Effect of Extracting Conditions and Phosphorylation on Characteristic and Functional Properties of Gelatin from the Skin of Unicorn leatherjacket

Phanngam Kaewruang

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Author Miss Phanngam Kaewruang

Major Program Food Science and Technology

Major Advisor :

…….....

(Prof. Dr. Soottawat Benjakul)

Examining Committee :

……..….…Chairperson

(Assist. Prof. Dr. Manee Vittayanont)

…….…..……...

(Prof. Dr. Soottawat Benjakul)

…….……..…...

(Dr. Tanong Aewsiri)

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

……..……..……

(Assoc. Prof. Dr. Teerapol Srichana)

Dean of Graduate School

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

…….…….....Signature

(Prof. Dr. Soottawat Benjakul)

Major advisor

…….…….....Signature

(Miss Phanngam Kaewruang)

Candidate

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

……..……. Signature

(Miss Phanngam Kaewruang)

Candidate

ชื่อวิทยานิพนธ์ ผลของสภาวะการสกัดและกระบวนการฟอสฟอริเลชันต่อคุณลักษณะและสมบัติเชิงหน้าที่ของเจลาตินจากหนังปลาวัว

ผู้เขียน นางสาวพรรณงาม แก้วเรือง

สาขาวิชา วิทยาศาสตร์และเทคโนโลยีอาหาร

ปีการศึกษา 2555

บทคัดย่อ

จากการสกัดเจลาตินจากหนังปลาวัวด้วยน้ำที่อุณหภูมิต่างๆ (45, 55, 65 และ 75 องศาเซลเซียส) ในสภาวะที่มีและไม่มีสารยับยั้งทริปซินจากถั่วเหลือง (100 ยูนิตต่อกรัมหนัง) เป็นระยะเวลา 12 ชั่วโมง พบว่า การสกัดเจลาตินในสภาวะที่มีการเติมสารยับยั้งทริปซินจากถั่วเหลืองมีผลให้ร้อยละของผลผลิตเจลาตินลดลงโดยไม่คำนึงถึงอุณหภูมิที่ใช้ในการสกัด ทั้งนี้อุณหภูมิสกัดที่เพิ่มขึ้นมีผลให้ ผลผลิตเจลาตินเพิ่มขึ้น ($P < 0.05$) โดยเจลาตินที่ได้จากการสกัดที่อุณหภูมิ 75 องศาเซลเซียสภายใต้สภาวะที่ไม่มีสารยับยั้งทริปซินจากถั่วเหลืองให้ผลผลิตเจลาตินสูงสุด (ร้อยละ 10.66±0.41) (โดยน้ำหนักแห้ง) เจลาตินที่ได้จากการสกัดที่อุณหภูมิ 55 องศาเซลเซียสภายใต้สภาวะที่ไม่มีสารยับยั้งทริปซินจากถั่วเหลืองมีปริมาณแอลฟาอะมิโนสูงสุด และสายโซ่บีตา และแอลฟามีความเข้มแถบเพิ่มสูงขึ้นเมื่ออุณหภูมิสูงขึ้น โดยเฉพาะอุณหภูมิที่สูงกว่า 55 องศาเซลเซียส การสกัดเจลาตินที่อุณหภูมิ 65 องศาเซลเซียสในสภาวะที่มีและไม่มีสารยับยั้งทริปซินจากถั่วเหลืองให้ความแข็งแรงของเจลสูงสุด (178.00±7.50 และ 170.47±1.30 กรัม ตามลำดับ) จากสเปคตรา FTIR บ่งชี้ว่าการสกัดที่อุณหภูมิ 55 องศาเซลเซียสภายใต้สภาวะที่ไม่มีสารยับยั้งทริปซินจากถั่วเหลืองเกิดการสูญเสียของเกลียวทริปเปิลเฮลิกซ์สูงสุด เจลาตินที่สกัดที่อุณหภูมิ 65 องศาเซลเซียส ในสภาวะที่มีและไม่มีสารยับยั้งทริปซินจากถั่วเหลือง มีความสามารถในการเป็นอิมัลซิไฟเออร์ ความคงตัวของอิมัลชันสูงสุด รวมทั้งมีความสามารถในการเกิดโฟม และความคงตัวของโฟมสูง เมื่อสกัดเจลาตินจากหนังปลาวัวด้วยน้ำที่อุณหภูมิต่างๆ (65 และ 75 องศาเซลเซียส) เป็นระยะเวลาต่างกัน (9, 12 และ 15 ชั่วโมง) พบว่าผลผลิต การเก็บเกี่ยวและปริมาณแอลฟาอะมิโนเพิ่มขึ้น แต่ค่าความแข็งแรงเจลลดลงเมื่ออุณหภูมิและเวลาในการสกัดเพิ่มขึ้น ($P < 0.05$) เจลของเจลาตินเจลที่ได้จากการสกัดภายใต้อุณหภูมิสูงและระยะเวลานานมีรูพรุนใหญ่แล ะโครงข่ายเจลมีขนาดใหญ่ ที่ระดับความเข้มข้นของเจลาตินเดียวกัน สมบัติเกี่ยวกับอิมัลชันและการเกิดโฟมของเจลาตินแตกต่างตามสภาวะการสกัด

จากการฟอสฟอริเลชันของเจลาตินจากหนังปลาวัว โดยการเติม โซเดียมไตรพอลิฟอสเฟต (STPP) ที่ระดับความเข้มข้นต่างๆ ในระหว่างขั้นตอนการปฏิบัติเบื้องต้นและระหว่างขั้นตอนการสกัด พบว่าเจลาตินที่มีการเติมฟอสเฟตในระหว่างขั้นตอนการปฏิบัติเบื้องต้นและระหว่างขั้นตอนการสกัดมีปริมาณฟอสเฟต 12.43 และ 18.25 ไมโครโมลต่อกรัมตัวอย่าง ตามลำดับ เมื่อวิเคราะห์ศักย์ซีต้า พบว่าเจลาตินที่ผ่านกระบวนการฟอสฟอริเลชันมีประจุสุทธิเป็นลบ สำหรับเจลาตินที่มีการเติมฟอสเฟตระหว่างขั้นตอนการปฏิบัติเบื้องต้น พบว่าการใช้ฟอสเฟตที่ระดับความเข้มข้นร้อยละ 0.2 ทำให้ได้เจลาตินที่มีค่าความแข็งแรงเจลสูงสุด (128.3 กรัม) เมื่อเปรียบเทียบกับเจลาตินที่มีการเติมฟอสเฟตระหว่างขั้นตอนการสกัด พบว่าการใช้ฟอสเฟตที่ระดับความเข้มข้นร้อยละ 0.08 ให้ค่าความแข็งแรงเจลสูงสุด (146.0 กรัม) เมื่อศึกษาโครงสร้างทางจุลภาค พบว่าเจลจากเจลาตินที่เติมฟอสเฟตที่ระดับความเข้มข้นร้อยละ 0.08 ระหว่างการสกัด มีลักษณะโครงสร้างที่ละเอียดและการจัดเรียงตัวที่แน่น และสามารถเ ซ็ตตัวที่อุณหภูมิ 4 องศาเซลเซียสได้เร็ว ที่สุดเมื่อเปรียบเทียบกับเจลาตินอื่น และเมื่อทำการฟอสฟอริเลชันโดยเติมฟอสเฟตในสารละลายเจลาติน ที่ระดับความเข้มข้นต่างๆ (ร้อยละ 0.25, 0.5, 0.75 และ 1 น้ำหนักต่อน้ำหนัก) เป็นระยะเวลา 1 และ 3 ชั่วโมง ที่อุณหภูมิ 65 องศาเซลเซียส พบว่าความเข้มข้นของฟอสเฟตและระยะเวลาที่เพิ่มขึ้นไม่ส่งผลต่อปริมาณฟอสเฟตที่จับกับโครงสร้างของเจลาติน การเติมฟอสเฟตที่ระดับความเข้มข้นร้อยละ 0.25 เป็นระยะเวลา 1 ชั่วโมง ให้ความแข็งแรงเจลสูงสุด ($P < 0.05$) จากการศึกษาผลของระดับพีเอชต่อกระบวนการฟอสฟอริเลชันและสมบัติเจลของเจลาติน พบว่าเจลาตินที่มีการฟอสฟอริเลชันที่พีเอช 9 ให้ความแข็งแรงเจลสูงสุด (204.3 กรัม) ($P < 0.05$) และมีลักษณะโครงสร้างเจลที่ละเอียดและมีการจัดเรียงตัวที่แน่น เจลาตินมีประจุสุทธิเป็นลบซึ่งสามารถเกิดเจลที่แข็งแรงโดยอันตรกิริยาระหว่างประจุ

จากการศึกษาผลของซิงค์คลอไรด์และแคลเซียมคลอไรด์ที่ระดับความเข้มข้นต่างๆ (2.5-40 ไมโครโมลาร์) ต่อความแข็งแรงเจลของเจลาตินปลาที่ผ่านกระบวนการฟอสฟอริเลชัน พบว่าค่าความแข็งแรงเจลเพิ่มขึ้นเมื่อความเข้มข้นของซิงค์คลอไรด์และแคลเซียมคลอไรด์เพิ่มขึ้น แต่อย่างไรก็ตามการเติมแคลเซียมคลอไรด์ให้ความแข็งแรงเจลที่สูงกว่าเมื่อเปรียบเทียบกับการเติมซิงค์คลอไรด์ เจลาตินปลาที่ผ่านกระบวนการฟอสฟอริเลชันที่มีการเติมแคลเซียมคลอไรด์ที่ระดับความเข้มข้น 20 ไมโครโมลาร์ ให้ค่าความแข็งแรงเจลเพิ่มขึ้นร้อยละ 15.7 เมื่อเปรียบเทียบกับเจลาตินควบคุม เมื่อศึกษาผลของอัตราส่วนระหว่างเจลาตินวัวกับเจลาตินปลาที่ผ่านกระบวนการฟอสฟอริเลชัน พบว่าความแข็งเจลลดลงเมื่อความเข้มข้นของเจลาตินปลาที่ผ่านกระบวนการฟอสฟอริเลชันเพิ่มขึ้น ($P < 0.05$) แต่อย่างไรก็ตามความแข็งเจลของเจลาตินวัวและเจลาตินผสมระหว่างเจลาตินวัวกับเจลาตินปลาที่ผ่านกระบวนการฟอสฟอริเลชันอัตราส่วน 7.5:2.5 ไม่แตกต่าง

กัน ดังนั้นเจลาตินปลามีสมบัติเจลที่ดีขึ้นภายหลังกระบวนการฟอสฟอริเลชันภายใต้สภาวะที่เหมาะสม ส่วนแคลเซียมคลอไรด์สามารถเหนี่ยวนำให้เกิดการเชื่อมประสานกันของเจลาตินปลาที่ผ่านกระบวนการฟอสฟอริเลชัน นอกจากนี้การผสมเจลาตินปลาที่ผ่านกระบวนการฟอสฟอริเลชันและเจลาตินวัวด้วยอัตราส่วนที่เหมาะสมเป็นอีกแนวทางหนึ่งในการปรับปรุงสมบัติของเจลาตินปลา

Thesis Title Effect of Extracting Conditions and Phosphorylation on Characteristic and Functional Properties of Gelatin from the skin of unicorn leatherjacket

Author Miss Phanngam Kaewruang

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ABSTRACT

Gelatins extracted from the skin of unicorn leatherjacket using water at different temperatures (45, 55, 65 and 75 ^{o}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 ^{o}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 ^{o}C without SBTI incorporated. The band intensity of β-chain and α-chains increased as the extraction temperature increased, particularly above 55 ^{o}C. Gelatin extracted at 65 ^{o}C with and without SBTI incorporation 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 ^{o}C in the absence SBTI. Gelatin extracted at 65 ^{o}C, either with or without SBTI, had the highest EAI and ESI with high foam expansion and stability. When gelatin from the skin of unicorn leatherjacket was extracted at different temperatures (65 and 75 ^{o}C) for various times (9, 12 and 15 h), yield, recovery and free amino group content of gelatin increased. Nevertheless, gel strength generally decreased as the extraction temperature and time increased ($P < 0.05$). 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.

Phosphorylation of gelatin from the skin of unicorn leatherjacket was carried out by incorporating sodium tripolyphosphate (STPP) at varying concentrations during pretreatment and extraction. Phosphate attached to gelatin during pretreatment and extraction was found at levels of 12.43 and 18.25 μmol/g, respectively. Based on zeta potential analysis, phosphorylated gelatin became negatively charged, reconfirming the charge modification mediated by phosphorylation. For gelatin phosphorylated during pretreatment, that using 0.2% STPP had the highest gel strength (128.3 g). Amongst all gelatins incorporated with STPP during extraction, that added with 0.08% STPP showed the highest gel strength (146.0 g). Gel of gelatin phosphorylated with 0.08% STPP during extraction exhibited a finer and more compact structure with smaller pores and was able to set at 4 °C at the fastest rate, compared with other gelatins. Gelatin from the skin of unicorn leatherjacket was also phosphorylated by incorporating STPP at various concentration (0.25, 0.50, 0.75 and 1.00% w/w) into gelatin solution for different times (1 and 3 h) at 65 °C. 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.

Gel properties of phosphorylated fish gelatin (PFG) as influenced by zinc chloride ($ZnCl_2$) and calcium chloride ($CaCl_2$) at various levels (2.5-40 μM) were determined. Gel strength of PFG increased with increasing concentrations of $ZnCl_2$ and $CaCl_2$. Nevertheless, a higher gel strength was found in gelatin gel added with $CaCl_2$, compared with $ZnCl_2$. PFG added with 20 μM $CaCl_2$ had the increase in gel strength by 15.7%, compared with control gel. When the effect of bovine gelatin (BG) to PFG ratios on the properties of the resulting gel was studied, hardness of gels decreased with increasing PFG content ($P < 0.05$). Nevertheless, no differences in hardness were found amongst gels with BG/PFG ratios of 10:0 and 7.5:2.5 ($P > 0.05$).

Therefore, fish gelatin could possess the better gelling property after being phosphorylated under the optimal condition. $CaCl_2$ could be used to induce aggregation of PFG and the mixing of PFG with bovine gelatin at an appropriate ratio could be another means to improve the properties of fish gelatin.

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CHAPTER 1

Introduction and review of literature

1.1 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 bovine and porcine skins and skeletons (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 contains 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 *et al.*, 1996). Apart from extraction method used, intrinsic factor such as indigenous collagenase in the skin also plays a vital role in hydrolysis of polypeptide, contributing to the decrease in functional properties of resulting gelatin (Intarasirisawat *et al.*, 2007).

Phosphorylation has been proven to be a useful method for improving the functional properties of food proteins (Sitohy *et al.*, 1995). Phosphorylation may introduce the phosphate to protein molecules. Phosphate groups can be attached to the oxygen of seryl, threonyl, aspartyl (β-carboxyl) and tyrosyl residues and via nitrogen to lysyl (ε-amino) and the imidazole group of histidine. Sodium tripolyphosphate (STPP) is approved as a food additive, which can improve water holding capacity of seafood, meat and poultry (Dziezak, 1990; Rasković, 2007). Due to the prevalence of negative charge, STPP can be used for phosphorylation of gelatin. Additionally, the protein cross-linking via the salt bridges induced by divalent cations can modify the properties of resulting gelatin.

Unicorn leatherjacket skin has been used for gelatin extraction (Ahmad and Benjakul, 2011a). However, severe degradation occurred during extraction at

50 °C, mainly caused by indigenous proteases (Ahmad *et al.*, 2011). 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 can be associated with the increased bloom strength of gelatin from the skin of unicorn leatherjacket, particularly in conjuction with the use of selected divalent cations. Nevertheless, there is no information about properties of gelatin from unicorn leatherjacket skin as affected by extraction temperature and time and the impact of phosphate treatment on gelling property of gelatin. Additionally, little information regarding the influence of divalents on gel property of phosphorylated fish gelatin and the effect of mixing bovine gelatin with phosphorylated gelatin from unicorn leatherjacket skin on textural properties has been reported. Therefore, better understanding on production of fish gelatin and quality improvement is needed, in which the better quality fish gelatin can be obtained.

1.2 Review of literature

1.2.1 Gelatin

Gelatin is one of commonly used food ingredient, obtained by the thermal denaturation of collagen (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 (Karim and Bhat, 2009). Acid treatment is most suitable for less fully cross-linked collagens commonly found in pig or fish skins, whereas alkaline treatment is appropriate for the more complex collagens found in bovine hides (Foegeding *et al.*, 1996).

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 (Eastoe and Leach, 1977). 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 non-covalent 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 (Bremner, 1992). Generally, at heating temperature more than T_s, the triple-stranded helix of collagen is also destroyed to a great extent and exists as the random coils. During the collagen to gelatin transition, many non-covalent bonds are broken along with some covalent inter-and intramolecular bonds and a few peptide bonds. This results in conversion of the helical collagen structure to a more amorphous form, known as "gelatin". These changes constitute denaturation of the collagen molecule but not to the point of a completely unstructured product. If the latter event happens, glue instead of gelatin is produced (Foegeding *et al.*, 1996). For fish collagen, T_s is 45 ^{o}C, while collagen from mammals has T_s of 60-65 ^{o}C (Belitz and Grosch, 1999).

1.2.2 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. All processes used for gelatin extraction have the direct impact on the yield and properties of gelatin obtained. The process has been optimized for different sources of raw materials (Cho *et al.*, 2005; Hou and Regenstein, 2004; Yang *et al.*, 2007).

1.2.2.1 Pretreatment processes

1.2.2.1.1 Removal of non-collagenous protein

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

1.2.2.1.2 Swelling of raw materials

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

1.2.2.1.2.1 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 *et al.*, 1996). The swelling process is designed to convert the collagen into a form suitable for extraction. A sufficient number of the covalent cross-links in the collagen must be broken in order to enable the release of free α-chains. The process is also designed to remove other organic substances, such as proteoglycan, blood, mucins, sugars, 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). Nevertheless, other acids including citric and sulfuric has been used for skin pretreatment before gelatin extraction (Gudmundsson and Hafsteinsson, 1997).

Moreover, type and concentration of acids affected the yield and properties of gelatin. Ahmad and Benjakul (2011a) 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. The concentration of H^+ used in processing of gelatin from cod skins affected yield and quality of resulting gelatin (Gudmundsson and Hafsteinsson, 1997). Gómez-Guillén and Montero (2001) reported that gelatin from megrim skin treated with 0.05 M acetic or 0.05 M propionic had the highest elastic modulus, viscous modulus, melting temperature, and gel strength. Gelatin obtained from skin swollen with citric acid exhibited the lowest turbidity, whereas propionic acid led to the most turbid gelatin solution. Giménez *et al.* (2005) reported that lactic acid (25 mM) could be an excellent substitute for acetic acid for the skin swelling process. The gelatin obtained showed similar properties to that prepared by using 50 mM acetic acid without the negative organoleptic properties. Gelatin obtained from the acid process is named as type A gelatin (Karim and Bhat, 2009).

Table 1. Acid pretreatment of fish skin before gelatin extraction.

Species	Acid	Condition	References
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	Jongjareonrak *et al.* (2006)
Channel catfish *(Ictalurus punctatus)*	Acetic acid	Soak in eight volumes (v/w) at 15 ^{o}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 at 4 ^{o}C	Benjakul *et al.* (2009)
Snakehead (*Channa striatus*), catfish (*Clarias batrachus*), pangasius catfish (*Pangasius sutchi*) and red tilapia (*Oreochromis niloticus*)	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)
Unicorn leatherjacket *(Aluterus monoceros)*	0.2 M phosphoric acid	Soak with a skin/solution ratio of 1:10 (w/v) for 24 h at 4 ^{o}C	Ahmad and Benjakul (2011a)
Grey triggerfish (*Balistes capriscus*)	0.05 M acetic acid	Soak with a skin/solution ratio of 1:10 (w/v) for 6 h at 4 ^{o}C	Jellouli *et al.* (2011)
Splendid squid *(Loligo formosana)*	0.05 M phosphoric acid	Soak with a skin/solution ratio of 1:10 (w/v) for 24 h at 4 °C	Nagarajan *et al.* (2012)

1.2.2.1.2.2 Alkaline process or type B gelatin

Type B gelatins are generally produced by alkali hydrolysis of bovine materials, which results in deamidation and a greater range of molecular weight species (Foegeding *et al.*, 1996). Alkaline pretreatment 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). Gelatin obtained from the alkaline process is named as type B gelatin. To increase the yield, alkaline process was used for gelatin extraction from cartilage. Cho *et al.* (2004) optimized the extraction condition for production of gelatin from shark cartilage using response surface methodology. Gelatin production has two important steps, including alkali treatment and hot-water extraction. The alkali treatment removes non-collagenous protein. Hot water extraction causes thermal hydrolysis, leading to the solubilization of gelatin. The predicted maximum yield of 79.9% for gelatin production was obtained when alkali treatment using 1.6 N NaOH for 3.16 days and hot-water extraction at 65 ºC for 3.4 h were implemented.

1.2.2.2 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). Heating is primarily required for gelatin extraction. Heat applied at temperature higher than transition temperature (T_{max}) is able to disrupt the bonds, mainly H-bond, stabilizes collagen structure (Figure 1). This results in conversion of the helical collagen structure to a more amorphous form, known as gelatin (Benjakul *et al.*, 2012). 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 (Johnston-Banks, 1990). 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 (Table 2).

Table 2. Extraction time and temperature used for fish gelatin production.

Species	Temperature	Time	References
Black tilapia *(Oreochromis mossambicus)* and red tilapia *(Oreochromis nilotica)*	45 ^{o}C	12 h	Jamilah and Harvinder (2002)
Yellowfin tuna *(Thunnus albacares)*	40-80 ^{o}C	1-9 h	Cho *et al.* (2005)
Dover sole *(S. vulgaris)*	45 ^{o}C	Overnight	Giménez *et al.* (2005)
Bigeye snapper *(Priacanthus macracanthus)* and brownstripe red snapper *(Lutjanus vita)*	45 ^{o}C	12 h	Jongjareonrak *et al.* (2006)
Yellowfin tuna *(Thunnus albacares)*	50 ^{o}C	18 h	Rahman *et al.* (2008)
Channel catfish skins	45 ^{o}C	7 h	Liu *et al.* (2008)
Snakehead (*Channa striatus*), catfish (*Clarias batrachus*), pangasius catfish (*Pangasius sutchi*) and red tilapia (*Oreochromis niloticus*)	45 ^{o}C	18 h	See *et al*. (2010)
Grass carp fish (*Ctenopharyngodon idella*)	60 ^{o}C	6 h	Zhang *et al.* (2011)
Splendid squid *(Loligo formosana)*	50-80 ^{o}C	12 h	Nagarajan *et al.* (2012)

Gelatin from Nile perch skin extracted at higher temperature yielded the lower gel strength, melting point, setting temperature and longer setting time (Muyonga *et al.*, 2004a). Yang *et al.* (2007) reported that gelatin from channel catfish skin showed the lower gel strength as extraction temperature increased from 60 to 75 ºC. Kittiphattanabawon *et al.* (2010) also 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. Moreover, Nagarajan *et al.* (2012) found that gelatin from the skin of splendid squid *(Loligo formosana)* extracted at higher temperature (80 ºC) had a relatively higher free amino group content and lower gel strength than gelatin extracted at lower temperatures (50, 60 and 70 ºC). Shorter chain fragments of gelatin could not form the junction zone, in which the strong network could be developed (Benjakul *et al.*, 2012) (Figure 1).

1.2.2.3 Recovery

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

1.2.3 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. However, gelatins from these sources have limited application as they have lower gel strength and their gels are less stable, compared with those from their mammalian

counterparts. The differences between fish and mammalian gelatin are mainly dependent on their molecular weight distribution and amino acid content, especially the content of imino acid (proline and hydroxyproline) (Johnston-Banks, 1990). Fish gelatin from both cold and warm water are generally produced by a mild acid treatment (type A gelatin) process (Gómez-Guillén and Montero, 2001). Gelatin can be extracted from different fish species (Table 3).

Table 3. Gelatin from different fish species.

Fish species	Portions	References
Flounder *(Platichthys flesus)*	Skin	Fernández *et al.* (2003)
Nile perch *(Lates niloticus)*	Skin and bone	Muyonga *et al.* (2004a)
Alaska pollock *(Theragra chalcogramma)*	Skin	Zhou and Regenstein (2005)
Horse mackerel *(Trachurus trachurus)*	Skin	Badii and Howell (2006)
Bigeye snapper *(Priacanthus macracanthus)*, brownstripe red snapper *(Lutjanus vitta)*	Skin	Jongjareonrak *et al.* (2006)
Atlantic salmon (*Salmo salar*)	Skin	Arnesen and Gildberg (2007)
Grass carp *(Ctenopharyngodon idella)*	Skin	Kasankala *et al.* (2007)
Baltic cod *(Gadus morhua)*, salmon *(Salmo salar)*, herrings *(Clupea harengus)*	Skin, head and backbone	Kołodziejska *et al.* (2008)
Yellowfin tuna	Skin	Rahman *et al.* (2008)
Cuttlefish (*Sepia pharaonis*)	Skin	Aewsiri *et al.* (2009)
Brownbanded bamboo shark (*Chiloscyllium punctatum*) and blacktip shark (*Carcharhinus limbatus*)	Skin	Kittiphattanabawon *et al*. (2010)
Grey triggerfish *(Balistes capriscus)*	Skin	Jellouli *et al.* (2011)
Grass carp (*Ctenopharyngodon idella*)	Scale	Zhang *et al.* (2011)
Carp *(Cyprinus carpio)*	Skin	Duan *et al.* (2011)
Splendid squid *(Loligo formosana)*	Skin	Nagarajan *et al.* (2012)

Furthermore, 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.

1.2.4 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 (Giménez *et al.*, 2005). Moreover, using minced skins instead of whole skins significantly shortened the extraction time of gelatins (Kołodziejska *et al.*, 2004).

The extraction yield of gelatin from skins ranged from about 5.5% to 21% of the starting weight of the raw material (Giménez *et al.*, 2005; Jamilah and Harvinder, 2002; Muyonga *et al.*, 2004a; Songchotikunpan *et al.*, 2008). The variation in such values depends on the differences in both composition and amount of soluble components in the skins (Muyonga *et al.*, 2004a), 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). Gómez-Guillén *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. Kołodziejska *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 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 (Kołodziejska *et al.*, 2004). Nagarajan *et al.* (2012) reported that yield of gelatin from the skin of splendid squid was increased when extraction temperature increased from 50 to 80 °C.

1.2.5 Impact of indigenous protease on fish gelatin extraction

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).

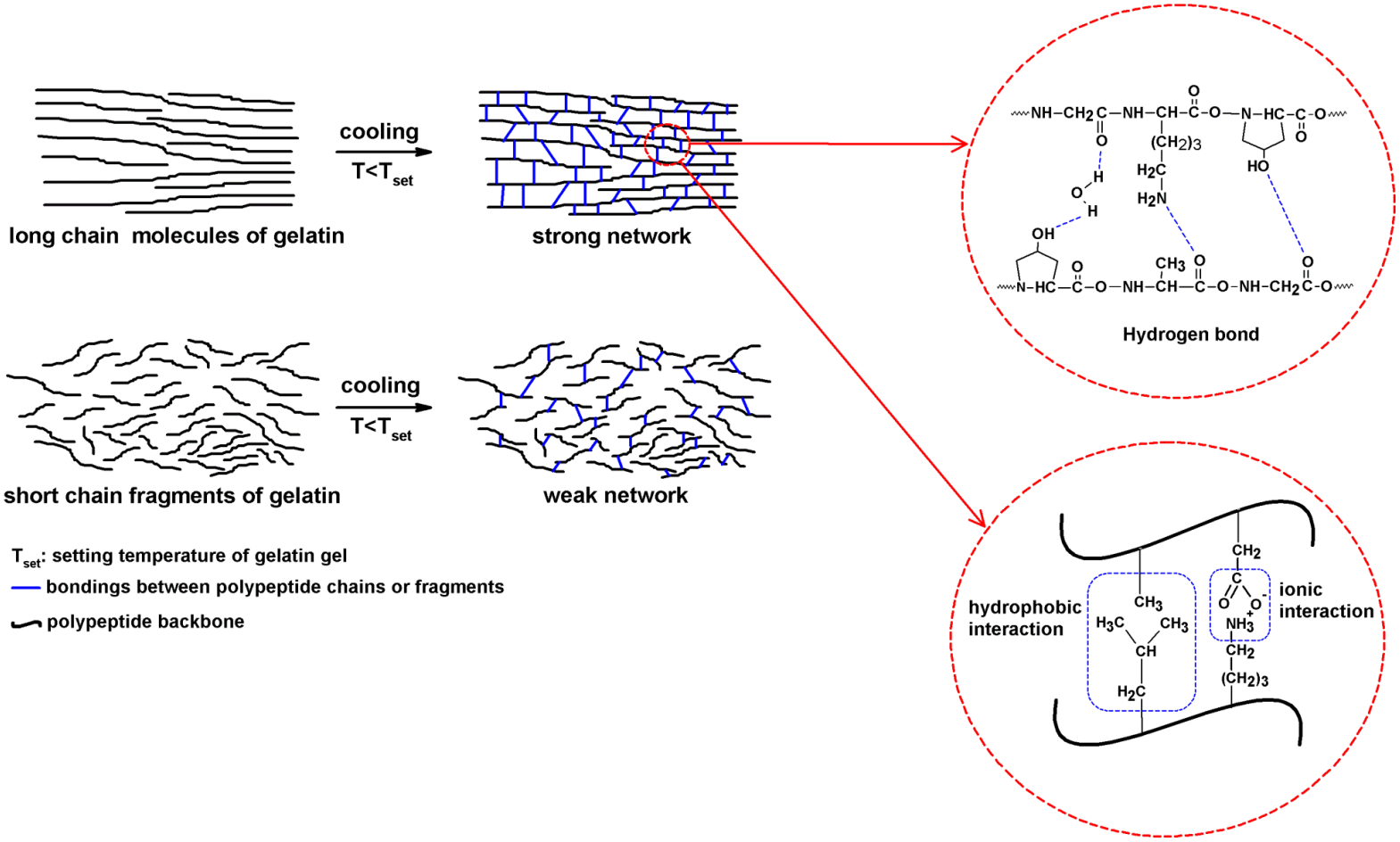


Figure 1. Gelatin network associated with hydrogen bond, hydrophobic interaction and ionic interaction.

Source: Benjakul *et al.* (2012)

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 ^{o}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 ^{o}C and pH 7.5 (Intarasirisawat *et al.*, 2007). With the addition of 0.001 mM soy bean trypsin inhibitor (SBTI), the degradation was markedly inhibited and β- and α-chains in gelatin were more retained when extracted at temperatures lower than 50 ^{o}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). Nalinanon *et al.* (2008) reported that the gelatin extracted by the typical process in the absence of protease inhibitor, SBTI, showed lower gel strength in comparison with gelatin extracted in the presence of SBTI. When gelatin was extracted from the skin of bigeye snapper skin in the presence of SBTI, β- and α-chains along with the polymerized components were retained (Nalinanon *et al.*, 2008). 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. Maximized degradation was found at pH 7 and 50 ^{o}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 (Ahmad and Benjakul, 2011b).

Gelatin molecules with the shorter chains generated by the serine protease are not able to form the strong inter-junction zones, especially via hydrogen bonding or other weak bonds such as by hydrophobic interactions or ionic

interactions. As a consequence, a weaker network was developed, in comparison with those from gelatins with a longer chain length, which are capable of aligning or self-aggregating more effectively (Figure 2).

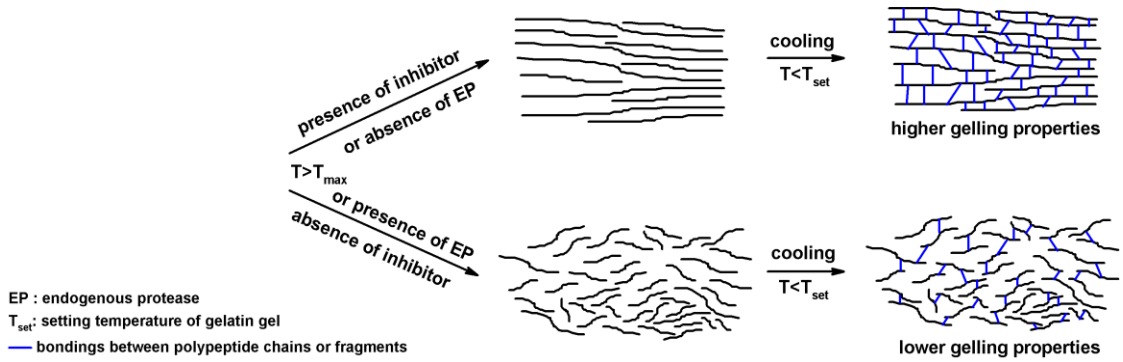


Figure 2. Impact of endogenous protease associated with fish skin on degradation of gelatin molecules.
Source: Benjakul *et al.* (2012)

1.2.6 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).

1.2.6.1 Solubility

Gelatin swells upon the contact with cold water, forming the large visible swollen particles. When heated above the melting point, the hydrated gelatin will rupture and go into solution, and form a gel upon cooling (Jamilah and Harvinder, 2002). Gelatin is practically insoluble in alcohol and non polar solvents such as carbon tetrachloride, petroleum ether and carbon disulfide. Although soluble in polyhydric alcohols such as sorbitol, mannitol and glycerin, in which water is usually present as an accessory solvent (Poppe, 1997). Due to high solubility of gelatins at pH 1-10, they can be used widely and effectively since solubility is a prerequisite for most functionalities of food proteins. Kittiphattanabawon *et al.* (2010)

reported high solubility in the wide pH range of gelatin from shark skin. Gelatin extracted at 120 °C for 30 min showed higher solubility in the wild pH range (1-10) (Sukkwai *et al.*, 2011). The solubility of the gelatin from bigeye snapper *(Priacanthus tayenus)* skin ranged from 87.37 to 94.28 % (Sukkwai *et al.*, 2011). Gelatins extracted from both skin of *P. tayenus* and *P. macracanthus* showed relative solubility greater than 90 % at all pHs tested (1-10) (Benjakul *et al.*, 2009). Difference in solubility of different gelatins might be due to the difference in molecular weight and the content of polar and non-polar groups in amino acids (Zayas, 1997).

1.2.6.2 Gelation

Collagen denatures at temperatures above 40 ^{o}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 gelatin gel is a reversibly crosslinked biopolymer network stabilized mainly by hydrogen bonded junction zones (Figure 1). Furthermore, hydrophobic interaction and ionic interaction are also involved in gelation of gelatin (Figure 1) (Benjakul *et al.*, 2012). 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 the association of polypeptide chains to form triple-helical collagen molecules can occur (Wong, 1989).

Gel formation, which is obtained by cooling gelatin aqueous solution, 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 (Kittiphattanabawon *et al.*, 2010; Nagarajan *et al.*, 2012). Gelatin containing more α-

chains would thus show higher gel strength (Johnston-Banks, 1990). Gelatin from shark skin extracted at 75 ^{o}C containing the large proportion of small peptides had much lower bloom strength than those extracted at 45 ^{o}C and 60 ^{o}C (Kittiphattanabawon *et al.*, 2010). These fragments cannot form the junction zone effectively. As a result, a poor gel is formed or those fragments cannot set at room temperature (Figure 1).

It is generally recognized that the imino acids, proline and hydroxyproline, are important in gelation (Kittiphattanabawon *et al.*, 2005). Imino acid content correlates with the gel strength of gelatin. Imino acids, especially hydroxyproline, are involved in gel formation by acting as an H-donor, so that a hydrogen bond can be formed with the adjacent chain possessing an H-acceptor (Figure 1). The hydroxyl group of the hydroxyproline plays a part in the stability of the helix by interchain hydrogen bonding via a bridging water molecule as well as by direct hydrogen bonding to a carbonyl group (Wong, 1989).

The gel strength of commercial gelatins ranges from 100 to 300, but gelatins with bloom values of 250–260 are the most desirable (Karim and Bhat, 2009). 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) (Gómez-Guillén *et al.*, 2002). Gómez-Guillén *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 (Gómez-Guillén *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. A lower number of hydrophobic amino acids in the commercial non-gelling cod gelatin was observed, 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 Gómez-Guillén, 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.

In general, fish gelatin cannot set at room temperature, unlike mammalian gelatin. Gelling temperature is known to be affected by habitat of animal used for gelatin extraction. Gelling and melting temperatures of cold water fish/mammalian gelatins are in general lower than the warm water fish/mammalian gelatins (Muyonga *et al.*, 2004a). Gómez-Guillén *et al.* (2002) reported that low melting and gelling points are related to the lower imino acid content and decreased

proline hydroxylation degree in the cold water fish gelatin. The lower imino acid content in cold water fishes reduces the propensity for intermolecular helix formation (Gilsenan and Ross-Murphy, 2000). The melting temperature of tropical fish gelatins was generally high with 22-28.9 °C for tilapia skin (Jamilah and Harvinder, 2002), 25-26 °C for red snapper and grouper bone gelatins (Shakila *et al.*, 2012), 26.5-25.9 °C for Nile perch bone gelatin (Muyonga *et al.*, 2004a) and the cold water cod skin with 8-10 °C (Gudmundsson and Hafsteinsson, 1997). Gelling temperatures of fish gelatins were 6 °C lower than that of mammalian gelatin. Gelling temperatures of Nile perch bone gelatin were 18.5-19 °C (Muyonga *et al.*, 2004a) and those of rohu and common carp skin gelatin were 18.52 °C and 17.96 °C, respectively (Ninan *et al.*, 2011). The difference was also related to the imino acid composition of gelatin. The imino acids were found to stabilize the ordered conformation when gelatin forms the gel network during gelling (Muyonga *et al.*, 2004a).

The textural parameters obtained from texture profile analysis (TPA) force/deformation curves have been well correlated with sensory evaluation of textural parameters (Munoz *et al.*, 1986), provides more information than "gel strength" measurements, and is useful for routine analysis of gel texture (Sanderson, 1990). Hardness of fish and mammalian gelatin increased significantly as the concentrations of gels increased. The hardness of bovine gelatin gels was highest (harder), followed by porcine then fish skin gels. The lower content of Pro and Hyp probably gave fish gelatin its low gel modulus, gelling and melting temperatures (Rahman and Al-Mahrouqi, 2009). Sanderson (1990) reported that by adding 0.25% gellan to 250 Bloom type A gelatin, hardness and springiness of the resulting gels increased. Wangtueai and Noomhorm (2009) reported that gelatin from the scales of lizardfish had a lower hardness than bovine gelatin. The value of hardness, gumminess and chewiness of the bovine gelatin were found to be higher than the lizardfish scales gelatin but there were insignificantly different in springiness and cohesiveness between the gels (Wangtueai and Noomhorm, 2009).

The use of gelatin in surimi directly affected the textural property of surimi gel. Kaewudom *et al.* (2012) reported that the addition of 10% bovine gelatin (BG) into surimi gel, the hardness, springiness, cohesiveness, gumminess, and

chewiness decreased. Nevertheless, surimi gel containing bovine gelatin or bovine/fish gelatin mix with FG/BG ratio of 1:1 or 1:2 in conjunction with MTGase 1.2 units/g surimi could render the gel with acceptability equivalent to the control gel.

1.2.6.3 Emulsifying and foaming properties

Emulsions and foams are heterogeneous systems consisting of one phase dispersed in another. An emulsion is a dispersion or suspension of two immiscible liquids, while a foam is a gas phase dispersed in a liquid (Hill, 1998). 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.*, 2005). 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). They found that the oil-in-water emulsion prepared with high average molecular weight fish gelatin (~120 kDa) was more stable than that prepared with a low average molecular weight fish gelatin (~50 kDa). 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. Aewsiri *et al.* (2009) studied the emulsifying properties, emulsion activity index (EAI) and emulsion stability index (ESI), and foaming properties, foam expansion (FE) and foam stability (FS) of gelatin from cuttlefish skin with and without bleaching using hydrogen peroxide. Emulsions containing gelatin from bleached dorsal and ventral skin were more stable than that of gelatins without bleaching. A longer bleaching time and higher hydrogen peroxide concentration led to a lower ESI of gelatin for all samples, except for gelatin from the dorsal skin, in

which the highest ESI was obtained when the skin was bleached with 5% H_2O_2 for 48 h ($p < 0.05$). Additionally, 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). For foam forming ability, gelatin from unbleached skin, both dorsal and ventral, had a slightly lower FE than gelatin extracted from bleached skin, while bleaching had no effect on FS of gelatin from the ventral skin but gelatin from the dorsal skin bleached with 5% H_2O_2 for 48 h showed the highest FS (Aewsiri *et al.*, 2009). Jongjareonrak *et al.* (2010) reported that foam capacity and foam stability of gelatin from farmed giant catfish was higher than that from calf skin. Gelatin from shark cartilage and pre-cooked tuna fin showed lower foam capacity and foam stability than gelatin from porcine skin (Aewsiri *et al.*, 2008; Cho *et al.*, 2004).

1.2.6.4 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 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).

1.2.7 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:

1.2.7.1 Orthophosphates

Orthophosphate is the larger and perhaps more important for food industry. It consists of one phosphorus atom tetrahedrally surrounded by four oxygens (Table 4). 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).

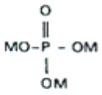
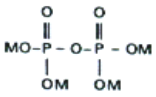
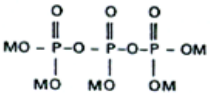
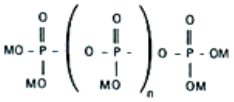
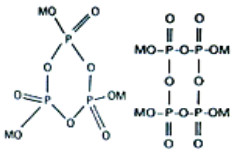
1.2.7.2 Condensed phosphates

Condensed phosphates are produced by heating mixtures of orthophosphates under controlled conditions. They are composed of two or more

phosphorus atoms linked through a 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).

Table 4. Classes, formulas, pH, solubility, and functions of several phosphates.

Class of phosphate and Basic structure[a]	Phosphate name	Generally accepted formula	pH (1% solution)	Solubility at 25% (g/100g water)	Function
Orthophosphate	Monosodium phosphate	NaH_2PO_4	4.6	87	Emulsifier, buffer
	Disodium phosphate	Na_2HPO_4	9.2	12	Emulsifier, buffer
	Disodium phosphate dihydrate	$Na_2HPO_4 .2H_2O$	9.1	15	Emulsifier, buffer
	Trisodium phosphate	Na_3PO_4	11.8	14	Emulsifier, buffer
	Monopotassium phosphate	KH_2PO_4	4.6	25	Water binding in meats
	Dipotassium phosphate	K_2HPO_4	9.3	168	Emulsifier, buffer
	Tripotassium phosphate	K_3PO_4	11.9	107	Emulsifier, buffer
	Monocalciun phosphate	$Ca(H_2PO_4) .H_2O$	3.8	-	Acidulant, leaving acid , dough condition, yeast food, nutrient
Condensed phosphates					
pyrophosphate	Sodium acid pyrophosphate	$Na_2H_2P_2O_7$	4.3	15	Emulsifier,buffer,sequestrant Water binding in meats
	Tetrasodium pyrophosphate	$Na_4P_2O_7$	10.3	8	Dispersant, coagulant, crystallization inhibitor in canned tuna
	Tetrapotassium pyrophosphate	$K_4P_2O_7$	10.5	187	Emulsifier, Water binding in meats, suspending agent
tripolyphosphate	Sodium tripolyphosphate	$Na_5P_3O_{10}$	9.9	15	Emulsifier, Water binding in meats
	Potassium tripolyphosphate	$K_5P_3O_{10}$	9.6	193	Emulsifier, Water binding in meats
Long-chain polyphosphates	Sodium polyphosphates, glassy, or Graham's Salt;	$(NaPO_3)_6 .Na_2O$	7.7	40[b]	Sequestrant, emulsifier Water binding in meats, suspending agent
	three chain lengths;	$(NaPO_3)_{13} .Na_2O$	6.9	40[b]	Sequestrant, emulsifier Water binding in meats, suspending agent
	Sodium hexametaphosphate has an average chain length of 13	$(NaPO_3)_{21} .Na_2O$	6.3	40[b]	Sequestrant, emulsifier Water binding in meats, suspending agent
Metaphosphate					
Tri- **Tetra-**	Sodium trimetaphosphate	$(NaPO_3)_5$	6.7	23	
	Sodium tetrametaphosphate	$(NaPO_3)_4 .4H_2O$	6.2	18	

[a]M stands for one equivalent of a metal ion or hydrogen

[b]Solubility is higher than 40% but this recommended for ease of preparation and use

Source: Dziezak (1990)

1.2.8 Phosphorylation of proteins

Inorganic phosphate (Pi) can be transferred to proteins by either O-esterification or N-amidation reactions (Matheis *et al.*, 1983). For 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. For N-amidation, Pi combines with the Ɛ-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, thereby affecting functional properties of proteins (Matheis and Whitaker, 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 STMP to modify serine and lysine in soy protein isolate under alkaline conditions. The reactions included phophoesterification of serine residues and phosphoramidation of lysine residues in soy protein (Figure 3). 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 and its hydrolysis in water produces only harmless Pi (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-esterification and N-amidation (Damodaran, 1997).

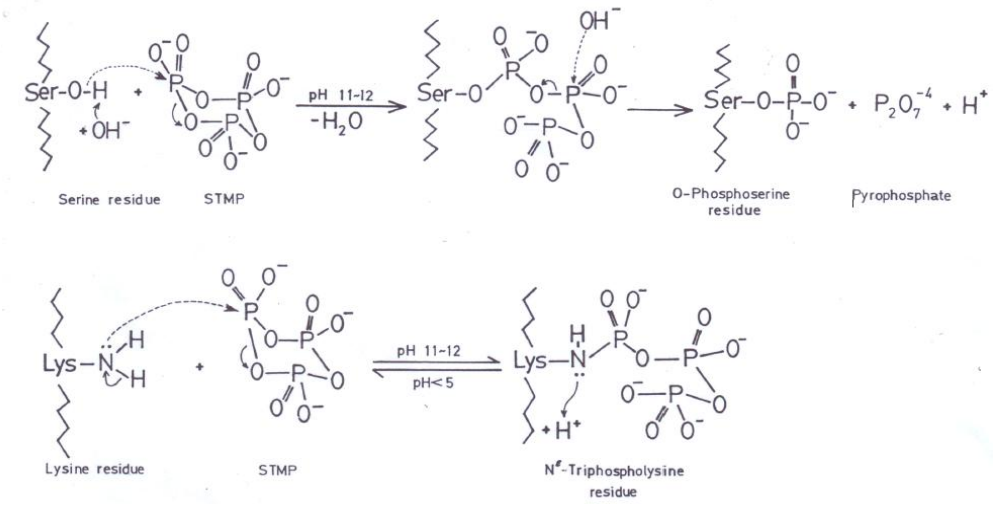


Figure 3. Phosphoesterification and phosphoramidation of soy protein with sodium trimetaphosphate (STMP) in alkali.

Source: Sung *et al.* (1983)

Phosphorylation has been proven to be a useful method for improving the functional properties of food proteins (Table 5) (Li *et al.*, 2010b). Li *et al.* (2003) phosphorylated egg white protein by dry-heating in the presence of phosphate. The phosphorylated protein had the improved heat stability, emulsifying properties, and gelling properties. Furthermore, the calcium phosphate solubilizing ability of egg white protein was enhanced by phosphorylation. Enomoto *et al.* (2008) reported that the functional properties of bovine serum albumin (BSA) such as heat stability and calcium phosphate solubilizing ability were improved by phosphorylation. Transparent gels of BSA with relatively high water-holding capacity were obtained by phosphorylation. Woo *et al.* (1982) approved that the electronegativity of phosphorylated protein was increased. Zhang *et al.* (2007) reported that the phosphorylation of soy protein isolate with sodium tripolyphosphate affected functional properties. The highest degrees of phosphorylation were found at pH 9. The emulsifying properties increased after soy protein isolate was phosphorylated. Additionally, Miedzianka and Pęksa (2013) reported that the phosphorylation of potato protein isolate with sodium trimetaphosphate (STMP) under slightly alkaline pH (8.0) improved functional properties (water and oil absorption capacity, emulsifying activity and foaming capacity).

Table 5. Changes in functional properties of phosphorylated-proteins.

Phosphorylated protein	Functional properties	References
Soy protein isolate	Solubility	Sung *et al.* (1983)
	Emulsifying properies	
Yeast homogenate	Solubility	Giec *et al.* (1989)
	Emulsifying properties	
Egg white protein	Heat stability	Li *et al.* (2003)
	Emulsifying properties	
	Gelling properties	
Whey protein isolate	Emulsifying properies	Li *et al.* (2005)
	Gelling properties	
	Water-holding capacity	
Soy protein isolate	Emulsifying properies	Zhang *et al.* (2007)
Bovine serum albumin	Heat stability	Enomoto *et al.*
Whey soy protein	Emulsifying properties	(2008)
	Foaming properties	Li *et al.* (2010a)
	Water and oil absorption capacity	
Potato protein isolate	Water and oil absorption capacity	Miedzianka and
	Emulsifying properties	Pęksa (2013)
	Foaming properties	

1.2.9 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, 1997b). 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, 1997a). Thus, these ions can be used to induce protein gelation. 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). 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). Additionally, 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 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.

The addition of divalent cations at an appropriate concentration could be a promising means to improve the gel property by enhancing the aggregation of proteins via salt bridges (Arfat and Benjakul, 2012). Mathew *et al.* (2009) reported that the binding of zinc salts to actomyosin from oil sardine induced conformational changes with the exposure of functional groups such as sulfhydryl groups, which

subsequently undergo oxidation to disulfide bond. Lau *et al.* (2000) reported that the hardness of gellan/gelatin mixed gels increased with increasing calcium ions until calcium concentration reached a critical level. Further increases in calcium resulted in a reduction of hardness. Brittleness, springiness and cohesiveness were very sensitive to low levels of added calcium (0-10 mM), but less sensitive to higher calcium concentrations. In general, the addition of calcium ions caused gels to be more brittle and less cohesive and springy (Lau *et al.*, 2000).

1.3 Objectives of study

1. To study the effect of proteases inhibitor and extracting temperature/time on yield and properties of gelatin from skin of unicorn leatherjacket.
2. To investigate the effect of phosphate incorporation on phosphorylation and functional properties of gelatin from skin of unicorn leatherjacket.
3. To investigate the effect of divalent cation on the gel forming ability of phosphorylated gelatin from skin of unicorn leatherjacket.
4. To study the gel property of phosphorylated gelatin mixed with commercial bovine gelatin from skin of unicorn leatherjacket.

CHAPTER 2

Molecular and functional properties of gelatin from the skin of unicorn leatherjacket as affected by protease inhibitor and extracting temperatures

2.1 Abstract

Gelatins extracted from the skin of unicorn leatherjacket at different temperatures (45, 55, 65 and 75 ^{o}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 ^{o}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 ^{o}C without SBTI incorporated. The band intensity of β-chain and α-chains increased as the extraction temperature increased, particularly above 55 ^{o}C. Gelatin extracted at 65 ^{o}C with and without SBTI incorporation 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 ^{o}C in the absence SBTI. Gelatin extracted at 65 ^{o}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.

2.2 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.*, 2011). 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 ºC 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.

2.3 Materials and methods

2.3.1 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).

2.3.2 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 -20ºC until use. The storage time was less than 2 months.

2.3.3 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 ^{o}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^{o}C.

2.3.4 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.

2.3.5 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 and Benjakul, 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.

2.3.6 Analyses

2.3.6.1 Determination of yield and recovery

2.3.6.1.1 Gelatin yield was calculated by the following equation:

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

2.3.6.1.2 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

2.3.6.2 Determination of chemical composition

2.3.6.2.1 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.

2.3.6.2.2 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.

2.3.6.2.3 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 85ºC 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.

2.3.6.3 Determination of functional properties

2.3.6.3.1 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 ^{o}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.

2.3.6.3.2 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.

2.3.6.3.3 Foaming properties

Foam expansion (FE) and foam stability (FS) of gelatin solutions were determined as described by Shahidi, Han and Synowiecki (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).

2.3.6.4 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 (45º 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 ºC.

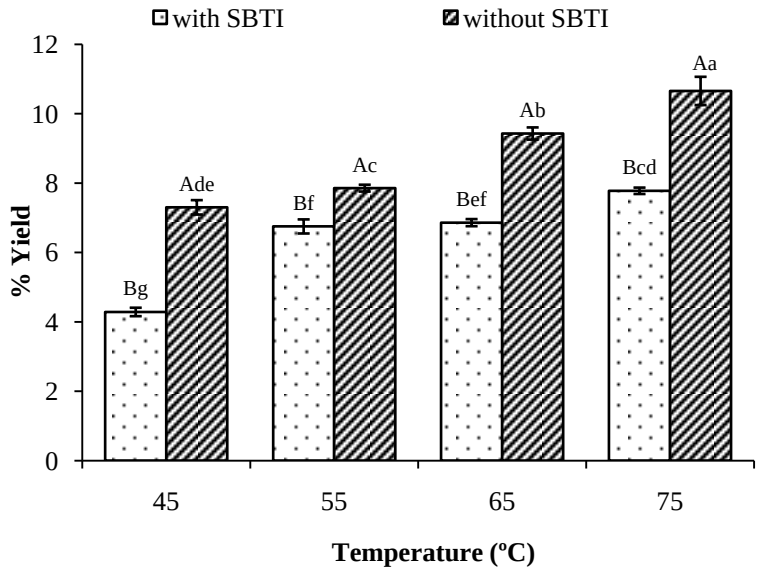
2.3.7 Statistical analysis

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)**.**

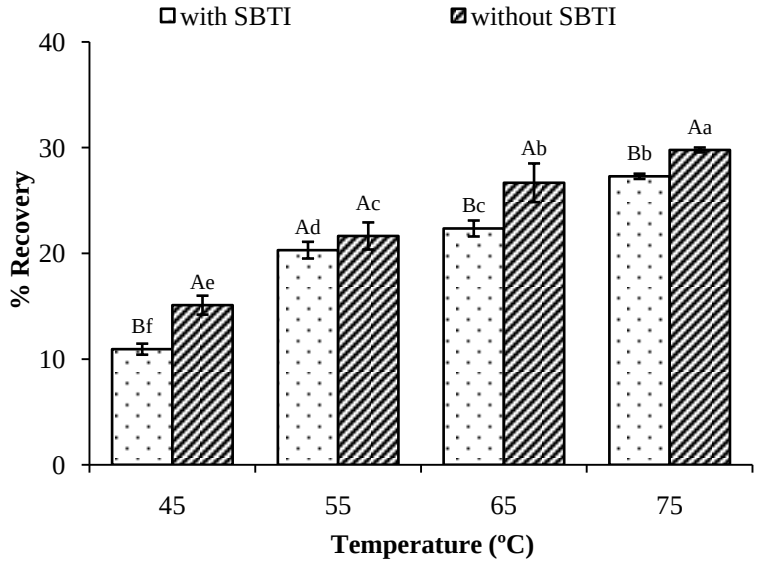
2.4 Results and discussion

2.4.1 Yield

Yield and recovery of gelatin from unicorn leatherjacket skin extracted at different temperatures (45-75 ^{o}C) in the presence and the absence of SBTI (100 units/g pretreated skin) for 12 h are shown in Figure 1. Both yield and recovery increased as the extraction temperatures increased from 45 to 75 ^{o}C ($P < 0.05$), except for the yield of gelatin extracted at 55 and 65 ^{o}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.* (2004a) 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 ^{o}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.



(A)



(B)

Figure 4. Effect of extracting temperature 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 indicate significant difference ($P < 0.05$).

2.4.2 α-amino group content

The α-amino group content of gelatin from unicorn leatherjacket skin extracted under different conditions is shown in Figure 2. The highest α-amino group content was observed in gelatin extracted at 55 ^{o}C in the absence of SBTI. The optimal temperature for indigenous proteases in the skin of unicorn leatherjacket was 50 ^{o}C (Ahmad *et al.*, 2011). However, a decrease in α-amino group content was found when SBTI was incorporated during extraction at 55 ^{o}C. This reconfirmed the role of indigenous proteases in hydrolysis of peptide chains at 55 ^{o}C. However, when heating temperatures of 65 and 75 ^{o}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 ^{o}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 ^{o}C.

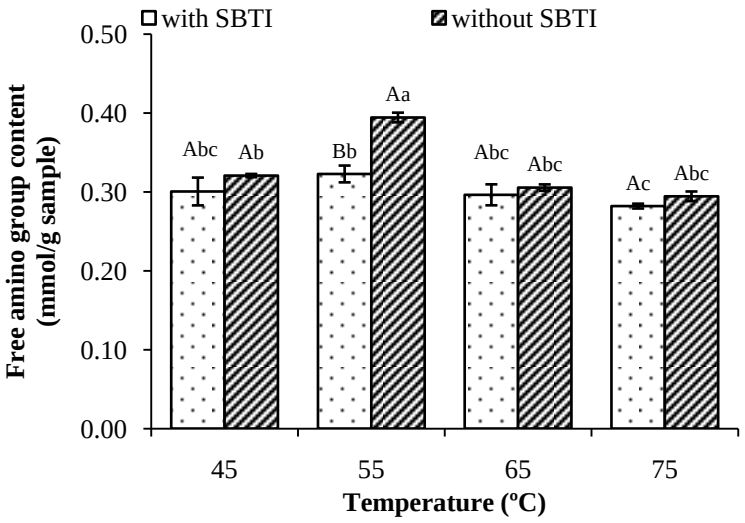


Figure 5. Effect of extracting temperature on free amino group content of gelatin from the skin of unicorn leatherjacket in the presence and the absence of SBTI at a level of 100 Units/g pretreated skins. Bars represent the standard deviation (n=3). Different uppercase letters on the bars within the same temperature indicate significant difference ($P < 0.05$). Different lowercase letters on the bars indicate significant difference ($P < 0.05$).

2.4.3 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.*, 2004b). 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 ^{o}C and 55 ^{o}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.* (2011) who reported that the highest degradation of β-chain and α-chains was obtained at 50 ^{o}C. When the SBTI was incorporated, gelatin extracted at 45 and 55 ^{o}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 ^{o}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 ^{o}C were used. These results were coincidental with those observed for α-amino group content (Figure 5).

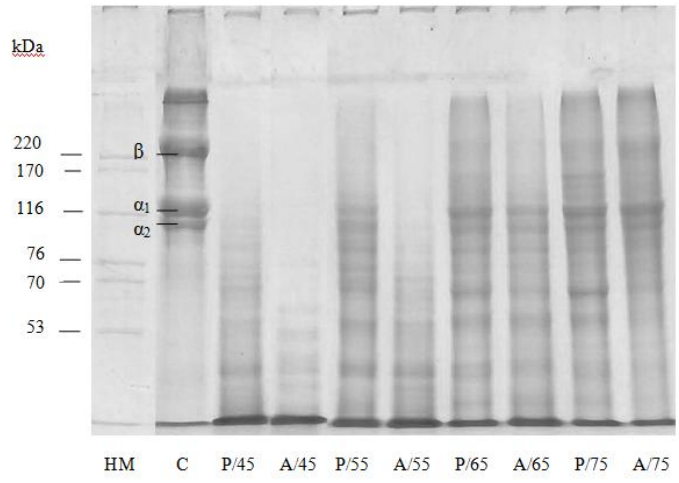


Figure 6. SDS-PAGE pattern of gelatin extracted from the skin of unicorn leatherjacket at different temperatures in the presence (P) and the absence (A) of SBTI at a level of 100 Units/g pretreated skins. C: collagen type I from calf skin. The numbers denote extraction temperature (^{o}C).

2.4.4 Gel strength

Gel strength of gelatin from unicorn leatherjacket skin extracted under various conditions is shown in Figure 7. Among all samples, gelatin extracted at 45 ^{o}C had the lowest gel strength ($P < 0.05$). Gelatin extracted at higher temperature up to 65 ^{o}C possessed higher gel strength ($P < 0.05$). Nevertheless, slight decrease in gel strength was found in gelatin extracted at 75 ^{o}C ($P < 0.05$). When gelatin was extracted at 45 or 55^{o}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.* (2011) 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 (Figure 6). Apart from chain length, the imino acid content, especially hydroxyproline content, affected gelation of gelatin (Aewsiri *et al.*, 2008; Benjakul *et al.*, 2009).

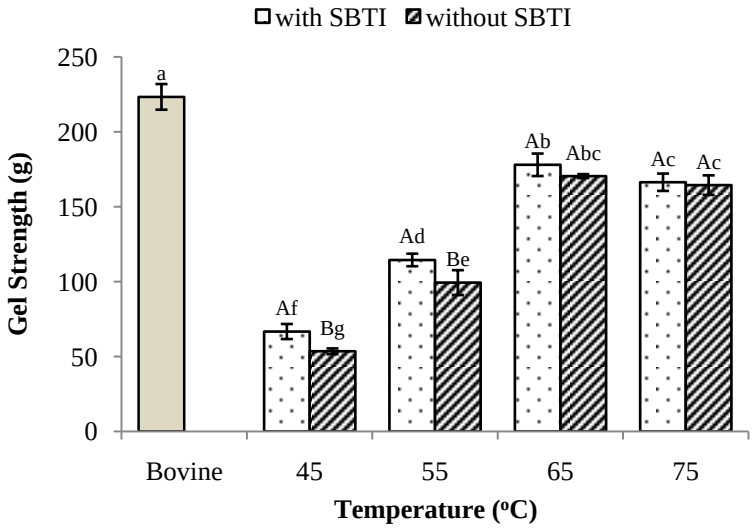


Figure 7. Gel strength of gelatin extracted from the skin of unicorn leatherjacket at different temperatures in the presence and the absence of SBTI at a level of 100 units/g pretreated skins. Bars represent the standard deviation (n=3). Different uppercase letters on the bars within the same temperature indicate significant difference ($P < 0.05$). Different lowercase letters on the bars indicate significant difference ($P < 0.05$).

2.4.5 FTIR spectra

FTIR spectra of gelatin extracted from unicorn leatherjacket skin in the presence and the absence of SBTI at different temperatures (45–75 ^{o}C) are depicted in Figure 8. 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.*, 2004b). Amide-I band of gelatins at 1700–1600 cm^{-1} was reported by Muyonga *et al.* (2004b). 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 (Yakimets *et al.*, 2005). The amide-I peak of gelatin extracted at 55 ^{o}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 generallyf 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 ^{o}C or 45 ^{o}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 (Figure 5) and the marked degradation of α- and β-chains (Figure 6). 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 $-NH_2$.

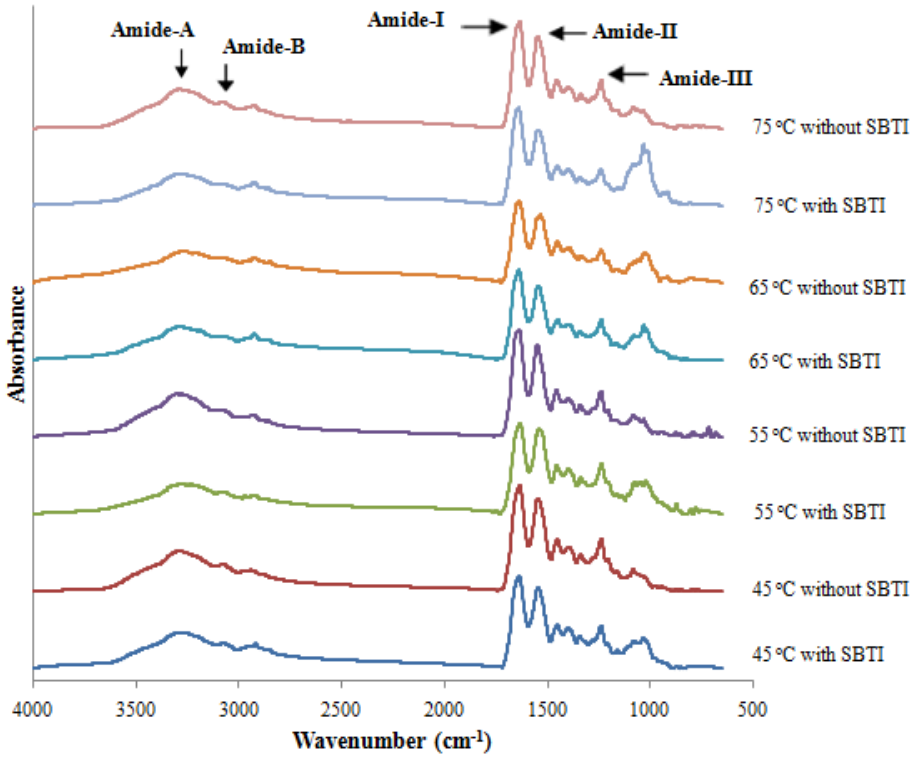


Figure 8. Fourier transform infrared spectra of gelatin extracted from the skin of unicorn leatherjacket at different temperatures in the presence and the absence of SBTI at a level of 100 units/g pretreated skins.

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.

2.4.6 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 6. EAI of all gelatin samples decreased as the concentration of gelatin increased ($P < 0.05$). Gelatin extracted at 45 ^{o}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 ^{o}C in the absence of SBTI, it had the highest degradation with shorter chain length than that extracted at 45 ^{o}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 ^{o}C in the presence or absence of SBTI and gelatin extracted at 75 ^{o}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.

Table 6. 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 (%)
45°C with SBTI	1	10.69 ± 0.09^{dA}	31.28 ± 1.30^{aA}	163.75 ± 1.77^{cC}	123.13 ± 0.88^{eC}
	2	7.92 ± 0.41^{cB}	32.21 ± 3.86^{abA}	176.25 ± 1.77^{cB}	133.13 ± 0.88^{eB}
	3	6.98 ± 0.51^{dC}	34.09 ± 3.45^{aA}	187.5 ± 0.00^{dA}	144.38 ± 4.42^{dA}
45°C without SBTI	1	28.04 ± 1.43^{aA}	19.96 ± 2.06^{bcB}	220.00 ± 1.77^{bC}	159.38 ± 4.42^{cC}
	2	12.13 ± 1.15^{aB}	25.86 ± 2.99^{cB}	229.38 ± 2.65^{bB}	191.25 ± 1.77^{bcB}
	3	9.12 ± 0.41^{aC}	36.77 ± 4.19^{aA}	241.25 ± 1.77^{cA}	200.63 ± 0.88^{cA}
55°C with SBTI	1	19.85 ± 1.88^{cA}	13.90 ± 0.59^{fC}	216.25 ± 5.30^{bC}	160.00 ± 5.34^{cC}
	2	11.22 ± 0.93^{abB}	16.58 ± 0.86^{dB}	231.88 ± 0.88^{bB}	182.50 ± 5.30^{dB}
	3	8.37 ± 0.32^{abC}	19.06 ± 1.76^{bA}	243.13 ± 0.88^{cA}	212.50 ± 0.00^{bA}
55°C without SBTI	1	23.75 ± 1.82^{bA}	15.41 ± 1.33^{efB}	215.00 ± 3.54^{bC}	149.38 ± 2.65^{dC}
	2	12.42 ± 1.82^{aB}	16.27 ± 1.06^{dAB}	228.13 ± 0.88^{bB}	187.50 ± 0.00^{cdB}
	3	8.98 ± 0.58^{aC}	18.07 ± 0.53^{bA}	241.25 ± 5.30^{cA}	212.50 ± 0.00^{bA}
65°C with SBTI	1	23.90 ± 1.52^{bA}	17.18 ± 0.61^{deC}	216.88 ± 2.65^{bC}	180.00 ± 5.30^{aC}
	2	12.32 ± 0.71^{aB}	32.07 ± 2.98^{abB}	230.00 ± 3.54^{bcdB}	196.25 ± 1.77^{bB}
	3	9.17 ± 0.38^{aC}	37.39 ± 1.16^{aA}	246.25 ± 5.30^{bcA}	216.25 ± 5.30^{bA}
65°C without SBTI	1	23.77 ± 0.84^{bA}	15.67 ± 0.54^{efC}	236.25 ± 1.77^{aC}	183.75 ± 1.77^{aC}
	2	10.10 ± 0.79^{bB}	27.69 ± 2.78^{bcB}	246.25 ± 1.77^{aB}	207.50 ± 3.54^{aB}
	3	7.91 ± 0.66^{bcC}	37.22 ± 3.89^{aA}	260 ± 3.54^{aA}	216.88 ± 2.65^{bA}
75°C with SBTI	1	18.36 ± 1.02^{cA}	21.39 ± 0.45^{bB}	230.63 ± 2.65^{aC}	183.75 ± 1.77^{aC}
	2	11.91 ± 0.89^{aB}	34.79 ± 3.53^{aA}	241.88 ± 0.88^{aB}	206.25 ± 5.30^{aB}
	3	7.45 ± 0.37^{cdC}	39.69 ± 3.48^{aA}	253.75 ± 5.30^{abA}	243.13 ± 4.42^{aA}
75°C without SBTI	1	8.73 ± 0.27^{dA}	19.00 ± 0.86^{cdA}	214.38 ± 6.19^{bC}	170.63 ± 2.65^{bC}
	2	7.56 ± 0.20^{cB}	19.06 ± 0.66^{dA}	229.38 ± 0.88^{bB}	183.13 ± 2.65^{dB}
	3	5.20 ± 0.47^{eC}	23.35 ± 3.91^{bA}	250.63 ± 4.42^{bcA}	210.63 ± 4.42^{bA}

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

2.4.7 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 6. 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 ^{o}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. FS is directly affected by protein concentration, which influences the thickness, mechanical strength and cohesiveness of film (Zayas, 1997). The stability of foams depends on various parameters, such as the rate of attaining equilibrium surface tension, bulk and surface viscosities, steric stabilisation, and electrical repulsion between the two sides of the foam lamella (Liu *et al.*, 2003). 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.

2.5 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.

CHAPTER 3

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

3.1 Abstract

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 ($P < 0.05$).

3.2 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 a 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 tonnes). 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, 2011a). 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^{o}C (Kaewruang *et al.*, 2013a). Thus, extraction at temperatures higher than 55^{o}C gave gelatin with higher gel strength (Kaewruang *et al.*, 2013a). 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.

3.3 Materials and methods

3.3.1 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).

3.3.2 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 h 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 h. 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 -20ºC until use.

3.3.3 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 (2011a) 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 h and the total operation time was 4 h. 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 h and the total operation time was 12 h. 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.

3.3.4 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). The mixture was incubated in a temperature controlled water bath (Memmert, Schwabach, Germany) and stirred continuously for various times (9, 12 and 15 h) at a speed of 150 rpm using an overhead stirrer equipped with propeller (RW 20.n, IKA® Werke GmbH & CO.KG, Staufen, Germany). 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 obtained gelatin was subjected to analyses.

3.3.5 Analyses

3.3.5.1 Determination of yield and recovery

3.3.5.1.1 Gelatin yield was calculated by the following equation:

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

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

3.3.5.1.2 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

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

3.3.5.2 Determination of chemical composition

3.3.5.2.1 *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 subtraction of the blank and expressed as mole L-leucine/ g sample.

3.3.5.2.2 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 85ºC for 1 h. 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 (15 µg protein) 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 h and destained with 30% (v/v) methanol and 10% (v/v) acetic acid for 2 h. Type I collagen was used as a standard.

3.3.5.3 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^{o} 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 25ºC to provide the background spectrum that was used to correct the computer generated spectra. Analysis of spectral data was done using the OPUS 3.0 data collection software programme (Bruker, Ettlingen, Germany).

3.3.5.4 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^{o}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 h 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 microstructure 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 h. The samples was then rinsed with distilled water for 1 h 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.

3.3.5.5 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.

3.3.5.6 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 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^{o}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.

3.3.6 Protein determination

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

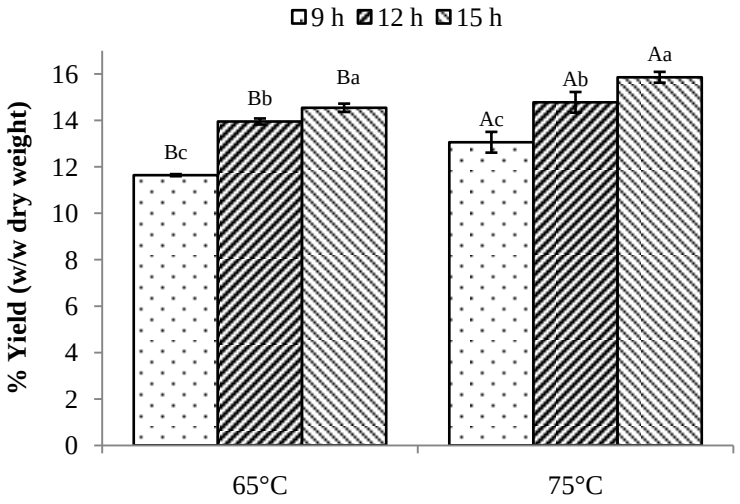
3.3.7 Statistical analysis

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)**.**

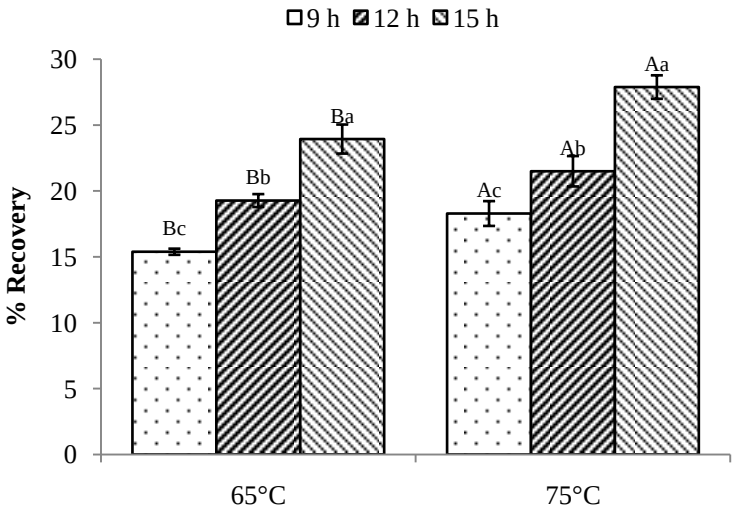
3.4 Results and discussion

3.4.1 Yield

The yield and recovery of gelatin from unicorn leatherjacket skin extracted with water at 65 or 75^{o}C for 9, 12 and 15 h are shown in Figure 9. Both yield and recovery of gelatin increased as the extraction temperature and time increased ($P < 0.05$) (Figure 9). The results suggest 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^{o}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^{o}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^{o}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^{o}C. However, decreased gelling properties were obtained, most likely associated with higher degradation at higher extraction temperatures.



(A)



(B)

Figure 9. 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 ($P < 0.05$).

3.4.2 α-amino group content

The α-amino group content of gelatin from unicorn leatherjacket skin extracted under different conditions is shown in Figure 10. 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 h 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 suggests 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.*, 2004a), 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 (Figure 9).

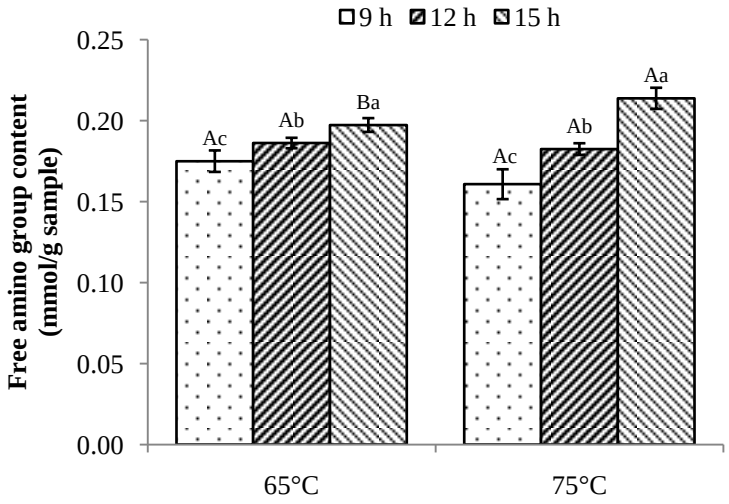


Figure 10. Free amino group content of gelatin from the skins of unicorn leatherjacket as affected by extraction temperatures and times. Bars represent the standard deviation (n=3). Different uppercase letters on the bars within the same extraction time indicate significant differences ($P < 0.05$). Different lowercase letters on the bars within the same extraction temperature indicate significant differences ($P < 0.05$).

3.4.3 Protein pattern

The protein patterns of gelatin extracted from unicorn leatherjacket skin at different temperatures for various times are shown in Figure 11. All gelatins contained α-chains as the major components. Among all of the samples, gelatin extracted under the milder condition (65^{o}C, 9 h) 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^{o}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^{o}C as shown by the decrease in band intensity of protein with a MW in the range of 64-75 kDa. Muyonga *et al.* (2004a) 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.

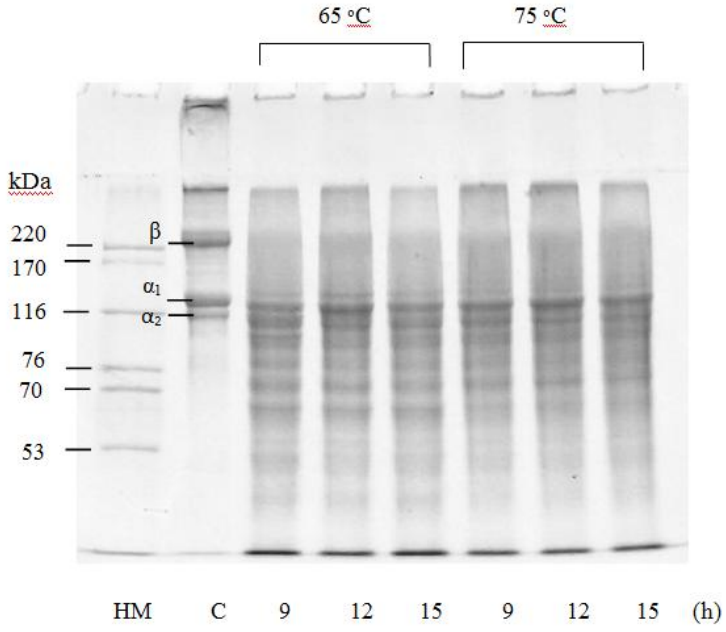


Figure 11. SDS-PAGE pattern of gelatin extracted from the skins of unicorn leatherjacket as affected by extraction temperatures and times. C: collagen type I from calf skin, HM: high MW markers.

3.4.4 Fourier transformed-infrared spectroscopy

FT-IR spectra of gelatins extracted with different conditions are shown in Figure 12. FTIR spectroscopy has been used to monitor the functional groups and secondary structure of gelatin (Muyonga *et al.*, 2004c). Spectra of gelatin displayed the major peaks in amide region. FTIR spectra of unicorn leatherjacket skin gelatin were similar to those found in other gelatins (Kittiphattanabawon *et al.*, 2010; Muyonga *et al.*, 2004b; 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 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^{o}C

for 9, 12 and 15 h 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 h, 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 h.

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 h and at 3286, 3286 and 3278 cm^{-1} for gelatin extracted at 75°C for 9, 12 and 15 h, 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 h and at 3074, 3086 and 3072 cm^{-1} for gelatin extracted at 75°C for 9, 12 and 15 h, respectively. Gelatin extracted for 15 h 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.

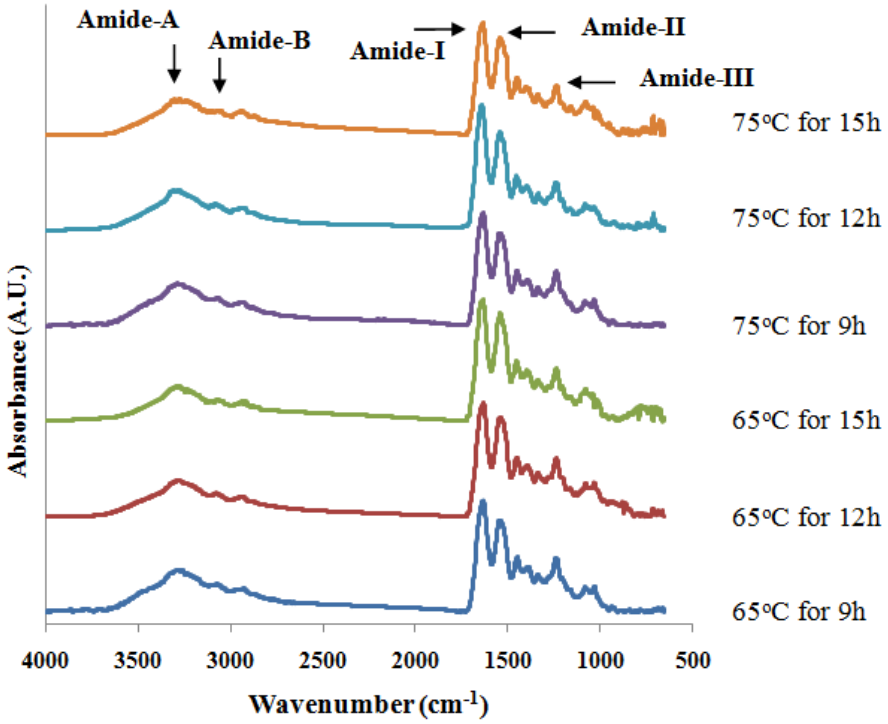


Figure 12 Fourier transform infrared spectra of gelatin extracted from the skins of unicorn leatherjacket as affected by extraction temperatures and times.

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3.4.5 Gel strength and microstructure

The gel strength of gelatin from the unicorn leatherjacket skin extracted at various temperatures for different times is shown in Figure 13. Gel strength generally decreased as the extraction temperature and time increased ($P < 0.05$), except for gelatin extracted for 9 and 12 h 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 h (88.1 g) ($P < 0.05$). This was probably associated with the highest degradation of this sample as indicated by it having the highest α-amino group content (Figure 10) and the highest amount of degradation (Figure 11). 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 (Figure 11), proteins with MW ranging from 20-60 kDa were found in gelatin extracted at 65°C. Those proteins disappeared when the extraction temperature of 75ºC was used. Due to the higher gel strength of gelatin extracted at 65ºC, 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.*, 2013a). 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.

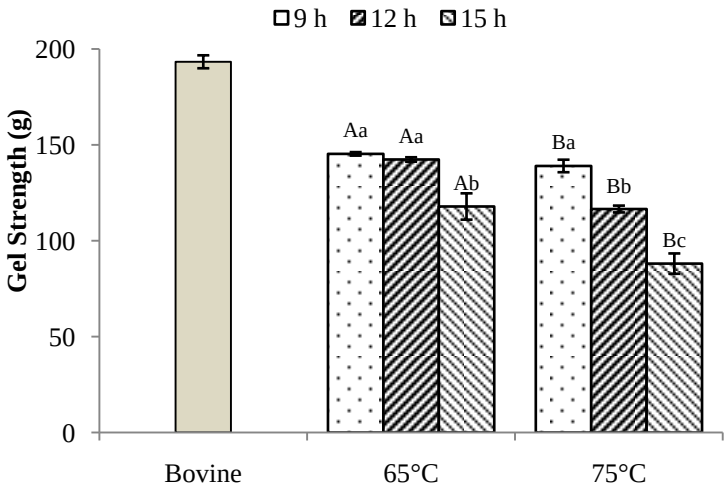


Figure 13. Gel strength of gelatin extracted from the skins of unicorn leatherjacket as affected by extraction temperatures and times. Bars represent the standard deviation (n=3). Different uppercase letters on the bars within the same extraction time indicate significant differences ($P < 0.05$). Different lowercase letters on the bars within the same extraction temperature indicate significant differences ($P < 0.05$).

The gel microstructures of gelatin from the skins of unicorn leatherjacket and commercial bovine gelatin are shown in Figure 14. 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 (Figure 13). 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^{o}C showed a uniform network with the thinner strands, whereas those extracted at 75^{o}C had larger strands with larger voids (Figure 14). The finer network structure of gels of gelatin extracted at 65^{o}C was in accordance with their higher bloom strength (Figure 13), compared with that of gelatin extracted at 75^{o}C, which had coarser gel structures. The coarser network of the gel might be easier to disrupt by the force applied.

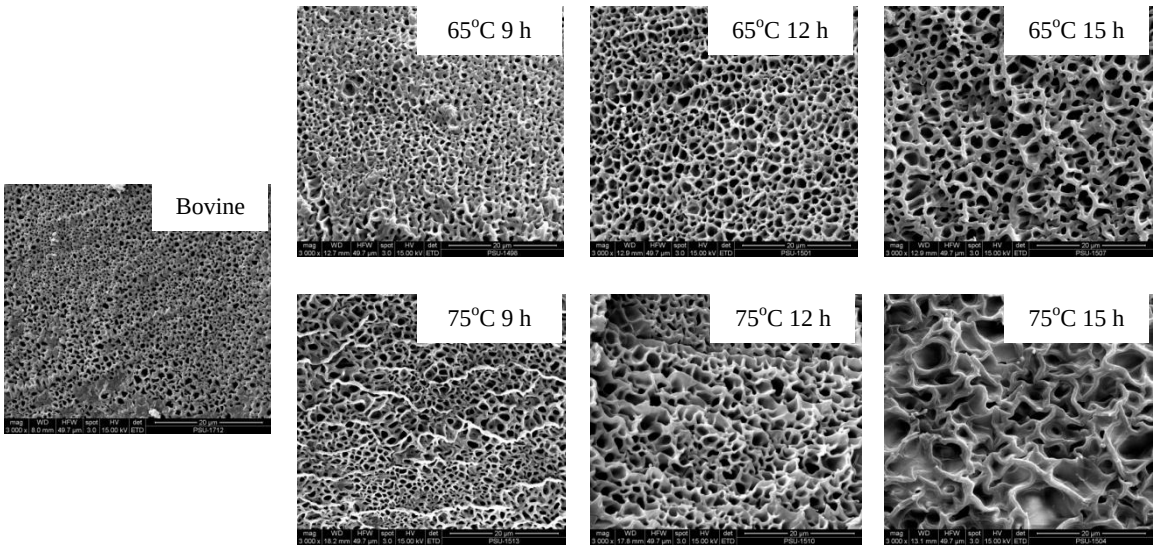


Figure 14. SEM microstructure of gels of gelatins from the skins of unicorn leatherjacket as affected by extraction temperatures and times. Magnification: 3000x.

3.4.6 Emulsifying properties

The emulsion activity index (EAI) and emulsion stability index (ESI) of gelatin are shown in Table 7. 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 ^{o}C showed higher EAI than that extracted at 65 ^{o}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 ^{o}C for 9 h, 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.

3.4.7 Foaming properties

Foam expansion (FE) and foam stability (FS) of the gelatins are shown in Table 7. 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). 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 ^{o}C for 12 h 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.

Table 7. 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 °C for 9 h	1	7.00 ± 0.64^{cA}	22.81 ± 2.55^{aB}	183.33 ± 0.67^{dC}	95.24 ± 5.39^{cC}
	2	4.17 ± 0.01^{bB}	23.02 ± 0.40^{aB}	193.81 ± 2.02^{deB}	114.29 ± 0.00^{cB}
	3	3.85 ± 0.05^{bcB}	34.69 ± 2.71^{aA}	231.61 ± 0.86^{abA}	139.72 ± 6.68^{cdA}
65 °C for 12 h	1	8.34 ± 0.63^{bcA}	17.89 ± 1.22^{bcB}	185.45 ± 0.37^{dC}	96.30 ± 5.24^{cC}
	2	5.06 ± 0.10^{aB}	20.17 ± 3.67^{abcB}	190.69 ± 0.31^{eB}	120.11 ± 6.58^{cB}
	3	4.88 ± 0.34^{aB}	30.96 ± 2.10^{abA}	227.08 ± 2.56^{bA}	157.37 ± 3.72^{bA}
65 °C for 15 h	1	8.24 ± 0.77^{bcA}	20.27 ± 1.35^{bB}	216.34 ± 0.93^{aC}	93.09 ± 2.70^{cC}
	2	4.20 ± 0.44^{bB}	20.97 ± 3.14^{abB}	223.50 ± 0.71^{aB}	117.50 ± 0.71^{cB}
	3	3.68 ± 0.26^{cB}	29.95 ± 2.75^{bA}	235.20 ± 3.64^{aA}	128.16 ± 2.61^{eA}
75 °C for 9 h	1	9.34 ± 0.88^{bA}	17.71 ± 0.79^{bcB}	180.56 ± 3.93^{dC}	97.22 ± 1.31^{cC}
	2	4.77 ± 0.07^{abB}	18.03 ± 1.07^{bcAB}	217.80 ± 2.55^{bB}	113.89 ± 4.40^{cB}
	3	4.65 ± 0.05^{abB}	21.80 ± 3.13^{cA}	234.01 ± 0.96^{aA}	135.17 ± 2.12^{deA}
75 °C for 12 h	1	8.44 ± 0.19^{bcA}	18.67 ± 0.27^{bcB}	194.08 ± 0.29^{cC}	145.82 ± 0.14^{aC}
	2	5.30 ± 0.34^{aB}	18.82 ± 1.59^{abcB}	196.87 ± 0.50^{dB}	152.48 ± 0.43^{aB}
	3	4.57 ± 0.44^{abC}	33.47 ± 2.11^{abA}	200.93 ± 0.03^{dA}	185.17 ± 3.46^{aA}
75 °C for 15 h	1	10.92 ± 1.51^{aA}	16.49 ± 0.61^{cB}	204.86 ± 3.02^{bC}	109.36 ± 4.76^{bC}
	2	4.92 ± 0.56^{aB}	16.61 ± 1.69^{cB}	212.71 ± 1.82^{cB}	134.62 ± 0.88^{bB}
	3	4.80 ± 0.88^{aB}	20.23 ± 1.71^{cA}	220.63 ± 2.24^{cA}	146.27 ± 2.13^{bcA}

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

3.5 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 was 65°C for 12 h, providing the best yield and gel strength.

CHAPTER 4

Effect of phosphorylation on gel properties of gelatin from the skin of unicorn leatherjacket

4.1 Abstract

Phosphorylation of gelatin from the skin of unicorn leatherjacket was carried out by incorporating sodium tripolyphosphate (STPP) at varying concentrations during pretreatment and extraction. Phosphate attached to gelatin during pretreatment and extraction was found at levels of 12.43 and 18.25 μmol/g, respectively. Based on zeta potential analysis, phosphorylated gelatin became negatively charged, reconfirming the charge modification mediated by phosphorylation. For gelatin phosphorylated during pretreatment, that using 0.2% STPP had the highest gel strength (128.3 g). Amongst all gelatins incorporated with STPP during extraction, that added with 0.08% STPP showed the highest gel strength (146.0 g). Slight differences in FTIR spectra were observed when gelatin was phosphorylated, compared with the control gelatin. Gel of gelatin phosphorylated with 0.08% STPP during extraction exhibited a finer and more compact structure with smaller pores and was able to set at 4 °C at the fastest rate, compared with other gelatins. Thus, the phosphorylation under the appropriate condition could improve gelling property of gelatin from skin of unicorn leatherjacket.

4.2 Introduction

Fish gelatin has been considered as a possible alternative to bovine gelatin, which may be associated with bovine spongiform encephalopathy (BSE) and foot-and-mouth disease crisis (Kittiphattanabawon *et al.*, 2010). In addition, gelatin from pig skin is not allowed for Judaism and Islam, whilst bovine gelatin is unacceptable for Hindu or Sikh. Owing to the limitation of gelatin from those sources, fish skin, which is a byproduct of fish processing industry, has been exploited as the starting material for gelatin. Gelatin from skin of different fish species has been intensively studied (Gómez-Guillén *et al.*, 2002; Muyonga *et al.*, 2004a). However, fish gelatin has poorer gel strength, compared with mammalian counterpart, due to its

lower imino acid content (Aewsiri *et al.*, 2009). Therefore, the improvement of gel properties of gelatin from marine sources, could widen the applications as food ingredient or for other uses.

Phosphorylation has been proven to be a useful method for improving the functional properties of food proteins (Sitohy *et al.*, 1995). Phosphorylation may introduce the phosphate to protein molecules. Modification of milk proteins by phosphorylation increased the net negative charge on the casein and whey proteins (Nayak *et al.*, 2006). The chemical phosphorylation, in which many phophoproteins are formed, occurs in nature. However, chemically phosphorylated food proteins have not been easily accepted by consumers or widely used in foods because of harsh reaction conditions, nonspecific chemical reagents, and the difficulty of removing unreacted reagent from the final product (Li *et al.*, 2010b). Sodium tripolyphosphate (STPP) is approved as a food additive, which can improve water holding capacity of seafood, meat and poultry (Dziezak, 1990; Rasković, 2007). Due to the prevalence of negative charge, STPP can be used for phosphorylation of gelatin, thereby modifying the properties of resulting gelatin.

Unicorn leatherjacket *(Aluterus monoceros)* belongs to the order Tetraodontiformes and is a member of the Monacanthidae family. This species has been used for frozen fillet production in Thailand, especially for the export. As a consequence, a large amount of skin has been produced as by-product. Its skin has been used as the raw material for gelatin production (Kaewruang *et al.*, 2013a). However, gelatin from the skin of unicorn leatherjacket still had the poor gel property, in comparison with commercial bovine gelatin (Kaewruang *et al.*, 2013a). Phosphorylation could be an promising method for quality improvement of gelatin from this species. Therefore, the objectives of this work were to phosphorylate the gelatin from the skin of unicorn leatherjacket by incorporating STPP during pretreatment and extraction processes, and to study the structural and gel properties of resulting gelatin.

4.3 Materials and methods

4.3.1 Chemicals/gelatin

All chemicals were of analytical grade. Sodium tripolyphosphate (STPP) ($Na_5O_{10}P_3$), 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).

4.3.2 Collection and preparation of fish skin

The skin of unicorn leatherjacket (*Aluterus monocerous)* was obtained from a dock, Songkhla, Thailand. 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 -20ºC until use. The storage time was less than 2 months.

4.3.3 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 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) for 6 h 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.

4.3.4 Phosphorylation and extraction of gelatin

Two processes were implemented for phosphorylation of gelatin as follows:

Process I. Swollen skin was soaked with STPP solution at different concentrations (0.2, 0.4 and 0.8%, w/v), using a skin/solution ratio of 1:5 (w/v) for 12 h at 4 °C. Thereafter, the skin was washed three times using twenty volumes for each washing. Thereafter, the treated skin was subjected to gelatin extraction using hot water (65 °C) at a skin/water ratio of 1:5 (w/v) for 12 h.

Process II. Swollen skins was mixed with distilled water containing STPP at different levels (0.04, 0.08 and 0.16%, w/v) at a skin/solution ratio of 1:5 (w/v). The extraction was performed at 65 ^{o}C for 12 h.

Gelatin prepared using typical process (without phosphorylation) was used as the control. After swelling process, the swollen skin was subjected to extraction using hot water (65 °C) with skin/ water ratio of 1:5 (w/v) for 12 h.

After extraction, the mixtures from both phosphorylation processes and from typical process were then centrifuged at 5,000 xg for 10 min using a refrigerated centrifuge model Avanti® J-E (Beckman Coulter, Inc., Palo Alto, CA, USA) to remove insoluble debris. The supernatant was collected and freeze-dried using a Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lynge, Denmark). All gelatins obtained were subjected to analyses.

4.3.5 Analyses

4.3.5.1 Determination of gel strength

Gelatin gel was prepared as per the method of Kaewruang *et al.* (2013a) with a slight modification. Gelatin sample was dissolved in distilled water at 60 ^{o}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 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.

4.3.5.2 Determination of phosphate content

Prior to analysis of phosphate content bound to gelatin, gelatin sample 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 changes for totally four times. The dialysed samples were then analysed for phosphate content following the method of Fiske and Subbarow (1925) with a slight modification. Gelatin (~1 g) sample was ashed at 550 ^{o}C to remove organic compounds using a muffle furnace (Fisher Scientific Model 550-58, Napean, Ontario, Canada). Thereafter, the inorganic residue was collected and 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 (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.

4.3.5.3 Measurement of zeta potential

Gelatin samples were dissolved in distilled water at a concentration of 0.5 mg/ml. Zeta potential analysis was performed using a zeta potential analyser (ZetaPALS, Brookhaven Instruments Co., Holtsville, NY, USA) at room temperature. Prior to analysis, the samples were adjusted to pH 7 using 1 M NaOH.

4.3.5.4 Determination of setting time

Setting time for gelatin was determined at 4 ^{o}C and room temperature (25-26 ^{o}C) according to the method of Muyonga *et al.* (2004a) with a slight modification. Gelatin solution (6.67%, w/v) was prepared in the same manner as

described previously. The solution (2 ml) was transferred to thin wall (12 mm × 75 mm) test tubes (PYREX®, Corning, NY, USA) and preheated at 60 ^{o}C for 10 min, followed by incubation in an ice bath (4 ^{o}C) and at room temperature (25-26 ^{o}C). An aluminum needle with the diameter and length of 0.1 and 25 cm, respectively, was inserted manually in the gelatin solution and raised every 10 s. The time at which the needle could not detach from the gelatin sample was recorded as the setting time.

4.3.5.5 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^{o} angle of incidence from the IR beam. For spectra analysis, freeze-dried gelatin 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 25ºC. Analysis of spectral data was done using the OPUS 3.0 data collection software programme (Bruker, Ettlingen, Germany).

4.3.5.6 Microstructure analysis

The microstructure 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.

4.3.6 Statistical analyses

All experiments were performed in triplicate 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).

4.4 Results and discussion

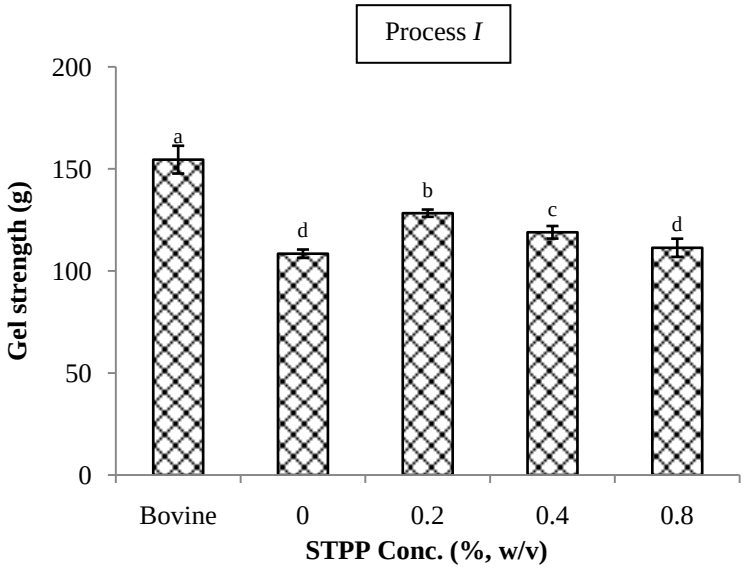
4.4.1 Gelling properties of phosphorylated gelatin

Gel strength of gelatin from the skin of unicorn leatherjacket incorporated without and with STPP during pretreatment (Process *I*) and extraction (Process *II*) is shown in Figure 15A and 15B, respectively. When comparing the gel strength of all gelatin samples with that of commercial bovine bone gelatin, all gelatins from unicorn leatherjacket skin had the lower gel strength ($P < 0.05$). Proline and hydroxyproline are thought to be responsible for gel strengthening through hydrogen bonding between free water molecules and the hydroxyl group of the hydroxyproline in gelatin (Fernández-Díaz *et al.*, 2001). The lower content of proline and hydroxyproline generally gives fish gelatin a low gel modulus, and low gelling and melting temperatures (Balti *et al.*, 2011). Amongst all gelatins incorporated with STPP during pretreatment (process *I*), that treated with 0.2% STPP had the highest gel strength (128.3 g) ($P < 0.05$). For gelatin prepared using process *II*, that incorporated with STPP at a level of 0.08% during extraction had the highest gel strength (146.0 g) ($P < 0.05$). The result indicated that the incorporation of STPP could play a role in the improvement of gel strength of gelatin from the skin of unicorn leatherjacket. Phosphate groups might attach with some amino acids, leading to phosphorylation of gelatin (Protein-OH + H_3PO_4 $\longrightarrow$ Protein-O-H_2PO_3 + H_2O) (Guo *et al.*, 2005). Phosphorylation may introduce the phosphate to gelatin molecules. As a result, ionic interaction between phosphate groups and $-NH_3^+$ of amino acids in gelatin chain could be augmented. Moreover, Li *et al.* (2005) reported the increased hardness,

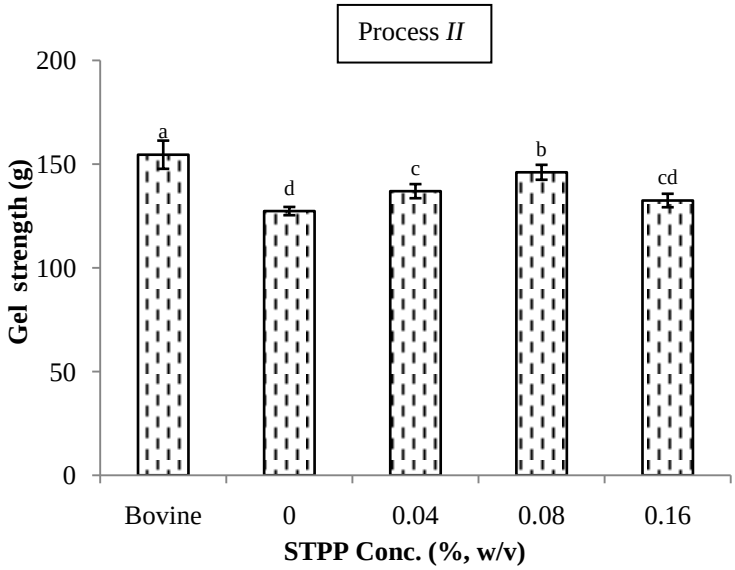
resiliency, compressibility, and water-holding capacity of phosphorylated egg white protein (PP-EWP). Nevertheless, at higher concentrations, 0.4% and 0.8% STPP for process *I* and 0.16% STPP for process *II*, gel strength decreased ($P < 0.05$). The decrease in gel strength might be due to the excessive phosphate group in gelatin or free phosphates, which more likely increased the electrostatic repulsions between protein molecules (Zhang *et al.*, 2007). Based on gel strength, the incorporation of 0.2% STPP during pretreatment in process *I* and 0.08% STPP during extraction in process *II* resulted in the increased gel strength of gelatin from the skin of unicorn leatherjacket.

4.4.2 Phosphate content and zeta potential

To verify the attachment of phosphate into gelatin, the dialysis of gelatin sample was carried out before analyses of phosphate content and zeta potential. Phosphate content in gelatin from tropical process was 4.85 µmol/g gelatin. Since the skin was swollen using phosphoric acid, phosphate might be incorporated to some extent into gelatin. Gelatins prepared by process *I* using 0.2% STPP and by process *II* with 0.08% STPP contained the bound phosphate of 12.43 and 18.25 µmol/g gelatin, respectively (Table 8). Phophate groups can be attached to the oxygen of seryl, threonyl, aspartyl (β-carboxyl) and tyrosyl residues and via nitrogen to lysyl (ε-amino) and histidyl (1 and 3) residues (Kunsheng *et al.*, 2007). The attached phosphate was more likely involved in gel formation by introducing negative charge to protein. As a result, ionic interaction of phosphorylated chains with positively charged adjacent chains could be enhanced, resulting in increased gel strength (Figure 15).



(A)



(B)

Figure 15. Gel strength of gelatin extracted from the skin of unicorn leatherjacket by process *I* (A) and process *II* (B) with different levels of STPP. Bars represent the standard deviation (n=3). Different letters on the bars indicate significant difference ($P < 0.05$). Process *I*: Phosphorylated during pretreatment; Process *II*: Phosphorylated during extraction.

Zeta potential analysis revealed that gelatin from typical process and gelatins incorporated with STPP using two processes had different charges (Table 8). The negative charge of gelatin was more likely attributed to the negative charge from phosphate, which could be attached to gelatin during pretreatment or extraction. When comparing two processes, it was noted that the addition of STPP during extraction was more effective in attachment of phosphate into gelatin, than during pretreatment. The process *II*, in which STPP was added during extraction for 12 h, might favour the attachment of phosphate into gelatin at high temperature. On the other hand, process *I* only allowed phosphate to attach to gelatin during pretreatment at lower temperature. Phosphorylation takes place at higher rate in proteins at higher temperature (Li *et al.*, 2004).

Table 8. Phosphate content and zeta potential of gelatin extracted from the skin of Unicorn leatherjacket as affected by phosphorylation processes

Processes	Phosphate content (μmol/g gelatin)	Zeta potential (mV)
Typical process	4.85 ± 1.09^{c}	2.38 ± 1.27^{c}
Process *I* *	12.43 ± 3.96^{b}	-4.24 ± 1.33^{b}
Process *II* *	18.25 ± 2.82^{a}	-6.40 ± 1.94^{a}

Mean ± SD (n = 3).

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

* Process *I*: Phosphorylated with 0.2% STPP during pretreatment.

* Process *II*: Phosphorylated with 0.08% STPP during extraction.

4.4.3 Setting time

Setting time required for the development of gels of gelatins from typical process (without phosphorylation) and those phosphorylated using the processes *I* and *II* under the optimal condition (Process *I*: 0.2% STPP, Process *II*: 0.08% STPP) at 4 ^{o}C are shown in Figure 16. The typical gelatin gel had a longer setting time at 4 ^{o}C than the phosphorylated gelatin and commercial bovine bone gelatin ($P < 0.05$). The longer setting time of the typical gelatin gel was coincidental with the poorer gel strength (Figure 15). Amongst all gelatins from skin of unicorn leatherjacket, gelatin prepared by process *II* using 0.08% STPP during extraction had the lowest setting times (1.46 min). Phosphate attached to gelatin might undergo ionic interaction with the positively charged residues of adjacent gelatin molecules, especially at low temperature. As a consequence, the gelatin chains were able to align or anneal more rapidly as indicated by the shorter setting time. Setting time was reported to be influenced by molecular properties of gelatin, including species, chain length, amino acid sequence and compositions (Kittiphattanabawon *et al.*, 2010). When setting was carried out at room temperature, all fish gelatins were not able to set (data not shown), regardless of phosphorylation. Conversely, bovine gelatin was able to set at room temperature with setting time of 24.30 min (data not shown). The result suggested that the nucleated polypeptides of unicorn leatherjacket skin gelatin might not be effectively generated during gelation process, more likely associated with the lower content of imino acids (Ahmad and Benjakul, 2011a). As a result, the junction zones could not be developed and the gel matrix could not be formed during setting at room temperature, even with the longer setting time.

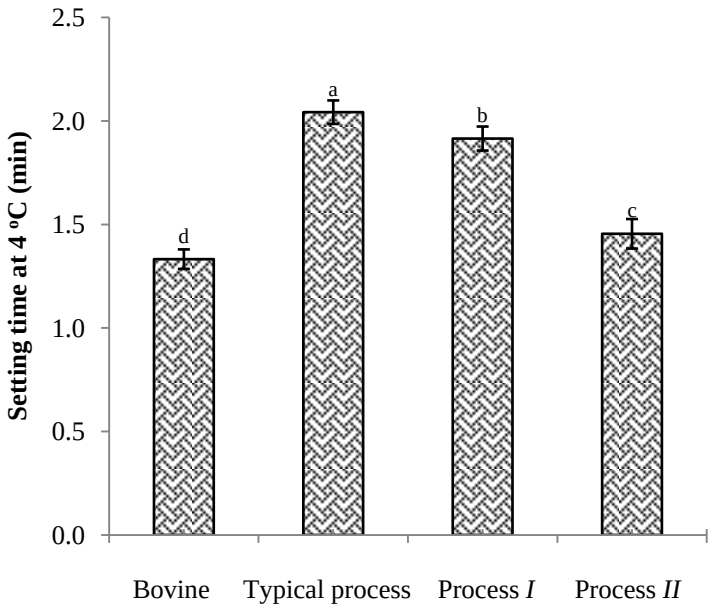


Figure 16. Setting time of gels of gelatin from the skins of unicorn leatherjacket at 4 °C as affected by phosphorylation processes. Bars represent the standard deviation (n=3). Different letters on the bars indicate significant difference ($P < 0.05$).

4.4.4 Fourier transformed-infrared spectroscopy of phosphorylated gelatin

FTIR spectra of gelatin from unicorn leatherjacket skin exhibited the characteristic peaks of Amide-A, Amide-B, Amide-I, -II, and -III (Figure 17). FTIR spectroscopy has been used to monitor the changes in the functional groups and secondary structure of gelatin samples (Muyonga *et al.*, 2004c). The absorption characteristics of Amide-A, commonly associated with N-H stretching vibration, occur in the wavenumber range of 3400-3440 cm^{-1} (Matmaroh *et al.*, 2011; Sai and Babu, 2001). Gelatins from typical process, processes *I* and *II* exhibited the amide-A bands at the wavenumber of 3311, 3301 and 3305 cm^{-1}, respectively. When NH group is involved in any bonding, the position starts to shift to lower frequencies. In amide-A region, the lower wavenumber was found in gelatin with phosphorylation, suggesting the involvement of an N-H group of gelatin in interaction with phosphate via ionic interaction. Amide-B (3081 cm^{-1}), represents assymetrical stretch of CH_2 (Abe and Krimm, 1972). This peak is involved with hydrogen bonding between free NH stretch coupled with hydrogen in polypeptide chain (Matmaroh *et al.*, 2011). In

this study, the higher amplitudes of amide-B as well as the lower wavenumber were found in phosphorylated gelatins (3060 cm^{-1}), compared to that obtained from the typical process (3093 cm^{-1}), indicating that the interaction between NH group of peptide chains and phosphates.

Amide-I peak is located in the wavenumber range of 1600-1700 cm^{-1}. Amide-I represents C=O stretching/hydrogen bonding coupled with COO. The absorption peak at amide-I region is characteristic for the coil structure of gelatin (Yakimets *et al.*, 2005). Its exact location depends on the hydrogen bonding and the conformation of protein structure (Uriarte-Montoya *et al.*, 2011). In the present study, the amide-I peak was observed in the range of 1644-1646 cm^{-1}. This observation confirmed the formation of hydrogen bond between N–H stretch and C=O of gelatin chains (Zanaboni *et al.*, 2000). Gelatin from typical process, processes *I* and *II* had the amide-II peak at the wavenumbers of 1544, 1546 and 1548 cm^{-1}, respectively. The amide-II vibration modes are attributed to an out-of-phase combination of CN stretch and inplane NH deformation modes of the peptide (Bandekar, 1992). Lower amplitude was found in gelatin prepared by process *II*, compared to others. The result suggested that N–H was more likely involved in bonding with phosphates during gelatin extraction process. In addition, amide-III was detected around the wavenumber of 1238-1240 cm^{-1} in all gelatins. The amide III represented the combination peaks between C-N stretching vibrations and N-H deformation from amide linkages as well as absorptions arising from wagging vibrations from CH_2 groups from the glycine backbone and proline side-chains (Jackson *et al.*, 1995). This indicated that the disorder of molecular structure due to transformation of an α-helical to a random coil structure, which occurred during extraction at high temperature. These changes were associated with loss of triple-helix state as a result of denaturation of collagen to gelatin (Muyonga *et al.*, 2004c). It was noted that gelatin prepared by process *II* had the peak at wavenumber of 990 cm^{-1}, while gelatin from process *I* showed a small peak. This was coincidental with the lower amplitude of Amide-II peak. However, there was no peak at the same wavenumber in gelatin prepared by the typical process. This peak was more likely due to phosphate groups attached to gelatin as affected by phosphorylation. Gadaleta, Paschalis, Betts,

Mendelsohn and Boskey (1996) reported that phosphate absorption region was at 900-1200 cm^{-1}. The peak amplitude was in agreement with the amount of phosphate incorporated into gelatin (Table 8).

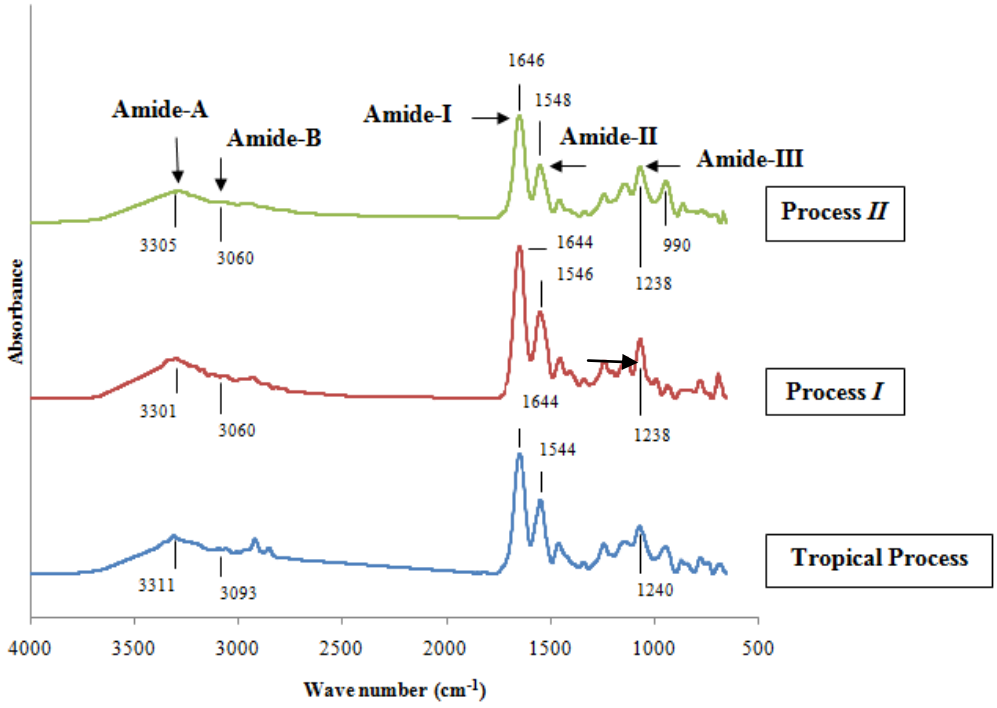


Figure 17. Fourier transform infrared spectra of gelatins extracted from the skin of unicorn leatherjacket as affected by phosphorylation processes. Process *I*: Phosphorylated with 0.2% STPP during pretreatment; Process *II*: Phosphorylated with 0.08% STPP during extraction.

4.4.5 Microstructure of gelatin gels

Microstructures of the gel of typical gelatin from unicorn leatherjacket skin (without phosphorylation) (A), gel of gelatin prepared by process *I* (B) and gel of gelatin prepared by process *II* (C) are shown in Figure 18. In general, gelatin gels were sponge or coral-like in structure. The typical gelatin gel had a much looser network with the larger voids, compared with gels from gelatins phosphorylated by both processes. These observations suggested that phosphates attached to gelatin might be involved in network formation, in which an ordered structure with finer strands could be developed. However, gel of gelatin prepared by process *II*

(0.08% STPP during extraction) exhibited a finer and more compact or denser network structure with smaller pores in the matrix than that of gelatin from process *I* (0.2% STPP during pretreatment). The finer and denser structure of gel network was not easy to disrupt by the force applied. This was in accordance with the higher gel strength of gelatin prepared by process *II* (Figure 15). Therefore, the arrangement and association of protein molecules in the gel matrix were governed by phosphorylation process.

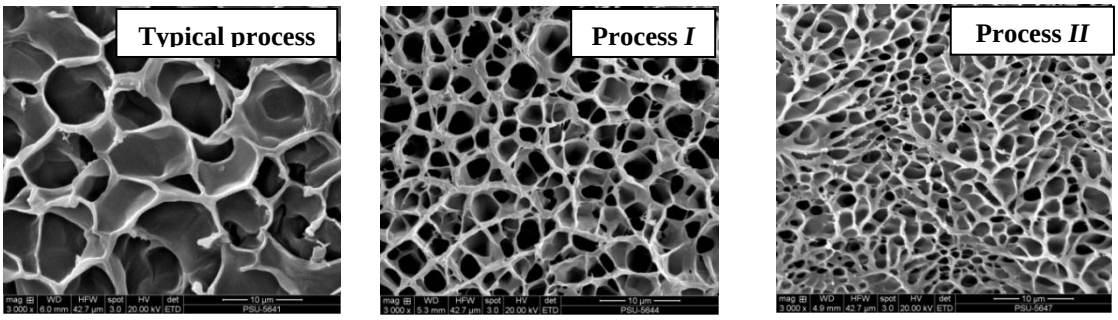


Figure 18. Microstructure of gels of gelatins from the skin of unicorn leatherjacket as affected by phosphorylation processes. Magnification: 3000x. Process *I*: Phosphorylated with 0.2% STPP during pretreatment; Process *II*: Phosphorylated with 0.08% STPP during extraction.

4.5 Conclusion

The phosphorylation of gelatins by addition of STPP during pretreatment or extraction could improve the gel properties of gelatin from skin of unicorn leatherjacket. Phosphorylated gelatin became negatively charged, thereby enhancing the aggregation of gelatin and strengthing gel. Gelatin incorporated with 0.08% STPP during extraction exhibited the improved gel properties, as indicated by higher gel strength and lower setting time. Therefore, the appropriate phosphorylation could be an promising process to improve gel property of fish gelatin.

CHAPTER 5

Characteristics and gelling property of phosphorylated gelatin from the skin of unicorn leatherjacket

5.1 Abstract

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.

5.2 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.*, 2013a). 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, 2011a). 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.* (2013b) 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.

5.3 Materials and methods

5.3.1 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).

5.3.2 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 -20ºC until use. The storage time was less than 2 months.

5.3.3 Pretreatment of skin

Removal of non-collagenous proteins and swelling of prepared skin were carried out according to the method of Ahmad and Benjakul (2011a). 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.

5.3.4 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 ^oC 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 refered to as "control gelatin".

5.3.5 Phosphorylation of gelatin

5.3.5.1 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.

5.3.5.2 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.

5.3.6 Analyses

5.3.6.1 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 changes for totally four times, followed by freeze-drying. 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 ^{o}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 (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.

5.3.6.2 Determination of gel strength

Gelatin gel was prepared as per the method of Kaewruang *et al.* (2013a). Gelatin sample was dissolved in distilled water at 60 ^{o}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 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.

5.3.6.3 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.

5.3.6.4 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^{o} 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 25ºC. Analysis of spectral data was done using the OPUS 3.0 data collection software programme (Bruker, Ettlingen, Germany).

5.3.6.5 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.

5.3.7 Statistical analyses

All experiments were performed in triplicate 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).

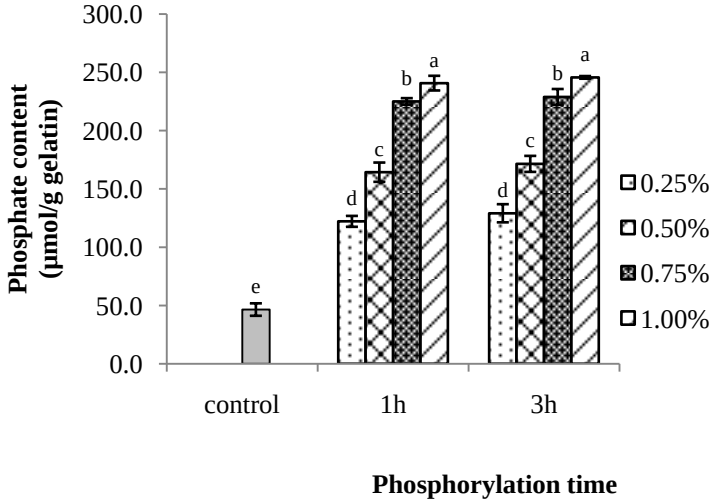
5.4 Results and discussion

5.4.1 Effect of STPP level and time on phosphorylation and gel strength of gelatin

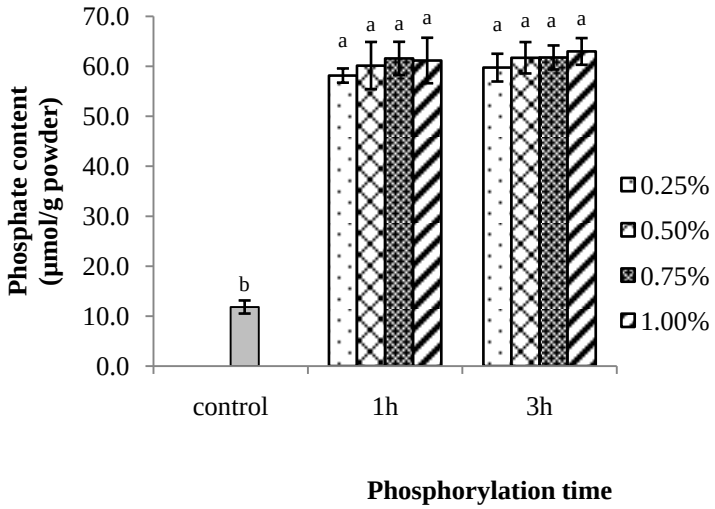
5.4.1.1 Phosphate content

Phosphate contents of gelatin phosphorylated by incorporating STPP at various levels for different times are shown in Figure 19. 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) (Figure 16A). 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 (Figure 16B). 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).



(A)



(B)

Figure 19. 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 ($P < 0.05$).

5.4.1.2 Gel strength

Gel strength is one of the most important functional properties of gelatin. Gel strength of gelatin phosphorylated with STPP at different concentrations for various times is presented in Figure 20. 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 of 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 °C for 1 h resulted in the increased gel strength of gelatin from the skin of unicorn leatherjacket.

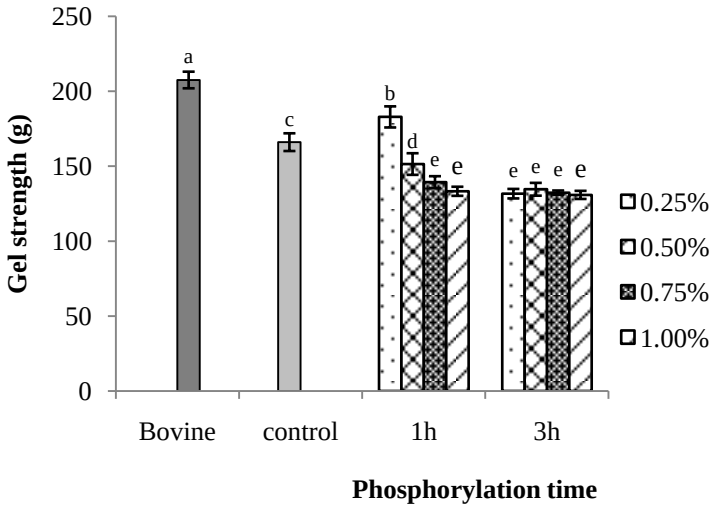


Figure 20. Gel strength of gelatins from the skin of unicorn leatherjacket phosphorylated using STPP at different levels for various times. Bars represent the standard deviation (n=3). Different letters on the bars indicate significant difference ($P < 0.05$).

5.4.2 Effect of pH on phosphorylation and gel strength of gelatin

5.4.2.1 Phosphate content and zeta potential

Phosphate contents of gelatin phosphorylated with 0.25% STPP at different pHs for 1 h are shown in Table 9. 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 9. 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 9. Phosphate content and zeta potential of gelatin from skin of Unicorn leatherjacket phosphorylated at different pHs

Concentration of phosphate	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 ($P < 0.05$).

5.4.2.2 Gel strength

Gel strength of gelatin phosphorylated with 0.25% STPP at various pHs for 1 h is depicted in Figure 21. 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. Sodium tripolyphosphate ($Na_5P_3O_{10}$) has a

pKa of 8.9 (Rasković, 2007). When the pH of STPP was adjusted to pH 11, phosphate groups turned to be negatively charged at high level. Their might cause the repulsion between phosphates incorporated into protein chains, thereby lowering the interaction. As a result, the decrease in gel strength of gelatin phosphorylated at pH 11 was observed. This plausibly resulted in the loosen network structure 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, role 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, which subsequently favoured the aggregation of gelatin chains. This could compensate with the lower H-bond mediated by imino acids in fish gelatin.

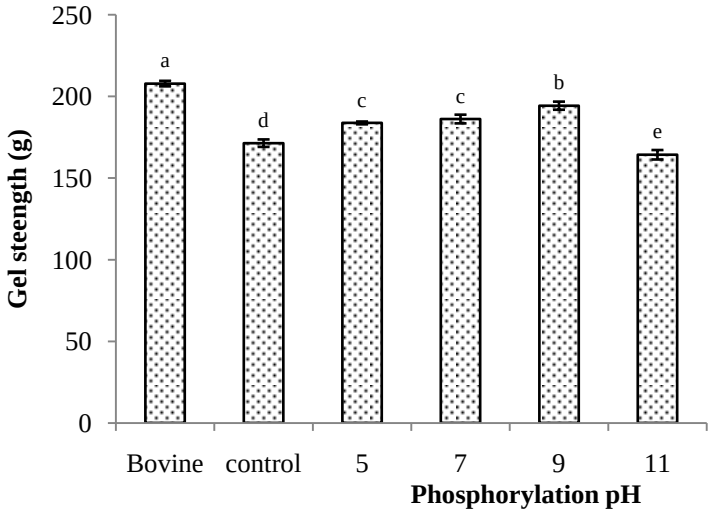


Figure 21. Gel strength of gelatins from the skin of unicorn leatherjacket phosphorylated using 0.25% STPP for 1 h at different pHs. Bars represent the standard deviation (n=3). Different letters on the bars indicate significant difference ($P < 0.05$).

5.4.3 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 Figure 22. 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.

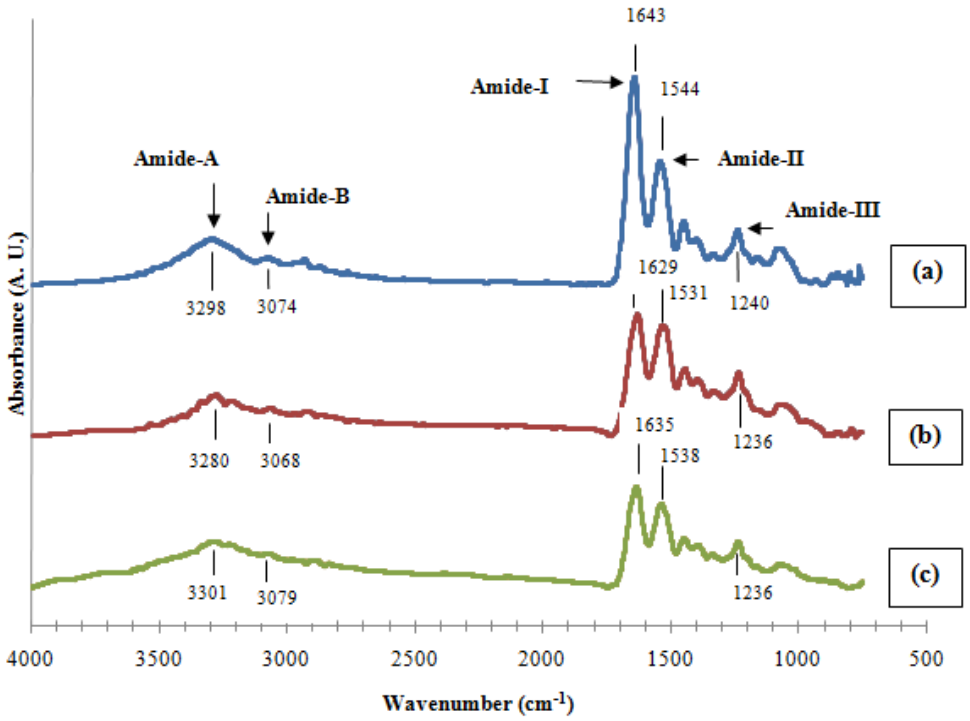


Figure 22. Fourier transform infrared spectra of gelatin extracted from the skin of unicorn leatherjacket without phosphorylation (a) and with phosphorylation using 0.25% STPP at pH 7 (b) and pH 9 (c).

5.4.4 Microstrutures of gel

Microstructures of gels of gelatin without and with phosphorylation using 0.25% STPP at pH 7 and 9 are illustrated in Figure 23. All gelatin gels showed a network of interconnection with a 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 (Figure 21). 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 controlled by phosphorylation condition.

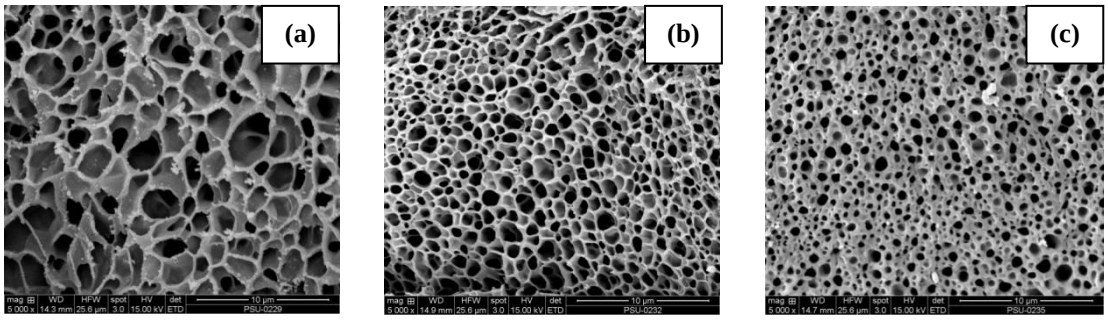


Figure 23. Microstructure of gels of gelatins from the skin of unicorn leatherjacket without phosphorylation (a) and with phosphorylation using 0.25% STPP at pH 7 (b) and pH 9 (c). Magnification: 5000x.

5.5 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.

CHAPTER 6

Impact of divalent salts and the ratios of bovine gelatin on gel properties of phosphorylated gelatin from skin of unicorn leatherjacket

6.1 Abstract

Impact of zinc chloride ($ZnCl_2$) and calcium chloride ($CaCl_2$) on gel strength of phosphorylated fish gelatin (PFG) from the skin of unicorn leatherjacket was investigated. Gel strength of PFG increased with increasing concentrations of $ZnCl_2$ and $CaCl_2$. Nevertheless, a higher gel strength was found in gelatin gel added with $CaCl_2$, compared with $ZnCl_2$. PFG added with 20 µM $CaCl_2$ had the increase in gel strength by 15.7%, compared with control gel. The effect of bovine gelatin (BG) to PFG ratios on the properties of the resulting gel was studied. Hardness of gels decreased with increasing PFG content ($P < 0.05$). Nevertheless, no differences in hardness were found amongst gels with BG/PFG ratios of 10:0 and 7.5:2.5 ($P > 0.05$). Therefore, $CaCl_2$ could be used to induce aggregation of PFG and the ratio of BG/PFG directly affected the properties of mixed gelatin.

6.2 Introduction

Gelatin is a protein derived from collagen by thermal denaturation or partial hydrolysis. It has rheological properties of thermo-reversible transformation between solution (sol) and gel form. Gelatins is composed of long chains of amino acids connected by peptide bonds (Rahman and Al-Mahrouqi, 2009). However, gelatin is almost completely lacking of tryptophan and is low in methionine, cystine and tyrosine (Jamilah and Harvinder, 2002). The quality of gelatin depends on its physical, chemical and structural characteristics. The most important physical properties of gelatin are gel strength and viscosity. The quality of gelatin is measured by the gel strength or Bloom value, classified as low (<150), medium (150-220) and high Bloom (220-300) (Johnston-Bank 1983).

Alternative gelatin has gained increasing attention in recent years as the demand for non-bovine and non-porcine gelatin has increased due to the BSE

(bovine spongiform encephalopathy) crisis as well as religious and social reasons. Pig skin gelatin is not acceptable for Judaism and Islam and beef gelatin is acceptable only if it has been prepared according to religious requirements (Badii and Howell, 2006). As a consequence, fish gelatin has gained increasing interest as the potential alternative for land animal counterpart. Nevertheless, fish gelatin generally possesses the poorer gelling property, compared with land animal counterpart. Therefore, the improvement of gel properties of gelatin from marine sources is needed, and the wider applications as food ingredient or for other uses can be achieved.

Phosphorylation of gelatin is another means to improve gel properties, in which phosphate could provide negatively charged domain for protein aggregation (Kaewruang *et al.*, 2013b). 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, 1997b). To augment the salt bridge between charged residues of phosphorylated gelatin, the introduction of divalents should be an effective approach. Divalent cations have a much greater effect on the gel properties than monovalent cations (Tang *et al.*, 1995). This is because divalent salt ions screen electrostatic interactions between the charged protein molecules (Yasuda *et al.*, 1986). Additionally, the mixing of fish gelatin with mammalian gelatin from acceptable sources at an appropriate ratio can bring about the improved gelling property of fish gelatin. Recently, Kaewudom *et al.* (2012) reported that the mixture of fish gelatin and bovine gelatin at a ratio of 1:1 or 1:2 in conjunction with MTGase could render surimi gel with acceptability equivalent to the control surimi gel.

The objectives of this study were to evaluate the effect of type and concentration of divalents on gel property of phosphorylated fish gelatin (PFG) from unicorn leatherjacket skin and to study the impact of bovine gelatin/PFG ratios on textural properties of mixed gelatins.

6.3 Materials and methods

6.3.1 Chemicals/gelatin

All chemicals were of analytical grade. Sodium tripolyphosphate was purchased from Sigma (St Louis, MO, USA). Zinc chloride ($ZnCl_2$) and calcium chloride ($CaCl_2$) were obtained from Lab-Scan (Bangkok, Hat Yai, Thailand). Food grade bovine bone gelatin with the bloom strength of 150-250 g was obtained from Halagel (Thailand) Co., Ltd. (Bangkok, Thailand).

6.3.2 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 -20ºC until use. The storage time was less than 2 months.

6.3.3 Preparation of phosphorylated fish gelatin (PFG)

6.3.3.1 Pretreatment of skin

Removal of non-collagenous proteins and swelling of prepared skin were carried out according to the method of Kaewruang *et al.* (2013a). 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 (28-32 °C) 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 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.

6.3.3.2 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^{o}C using a Beckman model Avanti J-E centrifuge (Beckman Coulter, Inc., Palo Alto, CA, USA) to remove insoluble materials. Soluble gelatin was subjected to phosphorylation.

6.3.3.3 Phosphorylation of gelatin

To gelatin solution, STPP was added to obtain final concentrations of 0.25 % (w/w gelatin). The solution was adjusted to pH 9 using 1 M NaOH. The solution was continuously stirred at 65 °C for 1 h. The solution was cooled and freeze-dried using a Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lynge, Denmark).

6.3.4 Study on the effect of $ZnCl_2$ and $CaCl_2$ at different concentrations on gel property of PFG

PFG was dissolved in distilled water at 60 ^{o}C to obtain the final concentration of 6.67% (w/v). The gelatin solution was stirred until the gelatin was solubilised completely. To gelatin solution, $ZnCl_2$ or $CaCl_2$ were added to obtain the different final concentrations (2.5, 5, 10, 20 and 40 µM). PFG without the addition of divalent salt was used as the control. The solution was continuously stirred at room temperature for 15 min and cooled in a refrigerator at 10 °C for 16-18 h for gel maturation. All gel samples were subjected to analyses.

6.3.5 The effect of bovine gelatin/PFG ratios on the gel properties of mixed gelatin

Bovine gelatin (BG) was mixed with PFG at different ratios (BG/PFG = 10:0, 7.5:2.5, 5:5, 2.5:7.5 and 0:10). Solutions of mixed gelatins at various BG/PFG ratios (6.67%, w/v) were prepared as previously described. Thereafter, $CaCl_2$ was

added at a final concentration of 20 µM. The gel samples were incubated at 10^{o}C for 16 h for gel maturation. The resulting gelatin gels were subjected to analyses.

6.3.6 Analyses

6.3.6.1 Determination of gel strength

Gelatin gel was prepared as per the method of Kaewruang *et al.* (2013a) with a slight modification. The dimension of sample was 3 cm in diameter and 2.5 cm in height. 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^{-1}.

6.3.6.2 TPA

TPA was measured using the Texture analyser (TA.XT Plus). Gelatin gel samples with 3 cm in diameter and 2.5 cm in height were used for testing. The gels were compressed by an aluminum probe (100 mm diameter plate) until the deformation reached 30% at a speed of 1.0 mm s^{-1}. The pause between the first and second compressions was 3 s. The testing was done immediately after the samples were removed from the refrigerator. Hardness, brittleness, springiness, cohesiveness, adhesiveness, gumminess and chewiness of samples were recorded.

6.3.6.3 Microstructure analysis

The microstructure 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.

6.3.6.4 Colour measurement

The colour of gelatin solutions (6.67% w/v) was measured by a Hunter lab colour metre (ColorFlex, HunterLab Reston, USA) and reported by the CIE system. L*, a* and b* parameters indicate lightness, redness/greenness and yellowness/blueness, respectively.

6.3.7 Statistical analyses

All experiments were performed in triplicate and a completely randomised design (CRD) was used. Data were presented as means ± standard deviation and the probability value less than 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).

6.4 Results and discussion

6.4.1 Effect of divalent salts on gel property of PFG

Gel strength of PFG added with different divalent salts ($ZnCl_2$ and $CaCl_2$) at various concentrations (2.5-40 µM) is shown in Figure 24. An increase in gel strength was observed when the concentrations of $ZnCl_2$ and $CaCl_2$ increased from 2.5 to 20 µM ($P < 0.05$). Nevertheless, no difference in gel strength was found between PFG containing $CaCl_2$ at levels of 2.5 and 5 µM ($P > 0.05$). Amongst all samples, PFG induced by $CaCl_2$ at a concentration of 20 µM had the highest gel strength (212.7 g) ($P < 0.05$) and it was equivalent to bovine gelatin ($P > 0.05$). The result suggested that divalents could act as the bridge between negatively charged residues, particularly phosphate group and COO^-. As a result, the interaction between gelatin chains could be enhanced, thereby strengthening gel network. Divalent ions were able to induce protein conformational changes and interacted with negative charges on the polypeptide chains of proteins (Ding *et al.*, 2011). At the same

concentration used, $CaCl_2$ resulted in the higher gel strength, compared with $ZnCl_2$. 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. Nevertheless, at higher concentration (40 µM) of both salts, gel strength of gelatin decreased. Cations at an excessive amount might occupy anionic sites of gelatin molecules, thus preventing the formation of linkages between adjacent site chains. As a result, the repulsive forces associated with dominant positive charge might reduce the formation of linkages, leading to weakened gel structures. Additionally, salts at high levels might compete in binding with water, causing the "salting out" effect. Gelatin might lose its solubility and the large aggregate could be formed along with non-uniform network. It was noted that both $CaCl_2$ and $ZnCl_2$ at low levels (2.5 and 5 µM) resulted in the lower gel strength, in comparison with control PFG gel (without divalent salts). At lower level, divalents more likely neutralised charge of gelatin chains and no divalents were available for bridging. Therefore, the formation of strong network could be achieved for PFG by incorporation with $CaCl_2$ at an appropriate concentration.

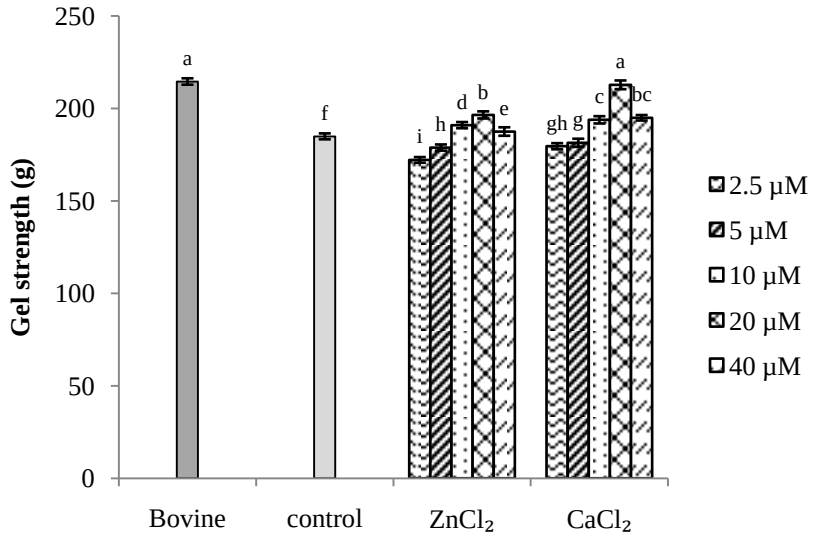


Figure 24. Gel strength of PFG containing $ZnCl_2$ or $CaCl_2$ at different concentrations. Bars represent the standard deviation (n=3). Different letters on the bars indicate significant difference ($P < 0.05$).

6.4.2 Effect of BG/PFG ratios on the gel properties of mixed gelatin

6.4.2.1 Gel strength

Gel strength is one of the most important functional properties of gelatin. Gel strength of mixed gelatin with different BG/PFG ratios are shown in Figure 25. The gel strength significantly decreased with increasing PFG proportion ($P < 0.05$). However, no differences in gel strength were found between gels with BG/PFG ratios of 10:0 and 7.5:2.5 ($P > 0.05$). This was most likely governed by the differences in the imino acid content. Mammalian gelatins generally have the high content of hydroxyproline and proline (Benjakul *et al.*, 2009). Fish gelatins have lower hydroxyproline contents (about 7-10% of the total amino acids), compared with that of bovine gelatin (14%) (Nalinanon *et al.*, 2008). Proline and hydroxyproline are thought to be responsible for the stability of the triple-helix of collagen structure through hydrogen bonding between free water molecules and the hydroxyl group of the hydroxyproline in gelatin (Fernández-Díaz *et al.*, 2001).

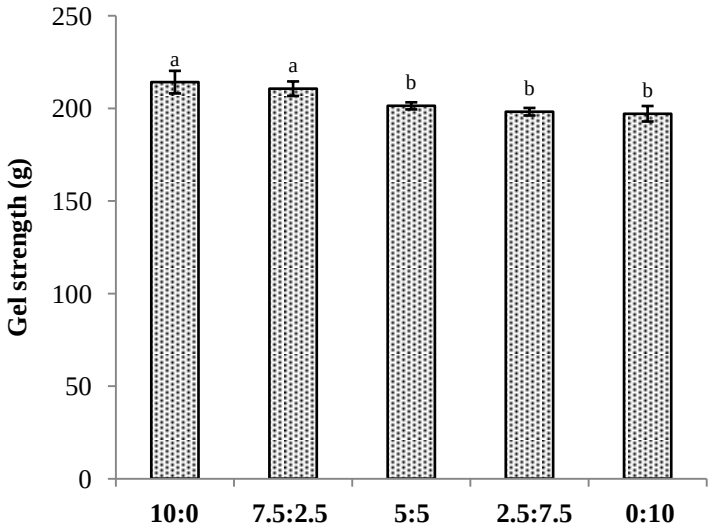


Figure 25. Gel strength of mixed gel with different BG/PFG ratios. Bars represent the standard deviation (n=3). Different letters on the bars indicate significant difference ($P < 0.05$).

6.4.2.2 Textural properties

The effects of BG/PFG ratios on gel hardness are shown in Figure 26(a). Hardness generally decreased as the proportion of PFG increased ($P < 0.05$). Nevertheless, no differences in hardness were found between gels with BG/PFG ratios of 10:0 and 7.5:2.5 ($P > 0.05$). The result was in agreement with gel strength (Figure 25).

No differences in springiness and cohesiveness were found amongst all gel samples with different BG/PFG ratios ($P > 0.05$) (Figure 26b and 26c). This result was in accordance with Wangtueai and Noomhorm (2009) who reported that the bovine and lizardfish scale gelatins were insignificantly different in springiness. Springiness (sometimes also referred to as "elasticity") is a perception of gel "rubberiness" in the mouth, and is a measure of how much the gel structure is broken down by the initial compression (Sanderson, 1990). If springiness is high, it requires more mastication energy in the mouth. High springiness will be obtained when the gel structure is broken into few large pieces during the first TPA compression, whereas low springiness results from the gel breaking into many small pieces. Muyonga *et al.* (2004a) reported that similar cohesiveness was observed between bovine, commercial fish skin and the Nile perch gelatin gels. Cohesiveness is often used as an indice of the ability of gel to maintain an intact network structure. It indicates how well the product withstands a second deformation relative to its behaviour during the first deformation.

Brittleness (or fracturability) of all gels was not detectable (data not shown). This result was in accordance with Rahman and Al-Mahrouqi (2009) who reported that fish and mammalian gelatin showed no brittleness.

Adhesiveness of gel increased with increasing PFG content ($P < 0.05$). Amongst all gels, that prepared from PFG exhibited the highest adhesiveness ($P < 0.05$). Adhesiveness is defined as the work necessary to overcome the attractive forces between the product and a specific surface. BG and mixed gelatin with BG/PFG ratios of 7.5:2.5 showed higher gumminess and chewiness, compared with others ($P < 0.05$). The higher gumminess was in accordance with the higher hardness of the formers

(Figure 26a). The higher hardness caused higher gumminess of gelatin (Shafiur Rahman and Al-Mahrouqi, 2009).

The differences in textural characteristics of mixed gelatin gels with various BG/PFG ratios could be explained by different amino acid concentrations, molecular weight distributions, as well as aggregation of protein chains (Benjakul *et al.*, 2009). Mammalian bovine gelatin had the higher content of Proline and Hydroxyproline, compared with fish skin gelatin (Gudmundsson, 2002). Hydroxyl groups of Hydroxyproline play a part in the stability of the helix by inter-chain hydrogen bonding via a bridging water molecule as well as direct hydrogen bonding to the carbonyl group (Wong, 1989). Gelatin, with the lower chain length, could not form the strong gel due to lower interjunction zones (Nagarajan *et al.*, 2012). The result suggested that PFG could be mixed with BG up to 25%, in which textural property was still comparable to BG. It was note that $CaCl_2$ at 20 µM also contributed to improvement of textural property of mixed gelatins, via salt bridging.

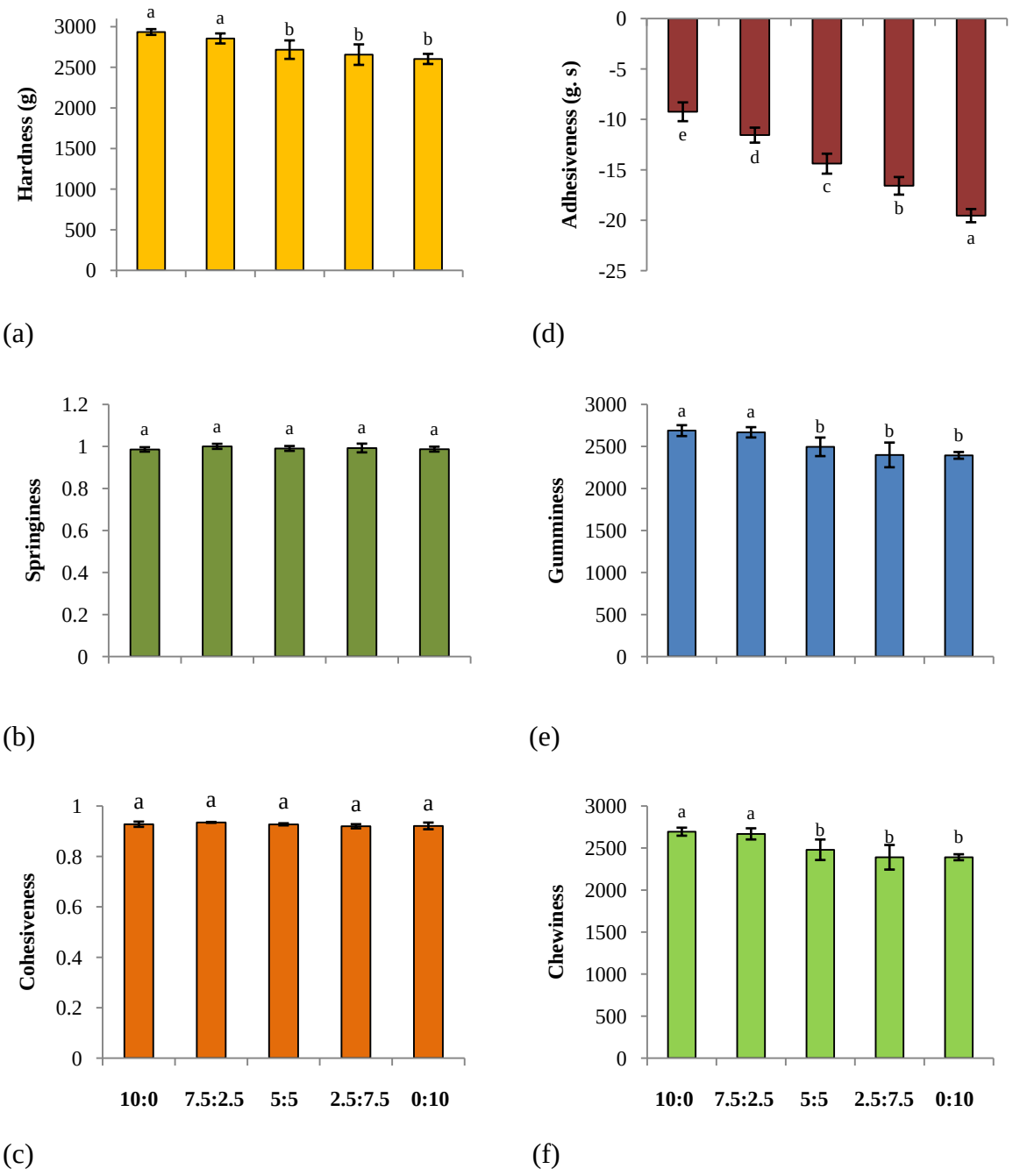


Figure 26. Hardness (a), springiness (b), cohesiveness (c), adhesiveness (d), gumminess (e) and chewiness (f) of mixed gel with different BG/PFG ratios. Bars represent the standard deviation (n=3). Different letters on the bars indicate significant difference ($P < 0.05$).

6.4.2.3 Colour

Differences in colour of gelatin solutions (6.67%) were observed as shown in Table 10. Higher L*-value of BG solution was observed, compared with others ($P < 0.05$) (Table 1). This was concomitant with the lower a* and b*-value of BG solution. The result suggested that PFG had more redness (a*-value) and yellowness (b*-value) than BG. The changes in colour of mixed gelatin varied with BG/PFG ratios. Lightness of mixed gelatin decreased with concomitant increases in redness and yellowness when PFG increased. Gelatin manufacture generally has a good process to clarify the impurities from the gelatin solution, such as chemical clarification and filtration processes (Ahmad and Benjakul, 2011a). PFG in the present study was not clarified or refined after extraction and phosphorylation. However, the colour did not affect functional properties of gelatin (Benjakul *et al.*, 2009).

Table 10. Colour of solutions of mixed gelatins with different BG/PFG ratios.

Sample (BG/PG)	L*	a*	b*
100:0	91.2 ± 0.3^{a}	-1.0 ± 0.0^{d}	21.2 ± 0.6^{c}
75:25	75.2 ± 0.6^{b}	3.0 ± 0.1^{c}	28.9 ± 0.4^{b}
50:50	73.3 ± 0.7^{c}	3.7 ± 0.2^{b}	31.0 ± 0.3^{a}
25:75	73.4 ± 0.2^{c}	3.7 ± 0.1^{b}	30.4 ± 0.2^{a}
0:100	67.3 ± 0.6^{d}	4.9 ± 0.1^{a}	30.5 ± 0.5^{a}

Mean ± SD (n = 3).

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

6.4.2.4 Scanning electron microscopy

The microstructures of gel with different BG/PFG ratios are shown in Figure 27. A highly interconnected and denser network was developed in BG gel. The larger strands were also observed in BG gel. For PFG gel, the coarser and non-uniform network was noticeable along with the larger voids. The thinner strands were obtained, compared with those from BG gels. Mixed gels turned to possess intermediate microstructure between BG and PFG gels, dependent upon ratio used. Larger voids were found in mixed gelatin with higher concentrations of PFG. This

was possibly correlated with low aggregation of peptide chains during gelation. Generally, the arrangement and association of protein molecules in the gel matrix directly contributed to the gel strength of gelatin (Benjakul *et al.*, 2009). Gel with fewer inter-junctions or thinner strands with looser network might be disrupted easily by an applied force, resulting in lower gel strength. Therefore, the ratio of BG/PFG had an impact on the network of gelatin gel, which directly determined textural property of gelatin gel.

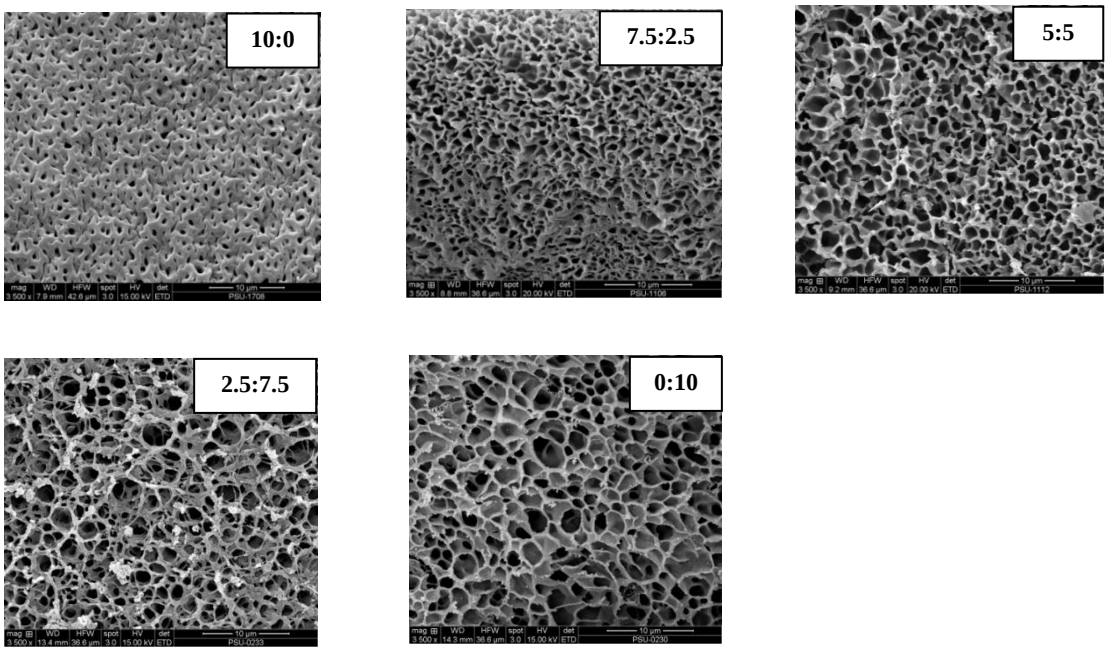


Figure 27. Microstructure of gels of mixed gelatin with different BG/PFG ratios. Magnification: 3500x.

6.5 Conclusion

PFG added with $CaCl_2$ at a concentration of 20 µM exhibited the improved gel properties. Textural characteristics of gel were governed by the BG/PFG ratio. PFG could be mixed with BG at a level of 25% without the negative effect on textural property. Therefore, the use of divalent and phosphorylated gelatin could be used to replace the bovine gelatin to some extent.

CHAPTER 7

Summary and future works

7.1 Summary

1. Extraction temperatures of 65 ^{o}C or 75 ^{o}C were suggested for gelatin extraction from the skin of unicorn leatherjacket, in which 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.

2. Longer extraction times resulted in the 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 was 65^{o}C for 12 hr, providing the best yield and gel strength.

3. The phosphorylation of gelatins by addition of STPP during pretreatment or extraction could improve the gel properties of gelatin from skin of unicorn leatherjacket to some degree. Phosphorylated gelatin became negatively charged, thereby enhancing the aggregation of gelatin. Gelatin incorporated with 0.08% STPP during extraction exhibited the improved gel properties, as indicated by higher gel strength and lower setting time.

4. The phosphorylation of gelatin using 0.25% STPP at pH 9 for 1 h yielded gelatin with improved gel strength. Finer and ordered network of gel was obtained for gelatin phosphorylated under the optimal condition.

5. Phosphorylated fish gelatin added with $CaCl_2$ at a concentration of 20 µM exhibited the improved gel properties. PFG could be mixed with BG at a level of 25% without the negative effect on textural property. Therefore, the use of divalent for phosphorylated gelatin and mixing of PFG with bovine gelatin could improve gel properties of FG.

7.2 Future works

1. Effect of phosphorylation of fish gelatin on functional properties such as emulsifying properties and foaming properties should be further studied.

2. The modification of fish gelatin using several methods should be investigated.

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VITAE

Name Miss Phanngam Kaewruang

Student ID 5311020024

Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Food Science and Technology)	Thammasat University	2009

List of Publication and Proceedings

Publications

1. Kaewruang, P., Benjakul, S. and Prodpran, T. 2013. Molecular and functional properties of gelatin from the skin of unicorn leatherjacket as affected by extracting temperatures. Food Chemistry. 138: 1431-1437.
2. Kaewruang, P., Benjakul, S., Prodpran, T. and Nalinanon, S. 2013. Physicochemical and functional properties of gelatin from the skin of unicorn leatherjacket *(Aluterus monoceros)* as affected by extraction conditions. Food Bioscience. In Press:

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