CHAPTER 9

PARTIAL PURIFICATION AND CHARACTERIZATION OF CYSTEINE PROTEINASE INHIBITOR FROM CHICKEN PLASMA

9.1 Abstract

A high-molecular-weight cysteine proteinase inhibitor (CPI) was purified from chicken plasma using polyethylene glycol (PEG) fractionation and affinity chromatography on carboxymethyl-papain-Sepharose-4B. The CPI was purified to 96.8 folds with a yield of 28.9%. Based on inhibitory activity staining for papain, CPI was shown to have an apparent molecular weight of 122 kDa. No inhibitory activity was obtained under reducing condition, indicating that CPI from chicken plasma was stabilized by disulfide bond. CPI was stable in the temperature ranges of 40-70°C for 10 min; however the inhibitory activity towards papain was lost more than 50% within 30 min of heating at 90°C. CPI was stable in the presence of salt up to 3%. The purified CPI exhibited the inhibitory activity toward autolysis of arrowtooth flounder and Pacific whiting natural actomyosin (NAM) in a concentration-dependent manner.

9.2 Introduction

Cysteine proteinase inhibitors (CPI) are widely distributed in many tissues and fluids of different living organisms (Turk and Bode, 1991). The CPI was divided into four major groups (based on their occurrence, sequence, and structure similarity) including stefins, cystatins, kininogens, and phytocystatins (Barrett *et al*., 1986; Turk *et al*., 2002). These inhibitors might protect the cells from inappropriate endogenous or external proteolysis and/or could be involved in the control mechanism responsible for intracellular or extracellular protein breakdown (Turk and Bode, 1991). Among four types of CPI, kininogen is widely investigated in mammalian plasma. The two different types of kininogens are classified based on the molecular mass; high-molecular-weight kininogen (HK) and low-molecular-weight kininogen (LK). HK has been purified from bovine (Komiya *et al*., 1974), canine (Mashiko and Takahashi, 1997), guinea-pig (Yamamoto,

1987), human (Nakayasu and Nagasawa, 1979), horse (Sugo *et al*., 1979), porcine (Mashiko *et al*., 1998) and rat plasma (Hayashi *et al*., 1985). Kininogens differ markedly from species to species in their molecular size, structure and mode of interaction with cysteine proteinases (Yoshida *et al*., 1989).

Endogenous cysteine proteinases in muscle of various fish species, e.g., Pacific whiting and arrowtooth flounder have been shown to cause severe and rapid textural degradation during thermal processing of surimi-based products (Wasson *et al*., 1992a; An *et al*., 1994b; Visessanguan and An, 2000). In order to utilize these soft-texture fish species, several food-grade proteinase inhibitors have been used. Plasma protein, a by-product from slaughtering process, has been used for an alternative source of inhibitors to solve these problems. So far, plasma proteins have received more attention for their potential role in protecting fish surimi proteins from proteolytic activities (Hamann *et al*., 1990). Benjakul and Visessanguan (2000) found that the protein with an apparent molecular weight of 60-63 kDa in porcine plasma protein (PPP) was the inhibitory component, which could inhibit trypsin and papain. Lee *et al*. (2000a) reported that a low-molecular-weight kininogen from pig plasma with a molecular weight of 55 kDa inhibited calpains, cathepsins B, L, L-like and papain.

Although proteinase inhibitors in various plasma have been intensively studied, a little information has been reported for those from avian plasma. Recently, Rawdkuen *et al*. (2004a) reported that chicken plasma proteins (CPP) and its fraction exhibited proteinase inhibitory activity and gel strengthening effect in heat-induced surimi gel. Therefore, purification and characterization should be achieved for better understanding of chicken plasma proteinase inhibitor. The objectives of this study were to purify and characterize cysteine proteinase inhibitor from chicken plasma and to investigate its inhibitory effect on autolysis of myofibrillar proteins from Pacific whiting and arrowtooth flounder.

9.3 Materials and Methods

Chemicals

Casein (sodium salt), N_{α}- benzoyl-*DL*-arginine-β-naphthylamide hydrochloride (BANA), ρ-dimethylamino-cinnam-aldehyde, *L*-cysteine, Triton X-100,

papain (from papaya latex), high and low-molecular-weight protein standards were purchased from Sigma Chemical Co. (St Louis, Mo, U.S.A). Polyethylene glycol (PEG-4000) was obtained from Fluka Chemika-Biochemika (Buchs, Switzerland). CNBr-activated Sepharose 4B was procured from Amersham Biosciences (Uppsala, Sweden). 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).

Preparation of chicken plasma

Chicken blood was obtained from a slaughterhouse in Hat Yai, Thailand. During collection, one-tenth volume of 3.8% (w/v) trisodium citrate 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 blood plasma was then frozen and kept at -18°C until used.

Preparation of carboxymethyl-papain-Sepharose

Carboxymethyl(CM)-papain-Sepharose was prepared according to the method of Anastasi *et al*. (1983) with a slight modification. Papain (60 mg) was incubated for 10 min at 25°C in 10 ml of 0.1 M phosphate buffer, pH 6.0 containing 1 mM EDTA and 2 mM cysteine prior to adding with iodoacetic acid at a final concentration of 10 mM. The mixture was incubated at 25°C for 10 min. The solution was dialyzed against 0.1 M $NaHCO_3$, pH 8.3 containing 0.5 M NaCl. The obtained *S*-carboxymethylated papain was coupled with 4 g (dry mass) of CNBr-activated Sepharose 4B according to the instructions of Amersham Biosciences.

Purification of cysteine proteinase inhibitor

Purification of cysteine proteinase inhibitor from chicken plasma was carried out in two steps: PEG fractionation and papain-affinity chromatography. Chicken plasma was subjected to fractionation with PEG at the level of 200-400g PEG/L as described by Rawdkuen *et al*. (2005c). The pellet obtained was re-dissolved in 20 mM phosphate buffer, pH 7.0 containing 100 mM NaCl (buffer I) at the ratio of 1:9 and then

dialyzed against the same buffer (buffer I) overnight at 4°C. The dialyzed sample was chromatographed on CM-papain-Sepharose-4B column (0.7x10 cm), which was equilibrated with buffer I, with a flow rate of 1 ml/min. The column was then washed with 2 column volume (CV) of buffer I to remove unabsorbed proteins and the nonspecifically bound proteins were removed by washing the column with 1 CV of the buffer I containing 1 M NaCl. The salt was removed with 1 CV of the buffer I before eluting the specifically bound proteins with 20 mM phosphate buffer pH 11.5, containing 0.1 M NaCl at the flow rate of 1 ml/min. The fractions of 2 ml were collected and A_{280} was monitored. Each fraction was adjusted to pH 7.0 with 1 M sodium phosphate buffer, pH 4.0 immediately and assayed for inhibitory activity. Fraction expressing inhibitory activity were pooled and used for further study.

Inhibitory activity assay

Proteinase inhibitory activity was determined with papain according to the method of Benjakul *et al.* (2001c) using BANA as a substrate. To 0.2 ml of 0.25 M sodium phosphate buffer (pH 6.0) containing 2.5 mM EDTA and 25 mM β-mercaptoethanol (βME), 0.1 ml of papain solution (50 μg/ml) in 25 mM sodium phosphate buffer (pH 7.0) and 0.2 ml of fractions were added. The mixture was pre-incubated at 37°C for 5 min. To initiate reaction, 0.2 ml of 1.0 mM BANA was added. After 10 min, the reaction was terminated by adding 1.0 ml of 2% HCl/ethanol. The color was developed by the addition of 1.0 ml of 0.06% *p*-dimethyl aminocinnamaldehyde/ethanol. The absorbance was measured at 540 nm. The inhibitory activity unit was defined as a decrease of 0.01 absorbance unit at 540 nm/ml/min under the assay condition.

Protein determination

Protein concentration of crude chicken plasma and its PEG fractions was measured by the Biuret method (Robinson and Hodgen, 1940). The Lowry method (Lowry *et al.*, 1951) was used to determine the protein content in papain-affinity fractions. Bovine serum albumin (BSA) was used as a standard protein.

SDS-substrate gel and staining for inhibitory components

Inhibitory activity staining was conducted using 10% SDS-substrate gels according to the method of Garcia-Carreno *et al.* (1993) with a slight modification. Chicken plasma and its fractions were mixed with the sample buffer in the presence or absence of 10% βME at a ratio of 1:1 (v/v). The samples were applied onto the gel without prior boiling. The proteins were separated on Mini-Protean II unit (Bio-Rad Laboratories, Hercules, CA) at a constant current of 30 mA for 90 min on ice.

Two identical gels were subjected to different stainings. One gel was fixed and stained for total proteins with Coomassie Brilliant Blue R-250. This gel was used as the control gel. Another gel was washed in 2.5% Triton X-100 for 15 min to remove SDS and to renature the proteins and then washed in distilled water. The gel was flooded with 50 ml of a mixture of 0.4 mg/ml papain in 0.1 M phosphate buffer, pH 6.0, containing 1 mM EDTA and 2 mM cysteine. The gel was incubated for 60 min at 4°C to allow papain to diffuse into the gel, and then washed with distilled water. The gel was incubated for 90 min at 37°C in 1% (w/v) casein in 0.1 M phosphate buffer, pH 6.0, and then rinsed with distilled water, fixed, and stained with Coomassie Brilliant Blue R-250 to develop inhibitory zones detected as dark band on a clear background. The apparent molecular weight of the proteinase inhibitors in the samples was estimated from the control gel by comparing the R_f with those of protein standards.

Thermal stability of purified CPI

The purified CPI (200 μl) was subjected to heating at different temperatures (40, 50, 60, 70, 80 and 90°C) for 10 min. The solution was immediately cooled in iced water, and then the inhibitory activity was determined. The residual inhibitory activity of heat-treated samples was expressed as the relative activity, compared with that of untreated sample.

The purified CPI (200 μl) was also incubated at 90°C for various times (10, 20, 30, 40, 50 and 60 min). The heat-treated samples were immediately cooled in iced water and tested for the remaining inhibitory activity.

Salt stability of purified CPI

The purified CPI (200 μl) was incubated at room temperature for 20 min in the presence of NaCl ranging from 0 to 3%. The mixture was determined for the inhibitory activity against papain. The residual inhibitory activity of CPI was reported as the relative activity, compared with that of untreated sample.

Preparation of natural actomyosin (NAM)

NAM from Pacific whiting and arrowtooth flounder muscles were prepared according to the method of Benjakul *et al*. (2001d). Fish muscle was chopped and then homogenized in chilled 0.6 M KCl, pH 7.0 at a ratio of 1:10 (w/v) using a Sorvall OMNI-MIXER (Ivan Sorvall Inc, Norwalk, CONN, USA) at a speed of 5. To avoid over-heating, the sample was placed in ice and homogenized for 20 sec, followed by a 20 sec rest interval for a total time of 4 min. The extract was centrifuged at 5,000*g* for 30 min at 4°C. Three volumes of chilled de-ionized water were added to precipitate NAM. The NAM was collected by centrifuging at 5,000*g* for 20 min at 4°C. The pellet was dissolved in 50 mM phosphate buffer, pH 7.0 containing 0.6 M NaCl to obtain the final protein concentration of 15 mg/ml. The NAM suspension was used immediately.

Inhibition of NAM autolysis by purified CPI

NAM suspension was incubated in a water bath at 55°C for Pacific whiting and 60°C for arrowtooth flounder for different times (0, 10, 20, 30, 60, 90 and 120 min). At time designated, the samples were mixed with the sample buffer at the ratio of 1:1 and then boiled for 3 min. The samples were subjected to SDS-PAGE according to the method of Laemmli (1970). Autolysis of NAM from both species in the presence of purified CPI at the level of 150 units/ml NAM was also monitored during incubation up to 120 min at optimal temperature.

To study the effect of purified CPI at different amounts on autolysis of NAM, CPI at levels of 0, 25, 50, 150, 250 and 400 units/ml NAM were added to NAM

suspension. The mixture was mixed thoroughly and then incubated in a water bath at 55°C for 60 min and 60°C for 30 min for NAM from Pacific whiting and arrowtooth flounder, respectively. Autolytic pattern of NAM was determined using SDS-PAGE according to the method of Laemmli (1970).

9.4 Results and Discussion

Purification of CPI from chicken plasma

CPI was purified from chicken plasma by two steps, PEG fractionation and affinity chromatography on CM-papain Sepharose 4B. The purification of CPI is summarized in Table 18. After PEG fractionation, total inhibitory activity of approximately 45% was remained, while 88.2% of protein was removed. PEG fractionation was used to remove other proteins and concentrated CPI from chicken plasma, leading to the higher purity of CPI (Rawdkuen *et al.*, 2005c). From the result, purity of 3.85-fold was obtained with PEG fractionation.

Table18. Purification of cysteine proteinase inhibitor from chicken plasma protein.

Steps	Volume (ml)	Total protein (mg)	Total inhibitory activity (units)	Specific inhibitory activity (unit/mg)	Yield (%)	Purity (fold)
CPP*	30	1006.5	52,200	51.86	100.00	1.00
PEG**	10	119.1	2,376	199.50	45.52	3.85
AF***	16	3.0	15,096	5,018.62	28.92	96.77

*Chicken plasma protein. **PEG fractionation was carried out by addition of 200-400 g/L. The precipitate was collected. ***CM-papain-Sepharose 4B.

PEG fraction was further purified using affinity chromatography on CM-papain Sepharose 4B. After washing and eluting with 20 mM phosphate buffer, pH 11.5, containing 0.1 M NaCl, a single protein peak (A_{280}) and a inhibitory peak were found at the same elution time (Figure 52). Fractions with inhibitory activity were pooled. Specific

inhibitory activity increased to 5,018.6 units/mg protein. Purification folds of 97 with a yield of 29% were obtained. Lee *et al.* (2000a) obtained an increase in purity of low-molecular-weight kininogen from pig plasma by 31,500 folds with three-step chromatography. High-molecular-weight kininogen was purified from porcine plasma to 867 folds with 43% recovery (Mashiko *et al.*, 1998). The procedure used could be used as a simple purification process for CPI from chicken plasma.

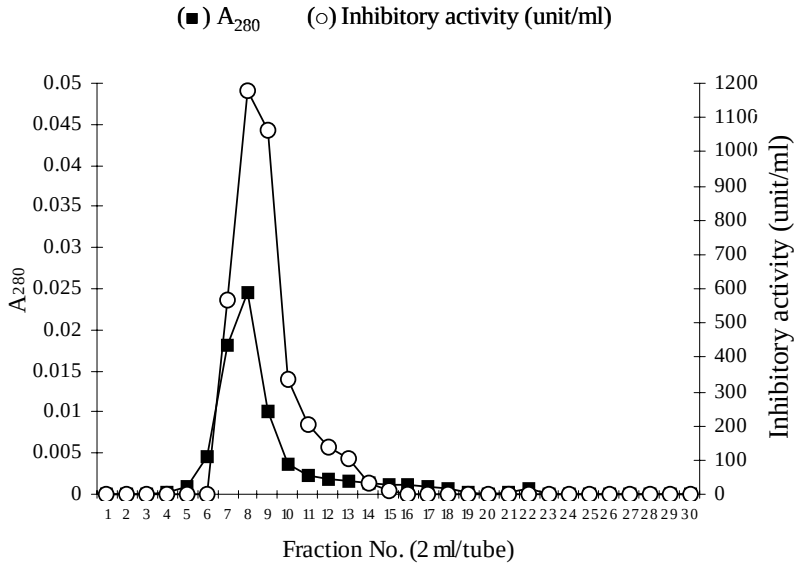

Figure 52. Elution profiles of cysteine proteinase inhibitor from chicken plasma on CM-papain-Sepharose-4B. PEG fraction was applied onto the column with a rate of 1 ml/min. After washing, elution was carried out with 20 mM phosphate buffer pH 11.5 containing 0.1 M NaCl at the flow rate of 1 ml/min.

Protein pattern and inhibitory activity staining of purified CPI

Based on the protein patterns under non-reducing condition (Figure 53A), PEG fraction contained several protein bands. The dominant protein band in this fraction had the molecular weight of 50 kDa. After being chromatographed with CM-papain-Sepharose-4B, most of proteins in PEG fraction were removed. However, the pooled CM-papain-Sepharose-4B fractions showed two protein bands on SDS-PAGE under non-reducing condition; one was major band with the molecular weight of 122 kDa, and the other was a very tiny band with the molecular weight of 116 kDa.

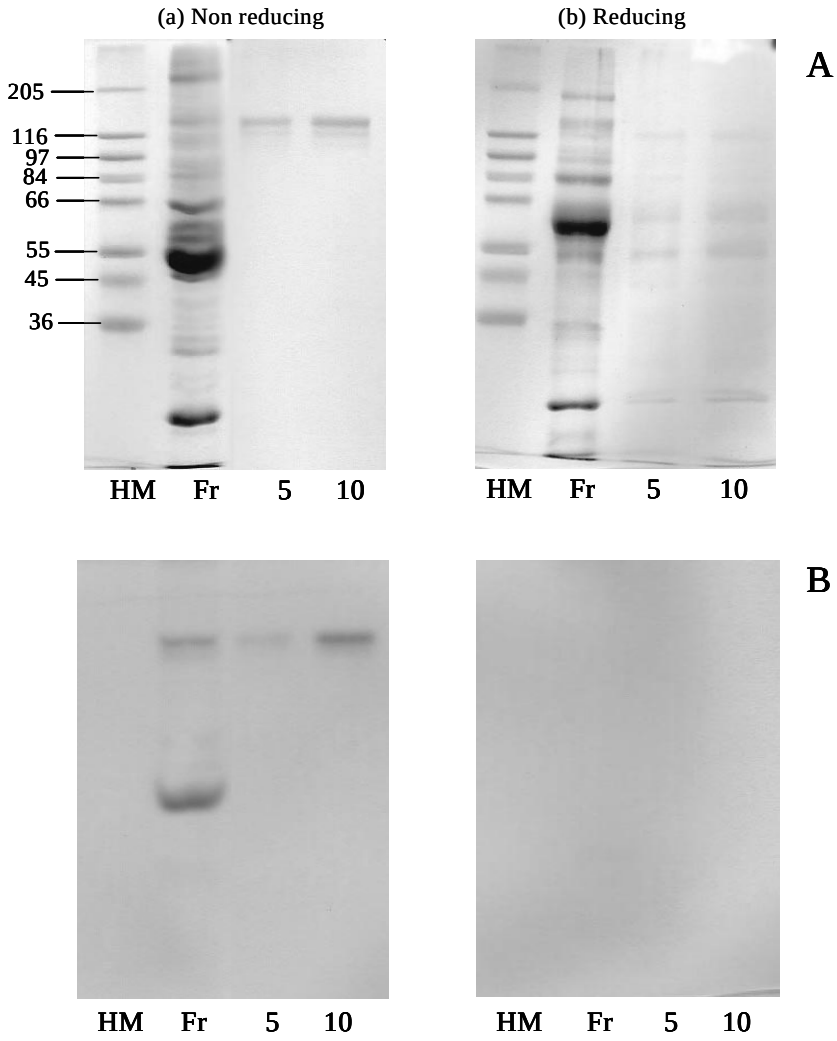

Figure 53. Protein pattern (A) and inhibitory activity staining for papain (B) of purified CPI from chicken plasma under non-reducing and reducing condition. HM: high-molecular-weight markers, Fr: 200-400g PEG/L fraction. Numbers designate the amount of protein loaded into the gel (μg).

Under reducing condition, the protein band with molecular weight of 122 kDa disappeared with the concomitant occurrence of proteins with molecular weight of 62, 55 and 23 kDa. This result suggested that the main component in pooled CM-papain-Sepharose-4B fractions was most likely stabilized by disulfide bond. Yamamoto (1987) reported that the molecular weight of guinea pig HK was 100 kDa. HK purified from dog plasma using zinc chelating Sepharose 6B and ion-chromatographies showed the molecular weight of 125 kDa and inhibited papain and ficin but did not inhibit bromelain (Mashiko

and Takahashi, 1997). Under non-reducing condition, purified HK from porcine plasma had the molecular weight of 116 kDa (Mashiko *et al*. 1998). Saito and Kitano (2000) reported that the papain inhibitory capacity of plasma protein is considerably different from species to species.

Inhibitory activity staining (Figure 53B) revealed that the protein with molecular weight of 122 kDa, the predominant protein in the pooled CM-papain-Sepharose-4B fractions, showed the inhibitory activity against papain. It can be concluded that this protein band was cysteine proteinase inhibitor. For the PEG fraction, the two major inhibitor bands with molecular weights of 122 kDa and 46 kDa were found, while only the protein band with molecular weight of 122 kDa from CM-papain-Sepharose-4B column showed inhibitory activity against papain. The result suggested that the 46 kDa protein might not be CPI since it could not bind to papain affinity column. This protein might be resistant to hydrolysis by papain under the assay condition. Under reducing condition, the inhibitory activity band with molecular weight of 122 kDa was not observed. This result suggested that CPI from chicken plasma was possibly stabilized by disulfide bond. Additionally, the resistant protein (46 kDa) was not found under reducing condition. It was postulated that this protein might be dissociated to smaller proteins, which could be hydrolyzed by papain.

Thermal stability of purified CPI

The thermal stability of purified CPI subjected to heating for 10 min at different temperatures ranging from 40 to 90°C is shown in Figure 54A. From the result, the inhibitory activity of CPI was not affected when heated at 40-50°C. A gradual decrease in relative inhibitory activity was observed at temperature above 50°C. When the CPI was heated at 70°C, the relative inhibitory activity of 90% was obtained. However, the inhibitory activity was decreased by 40% when the CPI was heated at 90°C for 10 min. At high temperature, proteinase inhibitor possibly underwent denaturation and lost their inhibitory activity. Lee *et al*. (2000a) also reported that L-kininogen from pig plasma had very high thermal stability and there was 90% of the inhibitory activity left after an incubation at 80°C for 30 min.

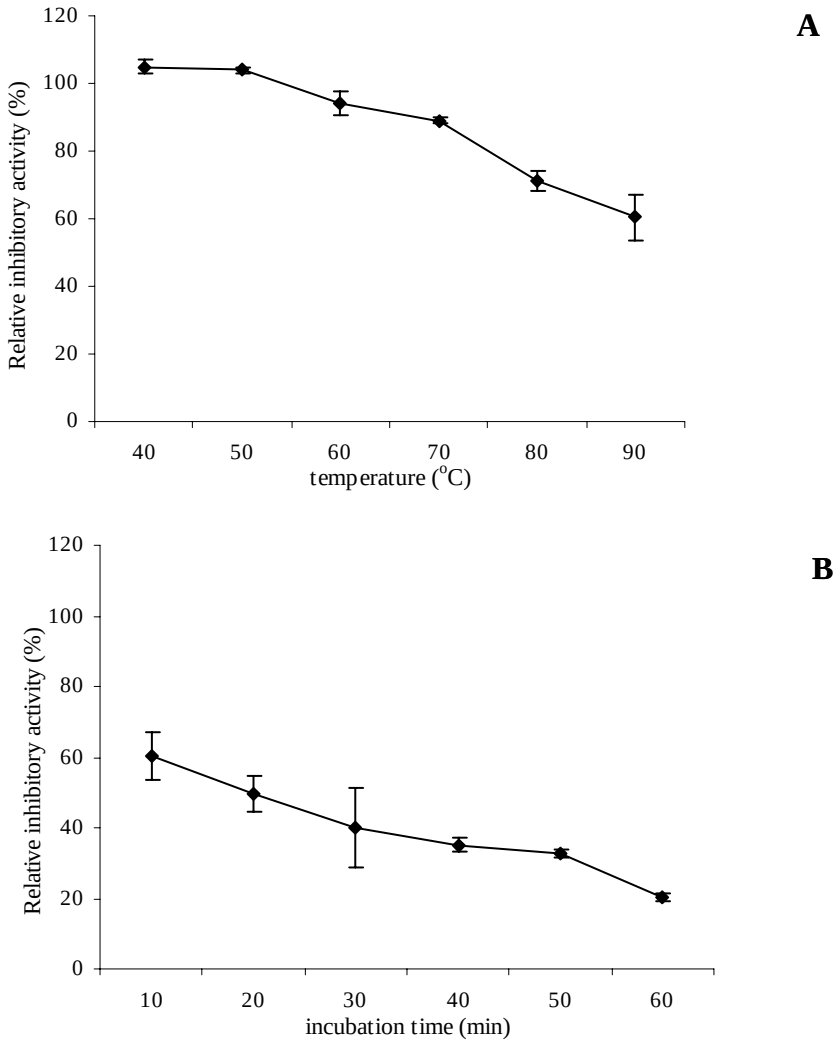

Figure 54. Effect of heating temperature (A) and time (B) on stability of purified CPI from chicken plasma. CPI was heated at different temperatures for 10 min (A) or heating at 90°C for various times (B). Residual inhibitory activity was determined using BANA as substrate.

Brzin *et al.* (1984) also reported that no significant change in the inhibitory activity of human cystatin C was observed after 10 min incubation at 80°C. When the purified CPI was heated at 90°C for various times (10-60 min), the relative inhibitory activity markedly decreased as the incubation time increased (Figure 54B). Relative inhibitory activity of less than 50% was found with the CPI heated at 90°C for 20 min and continuously decreased up to 60 min of incubation. From the result, it revealed that purified CPI might undergo thermal denaturation when heated for a longer time. Turk

and Bode (1991) reported that kininogens are thermally stable (at 90°C) for short periods of time. In general, thermal stability of the proteinase inhibitor mostly depended on its amino acid composition and conformation (Richard *et al*., 1999). After heating, a tightly coiled conformation with multiple disulfide bonds and/or a small and highly flexible structure exhibited the efficient refolding by cooling. These structural changes are considered to be responsible for the resistance to heat denaturation (Richard *et al*., 1999). The thermal degradation of Pacific whiting and arrowtooth flounder surimi gels was considered to be caused by endogenous cathepsin L at 55-60°C (An *et al*., 1994b; Visessanguan *et al*., 2003). In addition, heating at 90°C is commonly used in surimi gel cooking for 15-20 min. Thus, CPI from chicken plasma is thermal stable and could be applied in various thermal processes.

Salt stability of purified CPI

The effect of NaCl on inhibitory activity of purified CPI is shown in Figure 55. No marked changes in relative inhibitory activity were observed when NaCl was added up to 2.5% ($P>0.05$). However, a slight decrease in relative inhibitory activity was observed in the presence of NaCl at a level of 3%. The result suggested that NaCl at the high concentrations might either inhibit CPI activity. Salt has a number of effects on properties of protein including activity, conformational stability and solubility. These effects possibly arise from the binding of ions to specific sites on the protein that is able to screen charges on surface amino acid side chains and change the degree of hydration of the protein (Timasheff and Arakawa, 1997). Record *et al*. (1998) concluded that salt can destabilize proteins by at least three different means: a) by altering the interaction of water with the protein, i.e. preferential hydration or binding, b) by screening electrostatic repulsion between like-charged amino acid residues on the surface of a protein; and c) by specific binding to ion binding site in a protein. From this result, purified CPI showed high salt stability up to 3%. Thus, CPI from chicken plasma can be used for the surimi gel containing 2-3% NaCl without the severe loss in the inhibitory activity.

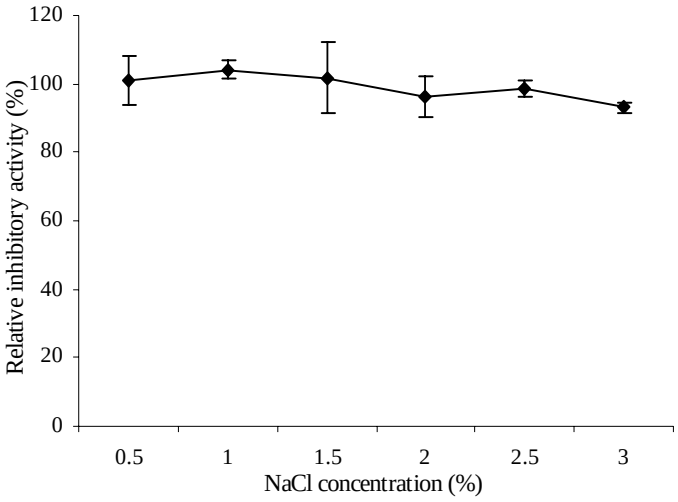

Figure 55. Effect of salt content on stability of purified CPI from chicken plasma. Residual inhibitory activity against papain was determined using BANA as substrate.

Autolysis of arrowtooth flounder and Pacific whiting NAM

Autolysis of NAM from both arrowtooth flounder and Pacific whiting incubated at 60 and 55°C, respectively, for different times is presented in Figure 56A. MHC of arrowtooth flounder NAM was completely hydrolyzed within 5 min of incubation. Actin band intensity was markedly decreased compared with that found in NAM without incubation and gradually decreased when the incubation time increased. High activity of cathepsin B, H and L, cysteine proteinases, have been reported in Pacific whiting and arrowtooth flounder muscles (An *et al.*, 1994b; Visessanguan *et al.*, 2003). These enzymes can degrade the myofibrillar proteins effectively, resulting in the decreased gel strength of surimi. Wasson *et al.* (1992a) reported that washing arrowtooth flounder minced fresh partially removed the enzyme and even low levels of residual proteinase caused the rapid degradation of MHC. Proteinase in arrowtooth flounder is closely associated with myosin and is resistant to removed by washing (Wasson *et al.*, 1992a). Apart from MHC and actin, tropomyosin and troponin-T were more degraded when the incubation time increased. After 120 min of incubation, troponin-T completely disappeared. Nevertheless, actin and tropomyosin still retained to a small extent after 120 min of incubation.

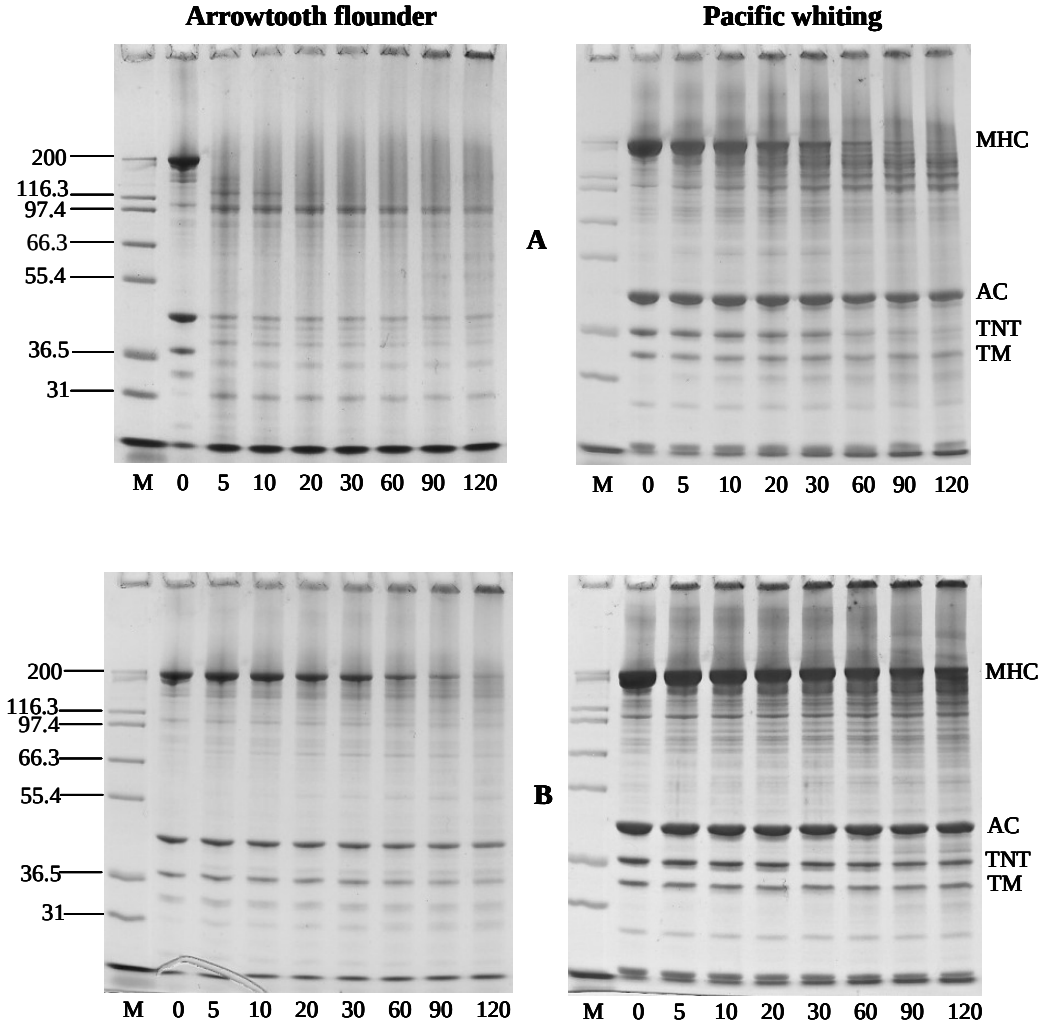

Figure 56. SDS-PAGE of natural actomyosin without (A) and with 150 units/ml NAM (B) incubated at 60 and 55°C for arrowtooth flounder and Pacific whiting, respectively. M: protein marker, MHC: myosin heavy chain, AC: actin, TNT: troponin-T, TM: tropomyosin. Numbers designate incubation time (min).

In the presence of purified CPI at the level of 150 units/ml NAM, autolysis of arrowtooth flounder and Pacific whiting NAM incubated at 60 and 55°C was markedly inhibited as evidenced by the more remaining proteins (Figure 56B). Generally, the MHC band intensity gradually decreased with increasing incubation time up to 120 min for both fish species. However, the MHC was still retained after 120 min in the presence of CPI. Greater band intensity of MHC was noticeable in NAM from Pacific whiting. This

result suggested that CPI at the level used could retard the proteolysis in both arrowtooth flounder and Pacific whiting NAM. However, with the excessive proteolytic activity in both NAM, especially from arrowtooth flounder, CPI with the amount used did not completely inhibit all proteinases. From the result, no changes in actin, troponin and tropomyosin were observed in Pacific whiting NAM added with CPI throughout 120 min of incubation. Nevertheless, slight decrease in those proteins was observed with increasing incubation time. The result suggested that arrowtooth flounder NAM contained a greater amount of heat-activated proteinases associated with the proteins, compared with Pacific whiting. Those proteinases caused more intensive degradation of the former even in the presence of CPI (150 units/ml NAM).

Effect of purified CPI on inhibition of NAM autolysis

Purified CPI inhibited the autolysis of Pacific whiting and arrowtooth flounder NAM at different degrees, depending on the level of CPI used (Figure 57). Generally, the greater inhibition was observed with increasing CPI levels added. When CPI level was greater than 150 units/ml NAM, no changes in MHC band intensity of arrowtooth flounder NAM were noticeable. However, MHC band intensity increased continuously in Pacific whiting NAM as CPI levels increased up to 400 units/ml NAM. The MHC, troponin-T and tropomyosin were completely hydrolyzed in the control sample of arrowtooth flounder NAM, while a small amount of actin still remained. Visessanguan *et al.* (2003) reported that cathepsin L was a predominant heat-activated proteinase in arrowtooth flounder. For Pacific whiting NAM, the band intensity of MHC increased as the concentration of CPI increased and was almost completely recovered when the CPI at a level of 400 units/ml NAM was added. The changes in actin, troponin-T and tropomyosin were not observed. The higher inhibition was obtained in Pacific whiting NAM, when compared with arrowtooth flounder. This was possibly caused by the higher amount of proteinases remaining in NAM of arrowtooth flounder. At the same level of CPI used, more efficacy in inhibiting the autolysis was found with NAM with lower proteinase activity. Though some proteinases could be removed by washing, some of them were still associated with the muscle. Cathepsin L was identified as the predominant proteinase in surimi wash water (Benjakul *et al.*, 1996, 1998). Lee *et al.* (2000b) reported that endogenous cathepsins in mackerel surimi was inhibited by the purified L-kininogen from pig plasma

and subsequently prevented the modori of surimi. Akpinar and An (2005) reported that r-soyacystatin was more effective than other proteinase inhibitors such as BPP for surimi application due to its small molecular mass. It should facilitate the diffusion into the muscle cells in surimi, resulting in more effective prevention of autolytic activity in surimi caused by cysteine proteinase.

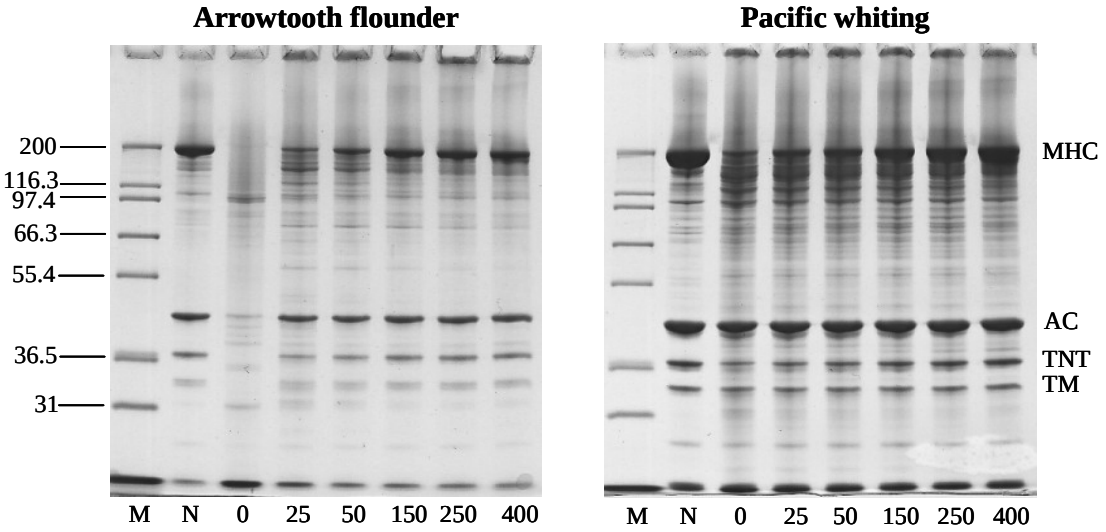

Figure 57. SDS-PAGE pattern of natural actomyosin added with purified CPI from chicken plasma at different levels. NAM was incubated at 60°C/30 min and 55°C/60 min for arrowtooth flounder and Pacific whiting, respectively. M: protein marker, N: natural actomyosin, MHC: myosin heavy chain, AC: actin, TNT: troponin-T, TM: tropomyosin. Numbers designate the CPI levels (unit/ml NAM). Protein concentration of NAM was 15 mg/ml.

9.5 Conclusion

Cysteine proteinase inhibitor from chicken plasma had a molecular weight of about 122 kDa. The CPI was quite stable to the heat treatment and was also stable at high salt concentration up to 2.5%. The purified CPI effectively inhibited the autolysis of myofibrillar proteins from both arrowtooth flounder and Pacific whiting NAM. The efficacy was in a concentration dependent manner.