J. Microbiol. Biotechnol. (2006), **16**(4), 524–530

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

© The Korean Society for Microbiology and Biotechnology

Purification, Characterization, and Inhibitory Activity of Glassfish (*Liparis tanakai*) Egg High Molecular Weight Protease Inhibitor Against Papain and Cathepsin

USTADI[1], SANG GUAN YOU[2], AND SANG MOO KIM[2]*

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

Received: July 25, 2005
Accepted: September 3, 2005

Abstract Two protease inhibitors of 67 and 18 kDa, respectively, were purified from the eggs of glassfish, *Liparis tanakai*, by affinity chromatography and electro-elution method. The high molecular weight (HMW) protein was purified with a specific inhibitory activity, yield, and purity of 18.46 U/mg, 0.07%, and 131.86 fold, respectively, and was further characterized: Optimal temperature and pH for inhibitory activity of the HMW glassfish egg protease inhibitor were 40°C and pH 6, respectively, and it was stable between 5°C and 50°C in the pH range of 5–6 with maximal stability at pH 6. It was shown to be a competitive inhibitor against papain with an inhibition constant (K_i) of 97.02 nM. Moreover, the 67 kDa protein inhibited cathepsin, a cysteine protease, more effectively than did an egg-white protease inhibitor. The HMW glassfish egg protease inhibitor was classified as a member of the family III (kininogen).

Key words: Glassfish egg, HMW protease inhibitor, K_i, kininogen

The inhibitor of cysteine protease was first isolated from chicken egg white in 1968 [30]. This inhibitor was further characterized as cystatin and the first member of the cystatin superfamily [3]. The cystatin superfamily is divided into three structurally related families; stefins, cystatins, and kininogens [3]. Family 1 (stefin) lacks both disulfide bridges and carbohydrates [3]. Stefin has a molecular mass of around 11 kDa, and is the smallest in the cystatin superfamily. This family includes cystatins A and B, found in different mammals; human [1, 27], cow [37], rat [34], sheep [28], and pig [20]. Family II (cystatin) is also single chained with one domain protein and is about 2 kDa larger than the family I inhibitor. The polypeptide chains of the cystatins contain two disulfide bonds near their C-terminus [3]. Family III (kininogen) consists of a N-terminal heavy chain combined with a variable length light chain. The heavy chain has three cystatin-like domains. Based on the length of the light chain [3], the kininogen is divided into two subfamilies; a high molecular weight kininogen (HMW kininogen, ~120 kDa) and a low molecular weight kininogen (LMW kininogen, ~68 kDa).

The interaction between proteases and their inhibitors was a target of intensive study for the last two decades. Protease inhibitors were purified from ovarian fluid carp [36], egg and muscle of chum salmon [40, 45], muscle of white croaker [29], Atlantic salmon and Arctic charr [23], and hake, Argentine anchovy, castaneta, rough sead, and sea trout [5]. The specific inhibitors of cysteine proteases are needed in preventing unwanted destructive proteolysis, which can be used in therapy and research [13, 42], pest toxin [10, 26], and the food industry [11, 12].

In industries of surimi-based product, commercial protease inhibitors are used to prevent the modori (gel softening) phenomenon and to maximize the gel strength of surimi. The most commonly used inhibitors are bovine plasma protein (BPP), chicken egg white, potato powder, and whey protein concentrate [11, 41]. Because there is some side effect on surimi-based products such as change of color [2], fish protease inhibitor is thought to be the best to prevent the modori phenomenon.

High molecular weight (HMW) cysteine protease inhibitors were purified from Atlantic salmon (*Salmo salar* L.) [33], plasma of steelhead trout [7], bowfin [8], Atlantic cod [25], and the sarcopterygian lungfish [21]. The HMW cysteine protease inhibitor from Atlantic salmon skin was purified and characterized, and its amino acid sequence

*Corresponding author
Phone: 82-233-640-2343; Fax: 82-33-640-2882;
E-mail: smkim@kangnung.ac.kr

was found to be homologous to kininogens [47]. In our previous study, HMW and LMW protease inhibitors were purified from glassfish egg [39]. In the present study, the HMW protease inhibitor of glassfish egg was further purified and characterized in order to have basic data for commercialization.

MATERIALS AND METHODS

Materials

Eggs from a mature glassfish, *Liparis tanakai*, were harvested immediately after capture and stored at −40°C until used. Papain, trypsin, cathepsin, azocasein, and protein molecular weight marker were purchased from Sigma Chemical Co. (St. Louis, MI, U.S.A.). The remaining chemicals used in this study were of the highest reagent grade. Carboxymethylated (CM)-papain immobilized resin was purchased from Calbiochem-Novabiochem Co. (La Jolla, CA, U.S.A.).

Purification of Protease Inhibitor

The HMW protease inhibitor from glassfish egg was purified by the modified method of Brillard-Bourdet *et al.* [6]. An 80 g sample of glassfish egg was homogenized in 240 ml of 50 mM sodium phosphate buffer, pH 6.5, containing 1 mM EDTA (buffer A). Cells and tissue debris were precipitated and removed from the homogenate by centrifugation at 27,390 ×*g* for 30 min at 4°C. The supernatant was loaded onto a CM papain immobilized resin column (1.0×10 cm) at a flow rate of 0.3 ml/min and washed with 50 mM sodium phosphate buffer, pH 6.5, containing 0.5 M NaCl and 1 mM EDTA (buffer B). Proteins were eluted from the column with 50 mM K_2HPO_4/NaOH (pH 11.5) at a flow rate of 1 ml/min. Fractions (1.4 ml each) were collected in tubes containing 0.25 ml of 250 mM KH_2PO_4, pH 4.5, to bring the pH to neutral.

Because there were two protein bands shown on SDS-PAGE, the electro-elution method with GeBA Gel Extraction Kit (Gene Bio Application Ltd., Kfar-Hanagig, Israel) was applied for further purification. After staining, the gel slices containing proteins were excised with a clean sharp scalpel and transferred to GeBAflex-tubes. Each tube (midi size) was filled with 800 µl of running buffer (0.025 M Tris-base, 0.192 M glycine, and 0.1% SDS), and then closed gently. The tube was placed on the supporting tray in a horizontal electrophoresis tank containing running buffer. Electric current was passed at 100 volt for 85 min. The polarity of the electric current was reversed for 2 min to release the protein from the membrane of the tube. Protein suspension was transferred to 1.5-ml microtubes and centrifuged at 14,000 ×*g* for 1 min to remove gel residues. The purified inhibitor was concentrated by ultrafiltration (cutoff 10 kDa, Millipore Corporation, Bedford, MA, U.S.A.).

Assay of Inhibitory Activity

Against Papain. Glassfish protease inhibitory activity was determined by measuring the degree of inhibition of papain activity using azocasein as the substrate. A 200 µl aliquot of 1.7 µg/ml inhibitor solution in buffer A was added to 100 µl of papain solution (0.1 U of activity) in buffer A. The combined solution was incubated at 37°C for 5 min and then added to 250 µl of 3.2 mg/ml azocasein substrate solution in buffer A. Following a 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 of inhibitor solution with 200 µl of buffer A. A blank was prepared by adding 700 µl of 20% TCA before adding 250 µl of substrate solution. For color development, the reaction mixture was centrifuged at 10,000 ×*g* for 5 min, and 720 µl of the supernatant was added to 800 µl of 1 M NaOH. The absorbance was measured at 440 nm. The inhibitory activity was calculated by the difference between papain activities with and without inhibitor. One unit of inhibitory activity was defined as a one unit decrease of papain activity [5].

Against Cathepsin L. Inhibitory activity against cathepsin L was measured using Z-Phe-Arg-NMec as the substrate. A 3 ng aliquot of cathepsin L in 500 µl of 0.1% Brij 35 was added to 250 µl of 340 mM sodium acetate buffer (pH 5.5) containing 60 mM acetic acid and 4 mM EDTA, and the mixture was left at 30°C for 1 min to activate cathepsin L. A 1 µl volume of 3 ng/µl inhibitor solution and 250 µl of 1 mM Z-Phe-Arg-NMec solution in dimethyl sulfoxide were added and then mixed immediately. Following 10 min incubation at 30°C, the reaction was stopped by adding 1 ml of 100 mM sodium monocloroacetate (pH 4.3) containing 30 mM sodium acetate and 70 mM acetic acid. Activity of the enzyme with and without inhibitor was determined by measuring the fluorescence of the free aminomethylcoumarin at 370 nm for excitation and at 460 nm for emission. The inhibitory activity was calculated by the difference between cathepsin activities with and without inhibitor. One unit of inhibitory activity was defined as a one unit decrease of cathepsin L activity [4].

Electrophoresis

The glassfish protease inhibitor was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and polyacrylamide gel electrophoresis (PAGE). Briefly, purified glassfish protease inhibitor was added to 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 a 4 min incubation at 95°C, 5 µl of the prepared sample was applied to a 12% SDS-polyacrylamide slab gel at pH 8.3 [17]. Sample buffer of PAGE [40] was composed of 15.5 ml of 1 mM Tris-HCl buffer, 2.5 ml of 1% bromophenol blue, 7 ml of water, and 25 ml of glycerol,

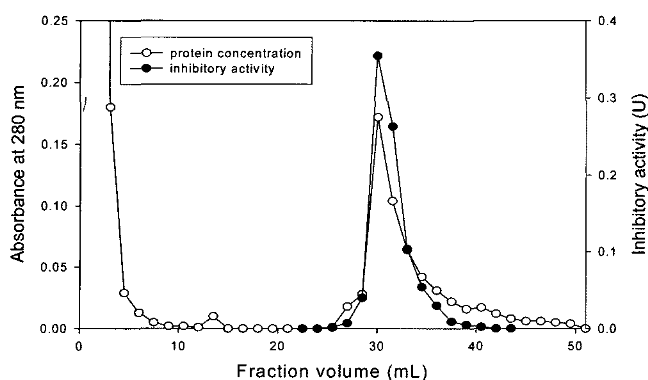

Fig. 1. CM-papain immobilized resin affinity chromatography pattern of HMW protease inhibitor purified from glassfish egg.

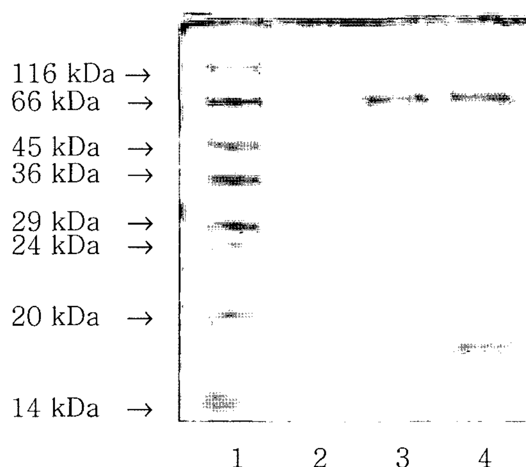

Fig. 2. SDS-PAGE pattern of a HMW glassfish egg protease inhibitor.

Lane 1, MW protein marker; lanes 2 and 3, protease inhibitor purified by electro-elution; lane 4, protease inhibitor purified by affinity chromatography.

pH 6.8. The further procedure of PAGE was the same as described above.

Thermal and pH Stabilities

The purified glassfish inhibitor was incubated for 30 min at various temperatures, ranging from 50°C to 80°C, and at varying pH values (pH range: 4–10) to determine the thermal and pH stabilities of the protein. Residual papain inhibitory activity was then determined at 37°C as described above.

Inhibitor Constant (K_i)

Aliquots of 0–30 μg/ml glassfish protease inhibitor, 0.5 mg/ml papain, and 0.1–6.4 mg/ml azocasein were prepared for the determination of kinetic parameters. K_m and V_{max} values for papain on azocasein were calculated using the hyperbolic regression analysis of Michaelis-Menten [22]. K_i was determined using a Dixon plot analysis [9]. The activity of glassfish egg inhibitor was measured at three azocasein concentrations (2, 1, and 0.5 times K_m).

RESULTS AND DISCUSSION

Purification of Protease Inhibitor

The fractionation pattern of glassfish protease inhibitor by affinity chromatography is shown in Fig. 1. There was one protein peak with high inhibitory activity (8.42 U/mg) against papain. However, on SDS-PAGE, there were two protein bands with MWs 67 and 18 kDa (Fig. 2). These two proteins were separated and purified by electro-elution using a GeBaflex-tube extraction kit. The specific inhibitory activity and purity of protease inhibitor with MW 67 kDa were 18.57 U/mg and 132.64-fold, respectively. The specific inhibitory activity and purity of protease inhibitor with MW 18 kDa were 19.70 U/mg and 140.71-fold, respectively, a little higher than those of the HMW protease inhibitor (Table 1). Because of the high yield, the higher molecular weight protein (HMW) was selected for further experiments in this study, and its properties were compared with other protease inhibitors. In another study, the molecular weight of the cysteine protease inhibitor from Atlantic salmon (*Salmo salar* L) skin was determined to be 52 kDa, and classified as a kininogen based on its amino acid sequence [47]. Cysteine protease inhibitor with MW 50 kDa was also isolated from the muscle of carp and inhibited both papain and calpain [35]. Family III of the cystatin superfamily is divided into two subfamilies; high (~120 kDa) and low (~68 kDa) molecular weight kininogens [3]. Therefore, based on its molecular weight, the purified glassfish egg protease inhibitor with MW 67 kDa might be a member of the kininogens.

Properties of the Protease Inhibitor

The optimal pH and temperature for inhibitory activity of HMW glassfish protease inhibitor were pH 6 (Fig. 3A) and

Table 1. Purification of HMW protease inhibitor from glassfish egg.

Purification step	Total protein (mg)	Total activity (unit)	Specific inhibitory activity (unit/mg)	Yield (%)	Purity (fold)
Egg extract	2,555.72	360.00	0.14	100.00	1.00
Affinity chromatography	0.25	2.11	8.42	0.59	60.14
Electro-elution: 18 kDa	0.012	0.23	19.70	0.06	140.71
67 kDa	0.014	0.26	18.57	0.07	132.64

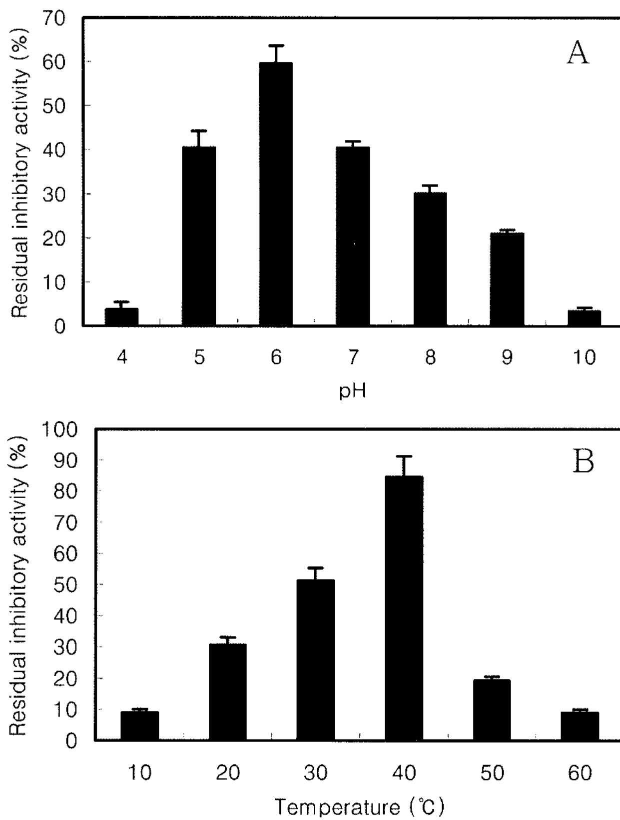

Fig. 3. The effect of temperature and pH on the inhibitory activity of a HMW glassfish egg protease inhibitor.

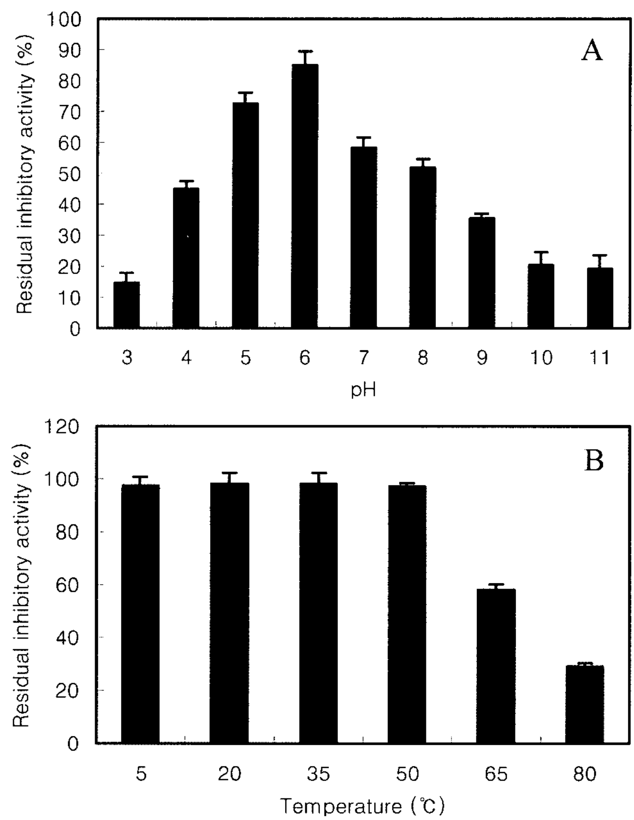

Fig. 4. The stability of an HMW glassfish egg protease inhibitor at different temperatures and pHs.

40°C (Fig. 3B), respectively. The HMW glassfish egg inhibitor was shown to be relatively stable within a pH range of 5 to 7, with maximal activity at pH 6. The residual inhibitory activity after preincubation at 37°C and pH 6 for 30 min was 85%, but more than 50% of inhibitory activity was lost at extreme pH (<pH 4 and >pH 9) (Fig. 3A). Another study has shown that human plasma kininogen with MW 83.5 kDa had high inhibitory activity against papain and cruzipain at pH 6.5 [38]. In contrast, the LMW glassfish egg inhibitor was shown to be relatively stable within a pH range of 7 to 10, with maximal activity at pH 8 (Fig. 4A). The residual inhibitory activity after preincubation at 37°C and pH 8 for 30 min was 70%, but no inhibition of papain was observed under acidic conditions (pH<6) [39].

The raw material surimi is the fresh muscle of fishes. During processing, it will come to be in the postmortem stage and decreases in pH. Because the fish muscle is weakly acidic, cysteine-like cathepsin may play an important role in softening the gel of surimi before proteases such as alkaline heat-stable proteases are active to cleave the myosin heavy chain and other proteins [18, 24, 19].

The 67 kDa glassfish egg protease inhibitor was even more stable at lower temperatures: Inhibitory activities of more than 90% were retained after 30-min incubations at 5, 20, 35, and 50°C. The inhibitory activity of 70% was lost when incubated at 80°C (Fig. 4B). In contrast, the LMW glassfish egg protease inhibitor was even more stable at higher temperatures: Its inhibitory activity of 60.8% and 40.1% were retained after 30-min preincubations at 65°C and 80°C, respectively [39]. Cathepsin B and L have been found to soften chum salmon [43, 44], tilapia [31], and mackerel [15]. The strength of surimi gel with cathepsin B and L decreased significantly after 2 h incubation at 55°C [16]. Because the HMW protease inhibitor from glassfish egg was stable at 50°C, this inhibitor might be able to inhibit heat-stable cathepsin B and L in the surimi gelling process.

To determine the K_i value of the HMW (67 kDa) glassfish egg protease inhibitor with papain, the velocity of azocasein hydrolysis by papain was measured with and without fish egg inhibitor at different azocasein concentrations, and the K_i was calculated using a Dixon plot of 1/V vs. [I] [9]. The result (Fig. 5) showed that the HMW glassfish egg protease inhibitor was a competitive inhibitor against papain, as the inhibitor concentration (X value) was the same (97.02 nM) for two different linear regressions based on the substrate concentrations, and the Y value (reciprocal of velocity) was at the reciprocal of V_{max}, 11.07 unit^{-1}. The K_i of the

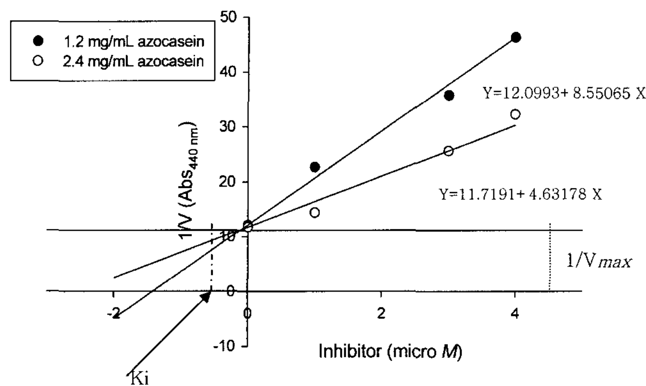

Fig. 5. Dixon plot of papain inhibition with a HMW glassfish egg protease inhibitor at different concentrations of azocasein.

HMW glassfish egg inhibitor (97.02 nM) was higher than the K_i of transgenic tomato cystatin (4.70 nM) [14] and the LMW glassfish egg inhibitor (4.70 nM) [39], and was lower than that of the human kininogen (170 nM) [32]. The K_i value of human kininogen was determined using cathepsin B with the fluorogenic peptide substrate, whereas the K_i value of the transgenic tomato cystatin and LMW glassfish egg inhibitor were determined using papain and azocasein as substrates. As the HMW glassfish egg inhibitor has a higher Ki, it would be less effective than the transgenic tomato inhibitor to inhibit papain at the same assay conditions.

As shown in Table 2, the HMW glassfish egg protease inhibitor was compared with the activity of chicken egg white cystatin. The HMW glassfish egg protease inhibitor was able to inhibit papain and cathepsin L. The relative inhibitory activity of the glassfish egg protease inhibitors against cathepsin L (26.47%) was significantly higher than chicken egg-white protease inihibitor (15.69%). Otherwise, there was no significant difference between the inhibitory activities of the two inhibitors against papain. In a recent study, the LMW glassfish egg inhibitor against cathepsin L (36.84 U/mg) was also significantly higher than that of egg-white inhibitor against cathepsin L (16.05 U/mg) [39]. Therefore, both of the glassfish egg protease inhibitors might be used to substitute commercial a chicken egg-white inhibitor to prevent the "modori" phenomenon in surimi-based products.

Table 2. Comparison of the inhibitory activity of a HMW protease inhibitor from glassfish egg and chicken egg-white against papain and cathepsin.

Protease inhibitor	Relative inhibitory activity (%)[d]	
	Papain	Cathepsin L
HMW glassfish egg	98.09[a]	26.47[b]
Chicken egg-white	97.13[a]	15.69[c]

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

Acknowledgment

This research was supported by a research fund of the Fisheries Research and Development Projects (Project No. 20020129) from the Korean Ministry of Marine Affairs and Fisheries.

REFERENCES

1. Abrahamson, M., A. Ritonja, M. A. Brown, A. Grubb, W. Machleidt, and A. J. Barrett. 1987. Identification of the probable inhibitory reactive sites for the cysteine protease inhibitor human cystatin C and chicken cystatin. *J. Biol. Chem.* **262:** 9688–9694.
2. An, H., M. Y. Peters, and T. A. Seymour. 1996. Roles of endogenous enzymes in surimi gelation. *J. Food Sci.* **7:** 321–326.
3. Barret, A. J., N. D. Rawlings, M. E. Davies, W. Macleidt, G.. Salvesen, and V. Turk. 1986. Cysteine protease inhibitors of the cystatin superfamily, pp. 515–569. *In* A. J. Barrett and D. Salvesen (eds.), *Proteinase Inhibitors*. Elsevier, Amesterdam.
4. Barrett, A. J. and H. Kirschke. 1981. Cathepsin B, Cathepsin H. and Cathepsin L, pp. 535–561. *In* A. J. Barrett (ed.), *Methodes in Enzymology,* **vol. 80**. Academic Press, San Diego, LA, U.S.A.
5. Borla, O. O., C. B. Martone, and J. J. Sánchez. 1998. Protease I inhibitor system in fish muscle: A comparative study. *Comp. Biochem. Physiol. B* **119:** 101–105.
6. Brillard-Bourdet, M., V. Nguyen, M. Ferrer-Di Martino, F. Gauthierand, and T. Moreau. 1998. Purification and characterization of a new cystatin from Taiwan cobra (*Naja naja atra*) venom. *Biochem. J.* **331:** 239–244.
7. Conlon, J. M. and K. R. Olson. 1993. Purification of a vasoactive peptide related to lysyl-bradykinin from trout plasma. *FEBS Lett.* **334:** 75–78.
8. Conlon, J. M., B. Platzack, L. E. Marra, J. H. Youson, and K. R. Olson. 1995. Isolation and biological activity of [Trp5]bradykinin from the plasma of the phylogenetically ancient fish, the bowfin and the longnosed gar. *Peptides* **16:** 485–489.
9. Dixon, M. and E. C. Webb. 1979. *Enzymes*. Logman Group Ltd., London, U.K.
10. Gruden, K., B. Štrukelj, T. Popoviè, B. Lenarèiè, T. Bevec, J. Brzin, I. Kregar, J. Herzog-Velikonja, W. J. Stiekema, D. Bosch, and M. A. Jongsma. 1998. The cysteine activity of Colorado potato beetle (*Leptinotarsa decemlineata* Say) guts, which is incentive to potato protease inhibitor, is inhibited by thyroglobulin type-1 domain inhibitors. *Insect Biochem. Mol. Biol.* **28:** 549–560.
11. Hammann, D. D., P. M. Amato, M. C. Wu, and E. A. Foegeding. 1990. Inhibition of modori (gel weakening) in

surimi by plasma hydrolysate and egg white. *J. Food Sci.* **55:** 665–669.
12. Sri, R., F. Tanuwidjaja, Y. Rukayadi, A. Suwanto, M. T. Suhartono, J. K. Hwang, and Y. R. Pyun. 2004. Study of thermostable chitinase enzymes from indonesian *Bacillus* K29-14. *J. Microbiol. Biotechnol.* **14:** 647–652.
13. Hernandez, A. A. and W. R. Roush. 2002. Recent advance in the synthesis, design and selection of cysteine protease inhibitors. *Curr. Opin. Chem. Biol.* **6:** 459–465.
14. Jacinto, T., K. V. S. Fernandes, O. L. T. Machado, and C. L. Siqueira Jr. 1998. Leaves of transgenic tomato plants overexpressing prosystemin accumalate high levels of cystatin. *Plant Sci.* **138:** 35–42.
15. Jiang, K., J. Lee, and H. Chen. 1994. Purification and characterization of cathepsin B from ordinary muscle of mackerel (*Scomber australasicus*). *J. Agric. Food Chem.* **42:** 1073–1079.
16. Jiang, S. T., J. J. Lee, and C. Y. Tsao. 1997. Mackerel cathepsin B and L effect on thermal degradation of surimi. *J. Food Sci.* **62:** 1–6.
17. Laemmli, U. K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophagety. *Nature* **227:** 265–275.
18. Lanier, T. C. 2000. Surimi gelation chemistry, pp. 237–266. *In* J. W. Park (ed.), *Surimi and Surimi Seafood.* Marcel Dekker Inc., New York, NY, U.S.A.
19. In, M.-J., E.-S. Jang, Y.-J. Kim, and N.-S. Oh. 2004. Purification and properties of an extracellular acid phytase from *Pseudomonas fragi* Y9451. *J. Microbiol. Biotechnol.* **14:** 1004–1008.
20. Lenarcic, B., A. Ritonja, I. Dolenc, V. Stoka, S. Berbic, J. Pungercar, B. Strukelj, and V. Turk. 1993. Pig leukocyte cysteine proteinase inhibitor (PLCPI), a new member of the stefin family. *FEBS Lett.* **336:** 289–292.
21. Li, Z., S. M. Secor, V. A. Lance, M. A. Masini, M. Vallarino, and J. M. Conlon. 1998. Characterization of bradykinin-related peptides generated in the plasma of six sarcopterygian species (African lungfish, amphiuma, coachwhip, bullsnake, gila monster, and Gray's monitor). *Gen. Comp. Endocrinol.* **112:** 108–114.
22. Michaelis, L. and M. L. Menten. 1913. Die Kinetik der invertinwerkung. *Biochem. Z.* **49:** 333–369.
23. Olenen, A., N. Kalkkinen, and L. Paulin. 2003. A new type of cysteine proteinase inhibitor - the salrin gene from Atlantic salmon (*Salmo salar* L) and Arctic charr (*Salvelinus alpinus*). *Biochemie* **1:** 001–005.
24. Otto, H. H. and T. Schirmeister. 1997. Cysteine proteases and their inhibitors. *Chem. Rev.* **97:** 133–171.
25. Platzack, B. and J. M. Conlon. 1997. Purification, structural characterization, and cardiovascular activity of cod bradykinins. *Am. J. Physiol.* **272:** R710–R717.
26. Jung, H. K. and S.-D. Kim. 2005. An antifungal antibiotic purified from *Bacillus megaterium* KL39, a biocontrol agent of red-pepper *Phytophthora*-blight disease. *J. Microbiol. Biotechnol.* **15:** 1001–1010.
27. Ritonja, A., W. Machleidt, and A. J. Barrett. 1985. Amino acid sequence of the intracellular cysteine protease inhibitor cystatin B from human liver. *Biochem. Biophys. Res. Commun.* **131:** 1187–1192.
28. Ritonja, A., T. H. Coetzer, R. N. Pike, and C. Dennison. 1996. The amino acid sequence, structure comparisons and inhibition kinetics of cathepsin L and sheep stefin B. *Comp. Biochem. Physiol. B Biochem. B.* **114:** 193–198.
29. Sangorrin, M. P., E. J. Folco, C. M. Martone, and J. J. Sánchez. 2001. Purification and characterization of a protease inhibitor from white croaker skeletal muscle (*Micropogon opercularis*). *Int. J. Biochem. Cell Biol.* **33:** 691–699.
30. Sen, L. J. and J. R. Whiteker. 1973. Some properties of a ficin-papain inhibitor from avian egg white. *Arch. Biochem. Biophys.* **158:** 623–632.
31. Sharekar, S. V., M. S. Gore, and V. Ninjoor. 1988. Purification and characterization of cathepsin B from skeletal muscle of fresh water fish, *Tilapia mossambica. J. Food Sci.* **53:** 1018–1023.
32. Sueyoshi, T., K. Enjyoji, T. Shimada, H. Kato, S. Iwanaga, Y. Bando, E. Kominami, and N. Katunuma. 1985. A new function of kininogens as thiol-proteinase inhibitor: Inhibition of papain and cathepsins B, H, and L by bovine, rat and human plasma kininogen. *FEBS* **182:** 193–195.
33. Synnes, M. 1998. Purification and characterization of two cysteine proteinase inhibitors from the skin of Atlantic salmon (*Salmo salar* L.). *Comp. Biochem. Physiol. B* **121:** 257–264.
34. Takio, K., E. Kaminami, N. Wakamatsu, N. Katunuma, and K. Titani. 1983. Amino acid sequence of rat liver proteinases inhibitor. *Biochem. Biophys. Res. Commun.* **115:** 902–908.
35. Toyohara, H., Y. Makinodan, and S. Ikeda. 1988. Detection of a cysteine protease inhibitor in carp muscle. *Nippon Suisan Gakkaishi* **54:** 157.
36. Tsai, Y. J., G. D. Chang, C. J. Huang, Y. S. Chang, and F. L. Huang. 1996. Purification and molecular cloning of carp ovarian cystatin. *Comp. Biochem. Physiol. B* **113:** 573–580.
37. Turk, B., I. Kriza, and V. Turk. 1992. Isolation and characterization of bovine stefin B. *Biol. Chem. Hoppe Seyler* **373:** 441–446.
38. Turk, B., V. Stoka, V. Turk, G. Johansson, J. J. Cazzulo, and I. Bjork. 1996. High-molecular-weight kininogen binds two molecules of cysteine proteinases with different rate constants. *FEBS Lett.* **391:** 109–112.
39. Ustadi, K. Y. Kim, and S. M. Kim. 2005. Purification and identification of a protease inhibitor from Glassfish (*Liparis tanakai*) Eggs. *J. Agric. Food Chem.* **53:** 7667–7672.
40. Walker, J. M. 2002. Nondenaturing polyacrylamide gel electrophoresis of protein, pp. 57–60. *In* J. M. Walker (ed.), *The Protein Protocols Handbook*, 2nd Ed. Huana Press, Totowa, NJ, U.S.A.
41. Weerasinghe V. C., M. T. Morrissey, and H. An. 1996. Characterization of active components in food grade proteinase inhibitor for surimi manufacture. *J. Food Chem.* **44:** 2584–2590.

42. Bae, E.-A., J.-E. Shin, S.-H. Park, and D.-H. Kim. 2004. Inhibitory effect of ginseng polysaccharides on rotavirus infection. *J. Microbiol. Biotechnol.* **14:** 202–204.
43. Yamashita, M. and S. Konagaya. 1990. Purification and characterization of cathepsin L from the white muscle of chum salmon, *Oncorhynchus keta. Comp. Biochem. Physiol.* **96B:** 247–252.
44. Yamashita, M. and S. Konagaya. 1990. Purification and characterization of cathepsin B from the white muscle of chum salmon, *Oncorhynchus keta. Comp. Biochem. Physiol.* **96B:** 733–737.
45. Yamashita, M. and S. Konagaya. 1991. Cysteine protease inhibitor in egg of chum salmon. *J. Biochem.* **110:** 762–766.
46. Yamashita, M. and S. Konagaya. 1991. A comparison of cystatin activity in various tissues of chum salmon (*Oncorhynchus keta*) between feeding and spawning migrations. *Comp. Biochem. Physiol. A* **100:** 749–751.
47. Ylonen, A., A. Rinne, J. Herttuainen, J. Bogwald, M. Jarvinen, and N. Kalkkinen. 1999. Atlantic salmon (*Salmo salar* L) skin contains a novel kininogen and another cysteine proteinase inhibitor. *Eur. J. Biochem.* **266:** 1066–1072.