Production and Characterization of Collagen, Gelatin and Gelatin Hydrolysate Powder from Scales of Spotted Golden Goatfish

Sira Chuaychan

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

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

……..Signature

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Candidate

ชื่อวิทยานิพนธ์ การผลิตและการจำแนกคุณลักษณะของคอลลาเจน เจลาติน และผงเจลาตินไฮโดรไลเสตจากเกล็ดปลาหนวดฤาษี
ผู้เขียน นายศิรา ช่วยจันทร์
สาขาวิชา วิทยาศาสตร์และเทคโนโลยีอาหาร
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บทคัดย่อ

จากการจำแนกคุณลักษณะของคอลลาเจนชนิดละลายในกรด (ASC) และชนิดที่ใช้เอนไซม์เปปซินสกัด (PSC) จากเกล็ดปลาหนวดฤาษี พบว่า ได้ผลผลิตร้อยละ 0.32 และ 1.22 ตามลำดับ(โดยฐานน้ำหนักเปียก) ทั้ง ASC และ PSC เป็นคอลลาเจนชนิด Type I ประกอบด้วยสายแอลฟา 1 แอลฟา 2 และสายบีตา ASC และ PSC ประกอบด้วยไกลซีนเป็นกรดอะมิโนหลักและมีกรดอิมิโน เท่ากับ 196 และ 198 หน่วยต่อ 1000 หน่วย T_{max} ของ ASC และ PSC มีค่าเท่ากับ 31.93 และ 32.95 องศาเซลเซียส ตามลำดับ จากสเปคตรา FTIR บ่งชี้ว่า การย่อยโดยเปปซินไม่มีผลต่อโครงสร้างเฮลิกซ์ชนิดทริปเปิลของ PSC ทั้ง PSC และ ASC มีการละลายสูงในช่วงพีเอชเป็นกรด (พีเอช 2-4) เกล็ดปลาหนวดฤาษีจึงอาจเป็นแหล่งวัตถุดิบทางเลือกของคอลลาเจน และกระบวนการสกัดที่ใช้มีผลต่อคุณลักษณะของคอลลาเจนเพียงเล็กน้อย

ก่อนสกัดเจลาติน เมื่อนำเกล็ดปลาหนวดฤาษีที่ผ่านการกำจัดโปรตีนที่ไม่ใช่คอลลาเจนมากำจัดแร่ธาตุโดยใช้กรดไฮโดรคลอริกที่ระดับความเข้มข้นต่างๆ (0.25-1 โมลาร์) เป็นเวลาต่างกัน (30-90 นาที) พบว่าสภาวะการกำจัดแร่ธาตุที่เหมาะสมคือ กรดไฮโดรคลอริกเข้มข้น 0.75โมลาร์ เป็นเวลา 30 นาที ซึ่งส่งผลให้มีปริมาณเถ้าเหลือเท่ากับร้อยละ 0.62 (โดยฐานน้ำหนักแห้ง) เมื่อนำเกล็ดปลาทั้งที่มีการกำจัดแร่ธาตุและไม่กำจัดแร่ธาตุมาตรวจสอบปริมาณแร่ธาตุโดยใช้ X-ray fluorescence spectrometer รวมทั้งตรวจสอบโครงสร้างโดยใช้กล้องจุลทรรศน์แบบส่องกราด (SEM) และ SEM-EDX พบว่า ภายหลังการกำจัดแร่ธาตุมีการกำจัดธาตุอินทรีย์ออกจากผิวชั้นนอก โดย Ca และ P ส่วนใหญ่ถูกกำจัดออกพร้อมกับการเพิ่มขึ้นของสารอินทรีย์ (C N และ O) แสดงว่าการกำจัดแร่ธาตุโดยการแช่กรดไฮโดรคลอริกสามารถกำจัดชั้นนอกของเกล็ดปลา ส่งผลให้การสกัดคอลลาเจนหรือเจลาตินมีประสิทธิภาพเพิ่มขึ้น

การสกัดเจลาตินจากเกล็ดปลาหนวดฤาษีภายใต้สภาวะต่างๆ พบว่า ผลผลิตและสมบัติของเจลาตินที่ได้แตกต่างกัน ผลผลิตเจลาตินซึ่งสกัดที่อุณหภูมิ 40, 60 และ 75 องศาเซลเซียส

เป็นเวลา 6 และ 12 ชั่วโมง มีค่าร้อยละ 2.3-2.6 8.6-9.3 และ 9.9-10.1 (โดยฐานน้ำหนักเปียก) ตามลำดับ เจลาตินทั้งหมดประกอบด้วยสายเปปไทด์บีตาและสายแอลฟาเป็นองค์ประกอบหลักและมีกรดอิมิโนสูง (182-192 หน่วยต่อ 1000 หน่วย) ความแข็งแรงเจลของเจลาตินลดลงในขณะที่สารละลายเจลาตินมีความขุ่นเพิ่มขึ้นเมื่ออุณหภูมิและระยะเวลาของการสกัดเพิ่มขึ้น อุณหภูมิในการเกิดเจลและอุณหภูมิหลอมตัวของเจลเจลาตินมีค่าเท่ากับ 18.7-20.1 และ 26.4-28.0 องศาเซลเซียส ตามลำดับ และอุณหภูมิทั้งสองลดลงเมื่ออุณหภูมิและเวลาในการสกัดเพิ่มขึ้น ดังนั้นควรสกัดเจลาตินที่อุณหภูมิ 75 องศาเซลเซียส เป็นเวลา 6 ชั่วโมง

จากการเตรียมและทดสอบกิจกรรมการต้านออกซิเดชันของเจลลาตินไฮโดรไลเสตจากเกล็ดปลาหนวดฤาษีที่มีระดับการย่อยแตกต่างกัน พบว่าเจลาตินไฮโดรไลเสตที่ระดับการย่อยร้อยละ 40 มีฤทธิ์การต้านออกซิเดชันสูงสุด ในการทำแห้งเจลาตินและเจลาตินไฮโดรไลเสต (ระดับการย่อยร้อยละ 40) แบบพ่นฝอย การศึกษาระดับของมอลโตเดกซ์ตรินเป็นสารช่วยยึดเกาะที่อัตราส่วนต่างๆ (1:0 2:1 1:1 และ 1:2 น้ำหนักต่อน้ำหนัก) ที่อุณหภูมิขาเข้าเท่ากับ 180 องศาเซลเซียส พบว่าเมื่อเพิ่มปริมาณของมอลโตเดกซ์ตรินทำให้ผลผลิตที่ได้เพิ่มขึ้น สัดส่วนของมอลโตเดกซ์ตรินที่เพิ่มขึ้นส่งผลให้ปริมาณน้ำตาลทั้งหมดและความขาวของผงเจลาตินและเจลาตินไฮโดรไลเสตเพิ่มขึ้น ส่วนค่า a^*, b^* และการเกิดสีน้ำตาลลดลง การละลายของผงเจลาตินเพิ่มขึ้นตามสัดส่วนของมอลโตเดกซ์ตริน เมื่อตรวจสอบโครงสร้างของผงเจลาตินและเจลาตินไฮโดรไลเสตโดยใช้กล้องจุลทรรศน์แบบส่องกราด (SEM) พบว่า ผงเจลาตินมีลักษณะทรงกลม ผิวเรียบและมีการหดตัวของอนุภาค ส่วนผงเจลาตินไฮโดรไลเสต มีความสม่ำเสมอและการหดตัวน้อยกว่า ฤทธิ์การต้านอนุมูลอิสระ DPPH ABTS และความสามารถรีดิวซ์เฟอริกของผงเจลาตินและผงเจลาตินไฮโดรไลเสตลดลง เมื่อมีการเพิ่มสัดส่วนของมอลโตเดกซ์ตรินเป็นสารช่วยยึดเกาะ

จากการศึกษาผลของอุณหภูมิขาเข้า (160 180 และ 200 องศาเซลเซียส) ของการทำแห้งแบบพ่นฝอย ต่อคุณลักษณะและฤทธิ์การต้านออกซิเดชันของผงเจลาตินไฮโดรไลเสตที่ผสมกับมอลโตเดกซ์ตริน (อัตราส่วน 2:1 น้ำหนักต่อน้ำหนัก) พบว่า ผลผลิตผงเจลาตินไฮโดรไลเสตและการเกิดสีน้ำตาลเพิ่มขึ้นเมื่ออุณหภูมิขาเข้าสูงขึ้น ผงเจลาตินไฮโดรไลเสตมีลักษณะผิวที่หดตัวเมื่ออุณหภูมิขาเข้าลดต่ำลง ผงเจลาตินไฮโดรไลเสตมีลักษณะทรงกลมและมีการหดตัวบางส่วนเมื่อใช้อุณหภูมิขาเข้าสูงขึ้น ฤทธิ์การต้านอนุมูลอิสระ ABTS DPPH และความสามารถรีดิวซ์เฟอริกของผงเจลาตินไฮโดรไลเสตลดลง เมื่ออุณหภูมิขาเข้าเพิ่มขึ้น การเติมผงเจลาตินไฮโดรไลเสตที่ทำแห้งด้วยอุณหภูมิขาเข้าเท่ากับ 180 องศาเซลเซียสลงในน้ำแอปเปิ้ล (ร้อยละ 1-5) สามารถเพิ่มฤทธิ์การ

ต้านอนุมูลอิสระ ABTS DPPH และความสามารถรีดิวซ์เฟอริกของน้ำแอปเปิ้ล ตามปริมาณของผงเจลาตินไฮโดรไลเสตที่เพิ่มขึ้น

Thesis Title Production and Characterization of Collagen, Gelatin and Gelatin Hydrolysate Powder from Scales of Spotted Golden Goatfish

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Major Program Food Science and Technology

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ABSTRACT

Acid soluble collagen (ASC) and pepsin soluble collagen (PSC) from the scales of spotted golden goatfish (*Parupeneus heptacanthus*) were characterized. Yields of ASC and PSC were 0.32 and 1.22% (based on wet weight basis), respectively. ASC and PSC were identified to be type I collagen, which consisted of α_1 and α_2 chains. β component was also found in both collagens. ASC and PSC contained glycine as the major amino acid and had high imino acid content (196 and 198 residues/1000 residues, respectively). T_{max} of ASC and PSC were 31.93 ºC and 32.95 ºC, respectively. FTIR spectra revealed that triple helical structure of PSC was not disrupted by pepsin digestion. Both ASC and PSC exhibited high solubility in very acidic pH range (pH 2–4). Therefore, spotted golden goatfish scale could be an alternative source of collagen and the characteristics of collagens were slightly affected by extraction process used.

Prior to gelatin extraction, scales were subjected to non-collagenous protein removal, followed by demineralization with HCl at different concentrations (0.25-1 M) for various times (30-90 min). The appropriate demineralization was pertained using 0.75 M HCl for 30 min, in which ash content was 0.62% (dry weight basis). The scales having non-collagenous protein removal without and with subsequent demineralization were analyzed for element contents using X-ray fluorescence spectrometer. Images of different scales were determined using scanning electron microscopy (SEM) and scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDX). Based on the images, an external layer rich in inorganic elements was removed. Most of Ca and P were eliminated with the coincidental increases in organic substances (C, N and O) after demineralization. Demineralization

therefore mainly removed the external layer of scales, which facilitated the further extraction of collagen or gelatin.

When gelatin was extracted from scales of spotted golden goatfish using various conditions, yields and properties varied. Yields of gelatin extracted at 45, 60 and 75 ^{o}C for various times (6 and 12 h) were 2.3-2.6%, 8.6-9.3% and 9.9-10.1% (on wet weight basis), respectively. All gelatins contained β-chain and α-chains as the predominant components and showed a high imino acid content (182-192 residues/1000 residues). Gel strength of gelatins decreased, whilst gelatin solution became more turbid as the extraction temperature and time increased. Gelling and melting temperatures of gelatin were 18.7-20.1 and 26.4-28.0^{o}C, respectively, and both temperatures decreased with increasing extraction temperatures and times. Thus, gelatin could be extracted from the scales at 75 ^{o}C for 6 h.

Gelatin hydrolysate from scale of spotted golden goatfish with different degrees of hydrolysis (DH) were prepared and examined for antioxidative activity. Gelatin hydrolysates with 40% DH exhibited the highest antioxidative activity ($p<0.05$). After spray drying at inlet temperature of 180 ^{o}C, characteristics and antioxidative activity of gelatin and gelatin hydrolysate (40% DH) powders using maltodextrin as a carrier agent at different ratios (1:0, 2:1, 1:1 and 1:2 (w/w)) were investigated. With increasing maltodextrin proportions, yield was increased. The resulting gelatin and gelatin hydrolysate powders had the increases in total sugar content and whiteness with coincidental decreases in a^*, b^*-values and browning intensity when maltodextrin proportion increased ($p<0.05$). Solubility of gelatin powder increased with increasing maltodextrin proportion ($p<0.05$). Based on SEM images, gelatin powder had spherical shape with both smooth surface and shrinkage. Sizes were varied, regardless of maltodextrin levels. For gelatin hydrolysate powder, uniform agglomerates with less shrinkage were formed when maltodextrin was incorporated. DPPH and ABTS radical scavenging activities and ferric-reducing antioxidant power of gelatin and gelatin hydrolysate decreased when maltodextrin was used as a carrier agent.

Effects of different spray drying temperatures (inlet temperatures of 160, 180 and 200 ^{o}C) on characteristics and antioxidative activity of gelatin hydrolysate from

spotted golden goatfish scale mixed with maltodextrin (2:1, w/w) were investigated. The yield of gelatin hydrolysate powder was increased, when inlet temperature increased ($p<0.05$). The resulting powders had the increases in browning intensity with increasing inlet temperatures ($p<0.05$). With lower inlet temperatures, the powder showed shriveled surface, whereas higher inlet temperature yielded the spherical powder with some shrinkage. ABTS and DPPH radical scavenging activities and ferric-reducing antioxidant power of gelatin hydrolysate decreased as inlet temperatures increased ($p<0.05$). Fortification of gelatin hydrolysate powder dried with inlet temperature of 180 ^{o}C (1-5%) into apple juice could increase ABTS, DPPH radical scavenging activities and ferric reducing antioxidant power in a dose-dependent manner ($P<0.05$).

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CHAPTER 1
INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

In various parts of the world, different fish species are being consumed daily in large quantities. As a result, a huge amount of byproducts, as large as 50–70% of original raw materials is generated in fish processing factories (Kittiphattanabawon *et al*., 2005). Improper disposal of these byproducts causes serious environment pollution with offensive odour. Generally these byproducts have been mainly used as animal feedstuff or fertilizer (Nagai and Suzuki, 2000a). Therefore, the maximized utilization of these byproducts, especially for the production of value-added products, is a promising solution. More than 30% of fish processing wastes consist of skin, scale and bone, which are very rich in collagen and have received increasing attention as collagen sources (Kittiphattanabawon *et al*., 2005). The main sources of type I collagen are generally from bovine or porcine origin (Ogawa *et al*., 2003). However, due to outbreak of Bovine Spongiform Encephalopathy (BSE), Transmissible Spongiform Encephalopathy (TSE), Foot and Mouth Disease (FMD) in pigs and cattle, the uses of collagen and collagen derived products from these sources have been limited (Jongjareonrak *et al*., 2005). Collagens from marine resources, especially from fish processing byproducts, have gained increasing attention as an alternative to mammalian collagen. Apart from collagen, gelatin, a denatured or partially hydrolyzed forms of collagen, has been widely used in various fields, e.g. food and pharmaceutical industries. Gelatin from fish scales has become increasing in demands to avoid the religious constraint. Additionally, it can serve as collagens derivative with bioactivity, especially antioxidative activity, which can prevent the damage of cells related with ageing. However, gelatin from fish skin has the limitation associated with fishy odor. Such a fishy offensive odor/flavor in skin is caused by the lipid oxidation (Sae-Leaw *et al*., 2013). To avoid the problem associated with undesirable flavor, fish scale can be used as the promising source of gelatin with negligible offensive odor/smell. However, fish scale has been known to render gelatin with lower yield. Therefore, the extraction process should be optimized. Additionally, some carrier can be added to improve the properties, especially solubility of gelatin

powder prepared by spray drying. During drying at higher temperature, those offensive odorous compounds could be removed to some degree. As a consequence, the gelatin powder obtained can be used as the instant powder for supplementation in drink without undesirable odor. The information gained can be of benefit for both fish processing industry and drink/food company, which can maximize the utilization of fish scales and the value added products can be launched.

1.2. Review of literature

1.2.1 Structure and composition of fish scale

Fish scale is composed of calcium-deficient hydroxyapatite ($Ca_{10}(OH)_2(PO_4)_6$) and extracellular matrix, mainly type I collagen fibers, which together form a highly ordered three-dimensional structure (Figure 1) (Lin *et al*., 2011). Each scale consists of two distinct regions: an external (osseous) layer and an internal fibrillary plate (Ikoma *et al*., 2003). In the upper external layer, collagen fibers are randomly arranged and embedded in a proteoglycan matrix. Within the fibrillary lower layer, in contrast, the collagen fibers are co-aligned and organized into lamellae that are superimposed to produce an orthogonal and/or a double-twisted plywood pattern (Ikoma *et al*., 2003). The collagen fibers are produced within the fibrillary layer by scleroblasts located at the base of the scales (Kemp *et al*., 2015). The fibers are organized through the cooperative involvement of microtubules and actin microfilaments that are subjected to consecutive alterations during the formation of plies of the basal plate (Ikoma *et al*., 2003; Zylberberg *et al*., 1988). In general, the spatial organization of collagen fibers is of key importance for the mechanical properties of different connective tissues (Ikoma *et al*., 2003; Weiner and Wagner, 1998).

Mineralization of scales occurs continuously throughout the life of fish. The external layer is initially mineralized with matrix vesicles, and then the internal layer is developed (Kemp *et al*., 2015). Needlelike or flaky crystals of apatite in random orientation are observed in the outer layer (Onozato and Watabe, 1979). Calcification of the internal layer, in contrast, occurs in the absence of matrix vesicles (Kemp *et al*., 2015), and the orientation of the crystallographic c-axis of apatite crystals parallel to collagen fibers in bony fish (*Leuciscus cephalus*) was reported (Bigi *et al*., 2001).

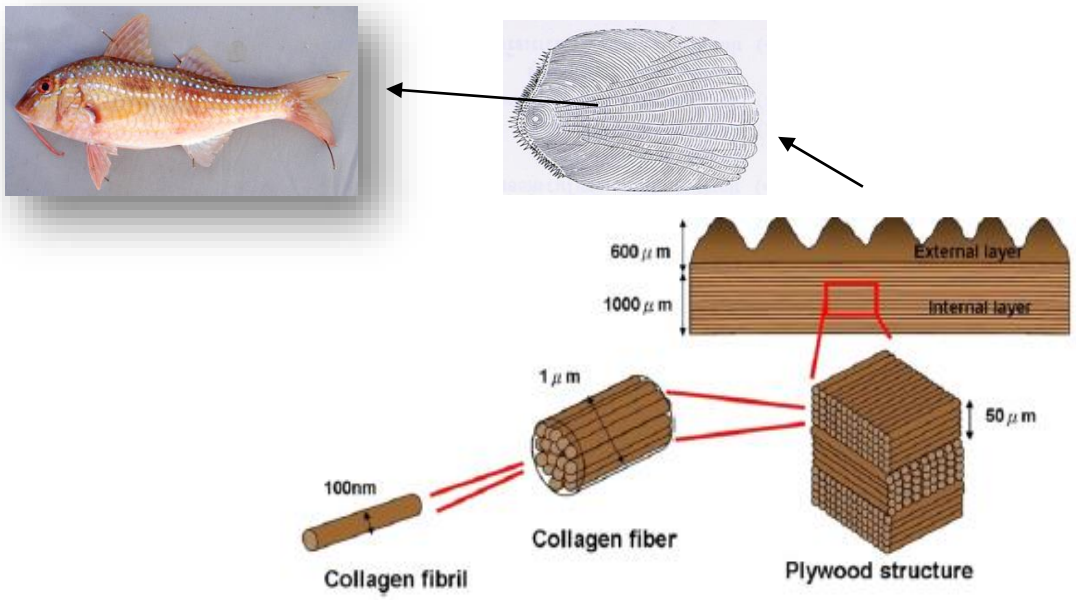

Figure 1 Structure of fish scale

Source: Lin *et al*. (2011)

1.2.2 Collagen

Collagen is abundant in tendons, skin, bone, etc. Additionally, it can be found in vascular system of animals and the connective tissue sheath surrounding muscle (Jongjareonrak *et al*., 2006). 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 coil is a left-handed helix with three residues per turn, and the three chains are twisted right-handed to form the triple helix held together by hydrogen bonding. 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 D, yielding a total molecular mass of about 300,000 D for collagen. (Foegeding *et al*., 1996).

Various types of collagen are observed among different organs and connective tissue layers of muscular tissue (Table 1).

Table 1. Type of collagen

Type	Molecule composition	Distribution
Fibrillar Collagen		
I	$[\alpha1(I)]_2$ [α2(I)]	skin, tendon, bone, dentin, ligaments, interstitial tissues
II	$[\alpha1(II)]_3$	Intervertebral disc, cartilage, vitreous humor
III	$[\alpha1(III)]_3$	Cardiovascular vessel, uterus, skin, muscle
V	[α1(V)][α2(v)][α3(V)]	Similar to type I, also cell cultures, fetal tissues; associates with type I
XI	[α1(XI)][α2(XI)][α3(XI)]	Cartilage, Intervertebral cartilage and bone enamel
Non-fibrillar collagens		
IV	[α1(IV)]2 [α2(IV)]	Basement membrane
VI	[α1(VI)][α2(VI)][α3(VI)]	Most interstitial tissues; associates with type I
VII	$[\alpha1(VII)]_3$	Epithelia
VIII	$[\alpha1(VIII)]_3$	Unknown, some endothelial cells
IX	[α1(IX)][α2(IX)][α3(IX)]	Cartilage; associates with type II
X	$[\alpha1(X)]_3$	Heterotrophic and mineralizing cartilage
XII	$[\alpha1(XII)]_3$	Ligaments, tendons and tooth

Source: Kuhn (2012)

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 (Chen *et al.*, 2016). Collagen molecules link end to end and adjacently form collagen fibers. Collagen has high contents of glycine (33%), proline

(12%) and the occurrence of 4-hydroxyproline (12%) and 5-hydroxylysine (1%) (Burghagen, 1999). Glycine generally represents mainly one-third of the total residues, and it is distributed uniformly at every third position throughout most of the collagen molecule. The repetitive occurrence of glycine is absent in the first 14 amino acid residues from the N-terminus and the first 10 from the C-terminus, with these end portions being termed "telopeptides" (Benjakul *et al*., 2010). Collagen is the only protein that is rich in hydroxyproline however, fish collagens have the lower hydroxyproline content than do mammalian counterpart (Chen *et al.*, 2016; Foegeding *et al*., 1996). Collagen is almost devoid of tryptophan and cysteine (Kittiphattanabawon *et al*., 2005).

The presence of proline stabilizes the helix structure by preventing rotation of the N-C bond. Hydroxyproline also stabilizes the collagen molecule. Collagen that contains small concentrations of both imino acids denature at lower temperatures than do those with larger concentrations (Foegeding *et al*., 1996). T_{max} of collagen of fish such as bigeye snapper (Nalinanon *et al*., 2007), unicorn leatherjacket (Ahmad *et al*., 2010) and seabass (Sinthusamran *et al*., 2013) were lower than that of bovine collagen (Ahmad *et al*., 2010). The higher T_{max} was in agreement with the higher imino acid content (Singh *et al*., 2011). The imino acid content of fish collagens therefore affects their thermal stability and correlates with the water temperature of their normal habitat (Foegeding *et al*., 1996).

1.2.3 Characteristics and molecular properties of collagens

Collagens in fish skin are generally belonging to type I. Collagens from skin of hake and trout were identified as type I (Ciarlo *et al*., 1997; Montero *et al*., 1991). Nagai *et al*. (2002) extracted the pepsin-solubilized collagen from the skin of ocellate puffer fish (*Takifuga rubripes*) and found that it comprised heterotrimer with a chain composition of $(\alpha 1)_2\alpha 2$. The denaturation temperature was 28 ^{o}C, about 9 ^{o}C lower than that of porcine collagen as determined by the changes in viscosity in 0.1 M acetic acid. From peptide mapping study using lysyl endopeptidase, peptide fragment patterns were different from porcine collagen, suggesting the difference in amino acid sequence among collagens from different species.

Collagens are most likely dissolved in the acidic pH ranges and the ionic strength affects the collagen solubility. Montero *et al*. (1999) reported that solubility of hake skin collagenous material in 0.5 M acetic acid was not greatly affected by pH between 1 to 5. However, it was found that the higher pH levels cause the solubility to decline. Moreover, at pH higher than 6, it practically becomes insoluble. The apparent viscosity of the skin collagenous material of hake declines progressively until at pH 5 where it was zero. The high solubility in 0.5 M acetic acid indicates a low degree of molecular cross-linking, or the predominance of weak bond in the skin collagenous material of hake (Montero *et al*., 1999). An increase in ionic strength of 0.5 M acetic acid in the solubility test from 0 to 0.17 by the addition of NaCl caused the increase in solubility of skin collagenous material. However, upon ionic strength of 0.17, the solubility decreased, and at 0.51, solubility was around zero. Matmaroh *et al*. (2011) reported that acid soluble collagen and pepsin soluble collagen from spotted golden goatfish scale (*Parupeneus heptacanthus*) had the decrease in solubility in the presence of NaCl (30 g/L). Minh Thuy *et al*. (2014) extracted acid soluble collagen from the scales of lizard fish (*Saurida spp*.) and horse mackerel (*Trachurus japonicus*). All acid soluble collagen showed high solubility at NaCl concentrations of 0.2–0.4 M. At low NaCl concentration, salt ions bind weakly to charged groups on proteins surface without affecting the hydration shell on those domains. This phenomenon contributed to the high solubility of ASC (Matmaroh *et al*., 2011)

1.2.4 Bone collagen

There is a wide distribution of molecular forms in fish bone collagen (Nagai and Suzuki 2000c). Subunit structures of collagen from bone of various species were quite different as follows: Japanese sea bass; (α1)2α2, horse mackerel; (α1)3 and ayu; α1α2α3. Li *et al*. (2013) extracted acid soluble collagen and pepsin soluble collagen from spanish mackerel (*Scomberomorous niphonius*) bone. Both collagens were type I and contained (α1)2α2. Wang *et al*. (2008) also used 0.5 M acetic acid to extract acid soluble collagen from deep-sea redfish (*Sebastes mentella*) bone and collagens was belonging to type I (α1)2α2. This observation was similar to

the findings reported for collagen from black drum (*Pogonia cromis*) and sheepshead seabream (*Archosargus probatocephalus*) bone (Ogawa *et al*., 2004).

1.2.5 Scale collagen

Fish scales are dermally derived, specifically in the mesoderm, and biocomposites of highly ordered type I collagen fibers and hydroxyapatite $Ca_{10}(OH)_2(PO_4)_6$ (Zhang *et al*., 2011). Scales are stiff and not easy to swell like the bone. Scale is generally decalcified using EDTA, prior to extraction using acid solution. Collagen from scale of snakehead (*Ophiocephalus Argus*) (Liu *et al*., 2009), scale of sardine (*Sardinops melanostictus*), red sea bream (*Pagrus major*) and Japanese sea bass (*Lateolabrax japonicas*) (Nagai *et al*., 2004) were extracted and characterized. Pati *et al*. (2010) reported that collagen from scale of rohu (*Labeo rohita*) and catla (*Catla catla*) had the similarity in property to mammalian counterpart. However, α1α2α3 heterotrimer was found in collagen from the scale of grass carp fish (*Ctenopharyngodon idellus*) (Li *et al*., 2008). Collagen from scale of carp (*Cyprinus carpio*) extracted with 0.5 M acetic acid was present as native form and had denaturation temperature around 28 ^{o}C (Duan *et al*., 2009). The abundance of glycine and high contents of alanine, proline, hydroxyproline and glutamic acid were reported in collagen from scale of sardine, red sea bream and Japanese sea bass (Nagai *et al*., 2004). Both ASC and PSC were rich in alanine, asparagine/asparatic acid, glutamine/glutamic acid and arginine. Imino acids (hydroxyproline and proline) were also found at the high content.

1.2.6 Extraction of fish collagen

In general, the raw material for collagen preparation contains a number of contaminants including noncollagenous proteins, lipids, and pigments, One or several pretreatments are used to remove the contaminants and increase the purity of extracted collagen. The removal of residual meats and cleaning are performed before further chemical pretreatments. Size reduction of raw material is also useful to facilitate the contaminant removal and collagen extraction. In general, alkaline solution, especially NaOH has been commonly used. Some raw materials such as bones from carp (Duan *et al*., 2009) and bigeye snapper (Kittiphattanabawon *et al*.,

2005) and scale from carp (Duan *et al.*, 2009) contain high amount of calcium. Those raw materials are effectively decalcified by using ethylenediaminetetraacetic acid (EDTA). Liu *et al*. (2012) also used 0.5 M EDTA for decalcification of scale from bighead carp *(Hypophthalmichthys nobilis)*. The decalcification can be also achieved by using inorganic acid, especially hydrochloric acid. As a consequence, the porous decalcified raw material with increased surface area can be readily subjected to collagen extraction (Simpson *et al*., 2012).

1.2.6.1 Acid solubilization process

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 using acidic condition, in which the positive charge of collagen polypeptides becomes dominant. As a consequence, the enhanced repulsion among tropocollagen can be achieved, leading to the increased solubilization (Simpson *et al*., 2012). Generally, tropocollagen is still in the form of triple helix with negligible changes. Fourier transform infrared (FTIR) spectra of collagens from the skins of carp and brownbanded bamboo shark and arabesque greenling extracted with acetic acid apparently revealed the triple-helical structure of collagen (Duan *et al*., 2009, Nalinanon *et al*., 2010, Kittiphattanabawon *et al*., 2010a). Collagen from total tissue can be isolated by direct extraction with organic acids (acetic, chloroacetic, citric, lactic acids) or inorganic acid (hydrochloric). The yield of extracted collagen depends on the animal species used and the age and parameters of extraction (Skierka and Sadowska, 2007). Collagen extraction from different species has been performed using acetic acid. Collagen extraction is generally achieved by 0.5 M acetic acid treatment at 4 ^{o}C for 24–48 h. Compared to simply prolonging the extraction time, the use of several consecutive extraction resulted in higher yield (Sadowska *et al*., 2003, Regenstein and Zhou 2007, Skierka and Sadowska 2007). Nalinanon *et al*. (2011) found that ASC yield from the skin of threadfin bream was increased from 12.32% to 34.90% (based on hydroxyproline content), when extended the extraction time from 6 to 48 h. The similar result was observed in collagen extraction from bigeye snapper skin, in which the yield increased from 7.3% to 9.3% (based on hydroxyproline

content) with increasing extraction time from 24 to 48 h (Nalinanon *et al*., 2007). Increasing the concentration of acetic acid from 0.1 to 0.5 M resulted in the increased yield of collagen extracted from minced cod skin, from 52% to 59% (dry weight). Re-extraction of residual matter with 0.5 M. acetic acid can be performed to increase the yield of acid soluble collagen, particularly from carp bone (Duan *et al*., 2009), bigeye snapper skin and bone (Kittiphattanabawon *et al.,* 2005). Chen *et al*. (2016) used 0.5 M acetic acid to extract acid soluble collagen from scale of tilapia with the yield of 3.2% (on a wet weight basis). Additionally, Minh thuy *et al*. (2014) extracted acid soluble collagen from the scale of lizard fish from Japan and Vietnam using 0.5 M acetic acid; the yield was 0.79% and 0.69% (on a dry weight basis), respectively. Nagai and Suzuki (2000c) extracted the acid-soluble collagen from various fish bones using EDTA solution to completely decalcify for 5 days, 10% butyl alcohol to remove fat and 0.5 M acetic acid to extract the acid-soluble collagen. The yields of the collagens were as follows: Japanese sea bass, 40.7%, horse mackerel, 43.5% and ayu, 53.6% on the basis of dry weight. The extraction conditions of collagen from different fish are summarized in Table 2.

1.2.6.2 Pepsin solubilization process

Generally, typical acid solubilization process renders a low yield of collagen. To tackle the problem, pepsin has been applied because it is able to cleave peptides specifically in telopeptide region of collagen, leading to increased extraction efficiency (Simpson *et al*., 2012) (Table 2). Use of pepsin as the aid for collagen extraction is a potential method for several reasons: (1) some of noncollagenous proteins are hydrolyzed and are easily removed by salt precipitation and dialysis, improving collagen purity; (2) hydrolyze those components and telopeptides of collagen to make the sample ready to solubilize in acid solution, resulting in improvement of extraction efficiency; (3) reduce antigenicity caused by telopeptide in the collagen which serve as the major problem in food and pharmaceutical applications (Werkmeister and Ramshaw 2000, Lee *et al*., 2001, Lin and Liu 2006, Cao and Xu 2008). After ASC extraction, the residues that represent the cross-linked molecules are further extracted in the presence of pepsin. The collagen obtained with pepsin treatment is referred to as “pepsin-soluble collagen, PSC” (Nagai *et al*., 2001,

Ogawa *et al*., 2003, Nalinanon *et al*., 2007). Pepsin, particularly pepsin from porcine stomach, has been used to maximize the extraction efficiency of collagen from several species such as largefin langbarbel catfish (Zhang *et al*., 2009), seaweed pipefish (Khan *et al*., 2009), deep-sea redfish (Wang *et al*., 2007), brownstripe red snapper (Jongjareonrak *et al*., 2005), ocellate puffer fish (Nagai *et al*., 2002), skate (Hwang *et al*., 2007), brownbanded bamboo shark (Kittiphattanabawon *et al*., 2010a), blacktip shark (Kittiphattanabawon *et al*., 2010c), cuttlefish (Nagai *et al*., 2001), octopus (Nagai *et al*., 2002b), scallop (Shen *et al*., 2007), paper nautilus (Nagai and Suzuki 2002), and common minke whale (Nagai *et al*., 2008). Skierka and Sadowska (2007) found that pepsin treatment of Baltic cod skin in acetic acid shortened the extraction time to 24 h and the solubility of collagen, after pepsin digestion increased from 55% to 90%. Liu *et al*. (2012) extracted PSC from bighead carp with the yield of 2.7% (on a dry weight basis). Zhang *et al*. (2010) also extracted PSC from scale of silver carp and the yield was 2.32% (on a dry weight basis).

Table 2 Extraction condition and some characteristics of collagen from different fish

Raw material	Species	Pretreatment[a]	Extraction[a]	%Yield (based on wet basis)	Collagen type	Molecular compositions	References
Skin	Bigeye snapper (*Priacanthus tayenus*, T) and *Priacanthus macracanthus*, M)	0.1 M NaOH for 6 h, followed by 10% butanol for 18 h	0.5 M acetic acid containing pepsin from tongol tuna stomach extract (TP) or porcine (PP) for 48 h	7.74 (T-PP) 8.73 (T-TP) 7.06 (M-PP) 7.29 (M-TP)	Type I	$(\alpha1)_2\alpha2$-heterotrimer	Benjakul *et al.* (2010)
	Largefin longbarbel catfish (*Mystus macropterus*)	0.1 M NaOH containing 0.5% non-ionic detergent for 24 h, followed by 15% (v/v) butanol for 24 h and 3% H_2O_2 for 24 h	0.5 M acetic acid for 24 h and re-extract with 0.5 M acetic acid for 12 h (ASC) 0.5 M acetic acid containing porcine pepsin for 30 h (PSC)	16.8[b] (ASC) 28.0[b] (PSC)	Type I	α1α2α3-herterotrimer	Zhang *et al.* (2009)
	Seaweed pipefish (*Syngnathus schlegeli*)	0.1 M NaOH for 36 h	0.5 M acetic acid for 3 days and re-extract with the same solution for 3 days (ASC) 0.5 M acetic acid containing porcine pepsin for 48 h (PSC)	5.5[b] (ASC) 33.2[b] (PSC)	Type I	$(\alpha1)_2\alpha2$ (ASC) and α1α2α3 (PSC) herterotrimer	Khan *et al.* (2009)
	Nile tilapia (*Oreochromis niloticus*)	0.1 M NaOH for 48 h, followed by 10 %(v/v) butanol for 24 h	0.5 M acetic acid for 3 days and re-extract with same solution for 2 days (ASC)	39.4[b] (ASC)	Type I	α1α2α3-herterotrimer	Zeng *et al.* (2009)

Table 2 Extraction condition and some characteristics of collagen from different fish (continued)

Raw material	Species	Pretreatment[a]	Extraction[a]	%Yield (based on wet basis)	Collagen type	Molecular compositions	References
Skin	Carp (*Cyprinus carpio*)	0.1 M NaOH for 6 h, followed by 1.0% detergent for 12 h	0.5 M acetic acid for 3 days (ASC)	41.3[b]	Type I	$(\alpha1)_2\alpha2$-heterotrimer	Duan *et al.* (2009)
Scale	Pacific saury (*Cololabis saira*)	50 mM Tris-HCl and 20 mM EDTA-Na for 1 week	500 mM acetic acid and 0.5% pepsin (w/v) for 2 days (PSC)	4[b]	Type I	$(\alpha1)_2\alpha2$-heterotrimer	Mori *et al.* (2013)
	Carp (*Cyprinus carpio*)	0.1 M NaOH for 6 h, followed by 0.5 M EDTA-2 Na for 24 h	0.5 M acetic acid for 4 days (ASC)	1.35[b]	Type I	$(\alpha1)_2\alpha2$-heterotrimer	Duan *et al.* (2009)
	Bighead carp (*Hypophthalmichthys nobilis)*	0.1 M NaOH for 36 h, followed by 0.5 M EDTA-2 Na for 5 days and 10 % (v/v) butyl alcohol for 36 h	0.5 M acetic acid containing 0.1% (w/v) pepsin at a sample/solution for 3 days	2.7[b] (PSC)	Type I	$(\alpha1)2\alpha2$-heterotrimer	Liu *et al*. (2012)
	Black drum (*Pogonia cromis*)	0.1M NaOH for 24 h and 0.1 M NaOH for 24 h.	0.5 M acetic acid for 3 days (ASC)	0.66	Type I	$(\alpha1)2\alpha2$-heterotrimer	Ogawa *et al*. (2004)
	Sheepshead seabream (*Archosargus probatocephalus*)	0.1 M NaOH for 24 h, re-soak with 0.1 M NaOH for 24 h.	0.5 M acetic acid for 3 days (ASC)	1.29	Type I	$(\alpha1)2\alpha2$-heterotrimer	Ogawa *et al*. (2004)

Table 2 Extraction condition and some characteristics of collagen from different fish (continued)

Raw material	Species	Pretreatment[a]	Extraction[a]	%Yield (based on wet basis)	Collagen type	Molecular compositions	References
Scale	Lizard fish *(Saurida spp.)* and Horse mackerel (*Trachurus japonicus)*	0.1 M NaOH for 6 h, followed by 0.5 M EDTA-2 Na for 24 h	0.5 M acetic acid for 4 days (ASC)	0.79 and 1.51	Type I	(α1)2α2-heterotrimer	Minh Thuy *et al.* (2008)
	Tilapia (Oreochromis niloticus)	0.1 M $NaHCO_3$ for 6 h	0.5 M acetic acid for 24 h. (ASC)	3.2	Type I	(α1)2α2-heterotrimer	Chen *et al.* (2016)
Bone	Bighead carp (*Hypophthalmichthys nobilis)*	0.1 M NaOH for 36 h, followed by 0.5 M EDTA-2 Na for 5 days and 10 % (v/v) butyl alcohol for 36 h	0.5 M acetic cid containing 0.1% (w/v) pepsin at a sample/solution for 3 days	2.9 (PSC)	Type I	(α1)2α2-heterotrimer	Liu *et al*. (2012)
	Carp (*Cyprinus carpio*)	0.1 M NaOH for 24 h, followed by 0.5 M EDTA-2Na for 5 days and 1.0% detergent for 12 h	0.5 M acetic acid for 3 days and re-extract with 0.5 M acetic acid for 2 days (ASC)	1.06[b]	Type I	$(α1)_2α2$-heterotrimer	Duan *et a* (2009)

[a] All procedures were performed at 4 ^oC; [b] base on dry basis; [c] NM: not mention

1.2.7 Gelatin

Gelatin is defined as a denatured protein derived from collagen by thermo-hydrolysis and has a rheological property of thermo-reversible transformation between sol and gel (Cho *et al*., 2004), which has been widely utilized in food, pharmaceutical, and photographic industries. Gelatin consists of random chains without triple helix. The process involves the disruption of non-covalent bonds and it is partially reversible during gelation process (Kittiphattanabawon *et al*., 2010). During the collagen to gelatin transition, many non-covalent bonds are broken along with some covalent inter-and intramolecular bonds (Schiff base and aldo condensation) and a few peptide bonds. This result is the conversion of helical collagen structure to a more amorphous form, known as "gelatin" (Figure 2). Generally, most of the commercial gelatins are made from the dermis or bone of porcine or bovine, however, because of the outbreak of bovine spongiform encephalopathy (BSE) and the foot-and mouth disease (FMD), the main sources of gelatin in many fields are limited to those of bovine or porcine dermis or bone (Zhang *et al*., 2011). Therefore, the study of gelatin from fish, such as skin, bone and scales has gained more attention. Gelatin from different kinds of fish skin and bone, such as grass carp fish skin (Kasankala *et al*., 2007), yellowfin tuna skin (Cho *et al*., 2005), shark cartilage (Cho *et al*., 2004), nile perch skin and bone (Muyonga *et al*., 2004), channel catfish skin (Liu *et al*., 2008), cod head (Arnesen and Gildberg, 2006), Atlantic salmon skin (Arnesen and Gildberg, 2007), flounder skin (Fernández-Díaz *et al*., 2003), etc. have been extracted and studied**.** Fish scales e.g. the scales of sardine scales (Nomura *et al*., 1996), the subtropical fish black drum and sheepshead scales (Ogawa *et al*., 2004) and deep-sea redfish scales (Wang *et al*., 2008) have been used for gelatin extraction.

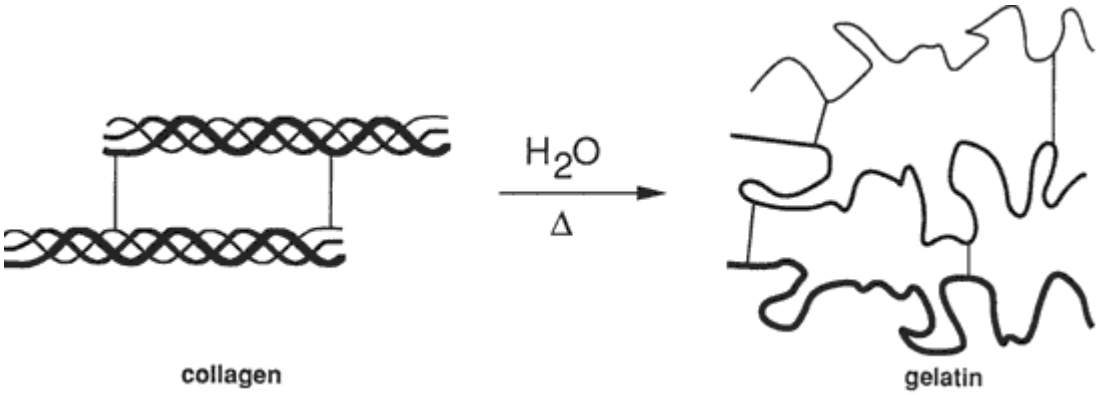

Figure 2. Conversion of collagen to gelatin

Source: Hansen *et al*. (1991)

1.2.8 Production of gelatin

1.2.8.1 Pretreatment of raw material

1.2.8.1.1 Removal of non-collagenous proteins

Prior to gelatin extraction from raw material, the pretreatment is practically implemented to increase purity of gelatin extracted. Alkaline solution has been used to remove considerable amounts of non-collagenous materials (Johns and Courts, 1977; Zhou and Regenstein, 2005) and break some interchain cross-links. Also, the process is able to inactivate proteases involved in degradation of collagen (Regenstein and Zhou, 2007). During alkaline pretreatment, the type of alkali does not make a significant different, but the concentration of alkali is critical (Zhou and Regenstein, 2005). Yoshimura *et al*. (2000) reported that alkali attacks predominantly the telopeptide region of the collagen molecule during pretreatment. Thus, some collagen can be solubilized by an alkaline solution. Long time and high concentration of alkaline pretreatment decreased the yield of gelatin from skin of channel catfish (Yang *et al.*, 2007). The concentration of alkali, time, and temperature used for pretreatment varied with raw materials as follows: 0.1 M NaOH for 3 h for seabass skin (Sinthusamran *et al*., 2014), 0.05 M NaOH for 4 h for skin of unicorn

leatherjacket (Kaewruang *et al*., 2013) and 0.025 M NaOH for 2 h for skin of bigeye snapper (Benjakul *et al*., 2009). Additionally, Akagunduz *et al*. (2014) used NaOH (4 g/l) for 1 h to remove non-collagenous proteins from scale of sea bream. Wangtueai and Noomhorm (2009) used 0.5 % NaOH for 3 h to remove non-collagenous protein from scale of lizard fish.

1.2.8.1.2 Removal of mineral

Unlike skins, scales are rich in Ca phosphate compounds such as hydroxyapatite and Ca carbonate. Therefore, the removal of Ca from fish scales is critical pretreatment in order to obtain gelatin with high purity and gel strength (Gomez-Guillen *et al.,* 2002). Demineralization of the raw material, especially bone or scale, aims to remove the calcium and other inorganic substances to facilitate the extraction of collagenous component (Waldner, 1977). The inorganic substances in raw material can be removed by treatment with dilute hydrochloric acid solution, whereby the calcium phosphate is dissolved as acid phosphates (Waldner, 1977). The fresh bone is commonly treated with hydrochloric acid solution, in which almost all minerals are completely removed. Gómez-Guillén *et al.* (2011) found that pre-treatment with 0.2 M EDTA resulted in decalcification more than 90%, with a gelatin yield of 22% and gel strength of 152 g. The property of gelatin from scale decalcified using EDTA was higher than those obtained from scale treated with 0.20 M HCl or 1.2 g/L citric acid. Depending on the nature of the material, temperature, and acid concentration, the demineralization time can be varied. HCl concentration used is generally in the range 2–6% (Waldner, 1977). Acid hydrolysis of the protein should be minimized during demineralization. High temperature should also be avoided since it can enhance the hydrolysis of protein. Bone from Nile perch (*Lates niloticus*) was demineralized with 3% HCl at room temperature prior to extraction using warm water (60^{o}C) (Muyonga *et al*., 2004). Demineralization of tuna fin (*Katsuwonus pelamis*) was carried out at room temperature using 0.6 N HCl (Aewsiri *et al*., 2008). Weng *et al*. (2014) used 0.05 M HCl solution at room temperature for 30 min to remove mineral of tilapia (*Tilapia zillii*) scale. Sha *et al*. (2014) also used 0.5 M HCl solution for 1 h to remove mineral of bighead carp scale. Additionally, Akagunduz *et al*. (2014) used 164 g/L EDTA for 12 h to remove mineral from sea bream scale.

1.2.8.1.3 Swelling process

The pretreatment via swelling is aimed to convert the collagen into a form suitable for extraction. The covalent cross-links in the collagen must be disrupted to enable the release of free α-chains during the extraction (Johnston-Banks, 1990). The process is able to remove other organic substances. Sulfuric and hydrochloric acids are often used with the addition of phosphoric acid to retard color development (Johnston-Banks, 1990). Additionally, the acid pretreatment can partially inactivate endogenous proteases involved in degradation. As a result, the enzymatic breakage of intrachain peptide bonds of collagen during extraction can be lowered (Zhou and Regenstein, 2005). Moreover, type of acid and concentration affected the yield and properties of gelatin. The concentration of H^+ used in processing of gelatin from cod skins affected yield and quality of resulting gelatin (Gudmundsson and Hafsteinsson, 1997). Megrim skin was treated with 0.05 M acetic acid and 0.05 M propionic acid prior to gelatin extraction using distilled water at 45^{o}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 (Gomez-Guillen and Montero, 2001). Gimenez *et al*. (2005) reported that use of lactic acid (25 mM) could be an excellent substitute for acetic acid for the skin swelling process. The gelatin obtained showed similar properties to that prepared by using 50 mM acetic acid without the negative organoleptic properties. Gelatin obtained from the acid process is known as type A gelatin. Additionally, Akagunduz *et al*. (2014) used acetic acid (2.85 mL/L) for 3 h to treat sea bream scale. Nikoo *et al*. (2014) used 0.05 M aetic acid for 3 h to swell skin of farmed amur sturgeon (*Acipenser schrenckii*). Apart from acetic acid, phosphoric acid has also been used for swelling process. Kaewruang *et al*. (2013) used 0.05 M phosphoric acid for 12 h to swell skin of unicorn leatherjacket. Nagarajan *et al*. (2012) used 0.05 M phosphoric acid for 24 h at 4 ^{o}C to swell skin of splendid squid (*Loligo formosana).*

1.2.8.2 Extraction

When the heat is applied, collagen fibrils shrink to less than one third of their original length at a critical temperature known as the shrinkage temperature (Foegeding *et al*., 1996). This shrinkage involves a disassembly of fibers and a collapse of the triple-helical arrangement of polypeptide subunits in the collagen molecule. When collagen is converted to gelatin, several noncovalent bonds are broken along with some covalent inter- and intramolecular bonds (Simpson *et al*., 2012). Heat applied at temperature higher than transition temperature (T_{max}) is able to disrupt the bonds, mainly H-bond, which stabilizes collagen structure. This results in conversion of the helical collagen structure to a more amorphous form known as gelatin. Different extraction conditions were implemented for different scales including lizardfish scales (78.5 ^{o}C for 3 h) (Wangtueai and Noomhorm, 2009), bighead carp scales (80 ^{o}C for 2 h) (Sha *et al*., 2014), sea bream scales (60 ^{o}C for 12 h) (Akagunduz *et al*., 2014), tilapia scales (80 ^{o}C for 1 h) (Weng *et al*., 2014) and fresh grass carp scale (60 ^{o}C for 6 h) (Zhang *et al.*, 2011). Gelatin from scales has varying yield, depending on species, pretreatment, etc. Process for gelatin extraction from fish scales including pretreatment condition are shown in Table 3.

Table 3 Extraction conditions of gelatin from scale of different fish.

Raw material	Pre-treatment	Extraction	References
Lizard fish scale (*Saurida spp.)*	Alkaline solution (0.51% NaOH) at room temperature for 3 h.	Water at 80 ºC for 3 h.	Wangtueai and Noomhorm, (2009)
Bighead carp scale (*Hypophthalmichthys nobilis*)	0.5 M HCl for 1 h.	Water at 80 ºC for 2 h.	Sha *et al*. (2014)
Grass carp fish scale	0.22% protease A 2G at 30.73 ºC for 5.52 h.	Water at 60 ºC for 6 h.	Zhang *et al.* (2011)
Seabream scale	5 % NaCl for 30 min., 4 g/l NaOH for 1 h., 146 g/l EDTA for 12 h. and 2.85 ml/l acetic acid for 3 h.	Water at 60 ºC for 12 h.	Akagunduz *et al.* (2014)
Tilapia scales	0.05 M NaOH, 25% alcohol and 1.5% H_2O_2 kept in a refrigerator at about 10 ºC overnight and treat with 0.05 M HCl for 30 min.	Water at 80 ºC for 1 h.	Weng *et al*. (2014)

1.2.8.3 Characteristics of fish gelatin

1.2.8.3.1 Gelling properties

Gel formation, which is obtained by cooling gelatin aqueous solution after the collagen is heated, is accompanied by some characteristic changes which have been ascribed to a partial regain of collagen triple-helix structure. Gelatins from different fish species have different gelling properties. Gelatins from flat-fish species (sole and megrim) presented the best gelling ability and the gels were more thermostable than those from cold-adapted fish (cod and hake) (Gomoz-Guillen *et al*., 2002). This different behaviour may be caused by the differences in amino acid composition, the α1: α2 collagen-chain ratio, and the molecular weight distribution (Kittiphattanabawon *at al*., 2005). Cod gelatin presented a lower alanine and imino acid content, and lower proline hydroxylation degree. Cod and hake gelatins had a low α1/α2 ratio (~1) and hake gelatin showed a highly significant decrease in β-components and other aggregates (Gomoz-Guillen *et al*., 2002). Squid gelatin showed viscoelastic properties between those from flat-fish and cold-adapted fish species (Gomoz-Guillen *et al*., 2002). Kittiphattanabawon *et al*. (2010) reported that gelatin from skin of two shark species showed different gelling property. Gelatin from blacktip shark had the poorer gel strength than that from brown banded bamboo shark. At the same extraction temperature, the α and β- chains were degraded in gelatin from blacktip shark. This was associated with the lower gel strength of gelatin from brown banded bamboo shark skin. Sinthusamran *et al*. (2014) also reported that extraction temperature played an important role in gelling property of gelatin from seabass skin. With higher extraction temperature, the gel strength became lower. Kaewruang *et al*. (2013) reported that the gels from gelatin extracted for a shorter time had more α-chains and higher gel strength. The content of α-chains and β-components influenced the gel strength of the gelatin, and the structure of gelatin was more stable with increasing amount of those components. The result was in agreement with Gómez-Guillén *et al*. (2002) who reported that gelatin extracted at higher temperatures showed a lower gel strength. Storage condition of raw material for gelatin production has been reported to affect gelling property of gelatin. Fernandez-Diaz *et al*. (2003) found that gelatin from the flounder (*Platichthys flesus*) skins frozen at –12 ^{o}C had

lower gel strength values when compared to that from fresh skins but showed the higher melting point value. Gelatin from frozen skins showed some high molecular weight aggregates. Gelatin from frozen skin showed less α and β-chains but more bands corresponding to lower molecular weight fragments. γ- component were less evident when both frozen at -12 ^{o}C and -20 ^{o}C and were especially pronounced in the case of skin frozen at -12 ^{o}C.

Fish gelatin has been known to possess the lower gel strength, compared to gelatin from porcine or bovine collagen (Norland, 1990). Fennandez-Diaz *et al*. (2001) studied the gel properties of gelatin from cod *(Gadus morhua*) and hake (*Merluccius merluccius*) as modified by the coenhancers, glycerol, salt and microbial transglutaminase. Gel strength was substantially increased by the addition of coenhancers although results varied, depending on the species. For gelatin from hake skin, the highest values were obtained with 10 mg/g of transglutaminase, whereas magnesium sulphate was more effective at both concentrations (0.1 and 0.5 M) in gelatin from cod skin. Jongjareonrak *et al*. (2006) reported that the increase in bloom strength of gelatin gel from bigeye snapper and brownstripe red snapper skin gelatin was observed with the addition of MTGase up to 0.005% and 0.01%, respectively. MTGase might catalyze the formation of ε-(γ-glutamyl) lysine linkages between protein chains. However, bloom strength decreased with further increase in MTGase concentration. This was in agreement with Gómez-Guillén *et al*. (2001) who reported that the increases in MTGase concentration from 0.015% to 0.075% lowered the bloom strength of megrim skin gelatin gel and Norziah *et al*. (2009) found that the addition of MTGase with the concentration of 0.5 and 1.0 mg/g into fish gelatin extracted from the wastes of fish herring species (*Tenualosa ilisha*) had higher gel strength than that without the addition of enzyme. Nevertheless, enzyme concentration above 1.0 mg/g gelatin resulted in a decreased gel strength. Additionally, Kaewruang *et al*. (2014) reported that an increase in gel strength was observed when the concentrations of $ZnCl_2$ and $CaCl_2$ increased from 2.5 to 20 mM. Divalent ions could act as the bridge between negatively charged residues, particularly phosphate groups and carboxyls. As a result, the interaction between gelatin chains could be enhanced, thereby strengthening the gel network.

1.2.8.3.2 Solubility

Gelatin solubility is dependent on the charge of the protein molecule of the gelatin. When pH is far from the isoionic pH of the gelatin, the better the solubility and performance of gelatin are obtained. Protein molecules have a net positive charge and behave as polycations at pH values below the IEP (De Kruif *et al*., 2004). Gelatin in solution is amphoteric, capable of acting either as an acid or as a base. In acidic solutions gelatin is positively charged and migrates as a cation in an electric field. In alkaline solutions, gelatin is negatively charged and migrates as an anion. The pH of the intermediate point, where the net charge is zero and no movement occurs, is known as the Isoelectric Point (IEP) (Janus *et al*., 1951). Type A gelatin has a broad isoelectric range between pH 7 and 9. Type B has a narrower isoelectric range between pH 4.7 and 5.4 (Maxey and Palme, 1967). Lassoued *et al*. (2014) reported that thornback ray gelatin have minimum solubility at pH 5. The differences in their amino acid composition, especially charged amino acids, affect the solubility of gelatin. At isoelectric point, the lack of electrostatic repulsion is noted. This promotes aggregation and precipitation via hydrophobic interactions (Zayas, 1997).

The addition of sodium chloride to gelatin solution was shown to increase the ionisation of gelatin (Thaimann, 1930). This effect is attributed to the formation of complex ions. The bearing of complex ion formation on the zwitter-ionic structure and solubility phenomena of proteins is pointed out. In addition, Damodaran (1996) reported that at low NaCl concentration, salt ions bind weakly to charged groups on proteins surface without affecting the hydration shell on those domains (Damodaran, 1996). Matmaroh *et al*. (2011) reported that the decrease in solubility of gelatin with the increase of NaCl concentration might be attributed to the salting-out effect (Bae *et al*., 2008; Matmaroh *et al*., 2011). These solubility behaviours of gelatin with changes in pH and NaCl concentrations may play a crucial role in their extraction and application (Montero *et al*., 1991).

1.2.9 Gelatin hydrolysate

1.2.9.1 Production of hydrolysate

Gelatin and collagen-derived hydrolysate and peptide are generally obtained by enzymatic proteolysis. Enzymatic hydrolysis of proteins brings about the formation of bioactive peptides and these can be obtained by hydrolysis of protein sources using appropriate proteolytic enzymes. Enzymatic conditions including temperature and pH for protein hydrolysis, must be optimized (Kim and Wijesekara, 2013). Enzymes have either endo- or exopeptidase activities. The former pertains to enzymes cleaving peptide bonds within the protein, resulting in many peptides but relatively few free amino acids. Exopeptidase activity refers to an attack on either end of the protein polypeptide chain, thus giving many free amino acids and few large peptides (Kristinsson, 2007). The general principle of the process is simple (Figure 3) but there are many factors that need to be carefully considered to control the process and produce a final product of consistent and good quality (Kristinsson, 2007). Proteolytic enzymes from microbes, plants and animals can be used for the hydrolysis process of marine proteins to release bioactive peptides (Kim and Wijesekara, 2013). Akagunduz *et al*. (2014) prepared scale gelatin hydrolysate using esperase under optimal conditions (pH 8, 60 ^{o}C) to obtain a maximum yield. Moreover, Ngo *et al*. (2010) using alcalase, pronase E, trypsin and pepsin to hydrolyze Nile tilapia (*Oreochromis niloticus*) scale gelatin. Peptide derived from Nile tilapia (*O. niloticus*) scale gelatin acts as a candidate against oxidative stress and could be used as a potential functional food ingredient. Gelatin hydrolysate can be prepared from skin gelatin such as blacktip shark gelatin using papaya latex enzyme (Kittiphattanabawon *et al*., 2012), tuna (*Thunnus spp.)* gelatin using alcalase (Aleman *et al*., 2011) and bigeye snapper skin (*Priacanthus macracanthus)* using pyloric caeca extract from bigeye snapper (Phanturat *et al*., 2010). Degree of hydrolysis has been shown to determine bioactivities of hydrolysate (Kittiphattanabawon *et al*., 2012). Klompong *et al*. (2007) reported that enzyme and levels of enzyme affected degree of hydrolysis. Recently, Sai-ut *et al*. (2013) used the protease from *Bacillus amyloliquefaciens* to produce gelatin hydrolysate and its proteolytic activity was equivalent to Alcalase. Senphan *et al*. (2014) also reported that gelatin hydrolysate from seabass skin with

antioxidant activity was prepared using protease from hepatopancreas of Pacific white shrimp.

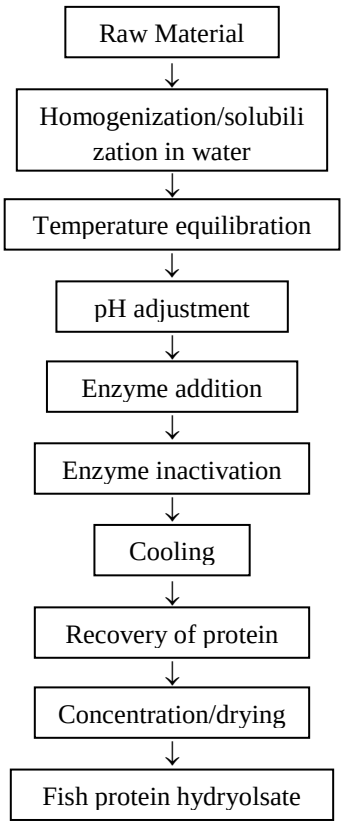

Figure 3 Flow chart for the production of fish protein hydrolysates.

Source: Kristinsson (2007)

1.2.9.2 Antioxidant activity

Peptides from fish processing byproducts have been known to exhibit bioactivities. The type of bioactive peptides generated from a particular protein is dependent on two factors: (a) the primary sequence of the protein substrate and (b) the specificity of the enzyme (s) used to generate such peptides (Harnedy and FitzGerald, 2012). Furthermore, different peptides can be generated by both acid and alkaline hydrolysis (Harnedy and FitzGerald, 2012). Although the structure–activity relationship of many bioactive peptides has not yet been fully established, several

structural features, especially sequence of amino acids, have been identified which appear to influence the biological action of peptides. Sequence of amino acid in an active peptide, Asp-Pro-Ala-Leu-Ala-Thr-Glu-Pro-Asp-Pro-Met-Pro-Phe, (1382.57 Da) isolated from nile tilapia (*Oreochromis niloticus*) scale gelatin was reported by Ngo *et al*. (2010). In addition, protective effect of nile tilapia skin hydrolysate on DNA damage caused by hydroxyl radicals generated was determined (Ngo *et al*., 2010). Gelatin peptides have repeated unique Gly–Pro–Ala sequence in their structure and it is presumed that the observed antioxidative and antihypertensive properties of gelatin peptides can be associated with their unique amino acid compositions (Kim *et al*., 1998). Gelatin hydrolysate from blacktip shark skin hydrolysate using papaya latex at levels of 500 and 1000 ppm could inhibit lipid oxidation in both β-carotene linoleate and cooked comminuted pork model systems. Gelatin hydrolysate from shark skin with DH of 40% effectively inhibited hydroxyl and peroxyl radical-induced DNA scission (Kittiphatanabawon *et al*., 2012; Kittiphatanabawon *et al*., 2013). Antioxidation activity and another biological activity of fish waste derived protein hydrolysates and peptides are shown in Table 4.

Table 4 Biological activity associated with fish waste derived protein hydrolysates and peptides

Sources	Peptide(s)	Mode of action	Reference
Hoki (*Johnius belengerii*) skin	His-Gly-Pro-Leu-Gly-Pro-Leu	Antioxidant activity	Mendis *et al*. (2005)
Pollack skin	Gly-Glu-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly- Pro-Hyp-Gly-Pro-Hyp-Gly	Antioxidant activity	Kim *et al*. (2001)
Sea bream scale gelatin	Gly-Tyr, Val-Tyr, Gly-Phe, Val-Ile-Tyr	ACE Inhibitory activity	Fahmi *et al*. (2004)
Blacktip shark skin gelatin	-	ACE-inhibitory, inhibition of LDL cholesterol, hydroxyl and peroxyl radical-induced DNA scission	Kittiphattanabawon *et al*. (2013)
Bigeye snapper (*Priacanthus macracanthus)* skin	-	(ABTS) radical scavenging activities, ferric reducing antioxidative power (FRAP) and DPPH	Phanturat *et al*. (2010)

Table 4 Biological activity associated with fish waste derived protein hydrolysates and peptides (continued)

Sources	Peptide(s)	Mode of action	Reference
Tuna frame	Gly-Asp-Leu-Gly-Lys-Thr-Thr-Thr-Val-Ser-Asn-Trp-Ser-Pro-Pro-Lys-Tyr-Lys-Asp-Thr-Pro	Antihypertensive activity	Lee *et al*. (2010)
Halibut (*Hippoglossus stenolepis*) skin	Gly-Pro , Ile-Pro , Pro-Pro, Ser-Pro and Leu-Pro	Antioxidant activity	Wang *et al*. (2015)
Sole frame (*Limanda aspera*)	Arg-Pro-Asp-Phe-Asp-Leu-Glu-Pro-Pro-Tyr	Antioxidant activity	Jun *et al*. (2004)
Tilapia (*Oreochromis niloticus*) skin gelatin	Glu-Gly-Leu (317.33 Da) and Tyr-Gly-Asp-Glu-Tyr (645.21 Da)	Hydroxyl radical scavenging activity, DPPH	Zhang *et al.* (2012)
Sole skin	-	Antioxidant activity	Gimenez *et al*. (2009)
The Pacific hake (*Merluccius productus*) skin	Ser-Pro, Gly-Pro, Pro-Pro, Ile-Pro and Leu-Pro	Antioxidant activity and DPP-IV inhibitory activity	Wang *et al*. (2015)

1.2.10 Spray-drying

1.2.10.1 Principle of spray drying technique

Spray drying is one of the best drying methods to convert directly the fluid materials into solid or semi-solid particles (Murugesan and Orsat, 2011). Spray drying is a unit operation by which a liquid product is atomized in a hot gas current to instantaneously obtain a powder. The gas generally used is air or more rarely an inert gas, particularly nitrogen gas. The initial liquid feeding can be a solution, an emulsion or a suspension (Gharasallaoui *et al*., 2007). It can be used to both heat-resistant and heat sensitive products.

Spray drying involves in the complex interactions of process, apparatus and feed parameters which all have an influence on the final product quality (Chegini *et al*., 2008). The spray drying process can produce a good quality final product with low water activity and reduce the weight, resulting in easy storage and transportation. The physicochemical properties of the final product mainly depend on inlet temperature, air flow rate, feed flow rate, atomizer speed, types of carrier agent and their concentration. Spray drying is often selected as it can process material very rapidly while providing relative control of the particle size distribution (Obon *et al*., 2009).

The spray dryer is a device used to produce dried foods. It takes a liquid stream and separates the solute or suspension as a solid and the solvent into a vapor. The solid is usually collected in a drum or cyclone. The liquid input stream is sprayed through a nozzle into a hot vapor stream and vaporized. A nozzle is usually used to make the droplets as small as possible to maximize the heat transfer and rate of water vaporization. The spray dryers can dry a product very quickly compared to other methods of drying. They also turn a solution or slurry into a dried powder in a single step, which can be the advantage for maximizing the profit and minimize the process (Chegini and Ghobadian, 2007; Murugesan and Orsat, 2011).

1.2.10.2 Factors influencing the properties of powder produced by spray drying

The spray drying parameters such as inlet temperature, air flow rate, feed flow rate, atomizer speed, types of carrier agent and their concentration have the influence on particle size, bulk density, moisture content, yield and hygroscopicity in spray dried foods (Chegini and Ghobadian, 2005; Chegini and Ghobadian, 2007; Yousefi *et al*., 2011).

1.2.10.2.1 Inlet temperature

Powder properties such as moisture content, bulk density, particle size, hygroscopicity and morphology were affected by inlet temperature. Normally, the inlet temperature used for spray dry technique is 150-220^{o}C. Chegini and Ghobadian (2005) studied the effect of inlet temperature (110-190^{o}C) on the moisture content of orange juice powder. It was found that at a constant feed flow rate, increasing the inlet air temperature reduced the residual moisture content. Fang *et al*. (2012) reported that higher drying temperature could remove the moisture more efficiently. The particles were larger with broader size variation when dried at higher temperatures. Accordance to Phisut (2012), moisture content is decreased with the increasing drying temperature, due to the faster heat transfer between the product and drying air. At higher inlet air temperatures, there is a greater temperature gradient between the atomized feed and drying air and it results in the greatest driving force for water evaporation (Phisut, 2012)

1.2.10.2.2 Air dry flow rate

Generally, the energy available for evaporation is varied according to the amount of drying air. The rate of air flow must be at a maximum in all cases. The movement of air decides the rate and degree of droplet evaporation by inducing the passage of spray through the drying zone and the concentration of product in the region of the dryer walls and finally extent the semi-dried droplets and thus re-enter the hot areas around the air disperser. A lower drying air flow rate causes an increase in the product heating time in drying chamber and enforces the circulatory effects

(Goula and Adamopoulos, 2004; Master, 1979; Oakley and Bahu, 2000). The moisture content in tomato powder was increased with an increase in drying air flow rate (Goula and Adamopoulos, 2005).

1.2.10.2.3 Atomizer speed

The atomizer is a critical component of the spray dryer. The degree of atomization influences the drying rate, the required particle residence time, particle size and particle size distribution, which in turn relates to dispersibility of the product for rehydration (Chegini and Taheri, 2013). At the same time, the increased atomizer speed applied on a droplet to spread on a larger surface has reduced the particle size and decreased the bulk density (Greenwald and King, 1981). Chegini and Taheri (2013) reported that a lower atomizer speed showed a higher density than with higher atomizer speed. At higher atomizer speed, smaller droplets are produced and more moisture is evaporated, resulting from the increased contact surface. Chegini and Ghobadian (2005) studied the effect of atomizer speed (10,000-25,000 rpm) on the properties of an orange juice powder. The residual moisture content was decreased when the atomizer speed increased. At higher atomizer speed, the smaller droplets were produced and more moisture was evaporated, resulting from an increased contact surface.

1.2.10.2.4 Feed flow rate

Higher flow rates imply a shorter contact time between the feed and drying air and making the heat transfer less efficient and thus caused the lower water evaporation (Phisut, 2012). The higher feed flow rate showed a negative effect on process yield. Chegini and Taheri (2013) reported that an increase in feed flow rate cause a decrease in product total solid with an increase in bulk density and particle size. Increasing feed flow rate reduced the percentage of insoluble solid, because of the higher droplet moisture content and thinned dried layer on the powder particles (Chegini and Taheri, 2013). The feed flow rate negatively affected the moisture content in the acai juice powder (Tonon *et al*., 2008).

1.2.10.3 Type and concentration of carrier agent

1.2.10.3.1 Functions

The addition of high molecular weight additives to the product before atomizing is widely used as an alternative way to increase Tg of powder (Truong *et al*., 2005). The use of carrier agents such as maltodextrins, gum Arabic, waxy starch, and microcrystalline cellulose, influenced the properties and stability of the powder (Phisut, 2012). Crystalline and amorphous forms of the same material powder show differences in particle size, particle shape, bulk density, physicochemical properties, chemical stability, water solubility and hygroscopicity (Yousefi *et al*., 2011). The addition of maltodextrin could increase the total solid content in the feed and thus reduce the moisture content of the product. It was suggested that maltodextrin could alter the surface stickiness of low molecular weight sugars such as glucose, sucrose and fructose and organic acids. This therefore facilitated drying and reduced the stickiness of the spray dried product. However, if the added maltodextrin was more than 10%, the resulting powders lost their attractive red-orange colour (Quek *et al*., 2007). Quek *et al*. (2007) investigated the effect of maltodextrin concentrations (0, 3 and 5%) on the properties of the watermelon juice powder. The addition of 5% maltodextrin to the feed appeared to give better results than addition of 3% maltodextrin. Maltodextrin was a useful drying aid in the spray drying process of watermelon juice.

1.2.10.3.2 Maltodextrin

Maltodextrin is a neutral dextrin enzymatically derived from starch with dextrose equivalent values < 20, consisting of (1→4 and 1→6)-α-D-glucopyranose-linked residues (Alevisopoulos *et al*., 1996). The formation of a maltodextrin network depends on the dissolution and quench temperature, time and polymer concentration. Loret *et al*. (2004) found that a ~30% (w/w) maltodextrin sample formed a network within 5 min at 25 ^{o}C, whereas 25% (w/w) maltodextrin gelled after ~50 min. Currently, maltodextrins used in food and herbal drug processing, have multifunctional properties: bulking and film formation, the ability to bind flavor and fat, and reducing the permeability of the wall matrix for oxygen

(Sansone *et al*., 2011). In general, the usage of maltodextrins with higher water solubility can contribute to a significant reduction in the apparent viscosity of feed dispersion in favor of the atomization and drying of the liquid feed (Pierucci *et al*., 2007). Since the food industry currently emphasizes the use of natural rather than synthetic ingredients, the formulations based on maltodextrins could be considered as favorable. However, the quantity of drying agents is limited by the organoleptic properties of the final products (Truong *et al*., 2005). The addition of drying aids in large amounts increases the cost and may also alter the original flavor and taste of the final product, thus risking consumer disapproval.

The concentration of the carrier agent also affected the moisture content of powder. Moisture content of powder was increased with the increase of maltodextrin concentration as reported by Goula and Adamopoulos (2008). The similar observation was attained in the maltodextrin addition on drying kinetics and stickiness of sugar and acid-rich foods during convective drying and it also lowered the drying rate (Adhikari *et al*., 2003). In contrast, the moisture content of spray dried watermelon juice powders was decreased when the maltodextrin increased. The addition of the drying additives has increased the total solids of the feed and reduced the amount of water evaporation. Maltodextrin has the capability to hurdle the sugars in the fruit powder that have the highly hygroscopic nature of absorbing the humidity in the surrounding air (Shrestha *et al*., 2007).

1.3 Objectives of study

1.3.1 To extract and characterize collagen from scale of spotted golden goatfish.

1.3.2 To study the impact of demineralization on morphology and inorganic element distribution of spotted golden goatfish scale.

1.3.3 To optimize the extraction process and characterize gelatin and gelatin hydrolysate from spotted golden goatfish scale.

1.3.4 To study drying condition and maltodextrin on characteristic and property of gelatin and gelatin hydrolysate powder.

1.3.5 To investigate the fortification of gelatin hydrolysate powder in apple juice.

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CHAPTER 2

CHARACTERISTICS OF ACID- AND PEPSIN- SOLUBLE COLLAGENS FROM SCALES OF SPOTTED GOLDEN GOATFISH *(PARUPENEUS HEPTACANTHUS)*

2.1 Abstract

Acid soluble collagen (ASC) and pepsin soluble collagen (PSC) from spotted golden goatfish (*Parupeneus heptacanthus*) scale were characterized. Yields of ASC and PSC were 0.32 and 1.22% (based on wet weight basis), respectively. ASC and PSC were identified to be type I collagen, which consisted of α_1 and α_2 chains. β component was also found in both collagens. ASC and PSC contained glycine as the major amino acid and had high imino acid content (196 and 198 residues/1000 residues, respectively). T_{max} of ASC and PSC from spotted golden goatfish scale were 31.93 ^{o}C and 32.95 ^{o}C, respectively. FTIR spectra revealed that triple helical structure of resulting PSC was not disrupted by pepsin digestion. ASC and PSC from spotted golden goatfish exhibited high solubility in very acidic pH range (pH 2–4). Therefore, spotted golden goatfish scale could be an alternative source of collagen and the characteristics of collagens were slightly affected by extraction process used.

2.2 Introduction

Fish are being consumed daily in large quantities and a huge amount of byproducts, as large as 50–70% of original raw materials, is generated during processing (Kittiphattanabawon *et al*., 2005). More than 30% of fish processing byproducts consist of skin, scale and bone, which are rich in collagen and have received increasing attention as collagen sources (Kittiphattanabawon *et al*., 2005). Collagen are generally extracted from bovine or porcine origins (Ogawa *et al*., 2004). However, due to the outbreak of Bovine Spongiform Encephalopathy (BSE), Transmissible Spongiform Encephalopathy (TSE), Foot and Mouth Disease (FMD) in pig and cattle as well as bird flu in poultry, collagens from marine resources, especially from fish processing byproducts, have gained increasing attention as an alternative to land animal collagens (Jongjareonrak *et al*., 2005a).

Collagen is the major structural protein in the connective tissue of vertebrates and constitutes about 30% of the total animal protein. Collagen is the fibrous protein, contributing to unique physiological functions of tissues in skins, tendons, bones and cartilages (Jongjareonrak *et al*., 2005b). Over 28 types of collagen have been identified as Type I–XXVIII from various animal tissues and each type has a distinctive amino acid sequence and molecular structure (Liu *et al*., 2010; Shoulders and Raines, 2009). Amongst all collagens, type I collagen has been found as the most common in fish and mammal. Collagen type I possesses a triple helical structure, in which an interchain hydrogen bonding between glycine and amide group in an adjacent chain is a key factor in stabilizing the collagen triple helix (Dai and Etzkorn, 2009; Shoulders and Raines, 2009). Type I collagen is a fibrous collagen and is the major type in skin, bone, scale and fins of various fish species (Ikoma *et al*., 2003).

Spotted golden goatfish (*Parupeneus heptacanthus*), is tropical and sub-tropical fish. Its flesh, particularly in fillet form, is very popular in Thailand and they are one of important exporting products. During dressing and descaling, scales are removed and those scales could be used for collagen extraction. Collagen with high market value can be used for medical and tissue engineering as well as bioengineered teeth, skin tissue (Neel *et al*., 2014; Lammers *et al*., 2009). Thus, this study aimed to extract and characterize collagens from spotted golden goatfish scales.

2.3 Materials and methods

2.3.1 Chemicals/enzyme

All chemicals were of analytical grade. Pepsin from porcine stomach mucosa (EC3.4.23.1; powderized; 516 U/mg dry matter) and 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). Folin–Ciocalteu's phenol reagent, acetic acid and tris(hydroxylmethyl) aminomethane were obtained from Merck (Darmstadt, Germany).

2.3.2 Collection and preparation of fish scales

Scales of spotted golden goatfish having the average body weight of 100-120 g/fish were obtained from a local market in Hat Yai, Thailand. The scales were placed in polyethylene bag, kept in ice and transported to the Department of Food Technology, Prince of Songkla University within 1 h. Scales were then washed with chilled water and drained. Cleaned scales were placed in polyethylene bags, and stored at -20 ^{o}C. The storage time was not longer than 2 months. Before use, fish scales were thawed using the running water for 20 min.

Scales of spotted golden goatfish showed 27.81% moisture, 0.72% fat, 28.64% protein and 42.64% ash contents. All proximate analyses were performed using AOAC method (2000). To calculate protein content, the conversion factor of 5.95 was used (Wang *et al*., 2008).

2.3.3 Extraction of acid soluble collagen (ASC)

To extract ASC, all processes were conducted at 4 ^{o}C. Scales were immersed in 0.1 M NaOH for 6 h at the ratio of 1:10 (w/v) to remove non-collagenous proteins. The mixtures were stirred continuously and the solution was changed every 3 h. Treated scales were subjected to washing using chilled water until wash water became neutral. Scales were then demineralized with 0.5 M EDTA-2Na solution (pH 7.4) for 48 h using the ratio of 1:10 (w/v). The solution was changed every 12 h. Thereafter, the demineralized fish scales were washed with 10 volumes of water. The prepare scales were subjected to ASC extraction with 0.5 M acetic acid with the ratio of 1:10 (w/v). During extraction, the mixture was stirred using an overhead stirrer model W20.n (IKA®-Werke GmbH & CO.KG, Stanfen, Germany) for 48 h. After being filtered through two layers of cheese cloth, the filtrate was collected. NaCl was added to filtrate to obtain the concentration of 2.5 M in the presence of 0.05 M tris (hydroxymethyl) aminomethane (pH 7.0) in order to salting out the collagen. The precipitate was collected by centrifugation at 15,000 g using a refrigerated centrifuge model Avanti® J-E (Beckman Coulter, Inc., Palo Alto, CA, USA) for 1 h. The pellet was dissolved in the minimum volume of 0.5 M acetic acid and subsequently dialyzed in 20 volumes of 0.1 M acetic acid for 48 h, followed by 20 volumes of distilled water for another 72 h. Collagen was freeze-dried by using a freeze-dryer (CoolSafe 55,

ScanLaf A/S, Lynge, Denmark). The matter termed ‘acid soluble collagen, ASC’ was kept at -40 ^{o}C until analyzed.

2.3.4 Extraction of pepsin soluble collagen (PSC)

Remaining matter from ASC extraction was further subjected to extraction using 0.5 M acetic acid in the presence of 1% pepsin (w/v) at a ratio of 1:10 (w/v) for 48 h. Thereafter, the mixture was filtrated, precipitated, dialyzed and freeze-dried as previously described. All processes were done at 4 ^{o}C. The collagen was referred to as ‘pepsin soluble collagen, PSC’.

2.3.5 Analyses of ASC and PSC

ASC and PSC from scales of spotted golden goatfish was subjected to the following analyses.

2.3.5.1 Yield

Yield of ASC and PSC was calculated based on the weight of starting material using the following equation.

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

2.3.5.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Both collagens were determined for protein patterns using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) following the method of Laemmli (1970) as modified by Sinthusamran *et al*. (2013). To quantify the band intensities of proteins, the public domain digital analysis software, ImageJ (ImageJ 1.42q, National Institutes of Health, Bethesda, MD, USA) was used.

2.3.5.3 Amino acid composition analysis

Both collagens were hydrolysed under reduced pressure in 3 N mercaptoethane sulphonic acid in the presence of 0.2% (v/v) 3-2(2-aminoethyl) indole for 22 h at 110 ^{o}C. The hydrolysates were neutralised with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot of 0.1 mL was applied to an amino acid

analyser (MLC-703; Atto Co., Tokyo, Japan). The content was expressed as residues/1000 residues.

2.3.5.4 Fourier transform infrared spectroscopy

FTIR spectra of both collagens were obtained using total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopic analysis as described by Sinthusamran *et al*. (2013). Freeze-dried collagens were placed onto the crystal cell. The spectra were acquired over the range of 650–4000 cm^{-1} with a resolution of 4 cm^{-1} for 32 scans against a background spectrum recorded from the clean empty cell at 25 ^{o}C using FTIR spectrometer (Model Equinox 55, Bruker, Ettlingen, Germany) equipped with a horizontal ATR trough plate crystal cell (45^{o} ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI, USA). Analysis of spectral data was carried out using the OPUS 3.0 data collection software programme (Bruker, Ettlingen, Germany).

2.3.5.5 Differential scanning calorimetry (DSC)

DSC of both collagens was run following the method of Kittiphattanabawon *et al*. (2005). The maximum transition temperature (T_{max}) and total denaturation enthalpy (ΔH) were estimated and reported.

2.3.5.6 Zeta potential analysis

Both collagens were dissolved in 0.5 M acetic acid to obtain a final concentration of 0.05% (w/v). Collagen solutions were determined for zeta potential using a model ZetaPALs (Brookhaven Instruments Co., Holtsville, NY, USA) as described by Sinthusamran *et al*. (2013).

2.3.5.7 Solubility test

Solubility of both collagens was determined by the method of Jongjareonrak *et al*. (2005) with a slight modification. Soluble protein content in the supernatant was determined by the Lowry method (Lowry *et al*., 1951) using bovine serum albumin as a standard. Relative solubility was calculated in comparison with that obtained at the pH giving the highest solubility.

2.4 Results and discussion

2.4.1 Yield of ASC and PSC

Yields (on a wet weight basis) of ASC and PSC from spotted golden goatfish scales were 0.32 and 1.22%, respectively. PSC showed the higher yield than ASC. The yield was increased, when pepsin was used for PSC extraction, compared with that of ASC. In general, ASC showed the low yield, more likely due to the cross-links mediated by the reaction of aldehyde with lysine and hydroxylysine at telopeptide region (Knott and Bailley, 1998). Fish scales were biocomposites of highly ordered type I collagen fibers with several cross-linked regions and hydroxyapatite ($Ca_5(PO_4)_3OH$) (Zylberberg *et al*., 1988). The higher yield of PSC revealed that pepsin facilitated the extraction of collagen from fish scale via the cleavage of telopeptide region. This could enhance the further extraction with acid. Pepsin was able to cleave specifically at the telopeptide region of collagen (Nalinanon *et al*., 2007). The cross-linked regions at the telopeptide could be cleaved by pepsin without damaging the integrity of the triple helix (Sato *et al*., 2000). The result was in agreement with Zhang *et al*. (2010) who found that pepsin (0.1%) potentially solubilised the collagen from scale of silver carp.

2.4.2 Protein patterns

Protein patterns of both collagens from scales of spotted golden goatfish determined under the reducing and non-reducing conditions are illustrated in Fig. 4. ASC and PSC consisted of two α chains, α_1 and α_2, with molecular weight of 118 and 105 kDa, respectively. β component was also found as the major component. Band intensity of β-chain was similar to that found for α-chain. Additionally, all collagens contained γ-chain to some extent. The presence of β-chain (dimer) and γ-chain (trimer) indicated that collagen from scales had the high cross-links. When protein patterns were determined under reducing and non-reducing conditions, similar patterns were observed between ASC and PSC. Therefore, ASC and PSC contained no disulphide bond. Collagens from scale of bighead carp (Liu *et al*., 2012), scale of subtropical fish black drum (*Pogonia cromis*), sheepshead seabream (*Archosargus probatocephalus*) (Ogawa *et al*., 2004) and scale of carp (*Cyprinus carpio*) (Duan *et*

al., 2009) had no disulphide bond. The ratio of α_1/α_2 chain was approximately 2:1, suggesting that collagens isolated from spotted golden goatfish scales were type I collagens.

In general, similar electrophoretic protein patterns were found between ASC and PSC. However, ASC had slightly higher molecular weight, compared with PSC. The cleavage of telopeptide region by pepsin could be removed some parts of that region, leading to a slight decrease in molecular weight of resulting PSC. The result was in accordance with Nalinanon *et al*. (2007) who reported the slight decrease in molecular weight of collagen extracted from skin of bigeye snapper with the aid of fish pepsin. Generally, no differences in protein pattern were observed between ASC or PSC from scale of spotted golden goatfish.

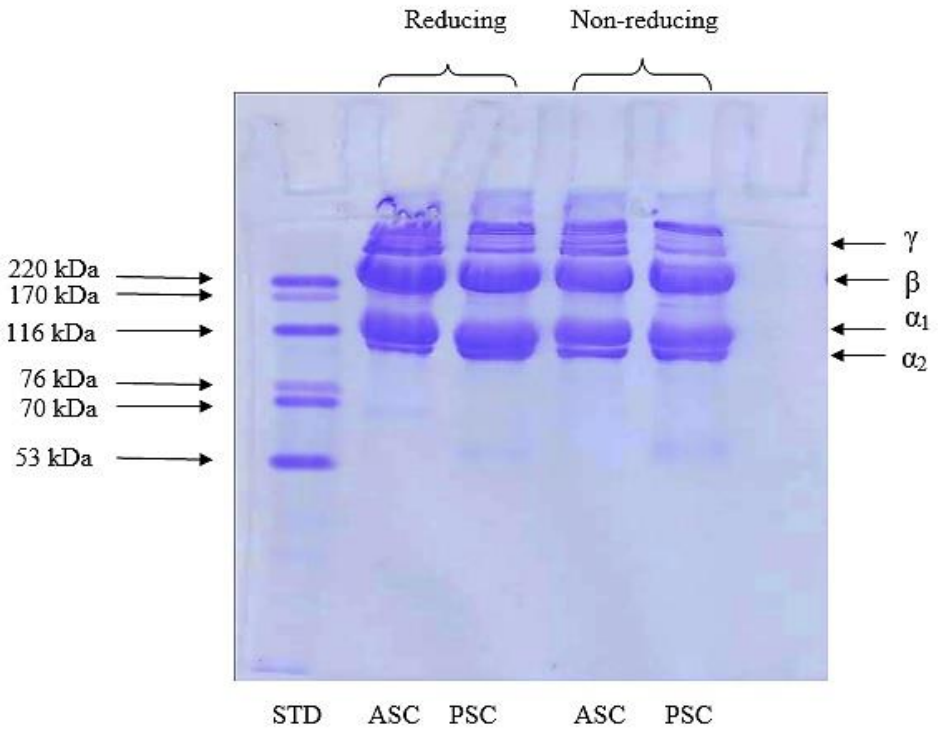

Figure 4 SDS polyacrylamide gel electrophoresis pattern of ASC and PSC from the scales of spotted golden goatfish under reducing and non-reducing conditions. STD: molecular weight marker

2.4.3 Amino acid composition

Amino acid composition of ASC and PSC from spotted golden goatfish scales, expressed as residues per 1000 total residues, is shown in Table 5. ASC and PSC had glycine as the most abundant amino acid (334 and 337 residues/1000 residues, respectively). In general, glycine represents about one-third of the total residues and is normally spaced at every third residue in collagen except for the first 10 amino acid residues at the C-terminus and the last 14 amino acid residues at the N-terminus (Jongjareonrak *et al*., 2005; Kittiphattanabawon *et al*., 2005). ASC and PSC were also rich in alanine. It was noted that cysteine was negligible in both collagens from spotted golden goatfish. This reconfirmed the absence of disulphide bond of all collagens as determined by SDS-PAGE (Figure 4). Total imino acid content of ASC and PSC from scales of spotted golden goatfish (196 and 198 residues per 1000 residues, respectively) were higher than those of bighead carp scale collagen (about 156 residues per 1000 residues) (Liu *et al*., 2012) and carp scale collagen (about 192 residues per 1000 residues) (Duan *et al*., 2009). The imino acids contribute to the thermal stability of collagens, which is one of the most important characteristics determining their potential use (Regenstein and Zhou, 2007). ASC and PSC are constructed in triple helix via hydrogen bonds. Pyrrolidine ring of those imino acids could also strengthen the triple helix (Foegeding *et al*., 1996). Moreover, hydroxyproline also contributes to the stabilisation of the triple helical structure by the formation of interchain hydrogen bond through the hydroxyl group (Kittiphattanabawon *et al*., 2005). The amino acid profiles of ASC and PSC were similar to those of type I collagen from scale of bighead carp (Liu *et al*., 2012), scale of deep sea redfish (Wang *et al*., 2008) and scale of lizard fish and horse mackerel (Minh-Thuy *et al*., 2014). Slight differences in amino acid composition between ASC and PSC might be owing to the removal of some portions of telopeptides induced by pepsin (Matmaroh *et al*., 2011). The amino acid compositions generally affect the properties of collagen (Liu *et al*., 2012).

Table 5 Amino acid composition of ASC and PSC from the scale of spotted golden goatfish (residues/1000 residues)

Amino acids	ASC	PSC
Alanine	131	131
Arginine	52	50
Aspartic acid/asparagine	44	41
Cysteine	1	1
Glutamic acid /glutamine	70	69
Glycine	334	337
Histidine	6	7
Isoleucine	8	8
Leucine	19	20
Lysine	27	26
Hydroxylysine	5	8
Methionine	14	14
Phenylalanine	14	12
Hydroxyproline	81	85
Proline	115	114
Tryptophan	0	0
Serine	36	32
Threonine	22	24
Tyrosine	3	2
Valine	18	19
Total	1000	1000
Imino acid	196	198

2.4.4 Zeta potential

The zeta potential of ASC and PSC from scales of spotted golden goatfish at different pHs is shown in Fig. 5. Both collagens had positive charge at acidic pHs (2-5), whilst they became the negatively charged in pH range of 6–9. Net charge became zero at pH values of 4.91 and 5.72 for ASC and PSC, respectively. Those pHs were assumed to be their isoelectric points (pI). The pIs of both collagens were observed at acidic pHs. Both collagens showed high content of acidic amino acids including glutamic acid and aspartic acid (Table 1). This might be associated with pI in acidic pH range. Pepsin more likely cleaved the telopeptide region of

collagen molecule, resulting in the difference in amino acid composition in α-chains. Amino acids in α-chain or β-chain, regulated by protonation and deprotonation at pH tested, determined the charge of collagens (Benjakul *et al*., 2010). Collagen from the swim bladders of yellowfin tuna prepared with crude tuna stomach extract had zero net charge at pH 6.05 and 5.93 for ASC and PSC, respectively (Kaewdang *et al*., 2014). Net charge of collagen from skin and swim bladder of seabass became zero at pH 6.46 and 6.64, respectively (Sinthusamran *et al*., 2013). The 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 (Kaewdang *et al*., 2014). ASC showed the higher contents of acidic amino acids (Table 5). This might be associated with the removal of telopeptide region cleaved by pepsin.

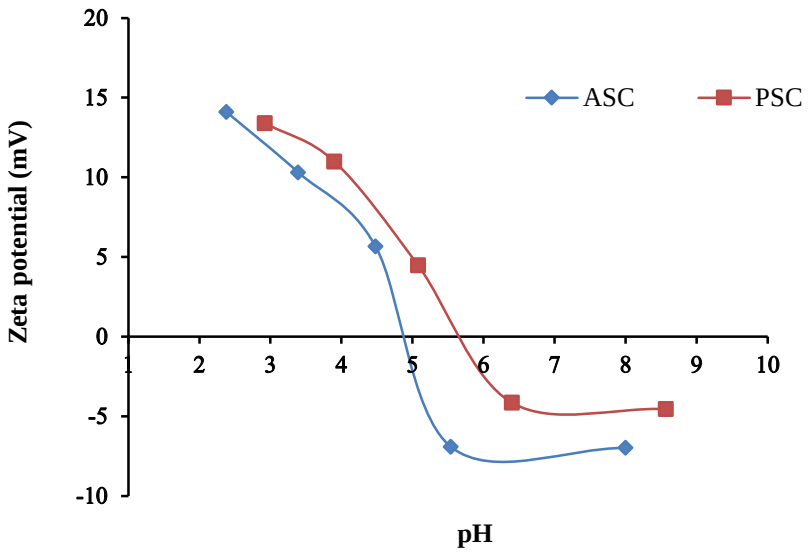

Figure 5 Zeta potential of ASC and PSC from scales of spotted golden goatfish. Bars represent the standard derivation (n=3).

2.4.5 Fourier-transform infrared spectra (FTIR)

FTIR spectra of ASC and PSC from scales of spotted golden goatfish had the characteristic peaks of Amide I, II, III, A and B (Fig. 6). Amide A is commonly arisen from N-H stretching vibration, occurs in the wavenumber range of 3400–3440 cm^{-1} (Sai and Babu, 2001). When the NH group of a peptide is involved in H-bond, the position is shifted to lower frequency (Doyle *et al*., 1975). The absorption

peaks of ASC and PSC from spotted golden goatfish scales were found at 3285 and 3312 cm^{-1}, respectively. Hydrolysis of telopeptide region by pepsin might increase free amino groups, as indicated by the higher wavenumber of PSC. The amide B bands of both collagens were found at wavenumbers of 3079 and 3100 cm^{-1}, Amide B is associated with the asymmetrical stretch of CH_2 (Muyonga *et al*., 2004), Higher amplitude of amide B peaks were found in PSC. It was found that PSC also showed the higher wavenumber than ASC. This result was in accordance with those of collagen from other fish scale (Matmaroh *et al*., 2011; Wang *et al*., 2008; Liu *et al*., 2012; Duan *et al*., 2009).

Amide I occurring at wavenumber of 1600–1700 cm^{-1}, is mainly associated with C=O stretching vibration or H-bond coupled with COO^- (Payne and Veis, 1988). The absorption peaks of ASC and PSC from spotted golden goatfish scales were found at 1641 and 1634 cm^{-1}. 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). Since the extraction was performed at low temperature, all collagens were most likely not thermally denatured during the extraction. Additionally, ASC and PSC exhibited the amide II band at wavenumbers of 1548 and 1544 cm^{-1} for spotted golden goatfish scales. Amide II generally appeared at 1550–1600 cm^{-1} and resulted from from N–H bending vibration coupled with C–N stretching vibration (Krimm and Bandekar, 1986). Bands of both Amide I and Amide II of PSC shifted to lower wavenumber, compared with those of ASC, suggesting higher proportion of hydrogen bond in PSC. After pepsin was applied, the non-helical telopeptide region was removed. As a result the proportion of triple helix of PSC stabilized with H-bond increased. Pepsin was able to cleave non-helical domain of telopeptide regions, As a result, molecular order of collagen structure was enhanced (Nalinanon *et al*., 2007).

Amide III bands were found at wavenumber of 1456 and 1459 cm^{-1} for ASC and PSC, respectively. The Amide III peak is associated with intermolecular interactions in collagen, involving C–N stretching and N–H in plane bending from amide linkages. Also it was arisen from wagging vibrations of CH_2 groups from the glycine backbone and proline side-chains (Plepis *et al*., 1996). The result indicated

that hydrogen bonds were involved in both collagens. The intensity ratio between Amide III band and 1450 cm^{-1} band has been used to elucidate the triple-helical structure of collagen (Plepis *et al*., 1996). The absorption ratios between the peak heights of amide III and peak of 1450 cm^{-1} were 1.01 and 1.01 for ASC and PSC from spotted golden goatfish scales. The absorption ratio of approximately 1.0 indicates the presence of triple helical structure of ASC and PSC (Benjakul *et al*., 2010; Heu *et al*., 2010). Therefore, ASC and PSC from spotted golden goatfish scales were still in triple-helical structure, though ASC and PSC might have a slight difference in functional group or interaction between chains.

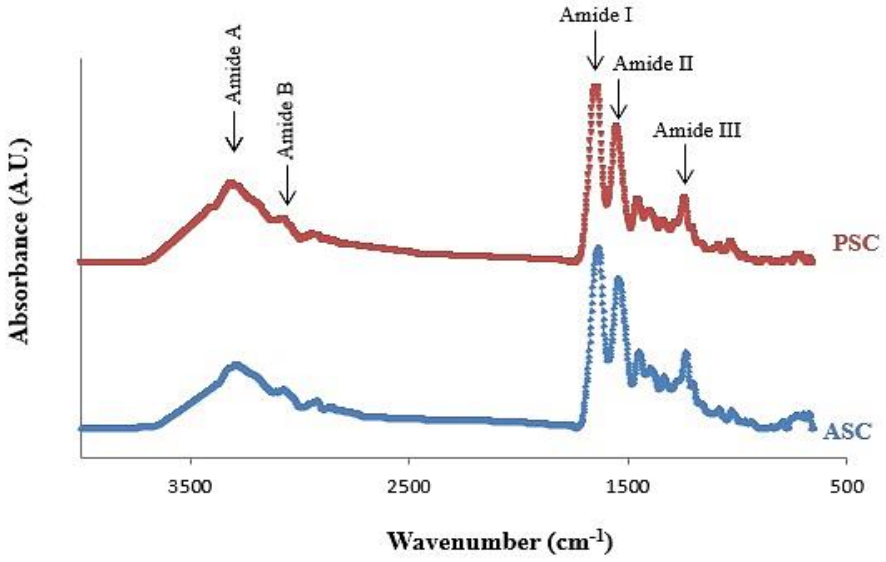

Figure 6 FTIR spectra of ASC and PSC from scales of spotted golden goatfish.

2.4.6 DSC thermogram

DSC thermograms of both collagens from spotted golden goatfish scale are depicted in Table 6. T_{max} values of ASC and PSC were 31.93 and 32.95 °C, respectively. ΔH of ASC and PSC were 1.31 and 1.85 J/g, respectively. The result suggested that slightly higher T_{max} and ΔH were found for PSC, compared with ASC. The removal of non-helical telopeptides plausibly resulted in the more ordered structure of PSC. As a consequence, higher energy was needed for destabilization. This was also reflected by slightly higher T_{max} of PSC. This was coincidental with the higher content of hydroxyproline content in PSC from spotted golden goatfish scales

(Table 1). Hydroxyl group of hydroxyproline could serve as hydrogen donor for binding between α-chains via H-bond. The higher imino acid content is associated with increasing thermal stability of collagens, governed by the pyrrolidine rings of proline and hydroxyproline and hydrogen bonding through the hydroxyl group of hydroxyproline (Benjakul *et al*., 2010). However, the T_{max} value might be also determined by the conformation and amino acid sequence of collagen (Sinthusamran *et al*., 2013). T_{max} of collagen varied with different sources, e.g. ASC from skin and swim bladder of seabass (33.3 and 35.0 °C, respectively) (Sinthusamran *et al*., 2013), PSC from the scale of bighead carp (35.2 °C) (Liu *et al*., 2012), ASC from skin of nile perch (36.0 °C) (Muyonga *et al*., 2004), ASC and PSC from skin of brownbanded bamboo shark (34.4 and 34.5 °C, respectively) (Kittiphattanabawon *et al*., 2010) and ASC and PSC from spotted golden goatfish (31.98 and 32.98 °C). Thermal transition or denaturation temperatures have been reported to be different, depending on fish species, habitat temperature, seasons, and age, etc (Duan *et al*., 2009; Muyonga *et al*., 2004).

Table 6 Maximum transition temperature (T_{max}) and total denaturation enthalpy (ΔH) of ASC and PSC from scales of spotted golden goatfish.

Collagens	T_{max} (°C)	ΔH (J/g)
ASC	31.93 ± 0.44^{b}	1.31 ± 0.37^{b}
PSC	32.95 ± 0.21^{a}	1.85 ± 0.65^{a}

* Values are presented as means ± SD (n=3)

** The different superscripts in the same column denote the significant differences ($p<0.05$).

2.4.7 Solubility

Solubility of ASC and PSC from scales of spotted golden goatfish as affected by pHs is shown in Fig. 7. ASC and PSC were solubilized in the pH range of 1–4 ($P<0.05$) with the highest solubility at pH 2 and 3, respectively, for ASC and PSC from spotted golden goatfish scales. It was noted that collagen became positively

charged in acidic pH range, while turned to be negatively charged at neutral or alkaline pHs. Zeta potential was higher in the acidic pH range than alkaline pH (Fig. 5). The collagen under acidic condition more likely underwent repulsion to a higher extent. It plausibly led to higher solubility. As the pH above 4, a sharp decrease in solubility was observed ($P<0.05$). Therefore, collagens lost the solubility in the neutral and alkaline pH range. Loss in solubility could be attributed to hydrophobic interaction amongst collagen molecules (Jongjareonrak *et al*. 2005a). This result was in accordance with those of collagen from scale of bighead carp (Liu *et al*., 2012) and collagen from scale of lizard fish and horse mackerel (Minh-Thuy *et al*., 2014). 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 solubility of collagens as a function of pHs were caused by the differences in the molecular properties and conformations of collagens (Kittiphattanabawon *et al*. 2005).

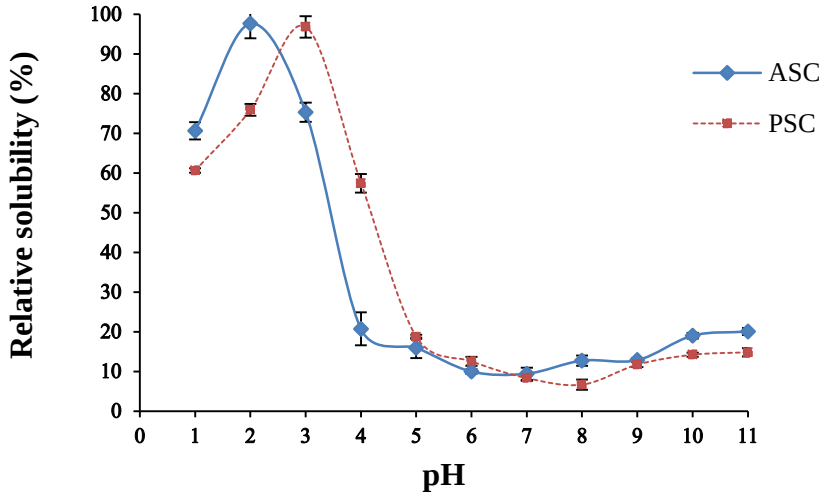

Figure 7 Relative solubility of ASC and PSC from scales of spotted golden goatfish at different pHs. Bars represent the standard derivation (n=3).

2.5 Conclusion

ASC and PSC from scales of spotted golden goatfish were identified as type I collagen, and maintained their triple helical structure. Pepsin digestion was able to increase the yield of collagen. ASC and PSC showed similar protein patterns and FTIR spectra. Collagens from spotted golden goatfish had the high thermal stability. Spotted golden goatfish scale collagen could be used as an alternative for mammalian counterpart collagens.

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CHAPTER 3

ELEMENT DISTRIBUTION AND MORPHOLOGY OF SPOTTED GOLDEN GOATFISH FISH SCALES AS AFFECTED BY DEMINERALIZATION

3.1 Abstract

Scales of spotted golden goatfish were subjected to non-collagenous protein removal followed by demineralization with hydrochloric acid at different concentrations (0.25, 0.5, 0.75 and 1 M) for various times (30, 60 and 90 min). The morphology and element composition/distribution of scales from spotted golden goatfish as influenced by demineralization conditions were determined. The appropriate demineralization was pertained using 0.75 M hydrochloric acid for 30 min, in which ash content was 0.62% (dry weight basis). The scales having non-collagenous protein removal without and with subsequent demineralization were analyzed for element contents using X-ray fluorescence spectrometer. Images of different scales were determined using scanning electron microscopy (SEM) and scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDX). Based on the images, an external layer rich in inorganic elements was removed. Most of Ca and P were eliminated with the coincidental increases in organic substances (C, N and O) after demineralization. Demineralization therefore mainly removed the external layer of scales, which facilitated the further extraction of collagen or gelatin.

3.2 Introduction

Fish collagen and gelatin have gained increasing attention since they can be produced from fish processing byproducts such as skin, scale and bones. They have been consumed widely, irrespective of religions. During dressing and descaling, scales are removed and those scales could be used as collagenous material. Basically, scales are rich in Ca phosphate compounds such as hydroxyapatite and Ca carbonate. The surface of a fish scale is an osseous layer consisting of randomly oriented collagen fibrils with many hydroxyapatite ($Ca_{10}(O_4)_6(OH)_2$) crystals. Thin layers of

the oriented collagen fibrils are piled up to form the fibrillary plate under the osseous layer. This plywood-like structure provides the high mechanical strength of scales (Okuda *et al*., 2011). Therefore, the removal of Ca from fish scales is necessary pretreatment for collagen or gelatin production (Gomez-Guillen *et al*., 2002). Demineralization aims to remove the calcium and other inorganic substances to facilitate the extraction of collagenous component (Waldner, 1977). The inorganic substances in scale can be removed with dilute hydrochloric acid (HCl) solution, whereby the calcium phosphate is dissolved as acid phosphates (Waldner, 1977). Depending on the nature of the material, temperature and acid concentration, the demineralization time can be varied. HCl concentration used is generally in the range of 2–6%. Acid hydrolysis of the protein should be minimized during demineralization. High temperature should also be avoided since it can enhance the hydrolysis of protein, especially under acidic condition (Waldner, 1977).

Fish scales have plywood-like structures of closely packed collagen fibre layers reinforced with a mineral phase of calcium-deficient hydroxyapatite (Ikoma *et al*., 2003). In general, the spatial organization of collagen fibres is of key importance for the mechanical properties of different connective tissues (Weiner *et al*., 1999). Spotted golden goatfish (*Parupeneus heptacanthus*) has been widely used for frozen fillet manufacturing in Thailand. The scales from this species can serve as alternative raw material for collagen or gelatin extraction, however the proper demineralization of scales is required. Thus, the better understanding on mineral composition/distribution in scale as affected by demineralization could bring about the effective extraction of collagen or gelatin from fish scale. Therefore, the aim of this study was to investigate the morphology and element composition/distribution of scales from spotted golden goatfish as influenced by demineralization.

3.3 Materials and methods

3.3.1 Collection and preparation of spotted golden goatfish scales

Scales of spotted golden goatfish with an average body weight of 100-120 g/fish were collected from Kingfisher Holding, Ltd., Songkhla Province, Thailand. The scales were packaged in polyethylene bag and transported in ice with a scale/ice ratio of 1:2 (w/w) to the Department of Food Technology, Prince of Songkla

University within 1 h. Upon arrival, scales were washed with chilled water and drained before being packaged in polyethylene bags. The samples were kept at -20 ^{o}C and the storage time was not longer than 2 months. Before use, fish scales were thawed using the running water for 20 min.

3.3.2 Remove non-collagenous proteins of spotted golden goatfish scales

Spotted golden goatfish scales were subjected to non-collagenous protein removal according to the method described by Weng *et al*. (2014) with a slight modification. Scales were firstly suspended in 0.1 M NaOH for 6 h at the ratio of 1:10 (w/v) with continuous stirring at 25 ^{o}C. The solution was changed every 3 h. Treated scales were washed with distilled water until wash water became neutral or faintly alkaline.

3.3.3 Demineralization of spotted golden goatfish scales under different conditions

Demineralization was performed according to the method described by Weng *et al*. (2014) with a slight modification. The prepared scales were soaked in HCl with different concentrations (0.25, 0.5, 0.75 and 1 M) for various times (30, 60 and 90 min) at the scale/solution ratio of 1:5 (w/v) with continuous stirring at 25 ^{o}C. After being demineralized, the scales were washed until the neutral or faintly acidic pH of wash water was obtained.

3.3.4 Determined for ash content

The resulting scales obtained from different demineralization conditions were determined for ash content (AOAC, 2000). The scales demineralized under the appropriate condition, showing the lowest ash content, were further analyzed.

3.3.5 Analyses of scales without and with different pretreatments

Different scales including 1) original scales 2) scales with non-collagenous protein removal and 3) scales having non-collagenous protein removal followed by the selected demineralization (using 0.75 M HCl for 30 min) were subjected to analyses.

3.3.5.1 Scanning electron microscopy

Scanning electron microscopy according to the method described by Lin *et al*. (2011) with slight modification. Microstructure of scale samples was visualized using a scanning electron microscopy (SEM). Scale was fixed with 2.5% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 12 h. The samples were rinsed with distilled water for 1 h and dehydrated in ethanol with a serial concentration of 50%, 70%, 80%, 90% and 100% (v/v). Dried samples were mounted on a bronze stub and sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA). The specimens were observed with a scanning electron microscope (JEOL JSM-5800 LV, Tokyo, Japan) at an acceleration voltage of 20 kV.

3.3.5.2 Scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDX)

Scanning electron microscopy with energy dispersive X-ray spectroscopy according to the method of Lin *et al.* (2011) with slight modification. Scale samples were also visualized using a field emission scanning electron microscope (FEI-XL30, FEI Company, Hillsboro, OR, USA) equipped with electron-dispersive X-ray spectroscopy (EDX). The samples were gold coated and observed with secondary electron mode at a 10 kV accelerating voltage. Elemental analysis was conducted on the cross section of the scale by EDX to verify the element content and distribution.

3.3.5.3 X-ray fluorescence spectrometer

X-ray fluorescence spectrometer according to the method of Lin *et al*. (2011) with slight modification. Elemental contents of different scale samples were determined by a X-ray fluorescence spectrometer (PW2400-Sequential WXRF Spectrometer, Philips, Eindhoven, The Netherlands). The scale was heated at high temperature (950 ^{o}C) for 2 h. The residues (0.5-1g) were analyzed using a flow and scintillation detector (PW2400-Sequential WXRF Spectrometer, Philips, Eindhoven, The Netherlands). Start angles (^{O}2T**)** were 12-130 and end angles (^{O}2T**)** were 18.6-146.98. kV of 24-60 and mA of 40-100 were used.

3.3.6 Statistical analysis

Demineralization was carried out in triplicate. All analyses were performed in duplicate, except for ash analysis, which was conducted in triplicate. Data were subjected to analysis of variance and mean comparison was done using Duncan's multiple range test. Analysis was performed using SPSS 11.0 for Windows (SPSS Inc., Chicago, IL, USA).

3.4 Results and discussion

3.4.1 Ash contents of scales demineralized under different conditions

Ash contents of spotted golden goatfish scale demineralized using different HCl concentrations and soaking times are shown in Table 7. Ash contents of scales generally decreased with increasing HCl concentrations ($p<0.05$), regardless of soaking time. However, when scales were treated with 0.5 HCl solution, the lower ash content was found as the soaking time of 1.5 h was used ($p<0.05$). The marked decrease in ash content was obtained when scales were demineralized using either 0.75 or 1 M HCl solution, irrespective of soaking time. The results indicated that the inorganic substances in scales could be removed by HCl solution, whereby the calcium phosphate was dissolved as acid phosphates (Waldner, 1977). Akagündüz *et al.* (2014) demineralized sea bream bones using 37% HCl solution. Additionally, demineralization of tuna fin (*Katsuwonus pelamis*) was carried out at room temperature using 0.6 N HCl (Aewsiri *et al*., 2008). Weng *et al*. (2014) used 0.05 M HCl solution at room temperature for 30 min to remove minerals of tilapia (*Tilapia zillii*) scale. Sha *et al*. (2014) remove mineral of bighead carp scale with 0.5 M HCl solution for 1 h. Scales generally consist of hydroxyapatite and calcium carbonate. It was reported that the removal of calcium from fish scales was critical pretreatment in order to obtain gelatin with high purity and gel strength (Gomez-Guillen *et al*., 2002). In the present study, demineralization using 0.75 M HCl and soaking time of 30 min was selected for further study, due to the less acid and shorter time consumed, in comparison with treatment using 1 M HCl.

Table 7 Ash contents of spotted golden goatfish scale demineralized under different conditions

HCl concentration (M)	Soaking time (h)	Ash (%, dry weight basis)
Control		48.69±0.76[a]
0.25	0.5	41.60±1.68[b]
	1	39.95±0.71[b]
	1.5	39.12±1.52[b]
0.5	0.5	23.12±1.02[c]
	1	22.17±1.24[c]
	1.5	14.70±0.93[d]
0.75	0.5	0.62±0.53[e]
	1	0.56±0.24[e]
	1.5	0.19±0.72[e]
1	0.5	0.17±0.78[e]
	1	0.11±0.13[e]
	1.5	0.08±0.22[e]

*Values and mean ± SD (n=3).

Different superscripts in the same column indicate the significant difference ($p<0.05$).

3.4.2 Element compositions of scales with different treatments

Elemental contents of different spotted golden goatfish scales as determined by a X-ray fluorescence spectrometer are shown in Table 8. Original scale and that with non-collagenous protein removal had similar element contents, in which oxygen, nitrogen, carbon and hydrogen constituted as the major organic matters. Calcium and phosphorous were found as the dominant inorganic matters. Calcium and phosphorous in scales were 19.64 and 8.43%, respectively. After non-collagenous protein removal process, most minerals as well as collagenous protein in the scale matrix were still retained in the scales. Nevertheless, phosphorous and sodium contents decreased, whilst magnesium and potassium contents increased ($P<0.05$). After demineralization, calcium and phosphorous contents were 0.16 and 0.11%. This result suggested that the inorganic substances in scales can be significantly removed

by treatment with dilute hydrochloric acid solution. Calcium phosphate was dissolved as acid phosphates (Waldner, 1977). Fang *et al*. (2014) reported that the surface of scales from *Carassius auratus* were rich in calcium, phosphorus, carbon and oxygen content, but had the low content of nitrogen, magnesium, sodium and sulphur. The marked decreases in both Ca and P were in accordance with the increased contents of H, C, N and O ($P<0.05$). The results suggested that organic matter, particularly collagens became more concentrated when demineralization was performed. It was noted that both aluminium and silicon contents increased after demineralization ($P<0.05$). When calcium and phosphorous were removed, those two elements were plausibly removed to a low extent. As a result, their proportions were increased.

Table 8 Element contents of spotted golden goatfish scale with different treatments

Elements	Concentration (%)		
	Original scale	Non-collagenous protein removal	Non-collagenous protein removal+demineralization
Oxygen	39.77 ± 0.12^{a}	38.65 ± 1.09^{a}	38.34 ± 0.37^{b}
Nitrogen	16.97 ± 0.67^{b}	16.75 ± 0.47^{b}	29.38 ± 1.24^{a}
Carbon	14.55 ± 1.03^{b}	14.36 ± 0.61^{b}	25.19 ± 0.93^{a}
Hydrogen	1.22 ± 0.83^{b}	1.21 ± 0.13^{b}	2.11 ± 1.21^{a}
Cacium	19.72 ± 0.14^{a}	19.64 ± 0.12^{a}	0.16 ± 0.42^{b}
Phosphorous	10.37 ± 0.64^{a}	8.43 ± 1.69^{b}	0.11 ± 0.37^{c}
Magnesium	0.38 ± 1.23^{b}	0.51 ± 1.27^{a}	0.14 ± 0.68^{c}
Sodium	0.21 ± 0.14^{a}	0.10 ± 0.16^{b}	0.05 ± 0.53^{c}
Iron	0.19 ± 0.12^{a}	0.18 ± 0.35^{a}	0.17 ± 0.11^{a}
Sulphur	0.11 ± 0.34^{a}	0.10 ± 0.42^{a}	0.09 ± 0.16^{b}
Silicon	0.10 ± 0.27^{c}	0.18 ± 0.48^{b}	3.05 ± 1.47^{a}
Potassium	0.05 ± 0.42^{c}	0.42 ± 0.82^{a}	0.36 ± 0.31^{b}
Aluminium	0.02 ± 0.21^{c}	0.10 ± 0.78^{b}	0.96 ± 0.37^{a}
Strontium	0.07 ± 0.74^{b}	0.09 ± 0.66^{a}	0.02 ± 0.34^{c}

*Values and mean ± SD (n=3).

Different superscripts in the same row indicate the significant difference ($p<0.05$).

3.4.3 SEM images of scales with different treatments

The microstructures of demineralized scale (soaked with 0.75 M HCl for 30 min) were visualized using a scanning electron microscopy (SEM) in comparison with the original scale (without treatment) and the scale with non-collagenous protein removal (Fig. 8). Fig 8A and Fig 8B illustrate the cross-section of original scale and that with non-collagenous protein removal, respectively. No marked difference was observed between both samples, in which a laminate structure was composed of an external layer with ridges and internal layers. The main building block of the scales is collagen fibres forming a plywood structure (Lin *et al*., 2011). The corrugated surface of external layer corresponds to the ridge structure of the surface, whereas the internal layers are characterized by lamellae (Lin *et al*., 2011). The central lamella of internal layers contains the collagen fibrils oriented in one direction. In the external layer, collagen fibres are randomly arranged and embedded in a proteoglycan matrix (Ikoma *et al*., 2003). Within the fibrillary lower layer, in contrast, the collagen fibres are co-aligned and organized into lamellae that are superimposed to produce an orthogonal and/or a double-twisted plywood pattern (Ikoma *et al*., 2003). The collagen fibres are produced within the fibrillary layer by scleroblasts located at the base of the scales (Onozato and Watabe, 1979; Ikoma *et al*., 2003). For the scale with non-collagenous protein removal, followed by demineralization using 0.75 M HCl solution for 30 min, the pronounced difference in microstructure was noticeable (Fig 8C) in comparison with the formers (Fig 8A and 8B). After being demineralized using HCl, the external layers containing calcium and phosphorus were removed. This result suggested that the collagen fibres were tightly packed and organized into lamellae (Liu *et al*., 2008), whilst inorganic matters were located mainly on the surface. The upper layer of the internal plate adjacent to the external layer is more easily mineralized as compared to the central or lower layer due to the formation of Mandl's corpuscles; however, this depends on the species and age of the fish (Okuda *et al*., 2009). Therefore, inorganic substances in raw material could be removed by treatment with HCl solution.

Similar surface between original scale and that with non-collagenous protein removal was observed (Fig 8D and 8E). Flaky crystals of apatite in random orientation were observed in the outer layer (Ikoma *et al*., 2003). Both samples

showed smooth and tightly packed surface. After demineralization, the cracks were generally found on the surface, indicating that some elements were removed by HCl solution (Fig 8F). The removal of calcium phosphate crystallites more likely led to porous structure. Meunier (1984) suggested that collagen layers in the fish scale are stiffened by mineralization. In the present study, the scale of spotted golden goatfish became less rigid after being demineralization using 0.75 M HCl for 30 min, suggesting the removal of minerals from scales.

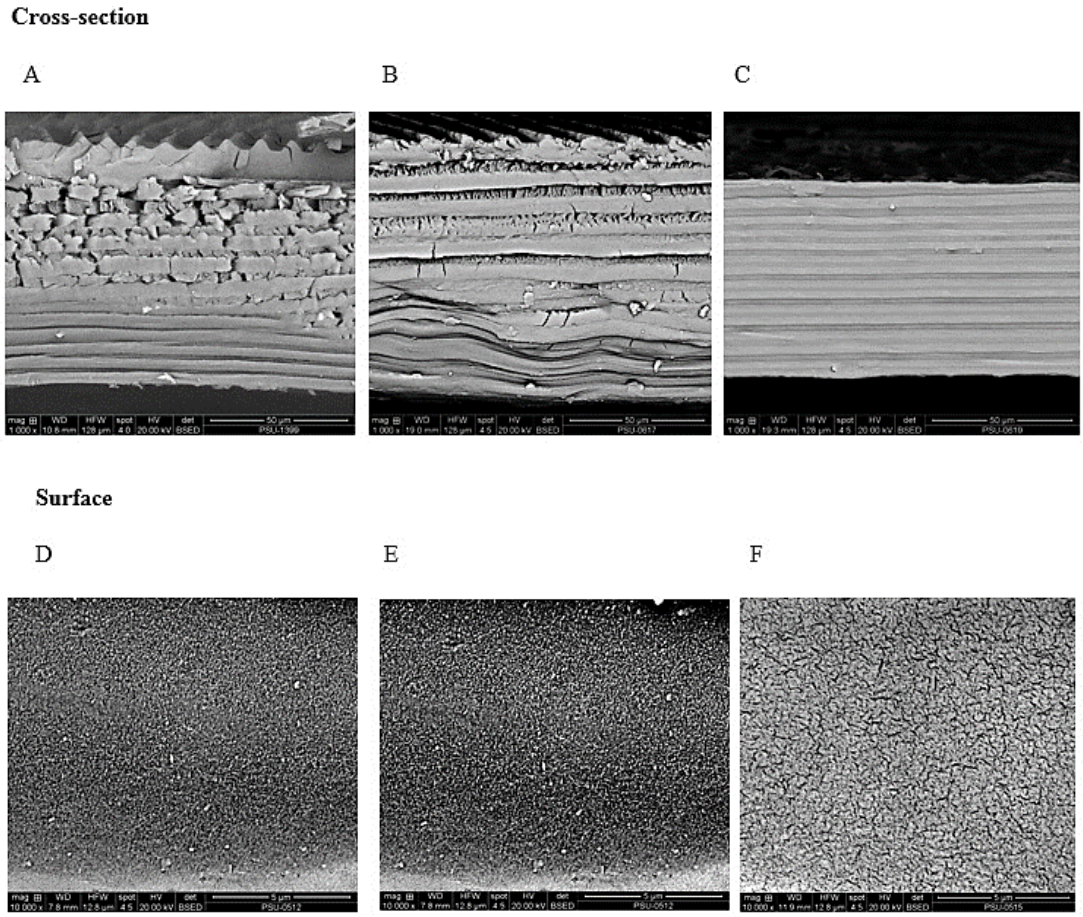

Figure. 8 SEM images of cross-section and surface of spotted golden goatfish scales with different treatments. Original scales (A, D); scale with non-collagenous protein removal (B, E) and scales with non-collagenous protein removal, followed by demineralization (0.75 M HCl, 30 min (C, F) (Magnification of cross-section was 1,000x and magnification of surface was 10,000x)

3.4.4 Scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDX) images of scales with different treatments

SEM-EDX images of cross-section of original scale and that with non-collagenous protein removal are shown in Fig 9 and Fig 10, respectively. The SEM-EDX result showed that calcium and phosphorous distributed at the external region was about 2-fold, compared to those found at the internal region. Calcium and phosphorous mapping was also reported on the cross section and the amount of these elements gradually decreased from the external to the internal layer (Lin *et al*., 2011). The higher amount and condensation of those elements might be responsible for the hardness of the external region. Fish scale contains collagen fibrils and calcium phosphates, particularly hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$; HAp) crystals (Okuda *et al*., 2009). Due to the similar image between both samples, the use of NaOH for non-protein removal did not have the effect on elements distributed in the scale.

Fig 11 shows the cross-section of scale subjected to non-collagenous protein removal with subsequent demineralization. From SEM-EDX image, carbon constituted as the most abundant element and oxygen and nitrogen were rich in the internal region. It was noted that Ca and P rich external layer was removed by demineralization. Fibrillary plate is constructed from lamellae composed of highly ordered collagen fibres (Ikoma *et al*., 2003). After scales were soaked with HCl solution (0.75 M) for 30 min, Ca was almost completely removed from scale. Phosphorous was not detected in demineralized scale. This result indicated that demineralization was effectively carried out and collagen fibrils were more concentrated.

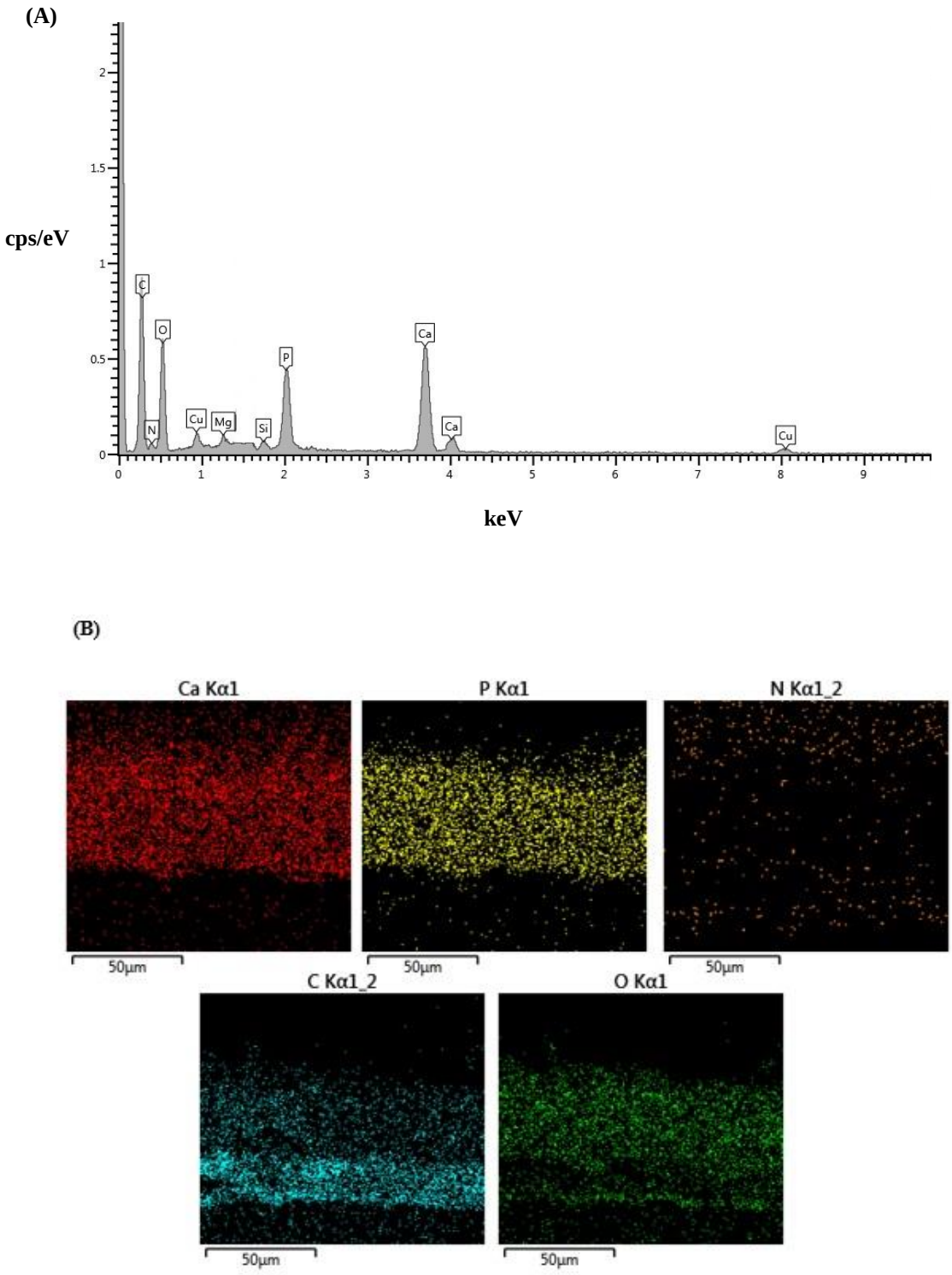

Figure. 9 Elemental profile (A) and mappings of different elements (B) of cross-section of original spotted golden goatfish scale as analyzed by SEM-EDX.

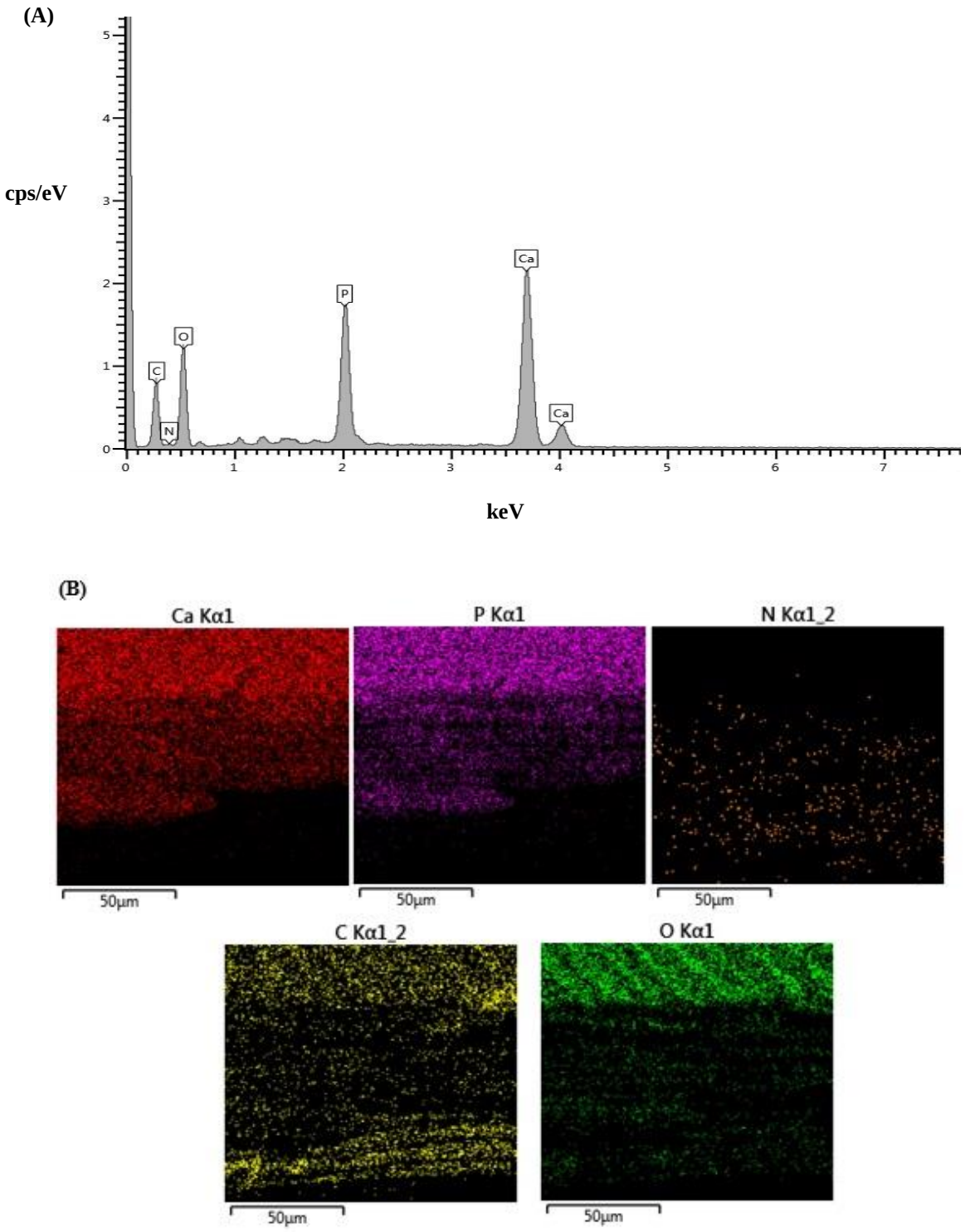

Figure. 10 Elemental profile (A) and mappings of different elements (B) of cross-section of spotted golden goatfish scale with non-collagenous protein removal as analyzed by SEM-EDX.

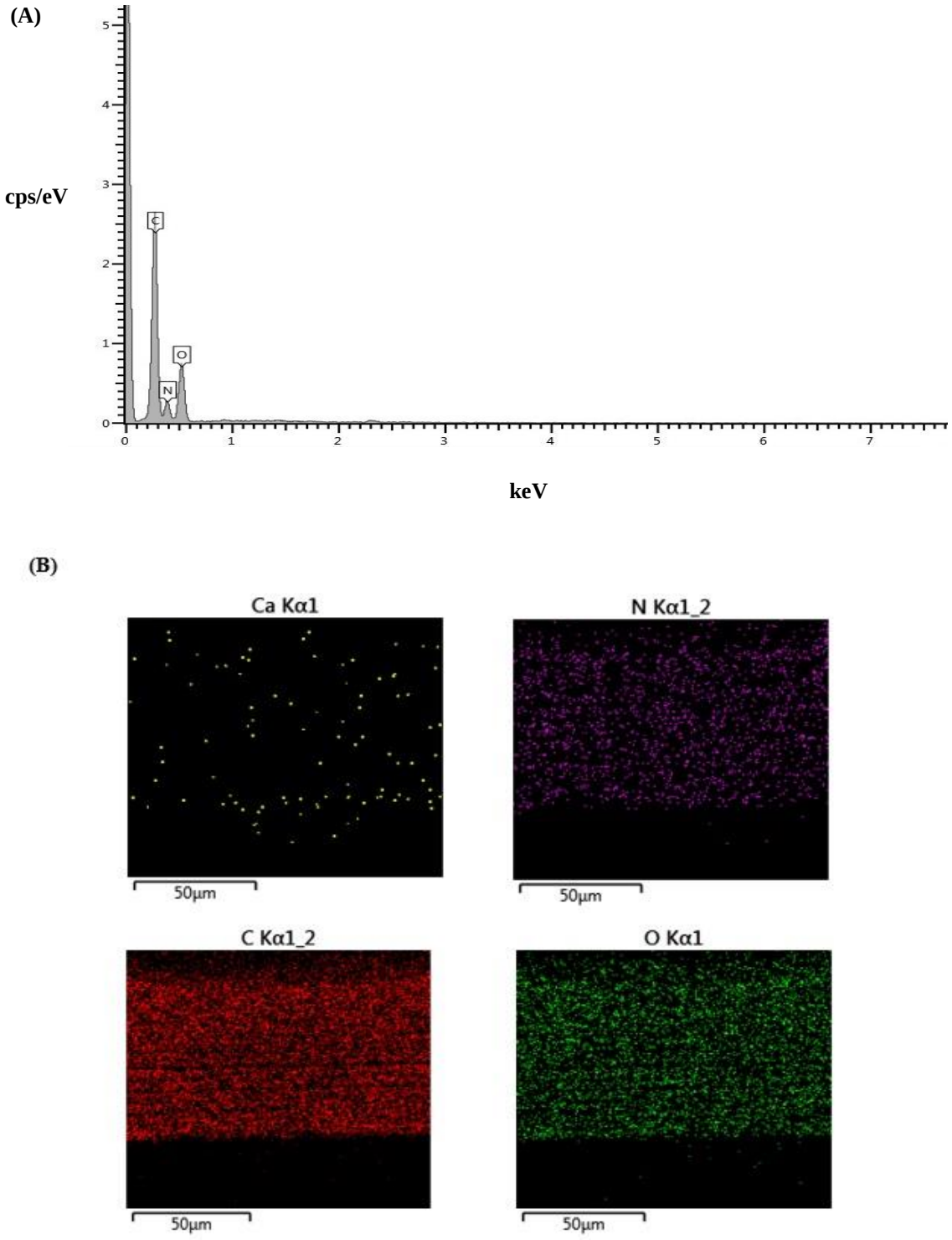

Figure. 11 Elemental profile (A) and mappings of different elements (B) of cross-section of spotted golden goatfish scale with non-collagenous protein removal and followed by demineralization (0.75 M HCl, 30 min) as analyzed by SEM-EDX.

3.5 Conclusion

Scale consisted of two distinct regions: an external (osseous) layer and an internal fibrillary plate. The use of 0.75 M HCl for 30 min with a scale/solution ratio of 1:5 (w/v) at 25 ^{o}C effectively demineralized the scales. Ca and P contents of scales drastically decreased after demineralization. Most of inorganic elements at external layer were removed by demineralization with coincidental increase in collagen fibrils. Thus, demineralization could favour the extraction of collagen or gelatin.

3.6 References

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CHAPTER 4

CHARACTERISTICS AND GELLING PROPERTY OF GELATIN FROM SCALE OF SPOTTED GOLDEN GOATFISH

4.1 Abstract

Characteristics and gelling properties of gelatin from the scale of spotted golden goatfish, as influenced by extraction conditions, were studied. Yields of gelatin extracted at 45, 60 and 75 ^{o}C for various times (6 and 12 h) were 2.3-2.6%, 8.6-9.3% and 9.9-10.1% (on wet weight basis), respectively. All gelatins contained β-chain and α-chains as the predominant components and showed a high imino acid content (182-192 residues/1000 residues). Gel strength of gelatins decreased, whilst gelatin solution became more turbid as the extraction temperature and time increased. Gelling and melting temperatures of gelatin were 18.7-20.1 and 26.4-28.0^{o}C, respectively, and both temperatures decreased with increasing extraction temperatures and times. Therefore, the scales of spotted golden goatfish had a potential as collagenous materials for gelatin extraction.

4.2 Introduction

Gelatin is a fibrous protein obtained from collagenous materials subjected to thermal denaturation or partial degradation. It has been widely used in food and non-food (photographic, cosmetic, and pharmaceutical) industries (Benjakul *et al*., 2009). Generally, gelatin is produced from skins and skeletons of land animals (Kaewruang *et al*., 2013). However, the occurrence of bovine spongiform encephalopathy (BSE) and foot-and-mouth disease (FMD) has led to the concerns for human health. Furthermore, porcine and bovine gelatins are prohibited for some religions. Fish gelatin, has therefore received increasing attention as the alternative for mammalian and avian gelatin. Fish skin, bone, and scale, considered as processing by-products, have become the potential collagenous material for gelatin production (Benjakul *et al*., 2012). Gelatin has several functional properties, including gelling, foaming, emulsifying and wetting properties (Balti *et al*., 2011). Generally, functional properties of gelatin and other food proteins are governed by many factors such as chain length or molecular weight, amino acid composition and hydrophobicity, etc

(Gomez-Guillen *et al*., 2002). Additionally, the quality of gelatin for a particular application is greatly influenced by the sources of raw material and species, and also by the processes used (Johnston-Banks, 1990).

Spotted golden goatfish (*Parupeneus heptacanthus*) has been widely used for frozen fillet manufacturing in Thailand. During descaling and dressing, a large amount of scale is produced as the low-value byproduct. Conversion of those remainders into a value added product, particularly for gelatin production, can pave the way for better utilization of limited fishery resources. Scale gelatins from several fish species, e.g., tilapia (Weng *et al*., 2014), bighead carp (Sha *et al*., 2014), grass carp fish (Zhang *et al*., 2011) have been extracted and characterized. Nevertheless, no information regarding gelatin from the scales of spotted golden goatfish as influenced by extracting conditions has been reported. Therefore, the aim of this study was to characterize gelatin from spotted golden goatfish scales extracted at various temperatures for different times.

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 spotted golden goatfish scales

Scales of spotted golden goatfish with an average body weight of 100-120 g/fish were collected from Kingfisher Holding, LTD., Songkhla Province, Thailand. The scales were packaged in polyethylene bag and transported in ice to the Department of Food Technology, Prince of Songkla University within approximately1 h. Upon arrival, scales were washed with tap water and drained before being placed in polyethylene bags. The samples were kept at -20 ^{o}C with the storage time not longer

than 2 months. Before use, fish scales were thawed using the running water for 20 min.

4.3.3 Pretreatment of spotted golden goatfish scales

Scales were suspended in 0.1 M NaOH for 6 h at the ratio of 1:10 (w/v). The mixture was continuously stirred to remove non-collagenous proteins. The solution was changed every 3 h. Treated scales were washed with tap water until wash water became neutral. Subsequently, the prepared scales were demineralized using 0.75 M HCl with a scale/solution ratio of 1:5 (w/v) for 30 min. The demineralization was performed at room temperature (28-30 ^{o}C) with the continuous stirring. Thereafter, the demineralized scales were washed until the neutral pH of wash water was obtained.

4.3.4 Extraction of gelatin

Demineralized scales were mixed with distilled water at a ratio of 1:10 (w/v). The extraction was conducted at different temperatures (45, 60 and 75 ^{o}C) for various times (6 and 12 h). The mixtures were stirred continuously. At the designated time, the mixtures were filtered using a Buchner funnel, with a Whatman No.4 filter paper (Whatman International, Ltd., Maidstone, England). Then, the filtrates were freeze-dried using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). Gelatin samples obtained were subsequently subjected to analyses.

4.3.5 Analyses

4.3.5.1 Yield

The yield of gelatin was calculated and expressed as the percentage based on the weight of the starting material as follows:

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

4.3.5.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

All gelatin samples were determined for protein patterns using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) following the method of Laemmli (1970) as modified by Sinthusamran *et al.* (2013). Gelatin samples were dissolved in 5% SDS solution. The mixtures were heated at 85 ^{o}C for 1 h using a temperature controlled water bath model W350 (Memmert, Schwabach, Germany). Solubilized samples were mixed at a 1:1 (v/v) ratio with sample buffer (0.5 M Tris–HCl, pH 6.8 containing 5% SDS and 20% glycerol). Samples were loaded onto a polyacrylamide gel made up of 7.5% separating gel and 4% stacking gel and subjected to electrophoresis at a constant current of 20 mA/gel. After electrophoresis, gels were stained with 0.05% (w/v) Coomassie blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid for 30 min. Finally, they were destained with 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 the proteins.

4.3.5.3 Fourier transform infrared (FTIR) spectroscopic analysis

FTIR spectra of all gelatin samples were obtained using total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopic analysis as described by Sinthusamran *et al*. (2013). Gelatin samples were placed onto the crystal cell. The spectra were acquired over the range of 650–4000 cm^{-1} with a resolution of 4 cm^{-1} for 32 scans against a background spectrum recorded from the clean empty cell at 25 ^{o}C using FTIR spectrometer (Model Equinox 55, Bruker, Ettlingen, Germany) equipped with a horizontal ATR trough plate crystal cell (45^{o} ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI, USA). Deconvolution was performed on the average spectra for the amide I and II bands, using a resolution enhancement factor of 1.8 and full height band width of 13 cm^{-1}. Analysis of spectral data was carried out using the OPUS 3.0 data collection software programme (Bruker, Ettlingen, Germany).

4.3.5.4 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 ^{o}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). The content was expressed as residues/1000 residues.

4.3.5.5 Determination of gel strength

Gelatin gel was prepared by the method of Kittiphattanabawon *et al*. (2010). Gelatin was dissolved in distilled water (60 ^{o}C) to obtain a final concentration of 6.67% (w/v). The solution was stirred until the gelatin is 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 ^{o}C) for 18 h prior to analysis. The gel strength was determined at 8–10 ^{o}C using a texture analyzer (Stable Micro System, Surrey, UK) with a load cell of 5 kg, cross-head speed of 1 mm/s, equipped with a 1.27 cm diameter flat-faced cylindrical Teflon® plunger. The maximum force (grams), taken when the plunger had penetrated 4 mm into the gelatin gels, was recorded.

4.3.5.6 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 measuring geometry included a 3.5 cm parallel plate and the gap set at 1.0 mm. The measurement was performed at a scan rate of 0.5 ^{o}C/min, frequency of 1 Hz, oscillating applied stress of 3 Pa during cooling from 40 to 5 ^{o}C and heating from 5 to 40 ^{o}C. The gelling and melting temperatures was calculated, where tan δ became 1 or δ was 45^{o}.

4.3.5.7 Determination of turbidity

The turbidity of gelatin solution was determined according to the method of Kittiphattanabawon *et al.* (2010). Gelatin was dissolved in distilled water (60 ^{o}C) to obtain a final concentration of 6.67% (w/v). The solution was stirred until the gelatin was completely solubilized. Turbidity was determined by measuring the absorbance at 360 nm using a spectrophotometer (UV-1800 Spectrophotometer, Shimadzu, Kyoto, Japan).

4.3.5.8 Microstructure analysis of gelatin gel

The microstructure of gelatin gel was visualized using a scanning electron microscopy (SEM). Gelatin gels (6.67% , w/v) having a thickness of 2–3 mm were fixed with 2.5% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 12 h. The samples were then rinsed with distilled water for 1 h and dehydrated in ethanol with a serial concentration of 50%, 70%, 80%, 90% and 100% (v/v). Dried samples were mounted on a bronze stub and sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA). The specimens were observed with a scanning electron microscope (JEOL JSM-5800 LV, Tokyo, Japan) at an acceleration voltage of 20 kV.

4.3.6 Statistical analysis

Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out using a Duncan's multiple range test. For pair comparison, a T-test was used. 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 Yield

The yields of gelatin from scales of spotted golden goatfish extracted with different conditions are shown in Table 9. Yields of gelatin increased as the extraction temperature increased ($P<0.05$). Yields of gelatin from the scales extracted at 45, 60 and 75 ^{o}C were 2.27-2.57%, 8.63-9.27% and 9.90-10.07% (on wet weight basis), respectively. Yield also increased as the longer extraction time was

implemented ($P<0.05$), except gelatin extracted at 75 ^{o}C, in which no difference was observed between samples extracted for 6 h and 12 h ($P>0.05$). When heat was applied, particularly at high temperatures, the bondings between α-chains in the native mother collagen were more effectively destroyed. As a consequence, the triple helix structure became amorphous and could be extracted into the medium with ease, leading to the higher yield. Increasing extraction time also provided more energy to destroy the bondings, in which more free α or β-chains were released from the skin complex (Sinthusamran *et al*., 2014). The result was in agreement with Nagarajan *et al.* (2012) who reported that the extraction yield of gelatin from splendid squid skin increased when the extraction temperature and time increased. Arnesen and Gildberg (2007) also found that the increasing extraction temperatures resulted in the increased yield of gelatin from Atlantic salmon skin. Different yields of fish gelatin have been reported for sea bream scale (9.55%) (Akagündüz *et al*., 2014), greater lizardfish skin (35.1%) (Taheri *et al*., 2009) and nile perch skin (64.3%) (Muyonga *et al*., 2004). It was noted that yields from the scale of spotted golden goatfish was much lower than those from fish skin. Scales had the compact and dense structure. Nevertheless, pretreatment, especially demineralization rendered the scale with looser structure (Chuaychan *et al.,* 2015). As a result, hot water could penetrate and extract gelatin from scales to a higher extent. The yield of gelatin depends on type of raw material and gelatin extraction process, including the pretreatment process (Kittiphattanabawon *et al*., 2010).

Table 9 Extraction yield (% wet weight basis), gel strength and turbidity of gelatin from the scale of spotted golden goatfish extracted at different temperatures for various times.

Temperature (ºC)	Time (h)	Yields (%)	Gel strength (g)	Turbidity (A_{360})
45	6	2.27 ± 0.12^{AaX}	286.61 ± 1.02^{BfZ}	0.791 ± 0.81^{AaX}
	12	2.57 ± 0.29^{BbX}	249.62 ± 0.53^{AeZ}	0.806 ± 0.83^{AaX}
60	6	8.63 ± 0.22^{AcY}	227.77 ± 1.52^{BdY}	0.939 ± 0.97^{AbY}
	12	9.27 ± 0.43^{BdY}	206.63 ± 0.91^{AcY}	1.194 ± 0.62^{BcY}
75	6	9.90 ± 1.76^{AeZ}	154.07 ± 1.20^{BbX}	1.493 ± 0.96^{AdZ}
	12	10.07 ± 0.93^{AeZ}	124.86 ± 1.38^{AaX}	1.700 ± 0.65^{BeZ}

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

Different uppercase letters (A and B) in the same column under the same temperature indicate significant differences ($P<0.05$). Different lowercase letters in the same column indicate significant differences ($P<0.05$). Different uppercase letters (X, Y and Z) in the same column under the same time indicate significant differences ($P<0.05$).

4.4.2 Protein patterns

Protein patterns of gelatin from scale of spotted golden goatfish extracted at different temperatures for various times are illustrated in Fig 12. All gelatin samples contained β- and α-chains with MWs of 192 and 123-112 kDa, respectively, as the major constituents. Similar protein patterns were observed between samples extracted at 45 and 60 ºC, except that α_2-chain of the latter showed the lower band intensity. For the scale extracted at 75 ºC, the lower band intensity was noticeable for most proteins, except for α_1-chain, which remained unchanged. However, no marked differences in protein patterns were observed between gelatin extracted for 6 and 12 h. The result suggested that degradation of protein chains was induced by the thermal process. The result was also in accordance with Kaewruang *et al*. (2013) who found the pronounced degradation of unicorn leatherjacket skin gelatin when extraction temperature increased. Content of α-chains in gelatin determined

functional properties of gelatin such as gelling, emulsifying and foaming properties (Gomez-Guillen *et al*., 2002). Therefore, extraction condition, both temperature and time, affected the constituents of gelatin from spotted golden goatfish scales.

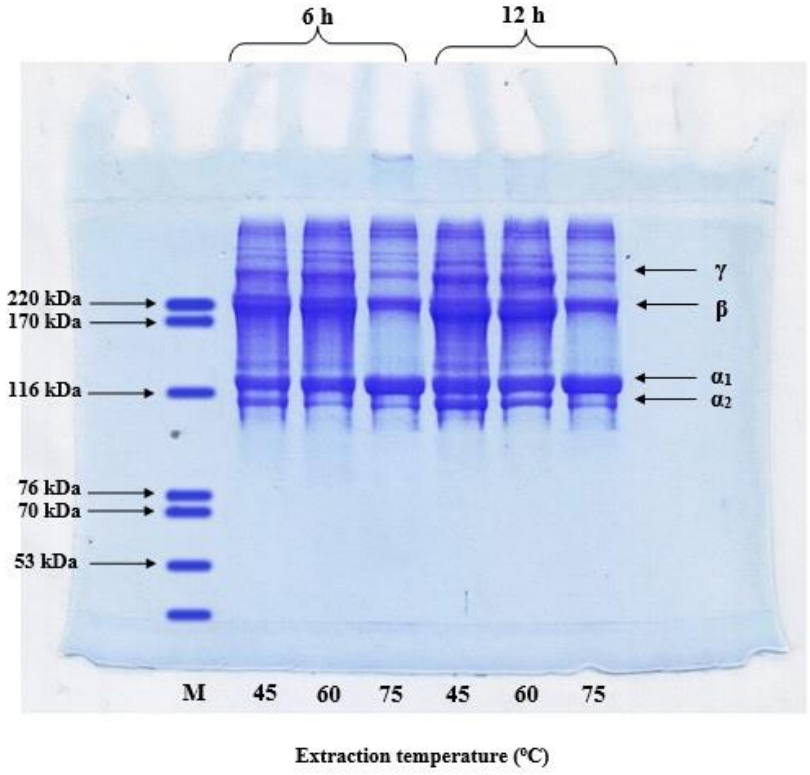

Figure 12 SDS-PAGE patterns of gelatin from scale of spotted golden goatfish extracted at different temperatures for various times. M denotes high molecular weight markers.

4.4.3 Fourier transform infrared (FTIR) spectra

FTIR spectra of gelatin from scale of spotted golden goatfish extracted at 45, 60 and 75 °C for different times (6 and 12 h) are shown in Fig. 13. FTIR spectroscopy has been used to determine the functional groups and secondary structure of gelatin (Muyonga *et al*., 2004a). Major peaks of gelatins were situated in Amine I, II, III, A and B regions. The amide I vibration mode is primarily a C=O stretching vibration coupled to contributions from the CN stretch, CCN deformation and in-plane NH bending modes (Bandekar, 1992). Similar amide I band was found between all gelatin samples, which appeared at wavenumbers of 1640-1643 cm^{-1}. Kittiphattanabawon *et al*. (2010) reported that with higher temperature and longer

time, the amide I band of gelatin from shark skin shifted to a higher wavenumber. (Kittiphattanabawon *et al*., 2010). The difference in Amine I of gelatin from spotted golden goatfish and other gelatin might be due to the different thermal stability of gelatin from different sources. It was found that α_1-chain of gelatin from scale of spotted golden goatfish show high thermal stability as evidenced by the maintainance of this component, regardless of extraction temperatures (Fig. 12). Sinthusamran *et al*. (2014) and Kaewruang *et al*. (2013) suggested that greater loss of triple helix was due to the disruption of interchain interaction induced by harsher condition. The amide II band of gelatin extracted at 45, 60 and 75ºC for both extraction times were observed at wavenumbers of 1540-1543 cm^{-1}. The amide II vibration mode is attributed to an out-of-phase combination of CN stretch and inplane NH deformation modes of the peptide group (Bandekar, 1992). All gelatin samples generally exhibited similar spectra, both in terms of wavenumbers and amplitude. Additionally, the absorption bands of all gelatins in the amide-III region appeared at the similar wavenumbers of 1539-1541 cm^{-1}. The amide III band represents the combination peaks between CN stretching vibrations and NH deformation from the amide linkages as well as the absorptions arising from wagging vibrations of CH_2 groups in the glycine backbone and proline side-chains. It has been used to indicate the disorder from an α-helical to a random coil structure (Friess and Lee, 1996).

Furthermore, amide A band of gelatin extracted at 45, 60 and 75 °C for 6 h were observed at 3290, 3292 and 3294 cm^{-1} and at 3291, 3294 and 3296 cm^{-1} for gelatin with extraction time of 12 h, respectively. The amide A band associated with the N–H stretching vibration showed the existence of hydrogen bonds. Generally, 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). With higher extraction temperature used, the amide A band shifted to higher wavenumber. Similar phenomenon was also found when the longer extraction time was used for gelatin extraction. When the higher temperature was used for extraction, the interchain between α- or β-chains was more disrupted. This was coincidental with higher yield (Table 9).

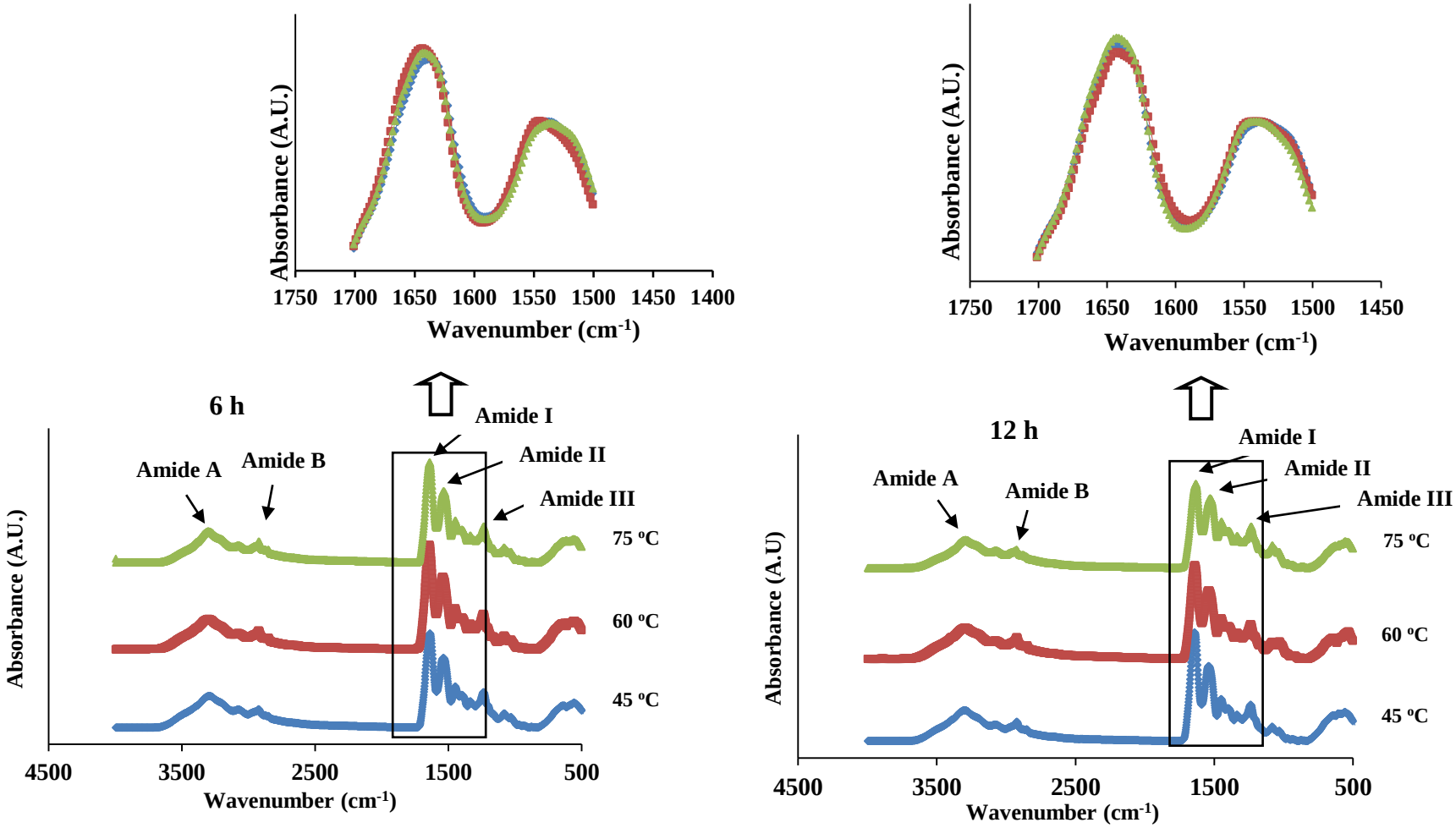

Figure 13 FTIR spectra of gelatin from scale of spotted golden goatfish extracted at different temperature for various times.

The amide B band were observed at wavenumbers of 3086, 3077 and 3075 cm^{-1} for gelatin extracted at 45, 60 and 75 °C for 6 h and 3081, 3076 and 3072 cm^{-1} for gelatin extracted at 45, 60 and 75 °C for 12 h, respectively. Gelatin extracted at higher temperature and longer time exhibited the lower wavenumbers. The amide B band vibration mode is asymmetric stretching vibration of =C–H as well as NH_3^+. This result suggested that the interaction of $-NH_3$ group between peptide chains was more pronounced in gelatin extracted at higher temperature for a longer time (Nagarajan *et al*., 2012). Therefore, the secondary structure and functional group of gelatins obtained from scales of spotted golden goatfish were affected by extraction temperature and time.

4.4.4 Amino acid composition

The amino acid compositions of gelatin extracted under different extraction conditions are shown in Table 10. All gelatin samples showed similar amino acid composition, in which glycine was the major amino acid (334–335 residues/1000 residues). Glycine is located at every third position of α-chains and represents nearly one third of total residues (Benjakul *et al*., 2009). All gelatin samples contained the imino acids, including proline (108-113 residues/1000 residues) and hydroxyproline (74-79 residues/1000 residues). Different imino acid contents of gelatin from various fish species were reported such as sea bream scale (185 residues/1000 residues) (Akagündüz *et al*., 2014), grass carp fish scales (157 residues/1000 residues) (Zhang *et al*., 2011) and cod skin (154 residues/1000 residues) (Arnesen and Gildberg, 2007).

Table 10 Amino acid compositions of gelatin from the scale of spotted golden goatfish extracted at different temperatures for various times.

Amino acids	Extraction temperature / time					
	45 ºC		60 ºC		75 ºC	
	6 h	12 h	6 h	12 h	6 h	12 h
Alanine	135	135	134	136	133	135
Arginine	53	54	54	53	54	53
Aspartic acid/asparagine	44	43	43	43	43	43
Cysteine	2	1	1	1	1	1
Glutamic acid /glutamine	71	72	72	72	72	75
Glycine	335	335	335	335	335	334
Histidine	6	6	7	6	7	8
Isoleucine	7	7	8	7	7	7
Leucine	17	19	19	19	19	19
Lysine	26	26	29	27	27	27
Hydroxylysine	6	6	6	7	6	6
Methionine	13	14	13	14	14	14
Phenylalanine	14	13	14	12	16	14
Hydroxyproline	79	78	76	77	75	74
Proline	113	112	109	110	109	108
Serine	36	36	37	37	36	38
Threonine	23	23	23	23	25	23
Tyrosine	2	2	2	3	3	3
Valine	18	18	18	18	18	18
Total	1000	1000	1000	1000	1000	1000
Imino acid	192	190	185	187	184	182

Gelatin, with a higher content of hydroxyproline, is believed to have higher viscoelastic properties and an ability to develop the strong gel structure (Gómez-Guillén *et al*., 2002). Hydroxyproline also plays an essential role in the stabilization of the triple helix strands of mother collagen via its hydrogen bonding ability through its −OH group (Benjakul *et al*., 2009). For gelatin, OH groups of hydroxyproline might be involved in hydrogen bondings between α- or β-chains thereby strengthening gel network. In the present study, it was found that both hydroxyproline and proline contents in gelatin decreased as the extraction temperature increased. The lowest imino acid content was obtained in gelatin extracted at 75 ºC.

Alanine was also found at the high content (133-136 residues/1000 residues). However, very low cysteine was obtained in gelatin from spotted golden goatfish scale. Collagen and gelatin have been known to be free of cysteine (Akagündüz *et al*., 2014). The result suggested that extraction temperature and time had the influence on amino acid composition of gelatin from scales of spotted golden goatfish

4.4.5 Gel strength of gelatin

Gel strength of gelatin extracted from spotted golden goatfish scales with varying extraction conditions is shown in Table 9. At the same extraction time, gel strength of gelatin decreased when the extraction temperature increased ($P<0.05$). For gelatin extracted at the same temperature, the lower gel strength was observed when the longer extraction time was used ($P<0.05$). Amongst all samples, that extracted at 45 ^{o}C for 6 h showed the highest gel strength (286.6 g). The decrease in gel strength was in accordance with the slight decreases in the β- and γ-chains band intensity (Fig. 12). The amount of the β- and γ-components was reported as the major factor governing gelation of gelatin (Taheri *et al*., 2009). Additionally, imino acids played a role in gel formation. Hydroxyl groups of hydroxyproline are involved in inter-chain hydrogen bonding via a bridging water molecule as well as direct hydrogen bonding to the carbonyl group (Benjakul *et al*., 2009). Different gel strength was reported for gelatin of different fish species including sea bream scale (126 g) (Akagündüz *et al*., 2014) and lizardfish scales (268 g) (Wangtueai and Noomhorm, 2009). It was noted that gelatin extracted at higher temperature, especially for 12 h, showed the lowest gel strength ($P< 0.05$). This coincided with the lowest imino acid content of this sample (182 residues/1000 residues). The difference in gel strength between the samples could be due to the differences in intrinsic characteristics, such as molecular weight distribution and amino acid composition.

4.4.6 Gelling and melting temperatures

Thermal transitions were monitored by changes in the phase angle (δ) of dissolved gelatin from scales of spotted golden goatfish during cooling (40-5 ^{o}C) and subsequent heating (5-40 ^{o}C) as shown in Fig 14A and B, respectively. The gelling temperatures of gelatin were in the range of 18.7- 20.1^{o}C. The formation of junction zones in the three-dimensional network of gelatin gel during cooling can be

monitored in term of changes of phase angle (Sinthusamran *et al*., 2014). Nagarajan *et al.* (2012) reported that the amount of γ-, β-, and α-chain components influenced gelling point of gelatin gel. Different gelling temperatures were reported for gelatin from different sources such as scale of sea bream (20.8 ^{o}C) (Akagündüz *et al*., 2014) and scale of grass carp (20.8 ^{o}C) (Zhang *et al*., 2011). Gelatin extracted at higher temperature, especially for a longer time, showed the lower gelling temperature. This was coincidental with the lower imino content of those samples (Table 10). Generally, fish gelatin has lower gelling and melting temperatures than mammalian counterpart (Karim and Bhat, 2009). This could be due to the lower contents of imino acids in fish gelatin (Gudmundsson, 2002).

The melting temperatures of gelatin from spotted golden goatfish scales ranged from 26.4 to 28.0 ^{o}C. Gelatin samples extracted at lower temperature and shorter time had the higher melting temperature. This suggested that gel could be maintained as semi-solid state for a longer time when heat was applied, e.g. during chewing in the mouth. Conversely, gelatin extracted at higher temperature showed the lower melting temperature. The higher melting temperatures of gelatin samples were in accordance with higher gel strength (Table 9). Gelatin from various sources with varying melting temperatures were reported such as grass carp scale (26.9 ^{o}C) (Zhang, *et al*., 2011) and sea bream scale (26.0 ^{o}C) (Akagündüz *et al*., 2014). Generally, thermal stability of gelatin gel has been shown to be directly correlated to the number and stability of Pro-rich regions in the collagen or gelatin molecules, which is considerably lower in cold water fish than in warm blooded animals. Temperature of the environment, where animals inhabit, affects the gelling and melting temperatures of the resultant gelatin (Gudmundsson, 2002).

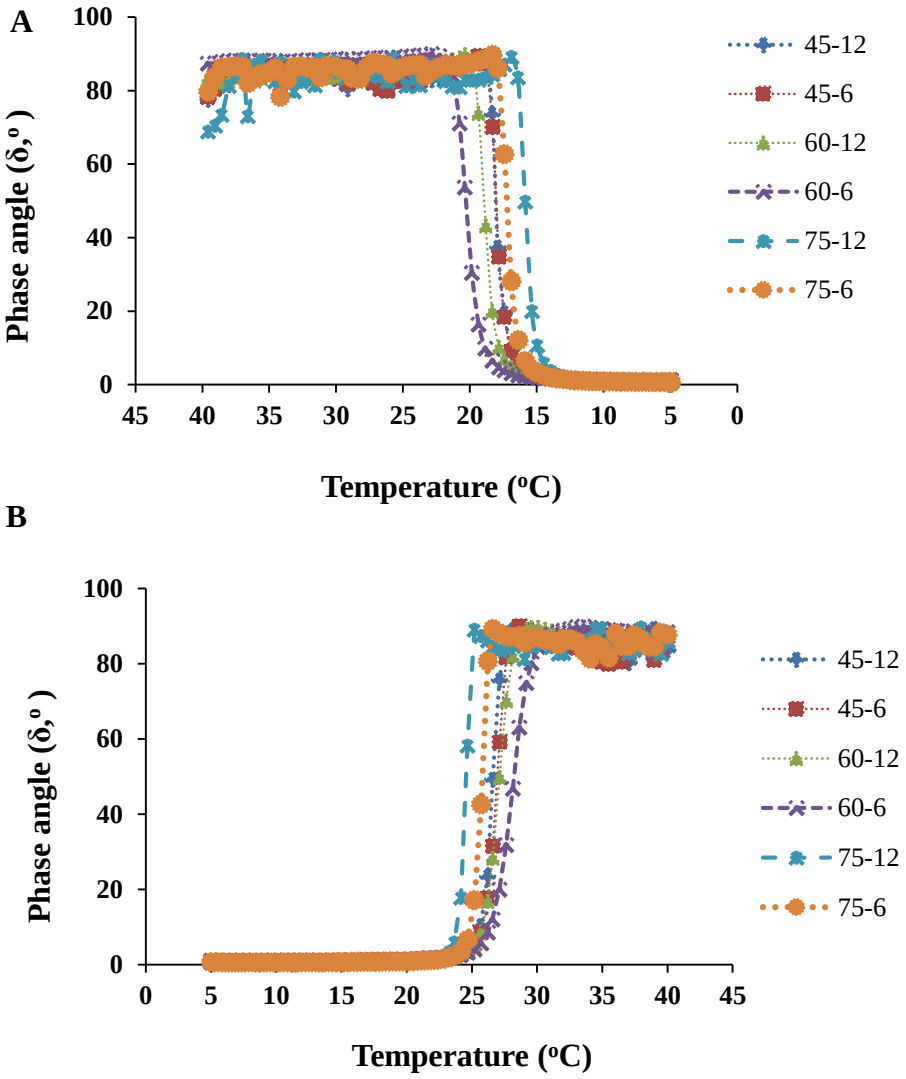

Figure 14 Changes in phase angle (δ, °) of gelatin solution (6.67%, w/v) from the scale of spotted golden goatfish extracted at different temperatures for various times during cooling (A) and subsequent heating (B).

4.4.7 Turbidity

Turbidity of solutions of gelatin extracted from spotted golden goatfish scales with different extraction conditions expressed as A_{360} is shown in Table 9. The increase in turbidity was noticed when the extraction temperature increased ($P<0.05$). At the same extraction temperature used, higher turbidity was observed for gelatin extracted for a longer time ($P<0.05$) except for gelatin extracted at 45 °C, in which no differences in turbidity were found between 6 and 12 h. It was noted that the turbidity

of gelatin solutions from spotted golden goatfish was higher than that of bovine gelatin solution ($p<0.05$). Random aggregation of gelatin molecules from spotted golden goatfish scales might occur to a high extent as evidenced by the increased turbidity. When proteins were subjected to high temperature, the aggregation could be induced, resulting in the increased turbidity (Johnson and Zabik, 1981). This was in agreement with the shift of amide B to the lower wavenumber (Fig. 13). Kittiphattanabawon *et al*. (2010) also reported that gelatin solutions from the skin of blacktip shark had the increased turbidity when the extraction temperature and time increased. In the present study, the clarification was not carried out, whilst commercial gelatin is commonly clarified using the activated charcoal (Tzokov *et al*., 1996). Therefore, the extraction condition affected the appearance of gelatin solution as well as resulting gel.

4.4.8 Microstructures of gelatin gels

Gel microstructures of gelatin from the spotted golden goatfish scales extracted at various temperatures for different times are shown in Fig. 15. For the same extraction time, gelatin extracted at higher temperature yielded the gel with the coarser network with larger voids. Furthermore, gel of gelatin extracted for longer time had the less uniformity with coarser structure. Amongst all gelatin gels, that of gelatin extracted at 45 ^{o}C for 6 h exhibited the finest gel network with high connectivity. Conversely, the largest strands and voids were found in gel from gelatin extracted at 75 ^{o}C for 12 h. In general, the finer network of gelatin gel was in accordance with the higher gel strength (Table 9). Gelatin extracted at lower temperatures for a shorter time most likely had chains with higher molecular weight (Fig. 12). Zhang *et al*. (2012) reported that the denser strands in gel structure were governed by the greater content of high molecular weight constituents (γ- and β-chains) in gelatin, whilst looser strands in gel matrix were found in gelatin containing smaller and shorter chains. Gelatin with longer chains could form the junction zones with ordered alignment. This led to the high aggregation with connectivity. The result revealed that the extraction condition had an impact on the molecular arrangement of gelatin chains in the network during gelation, thereby determining the properties of gels.

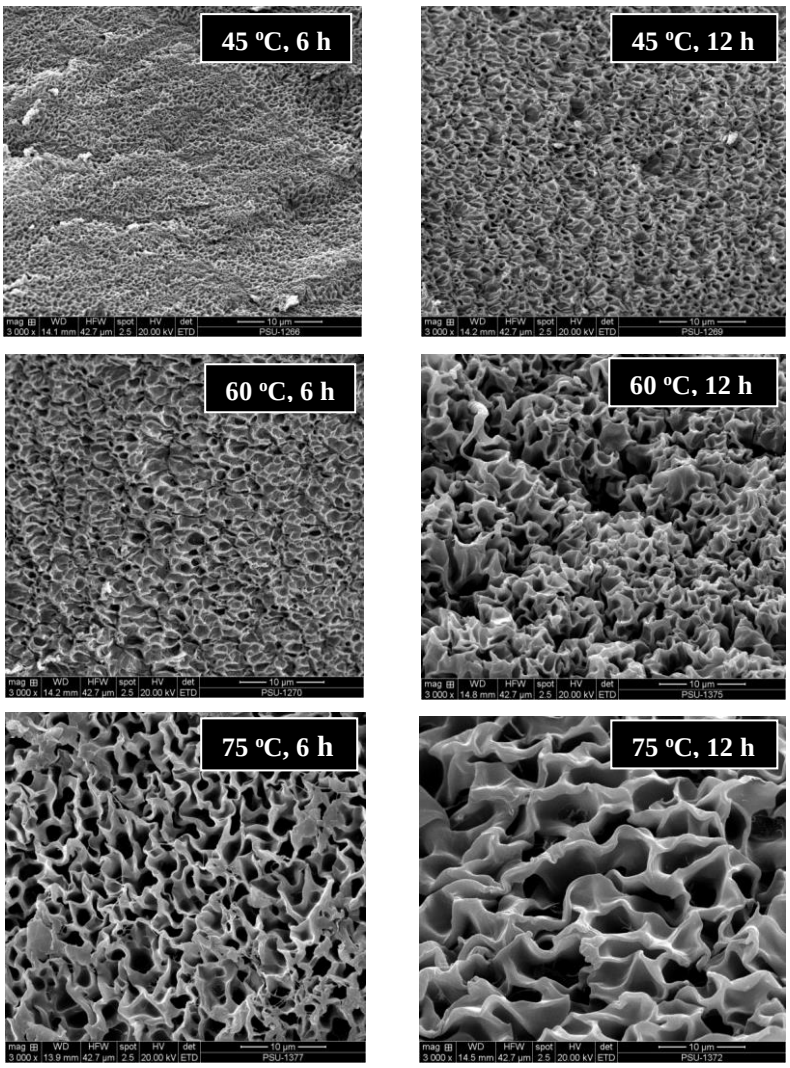

Figure 15 Microstructures of gelatin gel from the scale of spotted golden goatfish extracted at different temperatures for various times. Magnification: 3000x

4.5 Conclusion

Gelatin from the scale of spotted golden goatfish extracted at different temperatures and various times had varying characteristics and properties. Extraction of gelatin at higher temperature for longer time resulted in the higher yield, however gel became weaker. Therefore, the scale of spotted golden goatfish could serve as raw material for gelatin extraction and the extraction conduction should be appropriately selected, depending on the further uses.

4.6 References

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CHAPTER 5

EFFECT OF MALTODEXTRIN ON CHARACTERISTICS AND ANTIOXIDATIVE ACTIVITY OF SPRAY-DRIED POWDER OF GELATIN AND GELATIN HYDROLYSATE FROM SCALES OF SPOTTED GOLDEN GOATFISH

5.1 Abstract

Antioxidative activities of gelatin hydrolysate from scale of spotted golden goatfish as affected by degrees of hydrolysis (DH) were examined. Characteristics and antioxidative activity of gelatin and gelatin hydrolysate powders using maltodextrin as a carrier agent at different ratios (1:0, 2:1, 1:1 and 1:2 (w/w)) were investigated. Gelatin hydrolysates with 40% DH exhibited the highest antioxidative activity ($p<0.05$). With increasing maltodextrin proportions, yield was increased. The resulting powders had the increases in total sugar content and whiteness with coincidental decreases in *a**, *b**-values and browning intensity when maltodextrin proportion increased ($p<0.05$). Solubility of gelatin powder increased with increasing maltodextrin proportion ($p<0.05$). Based on SEM images, gelatin powder had spherical shape with both smooth surface and shrinkage. Sizes were varied, regardless of maltodextrin levels. For gelatin hydrolysate powder, uniform agglomerates with less shrinkage were formed when maltodextrin was incorporated. DPPH and ABTS radical scavenging activities and ferric-reducing antioxidant power of gelatin and gelatin hydrolysate decreased when maltodextrin was used as a carrier agent. Thus, maltodextrin levels used directly affected characteristics and antioxidative activity of gelatin and gelatin hydrolysate powders prepared by spray-drying process.

5.2 Introduction

Gelatin is a fibrous protein obtained from collagenous materials subjected to thermal denaturation or partial degradation. It has been widely used in food and non-food (photographic, cosmetic, and pharmaceutical) industries (Benjakul *et al*., 2009). Gelatin can be extracted from fish scales, the byproducts from fish

dressing or filleting process. Properties of gelatin can be affected by several factors. Drying condition is another factor determining the characteristics and functional properties of fish gelatin (Sae-Leaw *et al*., 2016). Although gelatin can exhibit several functional properties, especially gelation, the bioactivity is much lower than its hydrolysates. To enhance bioactivities, hydrolysis is implemented to release bioactive peptides. Gelatin hydrolysate from blacktip shark skin, prepared using papaya latex enzyme, possessed the increased antioxidant activities when assayed by DPPH and ABTS radical scavenging activity and ferric reducing antioxidant power (Kittiphattanabawon *et al*., 2012). Gelatin hydrolysate from seabass skin had the increases in antioxidation activity with increasing degree of hydrolysis (DH) (Senphan and Benjakul, 2014)

Drying is a process used for food preservation throughout the world. The most common methods applied in the food industry, apart from conventional air drying, are spray drying and freeze drying. Spray drying has been employed widely in food industries to produce dry powders and agglomerates. The advantages of spray drying include hygienic conditions during processing, low operational costs, and short contact time (Sagar and Suresh-Kumar, 2010). Spray-dried gelatin with low water activity and high storage stability was prepared by Sae-Leaw *et al*. (2016). During spray drying, sticky products can be generated, thereby adhering to the internal wall of drying chamber. This leads to the lower yield (Hennigs *et al*., 2001). The use of maltodextrin as an encapsulation or carrier agent in spray drying has been introduced to tackle the problem (Ratanasiriwat *et al*., 2013). It can also increase the glass transition temperature and stability during storage (Fazaeli *et al*., 2012)

However, drying conditions e.g. inlet temperature, etc. can affect the property of gelatin in different fashions (Sae-Leaw *et al*., 2016). Furthermore, high temperature might induce a loss in bioactivity of hydrolysate, particularly antioxidative activity. Nevertheless, there is a little information on characteristics and bioactivity of gelatin and gelatin hydrolysate prepared by spray drying. The objective of this study was to investigate the effect of maltodextrin levels on the properties and antioxidative activity of spray-dried gelatin and gelatin hydrolysate from spotted golden goatfish scales.

5.3 Materials and methods

5.3.1 Chemicals/enzyme

All chemicals were of analytical grade. 2,20 azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,4,6-tripyridyltriazine (TPTZ) were purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada). Maltodextrin was obtained from Zhucheng Dongxiao Biotechnology CO., LTD. (Shandong, China). Alcalase (EC 3.4.21.14, 2.4 L, 2.64 AU/g) was purchased from Brenntag Ingredients Public Company Limited (Bangkok, Thailand).

5.3.2 Collection and preparation of spotted golden goatfish scales

Scales of spotted golden goatfish with an average body weight of 100-120 g/fish were collected from Kingfisher Holding, LTD., Songkhla Province, Thailand. Scales were placed in polyethylene bag and embedded in ice using the scale/ice in ratio of 1:2 (w/w). The sample was transported in polystyrene box to the Department of Food Technology, Prince of Songkla University. Hat Yai, within 1 h.

5.3.3 Pretreatment of spotted golden goatfish scales

Scales were washed using a tap water and drained for 5 min. The scales were then suspended in 0.1 M NaOH for 6 h at the ratio of 1:10 (w/v). The mixture was continuously stirred using an overhead stirrer model W20.n (IKA®-Werke, GmbH & CO.KG, Stanfen, Germany) to remove non-collagenous proteins. The solution was changed every 3 h. Treated scales were washed with tap water until wash water became neutral. Subsequently, the prepared scales were demineralized using 0.75 M HCl with a scale/solution ratio of 1:5 (w/v). The demineralization was performed at room temperature (28-30 ^{o}C) with the continuous stirring for 30 min. Thereafter, the demineralized scales were washed until the neutral pH of wash water was obtained.

5.3.4 Extraction of gelatin

Demineralized scales were mixed with distilled water at a ratio of 1:10 (w/v). The extraction was conducted at 75 ^{o}C for 6 h in a temperature controlled water bath (Model W350, Memmert, Schwabach, Germany). The mixtures were stirred

continuously. The mixtures were then filtered using a Buchner funnel, with a Whatman No.4 filter paper (Whatman International, Ltd., Maidstone, England). The filtrate obtained was freeze-dried using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). Dried gelatin was placed in a polyethylene bag and kept at -20 ^{o}C until used.

5.3.5 Preparation of gelatin hydrolysate with different degrees of hydrolysis

Gelatin hydrolysates with different degrees of hydrolysis (DHs) from scale of spotted golden goatfish were prepared as per the method of Kittiphattanabawon et al. (2012) with slight modification. Gelatin (3 g) was dissolved in 80 mL of distilled water. The pH of mixture was adjusted to 8 with 1 M NaOH. The volume of solution was made up to 100 mL by distilled water previously adjusted to pH 8 to obtain a protein concentration of 3% (w/v). The hydrolysis reaction was started by the addition of Alcalase (EC 3.4.21.14, 2.4 L, 2.64 AU/g, Sigma-Aldrich, Inc., St. Louis, Mo., USA) at various amounts, which were calculated from the plot between log (enzyme concentration) and DH to obtain DH of 10%, 20%, 30% and 40%, respectively (Benjakul and Morrissey, 1997). After 1 h of hydrolysis at 50 ^{o}C, the enzyme was inactivated by heating at 90 ^{o}C for 15 min in a temperature controlled water bath (model W350, Memmert, Schwabach, Germany). The mixtures were then centrifuged at 5000g at room temperature for 10 min. The supernatants were freeze-dried using a freeze-dryer (CoolSafe 55, ScanLaf A/ S, Lynge, Denmark). The obtained powders referred to as ‘‘gelatin hydrolysate’’ were placed in polyethylene bag and stored at -20 ^{o}C. Gelatin hydrolysate samples were subsequently determined for antioxidative activities. Hydrolysate with DH exhibiting the highest activity (40% DH) was selected for further study.

5.3.6 Determination of antioxidative activity

5.3.6.1 ABTS radical scavenging activity

ABTS radical scavenging activity was assayed according to the method of Intarasirisawat *et al*. (2012). The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution. ABTS radical cation was

generated by reacting 7.4 mM ABTS with 2.6 mM potassium persulphate at a ratio of 1:1 (v/v). The mixture was stored in the dark for 12 h at room temperature. Prior to assay, ABTS radical cation was diluted with methanol to obtain an absorbance of 1.1 (±0.02) at 734 nm. Sample solution (150 µL) with different concentrations was mixed with 2850 µL of ABTS radical cation and the mixture was left at room temperature for 2 h in the dark. The absorbance was then read at 734 nm. A standard curve of Trolox ranging from 50 to 600 µM was prepared. The activity was expressed as µmol Trolox equivalents (TE)/g sample.

5.3.6.2 DPPH radical scavenging activity

DPPH radical scavenging activity was determined according to the method described by Intarasirisawat *et al.* (2012). Sample was dissolved in distilled water and then 1 mL of sample solution was mixed with 1 mL of 0.1 mM DPPH solution in 95% ethanol. The mixture was vortexed for 10 s and allowed to stand at room temperature for 30 min in the dark. The absorbance of the mixture was read at 517 nm. The blank was prepared in the same manner, except that distilled water was used instead of the sample. A standard curve was prepared using trolox in the range of 10–60 µM. The activity was expressed as µmol trolox equivalents (TE)/g sample.

5.3.6.3 Ferric reducing antioxidant power (FRAP)

FRAP was assayed following the method of Benzie and Strain (1996). Stock solutions included 30 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. A working solution was prepared freshly by mixing 25 mL of acetate buffer, 2.5 mL of TPTZ solution and 2.5 mL of $FeCl_3 \cdot 6H_2O$ solution. The mixed solution was incubated at 37 ^oC for 30 min in a temperature controlled water bath and was referred to as FRAP solution. A sample solution (150 µL) was mixed with 2850 µL of FRAP solution and kept for 30 min in dark at room temperature. The ferrous tripyridyltriazine complex (coloured product) was measured by reading the absorbance at 593 nm. Sample blank was prepared by omitting $FeCl_3$ from FRAP solution and distilled water was used instead. The standard curve was prepared using Trolox ranging from 50 to 600 µM. The activity was calculated after sample blank subtraction and was expressed as µmol Trolox equivalents (TE)/g sample.

5.3.7 Impact of maltodextrin on properties of dried gelatin and gelatin hydrolysate powder

Gelatin and gelatin hydrolysate were mixed with maltodextrin at different ratios (1:0, 1:1, 2:1 and 1:2 (w/w)). The mixtures were dissolved in distilled water to obtain a final concentration of 2% (w/v). The solutions were subjected to spray drying using a spray dryer (SD-06 Basic, North Yorkshire, England) equipped with a spry-drying chamber having the dimension of 500 mm height and 210 mm diameter. A spray nozzle type of two-liquid nozzle (0.5 mm in size) was used. A cyclone separator, a hot-air blower and an exhaust blower were equipped. The solutions were fed by a peristaltic pump at 485 mL h^{-1} into the chamber, atomised by the hot air (air velocity of 2 ms^{-1}) from the blower in a downward current flow mode, using the following process conditions: inlet temperature of 180 ^{o}C, outlet temperature of 80±2 ^{o}C, and an atomising pressure of 2.8 bars. The resultant powder was blown through the cyclone separator and collected in a container. The powder samples were transferred into a ziplock bag and kept in a plastic vacuum box prior to storage at -40 ^{o}C. The storage time was not longer than one month. All samples were subsequently subjected to analyses.

5.3.8 Analyses

5.3.8.1 Yield

The production yield was determined gravimetrically. The mass of the dry powder obtained at the end of the process was measured in comparison with that of total solids in the feed. Yield was expressed as a percentage (Xue *et al*., 2013).

5.3.8.2 Total sugar content

Total sugar content was determined by phenol-sulfuric acid method using glucose as a standard (Dubois *et al*., 1956). To 1 mL of sample solution, 1 mL of 5% (w/v) phenol in water was added, followed by 5 mL of sulfuric acid. The mixture was vortexed and allowed to stand for 10 min at room temperature. Then the solution was cooled in an ice bath for 15 min and the absorbance was read at 490 nm using a spectrophotometer (UV-1800 Spectrophotometer, Shimadzu, Kyoto, Japan).

The standard solution was prepared using glucose solution with different concentrations (0, 0.01, 0.03, 0.05, and 0.07 g/L).

5.3.8.3 Solubility

Samples (2 g) were mixed with distilled water (50 mL). The mixtures were stirred for 15 min at 25 ^{o}C. Thereafter, the mixtures were centrifuged at 3600g for 15 min at 25 ^{o}C. The undissolved debris was collected and dried. The soluble fraction was calculated by subtracting undissolved debris from total solid content of the mixture. The solubility was expressed as the percentage, relative to total solid content.

5.3.8.4 Scanning electron microscopic image

Microstructure of powder was visualised using a scanning electron microscope (SEM) (Quanta400, FEI, Tokyo, Japan) at an accelerating voltage of 15 kV. Prior to visualisation, the samples were mounted on brass stub and sputtered with gold in order to make the sample conductive.

5.3.8.5 Color

The color of samples was analysed using a Hunter lab colorimeter (Color Flex, Hunter Lab Inc., Reston, VA, USA). L^*, a^*, and b^*, indicating lightness/brightness, redness/greenness and yellowness/blueness, respectively, were recorded. The colorimeter was calibrated with a white standard. Total difference in color (ΔE*) was calculated according to the following equation:

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

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

5.3.8.6 Browning index

Browning index of solutions was determined according to the method of Benjakul *et al*. (2005). Samples (60 mg) were mixed with distilled water (1 mL). Appropriate dilution was made using distilled water. The intermediate and advanced

Maillard reaction products were measured using the UV/Vis spectrophotometer at 294 and 420 nm, respectively.

5.3.8.7 Antioxidative activities

Antioxidative activities were assayed as described previously.

5.3.9 Statistical analysis

Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out using a Duncan's multiple range test. For pair comparison, a T-test was used. 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 Antioxidative activities of gelatin hydrolysate from scale of spotted golden goatfish as affected by DHs

5.4.1.1 ABTS radical scavenging activity

ABTS radical scavenging activities of gelatin and gelatin hydrolysates with different DHs are shown in Fig. 16A. The increases in activity were observed in all gelatin hydrolysates with increasing DHs from 10 to 40% ($p<0.05$). Activities were 63.85, 75.46, 104.10 and 126.65 μmol TE/mg sample for hydrolysates with 10, 20, 30 and 40% DH, respectively. In general, the increases in ABTS radical scavenging activity were found in hydrolysate, compared with gelatin. The result suggested that peptides produced in hydrolysates with various DHs might be different in term of amino acid composition, sequence and chain length. Generally, all hydrolysates contained peptides or proteins, which were hydrogen donors and could react with the radicals to convert them to more stable products, thereby terminating the radical chain reaction (Khantaphant and Benjakul, 2008). The results indicated that antioxidative peptides were produced during the hydrolysis. Phanturat *et al*. (2010) reported that gelatin hydrolysates from bigeye snapper skin with DH ranging from 5% to 25% prepared using pyloric caeca extract from bigeye snapper had the increased ABTS scavenging activity with increasing DH. Gelatin hydrolysates from blacktip shark skin prepared using papaya latex enzyme with different DHs (10 to

40% DH) had increased ABTS scavenging activity with increasing DH (Kittiphattanabawon *et al*., 2012). Thus, gelatin hydrolysates, especially at 40% DH, had the ability to scavenge free radicals, thereby preventing lipid oxidation via a chain breaking reaction.

5.4.1.2 DPPH radical scavenging activity

DPPH radical scavenging activities of gelatin and gelatin hydrolysate with different DHs (10, 20, 30 and 40% DH) are shown in Fig. 16B. Gelatin hydrolysates prepared had the increases in DPPH radical scavenging activity when DH increased ($p<0.05$). Gelatin hydrolysates with 40% DH exhibited the highest activity (7.24 μmol TE/mg sample). For gelatin solution without hydrolysis, a much lower DPPH radical scavenging activity (0.494 μmol TE/mg sample) was found, compared with that obtained in the gelatin hydrolysates. During hydrolysis, a wide variety of smaller peptides and free amino acids is generated, depending on enzyme specificity (Khantaphant *et al*., 2008). Changes in size, level and composition of free amino acids and small peptides affect the antioxidative activity (Wu *et al*., 2003). Therefore, gelatin hydrolysate obtained could donate hydrogen atom to free radicals and become more stable diamagnetic molecule, leading to the termination of the radical chain reaction (Binsan *et al*., 2008). Nevertheless, the efficiency in hydrogen donation of peptides produced was governed by DHs.

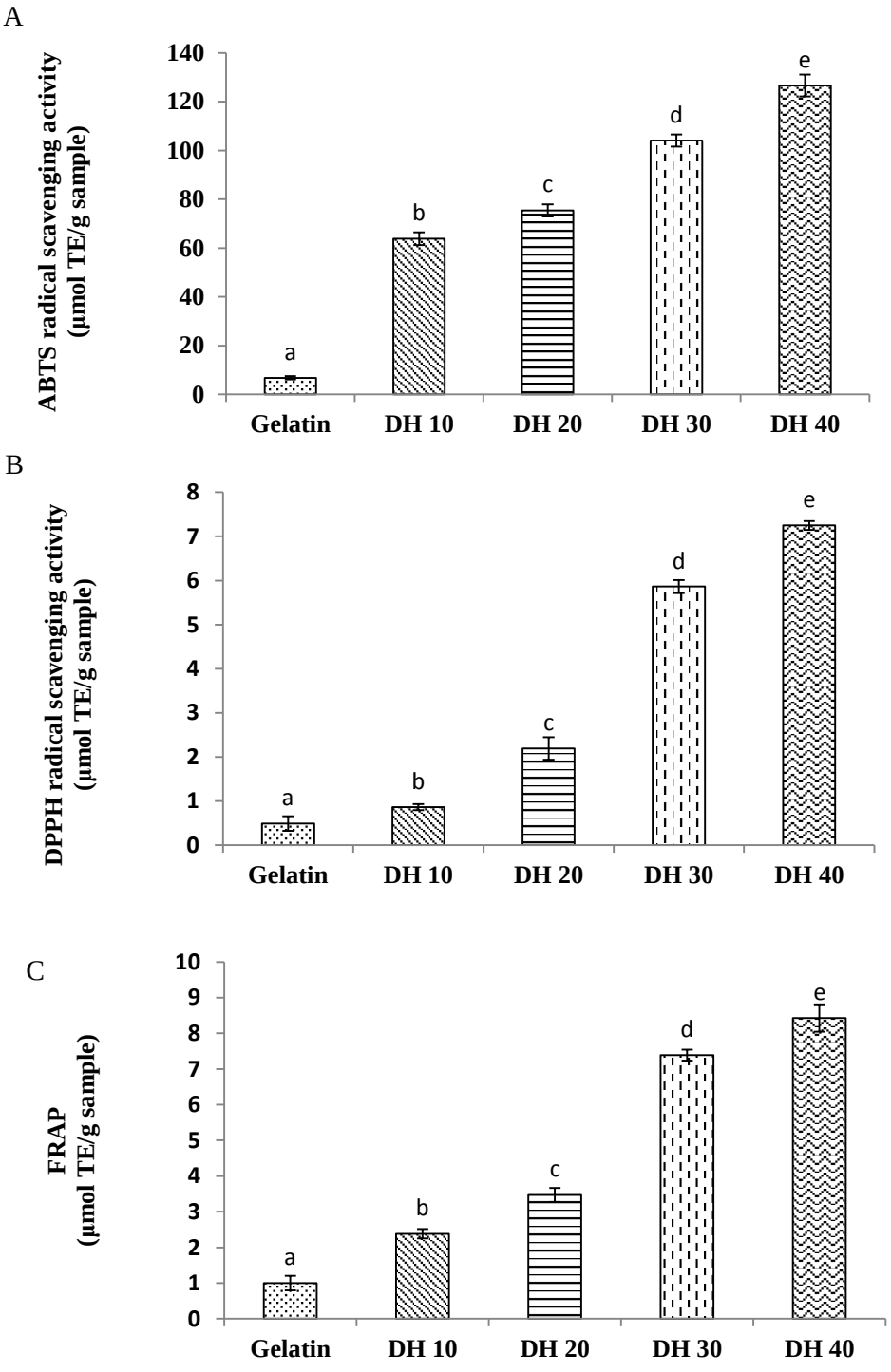


Figure 16 ABTS radical scavenging activity (A), DPPH radical scavenging activity (B) and ferric reducing antioxidant power (FRAP) (C) of gelatin and gelatin hydrolysates with different DHs. Bars represent standard deviation (n=3). Different letters on the bars denote significant differences ($P<0.05$).

5.4.1.3 Ferric reducing antioxidant power (FRAP)

Ferric reducing antioxidant power (FRAP) of gelatin and gelatin hydrolysates with different DHs are shown in Fig. 16C. FRAP of gelatin and gelatin hydrolysates with %DH of 10, 20, 30 and 40 was 0.85, 2.38, 3.47, 7.39 and 8.42 μmol TE/mg sample. The result was in agreement with DPPH and ABTS radical scavenging activities, the activities increased as DH increased. FRAP is generally used to measure the capacity of a substance in reducing TPTZ–Fe(III) complex to TPTZ–Fe(II) complex (Benzie *et al*., 1996; Binsan *et al*., 2008). The result suggests that gelatin hydrolysate with 40% DH possibly contained higher amounts of peptides, which were able to donate electrons to free radicals, thereby terminating the chain reaction. The greater reducing power indicated that hydrolysates could donate the electron to the free radical, leading to the prevention or retardation of propagation (Klompong *et al*., 2008). The DH determines the peptide chain length as well as the exposure of terminal amino groups of products obtained (Thiansilakul *et al*., 2007). Changes in size, level and composition of free amino acids of peptides also affected the antioxidative activity (Wu *et al*., 2003). Therefore, gelatin hydrolysate especially that with 40% DH, could provide the electron to radicals via reduction process, thereby impeding the oxidation.

5.4.2 Effect of maltodextrin on spray-dried powder of gelatin and gelatin hydrolysate

5.4.2.1 Yields

The yields of gelatin and gelatin hydrolysate powder containing maltodextrin at various ratios (1:0, 2:1, 1:1, and 1:2 (w/w)) are presented in Table 11. Without maltodextrin, the yields of 19.32% and 15.53%, respectively, were obtained for gelatin and gelatin hydrolysate powder. Gelatin hydrolysate with a shorter chain might be lost with ease during spray drying. When maltodextrin proportions increased, the yields of both gelatin and gelatin hydrolysate powders increased ($p<0.05$). Gelatin and gelatin hydrolysate powder containing maltodextrin at ratio 1:2 (w/w) had the highest yields (35.64% and 32.43%), compared to other ratios ($p<0.05$). Overall, gelatin hydrolysate powder had the lower yield, in comparison with gelatin powders, regardless of maltodextrin proportion used.

Table 11 Solubility and colour of gelatin and gelatin hydrolysate powder from spotted golden goatfish scales with different sample/maltodextrin ratios.

Samples	Gelatin : Maltodextrin (w/w)				Gelatin hydrolysate : Maltodextrin (w/w)			
	1:0	2:1	1:1	1:2	1:0	2:1	1:1	1:2
*L**	94.83 ± 0.43Db	95.73 ± 0.08Eb	96.75 ± 0.35Fb	96.6 ± 0.44Fb	87.29 ± 0.66Aa	91.39 ± 0.56Ba	92.54 ± 0.24Ca	93.02 ± 0.87Ca
*a**	-0.26 ± 0.06Gb	-0.45 ± 1.05Bb	-0.42 ± 0.86Ca	-0.41 ± 0.05Ca	-0.14 ± 0.97Fa	-0.77 ± 0.39Aa	-0.35 ± 0.57Db	-0.33 ± 0.12Eb
*b**	5.9 ± 0.83Da	2.50 ± 0.46Aa	2.21 ± 0.52Aa	1.92 ± 0.81Aa	14.53 ± 1.02Fb	11.80 ± 0.29Eb	5.52 ± 0.91Bb	5.69 ± 0.11Cb
Δ*E**	5.77 ± 0.51Ca	3.80 ± 0.15Aa	4.29 ± 0.66Ba	4.14 ± 0.53Ba	9.24 ± 0.24Db	9.42 ± 1.23Db	5.19 ± 0.41Cb	5.39 ± 0.63Cb
Yield (%)	19.32± 0.83Bb	23.45± 0.32Db	27.44± 0.74Fb	35.64± 0.21Hb	15.53± 0.56Aa	18.54± 0.61Ca	24.64± 0.87Ea	32.43± 0.95Ga
Solubility (%)*	11.70 ± 0.81Aa	15.35 ± 0.83Ba	17.54 ± 0.45Ca	26.88 ± 0.76Da	99.87 ± 0.64Fb	99.12 ± 0.96Fb	97.73 ± 0.69Eb	97.43 ± 0.52Eb

*Solubility was tested at 25 ^{o}C

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

Different lowercase letters within the same row under the same sample/maltodextrin ratio indicate significant difference ($p<0.05$).

Generally, gelatin and gelatin hydrolysate powders are hygroscopic products. However, gelatin hydrolysate was more hygroscopic due to the higher content of charged N- or C-termini. During drying at high temperatures, the greater evaporation of water proceeded, thus reducing the moisture of the powders and conversely increasing the capture of water molecules by the samples. When a dried powder was exposed to the environment, it rapidly assimilated the moisture. As a result, the product might be attached inside the drying chamber (Suhimi and Mohammad, 2011). In the presence of maltodextrin, maltodextrin particles are apparently bigger than the particles constituting the soluble gelatin and gelatin hydrolysate. Thus, maltodextrin addition directly increased the bulk densities, thereby lowering the loss of soluble solids. Caliskan and Nur Dirim (2013) found that the addition of maltodextrin increased the product yield by preventing the adhesion of sumac extract on the chamber walls. Therefore, maltodextrin had the influence on yields of gelatin and gelatin hydrolysate powders.

5.4.2.2 Total sugar contents

Total sugar contents in gelatin and gelatin hydrolysate powders containing maltodextrin at different ratios are shown in Fig 17. Both gelatin and gelatin hydrolysate powders had the increases in total sugar content with increasing maltodextrin proportions. Gelatin hydrolysate mixed with maltodextrin at a ratio 1:2 (w/w) showed the highest total sugar content, compared to others ($p<0.05$). Gelatin and gelatin hydrolysate without maltodextrin had total sugar content of 38.3 and 37.66 mg/g, respectively. This might be due to the presence of sugar in collagen. It was reported that collagen contained the carbohydrate. Glucose and galactose are attached to hydroxylysine residues of the peptide chain by O-glycosidic bonds to form 2-O-α-D-glucosyl-O-β-D-galactosyl-hydroxylysine and O-β-D-galactosyl-hydroxylysine (Belitz *et al*. 2009). In the absence of maltodextrin, the lowest total sugar content was noticeable ($p<0.05$). Maltodextrin consists of D-glucose units connected in chains of various lengths. It is typically composed of a mixture of chains that vary from 3 to 17 glucose units. The glucose units are primarily linked with α (1→4) glycosidic bonds (Barcelos *et al*., 2014). In the present study, the presence of maltodextrin was determined in term of total sugar using phenol–sulfuric acid method. The method

depends on dehydration of hydrolysed saccharides to furfural derivatives during reaction with concentrated sulfuric acid (DuBois *et al*., 1956; Albalasmeh *et al*., 2013). Further reaction of the furfural derivatives with phenol forms colored complexes that absorb light in the visible range, with a maximum absorbance at a wavelength of 490 nm (Albalasmeh *et al*., 2013). However, there was no difference in total sugar content between gelatin and gelatin hydrolysate powders when the same sample/maltodextrin ratio was used ($p>0.05$). The result confirmed the presence of maltodextrin in the powders, in which the content varied, depending on the proportion of maltodextrin added.

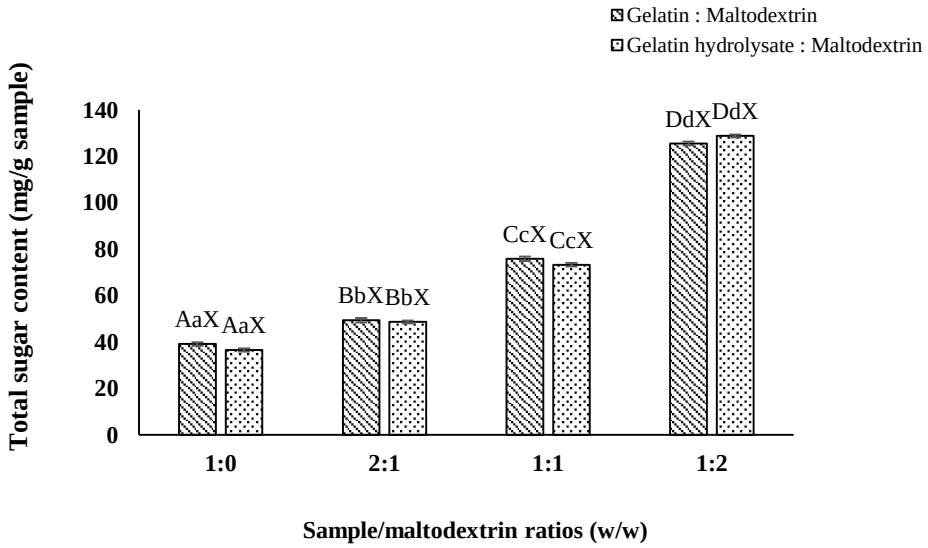


Figure 17 Total sugar content of gelatin and gelatin hydrolysate powders from the scale of spotted golden goatfish using different sample/maltodextrin ratios. Bars represent the standard deviation (n=3). Different uppercase letters (A, B, C and D) on the bars within the same samples indicate significant differences ($P<0.05$). Different lowercase letters on the bars indicate significant differences ($P<0.05$). Different uppercase letters (X and Y) on the bars within the same sample/maltodextrin ratio indicate significant differences ($P<0.05$).

5.4.2.3 Solubility

Solubility of gelatin and gelatin hydrolysate powders containing maltodextrin at various ratios is presented in Table 11. Gelatin powder showed the low solubility at room temperature. The increase in solubility was found when the maltodextrin proportion increased ($p<0.05$). Maltodextrin is more likely hydrophilic in nature, thereby enhancing the water solubility of powder. In general, gelatin is able to completely solubilized when the sufficient heat is applied. Heat could destroy the weak bonds stabilising the gelatin aggregate, particularly hydrogen bond. As a result, the gelatin could be dissolved readily. When it was dissolved at room temperature, a large proportion of gelatin was still undissolved as indicated by low solubility. High solubility of all gelatin hydrolysate powder was observed, regardless of gelatin hydrolysate/maltodextrin ratio. Hydrolysate generally has an excellent solubility, especially with high degree of hydrolysis (Klompong *et al*., 2007). High solubility of hydrolysates was due to the generation of low molecular weight peptides, which had more polar residues than the parent proteins. The enhanced ability to form hydrogen bonds with water increased the solubility of hydrolysate (Giménez *et al*., 2009). Enzymatic hydrolysis potentially affects the molecular size and hydrophobicity, as well as the polar and ionisable groups of protein hydrolysates (Kristinsson and Rasco, 2000). It was noted that maltodextrin had no marked impact on the solubility of resulting gelatin hydrolysate powders, suggesting high solubility of both gelatin hydrolysate and maltodextrin.

5.4.2.4 Color

The color of gelatin and gelatin hydrolysate powder from spotted golden goatfish scales using maltodextrin as a carrier agent at different ratios expressed as L^*, a^* and b^* is shown in Table 11. At all ratios of sample/maltodextrin used, gelatin powder exhibited higher L^*-value (lightness) than gelatin hydrolysate counterpart. Higher L^*-values were found when maltodextrin was incorporated. No difference in L^*-value was observed between powders with sample/maltodextrin ratios of 1:1 and 1:2 ($p>0.05$) for both gelatin and gelatin hydrolysate. a^* and b^*-values and ΔE^* of gelatin hydrolysate powder were higher than those of gelatin counterpart at all ratios of sample/maltodextrin. Those values decreased with

increasing maltodextrin proportion used. Carbonyl groups in maltodextrin might undergo Maillard products with free amino groups of gelatin hydrolysate to a higher content, compared to gelatin. The Maillard reaction involved in the formation of brown pigments comprises the condensation between a carbonyl group of reducing sugars, aldehydes or ketones and an amine group of free amino acids (such as amino acids, peptides and proteins) or any nitrogenous compound (Kim and Lee, 2009). As a consequence, gelatin powder more likely had higher whiteness with less yellowness, compared to gelatin hydrolysate powder. Therefore, maltodextrin had the influence on color of gelatin and gelatin hydrolysate powders.

5.4.2.5 UV-absorbance and browning intensity

The highest browning of gelatin and gelatin hydrolysate powders from spotted golden goatfish scales was obtained in the absence of maltodextrin as indicated by the highest A_{294} and A_{420} (Fig 18). At all sample/maltodextrin ratios, the gelatin hydrolysate powders showed higher browning intensity than gelatin powder. Free amino groups at a larger amount of gelatin hydrolysate were able to undergo glycation with indigenous carbonyl compound to a higher extent. Gelatin hydrolysate had higher content of free amino group, which readily underwent glycation. As a consequence, browning occurred to a higher extent, compared with gelatin. A_{294} has been used to indicate the formation of an uncoloured compound, which could be the precursor of the Maillard reaction (Ajandouz *et al*., 2001; Lerici *et al*., 1990). Lerici *et al*. (1990) found that heat treatment of a glucose–glycine mixture caused the marked increase in absorbance at 294 nm. Additionally, the increase in A_{420} was used as an indicator for browning development in the final stage of the browning reaction (Ajandouz *et al*., 2001; Benjakul *et al*., 2005). Gelatin and gelatin hydrolysate powders had the lower browning intensity as the maltodextrin at higher ratio was incorporated. This was coincidental with the higher whiteness (L^*-value) and lower a^* and b^*-values of powder when maltodextrin was used as a carrier agent (Table 11). Therefore, the use of maltodextrin could improve the whiteness of gelatin and gelatin hydrolysate powder in a dose dependent manner.

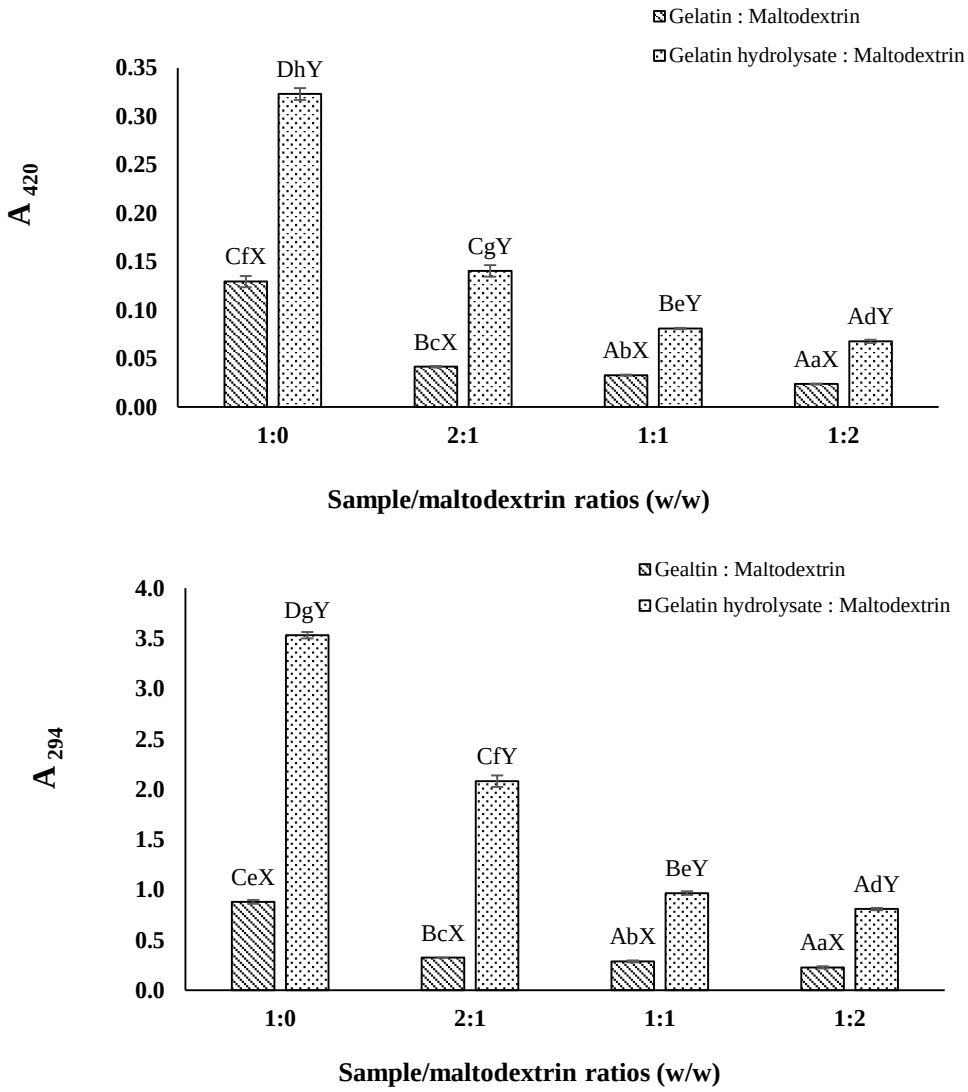
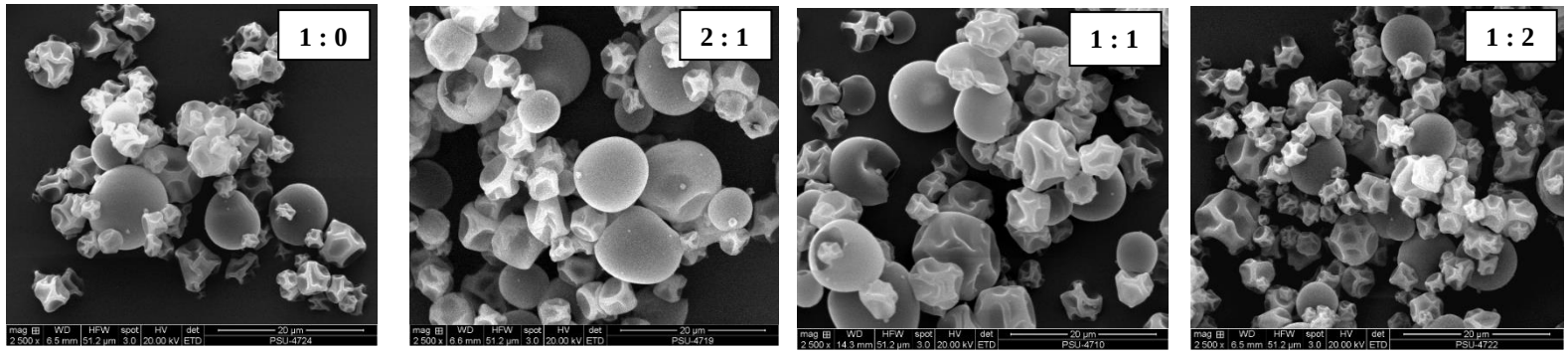


Figure 18 Absorbance at 420 and 294 nm of gelatin and gelatin hydrolysate powders from the scale of spotted golden goatfish using different sample/maltodextrin ratios. Bars represent the standard deviation (n=3). Different uppercase letters (A, B, C) on the bars within the same samples indicate significant differences ($P<0.05$). Different lowercase letters on the bars indicate significant differences ($P<0.05$). Different uppercase letters (X and Y) on the bars within the same sample/maltodextrin ratio indicate significant differences ($P<0.05$).

5.4.2.6 Scanning electron microscopic image

Microstructures of gelatin and gelatin hydeolysate mixed with maltodextrin at different sample/maltodextrin ratios are shown in Fig. 19. Gelatin powders were spherical in shape with varying sizes were noticeable. In general, the smaller particles had the shrinkage on the surface. There was no marked difference in morphology between gelatin powders with different sample/maltodextrin ratios. When the drying temperature is sufficiently high, moisture is evaporated very quickly and the surface becomes dry and hard. Hollow cannot deflate when vapor condenses within the vacuole as the particles moves into cooler regions of the dryer (Suhimi *et al*., 2011). Wrinkled surface is developed when rapid crust formation took place for droplets during the early stage of drying. All gelatin hydrolysate samples had smaller particles, compared with all gelatin counterpart, regardless of sample/maltodextrin ratio. Short chain peptides might not agglomerate into the large particles as indicated by small particles. In the presence of maltodextrin, the larger agglomerate was formed. In the presence of maltodextrin with longer chain length, higher ability to agglomerate along with gelatin hydrolysate during the drying process was obtained. Maltodextrin can significantly increase the glass transition temperature and reduce the hygroscopicity of dried products (Goula and Adamopoulos, 2008). However, there was no difference in morphology of all gelatin hydrolysate powders when different sample/maltodextrin ratios were used.

Gelatin : Maltodextrin



Gelatin hydrolysate : Maltodextrin

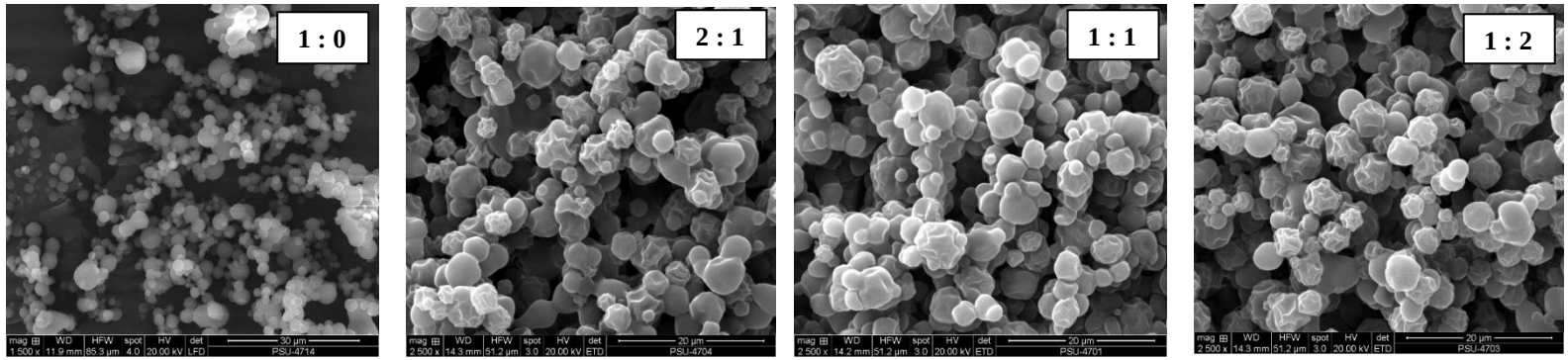


Figure 19 Microstructures of gelatin and gelatin hydrolysate powders from the scale of spotted golden goatfish using different sample/maltodextrin ratios. Magnification: 2500x

5.4.2.7 Antioxidant activities

Antioxidant activities of gelatin and gelatin hydrolysate powders using maltodextrin as a carrier agent at different levels are shown in Fig 20. The higher ABTS radical scavenging activity, DPPH radical scavenging activity and ferric reducing antioxidant power (FRAP) were found in gelatin hydrolysate powder, in comparison with those of gelatin powder when sample/maltodextrin ratio was used ($p<0.05$). Gelatin hydrolysates are breakdown products of enzymatic conversion of gelatin into smaller peptides, which are composed of free amino acids and short chain peptides exhibiting biological activity. DPPH and ABTS radical scavenging activities have been used to indicate the ability of antioxidants to donate a hydrogen atom or an electron to stabilise radicals, by converting it to the non-radical species (Kittiphattanabawon *et al*., 2012). Moreover, FRAP is generally used to measure the capacity of a substance in reducing TPTZ–Fe(III) complex to TPTZ–Fe(II) complex. The result suggested that gelatin hydrolysate contained higher amounts of peptides than gelatin. Those peptides were able to donate electrons to free radicals, thereby terminating the chain reaction (Kittiphattanabawon *et al*., 2012). However, the conformational changes of peptides in gelatin and gelatin hydrolysate might occur to some extent during the spray-drying process at high temperature. The changes in composition of free amino acids and/or peptides could affect the antioxidant activity (Thiansilakul *et al*., 2007; Sai-Ut *et al*., 2014). When maltodextrin was incorporated, the resulting powder of gelatin and gelatin hydrolysates showed the lower antioxidative activity. This suggested the dilution effect of maltodextrin present in gelatin and gelatin hydrolysate powders. Although maltodextrin yielded the powder with whiter colour, its incorporation directly lowed antioxidative activity of resulting powders.

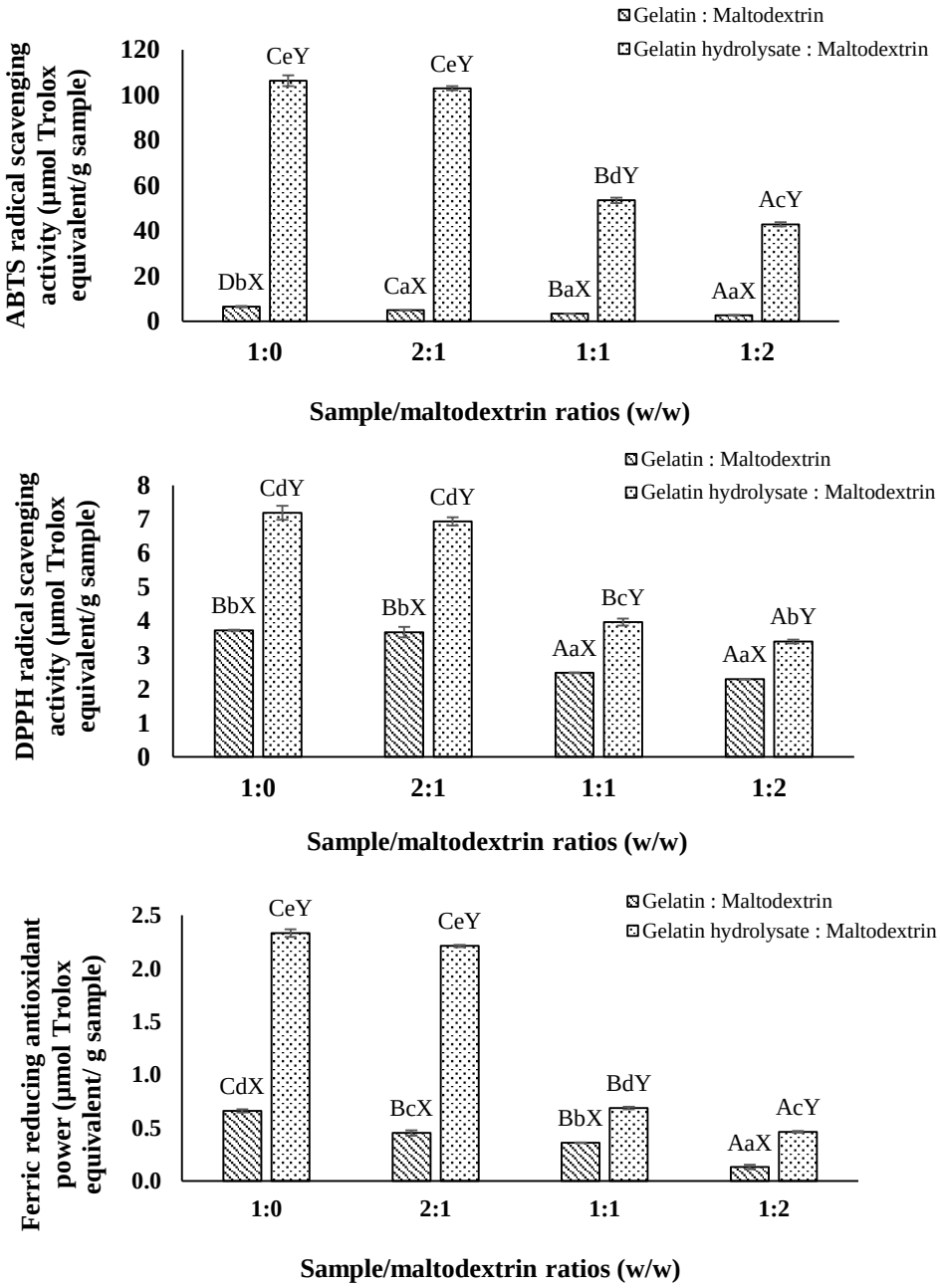


Figure 20 Antioxidant activity of gelatin and gelatin hydrolysate powders from the scale of spotted golden goatfish using different sample/maltodextrin ratios. Bars represent the standard deviation (n=3). Different uppercase letters (A, B, C and D) on the bars within the same samples indicate significant differences ($P<0.05$). Different lowercase letters on the bars indicate significant differences ($P<0.05$). Different uppercase letters (X and Y) on the bars within the same sample/maltodextrin ratio indicate significant differences ($P<0.05$).

5.5 Conclusion

Maltodextrin used as a carrier agent for gelatin and gelatin hydrolysate was able to increase the yield and improve the whiteness of gelatin and gelatin hydrolysate powders. All gelatin hydrolysate powder containing maltodextrin had higher solubility and antioxidant activities, compared with gelatin counterpart. However, maltodextrin exhibited the dilution effect on bioactivity of gelatin hydrolysate. Thus, gelatin hydrolysate powder using maltodextrin as a carrier agent at a ratio 2:1 (w/w) would be effective process to produce antioxidative activity of gelatin hydrolysate with high yield.

5.6 References

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CHAPTER 6

EFFECT OF DRYING CONDITION ON PROPERTIES, BIOACTIVITY AND SENSORY PROPERTIES OF GELATIN HYDROLYSATE POWDER FROM SPOTTED GOLDEN GOATFISH

6.1 Abstract

Effects of different spray drying temperatures (inlet temperatures of 160,180 and 200 ^{o}C) on characteristics, bioactivity and sensory properties of gelatin hydrolysate from spotted golden goatfish scale using maltodextrin as a carrier agent were investigated. The yield of gelatin hydrolysate powder was increased, when inlet temperature increased ($p<0.05$). The resulting powders had the increases in browning intensity with increasing inlet temperatures ($p<0.05$). With lower inlet temperatures, the powder showed shriveled surface, whereas higher inlet temperature yielded the spherical powder with some shrinkage as analyzed by scanning electron microscope. ABTS and DPPH radical scavenging activities and ferric-reducing antioxidant power of gelatin hydrolysate decreased as inlet temperatures increased ($p<0.05$). Fortification of gelatin hydrolysate powder (1-5% (w/v)) into apple juice could increase ABTS, DPPH radical scavenging activities and ferric reducing antioxidant power in a dose-dependent manner ($P<0.05$).

6.2 Introduction

Gelatin hydrolysates or hydrolyzed collagens have attracted increasing interest as potential ingredients for various health-promoting functional foods (Gómez-Guillén *et al*., 2011). Bioactive peptides with antioxidant properties derived from various proteins by enzymatic hydrolysis have gained attention for pharmaceutical, health food and processing/preservation industries. Antioxidant activity has been reported for gelatin hydrolysates prepared from various fish protein sources such as seabass skin (Senphan and Benjakul, 2014), blacktip shark skin (Kittiphattanabawon *et al*., 2012), tuna backbone (Je *et al*., 2007) and Atlantic cod (*Gadus morhua*) and Atlantic salmon (*Salmo salar*) frames (Liaset *et al*., 2000). In general, protein hydrolysates have an excellent solubility at high degree of hydrolysis, which is a substantially useful characteristic for many food applications and

influences other functional properties such as emulsifying and foaming properties (Gbogouri *et al*., 2004). Furthermore, the controlled enzymatic hydrolysis of proteins may produce a series of small peptides, which can modify and even improve the functional properties of proteins (Giménez *et al*., 2009).

Nevertheless, the drying process might affect the characteristics and bioactivity of hydrolysate. Rodríguez-Díaz *et al*. (2014) reported that drying at temperature higher than 180 ^{o}C lowered the FRAP values of hydrolysate from blue shark skin. Additionally, spray drying also increased browning of gelatin hydrolysate from blue shark skin, especially when inlet temperatures were increased (Rodríguez-Díaz *et al*., 2014). Recently, foods and beverages have been recognized as the key role in disease prevention and treatment. Thus, the production and consumption of functional foods or drinks has become the essential issue, due to the health benefit beyond the basic nutritional functions (Corbo *et al*., 2014). Fruit juices are of the choices due to their health benefits and the ease of consumption. The fortification of active ingredients having functional properties, especially from gelatin hydrolysate, is of interest for the development of functional beverages. The aims of this study were to investigate the effect of drying conditions on characteristics, antioxidative activity and sensory properties of gelatin hydrolysate and to characterize the apple juice fortified with gelatin hydrolysate from spotted golden goatfish scale.

6.3 Materials and methods

6.3.1 Chemicals/enzyme

All chemicals were of analytical grade. 2,20 azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,4,6-tripyridyltriazine (TPTZ) were purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada). Maltodextrin was obtained from Zhucheng Dongxiao Biotechnology CO., Ltd. (Shandong, China). Alcalase (EC 3.4.21.14, 2.4 L, 2.64 AU/g) was purchased from Sigma-Aldrich, Inc., (St. Louis, Mo, USA).

6.3.2 Collection and preparation of spotted golden goatfish scales

Scales of spotted golden goatfish with an average body weight of 100-120 g/fish were collected from Kingfisher Holding, Ltd., Songkhla Province,

Thailand. Scales were placed in polyethylene bag and embedded in ice using a scale/ice ratio of 1:2 (w/w). The sample was transported in polystyrene box to the Department of Food Technology, Prince of Songkla University. Hat Yai, within 1 h.

6.3.3 Pretreatment of spotted golden goatfish scales

Scales were washed using a tap water and drained for 5 min. The scales were then suspended in 0.1 M NaOH for 6 h at the ratio of 1:10 (w/v). The mixture was continuously stirred using an overhead stirrer model W20.n (IKA®-Werke, GmbH & CO.KG, Stanfen, Germany) to remove non-collagenous proteins. The solution was changed every 3 h. Treated scales were washed with tap water until wash water became neutral. Subsequently, the prepared scales were demineralized using 0.75 M HCl with a scale/solution ratio of 1:5 (w/v). The demineralization was performed at room temperature (28-30 ^{o}C) with the continuous stirring for 30 min. Thereafter, the demineralized scales were washed until the neutral pH of wash water was obtained.

6.3.4 Extraction of gelatin

Demineralized scales were mixed with distilled water at a ratio of 1:10 (w/v). The extraction was conducted at 75 ^{o}C for 6 h in a temperature controlled-water bath (Model W350, Memmert, Schwabach, Germany). The mixture was stirred continuously. The mixture was then filtered using a Buchner funnel, with a Whatman No.4 filter paper (Whatman International, Ltd., Maidstone, England). The filtrate obtained was freeze-dried using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). Dried gelatin was placed in a polyethylene bag and kept at -20 ^{o}C until used.

6.3.5 Preparation of gelatin hydrolysate

Gelatin hydrolysate from the scale of spotted golden goatfish was prepared as per the method of Kittiphattanabawon *et al.* (2012) with a slight modification. Gelatin (3 g) was dissolved in 80 mL of distilled water. The pH of mixture was adjusted to 8 with 1 M NaOH. The volume of solution was made up to 100 mL by distilled water previously adjusted to pH 8 to obtain a protein concentration of 3% (w/v). The hydrolysis reaction was started by the addition of

Alcalase. The amount of Alcalase was calculated from the plot between log (enzyme concentration) and DH to obtain DH of 40% as described by Benjakul and Morrissey (1997). After 1 h of hydrolysis at 50 ^{o}C, the enzyme was inactivated by heating at 90 ^{o}C for 15 min in a temperature-controlled water bath. The mixture was then centrifuged at 5000g using a centrifuge (Avanti® J-E, Beckman Coulter, Palo Alto, CA, USA) at room temperature for 10 min. The supernatant was freeze-dried using a freeze-dryer. The obtained powder referred to as ‘‘gelatin hydrolysate’’ was placed in polyethylene bag and stored at -20 ^{o}C.

6.3.6 Impact of different inlet temperatures on properties of gelatin hydrolysate powder

Gelatin hydrolysate was mixed with maltodextrin at a ratio of 2:1 (w/w). The mixtures were dissolved in distilled water to obtain a final concentration of 2% (w/v). The solution was subjected to spray drying using a spray dryer (SD-06 Basic, North Yorkshire, England) equipped with a spry-drying chamber having the dimensions of 500 mm height and 210 mm diameter. A spray nozzle type of two-liquid nozzle (0.5 mm in size) was used. A cyclone separator, a hot-air blower, and an exhaust blower, were equipped. The solution was fed by a peristaltic pump at 485 mL h^{-1} into the chamber, atomized by the hot air (air velocity of 2 ms^{-1}) from the blower in a downward current flow mode, using various process conditions: inlet temperature of 160, 180 and 200 ^{o}C, outlet temperature of 80±2 ^{o}C, and an atomizing pressure of 2.8 bars. The resultant powder was blown through the cyclone separator and collected in a container. The powder samples were transferred into a ziplock bag and kept in a plastic vacuum box prior to storage at -40 ^{o}C. The storage time was not longer than one month. All samples were subsequently subjected to following analyses.

6.3.7 Analyses

6.3.7.1 Yield

The yield of gelatin hydrolysate powder was determined gravimetrically according to the mass of total solids measured in the feed and the mass of the dry powder obtained at the end of the process. Yield was expressed as a percentage (Xue *et al*., 2013).

6.3.7.2 Solubility

Gelatin hydrolysate powder (2 g) was mixed with distillation water (50 mL). The mixture was stirred for 15 min at 25 ^{o}C. Thereafter, the mixture was centrifuged at 3600g for 15 min at 25 ^{o}C. The undissolved debris was collected and dried. The soluble fraction was calculated by subtracting undissolved debris from total solid content of the mixture. The solubility was expressed as the percentage, relative to total solid content.

6.3.7.3 Scanning electron micrograph

Microstructure of powder was visualized using a scanning electron microscope (SEM) (Quanta400, FEI, Tokyo, Japan) at an accelerating voltage of 15 kV. Prior to visualization, the samples were mounted on brass stub and sputtered with gold in order to make the sample conductive.

6.3.7.4 Color

The color of powder was analyzed using a Hunter lab colorimeter (Color Flex, Hunter Lab Inc., Reston, VA, USA). L^*, a^*, and b^*, indicating lightness/brightness, redness/greenness and yellowness/blueness, respectively, were recorded. The colorimeter was calibrated with a white standard. Total difference in color (ΔE^*) was calculated according to the following equation:

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

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

6.3.7.5 Browning index

Browning index of solutions was determined according to the method of Benjakul *et al*. (2005). Samples (60 mg) were mixed with distilled water (1 mL). Appropriate dilution was made using distilled water. The extents of intermediate and advanced Maillard reactions were measured using the UV/Vis spectrophotometer at 294 and 420 nm, respectively.

6.3.7.6 Antioxidant activities

6.3.7.6.1 ABTS radical scavenging activity

ABTS radical scavenging activity was assayed according to the method of Intarasirisawat *et al*. (2012). The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution. ABTS radical cation was generated by reacting 7.4 mM ABTS with 2.6 mM potassium persulphate at a ratio of 1:1 (v/v). The mixture was stored in the dark for 12 h at room temperature. Prior to assay, ABTS radical cation was diluted with methanol to obtain an absorbance of 1.1 (±0.02) at 734 nm. Sample solution (150 µL) was mixed with 2850 µL of ABTS radical cation and the mixture was left at room temperature for 2 h in the dark. The absorbance was then read at 734 nm. A standard curve of Trolox ranging from 50 to 600 µM was prepared. The activity was expressed as µmol Trolox equivalents (TE)/g sample.

6.3.7.6.2 DPPH radical scavenging activity

DPPH radical scavenging activity was determined according to the method described by Intarasirisawat *et al.* (2012). Sample was dissolved in distilled water and then 1 mL of sample solution was mixed with 1 mL of 0.1 mM DPPH solution in 95% (v/v) ethanol. The mixture was vortexed for 10 s and allowed to stand at room temperature for 30 min in the dark. The absorbance of the mixture was read at 517 nm. The blank was prepared in the same manner, except that distilled water was used instead of the sample. A standard curve was prepared using trolox in the range of 10–60 µM. The activity was expressed as µmol trolox equivalents (TE)/g sample.

6.2.7.6.3 Ferric reducing antioxidant power (FRAP)

FRAP was assayed following the method of Benzie and Strain (1996). Stock solutions included 30 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. A working solution was prepared freshly by mixing 25 mL of acetate buffer, 2.5 mL of TPTZ solution and 2.5 mL of $FeCl_3 \cdot 6H_2O$ solution. The mixed solution was incubated at 37 ^{o}C for 30 min in a temperature-controlled water bath and was referred to as FRAP solution. A sample solution (150 µL) was mixed with 2850 µL of FRAP

solution and kept for 30 min in dark at room temperature. The ferrous tripyridyltriazine complex (colored product) was measured by reading the absorbance at 593 nm. Sample blank was prepared by omitting $FeCl_3$ from FRAP solution and distilled water was used instead. The standard curve was prepared using Trolox ranging from 50 to 600 µM. The activity was calculated after sample blank subtraction and was expressed as µmol Trolox equivalents (TE)/g sample.

6.2.7.7 Volatile Compounds

Powder prepared using the condition yielding the highest solubility and antioxidant activity with low energy consumed were analyzed for volatile compounds in comparison with freeze-dried gelatin hydrolysate.

Volatile compounds in the samples were determined using a solid-phase micro-extraction gas chromatography mass spectrometry (SPME-GC-MS), following the method of Iglesias and Medina (2008) with a slight modification. One g of sample was mixed with 4 mL of deionized water and stirred continuously to disperse the sample. The mixture was heated at 60 ^oC in 20 mL headspace vial with equilibration time of 10 h. The SPME fiber (50/30 mm DVB/Carboxen^{TM} / PDMS Stable-Flex^{TM}) (Supelco, Bellefonte, PA, USA) was conditioned at 270 ^oC for 15 min before use and then exposed to the headspace. The 20 mL vial (Agilent Technologies, Palo Alto, CA, USA) containing the sample extract and the volatile compounds was allowed to absorb into the SPME fiber at 60 ^oC for 1 h. The volatile compounds were then desorbed in the GC injector port for 15 min at 270 ^oC.

GC–MS Analysis

GC–MS analysis was performed in a HP 5890 series II gas chromatography (GC), coupled with a HP 5972 mass-selective detector equipped with a splitless injector and coupled with a quadrupole mass detector (Hewlett Packard, Atlanta, GA, USA). Compounds were separated on a HP-Innowax capillary column (Hewlett Packard, Atlanta, GA, USA) (30 m×0.25 mm ID, with film thickness of 0.25 µm). The GC oven temperature programme was: 35^oC for 3 min, followed by an increase to 70 ^oC at 3 ^oC min^{-1}, then an increase of 10 ^oC min^{-1} to 200 ^oC, and finally an increase of 15 ^oC min^{-1} to a final temperature of 250 ^oC and holding for 10 min. Helium was employed as a carrier gas, with a constant flow of 1 mL/min. The injector was operated in the splitless mode and its temperature was set at 270 ^oC. Transfer line

temperature was maintained at 260 ^{o}C. The quadrupole mass spectrometer was operated in the electron ionization (EI) mode and source temperature was set at 250 ^{o}C. Initially, full scan mode data were acquired to determine appropriate masses for the later acquisition in scan mode under the following conditions: mass range of 25–500 amu and scan rate of 0.220 s/scan. All of the analyses were performed with ionization energy of 70 eV, filament emission current at 150 µA, and electron multiplier voltage at 500 V.

Analyses of Volatile Compounds

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

6.3.8 Study on characteristics and antioxidative activity of apple juice fortified with gelatin hydrolysate powder

Apple juice (Tipco F&B Co., Ltd., Ayutthaya, Thailand) was purchased from a local supermarket, Hat Yai, Thailand. Gelatin hydrolysate powder was added into apple juice to obtain different final levels (0, 1, 3 and 5%, (w/v)) and mixed well. The resulting apple juices were subjected to following analyses.

6.3.8.1 Color and browning intensity

The color of the apple juice sample was determined as previously described. After appropriate dilution, browning intensity of samples was measured at 420 nm using a spectrophotometer as mentioned above.

6.3.8.2 Turbidity

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

6.3.8.3 Antioxidative activities

All samples were determined for ABTS, DPPH radical scavenging activities and FRAP as mentioned above.

6.3.8.4 Sensory property

Sensory evaluation was performed by 30 panelists with ages ranging from 20 to 35 years, who were familiar with the consumption of apple juice. Samples were placed in a cup. Panelists were asked to evaluate for appearance, color, odor, flavor, taste and overall likeness using a nine-point hedonic scale, in which 1 = not like very much, 5 = neither like nor dislike and 9 = like extremely (Meilgaard *et al*., 2007).

6.3.9 Statistical analysis

The experiment was run in triplicate using different three lots of samples. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out using a Duncan's multiple range test. For pair comparison, a T-test was used. 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 Yields and characteristics of gelatin hydrolysate powder as affected by different inlet temperatures

6.4.1.1 Yields

The yields of gelatin hydrolysate powders containing maltodextrin prepared using various inlet temperatures (160, 180 and 200 ^{o}C) are presented in Table 12. Gelatin hydrolysate powder using inlet temperature of 200 ^{o}C exhibited the highest yield, compared to those dried at other inlet temperatures ($p<0.05$). The lowest yield was observed from gelatin hydrolysate powder with inlet temperature of 160 ^{o}C ($p<0.05$). The result suggested that higher drying temperature used for spray drying resulted in the faster drying rate and higher powder productivity. Increased drying rates caused the marked decrease of powder moisture (Cai and Corke, 2000). Additionally, the higher inlet air temperature led to a greater temperature gradient between the atomized feed and the drying air, resulting in a greater driving force for water evaporation. When the low inlet temperature was used, the atomized gelatin hydrolysate might not be dried completely and got stick on drying chamber. In addition, increasing temperatures showed the greater efficiency of heat and mass

transfer occurring in the drying system. The result was in accordance with Tonon, Brabet and Hubinger (2008) who reported that higher yield of acai (*Euterpe oleraceae Mart*.) powder was obtained at higher drying temperature. Therefore, inlet temperature directly had the influence on yields of gelatin hydrolysate powders.

Table 12 Yield and characterictics of gelatin hydrolysate powder from spotted golden goatfish scales prepared with different inlet temperatures

Parameters	Inlet temperatures (°C)		
	160	180	200
Yield (%)	15.73 ± 1.04a	19.56 ± 1.22b	20.03 ± 0.75c
Solubility (%)	99.73 ± 0.23a	99.87 ± 0.36a	99.89 ± 0.88a
*L**	94.02 ± 0.58c	91.59 ± 0.43b	86.69 ± 1.03a
*a**	-0.23 ± 0.73a	-0.19 ± 0.89b	-0.15 ± 0.96c
*b**	4.89 ± 1.31a	11.80 ± 0.37b	16.13 ± 0.67c
ΔE*	5.47 ± 0.79a	9.42 ± 0.14b	11.24 ± 0.72c
A_{294}	0.80 ± 0.21a	2.07 ± 0.06b	2.79 ± 0.07c
A_{420}	0.11 ± 0.21a	0.14 ± 0.53b	0.21 ± 0.09c

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

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

6.4.1.2 Solubility

Solubility of gelatin hydrolysate powders obtained from spray drying at various inlet temperatures is presented in Table 12. All gelatin hydrolysate powders showed high solubility (more than 99%) at room temperature. Generally, high solubility of gelatin hydrolysates was due to the generation of low molecular weight peptides by hydrolysis. Those peptides had more polar on charged residues than the parent proteins, with the ability to form hydrogen bonds with water, leading to the increased solubility (Giménez *et al*., 2009). Via spray-drying, the particle density was

decreased due to the steam formation in the drying droplet, causing expansion of the particle. Thus, powders tended to have hollow and porous structures, which were easy for solubilization in water (Rodríguez-Díaz *et al*., 2014). In addition, maltodextrin containing a number of hydrophilic groups, thus adsorbing more water. The phenomenon of water adsorption was attributed to the links between the hydrogen present in water molecules and the hydroxyl groups available in the amorphous regions of maltodextrin (Phisut, 2012). The result was agreement with Santhalakshmy *et al*. (2015) who found that spray-dried jamun fruit juice powder had high solubility, in which maltodextrin as carrier agent might lead to higher solubility of resulting powder.

6.4.1.3 Color

The color of gelatin hydrolysate powder with different inlet temperatures expressed as *L**, *a** and *b** is shown in Table 12. All gelatin hydrolysate powders produced had a white color. Gelatin hydrolysate powder using inlet temperature of 200 ^{o}C showed the lowest *L**-value, while that using inlet temperature of 160 ^{o}C had the highest *L**-value ($p<0.05$). The highest *a** and *b**-values and ΔE^* were found for gelatin hydrolysate prepared with inlet temperature of 200 ^{o}C. It was noted that *a** and *b**-values of gelatin increased with increasing inlet temperatures used for spray drying ($p<0.05$). Non-enzymatic interaction between reducing sugar and amino acid, peptide or protein known as the Maillard reaction was accelerated at higher temperature. Maltodextrin containing carbonyl groups might undergo Maillard products with free amino groups of gelatin hydrolysate, especially at higher inlet temperature. A spray drying process with a high ratio of surface area and volume of feed mixture contact with hot air in drying chamber could favor the browning reaction (Sae-Leaw *et al*., 2016). These results indicated that higher inlet temperature led to the browning color of resulting gelatin hydrolysate powder.

6.4.1.4 UV-absorbance and browning intensity

The browning intensity of gelatin hydrolysate powders prepared at various inlet temperatures as indicated by A_{294} and A_{420} is presented in Table 12. Among all samples, that prepared at 200 ^{o}C showed the highest A_{294} and $A_{420.}$ This was in agreement with the increases in *a**, *b** and ΔE^* when sample was dried at high

temperature. Therefore, spray drying of gelatin hydrolysate containing smaller peptides at higher inlet temperatures could enhance the Maillard reaction. For the intermediate compounds of the Maillard reaction, the absorbance at 294 nm was monitored. Similar trend was found, in which the sample prepared with higher inlet temperature showed the higher A_{294}. During spray drying, some intermediate products might undergo polymerization to form the brown pigments (Lertittikul *et al*., 2007). The higher A_{294} of sample prepared at 200 ^{o}C suggested the high rate of intermediate product formation along with the development of final brown products. Additionally, the increase in A_{420} nm was used as an indicator for browning development in the final stage of the browning reaction (Benjakul *et al*., 2005). Therefore, an amino acid–sugar complex could be formed more easily at high inlet temperature.

6.4.1.5 Scanning electron micrograph

SEM images of gelatin hydrolysate powder prepared by spray drying at different inlet temperatures are shown in Fig. 21. Powders produced at higher temperature (200 ^{o}C) showed spherical particles with some degree of shrinkage. It was noted that when low temperature (160 ^{o}C) was used, the particles had a shriveled surface. The results suggested that the use of higher inlet air temperature led to the formation of larger particles associated with higher swelling. When the temperature is low, the particle remains more shrunk and smaller (Phisut, 2012). When the drying temperature is sufficiently high and the moisture is evaporated very quickly, the skin becomes dry and hard (Phisut, 2012). As a result, the hollow particle cannot deflate when vapor condenses within the vacuole as the particle moves into cooler regions of the dryer (Tonon *et al*., 2008). However, when the drying temperature is lower, the skin remains moist. The hollow particle can subsequently deflate and shrivel as it cools. (Nijdam and Langrish, 2006). This was coincidental with Tonon *et al*. (2008) who found that the powder showed a shriveled surface when the inlet air temperature was low. Thus, inlet temperature used for spray drying had the influence on morphology of gelatin hydrolysate powders.

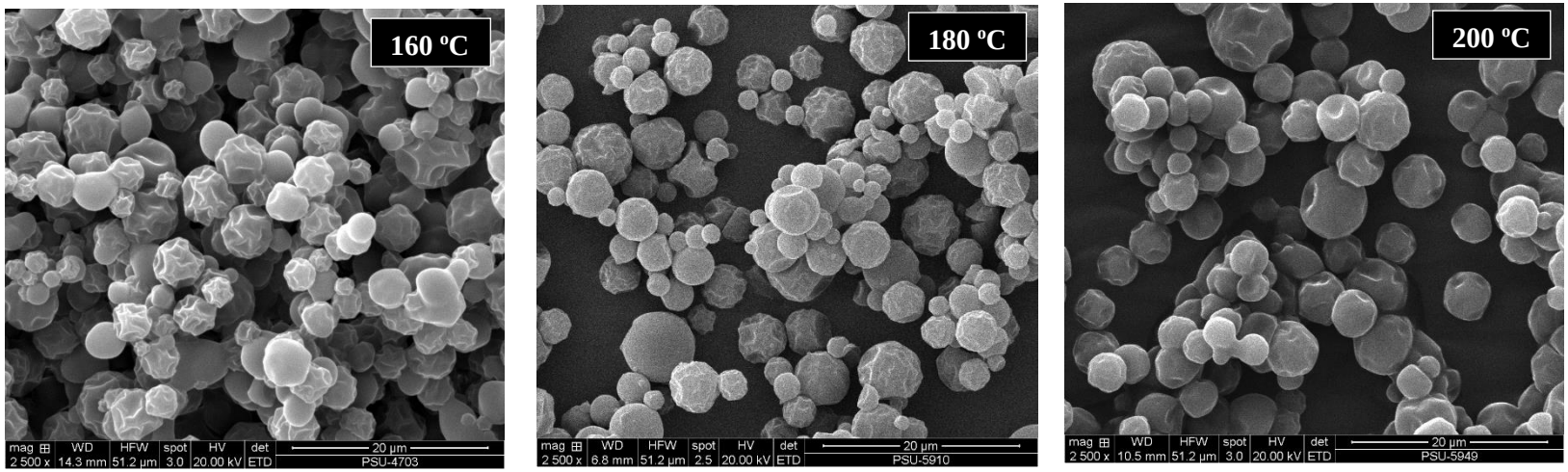

Figure 21 Microstructures of gelatin hydrolysate powder from the scale of spotted golden goatfish with different drying temperature. Magnification: 2500x

6.4.1.6 Antioxidant activities

Antioxidant activities of gelatin hydrolysate powders with various inlet temperatures are shown in Table 13. ABTS radical scavenging activity, DPPH radical scavenging activity and ferric reducing antioxidant power (FRAP) of gelatin hydrolysate powder decreased as inlet temperature increased ($p<0.05$). The results indicated that antioxidative peptides were partially destroyed during spray drying, particularly at high temperature. In addition, heat-sensitive amino acids such as tryptophan and methionine, which acted as strong radical scavengers, might be lost during the excessive heating (Sai-Ut *et al*., 2014). It was found that antioxidative activities were more retained when lower inlet temperature was used. Maltodextrin used as a carrier, could protect peptides, responsible for conferring antioxidant properties, against thermal damage during spray drying (Rodríguez-Díaz *et al*., 2014). Overall, antioxidative peptides in gelatin hydrolysate might loss the activity at high temperature caused by the changes in composition of free amino acids and/or peptides in gelatin hydrolysate.

Table 13 Antioxidative activities of gelatin hydrolysate powder from spotted golden goatfish scale prepared with different inlet temperatures

Antioxidative activities	Inlet temperatures (°C)		
	160	180	200
ABTS (µmol TE /g sample)	102.99 ± 0.89c	97.036 ± 1.51b	80.21 ± 1.63a
DPPH (µmol TE /g sample)	8.63 ± 0.17c	6.94 ± 0.36b	3.63 ± 0.24a
FRAP (µmol TE /g sample)	3.06 ± 0.78c	2.23 ± 0.29b	1.63 ± 1.04a

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

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

6.4.1.7 Volatile compounds

Gelatin hydrolysate powders using maltodextrin as a carrier agent and spray-dried at inlet temperature of 180 °C, which showed white color with high yield

and had high antioxidative activity, were determined for volatile compound in comparison with freeze-dried gelatin hydrolysate (Table 14)**.**

Table 14 Volatile compounds in gelatin hydrolysate powder from spotted golden goatfish scale prepared by spray drying with inlet temperature of 180 ^{o}C and freeze drying

Compounds	Abundance ($\times 10^7$)	
	Freeze-dried	Spray-dried
Hexanal	8.46	ND
Nonanal	19.99	ND
2-Decenal	13.38	2.31
1-Octanol	8.78	1.37
1-Hexanol	14.43	ND
1-Dodecanol	50.22	8.14

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

ND: not detectable.

Several aldehyde compounds, including nonanal, hexanal and 2-decenal were found in freeze-dried gelatin hydrolysate. Generally, aldehydes have been used as the index of lipid oxidation in a number of foods because they possess low threshold values and are the major contributors to the development of off-odor and off-flavor (Sae-Leaw *et al*., 2016). Hexanal and nonanal were not detected after the spray-drying. The result suggested that the decomposition or loss of hexanal and nonanal more likely took place at a high temperature used for spray drying. This result was in accordance with Sae-Leaw *et al*. (2016) who found that spray drying was effective in removal of the volatile secondary lipid oxidation products in gelatin from seabass skin. Alcohols, including 1-hexanol, 1-octanol and 1-dodecanol were found in both samples. Alcohols are known as the secondary products produced by

the decomposition of hydroperoxides of fatty acids. 1-hexanol was not found in spray-dried sample, while it was detected in freeze-dried sample. Although the spray drying could remove the low MW volatiles from the gelation solution, drying at high temperature could induce lipid oxidation to some extent. However the rate of removal was most likely much higher than the rate of formation. This was evidenced by the very low amount of volatiles in the spray-dried sample (Sae-Leaw *et al*., 2016). Therefore, Fish gelatin hydrolysate powder exhibited distinctly different profiles of odorous compounds, depending on the drying methods. Spray drying could be used as an alternative for removal of some odorous compounds, which contributed to the offensive fishy odor in the resulting gelatin hydrolysate.

6.4.2 Characteristics and properties of apple juice fortified with gelatin hydrolysate powder

6.4.2.1 Physical properties

Color, browning intensity and turbidity of apple juice fortified with gelatin hydrolysate powder from spotted golden goatfish scale prepared using inlet temperature of 180 ^{o}C at different levels are shown in Table 15. When gelatin hydrolysate powder was added in apple juice, *L**-value was decreased and *a**- and *b**-values were increased. Those changes were more pronounced when the concentration of gelatin hydrolysate powder increased ($P<0.05$). The increase in b*-value ($P<0.05$) indicated the increased yellowness of fortified apple juice, more likely caused by the color of gelatin hydrolysate added.

The increase in browning intensity of fortified apple juice was observed when the levels of gelatin hydrolysate increased ($P<0.05$). Fortification of gelatin hydrolysate resulted in the increased turbidity of apple juice. The highest turbidity was observed when added with 5% gelatin hydrolysate powder ($p<0.05$). Apple juice contains high amount of phenolic compounds (van der Sluis *et al*., 2002). Those phenolics might interact with gelatin hydrolysate, leading to the large aggregate as evidenced by the increased turbidity of apple juice. Thus, the addition of gelatin hydrolysate powder had the impact on physical property of apple juice to some degree.

Table 15 Physical properties and antioxidative activities of apple juice fortified with spotted golden goatfish scale gelatin hydrolysate at different levels

Parameters	Control	Levels of gelatin hydrolysate powder (%)		
		1	3	5
*L**	62.80 ± 0.35c	62.77 ± 0.38c	60.33 ± 0.03b	58.08 ± 0.03a
*a**	4.54 ± 0.13a	4.60 ± 0.04a	4.96 ± 0.06b	5.60 ± 0.09c
*b**	38.11 ± 0.13a	38.16 ± 0.16a	38.83 ± 0.23b	39.60 ± 1.11c
Δ*E**	3.92 ± 0.46a	3.94 ± 1.07a	5.68 ± 0.97b	7.53 ± 1.21c
Browning index (A_{420})	0.56 ± 0.00a	0.56 ± 0.00a	0.58 ± 0.01b	0.63 ± 0.02c
Turbidity (A_{660})	0.03 ± 0.00a	0.03 ± 0.00a	0.07 ± 0.00b	0.12 ± 0.00c
ABTS radical scavenging activity (μmol TE/mL)	82.53 ± 0.78a	98.43 ± 0.65b	107.21 ± 0.96c	118.46 ± 1.17d
DPPH radical scavenging activity (μmol TE/mL)	4.57 ± 0.03a	7.52 ± 0.76b	9.43 ± 0.29c	11.22 ± 0.47d
FRAP (μmol TE/mL)	1.66 ± 1.21a	3.01 ± 1.04b	5.57 ± 1.63c	8.67 ± 0.92d

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

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

6.4.2.2 Antioxidative activities

Antioxidant activities of apple juice fortified with gelatin hydrolysate powder at different concentrations are shown in Table 15. Apple juice had the increases in ABTS radical scavenging activity, DPPH radical scavenging activity and ferric reducing antioxidant power (FRAP) when the levels of gelatin hydrolysate powder increased ($p<0.05$). The fortification of gelatin hydrolysate powder with antioxidative activity (Table 15) in apple juice directly increased antioxidant activities of apple juice. Phanturat (2008) reported that apple juice fortified with gelatin hydrolysate from bigeye snapper skin prepared using Alcalase and Neutrase showed the higher antioxidant activity than the control. Apple juice has been reported as the good source of natural antioxidant such as flavonoids, etc (van der Sluis *et al*., 2002). Peptides in gelatin hydrolysates have been reported to possess antioxidative activities (Weng *et al*., 2014). Thus, fortification of gelatin hydrolysate powder could enhance the antioxidative activities of apple juice, mainly via providing antioxidative peptides.

6.4.2.3 Sensory properties

Sensory properties, including appearance, color, odor, flavor, taste and overall likeness scores of the control apple juice and those fortified with gelatin hydrolysate powder at different concentrations are presented in Table 16. The fortification of gelatin hydrolysate powder up to 50% had no effect on likeness scores of all attributes tested of apple juice ($P>0.05$). This might be due to the low abundance of volatile compounds in gelatin hydrolysate powder (Table 14) Yarnpakdee *et al*. (2012) reported that protein hydrolysate produced from Indian mackerel muscle with the proper pretreatment could be fortified into milk at a level of 0.2% without negative impact on sensory property. Additionally, apple juice had yellowish brown in color. The incorporation of gelatin hydrolysate powder at level tested had no effect on color. Therefore, gelatin hydrolysate powder from spotted golden goatfish scale could be added in apple juice to enhance antioxidative activities at a level of 5% (w/v) with no detrimental effect on sensory properties.

Table 5 Likeness scores of apple juice fortified with spotted golden goatfish scale gelatin hydrolysate powder at different levels

Attributes	Control	Levels of gelatin hydrolysate powder (%)		
		1	3	5
Appearance	7.37 ± 0.10a	7.47 ± 0.85a	7.17 ± 0.91a	7.60 ± 0.74a
Color	7.37 ± 0.16a	7.53 ± 1.11a	7.23 ± 1.25a	7.53 ± 0.86a
Odor	7.43 ± 1.04a	7.27 ± 1.26a	7.03 ± 1.35a	7.60 ± 0.73a
Flavor	7.37 ± 1.22a	7.40 ± 0.83a	7.20 ± 1.63a	7.17 ± 0.66a
Taste	7.23 ± 0.36a	7.30 ± 1.00a	7.23 ± 0.95a	7.27 ± 0.72a
Overall	7.30 ± 0.12a	7.37 ± 0.98a	7.20 ± 0.97a	7.43 ± 0.58a

Values are given as mean ± SD (n=3).

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

6.5 Conclusion

Spotted golden goatfish scale gelatin hydrolysate powder was produced by spray drying. Higher yield was gained with increasing inlet temperature. Nevertheless, higher inlet temperature enhanced browning intensity but lowered antioxidative activities of gelatin hydrolysate powder. The fortified gelatin hydrolysate powder prepared using the inlet temperature of 180 ^{o}C at a level of 5% in apple juice had no effect on likeness scores but increased antioxidative activities of apple juice.

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CHAPTER 7

CONCLUSION AND SUGGESTION

7.1 Conclusion

1. ASC and PSC from scales of spotted golden goatfish were identified as type I collagen, and maintained their triple helical structure. Pepsin digestion was able to increase the yield of collagen. ASC and PSC showed similar protein patterns and FTIR spectra. Collagens from spotted golden goatfish had the high thermal stability.

2. Scale consisted of two distinct regions: an external (osseous) layer and an internal fibrillary plate. The use of 0.75 M HCl for 30 min with a scale/solution ratio of 1:5 (w/v) at 25 ^{o}C effectively demineralized the scales. Ca and P contents of scales drastically decreased after demineralization. Most of inorganic elements at external layer were removed by demineralization with coincidental increase in collagen fibrils. Thus, demineralization could favor the extraction of collagen or gelatin.

3. Gelatin from the scale of spotted golden goatfish extracted at different temperatures and various times had varying characteristics and properties. Extraction of gelatin at higher temperature for longer time resulted in the higher yield, however gel became weaker. Therefore, the scale of spotted golden goatfish could serve as raw material for gelatin extraction and the extraction conduction should be appropriately selected, depending on the further uses.

4. Maltodextrin used as a carrier agent for gelatin and gelatin hydrolysate was able to increase the yield and improve the whiteness of gelatin and gelatin hydrolysate powders. All gelatin hydrolysate powder containing maltodextrin had higher solubility and antioxidant activities, compared with gelatin counterpart. However, maltodextrin exhibited the dilution effect on antioxidative activity of gelatin hydrolysate. Thus, production of gelatin hydrolysate powder using maltodextrin as a carrier agent at a ratio 2:1 (w/w) would be an effective process to yield gelatin hydrolysate with high yield and antioxidant activity.

5. Spotted golden goatfish scale gelatin hydrolysate powder could be produced by spray drying. Higher yield was gained with increasing inlet temperature. Nevertheless, higher inlet temperature enhanced browning intensity but lowered antioxidative activities of gelatin hydrolysate powder

6. The fortification of gelatin hydrolysate powder prepared using the inlet temperature of 180 ^{o}C at a level of 5 % in apple juice had no effect on likeness scores but increased antioxidative activities of apple juice.

7.2 Suggestion

1. Identification of collagen type using molecular or immunochemical techniques should be carried out.

2. The antioxidative peptides in hydrolysate should be purified and identified. In addition, the identified peptides should be synthesized to confirm their antioxidative activity.

3. Antioxidative activity of gelatin hydrolysate in cellular system and animal trial should be evaluated

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List of Publication and Proceedings

Publication

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