyses.

2.3.8.1 Yield and recovery

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

$$\text{Yield (\%)} = \frac{\text{Weight of lyophilized collagen (g)} \times 100}{\text{Weight of initial dry swim bladder (g)}}$$

$$\text{Recovery (\%)} = \frac{\text{Total hydroxyproline in collagen (mg)}}{\text{Total hydroxyproline in initial dry swim bladder (mg)}} \times 100$$

2.3.8.2 Hydroxyproline content

Hydroxyproline content was analysed according to the method of Bergman and Loxley (1963). Hydroxyproline content was calculated and expressed as mg/g sample.

2.3.8.3 Amino acid composition

ASC and PSC were hydrolysed under the reduced pressure in 4.0 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 0.4 ml was applied to an amino acid analyser (MLC-703; Atto Co., Tokyo, Japan). The content was expressed as residues/1000 residues.

2.3.8.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS–PAGE was performed by the method of Laemmli (1970). 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 to remove undissolved debris. Solubilised samples was 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, the 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 a mixture of 50 % (v/v) methanol and 7.5 % (v/v) acetic acid for 30 min and destained again with a mixture of 5 % (v/v) methanol and 7.5 % (v/v) acetic acid for 1 h. High-molecular-weight protein markers were used to estimate the molecular weight of proteins. Type collagen I from calf skin was used as standard. 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).

2.3.8.5 Fourier transform infrared (FTIR) spectra analysis

ASC and PSC were subjected to FTIR analysis using a horizontal ATR Trough plate crystal cell (45° ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI, USA) equipped with a Bruker Model Equinox 55 FTIR spectrometer (Bruker Co., Ettlingen, Germany) at room temperature. For spectra analysis, lyophilized samples were placed on the crystal cell and the cell was clamped into the mount of the FTIR spectrometer. The spectra in the range of 400-4000 cm^{-1} was ratioed and automatic signals gained were collected in 32 scans at a resolution of 4 cm^{-1} against a background spectrum recorded from the clean empty cell at 25 °C.

2.3.8.6 Differential scanning calorimetry (DSC)

ASC and PSC were rehydrated by adding the 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 calorimetre 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 25–50 °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. The total denaturation enthalpy (ΔH) was estimated by measuring the area of the DSC thermogram.

2.3.8.7 Solubility

Solubility of ASC and PSC was determined by the method of Jongjareonrak *et al*. (2005). The samples were dissolved in 0.5 M acetic acid to obtain a final concentration of 3 mg/ml and the mixture was stirred at 4 °C until collagens were completely solubilised.

ASC and PSC solutions (8 ml) were transferred to a 50 ml centrifuge tube (NUNC, Roskilde, Denmark) and the pH was adjusted with either 6 N NaOH or 6 N HCl to obtain the final pHs ranging from 1 to 10. The volume of solutions was made up to 10 ml by distilled water, previously adjusted to the same pH as the collagen solution. The solutions were centrifuged at 10,000 g at 4 °C for 30 min. Protein content in the supernatant was determined by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard. Relative solubility was calculated in comparison with that obtained at pH rendering the highest solubility (100%).

2.3.8.8 Zeta Potential analysis

ASC and PSC were dispersed in distilled water at a concentration of 0.5 mg/ml. The mixture was stirred at room temperature for 6 h. The zeta potential of each sample (20 ml) was measured using a zeta potential analyser (ZetaPALS, Brookhaven Instruments Co., Holtsville, NY, USA). Zeta Potential of samples adjusted to different pHs (1-10) 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 zeta potential of zero.

2.3.9 Statistical analyses

All experiments were performed in triplicate and a completely randomised design (CRD) was used. Data were presented as means ± standard deviation and a probability value of < 0.05 was considered significant. Analysis of variance (ANOVA) was performed and mean comparisons were done by Duncan's multiple range tests. Analysis was performed using SPSS 11.0 for Windows (SPSS Inc., Chicago, IL, USA).

2.4 Results and discussion

2.4.1 Proximate composition of swim bladder

Swim bladder of yellowfin tuna constituted around 3.5-5.5 % of body weight. It contained 83.33 % moisture, 12.09 % protein, 1.44 % fat and 0.29 % ash. Its hydroxyproline content was 1.46 mg/g sample, indicating the presence of collagen

in the swim bladder. Swim bladder of bighead carp (*Hypophthalmichthys nobilis*) had 75.2 % moisture content (Liu *et al*., 2012).

2.4.2 Yield and recovery of ASC and PSC

When ASC and PSC were extracted from the swim bladder of yellowfin tuna, the yields of 1.07 % and 12.10 % (on a dry weight basis) were obtained, respectively. The results indicated that collagen was not effectively extracted using 0.5 M acetic acid, as shown by the low yield of ASC. This result was in agreement with Singh *et al*. (2011) who reported the incomplete solubilisation of striped catfish skin collagen in 0.5 M acetic acid. Collagens in swim bladder were most likely cross-linked by covalent bonds through the condensation of aldehyde groups at the telopeptide region as well as the inter-molecular cross-linking, leading to a decrease in solubility of collagen under acidic condition (Foegeding *et al*., 1996). With further pepsin digestion, the cross-linked molecules at the telopeptide region were cleaved, resulting in further extraction with increased yield. Pepsin was able to cleave specifically at the telopeptide region of collagen (Nalinanon *et al*., 2007). The combined yield of ASC and PSC was 13.17 %. The collagen recovery of ASC and PSC were 61.08 %, suggesting that collagen around 60 % was extracted. Thus pepsin could be used as an aid for increasing the extraction yield of collagen from swim bladder of yellowfin tuna. The yields of ASC and PSC from balloon fish (*Diodon holocanthus*) skin were 4 % and 19.5 % (dry weight basis), respectively (Huang *et al*., 2011). Sinthusamran *et al*. (2013) reported that ASC of swim bladder from seabass was extracted with yield of 85.3 % (dry weight basis). The differences in extraction yield and collagen recovery between different species might be governed by varying cross-linking of collagen fibrils in raw material. It was suggested that swim bladder of yellowfin tuna had a high proportion of cross-links. This led to the lower extraction or solubilisation of collagen, despite of some cleavage mediated by pepsin.

2.4.3 Amino acid composition

The amino acid composition of both ASC and PSC from swim bladder of yellowfin tuna, expressed as residues per 1000 total residues, is shown in Table 5. ASC and PSC were rich in glycine (225 and 302 residues/1000 residues), followed by

alanine (102 and 118 residues/1000 residues). In general, glycine occurs uniformly at every third residue throughout most of collagen molecules, except for the first 14 amino acids from the N-terminus and the first 10 from the C-terminus (Foegeding *et al*., 1996). Glutamine and glutamic acid were also found at high level. No cysteine was found in both ASC and PSC. Relatively high contents of alanine and glutamic acid were observed and no cysteine was detected in other marine collagens (Jongjareonrak *et al*., 2005; Kittiphattanabawon *et al*., 2005). Glycine in ASC was lower than that found in PSC. This was in agreement with the lower hydroxyproline and proline found in ASC. The result suggested the contamination of proteins during extraction into collagens. This coincided with the higher leucine and lysine in ASC. Lysine generally constitutes at a low level in collagen (Jongjareonrak *et al*., 2005).

The imino acid (proline and hydroxyproline) contents of ASC and PSC from swim bladder of seabass were 128 and 169 residues/1000 residues, respectively. Imino acids are important for the structural integrity of collagen. Sinthusamran *et al*. (2013) reported that imino acid content of ASC from seabass swim bladder was 194 residues/1000 residues. The imino acid contents of ASC and PSC from the balloon fish skin were 179 and 174 residues/1000 residues, respectively (Huang*et et al*., 2011). Fish collagen generally has the lower imino acid content than mammalian counterpart (Foegeding *et al*., 1996). Imino acids contribute to the stability of the helix structure of collagen (Ikoma *et al*., 2003). The pyrrolidine rings of proline and hydroxyproline impose restrictions on the conformation of polypeptide chain and help to strengthen the triple helix (Wong, 1989). Zones of collagen molecules rich in hydroxyproline and proline are involved in the formation of junction stabilised by hydrogen bonding (Johnston-Banks, 1990). ASC showed the lower imino acid content than PSC, plausibly reflecting the varying strength of helix and the different content of cross-links.

Table 5. Amino acid compositions of ASC and PSC from swim bladder of yellowfin tuna (residues/1000 residue).

Amino acids	**ASC**	**PSC**
Alanine	102	118
Arginine	56	54
Aspartic acid/asparagine	69	50
Cysteine	0	0
Glutamic acid/glutamine	97	80
Glycine	225	302
Histidine	12	9
Isoleucine	25	15
Leucine	47	29
Lysine	44	27
Hydroxylysine	7	10
Methionine	18	15
Phenylalanine	22	17
Hydroxyproline	48	74
Proline	80	95
Serine	50	42
Threonine	42	32
Tryptophan	2	1
Tyrosine	15	6
Valine	38	25
Total	1000	1000
Imino acids	128	169

2.4.4 Protein patterns

Protein patterns of ASC and PSC from swim bladder of yellowfin tuna determined under non-reducing and reducing conditions are illustrated in Fig. 7. No differences in protein patterns between both ASC and PSC determined under both

conditions were observed. Thus, both ASC and PSC contained no disulphide bond. The result was in accordance with the absence of cysteine in ASC and PSC (Table 5). No disulphide bond was found in collagens from swim bladder of seabass (Sinthusamran *et al*., 2013), skin and bone of Spanish mackerel (*Scomberomorous niphonius*) (Li *et al*., 2013), skin of cobia (*Rachycentron canadum*) (Zeng *et al*., 2012), skin of balloon fish (*Diodon holocanthus*) (Huang *et al*., 2011), skin of brownbanded bamboo shark (*Chiloscyllium punctatum*) (Kittiphattanabawon *et al*., 2010) and skin of unicorn leatherjacket (*Aluterus monocerous*) (Ahmad and Benjakul, 2010).

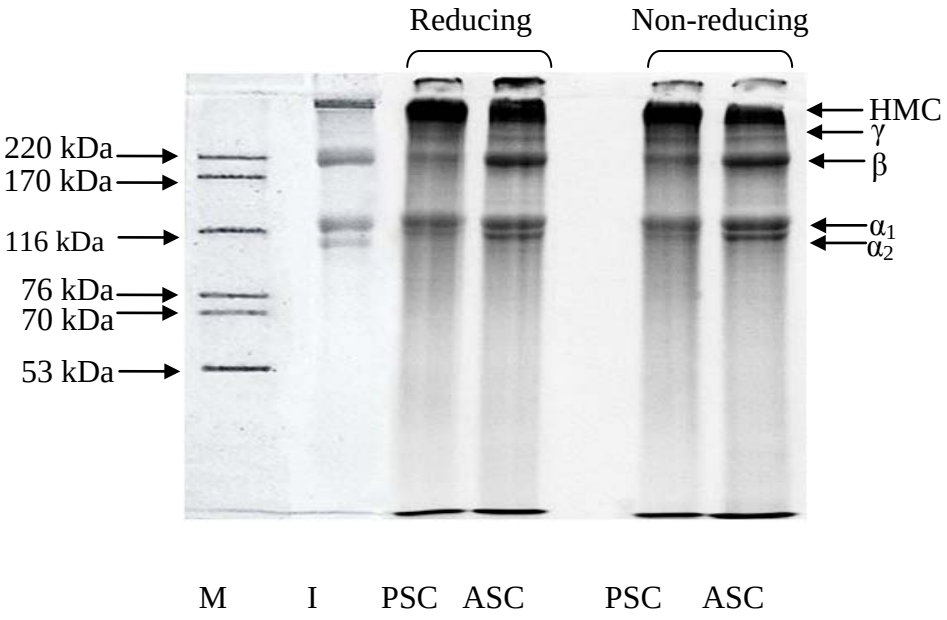

Figure 7. SDS-PAGE patterns of ASC and PSC from swim bladder of yellowfin tuna. I: type I collagen from calf skin. HMC: High molecular weight cross-links.

Both ASC and PSC contained α_1-chain and α_2-chain at a ratio of approximately 2:1, suggesting that they belonged to type I collagen. It was noted that ASC contained the higher band intensity of β-chains, compared with PSC. Both ASC and PSC also contained γ- chain to some extent. However, PSC consisted of cross-linked component to a higher extent than ASC. The result suggested that the large molecular weight intra- and inter-molecular cross-links of collagens were extracted with the aid of pepsin. This was coincidental with the increased extraction yield. Due

to the high proportion of cross-linked constituent, α-chains appeared with the lower band intensity. Similar electrophoretic protein patterns were found in ASC and PSC from the skin of large fin long barbel catfish (*Mystus macropterus*) (Zhang *et al*., 2009). Type I collagen was found in the skins of hake (Montero *et al*., 1990), trout (Montero *et al*., 1990), Nile perch (Muyonga *et al*., 2004) and bigeye snapper (Kittiphattanabawon *et al*., 2005). Collagen from swim bladder of seabass was also reported to be type I (Sinthusamran *et al*., 2013).

2.4.5 Fourier transform infrared (FTIR) spectra

FTIR spectra of ASC and PSC exhibited the characteristic peaks of Amide I, II, III as well as amide A and B (Fig. 8). The major peaks in the spectra of both ASC and PSC from swim bladder of yellowfin tuna were similar to those of collagen from others fish species (Li *et al*., 2013; Singh *et al*., 2011; Kittiphattanabawon *et al*., 2010; Wang *et al*., 2007). Similar FTIR spectra were observed between ASC and PSC. The absorption characteristics of Amide A, commonly associated with N-H stretching vibration, occurs in the wave number range of 3400–3440 cm^{-1} (Sai and Babu, 2001). When the NH group of a peptide is involved in a H-bond, the position is shifted to lower frequency (Doyle *et al*., 1975). The absorption peaks of ASC and PSC were found at 3275 and 3303 cm^{-1}, respectively. Hydrolysis of telopeptide region by pepsin in the stomach extract might cause some destruction of H-bonds with coincidental release of free amino groups, as indicated by the higher wavenumber of PSC. Additionally, the amplitude of PSC peak was slightly higher than that of ASC. Amide B bands of both ASC and PSC were observed at wavenumbers of 2919 and 2920 cm^{-1}, associated with the asymmetrical stretch of CH_2 (Muyonga *et al*., 2004). Slightly higher amplitude of amide B peak was found in PSC. This was related with the higher wavenumber in amide A. The sharp amide I band of ASC and PSC was observed at 1643 and 1648 cm^{-1}, respectively. This band is associated with C=O stretching vibration or H-bond coupled with COO^- (Payne and Veis, 1988). The amide I peak underwent a decrease in amplitude, followed by a broadening accompanied by the appearance of additional shoulders when collagen was heated at higher temperature (Bryan *et al*., 2007). Due to the similarity in the amplitude, both collagens were most likely not denatured during the

extraction. This was reconfirmed by the ratio of approximately 1 between amide III and 1454 cm^{-1} band of both collagens. The ratios of ASC and PSC were 0.99 and 1.01 respectively. Ratio of approximately 1 revealed the triple-helical structure of collagen (Plepis *et al.*, 1996). The amide II of both collagens appeared at 1544–1549 cm^{-1}, resulting from N–H bending vibration coupled with CN stretching vibration (Krimm and Bandekar, 1986). Thus, both ASC and PSC generally showed a similar secondary structure.

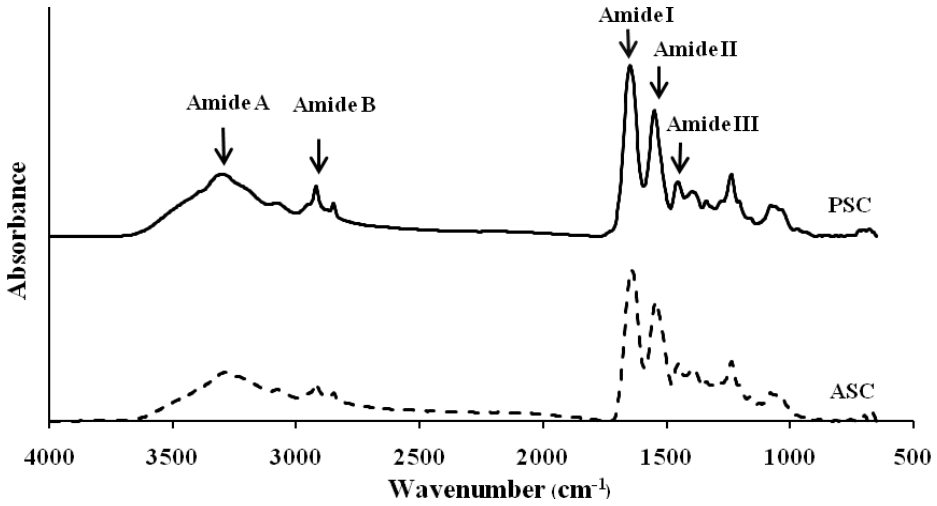

Figure 8. Fourier transform infrared spectra of ASC and PSC from swim bladder of yellowfin tuna.

2.4.6 Thermal transition

The maximum transition temperature (T_{max}) and enthalpy (ΔH) of ASC and PSC from swim bladder in deionised water were measured to indicate their thermal stability. T_{max} values of ASC and PSC were 32.97 ± 0.629 and 33.92 ± 0.216 °C, respectively. ΔH of 1.786 ± 0.034 and 0.354 ± 0.056 J/g ($P < 0.05$) were found for ASC and PSC, respectively. T_{max} of the latter was slightly higher than that of the former ($P < 0.05$). However, the former had the higher ΔH ($P < 0.05$). Thermal stability of collagen was governed by the pyrrolidine rings of proline and hydroxyproline and partially by hydrogen bonding through the hydroxyl group of hydroxyproline (Benjakul *et al.*, 2010). Slightly higher T_{max} was found for PSC,

compared for ASC. This was in accordance with the higher cross-links of PSC (Figure 7). The presence of imino acids, particularly hydroxyproline in ASC and PSC (128 and 169 residues/ 1000 residues) might contribute to stabilisation of triple helix structure through hydrogen bonding in coil-coiled α-chains (Bae *et al*., 2008). T_{max} and ΔH of PSC from swim bladder of bighead carp were observed at 37.3 °C and 1.39 J/g, respectively (Liu *et al*., 2012). The differences in T_{max} amongst collagens from different species were correlated with the different imino acid contents, body temperature and environmental temperature (Kittiphattanabawon *et al*., 2005; Nagai *et al*., 2008).

2.4.7 Zeta Potential

The zeta potential of ASC and PSC from swim bladder of yellowfin tuna at different pHs is shown in Fig. 9. At pH 2–5, both collagens were positively charged, whereas they had the negative charge in pH range of 7–11. Net charge of zero was found at pH value of 6.05 ± 0.781 and 5.93 ± 0.199 for ASC and PSC, respectively.

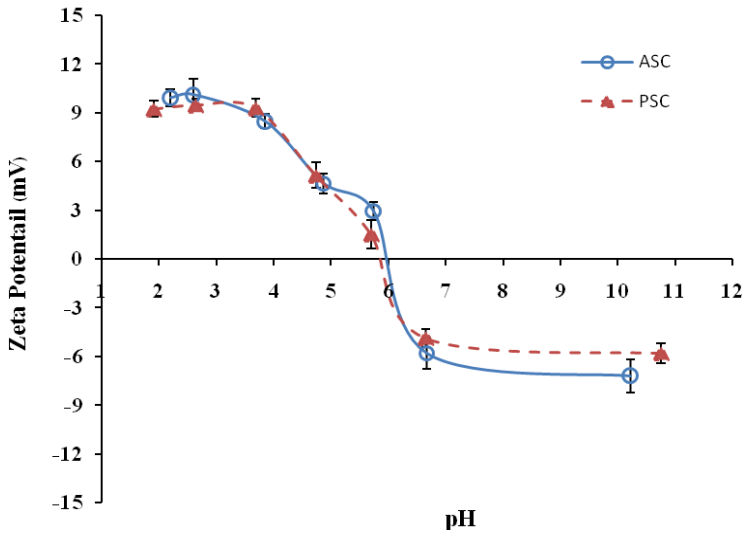

Figure 9. Zeta potential of ASC and PSC from swim bladder of yellowfin tuna at different pHs. Bars represent the standard deviation (n = 3).

The both collagen was not significantly different ($P > 0.05$). Those pHs were assumed to be their isoelectric points (pI). The pIs of ASC and PSC were found at acidic pH. This might be attributed to high content of acidic amino acids such as glutamic acid and aspartic acid. Pepsin might cleave the telopeptide region, leading to difference in amino acid composition in α-chains. This resulted in different charges of amino acids in α-chain or β-chain, regulated by protonation and deprotonation at pH tested (Benjakul *et al*., 2010). Collagens from skin of different fish showed varying pIs, e.g. 4.72 for striped catfish collagen (Singh *et al*., 2011), 6.40 for ornate threadfin bream collagen (Nalinanon *et al*., 2011) and 6.21 for bamboo shark collagen (Kittiphattanabawon *et al*., 2010). Additionally, the slight differences in pI between collagens from various fish species might be caused by the slight difference in their amino acid compositions and distribution of amino acid residues, particularly on the surface domains.

2.4.8 Solubility

Solubility of ASC and PSC from swim bladder of yellowfin tuna as affected by pHs is shown in Fig. 10. ASC and PSC were solubilised in the pH range of 1–6 with the highest solubility at pH 4 and 3, respectively. The decrease in solubility was observed in the pH range of 5–7, falling in the pI range of both collagens as determined by zeta potential analysis. Loss in solubility at a particular pH could be attributed to hydrophobic interaction amongst collagen molecules, and the total net charge of protein molecules becomes zero, especially at pI (Jongjareonrak *et al*., 2005). Slight increase in solubility was noticeable at pH 8 and 9. This was probably due to the repulsive effect of collagen molecules at pH above pI. The differences in the pH determining the solubility of collagens were caused by the differences in the molecular properties and conformations of collagens (Kittiphattanabawon *et al*., 2005). PSC and ASC had the different pH optimal for solubility. The removal of telopeptide regions might affect the protonation or deprotonation of charged amino and carboxyl groups, respectively. This could influence the repulsion of molecules associated with the different solubility.

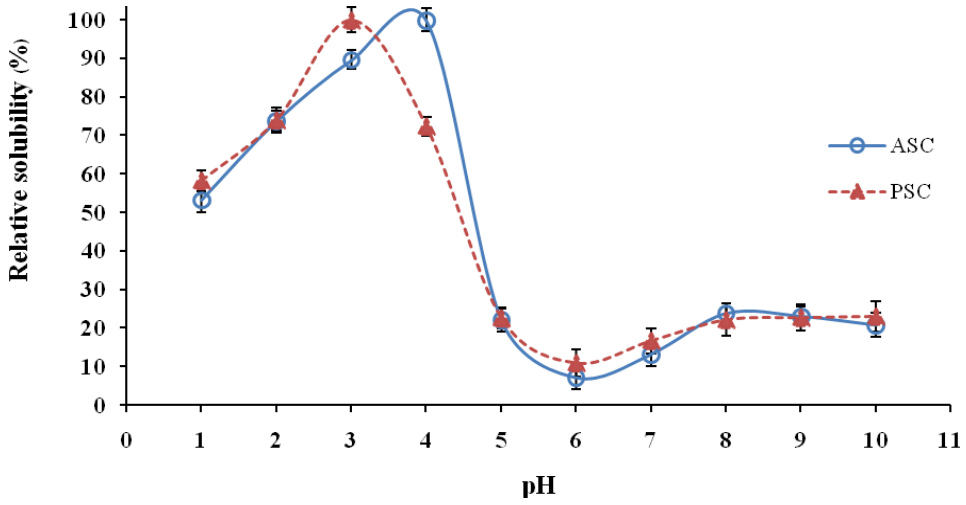

Figure 10. Relative solubility of ASC and PSC from swim bladder of yellowfin tuna at different pHs. Bars represent the standard deviation (n = 3).

2.5 Conclusions

Collagen type I could be extracted from swim bladder of yellowfin tuna using acetic acid (ASC) and with the aid of pepsin (PSC) with yields of 1.07 % and 12.10 %, respectively. Telopeptides of ASC were cleaved by pepsin, resulting in the increased yield of PSC. ASC and PSC were different in β-chain and cross-linked components. Thus, collagen from swim bladder of yellowfin tuna could serve as an alternative source of collagen for further application in food, neutraceutical and cosmetic industries. As a whole, the better use of swim bladder can be achieved.

2.6 References

Ahmad, M. and Benjakul, S. 2010. Extraction and characterisation of pepsin solubilised collagen from the skin of unicorn leatherjacket (*Aluterus monocerous*). Food Chem. 120: 817–824.

AOAC. 2000. Official methods of analysis. Arlington: Association of Official Analytical Chemists Inc.

Bae, I., Osatomi, K., Yoshida, A., Osako, K., Yamaguchi, A. and Hara, K. 2008. Biochemical properties of acid-soluble collagens extracted from the skins of underutilised fishes. Food Chem. 108: 49–54.

Bergman, I. and Loxley, R. 1963. Two improved and simplified methods for the spectrophotometric determination of hydroxyproline. Anal. Chem. 35: 1961–1965.

Bryan, M. A., Brauner, J. W., Anderle, G., Flach, C. R., Brodsky, B. and Mendelsohn, R. 2007. FTIR studies of collagen model peptides: Complementary experimental and simulation approaches to conformation and unfolding. J. Am. Chem. Soc. 129: 7877–7884.

Benjakul, S., Thiansilakul, Y., Visessanguan, W., Roytrakul, S., Kishimura, H., Prodpran, T. and Meesane, J. 2010. Extraction and characterisation of pepsin solubilised collagens from the skin of bigeye snapper (*Priacanthus tayenus* and *Priacanthus macracanthus*). J. Sci. Food Agric. 90: 132–138.

Department of Fisheries, Thailand. 2014. Summary of the import-export of fish of Thailand-year. 2013. www.fisheries.go.th/it-database/db-it/images/ import.xls.

Doyle, B. B., Blout, E. R. and Bendit, E. G. 1975. Infrared spectroscopy of collagen and collagen like polypeptides. Biopolymers. 14: 937–957.

Foegeding, E. A., Lanier, T. C. and Hultin, H. O. 1996. Collagen. *In* Food Chemistry. (Fennema, O. R., ed.). p. 902-906. Marcel Dekker. New York.

Jongjareonrak, A., Benjakul, S., Visessanguan, W., Nagai, T. and Tanaka, M. 2005. Isolation and characterisation of acid and pepsin-solubilised collagens from the skin of brownstripe red snapper (*Lutjanus vitta*). Food Chem. 93: 475–484.

Johnston-Banks, F. A. 1990. Gelatin. *In* Food Gels. (Harris, P., ed.). p. 233-285. Elsevier Applied Science. London.

Huang, Y. R., Shiau, C. Y., Chen, H. H. and Huang, B. C. 2011. Isolation and characterization of acid and pepsin-solubilized collagens from the skin of balloon fish (*Diodon holocanthus*). Food Hydrocolloid. 25: 1507-1513.

Ikoma, T., Kobayashi, H., Tanaka, J., Walsh, D. and Mann, S. 2003. Physical properties of type I collagen extracted from fish scales of *Pagrus major* and *Oreochromis niloticas*. Int. J. Biol. Macromol. 32: 199-204.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W. and Shahidi, F. 2010. Isolation and characterization of collagen from the cartilages of brownbanded bamboo shark (*Chiloscyllium punctat*um) and blacktip shark (*Carcharhinus limbatus*). LWT – Food Sci. Technol. 43: 792–800.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W., Nagai, T. and Tanaka, M. 2005. Characterisation of acid-soluble collagen from skin and bone of bigeye snapper (*Priacanthus tayenus*). Food Chem. 89: 363–372.

Klomklao S, Benjakul S, Visessanguan W, Simpson, B. K. and Kishimura H. 2005. Partitioning and recovery of proteinase from tuna spleen by aqueous two-phase systems. Proc. Biochem. 40: 3061.

Krimm, S. and Bandekar, J. 1986. Vibrational spectroscopy and conformation of peptides, polypeptides, and proteins. Adv. Protein Chem. 38: 181–364.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680–685.

Li, Z. R., Wang, B., Chi, C. F., Zhang, Q. H., Gong, Y. D., Tang, J. J., Luo, H. Y. and Ding, G. F. 2013. Isolation and characterization of acid soluble collagens and pepsin soluble collagens from the skin and bone of Spanish mackerel (*Scomberomorous niphonius*). Food Hydrocolloid. 31: 103-113.

Liu, D., Liang, L., Regenstein, J. M. and Zhou, P. 2012. Extraction and characterization of pepsin-solubilised collagen from fins, scales, skins, bones and swim bladders of bighead carp (*Hypophthalmichthys nobilis*). Food Chem. 133: 1441–1448.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265–275.

Montero, P., Borderias, J., Turnay, J. and Leyzarbe, M. A. 1990. Characterization of hake (*Merluccius merluccius L.*) and trout (*Salmoirideus Gibb*) collagen. J. Agric. Food Chem. 38: 604–609.

Muyonga, J. H., Cole, C. G. B. and Duodu, K. G. 2004. Characterisation of acid soluble collagen from skins of young and adult Nile perch (*Lates niloticus*). Food Chem. 85: 81–89.

Nagai, T., Suzuki, N. and Nagashima, T. 2008. Collagen from common minke whale (*Balaenoptera acutorostrata*) unesu. Food Chem. 111: 296–301.

Nagarajan, M., Benjakul, S., Prodpran, T., Songtipya, P. and Kishimura, H. 2012. Characteristics and functional properties of gelatin from splendid squid (*Loligo formosana*) skin as affected by extraction temperatures. Food Hydrocolloid. 29: 389-397.

Nalinanon, S., Benjakul, S., Kishimura, H. and Osako, K. 2011. Type I collagen from the skin of ornate threadfin bream (*Nemipterus hexodon*): Characteristics and effect of pepsin hydrolysis. Food Chem. 125: 500–507.

Nalinanon, S., Benjakul, S. and Kishimura, H. 2010. Collagens from the skin of arabesque greenling (*Pleurogrammus azonus*) solubilized with the aid of acetic acid and pepsin from albacore tuna (*Thunnus alalunga*) stomach. J. Sci. Food Agric. 90: 1492–1500.

Nalinanon, S., Benjakul, S., Visessanguan, W. and Kishimura, H. 2007. Use of pepsin for collagen extraction from the skin of bigeye snapper (*Priacanthus tayenus*). Food Chem. 104: 593–601.

Ogawa, M., Portier, R. J., Moody, M. W., Bell, J., Schexnayder, M. A. and Losso, J. N. 2004. Biochemical properties of bone and scale collagens isolated from the subtropical fish black drum (*Pogonia cromis*) and sheepshead seabream (*Archosargusprobato cephalus*). Food Chem. 88: 495–501.

Payne, K. J. and Veis, A. 1988. Fourier transform IR spectroscopy of collagen and gelatin solutions: Deconvolution of the amide I band for conformational studies. Biopolymers. 27: 1749–1760.

Plepis, A. M. D. G., Goissis, G. and Das-Gupta, D. K. 1996. Dielectric and pyroelectric characterization of anionic and native collagen. Polym. Eng. Sci. 36: 2932–2938.

Sadowska, M., Kolodziejska, I. and Niecikowska, C. 2003. Isolation of collagen from the skins of Baltic cod (*Gadus morhua*). Food Chem. 81: 257-262.

Sai, K. P. and Babu, M. 2001. Studies on Rana tigerina skin collagen. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 128: 81-90.

Rigby, B. J. 1968. Amino-acid composition and thermal stability of the skin collagen of the Antarctic ice-fish. Nature. 219: 166–167.

Rochdi, A., Foucat, L. and Renou, J. 2000. NMR and DSC studies during thermal denaturation of collagen. Food Chem. 69: 295–299.

Singh, P., Benjakul, S., Maqsood, S. and Kishimura, H. 2011. Isolation and characterisation of collagen extracted from the skin of striped catfish (*Pangasianodon hypophthalmus*). Food Chem. 124: 97–105.

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 Chem. 138: 2435-2441.

Wang, L., An, X., Xin, Z., Zhao, L. and Hu, Q. 2007. Isolation and characterization of collagen from the skin of deep-sea redfish (*Sebastes mentella*). J. Food Sci. 72: 450-455.

Wong, D. W. S. 1989. Mechanism and theory in food chemistry. Van Nostrand Reinhold. New York.

Zhang, M., Liu, W. and Li, G. 2009. Isolation and characterisation of collagens from the skin of large fin long barbel catfish (*Mystus macropterus*). Food Chem. 115: 826–831.

Zeng, S., Yin, J., Yang, S., Zhang, C., Yang, P. and Wu, W. 2012. Structure and characteristics of acid and pepsin-solubilized collagens from the skin of cobia (*Rachycentron canadum*). Food Chem. 135: 1975–1984.

CHAPTER 3

Characteristics of gelatin extracted from the swim bladder of yellowfin tuna (*Thunnus albacores*) as affected by alkaline pretreatments

3.1 Abstract

Gelatins extracted from the swim bladder of yellowfin tuna (*Thunnus albacores*) using various alkaline pretreatments were characterized. Alkaline mixtures (Na_2CO_3: NaOH) at different ratios (9:1, 8:2, 7:3 and 6:4) with a concentration of 4 % (w/v) were used. The corresponding gelatins termed "G1, G2, G3 and G4" had yields of 9.78, 14.91, 35.96, and 13.60 % (dry weight basis), respectively. All gelatins had α-chains as the major component. FTIR spectra of obtained gelatins revealed the significant loss of molecular order of the triple-helix. G3 having the highest imino acid content and exhibited the highest gel strength ($P < 0.05$), compared with others. Microstructure of G3 gel was finer with smaller voids, compared with others. With increasing proportion of NaOH, L^*- value of gelatin gel increased with coincidental decrease in ΔE^*- value. Gelling and melting temperatures of swim bladder gelatin were 12.3–15.1 and 21.3–22.3 °C, respectively.

3.2 Introduction

Gelatin is a fibrous protein extracted from mother collagen. It is an important functional biopolymer with several applications in many industrial fields, such as food, materials, pharmacy and photography. It is especially useful in the food and pharmaceutical industries owing to its unique chemical and physical characteristics (Rahman *et al*., 2008). Conversion of collagen into soluble gelatin can be achieved by heating collagen and the extraction process (temperature, time, concentration and pH) can influence the length, functional and biological properties of gelatin (Patil *et al*., 2000). There are two different processes to obtain gelatin from skins and bones of animals, an acid process (gelatin A with an isoelectric point at pH 6–9) and an alkaline process (gelatin B with an isoelectric point at pH 5) (Stainsby, 1987). Skin gelatins have been extracted from several fish species, e.g., seabass (Sinthusamran *et al*., 2014), tilapia (Niu *et al*., 2013), grey triggerfish (Jellouli *et al*.,

2011), brownbanded bamboo shark and blacktip shark (Kittiphattanabawon *et al*., 2010). Fish gelatins are normally produced by acid extraction method, whilst alkaline process is particularly designed for gelatin extraction from mammalian skins and bones (Schrieber and Gareis, 2007). However, alkaline process was used for gelatin extraction from tilapia skin. The liming process employed is a viable process for extraction of gelatin from tilapia skins with high bloom (Jamilah *et al*., 2011).

Tuna industry has been economically important for Thailand. Skipjack tuna, followed by yellowfin tuna and tongol tuna, account for over 80 % of Thai frozen tuna imports (Nalinanon *et al*., 2010). Thus, high amount of processing byproducts such as viscera, head, bone and swim bladder are discarded during canned tuna manufacturing. Amongst all tuna, only yellowfin tuna has swim bladder, which has been recently used for collagen extraction (Kaewdang *et al*., 2014). To fully exploit swim bladder, it can be used for the production of gelatin with high market value. Nevertheless, the acid process was not promising for gelatin extraction from yellowfin tuna swim bladder since it provided very low yield. Thus, alkaline process could be implemented to successfully extract gelatin from swim bladder of this species. The aim of this study was to characterise gelatin from yellowfin tuna swim bladder extracted by alkaline process.

3.3 Materials and methods

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

3.3.2 Collection and preparation of swim bladder

Swim bladders of yellowfin tuna (*Thunnus albacares*) were obtained from Tropical Canning Public Co., Ltd., Songkhla, Thailand. Swim bladders with the length of 8-12 cm were placed in polyethylene bags, inserted in ice using a sample/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Songkhla. Upon arrival, swim bladders were washed with distilled water, cut into pieces with the length of approximately 2 cm. The prepared samples were then placed in polyethylene bag and frozen at -20 °C. The samples were stored at -20 °C until used. The storage time was not longer than 3 months. Prior to extraction, frozen swim bladders were thawed using running water until the temperature was 0-2 °C.

3.3.3 Extraction of gelatin from swim bladder

Prepared swim bladders were subjected to alkaline pretreatments using different alkaline mixtures. Those included the mixtures of Na_2CO_3: NaOH with the ratios of 9:1, 8:2 7:3, and 6:4 (w/w). The solution of mixed alkali was prepared to obtain the final concentration of 4 % (w/v) pH of solutions were 10.35, 11.17, 11.70 and 12.02, respectively. Thereafter, swim bladders were mixed with solution at a ratio of 1:10 (w/v). The mixture was stirred for 12 h at room temperature (28–30 °C) using an overhead stirrer model W20.n (IKA®-Werke GmbH & CO.KG, Stanfen, Germany). The alkaline solution was changed every 6 h. The residues were washed with tap water until a neutral or faintly basic pH was obtained. Finally, the samples were mixed with distilled water at a ratio of 1:5 (w/v) at 60 °C. The mixtures were stirred for 24 h. The mixtures were then filtered using a Buchner funnel with a Whatman No. 4 filter paper (Whatman International, Ltd., Maidstone, England). The filtrates were freeze-dried using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The obtained gelatin powders using pretreatment with Na_2CO_3: NaOH at ratios of 9:1, 8:2 7:3, and 6:4 were referred to as “G1”, “G2”, “G3” and “G4”, respectively. All gelatin samples were calculated for extraction yield and were subjected to analyses.

3.3.4 Analyses

3.3.4.1 Yield

Gelatin yield was calculated by the following equation.

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

3.3.4.2 Amino acid analysis

Amino acid composition of gelatin samples were analysed according to the method of Nagarajan *et al*. (2012) with a slight modification. The samples were hydrolysed under reduced pressure in 4 M methanesulphonic acid containing 0.2 % (v/v) 3-2(2-aminoethyl) indole at 115 °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).

3.3.4.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS–PAGE was performed by the method of Laemmli (1970). 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). The mixtures were centrifuged at 8,500 g for 5 min using a microcentrifuge (MIKRO20, Hettich Zentrifugan, 170 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). 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, the 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 a mixture of 50 % (v/v) methanol and 7.5 % (v/v) acetic acid for 30 min and destained again with a mixture of 5 % (v/v) methanol and 7.5 % (v/v) acetic acid for 1 h. High-molecular-weight protein markers were used to estimate the molecular weight of proteins.

3.3.4.4 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 the gelatin was completely solubilised and then 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.

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

3.3.4.5 Determination of color of gelatin gel

The color of gelatin gels (6.67 % w/v) was measured with 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 for 10 min and calibrated with a white standard. The 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 the white standard (L^* = 93.6, a^*= -0.94 and b^* = 0.40).

3.3.4.6 Microstructure analysis of gelatin gel

The 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 25, 50, 70, 80, 90 and 100 % (v/v). Thereafter, samples were critical point dried. 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.

3.3.4.7 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). The 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 included a 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 measured, where tan δ became 1 or δ was 45°.

3.3.4.8 Fourier transform infrared (FTIR) spectroscopic analysis

FTIR spectra of the 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 a resolution of 4 cm^{-1} and the measurement range was between 400 and 4000 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).

3.3.5 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 a 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).

3.4 Results and discussion

3.4.1 Extraction yield

Yields of gelatin extracted from swim bladder of yellowfin tuna pretreated with various alkaline solutions were different. Yields of G1, G2, G3 and G4 were 9.78, 14.91, 35.96 and 13.60 % (on a dry weight basis), respectively. Amongst all samples, G3 had the highest yield ($P < 0.05$). It was noted that yield generally increased when the ratio of NaOH in the alkaline mixture increased. Pretreatment of swim bladder using the mixture of Na_2CO_3: NaOH (7:3) rendered the gelatin (G3) with the highest yield ($P < 0.05$). However, the use of Na_2CO_3: NaOH mixture (6:4) as the pretreatment medium resulted in the sharp decrease in yield (G4). In the presence of NaOH at high level, the solubilisation of collagen might take place to a higher extent. This was in agreement with the highest loss in hydroxyproline in the alkaline solution used for pretreatment (data not shown). The alkaline treatment was able to disrupt some cross-links of collagen molecules, causing the swelling of swim bladder. After heating, the loosen matrix of swim bladder might be easily disrupted. Hot water could penetrate and extract gelatin to a higher degree. Jamilah *et al*. (2011) reported that yields of 39.97, 32.06 and 26.23 % (on dry weight basis) were found for the gelatin extracted from the skins of red tilapia (*Oreochromis nilotica*), walking catfish (*Clarias batrachus*) and striped catfish (*Pangasius sutchi fowler*) when a liming process were implemented for 14 days. Different optimum alkaline solution for $Ca(OH)_2$ pretreatment upon different species might be governed by varying cross-linking of collagen fibrils in raw material. Swim bladder of yellowfin tuna more likely had a high proportion of cross-links. Acid pretreatment showed low efficacy in swelling the swim bladder and the yield of gelatin was very

low (data not shown). Thus, the appropriate alkaline mixture for pretreatment of swim bladder was Na_2CO_3: NaOH (7:3), in which the highest yield was obtained ($P < 0.05$).

3.4.2 Amino acid composition

Amino acid composition of gelatin extracted from the swim bladder with different alkaline pretreatments, expressed as residues per 1000 total amino acid residues, is shown in Table 6. Glycine was the predominant amino acid in all gelatin samples, ranging from 279 to 307 residues/1000 residues. This implied that gelatin obtained was more likely derived from its mother collagen. Collagen consists of one-third glycine in its molecule (Balti *et al*., 2011). It was noted that G3 and G4 had slightly higher glycine content than G1 and G2. Alanine is the second abundant amino acid in all gelatin samples (119-121 residues/1000 residues). Proline and hydroxyproline contents were 91-95 and 67-74 residues/ 1000 residues, respectively. Imino acids (proline and hydroxyproline) play a role in gelation of gelatin (Ahmad and Benjakul, 2011). Hydroxyproline contents of G1 and G2 (67-70 reidues/ 1000 residues) were lower than those of G3 and G4 (72-74 residues/1000 residues). G3 showed the highest imino acid content, compared with others. The super-helix structure of the gelatin, which is critical for the gel properties, is stabilised by steric restrictions. These restrictions are imposed by both the pyrrolidine rings of the hydroxyproline in addition to the hydrogen bonds formed between amino acid residues (Karim and Bhat, 2009).

From amino acid composition, all gelatins had low contents of cysteine (1 residues/1000 residues), histidine (7-8 residues/1000 residues), hydroxylysine (9-10 residues/1000 residues) and tyrosine (6-7 residues/ 1000 residues). This result was in agreement with Kittiphattanabawon *et al*. (2010) who reported that low contents of cysteine (1 residues/1000 residues), histidine (7 residues/1000 residues), hydroxylysine (5–6 residues/1000 residues) and tyrosine (2 residues/ 1000 residues) were observed in gelatins extracted from the skins of brownbanded bamboo shark and blacktip shark. Thus, alkaline treatment affected amino acid composition of gelatin from swim bladder to some extent.

Table 6. Amino acid composition of gelatins from the swim bladder of yellowfin tuna with different alkaline pretreatments

Amino acids	**Number of residues/ 1000 residues**			
	G1	**G2**	**G3**	**G4**
Alanine	119	120	121	121
Arginine	52	52	52	52
Asparagine/aspartic acid	54	52	49	50
Cysteine	1	1	1	1
Glutamine/glutamic acid	84	83	80	80
Glycine	291	279	307	307
Histidine	8	8	7	7
Isoleucine	16	16	14	14
Leucine	33	32	29	30
Lysine	30	28	26	26
Hydroxylysine	9	10	10	9
Methionine	17	17	17	17
Phenylalanine	17	17	16	16
Hydroxyproline	67	70	74	72
Proline	91	93	95	94
Serine	42	41	41	41
Threonine	33	31	30	31
Tyrosine	7	7	6	6
Valine	28	27	26	26
Total	1000	1000	1000	1000
Imino acids	158	163	169	166

3.4.3 Protein patterns

Protein patterns of gelatin from the swim bladder of yellowfin tuna with different alkaline pretreatments are shown in Fig.11. All samples had α-chain as

the major component. Proteins with MW lower than α-chain were noticeable. However, G4 showed the lowest band intensity of low MW proteins.

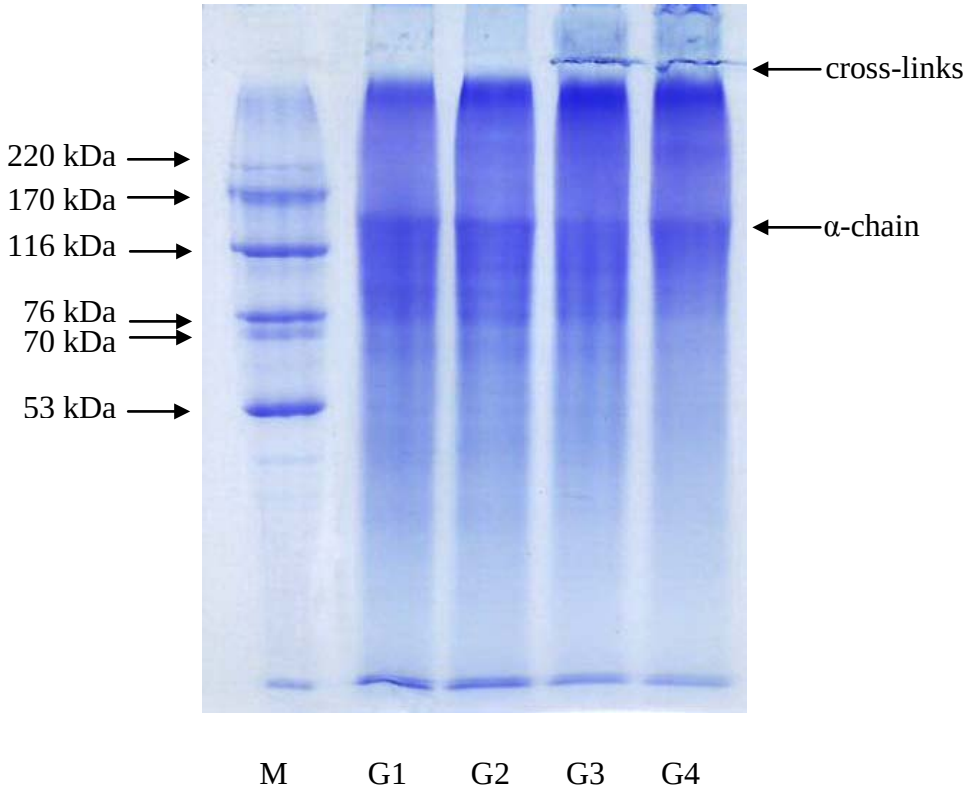

Figure 11. SDS–PAGE patterns of gelatin from the swim bladder of yellowfin tuna with different alkaline pretreatments. M denotes high molecular weight markers.
G1: Gelatin with pretreatment using the mixture of Na_2CO_3: NaOH (9:1)
G2: Gelatin with pretreatment using the mixture of Na_2CO_3: NaOH (8:2)
G3: Gelatin with pretreatment using the mixture of Na_2CO_3: NaOH (7:3)
G4: Gelatin with pretreatment using the mixture of Na_2CO_3: NaOH (6:4)

During conversion of collagen to gelatin, the inter- and intra-cross-links as well as peptide bonds are broken (Muyonga *et al*., 2004a). As a result, the gelatin obtained consists of a mixture of fragments with molecular weights in the range from 50 to 250 kDa. Higher molecular weight cross-links could be also found in the gelatin, especially G3 and G4. With alkaline treatment having high proportion of NaOH, a strong alkali, most of less cross-linked components were leached out. This

was obvious in G4 as evidenced by the lowest yield. After pretreatment, most of large MW cross-links retained in the matrix were extracted by hot water. As a result, those large components were recovered in G4 or G3. On the other hand, milder condition used for pretreatment of G1 or G2, less cross-linked components could be easily extracted but simultaneously degraded as shown by the larger bands of low MW proteins. Apart from pretreatment, extraction temperature was also reported to determine protein pattern of gelatin (Kittiphattanabawon *et al*., 2010). In addition to amino acid composition, the functional properties of gelatin are influenced by the distribution of the molecular weights, temperature of the fish habitat, type and sex of fish, as well as by the extraction process (Jongjareonrak *et al*., 2006). Alkaline pretreatment had therefore the profound effect as protein components in resulting gelatin.

3.4.4 Gel strength

Gel strengths of gelatin extracted from swim bladder of yellowfin tuna with varying pretreatment conditions are shown in Fig. 12. Gel strength is one of the most important functional properties of gelatin. Amongst all gelatin from swim bladder, the highest gel strength (72 g) was found in G3 ($P < 0.05$). As NaOH proportion in the alkaline mixture increased, the gel strength increased. However, the excessive amount of NaOH resulted in the lowered gel strength as shown in G4. According to the amino acid composition (Table 6), G3 also had higher imino acid content (169 residues/1000 residues). Imino acid content has been demonstrated to determine gel strength by introducing pyrrolidine rings for bridging between chains, apart from H-bondings (Sikorski, 2001). In the present study, swim bladder gelatin had a lower gel strength than fish gelatin (GF) and bovine gelatin (GB) (163-187 g) ($P < 0.05$). Ingeneral, fish gelatins have the lower gel strength than mammalian counterpart (Gilsenan and Ross-Morphy, 2000). Different gel strengths have been reported for gelatins from skin of different species including splendid squid (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

the molecular weight distribution (Gómez-Guillén *et al*., 2002). Gel strength (bloom) of commercial gelatins (pig skin and bovine gelatins) ranges from 100 to 300 g, but gelatin with bloom values of 250–300 g are most desired (Jellouli *et al*., 2011). Gelatin can be dissolved in warm water to form a thermo reversible gel. Upon cooling the aqueous solution of gelatin, it forms physical thermo-reversible gels due to partial recovery of collagen triple-helix structure (coil to helix transition). The three dimensional networks formed are responsible for the strength and rigidity of the gelatin gel (Karim and Bhat, 2009). The results suggested that α-chain along with degraded protein and large MW cross-links in G3 sample might align and interact each other to form the ordered network, leading to the strongest gel.

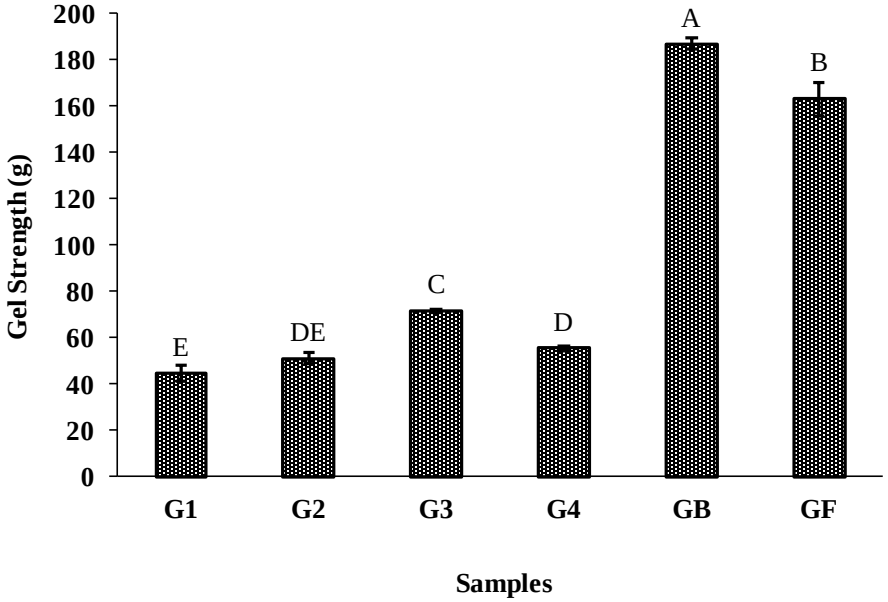

Figure 12. Gel strength of gelatin from the swim bladder of yellowfin tuna with different alkaline pretreatments. Different lowercase letters on the bars denotes significant differences ($P < 0.05$). Bars represent the standard deviations ($n = 3$).
GB: Commercial bovine gelatin
GF: Commercial fish gelatin
G1: Gelatin with pretreatment using the mixture of Na_2CO_3: NaOH (9:1)
G2: Gelatin with pretreatment using the mixture of Na_2CO_3: NaOH (8:2)
G3: Gelatin with pretreatment using the mixture of Na_2CO_3: NaOH (7:3)
G4: Gelatin with pretreatment using the mixture of Na_2CO_3: NaOH (6:4)

3.4.5 Microstructure of gelatin gels

The microstructures of gelatin gel from the swim bladder of yellowfin tuna (G1, G2, G3 and G4) and commercial gelatin (GB and GF) are shown in Fig. 13.

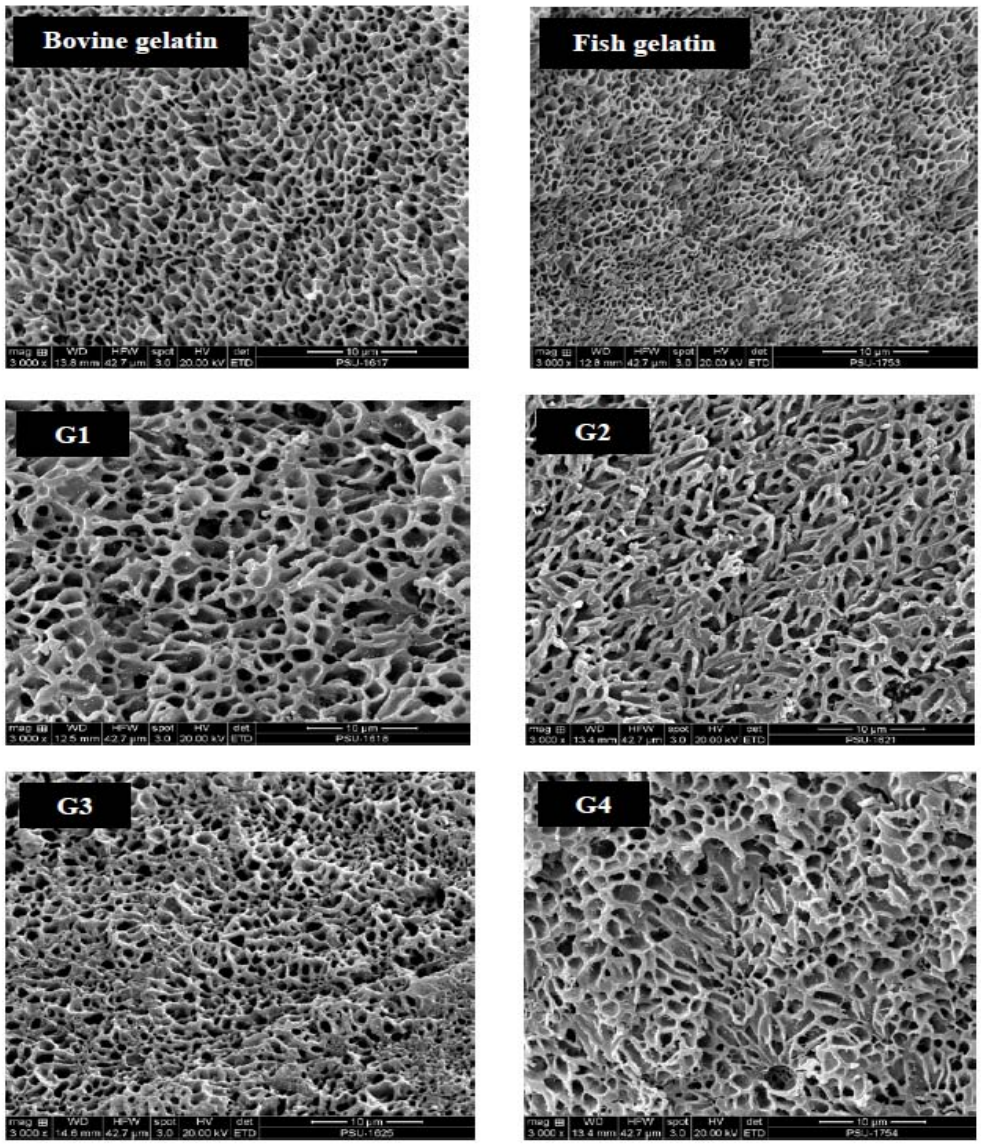

Figure 13. Microstructures of gelatin gel from the swim bladder of yellowfin tuna with different alkaline pretreatments. Magnification: 3000 x.
GB: Commercial bovine gelatin
GF: Commercial fish gelatin
G1: Gelatin with pretreatment using the mixture of Na_2CO_3: NaOH (9:1)
G2: Gelatin with pretreatment using the mixture of Na_2CO_3: NaOH (8:2)
G3: Gelatin with pretreatment using the mixture of Na_2CO_3: NaOH (7:3)
G4: Gelatin with pretreatment using the mixture of Na_2CO_3: NaOH (6:4)

All gelatin gels were sponge or coral-like in structure. Generally, the arrangement and association of protein molecules in the gel matrix directly contributed to the gel strength of the gelatin (Benjakul *et al*., 2009). The gel from GB and GF showed the finest gel network with very small voids, whilst that from swim bladder had larger strands with larger voids. The finer gel structure of commercial bovine and fish gelatins were in accordance with the higher gel strength (Fig. 12). Amongst all swim bladder gelatin, G3 had the finest network with the smallest voids. The denser structure was also noticeable. This was in accordance with highest gel strength (Fig. 12). It has been known that gelatin gel network was governed by the pretreatment conditions (Yang *et al*., 2008) and gelatin concentration (Yang and Wang, 2009). The result revealed that the pretreatment condition had an impact on the molecular weight distribution and peptides of gelatin, which also affected the arrangement of peptides in the network during gelation.

3.4.6 Color of gelatin gel

Gel color of gelatin extracted from the swim bladder of yellowfin tuna with different alkaline pretreatments is shown in Table 7. Highest L^*-value (lightness), a^*-value (redness) and b^*-value (yellowness) was observed for G4 gel, compared with other gels ($P < 0.05$) (Table 7). The lowest ΔE^* was also found in G4 ($P < 0.05$). The higher L^*-value of gelatin gel increased with increasing proportion of NaOH in alkaline mixture used for pretreatment. Higher b^*-value of G4 gel was probably related with enhanced Maillard reaction under alkaline condition during pretreatment. Those amino groups could undergo browning reaction along with carbonyl compounds in the swim bladder to a higher extent under strong alkaline condition. During extraction, those brown as yellow complex were extracted. Gelatin manufacturers generally have a good process to clarify the impurities from the gelatin solution, such as chemical clarification or a filtration process (Ahmad and Benjakul, 2011). It was reported that color did not affect the functional properties of gelatin (Benjakul *et al*., 2009). These results demonstrated that the pretreatment conditions had the impact on color of gelatin extracted from the swim bladder of yellowfin tuna.

Table 7. Gel color, gelling and melting temperatures of gelatin from the swim bladder of yellowfin tuna with different alkaline pretreatments

Samples	Color				Gelling point	Melting point
	L*	***a****	***b****	**Δ*E****	(˚C)	(˚C)
G1	20.37 ± 0.29^{D}	-1.64 ± 0.04^{D}	12.12 ± 0.30^{B}	74.31 ± 0.24^{C}	12.30 ± 0.30^{F}	21.31 ± 0.07^{F}
G2	24.98 ± 0.11^{C}	$-1.27 \pm 0.08B^{C}$	10.87 ± 0.13^{C}	69.41 ± 0.01^{D}	14.35 ± 0.05^{E}	21.74 ± 0.11^{E}
G3	28.41 ± 0.06^{B}	-2.09 ± 0.05^{E}	8.10 ± 0.23^{D}	65.65 ± 0.09^{E}	15.12 ± 0.15^{C}	22.28 ± 0.07^{C}
G4	33.11 ± 0.02^{A}	-0.96 ± 0.09^{AB}	14.87 ± 0.15^{A}	62.19 ± 0.02^{F}	14.74 ± 0.26^{D}	21.93 ± 0.03^{D}
GB	4.69 ± 0.11^{F}	-1.38 ± 0.43^{CD}	-1.94 ± 0.36^{E}	88.93 ± 0.12^{A}	23.23 ± 0.04^{A}	32.71 ± 0.10^{A}
GF	8.12 ± 4.46^{E}	-0.84 ± 0.33^{A}	-1.12 ± 1.26^{E}	85.49 ± 4.48^{B}	18.92 ± 0.06^{B}	25.78 ± 0.16^{B}

Mean ±SD (n = 3).
Different uppercase superscripts in the same column indicate significant differences ($P < 0.05$).

3.4.7 Gelling and melting temperatures

Thermal transitions were monitored by changes in the phase angle (δ) of dissolved gelatins during cooling (35–5 °C) and subsequent heating (5–35 °C). Gelling and melting temperatures were calculated (Table 7). The solution of G1, G2, G3 and G4 from yellowfin tuna swim bladder gelatin became gel at 12.30, 14.35, 15.12 and 14.74 °C and subsequently melted at 21.31, 21.74, 22.28 and 21.93 °C, respectively. GB and GF showed the higher gelling and melting temperatures than gelatins from swim bladder. GB and GF became gel at 23.23 and 18.92 °C and subsequently melted at 32.71 and 25.78 °C, respectively. Generally, the melting point was higher than the gelling point because gelatin gel absorbed energy when it was melting (Liu and Guo, 2008). Amongst all gelatins from swim bladder, G3 had the highest gelling and melting temperatures. This suggested that G3 underwent gelation most effectively and the gel network was strongest, plausibly with the larger numbers of bondings stabilising the gel network. This was coincidental with the highest gel strength of G3 samples. The gelling and melting temperatures of fish gelatins type B varied, e.g from the skin of channel catfish (22 and 25 °C) (Liu and Guo, 2008) and from skin of skate (16.12 and 19.3 °C) (Cho *et al*., 2006). The lower gelling and melting temperatures of gelatin from swim bladder could be related to lower content of imino acids and glycine. Glycine could server as hydrogen donor to from gel matrix. However, gelatin from different fish species exhibited different viscoelastic properties even though the amino acid composition was similar (Gómez-Guillén *et al*., 2002). Gelation is affected by the concentration, pH, molecular weight, relative content of α, β and γ-chain components, gel maturation time and temperature (Choi and Regenstein, 2000; Gómez-Guillén *et al*., 2002).

3.4.8 Fourier transform infrared (FTIR) spectra

FTIR spectra of gelatins with different alkaline pretreatments are shown in Fig.14. Generally, all gelatins showed a similar spectra. FTIR spectroscopy has been used to monitor the functional groups and secondary structure of gelatin (Muyonga *et al*., 2004a). Spectra of gelatin showed the major peaks in the amide region. Muyonga *et al*. (2004b) reported that amide I band with a 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 characteristic absorption bands in the amide I region of gelatins of G1, G2, G3 and G4 were noticeable at the wavenumbers of 1657.7, 1658.3, 1656.2 and 1658.7 cm^{-1}, respectively. The absorption in the amide I region is probably the most useful for infrared spectroscopic analysis of the secondary structure of proteins (Kittiphattanabawon *et al*., 2010). The absorption peak at amide I (1635 cm^{-1}) was characteristic for the coil structure of gelatin (Nagarajan *et al*., 2012). The spectral differences in amide I of different gelatin samples were largely attributed to different conformation of polypeptide chains. However, similar wavenumbers of Amide I were observed between samples. Therefore, availability of (C=O) group was in all samples similar.

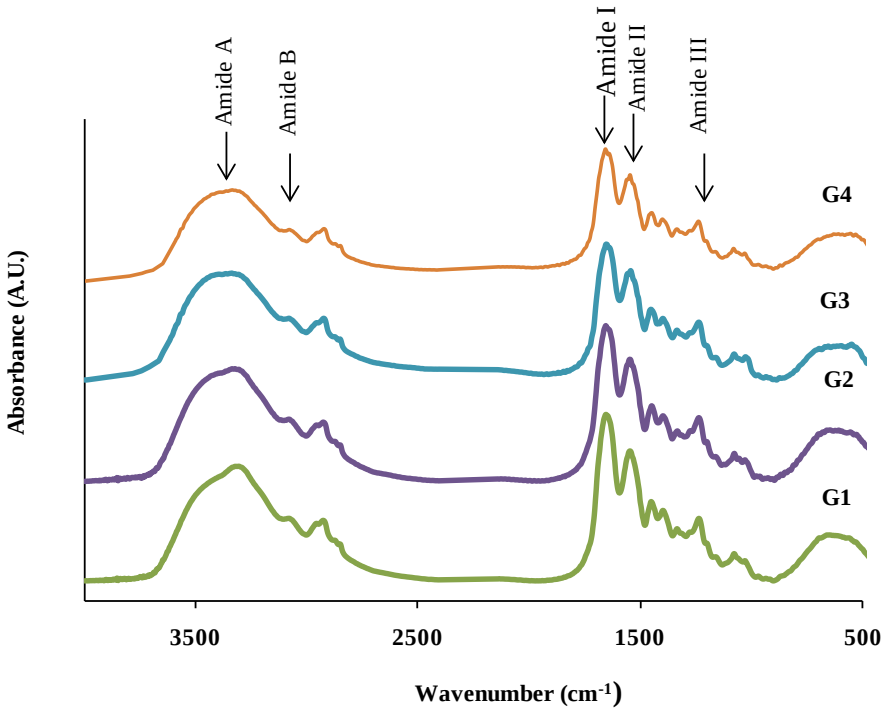

Figure 14. Fourier transform infrared spectra of gelatin from swim bladder of yellowfin tuna with different alkaline pretreatments.
G1: Gelatin with pretreatment using the mixture of Na_2CO_3: NaOH (9:1)
G2: Gelatin with pretreatment using the mixture of Na_2CO_3: NaOH (8:2)
G3: Gelatin with pretreatment using the mixture of Na_2CO_3: NaOH (7:3)
G4: Gelatin with pretreatment using the mixture of Na_2CO_3: NaOH (6:4)

The characteristic absorption bands of G1, G2, G3 and G4 in amide II region were noticeable at the wavenumbers of 1549.7, 1549.9, 1546.5 and 1547.9 cm^{-1}, respectively. The amide II vibration modes are attributed to an out-of-phase combination of 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 1239.8, 1240.2, 1240.5, 1239.7 cm^{-1} for G1, G2, G3 and G4, respectively. The amide III represented the combination peaks between C-N stretching vibrations and N-H deformation from amide linkages as well as absorptions arising from wagging vibrations from CH_2 groups from the glycine backbone and proline side-chains (Jackson *et al*., 1995). The greater disorder of molecular structure due to transformation of an α-helical to a random coil structure occurred during heating and these changes were associated with loss of triple-helix state as a result of denaturation of collagen to gelatin (Muyonga *et al*., 2004a). It was noted that all samples showed similar Amide II and III peaks. Moreover, the amide A band, arising from the stretching vibrations of N-H group, appeared at 3307.5, 3324.7, 3339.3 and 3339 cm^{-1} for G1, G2, G3 and G4, respectively. Normally, a free N-H stretching vibration occurs in the range of 3400-3440 cm^{-1}. When the N-H group of a peptide is involved in a hydrogen bond, the position is shifted to lower frequencies (Doyle *et al*., 1975). At amide A region, the lower amplitude as well as the lower wavenumber were found in G1, compared with others, whist the highest wavenumbers were observed for G3 and G4. Thus, it was suggested that N-H of peptide in G1 more likely underwent H-bonding to a higher extent, particularly during freeze-drying. The amide B was observed at 3082.1, 3082.3, 3082.1 and 3082.6 cm^{-1} for G1, G2, G3 and G4, respectively, corresponding to asymmetric stretch vibration of =C-H as well as $–NH^{3+}$. Thus, the secondary structure of gelatins obtained from the swim bladder of yellowfin tuna was affected by alkaline pretreatment to some extent.

3.5 Conclusion

Alkaline pretreatment directly determined yield and properties of gelatin from swim bladder of yellowfin tuna. The highest yield and gel strength was obtained when swim bladder was pretreated in the mixture of Na_2CO_3 and NaOH (7:3) at a concentration of 4 % (w/v) for 12 h prior to extraction.

3.6 References

Ahmad, M. and Benjakul, S. 2011. Characteristics of gelatin from the skin of unicorn leatherjacket (*Aluterus monoceros*) as influenced by acid pretreatment and extraction time. Food Hydrocolloid. 25: 381-388.

Arnesen, J. A. and Gildberg, A. 2007. Extraction and characterisation of gelatine from Atlantic salmon (*Salmo salar*) skin. Bioresource Technol. 98: 53-57.

Balti, R., Jridi, M., Sila, A., Souissi, N., Nedjar-Arroume, N., Guillochon, D. and Nasri, M. 2011. Extraction and functional properties of gelatin from the skin of cuttlefish (*Sepia officinalis*) using smooth hound crude acid protease-aided process. Food Hydrocolloid. 25: 943-950.

Bandekar, J. 1992. Amide modes and protein conformation. BBA-Protein Struct. M. 1120: 123-143.

Benjakul, S., Oungbho, K., Visessanguan, W., Thiansilakul, Y. and Roytrakul, S. 2009. Characteristics of gelatin from the skins of bigeye snapper, *Priacanthus tayenus* and *Priacanthus macracanthus*. Food Chem. 116: 445-451.

Boran, G., Mulvaney, S. J. and Regenstein, J. M. 2010. Rheological properties of gelatin from silver carp skin compared to commercially available gelatins from different sources. J. Food Sci. 75: 565–571.

Cho, S. M., Gu, Y. S. and Kim, S. B. 2005. Extracting optimization and physical properties of yellowfin tuna (*Thunnus albacares*) skin gelatin compared to mammalian gelatins. Food Hydrocolloid. 19: 221-229.

Cho, S.-H., Jahncke, M. L., Chin, K.-B. and Eun, J.-B. 2006. The effect of processing conditions on the properties of gelatin from skate (*Raja Kenojei*) skins. Food Hydrocolloid. 20: 810-816.

Choi, S. S. and Regenstein, J. 2000. Physicochemical and sensory characteristics of fish gelatin. J. Food Sci. 65: 194-199.

Doyle, B. B., Bendit, E. and Blout, E. R. 1975. Infrared spectroscopy of collagen and collagen-like polypeptides. Biopolymers. 14: 937-957.

Gennadios, A., Weller, C. L., Hanna, M. A. and Froning, G. W. 1996. Mechanical and barrier properties of egg albumen films. J. Food Sci. 61: 585–589.

Gilsenan, P. and Ross-Murphy, S. 2000. Rheological characterisation of gelatins from mammalian and marine sources. Food Hydrocolloid. 14: 191-195.

Gómez-Guillén, M., Turnay, J., Fernández-Dıaz, M., Ulmo, N., Lizarbe, M. and Montero, P. 2002. Structural and physical properties of gelatin extracted from different marine species: a comparative study. Food Hydrocolloid. 16: 25-34.

Jackson, M., Choo, L., Watson, P. H., Halliday, W. C. and Mantsch, H. H. 1995. Beware of connective tissue proteins: assignment and implications of collagen absorptions in infrared spectra of human tissues. BBA-Mol. Basis Dis. 1270: 1-6.

Jamilah, B., Tan, K., Umi Hartina, M. and Azizah, A. 2011. Gelatins from three cultured freshwater fish skins obtained by liming process. Food Hydrocolloid. 25: 1256-1260.

Jellouli, K., Balti, R., Bougatef, A., Hmidet, N., Barkia, A. and Nasri, M. 2011. Chemical composition and characteristics of skin gelatin from grey triggerfish (*Balistes capriscus*). LWT-Food Sci. Technol. 44: 1965-1970.

Jongjareonrak, A., Benjakul, S., Visessanguan, W. and Tanaka, M. 2006. Skin gelatin from bigeye snapper and brownstripe red snapper: chemical compositions and effect of microbial transglutaminase on gel properties. Food Hydrocolloid. 20: 1216-1222.

Kaewdang, O., Benjakul, S., Kaewmanee, T. and Kishimura, H. 2014. Characteristics of collagens from the swim bladders of yellowfin tuna (*Thunnus albacares*). Food Chem. 155: 264-270.

Karim, A. and Bhat, R. 2009. Fish gelatin: properties, challenges, and prospects as an alternative to mammalian gelatins. Food Hydrocolloid. 23: 563-576.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W. and Shahidi, F. 2010. Comparative study on characteristics of gelatin from the skins of brownbanded bamboo shark and blacktip shark as affected by extraction conditions. Food Hydrocolloid. 24: 164-171.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685.

Lavialle, F., Adams, R. G. and Levin, I. W. 1982. Infrared spectroscopic study of the secondary structure of melittin in water, 2-chloroethanol, and phospholipid bilayer dispersions. Biochem. 21: 2305-2312.

Liu, H. Y., Li, D. and Guo, S. D. 2008. Extraction and properties of gelatin from channel catfish (*Ietalurus punetaus*) skin. LWT-Food Sci. Technol. 41: 414-419.

Muyonga, J., Cole, C. and Duodu, K. 2004a. Characterisation of acid soluble collagen from skins of young and adult Nile perch (*Lates niloticus*). Food Chem. 85: 81-89.

Muyonga, J., Cole, C. and Duodu, K. 2004b. Extraction and physico-chemical characterisation of Nile perch (*Lates niloticus*) skin and bone gelatin. Food Hydrocolloid. 18: 581-592.

Nagarajan, M., Benjakul, S., Prodpran, T., Songtipya, P. and Kishimura, H. 2012. Characteristics and functional properties of gelatin from splendid squid (*Loligo formosana*) skin as affected by extraction temperatures. Food Hydrocolloid. 29: 389-397.

Nalinanon, S., Benjakul, S. and Kishimura, H. 2010. Collagens from the skin of arabesque greenling *(Pleurogrammus azonus*) solubilized with the aid of acetic acid and pepsin from albacore tuna (*Thunnus alalunga*) stomach. J. Sci. Food Agric. 90: 1492-1500.

Niu, L., Zhou, X., Yuan, C., Bai, Y., Lai, K., Yang, F. and Huang, Y. 2013. Characterization of tilapia (*Oreochromis niloticus*) skin gelatin extracted with alkaline and different acid pretreatments. Food Hydrocolloid. 33: 336-341.

Patil, R., Dalev, P., Mark, J., Vassileva, E. and Fakirov, S. 2000. Biodegradation of chemically modified gelatin films in lake and river waters. J. Appl. Polym. Sci. 76: 29-37.

Rahman, M. S., Al-Saidi, G. S. and Guizani, N. 2008. Thermal characterisation of gelatin extracted from yellowfin tuna skin and commercial mammalian gelatin. Food Chem. 108: 472-481.

Schrieber, R. and Gareis, H. 2007. Gelatine handbook: theory and industrial practice: John Wiley & Sons.

Sinthusamran, S., Benjakul, S. and Kishimura, H. 2014. Characteristics and gel properties of gelatin from skin of seabass (*Lates calcarifer*) as influenced by extraction conditions. Food Chem. 152: 276-284.

Sikorski, Z. E. 2001. Functional properties of proteins in food systems. *In* Chemical and Functional Properties of Food Proteins (Sikorski, Z. E., ed.). p. 113-136. PA: Technomic publishing Co., Inc. Lancaster.

Stainsby, G. 1987. Gelatin gels. *In* Advances in Meat Research. (Pearson, A. M., Dutson, T. R. and Bailey, A. J., eds.). p. 209–222. Van Nostrand Reinhold Company Inc. New York.

Steel, R. G. D. and Torrie, J. H. 1980. Principles and procedures of statistics: A biometrical approach (2nd ed.). McGraw-Hill. New York.

Tabarestani, H. S., Maghsoudlou, Y., Motamedzadegan, A. and Mahoonak, A. R. 2010. Optimization of physico-chemical properties of gelatin extracted from fish skin of rainbow trout (*Onchorhynchus mykiss*). Bioresource Technol. 101: 6207-6214.

Yang, H. and Wang, Y. 2009. Effects of concentration on nanostructural images and physical properties of gelatin from channel catfish skins. Food Hydrocolloid. 23: 577-584.

Yang, H., Wang, Y., Zhou, P. and Regenstein, J. M. 2008. Effects of alkaline and acid pretreatment on the physical properties and nanostructures of the gelatin from channel catfish skins. Food Hydrocolloid. 22: 1541-1550.

CHAPTER 4

Characteristics of gelatin from swim bladder of yellowfin tuna (*Thunnus albacores*) as influenced by extracting temperatures

4.1 Abstract

Gelatin was extracted from the swim bladder of yellowfin tuna (*Thunnus albacores*) at different temperatures (60, 70 and 80 °C) with the extraction yields of 35.6 %, 41.1 % and 47.3 % (dry weight basis), respectively. The α-chains of gelatin decreased with increasing extraction temperatures. Similar amino acid compositions were noticeable among all gelatins, in which glycine constituted the major amino acid. Imino acids ranged from 169 to 172 residues/1000 residues. The gel strength of gelatin extracted at lower temperature was higher than that of gelatins extracted at higher temperatures. Gelling and melting temperatures for swim bladder gelatin were 11.07–15.24 and 20.36–22.33 °C, respectively. Higher gelling and melting points were observed for gelatin extracted at lower temperatures. Microstructure of gel of gelatin extracted at 60 °C was finer with smaller voids, compared with others. FTIR spectra of obtained gelatins revealed the significant loss of molecular order of the triple-helix. Thus, extraction temperatures showed the direct impact on characteristics of gelatin from swim bladder.

4.2 Introduction

Gelatin is a fibrous protein obtained by partial denaturation or hydrolysis of collagen. Gelatin represents biopolymer with many applications in food, materials (for edible and biodegradable packaging), cosmetic, pharmaceutical and photographic industries (Jellouli *et al*., 2011). The source, type of collagen and processing conditions have the influence on the properties of the resulting gelatin (Kittiphattanabawon *et al*., 2010). Different types of gelatins have varying thermal and rheological properties such as gel strength, melting and gelling temperatures (Benjakul *et al*., 2012). These properties are governed by several factors such as chain length or molecular weight distribution, amino acid composition and hydrophobicity, etc. (Gómez-guillén *et al*., 2002; Norziah *et al*., 2009).

Commercial gelatins are produced mainly from porcine and bovine skins and bones by alkaline or acidic extraction (Benjakul *et al*., 2009). However, both Judaism and Islam forbid the consumption of any pork-related products, while Hindus do not consume cow-related products. Additionally, bovine gelatin has a high risk for bovine spongiform encephalopathy (Nagarajan *et al*., 2012). Furthermore, the need to exploit the fish processing byproducts has led to the production of gelatin as an alternative to mammalian counterpart (Gǿmez-guillén *et al*., 2011). Fish gelatin has been extracted mainly from fish skin such as seabass (Sinthusamran *et al*., 2014), cobia (Silva *et al*., 2014) skipjack tuna, dog shark and rohu (Shyni *et al*., 2014) and unicorn leatherjacket (Kaewruang *et al*., 2013), etc.

Among fish processing industries, canned tuna industry is economically important. Tuna including yellowfin, skipjack and tongol have been the important species for canning in Thailand with a large volume of raw materials used. Approximately two-thirds of the whole fish are utilized and the remainings involving the viscera, head, bone and swim bladder become the byproducts (Klomklao *et al*., 2004). Fish swim bladders can be used for production of “isinglass” (Weber *et al*., 2009). Recently, Kaewdang *et al*. (2014) reported that alkaline pretreatment was essential for gelatin extraction from yellowfin tuna swim bladder. However, no information on the effect of extracting temperature on characteristics and properties of gelatin has been reported. Therefore, the objectives of this investigation were to extract and characterize gelatin from the swim bladder of yellowfin tuna using different extraction temperatures.

4.3 Materials and methods

4.3.1 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). Food grade bovine bone gelatin with the bloom strength of 150–250 g. was obtained from Halagel (Thailand) Co., Ltd., (Bangkok, Thailand).

4.3.2 Collection and preparation of swim bladder

Swim bladders of yellowfin tuna (*Thunnus albacares*) were obtained from Tropical Canning Public Co., Ltd., Songkhla, Thailand. Swim bladders with the length of 8-12 cm were placed in polyethylene bags, inserted in ice using a sample/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Songkhla. Upon arrival, swim bladders were washed with distilled water and cut into pieces with the length of approximately 2 cm. The prepared samples were then placed in polyethylene bag and frozen at -20 °C. The samples were stored at -20 °C until used. The storage time was not longer than 3 months. Prior to extraction, frozen swim bladders were thawed using running water until the temperature was 0-2 °C

4.3.3 Extraction of gelatin from swim bladder

Prior to gelatin extraction, swim bladders were pretreated with alkaline solution as per the method of Kaewdang *et al*. (2014). Prepared swim bladders were added with the mixed alkaline solution (Na_2CO_3: NaOH; 7:3) having the concentration of 4 % (w/v) at a ratio of 1:10 (w/v). The mixture was stirred for 12 h at room temperature (28–30 °C) using an overhead stirrer model W20.n (IKA®-Werke GmbH & CO.KG, Stanfen, Germany). The alkaline solution was changed every 6 h. The residues were washed with tap water until a neutral or faintly basic pH was obtained.

To extract gelatin, alkali pretreated samples were soaked in distilled water with different temperatures (60, 70 and 80 °C) using a swim bladder/water ratio of 1:5 (w/v) in a temperature-controlled water bath (W350, Memmert, Schwabach, Germany) for 24 h with a continuous stirring at a speed of 150 rpm. The mixtures were then filtered using a Buchner funnel with a Whatman No. 4 filter paper (Whatman International, Ltd., Maidstone, England). The filtrates were freeze-dried using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The dry gelatin extracted from swim bladder from yellowfin tuna at 60, 70 and 80 °C was referred to as “G60”, “G70” and “G80”, respectively. All gelatin samples were weighed, calculated for extraction yield and subjected to analyses.

4.3.4 Analyses

4.3.4.1 Yield

Gelatin yield was calculated by the following equation.

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

where the weight of dry swim bladder was calculated by subtracting moisture content determined by AOAC (2000) from the initial wet weight.

4.3.4.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS–PAGE was performed by the method of Laemmli (1970). 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). The mixtures were centrifuged at 8,500 g for 5 min using a microcentrifuge (MIKRO20, Hettich Zentrifugan, 170 Germany) to remove undissolved debris. Solubilized samples were mixed at 1:1 (v/v) ratio with the 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, the 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 a mixture of 50 % (v/v) methanol and 7.5 % (v/v) acetic acid for 30 min and destained again with a mixture of 5 % (v/v) methanol and 7.5 % (v/v) acetic acid for 1 h. High-molecular-weight protein markers were used to estimate the molecular weight of proteins.

4.3.4.3 Amino acid analysis

Amino acid composition of gelatin samples was analyzed according to the method of Nagarajan *et al*. (2012) with a slight modification. The samples were hydrolyzed 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 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).

4.3.4.4 Fourier transform infrared (FTIR) spectroscopic analysis

FTIR spectra of the 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 a resolution of 4 cm^{-1} and the measurement range was between 400 and 4000 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).

4.3.4.5 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 the gelatin was completely solubilized and then 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.

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

4.3.4.6 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). The 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 included a 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 measured, where tan δ became 1 or δ was 45°.

4.3.4.7 Microstructure analysis of gelatin gel

The microstructure of gelatin gel 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 a serial concentration of 25, 50, 70, 80, 90 and 100 % (v/v). Then, samples were critical point dried. Dried samples were mounted on a bronze stub and sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA). The specimens were observed with a scanning electron microscope (JEOL JSM-5800 LV, Tokyo, Japan) at an acceleration voltage of 20 kV.

4.3.4.8 Determination of color of gelatin gel

The color of gelatin gels (6.67 % w/v) was measured with 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 for 10 min and calibrated with a white standard. The 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 the white standard (L^* = 93.6, a^*= -0.94 and b^* = 0.40).

4.3.5 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 a 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).

4.4 Results and discussion

4.4.1 Extraction yield

Yield of gelatin from the swim bladder of yellowfin tuna extracted at various temperatures was different. Increasing yield was obtained when the extraction temperatures increased ($P < 0.05$). Yield of 35.6 %, 41.1 % and 47.3 % (on dry weight basis) was found for G60, G70, and G80, respectively. This result was in agreement with Kaewruang *et al*. (2013), Duan *et al*. (2011) and Kittiphattanabawon *et al*. (2010) who reported the increasing yield of gelatin as the extraction temperature increased with higher temperatures, the bondings stabilizing α-chains in the native mother collagen were destroyed to a higher extant. As a consequence, the triple helix structure became amorphous and could be extracted into the medium with ease, leading to the higher yield (Sinthusamran *et al*., 2014). In addition, the higher energy applied could induce thermal hydrolysis of peptide chains, resulting in the formation of shorter peptides. As a result, those small peptides could be easily extracted into water. The yield and characteristics of gelatin are associated with the type of raw material and gelatin extraction process, including the pretreatment process and extraction temperatures. (Nagarajan *et al*., 2012; Kittiphattanabawon *et al*., 2010; Montero and Gǿmez-guillen, 2000).

4.4.2 Protein patterns

Protein patterns of gelatin from the swim bladder of yellowfin tuna extracted at different temperatures are shown in Fig. 15. The band intensity of α_1-chain and α_2-chain decreased with increasing extraction temperature. The decreases in α_1-chain band intensity were observed in G70 and G80, in comparison with that found in G60. Among all gelatin samples, G80 possessed the lowest α-chain band intensity. This might be caused by the degradation induced by the thermal process. Therefore, the extraction temperatures played a major role in protein components of resulting gelatin.

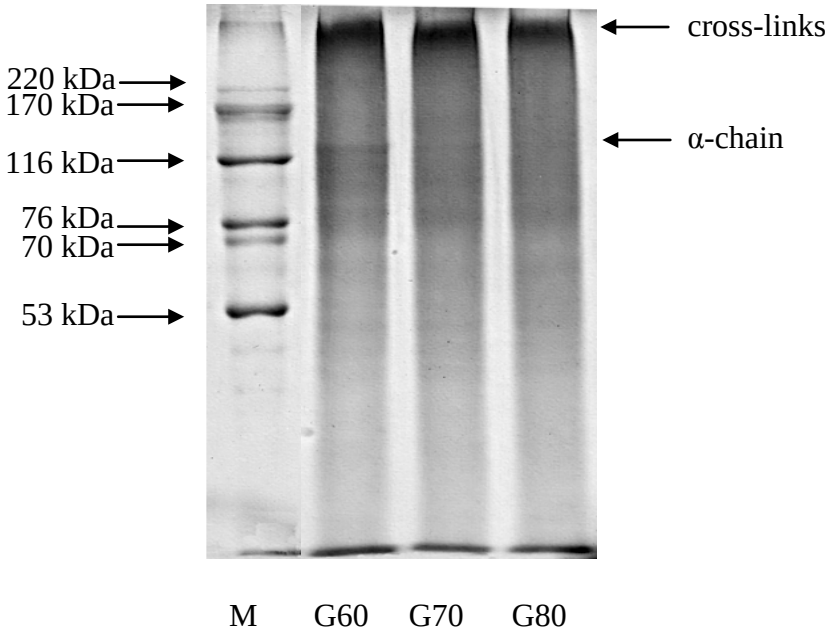

Figure 15. Protein patterns of gelatins from the swim bladder of yellowfin tuna extracted at different temperatures. M: high molecular weight markers. G60, G70 and G80 represent gelatin extracted from swim bladder at 60, 70 and 80 °C, respectively.

Kittiphattanabawon *et al*. (2010) reported that the gelatins extracted from the skins of brownbanded bamboo shark and blacktip shark with higher extraction temperature contained more peptides with the MW less than α-chain and the lower proportion of high MW (greater than β-chain) fractions, compared with those obtained from lower temperature extraction. Gelatins from splendid squid skin

with higher extraction temperatures contained a lower band intensity of the α-chains than those obtained with lower extraction temperature (Nagarajan *et al*., 2012). On the other hand, gelatin from skin of unicorn leatherjacket extracted at higher temperature (65-75 °C) had α-chain retained at higher level than that extracted at lower temperature (Kaewruang *et al*., 2013). This was due to the thermal inactivation of indigenous proteases in the skin of unicorn leatherjacket at high temperature. Generally, gelatins with a higher content of α-chains showed better functional properties including gelling, emulsifying and foaming properties (Gǿmez-guillén *et al*., 2002). In general, the formation of peptide fragments is associated with lower viscosity, low melting point, low setting point, high setting time, as well as decreased bloom strength of gelatin (Muyonga *et al*., 2004a). The results suggested that G70 and G80, which were extracted at higher temperatures, had the shorter chains as indicated by lower content of α-chain.

4.4.3 Amino acid composition

Amino acid compositions of gelatins from the swim bladder of yellowfin tuna extracted at different temperatures are shown in Table 8. Glycine was the predominant amino acid in all gelatin samples, ranging from 305 to 314 residues/1000 residues. This implied that gelatin obtained was derived from its mother collagen. Collagen consists of one-third glycine in its molecule (Balti *et al*., 2011). It was noted that G80 had the higher glycine content than G60 and G70. The higher glycine in G80 might be caused by free glycine, which was released to a high extent during extraction at high temperature. Alanine (121-122 residues/1000 residues) was found at high content. Alanine plays a role in viscoelastic property of gelatin (Gimeńez *et al*., 2005). Low contents of cysteine (1 residues/1000 residues), tyrosine (5-6 residues/1000 residues), histidine (7-8 residues/1000 residues) and hydroxylysine (10 residues/1000 residues) were observed in all gelatin samples. For imino acids, all gelatins contained proline and hydroxyproline contents of 95–99 and 72–74 residues/1000 residues, respectively.

Table 8. Amino acid compositions of gelatins from the swim bladder of yellowfin tuna extracted at different temperatures

Amino acids	Number of residues/1000 residues		
	G60	**G70**	**G80**
Alanine	121	121	122
Arginine	52	52	53
Aspartic acid/asparagine	49	48	46
Cysteine	1	1	1
Glutamic acid /glutamine	80	80	78
Glycine	307	305	314
Histidine	7	8	7
Isoleucine	14	14	13
Leucine	29	30	28
Lysine	26	26	26
Hydroxylysine	10	10	10
Methionine	17	16	16
Phenylalanine	16	16	16
Hydroxyproline	74	72	73
Proline	95	99	99
Serine	41	41	40
Threonine	30	30	30
Tyrosine	6	6	5
Total	1000	1000	1000
Imino acids	169	171	172

Regenstein and Zhou (2007) reported that glycine, alanine, proline and hydroxyproline are four of the most abundant amino acids in gelatin. The properties of gelatin are largely influenced by the amino acid composition and their molecular weight distribution (Gośmez-guilleń *et al*., 2009). When comparing the content of imino acids (proline and hydroxyproline), gelatin from swim bladder had the lower imino acid content than those from seabass skin (198-202 residues/1000 residues)

(Sinthusamran *et al*., 2014) and from carp skin (188-190 residues/1000 residues) (Duan *et al*., 2011). The imino acid content of fish collagens and gelatins correlates with the water temperature of their normal habitat (Foegeding *et al*., 1996; Regenstein and Zhou, 2007). It has been known that imino acid content, especially hydroxyproline content, affected functional properties of gelatin, especially gelling property (Aewsiri *et al*., 2008; Benjakul *et al*., 2009). Therefore, amino acid composition of gelatin from swim bladder was governed by extraction temperature.

4.4.4 Gel strength

Gel strength of gelatin from the swim bladder of yellowfin tuna extracted at different temperatures is presented in Fig 16. G60, G70 and G80 had the gel strength of 72, 64 and 51 g, respectively. The difference in gel strength between the samples could be due to the differences in intrinsic characteristics, especially molecular weight distribution. Protein degradation resulted in the formation of peptides with shorter chain length, which might show the lower ability to from the junction zone or anneal each other.

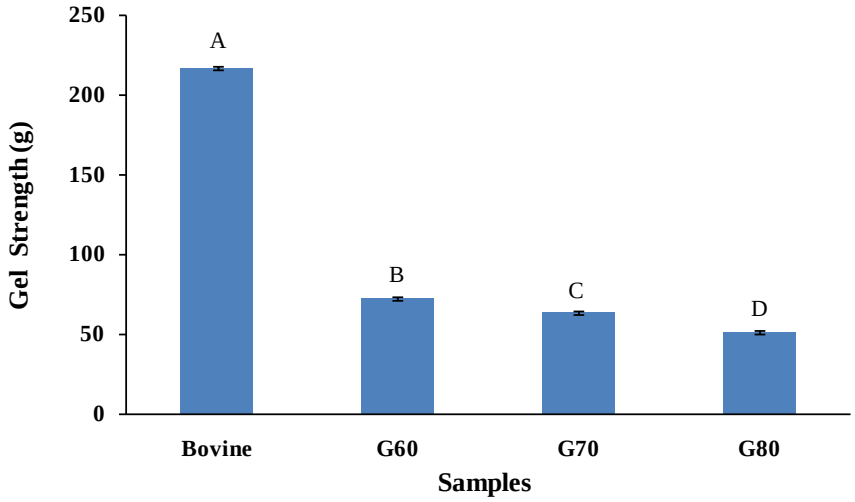

Figure 16. Gel strength of gelatin from the swim bladder of yellowfin tuna with different temperatures. Different uppercase letters on the bars denote significant differences ($P < 0.05$). Bars represent the standard deviations ($n = 3$). G60, G70 and G80 represent gelatin extracted from swim bladder at 60, 70 and 80 °C, respectively.

The longer chains in G60 could undergo aggregation to form gel network more effectively than G70 and G80. As a result, a stronger gel network could be formed as indicated by the higher gel strength. Bloom strength of commercial gelatins ranges from 100 to 300, but gelatins with bloom values of 250-260 are the most desirable (Holzer, 1996). Different gel strength was reported for gelatin from skin of different species including splendid squid (85–132 g) (Nagarajan *et al*., 2012), brownbanded bamboo shark and blacktip shark (206–214 g) (Kittiphattanabawon *et al*., 2010) and bigeye snapper (108 g) (Binsia *et al*., 2009).

4.4.5 Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of gelatins extracted using different temperatures are shown in Fig. 17. FTIR spectroscopy has been used as a well established technique to monitor the functional groups and secondary structure of gelatin (Kong and YU, 2007). The absorption bands were situated in the amide region. The absorption in the amide-I region, owing to C=O stretching vibration, is probably the most useful for infrared spectroscopic analysis of the secondary structure of proteins (Benjakul *et al*., 2009). It depends on the hydrogen bonding and the conformation of the protein structure (Benjakul *et al*., 2009; Uriarte-Montoyaetal *et al*., 2011). G60, G70 and G80 exhibited the amide-I bands at the wavenumbers of 1652.8, 1653.7 and 1652.9 cm^{-1}, respectively. The characteristic absorption bands of G60, G70 and G80 in amide-II region were noticeable at the wavenumbers of 1544.6, and 1545.5 and 1543.5 cm^{-1}, respectively. Amide-II arises from bending vibration of N-H groups and stretching vibrations of C-N groups. In addition, amide-III was detected at the wavenumbers of 1241.9, 1241.3 and 1240.8 cm^{-1} for G60, G70 and G80, respectively. The amide-III represents the combination peaks between C-N stretching vibrations and N-H deformation from amide linkages as well as absorptions arising from wagging vibrations from CH_2 groups from the glycine backbone and proline side-chains (Jackson *et al*., 1995). G80 had the lowest amplitude, whereas G60 exhibited the highest amplitude at amide-III region. This indicated that the greater disorder of molecular structure due to transformation of an α-helical to a random coil structure occurred at higher temperature. These changes were associated with loss of triple-helix state as a result of denaturation of collagen to gelatin (Muyonga *et al*., 2004b).

The result reconfirmed the higher degradation of gelatin extracted at higher temperatures.

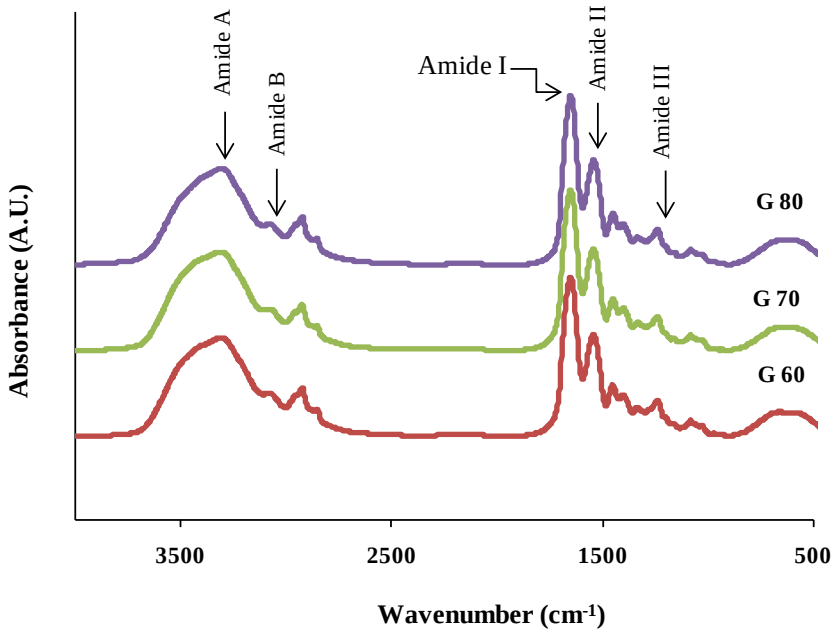

Figure 17. ATR-FTIR spectra of gelatins from the swim bladder of yellowfin tuna extracted at different temperatures. G60, G70 and G80 represent gelatin extracted from swim bladder at 60, 70 and 80 °C, respectively.

Amide-A band, arising from the stretching vibrations of the N-H group, appeared at 3338.3, 3339.1 and 3339.3 cm^{-1} for G60, G70 and G80, respectively. Amide-A represents NH-stretching coupled with hydrogen bonding. Normally, a free N-H stretching vibration is found in the range of 3400-3440 cm^{-1} (Muyonga *et al*., 2004b). When the N-H of a peptide is involved in a hydrogen bond, the position shifts to lower frequencies (Doyle *et al*., 1975). In amide-A region, the lower wavenumber was found in G60, suggesting the hydrogen bonding involvement of N-H in α-chain. On the other hand, the lower wavenumber with the concomitantly higher amplitude of amide-A observed in G80 could be associated with the higher degradation of gelatin and higher free amino groups. The amide B was observed at 3082.1, 3080.9 and 3081.8 cm^{-1} for G60, G70 and G80, respectively. Amide B corresponds to asymmetric stretch vibration of =C-H as well as $–NH_3^+$. Thus, the

secondary structure of gelatins obtained from the swim bladder of yellowfin tuna was affected to some degree by extraction temperature.

4.4.6 Gelling and melting temperatures

The gelling temperatures of all the gelatin samples were in the range of 11.07–15.24 °C (Table 9). Thermal transitions were monitored by changes in the phase angle (δ) of dissolved gelatins during cooling (35–5 °C) and subsequent heating (5–35 °C). It was found that G80 had the lowest gelling point (11.07 °C) ($P < 0.05$), while no difference in gelling point were observed between G60 and G70 ($P > 0.05$). In general, fish gelatin is not able to form gel at room temperature (Norland, 1990). It has been known that imino acid content is an essential factor governing gelation of gelatin (Gilsenan and Ross-murphy, 2000). However, the similar amino acid content was observed among all samples (169-172 residues/1000 residues). The result indicated that the gelling temperature was affected by the extraction temperature, more likely related with varying chain length. As a thermoreversible gel, gelatin gel starts melting when the temperature increases above a certain point, which is called the gel melting point (Karim and Bhat, 2009). The melting temperatures of gelatin gel from swim bladder were in the range of 20.36–22.33 °C. G80 had the lowest melting point (20.36 °C) ($P < 0.05$). Nevertheless, G60 and G70 showed similar melting points ($P > 0.05$). Typical melting points for fish gelatins ranged from 11 to 28 °C (Karim and Bhat, 2009). Gǿmez-guillen *et al*. (2002) reported that melting points of cod, hake, sole and megrim were 13.8, 14, 19.4 and 18.8 °C, respectively. Melting points of red and black tilapia skin gelatins were 22.4 and 28.9 °C, respectively (Jamilah and Harvinder, 2002). There was a relationship between melting point and molecular weight of gelatin. Low molecular weight gelatins melt at lower temperature than high molecular weight counterparts (Gilsenan and Ross-murphy, 2000). The results suggested that lower melting point of G80 was attributed to the lower molecular weight of peptide chains. Temperature of the environment also affects the gelling and melting temperatures of gelatin (Gudmundsson, 2002). Poorer gel strength of G80 (Fig. 16) was in accordance with lower gelling and melting points.

Table 9. Gelling and melting temperatures and gel color of gelatin from the swim bladder of yellowfin tuna extracted at different temperatures

Samples	Melting point	Gelling point	Color			
	(C°)	(C°)	*L**	*a**	*b**	*ΔE**
G60	22.33±0.42^{A}	15.24±0.27^{A}	27.98±0.57^{C}	-2.07±0.02^{C}	8.21±0.11^{C}	66.09±0.57^{A}
G70	22.05±0.45^{A}	14.86±0.24^{A}	42.79±0.47^{B}	-0.76±0.10^{B}	16.79±0.24^{B}	53.39±0.42^{B}
G80	20.36±0.27^{B}	11.07±0.58^{B}	45.79±0.78^{A}	-0.34±0.05^{A}	19.03±0.20^{A}	51.32±0.80^{C}

Mean ±SD (n = 3).
Different uppercase superscripts in the same column indicate significant differences ($P < 0.05$).

4.4.7 Microstructures of gelatin gels

The microstructures of gelatin gels from swim bladder with different extraction temperatures are illustrated in Fig. 18. In general, the conformation and chain length of the proteins in gel matrix directly contributed to the gel strength of gelatin (Benjakul *et al*., 2009). Gelatin extracted at 60 °C showed the finest gel network with small voids. Conversely, the coarser networks with the larger voids were found in gel of the gelatin extracted at higher temperatures.

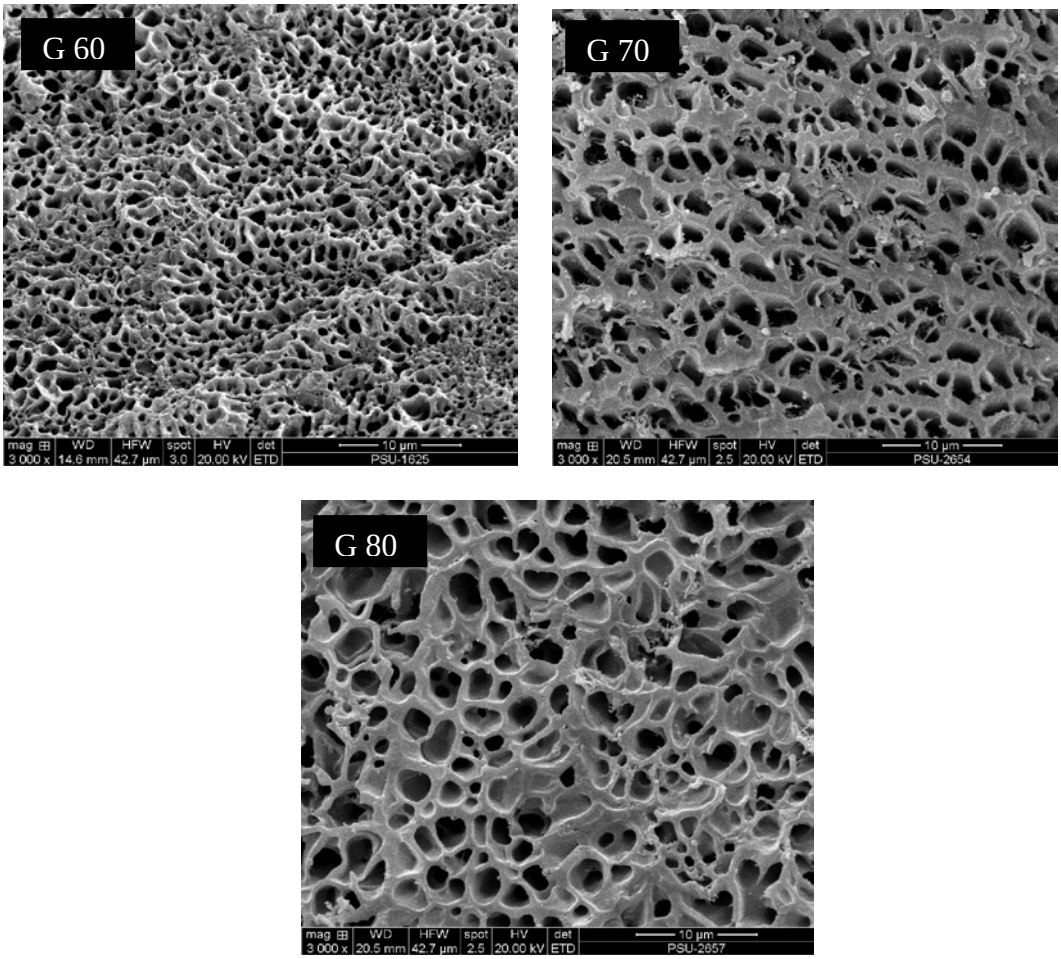

Figure 18. Microstructures of gelatin gel from the swim bladder of yellowfin tuna extracted at different temperatures. Magnification: 3000 x. G60, G70 and G80 represent gelatin extracted from swim bladder at 60, 70 and 80 °C, respectively.

The fine gel structure of gelatin extracted at lower temperature was in accordance with the higher gel strength (Fig. 17). It has been known that the microstructure of the gel is related to the physical properties. The gelatin gel network was governed by the pretreatment conditions (Yang *et al*., 2008) and gelatin concentration (Yang and Wang, 2009). Gelatin extracted at lower temperatures had the lower degradation, in which proteins with higher chain length were present. As a result, junction zones could be formed to a greater extent. This led to the high aggregation with a strong and ordered network. In the first stage of gel network formation, there is competition between intramolecular folding and intermolecular aggregate formation (Yang and Wang, 2009). For gelatin extracted at lower temperature, longer chains might undergo aggregation to a higher extent. Thus, the arrangement of peptides in the network during gelation as determined by chain length directly affected gel properties of gelatin.

4.4.8 Color

Color of the gelatin gel from swim bladder with different extraction temperatures expressed as *L**, *a** and *b** is shown in Table 9. Gel of gelatin extracted at lower temperatures (G60) showed the lower *L**-value (lightness) than others (G70 and G80) ($P < 0.05$). The higher redness (*a**-value) and yellowness (*b**-value) were found in the latters ($P < 0.05$). Generally, the increases in *L**, *a** and *b**-value of gelatin increased with increasing extraction temperatures. For yellowness (*b**-value), an increase was observed in all gelatin gels when the extraction temperatures increased ($P < 0.05$). This might be due to a non-enzymatic browning reaction taken place at the higher temperature, especially when the extraction time increased (Ajandouz and Puigserver, 1999). Among all the gelatin samples, those extracted at a lower temperature (60 °C) showed the highest total difference in the color value (*ΔE**) (66.09) with the lowest lightness (*L**-values). These results showed that the extraction temperatures had the impact on color of gelatin extracted from the swim bladder of yellowfin tuna.

4.5 Conclusion

Swim bladder from yellowfin tuna could be an alternative source of gelatin. Gelatin extracted at a higher temperature had the highest extraction yield, but possessed the poorer gel properties. Extraction conditions also affected the color of resulting gelatin. The appropriate extraction temperature for gelatin from swim bladder was 60 °C, providing the highest gel strength.

4.6 References

Aewsiri, T., Benjakul S., Visessanguan, W. and Tanaka, M. 2008. Chemical compositions and functional properties of gelatin from pre-cooked tuna fin. Int. J. Food Sci. Technol. 43: 685-693.

Ajandouz, E. H. and Puigserver, A. 1999. Nonenzymatic browning reaction of essential amino acids: effect of pH on caramelization and Maillard reaction kinetics. J. Agric. Food Chem. 47: 1786-1793.

AOAC. 2000. Official methods of analysis. Arlington: Association of Official Analytical Chemists Inc.

Balti, R., Jridi, M., Sila, A., Souissi, N., Nedjar-Arroume, N., Guillochon, D. and Nasri, M. 2011. Extraction and functional properties of gelatin from the skin of cuttlefish (*Sepia officinalis*) using smooth hound crude acid protease-aided process. Food Hydrocolloid. 25: 943-950.

Benjakul, S., Kittipahattanabawon, P. and Regenstein, J. M. 2012. Fish gelatin. *In* Food Biochemistry and Food Processing. (Simpson, B. K., Nollet, L. M. L., Toldrá. F., Benjakul, S., Paliyath, G. and Hui, Y. H., eds.). p. 388-405. John Wiley & Sons, Inc. Ames.

Benjakul, S., Oungbho, K., Visessanguan, W., Thiansilakul, Y. and Roytrakul, S. 2009. Characteristics of gelatin from the skins of bigeye snapper, *Priacanthus tayenus* and *Priacanthus macracanthus*. Food Chem. 116: 445-451.

Binsi, P. K., Shamasundar, B. A., Dileep, A. O., Badii, F. and Howell, N. K. 2009. Rheological and functional properties of gelatin from the skin of Bigeye snapper (*Priacanthus hamrur*) fish: Influence of gelatin on the gel-forming ability of fish mince. Food Hydrocolloid. 23: 132-145.

Boran, G., Mulvaney, S. J. and Regenstein, J. M. 2010. Rheological properties of gelatin from silver carp skin compared to commercially available gelatins from different sources. J. Food Sci. 75: E565-E571.

Duan, R., Zhang, J., Xing, F., Konno, K. and Xu, B. 2011. Study on the properties of gelatins from skin of carp (*Cyprinus carpio*) caught in winter and summer season. Food Hydrocolloid. 25: 368-373.

Foegeding, E. A., Lanier, T. C. and Hultin, H. O. 1996. Characteristics of edible muscle tissues. *In* Food Chemistry. (Fennema, O. R., ed.). p. 879-942. Marcel Dekker. New York.

Gennadios, A., Weller, C. L., Hanna, M. A. and Froning, G. W. 1996. Mechanical and barrier properties of egg albumen films. J. Food Sci. 61: 585-589.

Gilsenan, P. and Ross-Murphy, S. 2000. Rheological characterisation of gelatins from mammalian and marine sources. Food Hydrocolloid. 14: 191-195.

Giménez, B., Turnay, J., Lizarbe, M., Montero, P. and Gómez-Guillén, M. 2005. Use of lactic acid for extraction of fish skin gelatin. Food Hydrocolloid. 19: 941-950.

Gómez-Guillén, M. C., Giménez, B., López-Caballero, M. E. and Montero, M. P. 2011. Functional and bioactive properties of collagen and gelatin from alternative sources: A review. Food Hydrocolloid. 25: 1813-1827.

Gómez-Guillén, M. C., Pérez-Mateos, M., Gómez-Estaca, J., López-Caballero, E., Giménez, B. and Montero, P. 2009. Fish gelatin: a renewable material for developing active biodegradable films. Trends Food Sci. Technol. 20: 3-16.

Gómez-Guillén, M., Turnay, J., Fernández-Dıaz, M., Ulmo, N., Lizarbe, M. and Montero, P. 2002. Structural and physical properties of gelatin extracted from different marine species: a comparative study. Food Hydrocolloid. 16: 25-34.

Gudmundsson, M. 2002. Rheological properties of fish gelatins. J Food Sci. 67: 2172-2176.

Holzer, D. 1996. Gelatin production. US patent. 5,484,888.

Jackson, M., Choo, L., Watson, P. H., Halliday, W. C. and Mantsch, H. H. 1995. Beware of connective tissue proteins: assignment and implications of collagen absorptions in infrared spectra of human tissues. Biochim. Biophy. Acta. 1270: 1-6.

Jamilah, B. and Harvinder, K. 2002. Properties of gelatins from skins of fish-black tilapia (*Oreochromis mossambicus*) and red tilapia (*Oreochromis nilotica*). Food Chem. 77: 81-84.

Jellouli, K., Balti, R., Bougatef, A., Hmidet, N., Barkia, A. and Nasri, M. 2011. Chemical composition and characteristics of skin gelatin from grey triggerfish (*Balistes capriscus*). LWT-Food Sci. Technol. 44: 1965-1970.

Kaewdang, O., Benjakul, S., Kaewmanee, T. and Kishimura, H. 2014. Characteristics of gelatin extracted from the swim bladder of yellowfin tuna (*Thunnus albacores*) as affected by alkaline pretreatments. Ital. J. Food Sci. (Accepted).

Kaewruang, P., Benjakul, S., Prodpran, T. and Nalinanon, S. 2013. Physicochemical and functional properties of gelatin from the skin of unicorn leatherjacket (*Aluterus monoceros*) as affected by extraction conditions. Food Biosci. 2: 1-9.

Karim, A. and Bhat, R. 2009. Fish gelatin: properties, challenges, and prospects as an alternative to mammalian gelatins. Food Hydrocolloid. 23: 563-576.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W. and Shahidi, F. 2010. Comparative study on characteristics of gelatin from the skins of brownbanded bamboo shark and blacktip shark as affected by extraction conditions. Food Hydrocolloid. 24: 164-171.

Klomklao, S., Benjakul, S. and Visessanguan, W. 2004. Comparative studies on proteolytic activity of splenic extract from three tuna species commonly used in Thailand. J. Food Biochem. 28: 355-372.

Kong, J. and Yu, S. 2007. Fourier transform infrared spectroscopic analysis of protein secondary structures. Biochim. Biophys. Acta. 39: 549-559.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage. T4. Nature. 227: 680-685.

Montero, P. and Gómez-Guillén, M. C. 2000. Extracting conditions for megrim (*Lepidorhombus boscii*) skin collagen affect functional properties of the resulting gelatin. J. Food Sci. 65: 434-438.

Muyonga, J., Cole, C. and Duodu, K. 2004a. Extraction and physico-chemical characterisation of Nile perch (*Lates niloticus*) skin and bone gelatin. Food Hydrocolloid. 18: 581-592.

Muyonga, J., Cole, C. and Duodu, K. 2004b. Fourier transform infrared (FTIR) spectroscopic study of acid soluble collagen and gelatin from skins and bones of young and adult Nile perch (*Lates niloticus*). Food Chem. 86: 325-332.

Nagarajan, M., Benjakul, S., Prodpran, T., Songtipya, P. and Kishimura, H. 2012. Characteristics and functional properties of gelatin from splendid squid (*Loligo formosana*) skin as affected by extraction temperatures. Food Hydrocolloid. 29: 389-387.

Norland, R. E. 1990. Fish gelatin. *In* Fisheries Technology and Biotechnology for Increased Profitability. (Voight, M. N. and Botta, J. K., eds.) p. 325-333. Teechnomic Publishing Co., Lancaster, PA.

Norziah, M., Al-Hassan, A., Khairulnizam, A., Mordi, M. and Norita, M. 2009. Characterization of fish gelatin from surimi processing wastes: Thermal analysis and effect of transglutaminase on gel properties. Food Hydrocolloids. 23: 1610-1616.

Regenstein, J. M. and Zhou, P. 2007. Collagen and gelatin from marine by-products. *In* Maximising the Value of Marine By-products. (Shahidi, F., ed.). p. 279-303. Woodhead Publishing Limited. Cambridge.

Silva, R. S., Bandeira, S. F. and Pinto, L. A. 2014. Characteristics and chemical composition of skins gelatin from cobia (*Rachycentron canadum*). LWT-Food Sci. Technol. 57: 580-585.

Shyni, K., Hema, G. S, Ninan, G., Mathew, S., Joshy, C. and Lakshmanan, P. 2014. Isolation and characterization of gelatin from the skins of skipjack tuna (*Katsuwonus pelamis*), dog shark (*Scoliodon sorrakowah*), and rohu (*Labeo rohita*). Food Hydrocolloids. 39: 68-76.

Sinthusamran, S., Benjakul, S. and Kishimura, H. 2014. Characteristics and gel properties of gelatin from skin of seabass (*Lates calcarifer*) as influenced by extraction conditions. Food Chem. 152: 276-284.

Steel, R. G. D. and Torrie, J. H. 1980. Principles and procedures of statistics: A biometrical approach 2nd ed. McGraw-Hill. New York.

Uriarte-Montoya, M. H., Santacruz-Ortega, H., Cinco-Moroyoqui, F. J., Rouzaud-Sández O., Plascencia-Jatomea, M. and Ezquerra-Brauer, J. M. 2011. Giant squid skin gelatin: chemical composition and biophysical characterization. Food Res. Int. 44: 3243-3249.

Weber, P., Steinhart, H. and Paschke, A. 2009. Competitive indirect ELISA for the determination of parvalbumins from various fish species in food grade fish gelatins and isinglass with PARV-19 anti-parvalbumin antibodies. J. Agric. Food Chem. 57: 11328-11334.

Yang, H. and Wang, Y. 2009. Effects of concentration on nanostructural images and physical properties of gelatin from channel catfish skins. Food Hydrocolloid. 23: 577-584.

Yang, H., Wang, Y., Zhou, P. and Regenstein, J. M. 2008. Effects of alkaline and acid pretreatment on the physical properties and nanostructures of the gelatin from channel catfish skins. Food Hydrocolloids. 22: 1541-1550.

CHAPTER 5

Effect of ethanolic extract of coconut husk on gel properties of gelatin from swim bladder of yellowfin tuna

5.1 Abstract

The impacts of ethanolic extract from coconut husk (EECH) rich in tannic acid at different levels (0.25, 0.5, 0.75, 1, 2, 3 and 5 mg/g dry gelatin) on gel properties of gelatin from yellowfin tuna swim bladder were investigated. Gel strength of gelatin increased when EECH concentrations increased up to 0.5 mg/g ($P < 0.05$). Nevertheless, the gradual decrease in gel strength was found with increasing EECH levels. When EECH at different levels was incorporated, no marked changes in protein patterns determined by SDS-PAGE were observed, suggesting that most of bondings were hydrogen bond or other weak bonds. Gel matrix with uniformity and larger strands were observed with gels added with 0.5 EECH mg/g. Gelling and melting temperatures were also increased when EECH at a level of 0.5 mg/g was incorporated. The color of all gelatin gels slightly increased with increasing concentrations of EECH. Therefore, ethanolic extract from coconut husk at an appropriate level could act as a natural gel enhancer of gelatin.

5.2 Introduction

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). However, the outbreaks of bovine spongiform encephalopathy (BSE), foot-and-mouth disease (FMD) as well as bird flu have resulted in the 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 religious objections (Sadowska *et al*., 2003). The gelatin industry primarily uses mammalian skins and bones as raw materials. Byproducts from fish processing, such as fish skins have been recognized as a promising alternative material for gelatin extraction (Yang *et al*., 2007). The effects of the extraction conditions on gelatin yield and properties have

been reported for the skins of many fish species, including African catfish (Alfaro *et al*., 2014), cobia (Silva *et al*., 2014), unicorn leatherjacket (Kaewruang *et al*., 2013) and tilapia (Niu *et al*., 2013). Recently, Kaewdang *et al*. (2014) successfully extracted gelatin from swim bladder of yellowfin tuna using an alkaline pretreatment. However, gelatin from swim bladder showed low gel strength. Generally, gelatins of fish origin have poorer gel strength, compared with mammalian counterpart, due to their lower imino acid content (Grossman and Bergman, 1992). Therefore, the modification of fish gelatin has been developed gradually to conquer the problem. Chemical and physical treatments can be applied to modify the gelatin network through cross-linking of the gelatin chains to improve gel properties (Cao *et al*., 2007). Cross-linking agents including glutaraldehyde, genipin, carbodiimides, calcium salts and transglutaminase have been used to cross-link gelatin, (Chiou *et al*., 2006; Benjakul and Visessanguan, 2003). Physical treatments, such as UV- and γ-irradiation (Chambi and Grosso, 2006) and high pressure technology (Montero *et al*., 2002) have been also applied to enhance gelling property of gelatin.

Plant phenolics are defined as compounds possessing one or more aromatic ring bearing a hydroxyl substituent(s), and can be found in many foods and drinks from plant origin, e.g. fruits, vegetables, coffee (Parr and Bolwell, 2000). Phenolic compounds can interact with proteins through non-covalent and covalent interaction (Maqsood *et al*., 2013). Covalent bonding seems to play an important role in protein-phenol interaction, which is used to improve functional properties of proteins (Sarker *et al*., 1995). Coconut is considered as an important crop in tropical countries. Coconut husk is one of the major agro-industrial waste generated in the developing countries each year. A portion of this waste material is processed and used by the rope-making industry, but the majority remains unutilized (Dey *et al*., 2003). Coconut husk is the major sources of carbohydrate and phenolics (Suzuki *et al*., 1998; Sueli and Gustavo, 2007). Temdee and Benjakul (2014) used the extracts from kiam wood and cashew bark rich in tannic acid as the gel enhancer for gelatin. Phenolics in coconut husk could be used as the alternation protein cross-linker, which strengthens the gel network of gelatin, particularly from swim bladder. Therefore, the present study aimed to investigate the impact of the extract from coconut husk rich in

phenolic on the gel properties of gelatin extracted from swim bladder of yellowfin tuna.

5.3 Materials and methods

5.3.1 Chemicals

All chemicals were of analytical grade. Sodium dodecyl sulfate (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). Tannic acid was obtained from Sigma Chemical, Ltd, St. Louis, MO, USA with a purity of 98 %.

5.3.2 Collection and preparation of swim bladder

Swim bladders of yellowfin tuna (*Thunnus albacares*) were obtained from Tropical Canning Public Co., Ltd., Songkhla, Thailand. Swim bladders with the length of 8-12 cm were placed in polyethylene bags, inserted in ice using a sample/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Songkhla. Upon arrival, swim bladders were washed with tap water and cut into pieces with the length of approximately 2 cm. The prepared samples were then placed in polyethylene bag and frozen at -20 °C. The samples were stored at -20 °C until used. The storage time was not longer than 3 months. Prior to extraction, frozen swim bladders were thawed using a running water until the temperature was 0-2 °C.

5.3.3 Extraction of gelatin from swim bladder

Firstly, swim bladders were pretreated with alkaline solution as per the method of Kaewdang *et al*. (2014). Prepared swim bladders were added with the mixed alkaline solution (Na_2CO_3: NaOH ; 7:3) having the concentration of 4 % (w/v) at a ratio of 1:10 (w/v). The mixture was stirred for 12 h at room temperature (28–30 °C) using an overhead stirrer model W20.n (IKA®-Werke GmbH & CO.KG,

Stanfen, Germany). The alkaline solution was changed every 6 h. The pretreated samples were washed with tap water until a neutral or faintly basic pH was obtained.

To extract gelatin, alkali-pretreated samples were immersed in distilled water (60 °C) using a swim bladder/water ratio of 1:5 (w/v) in a temperature-controlled water bath (W350, Memmert, Schwabach, Germany). The extraction was performed for 24 h with a continuous stirring at a speed of 150 rpm. The mixture was then filtered using a Buchner funnel with a Whatman No. 4 filter paper (Whatman International, Ltd., Maidstone, England). The filtrate was freeze-dried using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The dried gelatin was placed in polyethylene bag and kept at 4 °C.

5.3.4 Preparation of coconut husk

5.3.4.1 Collection and preparation of coconut husk

Coconut husk was obtained from a local market in Hat Yai, Songkhla, Thailand and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand. Husk was prepared following the method of Vazquez-Torres *et al*. (1992) with slight modifications. Husk sample was dried at 60 °C in the cabinet rotary dryer for 16 h and then defibered. Husk sample was then subjected to grinding using a mill (IKA Labortechnik colloid mill, Selangor, Malaysia). The prepared sample was then sieved with the aid of sieve shaker (Model EVJ1, Endecotts Ltd., London, UK) having a sieve size of 6 mm (Woven wire sieves, Endecotts Ltd., London, UK). The coarse form was further blended using a blender (Panasonic, Model MX-898N, Berkshire, UK) and finally sieved to 80 mesh. The coconut husk powder obtained was further dried in a hot air oven (Memmert, Schwabach, Germany) at 105 °C overnight. The obtained powder was placed in a polythene bag, sealed and kept at room temperature until use.

5.3.4.2 Preparation of ethanolic extract from coconut husk

Coconut husk powder was subjected to extraction according to the method of Santoso *et al*. (2004) with a slight modification. Ten grams of husk powder were mixed with 250 ml of 80 % ethanol (w/v). The mixture was stirred at room

temperature (28–30 °C) using a magnetic stirrer (IKA-Werke, Staufen, Germany) for 3 h. The mixture was then centrifuged at 5000 x g for 30 min at room temperature using a RC-5B plus centrifuge (Beckman, JE-AVANTI, Fullerton, CA, USA). The supernatant was filtered using a Whatman No. 1 filter paper (Whatman International, Ltd., Maidstone, England). The filtrate was then evaporated at 40°C using an EYELA rotary evaporator N-1000 (Tokyo Rikakikai, Co. Ltd., Tokyo, Japan). To remove the residual ethanol, the extract was purged with nitrogen gas. The extract was then dried using a Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lynge, Denmark). Dried extract was powdered using a mortar and pestle and was kept in an amber bottle and stored in a desiccator at room temperature (28-30 °C) until use. The obtained powder was referred to as “ethanolic extract from coconut husk, EECH”.

5.3.4.3 Determination of total phenolic content

Total phenolic content was determined using a colorimetric method using the Folin–Ciocalteau reagent (Singleton *et al*., 1999). EECH was dissolved in ethanol to obtain a concentration of 0.5 mg/ml. Aliquot (100 µl) was mixed with 2.0 ml of 2 % sodium carbonate solution. One hundred µl of the Folin–Ciocalteau reagent (diluted 1:1 with water) were added and vortexed using a vortex Genie2 mixer (Scientific Industries, Bohemia, NY, USA) for 30 s. After 30 min, the absorbance of the resulting mixture was measured at 760 nm using a spectrophotometer (Model UV 1800, Shimadzu, Kyoto, Japan). Total phenolic content in EECH was calculated from a standard curve of tannic acid (0.01-0.1 mg/l) and expressed as g tannic acid equivalent/kg dry matter (Singleton *et al*., 1999).

5.3.4.4 Determination of tannic acid content

Tannic acid in EECH was determined using an HPLC equipped with a variable wavelength detector (VWD) following the method of Tian *et al*. (2009) with slight modifications. The HPLC system consisted of an Agilent 1100 series HPLC (Alginet, Wilmington, DE, USA), quaternary pump with the seal wash option, degasser, solvent, cabinet and preparative auto-sampler with thermostat equipped with a diode array detector. The separations were done using a Hypersil ODS C18 4.0 x 250 mm, 5 µm column (Cole-Parmer, London, UK). HPLC conditions were as

follows: mobile phase: 0.4 % formic acid: acetonitrile (85: 15), flow rate: 0.8 ml/min, temperature: 25 °C. Detection was carried out at 280 mm. The concentration of extracts was 25 mg/ml and each injection volume was 20 µl. Standard tannic acid was used for peak identification.

5.3.5 Preparation of gelatin gels containing EECH

The gelatin with the concentration of 6.67 % (w/v) was dissolved in 40 °C water bath with the aid of mechanical stirring until it was completely dissolved. To gelatin solution, EECH was added to obtain the final concentrations of 0.25, 0.5, 0.75, 1, 2, 3 and 5 mg/g dry gelatin. The mixtures were stirred using a magnetic stirrer (IKA Labortechnik stirrer, Selangor, Malaysia) for 1 h at room temperature (28–30 °C), followed by setting at 10 °C for 16–18 h. The resulting gels were subjected to analyses.

5.3.5.1 Determination of gel strength

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

5.3.5.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS–PAGE was performed by the method of Laemmli (1970). Gel 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). The mixtures were centrifuged at 8,500 g for 5 min using a microcentrifuge (MIKRO20, Hettich Zentrifugan, 170 Germany) to remove undissolved debris. Solubilized samples were mixed at 1:1 (v/v) ratio with the 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, the 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 a mixture of 50 % (v/v) methanol and 7.5 % (v/v) acetic acid for 30 min and destained again with a mixture of 5 % (v/v) methanol and 7.5 % (v/v) acetic acid for 1 h. High-molecular-weight protein markers were used to estimate the molecular weight of proteins.

5.3.5.3 Determination of color of gelatin gel

The color of gelatin gels was determined using 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 for 10 min and calibrated with a white standard.

The 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 the white standard (L^* = 93.6, a^*= -0.94 and b^* = 0.40).

5.3.6 Characterization of gelatin and gel incorporated with the selected EECH levels

5.3.6.1 Determination of gelling and melting temperatures

Gelling and melting temperatures of gelatin solutions containing EECH at the selected concentrations (0.5 and 5 mg/g) were measured following the method of Boran *et al*. (2010) using a controlled stress rheometer (RheoStress 1, HAAKE, Karlsruhe, Germany). The gelatin solutions (6.67 %, w/v) without and with EECH were prepared in the same manner as described previously. The solution was preheated at 35 °C for 30 min. The measuring geometry included a 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 measured, where tan δ became 1 or δ was 45°.

5.3.6.2 Determination of microstructure

The microstructure of gelatin gel without and with EECH (0.5 and 5 mg/g) were 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 a serial concentration of 25, 50, 70, 80, 90 and 100 % (v/v). Thereafter, samples were critical point dried. 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.

5.3.7 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 a 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).

5.4 Results and discussion

5.4.1 Composition of EECH

EECH had total phenolic content of 443.4 ± 1.47 g tannic acid equivalent/kg. Thus, coconut husk was an alternative source of phenolic compounds. Temdee and Benjakul (2014) reported that total phenolic contents of the extracts from kiam wood and cashew bark were 439 and 254 g tannic acid equivalent/kg, respectively. The differences in phenolic content might be attributed to the variation in tree species, plant nutrition and stage of plant growth (Tian *et al*., 2009).

As analyzed using the HPLC-diode array detector, tannic acid was found to be the major component in EECH (Fig. 19.) EECH contained tannic acid at

29.88 ± 1.01 g tannin/kg of dry wood extract. Ethanolic kiam wood and cashew bark extracts had tannic acid contents of 193 and 74.6 g/kg dry wood extract, respectively (Temdee and Benjakul, 2014). Woods of different trees mainly contain tannins (Fradinho *et al*., 2002). Apart from tannin, other components such as resin, lignan or lignin are also found in the wood or bark of trees (Akinpelu, 2001; Balange and Benjakul, 2009). The result indicated that coconut husks a waste from coconut, could serve as a source of tannic acid.

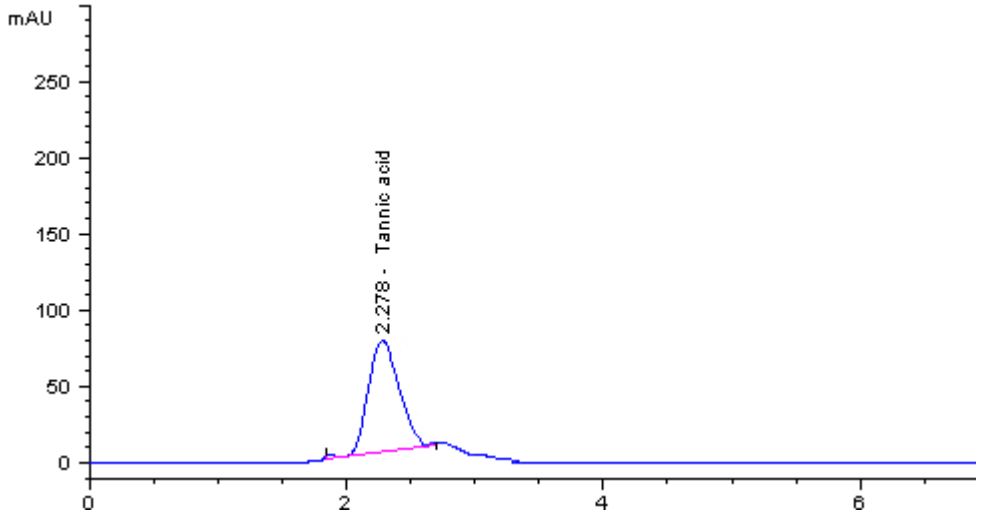

Figure 19. HPLC-diode array detector chromatogram of EECH

5.4.2 Effect of EECH addition on properties of gelatin from swim bladder

5.4.2.1 Gel strength

Gel strength is one of the important properties of gelatin gels, and the specific application of gel is determined by the range of gel strength values (Cho *et al*., 2005). The gel strength of gelatins from swim bladder of yellowfin tuna added with EECH at different concentrations is shown in Fig. 20. Gelatin gel showed the increase in gel strength as EECH levels increased up to 0.5 mg/g dry gelatin ($P < 0.05$). The gel strength of gelatin added with EECH at a level of 0.5 mg/g dry gelatin had the increase by 41.67 % ($P < 0.05$), compared with that of control. However, the lowered gel strength was observed as EECH concentration was above

0.5 mg/g dry gelatin ($P < 0.05$). The decrease in gel strength was in descending order when EECH levels increased from 0.75 to 5 mg/g dry gelatin ($P < 0.05$). These results indicate that phenolic compounds at the optimum concentration showed the enhancing effect on gel strength of gelatin from yellowfin tuna swim bladder. Nevertheless, the excessive amount of EECH containing tannic acid negatively affected gel strength of gelatin.

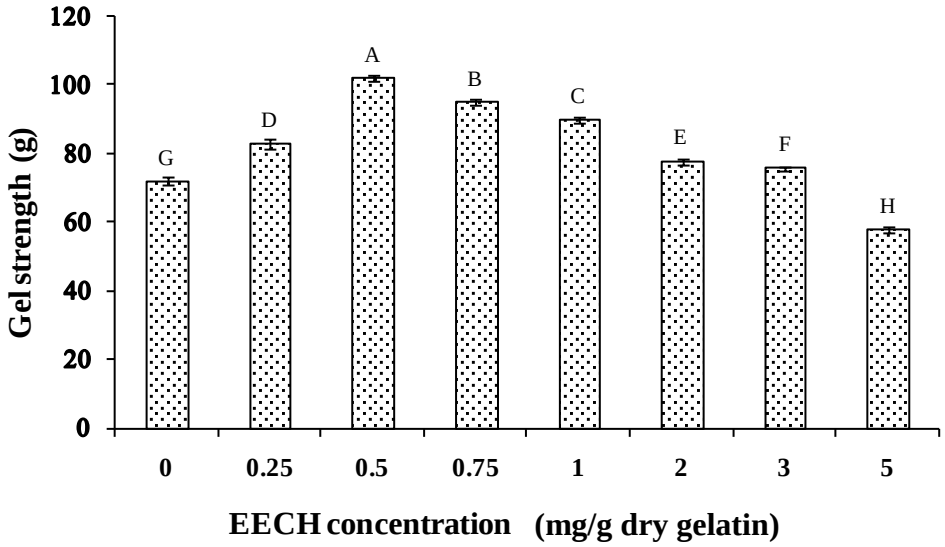

Figure 20. Gel strength of gelatin from the swim bladder of yellowfin tuna without and with EECH at different concentrations. Different uppercase letters on the bars denote significant differences ($P < 0.05$). Bars represent the standard deviations ($n = 3$).

Silber *et al*. (1998) postulated that protein could be precipitated by polyphenols when the number of polyphenol molecules interacted with a protein molecule at a critical level. The result was in accordance with Yan *et al*. (2011) who reported that gel strength of hydrogels cross-linked by gallic acid increased and then decreased as gallic acid concentration was above 20 mg/g dry gelatin. Among all gels, that incorporated with EECH at 5 mg/g dry gelatin exhibited the poorest gel strength ($P < 0.05$). At an appropriate level, phenolic compound, especially tannic acid, could induce the cross-linking, mainly via hydrogen bond or hydrophobic interaction. Such cross-links formed contributed to the stronger gel network as evidenced by the

increase in gel strength. Maqsood *et al*. (2013) reported that phenolic compound can act as network protein cross-linker. Excessive amount of EECH more likely induced the coagulation of gelatin, in which the ordered structure could not be formed. Similar result was found by Temdee and Benjakul (2014). Gelatin from cuttlefish skin had the decrease in gel strength when levels of ethanolic extracts from kiam wood and cashew bark increased. Therefore, EECH showed the gel strengthening effect when added at a level of 0.5 mg/g dry gelatin.

5.4.2.2 Protein patterns

Protein patterns of swim bladder gelatin gel incorporated with EECH at different concentrations are shown in Fig. 21. All samples had α-chains as the major components. Proteins with MW of 65 and 75 kDa were also found in gelatin gel samples. It was noted that the addition of EECH had no impact on protein patterns of gelatin gel, regardless of levels of EECH added. Although gel strength was increased as EECH at an appropriate level was incorporated, no changers in protein patterns were noticeable. EECH used in the present study was not oxidized to quinone. As a result, hydroxyl groups of tannic acid were still remained. Those hydroxyl groups of tannic acid in the reduced from could serve as hydrogen donor to gelatin chains, in which H-bonds were formed. Since tannic acid is a complex phenol with several hydroxyl groups per one molecular (Van *et al*., 1969), it could cross-link gelatin chains to form strong net work. However, under the condition for electrophoresis, SDS was able to destroy those weak bonds. As a consequence, there was no difference in protein patterns among gelatin gel added without and with EECH at various levels. Phenolic compounds have been oxidized under alkaline condition in the presence of oxygen (Strauss and Gibson, 2004). The quinone formed could induce the cross-linking of proteins such as gelatin (Temdee and Benjakul, 2014) or surimi (Balange and Benjakul, 2009). To shorten the preparation process and to reduce chemical used, the use of EECH without oxidation process could be a better choice for application, especially for human consumption.

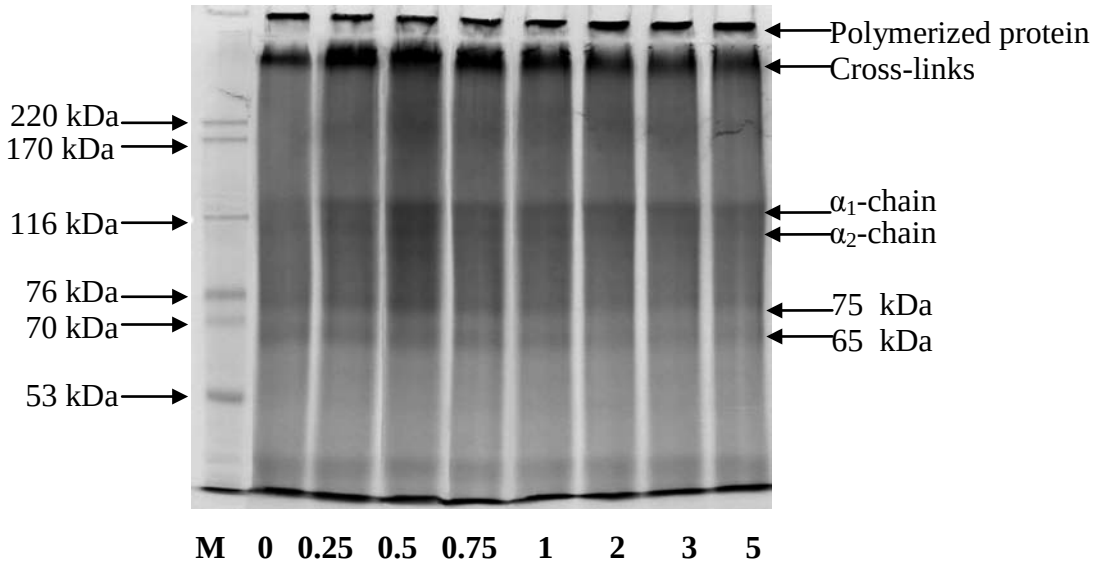

Figure 21. Protein patterns of gelatin gels from the swim bladder of yellowfin tuna without and with EECH at different concentrations. M: high molecular weight markers. Numbers denote the EECH concentration (mg/g dry gelatin).

5.4.2.3 Color

Lightness (*L**), redness (*a**) and yellowness (*b**) of gelatin gels without and with the addition of EECH (0.25-5 mg/g dry gelatin) are shown in Table 10. The decreases in *L** -values of gelatin gels were observed as the levels of EECH increased ($P < 0.05$). The coincidental increases in *a** and *ΔE** -values were noticeable as EECH levels increased ($P < 0.05$). The results indicated that the increased redness with the darker color of gelatin gels was obtained when EECH was added at high levels. O'connell and Fox (2001) reported that phenolic compounds were responsible for discoloration in cheese products. Temdee and Benjakul (2014) reported that gelatin gels from cuttlefish were darker and more redder when the levels of oxygenated ethanolic kiam wood extract and ethanolic cashew bark extract increased. The polymerized phenolic compound, especially tannin with its dark color most likely contributed to the dark color of gels. In the present study, the EECH was not oxidized and color was lighter than oxidized counterpart (data not shown). With the addition of EECH at a level of 0.5 mg/g dry gelatin, no change in *L**- value was observed, compared with that of the control ($P > 0.05$). Only slight increase in *a**- and

*ΔE** values were found. Thus, EECH could be used to increase gel strength of gelatin from yellowfin tuna swim bladder without the drastic changes in color of resulting gelatin gel.

Table 10. Color of gelatin gels from the swim bladder of yellowfin tuna without and with EECH (mg/g dry gelatin) at different concentrations

Samples	Color parameters			
	L*	***a****	***b****	***ΔE****
Control	28.41 ± 0.06^{A}	-2.09 ± 0.05^{F}	8.10 ± 0.23^{E}	65.65 ± 0.09^{F}
0.25	28.25 ± 0.31^{A}	3.38 ± 0.21^{E}	7.42 ± 0.14^{F}	65.86 ± 0.31^{EF}
0.5	28.14 ± 0.11^{A}	3.34 ± 0.02^{E}	7.49 ± 0.05^{F}	65.98 ± 0.11^{E}
0.75	28.23 ± 0.03^{A}	3.53 ± 0.16^{E}	8.28 ± 0.19^{E}	65.99 ± 0.03^{E}
1	27.81 ± 0.07^{A}	3.76 ± 0.07^{D}	12.56 ± 0.19^{D}	67.07 ± 0.04^{D}
2	27.69 ± 0.27^{AB}	4.18 ± 0.03^{C}	14.44 ± 0.11^{C}	67.58 ± 0.28^{C}
3	22.65 ± 0.07^{B}	5.03 ± 0.16^{B}	10.57 ± 0.33^{B}	71.92 ± 0.08^{B}
5	21.85 ± 0.16^{C}	8.10 ± 0.01^{A}	11.84 ± 0.12^{A}	73.22 ± 0.15^{A}

Mean ±SD (n = 3).
Different uppercase superscripts in the same column indicate significant differences ($P < 0.05$).

5.4.3 Characteristic of gelatin and gel incorporated with the selected EECH levels

5.4.3.1 Gelling and melting points

Thermal transitions of swim bladder in the presence of EECH at 0.5 or 5 mg/g were monitored by changes in the phase angle (δ) of gelatin solution during cooling (35–5 °C) and subsequent heating (5–35 °C). The gelling and melting point of gelatin as affected by the addition of EECH are shown in Fig. 22. Gelatin solution (control) and those added with EECH at levels of 0.5 and 5 mg/g became gel at 15.53, 16.43 and 14.36 °C and subsequently molt at 22.05, 23.47 and 21.86 °C, respectively. It was found that gelatin containing 5 mg EECH/g had the lowest gelling and melting point. Gelling point became lower when EECH at high level (5 mg/g) was

incorporated. This was coincidental with the lower melting point. The result suggested that the gelatin might undergo coagulation.

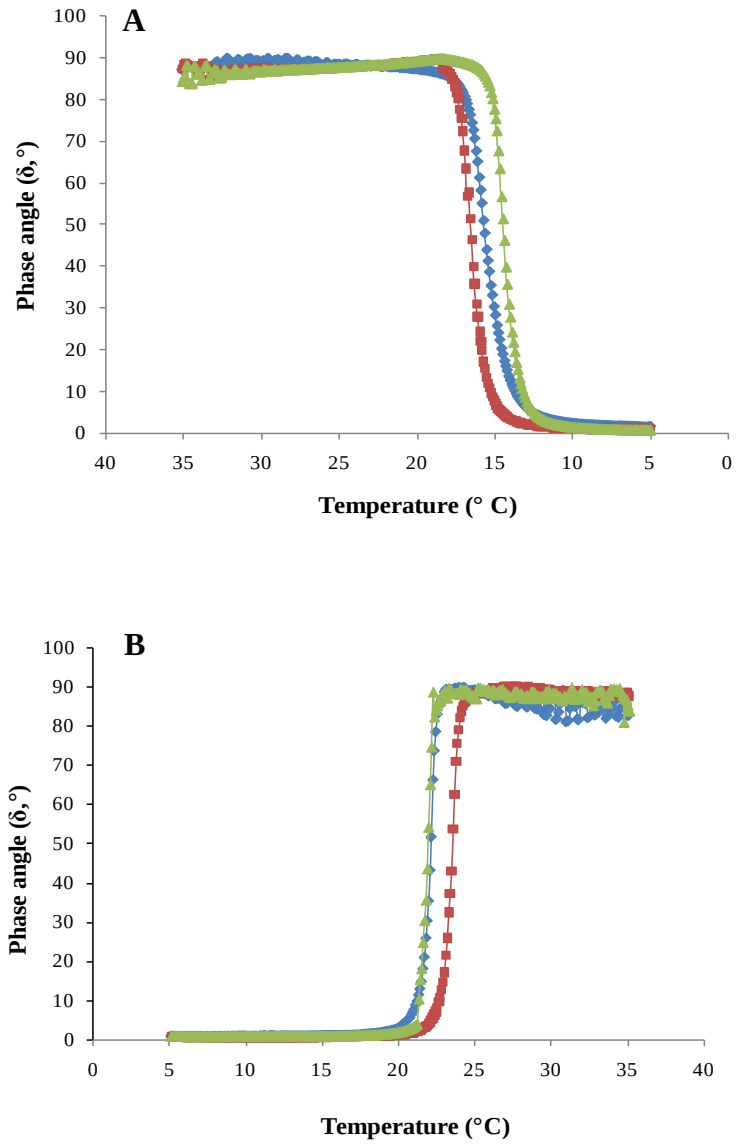

Figure 22. Changes in phase angle (δ,°) of gelatin solution (6.67 %, w/v) from the swim bladder of yellowfin tuna without and with EECH at different concentrations during cooling (A) and subsequent heating (B).

Such coagulated chains were not able to gel properly and required lower temperature for gelation. Such a gel was also not resistant to heat as indicated by lower melting temperature. In general, the increases of gelling and melting are due

to the increasing number of chemical junctions responsible for the formation of the amide bonds (Saito *et al*., 2007). Yan *et al*. (2011) reported that gelatin gels from walleye pollock skin added with gallic aicd (10-40 mg/g) and rutin (2-8 mg/g) had gelling temperature of 4-6°C and the melting temperature of 11-13 °C. Therefore, the addition of EECH at an appropriate level could increase gelling point, in which gel could be formed easier than the control. Also gel was maintained for a longer time as indicated by the higher melting temperature. Generally, the melting point was higher than the gelling point because gelatin gel absorbed energy when it was melting (Liu and Guo, 2008).

5.4.3.2 Microstructure of gelatin gels

Microstructures of gels of swim bladder gelatins without EECH (A) and those added with EECH at levels of 0.5 mg/g dry gelatin (B) and 5 mg/g dry gelatin (C) are illustrated in Fig. 23. All gelatin gels were sponge or coral-like in structure. Generally, the arrangement and association of protein molecules in the gel matrix directly contributed to the gel strength of the gelatin (Benjakul *et al*., 2009). The control gel showed the finest gel network with very small voids. When EECH at 0.5 mg/g was incorporated, the uniform structure with larger strands was formed. This was coincidental with the increase in gel strength (Fig. 20). However, non-uniform structure with disconnected large strands and large voids was obtained as EECH at 5 mg/g was present. Gelatin could undergo the coagulation more effectively in the presence of EECH at an excessive amount. The coagulated gelatin chains could not undergo the gelation with the ordered structure. This led to poor gel strength (Fig. 20). Therefore, the extracts from coconut husk could be used as the natural gel strengthening agents for gelatins when the appropriate level was used.

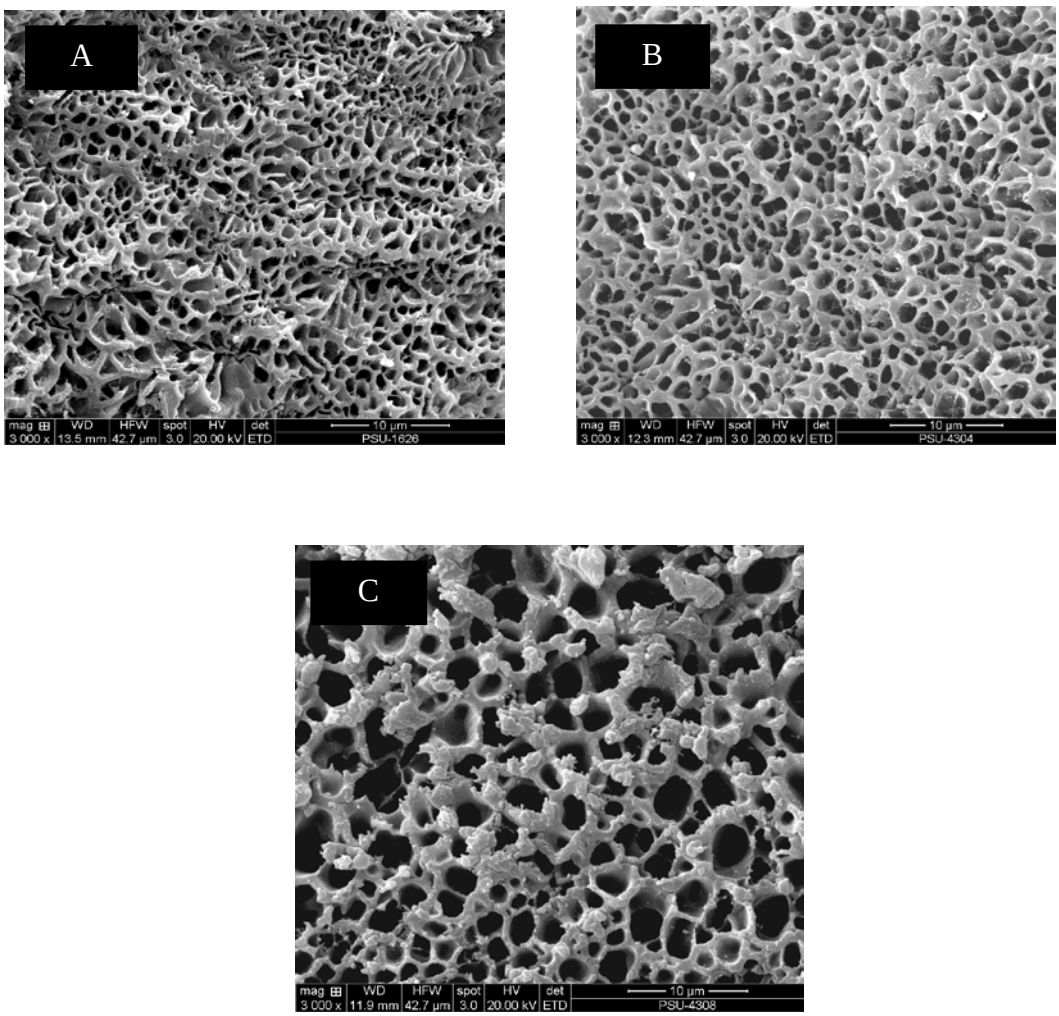

Figure 23. Microstructures of gelatin gel from the swim bladder of yellowfin tuna without and with EECH at different concentrations. Magnification: 3000 x. A: Gel without EECH, B: gel with 0.5 mg EECH/g dry gelatin, C: gel with 5 mg EECH/g dry gelatin.

5.5 Conclusion

Ethanolic extracts from coconut husk (EECH) could be used as the potential cross-linkers for strengthening the gel of yellowfin tuna swim bladder gelatin. EECH was able to increase gel strength most effectively when it was added at a level of 0.5 mg/g. EECH at an excessive amount (5 mg/g) showed the negative impact on gel strength. EECH incorporation at 0.5 mg/g showed no detrimental effect on color of gelatin gel.

5.6 References

Alfaro, A. T., Biluca, F. C., Marquetti, C., Tonial, I. B. and Souza, N. E. 2014. African catfish (*Clarias gariepinus*) skin gelatin: Extraction optimization and physical–chemical properties**.** Food Res. Int. DOI: 10.1016/j.foodres.2014.05.070.

Akinpelu, D. A. 2001. Antimicrobial activity of *Anacardium occidentale* bark. Fitoterapia. 72: 286–287.

Balange, A. and Benjakul, S. 2009. Enhancement of gel strength of bigeye snapper (*Priacanthus tayenus*) surimi using oxidised phenolic compounds. Food Chem. 113: 61–70.

Benjakul, S., Oungbho, K., Visessanguan, W., Thiansilakul, Y. and Roytrakul, S. 2009. Characteristics of gelatin from the skins of bigeye snapper, *Priacanthus tayenus* and *Priacanthus macracanthus*. Food Chem. 116: 445–451.

Benjakul, S. and Visessanguan, W. 2003. Transglutaminase-mediated setting in bigeye snapper surimi. Food Res. Int. 36: 253-266.

Boran, G., Mulvaney S. J. and Regenstein J. M. 2010. Rheological properties of gelatin from silver carp skin compared to commercially available gelatins from different sources. J. Food Sci. 75: 565-571.

Cao, N., Fu, Y. and He, J. 2007. Mechanical properties of gelatin films cross-linked, respectively, by ferulic acid and tannin acid. Food Hydrocolloid. 21: 575-584.

Cho, S. M., Gu, Y. S. and Kim, S. B. 2005. Extracting optimization and physical properties of yellowfin tuna (*Thunnus albacares*) skin gelatin compared to mammalian gelatins. Food Hydrocolloid. 19: 221-229.

Chambi, H. and Grosso, C. 2006. Edible films produced with gelatin and casein cross-linked with transglutaminase. Food Res. Int. 39: 458-466.

Chiou, B. S., Avena-Bustillos, R. J., Shey, J., Yee, E., Bechtel, P. J., Imam, S. H., Glenn, G. M. and Orts, G. W. 2006. Rheological and mechanical properties of cross-linked fish gelatins. Polymer. 47: 6379-6386.

Dey, G., Sachan, A., Ghosh, S. and Mitra, A. 2003. Detection of major phenolic acids from dried mesocarpic husk of mature coconut by thin layer chromatography. Ind. Crop Prod. 18: 171-176.

Fradinho, D. M., Neto, C. P., Evtuguin, D., Jorge, F. C., Irle, M. A., Gil, M. H. and de Jesus, J. P. 2002. Chemical characterisation of bark and of alkaline bark extracts from maritime pine grown in Portugal. Ind. Crop Prod. 16: 23–32.

Gennadios, A., Weller, C. L., Hanna, M. A. and Froning, G. W. 1996. Mechanical and barrier properties of egg albumen films. J. food sci. 61: 585-589.

Grossman, S. and Bergman, M. 1992. Process for the production of gelatin from fish skins. US patent 5,093,474.

Kaewdang, O., Benjakul, S., Kaewmanee, T. and Kishimura, H. 2014. Characteristics of gelatin extracted from the swim bladder of yellowfin tuna (*Thunnus albacores*) as affected by alkaline pretreatments. Ital. J. Food Sci. (In revision).

Kaewruang, P., Benjakul, S., Prodpran, T. and Nalinanon, S. 2013. Physicochemical and functional properties of gelatin from the skin of unicorn leatherjacket (*Aluterus monoceros*) as affected by extraction conditions. Food Biosci. 2: 1–9.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage. T4. Nature. 227: 680.

Liu, H. Y., Li, D. and Guo, S. D. 2008. Extraction and properties of gelatin from channel catfish (*Ietalurus punetaus*) skin. LWT-Food Sci. Technol. 41: 414-419.

Maqsood, S., Benjakul, S. and Shahidi, F. 2013. Emerging role of phenolic compounds as natural food additives in fish and fish products. Crit. Rev. Food Sci. Nutri. 53: 162-179.

Montero, P., Fernández-Díaz, M. D. and Gómez-Guillén, M. C. 2002. Characterization of gelatin gels induced by high pressure. Food Hydrocolloid. 16: 197-205.

Niu, L., Zhou, X., Yuan, C., Bai, Y., Lai, K., Yang, F. and Huang, Y. 2013. Characterization of tilapia (*Oreochromis niloticus*) skin gelatin extracted with alkaline and different acid pretreatments. Food Hydrocolloid. 33: 336-341.

O'connell, J. and Fox, P. 2001. Significance and applications of phenolic compounds in the production and quality of milk and dairy products: A review. Int. Dairy J. 11: 103–120.

Parr, A. J. and Bolwell, G. P. 2000. Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. J. Sci. Food Agric. 80: 985-1012.

Sadowska, M., Kolodziejska, I. and Niecikowska, C. 2003. Isolation of collagen from the skins of Baltic cod (*Gadus morhua*). Food Chem. 81: 257-262.

Saito, H., Taguchi, T., Aoki, H., Murabayashi, S., Mitamura, Y., Tanaka, J. and Tateishi, T. 2007. pH responsive swelling behavior of collagen gels prepared by novel cross-linkers based on naturally derived di- or tricarboxylic acids. Acta Biomaterialia. 3: 89-94.

Santoso, J., Yoshie-Stark, Y. and Suzuki, T. 2004. Anti-oxidant activity of methanol extracts from Indonesian seaweeds in an oil emulsion model. Fish. Sci. 70: 183-188.

Sarker, D. K., Wilde, P. J. and Clark, D. C. 1995. Control of surfactant-induced destabilization of foams through polyphenol-mediated protein–protein interactions. J. Agric. Food Chem. 43: 295–300.

Silber, M. L., Davitt, B. B., Khairutdinov, R. F. and Hurst, J. K. 1998. A mathematical model describing tannin-protein association. Anal. Biochem. 263: 46-50.

Silva, R. S. G., Bandeira, S. F. and Pinto, L. A. A. 2014. Characteristics and chemical composition of skins gelatin from cobia (*Rachycentron canadum*). LWT-Food Sci. Technol. 57: 580-585.

Singleton, V. L., Orthofer, R. and Lamuela-Raventos, R. M. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin–ciocalteu reagent. Method. Enzymol. 299: 152–178.

Steel, R. G. D. and Torrie J. H. 1980. Principles and procedures of statistics: A biometrical approach 2nd ed. McGraw-Hill. New York.

Strauss, G. and Gibson, S. M. 2004. Plant phenolics as cross-linkers of gelatin gels and gelatin-based coacervates for use as food ingredients. Food Hydrocolloid. 18: 81–89.

Sueli, R. and Gustavo, A. S. P. 2007. Ultrasound extraction of phenolic compounds from coconut (*Cocos nucifere*) shell powder. J. Food Eng. 80: 869–872.

Suzuki, S., Rodriguez, E. B., Saito, K., Shintani, H. and Iiyama, K. 1998. Compositional and structural characteristics of residual biomass from tropical plantations. J. Wood Sci. 44: 40-46.

Tabarestani, H. S., Maghsoudlou, Y., Motamedzadegan, A. and Sadeghi Mahoonak, A. R. 2010. Optimization of physico-chemical properties of gelatin extracted from fish skin of rainbow trout (*Onchorhynchus mykiss*). Bioresource Technol. 101: 6207–6214.

Tian, F., Li, B., Ji, B., Yang, J., Zhang, G., Chen, Y. and Luo, Y. 2009. Antioxidant and antimicrobial activities of consecutive extracts from *Galla chinensis*: The polarity affects the bioactivities. Food Chem. 113: 173–179.

Temdee, W. and Benjakul, S. 2014. Effect of oxidized kiam wood and cashew bark extracts on gel properties of gelatin from cuttlefish skins. Food Biosci. 7: 95–104.

Van Buren, J. P. and Robinson, W. B. 1969. Formation of complexes between protein and tannic acid. J. Agric. Food Chem. 17: 772-777.

Vazquez-Torres, H., Canche-Escamilla, G. and Cruz-Ramos, A. 1992. Coconut husk lignin. I. Extraction and characterization. J. Appl. Polym. Sci. 45: 633-644.

Yan, M., Li, B., Zhao, X. and Yi, J. 2011. Physicochemical properties of gelatin gels from walleye pollock (*Theragra chalcogramma*) skin cross-linked by gallic acid and rutin. Food Hydrocolloid. 25: 907-914.

Yang, H., Wang, Y., Jiang, M., Oh, J. H., Herring, J. and Zhou, P. 2007. 2-Step optimization of the extraction and subsequent physical properties of channel catfish (*Ictalurus punctatus*) skin gelatin. J. Food Sci. 72: 188–195.

CHAPTER 6

Effect of agar on gel properties of gelatin from swim bladder of yellowfin tuna (*Thunnus albacores*)

6.1 Abstract

The impacts of agar at different levels (5, 10, 15 and 20 % of gelatin substitution) on gel properties of gelatin from yellowfin tuna swim bladder were investigated**.** Gel strength, gelling and melting points continuously increased as agar concentration increased from 5 to 20 % of gelatine substitution ($P < 0.05$). Hardness increased, whereas cohesiveness decreased with increasing agar levels ($P < 0.05$). Nevertheless, there was no difference in springiness between sample containing 5-20 % agar ($P > 0.05$). No differences in protein patterns as determined by SDS-PAGE were observed, regardless of agar levels. L^* and a^* increased, but b^* and ΔE^* decreased as the level of agar increased. Gel matrix with uniformity and larger strands were observed with gels added with agar, compared with the control gelatin gel (without agar). Therefore, the addition of agar into gelatin affected the properties of mixed gelatin-agar gel.

6.2 Introduction

Gelatin is one of the most popular biopolymers, for food, pharmaceutical, cosmetic, and photographic applications because of its unique functional and technological properties (Karim and Bhat, 2009). Generally, the traditional sources of gelatin are bovine and porcine skins and bones. Nevertheless, gelatin from aquatic sources has been recognized to be free of the risk associated with bovine spongiform encephalopathy (BSE) and foot and mouth diseases (Sadowska *et al*., 2003). Additionally, it can be used to replace porcine gelatin, which is prohibited for Muslims or Jews as well as bovine gelatin, which is not consumed by Hindus (Karim and Bhat, 2009). As a consequence, fish gelatin has gained increasing interest as the potential alternative for land animal counterpart. Due to the abundance of skin, fin, scale and bones, etc., which are the byproducts from the fish processing industry, it would be of full benefit to utilize those resources as promising raw material for

gelatin production (Ahmad and Benjakul, 2011; Gómez-Guillén *et al*., 2002; Muyonga *et al*., 2004). Skin gelatins have been extracted from skins of several fish species, e.g., Amur sturgeon (Nikoo *et al*., 2014), seabass (Sinthusamran *et al*., 2014) and unicorn leatherjacket (Kaewruang *et al*., 2013). Recently, Kaewdang *et al*. (2014) successfully extracted gelatin from swim bladder of yellowfin tuna using an alkaline pretreatment. However, gelatin from swim bladder showed low gel strength. Fish gelatin generally has lower concentrations of proline and hydroxyproline than mammalian gelatin (Haug *et al*., 2004; Muyonga *et al*., 2004; Avena-Bustillos *et al*., 2006). Low gelling and melting temperatures and low gel strength (Gómez-Guillén *et al*., 2002; Lui *et al*., 2008) limit its applications. Therefore, the modification of fish gelatin is still needed to conquer the problem. Enzymic modification (Yi *et al*., 2006), the addition of some solutes (Elysee-Collen and Lencki, 1996), or mixing fish gelatin with other gelling agents (Haug *et al*., 2004; Pranoto *et al*., 2007) have been implemented to improve gel strength or increase gelling and melting points of gelatin gel.

Agar is a fibrous polysaccharide extracted from marine algae such as *Gelidium* sp. and *Gracilaria* sp. It is a mixture of agarose and agaropectin, which is slightly branched and sulfated (Rhim *et al*., 2011). This thermoplastic, biodegradable and biocompatible polysaccharide has shown high mechanical strength with moderate water resistance as packaging material (Freile-Pelegrín *et al*., 2007; Wu *et al*., 2009). It has been used in blends with other biopolymers such as starch, soy or milk protein to improve the mechanical and water vapor barrier properties of protein films (Tian *et al*., 2011; Wu *et al*., 2009). Therefore, the present study aimed to investigate the impact of the agar on the gel properties of gelatin extracted from swim bladder of yellowfin tuna.

6.3 Materials and methods

6.3.1 Chemicals

All chemicals were of analytical grade. Sodium dodecyl sulfate (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). A commercial agar powder was obtained from HiMedia Laboratories PvT., Ltd, Mumbai, India.

6.3.2 Collection and preparation of swim bladder

Swim bladders of yellowfin tuna (*Thunnus albacares*) were obtained from Tropical Canning Public Co., Ltd., Songkhla, Thailand. Swim bladders with the length of 8-12 cm were placed in polyethylene bags, inserted in ice using a sample/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Songkhla. Upon arrival, swim bladders were washed with tap water and cut into pieces with the length of approximately 2 cm. The prepared samples were then placed in polyethylene bag and frozen at -20 °C. The samples were stored at -20 °C until used. The storage time was not longer than 3 months. Prior to extraction, frozen swim bladders were thawed using tap water until the temperature was 0-2 °C.

6.3.3 Extraction of gelatin from swim bladder

Firstly, the prepared swim bladders were added with the mixed alkaline solution (Na_2CO_3: NaOH; 7:3) having the concentration of 4 % (w/v) at a ratio of 1:10 (w/v). The mixture was stirred for 12 h at room temperature (28–30 °C) using an overhead stirrer model W20.n (IKA®-Werke GmbH & CO.KG, Stanfen, Germany). The alkaline solution was changed every 6 h. The pretreated samples were washed with tap water until a neutral or faintly basic pH was obtained.

To extract gelatin, alkali-pretreated samples were immersed in distilled water (60 °C) using a swim bladder/water ratio of 1:5 (w/v) in a temperature-controlled water bath (W350, Memmert, Schwabach, Germany). The extraction was performed for 24 h with a continuous stirring at a speed of 150 rpm. The mixture was then filtered using a Buchner funnel with a Whatman No. 4 filter paper (Whatman International, Ltd., Maidstone, England). The filtrate was freeze-dried using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The dried gelatin was placed in polyethylene bag and kept at 4 °C.

6.3.4 Preparation of gelatin gels containing agar

Agar and gelatin samples were separately solubilized in distilled water at 95 °C and 60 °C, respectively. The agar was added at different levels (5, 10, 15 and 20 % of gelatin substitution), in which the total solid content in solution was 6.67 % (w/v). The mixtures were stirred using a magnetic stirrer (IKA Labortechnik stirrer, Selangor, Malaysia) for 10 min at room temperature (28-30 °C), followed by setting at 10 °C for 16-18 h. The resulting gels were subjected to analyses.

6.3.4.1 Determination of gel strength

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

6.3.4.2 Texture profile analysis

Textural profile analysis (TPA) of all gel samples was carried out using a Model TA-X Tplus texture analyzer (Stable Micro System, Surrey, England) and a cylinder probe with a diameter of (5 cm) was used.

6.3.4.3 Determination of gelling and melting temperatures

Gelling and melting temperatures of gelatin and gelatin-agar mix were measured following the method of Somboon *et al*. (2014) using a controlled stress rheometer (RheoStress RS 75, HAAKE, Karlsruhe, Germany). The gelatin solutions containing agar at the different levels (0, 5, 10, 15 and 20 % of gelatin substitution), with a total solid content of 6.67 % (w/v) were prepared in the same manner as described previously. All solutions were preheated at 60 °C for 30 min. The measuring geometry included a 6 cm parallel plate and the gap was set at 1.0 mm. The measurement was performed at a scan rate of 1 °C/min, frequency of 1 Hz, oscillating applied stress of 3 Pa during cooling from 60 to 5 °C and heating from 5 to 90 °C.

The gelling and melting temperatures were measured, where tan δ became 1 or δ was 45°.

6.3.4.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS–PAGE was performed by the method of Laemmli (1970). Gel 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). The mixtures were centrifuged at 8,500 g for 5 min using a microcentrifuge (MIKRO20, Hettich Zentrifugan, 170 Germany) to remove undissolved debris. Solubilized samples were mixed at 1:1 (v/v) ratio with the 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, the 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 a mixture of 50 % (v/v) methanol and 7.5 % (v/v) acetic acid for 30 min and destained again with a mixture of 5 % (v/v) methanol and 7.5 % (v/v) acetic acid for 1 h. High-molecular-weight protein markers were used to estimate the molecular weight of proteins.

6.3.4.5 Microstructure analysis

The microstructure of gelatin and gelatin-agar mixed gels 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 a serial concentration of 25, 50, 70, 80, 90 and 100 % (v/v). Subsequently, samples were critical point dried. 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.

6.3.4.6 Determination of color

The color of gelatin gels was determined using 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 for 10 min and calibrated with a white standard.

The 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 the white standard (L^* = 93.6, a^*= -0.94 and b^* = 0.40).

6.3.5 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 a 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).

6.4 Results and discussion

6.4.1 Gel strength

Gel strength of gelatins from swim bladder of yellowfin tuna added with agar at different levels is shown in Fig. 24. Gel strength continuously increased as agar levels increased from 5 to 20 %. The highest gel strength was found with gelatin gel containing 20 % agar (195 g). With addition of 20 % agar, the resulting gel had the increase in gel strength by 167.12 % ($P < 0.05$), compared with that of control gel (without agar addition). Gel strength is one of the most important functional

properties of gelatin. The gel strength of commercial gelatins varied between 100 and 300, but gelatins with values of 250–260 are the most desirable.

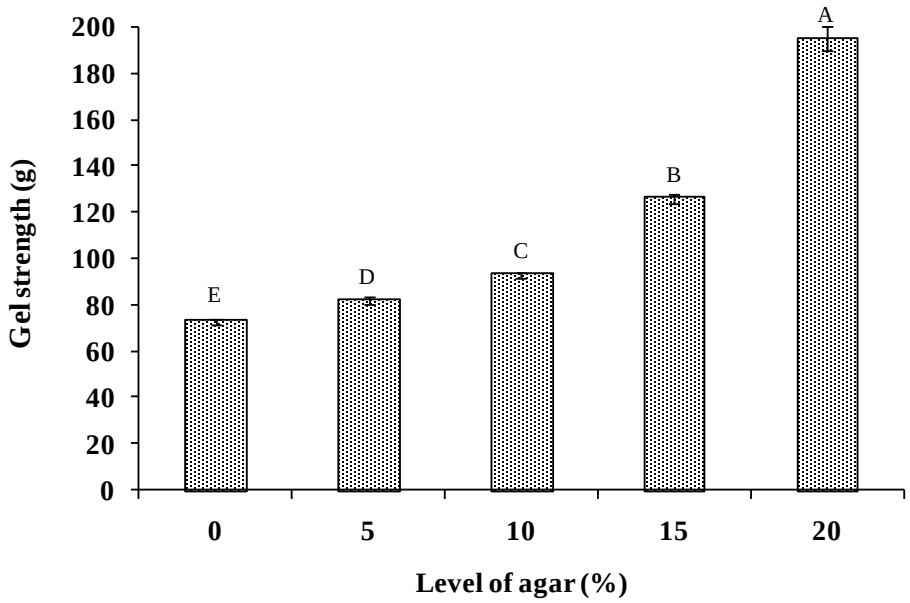

Figure 24. Gel strength of gelatin from the swim bladder of yellowfin tuna containing agar at different levels. Different uppercase letters on the bars denote significant differences ($P < 0.05$). Bars represent the standard deviations ($n = 3$).

Typically, fish gelatin has lower gel strength than mammalian gelatin due to their lower imino acid content (Grossman and Bergman, 1992). Agar is a fibrous polysaccharide, which is able to form the gel (Haug *et al*., 2004). Agar might align themselves along with gelatin in the gel matrix, in which the stronger network could be established. With higher level of agar, the agar was able to allocate or penetrate throughout gelatin network, thereby strengthening the developed network. These results indicate that agar showed the enhancing effect on gel strength of gelatin from yellowfin tuna swim bladder in dose dependent manner.

6.4.2 Texture profiles

Hardness, cohesiveness and springiness of gelatin gel and gelatin-agar mixed gels are shown in Fig. 25A, B and C, respectively. Hardness is related to the

strength of the gel structure under compression. With increasing agar levels, hardness of resulting gel increased ($P < 0.05$).

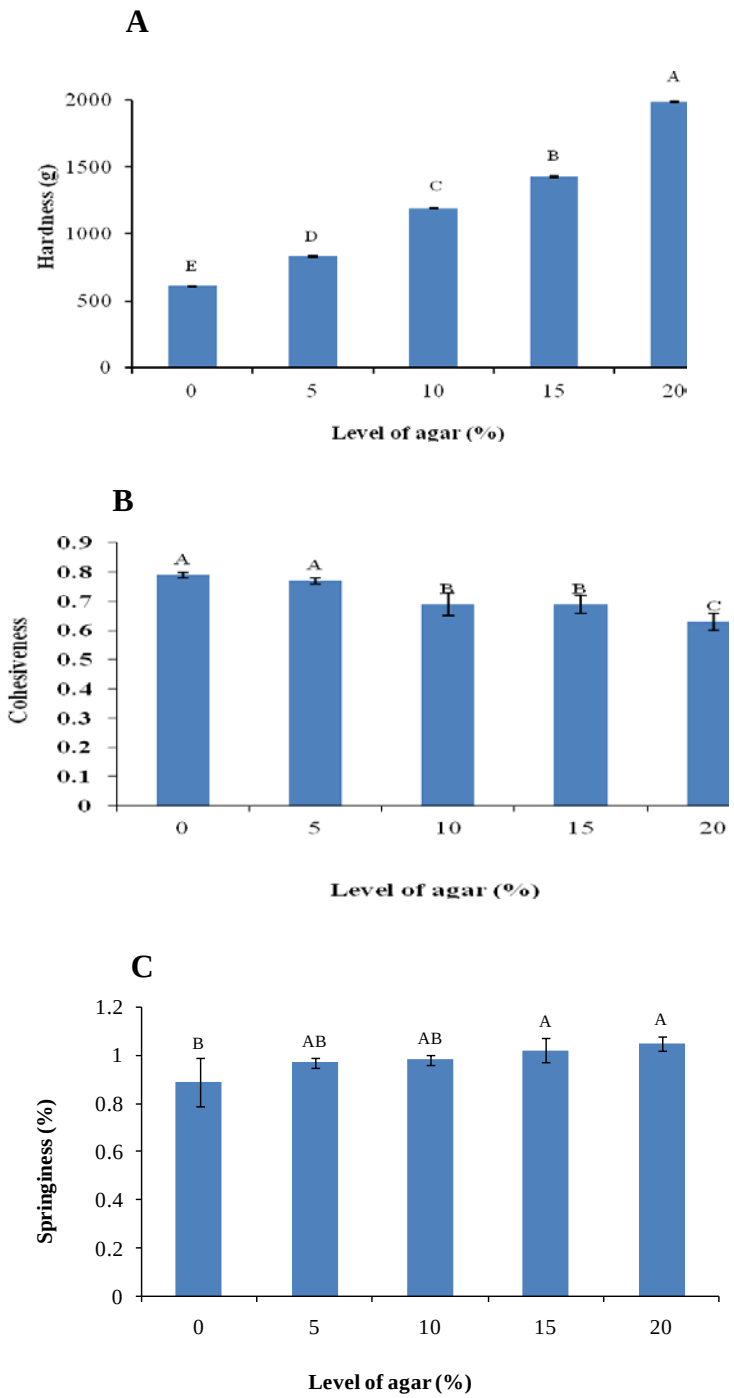

Figure 25. Hardness (A), cohesiveness (B) and springiness (C) of gelatin gels from swim bladder of yellowfin tuna containing agar at different levels. Different uppercase letters on the bars denote significant differences ($P < 0.05$). Bars represent the standard deviations ($n = 3$).

It was noteworthy that the strong interactions between two polymers in the mixed solution could undergo gelation in the synergistic fashion. For gelatin gel containing 20 % agar, hardness increased by 227.34 %, compared with that of the control gelatin gel.

Cohesiveness is a measure of the degree of difficulty in breaking down the gel's internal structure. Cohesiveness of the gelatin gel decreased as the levels of agar increased. Liu *et al*. (2007) reported that cohesiveness of gelatin gel increased with the addition of pectin at high content. These observations showed the internal structure of gels was more easier to break during the first compression when the ratio of the agar to gelatin increased. When gel matrix was broken during the first compression, less energy was required to break the remaining gel matrix during the second compression (Lau *et al*., 2000). The decrease in cohesiveness was in accordance with the increase in hardness, especially when the levels of agar added increased. The result suggested that gel became more brittle as the level of agar increased.

Springiness (sometimes also referred to as "elasticity") is a perception of gel "rubberiness" in the mouth, and is a measure of how much the gel structure is broken down by the initial compression (Sanderson, 1990). High springiness will be obtained when the gel structure is broken into few large pieces during the first TPA compression, whereas low springiness results from the gel breaking into many small pieces. Generally, less springy gels, such as low-methoxy pectin, carrageenan and agar gels would break down more easily during mastication than a firm and springy gelatin gel (Marshall and Vaisey, 1972). Springiness of gel increased slightly when agar at levels of 15 and 20 % was added, in comparison with the control gel. However, agar at levels of 5 and 10 % showed no impact on springiness of gelatin gel. When a small amount of calcium ions was added into mixed gels of gellan-gelatin, there was a rapid decrease in gel springiness, followed by a more gradual increase (Lau *et al*., 2000). The result suggested that the interaction between gelatin and agar, particularly at 15 and 20%, might favor the formation of gel with higher springiness, ever though agar showed the lower springiness than gelatin. Thus, the addition of agar had the impact on textural property of gelatin gel.

6.4.3 Gelling and melting points

Thermal transitions of swim bladder gelatin in the absence and presence of agar at difference levels were monitored by changes in the phase angle (δ) of gelatin solution during cooling (60–5 °C) and subsequent heating (5-90 °C). The gelling and melting points of gelatin as affected by the addition of agar are shown in Table 11. Gelatin solution (control) and those added with agar at levels of 5, 10, 15 and 20 % (gelatin substitution) became gel at 15.32, 15.69, 15.87, 25.32 and 26.95 °C. The corresponding gels molt at 22.28, 25.12, 27.45, 30.07 and 32.34 °C, respectively. It was found that gelatin had the higher gelling point and melting points when incorporated with agar. The addition of agar made the gelatin network more stable as indicated by higher melting point, in which higher energy was required to break down the network. Additionally gelatin and agar were able to form the gel at higher temperature.

Table 11. Gelling and melting temperatures of gelatin from the swim bladder of yellowfin tuna containing agar at different levels

Agar levels (%, gelatin substitution)	Gelling point (˚C)	Melting point (˚C)
0	15.32 ± 0.22^{D}	22.28 ± 0.44^{E}
5	15.69 ± 0.35^{CD}	25.12 ± 0.58^{D}
10	15.87 ± 0.28^{C}	27.45 ± 0.31^{C}
15	25.32 ± 0.12^{B}	30.07 ± 0.42^{B}
20	26.95 ± 0.09^{A}	32.34 ± 0.25^{A}

Mean ±SD (n = 3).
Different uppercase superscripts in the same column indicate significant differences ($P < 0.05$).

Currently, agar has been widely used in food and pharmaceutical industries as gelling and stabilizing agent (Boral and Bohidar, 2010). Therefore, the addition of agar could increase gelling and melting points, in which gel of gelatin could be formed more easily. Also gel was maintained for a longer time as indicated by the higher melting temperature. Generally, the melting point was higher than the gelling point because gelatin gel absorbed energy when it was melting (Liu and Guo, 2008). Therefore, mixing agar with gelatin from swim bladder could alter gelling

behavior of both components in the mixed system. Agar has been mixed with other polysaccharides, e.g., agar and k-carrageenan (Noziah *et al*., 2006), agar and locust bean gum (Selby and Wynne, 1973), agarose and galactomannan (Rees, 1972) and agar and gellan (Banerjee and Bhattacharya, 2011).

6.4.4 Protein patterns

Protein patterns of swim bladder gelatin gel incorporated with agar at different levels are shown in Fig. 26 All samples had α-chains as the major components. It was noted that the addition of agar had no impact on protein patterns of gelatin gel, regardless of levels of agar added. It was noted that gel strength was increased, when agar was incorporated, particularly at high level. Nevertheless, no changers in protein patterns were noticeable. Under the condition used for electrophoresis, SDS was able to destroy weak bonds. The result suggested that most bonding between gelatin and agar were weak bond, especially hydrogen bond. Agar contains a number of hydroxyl group, which could act as hydrogen donor (Noel *et al*., 1990). As a consequence, there was no difference in protein patterns among gelatin gel added without and with agar at various levels.

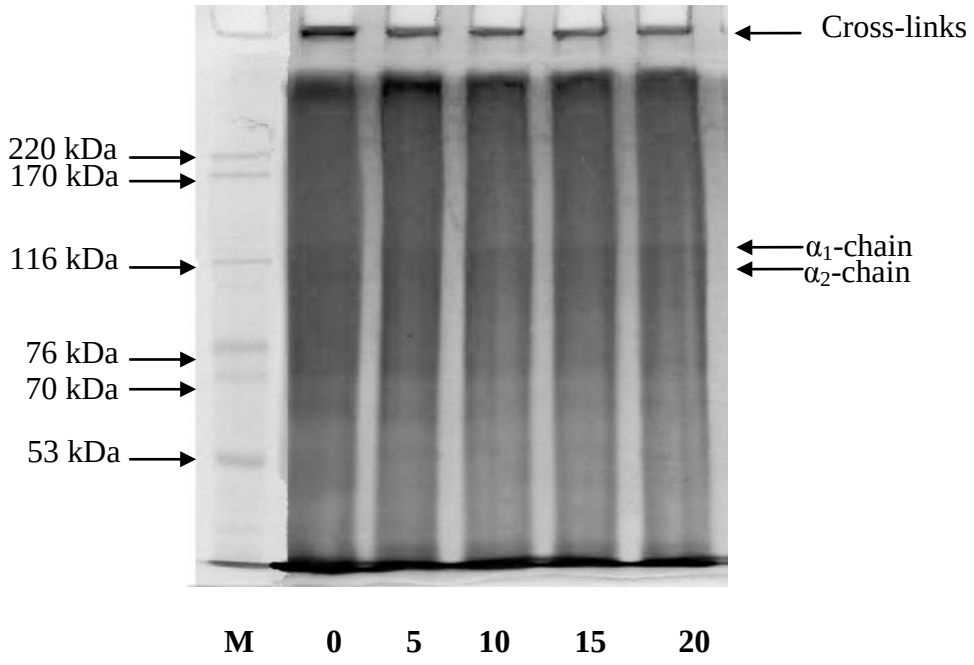

Figure 26. Protein patterns of gelatin gels from the swim bladder of yellowfin tuna containing agar at different levels. M: high molecular weight markers.

6.4.5 Microstructure of gelatin gels

Gel microstructures of gelatin from the swim bladder of yellowfin tuna in the absence and presence of agar at different levels are shown in Fig. 27.

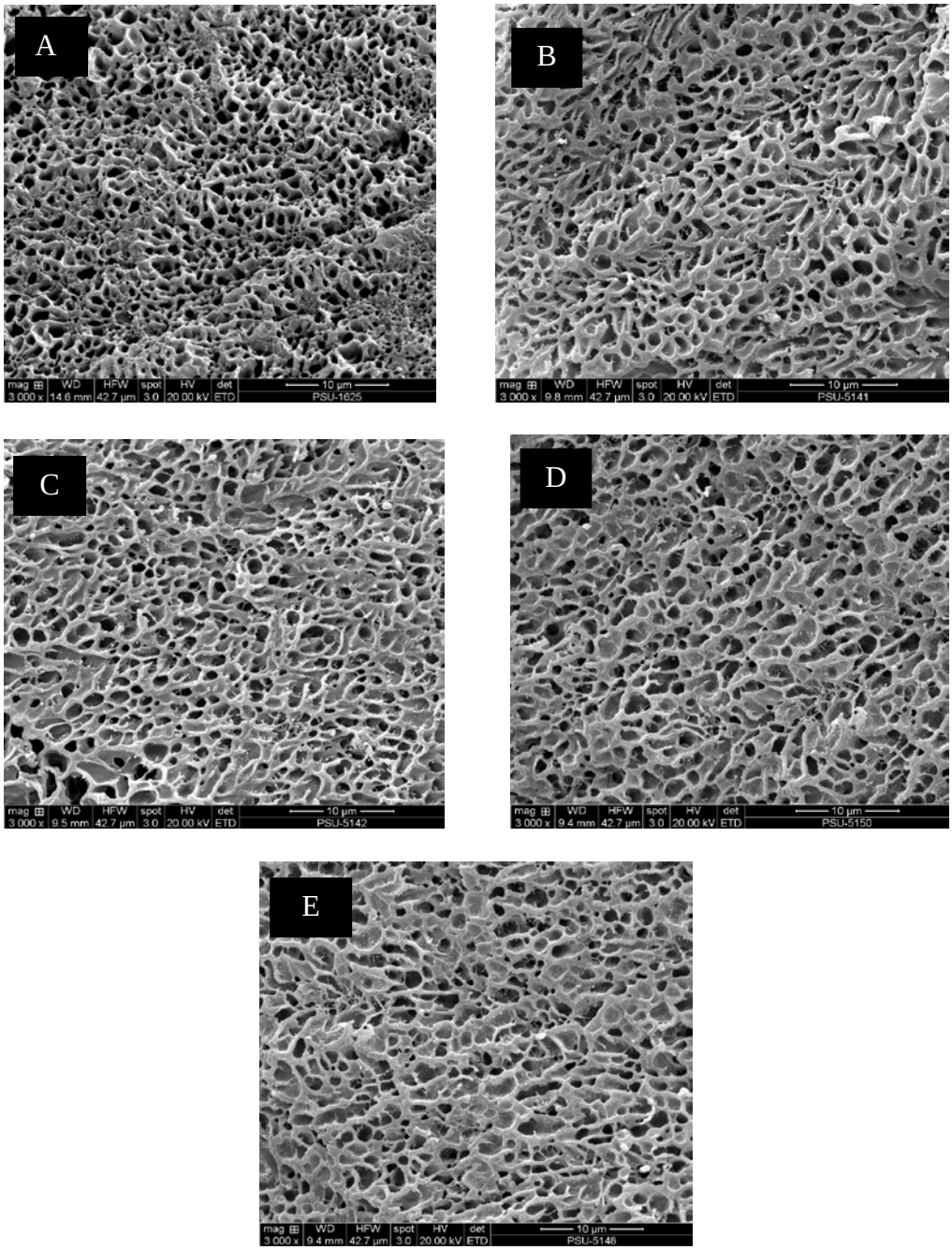

Figure 27. Microstructures of gelatin gel from the swim bladder of yellowfin tuna containing agar at different levels. A: Gelatin gel without agar, B: Gelatin gel with 5 % agar, C: Gelatin gel with 10 % agar, D: Gelatin gel with 15% agar and E: Gelatin gel with 20 % agar. Magnification: 3000 x.

All gelatin gels were sponge or coral-like in structure. Generally, the arrangement and association of protein molecules in the gel matrix directly contributed to gel strength of gelatin (Benjakul *et al*., 2009). The control gelatin gel showed the finest gel network with small voids. When agar at different levels was incorporated, the uniform structure was still observed, but the larger strands were developed. Similar microstructure of gelatin gel contaning agar at levels of 5-20 % were noticeable. Shrinivas *et al*. (2009) reported that bicontinuous mixed gel between 1% agarose and 5 to 30% of fish gelatin may have large pores in the gel structure and the water in these pores would contribute to high syneresis. Based on structure, the interaction between gelatin and agar took place and the larger strands were formed. Those large strands might be more resistant to force applied as indicated by the increased gel strength (Fig. 1) and hardness (Fig. 2A).

6.4.6 Color

Lightness (*L**), redness (*a**) and yellowness (*b**) of gelatin gels without and with the addition of agar (5 to 20 %, gelatin substitution) are shown in Table 12.

Table 12. Color of gelatin gels from swim bladder of yellowfin tuna containing agar at different levels

Agar levels (%, gelatin substitution)	Color parameters			
	L*	***a****	***b****	***ΔE****
0	28.31 ± 1.09^{C}	-2.74 ± 0.56^{E}	9.21 ± 0.11^{E}	65.90 ± 1.08^{A}
5	28.23 ± 0.81^{C}	2.36 ± 0.06^{D}	8.19 ± 0.19^{D}	65.92 ± 0.82^{A}
10	30.11 ± 0.19^{C}	5.08 ± 0.14^{C}	7.55 ± 0.41^{C}	64.18 ± 0.21^{A}
15	36.17 ± 0.40^{B}	8.02 ± 0.42^{B}	6.68 ± 0.26^{B}	58.47 ± 0.46^{B}
20	44.90 ± 1.81^{A}	10.63 ± 0.23^{A}	5.38 ± 0.51^{A}	50.31 ± 1.72^{C}

Mean ±SD (n = 3).
Different uppercase superscripts in the same column indicate significant differences ($P < 0.05$).

The increases in *L** and *a**-values of gelatin gels were observed as the levels of agar increased ($P < 0.05$). The coincidental decreases in *b** and *ΔE** -values were noticeable as the levels of agar increased ($P< 0.05$). These results demonstrated

that the added agar at different levels had the impact on color of gelatin extracted from the swim bladder of yellowfin tuna. With increasing level of agar, the gelatin network might be interrupted to some degree, in which syneresis might occur. The water released might enhance the higher light scattering as indicated by higher L^*-value. This coincided with the lower b^* and ΔE^* values.

6.5 Conclusion

Agar could be used to strengthen the gel of gelatin from yellowfin tuna swim bladder gelatin. Gel strength continuously increased as agar concentration increased from 5 to 20 %. The stronger gel with higher gelling and melting points was obtained with addition of agar.

6.6 References

Ahmad, M. and Benjakul, S. 2011. Characteristics of gelatin from the skin of unicorn leatherjacket (*Aluterus monoceros*) as influenced by acid pretreatment and extraction time. Food Hydrocolloid. 25: 381-388.

Avena-Bustillos, R. J., Olsen, C. W., Chiou, B., Yee, E., Bechtel, P. J. and McHugh, T. H. 2006. Water vapor permeability of mammalian and fish gelatin films. J. Food Sci. 71: 202–207.

Banerjee, S. and Bhattacharya, S. 2011. Compressive textural attributes, opacity and syneresis of gels prepared from gellan, agar and their mixtures. J. Food Eng. 102: 287–292.

Benjakul, S., Oungbho, K., Visessanguan, W., Thiansilakul, Y. and Roytrakul, S. 2009. Characteristics of gelatin from the skins of bigeye snapper, *Priacanthus tayenus* and *Priacanthus macracanthus*. Food Chem. 116: 445.

Boral, S. and Bohidar, H. B. 2010. Effect of ionic strength on surface-selective patch binding-induced phase separation and coacervation in similarly charged gelatin-agar molecular systems. J. Phys. Chem. 114: 12027–12035.

Elysee-Collen, B. and Lencki, R. W. 1996. Effect of ethanol, ammonium sulfate, and temperature on the phase behavior of type B gelatin. J. Agric. Food Chem. 44: 1651–1657.

Freile-Pelegrín, Y., Madera-Santana, T., Robledo, D., Veleva, L., Quintana, P. and Azamar, J. A. 2007. Degradation of agar films in a humid tropical climate: thermal, mechanical, morphological and structural changes. Polym. Degrad. Stabil. 92: 244-252.

Gennadios, A., Weller, C. L., Hanna, M. A. and Froning, G. W. 1996. Mechanical and barrier properties of egg albumen films. J. Food Sci. 61: 585-589.

Gómez-Guillén, M. C., Turnay, J., Fernandez-Diaz, M. D., Ulmo, N., Lizarbe, M. A. and Montero, P. 2002. Structural and physical properties of gelatin extracted from different marine species: a comparative study. Food Hydrocolloid. 16: 25-34.

Grossman, S. and Bergman, M. 1992. Process for the production of gelatin from fish skins. US patent 5,093,474.

Haug, I. J., Draget, K. I. and Smidsrod, O. 2004. Physical and rheological properties of fish gelatin compared to mammalian gelatin. Food Hydrocolloid. 18: 203-213.

Kaewdang, O., Benjakul, S., Kaewmanee, T. and Kishimura, H. 2014. Characteristics of gelatin extracted from the swim bladder of yellowfin tuna (*Thunnus albacores*) as affected by alkaline pretreatments. Ital. J. Food Sci. (Accepted).

Kaewruang, P., Benjakul, S. and Prodpran, T. 2013. Molecular and functional properties of gelatin from the skin of unicorn leatherjacket as affected by extracting temperatures. Food Chem. 138: 1431–1437.

Karim, A. A. and Bhat, R. 2009. Fish gelatin: properties, challenges, and prospects as an alternative to mammalian gelatins. Food Hydrocolloid. 23: 563–576.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage. T4. Nature. 227: 680.

Lau, M. H., Tang, J. and Paulson, A. T. 2000. Texture profile and turbidity of gellan/gelatin mixed gels. Food Res. Int. 33: 665-671.

Liu, H., Zhu, D. Sh., Xu, X. M., Guo, Sh. D. and Jin, Zh. Y. 2007. Rheological, textural and microstructure analysis of the high-methoxy pectin/gelatin mixed systems. J. Texture Stud. 38: 577-600.

Liu, H., Li, D. and Guo, S. 2008. Rheological properties of channel catfish (*Ictalurus punctaus*) gelatine from fish skins preserved by different methods. LWT- Food Sci. Technol. 41: 414-419.

Marshall, S. G. and Vaisey, M. 1972. Sweetness perception in relation to some textural characteristics of hydrocolloid gels. J. Texture Stud. 3: 173-185.

Muyonga, J. H., Cole, C. G. B. and Duodu, K. G. 2004. Extraction and physic chemical characterisation of Nile perch (*Lates niloticus*) skin and bone gelatin. Food Hydrocolloid. 18: 581-592.

Nikoo, M., Benjakul, S., Ehsani, A., Li, J., Wu, F., Yang, N., Xu, B., Jin, Z. and Xu, X. 2014. Antioxidant and cryoprotective effects of a tetrapeptide isolated from Amur sturgeon skin gelatin. J. Funct. Food. 7: 609 – 620.

Noel, T. R., Ring S. G. and Whittam M. A. 1990. Glass transitions in low-moisture foods. Trend. Food Sci. Technol. 1: 62-67.

Norziah, M. H., Foo, S. L. and Karim, A. A. 2006. Rheological studies on mixtures of agar (*Gracilaria changii*) and k-carrageenan. Food Hydrocolloid. 20: 204-217.

Pranoto, Y., Lee, C. M. and Park, H. J. 2007. Characterizations of fish gelatin films added with gellan and k-carrageenan. LWT- Food Sci. and Technol. 40: 766-774.

Rees, D. A. 1972. Polysaccharide gels-a molecular review. Chem. Ind. 16: 630–636.

Rhim, J. W., Lee, S. B. and Hong, S. I. 2011. Preparation and characterization of agar/clay nanocomposite films: the effect of clay type. J. Food Sci. 76: 40-48.

Sadowska, M., Kolodziejska, I. and Niecikowska, C. 2003. Isolation of collagen from the skins of Baltic cod (*Gadus morhua*). Food Chem. 81: 257–262.

Sanderson, G. R. 1990. Gellan gum. *In* Food Gels. (Harris, P., ed.). p. 201-232. Elsevier Science Publishing Co., Inc. New York.

Selby, H. H. and Wynne, H. H. 1973. Agar. *In* Industrial Gums. (Whistler, R. C. and BeMiller J. N., eds.). p. 29-48. Academic Press. New York.

Shrinivas, P., Kasapis, S. and Tongdang, T. 2009. Morphology and mechanical properties of bicontinuous gels of agarose and gelatin and the effect of added lipid phase. Langmuir. 25: 8763-8773.

Sinthusamran, S., Benjakul, S. and Kishimura, H. 2014. Characteristics and gel properties of gelatin from skin of seabass (*Lates calcarifer*) as influenced by extraction conditions. Food Chem. 152: 276–284.

Somboon, N., Karrila, T. T., Kaewmanee, T. and Karrila, S. J. 2014. Properties of gels from mixed agar and fish gelatin. Int. Food Res. J. 21: 485-492.

Steel, R. G. D. and Torrie J. H. 1980. Principles and procedures of statistics: A biometrical approach 2nd ed. McGraw-Hill. New York.

Tian, H., Xu, G., Yang, B. and Guo, G. 2011. Microstructure and mechanical properties of soy protein/agar blend films: effect of composition and processing methods. J. Food Eng. 107: 21-26.

Wu, Y., Geng, F., Chang, P. R., Yu, J. and Ma, X. 2009. Effect of agar on the microstructure and performance of potato starch film. Carbohyd. Polym. 76: 299-304.

Yi, J. B., Kim, Y. T., Bae, H. J., Whiteside, W. S. and Park, H. J. 2006. Influence of transglutaminase-induced cross-linking on properties of fish gelatin films. J. Food Sci. 71: 376–383.

CHAPTER 7

Summary and future works

7.1 Summary

1. Collagen could be extracted from swim bladder of yellowfin tuna using acetic acid (ASC) and with the aid of pepsin (PSC). Both ASC and PSC consisted of two α-chains (α_1 and α_2) and were characterised to be type I collagen.

2. Gelatin was extracted from the swim bladder using alkaline pretreatments. Pretreatment using the mixture of Na_2CO_3 and NaOH (7:3) at a concentration of 4 % (w/v) for 12 h prior to extraction was recommended, in which the resulting gelatin had high yield and gel strength.

3. Extraction temperature determined the yield and gel property of gelatin from swim bladder. The appropriate extraction temperature for gelatin from swim bladder was 60 °C, providing the highest gel strength.

4. Ethanolic extracts from coconut husk (EECH) could be used as the potential cross-linkers for strengthening the gel of swim bladder gelatin. EECH was able to increase gel strength most effectively when it was added at a level of 0.5 mg/g dry gelatin.

5. Agar could be used to strengthen the gel of gelatin from swim bladder. The stronger gel with higher gelling and melting points was obtained with addition of agar, especially at higher level.

7.2 Future works

1. Functional properties of gelatin from swim bladder should be studied.
2. Modification of gelatin from swim bladder using appropriate technology for property improvement should be investigated.
3. Applications of gelatin as food ingredients e.g. the stabilizer and gelling agent, in some selected foods or beverages should be further studied.

VITAE

Name Miss Onouma Kaewdang

Student ID 5511020017

Education Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Food Science and Technology)	Thaksin Unversity	2011

List of Publication and Proceedings

Publication

1. Kaewdang, O., Benjakul, S., Kaewmanee, T. and Kishimura, H. 2014. Characteristics of collagens from the swim bladders of yellowfin tuna (*Thunnus albacares*). Food Chemistry. 155: 264–270.

2. Kaewdang, O., Benjakul, S., Prodpran, T., Kaewmanee, T. and Kishimura, H. Characteristics of gelatin from swim bladder of yellowfin tuna (*Thunnus albacores*) as influenced by extracting temperatures. Italian Journal of Food Science (Accepted).

Proceeding

1. Kaewdang, O., Benjakul, S., Kaewmanee, T. and Kishimura, H. 2014. Characteristics of acid soluble- and pepsin soluble-collagens from swim bladder of yellowfin tuna (*Thunnus albacares*). The 16th Food Innovation Asia Conference, Bangkok, Thailand: 12th-13th June, 2014. Poster presentation.