Gelling Properties and Proteolysis of Goatfish (*Mulloidichthys martinicus*) Muscle

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ชื่อวิทยานิพนธ์ สมบัติการเกิดเจลและการย่อยสลายโปรตีนกล้ามเนื้อปลาเม็ดขนุน (*Mulloidichthys martinicus*)

ผู้เขียน นางสาวสุธาสินี ญาณภักดี

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บทคัดย่อ

จากการศึกษาองค์ประกอบทางเคมีและสมบัติทางความร้อนของกล้ามเนื้อปลาเม็ดขนุนพบว่ากล้ามเนื้อปลาประกอบด้วย โปรตีนไมโอฟิบริลร้อยละ 63.93 และมีโปรตีนซาร์โคพลาสมิคร้อยละ 30.98 จากการศึกษาด้วย Differential Scanning Colorimetry พบว่า T_{max} และเอนธาลปี (ΔH) ของพีคไมโอซินและแอกตินมีค่าลดลงในสภาวะที่มีการใช้เกลือโซเดียมคลอไรด์ร้อยละ 2.5 อย่างไรก็ตามการล้างไม่ส่งผลต่อการเปลี่ยนแปลงพีคไมโอซิน แต่ส่งผลให้ค่า T_{max} ของพีคแอกตินลดลง เมื่อศึกษาเชิงเปรียบเทียบสมบัติทางความร้อนและความสามารถในการจับเรียงตัวของแอกโตไมโอซินธรรมชาติที่สกัดจากปลาเม็ดขนุนสดและที่ผ่านการเก็บรักษาในน้ำแข็งเป็นเวลา 9 วัน พบว่า ไมโอซินของแอกโตไมโอซินธรรมชาติที่สกัดจากปลาสดมีความคงตัวต่อความร้อนสูงกว่าที่สกัดจากปลาที่ผ่านการเก็บรักษาเป็นเวลา 9 วัน โดยมีค่า T_{max} ที่สูงกว่าและมีค่าความไม่คงตัวต่อความร้อน (K_D) ต่ำกว่า เมื่อให้ความร้อนที่อุณหภูมิสูงกว่า 35 องศาเซลเซียส แอกโตไมโอซินธรรมชาติที่สกัดจากปลาสดมีค่าความขุ่น ปริมาณไฮโดรโฟบิกซิตีบริเวณพื้นผิว และปริมาณพันธะไดซัลไฟด์สูงกว่าแอกโตไมโอซินธรรมชาติที่สกัดจากปลาที่ผ่านการเก็บรักษานาน 9 วัน ระหว่างการให้ความร้อนถึง 75 องศาเซลเซียส อย่างไรก็ตามทุกดัชนีที่ทดสอบมีค่าต่ำกว่า เมื่อเปรียบเทียบกับแอกโตไมโอซินธรรมชาติที่สกัดจากปลาตาหวาน เมื่อตรวจสอบโครงข่ายโปรตีนของแอกโตไมโอซินธรรมชาติที่สกัดจากปลาตาหวานด้วยกล้องจุลทรรศน์แบบส่องผ่าน พบว่าโครงข่ายมีความละเอียดและเป็นระเบียบกว่าแอกโตไมโอซินธรรมชาติที่สกัดจากปลาเม็ดขนุน ดังนั้นความสามารถในการเกิดเจลโดยการเหนี่ยวนำด้วยความร้อนจึงขึ้นกับชนิดและความสดของปลา

จากการศึกษาความสามารถในการเกิดเจลจากซูริมิปลาเม็ดขนุนภายใต้การเซ็ตตัวหรือให้ความร้อนในสภาวะต่าง ๆ ร่วมกับแคลเซียมคลอไรด์ที่ระดับต่าง ๆ (0, 25 และ 50 มิลลิโมลต่อกิโลกรัม) พบว่าการเติมแคลเซียมคลอไรด์ที่ระดับ 50 มิลลิโมลต่อกิโลกรัม ร่วมกับการเซ็ตตัวที่อุณหภูมิ 40 องศาเซลเซียส นาน 30 นาที ตามด้วยการให้ความร้อนต่อที่อุณหภูมิ 90 องศาเซลเซียส นาน 20 นาที ส่งผลให้ค่าของแรงและระยะทางก่อนการเจาะทะลุของเจลซูริมิมีค่าสูงสุด ($P<0.05$)

โดยการเพิ่มขึ้นของความแข็งแรงของเจลสัมพันธ์กันกับการเพิ่มความสามารถในการอุ้มน้ำและการเชื่อมประสานของโปรตีน อย่างไรก็ตามแรงและระยะทางก่อนการเจาะทะลุของเจลซูริมิลดลงพร้อมกับมีการลดลงของค่าความขาวเมื่อระยะเวลาการเก็บรักษาปลาเม็ดขนุนเพิ่มขึ้น ($P<0.05$) ระหว่างการเก็บรักษาปลาเม็ดขนุนในน้ำแข็งเป็นเวลา 15 วัน พบว่าไมโอซินเส้นหนักเกิดการสูญเสียสภาพธรรมชาติแสดงด้วยการลดลงของกิจกรรมของเอนไซม์ Ca^{2+}-ATPase และเกิดการย่อยสลายของโปรตีนดังแสดงจากการจางหายของแถบไมโอซินเส้นหนัก ดังนั้นระยะเวลาในการเก็บรักษาในน้ำแข็งจึงเป็นปัจจัยสำคัญที่มีอิทธิพลต่อการเปลี่ยนแปลงทางเคมี-กายภาพและความสามารถในการเกิดเจลจากปลาเม็ดขนุน

จากการศึกษาการย่อยสลายตัวเองของปลาเม็ดขนุนที่ผ่านและไม่ผ่านการล้าง พบว่ากิจกรรมการย่อยสลายตัวเองเกิดขึ้นสูงสุดที่อุณหภูมิ 60 องศาเซลเซียส แสดงโดยปริมาณเปปไทด์ที่ละลายในกรดไตรคลอโรแอซิติกและการจางหายไปของแถบไมโอซินเส้นหนักปรากฏสูงสุดโดย กิจกรรมการย่อยสลายตัวเองของเนื้อที่ผ่านและไม่ผ่านการล้าง พบสูงสุดที่พีเอช 4 ส่วนที่พีเอช 7 พบกิจกรรมต่ำรองลงมา E-64 แสดงกิจกรรมการยับยั้งการย่อยสลายตัวเองได้สูงสุดในสภาวะกรด (พีเอช 4) ส่วน SBTI แสดงประสิทธิภาพในการยับยั้งในสภาวะที่เป็นกลาง (พีเอช 7) นอกจากนี้กิจกรรมย่อยสลายตัวเองเกิดสูงสุดในสภาวะที่มีเกลือร้อยละ 2 แต่จะลดลงเมื่อความเข้มข้นของเกลือเพิ่มสูงขึ้น (ร้อยละ 2.5-3.5) และกิจกรรมการย่อยสลายตัวเองของเนื้อปลาสูงขึ้นในสภาวะที่มีหนังปะปน

เมื่อใช้โปรตีนเวย์เข้มข้นที่ระดับต่าง ๆ (ร้อยละ 1, 2 และ 3 โดยน้ำหนัก) เพื่อลดการย่อยสลายตัวเองของเนื้อปลาเม็ดขนุน โดยศึกษาการยับยั้งการย่อยสลายตัวเองและสมบัติการเกิดเจลของซูริมิที่เติมโปรตีนเวย์ พบว่าการใช้โปรตีนเวย์เข้มข้นร้อยละ 3 สามารถยับยั้งการย่อยสลายตัวเองของซูริมิจากปลาเม็ดขนุนได้สูงสุดและกิจกรรมการยับยั้งเพิ่มขึ้นในสภาวะที่มีเกลือร้อยละ 2.5 ($P<0.05$) สำหรับค่าแรงและระยะทางก่อนการเจาะทะลุของเจลคามาโบโกะ (40/90°C) และเจลโมโดริ (60/90°C) ร่วมกับแคลเซียมคลอไรด์ที่ระดับ 50 มิลลิโมลต่อกิโลกรัม มีค่าเพิ่มขึ้นเมื่อระดับของโปรตีนเวย์เข้มข้นเพิ่มขึ้น ($P<0.05$) ระดับความเข้มแถบไมโอซินเส้นหนักเพิ่มขึ้นเมื่อระดับของโปรตีนเวย์เข้มข้นสูงขึ้น ซึ่งสัมพันธ์กับการเพิ่มขึ้นของความสามารถในการอุ้มน้ำ อย่างไรก็ตามค่าความขาวมีค่าลดลงเล็กน้อย ($P<0.05$) ที่ระดับของโปรตีนเวย์เข้มข้นเดียวกัน เจลคามาโบโกะที่มีการเติมแคลเซียมคลอไรด์มีสมบัติของเจลสูงกว่าเจลที่ไม่มีการใช้แคลเซียมคลอไรด์ ในทางตรงกันข้ามการเติมแคลเซียมคลอไรด์ส่งผลให้เจลโมโดริมีสมบัติลดต่ำลง เมื่อศึกษาโครงสร้างทางจุลภาคของเจลซูริมิที่เติมโปรตีนเวย์เข้มข้นร้อยละ 3 พบว่าโครงข่ายของเจลมีลักษณะหนาแน่นและเป็นระเบียบมากว่าเจลชุดควบคุม โดยไม่ขึ้นกับการเติมแคลเซียมคลอไรด์

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ABSTRACT

Chemical compositions and thermal properties of goatfish (*Mulloidichthys martinicus*) mince were determined. The mince contained 63.93% myofibrillar protein and 30.88% sarcoplasmic protein. Differential scanning colorimetric (DSC) study revealed that the maximum transition temperatures (T_{max}) and enthalpy (ΔH) of myosin and actin peaks shifted to the lower values in the presence of 2.5% NaCl. However, washing had no impact on the transition of myosin peak, but lowered T_{max} of actin peak. Thermal denaturation and aggregation of natural actomyosin (NAM) extracted from fresh (G_0) and 9 days-ice-stored goatfish (G_9) were comparatively studied. Myosin of G_0 had the higher thermal stability than that from G_9 as indicated by the higher T_{max} as well as the lower K_D value. At temperature above 35°C, G_0 exhibited the higher turbidity, surface hydrophobicity and disulfide bond formation than did G_9 during heating up to 75°C. However, the lower values of all parameters tested were found in goatfish NAM, when compared with bigeye snapper counterpart. As visualized by transmission electron microscopy, network strands of aggregates from bigeye snapper were finer and more uniform than those from goatfish. Therefore, thermal induced gelation was varied with fish species and freshness.

Gel forming ability of goatfish surimi prepared under several setting/heating conditions in the presence of $CaCl_2$ at different levels (0, 25 and 50 mmoles/kg) was investigated. The addition of 50 mmoles $CaCl_2$/kg in combination with setting at 40°C for 30 min, followed by heating at 90°C for 20 min resulted in the highest breaking force and deformation of surimi gel ($P<0.05$). The increased gel strength was associated with the increase in water holding capacity and protein cross-linking. Nevertheless, breaking force and deformation of surimi gels decreased with a concomitant decrease in whiteness as the storage time increased ($P<0.05$). Upon 15 days of iced storage, myosin heavy chain (MHC) underwent denaturation as evidenced by the decrease in

Ca^{2+}-ATPase activity and protein degradation as indicated by the disappearance of MHC. Therefore, storage time was a crucial factor determining the physico-chemical properties and gel forming ability of goatfish during iced storage.

Autolysis of goatfish mince and washed mince was studied. The highest autolytic activity was generally observed in mince and washed mince at 60°C as evidenced by the highest TCA- soluble peptide content and the greatest disappearance of MHC. Autolysis of both mince and washed mince was maximized at pH 4 and a lower autolytic activity was also observed at pH 7. E-64 showed the highest inhibition toward autolysis in the acidic condition (pH 4) and soybean trypsin inhibitor effectively inhibited the autolysis at neutral pH (pH 7). Autolysis was maximized in the presence of 2% NaCl, but decreased as the NaCl concentration (2.5-3.5%) increased and a higher autolysis was obtained in goatfish mince in the presence of skin.

To alleviate the autolysis in goatfish muscle, whey protein concentrate (WPC) at different levels (1, 2 and 3% w/w) was used. Autolysis inhibition and gel properties of goatfish surimi added with WPC were determined. The addition WPC at 3% exhibited the highest inhibitory activity toward autolysis of goatfish surimi. The inhibition was more pronounced in the presence of 2.5% NaCl ($P<0.05$). Breaking force and deformation of both kamaboko (40/90°C) and modori (60/90°C) gels added with 50 mmoles $CaCl_2$/kg increased with increasing WPC ($P<0.05$). MHC band intensity of all gels was more retained as WPC levels increased. The coincidental increase in water holding capacity was obtained, however the whiteness was slightly decreased ($P<0.05$). At all levels of WPC added, kamaboko gels added with $CaCl_2$ exhibited the superior properties to those without $CaCl_2$ ($P<0.05$). On the other hand, $CaCl_2$ addition resulted in the poorer gel properties of modori gel. The microstructure of surimi gels added with 3% WPC generally became denser and more ordered, compared with the control gel, irrespective of $CaCl_2$ addition.

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CHAPTER 1

INTRODUCTION

Surimi is washed fish mince and has been used as raw material for various types of products such as fish ball, fish bar as well as imitation products. Quality of surimi is determined by its gel forming ability, which is governed by both intrinsic and extrinsic factors. Different fish species exhibit the different gel properties, mainly due to the differences in protein aggregation as well as bondings stabilizing the gel networks (Benjakul *et al*., 2001a). Freshness of fish has been realized as the major factor affecting the surimi quality. Additionally, integrity of myofibrillar proteins is of prime importance for surimi production. Superior surimi gel quality cannot be produced from denatured or degraded myosin (Benjakul *et al*., 1997). Proteolysis of fish muscle has generally been recognized to result from endogenous proteinases and associated with the quality loss of fish flesh as well as the softening of gel products. Endogenous proteinases, especially heat activated and heat stable proteinases, have been known to play an essential role in surimi gel weakening (Benjakul *et al*., 2003b, e). Disintegration of surimi gels is caused by severe degradation of myofibrillar proteins, particularly myosin at temperature above 50°C (Jiang, 2000). A rapid breakdown of myofibrillar protein inhibits development of a three-dimensional gel network. To alleviate the gel weakening caused by endogenous proteinases, some protein additives such as bovine plasma proteins, porcine plasma proteins, chicken plasma proteins, egg white, potato extract, etc., have been widely used to enhance the gel quality via inhibition of proteolysis (Wasson *et al*., 1992; Reppond and Babbitt, 1993; Visessanguan *et al*., 2000a; Benjakul *et al*., 2004; Rawdkuen *et al*., 2004b). However, the applications of BPP and EW in surimi have recently been limited by the outbreak of mad cow disease and allergy problem, respectively. Additionally, the addition of blood plasma to surimi or surimi products rendered the end product with off-color and off-flavor (Rawdkuen *et al*., 2004b) as well as esthetic and religious objections. To improve the gel property, the enhancement of endogenous TGase by $CaCl_2$ addition is the promising alternative means, in which protein cross-linking via the formation

of ε-(γ-glutamyl lysine) isopeptide occurs (Seki *et al*., 1990). Formation of large aggregates is presumably a prerequisite to formation of elastic gel (Chan, 1992b).

Thailand is one of the largest surimi producer in Southeast Asia which has exported for several countries. In 2007, Thailand exported 23,646.24 tons of surimi with a value of 1,747.05 million baths (Fishery economic devision, 2008). In general, surimi is served as a potential raw material for a variety of seafood products, which become more increasingly popular due to their unique textural properties as well as high nutritional value. However, raw materials for surimi production such as threadfin bream (*Nemipterus spp*.), bigeye snapper (*Priacanthus spp*.) and lizardfish (*Saurida spp*.) have been decreasing. Goatfish is an abundant species in the Gulf of Thailand and has low commercial values. So far it has become more economically important as a raw material for surimi production. Nevertheless, goatfish surimi generally exhibited poor gel property. Similar to other underutilized fish species, this common problem is associated with the high endogenous proteolytic activity in the muscle (Greene and Babbitt, 1990). Nevertheless, no basic information on gelation of this species as affected by freshness has been reported. Additionally, autolysis study and the use of food grade protein additive, particularly whey protein concentrate, have not been carried out. The maximization of setting together with the addition of protein additive could lead to the improved gel quality. Therefore, the information gained can be applied to improve the quality of gel from goatfish surimi.

Literature Review

1. Fish muscle proteins

Protein is a major composition of fish muscle, ranging from 15 to 20% (wet weight), but protein content is reduced in a spawning period (Almas, 1981). Protein compositions of fish vary depending upon muscle type, feeding period and spawnig, etc. Apart from proteins, non-protein nitrogenous compounds such as TMA has a distinctive fishy, amine like odor, which directly affects the sensorial property of fish and fish products. TMA is derived from TMAO, which is abundant in marine fish (Cadwallader, 2000; Debevere and Boskou, 1996). In general, washing can remove those components and concentrate the desirable myofibrillar protein (Kudo *et al*., 1973). Fish muscle protein can be divided into three major groups on the basis of solubility characteristics including sarcoplasmic protein (water-soluble), myofibrillar protein (salt soluble) and stroma protein (insoluble) (Xiong, 1997).

1.1 Sarcoplasmic proteins

Sarcoplasmic proteins are located inside the sarcolemma and are soluble in low salt concentrations (<0.1 M KCl). Sarcoplasmic proteins comprise about 30-35% of the total muscle proteins (Xiong, 1997), including myoglobin, enzymes and other albumin (Sikorski, 1994). Sarcoplasmic enzymes influencing the quality of fish include the enzymes of the glycolytic pathway and the hydrolytic enzymes of the lysosomes (Sikorski *et al.,* 1990). Sarcoplasmic proteins have an adverse effect on the strength and deformability of myofibril protein gels via an interference with myosin cross-linking during gel matrix formation (Sikorski, 1994).

1.2 Myofibrillar proteins

The major proteins in fish muscle are myofibrillar proteins. These protein account for 65-75% of total proteins in muscle, compared with 52-56% in mammals (Mackie, 1994). The myofibrillar proteins are also mainly responsible for the water holding capacity of fish, for the textural development of fish product, as well as for the functional properties of fish minces and homogenate (Sikorski *et al*., 1990; Zayas, 1997). Myofibrillar proteins undergo changes during the rigor mortis and extended frozen storage (Shahidi, 1994). The texture of fish products and the gel-forming ability of fish minces and surimi may also be affected by the changes of myofibrillar proteins (Shahidi, 1994). Myofibrillar proteins are soluble in solution of neutral

salts with ionic strength less than 0.5 and are often called the "salted-soluble protein". Kalakowska *et al*. (1976) recommended that NaCl solution at 2.5-3.0% could be used for extraction of myofibrillar proteins. Myofibrillar proteins can be further divided into two subgroups as follows:

1.2.1 Contractile proteins

1.2.1.1 Myosin

Myosin is a large fibrous protein with a molecular weight of about 500,000 daltons (Ogawa *et al*., 1994). It is the most abundant myofibrillar component, constituting approximately 40-60% of total proteins content (Bechtel, 1986). Myosin consists of six polypeptide subunits, two large heavy chains and four light chains arranged into an asymmetrical molecule with two pear-shape globular heads attached to a long α-helical rod-like tail (Figure 1) (Xiong, 1997). The two globular heads with ATPase activity are relatively hydrophobic and are able to bind actin. The rod portion is relatively hydrophilic and is responsible for the assembly of myosin into thick filaments. Proteolytic treatment by trypsin cleave myosin at the hinge region yielding two fragments called heavy meromyosin or HMM (head portion) and light meromyosin or LMM (tail portion), respectively. HMM retains all enzymes activity and actin- binding ability. Treatment of HMM with papain results in the formation of two additional fragments termed S-1 (globular head) and S-2 (rod portion) (Xiong, 1997).

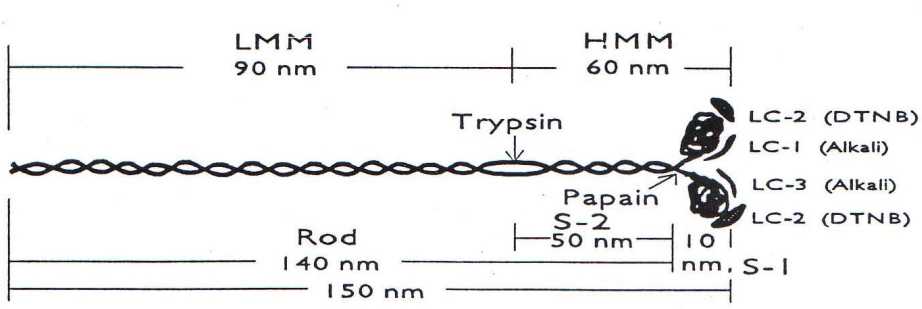


Figure 1 Schematic presentation of the myosin molecule. Light meromyosin (LMM), heavy meromyosin (HMM), rod, S-1 and S-2 subfragments of HMM, the light chains, and the hinge regions susceptible to trypsin and papain are indicated.

Source: Xiong (1997)

1.2.1.2 Actin

Actin is the second most abundant myofibrillar protein, constituting about 22% of the myofibrillar mass. Actin can exist either as monomer (G-actin) or in a fibrous form (F-actin). Normally, actin exists as double-helical filaments (F-actin) composed of polymerized globular monomers. Each monomer has a molecular weight of approximately 43,000 daltons (Xiong 1997). In muscle tissue, actin is naturally associated with tropomyosin and troponin complex. It also contains a myosin-binding site, which allows myosin to form temporary complex with it during muscle contraction or the permanent myosin-actin complex during rigor mortis development in postmortem meat (Sikorski, 1994).

1.2.1.3 Actomyosin

When actin and myosin are mixed *in vitro*, a complex, call actomysin, is formed. The complex is bound not by covalent, but by electrostatic bonding with the contribution of phosphate groups (Xiong, 1997). The reconstitute actomyosin produce from both component proteins demonstrates many biochemical and physicochemical features of myosin, however does not exhibit physicochemical and function features of F-actin (Kijowski, 2001). This complex can be dissociated by the addition of ATP or high ionic strengths (Xiong, 1997). Actomyosin is the main state of actin and myosin in postmortem muscle because ATP is depleted by postmortem metabolism (Ochai and Chow, 2000).

1.2.2 Regulatory proteins

Tropomyosin and troponin constituting 5% of total protein are the main proteins that play an important part in regulation of muscle contraction (Kijowski, 2001). Myofibrils also contain other regulatory protein at lower quantities. They are present in the myofibril filament structure, e.g. A-band, I band, Z-disc, namely, α-, β-, γ-actinin, C-, M-, H-, and X-protein paramyosin and others (Kijowski, 2001; Xiong, 1997).

1.2.2.1 Tropomyosin

Tropomyosin is a dimeric molecule consisting of two dissimilar subunits designated α- and β- tropomyosin with molecular weights of about 34,000 and 36,000 dalton, respectively. Each tropomyosin molecule is about 385 A° long and associates in head-to-tail fashion to form a filament that follows and associates with the coil of the F-actin filament

(McCormick, 1994) (Figure 2a). Tropomyosin is about 5% of myofibrillar protein. Each tropomyosin molecule consists of 7 molecules of G-actins (Foegeding *et al.,* 1996).

1.2.2.2 Troponin

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

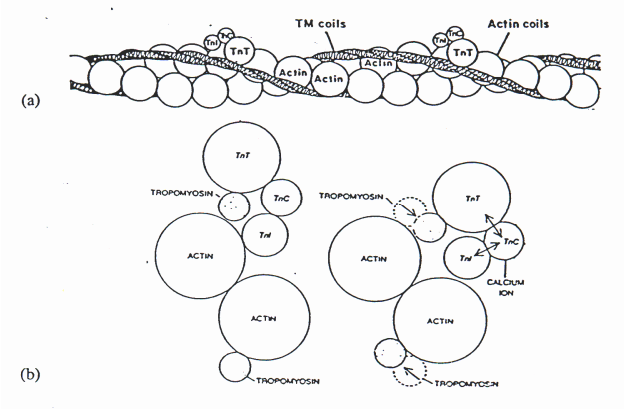


Figure 2 Thin filament of muscle formed by the filament of tropomyosin molecules wound in each of the two grooves of the actin helix (a) and proposed model for configuration of actin, tropomysin and troponin (Tn) subunits (b)

Tn T = troponin-tropomyosin subunit

Tn I = troponin-inhibitory subunit

Tn C = troponin-calcium-binding subunit

Source: McCormick (1994)

1.3 Stroma protein

The stroma is composed of connective tissue proteins, such as collagen and elastin, representing approximately 3% of total protein content of fish muscle (Suzuki, 1981). The stroma

is the residue after extraction of the sarcoplasmic and myofibrillar proteins. Generally, the stroma is insoluble in dilute solutions of hydrochloric acid or sodium hydroxide (Sikorski *et al.,* 1990).

2. Postmortem changes of fish proteins

Functional properties of muscle protein are closely associated with the integrity of proteins. Denaturation and degradation mainly contribute to the loss of those functionalities. Freshness is generally considered as the most crucial factor determining the final gel quality (Benjakul *et al*., 2003d). Surimi gel quality is affected by time and temperature of fish between capture and processing (Yean, 1993). Chilling is a means to preserve fish before processing and consumption. The growth of bacteria, enzymatic and chemical reactions are slowed down. However, the biochemical and physicochemical properties of fish and textural properties changes during cold storage have been reported (Chomnawang *et al*., 2007).

Among post-mortem changes, degradation of fish muscle caused by microbial and endogenous proteinases is a primary cause of quality losses during cold storage or handling (Haard *et al.,* 1994). The autolysis of nucleotides as well as nitrogenous compounds become more intense after prolonged storage, particularly under inappropriate condition (Gill, 1992). Benjakul *et al.* (1997) reported that MHC of Pacific whiting muscle was hydrolyzed continuously throughout iced storage. MHC decreased to 45% of the original content within 8 days, whereas no changes in actin were observed. Proteinases are able to hydrolyze the muscle proteins differently. An *et al.* (1994b) reported that among the Pacific whiting proteins, MHC was the most extensively hydrolyzed, followed by troponin-T, α- and β-tropomyosin, respectively.

Denaturation of muscle protein during postmortem storage is another phenomenon causing the changes of fish quality. Myofibrillar ATPase activities have been widely used as measure of myosin or actomyosin integrity. ATPase (E.C.3.6.1.8 pyrophosphohydrolase) is associated with the postmortem disappearance of ATP in fish muscle, leading to rigor mortis (Nambudiri and Gopakumar, 1992). Normally, Ca^{2+}-ATPase has been used as an indicator of myosin integrity (Benjakul *et al*., 1997). Myofibrillar adenosine triphosphate (ATPase) is located in the myosin head region, which has ability to hydrolyze ATP in the presence of Ca^{2+} ion.Therefore, a decrease of Ca^{2+}-ATPase activity implied conformational changes of myosin (Yongsawatdikul and Park,

2002). The loss in Ca^{2+}-ATPase activity of lizardfish during iced storage was associated with the proteolysis of myosin molecules (Benjakul *et al.* 2003d). Kamal *et al*. (1991) reported that myofibrillar ATPase activities of sardine ordinary and dark muscles decreased during extended iced storage of 10 days. Benjakul *et al*. (1997) found that the total SH content of natural actomyosin increased slightly after 2 days of iced storage, followed by a gradual decrease up to 8 days. A decrease in total SH group was reported to be due to formation of disulfide bonds through oxidation of SH group or disulfide interchanges (Hayakawa and Nagai, 1985). During the extended iced storage, many hydrophobic groups are exposed at the molecular surface as protein unfolds (Kato and Nakai, 1980), leading to the aggregation of protein. Changes of protein conformation and degree of unfolding have the impact on protein functionalities. Benjakul *et al*. (2003d) reported that gel strength of kamaboko made from lizardfish decreased rapidly during iced storage. Thus, pretreatment of fish, such as heading and eviscerating could retard the denaturation and physicochemical changes of fish protein during iced storage (Benjakul *et al.,* 2003d).

3. Gelation of fish muscle proteins

Fish muscle proteins typically undergo heat-induced gelation, which plays a role in texture development of many seafood gelly products. Among fish muscle proteins, myofibrillar proteins mainly contribute to functional properties including gelation (Benjakul *et al*., 2001a; Ko *et al*., 2007). Myosin or actomyosin are the major protein in myofibrils and mainly involve in gelation of fish proteins. Generally, myosin alone form excellent gels and actin has a synergistic or antagonistic on myosin gelation, depending upon the myosin/actin ratio in the gelling system (Grabowska and Sikorski, 1976; Matsumoto, 1980). A specific actin/myosin weight ratio (~1/15) favors the formation of a stronger gel than that from myosin alone (Ishioroshi *et al*., 1980; Yasui *et al.,* 1980). Gelation of fish protein generally involves denaturation and aggregation. Denaturation is a process, in which proteins undergo conformational changes, primarily unfolding without alteration of the amino acid sequence. Then, protein-protein interactions, known as association, aggregation and polymerization, take place and a three-dimensional network can be formed. Normally, gel are formed when partially unfolded proteins developed uncoiled polypeptide segments that interact at specific points to form a three dimensional cross-linked network (Zayas,

1997) and capable of holding water. Table 1 gives a summary of changes, which may occur during the heat denaturation of natural actomyosin.

Table 1 Conformational change occurring during the thermal denaturation of natural actomyosin

Temperature (°C)	Protein (s) or segment involved	Description of events
30-35	Native tropomyosin	Thermal dissociation from the F-actin backbone
38	F-actin	Super helix dissociates into single chain
40-45	Myosin	Dissociated into light and heavy chains
	Head	Possibly some conformational change
	Hinge	Helix to random coil transformation
45-50	Actin, myosin	Actin-myosin complex dissociates
50-55	Light meromyosin	Helix to coil transformation and rapid aggregation
>70	Actin	Major conformational changes in the G-actin monomer

Source: Ziegler and Aton (1984)

3.1 Denaturation or dissociation

Muscle proteins undergo structural and conformational changes during heating, leading to promoting the interaction among protein molecules (Xiong, 1997). Conformational changes of proteins occurring on heating are usually called denaturation (Tornberg, 2005). When protein denatures, both inner- and intramolecular bonds are disrupted or the protein is thought to change its conformation from a highly ordered state to a less ordered counterpart (Kilara and Harwarwakar, 1996). Thermal transition temperatures (T_{max}) represent point where conformational changes in protein structure take place. Generally, three major transition peaks are found in fish muscle, depending on species which related to the environmental at which fish live. Peak transition temperature (T_{max}) for the protein in cod muscle was reported by Thorainsdottir *et al.* (2002). The first transition (zone A) at 43.5°C is attributed to myosin (Figure 3). The second transition (zone B), which occurs at 59.3°C, was assigned to collagen or sarcoplasmic proteins. This peak is a smaller peak usually seen at a temperature intermediate between the myosin and actin denaturation peaks. The third transition (zone C) has been assigned to actin and is found at 73.6°C. Thermal denaturation of hake (*Merluccius hubbsi*) myofibrillar proteins of whole muscle

showed two T_{max}, 46°C and 75°C (Beas *et al.*, 1990). Myosin of cod (*Gadus morhua*) which lived in cold water was more labile, compared with that of snapper (*Lutjanus sebae*) which lived in tropical area (Davies *et al.*, 1988). However, fish muscle proteins are less stable than mammalian protein (Ogawa *et al.*, 1994). Kijowski and Mast (1988) reported that chicken breast muscle had T_{max} of 58.9, 68.7 and 78.7°C. In general, myosin is unstable and prone to denaturation than actin (Hasting *et al.*, 1985).

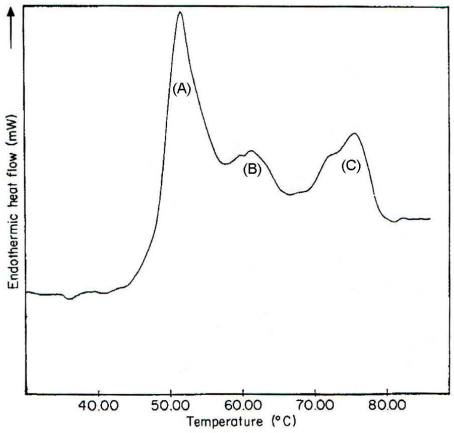


Figure 3 A typical thermal curve of whole muscle involving three major zones: myosin (A) subunits; sarcoplasmic proteins or collagen (B); actin (C)

Source: Adapted from Howell *et al.* (1991)

During the dissociation, protein is unfolded or denatured by modifying the charged groups through pH changes or addition of salt or water-competitive compounds under appropriate conditions (Zayas, 1997). Normally, addition of salt in combination of heating process is two major factors involved in denaturation and gelation of muscle proteins. Salts have been used as important ingredient for surimi gel production. Salts can disrupt the ionic linkages and assist in the dispersion of the proteins, which are necessary for the development of an elastic structure in heat-set-gel (Niwa, 1992). The salts ion (Na^+ and Cl^-) selectively bind to the oppositely charged groups exposed on the protein surface (Figure 4). As a consequence, the intermolecular ionic lingkage among the myofibrillar proteins are ruptured, and the proteins are dissolved in water because of their increased affinity for water (Lanier, 2000). The addition of salt shifted the denaturation transitions to lower temperatures and decreased the enthalpies of heat denaturation. Hastings *et al.* (1985) reported that salt treatment of herring muscle decreased transition temperature by 5-10 K

and peak area was also decreased by salt addition. This might cause a partial unfolding of proteins and increased sensitivity to denaturation (Park and Lanier, 1989). Park and Lanier (1990) reported that 3% salt addition in washed tilapia muscle shifted endothermic peaks to lower temperature when compared to the thermogram of the control.

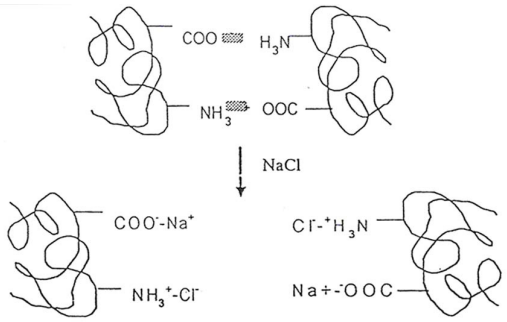


Figure 4 Model of salt linkage formation among myofibrillar protein

Source: Adaped from Niwa (1985)

3.2 Aggregation

Aggregation of protein results from the association of protein molecules Denatured proteins begin to interact noncovalently to form a fine elastic network when surimi sol is subjected to heating (Lanier, 2000). Differences in cross-linking of myosin heavy chain contribute to the differences in gel forming ability among the muscles of various fish (Benjakul *et al.*, 2001a). Different composition and structure of MHC among various fish species may be responsible for species-to-species differences in gelation (Visessanguan *et al*., 2000b). Samejima *et al.* (1981) reported that heat-induced gelation of myosin can be represented by two reactions. One is the aggregation of the globular head portion of the molecule which is complementary to and closely associated with the oxidation of SH groups and the other is the inevitable network formation by the thermal unfolding of the helical tail portion (Samejima *et al*., 1981). Aggregation of myosin during heating is solely due to the association of the head portions (Samejima *et al*., 1981). In the sol state, the rod chains have 100% helix-coil transition, which is sufficient to provide cross-links for a continuous network. The unfolding tends to prevent the gel from becoming progressively more tightly cross-linked. Chan *et al*. (1993) 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. Both HMM and LMM are involved in the thermal aggregation of cod and herring myosin. 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 aggregate at higher temperatures (Figure 5). Gill and Conway (1989) concluded that the tail rather head portion of myosin was involved in the thermal aggregation of cod myosin. Furthermore, Sano *et al.* (1994) reported that the natural actomyosin (NAM) molecule extracted from carp muscle began to unfold around 30°C and substantial unfolding occurred from 30°C to 50°C, which was mainly attributed to the interaction of LMM.

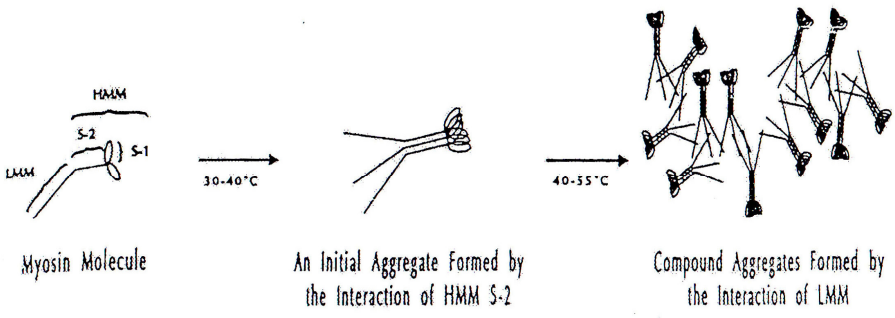


Figure 5 A schematic representation of the thermal aggregation of fish myosin

Source: Chan *et al.* (1993)

3.2.1 Factors affecting gelation

In general, the characteristics of gel are governed by many factors including temperature (Sano *et al.*, 1994), heating rate (Yongsawatdigul and Park, 1999), pH (Shikha *et al.*, 2006) and type of actomyosin used (Lefevre *et al.*, 2007). Muscle protein gel network is influenced by a number of factors as shown in Figure 6.

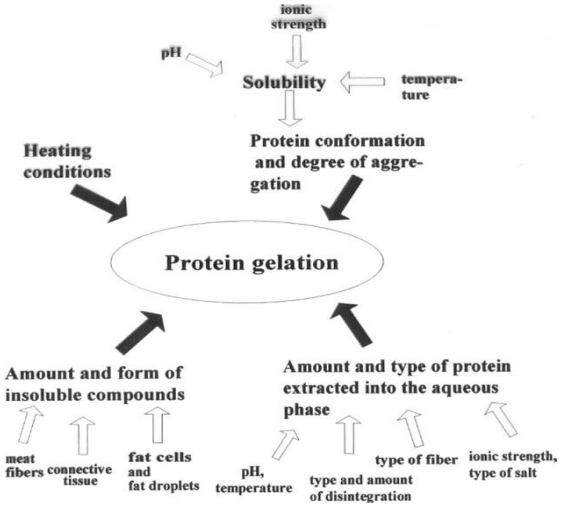


Figure 6 Factors affecting protein gelation of a meat batter

Source: Tornberg (2005)

3.2.1.1 Effect of heating rate and time

The heating rate or time of heating affect the unfolding and appear to influence the gel formed. When the rate of aggregation is slow to cause denaturation, heat-denatured proteins are allowed to algin in an ordered fashion to form a fine gel network, resulting in more elastic gels (Hermasson, 1979). Gill *et al.* (1992) demonstrated that slow heating rate yielded more elastic gel of herring muscle. Yongsawatdigul and Park (1996) indicated that the effect of heating rate on textural properties of fish myofibrillar proteins was species-dependent. Myofibrillar proteins of Alaska pollock formed stronger gels at slower heating rate (e.g. 0.5°C/min) while those of Pacific whitting exhibited higher shear stress and shear strain at higher heating rate (e.g. 30°C/min). This was attributed to the absence or presence of predominant endogenous enzyme in each species. Yongsawatdigul and Park (1999) concluded that slow heating rate activated endogenous protease associated with whiting myosin, leading to the hydrolysis of myosin and lowering gel elasticity. However, it promoted the aggregation of cod myosin and enabled aggregates to form a more elastic gel.

3.2.1.2 Effect of muscle type

Differences in thermal gelation properties of muscle protein relate to muscle type origin (red and white). In brown trout muscle, myosin extracted from white muscle form gels at lower temperature than red counterpart (Lefevre *et al*., 1998). Red muscle protein extracted from salmon had higher transition temperatures (Lefevre *et al*., 2007). Chaijan *et al.*

(2004) reported that surimi produced from mackerel and sardine exhibited poor gel quality, because of high content of dark muscle associated with high content of lipid and myoglobin. In addition, dark muscle also has a higher proteolytic activity than white muscle (Shimizu *et al*., 1992). Ochiai *et al.* (2001) suggested that high-quality surimi with higher gel strength and better whiteness can be obtained when dark muscle is removed as much as possible.

3.2.1.3 Effect of pH

The pH of the salted paste is one of the most important factors in producing a strong elastic kamaboko gel. Postmortem lowering of muscle pH induced the denaturation of the myofibrillar protein. The solubility was decreased with lowering of fish meat pH. Shimazu *et al.* (1954) reported that the gel strength was high at pH 6.5-7.0 for flying fish and became low by acidifying. The net charge of protein at its isoelectric point is equal to zero. The greater the net charge on the protein molecules, the greater the electrostatic repulsion between molecules is obtained, preventing the interactions required to form a gel matrix (Totosaus *et al*., 2002). Shikha *et al*. (2006) reported that gel strength produced from walleye pollack surimi decreased with decreasing pH value. Neutralization of acidified surimi (pH 6.0 reneutralized to pH 7.2) could improve gel strength, however it was considerably lower than original gel strength (pH 7.2). Park *et al.* (2008) found that the solubility of myosin purified from Pacific sardine was low at pH range of 2.5-5.0, which was possibly due to the pI of myosin (pH 4.6-6.2), resulting in the poor gel forming ability.

3.2.2 Formation of gel network

The formation of gel network during gelation process is associated with various bonds. Types and numbers of bonds directly affect the characteristics and properties of protein gels.

3.2.2.1 Hydrogen bonds and salt linkage

Hydrogen bonds are weaker dipole bonds not responsible for the gelation of myofibrillar proteins but are important in the stabilization of bound water within the hydrogel. Normally, hydrogen bonds between proteins are more numerous when the gel is colder, but it is destabized by heating (Lanier, 2000). A large amount of water molecules are hydrogen bonded to the polar amino acid residues, which are abundantly exposed on the molecular surface of heat-denatured proteins (Lanier, 2000). The tyrosine, serine, hydroxylproline and threonine residues

contain a hydroxyl group and the proline and hydroxyproline residue contains an imino group, both of which act as proton donors and acceptors (Niwa, 1992). Furthermore, calcium chloride has been use to enhance the gel strength of surimi. Calcium ion can form salt linkages between negatively charges localized on the adjacent proteins, resulting in the strengthening of surimi gel by calcium bridge (Wan *et al*., 1994).

3.2.2.2 Hydrophobic interaction

Hydrophobic interactions play an important role strengthening of surimi gel. Generally, hydrophobic interactions are formed by increasing temperature, at least to near 60°C (Lanier, 2000). About 25% of the amino acids that constitute the myosin molecule are hydrophobic amino acids such as alanine, valine, leucine, isoleucine, proline, tryptophan and phenylalanine. Normally, the interior portion of the folded protein chain has the greater density of hydrophobic amino acids. Conversely, the amino acids on the surface of the undenatured protein are largely hydrophilic. When a protein denatures (unfold) during heating, the hydrophobic core is exposed to the surface, leading to hydrophobic interaction between the hydrophobic residue. Sano *et al*. (1994) found that carp myosin started to unfold hydrophobic domains when heated to 30°C and extensively increased when heated to 50°C. The increase of hydrophobicity also indicated the involvement the hydrophobic interaction in gel formation (Chan *et al.,* 1992a). Yongsawatdikul and Park (2003) reported that the hydrophobic interaction of threadfin bream actomyosin took place at 70°C. Hydrophobic interaction is a major force involved in myosin aggregation at high temperature (Visessanguan *et al*., 2000b). Benjakul *et al*. (2001a) suggested that the higher surface hydrophobicity was in agreement with the higher aggregation of bigeye snapper natural actomyosin.

3.2.2.3 Disulfide bonds

During heating at high temperatures (cooking at >40°C), disulfide bonds are the predominant covalent bond contributing to gel formation of proteins. Generally, the rate of disulfide bond formation of most native protein is low. It can be increased by unfolding the native structure via applying high pressure, elevated temperature, shears or as a result of addition of component that affect the solvent quality (Visschers *et al*., 2005). In general, most of sulfhydryl groups (~68%) in myosin are located in the head portion of myosin (Lowry *et al*., 1969). S-1 (globular head) and rod (fibrillar tail) fractions of myosin primarily involved in the dimerization

and polymerization of myosin, respectively (Kishi *et al*., 1997). Benjakul *et al*. (2001) found that disulfide bonds in natural actomyosin from *P. tayenus* and *P. macacanthus* were formed at temperatures of 45°C and 40°C, respectively. Yongsawatdikul and Park (2003) found that reactive SH increased as heating progressed and decreased at 50°C, suggesting the formation of inter or intra molecular disulfide bonds of threadfin bream actomyosin at >50°C. Ko *et al*. (2007) reported that the decreases in the total SH content of tilapia actomyosin observed at 55°C and 65°C due to the formation of disulfide bonds.

3.2.2.4 Non disulfide covalent bond

The cross-linking reactions other than disulfide bonding involve in the setting phenomenon (Niwa, 1992) at temperature ranging from 0 to 40°C (Lanier, 1992). The unique setting ability of surimi is thought to mainly result from the enzymatically catalyzed formation of nondisulfide covalent bonds between protein molecules, known as induced by endogenous transglutaminase (TGase). TGase is a transferase that catalyzes the acyl transfer reaction between γ-carboxyamide groups of glutamine serves as the acyl donor and ε-amino groups of lysine acts as the acceptor, resulting in protein polymerization via ε-(γ- glutamyl) lysine cross-linkings (Folk, 1980) (Figure 7). Benjakul and Visessanguan (2003) found that setting of surimi paste with and without subsequent heating resulted in the increase in both breaking force and deformation of suwari and kamaboko gel from bigeye snapper surimi, respectively. However, TGase mediated cross-linking reaction of MHC depends upon species (Araki and Seki, 1993) and setting temperatures (Benjakul and Visessanguan, 2003). The maximum gel strength and MHC cross-liking ability of Alaska pollock surimi was found with setting temperature of 25°C, while the maximum cross-linking by endogenous TGase occurred at 40°C in croaker surimi (Kamath *et al.*, 1992). Benjakul *et al.* (2003a) reported that high temperature setting at 40°C for an appropriate times enhanced gel strength of surimi from some tropical fish. Additionally, endogenous TGase is a Ca^{2+}-dependent enzyme. The addition of Ca^{2+} to fish protein paste has been reported to activate TGase activity and the strengthened gels are obtained (Benjakul and Visessanguan, 2003; Lee and Park, 1998).

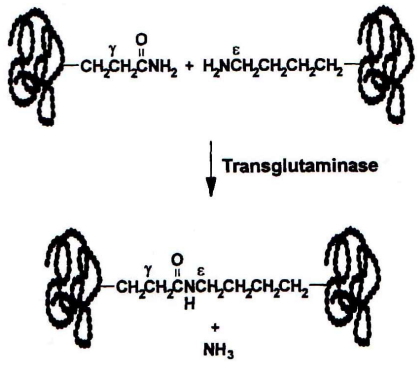


Figure 7 Cross-linking of proteins by transglutaminase

Source: An *et al.* (1996)

4. Proteolytic enzymes

Proteinase is the generic name given to those enzymes hydrolyzing the peptide bond in proteins and some synthetic substrates and coded as the EC 3.4.11-99. (Garcia-Carreno and Hernandez-Cortes, 2000).

4.1 Classification of proteinases

Proteinases may be classified based on their similarities to well characterized proteinases, as trypsin-like, chymotrypsin-like, etc., their pH profiles as acid, neutral or alkaline proteinases, substrate specificity and mechanism of catalysis (Haard and Simpson, 1994). Additionally, proteinases are classified according to their source (animal, plant, microbial), their catalytic action (endopeptidase or exopeptidase) and the nature of the catalytic site. In EC system for enzyme nomenclature, all proteinases belong to subclass 3.4, which is further divided into 3.4.11-19, the exopeptidases and 3.4.21-24, the endopeptidases or proteinases (Nissen, 1993). Endopeptidases cleave the polypeptide chain at particularly susceptible peptide bonds distributed along the chains, whereas exopeptidases hydrolyze one amino acid from N terminus (amino peptidases) or from C terminus (carboxypeptidases) (Figure 8).

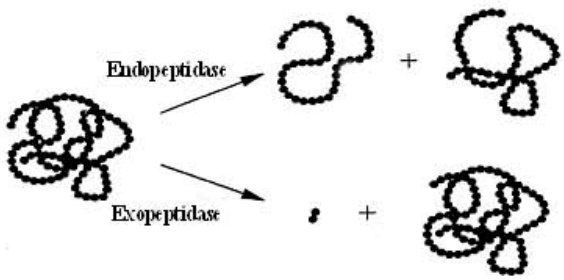


Figure 8 Action of endopeptidase and exopeptidase on protein structure

Source: An *et al*. (1996).

4.1.1 Endopeptidases

The four major classes of endopeptidases can be distinguished according to the chemical group of their active site, including serine proteinases, (EC 3.4.21), cysteine proteinases (EC 3.4.22), aspartic proteinases (EC 3.4.23) and metalloproteinases (EC 3.4.24) (Nissen, 1993).

Serine, cysteine and aspartic proteinases have serine, cysteine and aspartic acid side chains, respectively, as a part of the catalytic site (Garcia-Carreno, 1996). Modification or blocking of this side chain usually leads to complete inactivation of the enzyme and is a standard way of determining the nature of an unknown proteinase (Nissen, 1993). The serine proteinases have maximum activity at alkaline pH, while the closely related cysteine proteinases usually show maximum activity at more neutral pH values. The aspartic proteinases generally have maximum catalytic activity at acid pH. The metalloproteinases contain an essential metal atom, usually Zn and have optimum activity near neutral pH. Ca^{2+} generally stabilizes these enzymes and strong cheleating agents, such as EDTA inhibit the activity (Nissen, 1993).

4.1.2 Exopeptidases

The aminopeptidases (EC 3.4.11) are ubiquitous, but less readily available as commercial products, since many of them are intracellular or membrane bound. Carboxypeptidases are subdivided into serine carboxypeptidase (EC 3.4.16), metallocarboxypeptidases (EC 3.4.17) and cysteine carboxypeptidases (EC 3.4.18) according to the nature of the catalytic site (Nissen, 1993).

5. Muscle proteinases

Although proteolytic activity in skeletal muscle is generally much lower than in other tissues, it plays a significant role in protein turnover during growth and development of the live animal and in pathological conditions of the muscles (Kang and Lanier, 2000). These muscle proteinases are found in the sarcoplasmic (or soluble) component of muscle tissues, in association with cellular organelles, connective tissues, myofibrils and in the interfiber space (Asghar and Bhatti, 1987; Haard, 1992). The physicochemical/catalytic properties and levels of these enzymes are influenced by various factors such as age, diet, exercise, habitat temperature etc. Some of the proteinases which have been widely studied due to their influence on the quality of food products prepared from muscles include acid, neutral and alkaline proteinase (Kang and Lanier, 2000). Fish muscle contains a wide range of proteinases (Table 2) (Kolodziejska and Sikorski, 1996).

5.1 Classification of fish muscle proteinases

5.1.1 Acid proteinases

Lysosomal cathepsins are responsible for the intracellular protein degradation that is important in living tissue physiologically and pathologically. Cathepsins are recognized as families of endopeptidases and/or exopeptidases. Many cathepsins have acidic pH optimum although some are most active at neutral pH. Siebert (1958) reported the catheptic acitivity of muscle from various fish including cod, herring, sole, flounder, trout and carp. In carp muscle, three cathepsins were subsequently isolated, denoted as A, B and C (Makinodan and Ikeda, 1971). During spawning migration, salmon exhibited three to seven times higher activity of cathepsin B, D, H, and L compared with those measured during feeding migration (Yamashita and Konagaya, 1990). Additionally, cathepsin L is implicated in surimi gel degradation during cooking (An *et al*., 1994a). A list of cathepsins identified in muscle tissue and those known to be present in fish myosystems is summarized in Table 3 (Goll *et al.,* 1989).

Table 2 End opeptidases of fish muscle

Enzymes		Optimum		Effect of muscle proteins
		pH	Temperature (º C)	
Cysteine proteinases	Calcium-activate proteinase	6.9 - 7.5	30	Cleave of myofibrillar proteins to TCA soluble fragments, degradatiom of cytoskeletal proteins
	Cathepsin L	5.0 – 5.6	40 -50	Hydrolysis of most myofibrillar proteins, cleaving of telopeptides from type I collagen
	Cathepsin B	5.7 – 6.0		Slight hydrolysis of myosin, actin, nebulin and troponin T
	Cathepsin C	6.0 - 6.5		
	Heat-activated cysteine proteinase	6.0 – 8.5	55 - 65	Hydrolysis of myosin
Serine proteinases	Heat-activated trypsin - like proteinases	6.2 – 8.0	50 – 60	Hydrolysis of myosin
	Multicatalytic proteinases	6.0 –10.0	60 – 65	Hydrolysis of myosin
	Other trypsin – like proteinases	8.0 – 9.0	37 - 40	Hydrolysis of isolated myosin, disintegration of the cytoskeletal and contractile elements of intact myofibrils
Aspartyl proteinases	Cathepsin D	5.5	30	Hydrolysis of myosin
Metallo proteinases	Neutral proteinase	7.2	40	
	Heat stable alkaline proteinases	7.0 – 8.0	50	Hydrolysis of type I collagen, gelatin and other cytoskeletal matrix proteins
	Myosinase I and II	7.0	40	Hydrolysis of myosin

Source: Adapted from Kolodziejska and Sikorski (1996)

Table 3 Proteolytic enzymes associated with muscle lysosomes

Enzyme	Family	Activity	Fish
Cathepsin B_1 (cathepsin B)	Cysteine	Endopeptidase	Purified from muscle of several species; identified in many species
Cathepsin H (cathepsin B_3)	Cysteine	Endopeptidase	Identified in salmon muscle
Cathepsin L	Cysteine	Endopeptidase	Identified in salmon and mackerel muscle
Dipeptidyl peptidase I (cathepsin C)	Cysteine	Exopeptidase	Identified in muscle from several species
Cathepsin D	Aspartic	Endopeptidase	Purified and identified in muscle from many species
Carboxypeptidase A (cathepsins A and I)	Serine	Exopeptidase	Purified from several species and identified in muscle from many species
Cathepsin S	Cysteine	Endopeptidase	Tentatively identified in mackerel muscle

Source: Adapted from Goll *et al.* (1989)

a) Cathepsin A

Cathepsin A (EC3.4.16.1), now called carboxypeptidase A, is defined as an enzyme splitting carbobenzoxy-L-Glu-L-Tyr and is capable of splitting residues sequentially from the carboxyl terminus of peptides like glucagon. The exopeptidase has a pH optimum of 5-6 and is readily inactivated by heat and alkaline and normally has a molecular mass of 35 kDa. In mammals, the enzyme is believed to act synergistically with endopeptidases such as cathepsin D (Haard *et al*., 1994). Carp cathepsin A, when added simultaneously with cathepsin D, increases hydrolytic activity of the latter enzyme. Cathepsin A further hydrolyze the peptide products released by cathepsin D (Makinodan *et al.,* 1983).

b) Cathepsin B

Cathepsin B is the best known and most thoroughly investigated lysosomal thiol proteinase. It is now recognized that at least two enzymes have this activity including cathepsin B (B_1) and B_2. Cathepsin B_1 is a thiol endopeptidase, having a molecular weight of 24-28 kDa with a pI of 5.0-5.2 and has maximum activity at pH 6.0. In contrast, cathepsin B_2 is a

exopeptidase also called carboxypeptidase B, has a molecular weight of 47-52 kDa (Kang and Lanier, 2000). Cathepsin B_3, now called cathepsin H, is a 25 kDa glycoprotein which is very heat stable. Cathepsin B purified from carp (Makinodan and Ikeda, 1971), grey mullet (Bonete *et al*., 1984), tilapia (Sherekar *et al*., 1988) and mackerel (Matsumiya *et al*., 1989) had the molecular weights of 23-29 kDa and optimal pH range of 5.5-6.0.

c) Cathepsin C

Cathepsin C (EC 3.4.4.9), is an exopeptidase, also called dipeptidyl transferase and dipeptidyl-aminopeptidase, was first recognized as an enzyme which deaminates Gly-Phe-NH_2. It is unlikely to act on intact protein directly, but rather has the highest specific activity among all the lysosomal peptidase, suggesting that it further digests the resulting peptide fragments by cathepsin D (Huang and Tappel, 1971) Hameed and Haard (1985) reported that cathepsin C extract from Alantic squid muscle had molecular weight of 25 kDa and was Cl and sulfhydryl dependent, being inhibited by sulfhydryl enzyme inhibitors, such as iodoacetate, PCMB, and $HgCl_2$. From the sarcoplasmic fluid in Pacific whiting, cathepsin C showed optimum activity at pH 7.0, which was greater than the optimum activity at pH 6.0 in true cod (Erickson *et al*., 1983).

d) Cathepsin D

Cathepsin D (EC 3.4.4.23) is an endopeptidase, which plays a significant role in texture degradation during chilled storage in postmortem muscle. This enzyme is an aspartyl-type proteinase having several isomers with pIs of 5.7-6.8, optimum pH of 3.0-4.5 and broad range of activity on muscle substrates (Ogunro *et al*., 1979). Cathepsin D is strongly inhibited by pepstatin A, a specific inhibitor of carboxyl proteinases, whereas thiol proteinase inhibitors have negligible effects on enzyme activity (Bonete *et al*., 1984). This enzyme is capable of degrading titin, connectin, C-protein, M-protein, and myosin (both heavy and light chains), while actin, troponin T/I and tropomyosin are broken down slowly. Klomklao *et al.* (2008) reported that the predominant enzyme responsible for autolysis in true sardine was a cathepsin D, which exhibited the maximum activity at 55°C, pH 3.5 and was effectively inhibited by Pepstatin A.

e) Cathepsin H

Cathepsin H shows both thiol endopeptidase and aminopeptidase activities. It has molecular weight of 28 kDa. It has two multiple forms showing a maximum activity at pH 7.

Cathepsin H was reported to break down myosin two to three times faster than cathepsin B. In white muscle of spawning migration salmon, cathepsin H activity was three times higher than fish in feeding migration (Yamashita *et al*., 1990).

f) Cathepsin L

Cathepsin L (EC 3.4.22.15) is a typical cystein proteinase found in lysosomes which has several multiple forms (pI 5.8-6.1) and a wide range of pH (3.0-6.5) for activity. It is strongly inhibited by iodoacetate, leupeptin and antipain (Kang and Lanier, 2000). This enzyme is capable of hydrolyzing a broad range of proteins including myosin, actin, nebulin, cytosolic proteins, collagen and elastin (Kirschke and Barrett, 1987). Yamashita *et al*. (1990) reported that the enzyme responsible for postmortem softening of salmon meat during spawning migration was cathepsin L. Cathepsin L from both fillets and surimi of Pacific whiting has the highest activity at 55°C, and thus can degrade gel texture during conventional cooking of surimi seafood (An *et al*., 1994b). Among the highly active lysosomal cathepsin (B, H and L) in whiting fillets, cathepsin L was the predominant cathepsin in surimi, whereas cathepsin B was highest in the intact fillets. This indicates that cathepsin L remained after the extended washing process used for surimi manufacture, while the others were leached away from the myofibrillar proteins.

5.1.2 Neutral Ca^{2+}-activated proteinases

Neutral proteinases in skeletal muscle has been directed to the Ca^{2+}-activated, neutral endopeptidases known as CANP and recently as calpains (EC 3.4.33.17) (Kolodziejska and Sikorski, 1996). These enzymes are further subclassified into μ-calpain and m-calpain, which differ in sensitivity to calcium ions. Both are heterodimers: the large subunit and the small subunit for μ- and m-calpain, which have molecular weights near 80 kDa and 28 kDa, respectively (Cheret *et al*., 2006). The calcium requirement of μ- and m-calpain was 50-70 μM and 1-5 mM Ca^{2+}, respectively. Calpastatin is known to be the endogenous specific inhibitor of calpain (Kolodziejska and Sikorski, 1996).

5.1.3 Alkaline proteinases

Alkaline proteinases are located in the muscle sarcoplasm, microsomal fraction or are bound to myofibrils (Dahlmann and Reinauer, 1978). Alkaline proteinases are active at neutral to slightly alkaline pH and are unstable under acid condition (Simpson, 2000). The former proteinase was characterized as trypsinlike serine enzyme, which showed great capacity of

degrading intact myofibrils *in vitro* (Busconi *et al*., 1987). Generally, alkaline proteinases are heat stable and also become active at the neutral pH of fish meat paste. Makinodan and Ikeda (1971) reported that heat stable alkaline proteinases (HAP) are recognized to be the modori-inducing or gel softening factor in some fish. This enzyme mainly degrades the myofibrillar proteins, especially myosin (Okata *et al*., 1998; Cao *et al*., 2000). Benjakul *et al*. (2003c) reported that heat-stable alkaline proteinase purified from bigeye snapper had optimum pH and temperature for casein hydrolysis at 8.5 and 60°C, respectively.

5.2 Roles of proteolytic enzymes in fish muscle

Texture is considered to be one of most important quality attributes of fish muscle. It determines consumer acceptance and hence the marketability of the final products. The post-mortem softening of fish and fish products due to endogenous proteases is a serious problem. Currently, two proteolytic systems known to hydrolyze myofibrillar protein during postmortem storage of fish muscle are calpain and cathepsins (Cheret *et al*., 2006).

5.2.1 Effect of proteinases on fish muscle

Postmortem tenderization is one of the most unfavorable quality changes in fish muscle. A proteolytic degradation of myofibrillar and connective tissue component occurs during the extended storage. After death, fish muscle becomes susceptible to autolysis by endogenous muscle proteinases, resulting in the softening of fish flesh (An *et al*., 1996; Benjakul and Visessanguan, 2000). Muscle proteinases active at 5-7 are involved in postmortem degradation of muscle proteins. The ultimate pH of muscle tissue most likely drops from physiological pH (~6-7) due to the accumulation of lactic acid and hydrogen ions. The participation of various proteinases in autolysis process of fish depends on location of enzyme in cytosal and/or factors affecting tissue compartmentization, the presence of activator or inhibitor and susceptibility of the proteins responsible for muscle intregrity to *in situ* cleavage by the respective enzymes (Ladrat *et al*., 2003). Among endogenous proteinases, lysosomal cathepsin and a cytosolic calcium dependent enzyme including calpains play the most influential role in tissue softening of most fish in postmortem stage. Calpain are believed to initiate the disintegration of the Z line by a titin cleavage (Astier *et at*., 1991) which weakens the titin/α-actinin interaction and results in the release of intact α-actinin from Z-line (Papa *et al*., 1996) before the further proteolysis by proteasome. The muscle softening occurred in chum salmon during spawning migration owing to

the degradation of myofibrillar protein caused by lysosomal cysteine proteinases, particularbly cathepsin L, present in muscles (Yamashita and Konagaya, 1990).

5.2.2 Effect of proteinases on textural properties of fish gel

Gel weakening phenomenon is observed during cooking of surimi from some fish species. Temperature plays an important role in surimi gelation either by induction of network formation or by activation of endogenous enzymes. Proteolysis disintegration of surimi gels is enhanced at temperature above 50°C, in which the rapid and severe degradation of myofibrillar proteins, particulary myosin, take place (Kang and Lanier, 2000). Degree of proteolysis varies with fish species and is governed by the type of proteinases. As a consequence, gel weakening of surimi or mince induced by proteinases depends on species. A rapid breakdown of myofibrillar protein inhibits development of a three-dimensional gel network (Figure 9). Generally, cathepsin and heat-activated alkaline proteinases mainly contribute to a decrease in gel strength with a brittle and non-elastic gel (An *et al*., 1996).

High level of cysteine proteinase including cathepsin B, H, L and L-like have been observed in Pacific whiting and arrowtooth flounder, chum salmon and mackerel (Yamashita and Konagaya, 1990; Jiang *et al*., 1994; An *et al*., 1994a, b). When the Pacific whiting muscle was incubated at 60°C for 30 min prior to cooking at 90°C, most myosin heavy chain (MHC) was degraded, and surimi did not form a gel with measurable gel strength (Morrissey *et al.*, 1993). In Pacific whiting surimi and arrowtooth flounder muscle, the reduction of gel strength or softening of muscle tissue was attributed to degradation of myofibrillar components like myosin, actin, beta-tropomyosin/troponin-T and collagen by cathepsin L which was maximally active at 55°C (An *et al*., 1994b; Visessanguan *et al*., 2001).

Heat-stable alkaline proteinase is responsible for textural degradation of surimi gels. It has been found in muscle of fish including rainbow trout, sardine, white croaker, carp, common mackerel, cod, herring, bigeye snapper and Alantic salmon (Makinodan *et al*., 1984; Stoknes *et al*., 1993; Stokne and Rustad, 1995; Benjakul *et al*., 2003b). White croaker meat paste formed a poor elastic gel when heated around 60°C (Makinodan *et al*., 1985). Proteinases associated with surimi gel softening were classified as modori-inducing proteinase (Toyohara *et al*., 1990, Ramos-Martiner *et al*., 1999). Toyohara *et al.* (1990) reported that MHC degradation was caused by heat-stable serine proteinases termed the gel-degradation-inducing factor (GIF).

GIF could degrade MHC at pH 7.0 in the presence of NaCl. Cao *et al.* (1999) reported that myofibril-bound serine proteinase (MBP) from carp (*Cyprinus carprio*) had the optimum temperatures on myofibril autolysis and kamaboko gel degradation at 55 and 60°C, respectively. Additionally, actin, α-actinin and tropomyosin were also hydrolyzed by this enzyme to different degrees.

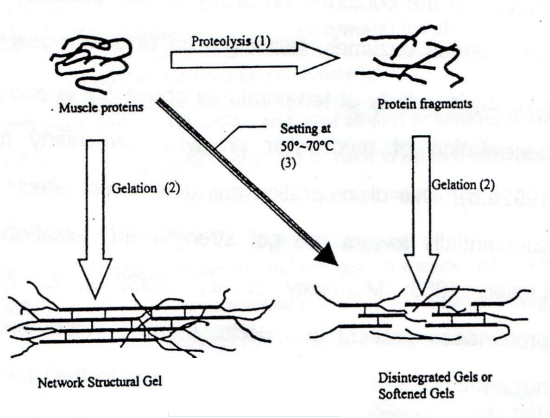


Figure 9 Proposed model of the gelation and disintegration of surimi gels. (1) proteolysis by calpains, cathepsin and other proteinasese during storage; (2) gelation; (3) proteolysis by heat activated proteinses (cathepsin B, L and L-like) during setting at 50-70°C

Source: Jiang (2000)

5.3 Application of proteinase inhibitors in surimi gel

Poor gel forming ability of surimi from some fish species has been known to be mediated by endogenous proteinases. Cathepsin and heat-stable alkaline proteinases were the major proteinase contributing to softening of gel from fish (Benjakul *et al*., 2003c; An *et al*., 1996). To alleviate this problem caused by endogenous proteinases, some protein additives have been widely used to prevent the protein degradation and enhance the gel quality. However, degree of proteinase inhibition was dependent upon the types of additives used (Morissey *et al*., 1993; Reppond and Babbitt, 1993).

5.3.1 Protein plasma

Blood plasma contains a variety of bioactive compounds including proteinases inhibitor and plasma transglutaminase (Benjakul *et al*., 2001b). Plasma contains more than 150 proteins and can be used as functional ingredients in food (Anderson and Lunden, 1979),

especially to increase surimi gel strength. Among the proteinase inhibitors, kininogen is a specific cystein proteinase inhibitor. Alpha-2-macroglublin (α_2M) acted as a nonspecific inhibitor for all four classes of proteinases (Starkey and Barrett, 1977). Additionally, plasma α_2M can form ε-(γ-glutamyl)-lysine bonds between γ-carboyamide groups of glutamine residues in proteins and several primary amine (Salvesen *et al*., 1989), resulting in the strengthening of surimi gel. Bovine plasma protein (BPP) has been reported to be more effective in both proteinases inhibitory activity and gel strengthening ability, compared with other cysteine proteinase inhibitors (Kang and Lanier, 1999). Seymour *et al*. (1997) reported that enhancement of Pacific whiting gel by BPP is due to the inhibition of proteolytic degradation by plasma proteins, protein cross-linking by transglutaminase and gelation of bovine serum albumin (BSA). In addition to BPP, porcine plasma protein (PPP) was shown to increase the gel strength of surimi (Benjakul *et al*., 2001b). Benjakul and Visessanguan (2000) found that PPP effectively inhibited the proteolytic acitivity of surimi from Pacific whiting. Proteins with apparent molecular weight of 60,000-63,000 Da appeared to exhibit inhibitory activity against papain and trypsin (Benjakul and Visessanguan, 2000). Porcine plasma is a crucial source of various proteinase inhibitors including proteinase inhibitor-1 (PI-1), proteinase inhibitor-2 (PI-2), protein inhibitor-3 (PI-3), proteinase inhibitor-4 (PI-4), postalbumin-1A (PO-1A) and postalbumin-1B (PO-1B) (Gahne and Juneja, 1985). However, the applications of BPP and PPP in surimi have recently been limited by the outbreak of mad cow diseases and/or the constraint of some religions, respectively. Recently, addition of chicken plasma protein was found to increase the gel properties of surimi from bigeye snapper (Rawdkuen *et al*., 2004a). Rawdkuen *et al.* (2004b) found that CPP showed the inhibitory effect on sarcoplasmic proteinases and autolysis of mince and washed mince from bigeye snapper as well as improved gel quality with 2% CPP addition. Cysteine proteinase inhibitor (CPI), partially purified from chicken (*Gallus gallus*) plasma using PEG fractionation and affinity chromatography on carboxymethyl–papain–Sepharose-4B, had the molecular mass of 122 kDa and exhibited the inhibitory activity towards papain (Rawdken *et al*., 2007). However, the addition of blood plasma to surimi or surimi products renders an end-product with off-color and off-flavour (Benjakul *et al*., 2001b).

5.3.2 Egg white

Egg white (EW) is the one of popular ingredient which has been used widely for surimi production. Two forms of egg white proteins are commonly used in surimi seafood

including liquid and dried. Egg white contains many proteins (~40 protein), but more than half of these are minor components. The major protein components in egg white are ovalbumin (54%), conalbumin (12%) and ovomucoid (11%) (Park, 2000). Several of them function as a proteinase inhibitors including ovomucoid, ovoinhibitor, ovomacroglobulin, which have inhibitory activity against serine proteinase (Nakamura and Doi, 2000). A cysteine proteinase inhibitor, cystatin, was also found in egg white. Weerashinge *et al*. (1996b) found that egg white contained more serine proteinase inhibitor than cysteine proteinase inhibitor. Benjakul *et al.* (2004d) found that proteolysis of lizardfish muscle could be partially inhibited by addition of 3% EW or BPP. Additionally, gelation of egg white protein is expected to support and bind other ingredients within the gel matrix of food products (Setser, 1992). Egg white has its gel-strengthening ability by composite reinforcing and water-binding capacity (Lee, 1984). Additon of EW, in conjunction with setting at 25°C, increased the breaking force of lizard surimi gel and high molecular weight cross-linked proteins (Yongsawatdikul and Piyahammaviboon, 2004). The level of frozen raw egg white commonly used in a surimi formulation is 3-10% (weight basis). However, egg white may have unavoidable negative effects on the quality of surimi seafood such as sulfurous odors. Moreover, some consumer may have allergic reaction to the noninhibitor component of egg white (Kang and Lanier, 2000).

5.3.3 Whey protein concentrate

Whey protein concentrate (WPC) has commonly been used as a protein supplement, foam stabilizer, filler/water binder, thickening, emulsifying and gelling agent (Morr and Foegeding, 1990). It can be used to improve texture and nutritional value of a variety of food, such as sausages, meat balls and low salt fish products (Rawdkuen and Benjakul, 2008). WPC consists of several protein components. β-lactoglobulin is the most abundant protein (50%); α-lactalbumin is the second major protein with 20% by weight; serum albumin and immonoglubulin each constitutes 10% of WPC by weight (Morr, 1985). Rawdkeun and Benjakul (2008) reported that 3% WPC addition could produce high gel quality of surimi from tropical fish via preventing protein degradation. WPC has been reported to protect myofibrillar proteins of surimi by acting as a true inhibitor or serving as an alternative substrate (Piyachomkwan and Penner, 1995). Although β-lactoglobulin and α-lactalbumin are the major components of WPC, they did not show any inhibitory activity toward papain and trypsin (Weerasinghe *et al*., 1996a). Akazawa *et al.* (1993)

reported that α-lactalbumin from WPC did not exhibit the inhibitory activity towards proteinase. Weerashinge *et al*. (1996a) reported that an unidentified high-molecular-weight protein (HMP) with apparent M_w of 101,000 Da showed inhibition toward papain and trypsin. Additionally, the strengthening of gel was found when WPC was added as gelling agent. Whey protein form thermo-irreverible gels, which hold the water and prevent moisture loss. The gelling properties of whey proteins are also responsible for maintaining moistureness and improving texture and mouthfeel in the resulting products.

OBJECTIVES

1. To characterize goatfish muscle and to study the thermal properties and aggregation of natural actomyosin from goatfish muscle during heat-induced gelation process.
2. To elucidate the effect of setting condition and $CaCl_2$ levels on gel properties of goatfish surimi.
3. To study the effect of ice storage on physicochemical properties changes and gel forming ability of goatfish surimi.
4. To characterize the predominant endogenous proteinases responsible for autolysis of goatfish muscle and to study the effect of washing and skin inclusion on autolysis.
5. To investigate the effects of whey protein concentrate (WPC) on autolysis inhibition and gel properties of goatfish surimi.

CHAPTER 2

MATERIALS AND METHODS

1. Materials

1.1 Fish sample and preparation

Goatfish (*Mulloidichthys martinicus*) with the size of 10 – 15 cm and weight of 50-100 g/fish were caught off the Songkhla- Pattani Coast along the Gulf of Thailand. The fish, off-loaded within 48 h after capture, were transported in ice with the fish/ice ratio of 1:2 (w/w) to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, fish were washed, headed and gutted. The flesh was separated manually, minced into homogeneity using a mincer with a hole diameter of 5 mm and kept on ice until use.

1.2 Chemicals

All chemicals were of analytical grade. Sodium chloride, trichloroacetic acid, Folin-Ciocalteu's phenol reagent and Coomassie Brilliant Blue R-250 were obtained from Merck (Darmstadt, Germany). Calcium chloride, β-mercaptoethanol (β-ME), ethylenediaminetetraacetic acid (EDTA), trans-epoxysuccinyl-L-leucyl-amido (4-guanidino) butane (E-64), pepstatin A, soybean trypsin inhibitor (SBTI), Adenosine 5′-triphosphate (ATP), ammonium molybdate, 5-5′-dithio-bis (2-nitrobenzoic acid) (DTNB), 1-anilinonaphthalene-8-sulphonic acid (ANS), guanidine thiocyanate, sodium hydrogen sulfite and L-tyrosine were procured from Sigma Chemical Co. (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS) and *N,N,N′,N′*-tetramethyl ethylene diamine (TEMED) were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Potassium chloride (KCl) and urea were obtained from Ajax Finechem (Wellington, Auckland, New Zealand). Whey protein concentrate (WPC) was obtained from Hilmar Ingredients (Hilmar, CA, USA).

2. Instruments

Instruments	Model	Company/City/Country
- pH meter	CG 842	Schott, Mainz, Germany
- Magnetic stirrer	BIG SQUID	IKA labortechnik, Stanfen, Germany
- Homogenizer	T25 basic	IKA labortechnik, Selangor, Malaysia
- Water bath	W350	Memmert, Schwabach, Germany
- Microcentrifuge	MIKR 020	Zentrifugan, Hettich, Germany
- Refrigerated centrifuge	RC-5B plus	Sorvall, Norwalk CT, USA
- Colorimeter	ColorFlex	HunterLab Reston, VA, USA
- Electrophoresis apparatus	Mini-Protein II	Bio-Rad, CA, USA
- Double-beam Spectrophotometer	RF-1501	Shimadzu, Kyoto, Japan
- Texture analyzer	TA-XT2	Stable Micro Systems, Surrey, UK
- Mixer	MK-K77	National, Tokyo, Japan
- Scanning electron microscope	JSM 5800LV	JEOL, Akishima, Japan
- Transmission electron microscope	JEM-2010	JEOL, Tokyo, Japan

3. Methods

3.1 Determination of chemical composition and thermal properties of goatfish mince

3.1.1 Chemical composition and protein pattern

3.1.1.1 Proximate analysis

Fish mince was determined for moisture, protein, ash and fat contents according to AOAC method (AOAC, 1999). The values were expressed as % (wet weight basis).

3.1.1.2 Determination of TVB and TMA

TVB and TMA contents were determined using the Conway microdiffusion assay according to the method of Conway and Byrne (1936). Sample (5 g) was mixed with 20 ml of 4% tricloroacetic acid (TCA). The mixtures were homogenized at 11,000 rpm using an Ultra Turrax homogenizer (IKA Labortechnink, Selangor, Malaysia) for 1 min. The homogenate was filtered using Whatman No. 4 filter paper and the filtrate was used for analysis. To determine the TMA content, formaldehyde was added to the filtrate to fix ammonia present in sample. TVB and TMA were released after addition of saturated K_2CO_3 and diffused into the boric

acid solution. The titration of solution was performed and the amount of TVB and TMA were calculated.

3.1.1.3 Determination of K-value

K-value was determined using ion exchange chromatography (Uchiyama and Kakuda, 1984). Sample (5 g) was homogenized in 10 ml of 10% perchloroacetic acid (PCA) for 2 min. The sample was centrifuged at 3,000×g for 5 min. The extraction steps were repeated for three times. The extract was subjected to ion exchange chromatography with Anion exchange resin-AG (R) 1-X4, 400 mesh Cl-form. Elution was carried out and the eluate was determined for ATP and derivatives. K-value was then calculated.

3.1.1.4 Determination of protein pattern

Protein patterns of goatfish mince was analyzed by SDS-PAGE according to the method of Laemmli (1970). To solubilize the protein, goatfish mince (3 g) was added with 27 ml of 5% SDS solution (85°C). The mixture was then homogenized using a homogenizer (IKA Labortechnik, Selangor, Malaysia) at a speed of 11,000 rpm for 1 min. The homogenate was incubated at 85°C for 1 h to dissolve total proteins. The sample was centrifuged at 7500×*g* for 15 min to remove undissolved debris. Protein concentration in goatfish mince was determined according to the method of Biuret (Robinson and Hodgen, 1940) using bovine serum albumin as standard. The samples (15 μg protein) were loaded onto the polyacrylamide gel made of 10% running gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA per gel, using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.02% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and destained with 50% methanol (v/v) and 7.5% (v/v) acetic acid, followed by 5% methanol (v/v) and 7.5% (v/v) acetic acid.

3.1.2 Determination of protein and non-protein nitrogenous compounds

Non-protein nitrogenous constituents, sarcoplasmic protein, myofibrillar protein, alkali-soluble protein and stromal proteins in goatfish mince were fractionated according to the method of Hashimoto *et al*. (1979). Nitrogen content in each fraction was measured by the Kjeldahl method (AOAC, 1999). Protein patterns of different fractions were determined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with 10% running gel and 4% stacking gel, as described previously.

3.1.3 Thermal properties of muscle proteins

3.1.3.1 Differential Scanning calorimetry (DSC)

Thermal transition of goatfish mince and washed mince in the absence and in the presence of 2.5% NaCl was determined using the differential scanning calorimetry (DSC) (Perkin-Elmer, Model DSCM, USA). The samples (15–20 mg wet weight) were placed in the DSC hermetic pans, assuring a good contact between the sample and the pan bottom. An empty hermetic pan was used as a reference. The samples were scanned at 10°C/min over the range of 20–100°C. T_{max} was measured and denaturation enthalpies (ΔH) were estimated by measuring the area under the DSC transition curve.

3.1.3.2 Thermal stability

Natural actomyosin (NAM) was extracted according to the method of Benjakul *et al.* (1997). A 3 ml of NAM solution (3-5 mg/ml) was incubated at different temperatures (0, 10, 20, 30, 40, 50 and 60°C). At definite times (0, 5, 10, 30 and 60 min), a sample solution was immediately cooled in iced water. The sample was then equilibrated at 25°C prior to Ca^{2+}-ATPase activity analyses. The inactivation rate constant (K_D) of actomyosin was calculated according to Arai *et al.* (1973) as follows:

$$K_D = \frac{\ln C_0 - \ln C_t}{t}$$

where C_0 = Ca^{2+}-ATPase activity before treatment; C_t = Ca^{2+}-ATPase activity after treatment for time t and t = treatment time (s)

3.2 Study on the thermal properties and aggregation of natural actomyosin (NAM) extracted from fresh and ice-stored goatfish

3.2.1 Thermal properties of NAM

3.2.1.1 Differential Scanning calorimetry

Thermal transition of NAM extracted from fresh and 9 days ice-stored goatfish were studied using the differential scanning calorimetry (DSC) as described in section 3.1.3.1.

3.2.1.2 Thermal stability

NAM solution (3-5 mg/ml) extracted from fresh and 9 days ice-stored goatfish were subjected to thermal stability study as described in section 3.1.3.2.

3.2.2 Aggregation of NAM

NAM extracted from goatfish stored in ice for 0 (G_0) and 9 days (G_9) were diluted to 1 mg/ml with chilled 50 mM potassium phosphate buffer containing 0.6 M KCl (pH 7.0). The solutions were heated at heating rate of 0.65°C/min from 20 to 75°C using digital thermoregulator (TH/150, Ratek, Australia). NAM extracted from bigeye snapper (*Priacanthus tayenus*) muscle (B) was also prepared and diluted to obtain the same concentration. The diluted sample was then subjected to heating in the same manner. The samples were taken every 5°C of temperature increment. At temperature designated, the sample were cooled immediately with iced water and subjected to the following analyse:

3.2.2.1 Turbidity measurement

Heated NAM solutions were placed in cuvette (light path length of 1 cm) and the turbidity was measured by monitoring the absorbance at 660 nm (Benjakul *et al*., 2001a).

3.2.2.2 Determination of surface hydrophobicity

Surface hydrophobicity was mearsured according to the method of Benjakul *et al*. (2001a) using 8-anilo-1-naphthalenseulfonic acid (ANS) as a probe. Heated NAM solutions were diluted to 0.125, 0.25, 0.5 and 1 mg/ml using the same buffer. To 2.0 ml of diluted NAM solution, 10 μl of 10 mM ANS dissolve in 50 mM potassium phosphate buffer (pH 7.0) was added and the mixtures were mixed thoroughly. Fluorescence intensity was measured using a FP–750 spectrofluorometer (Shimadzu, Kyoto, Japan) at the excitation and emission wavelength of 374 and 485 nm, respectively. Surface hydrophobicity was calculated from initial slope of plot of fluorescence intensity against protein concentration using linear regression analysis. The initial slope was referred to as S_0ANS.

3.2.2.3 Determination of total sulfhydryl group and disulfide bond contents

Total sulfhydryl group content was measured according to the method of Ellman (1959) as modified by Benjakul *et al.* (2001a). To 0.25 ml aliquot of heated NAM solutions, 3 ml of 0.2 M Tris- HCl buffer containing 8 M urea, 2% SDS and 10 mM EDTA (pH 6.8) and 0.25 ml of 0.1% DTNB in 0.2 M Tris-HCl buffer (pH 6.8) were added. The mixture was incubated at 40°C for 40 min and the absorbance at 412 nm was read using a UV-16001 spectrophotometer (Shimadzu, Kyoto, Japan). Reagent blank was prepared by replacing the sample with 50 mM potassium phosphate buffer containing 0.6 M KCl (pH 7.0). For sample

blank, the reaction was run in the same manner except 0.2 M Tris HCl (pH 6.8) was used instead of DTNB solution. Sulfhydryl group content was calculated using a molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$. Disulfide bond content was determined by using 2-nitro-5-thiosulfobenzoate (NTSB) assay as described Thannhauser *et al.* (1987). To 0.5 ml of NAM solutions, 3.0 ml of freshly prepared NTSB assay solution were added. The mixture was incubated in the dark at room temperature (25°C) for 25 min. Absorbance was then measured at 412 nm. Disulfide bond content was calculated from the absorbance using the extinction coefficient of 13,900 $M^{-1}cm^{-1}$.

3.2.2.4 Transmission electron micrograph

After the selected heat treatment, NAM solutions (B, G_0 and G_9) were diluted to 0.2 mg/mL with 50 mM potassium phosphate buffer containing 0.6 M KCl (pH 7). A drop of sample was fixed for 5 min on carbon-coated grid, negatively stained with 4% uranyl acetate for 5 min and washed with distilled water until the grid was cleaned. The specimens were visualized using in a JEOL JEM-2010 transmission electron microscope (JEOL Ltd., Tokyo, Japan) (80,000×) at an accelerating voltage of 160 kV.

3.3 Study on the some factors affecting gel properties of goatfish surimi

3.3.1 Effect of setting condition and $CaCl_2$ levels on gel properties of goatfish surimi

3.3.1.1 Surimi preparation

Goatfish mince

↓

Wash with cold water with fish/water ratio of 1:3 (w/v) for three times

↓

Filter through cheese cloth and centrifuge at 860 ×g for 5 min

↓

Add with of 4% sorbitol and 4% sucrose

↓

Mix for 5 min, pack and store at -20°C

↓

Frozen surimi

Figure 10 Scheme for surimi preparation

3.3.1.2 Surimi gel preparation

Frozen surimi was partially thawed at 4°C for 4–5 h to obtain the core temperature of 0-2°C, cut into small pieces with an approximate thickness of 1 cm and then placed in the mixer (Moulinex Masterchef 350, Paris, France). The moisture content of samples was then adjusted to 80% (w/w) and salt (2.5%, w/w) was added. $CaCl_2$ at different levels (0, 25 and 50 mmoles/kg) was added. The mixture was chopped for 4 min at 4°C. The paste was stuffed into a polyvinylidine chloride casing with a diameter of 2.5 cm and both ends were sealed tightly. The paste was incubated under different conditions as follows: 1) set at 25°C for 3 h, 2) set at 40°C for 30 min, 3) incubate at 60°C for 30 min and 4) without setting or pre-incubation.

Thereafter, the samples were heated immediately at 90°C for 20 min in a temperature controlled water bath (Memmert, Schwabach, Germany), followed by rapid cooling in iced water for 30 min. The gels obtained were stored at 4°C overnight prior to analyse as follows:

1. **Texture analysis**

Texture analysis of gel samples was carried out using a Model TA-XT2 texture analyzer (Stable Micro System, Surrey, UK). Gels were equilibrated at room temperature (25–30°C) for 2 h before analysis. Five cylindrical samples (2.5 cm in length) were prepared and tested. Breaking force (strength) and deformation (cohesiveness/elasticity) were measured by the texture analyzer equipped with a spherical plunger (5 mm diameter), with a depression speed of 60 mm/min and 60% compression.

2. **Determination of whiteness**

All gels were subjected to whiteness measurement using a HunterLab (ColorFlex, Hunter Associates Laboratory, VA, USA). Illuminant C was used as the light source of measurement. CIE L*, a* and b* values were measured. Whiteness was calculated using the following equation (Park, 1994):

$$\text{Whiteness} = 100-[(100-L^*)^2+a^{*2}+b^{*2}]^{1/2}$$

3. **Determination of expressible moisture content**

Expressible moisture content was measured according to the method of Ng (1987). Cylindrical gel samples were cut to a thickness of 5 mm, weighed (X) and placed between two pieces of Whatman paper (No. 1) at the bottom and one piece of paper on the top. A

standard weight (5 kg) was placed on the top of the sample for 2 min, and then the sample was removed from the papers and weighed again (Y). Expressible moisture content was calculated and expressed as percentage of sample weight as follows:

$$\text{Expressible moisture content} = [(X-Y)/X] \times 100$$

4. Determination of TCA-soluble peptide content

To 3 g of finely chopped gel samples, 27 ml of 5% TCA were added and homogenized for 1 min using an IKA Labortechnik homogenizer (Selangor, Malaysia) at a speed of 11,000 rpm for 2 min. The homogenate was incubated at 4°C for 1 h and centrifuged at 8000×g for 5 min using a Micro 20 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). TCA-soluble peptide content in the supernatant was measured as per the Lowry method (Lowry *et al*., 1951) and expressed as μmole tyrosine/g sample.

5. Protein pattern analysis

Protein patterns of gels were determined using SDS-PAGE according to method of Laemmli (1970) with 10% running gel and 4% stacking gel. To prepare the protein sample, 27 ml of 5% (w/v) SDS solution heated to 85°C were added into the gel samples (3 g). The mixture was then homogenized for 2 min at a speed of 11,000 rpm using a homogenizer. The homogenate was incubated at 85°C for 1 h to dissolve total proteins. The samples were centrifuged at 8500×g for 20 min to remove undissolved debris as mentioned before (section 3.1.1.4).

The condition for gel preparation treatment yielding the resulting gel with the highest breaking force and deformation was used for further study.

3.3.2 Effect of iced storage of goatfish on physicochemical properties and gel forming ability of surimi

Fresh whole goatfish were kept in ice with fish/ice ratio of 1:2 (w/w). Fish and ice were placed in a polystyrene box, which were kept at room temperature (28-30°C). The molten ice was removed and replaced with an equal amount of ice every 2 day. The stored fish were taken every 3 days for analyses:

3.3.2.1 Chemical and physicochemical analyses

1. Determination of TVB and TMA contents

TVB and TMA contents were determined using the Conway microdiffusion assay according to the method of Conway and Byrne (1936) as described in section 3.1.1.2.

2. Determination of Ca^{2+}-ATPase activity

ATPase activity was determined according to the method of Benjakul *et al*. (1997). NAM prepared as described by Benjakul *et al.* (1997) was diluted to 3-5 mg/ml with 0.6 M KCl (pH 7.0). Diluted NAM solution (0.5 ml) was added to 0.3 ml of 0.5 M Tris-maleate (pH 7.0). The mixture was added with 0.5 ml of 0.1 M $CaCl_2$ and 3.45 ml of distilled water. To initiate the reaction, 0.25 ml of 20 mM ATP was added. The reaction was conducted for 10 min at 25°C and terminated by 2.5 ml of chilled 15% trichloroacetic acid. The reaction mixture was centrifuged at 3500×g for 5 min and the inorganic phosphate liberated in the supernatant was measured by the method of Fisk and Subbarow (1925). Specific activity was expressed as μmole inorganic phosphate released/mg protein/min. A blank solution was prepared by adding chilled trichloroacetic acid prior to addition of ATP.

3. Determination of TCA-soluble peptide content

TCA-soluble peptide content of gel samples was measured as described in section 3.3.1.2.

4. Protein pattern

Protein patterns of gel samples were determined by SDS-PAGE as mentioned in section 3.1.1.4.

3.3.2.2 Determination of gel properties

Goatfish surimi was prepared from fish stored in ice for different times as described in section 3.3.2. Surimi gel was prepared with the condition yielding the highest breaking force and deformation (section 3.3.1.2). Prepared gels were subjected to analyses as described in section 3.3.1.2

3.4 Study on autolysis of goatfish muscle

3.4.1 Preparation of goatfish mince and washed mince

Goatfish mince was prepared as mentioned before. Washed mince was prepared according to the method of Toyohara *et al.* (1990) with a slight modification. Mince was homogenized with 5 volumes of 50 mM NaCl at a speed of 11,000 rpm for 2 min using an IKA Labortechnik homogenizer (Selangor, Malaysia) followed by centrifuging at 9600×g at 4°C for 10 min using a Sorvall Model RC-B Plus centrifuge (Newtown, CT, USA). Washing process was repeated twice. To minimize the denaturation, the preparation was conducted at temperature below 4°C.

3.4.2 Characterization of predominant endogenous proteinase in goatfish muscle

3.4.2.1 Effect of temperature on autolysis of goatfish mince and washed mince

Mince and washed mince (3 g) was incubated at different temperatures (30, 35, 40, 45 50, 55, 60, 65 and 70°C) in a temperature-controlled water bath (Memmert, Schwabach, Germany) for 60 min. Autolytic reaction was terminated by addition of 27 ml of cold 5% trichloroacetic acid (w/v). The mixture was homogenized at the speed of 11,000 rpm using a homogenizer (Model T25 basic, IKA, LABORTECHNIK, Selangor, Malaysia) for 1 min. The homogenate was subjected to centrifugation at 7500×*g* for 10 min using a microcentrifuge (Model MIKRO20, Hettich ZENTRIFUGEN, Germany). TCA-soluble peptide content in the supernatant obtained were determined by the Lowry method (Lowry *et al*., 1951) using *L*-tyrosine as a standard and expressed as μmole tyrosine/g sample. After the autolytic reaction, another sample was mixed with 27 ml of 5% SDS (85°C) and homogenized at a speed of 11,000 rpm for 1 min. The homogenate was then incubated at 85°C for 60 min. To remove undissolved debris, the mixture was centrifuged at 7500×*g* for 10 min. The supernatant was subjected to SDS-PAGE analysis.

3.4.2.2 Effect of pH on autolysis of goatfish mince and washed mince

Mince and washed mince (3 g) was homogenized with 9 ml of different buffers having various pHs. Those included 0.2 M McIlvaines' buffer (0.2 M Na-phosphate and 0.1 M Na-citrate) for pHs 2.0–7.0 and 0.1 M Na_2HPO_4–0.05 M $Na_2B_4O_7$ for pHs 8.0–11.0. The mixture was homogenized at a speed of 11,000 rpm for 1 min. The homogenate was incubated at the optimal temperature (section 3.4.2.1). Autolysis was terminated after 60 min of incubation by

addition of 18 ml of cold 5% TCA or 5% SDS solution (85°C) as previously described. TCA-soluble peptide content and SDS-PAGE protein patterns were determined, respectively.

3.4.2.3 Effect of various proteinase inhibitors on autolysis of mince and washed mince

Mince and washed mince (0.5 g) was homogenized with 1.5 ml of the buffer having the pH showing the maximal activity at a speed of 11,000 rpm for 1 min using a homogenizer. The homogenate was mixed with 2 ml of proteinase inhibitor solution to obtain the final designated concentration: 2 mM EDTA, 0.01 mM soybean trypsin inhibitor (SBTI), 0.01 mM E-64 and 1 μM and 10 μM pepstatin A (Benjakul *et al*., 2003e). The mixtures were allowed to stand in ice for 2 h, followed by incubation at optimal temperature for 2 h. Autolysis was terminated by addition of 2 ml of either 10% TCA or 10% SDS solution (85°C). TCA-soluble peptide content and SDS-PAGE protein patterns were then determined. The control was performed in the same manner, but the deionized water was added instead of protease inhibitor solution.

3.4.2.4 Effect of sodium chloride on autolysis of mince and washed mince

Mince and washed mince (3 g) were mixed with NaCl at different concentrations (2, 2.5, 3 and 3.5% w/w). The mixtures were incubated at the optimal temperature for 60 min. Autolysis was terminated by addition of 27 ml of either 5% TCA or 5% SDS solution (85°C). TCA-soluble peptide contents and SDS-PAGE protein patterns were determined.

3.4.2.5 Effect of skin on autolysis of mince

To prepare mince with skin, fillets were directly minced without prior skin removal. Mince with and without skin (3 g) was incubated at the optimal temperature for different times (0, 10, 30, 60, 90 and 120 min). Autolysis was terminated by addition of 27 ml of either 5% TCA or 5% SDS solution (85°C). TCA-soluble peptide contents and SDS-PAGE protein patterns were determined.

3.5 Study on the effect of whey protein concentrate (WPC) on autolysis inhibition and gel property of goatfish surimi

3.5.1 Effect of WPC on autolysis inhibition

To study the effect of WPC on surimi autolysis, frozen surimi was thawed using running water until core temperature reached 0-2°C. Thawed surimi (3 g) was mixed with WPC at different level (0, 1, 2 and 3% w/w) in the absence and in the presence of 2.5% NaCl. The mixture

was incubated at optimum temperature for 60 min. Autolysis was terminated by addition of 27 ml of either cold 5% TCA or 5% SDS solution (85°C). TCA-soluble peptide contents and SDS-PAGE protein patterns were then determined. Sample blank was performed in the same manner but the mixture was incubated in ice for 60 min prior to termination of autolysis. The autolysis inhibition was calculated as follows:

$$\%Inhibition = \frac{A-B}{A} \times 100$$

where *A* is TCA-soluble peptide content in sample without WPC and *B* is TCA- soluble peptide content in sample with WPC.

3.5.2 Effect of WPC on surimi gel properties

To prepare the gel, frozen surimi was tempered for 30 min in running water (25°C). The surimi was then cut into small pieces with an approximate thickness of 1 cm. The surimi was placed in a mixer (National Model MKK77, Tokyo, Japan). The moisture was adjusted to 80% and 2.5% NaCl was added. WPC was added to obtain the final concentrations of 0, 1, 2 and 3% in the presence and in the absence of $CaCl_2$ at the level rendering the highest breaking force and deformation of goatfish gel (section 3.3.1.2). The mixture was chopped for 4 min at 4°C to obtain a homogenous sol. Then, the sol was stuffed into a polyvinylidine chloride casing with a diameter of 2.5 cm and both ends were sealed tightly.

Kamaboko gels were prepared by incubating the sol at 40°C for 30 min, followed by heating at 90°C for 20 min. For modori gel, the sol was incubated at 60°C for 30 min, followed by heating at 90°C for 20 min. All gels were cooled in iced water and stored for 24 h at 4 °C prior to analyses as described in section 3.3.1.2.

Additionally, the control gel, gels with WPC at the level rendering the gel with the highest breaking force and deformation together with $CaCl_2$ were subjected to microstructure study. Microstructures of the gels were determined using scanning electron microscopy. Gel samples (0.25×0.25×0.25 cm) was fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 2 h and distilled water for 1 h at room temperature (Hayat, 1981). Fixed specimens were dehydrated in graded ethanol solution with serial concentrations of 50, 70, 80, 90 and 100% and critical point dried (Balzers mod. CPD 030, Blazers Process Systems, Liechtenstein) using CO_2 as

transition fluid. The prepared samples were mounted on copper specimen holders, sputter-coated with gold (Balzers mod. SCD 004) and examined on a JSM 5200 scanning electron microscope (JEOL, Ltd., Akishima, Japan).

4. Statistical analysis

All experiments were run in triplicate and CRD (Completely Randomized Design) was used. Analysis of variance (ANOVA) was performed and means comparisons were carried out by Duncan's multiple range tests (Steel and Torrie, 1980). Analyses were conducted using a SPSS package (SPSS 11.0 for windows, SPSS Inc, Chicago, IL, USA).

CHAPTER 3

RESULTS AND DISCUSSION

1. Chemical composition and thermal properties of goatfish muscle

1.1 Chemical composition and protein pattern

1.1.1 Chemical composition

Proximate composition of goatfish mince is shown in Table 4. Goatfish mince contained high moisture content, accounting for approximately 79.88%. Depending on the species and the nutritional status of the animal, the muscle of marine fish and invertebrate consists of 50-80% water (Sikorski *et al.*, 1990). Protein was found as the major constituent (17.62%), indicating that goatfish mince can be the good source of amino acids. Trace amounts of ash (1.32%) and lipid (0.28%) were found. The composition of muscle can vary with the season, size, sex, spawning and feeding condition (Pigott and Tuker, 1990). With low fat content, goatfish can be classified as the lean fish (Sikorski *et al*., 1990).

When the K-value, TVB and TMA contents were determined, very low values were observed. This indicated that goatfish samples were very fresh with negligible spoilage. Generally, K-value has been used as a freshness index of fish and is related to degradation of nucleotides by nucleotide-degrading enzymes. The K-value is the ratio of Ino and Hx to the sum of ATP, ADP, AMP, IMP, Ino and Hx. Thus, the low K-value obtained in goatfish mince indicated that the low amount of degradation product of nucleotide was formed. Furthermore, the low TVB and TMA contents suggested the low extent of spoilage in goatfish. In freshly caught marine fish, TVB and TMA contents are negligible (Oehlenschlager, 1992). Over time of storage, TVB and TMA contents generally increased, depending on the fish species, feeding habits, temperature and the storage conditions (Pacquit *et al*., 2007). Benjakul *et al.* (2002) reported that initial values of TVB in bigeye snapper samples were 5.0–5.4 mg N/100 g. The presence of TVB and TMA indicated some decomposition of nitrogenous compounds occurring during post-mortem handling and storage. The volatile amines such as TMA, NH_3 and DMA were responsible for fishery odor in unfresh fish (Sikorski *et al*., 1990).

Table 4 Chemical compositions of goatfish mince

Compositions (% wet wt.)	Contents
Moisture	79.88±0.17*
Protein	17.62±0.45
Fat	0.28±0.01
Ash	1.32±0.05
K-value	1.08±0.40
TVB (mgN/ 100g sample)	6.50±0.57
TMA (mgN/ 100g sample)	0.73±0.16

*Values are given as mean ± SD from triplicate determinations.

1.1.2 Protein and non-protein nitrogenous compounds

Based on solubility, goatfish muscle was fractionated into five fractions as shown in Table 5. Non-protein nitrogenous compounds were found at a low content (2.84 mgN/g muscle or 9.92% of total nitrogen content). Non–protein nitrogenous fraction included amino acid, imidazole, dipeptide, nucleotides, trimethylamine, urea and the products of postmortem changes (Sikorski, 1994; Foegeding *et al*., 1996). Generally, non-protein nitrogenous compounds in white muscle constitute 9-15% of total nitrogen content (Sikorski, 1994). These compounds, especially free amino acids, peptide, butaine and nucleotide play an essential role in unique or delicate taste of seafood (Sikorski, 1994). Sriket *et al*. (2007) reported that black tiger shrimp possess the superior acceptability in term of flavor and taste to white shrimp, due to the greater amount and different compositions of non-protein nitrogenous constituents.

Myofibrillar protein fraction constituted as the major protein in goatfish mince, accounting for 63.93% of total muscle proteins. Similar result was found in muscle from mackerel, which contained the myofibrillar protein of 69.9% of total muscle proteins (Chaijan *et al*, 2004). Sarcoplasmic protein was found as the second predominant protein in goatfish mince. Among the sarcoplasmic proteins, myoglobin was dominant in dark muscle, while palvalbumin was prevalent in white muscle of sardine and mackerel (Hashimoto *et al*., 1979). This result was in agreement with Hashimoto *et al*. (1979) who reported that myofibrillar and sarcoplasmic protein are the major proteins in fish muscle. Nevertheless, the difference in sarcoplamic and myofibrillar protein fraction varied, depending on the species of fish. This might contribute to the different functional

properties of meat from different species. The content of sarcoplasmic protein was generally high in pelagic fish such as sardine and mackerel (Chaijan *et al*., 2004; Hashimoto *et al*., 1979). That protein has an adverse effect on the development of myofibrillar protein gel (Sikorski, 1994). Alkaline soluble protein and stroma protein fractions of goatfish were found at low levels. Alkaline soluble proteins include the cross-linked myofibrillar proteins (Hashimoto *et al*., 1979). Stroma proteins consist not only of collagen and elastin, but also conectin and other proteins. Teleost and elasmobranch species generally contain 3 and 10% stroma, respectively (Sikorski and Borderies, 1990).

Table 5 Nitrogenous constituents of goatfish mince

Fractions	Nitrogen content (mg N/g muscle)
Non-protein nitrogen	2.84±0.06
Sarcoplasmic protein	7.96±0.11 (30.88)[*, †]
Myofibrillar protein	16.48±0.39 (63.93)
Alkali-soluble protein	1.14±0.02 (4.42)
Stroma protein	0.20±0.01(0.78)

[*]Values are given as mean ± SD from triplicate determinations.

[†]Values in the parenthesis represent the percentage of each protein fraction based on total protein content.

Protein patterns of different fractions of goatfish mince are illustrated in Figure 11. Myofibrillar protein fraction consisted of several protein bands. Myosin heavy chain (MHC), actin and tropomyosin were found as the dominant protein in myofibrillar protein fraction. Among those proteins, MHC constituted predominantly. For sarcoplasmic proteins, low molecular weight proteins were generally observed. This might be associated with its high water solubility. For alkaline soluble protein fraction, the smear protein band appeared at 45 kDa was observed. It was postulated that alkaline soluble proteins were most likely the cross-linked myofibrillar protein. In this present study, actin was possibly cross-linked and lost its solubility in salt solution. The results were in agreement with those of Hashimoto *et al*. (1979) who reported that alkaline soluble protein fraction of sardine and mackerel muscle exhibited a similar protein pattern to the myofibrillar

fraction. For the stroma protein fraction, protein band with molecular weight higher than MHC was found on the top of gel. Since most of sarcoplasmic proteins and connective tissues are generally removed during surimi processing, myofibrillar proteins in muscle become concentrated and thus play an essential role in muscle functional properties, especially gelation (Foegeding *et al*., 1996). The proportion of different proteins in goatfish mince might determine the gelation as well as gel properties of this species.

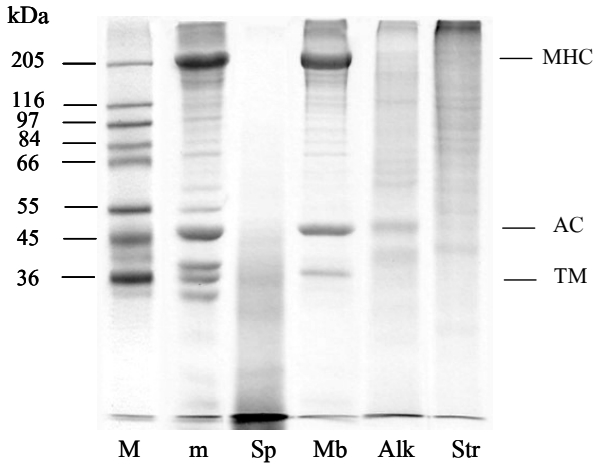


Figure 11 SDS-PAGE patterns of different protein fractions obtained from goatfish mince. M: high molecular-weight marker; m: mince (whole muscle); Sp: sarcoplasmic protein fraction; Mb: myofibrillar protein fraction; Alk: alkaline soluble protein fraction; Str: stroma protein fraction; MHC: myosin heavy chain; AC: actin; TM: tropomyosin.

1.2 Thermal properties of muscle proteins

1.2.1 Thermal denaturation

Thermal transition of proteins in goatfish mince and washed mince in the presence and absence of 2.5% NaCl were determined using differential scanning colorimetry (Table 6). DSC thermogram of goatfish revealed two major peaks with T_{max} of 49.7 and 71.1°C for mince and T_{max} of 50.3 and 67.7°C for washed mince. The observed T_{max} were similar to those found in various fish species, in which the first and second peaks were postulated to be the transitions of myosin and actin, respectively (Akahane *et al*., 1985). Whole cod muscle showed two maximal transitions on DSC thermogram with T_{max} at about 45 and 75 °C (Hasting *et al*., 1985) and whole muscle of fresh hake also showed two endothermic transitions with T_{max} values of 46 and 75°C

(Beas *et al*., 1990). Fresh bigeye snapper muscle proteins from two species had two major endothermic peaks with T_{max} at 47.7 and 70.6°C for *P. tayenus* and 47.6 and 69.9°C for *P. macracanthus*, respectively (Benjakul *et al*., 2002). Myosin and actin of lizardfish had the T_{max} of 49.55 and 70.94°C, respectively (Tammatinna, 2006).The differences in T_{max} among the fish species seems to be correlated with the habitat temperature of the fish (Akahane *et al*., 1985; Hasting *et al*., 1985; Davies *et al*., 1988). Enthalpy of the first and second peaks were 1.55 and 0.24 J/g for mince and 1.35 and 0.22 J/g for washed mince, respectively. This result indicated that T_{max} of the second peak was shifted to the lower value after washing. However, washing had no impact on T_{max} of the first peak, myosin peak. The result indicated that washing could destabilize actin in resulting washed mince. The removal of sarcoplasmic proteins via washing might lower protection effect of those proteins toward actin denaturation. Additionally, no differences in enthalpy were found between mince and washed mince ($P>0.05$). Lin *et al*. (2005) reported that T_{max} of both myosin and actin of horse mackerel shifted to lower temperature after air floatation washing. The lower temperature endothermic peak was obtained in DSC thermograms of leached muscle and muscle deplete sarcoplasmic protein of both silvertip and hammer-head shark (Lo *et al*., 1995). Chen (1995) suggested that the destabilized myosin was possibly due to the myosin rod unfolding which became easier after destruction of the sarcolemma of muscle during washing.

In the presence of 2.5% NaCl, T_{max} and ΔH of myosin and actin of both mince and washed mince shifted to the lower values. This suggested that both myosin and actin underwent thermal denaturation more easily in the presence of NaCl. NaCl might induce the conformational changes of myosin or actin molecules via the alteration of ionic interaction stabilizing the protein structure. T_{max} of free myosin and actin in shark surimi was reduced when NaCl concentration of surimi increased (Chen, 1995). The denaturation temperature of actin observed in water-washed chicken myofibrils was 16.7°C lower in samples treated with 4% NaCl than was sample without NaCl (Kijowski and Mast, 1988). Similar results were also observed by Wu *et al.* (1985) who found that the salt treatment could decrease the stability of surimi prepared from Atlantic croaker. At 1% salt level, T_{max} of the first peak shifted from 43°C to 38°C when 3% salt was added and the second and third peaks also shifted to lower temperatures. Salt might destabilize protein via the solubilization and dissociation of proteins, especially myosin to an individual molecules and a partial unfolding of the α–helix region of MHC (Chen, 1995). This was probably due to the effect

of Cl^- ion (Beas *et al*., 1991). It can rupture the electrostatic interaction between protein molecule and bind to the potentially active sites on a protein surface resulting in protein solubilization or dissociation (Kijowski and Mast, 1988). The loosened native structure of proteins was sensitive to heat input (Park and Lanier, 1990). Therefore, the addition of salt generally lowered the denaturation temperature and enthalpies of goatfish muscle. This affected the gelation of muscle proteins by influencing the unfolding and aggregation of protein molecules.

Table 6 T_{max} and enthalpy (ΔH) of goatfish mince and washed mince in the presence and absence of 2.5% of NaCl

Samples	Peak I		Peak II	
	T_{max} (°C)	ΔH (J/g)	T_{max} (°C)	ΔH (J/g)
Mince	49.7±0.3[a,*,†]	1.55±0.19[a]	71.1±0.4[a]	0.24±0.01[a]
Mince + 2.5% NaCl	47.9±0.4[b]	0.67±0.04[b]	68.5±0.5[b]	0.18±0.05[ab]
Washed mince	50.3±0.3[a]	1.35±0.33[a]	67.7±0.1[c]	0.22±0.06[a]
Washed mince + 2.5% NaCl	47.3±0.1[c]	0.54±0.09[b]	64.1±0.4[d]	0.10±0.04[b]

[*]Values are given as mean ± SD from triplicate determinations.

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

1.2.2 Thermal stability

The inactivation rate constants (K_D value) of natural actomyosin (NAM) from goatfish mince is shown in Table 7. Ca^{2+}-ATPase activity of myosin has been generally used to evaluate the thermal stability of fish protein. The K_D values of goatfish NAM generally increased as the incubation temperature increased ($P<0.05$). Non-significant increases in K_D values were noticeable at temperature below 20°C. At temperature ranges of 30-40°C, the substantial increases in K_D value were observed. At temperature above 40°C, K_D could not detectable, which was associated with the complete loss in Ca^{2+}-ATPase activity. This result implied that NAM, especially MHC of goatfish, was not stable at high temperatures, particularly at temperature above 40°C. MHC has been reported to possess Ca^{2+}-ATPase activity, which can be used as the indicator of MHC integrity (Benjakul *et al*., 1997). The result was in accordance with the completed denaturation of myosin observed at temperature 49.72°C by DSC. As a consequence, no measurement in K_D value at 50 and 60°C was achieved. Benjakul *et al.* (2002) reported that K_D

value from two species of bigeye snapper increased rapidly at 40-60°C. *Priacanthus macracanthus* NAM was more susceptible to thermal denaturation than that of *Priacanthus tayenus*. Generally, K_D value of NAM was significantly lower than that of myosin. Actin was suggested to play a protective role in the stability of myosin (Jiang *et al*., 1989). K_D values were detected at 50 and 60°C in bigeye snapper, whereas it was not detected in goatfish NAM. The differences in stability of various fish most likely resulted from the different intrinsic properties, amino acid composition as well as myosin/actin ratio (Jiang *et al*., 1989).

Table 7 Thermal inactivation rate constant (K_D) of natural actomyosin from goatfish muscle

Temperature (°C)	$K_D \times 10^5$ (S^{-1})
0	$0.89\pm0.29^{c,*,\dagger}$
10	1.43 ± 0.45^{c}
20	3.95 ± 0.32^{c}
30	16.03 ± 3.32^{b}
40	68.16 ± 6.29^{a}
50	ND**
60	ND

*Values are given as mean ± SD from triplicate determinations.

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

**ND: Not detectable.

2. Thermal properties and aggregation of natural actomyosin (NAM) as influenced by iced storage

2.1 Thermal properties of NAM

2.1.1 Thermal denaturation

Thermal transition of goatfish NAM extracted from goatfish muscle stored in ice for 0 (G_0) and 9 days (G_9) using DSC is shown in Table 8. NAM from fresh goatfish (G_0) exhibited two major peaks with the maximum transition temperatures (T_{max}) of myosin and actin of 47.4 and 63.5°C, respectively. After stored in ice for 9 days (G_9), T_{max} of myosin shifted to a lower level (45.5°C) with coincidental decrease in enthalpy ($P<0.05$). Lower T_{max} and enthalpies indicated

that myosin from ice-stored goatfish had lower thermal stability as compared with that from fresh fish. However, no differences in T_{max} and ΔH of actin were found between NAM from fresh and ice-stored goatfish ($P>0.05$). Rattanasatheirn *et al*. (2008) found that both T_{max} and ΔH of myosin peak of white shrimp shifted from 50.1 to 49.8°C and from 1.65 and 0.76 J/g, respectively, after kept in ice for 7 days. T_{max} of red claw fish for myosin head (50.2°C) and actin (72.6°C) showed a significant decrease after 7 days of iced storage to 46.3 and 69.7°C and dropped to 39.4 and 60.3°C after 14 days of iced storage (Tseng *et al.*, 2002). From the result, myosin was more susceptible to denaturation during extended iced storage. During storage, autolysis might take place, leading to the losses in protein integrity. Endogenous proteolytic enzymes including calpain and lysosomal proteinases might partially degrade MHC, leading to the ease of denaturation at lower temperature with less energy input. Moreover, the protein conformation changes during iced storage were not only caused by proteolysis but also chemical reaction, such as lipid oxidation. The lipid oxidation products could interact with muscle proteins resulting in the destabilized proteins (Tseng *et al*., 2002). Thus, the iced storage of goatfish had the influence on thermal property of muscle proteins from goatfish.

Table 8 T_{max} and enthalpy (ΔH) of natural actomyosin from fresh goatfish and goatfish stored in ice for 9 days

Samples	Peak I		Peak II	
	T_{max}(°C)	ΔH (J/g)	T_{max}(°C)	ΔH (J/g)
G_0	47.4±0.3[a,*, †]	0.22±0.03[a]	63.5±0.5[a]	0.05±0.03[a]
G_9	45.5±0.2[b]	0.06±0.02[b]	62.9±0.3[a]	0.05±0.02[a]

[*]Values are given as mean ± SD from triplicate determinations.

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

2.1.2 Thermal stability

The inactivation rate constants (K_D value) of natural actomyosin extracted from fresh goatfish (G_0) and goatfish stored in ice for 9 days (G_9) are shown in Table 9. No changes in K_D were observed for G_0 and G_9 when heated up to 30 and 20°C, respectively. A significant increase in K_D value was observed for G_0 and G_9 when the temperatures were 40 and 30°C, respectively. However, no K_D of both samples was detected at temperature range of 50-60°C. At

the same heating temperature, the higher K_D value was found in G_9 than was G_0. This result indicated that myosin from G_9 was unstable and underwent partial denaturation during handling and storage in ice. The result was in agreement with the DSC analysis (Table 8), which revealed that the lower enthalpy of G_9 was obtained in comparison with G_0. The changes were thought to be induced by proteolytic activity associated with muscle or microbial proteinases. Hydrolysis might result in the ease of comformational changes of proteins, particularly the head portion of MHC. Benjakul *et al.* (1997) found that the changes in troponin-tropomyosin complex of Pacific whiting muscle during ice storage was caused by proteolysis. Visessanguan and An (2000) reported that myosin from arrowtooth flounder in the presence of 2.5 mU papain exhibited the lower enthalpy required to induce myosin denaturation (1.9 J/g) than in the absence of papain (5.2 J/g). It was inferred that the longer storage time, the greater denaturation occurred. This might affect the functional properties of goatfish stored in ice.

Table 9 Thermal inactivation rate constant of natural actomyosin extracted from fresh goatfish and goatfish stored in ice for 9 days

Temperature (°C)	$K_D \times 10^5$ (S^{-1})	
	G_0	G_9
0	0.66±0.44^{bA,*,**,†}	0.93±0.22^{cA}
10	0.87±0.46^{bA}	1.03±0.45^{cA}
20	1.23±0.09^{bB}	2.36±0.39^{cA}
30	5.04±0.61^{bB}	9.70±0.85^{bA}
40	81.60±7.24^{aB}	120.03±5.75^{aA}
50	ND***	ND
60	ND	ND

†Values are given as mean ± SD from triplicate determinations.

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

**Different capital superscripts in the same row indicate significant differences ($P<0.05$).

***ND: Not detectable

2.2 Thermal aggregation of NAM

2.2.1 Turbidity of NAM

Turbidity development of NAM solution (1 mg/ml) extracted from fresh goatfish (G_0) and goatfish stored in ice for 9 days (G_9) as well as from bigeye snapper (B) were monitored from 20 to 75°C (Figure 12). Absorbance reading is commonly used to monitor the extent of protein aggregates (Benjakul *et al*., 2001a). NAM solution became more turbid as temperature increased, suggesting an increased protein aggregates. NAM solution consists of long filaments, in which approximately 1 mm long thin filaments of actin, tropomyosin and troponin are conjugated with a great number of myosin all along the filament (Benjakul *et al*., 2001a). Each myosin molecule is bound to the actin filament at its head portion with its tail portion sticking out (Sano *et al*., 1988). Thus, NAM molecules tend to interact each other and form protein aggregates upon heating. An increase in turbidity of G_0 and B was observed at 35°C while NAM of goatfish stored in ice for 9 days (G_9) was found at 30°C. This was related with the onset of denaturation, which was 41.3°C and 39.2°C, respectively (data not shown). Bigeye snapper NAM exhibited the higher rate in turbidity development than did goatfish NAM, both G_0 and G_9 at temperatures above 40°C. The differences in turbidity of myofibril or myosin solution among fish species were most probably due to the varying size and/or rate of aggregation (Chan and Gill, 1994). Muscle protein molecules can associate with one another through hydrophobic, electrostatic and hydrogen bond as well as disulfide bond (Chawla *et al*., 1996; Xiong, 1997). The relative contribution of each bond is different between aggregated proteins and the native counterparts (Xiong, 1997). The result suggested that NAM from bigeye snapper could undergo aggregation at a higher extent, compared to NAM extracted from goatfish. Bigeye snapper has been used as potential raw material for surimi production owing to the excellent gel formability of this species. However, goatfish yields the surimi with poor gel. Benjakul *et al.* (2001a) reported that the difference in aggregation of NAM from bigeye snapper of two species was due to the difference in amount of hydrophobic domain and in intermolecular disulfide bonds formation during heating process. When comparing NAM from goatfish, G_0 and G_9, the greater turbidity was observed in NAM extracted from fresh goatfish (G_0) than that stored in iced (G_9). The result suggested that G_0 underwent higher aggregation than did G_9. When proteins underwent denaturation or degradation during iced storage, they might lose their ability in aggregation, possibly due to the hindrance of reactive

groups or the shorter chain length caused by autolysis. Yongsawatdikul and Park (1999) reported that a slower heating rate (0.5°C/min) of myosin purified from Pacific whiting exhibited the lower aggregation than higher heating rates (2°C/min) which was caused by proteolysis action, while greater aggregation of myosin purified from cod was found when monitored by slow heating rate. Formation of large aggregates is presumably a prerequisite to formation of a good elastic gel (Chan *et al*., 1992b). Chan *et al.* (1992b) reported that the poorer aggregating ability of herring actomyosin reflected the inferior gelling properties of surimi. The differences in aggregation of NAM were postulated to be associated with the different gelling properties between species.

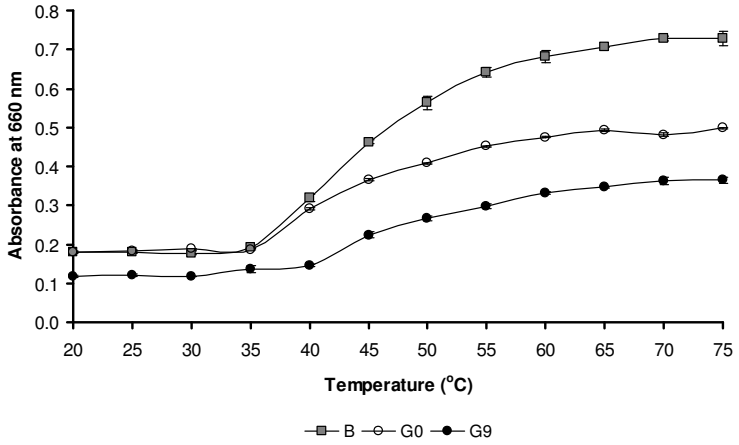


Figure 12 Turbidity of different NAM solutions (1 mg/ml) during heating from 20 to 75°C at 0.65°C/min. B, G_0 and G_9 represent NAM solutions extracted from bigeye snapper, fresh goatfish and goatfish stored in ice for 9 days, respectively. Bars represent standard deviation from triplicate determinations.

2.2.2 Surface hydrophobicity

Changes in surface hydrophobicity (S_0ANS) NAM solutions (1 mg/ml) of both G_0 and G_9 and bigeye snapper (B) were shown in Figure 13. S_0ANS continually increased after heating at the temperature above 35°C up to 60°C. Thereafter, slight decreases in S_0ANS were observed at temperature above 65°C for G_0 and G_9, and above 70°C for B sample. The increase in S_0ANS indicated the structural changes of NAM during heating. Upon heating, the aromatic hydrophobic amino acid residues, i.e. phenylalanine and tryptophan, could be exposed to a greater extent (Visessanguan *et al.*, 2000b). ANS, an effective fluorescent probe, has been found to bind

non-polar regions of protein (Wicker *et al*., 1986). A slight decrease in S_0ANS at temperatures above 65 or 70°C suggested that the hydrophobic interaction between proteins molecules took place. Higher S_0ANS in bigeye snapper NAM was found, compared with goatfish NAM, possibly caused by the differences in amino acid composition, especially hydrophobic amino acids. The exposure of hydrophobic domains has been suggested as a pre-requisite for formation of large myosin aggregates via hydrophobic interaction (Chan *et al*., 1992a). For goatfish NAM, S_0ANS of G_9 was higher than G_0 at temperature range of 20-35°C. Nevertheless, G_0 exhibited the higher S_0ANS than G_9 when heated above 40°C. With the sufficient heat, G_0 which had more native form of protein molecules, could undergo conformational changes or the exposure of interior molecules that hydrophobic portion is located, to the greater extent than G_9. The rate of the increase in S_0ANS in G_0 was higher than G_9 for further heating at temperature above 40°C. This result suggested that hydrophobic portion of G_9 might be less exposed during heating. This might be caused by the prior aggregation during iced storage. From the result, higher surface hydrophobicity was in good agreement with the higher aggregation as indicated by greater turbidity (Figure 12).

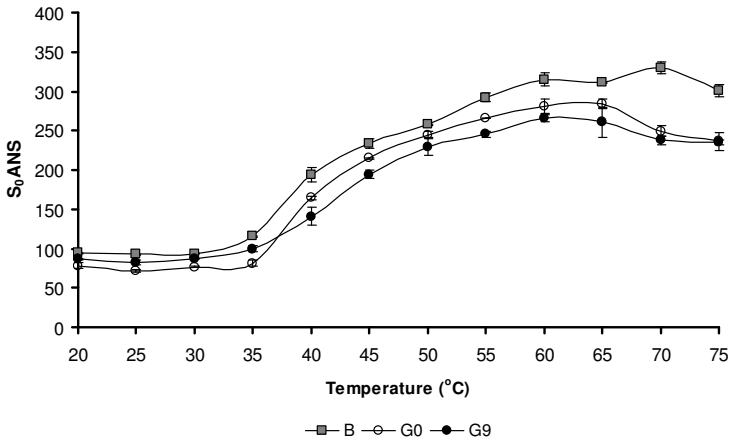


Figure 13 Surface hydrophobicity (S_0ANS) of different NAM solutions (1 mg/ml) during heating from 20 to 75 at 0.65°C/min. B, G_0 and G_9 represent NAM solutions extracted from bigeye snapper, fresh goatfish and goatfish stored in ice for 9 days, respectively. Bars represent standard deviation from triplicate determinations.

2.2.3 Total sulfhydryl group content and disulfide bond formation

Changes in total sulfhydryl group content (total SH) and disulfide bond formation of different NAM solutions after heating from 20 to 75°C were shown in Figure 14. The continuous decrease in total SH content was observed in all samples as heating temperatures increased starting from 35°C or above (Figure 14A). Decreases in total SH content were concomitant with the increased disulfide bond formation of all NAM samples. During heating, the formation of disulfide bond might be induced and involved in aggregation. This indicated that thermally exposed sulfhydryl groups could be oxidized to disulfide bond, especially at temperature above 40°C. A decrease in total SH groups was reported to be due to the formation of disulfide bond through oxidation of SH group or disulfide interchanges (Hayagawa and Nakai, 1985) (Figure 14B). Chan *et al*. (1995) reported that myosin contained 42 SH groups. Two type of SH groups on the myosin head portion, named SH_1 and SH_2, have been reported to be involved in ATPase activity of myosin (Benjakul *et al.*, 1997; Yamaguchi and Sekine, 1966). Another SH group (SH_a) localized in the light meromyosin contributes to oxidation (Sompongse *et al*., 1996). Niwa *et al*. (1992) reported that the disulfide bond formation of fish actomyosin and myosin occurred at temperature 40 and 45°C, respectively. Yongsawatdigul and Park (2003) reported that the disulfide bond formation in tilapia actomyosin required high temperature (above 50°C). Disulfide bond content was higher in NAM from bigeye snapper, in comparison with goatfish NAM at all temperatures tested. This was in accordance with the higher aggregation of the former NAM as indicated by the greater turbidity. This was possibly due to the differences in amino acid composition as well as the structural rigidity. After the storage in ice for 9 days, disulfide bond formation of G_9 was lower than G_0, especially, at temperature higher than 40°C. This result indicated that G_9 NAM possibly underwent oxidation during iced storage. As a result, during heating, the intermolecular disulfide bond formation could occur to a lower content, leading to the less aggregation. Benjakul *et al.* (2001a) reported that the hydrophobic interaction and disulfide bond formation determined the aggregation of protein as well as gel properties.

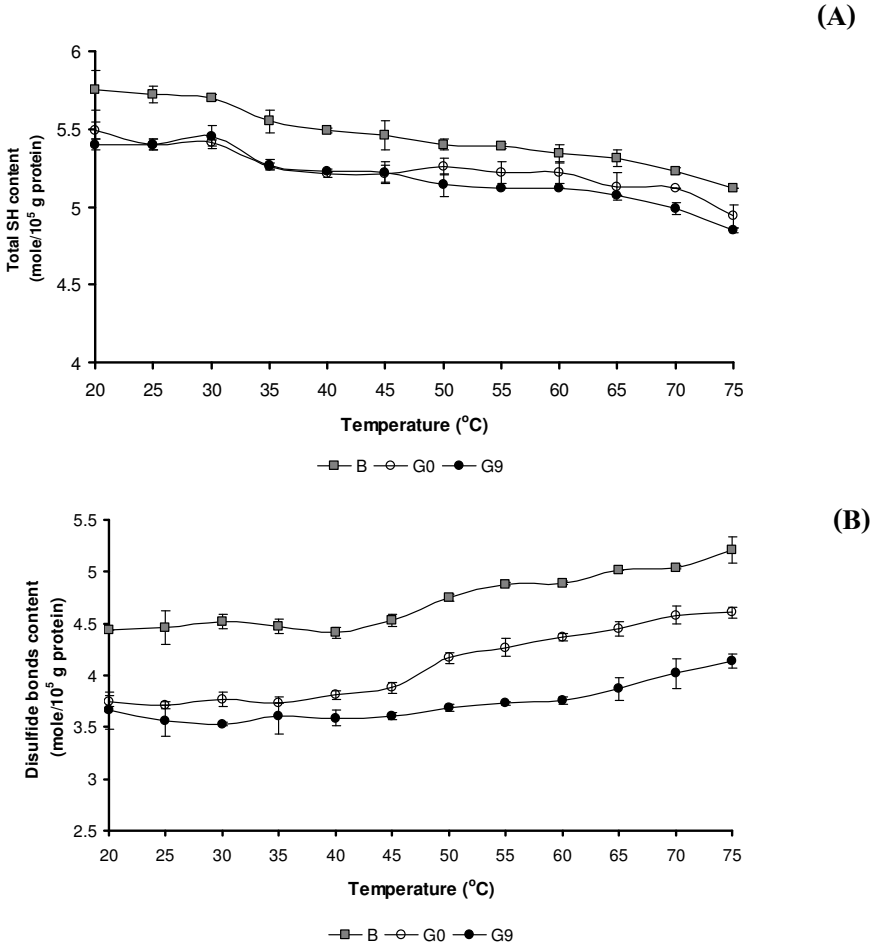


Figure 14 Total sulfhydryl group content (A) and disulfide bond content (B) of different NAM solutions (1 mg/ml) during heating from 20 to 75 at 0.65°C/min. B, G_0 and G_9 represent NAM solution extracted from bigeye snapper, fresh goatfish and goatfish stored in ice for 9 days. Bars represent standard deviation from triplicate determinations.

2.2.4 Protein Patterns

Protein patterns of mince and NAM are shown in Figure 15. Similar protein patterns of mince were observed between bigeye snapper and goatfish mince. However, mince from goatfish stored in ice for 9 days had the lower MHC band intensity. This result revealed that MHC was more degraded during iced storage. Slight decrease in actin band intensity was also observed in G_9. However, Benjakul *et al*. (1997) reported that MHC was more prone to proteolytic degradation than other proteins, i.e actin, troponin and tropomyosin. For the NAM, many proteins, particularly sarcoplasmic proteins, found in mince were largely removed by extracting process.

NAM contained myosin and actin as the major constituents. Other proteins naturally associated with myosin and actin including troponin, tropomyosin and C-proteins were also found in NAM (Benjakul *et al*., 2001a). No differences in protein patterns between NAM from G_0 and G_9 were observed. Although some degradation might occur in goatfish during iced storage, those small M_w peptide were most likely removed during extraction. As a consequence, the similar components were noticeable.

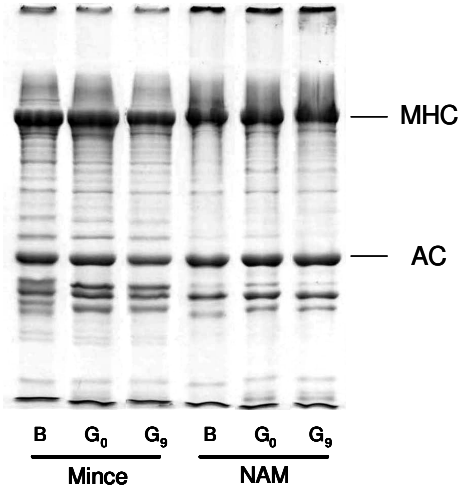


Figure 15 Protein patterns of mince and natural actomyosin (NAM) extracted from mince of goatfish and bigeye snapper. B: bigeye snapper, G_0: fresh goatfish; G_9: goatfish stored in ice for 9 days; MHC: myosin heavy chain; AC: actin.

2.2.5 Microstructure of heated NAM

The microstructure study of NAM from fresh goatfish and from goatfish stored in ice for 9 days as well as from bigeye snapper after heating to 75°C revealed that the aggregation of NAM occurred during heat treatment as depicted in Figure 16. The heated NAM exhibited the networks with the fibrous strand of each single actomyosin molecule. NAM network from fresh goatfish had the larger strand with the coarser structure, compared to that of NAM from bigeye snapper. Gelation is the result of protein denaturation, followed by the aggregation via inter-molecular covalent bonds and noncovalent interactions (Lee and Lanier, 1995). The thermal treatment produces enough protein denaturation to cause the interactions and the formation of a network structure (Totasaus *et al*., 2002). Large aggregates were formed by various bonds including hydrophobic interaction and disulfide bonds (Figures13 and 14B). The native actomyosin was found an arrowhead structure on the filaments and became shortened after heat

treatments (Ko *et al*., 2007). Looser protein network was formed in NAM from goatfish stored in ice for 9 days. This was coincidental with the lower turbidity development (Figure 12). From the result, the alignment of myofibrillar proteins as well as aggregation pattern induced by heat treatment was different between species. Moreover, the proteins from fish with lower freshness exhibited the lower gel forming ability, compared with the fresh counterpart. Benjakul *et al.* (2001a) noted that the differences in NAM gelation between two fish species were caused by different intrinsic properties of muscle proteins, mainly myosin and actin. Yongsawatdigul and Park (1999) reported that the intrinsic parameters, i.e. endogenous proteinases, were important in controlling gelation on fish myosin which led to lowering gel formation.

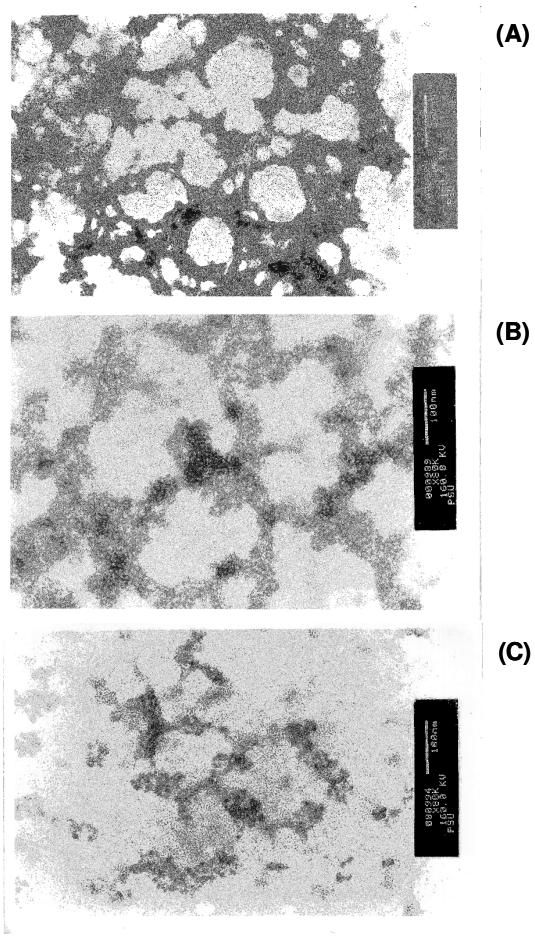


Figure 16 Transmission electron micrograph on aggregation of NAM from bigeye snapper (A), fresh goatfish (B) and goatfish stored in ice for 9 days (C) after heating at 0.65°C/min to 75°C. Heated NAM was cooled rapidly in iced water prior to TEM analysis. Magnification: 80,000×.

3. Effect of some factors on gel properties of goatfish surimi

3.1 Effect of setting condition and $CaCl_2$ levels on gel properties

3.1.1 Breaking force and deformation

Breaking force and deformation of goatfish surimi gel with $CaCl_2$ addition at different levels prepared under various heating conditions are shown in Figure 17. Regardless of $CaCl_2$ addition, the different heating condition for gel preparation resulted in different gel properties. The highest breaking force and deformation were observed in gel with prior setting (medium temperature setting: 25°C for 3 h or high temperature setting: 40°C for 30 min and followed by heating at 90°C for 20 min), while the lowest values were found in gel with prior incubation at 60°C for 30 min, followed by heating at 90°C for 20 min (modori gel). This result indicated that gel weakening was found in gel with pre-incubation at 60°C and gel strengthening was obtained when prior setting was used. Generally, setting is a phenomenon describing the increased gel strength after pre-incubation of surimi paste at a certain temperature between 5 and 40°C for a specific period of time (Lanier, 2000). In the absence of $CaCl_2$, gels with prior setting at 40°C showed the higher breaking force than those set at 25°C ($P<0.05$). For deformation, gel added with 25 or 50 mmoles $CaCl_2$/kg had the higher deformation when setting at 40°C was used, compared with setting at 25°C ($P<0.05$). This was probably because endogenous TGase in goatfish muscle was more active at 40°C than at 25°C. Furthermore, the alignment or aggregation of protein probably took place to a high extent with higher temperature setting. At sufficiently high temperature, both hydrophobic interaction and disulfide bond formation could occur (Niwa, 1992). Thereafter, unfolded proteins underwent more aggregation, via both disulfide bonds and hydrophobic interaction between molecules as the temperature was elevated to 90°C. Benjakul *et al.* (2004a) reported that high temperature setting at 40°C generally used for enhancing gel properties of surimi from tropical fish, i.e threadfin bream, bigeye snapper, barracuda and bigeye croaker. Setting under an appropriate condition contributed to improving gelling properties of surimi (Benjakul *et al*., 2003a). An improvement of textural properties is attributed to an endogenous transglutaminase (TGase) that catalyzes the cross-linking reaction of muscle proteins, especially myosin (Kimura *et al*., 1991). The enzyme catalyzes an acyl transfer reaction between γ-carboxy amide group of glutamyl residues in protein as the acyl donor and variety of primary amines as the acyl acceptor (Folk, 1980). The formation of ε-(γ-glutamyl) lysyl isopeptide bonds

between glutamine (acyl donors) and lysine (acyl acceptor) resulted in a covaltent cross-linking of muscle proteins correlates with the increase in gel strength (Rawdkuen *et al*., 2005). Benjakul and Visessanguan (2003) reported that setting at either 25 or 40 °C prior to heating at 90°C, resulted in the increase in both breaking force and deformation of surimi gel from bigeye snapper. From the results, setting of goatfish surimi sol prior to heating was crucial for gel strengthening. On the other hand, the modori-inducing proteinases, especially heat stable and heat activated proteinase, caused the soft and mushy gel texture.

When $CaCl_2$ was added, breaking force and deformation of all gels increased with addition of $CaCl_2$, excepted modori gel (60 /90°C). With 50 mmoles $CaCl_2$/kg addition, breaking force of gel with prior setting at 25°C, 40°C and directly heated gel increased by 36.2, 31.3 and 23.3%, respectively, compared with the control (no $CaCl_2$ addition). This result revealed that the addition of $CaCl_2$ and setting significantly improved the textural properties of goatfish surimi gels. Ca^{2+} could activate endogenous TGase activity. Additionally, setting directly promoted the conformational changes of TGase substrate, myofibrillar proteins and more available reactive groups of glutamine and lysine could be exposed (Hemung and Yongsawatdigul, 2003). From the result, the highest breaking force and deformation was found when high temperature setting together with the addition of 50 mmoles $CaCl_2$/kg was used. Morales *et al.* (2001) reported that the use of 0.2% $CaCl_2$ had no effect on the shear stress of surimi gel from Alantic croaker and Mexican flounder. Therefore, the combination of $CaCl_2$ and pre-setting was the promising means to improve the textural properties of surimi from goatfish.

For directly heated gel, gel added with $CaCl_2$ showed the superior breaking force and deformation to the control gel (without $CaCl_2$). Heating at 90°C might result in protein unfolding in which reactive groups with negative charges could be exposed. Ca^{2+} could bind with negative charge localized on the adjacent proteins, resulting the strengthening of surimi by calcium bridge (Wan *et al*., 1994). Furthermore, some cross-linkings induced by endogenous TGase in the temperature range of 25-40°C during raising the temperature up to 90°C might take place. Nevertheless, the decrease in breaking force was found in modori gels added with $CaCl_2$, especially at the higher concentration. $CaCl_2$ might progressively promote endogenous proteolytic activity of calpain, calcium activated proteinase, naturally found in fish muscle. The severe

degradation of myofibrillar proteins induced by proteinases resulted in poor quality of surimi gels (An *et al*., 1996).

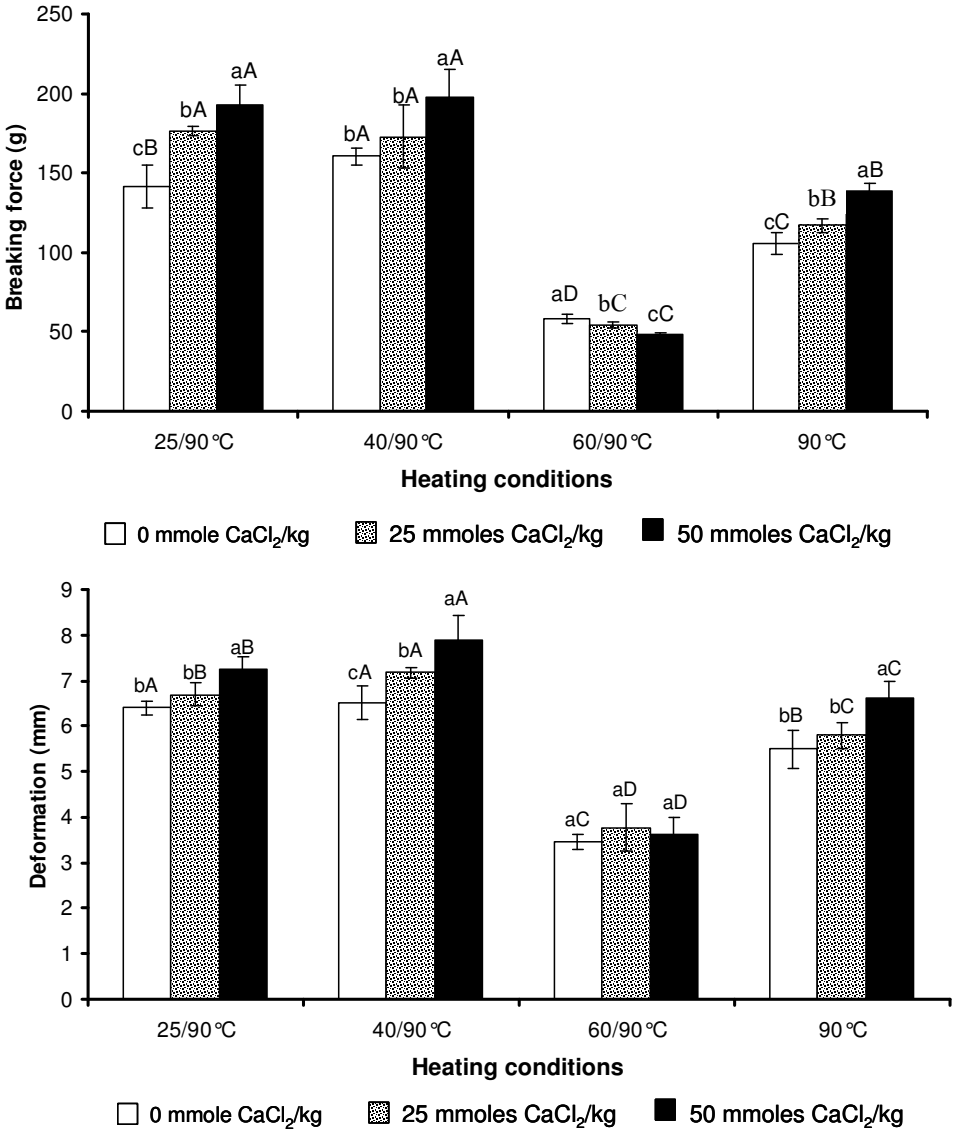


Figure 17 Breaking force and deformation of goatfish surimi gel added with $CaCl_2$ at different levels (0, 25 and 50 mmoles/kg) and prepared under various heating conditions. Bars represent the standard deviation from five determinations. Different letters within the same setting/ heating condition indicated significant differences ($P<0.05$). Different capital letters within the same $CaCl_2$ level indicated significant differences ($P<0.05$).

3.1.2 Whiteness

Whiteness is one factor determining surimi gel quality. The whiteness of goatfish surimi gel added with $CaCl_2$ at different levels (0, 25 and 50 mmole $CaCl_2$/kg) and prepared under various setting/ heating processes is shown in Table 10. The whiteness of all gels was in the range of 72.53-75.91%. Regardless of heating process, whiteness of all gels generally increased as the $CaCl_2$ level increased ($P<0.05$). Addition of $CaCl_2$ might form complex with some anion in the muscle. As a result, the formation of insoluble particles could be formed, leading to the light scattering in goatfish gels. Julavittayanukul *et al*. (2006) reported that the use of $CaCl_2$ at 50 mmoles/kg could increase the whiteness of surimi gel from bigeye snapper. Benjakul *et al.* (2004c) reported that an increase in whiteness was associated with the light scattering effect of insoluble calcium carbonate in surimi gel. At the same $CaCl_2$ level, a little difference in whiteness was observed among gel samples prepared under the different heating processes.

3.1.3 Expressible moisture content

Expressible moisture content of goatfish surimi gels containing $CaCl_2$ at different levels is shown in Table 10. Expressible moisture content decreased continuously as the $CaCl_2$ levels increased ($P<0.05$) of both gels with prior setting (medium and high temperature setting) and directly heated gel. Decrease in expressible moisture content indicated an increase in water-holding capacity of the gel. The results suggested that the higher amount of $CaCl_2$ could enhance the gelation of goatfish protein either by the activation of endogenous TGase or formation of Ca^{2+}-bridge between protein molecules, resulting in the greater ability of gel to hold more water. The higher expressible moisture content was found in directly heated gel, compared with gels subjected to prior setting ($P<0.05$). With rapid heating, the rate of protein aggregation was faster than protein dissociation, resulting in more intense coagulation and low affinity for water retention. When comparing expressible moisture content between gels set at medium and high temperature, the lower expressible moisture content was found in gel with high temperature setting. This reconfirmed that the optimal condition for the improvement of gel quality from goatfish was setting at high temperature in combination with 50 mmoles $CaCl_2$/kg.

For modori gel, the highest expressible moisture content was noticeable, compared to other gels. Expressible moisture content increased with increasing $CaCl_2$ levels ($P<0.05$). This result suggested that Ca^{2+} might activate endogenous proteinases, which could hydrolyze myofibrillar proteins. As a consequence, the network formed could be destroyed to some extent, leading to the lower ability to imbibe water. This result was in agreement with the lowest breaking force and deformation of modori gels, particularly those added with $CaCl_2$ at higher levels.

Table 10 Whiteness and expressible moisture content of goatfish surimi gel added with $CaCl_2$ at different levels and prepared under various heating conditions

Setting/heating conditions	$CaCl_2$ levels (mmoles/kg)	Whiteness (%)	Expressible moisture content (%)
25/90°C	0	$72.53\pm0.83^{bC,\dagger,*,**}$	4.73 ± 0.02^{aBC}
	25	75.39 ± 0.27^{aA}	4.20 ± 0.15^{bA}
	50	75.53 ± 0.15^{aA}	3.88 ± 0.17^{cB}
40/90°C	0	73.18 ± 0.36^{cB}	4.09 ± 0.06^{aC}
	25	75.11 ± 0.18^{bB}	4.14 ± 0.16^{aC}
	50	75.91 ± 0.17^{aA}	3.70 ± 0.03^{bB}
60/90°C	0	73.95 ± 0.20^{bA}	13.99 ± 1.51^{bA}
	25	75.47 ± 0.13^{aA}	15.27 ± 0.21^{abA}
	50	75.75 ± 0.41^{aA}	16.52 ± 1.03^{aA}
90°C	0	73.26 ± 0.19^{cB}	5.75 ± 0.25^{aB}
	25	74.90 ± 0.19^{bB}	4.74 ± 0.19^{bB}
	50	$75.440\pm.22^{aA}$	4.51 ± 0.03^{bB}

†Values are given as means ± SD from five determinations.

*Different letters within the same heating condition in the same column indicate the significant differences ($P<0.05$).

**Different capital letters within the same $CaCl_2$ level in the same column indicate the significant differences ($P<0.05$).

3.1.4 TCA-soluble peptide content

Protein degradation was monitored as TCA-soluble peptide content in different surimi gels added with $CaCl_2$ at various levels (Figure 18). Regardless of $CaCl_2$ addition, the highest TCA-soluble peptide content was observed in modori gel, compared with other gels, indicating that the highest degradation induced by endogenous proteinases associated with muscle. Generally, proteolytic activity in fish muscle is high at 50–60°C and causes rapid and severe degradation of myofibrillar proteins, particularly myosin (An *et al*., 1996). TCA-soluble peptides were also found in directly heated gel and gels with prior setting. It was suggested that proteolytic degradation still occurred during setting or heating, but at a lower degree. In the presence of 50 mmoles $CaCl_2$/kg, higher degradation of muscle protein was observed at high temperature, in comparison with medium temperature setting. Temperature of 40°C was close to the optimum temperature for most heat-activated fish proteinase (50–60°C) (Kang and Lanier, 2000). When comparing TCA-soluble peptide content between directly heated gel and gels with prior setting, the former contained the lower content than did the latter.

For gels with prior setting and directly heated gel, TCA-soluble peptide content decreased as $CaCl_2$ level increased ($P<0.05$). Cross-linked protein at higher levels of $CaCl_2$ might be more resistant to proteolysis. As a consequence, the lower degradation occurred. Moreover, Ca^{2+} at higher amount might bind with protein substrates and caused the conformational changes, in which proteinases could not hydrolyze those substrates easily. Conversely, the greater protein degradation was found in modori gel added with higher $CaCl_2$ levels as evidenced by the higher TCA-soluble peptide content. This was possibly due to the activation of calpains by $CaCl_2$ added. Although the use of 10 mM Ca^{2+} was essential to activate endogenous TGase via myosin cross-linking, it could activate calpain counterpart in squid paste (Park *et al*., 2003).

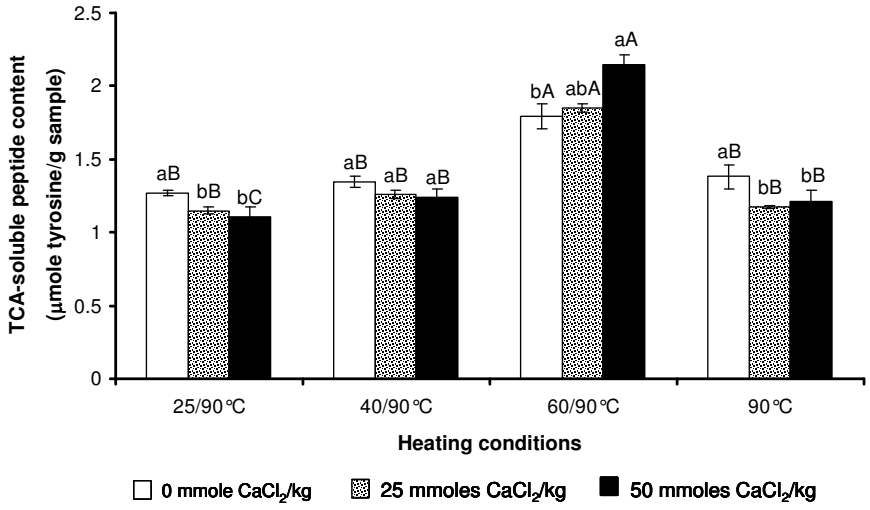


Figure 18 TCA-soluble peptide content of goatfish gels added with $CaCl_2$ at different levels and prepared under various heating conditions. Bars represent the standard deviation from five determinations. Different letters within the same heating condition indicated significant differences ($P<0.05$). Different capital letters within the same $CaCl_2$ level indicated significant differences ($P<0.05$).

3.1.5 Protein patterns

Protein patterns of different surimi gels with various $CaCl_2$ levels are depicted in Figure 19. MHC band intensity of all gels decreased to different degrees, compared to that found in surimi sol. For gels with prior setting, the decreases in MHC band intensity were noticeable as $CaCl_2$ levels increased. Decrease in MHC band intensity was presumed to be due to the cross-linking in the presence of Ca^{2+}- ion as evidenced by the formation of higher molecular weight protein molecules, which were observed on the top of polyacrylamide gels. The result indicated that MHC underwent more polymerization with the addition of $CaCl_2$, especially via non-disulfide covalent bonds. Cross-linked proteins were not dissociated in the mixture of SDS and mercaptoethanol used for SDS-PAGE analysis. However, no differences in actin band were observed in all gels, irrespective of $CaCl_2$ levels. Benjakul *et al.* (2004a) reported that the decreases in actin band of gel produced from tropical fish were found when used of $CaCl_2$ at very high level, particularly at 120 mmoles $CaCl_2$/kg. Nakahara *et al*. (1999) reported that endogenous TGase form carp could not cross-linked actin molecules due to the lack of reactive lysyl residue as the acyl acceptor. The result revealed that MHC was more preferable protein substrate for

polymerization induced by endogenous TGase, compared with actin. The formation of cross-linked protein (CP) corresponded with the increases in breaking force and deformation found in gels with prior setting (Figure 17). At the same level of $CaCl_2$ added, MHC was more retained in gel with medium temperature setting than that found in gel set at high temperature. This suggested the lower cross-linking of MHC in gel set at medium temperature. The more MHC retained, the less the breaking force and deformation were obtained (Figure 17). The higher TCA-soluble peptide content was observed in gel with high temperature setting. This result revealed that the proteolysis still occurred during setting at 40°C. Nevertheless, the rate of polymerization was higher than proteolysis in gel with prior setting (Benjakul and Visessanguan, 2003), leading to the strengthening of gel.

For modori gel, the disappearance of MHC was noticeable, regardless of $CaCl_2$ levels added. The decrease in MHC was concomitant with the formation of low molecular weight degradation peptides indicated by the high TCA-soluble peptide content (Figure 18). However, no difference in MHC band intensity of samples added with different $CaCl_2$ levels was found. Benjakul *et al*. (2003c) reported that heat stable alkaline proteinase from bigeye snapper mainly degrade MHC of natural actomyosin but not actin. An *et al*. (1994b) reported that myofibillar proteins of Pacific whiting underwent extreme textural softening upon heating due to an endogenous heat stable proteinase, which primarily hydrolyzed myosin. Some cross-linked proteins were also found in modori gel as shown by the appearance of polymerized proteins on the top stacking gel. For the directly heat gel, the decrease in MHC band intensity was found as the $CaCl_2$ increased. However, the MHC was retained to a greater extent, when compared with that found in gel with prior setting. This result reconfirmed that non-disulfide convalent bond mainly caused by endogenous TGase occurred during setting. Those cross-links were also formed in directly heated gel, but the extent was lower than gel with prior setting. Setting for a sufficient time allowed TGase to induce the protein cross-linking effectively. From the result, TGase played an essential role in gel enhancement of goatfish surimi. Thus, the use of $CaCl_2$ at appropriate level in combination with setting phenomenon could improve the gel forming ability of surimi for goatfish.

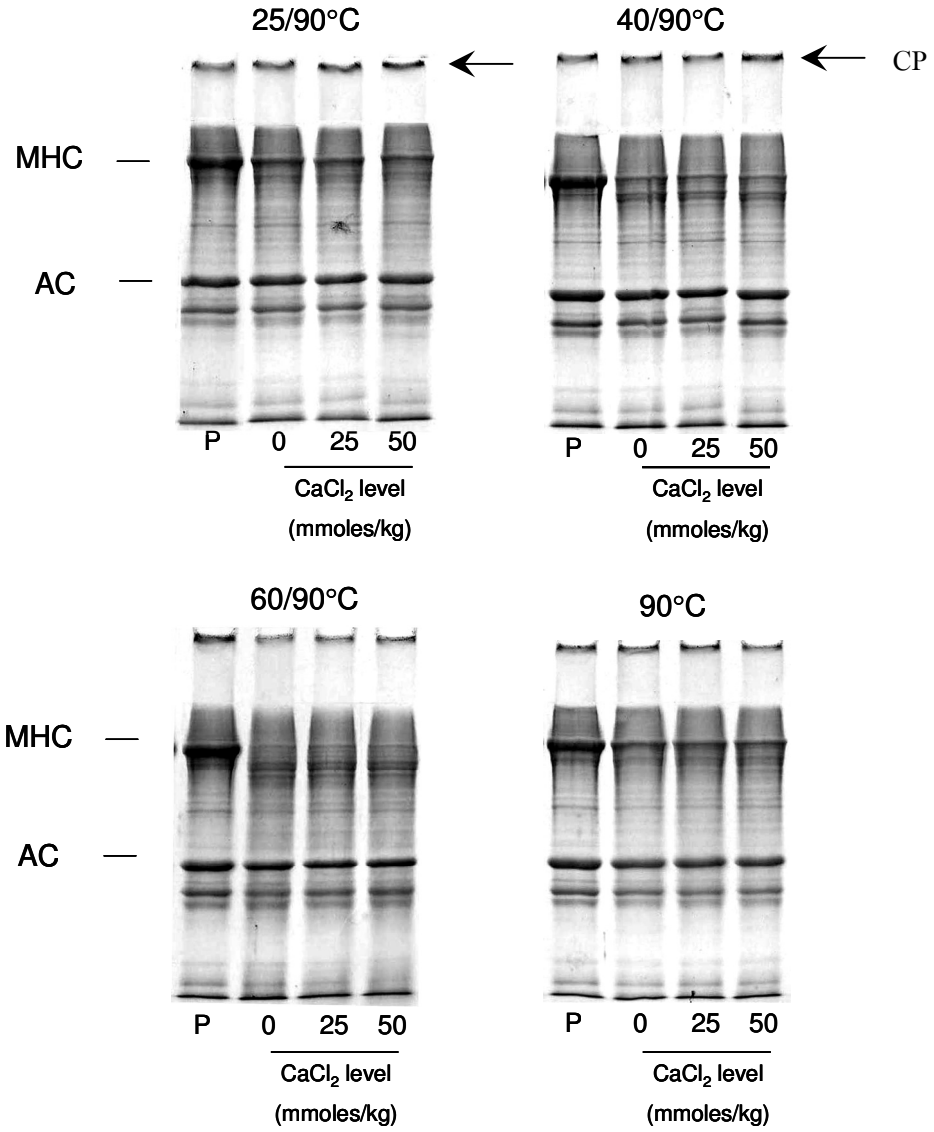


Figure 19 Protein patterns of goatfish surimi gels added with $CaCl_2$ at different levels and prepared under various heating conditions. P: surimi paste; MHC: myosin heavy chain; AC: actin; CP: cross-linked protein; Number designate $CaCl_2$ levels (mmoles/kg).

3.2 Effect of iced storage of goatfish on physicochemical properties and gel forming ability of surimi

3.2.1 Chemical and physicochemical changes during iced storage

3.2.1.1 Changes in TVB-N and TMA-N contents

Changes in TVB-N and TMA-N contents of goatfish muscle during iced storage are shown in Figure 20. The initial TVB-N content of 5.73 mg N/100 g sample in fish sample at day 0 suggested that fish muscle possibly underwent some deterioration during handling or storage prior to analysis. Riebroy *et al*. (2007) and Benjakul *et al.* (2003e) reported that bigeye snapper and lizardfish sample muscle had TVB-N contents of 5.53 and 10.01 mg N/ 100 g sample, respectively. Small amount of TMA-N (0.30 mg N/100 g sample) was also found in fish muscle at day 0. The presence of TMA indicated that some changes of TMAO to TMA mediated by some microorganisms occurred prior to iced storage. Both TVB-N and TMA-N contents increased as the storage time increased ($P<0.05$). The formation of TVB-N is generally associated with the growth of microorganism and can be used as an indicator of spoilage (Benjakul *et al*., 2002). The production of TMA in muscle during cold storage could be used as an indicator of bacterial activity (Gokodlu *et al*., 1998). Generally, TVB-N comprises mainly TMA-N and ammonia, which are produced by both microbial and endogenous enzymes. A number of specific spoilage bacteria such as *Shewanella putrefaciens, Photobacterium phosphoreum and Vibrionaceae,* typically use TMAO as an electron acceptor in anaerobic respiration, resulting in off-odor and off-flavor due to the formation of TMA (Gram and Huss, 1996; Huss, 1995). The formations of TVB-N and TMA-N in goatfish during iced storage were probably mediated by psychrotropic and psychrotolerant bacteria (Sasajima, 1973; Gokodlu *et al*., 1998). TVB-N content of 30 mg N/100 g sample is generally regarded as the fish acceptability limit (Sikorski *et al*., 1990). From the result, the storage of goatfish in ice longer than 12 days yield the fish with unacceptability for consumption. This was in accordance with the formation of slime on the fish surface from the visual observation and putrid smell. TVB-N content of 25 mg N/100 g sample was suggested to be a limit for acceptability of cod and temperature water fish (Cornell, 1975). Bennour *et al.* (1991) recommended a TMA-N content of 5 mg N/100 g as the rejection value for mackerel. Therefore, the extended storage of goatfish in ice caused the loss in quality.

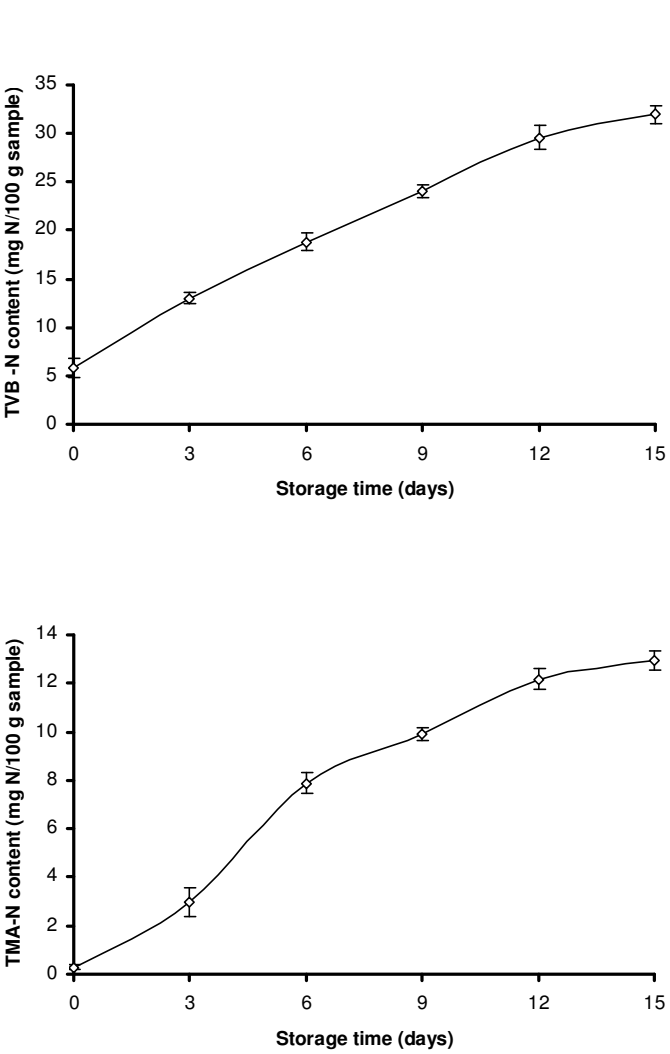


Figure 20 Changes in TVB-N (A) and TMA-N (B) contents of goatfish muscle during 15 days of iced storage. Bars represent the standard deviation from five determinations.

3.2.1.2 Change in Ca^{2+}- ATPase activity

The changes in Ca^{2+}-ATPase activity of natural actomyosin (NAM) extracted from goatfish during iced storage are depicted in Figure 21. A slight decrease in Ca^{2+}-ATPase activity of NAM extracted from goatfish was observed throughout the iced storage ($P<0.05$). This result indicated that MHC, particularly head portion, underwent some conformational changes during iced storage. Ca^{2+}-ATPase activity is a measure of ability of muscle tissue to hydrolyze adenosine triphosphate (ATP) in the presence of Ca^{2+} ion. ATPase is located in the myosin head region (Chan *et al*., 1995). Ca^{2+}-ATPase has been used as an indicator

of myosin integrity (Benjakul *et al*., 1997). The loss in Ca^{2+}-ATPase activity during iced storage was possibly associated with the proteolysis of myosin molecule (Ouali and Valin, 1981). Ca^{2+}-ATPase of ordinary muscle of sardine, during 6 day iced storage, decreased to 50% with concomitant degradation of MHC due to proteolysis (Seki *et al*., 1980). Moreover, the denaturation of myosin was possibly caused by the oxidation of SH groups or disulfide interchanges during ice storage. Yongsawatdikul and Park (2002) found that freshness of threadfin bream kept in ice decreased slightly during 12 days and actomyosin underwent conformational changes after storage in ice for 3 days. Benjakul *et al.* (2003d) reported that lizardfish underwent rapid physico-chemical changes caused by both denaturation and proteolytic degradation during iced storage. However, the degree of denaturation was dependent on fish species. The pretreatment of fish before iced storage could be another means to retard the denaturation of myosin during iced storage. Benjakul *et al.* (2002) reported that deheading and eviscerating could remove the proteinase localized in the viscera and prevent myosin from proteolytic degradation and denaturation.

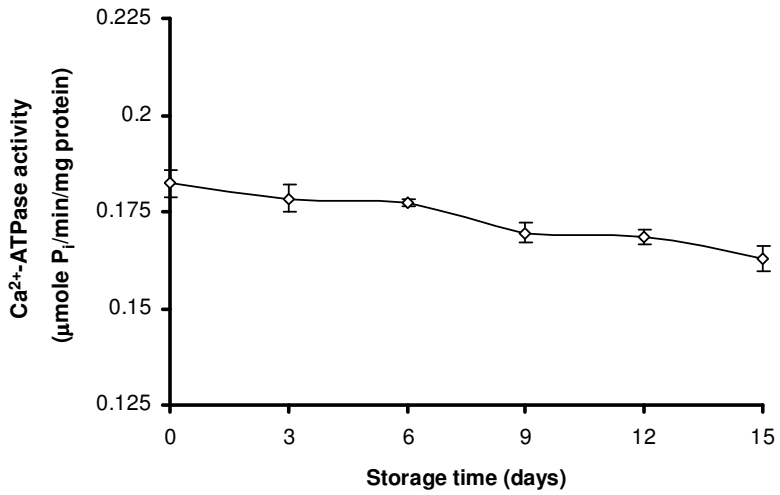


Figure 21 Changes in Ca^{2+}-ATPase of natural actomyosin extracted from goatfish muscle during 15 days of iced storage. Bars represent the standard deviation from three determinations.

3.2.1.3 Changes in TCA-soluble peptide content

TCA-soluble peptide content in goatfish muscle increased throughout 15 days of iced storage ($P<0.05$), suggesting protein degradation in fish muscle (Figure 22). At day 0, TCA-soluble peptide content in goatfish muscle was 0.43 μmole tyrosine/g sample. TCA-soluble peptide content detected at day 0 indicated the presence of endogenous oligopeptides and free amino acid as well as the degradation products accumulated during post-harvest handling (Benjakul *et al*., 1997). TCA-soluble peptide content increased sharply during the first 6 days of storage. The continuous increases were also found during 9 and 15 days of storage. This indicated that goatfish protein was susceptible to hydrolysis. The increase in protein degradation was presumed to be due to muscle proteinases, digestive proteinases as well as microbial proteinases. At the beginning of iced storage, endogenous enzymes are mainly involved in the gradual loss of fish freshness. Degradation of myofibrils occurred at 0°C by cathepsins and serine proteinase, though the hydrolysis rate was low (Tokiwa and Matsumiya, 1969; Busconi *et al*., 1989). Fish muscle contains several proteinases capable of hydrolyzing muscle proteins. An *et al.* (1994b) reported that cathepsin B was most active cysteine proteinase found in Pacific whiting fillets at < 37°C followed by cathepsin L and H. Caplain II, Ca^{2+}-activated neutral proteinase, may also contribute to autolytic degradation in post mortem tenderization of fish muscle (Jiang *et al.*, 1991). Since Ca^{2+} ion equilibrium fails after death, free ion is distributed throughout the tissue, resulting in the activation of Ca^{2+}-requiring proteinase in muscle cell (Etherington, 1984). Thereafter, bacterial enzymes predominate and lead to final spoilage (Pachedo-Aguilar *et al.*, 2000). The increases in TCA-soluble peptide content were in accordance with the increases in TVB and TMA contents of fish stored in ice for a longer time (Figure 20). Proteinase from *Psuedomonas marinoglutinosa* was reported to hydrolyze actomyosin at 0-2°C and the optimal pH was above 7.0. The degradation of muscle proteins, especially myosin, resulted in poor gelling properties of the surimi.

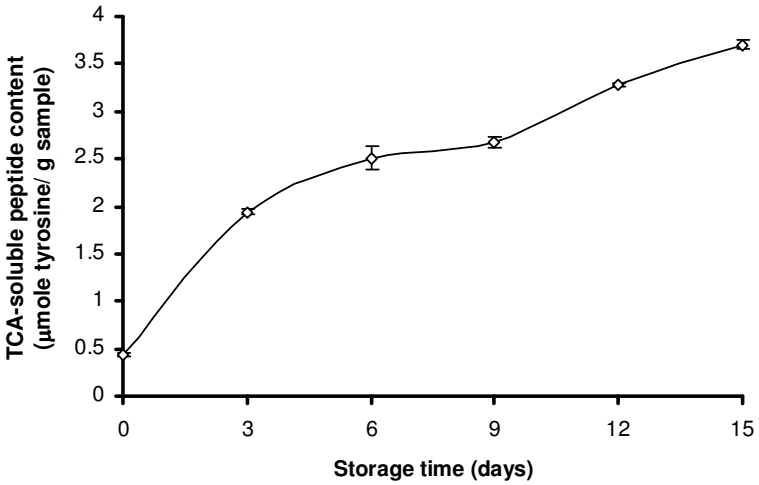


Figure 22 Changes in TCA-soluble peptide content of goatfish muscle during 15 days of iced storage. Bars represent the standard deviation from three determinations.

3.2.1.4 Changes in protein patterns

Protein patterns of goatfish kept in ice for up to 15 days are shown in Figure 23. SDS-PAGE pattern revealed that the band intensity of MHC slightly decreased as the storage time increased, corresponding to an increased TCA-soluble peptide content (Figure 22). This could be attributed to proteolysis mediated by endogenous proteinases and microbial proteinases during extended storage. Those enzymes were able to cleave muscle proteins extensively into the small peptide. However, no changes in actin band intensity were observed on SDS-PAGE. The result revealed that MHC was more susceptible to hydrolysis than actin. Benjakul *et al*. (1997) reported that MHC of Pacific whiting decreased almost 60% after fish was stored in ice for 8 days. Yongsawatdigul and Park (2002) found that a less MHC band intensity from threadfin bream was observed at day 12 of storage in ice. In the present study, whole fish were stored in ice. Thus, digestive proteinase might be involved in proteolysis during iced storage. Haard (1994) reported that viscera contained a variety of digestive proteinases, which played a role in the softening of abdominal tissue during post-mortem storage of fish. These results emphasized the importance of rapid and proper post harvest handling to ensure the good quality of goatfish.

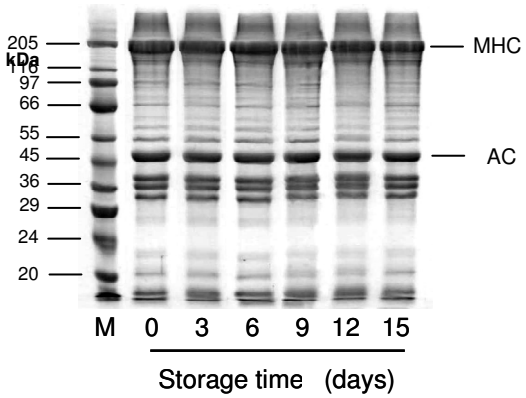


Figure 23 Protein patterns of goatfish muscle during 15 days of iced storage. M: high molecular weight marker. Numbers designate the storage times in ice (days).

3.2.2 Changes in surimi gel properties

3.2.2.1 Changes in breaking force and deformation

Breaking force and deformation of gels of surimi from goatfish kept in ice up to 15 days were shown in Figure 24. When the storage time increased, breaking force and deformation of surimi gel decreased ($P<0.05$). The highest breaking force and deformation of surimi gel was obtained when fresh fish was used as raw material. At day 15, breaking force was decreased by 57.4%, while deformation was decreased by 35.1%. It was postulated to be caused by the degradation and denaturation of proteins during iced storage. Yongsawatdigul and Park (2002) reported that almost 50% decrease in breaking force was observed in surimi prepared from threadfin bream stored in ice for more than 6 days. Ablett *et al.* (1991) found that white hake (*Urophycis tenuis*) kept on ice for 2 days yielded surimi with high gel quality. Thereafter gel strength decreased after fish was stored in ice up to 8 days.

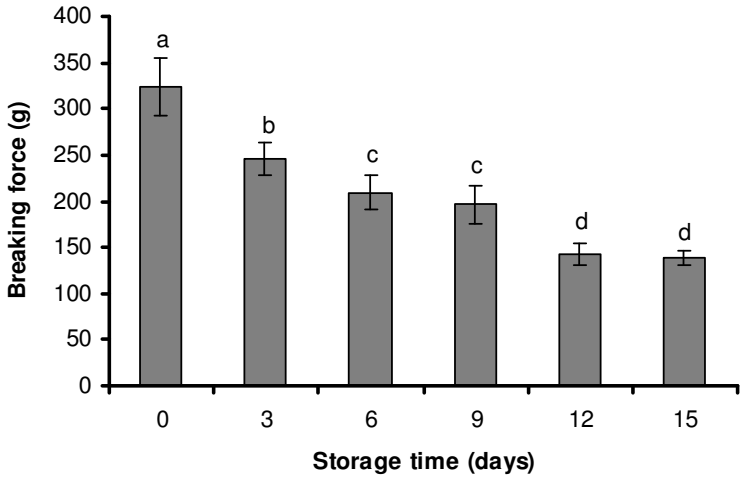
Furthermore, endogenous TGase, which functioned as a gel enhancer during setting process, was possibly inactive to some degree. Benjakul *et al.* (2003d) reported that the high proteinase activity and formaldehyde formed in whole lizardfish during iced storage might result in the inactivation of TGase. Huang *et al.* (1992) reported that ammonia formed during storage from enzymatic deamination of free amino acids or amino acid split from protein, oxidation of amines and decomposition of nucleic bases could partially inhibit the TGase activity in the mince kept for a longer time. Additionally, the formaldehyde formation during iced storage was able to induce protein aggregation and decreased protein solubility, resulting in the reduction

of gel strength (Careche *et al*., 1998). Chomnawang *et al.* (2007) reported that the lower breaking force of catfish stored in ice for 15 days was caused by the decrease in salt soluble protein (SSP). The decrease in gel forming ability of goatfish surimi was concomitant with the increase in TCA-soluble peptide content and the decrease in Ca^{2+}-ATPase activity as well as MHC band intensity (Figures 21 and 22). Myosin integrity is of paramount importance for gelation (An *et al*., 1996). From the result, freshness was a prime factor determining the gel properties of surimi from goatfish. Based on the denaturation of MHC as indicated by Ca^{2+}-ATPase, the fish should be stored up to 6 days to lower such a change and to maintain gelling property. Therefore, to obtain high quality of resulting gel, goatfish should be processed within 6 days of iced storage.

3.2.2.2 Changes in whiteness

Whiteness of gel of surimi from goatfish markedly decreased as the storage time of goatfish increased ($P<0.05$) (Table 11). During iced storage, the oxidized pigments in fish muscle, particularly metmyoglobin or methaemoglobin, possibly adducted to muscle and could not be totally removed by washing. As a result, surimi gel produced from fish kept for a longer time showed some discoloration. Benjakul *et al*. (2003d) reported that these oxidation products were capable of binding tightly with muscles, especially in the presence of formaldehyde. Those oxidation products could not be removed by washing. Since myoglobin is retained by the intracellular structure, it most likely contributes to the appearance more than haemoglobin during handling and storage (Haard, 1992). Lipid oxidation in muscle during ice storage might induce cross-linking of pigment proteins and muscle protein via the free radical process (Saeed *et al*., 1999). The oxidation products including aldehydes might undergo Maillard reaction, leading to the browner color of meat. During extended storage, blood and liquid from internal organs in whole fish could penetrate through the muscle, especially when autolysis proceeded and caused a looser muscle structure. Moreover, discoloration of gel produced from iced storage goatfish might be also caused by the lipid oxidation.

(A)



(B)

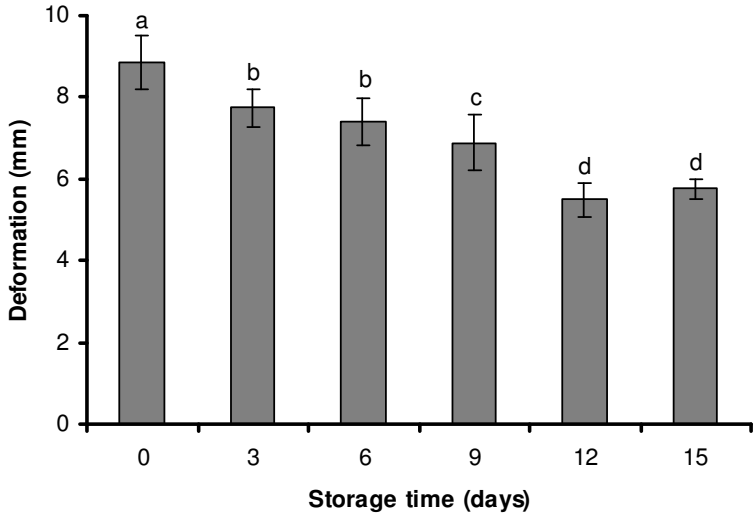


Figure 24 Breaking force (A) and deformation (B) of gel of surimi from goatfish during 15 days of iced storage. Surimi sols were added with $CaCl_2$ at a level of 50 mmoles/ kg and subjected to setting at 40°C for 30 min, followed by heating at 90°C. Bars represent the standard deviation from five determinations. Different letters on the bars indicate the significant differences ($P<0.05$).

Table 11 Whiteness of gels of surimi produced from goatfish during 15 days of iced storage

Storage time (days)	Whiteness
0	80.33±0.18 [a,*,†]
3	80.36±0.18 [a]
6	79.58±0.28 [b]
9	79.46±0.17 [b]
12	79.23±0.31 [b]
15	78.23±0.45 [c]

[*]Values are given as mean ± SD from five determinations.

[†] Different superscripts in the same column indicate significant differences (P<0.05).

3.2.2.3 Changes in expressible moisture content

The expressible moisture content in surimi gel prepared from goatfish stored in ice for up to 15 days as shown in Figure 25. No changes in expressible moisture content were observed within the first 9 days of storage (P>0.05). After 15 days of iced storage, the expressible moisture content was increased by 22.8%. This indicated that less water was imbibed in the gel matrix. When fish were kept for a long time, proteins were more denatured or degraded and lost their functional properties. Denatured protein induced by extended iced storage had the low affinity for water. Furthermore, the increases in expressible moisture content were closely associated with the decrease in breaking force and deformation (Figure 24). Proteinase possibly contributed to degradation to some degree. The degraded polypeptide could not form the strong three-dimensional matrix, which can imbibe water effectively. Additionally, the loss in TGase activity during iced storage was also presumed. During setting, TGase induced the non-disulfide covalent bond formation, leading to strengthened surimi gel with the ability to imbibe water. This result reconfirmed that the inferior gel quality was found when surimi was produced from goatfish stored in ice for a longer time.

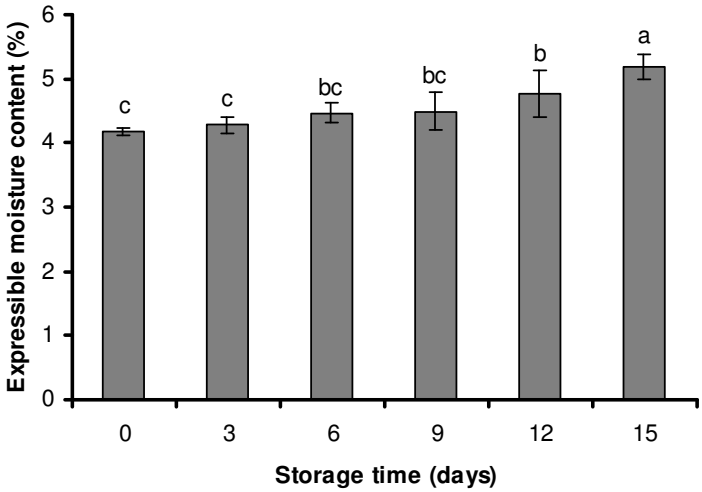


Figure 25 Expressible moisture content of gels of surimi from goatfish during 15 days of iced storage. Bars represent the standard deviation from five determinations. Different letters on the bars indicate the significant differences ($P<0.05$).

3.2.2.4 Changes in TCA-soluble peptide content

Changes in TCA-soluble peptide content of gels produced from goatfish stored in ice for up to 15 days are shown in Figure 26. TCA-soluble peptide content of gel increased markedly as the storage time increased ($P<0.05$). The result revealed that protein underwent degradation to a higher extent when fish with longer storage time were used. The result was in agreement with the increase in TCA-soluble peptides in fish meat and the decrease in MHC band intensity. Although washing was used to leach out the small molecular weight substances, it could not remove the degradation products completely. Furthermore, the degradation occurred during heat-induced gelation due to the action of endogenous proteinases associated with myofibrillar protein remaining after washing. Cathepsins are highly active in both post-mortem muscle condition and during heat-induced gelation, resulting in the myofibrillar protein and gel degradation (Visessanguan *et al*., 2001). Generally, denatured proteins are preferable substrate for proteolysis. As indicated by lowered Ca^{2+}-ATPase activity, protein underwent denaturation to a greater extent as storage time increased (Figure 21). Rawdkuen *et al.* (2004a) reported that proteins in surimi grade A with a less integrity were degraded more easily than those in surimi grade SA. The increase in TCA-soluble peptide content was in accordance with the lower gel strength of surimi produced from goatfish stored in ice for a longer time (Figure 24).

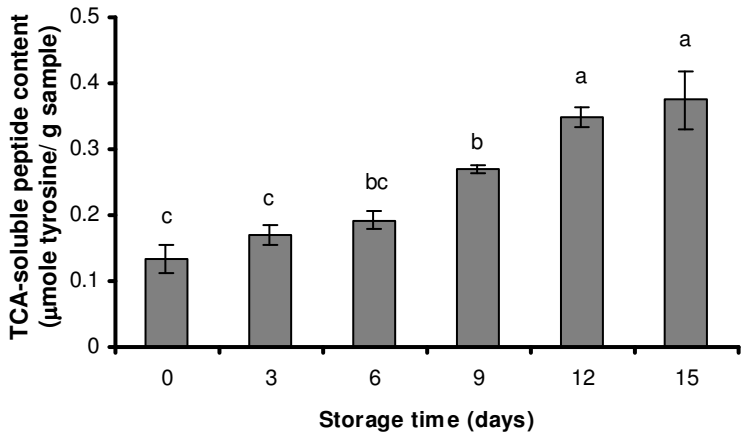


Figure 26 TCA-soluble peptide content of gels of surimi from goatfish during 15 days of iced storage. Surimi sols were added with $CaCl_2$ at level of 50 mmoles/ kg and subjected to setting at 40°C for 30 min, followed by heating at 90°C for 20 min. Bars represent the standard deviation from five determinations. Different letters on the bars indicate the significant differences ($P<0.05$).

3.2.2.5 Changes in protein patterns

Protein patterns of surimi gels from goatfish stored in ice for different times are shown in Figure 27. MHC band of gels almost disappeared when the surimi sols were subjected to setting and heating. This indicated that proteins underwent polymerization induced most likely by endogenous TGase in the presence of Ca^{2+} ion. High molecular weight proteins (HP) were observed on the top of stacking gel. The band intensity of MHC was slightly more retained with increasing storage time. The increased MHC band intensity of gel from goatfish stored for a longer time was associated with the decreased breaking force and deformation of resulting gels. This might be due to the denaturation of endogenous TGase as the storage time increased. This was evidenced by the lower band intensity of polymerized proteins in gel from goatfish kept for an extended period. Chantarasuwan (2001) reported that the activity of TGase in bigeye snapper mince decreased to 33.7% after 8 days of iced storage. This possibly led to a lower setting phenomenon in the poor quality goatfish. However, it was noted that the protein degradation (DP) band intensity was also retained in the gel. This indicated the formation of degradation peptides as induced by heat-activated proteinase. Also, it was presumed that the small

peptides could not be cross-linked by endogenous TGase effectively. Benjakul *et al.* (2008) reported that MHC was degraded after storage for 10 days contributed to the lowered gel strength of lizardfish. Generally, MHC with more integrity was a preferable substrate for endogenous TGase, leading to protein cross-linking during heat induced gelation. The degradation of MHC resulted in an inferior gel network formation, causing a lower elasticity with poor water-holding capacity in the gel matrix. From the result, the low gel forming ability produced from ice-stored goatfish was associated with the lower setting phenomenon and the enhanced protein degradation occurred during iced storage.

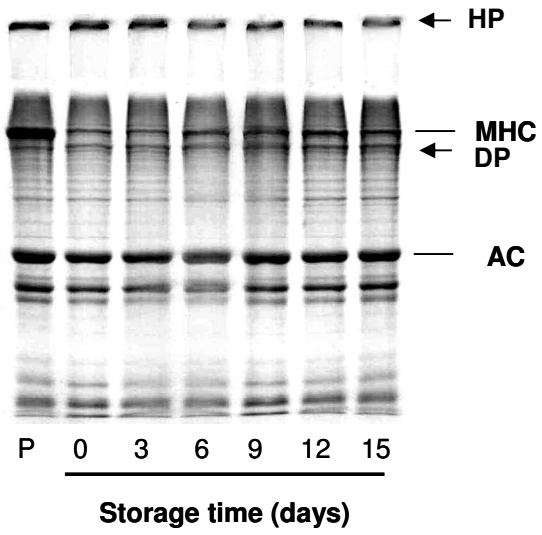


Figure 27 Protein patterns of gels of surimi from goatfish during 15 days of iced storage. Surimi sols were added with $CaCl_2$ at a level of 50 mmoles/ kg and subjected to setting at 40°C for 30 min, followed by heating at 90°C for 20 min. P: surimi paste; HP: high molecular weight proteins; DP: degradation peptides. Numbers designate the storage times in ice (days).

4. Autolysis of goatfish muscle

4.1 Effect of temperature on autolysis of goatfish mince and washed mince

Autolysis of goatfish mince and washed mince incubated at various temperatures was monitored and expressed in terms of TCA-soluble peptide (Figure 28). The highest autolysis was observed at 60°C. The activity decreased sharply at temperatures above 60°C, most likely due to thermal denaturation of endogenous proteinase. The result showed that temperature was a crucial factor affecting the autolysis. Proteolysis of fish muscle caused by endogenous proteinases has been widely reported. Cathepsin L caused severe texture degradation in Pacific whiting (An *et al*., 1994b), arrowtooth flounder (Visessanguang *et al*., 2003) and chum salmon (Saeki *et al*., 1995). Serine proteinases were found to be responsible for textural breakdown of threadfin bream (Toyohara and Shimizu, 1988) oval-filefish (Toyohara *et al*., 1990) and lizardfish (Suwansakornkul *et al*., 1993). When comparing the autolytic activity between mince and washed mince, higher activity was found in mince. Fish mince contained both sarcoplasmic proteinases and myofibril-associated proteinases. Washing the mince with 50 mM NaCl could remove sarcoplasmic proteinases to some degree. Therefore, the use of low molarity saline solution for mince washing was able to lower autolysis of resulting washed mince. Nevertheless, the proteinase associated with the myofibrils still remained as indicated by the protein degradation, which was still found in washed mince. Furthermore, washing not only removed the proteinases, but also the co-factor for proteolytic activity. This might lead to the lowered proteolysis of washed mince. Generally, those enzymes contributed to degradation of proteins, leading to poor gel network. Toyohara *et al.* (1990) reported that proteolytic degradation of fish gels could be classified into two types according to the localization of the gel degradation inducing factor (GIF) in muscle cells, sarcoplasmic GIF, which could be easily washed out from the myofibrils and myofibrillar GIF, which was tightly associated with myofibrils. These proteinases were responsible for gel softening (Toyohara *et al*., 1990; Kinoshita *et al*., 1990). Benjakul *et al.* (2003b) reported that the maximum autolysis of mince and washed mince from two species of bigeye snapper (*P. macracanthus and P. tayenus*) was found at 60°C. Myofibril-bound serine proteinase in carp (*Cyprinus carpio*) with an optimal temperature of 55°C were responsible for the degradation of MHC and other myofibrillar proteins such as α-actinin, β-actin and tropomyosin (Jiang *et al.*, 2006).

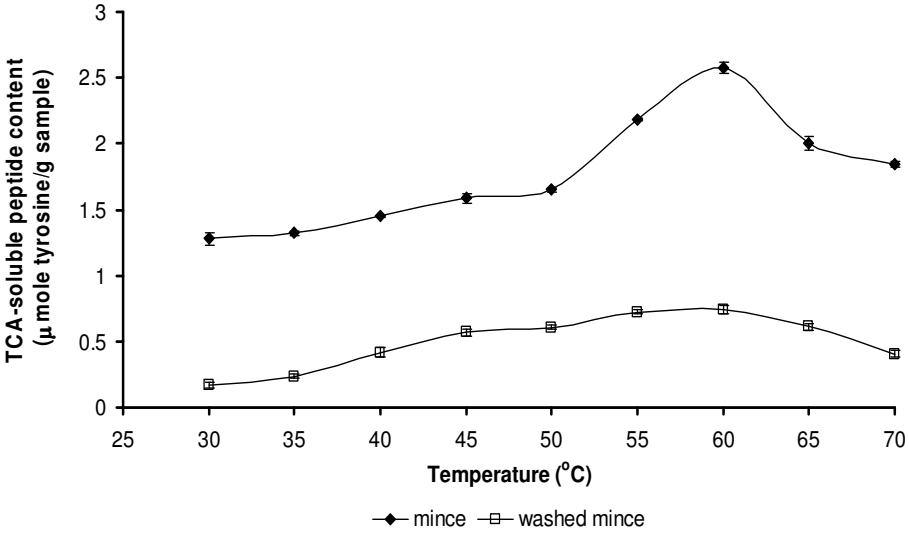


Figure 28 TCA-soluble peptide content of goatfish mince and washed mince incubated at different temperatures. Autolysis was conducted for 60 min at physiological pH (~7). Bars represent the standard deviation of triplicate determinations.

Autolysis patterns of goatfish mince and washed mince are shown in Figure 29. Degradation was more pronounced at temperature higher than 50°C, as evidenced by the marked decrease in MHC band intensity. For actin, no changes in band intensity were observed. Thus it was noted that MHC was more susceptible to degradation than other easily observable muscle protein bands with SDS-PAGE. However, MHC with high band intensity was found in mince incubated at 70°C, suggesting heat inactivation of endogenous proteinases. Cao *et al*. (1999) reported that myofibril-bound serine proteinase from lizardfish muscle hydrolyzed MHC at 55-60°C effectively, whereas α-actinin and actin were not degraded. Benjakul *et al.* (2003e) reported that actin was regarded as a proteolytically resistant protein in comparison with MHC, β-tropomyosin and troponin T. The decrease in protein band intensity was in agreement with the increase in TCA-soluble peptide content (Figure 28). A larger decrease in MHC band was found in mince than washed mince, indicating the higher autolysis in the former. Thermal activated proteinases contributed to degradation of myofibrils in mince, whereas those proteinases that are not removed by washing were mainly involved in degradation of MHC in washed mince. The results suggested that autolysis of goatfish mince could be minimized by thorough washing.

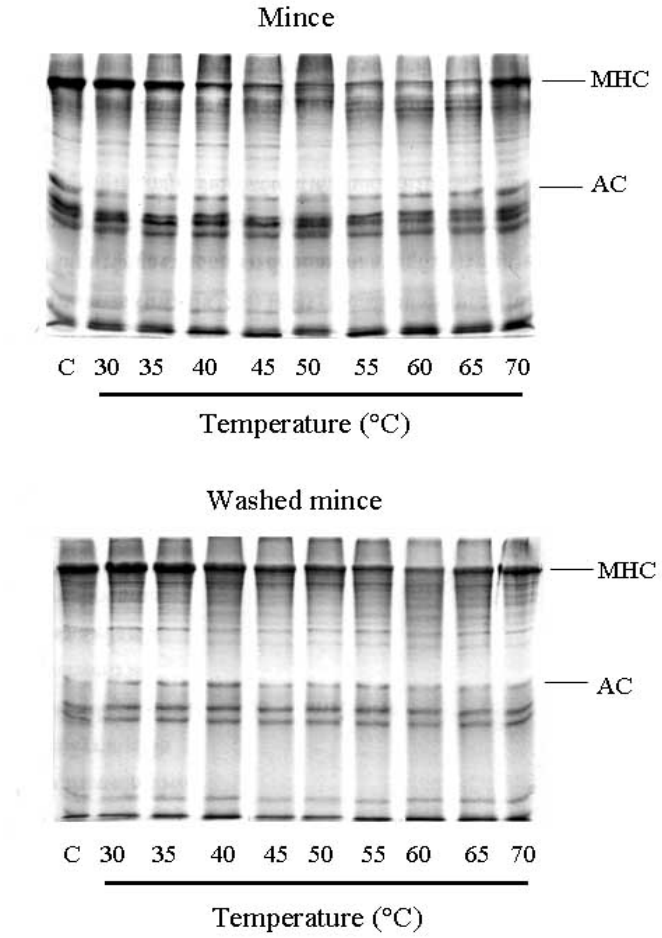


Figure 29 Autolytic SDS-PAGE pattern of goatfish mince and washed mince incubated at different temperatures for 60 min. Numbers designate the incubation temperatures (°C). MHC: myosin heavy chain; AC: actin; C: control (no incubation).

4.2 Effect of pH on autolysis of mince and washed mince

The pH profiles for the autolysis of mince and washed mince conducted at the optimal temperature (60°C) for 60 min are shown in Figure 30. For both mince and washed mince, major activity peak was found at pH 4 with a small activity peak at pH 7. Therefore, acidic proteinases were the major proteinases, while neutral proteinases were present at a low level. Cathepsin, especially cathepsin D, had an optimal pH range of 3.0-4.5 (Makinodan *et al*., 1982). Bonete *et al.* (1984) found that cathepsin D from grey mullet had maximum activity at pH 4.0 and was stable up to 45°C. Activity losses of 34 and 100 % were found at 50 and 70°C, respectively. Moreover, cathepsin L, a cysteine proteinase, was also presumed to exhibit its activity peak at acidic pH.

Cathepsin L has several forms and a wide range of pH (3.0-6.5) for activity (Kang and Lanier, 2000). Visessanguan *et al.* (2003) reported that a pre-dominant heat-activated proteinases in arrowtooth flounder displayed optimal activity at pH 5.0-5.5. Since calpain was not stable at temperature above 40°C (Wang *et al*., 1993), other proteinases might play a role in degradation at neutral and slightly alkaline condition. Among all proteinases (cathepsin D, neutral proteinase, calpain and alkaline proteinase), only alkaline proteinase could show activity at pH 6.8 and 60°C in meat paste (Makinodan *et al*., 1985). Autolysis of goatfish muscle was most likely mediated by heat-activated acidic proteinases at pH lower than 6. At the autolysis peak (pH 4), lower activity was found in washed mince, compared with mince. This reconfirmed that washing could remove some proteinases in muscle, resulting in lower amounts of proteinases.

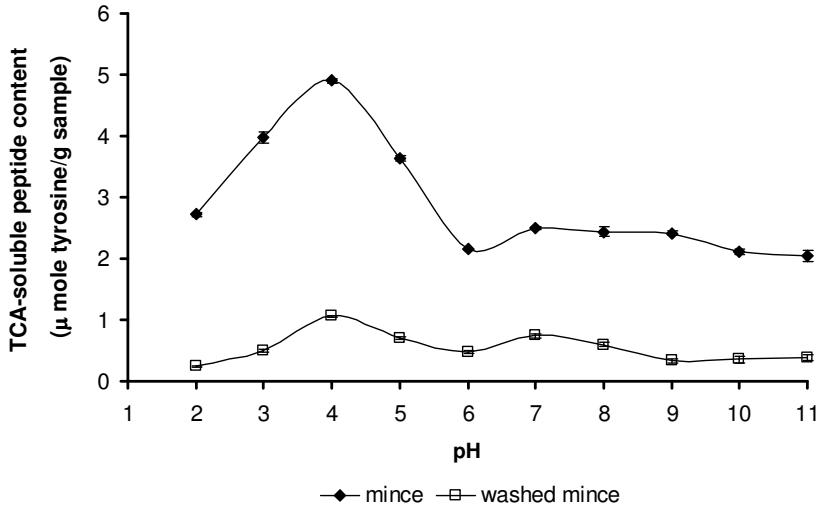


Figure 30 TCA-soluble peptide content of goatfish mince and washed mince incubated at different pH at 60°C for 60 min. Bars represent the standard deviation from triplicate determinations.

4.3 Effect of various proteinase inhibitors on autolysis of mince and washed mince

The effect of various proteinase inhibitors on autolysis of mince and washed mince at pH 4 and 7 is shown in Table 12. For autolysis at pH 4, E-64, a specific cysteine proteinase inhibitor, showed the highest inhibitory activity towards autolysis in goatfish mince (63.3%) and washed mince (54.0%). Inhibitors tested including 10 µM pepstatin A (an aspartic proteinase inhibitor) and SBTI (a serine proteinase inhibitor) also showed inhibition of autolysis at this pH. However, EDTA and metallo-proteinase inhibitor had little effect on inhibition of both samples. The results

suggested that different types of proteinases are involved in autolysis of goatfish muscle at acidic pH. Autotysis of lizardfish was inhibited by E-64 (Benjakul *et al*., 2003e). Klomklao *et al.* (2008) reported that an aspartic proteinase was the major proteinase in true sardine when tested at pH 3. For autolysis at pH 7, SBTI was the most effective proteinase inhibitor with 49.7% and 75.4% inhibition for mince and washed mince, respectively. Since the highest activity was found at acidic pH and autolysis was most strongly inhibited by E-64, cysteine proteinase including cathepsin could be the main proteinases in mince and washed mince. Additionally serine proteinase was another proteinase present in goatfish muscle but to a lesser extent. Myofibril-bound proteinase in lizardfish (*S. wanieso*) was classified as a trypsin–like proteinase, which showed optimal activity at 50-60°C and pH 7-8 and it was also inhibited by SBTI, leupeptin and *N-p*-tosyl-L-lysine chloromethyl ketone (TLCK) (Cao *et al*., 2000). Other inhibitors also showed inhibition towards autolysis, suggesting that goatfish contained a variety of proteinases, which had different characteristics.

Table 12 Effect of various proteinase inhibitors on autolysis of goatfish mince and washed mince

Inhibitors	Concentrations	% Inhibition			
		Mince		Washed mince	
		pH 4	pH 7	pH 4	pH 7
Control		$0^{e,*,\dagger}$	0^{f}	0^{d}	0^{d}
SBTI	0.01 mM	29.94 ± 1.06^{bC}	49.69 ± 0.02^{aB}	29.80 ± 2.98^{bcC}	75.44 ± 5.32^{aA}
E-64	0.01 mM	63.31 ± 0.38^{aA}	18.16 ± 2.43^{cD}	54.01 ± 1.12^{aB}	30.97 ± 5.02^{bC}
Pepstatin A	1 μM	13.94 ± 1.01^{cB}	12.20 ± 0.03^{dB}	26.05 ± 0.67^{cA}	22.78 ± 3.46^{cA}
Pepstatin A	10 μM	28.55 ± 2.88^{bA}	19.56 ± 0.99^{bB}	30.57 ± 3.86^{bA}	37.44 ± 11.20^{bA}
EDTA	2 mM	8.02 ± 3.44^{dB}	8.53 ± 0.35^{eB}	1.38 ± 0.04^{dC}	22.30 ± 0.51^{cA}

†Values are given as mean ± SD from triplicate determinations.

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

** Different capital superscripts in the same row indicate significant differences (P<0.05).

Protein patterns of mince and washed mince autolyzed at pH 4 and 7 in the presence of different proteinase inhibitors is shown in Figure 31. Degradation of MHC was more pronounced in mince than washed mince. For the same sample, higher degradation was found at pH 4 than pH

7. The results were in agreement with the higher TCA-soluble peptide content at pH 4 (Figure 30). The lowest band intensity of MHC was observed in the control (without proteinase inhibitor). MHC of both mince and washed mince were most retained in the presence of E-64 when tested at pH 4, while SBTI was the most effective inhibitor towards the degradation of MHC at pH 7. Greater retention of MHC band in samples with inhibitors gives a higher %inhibition (Table 12). Pepstatin A and EDTA had no impact on autolysis. The results reconfirmed that cysteine proteinase and serine proteinase were most likely responsible for protein degradation of this species. However, serine proteases most likely contribute practically more to the autolysis of surimi or mince products, which have the neutral pH. Suwansakornkul *et al*. (1993) reported that both cysteine and serine proteinases were responsible for MHC degradation of washed lizardfish mince and varied with species.

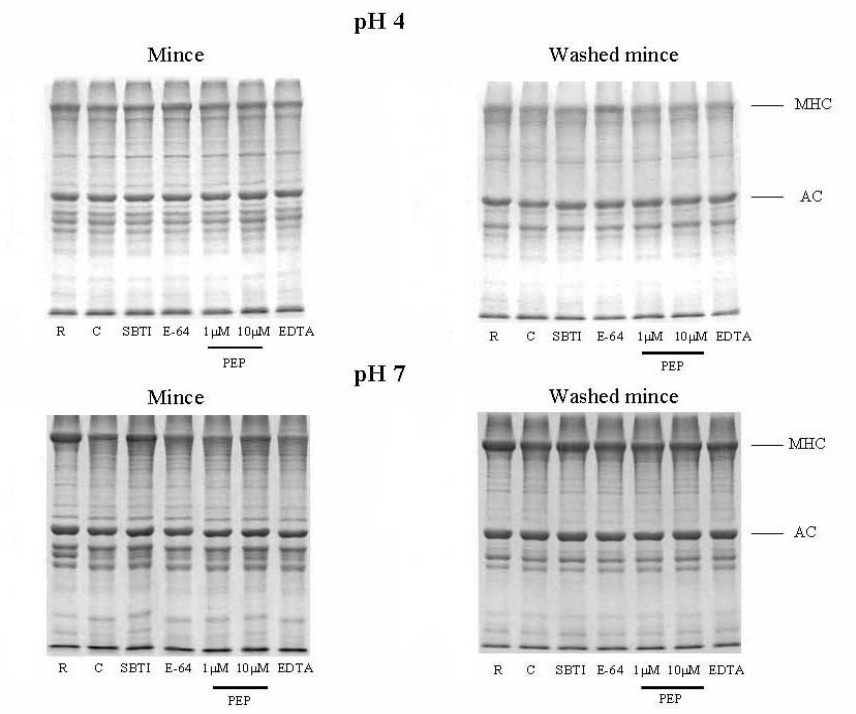


Figure 31 Autolytic SDS-PAGE patterns of goatfish mince and washed mince with addition of various proteinase inhibitors at pH 4 and 7. Autolysis was conducted at 60°C for 120 min. MHC: myosin heavy chain; AC: actin; M: high molecular weight marker. R: raw material (without inhibitor and no incubation); SBTI: soybean trypsin inhibitor; E-64: trans-epoxysuccinyl-L-leucyl-amido (4-guanidino) butane; PEP: pepstatin A; EDTA: ethylenediaminetetraacetic acid.

4.4 Effect of sodium chloride on autolysis of mince and washed mince

Autolysis as monitored by TCA soluble peptide content of mince and washed mince as affected by NaCl concentrations is shown in Table 13. TCA-soluble peptide content of mince increased in the presence of 2% NaCl, but decreased as 2.5-3.5% NaCl was incorporated ($P<0.05$). The enhancement of autolysis of mince containing 2% NaCl was possibly due to the increased solubilization of myofibrils, accompanied by the exposure of cleavage site of muscle proteins, especially MHC. When NaCl is added to proteins, Na^+ and Cl^- ions act as counter ions toward negatively and positively charged group, respectively, disturbing the native conformation of protein (Sikorski *et al*., 1994). Furthermore, Cl^- ions could activate some proteinases responsible for degradation of muscle proteins. Cathepsin C in muscle of squid (*Illex illcebrous*) was activated by Cl^- ion, but not by K^+ and Na^+ ion (Hameed and Haard, 1985). Kinoshita *et al.* (1990) reported that serine proteinase in threadfin bream muscle was activated in the presence of 2-4% NaCl. Nevertheless, TCA-soluble peptide content was decreased in the presence of NaCl concentration above 2%. The result suggested that NaCl at higher concentration might cause the denaturation of proteinases. Jiang *et al.* (1997) found that the loss of cathepsin B and L in mackerel surimi after grinding with 2.5% NaCl was due to the denaturation of enzymes. Ohkubo *et al.* (2005) reported that about 60% of the myofibrillar proteolytic activity from white croaker was suppressed in the presence of 0.5 M NaCl (~3%). For washed mince, NaCl at all levels tested had no impact on autolysis. Due to the removal of soluble proteinases, mainly sarcoplasmic proteinases, during washing, myofibril-associated proteinases remained were not affected by NaCl. Therefore, NaCl more likely had the inhibitory effect on sarcoplasmic proteinase, rather than myofibril-associated proteinase.

Protein patterns of mince and washed mince as affected by NaCl concentrations are shown in Figure 32. Similar MHC band intensity was observed for either mince or washed mince. However, retention of MHC in mince increased slightly as the NaCl concentration increased when compared with the control (without NaCl). Though the varying NaCl concentrations affected TCA-soluble peptide content in mince differently (Table 13), no obvious differences in protein patterns were found. It was postulated that small degradation peptides produced might not be detected by SDS-PAGE.

Table 13 Effect of NaCl on autolysis of goatfish mince and washed mince

NaCl concentrations (%)	TCA-soluble peptide content (μmole tyrosine/g sample)	
	Mince	Washed mince
0	2.38±0.04[b,*,†]	0.45±0.05[a]
2.0	2.68±0.04[a]	0.51±0.05[a]
2.5	2.03±0.04[c]	0.44±0.02[a]
3.0	1.99±0.08[c]	0.47±0.02[a]
3.5	1.98±0.02[c]	0.46±0.04[a]

*Values are given as mean ± SD from triplicate determinations.

† Different superscripts in the same column indicates the significant difference (P<0.05).

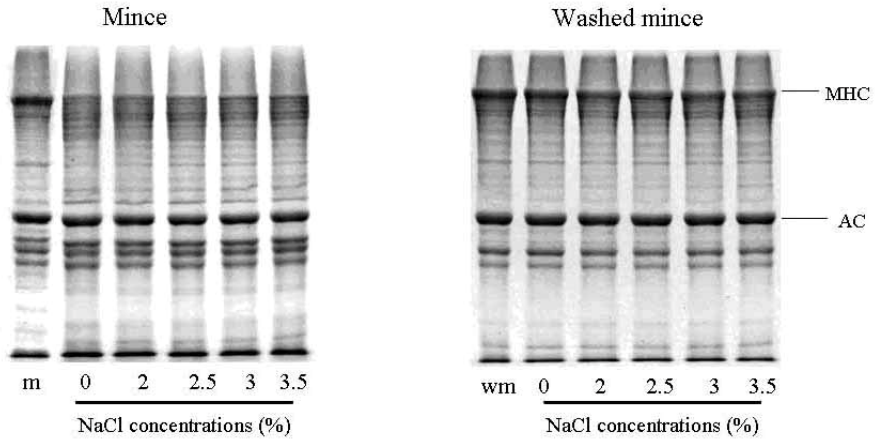


Figure 32 Autolytic patterns of goatfish mince and washed mince with addition of NaCl at different concentrations. Autolysis was conducted at 60°C for 60 min. Numbers designate NaCl concentrations (%). MHC: myosin heavy chain; AC: actin; m: mince; wm: washed mince.

4.5 Effect of skin on autolysis of mince

The effect of skin on autolysis of goatfish mince incubated at 60°C for different times is shown in Figure 33. At the same incubation times, TCA-soluble peptide content of mince with skin (4.63 μmole tyrosine/g sample) was greater than that of mince without skin (3.75 μmole tyrosine/g sample) (P<0.05). This result indicated that skin might contain proteinases, which were able to hydrolyze muscle proteins. It was noted that at incubation time of 0 min, the autolysis of

mince with skin exhibited the greater autolysis than that of mince. The rupture of muscle or skin cells during sample preparation might result in the releases of enzymes which were able to hydrolyze muscle protein to some extent prior to incubation. Endogenous skin proteinases of bigeye snapper (*Priacanthus macracanthus*) were classified as serine proteinases, which were strongly inhibited by SBTI (Intarasirisawat *et al*., 2007). Nalinanon *et al.* (2008) found that skin of bigeye snapper (*Priacanthus tayenus*) contained proteinase capable of degrading β, α_1 and α_2-yachains of gelatin. Longer incubation time resulted in higher TCA-soluble peptide content for both mince with and without skin ($P<0.05$), suggesting that the proteins or peptides underwent autolysis caused by thermal activated endogenous proteinases localized in either the muscle or associated with the skin to a larger extent. Therefore, contamination of mince with skin during surimi production may play role in enhanced degradation of myofibrils in goatfish surimi or mince products.

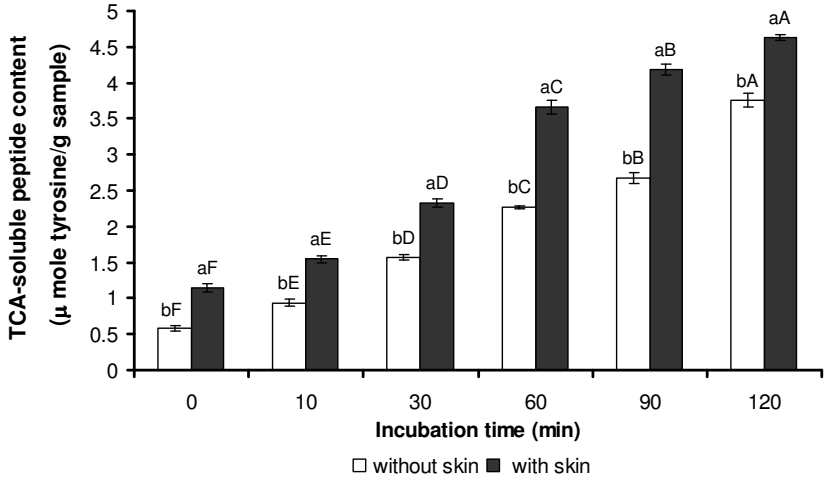


Figure 33 TCA-soluble peptide content of goatfish mince with and without skin incubated at 60°C for different times. Bars represent the standard deviation of triplicate determinations. Different letters in the same incubation time indicate significant differences ($P<0.05$). Different capital letters in the same sample indicate significant differences ($P<0.05$).

The protein patterns of goatfish mince with and without skin autolyzed at 60°C for different times are shown in Figure 34. MHC band intensity decreased when the incubation time increased with the concomitant occurrence of degradation products. This result was in agreement

with the increase in TCA-soluble peptide content of both mince with and without skin as incubation time increased. Actin was degraded to a much lower extent, compared to MHC. Within 30 min, a slightly lower intensity of MHC band was observed in mince with skin than mince without skin. However, no differences in protein patterns were found with increasing incubation time. It was suggested that mince with skin contained higher amount of proteinases, resulting in greater degradation of muscle. Therefore, endogenous skin proteinases, especially heat-activated proteinases or collagenolytic enzymes, might be involved in the degradation of muscle proteins in surimi contaminated with skins. Deskining prior to deboning or appropriate refining could be a means of reducing degradation induced by skin proteinases.

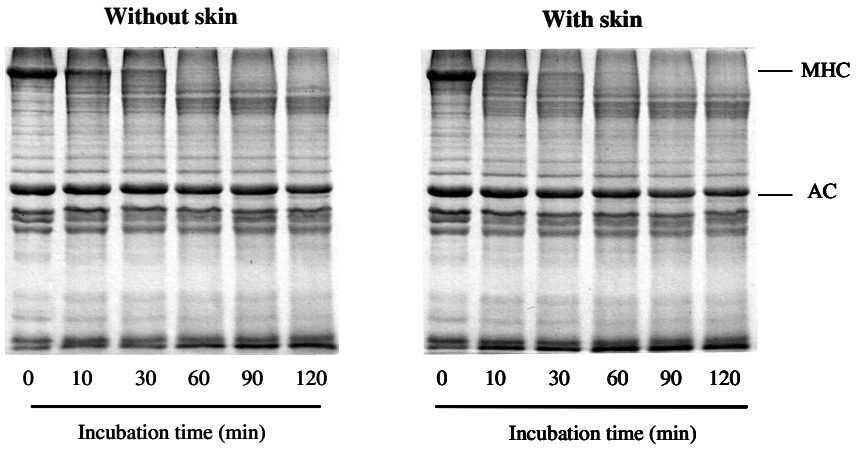


Figure 34 Autolytic patterns of goatfish mince without and with skin incubated at 60°C for different times. Numbers designate the incubation times (min). MHC: myosin heavy chain; AC: actin.

5. Effect of whey protein concentrate (WPC) on autolysis inhibition and gel properties of goatfish surimi

5.1 Effect of WPC on autolytic activity of goatfish surimi

Effect of WPC at different levels (0, 1, 2 and 3% w/w) on autolysis inhibition of goatfish surimi incubated at 60°C for 60 min in the absence and presence of 2.5% NaCl is shown in Figure 35. A greater degree of inhibition was observed when the level of WPC increased up to 3%, regardless of NaCl inclusion ($P<0.05$). The addition of 3% WPC showed the most effective inhibition toward autolysis, in which % inhibition of 76.4 and 78.3 % were obtained in surimi without and with 2.5% NaCl, respectively. This result suggested that the degradation of goatfish

surimi could be minimized by addition of WPC. WPC was reported to contain both serine and cysteine proteinase inhibitors (Weerasinghe *et al*., 1996a). It may protect myofibrillar proteins of surimi by acting as a true inhibitor or by serving as an alternative substrate, which effectively decreased the enzyme activity on myosin (Piyachomkwan and Penner, 1995). Cysteine and serine proteinase were predominant and capable of degrading muscle protein in goatfish (Table 12). Thus the use of WPC could provide the substantial protection against proteolysis in this species. Rawdkeun and Benjakul (2008) reported that goatfish (*Mulloidichthys vanicolenis*) surimi showed the highest autolysis inhibition by WPC when compared with other tropical fish surimi. Akazawa *et al.* (1993) also found that increasing WPC concentration to 3% reduced the enzyme activity of Pacific whiting surimi by approximately 80%, compared with the control. Yongsawatdikul and Piyadhammaviboon (2004) reported that the addition of WPC at 4% inhibited the autolysis of lizardfish surimi about 96% at 65°C. Degree of inhibition by WPC towards autolysis was varied among different fish species which was likely due to the differences in the type and amount of protienases predominantly found in their muscle (Rawdkuen and Benjakul, 2008). The greater degree of autolysis inhibition was observed in the presence of 2.5% NaCl. It was presumed that NaCl could solubilize muscle proteins, leading to the exposure of proteinases. As a consequence, proteinase inhibitor could bind with enzyme more effectively, resulting in higher inhibition. Benjakul *et al*. (2004d) found that the autolysis inhibition of lizardfish mince and washed mince by BPP and EW was more pronounced in the presence of 3% NaCl. Furthermore, the endogenous proteinases were partially inhibited by NaCl, possibly due to instability of enzyme in the presence of salt.

Autolytic patterns of surimi added with WPC at different levels in the absence and presence of 2.5% NaCl at 60°C are shown in Figure 36. The intensive degradation of MHC was observed in sample without the addition of WPC as indicated by the lowest band intensity retained. However, no changes in actin were noticeable. The result indicated that MHC was the preferable substrate for proteinases, which were activated at high temperature ranging from 60-65°C (Benjakul *et al*., 2003e). With the addition of WPC, MHC band intensity of surimi generally increased, irrespective of NaCl inclusion. The result indicated that WPC could inhibit the protein degradation to some extent. WPC at levels of 2 and 3% was suggested to be sufficient for inhibiting proteolysis in goatfish surimi. It was noted that slightly greater intensity of MHC band

was found in gel added with WPC in the presence of 2.5% NaCl. The result was in accordance with the autolysis study (Figure 35). As a result, it was reconfirmed that NaCl would induce inhibition via facilitating the interaction between proteinase associated with myofibrils and proteinase inhibitors in WPC via solubilize of muscle protein (Benjakul *et al.*, 2004d). Thus, it can be inferred that autolysis in goatfish surimi could be minimized by WPC in a concentration dependent manner and could function synergistically to inhibit proteolysis with NaCl addition.

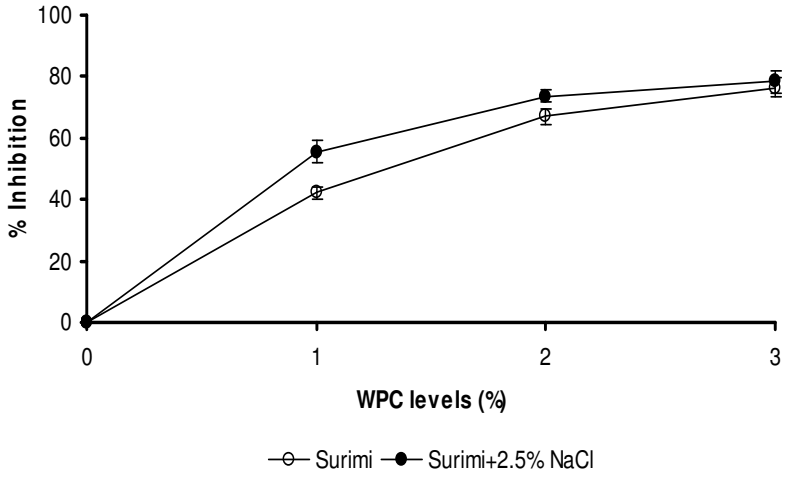


Figure 35 Effect of whey protein concentrate (WPC) at different levels on autolysis inhibition of goatfish surimi incubated at 60°C for 60 min in the absence and presence of 2.5% NaCl. Bars represent the standard deviation from three determinations.

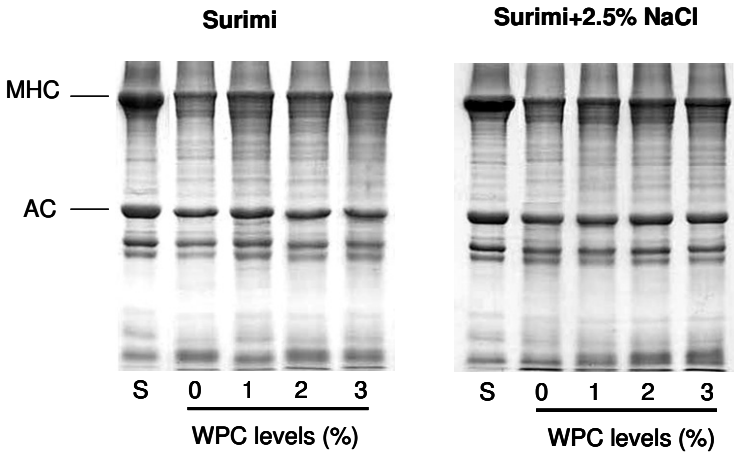


Figure 36 Protein patterns of surimi added with WPC at different levels in the absence and presence of 2.5% NaCl. Samples were incubated for 60 min at 60°C. S: surimi; MHC: myosin heavy chain; AC: actin; Numbers designate the levels of WPC added (%).

5.2 Effect of WPC on gel properties

5.2.1 Breaking force and deformation of surimi gel

Breaking force and deformation of both kamaboko (40/90°C) and modori (60/90°C) gels containing WPC at different levels in the absence and in the presence of 50 mmoles $CaCl_2$/kg are shown in Figure 37. The control gel (without WPC) had the lower breaking force and deformation when compared with samples added with WPC, regardless $CaCl_2$ addition ($P<0.05$). Breaking force and deformation increased with increasing WPC levels ($P<0.05$). Addition of 3% WPC resulted in the increased in breaking force by 49.9% and 115.8% for kamaboko and modori gel containing $CaCl_2$, respectively and by 45.1 and 157.3% for kamaboko and modori gel without $CaCl_2$, respectively. The result revealed that the strengthening of surimi gel could be achieved by the addition of WPC. WPC has been reported to enhance gel properties of some tropical fish by inhibiting the proteolysis or acting as the filler in the gel matrix (Rawdkuen and Benjakul, 2008). Akazawa *et al*. (1993) reported that the addition of WPC resulted in an improvement in heat-set gel texture and an apparent inhibition of proteolysis during gel formation caused by indigenous proteinases.

Lower breaking force and deformation were found in modori gel, compared with kamaboko gel. Generally, modori gel with softened texture is ascribed to the degradation of myosin by endogenous proteinases (An *et al*., 1996; Jiang *et al*., 1996). Gel structure disintegration reaction was observed at 50-60°C (An *et al*., 1996). Benjakul *et al*. (2004b) found that the lower breaking force and deformation were observed with modori gel (60/90°C) compared with kamaboko gel (40/90°C) from lizardfish surimi. Addition of $CaCl_2$ affected breaking force and deformation of kamaboko and modori gels differently. For kamaboko gel, at all levels of WPC, the addition of 50 mmoles $CaCl_2$/kg resulted in the superior gel quality as evidenced by the increases in breaking force and deformation obtained ($P<0.05$). This indicated that $CaCl_2$ played the essential role in setting, in which endogenous TGase was activated. Fish TGase has been found to be Ca^{2+}-dependent, however the requirement varies among fish species (Nozawa *et al*., 1997). Endogenous TGase has been reported to induce the formation of ε-(γ-glutamyl) lysine linkage during setting (Tsukamasa *et al*., 2002). Calcium compounds are commonly added in surimi or fish mince as a gel enhancer (Yamamoto *et al*., 1991). Julavittayanukul *et al*. (2006) found that breaking force and defomation of bigeye snapper surimi

increased with increasing $CaCl_2$ concentrations (0, 25 and 50 mmoles/kg). $CaCl_2$ in combination with WPC contributed to gel strengthening via enhancing myosin cross-linking and inhibiting proteolysis. Furthermore, cross-linked proteins formed during setting were more likely resistant to proteolysis. Therefore, the combination effect between cross-linking activity and proteolysis inhibition contributed to the improved gel strength of goatfish surimi.

At the same level of WPC added, modori gels containing $CaCl_2$ showed the lower breaking force and deformation than did those without $CaCl_2$ ($P<0.05$). This result indicated that $CaCl_2$ addition caused the adverse effect on modori gel. Some proteinases such as calpain, a calcium activated neutral proteinase, might be activated by calcium ion. Park *et al.* (2003) reported that $CaCl_2$ addition in squid paste resulted in the lower breaking force and deformation of resulting gel, compared with control (no addition of $CaCl_2$). The superior gel strength was obtained when the combination of $CaCl_2$ with calpain inhibitor was used (Park *et al*., 2003). However, the breaking force and deformation of modori gel was increased with the addition of WPC. This result confirmed that the strength of kamaboko and modori gels could be improved by the addition of WPC.

5.2.2 Whiteness of surimi gel

Whiteness of kamaboko and modori gel added with WPC at different levels in the presence and in the absence of $CaCl_2$ (50 mmoles/kg) is shown in Table 14. The slight decrease in whiteness was noticeable in all gels, except kamaboko gel without $CaCl_2$, as WPC levels increased ($P<0.05$). Protein additives generally have the different impact on surimi gel color, depending on the initial color of each protein additives (Benjakul *et al*., 2004d; Rawdkuen *et al.,* 2004a). WPC is predominantly light-cream-colored in nature. It might reduce the whiteness of surimi gel to some extent, especially when a higher amount was used. Ramirez *et al.* (2007) reported that the color attributes of strip mullet restructure fish product changed from a yellow hue to a slightly more reddish hue after adding of WPC. Regardless of heating processes, whiteness of both kamaboko and modori gels in the presence of 50 mmoles $CaCl_2$/kg was greater than that of gels without $CaCl_2$ ($P<0.05$). $CaCl_2$ might form complex with some anion in the muscle, leading to the light scattering in resulting gels (Benjakul *et al*., 2004c).

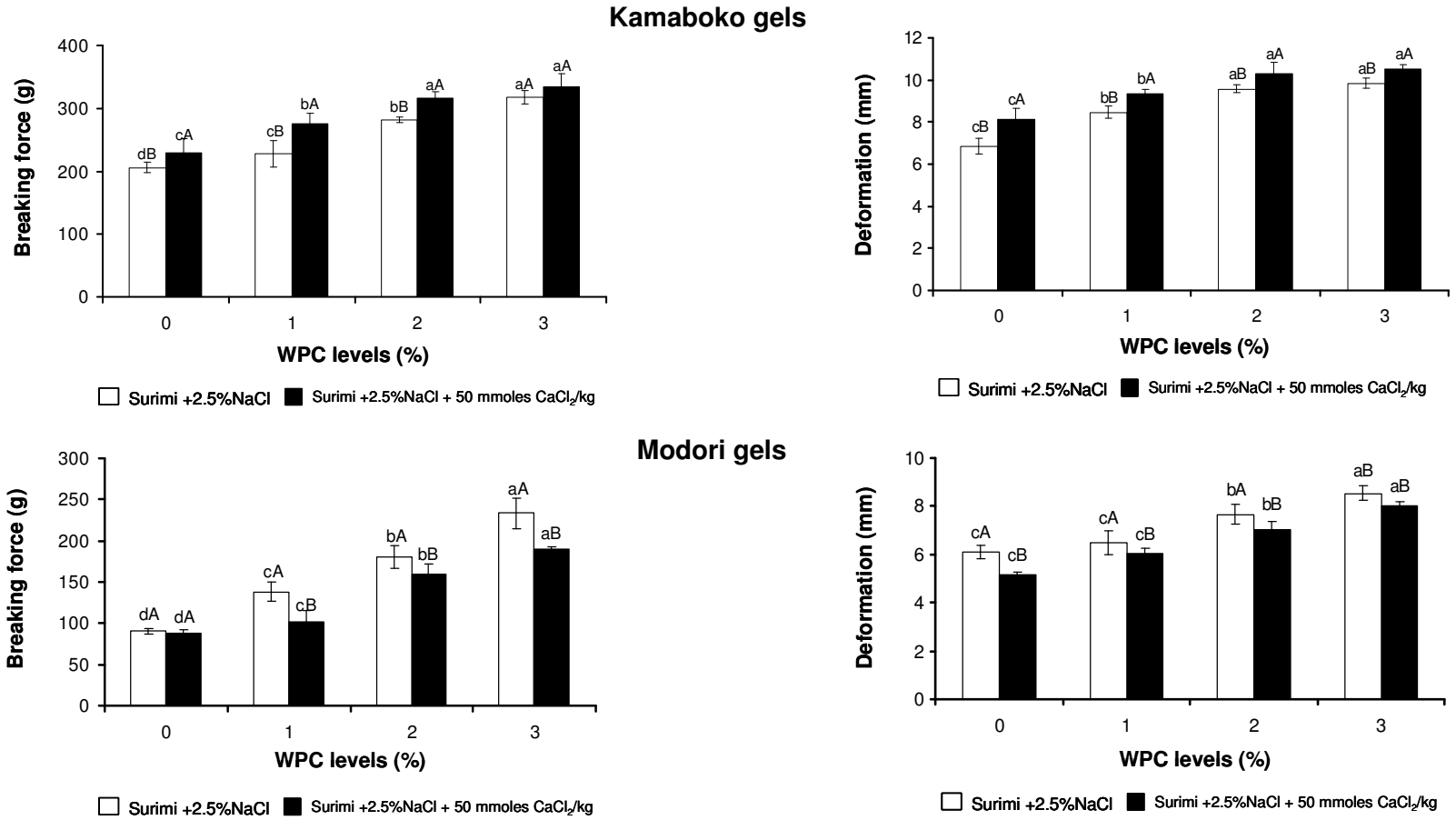


Figure 37 Breaking force and deformation of kamaboko and modori gels added with WPC at different levels in the absence and in the presence of 50 mmoles $CaCl_2$/kg. Bars represent the standard deviation from five determinations. Different letters on the bars within the $CaCl_2$ level indicate the significant differences ($P<0.05$). Different capital letters on the bars within the same level of WPC indicate the significant differences ($P<0.05$).

5.2.3 Expressible moisture content of surimi gel

Expressible moisture content of kamaboko and modori gels added with WPC at different levels in the presence and in the absence of $CaCl_2$ is shown in Table 14. Decreasing expressible moisture contents were obtained in all gel samples as WPC levels increased ($P<0.05$). Expressible moisture content of kamaboko and modori gels decreased by 18.9 and 35.9% with addition of 3% WPC and $CaCl_2$. Expressible moisture content decreased by 18.8 and 51.4% for kamaboko and modori gels added with only 3% WPC. Thus, addition of WPC increased water holding capacity of gel matrix. WPC could prevent protein degradation, leading to the well-ordered network with high water holding capacity. Additionally, WPC was able to bind water, resulting in a greater amount of water retained in the gel matrix.

In general, the greater expressible moisture content of modori gel was observed, compared with kamaboko gel, irrespective of WPC addition. This indicated the poor gel matrix with low water holding capacity as mediated by indigenous proteinases. For the kamaboko gel, higher water holding capacity was obtained when $CaCl_2$ was present at all levels of WPC used as evidenced by lower expressible moisture content ($P<0.05$). During setting, $CaCl_2$ most likely improved gel forming ability via the formation of non-disulfide covalent bonds. As a consequence, a stronger gel network formed could imbibe more water. This was in agreement with higher breaking force and deformation of gels in the presence of $CaCl_2$ (Figure 37). At the same level of WPC, the expressible moisture content of modori gel containing $CaCl_2$ was higher than that of gels without $CaCl_2$ ($P<0.05$). Ca^{2+} might activate endogenous proteinases, particularly calpain. As a result, the network formed could be destroyed to some extent, leading to the lower ability to imbibe water in gel network. Rawdkuen and Benjakul (2008) also found that the addition of WPC (0-3%) resulted in the increase water holding capacity of surimi gel from some tropical fish.

Table 14 Whiteness and expressible moisture contents of kamaboko and modori gels added with WPC at different levels in the presence and in the absence of 50 mmoles $CaCl_2$/kg

Samples	Treatments	WPC levels (%)	Whiteness	Expressible moisture content (%)
Kamaboko gel	Surimi+2.5% NaCl	0	73.38±0.69$^{aB, *,**,\dagger}$	4.46±0.24^{aA}
		1	73.09±0.14^{aB}	4.04±0.14^{bA}
		2	73.01±0.47^{aB}	3.83±0.10^{bcA}
		3	73.08±0.38^{aB}	3.62±0.25^{cA}
	Surimi+2.5% NaCl + 50 mmoles $CaCl_2$/kg	0	77.23±0.48^{aA}	3.60±0.20^{aB}
		1	76.14±0.18^{bA}	3.42±0.11^{aB}
		2	75.82±0.15^{cA}	3.16±0.13^{bB}
		3	75.57±0.35^{cA}	2.92±0.18^{cB}
Modori gel	Surimi+2.5% NaCl	0	74.21±0.38^{aB}	8.49±0.32^{aA}
		1	73.30±0.41^{aB}	4.75±0.08^{bB}
		2	72.71±0.19^{bB}	4.39±0.28^{bcB}
		3	72.44±0.37^{cB}	4.13±0.14^{cB}
	Surimi+2.5% NaCl + 50 mmoles $CaCl_2$/kg	0	76.38±0.37^{abA}	9.01±0.57^{aA}
		1	76.79±0.40^{aA}	6.29±0.17^{bA}
		2	76.13±0.30^{bA}	5.95±0.41^{bA}
		3	76.16±0.20^{bA}	5.78±0.39^{bA}

†Values are given as means ± SD from five determinations.

*Different superscripts within the same $CaCl_2$ levels of the same gel indicate the significant differences ($P<0.05$).

**Different capital superscripts within the same WPC levels of the same gel indicate significant differences ($P<0.05$).

5.2.4 TCA-soluble peptide content of surimi gel

TCA-soluble peptide content in kamaboko and modori gels containing WPC at different levels with and without $CaCl_2$ is present in Figure 38. The highest TCA-soluble peptide content was obtained in both kamaboko and modori gels without WPC addition ($P<0.05$). TCA-soluble peptide content decreased as WPC levels increased ($P<0.05$). This result suggested that WPC exhibited the inhibitory activity toward degradation of muscle protein. The degradation occurred during heat-induced gelation is considered to result from the action of endogenous proteinases (An *et al*., 1996), especially heat activated proteinases (Benjakul *et al*., 2003e). Heat activated alkaline proteinases in bigeye snapper classified as a serine proteinase contributed to degradation of gel texture at 60°C (Benjakul *et al*., 2003c). The enzymes induced modori gel of lizardfish was characterized to be heat activated alkaline cysteine proteinase (Benjakul *et al*., 2003e). High molecular weight protein (101,000 daltons) of WPC was reported as the effective inhibitor toward papain and trypsin (Weerasinghe *et al*., 1996b). For the kamaboko gel, the lower TCA-soluble peptide content was obtained in gels added with $CaCl_2$, compared with those without $CaCl_2$ ($P<0.05$). With addition of 3% WPC, TCA-soluble peptide content of gel without and with $CaCl_2$ decreased by 54.5% and 68.6%, respectively, compared with that of the control gel. This suggested that $CaCl_2$ and WPC could function synergistically to inhibit protein degradation. $CaCl_2$ more likely induced protein cross-linking mediated by TGase, resulting in the resistance to proteolysis. Additionally, Ca^{2+} could bind protein substrates and caused the conformation changes, in which proteinases could not hydrolyze easily. Nevertheless, TCA-soluble peptide content was higher in modori gels containing $CaCl_2$ than those without $CaCl_2$ ($P<0.05$). This reconfirmed that $CaCl_2$ might involve in gel softening mediated by proteinases. The addition of WPC, especially at higher levels, could lower protein degradation. The result was in accordance with the increases in breaking force and deformation of the gels added with 3% WPC, irrespective of $CaCl_2$ addition (Figure 37). The results reconfirmed that the improved gel strength of surimi was partially associated with lowering of proteolysis.

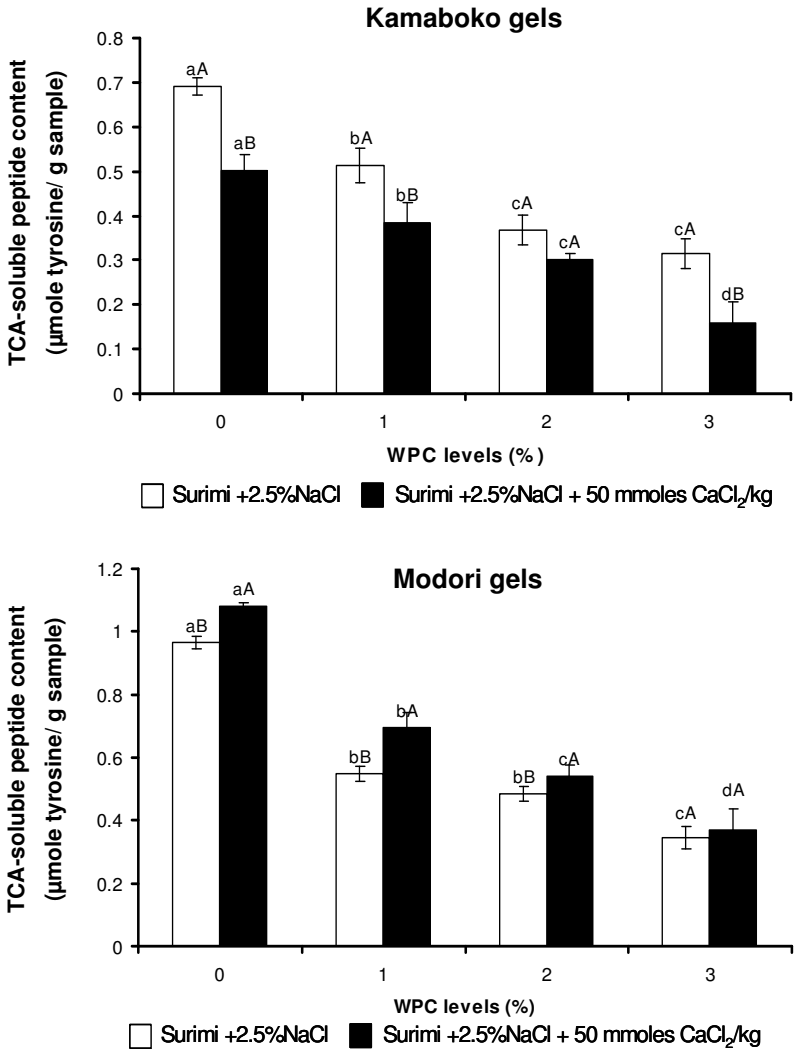


Figure 38 TCA-soluble peptide content of kamaboko and modori gels added with WPC at different levels in the absence and in the presence of 50 mmoles $CaCl_2$/kg. Bars represent the standard deviation from three determinations. Different letters on the bars within the $CaCl_2$ level indicate the significant differences ($P<0.05$). Different capital letters on the bars within the same level of WPC indicate the significant differences ($P<0.05$).

5.2.5 Protein patterns of surimi gel

Protein patterns of all surimi gels are shown in Figure 39. The intensive degradation of myosin heavy chain (MHC) was observed in the sample without the addition of WPC as indicated by the lowest of MHC band intensity retained in both kamaboko and modori gels, regardless of $CaCl_2$ addition. However, no changes in actin were observed in all treatments. MHC band intensity became increased with increasing levels of WPC, indicating that WPC could inhibit the degradation of MHC to some extent. At a level of 3% WPC, which rendered the highest breaking force and deformation, the highest MHC intensity was observed. This suggested that the proteolysis of goatfish gel could be minimized when 3% WPC was used. For the control gels, MHC band intensity of kamaboko gel was slightly higher than that of modori gel. Degrading products with molecular weight lower than 200 kDa were found in the latter, indicating the greater protein degradation.

When comparing protein patterns between gels with and without $CaCl_2$ addition, no obvious differences were found at the same level of WPC added. Occurrence of higher molecular weight protein cross-links were observed in kamaboko gel, regardless of $CaCl_2$ addition. The formation of cross-linked proteins coincided with higher breaking force and deformation of surimi gel (Figure 37). Conversely, lower protein polymerization took place in modori gels. Disappearance of MHC band in modori gel most likely resulted mainly from proteolysis. Additionally, TGase might be destabilized at 60°C, an incubation temperature for modori gel preparation. The changes in MHC, either polymerization or degradation, which were found in kamaboko and modori gels, were mirrored by the increases and decreases in gel strength, respectively. Thus, the addition of WPC could prevent the degradation of proteins in goatfish surimi, allowing a stronger three-dimensional gel network to be formed.

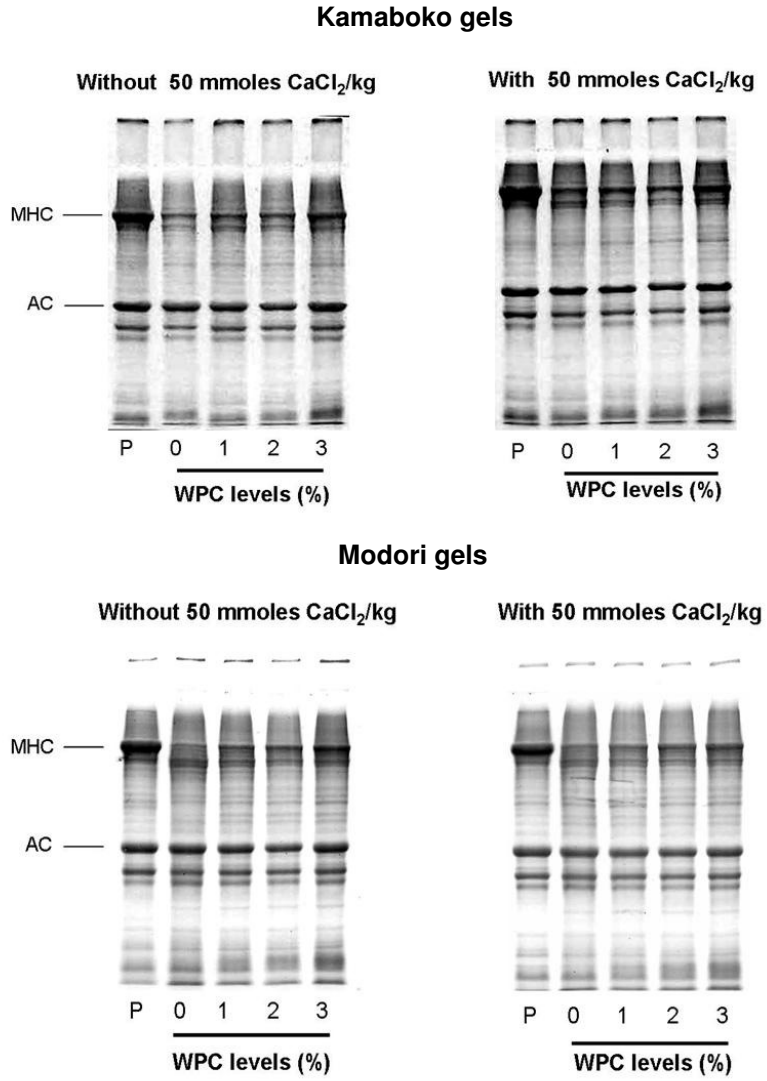


Figure 39 Protein patterns of kamaboko and modori gels added with WPC at different levels in the absence and in the presence of 50 mmoles $CaCl_2$/kg. P: surimi paste; MHC: myosin heavy chain; AC: actin; Numbers designate the amounts of WPC added (%).

5.2.6 Microstructure of surimi gel

The selected micrographs of different kamaboko and modori gels from goatfish surimi: 1) control gel, 2) gel added with 3% WPC and 3) gel added with 3% WPC and 50 mmoles $CaCl_2$/kg, visualized by SEM are shown in Figure 40. The microstructure of kamaboko gel without WPC showed the higher interconnected three-dimensional protein network, compared with the modori gel. When 3% WPC was added, more compact structure with smaller voids was obtained,

in comparison with the control gel. These observations suggested that WPC might distribute uniformly as the filler in the ordered network. However, surimi gel added with 3%WPC and $CaCl_2$ exhibited a finer compact structure with smaller voids, compared with that containing only WPC. This result indicated that $CaCl_2$ acted synergistically with WPC for gel improvement. Calcium ion therefore contributed to protein cross-linking during setting as evidenced by the stronger gel (Figure 37). Modori gels without WPC addition had a structure containing aggregates of sparse pack spherical proteins and arranged in clusters. A large porous matrix with some cavities on the surface was also observed. When 3% WPC was added, more compact structure with smaller clusters of aggregated protein was formed as evidenced by the smaller voids. This suggested that WPC was effective in inhibiting protein degradation. Similar structures were found between modori gel containing 3% WPC without and with $CaCl_2$ addition.

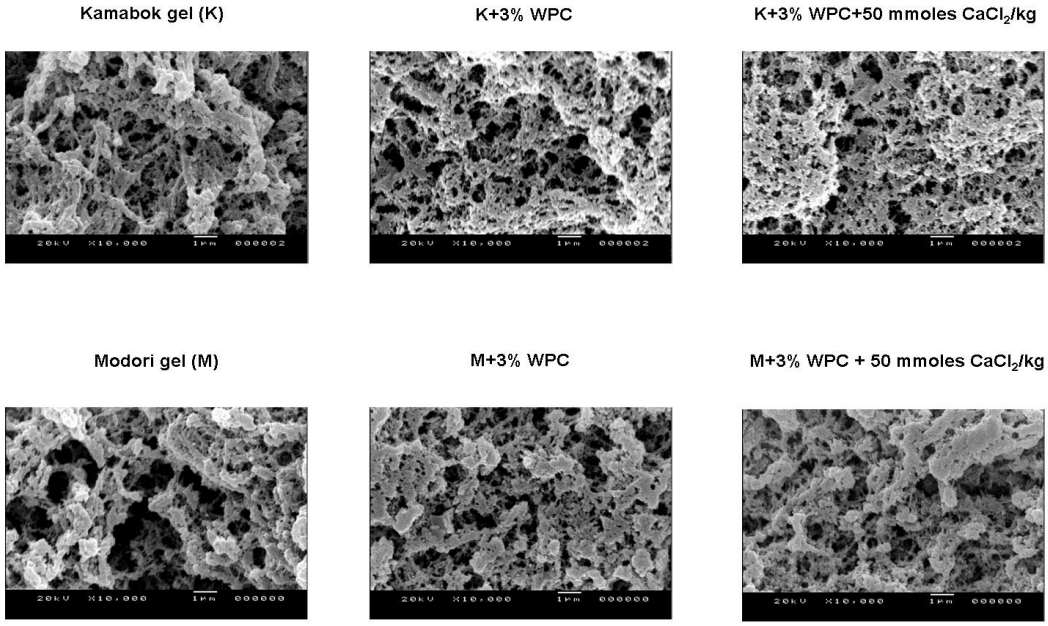


Figure 40 Microstructures of kamaboko and modori gels from goatfish surimi added without and with 3% WPC in the absence and in the presence of 50 mmoles $CaCl_2$/kg. Magnification: 10,000×.

CHAPTER 4

CONCLUSIONS

1. Goatfish meat was the good source of proteins, especially myofibrillar proteins. However, goatfish proteins exhibited the lower gel forming ability than did bigeye snapper proteins as indicated by the lower extent of aggregation of natural actomyosin via hydrophobic interaction and disulfide bonds during heating in the temperature range of 20-75°C. Proteins from unfreshed fish had the decrease in gelation determined by aggregation test.

2. Setting of goatfish surimi at 40°C yielded the gel with the improved properties, especially when 50 mmoles $CaCl_2$/kg was added. The increased gel strength was associated with MHC cross-linking via non-disulfide formation induced by endogenous TGase. Substantial decreases in breaking force and deformation as well as whiteness of surimi gel were found when unfresh goatfish were used as raw material, mainly caused by denaturation and degradation of muscle proteins during the extended storage.

3. Autolysis of goatfish took place at high temperature with the activity peak at 60°C. MHC was most susceptible to hydrolysis. At physiological pH, serine proteinase was a major contributor to protein degradation. However, cysteine proteinase was dominant at acidic pH. Inclusion of skin also resulted in the increased autolysis. Therefore, degradation of myofibrillar proteins of goatfish muscle was governed by endogenous proteinases as well as the contamination of skin. This was most likely associated with gel weakening of this species.

4. Autolysis of goatfish surimi caused by heat-activated proteinases was partially inhibited by the addition of WPC. The addition of WPC up to 3% (w/w) resulted in the increased breaking force and deformation with higher water holding capacity of surimi from goatfish. Addition of $CaCl_2$ at 50 mmoles/kg and 3% WPC effectively improved the gel strength of kamaboko gel. Thus, the use of WPC and $CaCl_2$ in goatfish surimi together with prior setting led to the improved gel-forming ability of goatfish surimi.

FUTURE RESEARCH

1. Effect of new protein additives on gelling ability and proteolysis inhibition of goatfish muscle should be further studied.
2. Alternative uses of goatfish meat should be expanded, particularly as the raw material for film formation or as emulsifier.
3. Autolysis process, which can be used to produce protein hydrolysate from goatfish meat with bioactivity, should be focused.

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APPENDIX

ANALYTICAL METHODS

1. Determination of moisture content (AOAC, 1999)

Method

1. Dry the empty dish and lid in the oven at 105 °C for 3 h and transfer to a desiccator to cool. Weigh the empty dish and lid.
2. Weigh about 3 g of sample to the dish. Spread the sample to the uniformity.
3. Place the dish with sample in the oven. Dry for 3 h at 105°C.
4. After drying, transfer the dish with partially covered lid to the desiccator to cool. Reweigh the dish and its dried sample.

Calculation

$$\text{Moisture content (\%)} = \frac{(W1-W2)}{W1} \times 100$$

where: W1 = weight (g) of sample before drying

W2 = weight (g) of sample after drying

2. Determination of protein content (AOAC, 1999)

Reagents

- Kjeldahl catalyst: Mix 9 parts of potassium sulphate (K_2SO_4) with 1 part of copper sulphate (Cu_2SO_4)
- Sulfuric acid (H_2SO_4)
- 40% NaOH solution
- 0.2 N HCl solution
- 4% H_3BO_3
- Indicator solution: Mix 100 ml of 0.1% methyl red (in 95% ethanol) with 200 ml of 0.2% bromocresol green (in 95% ethanol)

Method

1. Place sample (0.5-1.0 g) in digestion flask.
2. Add 5 g of Kjeldahl catalyst and 200 ml of conc. H_2SO_4
3. Prepare a tube containing the above chemical except sample as blank. Place flasks in inclined position and heat gently unit frothing ceases. Boil until solution clears.
4. Cool and add 60 ml of distilled water cautiously.
5. Immediately connect flask to digestion bulb on condenser and with tip of condenser immersed in standard acid and 5-7 drops of mix indicator in receiver. Rotate flask to mix content thoroughly; then heat until all NH_3 is distilled.
6. Remove receiver, wash tip of condenser and titrate excess standard acid distilled with standard NaOH solution.

Calculation

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

Where:
A = volume (ml) of 0.2 N HCl used sample titration
B = volume (ml) of 0.2 N HCl used in blank titration
N = Normality of HCl
W = weight (g) of sample
14.007 = atomic weight of nitrogen
6.25 = conversation factor

3. Determination of ash content (AOAC, 1999)

Method

1. Place the crucible and lid in the furnace at 550°C overnight to ensure that impurities on the surface of crucible are burned off.
2. Cool the crucible in the desiccator (30 min).
3. Weigh the crucible and lid to 3 decimal places.
4. 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.

5. Heat at 550°C overnight. During heating, do not cover the lid. Place the lid after complete heating to prevent loss of fluffy ash. Cool down in the desiccator.
6. Weigh the ash with crucible and lid when the sample turns to gray. If not, return the crucible and lid to the furnace for the further ashing.

Calculation

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

4. Determination of fat content (AOAC, 1999)

Reagents

- Petroleum ether

Method

1. Place the bottle and lid in the incubator at 105°C overnight to ensure that weight of bottle is stable.
2. Weigh about 3-5 g of sample to filter paper and wrap.
3. Take the sample into extraction thimble and transfer into soxhlet.
4. Fill petroleum ether about 250 ml into the bottle and take it on the heating mantle.
5. Connect the soxhlet apparatus and turn on the water to cool them and then switch on the heating mantle.
6. Heat the sample about 14 h (heat rate of 150 drop/min).
7. Evaporate the solvent by using the vacuum condenser.
8. Incubate the bottle at 80-90°C until solvent is completely evaporated and bottle is completely dry.
9. After drying, transfer the bottle with partially covered lid to the desiccator to cool. Reweigh the bottle and its dried content.

Calculation

$$\text{Fat content (\%)} = \frac{\text{Weight of fat}}{\text{Weight of sample}} \times 100$$

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

Reagent

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

Method

1. Weigh 3 g of Pacific white shrimp and homogenize in 27 ml of 5% TCA.
2. Keep the homogenate in ice for 1 h, and centrifuge at 5,000 x g for 5 min.
3. Measure tyrosine content in the supernatant using the Lowry's method and express as μmole tyrosine/ g sample.

6. Lowry' s procedure for quantitation of protein (Lowry *et al*., 1951)

Reagent

- A: 2% sodium carbonate in 0.1 N NaOH
- B: 0.5% $CuSO_4.5H_2O$ in 1% sodium citrate
- C: 2 N Folin phenol reagent diluted with deionized water (1:1 v/v)
- D: 50 ml reagent A + 1 ml reagent B
- Standard reagent : Bovine serum albumin (BSA) at concentration of 1 mg/ml

Method

1. Add 2 ml of reagent D to 200 μl of the standards and unknown and vortex immediately.
2. Incubate precisely for 10 min at room temperature.
3. Add 0.2 ml of reagent C (previously dilute 1: 1 with distilled water) and vortex immediately.
4. Incubate the mixture for 30 min at room temperature not longer than 60 min.
5. Read the absorbance at 750 nm (glass cuvate).
6. Plot standard curve and calculate the unknown.

Standard

- Bovine serum albumin (BSA) at concentration of 1 mg/ml (used to determine protein concentration).
- Tyrosine at a concentration of 1 mM
- Standard volumes (μl) : 0, 20, 40, 60, 100, 140, and 200

Standard curve of Lowry's procedure

Std. volume (μl)	Tyrosine (μl)	Distilled water (μl)
0	0	200
20	20	180
40	40	160
60	60	140
100	100	100
140	140	600
200	200	0

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

Reagent

- protein molecular weight standards
- 30% Acryamide - 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 0f 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 aliquot and store at -20°C
- 2% (w/v) Ammonium persulfate
- 1% (w/v) SDS
- - TEMED (*N, N, N′N′*- tetramethylenediamine)
- 0.5 M Tris- HCl, pH 6.8
- 1.5 M Tris- HCl, pH 8.8
- 0.1 M EDTA

- Electrode buffer: Dissolve 3 g of Tris, 14.4 g of glycine and 1 g of SDS in distilled water. Adjust to pH 8.3. Add distilled water to 1.1.
- Staining solution: Disslove 0.04 g of Coomassie blue R-250 in 100 ml of methanol. Add 15 ml of glacoial acetic acid and 85 ml of distilled water.
- Destaining solution I: 50% methanol- 7.5 glacial acetic acid.
- Destaining solution II: 5% methanol- 7.5% glacial acetic acid.

Method

Pouring the separating gel:

1. Assemble the minigel apparatus according to the manufacture's detailed instructions. Make sure that the glass and other components are rigorously clean and dry before assembly.
2. Mix the separating gel solution as shown in Table.
3. Transfer the separating gel solution by using a Pasture pipettes to the center of
4. sandwich, which is about 1.5 to 2 cm from the top of the shorter (front) glass plates.
5. Cover the top of the gel with a layer of water by gently squirting the water against the edge of one of the spacers. Allow the resolving gel to polymerized 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 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
10%SDS	0.35 ml	0.3 ml
Distilled water	0.7585 ml	0.9 ml
0.1M 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 final volume of 30 ml.
2. Incubate the mixture at 85 °C for 1 h.
3. Centrifuge at 3,500×g for 5 min at ambient temperature and collect the supernatant.

Loading the gel:

1. Dillute the protein with sample buffer at a ratio of 1:4 (v/v) in microcentrifuge tube and boil for 1 min at 100°C
2. Remove the comb without tearing the edge of polyacrylamide wells.
3. Fill the wells with electrode buffer.
4. Place the upper chamber over 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 syring 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 well.
7. Fill the remainder of the upper buffer chamber with additional electrode buffer.

Runing 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.

Dissembling 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 apacers out from the edge of the sandwich along its entire length.
3. Insert a spectula between the glass plates at one corner where the spacer is, 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 about 15 min.
3. Pour off the destaining solution I and cover the destaining solution II. Discard destaining solution and replace with fresh solution. Repeat unit the gel is clear except for the protein bands.

8. Biuret method quantitation of protein (Copeland *et al*., 1994)

Reagent

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

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

Method

1. Standard volumes (0, 100, 200, 300, 400 and 500 µl) were prepared.
2. For the unknown protein samples, add 500 µl of the samples and mix the contents of each tube well.
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. Using the average absorbance for the three samples of unknown, read the concentration of sample from the plot.

Preparation of standard curve of 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	0	500	10

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

Reagents

- 0.6 M KCl, pH 7
- Distilled water

Method

1. Homogenize 10 g of muscle in 100 ml chilled (4°C) 0.6 M KCl, pH 7.0 for 4 min.
2. Place the beaker containing the sample in ice.
3. Blend every 20 sec, followed by a 20 sec rest interval to avoid overheating during extraction.
4. Centrifuge the extract at 5,000xg for 30 min at 4°C.

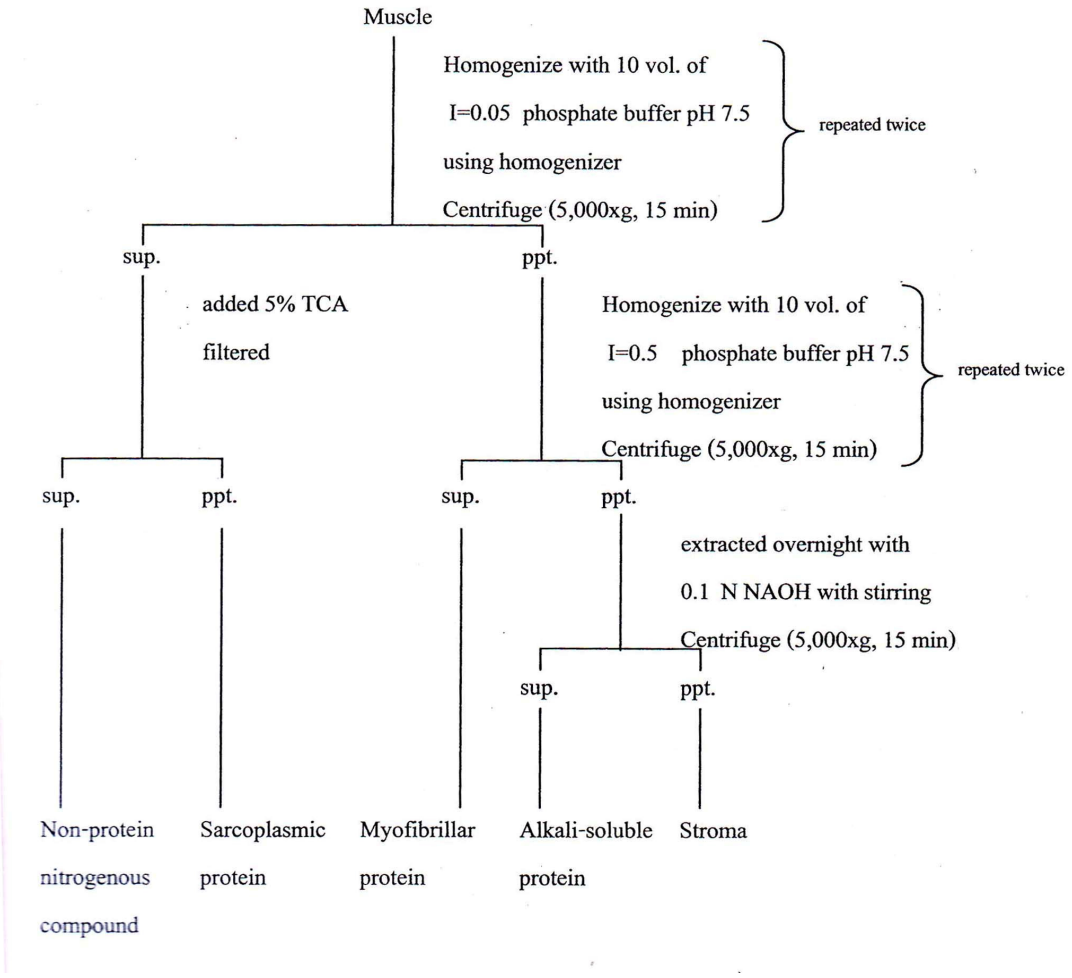
5. Add three volumes of chilled distilled water to precipitate actomyosin.
6. Collect actomyosin by centrifuging at 5,000xg for 20 min at 4°C.
7. Dissolve the pellet by stirring for 30 min at 4°C in an equal volume of chilled 0.6 M KCl, pH 7.

10. Fractionaion of muscle proteins (Hashimoto *et al.*, 1979)

Reagents

- 0.05 M phosphate buffer, pH 7.5
- 0. 5 M KCl phosphate buffer, pH 7.5
- Trichloroacetic acid
- 0.1 N NaOH

Method



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Yarnpakdee, S., Benjakul, S., Visessanguan, W. and Phatcharat, S. 2008. Combination effects of whey protein concentrate and calcium chloride on the properties of goatfish surimi gel. J. Muscle Foods (Submitted).

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