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Cysteine proteinase inhibitor from chicken plasma: Fractionation, characterization and autolysis inhibition of fish myofibrillar proteins

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Abstract

Cysteine proteinase inhibitor (CPI) from chicken plasma was fractionated by using polyethylene glycol (PEG-4000) or ammonium sulfate (AS). Addition of PEG, at the level of 200–400 g/l based on the original volume of plasma protein, was more effective to fractionate CPI than was using AS. Highest inhibitory activity and purification-fold were obtained in the PEG precipitate II (CPI fraction). The CPI fraction was stable in the temperature ranges of 40–90 °C for 10 min but extended incubation time at 90 °C markedly decreased the inhibitory activity of the CPI fraction. The fraction was stable in the broad pH ranges tested (3–10). NaCl concentrations of 0.5–3% did not affect the inhibitory activity of the CPI fraction. The CPI fraction effectively prevented the degradation of mince and washed mince from Pacific whiting; however, lower efficacy in inhibiting autolysis of the arrowtooth flounder mince and the washed mince was observed, suggesting differences in initial proteolytic activity between the two species. Therefore, the CPI fraction from chicken plasma could be an alternative food grade inhibitor for the surimi industry.

Keywords: Ammonium sulfate; Autolysis; Chicken plasma; Fractionation; Polyethylene glycol; Cysteine proteinase inhibitor

1. Introduction

Thailand is the largest poultry exporter in Asia and the fourth largest of the world in the last two years (Department of Livestock Development, 2004). As a consequence, a large amount of blood is produced each year in the slaughtering process. Approximately 4.5% of the live weight of an animal is collected blood, which contains 60–70% of plasma and 30–40% of suspended red cells (Fernando, 1992). Plasma is basically composed of protein (7%), water (91%) and a variety of salts and other low-molecular-weight compounds (1%) (Moure, Rendueles, & Diaz, 2003). Food grade blood fractions with high nutritive value can be obtained by ultrafiltration and spray-drying and can be used as ingredients in human foods, mainly as sources of iron and protein (Duarte, Carvalho Simoes, & Sgarbieri, 1999).

Plasma protein fractionation was the first process for large-scale protein purification developed by Cohn et al. (1946). The so-called Cohn process is based on the differential precipitation of plasma proteins from blood with ethanol. Nevertheless, the component obtained by alcoholic fractionation has a notable capacity for use, as a result of the denaturation of the protein during the process (Ristol, Rabaneda, & Lopez, 2002). Plasma fractionation by salt precipitation, typically using mineral salts such as ammonium sulfate (AS) and sodium sulfate, is also carried out; however, it requires a high concentration or cooling to

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avoid the denaturation (Burnouf, 1995). In order to avoid disadvantages of these techniques, polyethylene glycol (PEG) is an alternative precipitating agent for plasma protein fractionation. PEG has several advantages over other precipitants, including least denaturation of proteins at ambient temperatures, negligible temperature control required in the range 4–30 °C, relatively small amount of precipitant required compared with AS or organic solvents, and low residual PEG concentration in the precipitate since most of the PEG is retained in the supernatant (Sharma & Kalonia, 2004). The use of PEG is commonly exploited in large-scale protein preparation or purification for both therapeutic and food industries use (Burnouf, 1995; Lee, Aishima, Nakai, & Sion, 1987).

The presence of endogenous proteolytic enzymes in fish mince or surimi, especially lysosomal cathepsins, results in a decrease in gel strength with a brittle and non-elastic gel at temperatures around 60 °C. Cathepsin L, from both Pacific whiting and arrowtooth flounder, contributes to autolysis of myofibrillar proteins during post mortem storage (Benjakul, Seymour, Morrissey, & An, 1997; Visessanguan, Benjakul, & An, 2003). Moreover, cathepsin L was reported to be important in the thermal degradation of surimi gel from both fish species (An, Weerasinghe, Seymour, & Morrissey, 1994; Visessanguan & An, 2000). To alleviate these problems, inhibitors and other additives such as beef plasma protein (BPP), egg white and whey protein, have been used in Pacific whiting and arrowtooth flounder surimi to improve the gel forming ability (Morrissey, Wu, Lin, & An, 1993; Wasson, Reppond, Babbitt, & French, 1992; Weerasinghe, Morrissey, Chung, & An, 1996). Plasma proteins have been reported to exhibit proteinase inhibitory activity and gel strengthening ability in the heat-induced surimi gel (Benjakul & Visessanguan, 2000; Benjakul, Visessanguan, & Srivilai, 2001a, Benjakul, Visessanguan, & Srivilai, 2001b; Kang & Lanier, 1999; Lee, Tzeng, & Jiang, 2000a, Lee, Tzeng, Wu, & Jiang, 2000b). Proteinase inhibitors normally found in plasma include α_2-macroglobulin, α_1-antitrypsin, α_1-antichymotrypsin and inter α-trypsin inhibitor (Kent & Drohan, 2001). Due to the abundance of chicken blood during the slaughtering process in Thailand, chicken plasma can be a value-added product, as well as an alternative protein additive, for surimi. Recently, chicken plasma has been reported to strengthen the surimi gel (Rawdkuen, Benjakul, Visessanguan, & Lanier, 2004a, 2004b). However, the addition of blood plasma to surimi or surimi products renders an end-product with off-colour and off-flavour (Benjakul et al., 2001a, 2001b). To maximise the use of chicken plasma, the fractionation to concentrate proteinase inhibitor should be a promising means for obtaining the active fraction with a smaller discoloration problem. The objectives of this study were to fractionate and characterize the cysteine proteinase inhibitor from chicken plasma and to investigate the efficacy of the CPI fraction in inhibiting the autolysis of Pacific whiting and arrowtooth flounder muscles.

2. Materials and methods

2.1. Chemicals

Sodium dodecyl sulfate (SDS) was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Novex pre-cast gels and other electrophoresis reagents were purchased from Invitrogen life technologies (Carlsbad, CA, USA). N_α- benzoyl-DL-arginine-β-naphthylamide (BANA), ρ-dimethylamino-cinnamaldehyde, 2-mercaptoethanol (βME), papain (from papaya latex) and high and low-molecular-weight protein standards were procured from Sigma Chemical Co. (St Louis, Mo, USA). Polyethylene glycol (PEG-4000) was obtained from Fluka Chemika-Biochemika (Buchs, Switzerland). Ammonium sulfate, sodium chloride and trisodium citrate were purchased from Merck (Darmstadt, Germany).

2.2. Fractionation of cysteine proteinase inhibitor from chicken plasma

2.2.1. Preparation of chicken plasma

Chicken blood was obtained from a slaughterhouse in Hat Yai, Thailand. During collection, a one-tenth volume of 3.8% (w/v) trisodium citrate was added to prevent coagulation. The blood was centrifuged twice at 1500*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.

2.2.2. Polyethylene glycol (PEG-4000) fractionation

Fractionation of chicken plasma with PEG-4000 was carried out according to the method of Hao, Ingham, and Wickerhauser (1980) with a slight modification. The amount of PEG added was based on the original volume of plasma. All operations were conducted in a cold room at 4 °C. Initially, solid PEG (200 g) was added to chicken plasma (1 l) with gentle stirring. After the complete addition of PEG, the mixture was allowed to stand for 2 h at 4 °C. The precipitated protein was collected by centrifugation (7000*g*, 15 min, 4 °C) and was referred to as "*PEG precipitate I*". An additional PEG (200 g) was added into the supernatant obtained with gentle stirring. The mixture was allowed to dissolve and equilibrate as mentioned previously. The protein precipitate was collected in the same manner and referred to as "*PEG precipitate II*". *PEG precipitates III and IV* were subsequently obtained by adding PEG in increments of 200 g into the supernatant. Each mixture was stirred gently which allowed PEG to dissolve. After equilibrating, the supernatant obtained after a quadruple addition of PEG was referred to as "*PEG supernatant IV*". The precipitate from each fraction was redissolved in 10 mM phosphate buffer, pH 7.4, containing 0.9 mM $CaCl_2$ and 0.05 mM $MgCl_2 \cdot 6\,H_2O$ (Buffer A) at the ratio of 1:9 (w/v). All fractions were dialysed against the Buffer A overnight at 4 °C with 4-time changes of dialysis buffer to remove the residual PEG. All fractions were stored at 4 °C until used.

2.2.3. Ammonium sulfate (AS) fractionation

Chicken plasma was treated with solid AS to reach a concentration of 20% saturation. The mixture was left at 4 °C for 2 h and centrifuged at 7000*g* for 15 min at 4 °C. The precipitated protein was collected and referred to as "*AS precipitate I*". The 20% saturated AS supernatant was treated with solid AS to reach 40% saturation and the protein precipitate was "*AS precipitate II*". The proteins in the supernatant were further fractionated with 60% and 80% saturation and the pellets were referred to as "*AS precipitates III and IV*", respectively. The supernatant obtained after addition of 80% saturation AS was referred to as "*AS supernatant IV*". All precipitates were redissolved in Buffer A. The redissolved precipitate and the supernatant fraction were dialysed against Buffer A overnight at 4 °C with quadruple changes of dialysis buffer. All fractions were kept at 4 °C until used.

2.3. Characterization of cysteine proteinase inhibitor from chicken plasma

2.3.1. Determination of inhibitory activity of chicken plasma fraction toward papain

Proteinase inhibitory activity was determined with papain according to the method of Benjakul et al. (2001a), using BANA as a substrate. To 2.0 ml of 0.25 M sodium phosphate buffer (pH 6.0) containing 2.5 mM EDTA and 25 mM 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 was 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 2.0% HCl/ethanol. The colour was developed by 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 units at 540 nm/ml/min under the assay conditions.

2.3.2. Protein determination

Protein concentration of crude chicken plasma and the fractions was measured by the Biuret method (Robinson & Hodgen, 1940), using bovine serum albumin (BSA) as a standard.

2.3.3. 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, Dimes, and Haard (1993) with a slight modification. PEG precipitate II was mixed with the sample buffer in the absence of βME at a ratio of 1:1 (v/v). The samples were applied onto the gel without prior boiling. The proteins were separated on a 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 bands 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.

2.3.4. Thermal stability

The CPI fraction (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 the untreated sample.

The fraction (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.

2.3.5. pH stability

The CPI fraction (200 μl) was mixed with McIlvaine buffer (0.2 M sodium phosphate and 0.1 M sodium citrate) with different pH values (3, 4, 5, 6, 7 and 8) at a ratio of 1:1 (v/v). At the pH values of 9 and 10, glycine–NaOH buffer (0.1 M glycine and 0.1 M NaOH) was used. The mixture was then incubated at room temperature for 20 min prior to inhibitory activity assay.

2.3.6. Salt stability

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

2.4. Effect of the CPI fraction on inhibition of fish mince and washed mince autolysis

2.4.1. Mince and washed mince preparation

Frozen Pacific whiting and arrowtooth flounder fillets were obtained from Pacific Seafood (OR, USA) and a grocery store in Raleigh (NC, USA), respectively. Fillets were thawed using running water and minced using a blender. The mince was separated into two portions. Another portion was used for the washed mince preparation. Washed mince was prepared according to the method of Toyohara, Kinoshita, and Shimizu (1990). The mince was homogenized with 5 volumes of cold 50 mM NaCl at a speed of 1 for 2 min using a Sorvall OMNI-MIXER (Ivan Sorvall Inc, Norwalk, CONN, USA). The homogenate was centrifuged at 5000*g* for 10 min at 4 °C. The washing was repeated twice. The pellet was referred to as "washed mince".

2.4.2. Autolysis inhibition study

The inhibitory activity of the CPI fraction against mince and washed mince autolysis was measured according to the method of Morrissey et al. (1993). CPI fractions, at levels of 0%, 0.3%, 0.5%, 1%, 2% and 3%, were added to 3 g of mince or washed mince. The mixture was mixed thoroughly and then incubated in a water bath (Blue M electrical company, Blue Island, IL, USA) at 55 °C for 60 min or 60 °C for 30 min for Pacific whiting and arrowtooth flounder, respectively. Autolysis was terminated by addition of 27 ml of 5% SDS solution (85 °C). The homogenate was incubated at 85 °C in a water bath for 1 h to dissolve total proteins. Autolytic patterns of myofibrillar proteins were determined using SDS–PAGE according to the method of Laemmli (1970). Autolysis profiles of mince and washed mince of Pacific whiting, at 55 °C, and arrowtooth flounder, at 60 °C, from 0 to 120 min of incubation, were also investigated.

2.5. Statistical analysis

All experiments were conducted in triplicate. The analysis in each experiment was performed in triplicate. Means and standard deviations were then calculated.

3. Results and discussion

3.1. Fractionation of cysteine proteinase inhibitor from chicken plasma

3.1.1. Polyethylene glycol fractionation

The distribution of CPI in different fractions obtained from PEG precipitation is shown in Table 1. Among all precipitates obtained, PEG precipitate II had the highest inhibitory activity, followed by PEG precipitates I, III and IV, respectively. In addition, PEG precipitate II also showed the highest purification-fold (3.85). Approximately 70% of total inhibitory activities of chicken plasma were recovered in PEG precipitates I and II. From the result, the inhibitory activity (14%) still remained in the supernatant even though PEG at the highest concentration was added. After all step-wise fractionations, CPI values of 84% were obtained as the pellets. During fractionation, losses in protein were found. Proteins, especially those of low molecular weight, might be removed during dialysis. As a result, the dialyzed fractions contained less protein. This also helped to eliminate the contaminating proteins from the resulting fractions. Bovine blood plasma was fractionated into fribrinogen, immunoglobulins and albumin by PEG precipitation with high separation efficiencies (Lee et al., 1987). Gaertner and Puigserver (1992) reported that the modification of trypsin with PEG significantly improved the resistance to heat and detergent, as well as the catalytic action of enzymes. From the results, some losses of inhibitory activities were noticeable. This was probably due to the denaturation of those inhibitors during fractionation. High-molecular-weight PEG can significantly alter the tertiary and secondary structure of interferon alpha-2a, a model protein, rather than lower-molecular-weight PEG (Sharma & Kalonia, 2004).

Based on the protein patterns (Fig. 1), PEG precipitates I and II contained several protein bands similar to those of chicken plasma. Addition of PEG, at levels greater than 400 g/l, resulted in the complete removal of protein bands with the MW of 307 and 220 kDa, along with the precipitation of proteins with MW of 46, 56 and 61 kDa. Lee et al. (1987) reported that fibrinogen and immunoglobulin from bovine blood plasma were fractionated with 9.06% and 12.6% PEG concentration. A protein band with the MW of 122 kDa appeared in the PEG precipitate II but was rarely found in other fractions. Generally, a similar protein pattern was observed in PEG precipitates III, IV and supernatant IV, in which protein with MW of 46 kDa was the dominant protein. From this result, the PEG amount used was found to determine the protein compositions of resulting fractions.

Table 1
Fractionation of cysteine proteinase inhibitor from chicken plasma by polyethylene glycol (PEG-4000)

Fractions[a]	Volume (ml)	Total protein (mg)	Total inhibitory activity (units)	Specific inhibitory activity (U/mg)	Yield (%)	Purification (fold)
Crude CPP	30	1007	52,200	51.9	100	1.00
PEG precipitate I	24	326	15,408	47.3	29.5	0.91
PEG precipitate II	10	119	23,760	200	45.5	3.85
PEG precipitate III	8	50.6	2760	54.6	5.29	1.05
PEG precipitate IV	7	32.6	2142	65.7	4.10	1.27
PEG supernatant IV	42	54.2	7182	133	13.8	2.56

All values are averages from triplicate determinations.
[a] I–IV: fraction obtained by PEG fractionation: 0–200, 200–400, 400–600, 600–800 g/l precipitate, respectively, and 800 g/l supernatant.

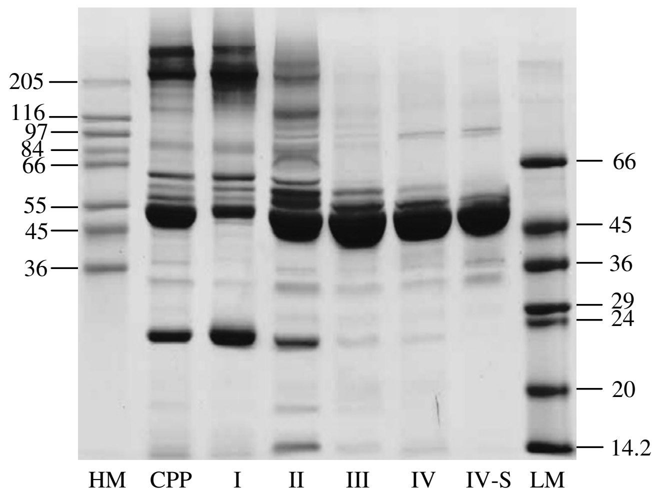

Fig. 1. Protein pattern of chicken plasma and different fractions from polyethylene glycol (PEG-4000) fractionation. HM: high-molecular-weight markers; LM: low-molecular-weight markers; CPP: chicken plasma; I–IV: fraction obtained by PEG fractionation: 0–200, 200–400, 400–600, 600–800 g/l precipitate, respectively, and 800 g/l supernatant (IV-S). Proteins (15 µg) were applied on 10% gel.

Hasko and Vassilyeva (1981) reported that 15% PEG-4000 was the most suitable plasma protein fractionator for obtaining a precipitate containing mainly IgG and a supernatant containing chiefly albumin.

3.1.2. Ammonium sulfate fractionation

CPI was found in varying amounts in different fractions obtained from AS precipitation (Table 2). AS precipitate II had the highest inhibitory activity, followed by AS precipitates III, IV, and I, respectively. Inhibitory activities of about 60% were recovered in precipitates II, III and IV. However, AS supernatant IV also contained 7% inhibitory activity. From the results, CPI was more likely concentrated in AS precipitate II. When comparing the inhibitory activities between selected fractions (precipitate II), it appeared that AS fractionation was less effective than PEG fractionation. Jiang, He, and Fountoulakis (2004) reported that the pellet of the 50% AS precipitation included the majority of plasma proteins and only a small percentage of albumin, while the 70% AS pellet mainly included albumin, serotransferrin, anti-trypsin and haptoglobin-1.

SDS–PAGE patterns of various fractions were different (Fig. 2). AS precipitates I and II contained mainly high-molecular-weight components. AS precipitate I consisted of proteins with MWs of 205 and 180 kDa as the major components. AS precipitate II had a protein pattern similar to that of crude chicken plasma, but the band intensities of proteins with MWs of 46 and 23 kDa decreased with the appearance of protein bands at MWs of 122 and 75 kDa. AS precipitate III also had a pattern similar to that of crude chicken plasma, except that the protein with MW of 46 kDa had an increase in band intensity. However, the band intensity of the 23 kDa protein was reduced. Similar protein patterns were observed between the precipitate IV and supernatant IV, in which protein with MW of 46 kDa was predominant. Nevertheless, protein with MW of 23 kDa was still retained in these fractions.

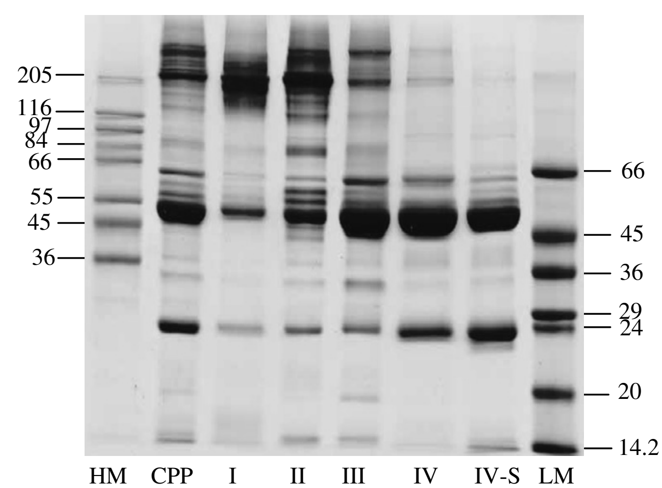

Fig. 2. Protein pattern of chicken plasma and different fractions from ammonium sulfate (AS) fractionation. HM: high-molecular-weight markers; LM: low-molecular-weight markers; CPP: chicken plasma; I-IV: fraction obtained by AS fractionation: 0–20%, 20–40%, 40–60%, 60–80% saturation precipitate, respectively, and 80% saturation supernatant (IV-S). Proteins (15 µg) were applied on 10% gel.

Table 2
Fractionation of cysteine proteinase inhibitor from chicken plasma by ammonium sulfate (AS)

Fractions[a]	Volume (ml)	Total protein (mg)	Total inhibitory activity (U)	Specific inhibitory activity (U/mg)	Yield (%)	Purification (fold)
Crude CPP	30	1007	52,200	51.9	100	1.00
AS precipitate I	7.6	84.9	4081	48.1	7.82	0.93
AS precipitate II	9	140	16,956	122	32.5	2.34
AS precipitate III	12	154	8928	58.1	17.1	1.12
AS precipitate IV	27	142	8262	58.4	15.8	1.13
AS supernatant IV	37	26.3	3885	148	7.44	2.85

All values are averages from triplicate determinations.
[a] I–IV: fraction obtained by AS fractionation: 0–20%, 20–40%, 40–60%, 60–80% precipitate, respectively, and 80% supernatant.

3.2. Characterization of cysteine proteinase inhibitor from chicken plasma

3.2.1. Protein pattern and inhibitory activity staining of CPI fraction

Due to the high CPI inhibitory activity, as well as high purity, PEG precipitate II was selected as the cysteine proteinase inhibitor-containing fraction (CPI fraction). Protein patterns and inhibitory activity staining for papain of CPI fraction are depicted in Fig. 3. Inhibitory activity staining revealed that the proteins with MWs of 46 and 122 kDa are the predominant proteins in the CPI fraction (Fig. 3B). Recently, the protein with MW of 46 kDa in the CPI fraction was identified as a papain-resistant protein, not a cysteine proteinase inhibitor, since it could not bind to a papain affinity column (Rawdkuen, Benjakul, Visessanguan, & Lanier, accepted for publication). Therefore, the protein with MW of 122 kDa in the CPI fraction was a cysteine proteinase inhibitor.

3.2.2. Thermal stability of CPI fraction

The thermal stability of the CPI fraction subjected to heating for 10 min in the temperature ranges of 40–90 °C is shown in Fig. 4A. This shows that the fraction was heat-stable, as evidenced by the remaining inhibitory activity at all temperature ranges tested. From the result, the incubation time might not be sufficient to cause the denaturation of CPI in the fraction. Lee et al. (2000a) also reported that L-kininogen from pig plasma had a very high thermal stability and there was 90% of the inhibitory activity left after a 30 min incubation at 80 °C.

When the CPI fraction was heated at 90 °C for various times (10–60 min), the relative inhibitory activity markedly decreased as the incubation time increased, especially in the range 10–50 min of incubation (Fig. 4B). Relative inhibitory activity of less than 50% was found with the CPI fraction heated at 90 °C for 50 and 60 min. This result shows that CPI might undergo thermal denaturation when a longer time for heating at high temperature is used. Generally, heating at 90 °C is used in the surimi gel cooking for 15–20 min. Thus, CPI was not completely inactivated at this temperature and could function as a proteinase inhibitor during the thermal gelation of surimi.

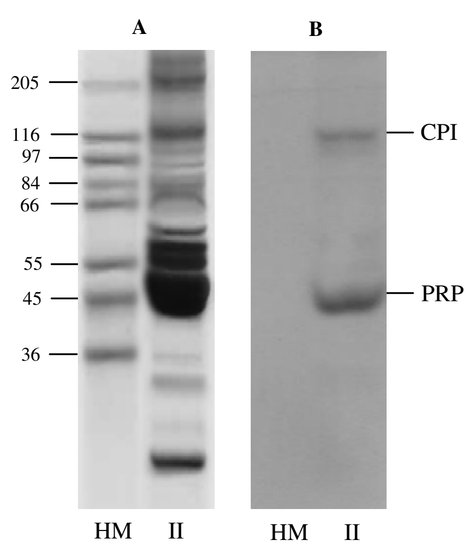

Fig. 3. Protein pattern (A) and inhibitory activity staining for papain (B) of cysteine proteinase inhibitor fraction from chicken plasma. HM: high-molecular-weight markers, II: 200–400 g PEG/l fraction. Proteins (15 μg) were applied on 10% gel. CPI: cysteine proteinase inhibitor; PRP: papain resistant protein.

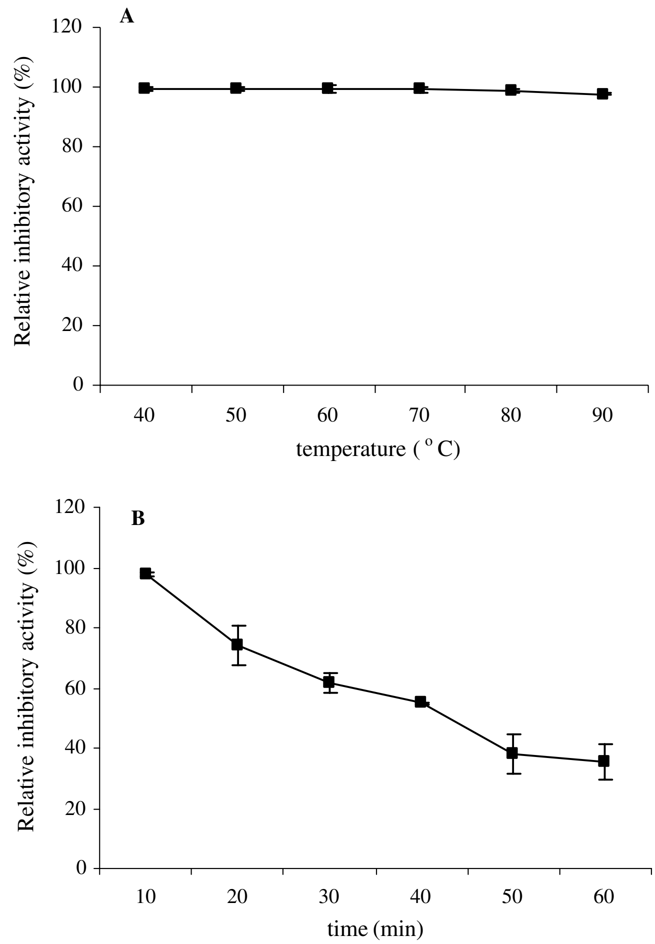

Fig. 4. Effect of heating temperature (A) and time (B) on stability of cysteine proteinase inhibitor fraction from chicken plasma. Fractions were incubated at different temperatures for 10 min (A) or heated at 90 °C for various times (B). Residual inhibitory activity against papain was determined using BANA as substrate. Bars represent the standard deviations from triplicate determinations.

3.2.3. pH stability of the CPI fraction

The pH stability of the CPI fraction is shown in Fig. 5. No changes in relative inhibitory activity were observed in the pH range tested. The inhibitory activity of the CPI fraction was stable over a broad range of pH from 3 to 10. This result was in agreement with Lee et al. (2000a) who reported that the inhibitory activity of L-kininogen from pig plasma was stable at pHs ranging from 3 to 10.5 and also was very reactive over a broad pH range.

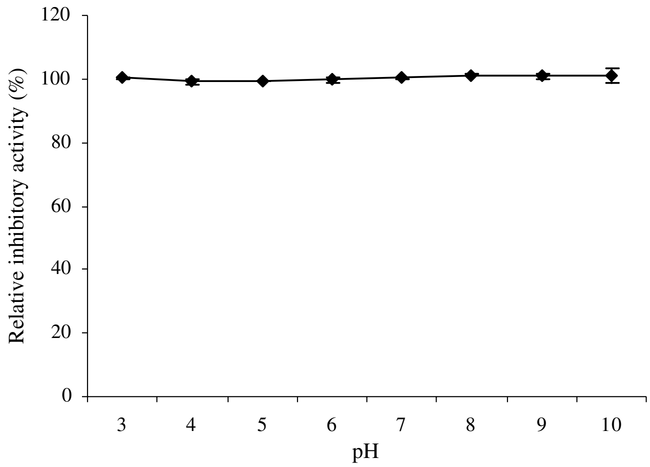

Fig. 5. Effect of pH on stability of cysteine proteinase inhibitor fraction from chicken plasma. Residual inhibitory activity against papain was determined using BANA as substrate. Bars represent the standard deviations from triplicate determinations.

Kos, Dolinar, and Turk (1992) reported that isolated kininogens from chicken egg white and plasma have pI values of 4.3–5.2. Normally, when pH is increased or decreased, away from the isoelectric point, the ionizable groups in proteins become increasingly charged to a point, where charge repulsion causes the protein molecules to unfold and may bring about different protein structures that could have modified functionalities, different from the native protein (Dill & Shortle, 1991). From this result, it was postulated that CPI might undergo reversible denaturation. When pH was adjusted to neutral, the native structure was regained and functionality was also recovered.

3.2.4. Salt stability of the CPI fraction

The effect of NaCl on inhibitory activity of the CPI fraction is shown in Fig. 6. No marked changes in relative inhibitory activity were observed when NaCl was added up to 3%. However, a slight increase in relative inhibitory activity was observed in the presence of NaCl at levels of 0.5–1.5%. The result suggested that NaCl, at low concentrations, might induce conformational changes of CPI, in a fashion by which CPI could work or inhibit the proteinase more effectively. Salt has a number of effects on properties of proteins, including activity, conformational stability and solubility. These effects possibly arise from the binding of ions to specific sites on the protein, screening charges on surface amino acid side chains and changing the degree of hydration of the protein (Timashaff & Arakawa, 1997). From the result, the CPI fraction showed high salt stability up to 3%, which might be useful in surimi-based products in which 2–3% salt is commonly used.

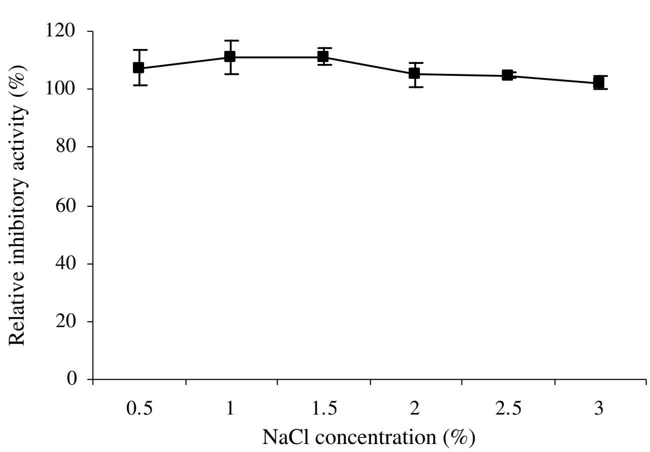

Fig. 6. Effect of salt content on stability of cysteine proteinase inhibitor fraction from chicken plasma. Residual inhibitory activity against papain was determined using BANA as substrate. Bars represent the standard deviations from triplicate determinations.

3.3. Effect of the CPI fraction on inhibition of fish mince and washed mince autolysis

3.3.1. Inhibition of fish mince autolysis

Autolysis of mince from arrowtooth flounder and Pacific whiting incubated at 60 and 55 °C, respectively, for different times is presented in Fig. 7A. Arrowtooth flounder mince showed very high autolytic activity as indicated by the complete disappearance of the myosin heavy chain (MHC) band after 5 min of incubation. Not only was MHC degraded at 5 min, but also actin was markedly hydrolyzed. The protein band with MW of 31 kDa slightly decreased when the incubation time increased and disappeared when heated for more than 60 min. Visessanguan et al. (2003) reported that cathepsin L was a predominant, heat-activated proteinase in arrowtooth flounder, which could hydrolyze myofibrillar proteins. For Pacific whiting mince (Fig. 7A), the autolytic pattern was similar to that of arrowtooth flounder. The MHC band completely disappeared when the incubation time was 10 min. However, actin was quite resistant to hydrolysis. Increasing degradation of troponin and tropomyosin was found when the incubation time increased and those proteins were completely hydrolyzed within 60 min. Cathepsin L was the most active cysteine protease in Pacific whiting (An et al., 1994). Fish mince contains both sarcoplasmic and myofibril-associated proteinases. As a consequence, higher autolysis generally occurs in mince, compared with washed mince, in which sarcoplasmic proteinases were removed (Benjakul, Seymour, Morrissey, & An, 1996). Therefore, fillets or mince containing high proteolytic activity produce low quality surimi products, due to degradation of myosin, needed to form a surimi gel (Morrissey et al., 1993).

Inhibition of autolysis of both arrowtooth flounder and Pacific whiting minces by the CPI fraction is shown in Fig. 7B. The CPI fraction, at concentrations up to 3%, was unable to inhibit the autolysis of arrowtooth flounder mince. However, the degradation of protein with MW of 31 kDa was completely suppressed when the CPI levels added were up to 1%. From these results, it can be concluded that the CPI fraction, at the levels used, was not enough to inhibit the myofibrillar degradation in

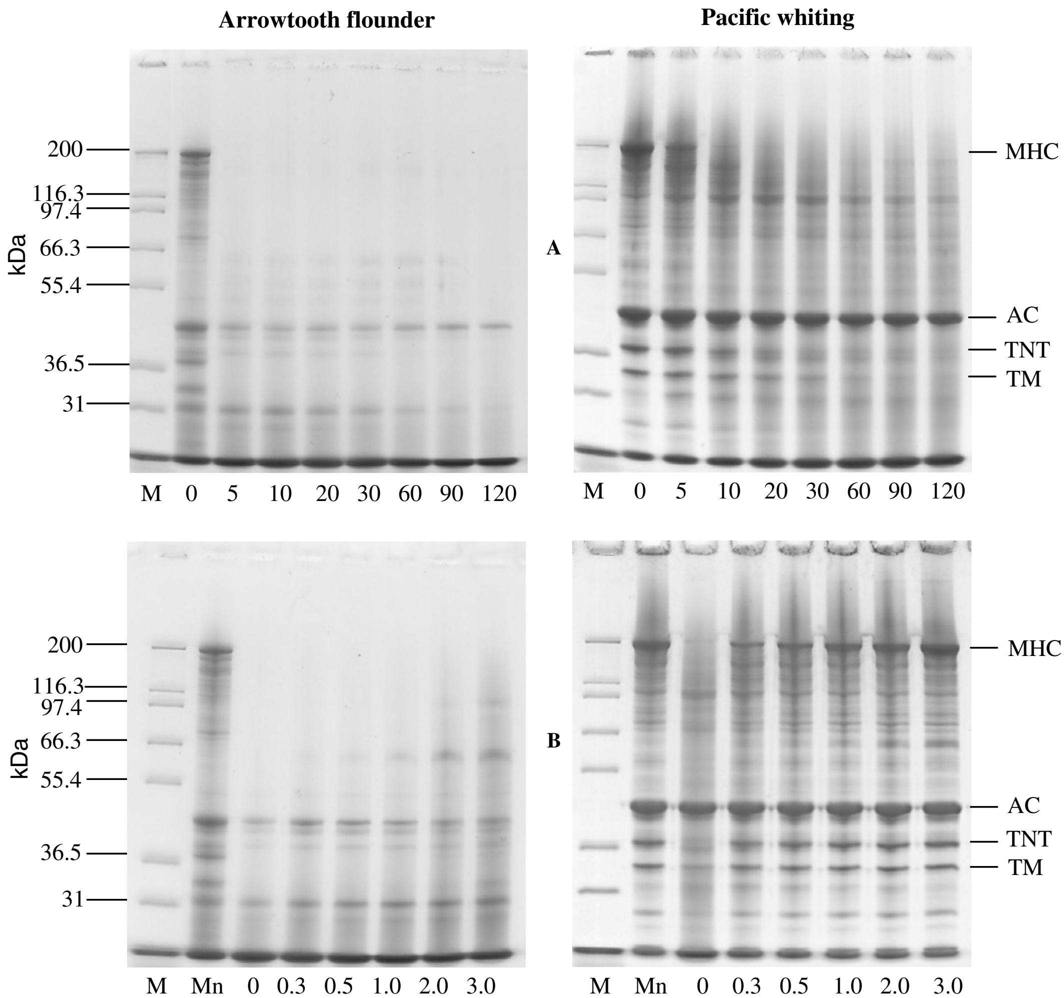

Fig. 7. Effect of cysteine proteinase inhibitor fraction from chicken plasma on autolysis inhibition of arrowtooth flounder and Pacific whiting mince. Mince was incubated at 60 °C for 30 min and 55 °C for 60 min for arrowtooth flounder and Pacific whiting, respectively. M: protein marker, Mn: mince, MHC: myosin heavy chain, AC: actin, TNT: troponin-T, TM: tropomyosin. Numbers (0–120) designate incubation time (min) (A). Numbers (0–3) designate CPI fraction concentration (%) (B).

arrowtooth flounder mince. This was presumed to be due to the excessive amount of proteinases in arrowtooth flounder mince, in which CPI was not sufficient for complete inhibition. For Pacific whiting mince, slight increase in the MHC band intensity was observed with increasing CPI fraction added. Moreover, the degradations of troponin and tropomyosin were inhibited when CPI fraction, at a level of 0.3%, was used. When the CPI fraction, at the level of 3%, was added, almost complete inhibition of MHC degradation was observed. Within the concentration range of CPI fraction used, the fraction would be more appropriate for inhibiting the autolysis of Pacific whiting mince rather than arrowtooth flounder mince. Morrissey et al. (1993) reported that more than 80% inhibition was obtained in Pacific whiting mince by treatment with 2% BPP. Wasson et al. (1992) successfully improved the gel strength of arrowtooth flounder surimi by using BPP and egg white. Catehpsin L was the main proteolytic enzymes in both arrowtooth flounder and Pacific whiting (An et al., 1994; Visessanguan et al., 2003). This result suggested that the CPI fraction could inhibit the catheptic enzymes to different degrees, depending upon the initial proteolytic activity of fish mince or muscle.

3.3.2. Inhibition of washed mince autolysis

The autolysis pattern of washed mince from arrowtooth flounder and Pacific whiting at different times of incubation at 60 and 55 °C is depicted in Fig. 8A. MHC band intensity in the arrowtooth flounder washed mince completely disappeared after 10 min of incubation. The degradation products from MHC autolysis were clearly observed at incubation times of 5–20 min, with the appearance of protein with MW of 98 kDa. The actin and tropomyosin slightly decreased when the incubation time increased and no marked changes were observed when the times were above 60 min. The troponin-T was degraded within 5 min with the appearance of a protein band with MW of 31 kDa. For Pacific whiting washed mince, MHC slightly decreased as the incubation times increased and almost disappeared when the incubation time reached 90 min. No

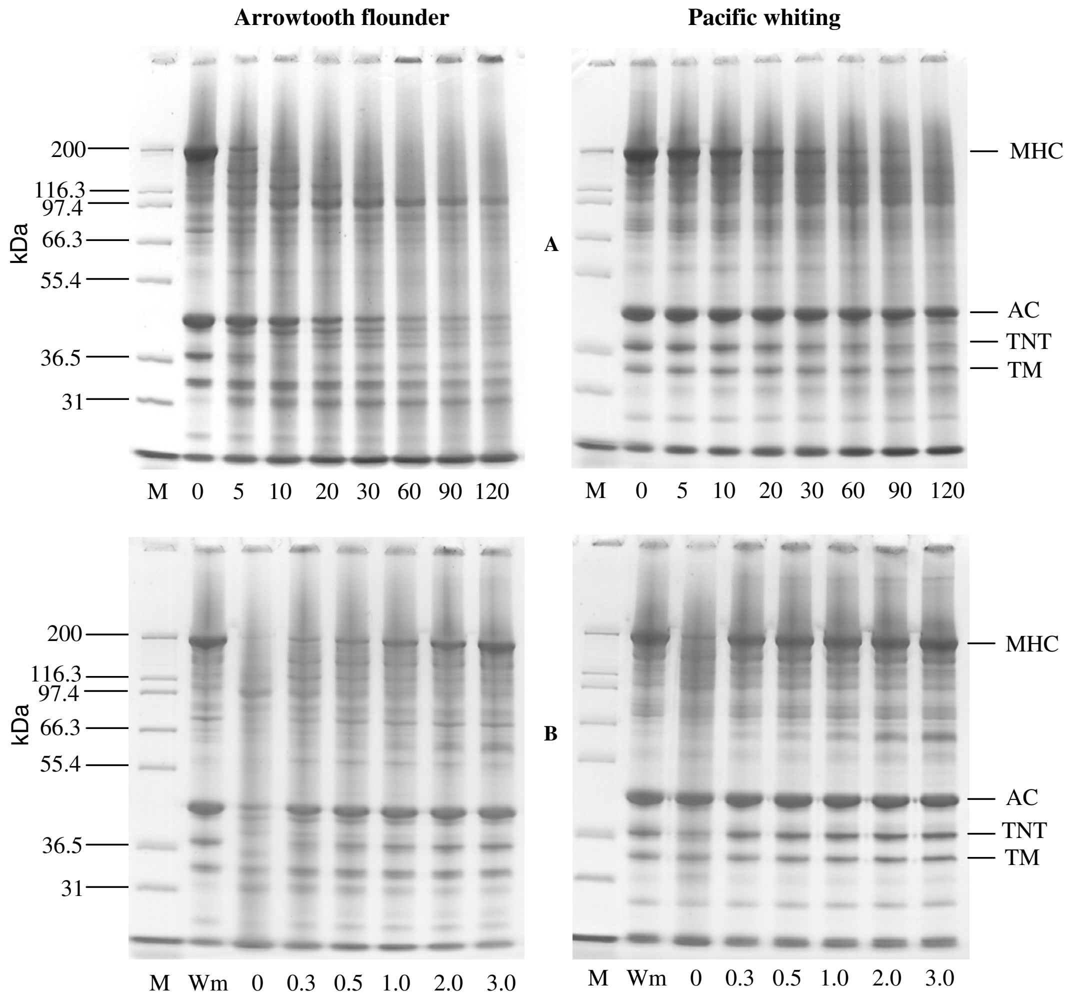

Fig. 8. Effect of cysteine proteinase inhibitor fraction from chicken plasma on autolysis inhibition of arrowtooth flounder and Pacific whiting washed mince. Washed mince was incubated at 60 °C for 30 min and 55 °C for 60 min for arrowtooth flounder and Pacific whiting, respectively. M: protein marker, Wm: washed mince, MHC: myosin heavy chain, AC: actin, TNT: troponin-T, TM: tropomyosin. Numbers (0–120) designate incubation times (min) (A). Numbers (0–3) designate CPI fraction concentrations (%) (B).

changes in actin band intensity were observed throughout 120 min of incubation. However, the MHC was more retained in both arrowtooth flounder and Pacific whiting washed minces (Fig. 8), when compared with that observed in the mince (Fig. 7). Washing could remove some endogenous proteinases which play an important role in the degradation of MHC (Benjakul et al., 1996). As a result, lower hydrolysis was found, as evidenced by the much greater MHC retained. Chang-Lee, Pacheco-Aguilar, Crawford, and Lampila (1989) reported that the protease activity in mechanically de-boned Pacific whiting flesh was reduced to 56.3% by washing and refining processes. An et al. (1994) reported that washing, during Pacific whiting surimi processing, removed cathepsin B and H. Cathepsin L was identified as the predominant proteinase in surimi wash water (Benjakul et al., 1996).

The autolysis pattern of washed mince from arrowtooth flounder and Pacific whiting incubated at 60 and 55 °C for 30 min, in the presence of the CPI fraction at different concentrations, is shown in Fig. 8B. MHC band intensity of arrowtooth flounder washed mince was more retained with increasing CPI fraction concentrations, suggesting the inhibitory activity of the CPI fraction towards autolysis of MHC. Actin, troponin-T and tropomyosin bands were recovered in the sample treated with CPI fraction, especially at the higher concentration. For Pacific whiting washed mince, no significant differences in MHC band intensity were observed in any samples treated with the CPI fraction. Furthermore, the degradations of actin, tropomyosin and troponin-T were also completely inhibited in the presence of 0.3% CPI fraction. This result indicated that only 0.3% could be enough for autolysis inhibition of Pacific whiting washed mince. Since the washing process could remove some endogenous proteinases, a smaller amount of CPI fraction was required for inhibition of autolysis. Reppond and Babbitt (1993) reported that addition of 2% potato inhibitor or bovine plasma or egg white increased stress values of arrowtooth flounder gel by three times but no differences were obtained among the various inhibitors. The greater inhibition of autolysis observed in washed mince from both species might be due to the lower amount of proteinases retained, which could be inhibited more efficiently by the CPI fraction.

4. Conclusion

Cysteine proteinase inhibitor from chicken plasma was successfully fractionated by using 200–400 g PEG/l. The CPI fraction obtained showed high inhibitory activity against papain. The CPI fraction was stable to various pHs, heating, and salt up to 3%. The CPI fraction effectively inhibited the autolytic activity of myofibrillar proteins from both arrowtooth flounder and Pacific whiting, especially in washed mince.

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