CHAPTER 4

COMBINATION EFFECTS OF CHICKEN PLASMA PROTEIN AND SETTING PHENOMENON ON GEL PROPERTIES AND CROSS-LINKING OF BIGEYE SNAPPER MUSCLE PROTEINS

4.1 Abstract

Effects of chicken plasma protein (CPP) in combination with setting on surimi gel properties and protein cross-linking were investigated. Addition of 0.5% CPP, 10 mM $CaCl_2$ and 200 units of thrombin/g CPP in combination with setting at 40°C for 30 min prior to heating at 90°C for 20 min resulted in the highest breaking force and deformation ($P<0.05$). Regardless of CPP addition, myosin heavy chain (MHC) in surimi proteins and natural actomyosin (NAM) underwent polymerization to some extent in presence of $CaCl_2$ and thrombin. The cross-linking of MHC in surimi proteins were markedly suppressed by the addition of EGTA and NH_4Cl, transglutaminase (TGase) inhibitors. No cross-linking of myosin was observed when CPP, $CaCl_2$ and thrombin were added, even with the prolonged incubation time. The result revealed that CPP showed no cross-linking activity and gel strengthening effect of CPP was attributed to its filler effect and proteolytic inhibitory activity.

4.2 Introduction

Surimi possesses the functionality including binding, gelling and emulsifying properties and can be used as a functional protein ingredient in several products (Lanier, 1986). The thermal aggregation of myosin molecule is a crucial process for developing the elastic gels (Sano *et al.*, 1988). The cross-linking and aggregation of protein molecules into three-dimensional solid-like networks results in elastic gel with high water holding capacity (Sano *et al.*, 1988). To increase the gel strength, setting has been widely implicated in surimi gel preparation. Setting is a phenomenon describing 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). Benjakul and Visessanguan (2003)

reported that the optimum condition for setting of surimi sol from bigeye snapper (*Priacanthus tayenus* and *Priacanthus macracanthus*) were 40°C and 25°C for 2 and 3 h, respectively. Setting at 25°C for an appropriate time also improves gelling properties of surimi produced from other tropical fish (Benjakul *et al*., 2003b). 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; Kishi *et al*., 1991).

Setting of surimi is enhanced by sufficient calcium content since endogenous TGase is a Ca^{2+} dependent enzyme (Kimura *et al*., 1991; Lee and Park, 1998). ε(γ-glutamyl) lysine dipeptide cross-links formed during setting generally correlates with the increase in gel strength (Imai *et al*., 1996). The formation of non-disulfide covalent cross-links catalyzed by an endogenous TGase resulted in the polymerization of myosin (Seki *et al*., 1990; Kimura *et al*., 1991; Benjakul and Visessanguan, 2003).

Plasma proteins are the promising sources of soluble proteins, which have been recognized as a useful ingredient in cooked meat products due to its excellent gelling properties (Caldironi and Ockerman, 1982). Additionally, it has been reported to exhibit proteinase inhibitory activity and gel strengthening ability during heat-induced gelation of surimi (Seymour *et al*., 1997; Benjakul and Visessanguan, 2000; Visessanguan *et al*., 2000). From our previous study, chicken plasma protein (CPP) was able to enhance the gel strength by acting as filler in surimi gel matrix and also as protease inhibitor (Rawdkuen *et al*., 2004a, b). To improve the gel strength of surimi, the appropriate addition of CPP in combination with proper setting induced by either endogenous or plasma TGase could be a promising approach. However no information regarding cross-linking activity of chicken plasma has been reported. Therefore, the objective of this study was to determine the cross-linking activity of chicken plasma towards bigeye snapper muscle and surimi proteins and to study the combination effect of CPP and setting on surimi gel properties.

4.3 Materials and Methods

Chemicals

Trisodium citrate and sodium chloride were purchased from Merck (Darmstadt, Germany). Adenosine 5'-triphosphate (ATP), β-mercaptoethanol (βME), ethylene glycol-bis (β-aminoethyl ether) *N*, *N*, *N'*, *N'* tetra acetic acid (EGTA), thrombin from bovine plasma and L-tyrosine were obtained from Sigma Chemical Co. (St Louis, MO, USA). Sodium dodecyl sulfate (SDS), *N*, *N*, *N'*, *N'* tetramethyl ethylene diamine (TEMED) and Coomassie Blue R-250 were obtained from Bio-Rad Laboratories (Hercules, CA, USA).

Chicken plasma protein preparation

Chicken blood was obtained from a slaughterhouse in Hat Yai, Songkhla, Thailand. During collection, one-tenth volume of trisodium citrate (3.8%) was added to prevent coagulation. The blood was centrifuged twice at 1,500*g* for 15 min at 4°C to remove red blood cells using a Sorvall Model RC-B Plus centrifuge (Newtown, CT, USA). The supernatant was then freeze-dried and kept at -18°C until used.

Natural actomyosin (NAM) preparation

NAM was prepared from bigeye snapper muscle according to the method of Nowsad *et al.*, (1994). Briefly, fish muscle (100g) was homogenized with 500 ml of cold 3.38 mM NaH_2PO_4, 15.5 mM Na_2HPO_4, pH 7.5 using an IKA homogenizer (Selangor, Malaysia) at a speed of 11,000 rpm for 2 min. The homogenate was centrifuged at 6,000*g* for 10 min. The washing was conducted for totally 6 times. NAM pellet was solubilized in a 500 ml of 0.8 M KCl, 3.38 mM KH_2PO_4, 20.5 mM Na_2HPO_4, pH 8.0 with continuous stirring. NAM was precipitated with 4 volumes of water. The mixture was centrifuged at 15,000*g* for 30 min and NAM pellet was collected. All the procedures were done in a cold room at 4°C.

Myosin preparation

Myosin was extracted according to the method described by Martone *et al.* (1986) with a slight modification. Bigeye snapper fillets was finely chopped and added with 10 volumes of buffer A (0.10 M KCl, 0.02% NaN_3 and 20 mM Tris-HCl, pH 7.5). After incubation on ice for 10 min with occasional stirring, washed muscle was recovered by centrifugation at 1,000*g* for 10 min. The pellet was suspended in 5 volumes of buffer B (0.45 M KCl, 5 mM βME, 0.2 M $Mg(CH_3COO)_2$, 1 mM EGTA and 20 mM Tris-maleate, pH 6.8), and ATP was added to a final concentration of 10 mM. The mixture was kept on ice for 1 h and centrifuged at 10,000*g* for 15 min. Supernatant was recovered, and 25 volumes of 1 mM $NaHCO_3$ were slowly added. The mixture was incubated for 15 min on ice. Precipitated myosin was collected by centrifugation at 12,000*g* and resuspended gently with 5 volumes of buffer C (0.5 M, 5 mM βME and 20 mM Tris-HCl, pH 7.5), followed by the addition of 3 volumes of 1 mM $NaHCO_3$. $MgCl_2$ was added to obtain the final concentration of 10 mM. The mixture was incubated overnight and centrifuged at 22,000*g* for 15 min. Myosin was recovered as the pellet. The purity of extracted myosin was estimated by SDS-PAGE.

Study on the effect of CPP in combination with $CaCl_2$ and thrombin on surimi gel properties

Frozen surimi from bigeye snapper grade SA (breaking force of 600-800 g; deformation of 12-14 mm) obtained from Man A Frozen Food Co, Ltd. (Songkhla, Thailand) was used. Frozen surimi was partially thawed at 4°C for 2-3 h, cut into small pieces and chopped by a Moulinex Masterchef 350 mixer (Paris, France). Surimi paste was added with 0.5% CPP and 10 mM $CaCl_2$ in presence of various levels of thrombin (0, 100, 150 and 200 units/g CPP). Surimi paste added with 0.5% CPP or with 10 mM $CaCl_2$ was used as the controls. The moisture of the mixture was adjusted to 80% with iced water and 2.5% NaCl was added. The mixture was chopped for 4 min. The surimi sol was stuffed into polyvinylidine casing with a diameter of 2.5 cm and both ends were sealed tightly. The sol was incubated at 40°C for 30 min, followed by heating at 90°C for 20 min in a temperature controlled water bath (Memmert, Schwabach, Germany). These gels were referred to as "kamaboko gel". For directly heated gel, surimi sol was subjected to

heating at 90°C for 20 min. After heating, all gels were cooled in iced water for 30 min and stored at 4°C overnight prior to analysis.

Determination of surimi gel compositions and properties

Texture analysis

Texture analysis of surimi gels was carried out using a Model TA-XT2 texture analyzer (Stable Micro System, Surrey, UK). Gels were equilibrated at room temperature (25°C) 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 probe (5 mm diameter, 60 mm/min depression speed).

Determination of autolysis in surimi gel

To 2 g of finely chopped gel samples, 18 ml of 5% TCA were added and homogenized for 2 min using an IKA homogenizer at a speed of 11,000 rpm. The homogenate was incubated at 4°C for 1 h and centrifuged at 8,000*g* for 5 min. TCA-soluble peptides in the supernatant were measured according to the Lowry method (Lowry *et al*., 1951) and expressed as μmole tyrosine/g sample (Morrissey *et al*., 1993).

Protein solubility

Solubility of surimi gel proteins was determined as described by Benjakul *et al*. (2001b). Finely chopped gel sample (1g) was solubilized with 20 ml of 20 mM Tris-HCl, pH 8.0 containing 1% SDS, 2% βME and 8 M urea. The mixture was homogenized for 1 min, boiled for 2 min and stirred for 4 h at room temperature (25°C) using a magnetic stirrer (IKA-Werke, Staufen, Germany). The mixture was centrifuged at 10,000*g* for 30 min. A 2 ml of cold trichloroacetic acid (TCA) (5%) was added to 10 ml of supernatant. The mixture was kept at 4°C for 18 h prior to centrifugation at 10,000*g* for 20 min. The precipitate was washed with 10% TCA, and solubilized in 0.5

M NaOH. Protein concentration was determined by the Biuret method (Robinson and Hodgen, 1940). Solubility of protein in surimi samples was expressed as the percentage of total protein in surimi gels solubilized directly in 0.5 M NaOH.

SDS-PAGE

SDS-PAGE analysis was performed according to the method of Laemmli (1970). To 2 g of sample, 18 ml of 5% SDS solution was added. The mixture was then homogenized using an IKA Labortechnik homogenizer at a speed of 11,000 rpm for 1 min. The homogenates was incubated at 85°C in a temperature controlled water bath for 1 h to dissolve total proteins. The sample was centrifuged at 10,000*g* for 5 min to remove undissolved debris. Solubilized samples were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris-HCl, pH 6.8 containing 4% SDS, 20% glycerol and 10% βME and boiled for 3 min. The samples (20 μg protein) were loaded into 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 Protean II unit electrophoresis system (Bio-Rad Laboratories, Inc, Richmond, CA, USA). After separation, the proteins were stained with Coomassie Brilliant Blue R-250 (0.02%) in 50% methanol and 7.5% acetic acid and destained with 50% methanol and 7.5% acetic acid, followed by 5% methanol and 7.5% acetic acid.

Scanning Electron Microscopy (SEM)

Microstructure of directly heated gel and kamaboko gel without and with 10 mM $CaCl_2$ or 0.5% CPP in combination with 10 mM $CaCl_2$ and 200 units of thrombin were determined. Samples with a thickness of 2-3 mm were fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffer, pH 7.2. The samples were then rinsed for 1 h in distilled water before being dehydrated in ethanol with a serial concentration of 50, 70, 80, 90 and 100 %. Dried samples were mounted on a bronze stub and sputter-coated with gold (Sputter coater SPI-Module, PA, USA). The specimens were observed with a scanning electron microscope (JEOL JSM-5800 LV, Tokyo, Japan) at an acceleration voltage of 10 kV.

Study on the effect of CPP on NAM and myosin cross-linking

NAM paste was added with 0.5% CPP in presence or absence of 10 mM $CaCl_2$. NAM containing 0 and 10 mM $CaCl_2$ was used as the controls. Addition of CPP at a level of 0.5% resulted in the highest breaking force and deformation (Rawdkuen *et al.*, 2004a). For $CaCl_2$, the level of 10 mM was found to increase the breaking force and deformation of kamaboko gels of bigeye snapper (Benjakul and Visessanguan, 2003). To study the effect of thrombin on cross-linking activity of CPP, NAM added with 0.5% CPP and 10 mM $CaCl_2$ was mixed with thrombin at various concentrations (100, 150 and 200 units/g CPP). All samples were incubated at 40°C for 30 min. Cross-linking of proteins was visualized by SDS-PAGE using 10% running gel and 4% stacking gel.

For myosin cross-linking study, myosin was diluted with buffer C to obtain the protein content of 10 mg/ml. CPP was added into myosin solution to obtain the final concentration of 3% protein, which was equivalent to the CPP concentration used in surimi. The reaction mixtures were prepared in the same manner with those described for NAM cross-linking study.

To study the effect of setting time on NAM and myosin cross-linking, sample treatment exhibiting the highest gel strength was chosen. Reaction mixture (0.5% CPP, 10 mM $CaCl_2$ and 200 units of thrombin/g CPP for NAM; 3% CPP, 10 mM $CaCl_2$ and 200 units of thrombin/g CPP for myosin) was incubated at 40°C for 0, 10, 30, 60, 90, and 120 min. All samples were analyzed for protein cross-linking by SDS-PAGE.

Study on the effect of TGase inhibitors on protein cross-linking

Surimi paste with and without 0.5% CPP was prepared as mentioned above. EGTA was added to the surimi paste to obtain a final concentration of 10 and 20 mM, whereas NH_4Cl with a final concentration of 125 and 250 mM was used. A control sample (without inhibitor) was also prepared. All gel samples were incubated at 40°C for 30 min, followed by heating at 90°C for 20 min. The gels were then subjected to analysis for the textural properties and protein patterns.

Statistical analysis

Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple-range test (Steel and Torrie, 1980). Analysis was performed using a SPSS package (SPSS 8.0 for windows, SPSS Inc, Chicago, IL).

4.4 Results and discussion

Effects of CPP in combination with thrombin and/or $CaCl_2$ on textural properties of surimi gels.

The highest breaking force and deformation of kamaboko gel from bigeye snapper surimi were obtained when 0.5% CPP was added in combination with 10 mM $CaCl_2$ and 150-200 units of thrombin/g CPP (Figure 14). Addition of 0.5% CPP or 10 mM $CaCl_2$ resulted in 33.7 and 62.3% increase in breaking force, respectively, compared with the control kamaboko gel (without additives). Breaking force of surimi gel increased by 90.6 and 103.8% with the addition of 0.5% CPP and 10 mM $CaCl_2$ or the combination of 0.5% CPP, 10 mM $CaCl_2$ and 200 units of thrombin/g CPP, respectively, compared with the control kamaboko gel. Marked increase in breaking force was found in the presence of both $CaCl_2$ and thrombin, especially with the higher level of thrombin ($P<0.05$). However, no differences in deformation were observed with different levels of thrombin used ($P>0.05$). Breaking force and deformation of kamaboko gels were 73.6 and 29.6% higher than those of directly heated gel, respectively. This indicated the essential role of setting in gel strengthening. Benjakul *et al.* (2001b) found that kamaboko gel from bigeye snapper in the presence of 0.5% porcine plasma protein (PPP) showed the maximum breaking force and deformation with concomitant increased non-disulfide covalent bonds catalyzed by both endogenous TGase in fish muscle and porcine plasma TGase. The increase in breaking force of kamaboko gel added with CPP, $CaCl_2$ and thrombin might result from activated-factor XIII, which catalyzed the covalent cross-linking of MHC via intermolecular $\varepsilon(\gamma$-glutamyl) lysine bond (Benjakul *et al.*, 2001b). Furthermore, addition of $CaCl_2$ could activate endogenous TGase in bigeye snapper surimi (Benjakul *et al.*, 2004a). 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 and also confirmed that endogenous TGase played an important role in gel enhancement of surimi from bigeye snapper.

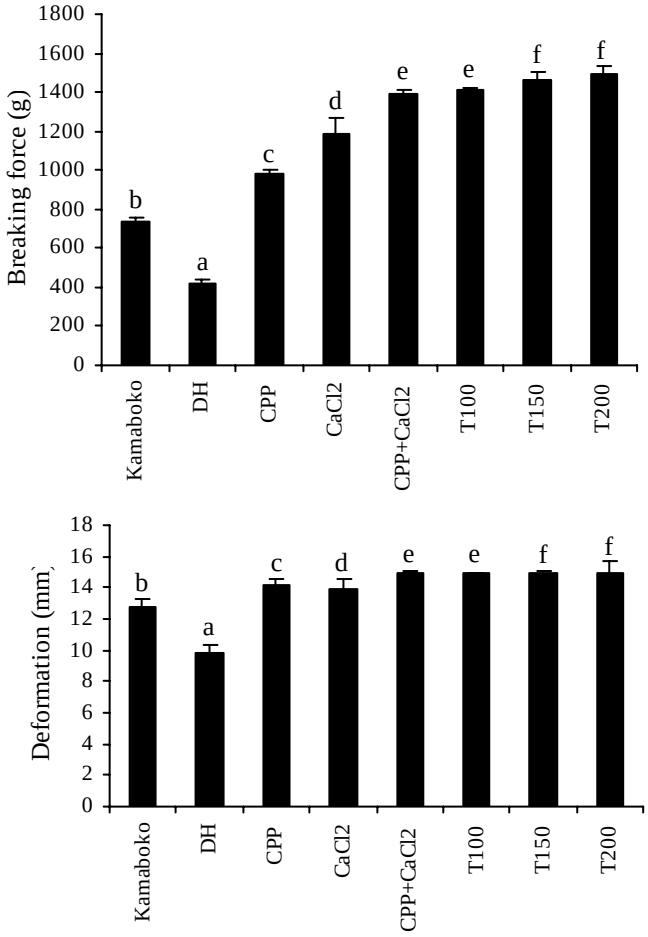

Figure 14. Breaking force and deformation of surimi gel added with chicken plasma protein in combination with thrombin and/or $CaCl_2$. DH: directly heated gel, T: thrombin. Bars represent the standard deviation from five determinations. Different letters indicate significant differences ($P<0.05$).

In presence of CPP alone, it was noted that breaking force and deformation of surimi gel increased ($P<0.05$). The filler effect of plasma protein in surimi gel matrix was suggested to contribute to strengthening of surimi gel (Rawdkuen *et al.*, 2004a). Foegeding *et al.* (1986) reported that myosin and fibrinogen interacted to form a gel, which was stronger than the gel produced by the individual protein. A fibrinogen-thrombin mixture was very reactive in forming a strong structure (Yoon *et al.*, 1999). Mixing fibrinogen-thrombin into low grade surimi enhanced gel properties. Jiang and Lee (1992)

reported that pig plasma Factor XIII was activated by thrombin and Ca^{2+} and activated Factor XIIIa catalyzed the covalent cross-linking of MHC. The addition of crude plasma Factor XIII (including thrombin) substantially increased the gel strength of minced mackerel. Plasma TGase can catalyze the formation of glutamyl-lysine bonds in myosin, myosin/actin, myosin/fibronectin, fibrin/fibronectin and fibrin/actin (Cohen *et al*., 1979; Kahn and Cohen, 1981).

Effects of CPP in combination with thrombin and/or $CaCl_2$ on protein solubility and TCA-soluble peptides of surimi gels.

Kamaboko gels added with CPP, $CaCl_2$ and thrombin had the lowest protein solubility. However, no significant differences in solubility were observed in gels with different levels of thrombin added ($P>0.05$) (Table 11). These results indicated that addition of CPP, $CaCl_2$ and thrombin in combination with setting effectively induced a large number of protein-protein interactions stabilized by non-disulfide covalent bond, which can not be destroyed by the mixture containing SDS, urea, and βME. Non-disulfide covalent bond formed might contribute to the strengthening of gel matrix. When comparing the solubility between directly heated gel and kamaboko gels, the former had a greater solubility than the later. Therefore, setting at 40°C for 30 min, followed by heating at 90°C for 20 min induced protein cross-linking. With the addition of CPP, $CaCl_2$ and thrombin, the solubility of kamaboko gel decreased to less than 30%. Apart from activation of factor XIII in plasma, thrombin might hydrolyze protein to some extent, resulting in the exposure of reactive groups for cross-linking induced by both endogenous and plasma TGase. TGase catalyzes the conversion of soluble protein to insoluble high molecular polymers through formation of covalent cross-links (De Backer-Royer *et al*., 1992; Traore and Meunier, 1992). The lowest TCA-soluble peptide was obtained in kamaboko gel added with CPP (Table 11). This result suggested that CPP had inhibitory activity towards degradation of protein. The degradation probably occurred during setting at high temperature (40°C), which was close to the temperature of gel weakening (50-60°C) mainly caused by indigenous proteases (An *et al*., 1996; Kang and Lanier, 2000; Benjakul *et al*., 2003b).

Table 11. Protein solubility and TCA-soluble peptides of surimi gels added with chicken plasma protein in combination with thrombin (T) and/or $CaCl_2$

Samples	Solubility (%)	Tyrosine (μmole/g sample)
Kamaboko gel (K)	67.14 ± 0.41*c**	1.05 ± 0.01f
Directly heated gel	78.09 ± 2.76d	0.85 ± 0.02d
K+0.5% CPP	66.84 ± 2.59c	0.18 ± 0.02a
K+10 mM $CaCl_2$	33.57 ± 1.15b	0.94 ± 0.02e
K+CPP/$CaCl_2$	32.91 ± 1.11b	0.24 ± 0.01b
K+CPP/$CaCl_2$/T100NIH***	28.45 ± 0.98a	0.26 ± 0.02bc
K+CPP/$CaCl_2$/T150NIH	27.76 ± 1.31a	0.27 ± 0.02c
K+CPP/$CaCl_2$/T200NIH	27.38 ± 1.48a	0.29 ± 0.03c

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

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

*** NIH: National Institute of Health-Units.

When comparing the degradation between directly heated gel and kamaboko gels, the former contained the lower amount of TCA-soluble peptides than the latter. Slight increase in TCA-soluble peptides in surimi gel added with thrombin was possibly owing to thrombin cleavage of an Arg_{37}-Gly_{38} peptide near the amino-terminals of the a-chains of Factor XIII (Takahashi *et al.*, 1986). Nevertheless, partial hydrolysis caused by thrombin might contribute to the increased cross-linking induced by TGase.

Effects of CPP in combination with thrombin and/or $CaCl_2$ on protein pattern and microstructure of surimi gels.

Protein patterns of different surimi gels are depicted in Figure 15. The kamaboko gels added with 10 mM $CaCl_2$ (lane 6A) showed the lowest intensity of MHC band when compared with other treatments. When 0.5% CPP was added (lane 5A), the higher amount of MHC was retained. This indicated interfering effect of CPP on MHC cross-linking. No MHC band was retained in gels added with 0.5% CPP in combination

with 10 mM $CaCl_2$ and thrombin at various concentrations (lane 4-6B), surimi gel added with CPP and $CaCl_2$ (lane 3B) and the gel added with 10 mM $CaCl_2$ (lane 6A). This indicated that cross-linking activity of MHC in surimi was mainly caused by endogenous TGase, which was activated by Ca^{2+}. As a result, MHC was almost disappeared in the kamaboko gel added with $CaCl_2$, regardless of CPP or thrombin addition. However, no marked changes in actin and tropomyosin were observed in all samples.

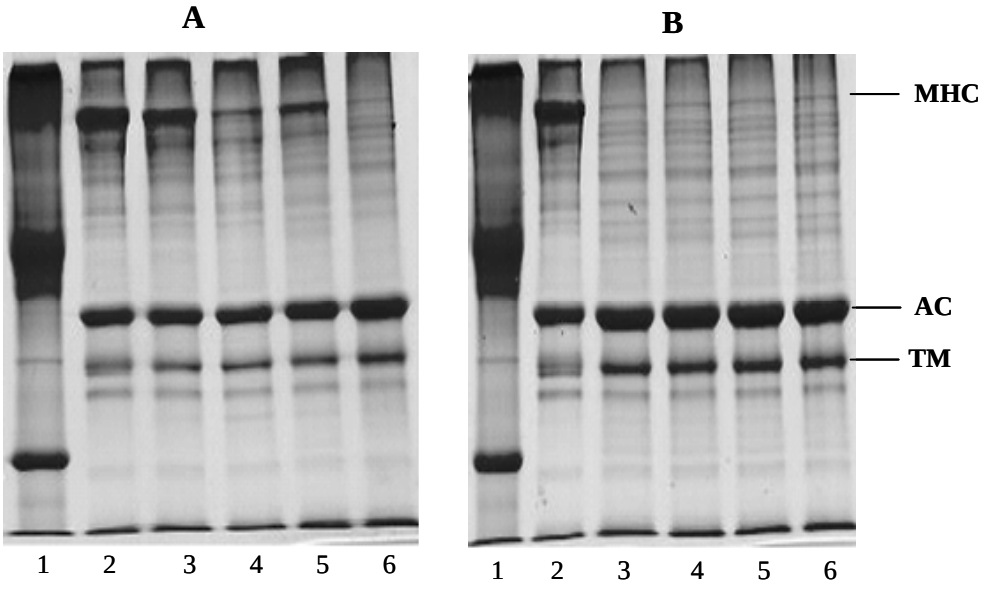

Figure 15. SDS-PAGE patterns of surimi gels added with chicken plasma protein in combination with thrombin and/or $CaCl_2$. Lane 1: CPP; lane 2: surimi sol; lane 3A: directly heated gel; lane 4A: kamaboko gel; lane 5A: kamaboko gel added with 0.5% CPP; lane 6A: kamaboko gel added with 10 mM $CaCl_2$; lane 3B: kamaboko gel added with 0.5% CPP and 10 mM $CaCl_2$; lane 4-6B: kamaboko gel added with 0.5% CPP, 10 mM $CaCl_2$ and 100, 150 and 200 units of thrombin/g CPP, respectively. MHC: myosin heavy chain, AC: actin, TM: tropomyosin.

The microstructure of bigeye snapper surimi gels prepared with different conditions was visualized by scanning electron microscope as shown in Figure 16. The kamaboko gel without additive (A) had a coarse and disordered cross-link structure with large cavities. For directly heated gel (B), more irregularly disordered and coarser fibrillar structures were observed. The fine cross-link structure of kamaboko was observed when added with 10 mM $CaCl_2$ (C). When 0.5% CPP, 10 mM $CaCl_2$ and 200 units of

thrombin were added (D), the finer and more ordered structure became more evident. The result suggested that activation of TGase by $CaCl_2$ addition improved the alignment and aggregation of protein, leading to the fine and ordered structure. CPP was possibly localized in the cavities, resulting in the finer structure of gel network.

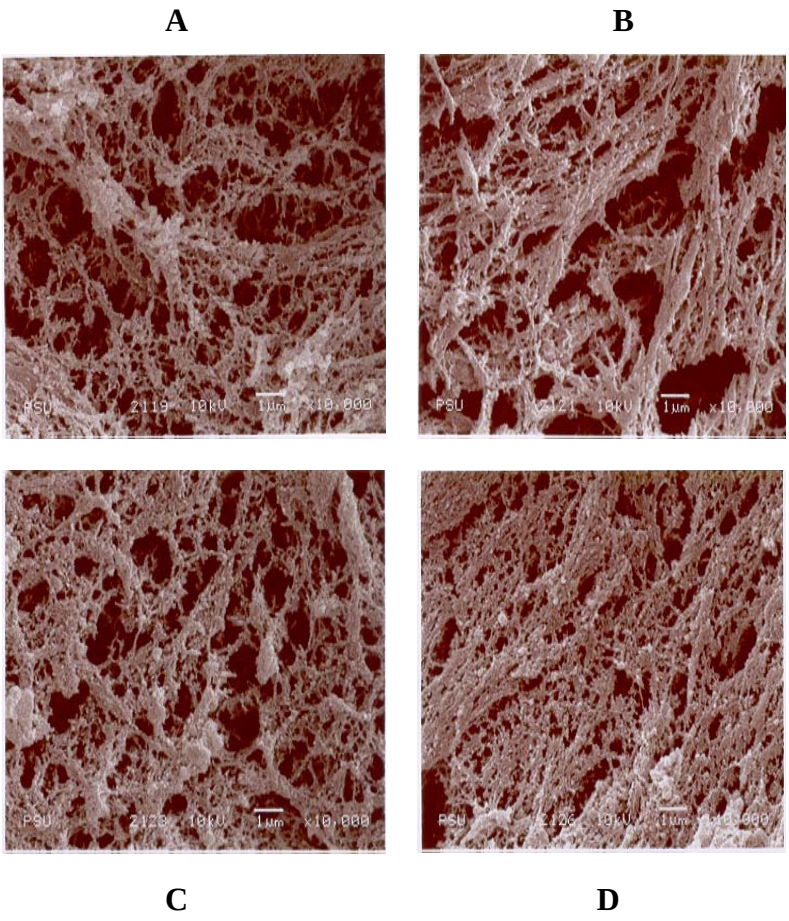

Figure 16. Electron microscopic image of surimi gels from bigeye snapper (magnification: 10,000X). A: kamaboko gel without additive; B: directly heated gel; C: kamaboko gel added with 10 mM $CaCl_2$; D: kamaboko gel added with 0.5% CPP, 10 mM $CaCl_2$ and 200 units of thrombin/g CPP.

Effect of CPP in combination with setting on natural actomyosin cross-linking

The effect of CPP in combination with $CaCl_2$, thrombin and setting on cross-linking of bigeye snapper NAM is demonstrated in Figure 17. The intensity of MHC band noticeably decreased when all NAM paste was added with $CaCl_2$, regardless of CPP

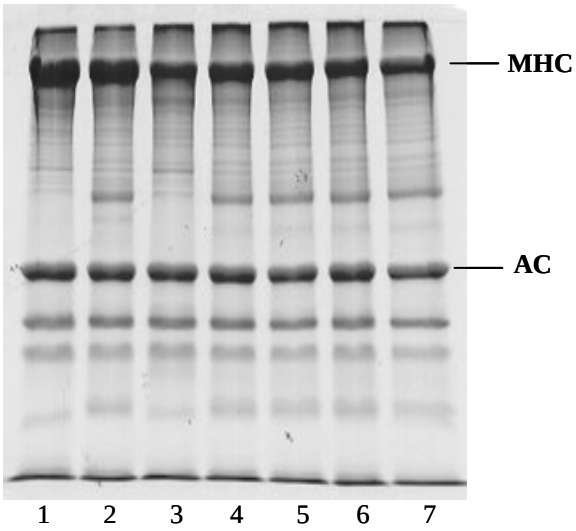

Figure 17. SDS-PAGE patterns of natural actomyosin (NAM) paste added with chicken plasma protein in combination with thrombin and/or $CaCl_2$. Lane 1: NAM; lane 2: NAM added with 0.5% CPP; lane 3: NAM added with 10 mM $CaCl_2$; lane 4: NAM added with 0.5% CPP and 10 mM $CaCl_2$: lane 5-7: NAM added with 0.5% CPP, 10 mM $CaCl_2$ and 100, 150, 200 units of thrombin/g CPP, respectively. MHC: myosin heavy chain, AC: actin.

and thrombin addition. Decrease in MHC was apparently observed in NAM added with $CaCl_2$ (lane 3). Slight decrease in MHC was obtained in the gel with CPP addition (lane 2). This result suggested that decrease in MHC in bigeye snapper NAM might cause by endogenous TGase rather than plasma TGase. Benjakul *et al.* (2004a) reported that addition of sarcoplasmic fraction from bigeye snapper into NAM in combination with setting at 40°C resulted in the cross-linking of MHC and higher cross-linking were observed when the higher amount of sarcoplasmic fraction and extended setting time were used. For NAM added with CPP, the presence of thrombin in the reaction mixture could induce cross-linking of MHC. Thrombin might hydrolyze and expose some reactive group of amino acid, which acts as an acyl acceptor or acyl donor in the formation of ε-(γ-glutamyl)lysyl cross-linking. Furthermore, plasma activated TGase possibly showed a synergetic effect with endogenous TGase on cross-linking of bigeye snapper NAM.

As indicated in Figure 18, the MHC in bigeye snapper NAM slightly decreased after 10 min of incubation with CPP, $CaCl_2$ and thrombin at 40°C (lane 3) and the decrease was more evident when the incubation time was extended to 2 h (lane 7). No distinct change in the density of actin was obtained in all samples. Myosin contains both acyl acceptor (γ-glutamine) and acyl donor (ε-lysine side chain) and is considered to be a good substrate for TGase (Gorman and Folk, 1980). Kim *et al*. (1993) reacted guinea pig liver TGase with beef actomyosin at 35°C for 10-120 min and found that myosin monomer concentration gradually decreased with simultaneous appearance of myosin polymers as the reaction time increased. No degradation occurred in bigeye snapper NAM with CPP addition during incubating process (data not shown). The cross-linking of MHC might have a steric hindrance to prevent proteolysis.

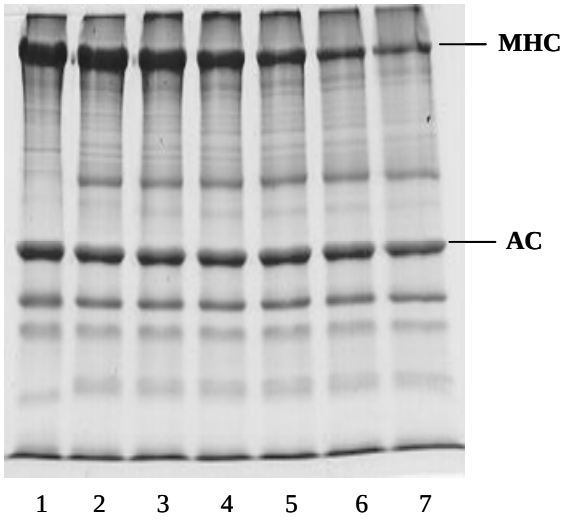

Figure 18. SDS-PAGE patterns of natural actomyosin (NAM) paste added with chicken plasma protein in combination with thrombin and/or $CaCl_2$ as affected by incubation time at 40°C. Lane 1: NAM; Lane 2-7 NAM added with 0.5% CPP, 10 mM $CaCl_2$ and 200 units of thrombin/g CPP incubated at 40°C for 0, 10, 30, 60, 90 and 120 min, respectively. MHC: myosin heavy chain, AC: actin.

Effect of TGase inhibitor on textural properties and electrophoretic patterns of surimi proteins.

The effects of TGase inhibitors on textural properties were investigated. The lowest breaking force and deformation were observed in kamaboko gel with and without CPP when 20 mM EGTA was added (Figure 19). Breaking force of kamaboko gels decreased by 45 and 60 % with the addition of 250 mM NH_4Cl and 20 mM EGTA, respectively, when compared with the control kamaboko gel.

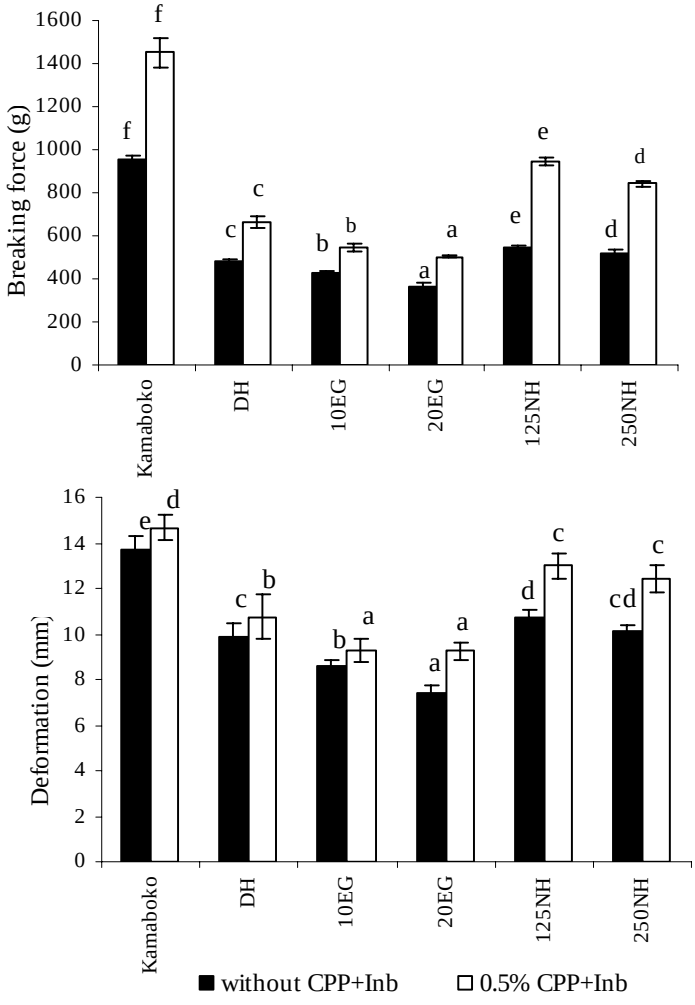

Figure 19. Breaking force and deformation of surimi gels added with/without chicken plasma protein in the presence of TGase inhibitor. DH: directly heated gel, EG: EGTA, NH: NH_4Cl, Inb: inhibitor. Bars represent the standard deviation from five determinations. Different letters indicate significant differences ($P<0.05$).

Both EGTA and NH_4Cl showed an inhibitory effect on gel formation in a concentration dependent manner. During acyl transfer reaction between γ- carboxyamide groups of glutamine residues and primary amine, ammonia is generated and an excess amount of ammonium ion should prevent further progress of the reaction (Ashie and Lanier, 2000). EGTA inhibits endogenous TGase activity through chelating Ca^{2+}, a divalent cation required for the activation of tissue TGase (Folk, 1980). These results suggested that endogenous TGase played an essential role in setting of surimi gel with and without CPP addition.

The higher breaking force and deformation were obtained in all samples when 0.5% CPP was added when compared with the sample without CPP addition (Figure 19). Without TGase inhibitors, kamaboko gel added with 0.5% CPP showed 51.7% and 7% higher in breaking force and deformation than that without CPP addition. In the presence of both CPP and endogenous TGase inhibitor, especially 20 mM EGTA, breaking force and deformation still increased. These results revealed that CPP showed an enhancing effect on kamaboko gel from bigeye snapper, which presumably acted as filler or binder in surimi gel network. This result confirmed our previous study (Rawdkuen *et al.*, 2004a).

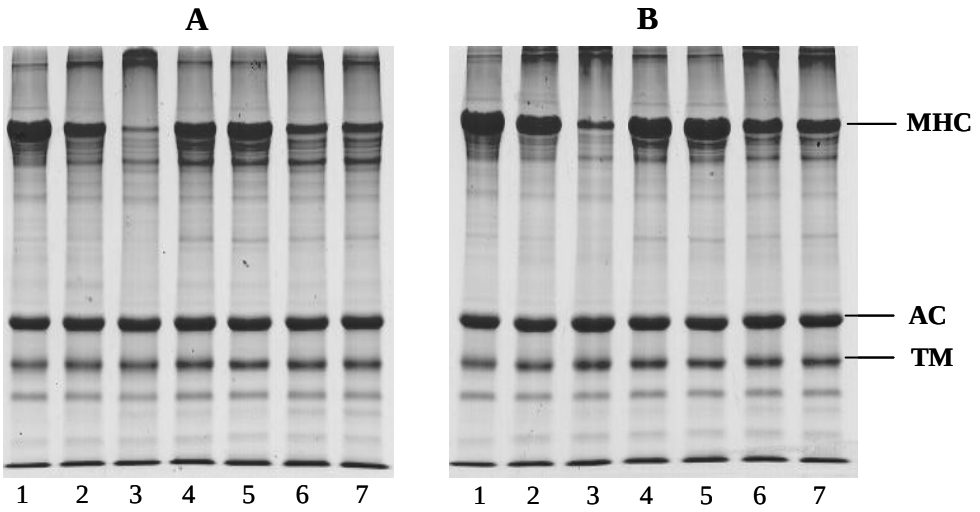

Figure 20. SDS-PAGE patterns of surimi gels without chicken plasma protein (A) and with chicken plasma protein (B) in the absence and presence TGase inhibitors. Lane 1: surimi; lane 2: directly heated gel; lane 3: kamaboko gel; lane 4-5: kamaboko gel added with 10 and 20 mM EGTA, respectively; lane 6-7: kamaboko gel added with 125 and 250 mM NH_4Cl, respectively. MHC: myosin heavy chain, AC: actin, TM: tropomyosin.

Regardless of CPP addition, MHC band intensity of samples mixed with EGTA and NH_4Cl was greater than that of the control (without TGase inhibitor addition) (Figure 20). From the result, intensity of MHC in kamaboko gel was more retained when CPP was added (Figure 20B), compared with that of gel without CPP addition (Figure 20A). This result confirmed that CPP showed an interfering effect on MHC cross-linking. Negligible TCA-soluble peptides content also revealed that proteolysis was inactivated in the sample with 0.5% CPP addition (data not shown). The results suggested that TGase involved in cross-linking of protein during setting of surimi sol either with or without CPP addition.

Effect of CPP in combination with setting on myosin cross-linking

Myosin from bigeye snapper muscle was incubated with CPP, $CaCl_2$, CPP/$CaCl_2$ or CPP/$CaCl_2$/thrombin at 40°C for 30 min (Figure 21). With the addition of 3% CPP, no myosin cross-linking was observed for all treatments. The results indicated that CPP could not induce myosin cross-linking, even in the presence of both $CaCl_2$ and thrombin, the factor XIII activators (Figure 8).

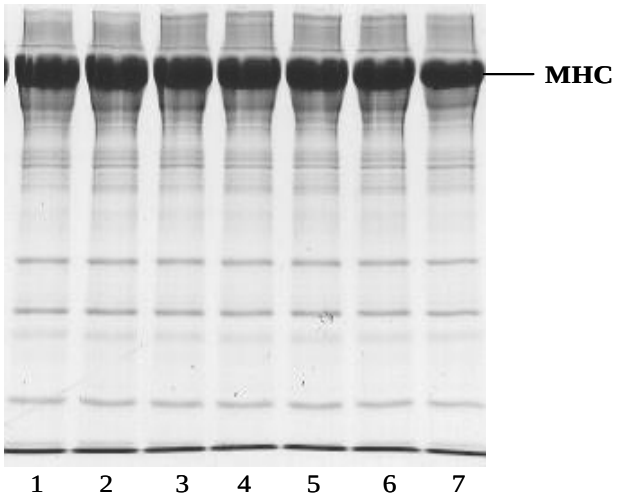

Figure 21. SDS-PAGE pattern of myosin added with chicken plasma protein in combination with thrombin and/or $CaCl_2$. Lane 1: myosin; lane 2: myosin added with 3% CPP; lane 3: myosin added with 10 mM $CaCl_2$; lane 4: myosin added with 3% CPP and 10 mM $CaCl_2$: lane 5-7: myosin added with 3% CPP, 10 mM $CaCl_2$ and 100, 150, 200 units of thrombin/g CPP, respectively. MHC: myosin heavy chain.

In the presence of CPP, $CaCl_2$ and thrombin, no visible change in MHC intensity of myosin incubated at 40°C was observed up to 120 min (Figure 22). These results indicated that CPP had no cross-linking activity under the condition tested. It has been known that activation of the plasma zymogens requires the presence of both thrombin and calcium ions (Lorand and Konishi, 1964). From the result, the lack of cross-linking activity of CPP might result from the insufficient TGase activators to convert zymogens into active enzyme. Furthermore, CPP TGase might require additional activating factors, which are different from mammal plasma TGase. Credo *et al.* (1978) reported that plasma Factor XIII is activated at non-physiologically high concentration (>100 mM) of $CaCl_2$. For human placenta Factor XIII, the maximum activity was reached at about 150 mM $CaCl_2$ (De Backer-Royer *et al.*, 1992). Cooke and Holbrook (1974) also reported that low thrombin concentration was not enough to completely convert the zymogens into Factor XIIIa.

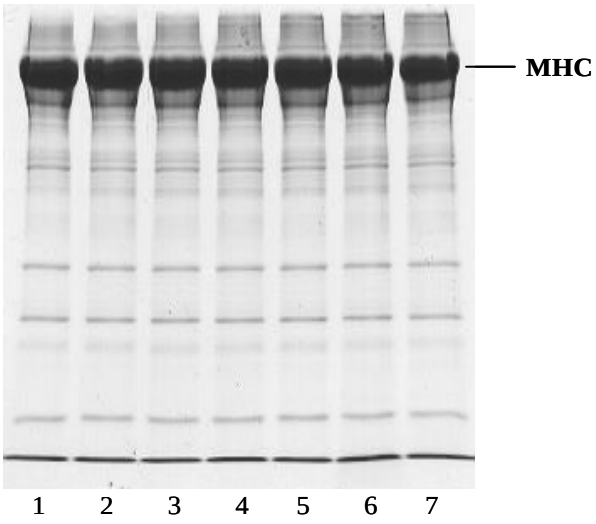

Figure 22. SDS-PAGE patterns of myosin added with chicken plasma protein in combination with thrombin and/or $CaCl_2$ as affected by incubation time at 40°C. Lane 1: myosin; Lane 2-7 myosin added with 3% CPP, 10 mM $CaCl_2$ and 200 units of thrombin/g CPP incubated at 40°C for 0, 10, 30, 60, 90 and 120 min, respectively. MHC: myosin heavy chain.

From the result, thrombin addition showed the strengthening effect on the surimi gel added with CPP and $CaCl_2$ as evidenced by the increased breaking force (Figure 14) and lowered solubility (Table 11). Nevertheless, no cross-linking of myosin was observed with the addition of thrombin in combination with CPP and $CaCl_2$ in pure myosin system. Thus, it was suggested that thrombin might partially hydrolyze the muscle proteins, leading to the conformational changes in fashion which endogenous TGase induced cross-linking effectively. As a result, the increased gel strength was found in the surimi, but not in pure myosin.

4.5 Conclusion

Addition of CPP in combination with $CaCl_2$ and setting effectively induced the cross-linking of surimi proteins as indicated by the increase in breaking force and deformation and the formation of ordered microstructure. CPP had no cross-linking activity and endogenous TGase played an important role in gel strengthening of surimi added with CPP.