Post-mortem Softening of Freshwater Prawn (*Macrobrachium rosenbergii*) Muscle: Role of Proteases and Prevention

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ชื่อวิทยานิพนธ์	การอ่อนตัวของกล้ามเนื้อกุ้งก้ามกรามภายหลังการตาย: บทบาทของเอนไซม์โปรตีเอส และการป้องกัน
ผู้เขียน	นายโชษณ ศรีเกตุ
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บทคัดย่อ

เนื้อกุ้งก้ามกรามได้รับการยอมรับจากผู้บริโภคว่าเป็นอาหารอันโอชะ เนื้อของกุ้งชนิดนี้ประกอบด้วยโปรตีนร้อยละ 83.2 (โดยน้ำหนักแห้ง) โดยมีโปรตีนไมโอไฟบริลร้อยละ 62.7 เป็นองค์ประกอบ และมีกรดกลูตามิก/กลูตามีน อาร์จินีน กรดแอสปาติก/แอสพาราจีน และไลซีนเป็นกรดอะมิโนหลักในกล้ามเนื้อ ปริมาณคอลลาเจนที่ละลายด้วยเปปซิน (PSC) และคอลลาเจนที่ไม่ละลาย (ISC) ในกล้ามเนื้อเท่ากับร้อยละ 0.63 และ 0.32 ตามลำดับ คอลลาเจนทั้งสองมีความคล้ายคลึงกับคอลลาเจน type V และมีไกลซีน โพรลีน ไฮดรอกซิโพรลีน และกรดแอสปาติก/แอสพาราจีนเป็นกรดอะมิโนหลัก

ระหว่างการเก็บรักษาในน้ำแข็งกล้ามเนื้อของกุ้งระยะก่อนวางไข่มีการย่อยสลายมากกว่า กุ้งระยะหลังวางไข่โดยพิจารณาจากการเพิ่มขึ้นของปริมาณเปปไทด์ที่ละลายในกรดไตรคลอโรอะซิติก (TCA-soluble peptide) และคอลลาเจนที่ละลายได้โดยความร้อน (heat soluble collagen) องค์ประกอบเหล่านั้นมีการเพิ่มขึ้นในกล้ามเนื้อของกุ้งทั้งสองระยะอย่างเด่นชัดหลังจาก 3 วันของการเก็บรักษา ($p < 0.05$) ในทางตรงกันข้ามปริมาณ ISC ค่าแรงเฉือน (shear force) และค่าความชอบทางเนื้อสัมผัส (texture liking) ของกุ้งทั้งสองลดลง ($p < 0.05$) แสดงให้เห็นถึงการอ่อนตัวของกล้ามเนื้อ อุณหภูมิสูงสุดในการสูญเสียสภาพธรรมชาติ (T_{max}) และเอนทัลปีของ PSC จากกุ้งทั้งสองระยะลดลงในช่วง 4 วันแรกของการเก็บรักษา ($p < 0.05$) บ่งบอกถึงการย่อยสลายหรือการสูญเสียสภาพธรรมชาติของคอลลาเจนในกล้ามเนื้อ จากการศึกษาโครงสร้างทางจุลภาคโดยใช้กล้องจุลทรรศน์แบบใช้แสงแสดงให้เห็นถึงการลดลงของการเชื่อมต่อระหว่างเซลล์กล้ามเนื้อของกุ้งสดที่สูงขึ้น รวมทั้งมีการเกิดช่องว่าง (gaping) ระหว่างเซลล์กล้ามเนื้อในตัวอย่างกุ้งที่ผ่านการให้ความร้อนเพิ่มขึ้นเมื่อตัวอย่างผ่านการเก็บรักษาเป็นเวลานานขึ้น

เนื้อกุ้งก้ามกรามประกอบด้วยโปรตีเอสที่มีกิจกรรมสูงสุดที่ พีเอช 5 และอุณหภูมิ 50 °ซ โดยใช้ ฮีโมโกลบินเป็นสับสเตรท ส่วนโปรติเอสจากตับอ่อนมีกิจกรรมสูงสุดที่ พีเอช 7 และอุณหภูมิ 60 °ซ เมื่อใช้เคซีนเป็นสับสเตรท จากการศึกษาผลของสารยับยั้งต่อกิจกรรมของโปรติเอส

พบว่าโปรติเอสชนิดซิสเตอีน และซีรีนเป็นเอนไซม์หลักในกล้ามเนื้อ และตับอ่อนตามลำดับ โปรติเอสจากกล้ามเนื้อสามารถย่อยสลายแอคโตไมโอซินธรรมชาติ (NAM) ได้น้อย แต่ไม่สามารถย่อยสลาย PSC ในทางตรงกันข้ามโปรติเอสจากตับอ่อนสามารถย่อยสลาย NAM และ PSC ได้อย่างมีประสิทธิภาพ นอกจากนั้นยังพบคาลเพน และคาเทปซิน L ในกล้ามเนื้อส่วนทริปซิน และไคโมทริปซินสามารถพบในตับอ่อน

ทริปซินสามารถพบในกล้ามเนื้อปล้องที่หนึ่งและสองในวันที่ 4 และหลังจาก 4 วันของการเก็บรักษาในน้ำแข็งตามลำดับ ปริมาณคอลลาเจนที่ละลายโดยความร้อนได้เพิ่มขึ้นอย่างต่อเนื่องระหว่างการเก็บรักษา อย่างไรก็ตามไม่มีการเปลี่ยนแปลงของทริปซิน และปริมาณคอลลาเจนที่ละลายได้โดยความร้อนในเนื้อกุ้งที่มีการกำจัดตับอ่อนก่อนการเก็บรักษา ดังนั้นการปลดปล่อยทริปซินจากตับอ่อนที่มีสมบัติในการย่อยคอลลาเจนจึงมีผลโดยตรงต่อการอ่อนตัวของกล้ามเนื้อกุ้งก้ามกรามระหว่างการเก็บรักษาในน้ำแข็ง เมื่อทำบริสุทธิ์ทริปซินจากตับอ่อนของกุ้งก้ามกรามโดยใช้เทคนิคโครมาโทรกราฟี ได้แก่ Q-Sepharose, Superdex 75 และ MonoQ พบว่ามีความบริสุทธิ์เพิ่มขึ้น 525 เท่า และมีผลผลิตร้อยละ 10.6 ทริปซินปรากฏเป็นแถบโปรตีนเดี่ยวบนเจล native-PAGE และมีน้ำหนักโมเลกุลเท่ากับ 17 กิโลดาลตัน เมื่อตรวจสอบโดย SDS-PAGE ทริปซินมีกิจกรรมสูงสุดที่ พีเอช 8.0 และอุณหภูมิ 55 °ซ เมื่อใช้ Boc-Val-Pro-Arg-MCA เป็นสับสเตรท และมีความคงตัวต่อความร้อนในช่วงอุณหภูมิต่ำกว่า 40 °ซ และช่วงพีเอช 7.0-11.0 ทริปซินสามารถถูกยับยั้งอย่างสมบูรณ์ด้วยสารยับยั้งเอนไซม์ทริปซินจากถั่วเหลือง (SBTI) TLCK และ Pefabloc SC และถูกยับยั้งเพียงบางส่วนด้วย EDTA ทริปซินมีความจำเพาะต่อการย่อย Boc-Val-Pro-Arg-MCA โดยมีค่า K_m เท่ากับ 0.24 ไมโครโมลาร์ และมีค่า k_{cat} เท่ากับ 607.56 ต่อวินาที ลำดับของกรดอะมิโนของปลายสายด้านหมู่อะมิโนจำนวน 20 หน่วยของทริปซิน คือ IVGGDEAAPGEFPHQISMQV ซึ่งมีลักษณะใกล้เคียงกับทริปซินจากกุ้งสายพันธุ์อื่น ๆ

เมื่อใช้สารสกัดจากพืชตระกูลถั่วที่มีสารยับยั้งทริปซินเพื่อการลดกิจกรรมของทริปซินที่ปลดปล่อยเข้าไปในส่วนของกล้ามเนื้อ พบว่าสารสกัดจากถั่วเหลือง และถั่วหรั่ง สามารถยับยั้งโปรติเอสจากตับอ่อนของกุ้งก้ามกรามสูงกว่าสารสกัดจากถั่วแดงญี่ปุ่น และถั่วแดง เมื่อฉีดสารสกัดจากถั่วเหลือง และถั่วหรั่งที่ระดับความเข้มข้นที่แตกต่างกันเข้าไปในส่วนหัว/อกของกุ้งก้ามกราม และติดตามการเปลี่ยนแปลงคุณภาพของกล้ามเนื้อกุ้งก้ามกรามระหว่างเก็บรักษาในน้ำแข็งเป็นระยะเวลา 10 วัน พบว่าตัวอย่างชุดควบคุม (ไม่มีการฉีดสารสกัดจากถั่ว) มีค่าพีเอช ปริมาณเปปไทด์ที่ละลายได้ในกรดไตรคลอโรอะซิติก คอลลาเจนที่ละลายได้โดยความร้อน กิจกรรมของโปรติเอส และปริมาณแบคทีเรียชอบเย็นเพิ่มขึ้นมากกว่าตัวอย่างที่ผ่านการฉีดด้วยสาร

สกัดจากถั่วเหลือง และถั่วหรั่ง ในทางตรงกันข้ามค่าแรงเฉือน และความชอบของตัวอย่างชุดควบคุมลดลง ($p < 0.05$) ซึ่งน่าจะเป็นผลจากการอ่อนตัวของกล้ามเนื้อกุ้ง นอกจากนี้แถบของไมโอซินเส้นหนักในตัวอย่างชุดควบคุมลดลงหลังจาก 6 วันของการเก็บรักษา แต่ไม่มีการเปลี่ยนแปลงของรูปแบบโปรตีนในตัวอย่างที่ฉีดด้วยสารสกัดจากถั่วเหลืองที่ระดับความเข้มข้น 2.5 มิลลิกรัมโปรตีนต่อมิลลิลิตร หลังจาก 10 วันของการเก็บรักษา ดังนั้นการฉีดสารสกัดจากพืชตระกูลถั่ว โดยเฉพาะสารสกัดจากถั่วเหลืองที่ความเข้มข้นเพียงพอ สามารถใช้เป็นวิธีในการลดการอ่อนตัวของกล้ามเนื้อกุ้งก้ามกราม และรักษาคุณภาพของกุ้งก้ามกรามระหว่างการเก็บรักษาในน้ำแข็ง

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ABSTRACT

Freshwater prawn meat has been known as the delicacy. The meat contained 83.2% protein (dry basis), 62.7% of which were myofibrillar proteins. Glutamic acid/glutamine, arginine, aspartic acid/asparagine and lysine were abundant amino acids in meat. Pepsin-soluble collagen (PSC) and insoluble collagen (ISC) contents were 0.63 and 0.32%, respectively. Both collagens were similar to type V collagen and contained glycine, proline, hydroxyproline and aspartic acid/asparagine as predominant amino acids.

During the iced storage, the muscle of pre-spawned prawn underwent higher degradation as indicated by the increases in trichloroacetic acid (TCA) soluble peptide and heat soluble collagen than did post-spawned counterpart. Those components in the muscle of both prawns increased markedly after 3 days of storage ($p<0.05$). Conversely, ISC content, shear force value and texture liking of both prawns decreased ($p<0.05$), indicating the softening of muscle. T_{max} and enthalpy of PSC from both prawns decreased during the first 4 days of storage ($p<0.05$), suggesting the degradation or denaturation of collagen in the muscle. Light microscopic study showed the lowering of intercellular connection of raw meat and higher gaping in cooked meat when the samples were stored for a longer time.

Freshwater prawn meat contained indigenous proteases, which exhibited the highest hydrolytic activities towards hemoglobin at pH 5 and 50 °C. Proteases from hepatopancreas had the highest activity on casein at pH 7 and 60 °C. Based on inhibitor study, cysteine protease and serine protease were dominant in muscle and hepatopancreas, respectively. Proteases from muscle rarely hydrolyzed natural actomyosin (NAM) and could not degrade PSC. Conversely, proteases from

hepatopancreas actively hydrolyzed NAM and PSC. Proteases from muscle showed calpain and cathepsin L activities but proteases from hepatopancreas mainly exhibited tryptic and chymotryptic activities.

Trypsin activity was found in the first and second segments of prawn abdomen at day 4 and after 4 days of iced storage, respectively. Heat soluble collagen content was continuously increased during the storage. Nevertheless, no changes in trypsin activity and heat soluble collagen content were obtained in the abdomen of prawn with the removal of hepatopancreas. Therefore, the release of trypsin-like collagenase from hepatopancreas was most likely responsible for the softening of prawn meat during iced storage. When trypsin from hepatopancreas of freshwater prawn (HFWP) was purified using a series of chromatographies including Q-sepharose, Superdex 75 and MonoQ columns, purity of 525-fold with a yield of 10.6% was obtained. Based on native-PAGE, the purified trypsin showed a single band. Trypsin had a molecular mass of 17 kDa as estimated by SDS-PAGE. The optimal pH and temperature for Boc-Val-Pro-Arg-MCA hydrolysis were 8.0 and 55 °C, respectively. Trypsin was stable to heat treatment up to 40 °C, and over a pH range of 7.0-11.0. Trypsin activity was strongly inhibited by soybean trypsin inhibitor, TLCK and Pefabloc SC but was partially inhibited by EDTA. Apparent K_m value of trypsin was 0.24 μM and k_{cat} value was 607.56 s^{-1} for Boc-Val-Pro-Arg-MCA. The N-terminal amino acid sequence of 20 residues of HFWP trypsin was IVGGDEAAPGEFPHQISMQV, which was highly homologous with those from other species of prawn.

To lower the activity of trypsin-like enzyme released into abdomen, legume extracts containing trypsin inhibitors were used. Soybean and bambara groundnut extracts showed a more effective inhibition towards proteases from the hepatopancreas of freshwater prawn than the extracts from adzuki and red kidney beans. When the cephalothorax of freshwater prawn was injected with soybean and bambara groundnut extracts at different concentrations, the quality of meat was monitored during 10 days of iced storage. The control sample (without treatment) had higher pH, TCA-soluble peptide content, heat soluble collagen content, proteolytic activities and psychrophilic bacteria count than did samples treated with soybean and bambara groundnut extracts. Conversely, shear force value and likeness scores of the

control sample decreased ($p<0.05$), more likely associated with softening of muscle. The decrease in myosin heavy chain in the control sample was found after 6 days of storage. However, there were no changes in protein patterns of samples treated with soybean extracts at 2.5 mg/mL after 10 days of storage. Therefore, the injection of legume seed extracts, especially soybean extract, at a sufficient concentration, could be a means to retard muscle softening and maintain the qualities of freshwater prawn during iced storage.

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

A freshwater prawn, *Macrobrachium rosenbergii,* has been cultured in farms to support the increasing consumer demand due to its delicacy and useful nutrients. However, freshwater prawn production in Thailand is not successful because farmers have to face many constraints related to inconsistent market price, fragile prawn biology, water supply problems and textural deterioration. Soft or mushy texture seems to be the most critical because it limits prawn shelf-life, thereby impeding its marketing. During postmortem handling and storage, fish and shellfish proteins can be degraded by endogenous and microbial proteases (Shigemura *et al.*, 2004). The autolysis of nucleotides as well as nitrogenous compounds becomes more intense after the prolonged storage, particularly with inappropriate conditions (Aubourg *et al.*, 2007 ; Selvakumar *et al.*, 2002).

Generally, fish and shellfish such as freshwater prawns are locally distributed in ice, which renders the prawns easily susceptible to a textural problem called "softening or mushiness". This deterioration is usually influenced by the activity of digestive enzymes during iced storage which accordingly limits a shelf-life up to less than 1 week (Pornrat *et al.*, 2007). The development of mushiness in ice-chilled prawns has been described as the gradual and sequential degradation of prawn tissue, including the perimysium and endomysium connective tissue, as well as the proteins localized in Z-line and H-zones, caused by the action of hepatopancreatic enzymes (Papadopoulos *et al.*, 1989; Pornrat *et al.*, 2007). Hepatopancreas extracts contain both peptidase and proteinase activity, such as aminopeptidase, gelatinolytic proteases, trypsin and chymotrypsin, and collagenolytic proteases (Aoki *et al.*, 2003). Among proteolytic enzymes, collagenase has the pronounced impact on the softening of muscle (Brauer *et al.*, 2003). Collagenases are defined as proteases capable of degrading the native triple helix of collagen under physiological conditions (Aoki *et al.*, 2003). Serine collagenases have been purified from hepatopancreas of several shrimp species, including *Pandalus eous* (Aoki *et al.*, 2003), *Penaeus vannamei* (Carlos Sainz *et al.*, 2004; Hernandez-Cortes *et al.*, 1997) and *Pandalus borealis* (Aoki *et al.*, 2004a). So far, little information on the softening phenomenon of freshwater prawn cultured in Thailand exists.

Therefore, the better understanding of the role of proteases, especially those with collagenolytic activity, in softening phenomenon would help the farmers or processors to prevent or retard the quality losses associated with those proteases during post-mortem handling or storage. As a consequence, the prime quality of freshwater prawn with high market value could be maintained and minimized the economical losses.

1.2 Review of Literature

1.2.1 Composition of fish and shellfish muscle

Fish and shellfish are rich in nutrients, especially protein and amino acids. The main constituents of fresh fish are water (65-80%), crude protein (40-53.5%, dry weight basis), fat (9.9-18.8%, dry weight basis), total carbohydrate (23.7-37.3%, dry weight basis) and inorganic substances (6.4-11.4%, dry weight basis) (Jabeen and Chaudhry, 2011). Protein, a major component of fish and shellfish muscle, ranges from 15 to 20% (wet weight) and can be changed during spawning period (Kong *et al.*, 2006; Schubring, 2009). Protein compositions and quality can be varied, depending upon muscle type, feeding period, time of harvesting and spawning stage, etc. (Chaijan *et al.*, 2011; Kong *et al.*, 2006; Kong *et al.*, 2007). Shrimp meat is an excellent source of protein (Yanar and Celik, 2006). However, chemical compositions of shrimps vary, depending on species. The differences in chemical composition among species of shrimp such as black tiger shrimp, *Penaeus monodon*, Pacific white shrimp, *Penaeus vannamei*, (Sriket *et al.*, 2007) red shrimp, *Aristeus antennatus*, pink shrimp, *Parapenaeus longirostris* and Norway lobster, *Nephrops norvegicus* (Rosa and Nunes, 2004) are shown in Table 1.

Table 1. Chemical composition of different shrimp and lobster

Compositions (%, wet weight basis)	Black tiger shrimp	Pacific white shrimp	Red shrimp	Pink shrimp	Norway Lobster
Moisture	80.5±0.3	77.2±0.2	74.5±0.7	74.6±0.7	75.2±0.9
Protein	17.1±0.6	18.8±0.2	21.4±0.2	20.8±0.3	20.4±0.4
Ash	0.9±0.0	1.5±0.1	2.0±0.1	1.90±0.1	2.0±0.1
Fat	1.2±0.4	1.3±0.1	0.1± 0.1	0.20± 0.0	0.1±0.0

Source: Adapted from Rosa and Nunes (2004) and Sriket *et al.* (2007)

1.2.2 Proteins and nitrogenous compounds of fish and shellfish muscle

There are different proteins in fish and shellfish muscles. These proteins perform different tasks and have varying properties (Joo *et al.*, 1999; Sikorski *et al.*, 1990). The proteins can be classified into three groups based on solubility as follows:

1.2.2.1 Sarcoplasmic proteins

The sarcoplasmic proteins usually refer to the proteins of the sarcoplasm as well as the components of the extracellular fluid and the sarcoplasm. They are located in the interstitial region between individual muscle fibers (Niamnuy *et al.*, 2008). Sarcoplasmic protein was found as the second predominant protein in fish and shrimp meats (black tiger and white shrimps), accounting about 30-35% of the total muscle protein (Jafarpour and Gorczyca, 2012; Sriket *et al.*, 2007). Despite their diversity, sarcoplasmic proteins share many common physicochemical properties. Most are of relatively low molecular mass, high isoelectric pH and have globular or rod-shaped structures. The sarcoplasmic proteins can be extracted by homogenizing the muscle tissue with water or solutions of neutral salts of ionic strength below 0.15. Enzymes are another sarcoplasmic proteins found in fish muscle or other organs. Among the sarcoplasmic enzymes influencing the quality of fish and shellfish, the hydrolytic enzymes of the lysosomes and digestive tract are the major contributors (Ladrat *et al.*, 2003).

1.2.2.2 Myofibrillar proteins

The myofibrillar proteins are main proteins in muscle fiber related with the water holding capacity and the other functional properties such as gelation, etc. (Han *et al.*, 2009). These proteins can be extracted from the muscle tissue with neutral salt solutions of ionic strength above 0.15, usually ranging from 0.30 to 0.70. Contractile proteins which are different in size and location in the muscle are listed in Table 2 (Jafarpour and Gorczyca, 2012).

Myofibrillar proteins constituted as the major proteins (56.8-64.3 % of total proteins) in black tiger and white shrimps (Sriket *et al.*, 2007).

Table 2. Contractile proteins in food myosystems

Protein	Relative Abundance (%)	Size (kDa)	Location
Myosin	50-60	470	Thick filaments
Actin	15-30	40-45	Thin filaments
Tropomyosin	5	33-36	Thin filaments
Troponins	5		Thin filaments
Troponin-C		17-18	
Troponin-I		21-23	
Troponin-T		31-37	
C-protein	-	135	Thick filaments
α-Actinin	-	95	Z-disc
Z-nin	-	300-400	Z-disc
Connective/Titin	5	700-1,000	Gap filaments
Nebulin	5	~600	N_2-line

Source: Adapted from Jafarpour and Gorczyca (2012)

- Myosin and Paramyosin

Myosin is a major protein in the contractile apparatus (Watabe, 2002) and makes up 50 to 58% of the myofibrillar fraction (Sikorski *et al.*, 1990). About one-third of the total protein in muscle is myosin, the predominant myofibrillar protein of the thick filament. Native molecular mass of myosin is about 500 kDa. Myosin consists of six polypeptide subunits, two large heavy chains and four light chains arranged into an asymmetrical molecule with two pear-shaped globular heads attached to long α-helical rod-like tail (Watabe, 2002; Xiong, 1997) (Figure 1). The long tail of the molecule consists of two polypeptides in a coiled α-helix-terminating in two globular heads at one end (Watabe, 2002). Myosin is a protein possessing ATPase activity. The globular head regions of myosin bind and hydrolyze ATP to ADP. The activity reaches its maximum with 3-5 mM Ca^{2+}. This activity is due to myosin alone and thus is not essentially affected by the presence of actin (Ochiai and Chow, 2000). Ca^{2+}-ATPase activity is a good parameter to estimate the quality or the extent of deterioration of protein in fish muscle (Watabe, 2002). Myosin ATPase is also largely affected by chemical modification of reactive SH residues (SH_1, SH_2).

Modification of SH_2 results in the inactivation of Ca^{2+}-ATPase (Ochiai and Chow, 2000).

When myosin is digested by trypsin or chymotrypsin for a short period, myosin is divided into two components, a rapid sediment component called H-meromyosin (HMM) and a slow sediment called L-meromyosin (LMM). When HMM is treated with papain, it is divided into a head and a neck part which are called S-1 and S-2, respectively (Suzuki, 1981). The myosin head contains the actin binding site, ATP site, alkali light chain site, and DTNB [(5,5-dithiobis)-2-(nitrobenzoic acid)] light chain site. The light chains bind to the α-helical regions of the heavy chain. The tail portion of the heavy chain molecule is responsible for its association into thick filaments (Watabe, 2002).

Paramyosin is one of the major muscle proteins found in mollusks (Thanonkaew *et al.*, 2006a). It consists of high basic amino acid and amide content, such as glutamine (20 to 23.5%), aspartic acid (12%), arginine (12%) and lysine (9%), but low in proline content. Paramyosin, a rod-shaped α-helical chains, consists of 2 subunits, which are 120 nm long with a molecular mass ranging from 95 to 125 kDa per subunit (Foegeding *et al.*, 1996). Thanonkaew *et al.* (2006b) reported that the paramyosin was the second predominant protein in cuttlefish muscle. Thermal stability of myofibrillar protein varies with species, and is affected by the temperature of habitat. T_{max} and enthalpy (ΔH) of myosin are different among species. T_{max} of myosin was reported as 51 and 50 °C for black tiger and white shrimps (Sriket *et al.*, 2007), 47 °C for goatfish, *Mulloidichthys martinicus* (Yarnpakdee *et al.*, 2009) and 50 °C for red claw clayfish (Tseng *et al.*, 2002).

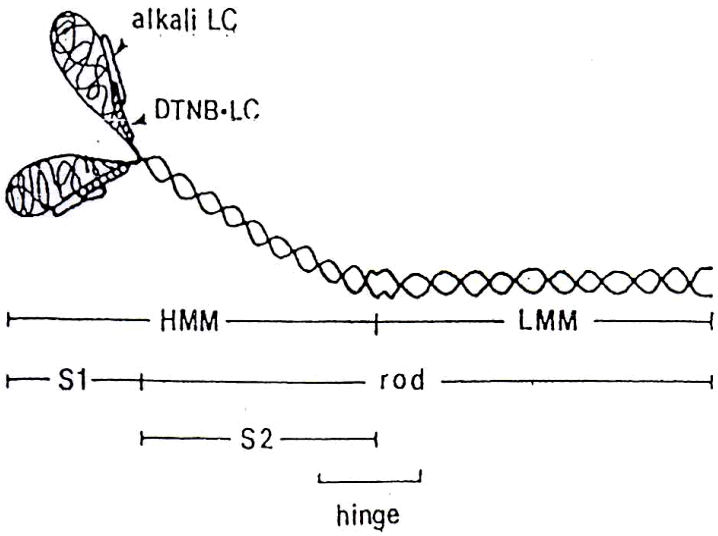

Figure 1. Model of myosin molecule
Source: Xiong (1997)

- Actin

Actin is about 15 to 20% of myofibrillar protein (Sikorski *et al.*, 1990). Actin is one of three major myofibrillar proteins of thin filaments. Each actin molecule, generally visualized as globular, has a molecular mass of about 40-45 kDa, called G-actin (Jafarpour and Gorczyca, 2012). Polymerized actin molecules via covalent interactions tends to be a helix filamentous molecules, called F-actin. Two F-actins wrap about each other, forming a double helix, called thin filament or I-band, which is associated with tropomyosin and troponin (McCormick, 1994).

- Tropomyosin

Tropomyosin, a rod-like molecule, consists of two polypeptide chains, each with a molecular mass range of 33-36 kDa, which associate to form a coiled helix. Each tropomyosin molecule is about 385 A° long and associates in head-to-tail fashion to form a filament that follows and associates with the coil of the F-actin filament (McCormick, 1994; Ochiai *et al.*, 2003). Tropomyosin is about 5% of myofibrillar protein. Each tropomyosin molecule consists of 7 molecules of G-actins (Foegeding *et al.*, 1996; Perry, 2001).

- Troponin

Troponin is an asymmetrical protein and consists of three subunits. Troponin T (molecular mass of 31-37 kDa), which is also bound to troponin subunits C and I, links the troponin molecule to the tropomyosin molecule in the I-band. Troponin C (molecular mass of 18 kDa) binds Ca^{2+} and confers Ca^{2+} sensitivity to the troponin-tropomyosin-actin complex. Troponin I (molecular mass of 21-23 kDa), the inhibitory subunit, binds tightly to troponin C and actin and only slightly to tropomyosin or troponin T (Jafarpour and Gorczyca, 2012; McCormick, 1994).

1.2.2.3 Stromal proteins

The stroma is composed of endomysium, perimysium, epimysium connective tissue proteins, such as collagen and elastin. The stroma is the residue after extraction of the sarcoplasmic and myofibrillar proteins. Generally, the stroma is insoluble in dilute solutions of hydrochloric acid or sodium hydroxide (Niamnuy *et al.*, 2008; Sikorski *et al.*, 1990).

Collagens in the muscle of marine animals play a key role in maintenance of meat texture. This protein is a unique protein, able to form insoluble fibers with a high tensile strength (Gelse *et al.*, 2003). The musculature of several crustaceans contains collagenous proteins (Brauer *et al.*, 2003; Sriket *et al.*, 2007). A collagen molecule is a helical α-chain. Each α-chain twists into a left-handed polyproline helix with three residues per turn. The helical α-chains contain a Gly-X-Y sequence, where x or y is often proline or hydroxyproline. The three helical α-chains are wound into a right-handed superhelix, which forms a molecule about 1.4 nm wide and 300 nm long. Fibrillar collagens contain about one-third glycine and one-quarter proline and hydroxyproline with a molecular mass of about 300,000 Da (Bhattacharjee and Bansal, 2005; Gineyts *et al.*, 2000; McCormick, 1994). Crustacean collagens are richer in several essential amino acids, making the biological values of such collagens significantly higher than bovine and other mammalian muscle collagens (Ashie and Simpson, 1997). Collagen content in animal tissue changes, depending upon the types of animals, animal's age and maturity (McCormick, 1994). The main difference between fish collagen and mammalian collagen is the varying

contents of the imino acids, proline and hydroxyproline (Solgaard *et al.*, 2008). Hydroxyproline and proline play a role in collagen stability (Rubin *et al.*, 2008).

At least 14 different collagen types have been identified. However, the major ones are types I, II III and V. Type I collagen is predominant in the epimysial membrane, whereas type II and III are in the perimysium and type III, IV and V are in the muscle endomysium (Brüggemann and Lawson, 2005). Type V like collagen is widely distributed in marine invertebrates, particularly crustaceans and mollusks (Sivakumar *et al.*, 2000b). The purification of type V collagen from crustaceans such as marine crab, *Scylla serrata* (Sivakumar *et al.*, 2000a) and kuruma prawn, *Penaeus japonicus* (Yoshinaka *et al.*, 1990) by pH and salt fractionations was reported. At least three types of collagen were found in the muscle of kuruma prawn. Two of them, called AR-I and AR-II collagens, were isolated from the pepsin-solubilized collagen (Yoshinaka *et al.*, 1990). The subunit composition of AR-I collagen, a major component, was proved to be $(\alpha\ I)_3$ homotrimer. Its amino acid composition was similar to that of type V collagen rather than type I collagen. AR-II collagen, a minor molecular component, was found to have disulfide bonds in the molecule. Its amino acid composition was similar to that of AR-I collagen. The major collagen in shrimp, *Penaeus indicus* (Sivakumar *et al.*, 1997), black tiger and white shrimps (Sriket *et al.*, 2007) was found to be a homotrimer of α 1 chain, similar to type V collagen of vertebreates.

Marine crustaceans like prawn has a flexible musculature and collagens with more crosslink and higher denaturation temperature than fish (Sivakumar *et al.*, 2000b). Different thermal transition temperatures (T_{max}) of fish and shellfish collagen have been reported for white shrimp (47 °C) (Brauer *et al.*, 2003), bigeye snapper, *Priacanthus tayenus* (32 °C) (Nalinanon *et al.*, 2007) and Pacific whiting (27 °C) (Kim and Park, 2004). However, Sato *et al.* (2002) reported that type V collagen was more susceptible to hydrolysis by proteinase than type I collagen.

1.2.3 Proteolytic enzymes in fish and shellfish

1.2.3.1 Classification of proteases

Enzymes that hydrolyze peptide bonds can be grossly grouped into two subclasses, exopeptidases and endopeptidases, depending on where the reaction takes place in the polypeptide substrate (Sternlicht and Werb, 2001). Exopeptidases cleave peptide bonds at the amino or carboxyl ends of the polypeptide chain, whereas endopeptidases cleave internal peptide bonds (Sternlicht and Werb, 2001). Regardless of the source, proteases can be classified on the basis of their similarity to well-characterized proteases, such as trypsin-like, chymotrypsin-like, chymosin-like or cathepsin-like (Klomklao, 2008). They may also be classified on the basis of their sensitivity to pH, including acid, neutral or alkaline proteases. They are also often classified according to their substrate specificity, the response to inhibitors or by their mode of catalysis (Simpson, 2000).

The standard method of classification proposed by the Enzyme Commission (EC) of the International Union of Biochemists (IUB) is based on the mode of catalysis. This divides the proteolytic enzymes into four groups: serine, cysteine, aspartic and metallo proteases. The name of each class is derived from a distinct catalytic group involved in the reaction (Rao *et al.*, 1998).

1.2.3.1.1 Serine proteases

Serine or alkaline proteases are so-named because they have a "super-reactive" serine in the active site (Simpson, 2000). Two distinct families can be classified according to their structural homology to trypsin and subtilisin. The trypsin family is the largest enzyme found in both mammalian and bacterial members. Some common examples are the pancreatic digestive enzymes such as trypsin, chymotrypsin and elastase; as well as the blood-clotting enzymes such as thrombin, plasmin and many complement enzymes. In contrast, the subtilisin family is only found in bacteria (Hamilton *et al.*, 2003).

Serine proteases are generally active at neutral and alkaline pH, with an optimal pH range of 7-11. Their molecular masses range between 18 and 35 kDa (Klomklao, 2008; Rao *et al.*, 1998). The isoelectric points of serine proteases are generally between pH 4 and 6. Trypsins (EC 3.4.21.4), mainly members of a large

family of serine proteases, specifically hydrolyze proteins and peptides at the carboxyl side of arginine and lysine residues (Klomklao *et al.*, 2006a). Trypsins play major roles in biological processes including digestion, activation of zymogens of chymotrypsin and other enzymes (Cao *et al.*, 2000a). Trypsins from fish resemble mammalian trypsins with respect to their molecular mass (22-30 kDa), amino acid composition and sensitivity to inhibitors. Their optimal temperature for hydrolysis ranged from 35 to 65°C. Heat stable and/or activated serine proteases were also reported (Ahmad *et al.*, 2011; Nalinanon *et al.*, 2008). These enzymes are synthesized as inactive zymogens or pro-enzymes which could be activated by proteolytic cleavage. Trypsin has been purified from many kinds of fish and shellfish (Aoki *et al.*, 2003; Klomklao *et al.*, 2010). The hepatopancreas of shrimp is known to have high proteolytic activities (Aoki *et al.*, 2003) and have ability to hydrolyze collagen (Aoki *et al.*, 2004a). The degradation of collagen depends upon the source of collagen as well as on the types of protease. Collagen molecules in the connective tissue generally undergo limited cleavage in the non-helical region by the various proteases, such as trypsin and chymotrypsin (Klomklao *et al.*, 2006b; Yamashita and Konagaya, 1991). Serine collagenases or trypsin-like proteinase were found in the intestines of Atlantic cod, *Gadus morhua* (Hernandez-Herrero *et al.*, 2003), filefish (Kim *et al.*, 2002) and the hepatopancreas of Northern shrimp, *Pandalus eous* (Aoki *et al.*, 2003) and king crab, *Paralithodes camtschaticus* (Rudenskaya *et al.*, 2004). Chymotrypsin was isolated from the hepatopancreas of Chinese shrimp, *Fenneropenaeus chinensis* (Shi *et al.*, 2008) and viscera of Monterey sardine, *Sardinops sagax* caerulea (Castillo-Yanez *et al.*, 2009).

Cleavage of these connective tissues by endogenous trypsin and chymotrypsin may lead to undesirable textural changes in fish and shellfish. Mushiness of fish and shellfish during ice storage was probably caused by the diffusion of digestive enzymes including trypsin and other proteolytic enzymes from autolyzed hepatopancreas and digestive tract (Ezquerra *et al.*, 1997; Felberg *et al.*, 2009). Trypsin activity was dependent on fish species and pH values, where the neutrality or higher pHs were optimal for hydrolytic activity (Hultmann and Rustad, 2004). The fish and shellfish serine collagenolytic enzyme was relatively stable within the pH range of 6-11 (Aoki *et al.*, 2003; Klomklao *et al.*, 2007).

1.2.3.1.2 Cysteine proteases

This family includes plant proteases such as papain, ficin and bromelain, several mammalian lysosomal cathepsins, the cytosolic calpains (calcium-activated) as well as several parasitic proteases. The most important cysteine proteases in mammals are cytoplasmic calpains and lysosomal cathepsins (Tetsumori, 2004). Calpains are cysteine proteases which need calcium ions for enzyme activation. Two types of calpain have been isolated which differ in their calcium requirement (μ-calpain and m-calpain) (Larsen *et al.*, 2004). The pH optimum is neutral to weakly alkaline (pH ~ 7.5) (Maddock *et al.*, 2005).

Calpain is primarily active within the first 24 hours postmortem (Camou *et al.*, 2007). Bahuaud *et al.* (2010a) reported that calpains could initiate the proteolytic degradation of myofibrils by causing hydrolysis of proteins, making them more susceptible to other proteases. Vertebrate muscle calpains have been shown to be very specific in digesting primarily tropinin-T, desmin, titin and nebulin and in attacking either vertebrate actin or myosin (Choi *et al.*, 2005). In contrast, fish calpains hydrolyzes myosin (specifically the myosin heavy chain) to form the lower molecular mass fragments (Delbarre-Ladrat *et al.*, 2004a). In crustacean muscle, calpains are associated with molt induced textural changes to the muscle and result in non-specific generalized digestion of the myofibrillar proteins (Kim *et al.*, 2005). Bonnal *et al.* (2001) showed that dystrophin can be used as a pertinent indicator of the early proteolytic process as it is highly sensitive to calpain action. Dystrophin, a subsarcolemmal actin-binding protein (ABP) located in costameric structures, ensures a link between the actin cytoskeleton and the extracellular matrix through an association with a glycoprotein complex.

Lysosomal cysteine proteases, generally known as the cathepsins, play an important role in many physiological processes such as protein degradation (Turk *et al.*, 2000). Cathepsins are mostly active at weakly acid pH values (pH 5). Among lysosomal enzymes, cathepsins B, C, H, L and S have been purified and characterized from fish and shellfish muscles and are the major proteases which participate in intracellular protein breakdown (Aoki *et al.*, 2004b; Pangkey *et al.*, 2000). Lysosomal membranes may lose their integrity under postmortem conditions, resulting in a release of catheptic enzymes into the sarcoplasm (Balti *et al.*, 2010; Zeece and Katoh,

1989). Although the muscle cathepsins generally are most active at pH 3-4, some of them retain fairly high activity up to pH 7.0. The activity of several cathepsins is negligible at low temperature (Balti *et al.*, 2010; Kolodziejska and Sikorski, 1997).

Cathepsins B, H and L activities of fish in spawning period were 3-7 times higher than those in feeding period, while the activities of metabolic enzymes decreased (Ashie and Simpson, 1997). In salmon muscle, the increased levels of cathepsins are considered to play an important role in the physiological changes occurring along with sexual maturation in spawning migration (Riley, 2005). Bahuaud *et al.* (2010b) suggested that cathepsins B and L were the main enzymes responsible for softening of Atlantic salmon (*Salmo salar* L.) muscle. Cathepsin L is activated at high temperature. Cathepsin L was a predominant proteinase responsible for autolysis of arrowtooth flounder muscle at high temperatures (Visessanguan *et al.*, 2001). In addition to hydrolyzing myofibrillar proteins, cathepsin L was reported to have high activity against various collagens. Thus, it is presumed to cause partial disintegration of the original extracellular matrix structure, which may play an important role in tissue softening of fish and shellfish.

1.2.3.1.3 Aspartic proteases

Aspartic proteases, commonly known as acidic proteases, depend on aspartic acid residues for their catalytic activity. Aspartic proteases are produced by a number of cells and tissues. Most of the aspartic proteases belong to the pepsin family. The pepsin family includes digestive enzymes such as pepsin and chymosin as well as lysosomal cathepsins D and processing enzymes such as rennin and certain fungal proteases (penicillopepsin, rhizopuspepsin, endothiapepsin) (Hughes *et al.*, 2003). These enzymes are active predominantly in the acidic range of pH 2-4. Rennin is one exception where activity is in the pH range of 5.5-7.5. Their molecular masses are in the range of 30-45 kDa (Zhao *et al.*, 2011).

Cathepsin D shows some activity in the lowest pH range prevailing postmortem in some fish. However, it is still uncertain whether it can be regarded as a very significant factor in softening of refrigerated fish of most species. Aoki (2000) detected cathepsin D in red or white muscle among 24 species, and no difference was found between red- and white-flesh fish, or fresh water fish. Wang *et al.* (2007) found

the low activity of cathepsin D in three species including Atlantic herring (*Clupea harengus* L.), Atlantic salmon (*Salmo salar* L.) and wolffish (*Anarhichas lupus* L.).

1.2.3.1.4 Metalloproteases

The metalloproteases include enzymes from a variety of origins, such as collagenases from higher organisms, toxins from snake venoms, and thermolysin from bacteria. They contain a zinc atom which is catalytically active. In some cases, zinc may be replaced by another metal such as cobalt or nickel without loss of activity (Carmeli *et al.*, 2004). The matrix metalloproteases (MMPs) are a family of zinc endopeptidases which are responsible for the degradation of collagen in extracellular fluids (Carmeli *et al.*, 2004).

Many metalloproteases contain the sequence of His-Glu-Xaa-Xaa-His (HGXXH), which provides two histidine ligands for the zinc, whereas the third ligand is either a glutamic acid (thermolysin, neprilysin, alanyl aminopeptidase) or a histidine (astacin) (Dauch *et al.*, 1995; Kadonosono *et al.*, 2007). The catalytic mechanism leads to the formation of a non-covalent tetrahedral intermediate after the attack of a zinc-bound water molecule on the carbonyl group of the scissile bond (Skiles *et al.*, 2004). This intermediate is further decomposed by transfer of the glutamic acid proton to the leaving group (Graycar, 1999).

1.2.3.2 Purification and characterization of proteases

Shrimp hepatopancreas contained serine protease (Oh *et al.*, 2000) and collagenolytic protease (Aoki *et al.*, 2003), while fish digestive tract and pancreas contained trypsin (Klomklao *et al.*, 2008) and chymotrypsin (Yang *et al.*, 2009). Moreover, fish pyloric caeca and intestine consisted of trypsin (Khantaphant and Benjakul, 2010; Lu *et al.*, 2008) and chymotrypsin (Caruso *et al.*, 2009; Simpson, 2000). Protease purified from shrimp *(Penaeus orientalis)* hepatopancreas had high proteolytic activity in the pH range of 7.0-9.5 at 70 °C (Oh *et al.*, 2000). The protease was stable at neutral and alkaline pH. K_m and V_{max} for hydrolysis of casein by the protease were determined to be 0.31% and 5.21s^{-1}, respectively. The N-terminal sequence of the protease showed higher homology with the collagenase of crab and trypsins from crustacea.

Two anionic trypsins (trypsin A and trypsin B) from the hepatopancreases of carp were purified (Cao *et al.*, 2000b). The purification procedures consisted of ammonium sulfate fractionation and chromatographies on DEAE-Sephacel, Untrogel AcA54 and Q-Sepharose. Trypsin A was purified to homogeneity with a molecular mass of approximately by 28 kDa, while trypsin B showed two bands of 28.5 kDa and 28 kDa on SDS-PAGE, both under reducing and non-renducing condition. Trypsin A and B had the optimum temperatures of 40 and 45 °C, respectively, and showed the optimal pH of 9 using Boc-Phe-Ser-Arg-MCA as substrate. Both enzymes were effectively inhibited by trypsin inhibitors.

Three gelatinolytic proteases (A1, A2, and B) were purified from the hepatopancreas of Northern shrimp (*Pandalus eous*) by several chromatographic steps involving hydroxyapatite column chromatography, gel filtration on Superdex75, and ion-exchange chromatography on a MonoQ column (Aoki *et al.*, 2003). Collagenolytic proteases A2 and B, but not protease A1, were demonstrated to digest native porcine type I collagen at 25 °C and pH 7.5. The pH optimum of enzyme A2 against DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg was found to be 11, whereas that of enzyme B was 8.5. The optimal temperature ranged from 40 to 45 °C for both enzymes. Both enzymes were strongly inhibited by PMSF and antipain.

Trypsin from the pyloric caeca of bigeye snapper was purified and characterized (Hau and Benjakul, 2006). The purification steps consisted of ammonium sulfate fractionation and SBTI-affinity chromatography. Trypsin had an apparent molecular mass of 23.8 kDa when analyzed using SDS-PAGE and substrate-gel electrophoresis. The trypsin fraction consisted of three isoforms as evidenced by the appearance of three different bands on native-PAGE. Optimal activity was observed at 55 °C and pH range of 8-11. The activity of trypsin fraction was completely inhibited by soybean trypsin inhibitor and was partially inhibited by E-64 and ethylenediaminetetraacetic acid. The apparent Michaelis-Menten constant (K_m) and catalytic constant (k_{cat}) were 0.312 mM and 1.06 s^{-1}, respectively when *N*α-Benzoyl-DL-arginine *p*-nitroanilide was used as a substrate. Trypsin from the pyloric caeca of bigeye snapper generally showed similar characteristics to other fish trypsins.

An anionic trypsin from North Pacific krill (*Euphausia pacifica*) (TRY-EP) was purified by ammonium sulfate precipitation, ion-exchange and gel-

filtration chromatography (Wu *et al.*, 2008b). The purified enzyme was identified as a trypsin by LC-ESI-MS/MS analysis. The relative molecular mass of TRY-EP was 33 kDa with isoelectric point of 4.5. The purified enzyme was active over a wide pH (6.0-11.0) and temperature (10-70 °C) range. Its optimum pH and temperature were 9.0 and 40–50°C, respectively. TRY-EP was stable between pH 6.0 and 11.0 and below 30 °C.

A collagenolytic serine proteinase (CSP) was purified from red sea bream (*Pagrus major*) skeletal muscle to homogeneity by ammonium sulfate fractionation and chromatographies including DEAE-Sephacel, Phenyl Sepharose and Hydroxyapatite (Wu *et al.*, 2010). The molecular mass of CSP was approximately 85 kDa as estimated by SDS-PAGE and gel filtration. Optimum temperature and pH of CSP were 40 °C and 8.0, respectively. CSP was specifically inhibited by serine proteinase inhibitors. The K_m and k_{cat} values of CSP for Boc-Leu-Lys-Arg-MCA were 3.58 μM and 0.13 s^{-1} at 37 °C, respectively.

Two chymotrypsins (chymotrypsin A and B) have been purified to homogeneity from the hepatopancreas of crucian carp (*Carassius auratus*) (Yang *et al.*, 2009) by ammonium sulphate fractionation and chromatographies on DEAE Sepharose, Sephacryl S-200 HR, Phenyl-Sepharose and SP-Sepharose. The molecular masses of chymotrypsin A and B were approximately 28 and 27 kDa, respectively, as determined by SDS-PAGE. Purified chymotrypsins also revealed single bands by native-PAGE. Optimal temperatures of chymotrypsin A and B were 40 and 50 °C, and optimal pHs were 7.5 and 8.0 using Suc-Leu-Leu-Val-Tyr-AMC as substrate. Both enzymes were effectively inhibited by serine proteinase inhibitors. Apparent K_m of chymotrypsin A and B were 1.4 and 0.51 M, and k_{cat} were 2.7 s^{-1} and 3.4 s^{-1}, respectively.

1.2.4 Role of proteases in muscle softening

The biochemical change caused by endogenous enzyme, including proteases, is the primary cause of quality loss in fish and shellfish during iced storage (Brauer *et al.*, 2003; Hultmann and Rustad, 2007). Moreover, proteases can be directly responsible for unusual textural defects in seafood, e.g. ‘gaping’ and

'mushiness' of bony fish and 'tail meat' softening of crustacean (Gornik *et al.*, 2009; Pornrat *et al.*, 2007). Postmortem fish and shellfish is generally susceptible to proteolysis by endogenous proteases, resulting in a soft or mushy texture (Gornik *et al.*, 2009; Jiang, 2000). Proteases in muscle and from digestive tract of fish and shellfish associated with the muscle softening are shown in Table 3.

Table 3. Endogenous proteases involved in muscle softening of fish and shellfish

Fish species	**Quality deterioration**	**Enzymes involved**	**Sources**
		Muscle proteases **:**	
Tilapia (*Oreochromis niloticus*)	Muscle softening	Serine and metalloproteases	Ishida *et al.* (2003)
Cod (*Gadus morhua*), Spotted wolffish (*Anarhichas minor*) and Atlantic salmon (*Salmo salar*)	Fillet softening and muscle gaping	Metalloproteases	Lødemel *et al.* (2004)
Silver carp (*Hypophthalmichthys molitrix*)	Muscle degradation	Myofibril-bound serine protease	Cao *et al.* (2004)
Atlantic salmon (*Salmo salar*)	Fillet softening	Cathepsin and collagenase-like enzyme	Hultmann and Rustad (2004)
Seabass (*Dicentrarchus labrax* L.)	White muscle deterioration	Calpain	Delbarre-Ladrat *et al.* (2004b)
White Croaker (*Argyrosomus argentatus*)	Muscle degradation	Trypsin-like enzyme	Cao *et al.* (2005)
Seabass (*Dicentrarchus labrax* L.)	Postmortem softening of fish muscle	Cathesin B and L	Che´ret *et al.* (2007)
Dark muscle of common carp (*Cyprinus carpio*)	Muscle softening	Metalloproteases	Wu *et al.* (2008a)
Red sea bream (*Pagrus major*)	Muscle softening, collagen degradation	Serine collagenolytic protease	Wu *et al.* (2010)

Table3. Endogenous proteases involved in muscle softening of fish and shellfish (continued)

Fish species	Quality deterioration	Enzymes involved	Sources
White shrimp (*Penaeus vannamei*)	Mushy texture	***Digestive proteases :*** Collagenolytic enzymes	Brauer *et al.* (2003)
Shrimp (*Penaeus orientalis*)	Mushy texture	Trypsin and collagenase-like enzyme from hepatopancreas	Oh *et al.* (2000)
Herring (*Clupea harengus*)	Belly burst	Serine collagenolytic enzyme (trypsin) leaked from pyloric caeca	Felberg *et al.* (2010)

The hydrolytic changes of collagen and of other extracellular matrix proteins are probably to some extent catalyzed by collagenase. Degradation at the interface between the connective tissue of the myocommata and the muscle cell causes the significant structural alterations within the muscle fiber. Among proteolytic enzymes, serine collagenase had the most impact on the softening of muscle (Brauer *et al.*, 2003). During postmortem storage, the activity of a collagenolytic enzyme was detected in the muscle of white shrimp, *Penaeus vannamei* (Brauer *et al.*, 2003). Ice-chilled storage of fish and shellfish had a gradual disintegration of collagenous protein leading to separation of the muscle fibers, causing the softening of meat (Kubota *et al.*, 2003). Gelatinolytic proteases with properties similar to collagenase have been proposed to participate in the metabolism of collagens and in the post mortem degradation of fish muscle during cold storage in species like red sea bream (Wu *et al.*, 2010; Yoshida *et al.*, 2009). Collagenase activity was found in fish muscle tissues including skeletal muscle of mackerel, *Scomber japonicus*, Japanese flounder, *Paralichthys olivaceus*, rainbow trout and common carp (Kubota *et al.*, 2003; Park *et al.*, 2002; Saito *et al.*, 2000; Wu *et al.*, 2008a). The mackerel (*Scomber japonicus*) collagenase fraction was shown to be optimally active at pH 7.5 and 55 °C (Park *et al.*, 2002). The metalloproteases with molecular masses of 64, 67 and 75 kDa were found

in dark muscle of common carp (Wu *et al.*, 2008a). The activity of these enzymes was highest at pH 7-9 and they were activated by calcium (Saito *et al.*, 2000; Wu *et al.*, 2008a; Yoshida *et al.*, 2009).

Disintegration of collagen type I and V is mainly responsible for the softening of fish muscle, presumably due to the action of autolytic collagenase enzymes (Kubota *et al.*, 2003; Yoshida *et al.*, 2009). These enzymes were presumably a major cause of "gaping" or breakdown of the myotome during long-term iced storage or short term storage at high temperature. For Atlantic cod, upon reaching 17°C, gaping is inevitable, presumably because of degradation of the connective tissue. The relatively short shelf-life of chilled shrimp associated with softening of tissue was due to the presence of collagenase (Brauer *et al.*, 2003). Hepatopancreas is the important source of the collagenolytic proteases in shrimp species (Aoki *et al.*, 2004a). Two proteases known to hydrolyze collagen of fish and shellfish muscle include matrix metalloprotease (MMP) and serine protease (Kubota *et al.*, 2003; Lødemel and Olsen, 2003). Heat-stable metalloproteases was identified in Pacific rockfish muscle (Bracho and Haard, 1995).

1.2.5 Postmortem changes of fish and shellfish muscle

In fish and shellfish, there are uncertainties regarding the underlying mechanisms and factors which contribute to the postmortem softening of the flesh. Many factors including type, size, molting and spawning stages and storage conditions caused the differences in postmortem phenomenon (Mishima *et al.*, 2005; Rosenvold and Andersen, 2003). Rigor mortis is one of the first postmortem changes and has the major influence on appearance and structure of the muscle. After death, Ca^{2+} flows out from the muscle endoplasmic reticulum and ATPase activity of the myosin increases. Thereafter, ATP degradation proceeds; actin and myosin combine to become actomyosin and the muscle shrinks. The accumulation of lactic acid by glycolysis induces a reduction in pH, which promotes the rigor mortis process (Cappeln and Jessen, 2002). Atlantic salmon fillet color was affected by postmortem glycolysis (pH drop) (Erikson and Misimi, 2008). The relationships between the degree of rigor mortis, slaughter method and the storage temperature of several kinds

of fish were also examined (Ando *et al.*, 2007; Bagni *et al.*, 2007; Kagawa *et al.*, 2002).

A softening phenomenon can take place within a day due to the collapse of Z-lines and fragmentation of myofibril (Delbarre-Ladrat *et al.*, 2006; Shigemura *et al.*, 2003). Moreover, the loss of elasticity due to the degradation of connectin is considered to be the causes of dissociation of myosin and actin filaments as well as disintegration of collagen fiber (Ando *et al.*, 2004; Kagawa *et al.*, 2002). Myofibrillar proteins in fish and shellfish muscle including bigeye snappers (*Priacanthus tayenus* and *P. macracanthus*) (Benjakul *et al.*, 2002), Monterey sardine (Pacheco-Aguilar *et al.*, 2000) and shrimp (*Pandalus borealis*) (Martinez *et al.*, 2001) became less stable during iced storage. Spawning stage has been reported to have the impact on the softening of fish and shellfish during extended storage (Aussanasuwannakul *et al.*, 2011; Ishida *et al.*, 2003; Salem *et al.*, 2006). The decrease in myosin content of pre-spawned flounder was observed at earlier stage of iced storage (Paredi and Crupkin, 2007). Pre-spawned hake showed a partially denatured myosin heavy chain at the beginning of storage (Pagano *et al.*, 2003). Generally, autolytic activity of muscle from pre-spawned hake was higher than that of post-spawned counterpart (Pagano *et al.*, 2003).

Softening of fish and shellfish muscle during iced storage is also associated with the weakening of endomysium and the collapse of collagen fibrils (Shigemura *et al.*, 2003; Shigemura *et al.*, 2004). Masniyom *et al.* (2005) reported that insoluble collagen content in sea bass (*Lates calcalifer*) decreased gradually during 21 days of the storage. High collagen content resulted in a firm meat, indicating the relationship between collagen content and texture property (Jonsson *et al.*, 2001; Kong *et al.*, 2008). Disintegration of the pericellular connective tissue of fish muscle was histologically observed in Pacific bluefin tuna (*Thunnus orientalis*) muscle during storage (Nakamura *et al.*, 2005). A change in microstructure of *M. rosenbergii* during storage due to proteolysis in the meat was reported (Pornrat *et al.*, 2007; Rowland *et al.*, 1982).

The initial deterioration of fish and shellfish during iced storage is related with hydrolytic reactions catalyzed by endogenous enzymes, which produce nutrients, allowing bacteria proliferation (Hernandez-Herrero *et al.*, 2003). Like other

marine species, endogenous and bacterial enzymes are involved in the deterioration of crustacean and influence its shelf-life and wholesomeness during refrigerated storage and shipping (Aubourg *et al.*, 2007; Múgica *et al.*, 2008; Piñeiro *et al.*, 2004). During the storage, autolysis of cephalothorax, where hepatopancreas and other internal organs are located, could take place, thereby releasing the active proteases into the muscle. Hepatopancreas extracts from crustacean contain both peptidase and proteases, such as trypsin, chymotrypsin and collagenases capable of degrading the native triple helix of collagen under physiological conditions (Aoki *et al.*, 2004a; Oh *et al.*, 2000). Freshwater prawns stored in ice had a maximum shelf-life up to 6 days (Begum *et al.*, 2011). The short shelf-life of prawns may result from the degradation of protein structure by their endogenous enzymes. Denaturation and degradation mainly contribute to the loss of functional properties. Firmness is generally considered as the most crucial factor determining fish quality (Benjakul *et al.*, 2003b; Dileep *et al.*, 2005). Therefore, it is important for the shrimp processing industry to develop a storage method to maintain high quality and freshness of shrimp.

1.2.6 Effect of pretreatment, chemicals and storage conditions on quality of fish and shellfish

1.2.6.1 Beheading and eviscerating

Pretreatment methods including beheading, eviscerating and/or gutting have been used to extend the shelf-life of fish and shellfish during storage. Beheading and evisceration could retard the muscle deterioration of bigeye snapper*, Priacanthus tayenus* and *P. macracanthus* (Benjakul *et al.*, 2002) and lizardfish (*Saurida tumbil*) (Benjakul *et al.*, 2003c) during storage in ice. Gutting was reported as the means to extend the shelf-life of sea bass (*Dicentrarchus labrax*) (Papadopoulos *et al.*, 2003) and sea bream (*Sparus aurata*) (Cakli *et al.*, 2006) during iced storage. Thepnuan *et al.* (2008) reported that the decapitation of shrimp could lower protein degradation caused by digestive and proteases of white shrimp kept under modified atmosphere packaging (MAP). Additionally, the decapitation could lower the aerobic plate count (APC) of Chinese shrimp (*Fenneropenaeus chinensis*) (Lu, 2009) and white shrimp (Thepnuan *et al.*, 2008) stored under MAP. Furthermore, gutting resulted in a

decrease of microbial load of seabream (*Sparus aurata*) (Tejada and Huidobro, 2002) and seabass (*Dicentrarchus labrax*) during iced storage (Paleologos *et al.*, 2004).

At the beginning of storage in ice, endogenous enzymes are mainly involved in the gradual loss of fish freshness. Thereafter, bacterial metabolism predominates and leads to final spoilage (Pacheco-Aguilar *et al.*, 2000). The hepatopancreas of crustacean (Aoki *et al.*, 2004a; Brauer *et al.*, 2003), pyloric caeca and intestine of fish (Klomklao, 2008; Simpson, 2000) are very rich in proteolytic and collagenolytic enzymes. The leakage of digestive enzymes also contributes to subsequent hydrolysis of muscle proteins. Therefore, pretreatment of fish and shellfish, including beheading and eviscerating prior to storage, could be another means to retard the deterioration caused by proteolysis.

1.2.6.2 Icing and chilling

Generally, fish meat softens rapidly during storage. The softening phenomenon indicates the deterioration of fish meat. The killing methods and storage conditions, affect the postmortem changes in fish (Álvarez *et al.*, 2009; Bagni *et al.*, 2007; Shigemura *et al.*, 2004). Therefore, it is important to delay or prevent the progression of this phenomenon for maintaining fish freshness. Substantial portion of the fish and shellfish is still preserved by traditional chilling and icing. Different types of novel refrigeration systems have been widely used for the preservation of seafood products at subzero temperatures (-4 to 0 °C) such as slurry ice or ozone-slurry ice combined refrigeration system (Álvarez *et al.*, 2009; Campos *et al.*, 2006; Pena *et al.*, 2009), and the use of a cooling agent, e.g. dry ice (solid carbon dioxide) or a combination of dry ice and iced water (Jeyasekaran *et al.*, 2004; Jeyasekaran *et al.*, 2006).

Storage temperature can limit softening by decreasing protease activity (Ando *et al.*, 2007). Superchilling is one of the few promising techniques with the potential to preserve the prime quality of fresh fish. Superchilling temperatures can be advantageous in maintaining food freshness and suppressing harmful microorganisms (Ando *et al.*, 2004; Ando *et al.*, 2005). Additionally, cold storage places a serious stress on living cells, resulting in generating of amino acids and sugars that could act as anti-freezing materials against cold temperatures. The shelf-life of various fish and

shellfish can be extended by storage at subzero temperatures. This technique can be used for fish, where productive fishing grounds are so far from ports and consumers and the normal icing is insufficient for maintaining good quality products prior to being landed and sold (Dalgaard and Huss, 1997). However, some negative effects on quality have also been found in superchilled fish and shellfish. The disadvantages of slurry ice on fish quality including cloudy eyes and development of dull color was reported (Medina *et al.*, 2009). Huidobro *et al.* (2001) reported that the cloudy eyes of seabream (*Sparus aurata*) stored in liquid ice (-2.2°C) significantly reduces the commercial value. The loss of characteristic bright colors and development of dull tones in the carapace of pink shrimp (*Parapenaeus longirostris*) stored in liquid ice was also reported (Huidobro *et al.*, 2002). Furthermore, Bahuaud (2008) reported freeze damage during superchilling. The upper layer of the super-chilled fillets showed freeze damage as characterized by the formation of large intra- and extracellular ice crystals during superchilling. Freeze damage due to superchilling accelerated the amount of detachments between myofibers and increased the amount of myofiber breakages during storage time. Superchilling accelerated the release of the proteolytic enzymes cathepsin B and L from the lysosomes, causing an acceleration of fish muscle degradation (Bahuaud *et al.*, 2008). Duun and Rustad (2008) found that myofibrillar proteins denatured more easily during superchilled than during chilled storage both in salmon and cod fillets and the amount of free amino acids increased more rapidly due to exoproteolytic activity. Duun and Rustad (2007) also found a higher liquid loss in superchilled samples, compared to ice chilled cod fillets.

The negative effect on texture of salmon (*Salmo salar*) (Gallart-Jornet *et al.*, 2007; Hansen *et al.*, 2009) and cod (*Gadus morhua*) (Wang *et al.*, 2008) during superchilling in combination with MAP has been reported. The negative effects such as loss in textural property, liquid loss and protein denaturation during superchilling from those reports indicated that the iced storage and chilling process still need to be used to maintain the quality of freshwater prawn meat during distribution and storage. The combination with other methods including the use of food grade additive with the storage at low temperature (icing and chilling storage) is still needed to maximize shelf-life extension.

1.2.6.3 Use of additives

Several additives have been used to maintain the quality of fish and shellfish during postmortem storage. Additionally, some compounds such as phosphate have been used for increasing the yield as well as improving the texture of some fish products.

1.2.6.4 Use of protease inhibitors

Since autolysis causes the loss in quality of fish and shellfish, food grade protease inhibitors have been applied to lower the degradation and softening of meat. Ishida *et al*. (2003) found that the reduction of breaking strength of stored tilapia was inhibited by the perfusion of leupeptin and benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk). Hopkins and Thompson (2001) reported that the use of various proteases inhibitors, especially cysteine protease inhibitors, was the most effective in preventing ageing of meat. EDTA, a bivalent metal ionic chelator, and 1, 10 phenanthroline, a specific inhibitor of metalloprotease, suppressed tenderization of flounder muscle (Kubota *et al.*, 2001). Kang and Lanier (2005) reported that the addition of crude recombinant soy cystatin (CRSC) was able to greatly inhibit the breakdown of myosin of ground arrowtooth flounder, compared with water-soaked samples. In order to maintain the quality of fish and shellfish during storage, protease inhibitors, especially natural and/or food grade inhibitor, have been paid attention in protecting muscle protein from proteolytic enzyme such as serine protease (Ayensa *et al.*, 2002; Choi *et al.*, 2005).

The most commonly food grade inhibitors used are dried beef plasma protein (BPP), egg white, milk whey and a white potato extract (Benjakul *et al.*, 2004). In general, these protease inhibitors have three complex forming domains which react with trypsin-like and chymotrypsin-like enzymes independently (Feeney and Osuga, 1988). These additives exert various degrees of inhibition towards the proteases responsible for weak gelation of surimi. Although food grade protease inhibitors have been widely used, unwanted side effects have been noticed, including modified color and/or taste (Benjakul *et al.*, 2001; Rawdkuen *et al.*, 2007).

Protease inhibitors can be obtained from various plants. Some of them have been proved as effective in preventing fish and shellfish protein degradation.

Protease inhibitors in plant organ are proteins or peptides capable of inhibiting catalytic activities of proteolytic enzymes. These inhibitors form stable complexes with target proteases, blocking, altering or preventing the access to the enzyme active site. Among those, serine protease inhibitors are the most widely studied, and have been isolated from soybean and other leguminous seeds (Bhattacharyya *et al.*, 2006; Fawole *et al.*, 2009; Garcia *et al.*, 2004).

Legume seed protease inhibitors belong to two families, Kunitz and Bowman-Birk. Both types of protease inhibitors are found in soybean (*Glycine max*) (Guillamo´n *et al.*, 2008). Protease inhibitors from grain legumes such as pea seed, common bean (*Phaseolus vulgaris* L.), cowpeas (*Vigna unguiculata*) and lentil (*Lens culinaris*) were characterized as members of the Bowman-Birk family (Galasso *et al.*, 2009; Lajolo and Genovese, 2002; Rahbé *et al.*, 2003), while those from *Delonix regia* seeds, chickpea (*Cicer arietinum* L.), kidney bean (*Phaseolus vulgaris*) and pea (*Lahtyrus sativus*) belonged to the Kunitz family (Pando *et al.*, 2001; Ramirrez *et al.*, 2002; Srinivasan *et al.*, 2005). Kunitz type inhibitors are proteins with molecular mass of 18-20 kDa that contains four cysteine residues forming two disulfide bridges and possess a single reactive site (an arginine residue) located in one of the protein loops (Sharma and Suresh, 2011). Bowman Birk type inhibitors on the other hand have molecular mass of 6-10 kDa and mostly contain a single polypeptide chain with a conserved and characteristic pattern of 14 cysteine residues forming seven intrachain disulfide bridges, which are responsible for maintaining stability and double headed structure of Bowman-Birk inhibitors (Clemente and Domoney, 2006; Devaraj and Manjunatha, 1999; Qi *et al.*, 2005).

Among serine protease inhibitors from leguminous seeds, soybean trypsin inhibitor (SBTI) is well characterized. It is relatively thermal stable whether under acidic or basic conditions (Jiang *et al.*, 2006). Soybean trypsin inhibitor inhibited the protease from tilapia (Yongsawatdigul *et al.*, 2000), tuna viscera (Klomklao *et al.*, 2011), bigeye snapper (Benjakul *et al.*, 2003a) and Crucian carp muscle (Jiang *et al.*, 2006). The inhibition of trypsin (Guillamon *et al.*, 2008) and gel weakening of threadfin bream surimi (Benjakul *et al.*, 2000) by legume seed extract was also reported.

Methods for applying the protease inhibitor have been reported to affect the uptake of those compounds. Poor penetration of the inhibitors into the fillets by soaking method was reported by Kang and Lanier (2005). Some protease inhibitors have been injected in fish muscle to clarify the role of these enzymes in postmortem tenderization. Kubota *et al*. (2001) demonstrated the involvement of protease in the postmortem tenderization of fish muscle by injecting protease inhibitors into blood vessels in the caudal portion of live flounders. This method seemed not to exclude the effect of blood fluid, in which factors inducing muscle softening may exist. Bleeding is believed to reduce the muscle softening when fish are killed (Ando *et al.*, 1999). Kang and Lanier (2005) successfully infused a recombinant cystatin into arrowtooth flounder muscle chunks by injection to achieve reduction of proteolytic activity during cooking, resulting in firming of the meat. Furthermore, Carvajal-Rondanelli and Lanier (2010) reported that low molecular weight protease inhibitors such as cystatin can be effectively diffused into intact fish muscle cells to minimize proteolytic activity and meat softening.

1.3 Objectives

1. To characterize the muscle of freshwater prawn.
2. To monitor the proteolysis and textural changes of freshwater prawn during iced storage.
3. To purify and characterize protease associated with the softening of freshwater prawn meat.
4. To isolate the protease inhibitor from legume seeds and use for prevention of protein degradation induced by freshwater prawn hepatopancreatic proteases.
5. To study the impact of injection of legume seed extract into cephalothorax on softening and quality changes of prawn meat during iced storage.

CHAPTER 2

Chemical and thermal properties of freshwater prawn (*Macrobrachium rosenbergii*) meat

2.1 Abstract

Chemical compositions and thermal properties of cultured freshwater prawn meat were studied. Freshwater prawn meat contained 83.2% protein (dry basis), 62.7% of which was myofibrillar proteins. Pepsin-soluble collagen (PSC) and insoluble collagen (ISC) contents were 0.63 and 0.32%, respectively. Both collagens were similar to type V collagen from porcine placenta. Glutamic acid/glutamine, arginine, aspartic acid/asparagine and lysine were abundant amino acids in freshwater prawn meat. Glycine, proline, hydroxyproline and aspartic acid/asparagine were predominant in both collagens. Freshwater prawn meat exhibited thermal transition temperatures (T_{max}) of 48.3 and 64.7 ºC, whereas T_{max} of PSC and ISC were 43.0 and 46.0 ºC, respectively. Textural changes in freshwater prawn meat during post-mortem storage on ice are dependent upon its compositional and thermal properties.

2.2 Introduction

In Thailand, freshwater prawn (*Macrobrachium rosenbergii*) has become an economically important species and is widely cultured in freshwater ponds. Prawn production in Thailand, however, is limited by changes in muscle texture which begins after 4 days of iced storage (Sriket *et al.*, 2010). Sriket *et al.* (2011a) reported that muscle proteases did not play a major role in muscle degradation of freshwater prawn. However, the serine-like collagenolytic proteases released from hepatopancreas during extended iced storage were reported to cause the muscle softening of this species (Sriket *et al.*, 2011b). Hepatopancreas of crustaceans including shrimp, prawn and crab etc., have been known as a good source of proteolytic enzymes but the impact on the muscle degradation is depending upon species as well as amino acid composition (Aoki *et al.*, 2003; Oh *et al.*, 2000).

Generally, the muscle tissues of freshwater prawn and marine prawn are not greatly different in taste. Textural property can be varied with species, more likely governed by composition and thermal property of tissue. The content and type of collagen has been

known to affect the textural property of fish and shrimp (Fauconneau *et al.*, 1995; Sato *et al.*, 1991). The understanding of the chemical and thermal properties of freshwater prawn meat could provide the nutritive values as well as the susceptibility to softening of this species. However, a little information regarding the chemical composition and thermal property of freshwater prawn, especially cultured in Thailand, has been reported. This study aimed to investigate the chemical compositions and thermal property of meat from freshwater prawn cultured in the Southern Thailand.

2.3 Materials and Methods

2.3.1 Chemicals

Sulfuric acid, isopropanol, chloramine T and *ρ*-dimethylamino-benzaldehyde were purchased from Merck (Darmstadt, Germany). Acrylamide, *N,N,N′,N′*-tetramethylethylenediamide (TEMED) and bis-acrylamide were obtained from Fluka (Buchs, Switzerland). Type I collagen from calf skin was procured from Sigma (St. Louis, MO, USA). Types II, III and V collagens from porcine cartilage, porcine skin and porcine placenta, respectively, were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

2.3.2 Sample collection and preparation

Freshwater prawns (*M. rosenbergii*) with the size of 30-35 prawns/kg were obtained from a farm in Phatthalung province, Thailand. Three different lots (5 kg each) were used for the study. The prawn were placed in ice with an ice/prawn ratio of 2:1 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla within approximately 2 h. Upon the arrival, the prawn were washed with clean cold water (4 °C) and deheaded. The shells were then peeled off manually. The prawns were deveined and the edible portions were ground to obtain uniformity using a blender (type AY46, Moulinex, Group SEB Thailand Ltd, Bangkok, Thailand). The samples were placed in polyethylene bags and kept in ice until the analyses. The storage time was not longer than 5 h.

2.3.3 Proximate analysis

Freshwater prawn meat was analyzed for moisture, ash, fat and protein contents according to AOAC (1999) methods described in the analytical numbers of 950.46,

928.08, 960.39 and 920.153, respectively. The values were expressed as % (wet weight basis).

2.3.4 Determination of protein and non-protein nitrogenous compounds

Non-protein nitrogenous constituents, sarcoplasmic protein, myofibrillar protein, alkali-soluble protein and stromal protein in freshwater prawn meat were fractionated according to the method of Hashimoto *et al.* (1979). Nitrogen content in each fraction was measured by the Kjeldahl method (AOAC, 1999). Protein patterns of different fractions were determined using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) with 10% running gel and 4% stacking gel, as described by Laemmli (1970). Samples (10 µg protein) determined by the Biuret test (Robinson and Hogden, 1940) were loaded. After separation, proteins were stained and destained following the method of Sriket *et al.* (2007).

2.3.5 Determination of collagen

Collagen was isolated into two different fractions, pepsin soluble (PSC) and insoluble (ISC) collagens, according to the method of Sato *et al.* (1988). Hydroxyproline content in each fraction was determined as described by Bergman and Loxley (1963). A factor of 11.42 was used to convert the amount of hydroxyproline to total collagen (Sato *et al*., 1986). Protein patterns of each fraction were determined according to the method of Laemmli (1970) and Sriket *et al.* (2007).

2.3.6 Amino acid analysis

Whole prawn meat, PSC and ISC were hydrolyzed under reduced pressure in 4.0 M methanesulfonic acid containing 0.2% (v/v) 3-2 (2-aminoethyl) indole at 115 ºC for 24 h. The hydrolysates were neutralized with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot (0.4 mL) was applied to an amino acid analyzer (MLC-703; Atto Co.,Tokyo, Japan).

2.3.7 Differential scanning calorimetry (DSC)

Thermal transitions of whole prawn meat, PSC and ISC isolated from freshwater prawn meat were measured using differential scanning calorimeter (DSC) (Perkin–Elmer, Model DSCM, Norwalk, CT, USA). The samples (15-20 mg) were placed in the DSC hermetic pans, assuring a good contact between the sample and the pan bottom. An empty hermetic pan was used as a reference. Calibration was made using Indium

thermogram. The samples were scanned at 1 ºC/min over the range of 20-100 °C. T_{max} was measured and the denaturation enthalpies (ΔH) were estimated by measuring the area under the DSC transition curve.

2.3.8 Statistical analysis

Data were subjected to analysis of variance and mean comparison was carried out using Duncan's multiple range test (DMRT) (Steel and Torrie, 1980). Statistical analyses were performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL).

2.4 Results and Discussion

2.4.1 Proximate composition

Chemical composition of freshwater prawn meat is shown in Table 4. Freshwater prawn meat had 77.5% moisture, 18.7% protein, 1.70% fat and 1.08% ash contents. Based on dry weight basis, protein constituted around 83.2% of meat. The high protein content in freshwater prawn meat indicated that freshwater prawn meat could be a good source of proteins and amino acids. The slight difference in chemical composition was observed between freshwater prawn meat and that reported for marine shrimps. Black tiger shrimp (*Penaeus monodon*) contained 80.5% moisture, 17.1% protein, 1.23% fat and 0.95% ash. Whereas Pacific white shrimp (*Penaeus vannamei*) consisted of 77.2% moisture, 18.8% protein, 1.30% fat and 1.47% ash (Sriket *et al.*, 2007). Rosa and Nunes (2004) also reported that proximate composition of the edible part of red shrimp (*Aristeus antennatus*), pink shrimp (*Parapenaeus longirostris*) and lobster (*Nephrops norvegicus*) was slightly different. Protein contents of red shrimp, pink shrimp and lobster were 21.4, 20.8 and 20.4%, respectively (Rosa and Nunes, 2004). Differences in proximate composition might be associated with the differences in nutritional value, sensory properties and shelf-life of different prawn.

Table 4. Proximate compositions of freshwater prawn meat and other shrimps

Compositions (%, wet weight basis)	Freshwater prawn	Black tiger shrimp#	Pacific white shrimp#	Red shrimp##	Pink shrimp##	Norway Lobster##
Moisture	77.5±0.7*	80.5±0.3	77.2±0.2	74.5±0.7	74.6±0.7	75.2±0.9
Protein	18.7±2.3 (83.2)**	17.1±0.6	18.8±0.2	21.4±0.2	20.8±0.3	20.4±0.4
Ash	1.1±0.2 (4.8)	0.9±0.0	1.5±0.1	2.0±0.1	1.90±0.1	2.0±0.1
Fat	1.7±0.3 (7.6)	1.2±0.4	1.3±0.1	0.1± 0.1	0.20± 0.0	0.1±0.0

*Means ± SD (n=3).

**Values in parenthesis represent % (dry weight basis).

#Sriket *et al.* (2007)

##Rosa and Nunes (2004)

2.4.2 Proteins and non-protein nitrogenous compounds

The contents of different proteins and non-protein nitrogenous components in freshwater prawn meat are shown in Table 5. Myofibrillar protein was the major protein in the freshwater prawn meat, accounting for 62.7% of total protein. Sarcoplasmic protein was found as the second predominant protein, constituting 31.6% of total protein. This finding was in agreement with Hashimoto *et al.* (1979) and Sriket *et al.* (2007) who reported that myofibrillar and sarcoplasmic proteins were the major proteins in fish and marine shrimp muscle. Alkali-soluble protein and stromal protein were found as the minor components in freshwater prawn meat. Alkali-soluble protein was most likely cross-linked myofibrillar protein (Hashimoto *et al.*, 1979) and collagen was presented as the major protein in stroma (Foegeding *et al.*, 1986). For non-protein nitrogenous compound, it constituted 4.96 mg N/g meat. Non-protein nitrogenous constituents, such as free amino acids, peptides, betaine and nucleotide, etc. play an essential role in the flavor of fish and shellfish (Sikorski *et al.*, 1990). Additionally, the changes in non-protein nitrogenous compounds could be used as quality index for monitoring shelf-life of fish (Ruiz-Capillas *et al.*, 2002).

Table 5. Nitrogenous constituents of freshwater prawn meat

Compositions	Nitrogen content (mg N/g meat)
Non-protein nitrogen	4.96±0.19*
Sarcoplasmic protein	7.72±0.63 (31.63) **
Myofibrillar protein	15.3±0.07 (62.68)
Alkali-soluble protein	0.85±0.11 (3.48)
Stromal protein	0.54±0.12 (2.21)

*Means ± SD (n=3).

**Numbers in parenthesis represent percentage distribution.

Protein patterns of whole prawn meat and different protein fractions from freshwater prawn meat are shown in Figure 2. Freshwater prawn meat had myosin heavy chain (MHC) as the dominant protein component. MHC is the major protein in myofibrillar protein (Shahidi, 1994). Actin was found as the second prevalent protein. For the myofibrillar protein fraction, two major protein bands, corresponding to MHC and actin, were observed (Figure 2). Generally, low-molecular-weight proteins were found in the sarcoplasmic protein fraction and protein with molecular mass of 26 kDa was predominant. For the alkali-soluble protein fraction, smear bands of proteins were found and proteins with molecular mass of 45 and 108 kDa were present as the major proteins. Stromal protein fraction contained proteins with molecular mass of 50 and 200 kDa. It was noted that there were some cross-links with high molecular mass protein in this fraction as evidenced by the presence of protein band on the stacking gel.

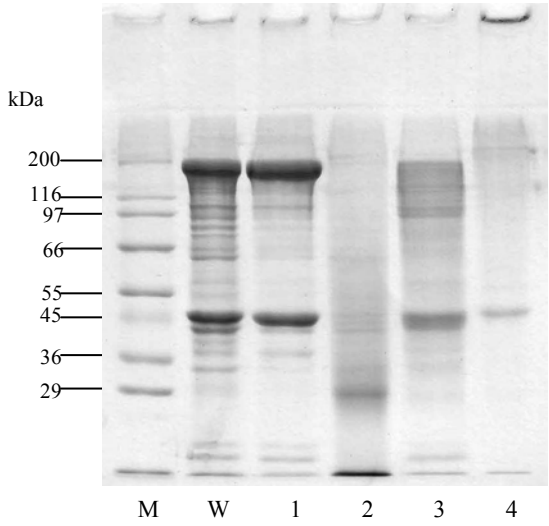

Figure 2. Protein patterns of freshwater prawn meat and various protein fractions. M; protein markers, W: whole meat; 1, 2, 3, 4: myofibrillar, sarcoplasmic, alkali-soluble and stromal protein fractions, respectively.

2.4.3 Collagen content

PSC and ISC from freshwater prawn meat constituted 0.63 and 0.32% (dry weight basis), respectively. PSC content was approximately two-fold higher than that of ISC. Nevertheless, PSC and ISC varied with shrimp species. Sriket *et al.* (2007) reported that PSC and ISC contents of black tiger shrimp were 0.36 and 0.48%, whereas white shrimps contained PSC and ISC of 0.83% and 3.32%, respectively. ISC was most likely associated

with the cross-linkings of collagen molecules, which have the influence on the texture of freshwater prawn meat (Montero and Borderias, 1990). Yoshinaka *et al.* (1989) reported that the collagen content in the abdominal muscle of seven species, including shrimp, prawn, lobster and squilla, varied among the species ranging from 1.1% to 6.2% of total tissue protein. Collagen content in pereiopod and thoracic muscles of four species of crab varied from 0.2% to 0.8%. A high collagen content was associated with a firm texture (Hatae *et al.*, 1986; Sato *et al.*, 1986). The distribution of soluble and insoluble collagen in fish muscle varies from species to species (Eckhoff *et al.*, 1998). The gaping score in fish samples correlated with the amount of insoluble collagen. The high ISC content was related with the less gaping (Espe *et al.*, 2004). Lost in textural quality of freshwater prawn meat during storage in ice can be attributed to the composition and low content of collagen in the muscle tissues.

The protein patterns of PSC and ISC of freshwater prawn meat are shown in Figure 3. PSC and ISC from freshwater prawn meat had patterns similar to those of porcine placenta collagen types V (Figure 3). Both PSC and ISC consisted of α-chain with molecular mass of 119 kDa as the dominant protein. Type V collagen consists of three chains of α1 (Foegeding *et al.*, 1986). Yoshinaka *et al.* (1989) reported that the major collagen from the crustacean muscle was similar to type V collagen from vertebrate muscle. Furthermore, protein band with molecular mass of 156 kDa was found as the minor proteins in both PSC and ISC.

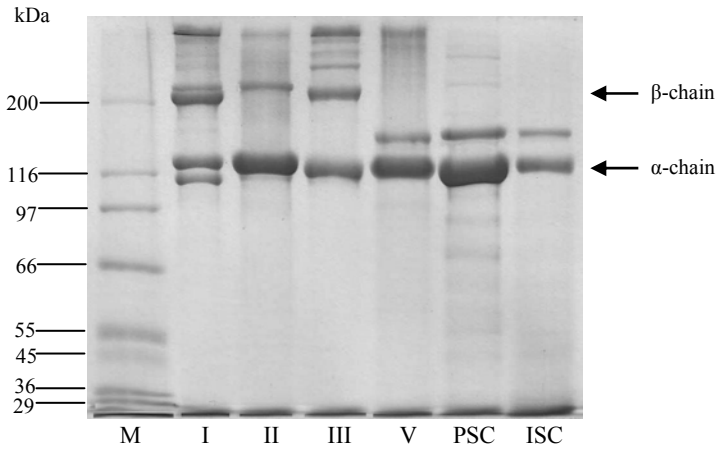

Figure 3. Protein patterns of PSC and ISC isolated from freshwater prawn meat. M: protein markers; I, II, III and V: calf skin collagen type I, porcine cartilage collagen type II, porcine skin collagen type III and porcine placenta collagen type V, respectively, PSC: pepsin-soluble collagen; ISC: insoluble collagen.

2.4.4 Amino acid compositions

The amino acid compositions of whole meat, PSC and ISC from freshwater prawn meat are shown in Table 6. Whole meat had glutamic acid/glutamine as the major amino acids (49.40 mg/g sample). Arginine, aspartic acid/asparagine, lysine and leucine were also found at high content (Table 6). These amino acids constituted more than 50% of the total amino acids. Glycine, alanine, serine and threonine have the sweet taste, while arginine, leucine, valine, methionine, phenylalanine, histidine and isoleucine give bitter taste (Sikorski *et al.*, 1990). High content (156.33 mg/g sample) of essential amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine) was found in whole meat. The ratio of essential amino acids (EAA) to nonessential amino acids (NEAA) was 0.98. Amino acid composition of freshwater prawn meat was slightly different from that of marine shrimp (black tiger shrimp and white shrimp) (Sriket *et al.*, 2007). The difference in amino acid compositions between marine and freshwater prawn might result in different taste and properties. It was noted that freshwater prawn meat contained a high content of arginine (34.91 mg/g sample) and lysine (27.11 mg/g sample).

Amino acid composition of PSC and ISC isolated from freshwater prawn meat is shown in Table 6. Generally, glycine was the most abundant amino acid (300-307 residues/1000 residues) in both PSC and ISC. Glycine has been reported to situate at every third position of α-chain except for the first 14 amino acids of N-terminal and the first 10 amino acids from C-terminal (Burghagen, 1999). PSC and ISC had high proline (100-108 residues/1000 residues), hydroxyproline (95-100 residues/1000 residues) and glutamic acid/glutamine (97-102 residues/1000 residues) contents. However, hydroxyproline content was higher in ISC (100 residues/1000 residues), compared with that of PSC (95 residues/1000 residues). Hydroxyproline has been reported to have the influence on meat firmness and thermal property. This result was in accordance with Sivakumar *et al.* (1997) who reported that glycine, proline and hydroxyproline were the major amino acids in marine prawn (*Penaeus indicus*) collagen. Nevertheless, hydroxyproline content found in both PSC and ISC of freshwater prawn in the present study was higher than that found in marine prawn (*P. indicus*) collagen (81 per 1000 residues) (Sivakumar *et al.*, 1997). In addition, PSC and ISC from freshwater prawn meat had imino acid content (proline and hydroxyproline content) of 195 and 208 residues/1000 residues, respectively. These values were much higher than those found in collagens from marine prawn muscle (171 residues/1000 residues) (Sivakumar *et al.*, 1997). Rubin *et al.* (2008) reported that imino acid content of animal collagens correlated with their habitat and thermal stability of collagen. The difference in amino acids

composition between freshwater prawn and other shrimp species might be a factor determining the differences in their sensory and textural properties.

Table 6. Amino acid composition of whole meat, PSC and ISC isolated from freshwater prawn meat

Amino acids	Whole meat	PSC	ISC
	mg/g sample	residue/1000 residues	residue/1000 residues
Alanine	18.68 (59)*	59	43
Arginine	34.91 (110)	48	23
Asp/Asn	33.81 (107)	48	48
Cysteine	0.18 (1)	0	0
Glu/Gln	49.40 (156)	102	97
Glycine	19.44 (61)	307	300
Histidine	7.40 (23)	5	9
Isoleucine	14.16 (45)	22	33
Leucine	24.60 (78)	47	48
Lysine	27.11 (86)	16	22
Hydroxylysine	0.21 (1)	24	3
Methionine	9.17 (29)	15	12
Phenylalanine	12.94 (41)	13	28
Hydroxyproline	0.47 (1)	95	100
Proline	9.78 (31)	100	108
Serine	13.11 (41)	37	42
Threonine	12.12 (38)	26	29
Tyrosine	11.59 (37)	7	17
Tryptophane	3.47 (11)	1	5
Valine	13.93 (44)	27	31
Total	316.47	1000	1000

* Values in parenthesis are expressed as residue/1000 residues

2.4.5 Thermal property of freshwater prawn meat

Thermal transitions of whole muscle, PSC and ISC isolated from freshwater prawn meat expressed as T_{max} and ΔH are shown in Table 7. DSC analysis was used to determine the thermal transition or unfolding temperature of protein and also to quantify the enthalpy of conformational transition (John and Shastri, 1998). Whole meat of freshwater

prawn showed two major transition peaks, corresponding to myosin and actin peaks. T_{max} of the first (48.33 °C) and second (64.66 °C) peaks with ΔH of 0.63 and 0.07 J/g, respectively, were observed. The result was in accordance with Sriket *et al.* (2007) who reported two major endothermic transitions with T_{max} of 51.28 and 66.20 °C for black tiger shrimp meat and 50.13 and 71.17 °C for white shrimp meat, respectively. Poulter *et al.* (1985) reported that T_{max} of the first and the second peaks of whole fish (*Gadus morhua*, *Siganus oramin* and *Sarotherodon aureiislniloticus*) meats were 41.70-52.70 and 72.6-73.8 °C, respectively. The differences in thermal properties between freshwater prawn meat and other species may be due to the differences in habitat, temperature, feed, etc. (Ezquerra Brauer *et al.*, 2003).

Table 7. T_{max} and enthalpy of whole meat, PSC and ISC isolated from freshwater prawn meat

Samples	T_{max} I (ºC)	ΔH (J/g)	T_{max} II (ºC)	ΔH (J/g)
Whole meat	48.3±1.22*	0.63±0.01	64.7±0.64	0.07±0.01
PSC	43.0±0.20	0.38±0.03	-	-
ISC	46.0±0.67	0.43±0.07	-	-

*Means ± SD (n=3).

For PSC from freshwater prawn, T_{max} was found at 43 °C with ΔH of 0.38 J/g (Table 7), indicating the temperature inducing the transition of triple helix to random coil of collagen. Nevertheless, Sivakumar *et al.* (1997) found that shrimp (*Penaeus indicus*) collagen had T_{max} of 37 °C. The difference in T_{max} among fish and shellfish species might be due to the difference in environment temperature they live. For ISC from freshwater prawn, the higher T_{max} (46 °C) with ΔH of 0.43 J/g was observed. The higher T_{max} value of ISC was in agreement with higher hydroxyproline content in ISC, compared with that of PSC (Table 3). Hydroxyproline and proline play a role in collagen stability (Rubin *et al.*, 2008).

2.5 Conclusion

Freshwater prawn meat was a good source of protein and amino acids. Freshwater prawn meat consisted of glutamic acid/glutamine, glycine and aspartic acid/asparagine at high content, indicating the umami or good taste of this species. Freshwater prawn meat also contained high content of arginine and lysine, indicating the more susceptibility to be hydrolyzed by trypsin from hepatopancreas during the extended

storage. Freshwater prawn meat had low collagen content, though its collagen showed higher thermal stability than other prawns. Chemical and thermal properties of freshwater prawn meat were shown to affect textural properties and may influence the softening of meat during extended storage in ice.

CHAPTER 3

Post-mortem changes of muscle from freshwater prawn (*Macrobrachium rosenbergii*) as influenced by spawning stages

3.1 Abstract

Post-mortem changes of the muscle from pre- and post-spawned freshwater prawn (*Macrobrachium rosenbergii*) were comparatively monitored during 7 days of iced storage. During the storage, the muscle of pre-spawned prawn had a greater value of trichloroacetic acid (TCA) soluble peptide, heat soluble collagen and pepsin soluble collagen (PSC) contents than did post-spawned counterpart. Those components in the muscle of both prawns increased markedly after 3 days of storage ($p < 0.05$). Conversely, insoluble collagen (ISC) content, shear force value and texture liking of both prawns decreased ($p < 0.05$), indicating the softening of muscle. No changes in protein patterns were observed, except the decreased band intensity of 66 kDa protein in water soluble fraction of both prawns was found after 3 days of storage. T_{max} and enthalpy of PSC from both prawns decreased during the first 4 days of storage ($p < 0.05$), suggesting the degradation or denaturation of collagen in the muscle. Light microscopic study showed the lowering of intercellular connection of raw meat and higher gaping in cooked meat when the samples were stored for a longer time. Therefore, post-mortem characteristics of prawn muscle was affected by storage time and spawning stages.

3.2 Introduction

Macrobrachium rosenbergii, a freshwater prawn, has been cultured worldwide to meet increasing consumer demands. However, freshwater prawn production in Thailand is still limited due to the inconsistent market price, water supply problems and rapid quality loss. Textural deterioration has been considered as the most crucial because it limits prawn shelf-life, thereby impeding its marketing. Generally, prawn are distributed or stored in ice. This practice renders the prawns easily susceptible to a textural problem called "softening", caused by the degradation of edible portion due to activity of proteolytic enzymes during iced storage (Lindner *et al.*, 1988). Softening of fish muscle during iced storage is one of the

important factors influencing the acceptability and market value. This phenomenon found in postmortem fish is associated with the decreased shear force and disintegration of microstructure. Many factors affecting the postmortem softening of fish muscle include fish species, size, handling and killing methods as well as spawning stage (Kubota *et al.*, 2000).

Spawning stage has been reported to have the impact on the postmortem softening of fish and shellfish during extended storage (Yamashita and Konagaya, 1991). The decrease in myosin content of pre-spawned flounder was observed at earlier stage of iced storage (Paredi and Crupkin, 2007). Pre-spawned hake showed a partially denatured myosin heavy chain at the beginning of storage (Roura and Crupkin, 1995). The autolytic activity of muscle from pre-spawned hake was higher than that of post-spawned counterpart (Prez-Borla *et al.*, 2002). Muscle proteins of post-spawned hake were more stable than those of pre-spawned hake during iced storage (Beas *et al.*, 1991). Muscle deterioration of fish during spawning was associated with the higher activity of cathepsin L (Salem *et al.*, 2007). Among proteolytic enzymes, collagenase has the greater impact on the softening of muscle (Brauer *et al.*, 2003). Disintegration of the pericellular connective tissue of fish muscle was histologically observed in ayu muscle during spawning stage (Ito *et al.*, 1992). Moreover, spawned female red claw crayfish had an inferior meat quality to male and non-spawning female during refrigerated storage (Kong *et al.*, 2006). Freshwater prawn is more susceptible to softening than the marine prawn, either wild or cultured (Nip and Moy, 1988). Nip and Moy (1988) reported that shelf-life of freshwater prawn stored in ice was not more than 3-4 days because of proteolytic activities. However, a little work regarding the effect of spawning stages on quality changes of freshwater prawn has been carried out. Thus, the objective of this investigation was to determine the effect of spawning stage on the changes of muscle from freshwater prawn cultured in Thailand during iced storage.

3.3 Materials and Methods

3.3.1 Chemicals

2-Propanol was purchased from Riedal-deHaen (Seelze, Germany). Trichloroacetic acid (TCA) was obtained from Merck (Darmstadt, Germany). Pepsin (EC 3.4.23.1, 542 U/mg solid), Bouin'solution, oxalic acid, orange G and methyl blue were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 4-Dimethylaminobenzaldehyde,

acrylamide, *N*, *N, N′ N′*-tetramethyl ethylene diamine (TEMED) and bis-acrylamide were purchased from Fluka (Buchs, Switzerland).

3.3.2 Sample collection and preparation

Pre- and post-spawned prawn (*Macrobrachium rosenbergii*), identified by a fully developed ovary and the eggs adhered to the underside of the abdomen, respectively, with the size of 30-35 prawn/kg were obtained from a farm in Phatthalung province, Thailand. The prawn were placed in ice with a prawn/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla within approximately 2 h. Upon arrival, the prawns were washed with clean water. The samples were kept in a styrene foam box containing crushed ice, with a prawn/ice ratio of 1:2 (w/w). The samples were placed and distributed uniformly between the layers of ice. The boxes containing samples and ice were kept at room temperature (28-30°C). To maintain the ice content, molten ice was removed and replaced with an equal amount of ice every day. Thirty prawns were taken every day for analyses. Prior to analyses, the prawns were beheaded, peeled and deveined. The meat, pooled as the composite sample, was kept in polyethylene bag and placed in ice until analyzed. Prawn meat was ground to obtain the uniformity and used as the composite sample. The prepared sample was then subjected to chemical analysis. For sensory analysis at day 0, 4 and 7, another thirty prawns were used. Three different lots of samples were used for the entire study.

3.3.3 Chemical analyses

3.3.3.1 Determination of TCA soluble peptide content

TCA-soluble peptide content was determined according to the method of Benjakul *et al.* (2002). Ground meat (3 g) was homogenized with 27 mL of 5% TCA using an IKA homogenizer (Selangor, Malaysia) at a speed of 11,000 rpm for 1 min. The homogenate was kept in ice for 30 min and centrifuged at 5000 × *g* for 20 min using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, CA, USA). Soluble peptides in the supernatant were measured according to the Lowry method (Lowry *et al.*, 1951) and expressed as μmol tyrosine/g muscle. For each sample, triplicate determinations were conducted.

3.3.3.2 Determination of collagen content

Collagen was isolated into two different fractions including pepsin-soluble (PSC) and insoluble (ISC) collagen fractions according to the method of Sato *et al.* (1988). Hydroxyproline content in each fraction was determined as described by Bergman and Loxley (1963). A factor of 11.42 was used to convert the amount of hydroxyproline to total collagen (Sato *et al.*, 1988). Protein patterns of each fraction were determined according to the method of Laemmli (1970). For each sample, the determinations were performed in triplicate.

3.3.3.3 Determination of heat soluble collagen

Heat soluble collagen content was extracted according to the method of Liu *et al.* (1996). Prawn meat (2 g) was homogenized with 8 mL of 25% Ringer's solution (a mixture of 32.8 mM NaCl, 1.5 mM KCl, and 0.5 mM $CaCl_2$). The homogenate was heated for 70 min at 77°C and centrifuged at 2,300 × *g* for 30 min at 4 °C. The extraction was repeated twice. The supernatants obtained were combined. The sediment and supernatants were hydrolyzed with 6 M HCl at 110 °C for 24 h. The collagen content of each fraction was determined as described previously. The amount of heat soluble collagen was expressed as a percentage of total collagen (collagen content in sediment plus that in the supernatant). For each sample, triplicate determinations were conducted.

3.3.3.4 SDS-polyacrylamide gel electrophoresis of water soluble and water insoluble proteins

To obtain the water soluble and water insoluble proteins, 3 g of meat was homogenized with 10 volumes of cold water using an IKA homogenizer at 11,000 rpm for 1 min. The homogenate was then centrifuged at 10,000 × *g* for 15 min at 4 °C using a refrigerated centrifuge. After centrifugation, the supernatant and the residue fractions were defined as 'water soluble' and water 'insoluble proteins', respectively. Protein patterns of both fractions were determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) made of 10% running gel and 4% stacking gel as described by Laemmli (1970).

To prepare the protein sample, 27 mL of 5% SDS solution heated to 85 °C were added to 3 mL of water soluble fraction or 3 g of water insoluble fraction. The mixture was then homogenized at 11,000 rpm for 2 min using an IKA homogenizer. The homogenate was incubated at 85 °C for 1 h to dissolve total proteins. The samples were centrifuged at

3,500 × *g* for 20 min to remove undissolved debris. Protein concentration was determined by the Lowry method (Lowry *et al.*, 1951) using bovine serum albumin as a standard. After separation, the gel was stained with 0.05% Coomassie Blue R-250 in 15% methanol and 5% acetic acid and destained with 30% methanol and 10% acetic acid.

3.3.4 Physical, thermal property and microstructure analyses

3.3.4.1 Differential scanning calorimetry (DSC) of pepsin-soluble collagen

Thermal transition of PSC extracted from pre- and post-spawned prawn at day 0, 4 and 7 of iced storage was determined using the differential scanning calorimetry (DSC) (Perkin-Elmer, Model DSCM, Norwalk, CT, USA). The samples (15-20 mg dry weight) were placed in the DSC hermetic pans, assuring a good contact between the sample and the pan bottom. An empty hermetic pan was used as a reference. Temperature calibration was done using the indium thermogram. The samples were scanned at 1ºC /min over the range of 20-50 ºC. T_{max} was measured and the denaturation enthalpies (ΔH) were estimated by measuring the area under the DSC transition curve. For each sample, DSC analysis was run in duplicate.

3.3.4.2 Determination of shear force

Shear force of prawn meats, raw and cooked, were measured using TA-XT2i texture analyzer equipped (Stable Micro Systems, Surrey, England) with a Warner-Bratzler shear apparatus (Brauer *et al.*, 2003). To prepare cooked prawn, deheaded and peeled prawns were steamed for 5 min, in which the core temperature 70 °C was obtained. After cooking, cooked prawn were cooled rapidly in iced-water and drained on the plastic screen for 3 min at 4 °C. The operating parameters consisted of a cross head speed of 10 mm/s and a 25 kg load cell. The shear force, perpendicular to the axis of muscle fibers, was measured at the second segment of prawn meat. The peak of the shear force profile was regarded as the shear force value. For each sample, ten determinations were run.

3.3.4.3 Determination of microstructure by light microscopy (LM)

Microstructures of raw and cooked meat of prawn stored in ice for 0, 4 and 7 days were determined as described by Ando *et al.* (2004) with a slight modification. Samples were prepared by cutting into a cube (4 x 4 x 4 mm) with a razor blade. The prepared samples were fixed in Bouin's fixative solution for 24 h at room temperature. After dehydration by serial concentration of 70-100% ethanol, prawn meat was embedded in resin (Technovit

7100, Kulzer Co., Weinheim, Germany). Thin sections (2 μm thick) were prepared by a microtome and stained with Mallory's trichrome staining solution (a mixture of 2% of orange G, 0.5% of methyl blue and 2% of oxalic acid). The microstructure was visualized using a light microscope (Olympus® Model AX-70, Tokyo, Japan).

3.3.5 Sensorial analysis

Cooked samples, pre- and post-spawned prawn, stored for 0, 4 and 7 days in ice were evaluated by 30 panelists using a 9-point hedonic scale (Mailgaard *et al.*, 1999): 1, dislike extremely; 2, dislike very much; 3, dislike moderately; 4, dislike slightly; 5, neither like nor dislike; 6, like slightly; 7, like moderately; 8, like very much; 9, like extremely. Panelists were the graduate students of the Food Science and Technology program, Prince of Songkla University, with the age of 25-30 years. They were familiar with prawn consumption and had no allergy. The samples were served on a white paper plate at room temperature. All samples were coded with three digit random numbers and presented at the same time in randomized order. The panelists were asked to assess samples for texture and overall liking. Panelists were instructed to rinse their mouths with water before starting and between sample evaluations. Evaluations were made in individual sensory evaluation booths.

3.3.6 Statistical analysis

The experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA) and mean comparison was carried out using Duncan's multiple range test (DMRT) (Steel and Torrie, 1980). For pair comparison, T-test was used. Statistical analyses were performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

3.4 Results and Discussion

3.4.1 Chemical changes of meat from pre- and post-spawned prawn during iced storage

3.4.1.1 Changes in TCA-soluble peptide content

TCA-soluble peptide content of pre- and post-spawned prawn meat during iced storage is shown in Figure 4A. Generally, slight increases in TCA-soluble peptide content of meat from pre- and post-spawned prawn were observed during the first 3 days of storage ($p<0.05$), followed by the marked increases at day 4. No changes in TCA-soluble

peptide content were noticeable during day 4-6 of storage ($p > 0.05$). Nevertheless, a slight decrease was found at day 7 ($p < 0.05$). This indicated the increase in proteolysis of prawn meat, especially after 3 days of storage. With extended storage, autolysis of gastrointestinal tract, mainly in cephalothorax might be enhanced. As a consequence, proteinases could be more released from cephalothorax to meat. Thus, small peptides were produced and could serve as nutrients for microorganisms. When microorganisms grew using those free amino acids or small peptides, the degradation mediated by increasing microorganisms could be much more enhanced. Hepatopancreas and digestive tract of crustaceans are very rich in proteolytic and collagenolytic enzymes (Tsai *et al.*, 1986). At the beginning of storage in ice, indigenous proteinases are mainly involved in the gradual loss of fish freshness. Thereafter, bacterial metabolism predominates and leads to final spoilage (Pacheco-Aguilar *et al.*, 2000). The slight decrease in TCA-soluble peptide content in all samples after 6 days of storage might be due to the leaching of small peptides by molten ice. This led to the lowered TCA-soluble peptide contents retained in the meat. Water uptake of prawn during storage in ice also contributed to the dilution of TCA-soluble peptide contents in prawn muscles. At the beginning, the muscle structure was more compact. As a result, water penetration and leaching effect was less. With the longer storage time, the muscle structure could be more looser caused by the increased autolysis and the water molecules were easily absorbed in prawn muscle. Therefore, leaching and diluting effect would be more pronounced in the prawn stored in ice for an extended time.

At the same storage time, pre-spawned prawn contained the higher TCA-soluble peptide content than did post-spawned prawn. This might be influenced by the higher proteolytic activities of pre-spawned prawn. Prez-Borla *et al.* (2002) reported that the autolytic activity of muscle extracts from pre-spawned hake was significantly higher than that of post-spawned hake.

3.4.1.2 Changes in collagen

Collagen content in prawn abdomen was in the range of 1.0-1.21 %. PSC and ISC contents of meat from pre- and post-spawned prawn stored in ice at day 0, 4 and 7 are shown in Table 8. PSC content in pre- and post-spawned prawn meat increased as the storage time increased ($p < 0.05$). Conversely, a continuous decrease in ISC content of both prawns was observed with increasing storage time ($p < 0.05$). The result was in agreement with Eckhoff *et al.* (1998) who reported that ISC content in Atlantic salmon decreased at day 15, compared to that found at day 0 but ASC (acid soluble collagen) content increased gradually

during storage in ice. ISC was most likely associated with the cross-linkings of collagen molecules, which have the influence on the texture of prawn meat (Montero and Borderias, 1990). PSC and ISC contents were different between the meat of black tiger prawn and Pacific white prawn (Sriket *et al.*, 2007). The decrease in ISC content of prawn meat during storage was probably a result of the cleavage of the triple helix caused by collagenase, neutral protease or acid protease (Pearson and Young, 1989). Sato *et al.* (1987) reported that proteases in fish muscle hydrolyzed nonhelical regions of collagen, leading to more solubilized collagen. Cathepsin L and serine proteases are capable of hydrolyzing major muscle structure proteins including collagen (Sato *et al.*, 1994). It was noted that the marked increase in PSC content was noticeable at day 4, which was in accordance with the increased TCA-soluble peptide content. Increase PSC together with the lowered ISC of prawn meat might lower the resistance to force applied, indicating the softening of prawn meat. When comparing PSC and ISC contents between both prawns, the lower ISC and higher PSC contents were found in pre-spawned prawn, compared with post-spawned counterpart ($p<0.05$). This might be associated with different textural property between both prawns. Fish and shellfish have the soft or mushy texture during spawning period due to the autolysis of protein for roe production (Kong *et al.*, 2007; Yamashita and Konagaya, 1991). Cathepsin L and serine proteases have ability to hydrolyze collagen and the extensively increased activities are found in muscle of spawning fish and shellfish (Sato *et al.*, 1994; Yamashita and Konagaya, 1991). The higher proteolytic activity in freshwater prawn meat was also found in pre-spawned prawn, as indicated by higher TCA-soluble peptide (Figure 4A). The higher proteolytic activity might contribute to the greater cleavage of collagen fibrils. This might lead to the loosen structure, in which pepsin could hydrolyze peptides at telopeptide region more effectively, as indicated by the higher PSC content with the concomitant decrease in ISC content. It was noted that the conversion rate of ISC to PSC in both prawns was slightly different. After 7 days of storage, PSC in pre-spawned and post-spawned prawn was increased by 22.48 and 25.37%, respectively. ISC was decreased by 28.72 and 27.75% in pre-spawned and post-spawned prawn, respectively.

PSC from both samples had the patterns similar to those of porcine cartilage collagen type II and V (Figure 6). Sato *et al.* (1989) reported that the major collagen from the crustacean muscle was similar to type V collagen from the vertebrate muscle. The solubility of rainbow trout type V collagen increased during storage in ice, while no change in type I was observed, which may suggest that type V collagen is involved in the rapid softening of fish muscle (Sato *et al.*, 1991).

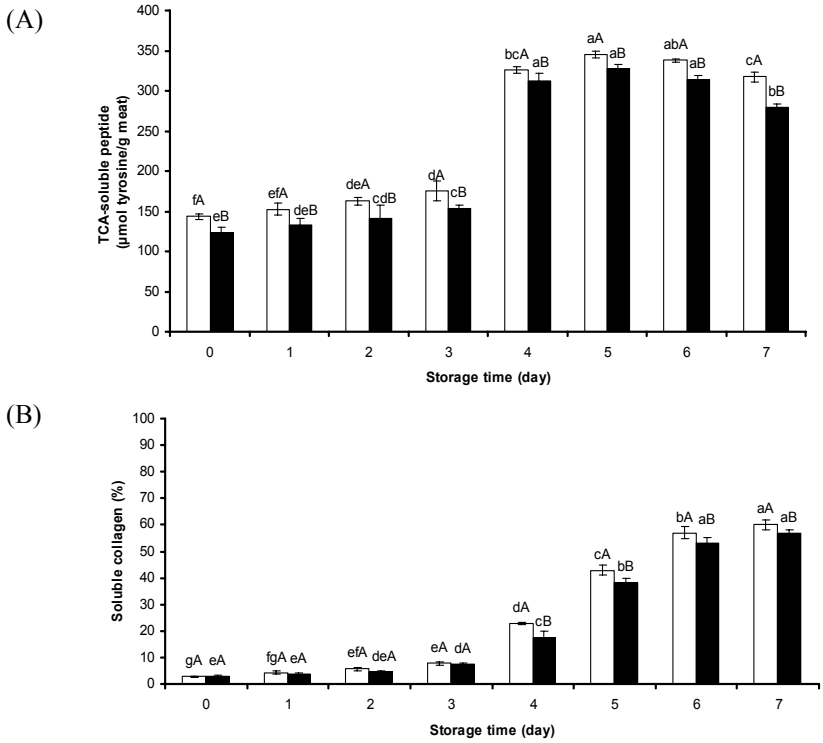

Figure 4. Changes in TCA-soluble peptide (A) and heat soluble collagen (B) contents of pre- and post-spawned freshwater prawn during iced storage. Bars represent the standard deviation (n=3). The different letters on the bars within the same prawn indicate significant differences ($p<0.05$). Different capital letters on the bars within the same storage time indicate the significant differences ($p<0.05$). □; pre-spawned, ■; post-spawned.

Table 8. PSC and ISC contents (% of 100% total collagen) of pre- and post-spawned prawn during iced storage

Storage time (day)	Pre-spawned prawn		Post-spawned prawn	
	PSC (%)	ISC (%)	PSC (%)	ISC (%)
0	56.09 ± 0.76^{cA}	43.91 ± 0.76^{aB}	52.03 ± 0.57^{cB}	47.97 ± 0.57^{aA}
4	66.64 ± 0.34^{bA}	33.36 ± 0.34^{bB}	63.62 ± 0.35^{bB}	36.38 ± 0.35^{bA}
7	68.70 ± 0.66^{aA}	31.30 ± 0.66^{cB}	65.23 ± 0.20^{aB}	34.77 ± 0.20^{cA}

Values are given as mean ±SD from triplicate determinations. Different superscripts in the same column indicate significant differences (($p<0.05$). Different capital superscripts in the same row within the same collagen type indicate the significant differences (($p<0.05$).

3.4.1.3 Changes in heat soluble collagen content

Changes in heat soluble collagen content in the meat of pre- and post-spawned prawn during iced storage of 7 days are shown in Figure 4B. Generally, heat soluble collagen content of both prawns increased with increasing storage time (($p<0.05$). Heat soluble collagen content of both prawn markedly increased after 3 days of storage (($p<0.05$). This was coincidental with the increase in TCA-soluble peptide content (Figure 4A) and the decrease in ISC content (Table 8). Collagen with lower content of cross-links as indicated by the lower ISC content of prawn stored in ice for a longer time could be more solubilized when heating was applied. Decreases in collagen cross-links might be associated with the increased autolysis caused by both indigenous and microbial proteinases.

Pre-spawned prawn meat contained more heat soluble collagen than did post-spawned prawn muscles during iced storage (($p<0.05$). The difference in the amount of heat soluble collagen between pre- and post-spawned prawns might be due to the differences in amounts of cross-linked collagen. After heat treatment, helix structure of collagen with lower cross-links could be disrupted much easily. This resulted in the higher extractability of collagen by heat as indicated by the increase soluble collagen content. Furthermore, hydrolyzed collagen with the shorter chain could be extracted with the aid of heat more easily. Cathepsin L and serine proteases are capable of hydrolyzing major muscle structure proteins such as collagen (Sato *et al.*, 1994). The increase in heat soluble collagen of pre- and post-spawned prawns might be associated with the muscle softening of both prawns during the extended iced storage.

3.4.1.4 Changes in protein patterns

Protein patterns of water soluble and water insoluble fractions of pre- and post-spawned prawns during the storage in ice for 7 days are shown in Figure 5. Generally, protein patterns of water soluble fraction, mainly containing sarcoplasmic protein and water insoluble fraction, consisting of myofibrillar protein from both prawns remained unchanged during 7 days of storage. However, the decrease in band intensity of protein with molecular mass of about 66 kDa in water soluble fraction was noticeable in both prawn kept in ice during 4-7 days of storage (Figure 5A and 5B). This change was in accordance with the substantial increase in TCA-soluble peptide contents of both prawns at day 4 of storage (Figure 4A). Kong *et al.* (2007) reported the decrease in a 69 kDa component and troponin-I of red claw crayfish during 10 days of storage at 2 °C. The result indicated that proteinases, either indigenous or microbial, were able to hydrolyze sarcoplasmic and myofibrillar proteins

differently. Among all proteins in the muscle, 66 kDa protein was susceptible to hydrolysis. Nevertheless, most proteins in prawn muscle were resistance to hydrolysis induced by proteinases. Additionally, both prawns possibly contained the negligible level of proteinases. The muscle softening of pre- and post-spawned prawns during iced storage might result from the degradation of collagen rather than myofibrillar and sarcoplasmic proteins.

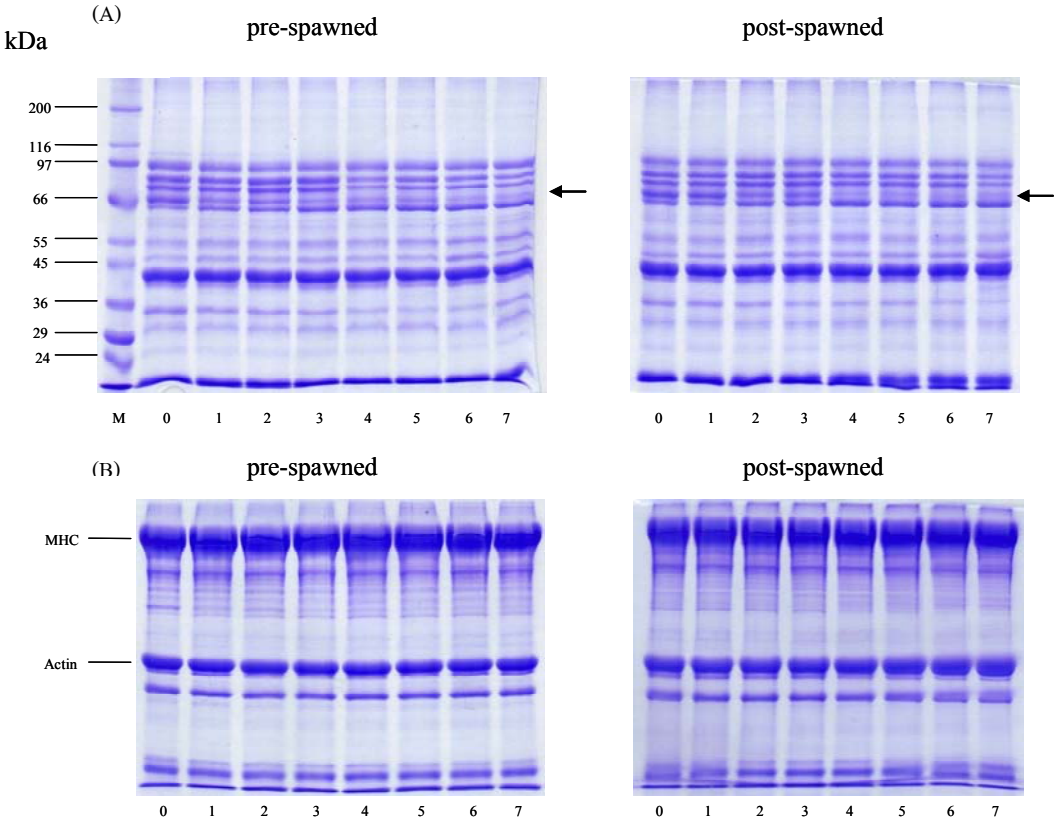

Figure 5. SDS-PAGE patterns of water soluble fraction (A) and water insoluble fraction (B) of pre- and post-spawned freshwater prawn during iced storage. Numbers designate the storage time (days). M: molecular weight marker. Arrow indicates 66-kDa protein band.

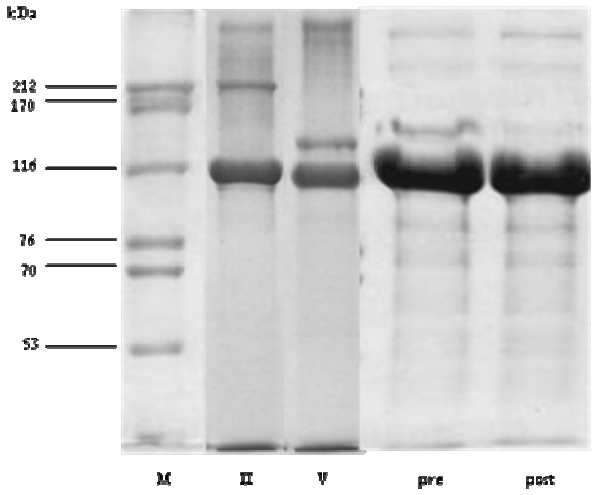

Figure 6. SDS-PAGE patterns of pepsin soluble collagen (PSC) of pre- and post-spawned freshwater prawn. M: molecular weight marker. II and V: porcine cartilage collagen type II and V, respectively. pre and post: PSC from pre-spawned prawn and PSC from post-spawned prawn, respectively.

3.4.2 Physical and microstructural changes of pre- and post-spawned prawn meat during iced storage

3.4.2.1 Changes in thermal transition of PSC

Thermal transitions of PSC extracted from the meat of pre- and post-spawned prawns stored in ice at day 0, 4 and 7 were determined using DSC (Table 9). T_{max} of PSC at day 0, 4 and 7 were 43.93, 40.82 and 40.35 °C for pre-spawned sample and 44.69, 41.05 and 40.64 °C for post-spawned sample, respectively. The decreases in enthalpy were found during the extended iced storage. Enthalpy of PSC from pre-spawned prawn and post-spawned prawn stored at day 0, 4 and 7 were 1.94, 1.42, 1.17 and 2.40, 1.37, 0.93 J/g, respectively. DSC analysis was used to determine the thermal transition or unfolding temperature of protein and also to quantify the enthalpy of conformational transition (John and Shastri, 1998). T_{max} and enthalpy of PSC from fresh pre-spawned sample (43.93 °C, 1.94 J/g) was lower than those of post-spawned sample (44.69 °C, 2.40 J/g). However, no difference in T_{max} and enthalpy between both prawns were observed at day 4 and 7 of storage ($p>0.05$), suggesting that collagen of both prawns required the similar temperature and energy for thermal denaturation. The decrease in T_{max} and enthalpy of both prawns was concomitant with the increase in PSC content (Table 8) and heat soluble collagen content (Figure 4B). The decreased T_{max} and enthalpy of both prawn during storage might be the result of degradation of collagen fibers, induced by proteolytic enzymes during storage. The

structures of endomysium and perimysium in the ice-chilled prawn (*Macrobrachium rosenbergii*) degraded gradually as the storage time increased, resulting in the mushy texture of prawn meat (Nip and Moy, 1988). Therefore, prawn meat was more prone to thermal denaturation as the storage time in ice increased.

Table 9. T_{max} and enthalpy of PSC of pre- and post-spawned prawn during iced storage

Storage time (day)	Pre-spawned prawn		Post-spawned prawn	
	T_{max} (°C)	ΔH (j/g)	T_{max} (°C)	ΔH (j/g)
0	43.93 ± 0.18^{aB}	1.94 ± 0.29^{aB}	44.69 ± 0.21^{aA}	2.40 ± 0.01^{aA}
4	40.82 ± 0.15^{bA}	1.42 ± 0.13^{bA}	41.05 ± 0.63^{bA}	1.37 ± 0.30^{bA}
7	40.35 ± 0.95^{bA}	1.17 ± 0.05^{bA}	40.64 ± 0.58^{bA}	0.93 ± 0.19^{bB}

Values are given as mean ±SD from triplicate determinations. Different superscripts in the same column indicate significant differences ($p < 0.05$). Different capital superscripts in the same row within the same parameter indicate the significant differences ($p < 0.05$).

3.4.2.2 Changes in shear force

Shear forces of raw and cooked meats from pre and post-spawned prawns are illustrated in Figure 7A and 7B, respectively. Generally, shear force of raw pre- and post-spawned meat gradually decreased during the first 4 days of iced storage and ($p < 0.05$) (Figure 7A). Thereafter, no changes in shear force were obtained up to the end of storage ($p > 0.05$). The higher shear force was observed in raw post-spawned samples, compared with that of pre-spawned samples throughout 7 days of storage ($p < 0.05$). The result suggested that shear force of prawn meat was governed by spawning stage. Due to the higher ISC content of post-spawned prawn, particularly in endomysium or epimysium, this cross linked collagen could strengthen the muscle fibers when force was applied. The degradation of prawn tissue caused by hepatopancreatic enzymes started from the perimysium, endomysium, the Z line and the H zones with concurrent degradation of the connective fibers and the sarcoplasm (Nip *et al.*, 1985). Additionally, it was most likely that the destruction of muscle fibers of pre- and post-spawned occurred continuously during the storage of 7 days ($p < 0.05$). Similar rate of decrease in shear force was noticeable between both prawns. Decrease in shear force was in agreement with higher TCA-soluble peptide contents (Figure 4A), higher PSC and lower ISC contents (Table 8). It was noted that the continuous decrease in shear force was observed within the first 4 days, while only slight increase in TCA soluble peptide content was detected. The degradation of muscle proteins might occur during the first 4 days of storage

but small peptides or free amino acid were produced at the low levels, which could not be detected in term of TCA-soluble peptides.

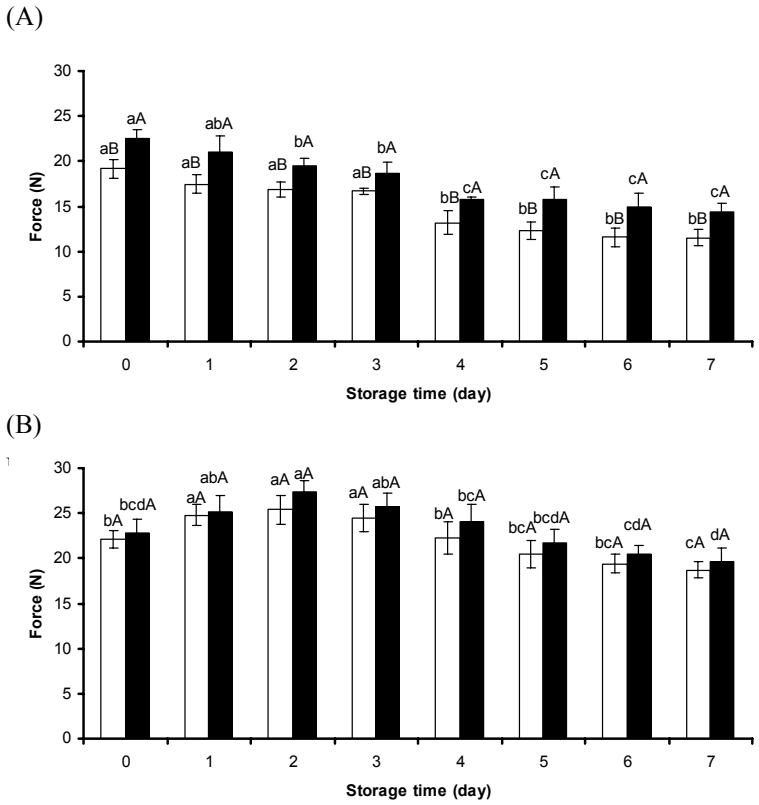

Figure 7. Changes in shear force of raw (A) and cooked (B) samples from pre- and post-spawned freshwater prawn during iced storage. Bars represent the standard deviation (n=3). The different letters on the bars within the same prawn indicate significant differences ($p < 0.05$). Different capital letters on the bars within the same storage time indicate the significant differences ($p < 0.05$). □; pre-spawned, ■; post-spawned.

For cooked meats, shear force of both prawn increased slightly during the first 3 days of storage. Thereafter, the gradual decrease in shear force was observed as the storage time increased ($p < 0.05$). The slight increases in shear force of cooked prawn during the first 3 days of storage in ice might be associated with the shrinkage of muscle fibers after heating. Collagens, which became more labile to heat treatment as indicated by lower T_{max} and enthalpy, could be more solubilized, favoring the shrinkage of muscle fibers. This resulted in the increased compactness of muscle and muscle bundles were more resistant to shearing. On the other hand, the decrease in shear force of both samples kept in ice for a long time might be caused by the destruction of muscle fibers caused by the intensive denaturation and degradation of connective tissues as indicated by the decrease in T_{max} and enthalpy of PSC

and the increase in heat soluble collagen content (Figure 4B) of both prawns during storage. In general, no differences in shear force were noticeable between both prawns throughout the storage of 7 days ($p > 0.05$). During cooking, the precipitation of muscle proteins, both myofibrillar and sarcoplasmic proteins, might occur at the same extent. This contributed to the similar shear force of cooked samples.

3.4.2.3 Changes in microstructure

Histological changes of raw and cooked meat of pre- and post-spawned prawns are presented in Figure 8A and 8B, respectively. At day 0, raw pre- and post-spawned prawns (Figure 8A) had the thin layers of connective tissues, both endomysium and perimysium, which connected well to the muscle fibers. Endomysium and perimysium were slightly separated from the muscle fibers at day 4 of storage. For both spawning stages, the obviously increased spacing or gaping between the muscle fibers of raw prawn was noticeable at day 7 of storage. This was in accordance with Nip and Moy (1988) who reported that ice-chilled whole freshwater prawn had a gradual disintegration of collagenous structures; perimysium and endomysium, leading to separation of the muscle fibers. Mizuta *et al.* (1997) also reported that muscle collagen of kuruma prawn disintegrates with increasing storage time. The damaged collagen in conjunctive tissue was more likely due to collagenolytic activities in prawn muscle during iced storage. This was in agreement with the decreased ISC with the increased PSC content in prawn stored in ice for an extended time (Table 8). The looser structure and disruption of muscle fibers found in samples stored in ice for a longer time were coincidental with the lower shear force of both prawns (Figure 7). Similar microstructures of raw meats between pre- and post-spawned prawns stored in ice for up to 4 days were found. However, the gaping or poorer intercellular connection was more pronounced in pre-spawned prawn after 7 days of storage in ice. Disintegration of the pericellular connective tissue of ayu muscle was also histologically observed in the spawning stage (Ito *et al.*, 1992).

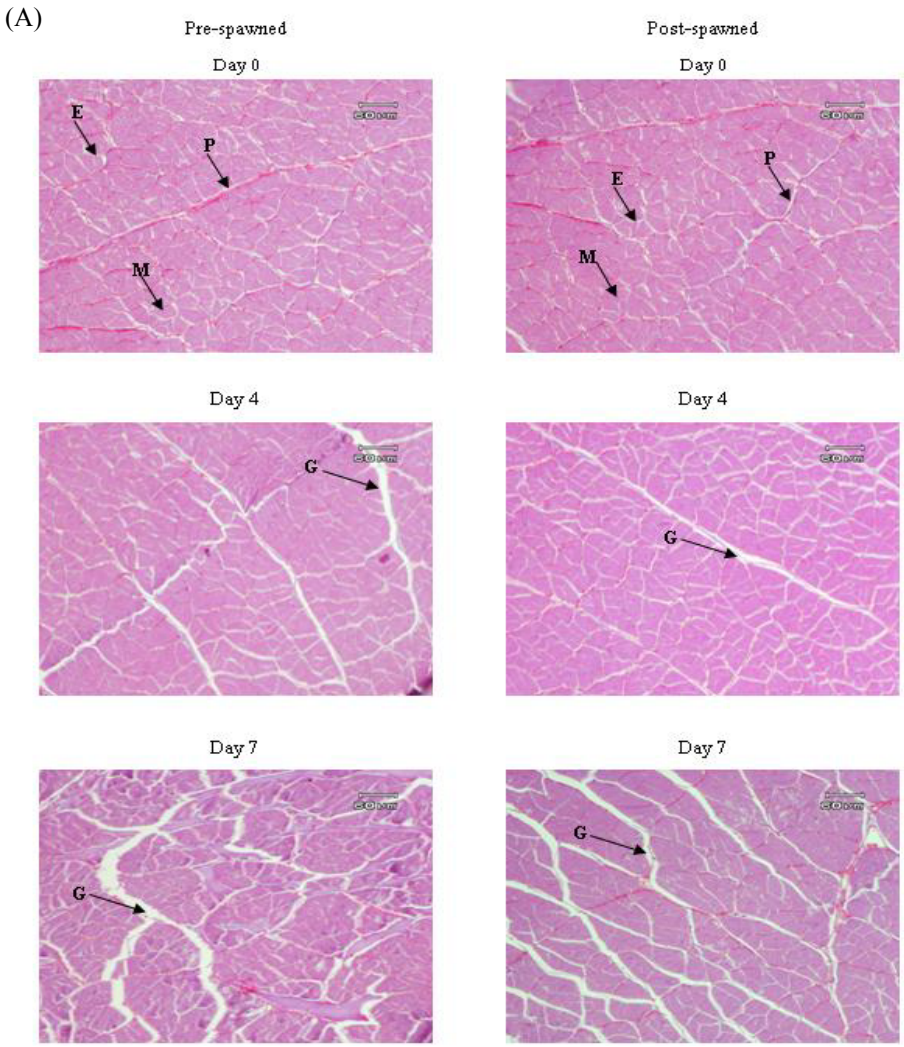

Figure 8. Histological images of raw (A) and cooked (B) meats from pre- and post-spawned freshwater prawn with different iced storage time. M: myofibrillar protein; P: perimysium; E: endomysium; G: gaping.

(B)

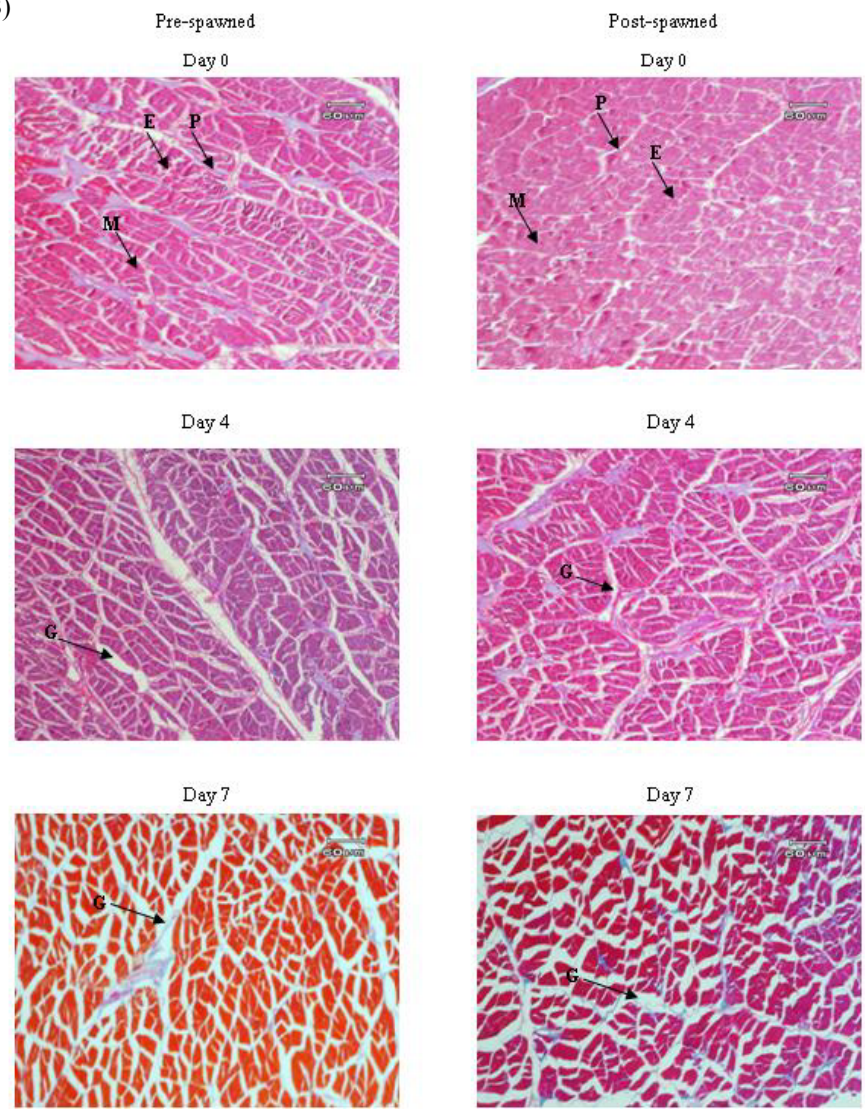

Figure 8. Histological images of raw (A) and cooked (B) meats from pre- and post-spawned freshwater prawn with different iced storage time. M: myofibrillar protein; P: perimysium; E: endomysium; G: gaping. (continued)

Cooked meats from pre- and post-spawned prawn had more compact muscle fiber arrangements with the larger gap (Figure 8B), compared with raw samples (Figure 8A). When the proteins underwent the thermal denaturation, the water was less imbibed or bound in their structure. The release of water from protein molecules might facilitate the muscle fiber to align closely, leading to the more compact structure. The compact fibers might be associated with the increased shear force values of cooked prawn meats during the first 3 days of iced storage (Figure 7B). However, the gaping between muscle fibers of cooked samples with increasing storage time was more pronounced in both prawns (Figure 8B). The

gaps might occur due to the degradation of collagen, especially in endomysium and perimysium, caused by proteolytic and collagenolytic activities. Those collagens which were more heat labile could be solubilized by heat to a higher extent, especially when storage time increased. The increased gaping was coincidental with the lowered shear force of sample kept in ice for 7 days (Figure 7). At the same storage time, no marked differences in microstructure of cooked meats between both prawns were observed. Nevertheless, cooked meat from post-spawned prawn tended to be denser in microstructure as manifested by the less gaping of muscle bundle, especially at day 0 of storage. Benjakul *et al.* (2008) found the denser structure of black tiger prawn and Pacific white prawn after cooking.

3.4.3 Sensorial changes of pre- and post-spawned prawn meat during iced storage

The similar texture and overall likeness between cooked pre- and post-spawned prawn meat were observed during 7 days of iced storage (Figure 9). For both attributes, the slight decreases were obtained in both prawns after 4 days of storage ($p < 0.05$) (Figure 9). The decrease in texture likeness of prawn stored for 7 days in ice was in agreement with the destruction of muscle fibers as evidenced by the increased gaping (Figure 8). Decreased texture likeness correlated well with the decreased shear force (Figure 7). Therefore, it was most likely that the lowered acceptability of both prawns mainly associated with the destruction of collagen in the connective tissue mediated by collagenolytic activities, which contributed to the softening of texture. Proteolysis of muscle proteins by proteolytic and/or collagenolytic enzymes was postulated as the main mechanism which contributes to postmortem mushiness in freshwater prawn (Nip and Moy, 1988). The result indicated that the extended iced storage was related with the loss in sensory property of freshwater prawn.

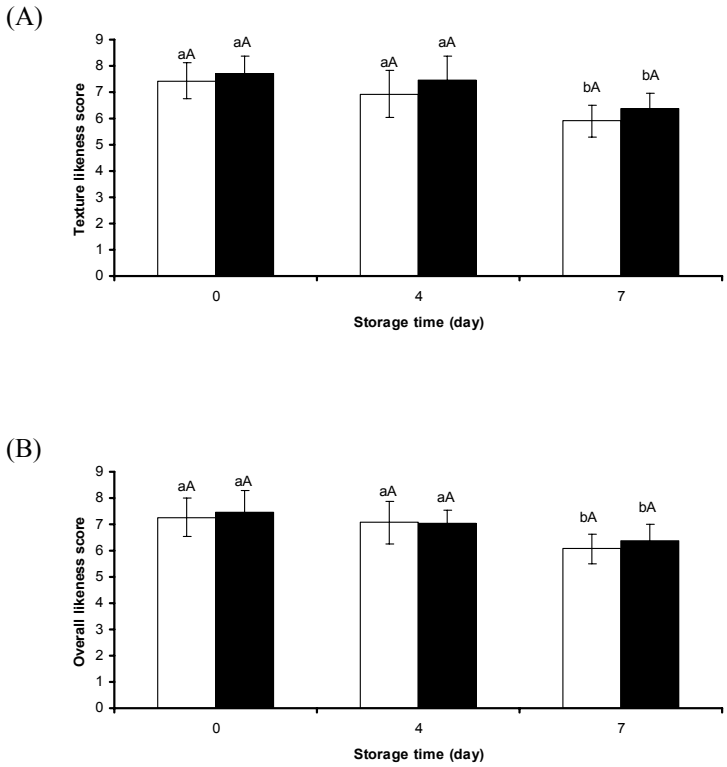

Figure 9. Changes in texture (A) and overall (B) likeness scores of cooked sample from pre- and post-spawned freshwater prawn during iced storage. Bars represent the standard deviation from thirty determinations. The different letters on the bars within the same prawn indicate significant differences ($p < 0.05$). Different capital letters on the bars within the same storage time indicate the significant differences ($p < 0.05$). □; pre-spawned, ■; post-spawned.

3.5 Conclusions

Both pre- and post-spawned freshwater prawn became softened during the extended iced storage. This was governed by the degradation and denaturation of collagen in endomysium and epimysium. This could cause the pronounced collapse of muscle structure, especially after heating. Spawning stage had the effect on heat soluble collagen, especially when the iced storage time increased. Conversion rate of ISC to PSC and heat soluble collagen contents were influenced by spawning stage. However, spawning stage showed no profound impact on the texture of prawn meat. Therefore, keeping freshwater prawns in ice for up to 3 days was recommended to lower the softening associated with the loss in textural quality.

CHAPTER 4

Characterization of proteolytic enzymes from muscle and hepatopancreas of freshwater prawn (*Macrobrachium rosenbergii*)

4.1 Abstract

Proteolytic enzymes in the crude extract (CE) from muscle and hepatopancreas of freshwater prawn (*Macrobrachium rosenbergii*) were characterized. CE from muscle exhibited the highest hydrolytic activities toward hemoglobin at pH 5 and 50 °C, while that from hepatopancreas had the highest activity on casein at pH 7 and 60 °C. Based on inhibitor study, cysteine protease and serine protease were dominant in CE from muscle and hepatopancreas, respectively. CE from muscle rarely hydrolyzed natural actomyosin (NAM) and could not degrade pepsin soluble collagen (PSC). Conversely, NAM and PSC were susceptible to hydrolysis by CE from hepatopancreas as evidenced by the marked decreases in band intensity. Activity staining using hemoglobin, casein and gelatin as substrates revealed that no proteolytic and gelatinolytic activities were observed in CE from prawn muscle, while CE from hepatopancreas exhibited the pronounced hydrolytic activities towards all substrates. CE from muscle showed calpain and cathepsin L activities but CE from hepatopancreas mainly exhibited the tryptic and chymotryptic activities. Serine proteases, mainly trypsin-like or chymotrypsin-like, from hepatopancreas were more likely responsible for the softening of prawn meat during postmortem storage via the degradation of both muscle and connective tissues.

4.2 Introduction

Freshwater prawn (*Macrobrachium rosenbergii*) has been served for useful nutrients along with its delicacy. However, freshwater prawn production in Thailand is still limited, mainly due to the rapid loss in textural quality known as softening or mushy texture. The softening in texture of fish and shellfish muscle is considered to be associated with the two major mechanisms including the degradation of myofibrillar protein and the breakdown of collagen (Ashie *et al.*, 1996). This deterioration is usually influenced by the proteolytic

enzymes during post-mortem storage which accordingly limits a shelf-life up to about 1 week or less (Lindner *et al.*, 1988).

Fish muscle softening has been reported to be caused by several proteases e.g. calpain in rainbow trout, *Oncorhynchus mykiss*, muscle (Salem *et al.*, 2004), cathepsin L in arrowtooth flounder, *Atheresthes stomias*, muscle (Visessanguan *et al.*, 2003), serine protease in red sea bream, *Pagrus major*, skeletal muscle (Wu *et al.*, 2010). Shrimp hepatopancreas was reported to have high proteolytic activities (Oh *et al.*, 2000) and most digestive enzymes in crustaceans were serine protease and metalloprotease (Garcia-Carreño *et al.*, 1994). Hepatopancreas from white shrimp (*Penaeus vannamei*) (Ezquerra *et al.*, 1997) and Northern shrimp (*Pandalus eous*) (Aoki *et al.*, 2003) possessed collagenolytic/gelatinolytic activities.

For freshwater prawn, a larger pancreas of this specie can be a crucial source of potential proteases, which may be released and contaminated into muscle during the extended post-mortem transportation and storage. However, indigenous proteases in muscle may be also involved in softening. Nevertheless, there is no information regarding proteolytic enzymes present in muscle and hepatopancreas of freshwater prawn and the hydrolytic activity of those enzymes towards muscle proteins and connective tissue. Therefore, the aims of this study were to characterize the proteases in the muscle and hepatopancreas and to investigate their hydrolytic activity toward muscle protein and collagen from freshwater prawn.

4.3 Materials and Methods

4.3.1 Chemicals

Hemoglobin, casein, tyrosine, α-*N*-benzoyl-DL-arginine-*ρ*-nitroanilide (DL-BAPNA), α-*N*-*ρ*-tosyl-*L*-arginine methyl ester (*L*-TAME), 1-(*L*-trans-epoxysuccinyleucylamino)-4-guanidinobutane (E-64), soybean trypsin inhibitor (SBTI), *N*-*ρ*-tosyl-L-lysine chloromethylketone (TLCK), *N*-tosyl-*L*-phenylalanine chloromethylketone (TPCK), pepstatin A, ethylenediaminetetraacetic acid (EDTA) and Tris (hydroxymethyl) aminomethane (Tris) were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), Coomassie Brilliant Blue R-250 and *N,N,N′,N′*-tetramethylethylene diamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). N-succinyl-Leu-Tyr-7-amido-4-methylcoumarin, Z-Arg-Arg-7-amido-4-methylcoumarin, Z-Phe-Arg-7-amido-4-methylcoumarin, L-Arginine-7-amido-4-methylcoumarin, dithiothreitol (DTT), *N*-succinyl-Ala-Ala-Phe-*ρ*-nitroanilide (SAAPNA) and *N*-Benzoyl-L-tyrosine ethyl ester

(BTEE) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals were of analytical grade.

4.3.2 Sample collection and preparation

Freshwater prawns (*M. rosenbergii*) with the size of 30-35 prawn/kg were obtained from a farm in Phatthalung province, Thailand. After capture, the prawns were ice-shocked, placed in ice with a prawn/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla, Thailand, within approximately 2 h. Upon arrival, the prawns were washed with clean water. Muscle and hepatopancreas were removed manually and powderised with liquid nitrogen using a blender (type AY46, Moulinex, Group SEB Thailand Ltd., Bangkok, Thailand). The powder was packed in polyethylene bag and stored at -20 °C until use. The samples were stored not longer than 1 month at -20 °C.

4.3.3 Preparation of crude extract from muscle and hepatopancreas of freshwater prawn

Muscle and hepatopancreas powder (50 g) was mixed with 100 mL of 0.01 M sodium phosphate buffer (pH 7.6) (Brauer *et al.*, 2003). The mixture was homogenized for 2 min using an IKA Labortechnik homogenizer (Selangor, Malaysia) at a speed of 11,000 rpm. During homogenization, the beaker containing the mixture was placed in an ice-water bath to maintain the temperature at 0-4 °C. The homogenate was stirred for 30 min at 4 °C, followed by centrifugation at 10,000 *x g* for 30 min using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Palo Alto, CA, USA). All procedures were carried out at 4 °C. The supernatant was referred to as 'crude extract; CE' and used for the assays of proteolytic activity.

4.3.4 Characterization of proteolytic enzymes in CE from muscle and hepatopancreas of freshwater prawn

4.3.4.1 pH and temperature profiles

Proteolytic activity of CE from muscle and hepatopancreas of freshwater prawn was determined over the pH ranges of 2-11 in the presence of 10 mM $CaCl_2$. The buffers used were McIlvaine's buffer (0.2 M Na-phosphate and 0.1 M Na-citrate) for pH 2-8 and glycine-NaOH buffer (50 mM) for pH 9-11. To initiate the reaction, CE (200 µL) was added into the assay mixtures containing 2 mg of hemoglobin (for pH 2-6) or casein (for pH

7-11), 200 µL of distilled water and 625 µL of reaction buffer. The mixture was incubated at 50 °C for 15 min. The enzymatic reactions were then terminated by adding 200 µL of cold 50% (w/v) trichloroacetic acid (TCA). Unhydrolyzed protein substrate was allowed to precipitate for 1 h at 4 °C, followed by centrifuging at 5000 *x g* for 10 min. The oligopeptide content in the supernatant was determined by the Lowry assay (Lowry *et al.*, 1951) using tyrosine as a standard. One unit of activity was defined as that releasing 1 µmole of tyrosine/min (µmol Tyr/min). Specific activity was expressed as units/mg protein. A blank was run in the same manner, except that CE was added after the addition of 50% TCA. To study the temperature profile, the activity was determined at various temperatures (20, 30, 40, 50, 60, 70 and 80 °C) at pH 5 for CE from muscle and at pH 7 for CE from hepatopancreas using hemoglobin and casein as the substrate, respectively.

4.3.4.2 Inhibitor study

Effects of different protease inhibitors toward the proteolytic activity of CE from muscle and hepatopancreas were determined as described by Klomklao *et al*. (2007). CE from muscle and hepatopancreas were incubated with an equal volume of protease inhibitor solutions to obtain the designated final concentrations (1 mM pepstatin A, 1 mM E-64, 10 mM EGTA, 10 mM EDTA, 5 mM SBTI, 5 mM PMSF, 10 mM TLCK and 10 mM TPCK). The mixture was incubated at room temperature (25-28 °C) for 15 min. The remaining proteolytic activity was determined at pH 5 and 50 °C (for CE from muscle) and at pH 7 and 60 °C (for CE from hepatopancreas) for 15 min. The percent inhibition was then calculated. The control was conducted in the same manner except that deionized water was used instead of inhibitors.

4.3.4.3 Hydrolysis of natural actomyosin (NAM) and pepsin soluble collagen (PSC)

CE (200 µL) of muscle and hepatopancreas, containing 0.01 unit protease activity determined by using hemoglobin and casein as substrates, respectively, was added to the pre-incubated reaction mixture containing 4 mg protein substrate (NAM or PSC) and 825 µL of 50 mM sodium acetate buffer pH 5.0 for CE from muscle and 50 mM Tris-HCl, buffer pH 7.0 for CE from hepatopancreas. The hydrolysis was conducted by incubating the mixture at 50 °C (for CE from muscle) and 60 °C (for CE from hepatopancreas) using NAM as substrate and at 25 °C using PSC as substrate for 0, 5, 10, 15, 30, 60 and 120 min. The control was performed in the same manner for 120 min except that distilled water was added

instead of CE. The reaction was terminated by adding the preheated solution containing 2% SDS, 8 M urea and 2% βME (85 °C). The mixture was further incubated at 85 °C for 60 min to solubilise total proteins. The solution was centrifuged at 5000 *x g* for 10 min at room temperature to remove the debris. The supernatant obtained (15 µg protein) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

4.3.4.4 Activity staining of CE from muscle and hepatopancreas

CE from muscle and hepatopancreas were separated on SDS-PAGE (Laemmli, 1970), followed by activity staining according to the method of Klomklao *et al.* (2004). CE from both muscle and hepatopancreas were mixed with sample buffer (0.125 M Tris–HCl, pH 6.8, containing 4% SDS and 20% (w/v) glycerol) at a ratio of 1:1 (v/v). Ten µg of proteins were loaded onto the gel made of 4% stacking and 12% separating gels. The electrophoresis was run at a constant current of 15 mA per gel by a Mini-Protein II Cell apparatus (Bio-Rad, Hercules, CA, USA). After electrophoresis, the gels were washed twice in 100 mL of 2.5% Triton X-100 for 15 min. The washed gel was then immersed in 100 mL of 50 mM Tris-HCl buffer, pH 7.5 containing 2% hemoglobin (a substrate for CE from muscle) and 2% casein (a substrate for CE from hepatopancreas) for 1 h with constant agitation at 0 °C to allow the substrate to penetrate into the gels. Thereafter, the gels were transferred to 2% hemoglobin in 0.5 M sodium acetate buffer (pH 5) or 2% casein (w/v) in 50 mM Tris-HCl buffer (pH 7) for CE from muscle and hepatopancreas, respectively. The mixtures were incubated at optimal temperature for 2 h with constant agitation. The gels were rinsed using cold distilled water for 15 min (two times). The gels were then fixed and stained with 0.125% Coomassie Blue R-250 in 50% methanol and 10% acetic acid and destained in 30% methanol and 10% acetic acid. Development of clear zones on blue background indicated proteolytic activity.

Activity staining toward gelatin, was performed according to the method described by Lødemel and Olsen (2003) with a slight modification. CE from muscle and hepatopancreas were mixed with same sample buffer as previously described. The separating gel contained 0.1% gelatin. The electrophoresis was run under the same condition as described above. After electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 15 min. Gels were incubated in 0.5 M sodium acetate (pH 5.0) for CE from muscle and 50 mM Tris-HCl (pH 7.0) for CE from hepatopancreas at 37°C for 6 h. All buffers used consisted of 10 mM $CaCl_2$ and 0.02% NaN_3. The gels were rinsed using cold distilled water

prior to staining as described above. The clear zones representing gelatinolytic activity were developed as previously described.

4.3.5 Proteolytic activities of CE from muscle and hepatopancreas using different synthetic substrates

For all assays determined, the assay buffers used were 0.5 M sodium acetate buffer (pH 5) for CE from muscle and 50 mM Tris-HCl buffer (pH 7) for CE from hepatopancreas.

4.3.5.1 Calpain activity

Total calpain activity was determined following the method of Sultan *et al.* (2000) with slight modifications. Twenty five μL of CE from muscle and hepatopancreas were added with 175 μL of substrate solution. Substrate solutions included 0.25 mM Suc-Leu-Tyr-AMC, 5 mM $CaCl_2$ and 1 mM DTT dissolved in assay buffers. The mixture was incubated at 37 ^{o}C for 15 min. The reaction was stopped by adding 200 μL of 0.2 M EDTA in 50 mM Tris-HCl (pH 9.0). Fluorescence intensity of 7-amino-4-methycoumarin (AMC) liberated in the supernatant was determined using a spectrofluorophotometer RF-1501 **(**Shimadzu**,** Kyoto**,** Japan**)** at an excitation wavelength of 355 nm and an emission wavelength of 480 nm. A blank was run in the same manner except that CE was added after the addition of stopping solution. One unit of calpain activity was defined as the amount causing an increase in fluorescence intensity per min. Calpain activity was expressed as fluorescence units/g sample (Cheret *et al.*, 2005).

4.3.5.2 Cathepsin B, L and H activities

Cathepsin activity of CE from muscle and hepatopancreas of freshwater prawn was determined using various synthetic substrates, including Z-Arg-Arg-AMC, Z-Phe-Arg-AMC and L-Arginine-AMC specific for cathepsin B, L and H, respectively, according to the method of Rifkin *et al.* (1991) with a slight modification. The reaction mixture contained 0.8 mL of assay buffer and 0.1 mL of 1 mM substrate. To initiate reaction, 0.1 mL of CE diluted properly with 0.1% Brij 35 was added into reaction mixture, and incubated at 37 ºC for 15 min. The reaction was terminated by adding 1.5 mL of the stopping reagent (methanol:n-butanol:deionized water = 35:30:35 (v/v/v)), followed by heating at 95 °C for 3 min. The fluorescence intensity of the liberated 7-amino-4-methylcoumarin (AMC) was measured by a spectrofluorophotometer at an excitation wavelength of 355 nm and an emission wavelength of 480 nm. One unit of cathepsin activity was defined as the amount causing an increase in

fluorescence intensity per min. Cathepsin activity was expressed as units/g sample (Cheret *et al.*, 2005).

4.3.5.3 Trypsin activity

Trypsin activity of CE from muscle and hepatopancreas was measured using BAPNA and TAME as substrates according to the methods of Hau and Benjakul (2006) and Hummel (1959), respectively. When BAPNA was used as a substrate, the diluted CE (200 µL) was added to 200 µL of distilled water and 1000 µL of assay buffers containing 10 mM $CaCl_2$. To initiate the reaction, 200 µL of BAPNA (2%) was added and mixed thoroughly. After incubation at 25 °C for 15 min, 200 µL of 30% acetic acid was added to terminate the reaction. Production of *ρ*-nitroaniline was measured by monitoring the absorbance of reaction mixture at 410 nm (A_{410}). Blank was run in the same manner except that BAPNA was added after the addition of acetic acid. Trypsin amidase activity was then calculated using the following formula:

$$\text{Trypsin activity (unit/mL)} = \frac{(A - A_0) \times \text{mixture volume (mL)} \times 1000}{8800 \times \text{reaction time (min)} \times 0.2}$$

where 8800 $cm^{-1}M^{-1}$ is the extinction coefficient of ρ-nitroaniline; A and A_0 are A_{410} of the sample and the blank, respectively. One unit of activity was defined as that releasing 1 mmol of *ρ*-nitroaniline per min.

When TAME was used as a substrate, CE with an appropriate dilution (20 µL) was mixed with 3.0 mL of 1 mM TAME in assay buffers containing 10 mM $CaCl_2$. The reaction was conducted at 25 °C for 15 min. Production of *ρ*-tosyl-arginine was measured by monitoring the increase in absorbance at 247 nm. Blank was prepared in the same manner, but reaction buffer was used instead of TAME solution. One unit of trypsin esterase activity was defined as the amount causing an increase of 1.0 in absorbance per min (Hummel, 1959). Both amidase and esterase activities were reported as units/g sample.

4.3.5.4 Chymotrypsin activity

Chymotrypsin activity of CE from muscle and hepatopancreas was evaluated using SAAPNA and BTEE as substrates according to Hummel (1959) with slight modifications. The diluted sample (20 µL) was added to 3.0 mL of 1 mM SAAPNA in assay buffers containing 10 mM $CaCl_2$. The reaction was conducted at 25 °C for 15 min. Production of *ρ*-nitroaniline was measured by monitoring the increase in absorbance at 410 nm (A_{410}). Blank was run in the same manner except that assay buffer was used instead of

SAAPNA solution. One unit of chymotrypsin amidase activity was defined as the amount causing an increase of 1.0 in absorbance at 410 nm per min.

Esterase activity was evaluated according to Hummel (1959) using BTEE as a substrate. CE with an appropriate dilution (20 μL) was mixed with 3.0 mL of 1 mM BTEE in assay buffer containing 10 mM $CaCl_2$. The reaction was conducted at 25 °C for 15 min. Production of benzoyl-tyrosine was measured by monitoring the increase in absorbance at 256 nm. Blank was prepared in the same manner, but assay buffer was used instead of BTEE solution. One unit of chymotrypsin esterase activity was defined as the amount causing an increase of 1.0 in absorbance at 256 nm per min. Both amidase and esterase activities were reported as units/g sample.

4.3.6 Statistical analysis

The experiments were run in triplicate with three different lots of samples. Data were subjected to analysis of variance and mean comparison was carried out using Duncan's multiple range test (DMRT) (Steel and Torrie, 1980). Statistical analyses were performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

4.4 Results and Discussion

4.4.1 Characteristics of proteolytic enzyme in CE from muscle and hepatopancreas of freshwater prawn

4.4.1.1 pH and temperature profiles

The pH profiles of CE from muscle and hepatopancreas of freshwater prawn are shown in Figure 10. CE from muscle exhibited a maximal proteolytic activity at pH 5 ($p<0.05$) (Figure 10A). The optimal pH for proteolytic enzyme in CE from muscle (Figure 10A) was slightly different from those of muscle protease (calpain) from grass shrimp, *Penaeus monodon* (pH 6.9) and tilapia, *Tilapia nilotica* (pH 7.5) (Wang *et al.*, 1993). Many proteases in muscle have the maximal activity in acidic pH ranges, however, some are most active at neutral pH (Haard *et al.*, 1994).

Maximal activity of proteolytic enzyme in CE from hepatopancreas was found at pH 7.0 ($p<0.05$) (Figure 10A). This result was similar to that of digestive proteases from mid gut glands of *Penaeus indicus* and lobster, *Nephrops norvegicus*, which had the optimal

pH at 7.7 and 8, respectively (Omondi and Stark, 2001). Digestive protease from mid gut gland of *Farfantepenaeus paulensis* showed the maximal activity at pH of 8 (Buarque *et al.*, 2009a). It was noted that the optimal pH (pH 7) of CE from freshwater prawn was close to physiological pH of prawn meat (6.5-6.8) (data not shown). This could be implied that proteases from hepatopancreas might be active during postmortem storage. For both CE from muscle and hepatopancreas, the marked decrease in activity was obtained at very acidic or alkaline pHs. The loss in activity of proteolytic enzymes at the harsh pH might be due to the denaturation of enzymes, mainly caused by the conformation change induced by repulsive force.

The effect of various temperatures on proteolytic activity of CE from muscle and hepatopancreas of freshwater prawn is depicted in Figure 10B. CE from the muscle of freshwater prawn exhibited the highest proteolytic activity at 50 °C ($p< 0.05$) (Figure 10B). The optimal temperature of muscle protease was similar to that of muscle protease from banded shrimp, *Penaeus japonicus* (50 °C) (Jiang *et al.*, 1992).

For the optimal temperature of proteolytic enzymes in CE from hepatopancreas, the highest proteolytic activity was observed at 60 °C ($p<0.05$) (Figure 10B). This result was different from that of crude protease from pink shrimp and shrimp (*Penaeus orientalis*), which had optimal temperatures of 70 and 45 °C, respectively (Buarque *et al.*, 2009b; Oh *et al.*, 2000). The differences in pH and temperature profiles between CE from muscle and hepatopancreas might be due to the differences in protease types between muscle and hepatopancreas. At very high temperature, thermal denaturation of proteolytic enzymes in CE resulted in the loss in activity.

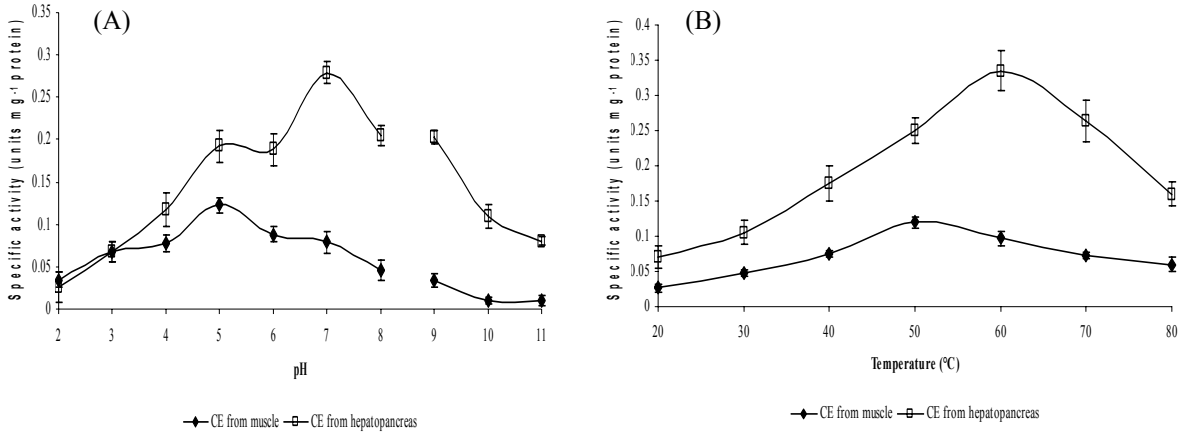

Figure 10. pH (A) and temperature (B) profiles of CE from muscle and hepatopancreas of freshwater prawn. For pH profile study, protease activity was determined by incubating reaction mixture at 50 °C at various pH values. For temperature profiles, protease activity was determined by incubating reaction mixture at pH 5 for CE from muscle and pH 7 for CE from hepatopancreas at various temperatures. Bars represent the standard deviation (n=3).

4.4.1.2 Effect of various protease inhibitors on proteolytic activity

The effect of various protease inhibitors on proteolytic activity of CE from muscle and hepatopancreas of freshwater prawn is shown in Table 10. E-64 (1 mM) showed the highest inhibition (76%) on protease activity of CE from muscle, followed by EGTA (10 mM). However, pepstatin A (1 mM) and EDTA (10 mM) exhibited a low inhibitory effect on proteolytic activity. All serine protease inhibitors (SBTI, PMSF, TLCK and TPCK) had no inhibitory effect. The inhibitory result revealed that cysteine protease was the major protease in CE from the muscle of freshwater prawn. Autolysis study of freshwater prawn muscle also confirmed the type of protease in the muscle (data not shown). Crustacean muscle including grass shrimp (*Penaeus monodon*) (Wang *et al.*, 1993) and lobster (*Homarus americanus*) (Beyette *et al.*, 1997) contained cysteine protease as the major enzyme.

Table 10. Effect of various protease inhibitors on inhibition of proteolytic activity in CE from muscle and hepatopancreas of freshwater prawn

Inhibitors	Concentration (mM)	% Inhibition	
		CE from muscle	CE from hepatopancreas
Pepstatin A	1	19.77±4.75	4.26±1.02*
E-64	1	76.56±3.11	22.08±2.44
EGTA	10	37.94±2.20	8.22±0.09
EDTA	10	13.55±3.12	8.54±2.14
SBTI	5	0	40.17±3.06
PMSF	5	0	42.10±2.46
TLCK	10	0	88.06±5.64
TPCK	10	0	52.14±4.21

CE from muscle and hepatopancreas (200 μL) was mixed with 200 μL of single protease inhibitor to obtain the designated final concentration. The residual proteolytic activity was measured using hemoglobin as substrate at pH 5 and 50 °C for CE from muscle and casein as substrate at pH 7 and 60 °C for CE from hepatopancreas.

*Values are mean ± standard deviation (n=3).

Inhibition of proteolytic activity in CE from hepatopancreas by several protease inhibitions is shown in Table 10. Among all protease inhibitors, TLCK (10 mM) exhibited the highest inhibition (88%), followed by TPCK (10 mM), PMSF (5 mM) and SBTI (5 mM), respectively. Pepstatin A and E-64 (1 mM) showed a low inhibitory effect toward proteases in CE from hepatopancreas. TLCK is the inhibitor specific for trypsin, while TPCK specifically inhibits chymotrypsin (Klomklao *et al.*, 2007). PMSF and SBTI has been known as the inhibitor specific to serine protease (Benjakul *et al.*, 2003). Pepstatin A and E-64 are the inhibitors specific for aspartic protease and cysteine protease, respectively (Klomklao *et al.*, 2004; Klomklao *et al.*, 2007). Based on the inhibitory study, serine protease was dominant in CE from hepatopancreas of freshwater prawn. The most active protease in hepatopancreas of shrimp (*Penaeus orientalis*) was identified to be trypsin-like enzyme (Oh *et al.*, 2000). Aoki *et al.* (2003) also reported that serine-collagenase was found in hepatopancreas of Northern shrimp (*Pandalus eous*). However, Lindner *et al.* (1988) reported that cysteine protease was the major enzyme and trypsin-like enzyme was the minor enzyme in the crude extract from hepatopancreas of *Macrobrachium rosebergii* in the intermolt stage. The result found in the present study was different from that of Linder *et al.* (1988). This was

probably due to the differences in types and concentration of protease inhibitors used as well as the assay condition for remaining activity.

4.4.1.3 Hydrolysis of different protein substrates by proteolytic enzyme in CE from muscle and hepatopancreas of freshwater prawn

Natural actomyosin

Protein patterns of natural actomyosin (NAM) incubated with CE from muscle and hepatopancreas for different times are shown in Figure 11A and B, respectively. NAM, extracted from freshwater prawn muscle, contained myosin heavy chain (MHC) and actin as major constituents. No degradation was observed in NAM incubated with CE from prawn muscle within the first 30 min of incubation (Figure 11A). However, the slight decrease in band intensity of MHC was observed at 60 and 120 min of hydrolysis with coincidental appearance of protein with molecular mass of 40 kDa. This result revealed that CE from muscle had very low proteolytic activity toward NAM. For the protein pattern of NAM incubated with CE from hepatopancreas, the rapid degradation of MHC was observed within 5 min of hydrolysis with the concomitant occurrence of proteins with lower molecular mass (81-94 kDa) (Figure 11B). No actin and tropomyosin were retained after hydrolysis with CE from hepatopancreas. This was in accordance with the increased band intensity of dye front. With increasing incubation time, no further changes in protein pattern were observed. Previously hydrolyzed proteins, with the peptides as the products, could not be further hydrolysis, even with the longer incubation time. Chain length and amino acid sequence have been known to govern the hydrolysis by protease. The different hydrolytic pattern of NAM incubated with CE from muscle and hepatopancreas might be caused by the different types of proteases present in both extracts. Generally, CE showed the higher proteolysis towards NAM, more likely due to the greater activity and higher amount of proteases in the extract. Aoki *et al.* (2003) reported that shrimp hepatopancreas had high proteolytic activities.

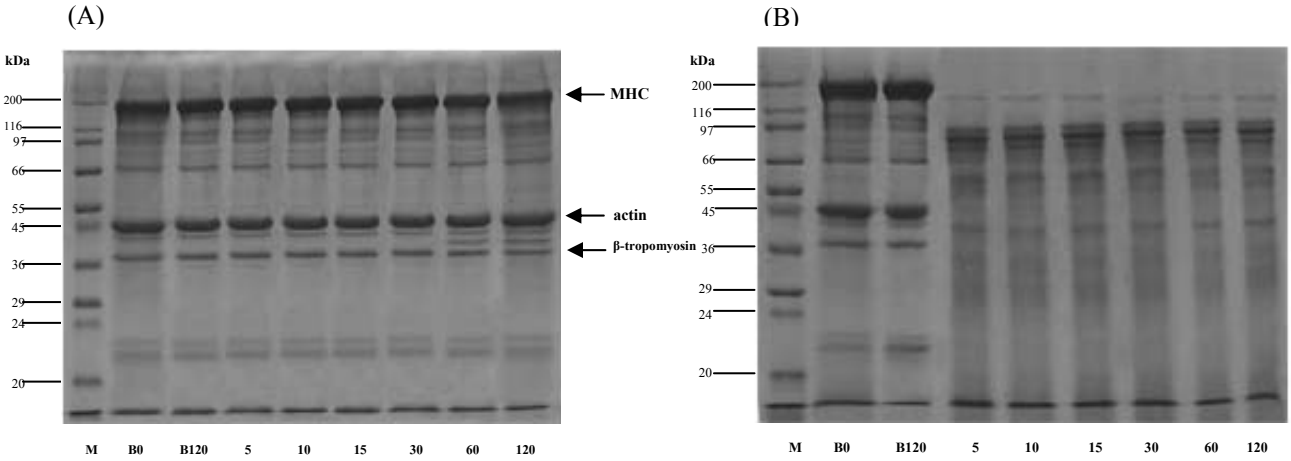

Figure 11. Protein pattern of natural actomyosin (NAM) incubated with CE from muscle (A) and hepatopancreas (B) of freshwater prawn. M, molecular weight marker; B0 and B120, NAM incubated without CE for 0 and 120 min, respectively. Numbers denoted the incubation time (min) at 50 °C for CE from muscle and 60 °C for CE from hepatopancreas.

Pepsin soluble collagen

Protein patterns of PSC incubated with CE from the muscle and hepatopancreas of freshwater prawn during the incubation at 25 °C for 0-120 min are shown in Figure 12A and B, respectively. No hydrolysis was observed in the PSC incubated with CE from the muscle during incubation of 120 min (Figure 12A). This result indicated that no collagenolytic protease was present in CE from freshwater prawn muscle. For the protein pattern of PSC incubated with CE from hepatopancreas, the marked degradation of α-chain and protein with molecular mass of 145 kDa was found after 5 min of hydrolysis with the concomitant occurrence of degradation products having the lower molecular mass (Figure 12B). The major degradation products had the molecular mass of 69 and 66 kDa. Collagen in freshwater prawn was more likely belonging to type V, where three identical α-chains aligned themselves in triple helix in structure (Sivakumar *et al.*, 1997). These results suggested that CE from the hepatopancreas had high collagenolytic activity. Endogenous serine collagenases and other proteases might be responsible for the degradation of collagenous fibrils in freshwater prawn muscle. This result was in agreement with Aoki *et al.* (2004) who found that proteases from Northern shrimp (*Pandalus eous*) were able to hydrolyze collagen. These enzymes may play a role in postmortem degradation of shellfish during storage (Bracho and Haard, 1995; Saito *et al.*, 2000).

Hepatopancreas of freshwater prawn was larger in size and contained a larger amount of proteases, which could be dispersed and contaminated in prawn meat during handling and storage. Proteases with collagenolytic activity might be able to hydrolyze the connective tissues, e.g. epimysium and endomysium etc. This might result in softening of meat of this specie during the extended post-mortem storage.

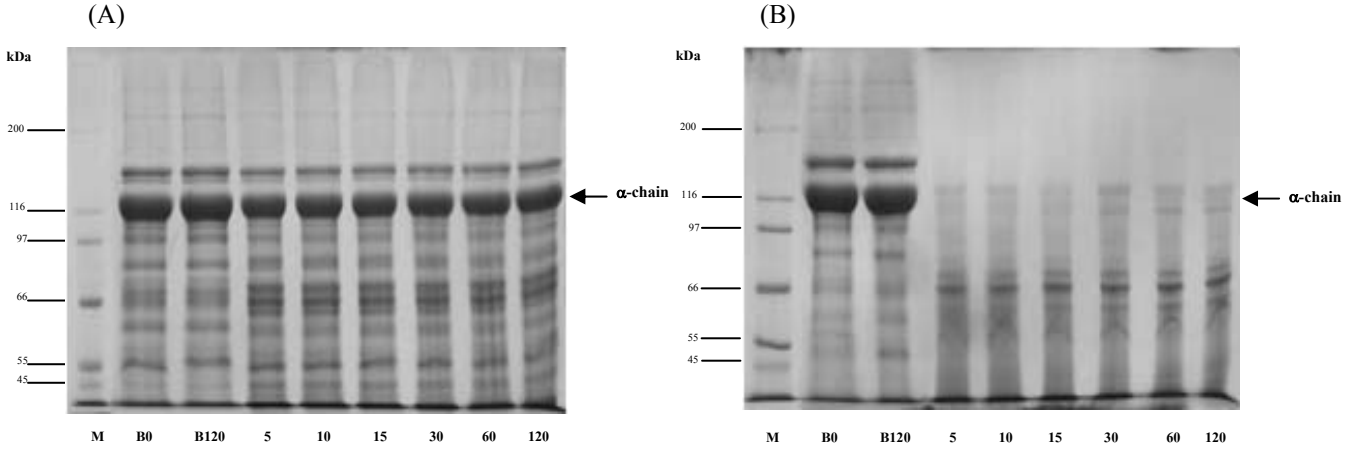

Figure 12. Protein pattern of pepsin soluble collagen (PSC) incubated with CE from muscle (A) and hepatopancreas (B) of freshwater prawn at 25 °C for different times. M, molecular weight marker; B0 and B120, PSC incubated without CE for 0 and 120 min, respectively. Numbers denoted the incubation time (min) at 25 °C.

4.4.1.4 Activity staining

Activity staining or substrate gel electrophoresis using hemoglobin, casein and gelatin as substrates of CE from muscle and hepatopancreas from freshwater prawn is shown in Figure 13A and B, respectively. CE from muscle showed no activity band for all substrates tested (Figure 13A). This result indicated that CE from muscle might possess very low proteolytic activity toward all substrates used. The result was in accordance with very low hydrolytic activities toward NAM and PSC of CE from the muscle (Figure 11A and 12A). However, CE from muscle might be denatured in the presence of SDS during electrophoresis, resulted in no activity band determined by activity staining. It was reported that the amount of gelatinolytic degrading enzymes varied from tissue to tissue (Lodemel and Olsen, 2003). Based on the result, protease from freshwater prawn muscle contained the negligible level of collagenolytic or gelatinolytic enzymes.

For activity staining of CE from hepatopancreas using three substrates, three major bands of proteolytic or gelatinolytic activities with molecular mass of 86, 41 and 17

kDa were observed (Figure 13B). This result was in agreement with the high hydrolytic activities on NAM and PSC of CE from hepatopancreas (Figure 11B and 12B). Hepatopancreas protease from Northern shrimp (*Pandalus borealis*) also showed the high hydrolytic activity on both collagen and myofibrillar protein (Aoki *et al.*, 2004). The results indicated that CE from heapatopancreas hydrolyzed myofibrillar protein (Figure 11B) collagen (Figure 12B), hemoglobin, casein and gelatin (Figure 13B) effectively. Therefore, proteases from hepatopancreas might be involved in the postmortem softening of freshwater prawn, especially with extended storage, which allowed the released proteases to penetrate into the muscle.

4.4.1.5 Identification of proteolytic enzyme in CE from muscle and hepatopancreas by using different synthetic substrates

Proteolytic activities of CE from muscle and hepatopancreas of freshwater prawn determined using different synthetic substrates are depicted in Table 11. N-succinyl-Leu-Tyr-AMC and Z-Phe-Arg-AMC, the substrates for calpain-like and cathepsin L-like proteinases, respectively, were hydrolyzed in the presence of CE from muscle of freshwater prawn (Table 11). Nevertheless, Z-Arg-Arg-AMC and L-Arginine-AMC, which are substrates for cathepsin B and cathepsin H, respectively, were not hydrolyzed by CE from muscle. For all substrates of serine protease (BAPNA, TAME for trypsin and SAAPNA, BTEE for chymotrypsin), they were not hydrolyzed by CE from the muscle. This result was in agreement with the inhibitor study, in which cysteine protease was the major protease in CE from muscle (Table 10). Calpain and cathepsin are the cysteine proteases, with cysteine in the active site. Calpain-like activity was found in crustacean muscle including red swap crayfish, *Procambarus clarkii* (Chen *et al.*, 2008) and grass prawn, *Penaeus monodon* (Wang *et al.*, 1993). Cathepsin L-like activity was also reported in many fish muscle such as Atlantic salmon (*Salmo salar* L.) (Bahuaud *et al.*, 2010) and arrowtooth flounder (*Atheresthes stomias*) (Visessanguan *et al.*, 2003). These enzymes were involved in post-mortem softening of fish muscle (Che´ret *et al.*, 2007).

For CE from hepatopancreas, proteolytic activities using various synthetic substrates are shown in Table 11. Only serine protease substrates (BAPNA and TAME synthetic substrates for trypsin and SAAPNA and BTEE synthetic substrates for chymotrypsin) were hydrolyzed (Table 11). Based on the specific synthetic substrates, trypsin-like and chymotrypsin-like proteases were dominant in CE from hepatopancreas of freshwater prawn. The greater esterase activity was observed, in comparison with amidase.

Oh *et al.* (2000) reported that shrimp (*Pandalus eous*) hepatopancreas contained serine protease and collagenolytic protease. It could be implied that serine-like protease, especially trypsin and chymotrypsin, might be responsible for the degradation of NAM and PSC (Figure 11B and 12B) of freshwater prawn muscle during postmortem storage.

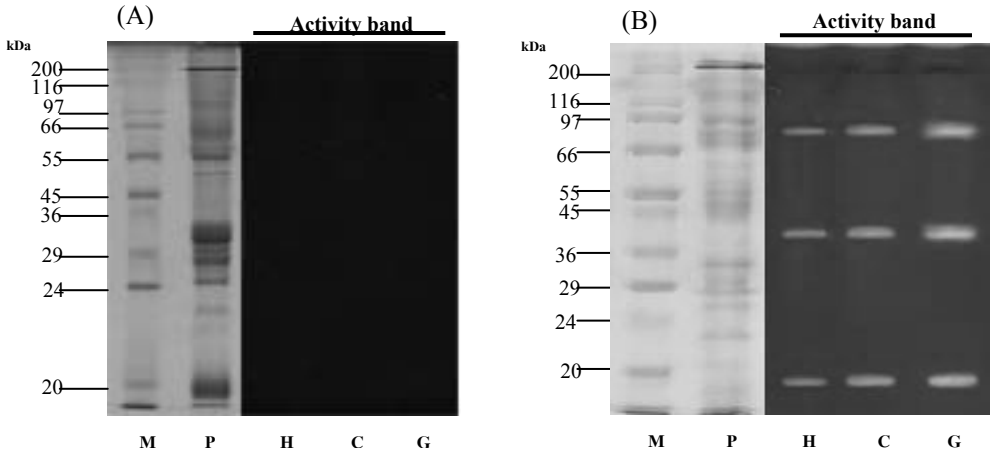

Figure 13. Protein patterns and activity staining of CE from muscle (A) and hepatopancreas (B) towards different substrates. The incubation was performed for 2 h using hemoglobin and casein as substrates at 50 °C and 60 °C for CE from muscle and hepatopancreas, respectively, and for 6 h using gelatin as substrate at 37 °C for both CE from muscle and hepatopancreas. M, molecular weight marker; P, protein bands determined by SDS-PAGE; H, hemoglobin; C, casein; and G, gelatin.

Table 11. Proteolytic activity towards different synthetic substrates of CE from muscle and hepatopancreas of freshwater prawn

Substrates	Proteolytic activity (units/g sample)	
	CE from muscle	CE from hepatopancreas
N-succinyl-Leu-Tyr-AMC	8.16±0.44*	ND†
Z-Arg-Arg-AMC	ND	ND
Z-Phe-Arg-AMC	111.29±1.60	ND
L-Arginine-AMC	ND	ND
BAPNA	ND	0.54±0.02
TAME	ND	8.50±0.03
SAAPNA	ND	0.005±0.00
BTEE	ND	0.51±0.07

†ND: non detectable

*Values are mean ± standard deviation (n=3).

4.5 Conclusions

CE from freshwater prawn muscle had very low proteolytic activity and no collagenolytic activity, while CE from hepatopancreas showed high hydrolytic activity on NAM and PSC. Based on protease inhibitors and specific synthetic substrates, cysteine proteases (calpain and cathepsin L) were dominant in prawn muscle, whereas serine proteases (trypsin and chymotrypsin) were predominant in CE from hepatopancreas of freshwater prawn. Serine-like proteases with collagenolytic activity from hepatopancreas were more likely responsible for the postmortem softening of freshwater prawn.

CHAPTER 5

Collagenolytic serine protease in freshwater prawn (*Macrobrachium rosenbergii*): characteristics and its impact on muscle during iced storage

5.1 Abstract

Proteolytic activity of crude protease extract (CE) from the hepatopancreas of freshwater prawn (*Macrobrachium rosenbergii*) was studied. Optimal activity of CE was found at pH 7 and 60 °C when casein was used as a substrate. The activity was strongly inhibited by 10 mM *N*-*ρ*-tosyl-L-lysine chloromethylketone (TLCK), suggesting that trypsin-like protease was dominant. CE also showed the collagenolytic activity toward pepsin soluble collagen extracted from prawn muscle. During extended iced storage of 4 days, proteolytic and trypsin activities was found in the first segment of prawn abdomen. These activities were detected in the second segment after 4 days of storage. Heat soluble collagen content was continuously increased during the storage. Nevertheless, no changes in proteolytic activity and heat soluble collagen content were obtained in the abdomen of prawn with the removal of hepatopancreas. Therefore, the release of trypsin-like collagenase from hepatopancreas was most likely responsible for the softening of prawn meat during iced storage.

5.2 Introduction

A major problem limiting freshwater prawn (*Macrobrachium rosenbergii*) shelf-life during iced storage is the deterioration or softening of texture. Shelf-life of freshwater prawn (*M. rosenbergii*) stored in ice was not more than 3-4 days (Nip and Moy, 1988). Softening of meat was developed within 4 days of iced storage and is most pronounced in the first segment of abdomen (Nip and Moy, 1988). Softening proceeded to the other sections toward the tail portion during the extended iced storage (Sriket *et al.*, 2010). This softening causes loosening and flaking of the cooked tissue of the prawn meat (Nip and Moy, 1988).

Generally, softening of fish and shellfish muscle during iced storage is associated with the weakening of endomysium and the collapse of collagen fibrils (Shigemura *et al.*, 2004). Sriket *et al.* (2010) reported that the muscle softening of freshwater

prawn during iced storage might result from the degradation of collagen situated in the non-helical region rather than myofibrillar and sarcoplasmic proteins. During the storage, autolysis of cephalothorax, where hepatopancreas and other internal organs are located, could take place, releasing the active proteases into the muscle. Hepatopancreas extracts from crustacean contain proteinases such as trypsin, chymotrypsin and collagenases, which are capable of degrading the native collagen under physiological conditions (Garcia-Carreño *et al.*, 1994). Among proteolytic enzymes, serine collagenase has the impact on the softening of muscle (Brauer *et al.*, 2003). Ezquerra *et al.* (1997) detected the activity of a collagenolytic enzyme in the muscle of white shrimp (*Penaeus vannamei*) during postmortem storage. Nevertheless, no information regarding the roles of proteolytic/collagenolytic enzymes from hepatopancreas in the development of mushiness in freshwater prawn during the extended iced storage exists. Therefore, the aims of this research were to characterize the proteases in the hepatopancreas of freshwater prawn and to monitor the collagenase-like enzymes released into prawn muscle during iced storage.

5.3 Materials and Methods

5.3.1 Chemicals

β-mercaptoethanol (βME), casein, tyrosine, α-*N*-benzoyl-DL-arginine-ρ-nitroanilide (DL-BAPNA), α-*N*-ρ-tosyl-L-arginine methyl ester (L-TAME), 1-(L-trans-epoxysuccinyleucylamino)-4-guanidinobutane (E-64), soybean trypsin inhibitor (SBTI), *N*-ρ-tosyl-L-lysine chloromethylketone (TLCK), *N*-tosyl-L-phenylalanine chloromethylketone (TPCK), pepstatin A, ethylenediaminetetraacetic acid (EDTA), Tris (hydroxymethyl) aminomethane (Tris) and trichloroacetic acid were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), Coomassie Brilliant Blue R-250 and *N,N,N′,N′*-tetramethylethylene diamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). All chemicals were of analytical grade.

5.3.2 Sample collection and preparation

Freshwater prawns (*M. rosenbergii*) with the size of 30-35 prawns/kg were obtained from a farm in Phatthalung province, Thailand. The prawn were placed in ice with a prawn/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla, Thailand, within approximately 2 h. Upon arrival, the prawns were washed with clean water. Hepatopancreas was removed manually from the

cephalothorax and powderized with liquid nitrogen. The powder was packed in polyethylene bag, immediately frozen and stored at -20 °C until used. The samples were stored not longer than 1 month at -20 °C.

5.3.3 Preparation of crude protease extract from hepatopancreas of freshwater prawn

Hepatopancreas powder (50 g) was mixed with 100 mL of 0.01 M sodium phosphate buffer (pH 7.6) (Brauer *et al.*, 2003). The mixture was homogenized for 2 min using an IKA Labortechnik homogenizer (Selangor, Malaysia) at a speed of 11,000 rpm. The homogenate was stirred for 30 min, followed by centrifugation at 10,000 *x g* for 30 min using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Palo Alto, CA, USA). All procedures were carried out at 4 °C. The supernatant was referred to as 'crude protease extract; CE' and used for the assays of proteolytic activities.

5.3.4 Characterization of CE

5.3.4.1 pH and temperature profiles

The proteolytic activity of CE from hepatopancreas of freshwater prawn was determined over the pH ranges of 2-11 in the presence of 10 mM $CaCl_2$. The buffers used were McIlvaine's buffer (0.2 M Na-phosphate and 0.1 M Na-citrate) for pH 2-8 and glycine-NaOH buffer (50 mM) for pH 9-11. To initiate the reaction, CE (200 µL) was added into the assay mixtures containing 2 mg of casein, 200 µL of distilled water and 625 µL of reaction buffer. The mixture was incubated at 60 °C for 15 min. The enzymatic reactions were then terminated by adding 200 µL of cold 50% (w/v) trichloroacetic acid (TCA). Unhydrolyzed protein substrate was allowed to precipitate for 1 h at 4 °C, followed by centrifuging at 5000 *x g* for 10 min. The oligopeptide content in the supernatant was determined by the Lowry assay (Lowry *et al.*, 1951) using tyrosine (99% purity) as a standard. One unit of activity was defined as that releasing 1 µmole of tyrosine per min (µmol Tyr/min). Specific activity was expressed as units/mg protein. A blank was run in the same manner, except that the enzyme was added after the addition of 50% TCA. To study the temperature profile, the activity was determined at various temperatures (20, 30, 40, 50, 60, 70 and 80 °C) with pH 7 using casein as a substrate.

5.3.4.2 Inhibitor study

Effects of different protease inhibitors toward the proteolytic activity of CE from hepatopancreas were determined as described by Klomklao *et al.* (2007). CE was incubated with an equal volume of protease inhibitor solutions to obtain the designated final concentrations (0.1 and 1 mM pepstatin A, 0.1 and 1 mM E-64, 5 and 10 mM EDTA, 0.1 and 1.0 mM SBTI, 0.1 and 10 mM PMSF, 5 and 10 mM TLCK and 5 and 10 mM TPCK). The mixture was incubated at room temperature (25-28 °C) for 15 min. The remaining proteolytic activity was determined at pH 7 and 60 °C for 15 min. The percent inhibition was then calculated using the following formula:

$$\% \text{ Inhibition} = 100 - \left(\frac{\text{As} \times 100}{\text{Ac}} \right)$$

where As and Ac are the proteolytic activity of sample treated with inhibitor and that of the control (sample without inhibitor), respectively. The control was conducted in the same manner except that deionized water was used instead of inhibitors.

5.3.4.3 Hydrolysis of pepsin soluble collagen (PSC)

CE (200 μL) containing 0.1 unit of trypsin determined by BAPNA assay was added to the reaction mixture containing 4 mg collagen and 825 μL of 50 mM Tris-HCl, pH 7.0. The hydrolysis was conducted by incubating the mixture at 25 °C for 0, 5, 10, 15, 30 and 60 min. The control was performed in the same manner for 60 min except distilled water was added instead of CE. The reaction was terminated by adding the preheated solution containing 2% SDS, 8 M urea and 2% βME (85 °C). The mixture was further incubated at 85 °C for 60 min to solubilize total proteins. The solution was centrifuged at 5000 x g for 10 min at room temperature to remove the debris. The supernatant was then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

To study the effect of various protease inhibitors on the collagen hydrolysis mediated by CE, the CE mixed with an equal volume of various protease inhibitors was prepared before initiation of hydrolysis as previously described.

5.3.5 Changes in protease activity and collagen of prawn muscle during iced storage

Freshwater prawns were separated into two groups including whole prawn without and with hepatopancreas removal. To remove hepatopancreas from the cephalothorax, the sharp knife was used to cut at the lower part of carapace with the width of

1 cm. The forcep was used to pull the hepatopancreas through the cut area. To reduce interfering effects of microbial enzymes, all prawn samples were soaked in 0.1 % sodium azide (NaN_3), used as the antimicrobial agent, at a prawn/solution ratio of 1:5 (w/v) for 10 min (Lichstein and Soule, 1944). The samples were drained on the screens for 3 min at 4 ^{o}C and placed in ice with a prawn/ice ratio of 1:2 (w/w). During storage**,** the molten ice was replaced with the same amount of ice every day. Thirty prawns were randomly taken for analyses every 4 days for up to 12 days. Prior to analyses, the prawns were beheaded, peeled and deveined. The first and second segments of prawn abdomen were collected and each segment was pooled as the composite sample. The prepared samples were kept in polyethylene bag and placed in ice until analyzed. Prior to analyses, prawn meat was ground to obtain the uniformity and used as the composite sample.

5.3.5.1 Total proteolytic activity

Total proteolytic activity of CE from prawn muscle was assayed using casein as a substrate according to the method of Klomklao *et al.* (2004). CE from prawn muscle was prepared in the same manner with CE from hepatopancreas. CE activity was assayed at pH 7 and 60 °C for 15 min. The activity was calculated and expressed as units/g meat.

5.3.5.2 Trypsin activity

Trypsin activity in prawn muscle was measured using BAPNA and TAME as substrates according to the methods of Hau and Benjakul (2006) and Hummel (1959), respectively. When BAPNA was used as a substrate, the diluted sample (200 µL) was added to 200 µL of distilled water and 1000 µL of 50 mM Tris-HCl, pH 7 containing 10 mM $CaCl_2$. To initiate the reaction, 200 µL of BAPNA (2 mg/mL) was added and mixed thoroughly. After incubation at 25 °C for 15 min, 200 µL of 30% acetic acid (v/v) was added to terminate the reaction. Production of ρ-nitroaniline was measured by monitoring the absorbance of reaction mixture at 410 nm (A_{410}). Blank was run in the same manner except BAPNA was added after the addition of acetic acid. Trypsin amidase activity was then calculated using the following formula:

$$\text{Trypsin activity (unit/mL)} = \frac{(A - A_0) \times \text{mixture volume (mL)} \times 1000}{8800 \times \text{reaction time (min)} \times 0.2}$$

where 8800 $cm^{-1}M^{-1}$ is the extinction coefficient of ρ-nitroaniline; A and A_0 are A_{410} of the sample and the blank, respectively. One unit of activity was defined as that releasing 1 mmol of ρ-nitroaniline per min.

When TAME was used as a substrate, the enzyme solution with an appropriate dilution (20 µL) was mixed with 3.0 mL of 1 mM TAME in 10 mM Tris-HCl buffer, pH 7. The reaction was conducted at 25 °C for 15 min. Production of ρ-tosyl-arginine was measured by monitoring the increase in absorbance at 247 nm. Blank was prepared in the same manner, but 10 mM Tris-HCl buffer (pH 7) was used instead of TAME solution. One unit of trypsin esterase activity was defined as the amount causing an increase of 1.0 in absorbance per min (Hummel, 1959). Both amidase and esterase activities were reported as m units/g meat.

5.3.5.3 Determination of heat soluble collagen

Heat soluble collagen content was extracted from prawn muscle according to the method of Liu *et al.* (1996). Prawn meat (2 g) was homogenized with 8 mL of 25% Ringer's solution (a mixture of 32.8 mM NaCl, 1.5 mM KCl, and 0.5 mM $CaCl_2$). The homogenate was heated for 70 min at 77 ^oC and centrifuged at 2,300 x g for 30 min at 4 °C. The extraction was repeated twice. The supernatants obtained were combined. The sediment and supernatants were hydrolyzed with 6 M HCl at 110 °C for 24 h. The collagen content of each fraction was determined according to the method of Sato *et al.* (1988). The amount of heat soluble collagen was expressed as a percentage of total collagen (collagen content in sediment plus that in the supernatant).

5.3.6 Statistical analysis

The experiments were run in triplicate with three different lots of samples. Data were subjected to analysis of variance (ANOVA) and mean comparison was carried out using Duncan's multiple range test (DMRT) (Steel and Torrie, 1980). Statistical analyses were performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

5.4. Results and Discussion

5.4.1 Characteristics of CE from hepatopancreas of freshwater prawn

5.4.1.1 pH and temperature profiles

pH profile of CE from hepatopancreas of freshwater prawn is shown in Figure 14A. CE exhibited a maximal proteolytic activity at pH 7 ($p< 0.05$). The proteolytic activity of CE sharply decreased when pH was below or above pH 7 ($p < 0.05$). At very acidic or

alkaline conditions, a decrease in electrostatic bonds might induce the conformational changes as well as unfolding of enzymes (Vojdani, 1996). This resulted in the loss in activity at very acidic or alkaline conditions. The optimal pH value of CE from hepatopancreas of freshwater prawn was similar to that of digestive proteases from mid gut glands of *Penaeus indicus* and lobster, *Nephrops norvegicus*, which had the optimal pH of 7.7 and 8, respectively (Omondi and Stark, 2001). Digestive protease from mid gut gland of *Farfantepenaeus paulensis* showed the maximal activity at pH of 8 (Buarque *et al.*, 2009). The optimal pH (pH 7) of CE from freshwater prawn was close to physiological pH of prawn meat (6.5-6.8). This could be implied that proteases from hepatopancreas might be active during postmortem storage.

The effect of various temperatures on proteolytic activity is depicted in Figure 14B. CE from the hepatopancreas of freshwater prawn exhibited the highest activity at 60 °C ($p< 0.05$). A sharp decrease in activity was observed as temperature increased, presumably as a result of thermal inactivation. At high temperature, unfolding of enzyme molecule occurred, leading to the loss in activity. This result was different from that of crude protease from pink shrimp and shrimp (*Penaeus orientalis*), which had optimal temperatures of 70 and 45 °C, respectively (Buarque *et al.*, 2009; Oh *et al.*, 2000).

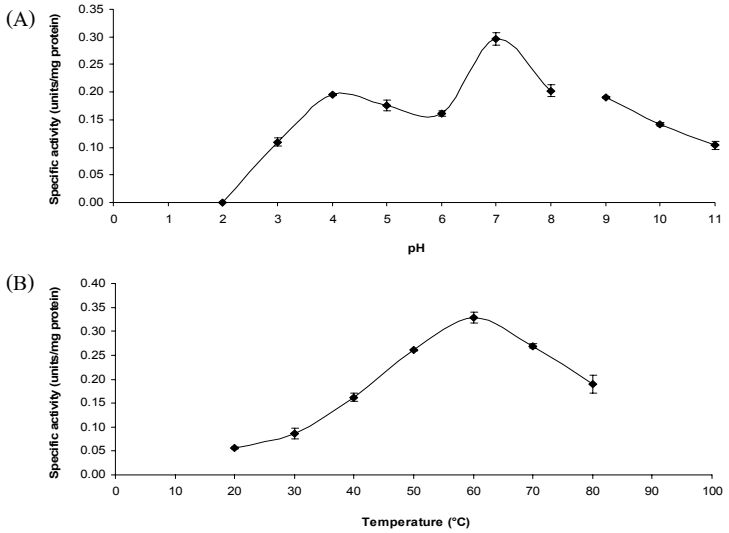

Figure 14. pH (A) and (B) temperature profiles of CE from hepatopancreas. For pH profile study, protease activity was determined by incubating reaction mixture at 60 °C at various pH values. For temperature profiles, protease activity was determined by incubating reaction mixture at pH 7 and various temperatures. Bars represent the standard deviation (n=3).

5.4.1.2 Effect of various protease inhibitors on proteolytic activity

The effect of various protease inhibitors on proteolytic activity of CE from the hepatopancreas of freshwater prawn is shown in Table 12. Among all protease inhibitors at a concentration of 10 mM, TLCK exhibited the highest inhibition (89%), followed by TPCK (55%), PMSF (44%) and SBTI (42%), respectively, while pepstatin A and E-64 at a level of 1 mM showed a low inhibitory effect. TLCK is the inhibitor specific for trypsin, while TPCK specifically inhibits chymotrypsin (Klomklao *et al.*, 2007). PMSF and SBTI has been known as the inhibitor specific to serine protease (Benjakul *et al.*, 2003). Pepstatin A and E-64 are the inhibitors specific for aspartic protease and cystein protease, respectively (Klomklao *et al.*, 2004; Klomklao *et al.*, 2007). The inhibitory result revealed that trypsin was the major protease of CE from hepatopancreas of freshwater prawn. The most active protease in hepatopancreas of shrimp (*Penaeus orientalis*) was identified to be trypsin-like enzyme (Oh *et al.*, 2000). Aoki *et al.* (2003) also reported that serine-collagenase was found in hepatopancreas of Northern shrimp (*Pandalus eous*).

Table 12. Effect of various inhibitors on % inhibition of protease activity in CE from hepatopancreas of freshwater prawn

Inhibitors	Concentration (mM)	% Inhibition
Control	-	0
Pepstatin A	0.1	$6.46\pm0.14^{*g}$
	1	7.24 ± 2.04^{g}
E-64	0.1	24.01 ± 1.23^{f}
	1	28.06 ± 2.42^{e}
EDTA	5	8.52 ± 1.22^{g}
	10	9.06 ± 5.46^{g}
PMSF	0.1	34.33 ± 1.09^{d}
	10	44.25 ± 2.28^{c}
SBTI	0.1	30.51 ± 0.27^{e}
	1.0	42.20 ± 2.17^{c}
TLCK	5	88.04 ± 0.68^{a}
	10	89.06 ± 0.14^{a}
TPCK	5	54.29 ± 3.14^{b}
	10	55.22 ± 5.66^{b}

CE from hepatopancreas (200 µL) was mixed with 200 µL of single protease inhibitor to obtain the designated final concentration. The residual proteolytic activity was measured using casein as substrates at pH 7 and 60 °C.

*Values are mean ± standard deviation (n=3).

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

5.4.1.3 Collagen hydrolysis by CE from hepatopancreas of freshwater prawn

Protein patterns of PSC hydrolyzed by CE from the hepatopancreas of freshwater prawn during the incubation at 25 °C for 0-60 min are shown in Figure 15. Marked degradation of α-chain of prawn PSC was found after 5 min of hydrolysis and the complete hydrolysis was noticed after 15 min with the concomitant occurrence of degradation products having the lower molecular weight. These results suggested that CE from the hepatopancreas exhibited high collagenolytic activity. Endogenous serine collagenases and other proteases might be responsible for the degradation of collagenous fibrils in freshwater prawn muscle. Proteases from Northern shrimp were able to hydrolyze beef collagen (Aoki *et al.*, 2004). In general, collagens in fish and shellfish muscle contain lower cross-links than those from warm-blooded vertebrates. Collagens from fish and shellfish are more heat labile than land animal collagen (Hernandez-Herrero *et al.*, 2003).

The effect of protease inhibitors on the degradation of PSC by CE from the hepatopancreas of freshwater prawn is shown in Figure 16. In the absence of protease inhibitor (control), the complete hydrolysis of α-chains was obtained. Among all protease inhibitors, TLCK exhibited the highest inhibitory effect as evidenced by the greatest amount of α-chains retained in PSC. PMSF, SBTI and TPCK also showed a high inhibition toward PSC hydrolysis by CE. The result was in agreement with that found in the *in vitro* study (Table 12). Therefore, it could be inferred that a serine protease with collagenolytic activity was the major protease in prawn hepatopancreas. Collagenolytic serine proteinases were found in hepatopancreas of Kamchatka crab (*Paralithodes camtschaticus*) (Klimova and Chebotarev, 1999) and Northern shrimp (Aoki *et al.*, 2003).

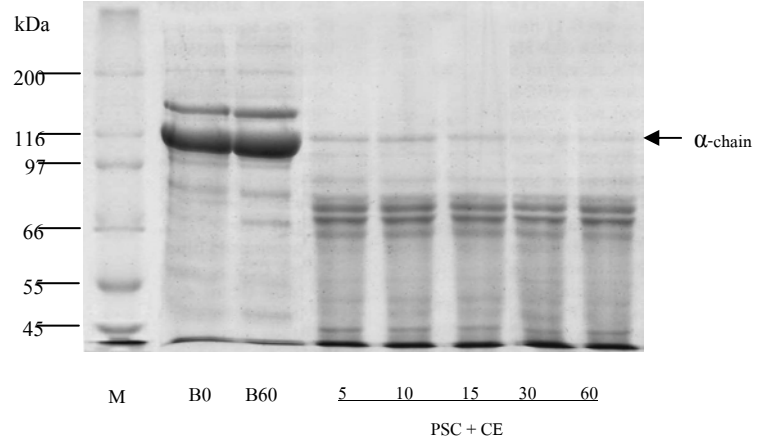

Figure 15. Protein patterns of PSC incubated with CE from hepatopancreas at 25 °C for different times. M: molecular weight marker, B0 and B60: PSC incubated without CE for 0 and 60 min, respectively. Numbers denoted the incubation time (min) at 25 °C.

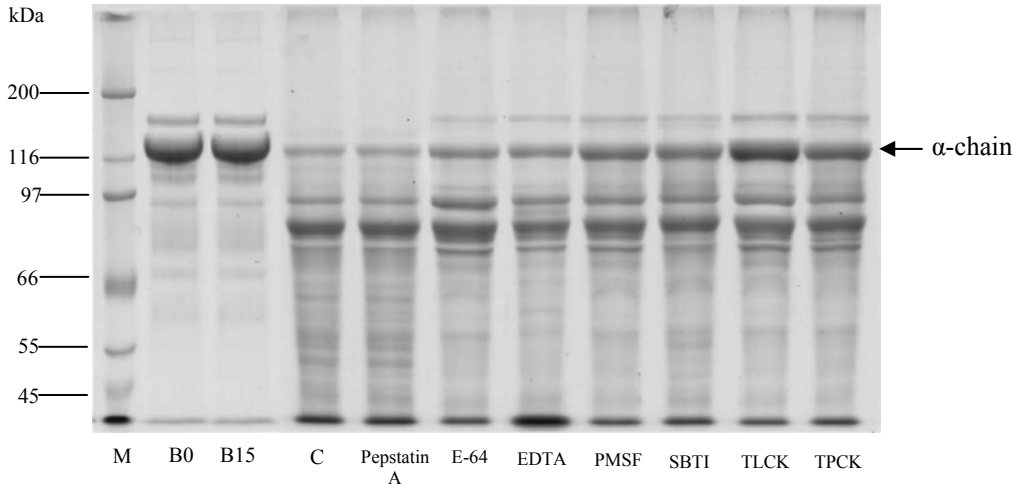

Figure 16. Protein patterns of PSC incubated without and with CE from hepatopancreas in the presence of various protease inhibitors at 25 °C for 15 min. M: molecular weight marker, B0 and B15: PSC incubated without CE for 0 and 15 min, respectively. C: PSC incubated with CE in the absence of inhibitors for 15 min. Concentration of inhibitors used: Pepstatin A (1 mM), E-64 (1 mM), EDTA (10 mM), PMSF (10 mM), SBTI (1.0 mM), TLCK (10 mM) and TPCK (10 mM).

5.4.2 Changes in indigenous proteolytic activity and heat soluble collagen content in freshwater prawn meat during iced storage

5.4.2.1 Changes in indigenous proteolytic activity in prawn meat

5.4.2.1.1 Total proteolytic activity

Total proteolytic activity of the first and second segments of abdomen from whole prawn without and with hepatopancreas removal during iced storage of 12 days is shown in Figure 17A. Generally, total proteolytic activity in both segments assayed using casein as a substrate increased when storage time increased ($p < 0.05$). Total proteolytic activity in all samples markedly increased after 4 days of storage ($p < 0.05$). At the same storage time, the first segment of whole prawn sample without hepatopancreas removal showed the highest total proteolytic activity, followed by the second segment of the same sample. Nevertheless, the negligible activity was observed in prawn with hepatopancreas removal. The result indicated that hepatopancreas was the major source of proteases, which could be released and contaminated in the meat, especially at the portion located close to cephalothorax, the first segment. Since prawn samples were treated with NaN_3 prior to

storage, microbial proteolytic activity was omitted. The hepatopancreas in the cephalothorax of shrimp was reported to have high proteolytic activities (Oh *et al.*, 2000). Therefore, the removal of hepatopancreas, mainly via beheading, could be an effective means to lower or prevent the degradation of meat associated with the development of mushy texture.

5.4.2.1.1 Trypsin activity

Trypsin activity in the first and second segments of abdomen from whole freshwater prawn samples without and with hepatopancreas removal during iced storage expressed as amidase and esterase activities was also monitored to confirm the leakage of trypsin or trypsin-like enzymes from hepatopancreas into the muscle during the extended storage. Amidase activity of the prawn meat is shown in Figure 17B. Freshwater prawn samples had no amidase activity at day 0 when BAPNA was used as substrate. At day 4 of iced storage, an amidase activity was found only in the first segment of abdomen from whole prawn sample (without hepatopancreas removal). It was suggested that trypsin-like enzyme might be released to prawn meat after the autolysis of prawn hepatopancreas. At day 8 and 12 of iced storage, the continuous increase in amidase activity was observed in the first segment of abdomen from whole prawn sample. However, very low amidase activity was found in the second segment of abdomen from whole prawn sample, indicating the less penetration of trypsin-like enzyme into this segment. The amidase activity was dominated in the first segment of abdomen from whole prawn sample during iced storage for 12 days, while no activity was detected in meat of prawn with heptopancreas removal. Removal of hepatopancreas could suppress the contamination of proteases from hepatopancreas of prawn into the prawn meat during iced storage.

For the esterase activity (Figure 17C), similar result was obtained in comparison with amidase. No esterase activity was observed in all prawn samples at day 0 when TAME was used as substrate. At day 4 of iced storage, esterase activity was observed in only the first segment of abdomen from whole prawn sample without hepatopancreas removal. The sharp increase in esterase activity was found in the first segment of abdomen from whole prawn sample ($p<0.05$) after 4 days of iced storage. Nevertheless, very low activity was found in the second segment. No esterase activity was observed in meat of prawn sample with hepatopancreas removal during iced storage. This was in agreement with the negligible level of amidase in prawn samples with hepatopancreas removal (Figure 17B). Released trypsin-like collagenase from hepatopancres as indicated by the increases in amidase and esterase activities more likely played a role in protein degradation, especially collagen in the prawn meat during the extended iced storage.

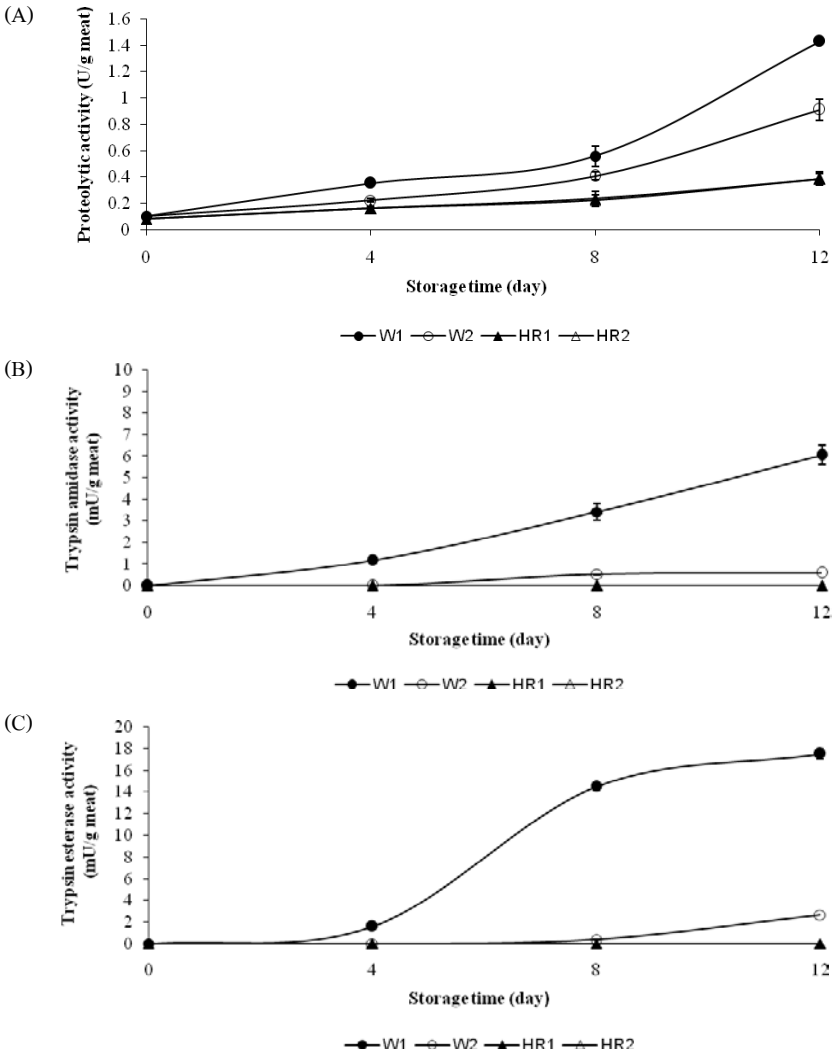

Figure 17. Total proteolytic activity (A) and trypsin activity assayed as amidase activity (B) and esterase activity (C) of the first and second segments of abdomen from freshwater prawn without and with hepatopancreas removal during 12 days of iced storage. W1; the first segment of abdomen from whole prawn, W2; the second segment of abdomen from whole prawn, HR1; the first segment of abdomen from whole prawn with hepatopancreas removal, HR2; the second segment of abdomen from whole prawn with hepatopancreas removal. Bars represent the standard deviation (n=3).

5.4.2.2 Changes in heat soluble collagen content of prawn meat

Changes in heat soluble collagen content of the first and second segments of abdomen from whole freshwater prawn without and with hepatopancreas removal during 12 days of iced storage are shown in Figure 18. Heat soluble collagen content in the first and second segments of abdomen from whole prawn samples increased with increasing storage

time ($p < 0.05$). Conversely, no changes in heat soluble collagen content were found in the samples with hepatopancreas removal throughout the storage of 12 days in ice. A marked increase in heat soluble collagen content was noticeable after 4 days of iced storage ($p<0.05$). This was coincidental with the increases in proteolytic activity in the meat (Figure 17), which was most likely from hepatopancreas. Collagen degradation can be explained as a result of specific collagenolytic enzyme on the collagen. Serine proteases are capable of hydrolyzing major muscle structure proteins such as collagen (Sato *et al.*, 1994). Collagen with lower content of cross-links could be more solubilized when heating was applied (Sriket *et al.*, 2010). When native collagen was hydrolyzed, the heat denaturation was more enhanced, as indicated by the increase in heat soluble collagen content.

At the same storage time, heat soluble collagen content was dominant in the first segment of abdomen from whole prawn meat, compared with the second segment ($p<0.05$). This might be results from the different penetration rate of collagenolytic enzyme from hepatopancreas during iced storage. Endogenous serine collagenases and other proteases might be responsible for the softening of prawn meat during the extended iced storage. Therefore, the removal of cephalothorax containing hepatopancreas could retard the degradation of collagen contributing to the soft texture of freshwater prawn during iced storage.

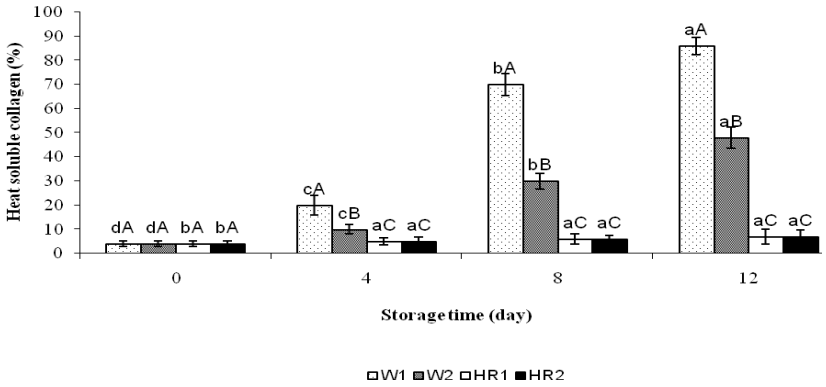

Figure 18. Heat soluble collagen content of the first and second segments of abdomen from freshwater prawn without and with hepatopancreas removal during 12 days of iced storage. W1; the first segment of abdomen from whole prawn, W2; the second segment of abdomen from whole prawn, HR1; the first segment of abdomen from whole prawn with hepatopancreas removal, HR2; the second segment of abdomen from whole prawn with hepatopancreas removal. The different letters on the bars within the same sample indicate significant differences ($p<0.05$). Different capital letters on the bars within the same storage time indicate the significant differences ($p<0.05$). Bars represent the standard deviation ($n=3$).

5.5 Conclusions

Freshwater prawn hepatopancreas contained serine collagenase-like enzyme, which was able to hydrolyze PSC. During iced storage, the protease from hepatopancreas possibly leaked from hepatopancreas and penetrated into the first segment or the second segment of abdomen. This was more likely responsible for the softening of prawn meat during extended iced storage. To alleviate such a problem, the prawn should be kept in ice less than 4 days or the decapitation should be performed prior to storage.

CHAPTER 6

Low molecular mass trypsin from hepatopancreas of freshwater prawn (*Macrobrachium rosenbergii*): characteristics and biochemical properties

6.1 Abstract

Trypsin was purified to homogeneity from hepatopancreas of freshwater prawn (*Macrobrachium rosenbergii*) using a series of chromatographies including Q-Sepharose, Superdex 75 and MonoQ columns. Freshwater prawn trypsin was purified to 525-fold with a yield of 10.6%. Based on native-PAGE, the purified trypsin showed a single band. Trypsin had a molecular mass of 17 kDa as estimated by SDS-PAGE. The optimal pH and temperature for Boc-Val-Pro-Arg-MCA hydrolysis were 8.0 and 55 °C, respectively. Trypsin was stable to heat treatment up to 40 °C, and over a pH range of 7.0-11.0. Trypsin activity was strongly inhibited by soybean trypsin inhibitor, N-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) and Pefabloc SC and was partially inhibited by ethylenediaminetetraacetic acid (EDTA). Apparent K_m value of trypsin was 0.24 μM and k_{cat} value was 607.56 s^{-1} for Boc-Val-Pro-Arg-MCA. The N-terminal amino acid sequence of 20 residues of freshwater prawn trypsin was IVGGDEAAPGEFPHQISMQV, which was highly homologous with those from other species of prawn. Freshwater prawn trypsin also showed high collagenolytic activity toward prawn, shrimp and fish collagens, suggesting its possible role in muscle softening of freshwater prawn during extended storage.

6.2 Introduction

Freshwater prawn is widely consumed in Thailand due to its delicacy. However, this species is not successfully marketable due to the loss in textural acceptability. During postmortem handling and storage, prawn meat becomes soft and mushy, probably as a result of indigenous proteases. Softening of meat was developed within 4 days of iced storage and was most pronounced in the first segment of abdomen (Sriket *et al.*, 2010). Sriket *et al.* (2011a) reported that proteases from freshwater prawn muscle had very low proteolytic activity and no collagenolytic activity, while protease from hepatopancreas showed high

hydrolytic activity on natural actomyosin (NAM) and pepsin soluble collagen (PSC) extracted from freshwater prawn muscle.

Shrimp hepatopancreas has been reported to have high proteolytic activities (Oh *et al.*, 2000) and most digestive enzymes in crustaceans were classified to be serine protease and metalloprotease (Garcia-Carreño *et al.*, 1994). Among proteolytic enzymes, serine collagenase has an impact on the softening of muscle (Ezquerra Brauer *et al.*, 2003). Hepatopancreas from white shrimp, *Penaeus vannamei* (Ezquerra *et al.*, 1997) and Northern shrimp, *Pandalus eous* (Aoki *et al.*, 2003) possessed collagenolytic/gelatinolytic activities. Recently, serine protease, mainly trypsin-like protease released from hepatopancreas, was reported to cause the softening of freshwater prawn muscle during extended iced storage (Sriket *et al.*, 2011b). Therefore, better understanding of trypsin from freshwater prawn, especially from the digestive organs, could pave a way to prevent or lower the activity of those proteases, thereby retarding the quality loss of freshwater prawn. Nevertheless, no information regarding the characteristics and biochemical properties of purified trypsin from hepatopancreas of freshwater prawn has been reported. Therefore, this study was aim to purify and characterize trypsin responsible for the muscle degradation from freshwater prawn.

6.3 Materials and methods

6.3.1 Chemicals

Q-Sepharose, Superdex 75 and MonoQ were obtained from Amersham Pharmacia Biotech AB (Uppsala, Sweden). t-Butyloxy-carbonyl-Val-Pro-Arg-4-methyl-coumaryl-7-amide (Boc-Val-Pro-Arg-MCA) and other synthetic fluorogenic peptide substrates (MCA-substrates) were purchased from Peptide Institute (Osaka, Japan). Pefabloc SC was obtained from Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid (EDTA), pepstatin A, soybean trypsin inhibitor, N-p-tosyl-L-lysine chloromethyl ketone (TLCK), N-tosyl-L-phenyl-alanine chloromethyl ketone (TPCK), 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), N-ethylmaleimide, β-mercaptoethanol (βME), bovine gelatin and bovine serum albumin were procured from Sigma Chemical Co. (St. Louis, MO, USA.). Protein molecular weight markers were obtained from Bio-Rad Laboratories (Hercules, CA, USA). All chemicals used were of analytical grade.

6.3.2 Sample preparation

Live freshwater prawns (*M. rosenbergii*) with a size of 30-35 prawn/kg were obtained from a farm in Phatthalung province, Thailand. The samples were ice-shocked with iced-water (0°C). The hepatopancreas was removed manually and immediately mixed with liquid nitrogen. The frozen sample was placed in a polyethylene bag, sealed and stored at -40 °C until use.

6.3.3 Preparation of hepatopancreas extract

Hepatopancreas were homogenized with three volumes of acetone at -20 °C for 3 min using an IKA homogenizer (Model T25, Selangor, Malaysia) according to the method of Klomklao *et al.* (2010a). The homogenate was filtered in vacuo on Whatman No. 4 filter paper. The residue obtained was then homogenized in two volumes of acetone at -20 °C for 3 min, and then the residue was air-dried in a vacuum desiccator until the sample was dry and free of acetone odor. Defatted hepatopancreas powder obtained was stored at -40 °C until use.

To prepare the hepatopancreas extract, the powder was suspended in 50 mM Tris–HCl, pH 7.5 containing 5 mM $CaCl_2$ referred to as ‘‘starting buffer; SB’’ at a ratio of 1:10 (w/v) and stirred continuously at 4 °C for 3 h. The suspension was centrifuged at 12000 *x g* for 10 min at 4 °C using a refrigerated centrifuge (Kubota, Model 7780II, Tokyo, Japan) to remove the tissue debris, and then the supernatant was referred to as ‘‘crude extract’’.

6.3.4 Purification of trypsin from freshwater prawn hepatopancreas

All purification processes were carried out at 4 °C in the laboratory of Faculty of Fisheries, Nagasaki University, Japan. Fractions obtained from all purification steps were subjected to the measurement of protein content and trypsin activity. Protein concentration during purification steps was determined by monitoring the absorbance of sample solution at 280 nm.

After filtration through a 0.2-µm filter, the sample was then applied to a Q-sepharose anion exchange column (3.0*x*45cm), which was equilibrated with approximately two-bed volumes of SB. The sample was loaded onto the column at a flow rate of 0.5 ml/min. The column was washed with SB until A_{280} was less than 0.05 and then eluted with a linear gradient of 0.0–0.6M NaCl in SB at a flow rate of 0.5 mL/min. Fractions of 5 mL were collected and the fractions with trypsin activity were pooled and concentrated by ultrafiltration with YM-10 membrane (Millipore, Bedford, MA, USA).

The concentrated Q-sepharose fractions were filtered with a 0.2-µm filter and further subjected to Superdex 75 column (2.6*x*60cm) previously equilibrated with approximately two-bed volumes of SB. The elution was performed with the same buffer at a flow rate of 0.5 ml/min. Fractions of 6 mL were collected and those with trypsin activity were pooled and further purified by MonoQ anion exchange column previously equilibrated with SB. The sample was loaded onto the column at a flow rate of 0.5 ml/min. The column was washed with SB until A_{280} was less than 0.05 and then eluted with a linear gradient of 0.0–0.6M NaCl in SB at a flow rate of 0.5 ml/min. Fractions of 0.5 mL were collected and the fractions with trypsin activity were pooled, dialyzed with SB for 12 h and used for further study.

6.3.5 Trypsin activity assay

Trypsin activity was measured by the method of Yoshida *et al.* (2009) using Boc-Val-Pro-Arg-MCA as a substrate. Appropriately diluted enzyme (100 µL) was added to 800 µL of 50 mM Tris–HCl buffer (pH 8.0). The reaction was immediately initiated by the addition of 100 µL of 50 µM substrate and incubated at 55 °C for 10 min. To stop the reaction, 1.5 mL of the stopping agent (methyl alcohol:n-butyl alcohol:distilled water = 35:30:35, v/v/v) was added. The fluorescence intensity of liberated 7-amino-4-methylcoumarin (AMC) was measured by a spectrofluorometer (Model RF-1500*,* Shimadzu Co., Kyoto, Japan) at the excitation wavelength of 380 nm and the emission wavelength of 450 nm. One unit of enzyme activity was defined as the amount of the enzyme, which released 1 µmol AMC per min.

6.3.6 pH and temperature profile

Trypsin activity was assayed over the pH range of 4.0–11.0 (50 mM acetate buffer for pHs 4.0–7.0; 50 mM Tris–HCl buffer for pHs 7.0–9.0 and 50 mM glycine–NaOH for pHs 9.0–11.0) at 55 °C for 10 min. For temperature profile study, the activity was assayed at different temperatures (20, 30, 40, 50, 60, 70 and 80 °C) for 10 min at pH 8.0.

6.3.7 pH and thermal stability

The effect of pH on enzyme stability was evaluated by measuring the residual activity after incubation at various pHs for 30 min at 30 °C. Enzyme solution was mixed with different buffers mentioned above at a ratio of 1:1 (v/v). For thermal stability, enzyme solution was mixed with 100 mM Tris–HCl, pH 8.0 at a ratio of 1:1 (v/v) and incubated at

different temperatures (20, 30, 40, 50, 60, 70 and 80 °C) for 15 min in a temperature controlled water bath (Thermo Minder Mini-80, Taiyo, Tokyo, Japan). Thereafter, the treated samples were suddenly cooled in iced water. The residual activity was assayed using Boc-Val-Pro-Arg-MCA as a substrate at pH 8.0 and 55 °C for 10 min.

6.3.8 Effect of inhibitors

The effect of inhibitors on trypsin activity was determined according to the method of Klomklao *et al.* (2007). Enzyme solution was mixed with an equal volume of protease inhibitor solution to obtain the final concentration designated (0.1 mM E-64, 1.0 g/L soybean trypsin inhibitor, 5 mM pefabloc SC, 5 mM TLCK, 5 mM TPCK, 1 mM pepstatin A and 10 mM EDTA). The mixture was allowed to stand at room temperature (26–28 °C) for 15 min. Thereafter, the remaining activity was measured and percent inhibition was calculated.

6.3.9 Determination of protein concentration

Protein concentration was determined by monitoring the absorbance of sample solution at 260 and 280 nm. The protein content was then calculated as described by Stoscheck (1990).

Protein content (mg/mL) = $(1.55 \times A_{280}) - (0.76 \times A_{260})$

where 1.55 and 0.76 are extinction coefficients of protein at A_{280} and of nucleic acid at A_{260}, respectively.

6.3.10 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS–PAGE was performed according to the method of Laemmli (1970). Protein solutions were mixed at 1:1 (v/v) ratio with the SDS–PAGE sample buffer (0.125 M Tris–HCl, pH 6.8; 4% SDS; 20% glycerol; 10% β-mercaptoethanol) and boiled for 3 min. The samples (10 µg) were loaded onto the gel made of 4% stacking and 15% separating gels and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-Protean II Cell apparatus (Atto Co., Tokyo, Japan). After electrophoresis, the gels were stained with 0.1% Coomassie brilliant blue R-250 in 50% methanol and 7% acetic acid and destained with 7% acetic acid.

Native-PAGE was performed using 15% separating gels in a similar manner, except that the sample was not heated and SDS and reducing agent were left out.

6.3.11 Activity staining

Activity staining of purified trypsin towards bovine gelatin was performed according to the method described by Lødemel and Olsen (2003) with a slight modification. Purified trypsin was mixed with sample buffer (0.125 M Tris–HCl, pH 6.8; 4% SDS; 20% glycerol) at a ratio of 1:1 (v/v). The mixture containing 1µg protein was loaded onto the gel made of 4% stacking and 15% separating gels containing 0.1% bovine gelatin. The electrophoresis was conducted at 4 °C. After electrophoresis, the gel was washed twice in 2.5% Triton X-100 for 15 min. The remaining Triton X-100 was removed by washing the gel (three times) with 50 mM Tris-HCl buffer pH 7.0. Gel was incubated in 50 mM Tris-HCl, pH 8.0 containing 5 mM $CaCl_2$ and 0.02% NaN_3 at 37 °C for 6 h. The gel was rinsed using cold distilled water prior to staining and destaining as described above. Development of clear zones on blue background indicated gelatinolytic activity.

6.3.12 Determination of N-terminal amino acid sequence

Purified trypsin was subjected to SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was briefly stained by Coomassie Brilliant Blue (CBB) and the protein bands were excised. The N-terminal amino acid sequence of trypsin was determined using a protein sequencer (Shimadzu, Model PPSQ-33A, Kyoto, Japan).

6.3.13 Substrate specificity

To study the substrate specificity of the trypsin, various synthetic fluorogenic MCA-substrates were used and the activity of trypsin toward different substrates was determined as previously described.

6.3.14 Kinetic studies

The activity was assayed with different final concentrations of Boc-Val-Pro-Arg-MCA and Boc-Leu-Lys-Arg-MCA. The final enzyme concentration for the assay was 0.015 µg/mL. The determinations were repeated twice and the respective kinetic parameters including V_{max} and K_m were evaluated by plotting the data on a Lineweaver–Burk double-reciprocal graph (Lineweaver and Burk, 1934). Values of turnover number (k_{cat}) were calculated from the following equation: $V_{max}/[E] = k_{cat}$, where [E] was the active enzyme concentration.

6.3.15 Hydrolysis of various collagens by purified trypsin

Purified trypsin (100 μL) with the activity of 0.01 unit determined by Boc-Val-Pro-Arg-MCA assay was added to the reaction mixture containing 4 mg collagen and 800 μL of 50 mM Tris–HCl, pH 7.0, containing 5 mM $CaCl_2$. Pepsin soluble collagen (PSC) from freshwater prawn, PSC from white shrimp and acid soluble collagen (ASC) from yellowtail meats were isolated according to the method of Sriket *et al.* (2007). The hydrolysis was conducted by incubating the mixture at 25 °C for 0, 5, 10 and 15 min. The control was performed in the same manner for 15 min except distilled water was added instead of trypsin. The reaction was terminated by adding the preheated solution containing 2% SDS, 8 M urea and 2% βME (85 °C). The mixture was further incubated at 85 °C for 60 min to solubilize total proteins. The solution was centrifuged at 5000 *x g* for 10 min at room temperature to remove the debris. The supernatant was then subjected to SDS-PAGE using 7.5% separating gel and 4% stacking gel.

6.3.16 Statistical analysis

The experiments were run in triplicate determinations, except for kinetic study, which was carried out in duplicate. Data were subjected to analysis of variance (ANOVA) and mean comparison was carried out using Duncan's multiple range test (DMRT) (Steel and Torrie, 1980). Statistical analyses were performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

6.4. Results and discussion

6.4.1 Purification of trypsin from hepatopancreas of freshwater prawn

Purification steps of trypsin from hepatopancreas of freshwater prawn are summarized in Table 13. The purification of trypsin was started by separating the crude extract using Q-Sepharose anion exchange column. After separation, the fractions with pronounced trypsin activity were pooled and purity of 14.3-fold was observed. A single activity peak was obtained for the pooled Q-Sepharose fractions upon the subsequent chromatography on Superdex 75 column (Figure19). The yield of 23.8% and the purity of 27.7-fold were observed after this gel filtration. The pooled fractions with trypsin activity were then subjected to MonoQ anion exchange column. Ion-exchange chromatography was used to remove contaminating proteins and to separate different trypsin isoforms (Klomklao *et al.*, 2006b). Pooled fractions from single activity peak obtained after MonoQ anion

exchange column showed high purity of 525-fold with 10.6% yield. The results showed that Q-Sepharose anion exchange, Superdex 75 and MonoQ anion exchange chromatographies effectively removed the contaminants from the crude extract. As a consequence, the purified trypsin was obtained.

Table 13. Purification of trypsin from hepatopancreas of freshwater prawn

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Purity (fold)	Yield (%)
Crude enzyme	1418.59	5827	0.24	1.0	100
Q-Sepharose	1037.45	301.9	3.44	14.3	73.1
Superdex 75	337.27	50.76	6.64	27.7	23.8
MonoQ	150.09	1.19	126	525	10.6

Trypsin activity was assayed at pH 8 and 55 °C for 10 min using Boc-Val-Pro-Arg-MCA as substrate.

6.4.2 Purity and molecular mass

Based on native gel-electrophoresis, there was only single protein band (Figure 20A), indicating the purity of trypsin. Trypsin from hepatopancreas of freshwater prawn also showed a single band on SDS–PAGE (Figure 20B). Molecular mass of trypsin from hepatopancreas of freshwater prawn was estimated to be 17 kDa. This value was in accordance with that determined by Superdex 75 column (data not shown). The result suggested that trypsin from hepatopancreas of freshwater prawn was monomeric. However, the molecular mass of trypsin was quite different from those of mammalian fish and shrimp trypsins. Trypsins purified from black tiger prawn (27-29 kDa) (Lu *et al.*, 1990), white shrimp (*Penaeus setiferus*) (24 kDa) (Gates, 1969) and crayfish (*Procambarus clarkia*) (23.8, 27.9, 24.8 and 31.4 kDa) (Kim *et al.*, 1992) have been reported. The activity band was confirmed by substrate gel electrophoresis, in which clear band with the same molecular mass appeared on the blue background (Figure 20C). The result indicated that purified freshwater prawn hepatopancreas trypsin was able to hydrolyze gelatin. Aoki *et al.* (2003) reported that four gelatinolytic proteases were found in hepatopancreas of Northern shrimp (*Pandalus eous*). Generally, trypsins have molecular masses between 20 and 25 kDa (Klomklao, 2008). The molecular mass of trypsin-like enzymes from crustacea varies widely. This present study

therefore firstly reported the trypsin with low molecular mass (17 kDa) in crustacean and fish. Nevertheless, the possibility that autolytic degradation causing such a low molecular mass should not be excluded (Lu *et al.*, 2008).

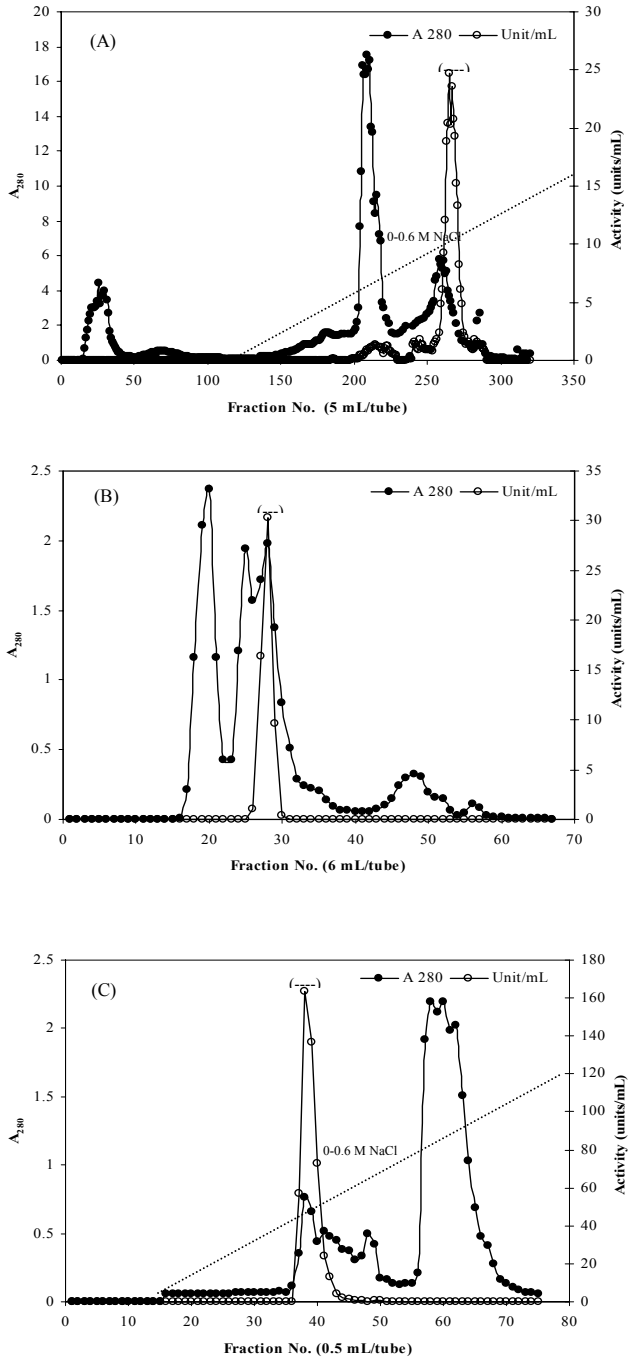

Figure 19. Purification of trypsin from hepatopancras of freshwater prawn. (A) Elution profile of trypsin on Q-Sepharose anion exchange column; (B) Superdex 75 gel filtration column and (C) MonoQ anion exchange column. Fractions under the bars were pooled.

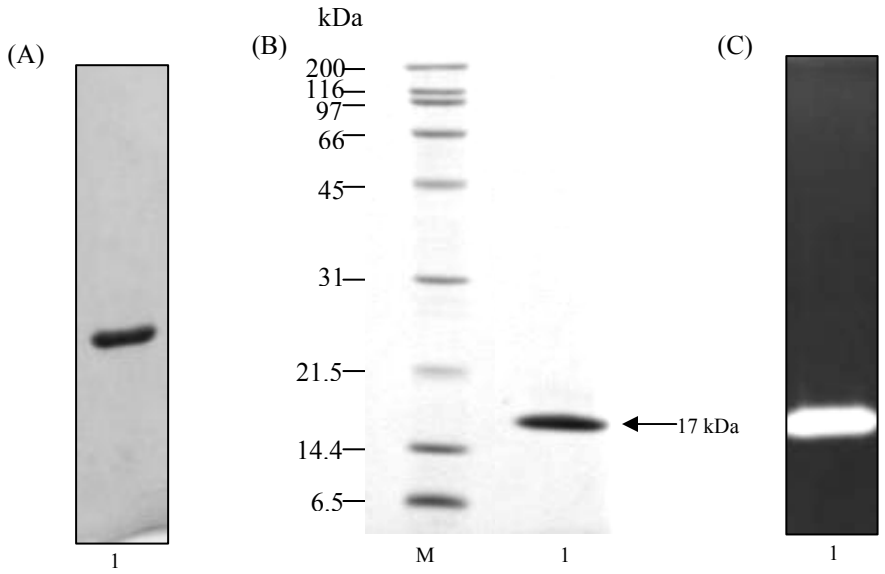

Figure 20. Protein pattern from native-PAGE (A), SDS-PAGE (B) and activity staining (C) of purified trypsin from hepatopancreas of freshwater prawn. M, molecular weight standard; lane 1, trypsin.

6.4.3 pH and temperature profile

Purified trypsin from freshwater prawn hepatopancreas had the increases in activity as the pH increased and reached the maximum at pH 8.0 ($p<0.05$) (Figure 21A). The optimal pH of purified trypsin from freshwater prawn hepatopancreas was in accordance with those of trypsins from other fish and shellfish (Castillo-Yanez *et al.*, 2005; Hjelmeland and Raa, 1982; Jiang *et al.*, 1991). Activity of trypsin decreased when pHs were higher than the optimal pH. The lowered activity at very acidic and alkaline pH was plausibly due to the conformational changes of enzyme under harsh condition (Klomklao *et al.*, 2010b).

Temperature profile of trypsin from hepatopancreas of freshwater prawn is depicted in Figure 21B. The optimal temperature of trypsin was 55 °C ($p<0.05$) when Boc-Val-Pro-Arg-MCA was used as a substrate. The optimal temperature of purified trypsin from hepatopancreas of freshwater prawn was similar to that of trypsin from other crustacean, which ranges from 50 to 60 °C (Jiang *et al.*, 1991; Maeda-Martinez *et al.*, 2000). However, the optimal temperature of purified trypsin from hepatopancreas of freshwater prawn was higher than those of trypsins obtained from hepatopancreas from other fish and shellfish such as Japanese sea bass (*Lateolabrax japonicus*) (40–45 °C) (Cai *et al.*, 2011), carp (40–45 °C) (Cao *et al.*, 2000) and crab (45 °C) (Galgani and Nagayama, 1987). The differences in optimal temperature might be governed by habitat, environment and genetic (Klomklao *et al.*,

2006a). A sharp decrease in activity of trypsin from hepatopancreas of freshwater prawn at temperature above 60 °C was most likely due to thermal denaturation.

6.4.4 pH and thermal stability

The pH stability of trypsin from hepatopancreas of freshwater prawn is shown in Figure 21C. Trypsin from hepatopancreas of freshwater prawn was stable in pH values ranging from 7.0 to 11.0. The inactivation was more pronounced at pH below 7. A completely loss in activity was observed after incubating trypsin at pH 4. (Osnes and Mohr, 1985) reported that trypsins from krill, *Euphausia superba*, were inactivated at pH 4.5. Fish trypsins are stable only in alkaline pH (Simpson, 2000). The stability of trypsin at a particular pH might be related to the balance of charge of the enzyme (Castillo-Yanez *et al.*, 2005). A similar pH effect on activity has been reported for trypsin from several fish species (Cai *et al.*, 2011; Castillo-Yanez *et al.*, 2005; Klomklao *et al.*, 2009). Thus, trypsin from hepatopancreas of freshwater prawn might undergo the denaturation under the acidic conditions, where the conformational change took place and enzyme could not bind to the substrate properly. Inactivation at acidic pH is a common phenomenon for anionic trypsins (Kim *et al.*, 1992).

Thermal stability of trypsin from hepatopancreas of freshwater prawn is depicted in Figure 21D. Trypsin from hepatopancreas of freshwater prawn was stable for 15 min when heated up to 40 °C (Figure 21D). The loss in activity was noticeable after incubation at temperature above 40ºC for 15 min. When the enzyme was heated at sufficiently high temperature, the configuration could be altered. As a consequence, the lowered activity could be attained. In the presence of substrate during activity assay, the enzyme might be stabilized to some extent. On the other hand, the enzyme was directly subjected to heat treatment in the absence of substrate for thermal stability study. No trypsin activities remained after heat treatment at 80 °C. After heat treatment at 60 °C, activities of approximately 60% were retained. At high temperatures, enzyme more likely underwent denaturation and lost their activity. The results were in agreement with Kim *et al.* (1992) who reported that trypsin activity of crayfish hepatopancreas was stable up to 40 °C and activity almost disappeared when heated at temperatures higher than 60 °C. Trypsin from the hepatopancreas of Atlantic blue crab was stable at temperatures ranging from 30 to 50 °C for 30 min but the activity was rapidly lost at temperature above 50 °C (Dendinger and O'Connor, 1990). Brun and Wojtowicz (1976) reported that trypsin activity of rock crab hepatopancreas was reduced by 60% after incubation at temperatures 50 °C for 20 min.

Enzymes are inactivated at high temperature due to the partial unfolding of their molecules. The mechanism for increasing thermal stability of proteins appears to be due to strengthening of hydrophobic interactions and disulfide bonds in the interior of the protein molecule (Kim *et al.*, 1994). Numerous disulfide linkages, as well as stronger hydrophobic interactions in the interior of the protein contribute to protein thermal stability. Disulfide bonds may stabilize a folded conformation of trypsin (Klomklao *et al.*, 2009). Fish and shellfish trypsins had the lower thermal stability than those from the mammal, possibly caused by the lower number of intramolecular disulfide bonds in fish and shellfish trypsins, compared with mammal counterpart (Klomklao *et al.*, 2009; Lu *et al.*, 1990). When internal temperature during cooking was not sufficiently high, trypsin is still retained and cause substantial softening of shrimp meat during storage or distribution.

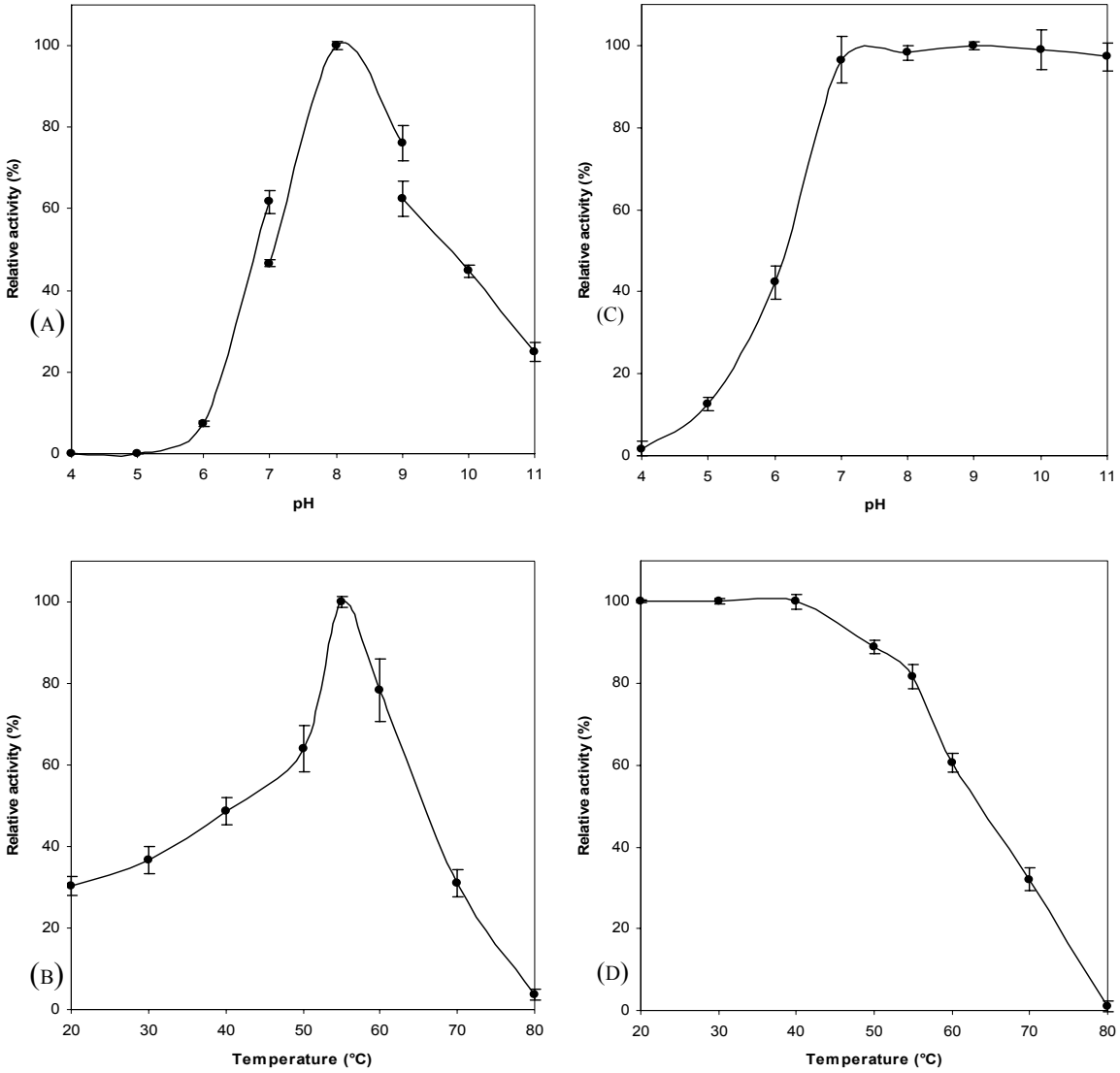

Figure 21. pH and temperature profiles (A and B) and pH and thermal stabilities (C and D) of purified trypsin from hepatopancreas of freshwater prawn.

6.4.5 Effect of inhibitors

Effect of various inhibitors on activity of trypsin from hepatopancreas of freshwater prawn was determined as shown in Table 14. Trypsin was markedly inhibited by trypsin inhibitors such as soybean trypsin inhibitor (99.23% inhibition), TLCK (97.94% inhibition) and pefabloc SC (99.35% inhibition). No differences in %inhibition were found among those three inhibitors ($p>0.05$). Soybean trypsin inhibitor is a single polypeptide that forms a stable stoichiometric enzymically inactive complex with trypsin, thereby reducing the availability of trypsin. TLCK is competitions inhibitor for trypsin. When the enzyme binds to TLCK at active site, the substrate cannot be hydrolyzed. Pefabloc SC or 4-(2-Aminoethyl)-benzenesulfonylfluoride hydrochloride (AEBSF) is an irreversible proteinase inhibitor with broad specificity for serine proteinases. TPCK is a competitive inhibitor of chymotrypsin. Inhibitors for cysteine and aspartic proteases involving E-64 and pepstatin A did not show inhibitory effects towards trypsin activity. No inhibition was also observed when TPCK, a specific inhibitor for chymotrypsin, was used. The result confirms that this purified enzyme was serine proteases, most likely trypsin. Kim *et al.* (1992) reported that the activities of trypsins from hepatopancreas of crayfish were inhibited by soybean trypsin inhibitors and TLCK. The enzymatic activities of two trypsins from the hepatopancreas of Japanese sea bass were also effectively inhibited by pefabloc SC (Cai *et al.*, 2011). EDTA, which chelates the metal ions required for enzyme, partially lowered trypsin activity (9.71% inhibition). Removal of calcium ion might affect trypsin structure, resulting in some losses in activity. The result suggests that trypsin most likely required metal ions as cofactors for activity. It has been reported that calcium ion is required for fish and crustacean trypsins (Klomklao *et al.*, 2009; Zwilling *et al.*, 1969). However, it was noted that soybean trypsin inhibitor inhibited trypsin from hepatopancreas of freshwater prawn effectively. Thus the extracts from soybean or other legumes containing trypsin inhibitor could be used to inhibit the proteolytic activity from hepatopancreas, which was more likely released to flesh and caused the softening of this species.

Table 14. Effect of various inhibitors on the activity of purified trypsin from hepatopancreas of freshwater prawn

Inhibitors	Concentration	% Inhibition
Control	-	0
Pestain A	1 mM	0
E-64	0.1 mM	0
SBTI	1 g/L	99.23±0.02[*a]
TLCK	5 mM	97.94±0.01[a]
PefablocSC	5 mM	99.35±0.03[a]
TPCK	5 mM	0
EDTA	10 mM	9.71±0.17[b]

Enzyme solution was incubated with the same volume of inhibitor at 25 °C for 15 min and the residual activity was determined using Boc-Val-Pro-Arg-MCA as substrate for 10 min at pH 8 and 55 °C.

[*]Means ± SD (n=3).

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

6.4.6 Substrate specificity

Among all fluorescent MCA-substrates tested, Boc-Val-Pro-Arg-MCA and Boc-Leu-Lys-Arg-MCA were hydrolyzed by purified trypsin freshwater prawn more effectively than other specific synthetic substrates. The result suggested that the substrates containing arginine (Boc-Phe-Ser-Arg-MCA) or lysine (Boc-Glu-Lys-Lys-MCA and Boc-Vla-Leu-Lys-MCA) residues at P1 site were more preferable for trypsin (Table 15). Nevertheless, the substrate containing arginine residue was more hydrolyzed than that with lysine residue (Table 15). Similar result was obtained for other fish trypsins (Cai *et al.*, 2011). However, chymotrypsin substrates (Suc-Leu-Leu-Val-Tyr-MCA and Suc-Ala-Ala-Pro-Phe-MCA), cathepsin L substrate (Z-Phe-Arg-MCA) and aminopeptidase substrate (Arg-MCA) were not hydrolyzed by freshwater prawn trypsin. These results strongly indicated that the purified enzyme from hepatopancreas of freshwater prawn was trypsin.

Since trypsin from heapatopancreas of freshwater prawn could cleave at arginine and lysine residues, this enzyme might be responsible for muscle softening of this species during extended iced storage. Freshwater prawn meat had a high content of arginine (34.91 mg/100 g) and lysine (27.11 mg/mg) (Sriket *et al.*, 2011c).

Table 15. Substrate specificity of purified trypsin from hepatopancreas of freshwater prawn on synthetic fluorogenic substrates

Substrates (10 μM)	Relative activity (%)
Boc-Val-Pro-Arg-MCA	100±3.06[*b]
Boc-Phe-Ser-Arg-MCA	72.6±4.45[c]
Boc-Leu-Lys-Arg-MCA	120±2.88[a]
Boc-Glu-Lys-Lys-MCA	38.1±5.43[d]
Boc-Val-Leu-Lys-MCA	17.4±3.55[e]
Arg-MCA	0
Z-Phe-Arg-MCA	0
Suc-Leu-Leu-Val-Tyr-MCA	0
Suc-Ala-Ala-Pro-Phe-MCA	0

Trypsin activity was assayed at pH 8 and 55 °C for 10 min using various MCA-substrates.

[*]Means ± SD (n=3).

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

6.4.7 Kinetic study

Kinetic data for purified trypsin from hepatopancreas of freshwater prawn are summarized in Table 16. K_m and k_{cat} for the hydrolysis of Boc-Val-Pro-Arg-MCA were 0.24 μM and 607.5 s^{-1}, respectively, and the corresponding values for the hydrolysis of Boc-Leu-Lys-Arg-MCA were 0.56 μM and 26.1 s^{-1}, respectively. The catalytic efficiencies (k_{cat}/K_m) for the hydrolysis of Boc-Val-Pro-Arg-MCA and Boc-Leu-Lys-Arg-MCA were calculated to be 2531.5 and 46.72 s^{-1} μM^{-1}, respectively.

When comparing K_m of purified trypsin from hepatopancreas of freshwater prawn with two trypsins from Japanese sea bass hepatopancreas, it was found that trypsin from hepatopancreas of freshwater prawn had the lower K_m values than trypsins from Japanese sea bass (Table 16). The result indicated that trypsin from hepatopancreas of freshwater prawn had a higher affinity for Boc-Val-Pro-Arg-MCA than did sea bass trypsins. For the k_{cat} value, trypsin from hepatopancreas of freshwater prawn also showed higher k_{cat} value on both Boc-Val-Pro-Arg-MCA and Boc-Leu-Lys-Arg-MCA than did trypsins from hepatopancreas of Japanese sea bass. Trypsin from hepatopancreas of freshwater prawn also had higher catalytic efficiencies on both Boc-Val-Pro-Arg-MCA and Boc-Leu-Lys-Arg-MCA than did trypsin from Japanese sea bass. Thus, hepatopancreas trypsin from freshwater

prawn might have higher hydrolytic activity on muscle proteins than did trypsins from hepatopancreas of Japanese sea bass.

Table 16. Kinetic parameters of purified trypsin from hepatopancreas of freshwater prawn and other fish trypsins

Substrates	Trypsins	K_m (μM)*	k_{cat} (s^{-1})*	k_{cat}/K_m (s^{-1} μM^{-1})
Boc-Val-Pro-Arg-MCA	Freshwater prawn	0.24	607.56	2531.51
	Sea bass Trypsin A**	0.58	5.51	9.45
	Sea bass Trypsin B**	0.59	25.75	43.58
Boc-Leu-Lys-Arg-MCA	Freshwater prawn	0.56	26.16	46.72
	Sea bass Trypsin A**	0.39	5.42	14.07
	Sea bass Trypsin B**	0.82	12.19	14.86

*K_m and k_{cat} values of all trypsins were determined at 37 °C under optimal pH.

**Cai *et al.* (2011)

6.4.8 N-terminal sequencing

The N-terminal amino acid sequence of trypsin from hepatopancreas of freshwater prawn was aligned to compare with those of other trypsins as depicted in Figure 22. The N-terminal amino acid sequence of the first 20 amino acids of purified trypsin was determined to be IVGGDEAAPGEFPHQISMQV (Figure 22). When N-terminal amino acid sequences of purified trypsin were compared with those of other shrimp, fish and three mammals, it was found that the sequence of the purified trypsin from hepatopancreas of freshwater prawn displayed high homology to that of black tiger prawn trypsin, especially the sequence from the first to fourth (IVGG) and ninth to thirteenth (PGEFP) residues. However, trypsin from hepatopancreas of freshwater prawn and all fish trypsins had a charged Glu residue at position 6, whereas Thr is most common in mammalian pancreatic trypsin (Figure 22). Moreover, the sequences of trypsin from hepatopancreas of freshwater prawn and other trypsins started with IVGG after the proteolytic cleavage of inactive trypsinogen. The result confirmed that trypsin from hepatopancreas of freshwater prawn was most likely a member of the trypsin family.

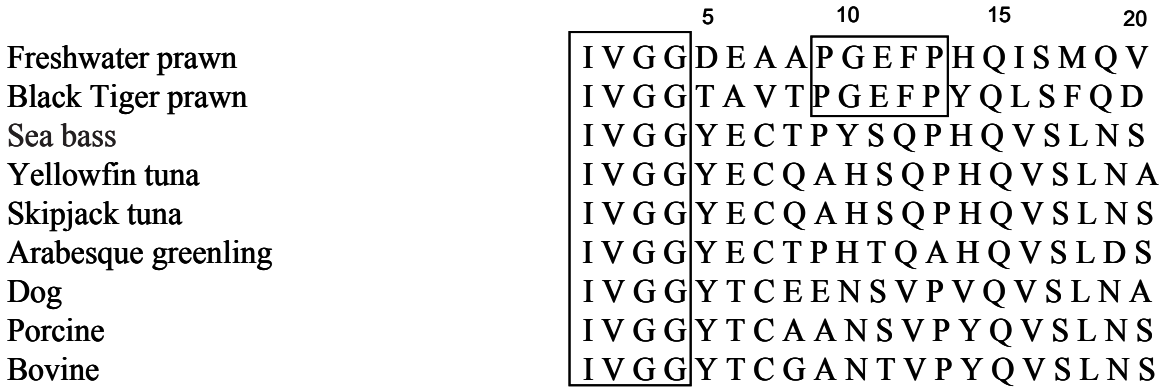

Figure 22. Comparison of N-terminal amino acid sequence of the purified trypsin from hepatopancreas of freshwater prawn with other trypsins. Black tiger prawn (Lu *et al.*, 1990), sea bass (Cai *et al.*, 2011), yellowfin tuna (Klomklao *et al.*, 2006b), skipjack tuna (Klomklao *et al.*, 2009), arabesque greenling (Kishimura *et al.*, 2006), dog (Pinsky *et al.*, 1985), porcine (Hermodson *et al.*, 1973) and bovine (Walsh, 1970). Same amino acid residues were boxed.

6.4.9 Collagen hydrolysis by purified trypsin from hepatopancreas of freshwater prawn

Protein patterns of PSC extracted from freshwater prawn and white shrimp muscle incubated with purified trypsin from hepatopancreas of freshwater prawn at 25 °C for 0–15 min are shown in (Figure 23A). Marked degradation of α-chain was found after 5 min of hydrolysis, with concomitant occurrence of degradation products having a lower molecular mass (Figure 23A). Protein pattern of ASC type I extracted from yellowtail muscle incubated with purified trypsin from hepatopancreas of freshwater prawn is depicted in Figure 23B. The degradations of β and α-chains were observed after 5 min of incubation. The decrease in band intensity of β and α-chains became more pronounced with increasing incubation time.

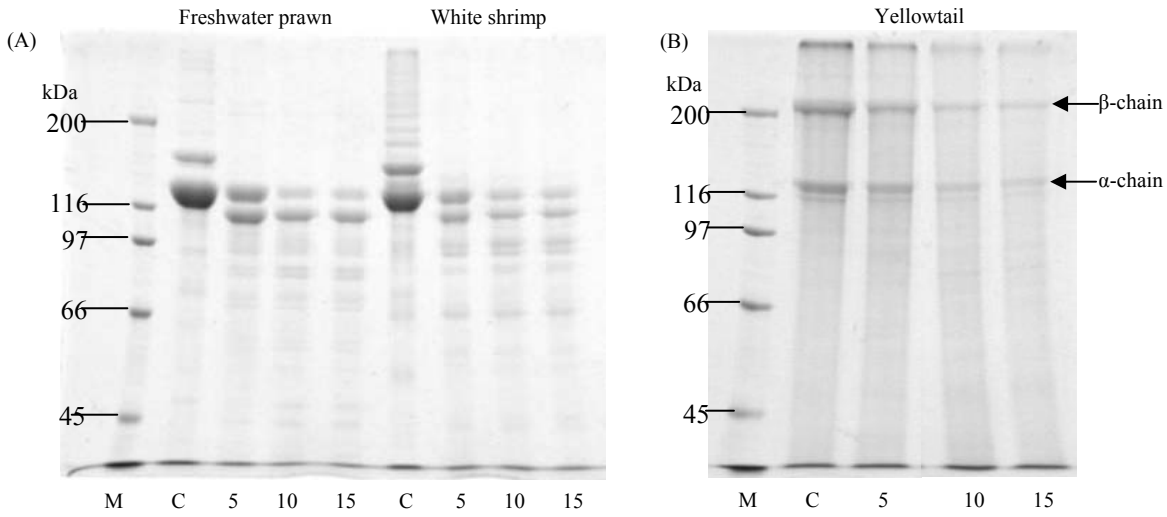

Figure 23. Protein patterns of pepsin soluble collagen from freshwater prawn and white shrimp (A) and acid soluble collagen from yellowtail (B) incubated with trypsin from hepatopancreas of freshwater prawn at 25 °C for different times. M: molecular weight marker, C: collagen incubated without trypsin for 15 min. Numbers denote the incubation time (min) at 25 °C.

These results suggested that trypsin from hepatopancreas of freshwater prawn had high collagenolytic activity toward prawn and fish collagens. This result was in agreement with Aoki *et al.* (2003) who found that proteases from Northern shrimp (*Pandalus eous*) were able to hydrolyze collagen. In general, collagens in fish and shellfish muscle contain lower cross-links than those from warm-blooded vertebrates. Collagens from fish and shellfish are more heat labile than land animal collagen (Hernandez-Herrero *et al.*, 2003). Therefore, trypsin released from freshwater prawn hepatopancreas may play a role in postmortem degradation of prawn muscle during extended iced storage.

6.5 Conclusion

Low molecular mass trypsin from hepatopancreas of freshwater prawn showed high hydrolytic activity at pH 8.0 and 55 °C. Based on substrate specificity, inhibitor study and N-terminal sequencing, enzyme purified was found to belong to trypsin family. Trypsin from freshwater prawn also showed high hydrolytic activity toward prawn collagen. Therefore, trypsin released from hepatopancreas more likely play a role in muscle softening of this species during extended iced storage.

CHAPTER 7

Effect of legume seed extracts on the inhibition of proteolytic activity and muscle degradation of freshwater prawn (*Macrobrachium rosenbergii*)

7.1 Abstract

Trypsin inhibitors in the extracts from soybean (*Glycine max*), adzuki bean (*Vigna angularis*), bambara groundnut (*Vigna subterranea*) and red kidney bean (*Phaseoulus vulgaris*) varied in amount and molecular mass. The soybean extract had the highest level of trypsin inhibitor with molecular mass of 21 kDa, followed by bambara groundnut extract possessing trypsin inhibitor with molecular mass of 15 kDa. Both extracts showed a more effective inhibition towards crude protease extract (CE) from the hepatopancreas of freshwater prawn (*Macrobrachium rosenbergii*) than the extracts from adzuki and red kidney beans. Activity staining also reconfirmed the higher inhibitory activity on CE from hepatopancreas by the extracts from both soybean and bambara groundnut. The extracts from all seeds were able to inhibit the degradation of freshwater prawn meat containing CE in a concentration dependent manner. Based on inhibitor study, the extracts from soybean and bambara groundnut can be a potential aid to suppress the muscle softening of freshwater prawn, mediated by trypsin-like proteases released from hepatopancreas, during extended iced storage.

7.2 Introduction

Muscle softening is a term used to describe the degradation of fish and shellfish meat during post-mortem storage. This phenomenon is mainly caused by endogenous proteolytic enzymes in fish and shellfish (1988). According to the hydrolysis mechanism, proteases are classified as serine, cysteine, aspartic and metallo proteases (Garcí a-Carreno, 1992). Among these proteases, serine-like proteases are mostly associated with muscle softening in fish and shellfish (Ezquerra Brauer *et al.*, 2003) including common carp (*Cyprinus carpio*) (Wu *et al.*, 2008), red sea bream (*Pagrus major*) (Wu *et al.*, 2010) and Mexican flounder (*Cyclopsetta chittendeni*) (Ramirrez *et al.*, 2002). Recently, serine protease has been reported to contribute to the softening of freshwater prawn (*Macrobrachium*

rosenbergii) during iced storage (Sriket *et al.*, 2011b). Freshwater prawn is widely consumed in Thailand due to its delicacy. However, this species is not successfully marketable because of the textural problem. Serine collagenolytic protease released from hepatopancreas was detected in freshwater prawn meat with increasing storage time (Sriket *et al.*, 2011b). This enzyme cleaved collagen and degraded myofibrillar protein effectively, leading to muscle softening (Sriket *et al.*, 2011a; Sriket *et al.*, 2011b). In order to tackle the softening problem in freshwater prawn meat, the use of protease inhibitors, especially food grade or natural inhibitors, can be a promising means to alleviate such a problem and can maintain the textural quality of this species. Several protein additives containing natural protease inhibitors including beef plasma protein, potato powder and egg white have been used to improve the properties of surimi gels (Porter *et al.*, 1993; Reppond and Babbitt, 1993). However, the extracts of leguminous seeds containing protease inhibitors were also reported to prevent modori gel of surimi (Benjakul *et al.*, 1999; Benjakul *et al.*, 2000a). Nevertheless, no information regarding the use of trypsin inhibitors from leguminous seed on the retardation of muscle softening of freshwater prawn has been reported. Therefore, the aims of this research were to characterize the protease inhibitors from leguminous seeds and to investigate the inhibitory effect on the degradation of muscle proteins in freshwater prawn mediated by proteases from hepatopancreas, more likely released during extended storage.

7.3 Materials and methods

7.3.1 Chemicals

β-mercaptoethanol (βME), casein, tyrosine, α-*N*-benzoyl-DL-arginine-*ρ*-nitroanilide (DL-BAPNA) and *N*-succinyl-Ala-Ala-Phe-*ρ*-nitroanilide (SAAPNA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Tris (hydroxymethyl) aminomethane (Tris) and trichloroacetic acid were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), Coomassie Brilliant Blue R-250 and *N,N,N′,N′*-tetramethylethylene diamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). All chemicals were of analytical grade.

7.3.2 Preparation of extract from legume seeds

Soybean (*Glycine max*), adzuki bean (*Vigna angularis*), red kidney bean (*Phaseoulus vulgaris*) and bambara groundnut (*Vigna subterranea*) were purchased from the market in Hat Yai, Songkhla, Thailand. The seed extracts were prepared according to the method of Benjakul *et al.* (2000b) with slight modifications. Dry seeds were ground using a

coffee mill to a particle size of 20 mesh. The seed flour was defatted by mixing with 5 volumes of hexane (w/v) for 10 min and filtered through Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, England). The sediment was rinsed with hexane 3 times to remove the residual oil and the defatted seed flour was air-dried at room temperature (28–30 °C). The defatted seed flour was mixed with 0.15 M NaCl at a ratio of 1:10 (w/v) and shaken at180 rpm at room temperature for 3 h using a shaker (Heidolph UNIMAX 1010, Schwabach, Germany). The extracts were recovered by centrifuging at 5000 g for 30 min at 4 °C using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Palo Alto, CA, USA). To partially purify the protease inhibitors, the extracts were heated at 90 °C for 10 min and cooled down with iced water. The coagulated debris was removed by centrifuging at 8000 *x g* for 5 min. The supernatants were freeze-dried using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark) and used for further study.

7.3.2.1 Trypsin and chymotrypsin inhibitory activity assays

Trypsin (from bovine pancreas) inhibitory activity was determined using BAPNA as a substrate according to the method of Benjakul *et al.* (2000b) with slight modifications. One unit of trypsin inhibitory activity was defined as the amount of inhibitor, which reduces the trypsin activity by 1 U. One unit of trypsin activity was defined as an increase of 0.01 A410 due to *ρ*-nitroaniline released.

For the determination of chymotrypsin (from bovine pancreas) inhibitory activity, the assay was performed using SAAPNA as a substrate according to the method of Hummel (1959) with slight modifications. One unit of chymotrypsin inhibitory activity was defined as the amount of inhibitor, which reduces the chymotrypsin activity by 1 U. One unit of chymotrypsin activity was defined as an increase of 0.01 A410 due to *ρ*-nitroaniline released.

7.3.2.2 Inhibitory activity staining of trypsin inhibitor from legume seed extracts

Extracts from various legume seeds were separated on SDS–PAGE, followed by inhibitory activity staining using casein as a substrate according to the method of Benjakul *et al.* (2000b). Proteins (15 µg), determined by the Biuret method (Robinson and Hogden, 1940), were loaded onto the gel made of 4% stacking and 12% separating gels. The electrophoresis was run at a constant current of 15 mA per gel by a Mini-Protein II Cell apparatus (Bio-Rad, Hercules, CA, USA). After electrophoresis, the gels were washed in

2.5% Triton X-100 for 15 min to remove SDS and renature the proteins. The gels were then washed with distilled water before soaking in trypsin (from bovine pancreas) solution (0.5 mg/mL) at 4 °C for 1 h. The gels were then washed again with distilled water and incubated with 1% casein in 50 mM Tris–HCl, pH 7.5 containing 10 mM $CaCl_2$ for 2 h at 37 °C. The gels were washed again with distilled water, fixed and stained with Coomassie Blue R-250. After destaining, the bands with inhibitory activity were compared with the control gel and molecular weight markers, directly subjected to staining and destaining after electrophoresis.

7.3.3 Inhibitory activity of legume seed extracts toward proteases from hepatopancreas of freshwater prawn

7.3.3.1 Preparation of crude extract from hepatopancreas of freshwater prawn

Freshwater prawns (*M. rosenbergii*) with the size of 30–35 prawn/kg were obtained from a farm in Phatthalung province, Thailand. The prawn were placed in ice with a prawn/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla, Thailand, within approximately 2 h. Upon arrival, the prawns were washed with clean cold water. Hepatopancreas was removed manually from the cephalothorax and powderized with liquid nitrogen. The powder was packed in polyethylene bag, immediately frozen and stored at -20 °C until used. The samples were stored for not longer than 1 month at -20 °C.

To prepare the crude extract, hepatopancreas powder (50 g) was mixed with 100 mL of 0.01 M sodium phosphate buffer (pH 7.6) (Sriket *et al.*, 2011b). The mixture was homogenized for 2 min using an IKA Labortechnik homogenizer (Selangor, Malaysia) at a speed of 11,000 rpm. The homogenate was stirred for 30 min, followed by centrifugation at 10,000 g for 30 min using a refrigerated centrifuge. All procedures were carried out at 4 °C. The supernatant was referred to as ‘crude extract; CE’ and used for the assays of proteolytic activities.

7.3.3.2 Protease inhibition assay

The effects of extracts from different legume seeds toward the proteolytic activity of CE from hepatopancreas were determined using casein as substrate. CE was mixed with an equal volume of legume seed extract solutions to obtain the designated final concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 mg protein/mL). The mixture was incubated at room temperature (25–28 °C) for 15 min. The remaining proteolytic activity was determined at pH

7 and 60 °C for 15 min (Sriket *et al.*, 2011b). The percent inhibition was then calculated using the following formula:

$$\% \text{ Inhibition} = 100 - \left(\frac{\text{As} \times 100}{\text{Ac}} \right)$$

where As and Ac are the proteolytic activity of sample treated with legume seed extract and that of the control, respectively. The control was conducted in the same manner except that deionized water was used instead of legume seed extracts.

7.3.3.3 Activity staining

CE from hepatopancreas was pre-incubated with various legume seed extracts for 15 min at room temperature before separating using SDS–PAGE. The mixtures (20 μg protein) were loaded onto the gel and subjected to electrophoresis as previously described. After electrophoresis, the gels were washed twice in 100 mL of 2.5% Triton X-100 for 15 min. The washed gel was then immersed in 100 mL of 50 mM Tris–HCl buffer, pH 7.5, containing 1% casein for 1 h with constant agitation at 0 °C to allow the substrate to penetrate into the gels. Thereafter, the gels were transferred to 1% casein (w/v) in 50 mM Tris–HCl buffer containing 10 mM $CaCl_2$ (pH 7). The mixtures were incubated at 60 °C for 2 h with constant agitation. The gels were rinsed using cold distilled water for 15 min (twice). The gels were then fixed and stained with 0.125% Coomassie Blue R-250 in 50% methanol and 10% acetic acid and destained in 30% methanol and 10% acetic acid. The development of clear zones on blue background indicated proteolytic activity. The inhibition of proteolytic activity by each legume seed extract was identified by the reduction of clear zones, compared with that of control (without legume seed extract).

7.3.4 Effect of legume seed extracts on the hydrolysis of natural actomyosin (NAM) and pepsin soluble collagen (PSC) mediated by hepatopancreas crude extract

7.3.4.1 Preparation of natural actomyosin (NAM) and pepsin soluble collagen (PSC)

NAM was prepared according to the method of Benjakul *et al.* (1997). Pepsin soluble collagen (PSC) from freshwater prawn meat was isolated according to the method of Sriket *et al.* (2007).

7.3.4.2 Inhibition of hydrolysis of NAM and PSC mediated by hepatopancreas proteases by legume seed extracts

CE (200 μL) was mixed with an equal volume of legume seed extracts to obtain the different concentrations (0.5, 1.0 and 1.5 mg protein/mL). The mixtures were then added into the suspension of NAM or PSC (4 mg in 825 μL of 50 mM Tris–HCl, pH 7.0). The hydrolysis was conducted by incubating the mixture at 60 °C for NAM and at 25 °C for PSC. The hydrolysis time was 60 min. The control was performed in the same manner for 60 min except distilled water was added instead of legume seed extracts. The reaction was terminated by adding the preheated solution containing 2% SDS, 8 M urea and 2% βME (85 °C). The mixtures were further incubated at 85 °C for 60 min to completely solubilize total proteins. The mixtures were centrifuged at 5000g for 10 min at room temperature to remove the debris. The supernatants were then subjected to SDS–PAGE using 4% stacking gel and 10% (for NAM) and 7.5% (for PSC) separating gel, as previously described.

7.3.5 Statistical analysis

The experiments were run in triplicate with three different lots of samples. Data were subjected to analysis of variance and mean comparison was carried out using Duncan's multiple range test (DMRT) (Steel and Torrie, 1980). Statistical analyses were performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

7.4. Results and discussion

7.4.1 Trypsin and chymotrypsin inhibitors from legume seed extracts

Trypsin and chymotrypsin inhibitory activities in the extracts from various legume seeds are shown in Table 17. Generally, all legume seed extracts contained trypsin inhibitors, but the amount varied with seed variety. Among all seed extracts, soybean extract showed the highest trypsin inhibitory activity (37.19 unit/mg protein) ($p< 0.05$), followed by bambara groundnut extract (32.55 unit/mg protein). Lower trypsin inhibitory activity was found in extracts from red kidney bean (18.76 unit/mg protein) and adzuki bean (18.24 unit/mg protein) ($p<0.05$) (Table 17). Trypsin inhibitors in legume seeds can be varied in types and levels, depending on the cultivars, climate, processing, etc (Van der Poel, 1990). The higher trypsin inhibitory activity of soybean extract, compared with other legume seed extracts (white cowpea, mungbean and peanut) was also reported (Benjakul *et al.*, 1999).

For chymotrypsin inhibitory activity, it was found only in red kidney bean extract (12.62 unit/mg protein) (Table 17), while the extracts from other seeds had no inhibitory activity. This result was coincidental with Wu and Whitaker (1990) who reported

that both tryspin and chymotrypsin inhibitors were present in red kidney bean. Nevertheless, Ramirez *et al.* (2002) found chymotrypsin inhibitors in both soybean and red kidney bean extracts. The difference in types and amount of inhibitors between the present result and other reports might be due to the different cultivars, climate, variety or other factors, which can influence the production of inhibitors in legume seeds. Moreover, different extraction processes might be another factor determining the types of inhibitors in the resulting extract.

Table 17. Trypsin and chymotrypsin inhibitors in different legume seed extracts

Seed extracts	Trypsin inhibitory activity* (units/mg protein)	Chymotrypsin inhibitory activity* (units/mg protein)
Soybean	$37.19 \pm 0.09^{\dagger a\dagger\dagger}$	ND
Adzuki bean	18.24 ± 0.21^{c}	ND
Red kidney bean	18.76 ± 0.05^{c}	12.62 ± 2.24
Bambara groundnut	32.55 ± 2.41^{b}	ND

ND: non-detectable.

* Trypsin inhibitor activity was assayed using BAPNA as a substrate and chymotrypsin inhibitor activity was assayed using SAAPNA as a substrate.

† Values are mean ± standard deviation (n = 3).

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

7.4.2 Protein pattern of extracts and trypsin inhibitors from various legume seeds

Protein patterns of the extracts from soybean, adzuki bean, red kidney bean and bambara groundnut are shown in Figure 24A. Soybean extract consisted of several protein bands with the major band having molecular mass ranging from 50 to 95 kDa. Liu *et al.* (2007) reported that 7S protein with molecular mass ranging from 44 to 91 kDa was found in soybean. Two major protein bands of adzuki bean extract were observed with molecular mass of 49 and 55 kDa. The extract of red kidney bean contained protein with 45 and 97 kDa as the dominant protein, while the extract of bambara groundnut showed two major protein bands with molecular mass of 47 and 57 kDa.

Based on trypsin inhibitory activity staining, trypsin inhibitors with different apparent molecular mass were found in different legume seed extracts (Figure 24B). For soybean extract, trypsin inhibitor with the molecular mass of 21 kDa was noticeable. An inhibitor with the apparent molecular mass of 14 kDa was found in adzuki bean extract. Trypsin inhibitors with 18 and 15 kDa were found in the extracts of red kidney bean and bambara groundnut, respectively. Generally, protease inhibitors found in legume seed have

molecular mass ranging from 14 to 66 kDa (Garcia-Carreno *et al.*, 1996). Soybean has been known to contain two main classes of serine protease inhibitors, namely Kunitz-type inhibitors and Bowman-Birk inhibitors (Benjakul *et al.*, 1999). Bowman-Birk type inhibitors have molecular mass of 8 to 10 kDa, whereas Kunitz type inhibitors usually have molecular mass of 18 to 22 kDa (Laskowski Jr and Qasim, 2000). Two protease inhibitors, including trypsin-chymotrypsin inhibitor and trypsin inhibitor (Bowman-Birk type) with molecular mass of 15 and 10.5 kDa, respectively, were found in pigeon pea (Godbole *et al.*, 1994). A Kunitz type trypsin inhibitor with molecular mass of 19.81 kDa was present in *Cassia obtusifolia*, seed (Liao *et al.*, 2007). Recently, Klomklao *et al.* (2010) also reported the Bowman-Birk type trypsin inhibitor in adzuki bean.

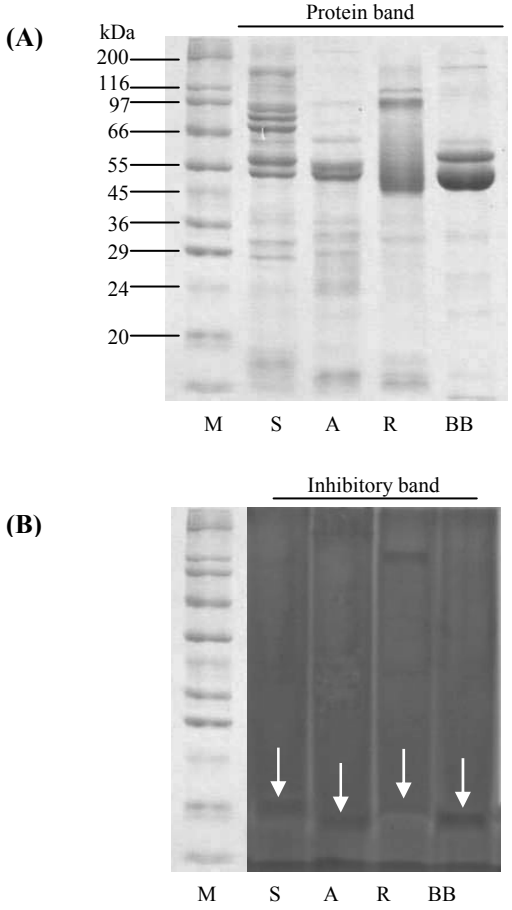

Figure 24. Protein pattern (A) and inhibitory activity staining (B) of extracts from different legume seeds. M, molecular weight marker; S, soybean extract; A, adzuki bean extract; R, red kidney bean extract; BB, bambara groundnut extract. Arrows indicated the inhibitor bands.

7.4.3 Inhibitory activity of legume seed extracts on proteolytic activity of CE from freshwater prawn hepatopancreas

Inhibition toward proteolytic activity of CE from freshwater prawn hepatopancreas by the extracts from legume seed extracts at different concentrations is depicted in Figure 25. The extracts from various legume seeds showed the different inhibitory activities against protease in CE (Figure 25). Generally, inhibitory activity of all legume seed extracts increased with increasing concentrations up to 1.0 mg protein/mL and remained unchanged at the higher concentrations (1.5-2.5 mg protein/mL). At the same concentration used, soybean extract showed the highest inhibitory activity ($p < 0.05$), followed by the extracts of bambara groundnut, red kidney bean and adzuki bean, respectively. It was noted that soybean and bambara groundnut extracts at a concentration of 1.0 mg protein/mL inhibited protease activity in CE from freshwater prawn hepatopancreas more than 60 % (Figure 25). The result indicated that extracts from soybean and bambara groundnut were able to inhibit the proteolytic activity, mainly trypsin-like enzyme, from freshwater prawn hepatopancreas effectively. Serine protease mainly trypsin-like protease was reported to be the major proteases in freshwater prawn hepatopancreas, causing the softening of muscle during extended iced storage (Sriket *et al.*, 2011b). The high inhibitory activity was in agreement with the high amount of trypsin inhibitor present in soybean and bambara groundnut extracts (Table 17). The higher inhibitory activity of extracts from soybean and bambara groundnut reflected the higher specificity of inhibitors in both extracts in inhibiting proteases in CE from hepatopancreas of freshwater prawn, compared with the extracts of red kidney bean and adzuki bean. Thus the extracts from soybean and bambara groundnut can be the potential inhibitors for proteases from hepatopancreas, which was more likely released to flesh and caused the softening of this species.

The activity staining of CE from freshwater prawn hepatopancreas in the absence and presence of extracts from various legume seeds is shown in Figure 26. Generally, three major bands with proteolytic activities having molecular mass of 86(a), 41(b) and 17(c) kDa were observed in CE without legume seed extracts (lane C) (Figure 26). In the presence of 5 mM TLCK, those activity bands were completely inhibited (data not shown). From inhibitor study using different protease inhibitors (TLCK, TPCK, EDTA, E-64 and pepstatin A), TLCK, a specific trypsin inhibitor, showed the highest inhibitory activity toward protease in freshwater prawn hepatopancreas (Sriket *et al.*, 2011b). Therefore, those three activity bands most likely represented trypsins.

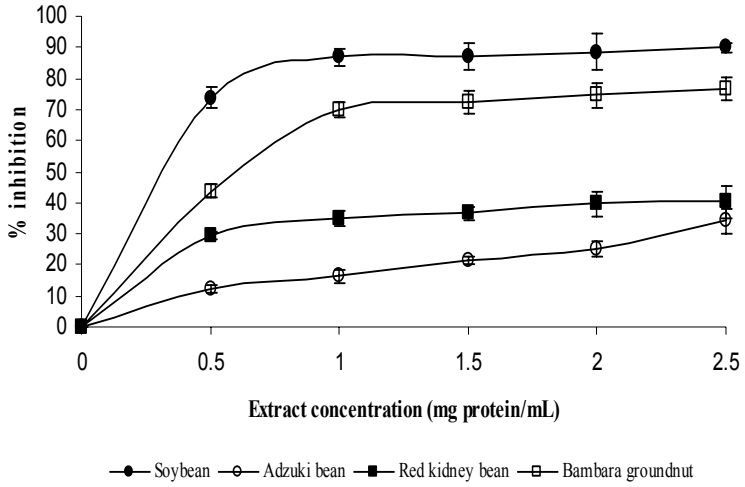

Figure 25. Effect of extracts from different legume seeds at various concentrations on the inhibition of proteolytic activity from freshwater prawn hepatopancreas. The reaction was performed for 1 h at 60 ºC using casein as substrate. Bars represent standard deviation (n = 3).

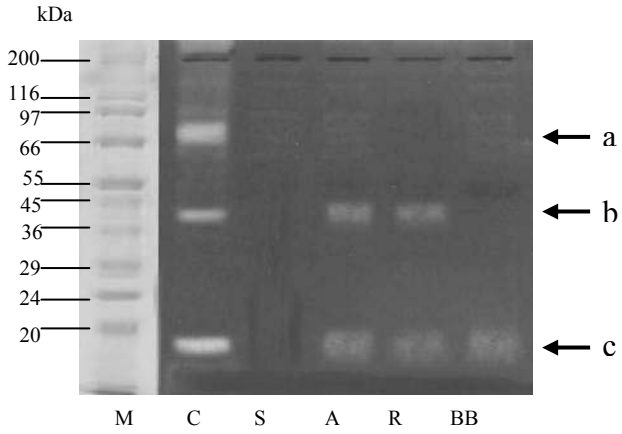

Figure 26. Activity staining of CE from freshwater prawn hepatopancreas in the absence and presence of the extracts from different legume seeds. The incubation was performed for 2 h at 60 ºC using casein as substrate. The concentration of extracts was 1.5 mg protein/mL. M, molecular weight marker; C, CE without legume extract; S, CE with soybean extract; A, CE with adzuki bean extract; R, CE with red kidney bean extract; BB, CE with bambara groundnut extract.

Inhibitory effect of legume seed extracts on proteolytic activity of CE was visualized by a decrease in the intensity of active proteolytic bands. Proteolytic bands of CE were decreased with the addition of legume seed extracts (lane S, A, R and BB). However, the degree of inhibition varied with seed extracts. The extract from soybean suppressed all

proteolytic bands of CE completely, while bambara groundnut extract dramatically inhibited bands with molecular mass of 86 and 41 kDa and partially inhibited the activity band with molecular mass of 17 kDa. Both extracts from red kidney bean and adzuki were able to inhibit the activity band with molecular mass of 86 kDa (a) completely. Activity bands with molecular mass of 41 and 17 kDa were partially inhibited by the extracts from red kidney bean and adzuki bean. The high inhibition on digestive proteases from tilapia (*Oreochromis niloticus*) by soybean extract was also reported (Moyano Lopez *et al.*, 1999).

The higher inhibition of proteolytic activity band of CE from hepatopancreas by the extracts from soybean and bambara groundnut was in accordance with the higher trypsin inhibitor found in both extracts (Table 17), compared with the extracts from red kidney bean and adzuki bean. However, the efficacy of trypsin inhibitors in all extracts tested varied, especially in term of specificity toward the proteases present in the CE. Soybean extract could inhibit all three dominant proteases in CE and was shown as the most potential inhibitor to control proteolysis caused by proteases from hepatopancreas.

7.4.4 The inhibition of degradation of NAM and PSC from freshwater prawn muscle mediated by proteases in CE by various legume seed extracts

Protein patterns of NAM incubated with CE from hepatopancreas in the absence and presence of legume seed extracts at different concentrations at 60 °C for 1 h are shown in Figure 27. NAM, extracted from freshwater prawn muscle, contained myosin heavy chain (MHC) as the most dominant protein, followed by actin. The pronounced degradation of both MHC and actin in NAM was observed in the absence of legume seed extracts (lane C) (Figure 27), indicating the high hydrolytic activity of CE toward muscle proteins. No degradations of MHC and actin were observed in NAM incubated with CE in the presence of soybean extract at all concentrations used (0.5-1.5 mg protein/mL). At a level of 1.5 mg protein/mL, the band intensity of MHC and actin of NAM added with extract from bambara groundnut was similar to that observed in NAM added with soybean extract (0.5-1.5 mg protein/mL). When the extracts of adzuki bean and red kidney bean were incorporated, the degradation of both MHC and actin was inhibited, depending upon the concentration used. At the same concentration used, the former exhibited slightly higher inhibitory activity as evidenced by higher band intensity of MHC and actin retained (Figure 27). However, no marked differences in band intensity were observed in NAM added with the extract of adzuki bean at level of 1.0 and 1.5 mg protein/mL. The result suggested that legume seed extracts could prevent the degradation of muscle proteins caused by proteases released from

hepatopancreas, particularly when the storage or distribution time increased. Soybean extract was shown to have the highest efficacy in prevention of such a degradation.

When PSC was incubated with CE from freshwater prawn hepatopancreas at 25 °C for 1 h, the degradation of α-chain took place (Figure 28). The coincidental formation of bands with molecular mass of 69 and 66 kDa was observed. In the presence of legume seed extracts at different concentrations, the most retained α-chain was found in PSC incubated with CE in the presence of soybean extract. However, the degradation still occurred to some extent, even in the presence of soybean extract (1.5 mg protein/mL) as shown by the occurrence of protein with molecular mass of 69 and 66 kDa. For PSC incubated with CE in the presence of extracts from red kidney bean and bambara groundnut, inhibition of collagen hydrolysis was in a concentration dependent manner. In general, the extracts with higher concentrations yielded the higher inhibitory effect as indicated by higher band intensity of α-chain retained (Figure 28). At a level of 1.5 mg protein/mL, the extract of adzuki bean had the lowest efficiency in prevention of α-chain hydrolysis caused by proteases in CE of hepatopancreas. Therefore, the preventive effect of seed extracts varied with variety and soybean extract was shown to have the highest inhibitory effect on PSC degradation caused by CE. Nevertheless, the extract of bambara groundnut could be also used to prevent such a phenomenon, but the sufficient concentration was required.

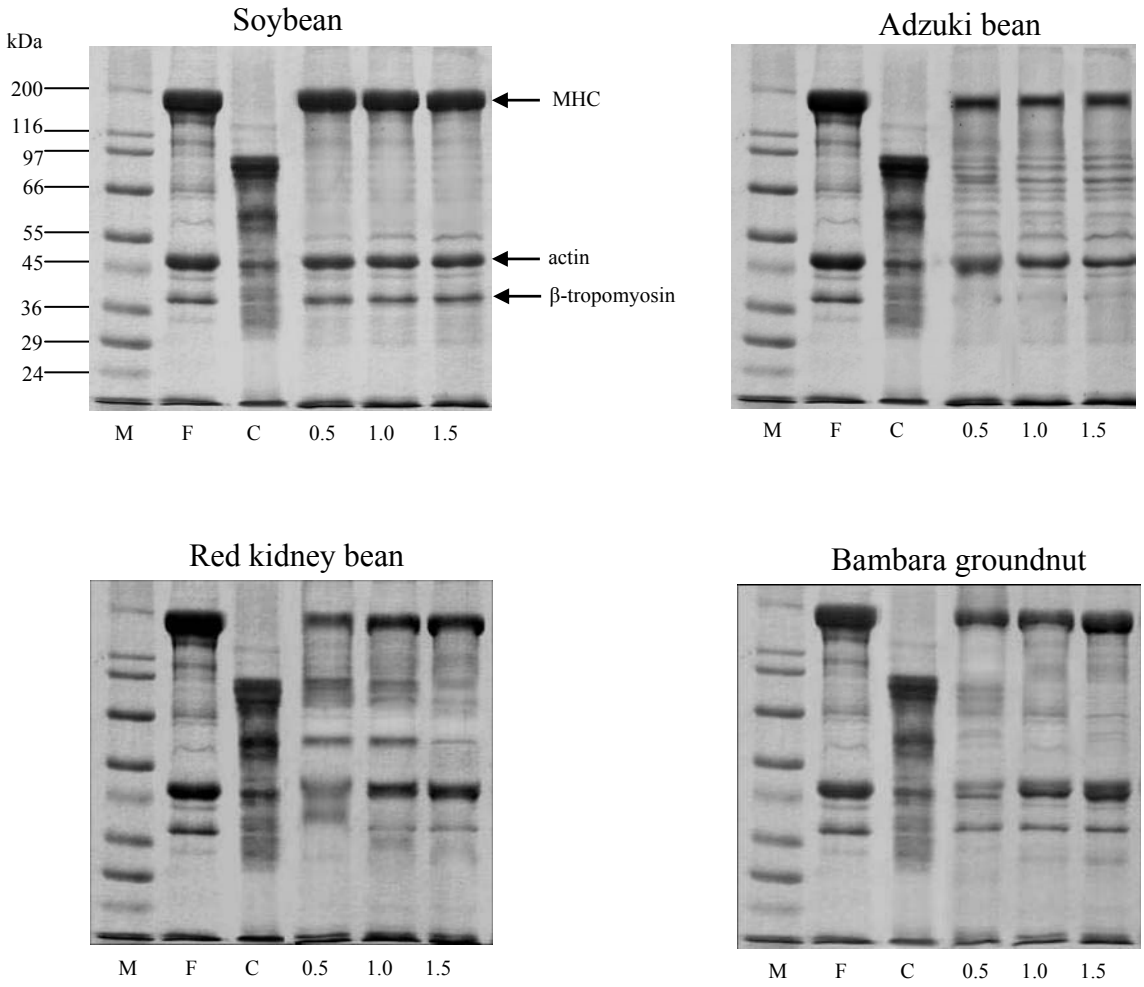

Figure 27. Protein pattern of natural actomyosin (NAM) incubated with CE from hepatopancreas in the absence and presence of different legumes extracts at various concentrations at 60 °C for 1 h. M, molecular weight marker; F, NAM without incubation; C, NAM incubated with CE (without legume extract). Numbers denoted the concentration of legume extract used (mg protein/mL). MHC, myosin heavy chain.

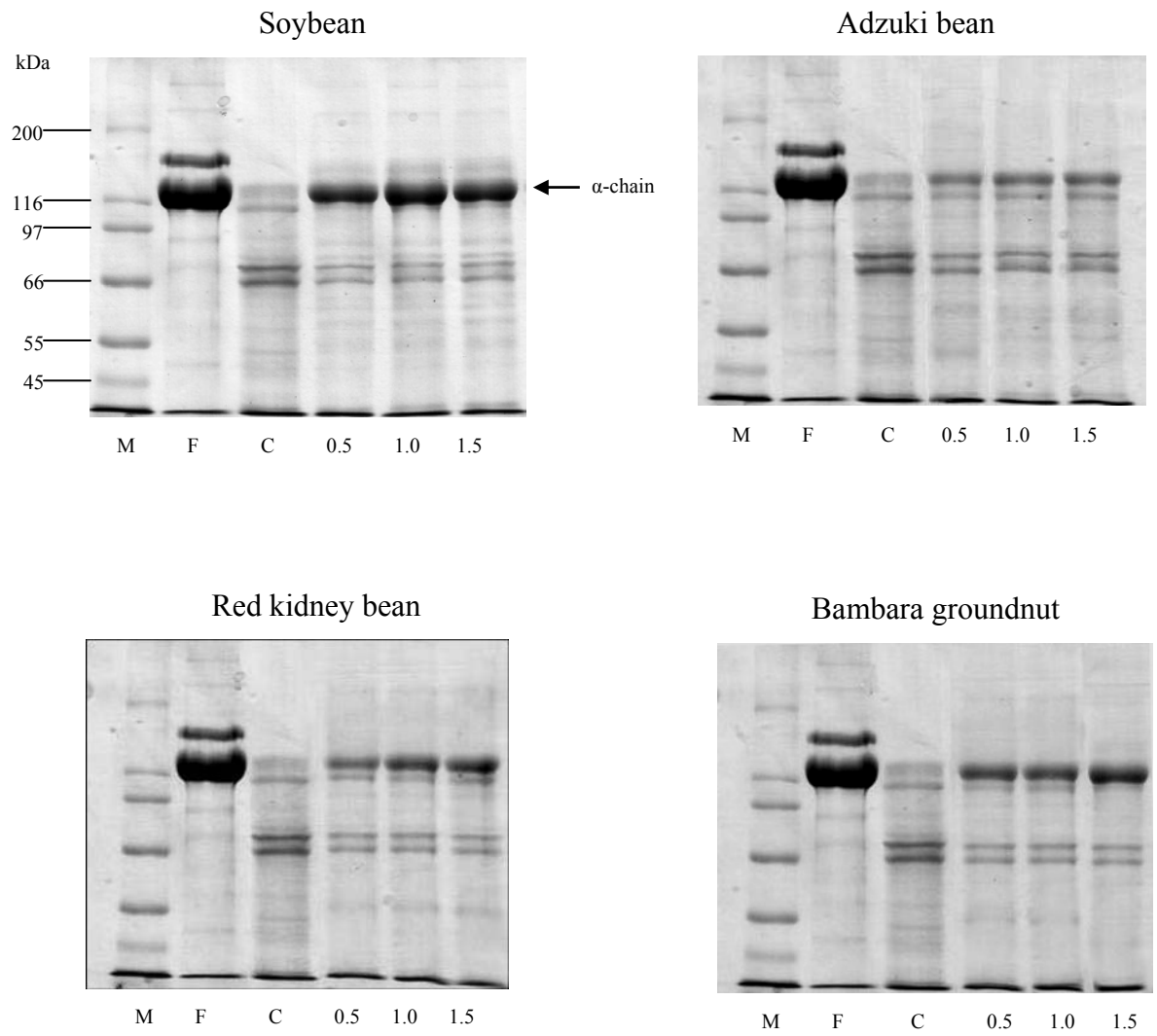

Figure 28. Protein pattern of pepsin soluble collagen (PSC) incubated with CE from hepatopancreas in the absence and presence of different legumes extracts at various concentrations at 25 °C for 1 h. M, molecular weight marker; F, PSC without incubation; C, PSC incubated with CE (without legume extract). Numbers denoted the concentration of legume extract used (mg protein/mL).

7.5 Conclusions

Extracts from all legume seeds used in this study contained trypsin inhibitors at different contents. Inhibition toward proteases in hepatopancreas depended on specificity and concentration of the extracts used. The extracts of soybean (0.5 mg protein/mL) and bambara groundnut (1.5 mg protein/mL) suppressed the degradation of NAM and PSC mediated by CE from freshwater prawn hepatopancreas effectively, while the extracts from red kidney bean showed the lower inhibitory effect. The extract of adzuki bean had the lowest

inhibitory activity even at the highest concentration used. Legume seed extract, especially from soybean, can be used to prolong the shelf life of freshwater prawn by retarding the degradation, in which firm texture and consumer acceptability can be maintained during handling and distribution.

CHAPTER 8

Retardation of post-mortem changes of freshwater prawn (*Macrobrachium rosenbergii*) stored in ice by legume seed extracts

8.1 Abstract

Meat quality of freshwater prawn (*Macrobrachium rosenbergii*) treated with soybean and bambara groundnut extracts at different concentrations are monitored during 10 days of iced storage. During the storage, the control sample (without treatment) had higher pH, TCA-soluble peptide content, heat soluble collagen content, proteolytic activities and psychrophilic bacteria count than did samples treated with soybean and bambara groundnut extracts. Conversely, shear force value and likeness scores of the control sample decreased ($p<0.05$), more likely associated with softening of muscle. The decrease in myosin heavy chain in the control sample was found after 6 days of storage. However, no changes in protein patterns of samples treated with soybean extracts at 2.5 mg/mL were found after 10 days of storage. Therefore, the injections of legume seed extracts, especially soybean extract, at a sufficient concentration, could be a means to retard muscle softening and maintain the qualities of freshwater prawn during iced storage.

8.2 Introduction

Freshwater prawn (*Macrobrachium rosenbergii*) had been cultured worldwide to meet increasing consumer demands. However, the production and consumption of freshwater prawn in Thailand are still limited due to the inconsistent market price and rapid post-mortem quality loss. Textural deterioration has been considered as the most crucial problem limiting prawn shelf-life and impeding its marketing. Generally, freshwater prawn are distributed or stored in ice, where “softening” caused by proteolytic enzymes occurs (Sriket *et al.*, 2010). Softening of fish and shellfish muscle during iced storage is one of the important factors influencing the acceptability and market value. This phenomenon found in post-mortem freshwater prawn is associated with the development of mushy texture along with decreased shear force and disintegration of microstructure (Sriket *et al.*, 2010).

The degradations of myosin heavy chain (MHC) and collagen of freshwater prawn meat by endogenous proteases were reported (Sriket *et al.*, 2011a; Sriket *et al.*, 2011c). Muscle deterioration of freshwater prawn meat during iced storage was mediated by trypsin-like enzyme released from its hepatopancreas (Sriket *et al.*, 2011c). During the storage, autolysis of cephalothorax, where hepatopancreas and other internal organs are located, could take place, releasing the active proteases into the muscle. Recently, soybean and bambara groundnut extracts containing trypsin inhibitors have been used to prevent muscle degradation mediated by crude proteases from hepatopancreas of freshwater prawn (Sriket *et al.*, 2011b). However, no information regarding the effect of legume seed extracts on proteolytic activities and quality changes of freshwater prawn during iced storage has been reported. Therefore, the aim of this study was to investigate the effect of soybean and bambara groundnut extracts on the inhibition of proteolytic activities and the retardation of quality changes of freshwater prawn cultured in the Southern Thailand during iced storage.

8.3 Materials and methods

8.3.1 Chemicals

β-mercaptoethanol (βME), casein, tyrosine and α-*N*-ρ-tosyl-*L*-arginine methyl ester (*L*-TAME) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Tris (hydroxymethyl) aminomethane (Tris) and trichloroacetic acid were procured from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), Coomassie Brilliant Blue R-250 and *N,N,N′,N′*-tetramethylethylene diamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). All chemicals were of analytical grade.

8.3.2 Preparation of protease inhibitors from legume seeds

Soybean (*Glycine max*) and bambara groundnut (*Vigna subterranea*) were purchased from a market in Hat Yai, Songkhla, Thailand. The seed extracts were prepared according to the method of Benjakul *et al.* (2000) with slight modifications. Dry seeds were ground using a coffee mill to a particle size of 20 mesh. The seed flour was defatted by mixing with 5 volumes of hexane. The mixture was stirred for 10 min and filtered through Whatman No.1 filter paper (Whatman International Ltd., Maidstone, England). The retentate was rinsed with hexane 3 times to remove the residual oil and the defatted seed flour was air-dried at room temperature (28-30 °C).

To prepare the extracts containing protease inhibitors, the defatted seed flour was mixed with 0.15 M NaCl at a ratio of 1: 10 (w/v) and shaken at 180 rpm at room

temperature for 3 h using a shaker (Heidolph UNIMAX 1010, Schwabach, Germany). The extracts were recovered by centrifuging at 5000 *x g* for 30 min at 4 °C using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Palo Alto, CA, USA). To partially purify the protease inhibitors, the extracts were heated at 90 °C for 10 min and cooled down with iced water. The coagulated debris was removed by centrifuging at 8000 *x g* for 5 min. The supernatants were freeze-dried using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark) and used for further study. Extract powders from soybean and bambara groundnut contained trypsin inhibitor at levels of 37.19 and 32.55 (U/mg protein), respectively (Benjakul *et al.*, 1997).

8.3.3 Effect of protease inhibitor treatments on the quality of prawn during iced storage

Freshwater prawns with the size of 30-35 prawn/kg were obtained from a farm in Phattalung province, Thailand. The prawn were placed in ice with a prawn/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla, Thailand, within approximately 2 h. Upon arrival, the prawns were washed with clean cold water (2-5 °C). Whole prawn was injected with the extracts containing protease inhibitor from soybean and bambara groundnut. Extracts powder were dissolved in sterile distilled water to obtain the designated concentrations. The solutions (~300 μL) with the concentrations ranging from 1.5 to 2.5 mg protein/mL were evenly injected at the lower part of cephalothorax portion with a 20-gauge hypodermic needle throughout the samples. Prawn samples were stored in ice using prawn/ice ratio of 1:2 (w/w). Samples were taken every 2 days up to 10 days for analyses. Molten ice was removed every day and the ice was added to maintain a prawn/ice ratio during the storage.

8.3.4 Chemical and enzymatic analysis

8.3.4.1 pH determination

The pH of prawn muscle was measured as described by Benjakul *et al.* (1997). Prawn muscle was homogenized using an IKA Labortechnik homogenizer (Selangor, Malaysia) with 10 volumes of deionized water (w/v), and the pH was measured using a pH meter (Sartorius, Goetingen, Germany).

8.3.4.2 Determination of TCA soluble peptide content

TCA-soluble peptide content of prawn muscle was determined according to the method of Benjakul *et al.* (2002a). Ground meat (3 g) was homogenized with 27 mL of cold 5% TCA using a homogenizer at a speed of 11,000 rpm for 1 min. The homogenate was kept in ice for 30 min and centrifuged at 5000 *x g* for 20 min at 4 °C using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, CA, USA). Soluble peptides in the supernatant were measured according to the Lowry method (Lowry *et al.*, 1951) and expressed as μmol tyrosine/g muscle.

8.3.4.3 Determination of heat soluble collagen content

Heat soluble collagen content was extracted from prawn muscle according to the method of Liu *et al.* (1996). Prawn meat (2 g) was homogenized with 8 mL of 25% Ringer's solution containing 32.8 mM NaCl, 1.5 mM KCl, and 0.5 mM $CaCl_2$. The homogenate was heated for 70 min at 77 ^{o}C and centrifuged at 2,300 *x g* for 30 min at 4 °C. The extraction was repeated twice. The supernatants obtained were combined. The sediment and supernatants were hydrolyzed with 6 M HCl at 110 °C for 24 h. The collagen content of each fraction was determined according to the method of Sato *et al.* (1988). The amount of heat soluble collagen was expressed as a percentage of total collagen (collagen content in sediment plus that in the supernatant).

8.3.4.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein patterns of prawn samples treated without and with soybean and bambara groundnut extracts at various concentrations during iced storage of 10 days were analyzed. Prawn sample (3 g) was homogenized with 27 mL of 5% SDS at a speed of 11,000 rpm for 1 min using a homogenizer. The homogenate was heated at 85 °C for 1 h, followed by centrifugation at 7,500 *x g* for 15 min at room temperature. The protein concentration of supernatant was determined by the Biuret method (Robinson and Hogden, 1940). SDS–PAGE was performed using 4% stacking gel and 10% running gel according to the method of Laemmli (1970) with a vertical gel electrophoresis unit (Mini-Protein II; Bio-Rad Laboratories, Richmond, CA, USA). The electrophoresis was carried out at 15 mA. After separation, protein bands were stained using Coomassie Brillant Blue R-250 (0.2%) in 25% methanol and 10% acetic acid. Destaining was performed using 30% methanol and 10% acetic acid.

8.3.4.5 Total proteolytic and trypsin activities

Total proteolytic activity of crude protease extract (CE) from prawn muscle obtained from whole prawn, whose cephalothoraxes were injected with soybean and bambara groundnut extracts at different concentrations, was assayed using casein as a substrate according to the method of Sriket *et al.* (2011a). The activity of CE was determined at pH 7 and 60 °C for 15 min. The activity was calculated and expressed as units/g meat. One unit was defined as the amount of protease causing the release of 1 μmol of tyrosine/min under the assay condition.

Trypsin activity of CE from prawn muscle from different treatments was measured using TAME as a substrate according to the methods of Hummel (1959). The enzyme solution with an appropriate dilution (20 μL) was mixed with 3.0 mL of 1 mM TAME in 10 mM Tris-HCl buffer, pH 7. The reaction was conducted at 25 °C for 15 min. Production of *ρ*-tosyl-arginine was measured by monitoring the increase in absorbance at 247 nm. Blank was prepared in the same manner, but 10 mM Tris-HCl buffer (pH 7) was used instead of TAME solution. One unit of trypsin activity was defined as the amount of protease, causing an increase of 1.0 in absorbance/min (Hummel, 1959). Trypsin activity was reported as units/g meat.

8.3.5 Physical analysis

Shear force of prawn meats was measured using a TA-XT2i texture analyzer (Stable Micro Systems, Surrey, England) equipped with a Warner-Bratzler shear apparatus (Brauer *et al.*, 2003). The operating parameters consisted of a cross head speed of 10 mm/s and a 25 kg load cell. The shear force, perpendicular to the axis of muscle fibers, was measured at the portion between the first and second segment of prawn meat. The peak of the shear force profile was regarded as the shear force value.

8.3.6 Sensory evaluation

At day 0, 4 and 8 of storage, prawn samples with different treatments were deheaded, peeled and steamed for 7 min, in which the core temperature of 70 °C was obtained. After cooking, cooked prawn were cooled rapidly in iced water and drained on the plastic screen for 3 min at 4 °C. The samples were placed on a stainless steel tray and covered with aluminum foil. The cooked samples were evaluated by 30 panelists, the graduate students of the Food Science and Technology program, Department of Food Technology, Prince of Songkla University with the age of 25–30 years. Panelists were acquainted with shrimp consumption and had no allergies to shrimp. Nine-point hedonic scale was used to

evaluate samples, where 9=like extremely; 7=like moderately; 5= neither like or nor dislike; 3=dislike moderately; 1=dislike extremely (Mailgaard *et al.*, 1999). All panelists were asked to evaluate for texture and overall likeness. Samples were presented in plates coded with random three-digit numbers.

8.3.7 Microbiological analysis

Psychrophilic bacteria count was determined following the method of Cousin *et al.* (1992). Whole prawn samples (50 g) were weighed into a stomacher bag containing 450 mL of peptone water. Blending was performed in a Stomacher (IUL Instrument, city, Spain) for 2 min. Peptone water was used for diluting the samples. Thereafter, the sample diluted in serial 10-fold steps was used for aerobic plate count (APC). APC was done by the spread plate technique on plate count agar (PCA-Merck). The plates were incubated at 10 °C for 7 days and the number of psychrophilic bacteria was recorded.

8.3.8 Statistical analysis

The experiments were run in triplicate with three different lots of samples. Data were subjected to analysis of variance and mean comparison was carried out using Duncan's multiple range test (DMRT) (Steel and Torrie, 1980). For all parameters, all determinations were carried in triplicate, except for shear force, where the analysis was conducted with 10 determinations. Statistical analyses were performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

8.4 Results and discussion

8.4.1. Changes in pH

pH of meat from the control freshwater prawn and those treated with soybean and bambara groundnut extracts at various concentrations during iced storage of 10 days is shown in Figure 29A. A gradual increase in the pH was observed in all samples up to 10 days of iced storage ($p < 0.05$) (Figure 29A). Generally, the pH of the control sample increased sharply after 4 days of iced storage. For samples treated with soybean and bambara groundnut extracts, pH increased gradually up to 10 days of storage and no differences in pH among those treatments were found ($p > 0.05$). The differences in pH changes between the control and samples treated with soybean and bambara groundnut extracts suggested that the treatment with legume seed extracts more likely retarded the formation of volatile bases

produced by either endogenous or microbial enzymes. Benjakul *et al.* (2002b) reported that the decomposition of nitrogenous compounds resulted in an increase in pH of fish flesh. The increase in pH ranging from 0.1 to 0.2 units suggested that the quality of fish muscle was still acceptable but the increase higher than 0.2 units indicated the deteriorated samples (Fatima and Qadri, 1985). Rahaman *et al.* (2001) reported that the pH value of shrimp (*Penaeus monodon*) varied from 6.63 to 7.95 during 10 days of iced storage. Changes in pH might be different, depending on a variety of factors such as buffering capacity of meat (Pacheco-Aguilar *et al.*, 2000) and the liberation of inorganic phosphate and ammonia due to the enzymatic degradation of ATP (Sikorski *et al.*, 1990), etc. Since the treatment of prawn with soybean and bambara groundnut extracts could lower the increase in pH throughout the storage, both extracts might act as a preservative to some degree. As a result, prawn quality could be maintained to some extent.

8.4.2. Changes in TCA-soluble peptide content

TCA-soluble peptide contents of freshwater prawn treated with soybean and bambara groundnut extracts at various levels in comparison with the control sample during 10 days of iced storage are shown in Figure 29B. Within the first four days, the control sample contained slightly higher TCA-soluble peptide content than did samples treated with soybean and bambara groundnut extracts ($p<0.05$). After 4 days of storage, the marked increases in TCA-soluble peptide content were observed for the control sample ($p<0.05$), but only slight increases were noticeable in the samples treated with both legume extracts at all concentrations used. The slight decreases in TCA-soluble peptide content of all samples were found after 8 days of storage, but the marked decrease was found in the control. The peptides generated might be leached out together with molten ice, leading to the lower TCA-soluble peptide content. Water uptake of prawn during storage in ice plausibly contributed to the dilution of TCA-soluble peptides in prawn muscles. At the same storage period, TCA-soluble peptides of control sample were generally higher than those of samples treated with legume extracts throughout the storage ($p<0.05$). This was in agreement with the higher pH of the control sample in comparison with other samples (Figure 29A). The result suggested that the control sample might contain higher activity of proteases, especially trypsin-like proteases. However, no marked differences in TCA-soluble peptide content were found between the sample treated with soybean and bambara groundnut extracts ($p>0.05$). Therefore, samples treated with soybean and bambara groundnut extracts had the lower protein degradation. In the presence of extracts containing trypsin inhibitor, the activity of trypsin-like proteases

released from hepatopancreas could be retarded. Trypsin like enzyme released from hepatopancreas to the first abdomen of freshwater prawn was reported (Sriket *et al.*, 2011c). The results suggested that the freshwater prawn treated with soybean and bambara groundnut extracts had the lower activity of endogenous and microbial proteases. It could be implied that injection of soybean and bambara groundnut extracts at cephalothoraxes could be a means to prevent protein degradation by proteolytic enzymes.

8.4.3 Changes in heat soluble collagen content

Generally, heat soluble collagen content of the control sample markedly increased with increasing storage time ($p<0.05$) (Figure 29C). On the other hand, no changes in heat soluble collagen content of prawn meat treated with soybean and bambara groundnut extracts were observed up to 6 days of iced storage ($p>0.05$), regardless of concentration used (Figure 29C). This was coincidental with the increase in TCA-soluble peptide content in the control sample (Figure 29B). Collagen with lower cross-link as indicated by the lower ISC content of prawn stored in ice for a longer time could be more solubilized when heating was applied (Sriket *et al.*, 2010). Decreases in collagen cross-links might be associated with the increased autolysis caused by both indigenous and microbial proteases.

Prawn meat without legume seed extract injection contained more heat soluble collagen than did those treated with legume extracts during iced storage ($p<0.05$). The difference in the amount of heat soluble collagen among prawn samples might be due to the differences in collagenolytic activity. Samples treated with legume extracts containing protease inhibitor might have the lower collagenolytic activity. After heat treatment, helix structure of collagen with lower cross-links could be disrupted much easily. This resulted in the higher extractability of collagen by heat as indicated by the increase in soluble collagen content. Furthermore, hydrolyzed collagen with the shorter chain could be extracted with the aid of heat more easily. Trypsin like collagenolytic proteases released from freshwater prawn hepatopancreas is capable of hydrolyzing collagen (Sriket *et al.*, 2011c). The increase in heat soluble collagen of the control sample might be related with the muscle softening of prawns during the extended iced storage. However, at day 8 and 10 of the storage, the slight increase in heat soluble collagen in samples treated with soybean and bambara groundnut extracts were observed. The use of legume seed extracts to lower the degradation of myofibrillar protein and collagen caused by proteolytic and collagneolytic enzymes from freshwater prawn hepatopancreas has been reported (Sriket *et al.*, 2011b). Therefore, the injection of

soybean and bambara groundnut extracts containing trypsin inhibitor was able to retard collagenolytic activity and muscle degradation of freshwater prawn meat during iced storage.

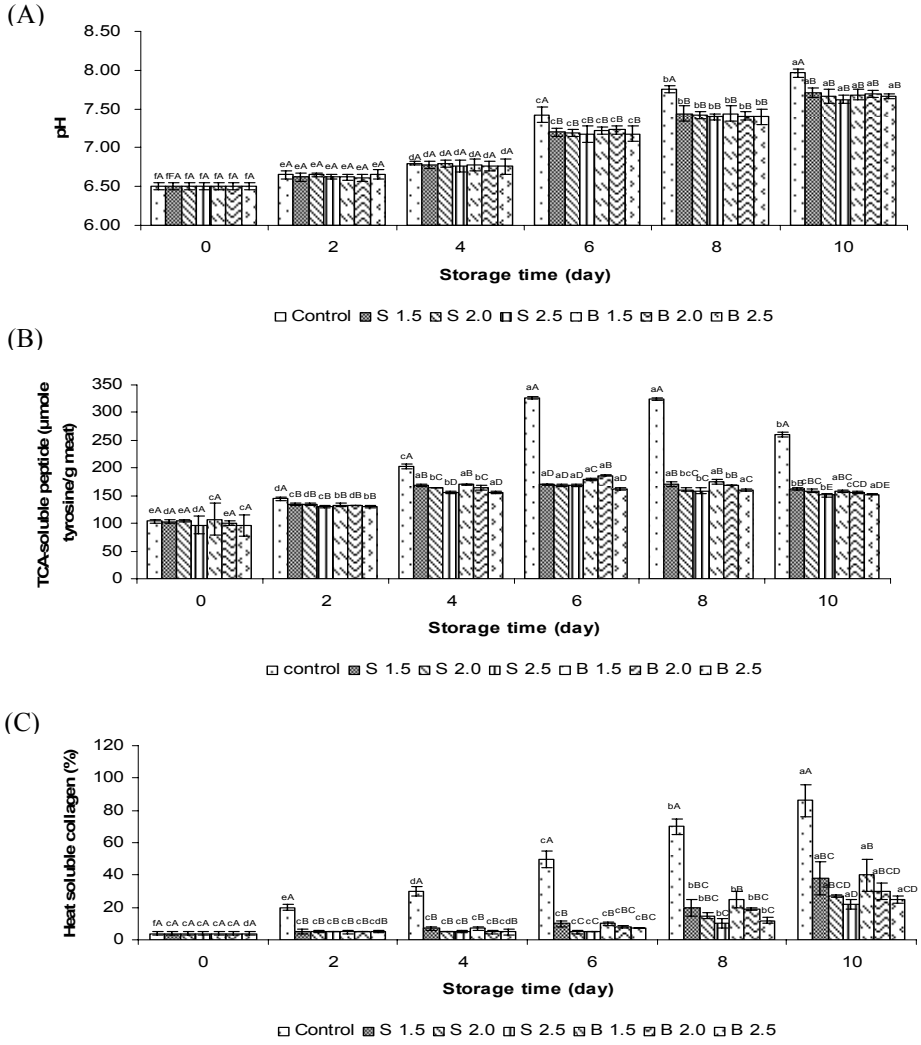

Figure 29. Changes in pH (A), TCA-soluble peptide (B) and heat soluble collagen (C) contents of freshwater prawn treated without and with soybean and bambara groundnut extracts at different concentrations during iced storage. Bars represent the standard deviation (n=3). The different lower case letters on the bars within the same treatment indicate significant differences ($p < 0.05$). Different upper case letters on the bars within the same storage time indicate the significant differences ($p < 0.05$). Control, sample without treatment; S, soybean; B, Bambara groundnut. Numbers denoted the concentration of legume extract used (mg protein/mL).

8.4.4 Changes in protein patterns

Protein patterns of muscle proteins of all samples remained unchanged during the first 6 days of iced storage (Figure 30). However, the slight decrease in band intensity of myosin heavy chain (MHC) of the control sample was observed at day 6 of the storage. MHC was susceptible to proteolytic degradation than other muscle proteins, e.g. actin, troponin and tropomyosin (Benjakul *et al.*, 1997). The pronounced decrease in band intensity of MHC was noticeable in the control sample kept in ice from day 8 to 10 (Figure 30). Nevertheless, slight decrease in band intensity of MHC was found in samples treated with soybean and bambara groundnut extracts at the end of the storage. It was noted that MHC in prawn sample treated with soybean extract at the highest concentration (2.5 mg protein/mL) was more retained. Therefore, pretreatment of prawn by legume extracts injection could be another means to retard the muscle deterioration caused by proteolysis. The retardation of muscle protein degradation induced by crude protease extract was achieved by legume seed extracts containing trypsin inhibitor (Sriket *et al.*, 2011b).

8.4.5 Total proteolytic and trypsin activities

Total proteolytic activity of the first segment of abdomen from prawn meat treated with soybean and bambara groundnut extracts at different levels and the control sample during iced storage is shown in Figure 31A. No changes in total proteolytic activity assayed using casein as a substrate were observed in all samples within the first 2 days of iced storage ($p>0.05$) (Figure 31A). After 2 days of iced storage, total proteolytic activity in the control sample increased when storage time increased ($p< 0.05$). For the samples treated with soybean and bambara groundnut extracts, the gradual increases in total proteolytic activity were obtained. The increase in total proteolytic activity in the control sample was coincidental with the increase in TCA-soluble peptide and heat soluble collagen contents (Figure 29B and C). At the end of storage, the sample treated with the soybean extract at the highest concentrations (2.5 mg protein/mL) showed the lowest total proteolytic activity. Therefore, the use of legume extracts, particularly soybean extract at high concentration, could be an effective means to inhibit proteases causing the degradation of freshwater prawn meat.

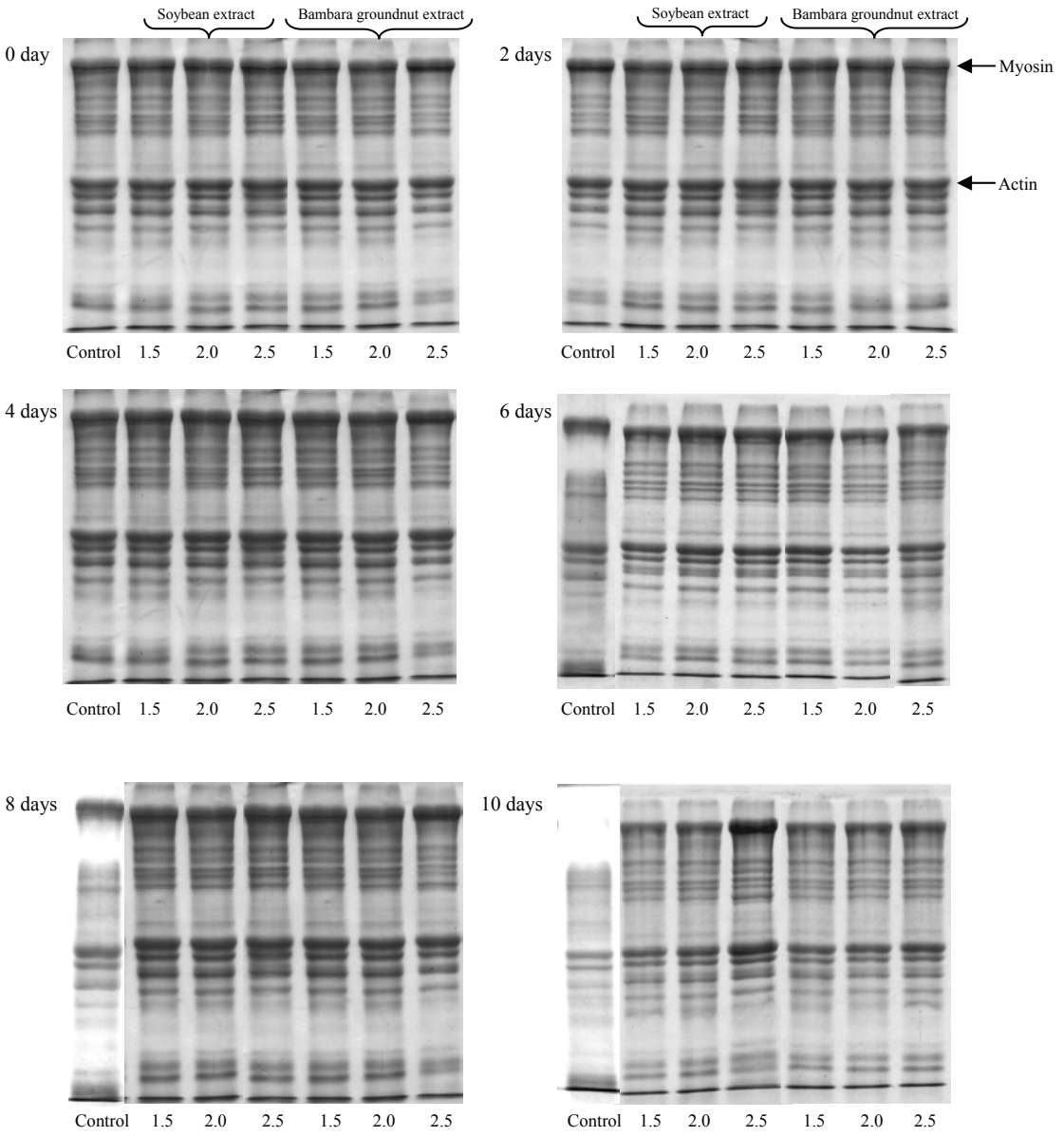

Figure 30. SDS-PAGE patterns of freshwater prawn meat treated without and with soybean and bambara groundnut extracts at different concentrations during iced storage. Numbers denoted the concentration of legume extract used (mg protein/mL).

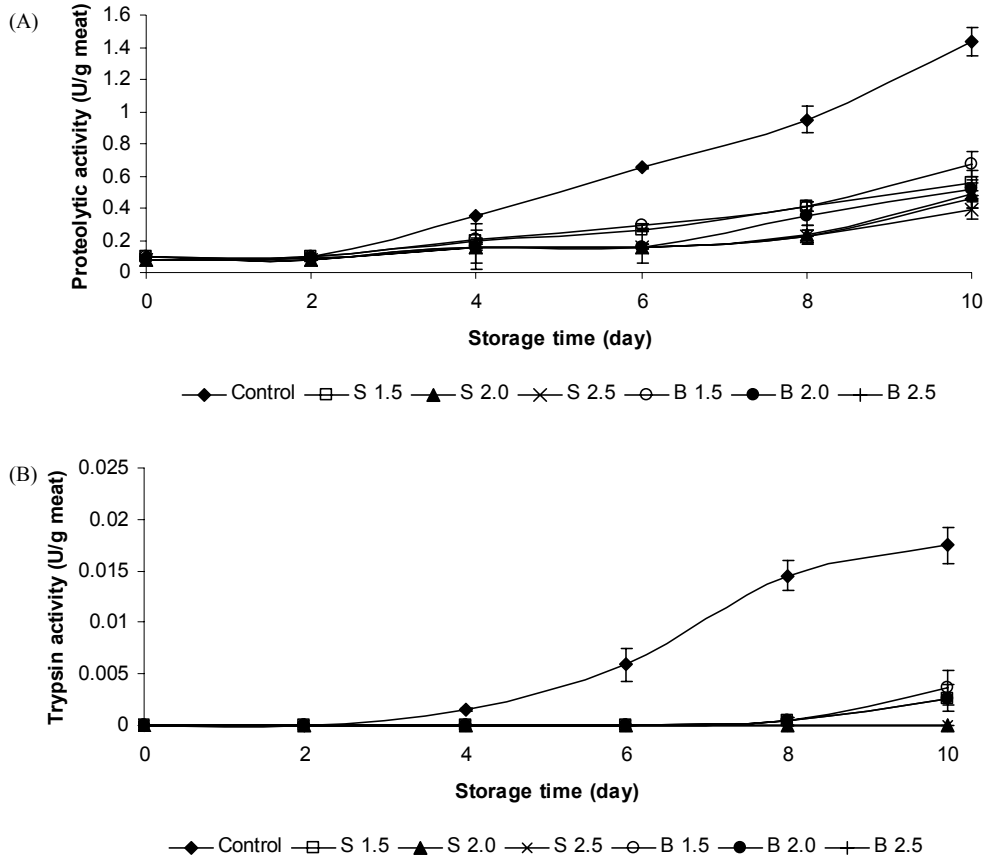

Figure 31. Total proteolytic activity (A) and trypsin activity (B) of meat from freshwater prawn treated without and with soybean and bambara groundnut extracts at different concentrations during iced storage. Bars represent the standard deviation (n = 3). Control, sample without treatment; S, soybean; B, Bambara groundnut. Numbers denoted the concentration of legume extract used (mg protein/mL).

When trypsin activity in the first segment of abdomen was monitored during 10 days of iced storage, no trypsin activity was detectable in all samples within the first 2 days. At day 4, trypsin activity was found only in the control sample (Figure 31B). Typsin-like enzyme released into prawn meat after the autolysis of prawn hepatopancreas during extended iced storage was previously reported by Sriket *et al.* (2011c). After day 4 of iced storage, the continuous increase in trypsin activity was observed in the control samples ($p<0.05$). The trypsin activity was dominated in the control sample during iced storage of 10 days, while no activity was detected in prawn meat treated with soybean and bambara groundnut extracts at high concentrations (2.5 mg protein/mL) during the storage. However, very low trypsin activity was found in the sample treated with soybean and bambara

groundnut extracts at the lowest concentration (1.5 mg protein/mL) at the end of the storage. The result indicated that inhibition of trypsin by legume extracts depends on the concentration used. Therefore, injection of soybean and bambara groundnut extracts could suppress the proteases released from hepatopancreas into prawn meat during iced storage.

8.4.6. Changes in shear force

Changes in shear force of freshwater prawn treated with soybean and bambara groundnut extracts at different concentrations and the control sample during 10 days of iced storage are shown in Figure 32. Shear force of the control freshwater prawn decreased when storage time increased up to 10 days ($p<0.05$). No changes in shear force were observed in freshwater prawn treated with legume extracts during the first 4 days of storage ($p > 0.05$). It was most likely that the destruction of muscle fibers of the control sample was more pronounced, compared with sample treated with soybean and bambara groundnut extracts. The degradation of muscle tissue caused by hepatopancreatic enzymes started from the perimysium, endomysium, the Z line and the H zones with concurrent degradation of the connective fibers and myofibrillar proteins (Sriket *et al.*, 2010). During 2-10 days of storage, shear force of the control sample was the lowest, when compared with other samples ($p<0.05$). The lowered shear force of the control sample kept in ice for a long time might be caused by the destruction of muscle fibers and connective tissues. This was in agreement with the higher TCA soluble peptide content (Figure 29B), heat soluble collagen content (Figure 29C) and the proteolytic activities (Figure 31A and B) of the control sample. The result suggested the role of legume extracts in prevention of serine proteases released from hepatopancreas in hydrolysis of muscle proteins in freshwater prawn. However, at day 8 to day 10 of the storage, the slightly decreased shear force of samples treated with soybean and bambara groundnut, regardless of concentration used, was observed. Therefore, the treatment of freshwater prawn with soybean and bambara groundnut extracts could maintain the texture of freshwater prawn during iced storage.

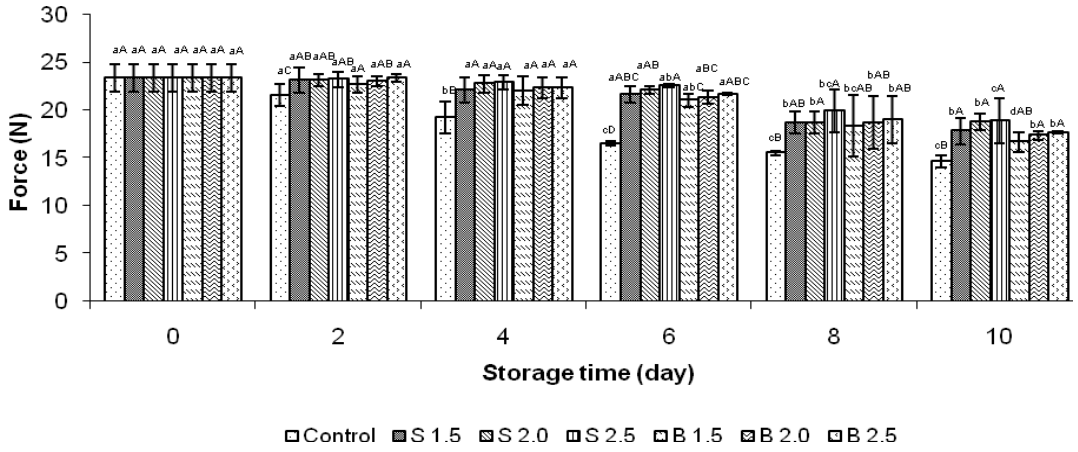

Figure 32. Changes in shear force of freshwater prawn meat treated with soybean and bambara groundnut extracts at different concentrations during iced storage. Bars represent the standard deviation (n = 3). The different lower case letters on the bars within the same treatment indicate significant differences ($p < 0.05$). Different upper case letters on the bars within the same storage time indicate the significant differences ($p<0.05$). Control, sample without treatment; S, soybean; B, Bambara groundnut. Numbers denoted the concentration of legume extract used (mg protein/mL).

8.4.7 Changes in likeness

At the beginning of the storage, the similar texture (Figure 33A) and overall likeness (Figure 33B) was found between the control and those treated with soybean and bambara groundnut extracts (Figure 33). For both attributes, the slight decreases were obtained in the control sample after 4 days of storage ($p < 0.05$), whereas the likeness of samples treated with soybean and bambara groundnut extracts at all concentrations used remained unchanged up to day 8 (Figure 33). Furthermore, odor, taste and favor likeness scores of samples were not affected by the treatment using legume extracts, regardless concentration used during the storage (data not shown). The decrease in texture likeness of the control sample stored for 8 days in ice was in agreement with the destruction of muscle fibers as evidenced by the decrease in shear force (Figure 32). Decrease in texture likeness of shrimp meat during iced storage correlated well with the decreased shear force (Sriket *et al.*, 2010). It was most likely that the lowered acceptability of both attributes (Figure 33A and B) of the control sample was mainly associated with the destruction of collagen in the connective tissue mediated by collagenolytic enzyme, which contributed to the softening.

Proteolysis of muscle proteins by serine collagenolytic enzymes released from hepatopancreas was postulated as the main mechanism, causing mushiness in freshwater prawn (Sriket *et al.*, 2011c). The result indicated that the extended iced storage was related with the loss in sensory property of freshwater prawn. Therefore, the use of soybean and bambara groundnut extracts effectively maintained the sensory properties of freshwater prawn stored in ice.

8.4.8 Changes in psychrophilic bacteria count

At the beginning of storage, psychrophilic bacterial count of all samples was 2.7 log colony forming units (CFU)/g, regardless of treatments (Figure 34). As the storage time increased, a continuous increase in psychrophilic bacteria count was observed in the control samples ($p<0.05$). The lower rate of increase was observed in the samples treated with soybean and bambara groundnut extracts, irrespective of concentrations used. The result suggested that soybean and bambara groundnut extracts could retard the microbial growth in freshwater prawn during the extended iced storage. The lower amount of peptide generated in prawn sample treated with legume seed extracts might not favor the growth of microorganisms. Psychrophilic bacteria count of the control sample exceeded 10^5cfu/g during the storage, which is the limit for acceptability (Van Schothorst, 1998), while the samples treated with soybean and bambara groundnut extracts did not exceed the limit. Therefore, the injection of soybean and bambara groundnut extracts into cephalothorax could retard the microbial growth as well as maintain the quality of freshwater prawn meat during iced storage.

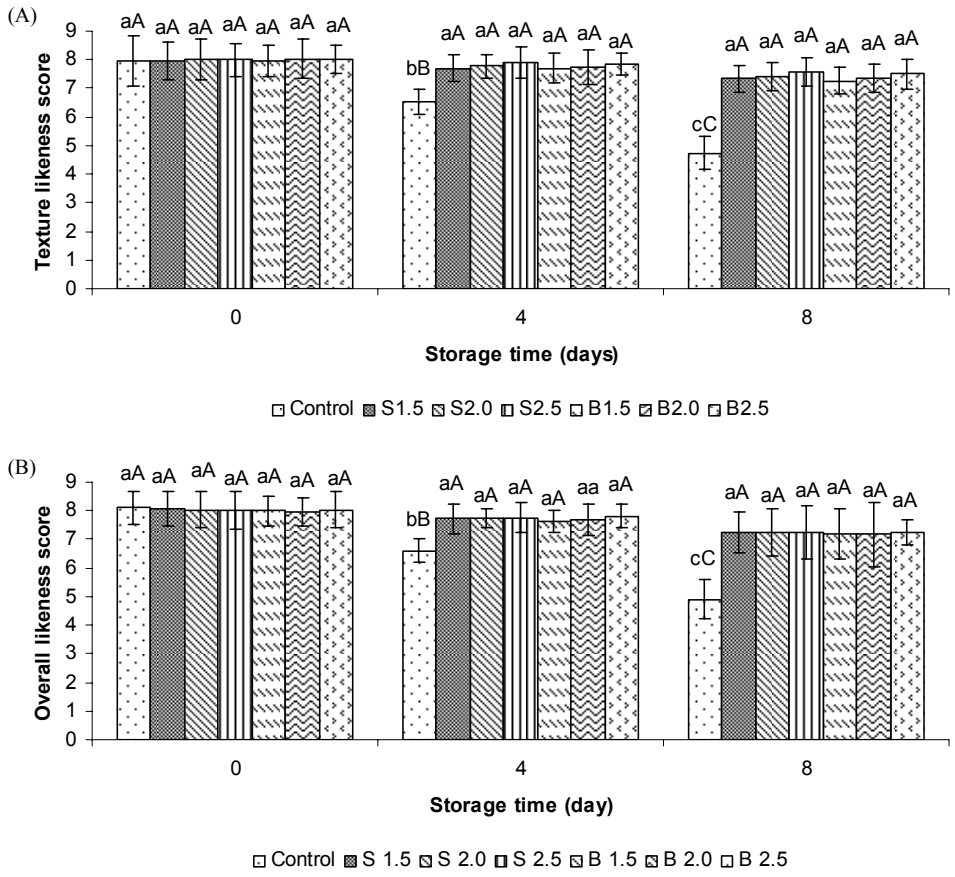

Figure 33. Changes in texture (A) and overall (B) likeness scores of freshwater prawn treated without and with soybean and bambara groundnut extracts at various concentrations during iced storage. Bars represent the standard deviation from thirty determinations. The different lower case letters on the bars within the same treatment indicate significant differences ($p<0.05$). Different upper case letters on the bars within the same storage time indicate the significant differences ($p<0.05$). Control, sample without treatment; S, soybean; B, Bambara groundnut. Numbers denoted the concentration of legume extract used (mg protein/mL).

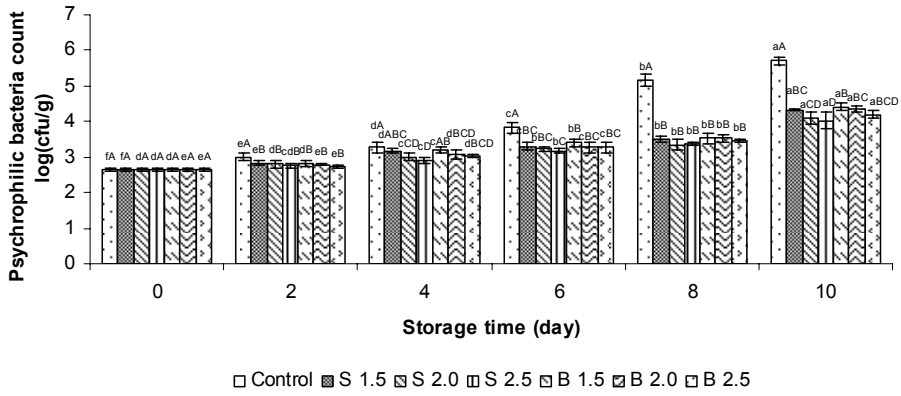

Figure 34. Changes in psychrotrophic bacteria count of freshwater prawn treated without and with various concentrations of soybean and bambara groundnut extracts at various concentrations during iced storage. Bars represent the standard deviation (n = 3). The different lower case letters on the bars within the same treatment indicate significant differences ($p<0.05$). Different upper case letters on the bars within the same storage time indicate the significant differences ($p<0.05$). Control, sample without treatment; S, soybean; B, Bambara groundnut. Numbers denoted the concentration of legume extract used (mg protein/mL).

8.5 Conclusion

Soybean and bambara groundnut extracts were able to inhibit the degradation of freshwater prawn, dependent on the concentration used. The extracts of soybean and bambara groundnut (1.5-2.5 mg protein/mL) suppressed the degradation of freshwater prawn meat effectively as indicated by the lower TCA-soluble peptide, heat soluble collagen content and proteolytic activities than the control sample. Freshwater prawn meat treated with legume seed extracts showed higher shear force value, texture likeness and overall likeness scores than did the control sample, throughout the storage. Legume extracts, especially from soybean, could be used to prolong the shelf-life of freshwater prawn by retarding the muscle degradation, in which firm texture and consumer acceptability could be maintained during iced storage up to 8-10 days.

CHAPTER 9

SUMMARY AND FUTURE WORKS

9.1 Summary

1. Freshwater prawn meat became softened during the extended iced storage as indicated by the increases in heat soluble collagen, PSC contents as well as the collapse of muscle structure especially collagen fiber after heating. To avoid the loss in textural quality, freshwater prawn was recommended to be stored in ice for up to 3 days.
2. Cysteine proteases in freshwater prawn meat had very low proteolytic activities, while serine proteases from hepatopancreas showed high hydrolytic activity on myofibrillar and collagenous proteins.
3. Trypsin-like protease with high hydrolytic activity on PSC released from hepatopancreas into the first segment of freshwater prawn abdomen was more likely responsible for the softening of meat during extended iced storage.
4. Purified trypsin from hepatopancreas of freshwater prawn showed high hydrolytic activity at pH 8.0 and 55 °C and was able to hydrolyze prawn collagen. The trypsin released from hepatopancreas was directly involved in muscle softening during iced storage.
5. The extracts of soybean and bambara groundnut (1.5-2.5 mg protein/mL) suppressed the degradation of freshwater prawn meat effectively. Legume extracts, especially from soybean, could be used to prolong the shelf-life of freshwater prawn by retarding the muscle degradation, in which firm texture and consumer acceptability could be maintained during iced storage up to 8-10 days.

9.2 Future works

1. Purification and characterization of collagen type V from freshwater prawn muscle should be further investigated.
2. Different factors influencing trypsin-like collagenolytic activity from hepatopancreas should be further investigated, in which promising and effective preventive method can be obtained.
3. Other legume seeds containing trypsin inhibitor grown in Thailand should be further studied to prevent the quality loss of freshwater prawn and other fish species during storage and distribution.

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List of Publications and Proceedings

Publications

1. Sriket, C., Benjakul, S. and Visessanguan, W. 2010. Post-mortem changes of muscle from freshwater prawn (*Macrobrachium rosenbergii*) as influenced by spawning stages. LWT Food Sci. Technol. 43: 608-616.
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7. Sriket, C., Benjakul, S., Visessanguan, W., Hara, K. and Yoshida, A. Retardation of post-mortem changes of freshwater prawn (*Macrobrachium rosenbergii*) stored in ice by legume seed extracts. Food Chem. Submitted.

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1. Sriket, C., Benjakul, S. and Visessanguan, W. 2009. Post-mortem changes of muscle from freshwater prawn (*Macrobrachium rosenbergii*) as influenced by spawning stages. The CHE-USDC Congress II. A-One The Royal Cruise Hotel, Pattaya, Chon Buri, Thailand, August, 2009. Oral presentation.
2. Sriket, C., Benjakul, S. and Visessanguan, W. 2010. Proteases from muscle and hepatopancreas of freshwater prawn (*Macrobrachium rosenbergii*): characteristics and the effect on muscle degradation. The 7th IMT-GT UNINET and 3rd joint International PSU-UNS Conference, on Bioscience for Future, Prince of Songkla University, Hat Yai, Songkhla, Thailand, October 7-8, 2010. Oral presentation.
3. Sriket, C., Benjakul, S. and Hara, K. 2011. Inhibition of proteolytic activity and muscle degradation of freshwater prawn (*Macrobrachium rosenbergii*) by legume seed extracts. The 62nd Pacific Fisheries Technologists Conference. Vancouver, British Columbia, Canada. February 13-16, 2011. Poster presentation.