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Purification and Identification of a Protease Inhibitor from Glassfish (*Liparis tanakai*) Eggs

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Two protease inhibitors of 67 and 18 kDa, respectively, were purified from glassfish, *Liparis tanakai*, eggs by affinity chromatography. The smaller protein was purified with a yield and purity of 0.25% and 49.69-fold, respectively, and was characterized for further study. The glassfish egg protease inhibitor exhibited stability between 50 and 65 °C in an alkaline environment (pH 8). It was shown to be a noncompetitive inhibitor against papain, with an inhibitor constant (K_i) of 4.44 nM. Potent glassfish protease inhibitor with N-Val-Gly Ser-Met-Thr-Gly-Gly-Phe-Thr-Asp-C amino acid residues was synthesized and its inhibitory activity was compared. Moreover, the 18-kDa protein inhibited cathepsin, a cysteine protease, more effectively than did egg white protease inhibitor, whereas the reverse was true for papain. Glassfish egg protease inhibitor is classified as a member of the family I cystatins.

KEYWORDS: Glassfish egg; protease inhibitor; stability; cysteine protease; inhibitor constant

INTRODUCTION

The changes in functional and organoleptic properties of fish muscle are a consequence of proteolytic activity (*1*−*3*). Cathepsin is a member of the cysteine proteases, among which cathepsins B and L were shown to cause softening in chum salmon (*4*). A thermally stable alkaline protease has been hypothesized to be responsible for the heat-induced softening of surimi gel that occurs between 55 and 60 °C (*5*). Cathepsin L was found to be the predominant proteinase involved in heat-induced degradation of the myofibrillar protein in Pacific whiting surimi (*6*). The addition of protease inhibitors, such as cystatin, to lower grade surimi may improve its usefulness by inhibiting endogenous cysteine proteases (*7*).

Cystatins are cysteine protease inhibitors that are widely distributed in animal tissues and body fluids. Cystatins are classified into three groups according to their molecular structures (*8*). Family I cystatins lack disulfide bonds and include A (*9*), B (*10*), and rat cystatin β (*11*). Family II cystatins are characterized by two disulfide bonds and include human cystatin S (*12*), chicken cystatin (*13*), mouse cystatin C (*14*), and rat cystatin A (*15*). Members of families I and II have molecular masses ranging from 10 to 20 kDa. Family III cystatins include kininogens (*16*), which are single-chain glycoproteins containing three cystatin-like domains with molecular masses ranging from 68 to 120 kDa (*17*).

Cystatins have been purified from the ovarian fluid of carp (*18*), chum salmon egg (*19*), Atlantic salmon, and Arctic charr (*20*). Protease inhibitors in the eggs of fishes are thought to be involved in protection from microorganisms, the process of embryogenesis, and the regulation of early embryonic growth (*21*). Cystatins may also contribute to the defense against the viral proteases that are necessary for virus replication (*22*).

There is a strong demand for natural fish protease inhibitors that can be used to prevent the deterioration of surimi-based products and fish meat. Therefore, the objective of this study was to purify and identify such a protease inhibitor from glassfish egg.

MATERIALS AND METHODS

Materials. Eggs from a mature glassfish, *Lipari tanakai*, were harvested immediately after capture and were stored at −40 °C until analysis. Papain, trypsin, cathepsin, azocasein, and protein molecular weight markers were purchased from Sigma Chemical Co. (St. Louis, MO). The remaining chemicals used in this study were reagent grade. Sephacryl HR 100, CM Sepharose, and CNBr-activated Sepharose 4B were purchased from Amersham Pharmacia Biotech, Ltd. (Uppsala, Sweden).

Purification of Protease Inhibitor. A 250-g sample of fish eggs was 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 10 000*g* for 25 min. To further purify the extract, the supernatant was incubated for 10 min at 80 °C, cooled to room temperature, and centrifuged at 10 000*g* for 25 min. The extract was fractionated with ammonium sulfate (40% to 80% saturation). The precipitated fraction was dissolved in buffer A and dialyzed overnight against a 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

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from the column using a linear gradient from 0 to 1 M NaCl in sodium acetate buffer (pH 5.5) at a flow rate of 1 mL/min. Fractions containing greater than 50% of maximal peak 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). The concentrate was then loaded onto a Sephacryl column (2.6 × 90.0 cm) equilibrated with buffer C and was eluted at a flow rate of 0.2 mL/min.

Affinity Chromatography. A 7-g aliquot of CNBr-activated Sepharose 4B was washed and reswelled 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 of blocking agent (0.2 M glycine, pH 8.0) and was stirred overnight at 4 °C. The gel was washed first with 0.1 M acetate buffer (pH 4.0, 0.5 M NaCl) and then with the coupling buffer. Finally, the gel was poured into a 1.0 cm × 20.0 cm column and was equilibrated with buffer A. A 50-mL aliquot of crude extract of fish eggs 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 a flow rate of 0.3 mL/min.

Inhibitory Activity Assay. 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 N NaOH. The absorbance was measured at 440 nm. The inhibitory activity was calculated using the difference between papain activities with and without inhibitor. One unit of inhibitory activity was defined as a one-unit decrease of papain activity (*23*).

Determination of Inhibition 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 acting on azocasein were calculated using the hyperbolic regression analysis of Michaelis−Menten (*24*). K_i was determined using a Dixon plot analysis (*25*). The activity of glassfish egg inhibitor was measured at three azocasein concentrations (2, 1, and 0.5 times K_m).

Heat and pH Stability. The purified glassfish inhibitor was incubated for 30 min at temperatures ranging from 5 to 80 °C and at varying pH values (pH range 4−10) to determine the heat and pH stability of the protein. Residual papain inhibitory activity was then determined at 37 °C as described above.

Electrophoresis. The glassfish protease inhibitor was analyzed using sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis (PAGE). Briefly, purified glassfish protease inhibitor was added to sample buffer (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% polyacrylamide slab gel at pH 8.3 (*26*).

Amino Acid Sequence. Following SDS−PAGE, protein bands were transferred to a poly(vinylidene difluoride) (PVDF) membrane (*27*). The amino acid sequence of the 18-kDa glassfish protease inhibitor was determined using a PERKIN 491 protein sequencer (Perkin-Elmer Inc., Wellesley, MA).

Synthesis of Glassfish Egg Protease Inhibitor. The glassfish egg protease inhibitor was synthesized using an automated peptide synthesizer (Peptron III-R24, Peptron, Daejeon, Korea) according to the Fmoc solid-phase method. After deprotection of the synthesized inhibitor from the resin, the peptide was purified and analyzed by reverse-phase HPLC using a Waters 2690 Separations Module with a C18 analytical RP column (Waters, Milford, MA). The glassfish egg protease inhibitor was identified by mass spectrometry (HP 1100 Series LC/MSD, Hewlett-Packard, Roseville, CA).

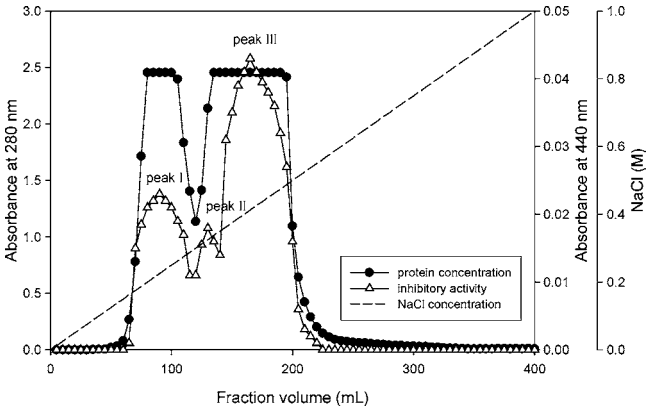

Figure 1. CM Sepharose cation exchange chromatography pattern of glassfish egg protease inhibitor.

Table 1. Purification of the Protease Inhibitor from Glassfish Egg by Ion Exchange and Gel Permeation Chromatography

purification step	total protein (mg)	total inhibitory activity (U)	specific inhibitory activity (U/mg)	yield (%)	purification (fold)
heated extract	20031.00	1840.00	0.09	100.00	1.00
40–80% $(NH_4)_2SO_4$	1504.60	465.00	0.31	25.30	3.38
CM Sepharose (peak III)	29.50	41.50	1.41	2.30	15.36
Sephacryl HR-100 (peak II)	1.09	1.56	1.43	0.10	15.57

Protein Concentration. Protein concentrations were measured using a protein kit (Bio-Rad Lab. Inc., Hercules, CA) according to the manufacturer's instructions. Bovine serum albumin was used as the calibration standard. The relative protein contents of the chromatographically separated fractions were estimated by measuring absorbance at 280 nm.

RESULTS AND DISCUSSION

Protease Inhibitor Purification. The conventional purification of glassfish egg protease inhibitor is summarized in **Table 1**. As the inhibitor was shown to be heat-stable, the fish egg extract was heated to remove other enzymes or inhibitors (*28*). When the heated extract was precipitated by ammonium sulfate, the specific activity and purification were 0.31 U/mg and 3.38-fold, respectively.

The fractionation pattern of protease inhibitor by CM Sepharose chromatography is shown in **Figure 1**. There were two protein and three inhibitory peaks, with peak III showing the highest inhibitory activity (1.41 U/mg) and a purification of 15.36-fold (**Table 1**). In another study, a similar pattern was obtained for a protease inhibitor isolated from ovarian carp using CM-TSK chromatography (*18*). Specific activities between 1 and 7 U/mg were obtained for pooled azocaseinolytic-active ion exchange chromatography fractions of proteases isolated from different fish species (*23*), which were comparable to the results in this study. However, the activity and purification of the glassfish protease inhibitor was lower than the 3.8 U/mg and 66-fold obtained for a chum salmon egg protease inhibitor (*19*).

A single protein and three papain inhibitory peaks (I, II, and III) were obtained from Sephacryl HR 100 chromatography (**Figure 2**). Two protein bands with molecular mass of 67 and 18 kDa, respectively, from SDS−PAGE analysis corresponded with the peak that had the highest inhibitory activity (peak II) (**Figure 3A**). The application of heat to the glassfish egg extract

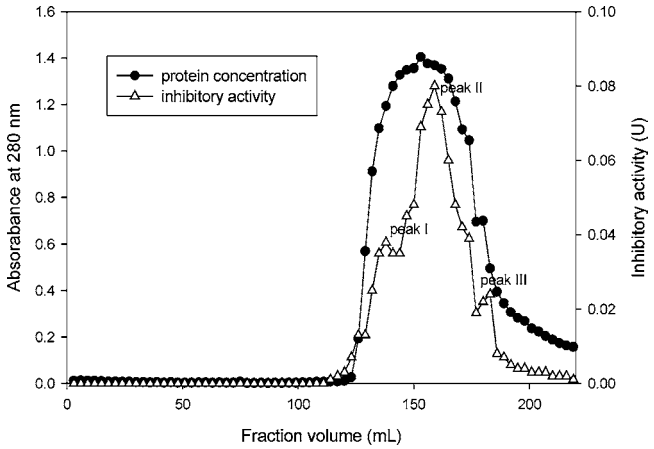

Figure 2. Sephacryl HR 100 gel permeation chromatography pattern of glassfish egg protease inhibitor.

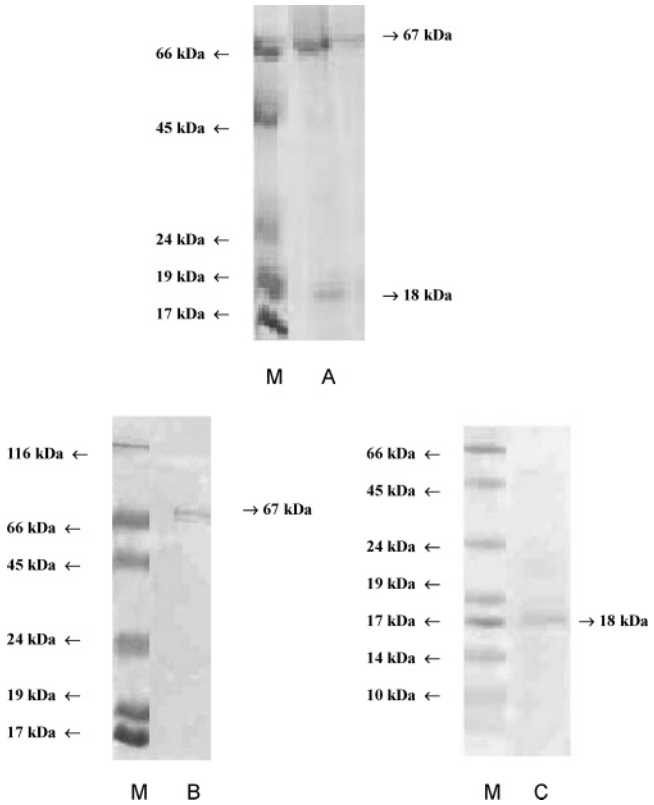

Figure 3. SDS–PAGE pattern of the purified glassfish egg inhibitor.

Table 2. Purification of the Protease Inhibitor from Glassfish Egg by Affinity Chromatography

purification step	total protein (mg)	total inhibitory activity (U)	specific inhibitory activity (U/mg)	yield (%)	purification (fold)
egg extract	23437.50	1875.00	0.08	100.00	1.00
affinity chromatography	1.18	4.69	3.97	0.25	49.69

during the first step of purification eliminated heat-labile proteins and resulted in an increase of specific inhibitory activity from 0.08 U/mg (**Table 2**) to 0.09 U/mg (**Table 1**). However, the structures of the proteins may have been changed from globular forms (tertiary and quaternary structures) to linear forms (secondary and primary structures) by heat denaturation. In general, the linear forms of polypeptides are not effectively separated by gel permeation chromatography (*29*). In this study,

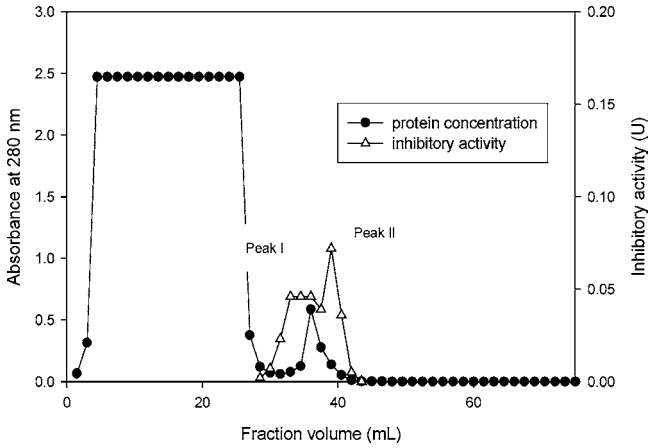

Figure 4. Affinity chromatography pattern of glassfish egg protease inhibitor.

the SDS−PAGE results indicate that the glassfish egg protease inhibitor is a dimer consisting of two peptides with molecular masses of 67 and 18 kDa that could not be separated by ion exchange or gel permeation chromatography.

A CNBr-activated Sepharose 4B-papain column was used to purify the glassfish egg protease inhibitor because cystatin-like protease inhibitors bind to active sites in the matrix. There were two protein peaks from affinity chromatography, but only the 18-kDa protein showed two inhibition activity peaks (**Figure 4**). The 67-kDa protein corresponded to peak I (fraction volume, 36 mL) of the affinity chromatography pattern, and the 18-kDa protein corresponded to peak II (fraction volume, 39 mL) (**Figure 3B,C**). As the 18-kDa protease inhibitor exhibited strong inhibitory activity against papain, it was selected for further experiments in this study.

Approximately 170 μg of purified protease inhibitor was obtained from 250 mg of glassfish egg, with a yield and purity of 0.25% and 49.69-fold, respectively (**Table 2**). These results indicate that a single purification step using CNBr-activated Sepharose 4B coupled with papain is an effective way to purify protease inhibitor from glassfish egg. In comparison, Yamashita and Konagaya (*19*) isolated a 16-kDa protease inhibitor from chum salmon eggs with a purity of 78-fold and 1.3% recovery. The single-step purification used in the current study not only saved time but also resulted in higher yields (0.25% vs 0.10%) as compared with conventional purification methods using ion exchange and gel permeation chromatography.

Amino Acid Sequence. The amino acid sequence of the 18-kDa inhibitor consisted of about 141 amino acid residues with 10 N-terminals based on the Edman degradation method: Val, Gly, Ser, Met, Thr, Gly, G, Phe, Thr, and Asp. Based on N-terminals, a potent protease inhibitor with N-Val-Gly-Ser-Met-Thr-Gly-Gly-Phe-Thr-Asp-C amino acid residues was synthesized. Among 10 amino acid residues, five were unique, and five of these were the same amino acids found in a protease inhibitor from chum salmon (*19*). When the amino acid sequence of glassfish egg inhibitor was compared with those of human cystatin C (*30*), human cystatin S (*31*), and chicken cystatin (*13*), no apparent similarities between the sequences were found. In fact, the cystatins and the fish egg inhibitor only had three amino acids in common. As the glassfish egg inhibitor did not contain cysteine residues necessary for disulfide bonding, it was classified as family I cystatin. In contrast, chum salmon egg inhibitor exhibits disulfide bonding and is thus a member of the cystatin family II (*19*).

Table 3. Comparison of Inhibitory Activity of Glassfish Egg Protease Inhibitor with Others against Papain and Cathepsin Proteases

	specific inhibitory activity (U/mg)[a]	
inhibitor	papain	cathepsin
natural glassfish egg	19.7 b	36.84 a
synthesized	16.98 b	32.76 a
egg white	37.71 a	16.05 b
chymotrypsin potato i	2.00 c	4.12 c

[a] Means in the same column with different letters are significantly different ($p < 0.05$). Mean values were obtained from four replications.

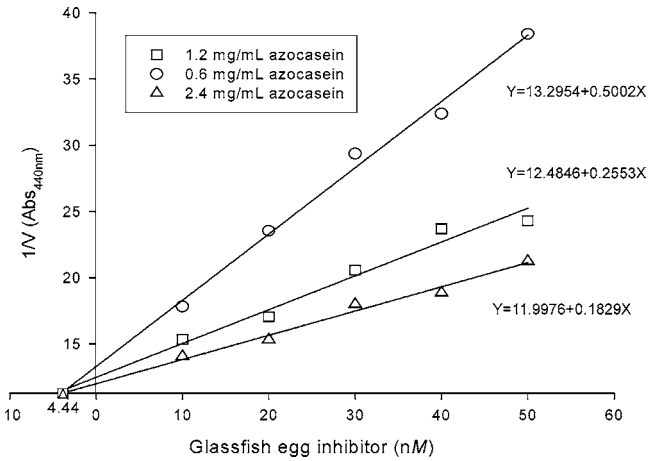

Figure 5. Dixon plot of papain inhibition with glassfish egg protease inhibitor at different concentrations of azocasein.

Properties of the Protease Inhibitor. The glassfish egg protease inhibitor was able to inhibit the cysteine proteases papain and cathepsin (**Table 3**) but did not inhibit trypsin, which is a serine protease (data not shown). The glassfish egg inhibitor demonstrated higher inhibitory activity against cathepsin than against papain. The fish egg inhibitor may be more effective against cathepsin because the latter is a fish muscle protease, while papain is a plant protease.

The inhibitory activity of the glassfish egg inhibitor was compared with the activities of other inhibitors, as shown in **Table 3**. The specific inhibitory activity of the natural glassfish egg inhibitor against papain was not significantly different from that of the synthesized form of the inhibitor (19.97 vs 16.98 U/mg, respectively), but the activities of both were lower than the specific activity of egg white inhibitor (37.71 U/mg), which is a member of the cystatin family II (*31*). In contrast, the specific inhibitory activities of the natural (36.84 U/mg) and synthesized (32.76 U/mg) glassfish egg inhibitors against cathepsin were significantly higher than that of egg white inhibitor against cathepsin (16.05 U/mg). On the basis of these data, the glassfish egg protease inhibitor appears to be more suitable than egg white inhibitor for use as an inhibitor of fish gel proteolysis in the surimi industry.

To determine the K_i value of glassfish egg inhibitor with papain, the velocities of azocasein hydrolysis by papain were 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] (*25*). The results (**Figure 5**) showed that glassfish protease inhibitor was a noncompetitive inhibitor against papain, as the inhibitor concentrations (X values) were the same (4.44 nM) for three different linear regressions based on the substrate concentrations and the Y value (inverse of velocity) was at the inverse of V_{max}, 11.07 unit^{-1}. The K_i of glassfish egg inhibitor (4.44 nM) was higher than the K_i of chum salmon egg inhibitor (0.1 nM) (*19*) and tomato cystatin (1.45 nM) (*32*) and was lower than those of transgenic tomato cystatin (4.70 nM) (*33*) and cowpea cystatin (6.10 nM) (*34*) (**Table 4**). The values for chum salmon egg inhibitor, cowpea cystatin, and tomato cystatin were determined using papain with the substrate BANA, whereas the value of the transgenic tomato protease inhibitor was determined using papain and azocasein as substrates. Azocasein was also used in the current study. As the glassfish egg inhibitor has a lower K_i, it would be more effective than the transgenic tomato inhibitor at inhibiting papain under the same assay conditions.

Table 4. Inhibitor Constant (K_i) of Glassfish Proteases Inhibitor

source	K_i (nM)
glassfish	4.44[a]
salmon	0.10 (*19*)[b,c]
tomato cystatin	1.45 (*32*)[b]
tomato transgenic cystatin	4.70 (*34*)[a]
cowpea cystatin	6.10 (*33*)[b]

[a] Azocasein as substrate. [b] 1.4 μM benzoyl-DL-arginine-β-naphthylamide (BANA) as a substrate. [c] The number in parentheses is the cited reference.

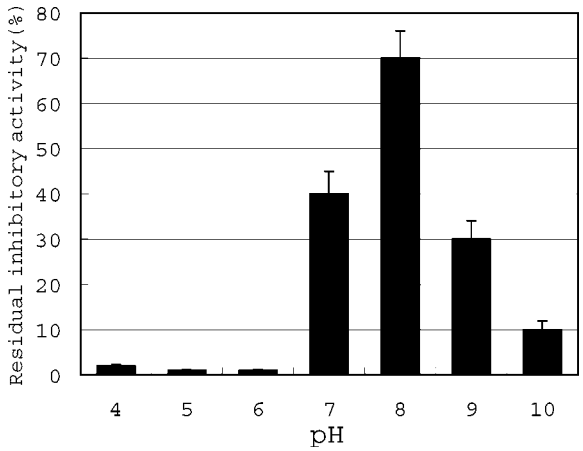

Figure 6. The stability of glassfish egg protease inhibitor at different pHs.

The glassfish egg inhibitor was shown to be relatively stable within a pH range of 7−10, with maximal activity at pH 8 (**Figure 6**). The residual inhibitory activity after incubation at 37 °C and pH 8 for 30 min was 70%, but no inhibition of papain was observed under acidic conditions (pH <6). Other research has shown that tomato cystatin lost 10% of its inhibitory activity after incubation within a pH range of 4−11 for 24 h at 4 °C (*32*). An alkaline protease inhibitor from *Actinomycetes* was stable at pH 5−12 after a 1-h incubation at room temperature (*35*).

The glassfish egg protease inhibitor was even more stable at higher temperatures (**Figure 7**). Inhibitory activities of 60.8 and 40.1% were retained after 30-min incubations at 65 and 80 °C, respectively. Ninety percent of the inhibitory activity of the glassfish egg inhibitor was lost when incubated at <20 °C. When the glassfish egg protease inhibitor was incubated for 30 min at 60 °C, it retained more inhibitory activity than did an alkaline protease inhibitor of *Actinomycetes* subjected to the same conditions (60% vs 40%, respectively) (*35*). Moreover, glassfish protease inhibitor at 60 °C was shown to inhibit endogenous fish muscle proteases such as cathepsin B, H, and L, with optimal activity between 50 and 60 °C (6).

Most of fish muscle cathepsins are also active at a range of pH 5−8 (*3*). There are two major groups that cause softening

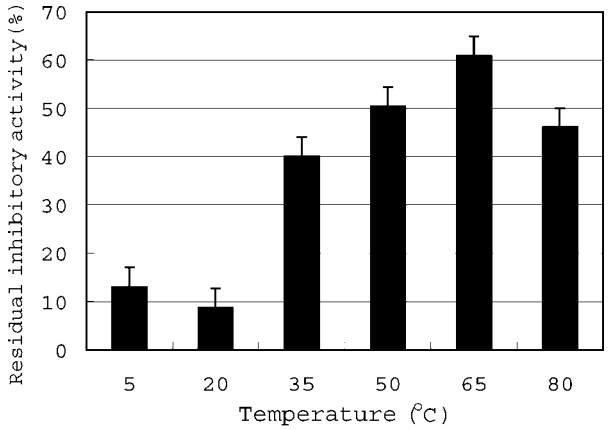

Figure 7. The stability of glassfish egg protease inhibitor at different temperatures.

in the surimi gel. Those are cathepsins (*36*−*38*) and heat stable alkaline protease (*39*). Because its optimal inhibitory activity of protease inhibitor from glassfish egg is under neutral to weakly alkaline conditions and at a temperature ranging from 50 to 70 °C, the Modori phenomenon might be prevented strongly in the surimi gelling process by this inhibitor. But low recovery or purification yield of glassfish protease inhibitor, 0.25% (**Table 2**), was one problem for application in surimi industries. Therefore, the production of a large amount of inhibitor can be achieved by recombinant technology.

In conclusion, the molecular masses of glassfish egg protease inhibitors are 18 and 67 kDa. These inhibitors was not separated with ion exchange and gel permeation chromatography but were resolved using a single-step purification with a CNBr-activated Sepharose 4B-papain affinity column. The yield and purity of the 18-kDa protease inhibitor were 0.25% and 49.69-fold, respectively. Glassfish egg inhibitor is classified as a member of cystatin family I because of its lack of disulfide bonding. The K_i of glassfish egg protease inhibitor against papain was 4.44 nM, and its inhibitory activity was shown to be stable within a temperature range of 50−65 °C at pH 8. The results of this study indicate that glassfish egg inhibitor can be used to effectively inhibit cysteine proteases that cause deterioration of fish meat during the surimi gelling process.

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