Physicochemical Changes of Seabass (*Lates calcarifer*) Muscle Proteins during Iced and Frozen Storage

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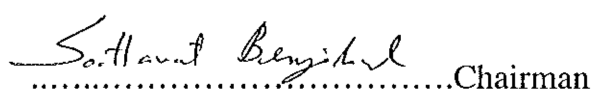
..............................Chairman	 ....Chairman
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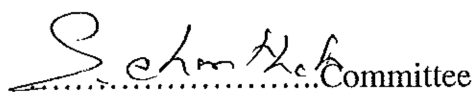
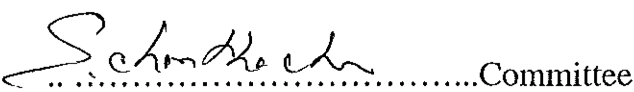
 ....Committee	 ....Committee
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	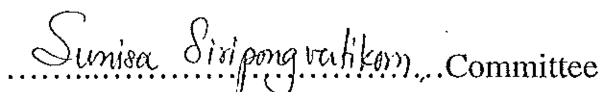 ....Committee
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	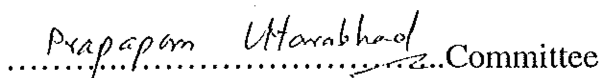 ....Committee
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ชื่อวิทยานิพนธ์	การเปลี่ยนแปลงเชิงเคมี-กายภาพของโปรตีนกล้ามเนื้อปลากะพงขาว (*Lates calcarifer*) ระหว่างการเก็บรักษาในน้ำแข็งและการแช่แข็ง
ผู้เขียน	นางสาวศิริพร เรียบร้อย
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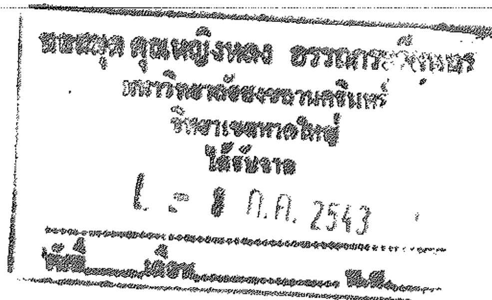

บทคัดย่อ

จากการศึกษาองค์ประกอบทางเคมีและความคงตัวของโปรตีนกล้ามเนื้อปลากะพงขาว (*Lates calcarifer*) พบว่า องค์ประกอบไนโตรเจนและกรดอะมิโนจากกล้ามเนื้อส่วนหลังและกล้ามเนื้อส่วนท้องมีลักษณะใกล้เคียงกัน เมื่อทำการทดสอบความคงตัวของสารสกัดแอกโตไมโอซินจากกล้ามเนื้อปลากะพงขาว พบว่า inactivation rate constant (K_D) ของเอนไซม์ Ca^{2+}-ATPase มีความคงตัวที่อุณหภูมิ 0-20 องศาเซลเซียส และพีเอช 6.5-7.5

จากการศึกษาการเปลี่ยนแปลงเชิงเคมี-กายภาพของแอกโตไมโอซินในรูปอิสระและรูปที่จับกับกล้ามเนื้อปลากะพงขาวระหว่างการเก็บรักษาในน้ำแข็งเป็นเวลา 10 วัน พบว่า ปริมาณหมู่ซัลไฮดริลของสารสกัดแอกโตไมโอซินลดลง อย่างไรก็ตามไม่มีการเปลี่ยนแปลงของปริมาณหมู่ซัลไฮดริลในสารสกัดแอกโตไมโอซินที่เติมสารประกอบโซเดียมโดเดซิลซัลเฟต (30 ไมโครโมล/กรัม โปรตีน) ยูเรีย (30 ไมโครโมล/กรัม โปรตีน) และเบต้า-เมอแคปโตเอธานอล (30 ไมโครโมล/กรัม โปรตีน) เมื่อระยะเวลาการเก็บรักษาเพิ่มขึ้นไม่มีการเปลี่ยนแปลงของกิจกรรมเอนไซม์ Mg^{2+}-ATPase และ Mg^{2+}-Ca^{2+}-ATPase ในแอกโตไมโอซินทั้งชุดควบคุมและชุดที่เติมสารประกอบชนิดต่าง ๆ แต่พบว่ากิจกรรมของเอนไซม์ Ca^{2+}-ATPase ลดลง และ กิจกรรมของเอนไซม์ Mg^{2+}-EGTA-ATPase เพิ่มขึ้น รวมทั้งมีการสูญเสีย Ca^{2+}-sensitivity ระหว่างการเก็บรักษา เมื่อศึกษาการเก็บรักษากล้ามเนื้อปลากะพงขาวในน้ำแข็งพบว่ามีการเปลี่ยนแปลงเชิงเคมี-กายภาพของแอกโตไมโอซินเล็กน้อย แต่กล้ามเนื้อปลากะพงขาวเกิดการย่อยสลายของ

โปรตีน กิจกรรมของเอนไซม์ Ca^{2+}-ATPase ลดลง ขณะที่กิจกรรมของเอนไซม์ Mg^{2+}-EGTA-ATPase เพิ่มขึ้น หลังจากการเก็บรักษาในน้ำแข็งเป็นระยะเวลา 10 วัน ($p<0.05$) แอกโตไมโอซินอิสระสามารถสูญเสียสภาพเร็วกว่าแอกโตไมโอซินที่จับกล้ามเนื้อปลากะพงขาวระหว่างการเก็บรักษาในน้ำแข็ง

ในระหว่างการเก็บรักษาภายใต้สภาวะแช่แข็งแอกโตไมโอซินอิสระและแอกโตไมโอซินที่จับกับกล้ามเนื้อปลากะพงขาวมีกิจกรรมของเอนไซม์ Ca^{2+}-ATPase และปริมาณหมู่ซัลไฮดริลลดลงหลังการเก็บรักษาที่อุณหภูมิ –18 องศาเซลเซียส เป็นระยะเวลา 4.5 เดือน ($p<0.05$) และพบว่าเกิดการเปลี่ยนแปลงเล็กน้อยในตัวอย่างที่ทำการเก็บรักษาที่อุณหภูมิ –80 องศาเซลเซียส นอกจากนี้เมื่อทำการเปรียบเทียบการทำละลายที่สภาวะแตกต่างกันภายหลังการเก็บที่อุณภูมิ –18 องศาเซลเซียส และ –80 องศาเซลเซียส พบว่า การทำละลายอย่างช้า ส่งผลให้กิจกรรมของเอนไซม์ α-glucosidase (AG) และ β-*N*-acetylglucosaminidase (NAG) เพิ่มขึ้นในอัตราที่สูงกว่าการทำละลายอย่างรวดเร็ว ซึ่งบ่งชี้ถึงการรั่วของไลโซโซมอันเกิดจากการแช่แข็งและการทำละลายไม่เหมาะสม นอกจากนี้โปรตีนกล้ามเนื้อมีการเปลี่ยนแปลงเชิงเคมี-กายภาพเพิ่มขึ้น เมื่อจำนวนรอบของการแช่แข็ง-ทำละลายเพิ่มขึ้น ดังนั้นการแช่แข็งและทำละลายอย่างรวดเร็ว โดยหลีกเลี่ยงการแช่แข็ง-ทำละลายซ้ำ จึงเป็นวิธีการในการรักษาโปรตีนจากกล้ามเนื้อปลากะพงขาว

Thesis Title	Physicochemical Changes of Seabass (*Lates calcarifer*) Muscle Proteins during Iced and Frozen Storage
Author	Miss Siriporn Riebroy
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Abstract

Compositions and properties of seabass (*Lates calcarifer*) muscle proteins were studied. The dorsal and ventral muscle had the similar nitrogenous compositions and amino acid profile. Base on the inactivation rate constant (K_D) of Ca^{2+}-ATPase, seabass actomyosin was stable at 0-20°C and pH 6.5-7.5.

Physicochemical changes of free and intact actomyosin from seabass muscle during 10 days of iced storage were investigated in the absence and presence of various chemical reagents. The sulhydryl content in actomyosin solution decreased significantly ($p<0.05$) throughout the storage. However, no changes in sulhydryl content were obtained in actomyosin solution added with sodium dodecyl sulfate (30 μmol/g protein), urea (30 μmol/g protein), or β-mercaptoethanol (30 μmol/g protein) ($p>0.05$). No changes in Mg^{2+}, or Mg^{2+}-Ca^{2+}-ATPase activities of actomyosin were found in the absence or presence of chemical reagents ($p>0.05$). Nevertheless, a significant decrease in Ca^{2+}-ATPase activity and a significant increase in Mg^{2+}-EGTA-ATPase activity were observed ($p<0.05$) with a loss of Ca^{2+}-sensitivity when the storage time increased. For sea bass muscle kept in ice, slight physicochemical changes in actomyosin were observed, though the muscle underwent degradation as well as decomposition. However, Ca^{2+}-ATPase activity decreased, whereas the Mg^{2+}-EGTA-ATPase activity increased significantly after 10 days of storage (p

<0.05). The results suggest that free actomyosin undergoes denaturation to higher extent, compared to the intact actomyosin.

During frozen storage, the significant decrease in Ca^{2+}-ATPase activity and sulhydryl content in free and intact actomyosin was obtained, especially after 4.5 months of storage at -18°C ($p<0.05$). However less changes were found in the samples stored at -80°C. Lysosomal α-glucosidase (AG) and β-*N*-acetylglucosaminidase (NAG) activities increased significantly ($p<0.05$) to higher extent with the slow freezing and thawing rate. This suggests the leakage of lysosome caused by improper freezing and thawing. Moreover, muscle proteins were prone to physicochemical changes as well as cell damages as freeze-thaw cycles increased. Therefore, rapid freeze-thawing without repetition is a means to preserve seabass muscle proteins.

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List of Abbreviation

^{o}C	=	degree Celsius
CFU	=	colony forming unit
CFU/g muscle	=	colony forming unit per gram muscle
g	=	gram
x g	=	gravitational force
hr	=	hour
l	=	litre
mg/ml	=	milligram per millilitre
min	=	minute
ml	=	millilitre
nm	=	nanometre
µmol	=	micromole
p	=	pico (10^{-12})

Chapter 1

Introduction

After harvested, fish undergoes deterioration via microbial and chemical reactions. Protein is one of major constituents, which is prone to deterioration and changes. Fish proteins can be degraded by endogenous proteases. Protein denaturation can also be occurred during handling and cold storage (Haard *et al.*,1994). Those proteins, particularly myofibrillar proteins, undergo aggregation during frozen storage, resulting in the quality loss. Due to those changes of proteins, the fish muscle will loss the functional properties, eating quality as well as acceptability (Mackie, 1993; Jiang *et al.*,1988). Study on physicochemical changes to find a means to prevent the loss in fish quality should be taken into consideration.

Seabass, also called giant sea perch, is an economically important fish in many countries in tropical areas of western Pacific and Indian ocean (Chomdej, 1986). In Thailand, a lot of seabass farms are located, particularly in the south. Seabass is generally available and sold in the local market. Moreover, it has been sold in the south east market such as Singapore and Malaysia. Normally, it is transported in ice or as a frozen fish (Kritayanawat, 1992). However, few information about the changes in physicochemical and biochemical properties during handling and transportation of seabass has been reported.

Literature Review

Seabass

Seabass (*Lates calcarifer*), also called pla kapong in Thailand, kakap in Indonesia, siakap in Malaysia and Singapore, apahap in the Philippines, and barramundi in Australia and Papua New Guinea, has many characteristics favorable to coastal aquaculture (Chomdej, 1986).

The body of seabass is oblong-elongated, compressed with a deep caudal peducle. Head is pointed with concave dorsal profile becoming convex in front of dorsal fin. Mouth is large with slightly oblique lower jaw. Upper jaw reachs to behind eye. Lower edge of pre-operculum has strong spine, while the operculum has a small spine with a serrated flap above the origin of lateral line. Dorsal fin of seabass contains 7-9 spines with 10-11 soft rays and a very deep notch dividing spine from soft part of fin. The anal fin is round with three spines and 7-8 soft rays. Scales are large and ctenoid. Adult has olive brown color with a silvery belly. During juvenile stage, fish has 3-4 black transverse bars which disappear when the fish grow to adult (Sirimontaporn, 1989).

Chemical compositions of fish

As expected for the flesh fish, the main constituents of flesh are water, protein and fat. The relative amounts of these components are generally within the range found in mammals (Mackie, 1994). Stanby (1963) reported that fish generally contain 74.8% water, 19.0% protein and 1.0% fat.

Protein is a major composition of fish muscle. Protein content is in the range of 15-20% (wet weight), but protein content is reduced in spawning period (Almas, 1981). Protein compositions of fish vary, depending on muscle type, feeding period, and spawning, etc.

Hashimoto *et al.*(1979) found that white muscle from sardine (*Sardinops melanosticta*) contained a larger amount of myofibrillar protein than dark muscle (Table 1).

Table 1. Protein compositions of sardine (mgN/g muscle)

Compositions	Muscle type	
	Dark muscle	White muscle
Non-protein nitrogenous compound	3.22	4.95
Sarcoplasmic	7.01	10.33
Myofibrillar	15.10	17.55
Alkali-soluble	1.55	1.35
Stroma	0.55	4.95

Source : Hashimoto *et al.* (1979)

Fish protein composition can be divided into 3 main groups as follows :

1. Myofibrillar protein

Myofibrillar proteins are the major proteins in fish muscle. Due to their association with the textural properties of flesh, these proteins have received a greatest attention. Normally, these proteins account for 65-75% of total protein in muscle, compared with 52-56% in mammals (Table2). However, very different

arrangement of cells is found in fish muscle (Figure1). Instead of being connected to tendons, the cells are bound together and parallel to one another, to form the segments or myotomes of muscle.

Table 2. Skelatal muscle proteins in the flesh of fish and mammals.

Protein groups	% composition	
	Fish	Mammals
Sarcoplasmic	20-35	30-35
Myofibrillar	65-75	52-56
Connective tissue	3-10	10-15

Source : Mackie (1994)

These blocks of muscle cells are shaped like the letter "W" and are oreinted across the midplane of the fish with their central part directed toward the head of the fish. The end of the cells are attached to sheets of connective tissue called "mycommata" which separate one block of cells or myotomes from another (Mackie, 1994).

Myofibrillar protein can be further divided into three subgroups as follows :

- Myosin

Myosin is a large fibrous protein with a molecular weight of about 500,000 daltons (Ogawa *et al.*, 1994). It is the most abundant myofibrillar component, constituting approximately 40-60% of total protein content (Bechtel, 1986).

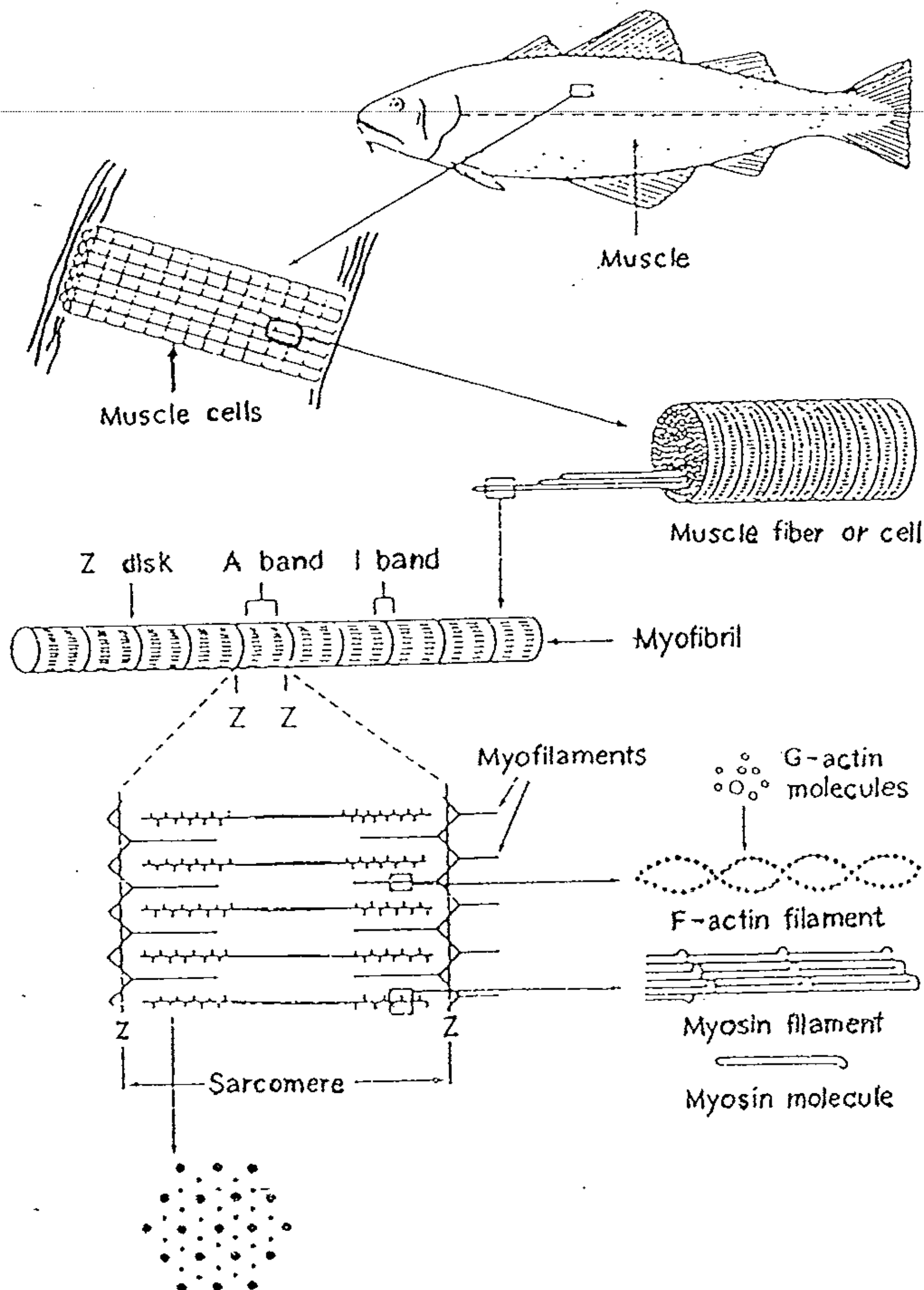


Figure 1. Fish muscle cell arrangement.

Source : Mackie (1993)

Myosin consists of six polypeptide subunits, two large heavy

chains and four light chains arranged into an asymmetrical molecule with two pear-shaped globular heads attached to long α-helical rodlike tail (Xiong, 1997) (Figure 2). The molecular structure of myosin comprises two globular heads (S-1s) and a double-stranded α-helical rod. The S-1 globular heads in myosin have ATPase activity and actin binding ability. The α-helical rod forms a filament (Ogawa *et al.*, 1994). The myosin molecule has a relatively high content of α-helix. The domain structure is S-1, S-2 and LMM (Light meromyosin). For fish myosin, the rod was found to be as labile as S-1 (Wright and Wilding, 1984).

The myosin molecule has a relatively high content of α-helix. The 73% of the total helicity of myosin is occupied by α-helicity of the rod (Ogawa *et al.*, 1993) (Table 3).

Table 3. The α-helical content of myosin and the fragment

Proteins	α-Helical content(%)
Myosin	-
S-1	24
Rod	73

Source : Ogawa *et al.* (1993)

- Actin

Actin is the second most abundant myofibrillar protein, constituting about 22% of myofibrillar mass. This protein

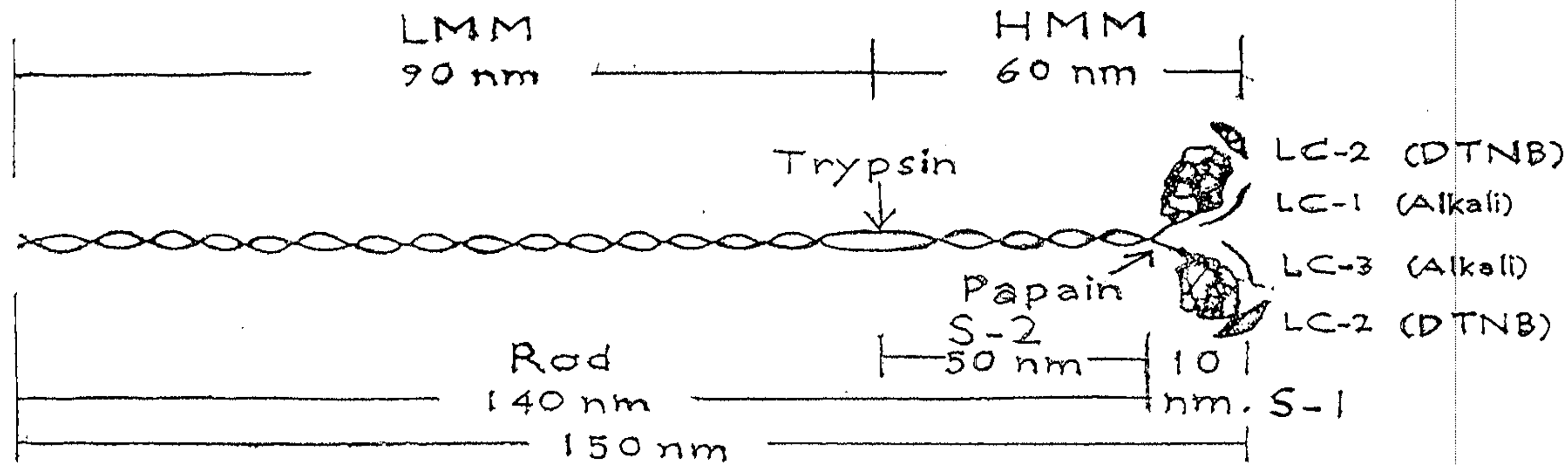
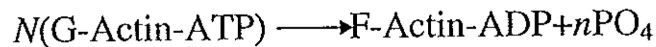


Figure 2 : Schematic representation of the myosin molecule. Light meromyosin(LMM), heavy meromyosin(HMM), rod, S-1 and S-2 subfragments of HMM, the light chains, and the hinge regions susceptible to trypsin and papain are indicated.

Source : Xiong (1997).

has a molecular weight of 42,000 daltons. In muscle tissue, actin is naturally associated with tropomyosin and the troponin complex. It also contains a myosin binding site, which allows myosin to form temporary complex with it during muscle contraction or the permanent myosin-actin complex during rigor mortis development in postmortem (Xiong, 1997).

When actin is in its monomer form, it is called G actin. Actin molecules polymerize together, forming actin filaments referred to as F actin (Bechtel, 1986).



$$N(\text{G-Actin-ATP}) \longrightarrow \text{F-Actin-ADP} + n\text{PO}_4$$

- Troponin

Troponin is a thin filament protein. This protein has a molecular weight of 76,000 daltons. Troponin, accounting for 5% of myofibrllar proteins, consists of three subunits designated troponin C (for calcium binding), troponin I (for inhibition), and troponin T (for binding with tropomyosin). Each subunit of troponin has distinct functions. Troponin C is a calcium binding protein and confers calcium regulation to the contractile process via the thin filament. Troponin I, when tested without the other subunits, strongly inhibits ATPase activity of actomyosin. Troponin T functions to provide a strong association site for binding of troponin to tropomyosin (Foegeding *et al.*, 1996).

- Tropomyosin

Tropomyosin, representing 5% of myofibrillar protein, is composed of two alpha-helical polypeptides wound together into a two-stranded, coiled-coil supersecondary structure. It resembles the tail or rod portion of myosin molecule. In skelatal muscle, two polypeptides, alpha- and beta-tropomyosin, can combine to form a tropomyosin dimer. The alpha -and beta-tropomyosin polypeptides have molecular weights of 37,000 and 33,000 daltons respectively. They are found in muscle as the alpha-alpha or beta-beta homodimers and the alpha-beta heterodimer. Tropomyosin aggregates end-to-end and binds to actin filaments along each groove of the actin double helix such that each molecule interacts with seven G-actin monomers. Tropomyosin and troponin are combined in a complex that regulates interaction of myosin with the thin filament (Foegeding *et al.*, 1996).

2. Sarcoplasmic proteins

Sarcoplasmic proteins or water soluble proteins make up 20-35% of the total protein content in muscle (Mackie, 1994). They are present within the cell, usually at concentrations as high as 20%. Enzymes responsible for the metabolism of the cell are available in this fraction. Myoglobin is also found in this fraction.

3. Stroma protein

Stroma protein is connective tissue protein of fish flesh, representing approximately 3% of the total protein content of muscles.

This is a reflection of the different structural arrangements of muscle cells in fish, compared to mammals. Connective tissue proteins are mainly collagens (Mackie, 1994).

Thermal stability of muscle proteins

Thermal stability is one of the most important properties of muscle proteins. Fish myosin is very unstable in comparison with that of mammal (Connell, 1961; Ogawa *et al.*, 1994). This dependence is more important for fish muscle than for mammalian muscle because of its low collagen content (Brown, 1986). Jonhston *et al.* (1973) found a relationship between the thermal stability of fish myosin and the environment temperature in which the species live. The myosin of cod (*Gadus morha*) which lived in cold water was more labile, compared with that of snapper (*Lutjanus sebae*) which lived in tropical area (Davies *et al.*, 1988).

The transition due to denaturation is often referred to in term of its peak maximum temperature (T_{max}). Peak transition temperatures (T_{max}) for the proteins present in different fish muscle were reported by Poulter *et al.* (1985) (Table 4). T_{max} of the first peak is assumed to correspond to myosin denaturation. Peak 2 is the small peak usually seen at a temperature intermediate between the myosin and actin denaturation peaks. Peak 3 is the most stable transition observed in the thermograms and is assumed to correspond to actin denaturation.

The T_{max} of myosin was different among fish species. The highest T_{max} for all species (e.g. sardine, stone flounder, walleye pollack, sea bream, carp, greenling, bigeye tuna, and yellow tail), except rainbow trout was approximately as high as T_{max} (46 °C) for rabbit (Ogawa *et al.*,

1993). Thermal denaturation of hake (*Merluccius hubbsi*) myofibrillar proteins of whole muscle showed two T_{max}, 46°C and 75°C (Beas *et al.*, 1990).

Table 4. Peak transition (T_{max}) for the proteins present in different fish muscle.

Samples	T_{max}(°C)		
	Peak 1	Peak 2	Peak 3
Cod	41.7±2.0	53.2±0.2	73.8±0.2
Tilapia	52.7±0.3	-	73.2±0.4
Siganus	46.4±0.5	57.1±0.5	72.6±0.5

All values are the means ±standard deviation.

Source : Poulter *et al.* (1985).

Thermal denaturation of the myosin was dependent on pH and ionic strength (Stabursvik and Martens, 1980; Wright and Wilding, 1984). Davies *et al.* (1988) found that snapper myosin showed the distinct peak with relatively high T_{max}. As the pH was increased, the transition peak became distinguishably less stable, as shown with a sharp decrease in T_{max}.

Physicochemical changes of muscle proteins during iced storage

Physicochemical changes of muscle proteins

Among post-harvest changes, degradation of fish muscle caused by endogenous proteases is a primary cause of quality losses during cold storage or handling (Haard *et al.*, 1994). Pacific whiting

muscle protein underwent denaturation and degradation during iced storage (Benjakul *et al.*, 1997). ATPase (E.C.3.6.1.8, ATP pyrophosphohydrolase) is associated with the postmortem disappearance of ATP in fish muscle, leading to rigor mortis (Nambudiri and Gopakumar, 1992). Kamal *et al.* (1991) reported that myofibrillar ATPase activities of sardine ordinary and dark muscles decreased during extended iced storage of 10 days.

During iced storage of Pacific whiting muscle, no changes in Ca^{2+}-ATPase, Mg^{2+}-Ca^{2+}-ATPase or Mg^{2+}-ATPase were observed, but Mg^{2+}-EGTA-ATPase activity gradually increased during iced storage (Benjakul *et al.*, 1997) (Figure 3). Ca^{2+}-ATPase activity is a good indicator of the integrity of the myosin molecule (Roura and Crupkin, 1995). Mg^{2+}-ATPase and Mg^{2+}-Ca^{2+}-ATPase activities are indicative of the integrity of the actin-myosin complex in the presence of endogenous or exogenous Ca^{2+} ions, respectively. Mg^{2+}-EGTA-ATPase activity indicates the integrity of the tropomyosin-troponin complex (Ouali and Valin, 1981; Ebashi and Endo, 1968; Watabe *et al.*, 1989).

Roura and Crupkin (1995) reported the enzymatic activities of myofibrils from pre- and post-spawned hake during iced storage. Mg^{2+}-ATPase, Mg^{2+}-Ca^{2+}-ATPase, and Ca^{2+}-ATPase activities of myofibrils were higher in post-spawned than those in pre-spawned fish. ATPase activities of myofibrils from post-spawned hake were not changed during iced storage for 10 days. For pre-spawned myofibrils, Mg^{2+}-ATPase was retained after 7 days of iced storage, while enzymatic activities of the others gradually decreased during iced storage. Seki and Narita (1980) found that EGTA-ATPase activity of the myofibrils from minced carp muscle decreased rapidly, while Ca^{2+}-ATPase and Mg^{2+}-ATPase

activities decreased gradually during iced storage with the exception that Mg^{2+}-ATPase activity in the presence of EGTA increased. The increase in this ATPase activity was accompanied with a loss of Ca^{2+}-sensitivity of myofibrils during iced storage for 16 days. Chalmers *et al.* (1992) showed that the apparent viscosity and Ca^{2+}-ATPase activity of actomyosin isolated from cod (*Gadus morhua*) tended to decrease slightly during aging of the fish in ice.

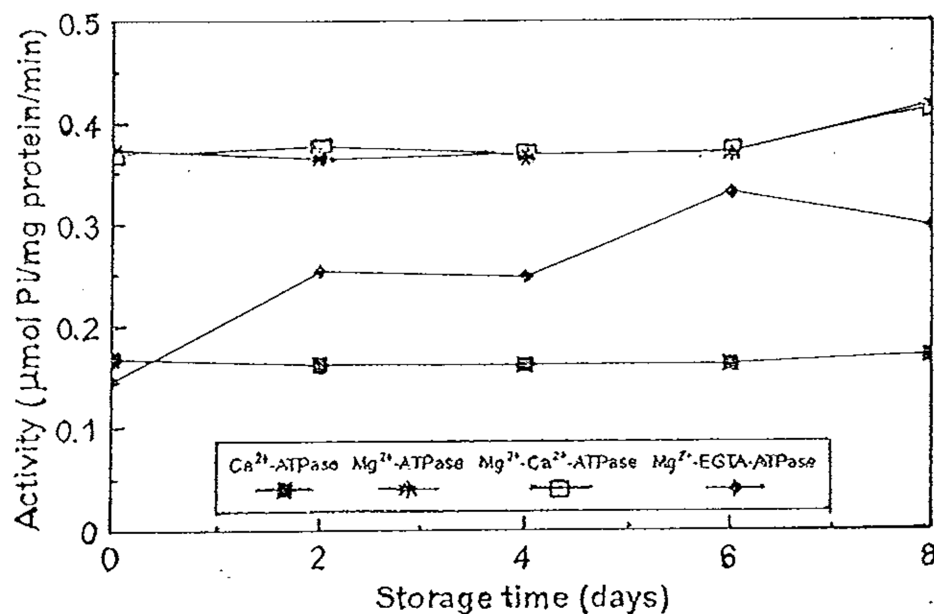


Figure 3 : ATPase activities of actomyosin from Pacific whiting stored in iced. One unit of activity was defined as that releasing 1 µ mole Pi/mg protein/min.

Source : Benjakul *et al.* (1997).

Ca^{2+}-sensitivity was reported to be a good indicator of Ca^{2+} regulation of myofibrillar proteins (Ruara and Crupkin, 1995) and was dependent upon the affinity of the troponin molecule for Ca^{2+} ion (Ebashi *et al.*, 1968). The decrease in both Ca^{2+}-binding capacity and Ca^{2+}-sensitivity was shown to be caused by proteolysis (Tokiwa and Matsumiya, 1969). Additionally, oxidation of thiol groups of myosin has been shown to reduce Ca^{2+}-sensitivity and modify actin-myosin interactions (Seki *et al.*, 1979). Therefore, loss of Ca^{2+}-sensivity in myofibrils from pre-spawned fish could be related to an increment in proteinase activity, which selectively degrades myosin during gonadal maturity (Ruara and Crupkin, 1995).

The denaturation of muscle proteins is caused by the oxidation of thiol (SH) groups of muscle. Hamada *et al.* (1977) reported the oxidation of carp and rabbit actomyosin during 14 days of storage at 4°C. The rabbit actomyosin had little changes in SH content and disulfide bond contents. Carp actomyosin was dramatically decreased in SH content with an increase in disulfide bond. Benjakul *et al.* (1997) found that total SH content of actomyosin increased slightly after 2 days of iced storage, followed by a gradual continued decrease up to 8 days. A decrease in total SH group was reported to be due to formation of disulfide bonds through oxidation of SH groups or disulfide interchanges (Hayakawa and Nakai, 1985). Chan *et al.* (1995) reported that myosin contained 42 SH groups. Two types of SH groups on the myosin head portion (SH_1 and SH_2) have been reported to be involved in ATPase activities of myosin (Yamaguchi and Sekine, 1966). Another SH group (SH_a) was localized in the light meromyosin region of myosin molecule and was responsible for oxidation of MHC (myosin heavy chain) and its

dimer formation resulted in an increase in Mg^{2+}-EGTA-ATPase activity of carp actomyosin during iced storage (Sompongse *et al.*, 1996a,b).

Degradation of muscle proteins

Protease is closely associated with degradation of muscle proteins. An *et al.* (1994) reported that among the Pacific whiting proteins, MHC was the most extensively hydrolyzed, followed by troponin-T and α-and β-tropomyosin. Degradation of myofibrils occurred at 0°C, possibly caused by cathepsins and serine proteinases (Benjakul *et al.*, 1997). Microbial proteases may also be a potential cause of proteolytic degradation. Protease from *Pseudomonas marinoglutinosa* was reported to hydrolyze actomyosin at 0-2°C and the optimal pH was above 7.0 (Venugopal *et al.*, 1983). Benjakul *et al.* (1997) showed that MHC of Pacific whiting muscle was hydrolyzed continuously throughout iced storage. MHC decreased to 45% of the original content within 8 days, whereas no changes in actin were observed on SDS-PAGE.

The myofibrillar proteins of bovine muscle stored at 2°C contained 95,000 dalton component, suggesting involvement of calcium activated factor (CAF). Gradual disappearance of troponin-T and gradual appearance of 30,000 daltons component were also observed (Koohmaraie *et al.*, 1984). Chalmer *et al.* (1992) found that the ratio of myosin heavy chain to actin in the cod actomyosin was at a maximum after 3 days of storage (early post-rigor mortis condition).

Physicochemical changes of muscle proteins during frozen storage

Physicochemical changes of muscle proteins

During frozen storage of fish, deterioration caused by microorganisms and some biochemical processes are retarded. The palatability of frozen fish stored for extended times is normally limited by losses in either flavor and/or texture (Haard, 1992). When muscle tissue is frozen and stored in the frozen state, it inevitably losses some of these special eating quality, usually observed as a loss in juiciness and increase in toughness (Mackie, 1993).

Fennema (1982) noted that all proteins would be expected to have an optimum stability at a temperature just above the freezing of water. At higher temperatures, thermal denaturation occurred as a result of destabilization of hydrogen bonds and increased molecular motion. At lower temperatures, despite enhanced intramolecular hydrogen bonding, hydrophobic interactions would be weaken to the point that a net destabilization may be expected to occur. This optimum temperature of stability might actually be below freezing point, but the onset of ice crystal formation could induce changes in the protein environment that affects denaturation. Figure 4 summarizes some of the changes which may occur in a muscle protein system during freezing and frozen storage (MacDonald and Lanier, 1991). The denaturation of carp myosin in KCl solution was found during frozen storage. Myosin in the ionic strength ranging from 0.2 to 0.6 was denatured faster at -5°C than at -20°C (Okada *et al.*, 1985). Itoh *et al.*(1990) found a marked denaturation of carp myosin (in presence of 0.6M KCl) during the slow freezing process.

LeBlanc and LeBlanc (1992) reported that the aldehyde content increased in cod fillets at warmer frozen storage temperatures. Aldehydes are formed by the oxidation of ε-NH_2- group of lysine. Displacement of an ε-NH_2- group by oxidation to aldehyde or ketones results in the loss of water from the terminal group. A number of these new hydrophobic groups, coupled with the increase in other hydrophobic parameters, could twist the protein and increase hydrophobic interactions.

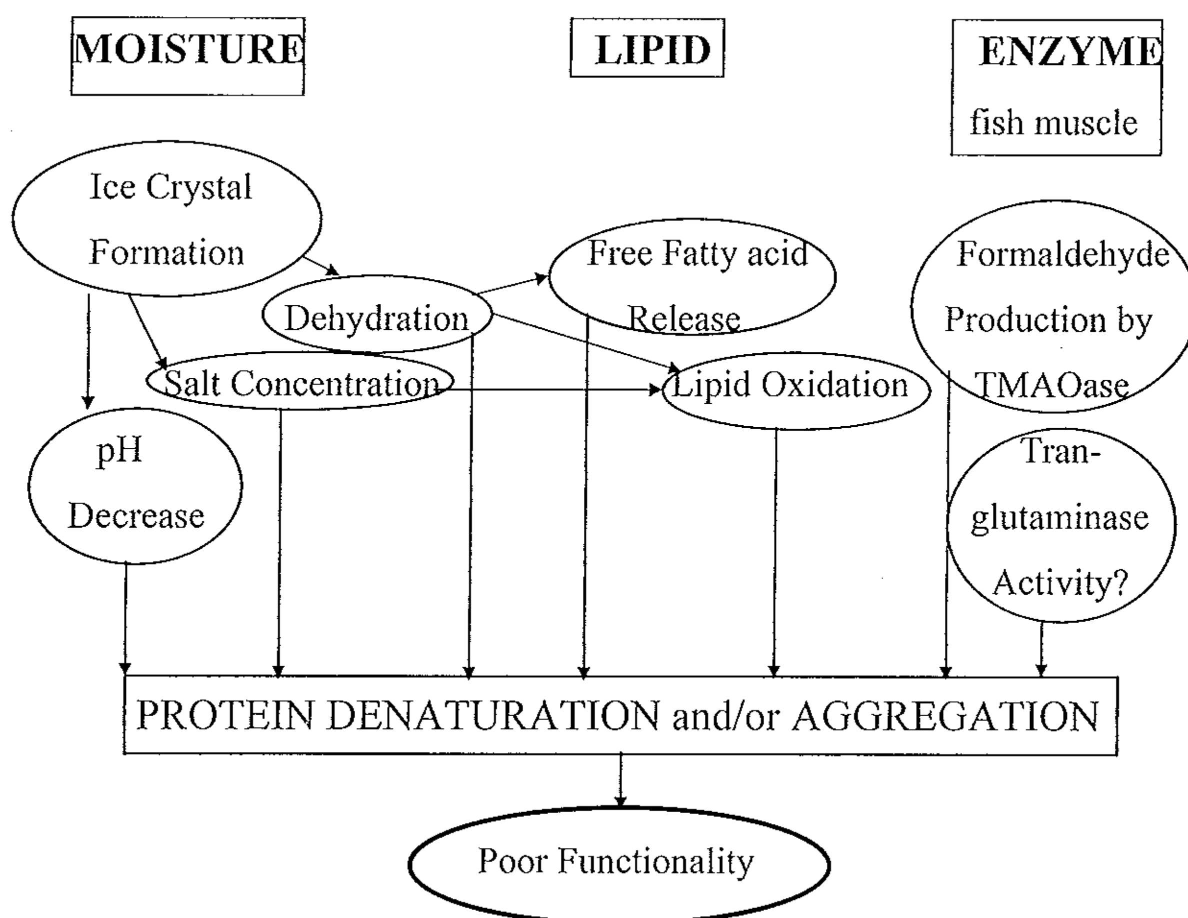


Figure 4 : Factors affecting muscle protein denaturation during frozen storage.

Source : MacDonald and Lanier (1991)

Jiang and Lee (1995) found protein denaturation of fish muscle during frozen storage at -20°C. Muscle having higher free amino nitrogen content was more prone to protein denaturation. The predominant free amino acids in the muscle of frozen mackerel, amberfish, and mullet were histidine, lysine, alanine, and taurine, whereas proline, glycine, alanine, histidine, and taurine were abundant in carp. The muscle protein with high level of free histidine and lysine was relatively unstable, while protein of fish muscle with high levels of free glycine, proline, and alanine was stable during frozen storage at -20°C.

The SH groups are known to be oxidized to disulfide bonds during denaturation of proteins. This form of covalent bonding occurs during frozen storage of muscle protein (Mackie, 1993). The milkfish actomyosin was denatured during frozen storage by formation of disulfide, hydrogen and hydrophobic bonds. More disulfides were formed than other bonds in actomyosin stored at -20°C than at -35°C (Jiang *et al.*, 1988). The total SH of fish actomyosin decreased during frozen storage, suggesting the formation of disulfides occurred at a much faster rate at -20°C than -40°C (Jiang *et al.*, 1989).

Jiang *et al.* (1985) reported a decrease in Ca^{2+}-ATPase activity of actomyosin during frozen storage. Muscle proteins of frozen mackeral was the most unstable, compared to those of amberfish, mullet, and carp, respectively. Nambudiri and Gopakumar (1992) reported the decrease in ATPase and Lactate dehydrogenase (LDH) activities in a variety of fish stored at -20°C during 180 of days storage. Both of enzymes were useful indices to detect early deterioration in frozen fish.

Kamal *et al.* (1991) found that myofibrillar Ca^{2+}-ATPase activity of the ordinary and dark muscle of sardine more decreased at –

20°C than that at -80°C. Azuma and Konno (1998) studied the freeze denaturation of carp myofibrils and found that Ca^{2+}-ATPase activity slowly decreased during frozen storage. The Mg^{2+}-ATPase activity increased to a higher extent, compared to Mg^{2+}-Ca^{2+}-ATPase activity, suggesting the actin-myosin interaction in the frozen storage was controlled by the regulatory proteins and Ca^{2+}.

Effect of freezing and thawing on enzyme leakage in muscle tissue.

Apart from the primary quality of the fresh fish, the quality of frozen fish depends freezing, thawing and storage conditions (Nilsson and Ekstrand, 1995). Freezing and thawing also affect the membrane structure of muscle cells (Fennema, 1978). The disintegration of membrane structures can be measured by the activity of one or more enzymes in muscle tissue fluids. The leakage of enzymes are regarded as markers of membrane damage. The activity of lysosomal enzymes e.g. acid phosphatase (AP : E.C.3.2.1.3.2), *β-N*-acetylglucosaminidase (NAG : E.C.3.2.1.30) and α-glucosidase (AG : E.C. 3.2.1.20) in the centrifugal tissue fluid has been used to differentiate frozen from fresh fish (Rehbein, 1978; 1979). Rehbein *et al.* (1978) studied the specific activities of the lysosomal enzymes, α-glucosidase and *β-N*-acetylglucosaminidase from fillets of cod, saithe, red fish and haddock after freezing/thawing. The enzyme activity in the pressed juice/extract increased with the freezing/thawing process (six to nine times for α-glucosidase and three to five times for *β-N*-acetylglucosaminidase).

Kitamikado *et al.* (1990) reported that the enzyme activity in blood from the fresh carp (4°C, 0-1 day) was close to zero and remained

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at low activity (less than 0.2 mU/ml) for up to 5 days. The enzyme activity in blood from the frozen-thawed carp, even when stored at -20°C for 1 day, increased dramatically, being more than 100-fold higher than that in fresh fish (0-1 day). The activity of marker enzymes in rainbow trout muscle tissue including lysosomal α-glucosidase and *β-N*-acetylglucosaminidase and acid phosphatase increased after iced storage and also with a freezing-thawing cycle. The increase in enzyme leakage was dependent on the duration of iced storage in relation to a subsequent freezing process (Nilsson and Ekstrand, 1993) (Table 5). The slowest freezing rate caused the highest enzyme leakage.

Table 5 : The activity of α-glucosidase (AG), β-*N*-acetylglucosaminidase(NAG) and acid phosphatase (AP) in centrifugal tissue fluid (CTF) expressed as pkatal per gram of muscle tissue of rainbow trouts kept on ice.

Day on ice	AG (pkatal/g of muscle)		NAG (pkatal/g ofmuscle)		AP (pkatal/g of muscle)	
	iced	freeze/thawed	iced	freeze/thawed	Iced	freeze/thawed
3	6.6	28.7	30.3	97.3	691	1330
6	6.8	24.9	29.1	77.9	913	1260
10	7.9	20.2	37.1	94.5	548	1105
14	6.7	31.6	28.5	114.0	683	1385

Source : Nilsson and Ekstran (1993).

Nilsson and Ekstrand (1995) reported that the frozen storage and thawing methods affected biochemical properties of rainbow trout. Slow thawing, in air at 5°C, resulted in higher NAG activity in CTF, compared to fast thawing, at 25°C in water. After 3 months of storage, a higher NAG activity in CTF and a larger volume of CTF were found in sample stored at -18°C, compared to that obtained in samples stored at -40°C.

Objectives

1. To study the biochemical and physicochemical properties of seabass muscle proteins during iced and frozen storage.

2. To study the effect of freezing and thawing method on seabass muscle tissue.

Chapter 2

Materials and Methods

1. Samples and preparation

Seabass were caught from Koa Yo, Songkhla. The fish were killed by ice shocking and transported to Department of Food Technology on ice with a ratio of ice to fish 1:2 within 1 hr. Then the fish were immediately washed and filleted. The dorsal and ventral muscle were carefully excised from those specimens and used for chemical composition analysis and other studies.

2. Chemicals

Ammonium molybdate, 5-5'-dithio-bis(2-nitrobenzoic acid) (DTNB), ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), $CaCl_2$, adenosine 5'-triphosphate (disodium salt) 2,4,6-trinitrobenzenesulfonic acid (TNBS), L-leucine, *p*-nitrophenyl-α-glucopyranoside *p*-nitrophenyl-*N*-β-D-glucose, sodium dodecyl sulfate (SDS) and β-mercaptoethanol (βME) were purchased from Sigma (MO, USA). N,N,N',N'-tetramethylethylenediamine (TEMED), acrylamide, bis-acrylamide, and urea were obtained from Fluka (Switzerland).

3. Instruments

Instruments	Model	Company
Differential scanning calorimeter	DSC7	Perkin-Elmer, USA
Double-beam spectrophotometer	UV-16001	SHIMADZU, Australia
Balance	AB 204	MRTTLER TOLEDO, Switzerland
Electrophoresis apparatus	Mini-Protein II	Bio-Rad, USA
Homogenizer	T 25	Ultra turrax, Malaysia
Low temperature incubator	MIR-153	Sanyo, Japan
Magnetic stirrer	RO 10 power	KIKAL labortechnik, Germany
Muffle furnace	550-126	Fisher Scientific, France
pH meter	Denver 15	Scientific, USA
Refrigerated centrifuge	RC-5B plus	Sorvall, USA
Stomacher	M.400	SEWARD, UK
Water bath	W 350	Memmert, Germany

4. Determination of protein compositions and properties of seabass muscle proteins

4.1 Chemical compositions of seabass muscle

Chemical compositions of dorsal and ventral muscle were determined as follows :

- Protein (AOAC, 1991)
- Moisture (AOAC, 1991)
- Ash (AOAC, 1991)
- Lipid (Bligh and Dyer, 1952)
- Sodium chloride (AOAC, 1991)
- Amino acid (analyzed by Thailand Institute of Scientific and Technological Research)

4.2 Nitrogenous compositions of seabass muscle

Fractionation of dorsal and ventral muscle was carried out according to the method of Hashimoto *et al.* (1972). As shown in Figure 5, each fraction contained different compositions, e.g. non-protein nitrogen, sarcoplasmic protein, myofibrillar protein, alkali-soluble protein, and stroma. Nitrogen content in each fraction was measured by Kjeldahl method (AOAC, 1991).

4.3 Thermal denaturation

Thermal denaturation of muscle proteins were studied using Differential Scanning Colorimetry (DSC). Seabass muscle was subjected to different pHs and salt concentrations before analysis.

- pH

Twenty-five grams of muscle were mixed and homogenized with 5 ml of 1.2 M phosphate buffer (KH_2PO_4/K_2HPO_4) with different pHs (5, 6, 7, 8) at 4°C. Then, prepared samples were run for DSC analysis.

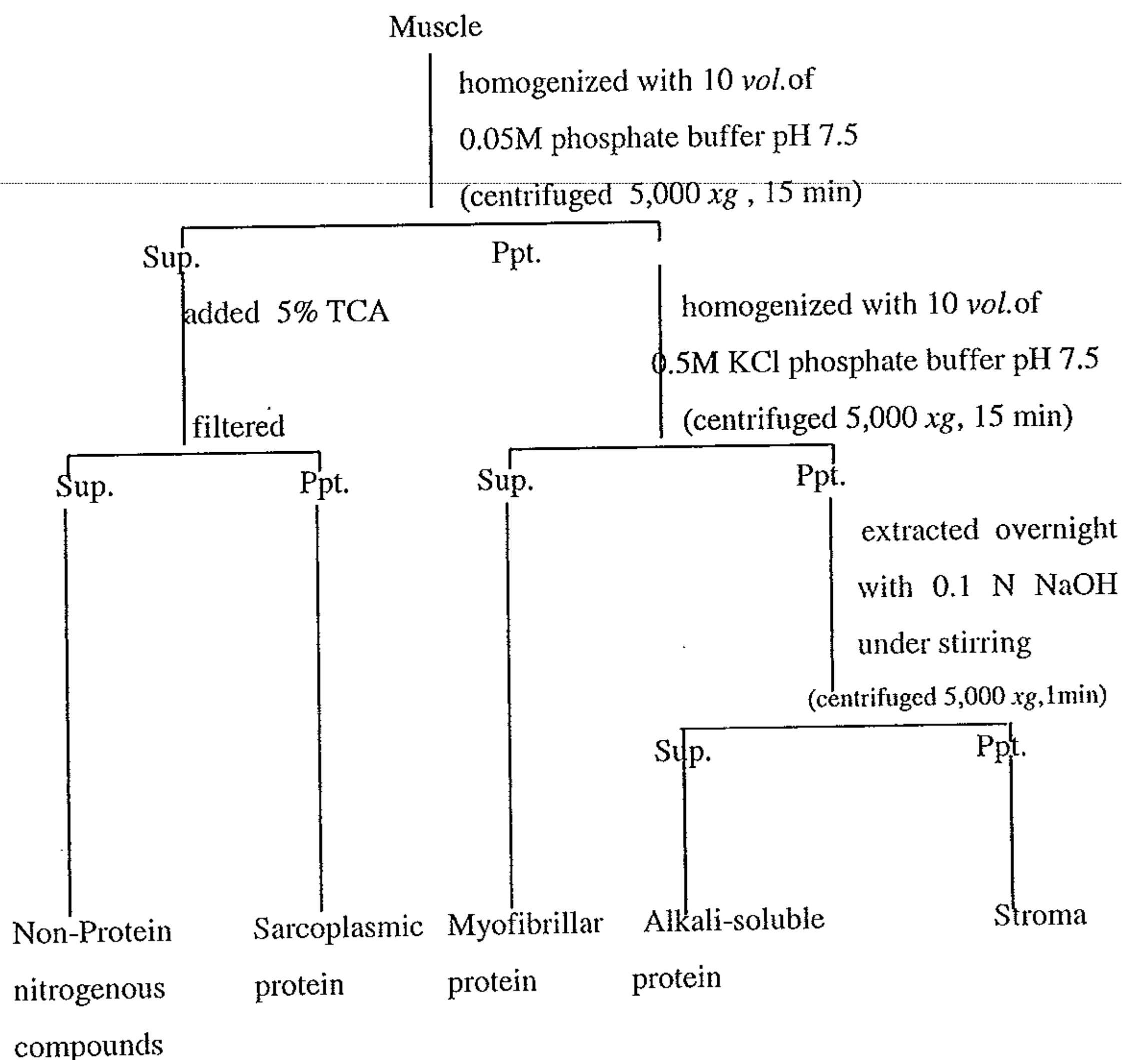


Figure 5 : Fractionation procedure of muscle proteins

Source : Hashimoto *et al.* (1972)

- Salt concentration

The seabass muscle (25 g) were homogenized with 15 ml of KCl solutions at different concentrations (0.06, 0.6, 1.0, and 1.2 M) at 4°C. Subsequently, prepared samples were subjected to DSC analysis.

T_{max} was measured and the enthalpy of denaturation (ΔH) was calculated from thermogram

4.4 Stability of seabass actomyosin

The actomyosin solution was prepared according to the method of Benjakul *et al.* (1997) and subjected to different temperatures and pHs.

4.4.1 Effect of the temperature

Actomyosin solution (1.0-5.0 mg/ml) was incubated at various temperatures (0, 10, 20, 30, 40, 50 and 60°C). At a definite time interval (0, 5, 10, 20, 30 and 60 min). The solutions were cooled down immediately in iced water for 5 min and then kept at 25°C for 5 min.

4.4.2 Effect of pH

To investigate the effects of pH on the stability of seabass actomyosin, actomyosin solution (1.0-1.5 mg/ml) was subjected to different pHs (4.5, 5.5, 6.5, 7.5, 8.5, and 9.5). 0.2M McIlvain buffers (0.2M Na phosphate, 0.1M Na citrate) with different pHs were added into actomyosin with a ratio of buffer to actomyosin 1 : 1. The mixtures were then incubated at 25°C for 0, 5, 10, 20, 30 and 60 min.

Actomyosin exposed to different conditions were 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)

5. Physicochemical changes of seabass actomyosin during iced and frozen storage

5.1 Seabass actomyosin changes during iced storage

Actomyosin was prepared as mention above. Chemical reagents including sodium dodecyl sulfate (SDS), urea, and *β*-mercaptoethanol (βME) were added into the actomyosin solution to obtain a final concentration of 30 μmol/g protein. The prepared samples were kept on ice for 14 days.

Actomyosin samples were taken for analysis every 2 days. The analysis was run as follows :

1) Total sulhydryl group (Sompongse *et al.*, 1996b)

2) ATPase activity (Benjakul *et al.*, 1997)

Ca^{2+}-ATPase, Mg^{2+}-ATPase, Mg^{2+}-Ca^{2+}-ATPase, and Mg^{2+}-EGTA-ATPase activities were measured according to the method of Benjakul *et al.* (1997). The Ca^{2+}-sensivity was calculated according to Seki and Narita (1980) as follows :

$$Ca^{2+}\text{sensitivity} = \left[1 - \frac{Mg^{2+}\text{-EGTA-ATPase}}{Mg^{2+}\text{-}Ca^{2+}\text{-ATPase}} \right] \times 100$$

3) Solubility (Hamada *et al.*, 1977; Jiang *et al.*, 1988)

Samples were solubilized with different solvents and fractionated into 4 different fractions are follows:

- Salt-soluble fraction (I)
- Urea-soluble fraction (II)
- $NaBH_4$-soluble fraction (III)
- Insoluble fraction (IV)

Each fractions were subjected to protein determination by Biuret's method (Copeland, 1994).

4) Free α-amino acid (Benjakul and Morrissey, 1997)

5) Electrophoretic analysis

To investigate the molecular weight of actomyosin, samples kept at day 0, 4, 8, 12, and 14 were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The analysis was carried out according to the method of Laemmli (1970) using 4% stacking gel and 10% separating gel.

5.2 Seabass actomyosin changes during frozen storage

Seabass actomyosin was prepared and various chemical reagents were added as described in 5.1. Actomyosin samples were stored at either -18 or -80°C for 6 months. The samples were taken for analysis every 2 weeks. All chemical analysis were performed as shown in 5.1.

5.3 Effect of freeze-thawing on seabass actomyosin

Seabass actomyosin was frozen to obtain the core temperature of either -18 or -80°C. Subsequently, the samples were thawed with two different thawing methods as follows :

1) Thawing with tap water (25-26°C)

2) Thawing at room temperature (28-30°C)

The samples were subjected to 1, 2, and 3 freeze-thaw cycles. Thawed sample were determined for physicochemical changes as appeared in 5.1.

6. Physicochemical changes of seabass muscle during iced and frozen storage

6.1 Seabass muscle during iced storage

Seabass dorsal fillets were sliced into a thickness of approximately 1 cm. The slices obtained were packaged in polyethylene bag and kept on ice (0°C) for 14 days. Samples were removed at 0, 2, 4, 6, 8, 10, 12 and 14 days of storage for analysis as follows :

6.1.1 Chemical composition

pH and lactic acid was measured according to Lees (1975). Ammonia was analyzed according to AOAC (1984). Total Volatile Base (TVB), trimethylamine (TMA) and formaldehyde were determined as described by NG (1987).

6.1.2 Physical property

Expressible moisture was measured according to Wierbicki and Deatherage (1985).

6.1.3 Microbiological property

Psychrophillic bacteria was measured according to the method of Gilliland *et al.*(1984).

6.1.4 Physicochemical changes of actomyosin

Actomyosin was analyzed for physicochemical changes as mentioned in 5.1.

6.2 Seabass muscle during frozen storage

Samples were stored at either -18 or -80°C and removed every 2 weeks for analysis. All analysis were carried out as described in 5.1.

6.3 Effect of freeze-thawing on seabass muscle

The samples were frozen at either -18 or -80°C, and thawed with two different thawing methods as follows :

1) Thawing with tap water (25-26°C)

2) Thawing at room temperature (28-30°C)

The samples were subjected to freeze-thawing with different cycle (1, 2, and 3). The samples were analyzed as described in 5.1. In addition, the samples were centrifuged at 20,000 *xg* for 60 min and centrifugal tissue fluid (CTF) was measured for α-glucosidase and *β*-*N*-Acetyl-glucosaminidase according to the method of Nilsson and Ekstrand (1993).

Chapter 3

Results and Discussion

Chemical compositions and properties of seabass muscle

Chemical compositions

The dorsal and ventral seabass muscle had the similar chemical compositions as shown in Table 6. The sample contained high moisture content (~80-81%). The protein content of dorsal and ventral muscle were 16.04 and 15.7 %, respectively. This result indicated that seabass was a potential source of protein. Mackie (1994) reported that the main constituents of flesh are protein and fat. Generally, the fish muscle contains 19.0 % protein and 1.0% fat (Stanby, 1963).

Table 6. Chemical compositions of seabass muscle

Chemical compositions (% wet basis)	Muscles	
	Dorsal	Ventral
Protein	16.04±0.02*	15.71±0.07
Ash	1.20±0.05	1.26±0.13
Moisture	80.63±0.39	80.80±0.30
Fat	3.24±0.15	3.65±0.27
sodium chloride	0.18±0.07	0.19±0.12

* Mean ± SD from triplicate determinations.

Seabass muscles consisted of a large amount of essential amino acids. Glutamic acid was found to be the most predominant amino acid in muscle, followed by aspartic acid. It has been reported that both glutamic acid and aspartic acid are the essential contributor for a sensory taste for food. Aspartic acid, alanine, luecine, lysine, arginine, and glycine were found at a high extent ranging from 1,020 to 1,726 mg/100 g muscle (Table 7). These amino acids were presumably associated with the properties of seabass muscle.

Table 7. Amino acid compositions of seabass muscle*

Amino acid	Muscles	
(mg/100 g sample)	Dorsal	Ventral
Aspartic acid	1726.29	1706.95
Threonine	749.82	750.15
Serine	678.33	664.43
Glutamic acid	2829.97	2823.19
Proline	538.31	604.09
Glycine	761.80	735.19
Alanine	1035.03	1082.02
Cystine	113.67	141.36
Valine	714.81	790.13
Methionine	443.19	443.18
Isoleucine	643.26	706.17
Luecine	1301.73	1314.43
Phenylalanine	660.41	662.26
Histidine	410.00	403.10
Lysine	1548.75	1552.71
Arginine	1020.64	1056.79
Tryptophan	122.77	183.73

* Analyzed by Thailand Institute of Science and Technological Research

Nitrogenous compositions

Nitrogenous compositions of seabass muscle is shown in Table 8. The dorsal muscle contained slightly higher non protein nitrogen, compared to the ventral muscle. Dorsal muscle had a lower content of sarcoplasmic protein and stroma. However, it contained a slightly larger content of myofibrillar and alkali-soluble fraction. Among proteins and other nitrogenous components in muscle, myofibrillar protein was found to be a major constituent. This result was in agreement with Mackie (1994) who reported that myofibrillar proteins are main nitrogenous constituents in fish muscle. Generally, protein compositions of fish vary, depending on muscle type, feeding period, and spawning (Mackie, 1994). Non protein nitrogen content in sardine white muscle was higher than dark muscle (Hashimoto *et al.*, 1979). Stroma content could be related to the stiffer structure of the muscle (Hashimoto *et al.*, 1972).

Protein pattern of each fraction determined using SDS-PAGE revealed that similar protein compositions were observed between dorsal and ventral muscle of seabass. As shown in Figure 6, most of sarcoplasmic proteins had the low molecular weight with the association of high water solubility of this fraction. Sarcoplasmic protein in sardine dark and white muscles differed from each other due to the differences in myoglobin and parvalbumin. The enzymes responsible for the metabolism of the cell are also available in this fraction (Mackie, 1994).

The myofibrillar fraction showed several bands with the major bands of myosin heavy chain (MW 205,000) and actin (MW 45,000). Troponin (MW 76,000) and tropomyosin (MW 37,000 and 33,000) were also found at high extent. For alkali-soluble fraction, major protein bands were observed at MW of 45,000, 36,000 and 20000 dalton. Hashimoto *et*

al. (1972) reported that pattern of alkali-soluble fraction from sardine and mackerel muscle was similar to that of myofibrillar fraction.

Table 8. Nitrogenous compositions of seabass muscle

Compositions (mg N/g wet muscle)	Muscles	
	Dorsal	Ventral
Non-protein nitrogen	2.94 ± 0.09*	2.28 ± 0.19
Sarcoplasmic	5.72 ± 0.36	9.43 ± 1.70
	(34.43)**	(36.08)
Myofibrillar	15.79 ± 0.13	13.46 ± 0.97
	(60.73)	(59.19)
Alkali-soluble	1.26 ± 0.14	0.98 ± 0.03
	(4.71)	(3.75)
Stroma	0.14 ± 0.01	0.26 ± 0.02
	(0.49)	(0.98)

* Mean ± SD from triplicate determinations.

** Numbers in parentheses represent percentage distribution.

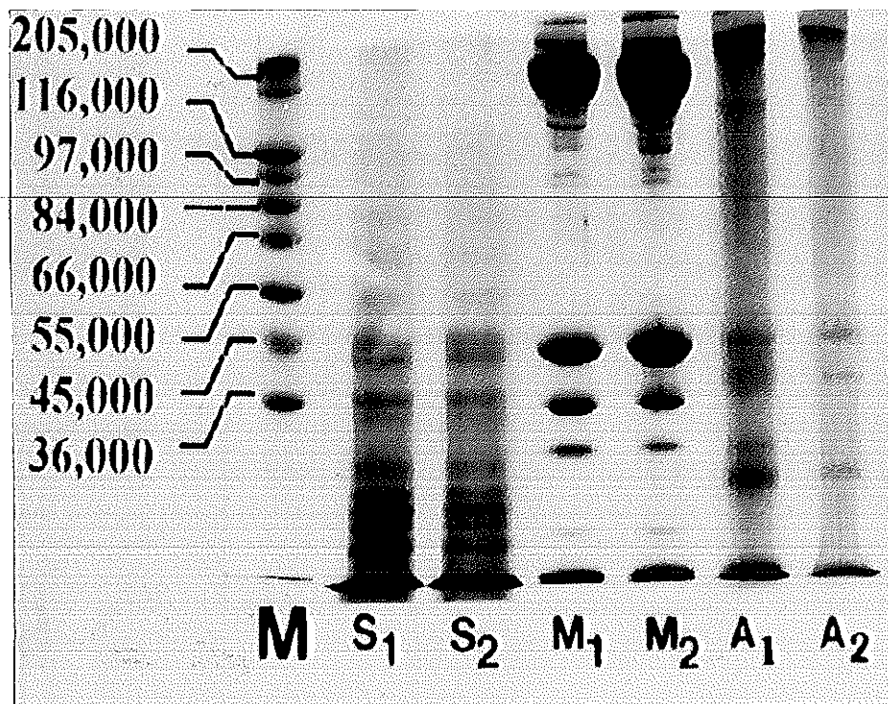


Figure 6 : SDS-gel electrophoretic patterns of seabass muscle protein fractions (12% gel).

From left to right : high molecular weight standard, M; sarcoplasmic fraction from dorsal (S_1) and ventral (S_2) muscles; myofibrillar fraction from dorsal (M_1) and ventral (M_2) muscles; alkali-soluble fraction from dorsal (A_1) and ventral (A_2) muscles.

Thermal denaturation

In order to determine thermal denaturation, the dorsal and ventral seabass muscles were subjected to DSC analysis. The similar transition profiles between dorsal and ventral muscle were found. Three major peaks were obtained with the T_{max} of 45.15-45.28, 54.48-54.44, and 73.58-73.81°C (Table 9). The first peak was presumed to be myosin peak, while the third peak was postulated to represent actin peak. Poulter *et al.*(1985) studied the heat stability of fish muscle proteins and found that T_{max} (41.70-52.70°C) of the first peak was the singlet or double peak, which was assumed to correspond to myosin denaturation. Peak 2 with T_{max} of 53.20-57.10°C was the small peak usually seen at a temperature

intermediate between the myosin and actin denaturation peak. Peak 3 with a T_{max} of 72.60-73.80°C was the most stable transition observed in the thermograms and was assumed to correspond to actin denaturation.

For both dorsal and ventral muscle, T_{max} of first peak was shifted to a lower temperature when exposed to more acidic or alkaline pHs, indicating that myosin was more prone to denaturation. T_{max} of the third peak was also shifted to lower temperature, but lesser extent was observed, compared to the first peak. The result suggested that actin was more stable to thermal denaturation under either acidic or alkaline condition than myosin. Myosin transitions observed by DSC appeared to be strongly dependent upon pH and ionic strength both in mammalian (Stabursvik and Martens, 1980; Wright and Wilding, 1984) and fish protein (Davies *et al.*, 1988; Beas *et al.*, 1990).

Transition temperatures of the dorsal and ventral muscle protein in different concentration of KCl solution are shown in Table 10. As the concentration of KCl solution increased up to 1.0 M, all three peak were observed at lower temperatures. No marked decrease in T_{max} was found at concentration higher than 1.0 M KCl. The decrease in the peak transition suggested an additional effect of chloride anion (Beas *et al.*, 1990). However, the presence of minor proteins (e.g. tropomyosin, troponin, and actinins) possibly contributed to the total endotherm. Since troponin and tropomyosin are thermostable proteins (T_{max} is greater than 85°C in mammalian muscle), these proteins could contribute to the third peak transition (Wagner, 1986; Beas *et al.*, 1990). From the result, third peak seemed to represent the most stable protein observed among three peaks. Therefore, different proteins had different thermal stability.

Table 9. Peak transition temperatures (T_{max}) for the seabass muscle proteins at different pHs.

Samples	T_{max} (°C)		
	Peak I	Peak II	Peak III
Dorsal muscle	45.28 ± 2.38*	54.48 ± 0.15	73.81 ± 1.83
Ventral muscle	45.15 ± 2.33	54.64 ± 0.47	73.58 ± 1.35
Dorsal muscle (pH 5.0)	40.14 ± 0.06	51.62 ± 0.38	69.99 ± 0.92
Ventral muscle (pH 5.0)	40.53 ± 0.41	51.08 ± 0.84	70.00 ± 1.40
Dorsal muscle (pH 6.0)	44.14 ± 0.45	54.72 ± 0.69	72.93 ± 0.44
Ventral muscle (pH 6.0)	44.03 ± 0.24	53.88 ± 0.50	72.94 ± 0.55
Dorsal muscle (pH 7.0)	45.13 ± 0.80	54.33 ± 0.15	73.37 ± 0.23
Ventral muscle (pH 7.0)	45.04 ± 0.06	54.30 ± 0.21	73.624 ± 0.54
Dorsal muscle (pH 8.0)	39.75 ± 0.47	49.95 ± 0.21	69.47 ± 1.02
Ventral muscle (pH 8.0)	39.48 ± 1.03	49.43 ± 0.89	69.98 ± 0.39

- Mean ± SD from triplicate determinations.

Table 10. Peak transition temperatures (T_{max}) for the seabass muscle proteins at different KCl concentrations.

Samples	T_{max} (°C)		
	Peak I	Peak II	Peak III
Dorsal muscle (0.06 M KCl)	45.28 ± 0.20*	54.477 ± 0.34	73.81 ± 0.28
Ventral muscle (0.06 M KCl)	45.15 ± 0.42	54.64 ± 0.60	73.58 ± 0.35
Dorsal muscle (0.6 M KCl)	42.79 ± 0.38	53.92 ± 0.47	67.60 ± 0.51
Ventral muscle (0.6 M KCl)	44.18 ± 0.40	53.79 ± 0.24	68.87 ± 0.36
Dorsal muscle (1.0 M KCl)	41.45 ± 0.26	52.37 ± 0.47	67.22 ± 0.50
Ventral muscle (1.0 M KCl)	41.85 ± 0.44	51.59 ± 0.34	66.46 ± 0.24
Dorsal muscle (1.2 M KCl)	41.51 ± 0.52	51.39 ± 0.60	66.99 ± 0.31
Ventral muscle (1.2 M KCl)	41.04 ± 0.63	51.04 ± 0.47	66.58 ± 0.54

* Mean ± SD from triplicate determinations.

Stability of seabass actomyosin

The inactivation rate constants of actomyosin Ca^{2+}-ATPase activity were frequently used for evaluating the thermal stability of fish muscle proteins (Suzuki, 1981; Seki, 1977; Tsai *et al.*, 1989). The stability of myofibrillar proteins was considered to be highly related to the living environment temperatures of fish (Seki, 1977; Connell, 1961). From the result, it was found that temperature considerably affected the

stability of seabass actomyosin. Seabass actomyosin was most stable up to 20°C. At higher temperature, protein was more susceptible to denaturation (Table 11). As monitored by inactivation rate, protein underwent thermal denaturation more severely as the temperature increased. Therefore, temperature is a crucial factor affecting the muscle protein denaturation.

Table 11. Effect of temperatures on the inactivation rate constant of seabass actomyosin Ca^{2+}-ATPase.

Temperature (°C)	K_D x 10^{-5} . s^{-1}
0	0.13 ± 0.01[f*]
10	0.21 ± 0.03[f]
20	0.52 ± 0.01[e]
30	5.20 ± 0.06[d]
40	10.29 ± 0.04[c]
50	25.41 ± 0.07[b]
60	40.94 ± 0.02[a]

* The same letters in the same column indicates no significant differences ($p>0.05$).

The effect of pH on the stability of seabass actomyosin was also investigated (Table 12). At tested temperature, 25°C, seabass actomyosin was stable at pH 6.5 and 7.5. At very high acidic or alkaline pHs, the muscle protein was more denatured. Tsai *et al.* (1989) reported that the actomyosin of milkfish, tilapia hybrid, tilapia, and carp were stable at 6.5-7.9, which were almost the same as those of other species. The pH of animal muscle is around 7.4, when alive or immediately after slaughtered (Fukada, 1987). After death, the formation of lactic acid

resulting from anaerobic glycolysis decreases the muscle pH to 6.3-6.1, where the myofibrillar proteins have maximum ATPase activity. The hydrolysis of ATP is accelerated and consequently causes an irreversible muscle contraction (Tsai *et al.*, 1989). The pH of muscle further decreases to 5.4-5.7. Because of the low pH, the glycolytic enzyme activities are greatly inhibited and glycolysis ceases (Tsai *et al.*, 1989). From the results, the stability of seabass muscle proteins decreased much more at either higher temperature or drastic pH condition.

Table 12. Effect of pH on the inactivation rate constant of seabass actomyosin Ca^{2+}-ATPase.

pH	$K_D \times 10^{-5} . s^{-1}$
4.5	$25.14 \pm 0.02^{a*}$
5.5	19.66 ± 0.07^{b}
6.5	1.27 ± 0.02^{e}
7.5	1.56 ± 0.03^{e}
8.5	12.46 ± 0.05^{d}
9.5	15.41 ± 0.05^{c}

* The same letter in the same column indicates no significant differences ($p > 0.05$).

Physicochemical changes of seabass actomyosin during iced and frozen storage

Changes of seabass actomyosin during iced storage.

Solubility

After 14 days of iced storage, salt-soluble fraction decreased to 61.96%, compared to that of original sample (Table 13). This indicated that salt-soluble proteins underwent denaturation and aggregation via some bonding generated during storage. The quantity of urea-soluble fraction (II) of seabass actomyosin increased during 6 days of iced storage, but decreased with prolonged storage. The increase in urea-soluble fraction indicated formation of hydrogen and hydrophobic bonds during storage (Jiang *et al.*, 1988; 1989). After 8 days of iced storage, the quantity of the total $NaBH_4$-soluble (III) and insoluble fractions (IV) of seabass actomyosin increased markedly, while urea-soluble fraction gradually decreased. These data indicated that denaturation occurred by formation of disulfide, hydrogen and hydrophobic bonds. Disulfide bond was a major contributor of aggregation, particularly during extended storage.

Table 13 : The solubility of seabass actomyosin during iced storage*

Storage time (days)	Fractions**			
	I	II	III	IV
0	9.91^{a***} ± 0.20	0.32^{de} ± 0.06	0.30^{e} ± 0.10	0.48^{d} ± 0.20
	(100.00)	(3.23)	(3.03)	(4.84)
2	9.87^{a} ± 0.11	0.37^{d} ± 0.12	0.30^{e} ± 0.18	0.49^{d} ± 0.61
	(99.60)	(3.73)	(3.03)	(4.94)
4	9.80^{a} ± 0.06	0.42^{c} ± 0.38	0.31^{e} ± 0.30	0.49^{d} ± 0.14
	(98.89)	(4.24)	(3.13)	(4.94)
6	9.65^{a} ± 0.16	0.58^{a} ± 0.49	0.30^{e} ± 0.28	0.49^{d} ± 0.34
	(97.38)	(5.85)	(3.04)	(4.95)
8	9.18^{b} ± 0.25	0.50^{b} ± 0.08	0.38^{d} ± 0.39	0.96^{c} ± 0.36
	(92.63)	(5.05)	(3.83)	(9.69)
10	6.75^{c} ± 0.18	0.44^{c} ± 0.35	0.97^{c} ± 0.18	2.87^{b} ± 0.15
	(68.11)	(4.44)	(9.79)	(28.96)
12	6.28^{d} ± 0.95	0.29^{e} ± 0.60	1.51^{b} ± 0.36	2.93^{ab} ± 0.42
	(63.37)	(2.93)	(15.24)	(29.57)
14	6.14^{d} ± 0.56	0.24^{e} ± 0.49	1.67^{a} ± 0.08	2.98^{a} ± 0.21
	(61.96)	(2.42)	(1.69)	(30.07)

* The solubility is expressed as mg of soluble protein per milliliter.

** I : salt soluble ; II : Urea soluble ; III : $NaBH_4$ soluble ; IV : insoluble fractions. Values in parenthesis represent solubility percentage.

*** The same letter in the same column indicates no significant differences ($p<0.05$).

Total SH content

A decrease in total sulhydryl content of seabass actomyosin was observed, particularly during day 8 and 14 (Figure 7). This suggested the formation of disulfide bond in seabass actomyosin during prolonged storage in ice. At day 14, total sulhydryl content decreased to 73.13%. The

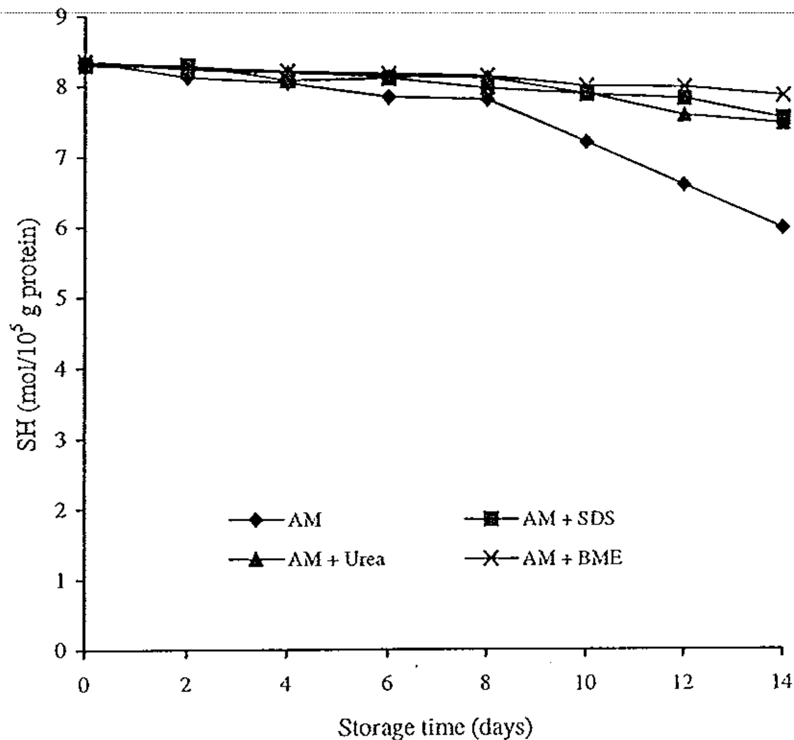


Figure 7 : Total sulhydryl group content of seabass actomyosin during iced storage. Total SH was calculated from absorbance at 412 nm using the molar extinction coefficient of 13,600 M^{-1}. cm^{-1} for 2-nitro-5-thiobenzoic acid.

decrease in total sulhydryl group was reported to be due to formation of disulfide bonds through oxidation of sulhydryl groups or disulfide interchanges (Hayakawa and Nakai, 1985). This confirmed results of Hamada *et al.* (1977) that the sulhydryl content of carp actomyosin decreased, while disulfide bonds increased. Sompongse *et al.* (1996b)

also reported that the total SH group content of carp actomyosin decreased constantly, especially after 3 days of storage in ice. Myosin head portions (SH_1 and SH_2) have been reported to be involved in ATPase activities of myosin (Chen *et al*, 1995; Yamaguchi and Sekine, 1966). In presence of βME, a reducing agent, a lowest change in total SH content during 14 days was found. This result coincided with a sharp decrease in total SH content in the absence of reducing agent. This indicated a significance of disulfide bond during iced storage. In presence of either SDS or urea, the reduction of total SH content was retarded, though both compounds are not known as reducing agents. This result suggested that the formation of hydrogen or hydrophobic interaction possibly accelerated the oxidation of sulhydryl group via some changes of protein conformation, in which free sulhydryl group in protein molecule could be oriented in the appropriate position for disulfide formation.

ATPase activity

An enzymatic activity is often a sensitive indicator of changes in the protein configuration. The changes of Ca^{2+}- ATPase activity of seabass actomyosin during iced storage are shown in Figure 8. The activity decreased sharply after 8 days of storage, but no changes in actomyosin added with chemical reagents, particularly the sample treated with βME, were observed. The decrease in Ca^{2+}-ATPase activity was coincident with a decrease in SH group (Figure 7). Therefore, disulfide formation in myosin globular head possibly involved in a loss of activity. Ca^{2+}-ATPase activity is considered to be a good indicator of myosin functionality in the actomyosin complex (Roura *et al.*, 1990) and

integrity of the myosin molecule (Roura and Crupkin, 1995). The reactive SH, SH_1, on the head portion of myosin may contribute to those changes (Sompongse *et al.*, 1996b)

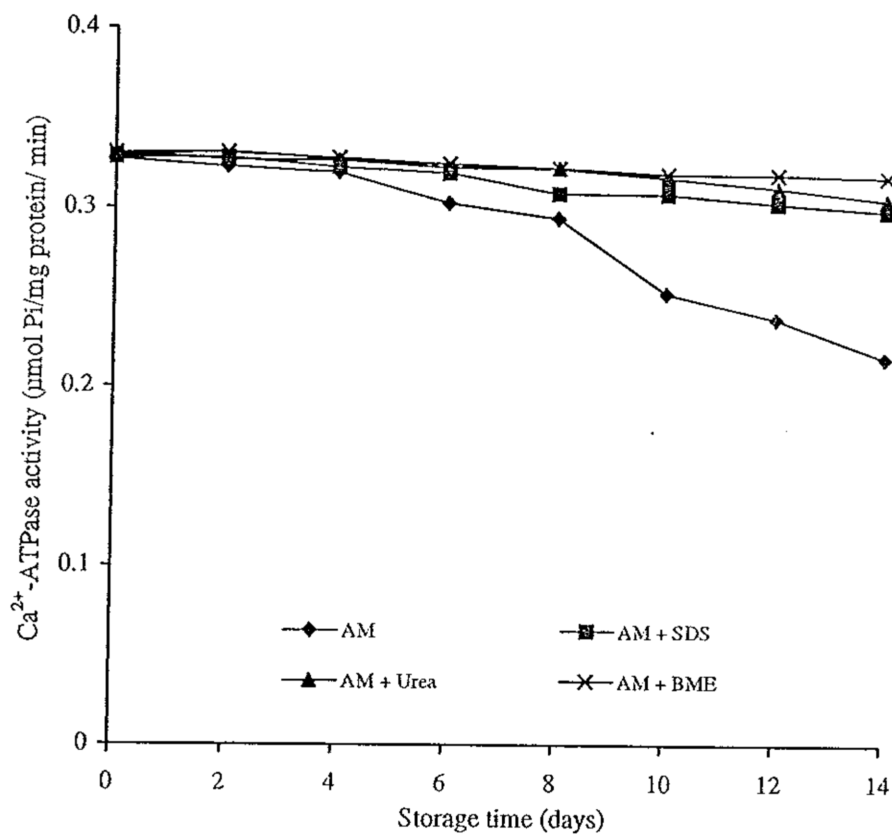


Figure 8 : Ca^{2+}-ATPase activity of seabass actomyosin during iced storage. One unit activity was defined as that releasing 1 μ mol Pi/mg protein/min.

No changes in Mg^{2+}-ATPase and Mg^{2+}-Ca^{2+}-ATPase activities of seabass actomyosin in presence or absence of chemical reagents were found (Figures 9, 10). Mg^{2+}-ATPase activity was found in the sarcoplasmic recticulum, near both ends of A band, mitochondria, and nucleaus membrane (Tachibana *et al.*, 1993). The Mg^{2+}-ATPase activity functions through regulatory proteins such as troponin and tropomyosin, thus inducing muscle contraction (Ebasi and Endo, 1968). Mg^{2+}-ATPase is responsible for interaction between actin and myosin in the presence of endogenous Ca ion (Roura *et al.*, 1990). Roura and Crupkin (1995) reported that this enzyme activity was low in myofibrils from pre-spawned hake.

Mg^{2+}-EGTA-ATPase activity of seabass actomyosin in absence or presence of chemical reagents is shown in Figure 11. The activity increased significantly in the control ($p<0.05$) but no changes in actomyosin added with chemical reagents ($p>0.05$) were observed. These result was in accordance with Benjakul *et al.*(1997) who reported the increase in Mg^{2+}-EGTA-ATPase activity of Pacific whiting myofibrillar proteins during 8 days of iced storage. The change in Mg^{2+}-EGTA-ATPase indicates the change of troponin-tropomyosin complex (Ebashi and Endo, 1968; Watabe *et al.*, 1989). Additionally, proteinase in muscle could play a partial role in these changes. Mg^{2+}-EGTA-ATPase activity of myofibrils was reported to increase by treatment with lysosomal protease (Ouali and Valin, 1981).

Ca^{2+}-sensitivity of seabass actomyosin sharply decreased with storage time. However, no changes were observed in the samples added with chemical reagents (Figure 12). Modifications in actin-myosin interaction could cause loss of Ca^{2+}-sensitivity.. From the result, the

changes in ATPase activities and the loss of Ca^{2+}-sentivity of the myofibril during iced storage, especially in control may be due to the modification of actin-myosin interaction by the oxidation of the thiol groups of of myosin moiety (Seki *et al.*, 1979 ; Sompongse *et al*, 1996b ; Benjakul *et al.*, 1997). Ca^{2+}-sensitivity was reported to be a good indicator of Ca^{2+}-regulation of myofibrillar proteins (Roura and Crupkin, 1995). The Ca^{2+}-sensitivity of myofibrillar proteins was attributed to the activity of native tropomyosin (Ebashi *et al.*, 1986). The decrease in Ca^{2+}-binding and Ca^{2+}-sensitivity was shown to be caused by proteolysis (Tokiwa and Matsumiya, 1969). However, the loss of Ca^{2+}-sensitivity of myofibrillar proteins during freezing was considered to result from the modification of actin-myosin interaction caused by the oxidation of SH in myosin, instead of the hydrolysis of tropomyosin and troponins by protease (Seki *et al.*, 1978; 1979).

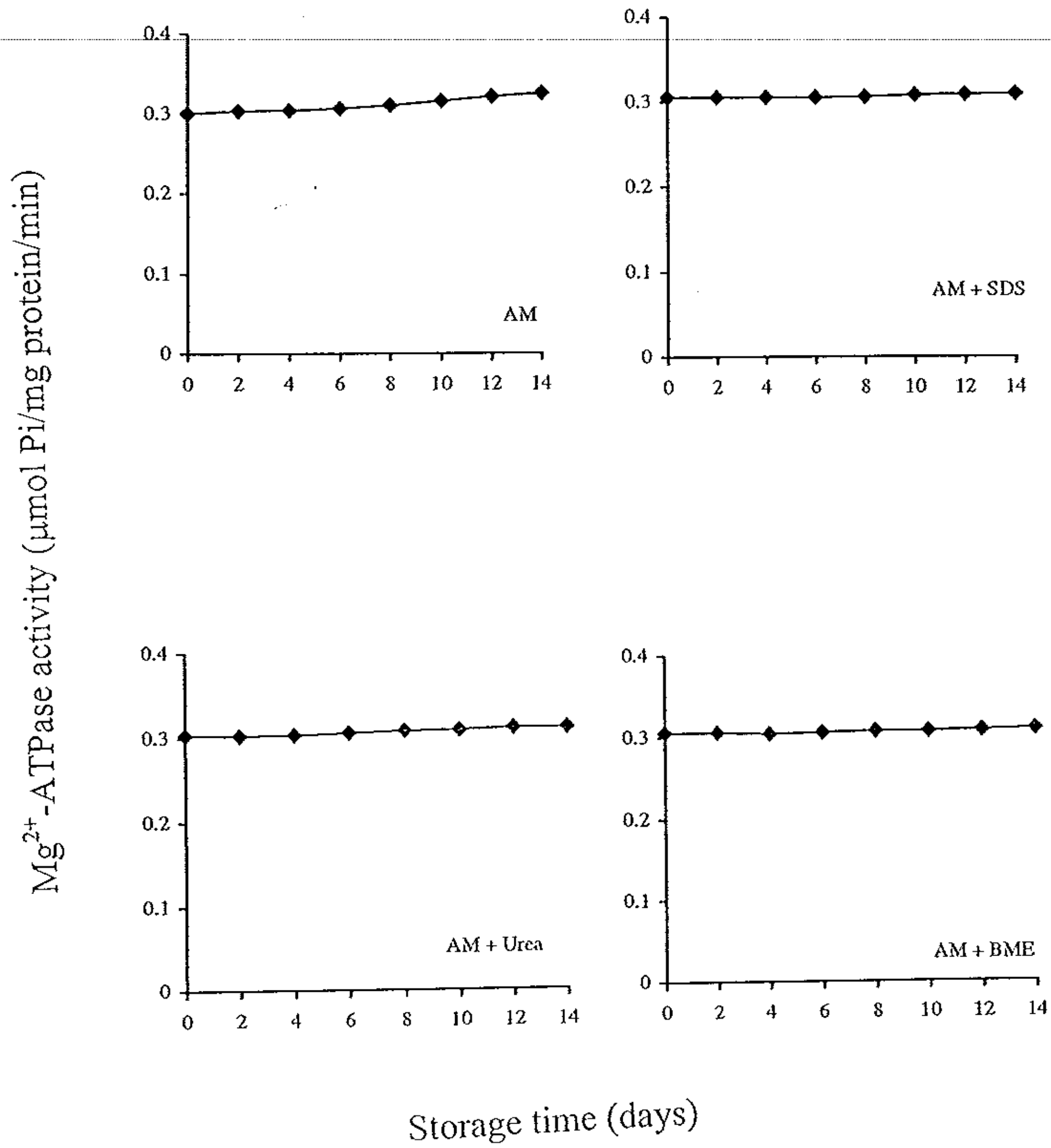


Figure 9 : Mg^{2+}-ATPase activity of seabass actomyosin during iced storage. One unit activity was defined as that releasing 1 µmol Pi/mg protein/min.

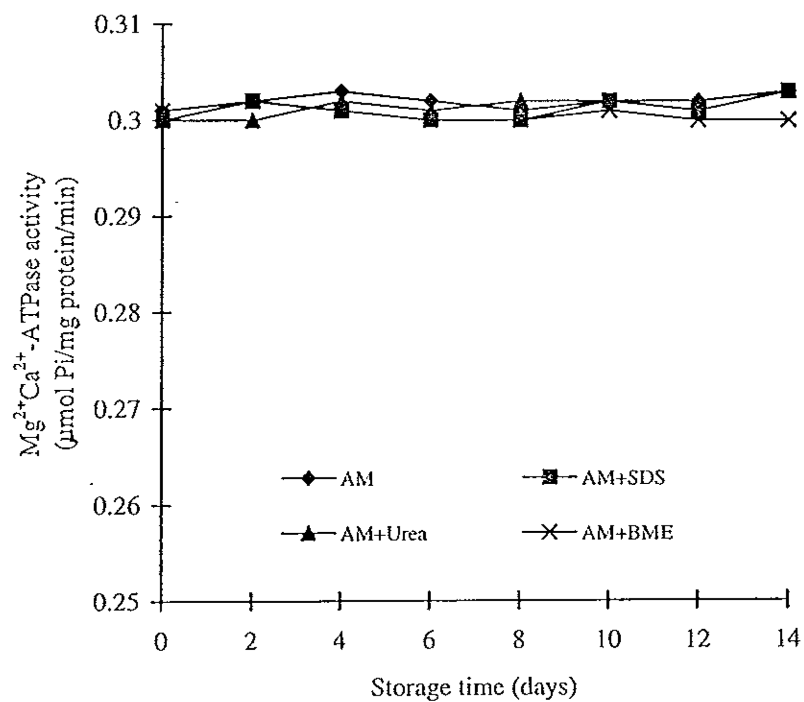


Figure 10 : Mg^{2+}-Ca^{2+}-ATPase activity of seabass actomyosin during iced storage. One unit activity was defined as that releasing 1 µ mol Pi/mg protein/min.

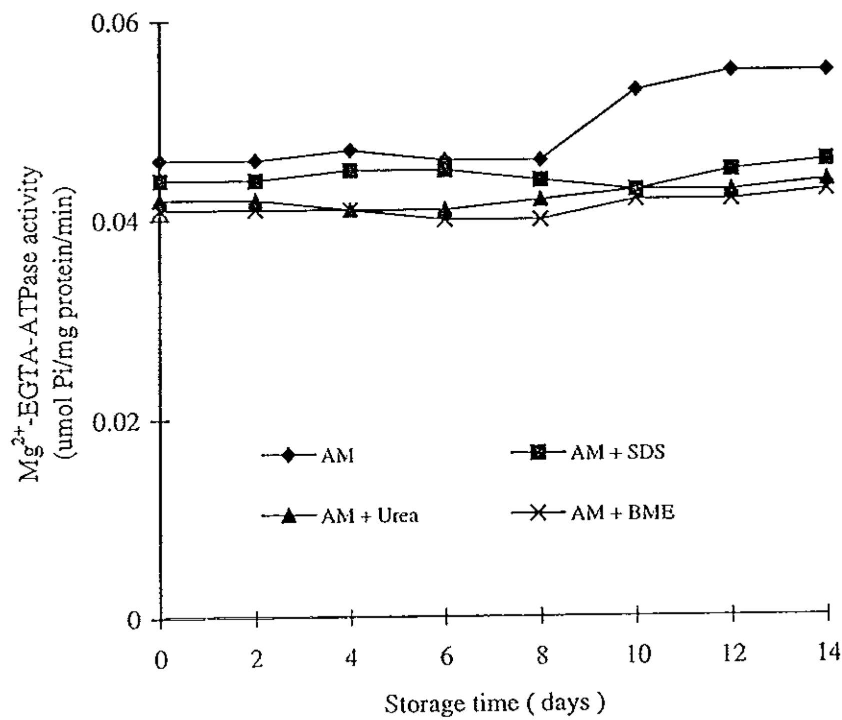


Figure 11 : Mg^{2+}-EGTA-ATPase activity of seabass actomyosin during iced storage. One unit of activity was defined as that releasing 1 μmol Pi/mg protein/min.

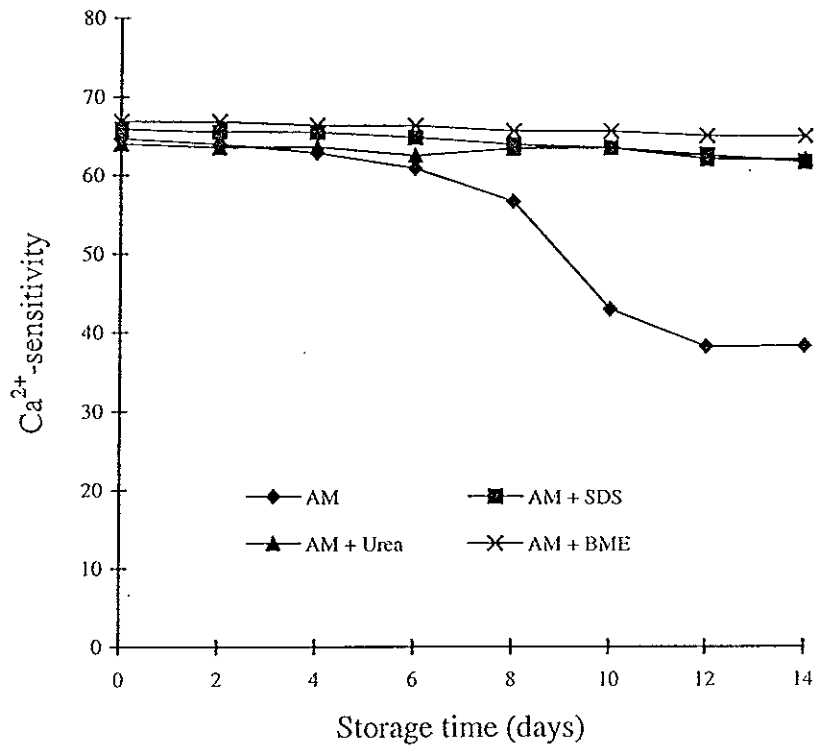


Figure 12 : Ca^{2+}-sensitivity of seabass actomyosin during iced storage.

Free α - amino acid

The change in the free α - amino acid of all samples are given in Table 14. No significant changes in free α - amino acid were observed in samples added with SDS, urea or βME, while free α-amino acid in actomyosin increased approximately 3 times after 14 days storage ($p < 0.05$). During storage, seabass actomyosin could undergo autolysis caused by endogenous enzymes. Those enzymes contributed to the hydrolysis of proteins, releasing more free α-amino acid. This result coincided with a decrease in myosin heavy chain band as shown in Figure 13. From the result, it was postulated that myofibril associated proteinase mainly played an essential role in degradation, instead of sarcoplasmic proteinases. This was due to the removal of sarcoplasmic proteins during actomyosin preparation. Therefore, some proteolysis occurred during iced storage, partially resulting in physico-chemical changes of muscle protein.

Table 14 : Free α- amino acid of seabass actomyosin during iced storage.

Storage time (days)	Treatment*			
	AM	AM + SDS	AM + Urea	AM + BME
0	$0.64^{c**} \pm 0.92$	$0.62^{a} \pm 0.45$	$0.62^{a} \pm 0.06$	$0.63^{a} \pm 0.62$
2	$0.68^{c} \pm 0.44$	$0.63^{a} \pm 0.69$	$0.63^{a} \pm 0.65$	$0.63^{a} \pm 0.84$
4	$0.64^{c} \pm 0.38$	$0.63^{a} \pm 0.02$	$0.65^{a} \pm 0.48$	$0.63^{a} \pm 0.06$
6	$0.66^{c} \pm 0.20$	$0.63^{a} \pm 0.83$	$0.63^{a} \pm 0.31$	$0.63^{a} \pm 0.12$
8	$0.99^{b} \pm 0.55$	$0.65^{a} \pm 0.65$	$0.65^{a} \pm 0.02$	$0.64^{a} \pm 0.67$
10	$1.12^{ab} \pm 0.95$	$0.68^{a} \pm 0.92$	$0.65^{a} \pm 0.16$	$0.68^{a} \pm 0.52$
12	$1.84^{a} \pm 0.68$	$0.82^{a} \pm 0.39$	$0.70^{a} \pm 0.09$	$0.68^{a} \pm 0.84$
14	$1.87^{a} \pm 0.20$	$0.85^{a} \pm 0.41$	$0.72^{a} \pm 0.76$	$0.70^{a} \pm 0.91$

AM : seabass actomyosin ; AM + SDS : actomyosin was added with sodium dodecyl sulfate ; AM + urea : actomyosin was added with urea ; AM + BME : actomyosin was added with β-mercaptoethanol.

** The same letter in the same column indicates no significant differences ($p > 0.05$).

SDS-PAGE patterns of seabass actomyosin during iced storage

A decrease in myosin heavy chain (MHC) was observed in sample kept for 14 days (Figure 13). The decrease in MHC was postulated to be due to either degradation caused by proteolysis or cross-linking of MHC during iced storage. Sompongse *et al.* (1996a) found a decrease in MHC of actomyosin after 12 days storage in ice, suggesting the polymer formation through disulfide forming. In presence of denaturing agents e.g., SDS, urea, and βME, no changes in protein pattern were observed. This was postulated that those chemicals caused the denaturation of either enzymes or proteins. As a result, proteinase activity was inhibited. In addition, aggregation could be retarded since those chemicals were known to prevent bond formation. Therefore, physicochemical changes in seabass actomyosin could be caused by either proteolysis or denaturation.

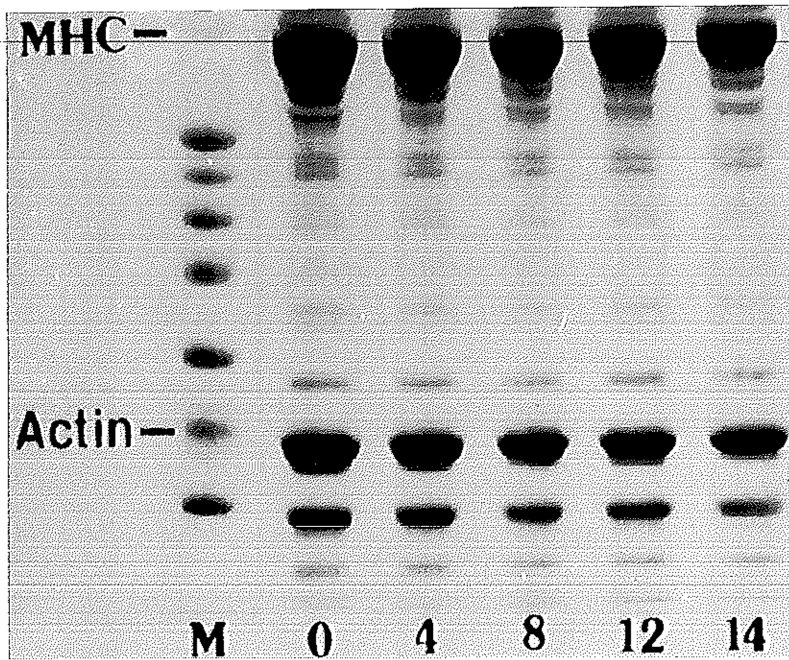


Figure 13 : SDS-PAGE pattern of seabass actomyosin during iced storage. Ten μg protein were applied on 10% polyacrylamide gel. Numbers designate storage time (days). M, high molecular weight standard ; MHC, myosin heavy chain.

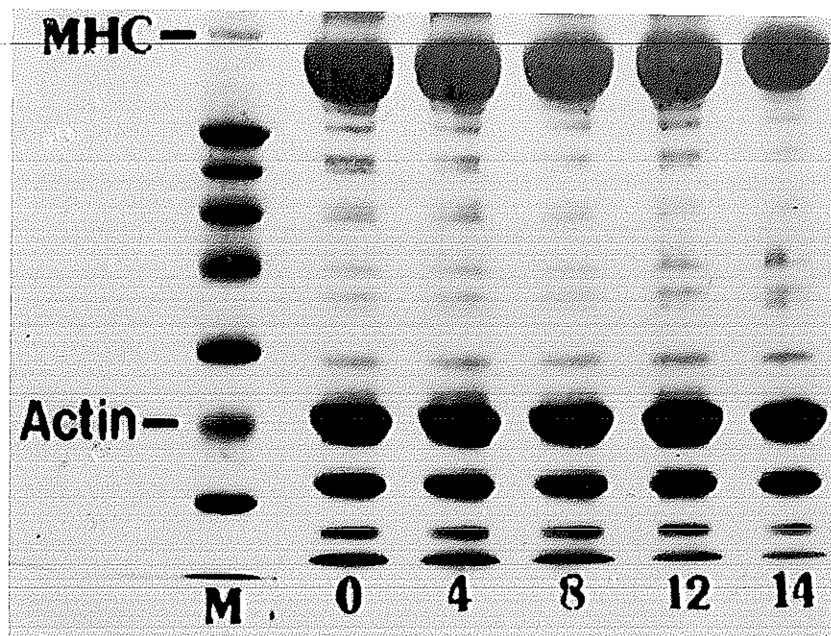


Figure 14 : SDS-PAGE pattern of seabass actomyosin added with sodium dodecyl sulfate during iced storage. Ten μg protein were applied on 10% polyacrylamide gel. Numbers designate storage time (days). M, high molecular weight standard ; MHC, myosin heavy chain.

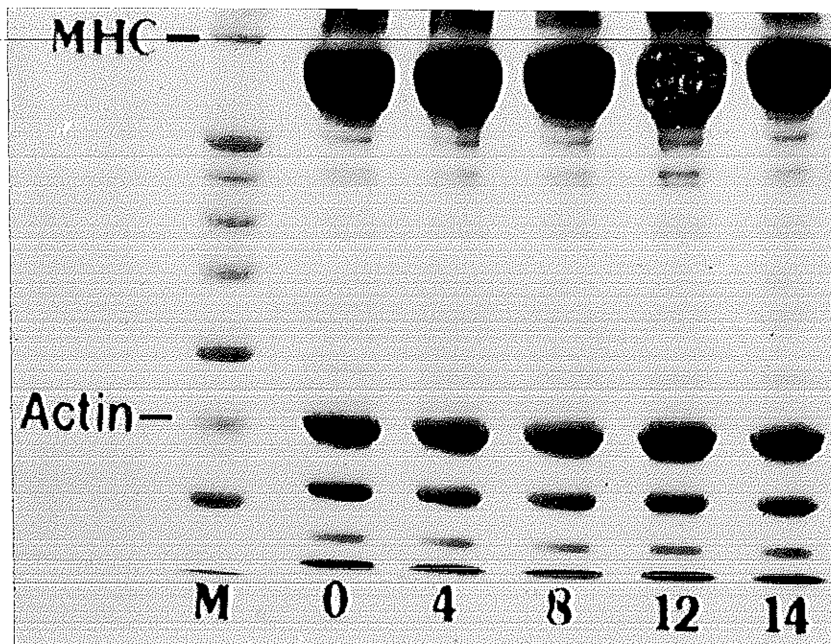


Figure 15 : SDS-PAGE pattern of seabass actomyosin added with urea during iced storage. Ten µg protein were applied on 10% polyacrylamide gel. Numbers designate storage time (days). M, high molecular weight standard ; MHC, myosin heavy chain.

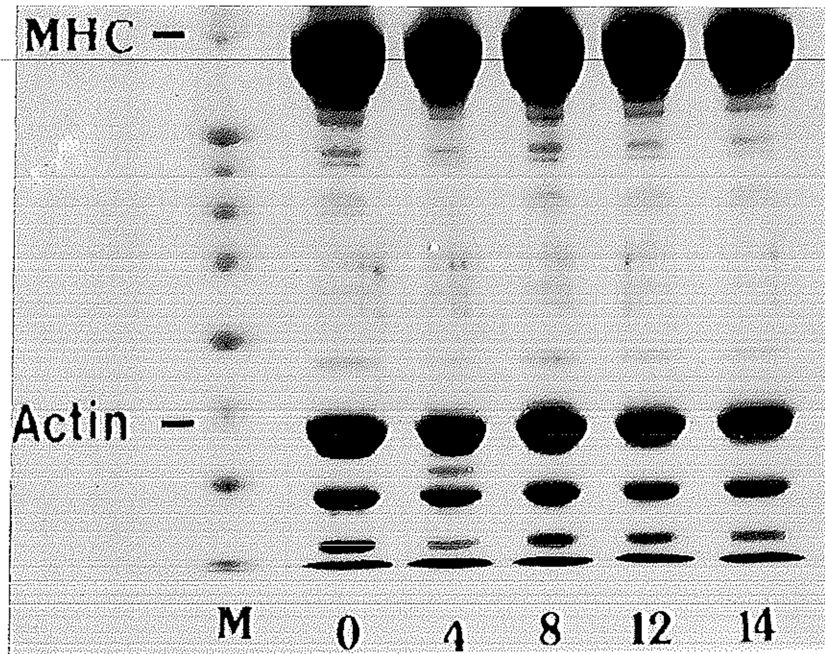


Figure 16 : SDS-PAGE pattern of seabass actomyosin added with β-mercaptoethanol during iced storage. Ten μg protein were applied on 10% polyacrylamide gel. Numbers designate storage time (days). M, high molecular weight standard ; MHC, myosin heavy chain.

Seabass actomyosin during frozen storage

Solubility

The solubility of seabass actomyosin is shown in Table 15. After 6 months of frozen storage, salt-soluble fraction (I) of seabass actomyosin stored at –18°C and –80°C decreased to 56.23 and 92.76%, respectively. During storage, the quantity of salt-soluble fraction of seabass actomyosin decreased with the duration of storage. The decreasing rate of the salt-soluble fraction (I) was higher when stored at -18°C than that stored at -80°C. This indicated that the salt soluble protein underwent denaturation via aggregation, resulting in the loss of solubility. After freezing, the quantity of urea-soluble fraction (II) at -18°C was higher than that at –80°C. The quantity of urea-soluble fraction of seabass actomyosin increased during storage. At -18°C, it increased during 2.5 month of storage, but decreased after 3 months. The decrease in urea-soluble proteins might be due to the formation of the disulfides. The increase in urea-soluble fraction indicated the formation of hydrogen and hydrophobic bond during storage. The total $NaBH_4$-soluble (III) and insoluble fractions (IV) of seabass actomyosin stored at -18°C increased, particularly after 3 months of storage. The increase in $NaBH_4$-soluble proteins indicated the presence of disulfide bond increased, particularly when the time increased. The disulfide bond formed in seabass actomyosin stored at -18°C was higher than that stored at -80°C. This confirmed results of Jiang *et al.*(1988) who reported that the decrease in salt soluble fraction of milkfish actomyosin was higher at –20°C than that at –35°C. When compared insoluble fraction between samples stored at –18°C and –80°C, it was found that samples kept at

Table 15: The solubility of seabass actomyosin during frozen storage.

Storage time (months)	Fractions*							
	-18°C				-80°C			
	I	II	III	IV	I	II	III	IV
0	$10.19^{a**} \pm 0.16$	$0.31^{cd} \pm 0.01$	$0.09^{h} \pm 0.06$	$0.27^{c} \pm 0.10$	$10.22^{a} \pm 0.00$	$0.28^{f} \pm 0.01$	$0.09^{c} \pm 0.00$	$0.27^{g} \pm 0.00$
	(100.00)	(3.04)	(0.88)	(2.65)	(100.00)	(2.74)	(0.88)	(2.64)
0.5	$10.19^{a} \pm 0.18$	$0.31^{ab} \pm 0.01$	$0.09^{h} \pm 0.01$	$0.27^{c} \pm 0.05$	$10.21^{ab} \pm 0.00$	$0.28^{f} \pm 0.00$	$0.09^{c} \pm 0.01$	$0.28^{g} \pm 0.02$
	(99.98)	(3.04)	(0.88)	(2.65)	(99.90)	(2.76)	(0.90)	(2.74)
1.0	$10.18^{a} \pm 0.10$	$0.32^{ab} \pm 0.00$	$0.09^{h} \pm 0.00$	$0.27^{c} \pm 0.09$	$10.21^{ab} \pm 0.02$	$0.29^{f} \pm 0.00$	$0.09^{c} \pm 0.00$	$0.28^{g} \pm 0.00$
	(99.86)	(3.14)	(0.89)	(2.66)	(99.89)	(2.84)	(0.90)	(2.75)
1.5	$10.18^{a} \pm 0.06$	$0.32^{ab} \pm 0.01$	$0.09^{h} \pm 0.04$	$0.27^{c} \pm 0.09$	$10.21^{ab} \pm 0.01$	$0.29^{f} \pm 0.01$	$0.09^{c} \pm 0.01$	$0.28^{g} \pm 0..03$
	(99.87)	(3.14)	(0.89)	(2.66)	(99.87)	(2.94)	(0.92)	(2.78)
2.0	$10.18^{a} \pm 0.14$	$0.32^{ab} \pm 0.03$	$0.09^{h} \pm 0.07$	$0.27^{c} \pm 0.00$	$10.14^{ab} \pm 0.09$	$0.30^{ef} \pm 0.00$	$0.09^{c} \pm 0.00$	$0.34^{fg} \pm 0.06$
	(99.86)	(3.15)	(0.89)	(2.68)	(99.22)	(2.94)	(0.92)	(3.33)
2.5	$10.12^{a} \pm 0.05$	$0.38^{a} \pm 0.00$	$0.09^{h} \pm 0.05$	$0.28^{c} \pm 0.01$	$10.10^{ab} \pm 0.08$	$0.32^{ef} \pm 0.02$	$0.09^{bc} \pm 0.01$	$0.35^{fg} \pm 0.02$
	(99.27)	(3.73)	(0.89)	(2.75)	(98.83)	(3.13)	(0.92)	(3.42)
3.0	$9.18^{b} \pm 0.08$	$0.24^{bc} \pm 0.08$	$0.18^{g} \pm 0.00$	$1.26^{b} \pm 0.00$	$10.10^{ab} \pm 0.06$	$0.33^{ef} \pm 0.01$	$0.10^{bc} \pm 0.00$	$0.35^{fg} \pm 0.00$
	(90.05)	(2.36)	(1.77)	(12.37)	(97.93)	(3.22)	(0.98)	(3.44)

*The solubility is expressed as mg of soluble protein per millilitre. Values in parentheses represent solubility percentage.
**The same letter in the same column indicates no significant differences (*p*>*0.05*).

Table 15: The solubility of seabass actomyosin during frozen storage (continued).

Storage time (months)	Fractions*							
	-18°C				-80°C			
	I	II	III	IV	I	II	III	IV
3.5	$9.14^{b**} \pm 0.08$	$0.23^{cd} \pm 0.02$	$0.28^{f} \pm 0.05$	$1.22^{b} \pm 0.00$	$10.07^{cd} \pm 0.03$	$0.34^{ab} \pm 0.05$	$0.10^{bc} \pm 0.02$	$0.35^{gf} \pm 0.04$
	(89.70)	(2.26)	(2.75)	(11.97)	(98.53)	(0.98)	(0.98)	(3.42)
4.0	$8.64^{c} \pm 0.38$	$0.19^{cde} \pm 0.06$	$0.34^{e} \pm 0.02$	$1.69^{b} \pm 0.02$	$10.03^{d} \pm 0.05$	$0.34^{ab} \pm 0.00$	$0.11^{bc} \pm 0.01$	$0.38^{efg} \pm 0.04$
	(84.79)	(1.80)	(3.34)	(16.58)	(98.14)	(3.36)	(1.08)	(3.72)
4.5	$8.14^{d} \pm 0.14$	$0.18^{cde} \pm 0.03$	$0.44^{d} \pm 0.02$	$2.11^{b} \pm 0.03$	$9.84^{e} \pm 0.08$	$0.35^{a} \pm 0.02$	$0.12^{bc} \pm 0.00$	$0.55^{c} \pm 0.01$
	(79.88)	(1.77)	(4.32)	(2.07)	(96.28)	(3.42)	(1.17)	(5.38)
5.0	$6.15^{e} \pm 0.05$	$0.17^{cde} \pm 0.00$	$0.62^{c} \pm 0.01$	$3.93^{a} \pm 0.05$	$9.80^{e} \pm 0.13$	$0.36^{a} \pm 0.04$	$0.12^{b} \pm 0.06$	$0.59^{b} \pm 0.02$
	(60.35)	(1.67)	(6.08)	(33.27)	(95.89)	(3.52)	(1.20)	(5.41)
5.5	$6.01^{e} \pm 0.10$	$0.15^{cde} \pm 0.06$	$0.76^{b} \pm 0.01$	$3.88^{a} \pm 0.10$	$9.51^{f} \pm 0.07$	$0.35^{ab} \pm 0.01$	$0.16^{a} \pm 0.03$	$0.85^{a} \pm 0.07$
	(58.98)	(1.47)	(7.46)	(38.08)	(93.05)	(3.46)	(1.57)	(8.32)
6.0	$5.73^{e} \pm 0.16$	$0.13^{f} \pm 0.08$	$0.98^{a} \pm 0.04$	$4.03^{a} \pm 0.16$	$9.48^{f} \pm 0.03$	$0.31^{de} \pm 0.08$	$0.22^{a} \pm 0.08$	$0.86^{a} \pm 0.06$
	(56.23)	(1.28)	(9.62)	(39.55)	(92.76)	(3.03)	(2.15)	(8.41)

*The solubility is expressed as mg of soluble protein per millilitre. Values in parentheses represent solubility percentage.
**The same letter in the same column indicates no significant differences ($p>0.05$).

-18°C had a much higher insoluble fraction. This result suggested the presence of non disulfide covalent bonds formed, particularly at warmer temperature.

Total SH content

The total SH content in all samples decreased throughout storage, especially after 4 months of frozen storage at -18°C. However, the control showed the highest decreasing rate, compared to other samples which were added with chemical reagents (Figure 17A). Additionally, sample added with βME showed a lowest decreasing rate throughout the storage. This suggested the crucial role of disulfide bond during frozen storage. During frozen storage, the reactive SH generally decreased. This was presumed due to either the oxidation of SH or the formation of hydrogen and hydrophobic bonds, which masked the reactive SH structure of actomyosin molecules (Jiang *et al.*, 1988; 1989). However, the total SH of samples stored at -80°C slightly decreased during storage, but no significant changes in sample added with βME were observed during prolonged storage ($p>0.05$) (Figure 17B).

Based on the changes in the total SH and $NaBH_4$-soluble and insoluble fractions of actomyosin during frozen storage, the oxidation of SH to SS was faster at -18°C than at -80°C. Disulfide bond formation was accelerated at high temperature, compared to low temperature. The total SH content of milkfish actomyosin stored at –20°C more decreased than that at –35°C (Jiang *et al.*, 1988). During prolonged storage, the tertiary structure of actomyosin was changed by formation of disulfide, hydrogen and hydrophobic bond. The reactive SH groups masked in molecules were gradually exposed and oxidized to disulfide (Jiang *et al.*, 1988;

1989). Therefore, it can be concluded that disulfide bonds contributed to protein denaturation during frozen storage.

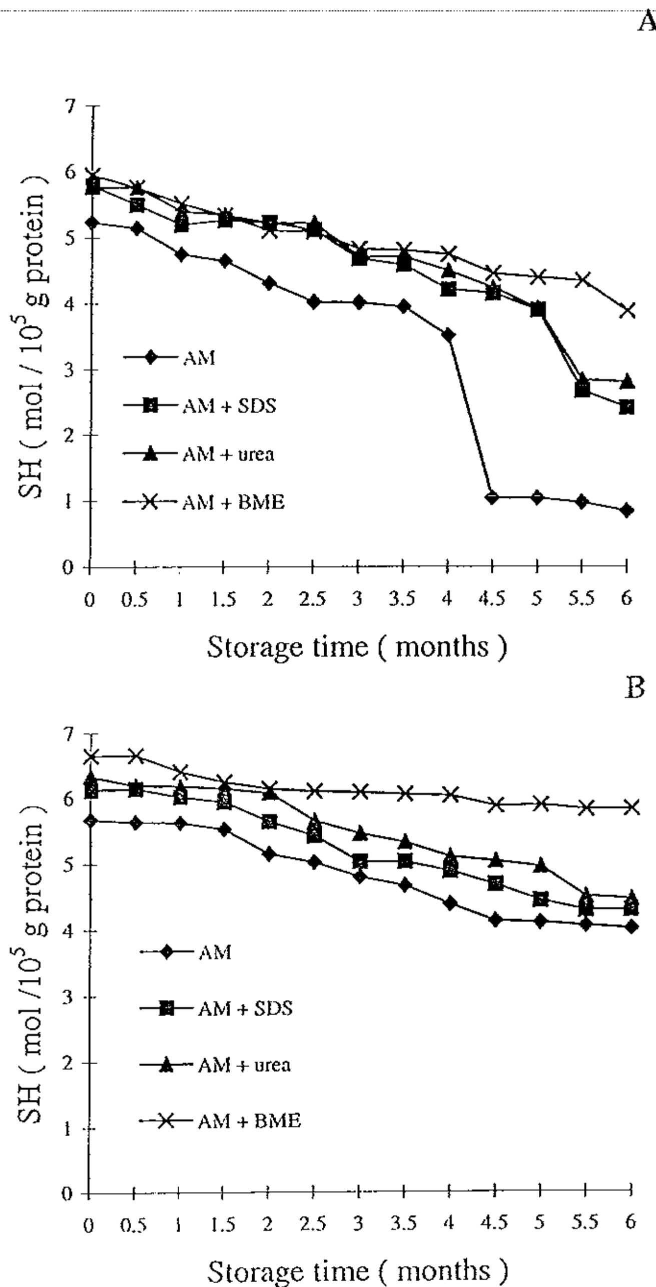


Figure 17 : Total sulhydryl group content of seabass actomyosin during frozen storage. Total SH was calculated from absorbance at 412 nm using the molar extinction coefficient of 13,600 M^{-1} cm^{-1} for 2-nitro-5-thiobenzoic acid.

A : -18°C ; B : -80°C

ATPase activity

After 4 months of storage, Ca^{2+}-ATPase activity of seabass actomyosin stored at -18°C decreased sharply (Figure 18). Actomyosin added with βME showed quite constant ATPase activity throughout the storage, while actomyosin added with either SDS or urea exhibited similar activity. However, no significant changes during 6 months of storage at –80°C were observed. In general, the result was in accordance with those observed for total sulhydryl group. The decrease in Ca^{2+}-ATPase activity was reported to be highly related to the oxidation of SH (Hamada *et al.*, 1977; Jiang *et al.*, 1988; 1989). The decrease in the SH and increase in insoluble fraction during frozen storage (Figure 17 and Table 15) indicated that the loss of Ca^{2+}-ATPase activity might be due to the oxidation of SH on myosin globular head. Suzuki (1976) and Hatano (1968) postulated that the loss of enzymic activity of actomyosin during frozen storage was due to the tertiary structural changes caused by iced crystallization.

No changes in Mg^{2+}-ATPase and Mg^{2+}-Ca^{2+}-ATPase activities in all samples during storage at -18°C and -80°C were found (Figure 19 and 20). Mg^{2+}- and Mg^{2+}-Ca^{2+}-ATPase activities are indicative of the integrity of the actin-myosin complex in the presence of endogenous or exogenous Ca^{2+}ions, respectively.

After 4 months of storage, Mg^{2+}-EGTA-ATPase activity increased with a loss of Ca^{2+}-sensitivity in seabass actomyosin, particularly in the control stored at -18°C (Figure 21 and 22). No changes were found in seabass actomyosin added with chemical reagents. The Ca^{2+}-sensitivity decreased slightly during the first 4 months of storage at -18°C, whereas no marked changes in Ca^{2+}-sensitivity were found in

those stored at –80°C. Jiang *et al.* (1989) reported that the activity of Mg^{2+}-EGTA-ATPase of tilapia actomyosin increased gradually during frozen storage at –20°C and –40°C. The loss Ca^{2+}-sensitivity of actomyosin occurred in actomyosin stored at –20°C was greater than that at –40°C, and then decreased rapidly up to 6 months of storage. From the result, it appeared that the loss of Ca^{2+}-sensitivity of actomyosin stored at -18°C was observed more obviously than those obtained at -80°C.

The Ca^{2+}-sensitivity of myofibrillar proteins is attributed to the activity of native tropomyosin (Ebashi *et al.*, 1968). The loss of Ca^{2+}-sensitivity of myofibrillar proteins was considered to result from the modification of actin-myosin interaction caused by the oxidation of SH in myosin, instead of the hydrolysis of tropomyosin and troponins by protease (Seki *et al.*, 1978 ; Seki *et al.*, 1979). In this study, an increase in the insoluble fractions (Table 15), a decrease in total SH (Figure 17), and the loss of Ca^{2+}-sensitivity (Figure 22) suggested that the oxidation of SH in actomyosin during frozen storage occurred at a much faster rate at -18°C than at -80°C.

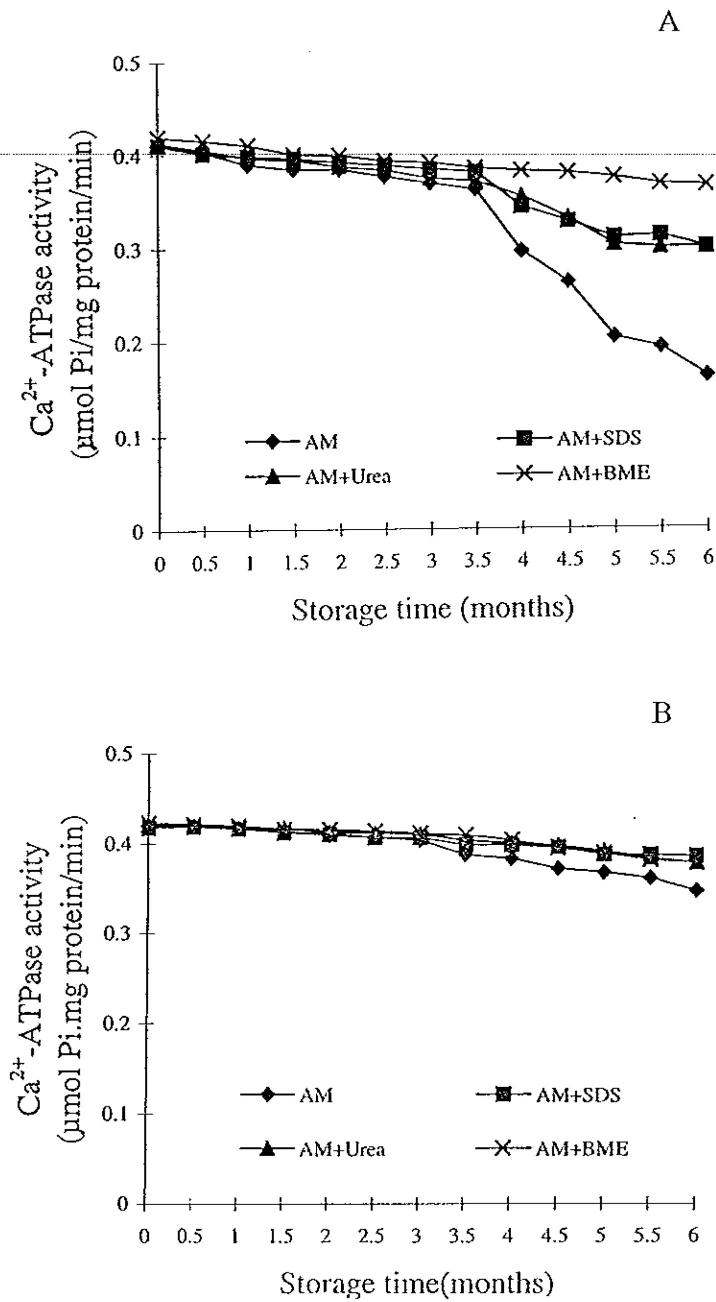


Figure 18 : Ca^{2+}-ATPase activity of seabass actomyosin during frozen storage. One unit of activity was defined as that releasing 1 µmol Pi/mg protein/min.

A : -18°C ; B : -80°C

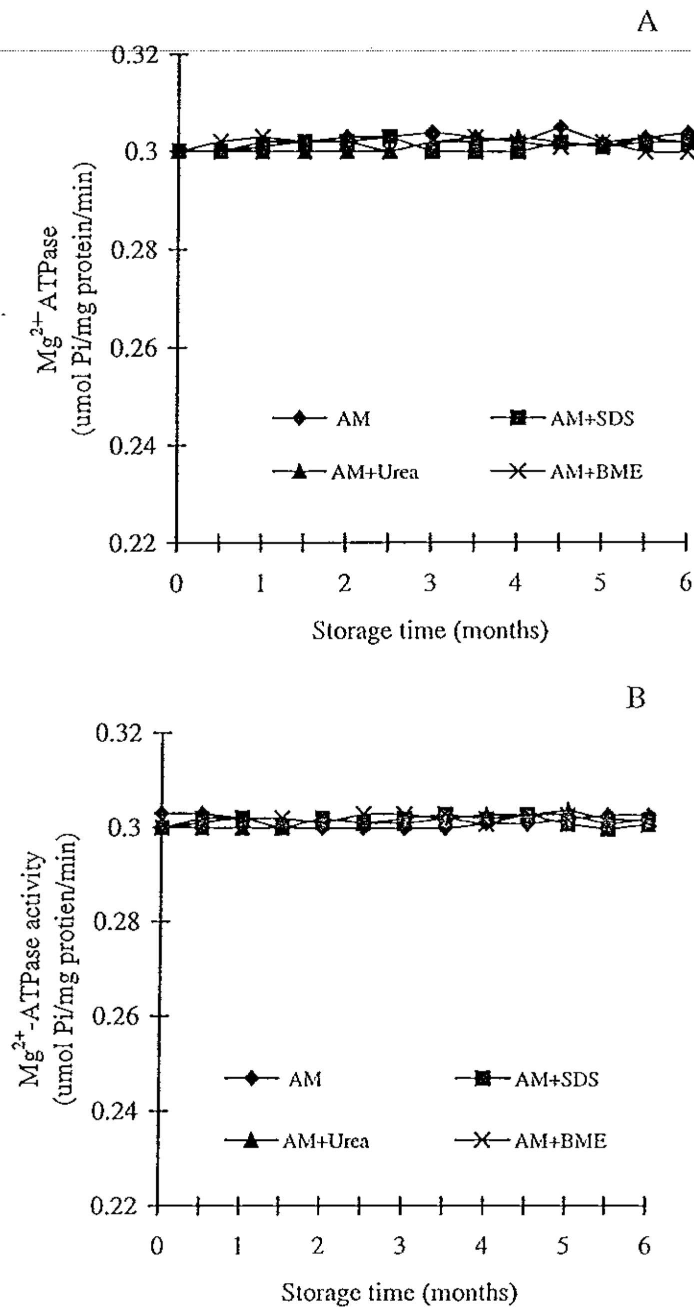


Figure 19 : Mg^{2+}-ATPase activity of seabass actomyosin during frozen storage. One unit of activity was defined as that releasing 1 µmol Pi/mg protein/min.

A :-18°C; B : -80°C

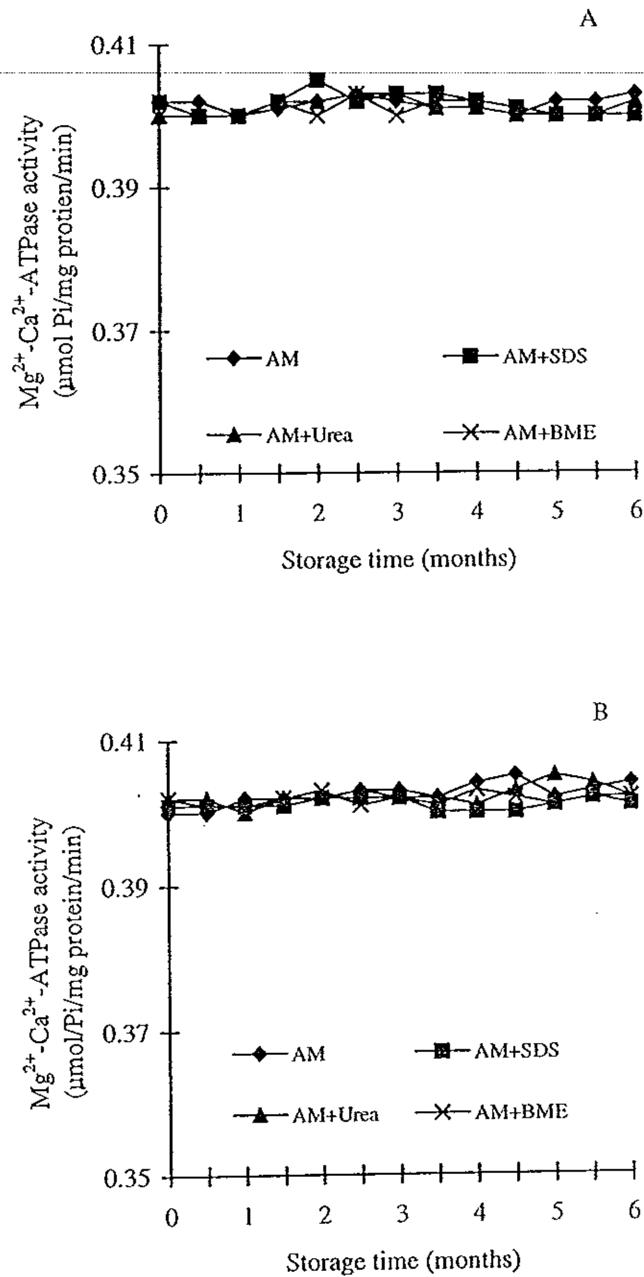


Figure 20 : Mg^{2+}-Ca^{2+}-ATPase activity of seabass actomyosin during frozen storage. One unit of activity was defined as that releasing 1 µmol Pi/mg protein/min.

A :-18°C ; B : -80°C

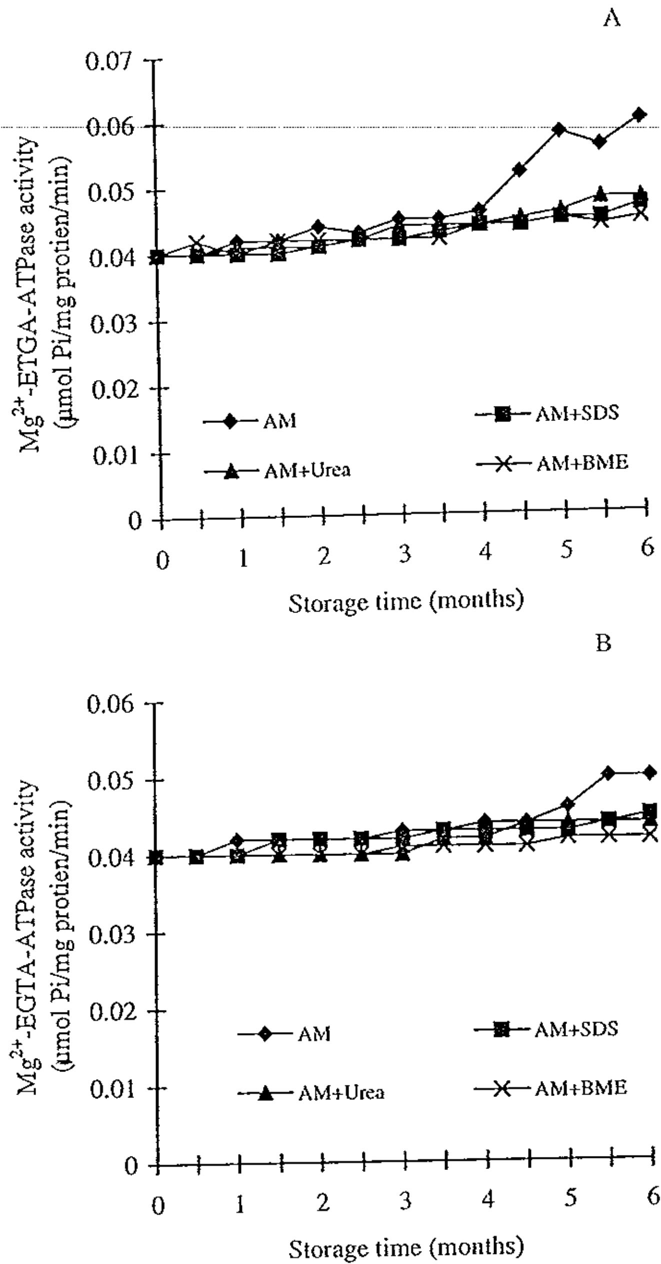


Figure 21 : Mg^{2+}-EGTA-ATPase activity of seabass actomyosin during frozen storage. One unit of activity was defined as that releasing 1 µmol Pi/mg protein/min.

A : -18°C; B : -80°C

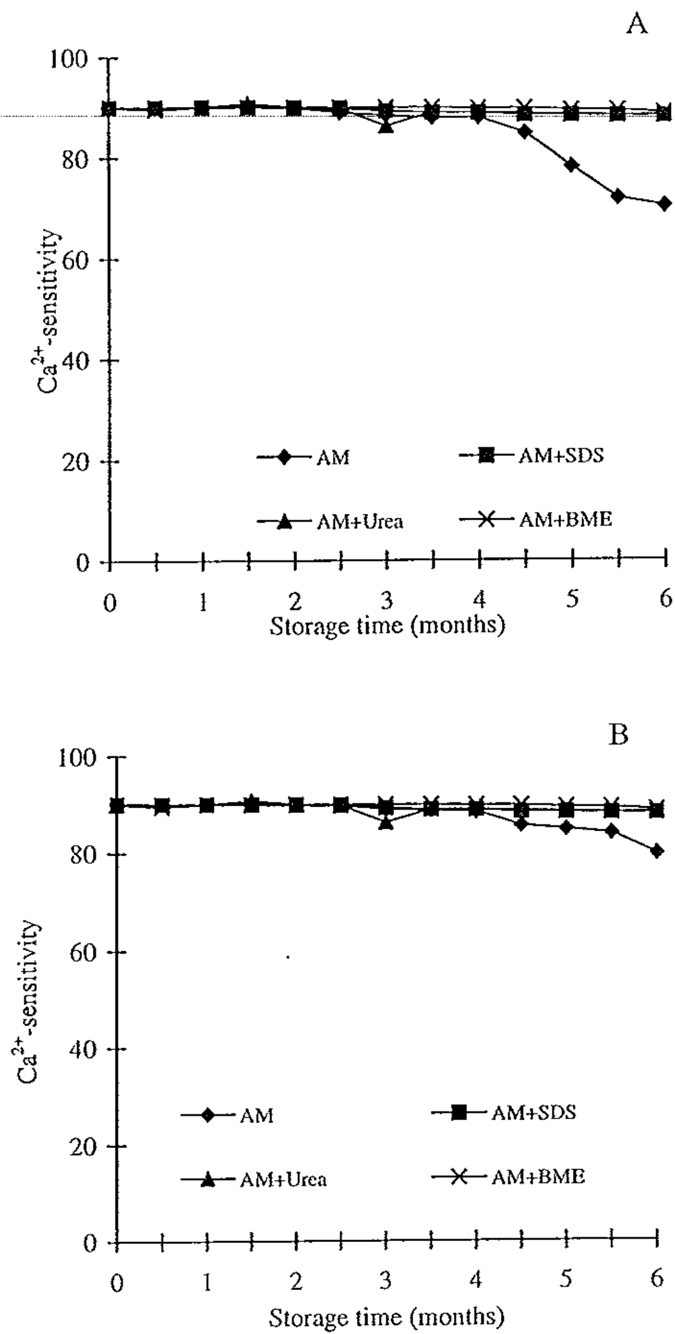


Figure 22 : Ca^{2+}-sensitivity of seabass actomyosin during frozen storage.

A : -18°C ; B : -80°C

Effect of freeze-thawing on seabass actomyosin.

Solubility

The effect of freeze-thawing methods on the stability of seabass actomyosin was investigated. As shown in the Table 16, the salt-soluble fraction (I) of frozen seabass actomyosin (-18°C and -80°C) thawed with tap water were higher than that thawed at room temperature ($p<0.05$). When thawed at room temperature, the quantity of 8M urea-soluble fraction (II) of seabass actomyosin frozen at -18°C was higher than that frozen at -80°C. The $NaBH_4$-soluble fraction (III) and insoluble fraction (IV) increased significantly in sample stored at –18°C and thawed at room temperature ($p<0.05$), compared with flesh actomyosin. The proteins soluble in fraction III was considered to result from the formation of disulfide. The quantity of III and IV fraction proteins of all samples was much lower than that of urea-soluble protein (II). These data indicated that denaturation occurred mostly via hydrogen and hydrophobic bond. Nevertheless, disulfide was also found during freeze-thawing process. Apparently, the solubility of frozen seabass actomyosin was affected by different thawing methods. This might be because the actomyosin is very unstable and denaturation easily occurred during frozen storage. Denaturation found in samples thawed at room temperature may result from long exposure to respective temperature. Under the same thawing method, samples frozen at lower temperature (-80°C) were less denatured, compared to those frozen at higher temperature (-18°C). For the study on freeze-thaw cycles, the results indicated that protein was more denatured when samples were subjected to more freeze-thaw cycles.

Table 16 : Effect of freeze/thawing method on the solubility of seabass actomyosin.

Storage temperature(°C)	Thawing method	Freeze-thaw cycle	Fractions*			
			I	II	III	IV
unfrozen			10.05^{a**} ± 0.03	1.75^{i} ± 0.05	0.12^{e} ± 0.03	0.22^{i} ± 0.04
-18	Tap water	1	9.98^{ab} ± 0.00	1.77^{hi} ± 0.02	0.15^{e} ± 0.05	0.24^{i} ± 0.08
		2	9.76^{c} ± 0.05	1.84^{ghi} ± 0.03	0.22^{d} ± 0.02	0.32^{h} ± 0.01
		3	9.56^{d} ± 0.02	1.88^{fg} ± 0.00	0.28^{c} ± 0.00	0.42^{g} ± 0.01
	Air temperature	1	8.95^{e} ± 0.08	1.85^{fgh} ± 0.06	0.32^{bc} ± 0.10	1.02^{e} ± 0.05
		2	8.54^{f} ± 0.03	1.98^{de} ± 0.02	0.44^{b} ± 0.06	1.18^{bc} ± 0.00
		3	8.30^{g} ± 0.05	2.16^{b} ± 0.03	0.45^{b} ± 0.00	1.23^{b} ± 0.06
-80	Tap water	1	9.84^{bc} ± 0.07	1.80^{ghi} ± 0.05	0.19^{de} ± 0.03	0.31^{h} ± 0.02
		2	9.08^{e} ± 0.03	1.82^{ghi} ± 0.09	0.32^{bc} ± 0.05	0.92^{f} ± 0.10
		3	8.95^{e} ± 0.00	1.94^{ef} ± 0.02	0.35^{b} ± 0.00	0.90^{f} ± 0.01
	Air temperature	1	8.63^{f} ± 0.08	2.05^{cd} ± 0.10	0.35^{b} ± 0.10	1.11^{d} ± 0.06
		2	8.50^{f} ± 0.10	2.09^{bc} ± 0.06	0.38^{b} ± 0.10	1.17^{c} ± 0.08
		3	7.21^{h} ± 0.09	2.47^{a} ± 0.10	0.65^{a} ± 0.05	1.81^{a} ± 0.08

* The solubility is expressed as mg of soluble protein per milliliter. ** The same letter in the same column indicates no significant differences (*p*<*0.05*).

Total SH content

Freeze/thaw cycle affected the total SH content of seabass actomyosin (Figure 23). The total SH content of samples with 3 freeze/thaw cycles decreased markedly, compared to other samples. When compared the effect of freezing temperature, the seabass actomyosin frozen at -80°C had higher total SH content for both thawing methods than those frozen at -18°C ($p<0.05$).

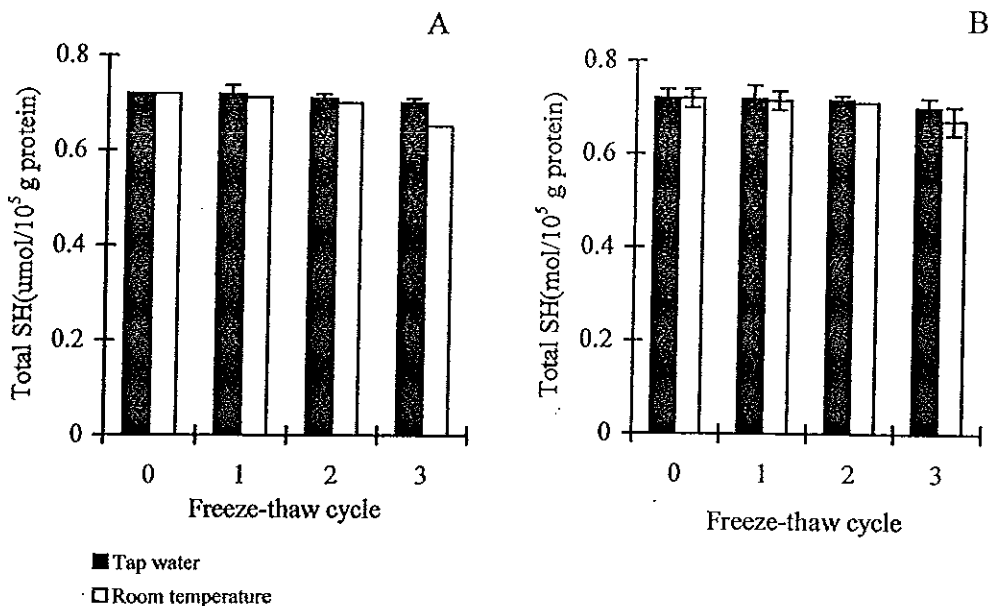


Figure 23 : Effect of freeze/thaw cycle on total sulhydryl content of seabass actomyosin. Total SH was calculated from absorbance at 412 nm using the molar extinction coefficient of 13,600 M^{-1} cm^{-1} for 2-nitro-5-thiobenzoic acid.

A : -18°C ; B : -80°C

This data indicated that freezing temperature had an essential role in actomyosin denaturation. Since a marked difference between samples frozen at -18°C with different thawing methods was obtained, particularly at 3 cycles, it also indicated that thawing method was another factor affecting the change of SH content. Thus freezing temperature, thawing method as well as freeze/thaw cycles directly affected the physico-chemical properties of seabass muscle protein.

ATPase activity

Changes in the ATPase activities of seabass actomyosin after freeze-thawing are shown in Table 17. Ca^{2+}-ATPase activity of actomyosin frozen at the both temperatures decreased significantly with the increase in freeze-thaw cycles ($p<0.05$). The decrease in Ca^{2+}-ATPase activity of samples thawed at room temperature was higher than that thawed by tap water ($p<0.05$). No changes in Mg^{2+}-ATPase activity of all samples were observed ($p>0.05$). However, the Mg^{2+}-Ca^{2+}-ATPase activity of samples decreased after freeze-thaw process ($p<0.05$). No changes in Mg^{2+}-ATPase activity were found in all samples tested. However, a significant increase in Mg^{2+}-EGTA-ATPase was found when freeze-thaw cycles increased ($p<0.05$). The largest changes in Ca^{2+}-ATPase and Mg^{2+}-EGTA-ATPase activities were observed in the samples subjected to freezing at -18°C and thawing at room temperature. This results indicated that myosin underwent denaturation either via aggregation or conformation changes, as indicated by the decrease in Ca^{2+}-ATPase activity. Additionally, troponin-tropomyosin complex was presumed to be affected to some extent, as indicated by a sharp increase in Mg^{2+}-EGTA-ATPase. An increase in Mg^{2+}-EGTA-ATPase activity

was concomitant with a decrease in Ca^{2+}-sensivity. This result suggested that freeze-thaw process directly caused the changes of muscle protein, particularly on myosin and troponin-tropomyosin complex.

Table 17 : Effect of freeze-thawing on ATPase activities of seabass actomyosin.

Storage Temperature (°C)	Thawing method / freeze-thaw cycle	ATPase activities* (µmol Pi/mg protein/min)			
		Ca^{2+}	Mg^{2+}	Mg^{2+}-Ca^{2+}	Mg^{2+}-EGTA
Unfrozen	-	$0.32^{**a}\pm0.02$	$0.48^{ab}\pm0.05$	$0.55^{a}\pm0.01$	$0.04^{h}\pm0.01$
-18	Tap water/1	$0.32^{a}\pm0.05$	$0.46^{ab}\pm0.02$	$0.50^{d}\pm0.05$	$0.04^{gh}\pm0.03$
	2	$0.30^{ab}\pm0.08$	$0.45^{bc}\pm0.11$	$0.50^{e}\pm0.00$	$0.04^{gh}\pm0.05$
	3	$0.29^{bc}\pm0.12$	$0.45^{bc}\pm0.07$	$0.46^{e}\pm0.02$	$0.05^{ef}\pm0.02$
	Room temp/1	$0.29^{bc}\pm0.10$	$0.43^{bc}\pm0.06$	$0.42^{f}\pm0.02$	$0.05^{e}\pm0.05$
	2	$0.20^{d}\pm0.12$	$0.44^{bc}\pm0.11$	$0.43^{f}\pm0.09$	$0.07^{b}\pm0.05$
	3	$0.15^{e}\pm0.06$	$0.43^{bc}\pm0.08$	$0.38^{h}\pm0.06$	$0.09^{a}\pm0.02$
-80	Tap water/1	$0.32^{a}\pm0.10$	$0.46^{ab}\pm0.03$	$0.55^{a}\pm0.06$	$0.04^{h}\pm0.00$
	2	$0.32^{a}\pm0.04$	$0.46^{ab}\pm0.09$	$0.52^{ab}\pm0.05$	$0.04^{gh}\pm0.06$
	3	$0.30^{ab}\pm0.02$	$0.45^{ab}\pm0.12$	$0.52^{ab}\pm0.08$	$0.05^{fg}\pm0.10$
	Room temp/1	$0.29^{bc}\pm0.11$	$0.45^{bc}\pm0.07$	$0.47^{e}\pm0.05$	$0.05^{e}\pm0.07$
	2	$0.27^{c}\pm0.00$	$0.45^{bc}\pm0.05$	$0.42^{f}\pm0.03$	$0.06^{d}\pm0.02$
	3	$0.20^{d}\pm0.06$	$0.45^{bc}\pm0.03$	$0.40^{g}\pm0.06$	$0.06^{c}\pm0.00$

*The ATPase activities are expressed as µmol Pi/ mg protein/min.

**The same letter in the same column indicates no significant differences ($p>0.05$).

The loss of Ca^{2+}-sensitivity of seabass actomyosin was dominant in sample frozen at –18°C and thawed at room temperature (Figure 24). The loss was much higher as the freeze-thaw cycles increased. Therefore, freezing at low temperature and thawing with the fast method effectively prevented the loss of Ca^{2+}-sensitivity.

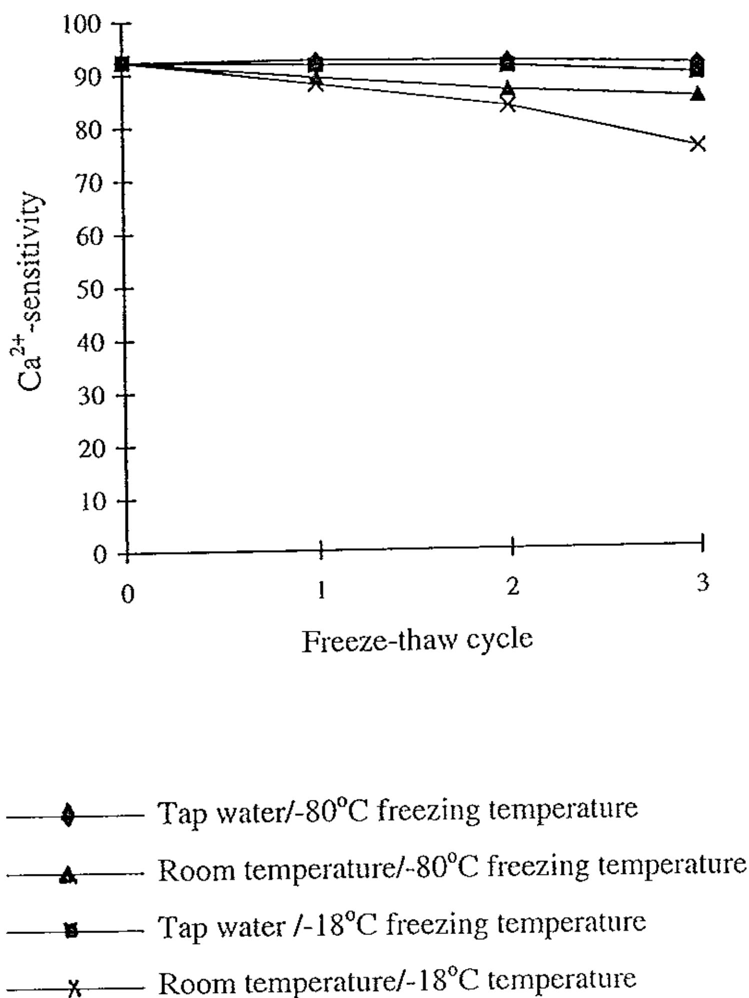


Figure 24 : Effect of freeze-thawing on Ca^{2+}-sentivity of seabass actomyosin.

Physicochemical changes of seabass muscle during iced and frozen storage

Seabass muscle during iced storage

Chemical analysis

pH of seabass muscle decreased slightly during 6 days of iced storage. However, pH of muscle gradually increased with further storage (Figure 25). The accumulation of lactic acid of muscle increased within 6 days of storage. Subsequently, lactic acid was decreased. The changes of pH in seabass muscle would normally be expected to reflect the changes in lactic acid concentration. ATP degradation and lactic acid accumulation were faster in the tilapia which were held at ambient temperature (Curran *et al.*, 1986). It is known that the pH of fish killed in relaxed conditions has a tendency to fall until the post-rigor state occurs. However, in fish which have struggled during capture, the minimal pH can be reached with in the first day of post-mortem storage (Chalmers *et al.*, 1988). The increase in pH of muscle during iced storage was probably due to spoilage of the fish (Chang and Regenstein, 1997). From the result, the increase in pH at the extended storage coincided with the increase in either TVB or TMA.

The ammonia content of seabass muscle during iced storage is shown in Figure 26. During storage, the ammonia content increased significantly after 10 days of storage ($p<0.05$). The majority of ammonia formed in most stored fish was originated from enzymatic deamination of free amino acids or amino acid split from proteins, from oxidation of amines, and decomposition of nucleic bases (Huang *et al.*, 1992). However, ammonia precursors can react with malonaldehyde, producing

low TBA values and low ammonia content in fish (Huang *et al.*, 1993). Thus, an increase in ammonia content, particularly after 10 days reflected the decomposition of muscle. This was concomitant with an increase with TVB.

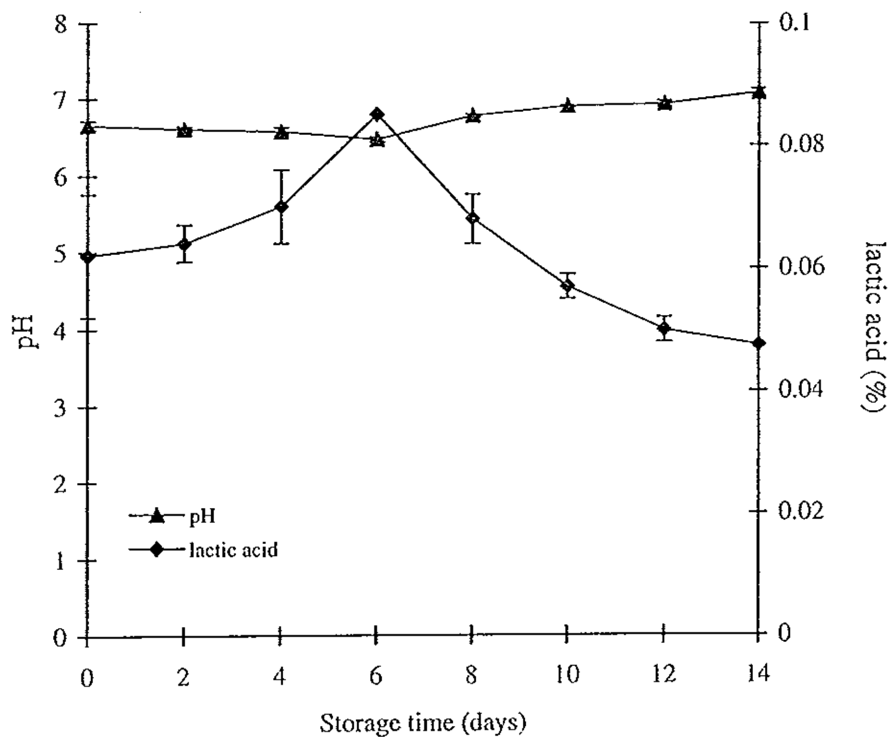


Figure 25 : Changes in pH and lactic acid accumulation in the seabass muscle during iced storage.

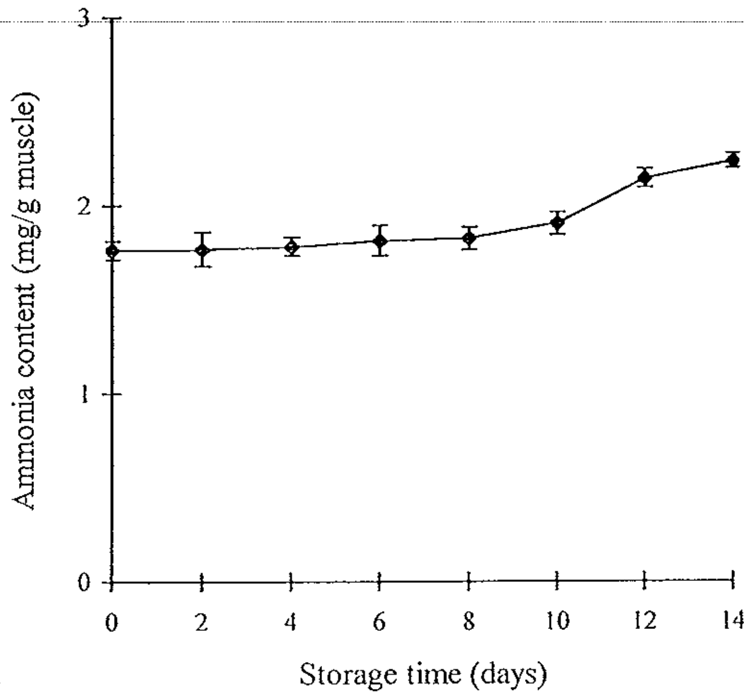


Figure 26 : Changes of ammonia content in seabass muscle during iced storage.

The changes in TVB and TMA content are shown in Figure 27. Both TVB and TMA slightly increased during first 8 days of storage. After 10 days of storage, TVB and TMA significantly increased ($p <0.05$). Trimethylamine oxide (TMAO) is generally present in seawater fish. TMA is formed from TMAO by bacterial reduction during iced storage. Magnusson and Martinsdottir (1995) reported that much more TMA was formed in the flesh fish kept on ice after 21 days of storage. The spoilage bacteria use TMAO as an electron acceptor instead of oxygen (anaerobic respiration) when oxygen concentration is low. TVB

determination included both TMA and ammonia and is a better estimator during later stages of spoilage (Huss, 1988). Bennor *et al.* (1991) reported that a large increase in TVB was found in mackerel after 10 days of chilled storage. The TVB in sardine increased after 16 days of storage at 0°C, indicating the end of lag phase of microorganism. This result suggested that spoilage caused by bacteria occurred, particularly when storage time increased. The increase in both TVB and TMA was in accordance with the increase in pH and ammonia content. Those volatile compounds were known to cause the offensive odor in the fish muscle.

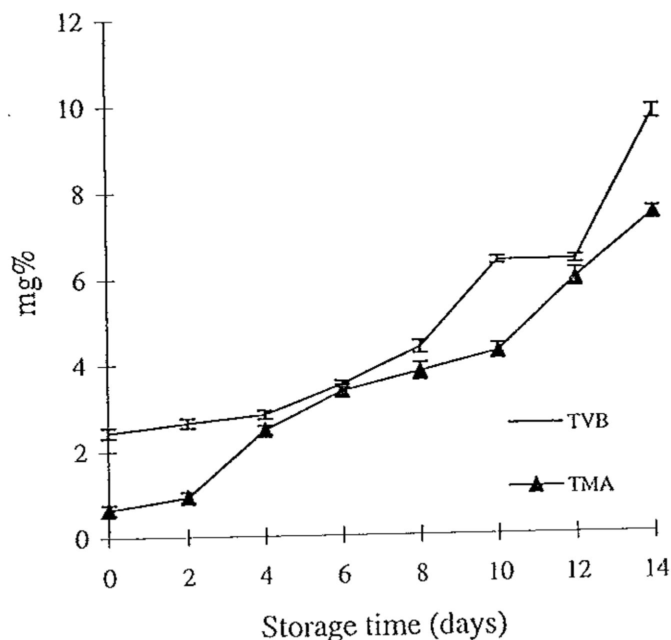


Figure 27 : Changes in TVB and TMA in seabass muscle during iced storage.

Changes in microbiology load

The initial psychrotrophic microbial population in seabass muscle was approximately 10^4 CFU/g (Figure 28). After 8 days, the significant increase in number of bacteria was observed ($p<0.05$). The increased microbial load possibly caused a spoilage of muscle, particularly via the degradation or deamination of protein. Psychrotropic bacteria in seabass muscle could be found as a normal flora or contaminated from environment. Psychrotropic bacteria are part of natural microflora in aquaculture channel catfish (*Ictalurus punctatus*) and rainbow trout (*Oncorhynchus mykiss*). The population of bacteria increased during storage at 2 to 4°C (Fernandes *et al.*, 1998). Gram-negative bacteria became predominant with the highest proportion of the genera *Pseudomonas/Alteromonas/Alcaligenes* in cod fillet kept on ice (Magnusson and Martinsdottir, 1995). Hong *et al.* (1996) also found that the lactic acid bacteria increased in Atlantic mackerel (*Scomber scombrus*) during 21 days of storage at 2°C. The lactic acid bacteria can generate the larger amounts of unflavorable breakdown products such as ammonia, ester and volatile sulfur compounds.

Expressible moisture

The expressible moisture of seabass muscle during iced storage increased after 8 days of iced storage ($p<0.05$) (Figure 29). The expressible moisture refers to as an amount of water exuded when the external force is applied (Muyonga and Regenstein, 1997). The increase in expressible moisture coincided with the increased degradation (Figure 30). This was postulated that degradation of muscle protein possibly led to the loss of protein configuration which can imbibe the water, causing a

release of free water. Water that can be incorporated in the muscle is of great economic significance (Muyonga and Regenstein, 1997). Additionally, expressible moisture is an indirect indicator of protein integrity during storage (Huss, 1988). The changes in pH during storage resulted in increased expressible fluids (Hong *et al.*, 1996).

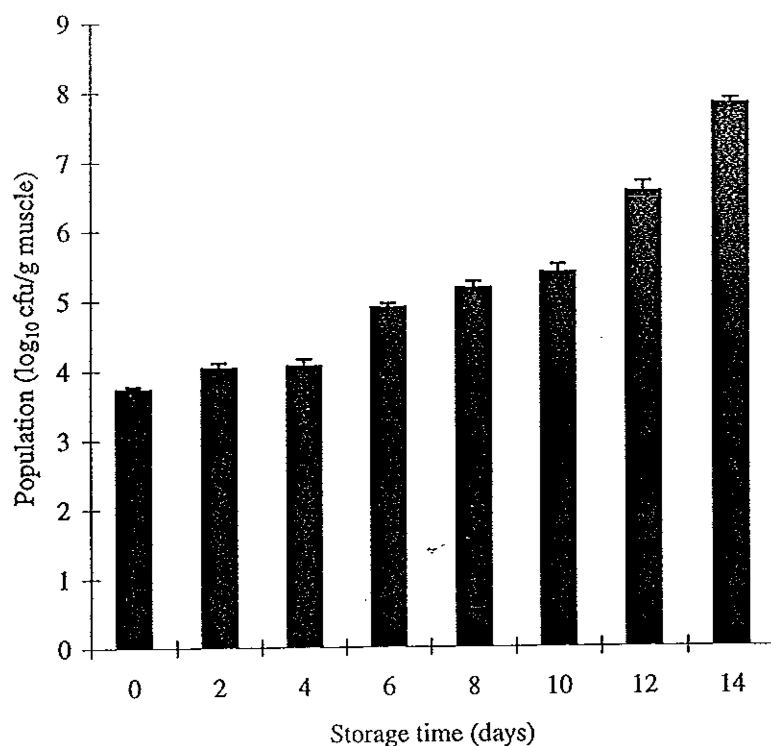


Figure 28 : Population of psychrotrophic microorganisms (log_{10}CFU/g) in seabass muscle during iced storage.

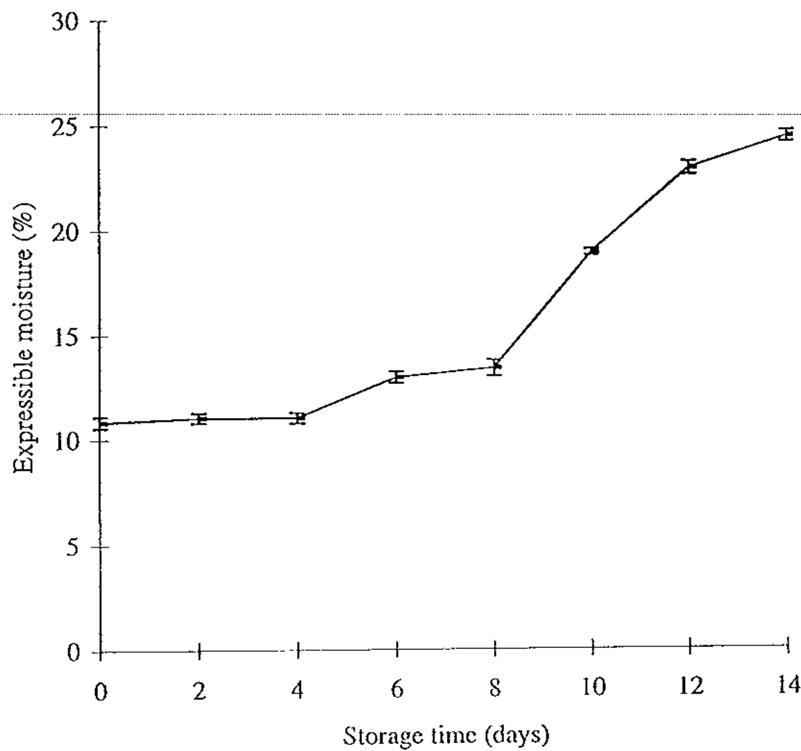


Figure 29 : Changes in expressible moisture in seabass muscle during iced storage.

Autolytic degradation

Figure 30 shows the autolytic degradation products in seabass muscle during iced storage. The degradation products found at day 0 indicated the endogenous peptides in muscle, which could be accumulated during post harvest handling. No marked changes in patterns of muscle proteins analyzed by SDS-PAGE were observed (Figure 31), throughout storage, though some degradation products were found (Figure 30). This result suggested that muscle proteins underwent degradation to small extent, which could not be detected by SDS-PAGE. However, myosin heavy chain in Pacific whting muscle decreased significantly within 8 days of iced storage and no changes in actin was

observed (Benjakul *et al.*, 1997). This was possibly due to the differences in susceptibility of protein to hydrolysis as well as between species. Furthermore, the proteases in different muscles would be belong to different groups with different hydrolytic property. The proteases have recognized to act on muscle during postmortem storage (Venugopal *et al.*, 1983). An *et al.* (1994) reported that cathepsin B was the most active

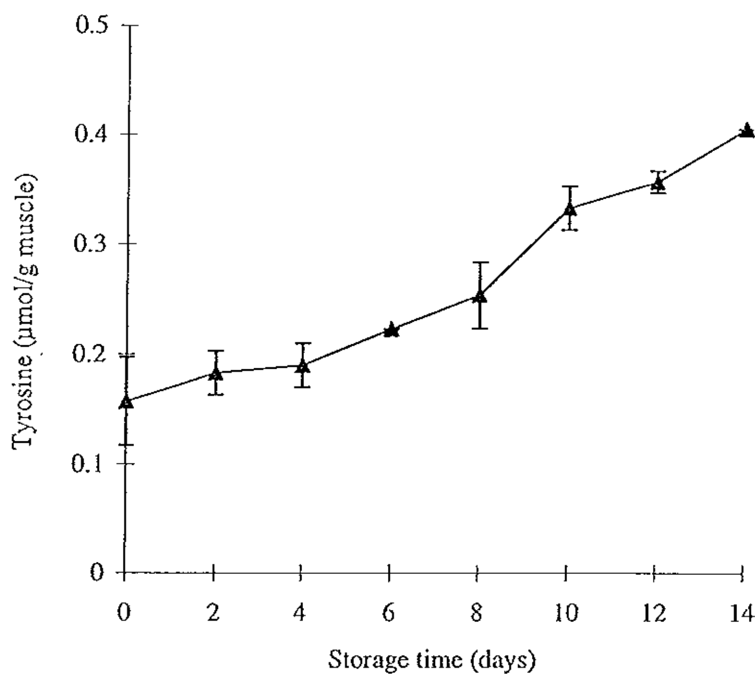


Figure 30 : Autolytic dagradation products in seabass muscle during iced storage as indicated by TCA-soluble tyrosine.

Cysteine protease found in Pacific whiting fillets. The results indicated that proteolysis may involve in changes of seabass muscle proteins during iced storage.

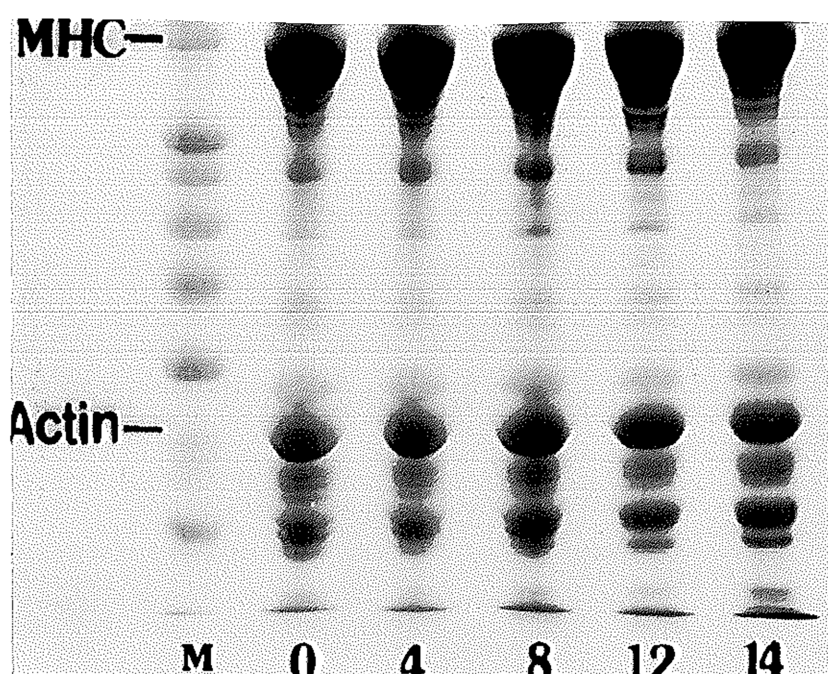


Figure 31: SDS-PAGE patterns of seabass muscle proteins during iced storage. Ten μg proteins were applied on 10% polyacrylamide gel. Numbers designated storage days. M, high molecular weight standard : MHC, myosin heavy chain.

Solubility

Solubility of actomyosin extracted from seabass muscle in different chemical solutions during iced storage was tested (Table 18). A salt-soluble fraction (I) decreased after 10 days of storage with an increased in $NaBH_4$ (III) and insoluble fractions (IV) ($p<0.05$). The salt-soluble fraction consists of myofibrillar proteins and sarcoplasmic proteins(Jiang *et al.*, 1985). The decrease in salt-soluble fraction indicated some bonding formation in myofibrillar protein. The increase in $NaBH_4$-soluble and insoluble fractions were considered to result from the formation of disulfides (Hamada *et al.*, 1977, Jiang *et al.*, 1988). These results suggested that denaturation of seabass myofibrils occurred by formation of disulfide. Hamada *et al.*(1977) reported that the salt-soluble fraction of carp actomyosin decreased during 16 days of storage at 4°C.

Total SH content

No changes in the total sulhydryl content of actomyosin extracted from seabass muscle were obtained in the first 10 days storage but a slight decrease was noted thereafter (Figure 32). The decrease in sulhydryl content was associated with an increase in $NaBH_4$-soluble fraction, particularly after 12 days of storage. A decrease in total SH group was reported to be due to formation of disulfide bonds through oxidation of SH groups or disulfide interchanges (Hayakawa and Nakai, 1985). Oxidation of thiol groups of myosin has been shown to reduce Ca^{2+}-sensitivity and modified actin-myosin interaction (Seki *et al.*, 1979). Benjakul *et al.*(1997) found that the total SH content in actomyosin of

Pacific whiting muscle increased slightly after 2 days of iced storage followed by gradual continued decrease up to 8 days

Table 18. The solubility* of actomyosin from seabass muscle during iced storage.

Storage time (days)	Fractions**			
	I	II	III	IV
0	9.56[a***] ± 0.09 (100.00)	0.28[c] ± 0.10 (2.93)	0.08[a] ± 0.02 (0.84)	0.10[a] ± 0.07 (1.04)
2	9.52[a] ± 0.07 (99.58)	0.30[c] ± 0.09 (3.14)	0.10[a] ± 0.04 (1.05)	0.10[a] ± 0.05 (1.05)
4	9.50[a] ± 0.12 (99.37)	0.32[c] ± 0.05 (3.35)	0.10[a] ± 0.06 (1.05)	0.10[a] ± 0.08 (1.05)
6	9.50[a] ± 0.01 (99.36)	0.31[c] ± 0.10 (3.24)	0.09[a] ± 0.01 (0.95)	0.10[a] ± 0.08 (1.05)
8	9.46[a] ± 0.05 (98.95)	0.32[c] ± 0.03 (3.35)	0.12[ab] ± 0.03 (1.26)	0.12[a] ± 0.05 (1.26)
10	9.21[b] ± 0.09 (96.33)	0.26[b] ± 0.01 (2.72)	0.25[c] ± 0.05 (2.62)	0.30[b] ± 0.06 (3.14)
12	8.84[c] ± 0.07 (92.47)	0.20[a] ± 0.03 (2.09)	0.48[d] ± 0.09 (5.02)	0.50[c] ± 0.05 (5.23)
14	8.70[c] ± 0.10 (91.00)	0.18[a] ± 0.05 (1.88)	0.54[e] ± 0.05 (5.65)	0.60[d] ±0.08 (6.28)

*The solubility is expressed as mg of soluble protein per milliliter

** I : salt soluble ; II : urea soluble ; III : $NaBH_4$; IV : insoluble fractions. Values in perentheses represent solubility percentage.

*** The same letter in the same column indicates no significant differences ($p>0.05$).

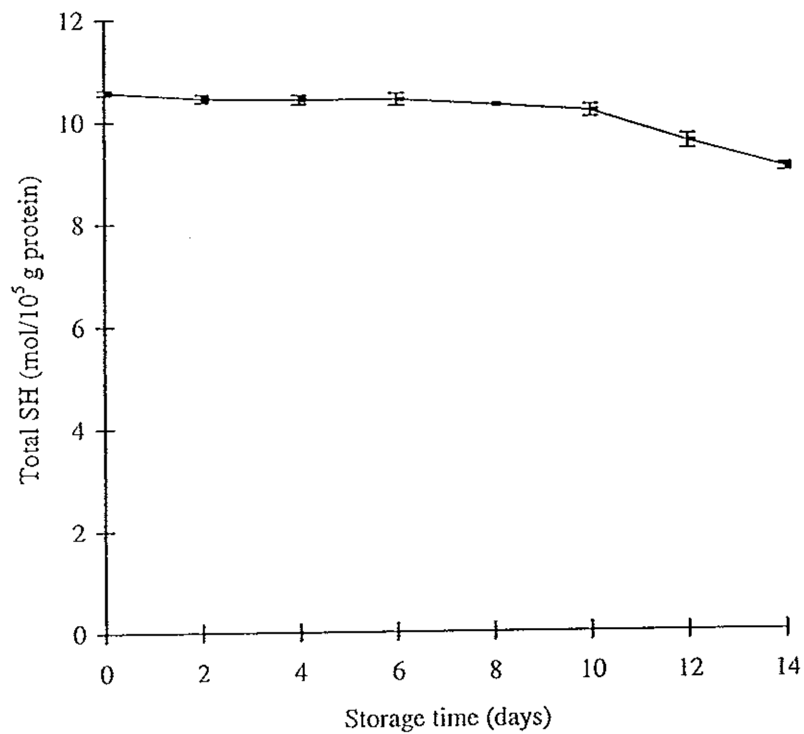


Figure 32 : Total sulhydryl content of actomyosin from seabass muscle during iced storage. Total SH was calculated from absorbance at 412 nm using the molar extinction coefficient of 13,600 M^{-1} cm^{-1} for 2-nitro-5-thiobenzoic acid.

ATPase activity

No changes in Ca^{2+}-ATPase, Mg^{2+}-ATPase and Mg^{2+}-Ca^{2+}-ATPase activities were observed ($p > 0.05$) (Figure 33). However, Mg^{2+}-EGTA-ATPase activity gradually increased during iced storage with a loss of the Ca^{2+}-sensitivity (Figure 34). These results indicated that the integrity of the tropomyosin-troponin complex was more affected by iced storage than actin-myosin interaction in muscle. This results confirmed an increase in Mg^{2+}-EGTA-ATPase activity of Pacific whiting muscle proteins during iced storage (Benjakul *et al.*, 1997). When compared with the changes in ATPase activity of actomyosin stored in ice, it was found that less changes was observed in actomyosin extracted from seabass muscle. This suggested that actomyosin in solution was more susceptible to denaturation than actomyosin in the intact muscle.

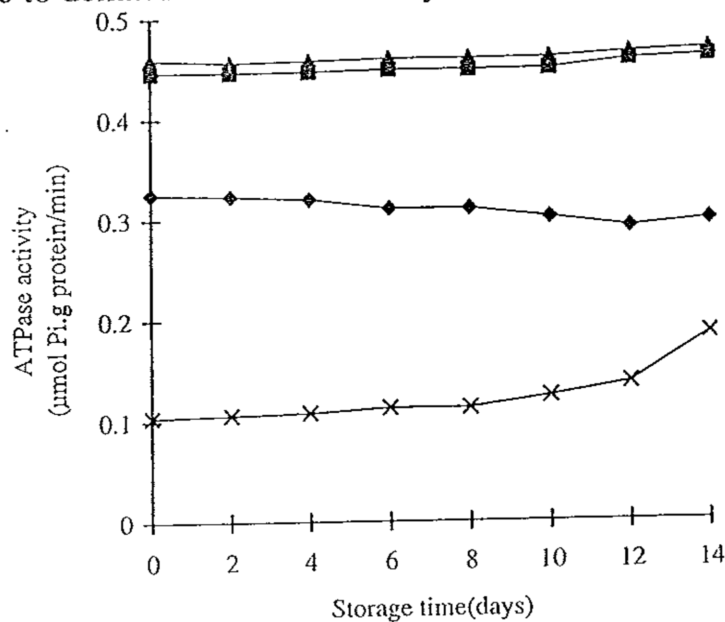


Figure 33 : ATPase activities of actomyosin from seabass muscle during iced storage. One unit of activity was defined as that releasing 1 μmol Pi/mg protein/min. (—♦— Ca^{2+}-ATPase : —■— Mg^{2+}-ATPase: —▲— Mg^{2+}-Ca^{2+}-ATPase : —×— Mg^{2+}-EGTA-ATPase)

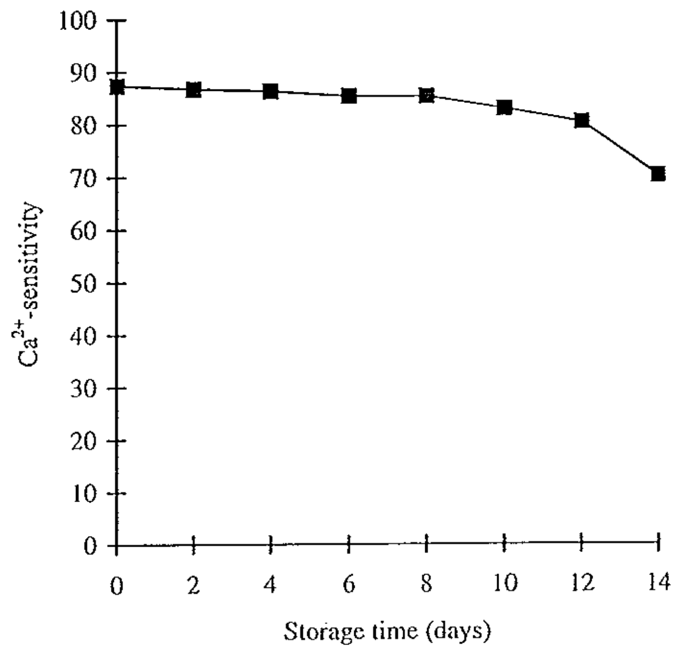


Figure 34 : Ca^{2+}-sensitivity of actomyosin extracted from seabass muscle during iced storage.

Free α- amino acid

Free α-amino acid of actomyosin extracted from seabass muscle increased after 12 days of storage (Figure 35). The increase in free α-amino acid was considered to be caused by hydrolysis of muscle proteins. Therefore, the proteases possibly resulted in the degradation of muscle proteins, resulting in an increase in free α-amino acid. The proteases involved in degradation of seabass muscle were both sarcoplasmic proteases and myofibril associated proteases. Furthermore, proteases from bacteria contaminated into fish muscle were also expected to play a role in this phenomenon.

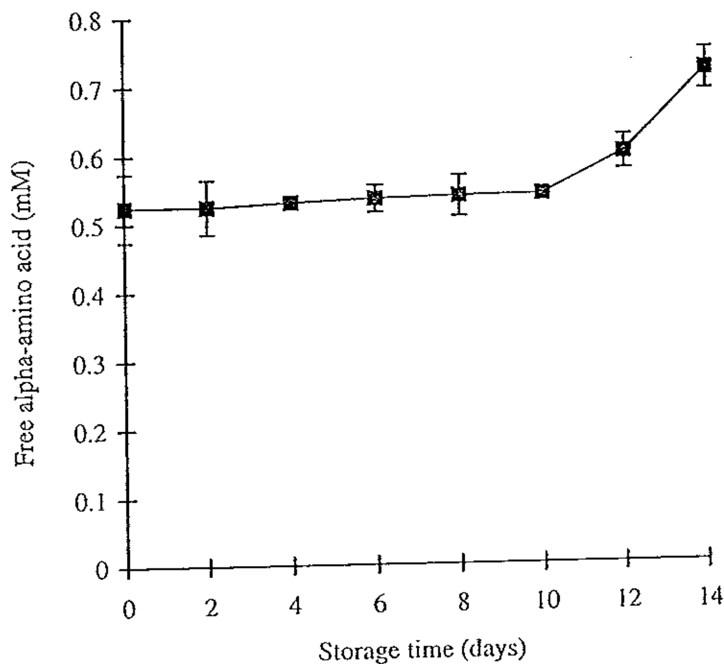


Figure 35 : Changes in free α-amino acid of actomyosin extracted from seabass muscle during iced storage.

Seabass muscle during frozen storage

Formaldehyde

The levels of formaldehyde in the seabass muscle stored at -18 and -80°C were determined (Figure 36). Formaldehyde content of seabass muscle increased sharply after 2.5 months of storage ($p<0.05$). More increase in formaldehyde was observed in samples kept at –18°C, compared to those kept at -80°C. This result was in agreement with Connell (1978) who reported the increase in formaldehyde content during frozen storage of cod muscle. Formaldehyde is an enzymatic product from trimethylamineoxide. Formaldehyde can be formed in fish muscle during storage, especially in gadoid family (Ragnarsson and Regenstein, 1989). During frozen storage, covalent methylene crosslinks was found with resulting increase in molecular weight and polymers that are insoluble in hydrogen bond breaking solvents (Connell, 1975). Since seabass muscle contained high protein and formaldehyde concentrations, there is a tendency for formaldehyde formed to bind reversibly or irreversibly to single amino acid residues. This presumably resulted in the changes in texture, particularly toughening of fish muscle.

Solubility

The solubility of actomyosin extracted from seabass muscle is shown in Table 19. Salt-soluble fraction (I) of actomyosin from seabass muscle stored at -18°C more decreased after 4.5 months of storage, compared to that stored at -80°C. The quantity of urea-soluble fraction (II) of actomyosin from muscle stored at -18 and -80°C increased during the first month of storage but decreased during prolonged storage. During the early stage of freeze denaturation of fish proteins, both

myosin and actin apparently form an insoluble fraction that accounts for observed decrease in protein solubility (Jiang and Lee, 1985). The increase in the urea-soluble fraction indicated formation of hydrogen and hydrophobic bonds during storage. After 4.5 months of storage, the decrease in urea-soluble fraction might be due to the formation of the disulfides, which consequently caused incomplete disruption of hydrogen and hydrophobic bonding. The $NaBH_4$-soluble (III) and insoluble fractions (IV) of both samples stored at –18 and –80°C increased during storage. The increase in fraction III and IV indicated formation of disulfide bonds. These results suggested that protein underwent denaturation during frozen storage, mainly caused by formation of disulfide, hydrogen, and hydrophobic bonds.

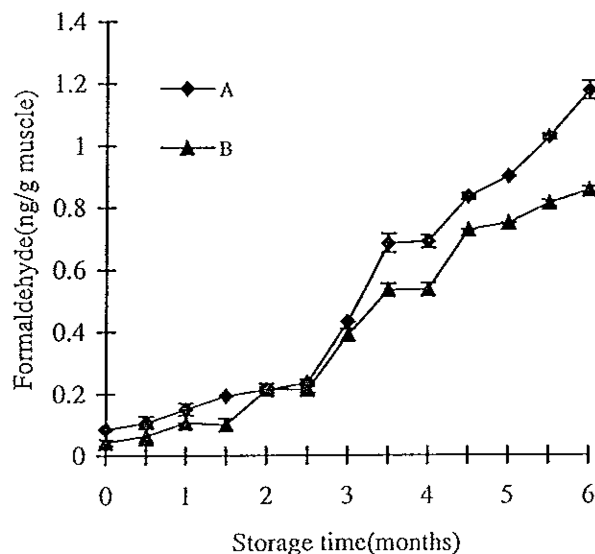


Figure 36 : Changes in formaldehyde content of seabass muscle during frozen storage. A : -18°C ; B : -80°C.

Table 19 : The solubility of actomyosin from seabass muscle during frozen storage.

Storage time (months)	Fractions*							
	-18°C				-80°C			
	I	II	III	IV	I	II	III	IV
0	$10.19^{a**} \pm 0.16$	$0.31^{cd} \pm 0.01$	$0.09^{h} \pm 0.06$	$0.27^{c} \pm 0.10$	$10.22^{a} \pm 0.00$	$0.28^{f} \pm 0.01$	$0.09^{c} \pm 0.00$	$0.27^{g} \pm 0.00$
	(93.85)	(2.85)	(0.81)	(2.49)	(94.08)	(2.61)	(0.80)	(2.51)
0.5	$10.19^{a} \pm 0.18$	$0.31^{ab} \pm 0.01$	$0.09^{h} \pm 0.01$	$0.27^{c} \pm 0.05$	$10.21^{ab} \pm 0.00$	$0.28^{f} \pm 0.00$	$0.09^{c} \pm 0.01$	$0.28^{g} \pm 0.01$
	(93.80)	(2.88)	(0.84)	(2.48)	(93.99)	(2.60)	(0.80)	(2.61)
1.0	$10.18^{a} \pm 0.10$	$0.32^{ab} \pm 0.00$	$0.09^{h} \pm 0.00$	$0.27^{c} \pm 0.09$	$10.21^{ab} \pm 0.02$	$0.29^{f} \pm 0.00$	$0.09^{c} \pm 0.00$	$0.28^{g} \pm 0.00$
	(93.78)	(2.94)	(0.83)	(2.45)	(93.97)	(2.67)	(0.79)	(2.57)
1.5	$10.18^{a} \pm 0.06$	$0.32^{ab} \pm 0.01$	$0.09^{h} \pm 0.04$	$0.27^{c} \pm 0.00$	$10.21^{ab} \pm 0.01$	$0.29^{f} \pm 0.01$	$0.09^{c} \pm 0.01$	$0.28^{g} \pm 0.03$
	(93.77)	(2.95)	(0.82)	(2.46)	(93.95)	(2.67)	(0.81)	(2.57)
2.0	$10.18^{a} \pm 0.14$	$0.32^{ab} \pm 0.03$	$0.09^{h} \pm 0.07$	$0.27^{c} \pm 0.01$	$10.14^{ab} \pm 0.09$	$0.30^{ef} \pm 0.00$	$0.09^{c} \pm 0.00$	$0.34^{gf} \pm 0.06$
	(93.75)	(2.93)	(0.83)	(2.49)	(93.32)	(2.74)	(0.83)	(3.11)
2.5	$10.12^{a} \pm 0.05$	$0.38^{a} \pm 0.00$	$0.09^{h} \pm 0.05$	$0.28^{c} \pm 0.01$	$10.10^{ab} \pm 0.08$	$0.32^{ef} \pm 0.02$	$0.09^{bc} \pm 0.01$	$0.35^{gf} \pm 0.02$
	(93.14)	(3.49)	(0.84)	(2.53)	(92.99)	(2.93)	(0.85)	(3.23)
3.0	$9.18^{b} \pm 0.08$	$0.24^{bc} \pm 0.08$	0.18 ± 0.00	$1.26^{b} \pm 0.00$	$10.10^{ab} \pm 0.06$	$0..33^{ef} \pm 0.01$	$0.10^{bc} \pm 0.00$	$0.35^{gf} \pm 0.00$
	(84.53)	(2.24)	(1.66)	(9.75)	(92.83)	(3.03)	(0.89)	(3.25)

* The solubility is expressed as mg of soluble protein per milliliter ; Value in parenthesis represent solubility percentage.

** The same latter in the same column indicates no significant differences (*p<0.05*).

Table 19 : The solubility of actomyosin from seabass muscle during frozen storage (continued).

Storage time (months)	Fractions*							
	-18°C				-80°C			
	I	II	III	IV	I	II	III	IV
3.5	$9.14^{b**} \pm 0.08$	$0.23^{cd} \pm 0.02$	$0.28^{f} \pm 0.05$	$1.22^{b} \pm 0.00$	$10.07^{cd} \pm 0.03$	$0.34^{ab} \pm 0.05$	$0.10^{bc} \pm 0.02$	$0.35^{gf} \pm 0.04$
	(84.12)	(2.11)	(2.56)	(11.23)	(92.69)	(3.13)	(0.94)	(3.24)
4.0	$8.64^{c} \pm 0.38$	$0.19^{cde} \pm 0.06$	$0.34^{e} \pm 0.02$	$1.69^{b} \pm 0.02$	$10.03^{d} \pm 0.05$	$0.34^{ab} \pm 0.00$	$0.11^{bc} \pm 0.01$	$0.38^{efg} \pm 0.04$
	(79.51)	(1.80)	(3.14)	(15.55)	(92.29)	(3.15)	(1.03)	(3.53)
4.5	$8.14^{e} \pm 0.14$	$0.18^{cde} \pm 0.03$	$0.44^{d} \pm 0.02$	$2.11^{b} \pm 0.03$	$9.84^{e} \pm 0.08$	$0.35^{a} \pm 0.02$	$0.12^{bc} \pm 0.00$	$0.55^{c} \pm 0.01$
	(74.89)	(1.61)	(4.07)	(19.43)	(90.56)	(3.26)	(1.09)	(5.09)
5.0	$6.15^{f} \pm 0.05$	$0.17^{cde} \pm 0.00$	$0.62^{c} \pm 0.01$	$3.93^{a} \pm 0.05$	$9.80^{e} \pm 0.13$	$0.36^{a} \pm 0.04$	$0.12^{b} \pm 0.06$	$0.59^{b} \pm 0.02$
	(56.56)	(1.56)	(5.67)	(36.21)	(90.20)	(3.27)	(1.12)	(5.41)
5.5	$6.01^{f} \pm 0.10$	$0.15^{cde} \pm 0.06$	$0.76^{b} \pm 0.01$	$3.88^{a} \pm 0.10$	$9.51^{f} \pm 0.07$	$0.35^{ab} \pm 0.01$	$0.16^{a} \pm 0.03$	$0.85^{a} \pm 0.07$
	(55.89)	(1.37)	(7.03)	(35.71)	(87.53)	(3.19)	(1.44)	(7.84)
6.0	$5.73^{f} \pm 0.16$	$0.13^{f} \pm 0.08$	$0.98^{a} \pm 0.04$	$4.03^{a} \pm 0.16$	$9.48^{f} \pm 0.03$	$0.31^{de} \pm 0.08$	$0.22^{a} \pm 0.08$	$0.86^{a} \pm 0.06$
	(52.70)	(1.18)	(9.02)	(38.00)	(87.23)	(2.86)	(2.03)	(7.88)

* The solubility is expressed as mg of soluble protein per milliliter ; Value in parentheses represent solubility percentage.

** The same latter in the same column indicate no significant differences ($p<0.05$).

Total SH content

Total sulhydryl content gradually decreased in seabass muscle stored at -18°C after 4 months (Figure 37). No changes in sulhydryl content were found in sample kept at –80°C. This result suggested that SH content was more retained, when kept the muscle protein at lower temperature, indicating less denaturation of protein. Buttkus (1972) proposed the formation of intermolecular S-S bonds as major cause of aggregation. A decrease in total SH content of tilapia hybrid actomyosin was reported to be due to formation of disulfides bonds (Jiang *et al.*, 1989). LaBlanc and LaBlanc (1992) reported that the total SH content more decreased in myofibrils of cod fillets stored at -12°C, compared to that stored at -15 and -30°C. The denaturation process subsequently leads to increased hydrophobicity and concomitant decreased solubility (Nakai, 1983). The decrease in salt-soluble fraction and total SH content probably activated the exposed hydrophobic sites that cause aggregation of protein molecule.

ATPase activity

ATPase activities of seabass muscle during frozen storage are presented in Figure 38. The Ca^{2+}-ATPase activity more decreased in actomyosin extracted from seabass muscle kept at -18°C, compared to those kept at -80°C. A marked decreased in Ca^{2+}-ATPase activity was observed after 4 months of storage at –18°C. The decrease in Ca^{2+}-ATPase activity was reported to be highly related to the oxidation of sulhydryl group (Jiang *et al.*, 1989). Jiang *et al.* (1985) reported that the Ca^{2+}-ATPase activity of actomyosin extracted from amberfish stored at -20°C more decreased during frozen storage, compared to that stored at -

40°C. The loss of Ca^{2+}-ATPase activity might be due to the oxidation of SH on active site of actomyosin. A change in Ca^{2+}-ATPase activity of actomyosin extracted from seabass muscle kept at both temperatures was almost concordant with changes in the total SH content and insoluble fraction (Figure 37 and Table 19).

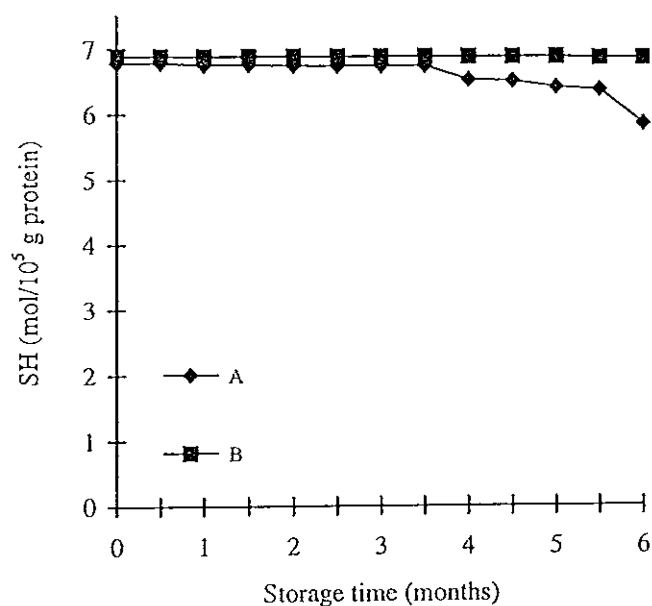


Figure 37 : Total sulhydryl group content of actomyosin from seabass muscle during frozen storage. Total SH was calculated from absorbance at 412 nm using the molar extinction coefficient of 13,600 M^{-1} cm^{-1} for 2-nitro-5-thiobenzoic acid.

A : -18°C ; B : -80°C

No changes in Mg^{2+}-ATPase and Mg^{2+}-Ca^{2+}-ATPase activities were found, but Mg^{2+}-EGTA-ATPase activity slightly increased during frozen storage at –18°C, particularly after 4 months with a loss of Ca^{2+}-sensitivity (Figure 39).

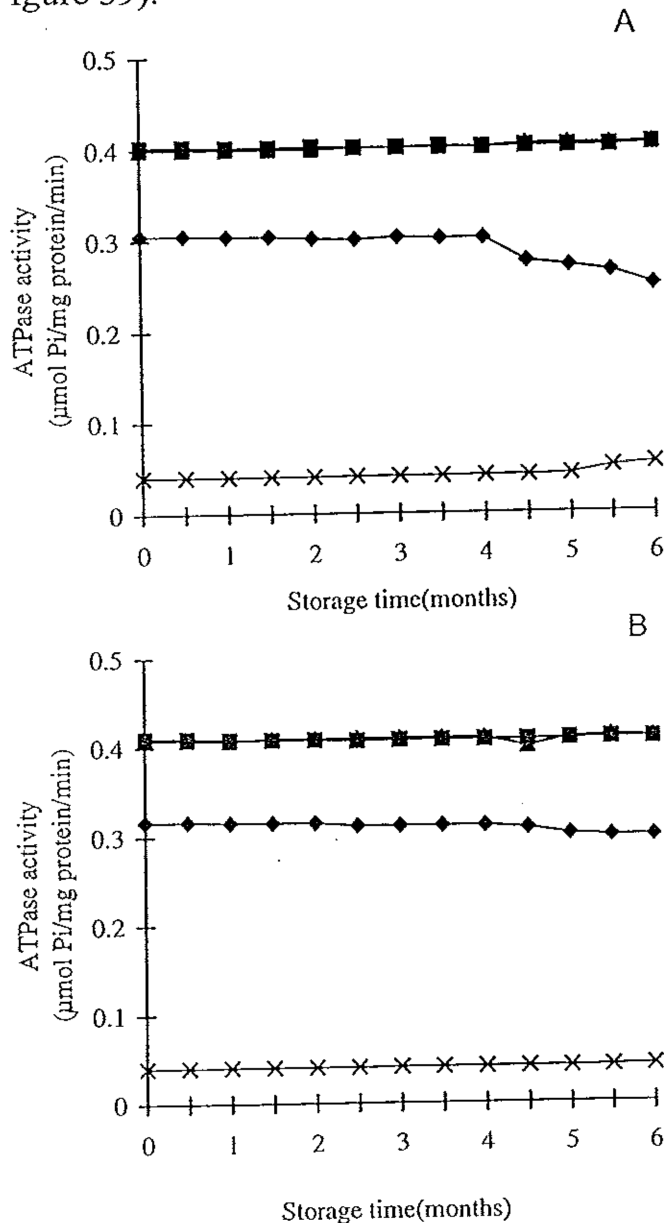


Figure 38 : ATPase activities of actomyosin from seabass muscle during frozen storage. One unit of activity was defined as that releasing 1 µmol Pi/mg protein/min. (◆ Ca^{2+}-ATPase ; ■ Mg^{2+}-ATPase ; ▲ Mg^{2+}-Ca^{2+}-ATPase ; × Mg^{2+}-EGTA-ATPase).

A : -18°C ; B : -80°C

The Ca^{2+}-sensitivity of actomyosin extracted from unfrozen seabass muscle was quite high, but decreased significantly after during 4 months of storage at -18°C (*p<0.05*). Therefore, the loss of Ca^{2+}-sensitivity of actomyosin extracted from seabass muscle stored at -18°C was faster than that at -80°C. From the result, the seabass muscle proteins underwent more severe denaturation after 4 months of storage at –18°C.

Frozen storage temperature and duration are important factors affecting fish muscle quality (Jiang *et al.*, 1985). Therefore, the lower storage temperature and shorter period of frozen storage retarded the protein denaturation.

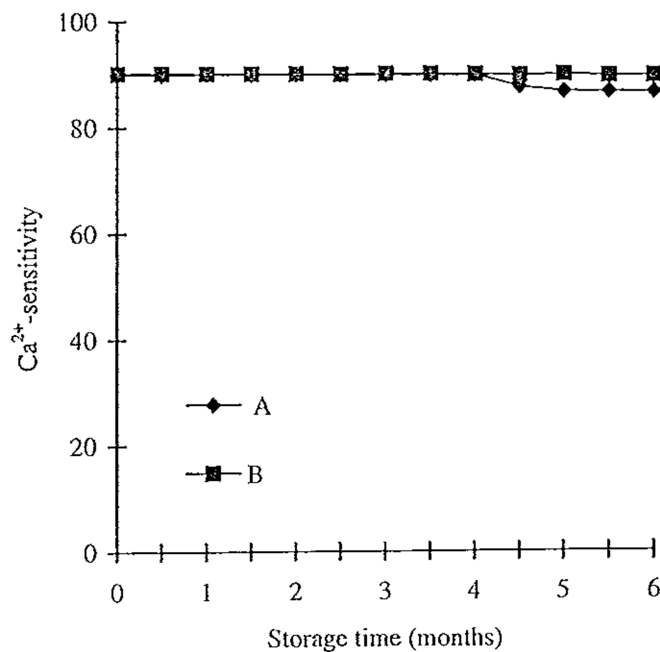


Figure 39 : Ca^{2+}-sensitivity of actomyosin extracted from seabass muscle during frozen storage.

A : -18°C ; B : -80°C

Effect of freezing and thawing on physico-chemical changes in seabass muscle.

Solubility

The effect of freeze-thawing on solubility of frozen seabass protein is shown in Table 20. At the same freezing temperature, the salt-soluble fraction (I) of actomyosin extracted from seabass muscle thawed by tap water was higher than that thawed at room temperature ($p<0.05$). The quantity of salt-soluble fraction of actomyosin extracted from seabass muscle stored at -18°C more decreased, when compared to that stored at -80°C. The differences in salt-soluble proteins of actomyosin extracted from seabass muscle subjected to different freeze-thaw cycles were observed ($p<0.05$). The more freeze-thaw cycles, the less solubility was found. Jiang *et al.* (1985) found the salt-soluble proteins of actomyosin from mackerel thawed by 20°C running water was higher than that thawed at room temperature. These indicated that the thawing methods directly affected the denaturation of muscle protein.

No significant differences in the urea-soluble fraction (II) of actomyosin were obtained in seabass muscle thawed by tap water and at room temperature ($p>0.05$). The $NaBH_4$-soluble (III) and insoluble fractions (IV) of actomyosin extracted from seabass muscle thawed at room temperature was higher than that thawed by tap water. The increase in fraction III and IV was considered to be due to formation of disulfide bonds. These results indicated that the disulfide bonds occurred after refreezing and thawing. This suggested that the denaturation occurred in samples thawed at room temperature because of long exposure to respective temperature.

Table 20 : Effect of freeze/thawing method on the solubility of actomyosin from seabass muscle.

Storage temperature (°C)	Thawing method	Freeze-thaw cycle	Fractions* I	II	III	IV
unfrozen			$10.12^{a**} \pm 0.05$	$0.14^{b} \pm 0.07$	$0.08^{c} \pm 0.03$	$0.12^{d} \pm 0.04$
-80	Tap water	1	$10.10^{a} \pm 0.00$	$0.14^{b} \pm 0.02$	$0.08^{c} \pm 0.05$	$0.14^{d} \pm 0.08$
		2	$10.00^{a} \pm 0.07$	$0.14^{b} \pm 0.03$	$0.10^{c} \pm 0.02$	$0.16^{d} \pm 0.06$
		3	$9.90^{a} \pm 0.02$	$0.16^{b} \pm 0.08$	$0.12^{c} \pm 0.04$	$0.28^{c} \pm 0.07$
	Room temperature	1	$10.06^{a} \pm 0.08$	$0.15^{b} \pm 0.06$	$0.10^{c} \pm 0.10$	$0.15^{d} \pm 0.05$
		2	$9.92^{a} \pm 0.03$	$0.16^{b} \pm 0.04$	$0.10^{c} \pm 0.06$	$0.28^{c} \pm 0.03$
		3	$9.28^{b} \pm 0.09$	$0.25^{b} \pm 0.09$	$0.28^{b} \pm 0.03$	$0.55^{a} \pm 0.06$
-18	Tap water	1	$10.07^{a} \pm 0.00$	$0.15^{b} \pm 0.05$	$0.10^{c} \pm 0.03$	$0.14^{d} \pm 0.02$
		2	$10.00^{a} \pm 0.06$	$0.18^{b} \pm 0.09$	$0.13^{c} \pm 0.05$	$0.25^{c} \pm 0.02$
		3	$9.34^{b} \pm 0.04$	$0.30^{ab} \pm 0.03$	$0.32^{b} \pm 0.08$	$0.50^{a} \pm 0.01$
	Room temperature	1	$9.97^{ab} \pm 0.08$	$0.20^{b} \pm 0.10$	$0.16^{c} \pm 0.10$	$0.13^{d} \pm 0.06$
		2	$9.02^{b} \pm 0.10$	$0.36^{ab} \pm 0.06$	$0.36^{b} \pm 0.10$	$0.52^{b} \pm 0.05$
		3	$8.94^{c} \pm 0.03$	$0.43^{a} \pm 0.10$	$0.48^{a} \pm 0.05$	$0.59^{a} \pm 0.08$

* The solubility is expressed as mg of soluble protein per milliliter. ** The same letter in the same column indicates no significant differences ($p>0.05$).

Total SH content

As shown in Figure 40, the total SH content of sample thawed at room temperature more decreased, when compared to that thawed by tap water. Generally, the total SH content of sample stored at -18°C decreased significantly after refreezing and thawing at room temperature ($p<0.05$). Increase in $NaBH_4$-soluble and insoluble fractions and decrease in the total SH (Table 20 and Figure 40) indicated the formation of disulfides in samples, particularly for sample frozen at –18°C and thawed at room temperature. Moreover, the repeated freeze-thaw process increased those changes, especially for the harsh conditions.

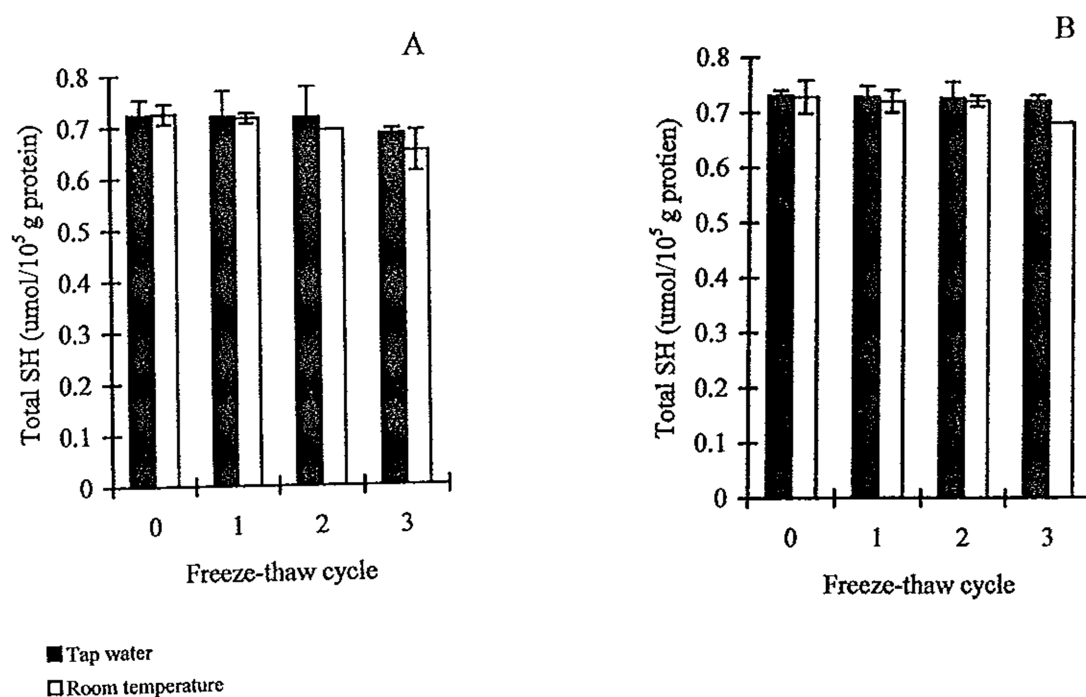


Figure 40 : Total sulhydryl group content of actomyosin from seabass muscle subjected to different freeze-thaw cycle. Total SH was calculated from absorbance at 412 nm using the molar extinction coefficient of 13,600 M^{-1} cm^{-1} for 2-nitro-5-thiobenzoic acid. A : -18°C ; B : -80°C

ATPase activities

The ATPase activities of actomyosin extracted from seabass muscle subjected to different freeze-thaw process is shown in Figure 41. The Ca^{2+}-ATPase activity of actomyosin from seabass muscle thawed by tap water decreased to a higher extent, compared to that of sample thawed at room temperature, particularly with a higher freeze-thaw cycle. No significant differences in the Ca^{2+}-ATPase activity of actomyosin were obtained in seabass muscle stored at -18 and -80°C. The Ca^{2+}-ATPase activity is an indicator of the integrity of the myosin molecule. The decrease in total SH content (Figure 40) indicated that the loss of Ca^{2+}-ATPase activity might be due to the oxidation of sulhydryl groups on the active site of actomyosin (Jiang *et al.*, 1989). Therefore, the decrease in the Ca^{2+}-ATPase activity was probably due to oxidation of sulhydryl groups.

No changes in Mg^{2+}-ATPase and Mg^{2+}-Ca^{2+}-ATPase activities were observed, but Mg^{2+}-EGTA-ATPase activity slightly increased in samples thawed at room temperature, particularly with a higher freeze-thaw cycle. However, no increase in Mg^{2+}-EGTA-ATPase activity of samples stored at -18 and -80°C and thawed by tap water was observed.

Ca^{2+}-sensitivity of all samples is shown in Figure 42. The Ca^{2+}-sensivity of sample thawed at room temperature more decreased, compared to that thawed by tap water. This result coincided with the increase in Mg^{2+}-EGTA-ATPase activity. The Ca^{2+}-sensitivity of sample stored at -18°C decreased after refreezing and thawing at room temperature. The loss of Ca^{2+}-sensitivity of samples after freeze-thawing was considered to result from the modification of actin-myosin interaction caused by the oxidation in myosin molecule. These results

suggested that the collapse of seabass muscle occurred during thawing accelerated the biochemical deterioration on refreezing.

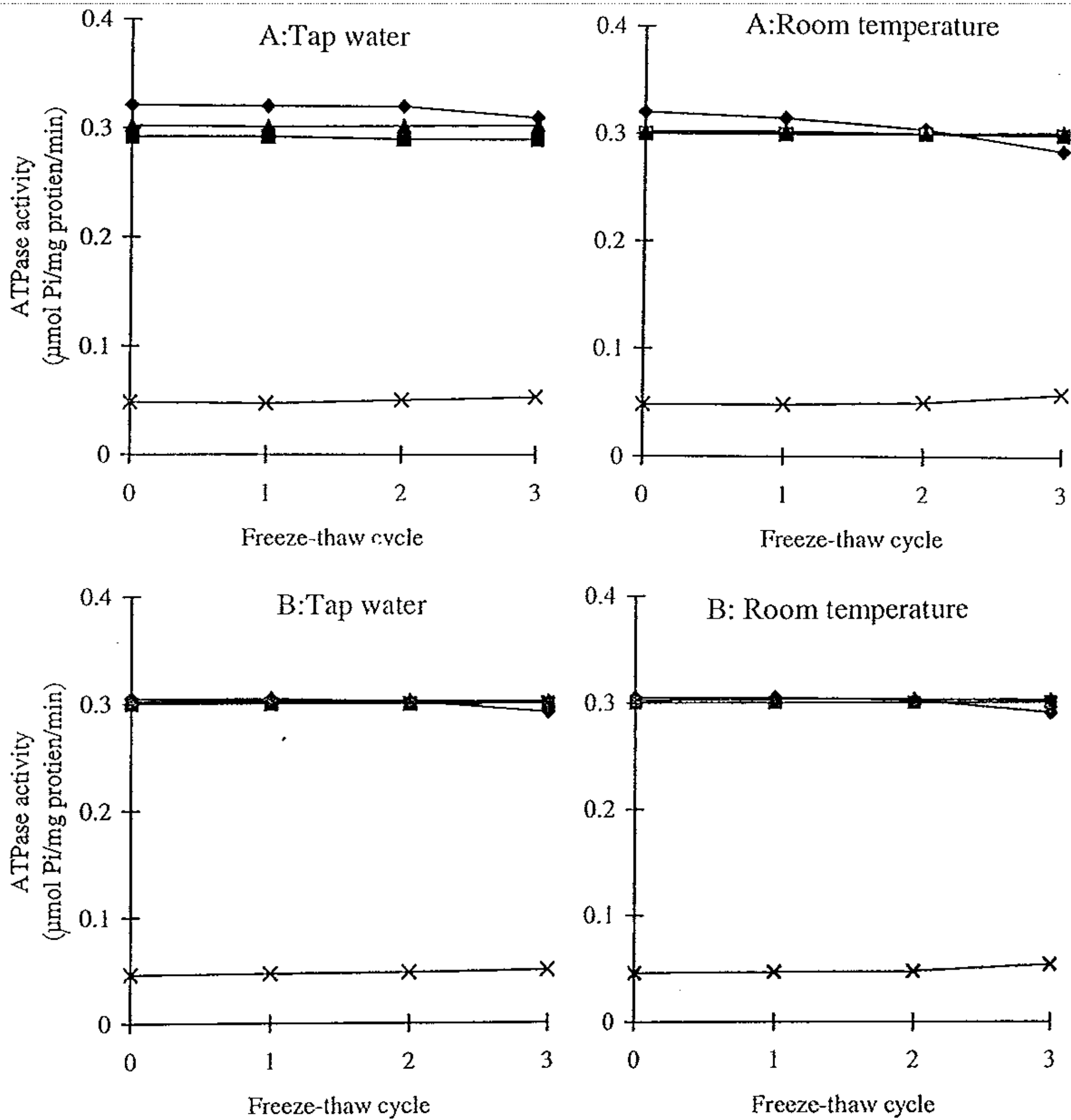


Figure 41 : ATPase activities of actomyosin from seabass muscle subjected to freeze-thaw cycles. One unit of activity was defined as that releasing 1 µmol Pi/mg protein/min.

(Ca^{2+}-ATPase : Mg^{2+}-ATPase : Mg^{2+}-Ca^{2+}-ATPase : Mg^{2+}-EGTA-ATPase)

A : -18°C ; B : -80°C

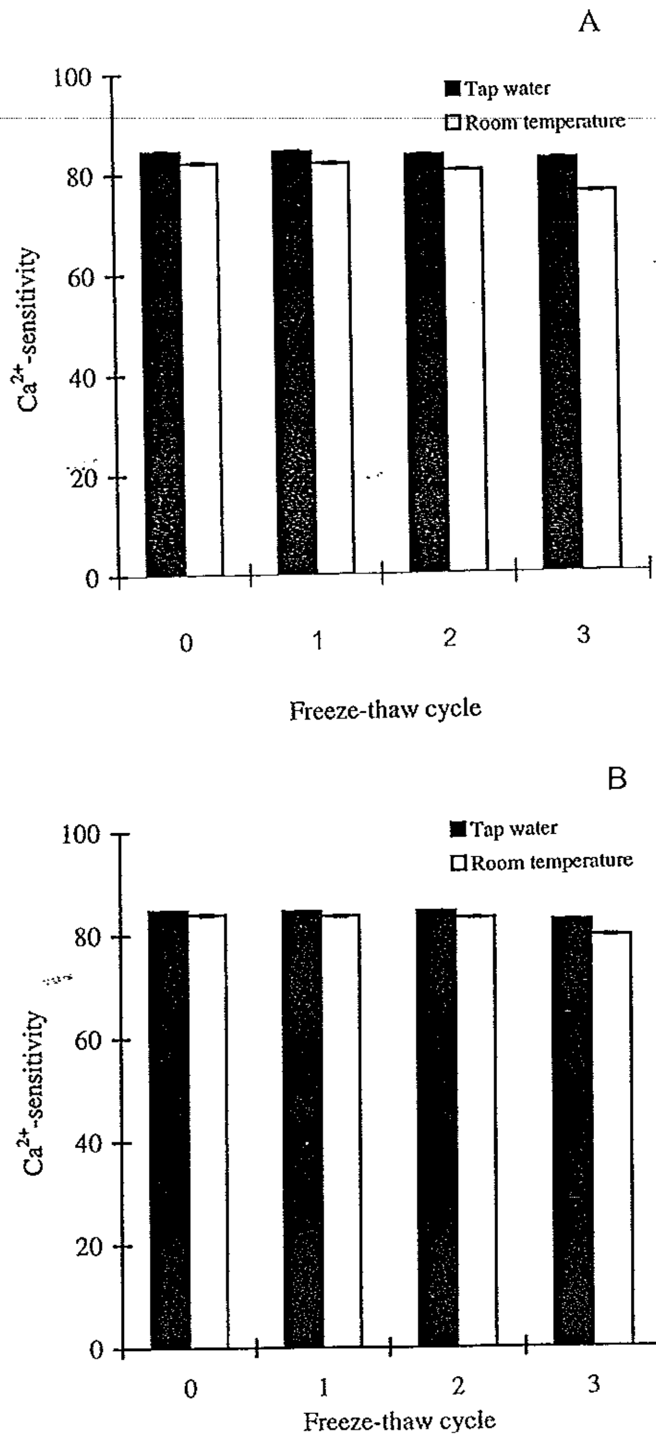


Figure 42 : Ca^{2+}-sensitivity of actomyosin extracted from seabass muscle subjected to different freeze-thaw cycles.

A : -18°C ; B : -80°C

Effect of freezing and thawing on enzyme leakage in muscle tissue of seabass

Enzyme activities in the centrifugal tissue fluid (CTF) from seabass muscle samples subjected to different freeze-thawing methods as well as different freeze-thaw cycles were measured. The activity of α-glucosidase (AG) is shown in Figure 43. The AG activity more increased in seabass muscle thawed at room temperature, compared to that thawed by tap water. The leakage of enzyme into the interstitial fluid seemed to increase sharply when the freeze-thaw cycle increased. At higher freezing temperature (-18°C), a higher AG activity was observed in the fluid. This results suggested that both freezing temperature and thawing method influentially affected the integrity of muscle cell. The activity of AG from rainbow trout muscle thawed at 30°C was higher than that thawed at 5°C (Nilsson and Ekstran, 1994). Rehbein *et al.* (1987) reported that the activity of AG in cod and saithe sharply increased after freeze-thawing.

The activity of *β*-N-acetylglucosaminidase (NAG) in seabass muscle is shown in Figure 44. The NAG activity increased with increasing freeze-thaw cycle for all samples. Thawing at room temperature resulted in a higher NAG activity, compared to thawing by tap water ($p<0.05$).

After thawing by either tap water or air at room temperature, samples frozen at –18°C had a higher NAG activity, compared to that frozen at –80°C ($p<0.05$). This indicated that freezing temperature had marked effect on muscle membrane. Lower freezing temperature caused less cell leakage. For thawing method, the faster method resulted in a lower muscle cell damage, as indicated by less AG or NAG activities.

From the result, both AG and NAG found in the fresh samples indicated improper handling or preparation of fish, which caused some damage of cell. However, the activities were very low.

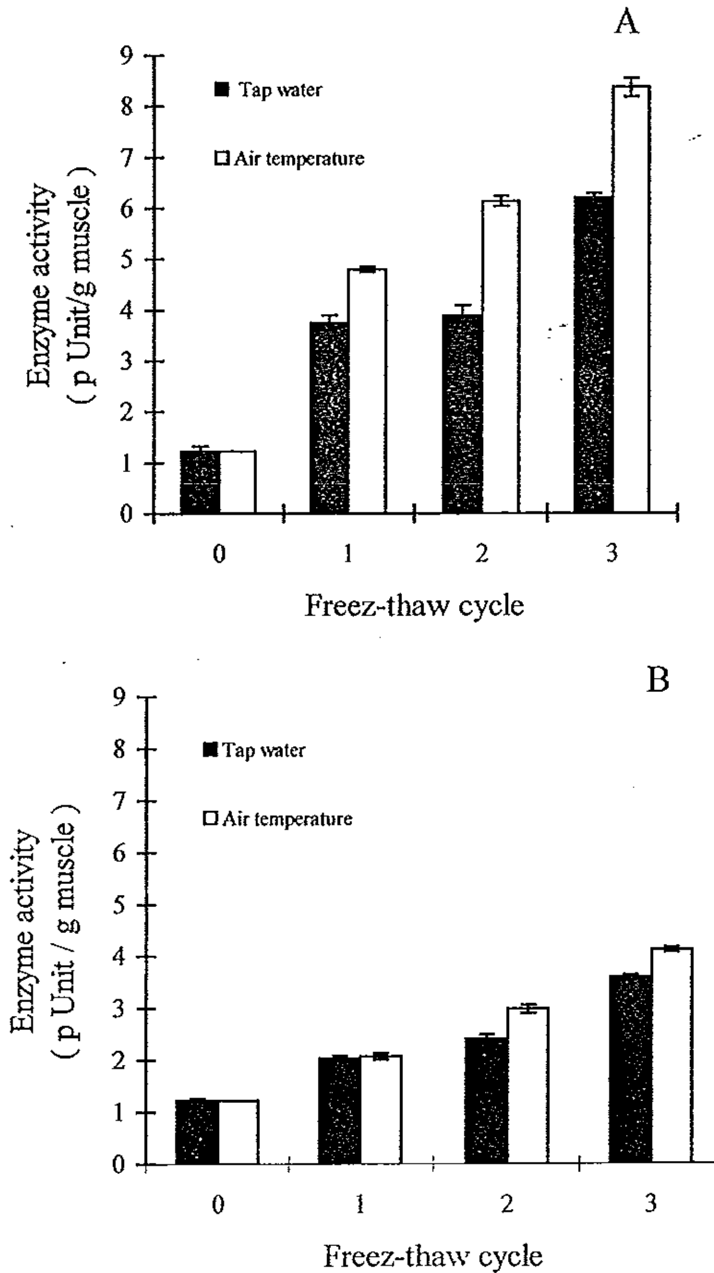


Figure 43 : α-glucosidase (AG) activity in centrifugal tissue fluid (CTF) from seabass muscle subjected to different freeze-thaw cycles.

A : -18°C ; B : -80°C

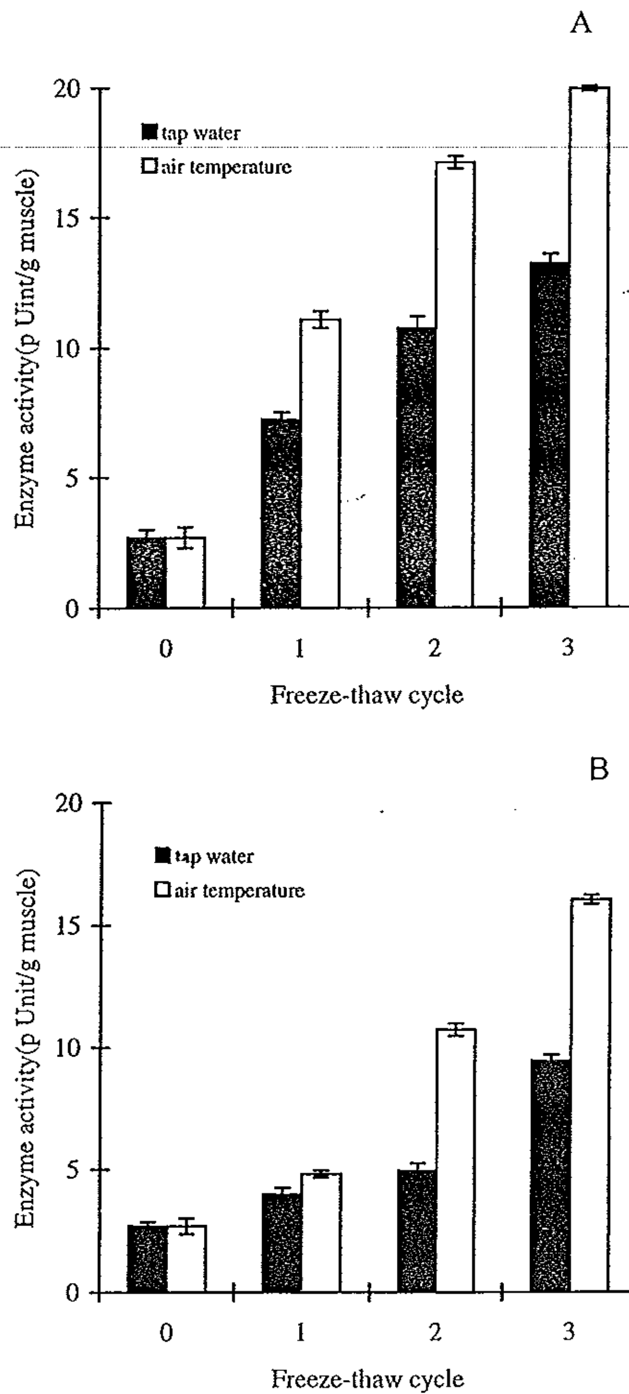


Figure 44 : β-N-acetylglucosaminidase (NAG) activity in centrifugal tissue fluid (CTF) from seabass muscle subjected to different freeze-thaw cycles.

A : -18°C ; B : -80°C

Chapter 4

Conclusion

1. Seabass muscle proteins underwent denaturation and degradation during iced storage, particularly after10 days.

2. Seabass muscle proteins were susceptible to denaturation when kept at -18°C, compared to -80°C, especially after 4.5 months of storage.

3. Slow freezing and thawing of seabass muscle with a repeated freeze-thaw cycle caused more cell damage as well as physicochemical changes.

References

Almas, K.A. 1981. Chemistry and microbiology of fish proceeding. Dept. of Biochemistry, Norwegian Inst. of Technol., Univ. of Trondheim. Norway.

An, H., Weerasinghe, V., Seymour, T.A., and Morrissey, M. 1994. Cathepsin degradation of Pacific whiting surimi proteins. J. Food Sci. 59 : 1013-1017.

A.O.A.C. 1991. Official Method of Analysis 14th ed. Association of Agricultural Chemists. Washington, DC.

Azuma, Y. and Konno, K. 1998. Freeze danaturation of carp myofibrils compared with thermal danaturation. Fish. Sci. 64 : 287-290.

Beas, V.E., Wagner, J.R., Crupkin, M., and Anon, M.C. 1990. Thermal denaturation of hake (*Merluccius hubbsi*) myofibrillar proteins. A differential scanning calorimetric and electrophoretic study. J. Food Sci. 55 : 683-696.

Beas, V.E., Wagner, J.R., Anon, M.C., and Crupkin, M. 1991. Thermal denaturation in fish muscle proteins during gelling : effect of spawning condition. J. Food Sci. 56 : 281-284.

Bechtel, J.P. 1986. Muscle development and contractile proteins. *In* Muscle as Food. (ed. J.P. Bechtel) pp. 1-35. London : Academic Press.

Beck, C. and Tappel, A.L. 1967. An automated multiple enzyme monitor for column chromatography. Anal. Biochem. 21 : 208-218.

Benjakul, S. and Morrisey, M.T. 1997. Protein hydrolysates from Pacific whiting solid wastes. J. Agric. Food Chem. 45 : 3423-3430.

Benjakul, S., Seymour, T.A., Morrissey, M.T., and An, H. 1997. Physicochemical changes in Pacific whiting muscle proteins during iced storage. J. Food Sci. 62 : 729-733.

Bligh, E.G. and Dyer, W. J. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37 : 911-917.

Brown, W.D. 1986. Fish muscle as food. *In* Muscle as Food (ed. P.J. Bechtel). pp.36-54 London : Academic Press.

Buttkus, H. 1971. The sulhydryl content of rabbit and trout myosins in relation to protein stability. Can. J. Biochem. 49 : 97-107.

Chalmer, M., Careche, M., and Mackies, I.M. 1992. Properties of actomyosin isolated from cod (*Gadus morhua*) after various periods of storage in ice. J. Sci. Food Agric. 58 : 375-383.

Chan, J.K., Gill, T.A., Thompson, J.W., and Singer, D.S. 1995. Herring surimi during low temperature setting, physicochemical and textural properties. J. Food Sci. 60 : 1248-1253.

Connell, J.J. 1961. The relative stabilities of the skeletal muscle myosin of some animals. J. Biochem. 80 : 503-509.

Connell, J.J. 1975. The role of formaldehyde as a protein crosslinking agent acting during the frozen storage of cod. J. Sci. Food Agric. 26 : 1925-1929.

Connell, J.J., Laird, W.M., Mackie, I.M., and Ritchie, A.H. 1978. Changes in proteins during frozen storage of cod as detected by SDS-electrophoresis. Proc. : V Int. Congress Food Sci. Technol.

Copeland, R.A. 1994. Methods of protein analysis : a practical giude to laboratory protocols. New York : Chapman & Hall. pp. 228.

Davies, J.R., Bardsley, R.O., and Ledward, D.A. 1988. Myosin thermal stability in fish muscle. J. Sci. Food Agric. 45 : 61-68.

Ebashi, S. and Endo, M. 1968. Calcium and muscle contraction. Prog. Biophys. Mol. Biol. 18 : 123-183.

Ebashi, S. Kodama, A., and Ebashi, M. 1968. Troponin I. Preparation and physiological function. J. Biochem. 64 : 465-477.

Foegeding, E.A., Lanier, T.C., and Hultin, H.O. 1996. Characteristics of edible muscle tissue. *In* Food Chemistry (ed. O.R. Fennema) pp. 879-942. New York : Marcel Dekker.

Gilliland, S.E., Michener, H.D., and Kraft, A.A. 1984. Psychrotrophic microoganisms. *In* Compendium of methods for the microbiological examination of foods (ed. M.L. Speck) pp. 173-178. Washington, DC : American Public Health Association, Inc.

Haard, N.F. 1990. Influence of holding fillets from undersize Atlantic cod (*Gadus morhua*) at 0°C or -3°C on the yield and quality of surimi. *In* Engineered Seafood Including Surimi (ed. R.E. Martin & R.L. Collette) pp. 89-111. NJ : Noyes Corporation Park Ridge.

Haard, N.F. 1992. Biochemical reactions in fish muscle during frozen storage. *In* Seafood Science and Technology (ed. E.G. Bligh) pp. 176-209. Canada : Fishery News Books.

Haard, N.F., Simpson, B.K., and Sikorski, Z.E. 1994. Biotechnological applications of seafood proteins and other nitrogenous compounds Ch.13, *In* Seafood Proteins (ed. Z.E. Sikorski, B.S. Pan, and F. Shihidi), p.194-216. Chapman & Hall, Inc., New York.

Hamada, I., Tsuji, K., Nakayama, T., and Niwa, E. 1977. Oxidative denaturation of actomyosin. Bull. Jap. Soc. Sci. Fish. 43 : 1105-1109.

Hatano, S. 1968. Effects of freezing and storage on the enzyme activities. Refigeration (Tokyo). 43 : 14-20.

Hashimoto, K., Watabe, S., Kono, M., and Shiro, K. 1979. Muscle protein composition of sardine and mackerel. Bull. Jpn. Soc. Sci. Fish. 45 : 1435-1441.

Hayakawa, S. and Nakai, S. 1985. Contribution of hydrophobicity, net charge and sulhydryl groups to thermal properties of ovalbumin. Can. Inst. Food Sci. Technol. J. 18 : 290-295.

Huang, G.C., Ushino, H., Watabe, S., Iwamoto, M., and Hashimoto, K. 1991. The effect of thermal acclimation on rigor mortis progress of carp stored at different temperatures. Nippon Suisan Gakkaishi. 57 : 541-548.

Huang, Y., Kouadio, K., and Gates, K.W. 1992. Effect of packaging on chemical changes and quality of refrigerated spot (*Leiostomus xanthurus*). J. Aqua. Food Prod. Technol. 1(2) : 111-127.

Huang, Y., Kouadio, K., and Gates, K.W. 1993. Microbiological, sensory and chemical assessment of pre-packaged weakfish

during iced storage. J. Aqua. Food Prod. Technol. 2 (1) : 135-144.

Itoh, M., Inoue, N., and Shinano, H. 1991. Denaturation of carp myosin B during slow freezing. Nippon Suisan Gakkaishi. 57 : 319-323.

Jacobson, A.L. and Henderson, J. 1973. Temperature sensitivity of myosin and actomyosin. Can. J. Biochem. 51 : 71-86.

Jarenback, L. and Liljmark, A. 1975. Ultrastructural changes during frozen storage of cod (*Gadus morhua* L.). II. structure of extracted myofibrillar proteins and myofibril residues. J. Food Technol. 10 : 309-325.

Jiang, S.T., Ho, M.L., and Lee, T.C. 1985. Optimization of the freezing conditions on mackerel and amberfish for manufacturing minced fish. J. Food Sci. 50 : 727-732.

Jiang, S.T., Hwang, D.C., and Chen, C.S. 1988. Effect of storage temperatures on the formation of disulfides and denaturation of milkfish actomyosin (*Chanos chanos*). J. Food Sci. 53 : 1333-1335.

Jiang, S.T. and Lee, T.C. 1985. Changes in free amino acids and protein denaturation of fish muscle during frozen storage. J. Agric. Food Chem. 33 : 839-844.

Jiang, S.T., San, P.C., and Japit, L.S. 1989. Effect of storage temperatures on the formation of disulfides and denaturation of tillapia hybrid actomyosin (*Tilapia nilotica* x *Tilapia aurea*). J. Agric. Food Chem. 37 : 633-636.

Johnston, I.A. and Frearson, N.G. 1973. The effects of environmental temperature on properties of myofibrillar adenosine triphosphate from various species of fish. J. Biochem. 133 : 735-738.

Kamal, M., Watabe, S., and Hashimoto, K. 1991. Postmortem changes in myofibrillar ATPase of sardine ordinary and dark muscles. Nippon Suisan Gakkaishi. 57 : 1177-1184.

Kitamikado, M., Yuan, C.H., and Ueno, R. 1990. An enzymatic method designed to differentiate between fresh and frozen-thawed fish. J. Food Sci. 55 : 74-76.

Koohmaraie, M., Kennick, W.H., Elgasim, E.A., and Anglemier, A.F. 1984. Effects of postmortem storage on muscle protein degradation : analysis by SDS-polyacrylamide gel electrophoresis. J. Food Sci. 49 : 292-293.

LaBlanc, E.I. and LaBlanc, R. 1992. Denaturation of hydrophobicity and reactive groups in proteins of cod (*Gadus morhua*) muscle during frozen storage. Food Chem. 43 : 3-11.

Leammli, U.K. 1970. Cleavage of structure protein during the assembly of head bacteriophage T4. Nature. 277 : 680-685.

Lee, C.L. 1995. A revive of the barramundi aquaculture in tropical Australia. School of Biological Sciences, Northern Territory University. Darwin, Australia. 14 pp.

Lees, R. 1975. Food analysis : analytical and quality control methods for the food manufacture and buyer. London : Hill books.

Lim, H.K. and Haard, N.F. 1984. Protein insolubilization in frozen Greeland halibut (*Reinhardtius hippoglossides*). J. Food Biochem. 8 : 163-187.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. J. Biol Chem. 193 : 265-275.

MacDonald, G.A. and Lanier, T.C. 1991. Carbohydrates as cryoprotectants for meats and surimi. Food Technol 45(3) : 150-159.

MacDonald, G.A. and Lanier, T.C. 1994. Actomyosin stabilization to freeze-thaw and heat denaturation by lactate salts. J. Food Sci. 59 : 101-105.

Mackie, I.M. 1993. The effects of freezing on flesh proteins. Food Rev. Inter. 9 : 575-610.

Mackie, I.M. 1994. Fish Protein. *In* New and developing sources of food proteins. (ed. B.F.J. Hudson) pp. 95-143. London : Chapman & Hall.

Magnusson, H. and Martinsdottir, E. 1995. Storage quality of fresh and frozen-thawed fish in ice. J. Food Sci. 60 : 273-278.

Milanesi, A.A., and Bird, J.W. 1972. Lysosomal enzymes in aquatic species II. distribution and particle properties of thermally acclimated muscle lysosomes of rainbow trout, *Salmo gairdneri*. Comp. Biochem. Physiol. 41 : 573-591.

Morrissey, M.T., Wu, J.W., Lin, D.D., and An, H. 1993. Protease inhibitor effects on torsion measurements and autolysis of Pacific whiting of surimi. J. Food Sci. 61 : 270-274.

Muyonga, J.H. and Regenstein, J.M. 1997. Bacterial and autolytic changes in water retention and adenosine nucleotides of cod mince. J. Food Sci. 62 : 1139-1188.

Nambudiri, D.D. and Gopakumar, K. 1992. ATPase and lactate dehydrogenase activities in frozen stored fish muscle as indices of cold storage deterioration. J. Food Sci. 57 : 72-76.

Ng, C.S. 1987. Determination of trimethylamine oxide (TMAO-N), trimethylamine (TMA-N), total volatile basis nitrogen (TVB-N) by Conway's method. *In* Laboratory manual on analytical methods and procedures for fish and fish products (ed. H. Hasegawa) pp. B3.1-3.8. Marine fisheries research department. SEAFDEC. Singapore.

Ng, C.S. 1987. Determination of formaldehyde in fish meat using Nash's reagent. *In* Laboratory manual on analytical methods and procedures for fish and fish products (ed. H. Hasegawa). pp. B5.1-5.4. Marine fisheries research department. SEAFDEC. Singapore.

Nilsson, K. and Ekstrand, B. 1993. The effect of storage on ice and various freezing treatments on enzyme leakage in muscle tissue of rainbow trout (*Oncorhynchus mykiss*). Z Lebensm Unters Forsch. 97 : 3-7.

Nilsson, K. and Ekstrand, B. 1994. Enzyme leakage in muscle tissue of rainbow trout (*Oncorhynchus mykiss*) related to various thawing treatments. Z Lebensm Unters Forsch. 98 : 253-257.

Nilsson, K. and Ekstrand, B. 1995. Frozen storage and thawing methods affect biochemical and sensory attributes of rainbow trout. J. Food Sci. 60 : 627-635.

Ofstad, R., Egelandsdal, B., Kidmen, S., Myklebust, R., Olsen, R.L., and Hermannsson, A. 1996. Liquid loss as affected by *post mortem* ultrastructural changes in fish muscle : cod (*Gadus morhua* L) and salmon (*Salmo salar*). J. Sci. Food Agric. 71 : 301-312.

Okada, T., Ohta, F., Inoue, N., and Akiba, M. 1985. Denaturation of carp myosin B in KCl solution during frozen storage. Bull. Jap. Soc. Sci. Fish 51: 1887-1892.

Ogawa, M., Ehara, T., Tamiya, T., and Tsuchiya, T. 1993. Thermal stability of fish myosin. Comp. Biochem. Physiol. 106 : 517-521.

Ogawa, M., Tamiya, T., and Tsuchiya, T. 1994. Structural changes of carp myosin during heating. Fish. Sci. 60 : 723-727.

Ogawa, M., Kanamaru, J., Miyashita, H. Tamiya, T., and Tsuchiya, T. 1995. Alpha-helical structure of fish actomyosin : changes during setting. J. Food Sci. 60 : 297-299.

Ouali, A. and Valin, C. 1981. Effect of muscle lysosomal enzyme and calcium activated neutral proteinase on myofibrillar ATPase activity with aging change. Meat Sci. 5 : 233-245.

Poulter, R.G., Ledward, D.A., Godber, S., Hall, G., and Rowlands, B. 1985. Heat stability of fish muscle proteins. J. Food. Technol. 20 : 203-217.

Ragnarsson, K. and Regenstein, J.M. 1989. Changes in electrophoretic patterns of gadoid and non-gadoid fish muscle during frozen storage. J. Food Sci. 54 : 819-823.

Rehbein, H. 1979. Development of an enzymatic method to differentiate fresh and sea-frozen and thawed fish fillets. Z. Lebensm. Unters. Forsch. 169 : 263.

Rehbein, H., Kress, G., and Schreiber, W. 1978. An enzymic method for differentiating thawed and fresh fish fillets. J. Sci. Food Agric. 29 : 1076-1082.

Roura, S.I. and Crupkin, M. 1995. Biochemical and functional properties of myofibrils from pre- and post-spawned hake (*Merluccius hubbsi Marini*) stored in ice. J. Food Sci. 60 : 269-272.

Roura, S.I., Monteccia, C., Goldemberg, A.L., Trucco, R.E., and Crupkin, M. 1990. Biochemical and physicochemical properties of actomyosin from pre and post-spawned hake (*Merluccius hubbsi Marini*) stored on ice. J. Food Sci. 55 : 688-692.

Sawicki, E., Hauser, T.R., Stanley, T.W., and Elbert, W. 1961. The 3-methyl-2-benzothiazolone hydrazone test sensitive new methods for the detection, rapid estimation, and determination of aliphatic aldehydes. Anal. Chem. 33 : 93-96.

Seifter, S., Gallop, M.P., Michaels, S., and Meilman, E. 1960. Analysis of hydroxamic acids and hydrazides ; preparation and properties of dinitrophenyl derivatives of hydroxamic acids, oximes, hydrazides, and hydrazones. J. Biol. Chem. 235 : 2613-2618.

Seki, N. 1977. Extraction of fish muscle protein. *In* Fish Proteins (*Gyoniku Tanpakushitsu*) pp.7-23. Tokyo : Koseisha.

Seki, N., Ikeda, M., and Narita, N. 1979. Changes in ATPase activities of carp myofibril during ice-storage. Bull. Jap. Soc. Sci. Fish. 45 : 791-799.

Seki, N. and Narita, N. 1980. Changes in ATPase activities and other properties of carp myofibrillar proteins during iced storage. Bull. Jap. Soc. Sci. Fish. 46 : 207-213.

Shimomura, H., Takahashi, T., Morishita, T., and Ueno, R. 1987. Investigation of the differentiation of frozen-thawed fish from unfrozen fish by comparison of lysosomal enzyme activity. Nippon Suisan Gakkaishi. 53 : 1841-1845.

Sompongse, W., Itoh, Y., and Obatake, A. 1996. Effect of cryoprotectants and a reducing reagent on the stability of actomyosin during storage. Fish. Sci. 62 : 73-79.

Sompongse, W., Itoh, Y., and Obatake, A. 1996. Role of SH_a in the polymerization of myosin heavy chain during iced storage of carp actomyosin. Fish. Sci. 62 : 110-113.

Srinivasan, S., Xiong, Y.L., and Blanchard, S. 1997. Effects of freezing and thawing methods and storage time on thermal properties of freshwater prawns (*Macrobrachium rosenbergii*). J. Sci. Food Agric. 75 : 37-44.

Stabursvik, E. and Marten, H. 1980. Thermal denaturation of proteins in post rigor muscle tissue as studied by differential scanning calorimetry. J. Sci. Food Agric. 31 : 1034-1042.

Stanby, M.E. 1962. Proximate composition of fish. FAO Symp. Fish in nutrition. London : Fishing News (Books).

Suzuki, T. 1981. Fish and Krill Proteins, Processing Technology. pp. 1-45. London : Applied Science.

Tachibana, K., Misima, T., and Tsuchimoto, M. 1993. Changes of ultrastructure and cytochemical Mg^{2+}-ATPase activity in ordinary muscle of cultured and wild red sea bream during storage in ice. Nippon Suisan Gakkaishi. 54 : 721-727.

Tokiwa, T. and Matsumiya, J. 1969. Fragmentation of fish myofibril. Effect of storage condition and muscle cathepsin. Bull. Jap. Soc. Sci. Fish. 35 : 1099-1109.

Tsai, S.F., Wang, J.H., and Jiang, S.T. 1989. Stability of muscle proteins from some subtropical fish. J. Agric. Food Chem. 37 : 749-752.

Ueno, R., Yuan, C., and Horiguchi, Y. 1988. Study of the distribution of β-*N*-acetylglucosaminidase in fish blood. Nippon Suisan Gakkaishi. 54 : 95-101.

Umemoto, S. 1966. A modified method for estimation of fish muscle protein by Biuret method. Bull. Jap. Soc.Sci. Fish. 32 : 427-435.

Venugopal, V., Alur, M.D., and Lewis, N.F. 1983. Extracellular protease from *Pseudomonas marinoglutinosa* : some properties and its action on fish actomyosin. J. Food Sci. 48 : 671-702.

Wagner, J.R. and Anon, M.C. 1986. Effect of frozen storage on protein denaturation in bovine muscle I. Myofibrillar ATPase activity and differential scanning calorimetric studies . J. Food Technol. 21 : 9.

Watabe, S., Ushino, H., Iwamoto, M., Yamanako, H., and Hashimoto, K. 1989. Temperature-dependency of rigor-mortis of fish muscle : Myofibrillar Mg^{2+}-ATPase activity and Ca^{2+} uptake by sarcoplasmic reticulum. J. Food Sci. 54 : 1107-1115.

Wierrbicki, E. and Deatherage, F.E. 1985. Determination of water holding capacity of fresh meats. J. Agric. Food Chem. 58 : 387.

Wright, D.J. and Wilding, P. 1984. Differential scanning calorimetric study of muscle and its proteins : myosin and subfragments. J. Sci. Food Agric. 35 : 357-372.

Yamagushi, M. and Sekine, T. 1966. Sulhydryl groups involved in the active site of myosin. A adenosine triphosphate. I. Specific blocking of the SH group responsible for the inhibitory phase in "B : phasic response" of the catalytic activity. J. Biochem. 59 : 24-33.

Yoshikawa, K., Inoue, N., Kawai, Y., and Shinano, H. 1995. Changes of the solubility and ATPase activity of carp myofibrils during frozen storage at different temperatures. Fish. Sci. 61 : 804-812.

Yoshikawa, K., Inoue, N., Kawai, Y., and Shinano, H. 1995. Subunit components in salt-soluble and insoluble fractions of carp myofibrils during frozen storage. Fish. Sci. 61 : 813-816.

Zhuang, R.Y., Huang, Y., and Beuchat, L.R. 1996. Quality changes during refrigerated storage of packaged shrimp and catfish fillets treated with sodium acetate lactate or propryl gallate. J. Food Sci. 61 : 241-244.

Appendices

Appendix 1
Analytical Methods

Moisture content (AOAC, 1991)

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 hrs. 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 = weight (g) of sample before drying

W_2 = weight (g) of sample after drying

Ash (AOAC, 1991)

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 burnt off. Cool the crucible in the desiccator (30 mins).
2. Weigh the crucible and lid to 3 decimal places.
3. Weigh about 5 g sample into the 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 with 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 to 3 decimal places.
6. Ash must be white or light gray. If not, return the crucible and lid to the furnace for further ashing.

Calculation

$$\% \text{ Ash content} = \frac{\text{Weight of ash x 100}}{\text{Weight of sample}}$$

Protein (AOAC, 1991)

Reagents

- Kjeldahl catalyst : Mix 9 parts of potassium sulphate (K_2SO_4) anhydrous, nitrogen free with 1 parts of copper sulphate (Cu_2SO_4)
- Sulfuric (H_2SO_4)
- 40% NaOH solution (w/v)

- 0.02N 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 weighed sample (0.5-1.0 g) in digestion flask.
2. Add 5 g of kjeldahl catalyst, and 20 ml of conc. H_2SO_4.
3. Prepare a tube containing the above chemicals except sample as blank. Place flask 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 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 distillate with standard NaOH solution.

Calculation

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

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

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

N = Normality of HCl

W = weight (g) of sample

14.007 = atomic weight of nitrogen

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

Salt (Chloride as Sodium Chloride) (AOAC, 1991)

Reagents

- 0.1N Silver nitrate standard solution
- 0.1N Ammonium thiocyanate standard solution
- 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 0.1N $AgNO_3$ solution, more than enough to precipitate all Cl as AgCl, and then add 20 ml HNO_3. Boil gently on hot plate or sand bath until all solids except AgCl are dissolved (usually 15 min). Cool and add 50 ml indicator, and titrate with 0.1N NH_4SCN solution until solution becomes permanent light brown. Subtract volume (ml) of 0.1 N NH_4SCN used from volume (ml) of 0.1N $AgNO_3$ added and calculated difference as NaCl. With 10 g sample, each ml 0.1N $AgNO_3$ = 0.058% NaCl

Lipid (Bligh and Dyer, 1952)

Reagents

- Chloroform
- Methanol
- Sodiumsulfate anhydrous

Method

1. Weigh 50 g of sample, add 50 ml of distilled water, 100 ml of chloroform, and 200 ml of methanol.
2. Homogenize for 2 min.
3. Add 100 ml of chloroform and homogenize for 1 min.
4. Add 100 ml of distilled water and homogenize for 30 sec.
5. Centrifuge at 2000 *xg* for20 min
6. Remove chloroform layer
7. Add sodiumsulfate anhydrus and mix thoroughly and filter
8. Evaporate chloroform (T ~ 50°C)
9. Collect fat and weight

Determination of Ammonical nitrogen (AOAC, 1991)

Reagents

- MgO
- 4% H_3BO_3
- Indicator solution : Mix 100 ml of 0.1% methyl red (in 95% ethanol) with 200 ml of 0.2% bromocresol green (in 95% ethanol)
- 0.1 N H_2SO_4

Method

1. Weigh 4 g of sample into kjeldahl flask with 25 ml of H_2O.
2. Add 3 g of MgO and connect flask with condenser by kjeldahl connecting bulb.
3. Distill 100 ml into measured 50 ml of 4% H_3BO_3.
4. Titrate with standard acid (0.1 N H_2SO_4)

Calculation

$$\text{Ammonical nitrogen (mg/g sample)} = \frac{14 \times N \times V}{W}$$

where :

N = Normality of H_2SO_4

V = volume (ml) of 0.1 N H_2SO_4 used in sample titration

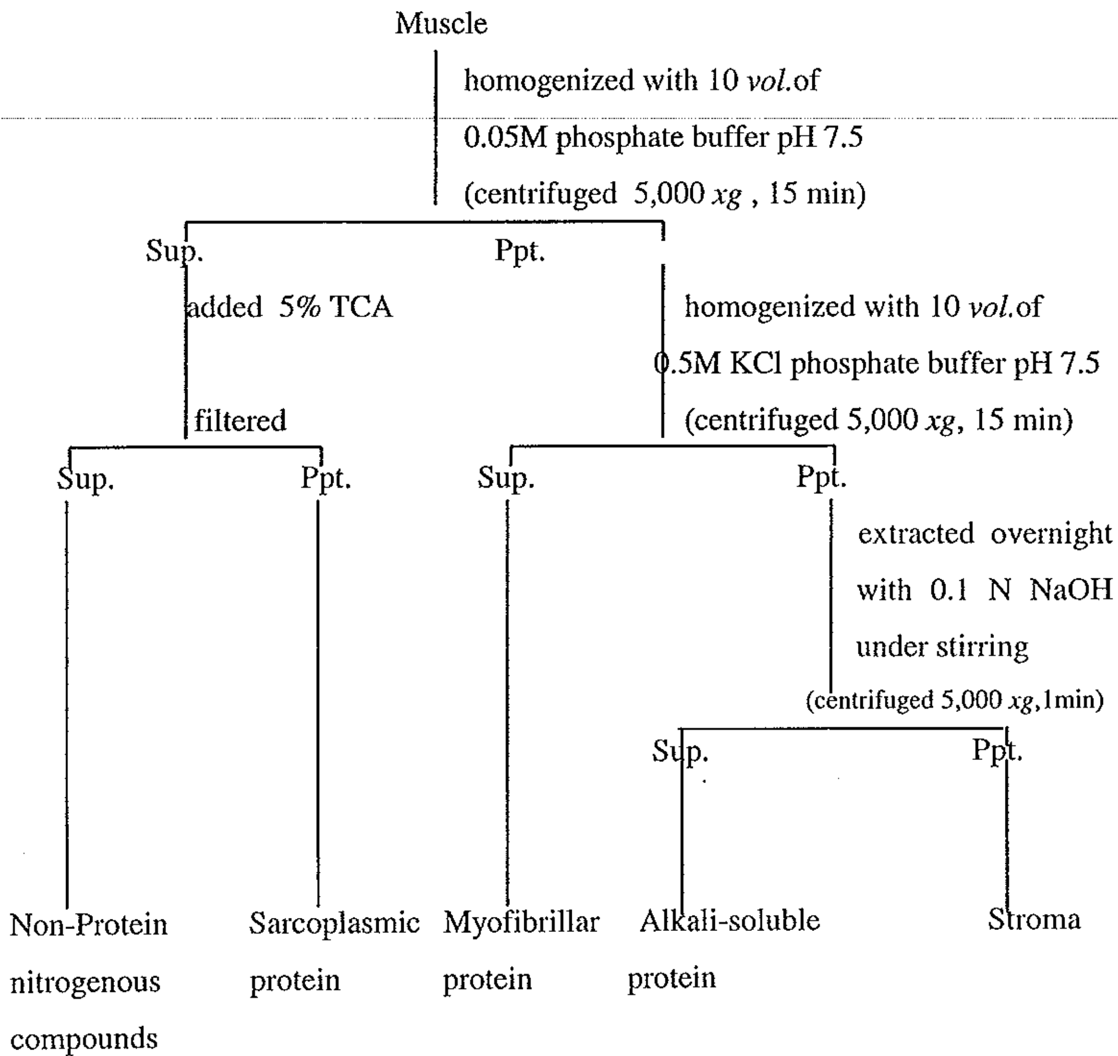
W = wieght of sample

Determination of protein composition (Hashimoto *et al.*, 1979)

Reagents

- 0.05M phosphate buffer, pH 7.5
- 0.5M KCl . phosphate buffer, pH 7.5
- Trichloroacetic acid
- 0.1N NaOH

Method



pH and lactic acid accumulation determination (Lees, 1975)

Reagents

- Phenolphthalein indicator
- 0.1 N NaOH

Method

1. Weigh 1-2 g of sample and add 150 ml of distilled water. Mix by magnetic stirrer.
2. Measure pH using pH meter
3. Titrate using 0.1 N NaOH and phenolphthale as indicator
4. Add the caustic solution-dropwise near the endpoint, making sure the end color dose not fade.

Calculation

$$\text{Lactic acid (\%)} = \frac{0.90 \times N \times V}{W}$$

where : N = Normality of NaOH

V = volume (ml) of 0.1 N NaOH used in sample titration

W = weight (g) of sample

Measurement of expressible moisture (Hasegawa, 1987)

Method

1. Place the muscle sample (X g) between 2 filter paper on top and three filter paper on the bottom.
2. Increase the pressure to 10 kg/cm^2 within 30 sec.

3. Maintain at 10 kg/cm^2 constant pressure for 2 min, then remove the sample, and weigh the pressed sample (Z g).

Calculation

$$\text{Expressible moisture (\%)} = \frac{(X - Z) \times 100}{X}$$

Determination of trimethylamine (TMA-N), total volatile basic nitrogen (TVB-N) by Conway's method (NG, 1987)

Reagents

- Inner ring solution (1% boric acid solution containing indicator) :Take 10 g of boric acid in 1 litre flask, add 200 ml of ethanol. After dissolving boric acid, add 10 ml of mixed indicator solution, then make up to 1 litre with distilled water.
- Mixed indicator solution : Dissolve bromocresol green (BCG) 0.01 g and methyl red (MR) 0.02 g in 10 ml of ethanol.
- 0.02N HCl : Dilute 20 ml of 1N HCl standard solution with distilled water and make up to 1000 ml.
- Saturated K_2CO_3 solution : Take 60 g of potassium carbonate (K_2CO_3), and add 50 ml of distilled water. Boil gently for 10 min. After cooling down, obtain filtrate through filter paper.
- 50% K_2CO_3 solution : Dilute saturated K_2CO_3 solution twice with distilled water.

- 4% trichloroacetic acid (CCl_3COOH) (TCA) solution : Dissolve 40 g of TCA in 960 ml of distilled water.
- Sealing agent : Take 3 g Tragacanth gum, add 30 ml of distilled water, 15 ml of glycerine and 15 ml of 50% saturated K_2CO_3 solution and mix well.
- Neutralized 10% formaldehyde solution : Add 10 g of $MgCO_3$ to 100 ml of formaline (35% formaldehyde solution) and shake in order to neutralize the acidity of formaline. Filter and dilute filtrate 3 times with distilled water.

Method

Sample extraction :

1. Take 2 g of fish meat in a mortar and grind well.
2. Add 8 ml of 4% TCA solution and grind well.
3. Stand for 30 min at ambient temperature with occasional grinding.
4. Filter through filter paper (Whatman No. 41) (or centrifuge at 3,000 rpm, for 10 min).
5. Keep the filtrate in -20°C freezer if necessary.

Determination of TVB-N :

1. Apply sealing agent to Conway's unit.
2. Pipette 1 ml of inner ring solution into inner ring.
3. Pipette 1 ml of sample extract into outer ring.
4. Slant the Conway's unit with cover.
5. Pipette 1 ml of saturated K_2CO_3 solution into outer ring.

6. Close the unit.
7. Mix gently.
8. Stand for 60 min. at 37°C in incubator.
9. Titrate inner ring solution with 0.02N HCl using a micro-burette until green color turns to pink.
10. Do blank test using 1 ml of 4% TCA instead of sample extract.

Determination of TMA-N :

1. Apply sealing agent to Conway's unit.
2. Pipette 1 ml of inner ring solution into inner ring.
3. Pipette 1 ml of sample extract into outer ring.
4. Pipette 1 ml of neutralized 10% formaldehyde into outer ring.
5. Slant the Conway's unit with cover.
6. Pipette 1 ml of saturated K_2CO_3 solution into outer ring.
7. Close the unit.
8. Mix gently.
9. Stand for 60 min. at 37°C in incubator.
10. Titrate inner ring solution with 0.02N HCl using a micro-burette until green colour turns to pink.
11. Do blank test using 1 ml of 4% TCA instead of sample extract.

Calculation

$$\underset{(\text{ mg N/ 100 g })}{\text{TMA-N or TVB-N}} = \frac{(V_S - V_B) \times (N_{HCl} \times A_N) \times V_E \times 100}{W_S}$$

where : V_S = Titration volume of 0.02N HCl for sample extract (ml)

V_B = Titration volume for blank (ml)

N_{HCl}= Normality of HCl (=0.02N x f, factor of HCl)

A_N = Atomic weight of Nitrogen(x 14.00)

W_S = Weight of muscle sample (g)

V_E = Volume of 4% TCA used in extraction

Determination of formaldehyde (NG, 1987)

Reagents

- 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.
- Formaldehyde standard stock solution : Pipette 0.3 ml of 35% formaldehyde and fill up to 100 ml to get approximately 1,000 ppm solution in distilled water. This aqueous solution is stable for several months.
- Formaldehyde standard stock solution : Dilute the stock solution 100 times as follows : Pipette 10 ml of the stock solution (approximately 1,000 ppm) and make up to 100 ml with distilled water to get approx. 100 ppm solution. Ten ml of 100 ppm solution is diluted 10 times with distilled water in the volumetric flask. This final dilute gives approx. 10 ppm

solution of formaldehyde. This dilute is not stable, so, it is necessary to be renewed in each series of determination.

- 0.1N 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.
- Sodium bisulfite solution (approximately 0.1N) : Dissolve 5.2 g of $NaSHO_3$ in distilled water and make up to 1 litre.
- 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 litre.
- 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 of 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 1N or 0.1N KOH dropwise, and make up to 50 ml with distilled water.

2. Stand in water bath (60°C) for 15 min.
3. Cool the solution in running water.
4. 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

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

Where : A = Reading from calibration curve (μg)

f = factor of formaldehyde of standard solution.

Measurement of autolytic degradation products (Morrissey *et al.*, 1993 ; Benjakul *et al.*, 1997)

Reagents

- 5% Trichloroacetic acid (TCA) (w/v)
- Tyrosine

Method

1. Weigh 3 g of fish muscle and homogenize in 27 ml of 5% TCA.
2. Keep on ice for 1 hr, and centrifuge at 5,000 *xg* for 5 min.
3. Measure tyrosine in the supernatant was measured as an index of autolytic degradation products and express as μmol tyrosine/g muscle.

Lowry's procedure for quantitation of proteins (with a slight modified Lowry *et al.*, 1951)

Reagents

- A : 2% sodium carbonate in 0.1N NaOH
- B : 0.5% $CuSO_4.5H_2O$ in 1% sodium citrate
- C : 1N Folin Phenol reagent
- D : 1 ml reagent B + 50 ml reagent A (or similar ratio)
- Standard : Bovine Serum Albumin (BSA) at concentration of 1 mg/ml

Method

1. Add 2 ml reagent D to each of the standards and unknown tubes (sample = 200 ml). 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 H_2O) 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.

Standard : Bovine Serum Albumin (BSA) at concentration of 1 mg/ml

BSA(µl)	0	20	40	60	100	140	200
H_2O(µl)	200	180	160	140	100	60	0

Biuret method for quatitation of proteins (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 a 1-liter volumetric flask and bring to 1 liter with distilled water, transfer to a plastic bottle for storage.
- Distilled water
- Standard curve : 10 mg/ml bovine serum albumin (BSA)

Method

1. To each of eight disposable cuvettes, add the following reagents according to the table
2. To tubes 6-8, 50 µl of protein sample are added. Mix the contents of each tube well by using the closed end of the capillary tube as a 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 tubes 1-5, plot the absorbance at 540 nm as a function of effective BSA concentration, and calculate the best fit straight line from the data. Then, using the average absorbance for the three sample of unknown, read the concentration of sample from the plot.

Table : Experimental set up for the Biuret's assay.

Tube Number	Water	10 mg/ml BSA	Effective BSA

	(μl)	(μl)	Concentration (mg/ml)
1	500	0	0
2	400	100	20
3	300	200	40
4	200	300	60
5	100	400	80
6	450	0	unknown
7	450	0	unknown
8	450	0	unknown

Preparation of actomyosin (MacDonald and Lanier, 1994)

Reagents

- 0.6M KCl, pH 7.0
- 1.2M KCl, pH 7.0
- Distilled water

Method

1. Homogenize 4 g of muscle in 40 ml chilled (4°C) 0.6M 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 30 min at 0°C.
4. Add three volumes of chilled deionized water to precipitate actomyosin.

5. Collect actomyosin by centrifuging at 5,000 *xg* for 20 min at 0°C.
6. Dissolve the pallet by stirring for 30 min at 0°C in an equal volume of chilled 1.2M KCl, pH 7.0.
7. Remove undissolved material from the preparation by centrifugating at 5,000 *xg* for 20 min at 0°C.

Solubility (Hamada *et al.*, 1977; Jiang *et al.*, 1988)

Reagents

- Solution I : containing 8 M urea, 6 mM EDTA and 0.6 M KCl
- Solution II : containing 0.5% $NaBH_4$, 6 mM EDTA, 8 M urea and 0.6 M KCl.

Method

1. Thaw the sample to 0°C with running tap water (25°C) and centrifuge at 15,000 *xg*, 0°C, for 1 hr. The collected supernatant was defined as salt-soluble fraction (I), which was considered to be native proteins.
2. Add 5 ml of solution I into precipitate and stir for 30 min at 25°C. Centrifuge the mixture at 15,000 *xg*, 0°C, for 1 hr. The supernatant was defined as urea-soluble fraction (II), which considered to be aggregated by formation of hydrogen and hydrophobic bonds.
3. Add 5 ml of solution II into precipitate and stir for 20 min at 25°C. Centrifuge the mixture at 15,000 *xg*, 0°C, for 1 hr. The supernatant was defined as $NaBH_4$-soluble fraction (III),

which was aggregated by formation of disulfides, and final residue, as insoluble fraction (IV).

Total SH content (Sompongse *et al.*, 1996a)

Reagents

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

Method

1. Mix actomyosin (1 ml, 0.4%) with 9 ml of 0.2M Tris-HCl.
2. Take 4 ml-aliqout of of the mixture and add with 0.4 ml of 0.1% DTNB solution. Incubate the mixture at 40°C for 25 min.
3. Measure the absorbance at 412nm with spectrophotometer
4. Prepare a blank by replacing the sample with 0.6M KCl, pH 7.0.
5. Calculate SH content from the absorbance using the molar extinction of 13,600 M^{-1} cm^{-1} and express as mol/10^5 g protein.

Actomyosin ATPase activity and protein (Benjakul *et al.*, 1997)

Reagents

- 0.5M Tris maleate
- 0.1M $CaCl_2$
- 0.1M $MgCl_2$
- 0.01M EGTA

- 20mM ATP
- 15% Trichloroacetic acid

Method

1. Dilute the prepared actomyosin to 2.5-4 mg/ml with 0.6M KCl, pH 7.0. Add 1 ml of the diluted solution to 0.6 ml of 0.5M Tris-maleate, pH 7.0.
2. To that mixture, add one of the following solutions for each ATPase activity assay to a total volume of 9.5 ml :10mM $CaCl_2$ for Ca^{2+}-ATPase ; 2mM $MgCl_2$ for Mg^{2+}-ATPase ; 0.1mM $CaCl_2$ and 2mM $MgCl_2$ for Mg^{2+}-Ca^{2+}-ATPase ; and 2mM $MgCl_2$ and 0.5mM EGTA for Mg^{2+}-EGTA-ATPase.
3. To each assay solution, add 0.5 ml of 20 mM ATP to initiate the reaction. Terminate reaction by adding 5 ml chilled 15% (w/v) trichloroacetic acid (TCA) after precisely 10 min at 25°C.
4. Centrifuge the reaction mixture at 3,500 *xg* for 5 min. Measure inorganic phosphate liberated in the supernatant.

Free α-amino acid (Benjakul and Morrissey, 1997)

Reagents

- 0.2125M phosphate buffer, pH 8.2
- 0.01% 2,4,6-Trinitrobenzenesulfonic acid (TNBS) solution
- 0.1M sodium sulfite
- L-leucine

Method

1. Mix properly diluted samples (125μl) thoroughly with 2.0 ml of 0.2125M phosphate buffer, pH 8.2.
2. Add 1.0 ml of 0.01% TNBS solution.
3. Place the mixtures in a water bath at 50°C for 30 min in the dark.
4. Terminate the reaction by adding 2.0 ml of 0.1M sodium sulfite.
5. Cool the mixtures down at ambient temperature for 15 min.
6. Measure the absorbance at 420 nm and express α-amino acid in term of L-leucine.

Measurement of α-glucosidase (E.C.3.2.1.20) and *β-N*-Acetyl-glycosaminidase (E.C.3.2.1.30) (Nilsson and Ekstrand, 1993).

<u>Reagents</u>

- 0.05M Sodium citrate buffer, pH 4.0
- 0.1M Sodium citrate buffer, pH 4.5
- 1.0M NaCl
- 0.6M KCl
- 0.2M KOH
- 0.3M KOH
- Substrate : 4.2mM *p*-nitrophenyl-α-glucopyranoside solution
  1.0mM *p*-nitrophenyl-*N*-acetyl-*β*-D-glucose amide solution

Method

Sample preparation :

Centrifuge the fish slices at 22,000 *xg* for 60 min and collect the fluid with a Pasteur pipette for determination of volume, protein concentration and enzyme activity.

Enzyme assay :

α-glucosidase activity

1. Mix 0.3 ml of 0.05M sodium citrate buffer, pH 4.0 with 0.2 ml of 1.0M NaCl, 0.2 ml of 4.2 mM *p*-nitrophenyl-α-glucopyranoside solution.
2. Add 0.8 ml of sample solution (CTF) adjusted with distilled water to a total protein content of about 5 mg/ml.
3. Incubate mixture at 37°C for 60 min.
4. Stop reaction by addition of 1 ml of 0.2M KOH, and measure the absorbance immediately (λ = 405nm, E_M = 19,500 M^{-1} cm^{-1}).

β-N-Acetyl-glycosaminidase activity

1. Mix 0.3 ml of 0.1M sodium citrate buffer, pH 4.5, 0.2 ml of 0.6M KCl , 0.2ml of 1.0mM *p*-nitrophenyl-*N*-acetyl-*β*-D-glucose amide solution and 0.5 ml of appropriately diluted sample (protein content in the final mixture about 2 mg).
2. Incubate the reaction mixture at 37°C for 30 min, and stop the reaction by addition of 1 ml of 0.3M KOH.
3. Measure the absorbance as described for α-glucosidase.

Electrophoresis (SDS-PAGE) (Leammli, 1970)

Reagents

- Protein molecular weight standards
- 30% Acrylamide/0.8% bisAcrylamide
- Sample buffer : Mix 30 ml of 10% of SDS, 10 ml of glycerol, 5 ml of β-Mercaptoethanol, 12.5 ml of 50 mM Tris-HCl, pH 6.8, and 5-10 mg Bromphenol 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'-tetramethylethylenediamine)
- 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. Adjust to pH 8.3. Add distilled water to 1 litre 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 table.
3. Transfer the separating gel solution using a Pasture pipette to the center of sandwich along an edge of one of the spacers until the height of the solution in the 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 against the edge of one of the spacers. Allow the resolving gel to polymerize fully (usually 30-60 min).

Reagents	12 % gel	10 % gel
30% Acrylamide/bis	1.400 ml	1.167 ml
1.5 M Tris-HCl buffer, pH 8.8	0.875 ml	0.875 ml
1 % SDS	0.350 ml	0.350 ml
Distilled water	0.525 ml	1.340 ml
2 % Ammomium persufate	0.350 ml	0.350 ml
TEMED	5 ul	5 ul

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	Volume
30% Acrylamide/bis	0.4 ml
0.5 M Tris-HCl buffer, pH 6.8	1.0 ml
1 % SDS	0.3 ml
Distilled water	1.1 ml
0.1 M EDTA	0.8 ml
2 % Ammomium persufate	0.4 ml
TEMED	5 ul

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 hr.
3. Centrifuge at 3,500 *xg* 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 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 buffer 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 flate-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 cathode of the gel apparatus and run at 50 v and 150 v.
2. After the bromphenol 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 sanwich along its entire length.
3. Insert a spatula between the glass plates at one corner where the spacer was, and gently pry the two 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 hr 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 ~30min.
3. Pour off the destaining solution I and cover the destaining solution II. Discard destaing solution and replace with fresh solution. Repeat until the gel is clear except for the protein bands.

Psycrhotrophic microorganisms analysis (Gilliland *et al.*, 1984)

Ragents and media

- 0.85% normal saline

- plate count agar

Method

1. Weigh 10 g of sample into sterized beaker.
2. Add 90 ml of 0.85% normal saline solution and blend for 1 min by stomacher.
3. Dilute mixture to 1:100, 1:1,000, and 1:10,000 in 0.85% normal saline.

4.Pipette 1 ml of diluted mixture into sterized plate. Add 15 ml of plate count agar (PCA).

5. Incubate at 10°C for 7 days.

6. Count the colony and reported the psychrotrophic microoganisms as colony forming unit per gram sample (CFU/g sample)

Calculation

CFU/ g sample = Average number of colonies x dilution factor

Appendix 2

Analysis of variance

Table 1-A : Analysis of variance for effect of temperature on the inactivation rate constant of seabass actomyosin Ca^{2+}-ATPase.

SV	DF	SS	MS	F
Treatment	6	4432.900	738.800	66190.98**
Error	14	0.200	0.000	
Total	20	4433.100		

CV = 0.9%

** = significant at 1% level

Table 2-A : Analysis of variance for effect of pH on the inactivation rate constant of seabass actomyosin Ca^{2+}-ATPase.

SV	DF	SS	MS	F
Treatment	6	1396.400	279.300	7589.34**
Error	14	0.400	0.000	
Total	20	1396.900		

CV = 1.5%

** = significant at 1% level

Table 3-A : Analysis of variance for the salt-soluble fraction (I) of seabass actomyosin during iced storage.

SV	DF	SS	MS	F
Treatment	7	62.772	8.967	131.391**
Error	16	1.092	0.068	
Total	23	63.864		

CV = 1.5%

** = significant at 1% level

Table 4-A : Analysis of variance for the urea-soluble fraction (II) of seabass actomyosin during iced storage.

SV	DF	SS	MS	F
Treatment	7	0.389	0.055	62.312**
Error	16	0.014	0.089	
Total	23	0.403		

CV = 9.5%

** = significant at 1% level

Table 5-A : Analysis of variance for the $NaBH_4$-soluble fraction (III) of seabass actomyosin during iced storage.

SV	DF	SS	MS	F
Treatment	7	7.161	1.023	1186.775**
Error	16	0.014	0.008	
Total	23	7.715		

CV = 3.0%

** = significant at 1% level

Table 6-A : Analysis of variance for the insoluble fraction (IV) of seabass actomyosin during iced storage.

SV	DF	SS	MS	F
Treatment	7	31.203	4.458	1648.439**
Error	16	0.043	0.002	
Total	23	31.246		

CV = 4.5%

** = significant at 1% level

Table 7-A : Analysis of variance for total sulhydryl content of seabass actomyosin during iced storage.

SV	DF	SS	MS	F
Treatment	31	23.604	0.761	69.71**
Time (T)	7	12.012	1.716	157.10**
Treat (A)	3	5.662	1.887	172.79**
T x A	21	5.930	0.282	25.85**
Error	64	0.699	0.011	
Total	95	24.303		

CV = 1.3%

** = siginificant at 1% level

Table 8-A : Analysis of variance for Ca^{2+}-ATPase activity of seabass actomyosin during iced storage.

SV	DF	SS	MS	F
Treatment	31	0.066	0.002	76.48**
Time (T)	7	0.022	0.003	113.29**
Treat (A)	3	0.024	0.008	287.28**
T x A	21	0.020	0.001	34.10**
Error	64	0.002	0.000	
Total	95	0.068		

CV = 1.7%

** = significant at 1% level

Table 9-A : Analysis of variance for Mg^{2+}-ATPase activity of seabass actomyosin during iced storage.

SV	DF	SS	MS	F
Treatment	31	0.001	0.000	9.28**
Time (T)	7	0.000	0.000	20.98**
Treat (A)	3	0.000	0.000	3.62**
T x A	21	0.000	0.000	6.19**
Error	64	0.000	0.000	
Total	95	0.001		

CV = 0.5%

** = significant at 1% level

Table 10-A : Analysis of variance for Mg^{2+}-Ca^{2+}-ATPase activity of seabass actomyosin during iced storage.

SV	DF	SS	MS	F
Treatment	31	0.001	0.001	4.01**
Time (T)	7	0.003	0.002	7.38**
Treat (A)	3	0.002	0.002	6.94**
T x A	21	0.002	0.003	2.46**
Error	64	0.000	0.000	
Total	95	0.000		

CV = 0.3%

** = significant at 1% level

Table 11-A : Analysis of variance for free α-amino acid of seabass actomyosin during iced storage.

SV	DF	SS	MS	F
Treatment	31	8.915	0.288	182.96**
Time (T)	7	2.040	0.292	185.42**
Treat (A)	3	3.102	1.034	657.82**
T x A	21	3.773	0.180	114.30**
Error	64	0.101	0.002	
Total	95	9.016		

CV = 5.2%

** = significant at 1% level

Table 12-A : Analysis of variance for salt-soluble fraction (I) of seabass actomyosin during frozen storage.

SV	DF	SS	MS	F
Treatment	31	139.34	5.57	151.91**
Time (T)	7	70.66	5.89	160.48**
Treat (A)	3	28.88	28.88	787.13**
T x A	21	39.80	3.32	90.39**
Error	64	1.91	0.04	
Total	95	141.24		

CV = 2.0%

** = significant at 1% level

Table 13-A : Analysis of variance for urea-soluble fraction (II) of seabass actomyosin during frozen storage.

SV	DF	SS	MS	F
Treatment	25	0.358	0.014	128.33**
Time (S)	12	0.076	0.006	56.67**
Temp (T)	1	0.092	0.092	824.62**
S x T	12	0.190	0.016	141.96**
Error	52	0.006	0.000	
Total	77	0.364		

CV = 3.7%

** = significant at 1% level

Table 14-A : Analysis of variance for $NaBH_4$-soluble fraction (III) of seabass actomyosin during frozen storage.

SV	DF	SS	MS	F
Treatment	25	4.109	0.164	515.26**
Time (S)	12	2.028	0.169	529.83**
Temp (T)	1	0.831	0.831	2605.35**
S x T	12	1.250	0.104	326.51**
Error	52	0.017	0.000	
Total	77	4.125		

CV = 8.3%

** = significant at 1% level

Table 15-A : Analysis of variance for insoluble fraction (IV) of seabass actomyosin during frozen storage.

SV	DF	SS	MS	F
Treatment	25	110.10	4.40	181.56**
Time (S)	12	54.71	4.56	187.97**
Temp (T)	1	22.29	22.29	919.09**
S x T	12	33.09	2.76	113.70**
Error	52	1.26	0.02	
Total	77	111.36		

CV = 16.0%

** = significant at 1% level

Table 16-A : Analysis of variance for insoluble fraction (IV) of seabass actomyosin during frozen storage.

SV	DF	SS	MS	F
Treatment	25	110.10	4.40	181.56**
Time (S)	12	54.71	4.56	187.97**
Temp (T)	1	22.29	22.29	919.09**
S x T	12	33.09	2.76	113.70**
Error	52	1.26	0.02	
Total	77	111.36		

CV = 16.0%

** = significant at 1% level

Table 17-A : Analysis of variance for Ca^{2+}-ATPase activity of freeze-thawing seabass actomyosin.

SV	DF	SS	MS	F
Treatment	15	0.0411	0.0027	90.05**
Cycle (S)	3	0.0226	0.0075	247.92**
Thaw (M)	1	0.0084	0.0084	276.86**
Temp (T)	1	0.0014	0.0014	49.16**
C x M xT	3	0.0002	0.0007	2.40ns
C x M	3	0.0064	0.0021	70.13**
C x T	3	0.0016	0.0005	18.50**
M x T	1	0.0002	0.0002	7.98**
Error	32	0.0009	0.0003	
Total	47	0.0420		

CV = 1.9%

** = significant at 1% level; ns = not significant

Table 18-A : Analysis of variance for Mg^{2+}-ATPase activity of freeze-thawing seabass actomyosin.

SV	DF	SS	MS	F
Treatment	15	0.1735	0.0115	52.57**
Cycle (S)	3	0.0784	0.0261	118.82**
Thaw (M)	1	0.0519	0.0519	236.01**
Temp (T)	1	0.0148	0.0148	67.59**
C x M xT	3	0.0170	0.0056	25.83**
C x M	3	0.0051	0.0017	7.75**
C x T	3	0.0043	0.0043	19.94**
M x T	1	0.0017	0.0005	2.62ns
Error	32	0.0007	0.0002	
Total	47	0.1806		

CV = 3.0%

** = significant at 1% level; ns = not significant

Table 19-A : Analysis of variance for Mg^{2+}-EGTA-ATPase activity of freeze-thawing seabass actomyosin.

SV	DF	SS	MS	F
Treatment	15	0.0041	0.0002	50.03**
Cycle (S)	3	0.0017	0.0005	103.09**
Thaw (M)	1	0.0006	0.0006	124.09**
Temp (T)	1	0.0003	0.0003	57.31**
C x M xT	3	0.0004	0.0001	26.91**
C x M	3	0.0003	0.0001	22.02**
C x T	3	0.0001	0.0001	30.00**
M x T	1	0.0004	0.0001	27.65**
Error	32	0.0001	0.0000	
Total	47	0.0063		

CV = 5.6%

** = significant at 1% level

Table 20-A : Analysis of variance for change in pH of seabass muscle during iced storage

SV	DF	SS	MS	F
Treatment	7	0.940	0.340	123.52**
Error	16	0.017	0.001	
Total	23	0.957		

CV = 0.5%

** = significant at 1% level

Table 21-A : Analysis of variance for change in lactic acid accumulation of seabass muscle during iced storage

SV	DF	SS	MS	F
Treatment	7	18.5279	2.6468	184.17**
Error	16	0.2300	0.0144	
Total	23	18.7579		

CV = 2.4%

** = significant at 1% level

Table 22-A : Analysis of variance for change in pH of seabass muscle during iced storage

SV	DF	SS	MS	F
Treatment	7	0.700	0.100	76.77**
Error	16	0.021	0.001	
Total	23	0.721		

CV = 1.9%

** = significant at 1% level

Table 23-A : Analysis of variance for change in TVB of seabass muscle during iced storage

SV	DF	SS	MS	F
Treatment	7	127.220	18.170	31.03**
Error	16	9.370	0.590	
Total	23	136.590		

CV = 14.3%

** = significant at 1% level

Table 24-A : Analysis of variance for change in TMA of seabass muscle during iced storage

SV	DF	SS	MS	F
Treatment	7	112.48	16.070	142.15**
Error	16	1.81	0.110	
Total	23	114.29		

CV = 0.5%

** = significant at 1% level

Table 25-A : Analysis of variance for change in psychrotrophic microorganisms of seabass muscle during iced storage

SV	DF	SS	MS	F
Treatment	7	55.954	7.993	1693.23**
Error	16	0.075	0.004	
Total	23	56.029		

CV = 1.2%

** = significant at 1% level

Table 26-A : Analysis of variance for change in expressible moisture of seabass muscle during iced storage

SV	DF	SS	MS	F
Treatment	7	623.575	89.082	665.580**
Error	16	2.141	0.134	
Total	23	625.717		

CV = 2.4%

** = significant at 1% level

Table 27-A : Analysis of variance for change in pH of seabass muscle during iced storage

SV	DF	SS	MS	F
Treatment	7	0.112	0.016	146.02**
Error	16	0.002	0.000	
Total	23	0.114		

CV = 3.4%

** = significant at 1% level

Table 28-A : Analysis of variance for salt-soluble fraction (I) of actomyosin from seabass muscle during frozen storage.

SV	DF	SS	MS	F
Treatment	25	3.1343	0.1254	97.00**
Storage time (S)	12	1.3841	0.1153	89.24**
Temperature (T)	1	0.5021	0.5021	388.47**
S x T	12	1.248	0.1040	80.47**
Error	52	0.0672	0.0013	
Total	77	3.0215		

CV = 0.4%

** = significant at 1% level

Table 29-A : Analysis of variance for urea-soluble fraction (II) of actomyosin from seabass muscle during frozen storage.

SV	DF	SS	MS	F
Treatment	25	0.1620	0.0065	13.61**
Storage time (S)	12	0.1032	0.0086	18.07**
Temperature (T)	1	0.0530	0.0530	111.38**
S x T	12	0.0057	0.0005	1.00ns
Error	52	0.0248	0.0005	
Total	77	0.1868		

CV = 10.0%

** = significant at 1% level; ns = not significant

Table 30-A : Analysis of variance for $NaBH_4$-soluble fraction (III) of actomyosin from seabass muscle during frozen storage.

SV	DF	SS	MS	F
Treatment	25	1.2587	0.0504	268.29**
Storage time (S)	12	0.6294	0.0525	279.50**
Temperature (T)	1	0.2948	0.2948	1570.71**
S x T	12	0.3345	0.0279	148.55**
Error	52	0.0098	0.0002	
Total	77	1.2685		

CV = 5.9%

** = significant at 1% level

Table 31-A : Analysis of variance for insoluble fraction (IV) of actomyosin from seabass muscle during frozen storage.

SV	DF	SS	MS	F
Treatment	25	1.0143	0.0406	188.01
Storage time (S)	12	0.8396	0.0700	324.21
Temperature (T)	1	0.0998	0.0998	462.43
S x T	12	0.0749	0.0062	28.93
Error	52	0.0112	0.0002	
Total	77	1.0256		

CV = 4.6%

** = significant at 1% level

Table 32-A : Analysis of variance for AG activity of actomyosin from seabass muscle during frozen storage.

SV	DF	SS	MS	F
Treatment	15	259.70	17.30	202.70**
Cycle (S)	3	175.70	58.60	685.72**
Thaw (M)	1	21.00	21.00	246.39**
Temp (T)	1	26.30	26.30	308.00**
C x M xT	3	19.40	6.50	75.68**
C x M	3	10.20	3.40	39.96**
C x T	3	0.00	0.00	<1
M x T	1	7.00	2.30	27.29**
Error	32	2.70	0.10	
Total	47	262.50		

CV = 7.9%

** = significant at 1% level

Table 32-A : Analysis of variance for NAG activity of actomyosin from seabass muscle during frozen storage.

SV	DF	SS	MS	F
Treatment	15	1668.00	111.00	90.89**
Cycle (S)	3	1161.00	387.00	315.72**
Thaw (M)	1	120.00	120.00	98.22**
Temp (T)	1	222.00	222.00	181.10**
C x M xT	3	24.00	8.00	6.66**
C x M	3	61.00	20.00	16.61**
C x T	3	78.00	26.00	21.30**
M x T	1	0.00	0.00	< 1
Error	32	39.00	1.00	
Total	47	1707.00		

CV = 12.2%

** = significant at 1% level

Vitae

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