Role of Endogenous Transglutaminase in Setting of Surimi from Some Tropical Fish

Chakkawat Chantarasuwan

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Author	Mr. Chakkawat Chantarasuwan
Major Program	Fishery Products Technology

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ชื่อวิทยานิพนธ์ บทบาทของเอนไซม์ทรานส์กลูทามิเนสในกล้ามเนื้อต่อการเซ็ทตัวของซูริมิจากปลาในเขตร้อนบางชนิด

ผู้เขียน นายจักรวรรดิ์ จันทรสุวรรณ

สาขาวิชา เทคโนโลยีผลิตภัณฑ์ประมง

ปีการศึกษา 2543

บทคัดย่อ

ซูริมิจากปลาในเขตร้อน 4 ชนิด ประกอบด้วยปลาทรายแดง (*Nemipterus bleeker*) ปลาตาหวาน (*Priacanthus tayenus*) ปลาเข็ม (*Sphyraena jello*) และปลาจวด (*Pennahai macrophthalmus*) มีองค์ประกอบทางเคมีที่ใกล้เคียงกัน โดยมีโปรตีนไมโอไฟบริลเป็นองค์ประกอบหลัก จากการศึกษาสภาวะที่เหมาะสมในการเซ็ทตัวของซูริมิทั้ง 4 ชนิด พบว่าเจลแอชชิซึ่งเตรียมโดยการเซ็ทตัวที่อุณหภูมิ 40 องศาเซลเซียส เป็นระยะเวลาที่เหมาะสม ซึ่งแตกต่างตามชนิดของปลา ตามด้วยการให้ความร้อนที่อุณหภูมิ 90 องศาเซลเซียส 20 นาที ให้ค่าแรง (Breaking force) และระยะทางก่อนเจาะทะลุ (Deformation) สูงกว่าเจลซูริมิที่ผ่านการให้ความร้อนโดยตรง (เจลซูริมิที่ไม่ได้ผ่านการเซ็ตตัว) ($p<0.05$) ดังนั้นการเซ็ทตัวจึงมีบทบาทสำคัญต่อคุณสมบัติของเจล ซึ่งอาจเป็นผลจากการทำงานของเอนไซม์ทรานส์กลูทามิเนสที่มีในกล้ามเนื้อ เจลซูริมิที่มีความแข็งแรงของเจลสูงมีการละลายต่ำในสารละลายผสมของโซเดียมโดเดซิลซัลเฟต (ร้อยละ 1) ยูเรีย (8 โมลาร์) และเบต้า-เมอแคปโตเอธานอล (ร้อยละ 2) แสดงถึงการเกิดพันธะโควาเลนท์ชนิดที่ไม่ใช่พันธะไดซัลไฟด์ซึ่งก่อให้เกิดความแข็งแรงของเจล ค่าแรงและระยะทางก่อนเจาะทะลุเพิ่มขึ้นเมื่อความเข้มข้นของแคลเซียมคลอไรด์เพิ่มขึ้น บ่งบอกถึงความต้องการแคลเซียมอิออนสำหรับกระตุ้นการทำงานของเอนไซม์ทรานส์กลูทามิเนส ส่วนการเติมสารบล็อคหมู่ซัลฟ์ไฮดริล (N-ethylmaleimide; NEM) แอมโมเนียมคลอไรด์ (NH_4Cl) และสารจับโลหะ (Ethylenediaminetetraacetic acid; EDTA) ทำให้ความสามารถในการเกิดเจลลดลง สำหรับพอลิฟอสเฟต (Sodium hexametaphosphate หรือ Penta-sodiumtriphosphate) มีผลลดความแข็งแรงของเจลซูริมิเล็กน้อย ภายใต้สภาวะที่เหมาะสมต่อการเซ็ทตัวของซูริมิทั้ง 4 ชนิด พบว่า ซูริมิที่ได้จากปลาตาหวานและปลาจวดให้ผลในการ

เซ็ทตัวที่ดีกว่าซูริมิจากปลาชนิดอื่น โดยการเซ็ทตัวมีความสัมพันธ์กับกิจกรรมเอนไซม์ทรานส์กลูทามิเนส อย่างไรก็ตามซูริมิจากปลาตาหวานมีกิจกรรมของเอนไซม์ทรานส์กลูทามิเนสสูงสุด

เอนไซม์ทรานส์กลูทามิเนสจากกล้ามเนื้อปลาตาหวานมีพีเอชและอุณหภูมิที่เหมาะสมเท่ากับ 6.0 และ 40 องศาเซลเซียส ตามลำดับ แคลเซียมอิออน และสารรีดิวซ์ (β-mercaptoethanol และ Dithiothreitol) สามารถกระตุ้นกิจกรรมของเอนไซม์ ส่วน NEM NH_4Cl และ EDTA มีผลยับยั้งกิจกรรมของเอนไซม์ นอกจากนี้สารพอลิฟอสเฟตมีผลยับยั้งกิจกรรมของเอนไซม์ อันอาจมีสาเหตุมาจากความสามารถในการจับแคลเซี่ยมอิออนของฟอสเฟต เป็นผลให้ปริมาณของแคลเซี่ยมอิออนมีไม่เพียงพอต่อการกระตุ้นการทำงานของเอนไซม์ เอนไซม์ทรานส์กลูทามิเนสจากกล้ามเนื้อปลาตาหวานไม่คงทนต่อความร้อนที่อุณหภูมิสูงกว่า 40 องศาเซลเซียส โดยเฉพาะเมื่อใช้ระยะเวลาการให้ความร้อนเพิ่มขึ้น และมีความคงทนในช่วงพีเอช 5.0-7.0 โดยมีกิจกรรมสัมพัทธ์มากกว่าร้อยละ 90 เอนไซม์ทรานส์กลูทามิเนสที่สกัดจากกล้ามเนื้อปลาตาหวานมีผลช่วยเพิ่มการเชื่อมประสานของโปรตีนแอกโตไมโอซิน

กิจกรรมของเอนไซม์ทรานส์กลูทามิเนสในเนื้อปลาตาหวานบดลดลงภายหลังการเก็บรักษาในน้ำแข็งนาน 2 วัน ($p<0.05$) โดยมีระดับกิจกรรมเหลือร้อยละ 33.56 ภายหลังการเก็บรักษาในน้ำแข็งเป็นเวลา 8 วัน ($p<0.05$) กิจกรรมของเอนไซม์ทรานส์กลูทามิเนสลดลงเมื่อจำนวนรอบในการแช่แข็งและทำละลายเพิ่มขึ้น นอกจากนี้กระบวนการล้างเนื้อปลาบดมีผลชะล้างเอนไซม์ทรานส์กลูทามิเนสออกจากกล้ามเนื้อ โดยเฉพาะเมื่อจำนวนครั้งในการล้างและปริมาณโซเดียมคลอไรด์เพิ่มขึ้น ดังนั้น กระบวนการผลิตซูริมิมีผลโดยตรงต่อกิจกรรมเอนไซม์ทรานส์กลูทามิเนสและส่งผลให้การเซ็ทตัวลดลง

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Abstract

Surimi from four tropical fish species including threadfin bream (*Nemipterus bleekeri*), bigeye snapper (*Priacanthus tayenus*), barracuda (*Sphyraena jello*) and bigeye croaker (*Pennahai macrophthalmus*) contained similar compositions. Myofibrillar protein was found to be a major constituent. Setting phenomenon was observed in all surimi tested. Ashi gel prepared by setting at 40°C for a proper time, followed by heating at 90°C for 20 min rendered the higher force and deformation, compared to that prepared by direct heating (without setting) ($p<0.05$). This result suggested the essential role of setting, possibly caused by endogenous transglutaminase (TGase). Increased gel strength was associated with decreased solubility in the mixture of sodium dodecyl sulfate (1%), urea (8 M) and β-mercaptoethanol (2%), suggesting that non-disulfide covalent bonds were formed and contributed to enhanced gel strength. Gel force and deformation increased when $CaCl_2$ concentration increased, indicating the presence of Ca^{2+} dependent TGase. TGase inhibitors including sulfhydryl blocking agent (*N*-ethylmaleimide; NEM), NH_4Cl and metal chelator (ethylenediaminetetraacetic acid; EDTA) reduced the gel forming ability of surimi. Addition of polyphosphate (sodium hexametaphosphate or penta-sodiumtriphosphate) slightly reduced the gel strength of surimi. Among surimi tested, surimi produced from bigeye snapper and bigeye croaker showed the superior setting to others. Setting was

associated well with TGase activity. However, surimi from bigeye snapper had the highest TGase activity.

Endogenous TGase from bigeye snapper muscle had the optimum pH and temperature at 6.0 and 40°C, respectively. The activity was activated by calcium ion and reducing agents (β-mercaptoethanol and dithiothreitol), but was inhibited by NEM, NH_4Cl and EDTA. Decreased activity of bigeye snapper muscle TGase was observed with the addition of polyphosphate. It was presumed that phosphate may chelate Ca^{2+}, resulting in the less availability of Ca^{2+}. Bigeye snapper muscle TGase was not stable when heated at temperature higher than 40°C, particularly with an increasing time. The bigeye snapper muscle TGase was stable at pH ranging from 5.0 to 7.0, in which more than 90% activity was retained. Extracted TGase from bigeye snapper effectively induced the cross-linking of actomyosin.

During iced storage of bigeye snapper mince, muscle TGase activity decreased significantly after 2 days of storage ($p<0.05$) and decreased to 33.56% after 8 days. TGase activity was decreased ($p<0.05$) with freeze-thawing process, especially with a repeated cycle. During washing process, TGase was washed out to a high extent, particularly when washing cycles increased. The presence of NaCl in wash water accelerated the removal of TGase from the muscle. Therefore, surimi process detrimentally affected the TGase activity, leading to the decreased setting phenomenon.

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Chakkawat Chantarasuwan

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Chapter 1

Introduction

"Surimi" is Japanese term denoting the washed mince, used as starting material for Japanese surimi-based products, generally known as "kamaboko". However, surimi is at present known as a wet concentrate of fish muscle proteins. Normally, fish is mechanically deboned and water-washed. Before freezing, cryoprotectants are added to prevent protein denaturation.

Quality of surimi gel is a prime factor for processor to concern. It depends upon many factors, particularly protein integrity, freshness, etc. Additionally, endogenous enzymes play an essential role in gelation as well as gel properties. Proteinases have been known to impede the gel quality by causing the gel weakening. Therefore, some commercial proteinase inhibitors are added to prevent this phenomenon. However, some enzymes, especially transglutaminase, can enhance the gel quality by inducing ε-(γ-glutamyl) lysine linkage. This non-disulfide covalent bond can improve the gel strength of surimi. Since different surimi has the different gel quality, it is postulated that the different amounts of endogenous transglutaminase in fish muscle lead to different setting characteristic as well as final gel quality. It has been reported that different transglutaminase activities were obtained among different fish species (Lanier, 1992). However, very few information regarding endogenous transglutaminase in tropical fish muscle and its role in gelling property of surimi has been reported. To maximize the transglutaminase activity is another promising means for surimi gel quality improvement. The information gain will be beneficial for surimi industries in Thailand as well as ASEAN countries.

Literature Review

1. Surimi

Surimi is minced fish which has been extracted with water to remove strong flavor, pigments, and nonfunctional proteins and subsequently dewatered to reduce the moisture content to approximately that of intact fish muscle (Suzuki, 1981). When fish flesh is separated from bones and skin (usually mechanically), it is called "minced fish" (Figure 1). The mince is used as the starting material for surimi production and as ingredient for some processed fish products, such as fish sticks, fish cakes, etc. The removal of water-soluble proteins, fat, connective tissues and undesirable muscle components such as blood and pigments is needed for surimi production (Suzuki, 1981). These constituents are thought to interfere with gel formation (Lee, 1984; Hultin, 1985; Roger and Wilding, 1990). Washing also functions to concentrate the desirable myofibrillar proteins, particularly myosin (Kudo *et al.*, 1984). Myosin has been known as the predominant protein involved in gelation (Itawa *et al.*, 1977; Shimizu *et al.*, 1983; Akahane *et al.*, 1984; Sano *et al.*, 1988, 1989). The use of fresh fish for the surimi production is also essential since tissue autolysis by endogenous proteolytic enzymes during storage reduces the level of extractable actomyosin available for gelation (Makinodan *et al.*, 1980; Kim *et al.*, 1982).

After the minced fish is water-washed to remove fat and water-soluble components, it becomes "raw surimi". This raw surimi is a wet concentrate of the myofibrillar proteins and possesses enhanced gel-forming, water-holding, fat-binding, and other functional properties relative to minced fish (Buttkus, 1970; Matsumoto, 1979; Arakawa and Timasheff, 1982; Sun and Wang, 1984; Slade and Levine, 1988; Okada, 1992).

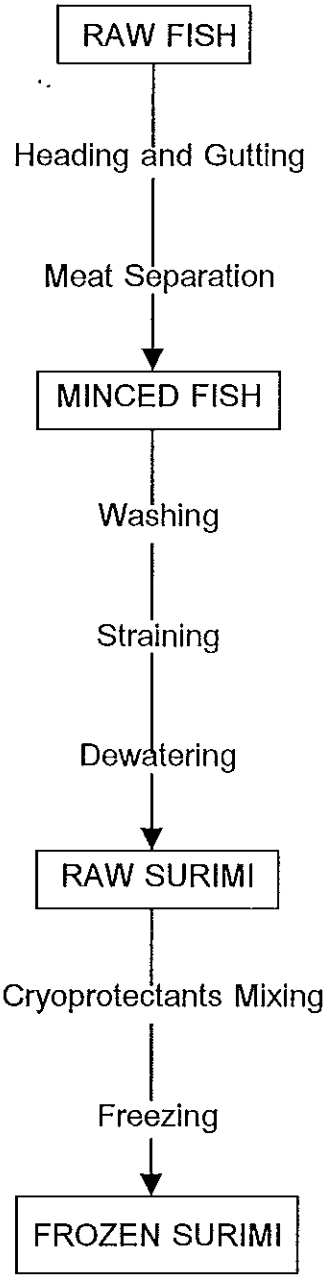

Figure 1. Scheme for surimi production

Source: Adapted from Okada (1992).

However, the myofibrillar proteins in the raw surimi will lose their functional properties rapidly when they are frozen. It is crucial to prevent denaturation of myofibrillar proteins, which directly affect the gel-forming ability. Therefore, the raw surimi is generally mixed with cryoprotectants such

as sugars or sugar alcohols and quick-frozen into a block form, "frozen surimi". The myofibrillar proteins in the frozen surimi will retain their functional properties for many months if properly stored. Since frozen surimi is conveniently and economically transported, stored, and handled, the term "surimi" generally refers to frozen surimi.

Surimi can be used to produce a variety of products. Consumption is usually focused on four major product forms, namely: chikuwa, kamaboko, satsuma-age and hampen (Kano, 1992). Imitation shellfish meats, such as shrimp, clams, scallops, crabmeat, and others, which possess textural properties similar to the natural shellfish products, can also be made from surimi.

2. Raw materials

The species most commonly used in the manufacture of surimi is Alaska pollock, or walleye pollock (*Theragra chalcogramma*) (Holmes *et al.*, 1992). In the South East Asia, the species of fish for surimi production are threadfin bream (*Nemptera spp.*), bigeye snapper (*Priacanthus spp.*), baracuda (*Sphyparena spp.*) and croaker (*Pennahia, Johnius spp.*) (Morrissey and Tan, 2000). Other species such as cods, lizardfish, barracuda, conger eel, wolf herring, croakers, jewfish and shark are also used for surimi production (Kano, 1992). In Japan, in addition to Alaska pollock, several species are often used for surimi products (Table 1). Surimi production in Thailand is based on threadfin bream (*Nemipterus japonicus*) because it is the most abundant material and it also exhibits the proper characteristics for processing export-quality surimi.

Generally, fish commonly used for surimi production should be lean and white-flesh fish. However, gel forming ability, an index of surimi quality, varies according to species and season (Shimizu *et al.*, 1981; Itoh *et al.*, 1995).

Table 1. Utilization of various species for surimi products

Species	Mainly used for
hake (*Merluccius*)	chikuwa
southern blue whiting (*Micromessistius australis*)	high-grade kamaboko
blue whiting (*Sillago parvisquamis*)	not specified
Pacific whiting (*Merluccius productus*)	not specified
Atlantic menhaden (*Brevoortia tyrannus*)	not specified
atka mackerel (*Pleurogrammus azonus*)	satsuma-age
horse mackerel (*Carangidae*)	chikuwa, satsuma-age
sardine (*Sardina pilchardus*)	satsuma-age, chikuwa and others
yellow croaker (*Pseudosciaena polyactis*	high-grade kamaboko
alfonsino (*Beryx splendens*)	kamaboko, chikuwa
threadfin bream (*Nemipterus virgatus*)	kamaboko, chikuwa
silver jewfish (*Argyrosomus argentatus*)	all products
hairtail (*Trichiurus lepurus*)	satsuma-age
pike eel (*Muraenesox cinereus*)	products other than kamaboko and chikuwa
lizardfish (*Saurida undosquamis*)	kamaboko, chikuwa
blue shark (*Prionace glauca*)	hampen

Source: Kano (1992)

Ni *et al.* (1999) reported that freshwater fish and some marine fish species are generally less desirable as a raw material for surimi-based products because of their poor gelling ability in comparison with walleye pollack surimi. In particular, carp and salmon are typical fish of poor gelation, resulting primarily from the lack of setting response and from modori (gel weakening) (Ni *et al.*, 1998; Wan *et al.*, 1995).

3. Gelation

The process of protein gelation involves two steps, an initial phase in which proteins are thermally denatured with concomitant conformational changes, and the second phase in which the denatured proteins are aggregated. A balance of attractive and repulsive forces is essential for aggregation. This aggregation process, ultimately responsible for the formation of a three dimensional structure, requires the participating molecules to interact at specific point. At least three cross-links (bonds) or intertwinements per polymer segment are formed (Figure 2c). A network can not be formed without three cross-links (Figure 2a, b) (Niwa, 1992). The resultant molecular orientation necessary to facilitate these interactions may proceed at a relatively slow rate (Hermansson, 1979). Gelation is a controlled process in which the final network displays some degree of order, while coagulation is the process in which structure is totally random (Tombs, 1974; Stone and Stanley, 1992). The degree of ordering in gelation is controlled by a number of factors such as thermal treatment. A high heating rate allows insufficient time for molecules to suitably orient themselves, resulting in a discontinuous matrix of gel (Mulvihill and Arai, 1987). Other factors contributing to the nature of the gel include protein species, concentration, pH and the ionic environment (Ferry, 1948; Kinsella, 1984).

Proteins priminarily involved in gelation include myosin (Samejima *et al.*, 1969; Taguchi *et al.,* 1978; Samejima *et al.,* 1981) and actomyosin (Itoh *et al.,* 1979; Yasui *et al.,* 1980). The role of actin in gelation is still in dispute since an isolated molecule has been observed to form a 'curdy matter' on heating (Sano *et al.,* 1989). When actin was incorporated into myosin solutions at a suitable level, it appeared to confer increased gel firmness (Yasui *et al.*, 1980; Sano *et al.,* 1989; Hirahara *et al.,* 1990).

Fish protein composition can be divided into three main groups as myofibrillar protein, sarcoplasmic protein and stroma protein. Myofibrillar

protein can be further divided into four subgroups as myosin, actin, troponin, and tropomyosin. Myosin is the most abundant myofibrillar component, constituting approximately 40-60% of total protein content (Bechtel, 1986). Myosin has a molecular weight of approximately 520,000 dalton and is composed of two heavy chains and four light chains (Figure 3). The molecule is cleaved to head and tail portions, subfragment-1 (S1) and rod, or heavy meromosin (HMM) and light meromyosin (LMM), by limited proteolysis. The importance of myosin as the major protein contributing to thermally induced gelation of muscle proteins has been well established (Seki *et al.*, 1998). The reactions comprise protein unfolding, aggregation, and noncovalent formation of a gel network (Niwa *et al.*, 1981; Lanier *et al.*, 1982; Gill and Conway, 1989).

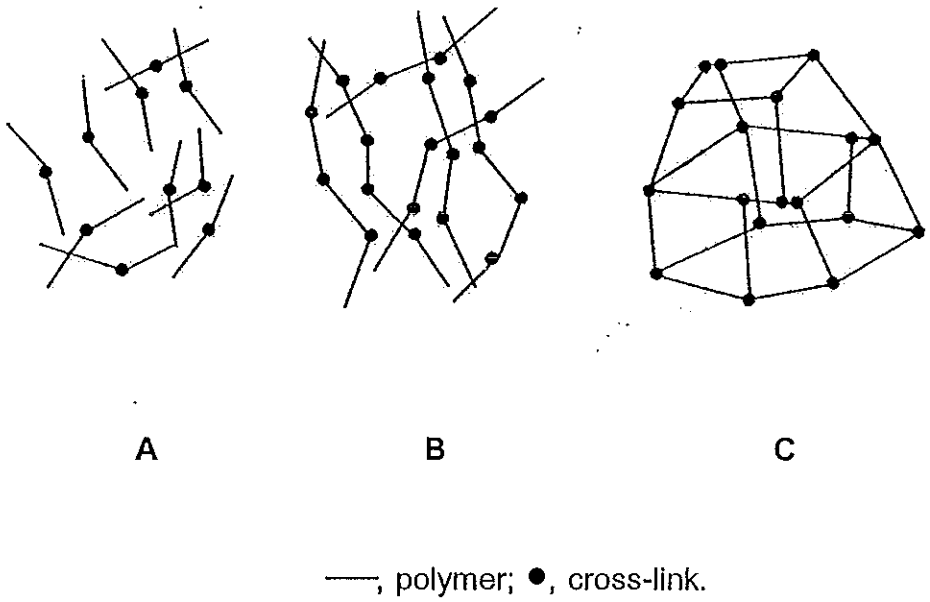

Figure 2. Formation of a gel network structure
Source: Niwa (1992)

The texture of kamaboko-type thermostable gels made from surimi is determined by interactions or bonds that occur among myofibrillar proteins

after solubilization with salt. Gelling involves unfolding of the proteins and the establishment of bonds among them. It is believed that hydrogen bonds

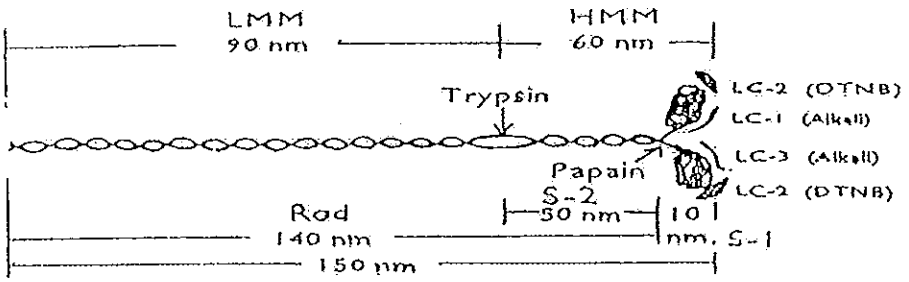

Figure 3. Myosin molecule.

Source: Xiong (1997).

(Suzuki, 1981; Beas *et al.*, 1988), hydrophobic interactions (Sano, 1988; Beas and Crupkin, 1990), disulfide bridges (Ishioroshi *et al.*, 1981; Sano, 1988; Roussel and Cheftel, 1990), and non-disulfide covalent bonds (Numakura *et al.*, 1985; Seki *et al.*, 1990) intervene differently in the formation of the network, depending on the various parameters involved. Niwa (1992) recently reviewed the importance of the various bonds in gel network formation. The factors determining the number and kind of interactions or bonds include not only the species from which the surimi is derived (Suzuki, 1981; Shimizu, 1985) but also the heat conditions in which the gel is made (Ishikawa, 1978; Akahane and Shimizu, 1990; Lee *et al.*, 1990a; Yamazawa, 1990). Chan *et al.* (1992) reported that thermal aggregation of fish myosin was coincidental with an increase in the surface hydrophobicity of the unfolded domains of myosin molecules and was affected by the temperature at which these domains unraveled. Temperature and ionic strength have a profound effect on the hydrophobic interaction of proteins. The destabilization of the hydrophobic core of proteins and the strength of intermolecular hydrophobic interactions

are dependent on both temperature and salt concentration (Niwa, 1982). Hydrogen and other polar bondings are well known to be unstabilized at high temperature but stabilized at low temperature, in contrast to hydrophobic interaction (Gill *et al.*, 1992).

As the temperature is increased over 45-50°C, gel network (suwari) is partially disrupted to form a 'broken net' (modori), This process is species dependent (Shimizu *et al.*, 1981). The action of proteases has been found to promote this gel weakening (Lanier *et al.*, 1981; Lee, 1984). However, in systems without enzymes or containing enzyme inhibitors, softening was still apparent (Itawa *et al.*, 1974; Lee, 1984). Once the temperature is increased above 65-70°C, the gel becomes ordered and non-transparent. This stage is referred to as kamaboko (Suzuki, 1981). In this final stage, the cohesiveness and elasticity of the gel is enhanced. The magnitude is also determined by the slow ordering of the protein network that occurs during setting (Lanier *et al.*, 1982; Niwa *et al.*, 1983; Akahane and Shimizu, 1990). Figure 4 shows changes in rheological properties of myosin and actomyosin during heating and delineates the three phases of texture formation (Sano *et al.*, 1988).

4. Suwari (setting)

Meat paste left for a period of time after grinding loses its plasticity and turns into gel, called "suwari" or setting. The degree of suwari forming depends on the fish species. Okada (1959) has made an extensive research on various species of fish and compiled the results as shown in Table 2.

The first evidence for the mechanisms of the setting process was supplied by Connell (1960) who examined the aggregation of cod myosin solutions under various conditions of protein concentration, pH and ionic strength at a temperature of 0°C. Connell (1960) concluded that a denaturation step proceeded by a comparatively rapid side-to-side

aggregation process. These events were linked with the inactivation of ATPase activity.

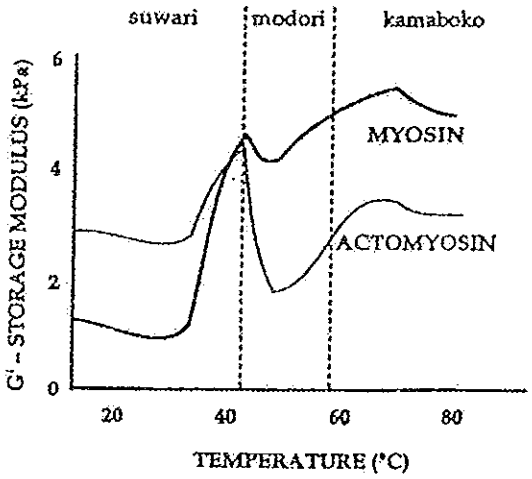

Figure 4. Changes in rheological properties of actomyosin and myosin during heating.

Source: Sano *et al.* (1988).

Subsequent research helped to clarify Connell's earlier observations, indicating that non-covalent protein-protein interactions arose as a consequence of the unfolding of the α-helix of heavy myosins (Liu *et al.*, 1982; Niwa, 1975; Niwa *et al.*, 1981,1982b, 1983; Gill and Conway, 1989; Sano *et al.*, 1990). This conformational change, derived from the dissociation and solubilization of the myosin filaments, was initiated by temperature and controlled by pH, Ca^{2+} concentration and ionic strength (Liu *et al.*, 1982; Nishimoto *et al.*, 1987; Saeki *et al.*, 1988). A wealth of research has focused on the contribution of hydrophobic interactions in setting (Niwa, 1975; Niwa *et al.*, 1981, 1983; Gill and Conway, 1989; Akahane and Shimizu, 1990; Wan *et al.*, 1992). Differences species had different degree of surface hydrophobicity and this was suggested to be related to the differences in setting (Niwa *et al.*,

1981). Suwari gel is formed by retaining water in the molecule linkage formed by hydrophobic bond and hydrogen bond.

Table 2. Suwari forming capacity of different fish species

Suwari forming capacity	Fish species
Easy	Sardine (*Sardinops melanosticta*)
	Anchovy (*Engraulis japonica*)
	Alaska pollack (*Theragra chalcogramma*)
	Lizard fish (*Saurida undosquamis*)
	Cutlass fish (*Trichiurus lepturus*)
	Horse mackerel (*Trachurus japonicus*)
	Mackerel (*Scomber japonicus*)
Normal	Sea bass (*Lateolabrax japonicus*)
	Skipjack (*Katsuwonus pelamis*)
	Cod (*Gadus morhua macrocephalus*)
	Bigeye tuna (*Parathunnus sibi*)
Difficult	Sharks
	Carp (*Cyprinus carpio*)
	Black marlin (*Makaira mazara*)
	Sharp-toothed eel (*Muraenesox cinereus*)
	Croaker (*Argyrosomus argentatus*)
	Squids (*Todarodes pacificus* etc.)

Source: Okada (1959)

Gill and Conway (1989) identified the HMM S-2 and LMM regions of the tail as those engaged in the interactions. It was noted that at 30-44°C only the LMM formed gels, while HMM formed a curd-type structure (Sano *et al.*, 1990).

Itoh *et al.* (1979) working with actomyosin and Niwa *et al.* (1982) working with myosin both concluded that sulfhydryl groups were prominent in gelation at 40°C. Conversely, Taguchi *et al.* (1987) reported that at setting temperatures (30-40°C), the HMM S-1 fragments were thermally aggregated, whereas only the LMM displayed a similar behavior when heated above 50°C. Other forms of interaction, such as hydrogen bonding, are also present during setting. Like disulfide bond, they are not thought to regulate the suwari structure, but instead impart an elasticity to the system (Niwa *et al.*, 1982).

Gill and Conway (1989) observed that actin, along with other low molecules weight species, only became involved in the polymerizing process through hydrophobic interactions when half of the myosin heavy chain molecules were cross-linked. Rheological studies on myosin have demonstrated little variation in gel elasticity during setting as a result of the inclusion of F-actin (Sano *et al.*, 1989). This would imply that F-actin, although interacting with myosin, does not play a meaningful role in establishing the protein network during setting.

Niwa *et al.* (1989) proposed that during heating (40°C) of actomyosin solutions of easily setting species, the heat-labile heavy myosin chains initially dissociated from actin, troponin, tropomyosin and the light chains, and rapidly aggregated to form suwari. As heating at 40°C was continued, the actin and tropomyosin were observed to reassociate, however, the nature of the interaction was not determined.

Numakura *et al.* (1990) showed that a 150 kDa component was produced and troponin T was markedly reduced in walleye pollack surimi during a setting at 30°C. The 150 kDa component was referred to as a proteolytic fragment of myosin heavy chain (Kamath *et al.*, 1992). Lee *et al.* (1990b) and Kamath *et al.* (1992) also reported that a considerable degree of proteolysis was detected on SDS-PAGE even at optimum setting temperatures for surimi pasts of threadfin bream, hoki, walleye pollack and

Atlantic croaker. The proteolysis as well as the cross-linking of myosin during the setting affected the textural properties of final cooked gels (Takeda and Seki, 1996).

Seki *et al.* (1990) and Kimura *et al.* (1991) have provided evidence for an enzyme-catalyzed cross-linking during setting. Transglutaminase (TGase), an enzyme present in water soluble fraction extracted from fish species plays a crucial role in catalyzing the formation of ε-(γ-glutamyl) lysine (Tsukamasa and Shimizu, 1991). Gel was considerably strengthened by the addition of sarcoplasmic proteins to the Alaska pollack surimi, from which they were washed away (Arai and Seki, 1989), and the gel was considerably weakened by the removal of TGase (Nowsad *et al.*, 1994, 1995).

The setting of fish flesh is thought to be due to the polymerization of myosin heavy chain catalyzed by TGase (Urakami *et al.*, 1967). Takeda and Seki (1996) have considered that the setting is attributed to the modification of thermal myosin gelation by TGase-catalyzed myosin cross-linking (Seki *et al.*, 1990; Kimura *et al.*, 1991; Kamath *et al.*, 1992; Wan *et al.*, 1995).

Seki *et al.* (1998) carried out enzymatic labelling of a fluorescent amine on carp myosin to find a fluorophore-binding site. When myosin was incubated with endogenous TGase from carp dorsal muscle in the presence of 4 mM monodansylcadaverine (MDC) at 25°C for 1 h, the MDC was covalently incorporated into the heavy chain but not to the light chains, and completely inhibited the heavy chain cross-linking. The results indicated the enzymatic dimerization site was located on S2 heavy chain portions of myosin molecule.

The quality of directly cooked gel is in most case poorer than those with prior setting, without modori phenomenon. The lower quality of such directly cooked gels is thought to be due to rapid formation of disulfide and hydrophobic protein-protein bonds in the absence of the conditions required for the protein to orient to form a network. Protein coagulation becomes more prevalent, compared to set gels (Niwa, 1985; Alvarez *et al.*, 1999a).

5. Modori

Modori is a common term for thermal gel degradation occurring when surimi paste is incubated at temperatures close to 60°C. Modori has been reported to occur at low temperatures (5°C) (Nomura *et al.*, 1993) and at higher temperatures (50°C or 60°C) (Tsukamasa and Shimizu, 1991; Alvarez *et al.*, 1999b). The degree of modori varies, depending on the fish species. For round herring (*Etrumeus micropus*) and filefish (*Navodon modestus*), the gel was nearly completely destroyed, but gel became stronger with shark (*Cynias manazo*), chicken and swordfish (*Makaira mazara*) (Suzuki, 1981).

Niwa (1992) described three mechanisms responsible for modori: proteolytic degradation due to enzymes, thermal coagulation of myofibrillar proteins during heating and involvement of specific proteins. Enzymatic degradation of the gel with breakdown of myosin heavy chain has been shown to depend on species and season (Au *et al.*, 1994). For the same species, there were different types of heat activated proteolytic enzymes of myofibrillar and/or sarcoplasmic origin that produce modori at different temperatures (Itoh *et al.*, 1995). Another type of modori-inducing enzymes was involved in the degradation of fish species infested with parasites of myxosporidia spp. (Niwa, 1992). Hydrophobic interactions and disulfide bridges in myofibrillar protein have been found to increase at modori temperatures (Sano, 1988). Such increase was reported in sardine surimi paste set at 60°C (Careche *et al.*, 1995). The mechanism of particular proteins that induce modori is not understood. It was hypothesized to be due to a non-enzymatic interaction between such proteins and myofibrillar proteins (Iwata *et al.*, 1977).

Rheological studies on surimi (Montejano *et al.*, 1984) and actomyosin (Wu *et al.*, 1985) placed the modori phenomenon within the temperature range of 52-64°C. This appeared as a loss of the apparent rigidity modulus. Subsequent studies comparing carp myosin and actomyosin systems from 46

to 53°C revealed that only the latter showed a decline in rigidity (Sano *et al.*, 1989), which was attributed to the dissociation of myosin from actin and the possible fragmentation of the actin filament (Sano *et al.*, 1988, 1989).

Itoh *et al.* (1989) reported that the degradation of gels occurred in unwashed meat gels heated at 60°C and in washed meat gel heated at 40 and 60°C caused by two types of proteolytic factor. The sarcoplasmic type (Sp) can be removed by washing the meat. The myofibrillar type (Mf), which still remains in washed meat, was proposed to be responsible for textural degradation.

In sardine surimi, the modori effect is mainly attributed to heat denaturation of myofibrillar proteins (Toyohara and Shimizu, 1988). Tsukamasa and Shimizu (1991b) described two types of proteinase-independent modori in sardine meat. Thermal-scanning rigidity monitoring of sardine paste showed a zone of minimum rigidity values (between 46°C and 50°C), indicating the occurrence of modori at high temperatures. When setting at 40°C was prolonged, the gel strength and texture profile analyses of the set gels decreased, indicating with poorer texture characteristics (Alvarez and Tejada, 1997).

6. Kamaboko

Further heating, above 60-70°C, produces an increase in strength (firmness). The rigidity of the previously formed elastic network is enhanced as aggregation continues (Roussel and Cheftel, 1990; Sano *et al.*, 1990). Non-transparent elastic kamaboko gel is formed when the meat paste is heated after passing the modori temperature zone. This can be attributed to a network formation of the fibrous myofibrillar protein molecules. The configuration of the protein is changed by heat with an interaction of a radical group on the molecular surface, forming a network far stronger than that of suwari gel. Molecular level and bigger networks exist in kamaboko, and these

structures are contributed to the characteristic elasticity. The tail region had been predominantly involved in cross-linking interactions at lower temperatures, while the globular head portion (HMM S-1) of myosin take action above 60-70°C (Liu *et al.*, 1982; Taguchi *et al.*, 1987; Sano *et al.*, 1990).

Sano *et al.* (1990) studied the dynamic viscoelastic behaviors and turbidity of isolated carp HMM and LMM and concluded that the initial development of gel elasticity was attributable mainly to the LMM and a second stage was due to HMM. Gill and Conway (1989) also showed that the tail of the myosin molecule rather than the head was involved in thermal aggregation. On the other hand, Taguchi *et al.* (1987) reported that the extent of aggregation for S1 was higher than for HMM and LMM at the initial stage.

Both HMM and LMM are involved in thermal aggregation of cod and herring myosins. Thermal aggregation may be initiated by the unfolding and interaction of HMM S-2, and further aggregation may be mediated through the interaction of LMM to form clusters of aggregates at higher temperatures (Figure 5) (Chan *et al.*, 1993).

Initial studies performed by Itoh *et al.* (1979) on carp actomyosin solutions demonstrated a concomitant increase in the level of oxidized sulfhydryl groups (estimated from the reduction in SH content) with increasing temperature up to 80°C. This implies that S-S interchanges between protein molecules were important in the development of gel structure. Disulfide interchanges (SH-SS) were also associated with the development of network structure. The addition of cysteine and cystine to actomyosin solutions and assorted fish meat pastes was found to improve the gel strength (Itoh *et al.*, 1979)

The involvement of the head region through SH group oxidation was only recently proposed, and the order of interaction (tail or head first) still appeared in doubt (Taguchi *et al.*, 1987). Sano *et al.* (1990) proposed that the

bulk of the protein-protein interactions over the range 50-80°C were those of the head-head type. However, no mention of S-S interchanges were made, in stead, hydrophobic interactions were tentatively considered since the conformation of the protein molecule was changed further by heating.

On heating actomyosin solutions from 53 to 80°C, Sano *et al.* (1989) noted that the rate of elasticity development increased with F-actin content. This was attributed to the dissociation of actin filaments (Sano *et al.*, 1989). However, F-actin alone formed a viscous and curdy sol when exposed to the same thermal treatment

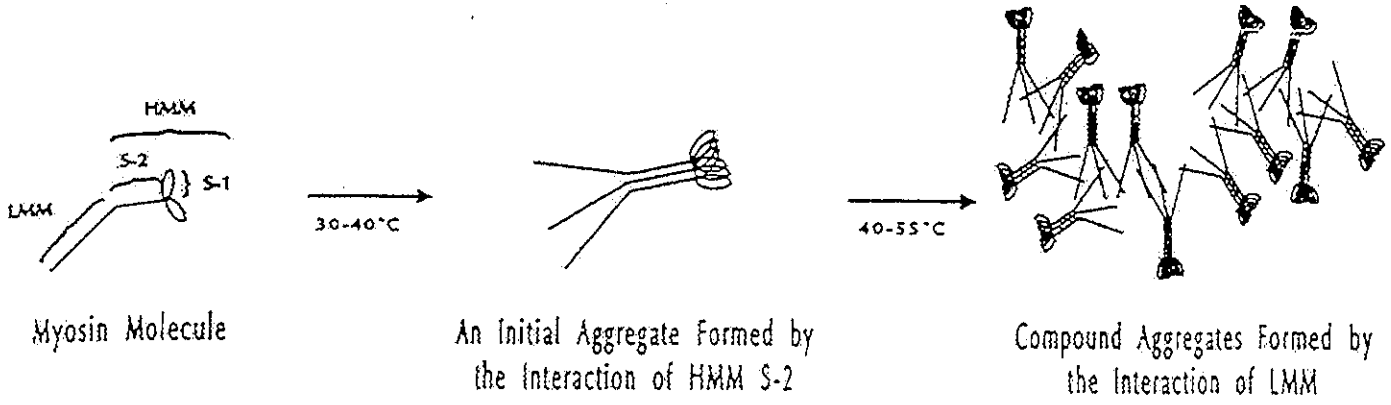

Figure 5. A schematic representation of the thermal aggregation of fish myosin.

Source: Chan *et al.* (1993)

7. Transglutaminase (TGase)

TGase is a transferase, having the systematic name as protein-glutamine γ-glutamyltransferase (EC 2.3.2.13). It catalyzes the acyl transfer reaction between γ-carboxyamide groups of glutamine residues in proteins, peptides, and various primary amines. When the ε-amino group of lysine acts as acyl acceptor, it results in polymerization and inter- or intra-molecular cross-linking of protein via formation of ε-(γ-glutamyl) lysine linkages. This occurs through exchange of the ε-amino group of the lysine residue for ammonia at the carboxyamide group of a glutamine residue in the protein molecule(s) (Figure 6). In the absence of primary amines, water may act as the acyl acceptor, resulting in deamination of γ-carboxyamide groups of glutamine to form glutamic acid. Formation of covalent cross-links between protein is the basis of the ability of TGase to modify the physical properties of protein foods (Ashie and Lanier, 2000).

TGase has a broad specificity for primary amine acceptors (peptide-bound lysine or polyamines) (Folk and Finlayson, 1977). In contrast, relatively few proteins contain glutamine residues that form acyl-enzyme intermediates. This capability is influenced by the amino acid sequence (including charge) surrounding the susceptible glutamine residue and local secondary structures that are not well defined (Folk, 1980). Proteolysis of a nonreactive protein can convert it to a transglutaminase substrate (Greenberg *et al.*, 1991).

TGase activity in tissues may be determined by one of several mechanisms including: amine incorporation into substrates using monodansylcadaverine, hydroxamate or radioactive putrescine (Folk and Cole, 1966; Folk and Chung, 1985; Lorand *et al.*, 1969), disappearance of amino groups by trinitrobenzenesulfonate or fluorescence intensity methods (Ikura *et al.*, 1980); increase in molecular weight of substrate by sodium dodecylsulfate-polyacylamide gel electrophoresis (SDS-PAGE) (Ikura *et al.*, 1980); release of

ammonia (Backer-Royer *et al.*, 1992); or measurement of functional effects such as viscosity and gel strength (Sakamoto *et al.*, 1994). Ohtusuka *et al.* (1996) have also developed an enzyme-linked immunosorbent assay (ELISA) for specifically estimating microbial TGase activity in surimi-based products.

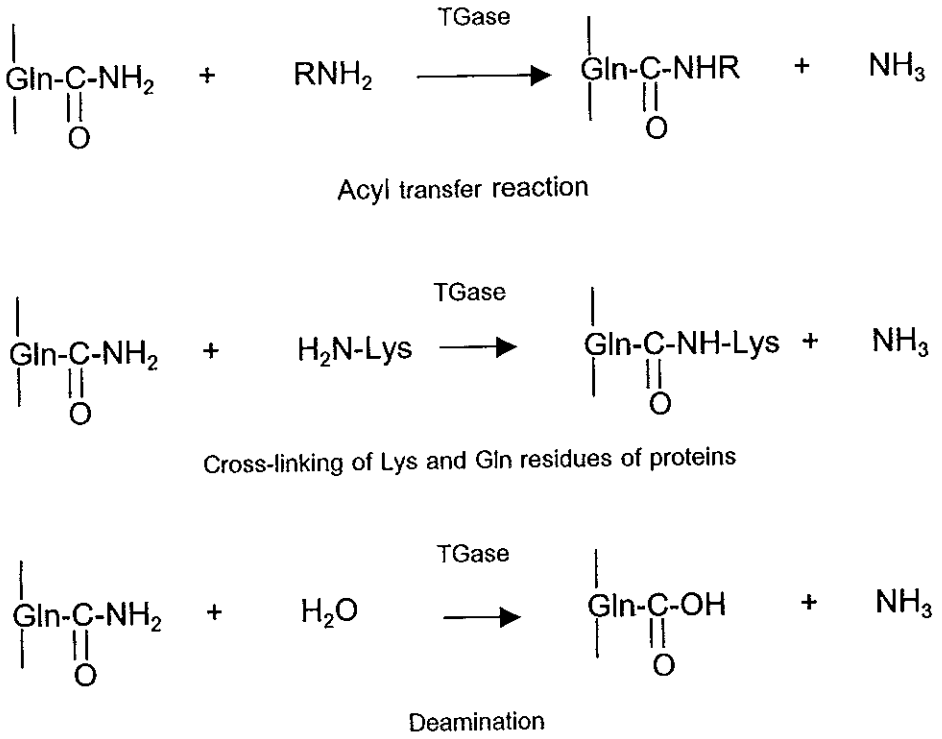

Figure 6. Reaction catalyzed by TGase

Source: Ashie and Lanier (2000)

8. Sources of transglutaminase

TGase has been found in tissues of various species including mammals, birds, fish and shellfish, microorganisms, and plants (Table 3). The four best-characterized TGases and their terminology are shown in Table 4.

Table 3. Sources and characteristics of some TGases

Sources	Mol. Wt. (kDa)	Optimum Temp. (°C)	Optimum pH
Mammal			
Human plasma factor XIII	300-350[t]		
Bovine factor XIIIa			
Guinea pig liver	75-85[m]		8.0
Rabbit liver	80[m]		
Plant			
Pea seedlings			
Alfalfa	39[m]		
Microbial			
Streptoverticillium mobaraense	40[m]	50	6.0-7.0
Physarum polycephalum	77[d]		
Streptoverticillium ladakanum	37.5	50	6.0
Seafoods			
Red sea bream liver	78	55	9.0-9.5
Carp muscle	80		
Walleye pollack liver	77	50	9.0
Lobster muscle	200		
Japanese oyster	84/90	40/25	8.0
Limulus hemocyte	86		
Scallop	80		
Botan shrimp	80		
Squid	80		
Rainbow trout	80		
Atka mackerel	80		

[t]tetramer; [m]monomer; [d]dimer.

Source: Adapted from Ashie and Lanier (2000).

Table 4. Transglutaminase terminology

Type	Alternative designations
Plasma	Fibrin-stabilizing factor, Laki-Lorand factor, fibrinoligase, factor XIIIa
Tissue	TG_C, erythrocyte, cellular, endothelial, cytoplasmic, type II, liver, tTG
Keratinocyte	TG_K, particulate, type I
Epidermal	TG_E, callus, bovine snout

Abbreviation: TG, transglutaminase; TG_K, keratinocyte transglutaminase; TG_C, tissue transglutaminase; TG_E, epidermal transglutaminase

Source: Greenberg *et al.* (1991)

8.1 Plasma transglutaminase (Factor XIII)

Role of the plasma TGase of higher animals (also referred to as fibrinoligase or factor XIIIa) has been well established as cross-linking of the fibrin clot hemostasis. The zymogen factor XIII is activated by the protease thrombin, another component of plasma that also induces clotting of fibrinogen.

Addition of beef plasma to surimi-based products may also enhance isopeptide cross-link, as evidenced by the proportionately greater increase in strength of Pacific whiting surimi gels containing plasma when subjected to low temperature setting (Lanier and Kang, 1999).

The effectiveness of bovine plasma protein (BPP) may be attributed to one or more mechanism. Plasma contributed to enhanced gelation of Pacific whiting surimi by inhibition of fish protease and also by other gel-enhancing factors in the plasma (Kang and Lanier, 1999). In addition to the

protease inhibitory activity due to the presence of α_2M and Kininogen (Hamann *et al.*, 1990; Morrissey *et al.*, 1993), plasma has protein cross-linking activity from both PTGase and α_2M (Seymour *et al.*, 1997). This could contribute to strengthening of the gel by myosin and/or fibrinogen cross-linking and could also reduce the availability of myosin as a substrate for protease action (Lorand, 1983; Seki *et al.*, 1990; Kimura *et al.*, 1991; Sakamoto *et al.*, 1995). Additionally, fibrinogen and serum albumin have been shown to form gel synergistically with myosin during heating (Foegeding *et al.*, 1996).

8.2 Liver transglutaminase

Mammal and fish livers were found to be the source of TGase, generally termed liver TGase. The molecular weight of pollack liver TGase was estimated to be 77 kDa from SDS-PAGE mobility under the reduced condition. This is similar to that of guinea pig liver (76.6 kDa) (Ikura *et al.*, 1988), red sea bream liver TGase (78 kDa) (Yasueda *et al.*, 1994) and partially purified carp muscle TGase (80 kDa) (Kishi *et al.*, 1991). In contrast, the molecular weight of Ca^{2+}-independent microbial TGase was one half (38 kDa) (Ando *et al.*, 1989) that of the tissue-type TGase of these vertebrates. The concentration of Ca^{2+} to express full walleye pollack liver TGase activity was 3 mM. Thus, this TGase requires higher Ca^{2+} than red sea bream liver TGase liver, which required 0.5 mM Ca^{2+} for its maximum activation (Kumazawa *et al.*, 1996).

Kumazawa *et al.* (1996) reported that the activity of TGase from walleye pollack (*Theragra Chalcogramma*) liver was inhibited by sulfhydryl reagent. This enzyme was a thiol enzyme, the same as mammalian TGases. This purified TGase catalyzed the gelation of myosin B solution, possibly through the polymerization of myosin heavy chains.

8.3 Microbial transglutaminase

TGases have been found in microorganisms. Ando *et al.* (1989) isolated microorganism (*Streptoverticillium mobaraense*) that produced a TGase which did not require calcium ions for activity. This microbial TGase (MTGase) has been applied to polymerize rabbit myosin, carp myosin, beef myosin and actin (Nonaka *et al.*, 1989, 1994; Muguruma *et al.*, 1990; Kato *et al.*, 1991). MTGase has been shown to be useful in strengthening surimi gels during the setting reaction (Seguro *et al.*, 1995; Sakamoto *et al.*, 1995). The gel-forming and viscoelastic properties of surimi-based products were improved by a treatment with MTGase (Seguro *et al.*, 1995). Tsukamasa and Shimizu (1990) reported that the strong gel-forming ability of sardine was due to the formation of the non-disulfide bond, which was shown to be due to the action of TGase (Tsukamasa *et al.*, 1993). Ca^{2+}-independent microbial TGase from *Streptoverticillium mobaraense* (Nonaka *et al.*, 1989; Huang *et al.*, 1992; Gerbeg *et al.*, 1994) or from *Streptoverticillium ladakanum* (Tsai *et al.*, 1995,1996a, b) has shown potential to increase the gel strength of fish surimi.

Addition of microbial TGase to surimi significantly increases its gel strength, particularly when the surimi has lower natural setting ability (presumably due to lower endogenous TGase activity) (Lee *et al.*, 1997; Seguro *et al.*, 1995; Kumazawa *et al.*, 1993a). An increase in non-disulfide polymerization and formation of Ɛ-(γ-glutamyl) lysine dipeptides was found with increase in setting time and microbial TGase concentration (Tsukamasa and Shimizu, 1990). At equal levels of Ɛ-(γ-glutamyl) lysine content, gels prepared with added microbial TGase displayed higher gel stress (Lee *et al.*, 1997). Since these gels achieved this level of isopeptide content more rapidly than pastes gelled with endogenous TGase only, it was concluded that the rate of myosin polymerization may also be a factor influencing gel strength, not isopeptide content alone. Yasunaga *et al.* (1996) observed that increased isopeptide content was concomitant with increased gel strength and increased

microbial TGase added. The relatively higher gel strength with an increasingly lower gel strain produced gels that differed in texture from those produced by setting without additives (Abe, 1994).

8.4 Endogenous transglutaminase

Endogenous TGases were found in fish flesh. These enzymes induced the Ε-(γ-glutamyl) lysine cross-linking in fish proteins and change textural properties of fish sol during processing (Seki *et al.*, 1990; Tsukamasa and Shimizu, 1990, 1991a; Kimura *et al.*, 1991; Sato *et al.*, 1992; Kumazawa *et al.*, 1993b; Sakamoto *et al.*, 1995). Endogenous TGase is water soluble and can be removed if washing is too extensive (Nowsad *et al.*, 1994). TGase activities in fish muscles are shown in Table 5. Fish muscles contained TGase

Table 5. TGase activity level in the various fish muscles and surimis

Species	Activity level Unit/g of muscle (wet weight)*
White croaker	2.41
Carp	1.14
Sardine	0.83
Walleye pollack	0.41
Chum salmon	0.33
Atka mackerel	0.23
Rainbow trout	0.10
Walleye pollack surimi	0.33
Chum salmon surimi	0.05

* The values are means of three independent experiments.

Source: Araki and Seki (1994)

in an activity range of 2.41-0.10 unit/g of wet weight. TGase is thus widely distributed in fish muscles regardless of fish species (Araki and Seki, 1993).

TGase is a water–soluble enzyme and thus its content can vary greatly with the type and extent of process used during surimi manufacture. Nowsad *et al.* (1995) showed that the sarcoplasmic fraction of fish can actually enhance the gelling ability when added back to surimi because of its higher TGase activity. It is likely that different fish species, and perhaps different individuals within species, could vary in natural content of the enzyme, possibly affected by habitat, feed, and physiological condition. This possibility has not been well investigated to date. α_2-macroglobulin component of fish blood plasma (or added beef plasma) is postulated to has the ability to form ε-(γ-glutamyl) lysine cross-links in fish protein (Kang and Lanier, 1999). It has been shown that in certain species, such as salmon, the water-soluble fraction of muscle also contains factors that inhibit TGase activity (Wan *et al.*, 1994).

Seki *et al.* (1990; 1998), Kumura *et al.* (1991), Kamath *et al.* (1992), Wan *et al.* (1994) reported that an endogenous TGase was largely responsible for the setting phenomenon. Wan *et al.* (1995) confirmed the essential role of TGase in the setting of walleye pollack surimi paste, compared with that of salmon, non-setting fish. The TGase is calcium-dependent enzyme that catalyzes the cross-linking of certain glutamine and lysine side chains in myosin heavy chains in salted surimi paste during setting prior to cooking. In easy setting fish species, the thermal gelation of myosin is modified to various extents by the formation of covalent cross-links, depending on the enzymatic activity in the surimi paste during the setting.

Hossain *et al.* (1998) reported that in the presence of the TGase extracted from Alaska pollack surimi, the amount of dansyl-glutamine incorporated into muscle protein increased with the prolongation of incubation in the fish flesh sols. Large increment was found in Alaska pollack and Pacific

mackerel but not so large in horse mackerel. Tsukamasa and Shimizu (1990) have also detected TGase activity in fish muscle including sardine, Pacific mackerel, red sea bream, horse mackerel, ayu, carp, silver eel and Japanese Spanish mackerel.

Dimerization of carp myosin heavy chains at the initial stage of endogenous TGase-catalyzed cross-linking was investigated under the similar condition to that of setting in fish meat gelation, with 0.5 M NaCl and 5 mM $CaCl_2$ at pH 7.0 and 25°C (Seki *et al.*, 1998).

Araki and Seki (1993) estimated that the ratio of TGase unit to actomyosin in fish muscle was roughly 0.093-0.004 unit TGase to 5 mg actomyosin.

9. Factors affecting synergistic and inhibitory activity of transglutaminase

The setting temperature and duration may be critical for surimi derived from certain species. Incubation of surimi sols from Alaska pollack (*Theragra chalcogramma*) and Atlantic croaker (*Micropogan undulatus*) surimi at 40°C for 2-3 h increased gel strength, however incubation beyond this period resulted in reduced gel strength (Kamath *et al.*, 1992). When the setting temperature was raised to 50°C, the gels were progressively weakened. The gel-weakening phenomenon, referred to as "modori," has been attributed to the activity of thermostable proteases that are optimally active at 50-60°C (Seki *et al.*, 1995; Itoh *et al.*, 1989). Thus the gel-weakening activity of these proteases may compete with gel strengthening due to protein cross-linking by TGase. Addition of exogenous TGase could possibly offset somewhat the effects of the heat-stable proteases because the isopeptide bonds formed by TGase are supposedly resistant to proteolytic attack (Lorand, 1983). Generally, a protease inhibitor substance, such as beef plasma or egg white, is added to eliminate gel weakening by proteases and thus maximize the impact of TGase cross-linking on gel texture. Some muscle components may control the

activity of the enzyme in vitro. Chum salmon muscle was found to contain large amounts of anserine that inhibited TGase activity, preventing cross-linking and low-temperature gelation of actomyosin (Wan *et al.*, 1995). Several other fish species, such as yellowfin tuna, bluefin tuna, little tuna, skipjack, and black marlin, have been identified as having poor gelling qualities; these also contain large amounts of anserine (Suyama *et al.*, 1970; Shirai *et al.*, 1989). This potential regulatory activity of anserine may not be present in all species as some fish have good gelling ability without TGase (Ashie and Lanier, 2000).

The presence of certain ingredients in the meat paste may also influence the characteristics of the gels formed by TGase catalysis. When wheat and soy proteins were added to salted Alaska pollock surimi paste and allowed to set at 10°C for up to 72 h, the presence of these proteins decreased the setting response of the gels (Yamashita and Seki, 1996). Similar reductions of the setting response have been observed with the addition of 10% whole egg, albumen, or yolk to walleye pollock surimi (Yamashita and Seki, 1995). Addition of L-lysine also suppressed myosin cross-linking by TGase, as evidenced by reduced breaking force and strain of kamaboko prepared from Alaska pollock surimi (Liu *et al.*, 1995).

Because the tissue TGase is calcium dependent, added ingredients that affect calcium availability can also affect the cold setting reaction. Matsukawa *et al.* (1996) showed that addition of pyrophosphate diminished the setting response of pollock surimi. However, addition of a small amount of calcium salts to a surimi paste seems to remove any influence of phosphate on cross-linking and gel strengthening. Cross-linking activity of microbial TGase, which is insensitive to calcium level, is not affected by added phosphates. Wan *et al.* (1994) reported that gel strength of salted meat paste did not increase during setting in the absence of calcium ion, and the major effect of the calcium ion in the gelation was to activate intrinsic TGases.

Kumazawa *et al.* (1995) found that NH_4Cl and EDTA had inhibitory effect on myosin heavy chain cross-linking.

Objectives

1. To study the setting phenomenon in surimi from some tropical fish.
2. To study the role of endogenous transglutaminase in the setting of surimi.
3. To study endogenous transglutaminase activity as affected by fish handling and surimi processing conditions.

Chapter 2

Materials and Methods

1. Samples and preparation

Commercially frozen surimi (grade A) produced from threadfin bream (*Nemipterus bleekeri*), bigeye snapper (*Priacanthus tayenus*), barracuda (*Sphyraena jello*) and bigeye croaker (*Pennahai macrophthalmus*) were purchased from Man A Frozen Foods Co, Ltd., Maung, Songkhla. The surimi were cut into blocks (0.5 kg), placed in polyethylene bags and kept at –20°C until used.

Bigeye snapper (*Priacanthus tayenus*) were purchased from the dock in Songkhla. The fish, off-loaded approximately 36-48 h after catching, were transported to Department of Food Technology, Prince of Songkla University, in ice with a fish/ice ratio of 1:2 (w/w) within 1 h. Then the fish were immediately washed and the muscle were carefully excised from those specimens. The muscle was kept at –80°C until used.

2. Chemicals

Monodansylcadaverine (MDC), *N,N'*-dimethylated casein, $CaCl_2$, NH_4Cl, *N*-ethylmaleimide (NEM), sodium dodecyl sulfate (SDS), dithiothreitol (DTT), β-mercaptoethanol (βME), brilliant blue R, *N,N*-dimethylformamide, glycerol and high molecular weight markers were purchased from Sigma (St. Louis. Mo., U.S.A.). Coomassie blue R-250, penta-sodium triphosphate, sodium hexametaphosphate, tris (hydroxymethyl) aminomethane, Folin-Ciocalteu's phenol reagent, ethylene diamine tetraacetic acid (EDTA) and trichloroacetic acid (TCA) were obtained from Merck (Darmstadt, Germany).

N,N,N',N'-tetramethylethylenediamine (TEMED), Acrylamide, bis-acrylamide and urea were obtained from Fluka (Buchs, Switzerland).

3. Instruments

Instruments	Model	Company
Electrophoresis apparatus	Mini-Protein II	Bio-Rad, USA
Double-beam spectrophotometer	UV-16001	SHIMADZU, Australia
pH meter	Denver 15	Scientific, USA
Refrigerated centrifuge	RC-5B plus	Sorvall, USA
Water bath	W 350	Memmert, Germany
Magnetic stirrer	RO 10 power	KIKAL labortechnik, Germany
Homogenizer	T25	Ultra turrax, Malaysia
Balance	AB 204	METTLER TOLEDO, Switzerland
Texture analyzer	TA-XT2	Stable Micro Systems, England
Spectofluorometer	FP-750	JASCO, Japan
Freeze-Dryer	Dura-Top™ µp	FTS systems, USA
Basket centrifuge	CE 21 K	Grandimpianti, Italy
Mixer	MK-K77	National, Japan
Scanning Electron Microscope	JSM5800LV	JEOL, Japan

4. Determination of chemical compositions of surimi

4.1 Chemical compositions of surimi

Chemical compositions of surimi were determined as follows:

- Protein (AOAC, 1991)
- Moisture (AOAC, 1991)
- Ash (AOAC, 1991)
- Fat (AOAC, 1991)
- pH (Benjakul *et al.*, 1997)

4.2 Nitrogenous constituents in surimi

Fractionation of surimi was carried out according to the method of Hashimoto *et al.* (1979) (Figure 7). Each fraction containing different compositions, e.g. non-protein nitrogen, sarcoplasmic protein, myofibrillar protein, alkali-soluble protein and stroma was subjected to nitrogen determination using Kjeldahl method (AOAC, 1991).

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

The SDS-PAGE was performed according to the method of Laemmli (1970) using 4% stacking gel and 10% separating gel. The protein band was stained with Coomassie Brilliant Blue.

5. Study on setting phenomenon of surimi made from different fish species

5.1 Preparation of surimi gels

Frozen surimi was partially thawed at 4°C overnight (8-10 h). Surimi was then cut into small pieces and mixed with 2.5% salt in a mixer. The moisture content was adjusted to 80% with ice. During chopping, the

temperature was maintained below 10°C. The surimi sol was then stuffed into casing with a diameter of 2.5 cm.

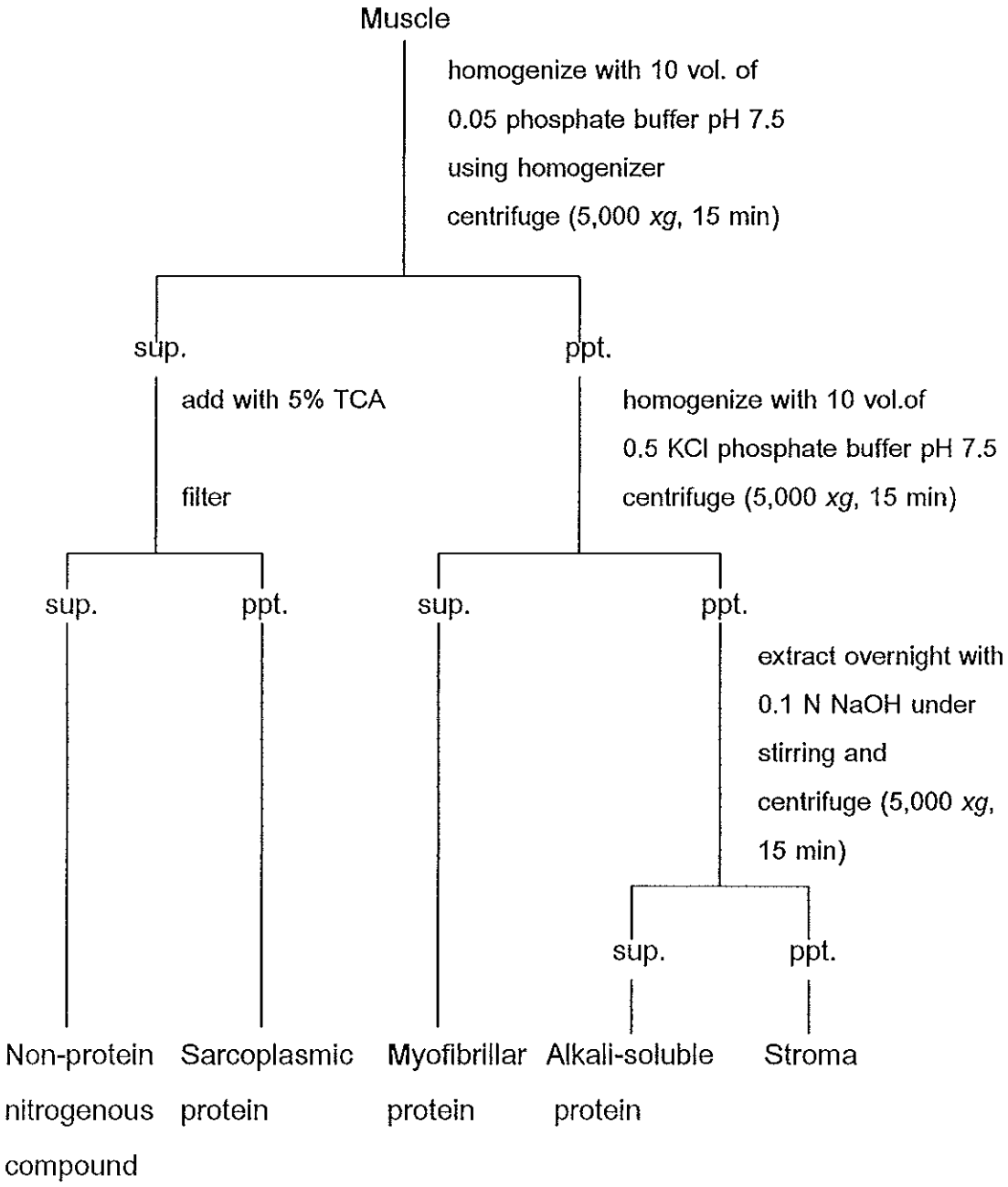

Figure 7. Fractionation procedure of muscle proteins

Source: Hashimoto *et al.* (1979)

5.2 The setting phenomenon of surimi

All prepared sol samples were subjected to different setting conditions. Suwari gel is defined as gel prepared by setting without heating, while ashi gel is referred to as the gel prepared by setting, followed by heating at 90°C for 20 min.

5.2.1 Suwari gels

The sol was subjected to setting at 25°C for various times (0.5, 1, 2, 3, 5 and 8 h) and at 40°C for different times (0.5, 1, 1.5, 2, 2.5 and 3 h). After setting, the gel was cooled down immediately using iced water.

5.2.2 Ashi gels

Ashi gels were prepared by setting the sol under the different conditions which was mentioned before (5.2.1). Then, suwari gels were heated at 90°C for 20 min. All gels were cooled down in iced water immediately and subjected to analysis.

5.3 Determination of surimi gel properties

Properties of surimi gels were tested. Force and deformation were measured by texture analyzer. Solubility of surimi gel was determined in solvent mixtures (20 mM tris-HCl containing 1% sodium dodecyl sulfate, 8 M urea and 2% β-mercaptoethanol, pH 8) according to the procedure of Roussel and Cheftel (1990).

Gel strength and solubility were used as the criterion for selecting of setting condition. Optimum setting time for either high or medium temperature setting of each surimi, which rendered the highest gel strength with lowest solubility, was chosen for further study.

Suwari gels prepared under optimum setting conditions were analyzed for TCA-soluble peptides according to the method of Morrissey *et al.* (1993). Protein pattern of suwari gels was also determined using SDS-PAGE.

Microstructures of suwari, ashi and directly heated gel of surimi from fish species, which exhibited the highest gel strength, were determined using scanning electron microscopy.

6. The effect of some chemicals on setting phenomenon

To study the effect of some chemicals on setting, surimi which exhibited the highest setting phenomenon was used.

6.1 Effect of calcium ion

Different amounts of $CaCl_2$ (0, 10, 20, 50, 80 and 120 mM) were added into surimi sol prior to setting at 40°C. The optimum setting time was used.

6.2 Effect of inhibitors

Some TGase (TGase) inhibitors were also added into surimi gel to verify the role of TGase in gel enhancement. Inhibitors and concentrations used are shown as follows:

- *N*-ethylmaleimide (NEM) (0, 1, 3, 5 and 10 mM)
- Ammonium chloride (NH_4Cl) (0, 0.05, 0.1, 0.2 and 0.5 M)
- Ethylenediaminetetraacetic acid (EDTA) (0, 0.5, 1, 3 and 5 mM)

6.3 Effect of polyphosphates

The effect of polyphosphate on setting was also studied. Different polyphosphates were added into surimi sol at different concentrations as follows:

- Sodium hexametaphosphate (0, 0.1, 0.3 and 0.5 % w/w)
- Penta-sodium triphosphate (0, 0.1, 0.3 and 0.5 % w/w)

Properties of suwari gels added with different chemicals were tested for force and deformation, solubility (Roussel and Cheftel, 1990) and protein pattern using SDS-PAGE (Laemmli, 1970).

7. Characterization of endogenous transglutaminase

7.1 Preparation of crude endogenous transglutaminase from fish muscle

Fish muscle was minced and homogenized with 2 volumes of extraction buffer (20 mM Tris-HCl containing 10 mM βME, pH 7.5) using a homogenizer. The homogenate was centrifuged at 16,000 *xg* for 20 min. The supernatant was further centrifuged at 18,000 *xg* for 60 min, followed by freeze-drying at –80°C. Dried sample with a yield of 9.47 % (w/w) was then kept at –80°C until used. To prepare enzyme solution, the dried powder was dissolved in distilled water to obtain the final concentration of 0.1 g/mL (Kishi *et al.*, 1991).

7.2 pH and temperature profile of endogenous transglutaminase

7.2.1 Effect of pH

To investigate the effect of pH on TGase activity, 0.2 mL of extract (0.1 g/mL) was mixed with 2.4 mL of 0.2 M McIlvaine buffers (0.2 M Na-phosphate, 0.1 M Na-citrate) with different pHs (2, 3, 4, 5, 6, 7, 8, 9 and 10). The mixtures were then preincubated at 25°C for 15 min prior to enzyme activity assay. TGase activity was determined by MDC-incorporating method as described Takagi *et al.* (1986).

7.2.2 Effect of the temperature

TGase activity of crude extract (0.1 g/mL) was assayed under optimum pH at different temperatures as follows: 20, 25, 30, 35, 40, 45, 50, 55 and 60°C. TGase activity was measured by MDC-incorporation into *N,N'*-dimethylated casein as mentioned above.

7.3 Effect of chemicals on transglutaminase activity

Different chemicals were added to crude extract (0.1 g/mL) to obtain the different final concentrations as follows:

- $CaCl_2$ (0, 1, 3, 5 and 8 mM)
- NaCl (0.1, 0.2, 0.4 and 0.6 M)
- NEM, NH_4Cl, EDTA (0, 0.5, 1 and 3 mM)
- β-mercaptoethanol, DTT (0, 1, 5 and 10 mM)
- Sodium hexametaphosphate, Penta-sodium triphosphate (0, 0.1, 0.2 and 0.3%)

The mixtures were mixed thoroughly and kept at room temperature for 15 min prior to enzyme activity assay.

7.4 pH stability

To investigate the effect of pH on the stability of crude TGase, the extract (0.1 g/mL) was subjected to different pHs (2, 3, 4, 5, 6, 7, 8, 9 and 10) by mixing the extract with 0.2 M McIlvaine buffers (0.2 Na-phosphate, 0.1 M Na-citrate) with different pHs at a ratio of buffer to crude extract of 1:1(v/v). The mixtures were then incubated at 25°C for 10 min. The residual activity was measured by MDC-incorporating activity assay (Takagi *et al.* 1986).

7.5 Thermal stability

Crude extract (0.1 g/mL) was incubated at various temperatures (30, 40, 50 and 60°C). At a definite time interval (0, 10, 30 and 60 min), the enzyme solutions were cooled immediately in iced water for 3 min. The residual activity was determined using MDC-incorporating activity assay (Takagi *et al.*, 1986).

7.6 Cross-linking of actomyosin by endogenous transglutaminase

The actomyosin solution was prepared according to the method of Benjakul *et al.* (1997). Crude TGase were added to actomyosin solution (2 mg/mL) to obtain different concentrations (0, 0.01, 0.03, 0.05, 0.08, 0.1 and 0.15 g/mL), MDC-incorporating activity assay was performed under the optimum condition using dimethylated casein. Degradation products in crude extract as well as those occurred during TGase activity assay were measured in term of TCA-soluble peptide according to the method of Morrissey *et al.* (1993).

To investigate the crosslinking of actomyosin by TGase, assay mixtures were subjected to sodium dodesylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 4% stacking gel and 10% separating gel (Laemmli, 1970).

8 Effect of storage and processing on transglutaminase activity in fish mince.

8.1 Effect of iced storage on transglutaminase activity in fish mince

The mince packed in polyethylene bags and kept in ice was taken at day 0, 2, 4, 6 and 8 for TGase activity measurement. Crude TGase was extracted as mentioned before (7.1) and subjected to MDC-incorporating activity assay under the optimum condition. The activity was expressed as units/g mince.

8.2 Effect of freeze-thawing on transglutaminase activity in fish mince

Fish mince was frozen at –20°C for 24 h. The samples were then thawed with running water (26-28°C) until the temperature reached 2°C. The samples were subjected to 0, 1, 2 and 3 freeze-thaw cycles. Samples obtained from different freez-thaw cycles were used for TGase extraction. The activity was determined by MDC-incorporating method.

8.3 Effect of washing on transglutaminase activity in fish mince

Mince was washed with NaCl solutions at different concentrations (0, 0.1, 0.2 and 0.3%) with different washing cycles (1, 2 and 3 times). The washed mince obtained from different washing conditions was determined for remaining TGase activity.

9. Statistical analysis

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

Chapter 3

Results and Discussion

1. Compositions of surimi from some tropical fish.

1.1 Chemical compositions

Surimi from four fish species had the similar chemical compositions as shown in Table 6. All surimi samples contained high moisture content ranging from 76.99 to 78.09%. Protein content was found between 16.79 and 18.60%, while very low fat content was observed. During washing process, fish proteins mainly myofibrillar proteins were concentrated with a concomitant removal of fat, blood as well as other flavoring compounds. As a result, fat content was very low. Additionally, all fish used were lean fish, which contained a low fat content. Mackie (1994) reported that the main constituents of flesh are protein and fat. The result was in accordance with Ching and Leinot (1993) who reported that surimi contains 18-21% protein, 75-78% moisture, 0-2 % fat, sugar (4% saccharose, 4% sorbitol) and polyphosphates (0.2-0.3%). Ash in surimi possibly represented inorganic matter, mainly from phosphate salt added in commercially produced surimi to enhance water binding capacity as well as to prevent protein denaturation during frozen storage. All samples had pH of 7.0. Very low or high pHs directly affect gel-forming ability of fish muscle or surimi.

1.2 Nitrogenous constituents

Nitrogenous compositions of surimi from four fish species are shown in Table 7. All surimi had the similar nitrogenous composition profile. Among proteins and other nitrogenous components in surimi, myofibrillar protein was

Table 6. Proximate compositions of surimi from different fish.

Samples		Constituent (% wet weight basis)			
	pH	Moisture	Protein	Fat	Ash
Threadfin bream surimi	7.00±0.00ns	78.09±0.32[a]b[b]	16.79±0.03a	0.19±0.01b	0.53±0.03ns
Bigeye snapper surimi	7.00±0.00ns	77.32±0.07a	18.60±0.24c	0.17±0.01a	0.41±0.15ns
Barracuda surimi	7.00±0.01ns	77.25±0.08a	17.27±0.45ab	0.20±0.00c	0.43±0.00ns
Bigeye croaker surimi	7.00±0.01ns	76.99±0.16a	18.01±0.64bc	0.39±0.01d	0.41±0.03ns

[a]Mean ± standard deviation from triplicate determinations.

[b]The different superscripts in the same column denote the significant differences ($p<0.05$)

found to be a major constituent ranging from 71.20 to 74.10%. Bigeye croaker was found to contain the highest myofibrillar fraction, compared to other surimi samples. Suzuki (1981) and Mackie (1994) reported that myofibrillar proteinswere main nitrogenous constituents in fish muscle. Myofibrillar proteins cover 66-77% of the total protein in fish meat and play an important role in coagulation and gel forming of fish meat (Careche *et al.*, 1995). Sarcoplasmic fractions found in surimi indicated that some water soluble proteins were remained unwashed. This was probably due to the entrapment of those proteins in the large particle size of mince. Insufficient washing was also presumed. Stroma and alkali-soluble proteins were also found in surimi in the range of 1.71-2.70% and 3.44-3.50%, respectively. Stroma represents connective tissues, primarily collagen. Stroma are almost totally insoluble in water or saline and do not participate in gel formation (Lanier, 2000). Large

Table 7. Nitrogenous compositions of surimi from different fish.

Samples	Compositions				
	Non-protein nitrogen	Sarcoplasmic	Myfibrillar	Alkali-soluble	Stroma
Threadfin bream surimi	0.27±0.01[a]b[b]	2.01±0.17b (7.43)*	18.41±0.64b (72.08)	4.50±0.06c (19.10)	2.13±0.07b (7.28)
Bigeye snapper surimi	0.28±0.01b	1.76±0.37b (6.89)	17.45±0.42a (74.10)	4.11±0.09b (14.04)	2.22±0.07b (8.69)
Barracuda surimi	0.29±0.01c	1.26±0.19a (5.35)	17.14±0.21a (72.83)	3.44±0.10a (14.60)	1.71±0.06a (7.26)
Bigeye croaker surimi	0.25±0.00a	1.23±0.14a (4.20)	20.84±0.63c (71.20)	4.50±0.34c (15.37)	2.70±0.12c (9.22)

[a]Mean ± standard deviation from triplicate determinations.

[b]Different letters in the same column denote the significant differences (p<0.05).

*Numbers in parenthesis represent percentage distribution.

connective tissues are normally removed using strainer in surimi process. Therefore, stroma found in surimi was possibly located in the outer side of cellular fibril. However, fish have only a small percentage of stroma protein relative to the myofibrillar protein. Therefore, the presence of collagen has a negligible effect on the gelling ability of surimi. For non-protein components, they were found at very low content. These components may include trimethylamine oxide, ammonia and other low molecular weight compound, which can contribute to the flavor of surimi (Hashimoto *et al.*, 1979).

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

Protein pattern of surimi determined using SDS-PAGE revealed that similar protein compositions were observed among surimi samples. As depicted in Figure 8, myosin heavy chain was found to constitute as a major muscle protein, which appeared at MW of 200,000 dalton. Actin was found to be the second abundant protein in surimi with a MW of 45,000 dalton. Tropomyosin was also found in all surimi samples.

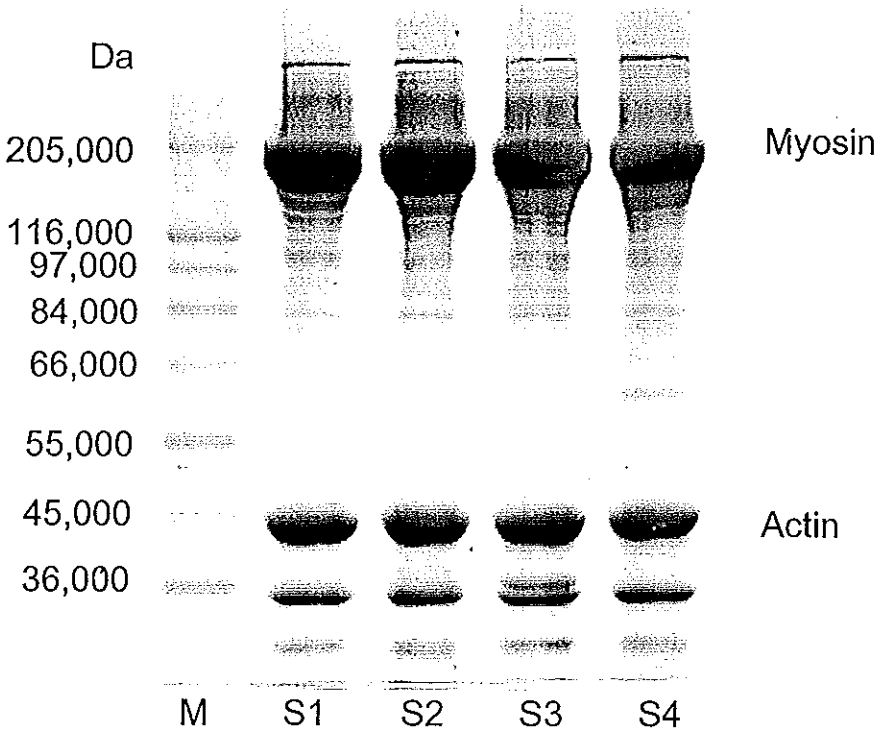

Figure 8. SDS-PAGE pattern of protein in surimi from some tropical fish. M; high molecular weight standard (dalton), S1; threadfin bream surimi, S2; bigeye snapper surimi, S3; barracuda surimi and S4; bigeye croaker surimi.

2. The setting phenomenon of surimi from different fish

Gel properties of barracuda surimi as affected by setting

Force and deformation of suwari and ashi gels prepared under different setting conditions are depicted in Figure 9 and 10, respectively. For suwari gel prepared at medium setting temperature (25°C), both force and deformation increased when setting time increased ($p<0.05$). At high temperature setting (40°C), both force and deformation increased with increasing setting time up to 1.5 h. Force decreased markedly when setting time was longer than 2 h, but no changes in deformation were observed. This was presumed to be due to protein degradation caused by endogenous proteinases. Proteolysis was detected during setting (Kamath *et al.*, 1992; Lu *et al.*, 1992).

Numakura *et al.* (1990) and Kamath *et al.* (1992) reported that walleye pollack surimi paste had the optimum temperature of setting at 25°C. No setting occurred obove 40°C. Marked depression of a setting response above 40°C was caused by the inactivation of TGase in the surimi paste. Thus, TGase-catalyzed formation of myosin heavy chain polymers was also depressed at higher temperatures.

Ashi gels were prepared by setting under the same condition which was mentioned before. Then, suwari gel was heated at 90°C for 20 min. Properties of ashi gels were similar to suwari gels. However, much higher force and deformation were observed with ashi gel. This was possibly because heating process induced protein aggregation formed by different bonding, including hydrophobic interactions, disulfide bonds, etc. As a result, ashi gel exhibited stronger gel network, compared to suwari gel. This result was in agreement with Alvarez and Tejada (1997) who reported that heating of suwari gels to produce kamaboko gels caused further aggregation of a structure formed in the setting stage, due probably to large-scale formation of disulfide bridges and hydrophobic interactions at high temperatures.

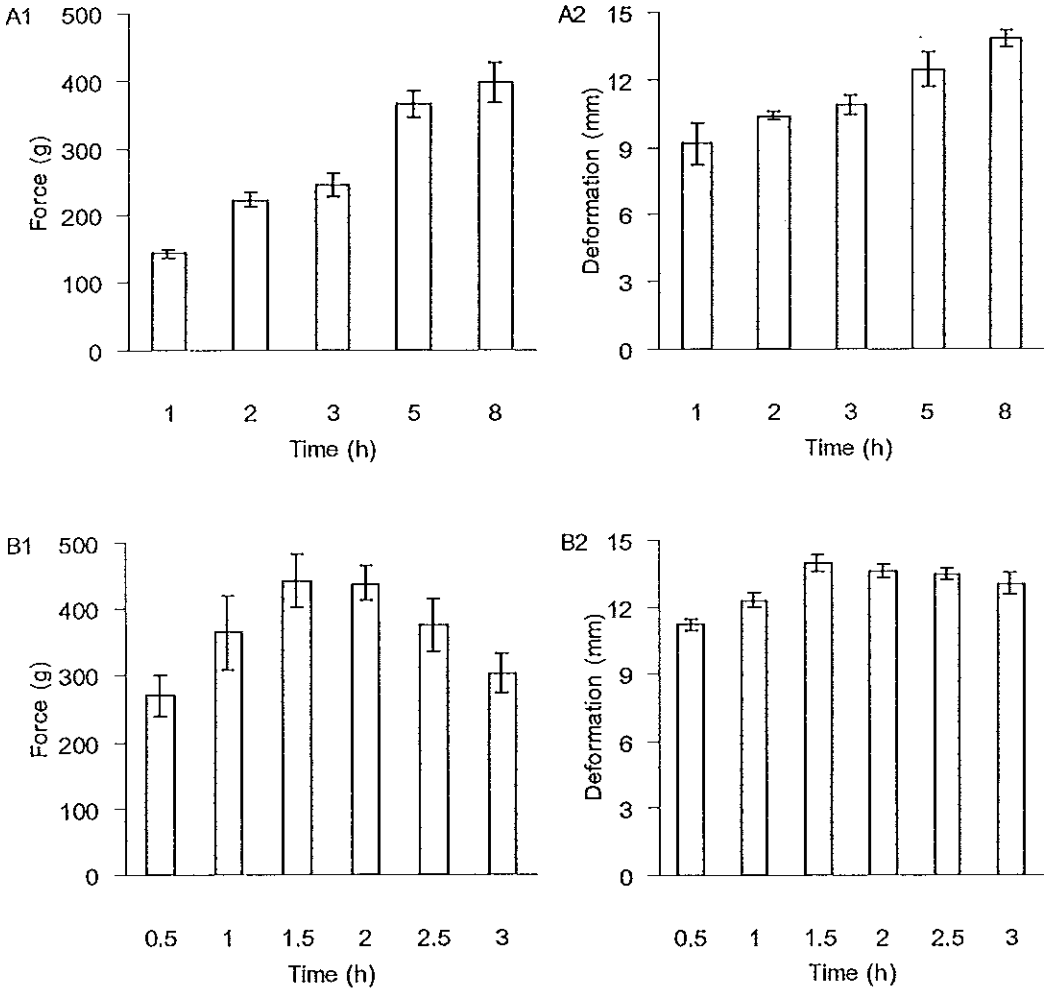

Figure 9. Force and deformation of barracuda suwari gel. A1, A2; setting at 25°C, B1, B2; setting at 40°C

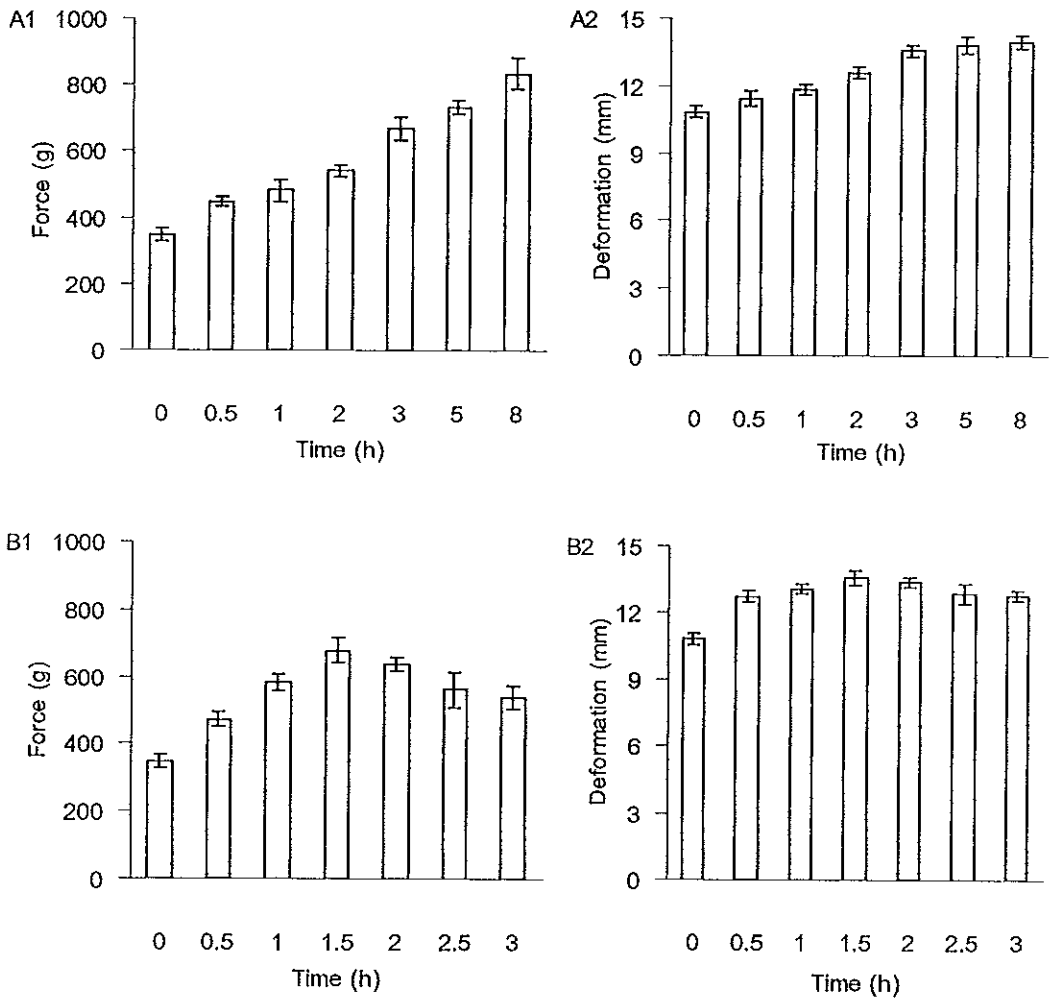

Figure 10. Force and deformation of barracuda ashi gel. A1, A2; setting at 25°C and followed by heating 90°C for 20 min. B1, B2; setting at 40°C and followed by heating 90°C for 20 min.

Gel properties of bigeye snapper surimi as affected by setting

For medium temperature setting (25°C), both force and deformation increased when the setting time increased ($p<0.05$) (Figure 11A). It was postulated that endogenous transglutaminase induced cross-linking to a higher extent at 25°C, particularly when setting time increased, while no marked proteolytic activity was found at this temperature. Mostly, modori-inducing proteolytic activity was observed at temperatures around 50-60°C (An *et al.*, 1995). As a consequence, the degradation of protein was negligible during medium temperature setting.

The suwari gels from bigeye snapper prepared at high temperature (40°C) had the similar characteristic to those from barracuda (Figure 11B). It was noted that force and deformation increased as the setting time increased, but decrease in force and deformation was found when setting time was longer than 2 h ($p<0.05$), suggesting that some degradation presumably occurred.

The properties of ashi gel from bigeye snapper were in accordance with those of suwari gel (Figure 12). However, the ashi gel had higher force and deformation than the suwari gel.

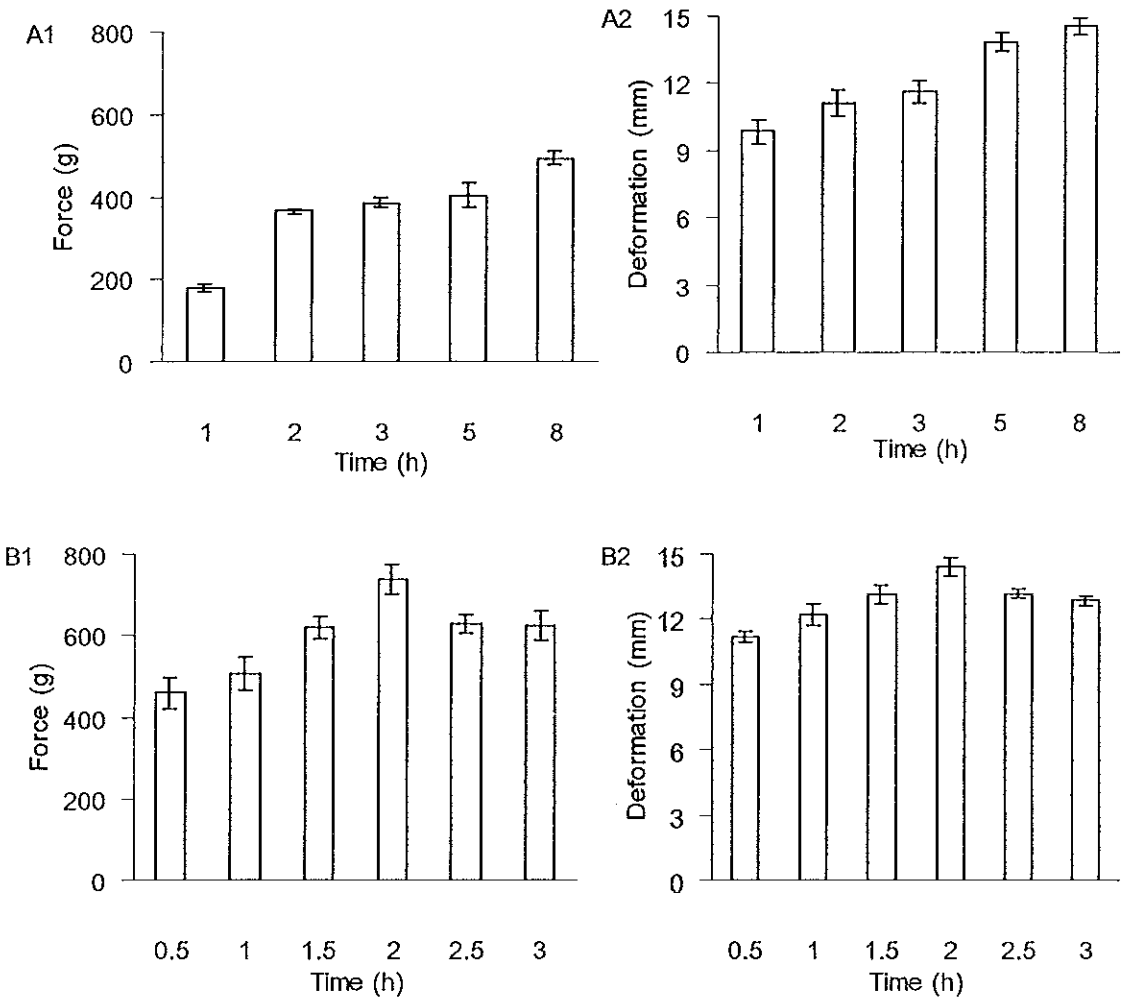

Figure 11. Force and deformation of bigeye snapper suwari gel. A1, A2; setting at 25°C, B1, B2; setting at 40°C

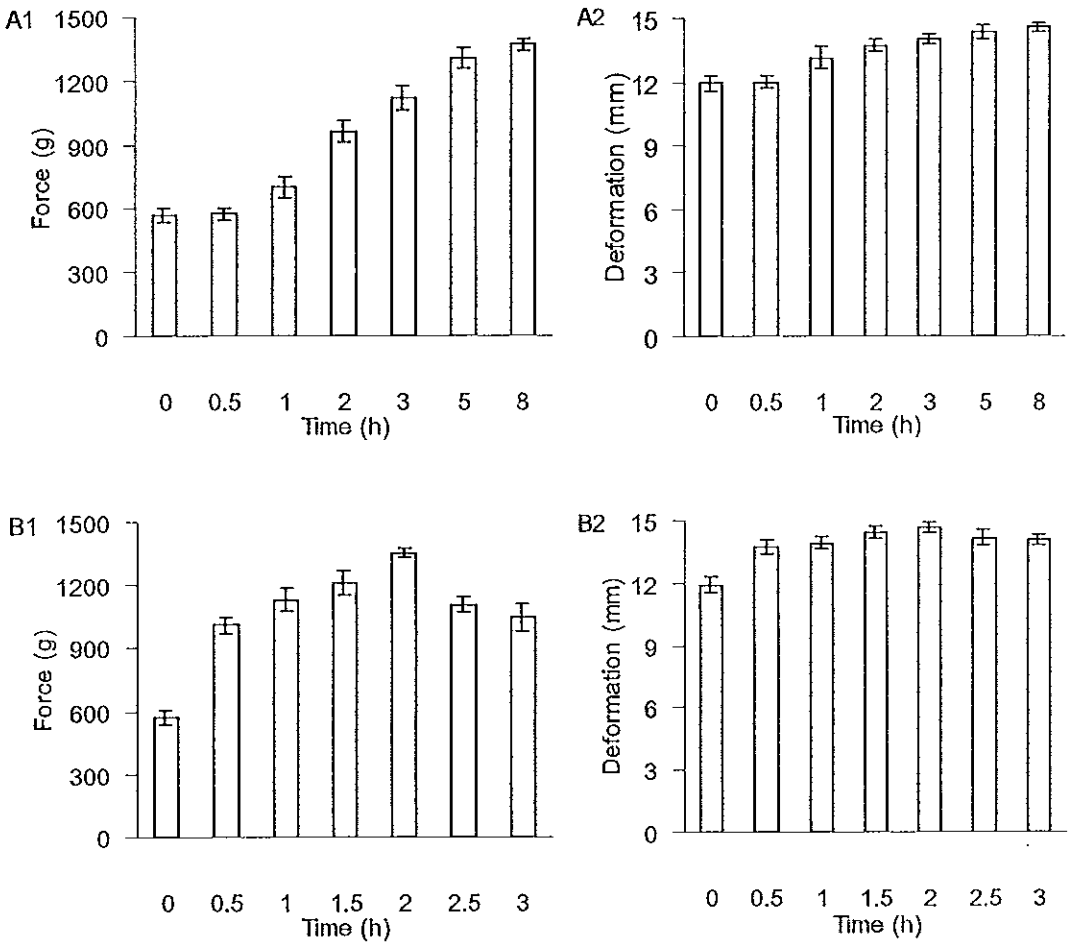

Figure 12. Force and deformation of bigeye snapper ashi gel. A1, A2; setting at 25°C and followed by heating 90°C for 20 min. B1, B2; setting at 40°C and followed by heating 90°C for 20 min.

Gel properties of threadfin bream surimi as affected by setting

For suwari gel from threadfin bream prepared at medium setting temperature (25°C), both force and deformation increased as setting time increased (p<0.05) (Figure 13). For high setting temperature (40°C), force and deformation decreased when setting time was longer than 1 h (p<0.05). Since threadfin bream was reported to contain a high amount of proteinase, those enzyme might play an essential role in hydrolyzing the muscle proteins, which contribute to gel formation. Modori-inducing proteinase (MIP) in threadfin bream were classified into four types according to location and optimal temperature: Sp-50-MIP, Sp-60-MIP, Mf-50-MIP and Mf-60-MIP (Kinoshita *et al.*, 1990). These proteinases caused gel weakening (Kinoshita *et al.*, 1992).

For ashi gel (Figure 14), it was shown that ashi gel prepared by setting at 25°C prior to heating had a much higher breaking force, particularly at a higher setting time, compared to those prepared by setting at 40°C before heating. This result reconfirmed that proteinase activated at high temperature should actively function in protein cleavage, resulting in the poor gel quality.

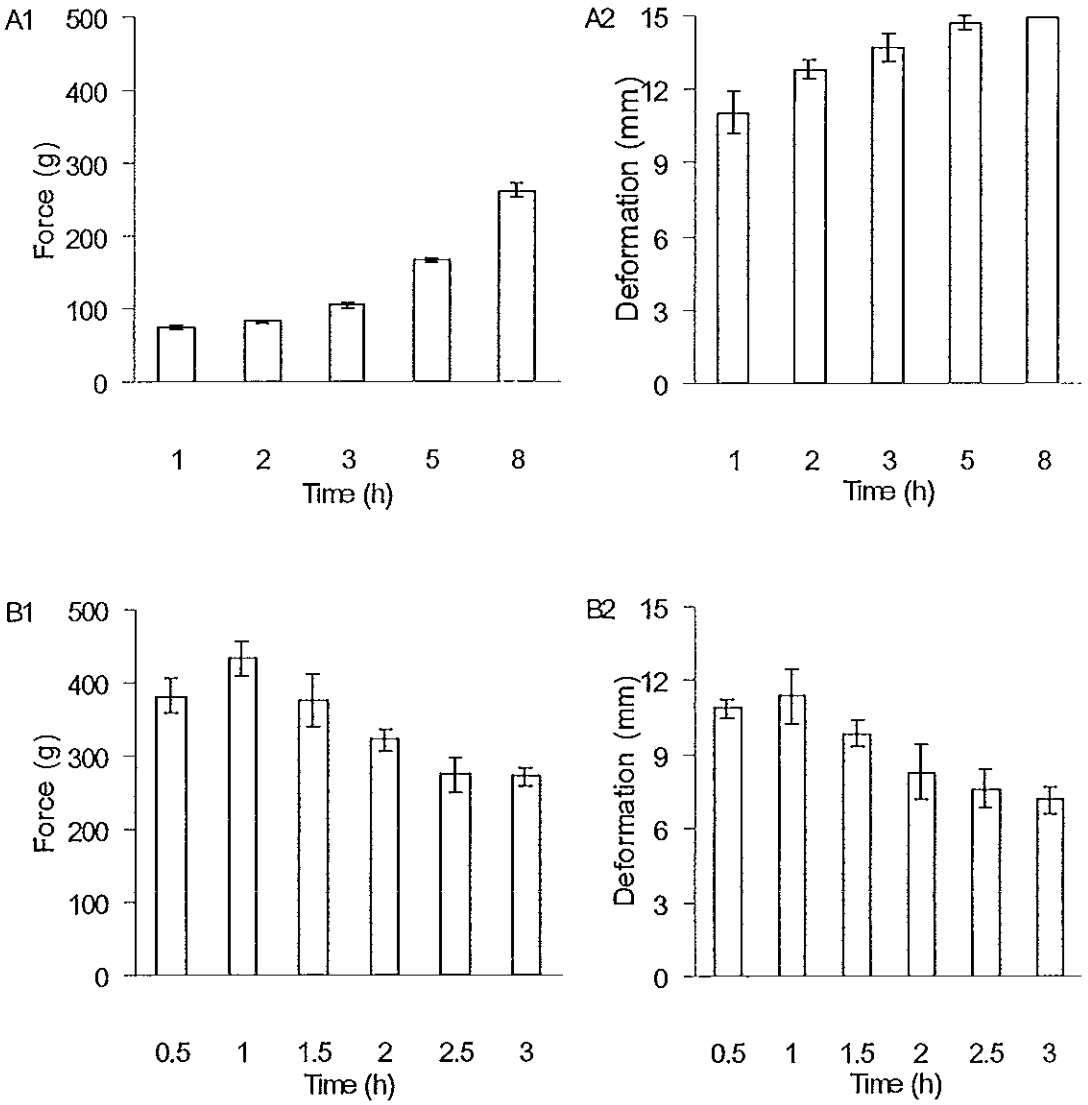

Figure 13. Force and deformation of threadfin bream suwari gel. A1, A2; setting at 25°C, B1, B2; setting at 40°C

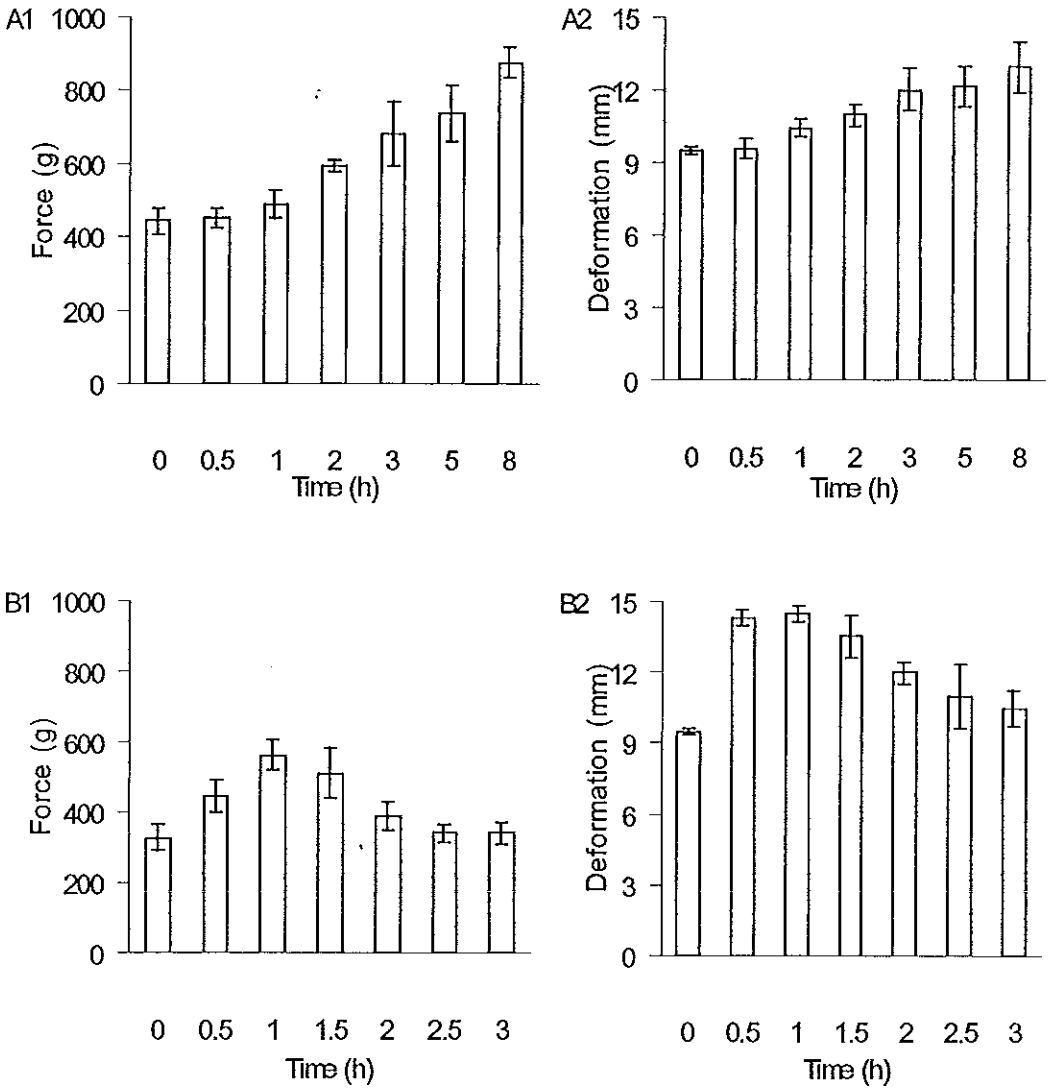

Figure 14. Force and deformation of threadfin bream ashi gel. A1, A2; setting at 25°C and followed by heating 90°C for 20 min. B1, B2; setting at 40°C and followed by heating 90°C for 20 min.

Gel properties of bigeye croaker surimi as affected by setting

The suwari gel from bigeye croaker prepared at different setting temperatures showed different force and deformation (Figure 15). Generally, suwari gel prepared at 40°C rendered the higher breaking force, compared to those prepared at 25°C. Suwari gels prepared at both setting temperature had increased breaking force as the setting time increased ($p<0.05$). No decrease in breaking force was observed when gel was set at 40°C for a longer time. This suggested that negligible proteolytic activity involved during high temperature setting. Interestingly, ashi gel preincubated at both setting temperatures showed the similar breaking force, through marked differences in breaking force were observed in suwari gel prepared at different setting temperatures (Figure 16). Therefore, setting at two different temperatures resulted in the similar final gel properties when the proper setting time was used. Generally, setting at 25°C took a longer time than at 40°C to obtain the some breaking force of ashi gel.

From the result, setting at either high or medium temperature for a proper time resulted in the increase in surimi gel quality, as indicated by an increase in breaking force and deformation. These results were in agreement with many researchers who found that setting of surimi paste, which was carried out below approximately 30°C prior to heating at 90°C, involved a process where myosin heavy chains were cross-linked to form a fine elastic network and resulted in the formation of gels with greater firmness. (Numakura *et al.*, 1985; Sano *et al.*, 1990; Careche *et al.*, 1995) The suwari and ashi gel prepared by setting at 35 and 40°C gave the highest folding test scores (Careche *et al*, 1995). Alvarez and Tejada (1997) reported that the gel strength of sardine was much higher in kamaboko gels than in the corresponding suwari gels. This would indicate that at cooking temperature, protein-protein bonds were established which strengthened the network previously formed by setting. Kumazawa *et al.* (1995) reported that the gel

strength of kamaboko made from FA grade surimi with 3% NaCl increased as setting time increased.

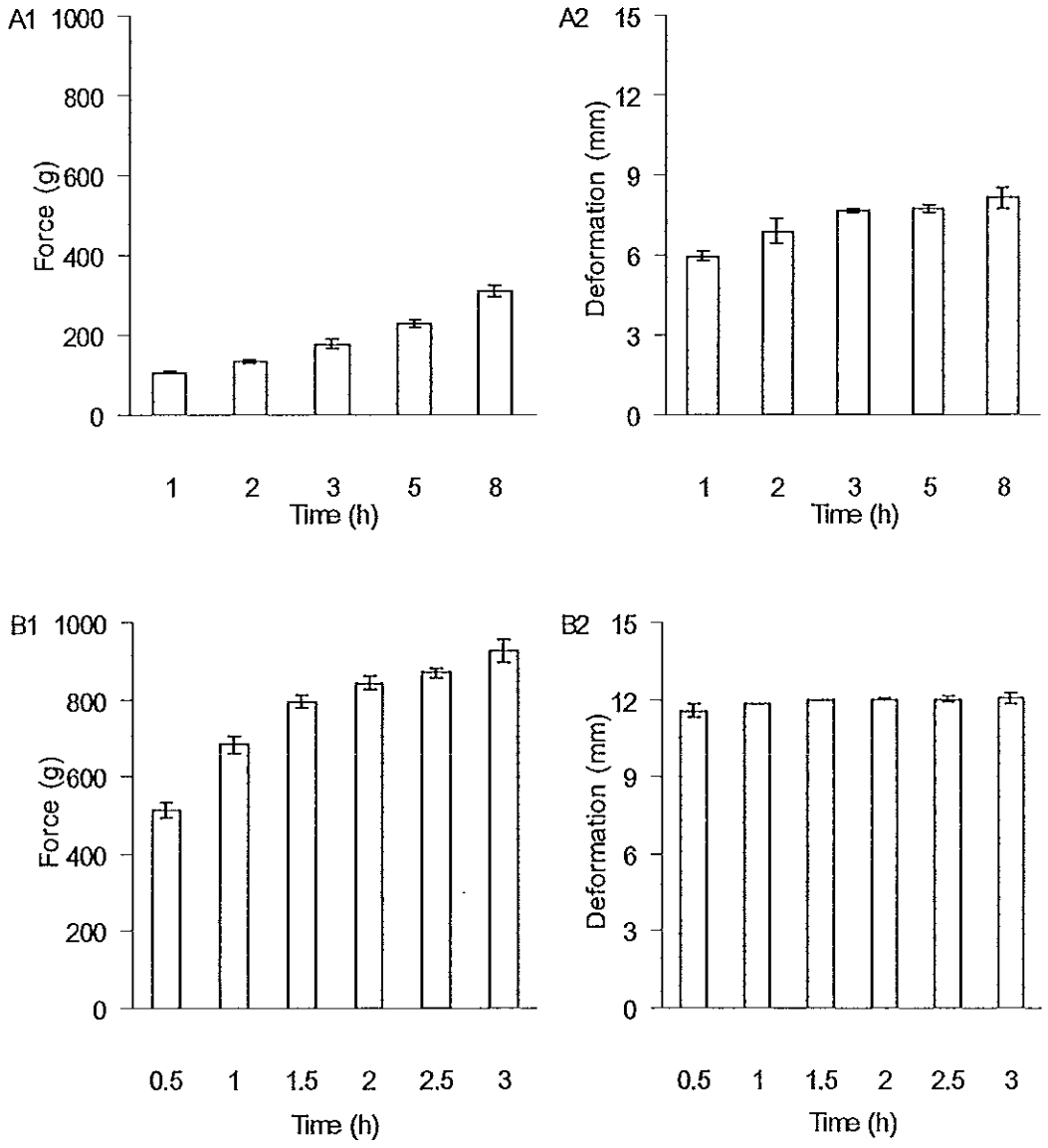

Figure 15. Force and deformation of bigeye croaker suwari gel. A1, A2; setting at 25°C, B1, B2; setting at 40°C

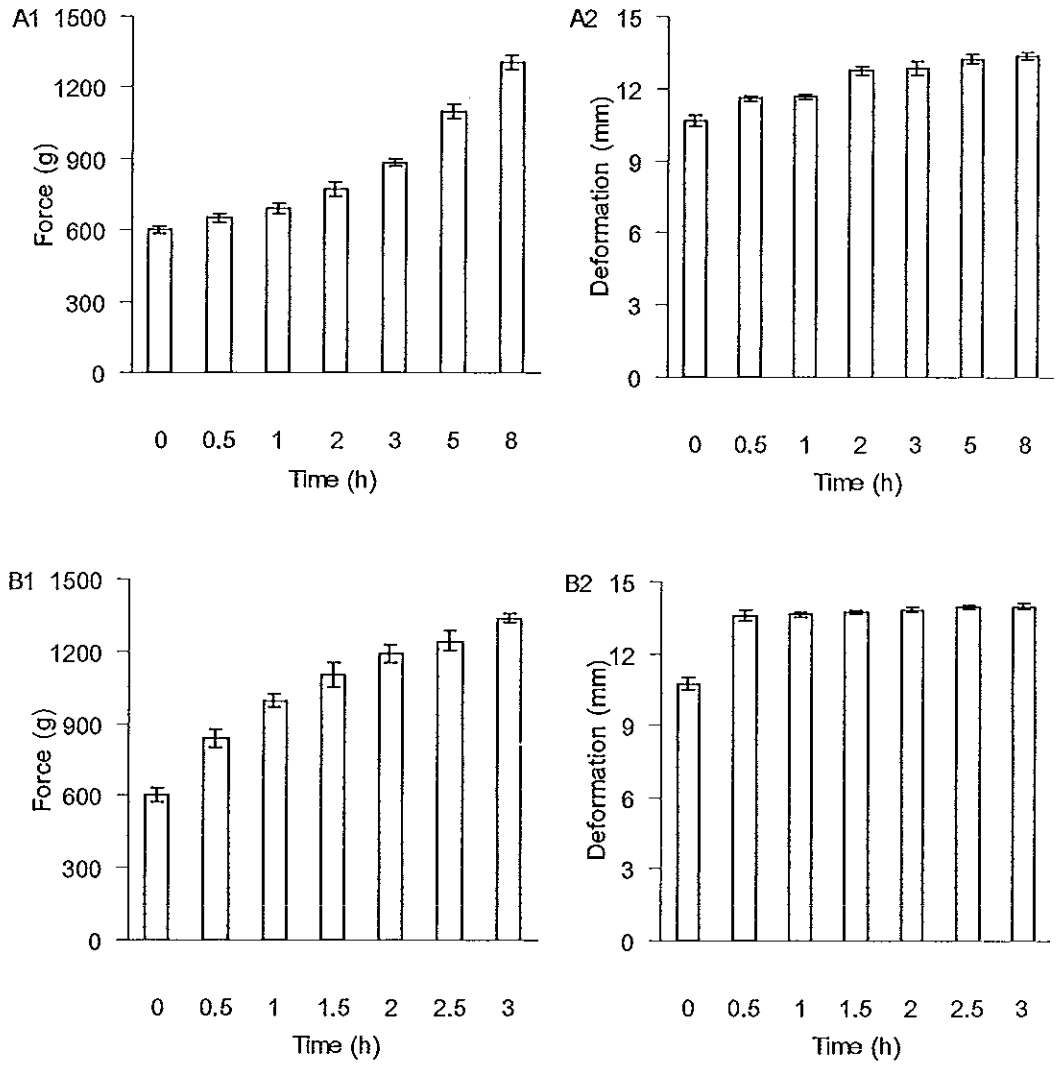

Figure 16. Force and deformation of bigeye croaker ashi gel. A1, A2; setting at 25°C and followed by heating 90°C for 20 min. B1, B2; setting at 40°C and followed by heating 90°C for 20 min.

Jiang *et al.* (1998) reported that the inter/intramolecular linkages of muscle proteins were gradually constructed during setting and accordingly the gel strength increased. However at the same time, the muscle proteins were also gradually hydrolyzed by endogenous proteases. Therefore, the optimal setting time needs to be determined for the individual surimi.

Solubility of suwari and ashi gel

For all surimi tested, the solubility of suwari and ashi gel prepared by setting at 25°C decreased as setting time increased ($p<0.05$) (Table 8). The rate of decrease was lowest in surimi from threadfin bream, compared to other surimi. This result was in agreement with the lowest gel strength observed in surimi from threadfin bream. Mixtures containing sodium dodesyl sulfate, urea and β-mercaptoethanol were used to solubilize proteins via destroying all bonds, except non-disulfide covalent bond, particularly ε-(γ-glutamyl) lysine linkage. Therefore, the low solubility indicated the presence of such a linkage, which was formed to a higher extent when setting time increased. It has been known that endogenous transglutaminase play a crucial role in ε-(γ-glutamyl) lysine linkage formation (Kamath *et al.*, 1992). From this result, ε-(γ-glutamyl) lysine linkage was presumed to be a major contributor for strengthening the gel matrix, which was associated with the low solubility.

For suwari and ashi gel prepared at high temperature setting (40°C) (Table 9), the solubility of all surimi gels except bigeye croaker surimi decreased up to particular period of setting, thereafter solubility became increased. However, solubility of both suwari and ashi from bigeye croaker decreased continuously as the setting time increased. This was coincidental with the increase in breaking force when setting time increased. The increase in solubility observed after setting for a long time, particularly at 40°C, was postulated to be due to the smaller peptides produced by proteolysis. The

Table 8. Solubility of suwari and ashi gels of surimi from some tropical fish prepared by setting at 25°C for different times.

Setting time (h)	Solubility (%)							
	Setting at 25°C				Setting at 25°C and followed heating 90°C for 20 min			
	Threadfin bream	Bigeye snapper	Barracuda	Bigeye croaker	Threadfin bream	Bigeye snapper	Barracuda	Bigeye croaker
Control	100.00f	100.00f	100.00e	100.00f	100.00f	100.00f	100.00f	100.00h
0	98.28±0.55e	98.48±1.10ef	99.78±0.89e	97.47±0.83e	90.83±0.84e	89.71±1.47e	87.21±1.16e	89.80±0.68g
0.5	95.06±0.71d	97.53±1.06e	97.86±1.13e	93.95±0.59d	89.72±0.48e	86.27±1.70d	86.28±0.67e	86.67±0.68f
1	93.05±0.47c	94.61±0.85d	94.57±1.35d	92.63±1.26d	87.22±1.27d	83.33±1.70c	85.66±1.78e	85.49±0.68e
2	92.22±0.48bc	90.69±0.84c	87.98±0.67c	90.39±1.94c	85.55±0.48c	78.43±1.69b	81.78±1.78d	81.57±0.67d
3	91.95±0.47bc	89.22±0.84c	86.44±0.67c	83.35±0.78b	84.45±0.48c	77.45±1.69b	75.97±1.34c	74.90±0.67c
5	91.11±0.49b	85.78±0.85b	81.78±1.78b	82.11±0.42b	80.28±0.96b	72.55±1.70a	73.74±1.44b	67.85±0.68b
8	85.55±2.30a	78.43±1.70a	74.33±2.19a	77.64±0.91a	78.33±0.84a	71.57±1.69a	71.43±0.60a	65.10±0.67a

Mean ± SD from triplicate determinations

Dfifferent letters in the same column denote a significant difference ($p<0.05$).

Table 9. Solubility of suwari and ashi gels of surimi from some tropical fish prepared by setting at 40°C for different times.

Setting time (h)	Solubility (%)							
	Setting at 40°C				Setting at 40°C and followed heating 90°C for 20 min			
	Threadfin bream	Bigeye snapper	Barracuda	Bigeye croaker	Threadfin bream	Bigeye snapper	Barracuda	Bigeye croaker
Control	100.00h	100.00f	100.00g	100.00h	100.00f	100.00f	100.00e	100.00h
0	98.28±0.55g	98.48±1.10e	99.78±0.89g	97.47±0.83g	90.83±0.84e	89.71±1.47e	87.21±1.16d	89.80±0.68g
0.5	94.25±0.80f	83.69±0.85d	87.21±1.16f	92.04±0.48f	78.61±0.49a	83.26±1.76d	73.64±1.35bc	81.57±0.68f
1	80.55±0.48a	81.73±0.85d	75.97±1.34c	86.67±0.68e	77.78±0.48a	74.51±1.70c	72.87±1.34bc	74.51±0.67e
1.5	85.27±0.47b	76.00±1.12c	70.15±0.68a	85.09±0.68d	80.55±0.48b	71.57±1.70b	68.21±1.34a	72.55±0.68d
2	87.78±0.47c	68.49±1.70a	72.87±1.34b	76.08±1.36c	83.61±0.49c	65.20±0.85a	71.04±0.19ab	71.37±0.67c
2.5	90.56±0.96d	71.43±1.70b	78.68±1.78d	66.67±0.68b	84.72±0.96c	66.67±0.85a	75.19±1.34c	67.85±0.68b
3	91.95±0.48e	78.26±3.00c	81.78±1.78e	65.10±0.67a	89.17±1.67d	76.47±2.94c	75.58±4.19c	65.49±0.68a

Mean ± SD from triplicate determinations

Dfifferent letters in the same column denote a significant difference ($p<0.05$).

high proportion of remaining insoluble protein may indicate that non-disulfide covalent bonds played an important in the formation of suwari and kamaboko gels (Careche *et al.*, 1995).

From the results, suwari gel could be prepared at either high or medium temperature with optimum setting time as follows: threadfin bream; 25°C for 8 h and 40°C for 1 h, bigeye snapper; 25°C for 8 h and 40°C for 2 h, barracuda; 25°C for 8 h and 40°C for 1.5 h, bigeye croaker; 25°C for 8 h and 40°C for 3 h.

In fact, a gel exhibited less solubility did not necessarily imply that a larger number of bonds were involved in network formation but rather that the proteins were linked by non-disulfide covalent bonds which would intervene in the formation of the network without being ruptured in the conditions of solubilization. In such conditions, greater network solubility would mean a weaker structure, which is generally consistent with the textural properties (Careche *et al.*, 1995).

Protein degradation in suwari gel from some tropical fish

During setting process, the degradation of muscle protein occurred (Table 10). The results revealed that protein underwent degradation to a higher extent at high temperature setting (40°C), compared to medium temperature setting (25°C). At high temperature, some endogenous proteinase, such as cathepsin was active and hydrolyzed muscle protein. The protease activity in Pacific whiting, as other species, had an adverse effect on gel forming properties of surimi (An *et al.*, 1994). A rapid breakdown of the myofibrillar protein inhibits development of a thee-dimensional gel network during heat setting of surimi based products (Morrissey *et al.*, 1993). Myosin is the most important component contributing to formation of surimi gels. The presence and concentration of intact myosin determined the gel strength of surimi (Niwa, 1992). The differences in degradation of suwari gel

were possibly due to the differences in susceptibility of protein to hydrolysis. Furthermore, the proteinases in different muscles possibly belonged to different groups with different hydrolytic property.

Among all surimi tested, threadfin bream surimi was most prone to degradation. As a result, it was more susceptible to modori, particularly during setting at high temperature. From the result, it was noted that degradation of protein occurred during setting. Therefore, setting time should be enough for enhancing the cross-linking induced by TGase, but should not be too long for proteolysis, which became dominant at a longer setting time. However, this phenomenon depended on fish species.

Table 10. TCA-soluble peptides in suwari gel from some tropical fish

Samples	Tyrosine (μmol/10 g suwari gel)	
	Setting at 25°C	Setting at 40°C
Threadfin bream surimi	1.74±0.01[a]d[b]	2.90±0.01d
Bigeye snapper surimi	1.15±0.01c	2.72±0.01c
Barracuda surimi	0.81±0.00b	1.22±0.01a
Bigeye croaker surimi	0.52±0.00a	1.95±0.01b

[a]Mean ± standard deviation from triplicate determinations

[b]Different letters in the same column denote the significant differences ($p<0.05$).

Protein pattern in suwari and ashi gel of surimi from some tropical fish

Protein patterns of suwari gels prepared at medium and high temperature setting are depicted in Figure 17 and 18. It was found that myosin band was much more decreased with high temperature setting. However, the rate of decrease was dependent upon fish species. This result indicated that high temperature setting was more efficient in protein cross-linking than medium temperature setting. Nevertheless, some lower molecular weight peptides or proteins were observed, particularly in suwari prepared at 40°C. This suggested that some degradation possibly occurred during high temperature setting.

The cross-linking formed during setting was dependent on fish species, suggesting the differences in TGase activity as well as different protein conformation, which could align in different fashion. At both setting temperatures, surimi from bigeye snapper and barracuda were found to undergo cross-link most effectively, as observed by the lowest myosin band remained. For threadfin bream surimi, the degradation protein bands were found to a highest extent with 40°C setting, compared to other surimi. This results was coincidental with gel strength and solubility result. Therefore, the temperature and time of setting should be maximized to reduce the degradation but to maximize the cross-linking.

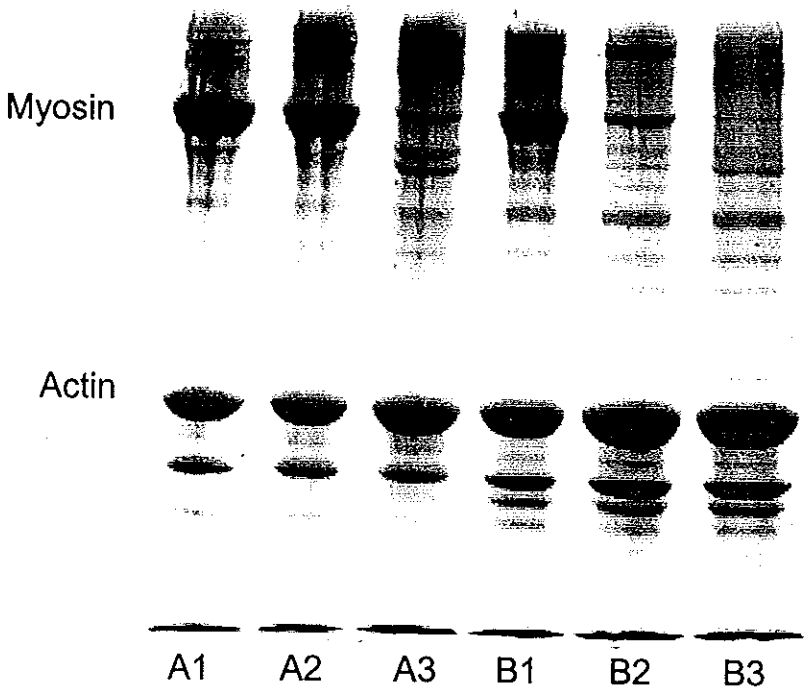

Figure 17. SDS-PAGE protein pattern of suwari gel from bigeye snapper and bigeye croaker prepared under optimum conditions. A1, A2, A3; suwari gel from bigeye croaker surimi, no setting, setting at 25°C and setting at 40°C, respectively. B1, B2, B3; suwari gel from bigeye snapper surimi, no setting, setting at 25°C and setting at 40°C, respectively.

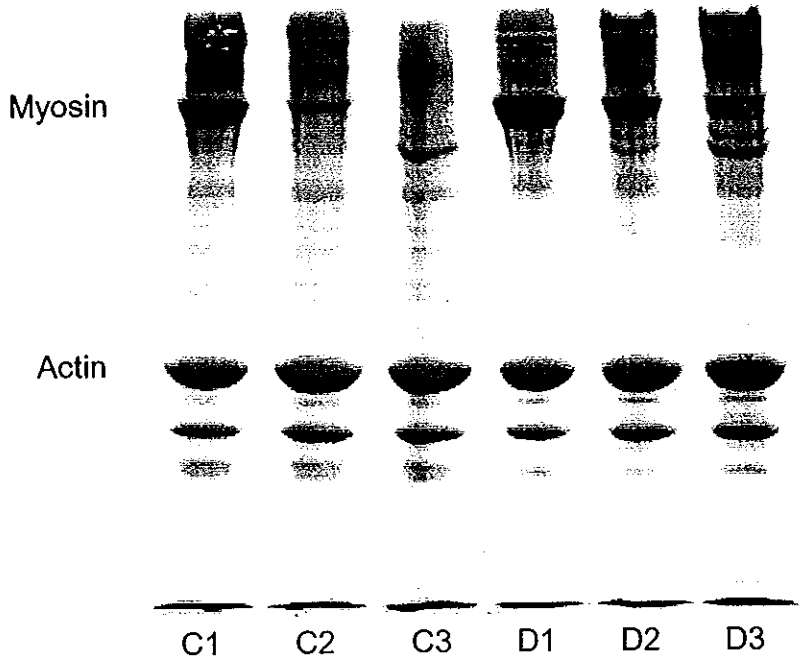

Figure 18. SDS-PAGE protein pattern of suwari gel from barracuda and threadfin bream prepared under optimum conditions. C1, C2, C3; suwari gel from barracuda surimi, no setting, setting at 25°C and setting at 40°C, respectively. D1, D2, D3; suwari gel from threadfin bream surimi, no setting, setting at 25°C and setting at 40°C, respectively.

Transglutaminase activity in surimi from some tropical fish

TGase activity in surimi from different fish was measured (Table 11). It was found that bigeye snapper surimi had the highest activity (15.95 munit/g), while barracuda surimi contained the lowest activity. Though some TGase was removed during washing, it was still remained in the surimi and functioned as gel enhancer via inducing non-disulfide covalent bond. Araki and Seki (1993) reported that surimi from walleye pollack contained a lower TGase activity than fish muscle.

Table 11. Endogenous transglutaminase activities of surimi from some tropical fish.

Samples	TGase activity (munits/g)
Threadfin bream surimi	14.69±0.01[a] c[b]
Bigeye snapper surimi	15.95±0.08d
Barracuda surimi	7.97±0.03a
Bigeye croaker surimi	12.40±0.18b

[a]Mean ± standard deviation from triplicate determinations

[b]Different letters in the same column denote the significant differences ($p<0.05$).

Since bigeye snapper surimi rendered higher gel-forming ability, compared to surimi from other fish as well as showed high transglutaminase activity, it was used for further study.

Microstructure of different gels from bigeye snapper surimi

The microstructure of protein gels from bigeye snapper surimi prepared with different conditions including setting at 40°C (suwari gel), setting at 40°C and followed by heating at 90°C for 20 min (ashi gel) and directly heated gel (90°C for 20 min) without setting were visualized by scanning electron microscopy as shown in Figure 19, 20 and 21, respectively. All gels had a three-dimensional network with different microstructure. The ashi gel had the finest and odered fibrillar structure. Consequently, it was suggested that this network structure was formed through intermolecular ε-(γ-glutamyl) lysine cross-links induced by TGase in co-operation with protein aggregation via hydrophobic interaction, disulfide bond and/or other interactions during heating process. Since directly heated gel had a coarser fibrillar structure than ashi gel, ε-(γ-glutamyl) lysine cross-links formed during setting would contribute to improving a gel network structure.

When surimi sol was directly cooked without prior setting, although the ordered fibrillar structure was lacking, some fibrillar structures were observed. The rate of intermolecular disulfide bonds and hydrophobic interactions involved in gel formation at cooking temperature had been modified after prolonged cooking (Alvarez *et al.*, 1999a).

For suwari gel, protein aggregation with slightly a courser network was observed, compared to ashi gel. Less three-dimensional structures, typical for gels with good texture characteristics, were found, compared to ashi gel. Although some fibrillar structures were formed when setting was prolonged, additional alignment of protein molecules needed to take place to form a strong network. However, more ordered network in suwari gel was noted, compared to directly heated gel.

Protein-protein bonds of varying heat stability are formed at different rates among the major proteins in set gels made in different time or

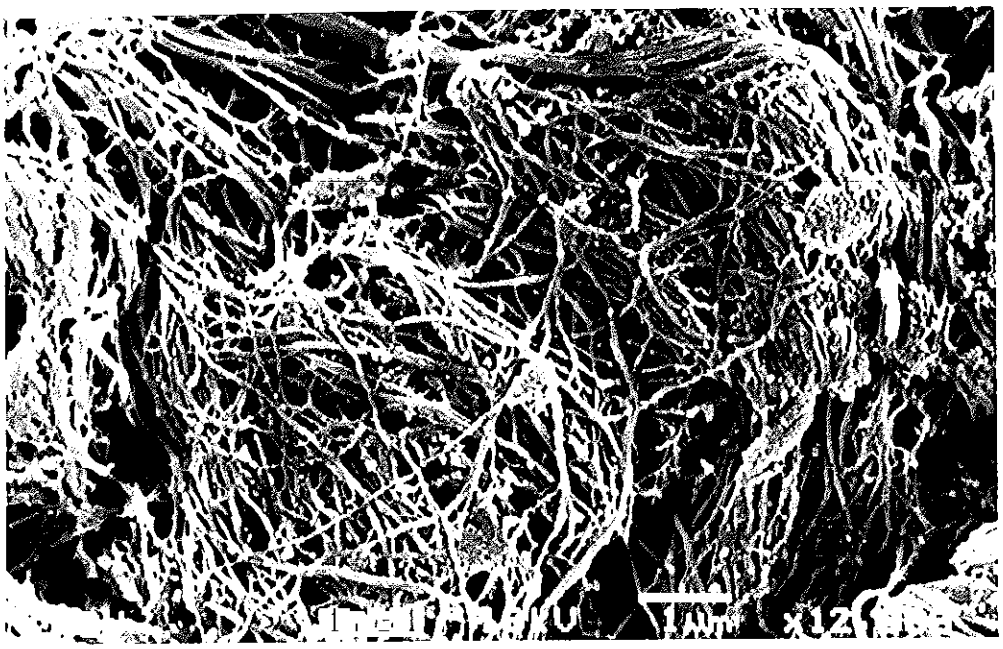

Figure 19. Microstructure of suwari gel from bigeye snapper

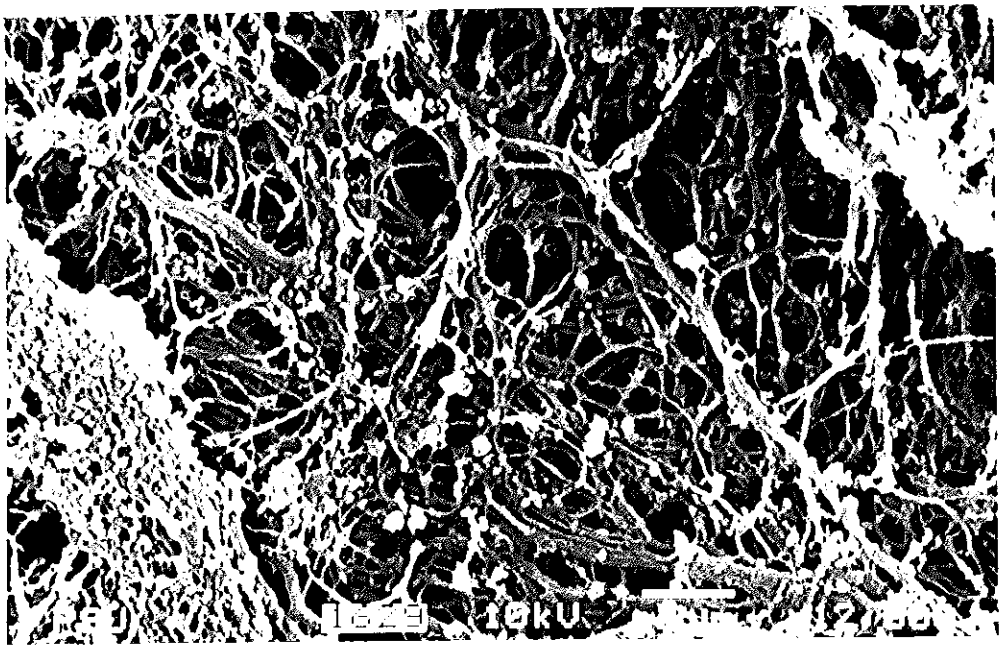

Figure 20. Microstructure of ashi gel from bigeye snapper

temperature conditions. This could explain the possibility of formation of different types of networks during cooking at higher temperatures (Alvarez *et al.*, 1999a).

Generally, ashi gel possessed the finest fibrillar structure, which can imbibe more water, leading to a strongest gel network. This coincided with highest gel strength as well as the lowest solubility

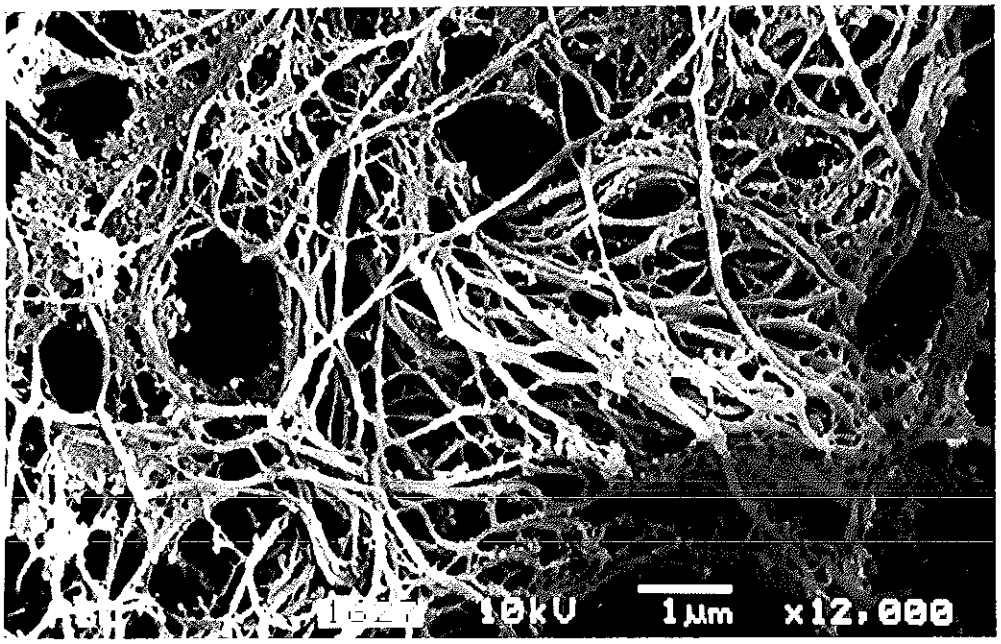

Figure 21. Microstructure of directly heated gel from bigeye snapper

3. Effect of some chemicals on setting phenomenon

3.1 Effect of calcium ion

It has been known that endogenous TGase is a Ca^{2+} dependent enzyme, which catalyzes the intra- and intermolecular cross-linking of certain proteins by γ-glutamyl-ε-lysine side chain bridges (Kimura *et al.*, 1991; Wan *et al.*, 1994; Zhu *et al.*, 1995). The effect of $CaCl_2$ on suwari gel strength is depicted in Figure 22. Gel force increased ($p<0.05$) when $CaCl_2$ concentration

increased, suggestion the presence of Ca^{2+} dependent TGase. However, no changes in deformation were observed even at a high concentration of $CaCl_2$. This result was in agreement with Saeki *et al.* (1995) who reported that gel formation of white croaker surimi was accelerated with an increase in Ca^{2+} content. Kimura *et al.* (1991) also reported that Ca^{2+} was essential for the cross-linking reaction of myosin heavy chain and formation of suwari and kamaboko gel. Although TGase requires Ca^{2+} for expression of enzymatic activity, Ca^{2+} requirement could be partially or fully filled with other cations (Yasueda *et al.*, 1994).

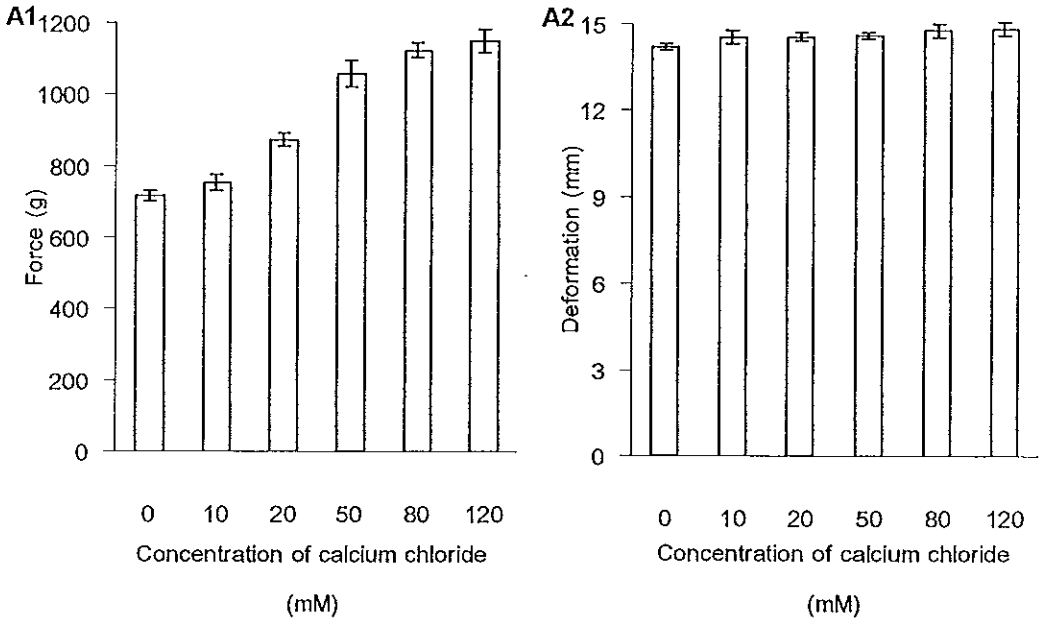

Figure 22. Force (A1) and deformation (A2) of bigeye snapper suwari gel added with various $CaCl_2$ concentrations and set at 40°C for 2 h.

Table 12 shows the solubility of suwari gel from bigeye snapper added with various concentration of $CaCl_2$. It was found that solubility decreased when $CaCl_2$ concentration increased ($p<0.05$). The result was coincidental with an increase in breaking force. This result reconfirmed the role of TGase in protein cross-linking during setting. ε-(γ-glutamyl) lysine was presumed to be formed to a higher content when higher Ca^{2+} concentration was added, resulting in the lower solubility in solution containing β-ME, urea and SDS.

Table 12. Solubility of bigeye snapper suwari gel added with various $CaCl_2$ concentrations.

Concentration of $CaCl_2$ (mM)	Solubility (%)*
Control	100e[b]
0	67.71±1.81[a]d
10	66.67±1.80cd
20	64.34±1.53c
50	60.94±1.56b
80	59.13±0.43ab
120	57.45±1.20a

Control : solubility in 0.5 N NaOH

[a]Mean ± standard deviation from triplicate determinations.

[b]The different superscripts in the same column denote the significant differences ($p<0.05$).

*Solubility in sovent mixtures (20 mM Tris-HCl containing 1% SDS, 8 M urea and 2% β-ME, pH 8.0)

The cross-linking reaction of myosin heavy chain was accelerated and the larger cross-linked myosin heavy chains were formed as the Ca^{2+} content increased (Saeki, 1996; Autio *et al.*, 1985).

Electrophoretic pattern of suwari gel added with different concentration of $CaCl_2$ is shown in Figure 23. At high temperature setting, myosin band decreased markedly when a higher concentration of $CaCl_2$ was used. SDS-PAGE analysis revealed that more polymerization of myosin was found as higher concentration of $CaCl_2$ was added. No myosin band was observed in the gel added with higher Ca^{2+} concentration. Therefore, Ca^{2+} induced the enzymatic polymerization between the myosin heavy chain. In addition, actin band slightly decreased when a higher concentration of $CaCl_2$ was added. The result suggested that myosin heavy chain was more susceptible to cross-linking induced by endogenous TGase, compared to actin. However, actin could also be used as a substrate for TGase. Kimura *et al.* (1991) reported that the amount of myosin heavy chain in mixture of myosin B and crude suwari-promoting enzyme decreased and the formation of high-molecular weight components, which could not enter the SDS-PAGE gels, increased with increasing Ca^{2+} concentrations.

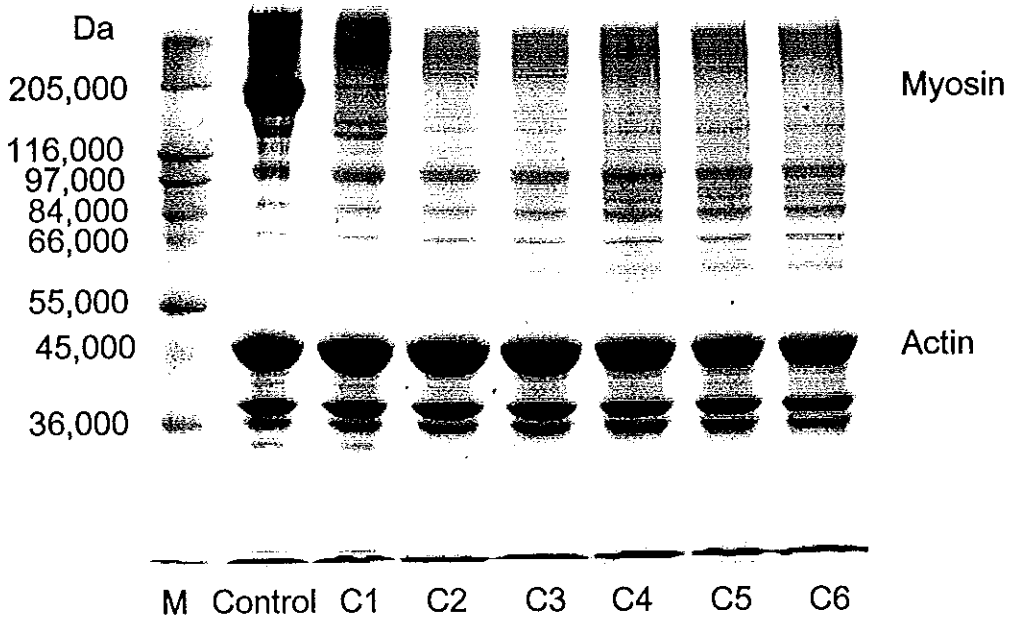

Figure 23. SDS-PAGE pattern of protein in bigeye snapper suwari gel added with various $CaCl_2$ concentrations and set at 40°C for 2 h. M; high molecular weight standard, Control; no setting and no $CaCl_2$ addition, C_1; 0, C_2; 10, C_3; 20, C_4; 50, C_5; 80, C_6; 120 mM $CaCl_2$

3.2 Effect of inhibitors

- NEM

Breaking force and deformation of suwari decreased with addition of NEM (Figure 24). Continuous decrease in breaking force and deformation was found when NEM was added up to 5 mM and sharp decrease was observed with a NEM concentration of 10 mM. Presumably, the decrease in the breaking force and deformation upon the addition of NEM would also be induced by either inhibiting of endogenous TGase or restricting the formation of the SS bonds. NEM is known as SH blocking agent. NEM could effectively inactivate TGase via blocking SH group in the TGase active site. Also, disulfide bond formation may be retarded. This led to a decrease in gel-forming ability of surimi in presence of NEM. The result was in agreement with Nowsad *et al.* (1994) who reported that the elasticity of the suwari gel with NEM added decreased, particularly with a higher concentration of NEM.

Solubility of suwari gel increased when high concentration of NEM was added ($p<0.05$) (Table 13). This was probably due to the inhibition of endogenous TGase by NEM, leading to a lower non-disulfide covalent bond formation. From the result, it indicated that NEM should be more influential in TGase inhibiting rather than prevention of disulfide bond formation. This was because non-disulfide covalent bonds formation was impeded at a higher concentration of NEM, as appeared by a higher solubility in solution containing β-ME, urea and SDS.

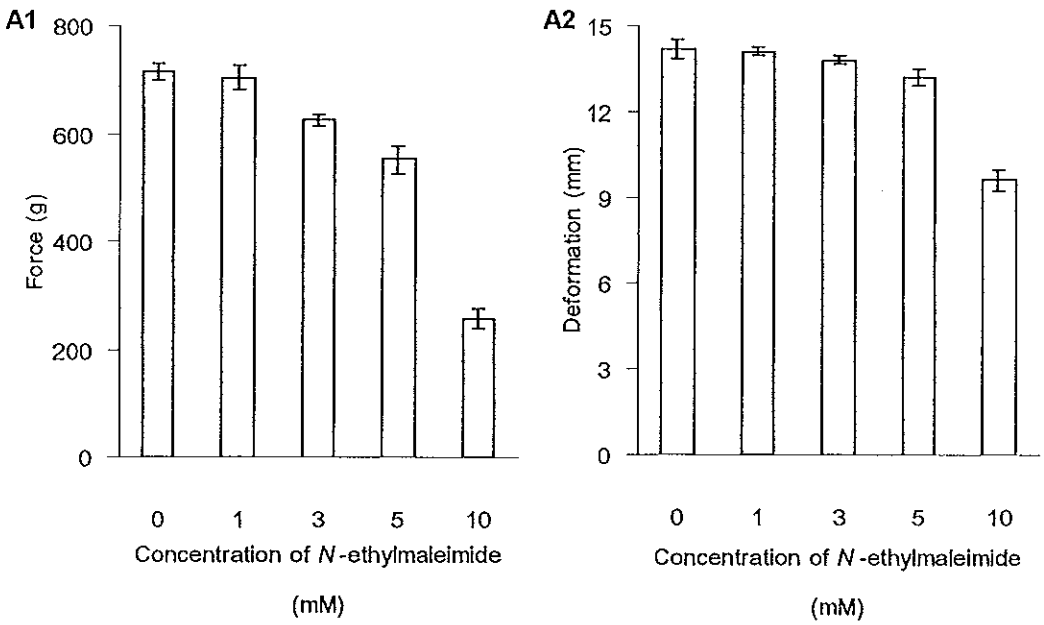

Figure 24. Force (A1) and deformation (A2) of bigeye snapper suwari gel added with various *N*-ethylmaleimide concentrations and set at 40°C for 2 h.

Table 13. Solubility of bigeye snapper suwari gel added with various *N*-ethylmaleimide concentrations.

Concentration of *N*-ethylmaleimide (mM)	Solubility (%)*
Control	100e[b]
0	67.71±1.81[a]a
1	70.30±1.56a
3	81.25±3.12b
5	86.08±1.58c
10	90.63±3.12d

Control: solubility in 0.5 N NaOH

[a]Mean ± standard deviation from triplicate determinations.

[b]The different letters in the same column denote the significant differences ($p<0.05$).

*Solubility in sovent mixtures (20 mM tris-HCl containing 1% SDS, 8 M urea and 2% β-ME, pH 8.0)

SDS-PAGE analysis (Figure 25) showed that myosin heavy chain band increased as NEM concentration increased, whereas no myosin band remained in suwari gel without NEM. At 10 mM NEM, more myosin heavy chain were still not polymerized. This was presumed to be due to the lower TGase activity available for inducing cross-linking. This result was in agreement with Nowsad *et al.* (1994) who reported that the polymerization of myosin heavy chain was suppressed with addition of NEM.

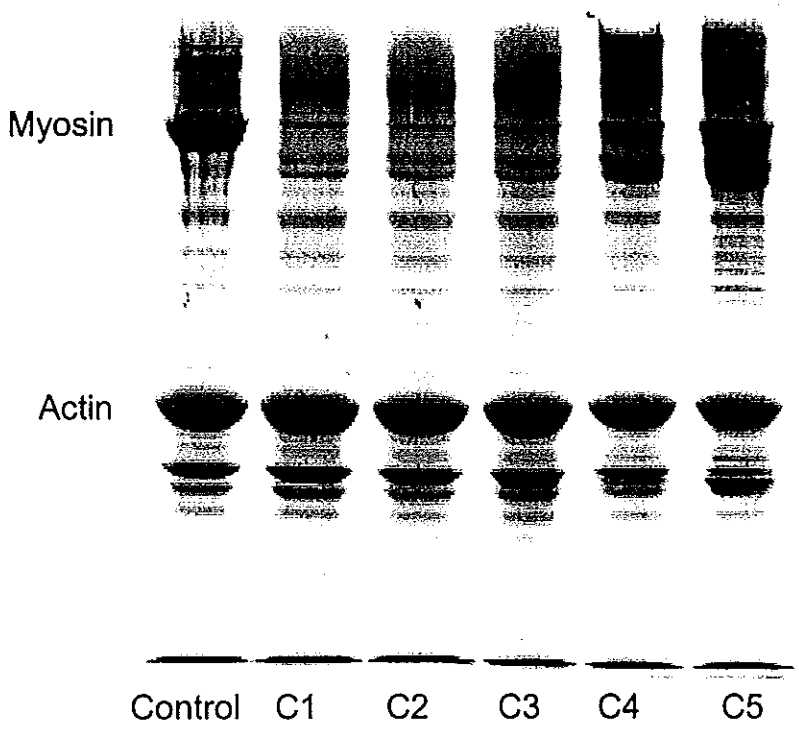

Figure 25. SDS-PAGE pattern of protein in bigeye snapper suwari gel added with various NEM concentrations. Control; no setting and no NEM addition, C_1; 0, C_2; 1, C_3; 3, C_4; 5, C_5; 10 mM NEM

- NH_4Cl

The Figure 26 shows force and deformation of suwari gel added with various concentrations of NH_4Cl. It was found that breaking force and deformation decreased when NH_4Cl concentration increased ($p<0.05$). This result was in accordance with Nowsad *et al.* (1993) who reported that the breaking force and breaking strain tended to decrease with the increase of the mole fraction of NH_4Cl and various amine salts added. This result reconfirmed the role of endogenous TGase in gel setting.

The setting of surimi paste was suppressed by the addition of ammonium salt (0.1-1.0 M NH_4Cl) which is known as an inhibitor of TGase (Wan *et al.*, 1992; Takagi *et al.*, 1986). Effect of NH_4^+ on depression of the reactions of TGase-mediated protein cross-linking and amine incorporation was reported (Wan *et al.*, 1992). Additionally, the elasticity of protein hydrogel was also affected by the sort of ions added. Those ions influence protein-protein interactions through altering the sterio-structure of water (Shimada and Matsushita, 1981).

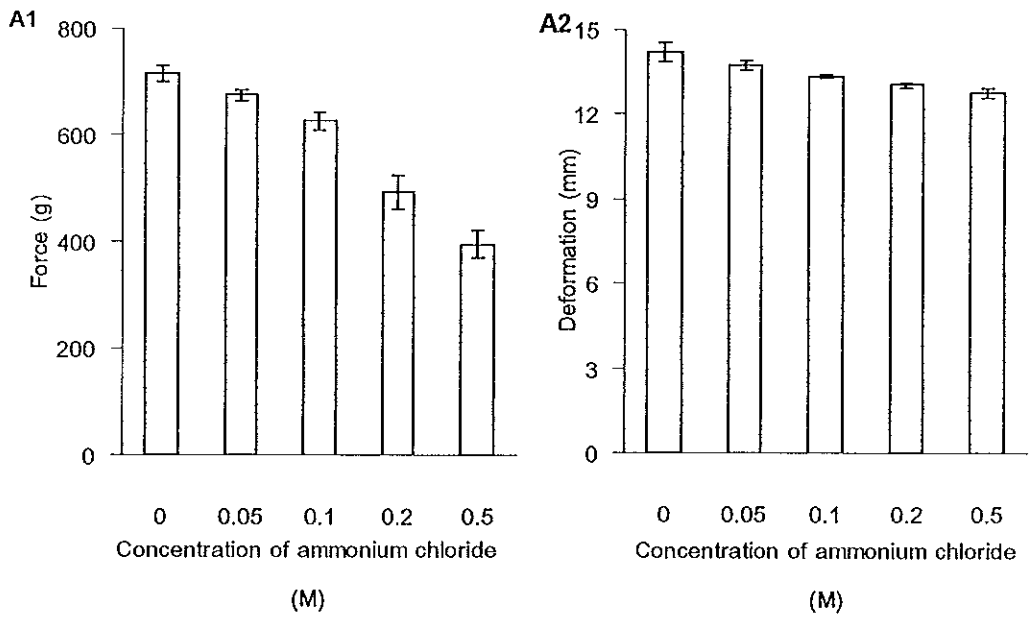

Figure 26. Force (A1) and deformation (A2) of bigeye snapper suwari gel added with various NH_4Cl concentrations and set at 40°C for 2 h.

Solubility of suwari gel increased, when NH_4Cl concentration increased (Table 14). This result suggested that a lower amount of ε-(γ-glutamyl) lysine was formed as NH_4Cl concentration increased. This possibly resulted from a lower activity of TGase in a presence of NH_4Cl, TGase inhibitor.

Table 14. Solubility of bigeye snapper suwari gel added with various NH_4Cl concentrations.

Concentration of NH_4Cl (M)	Solubility (%)*
Control	100e[b]
0	67.71±1.81[a]a
0.05	76.69±1.58b
0.1	88.90±1.59c
0.2	91.15±0.90c
0.5	93.75±1.56d

Control: solubility in 0.5 N NaOH

[a]Mean ± standard deviation from triplicate determinations.

[b]The different letters in the same column denote the significant differences ($p<0.05$).

*Solubility in sovent mixtures (20 mM Tris-HCl containing 1% SDS, 8 M urea and 2% β-ME, pH 8.0)

SDS-PAGE (Figure 27) was performed to determine the protein composition of suwari gel added with various NH_4Cl concentrations. Myosin heavy chain band increased as NH_4Cl increased. NH_4^+ is a product from cross-linking reaction induced by TGase (Park, 2000). A higher amount of NH_4Cl acted as a responding inhibitor of TGase. This has been considered to

be conclusive evidence that the formation of cross-linked myosin heavy chain was catalyzed by the TGase.

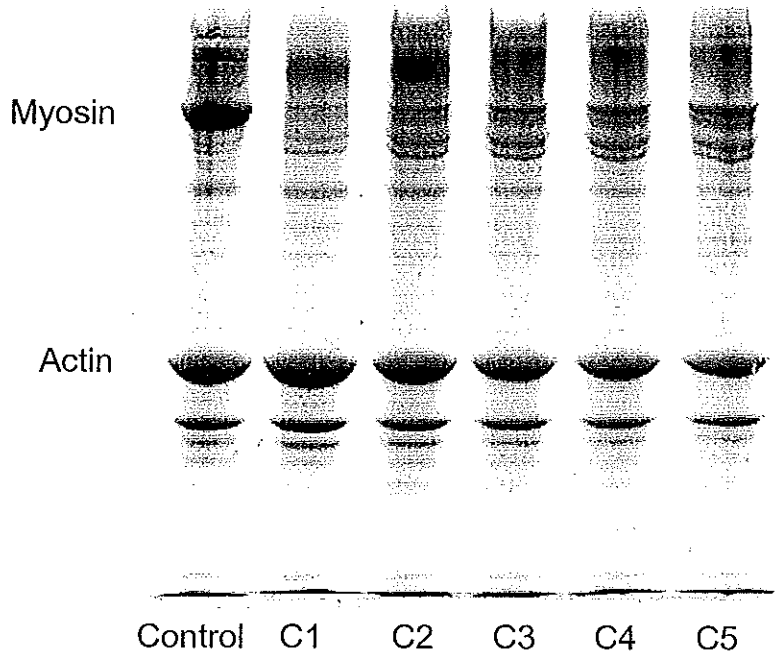

Figure 27. SDS-PAGE pattern of protein in bigeye snapper suwari gel added with various NH_4Cl concentrations. Control; no setting and no NH_4Cl addition, C_1; 0, C_2; 0.05, C_3; 0.1, C_4; 0.2, C_5; 0.5 mM NH_4Cl

- EDTA

The endogenous TGase requires Ca^{2+} to be activated, and thus can be inhibited with EDTA (Gilleland *et al.*, 1997; Kumazawa *et al.*, 1995). In this study, EDTA was found to decrease the breaking force and deformation, particularly when concentration increased (Figure 28). In presence of high amount of EDTA, Ca^{2+} was not available for TGase activation, As a result, non-disulfide covalent bond formation was reduced, leading to a low breaking force and deformation. This result revealed the role of endogenous TGase in gel setting and was in agreement with Kumazawa *et al.* (1995) who found that

the surimi gel strength was completely inhibited in the presence of 5 mM EDTA.

The solubility of suwari gel from bigeye snapper surimi added with various concentrations of EDTA is shown in Table 15. It was found that the solubility increased when EDTA concentration increased. The solubility of suwari gel with 5 mM EDTA was 84.38%, while solubility of suwari gel without EDTA was 67.71%. These results suggested that lower amount of ε-(γ-glutamyl) lysine was formed in presence of EDTA, which was possibly associated with a lower activity of endogenous TGase. SDS-PAGE revealed that myosin heavy chain in suwari gel increased as EDTA concentration increased (Figure 29). EDTA is a chelating agent which can bind with Ca^{2+}, resulting in a less available Ca^{2+} for activation of TGase. Generally, EDTA had inhibitory effects on myosin heavy chain cross-linking (Jiang *et al.*, 1992; Tsai *et al.*, 1996a; Gilleland *et al.*, 1997).

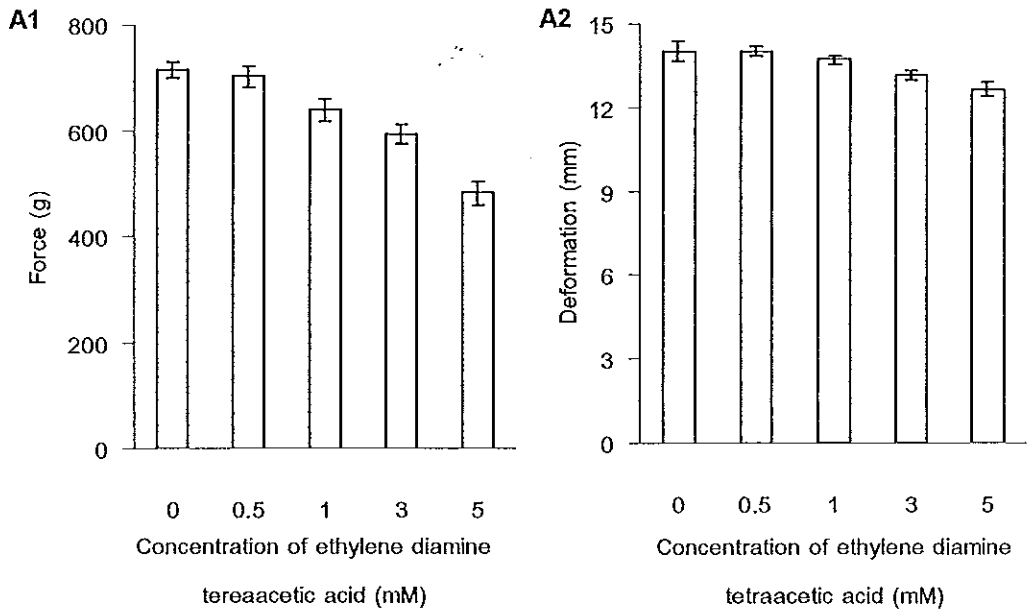

Figure 28. Force (A1) and deformation (A2) of bigeye snapper suwari gel added with various EDTA concentrations and set at 40°C for 2 h.

Table 15. Solubility of bigeye snapper suwari gel added with various EDTA concentrations.

Concentration of EDTA (mM)	Solubility (%)*
Control	100e[b]
0	67.71±1.81[a]a
0.5	70.84±1.81b
1	75.65±1.12c
3	78.59±0.80c
5	84.38±3.12d

Control: solubility in 0.5 N NaOH

[a]Mean ± standard deviation from triplicate determinations.

[b]The different superscripts in the same column denote the significant differences ($p<0.05$).

*Solubility in sovent mixtures (20 mM Tris-HCl containing 1% SDS, 8 M urea and 2% β-ME, pH 8.0)

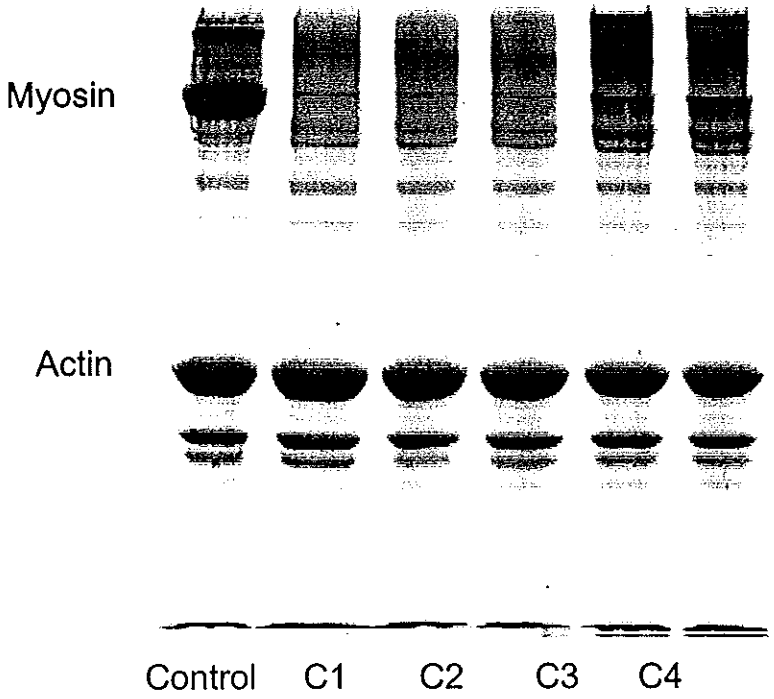

Figure 29. SDS-PAGE pattern of protein in bigeye snapper suwari gel added with various EDTA concentrations. Control; no setting and no EDTA addition, C_1; 0, C_2; 0.5, C_3; 1.0, C_4; 3.0, C_5; 5.0 mM EDTA

3.3 Effect of polyphosphate

Sodium hexametaphosphate and penta-sodiumtriphosphate were found to decrease breaking force and deformation of suwari gel ($p<0.05$) (Figure 30 and Figure 31). Polyphosphate possibly acted as an chelator for Ca^{2+}, leading to a decrease in TGase activity. Polyphosphates are commonly added in the form of a pyrophosphate (or diphosphate) compound and in a more polymerized form (tripolyphosphate or hexametaphosphate). The latter two compounds act as chelating agents for divalent heavy metals (Han-Ching and Leinot, 1993). Therefore, the cross-linking induced by Ca^{2+}-dependent

TGase was reduced a presence of polyphosphate. As a result, gel strength was decreased, particularly at a higher amount of phosphate. Solubility of suwari gel increased when sodium hexametaphosphate and penta-sodiumtriphosphate concentration increased (Table 16), suggesting a lower non disulfide covalent bonds were formed. With addition of sodium hexametaphosphate or penta-sodiumtriphosphate, myosin heavy chain in suwari gel slightly increased (Figure 32 and 33). The results revealed that myosin heavy chain underwent polymerization to a lower extent in a presence of phosphate compounds. Therefore, excess calcium salt should be added to compensate the Ca^{2+}, which is chelated with phosphates.

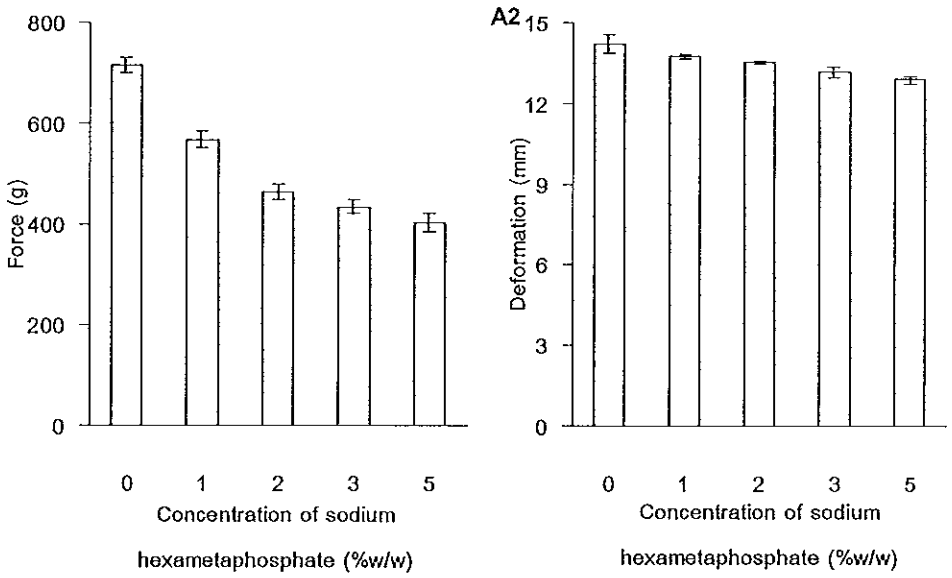

Figure 30. Force (A1) and deformation (A2) of bigeye snapper suwari gel added with various sodium hexametaphosphate concentrations and set at 40°C for 2 h.

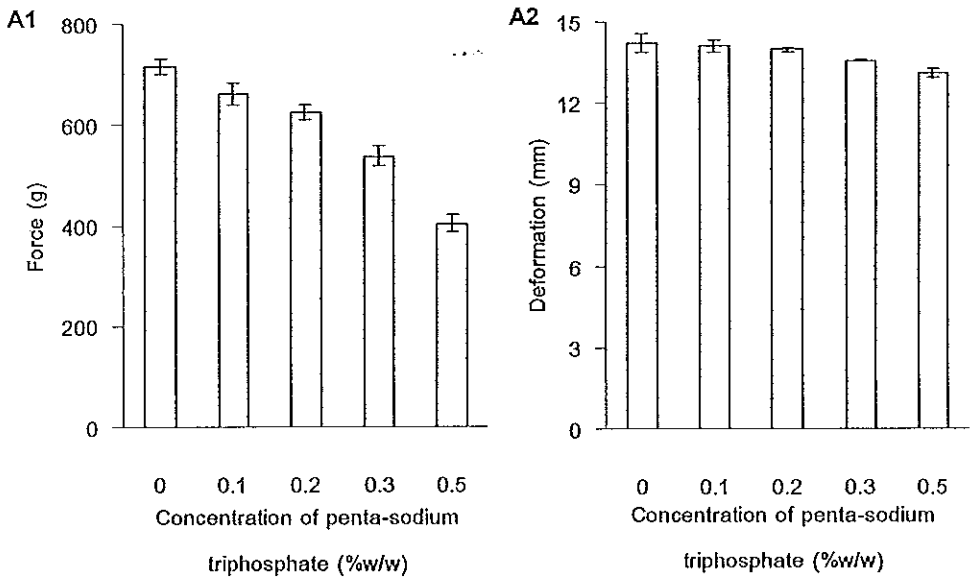

Figure 31. Force (A1) and deformation (A2) of bigeye snapper suwari gel added with various penta-sodiumtriphosphate concentrations and set 40°C for 2 h.

Table 16. Solubility of bigeye snapper suwari gel added with polyphosphate at different concentrations.

Concentration (% w/w)	Solubility (%)	
	Sodium hexametaphosphate	Penta sodium triphosphate
Control	100e[b]	100f[b]
0	67.71±1.81[a]a	67.71±1.81[a]a
0.1	77.09±1.81b	82.29±1.81b
0.2	89.59±1.81c	85.42±1.80c
0.3	91.15±0.90c	91.67±1.80d
0.5	94.27±0.90d	94.79±3.12e

Control: solubility in 0.5 N NaOH

[a]Mean ± standard deviation from triplicate determinations.

[b]The different superscripts in the same column denote the significant differences ($p<0.05$).

*Solubility in sovent mixtures (20 mM Tris-HCl containing 1% SDS, 8 M urea and 2% β-ME, pH 8.0)

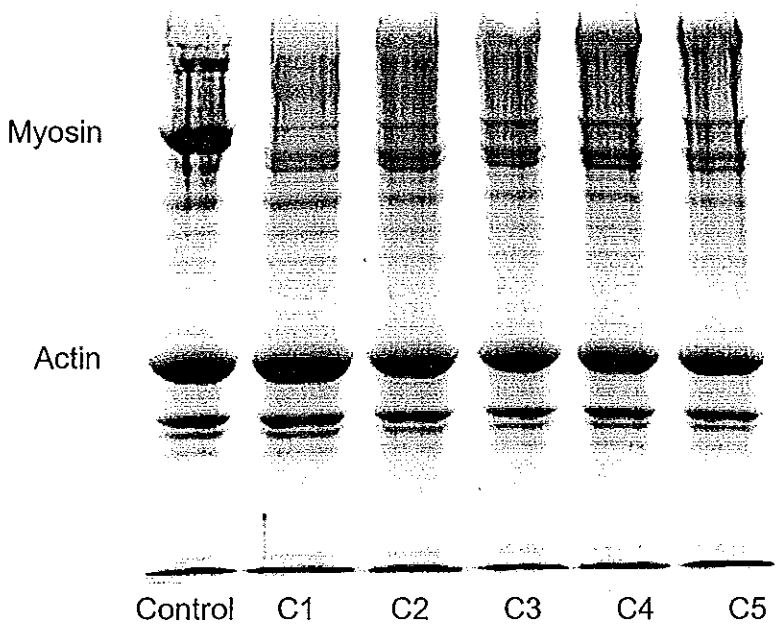

Figure 32. SDS-PAGE pattern of protein in bigeye snapper suwari gel added with various sodium hexametaphosphate concentrations. Control; no setting and no sodium hexametaphosphate addition, C_1; 0, C_2; 0.1, C_3; 0.2, C_4; 0.3, C_5; 0.5 (% w/w)

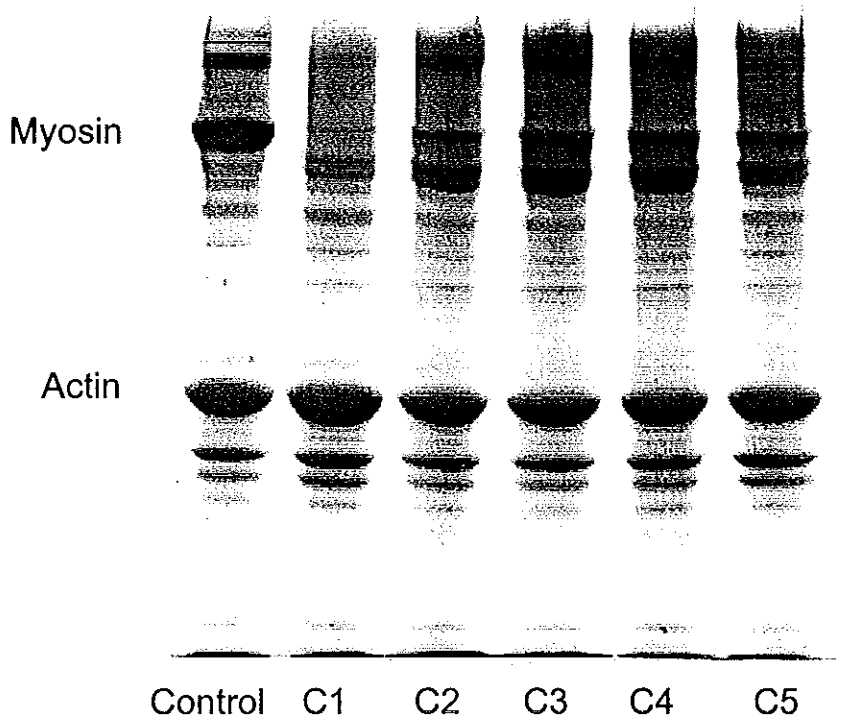

Figure 33. SDS-PAGE pattern of protein in bigeye snapper suwari gel added with various penta-sodiumtriphosphate concentrations. Control; no setting and no penta-sodiumtriphosphate addition, C_1; 0, C_2; 0.1, C_3;0.2, C_4; 0.3, C_5; 0.5 (% w/w)

4. Characterization of endogenous transglutaminase

4.1 pH profile of bigeye snapper muscle transglutaminase

The effect of various pHs (2, 3, 4, 5, 6, 7, 8, 9 and 10) on TGase activity was studied. The highest activity was observed at pH 6.0 (Figure 34). The marked decrease in activity was found in acidic and alkali pH ranges. This result suggested that the bigeye snapper muscle TGase exhibited high activity in the neutral pH ranges, which and the common pH of surimi (Table 6). However, Kumazawa *et al.* (1996) reported that the optimum pH of TGase from walleye pollack liver was 9.0. The optimum pH for purified TGase from *Streptoverticillium ladakanum*was was 5.0-7.5 (Tsai *et al.*, 1990; Ho *et al.*, 2000). Yasueda *et al.* (1994) reported that the maximum activity of tissue-type TGase from red sea bream (*Pagrus major*) was between pH 9.0 and 9.5 at 37°C. Thus, this enzyme had a more basic optimum pH than guinea pig TGase, which had its maximum activity at pH 8.0 under the same assay conditions (Yasueda *et al.*, 1994). The differences in optimum pH of TGase from different sources were probably due to the differences in conformation of individual enzyme at different pHs. As a consequence, pH which rendered the highest activity varied among different sources.

4.2 Temperature profile of bigeye snapper muscle transglutaminase

Optimal temperature of bigeye snapper muscle TGase was found to be 40°C at pH 6.0 (Figure 35). The activity increased as temperature increased and reached a maximum at 40°C. The activity sharply decreased at temperature higher than 40°C. The loss in activity at high temperature was caused by the thermal denaturation. Therefore, temperature was critical to maximize the setting phenomenon. Temperature of 40°C used in this study was coincidental with optimum temperature for endogenous TGase. As a

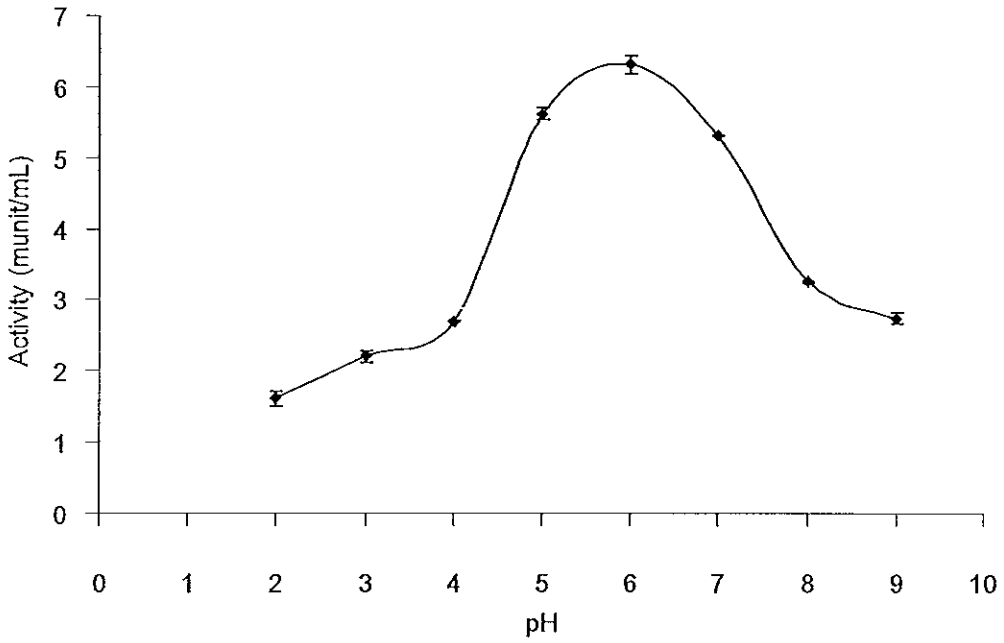

Figure 34. pH profile of bigeye snapper muscle TGase

result, TGase could exhibit full activity at 40°C and cross-linking could be formed effectively.

The optimal temperature of tissue-type TGase from red sea bream was 55°C at pH 7.5 (Yasueda *et al.*, 1994). Kumazawa *et al.* (1996) reported that the activity of purified TGase from walleye pollack liver was maximal at around 50°C.

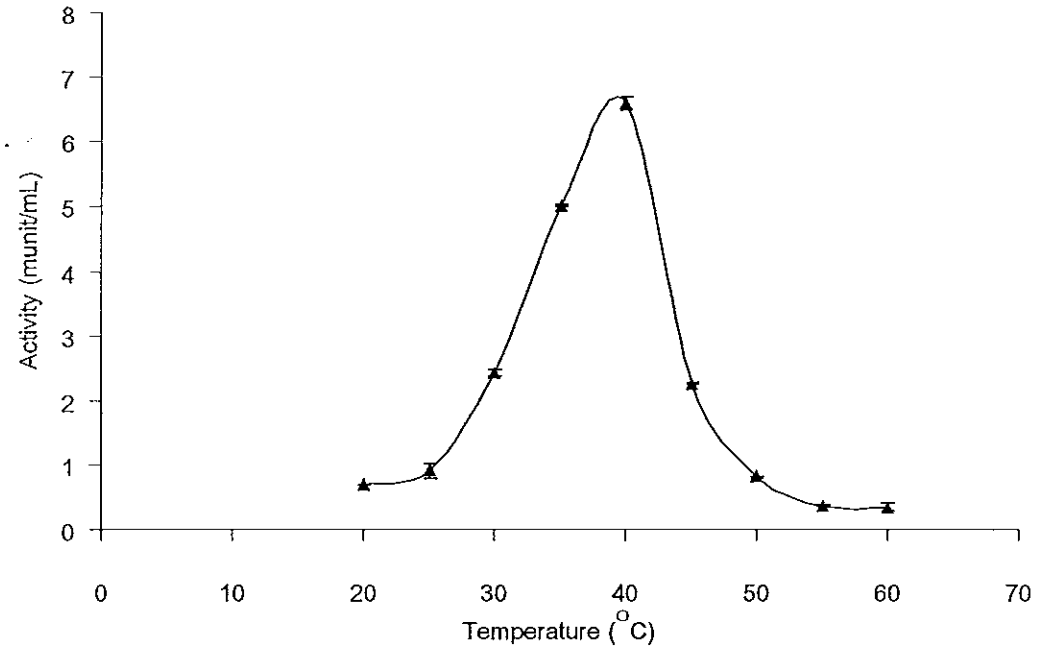

Figure 35. Temperature profile of bigeye snapper muscle TGase

4.3 Effect of some chemicals on bigeye snapper muscle transglutaminase activity

Effect of $CaCl_2$ on bigeye snapper muscle TGase activity

The extracted TGase was activated by Ca^{2+} (Table 17). TGases from animal sources are generally calcium dependent (De Baker-Royer *et al.*, 1992; Ho *et al.*, 2000). The calcium ion was considered to induce the conformation change, which consequently exposed the substrate to cysteine residue located in the active site (Ashie and Lanier, 2000). Bigeye snapper muscle TGase was more activated with an increase in the Ca^{2+} concentration and fully activated with a Ca^{2+} concentration above 5 mM. From the result, it can be inferred that Ca^{2+} functioned as TGase activator even at a low concentration (1 mM).

Table 17. Effect of $CaCl_2$ on bigeye snapper muscle TGase activity

Concentration (mM)	Relative activity (%)
0	100a[b]
1	206.65±3.47[a]b
3	213.11±1.58c
5	225.52±0.89d
8	224.12±2.50d

[a]Mean ± standard deviation from triplicate determinations.

[b]Different letters in the same column denote the significant differences ($p<0.05$).

Effect of NaCl on bigeye snapper muscle TGase acitvity

Table 18 shows the effect of NaCl on activity of TGase from bigeye snapper muscle. No changes in activity were found when NaCl was added up to 0.4 M. However, a non significant decrease in activity was observed at a high concentration (0.6 M). Therefore, TGase activity still remained in the surimi sol with NaCl at a level of approximatly 0.45 M, which isvcommonly used for gel preparation. This result was in agreement with Kishi *et al.* (1991) who reported that no effect of NaCl on TGase activity was obtained when NaCl was added up to 1 M.

Effect of some inhibitors on bigeye snapper muscle TGase activity

NEM exhibited a strong inhibitory effect on TGase activity as shown in Table 19. When 0.5 mM NEM was added, the activity was reduced to 54.05 %. At 3 mM NEM, the relative activity was 38.69 %. TGase is a sulfhydryl enzyme with a seemingly conserved pentapeptide (Tyr-Gly-Gln-Gys-Trp) active site sequence and is readily inactivated by sulfhdryl reagents, which

alkylate free sulfhydryl groups (Klein *et al.*, 1992). This result was in accordance with the decrease in breaking force in the presence of NEM (Figure 24).

Table 18. Effect of NaCl on bigeye snapper muscle TGase activity

Concentration of NaCl (M)	Relative activity (%)
0	100b[b]
0.1	99.77±2.20[a]ab
0.2	100.09±2.73b
0.4	99.70±0.23ab
0.6	96.82±0.26a

[a]Mean ± standard deviation from triplicate determinations.

[b]Different letters in the same column denote the significant differences (p<0.05).

Table 19. Effect of some inhibitors on bigeye snapper muscle TGase activity

Inhibitors	Concentration (mM)	Relative activity (%)
NEM	0	100d[b]
	0.5	54.24±0.29[a]c
	1	46.55±2.97b
	3	38.69±0.57a
NH_4Cl	0	100c[b]
	0.5	76.18±0.48[a]b
	1	72.73±4.76b
	3	66.47±0.88a
EDTA	0	100c[b]
	0.5	41.13±1.36[a]b
	1	26.52±0.90a
	3	25.35±1.16a

[a]Mean ± standard deviation from triplicate determinations.

[b]Different letters in the same column denote the significant differences (p<0.05).

The effect of NH_4Cl at different concentrations on TGase activity is shown in Table 19. At the same concentration, inhibitory activity of NH_4Cl was lower than NEM. Generally, inhibitory activity was higher as the concentration of NH_4Cl increased. This result was in agreement with Nowsad *et al.* (1993) who reported that the activity rapidly decreased with the increase in the mole fraction of ammonium chloride. By the addition of ammonium sulfate, the cross-linking reaction was inhibited in a dose-dependent manner and was completely stopped when the concentration was higher than 5 mM

(Takagi *et al.*, 1986). These results suggested that TGase activity was inhibited by ammonium chloride.

The activity was also inhibited by EDTA (Table 19), High inhibition was observed when high concentration of EDTA was added. EDTA, a chelator, caused a less availability of Ca^{2+}, which was needed for enzyme activation. As a result, TGase activity was reduced. This result reconfirmed the role of Ca^{2+} in endogenous TGase activity. Reduced activity caused by EDTA might lead to a less polymerization of myosin. As shown in Figure 29, more myosin heavy chain was retained with the addition of EDTA. Gilleland *et al.* (1997) reported that EDTA prevented myosin polymerization. These results clearly indicated that bigeye snapper muscle TGase requires Ca^{2+} to fully activate the activity.

Effect of some reducing agents on bigeye snapper muscle transglutaminase activity

The activity of TGase from bigeye snapper increased when β-ME and DTT were added (Table 20). At the same concentration, DTT showed a higher effectiveness in activition of TGase activity. The result clearly indicated that sulfhydryl group in active site was important for TGase activity. This result was coincidental with the decreased activity in presence of sulfhydryl blocking agent such as NEM (Table 19).

Effect of polyphosphate on bigeye snapper muscle transglutaminase activity

Decrease in activity of bigeye snapper muscle TGase was observed with the addition of sodium hexametaphosphate and penta-sodiumtriphosphate (Table 21). It was presumed that phosphate might chelate Ca^{2+}, resulting in the less availability of Ca^{2+}. As a consequence, insufficient

Ca^{2+} could be used for full activation of enzyme activity. Ashie and Lanier (2000) reported that guanosine triphosphate in micromolar concentrations inhibited guinea pig liver TGase. The most likely explanation was its function as a metal chelator (Park, 2000). The result was in accordance with the decreas in breaking force and reduced polymerization of myosin heavy chain in presence of phosphates (Figure 30 and 31).

Table 20. Effect of reducing agents on bigeye snapper muscle TGase activity

Reducing agents	Concentration (mM)	Relative activity (%)
βME	0	100c[b]
	1	102.78±0.67[a]b
	5	122.99±1.18a
	10	123.60±1.18a
DTT	0	100d[b]
	1	144.40±0.90[a]c
	5	156.23±0.63b
	10	161.61±0.97a

[a]Mean ± standard deviation from triplicate determinations.

[b]Different letters in the same column denote the significant differences ($p<0.05$).

Table 21. Effect of polyphosphate on bigeye snapper muscle TGase activity

Concentration (%w/w)	Relative activity (%)	
	Sodium hexametaphosphate	Penta-sodium triphosphate
0	100d[b]	100c
0.1	88.38±0.28[a]c	100.27±1.75c
0.2	56.34±0.51b	81.01±1.08b
0.3	47.17±1.34a	64.53±0.03a

[a]Mean ± standard deviation from triplicate determinations.

[b]Different letters in the same column denote the significant differences (p<0.05).

4.4 Thermal stability

The thermal stability of the bigeye snapper muscle TGase (0.1g/mL) was determined at 30-60°C for 0-60 min (Figure 36). When heated at 30 and 40°C, the activity decreased continuously with an increased heating time. At 50 and 60°C, the activity sharply decreased within 10 min and no activity was found after heating for 30 min. From the result, it indicated that a loss in TGase activity occurred to a higher extent when high temperature setting was applied. However, TGase could be inactivated at medium setting temperature with a lower rate. Therefore, setting temperature should not exceed 40°C to prevent the inactivation of TGase, and to avoid the activation of heat-activated proteinase, commonly active at 50-60°C.

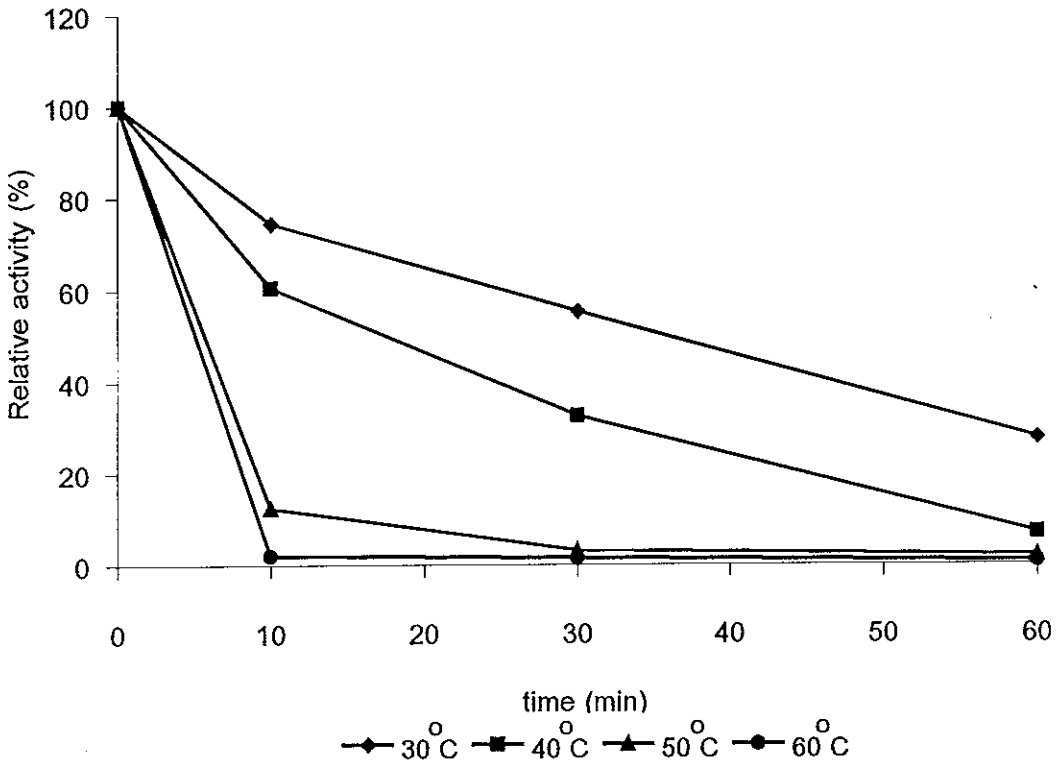

Figure 36. Thermal stability of bigeye snapper muscle

4.5 pH stability

The bigeye snapper muscle TGase was stable at pH ranging from 5.0 to 7.0, in which more than 90% activity was retained. This result suggested that the bigeye snapper muscle TGase was quite resistant to denaturation in neutral pH. TGase was not stable at acidic and alkali pH. This was postulated to be a result of irreversible denaturation of enzyme exposed to those harsh environments.

Kumazawa *et al.* (1996) reported that purified TGase from walleye pollack liver decreased during incubation at 40°C with incubation time.

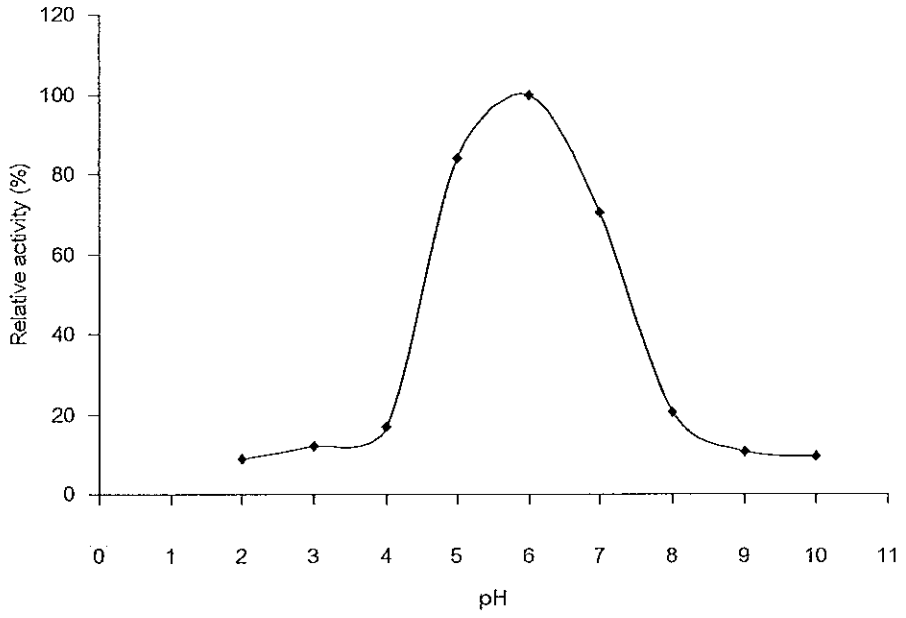

Figure 37. pH stability of TGase from bigeye snapper muscle.

4.6 Cross-linking of actomyosin

The effect of TGase on MDC-incorporation of actomyosin and *N,N'*-dimethylated casein was investigated (Figure 38). The result showed that the increased TGase activity was observed with an increasing concentration of bigeye snapper muscle extract. From the result, the rate of MDC-incorporation into *N,N'*-dimethylated casein induced by TGase was higher than that into actomyosin. These results provided strong indication that the rate of TGase-mediated cross-linking depended upon the substrate specificity. Generally, acyl transfer reaction was TGase concentration dependent manner regardless of substracte (Ashie and Lanier, 2000).

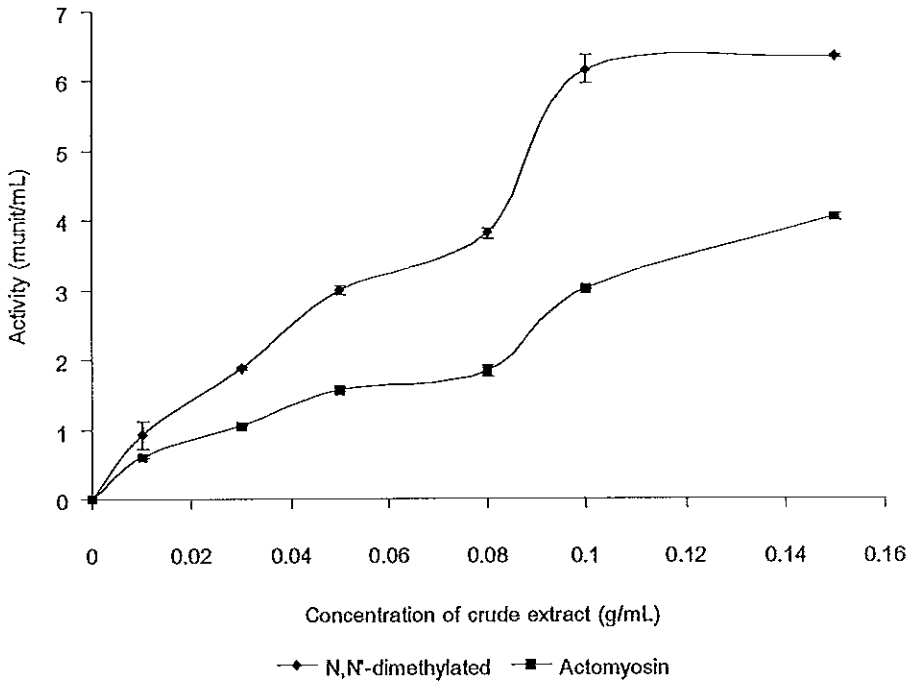

Figure 38. Effect of different concentrations of bigeye snapper muscle extract on MDC-incorporation into different substrates

Araki and Seki (1993) reported that when the same amount of carp TGase in the activity unit was used in reaction with different fish actomyosins, the rates of polymerization were, however, significantly different among the source of actomyosin. Niwa *et al.* (1995) reported that activities expressed as the amount of MDC-incorporated in actomyosin during incubation of the paste at 40°C for 1 h were 0.03, 0.04, 0.03 and 0.04 nmole/g protein for common mackerel (*Scomber japonicus*), black scraper (*Navodon modestus*), Spanish mackerel (*Scomberomorus niphonius*) and hair tail (*Trichiurus lepturus*), respectively.

The changes in SDS-PAGE patterns of actomyosin incubated with various concentrations of bigeye snapper muscle extract are shown in Figure 39. Myosin heavy chains decreased as the extract added increased. No myosin band was detected with addition of 0.15 g extract/mL. The results revealed that crude extract contained TGase, which induced polymerization of myosin effectively. When crude extract was added at a concentration of 0.1 g/mL, a longer incubation time at 40°C rendered the higher cross-linking, as indicated by a decrease in myosin heavy chain content (Figure 40). No myosin band was detected with the incubation time of 90 min, suggestion that all myosin heavy chains were polymerized within a sufficient time of reaction.

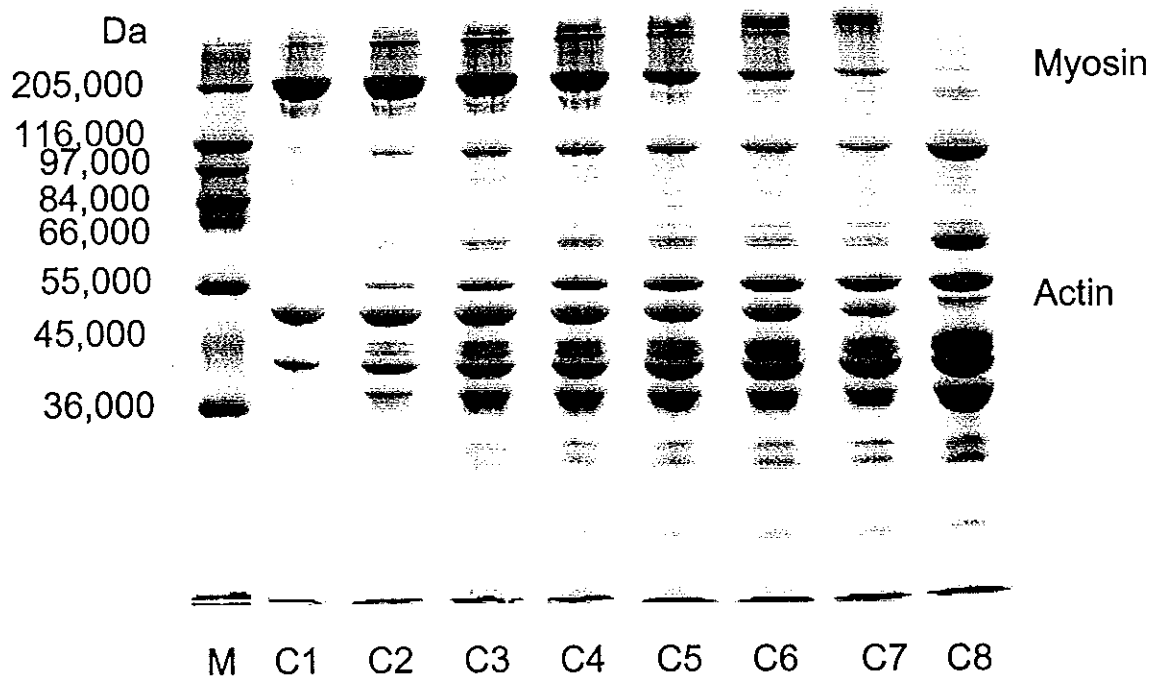

Figure 39. SDS-PAGE pattern of actomyosin added with different concentrations of TGase extract from bigeye snapper muscle and incubated at 40°C for 30 min. M; high molecular weight standard C1, C2, C3, C4, C5, C6, C7; reaction mixture contained TGase extract from bigeye snapper muscle , 0, 0.01, 0.03, 0.05, 0.08, 0.1 and 0.15 g/mL, respectively. C8; TGase extract from bigeye snapper muscle.

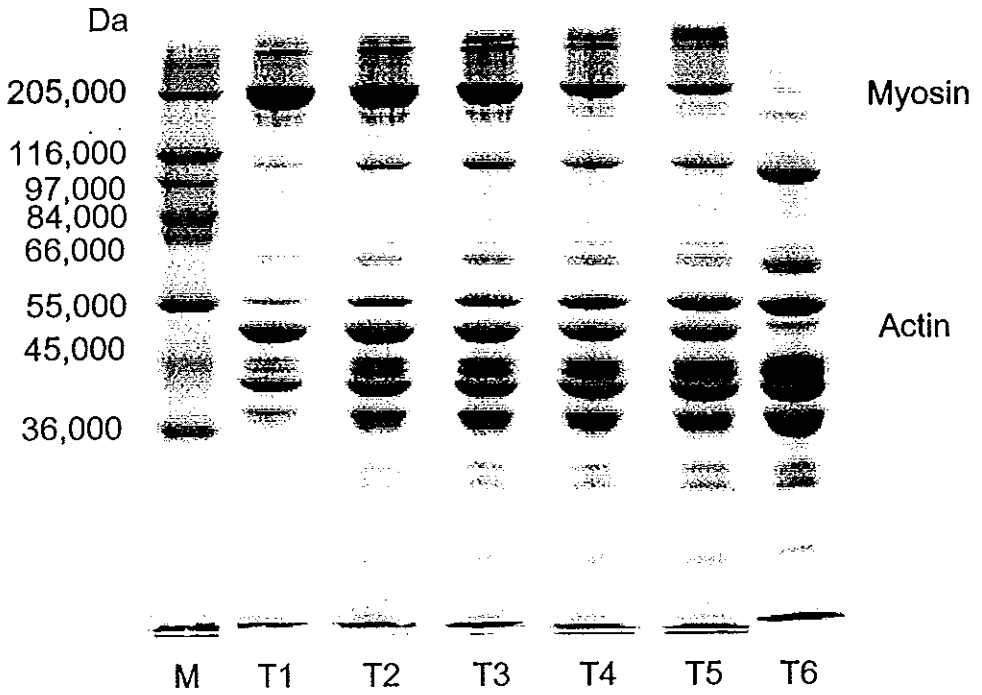

Figure 40. SDS-PAGE pattern of actomyosin added with 0.1 g/mL TGase extract from bigeye snapper muscle and incubated at 40°C for different times. M; high molecular weight standard, T1, T2, T3, T4, T5; 0, 15, 30, 60 and 90 min, respectively. T6; TGase extract from bigeye snapper muscle

To verify if the disappearance of myosin heavy chain band was mainly induced by the polymerization caused by TGase from bigeye snapper muscle extract, protein degradation resulted from endogenous proteinase was determined (Table 22 and 23). No significant differences in autolytic products were obtained (p>0.05) with an increase in bigeye snapper muscle extract concentration as well as with an increased incubation time. This reconfirmed that no significant proteolysis took place during incubation at 40°C, even for a longer time and higher concentration of bigeye snapper muscle extract. Therefore, extract from bigeye snapper muscle played an essential role in cross-linking of myosin, rather than degrading of myosin.

Table 22. TCA-soluble peptides in actomyosin added with different concentration of bigeye snapper muscle TGase extract

Concentrations of TGase (g/mL)	Tyrosine (μmol/g actomyosin)
0	5.44±3.59[a] a[b]
0.01	30.21±2.18ab
0.03	27.98±2.34ab
0.05	34.19±7.12bc
0.08	45.08±14.99bc
0.10	45.85±28.10bc
0.15	56.73±14.99c

[a]Mean ± standard deviation from triplicate determinations.

[b]Different letters in the same column denote the significant differences (p<0.05).

Table 23. TCA-soluble peptides in actomyosin added with bigeye snapper Muscle TGase extract and incubated for different times.

Incubation time (min)	Tyrosine (μmol/g actomyosin)
0	139.34±0.82[a] a[b]
15	140.58±2.25a
30	148.74±2.30b
60	140.58±1.82a
90	143.93±4.40a

[a]Mean ± standard deviation from triplicate determinations.

[b]Different letters in the same column denote the significant differences (p<0.05).

5. Effect of storage and processing on transglutaminase activity

5.1 Effect of storage time on TGase activity in bigeye snapper mince

The activity of TGase in bigeye snapper mince during iced storage is shown in Table 24. During storage, the activity decreased singificantly after 2 days of storage (p<0.05). Activity decreased to 33.56% after 8 days of iced storage. The result indicated that TGase was not stable during storage. This possibly led to a lower setting phenomenon in the poor quality fish in combination with a poor gel forming ability due to the protein degradation occurred during iced storage. The decrease in TGase activity was possibly due to the denaturation of TGase, which was possibly associated with proteolysis. Moreover, ammonia formed during storage fish from enzymatic deamination of free amino acids or amino acid split from protein, from oxidation of amines and decomposition of nucleic bases (Huang *et al.*, 1992) might partially inhibit the TGase activity in the mince kept for a longer time.

Ammonium ion produced from TGase reaction should prevent further progress of the reaction (Takagi *et al.*, 1986). Therefore, ammonia is known as an inhibitor of transglutaminase (Wan *et al.*, 1992).

Table 24. Relative activity of TGase in bigeye snapper mince during iced storage.

Storage time (days)	Relative activity (%)
0	100e[b]
2	86.65±0.20[a]d
4	76.70±0.46c
6	54.17±0.38b
8	33.56±0.26a

[a]Mean ± standard deviation from triplicate determinations.

[b]Different letters in the same column denote the significant differences ($p<0.05$).

5.2 Effect of freeze-thawing on transglutaminase activity in bigeye snapper mince.

The MDC incorporating activity of TGase in bigeye snapper mince subjected to different freeze-thaw cycles is shown in Table 25. The activity decreased significantly ($p<0.05$) when the freeze-thaw cycles increased. Freeze-thawing process has been known to accelerate the denaturation of protein via some bondings. Also, change in ionic strength during freeze-thawing could induce the denaturation of TGase in the muscle. As a result, loss in TGase activity could be obtained, particularly when freeze-thawing was repeated.

Frozen storage is an important preservation method for fish and fish products. The quality deterioration can be found during freezing and frozen storage due to the osmotic removal of water, denaturation of protein and mechanical damage (Thyholt and Isakssom, 1997; Benjakul and Bauer, 2000). The enzyme and other components are released (Nilsson and Ekstrand, 1993; 1995; Benjakul and Bauer, 2000). Thawing method played an important role in membrane disintegration as well as the sensory attributes (Nilsson and Ekstrand, 1994; 1995). The freeze-thaw process was found to be detrimental to overall physicochemical and textural quality (Srinivasan *et al.*, 1997; MacDonald *et al.*, 1992). The loss in protein functionality and gel-forming ability in fish meat and surimi during frozen storage is attributable to the denaturation of myofibrillar protein (Matsumoto and Noguchi, 1992; Atsumi *et al.*, 1995; Ragnarsson and Regenstein, 1989). Moreover, it has been reported that the activities of many enzymes are greatly affected by freezing and frozen storage (Matsumoto and Noguchi, 1992).

Table 25. Relative activity of TGase from bigeye snapper mince as affected by different freeze-thaw cycles

Freeze-thaw cycles	Relative activity (%)
0	100e[b]
1	96.99±0.74[a]d
2	80.45±0.01c
3	65.85±0.98b
4	58.94±0.14a

[a]Mean ± standard deviation from triplicate determinations.

[b]Different letters in the same column denote a significant difference ($p<0.05$).

5.3 Effect of washing on transglutaminase activity in bigeye snapper mince

Washing is an important process to remove water soluble proteins, fat as well as other constituents, but to concentrate the myofibrillar proteins, which play a crucial role in gel formation. During washing, TGase can be removed to some extent. TGase is a water-soluble enzyme and would be mostly washed away during preparation of myosin and actomyosin (Nowsad *et al.*, 1994; Kudo *et al.*, 1973). The effect of washing cycles (0, 1, 2 and 3 times) and concentrations of NaCl (0, 0.1, 0.2 and 0.3 %) on TGase activity remained in washed mince is shown in Table 26. It was found that the activity decreased when washing cycle and concentration of NaCl increased ($p<0.05$). Generally, TGase could be washed away to a higher extent with an increasing washing cycles. NaCl, which is commonly used to remove water from washed mince, was able to extract TGase from mince effectively. The removal of TGase was dominant when a higher concentration of NaCl was used. NaCl may unfold the protein structure and accommodate the exposure of enzyme, leading to the easy release during washing process. Therefore, a concentration of NaCl used to facilitate the water removal should be taken into consideration.

Table 26. Relative activity of TGase from bigeye snapper mince affected by washing.

Washing cycles	Concentration of NaCl (%w/v)			
	0	0.1	0.2	0.3
Control	100C[b]ns[c]	100Dns	100Dns	100Cns
1	78.11±1.50[a]Bc	64.29±0.78Cc	54.61±0.16Cb	44.85±1.09Ba
2	77.70±0.62Bd	57.83±0.56Bc	44.68±0.02Bb	43.77±0.33ABa
3	73.80±0.96Ac	53.92±0.36Ab	44.13±0.29Aa	43.31±0.71Aa

Control: no washed

[a]Mean ± standard deviation from triplicate determinations.

[b]Dfifferent capital letters in the same column denote the significant differences (p<0.05).

[c]Dfifferent letters in the same row denote the significant differences (p<0.05).

Chapter 4

Conclusions

1. Suwari gel from surimi produced from four different fish species could be prepared at either high or medium temperature with optimum setting time as follows: threadfin bream: 25°C for 8 h and 40°C for 1 h, bigeye snapper: 25°C for 8 h and 40°C for 2 h, barracuda: 25°C for 8 h and 40°C for 1.5 h, bigeye croaker: 25°C for 8 h and 40°C for 3 h.
2. The cross-linking formed during setting was dependent upon fish species, suggesting the differences in TGase activity as well as different protein conformation, which can align in different fashion.
3. Among surimi from different fish, bigeye snapper surimi had the highest TGase activity (5.70 munit/ml), while barracuda surimi contained the lowest activity.
4. Endogenous transglutaminase induced the non-disulfide covalent bond in surimi gel. This enzyme was activated by Ca^{2+} and was inhibited by sulfhydryl blocking agent (NEM), NH_4Cl and metal chelator (EDTA). Reducing agent (DTT, β-ME) increased TGase activity. Sodium hexametaphosphate or penta-sodiumtriphosphate partially reduced TGase activity.
5. Optimal pH and temperature of bigeye snapper muscle TGase were 6.0 and 40°C, respectively. Bigeye snapper muscle TGase activity was not thermal stable, particularly at temperature higher than 40°C. The crude TGase was stable at pH ranging from 5.0 to 7.0. TGase effectively induced cross-linking of actomyosin.
6. The activity of TGase in bigeye snapper mince decreased continuously during iced storage up to 8 days. TGase activity in bigeye snapper mince

subjected to different freeze-thaw cycles was decreased when the freeze-thaw cycles increased. TGase activity also decreased when washing cycle and concentration of NaCl in wash water increased.

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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 h. or overnight at 105°C.
4. After drying, transfer the dish with partially covered lid to the desiccator to cool. Reweigh the dish and its dried content.

Calculation

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

Where : W_1 = 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 burn 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 until turning to gray. If not, return the crucible and lid to the furnace for further ashing.

Calculation

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

Protein (AOAC, 1991)

Reagents

- Kjeldahl catalyst : Mix 9 parts of potassium sulphate (K_2SO_4) anhydrus, nitrogen free with 1 parts of copper sulphate ($CuSO_4$)
- Sulfuric acid (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 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 chemical except sample as blank. Place flasks in inclined position and heat gently until frothing ceases. Boil briskly until solution clears.
4. Cool and add 60 mL distilled water cautiously.
5. Immediately connect flask to digestion bulb on condenser, and with tip of condenser immersed in standard acid and 5-7 indicator in 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 sample titration
- B = volume (mL) of 0.02N HCl used in blank titration
- N = Normality of HCl
- W = weight (g) of sample
- 14.07 = atomic weight of nitrogen
- 6.25 = the protein-nitrogen conversion factor for fish and its by-products

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

Method

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

Solubility (Roussel and Cheftel, 1990)

Reagent

- Tris-HCl buffer (pH 8.0) containing 1% (w/v) SDS, 8 M urea and 2% (v/v) β-mercaptoethanol
- 0.5 M NaOH
- 50% trichloroacetic acid (TCA)

Method

To a weighed amount (1 g) of sample (mince or gel), cut into small pieces, in a 100 mL conical flask, 20 mL of solvent was added, then homogenize for 1 min. Heat in boiling water bath for 2 min, then incubate at 40°C for 4 h. All samples were then centrifuged at 12,100 *xg* for 30 min in a Sorvall-RC2 centrifuge. To 10 mL of the supernatant (soluble fraction), cold 50% (w/v) TCA was added to give a final TCA concentration of 10%. Samples were kept at 0-3°C for 18 h, centrifuged at 1000 *xg* for 20 min in a table-top clinical centrifuge and the supernatant removed. The precipitate was washed with 10% TCA and solubilized in 0.5 M NaOH. Protein content was estimated by the biuret method using BSA as standard.

Samples were also solubilized in 0.5 M NaOH. Protein content in 0.5 M NaOH extract was used as reference value, i.e., 100%.

Measurement of TCA-soluble peptide (Morrissey *et al.*, 1993)

Reagents

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

Method

1. Weigh 3 g of sample and homogenize in 27 mL of 5% TCA for 3 min.
2. Keep on ice for 1 h, and centrifuge at 5,000 *xg* for 5 min.
3. Meadure tyrosin in the supernatant was measured as an index of autolytic degradation products and express as µmol tyrosine/g sample.

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

Reagents

- A: 2% sodium carbonate in 0.1 N NaOH
- B: 0.5% $CuSO_4.5H_2O$ in 1% sodium citrate
- C: 1 N Folin Phenol reagent
- D: 1 mL reagent B + 50 mL reagent A (or similar ratio)
- Standard reagent: 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 µL). Vortex immediately.
2. Incubate precisely 10 min at room temperature.
3. Add 0.2 mL reagent C (previously diluted 1 : 1 with distilled H_2O) and vortex immediately.

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Method

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2. Incubate precisely 10 min at room temperature.
3. Add 0.2 mL 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.

Standards

Bovine serum albumin (BSA) at concentration of 1 mg/mL (used to determine protein concentration)

Tyrosine at concentration 1 mM (used to determine enzymatic activity)

Standard volumes (µL): 0, 20, 40, 60, 100, 140 and 200

Biuret method quantitation of proteins (Copeland, 1994)

Reagents

- Biuret reagent: Combine 1.50 g $CuSO_4$.5H2O, 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 reagent: 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 were 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 (μL)	10 mg/mL BSA (μL)	Effective BSA concentration (mg/mL)
1	500	0	0
2	400	100	2
3	300	200	4
4	200	300	6
5	100	400	8
6	450	0	unknown
7	450	0	unknown
8	450	0	unknown

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

Reagents

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

Method

1. Homogenize 4 g of muscle in 40 mL chilled (4°C) 0.6 M KCl, pH 7.0 for 4 min.

2. Place the beaker containing the sample in ice. Each 20 sec of blending was followed by a 20 sec rest interval to avoid overheating during extraction.
3. Centrifuge the extract at 5,000 *xg* for 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 1.2 M KCl, pH 7.0.

MDC-incorporating activity assays (Takagi *et al.*, 1986)

Reagent

- 0.2 M dithiothreitol (DTT)
- 0.1 M Tris-HCl, pH 7.5
- 0.1 M $CaCl_2$
- 0.5 mM mododancylcadaverine (MDC)
- 2 mg/mL *N,N'*-dimethylated casein
- 1.0 M ammoniumsulfate

Method

1. To prepare enzyme solution, the dried powder was dissolved in distilled water, to obtain the final concentration of 0.1 g/mL.
2. The mixture reaction contained 100 µL DTT, 2.4 mL Tris-HCl, 0.4 mL $CaCl_2$, 100 µL MDC and 0.4 mL *N,N'*-dimethylated casein. The mixtures were mixed by vortex.
3. The reaction was started by the addition of enzyme solution 0.2 mL, and the mixture was incubated at 40°C for 30 min.

4. The reaction was stopped by the addition of 0.2 mL of 1.0 M ammonium sulfate.
5. The intensity of fluorescence of MDC incorporated into *N,N'*-dimethylated casein was measured with excitation and emission wavelengths set at 350 and 480 nm, respectively.

Calculation

$$(MDC)_{incorporated} = \frac{\Delta f}{13 \times I_0} \times (MDC)_{total}$$

* $\Delta f = I_{sample} - I_0$

I_0 = The fluorescence intensity of the above mixture without the enzyme.

13 = Enhancement factor

$$\text{Enzyme activity (unit/mL)} = \frac{(MDC)_{incorporated}}{\text{Incubation time (min)} \times \text{reaction mixture (mL)}}$$

One unit of the enzyme was defined as the amount that incorporated 1 nmol of MDC into *N,N'*-dimethylated casein per min at 40°C.

Electrophoresis (SDS-PAGE) (Leammli, 1970)

Reagents

- Protein molecular weight standards
- 30% Acrylamide-0.8% bis Acrylamide
- Sample buffer: Mix 30 mL of 10% of SDS, 10 mL of glycerol, 5 mL of β-Mercaptoethanol, 12.5 mL of 50 mM Tris-HCl, pH 6.8, and 5-10 mg Bromophenol blue (enough to give dark blue color to the

solution). Bring the volume to 100 mL with distilled water. Divide into 1 mL aliquots, and stroed at –20°C.

- 2% (w/v) Ammonium persulfate
- 1% (w/v) SDS
- TEMED (*N,N,N'N'*-tetramethylenediamine)
- 0.5 M Tris-HCl, pH 6.8
- 1.5 M Tris-HCl, pH 8.8
- 0.1 M EDTA
- Electrode buffer: Dissolve 3 g of 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 liter total volume.
- Staining solution: Dissolve 0.04 g of Coomassie blue R-250 in 100 mL of methanol. Add 15 mL of glacial acetic acid and 85 mL of distilled water.
- Destaining solution I: 50% methanol-7.5% glacial acetic acid
- Destatning solution II: 5% methanol-7.5% galcial acetic acid

Method

Pouring the separating gel:

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

against the edge of one of the spacers. Allow the resolving gel to polymerize fully (usually 30-60 min).

Pouring the stacking gel:

1. Pour off completely the layer of isobutyl alcohol.
2. Prepare a 4% stacking gel solution by adding as defined in table.
3. Transfer stacking gel solution to tickle into the center of the sandwich along an edge of one of the spacers.
4. Insert a comb into the layer of stacking gel solution by placing one corner of the comb into the gel and slowly lowering the other corner in. Allow the stacking gel solution to polymerize 30 to 45 min at room temperature.

Reagents	10% running gel		4% stacking gel	
30% Acrylamide-bis	1.167	mL	0.4	mL
1.5 M Tris-HCl buffer, pH 8.8	0.875	mL	-	
0.5 M Tris-HCl buffer, pH 6.8	-		1.0	mL
1% SDS	0.35	mL	0.3	mL
Distilled water	0.7585	mL	0.9	mL
0.1 EDTA	-		0.8	mL
2% Ammonium persulfate	0.35	mL	0.4	mL
TEMED	6	µL	5	µL

Sample preparation:

1. Weigh 3 g of sample and homogenize with 5% (w/v) SDS in a final volume of 30 mL.
2. Incubate the mixture at 85°C for 1 h.
3. Centrifuge at 3,500 *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 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 bromophenol blue tracking dye has reached the bottom of the separating gel, disconnect the power supply.

Disassembling the gel:

1. Remove the upper buffer chamber and the attached sandwich.
2. Orient the gel so that the order of the sample well is known, remove the sandwich from the upper buffer chamber, and lay the sandwich

on a sheet of absorbent paper or paper towels. Carefully slide the spacers out from the edge of the sandwich along its entire length.

3. Insert a spatula between the glass plates 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 h or more on a rotary rocker.
2. Pour off the staining solution and cover the gel with a solution of destaining solution I. Agitate slowly for ~15 min.
3. Pour off the destaining solution I and cover the destaining solution II. Discard destaining solution and replace with fresh solution. Repeat until the gel is clear except for the protein bands.

Appendix 2

Analysis of variance

Table 1-A. Analysis of variance for pH of surimi from some tropical fish.

SV	DF	SS	MS	F
Treatment	3	0.00013	0.000044	0.533**
Error	8	0.00067	0.000083	
Total	11	0.00080		

** = Significant at 1% level

Table 2-A. Analysis of variance for moisture of surimi from some tropical fish.

SV	DF	SS	MS	F
Treatment	3	2.015	0.000044	19.099**
Error	8	0.281	0.000083	
Total	11	2.296		

** = Significant at 1% level

Table 3-A. Analysis of variance for ash surimi from of some tropical fish.

SV	DF	SS	MS	F
Treatment	3	0.027	0.0091	1.556**
Error	8	0.047	0.0058	
Total	11	0.074		

** = Significant at 1% level

Table 4-A. Analysis of variance for protein of surimi from some tropical fish.

SV	DF	SS	MS	F
Treatment	3	5.699	1.900	11.293**
Error	8	1.346	0.168	
Total	11	7.045		

** = Significant at 1% level

Table 5-A. Analysis of variance for fat of surimi from some tropical fish.

SV	DF	SS	MS	F
Treatment	3	0.091	0.03	748.417**
Error	8	0.00032	0.00004	
Total	11	0.091		

** = Significant at 1% level

Table 6-A. Analysis of variance for non-protein Nitrogenous compound of surimi from some tropical fish.

SV	DF	SS	MS	F
Treatment	3	0.0067	0.0022	25.478**
Error	8	0.0017	0.000087	
Total	11	0.0084		

** = Significant at 1% level

Table 7-A. Analysis of variance for sarcoplasmic protein of surimi from some tropical fish.

SV	DF	SS	MS	F
Treatment	3	2.623	0.874	15.597**
Error	8	1.121	0.056	
Total	11	3.744		

** = Significant at 1% level

Table 8-A. Analysis of variance for myofibrillar protein of surimi from some tropical fish.

SV	DF	SS	MS	F
Treatment	3	50.653	16.884	66.159**
Error	8	5.104	0.255	
Total	11	55.757		

** = Significant at 1% level

Table 9-A. Analysis of variance for alkali-soluble protein of surimi from some tropical fish.

SV	DF	SS	MS	F
Treatment	3	4.474	1.491	44.346**
Error	8	0.673	0.034	
Total	11	5.147		

** = Significant at 1% level

Table 10-A. Analysis of variance for stroma of surimi from some tropical fish.

SV	DF	SS	MS	F
Treatment	3	2.960	0.987	149.120**
Error	8	0.132	0.0066	
Total	11	3.092		

** = Significant at 1% level

Table 11-A. Analysis of variance for force of threadfin bream (*Nemipterus bleekeri*) suwari gel set at 25°C for different times.

SV	DF	SS	MS	F
Treatment	4	194901.060	48725.264	1803.215**
Error	35	945.746	27.021	
Total	39	195846.800		

** = Significant at 1% level

Table 12-A. Analysis of variance for deformation of threadfin bream (*Nemipterus bleekeri*) suwari gel set at 25°C for different times.

SV	DF	SS	MS	F
Treatment	4	79.489	19.872	79.981**
Error	35	8.696	0.248	
Total	39	88.185		

** = Significant at 1% level

Table 13-A. Analysis of variance for force of threadfin bream (*Nemipterus bleekeri*) suwari gel set at 40°C for different times.

SV	DF	SS	MS	F
Treatment	5	167334.020	33466.805	59.294**
Error	42	23705.618	564.419	
Total	47	191039.638		

** = Significant at 1% level

Table 14-A. Analysis of variance for deformation of threadfin bream (*Nemipterus bleekeri*) suwari gel set at 40°C for different times.

SV	DF	SS	MS	F
Treatment	5	120.103	24.021	39.582**
Error	42	25.488	0.607	
Total	47	145.590		

** = Significant at 1% level

Table 15-A. Analysis of variance for force of threadfin bream (*Nemipterus bleekeri*) ashi gel set at 40°C for different times.

SV	DF	SS	MS	F
Treatment	6	398322.25	66387.042	34.027**
Error	49	95600.739	195.1035	
Total	55	493922.99		

** = Significant at 1% level

Table 16-A. Analysis of variance for deformation of threadfin bream (*Nemipterus bleekeri*) ashi gel set at 40°C for different times.

SV	DF	SS	MS	F
Treatment	6	122.805	20.467	38.156**
Error	49	26.284	0.536	
Total	55	149.089		

** = Significant at 1% level

Table 17-A. Analysis of variance for force of barracuda (*Sphyraena jello*) suwari gel set at 25°C for different times.

SV	DF	SS	MS	F
Treatment	4	350495.87	87623.966	258.991**
Error	35	11841.47	338.328	
Total	39	362337.34		

** = Significant at 1% level

Table 18-A. Analysis of variance for deformation of barracuda (*Sphyraena jello*) suwari gel set at 25°C for different times.

SV	DF	SS	MS	F
Treatment	4	105.071	26.268	72.193**
Error	35	12.735	0.364	
Total	39	117.806		

** = Significant at 1% level

Table 19-A. Analysis of variance for force of barracuda (*Sphyraena jello*) suwari gel set at 40°C for different times.

SV	DF	SS	MS	F
Treatment	5	195614.59	39122.917	26.74**
Error	42	61450.015	1463.096	
Total	47	257064.60		

** = Significant at 1% level

Table 20-A. Analysis of variance for deformation of barracuda (*Sphyraena jello)* suwari gel set at 40°C for different times.

SV	DF	SS	MS	F
Treatment	5	40.942	8.188	69.845**
Error	42	4.924	0.117	
Total	47	45.866		

** = Significant at 1% level

Table 21-A. Analysis of variance for force of barracuda (*Sphyraena jello*) Ashi gel set at 25°C for different times.

SV	DF	SS	MS	F
Treatment	5	195614.59	39122.917	26.74**
Error	42	61450.015	1463.096	
Total	47	257064.60		

** = Significant at 1% level

Table 22-A. Analysis of variance for force of barracuda (*Sphyraena jello*) ashi gel set at 25°C for different times.

SV	DF	SS	MS	F
Treatment	6	1410370.3	235061.71	271.192**
Error	49	42471.837	866.772	
Total	55	1452842.1		

** = Significant at 1% level

Table 23-A. Analysis of variance for deformation of barracuda (*Sphyraena jello*) ashi gel set at 25°C for different times.

SV	DF	SS	MS	F
Treatment	6	75.171	12.529	145.098**
Error	49	4.231	0.8635	
Total	55	79.402		

** = Significant at 1% level

Table 24-A. Analysis of variance for force of barracuda (*Sphyraena jello*) ashi gel set at 40°C for different times.

SV	DF	SS	MS	F
Treatment	6	574053.76	95675.627	97.056**
Error	49	48303.015	985.776	
Total	55	622356.78		

** = Significant at 1% level

Table 25-A. Analysis of variance for deformation of barracuda (*Sphyraena jello*) ashi gel set at 40°C for different times.

SV	DF	SS	MS	F
Treatment	6	39.125	6.521	77.1**
Error	49	4.144	0.08458	
Total	55	43.269		

** = Significant at 1% level

Table 26-A. Analysis of variance for force of bigeye croaker (*Pennahai macrophthalmus*) suwari gel set at 25°C for different times.

SV	DF	SS	MS	F
Treatment	4	156896.98	39224.246	407.516**
Error	25	2406.303	96.252	
Total	29	159303.29		

** = Significant at 1% level

Table 27-A. Analysis of variance for deformatiom of bigeye croaker (*Pennahai macrophthalmus*) suwari gel set at 25°C for different times.

SV	DF	SS	MS	F
Treatment	4	17.585	4.396	50.865**
Error	25	2.161	0.086	
Total	29	19.746		

** = Significant at 1% level

Table 28-A. Analysis of variance for force of bigeye croaker (*Pennahai macrophthalmus*) suwari gel set at 40°C for different times.

SV	DF	SS	MS	F
Treatment	5	694824.91	138964.98	346.747**
Error	30	12023.015	400.767	
Total	35	706847.93		

** = Significant at 1% level

Table 29-A. Analysis of variance for deformation of bigeye croaker (*Pennahai macrophthalmus*) suwari gel set at 40°C for different times.

SV	DF	SS	MS	F
Treatment	5	0.993	0.199	10.869**
Error	30	0.548	0.018	
Total	35	1.541		

** = Significant at 1% level

Table 30-A. Analysis of variance for force of bigeye croaker (*Pennahai macrophthalmus*) ashi gel set at 25°C for different times.

SV	DF	SS	MS	F
Treatment	6	2430924	405154.0	692.639**
Error	35	20473.0	584.942	
Total	41	2451397		

** = Significant at 1% level

Table 31-A. Analysis of variance for deformation of bigeye croaker (*Pennahai macrophthalmus*) ashi gel set at 25°C for different times.

SV	DF	SS	MS	F
Treatment	6	36.761	6.127	174.674**
Error	35	1.228	0.35	
Total	41	37.989		

** = Significant at 1% level

Table 32-A. Analysis of variance for force of bigeye croaker (*Pennahai macrophthalmus*) ashi gel set at 40°C for different times.

SV	DF	SS	MS	F
Treatment	6	2357428	392905	307.071**
Error	35	44783.4	1279.524	
Total	41	2402211		

** = Significant at 1% level

Table 33-A. Analysis of variance for deformation of bigeye croaker (*Pennahai macrophthalmus*) ashi gel set at 40°C for different times.

SV	DF	SS	MS	F
Treatment	6	49.413	8.236	299.284**
Error	35	0.963	0.028	
Total	41	50.377		

** = Significant at 1% level

Table 34-A. Analysis of variance for force of bigeye snapper (*Priacanthus tayenus*) suwari gel set at 25°C for different times.

SV	DF	SS	MS	F
Treatment	4	422.847.18	105711.79	387.220**
Error	35	9555.066	273.002	
Total	49	432402.24		

** = Significant at 1% level

Table 35-A. Analysis of variance for deformation of bigeye snapper (*Priacanthus tayenus*) suwari gel set at 25°C for different times.

SV	DF	SS	MS	F
Treatment	4	132.267	30.817	131.733**
Error	35	8.188	0.234	
Total	49	131.455		

** = Significant at 1% level

Table 36-A. Analysis of variance for force of bigeye snapper (*Priacanthus tayenus*) suwari gel set at 40°C for different times.

SV	DF	SS	MS	F
Treatment	5	394480.91	78896.182	69.033**
Error	42	48000.383	1142.877	
Total	47	442481.75		

** = Significant at 1% level

Table 37-A. Analysis of variance for deformation of bigeye snapper (*Priacanthus tayenus*) suwari gel set at 40°C for different times.

SV	DF	SS	MS	F
Treatment	5	47.057	9.411	58.632**
Error	42	6.742	0.161	
Total	47	53.799		

** = Significant at 1% level

Table 38-A. Analysis of variance for force of bigeye snapper (*Priacanthus tayenus*) ashi gel set at 25°C for different times.

SV	DF	SS	MS	F
Treatment	6	5363038.6	893839.76	484.936**
Error	49	90317.377	1843.212	
Total	55	5453356.0		

** = Significant at 1% level

Table 39-A. Analysis of variance for deformation of bigeye snapper (*Priacanthus tayenus*) ashi gel set at 25°C for different times.

SV	DF	SS	MS	F
Treatment	6	54.921	9.154	78.120**
Error	49	5.742	0.117	
Total	55			

** = Significant at 1% level

Table 40-A. Analysis of variance for force of bigeye snapper (*Priacanthus tayenus*) ashi gel set at 40°C for different times.

SV	DF	SS	MS	F
Treatment	6	2849773.9	474962.31	224.234**
Error	49	103697.82	2116.282	
Total	55	2953471.7		

** = Significant at 1% level

Table 41-A. Analysis of variance for deformation of bigeye snapper (*Priacanthus tayenus*) ashi gel set at 40°C for different times.

SV	DF	SS	MS	F
Treatment	6	38.827	6.471	68.127**
Error	49	4.654	0.09499	
Total	55	43.481		

** = Significant at 1% level

Table 42-A. Analysis of variance for solubility of threadfin bream (*Nemipterus bleekeri*) suwari gel set at 25°C for different times.

SV	DF	SS	MS	F
Treatment	7	421.553	60.222	78.557**
Error	16	12.266	0.767	
Total	23	433.818		

** = Significant at 1% level

Table 43-A. Analysis of variance for solubility of threadfin bream (*Nemipterus bleekeri*) suwari gel set at 40°C for different times.

SV	DF	SS	MS	F
Treatment	7	895.233	127.890	361.183**
Error	16	5.665	0.354	
Total	23	900.898		

** = Significant at 1% level

Table 44-A. Analysis of variance for solubility of threadfin bream (*Nemipterus bleekeri*) ashi gel set at 25°C for different times.

SV	DF	SS	MS	F
Treatment	7	960.202	137.172	237.362**
Error	16	9.246	0.578	
Total	23	969.449		

** = Significant at 1% level

Table 45-A. Analysis of variance for solubility of threadfin bream (*Nemipterus bleekeri*) ashi gel set at 40°C for different times.

SV	DF	SS	MS	F
Treatment	7	1163.109	166.158	249.972**
Error	16	10.635	0.665	
Total	23	1173.744		

** = Significant at 1% level

Table 46-A. Analysis of variance for solubility of barracuda (*Sphyraena jello*) suwari gel set at 25°C for different times.

SV	DF	SS	MS	F
Treatment	7	1822.191	260.313	172.239**
Error	16	24.182	1.511	
Total	23	1846.372		

** = Significant at 1% level

Table 47-A. Analysis of variance for solubility of barracuda (*Sphyraena jello*) suwari gel set at 40°C for different times.

SV	DF	SS	MS	F
Treatment	7	2779.522	397.075	266.985**
Error	16	23.796	1.487	
Total	23	2803.318		

** = Significant at 1% level

Table 48-A. Analysis of variance for solubility of barracuda (*Sphyraena jello*) ashi gel set at 25°C for different times.

SV	DF	SS	MS	F
Treatment	7	1796.260	256.609	166.460**
Error	16	24.665	1.542	
Total	23	1820.925		

** = Significant at 1% level

Table 49-A. Analysis of variance for solubility of barracuda (*Sphyraena jello*) ashi gel set at 40°C for different times.

SV	DF	SS	MS	F
Treatment	7	2316.306	330.901	101.179**
Error	16	52.327	3.270	
Total	23	2368.633		

** = Significant at 1% level

Table 50-A. Analysis of variance for solubility of bigeye croaker (*Pennahai macrophthalmus*) suwari gel set at 25°C for different times.

SV	DF	SS	MS	F
Treatment	7	1311.035	187.291	187.086**
Error	16	16.018	1.001	
Total	23	1327.053		

** = Significant at 1% level

Table 51-A. Analysis of variance for solubility of bigeye croaker (*Pennahai macrophthalmus*) suwari gel set at 40°C for different times.

SV	DF	SS	MS	F
Treatment	7	3694.719	527.817	879.127**
Error	16	9.606	0.600	
Total	23	3704.325		

** = Significant at 1% level

Table 52-A. Analysis of variance for solubility of bigeye croaker (*Pennahai macrophthalmus*) ashi gel set at 25°C for different times.

SV	DF	SS	MS	F
Treatment	7	2858.018	408.288	1012.672**
Error	16	6.451	0.403	
Total	23	2864.469		

** = Significant at 1% level

Table 53-A. Analysis of variance for solubility of bigeye croaker (*Pennahai macrophthalmus*) ashi gel set at 40°C for different times.

SV	DF	SS	MS	F
Treatment	7	2944.310	420.616	1045.787**
Error	16	6.435	0.402	
Total	23	2950.745		

** = Significant at 1% level

Table 54-A. Analysis of variance for solubility of bigeye snapper (*Priacanthus tayenus*) suwari gel set at 25°C for different times.

SV	DF	SS	MS	F
Treatment	7	1126.275	160.896	158.884**
Error	16	16.203	1.013	
Total	23	1142.477		

** = Significant at 1% level

Table 55-A. Analysis of variance for solubility of bigeye snapper (*Priacanthus tayenus*) suwari gel set at 40°C for different times.

SV	DF	SS	MS	F
Treatment	7	2826.839	403.864	173.053**
Error	16	37.337	2.334	
Total	23	2864.176		

** = Significant at 1% level

Table 56-A. Analysis of variance for solubility of bigeye snapper (*Priacanthus tayenus*) ashi gel set at 25°C for different times.

SV	DF	SS	MS	F
Treatment	7	1900.911	271.559	111.593**
Error	16	38.935	2.433	
Total	23	1939.846		

** = Significant at 1% level

Table 57-A. Analysis of variance for solubility of bigeye snapper (*Priacanthus tayenus*) ashi gel set at 40°C for different times.

SV	DF	SS	MS	F
Treatment	7	2999.842	428.549	155.058**
Error	16	44.221	2.764	
Total	23	3044.063		

** = Significant at 1% level

Table 58-A. Analysis of variance for TCA-soluble peptide of threadfin bream (*Nemipterus bleekeri*) surimi gel set at optimum conditions.

SV	DF	SS	MS	F
Treatment	2	12.814	6.407	1087.994**
Error	6	0.035	0.0059	
Total	8	12.849		

** = Significant at 1% level

Table 59-A. Analysis of variance for TCA-soluble peptide of bigeye snapper (*Priacanthus tayenus*) surimi gel set at optimum conditions.

SV	DF	SS	MS	F
Treatment	2	11.154	5.577	1678.766**
Error	6	0.02	0.0033	
Total	8	11.174		

** = Significant at 1% level

Table 60-A. Analysis of variance for TCA-soluble peptide of barracuda (*Sphyraena jello*) surimi gel set at optimum conditions.

SV	DF	SS	MS	F
Treatment	2	2.323	1.162	823.283**
Error	6	0.0085	0.0014	
Total	8	2.332		

** = Significant at 1% level

Table 61-A. Analysis of variance for TCA-soluble peptide of bigeye croaker (*Pennahai macrophthalmus*) surimi gel set at optimum conditions.

SV	DF	SS	MS	F
Treatment	2	6.140	3.070	7468.000**
Error	6	0.0025	0.00041	
Total	8	6.143		

** = Significant at 1% level

Table 62-A. Analysis of variance for TCA-soluble peptide of surimi gel from some tropical fish set at 25°C .

SV	DF	SS	MS	F
Treatment	3	2.489	0.830	279.678**
Error	8	0.024	0.003	
Total	11	2.513		

** = Significant at 1% level

Table 63-A. Analysis of variance for TCA-soluble peptide of surimi gel from some tropical fish set at 40°C .

SV	DF	SS	MS	F
Treatment	3	5.329	1.776	334.633**
Error	8	0.042	0.0053	
Total	11	5.371		

** = Significant at 1% level

Table 64-A. Analysis of variance for TGase activity of surimi from some tropical fish.

SV	DF	SS	MS	F
Treatment	3	110.945	36.982	462.270**
Error	8	0.640	0.08	
Total	11	111.585		

** = Significant at 1% level

Table 65-A. Analysis of variance for force of bigeye snapper suwari gel added with various $CaCl_2$ concentrations and set at 40°C for 2 h.

SV	DF	SS	MS	F
Treatment	5	1381508.9	276301.78	417.652**
Error	30	19846.801	661.560	
Total	35	1401355.7		

** = Significant at 1% level

Table 66-A. Analysis of variance for deformation of bigeye snapper suwari gel added with various $CaCl_2$ concentrations and set at 40°C for 2 h.

SV	DF	SS	MS	F
Treatment	5	1.476	0.295	13.790**
Error	30	0.642	0.02141	
Total	35	2.118		

** = Significant at 1% level

Table 67-A. Analysis of variance for solubility of bigeye snapper suwari gel added with various $CaCl_2$ concentrations and set at 40°C for 2 h.

SV	DF	SS	MS	F
Treatment	5	3836.663	639.444	347.007**
Error	14	25.798	1.843	
Total	19	3862.462		

** = Significant at 1% level

Table 68-A. Analysis of variance for force of bigeye snapper suwari gel added with various *N*-ethylmaleimide concentrations and set at 40°C for 2 h.

SV	DF	SS	MS	F
Treatment	4	842478.28	210619.57	559.407**
Error	25	9412.633	376.505	
Total	29	851890.91		

** = Significant at 1% level

Table 69-A. Analysis of variance for deformation of bigeye snapper suwari gel added with various *N*-ethylmaleimide concentrations and set at 40°C for 2 h.

SV	DF	SS	MS	F
Treatment	4	89.332	22.333	294.642**
Error	25	1.895	0.07580	
Total	29	91.226		

** = Significant at 1% level

Table 70-A. Analysis of variance for solubility of bigeye snapper suwari gel added with various *N*-ethylmaleimide concentrations and set at 40°C for 2 h.

SV	DF	SS	MS	F
Treatment	5	2262.392	452.478	97.862**
Error	12	55.484	4.624	
Total	17	2317.876		

** = Significant at 1% level

Table 71-A. Analysis of variance for solubility of bigeye snapper suwari gel added with various NH_4Cl concentrations and set at 40°C for 2 h.

SV	DF	SS	MS	F
Treatment	5	2134.589	426.918	222.080
Error	12	23.068	1.922	
Total	17	2157.658		

** = Significant at 1% level

Table 72-A. Analysis of variance for force of bigeye snapper suwari gel added with various EDTA concentrations and set at 40°C for 2 h.

SV	DF	SS	MS	F
Treatment	4	216250.42	54062.604	111.500**
Error	25	12121.685	484.867	
Total	29	228372.10		

** = Significant at 1% level

Table 73-A. Analysis of variance for deformation of bigeye snapper suwari gel added with various EDTA concentrations and set at 40°C for 2 h.

SV	DF	SS	MS	F
Treatment	4	9.697	2.424	46.304**
Error	25	1.309	0.05236	
Total	29	11.006		

** = Significant at 1% level

Table 74-A. Analysis of variance for solubility of bigeye snapper suwari gel added with various EDTA concentrations and set at 40°C for 2 h.

SV	DF	SS	MS	F
Treatment	5	2021.520	404.304	133.388**
Error	12	36.372	3.031	
Total	17	2057.893		

** = Significant at 1% level

Table 75-A. Analysis of variance for force of bigeye snapper suwari gel added with various sodium hexametaphosphate concentrations and set at 40°C for 2 h.

SV	DF	SS	MS	F
Treatment	4	356837.24	89209.310	379.868**
Error	25	5871.076	234.843	
Total	29	367208.32		

** = Significant at 1% level

Table 76-A. Analysis of variance for deformation of bigeye snapper suwari gel added with various sodium hexametaphosphate concentrations and set at 40°C for 2 h.

SV	DF	SS	MS	F
Treatment	4	5.027	1.257	34.435**
Error	25	0.912	0.03650	
Total	29	5.939		

** = Significant at 1% level

Table 77-A. Analysis of variance for solubility of bigeye snapper suwari gel added with various sodium hexametaphosphate concentrations and set at 40°C for 2 h.

SV	DF	SS	MS	F
Treatment	4	2146.399	429.280	225.555**
Error	25	22.839	1.903	
Total	29	2169.238		

** = Significant at 1% level

Table 78-A. Analysis of variance for force of bigeye snapper suwari gel added with various penta-sodiumtriphosphate concentrations and set at 40°C for 2 h.

SV	DF	SS	MS	F
Treatment	4	387286.57	96821.643	306.962**
Error	25	7885.485	315.419	
Total	29	395172.06		

** = Significant at 1% level

Table 79-A. Analysis of variance for deformation of bigeye snapper suwari gel added with various penta-sodiumtriphosphate concentrations and set at 40°C for 2 h.

SV	DF	SS	MS	F
Treatment	4	6.333	1.5837	38.202**
Error	25	1.036	0.04144	
Total	29	7.369		

** = Significant at 1% level

Table 80-A. Analysis of variance for solubility of bigeye snapper suwari gel added with various penta-sodiumtriphosphate concentrations and set at 40°C for 2 h.

SV	DF	SS	MS	F
Treatment	4	1945.273	389.055	143.329**
Error	25	32.573	2.714	
Total	29	1977.846		

** = Significant at 1% level

Table 81-A. Analysis of variance for pH profile of bigeye snapper muscle TGase.

SV	DF	SS	MS	F
Treatment	7	65.272	9.325	1585.617**
Error	16	0.094	0.0059	
Total	23	65.366		

** = Significant at 1% level

Table 82-A. Analysis of variance for temperature profile of bigeye snapper muscle TGase.

SV	DF	SS	MS	F
Treatment	8	119.732	14.967	4525.666**
Error	18	0.060	0.0033	
Total	26	119.792		

** = Significant at 1% level

Table 83-A. Analysis of variance for effect of $CaCl_2$ on bigeye snapper muscle TGase activity.

SV	DF	SS	MS	F
Treatment	4	33785.0	8446.249	1957.603**
Error	10	43.146	4.315	
Total	14	33823.1		

** = Significant at 1% level

Table 84-A. Analysis of variance for effect of NaCl on bigeye snapper muscle TGase activity.

SV	DF	SS	MS	F
Treatment	4	23.167	5.792	2.332**
Error	10	24.838	2.484	
Total	14	48.004		

** = Significant at 1% level

Table 85-A. Analysis of variance for effect of *N*-ethylmaleimide on bigeye snapper muscle TGase activity.

SV	DF	SS	MS	F
Treatment	3	8605.188	2268.396	983.309**
Error	8	18.455	2.307	
Total	11	6823.644		

** = Significant at 1% level

Table 86-A. Analysis of variance for effect of NH_4Cl on bigeye snapper muscle TGase activity.

SV	DF	SS	MS	F
Treatment	3	1935.827	645.276	109.099**
Error	8	47.317	5.915	
Total	11	1983.143		

** = Significant at 1% level

Table 87-A. Analysis of variance for effect of EDTA on bigeye snapper muscle TGase activity.

SV	DF	SS	MS	F
Treatment	3	11175.8	3725.255	3730.789**
Error	8	7.988	0.999	
Total	11	11183.8		

** = Significant at 1% level

Table 88-A. Analysis of variance for effect of β-ME on bigeye snapper muscle TGase activity.

SV	DF	SS	MS	F
Treatment	3	1451.660	483.887	599.729**
Error	8	6.455	0.807	
Total	11	1458.114		

** = Significant at 1% level

Table 89-A. Analysis of variance for effect of DTT on bigeye snapper muscle TGase activity.

SV	DF	SS	MS	F
Treatment	3	7044.797	2348.266	4373.206**
Error	8	4.296	0.537	
Total	11	7049.093		

** = Significant at 1% level

Table 90-A. Analysis of variance for effect of sodium hexametaphosphate on bigeye snapper muscle TGase activity.

SV	DF	SS	MS	F
Treatment	3	5731.706	1910.569	3600.997**
Error	8	4.245	0.531	
Total	11	5735.951		

** = Significant at 1% level

Table 91-A. Analysis of variance for effect of penta-sodium triphosphate on bigeye snapper muscle TGase activity.

SV	DF	SS	MS	F
Treatment	3	2653.916	884.639	837.838**
Error	8	8.447	1.056	
Total	11	2662.363		

** = Significant at 1% level

Table 92-A. Analysis of variance for thermal stability of crude TGase from bigeye snapper muscle.

SV	DF	SS	MS	F
Treatment	12	37299.8	3108.317	15642.5**
Error	26	5.166	0.199	
Total	38	37305.0		

** = Significant at 1% level

Table 93-A. Analysis of variance for pH stability of crude TGase from bigeye snapper muscle.

SV	DF	SS	MS	F
Treatment	8	32679.3	4084.913	1364.925**
Error	18	53.870	2.993	
Total	26	32733.2		

** = Significant at 1% level

Table 94-A. Analysis of variance for effect of different concentrations of crude extract on MDC-incorporation into *N,N'*-dimethylated casein substrates.

SV	DF	SS	MS	F
Treatment	6	108.880	18.147	1411.412**
Error	14	0.180	0.013	
Total	20	109.060		

** = Significant at 1% level

Table 95-A. Analysis of variance for effect of different concentrations of crude extract on MDC incorporation into actomyosin substrates.

SV	DF	SS	MS	F
Treatment	6	35.142	5.857	3297.511**
Error	14	0.025	0.0018	
Total	20	35.167		

** = Significant at 1% level

Table 96-A. Analysis of variance for TCA-soluble peptides in actomyosin added with different concentrations of bigeye snapper muscle crude extract.

SV	DF	SS	MS	F
Treatment	6	4915.006	819.168	4.368**
Error	14	2625.629	187.545	
Total	20	7540.635		

** = Significant at 1% level

Table 97-A. Analysis of variance for TCA-soluble peptides in actomyosin added with of bigeye snapper muscle crude extract and incubated for different times.

SV	DF	SS	MS	F
Treatment	4	174.771	43.693	6.486**
Error	10	67.368	6.737	
Total	14	242.139		

** = Significant at 1% level

Table 98-A. Analysis of variance for relative activity of TGase in bigeye snapper mince during iced storage.

SV	DF	SS	MS	F
Treatment	4	8400.896	2100.224	22698.6**
Error	10	0.925	0.093	
Total	14	8401.821		

** = Significant at 1% level

Table 99-A. Analysis of variance for relative activity of TGase from bigeye snapper mince as affect by different freeze-thaw cycles.

SV	DF	SS	MS	F
Treatment	4	3994.842	998.711	3302.232**
Error	10	3.024	0.302	
Total	14	3997.867		

** = Significant at 1% level

Table 100-A. Analysis of variance for relative activity of TGase from bigeye snapper mince effected by wash.

SV	DF	SS	MS	F
Treatmentment	15	22934.3	1528.956	3742.836**
Contration (C)	3	4294.434	1431.478	3504.212**
Time washed (T)	3	17073.6	5691.200	13931.900**
C x T	9	1566.308	174.034	426.030**
Error	32	13.072	0.409	
Total	47	22947.4		

** = Significant at 1% level

Vitae

Name Mr. Chakkawat Chantarasuwan

Birth date 10 January 1975

Education Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Food Science and Technology)	Rajamangala Institute of Technology	1996