รายละเอียดโครงการวิจัยย่อยที่ 1

ชื่อโครงการวิจัยย่อยที่ 1 **ผลของสภาวะการสกัดต่อคุณลักษณะและสมบัติเชิงหน้าที่ของเจลาตินจากหนังปลาวัว (*Aluterus monoceros*)**
(Effect of extracting conditions on characteristic and functional properties of gelatin from the skin of unicorn leathrjacket (*Aluterus monoceros*))

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บทคัดย่อ

จากการศึกษาผลผลิตและสมบัติของเจลาตินที่สกัดจากหนังปลาวัว (*Aluterus monoceros*) ซึ่งสกัดที่อุณหภูมิต่างๆ (45, 55, 65 และ 75 °ซ) ในสภาวะที่มีและไม่มีสารยับยั้งทริปซินจากถั่วเหลือง (SBTI) ที่ระดับความเข้มข้น 100 ยูนิต/กรัม หนังที่ผ่านการพรีทรีตท์เป็นเวลา 12 ชั่วโมง พบว่า การเติม SBTI มีผลให้ผลผลิตลดลงโดยไม่ขึ้นกับอุณหภูมิการสกัด การใช้อุณหภูมิสูงมีผลให้ผลผลิตสูงขึ้น เจลลาตินที่สกัดจากหนังที่อุณหภูมิ 75 °ซ ในสภาวะที่ไม่มี SBTI ให้ผลผลิตสูงสุด (ร้อยละ 10.66±0.14) (น้ำหนักแห้ง) ปริมาณหมู่อะมิโนที่ตำแหน่งแอลฟ่าสูงสุด เมื่อสกัดเจลาตินที่อุณหภูมิ 55 °ซ ในสภาวะที่ไม่มี SBTI แถบสายโซ่แอลฟ่าและบีต้าเพิ่มขึ้น เมื่ออุณหภูมิการสกัดสูงกว่า 55 °ซ เจลาตินที่สกัดที่อุณหภูมิ 65 °ซ ทั้งในสภาวะที่มีและไม่มี SBTI มีความแข็งแรงเจลสูงสุด (178.00±7.50 กรัม และ 170.47±1.30 กรัม ตามลำดับ) จากการตรวจสอบสเปกตราของ FTIR พบว่ามีการสูญเสียทริปเปิ้ลเฮลิกซ์ และมีการสลายตัวของเจลาตินสูงสุดเมื่อสกัดที่อุณภูมิ 55 °ซ ในสภาวะที่ไม่มี SBTI เจลาตินที่สกัดที่อุณหภูมิ 65 °ซ ทั้งในสภาวะที่มีและไม่มี SBTI มีค่า EAI และ ESI สูงสุด รวมทั้งมีค่าการเกิดฟองความคงตัวของฟองสูง ดังนั้นการสกัดเจลาตินจากหนังปลาวัวที่อุณหภูมิสูงเพียงพอสามารถให้เจลาตินที่มีสลายตัวต่ำ

จากการศึกษาสมบัติทางเคมี-กายภาพ และสมบัติเชิงหน้าที่ของเจลาตินจากหนังปลาวัวซึ่งสกัดที่อุณหภูมิต่าง ๆ เป็นเวลาต่างกัน พบว่า ผลผลิต ปริมาณการเก็บเกี่ยว และปริมาณหมู่อะมิโนอิสระของเจลาตินเพิ่มขึ้น แต่ความแข็งแรงเจลลดลงเมื่ออุณหภูมิและเวลาในการสกัดมากขึ้น เจลาตินที่ได้มีสายโซ่แอลฟา 1 และแอลฟา 2 เป็นองค์ประกอบหลัก จากสเปกตราของ FTIR พบว่า เจลาตินทั้งหมดมีการสูญเสียโครงสร้างทริปเปิลฮีลิกซ์ เจลของเจลาตินที่สกัดที่อุณหภูมิสูงเป็นเวลานานมีเส้นสายโปรตีนขนาดใหญ่รวมทั้งมีช่องอากาศขนาดใหญ่กว่าเจลาตินที่สกัดที่อุณหภูมิต่ำกว่า ที่ระดับของเจลาตินเดียวกัน สมบัติการเป็นอีมัลซิไฟเออร์และการเกิดฟองแปรเปลี่ยนตามสภาวะการสกัด EAI ของเจลาตินลดลงเมื่อความเข้มข้นของเจลาตินเพิ่มขึ้น อย่างไรก็ตาม ESI สูงสุดเมื่อระดับเจลาตินเท่ากับ ร้อยละ 3 นอกจากนี้พบว่า FE และ FS ของเจลาตินเพิ่มขึ้นเมื่อระดับความเข้มข้นเพิ่มขึ้น

จากการศึกษาคุณลักษณะและสมบัติการเกิดเจลของเจลาตินจากหนังปลาวัว ที่ผ่านการเติมหมู่ฟอสเฟตด้วยโซเดียมไตรพอลิฟอสเฟต (STPP) ที่ระดับความเข้มข้นต่างๆ (0.25, 0.50, 0.75และ 1 น้ำหนัก/น้ำหนัก)เป็นระยะเวลาต่างๆกัน (1 และ 3 ชั่วโมง) ที่อุณหภูมิ 65 °ซ พบว่า ระดับ STPP และระยะเวลาที่เพิ่มขึ้นไม่มีผลต่อการเพิ่มปริมาณฟอสเฟตที่จับกับเจลาติน ความแข็งแรงเจลสูงสุดสำหรับเจลาตินที่ผ่านการเติมหมู่ฟอสเฟตโดยใช้ STPP ร้อยละ 0.25 เป็นเวลา 1 ชั่วโมง เมื่อศึกษาผลของพีเอช (5, 7, 9 และ 11) ต่อการเติมหมู่ฟอสเฟตและสมบัติของเจลาตินพบว่า เจลาตินที่ผ่านการเติมฟอสเฟตที่พีเอช 9 ให้ความแข็งแรงเจลสูงสุด (204.3 กรัม) และโครงข่ายเจลที่ละเอียดและแน่นโดยมีช่องอากาศขนาดเล็ก เจลาตินมีประจุเป็นลบ (-3.89 mV) และสามารถเกิดอันตรกิริยาไอออนิกที่ระดับสูงขึ้นส่งผลให้เจลมีความแข็งแรงมากขึ้น ดังนั้นการเติมหมู่ฟอสเฟตภายใต้สภาวะที่เหมาะสมสามารถปรับปรุงสมบัติการเกิดเจลของเจลาตินจากหนังปลาวัวได้

Abstract

Gelatins extracted from the skin of unicorn leatherjacket (*Aluterus monoceros*) at different temperatures (45, 55, 65 and 75 °C) in the presence and the absence of soybean trypsin inhibitor (SBTI;100 units/g pretreated skin) for 12 h were characterised. In general, the addition of SBTI resulted in the lower yield, regardless of extraction temperature. Higher yield was obtained when higher extraction temperature was used (P<0.05). Gelatin

from skin extracted at 75 °C in the absence of SBTI showed the highest yield (10.66±0.41%) (based on dry weight). The highest α-amino group content was observed in gelatin extracted at 55 °C without SBTI incorporated. The band intensity of β-chain and α-chains increased as the extraction temperature increased, particularly above 55 °C. Gelatin extracted at 65 °C with and without SBTI incorporaton exhibited the highest gel strength (178.00±7.50 g and 170.47±1.30 g, respectively). FTIR spectra indicated that a greater loss of molecular order of triple helix with a higher degradation was found in gelatin extracted at 55 °C in the absence SBTI. Gelatin extracted at 65 °C, either with or without SBTI, had the highest EAI and ESI with high foam expansion and stability. Thus, the extraction of gelatin from the skin of unicorn leatherjacket at temperature sufficiently high could render the gelatin with less degradation.

Physicochemical and functional properties of gelatin from the skin of unicorn leatherjacket extracted at different temperatures for various times were determined. Yield, recovery and free amino group content of gelatin increased, but gel strength generally decreased as the extraction temperature and time increased ($P < 0.05$). All gelatins contained α_1 and α_2 chains as the predominant components. FTIR spectra of all gelatins showed a significant loss of the triple-helix. Gels of gelatin extracted at higher temeprature for longer time had larger strands with larger voids. At the same level of gelatin, emulsifying and foaming properties varied with extraction conditions. Emulsion activity index (EAI) of all gelatins decreased with increasing concentrations ($P < 0.05$). Nevertheless, the highest emulsion stability index (ESI) was observed at a level of 3% ($P < 0.05$). Foam expansion (FE) and foam stability (FS) of gelatin generally increased as the concentration increased ($P < 0.05$).

Characteristics and gelling property of gelatin from the skin of unicorn leatherjacket phosphorylated with sodium tripolyphosphate (STPP) at various concentration (0.25, 0.50, 0.75 and 1.00% w/w) for different times (1 and 3 h) at 65 °C were studied. With increasing STPP concentration and time, no increase in bound phosphate was observed. Highest gel strength was obtained for gelatin phosphorylated using 0.25% STPP for 1 h ($P < 0.05$). When the effect of pH (5, 7, 9 and 11) on phosphorylation and gel property of gelatin was investigated, gelatin phosphorylated at pH 9 had the highest gel strength (204.3 g) ($P < 0.05$) and exhibited a finer and more compact network structure with smaller pores. Gelatin became negatively charged (-3.89 mV) and might undergo ionic interaction to a higher extent, thereby strengthening gel network. Thus, the phosphorylation under the appropriate condition could improve gelling property of gelatin from the skin of unicorn leatherjacket.

Chapter 1

Introduction and Literature Review

General Introduction

Gelatin, the denatured form of collagen, has been widely used in the food industry, pharmaceuticals, photography and other technical applications (Kittiphattanabawon *et al*., 2005). Generally, gelatin is produced from skins and skeletons of bovine and porcine (Gilsenan and Ross-Murphy, 2000). However, the occurrence of bovine spongiform encephalopathy (BSE) and foot-and-mouth disease (FMD) have caused major concerns for human health. Furthermore, porcine and bovine gelatins are prohibited for some religions (Sadowska *et al.,* 2003). Therefore, fish by-products, especially skin, which contain 30% of total material, become the potential alternative raw material for gelatin production (Shahidi, 1994). However, fish gelatins have lower bloom strength, compared with mammalian gelatin, due to its lower imino acid content (Foegeding and Lanier, 1996). Indigenous proteinase or endogenous collagenase also plays

a vital role in hydrolysis of polypeptide, contributing to the decrease in functional properties of resulting gelatin. Hence, the appropriate extracting temperature and the use of particular protease inhibitor to lower the autolysis can be an alternative way to improve functional properties of gelatin. In addition, the incorporation of phosphate into gelatin during pretreatment or extraction can be associated with the increased bloom strength of gelatin from the skin of unicorn leatherjacket, particularly in conjuction with the use of divalent cations. As a consequence, gelatin from marine sources can be utilized more widely as the food ingredient or other applications. Nevertheless, there is no information about gelatin extraction as affected by temperature and time and the role of phosphate treatment on properties of gelatin from unicorn leatherjacket *(Aluterus monoceros)* skin.

In general, fish gelatin shows the poorer gel forming ability, compared with mammalian counterpart. Therefore, the extraction under the appropriate condition can be another means to obtain the fish gelatin with the properties comparable to commercial gelatins. The degradation caused by indigenous gelatinase has been reported to be a factor lowering the gelling property of fish gelatin. Thus the use of protease inhibitor can help in lowering such a degradation of resulting gelatin. To improve gel strength of fish gelatin, the modification of gelatin by the selected phosphorylation process in conjunction with the addition of divalents can be a promising approach to enhance the gel strength of gelatin, mainly via the increased salt bridge between gelatin chains. The improved gelling property and other functional properties can maximize the use of fish gelatin and it will be widely used.

Literature Review

Gelatin and its composition

Gelatin is commonly used food ingredient to improve the elasticity, consistency and stability of foods (Bailey and Light, 1989). It is obtained by thermal denaturation or physical and chemical degradation of collagen. Gelatin consists of random chains without triple helix. Depending on the method in which collagens are pre-treated, two different types of gelatin with different characteristics including type-A, acid-treated collagen, and type-B, an alkaline treated counterpart, can be produced (Johnston-Banks, 1990). Acid treatment is most suitable for less fully cross-linked collagens commonly found in pig or fish skins, whereas alkaline treatment is appropriate for the more complex collagens found in bovine hides (Foegeding and Lanier, 1996).

Gelatin contains approximately 1,050 amino acids per alpha chain. The protein is made up of peptide triplets Gly-X-Y, where X and Y can be any one of the amino acids

but proline has a preference for the X position and hydroxyproline for the Y position (Bailey and Light, 1989). Serine, threonine, aspartic acid and glutamic acid predominate in alkaline-processed gelatins and alanine is dominant in acid processed ones. Furthermore, the amino acid composition of gelatin, is almost completely lacking in tryptophan and is low in methionine, cystine and tyrosine (Jamilah and Harvinder 2002).

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

Production of gelatin

Production of gelatin involves three steps involving 1) pretreatment of the raw material, the removal of noncollagenous components from the stock (skin and bones), 2) extraction of the gelatin, the conversion of collagen to gelatin by heating in the presence of water and 3) recovery of gelatin in the final form (Johnston-Banks, 1990; Foegeding and Lanier, 1996).

1. Pretreatment processes

1.1 Acid process or type A gelatin

Acid hydrolysis is a milder treatment that effectively solubilizes collagens of animals slaughtered at a young age, such as pigs (Foegeding and Lanier, 1996). The pretreatments process is designed to convert the collagen into a form suitable for extraction. A sufficient number of the covalent cross-links in the collagen must be broken in order to enable the release of free α-chains. The process is also designed to remove other organic substances, such as proteoglycan, blood, mucins, sugars, etc., that also occur naturally in the raw material. It is optimized by each manufacturer to yield gelatin with the required physical and chemical properties (Johnston-Banks, 1990). Normally, 18-

24 h soaking in dilute acid is sufficient to bring about the conversion. Sulphuric and hydrochloric acids are used, often with the addition of phosphoric acid to retard color development (Johnston-Banks, 1990). Different kinds of acids have been used for pretreatment of fish skin before extractions. Phosphoric acid and acetic acid have been used in fish skin pretreatment (Table 1).

Table 1 Acid pretreatment of fish skin before extraction

Species	Acid	Condition	Reference
Bigeye snapper and brownstripe red snapper	0.05 M acetic acid	Soak with a skin/solution ratio of 1:10 (w/v) for 3 h at room temperature (25 °C) with a gentle stirring	Jongjareonrak *et al.* (2006)
Channel catfish *(Ictalurus punctatus)*	Acetic acid	Soak in eight volumes (v/w) at 15 °C for 18 h	Liu *et al.* (2008)
Bigeye snapper *(Priacanthus tayenus)* and *(Priacanthus macracanthus)*	0.2 M Acetic acid	Soak with a skin/solution ratio of 1:10 (w/v) for 2 h with a gentle stirring at 4 °C	Benjakul *et al.* (2009)
Snakehead (*Channa striatus*), catfish (*Clarias batrachus*), pangasius catfish (*Pangasius sutchi*) and red tilapia (*Oreochromis niloticus*) Unicorn leatherjacket *(Aluterus monoceros)*	0.05 N acetic acid	Soak with a skin/solution ratio of 1:6 (w/v) at room temperature for 3 h	See *et al.* (2010)
Grey triggerfish (*Balistes capriscus*)	0.2 M Phosphoric acid	Soak with a skin/solution ratio of 1:10 (w/v) for 24 h with a gentle stirring at 4 °C	Ahmad and Benjakul (2011)
	0.05 M acetic acid	Soak with a skin/solution ratio of 1:10 (w/v) for 6 h at 4 °C with a gentle stirring	Jellouli *et al.* (2011)

Acid pretreatment has been reported to affect properties of resulting gelatin (Ahmad and Benjakul, 2011). Ahmad and Benjakul (2011) reported that the gel strength of gelatin from unicorn leatherjacket skin pretreated with phosphoric acid was higher than that of gelatin from skin pretreated with acetic acid.

1.2 Alkaline process or type B gelatin

Type B gelatins are produced by alkali hydrolysis of beef materials, which results in deamidation and a greater range of molecular weight species (Foegeding and Lanier, 1996). Alkaline pretreatment are normally applied to bovine hide and ossein. Lime is most commonly used for this purpose; it is relatively mild and does not cause significant damage to the raw material by excessive hydrolysis. Unfortunately, 8 weeks or more are required for complete treatment. Concentrations of up to 3% lime are used in conjunction with small amounts of calcium chloride or caustic soda. Frequent renewal of the liquors is practiced in order to remove extracted impurities and to maintain the degree of alkalinity present. If caustic soda is used, a 10-14 day pretreatment is possible (Johnston-Banks, 1990).

2. Extraction

The extraction process is designed to obtain the maximum yield in combination with the most desirable properties. The optimization can be achieved by controlling pH, temperature and the extraction time (Saunders and Ward, 1955).

To extract older collagens at neutral pH, a substantial proportion of the cross-links need to be cleaved, necessitating a longer liming pretreatment. If shorter liming times are used, then a lower extraction pH is necessary in order to achieve acceptable conversion rates. More efficient pretreatment conditions also allow the manufacturer to use lower extraction temperatures, resulting in gelatins of greater gel strength (bloom). Shorter treatments generally require higher extraction temperatures if neutral pH levels are chosen, yielding gelatins with lower gel strength (Johnston-Banks, 1990). Fish gelatin has been extracted using different extraction temperatures and times, depending on fish species and raw materials used. Kittiphattanabawon *et al.* (2010) reported that shark gelatin extracted at 75 ^{o}C showed the highest degradation peptides, while gelatin extracted at 45 ^{o}C had the highest content of α-chains.

3. Recovery

The gelatins are filtered to remove suspended insolubles such as fat or unextracted collagen fibres. This is usually performed using materials such as diatomaceous earth to give solutions of high clarity. The final stage is evaporation, sterilization and drying. These are performed as quickly as possible to minimize loss of properties (Johnston-Banks, 1990).

Fish gelatin

Gelatin from marine sources (fish skin, scales, bone and fins) has been paid increasing attention as a possible alternative to bovine and porcine gelatin. Fish gelatin is generally produced by a mild acid treatment (type A gelatin) process (Go´mez-Guille´n and Montero, 2001). Gelatin can be extracted from different fish species.

Extraction yield

In general, extraction yield of fish gelatin is lower than mammalian counterpart. The lower extraction yield of fish gelatin could be due to the loss of extracted collagen through leaching during the series of washing steps or due to incomplete hydrolysis of the collagen (Jamilah and Harvinder, 2002). The yield and properties of gelatin depend on the kind of raw material, pre-treatment and parameters of the process. Nalinanon *et al.* (2008) developed the pepsin-aided process in combination with an appropriate protease inhibitor (pepstatin A) to extract gelatin with a higher yield from bigeye snapper (*Priacanthus tayenus*) skin. The process markedly increased the yield from 22.2% to 40.3% (yield was calculated based on the hydroxyproline content of the gelatin in comparison with that of the skin prior to extraction).

Fish skins are especially suitable as a source of gelatin because it is easily extracted with high yield at relatively moderate temperature, usually at or below 50 °C (Gimenez *et al*., 2005). Moreover, using minced skins instead of whole skins significantly shortened the extraction time of gelatins (Kołodziejska *et al*., 2004).

Fish skin preserved by different methods yielded the gelatin with different properties. Liu *et al*. (2008) extracted and characterized gelatin from channel catfish skins preserved using different methods. Gelatin from dried channel catfish skin exhibited higher gel strength, associated with the large α-chain content of gelatin from the dried skins. Gelling and melting points of gelatin from dried channel catfish skin were similar to those of gelatin from fresh skin, but distinctly different from those from frozen skin.

The extraction yield of gelatin from skins ranged from about 5.5% to 21% of the starting weight of the raw material (Gimenez *et al*., 2005; Jamilah and Harvinder, 2002; Muyonga *et al*., 2004; Songchotikunpan *et al*., 2008). The variation in such values depends on the differences in both the composition and the amount of soluble components in the skins (Muyonga *et al*., 2004), which vary with the species and the age of the fish. In addition, the variation in extraction method can also have an effect on yields. The wide range in gelatin yields could also be attributed to differences in collagen content of the raw material (Songchotikunpan *et al*., 2008). Gomez-Guillen *et al*., (2002) reported that the extraction yield varied slightly among the fish species (sole: 8.3%; megrim: 7.4%; cod: 7.2%; hake: 6.5%). Increasing temperature for extraction increased the yield of gelatin and the temperature required varied with species. Apart from extraction temperature, the sufficient time is also required for the higher yield. Kolodziejska *et al*. (2008) studied the optimal conditions (time and temperature) for gelatin extraction from different kinds of fish offal. Depending on the raw material, 30-100% of collagen was solubilized during heating for 15 min at 45 °C. The increase in the thermal solubility of collagen was very small or was not observed when extraction time at 45 °C was longer than 45–60 min. Increasing the extraction temperature to 70 °C also did not affect collagen solubility, with

the exception of fresh salmon skins. For fish skins, a temperature of 45 °C and extraction time of 15-60 min depending on the kind of skins, were established as optimal conditions for extraction of gelatin (Koloziejska *et al*., 2004). The final stage is evaporation, sterilization and drying. These are performed as quickly as possible to minimize loss of properties (Johnston-Banks, 1990).

Impact of indigenous protease on gelatin

Proteolysis induced by heat-activated and heat-stable indigenous proteases associated with skin matrix can contribute to the destabilisation as well as disintegration of collagen structure by disrupting the intra- and intermolecular cross-links (Wu *et al.,* 2008). Collagenolytic enzymes have the unique ability to catalyze the hydrolysis of collagen and gelatin (Sovik and Rustard, 2006). They can cleave triple-helical collagen at a single site, resulting in the formation of fragments corresponding to 1/4 and 3/4 of its initial length (Sano *et al.,* 2004). Collagenases are classified into two major groups, metallocollagenases and serine collagenases (Aoki *et al.,* 2003). Furthermore, non-collagenase proteinases can cleave the collagen molecule in the telopeptide region and contribute to destabilization of the collagen molecule by disrupting the region, in which intermolecular cross-links are formed (Bornstein and Traus, 1979).

Heat-activated serine protease in bigeye snapper skin was involved in the drastic degradation of the β- and α-chains of the gelatin extracted at 60 °C (Intarasirisawat *et al.,* 2007). These enzymes are bound with matrix components such as collagens (Woessner, 1991). The proteolytic degradation of high molecular weight components caused by indigenous proteases during extraction of gelatin at high temperature resulted in adverse effects on gel-forming properties of resulting gelatin (Intarasirisawat *et al.,* 2007). The proteolytic breakdown of collagen structure is most likely related to the disintegration of connective tissues, which has been implicated in quality deterioration of products.

The maximal autolytic activity of bigeye snapper skin was observed at 60 °C and pH 7.5 (Intarasirisawat *et al*. 2007). With the addition of 0.001 mM SBTI, the degradation was markedly inhibited and β- and α- chains in gelatin were more retained when extracted at temperatures lower than 50 °C. However, a lower yield was obtained. Therefore, heat-activated serine proteinase, most likely collagenase, involved in the degradation and affected the yield of gelatin from bigeye snapper skin (Intarasirisawat *et al*. 2007). Additionally, Ahmad *et al*. (2011) reported that serine protease was the major enzyme in pretreated skin from unicorn leatherjacket and was involved in the drastic degradation of collagen/gelatin at high temperature, used for gelatin extraction. Maximised degradation was found at pH 7 and 50 °C. Degradation was markedly inhibited by 0.04 mM soybean trypsin inhibitor (SBTI), with coincidental maintainance of β- and α-

chains. As a result, the gel strength and emulsifying activity were increased, however the extraction yield was lowered.

Structural and rheological properties

Fish and mammalian gelatins have a polydisperse molecular weight distribution related to the collagen structure and production process. In addition to different oligomers of the alpha subunits, intact and partially hydrolyzed alpha-chains are also present, giving rise to a mixture containing molecules of different molecular weights (Foegeding and Lanier, 1996). Chiou *et al*. (2006) reported that pollock and salmon gelatins had slightly different molecular weight profiles compared to porcine gelatin, and that the fish gelatins had chains with slightly lower molecular weights. In addition, the fish gelatins contained lower molecular weight species that were not present in the porcine gelatin.

Structure and functional group of gelatin is governed by the processes used for pretreatment and extraction. Ahmad and Benjakul (2011) reported the changes in the functional groups and secondary structure of gelatin extracted from the skin of unicorn leatherjacket pretreated with different acids (0.2 M acetic acid or 0.2 M phosphoric acid) and extracted with distilled water at 45 ^{o}C for various times (4 and 8 h) using FTIR spectra. Spectra of gelatin from skin pretreated with acetic acid for 4 and 8 h (GAA4 and GAA8) and gelatin from skin pre-treated with phosphoric acid for 4 and 8 h (GPA4 and GPA8) displayed the major peaks in amide region.

Amide I peaks of GAA8 and GPA8 shifted to higher frequency, compared to those of GAA4 and GPA4. This indicated the greater loss of molecular order due to thermal uncoupling of intermolecular cross-links as the longer extraction was used. Since, GAA8 and GPA8 contained a higher amount of unordered and low molecular weight peptides. GPA8 also had the high intensity peaks at wavenumbers of 1144.04, 1119.62, 1063.38 and 985.59 cm^{-1}. The occurrence of those peaks was coincidental with more degradation of peptide chains in GPA8 sample. In addition, the prominent absorption bands in GPA4 and GPA8 around the wavenumber of 1074.06 and 1063.38 cm^{-1} respectively, most likely arose from asymmetric stretching vibrations of phosphate groups of phosphorylated proteins coupled to -CH_2 of the amino acid residues (Jackson *et al.*, 1995).

At amide A region, the lower amplitude as well as the lower wavenumber were found in GPA8, compared to GPA4. GPA8 might also form new covalent inter-molecular cross-links during freeze-drying. Thus, the secondary structure of gelatins obtained from the skin of unicorn leatherjacket was affected by acid pretreatment and extraction time. The low molecular weight peptides formed during the extraction for long time were more likely able to form covalent cross-links during freeze-drying process. Additionally rheological properties of fish and mammalian gelatin are comparatively different. Haug *et al*. (2004) found that the main difference between fish and mammalian gelatins is the

content of the imino acids, proline and hydroxyproline, which stabilize the ordered conformation when gelatin forms a gel network. The lower content of proline and hydroxyproline gives fish gelatin with a low gel modulus, and low gelling temperature. The super-helix structure of the gelatin gel, which is critical for the gel properties, is stabilized by steric restrictions.

Functionality of gelatin

The functional properties of gelatin are related to their chemical characteristics. The gel strength, setting behavior and melting point of gelatin depend on their molecular weight distribution and the amino acid composition (Johnston-Banks, 1990)

1. Gelation

Collagen denatures at temperatures above 40 °C to a mixture of random-coil single, double, and triple strands. Upon controlled cooling below the melting temperature, T_m, the reformation of the helical form occurs (Wong, 1989). The energy barrier for refolding is ~ 4 KJ/mole. The initial refolding is rapid and involves the Gly-I-I regions of the polypeptide chain, forming a single turn of a left-handed helix. This "nucleation" along the polypeptide chain is structurally stabilized by a certain type of water bridging. The "nucleated" polypeptide then (1) folds back into loops, with the nucleated regions aligned to form triple strands, or (2) has its nucleated region aligned with that of the other nucleated polypeptide chain. At high enough concentrations, interchain alignment becomes possible and association of polypeptide chains to form triple-helical collagen molecules can occur (Wong, 1989).

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. These are governed by molecular weight, as well as by complex interactions determined by the amino acid composition and the ratio of α/β-chains present in the gelatin (Cho *et al*., 2004). In addition, there is a strong correlation between gel strength and the α-chain content in gelatin. Gelatin containing more α-chains would thus show higher gel strength (Johnston-Bank, 1990).

It is generally recognized that the imino acids, proline and hydroxyproline, are important in gelation (Kittiphattanabawon *et al.,* 2005). The molecular weight distribution is also important in determining the gelling behavior of gelatin (Johnston-Bank, 1990). Gelatin from shark skin extracted at 75 °C containing the large proportion of small peptides had much lower bloom strength than extracted at 45 °C and 60 °C (Kittiphattanabawon *et al.,* 2010).

The gel strength of commercial gelatins ranges from 100 to 300, but gelatins with bloom values of 250–260 are the most desirable (Holzer, 1996). Gelatins from different

fish species have different characteristics and 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) (Go´mez-Guille´n *et al*., 2002). Go´mez-Guille´n *et al.* (2002) reported the different rheological characteristics (viscoelasticity and gel strength) and chemical/structural properties of gelatins extracted from the skins of several marine species. Gelatins from sole and megrim (flat-fish) showed the best gelling ability, and the gels were more thermostable than those from cod and hake (cold-adapted fish). This difference in behavior was explained based on the amino acid composition, the $\alpha 1/\alpha 2$ collagen-chain ratio, and the molecular weight distribution. Although the amino acid composition is important for determining the gelling properties of a given gelatin, the average molecular weight and, more specifically, the distribution of α-, β-, or γ-chains, also affect the physical properties of gelatin (Go´mez-Guille´n *et al.* 2002). Fish gelatin typically has a bloom value ranging from 0 to 270 g (tested under the conditions of the standard bloom test), compared to the high bloom values for bovine or porcine gelatin, which have bloom values of 200-240 g. Some species of warm-water fish gelatins have been reported to exhibit relatively high bloom values, close to that of high bloom pork gelatin. Bloom values ranging from 128 to 273 g have been reported for tilapia gelatin (Jamilah and Harvinder, 2002; Zhou *et al*., 2006).

The wide range of bloom values found for various gelatins arises from differences in proline and hydroxyproline content in collagens of different species, and is also associated with the temperature of the habitat of the animals. Badii and Howell (2006) showed that hydrophobic amino acids (Ala, Val, Leu, Ile, Pro, Phe, and Met) could also contribute to the high bloom value of tilapia fish gelatin. They found a lower number of hydrophobic amino acids in the commercial non-gelling cod gelatin, compared to tilapia and horse mackerel gelatin. The extraction conditions may affect the hydrophobic amino acid composition and distribution, which influences the physical properties of gelatin, even more than the imino acid content (Montero and Go´mez-Guille´n, 2000).

The main differences in the properties of mammalian and fish gelatins are that fish gelatins have lower gelling and melting temperatures, but relatively higher viscosities (Leuenberger, 1991). Typical gelling and melting points for porcine and bovine gelatins range from 20 to 25 °C and 28 to 31 °C, respectively (Gilsenan and Ross-Murphy, 2000). In comparison, typical gelling and melting points for fish gelatins range from 8 to 25 °C and 11 to 28 °C, respectively. The wide range of gelling temperatures is greatly influenced by the origin of the raw material used in the process. Melting temperatures of gelatins derived from the skins of cold-water fish are significantly lower than those of collagens and gelatins from the skins of mammals and fish living in warm-waters, due to the lower imino acid contents and less proline hydroxylation (Go´mez-Guille´n *et al*., 2002).

Consequently, cold-water fish gelatins behave as a viscous liquid at room temperature, which limits their use in many applications.

2. Emulsifying and foaming properties

Gelatin is a relatively high molecular weight protein with amphiphilic nature. The relatively high isoelectric point (pI≥7.0) of Type A gelatin means that it should be possible to create oil-in-water emulsions that have a positive charge over a wider range of pH values than conventional protein emulsifiers, such as soy, casein or whey proteins (Dickinson and Lopez, 2001). Consequently, Type A gelatin may be suitable for creating oil-in-water food emulsions with high oxidative stability since it could repel iron ions from oil droplet surfaces over most of the pH range typically found in foods (Surh *et al.,* 2006). Some previous studies have shown that gelatin is surface-active and is capable of acting as an emulsifier in oil-in-water emulsions (Lobo, 2002). However, gelatin often produces relatively large droplet sizes during homogenization (Dickinson and Lopez, 2001; Lobo, 2002). Therefore, it has to be either hydrophobically modified by attachment of nonpolar side-groups (Toledano and Magdassi, 1998) or used in conjunction with anionic surfactants to improve its effectiveness as an emulsifier (Surh *et al*., 2006). Fish gelatin can function as emulsifier. Surh *et al*. (2006) studied the properties and stability of oil-in-water emulsions stabilized by fish gelatin, and determined the influence of gelatin molecular weight (low molecular weight and high-molecular weight fish gelatin) in 20 wt% corn oil-in-water emulsions (pH 3.0, 10 mM imidazole-acetate buffer). Emulsions with mono-modal particle size distributions and small mean droplet diameters (d_{43}~0.35 mm for low molecular weight and 0.71 mm for high-molecular weight fish gelatin) could be produced at protein concentrations of ≥4.0 wt % for both molecular weight fish gelatins. The number of large droplets and the amount of destabilized oil was less in the high-molecular weight fish gelatin emulsions than in the low molecular weight fish gelatin emulsions. This effect may be attributed to the fact that the thickness of an adsorbed gelatin membrane increases with increasing molecular weight. Emulsions of both low- and high-molecular weight fish gelatins were fairly stable when subjected to high salt concentrations (250 mM sodium chloride), thermal treatments (30 or 90 $^{\circ}$C for 30 min), and different pH values (pH 3–8), demonstrating that fish gelatin may have limited use as a protein emulsifier for oil-in-water emulsions (Surh *et al*. 2006).

Aewsiri *et al.* (2011) determined emulsifying property of cuttlefish *(Sepia pharaonis)* skin gelatin modified with *N*-hydroxysuccinimide esters of various fatty acids including capric acid (C10:0), lauric acid (C12:0), and myristic acid (C14:0) at different molar ratios. Fatty acid esters were incorporated into gelatin as indicated by the decrease in free amino group content. Gelatin modified with fatty acid ester had the increased surface hydrophobicity and emulsifying property with coincidental decrease in surface tension.

Gelatin modified with fatty acid ester of C14:0 showed the highest surface activity, especially with the high degree of modification. Emulsion stabilized by gelatin modified with fatty acid ester of C14:0 had a smaller mean particle diameter with higher stability, compared with that stabilized by the control gelatin (without modification). Emulsion stabilized by modified gelatin remained stable at various pH (3-8) and salt concentrations (NaCl 0-500 mM). Emulsion was also stable after being heated at 50-90 °C for 30 min.

3. Sensory properties

As a thermo-reversible gel, gelatin gels will start melting when the temperature increases above a certain point, which is called the gel melting point, and is usually lower than human body temperature. This melt-in-the-mouth property has become one of the most important characteristics of gelatin gels, and is widely exploited in the food and pharmaceutical industries. The rheological properties of thermoreversible gelatin gels are primarily a function of temperature (below the melting point of the gel) and the concentration of gelatin for a given gelatin type (Zhou *et al*., 2006). Upon cooling, the random coils undergo a coil to helix transition (Kuijpers *et al*., 1999) and they attempt to reform the original structure (Mackie *et al.,* 1998). The resulting three dimensional network is responsible for the strength and integrity of the gelatin gel.

Choi and Regenstein (2000) studied the physicochemical differences between pork and fish gelatin and the effect of melting point on the sensory characteristics of a gelatin-water gel. Quantitative descriptive analysis (QDA) was performed to determine the effect of the melting point on the sensory characteristics of gelatin gels. Flavored fish gelatin dessert gel product had less undesirable off-flavors and off-ordors, with more desirable release of flavor and aroma than the same product produced with pork gelatin. The lower melting temperature of fish gelatin seems to assist in the release of fruit aroma, fruit flavor, and sweetness. In contrast, since pork gelatin melts more slowly than fish gelatin in the mouth, the perceived viscosity of pork gelatin might be expected to be higher than that of the fish gelatin under the same conditions (Karim and Bhat, 2009).

Phosphate compounds

Phosphates are compounds prepared from phosphoric acid where the acid has been partially or fully neutralized with alkali metal ions, predominately sodium, potassium, or calcium (Dziezak, 1990). Phosphates can be divided into two general classes:

1. Orthophosphates

Orthophosphate is the larger and perhaps more important group to the food industry. It consists of one phosphorus atom tetrahedrally surrounded by four oxygens. It

can form straight-chain and cyclic polymers. These compounds have three valences that can be filled by hydrogen atoms, alkali metal cations, or a combination of hydrogens and metal cations. Monobasic orthophosphates have one alkali metal ion and two hydrogens; dibasic orthophosphates have two metal ions, one hydrogen and tribasic orthophosphates are fully neutralized with three metal ions (Dziezak, 1990).

2. Condensed phosphates

Condensed phosphates are produced by heating mixtures of orthophosphates under controlled conditions. They are composed of two or more phosphorus atoms linked through shared oxygen. This group includes straight-chain phosphates called polyphosphates and rings, termed metaphosphates (Dziezak, 1990).

Pyrophosphates are the simplest as they have a two-phosphorus chain. Tripolyphosphates are next in the series with three phosphorus atoms and are followed by long-chain polyphosphates which have four or more phosphorus atoms. Pyrophosphates and tripolyphosphates are crystalline materials unlike long-chain polyphosphates, which are amorphous or glassy. Long-chain polyphosphates are not pure compounds but instead mixtures of many polyphosphates of varying chain lengths (Ellinger, 1977). The metaphosphates are pure crystalline compounds, which are composed of six- or eight- membered rings. Presently there are two metaphosphates, sodium trimetaphosphate and sodium tetrametaphosphate; only the first is used commercially (Dziezak, 1990).

Phosphorylation of proteins

Inorganic phosphate (Pi) can be transferred to proteins by either O- or N-esterification reactions (Matheis *et al.,* 1983). In O-esterification, Pi reacts with the primary or secondary hydroxyl on serine or threonine respectively, or with the weakly acidic hydroxyl on tyrosine, forming a -C-O-Pi bond. In N-esterification, Pi combines with the ε-amino group of lysine, the imidazole group of histidine, or the guanidino group of arginine, forming a -C-N-Pi bond. The N-bound phosphates are acid labile and are readily hydrolyzed below pH 7. Proteins containing O-bound phosphates are acid stable and are the modification of choice for food proteins, since the pH of most foods is 3-7. The introduction of phosphoryl residues increases the negative charge and hydration and changes the functional properties of proteins (Matheis *et al.,* 1984).

In general, sodium trimetaphosphate (STMP) seem to be the main suitable reagents for large-scale phosphorylation of food proteins. Sung *et al*. (1983) used sodium trimetaphosphate (STMP) to modify serine and lysine in soy isolate under alkaline conditions. About 40% of the total serine residues were phosphorylated with no protein cross-linking, and the isolate displayed increased solubility and emulsifying properties, particularly under acidic conditions. Giec *et al*. (1989) also reported the phosphorylation

of a yeast homogenate using STMP to remove substantially the nucleic acid and prepare a protein isolate with markedly improved functional properties including solubility and emulsifying properties. STMP is an FDA-approved food additive, does not cause protein cross-linking, and its hydrolysis in water produces only harmless Pi. However, phosphorylation with STMP occurs at alkaline pH, which could lead to undesirable reactions and products as described earlier (Giec *et al*. 1989). STMP shows potential for food protein modification, but further research is needed to determine the exact incorporation of covalently bound Pi, to avoid alkaline pH conditions, and to move its specificity toward O-esterification rather than a mixture of O- and N-esterifications (Giec *et al*. 1989).

Ion-induced gelation

Network formation via salt-mediated interactions of the soluble proteins can take place at a low temperature depending on the types of protein used and gelation time required (Hongsprabhas and Barbut, 1997a). Divalent salt ions screen electrostatic interactions between the charged protein molecules (Yasuda *et al.,* 1986). Nevertheless, divalent cations such as Ca^{2+} and Mg^{2+} have the cross-linking effect towards negatively charged carboxylic acid groups of protein molecules (Hongsprabhas and Barbut, 1997b). Thus, these ion can be used to form protein gels . Causeret *et al.* (1991) postulated that the Ca^{2+} ions could cause the development of ionic bridges between phosphate groups in phosphovitin, lipovitelinin and low density lipoprotein in egg yolk. Grizzuti and Perlmann (1973) confirmed that phosphovitin present in the egg yolk had the capacity of binding calcium and magnesium.

Zn^{2+} has been used to produce pidan with no black spots on the egg shell, and the color of the pidan's albumen and yolk was more stable (Chen and Su, 2004). Apart from pidan white, the properties of yolk have been taken into consideration by the consumers. Different ions used in the pickling solution might contribute to the development of pidan yolk differently, leading to the varying characteristics of pidan yolk (Chi and Tseng, 1998).

Ganasen and Benjakul (2010) monitored physical property and microstructure of pidan yolk during pickling in the presence of different divalent ($CaCl_2$, $MgCl_2$) and monovalent (KCl) cations at different levels (2 and 5 g kg^{-1}) up to 3 weeks, followed by ageing for another 3weeks. Pidan prepared following the commercial process, in which PbO_2 or $ZnCl_2$ at a level of 2 g kg^{-1} was incorporated, was also tested. Pidan prepared using PbO_2 showed the highest hardness, followed by that prepared using $ZnCl_2$. Those using $CaCl_2$ or $MgCl_2$ had the weak gel of Pidan white and turned to be liquefied with longer aging period. Therefore, the gel stabilization was dependent on type of divalent cations used.

Research Objectives

- To study the effect of proteases inhibitor and extracting temperature/time on yield and properties of gelatins.
- To investigate the effect of phosphate incorporation on yield and functional properties of gelatin.
- To investigate the effect of divalent cation on the gel forming ability of gelatin.
- To study the gel property of phosphorylated gelatin mixed with commercial bovine gelatin.

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Chapter 2

Molecular and functional properties of gelatin from the skin of unicorn leatherjacket (*Aluterus monocerous*) as affected by extracting temperatures

Abstract

Gelatins extracted from the skin of unicorn leatherjacket (*Aluterus monocerous*) at different temperatures (45, 55, 65 and 75 °C) in the presence and the absence of soybean trypsin inhibitor (SBTI;100 units/g pretreated skin) for 12 h were characterised. In general, the addition of SBTI resulted in the lower yield, regardless of extraction temperature. Higher yield was obtained when higher extraction temperature was used ($P<0.05$). Gelatin from skin extracted at 75 °C in the absence of SBTI showed the highest yield (10.66±0.41%) (based on dry weight). The highest α-amino group content was observed in gelatin extracted at 55 °C without SBTI incorporated. The band intensity of β-chain and α-chains increased as the extraction temperature increased, particularly above 55 °C. Gelatin

extracted at 65 °C with and without SBTI incorporaton exhibited the highest gel strength (178.00±7.50 g and 170.47±1.30 g, respectively). FTIR spectra indicated that a greater loss of molecular order of triple helix with a higher degradation was found in gelatin extracted at 55 °C in the absence of SBTI. Gelatin extracted at 65 °C, either with or without SBTI, had the highest EAI and ESI with high foam expansion and stability. Thus, the extraction of gelatin from the skin of unicorn leatherjacket at tempertaure sufficiently high could render the gelatin with less degradation.

Keywords: *Unicorn leatherjacket, Gelatin, Extraction temperature, Soybean trypsin inhibitor, Gel strength*

1. Introduction

Gelatin, the denatured form of collagen, has been widely used in the food industry, pharmaceuticals, photography and other technical applications (Kittiphattanabawon *et al.*, 2005). Generally, gelatin is produced from skins and skeletons of land animals (Gilsenan and Ross-Murphy, 2000). However, the occurrence of bovine spongiform encephalopathy (BSE) and foot-and-mouth disease (FMD) has caused major concerns for human health. Furthermore, porcine and bovine gelatins are prohibited for some religions (Sadowska *et al.*, 2003). Therefore, fish processing by-product, especially skin, which contains 30% of total material, has become the potential alternative raw material for gelatin production (Shahidi, 1994).

Unicorn leatherjacket *(Aluterus monoceros)* belongs to the order Tetraodontiformes and is a member of the Monacanthidae family (Ahmad and Benjakul, 2011a). This species has been used for fillet production in Thailand and other countries in South-east Asia. As a consequence, a large amount of skin has been produced as by-product, which can be further used for gelatin production. This leads to the increase in revenue for fish processing industry.

Indigenous proteases play a vital role in hydrolysis of polypeptide, contributing to the decrease in functional properties of resulting gelatin (Ahmad *et al*., 2011c). These enzymes are bound with matrix components such as collagens (Woessner, 1991). Recently, Ahmad and Benjakul (2011b) reported that the use of soybean extract containing trypsin inhibitor during extraction at 50 °C was able to prevent the degradation of gelatin to some degree, suggesting the remaining proteases in the skin. Hence, the inactivation of those proteases using the sufficiently high temperature without thermal degradation might be another approach to maintain those chains, leading to the improved functional properties of resulting gelatin. As a consequence, gelatin from marine sources can be utilised more widely as the food ingredient or for other applications. Nevertheless, there is no information about the impact of extraction temperatures, particullry in

conjunction with the use of protease inhibitor, on properties of gelatin from unicorn leatherjacket skin. Therefore, the objective of this investigation was to study the impact of extraction temperature on chemical composition and functional properties of gelatin from the skin of unicorn leatherjacket in the presence and absence of protease inhibitor.

2. Materials and methods

2.1. Chemicals, collagen and gelatin

All chemicals were of analytical grade. Phosphoric acid was obtained from Lab-Scan (Bangkok, Thailand). Sodium dodecyl sulphate (SDS) and Coomassie Blue R-250 were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Type I collagen from calf skin was purchased from Elastin Products Co., Inc. (Owensville, MO, USA). Food grade bovine bone gelatin was obtained from Halagel (Thailand) Co., Ltd. (Bangkok, Thailand).

2.2. Collection and preparation of fish skin

The skin of unicorn leatherjacket (*Aluterus monocerous)* was obtained from a dock, Songkhla, Thailand. Three different lots of skin were collected. For each lot, all skins were pooled and used as the composite sample. The sample was stored in ice with a skin/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Upon arrival, the skin was washed with iced tap water (0-2°C) and cut into small pieces (0.5×0.5 cm^2), placed in polyethylene bags and stored at -20°C until use. The storage time was less than 2 months.

2.3. Preparation of soybean extract containing trypsin inhibitor

Soybean was ground using a blender (Model MX-T2GN, National, Taipei, Taiwan). The seed flour was defatted by mixing with hexane at the ratio of 1:5 (w/v) for 10 min. The mixture was filtered through a Whatman No.1 filter paper and the retentate was rinsed with hexane 3 times to remove the residual oil. The defatted sample was air-dried at room temperature (28-30°C) until dry and free of hexane odour.

To extract trypsin inhibitor, defatted sample was mixed with 0.15 M NaCl with a ratio of 1:10 (w/v). The mixture was shaken at 180 rpm at room temperature for 3 h using a shaker (Heidolph UNIMAX 1010, Schwabach, Germany). The supernatant was recovered by centrifuging the mixture at 5,000 xg for 30 min. Thereafter, the supernatant was heated at 90 °C for 10 min and then cooled using iced water. The mixture was then centrifuged at 8000 xg for 15 min. The resulting supernatant was freeze-dried using a Model Coolsafe 55 freeze dryer (Scanvac, Coolsafe, Lynge, Denmark) and the obtained powder was used as crude trypsin inhibitor. Crude trypsin inhibitor was subjected to the measurement of trypsin inhibitor as described by Benjakul *et al*. (2000). One unit of trypsin inhibitor activity was defined as the amount of inhibitor, which reduced trypsin activity by one unit. Activity of trypsin was determined using BAPNA as a substrate and the absorbance at 410 nm due

to *p*-nitroaniline released was measured. One unit of trypsin was defined as an increase of 0.01 absorbance unit/min at pH 7 and 37°C.

2.4. Non-collagenous protein removal and swelling of skin

Removal of non-collagenous proteins and swelling were carried out according to the method of Ahmad and Benjakul (2011a) with a slight modification. Fish skin (0.5x0.5 cm^2) was soaked in 0.05 M NaOH with a skin/alkaline solution ratio of 1:10 (w/v) to remove non-collagenous proteins. The mixture was stirred continuously for 4 h at room temperature using an overhead stirrer equipped with a propeller (RW 20.n, IKA Labortechnik, Germany) at a speed of 150 rpm. The alkaline solution was changed every 2 h. Alkaline-treated skin was washed with tap water until neutral or faintly basic pH of wash water was obtained. The alkaline-treated skin was soaked in 0.1 M phosphoric acid with a skin/solution ratio of 1:10 (w/v) for 12 h with a gentle stirring at room temperature. The acidic solution was changed every 6 h. Acid-treated skin was washed thoroughly with tap water until wash water became neutral or faintly basic.

2.5. Extraction of gelatin

The swollen skin was mixed with distilled water at a ratio of 1:5 (w/v) at different temperatures (45, 55, 65 and 75 °C) in the absence and the presence of trypsin inhibitor at a level of 100 Units trypsin inhibitor/1 g swollen skins (Ahmad et al., 2011b). The mixture was incubated in a temperature controlled water bath (Memmert, Schwabach, Germany) and stirred continuously for 12 h. The extract was centrifuged at 5,000 xg for 10 min to remove insoluble material. The supernatant was collected, freeze-dried and subjected to analyses.

2.6. Analyses

2.6.1. Determination of yield and recovery

2.6.1.1. Gelatin yield was calculated by the following equation:

Yield % = [weight of dry gelatin (g)/weight of initial skin (g)] ×100

2.6.1.2. Recovery of gelatin was calculated as follows:

Recovery (%) = [hydroxyproline content of supernatant (g/ml) × volume of supernatant (ml)]/[hydroxyproline content of initial skin (g/g) × weight of initial skin (g)] ×100

2.6.2. Determination of chemical composition

2.6.2.1. Hydroxyproline content

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

2.6.2.2. α – amino group content

The α-amino group content was determined according to the method of Benjakul and Morrissey (1997). Properly diluted sample (125 µl) was mixed thoroughly with 2.0 ml of 0.2125 M phosphate buffer, pH 8.2, followed by the addition of 1.0 ml of 0.01 % TNBS solution. The mixture was then placed in a temperature controlled water bath at 50 °C for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulphite. The mixture was cooled down at room temperature for 15 min. The absorbance was measured at 420 nm using a double beam spectrophotometer (model UV-1800, Shimadzu, Kyoto, Japan) and α-amino group content was expressed in terms of L-leucine.

2.6.2.3. Protein patterns

SDS-PAGE was performed by the method of Laemmli (1970). The samples were dissolved in 5% SDS and the mixtures were incubated at 85°C for 1 h. Solubilised samples were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris HCl, pH 6.8, containing 4% SDS and 20% glycerol). The mixtures were boiled in boiling water for 2 min. Samples (15 µg protein) were loaded onto polyacrylamide gels comprising a 7.5% running gel and a 4% stacking gel and subjected to electrophoresis at a constant current of 15mA/gel using a Mini Protein II unit. After electrophoresis, gel was stained with 0.05% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. Type I collagen was used as a standard.

2.6.3. Determination of functional properties

2.6.3.1. Gel strength

Gelatin gel was prepared as per the method of Fernández-Díaz *et al*. (2001) with a slight modification. Gelatin sample was dissolved in distilled water at 60 °C to obtain the final concentration of 6.67% (w/v). The gelatin solution was stirred until the gelatin was solubilised completely and cooled in a refrigerator at 10 °C for 16-18 h for gel maturation. The dimension of the sample was 3 cm in diameter and 2.5 cm in height. The maximum force (in gram) was recorded when the penetration distance reached 4 mm. The speed of the plunger was 0.5 mm/s.

2.6.3.2. Emulsifying properties

Emulsion activity index (EAI) and emulsion stability index (ESI) of gelatin samples were determined according to the method of Pearce and Kinsella (1978) with a slight modification. Soybean oil (2 ml) and gelatin solution (1, 2 and 3%, w/v, 6 ml) were homogenised (model T25 basic, IKA LABORTECHNIK, Selangor, Malaysia) at a speed of 20,000 rpm for 1 min. Emulsion was pipetted out at 0 and 10 min and 100-fold diluted with 0.1% SDS. The mixture was mixed thoroughly for 10 s using a vortex mixer (Vortex-

Genie 2, Scientific Industries, Bohemia, NY, USA). A_{500} of the resulting dispersion was measured using a spectrophotometer (UV-160, Shimadzu, Kyoto, Japan). EAI and ESI were calculated by the following formula:

$$EAI\ (m^2/g) = (2 \times 2.303 \times A \times DF) / \ l \phi C$$

where A = A_{500}, DF = dilution factor (100), l = path length of cuvette (m), ø = oil volume fraction and C = protein concentration in aqueous phase (g/m^3)

$$ESI\ (min) = A_0 / \Delta A \times \Delta t$$

where A_0 and A_{10} = absorbance at 500 nm measured at time 0 and 10 min after emulsification, $\Delta A = A_0 - A_{10}$ and Δt = 10 min.

2.6.3.3. Foaming properties

Foam expansion (FE) and foam stability (FS) of gelatin solutions were determined as described by Shahidi *et al*. (1995) with a slight modification. Gelatin solution (1, 2 and 3%, w/v) was transferred into 100 ml cylinders and homogenised using a homogeniser (model T25 basic, IKA LABORTECHNIK, Selangor, Malaysia) at 13,400 rpm for 1 min at room temperature. The sample was allowed to stand for 0, 30 and 60 min. FE and FS were then calculated using the following equations:

$$FE\ (\%) = (V_T / V_0) \times 100$$
$$FS\ (\%) = (V_t / V_0) \times 100$$

where V_T is total volume after whipping; V_0 is the original volume before whipping and V_t is total volume after leaving at room temperature for different times (30 and 60 min).

2.6.4. Fourier transform infrared (FTIR) spectroscopy

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

2.7. Statistical analysis

Completely randomized design (CRD) was used throughout the study. All experiments and analyses were run in triplicate. Data were subjected to Analysis of

Variance (ANOVA) and differences between means were evaluated by Duncan's multiple range tests. For pair comparison, *t*-test was used (Steel and Torrie, 1980). Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for window: SPSS Inc, Chicago, IL, USA).

3. Results and discussion

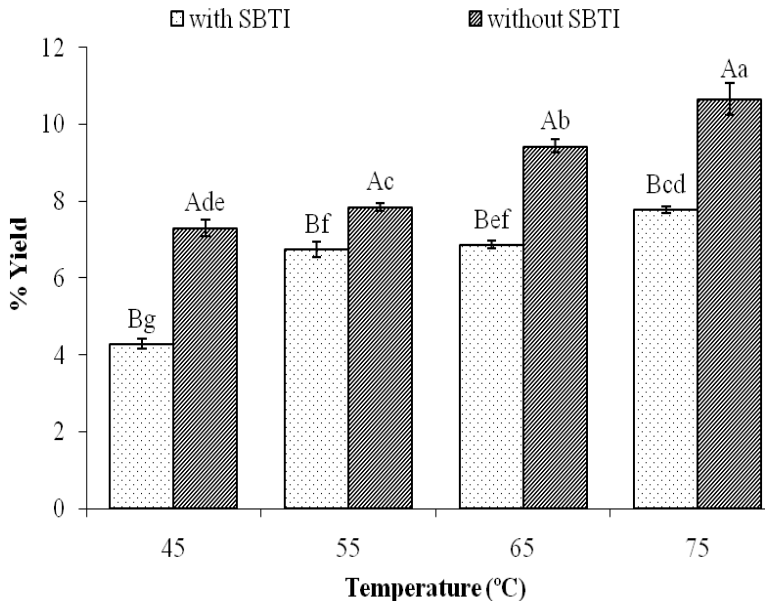
3.1. Yield

Yield and recovery of gelatin from unicorn leatherjacket skin extracted at different temperatures (45-75 °C) in the presence and the absence of SBTI (100 units/g pretreated skin) for 12 h are shown in Fig. 1. Both yield and recovery increased as the extraction temperatures increased from 45 to 75 °C ($P < 0.05$), except for the yield of gelatin extracted at 55 and 65 °C in the presence of SBTI, which showed the similar value ($P > 0.05$). This result was in agreement with Arnesen and Gildberg (2007) and Muyonga *et al*. (2004b) who reported that the increasing extraction temperatures resulted in the increased yield of gelatin from Atlantic salmon skin as well as Nile perch skin and bone. The higher temperatures used for extraction more likely provided higher energy for destroying the hydrogen bonds stabilising the collagen localised in the skin matrix. As a consequence, the collagen underwent denaturation to a higher extent, thereby yielding higher amount of gelatin (Wong, 1989). During the transition of collagen to gelatin, inter- and intra-molecular hydrogen bondings and covalent crosslinks were cleaved, leading to helix-to-coil transition. In addition, some amide bonds in the elementary chains of collagen molecules also undergo hydrolysis (Bailey and Light, 1989). Similar result was noticeable for the recovery. In the absence of SBTI, gelatin extracted at 75 °C showed 10.66±0.41 % yield and 29.79±0.23 % recovery. The recovery represented the percentage of collagen denatured and converted to the obtained gelatin, whilst yield represented the amount of solid released from pretreated skin matrix. Nevertheless, gelatin yield and recovery decreased when the SBTI was incorporated during the extraction. SBTI most likely inhibited the activity of indigenous serine proteases associated with the skin matrix, thereby preventing the cleavage of peptide chains. Generally, the small peptides could be extracted into hot water used as extraction medium with ease. Conversely, long chain peptides aligned in the skin matrix tightly and were not disrupted easily by heat.

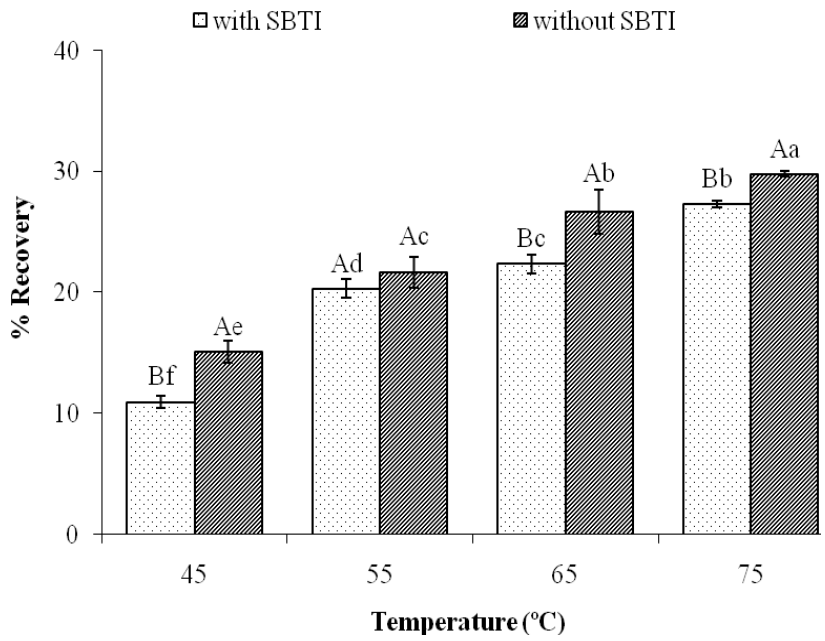
3.2. α-amino group content

The α-amino group content of gelatin from unicorn leatherjacket skin extracted under different conditions is shown in Fig. 2. The highest α-amino group content was observed in gelatin extracted at 55 °C in the absence of SBTI. The optimal temperature for indigenous proteases in the skin of unicorn leatherjacket was 50 °C (Ahmad *et al*., 2011c). However, a decrease in α-amino group content was found when SBTI was incorporated during extraction at 55 °C. This reconfirmed the role of indigenous proteases

in hydrolysis of peptide chains at 55 °C. However, when heating temperatures of 65 and 75 °C were used, the decreases in α-amino group content were obtained. This suggested thermal denaturation of indigenous proteases at sufficiently high temperature. It was noted that no difference in α-amino group content was observed when gelatin extraction was conducted in the presence and absence of SBTI, at temperature higher than 55 °C. This indicated that proteases were more likely inactivated at high temperature. Therefore, hydrolysis of gelatin could be minimised by heating the pretreated skin at temperatures higher than 55 °C.



(A)



(B)

Figure 1. Effect of extracting temperatures on yield (A) and recovery (B) of gelatin from the skin of unicorn leatherjacket in the presence and the absence of SBTI at a level of 100 units/g pretreated skins. Bars represent the standard deviation (n=3). Different uppercase letters on the bars within the same temperature indicate significant difference ($p<0.05$). Different lowercase letters on the bars within the same condition of SBTI addition indicate significant difference ($p<0.05$).

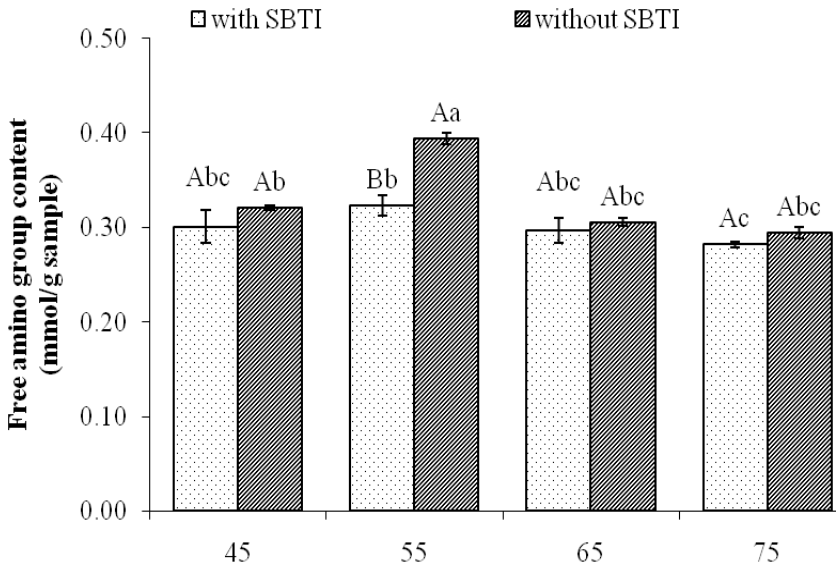
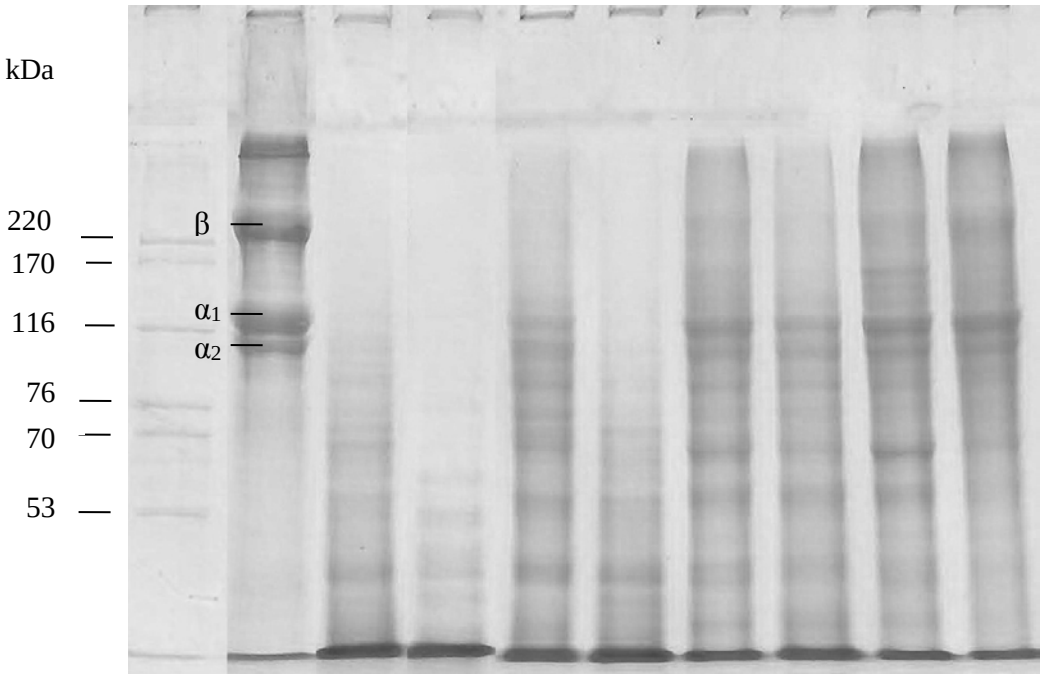


Figure 2. Effect of extracting temperatures on free amino group content of gelatin from the skin of unicorn leatherjacket in the presence and the absence of SBTI at a

level of 100 Units/g pretreated skins. Bars represent the standard deviation (n=3). Different uppercase letters on the bars within the same temperature indicate significant difference ($p<0.05$). Different lowercase letters on the bars within the same condition of SBTI addition indicate significant difference ($p<0.05$).

3.3. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

During conversion of collagen to gelatin, the inter- and intra-molecular bonds linking collagen chains as well as some peptide bonds are broken (Muyonga *et al*., 2004a). In the present study, the extraction at higher temperature did not cause the severe degradation, suggesting the high thermal stability of collagen of this species. When gelatins were extracted at 45 °C and 55 °C, no α-, β- and γ -chains were retained. This was more likely caused by the severe hydrolysis at temperature close to the optimal for indigenous proteases. This result was in accordance with Ahmad *et al*. (2011c) who reported that the highest degradation of β-chain and α-chains was obtained at 50 °C. When the SBTI was incorporated, gelatin extracted at 45 and 55 °C had more protein bands retained, especially α-chains. This indicated that SBTI was able to prevent degradation of α-chains to some degree. Nevertheless, the band intensity of β-chain and α-chains increased as the extraction temperature increased, particularly above 55 °C. This might be caused by the inactivation of the indigenous proteases at higher temperature. No differences in protein patterns were observed in gelatin extracted without and with SBTI incorporated ($P>0.05$) when extraction temperatures of 65 and 75 °C were used. These results were coincidental with those observed for α-amino group content (Fig 2).



HM C P/45 A/45 P/55 A/55 P/65 A/65 P/75 A/75

Figure 3. SDS-PAGE pattern of gelatin extracted from the skin of unicorn leatherjacket at different temperatures in the presence (P) and the absence (A) of SBTI at a level of 100 Units/g pretreated skins. C: collagen type I from calf skin. The numbers denote extraction temperature (°C).

3.4. Gel strength

Gel strength of gelatin from unicorn leatherjacket skin extracted under various conditions is shown in Fig. 4. Among all samples, gelatin extracted at 45 °C had the lowest gel strength ($P<0.05$). Gelatin extracted at higher temperature up to 65 °C possessed higher gel strength ($P<0.05$). Nevertheless, slight decrease in gel strength was found in gelatin extracted at 75 °C ($P<0.05$). When gelatin was extracted at 45 or 55°C, higher gel strength was obtained in samples extracted in the presence of SBTI ($P<0.05$). This indicated that SBTI might inhibit the degradation caused by indigenous proteases to some degree. Gelatin with the lower hydrolysis more likely had the longer chain. As a result, the interaction via inter-junction zone could take place, in which the stronger network was developed as indicated by the higher gel strength. Ahmad *et al*. (2011c) reported that the use of SBTI, as a protease inhibitor, during the extraction of gelatin from unicorn leatherjacket skin, generally increased the gel strength of resulting gelatin. This was mainly due to the maintenance of chain length, which was a prerequisite for better gelation. The difference in gel strength might be governed by molecular weight distribution, as well as the aggregation between gelatin molecules. The increase in gel strength was found when the extraction temperature increased. This correlated well with the presence of higher MW proteins or peptides (Fig. 3). Apart from chain length, the imino acid content, especially hydroxyproline content, affected gelation of gelatin (Aewsiri *et al*., 2008; Benjakul *et al*., 2009).

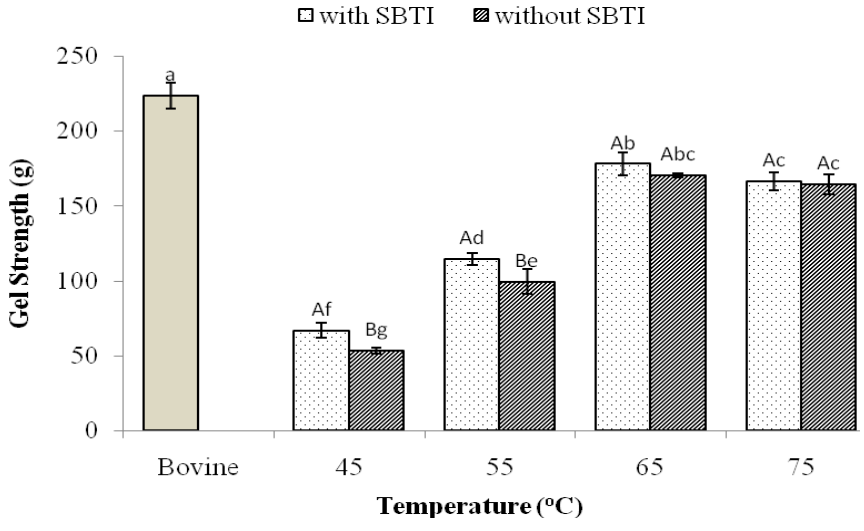


Figure 4. Gel strength of gelatin extracted from the skin of unicorn leatherjacket at different temperatures in the presence and the absence of SBTI at a level of 100 units/g pretreated skins. Bars represent the standard deviation (n=3). Different uppercase letters on the bars within the same temperature indicate significant difference ($p<0.05$). Different lowercase letters on the bars indicate significant difference ($p<0.05$).

3.5. FTIR spectra

FTIR spectra of gelatin extracted from unicorn leatherjacket skin in the presence and the absence of SBTI at different temperatures (45–75 °C) are depicted in Fig. 5. FTIR spectroscopy has been used to study changes in the functional groups and secondary structure of gelatin. All gelatin samples had the major peaks in amide region, but showed slight differences in the spectra. FTIR spectra of unicorn leatherjacket skin gelatin were similar to those found in other gelatins (Muyonga *et al*., 2004a). Amide I band of gelatins at 1700–1600 cm^{-1} was reported by Muyonga *et al*. (2004a). 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; Lavialle *et al*., 1982). The absorption peak at amide I was characteristic for the coil structure of gelatin *et al*., 2005). The amide I peak of gelatin extracted at 55 °C in the absence SBTI showed the higher amplitudes, compared that of other gelatins. This indicated the greater loss of triple helix and enhanced hydrolysis of collagen caused by heat-activated indigenous proteases during the extraction.

Amide II band of gelatins at 1560–1500 cm^{-1} was reported by Yakimets *et al*. (2005). 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; Lavialle *et al*., 1982). The amide II band is generally considered to be much more sensitive to hydration than to secondary structure change (Wellner *et al*., 1996). Small amide III bands of all gelatins were observed at 1234-1237 cm^{-1} which indicated the disorder in gelatin molecules and were associated with loss of triple helix state (Friess and Lee, 1996).

Furthermore, amide A peak was found at 3251-3296 cm^{-1}, representing NH-stretching coupled with hydrogen bonding. Normally, a free NH-stretching vibration occurs in the range of 3400-3440 cm^{-1}. When the NH group of a peptide is involved in a hydrogen bond, the position is shifted to lower frequencies (Doyle *et al*., 1975). Gelatin extracted at 55 °C or 45 °C in the absence of SBTI showed higher amplitude than others. This indicated

the higher free amino groups caused by the pronounced hydrolysis at these temperatures. This was in agreement with the higher α-amino group content (Fig. 2) and the marked degradation of α- and β-chains (Fig. 3). In the presence of SBTI, the lower amplitude for amide-A peak was found. This confirmed the role of SBTI in prevention of degradation. Amide B peak was found at 3072-3098 cm^{-1}, representing CH stretching and -NH_2.

Amplitude of peaks at wavenumbers of 1079 cm^{-1} and 1030 cm^{-1} increased when the gelatins were extracted with the addition of SBTI. Those peaks more likely represent the primary amines with a corresponding CN band at 1079 cm^{-1} and for compounds with primary and tertiary alpha carbon at 1030 cm^{-1} (Tellez *et al*., 2000). Those peaks might represent SBTI incorporated during gelatin extraction.

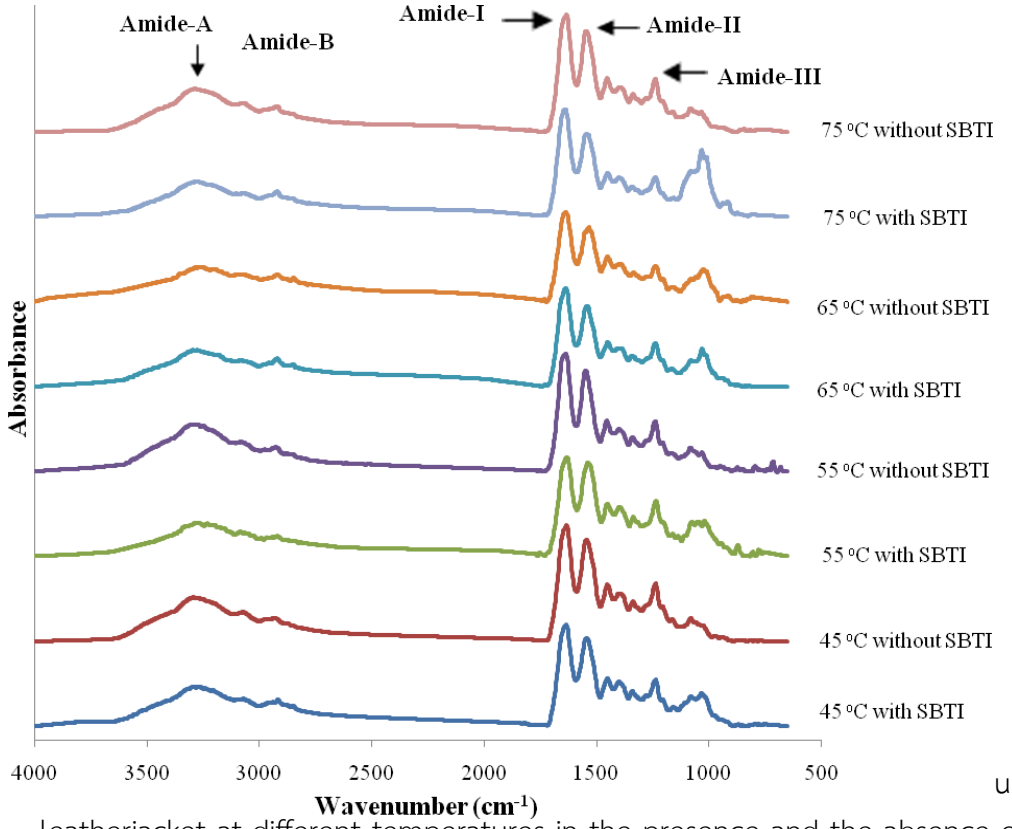


Figure unicorn leatherjacket at different temperatures in the presence and the absence of SBTI at a level of 100 units/g pretreated skins.

3.6. Emulsifying properties

Emulsion activity index (EAI) and emulsion stability index (ESI) of gelatin from the skin of unicorn leatherjacket extracted under different conditions are shown in Table 1. EAI of all gelatin samples decreased as the concentration of gelatin increased ($P < 0.05$). Gelatin extracted at 45 °C in the absence SBTI showed the highest EAI, compared with others ($P < 0.05$), when the same concentration of gelatin was used. This gelatin sample might contain a larger amount of short chain peptides, which were able to migrate to the

interface effectively and localised surrounding oil droplet at the faster rate than others with the larger size. Additionally, this sample might have more charged groups, especially amino or carboxyl groups at the end of peptides, thereby having the ability to facilitate the stabilisation of oil droplets via electrostatic repulsion. High solubility of protein in the dispersed phase increases the emulsifying efficiency, because the protein molecules should be able to migrate to the surface of the fat droplets rapidly (Sikorski, 2001). For gelatin extracted at 55 °C in the absence of SBTI, it had the highest degradation with shorter chain length than that extracted at 45 °C. The small peptides with higher polarity were preferably localised in the aqueous phase. As a result, the less peptides were situated at the interface. ESI of all gelatin samples increased as the concentration of gelatin increased ($P < 0.05$). The stabilisation of emulsion against coalescence/flocculation is greatly dependent on the force of electrostatic repulsions between the adsorbed proteins on the interfacial protein film (Aewsiri *et al*., 2009). Among all samples, gelatin extracted at 65 °C in the presence or absence of SBTI and gelatin extracted at 75 °C with SBTI had the higher ESI. Those samples contained the longer chain peptides, in which the thicker and stronger film surrounding oil droplets could be formed. The result suggested that extraction condition directly affected emulsifying property of gelatin.

3.7. Foaming properties

Foam expansion (FE) and foam stability (FS) of gelatin from the skin of unicorn leatherjacket extracted under various conditions are shown in Table 1. FE and FS of all gelatin samples increased as the concentration of gelatin increased ($P < 0.05$). Foams with higher concentration of proteins were denser and more stable owing to an increase in the thickness of interfacial films (Zayas, 1997). In general, the foaming ability of proteins is correlated with their film-forming ability at the air-water interface. When proteins rapidly adsorb at the newly created air-liquid interface during bubbling and undergo unfolding and molecular rearrangement at the interface, the better foam ability can be obtained, compared with proteins that adsorb slowly and resist unfolding at the interface (Damodaran, 1997). Gelatin extracted at 45 °C in the presence of SBTI had the lowest FE ($P<0.05$), while other gelatins showed similar FE. FS increased with protein concentrations ($P < 0.05$), depending on the protein surface properties.

Table 1. Emulsifying and foaming properties of gelatin from the skin of unicorn leatherjacket extracted at different extraction temperatures in the presence and the absence of SBTI at a level of 100 units/g pretreated skin.

Samples	Concentration (%w/v)	Emulsion activity index (m^2/g)	Emulsion stability index (min)	Foam expansion (%)	Foam stability (%)
45ºC with SBTI	1	10.69±0.09dA	31.28±1.30aA	163.75±1.77cC	123.13±0.88eC
	2	7.92±0.41cB	32.21±3.86abA	176.25±1.77cB	133.13±0.88eB
	3	6.98±0.51dC	34.09±3.45aA	187.5±0.00dA	144.38±4.42dA
45ºC without SBTI	1	28.04±1.43aA	19.96±2.06bcB	220.00±1.77bC	159.38±4.42cC
	2	12.13±1.15aB	25.86±2.99cB	229.38±2.65bB	191.25±1.77bcB
	3	9.12±0.41aC	36.77±4.19aA	241.25±1.77cA	200.63±0.88cA
55ºC with SBTI	1	19.85±1.88cA	13.90±0.59fC	216.25±5.30bC	160.00±5.34cC
	2	11.22±0.93abB	16.58±0.86dB	231.88±0.88bB	182.50±5.30dB
	3	8.37±0.32abC	19.06±1.76bA	243.13±0.88cA	212.50±0.00bA
55ºC without SBTI	1	23.75±1.82bA	15.41±1.33efB	215.00±3.54bC	149.38±2.65dC
	2	12.42±1.82aB	16.27±1.06dAB	228.13±0.88bB	187.50±0.00cdB
	3	8.98±0.58aC	18.07±0.53bA	241.25±5.30cA	212.50±0.00bA
65ºC with SBTI	1	23.90±1.52bA	17.18±0.61deC	216.88±2.65bC	180.00±5.30aC
	2	12.32±0.71aB	32.07±2.98abB	230.00±3.54bcdB	196.25±1.77bB
	3	9.17±0.38aC	37.39±1.16aA	246.25±5.30bcA	216.25±5.30bA
65ºC without SBTI	1	23.77±0.84bA	15.67±0.54efC	236.25±1.77aC	183.75±1.77aC
	2	10.10±0.79bB	27.69±2.78bcB	246.25±1.77aB	207.50±3.54aB
	3	7.91±0.66bcC	37.22±3.89aA	260±3.54aA	216.88±2.65bA
75ºC with SBTI	1	18.36±1.02cA	21.39±0.45bB	230.63±2.65aC	183.75±1.77aC
	2	11.91±0.89aB	34.79±3.53aA	241.88±0.88aB	206.25±5.30aB
	3	7.45±0.37cdC	39.69±3.48aA	253.75±5.30abA	243.13±4.42aA
75ºC without SBTI	1	8.73±0.27dA	19.00±0.86cdA	214.38±6.19bC	170.63±2.65bC
	2	7.56±0.20cB	19.06±0.66dA	229.38±0.88bB	183.13±2.65dB
	3	5.20±0.47eC	23.35±3.91bA	250.63±4.42bcA	210.63±4.42bA

Mean ± SD (n = 3). Different lowercase letters in the same column within the same concentration indicate significant differences (P<0.05).
Different uppercase letters in the same column within the same gelatin sample indicate significant differences (P<0.05)

FS is directly affected by protein concentration, which influences the thickness, mechanical strength and cohesiveness of film (Zayas, 1997). The stability of foams depends on various parameters, such as the rate of attaining equilibrium surface tension, bulk and surface viscosities, steric stabilisation, and electrical repulsion between the two sides of the foam lamella *et al*., 2003). Gelatin extracted at 45 ^{o}C in the presence of SBTI also exhibited the lowest FS, compared with others ($P<0.05$). Foaming property of gelatin was therefore governed by many factors including chain length, hydrophobicity, etc., which was influenced by the extraction condition.

4. Conclusion

Addition of SBTI during extraction of gelatin from the skin of unicorn leatherjacket at 45-55 ^{o}C could prevent degradation to some extent, but the resulting gelatin still had poor gelation property. Extraction at 65 ^{o}C or 75 ^{o}C was suggested for gelatin extraction from the skin of unicorn leatherjacket, and SBTI was not required. The gelatin with higher yield and better gel strength could be obtained from the skin at high temperature extraction, in which indigenous proteases were mostly inactivated.

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Chapter 3

Physicochemical and functional properties of gelatin from the skin of unicorn leatherjacket *(Aluterus monoceros)* as affected by extraction conditions

Abstract

Physicochemical and functional properties of gelatin from the skin of unicorn leatherjacket (*Aluterus monoceros*) extracted at different temperatures for various times were determined. Yield, recovery and free amino group content of gelatin increased, but gel strength generally decreased as the extraction temperature and time increased ($P < 0.05$). All gelatins contained α_1 and α_2 chains as the predominant components. FTIR spectra of all gelatins showed a significant loss of the triple-helix. Gels of gelatin extracted at higher temeprature for longer time had larger strands with larger voids. At the same level of gelatin, emulsifying and foaming properties varied with extraction conditions. Emulsion activity index (EAI) of all gelatins decreased with increasing concentrations ($P < 0.05$). Nevertheless, the highest emulsion stability index (ESI) was observed at a level of 3% ($P < 0.05$). Foam

expansion (FE) and foam stability (FS) of gelatin generally increased as the concentration increased ($P < 0.05$).

Keywords: *Unicorn leatherjacket, Gelatin, Gel strength, Aluterus monoceros, Emulsions, Foams*

1. Introduction

Gelatin is defined as a denatured protein derived from collagen by thermo-hydrolysis and the thermo-reversible transformation between sol and gel determines the property of gelatin (Cho *et al*., 2004). Gelatin has been widely used in the food, pharmaceutical, and photographic industries. Generally, the traditional sources of gelatin are bovine and porcine skins and bones. Nevertheless, gelatin from aquatic sources has been recognized to be free of infectious agents including the risk of contamination with bovine spongiform encephalopathy (BSE) and foot and mouth diseases (Sadowska *et al*., 2003). Additionally, it can be used to replace porcine gelatin, which is prohibited for Muslims or Jews and bovine gelatin, which is not consumed by Hindus (Karim and Bhat, 2009) and also usually not acceptable to Jews and Muslims.

Conversion of collagen into soluble gelatin is due to the cleavage of a number of intra- and intermolecular cross-linking bonds in collagen. As a result, the gelatin obtained generally has molecular weights lower than native collagen and constitutes a mixture of fragments with molecular weights in the range of 16–150 kDa (Asghar and Henrickson, 1982). The degree of conversion of collagen into gelatin depends on the raw material, pretreatment and processing parameters including temperature, time, and pH (Kołodziejska *et al*., 2008).

Unicorn leatherjacket has been used for fillet production in Thailand, especially for export as frozen fillets. Moreover, in Vietnam, annual production is about 34% (2.38 million tonnes) of the total catch (7 million tones). As a consequence, a large amount of skin is produced as a by-product. The skins of unicorn leatherjacket have been used for gelatin extraction (Ahmad and Benjakul, 2011). Due to the presence of indigenous proteases in the skin, the extraction at sufficiently high temperature, which was able to inactivate those proteases, yielded gelatin with less degradation. The pronounced degradation was found in gelatin from skins extracted at 45 and 55°C (Kaewruang *et al*., 2012). Thus, extraction at temperatures higher than 55°C gave gelatin with higher gel strength (Kaewruang *et al*., 2012). Increasing extraction time at an appropriate temperature may be an approach to increase yield, while still maintains the functional properties of gelatin. Nevertheless, there is no published information regarding the effect of extraction times, especially at high temperature, on gelatin from the skin of unicorn leatherjacket. Therefore, this investigation aimed to

elucidate the effect of extraction conditions, temperature and time, on the physical-chemical and functional properties of gelatin from the skin of unicorn leatherjacket.

2. Materials and methods

2.1. Chemicals, collagen and gelatin

All chemicals were of analytical grade unless otherwise noted. Phosphoric acid was obtained from Lab-Scan (Bangkok, Thailand). Sodium dodecyl sulfate (SDS), 2,4,6-trinitrobenzenesulfonic acid (TNBS), sodium sulfite and Coomassie Blue R-250 were purchased from Bio-Rad Laboratories (Hercules, CA, USA). L-leucine and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). High molecular weight markers were obtained from GE Healthcare UK (Little Chalfont, Buckinghamshire, UK). It consisted of myosin (220 kDa), α2- macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), lactate dehydrogenase (70 kDa) and glutamic dehydrogenase (53 kDa). Type I collagen from calf skin was purchased from Elastin Products Co., Inc. (Owensville, MO, USA). Food grade bovine bone gelatin (type B) with a bloom strength of 150-250 g was obtained from Halagel (Thailand) Co., Ltd. (Bangkok, Thailand).

2.2. Collection and preparation of fish skin

The skins of unicorn leatherjacket (*Aluterus monocerous)* were obtained from a dock in Songkhla, Thailand. The fish (250-330 g/fish) were skinned manually by the workers after off-loading, which was approximately 48 hr after capture. The skin samples were stored in ice with the approximately skin/ice ratio of 1:2 (w/w). Three different lots of skin (4-5 kg each) were obtained during March through May, 2012 and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 hr. Upon arrival, the skins were washed with iced tap water (0-2°C) and cut into small pieces (0.5×0.5 cm^2) with a scissor, placed in polyethylene bags and stored for no longer than 2 months at -20°C until use.

2.3. Non-collagenous protein removal and swelling of skin

Removal of non-collagenous proteins and swelling were done according to the method of Ahmad and Benjakul (2011) with a slight modification. Fish skin (0.5x0.5 cm^2) without scale removal was soaked in 0.05M NaOH with a skin/alkaline solution ratio of 1:10 (w/v) to remove non-collagenous proteins. The mixture was stirred continuously at room temperature using an overhead stirrer equipped with a propeller (RW 20.n, IKA Labortechnik, Staufen, Germany) at a speed of 150 rpm. The alkaline solution was changed every two hr and the total operation time was 4 hr. Alkaline-treated skin was washed with tap water until neutral or faintly basic pH of wash water using a pH meter (Schott, Mainz, Germany) was obtained. For swelling, the alkaline-treated skin was soaked in 0.05M phosphoric acid with a

skin/solution ratio of 1:10 (w/v) with a gentle stirring at a speed of 150 rpm using the overhead stirrer at room temperature. The acidic solution was changed every 6 hr and the total operation time was 12 hr. Owing to the thick and tough skin, alkaline and acid pretreatment with these long times were used. Acid-treated skin was washed thoroughly with tap water until the wash water became neutral or faintly acidic.

2.4. Extraction of gelatin

The swollen skin was mixed with distilled water at a ratio of 1:5 (w/v) at different temperatures (65 and 75°C). Those temperatures were reported to be able to inactivate the indigenous proteases in the skin of unicorn leatherjacket skin (Kaewruang *et al*., 2013). The mixture was incubated in a temperature controlled water bath (Memmert, Schwabach, Germany) and stirred continuously for various times (9, 12 and 15 hr) at a speed of 150 rpm using the overhead stirrer. The mixtures were centrifuged at 5,000 xg for 10 min at 25°C using a Beckman model Avanti J-E centrifuge (Beckman Coulter, Inc., Palo Alto, CA, USA) to remove insoluble material. The supernatant was collected and freeze-dried using a Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lynge, Denmark). The gelatin obtained was used for the further studies.

2.5. Analyses

2.5.1. Determination of yield and recovery

2.5.1.1. Gelatin yield was calculated by the following equation:

$$\textbf{Yield \%} = [\text{ weight of dry gelatin (g)/weight of initial dry skin (g) }] \times 100$$

Initial dry skin weight was obtained by weighing the whole skin without scale removal after drying at 105°C for 12 hr using a hot-air oven (Memmert, Schwabach, Germany).

$$\textbf{Recovery (\%)} = [\text{hydroxyproline content of supernatant (g/ml)} \times \text{volume of supernatant (ml)}] / [\text{ hydroxyproline content of initial skin (g/g)} \times \text{weight of initial skin (g) }] \times 100$$

Hydroxyproline content in both the supernatant and the initial dry skins was determined according to the method of Bergman and Loxley (1963).

2.5.2. Determination of chemical composition

2.5.2.1. α – amino group content

The α-amino group content was determined according to the method of Benjakul and Morrissey (1997). A properly diluted sample (125 µl) was mixed thoroughly with 2.0 ml of 0.2 M disodium hydrogen phosphate buffer, pH 8.2, followed by the addition of 1.0 ml of 0.01% TNBS solution. The mixture was then placed in the temperature controlled water bath at 50°C for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1M sodium sulfite. The mixture was cooled at room temperature for 15 min. The absorbance was measured at 420 nm using a double beam spectrophotometer (UV-160, Shimadzu, Kyoto, Japan). The sample blank was prepared in the same manner except that distilled water was used instead of the 0.01% TNBS solution. L-leucine standard solutions with concentrations ranging from 0.5 to 5.0 mM, were used. The α-amino group content was calculated after substraction of the blank and expressed as mole L-leucine/ g sample.

2.5.2.2. Protein patterns

SDS-PAGE was done using the method of Laemmli (1970). The samples were dissolved in 5% SDS and the mixtures were incubated at 85°C for 1 hr. Solubilized samples were mixed at a 1:1 (v/v) ratio with the sample buffer (0.5M Tris HCl, pH 6.8, containing 4% SDS and 20% glycerol). The mixtures were boiled in boiling water for 2 min. Samples (total amount of protein 15 µg as determined by the Biuret method using BSA as a standard) were loaded onto the polyacrylamide gels comprising a 7.5% running gel and a 4% stacking gel and electrophoresis was done at a constant current of 15mA/gel using a Mini Protein II unit (Bio-Rad, Hercules, CA, USA). After electrophoresis, the gel was stained with 0.05% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid for 4 hr and destained with 30% (v/v) methanol and 10% (v/v) acetic acid for 2 hr. Type I collagen was used as a standard.

2.5.3. Fourier transform infrared (FTIR) spectroscopy

FTIR analysis was done using a Bruker Model EQUINOX 55 FTIR spectrometer (Bruker, Ettlingen, Germany) equipped with a deuterated L-alanine triglycine sulphate (DLATGS) detector. The horizontal attenuated total reflectance (HATR) accessory was mounted in the sample compartment. The internal reflection crystal (Pike Technologies, Madison, WI, USA), made of zinc selenide, had a 45° angle of incidence from the IR beam. For spectra analysis, the samples were placed onto the crystal cell and the cell was clamped into the mount of the FTIR spectrometer. The spectra in the range of 650 – 4000 cm^{-1} with automatic signal gain were collected and averages for 32 scans at a resolution of 4 cm^{-1} and were recorded using the clean empty cell at 25°C. Normalisation was performed to obtain the same weight and

enable to make the comparison between spectra. Analysis of spectral data was done using the OPUS 3.0 data collection software programme (Bruker, Ettlingen, Germany).

2.5.4. Gel strength and microstructure

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

The microstructures of gelatin gels were visualized using scanning electron microscopy (SEM). Gelatin gels having a thickness of 2-3 mm were fixed with 2.5% (v/v) glutaraldehyde for 12 hr. The samples was then rinsed with distilled water for 1 hr and dehydrated in ethanol solutions 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 using a scanning electron microscope (Quanta 400, FEI, Eindhoven, Netherlands) at an acceleration voltage of 10 kV.

2.5.5. Emulsifying properties

The emulsion activity index (EAI) and emulsion stability index (ESI) of gelatin samples were determined according to the method of Pearce and Kinsella (1978) with a slight modification. Soybean oil (2 ml) and gelatin solution (1, 2 and 3%, w of powder/v, 6 ml) were homogenized at a speed of 20,000 rpm for 1 min using a homogeniser (model T25 basic, IKA LABORTECHNIK, Selangor, Malaysia). The emulsion was pipetted out at 0 and 10 min and diluted 100-fold with 0.1% SDS. The mixture was mixed thoroughly for 10 s using a Vortex mixer (Vortex-Genie 2, Scientific Industries, Bohemia, NY, USA). The A_{500} of the resulting dispersion was measured using the spectrophotometer. EAI and ESI were calculated using the following formulas:

$$EAI\ (m^2/g) = (2 \times 2.303 \times A \times DF) / \ l \phi C$$

where A = A_{500}, DF = dilution factor (100), l = path length of cuvette (0.01 m), ø = oil volume fraction (0.25) and C = protein concentration in aqueous phase (g/m^3)

$$ESI\ (min) = A_0 / \Delta A \times \Delta t$$

where A_0 and A_{10} = absorbance at 500 nm measured at time 0 and 10 min after emulsification, $\Delta A=A_0-A_{10}$ and Δt = 10 min.

2.5.6. Foaming proerties

Foam expansion (FE) and foam stability (FS) of gelatin solutions were determined as described by Shahidi *et al*. (1995) with a slight modification. Gelatin solutions (1, 2 and 3%, w/v) were transferred into 100 ml cylinders and homogenized at 13,400 rpm using the homogeniser for 1 min at room temperature (28-32°C). The sample was allowed to stand for 0 and 30 min. FE and FS were then calculated using the following equations:

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

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

where V_T is total volume after whipping; V_0 is the original volume before whipping and V_t is total volume after standing at room temperature for 30 min.

2.6. Protein determination

Protein content was determined by the Biuret method (Robinson and Hodgen, 1940), using bovine serum albumin (0-10 mg dry weight/ml) as a standard.

2.7. Statistical analysis

All experiments were run in triplicate using three different lots of samples. For each lot, all skins were pooled and used as a composite sample. The experiments and analyses were run in triplicate. (n=3). A Completely Randomized Design (CRD) was used throughout the study. Data were subjected to Analysis of Variance (ANOVA) and differences between means were evaluated using Duncan's multiple range tests. For pair comparisons, a *t*-test was used (Steel and Torrie, 1980). Statistical analysis was done using the Statistical Package for Social Sciences (SPSS 17.0 for Windows: SPSS Inc, Chicago, IL, USA)**.**

3. Results and discussion

3.1. Yield

The yield and recovery of gelatin from unicorn leatherjacket skin extracted with water at 65 or 75°C for 9, 12 and 15 hr are shown in Fig. 1. Both yield and recovery of gelatin increased as the extraction temperature and time increased ($P < 0.05$) (Fig. 1). The results suggested that gelatin, a denatured or partially hydrolyzed collagen, was extracted from pretreated skin to a higher extent as higher heat was applied. Yields of gelatin from the skin

extracted at 65 and 75°C were 11.6-14.5% and 13.1-15.9% (based on dry weight), respectively. The recovery of gelatin extracted at 65 and 75°C were 15.4-23.9% and 18.3-27.9% of total hydroxyproline, respectively. When the gelatin solution was freeze-dried, the hydroxyproline contents of the gelatin powders ranged from 93.4 to 96.0 mg/g powder (data not shown). Collagen from unicorn leatherjacket skin showed a T_{max} of 35.8°C (Ahmad *et al*., 2010). In the present study, temperatures above T_{max} were used, thereby facilitating the conversion of collagen to gelatin. The results suggested that the yield of gelatin could be increased by raising the extraction temperature. Kittiphattanabawon *et al*. (2010) also reported that higher yields were obtained for gelatin extracted from shark skin when temperature increased from 45 to 75°C. However, decreased gelling properties were obtained, most likely associated with higher degradation at higher extraction temperatures.

3.2. α-amino group content

The α-amino group content of gelatin from unicorn leatherjacket skin extracted under different conditions is shown in Fig. 2. At the same extraction temperature, an increase in α-amino group content was observed in gelatin when extraction time increased ($P < 0.05$). When the extraction time of 15 hr was used, the higher α-amino group content was found in gelatin extracted at 75°C, compared with 65°C ($P < 0.05$). The results suggested that thermal degradation was more pronounced at higher extraction temperatures and time as evidenced by the increased α-amino group content. Gelatin from Nile perch skin (Muyonga et al., 2004b), yellowfin tuna skin (Cho *et al*., 2005), and blacktip and brownbanded bamboo shark skin (Kittiphattanabawon *et al*., 2010) contained a higher amount of degradation peptides when higher extraction temperatures were employed. Coincidently, the increased α-amino group content was in agreement with increasing yield and recovery (Fig. 1).

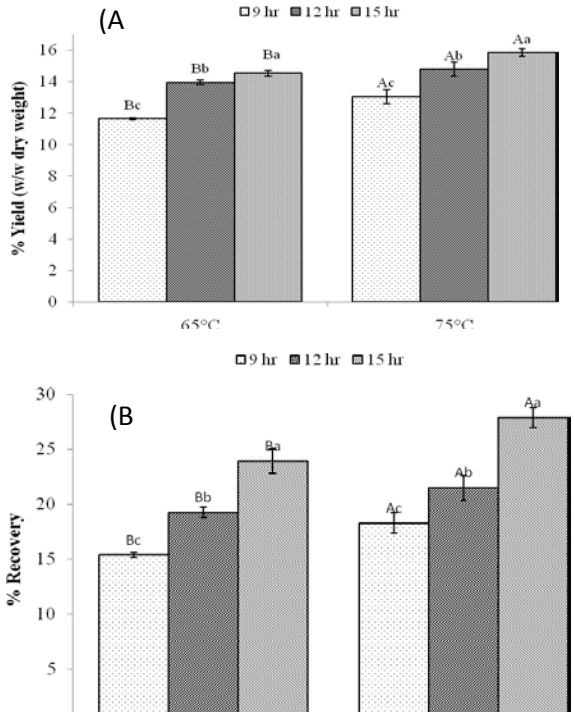


Figure 1 Yield (A) and recovery (B) of gelatin from the skins of unicorn leatherjacket as affected by extraction temperatures and times. Bars represent the standard deviation (n=3). Different uppercase letters on the bars within the same extraction time indicate significant differences ($P < 0.05$). Different lowercase letters on the bars within the same extraction temperature indicate significant differences ($P < 0.05$).

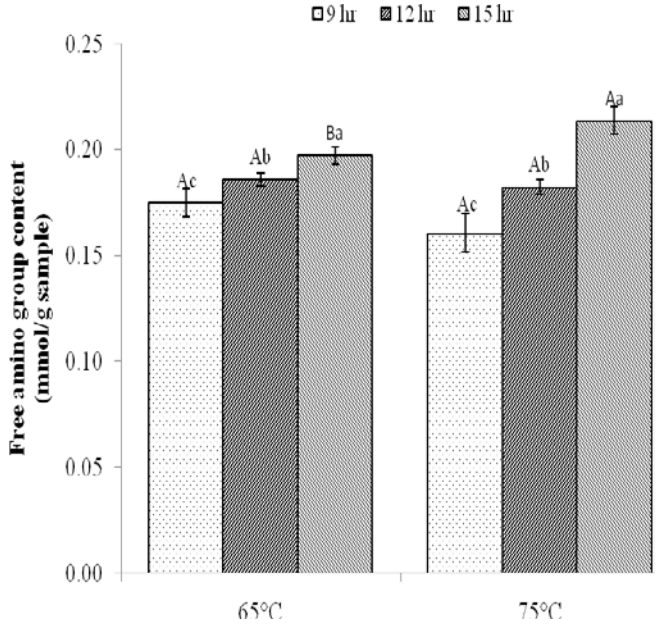
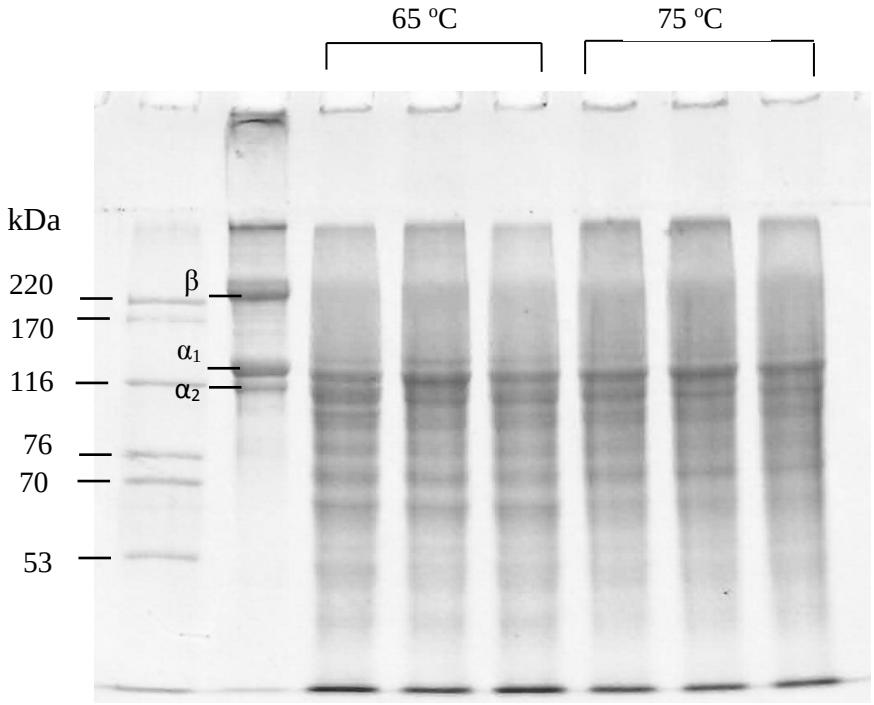


Figure 2 Free amino group content of gelatin from the skins of unicorn leatherjacket as affected by extraction temperatures and times. Bars represent the standard deviation (n=3). Different uppercase letters on the bars within the same extraction time indicate significant differences ($P < 0.05$). Different lowercase letters on the bars within the same extraction temperature indicate significant differences ($P < 0.05$).

3.3. Protein pattern

The protein patterns of gelatin extracted from unicorn leatherjacket skin at different temperatures for various times are shown in Fig. 3. All gelatins contained α-chains as the major components. Among all of the samples, gelatin extracted under the milder condition (65°C, 9 hr) showed a higher α_2-chain content (as observed visually) than the others. However, the α_2-chain was more degraded with the higher extraction temperature and longer times. It was noted that bands with MW of approximately 64, 70 and 75 kDa were also found, mainly in the sample extracted at 65°C. Nevertheless, low molecular weight molecules were also observed, suggesting that hydrolysis of peptides took place to some degree during extraction. During gelatin extraction, the conversion of collagen to gelatin with varying molecular mass took place, due to the cleavage of inter-chain cross-links (Zhou *et al*., 2006). More drastic degradation occurred in gelatin extracted at 75°C as shown by the decrease in band intensity of protein with a MW in the range of 64-75 kDa. Muyonga *et al*. (2004b) reported that gelatin from Nile perch skin and bone with higher extraction temperatures contained more peptides with MW less than the α-chain. The lower proportion of high MW (greater than the β-chain) fractions than those obtained using the lower extraction temperature was noticeable. The result was also in accordance with Kittiphattanabawon *et al*. (2010) who found a pronounced degradation of shark skin gelatin when extraction temperature increased. However, the degree of degradation depended on the species of shark.



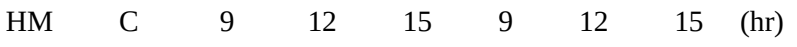


Figure 3 SDS-PAGE pattern of gelatin extracted from the skins of unicorn leatherjacket as affected by extraction temperatures and times. C: collagen type I from calf skin, HM: high MW markers.

3.4. Fourier transformed-infrared spectroscopy

FT-IR spectra of gelatins extracted using different conditions are shown in Fig. 4. FTIR spectroscopy has been used to monitor the functional groups and secondary structure of gelatin (Muyonga *et al*., 2004c). Spectra of gelatin showed the major peaks in the amide region. FTIR spectra of unicorn leatherjacket skin gelatin were similar to those found with other gelatins (Kittiphattanabawon *et al*., 2010; Muyonga *et al*., 2004a; Nagarajan *et al*., 2012). The absorption in the amide-I region, owing to C=O stretching vibration, is probably the most useful for infrared spectroscopic analysis of the secondary structure of proteins (Bandekar, 1992; Benjakul *et al*., 2009; Surewicz and Mantsch, 1988). Its exact location depends on the hydrogen bonding and the conformation of the protein structure (Benjakul *et al*., 2009; Uriarte-Montoya *et al*., 2011). In the present study, the amide-I peak was observed at 1631-1641 cm^{-1}, which is in agreement with Yakimets *et al*. (2005) who stated that the absorption peak at 1633 cm^{-1} was characteristic of the coiled structure of gelatin. The characteristic absorption bands in the amide-I region of gelatins extracted at 65°C for 9, 12 and 15 hr were noticeable at the wavenumbers of 1631, 1632 and 1635 cm^{-1} and were detected at the wavenumbers of 1631, 1635 and 1641 cm^{-1} for gelatins extracted at 75°C for 9, 12 and 15 hr, respectively. The amide-I peak of gelatin extracted for a longer time appeared at higher wavenumber, compared with those with the shorter time. This indicated the greater loss of triple helix due to the enhanced disruption of interchain interaction induced by harsher condition. Extraction at higher temperature with longer time led to the pronounced destruction of triple-helix via breaking down H-bonds between α-chains. All major absorption bands were shifted to the higher wavenumber when compared with those of collagen (Kittiphattanabawon *et al*., 2010). The greater disorder of the molecular structure owing to transformation of an α-helical to a random coil structure occurred during heating and these changes were associated with loss of triple-helix state as a result of denaturation of collagen to gelatin (Muyonga *et al*., 2004c).

The characteristic absorption bands of all gelatins in the amide-II region were noticeable at the wavenumbers of 1539-1541 cm^{-1}. In addition, the amide-III was detected at

the wavenumber of 1236 cm^{-1} for all gelatins. It was noted that the lowest amplitude of amide-III was found in gelatin extracted at 75°C for 15 hr.

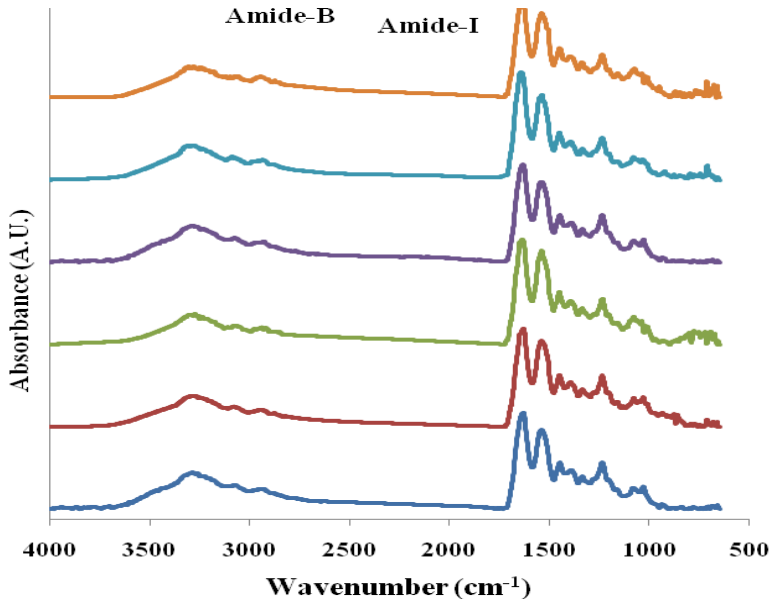


Figure 4 Fourier transform infrared spectra of gelatin extracted from the skins of unicorn leatherjacket as affected by extraction temperatures and times.

Moreover, the amide-A band, arising from the stretching vibrations of N-H group, were seen at 3286, 3280 and 3273 cm^{-1} for gelatin extracted at 65°C for 9, 12 and 15 hr and at 3286, 3286 and 3278 cm^{-1} for gelatin extracted at 75°C for 9, 12 and 15 hr, respectively. The amide-A band is associated with the N–H stretching vibration showed the existence of hydrogen bonds. Normally, a free N–H stretching vibration occurs in the range of 3400–3440 cm^{-1}. When the N–H group of a peptide is involved in a H-bond, the position is shifted to lower frequencies (Doyle *et al*., 1975). The amide-A also tends to join with the CH_2 stretch peak when carboxylic acid groups exist in a dimeric inter-molecular interaction (Kemp, 1987). The shift to the lower wavenumber of the amide-A observed in gelatin extracted for a longer time was plausibly associated with a more pronounced degradation of gelatin. These free amino group released from the degraded gelatins might undergo interaction with other reactive groups, resulting in the decreased wavenumber of the amide-A. The amide-B was observed at wavenumbers of 3076, 3080 and 3066 cm^{-1} for gelatin extracted at 65°C for 9, 12 and 15 hr and at 3074, 3086 and 3072 cm^{-1} for gelatin extracted at 75°C for 9, 12 and 15 hr, respectively. Gelatin extracted for 15 hr showed the lowest wavenumber for the amide-B

peak, suggesting the interaction of -CH_2 groups between peptide chains. Thus, it can be concluded that the secondary structure and functional group of gelatins obtained from the skin of unicorn leatherlacket was affected by extraction temperature and time.

3.5. Gel strength and microstructure

The gel strength of gelatin from the unicorn leatherjacket skin extracted at various temperatures for different times is shown in Fig. 5. Gel strength generally decreased as the extraction temperature and time increased ($P < 0.05$), except for gelatin extracted for 9 and 12 hr at 65°C, which showed similar values ($P > 0.05$). The result was in agreement with Gómez-Guillén *et al*. (2002) who reported that gelatin extracted at higher temperatures showed a lower gel strength. Among all samples, the lowest gel strength was observed for gelatin extracted at 75°C for 15 hr (88.1 g) ($P < 0.05$). This was probably associated with the highest degradation of this sample as indicated by its having the highest α-amino group content (Fig. 2) and the highest amount of degradation (Fig. 3). In general, chain length of gelatin molecules is the major factor governing the gelation process. The interconnection of gelatin moleculars is more favorable, when long chains are present (Giménez *et al*., 2005). It was noted 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. Based on the protein patterns (Fig. 3), proteins with MW ranging from 20-60 kDa were found in gelatin extracted at 65°C. Those proteins disappeared when the extraction temperature of 75°C was used. Due to the higher gel strength of gelatin extracted at 65°C, it was presumed that those proteins might partially contribute to gelation of gelatin. However, gelatin from the skin of unicorn leatherjacket had a lower gel strength than bovine gelatin (193 g). This might be due to the higher content of hydroxyproline in bovine gelatin (Kaewruang *et al*., 2012).

According to Arnesen and Gildberg (2002), the low hydroxyproline content in fish skin gelatin was associated with low gel strength. Apart from hydroxyproline content, the purity of gelatin was considered as another factor governing the gel strength. In the present study, the purity of gelatin was in the ranges of 93-96%, calculated using the conversion factor of 9.96. The conversion factor determined based on the hydroxyproline content in collagen extracted from the skin of unicorn leatherjacket (Ahmad *et al*., 2010). Thus, gel strength of gelatin from the skin of unicorn leatherjacket was directly influenced by extraction conditions.

The gel microstructures of gelatin from the skins of unicorn leatherjacket and commercial bovine gelatin are shown in Fig. 6. Generally, the arrangement and association of protein molecules in the gel matrix directly contributed to the gel strength of the gelatin (Benjakul *et al*., 2009). The gel from bovine gelatin showed the finest gel network with very

small voids, while that from unicorn leatherjacket skin had larger strands with larger voids. The finer gel structure of commercial bovine gelatin was in accordance with the higher bloom strength (Fig. 5). All gelatin gels were sponge or coral-like in structure. The gel network became coarser with larger voids as the extraction temperature and time increased. Gelatin extracted at 65°C showed a uniform network with the thinner strands, whereas those extracted at 75°C had larger strands with larger voids (Fig. 6). The finer network structure of gels of gelatin extracted at 65°C was in accordance with their higher bloom strength (Fig. 5), compared with that of gelatin extracted at 75°C, which had coarser gel structures. The coarser network of the gel might be easier to disrupt by the force applied.

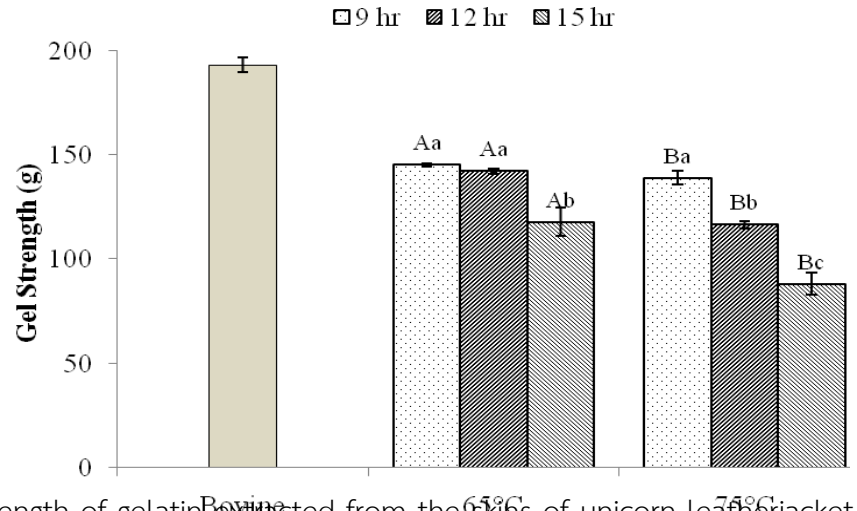


Figure 5 Gel strength of gelatin extracted from the skins of unicorn leatherjacket as affected by extraction temperatures and times. Bars represent the standard deviation (n=3). Different uppercase letters on the bars within the same extraction time indicate significant differences ($P < 0.05$). Different lowercase letters on the bars within the same extraction temperature indicate significant differences ($P < 0.05$).

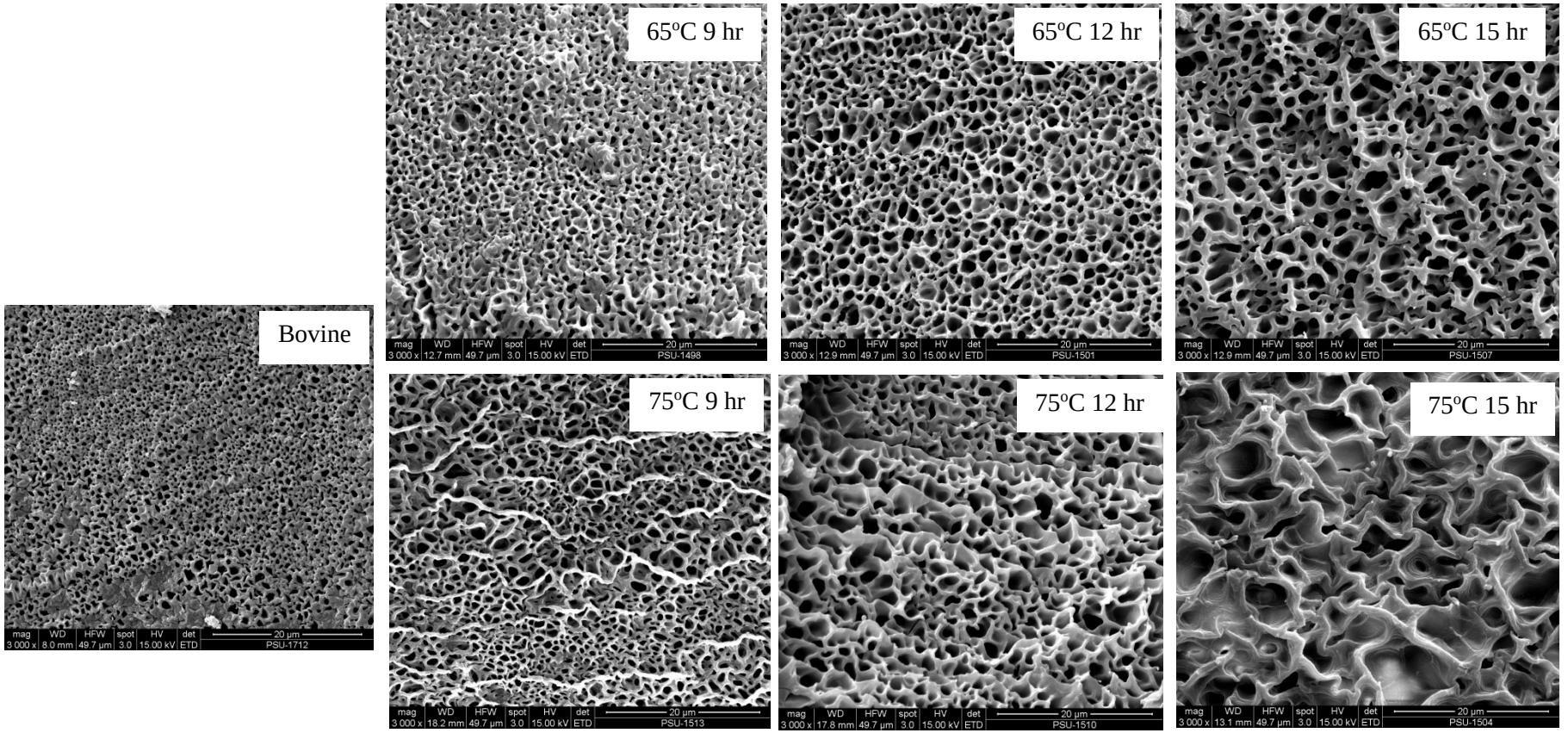


Figure 6 SEM microstructure of gels of gelatins from the skins of unicorn leatherjacket as affected by extraction temperatures and times. Magnification: 3000x.

3.6. Emulsifying properties

The emulsion activity index (EAI) and emulsion stability index (ESI) of gelatin are shown in Table 1. The EAI of all gelatins at 1% were higher than those found at higher concentrations (2% and 3%) ($P < 0.05$). However, no difference in EAI was observed between 2 and 3% sample ($P > 0.05$). At high concentrations, gelatin molecules, which have a high hydrophilicity might interact with each other. Thus, less gelatin was available to be localized at the oil-water interface. At a level of 1%, gelatin extracted at 75°C showed higher EAI than that extracted at 65°C. The shorter chains in the former might migrate to the oil-water interface faster, in which case the emulsion could be stabilized to a higher degree. For ESI, the higher ESI was found when a concentration of 3% was used ($P < 0.05$), except for gelatin extracted at 75°C for 9 hr, which showed similar values between 2 and 3% ($P > 0.05$). Proteins at higher concentrations might form a stronger layer surrounding the oil droplets, thereby preventing emulsion collapse (Yamauchi *et al*., 1980). The stabilization of an emulsion against coalescence/flocculation is greatly dependent on the force of electrostatic repulsions between the adsorbed proteins on the interfacial protein film (Aewsiri *et al*., 2009). The result suggested that extraction conditions had an influence on emulsifying activity of resulting gelatin.

3.7. Foaming properties

Foam expansion (FE) and foam stability (FS) of the gelatins are shown in Table 1. Foam formation is generally controlled by transportation, penetration and reorganization of protein molecules at the air-water interface (Hailing and Walstra, 1981). A protein must be capable of migrating rapidly to the air-water interface, unfolding and rearranging at the interface to show good foaming ability (Hailing and Walstra, 1981). FE and FS of all gelatin samples generally increased with increasing concentrations ($P < 0.05$). Foams with higher protein concentrations are denser and more stable because of an increase in the thickness of the interfacial films (Zayas, 1997). The stability of foams depends on various parameters, such as the rate of attaining equilibrium surface tension, bulk and surface viscosities, steric stabilization, and electrical repulsion between the two sides of the foam lamella (Liu *et al*., 2003).

Table 1 Emulsifying and foaming properties of gelatin from the skin of unicorn leatherjacket extracted at different temperatures for various times.

Samples	Concentration (% w/v)	Emulsion activity index (m^2/g)	Emulsion stability index (min)	Foam expansion (%)	Foam stability (%)
65 ºC for 9 h	1	7.00 ± 0.640cA	22.8 ± 2.55aB	183 ± 0.7dC	95.2 ± 5.39cC
	2	4.17 ± 0.011bB	23.0 ± 0.40aB	193 ± 2.0deB	114 ± 0.0cB
	3	3.85 ± 0.051bcB	34.7 ± 2.71aA	231 ± 0.9abA	139 ± 6.7cdA
65 ºC for 12 h	1	8.34 ± 0.631bcA	17.9 ± 1.22bcB	185 ± 0.4dC	96.3 ± 5.24cC
	2	5.06 ± 0.103aB	20.2 ± 3.67abcB	190 ± 0.3eB	120 ± 6.6cB
	3	4.88 ± 0.341aB	30.9 ± 2.10abA	227 ± 2.6bA	157 ± 3.7bA
65 ºC for 15 h	1	8.24 ± 0.770bcA	20.3 ± 1.35bB	216 ± 0.9aC	93.1 ± 2.70cC
	2	4.20 ± 0.442bB	20.9 ± 3.14abB	223 ± 0.7aB	117 ± 0.7cB
	3	3.68 ± 0.261cB	29.9 ± 2.75bA	235 ± 3.6aA	128 ± 2.6eA
75 ºC for 9 h	1	9.34 ± 0.882bA	17.7 ± 0.79bcB	180 ± 3.9dC	97.2 ± 1.31cC
	2	4.77 ± 0.072abB	18.0 ± 1.07bcAB	217 ± 2.6bB	113 ± 4.4cB
	3	4.65 ± 0.053abB	21.8 ± 3.13cA	234 ± 1.0aA	135 ± 2.1deA
75 ºC for 12 h	1	8.44 ± 0.191bcA	18.7 ± 0.27bcB	194 ± 0.3cC	145 ± 0.1aC
	2	5.30 ± 0.341aB	18.8 ± 1.59abcB	196 ± 0.5dB	152 ± 0.4aB
	3	4.57 ± 0.442abC	33.5 ± 2.11abA	200 ± 0.0dA	185 ± 3.5aA
75 ºC for 15 h	1	10.9 ± 1.51aA	16.5 ± 0.61cB	204 ± 3.0bC	109 ± 4.8bC
	2	4.92 ± 0.560aB	16.6 ± 1.69cB	212 ± 1.8cB	134 ± 0.9bB
	3	4.80 ± 0.881aB	20.2 ± 1.71cA	220 ± 2.2cA	146 ± 2.1bcA

Mean ± SD (n = 3).

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

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

In general, gelatin extracted for increasing times had the higher foam expansion than when a level of 1% was used ($P < 0.05$). This might be due to the better migration of shorter peptides to the air-water interface. Among all samples, gelatin extracted at 75°C for 12 hr had the highest FS ($P < 0.05$). The result suggested that molecular properties of gelatin as affected by extraction condition played a role in foam stabilization.

4. Conclusions

Gelatin from unicorn leatherjacket skin extracted at 65°C showed the higher gel strength than that extracted at 75°C. Longer extraction times resulted in increased yield, but poorer gelling properties, most likely associated with higher degradation. Extraction conditions also affected the interfacial properties of the resulting gelatin. The appropriate extraction conditions for gelatin from unicorn leatherjacket were 65°C for 12 hr, providing the best yield and gel strength.

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Chapter 4

Characteristics and gelling property of phosphorylated gelatin from the skin of unicorn leatherjacket (*Aluterus monocerous*)

Abstract

Characteristics and gelling property of gelatin from the skin of unicorn leatherjacket (*Aluterus monocerous)* phosphorylated with sodium tripolyphosphate (STPP) at various concentration (0.25, 0.50, 0.75 and 1.00% w/w) for different times (1 and 3 h) at 65 °C were studied. With increasing STPP concentration and time, no increase in bound phosphate was observed. Highest gel strength was obtained for gelatin phosphorylated using 0.25% STPP for 1 h ($P < 0.05$). When the effect of pH (5, 7, 9 and 11) on phosphorylation and gel property of gelatin was investigated, gelatin phosphorylated at pH 9 had the highest gel strength (204.3 g) ($P < 0.05$) and exhibited a finer and more compact network structure with smaller pores. Gelatin became negatively charged (-3.89 mV) and might undergo ionic interaction to a higher extent, thereby strengthening gel network. Thus, the phosphorylation under the appropriate condition could improve gelling property of gelatin from the skin of unicorn leatherjacket.

Keywords: *Unicorn leatherjacket, Phosphorylation, Gelling property, Gelatin, Sodium tripolyphosphate*

1. Introduction

Gelatin, the denatured form of collagen, has been widely used in the food and pharmaceutical industries as well as for other technical applications (Kaewruang *et al*. 2013b). Generally, gelatin is produced from skins and skeletons of bovine and porcine (Kittiphattanabawon *et al*., 2005). However, the occurrence of bovine spongiform encephalopathy (BSE) and foot-and-mouth disease (FMD) have led to the major concern for human health. Furthermore, porcine and bovine gelatins are prohibited for some religions (Sadowska *et al*., 2003). As a consequence, fish gelatin has gained increasing interest as the potential alternative for land animal counterpart. Fish skin, fin, scale and bones, etc., are the abundant by-product from the fish processing industry. They have been used as promising raw material for gelatin production (Ahmad and Benjakul, 2011). However, fish gelatins have lower gel strength, compared with mammalian counterpart, more likely due to the lower imino acid content (Foegeding *et al*., 1996).

Phosphorylation has been used to improve the functional properties of food proteins. Water solubility, emulsifying activity and gel-forming properties of food proteins are improved by phosphorylation (Li *et al*., 2004). Zhang *et al*. (2007) reported that phosphorylation of soy protein isolate with sodium tripolyphosphate (STPP) could improve its functional properties. Recently, Kaewruang *et al*. (2013a) reported that phosphorylation of gelatin by incorporation of STPP during extraction at an appropriate level was able to increases gel strength of resulting gelatin. Nevertheless, phosphorylation of gelatin might be governed by several factors, e.g. amount of phosphate, pH, etc. Additionally, the level of phosphates bound to gelatin might affect gelation of gelatin. Therefore, the objective of this investigation was to study the effect of phosphate level, time and pH on phosphorylation and gel properties of gelatin from the skin of unicorn leatherjacket.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade. Sodium tripolyphosphate (STPP), ammonium molybdate and glutaraldehyde were purchased from Sigma (St Louis, MO, USA). Food grade bovine bone gelatin with the bloom strength of 150-250 g was obtained from Halagel (Thailand) Co., Ltd. (Bangkok, Thailand).

2.2. Collection and preparation of fish skin

The skin of unicorn leatherjacket (*Aluterus monocerous)* was obtained from a dock, Songkhla, Thailand, stored in ice with a skin/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Upon arrival, the skin was washed with iced tap water (0-2°C) and cut into small pieces (0.5×0.5 cm^2), placed in polyethylene bags and stored at -20°C until use. The storage time was less than 2 months.

2.3. Pretreatment of skin

Removal of non-collagenous proteins and swelling of prepared skin were carried out according to the method of Ahmad and Benjakul (2011). Fish skin (0.5x0.5 cm^2) was soaked in 0.05 M NaOH with a skin/solution ratio of 1:10 (w/v) to remove non-collagenous proteins. The mixture was stirred continuously at room temperature using an overhead stirrer equipped with a propeller (RW 20.n, IKA Labortechnik, Germany) at a speed of 150 rpm. The alkaline solution was changed every 2 h for totally 4 h. Alkaline-treated skin was washed with tap water until neutral or faintly basic pH of wash water was obtained. For swelling process, the alkaline-treated skin was soaked in 0.05 M phosphoric acid with a skin/solution ratio of 1:10 (w/v) with a gentle stirring at room temperature. The acidic solution was changed every 3 h for totally 6 h. Swollen skin was washed thoroughly with tap water until wash water became neutral or faintly acidic.

2.4. Extraction of gelatin

The swollen skin was mixed with distilled water (65 °C) at a ratio of 1:5 (w/v). The mixture was incubated at 65 °C for 12 h in a temperature controlled water bath (Memmert, Schwabach, Germany) and stirred continuously at a speed of 150 rpm using the overhead stirrer equipped with propeller. The mixture was centrifuged at 5,000 xg for 10 min at 25°C using a Beckman model Avanti J-E centrifuge (Beckman Coulter, Inc., Palo Alto, CA, USA) to remove insoluble material. The solution was freeze-dried using a Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lynge, Denmark). The resulting gelatin was referred to as "control gelatin"

2.5. Phosphorylation of gelatin

2.5.1. Effect of STPP levels and times

To gelatin solution obtained after extraction, STPP was added to obtain final concentrations of 0.25, 0.50, 0.75 and 1.00 % (w/w gelatin). All solutions were adjusted to pH 7 using 1 M HCl. The solution was continuously stirred at 65 °C for different times

(1 and 3 h). The solution was cooled and freeze-dried. The gelatin samples were subjected to analyses of phosphate content and gel strength.

2.5.2. Effect of pHs

To study the effect of pH on phosphorylation, gelatin solution containing 0.25% STPP (w/w gelatin) was adjusted to various pH (5, 7, 9 and 11) using 1 M NaOH or 1 M HCl. The mixture was continuously stirred at 65 °C for 1 h. The solution was cooled and freeze-dried. Gelatins phosphorylated at different pHs were subjected to analyses of phosphate content and zeta potential and gel strength. Gelatins phosphorylated at the selected pHs were determined for FTIR spectra and microstructure.

2.6. Analyses

2.6.1. Determination of inorganic phosphate content

To determine the bound phosphate, gelatin samples were dissolved in distilled water to obtain the concentration of 0.5% (w/v). Then, gelatin solution was dialysed against 20 volumes of distilled water overnight. The dialysis water was changed every 3 h for totally four times. The resulting dialysate was then freeze-dried. Both non-dialysed and dialysed samples were determined for phosphate content.

Inorganic phosphate content was determined according to the method of Fiske and Subbarow (1925) with a slight modification. Gelatin sample (~1 g) was ashed at 550 °C to remove organic compounds using a muffle furnace (Fisher Scientific Model 550-58, Napean, Ontario, Canada) for 9 h. Thereafter, the inorganic residue was dissolved in 85% nitric acid. The solution was adjusted to 10 ml with distilled water. An aliquot of solution was subjected to phosphate determination using a spectrophotometric method, in which ammonium molybdate was used and the colored reaction mixture was monitored at the absorbance at 640 nm (Fiske and Subbarow, 1925). Potassium dihydrogen phosphate (KH_2PO_4) solutions with the concentration range of 0-1 mM were used for standard curve preparation. Phosphate content was expressed as μmol/g gelatin.

2.6.2. Determination of gel strength

Gelatin gel was prepared as per the method of Kaewruang *et al*. (2013b). Gelatin sample was dissolved in distilled water (60 °C) to obtain the final concentration of 6.67% (w/v). The gelatin solution was stirred using a magnetic stirrer (IKA-Werke, GMBH & COKG, Staufen, Germany) at a medium speed at room temperature until the gelatin was solubilised completely. Gelatin solution was cooled in a refrigerator at 10 °C for 16-18 h for gel maturation. The dimension of gel sample was 3 cm in diameter and 2.5 cm in height. The gel strength was determined using a Model TA-XT2 Texture Analyser (Stable Micro System, Surrey, UK) with a load cell of 5 kN and equipped with a 1.27 cm diameter flat faced cylindrical Teflon® plunger. The maximum force (in gram)

was recorded when the penetration distance reached 4 mm. The speed of the plunger was 0.5 mm/s.

2.6.3. Measurement of zeta potential

Gelatin samples were dissolved in distilled water at a concentration of 0.5 mg/ml. Prior to analysis, the samples were adjusted to pH 7. Zeta potential was measured using a zeta potential analyser (ZetaPALS, Brookhaven Instruments Co., Holtsville, NY, USA) at room temperature.

2.6.4. Fourier transform infrared (FTIR) spectroscopy

FTIR analysis was performed using a Bruker Model EQUINOX 55 FTIR spectrometer (Bruker, Ettlingen, Germany) equipped with a deuterated L-alanine triglycine sulphate (DLATGS) detector. The horizontal attenuated total reflectance (HATR) accessory was mounted in the sample compartment. The internal reflection crystal (Pike Technologies, Madison, WI, USA), made of zinc selenide, had a 45° angle of incidence from the IR beam. For spectra analysis, the samples were placed onto the crystal cell and the cell was clamped into the mount of the FTIR spectrometer. The spectra in the range of 650 – 4000 cm^{-1} with automatic signal gain were collected and averages for 32 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean empty cell at 25°C. Analysis of spectral data was done using the OPUS 3.0 data collection software programme (Bruker, Ettlingen, Germany).

2.6.5. Microstructure analysis

The microstructures of gelatin gels were visualised using a scanning electron microscopy (SEM). Gelatin gels having a thickness of 2-3 mm were fixed with 2.5% (v/v) glutaraldehyde for 12 h. The samples were then rinsed with distilled water for 1 h and dehydrated in ethanol solution 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 using a scanning electron microscope (Quanta 400, FEI, Eindhoven, Netherlands) at an acceleration voltage of 10 kV.

2.7. Statistical analyses

All experiments were performed in triplicate using and a completely randomised design (CRD) was used. Data were presented as means ± standard deviation and the probability value of $P<0.05$ was considered significant. Analysis of variance (ANOVA) was performed and mean comparisons were done by Duncan's multiple range test (Steel and Torrie, 1980). Analysis was performed using an SPSS package (SPSS 17.0 for windows, SPSS Inc, Chicago, IL, USA).

3. Results and discussion

3.1. Effect of STPP level and time on phosphorylation and gel strength of gelatin

3.1.1. Phosphate content

Phosphate contents of gelatin phosphorylated by incorporating STPP at various levels for different times are shown in Fig. 1. Gelatin phosphorylated with STPP had higher phosphate content than the control gelatin (without phosphorylation) ($P < 0.05$). Phosphate content in gelatin without dialysis increased with increasing levels of STPP used (0.25-1.00 %w/w) (Fig. 1A). No difference in phosphate content was observed between gelatin phosphorylated for 1 h and 3 h, when the same STPP level was used. Nevertheless, after dialysis, similar phosphate content (58.2-63.0 μmol/ g gelatin powder) was obtained in all samples, regardless of STPP level and time used (Fig. 1B). The result suggested that STPP at concentration above 0.25% (w/w) had no effect on incorporation of phosphate into gelatin. Due to the limited degree of phosphorylation of gelatin for phosphate incorporation, the excessive amount was present in the free form and could be removed by dialysis process. Phosphate groups can be attached to the oxygen of seryl, threonyl, aspartyl (β-carboxyl) and tyrosyl residues of proteins (Zhang *et al*., 2007)

3.1.2. Gel strength

Gel formation is the most important functional properties of gelatin. Gel strength of gelatin phosphorylated with STPP at different concentrations for various times is presented in Fig. 2. For gelatin phosphorylated with STPP for 1 h, the highest gel strength was observed as STPP at a level of 0.25% was used ($P < 0.05$). However, the decrease in gel strength was found as the level of STPP increased ($P < 0.05$). Since gelatin was not dialysed to remove free phosphate, those phosphates might interfere or prevent the interaction of gelatin chains. The excessive phosphate in gelatin or free phosphates more likely increased the electrostatic repulsions between protein molecules (Zhang *et al*., 2007). This was evidenced by the lower gel strength. Gelatin phosphorylated with 0.25% STPP might provide the negative charge via phosphate groups at an appropriate level, thereby enhancing the aggregation with the positively charged adjacent gelatin chains via ionic interaction. It was found that this gelatin had the higher gel strength (182.9 g) than the control gelatin (166.0 g). For gelatin phosphorylated with STPP for 3 h, all samples had the lower gel strength (130.8-134.6 g), irrespective of STPP levels used. With a longer time (3 h), phosphates, both bound and free forms, might modify the gelatin chains, in the fashion which did not favour the aggregation between chains. For the sample phosphorylated for 3 h, phosphorylated gelatin chains might undergo aggregation to form the larger bundles, in which the finer and non-uniform gel network could not be developed. Based on gel strength, the

phosphorylation of gelatin using 0.25% STPP at 65 ℃ for 1 h resulted in the increased gel strength of gelatin from the skin of unicorn leatherjacket.

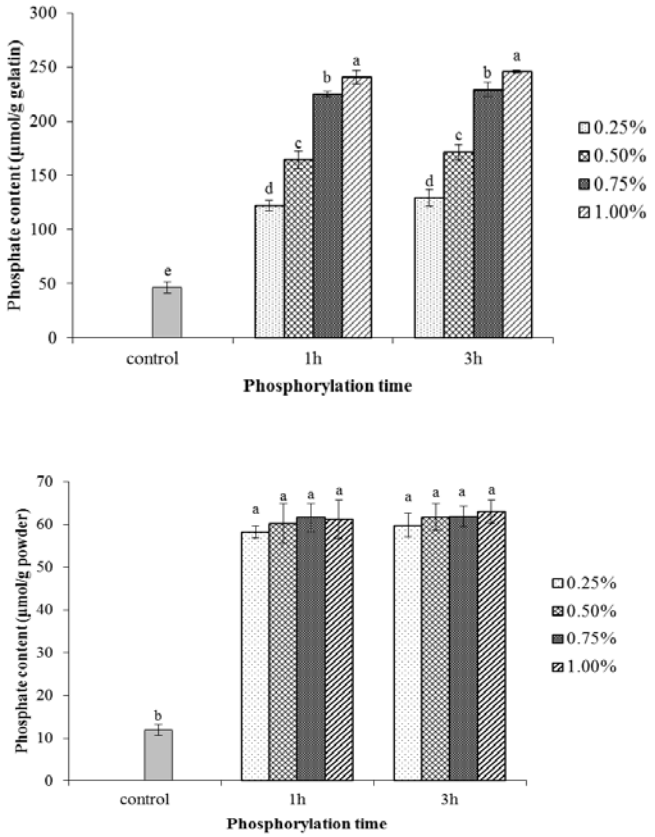

Figure 1 Phosphate content of gelatins from the skin of unicorn leatherjacket phosphorylated using STPP at different levels for various times without dialysis (A) and after dialysis (B). Bars represent the standard deviation (n=3). Different letters on the bars indicate significant difference ($p < 0.05$).

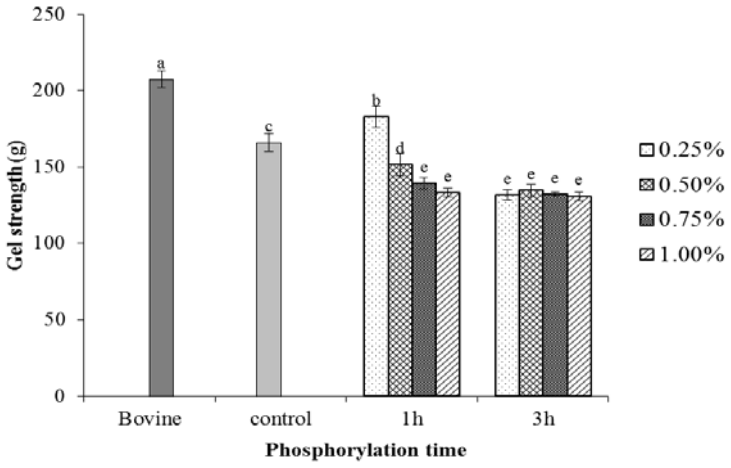

Figure 2 Gel strength of gelatins from the skin of unicorn leatherjacket phosphorylated using STPP at different levels for various times. Bars represent the standard deviation (n=3). Different letters on the bars indicate significant difference ($p < 0.05$).

3.2 Effect of pH on phosphorylation and properties of gelatin

3.2.1. Phosphate content and zeta potential

Phosphate contents of gelatin phosphorylated with 0.25% STPP at different pHs for 1 h are shown in Table 1. Increasing phosphate contents were noticeable as pH increased ($P < 0.05$). The results were in accordance with Li *et al*. (2004) who reported that the level of phosphorus in modified potato protein isolate increased with increasing pH. High energy phosphate compounds could react with OH and NH_2 groups on the side chains of proteins. Free OH groups in the protein molecules showed higher reactivity only in the alkaline conditions, whereas free NH_2 groups, with higher reacting activity, could react in both neutral and alkaline conditions (Miedzianka and Pęksa, 2013). As a result, phosphate could be attached to gelatin at higher extent in the alkaline pH range. It was noted that phosphate bound in control gelatin was more likely from phosphoric acid pretreatment during swelling process, used for production of control gelatin.

Zeta potential of phosphorylated gelatin and control gelatin (without phosphorylation), at pH 7 is shown in Table 1. The negative charge was observed in gelatin phosphorylated with STPP in alkaline condition. The higher negative charge was noticeable when higher pH was used. The result reconfirmed that phosphate groups were preferably attached to gelatin under alkaline condition, thereby providing negative charge to the resulting gelatin. For gelatin phosphorylated with STPP at neutral or acidic pH, positive charge was dominant. This was governed by positively charged side chains of gelatin, which were more prevalent, compared to negatively charged phosphates attached to gelatin.

Table 1 Phosphate content and zeta potential of gelatin from skin of Unicorn leatherjacket phosphorylated at different pHs

Concentration of STPP	pH	Phosphate content (μmol/g gelatin)	Zeta Potential (mV)
0.25 (%w/w)	5	36.72 ± 6.71^{d}	6.34 ± 0.54^{a}
	7	57.29 ± 3.32^{c}	4.23 ± 0.80^{b}
	9	128.35 ± 7.08^{b}	-3.89 ± 0.65^{c}

	11	216.42±10.56[a]	-5.64±0.98[d]
0 (control)	-	13.07±1.47[e]	3.62±0.80[b]

Mean ± SD (n = 3).

Different superscripts within the same column indicate the significant differences ($p < 0.05$).

3.2.2. Gel strength

Gel strength of gelatin phosphorylated with 0.25% STPP at various pHs for 1 h is depicted in Fig. 3. Amongst all samples, gelatin phosphorylated at pH 9 had the highest gel strength (194.3 g) ($P < 0.05$), compared with those prepared under other pHs. Some phosphate groups might attach with gelatin chains, leading to phosphorylation of gelatin. Guo *et al*. (2005) reported that phosphorylation might introduce the ionic interaction between phosphate groups and $-NH_3^+$ of amino acids, thus increasing the crosslinks of proteins. The decrease in gel strength of gelatin phosphorylated at pH 11 was probably due to the increased repulsion between protein molecules, as governed by the dominant negative charge. This might result in the loosen network of gelatin gel. However, phosphorylated gelatin (194.3 g) had the lower gel strength than did commercial bovine gelatin (207.8 g) ($P < 0.05$). The main structural difference between fish and mammalian gelatins is the imino acid contents (proline and hydroxyproline), where the mammalian gelatins have the higher amount (Gudmundsson, 2002). Hydroxyl groups of amino acids, especially hydroxyproline, play a role in inter-chain hydrogen bonding via a bridging water molecule as well as direct hydrogen bonding to the carbonyl group (Wong, 1989). Thus, the incorporation of phosphate into gelatin was able to introduce the negatively charge residues. As a result, those gelatin chains underwent aggregation with the positively charged chains via ionic interaction. This could compensate with the lower H-bond mediated by imino acids in fish gelatin.

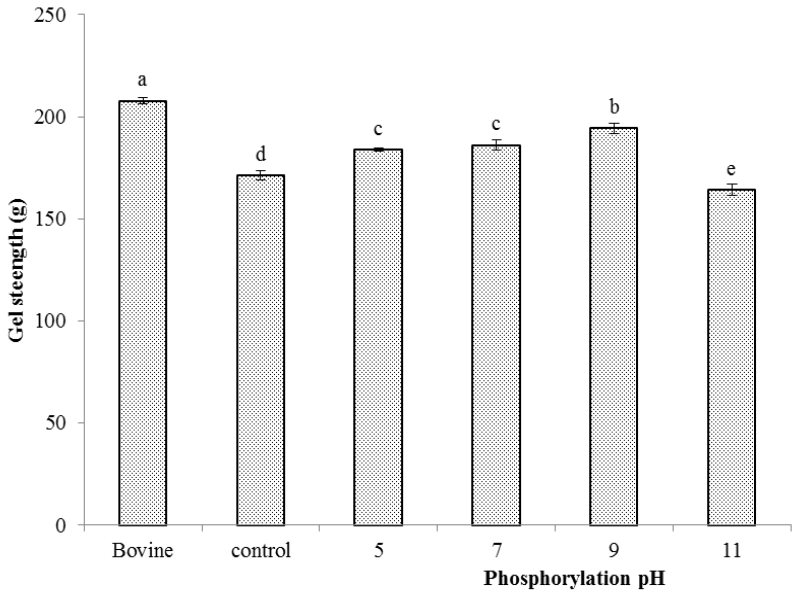

Figure 3 Gel strength of gelatins from the skin of unicorn leatherjacket phosphorylated using 0.25% STPP for 1 h at different pHs. Bars represent the standard deviation (n=3). Different letters on the bars indicate significant difference ($p < 0.05$).

3.2.3. FTIR spectra of gelatin

FTIR spectroscopy has been used to study changes in functional groups and secondary structure of gelatin (Nagarajan *et al*., 2012). FTIR spectra of gelatin phosphorylated without and with 0.25% STPP at various pH (7 and 9) are shown in Fig. 4. All gelatin samples had the major peaks in amide region, but showed slight differences in the spectra. Amide-I peak (representing C=O stretching vibration coupled with C–N stretch and CCN deformation) of control gelatin, and those phosphorylated at pH 7 and 9 were found at wavenumbers of 1643, 1629 and 1635 cm^{-1}, respectively. The result suggested that the hydrogen bonding and the conformation of protein structure might be slightly different (Bandekar, 1992). Yakimets *et al*. (2005) reported that the amide-I was characteristic for the coil structure of gelatin. The change in amide-I band of gelatin suggested that phosphate incorporated might affect the helix coil structure of gelatin mainly via the increased repulsion between charged residues in gelatin chains. For amide-II peak, control gelatin, those phosphorylated at pH 7 and pH 9 had the peak at the wavenumbers of 1544, 1531 and 1538 cm^{-1}, respectively. The amide-II vibration mode is attributed to combination of the N–H in plane bend and the C–N stretching vibration with smaller contributions from the C–O in plane bend and the C–C and N–C stretching vibrations (Jackson *et al*., 1995). In addition, amide-III peak (the combination peaks between C–N stretching vibrations and N–H deformation from amide linkages as well as absorptions arising from wagging vibrations of CH_2 groups from the glycine backbone and proline side-chains) was detected at a wavenumber of 1240 cm^{-1} and 1236 cm^{-1} for control gelatin and phosphorylated gelatin, respectively. The shift to lower wavenumbers of amide-I, II and III regions observed in gelatin phosphorylated at both pHs suggested that phosphates were more likely attached to functional groups, e.g. carbonyl, amino groups of gelatin. The phosphorylation using STPP at pH 7 and 9 also resulted in decreases in peak amplitude of amide I and II bands. These changes are indicative of the lowering of those reactive groups via the attachment with phosphate groups.

Amide-A peak (representing NH-stretching coupled with hydrogen bonding) appeared at wavenumbers of 3298, 3280 and 3301 cm^{-1} for control gelatin and gelatins phosphorylated at pH 7 and pH 9, respectively. Amide-B was observed at wavenumbers of 3074, 3068 and 3079 cm^{-1} for control gelatin and gelatins

phosphorylated at pH 7 and pH 9, respectively, corresponding to the asymmetric stretching vibration of =C–H as well as $–NH_3^+$ (Nagarajan *et al*., 2012). The phosphorylated gelatin had the lower amplitude of amide-A band than the control gelatin, suggesting the attachment of phosphates with NH_3^+ of side chain of gelatin. Phosphate attached to gelatin at an appropriate level played a role in gel formation, mainly favouring ionic interaction between chains. This resulted in the increased gel strength.

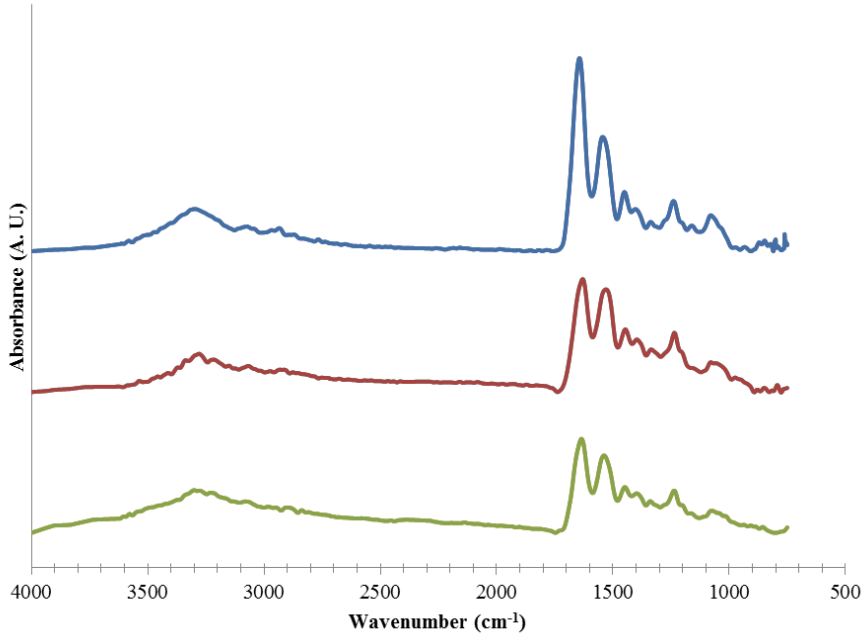

Figure 4 Fourier transform infrared spectra of gelatin extracted from the skin of unicorn leatherjacket without phosphorylation (a) and with phosphorylation using 0.25% STPP at pH 7 (b) and pH 9 (c).

3.2.4. Microstrutures of gel

Microstructures of gels of gelatin without and with phosphorylation using 0.25% STPP at pH 7 and 9 are illustrated in Fig. 5. All gelatin gels showed a network of interconnection with fairy uniform strands. The gelatin phosphorylated at pH 9 exhibited the finest gel network with very small voids. These observations suggested that phosphates attached to gelatin might be involved in network formation, in which more order and denser structure with finer strands could be developed. This was in

accordance with the higher gel strength of gelatin phosphorylated at pH 9 (Fig. 3). For gel of gelatin phosphorylated at pH 7, the larger voids with coarser network were observed, in comparison with that prepared from gelatin phosphorylated at pH 9. Therefore, the arrangement and association of gelatin molecules in the gel matrix were governed by modified gelatin structure, which was mediated by phosphorylation condition.

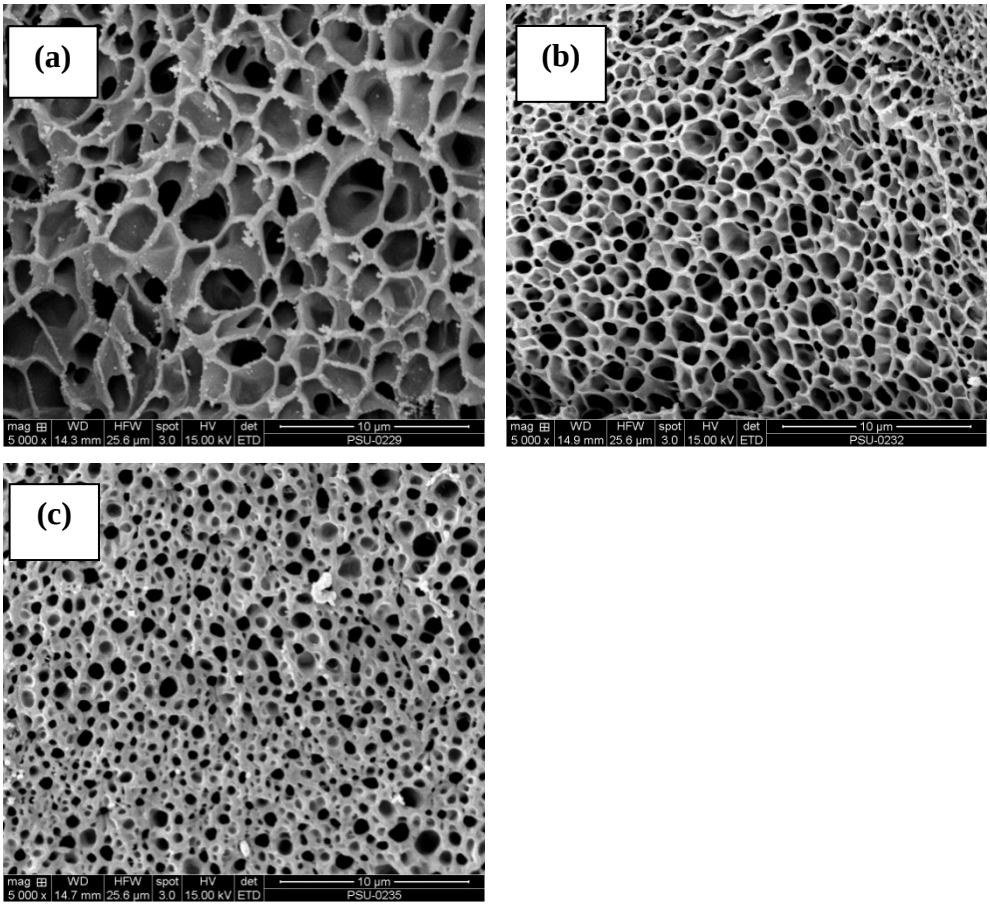

Figure 5 Microstructure of gels of gelatins from the skin of unicorn leatherjacket without phosphorylation (a) and with phosphorylation using 0.25% STPP at pH 7 (b) and pH 9 (c). Magnification: 5000x.

4. Conclusion

The phosphorylation of gelatin using 0.25% STPP at pH 9 for 1 h yielded gelatin with improved gel strength. This was the result of increasing negatively charge residues in gelatin. Finer and ordered network of gel was achieved for gelatin phosphorylated under the optimal condition.

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