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Purification and Characterization of a Cysteine Protease Inhibitor from Chum Salmon (*Oncorhynchus keta*) Plasma

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A cysteine protease inhibitor (CPI) in chum salmon (*Oncorhynchus keta*) plasma (CSP) was detected after performing inhibitory activity staining against papain under nonreducing condition. The CPI was purified from CSP by affinity chromatography with a yield and purification ratio of 0.94% and 30.36-fold, respectively. CSP CPI had a molecular mass of 70 kDa based on the results of SDS−PAGE and Sephacryl S-100 gel filtration. CSP CPI was a glycoprotein based on the periodic acid−Schiff (PAS) staining of the SDS−PAGE gel and classified as a kininogen. CSP CPI was stable in the pH range of 6.0–9.0 with maximal stability at pH 7.0. CSP CPI presented thermal stability at temperatures below 50 °C and exhibited maximal activity at temperatures of 20–40 °C. CSP CPI was determined to be a noncompetitive inhibitor against papain, with an inhibitor constant (K_i) of 105 nM.

KEYWORDS: Chum salmon plasma; cysteine protease inhibitor; inhibitory activity staining; purification; characterization

INTRODUCTION

Blood is a medium for transporting nutrients, metabolic waste products, and gases around the body (*1*). It is the main byproduct from the slaughtering process, which can be fractionated into plasma (65–70%) and cellular mass (35–40%) on a volume basis (*2*). Plasma comprises a diverse range of biologically valuable proteins and irons in the form of heme Fe (*3*). It is traditionally used in the meat industry, including sausage and bologna products, to provide emulsification properties and to hold moisture, fat, and other ingredients (*4*).

Nowadays, plasma has been reported to exhibit protease inhibitory activity and gel strengthening ability during heat-induced gelation of surimi (*5–11*). Porcine plasma protein (PPP) and bovine plasma protein (BPP) were applied to inhibit the surimi autolysis of bigeye snapper (*5*), mackerel (*6*), and Pacific whiting (*7*). A protease inhibitor with the molecular mass of 63–65 kDa (*8*) and an L-kininogen (*6*) were found to be contained in PPP. α_2-Macroglobulin (α_2-M), a nonspecific inhibitor (*12*), was found to exist in BPP (*9*). Chicken plasma protein (CPP) was applied to bigeye snapper and lizardfish surimi to prevent gel weakening (*10, 11*). A cysteine protease inhibitor (CPI) with the molecular mass of 122 kDa was purified from chicken plasma (*13*).

However, the outbreak of bovine spongiform encephalopathy (BSE) and avian influenza (AI) significantly limited the application of mammal and avian plasma and their protease inhibitors, especially in the food industry. Therefore, various researches were carried out so as to seek safe and economical alternatives. Since the past decade, CPIs have been isolated and characterized from fish, such as egg (*14, 15*) and skin (*16*) of chum salmon, ovarian fluid of carp (*17*), and eggs of Alaska pollock (*18*) and glassfish (*19*). Fish CPIs are important in pathological processes, fish disease prevention, and cure (*20*).

Until now, no reports on CPIs from fish plasma were available. Capture fisheries and aquaculture supplied the world with about 106 million tons of food fish in 2004 according to FAO's reports (*21*). The fish blood from food fish was calculated to be around 5 million tons on the basis that blood occupied 5% of the total weight (*2*). Generally, fish blood is regarded as a waste or pollutant, which is rinsed during surimi processing (*10, 11*). Studies on CPIs from fish plasma are helpful for the treatment or reutilization of this material. The objectives of this study were to purify and characterize the cysteine protease inhibitor from chum salmon plasma and to utilize it as an additive for preventing the deterioration of surimi-based products by inhibiting their endogenous proteases.

MATERIALS AND METHODS

Materials. Chum salmon (*Oncorhynchus keta*) was obtained alive from the Regional Inland Fisheries Research Institute in Korea. Papain (from papaya latex, 18 units/mg), cathepsin L (from human liver, 4 units/mg), trypsin (from porcine pancreas, 14 units/mg), azocasein, *N*-carbobenzoxyphenylalanine-arginine-7-amido-4-methylcoumarin (Z-Phe-Arg-NMec), casein, protein markers, and chicken egg white cystatin were purchased from Sigma (St. Louis, MO). Cyanogen bromide-

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(CNBr-) activated Sepharose 4B was purchased from Amersham Biosciences (Uppsala, Sweden). The other chemicals used in this study were of analytical grade.

Preparation of Chum Salmon Plasma. Chum salmon blood was collected by an intracaudal vein puncture. One-tenth volume of 3.8% (w/v) trisodium citrate was added to the blood to prevent coagulation. The blood was centrifuged twice at 1500*g* with a Combi-514R Centrifuge (Hanil Science Co., Korea) for 15 min at 4 °C to remove red blood cells. The supernatant was regarded as chum salmon plasma (CSP) and was frozen and kept at −40 °C until used.

Preparation of CNBr−Papain−Sepharose. A 3 g aliquot of CNBr-activated Sepharose 4B was washed on a sintered glass filter (porosity G3) with 600 mL of 1 mM ice-cold HCl. Then, 20 mL of 5 mg/mL papain solution in coupling buffer (0.2 M $NaHCO_3$ containing 0.5 M NaCl, pH 8.3) was mixed with the gel in an end-over-end mixer overnight at 4 °C. The gel was then transferred to 40 mL of blocking agent (0.2 M glycine, pH 8.0) and stirred overnight at 4 °C. After being washed with washing buffer (0.1 M sodium acetate containing 0.5 M NaCl, pH 4.0) and coupling buffer for three times, the gel was poured into a 1.0 × 20 cm column and equilibrated with acetate buffer [50 mM sodium acetate containing 25 mM NaCl, 1 mM β-mercaptoethanol (β-ME), and 1 mM EDTA, pH 6.0].

SDS−Substrate Gel and Staining for Inhibitory Proteins. Inhibitory proteins in CSP were analyzed using 10% separating gel and 5% stacking gel according to the modified method of Garcia-Carreno et al. (*22*). CSP diluted ten times with acetate buffer was mixed at a ratio of 1:1 (v/v) with the sample buffer (100 mM Tris-HCl containing 2.5% SDS, 0.01% bromophenol blue, and 2% glycerol, pH 6.8) containing 10% β-ME or not. The samples were loaded onto the gel without 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 prepared: one gel was fixed and stained for total proteins with Coomassie Brilliant Blue R-250 as a control, and the other was stained for inhibitory proteins as follows. The gel was washed in 100 mL of 2.5% (w/v) Triton X-100 for 15 min to remove SDS and to renature proteins (*23*). Then the gel was washed with distilled water and was transferred to 20 mL of 0.4 mg/mL papain in acetate buffer. After a 1 h incubation at 4 °C to allow the enzyme to diffuse in, the gel was washed with distilled water and transferred to 50 mL of 1% (w/v) casein solution in acetate buffer. After a 1.5 h incubation at 37 °C, the gel was rinsed with distilled water, fixed, and stained with Coomassie Brilliant Blue R-250 to develop inhibitory zones, which were detected as dark bands on a clear background.

Purification of the Cysteine Protease Inhibitor. CSP diluted five times with acetate buffer was filtered through a 0.45 μm membrane (Millipore) and loaded onto the preequilibrated CNBr−papain−Sepharose 4B column at a flow rate of 0.3 mL/min at 4 °C. The column was then washed with 3 column volumes (CV) of acetate buffer to remove unabsorbed proteins and with 2 CV of acetate buffer containing 1 M NaCl to remove nonspecifically bound proteins. Then the salt was removed with 3 CV of acetate buffer. Finally, the column was eluted with elution buffer (20 mM sodium phosphate containing 25 mM NaCl and 1 mM EDTA, pH 11.5) at a flow rate of 0.3 mL/min. Each fraction was monitored at 280 nm, adjusted to pH 6.0 with 0.25 M sodium phosphate solution (pH 4.0), and assayed for inhibitory activity against papain. Fractions containing higher than 50% of maximal inhibitory activity were pooled, dialyzed against acetate buffer, and used for further study.

Determination of Molecular Mass. The molecular mass of CSP CPI was determined by SDS−PAGE under reducing condition. The main procedure is the same as described above. The differences were that CSP CPI in the sample buffer containing 10% β-ME was boiled for 5 min before it was loaded onto the gel. The electrophoresis was run at 150 V for 1 h at room temperature. The molecular mass of CSP CPI was estimated by comparison with protein markers.

In order to determine native molecular mass, CSP CPI was chromatographied using FPLC (ÄKTAprime plus, Uppsala, Sweden) with a Sephacryl S-100 column (1.6 × 60 cm). Loading and elution of CSP CPI were performed at a flow rate of 0.5 mL/min at 4 °C. The elution volume (V_e) of each protein marker was determined by monitoring absorbance at 280 nm. The native molecular mass of CSP CPI was estimated from calibration curves obtained by plotting the log molecular mass of each protein marker versus the corresponding V_e (*24*).

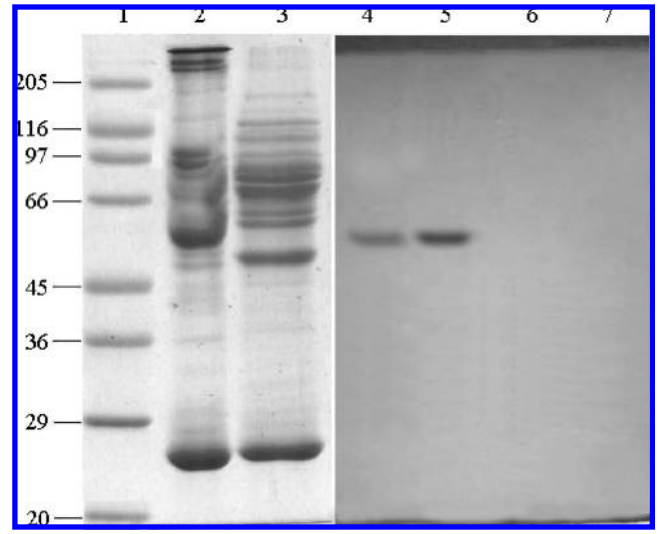

Figure 1. Inhibitory activity staining of chum salmon plasma against papain: lane 1, protein marker; lanes 2 and 3, 25 μg of chum salmon plasma protein under nonreducing and reducing conditions, respectively; lanes 4 and 5, 12.5 and 25 μg of inhibitory activity stained chum salmon plasma protein under nonreducing condition; lanes 6 and 7, 12.5 and 25 μg of inhibitory activity stained chum salmon plasma protein under reducing condition.

PAS Staining for the Cysteine Protease Inhibitor. Periodic acid−Schiff reagent (PAS) staining was performed according to the method of Zacharius et al. (*25*). After electrophoresis, the SDS−PAGE gel was immersed in 12.5% (w/v) trichloroacetic acid (TCA) solution for 15 min. Then the gel was washed with distilled water, transferred to 1% periodic acid solution, and kept for 1 h at 4 °C. After being washed thoroughly with distilled water to remove the periodic acid, the gel was incubated with Schiff reagent in the dark for 1 h at 4 °C. Finally, the gel was washed with 0.5% (w/v) sodium metabisulfite solution and distilled water. The glycoprotein was detected as a meganta band on a clear background.

Determination of the Inhibitory Activity of the Cysteine Protease Inhibitor. Inhibitory activity of CSP CPI was determined using papain as the enzyme and azocasein as the substrate (*26*). A 100 μL aliquot of papain solution (0.25 mg/mL in acetate buffer) was added with 200 μL of acetate buffer or CSP CPI in acetate buffer (0.04 mg/mL). The combined solution was incubated at 37 °C for 5 min, and then 250 μL of azocasein solution (3.2 mg/mL in acetate buffer) was added to initiate the reaction. After a 30 min incubation at 37 °C, 700 μL of 20% TCA was added to the solution to stop the reaction, followed by a centrifugation at 10000*g* for 5 min. A 720 μL supernatant was added to 800 μL of 1 N NaOH for 15 min to develop color. The absorbance was determined at 440 nm. The inhibitory activity was determined using the difference between papain activities with and without inhibitor. One unit of inhibitory activity was defined as a decrease of 1.0 in absorbance at 440 nm under the assay condition.

Cathepsin L was also used to determine the inhibitory activity of CSP CPI for comparison with other inhibitors, and Z-Phe-Arg-NMec was used as the substrate (*26*). A 3 ng aliquot of cathepsin L in 500 μL of 0.1% Brij 35 was added with 250 μL of assay buffer (0.34 M sodium acetate containing 60 mM acetic acid and 4 mM EDTA, pH 5.5) or CSP CPI in assay buffer (0.04 mg/mL). The combined solution was incubated for 1 min at 30 °C, and then 250 μL of 1 mM Z-Phe-Arg-NMec in dimethyl sulfoxide (DMSO) was added to initiate the reaction. After a 10 min incubation at 30 °C, 1 mL of stopping solution (0.1 M sodium monochloroacetate, 30 mM sodium acetate, and 70 mM acetic acid, pH 4.3) was added to the solution. The liberated aminomethylcoumarin (AMC) was determined using a RF-5301 fluorescence

Table 1. Purification of the Cysteine Protease Inhibitor from Chum Salmon Plasma by Affinity Chromatography

purification step	total protein (mg)	total inhibitory activity (units)	specific inhibitory activity (units/mg)	yield (%)	purification (*x*-fold)
chum salmon plasma	750.15	94.88	0.13	100	1.00
affinity chromatography	0.23	0.89	3.84	0.94	30.36

spectroscope (Shimadzu Co., Japan) with excitation and emission wavelengths of 340 and 460 nm, respectively. The inhibitory activity was determined using the difference between cathepsin L activities with and without inhibitor. One unit of protease activity was defined as the amount of enzyme required to produce 1 μmol of AMC/min at 30 °C. One unit of inhibitory activity was defined as one unit decrease of cathepsin L activity.

pH Stability, Thermal Stability, and Activity of the Cysteine Protease Inhibitor. pH and thermal stabilities of CSP CPI were determined according to the method of Tzeng et al. (*27*). CSP CPI in various buffers, pH 3.0−5.0 (50 mM sodium citrate buffer), pH 6.0−8.0 (50 mM sodium phosphate buffer), pH 9.0−10.0 (50 mM Clark and Lubs buffer), and pH 11.0 (50 mM disodium phosphate and sodium hydroxide buffer), was incubated at 37 °C for 30 min, and then an equal volume of 0.2 M acetate buffer (pH 6.0) was added. The residual inhibitory activity against papain was determined as described above. CSP CPI in acetate buffer was incubated at temperatures ranging from 4 to 80 °C for 30 min. After being cooled in ice water for 30 min, the residual inhibitory activity against papain was determined as described above. Thermal activity of CSP CPI was determined by carrying out the inhibitory activity assay at each temperature (4–80 °C) at pH 6.0.

Protein Concentration. Protein concentration of CSP was determined according to the method of Robinson and Hodgen (*28*). Protein concentration of papain affinity fractions was determined using a Bio-Rad kit according to the manufacturer's instructions (Hercules, CA). Bovine serum albumin (BSA) was used as a standard protein.

Kinetics. The inhibition type of CSP CPI against papain was determined by Dixon plot analysis (*29*). Aliquots of 7–42 μg/mL CSP CPI, 0.25 mg/mL papain, and 0.8–3.2 mg/mL azocasein were prepared to determine kinetic parameters. A Dixon plot was made by plotting the inverse papain activity (1/*V*) versus the CPI concentration (nanomolar) at three different azocasein concentrations. The inhibition constant (K_i) was determined by the intersection of those three lines in the Dixon plot.

RESULTS AND DISCUSSION

Identification of Inhibitory Proteins. CSP was used for the first time as a resource to isolate CPI so as to utilize it to prevent the deterioration of surimi-based products. Inhibitory activity staining against papain was performed to detect CPI in CSP for further purification. All proteins in CSP were shown in **Figure 1**, lanes 2 and 3. The protein pattern of CSP on the electrophorized gel was significantly discriminated dependent upon the presence or absence of β-ME, a reducing agent, in the sample buffer. Under nonreducing condition (without β-ME in the sample buffer), four groups of proteins corresponding to different ranges of molecular masses, i.e., lower than 29 kDa, between 45 and 66 kDa, between 66 and 116 kDa, and higher than 205 kDa, were clearly observed. However, under reducing condition (with 10% β-ME in the sample buffer), only the protein band with the molecular mass less than 29 kDa was similar to that under nonreducing condition. Other groups of protein bands were substituted with protein bands with molecular masses between 116 and 45 kDa. Thus, both under nonreducing and reducing conditions, inhibitory activity staining was performed to detect inhibitory proteins in CSP (**Figure 1**, lanes 4–7). One inhibitory zone corresponding to the molecular mass of 55 kDa was detected on the gel under nonreducing condition. The color depth of the inhibitory zone increased due to the increase in CSP loading amounts (**Figure 1**, lanes 4 and 5). Therefore, the protein corresponding to the molecular mass of 55 kDa under nonreducing condition might be a CPI in CSP. Contrarily, no inhibitory zones were detected on the gel under reducing conditions regardless of CSP loading amounts (**Figure 1**, lanes 6 and 7). Similar results were found during the inhibitory activity staining of CPI from chicken plasma (*13*). It was suggested that the active form of CSP CPI was possibly stabilized by a disulfide bond, which would be further discussed after CPI was purified from CSP.

Purification of the Cysteine Protease Inhibitor. A CPI was purified from CSP by one step of affinity chromatography on CNBr−papain−Sepharose 4B (**Table 1**). Affinity chromatography was widely used in the purification of CPIs from a lot of resources due to its simplicity and high purification capacity (*13–16, 19, 30–32*). Because there are more than 100 kinds of proteins in plasma (*2, 3*), two washing buffers in which the second contained 1 M NaCl were used to remove the unabsorbed and nonspecifically bound proteins. After washing and eluting, a single protein peak (Abs_{280}) together with an inhibitory activity peak against papain was fractionated (**Figure 2**). Fractions containing higher than 50% of maximal inhibitory activity were pooled. Specific inhibitory activity, purification ratio, and yield of CPI purified by CNBr−papain−Sepharose 4B chromatography were 3.84 units/mg, 30.36-fold, and 0.94%, respectively. The single step purification in this study resulted in higher yield than that of CPI purification from glassfish egg, which was only 0.25% (*19*). The purity of CPI in chicken plasma increased about 25-fold after purification by carboxymethyl- (CM-) papain−Sepharose 4B chromatography (*13*), which was a little lower than 30.36-fold in this study. A kininogen was even purified from PPP by a series of DEAE-Sepharose, CM-Sepharose, and Sephacryl S-200 chromatographies (*6*). Acidification, DEAE-TSK, and CM-TSK chromatographies were used for the cystatin purification from carp ovarian fluid (*17*). Compared with those performances, the purification procedure of CSP CPI on CNBr−papain−Sepharose 4B in this study was simpler and more time saving and, therefore, could be used as an effective purification process for CPI from CSP.

Electrophoresis. SDS−PAGE of CSP CPI was run under both reducing and nonreducing conditions (**Figure 3**). Only under reducing condition can molecular masses of proteins be

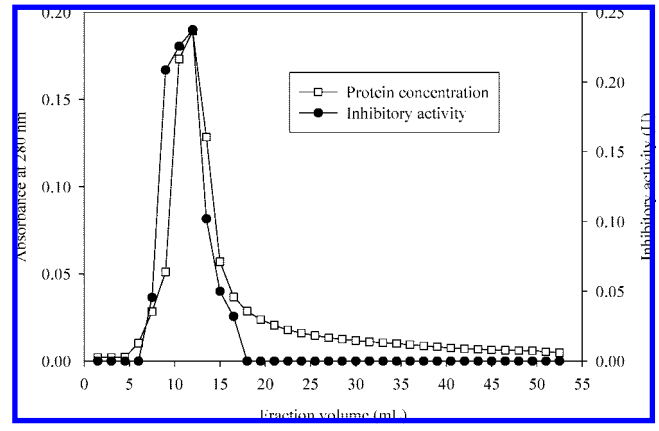

Figure 2. Elution profiles of the cysteine protease inhibitor from chum salmon plasma on CNBr−papain−Sepharose 4B.

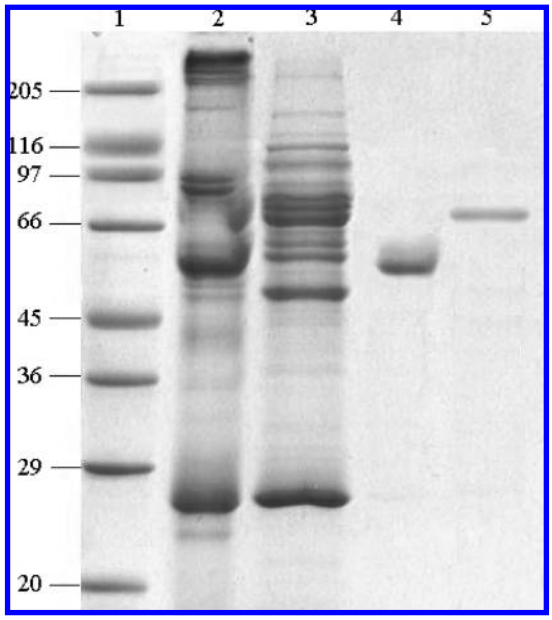

Figure 3. Protein pattern of the cysteine protease inhibitor: lane 1, protein marker; lanes 2 and 3, 25 μg of chum salmon plasma protein under nonreducing and reducing conditions, respectively; lanes 4 and 5, 5 μg of purified cysteine protease inhibitor under nonreducing and reducing conditions, respectively.

determined by SDS−PAGE, in which proteins migrate in the gel according to their molecular masses (*33*). The molecular mass of CSP CPI was determined to be 70 kDa by SDS−PAGE under reducing condition (**Figure 3**, lane 5), which was coincident with the result estimated by Sephacryl S-100 gel filtration chromatography. SDS−PAGE of CSP CPI under nonreducing condition was also run (**Figure 3**, lane 4). One protein band corresponding to a molecular mass of 55 kDa was observed, which coincided with that of the stained protein band under nonreducing condition (**Figure 1**, lanes 4 and 5). Therefore, the stained protein and CSP CPI were concluded to be the same protein in CSP. β-ME in the sample buffer reduced the intrachain disulfide bonds and might cause unfolding of proteins in CSP (*33*), which decreased the running speed of CPI under reducing condition more than under nonreducing condition during electrophoresis. For the same reason, CSP CPI under reducing condition might not be renatured on the electrophorized gel. Hence, no stained protein band was detected on the gel (**Figure 1**, lanes 6 and 7).

PAS Staining. Most of the proteins in blood plasma were found to be glycoproteins (*2*). PAS staining was proceeded to determine whether CSP CPI was glycosylated or not. A magenta band was observed where CSP CPI was on the gel under nonreducing or reducing conditions (**Figure 4**, lanes 2 and 3), which indicated that CSP CPI was a glycoprotein. A CPI with a molecular mass of 76 kDa was purified from Atlantic salmon skin and consisted of a single polypeptide chain, which was found to be both N- and O-glycosylated (*16*). The chum salmon egg inhibitor, with a molecular mass of 72.6 kDa, consisted of two protein bands with molecular masses of 54 and 18.6 kDa, respectively (*15*). Both above CPIs were classified as kininogens, the family III of cysteine protease inhibitors which are glycoproteins with molecular mass ranging from 50 to 120 kDa (*34*). Because CSP CPI was a glycoprotein with a molecular mass of 72 kDa (within the range of 50–120 kDa), it was tentatively classified as a kininogen.

Inhibitory Activity of the Cysteine Protease Inhibitor. Papain from plant and cathepsin L from mammal were used to determine the inhibitory activity of CSP CPI. Because cathepsin L was highly unstable and sensitive, only papain was used for other characterizations of CSP CPI. CSP CPI inhibited the cysteine proteases such as papain and cathepsin L (**Table 2**) but not trypsin, a serine protease (data not shown). The inhibitory activity of CSP CPI was compared with those of other inhibitors, whose inhibitory activities were determined using the same method as in this study. CSP CPI possessed higher specific inhibitory activity against papain than did that of the chum salmon egg inhibitor, whereas both were similar to the inhibitory activity against cathepsin L (*15*). Chicken egg white cystatin possessed higher specific inhibitory activity against papain but significantly lower specific inhibitory activity against cathepsin L than did CSP CPI. Therefore, CSP CPI appeared to be more suitable than chicken egg white cystatin to inhibit cathepsin L such as in surimi processing.

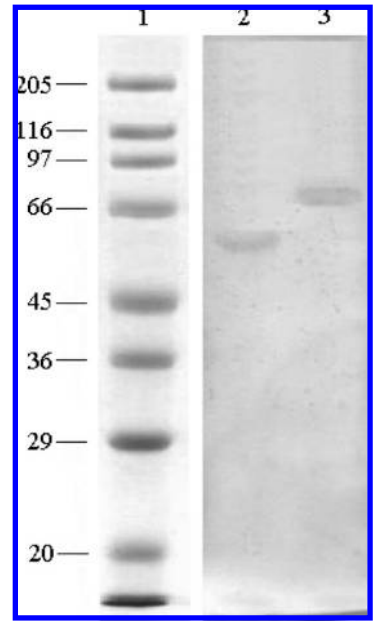

Figure 4. PAS staining of the cysteine protease inhibitor: lane 1, protein marker; lanes 2 and 3, 5 μg of purified cysteine protease inhibitor under nonreducing and reducing conditions, respectively.

Table 2. Comparison of Inhibitory Activity of the Cysteine Protease Inhibitor with Others against Papain and Cathepsin L[a]

	specific inhibitory activity (units/mg)	
inhibitor	papain	cathepsin L
CSP CPI	9.74 ± 0.25	27.56 ± 0.36
chum salmon egg inhibitor[b]	4.67 ± 0.14	28.02 ± 0.17
chicken egg white cystatin	36.65 ± 0.31	15.83 ± 0.25

[a] Values are given as the mean ± standard deviation from triplicate determinations. [b] Kim et al. (*16*).

pH and Thermal Stability and Thermal Activity of the Cysteine Protease Inhibitor. pH and thermal stability and thermal activity of CSP CPI were important for its practical application. Because azocasein, the substrate for papain, can only be dissolved in a narrow pH range (*26*), the pH activity of CSP CPI was not determined. As shown in **Figure 5**, CSP CPI was relatively stable under a wide pH range of 6.0–9.0, with maximal stability at pH 7.0, which was more stable than the chum salmon egg inhibitor with a stable pH range of 6.0–7.0 and maximal stability at pH 6.0 (*15*). The inhibitory activity of CSP CPI decreased under extreme acidic (pH <4.0) and alkaline conditions (pH >10.0) (**Figure 5**), which was still superior to that of the chum salmon egg inhibitor with nearly no activity

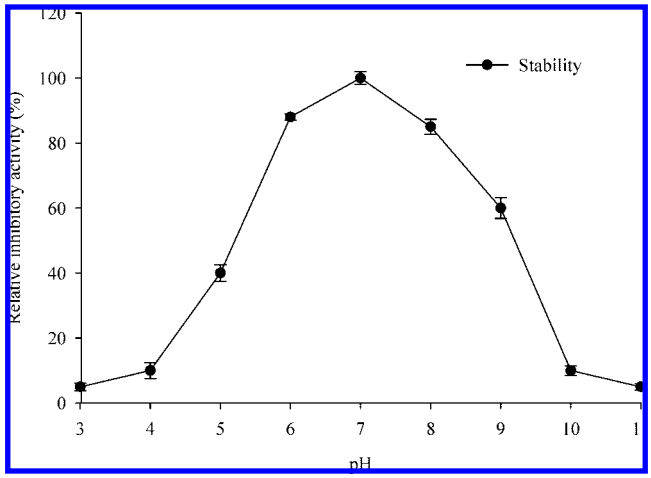

Figure 5. pH stability of the cysteine protease inhibitor against papain. Bars represent the standard deviation from triplicate determinations.

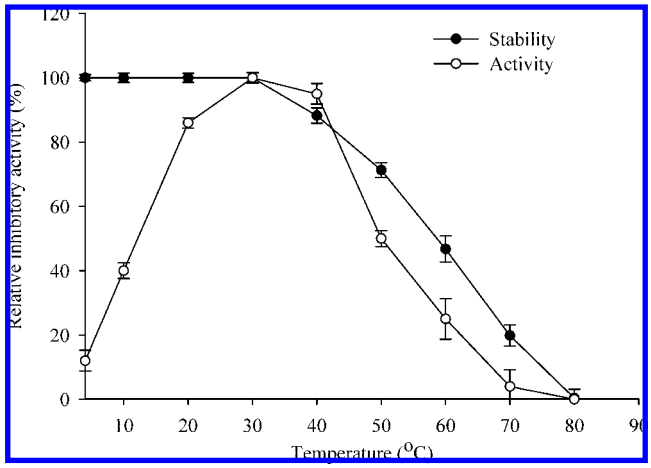

Figure 6. Thermal stability and activity of the cysteine protease inhibitor against papain. Bars represent the standard deviation from triplicate determinations.

below pH 4 and above pH 8 (*15*) and was also superior to that of the glassfish egg inhibitor (*19*) with no activity below pH 6. As most endogenous cysteine proteases are active at weak acidic pH with maximal activity around pH 7.0 (*34, 35*), the pH profiles of CSP CPI facilitated its practical application in the inhibition of endogenous cysteine proteases, such as fish disease cure and prevention and gel weakening prevention in surimi processing.

CSP CPI was relatively stable at temperatures below 40 °C (**Figure 6**). Seventy percent of inhibitory activity of CSP CPI was still retained after a 30 min incubation at 50 °C. In contrast, the chum salmon egg inhibitor presented stability only at temperatures lower than 35 °C (*15*), and partially purified CPI from chicken plasma presented stability after heating for only 10 min (*13*). Inhibitory activity of CSP CPI decreased to 45% and 18% when incubated at 60 and 70 °C for 30 min, respectively. The CPIs from Atlantic salmon were relatively stable up to 80 °C in which most of its inhibitory activity was retained (*16*). The glycosylation of CPIs might contribute to their thermal and pH stabilities (*16, 36*).

Besides thermal stability, the thermal activity of CSP CPI was also determined so as to better understand and utilize this inhibitor. To our best knowledge, this kind of information was not provided by other studies on plasma CPIs. CSP CPI was relatively active at temperatures of 20–40 °C (**Figure 6**). Even at 10 and 50 °C, CSP CPI still possessed 50% of its maximal inhibitory activity. Temperature (50–70 °C) can activate endogenous cysteine proteases naturally occurring in fish muscle and cause structure disintegration of surimi-based products (*35*). Thus, CSP CPI might be applied to inhibit the endogenous cysteine proteases in fish muscle, such as cathepsins B, L, and H, to enhance the gel strength of surimi-based products.

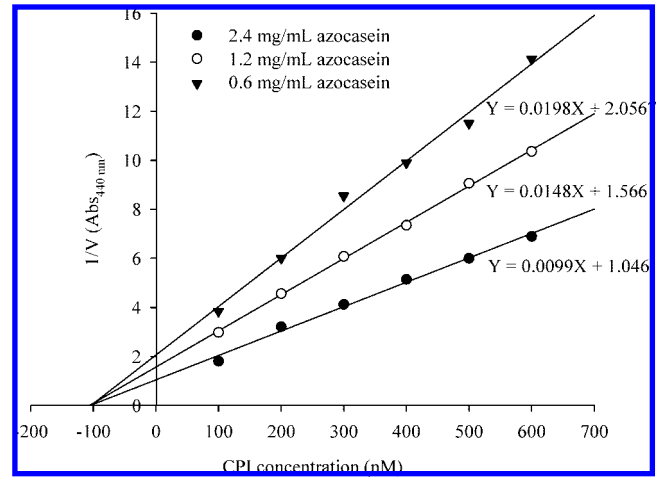

Figure 7. Dixon plot of the cysteine protease inhibitor against papain at different concentrations of azaocasein.

Inhibition Type of the Cysteine Protease Inhibitor. At different concentrations of CSP CPI, the hydrolysis rates of azocasein by papain were determined to make the Dixon plot (**Figure 7**). Because the three lines [$1/V(\mathrm{Abs}_{440\mathrm{nm}})$ vs CPI concentration (nanomolar)] intersected on the negative *X*-axis (*29*), the inhibition of CSP CPI against papain was concluded to be in a noncompetitive matter with a K_i value of 105 nM. The 105 nM of CSP CPI compared favorably with 150 nM of purified CPI with a molecular mass of 13 kDa from rabbit skeletal muscle (*31*). Lower K_i values were found for CPIs from chum salmon egg (0.1 and 0.06 nM), which were analyzed using benzoyl-DL-arginine naphthylamide as the substrate for papain (*14*). The relatively lower K_i value of CSP CPI against papain indicated that CSP CPI is a tightly binding inhibitor of cysteine proteases.

In summary, a cysteine protease inhibitor with the molecular mass of 70 kDa was purified, for the first time, from chum salmon plasma by affinity chromatography with a yield and purification ratio of 0.94% and 30.36-fold, respectively. The cysteine protease inhibitor was a glycoprotein and was classified as a kininogen. It showed high pH stability, high thermal stability, and activity, and it was a noncompetitive inhibitor against papain with a low K_i value of 105 nM. The results of this study indicate that CSP CPI can be used in surimi processing to prevent gel weakening.

LITERATURE CITED

(1) Zhou, W. T.; Fujita, M.; Yamamoto, S. Effects of ambient temperatures on blood viscosity and plasma protein concentration of broiler chickens. *J. Therm. Biol.* **1999**, *24*, 105–112.

(2) Halliday, D. A. Blood—A source of proteins. *Proc. Biochem.* **1973**, *8*, 15–17.

(3) Duarte, R. T.; Carvalho Simoões, M. C.; Sgarbieri, V. C. Bovine blood components: fractionation, composition, and nutritive value. *J. Agric. Food Chem.* **1999**, *47*, 231–236.

(4) Chang, Y. C.; Sun, H. L.; Chang, H. M. Effect of calcium and thrombin on the turbidity changes and the gelation properties of swine plasma. *Food Res. Int.* **1999**, *32*, 553–558.

(5) Benjakul, S.; Visessanguan, W.; Srivilai, C. Porcine plasma protein as proteinase inhibitor in bigeye snapper (*Priacanthus tayenus*) muscle and surimi. *J. Sci. Food Agric.* **2001**, *81*, 1039–1046.

(6) Lee, J. J.; Tzeng, S. S.; Wu, J.; Jiang, S. T. Inhibition of thermal degradation of mackerel surimi by pig plasma protein and l-kininogen. *J. Food Sci.* **2000**, *65*, 1124–1129.
(7) Kang, I. S.; Lanier, T. C. Bovine plasma protein functions in surimi gelation compared with cysteine protease inhibitors. *J. Food Sci.* **1999**, *64*, 842–846.
(8) Benjakul, S.; Visessanguan, W. Pig plasma protein: potential use as proteinase inhibitor for surimi manufacture; inhibitory activity and the active components. *J. Sci. Food Agric.* **2000**, *80*, 1351–1356.
(9) Weerasinghe, V. C.; Morrissey, M. T.; An, H. Characterization of active components in food-grade proteinase inhibitors for surimi manufacture. *J. Agric. Food Chem.* **1996**, *44*, 2584–2590.
(10) Rawdkuen, S.; Benjakul, S.; Visessanguan, W.; Lanier, T. C. Chicken plasma protein affects gelation of surimi from bigeye snapper (*Priacanthus tayenus*). *Food Hydrocolloids* **2004**, *18*, 259–270.
(11) Rawdkuen, S.; Benjakul, S.; Visessanguan, W.; Lanier, T. C. Chicken plasma protein: protease inhibitory activity and its effect on surimi gel properties. *Food Res. Int.* **2004**, *37*, 156–165.
(12) Freedman, S. J. The role of α_2-macroglobulin in furunculosis: a comparison of rainbow trout and brood trout. *Comp. Bicochem. Physiol., Part B: Biochem. Mol. Biol.* **1991**, *4*, 549–553.
(13) Rawdkuen, S.; Benjakul, S.; Visessanguan, W.; Lanier, T. C. Partial purification and characterization of cysteine proteinase inhibitor from chicken plasma. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **2006**, *144*, 544–552.
(14) Yamashita, M.; Konagaya, S. Cysteine protease inhibitor in egg of chum salmon. *J. Biochem.* **1991**, *110*, 762–766.
(15) Kim, K. Y.; Ustadi; Kim, S. M. Characteristics of the protease inhibitor purified from chum salmon (*Oncorhynchus keta*) eggs. *Food Sci. Biotechnol.* **2006**, *15*, 28–32.
(16) Ylönen, A.; Rinne, A.; Herttuainen, J.; Bøgwald, J.; Järvinen, M.; Kalkkinen, N. Atlantic salmon (*Salmo salar* L.) skin contains a novel kininogen and another cysteine protease inhibitor. *Eur. J. Biochem.* **1999**, *266*, 1066–1072.
(17) Tsai, Y. J.; Chang, G. D.; Huang, C. J.; Chang, Y. S.; Huang, F. L. Purification and molecular cloning of carp ovarian cystatin. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **1996**, *113*, 573–580.
(18) Ustadi; Kim, K. Y.; Kim, S. M. Protease inhibitor in fish eggs: A comparative study. *J. Ocean Univ. China* **2005**, *4*, 198–204.
(19) Ustadi; Kim, K. Y.; Kim, S. M. Purification and identification of a protease inhibitor from glassfish (*Liparis tanakai*) eggs. *J. Agric. Food Chem.* **2005**, *53*, 7667–7672.
(20) Bjorklund, H. V.; Johansson, T. R.; Rinne, A. Rhabdovirus-induced apoptosis in a fish cell line is inhibited by a human endogenous acid cysteine protease inhibitor. *J. Virol.* **1997**, *71*, 5658–5662.
(21) Nomura, I. *The state of world fisheries and aquaculture 2006*; Food and Agriculture Organization of the United Nations: Rome, Italy, 2007; p 3.
(22) Garcia-Carreno, F. L.; Dimes, L. E.; Haard, N. F. Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors. *Anal. Biochem.* **1993**, *214*, 65–69.
(23) Izquierdo-Pulido, M. L.; Haard, T. A.; Hung, J.; Haard, N. F. Oryzacystatin and other proteinase inhibitors in rice grain: Potential use as a fish processing aid. *J. Agric. Food Chem.* **1994**, *42*, 616–622.
(24) Pisani, F. M.; Rella, R.; Raia, C. A.; Rozzo, C.; Nucci, R.; Gambacorta, A.; De Rosa, M.; Rossi, M. Thermostable β-galactosidase from the Archaebacterium *Sulfolobus solfataricus*: purification and properties. *Eur. J. Biochem.* **1990**, *187*, 321–328.
(25) Zacharius, R. M.; Zell, T. E.; Morrison, J. H.; Woodlock, J. J. Glycoprotein staining following electrophoresis on acrylamide gels. *Anal. Biochem.* **1969**, *30*, 148–152.
(26) Sarath, G.; Motte, R. S.; Wagner, F. W. Protease assay methods. In *Proteolytic enzymes: a practical approach*; Beynon, B. J., Ed.; Oxford University Press: Oxford, England, 1989; p 28.
(27) Tzeng, S. S.; Chen, G. H.; Jiang, S. T. Expression of soluble thioredoxin fused-carp (*Cyprinus Carpio*) ovarian cystatin in *Escherichia coli*. *J. Food Sci.* **2002**, *67*, 2309–2316.
(28) Robinson, H. W.; Hodgen, C. G. The biuret reaction in the determination of serum protein. I. A study of the condition necessary for the production of the stable color which bears a quantitative relationship to the protein concentration. *J. Biol. Chem.* **1940**, *135*, 707–725.
(29) Dixon, M.; Webb, E. C. *Enzymes*; Logman Group Ltd.: London, 1979; p 556.
(30) Tsushima, H.; Ueki, A.; Mine, H.; Nakajima, N.; Sumi, H.; Hopsu-Havu, V. K. Purification and characterization of a cystatin-type cysteine proteinase inhibitor in the human hair shaft. *Arch. Dermatol. Res.* **1992**, *284*, 380–385.
(31) Matsuishi, M.; Okitani, A. Purification and properties of cysteine proteinase inhibitors from rabbit skeletal muscle. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **2003**, *136*, 309–316.
(32) Rassam, M.; Laing, W. A. Purification and characterization of phytocystatins from kiwifruit cortex and seeds. *Phytochemistry* **2004**, *65*, 19–30.
(33) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature* **1970**, *227*, 680–685.
(34) Otto, H. H.; Schirmeister, T. Cysteine proteases and their inhibitors. *Chem. Rev.* **1997**, *97*, 133–171.
(35) An, H.; Peters, M. Y.; Seymour, T. A. Roles of endogenous enzymes in surimi gelation. *Trends Food Sci. Technol.* **1996**, *7*, 321–327.
(36) Tzeng, S. S.; Jiang, S. T. Glycosylation modification improved the characteristics of recombinant chicken cystatin and its application on mackerel surimi. *J. Agric. Food Chem.* **2004**, *52*, 3612–3616.

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