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Characteristics of the Protease Inhibitor Purified from Chum Salmon (*Oncorhynchus keta*) Eggs

Keun Yeong Kim[1], Ustadi[2] and Sang Moo Kim[1]*

[1]*Faculty of Marine Bioscience and Technology, Kangnung National University, Gangneung, Gangwon 210-702, Korea*
[2]*Fisheries and Marine Department, Agriculture Faculty, Gadjah Mada University, Bulaksumur, Yogyakarta, Indonesia*

Abstract Protease inhibitor of 72.6 kDa was successively purified from chum salmon (*Oncorhynchus keta*) eggs by ion exchange, gel permeation, and affinity chromatographies. Protease inhibitor was purified with yield and purification fold of 1.50% and 58.11, respectively. SDS-PAGE results showed purified protease inhibitor consisted of two protein subunits of 54.0 and 18.6 kDa. Chum salmon inhibitor exhibited stability between 20 and 40°C in weak acid environment (pH 6), and inhibited papain and cathepsin, members of cysteine protease, but not chymotrypsin. The protein inhibited cathepsin more effectively than did egg white protease inhibitor, whereas the reverse was true for papain. These results indicate chum salmon egg inhibitor is heterodimer, thus the inhibitor was classified as cysteine protease inhibitor.

Keywords: protease inhibitor, chum salmon egg, cysteine protease

Introduction

The cysteine protease inhibitors, which are widely distributed in animal muscle and body fluid, are classified into three families based on their structural complexities (1). Family I cystatins lack disulfide bonds and include cystatins A (2) and B (3), and rat cystatin β (4). Family II cystatins are characterized by two disulfide bonds and include human cystatin S (5), chicken egg white cystatin (6), mouse cystatin (7), and rat cystatin (4). Molecular weights of Family I and II cystatins range from 10 to 20 kDa. Family III cystatins include kininogens, which are single-chain glycoproteins containing three cystatin-like domains with molecular weights ranging from 68 to 120 kDa (8). Protease inhibitors of the fish eggs are thought to be involved in the protection of eggs from microorganisms, embryogenesis, and the regulation of early embryonic growth. Cystatins may also contribute to the defense against viral proteases that are necessary for virus replication (9). In addition, cysteine protease inhibitors have also been used for medical treatment of parasite diseases (10) and malaria (11), as well as prevention of modori phenomenon, in which endogenous protease causes deterioration of the gel quality of surimi-based product (12). Some food-grade protease inhibitors such as egg white, bovine plasma protein, potato powder, and whey protein have been used to prevent modori phenomenon (13); however, they cause side effects such as color changes in surimi-based products (12). Cysteine protease inhibitors were purified from ovarian fluid carp (14), egg and muscle of chum salmon caught in Pacific Ocean near Japan (9, 15), muscle of white croaker (16), and Atlantic salmon and Arctic charr (17). There is a strong demand to prevent the quality deterioration of surimi-based product or fish meat (18). One of the most efficient methods is to inhibit digestive fish muscle proteases. Therefore, the objective of this study was to purify and characterize the protease inhibitor from the eggs of chum salmon, and to develop a novel inhibitor preventing modori phenomenon in surimi-based products.

*Corresponding author: Tel: 82-33-640-2343; Fax: 82-33-647-9535
E-mail: smkim@kangnung.ac.kr
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Materials and Methods

Materials The eggs from a mature chum salmon, *Oncorhynchus keta*, were harvested immediately after catching in the coast of East Sea, Korea and were stored at -40°C until used. Papain, trypsin, cathepsin, azocasein, and protein molecular weight markers were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The remaining chemicals used in this study were of first reagent grade. CM Sepharose and CNBr-activated Sepharose 4B were purchased from Amersham Pharmacia Biotech, Ltd. (Uppsala, Sweden).

Purification of protease inhibitor Purification of protease inhibitor was purified by the modified method of Moon and Kim (19). Fish eggs (250 g) were homogenized in 1 L of 25 mM sodium phosphate buffer, pH 7.0, containing 50 mM NaCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol (buffer A). Cell and tissue debris were precipitated and removed from the homogenate by centrifugation at 10,730×g for 25 min. To further purify the extract, the supernatant was dialyzed overnight against 50 mM sodium acetate buffer, pH 5.5, containing 50 mM NaCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol (buffer B). The dialyzed fraction was applied to a CM-Sepharose column (2.6×30.0 cm) equilibrated with buffer B. Proteins were eluted from the column using a linear gradient of 0 to 1 M NaCl in sodium acetate buffer (pH 5.5) at 1 mL/min. Fractions containing higher than 50% of maximal inhibitory activity were pooled and dialyzed against a 25 mM sodium phosphate buffer, pH 7.5, containing 0.15 M NaCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol (buffer C). The pooled fractions were concentrated by

ultrafiltration using a 10-kDa cutoff membrane (Amicon Co., Beverly, MA, USA). The concentrate was then loaded onto a Sephacryl column (2.6×60.0 cm) equilibrated with buffer C and was eluted at 0.2 mL/min.

Affinity chromatography Aliquot (7 g) of CNBr-activated Sepharose 4B was washed and re-swelled on a glass filter (size G3) with 1.5 L of 1 mM HCl. Subsequently, 20 mL of 5 mg/mL papain solution in coupling buffer (1 M $NaHCO_3$, pH 8.3, 0.5 M NaCl) was mixed with the gel and stirred overnight at 4°C. The gel was then transferred to 40 mL blocking agent (0.2 M glycine, pH 8.0) and stirred overnight at 4°C. The gel was sequentially washed with 0.1 M acetate buffer (pH 4.0, 0.5 M NaCl) and the coupling buffer. Finally, the gel was poured into a column (1.0×20.0 cm), which was then equilibrated with buffer A. Aliquot (50 mL) of the pooled fractions purified by Sephacryl gel permeation chromatography was loaded onto the affinity column and washed with buffer A. The protease inhibitor was eluted with 50 mM trisodium phosphate buffer, pH 10, containing 50 mM NaCl at 0.3 mL/min.

Inhibitory activity assay Chum salmon protease inhibitory activity was determined by measuring the degree of inhibition of papain activity using azocasein as the substrate. Aliquot (200 μL) of 1.7 μg/mL inhibitor solution in buffer A was added to 100 μL papain solution (0.1 U activity) in buffer A. The combined solution was incubated at 37°C for 5 min and added to 250 μL of 3.2 mg/mL azocasein substrate solution in buffer A. Following 30 min incubation at 37°C, the reaction was stopped by adding 700 μL of 20% trichloroacetic acid (TCA). A control was prepared by substituting 200 μL inhibitor solution with 200 μL buffer A. A blank was prepared by adding 700 μL of 20% TCA before adding 250 μL substrate solution. For color development, the reaction mixture was centrifuged at 10,000×g for 5 min, and 720 mL supernatant was added to 800 μL of 1 N NaOH. The absorbance was measured at 440 nm. Inhibitory activity was calculated based on the difference between papain activities with and without inhibitor. One unit each of the papain and inhibitory activities were defined as the amount of 0.1 μM azocasein hydrolyzed per min and one unit decrease of papain activity, respectively (13, 20).

Heat and pH stability The purified chum salmon inhibitor was incubated for 30 min at temperatures ranging from 5 to 80°C and at varying pH values (pH range: 2 - 8) to determine the heat and pH stabilities of the protein. Residual papain inhibitory activity was then determined at 37°C as described above.

Electrophoresis The chum salmon protease inhibitor was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and polyacrylamide gel electrophoresis (PAGE, native gel). Briefly, purified chum salmon protease inhibitor was added to the sample buffer of SDS-PAGE (100 mM Tris-HCl, pH 6.8, 2.5% SDS, 0.01% bromophenol blue, 2% glycerol, and 10% β-mercaptoethanol) to give a final protein concentration of 1.5 mg/mL. After 4 min incubation at 95°C, 5 μL prepared sample was applied to a 12% SDS-polyacrylamide slab gel at pH 8.3 (21). Sample buffer of PAGE (22) was composed of 15.5 mL of 1 mM Tris-HCl buffer, 2.5 mL of 1% bromophenol blue, 7 mL water, and 25 mL glycerol, pH 6.8. Mixture of the sample buffer of PAGE and chum salmon protease inhibitor at a final protein concentration of 1.5 mg/mL was applied to a 12% polyacrylamide slab gel at pH 8.3 (21).

Protein assay Protein assay was performed using a protein kit (Bio-Rad Lab. Inc., Hercules, CA, USA) according to the manufacturer's instructions. Bovine serum albumin was used as the calibration standard. The relative protein concentration of the chromatographically separated fraction was estimated by measuring absorbance at 280 nm.

Results and Discussion

Purification of protease inhibitor The purification of chum salmon egg protease inhibitor is summarized in Table 1. The fractionation pattern of protease inhibitor determined by CM Sepharose chromatography is shown in Fig. 1. Two protein peaks (CM I and CM II) showing inhibitory activity were observed, with the inhibitory activity of the second peak (CM II) being higher than the first one (CM I).

A similar pattern was obtained from an ovarian carp-isolated protease inhibitor through CM-TSK chromatography (14). Specific inhibitory activities between 1 and 7 U/mg were observed in the pooled azocaseinolytic-active ion exchange chromatography fractions of the protease inhibitors isolated from different fish species (20), which were comparable to the results of this study. Synnes (23) reported that ion exchange chromatography of Atlantic salmon skin protease inhibitor showed two peaks, in which the second peak had higher inhibitory activity. Therefore, the second peak (CM II) of CM-Sepharose chromatography was chosen for further purification by Sephacryl gel permeation chromatography; only one protein inhibitory peak was detected, with two protein bands on SDS-PAGE (data not shown). Therefore, pool of Sephacryl chromatography peak was further fractionated

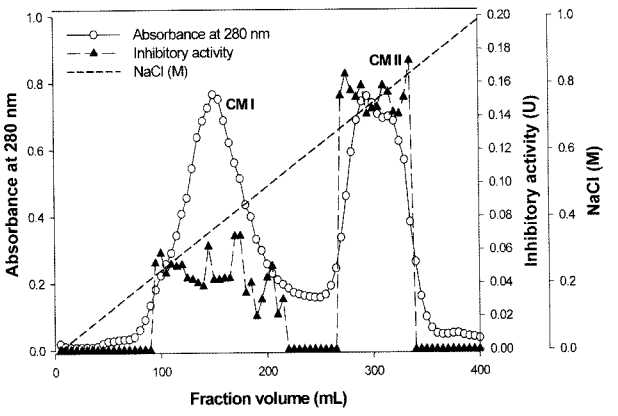

Fig. 1. CM Sepharose chromatography pattern of chum salmon egg protease inhibitor.

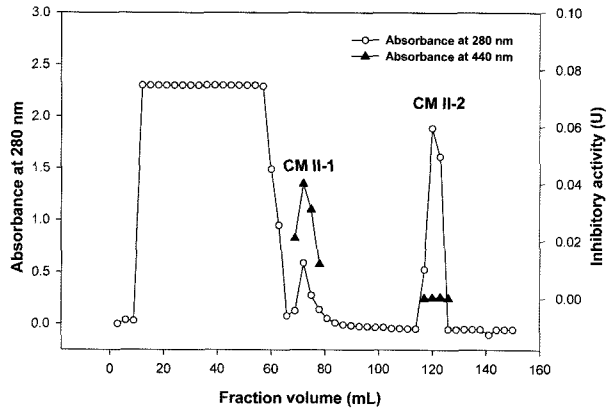

Fig. 2. CNBr-Sepharose 4B chromatography pattern of chum salmon egg protease inhibitor.

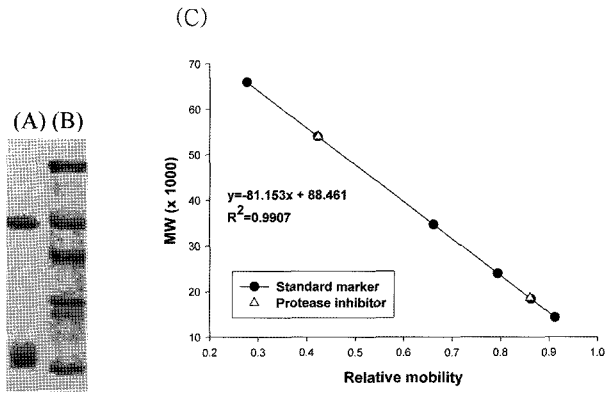

Fig. 3. SDS-polyacrylamide gel electrophoresis of chum salmon egg protease inhibitor. (A), SDS-PAGE of chum salmon egg protease inhibitor (54 kDa and 18.6 kDa); (B), Standard marker: bovine serum albumin (66 kDa), egg albumin (54 kDa), pepsin (34.7 kDa), trypsinogen (24 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.3 kDa); (C), Estimation of MW of protease inhibitor; (● : standard marker, △ : CM II-1).

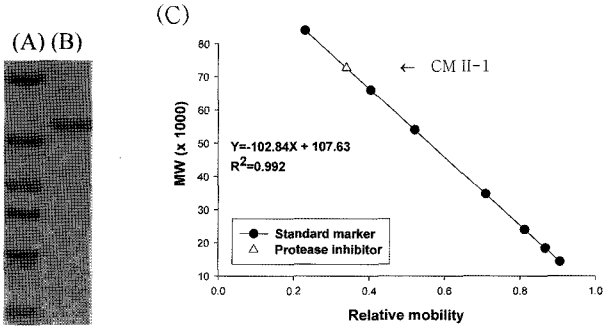

Fig. 4. Polyacrylamide gel electrophoresis of chum salmon egg protease inhibitor. (A) Standard marker: bovine serum albumin (84 kDa) bovine serum albumin (66 kDa), egg albumin (54 kDa), pepsin (34.7 kDa), trypsinogen (24 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.3 kDa); (B) chum salmon egg protease inhibitor (72.6 kDa); (C), Estimation of MW of protease inhibitor.

by affinity chromatography, and, among the two protein peaks (elution volumes, 72 and 117 mL) had no inhibitory activity obtained, only the smallest peak showed inhibitory activity against papain (Fig. 2). The specific inhibitory activity and purification fold of the smallest peak were 4.67 U/mg and 58.11, respectively (Table 1), higher than the specific inhibitory activity of protease inhibitor (3.8 U/mg) of from chum salmon caught in Japan (9). However, the purification yield and fold (1.5% and 58.11, respectively) of protease inhibitor from chum salmon egg captured in the coast of East Sea, Korea of this study were lower than 1.9% and 93 fold of salmon caught in the coast of the Pacific Ocean, Japan (9). Purification of Atlantic salmon skin protease inhibitor showed similar result on affinity chromatography in that the first peak has higher inhibitory activity than the second one (23).

Electrophoresis SDS-PAGE and PAGE (native gel) analyses of the smallest peak are shown in Fig. 3 and 4, respectively. SDS-PAGE analysis showed two protein bands with MW 54 and 18.6 kDa, whereas PAGE analysis showed only one protein band with MW 72.6 kDa, indicating that chum salmon egg protease inhibitor is a heterodimer protein. SDS-PAGE analysis of the protease inhibitor purified from chum salmon caught in Japan resulted in two protein bands with MW 11 and 16 kDa classified as cystatin (9). Synnes (23) reported that MW of protease inhibitor from Atlantic salmon (*Salmon salar* L) skin was 76 kDa and classified it as a kininogen (24, 25). Kininogn, a single chain glycoprotein consisting of two chains, heavy and light, connected by a single disulfide bond, is easily converted into two-chain forms (26). Therefore, based on these findings, cysteine protease inhibitor from salmon egg with MW 72.6 kDa was tentatively classified as a kininogen.

Properties of the protease inhibitor The chum salmon egg protease inhibitor inhibited the cysteine proteases such as papain, and cathepsin (Table 2), but not trypsin, a serine protease (data not shown). Furthermore, it demonstrated higher inhibitory activity against cathepsin than against

Table 1. Purification of cysteine protease inhibitor from chum salmon egg by ion exchange and CNBr-Sepharose 4B-coupled papain affinity chromatography

Purification step	Total protein (mg)	Total inhibitory activity (U)	Specific Inhibitory activity (U/mg)	Yield (%)	Purity (fold)
Extract	1,046.00	84.00	0.08	100.00	1.00
CM Sepharose	5.72	11.60	2.02	13.75	15.14
CNBr Sepharose 4B (CM II-1)	0.27	1.26	4.67	1.50	58.11

Table 2. Comparison of inhibitory activity of chum salmon egg protease inhibitor with chicken egg white and chymotrypsin potato I against papain and cathepsin L

Inhibitor	Specific inhibitory activity (U/mg)[d]	
	Papain	Cathepsin L
Salmon egg	4.67[c]	28.02[a]
Chicken egg white	37.71[a]	16.05[b]
Chymotrypsin potato I	2.00[c]	4.12[c]

[a,b,c]Means in the same column with different superscripts are significantly different ($p<0.05$).
[d]Mean values obtained from four replications.

papain, possibly because the cathepsin is a fish muscle protease, while papain is a plant protease (9). Although protease inhibitor from chum salmon caught in Japan inhibited papain and cathepsin L, the specific inhibitory activity against papain, 3.8 U/mg (9), was lower than 4.67 U/mg of protease inhibitor from chum salmon caught in Korea in this study (Table 1).

The chum salmon egg protease inhibitor was relatively stable at low temperatures (Fig. 5). Inhibitory activity of 80% was retained after 30 min incubation at 35°C. Inhibitory activity of chum salmon egg inhibitor decreased by 60 and 70% when incubated at 50 and 65°C, respectively. Although most of the residual inhibitory activity of salmon skin inhibitor remained after incubation at 70°C for 30 min, 60% residual inhibitory activity was lost after incubation at 80°C for 30 min (23). Forty percent residual inhibitory activity of *Actinomycetes* protease inhibitor remained after incubation at 60°C for 30 min (27). Moreover, chum salmon protease inhibitor at 60°C was shown to inhibit endogenous fish muscle proteases such as cathepsins B, H, and L, with optimal activity at 50 -60°C (12).

The chum salmon egg inhibitor was relatively stable within a pH range of 6 to 7, with maximal stability at pH 6 (Fig. 5). The residual inhibitory activity after incubation at 37°C and pH 7 for 30 min was 86.4%; however, no inhibition of papain was observed under extreme acid (pH <4) and alkaline (pH>8) conditions, as similarly observed by other research group, who showed that protease inhibitor from milt chum salmon was relatively stable within a pH range 5 to 7, and no inhibition of papain was observed under extreme acid and alkaline conditions (28). On the other hand, protease inhibitor from chum salmon

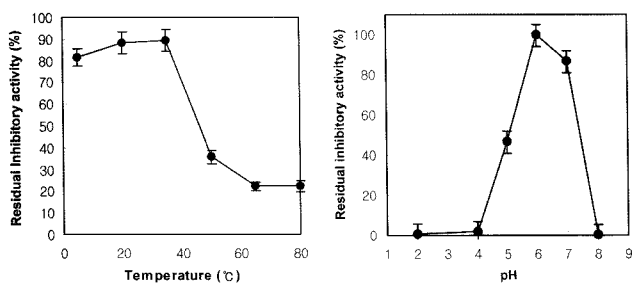

Fig. 5. Effects of temperature and pH on the stability of chum salmon egg protease inhibitor.

caught in Japan was relatively stable within a pH range of 2 to 7, with maximal activity at pH 3. Most endogenous cysteine proteases in fish muscle are also active at weak acid pH, with significant activity at around pH 7 (29). Therefore, salmon egg protease inhibitor with MW 72.6 kDa in this study could better inhibit cysteine protease in fish muscle and surimi product than that from chum salmon caught in Japan (9). The inhibitory activity of chum salmon egg inhibitor was compared with those of other inhibitors (Table 2). The specific inhibitory activity of chum salmon egg protease inhibitor against cathepsin (28.02 U/mg) was higher than against papain (4.67 U/mg). In contrast, the egg white inhibitor, which is also a member of the cystatain family II (25), demonstrated lower inhibitory activity against cathepsin (16.05 U/mg) than against papain (37.71 U/mg) (Table 2). The specific inhibitory activity of chymotrypsin potato, which is a member of the serine protease inhibitor, against papain and cathepsin were 2.00 and 4.12 U/mg, respectively. Cathepsin, an endogenous protease in fish muscle, plays an important role in softening surimi gel induced by heating (modori phenomenon). Therefore, the chum salmon egg protease inhibitor could be used as a substitute for chicken egg white, which is presently the most commonly used commercial inhibitor, to prevent modori phenomenon in the surimi-based product.

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