Characterization and Changes during Frozen Storage of the Muscles of Soft Shell and Hard Shell Mud Crabs

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ชื่อวิทยานิพนธ์	การจำแนกคุณลักษณะ และการเปลี่ยนแปลงระหว่างการเก็บรักษาในสภาวะแช่แข็ง ของกล้ามเนื้อปูดำและปูนิ่ม
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บทคัดย่อ

กล้ามเนื้อเชียงและกล้ามเนื้อก้ามของปูนิ่มมีปริมาณโปรตีนต่ำกว่ากล้ามเนื้อของปูดำแต่มีปริมาณความชื้นและเกลือสูงกว่ากล้ามเนื้อของปูดำ กล้ามเนื้อของปูทั้งสองชนิดมีปริมาณไฮดรอกซีโปรลีน 7.92 ถึง 8.88 มิลลิกรัมต่อกรัมน้ำหนักเปียก อย่างไรก็ตามกล้ามเนื้อก้ามของปูนิ่มประกอบด้วยไฮดรอกซีโปรลีนปริมาณต่ำ (2.75 มิลลิกรัมต่อกรัมน้ำหนักเปียก) โปรตีนซาร์โคพลาสมิกและโปรตีนที่ละลายได้ในด่างจัดเป็นองค์ประกอบไนโตรเจนหลักในกล้ามเนื้อของปูทั้งสองชนิด ยกเว้นกล้ามเนื้อก้ามของปูนิ่มซึ่งมีปริมาณซาร์โคพลาสมิกเป็นองค์ประกอบหลัก Inactivation rate constant (K_D) ของแอคโตไมโอซินธรรมชาติของกล้ามเนื้อปูทั้งสองชนิดเพิ่มขึ้นเมื่อให้ความร้อนที่อุณหภูมิ 40 ถึง 50 องศาเซลเซียส โดยกล้ามเนื้อก้ามของปูดำมีความคงตัวต่อความร้อนต่ำที่สุด ไมโอซินของกล้ามเนื้อเชียงมีค่า T_{max} เท่ากับ 47.58 ถึง 48.08 องศาเซลเซียส และมีค่าเอนทาลปี 0.17 ถึง 0.18 จูลต่อกรัม สำหรับโครงสร้างทางจุลภาคของกล้ามเนื้อปูดำและปูนิ่มนั้นพบว่ามีความแตกต่างกัน กล้ามเนื้อเชียงของปูดำและปูนิ่มมีลักษณะเป็นมัดกล้ามเนื้อที่เรียงติดกันอย่างเป็นระเบียบ ส่วนกล้ามเนื้อก้ามของปูทั้งสองชนิดมีรอยแยกระหว่างมัดกล้ามเนื้อที่ชัดเจน และโครงสร้างที่มีลักษณะคล้ายฟองน้ำสามารถพบได้ในกล้ามเนื้อก้ามของปูนิ่ม แร่ธาตุที่เป็นองค์ประกอบหลักของปูดำและปูนิ่มคือแคลเซียมและแมกนีเซียม (240.5 ถึง 699.2 พีพีเอ็ม) อย่างไรก็ตามปริมาณแร่ธาตุทั้งสองชนิดในกล้ามปูนิ่มมีเพียงหนึ่งในสามของแร่ธาตุที่พบในกล้ามเนื้อของปูดำ ทองแดง เหล็ก และสังกะสีเป็นแร่ธาตุที่พบน้อยกว่า 50 พีพีเอ็ม

กิจกรรมของโปรตีเนสของกล้ามเนื้อปูดำและปูนิ่มมีค่าสูงสุดที่อุณหภูมิ 65 องศาเซลเซียส และพีเอช 8 ยกเว้นโปรตีเนสจากกล้ามเนื้อก้ามของปูนิ่มซึ่งมีกิจกรรมสูงสุดที่อุณหภูมิ 60 องศาเซลเซียส และ พีเอช 5.5 กิจกรรมของโปรตีเนสจากกล้ามเนื้อของปูดำและปูนิ่มสามารถถูกยับยั้งได้โดย soybean trypsin inhibitor เข้มข้น 0.01 มิลลิโมลาร์ ในขณะที่กิจกรรมกล้ามเนื้อก้ามของปูนิ่มนั้นถูกยับยั้งได้ด้วย pepstatin A เข้มข้น 1 ไมโครโมลาร์

จากการศึกษาการเปลี่ยนแปลงทางฟิสิกส์-เคมีของกล้ามเนื้อปูดำและปูนิ่มระหว่างการเก็บรักษาที่อุณหภูมิ -20 องศาเซลเซียส เป็นเวลา 12 สัปดาห์ พบว่าแอคโตไมโอซินธรรมชาติของปูดำและ

ปูนิ่มมีกิจกรรมของ Ca^{2+}-ATPase ลดลง และมีค่า surface hydrophobicity เพิ่มขึ้น ตามระยะเวลาการเก็บรักษาที่เพิ่มขึ้น การเพิ่มขึ้นของพันธะไดซัลไฟด์มีความสัมพันธ์กับการลดลงของหมู่ซัลฟ์ไฮดริลระหว่างการเก็บรักษาในขณะแช่แข็ง TBARS และฟอร์มัลดีไฮด์ในกล้ามเนื้อปูดำและปูนิ่มเพิ่มขึ้นตามระยะเวลาการแช่แข็งที่เพิ่มขึ้น โดยเฉพาะอย่างยิ่งในกล้ามเนื้อก้ามของปูนิ่ม การละลายของกล้ามเนื้อปูทั้งสองชนิดลดลงตลอดระยะเวลาการแช่แข็งนาน 12 สัปดาห์ โดยทั่วไปพีเอชของกล้ามเนื้อปูนิ่มมีค่าเพิ่มขึ้นภายหลังสัปดาห์ที่ 1 และ 2 ของการเก็บรักษา สำหรับน้ำหนักที่สูญเสียจากการให้ความร้อนของกล้ามเนื้อปูทั้งสองชนิดพบว่ามีค่าเพิ่มขึ้นเมื่อระยะเวลาการเก็บรักษานานขึ้น

การแช่แข็ง-ทำละลายมีผลเร่งการเปลี่ยนแปลงทางฟิสิกส์-เคมีของกล้ามเนื้อปูดำและปูนิ่ม เมื่อจำนวนรอบของการแช่แข็ง-ทำละลายเพิ่มขึ้นค่าการละลายลดลงอย่างต่อเนื่อง ส่วนแอคโตไมโอซินธรรมชาติของปูทั้ง 2 ชนิด มีกิจกรรม Ca^{2+}-ATPase ลดลง ปริมาณฟอร์มัลดีไฮด์ TBARS และน้ำหนักที่สูญเสียจากการให้ความร้อนเพิ่มขึ้นเมื่อจำนวนรอบของการแช่แข็ง-ทำละลายเพิ่มขึ้น การเพิ่มขึ้นของพันธะ ไดซัลไฟด์มีความสัมพันธ์กับการลดลงของหมู่ซัลฟ์ไฮดริล สำหรับค่า surface hydrophobicity ของแอคโตไมโอซินธรรมชาติจากกล้ามเนื้อก้ามของปูดำมีค่าเพิ่มขึ้นตามจำนวนรอบของการแช่แข็ง-ทำละลาย ส่วนแอคโตไมโอซินธรรมชาติจากกล้ามเนื้อเชียงมี surface hydrophobicity ลดลงเมื่อผ่านการแช่แข็ง-ทำละลาย 5 รอบ สำหรับค่าพีเอชของกล้ามเนื้อปูทั้งสองชนิดมีค่าลดลงเพียงเล็กน้อยเมื่อจำนวนรอบของการแช่แข็ง-ทำละลายเพิ่มขึ้น

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Abstract

Both chunk and claw muscles of soft shell mud crabs contained lower protein content but higher moisture and salt contents than those from hard shell mud crab. Hydroxyproline content ranged from 7.92 to 8.88 mg/g wet muscle in all samples, except claw muscle from soft shell mud crab, which contained considerably low hydroxyproline content (2.75 mg/g wet muscle). Sarcoplasmic and alkali-soluble proteins were found to be the major constituents in all muscles, except claw muscle from soft shell mud crab, in which sarcoplasmic protein was the dominant component. Inactivation rate constant (K_D) of natural actomyosin (NAM) Ca^{2-}-ATPase increased markedly when heated at 40 and 50 °C. Among NAM from all muscles, that of hard shell mud crab claw showed the lowest thermal stability. T_{max} from myosin of chunk muscle were 47.58 - 48.08 °C with enthalpy of 0.20 - 0.21 J/g, whereas myosin from claw muscles had lower T_{max} (45.00 to 47.48 °C) with lower enthalpy (0.17 - 0.18 J/g). Chunk muscle bundles of hard and soft shell mud crabs aligned orderly, while claw muscles of both crabs had partial disintegrations and the porous structure was observed in that from soft shell crab. Calcium and magnesium were found to be major minerals in the muscles of hard and soft shell mud crabs (240.5 to 699.2 ppm). However, approximately one-third of both minerals were observed in the muscle of soft

shell mud crab, compared to that of hard shell mud crab. Copper, Iron and Zinc were found to be the trace minerals with the amount less than 50 ppm.

Maximum caseinolytic activities of proteinases from all muscles were observed at 65 °C and pH 8.0, however soft shell mud crab claw muscle proteinase had the highest activity at 60 °C and pH 5.5. Proteinase activities from all muscles tested were inhibited markedly by 0.01 mM soybean trypsin inhibitor, but the activity of proteinase from soft shell mud crab claw muscle was inhibited by 1 μM pepstain A.

Physiochemical changes of muscle from hard and soft shell mud crabs were monitored up to 12 weeks at -20 °C. Ca^{2+}-ATPase NAM from both crabs decreased continuously with increasing surface hydrophobicity as the storage time increased. Disulfide bond formation was observed with the coincidental decrease in sulfhydryl content during extended storage. TBARS and formaldehyde contents of all samples, especially soft shell mud crab claw muscle, increased throughout the storage. In general, solubility of all samples decreased continuously throughout 12 weeks of frozen storage. The pH of all samples decreased during frozen storage, except chunk and claw muscles of soft shell mud crab, whose pH increased between first and second week of the storage. Cooking loss of all crab muscles increased as storage time increased.

Freezing-thawing affected the physicochemical changes of hard and soft shell mud crab muscles. Ca^{2+}-ATPase activity of NAM and surface hydrophobicity decreased continuously while formaldehyde contents, TBARS and cooking loss increased as the freeze-thaw cycles increased. Decrease in sulfhydryl content was coincidental with the increase in disulfide bond formation. Surface hydrophobicity of NAM from the claw muscle of hard shell mud crab increased continuously as the freeze-thaw cycles increased, while that from chunk muscle decreased slightly

with 5 freeze-thaw cycles. Slight decrease in pH was detected in all samples as freeze-thaw cycles increased.

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Nuntapol Sutthipan

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Chapter 1

Introduction

Frozen storage of fish is an important preservation method, however deterioration in texture, flavor, and color of fish muscle has frequently occurred by poor conditions during the process (Hale and Waters, 1988). Freezing slows enzyme activity and inhibits microorganism growth but does not decrease lipid oxidation (Pigott and Tucker, 1990b). The extent of quality loss of marine frozen food is dependent upon many factors, which include storage temperature and time, rate of freezing-thawing, and temperature fluctuations and freeze-thaw abuse (Srinivasan *et al.*, 1997a). Fish quality prior to freezing has been recognized as the important factor affecting the final frozen product. After death, proteinases are related in accelerated postmortem degradation which results in fish flesh deterioration (Ladrat *et al.*, 2000).

Mud crab (*Scylla serrata*), a popular seafood, is farmed on a commercial scale in many tropical countries. Mud crabs are now monocultured in increasing density to supply the growing market (Catacutan, 2002). Naturally, the crab grows by molting process during its life time and the crab that has just molted is called soft shell crab. After molting, soft shell crab has to be taken out from seawater immediately to prevent hardening (Dassow, 1968). Soft shell crab becomes more popular and usually purchased at the higher value, compared to hard shell crab. Freezing is an essential method commonly used to prevent the deterioration of both hard and soft shell crabs during the storage or distribution. However, few information about chemical compositions and the changes in physicochemical properties during frozen storage of hard and soft shell mud crabs has been

reported. Thus, the information gained will be beneficial for frozen hard and soft shell crabs industries to maintain the prime quality with the high market value.

Literature Review

1. Mud crab

Scientific names of mud crab are *Scylla serrata* de Haan, *Scylla oceanica Estampador*, etc. Common names are Serrated swimming crab, Edible Mud crab, Pacific Samoan crab, Mangrove crab, etc. (Bhavanishanker and Subramoniam, 1997).

There are some distinguishing features of mud crab. Basal segment of antenna produced into a small lobule at antero-external angle; flagellum small. Antero-lateral borders cut into nine large teeth. Hand inflated and smooth; Carapace perfectly smooth excepting a curved transverse ridge crossing either protogastric and bronchial region. Front four-dentate; antero-lateral borders are cut into nine acuminate teeth of equal size. Three spines on anterior border of arm and two on posterior border; wrist with a stout spine at inner angle and two smaller ones on outer surface; palm very swollen, armed with three spines, one basal and others terminal, arranged side. Ambulatory legs are unarmed (Bhavanishanker and Subramoniam, 1997).

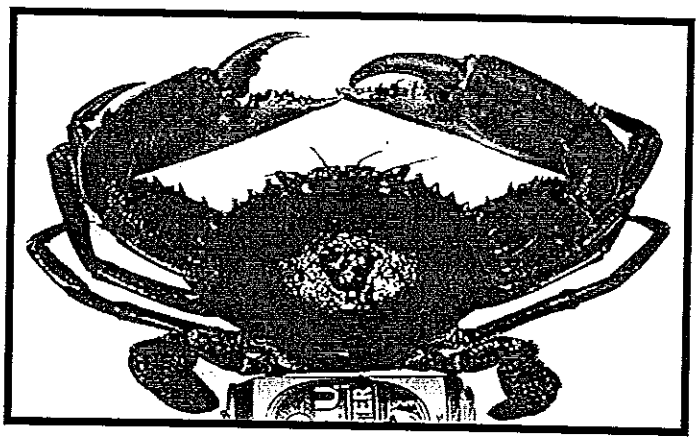


Figure 1 Mud crab (*Scylla serrata*)

Source : Bhavanishanker and Subramoniam (1997)

Both temperature and salinity affect the nitrogen metabolism of mud crabs. At low salinity (10 ptt), there is a catabolism of amino acid and the formation of ammonia to reduce osmolality. At high salinity (40 ptt), there is an occurrence of urea synthesis and a shift of the nitrogen excretory pattern (Chen and Chia, 1996a). Similar studies have shown that oxygen uptake (O_2 mg/g/h), ammonia-N excretion, urea-N excretion, organic-N excretion and total nitrogen excretion (N mg/g/h) increased with increasing temperature and salinity levels (Chen and Chia, 1996b).

2. Soft shell and hard shell crabs

Crabs are members of the *Crustacea* class and the order *Decapoda*. Common biological characteristics of these invertebrates are a hardened outer shell or exoskeleton, five pairs of jointed legs, gills for respiration, and an open circulatory system in which the blood or heamolymph bates the tissues in open channels or sinuses before returning to the single-chambered heart. Generally, crab grows by successive molts of its shell during its lifetime. Crabs are segmented animals with a chitinous exoskeleton. Beneath this rigid cover, the internal organs like gut, nervous system and glands are embedded in the heamolymph of the body cavity (Yoshinaka *et al.*, 1989).

Soft shell crabs are crabs that have just molted (Dassow, 1968). Hard and soft snow crabs are mainly distinguished on the basis of visual appearance and physical feel when touched by hand. The crust of a soft crab is much more transparent and softer than that of hard snow crab. The most pronounced characteristic of a soft snow crab is a considerable amount of free body fluid (FBF) in its appendages, which can flow out from appendages when the crust is out. Soft snow crab muscle have a higher water content and a lower protein content than hard shell crab muscle. Salinity of the free body fluid recovered

from the meropodites of the soft snow crab was significantly higher than that of the hard snow crab (Mizuta *et al.*, 2001).

Commercially, soft crabs can be obtained by holding hard shell crabs in floats until the molt occurs. The soft crabs are removed from the water within a few h and are graded for size. The crabs may be held in cool storage for 2 to 3 days before processing. The crabs then are killed, eviscerated, washed, wrapped individually with parchment, packed in a carton, and frozen (Dassow, 1968).

3. Growth and Molting

Crabs are invertebrates, which lack a spinal column. Instead, the crab has a rigid exoskeleton (hard shell). The shell grows in discrete stages interspersed by molting, but the growth of internal tissue is more continuous. In order for the crab to grow larger, it must periodically shed its smaller shell through a process known as molting (ecdysis). Early in its molting cycle, the crab slowly forms a new "soft" shell underneath its existing hard shell. There are certain marks or "signs" that allow experienced watermen to know how soon the crab will molt. When a crab has grown sufficiently to require a larger shell, the following events occur (Fig. 2) (Havens and McConaugha, 1990).

3.1 Precdysis (pre-molt or "peeler" stage)

In this stage, molting hormones are released. The hypodermis detaches from the existing hard shell. The hypodermis is a layer of cells directly beneath the shell. The hypodermis produces enzymes which begin to dissolve the shell components. Much of the existing shell is recycled causing it to become thin. Inorganic salts are resorbed from the shell and stored internally. A new inner "soft" shell slowly forms underneath the existing shell. When this new shell has fully formed, the crab will be ready to molt (Havens and McConaugha, 1990).

3.2 Ecdysis (Molting or "busting" stage)

The crab stops eating and seeks shelter in order to avoid predation. During this process the crab is highly vulnerable to predators. The crab rapidly absorbs water which causes its tissues to swell and split the old shell open across the back between the lateral spines. Fracture planes in the claws split open to allow the claws to be pulled through. The crab begins a slow arduous process of backing out of its old shell, which is then discarded. The newly molted crab pumps water into its tissues in order to inflate the new shell to its new size. The new shell will be roughly one-third (33%) larger than the old shell. The new shell reaches its full size within 6 h after molting (Havens and McConaugha, 1990).

3.3 Postecdysis (postmolt or "soft crab" stage)

The salvaged inorganic salts are rapidly redeposited to help thicken and harden the new shell. The new shell will only harden in water (the hardening process stops if the crab is removed from the water) and will take approximately two to four days to fully harden (Havens and McConaugha, 1990).

Over time, as the crab slowly grows inside its new shell, tissue water is replaced with protein. Once there is no more room left to grow inside this shell, the whole molting process starts over again (Havens and McConaugha, 1990)

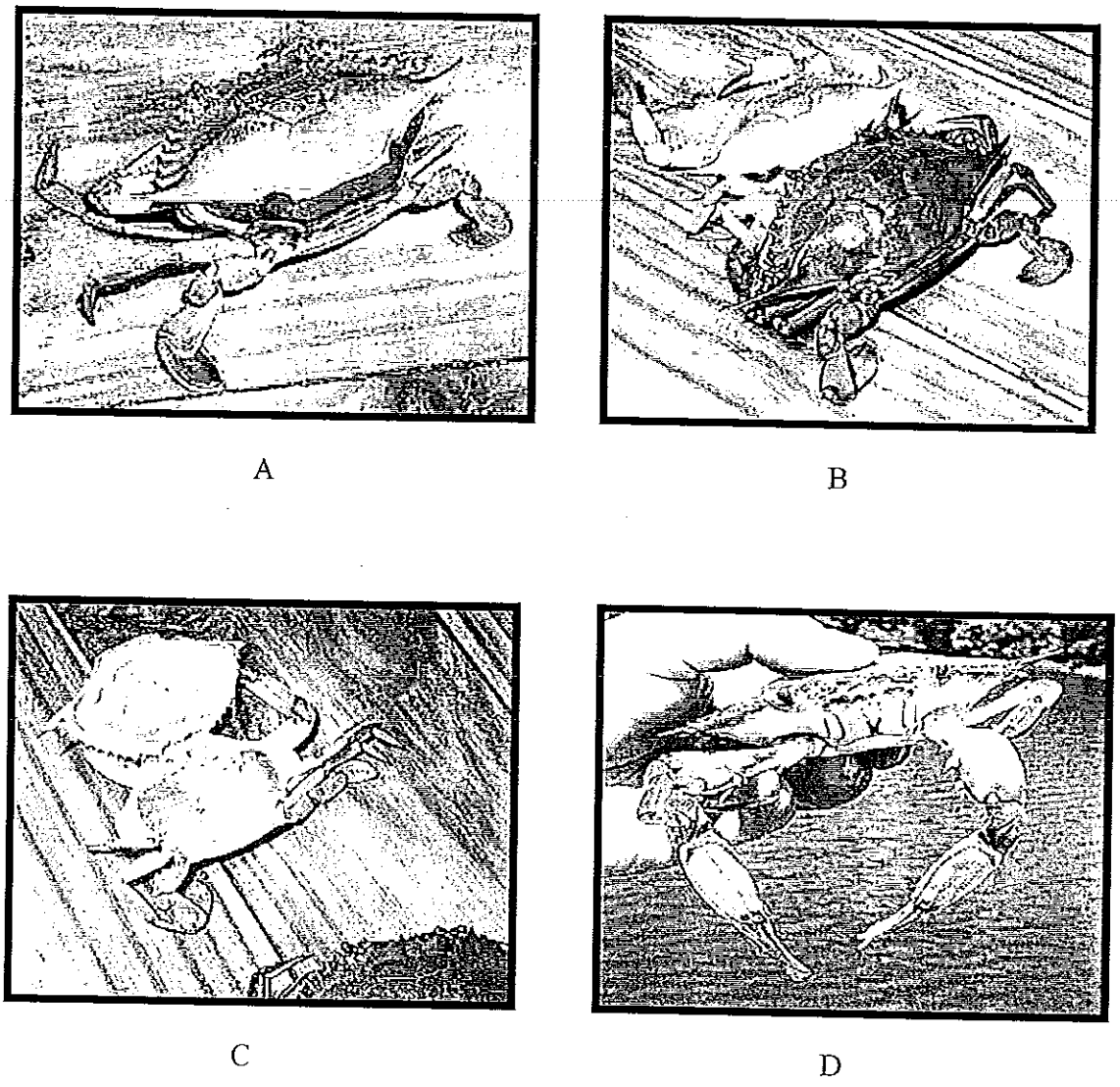


Figure 2 Molting process

A = Crab has just started to molt.

B = Crab has successfully molted.

C = The old gills are left behind.

D = Crab is very tired and weak after molting.

Source : Havens and McConaugha (1990)

Molting signs in a Molting cycle (Havens and McConaugha, 1990)

1. **Hard shell – very hard shell** - 14 to 50 days prior to molt, depending on crab size.
2. **White sign peeler** (green, snot, fat or heavy crab) - Two weeks prior to molt.
3. **Pink sign peeler** (pink or medium peeler) - one week prior to molt.
4. **Red sign peeler** (red or ripe peeler) - Two days prior to molt.
5. **Rank peeler** - Hour prior to molt.
6. **Buster** - In the process of shedding its old shell.
7. **Soft shell** - Smooth, soft shell; immediately following molt.
8. **Paper shell** - Slightly stiff shell; 12 h after molt.
9. **Buckram** (or tinback) - Semi-stiff, crinkly-hard or leathery shell; one day (24 h) after molt.
10. **Hard shell** - four days after molt. Known as "whitery".

4. Chemical compositions of crabs

Crab meat is quite high in protein content ranging from 16 to 20 %, and low in fat, from 0.8 to 1.6 % of the weight of the cooked meat ready to eat (Dassow, 1968) (Table 1). Crabs are slightly higher in protein, while mollusks, especially oysters, have the lower protein content of 8 – 9 %.

The total mineral content is about the same in crab but is more variable than in most fish species from 1.2 to 1.6 % by weight of the cooked crab meat. The natural sodium content is much higher than in most fish, about 200 mg. per 100 gm. of cooked crab meat (330 mg. per 100 gm. of uncooked crab meat) as compared to 60 to 70 mg. per 100 gm. of fish flesh (Table 2) (Dassow, 1968; Pigott and Tucker, 1990a). The contents of calcium, megnesium, phosphorus, and iron in crab meat are similar to those in fish fillets (Dassow, 1968) (Table 2).

Table 1 Proximate composition of some species (raw edible portion)

Species	Water (%)	Protein (%)	Lipid (%)	Cal / 100 g
Cod	81.2	17.6	0.3	78
Mackerel Atlantic	67.2	19.0	12.2	191
Salmon, Atlantic	74.5	17.2	5.5	125
Fish (in general)	74.8	19.0	1.0	-
Crab, blue	78.8	16.4	0.8	78
Oysters	82.2	8.6	2.4	66 – 91
Scallops	77.9	15.1	1.0	81
Beef, ground	60.2	17.9	21.2	268
Chicken	63.7	19.3	16.3	203

Source : Adapted from USDA (1986 Referred by Pigott and Tucker, 1990a)

Crab meat contains about 125 mg. % of cholesterol, a value about twice or more as high as that of fish fillets or chicken meat. Moreover, USDA (1986 cited by Pigott and Tucker, 1990a) reported that crabs are lower in fat content, contained only 1.3 % of edible portion, while some fishes, especially, tuna and salmon have higher fat content (about 4.9 – 10.4 %). Saturated fatty acids which present in crab are also found at a low level.

Table 2 Selected minerals in seafoods and other foods

Food	Na	Ca	Fe	Zn	Cu
	(mg / 100 g)	(mg / 100 g)	(mg / 100 g)	(mg / 100 g)	(mg / 100 g)
Cod	90	15	4.8	10.5	2.5
Herring	105	58	10.9	7.4	1.7
Salmon, king	42	20	9	8	4
Clam	316	83	69	30	2.5
Crab	330	60	52	28	5.7
Oyster	386	111	82	232	63
Beef	65	9	22	-	-
Chicken	12	58	13	-	-

Source : Adapted from USDA (1986 Referred by Pigott and Tucker, 1990a)

5. Proteins and other nitrogenous compounds of marine muscle

There are different proteins in the marine muscle. These proteins perform different tasks and differ in their properties. The proteins can be classified into these groups based on solubility as follows:

5.1 The sarcoplasmic proteins

The sarcoplasmic proteins usually refers to the proteins of the sarcoplasm as well as the components of the extracellular fluid and the sarcoplasm. The sarcoplasmic proteins are extracted by homogenizing the muscle tissue with water or solutions of neutral salts of ionic strength below 0.15. Among the sarcoplasmic enzymes influencing the quality of fish, the enzymes of the glycolytic pathway and the hydrolytic enzymes of the lysosomes are found to be important (Sikorski *et al.*, 1990a).

5.2 The myofibrillar proteins

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. The myofibrillar proteins are related with the water holding capacity and the other functional properties of proteins such as gelation, etc (McCormick, 1994). Other myofibrillar proteins are listed in Table 3 which are different in size and location in the muscle (Ashie and Simpson, 1997).

Table 3 Contractile proteins in food myosystems

Protein	Relative Abundance (%)	Size (kDa)	Location
Myosin	50 - 60	470	Thick filaments
Actin	15 - 30	43 - 48	Thin filaments
Tropomyosin	5	65 - 70	Thin filaments
Troponins	5		Thin filaments
Troponin - C		17 - 18	
Troponin - I		20 - 24	
Troponin - T		37 - 40	
C - protein	-	140	Thick filaments
α - Actin	-	180 - 206	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 Ashie and Simpson (1997)

The repeating contractile unit of the myofibril is the sarcomere (Fig. 3). Skeletal muscle generally consists of two sets of thin filaments each anchored at one end in a structure: the Z-disk and interdigitating thick filaments. The thick filaments are anisotropic. They refract birefringent light, and are called A-bands (McCormick, 1994).

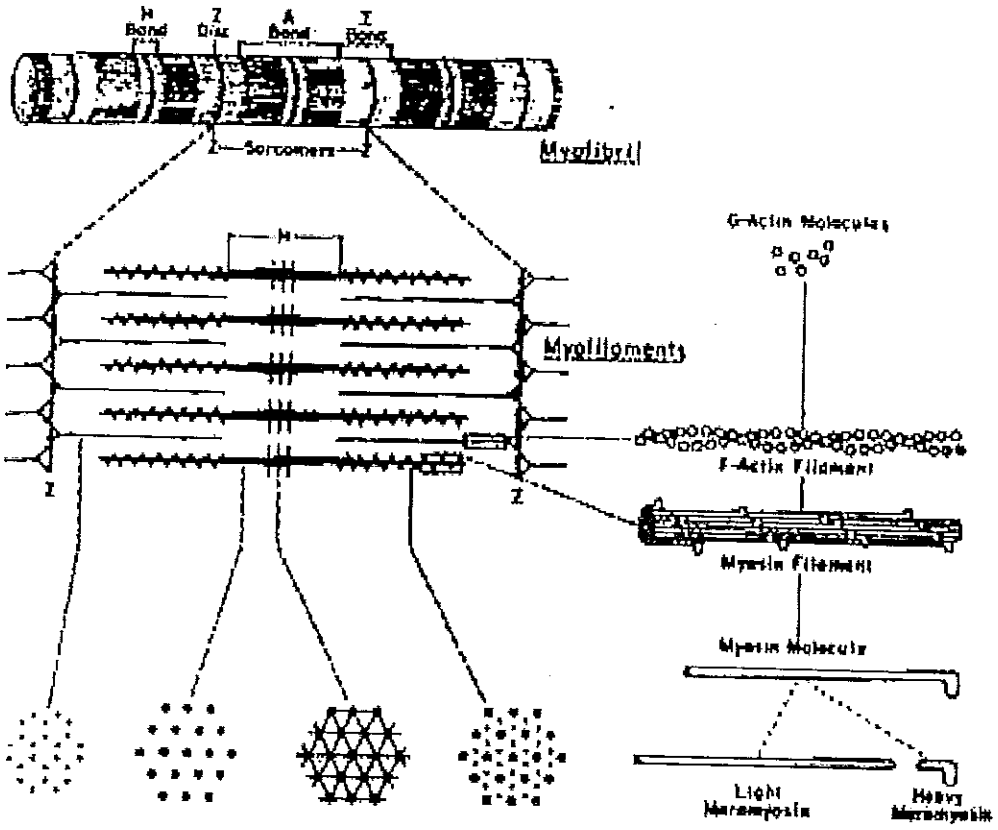


Figure 3 Diagram of the organization of skeletal muscle

Source : McCormick (1994)

5.2.1 Myosin and Paramyosin

Myosin makes up 50 to 58 % of the myofibrillar fraction (Sikorski *et al.*, 1990a). About one-third of the total protein in muscle is myosin, the predominant myofibrillar protein of the thick filament. Native molecular weight of myosin is about 500,000 dalton. The long tail of the molecule consists of two polypeptides in a coiled alpha-helix terminating in two globular heads at one end (McCormick, 1994).

Myosin is an enzyme possessing ATPase activity. The globular head regions of myosin bind and hydrolyze ATP to ADP. Digestion with trypsin cleaves the long tail of the molecule producing two fragments: light meromyosin and heavy meromyosin. The latter contains the globular head. Further treatment with papain cleaves one or both globular heads into subfragments. Treatment with papain produces a long tail-like fragment known as the myosin rod, whereas trypsin cleavage of the myosin rod produces light meromyosin and subfragment 2 (McCormick, 1994).

Paramyosin has particularly amino acid composition and has been extracted from various species of marine mollusks. It consists of high basic amino acid and amide content, such as glutamine (20 to 23.5 %), aspartic acid (12%), argenine (12%), and lysine (9%), but low in proline content. Paramyosin, a rod-shaped alpha-helical chains, consists of 2 subunits which are 120 nm long with a molecular weight ranging from 95,000 to 125,000 dalton per subunit (Foegeding *et al.*, 1996).

5.2.2 Actin

Actin is about 15 to 20 % of myofibrillar protein (Sikorski *et al.*, 1990a). Actin is one of three major myofibrillar proteins of thin filaments. Each actin molecule, generally visualized as globular, has a molecular weight of about 40,000 dalton, called G-actin. 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).

5.2.3 Tropomyosin

Tropomyosin, a rod-like molecule, consists of two polypeptide chains, each with a molecule weight range of 34,000 - 36,000 dalton, 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) (Figure 4a). Tropomyosin is about 5 % of myofibrillar protein. Each tropomyosin molecule consists of 7 molecules of G-actins (Foegeding *et al.*, 1996).

5.2.4 Troponin

Troponin is an asymmetrical protein and consists of three subunits. Troponin T (molecular weight of 37,000 dalton), 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 weight of 18,000 dalton) binds Ca^{2+} and confers Ca^{2+} sensitivity to the troponin-tropomyosin-actin complex. Troponin I (molecular weight of 23,000 dalton), the inhibitory subunit, binds tightly to troponin C and actin and only slightly to tropomyosin or troponin T (McCormick, 1994) (Figure 4b).

5.3 The proteins of the stroma

The stroma is composed of the main connective tissue proteins, such as collagen and elastin. The stroma is the residue after extraction of the sarcoplasmic and myofibrillar proteins. Furthermore, the stroma is insoluble in dilute solutions of hydrochloric acid or sodium hydroxide (Sikorski *et al.*, 1990a).

5.3.1 Collagen

Collagens in the muscle of marine animals play a key role in maintenance of meat texture. The musculature of several crustaceans has been shown to contain collagenous proteins (Yoshinaka *et al.*, 1989). The amino acid composition of crustacean collagens is richer in several essential amino acids making the biological value of such collagens significantly higher than bovine and other mammalian muscle collagens (Sikorski *et al.*, 1984 cited by Ashie and Simpson, 1997). A collagen molecule is a helical alpha chain. Each alpha chain twists into a left-handed polyproline helix with three residues per turn. The helical alpha chains contain a GLY-X-Y sequence repeated 340 + 2 times per molecule, where X or Y are often proline or hydroxyproline (Figure 5A). The three helical alpha chains are wound into a right-handed superhelix which forms a molecule about 1.4 nm wide and 300 nm long. Fibrillar collagens are, thus, about one-third glycine and one-quarter proline and hydroxyproline with a molecular weight of about 300,000 dalton (Figure 5B). Collagen content in animal tissue changes, depending upon the types of animals, animals age and maturity. Collagen cross-link concentrations increase with age of all species. The steady increase in mature collagen cross-linking is due to progressive and ongoing cross-linking reactions that occur within fibrillar collagen and with the slowing of collagen synthesis rates as animals reach maturity (McCormick, 1994).

At least 14 different collagen types have been identified. However, the major ones are types II and III. Type I collagen is predominant in the epimysial membrane, type II and III in the perimysium, and type III, IV, and V in the muscle endomysium (Hultin, 1985). Sivakumar *et al.* (2000) proposed that type V like collagens were widely distributed in marine invertebrates, particularly crustaceans and molluses.

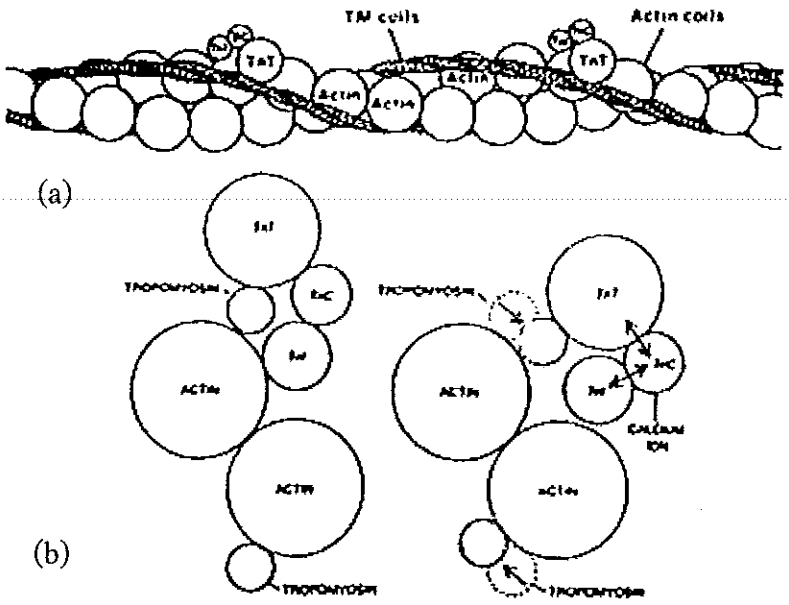


Figure 4 (a) A thin filament of muscle formed by the filament of tropomyosin molecules wound in each of the two grooves of the actin helix. In this schematic diagram only the actin helix are shown. (b) Proposed model for configuration of actin, tropomyosin and troponin (Tn) subunits.

Tn T = troponin-tropomyosin subunit

Tn I = troponin-inhibitory subunit

Tn C = troponin-calcium-binding subunit

Source : McCormick (1994)

The crab leg muscle collagen is involved in the provision of flexibility and support and to maintain the balance during locomotion and the claw muscle collagen performs similar functions to grip prey. The abdominal muscle, on the other hand, is involved in normal growth and development and metabolism (Yoshinaka *et al.*, 1989). The musculature of crab only contracts but does not bend, as opposed to that of marine crustaceans like prawn which have a

flexible musculature and collagens with more crossliking and higher denaturation temperature (Sivakumar *et al.*, 2000).

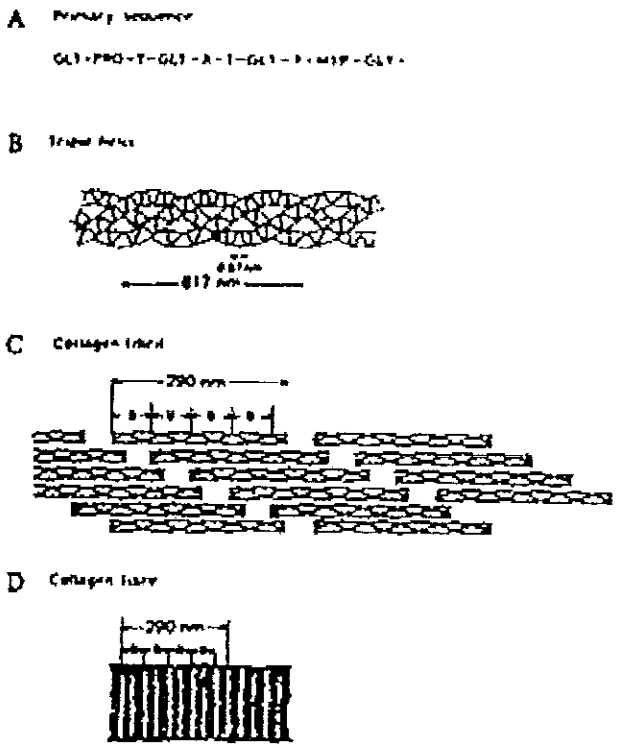


Figure 5 Collagen

A = Collagen amino acid sequence

B = Super helix of the three helical alpha chains

C = Collagen filament

D = Collagen fiber structure

Source : McCormick (1994)

5.3.2 Elastin

Elastin is a rubberlike connective tissue protein which confers elasticity and stretchability to tissues. It is heavily cross-linked with desmosine

and isodesmosine cross-links which arise from lysine residues. Elastin is noted for its extreme insolubility and resistance to physical disruption by heat. It occurs only in small quantities in skeletal muscle. However, elastin is hardly found in marine muscle (McCormick, 1994).

6. Proteolysis in marine muscle

Proteinases are associated with the accelerated postmortem degradation which results in fish flesh deterioration. Moreover, degraded tissue by proteolysis is considerred to be an excellent medium for bacterial growth, inducing microbial spoilage. Types of proteinases are varied considerably with types of muscle. Calcium-dependent proteinases or calpains were involved in postmortem proteolytic activity at neutral pH of sea bass (*Dicentrarchus labrax* L.) muscle (Ladrat *et al.*, 2000). Cathepsin D mainly degraded myosin, actin, and tropomyosin of herring (*Clupea harengus*) muscle at pH 4.23 (Nielson and Nielsen, 2001). Visessanguan *et al.* (2001) reported the involvement of heat-activated proteinases in the degradation of arrowtooth flounder muscle. Proteolytic activities in marine muscle vary greatly according to harvesting season, gender maturation, and spawning. Love (1970) reported that cathepsin played an important role in myofibrillar protein turnover of herring muscle during spawning cycle.

Molting cycle of the Bermuda land crab influenced the activities of proteinases in the crab muscle. Muscle protein decreased 40 % during proecdysis and was restored following ecdysis (Mykles and Skinner, 1982).

6.1 Proteinases

The great numbers of enzymes are found in marine muscle. However, proteinases that play important roles in the texture change of postmortem fish muscle are cathepsins and calpains. They are the major proteinases in tenderization of postmortem muscle (Jiang, 2000). Ward (1983) classified proteases

into two main groups as exopeptidases and endopeptidases, according to the location of proteolysis activity on the peptide chains. Exopeptidases, such as amino peptidases, and carboxypeptidases, have hydrolytic action on the terminus peptide bond. Endopeptidases or proteinases cleave peptide bonds internally in the peptides.

Furthermore, endopeptidase or proteinase can be classified into four groups via the nature of the catalytic residues at the active sites, which are serine, cysteine, aspartic, and metallo proteases. Their activities are controlled by specific endogenous inhibitors, activators, pH, temperature of the environment (Jiang, 2000), type of enzyme, enzyme concentration, and time of reaction (Arai and Fujimaki, 1991).

6.1.1 Serine proteinase (3.4.21)

Serine proteinase is widely distributed in various tissues and body fluids. The maximum activity is at neutral to alkaline pH. The activity can be inhibited by phynylmethanesulfonyl fluoride (PMSF) (Bond and Butler, 1987). Ishida *et al.* (1995) reported that two kinds of serine proteinases, type I and type II, were purified from salted muscle of anchovy (*Engraulis japonica*). Molecular weight of type I and type II, were estimated to 25,000 and 37,000, respectively. Optimal pH and temperature for type I activities were pH 6 to 8, 45 °C and pH 7.0 to 7.5, 50 °C for type II.

Choi *et al.* (1999) isolated two proteinases with molecular weights of 112,000 and 90,500 daltons from Atlantic menhaden muscle. Both of them were most likely tryptic serine type proteinases.

6.1.2 Cysteine proteinase (3.4.22)

Cysteine proteinase plays an important role in protein degradation of the tissue muscle (Hopkins and Thompson, 2001). Their active sites contain sulfhydryl groups. The reducing agents such as hydrogen cyanide (HCN) and

cysteine can activate enzyme activity, while diisopropyl fluorophosphate (DEP) inactivates the activity by blocking sulfhydryl groups in the active sites (Ward, 1983). Hopkins and Thompson (2001) reported that trans-Epoxysuccinyl-L-Leucylamido (4-Guanidino)-Butane (E-64) inhibited the activity of cysteine proteinase.

6.1.2.1 Calpain

Calpains are referred to as calcium-activated neutral proteases (CANP), which have the great activity at neutral pH (Jiang, 2000). Calpains were inhibited by Fe^{2+}, Fe^{3+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, or Hg^{2+} (Wang *et al.*, 1993). Klee (1988) reported that Hg^{2+} binded to SH groups of the target enzyme and subsequently inhibited the enzymatic activity.

On the basis of the calcium requirement, there were two types of calpains isolated from animal tissues, i.e., μ-calpain and m-calpain, which required 5 to 50 and 150 to 1000 μM Ca^{2+} for half-maximal activity, respectively. Only m-calpain was found in carp muscle, egg, erythrocytes, and lobster claw and abdominal muscles (Wang *et al.*, 1993). The Ca^{2+} - dependent proteinases (CDP) purified from crustacean muscle appeared to have a more direct role in myofibril protein turnover, while CDPs from vertebrate muscle hydrolyzed neither actin nor myosin. Moreover, Crustacean CDP also differed from vertebrate CDP in its stability. In the presence of Ca^{2-}, vertebrate CDP underwent a rapid autodegradation (Mykles and Skinner, 1982). Ladrat *et al.* (2000) isolated three groups of enzymes by anion-exchange chromatography from seabass white muscle and was classified to be calpain-like enzymes.

6.1.2.2 Cathepsin

Lysosomes are sources of about 13 cathepsins which play the key roles in the protein turnover *in vivo* and in the postmortem rheological changes of fish muscle. Among these lysosomal enzymes, cathepsins B, D, H, L,

L-like, and X have been purified and characterized from fish and shellfish muscles (Jiang *et al.*, 1993). The lysosomal cathepsins B, D, H, L, L-like, and X are cysteine proteinase and papainlike enzymes (Lee *et al.*, 1996)

Cathepsin B is considered to be both endopeptidase and dipeptidyl carboxypeptidase enzyme (Kirschke and Barrett, 1987 cited by Yamashita and Konagaya, 1990b). Cathepsin B and L caused the softening phenomenon of post-mortem muscle of chum salmon (Yamashita and Konagaya, 1991). Cathepsin L was found to be the predominant proteinase involved in heat-induced degradation of the myofibrillar protein in Pacific whiting surimi (An *et al.*, 1994b). Mackerel cathepsin L hydrolyzed myosin, troponin T, troponin I, and tropomyosin. Mackerel cathepsin B did not hydrolyze any of the myofibrils from mackerel white muscle (Aoki and Ueno, 1997).

Cathepsin L was considered to be a main cause of fish tissue degradation and softening of chum salmon (Yamashita *et al.*, 1990a) and Pacific whiting (Seymour *et al.*, 1994; An *et al.*, 1994a). Cathepsin L degraded proteins at least 10 times more rapidly than the other cysteine proteinases, including cathepsins B and H (Bohley and Seglen, 1992). Kirschke and Barrett (1987) reported cathepsin L had very high activity against various collagens and elastin. Therefore, it may play an important role in tissue softening of fish.

Cathepsins B and L caused the tenderization of postmortem muscle of salmon. Cathepsin L was capable of hydrolyzing the major muscle structural proteins, such as connectin, nebulin, myosin, collagen, α-actinin, and troponins T and I. Although cathepsin B hydrolyzed connectin, nebulin, myosin, the hydrolytic action was limited to only these proteins (Yamashita and Konagaya, 1991). Visessanguan *et al.* (2001) reported cathepsins L played an important role in the autolysis of arrowtooth flounder muscle. The enzymes exhibited the maximum activity at 60 °C and were inhibited by E-64.

6.1.3 Aspartic proteinase (3.4.23)

Aspartic proteinase has optimal pHs at acidic pH and are found in spleen and liver. Cathepsin D is a major lysosomal aspartic proteinase which has been purified and characterized from a variety of tissues (Kirchke and Barrett, 1987). Cathepsin D is involved in the cellular degradation of proteins (Reddi *et al.*, 1972) and has optimal pH at 3.2 (Makinodan *et al.*, 1984). Pepstatin A inhibits the proteolytic activity of aspartic proteinases completely (Yamashita and Konagaya, 1990a).

Cathepsin D may involve in the postmortem degradation of muscle proteins and thereby contribute to textural changes of fish. Cathepsin D from fish muscle is capable of cleaving muscle myofibrillar protein at pH as high as 6 – 6.5. This is far from the optimal pH of 2.5 – 3.5 (Goldman-Levkovitz *et al.*, 1995). Nielsen and Nielsen (2001) observed that cathepsin D from herring (*Clupea harengus*) muscle was a monomer with a molecular weight of 38,000 to 39,000 and had optimal activity at pH 2.5. It was inhibited by pepstatin. Furthermore, the first 21 amino acid residues of the N-terminal showed high similarity to cathepsin D from Antarctic icefish liver (*Choinodraco hamatus*) and trout ovary (*Oncorhynchus mykiss*).

6.1.4 Metallo proteinase (3.4.24)

Metallo proteinase had optimal activity at neutral pH (pH 7.2). Its optimal temperature was 40 °C. Metallo proteinases were inhibited by EDTA and O-phenanthroline (Makinodan *et al.*, 1982).

6.2 Collagenase

Collagenases are strictly defined as proteases capable of degrading native triple helix of collagen. Two classes of proteases with collagenolytic activity have been reported and are thought to play different physiological functions. 1.) Metallocollagenases are zinc-containing enzymes, requiring Ca^{2+} for stability. Their

molecular masses were found to vary from 30,000 to 150,000. 2.) Serine collagenolytic proteases are probably involved in food digestion rather than in morphogenesis. The range of molecular mass for each enzyme was found to be 24,000 – 36,000, isolated from the hepatopancreas of the fiddler crab (Stricklin *et al.*, 1977). A serine proteolytic collagenase from the digestive tissues of fiddler crab was considerably different from vertebrate and bacterial collagenases (Eisen and Jeffrey, 1964 cited by Sivakumar *et al.*, 1999).

Sivakumar *et al.* (1999) isolated a collagenolytic metalloprotease from hepatopancreatic digestive tissue of the marine crab, *Scylla serrata*. This enzyme differed considerably from other serine proteolytic collagenases of crustaceans, but closely resembled the metalloproteases of vertebrates. The enzyme had a molecular mass of 55,000 and was active against native type I collagen solution, casein, haemoglobin, and bovine serum albumin.

7. Deterioration of seafoods during frozen storage

The extent of quality loss of frozen marine food is dependent upon many factors including storage temperature and time, rate of freezing-thawing, and temperature fluctuations and freeze-thaw abuse (Srinivasan *et al.*, 1997a).

7.1 Temperature and time of frozen storage

Decreasing storage temperature reduces the rate of reactions which contribute to quality loss. Excellent storage life is observed at very low temperatures (Haard, 1992). Zayas (1997) studied the solubility in a 0.6 M KCl solution of frozen beef muscle (with fast and slow freezing methods) stored at -20 °C, -10 °C, and -5 °C for 40 – 45 weeks. The loss of solubility of myofibrillar proteins was greater at the higher storage temperature. At -5 °C, the solubility of proteins decreased after the first week; at -10 °C the solubility decreased

significantly after 6 weeks, while at -20 °C, solubility decreased slowly within 13 weeks of storage.

Jiang *et al.* (1988) observed that denaturation of milkfish actomyosin increased at a much higher rate at -20 °C than at -35 °C. The protein denaturation during freezing and subsequent storage was mainly caused by formation of disulfide. The loss of Ca^{2+} - ATPase and Mg^{2+} - ATPase activities and Ca^{2+}-sensitivity were due to the oxidation of SHs on the active site of actomyosin and the formation of the disulfides. The formation of the disulfides occurred in actomyosin molecules during frozen storage at a much higher content at -20 °C than at -35 °C. Licciardello *et al.* (1982) studied the effect of frozen storage temperature on shelf - life of red hake and found that frozen storage at -28 °C was able to keep the quality of frozen red hake muscle up to 100 weeks, while storage temperature at -10 °C resulted in the loss of the protein functionality within 10 weeks.

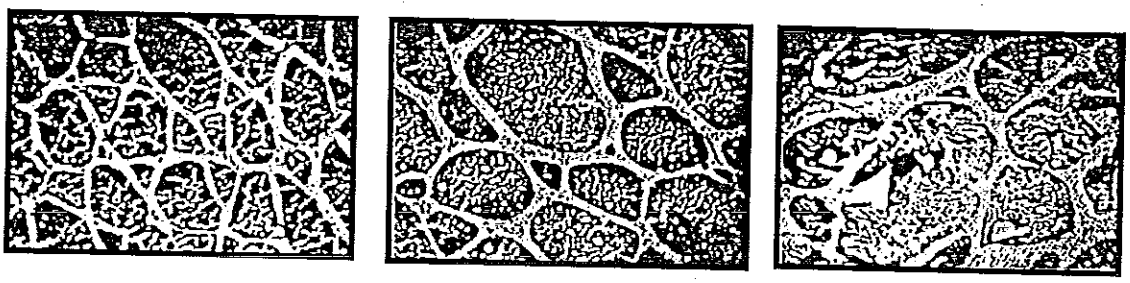
7.2 Rate of freezing

Fast freezing is necessary to maintain the basic physical and chemical properties of the product. However, large products such as whole fish take longer to freeze than small items or packaged prepared foods. This is due to the slower rate of the larger products to be frozen. In addition, the lack of strong connective and other interstitial tissues causes fish to have a rather delicate texture, which is easily altered (Pigott and Tucker, 1990c).

Bello *et al.* (1982) studied the effect of freezing rate on quality of trout skeletal muscle. The fast and slow freezing rates affected the quality of the muscle differently as shown in figure 6.

Major cell damage has resulted from the slow freezing. This is caused by the seeding and growth during freezing of large water crystals that expand and rupture the cell walls. The slowly frozen fish loses considerable weight when

thawed due to the loss of moisture through ruptured cell walls. Not only is the texture adversely affected, but there is a significant economic loss due to weight decrease. Fast freezing results in small crystals that cause minor cell wall damage. Thus, the maintainance of high quality in a frozen product depends on the temperature lowering which should be fast enough to pass through the zone of maximum crystal formation as rapidly as possible (Bello *et al.*, 1982).



A B C

Figure 6 Electron micrograph of cross-sections of trout skeletal muscle

A = Fresh trout skeletal muscle

B = Fast frozen

C = Slow frozen

Source : Bello *et al.* (1982)

Reid *et al.* (1986) studied biochemical/chemical change in fast versus slow-frozen Pacific rockfish stored at -5 °C and -20 °C. Storage temperature had a much larger influence on deteriorative processes than freezing rate. Lower storage temperature (-20 °C) was able to preserve more water retention in the muscle than higher storage temperature (-5 °C). Furthermore, the decrease in Ca^{2+} - ATPase was larger when the muscle was stored at higher storage temperature.

7.3 Temperature fluctuation

Temperature fluctuation or abuse generally occurs during transportation, storage or consumption. This can lead to the deterioration of fish quality (Benjakul and Bauer, 2000). Temperature fluctuation during storage has the adverse effects, not only on the quality of product due to enzyme action and the freeze-thaw crystal growth, but via moisture movement from the surface of the product. This movement cause surface desiccation (freezer burn) as well as unsightly appearance of the product (Pigott and Tucker, 1990c).

The ice crystals are allowed to grow during the fluctuation, causing additional cell damage and subsequent water loss when the product is thawed. Thus, a system must be designed and operated not only to maintain low temperature during freezing but to prevent significant temperature fluctuations during frozen storage (Pigott and Tucker, 1990c).

7.4 Thawing

Freezing and thawing also affect the membrane structures of muscle cells. The disintegration of membrane structures can be measured by the activity of one or more enzymes in muscle tissue fluids. Normally enzymes in fresh tissue are retained in intracellular organelles. The leaked enzymes are regarded as markers of membrane demage and the activity of lysosomal enzymes in the centrifuged tissue fluid has been used to differentiate frozen from fresh fish. (Rehbein *et al.*, 1978). Membrane integrity was estimated as the volume of centrifuged tissue fluid (CTF) and by lysosomal ß-N-acetyl-glucosaminidase (NAG) activity in CTF (Nilsson and Ekstrand, 1995). Slow thawing of rainbow trout, in air at 5 °C, resulted in higher NAG activity in CTF and a larger volume of CTF than fast thawing, at 25 °C in water.

The disintegration of membrane structures increased as the number of freeze-thaw cycles increased. Benjakul and Bauer (2000,2001) reported that when

the number of freeze-thaw cycles of cod and catfish increased, the activities of α-glucosidase (AG) and ß -N-acetyl-glucosaminidase increased. The loss of Ca^{2+} - ATPase and Mg^{2+}- Ca^{2+}- ATPase increased with increasing freeze-thaw cycles. However, Mg^{2+}- EGTA – ATPase activity increased with the loss of Ca^{2+}- sensitivity, indicating the denaturations of tropomyosins and/or troponins (Okitani *et al.*, 1980; Benjakul *et al.*, 1997).

Benjakul and Bauer (2001) reported that TBARS in catfish fillet increased when the number of freeze-thaw cycle increased. A significant increase in TBARS was found when the thawed samples were stored for 3 days at 4 °C. Therefore, the freeze-thaw cycles played an essential role in the oxidation acceleration. This was due to the fact that the freeze-thawing caused some loss of integrity in the muscle system. The ice crystals formed could injure the cell and caused the release of pro-oxidants for lipid oxidation, especially free iron (Benjakul and Bauer, 2001).

8. Changes in muscle during frozen storage

Freezing and storage in the frozen state is an effective method for preventing microbial spoilage of foods. However, changes in the functional properties of protein of animal origin have been observed (Wagner and Anon, 1986). The protein denaturation, in particular myofibrillar proteins, is the main factor of meat quality deterioration during frozen storage (Zayas, 1997). Badii and Hawell (2001) observed that frozen storage of cod and haddock muscle at 10 °C led to the denaturation of myosin causing a reduction in solubility and the selective precipitation of proteins.

8.1 Changes of protein

8.1.1 Protein denaturation

8.1.1.1 Surface hydrophobicity

Extended frozen storage caused the severe changes in tertiary conformation of actomyosin. As a result, an exposure of the interior in molecule occurred, leading to an increase in surface hydrophobicity. The increase in surface hydrophobicity possibly caused the association of hydrophobic groups via hydrophobic interaction. The surface hydrophobicity generally decreased with increasing frozen storage period (Benjakul and Bauer, 2000). Badii and Howell (2002) reported the initial increase in surface protein hydrophobicity of cod muscle in the first month before decreasing during the frozen storage at -10 and -30 °C.

8.1.1.2 ATPase activity

Ca^{2+}- ATPase activity can be used as an indicator for the integrity of the myosin molecule, while Mg^{2+}- Ca^{2+}- ATPase activity indicates the integrity of the actin-myosin complex in the presence of exogenous Ca^{2+} ions. Mg^{2+}- EGTA – ATPase activity is indicative of the integrity of the troponin-tropomyosin complex. Ice crystals and the increase in ionic strength of the system during frozen storage caused myosin denaturation and the disruption of the actin-myosin complex, as indicated by the decrease in both Ca^{2+}-ATPase and Mg^{2+}- Ca^{2+} - ATPase activities (Benjakul and Bauer, 2000). Ang and Hultin (1989) reported that crosslinking of cod myosin stored at -25 °C and -80 °C was related to the decrease in ATPase activity. Holmquist *et al.* (1984) found that Ca^{2+} - ATPase activity values were higher in the red hake fillets than in the red hake mince and surimi during 10 weeks of storage at -5 °C. Nambudiri and Gopakumar (1992) reported that the activities of adenosine triphosphatase (ATPase)

and lactate dehydrogenase (LDH) from the muscle of fresh water and brackish water fish decreased during frozen storage (-20 °C) over a period of 180 days.

8.1.2 Aggregation

The denaturation and aggregation of protein started from the formation of disulfide bonds followed by a rearrangement of hydrophobic and hydrogen bonded regions on an intra- and inter- molecular basis (Buttkus, 1974).

8.1.2.1 hydrophobic interaction

In general, the native protein structure is stabilised by many forces, including hydrogen bonding, dipole-dipole interaction, electrostatic interactions, and disulphide linkages. Protein can undergo unfolding, which increases with temperature abuse, eg. cyclic freezing and thawing (Srinivasan, 1997a, 1997b). Unfolding of the molecules leads to cross-linking and the formation of aggregates. As the result of these changes, a significant deterioration of the functional properties of the fish meat may occur, such as lossing of water retention, gel-forming ability, and lipid-emulsifying capacity. Moreover, ice crystallization may disturb the water structures surrounding the areas of hydrophobic interactions in proteins. It may also disrupt the water-mediated hydrophobic-hydrophilic interactions, which participate in buttressing the native conformation of protein molecules (Sikorski and Kolakowska, 1990b). Badii and Howell (2001) found that frozen storage of cod and haddock had the increased hydrophobicity of their muscle proteins. This could be attributed to the unfolding of proteins and exposure of hydrophobic aliphatic and aromatic amino acids. Hydrophobic interactions between the exposed groups resulted in a decrease in solubility of proteins and formation of aggregate. These more changes were found in cod and hoddock fillets stored at -10 °C compared with fish stored at -30 °C.

8.1.2.2 Disulfide bond

The level of reactive SH groups decreases considerably during frozen storage. This demonstrates that more disulfide bonds are formed in the muscle during frozen storage (Zayas, 1997). Disulfide bridges are the important covalent bonds which relates to aggregation of protein (Sikorski and Kolakowska, 1990b). The formation of disulfide bonds via oxidation of SH groups or disulfide interchanges is coincidental with the decrease in total and surface SH contents (Hayakawa and Nakai, 1985). SH groups located in the head portion of myosin have been found to play an important role in ATPase activity. Another SH group, which is localized in the light meromyosin region of the myosin molecule, was reported to be involved in the oxidation of myosin heavy chain and its dimer formation, leading to an increase in Mg^{2+}- EGTA – ATPase activity (Benjakul *et al.*, 1997).

8.2 Lipid oxidation and hydrolysis

By increasing the concentration of salts in the unfrozen pools of water, it may decrease the hydration of proteins, as the concentrated inorganic ions compete for the water molecules. Furtheremore, inorganic salts may participate in the freeze denaturation of proteins by catalyzing the hydrolysis and autooxidation of lipids (Sikorski and Kolakowska, 1990b).

Extensive phospholipid hydrolysis in frozen fish results in the formation of free fatty acids and loss of phospholipid. Fatty acid formation may contribute to texture toughening by influencing protein denaturation and to flavor deterioration by enhancing lipid oxidation. Less information is available concerning hydrolysis of triglycerides in frozen fish, however phospholipid hydrolysis is more generally occurred (Harrd, 1992). Srinivasan *et al.* (1997b) reported the TBARS of prawn samples increased gradually during the first three freeze-thaw cycles and then rapidly after four cycles. Thus, freeze-thaw cycles coincided with a release of

prooxidants from the muscle, enhanced lipid oxidation rate. Intramuscular fat deposits could be oxidized and/or hydrolyzed into free fatty acids during frozen storage (Jiang and Lee, 1985). In general, the fatty acids may be less available for interactions with proteins, as they preferentially associate with the lipid phase. However, oxidized fatty acids and secondary products of autooxidation, especially aldehydes, are able to form covalent bonds with reactive protein groups. They are much more damaging to protein solubility than the acids themselves, and may contribute to the formation of aggregates by binding to proteins due to hydrophobic interactions (Sikorski and Kolakowska, 1990b).

8.3 Formaldehyde formation

Formaldehyde formation and its reaction with muscle proteins during frozen storage is considered to be a major factor affecting texture and functionality deterioration (Tejada *et al.*, 2002). Formaldehyde and dimethylamine (DMA) are the products of enzymatic convertion of trimethylamine oxide (TMAO) by TMAO demethylase. Formaldehyde is associated with the increased denaturation of myosin, increased loss of solubility, and increased surface hydrophobicity, resulting in protein aggregation through noncovalent interactions (Zayas, 1997). Formaldehyde causes the crosslinking of myosin, leading to the loss of water holding capacity of myosin and toughening of the tissue during frozen storage (Regenstein and Regenstein, 1991). Sotelo *et al.* (1995b) reported that formaldehyde showed the detrimental effect on the texture and quality of frozen cod muscle. Badii and Howell (2002) observed that frozen stored cod and haddock, stored at -10 ^{0}C and -30 ^{0}C for up to 30 weeks, produced dimethylamine and formaldehyde, which increased with a higher storage temperature and prolonged time of storage.

Sotero *et al.* (1995a) found that dimethylamine and trimethylamine in whole hake were both produced at -5 °C. At -12 °C, there was a small increase

in dimethylamine and none of trimethylamine, while at -20 °C none of the amines was produced. Rehbein (1988) found a positive relationship between texture toughening and dimethylamine formation of gadoid minces during frozen storage at -8 °C for 6 months. As the longer storage time, texture toughening was directly related to the increase in dimethylamine. Tejada *et al.* (2002) observed that addition of sardine (*Sardina pilchardus*) to hake (*Merluccius merluccius*) minces reduced the undisirable effect of formaldehyde during frozen storage at -20 °C for 1 year.

Oxygen or potential oxidants, such as oxidized lipids, have been reported to be inhibitors of TMAOase. Thus, they are able to inhibit the TMAO decomposition to dimethylamide and formaldehyde (Careche and Tejada, 1990). The non-enzymatic reduction of TMAO to trimethylamine (TMA), dimethylamine (DMA), and formaldehyde also occurs in the presence of cysteine and either ferrous ion or haemoglobin as a catalyst (Rehbein, 1988).

Objectives

1. To compare the physical properties and chemical composition between the muscles of hard and soft shell mud crabs

2. To characterize the sarcoplasmic proteinases from the muscles of hard and soft shell mud crabs

3. To study physico-chemical changes of the muscles of hard and soft shell mud crabs as affected by frozen storage time and freeze-thaw cycles

Chapter 2

Materials and Methods

1. Chemicals

Sodium dodecyl sulfate (SDS), sodium caseinate, pepstatin A, 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), bovine serum albumin, ß-mercaptoethanol (BME), glycerol, ammonium molybdate, 5-5'-dithio-bis (2-nitrobenzoic acid) (DTNB), adenosine 5'-triphosphate (ATP), high molecular weight markers, and *N,N*-dimethylformamide were purchased from Sigma (St. Louis, Mo., U.S.A.). Coomassie blue R-250, penta-sodium triphosphate, sodium hexametaphosphate, tris (hydroxymethyl) aminomethane, Folin-Ciocalteu's phenol reagent, ethylene diamine tetraacetic acid (EDTA) and trichloroacetic acid (TCA) were obtained from Merck (Darmstadt, Germany). Acrylamide, *N,N, N', N'*-tetramethylethylenediamine (TEMED), $CaCl_2$, soybean trypsin inhibitor, iodoacetic acid, bis-acrylamide and urea were obtained from Fluka (Buchs, Switzerland).

2. Instruments

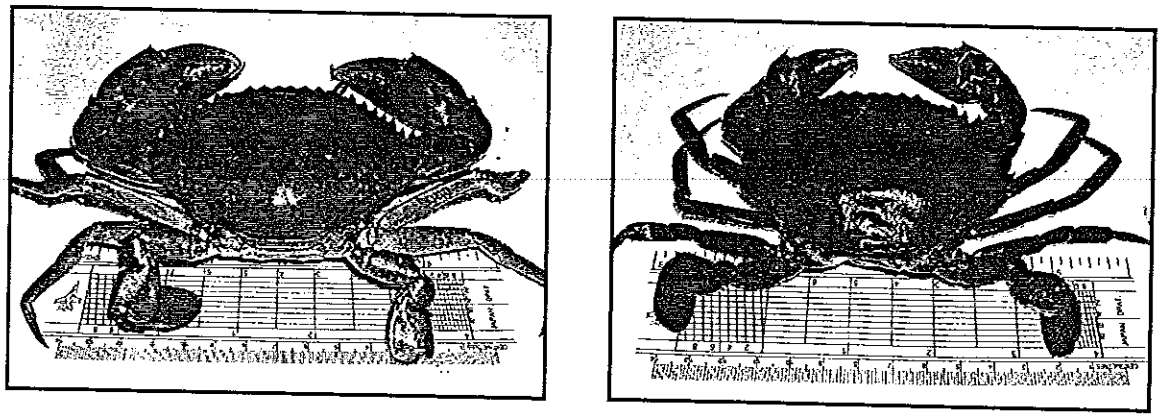
Instruments	Model	Company
Electrophoresis apparatus	Mini-Protein II	Bio-Rad, USA
Double-beam spectrophotometer	UV-16001	SHIMADZU, Australia
pH meter	pH 500	CyberScan, Singapore
Refrigerated centrifuge	RC-5B plus	Sorvall, USA
Water bath	W 350	Memmert, Germany

Instruments	Model	Company
Magnetic stirrer	RO 10 power	KIKAL labortechnik, Germany
Homogenizer	IKA	labortechnik, Malaysis
Balance	AB 204	METTLER TOLEDO, Switzerland
Spectrofluorometer	RF-1501	SHIMADZU, Japan
Scanning Electron Microscope	JSM5800LV	JEOL, Japan
Differential Scanning Calorimeter	DSC7	PerkinElmer, Japan

3. Methods

1. Sample preparation

Alive hard shell mud crabs (*Scylla serrata*) and soft shell mud crabs with the average weight ranging from 150 to 180 grams (Figure 7) were purchased from a farm in Kantan, Trang, Thailand. Soft shell mud crabs were obtained after 24 h molting. The crabs were kept in the plastic basket covered with the wet colth to keep the crabs moist. All samples were transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla. The samples were then excised into claw and chunk portions (Figure 8). Fresh meat from each portions was collected for analysis. Whole crabs were used for the study on changes during frozen storage and freeze-thawing process. Three different lots of crabs were used for each study.



a b

Figure 7 Hard (a) and soft (b) shell mud crabs

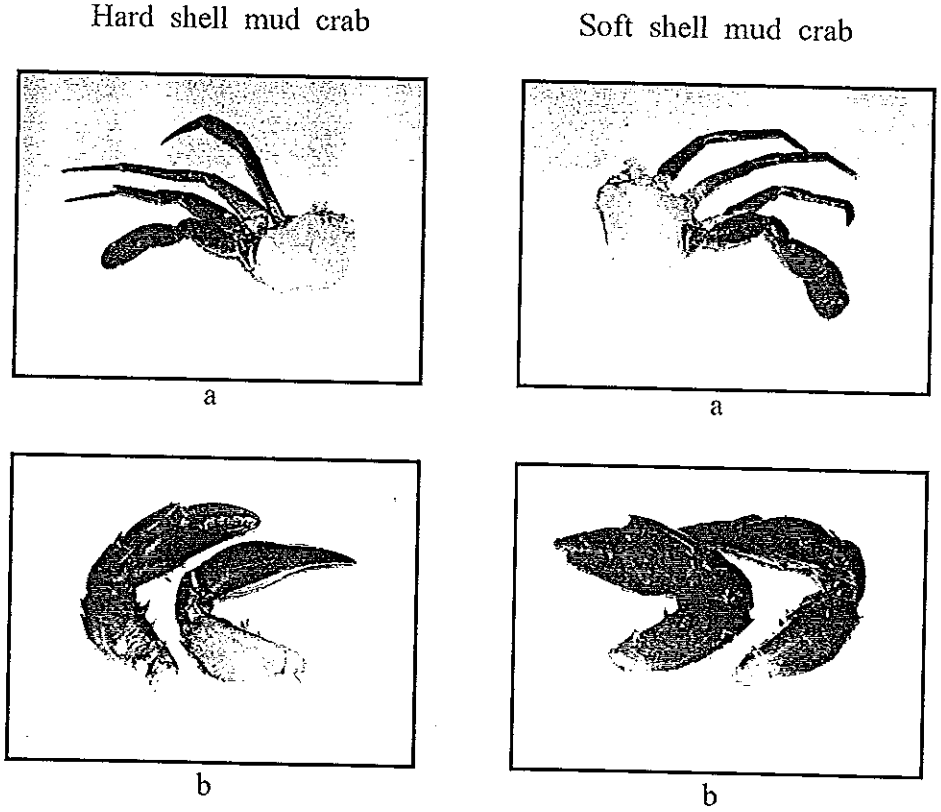


Figure 8 Chunk (a) and claw (b) muscles of hard and soft shell mud crabs

2. Characterization of crab muscles.

2.1 Chemical compositions of hard and soft shell mud crabs

2.1.1 Proximate composition

Chunk and claw muscles from both hard and soft shell mud crabs were subjected to analysis as follows:

2.1.1.1 Proximate analysis

- Protein (AOAC, 1999)
- Fat (AOAC, 1999)
- Moisture (AOAC, 1999)
- Ash (AOAC, 1999)
- $NaCl_2$ (AOAC, 1999)

2.1.1.2 Determination of hydroxyproline

Hydroxyproline content was determined according to the method of Bergman and Loxley (1963) with a slight modification. The samples were hydrolyzed with 6 M HCl at 110 °C for 24 h in an oil bath (model B-490, BUCHI, Flawil, Switzerland). The hydrolysate was clarified with activated carbon and filtered through Whatman No.4 filter paper. The filtrate was neutralized with 10 M and 1 M NaOH to obtain the pH of 6.0-6.5. The neutralized sample (0.1 ml) was transferred into a test tube and isopropanol (0.2 ml) was added. The mixtures were mixed well. A 0.1 ml of oxidant solution (the mixture of 7 % (w/v) chlororamine T and acetate/citrate buffer, pH 6 at a ratio of 1:4 (v/v) was added and mixed thoroughly. A 1.3 ml of Ehrlich's reagent solution (the mixture of solution A (2 g of *p*-dimethylamino-benzaldehyde in 3 ml of 60 % (v/v) perchloric acid (w/v) and isopropanol at a ratio of 3:13 (v/v) was added. The mixtures were mixed and heated at 60 °C for 25 min in a water bath (Memmert, Schwabach, Germany) and then cooled for 2-3 min in a running water. The solution was diluted to 5 ml with isopropanol. Absorbance was measured against water at 558

nm. A hydroxyproline standard solution with the concentration ranging from 10 to 60 mg/kg was also included. Hydroxyproline content was calculated and expressed as mg/g sample.

2.1.2 Nitrogenous constituents

Fractionation of mud crab and soft shell mud crab muscles was carried out according to the method of Hashimoto *et al.* (1979) as shown in Figure 9. Each fraction containing different composition, e.g. non-protein nitrogen, sarcoplasmic protein, myofibrillar protein, alkaline-soluble protein, and stroma, was subjected to the analysis of nitrogen content using Kjeldahl method (AOAC, 1999). Pattern and molecular weight of proteins in each fraction was performed by SDS-PAGE according to the method of Laemmli (1970) using 4 % stacking gel and 10 % running gel.

2.2 Thermal properties of crab muscle

2.2.1 Thermal inactivation rate constants (K_D)

Natural actomyosin (NAM) from crab muscles were extracted and subjected to Ca^{2+}-ATPase activity analysis according to the method of Benjakul *et al.* (1997). The inactivation rate constants (K_D) of actomyosin was calculated as described by Tsai *et al.* (1989) as follows:

$$(K_D) = (ln\ C_o - ln\ C_t) / t$$

Where: C_o = Ca^{2+}-ATPase activity before incubation

C_t = Ca^{2+}-ATPase activity after t second of incubation

t = Incubation time (second)

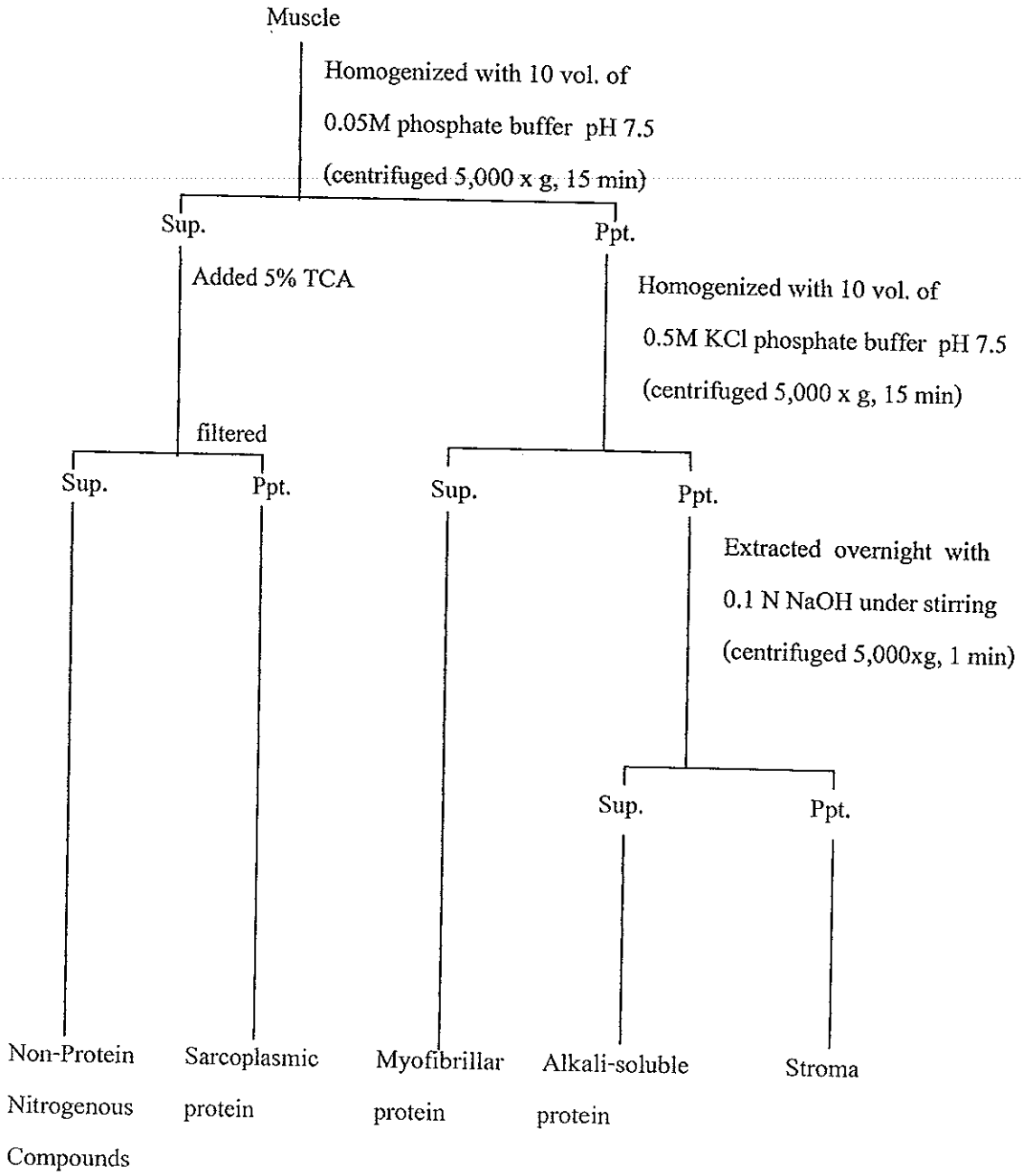


Figure 9 Fractionation procedure of muscle proteins

Source : Hashimoto *et al.* (1979)

2.2.2 Thermal denaturation of crab muscle protein

Thermal denaturation of crab muscle protein was investigated by monitoring Tmax of transition and denaturation enthalpy. All samples were scanned at 10 °C/min over the range 0-100 °C using differential scanning calorimetry (DSC) (model DSC 7, PerkinElmer, Japan).

2.3 Microstructure of crab muscle

Microstructure of crab muscle was determined using scanning electron microscopy according to the method of Nip and Moy (1988). The muscle tissue was fixed in 0.1 M sodium phosphate buffer containing 2.5 % glutaraldehyde and the fixed specimen was washed in 0.1 M sodium phosphate buffer. The sample was dehydrated in a graded series of ethanol (from 50 %, 70 %, 85 %, 95 % to 100 %). Dehydrated sample was coated with gold-palladium and viewed with a scanning electron microscope (model JSM5800LV, JEOL, Japan).

2.4 Mineral contents

Mineral contents in crab muscle were determined by Atomic Absorption Spectrometer according to the method of AOAC (1999). Mineral contents were determined as follows:

1. Copper
2. Calcium
3. Magnesium
4. Iron
5. Zinc

3. Characterization of sarcoplasmic proteinase from hard and soft shell mud crabs

3.1 Preparation of crude proteinase

The frozen hard and soft shell mud crabs were randomly selected from the freezer and separated into 2 portions, chunk and claw muscles. After being thawed with running water (25 °C) until the temperature reached 0-2 °C, the muscles were separated from each portions. To 15 g of each muscles, 22.5 ml of cold distilled water were added. No water was added in the muscle from claw portion of soft shell mud crab. The mixture was homogenized at speed of 1 for 30 sec using a homogenizer (model IKA, labortechnik, Malaysis) and centrifuged at 5,000 x g for 30 min at 4 °C using a Sorvall Model RC-B Plus centrifuge. The obtained crabs fluid was used as crude enzyme. All preparation procedures were carried out at 4 °C.

3.2 Optimum condition study

3.2.1 Temperature profile

Proteolytic activity was measured using casein-TCA Lowry methods. The activity assay was carried out at pH 3.0, 5.5 using 0.2 M McIlvaine's buffer and 8.5 using 0.1 M NaH_2PO_4 - 0.05 M Na_2BrO_7 buffer at various temperature (25, 30, 35, 40, 45, 50, 55, 60, 65 and 70 °C). Optimum temperature was then chosen for the next study.

3.2.2 pH profile

The pH profile was studied over the pH range of 3.0 to 10.0 (3, 4, 5, 6, 6.5, 7, 7.5, 8, 8.5, 9 and 10) under optimum temperature. McIlvaine's buffer was used for pH ranges 2.5-8.0 and 0.1 M NaH_2PO_4 - 0.05 M Na_2BrO_7 buffer was used for the pH range of 8.0-10.0.

3.3 Inhibitory study

Proteinase was classified using various types of inhibitors. The tested inhibitors were mixed with the proteinase to obtain the final concentration designated (1 μM pepstatin A, 10 mM EDTA, 0.01 mM soybean trypsin inhibitor, 10 mM iodoacetic acid and 10 μM trans – Epoxysuccinyl – L – Leucylamido (4 - Guanidino) – Butane (E - 64)) at room temperature (26-28 °C) for 15 min. The remaining activity was determinaed by casein-TCA-Lowry method (An *et al.*, 1994a).

4. Changes in chemical and physicochemical properties of hard and soft shell mud crab muscles during frozen storage

4.1 Freezing and frozen storage

Seven crabs were packed in a polyethylene bag and stored at -20 °C. The samples were randomly taken for physicochemical analysis after 0, 1, 2, 4, 6, 8, 10 and 12 weeks of storage.

4.2 Preparation of natural actomyosin (NAM)

NAM was extracted according to the method as Benjakul *et al.* (1997). Crab muscle was homogenized with 0.6 M KCl pH 7.0 at a ratio of 1:10 (w/v) for 4 min. To avoid heating during the process, each 20 sec of homogenization was followed by a 20 sec rest interval and the homogenate was kept in ice during the extraction process. The homogenate was centrifuged at 5,000 x g for 30 min at 4 °C. The supernatant was then mixed with three volume of chilled water to precipitate NAM. The NAM was collected by centrifuging at 5,000 x g for 30 min at 4 °C. An equal volume of chilled 0.6 M KCl pH 7.0 was added to dissolve the pellet and then stirred for 30 min at 4 °C. Undissolved material were removed by centrifuging at 5,000 x g for 20 min at 4 °C. Protein concentration in NAM was determined using the Biuret method.

4.3 Determination of chemical and physicochemical properties of NAM

NAM was analyzed as follows:

4.3.1 Ca^{2+}-ATPase activity according to Benjakul *et al.* (1997)

4.3.2 Total sulfhydryl content according to Benjakul *et al.* (1997)

4.3.3 Disulfide bond content according to Thannhauser *et al.* (1987)

4.3.4 Surface hydrophobicity according to Benjakul *et al.* (1997)

4.4.5 Formaldehyde according to Nash (1953)

4.4.6 Solubility in 0.6 M KCl according to Jiang *et al.* (1988)

4.4.7 Thiobarbituric acid reactive substances (TBARS) according to Buege and Aust (1978).

4.4.8 pH according to Benjakul *et al.* (1997)

4.4.9 Cooking loss was determined by steaming crab meat for 3 minutes. Cooked crab meat was immediately cooled down in ice. Weight loss was calculated with the following equation:

$$\text{Cooking loss (\%)} = \frac{A - B}{A} * 100$$

Where : A = weight before steaming

B = weight after steaming

5. Changes in chemical and physicochemical properties of hard and soft shell mud crab muscles with different freeze-thaw cycles

Crabs were subjected to freeze-thawing at 0, 1, 2, 3, and 5 cycles. Frozen crabs were thawed using a running water until the core temperature reached 0 to -2 °C. The meat from claw and chunk portion was collected for analysis as described in section 4.

6. Statistical analysis

Data was subjected to analysis of variance (ANOVA) and mean comparison was carried out using Duncan's Multiple Range Test (DMRT). Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for windows: SPSS Inc.).

Chapter 3

Results and Discussion

1. Chemical compositions and properties of hard and soft shell mud crabs

1.1 Chemical compositions

Chemical compositions of hard and soft shell mud crabs are shown in Table 4. Both chunk and claw muscles of soft shell mud crab contained the lower protein contents but higher moisture contents than those of hard shell mud crab ($P<0.05$). Claw muscles of hard and soft shell mud crabs contained the higher moisture content (84.38% and 94.76%) than chunk muscles of both crabs (78.69% and 82.37%). From the result, water was considered to be the main constituent in the soft shell mud crab muscle, in which varied depending on the location of the muscle. Claw muscle of soft shell mud crab had the highest moisture content (94.76%). This result was in agreement with Mizuta *et al.* (2001) who reported that water content in the muscle of the soft snow crab was significantly higher than that of the hard snow crab. A soft snow crab contained a considerable amount of free body fluid (FBF) in its appendages which was 33.80% to 37.30% (w/w), while hard snow crab appendages contained FBF only 5.00% to 6.08% (w/w). Salt content in the soft shell mud crab muscle varied from 1.10% to 1.70%, whereas the hard shell mud crab muscle had a lower salt content ranging from 0.70% to 1.38%. High content of inorganic substances was observed in the claw soft shell mud crab as indicated by high ash content (2.17%). The high moisture, ash, salt and low protein contents of the soft shell mud crab muscle seemed to be partially caused by the absorption of seawater. For fat content, it ranged from 0.12% to 0.24% in the soft shell mud crab muscle and 0.18% to 0.28% in the hard shell mud crab muscle.

Table 4 Chemical compositions of mud crab muscles

Chemical compositions (% wet basis)	Hard shell crab chunk	Hard shell crab claw	Soft shell crab chunk	Soft shell crab claw
Protein	15.61 ± 0.01*[a]	14.31 ± 0.05[b]**	12.87 ± 0.05[c]	3.05 ± 0.02[d]
Ash	1.59 ± 0.04[c]	1.67 ± 0.09[b]	1.56 ± 0.03[d]	2.17 ± 0.01[a]
Moisture	78.69 ± 0.06[d]	84.38 ± 0.39[b]	82.37 ± 0.17[c]	94.76 ± 0.06[a]
Fat	0.28 ± 0.01[a]	0.18 ± 0.02[c]	0.24 ± 0.01[b]	0.12 ± 0.01[d]
Sodium chloride	0.70 ± 0.03[d]	1.38 ± 0.09[b]	1.10 ± 0.03[c]	1.70 ± 0.06[a]

* mean ± standard deviation from triplicate determinations

** Values with the different letters in the same row are significantly different ($P \leq 0.05$)

1.2 Hydroxyproline contents

Table 5 shows the content of hydroxyproline in the muscle of hard and soft shell mud crabs. Generally, by wet basis, hydroxyproline contents in muscle of hard shell mud crab was higher than those of soft shell mud crab muscle. When comparing the hydroxyproline content in chunk and claw muscles of both crabs, hydroxyproline content in the chunk muscle was higher than that in the claw muscle. Among all samples, chunk muscle of hard shell mud crab had the highest hydroxyproline content. The claw muscle of soft shell mud crab had the lowest hydroxyproline content ($P<0.05$), ranging from 2.80 to 2.70 mg/g wet tissue. Low hydroxyproline content might be due to the lowest protein content with the highest moisture content in the claw of soft shell mud crab. Base on dry basis, similar hydroxyproline content was found between soft and hard shell mud crab claw and between soft and hard shell mud crab chunk. However, claw

protein contained higher hydroxyproline content than chunk protein. Chunk muscle is located at the joint connected between leg and abdominal muscle and its collagen is involved in the flexibility and maintaining the balance during locomotion. Claw muscle collagen, on the other hand, is involved in the functions to grip the prey (Yoshinaka *et al.*, 1989). The musculature of several crustaceans was found to contain collagenous proteins (Yoshinaka *et al.*, 1989). Collagens in the marine animal muscle plays an important role in maintenance of meat texture (Sato *et al.*, 1986). Sivakumar *et al.* (2000) found the levels of hydroxyproline in abdominal and leg muscles of mud crab at 0.28 and 0.38 mg/g wet tissue, respectively. The crab leg muscle collagens were highly crosslinked and stabilized by more bound carbohydrates, as compared to the abdominal muscle collagen. Analysis of amino acid composition revealed a close similarity to known type V collagens and the leg muscle collagen was characterized by more lysine hydroxylation and slightly reduced glycine content (Yoshinaka *et al.*, 1989).

Table 5 Hydroxyproline content in hard and soft shell mud crab muscles

Sample	Hydroxyproline content (mg/g muscle)	
	wet basis	dry basis
Hard shell crab chunk	8.88 ± 0.14*[a]	41.65 ± 0.66[a]**
Hard shell crab claw	8.26 ± 0.17[b]	52.88 ± 1.11[b]
Soft shell crab chunk	7.92 ± 0.12[c]	44.94 ± 0.67[c]
Soft shell crab claw	2.75 ± 0.05[d]	52.48 ± 0.91[d]

* mean $\pm$ standard deviation from triplicate determinations

** Values with the different letters in the same column are significantly different ($P \leq 0.05$)

1.3 Nitrogenous compositions and SDS-PAGE pattern of the fractions

Nitrogenous compositions of hard and soft shell mud crab muscles are shown in Table 6. Surprisingly, myofibrillar protein was not the major constituent in the muscle of hard and soft shell mud crabs. Its content was lower than alkali-soluble and sarcoplasmic proteins for both chunk and claw muscles of hard shell mud crab. Myofibrillar proteins showed the lowest content among other protein components in the muscle of soft shell mud crab ($P<0.05$). Hashimoto *et al.* (1979), Suzuki (1981) and Mackie (1994) found that myofibrillar protein was the main nitrogenous component in fish muscle. Sarcoplasmic protein seemed to be the major component in the muscle of hard and soft shell mud crabs, compared to other nitrogenous proteins. Sarcoplasmic protein content in soft shell mud crab muscle was 78.27% for the claw muscle and 44.65% for the chunk muscle. For hard shell crab muscle, sarcoplasmic protein content (38.00% to 39.33%) was similar to the content of alkaline-soluble protein (39.80% - 40.73%). From the result, it was suggested that proteins underwent the changes during molting period, in which the content of myofibrillar and alkaline-soluble proteins decreased while sarcoplasmic and stroma protein contents increased. This might be caused by the activities of proteinases in crab muscle, especially in the molting period.

Protein pattern of hard and soft shell crab muscle determined using SDS-PAGE are shown in Figure 10. The result was in agreement with the data of nitrogen composition as shown in Table 6. Myofibrillar protein was not the major component in both crab muscles. Therefore, myosin heavy chain, appeared at MW of 200,000 dalton, was not a major muscle protein as found in many fish species. Nevertheless, similar protein patterns of hard shell crab chunk (S1), hard shell crab claw (S2) and soft shell crab chunk (S3) were observed. Actin appeared at MW of 45,000 dalton was considered to be the major protein band.

Table 6 Nitrogenous composition of soft and hard shell mud crab muscles (mg N / g wet muscle)

Mud Crab	Muscle type	Non-protein N	Protein N			
			Myofibrillar	Sarcoplasmic	Alkali-soluble	Stroma
Hard shell crab	Chunk	5.87±0.04**[a]	2.76 ± 0.51*** (13.63)*	7.97 ± 2.27[b] (39.33)	8.06 ± 0.76[a] (39.80)	1.47 ± 0.27[c] (7.24)
	Claw	5.47 ± 0.36[b]	2.44 ± 0.06[b] (12.56)	7.39 ± 0.86[c] (38.00)	7.92 ± 0.27[b] (40.73)	1.69 ± 0.31[b] (8.70)
Soft shell crab	Chunk	5.40 ± 0.09[c]	1.70 ± 0.94[c] (8.54)	8.86 ± 2.03[a] (44.65)	7.53 ± 1.34[c] (37.93)	1.76 ± 0.14[a] (8.88)
	Claw	0.80 ± 0.05[d]	0.23 ± 0.03[d] (3.79)	4.78 ± 0.11[d] (78.27)	0.27 ± 0.02[d] (4.47)	0.82 ± 0.26[d] (13.48)

* Numbers in parentheses represent percentage distribution

** mean ± standard deviation from triplicate determinations

*** Values with the different letter in the same column are significantly different ($P \leq 0.05$)

For the protein pattern of soft shell crab claw (S4), not only the lowest intensity of myosin heavy chain but also actin, paramyosin (MW of 95,000 dalton) and tropomyosin (MW of 34,000 dalton) were hardly found. Among all samples, the claw of soft shell crab had the protein bands at MW of 75,000 dalton as the major protein band. This protein might play an important role in making the differences in functional properties of the muscle between hard shell crab and soft shell crab.

The muscle proteins were fractionated into different fractions based on different solubility (Figure 11 and 12). Actin was found to be the dominant

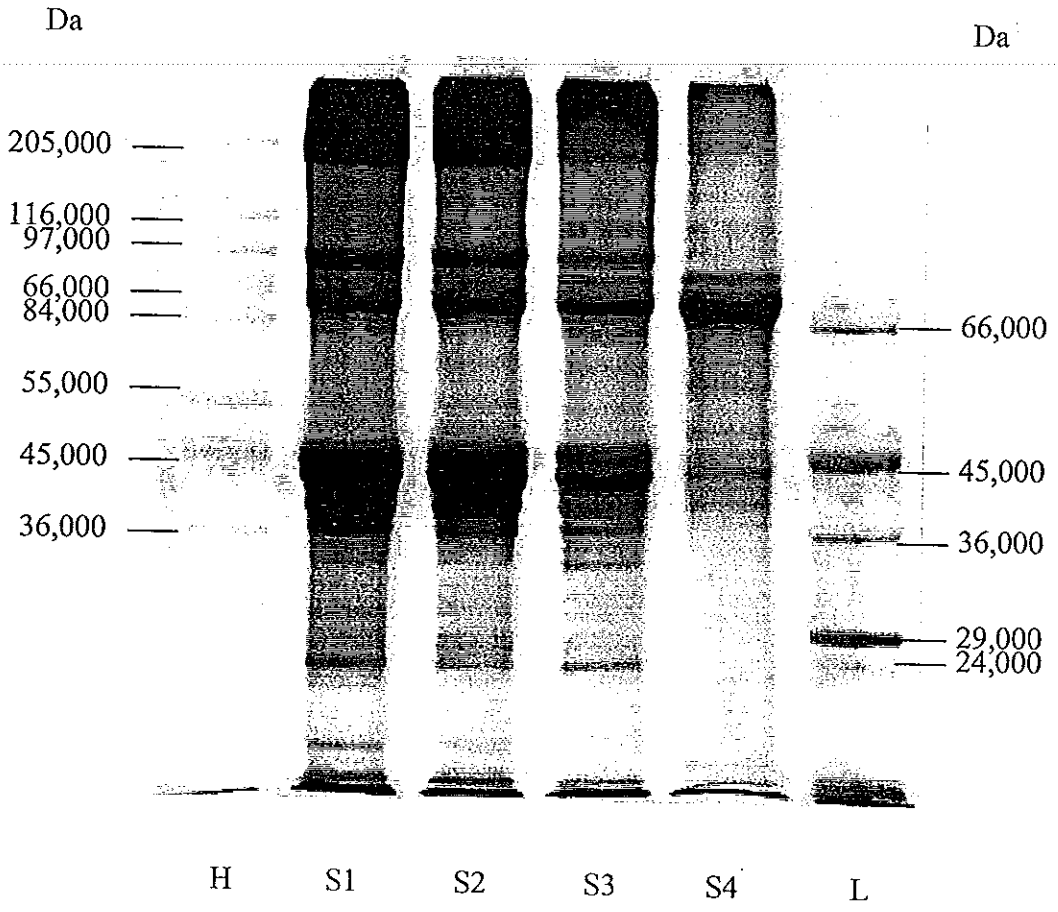


Figure 10 SDS-PAGE pattern of protein in the muscles of hard and soft shell mud crabs. Twenty g protein were applied on 10 % polyacrylamide gel. H; high molecular weight standard (dalton), S1; hard shell crab chunk muscle, S2; hard shell crab claw muscle, S3; soft shell crab chunk muscle S4; soft shell crab claw muscle and L; low molecular weight standard (dalton).

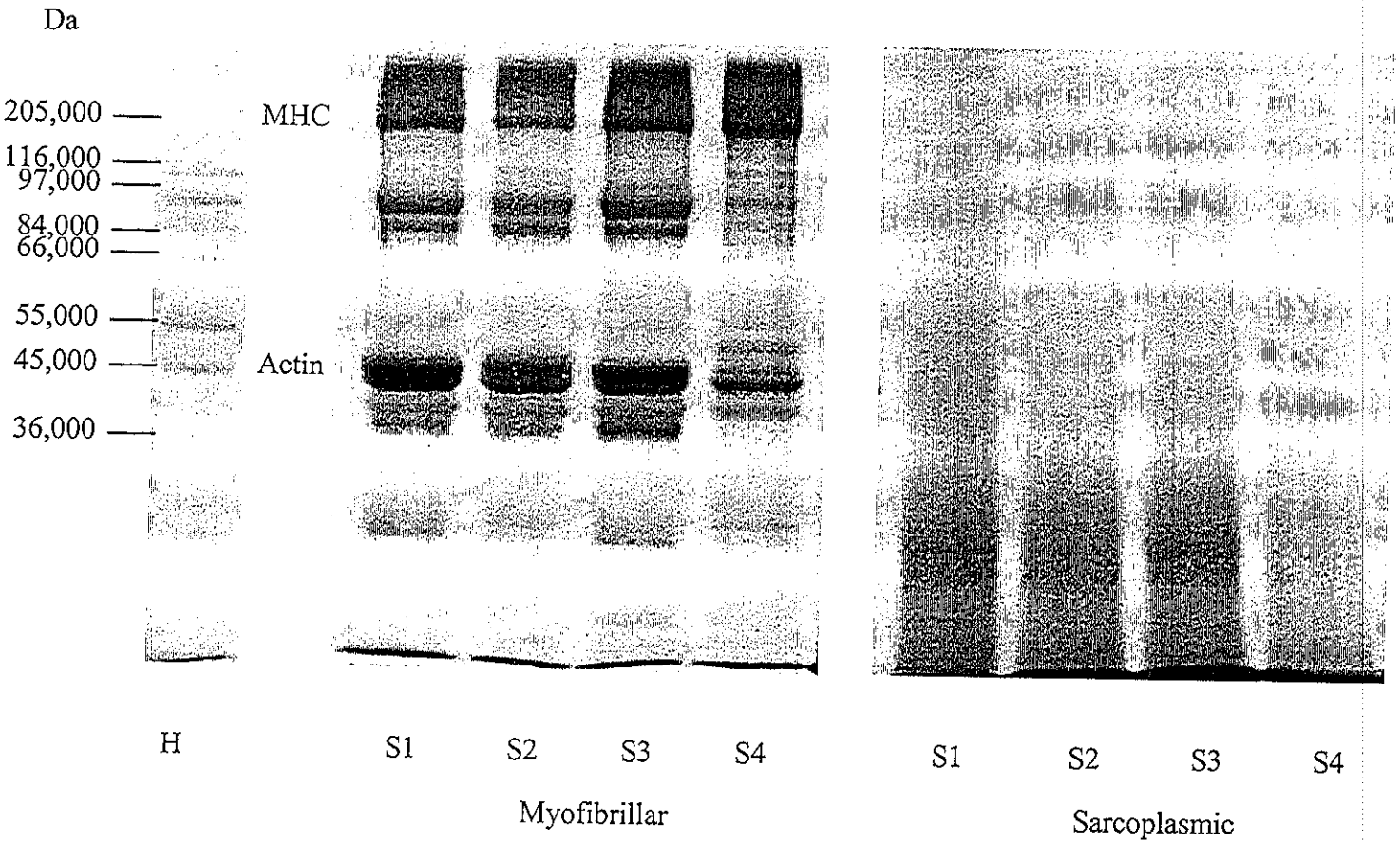


Figure 11 SDS-PAGE pattern of myofibrillar and sarcoplasmic proteins of muscles of hard and soft shell mud crabs. Twenty μg protein were applied on 10 % polyacrylamide gel. H; high molecular weight standard (dalton), S1; hard shell crab chunk muscle, S2; hard shell crab claw muscle, S3; soft shell crab chunk muscle and S4; soft shell crab claw muscle.

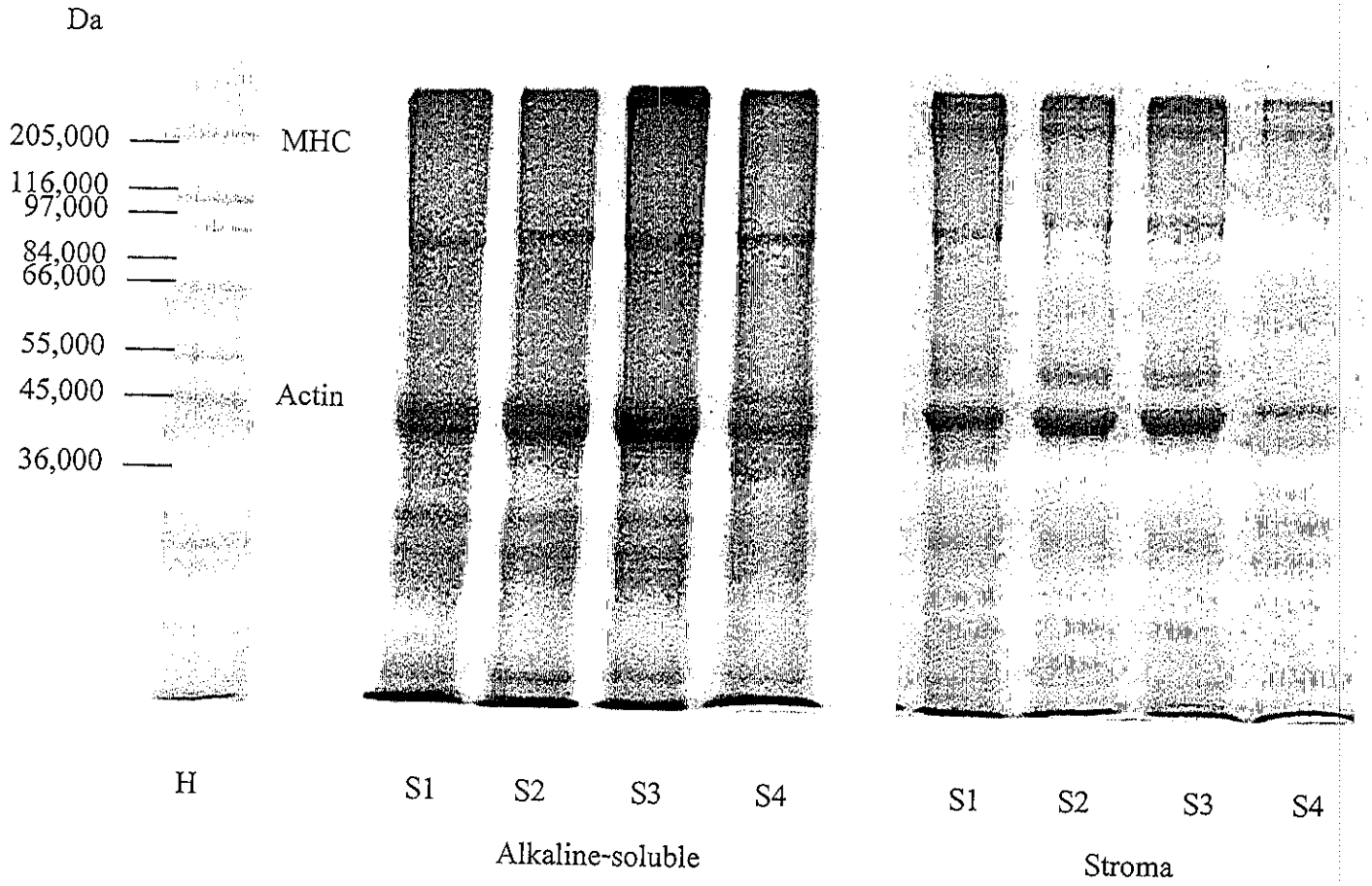


Figure 12 SDS-PAGE pattern of akali-soluble protein and stroma proteins of muscles of hard and soft shell mud crabs. Twenty μg protein were applied on 10 % polyacrylamide gel. H; high molecular weight standard (dalton), S1; hard shell crab chunk muscle, S2; hard shell crab claw muscle, S3; soft shell crab chunk muscle and S4; soft shell crab claw muscle.

protein in myofibrillar fraction of S1, S2 and S3 samples while myosin heavy chain was found to be the second abundant protein of those samples as shown in Figure 11. However, similar band intensity between actin and myosin heavy chain in S4 sample were observed (Figure 11). Sarcoplasmic protein patterns of all samples were not different. Low molecular weight proteins were dominant and associated with high water solubility of this fraction (Figure 11). Protein patterns of alkali-soluble and stroma protein of S4 sample were different from the others (Figure 12) as evidenced by the appearance of protein band with low intensity. The result suggested that most of proteins in alkaline-soluble and stroma fractions of S4 had the low molecular weight.

1.4 Thermal inactivation rate constant (K_D) of natural actomyosin

In this study, the inactivation rate contant (K_D values) of Ca^{2+}-ATPase of all NAMs increased as the incubation temperature increased (Table 7; Figure 13). K_D of Ca^{2+}-ATPase from soft shell mud crab NAM was not determined since the insufficient amount of NAM was obtained by the procedure used. K_D of Ca^{2+}-ATPase from all samples increased remarkedly at 40 and 50 °C ($P<0.05$) (Table 7). At 40 °C, Ca^{2+}-ATPase activity of hard shell mud crab claw NAM was inactivated at the highest rate compared to the other samples. The result indicated that NAM from the claw muscle of hard shell mud crab was more susceptible to thermal denaturation than those of the chunk muscle from both hard and soft shell mud crabs. However, Ca^{2+}-ATPase inactivation rates of NAM from the chunk muscle of both crabs were almost the same. It was reported that the stability of muscle proteins varied with species and storage conditions (Suyama and Konosu, 1987; tsai *et al.*, 1989). When NAMs from different muscle were heated at 40 °C for different times, the decrease in Ca^{2+}-ATPase activity was observed as the heating time increased (Fig. 13). For the same heating time, Ca^{2+}-

ATPase of NAM from claw muscle was much lower than that of other samples. This result showed that claw muscle was much more unstable to heat treatment than chunk muscle. The inactivation rate constants (K_D) of actomyosin and myosin Ca^{2+}-ATPase activities were often used for evaluating the thermal stability of fish muscle protein (Suzuki, 1981; seki, 1977; Tsai *et al.*, 1989).

Table 7 Effect of temperatures on the inactivation rate constant of Ca^{2+}-ATPase of NAM from hard and soft shell mud crabs**

temp.	$K_D \times 10^5$ / s		
°C	Hard crab chunk	Hard crab claw	Soft crab chunk
0	$0.00 \pm 0.00^{*a,A}$	$0.00 \pm 0.00^{a,A,***}$	$0.00 \pm 0.00^{a,A}$
10	$0.22 \pm 0.02^{b,B}$	$0.89 \pm 0.02^{b,A}$	$0.20 \pm 0.01^{b,C}$
20	$0.58 \pm 0.10^{c,B}$	$1.31 \pm 0.10^{c,A}$	$0.52 \pm 0.03^{c,C}$
30	$2.09 \pm 0.08^{d,C}$	$3.599 \pm 0.04^{d,A}$	$2.23 \pm 0.11^{d,B}$
40	$21.89 \pm 0.12^{e,B}$	$47.43 \pm 0.08^{e,A}$	$29.85 \pm 0.31^{e,C}$
50	$316.9 \pm 0.10^{f,C}$	$330.2 \pm 0.02^{f,B}$	$331.2 \pm 0.02^{f,A}$
60	$316.9 \pm 0.03^{f,C}$	$330.2 \pm 0.01^{f,B}$	$331.2 \pm 0.01^{f,A}$

Note: * mean ± standard deviation from triplicate determinations.

** NAM was heated to different designated temperatures for 30 min. prior to sudden cooling in iced water.

*** The first superscript in the same column indicate the significant differences ($P<0.05$). The second superscript in the same row indicate the significant differences ($P<0.05$).

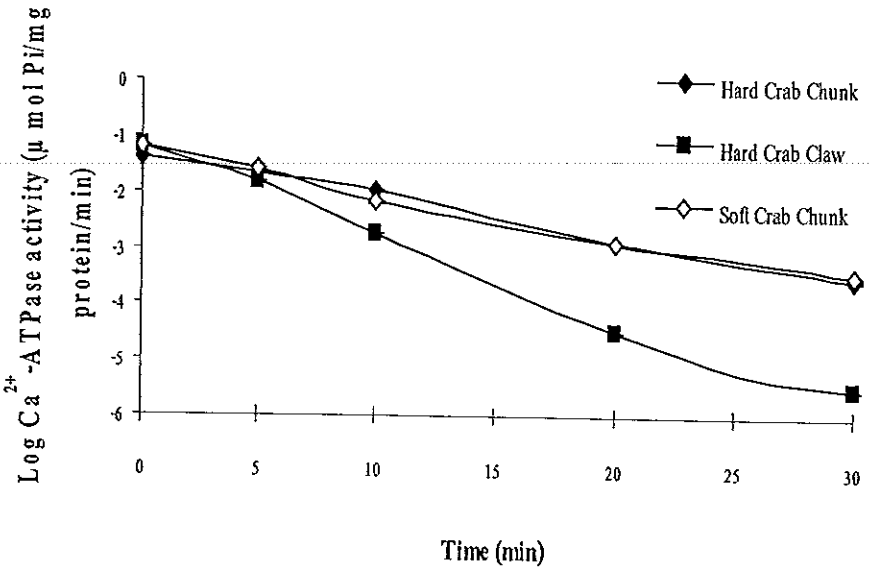


Figure 13 Logarithm of Ca^{2+}-ATPase activities of NAM from hard and soft shell mud crab as a function of incubation time at 40 °C

1.5 Thermal denaturation of hard and soft shell mud crab muscle proteins

DSC peak transition temperature (T_{max}) and enthapy of hard and soft shell mud crab muscle are shown in Table 8. Three major peaks with T_{max} values of 45.00 – 48.08 °C, 72.41 – 76.27 °C and 83.11 - 87.34 °C were obtained. The first two peaks were postulated to represent the transition of myosin and actin, respectively. Benjakul *et al.* (2000) reported two major endothermic transitions during thermal denaturation of threadfin bream muscle. The peaks at 49.89 and 73.16 °C were postulated to correspond to myosin and actin, respectively. The third peak with T_{max} of 83.11-87.34 °C was possibly attributed to the most heat stable protein of mud crab muscle. DSC analysis was used to determine the thermal transition or unfolding temperature of protein and also to quantify the enthapy of conformational transition (John and Shastri, 1998).

Table 8 Peak transition temperature (T_{max}) and enthalpy of hard and soft shell mud crab muscles

Samples	Muscle	Peak 1		Peak 2		Peak 3	
		Tmax* (°C)	Δ H* (J/g)	Tmax (°C)	Δ H (J/g)	Tmax (°C)	Δ H (J/g)
Hard shell mud crab	chunk	$48.08 \pm 0.80^{*a}$	0.20 ± 0.02^{b}**	76.27 ± 0.25^{a}	0.25 ± 0.02^{c}	83.72 ± 0.85^{bc}	0.16 ± 0.05^{b}
	claw	45.00 ± 0.17^{c}	0.17 ± 0.03^{d}	72.41 ± 0.08^{c}	0.40 ± 0.05^{b}	84.16 ± 0.17^{b}	0.10 ± 0.01^{d}
Soft shell mud crab	chunk	47.58 ± 0.38^{b}	0.21 ± 0.06^{a}	75.07 ± 0.12^{b}	0.50 ± 0.18^{a}	83.11 ± 1.33^{c}	0.14 ± 0.09^{c}
	claw	47.48 ± 0.17^{b}	0.18 ± 0.01^{c}	ND***	ND	87.34 ± 0.17^{a}	0.26 ± 0.04^{a}

* mean ± standard deviation from triplicate determinations

** Values with the different letter in the same column are significantly different ($P \leq 0.05$)

*** Not detectable

When the chunk muscle of hard and soft shell mud crabs were compared, T_{max} of all peaks of hard shell mud crab were higher than those of the soft shell mud crab. Therefore, proteins in the chunk muscle of soft shell mud crab were more likely to be sensitive to the thermal degradation than those of the chunk muscle of hard shell mud crab. This result might be due to the higher sodium chloride content in the soft shell mud crab muscle, which caused more rapid denaturation of the proteins. Park and Lanier (1989), Beas *et al.* (1991) and Benjakul *et al.* (2000) reported a decrease in denaturation enthalpy and the shift of T_{max} to the lower temperature of tilapia or hake surimi and threadfin bream muscle protein with salt addition (NaCl 2.5% - 3.0%). Nevertheless, peak 1 and peak 3 of claw muscle of soft shell mud crab had higher T_{max} and enthalpy than those of the claw muscle of hard shell mud crab. However, peak 2 was not detected in the thermogram of soft shell mud crab claw. This result was in agreement with the low intensity of actin as shown in SDS-PAGE (Figure 10). As a result, the endothermic transition of actin from soft shell mud crab claw was not observed. Furthermore, the protein band with the MW of 75,000 dalton of soft shell mud crab was found to be the largest band among the other protein bands. Also, the highest T_{max} of peak 3 was observed in soft shell mud crab claw. Thus, the protein with MW of 75,000 dalton was presumed to contribute to the thermal stability of soft shell mud crab claw muscle. However, more study has to be taken to verify this speculation.

1.6 Microstructure of hard and soft shell mud crabs

The microstructures of chunk and claw muscles of hard and soft shell mud crabs are shown in Figure 14. The bundle of chunk muscle from hard and soft crabs were connected orderly. The structure of claw muscle of both crabs had more partial disintegrations than those observed in the chunk muscle. Among

all samples tested, sponge-like structure was found only in the claw of soft shell crab. These histological characteristics of the soft shell crab muscle suggest a structural weakening of muscle fibers or connective tissue, possibly due to physiological phenomena related to molting, such as the uptake of seawater (Mizuta *et al.*, 2001). The structure was correlated well with the high moisture content and low protein content. The much more released fluid, compared to other samples, might be owing to the sponge like structure which could not hold water in such a structure. The soft shell crab muscle was juicier, saltier and less firm than the hard shell carb muscle (Mizuta *et al.*, 1994).

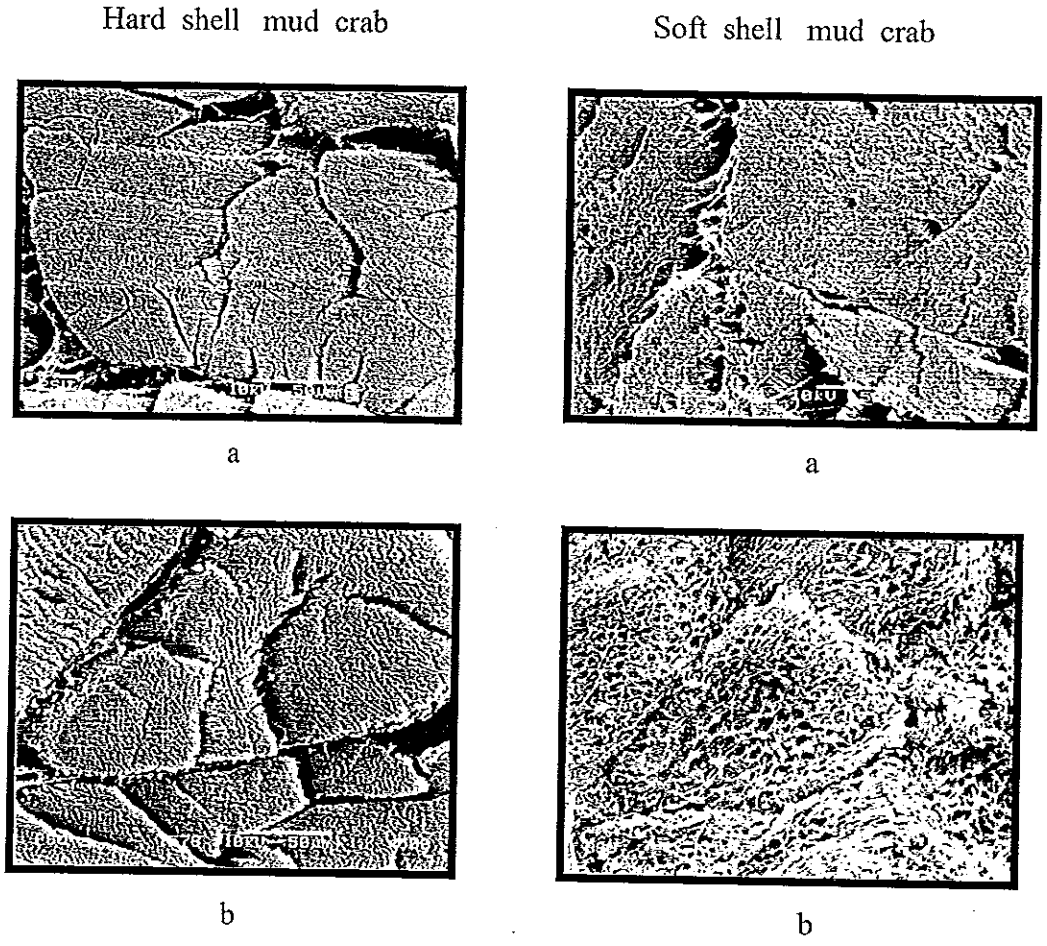


Figure 14 Microstructure of hard and soft shell mud crabs ; a, chunk muscle tissue; b, claw muscle tissue. Bars = 50 μm.

1.7 Mineral contents

Mineral composition of hard and soft shell mud crabs are shown in Table 9. Among all minerals of hard and soft shell crabs, calcium and magnesium were shown to be the major minerals ranging from 240.5 to 699.2 mg/kg wet tissue and from 403.6 to 428.2 mg/kg wet tissue, respectively. Copper, Iron and Zinc were considered to be trace constituents which were not higher than 50 mg/kg wet tissue.

From the result, one-third of calcium was obtained in soft shell mud crab, compared to that found in hard shell mud crab. However, no marked changes in magnisium were observed between both crabs. Approximately 50 % of Fe content was found in soft shell mud crab, compared to that found in hard shell mud crab. Slightly lower Ca and Zn contents were observed in soft shell mud crab, compared to those in hard shell mud crab. Growth stage in the life cycle of mud crabs also affected the mineral contents in the muscle. This result was in agreement with Scott-fordsmand and Depledge (1997) who reported the lower whole body calcium concentration in newly molted shore crabs (*Carcinus maenas*) compared to premolt shore crabs. Additionally, the copper and zinc content of postmolt stage of shore crab decreased by 25% and 24% on the whole body basis as crabs entered the paper shell stage from the newly molted stage. *S. Serrata* is a large portunid crab, which matures and spawns in seawater, spends post-larval and juvenile phases in brackish water, and then returns to the sea as a pre-adult (Davenport and Wong, 1987). Changes in salinity may disrupt the osmotic balance of decapod crustaceans (Chen and Chia, 1997).

Table 9 Mineral contents in the muscles of hard and soft shell mud crabs (mg/kg)

Mud crab	Muscle Type	Ca	Mg	Cu	Fe	Zn
Hard shell mud crab	Chunk	699.2 ± 82.72*[a]	406.6 ± 9.89[c]**	20.43 ± 3.55[a]	13.09 ± 12.96[a]	36.57 ± 8.56[a]
	Claw	644.5 ± 52.90[b]	418.7 ± 14.95[b]	17.82 ± 0.96[b]	10.07 ± 4.77[b]	33.51 ± 14.33[b]
Soft shell mud crab	Chunk	240.5 ± 25.83[d]	403.6 ± 5.21[d]	17.68 ± 5.16[b]	4.452 ± 2.56[c]	27.40 ± 3.01[c]
	Claw	252.7 ± 98.91[c]	428.2 ± 58.22[a]	16.38 ± 1.61[c]	5.358 ± 1.00[c]	13.06 ± 5.51[d]

* mean ± standard deviation from triplicate determinations

** Values with the different letter in the same column are significantly different ($P \leq 0.05$)

2. Characterization of sarcoplasmic proteinases in the muscles of hard and soft shell mud crabs

2.1 Temperature and pH profile of sarcoplasmic proteinases

The effects of temperature on proteinase activity are shown in Figure 15 and 16. The optimum temperature for caseinolytic activity of proteinases from hard shell mud crab chunk and claw muscles as well as soft shell mud crab chunk muscle was 65 °C when assayed at pH 5.5 and 8.5 ($P<0.05$). At pH 3.0,

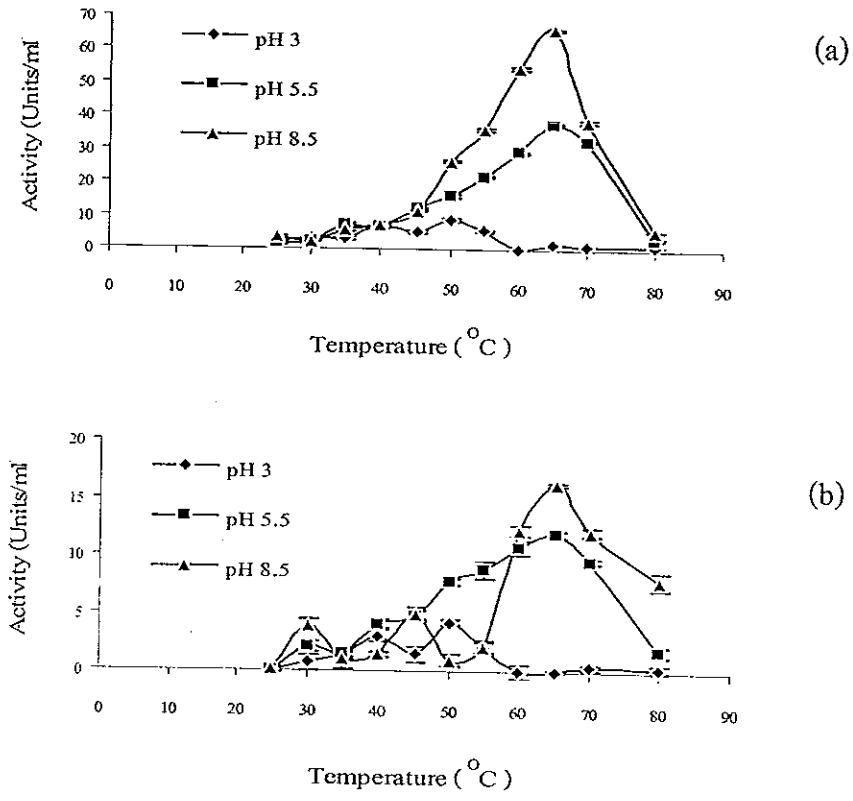


Figure 15 Temperature profiles of sarcoplasmic proteinases from the muscles of hard mud crab chunk (a) and hard mud crab claw (b). Proteinase activity was determined by incubating reaction mixture at pH 3.0, 5.5 and 8.5 at various temperatures. TCA-soluble peptides released were determined by the Lowry assay and activity was expressed as units/ml.

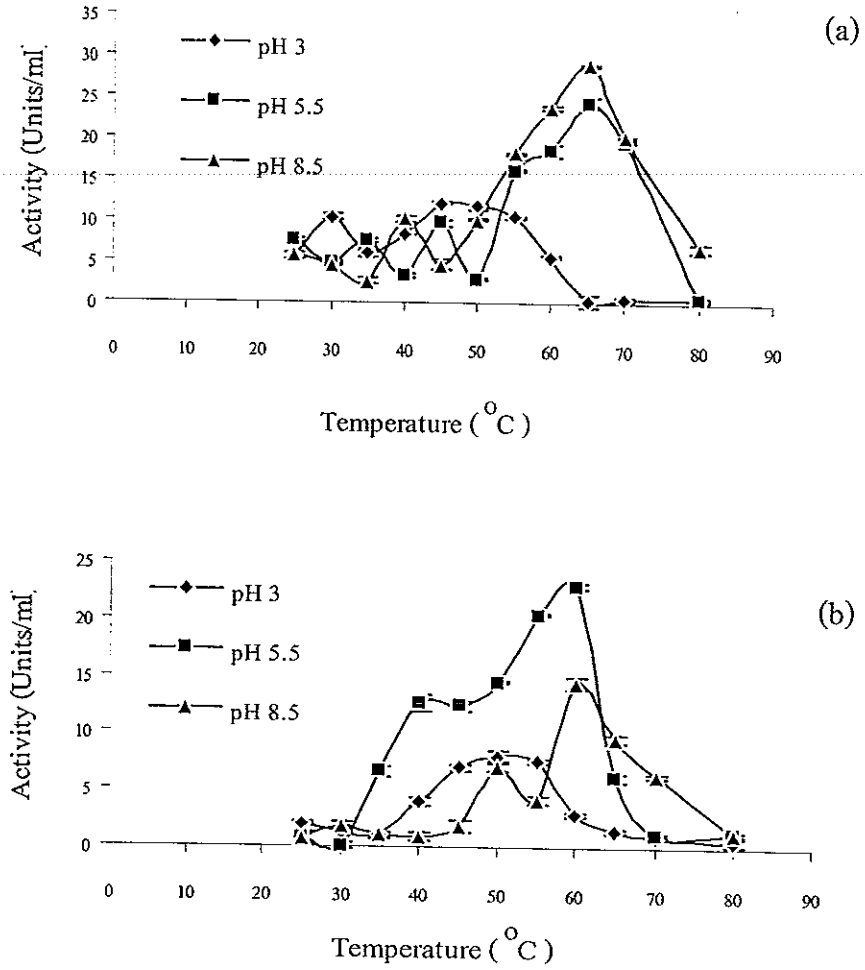


Figure 16 Temperature profiles of sarcoplasmic proteinases from the muscles of soft shell mud crab chunk (a) and soft shell mud crab claw (b). Proteinase activity was determined by incubating reaction mixture at pH 3.0, 5.5 and 8.5 at various temperatures. TCA-soluble peptides released were determined by the Lowry assay and activity was expressed as units/ml.

small peak with the optimum temperature of 50 °C was found. Jayasankar and Subramoniam (1997) reported the proteolytic activity of the seminal plasma of mud crab (*S. serrata*) which was highly active in the pH range 7.0 - 9.0, exhibiting maximum activity at pH 8.0. Benjakul *et al.* (2003a) purified a heat-

stable alkaline proteinase with optimal pH of 8.5 and optimal temperature at 60 °C in bigeye snapper muscle. Heat-stable alkaline proteinase was one of the most active proteinases in fish muscle (An *et al.*, 1996; Lin and Lanier, 1980; Boye and Lanier, 1988).

From the result, optimum temperature of sarcoplasmic proteinase in soft shell mud crab claw muscle was 60 °C at pH 5.5 and 8.5 ($P<0.05$) as shown in Figure 16 (b). However, at this optimum temperature tested, the activity of proteinase from soft shell mud crab claw muscle assayed at pH 5.5 was significantly higher than that observed at pH 8.5 (Figure 17). This sarcoplasmic proteinases in the claw muscle of soft shell mud crab was possibly cathepsin B L or D. Yamashita and Konagaya (1990b) reported that cathepsin B in chum salmon had an optimal pH of 5.7 at 30 °C. Aranishi and Ogata (1997) found that cathepsin L, purified from carp muscle, exhibited the maximum activity for Z-Phe-Arg-MCA at pH 5.5 – 6.0 and 50 °C. Wasson (1992) reported the highest proteolytic activity of cathepsin D from flounder muscle at pH 5.5 and 30 °C.

For pH profile study, the proteinases from all samples except soft crab claw muscle had the maximum activity at pH 8 ($P<0.05$). However, the activity peak was observed at pH 5.5 for the proteinase from soft shell mud crab claw muscle with a small peak at pH 8.0. Therfore it can be concluded that all muscles consisted of heat activated alkaline proteinase. Nevertheless, soft shell mud crab claw muscle contained cathepsin as the major proteinase and had some alkaline heat activated proteinase as the minor enzyme.

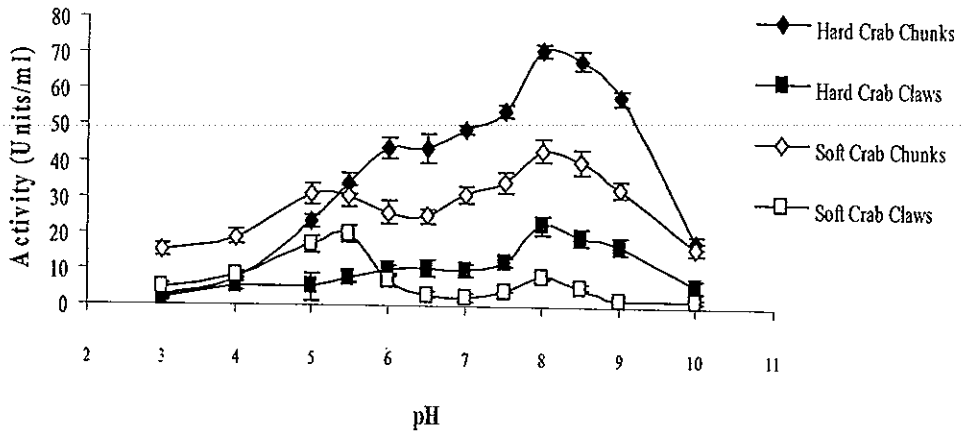


Figure 17 pH profiles of sarcoplasmic proteinases from hard and soft shell mud crab muscles. Proteinase activity was determined by incubating reaction mixture at 65 °C for proteinase from hard shell crab chunk, hard shell crab claw and soft shell crab chunk muscles, and at 60 °C for proteinase from soft shell crab claw muscle at various pHs. TCA – soluble peptides released were determined by the Lowry assay and activity was expressed as units/ml.

2.2 Effect of inhibitors on activity of sarcoplasmic proteinase from the muscles of hard and soft shell mud crabs

Specific proteinase inhibitors have been used to classify the groups of proteinases according to the nature of catalytic residues at the active sites (Asghar and Bhatti, 1987). The effect of various inhibitors on sarcoplasmic proteinase of hard and soft shell mud crabs is presented in Table 10. Proteinases from chunk and claw muscle of hard shell mud crab were predominantly inhibited by 0.01 mM soybean trypsin inhibitor ($P<0.05$). For soft shell mud crab, 0.01 mM soybean trypsin inhibitor also inhibited the activity of proteinase from the chunk muscle, while proteinase from the claw muscle was mainly inhibited by 1 μM pepstatin A ($P<0.05$). Soybean trypsin inhibitor is considered to be serine

proteinase inhibitor (Birk, 1976; North, 1989). E-64 and iodoacetic acid are cysteine proteinase inhibitor (Hanada *et al.*, 1978; North, 1989). Pepstatin A inhibits activities of most aspartic proteinases (Umezawa, 1976). EDTA is able to inhibit metallo-proteinases (Tsuchiya *et al.*, 1994). Since proteinase activity from the muscle of hard shell mud crab chunk, hard shell mud crab claw and soft shell mud crab chunk were inhibited by various proteinase inhibitors, multicatalytic proteinase or proteasome might present in those muscles. (Folco *et al.*, 1989; Kinoshita *et al.*, 1990; Stoknes and Rustard, 1995). Pepstatin A was able to inhibit the activity of proteinase from the claw muscle of soft shell mud crab markedly. Thus, the result indicated the presence of aspartic protease in the claw muscle of soft shell mud crab. Cathepsin D, considered to be in the group of aspartic proteinase, had optimal condition at pH 5.5 and 30 °C (Makinodan *et al.*, 1983; Wasson, 1992). Nevertheless, Draper and Zeece (1989) reported the higher optimal temperature of the cathepsin D from carp muscle which was 50°C. Cathepsin D was able to degrade myosin (both heavy and light chains), actin and the proteins of the regulatory complex (tropomyosin and troponin T and I) (Zeece and Kotoh, 1989; Zeece *et al.*, 1986). The high degradation activity of the proteinases in the claw muscle of soft shell mud crab was evidenced by the lighter bands of myosin, actin and troponin T with a concomitant appearance of 200, 40 and 37 kDa bands, respectively. Therefore, this proteinase might play an essential role in protein ture-over during molting process.

From the result, EDTA also showed the high inhibition on proteinase from all samples except that from soft shell mud crab claw muscle. This might be due to the chelating properties towards various ions which may be required for activition of enzyme. Therefore, various types of proteinase might be localized in the muscles of both crabs with different major proteinases. The further purification and characterization may be needed.

Table 10 Effect of inhibitors on the sarcoplasmic proteinases activity

Inhibitors	Concentration	% Inhibition			
		Hard shell mud crab		Soft shell mud crab	
		Chunk	Claw	Chunk	Claw
Control		0	0	0	0
Soybean trypsin inhibitor	0.01 mM	$58.74 \pm 3.11^{*c}$	$84.22 \pm 3.50^{a**}$	81.06 ± 0.22^{b}	28.64 ± 1.70^{d}
E - 64	10 μM	17.90 ± 1.59^{c}	37.59 ± 4.01^{a}	14.23 ± 2.78^{d}	19.21 ± 3.69^{b}
Iodoacetic acid	1 mM	22.30 ± 2.90^{c}	39.21 ± 4.13^{a}	3.32 ± 2.54^{d}	25.21 ± 0.40^{c}
Pepstatin A	1 μM	8.88 ± 1.48^{d}	53.09 ± 2.22^{b}	30.03 ± 0.92^{c}	86.96 ± 1.58^{a}
EDTA	10 mM	44.65 ± 1.33^{c}	55.80 ± 8.31^{a}	54.13 ± 0.68^{b}	11.20 ± 8.97^{c}

* mean ± standard deviation from triplicate determinations.

** Values with the same letter in the same row are not significantly different ($P>0.05$)

3. Changes in physicochemical of hard and soft shell mud crabs during frozen storage

3.1 Changes in Ca^{2+}-ATPase activity during frozen storage

Ca^{2+}-ATPase activity of NAM from all samples decreased throughout 12 weeks of frozen storage as shown in Figure 18 ($P<0.05$). The marked decrease in Ca^{2+}-ATPase was observed in the samples of hard and soft shell mud crabs within 2 weeks of frozen storage while Ca^{2+}-ATPase of hard shell mud crab claw decreased markedly within the first 4 week of frozen storage. Therefore, no considerable changes were observed up to 12 weeks of storage. Differences in the decrease of Ca^{2+}-ATPase activity among crab samples were probably due to different susceptibility to freeze denaturation of muscle proteins between pre- and post- molt stage as well as the differences in native structure of chunk and claw muscle protein. Ca^{2+}-ATPase activity is able to used as an indicator for the integrity of myosin molecules (Benjakul *et al.*, 1997; Auh *et al.*, 1999). The globular heads of myosin are responsible for Ca^{2+}-ATPase activity. A decrease in activity during extended frozen storage indicated the denaturation of myosin, especially in the head region. The decrease in ATPase activity was possibly associated with the oxidation of sulfhydryl on myosin globular head (Jiang *et al.*, 1988). Based on the decrease in Ca^{2+}-ATPase activity, myosin from claw muscle was found to undergo denaturation to a highest extent, compared to those of chunk muscle of both hard and soft shell mud crabs. When Ca^{2+}-ATPase activity of chunk muscle from hard and soft shell mud crabs were compared, that of soft shell mud crab was slightly lower, suggesting the higher susceptibility to denaturation of myosin of soft shell mud crab. Seo *et al.* (1997) reported the continuous decrease in Ca^{2+}-ATPase activity of myctophild fish during frozen storage and the degree of decrease depended on species. The decrease in Ca^{2+}-ATPase activity in carp myofibril was found during storage at -20 °C up to 60

days (Azuma and Konno., 1998). Nambudiri and Gopakumar (1992) also observed the decrease in ATPase activity of fresh water and brackish fish during -20 °C for 180 days. However, the loss of Ca^{2+}-ATPase activity is not necessarily synonymous with aggregation because total loss in ATPase activity was caused by the denaturation of active site without aggregation (Ramirez *et al.*, 2000).

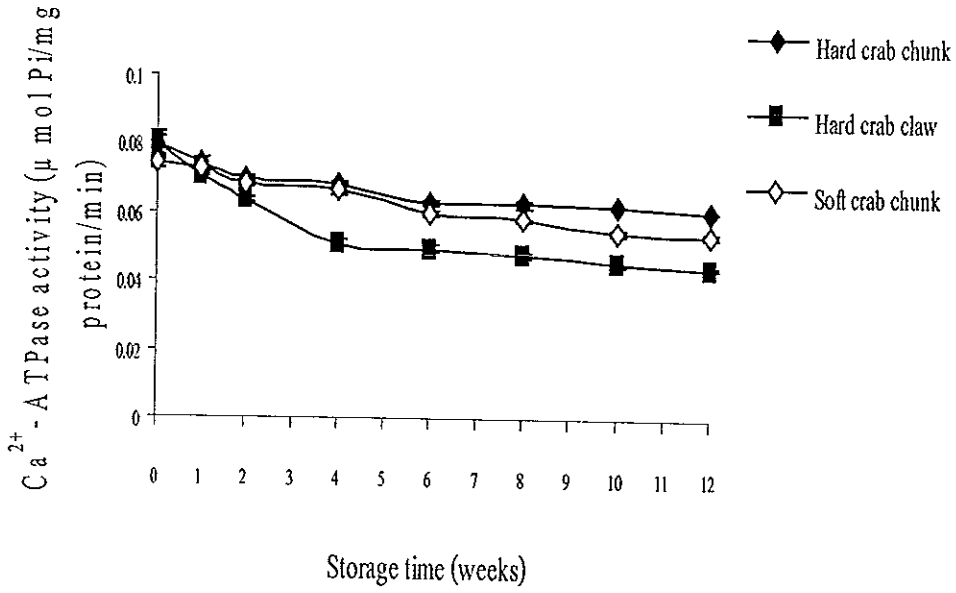


Figure 18 Changes in Ca^{2+}-ATPase activities of NAM extracted from hard and soft shell mud crabs during frozen storage at -20 °C for 12 weeks. Bars indicate standard deviation from triplicate determinations.

3.2 Changes in total sulfhydryl contents during frozen storage

Sulfhydryl content of NAM from hard and soft shell mud crabs decreased during frozen storage up to 12 weeks as shown in figure 19. For all samples, the marked decrease in sulfhydryl content was observed in the first week of storage and only slight decrease was found up to the end of storage ($P<0.05$). Sulfhydryl

content in the chunk and claw muscles of hard shell mud crab decreased to a higher extent, compared to the chunk muscle of soft shell mud crab, especially with the extented storage time. However, no differences in total sulfhydryl content in chunk and claw muscles of hard shell mud crab were observed. The differences in sulfhydryl content between hard and soft shell mud crab during frozen storage were probably caused by the difference in susceptibility in sulfhydryl oxidation of myofibrillar proteins. Jiang *et al.* (1988; 1989) reported that the reactive sulfhydryl generally decreased during frozen storage. The

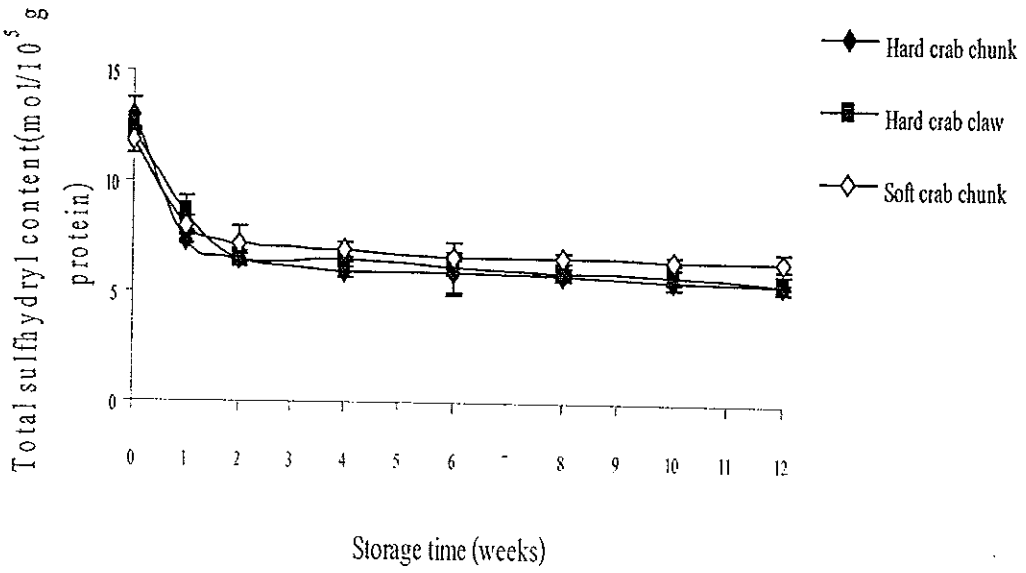


Figure 19 Changes in sulfhydryl content of NAM extracted from hard and soft shell mud crabs during frozen storage at -20 °C for 12 weeks. Bars indicate standard deviation from triplicate determinations.

decrease in sulfhydryl content probably due to either the oxidation of sulfhydryl, disulfide interchanges (benjakul and Bauer, 2000) or the formation of hydrogen and hydrophobic bonds, which masked the reactive sulfhydryl structure of actomyosin molecules. Ramirez *et al.* (2000) reported that continuous decrease of

total sulfhydryl groups of tilapia during 15 day of storage at -20 °C which declined 33% of the initial value.

3.3 Changes in disulfide bond contents during frozen storage

Changes in disulfide bonds content of NAM from hard and soft shell mud crabs during frozen storage was shown in Figure 20 ($P<0.05$). The disulfide bond content of all samples increased gradually throughout the frozen storage.

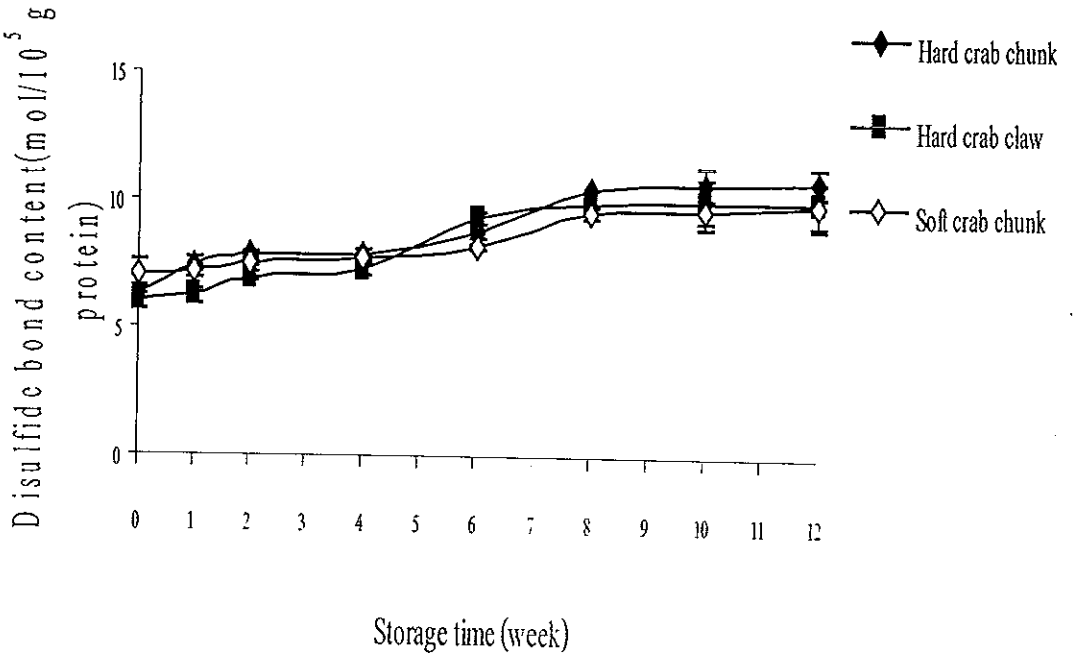


Figure 20 Changes in disulfide bonds content of NAM extracted from hard and soft shell mud crabs during frozen storage at -20 °C for 12 weeks. Bars indicate standard deviation from triplicate determinations.

Generally, no marked differences in disulfide bond content were found among crab samples. However, the sharp increase in disulfide bond content was found in

the sample of hard shell mud crab chunk during the first week of frozen storage while the other samples had a slight increase. Nevertheless, after the fifth week of frozen storage, soft shell mud crab chunk contained the lowest content of disulfide bonds which was generally coincidental with the highest sulfhydryl groups remained in their sample. The increase in disulfide bond was in accordance with the decrease in sulfhydryl content, suggesting the oxidation of sulfhydryl groups to disulfide bonds during extended storage. Benjakul *et al.* (2003b) reported the disulfide bond formation with the concomitant decrease in sulfhydryl groups in the muscle of croaker, lizardfish, threadfin bream and bigeye snapper during storage at -18 °C up to 24 weeks. Ramirez *et al.* (2000) also reported the decrease of sulfhydryl contents of myosin from *Tilapia miolotica* with the concomitant formation of disulfide bonds during frozen storage. The oxidation of sulfhydryl on the globular heads of myosin was possibly associated with the decrease in ATPase activity (Hamada *et al.*, 1977; Jiang *et al.*, 1988). However, the degree of sulfhydryl oxidation and Ca^{2+}-ATPase reduction were different, depending on species (Benjakul *et al.*, 2003b).

3.4 Changes in surface hydrophobicity during frozen storage

The changes in surface hydrophobicity were observed throughout the frozen storage up to 12 week as shown in Figure 21. During 4 weeks of storage, no changes in surface hydrophobicity of all samples were detected ($P<0.05$). Between 6 and 8 weeks, surface hydrophobicity of NAM from all samples increased, suggesting the conformation changes with exposure of hydrophobic portions. Hard mud crab claw had the highest increase in surface hydrophobicity, followed by soft shell mud crab chunk and hard shell mud crab chunk, respectively. Thereafter, surface hydrophobicity of all samples decreased gradually up to 12 weeks. The exposed hydrophobic residues might undergo aggregation via

hydrophobic interaction. The result suggested that protein from claw muscle seemed to be more susceptible to denaturation by freezing than that from the chunk muscle of both hard and soft shell mud crabs. Multilangi *et al.* (1996) reported that the increase in surface hydrophobicity indicated an exposure of the interior of the molecule due to denaturation on degradation. Badii and Howell (2001) reported that the increase in hydrophobicity of proteins during frozen storage could be attributed to the unfolding of proteins and exposure of hydrophobic aliphatic and aromatic amino acids.

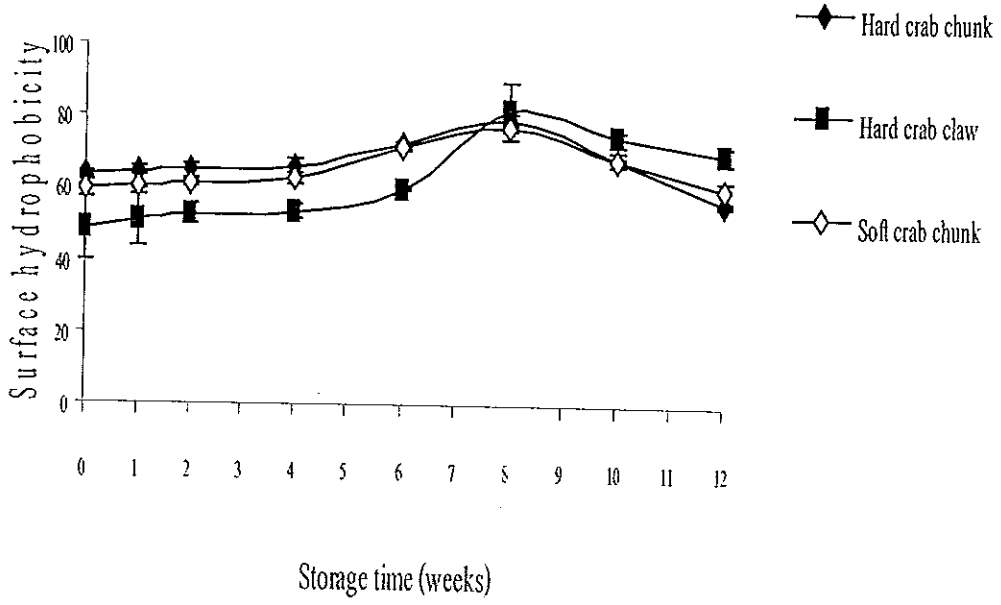


Figure 21 Changes in surface hydrophobicity of NAM extracted from hard and soft shell mud crabs during frozen storage at -20 °C for 12 weeks. Bars indicate standard deviation from triplicate determinations.

3.5 Changes in formaldehyde during frozen storage

Formaldehyde content in the muscle of crab during frozen storage is shown in Figure 22. Formaldehyde formation in all samples increased continuously during storage up to 12 weeks ($P<0.05$). However, the drastic increase in formaldehyde contents was found in the claw and chunk muscles of hard and soft shell mud crabs between the first 2 weeks. Thereafter, slight increase was observed up to 12 weeks of frozen storage. However, claw muscles of hard and soft shell mud crabs tended to have higher formaldehyde contents than chunk muscles. Claw muscle of soft shell mud crab had the highest formaldehyde content, compared to other samples. Formaldehyde formation and its reaction with muscle proteins in lean fish species during frozen storage is considered to be a major factor affecting texture and functionality deterioration (Tejada *et al.*, 2002). Formaldehyde is able to react with protein, resulting in the conformational changes, insolubilization and loss of extractability. The formaldehyde is known as an effective cross-linker via methylene bridge (Sikorski *et al.*, 1990a). Trimethylamine-*N*-oxide demethylase (TMAOase) is capable of catalyzing the conversion of trimethylamine oxide (TMAO) to dimethylamine (DMA) and formaldehyde (Gill and Paulson, 1982). This enzyme is concentrated in the internal organs and red muscle (Gill and Paulson, 1982; Rehbein and Schreiber, 1984). Takunaga (1970) reported that a high content in formaldehyde was commonly found in red muscle, compared to white muscle. From this result, the higher formaldehyde content of the claw muscle was coincidental with the highest decrease in Ca^{2+}-ATPase activity (Figure 18) and the highest surface hydrophobicity (Figure 21). Thus, formaldehyde might induce the conformational

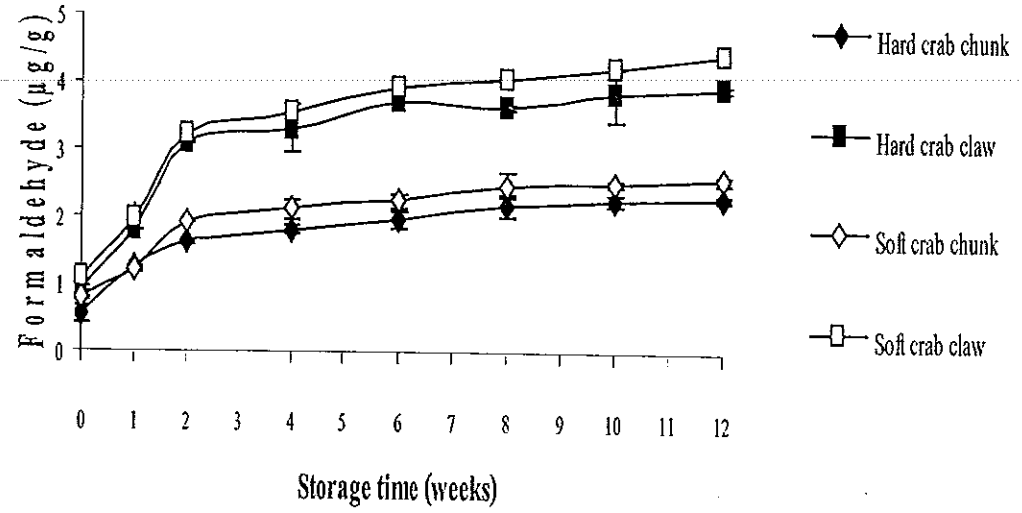


Figure 22 Changes in formaldehyde from chunk and claw muscles of hard and soft shell mud crabs during frozen storage at -20 °C for 12 weeks. Bars indicate standard deviation from triplicate determinations.

changes of myosin, especially the globular head region, leading to the decrease in Ca^{2+}-ATPase activity and the increase in the surface hydrophobicity. This result was in agreement with Aug and Hultin (1989) who reported that formaldehyde at concentrations occurring in cod myosin stored at -25 °C and -80 °C enhanced the protein denaturation as evidenced measured by the loss in solubility, the decrease in ATPase activity and the increase in surface hydrophobicity.

3.6 Changes in solubility during frozen storage

The decrease in solubility of crab proteins during frozen storage is shown in Figure 23. The solubility of all samples decreased continuously throughout 12 weeks of frozen storage ($P<0.05$). The lowest solubility was found in the chunk

muscle of hard shell mud crab, especially after 6 weeks of the frozen storage. The considerable decrease in solubility of chunk muscle of hard shell mud crab was related to high amount of disulfide bond formed during frozen storage as shown in Figure 20. A decrease in protein solubility was coincidental with an increase in hydrophobicity and the formation of non-covalently and covalently linked aggregates (Badii and Howell, 2002). Salt solubility of myofibrillar proteins was decreased progressively during frozen storage due to protein aggregation (Matsumoto and Noguchi, 1992). Disulfide and nondisulfide covalent bonds caused

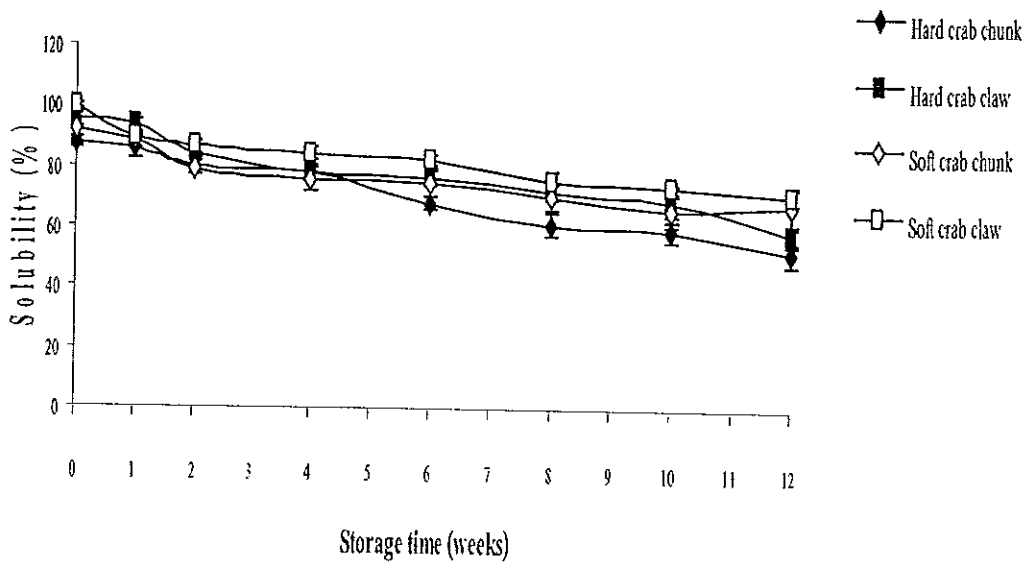


Figure 23 Changes in solubility of chunk and claw muscles of hard and soft shell mud crabs during frozen storage at -20 °C for 12 weeks. Bars indicate standard deviation from triplicate determinations.

the cross-linking of myosin heavy chain contributing to the formation of high-molecular-weight polymer and aggregates (Ragnarsson and Regenstein, 1989; Tejada *et al.*, 1996).

3.7 Changes in lipid oxidation during frozen storage

TBARS in muscle of all samples increased during 12 weeks of frozen storage at -20 °C ($P<0.05$) (Figure 24). No differences in TBARS were detected among all samples, except claw muscle of soft shell mud crab, which had a higher TBARS after 4 weeks of frozen storage. Although fat content in claw muscle of soft shell mud crab was presented at the lowest content (0.12%) (Table 4), TBARS content in this portion tended to increase to a higher extent during frozen storage. This was possibly due to the susceptibility to denaturation of claw muscle of soft shell mud crab during freezing process. Thus, lipid oxidation could be accelerated by catalysts released from the muscle cells. Benjakul and Bauer (2001) reported that lipid oxidation can be induced by frozen storage. The ice crystals formed during freezing process could cause cell injure and release pro-oxidants for lipid oxidation, especially free iron (Benjakul and

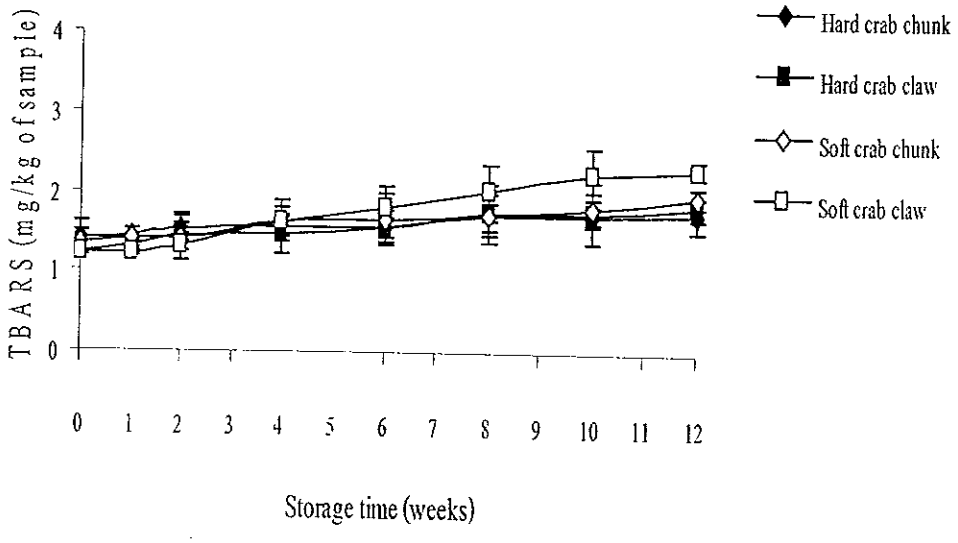


Figure 24 Changes in TBARS of chunk and claw muscles of hard and soft shell mud crabs during frozen storage at -20 °C for 12 weeks. Bars indicate standard deviation from triplicate determinations.

Bauer, 2001). Free iron is able to take part in electron transfer reactions with molecular oxygen, generating a superoxide anion production (Harris and Tall, 1994). Simeonidou *et al.* (1997) reported the differences in TBARS value in whole and fillets of horse mackerel and Mediterranean hake. Ben-Gigirey *et al.* (1999) reported the increase in TBARS value in albacore tuna during 1 years of storage at -18 °C and -25 °C.

3.8 Changes in pH during frozen storage

Changes in pH were observed throughout the frozen storage up to 12 weeks as shown in Figure 25. pH of all samples were quite stable during the first week of frozen storage ($P<0.05$). The increase in pH of chunk and claw muscles of soft shell mud crab was found at the second week of the storage while a slight decrease in pH was found in the chunk muscle of hard shell mud crab at the same storage period. The increase in pH of fresh fish was due to the decomposition of nitrogenous compounds (Sikorski *et al.*, 1990b) and formation of DMA from TMAO (Rodger *et al.*, 1980). The libration of inorganic phosphate and ammonia due to the enzymatic degradation of ATP was also associated with the changes in pH. After 2 weeks, pH of all samples decreased gradually up to 12 weeks. Olley *et al.* (1962) reported the decrease in pH was coincidental with the enzymatic hydrolysis of neutral fats and phospholipids, leading to the formation of free fatty acids. From the result, it was postulated that the decomposition of nitrogenous compounds and the enzymatic degradation of ATP occurred in the first two weeks of stored at -20 °C. Subsequently, the gradual enzymatic hydrolysis of lipids in the muscle of crab took place throughout the frozen storage.

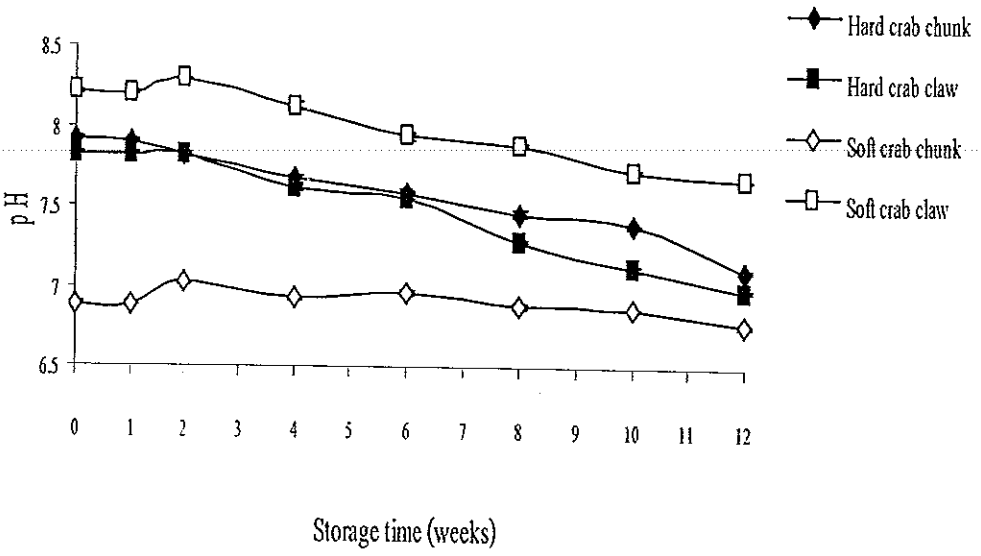


Figure 25 Changes in pH of chunk and claw muscles of hard and soft shell mud crabs during frozen storage at -20 °C for 12 weeks. Bars indicate standard deviation from triplicate determinations.

3.9 Changes in cooking loss during frozen storage

Cooking loss in crab muscles was monitored during frozen storage (Figure 26). Cooking loss increased as the storage time increased up to 12 weeks ($P<0.05$). However, the higher increasing rate was found in claw muscles, compared to the chunk muscles. Among all samples, the claw muscle of soft shell mud crab showed extremely high cooking loss. After 12 weeks of frozen storage, cooking loss of soft shell mud crab claw muscle increased to 47.43 %. Cooking loss of 19.40 % was found in hard shell mud crab claw muscle. In chunk muscle, the cooking loss increased to approximately 7.82 - 9.63 %. Aggregation and denaturation of protein in crab muscle were induced by heating, leading to the loss in water holding capacity of the protein. Additionally, denatured proteins formed during frozen storage could be susceptible to heat denaturation, causing the severe aggregation of protein. As a result, drastic

cooking loss was observed. Siddaiah *et al.* (2001) reported the expressible water of silver crap mince increased significantly throughout the storage of 180 days at -18 °C. Benjakul *et al.* (2003b) found that extended frozen storage caused the disintegration of muscle by ice formation, resulting in the increase of expressible drip in fish muscle. Shenouda (1980) reported that the changes in the water holding capacity of fish were due to surface dehydration of proteins during frozen storage of fish. Badii and Howell (2002) reported that changes in water content of cod and haddock fillets during frozen storage at -10 °C and -30 °C also contributes to changes in protein conformation and denaturation as indicated by increased protein hydrophobicity and decreased protein solubility of fish muscle.

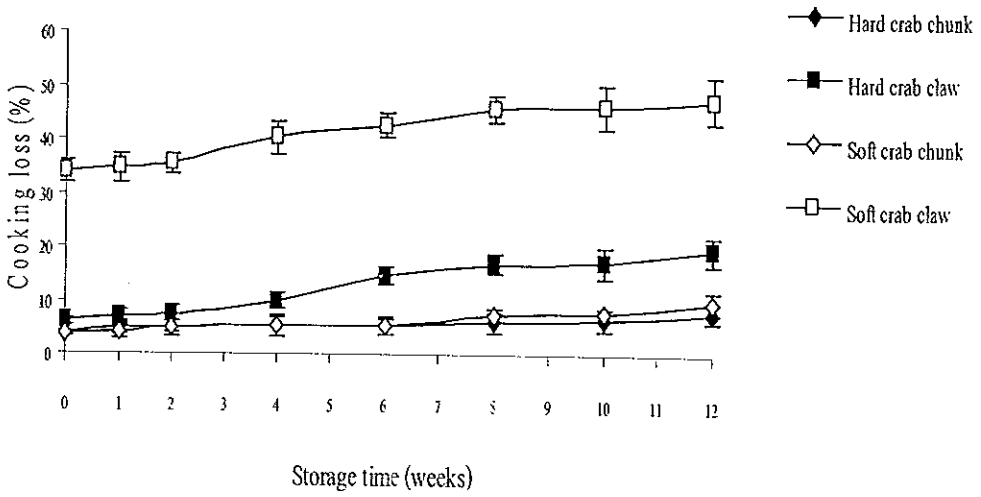


Figure 26 Changes in cooking loss of chunk and claw muscles of hard and soft shell mud crabs during frozen storage at -20 °C for 12 weeks. Bars indicate standard deviation from triplicate determinations.

4. Effect of freezing and thawing on physico-chemical changes in hard and soft shell mud crab muscles

4.1 Changes in Ca^{2+} -ATPase activity of NAM in crabs subjected to multiple freeze-thaw cycles

Ca^{2+} -ATPase activity of all samples decreased slightly with increasing freeze-thaw cycle as shown in figure 27. The Ca^{2+}-ATPase activity of NAM extracted from hard shell mud crab claw muscle decreased to the highest extent, compared to the others. After 5 freeze-thaw cycles, Ca^{2+}-ATPase activity decreased by 21.65 % in hard shell mud crab claw and 19.84 % and 10.72 % in the chunk muscles of soft shell mud crab and hard shell mud crab, respectively, when compared to that found in fresh muscles ($P<0.05$). Benjakul and Bauer (2000) reported that Ca^{2+}-ATPase activity of NAM extracted from cod fillets decreased as the freeze-thaw cycles increased. After freeze-thaw process, myosin was denatured, caused by ice crystallization, which induced the tertiary structural changes and the loss of Ca^{2+} -ATPase activity (Jiang *et al.*, 1988).

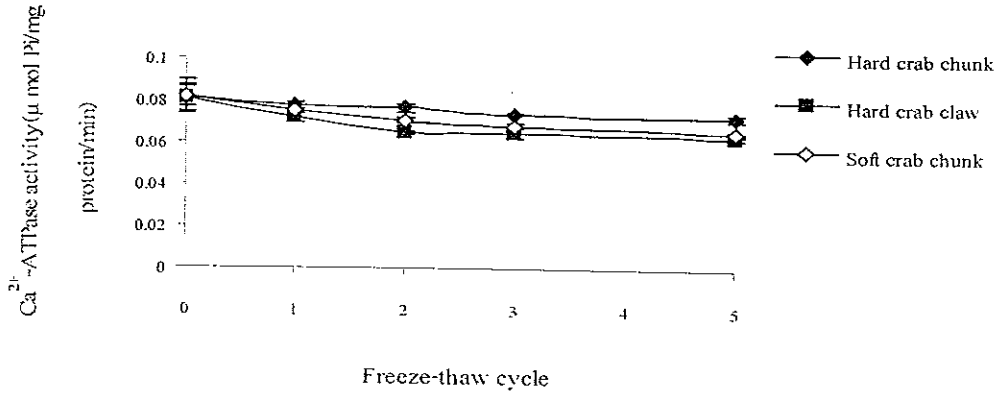


Figure 27 Changes in Ca^{2+} -ATPase activity of NAM extracted from chunk and claw muscles of hard and soft shell mud crabs subjected to different freeze-thaw cycles. Bars indicate standard deviation from triplicate determinations.

4.2 Changes in total sulfhydryl contents of NAM in crabs subjected to multiple freeze-thaw cycles

Total sulfhydryl contents of all samples decreased continuously as the number of freeze-thaw cycles increased (figure 28). It was found that total sulfhydryl contents of all sample decreased sharply after one cycle of freezing-thawing ($P<0.05$). However, no changes in total sulfhydryl content were observed with freeze-thaw cycle of 2-5. This result was in agreement with Benjakul and Bauer (2000) who reported that total sulfhydryl groups of NAM extracted from cod fillets decreased after the first freeze-thaw cycle and the constant of total sulfhydryl contents was generally observed after two cycles of freezing-thawing. The decrease in total sulfhydryl contents was due to the oxidation of sulfhyldryl groups between inter- or intra- proteins (Huidobro *et al.*, 1998). Furthermore, the loss of total sulfhyldryl contents might be caused by the interaction between the exposed sulfhydryl groups of protein and additives or small molecular compounds such as peptide (Lian *et al.*, 2000).

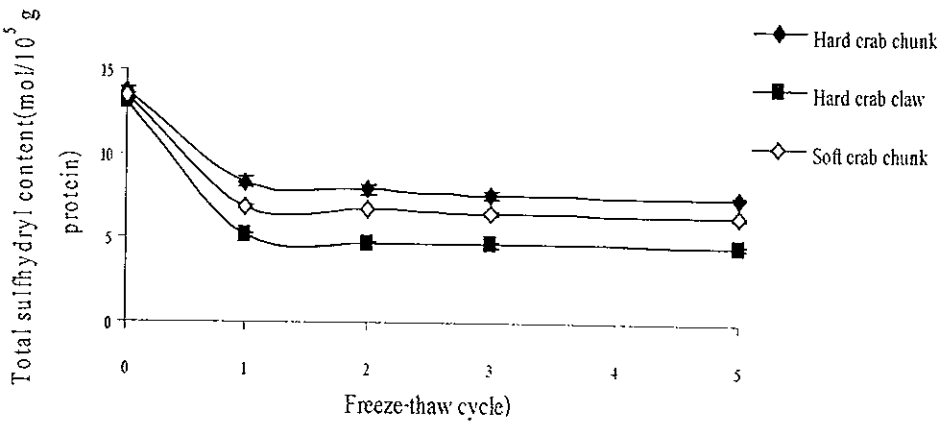


Figure 28 Changes in total sulfhydryl contents of NAM extracted from chunk and claw muscles of hard and soft shell mud crabs subjected to different freeze-thaw cycles. Bars indicate standard deviation from triplicate determinations.

4.3 Changes in disulfide bond contents of NAM in crabs subjected to multiple freeze-thaw cycles

Changes in disulfide bond in crab muscles with different freeze-thaw cycles are shown in Figure 29. Disulfide bond formation of all samples increased when freeze-thaw cycles increased ($P<0.05$). Disulfide bond formation in claw muscle of hard shell mud crab increased rapidly after the first cycle of freezing-thawing. Nevertheless, disulfide bond formation in chunk muscles of hard and soft shell mud crabs were quite constant after the first cycle of freeze-thawing and the increase was observed with increasing freeze-thaw cycles. The increase in disulfide bond content was concomitant with the decrease in total sulfhydryl content (figure 28) and Ca^{2+}-ATPase activity (figure 27). This result indicated that freeze-thaw cycles induced the changes of actomysin structure, leading to the exposure of sulfhyldryl groups, which were oxidized to disulfide bond. Benjakul and Bauer (2000) reported that freeze-thaw cycles accelerated the formation of disulfide bonds via oxidation of sulfhydryl groups. Ramirez *et al.* (2000) reported that head-to-head interactions of myosin via disulfide bonds during frozen storage induced the aggregation.

4.4 Changes in surface hydrophobicity of NAM in crabs subjected to multiple freeze-thaw cycles

Surface hydrophobicity of NAM extracted from crabs subjected to multiple freeze-thaw cycles is shown in figure 30. The surface hydrophobicity of NAM from hard shell crab claw muscle increased continuously as the cycles of freezing-thawing increased ($P<0.05$), while fluctuations in surface hydrophobicity were found in chunk muscles of hard and soft shell mud crabs. A slight decrease in surface hydrophobicity was observed in chunk muscle of soft shell mud crab when the sample was subjected to freezing-thawing up to three cycles.

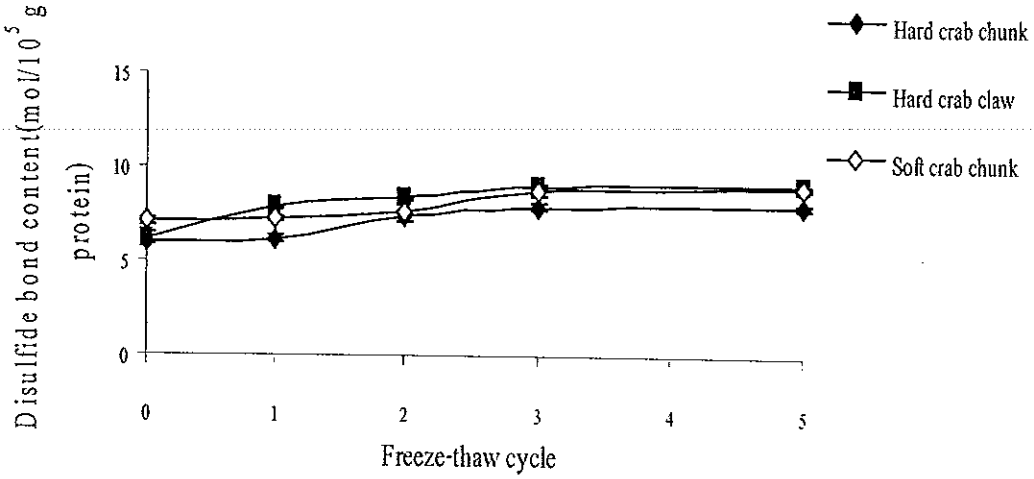


Figure 29 Changes in disulfide bond contents of NAM extracted from chunk and claw muscles of hard and soft shell mud crabs subjected to different freeze-thaw cycles. Bars indicate standard deviation from triplicate determinations.

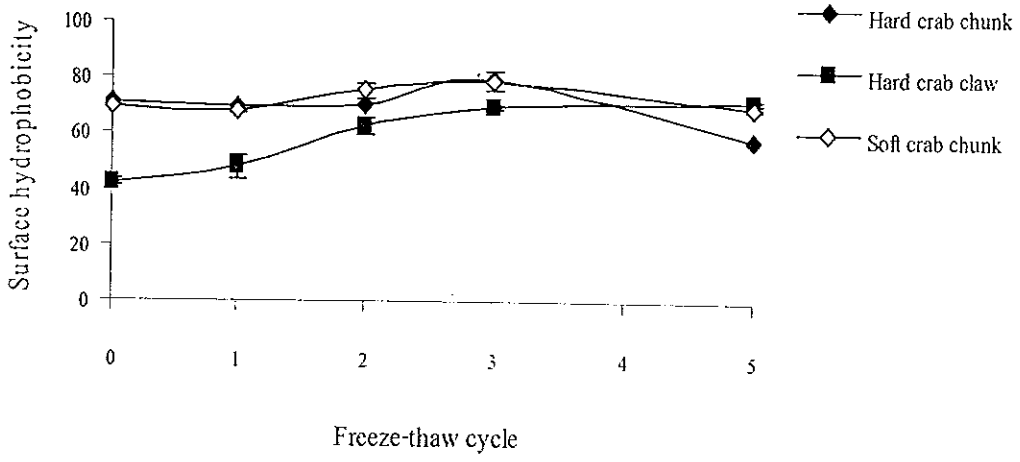


Figure 30 Changes in surface hydrophobicity of NAM extracted from chunk and claw muscles of hard and soft shell mud crabs subjected to different freeze-thaw cycles. Bars indicate standard deviation from triplicate determinations.

Subsequently, surface hydrophobicity decreased with five freeze-thaw cycles. The surface hydrophobicity of hard shell mud crab claw muscle increased continuously and reached the plateau with 3 cycles.

During freeze-thawing, conformational changes of crab proteins occurred. Hydrophobic regions located inside the protein molecules were exposed. This changes were related to an increase in surface hydrophobicity of crab proteins. The exposure of hydrophobic aliphatic and aromatic amino acids led to the increase in hydrophobicity of proteins (Badii and Howell, 2001; Multilangi *et al.*, 1996). Benjakul and Bauer (2000) also found that the freeze-thaw process induced cod protein denaturation which caused increased surface hydrophobicity. The subsequent decrease in surface hydrophobicity was associated with the formation of hydrophobic interaction.

4.5 Changes in formaldehyde contents of crab muscle subjected to multiple freeze-thaw cycles

Formaldehyde content of crab muscles increased continuously when freeze-thaw cycle increased as shown in Figure 31. Generally, formaldehyde contents of all samples increased gradually when the muscles were subjected to the first cycle of freezing-thawing. The formaldehyde contents increased sharply when all crab muscle samples were subjected to the second cycle of freeze-thaw cycle ($P<0.05$). The formaldehyde content began to increase slowly with 3 freeze-thaw cycles and no marked increases were found with five cycles of freezing-thawing. Claw muscle of soft shell mud crab had the highest formaldehyde content with 3 and 5 cycles of freezing-thawing. This result indicated that claw muscle of soft shell mud crab might have the highest activity of trimethylamineoxide demethylase, especially with increasing freeze-thaw cycles. Formaldehyde was considerated to be an enzymatic product from trimethylamine oxide and can be

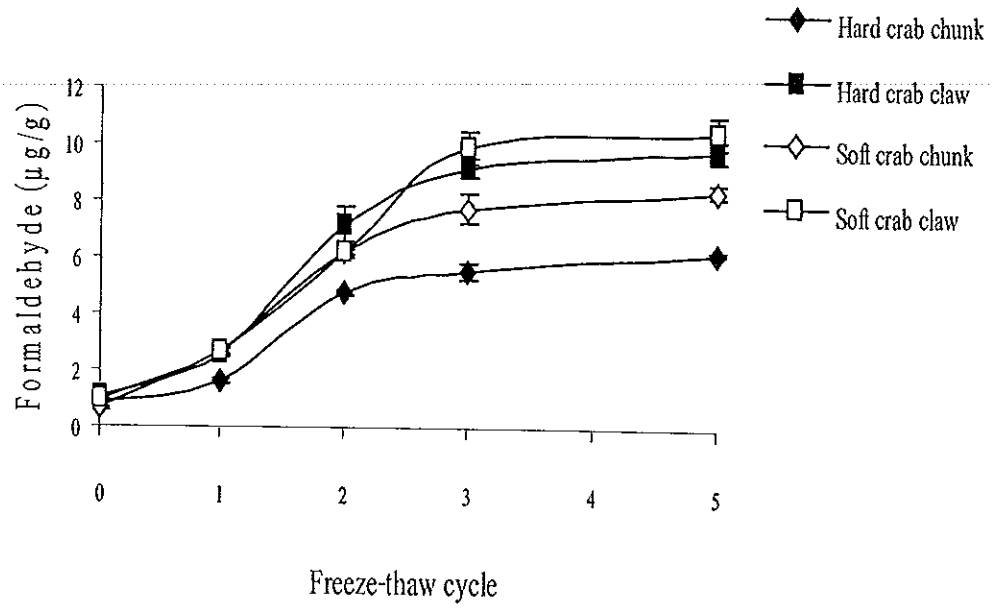


Figure 31 Changes in formaldehyde contents of chunk and claw muscles of hard and soft shell mud crabs subjected to different freeze-thaw cycles. Bars indicate standard deviation from triplicate determinations.

formed in fish muscle during frozen storage, especially in gadoid family (Ragnarsson and Regenstein, 1989). However, TMAOase has been found in muscle of red hake (Phillippy and Hultin, 1993), Alaska pollack (Kimura *et al.*, 2000), and lizard fish (Benjakul *et al.*, 2003c).

4.6 Changes in solubility of crab muscle subjected to multiple freeze-thaw cycles

The changes in solubility in 0.6 M KCl after freeze-thawing with different cycles are shown in Figure 32. Solubility of all samples decreased continuously when the number of freeze-thaw cycles increased ($P<0.05$). The decrease in solubility of all samples, except the sample of soft shell mud crab claw, were

shown to coincide with the increase in disulfide bonds (Figure 29) and formaldehyde formation (Figure 31) when the samples were subjected to repeated freeze-thaw process. Aug and Hultin (1989) reported that loss of solubilization of Gadoid proteins was caused by formaldehyde-mediated cross-liking of protein. Furthermore, disulfide bond formation was also related to the loss of protein extractability during frozen storage of halibut mince (Lim and Haard, 1984). Nevertheless, among all samples, soft shell mud crab claw showed the lowest decrease in solubility. This might be caused by the lowest myofibrillar fraction in this sample (Table 6). Therefore, the loss of solubility due to cross-linking of protein would occur at the lowest extent. This result was in agreement with Owusu-Ansah and Hultin (1992) who reported that contractile proteins such as

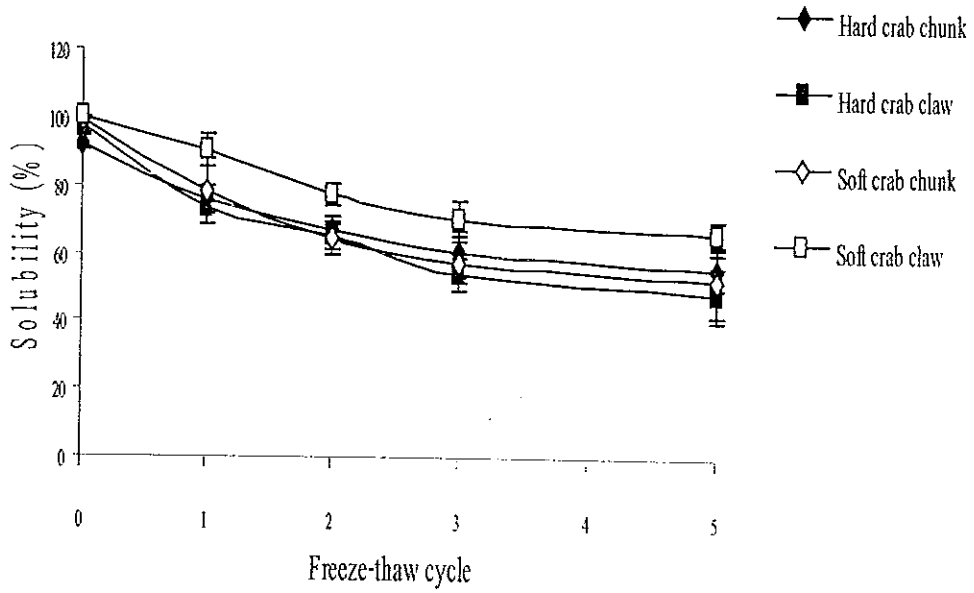


Figure 32 Changes in solubility of chunk and claw muscles of hard and soft shell mud crabs subjected to different freeze-thaw cycles. Bars indicate standard deviation from triplicate determinations.

myosin heavy chain was more susceptible to insolubilization than sarcoplasmic proteins. Benjakul and Bauer (2000) reported that the loss of salt-soluble protein resulted from the denaturation of proteins induced by the freeze-thaw process.

4.7 Changes in TBARS contents of crab muscle subjected to multiple freeze-thaw cycles

TBARS of all samples increased as the freeze-thaw cycles increased as shown in Figure 33 ($P<0.05$). This result was in agreement with Benjakul and Bauer (2001) who reported that TBARS in catfish fillet increased when the number of freeze-thaw cycles increased. Among all samples, the TBARS of soft shell mud crab claw muscle increased at the highest rate and reached to the highest amount after being subjected to two freezing-thawing cycles. The marked

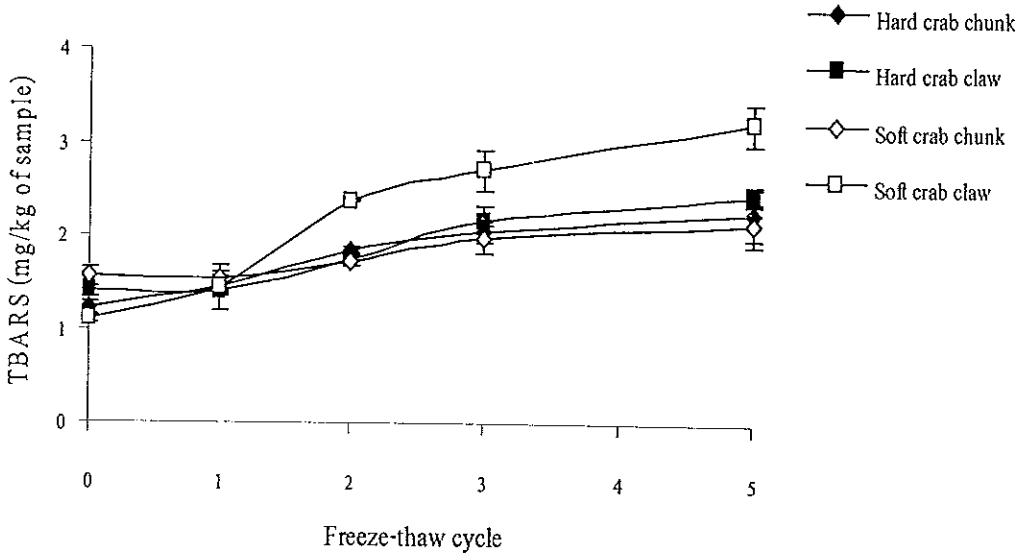


Figure 33 Changes in TBARS contents in chunk and claw muscles of hard and soft shell mud crabs subjected to different freeze-thaw cycles. Bars indicate standard deviation from triplicate determinations.

increase in TBARS in soft shell mud crab claw muscle during freeze-thawing indicated that the cells of this sample muscle were the most susceptible to injury by repeating freeze-thaw process, compared to the others. As a result, the release of pro-oxidations from the cells occurred to the highest extent and lipid oxidation was induced. Srinivasan *et al.* (1997a) found that the accelerated TBARS value of raw prawn samples after freeze-thaw cycles was due to the release of oxidative enzymes and pro-oxidants from various cellular organelles. Benjakul and Bauer (2001) reported that pro-oxidants, such as free iron, could be released from injured cells by ice crystals formed during freeze-thaw cycles.

4.8 Changes in pH of crab muscle subjected to multiple freeze-thaw cycles

A slight decrease in pH was detected in all samples as freeze-thaw cycles increased (Figure 34). Therefore, freezing-thawing might induce fat hydrolysis. Olley *et al.* (1962) reported that the formation of free fatty acids was

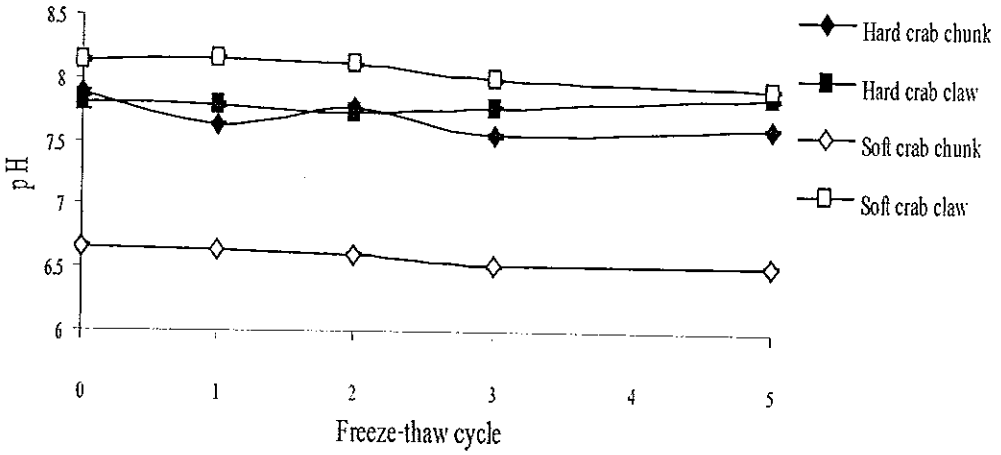


Figure 34 Changes in pH in chunk and claw muscles of hard and soft shell mud crabs subjected to different freeze-thaw cycles. Bars indicate standard deviation from triplicate determinations.

related to enzymatic hydrolysis of neutral fats and phospholipids, leading to the decrease in pH value.

4.9 Changes in cooking loss of crab muscle subjected to multiple freeze-thaw cycles

Cooking loss of crabs subjected to different freeze-thaw cycles is shown in Figure 35. Weight loss of cooked crab meats increased as freezing-thawing increased. The cooking loss of all samples increased sharply when the samples were subjected to more than one cycle of freeze-thawing ($P<0.05$). However, chunk muscles of hard and soft crabs had the constant cooking loss after being subjected to three and five cycles of freezing-thawing. Srinivasan *et al.* (1997b) found that the cooking loss of freeze-thaw abused prawns increased to 15.2 to 17.8%, compared to the fresh cooked prawn, which had cooking loss of 11.7%. Nevertheless, among of all samples, cooked soft shell mud crab claw muscle had

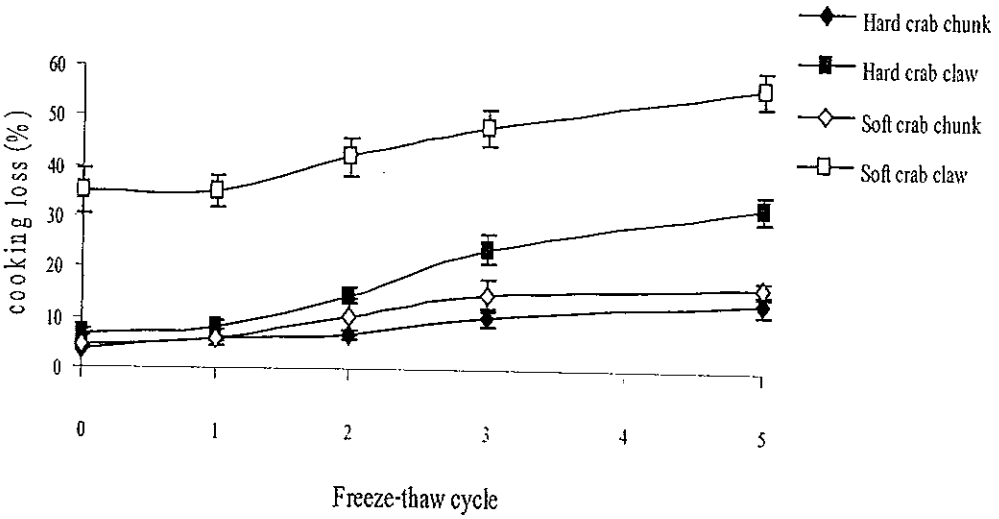


Figure 35 Changes in cooking loss in chunk and claw muscles of hard and soft shell mud crabs affected by different freeze-thaw cycles. Bars indicate standard deviation from triplicate determinations.

the highest weight loss. The highest cooking loss of soft shell mud crab claw muscle might be associated with the highest water content of this sample (Table 4), leading to the release of water during cooking. However, multiple freeze-thaw cycles induced cooking loss might be associated with the formaldehyde formation (Figure 31). Formaldehyde is able to induce the aggregation of protein in fish muscle and the loss in water holding capacity (Benjakul *et al.* 2004). Nowsad *et al.* (2000) found that proteins in surimi which was freeze-thawed repeatedly lost their functionality, such as gelation and water-holding capacity. However, ice crystal formed was also considered to cause the tissue damage and the leakage of water insided the cells easily. As a consequence, weight loss of cooked muscle increased as freeze-thaw cycle increased.

Chapter 4

Conclusion

1. Chemical compositions, physical properties and structure of the muscles from hard and soft shell mud crabs were different. Distinguished features of soft shell mud crab muscle were high water content and low protein content. The porous structural muscle with low water holding capacity was found in claw muscle of soft shell mud crab.
2. Muscle of soft shell mud crab was generally more susceptible to thermal denaturation than muscle of hard shell mud crab. Furthermore, claw muscle was more sensitive to denaturation than chunk muscle. Thus, mild heat processing should be applied for soft shell mud crab muscle, especially its claws to avoid the loss in quality caused by thermal denaturation.
3. Major proteinase in hard and soft shell mud crab was serine proteinase, which had the optimum temperature and pH at 65 °C and 8.0, respectively. However, major proteinase in soft shell mud crab claw muscle was aspartic proteinase, which showed the maximum activity at 60 °C and pH 5.5. The handling process of both hard shell and soft shell mud crabs should be performed in the way which minimizes the proteolytic activity such as lowering the storage temperature, etc.
4. Hard and soft shell mud crab muscles underwent chemical changes, especially protein denaturation during frozen storage at -20 °C for 12 weeks. Greater changes were observed with increasing freeze-thaw cycles. However, soft shell mud crab muscle was more susceptible to denaturation during frozen storage than the muscle of hard shell mud crab muscle. Therefore, different freezing

process or thawing process should be used for different crabs to obtain the prime quality of their frozen products.

Chapter 5

Suggestions

1. Fast freezing should be used to prevent the drastic chemical changes and protein denaturation during frozen storage. Chilling prior to freezing process would reduce the freezing time, resulting in the better quality of frozen crabs.
2. Modified atmosphere should be applied to frozen soft shell mud crabs to prevent lipid oxidation. The efficiency of this technique should be studied further.
3. Microbial study should be conducted to estimate shelf life of both hard shell and soft shell mud crabs. Furthermore, microbial standard should be established for both hard shell and soft shell mud crabs.

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Appendix 1

Analytical Methods

1. Moisture content (AOAC, 1999)

Method

1. Dry the empty dish and lid in the oven at 105 °C for 30 min and transfer to desiccator to cool (30 min). Weigh the empty dish and lid.
2. Weigh about 5 g of sample to the dish. Spread the sample with spatula.
3. Place the dish with sample in the oven. Dry for 16 h. or overnight at 105 °C
4. After drying, transfer the dish with partially covered lid to the desiccator to cool. Reweigh the dish and its dried content.

Calculation

$$\% \text{ Moisture} = \frac{(W_1 - W_2) \times 100}{W_1}$$

Where: W_1 = weigh (g) of sample before drying

W_2 = weigh (g) of sample after drying

2. Ash (AOAC, 1999)

Method

1. The crucible and lid is firstly placed in the furnace at 550 °C overnight to ensure that impurities on the surface of crucible is burn off. Cool the crucible in the desiccator (30 min).
2. Weigh the crucible and lid to 3 decimal places.

3. Weigh about 5 g sample into crucible. Heat over low bunsen flame with lid half covered. When fumes are no longer produced, place crucible and lid in furnace.
4. Heat at 550 °C overnight. During heating, do not cover the lid. Place the lid on after complete heating to prevent loss of fluffy ash. Cool down in the desiccator.
5. Weigh the ash with crucible and lid until turning to gray. If not, return the crucible and lid to the furnace for further ashing.

Calculation

$$\% \text{ Ash content} = \frac{\text{Weigh of ash} \times 100}{\text{Weigh of sample}}$$

3. Protein (AOAC, 1999)

Reagents

- Kjedahl catalyst: Mix 9 part of potassium sulphate (K_2SO_4) anhydrous, nitrogen free with 1 part of copper sulphate ($CuSO_4$).
- Sulfuric acid (H_2SO_4)
- 40% NaOH solution (w/v)
- 0.02 N HCl solution
- 4% (H_3BO_3) solution (w/v)
- indicator solution: Mix 100 ml of 0.1% methyl red (in 95% ethanol) with 200 ml of 0.2% bromocresol green (in 95% ethanol)

Method

1. Place sample (0.5-1.0 g) in digestion flask.
2. Add 5 g Kjeldahl catalyst, and 200 ml of conc. H_2SO_4.

3. Prepare a tube containing the above chemical except sample as blank. Place flasks in inclined position and heat gently until frothing ceases. Boil briskly until solution clears.
4. Cool and add 60 ml distilled water cautiously.
5. Immediately connect flask to digestion bulb on condenser, and with tip of condenser immersed in standard acid and 5-7 indicator in the receiver. Rotate flask to mix content throughly: then heat until all NH_3 has distilled.
6. Remove receiver, wash tip of condenser, and titrate excess standard acid in distilled with standard NaOH solution.

Calculation

$$\% \text{ Protein} = \frac{(A-B) \times 1.4007 \times 6.25}{W}$$

Where: A = volume (ml) of 0.02 N HCl used sample titration

B = volume (ml) of 0.02 N HCl used blank titration

N = Normality of HCl

W = weigh (g) of sample

14.007 = atomic weigh of nitrogen

6.25 = the protein-nitrogen conversion factor for fish and its by products

4. Biuret method quantitation of protein (Copeland, 1994)

Reagents

- Biuret reagent: combine 1.50 g $CuSO_4.5H_2O$, 6.00 g sodium potassium tartrate, and 500 ml distilled water in a beaker and stir, add while stirring 300 ml of 10% NaOH (w/v), transfer to plastic bottle for storage.
- Distilled water

- Standard reagent: 10 mg/ml bovine serum albumin (BSA)

Method

1. To each of eight disposable cuvette, add the following reagents according to the table.
2. To tubes 6-8, 50 µl of protein sample were added, mix the contents of each tube well by using the closed end of the capillary tube as stirring rod.
3. Add 2.0 ml of the biuret reagent to each tube, and mix well.
4. Incubate the mixture at room temperature for 30-45 min, then read the absorbance of each tube at 540 nm.
5. For tube 1-5, plot the absorbance at 540 nm as a function of effective BSA concentration, and calculate the best fit straight line from data. Then, using the average absorbance for the three sample of unknown read the concentration of sample from the plot.

Table 1-A Experimental set up for the Biuret's assay

Tube number	Water (µl)	10 mg/ml BSA (µl)	Effective BSA Concentration (mg/ml)
1	500	0	0
2	400	100	2
3	300	200	4
4	200	300	6
5	100	400	8
6	450	0	unknown
7	450	0	unknown
8	450	0	unknown

5. Preparation of natural actomyosin (Benjakul *et al.*, 1997)

Reagents

- 0.6 M KCl, pH 7.0
- Distilled water

Method

1. Homogenize 10 g of muscle in 100 ml chilled (4 °C) 0.6 M KCl, pH 7.0 for 4 min.
2. Place the beaker containing the sample in ice. Each 20 sec of blending was followed by a 20 sec rest interval to avoid overheating during extraction.
3. Centrifuge the extract at 5,000 xg for 20 min at 0 °C in an equal volume of chilled 0.6 M KCl, pH 7.0.

6. Lowry's method for protein quantitation (with a slight modified Lowry *et al.*, 1951)

Reagents

- A: 2% Sodium carbonate in 0.1 N NaOH
- B: 0.5% $CuSO_4.5H_2O$ in 1% Sodium citrate
- C: 1 ml Reagent B + 50 ml Reagent A (or similar ratio) (make up immediately before use)

Standard: Bovine serum albumin (BSA) at concentration of 1 mg/ml

Sample Volume: 200 µl

Method

1. Add 2 ml reagent D to each of the standards and unknown tubes. Vortex immediately.
2. Incubate precisely 10 min at room temperature.

3. Add 0.2 ml (200 µl) reagent C (previously diluted 1:1 with distilled water) and vortex immediately.
4. Incubate 30 min at room temperature. (Sample incubated longer than 60 min. Should be discarded).
5. Read absorbance at 750 nm.
6. Plot standard curve and calculate the unknown.

Standards

BSA: 1 mg/ml (used to determine protein concentration)

Tyrosine: 1 mM (used to determine enzymatic activity)

Table 2-A Experimental set up for the Lowry's method

Tube Number	Water (µl)	10 mg/ml BSA (µl)	Effective BSA Concentration (mg/ml)
1	200	0	0
2	180	20	0.1
3	160	40	0.2
4	140	60	0.3
5	100	100	0.5
6	60	140	0.7
7	0	200	1.0

Table 3-A Experimental set up for the Bradford's method

Tube Number	Water (µl)	10 mg/ml BSA (µl)	Effective BSA Concentration (mg/ml)
1	100	0	0
2	90	10	10
3	80	20	20
4	60	40	40
5	40	60	60
6	20	80	80
7	0	100	100

7. Electrophoresis (SDS-PAGE) (Leamli, 1970)

Chemical reagents

- Protein molecular weight standards
- 30% Acrylamide-0.8% bis Acrylamide
- Sample buffer: Mix 30 ml of 10% of SDS, 10 ml of glycerol, 5 ml of ß-Mercaptoethanol, 12.5 ml of 50 mM Tris-HCl, pH 6.8, and 5-10 mg Bromophenol blue (enough to give dark blue color to the solution). Bring the volume to 100 ml with distilled water. Divide into 1 ml aliquots, and stored at -20 °C.
- 2% (w/v) Ammonium persulfate
- 1% (w/v) SDS
- TEMED (*N,N,N',N'*-tetramethylenediamine)
- 0.5 M Tris-HCl, pH 6.8
- 1.5 M Tris-HCl, pH 8.8

- 0.1 M EDTA
- Electrode buffer: Dissolve 3 g of Tris, 14.4 g of glycine and 1 g of SDS in distilled water to 1 liter total volume.
- Staining solution: Dissolve 0.04 g of Coomassie blue R-250 in 100 ml of methanol. Add 15 ml of glacial acetic acid and 85 ml of distilled water.
- Destaining solution I: 50% methanol-7.5% glacial acetic acid
- Destaining solution II: 5% methanol-7.5% glacial acetic acid

Method

Pouring the separating gel:

1. Assemble the minigel apparatus according to the manufacturer's detailed instructions. Make sure that the glass and other components are rigorously clean and dry before assembly.
2. Mix the separating gel solution by adding, as defined in following Table
3. Transfer the separating gel solution using a Pasture pipette to the center of sandwich is ~1.5 to 2 cm from the top of the shorter (front) glass plate.
4. Cover the top of the gel with a layer of isobutyl alcohol by gently squirting the isobutyl alcohol by gently squirting the isobutyl alcohol against the edge of one of the spacers. Allow the resolving gel to polymerize fully (usually 30-60 min).

Pouring the stacking gel:

1. Pour off completely the layer of isobutyl alcohol.
2. Prepare a 4% stacking gel solution by adding as defined in table.
3. Transfer stacking gel solution to tickle into the center of the sandwich along an edge of one of the spacers.

4. Insert a comb into the layer of stacking gel solution by placing one corner of the comb into the gel and slowly lowering the other corner in. Allow the stacking gel solution to polymerize 30 to 45 min at room temperature.

Reagents	10% running gel	4% stacking gel
30% Acrylamide-bis	1.167 ml	0.4 ml
1.5 M Tris-HCl buffer, pH 8.8	0.875 ml	-
0.5 M Tris-HCl buffer, pH 6.8	-	1.0 ml
1% SDS	0.35 ml	0.3 ml
Distilled water	0.758 ml	0.9 ml
0.1 EDTA	-	0.8 ml
2% Ammonium persulfate	0.35 ml	0.4 ml
TEMED	6 µl	5 µl

Sample preparation:

1. Weigh 3 g of sample and homogenize with 5% (w/v) SDS in a final volume of 30 ml.
2. Incubate the mixture at 85 °C for 1 h.
3. Centrifuge at 3,500 x g for 5 min at ambient temperature and collect supernatant.

Loading the gel:

1. Dilute the protein to be 4:1 (v/v) with sample buffer in microcentrifuge tube and boil for 1 min at 100 °C.
2. Remove the comb without tearing the edge of the polyacrylamide wells.
3. Fill the wells with electrode buffer.

4. Place the upper chamber over chamber over the sandwich and lock the upper buffer chamber to the sandwich. Pour electrode buffer into the lower buffer chamber. Place the sandwich attached to the upper buffer chamber into the lower chamber.
5. Fill the upper buffer chamber with electrode buffer so that the sample wells of the stacking gel are filled with buffer.
6. Use a 10-25 µl syringe with a flat-tipped needle, load the protein sample into the wells by carefully applying the sample as a thin layer at the bottom of the well.
7. Fill the remainder of the upper buffer chamber with additional electrode buffer.

Running the gel:

1. Connect the power supply to the anode and cathodes of the gel apparatus and run at 50 V and 150 V.
2. After the bromophenol blue tracking dye has reached the bottom of the separating gel, disconnect the power supply.

Disassembling the gel:

1. Remove the upper buffer chamber and the attached sandwich.
2. Orient the gel so that the order of the sample well is known, remove the sandwich from the upper buffer chamber, and lay the sandwich on a sheet of absorbent paper or paper towels. Carefully slide the spacers out from the edge of the sandwich along its entire length.
3. Insert a spatula between the glass plates apart.
4. Remove the gel from the lower plate. Place the plate with the gel attached into the shallow dish of fixing agent or dye and swishing the plate.

Staining the gel:

1. Place the gel in a small plastic box and cover with the staining solution. Agitate slowly for 1 h or more on a rotary rocker.
2. Pour off the staining solution and cover the gel with a solution of destaining solution I. Agitate slowly for ~15 min.
3. Pour off the destaining solution I and cover the destaining solution II. Discard destaining solution and replace with fresh solution. Repeat until the gel is clear except for the protein bands.

8. Determination of Ca^{2+}-ATPase (Benjakul *et al.*, 1997)

Reagents

1. 0.6 M KCl, pH 7.0
2. 0.5 M Tris-maleate, pH 7.0
3. 0.1 M $CaCl_2$
4. 0.01 M EGTA
5. 15 % Trichloroacetic acid
6. Distilled water
7. 20 mM ATP

Method

1. Pipette 0.5 ml of natural actomyosin solution (NAM) (4 mg/ml in0.6 M KCl, pH 7.0)
2. Add the following solution as shown in the table
3. Incubate for 30 min at 25 °C
4. Stop reaction using 2.5 ml chilled 15 % tricholroacetic acid.
5. Centrifuge at 6,500 rpm for 5 min.
6. Measure inorganic phosphate in the supernatant.
7. Blank:

- NAM + Tris maleate + water (500+300+3950 µl)
- Add 2.5 ml, 15 % TCA
- Add 250 µl, 20 mM ATP
- Measure inorganic phosphate

Solution	Determination
	Ca^{2+}-ATPase
1. 0.5 M Tris-maleate, pH 7.0 (keep in 4 °C)	300
2. 0.1 M $CaCl_2$	500
3. 0.01 M EGTA	0
4. NAM (1.5-3.0 mg/ml)	500
5. Distilled water	3450
6. 20 mM ATP	250

9. Determination of total sulfhydryl content (Benjakul *et al.*, 1997)

Reagents

1. 0.1 % 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB)
2. 0.2 M Tris-HCl buffer, pH 6.8 (containing 8 M urea, 2 % SDS, and 10 mM EDTA)

Method

1. Mix actomysin (1 ml, 4 mg/ml) with 9 ml of 0.2 M Tris-HCl.
2. Take 4 ml-aliquot of the mixture and add with 0.4 ml of 0.1 % DTNB solution.
3. Measure the absorbance at 412 nm with spectrophotometer.
4. Prepare a blank by replacing the sample with 0.6 M KCl, pH 7.0.

5. Calculate SH content from the absorbance using the molar extinction coefficient of 13,600 $M^{-1} cm^{-1}$ and express as mol/10^5 g protein.

10. Determination of disulfide bond (Thannhauser *et al.*, 1987)

Reagents

1. 5-5'-dithiobis-2-nitrobenzoic acid (Eliman's reagent)
2. Na_2SO_3
3. Glycine
4. Sodium sulfite
5. EDTA

NTSB preparation:

1. Dissolve 100 mg of Eliman's reagent (0.235 mmol) in 10 ml of 1 M Na_2SO_3.
2. Adjusted pH to 7.5.
3. Incubate the bright red solution and at 38 °C, and bubble the oxygen through it with a gas dispersion tube until the color turns to be yellow.
4. Store this stock solution for up to 1 year at -20 °C .

NTSB assay solution:

The NTSB assay solution is prepared from the stock solution by diluting it 1:100 with a freshly prepared solution that is 2 M in guanidine thiocyanate, 50 mM in glycine, 100 mM in sodium sulfite, and 3 mM EDTA. The pH should be adjusted to 9.5. The assay solution is used directly to measure disulfide bond concentrations and is stable for up to 2 weeks when stored at room temperature.

Method

1. Add 10-200 µl of protein solution into 3 ml of the NTSB assay solution.

2. Incubate the reaction mixture in the dark for 25 min.
3. Measure the absorbance at 412 nm against a blank.
4. Calculate disulfide bond concentration from the absorbance using the molar extinction coefficient of 13,900 M^{-1} cm^{-1} and express as mol/10^6 g protein.

11. Determination of surface hydrophobicity (Benjakul *et al.*, 2000)

Reagents

1. 10 mM phosphate buffer, pH 6.0 containing 0.6 M NaCl
2. 8 mM 1-anilinonaphhalene-8-sulphonic acid (ANS) in 0.1 M phosphate buffer, pH 7.0

Method

1. Prepare actomyosin in 10 mM phosphate buffer, pH 6.0 containing 0.6 M NaCl and dilute to 0.1, 0.2, 0.3, 0.5, 0.9 mg/ml protein using the same buffer.
2. Incubate the diluted protein (2ml) at 20°C for 10 min.
3. Add 10 µl of 8 mM ANS in 0.1 M phosphate buffer, pH 7.0.
4. Measure the fluorescence intensity of ANS-protein conjugates using an spectrophotometer at excitation wavelength 374 nm and emission wavelength 485 nm.

Calculation

Protein hydrophobicity was calculated from initial slopes of plots of relative fluorescence intensity vs. protein concentration using linear regression analysis. The initial slope was referred to as SoANS.

12. Determination of solubility in 0.6 M KCl (Benjakul and Bauer, 2000)

Reagents

1. 0.6 M KCl, pH 7.0
2. 50 % Trichloroacetic acid
3. 100 % Trichloroacetic acid
4. 0.5 M NaOH

Method

1. Weigh 2 g sample.
2. Add 18 ml of 0.6 M KCl (pH 7.0) and homogenize for 30 s.
3. Stir the homogenate at room temperature (25-27°C) for 4 h, and centrifuge at 12,000 x g for 20 min at 4 °C.
4. To 10 ml of the supernatant, and cold 50 % (w/v) trichloroacetic acid to obtain the final concentration of 10%.
5. Wash the precipitate with 10 % Trichloroacetic acid and solubilize in 0.5 M NaOH.
6. Determine the protein content using the Biuret method.

13. pH determination (Benjakul *et al.*, 1997)

Method

1. Weigh 5 g of sample
2. Add 10 volumes of distilled water (w/v).
3. Homogenize for 2 min.
4. Measure pH using pH meter.

14. Salt (Chloride as Sodium Chloride) (AOAC, 1999)

Reagents

1. 0.1 N Silver nitrate standard solution
2. 0.1 N Ammonium thiocyanate standard solution
3. Ferric indicator: Saturated solution of $FeNH_4(SO_4)_2.H_2O$

Method

1. Weigh 10 g of sample into 250 ml erlenmeyer or beaker.
2. Add known volume of $AgNO_3$ solution, more than enough to prepitate all Cl as AgCl, and then add 20 ml HNO_3. Boil gently on hot pate or sand bath until all solids except AgCl are dissolved (usually 15 min). Cool and add 50 ml indicator, and titrate with 0.1 N NH_4SCN solution until solution becomes permanent light brown. Subtract volume (ml) of 0.1 N NH_4SCN used from volume (ml) of 0.1 NA gNO_3 = 0.058 % NaCl.

15. Determination of formaldehyde (Nash, 1953)

Reagents

1. Acetylacetone reagent (Nash,s reagent): Dissolve 150 g of ammonium acetate, 3 ml of acetic acid and 2 ml of acetylacetone in distilled water and make up to 1 litre.
2. Formaldehyde standard stock solution: Pipette 0.3 ml of 35% formaldehyde and fill up to 100 ml to get approximately 1,000 mg/kg solution in distilled water. This aqueous solution is stable for several months.
3. Formaldehyde standard stock solution: Dilute the stock solution 100 times as follows: Pipette 10 ml of the stock solution (approximately 1,000 mg/kg) and make up to 100 ml with distilled water to get

apprx. 100 mg/kg solution. Ten ml of 100 mg/kg solution is diluted 10 time with distilled water in the volumetric flask. This final dilute gives approx. 10 mg/kg solution of formaldehyde. This dilute is not stable, so it is necessary to be renewed in each series of determination.

4. 0.1 N Sodium thiosulfate standard solution: Dissolve 25 g of $Na_2S_2O_3.H_2O$ in distilled water which is cooled after boiling, and make up to 1 litre. Standardize after standing 1 to 2 days.
5. Sodium bisulfite solution (approximately 0.1 N): Dissolve 5.2 g of Na_2SHO_3 in distilled water and make up to 1 litre.
6. Iodine solution (approximately 0.1 N): Dissolve 12.7 g of I_2 and 40 g of KI in 25 ml of distilled water and make up to 1 liter.
7. 1.5 % Starch solution: Weigh 1.5 g of starch and add 100 ml of distilled water, then boil the solution for 30 sec.

Method

Sample preparation:

1. Weigh 5 g mince meat accurately in a 30-50 ml beaker.
2. Add 20 ml of 5% TCA solution and homogenize well with homogenizer.
3. Stand in an ambient temperature for 30 min.
4. Filter the supernatant with filter paper, Whatman No. 41.
5. Add 10 ml of 5% TCA solution to the residue, homogenize again, then filter.
6. Neutralize the combined filtrate to pH 6.0-6.5 by using pH meter with 1 N or 0.1 N KOH dropwise, and make up to 50 ml with distilled water.
7. Mix 3 ml of the solution with 3 ml acetylacetone reagent.

8. Stand in water bath (60 °C) for 15 min.
9. Cool the solution in running water.
10. Measure the absorbance of the solution against the blank solution at 412 nm (Blank solution contains distilled water instead of the neutralized filtrate).

Calculation of formaldehyde content

$$\text{Formaldehyde } (\mu g/g) = \frac{A \times (\text{Total make up vol. Of filtrate})}{(\text{vol. Of filtrate used}) \times (\text{Weigh of sample})}$$

Where: A = Reading from calibration curve (μg)

Appendix 2

Analysis of variance

Table 1-B Analysis of variance for Logarithm of Ca^{2+}-ATPase activities of NAM from hard and soft shell mud crab as a function of incubation time at 40 °C

SV	DF	SS	MS	F
Treatment	2	3.25	3.25	201618.12**
Muscle	5	117.28	23.46	1453552.67**
Crab* Muscle	10	7.92	.79	49065.37**
Error	36	5.809E-04	1.614E-05	
Total	53	131.70		

** Significant at 1% level

Table 2-B Analysis of variance for effect of inhibitors on the sarcoplasmic proteinases activity

SV	DF	SS	MS	F
Crab	1	829.29	829.29	133.13**
Muscle	1	544.03	544.03	87.34**
Crab* Muscle	1	4550.79	4550.79	730.56**
Error	8	49.83	6.23	
Total	11	5973.94		

** Significant at 1% level

Table 3-B Analysis of variance for temperature profiles of sarcoplasmic proteinases from hard and soft shell crab muscles.

Samples	SV	DF	SS	MS	F
Hard shell crab	pH	2	6702.23	3351.12	31265.72**
chunk	Temp	10	12014.64	1201.47	11209.59**
	pH* Temp.	20	8621.90	431.10	4022.09**
	Error	66	7.07	.11	
	Total	98	27345.85		
Hard shell crab	pH	2	553.71	276.86	29.82**
claw	Temp	10	1012.82	101.28	10.91**
	pH* Temp.	20	1000.96	50.05	5.3**
	Error	66	612.77	9.28	
	Total	98	3180.26		
soft shell crab	pH	2	596.41	298.21	4223.26**
chunk	Temp	10	2218.04	3141.26	3141.25**
	pH* Temp.	20	2680.72	134.04	1898.26**
	Error	66	4.66	7.061E-02	
	Total	98	14855.53		
Soft shell crab	pH	2	583.76	291.88	2602.73**
claw	Muscle	10	1635.05	165.31	1474.05**
	pH* Muscle	20	1167.10	58.36	520.36**
	Error	66	7.40	.112	
	Total	98	3411.31		

** Significant at 1% level

Table 4-B Analysis of variance for pH profiles of sarcoplasmic proteinases from hard and soft shell crab muscles.

Samples	SV	DF	SS	MS	F
Hard shell crab chunk	pH	11	33862.96	3078.45	1033.27**
	Muscle	1	23122.11	23122.11	7760.84**
	pH* Muscle	10	10740.85	1074.09	360.51**
	Error	42	125.13	2.98	
	Total	53	33988.09		
Hard shell crab claw	pH	11	3391.99	308.36	69.38**
	Muscle	1	2465.44	2465.44	554.73**
	pH* Muscle	10	926.55	92.66	20.85**
	Error	42	186.67	4.44	
	Total	53	3578.65		
soft shell crab chunk	pH	11	2929.40	2929.40	35.77**
	Muscle	1	333.43	333.43	44.78**
	pH* Muscle	10	2595.96	259.60	34.86**
	Error	24	178.781	7.45	
	Total	35	3108.10		
Soft shell crab claw	pH	11	1356.81	123.35	65.63**
	Muscle	1	423.86	423.86	225.51**
	pH* Muscle	10	932.96	93.30	49.64**
	Error	24	45.11	1.88	
	Total	35	1401.92		

** Significant at 1% level

Table 5-B Analysis of variance for changes in physico-chemical properties of NAM from hard and soft shell mud crabs during frozen storage at -20 °C for 12 weeks.

Properties	SV	DF	SS	MS	F
Ca^{2+}-ATPase	Crab	1	194E-04	1.894E-04	67.09**
	Muscle	1	1.441E-03	1.441E-03	510.20**
	Week	7	5.432E-03	7.759E-04	274.73**
	Crab* Muscle	0	.000		
	Crab* Week	7	6.305E-05	9.007E-06	3.19**
	Muscle*Week	7	5.067E-04	7.238E-05	25.63**
	Crab* Muscle *Week	0	.000		
	Error	48	1.355E-04	2.8245E-06	
	Total	71	7.545E-03		
Cooking loss	Crab	1	5020.23	5020.23	1198.933**
	Muscle	1	10356.133	5020.23	2473.25**
	Week	7	1015.999	145.14	34.66**
	Crab* Muscle	1	4695.82	4695.82	1121.46**
	Crab* Week	7	9.43	1.347	$.322^{ns}$
	Muscle*Week	7	335.88	47.98	11.46**
	Crab* Muscle *Week	7	7.60	1.09	$.26^{ns}$
	Error	64	267.98	4.19	
	Total	95	21709.07		

Properties	SV	DF	SS	MS	F
Disulfide bond	Crab	1	1.934	1.934	10.88**
	Muscle	1	3.71	3.71	20.85**
	Week	7	128.39	18.34	103.17**
	Crab* Muscle	0	.000		
	Crab* Week	7	3.73	.533	2.999**
	Muscle*Week	7	3.13	.49	2.52**
	Crab* Muscle *Week	0	.000		
	Error	48	8.53	.18	
	Total	71	165.73		
Formaldehyde	Crab	1	1.59	3.71	101.93**
	Muscle	1	40.54	40.54	2599.25**
	Week	7	64.83	9.26	593.85**
	Crab* Muscle	1	5.449E-03	5.449E-03	.349**
	Crab* Week	7	.22	3.136E-02	2.011**
	Muscle*Week	7	5.71	.82	52.28**
	Crab* Muscle *Week	7	.109	1.551E-02	.995**
	Error	64	.998	1.560E-02	
	Total	95	113.998		

Properties	SV	DF	SS	MS	F
Hydrophobicity	Crab	1	46.09	46.09	4.39**
	Muscle	1	369.87	369.87	35.25**
	Week	7	3810.73	544.39	51.89**
	Crab* Muscle	0	.000		
	Crab* Week	7	89.18	12.74	1.21**
	Muscle*Week	7	1278.5	182.66	17.41**
	Crab* Muscle *Week	0	.000		
	Error	48	503.61	10.49	
	Total	71	304855.46		
pH	Crab	1	.25	.25	1905.15**
	Muscle	1	6.03	6.03	46310.69**
	Week	7	4.17	.60	4576.64**
	Crab* Muscle	1	8.83	8.83	67837.95**
	Crab* Week	7	.627	8.953E-02	687.60**
	Muscle*Week	7	.33	4.666E-02	358.38**
	Crab* Muscle *Week	7	.13	1.780E-02	136.72**
	Error	64	8.333E-03	1.3023E-04	
	Total	95	20.37		

Properties	SV	DF	SS	MS	F
Solubility	Crab	1	569.88	569.88	56.68**
	Muscle	1	1018.61	1018.61	101.30**
	Week	7	9925.94	1417.99	141.02**
	Crab* Muscle	1	2.28	2.28	.23^{ns}
	Crab* Week	7	462.28	66.04	6.57**
	Muscle*Week	7	59.79	8.54	.85^{ns}
	Crab* Muscle *Week	7	130.17	18.60	1.85**
	Error	64	643.54	10.06	
	Total	95	12812.48		
Sulfhydryl group	Crab	1	4.43	4.43	18.07**
	Muscle	1	.55	.55	2.24**
	Week	7	265.36	37.91	154.74**
	Crab* Muscle	0	.000		
	Crab* Week	7	5.12	.75	2.99**
	Muscle*Week	7	2.77	.40	1.61**
	Crab* Muscle *Week	0	.000		
	Error	48	11.76	.25	
	Total	71	326.59		

Properties	SV	DF	SS	MS	F
TBARS	Crab	1	.29	.29	6.80**
	Muscle	1	1.265E-02	1.265E-02	.295 ns
	Week	7	5.13	.74	17.16**
	Crab* Muscle	1	.14	.14	3.22**
	Crab* Week	7	.59	8.487E-02	1.98**
	Muscle*Week	7	.58	8.250E-02	1.92**
	Crab* Muscle *Week	7	.11	1.627E-02	.38 ns
	Error	64	2.75	4.289E-02	
	Total	95	9.63		

** Significant at 1% level

ns Not significant (P>0.05)

Table 6-B Analysis of variance for effect of freezing and thawing on physico-chemical changes in hard and soft shell mud crab muscles

Properties	SV	DF	SS	MS	F
Ca^{2+}-ATPase	Crab	1	1371E-04	1371E-04	13.91**
	Muscle	1	3.961E-04	3.961E-04	40.19**
	Cycle	4	1.215E-03	3.037E-04	30.81**
	Crab* Muscle	0	.000		
	Crab* Cycle	4	5.212E-05	1.303E-05	1.32**
	Muscle*Cycle	4	1.054E-04	2.635E-05	2.67*
	Crab* Muscle *Cycle	0	.000		
	Error	30	2.957E-04	9.857E-06	
	Total	44	1.938E-03		
Cooking loss	Crab	1	6036.78	6036.78	478.59**
	Muscle	1	6036.78	6582.88	1037.44**
	Cycle	4	2469.63	617.41	97.30**
	Crab* Muscle	1	6036.78	2118.09	333.80**
	Crab* Cycle	4	7.15	1.79	$.28^{ns}$
	Muscle*Cycle	4	365.51	91.38	14.40**
	Crab* Muscle *Cycle	4	38.40	9.60	1.51**
	Error	40	253.81	6.35	
	Total	59	14872.26		

Properties	SV	DF	SS	MS	F
Disulfide bond	Crab	1	5.92	5.92	117.17**
	Muscle	1	7.43	7.43	155.12**
	Cycle	4	31.97	7.99	167.54**
	Crab* Muscle	0	.000		
	Crab* Cycle	4	.56	.14	2.95**
	Muscle*Cycle	4	1.80	.45	9.45**
	Crab* Muscle *Cycle	0	.000		
	Error	30	1.43	4.772E-02	
	Total	44	47.54		
Formaldehyde	Crab	1	40.85	40.85	433.98**
	Muscle	1	5.56	5.56	59.06**
	Cycle	4	384.41	96.10	1020.94**
	Crab* Muscle	1	37.16	37.16	394.81**
	Crab* Cycle	4	35.44	8.86	94.12**
	Muscle*Cycle	4	109.47	27.37	290.74**
	Crab* Muscle *Cycle	4	37.23	9.31	98.87**
	Error	40	3.77	9.413E-02	
	Total	59	653.88		

Properties	SV	DF	SS	MS	F
Hydrophobicity	Crab	1	44.55	44.55	4.35**
	Muscle	1	870.94	870.94	85.10**
	Cycle	4	1534.84	383.71	37.49**
	Crab* Muscle	0	.000		
	Crab* Cycle	4	171.40	42.85	4.19**
	Muscle*Cycle	4	1565.33	391.33	38.24**
	Crab* Muscle *Cycle	0	.000		
	Error	30	307.05	10.24	
	Total	44	4814.50		
pH	Crab	1	2.56	2.56	9303.76**
	Muscle	1	9.23	9.23	33555.21**
	Cycle	4	.19	4.769E-02	173.43**
	Crab* Muscle	1	7.05	7.05	25643.93**
	Crab* Cycle	4	7.042E-02	1.761E-02	64.02**
	Muscle*Cycle	4	5.554E-02	1.389E-02	50.50**
	Crab* Muscle *Cycle	4	8.878E-02	2.219E-02	80.71**
	Error	40	1.100E-02	2.750E-04	
	Total	59	19.26		

Properties	SV	DF	SS	MS	F
Solubility	Crab	1	457.68	457.68	16.20**
	Muscle	1	158.78	158.78	5.64**
	Cycle	4	8796.61	219.15	78.13**
	Crab* Muscle	1	399.52	399.52	14.12**
	Crab* Cycle	4	36.95	9.24	.33 ^{ns}
	Muscle*Cycle	4	10.22	2.56	.09 ^{ns}
	Crab* Muscle *Cycle	4	205.31	51.33	1.82**
	Error	20	562.96	28.15	
	Total	39	10628.03		
Sulfhydryl group	Crab	1	8.02	8.02	283.48**
	Muscle	1	48.04	48.04	1697.98**
	Cycle	4	348.93	87.23	3083.54**
	Crab* Muscle	0	.000		
	Crab* Cycle	4	1.34	.34	11.84**
	Muscle*Cycle	4	7.51	1.88	66.35**
	Crab* Muscle *Cycle	0	.000		
	Error	30	.85	2.829E-02	
	Total	44	410.54		

Properties	SV	DF	SS	MS	F
TBARS	Crab	1	.50	.50	24.43**
	Muscle	1	.76	.76	36.87**
	Cycle	4	12.04	3.01	146.41**
	Crab* Muscle	1	.38	.38	18.45**
	Crab* Cycle	4	.20	4.885E-02	2.38**
	Muscle*Cycle	4	1.26	.314	15.28**
	Crab* Muscle *Cycle	4	1.20	.300	14.59**
	Error	40	.82	2.055E-02	
	Total	59	17.15		

** Significant at 1% level

ns Not significant (P>0.05)

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