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Purification and determination of inhibitory activity of recombinant soyacystatin for surimi application

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A recombinant soyacystatin (r-soyacystatin) was tested for its inhibitory activity against cysteine proteinase of Pacific whiting and its activity was compared to that of egg white cystatin. A recombinant soyacystatin expressed in *Escherichia coli* was purified to electrophoretic homogeneity using phenyl-Sepharose and DEAE-Sepharose. Native egg white cystatin was purified by using affinity chromatography on CM-papain-Sepharose generated in our lab. Egg white cystatin and soyacystatin were tested for proteinase inhibitory activity against commercial papain and also cathepsin L purified from Pacific whiting muscle. The r-soyacystatin exhibited papain-like protease inhibition activity comparable to that of the egg white cystatin, which could inhibit papain and Pacific whiting cathepsin L. The r-soyacystatin subsequently inhibited the autolytic activity of Pacific whiting surimi.

Keywords: Autolytic activity / Egg white cystatin / Pacific whiting / Soyacystatin / Surimi

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1 Introduction

Cystatins are potent inhibitors of cysteine proteinases found in animal and plant tissues and human biological fluids [1]. They inhibit cysteine proteinases, such as cathepsins B, H, and L and several structurally similar plant proteinases, such as papain and actinidin, by making the reactive site of the enzyme inaccessible to substrates and to the thiol group reagents [2, 3]. They form tight reversible complexes with the proteinases with the dissociation constants typically in the nanomolar ratio [1, 2]. The cystatin superfamily is grouped into four different families based on their occurrence, sequence, and structure similarity. Cystatin family I, stefin, is known to have the smallest molecular mass of ~11 kDa. It has no intramoleculer disulfide bonds and glycosylation. Cystatin family II exists in the secrata and tissues of mammalian and avian origin. It has a molecular mass of ~13 kDa with two disulfide bridges [1]. Cystatin family III, also called kininogens, has the highest molecular masses of 70 kDA consisting of heavy and light chains and existing in mammalian blood [1]. Cystatin family 4 was recently discovered and found in plants [4]. Like family I, the members of this family do not have a disulfide bond. However, the amino acid sequence is closely related to cystatins of family II. Cystatin from a plant source, therefore, is classified as independent family referred to as "phytocystatin" [4, 5] and has been identified in seeds, leaves, roots, and fruits [5–19].

Phytocystatin shows a wide inhibition spectrum against cysteine proteinases from plant and animal origin. Abe *et al.* [20] reported that corn cystatin inhibited various cysteine proteinases, including cathepsins H and L and papain. It also weakly inhibits cathepsin B. Izquierdo-Pulido *et al.* [21] reported that cystatin isolated from rice was inhibitory against heat-activated arrowtooth flounder proteinase. Recently, cystatins have received more attention for their potential role in protecting fish surimi proteins from proteolytic activities [15, 22–24, 26]. Surimi is minced fish meat that has a unique functionality such as gel-forming ability, water- and oil-binding properties [22]. These characteristics make surimi the main ingredient for a wide range of seafood analogs, such as artificial crab. Alaska pollock has been the species used most for surimi manufacturing. Because of the maximized annual catch of Alaska pollock and its relatively higher price, some underutilized species have been used to produce surimi, such as arrowtooth flounder, hairtail, mackerel, and Pacific whiting. However, these fish species suffer from high levels of endogenous protease activity which cause soft texture [27,

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Abbreviations: **AS**, ammonium sulfate; **BANA**, *N*-benzoyl-L-arginine-2-naphthylamide; **BPP**, beef plasma protein; **IPTG**, isopropyl-β-D-thiogalactopyranoside; **LB**, Luria-Bertani

28]. In the last few years, Pacific whiting has been successfully utilized in surimi production because of the widespread availability in the U.S. Northwest coast and the low price. On the other hand, Pacific whiting suffers from *postmortem* softening as a result of hydrolysis of myofibrillar proteins by endogenous proteinase and becomes susceptible to autolysis by endogenous muscle proteinases. The degradation of myofibrillar proteins causes adverse effects on surimi quality and lowers the gel strength [27]. It was shown that cathepsin L was the major source of proteolytic activity in Pacific whiting surimi [30]. In order to alleviate the proteolytic activities of the fish muscle, food grade protease inhibitors, such as egg white, potato powder, and beef plasma protein (BPP) have been used in surimi production but their use has been limited due to their adverse effects on organoleptic properties of surimi. It was reported that specific cysteine proteinase inhibitors, such as egg white cystatin, reduced the protease activity at a negligible level without causing noticeable sensory defects in surimi [23, 29].

Although cysteine proteinase inhibitors are widely found in nature, their levels in natural sources are low. For example, to obtain enough inhibitor for the characterization of stefin A [30] or pig L-kininogen [31], a large amount of fresh blood is required. Likewise, kilograms of rice seeds and liters of egg white yielded only microgram quantities of oryzacystatin [5] and egg white cystatin [32], respectively. In addition, it is rather difficult and time-consuming to isolate cysteine proteinase inhibitors directly from natural sources. A more efficient approach to obtain amounts of inhibitors is to produce these proteins in bacterial expression systems. During the past few years, many bioactive proteins have been expressed in bacteria by using recombinant DNA techniques.

r-Soyacystatin from cloned *Escherichia coli* is a reversible noncompetitive inhibitor of papain [15]. It was found that recombinant soyacystatin was more inhibitory than E-64 [33]. We obtained a sample of culture containing more efficient vector pET28a and soyacystatin gene which we cultured and then purified r-soyacystatin. The objectives of this study were to purify soyacystatin expressed in *E. coli* and compare the inhibition efficiency to that of purified egg white cystatin against purified Pacific whiting cathepsin L.

2 Materials and methods

2.1 Materials

Papain, Sepharose 6B, Brij 35 (30% w/v), glycerol, *N*-benzoyl-L-arginine-2-naphthylamide (BANA), *N*-carbobenzoxy-Phe-Arg-7-amido-4-methylcoumarin (Z-Phe-Arg-Nmec), L-*trans*-epoxysuccinylleucylamido (4-guadino) butane (E-64), DMSO, β-mercaptoethanol (βME), *p*-dimethylaminocinnamaldehyde, Trizma base, Tricine, ammonium sulfate (AS), dithioerythritol, bovine serum albumin (BSA), kanamycin, isopropyl-β-D-thiogalactopyranoside (IPTG), L-tyrosine, Luria-Bertani broth (LB broth), low-molecular-weight standards including aprotinin (M_r 6500), α-lactalbumin (M_r 14200), trypsin inhibitor (M_r 20000), trypsinogen (M_r 24,000), carbonic anhydrase (M_r 29000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36000), ovalbumin (M_r 45000), and albumin (M_r 66000), were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Iodoacetic acid was obtained from Calbiochem (San Diego, CA, USA). Phenyl-Sepharase 6 Fast Flow, diethylaminoethyl (DEAE) Sepharose Fast Flow, and butyl-Sepharose 4B were purchased from Pharmacia (Piscataway, NJ, USA). Cloned *E. coli* containing soyacystatin gene was obtained by the courtesy of Dr. Hisashi Koiwa of Purdue University (West Lafayette, IN). Pacific whiting surimi was from a local processing company in Astoria, OR, USA. The stock solution of synthetic substrates and E-64 were prepared in DMSO and stored at -20°C until used. Fresh surimi samples without any inhibitor were obtained from a local commercial processor and transported in ice. They were packaged in individual plastic bags and kept frozen at -18°C.

2.2 Methods

2.2.1 Purification of soyacystatin

E. coli containing the soyacystatin gene (pETNM$^{8-103}$) was isolated as a single colony on agar plates by streaking. The recombinant cells were grown in small scale in 5 mL LB broth with 50 μg/L kanamycin overnight at 37°C under vigorous shaking. The following day it was inoculated into a large media (250 mL LB broth with 50 μg/L kanamycin) and allowed to grow until A_{600} reached 0.6 (generally 3–4 h after inoculation into a large culture). Finally, it was induced with 0.4 mM IPTG (final concentration) and incubated for 16 h at room temperature. The cells were harvested by centrifugation at $4000 \times g$ for 30 min using a Sorvall refrigerated centrifuge SS-34 rotor (DuPont, Newtown, CT, USA). Harvested cells were sonicated using Sonicor, Model UP-400 with ultrasonic probe (Copiague, NY, USA), in 10 mL 10-fold diluted McIlvaine's buffer (0.2 M sodium phosphate, 0.1 M sodium citrate, pH 7). Sonicated cell extract was used to purify the recombinant soyacystatin using phenyl-Sepharose and DEAE column with two-step purification [34] and was loaded onto phenyl-Sepharose column and equilibrated with 20 mM potassium phosphate, pH 6, containing 20% saturated AS. Elution was initiated with 15% saturated AS in 20 mM potassium phosphate buffer pH 6. When A_{280} of the fraction started to decrease, the elution buffer was changed to 10% saturated AS in the same buffer. Fractions were analyzed for protein concentra-

tion by measuring A_{280} and the presence of cystatin bands on SDS-Tricine PAGE. The fractions which had a visible cystatin band were combined. The sample was loaded onto a DEAE column equilibrated with 10 mM Tris, pH 8.8. After loading the sample, the column was washed with 10 mM Tris, pH 8.8, overnight and eluted with a linear gradient of 0 – 0.4 M NaCl in10 mM Tris, pH 8.8. The fractions which had a cystatin band on SDS-Tricine PAGE, were combined. The activity of combined fractions was analyzed for inhibitory activity as described in Section 2.2.4.

2.2.2 Purification of egg white cystatin

Egg white cystatin was purified according to Anastasi *et al.* [32] by using a CM-papain-Sepharose column from 12 eggs. The column was prepared according to the method of Axen and Ernback [35]. Papain (100 mg) was activated with 2 mM dithioerythritol and 1 mM disodium EDTA in 10 mL 0.1 M sodium phosphate, pH 6, for 10 min at 20°C and allowed to react with 10 mM iodoacetic acid. After activating Sepharose resin with CNBr, the resin was washed with cold 500 mL 0.1 M $NaHCO_3$, solution, pH 9.0. Activated papain solution was stirred with the Sepharose 6B overnight at room temperature for coupling. The resin was washed with 500 mL 0.01 M sodium acetate, solution, pH 4.1; 400 mL 0.1 M sodium phosphate, solution, pH 7.6, containing 1 M NaCl; 200 mL 0.1 M sodium phosphate, pH 7.6, containing 15 g/L glycine; 400 mL 0.1 M sodium phosphate, pH 7.6, containing 1 M NaCl; and finally 500 mL 0.01 M sodium acetate, pH 4.1. The purified egg white [32] was blended with an equal volume of 0.25% w/v NaCl. The pH of the solution was adjusted to 6 – 6.5 with 5 M sodium formate buffer, pH 3. To remove ovomucin from the egg white, the solution was centrifuged at $2100 \times g$ for 30 min. CM-papain-Sepharose (25 mL) was equilibrated with 50 mM phosphate buffer, pH 6.5, containing 0.5 M NaCl and 0.1% Brij. The centrifuged egg white solution was stirred with the equilibrated CM-papain-Sepharose overnight at 4°C. The resin was washed with 50 mM phosphate buffer, pH 6.5, containing 0.5 M NaCl and 0.1% Brij until A_{280} was less than 0.05. The CM-papain-Sepharose was packed onto a column and washed with two bed volumes of 50 mM phosphate buffer, pH 6.5, containing 0.5 M NaCl and 10% v/v glycerol. The bound protein was eluted with 50 mM phosphate buffer, pH 11.5, containing 0.5 M NaCl and 10% v/v glycerol. Fractions (2 mL) showing inhibitory activity against papain were combined and the pH was adjusted to 7.4 with 5 M sodium formate buffer, pH 3.

2.2.3 Purification of cathepsin L from Pacific whiting muscle

Cathepsin L was purified from parasitized Pacific whiting muscle according to the method of Seymour *et al.* [37]. To obtain sarcoplasmic fluid, 400 g finely chopped fish fillet was centrifuged at $5000 \times g$ for 30 min. The supernatant was combined with an equal volume of McIlvaine's buffer (0.2 M sodium phosphate and 0.1 M sodium citrate), pH 5.5, and the mixture was heat-treated at 60°C for 3 min in 100 mL fractions. The heat-treated mixture was centrifuged at $7000 \times g$ for 15 min and the supernatant was dialyzed overnight against 20 mM Tris buffer, pH 7.5, at 4°C. After dialysis, saturated ammonium sulfate was added to adjust the concentration to 1 M. The sample was loaded onto the butyl-Sepharose column which was previously equilibrated with 20 mM Tris, pH 7.5, containing 1 M ammonium sulfate. After loading the sample, the column was washed with equilibration buffer until A_{280} was less than 0.05. The sample was eluted with 20 mM Tris, pH 5.5. Fractions (5 mL) were pooled on the basis of activity and protein content [36].

2.2.4 Assay of enzyme inhibitory activity

Inhibitory activity of purified cystatins against papain was measured by the method of Abe *et al.* [20] with a slight modification. The concentration of this enyzme was determined by active site titration with E-64. Papain solution (10 μg/mL) was activated by incubation in 25 mM sodium phosphate, pH 7, containing 20 mM β-mercaptoethanol at 40°C for 10 min. An aliquot (0.2 mL) of assay buffer (0.25 M sodium phosphate, pH 6, containing 2.5 mM EDTA) was mixed with 0.1 mL of the activated papain. After preincubation of the mixture with 0.2 mL inhibitor at 40°C for 5 min, the reaction was initiated by adding 0.2 mL BANA and incubated at 40°C for 10 min. The reaction was stopped by adding 1 mL 2% v/v HCl in ethanol, and the reaction color was developed by adding 1 mL 0.06% w/v *p*-dimethylaminocinnamaldehyde in ethanol. Reaction products with bright magenta color were measured at 540 nm. A reaction blank was prepared by substituting cystatin by water. Inhibitory activity was measured as decrease in BANA-hydrolyzing activity by comparing papain activity measured with and without cystatin samples. One "unit" of inhibitory activity was defined as changes in absorbance at 540 nm per hour. Inhibitory activity of cystatins against cathepsin L was measured according to Barrett and Kirschke [37] with a slight modification. This assay was standardized by measuring the absolute concentration of cathepsin L which was determined by active-site titration with E-64. The inhibitory activity was determined directly by measuring the residual activities using the fluorescent substrate Z-Phe-Arg-Nmec. Cathepsin L (25 μL from 4.00 μg/mL in 0.1% Brij solution) was mixed with 25 μL of a suitably diluted inhibitor solution. To the enzyme inhibitor solution, 50 μL buffer (0.2 M sodium phosphate, 0.1 M citrate, 4 mM disodium EDTA, and 8 mM dithioerythritol, pH 7) was added. The r-soyacystatin, egg white cystatin, and *E. coli* cell lysates were tested as inhibitors. After incubation at 30°C for 5 min, 500 μL 0.1% Brij solution and 200 μL assay buffer (340 mM sodium acetate, 60 mM acetic acid, 4 mM disodium EDTA,

and 8 mM dithioerythritol, pH 5.5) were added. The reaction mixture was re-equilibrated at 30°C for 1 min. After preincubation, 250 μL 20 μM substrate was added to start the reaction and the reactiom mixture was incubated for 10 min at 30°C. To stop the reaction, 1 mL 5 mM iodoacetic acid was added to the reaction mixture. The fluorescence of the free aminomethylcoumarin was determined by emission at 460 nm and excitation at 370 nm using Perkin Elmer Luminescence Spectrometer Model (Norwalk, CT, USA). One "unit" of inhibitory activity was defined as the amount of cystatin inhibiting one unit of proteolytic activity that hydrolyzed the fluorogenic substrate and released 1 nmol of methylcoumarin within 1 min at 30°C.

2.2.5 Autolysis assay

An autolysis assay was carried out according to Morrissey *et al.* [38]. Finely chopped Pacific whiting surimi (3 g) was mixed with 3 mL inhibitor solutions to obtain a final designated concentration (0.041% w/w and 0.052% w/w soyacystatin cell extract, 1% w/w BPP, and 0.1 mM E-64). Mixtures were incubated at 55°C for 60 min. The autolytic reaction was stopped by adding 5% w/v cold TCA solution. To precipitate unhydrolyzed proteins, the solution mixture was incubated at 4°C for 15 min and centrifuged at 6100 × *g* for 15 min. The supernatant containing TCA-soluble proteins was analyzed for oligopeptide contents using the Lowry assay [39]. Autolytic activity was expressed as nmol tyrosine released per min. Sample blanks containing all components were kept on ice and used to correct for oligopeptide content originating from Pacific whiting, Pacific whiting surimi, and proteinase inhibitors. For control we used 0.1 mM E-64 and BPP. All samples were run in duplicate.

$$\% \text{ Inhibition} = \frac{(C_{55} - C_0) - (I_{55} - I_0)}{(C_{55} - C_0)} \times 100 \qquad (1)$$

C_{55} = nmole tyrosine/min surimi without inhibitor at 55°C
C_0 = nmole tyrosine/min surimi without inhibitor at 0°C
I_{55} = nmole tyrosine/min surimi with inhibitor at 55°C
I_0 = nmole tyrosine/min surimi with inhibitor at 0°C

2.2.6 Gel electrophoresis and protein content

SDS-PAGE gels (15%) were performed according to Laemmli [40] and 16.5% Tricine SDS-PAGE gel was performed according to Schagger and Jagow [41]. Since soyacystatin has a low molecular mass, a better separation of cystatin compounds was observed with Tricine SDS-PAGE than with the traditional Laemmli's SDS-PAGE system. Protein samples were boiled for 5 min in SDS-PAGE treatment buffer (0.125 M Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, 1:1 v/v) and applied on 15% and 16.5% polyacrylamide gels. Gels were run under a constant voltage of 150 V (on ice to prevent overheating) using a Bio-Rad Mini-Protean II unit (Bio-Rad, Hercules, CA, USA). Protein contents were determined according to Lowry *et al.* [39] using bovine serum albumin as a standard.

3 Results and discussion

3.1 Purification of r-soyacystatin and egg white cystatin

r-Soyacystatin was purified from *E. coli* overexpressing BL21 (DE3) pETNM$^{8-103}$ gene and its properties were compared to egg white cystatin purified from egg white. The recombinant cells were grown in LB broth. After IPTG induction, a high level of soluble r-soyacystatin was expressed as the major protein component in *E. coli* BL21 (DE3) pETNM$^{8-103}$ cells (Fig. 1). The r-soyacystatin was purified to electrophoretic homogeneity by 20–10% saturated ammonium sulfate, phenyl-Sepharose, and 0–0.4 M NaCl DEAE chromatograms. The purity of r-soyacystatin on each of the purification step is shown on SDS-PAGE (Fig. 1). The molecular mass of r-soyacystatin was estimated to be appximately 10.8 kDa. As shown in Fig. 1, the r-soyacystatin constituted a high percentage of the total cell protein. Approximately 20 mg purified cystatin was obtained from 213 mg proteins of *E. coli* cells with a specific activity of 14 957 U/mg (Table 1). The purification used provided a simple purification protocol with a high yield of r-soyacystatin, which indicated a high potential for this protocol to be used in a commercial application.

Egg white cystatin was purified by affinity chromatography. For this study, CM-papain-Sepharose, which was

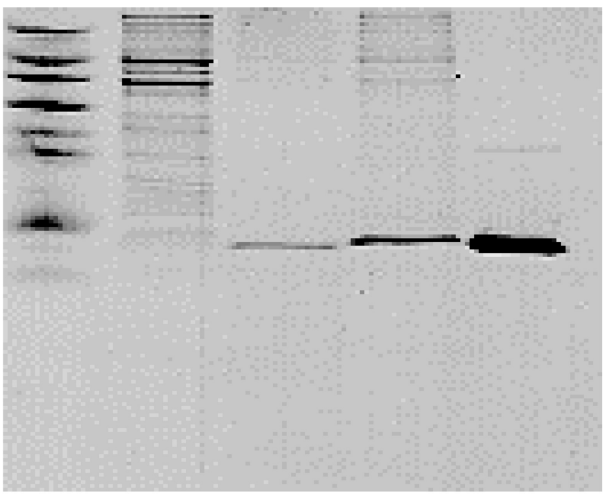

Figure 1. Various stages of purification of recombinant soyacystatin on SDS-tricine PAGE. (1) 5 μg uninduced recombinant *E. coli* cell lysate; (2) 5 μg induced recombinant *E. coli* cell lysate; (3) 5 μg of ammonium sulfate precipitated *E. coli* cell lysate; (4) 5 μg purified recombinant soyacystatin; (M) low-molecular-weight standards.

Table 1. Purification of soyacystatin

Steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purity
E. coli lysate[a)]	213	753 219	3 536	100	1.0
DEAE	20.0	299 130	14 957	39.7	4.3

a) The starting volume was 500 mL cultivated broth.
The inhibitory activity was measured as decrease in BANA hydrolyzing activity. One unit of inhibitory activity was defined as the changes in absorbance at 540 nm per hour.

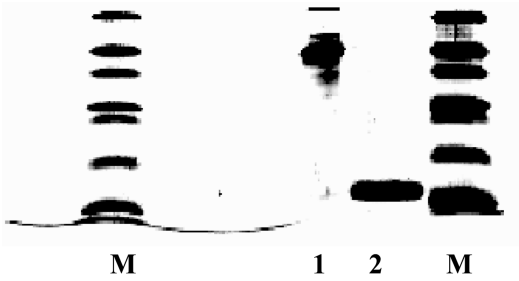

Figure 2. Egg white purification step on the SDS-PAGE. (M) Low molecular mass marker; (1) egg white proteins; (2) purified egg white cystatin; (M) low-molecular-mass marker.

effective in isolating cystatin from numerous egg white proteins, was used as the affinity media. By taking advantage of the instability of cysteine proteinase under alkaline conditions, the bound cystatin was eluted from CM-papain-Sepharose by increasing the pH to 11.5. Egg white cystatin was purified from 12 pooled egg whites and the pure egg white cystatin is shown in Fig. 2. Approximately 5 mg purified cystatin was obtained from 29 700 mg egg white proteins with a 240-fold purification.

3.2 Purification of cathepsin L

Two forms (P-I and P-II) of cathepsin L were purified from Pacific whiting muscle (Fig. 3). The heat treatment step served to inactivate and remove heat-labile proteolytic enzymes, myofibrillar and sarcoplasmic proteins. Hydrophobic chromatography on butyl-Sepharose was effective in separating the activity of two peaks designated as P-I and P-II [36]. Fractions from both peaks containing active enzymes as analyzed by their proteolytic activity were pooled and applied on SDS-PAGE. As seen on the SDS-PAGE gel, the P-I fraction contained the low-molecular-mass component while P-II showed only one band whose molecular mass was 28.8 kDa (Fig. 2). An *et al.* [42] reported that the P-I was complexed with an inhibitor while the P-II was a free form of the enzyme. An *et al.* [42] showed that low pH treatment (pH 3.3) increased the activity of P-I by dissociating the proteinase inhibitor complex;

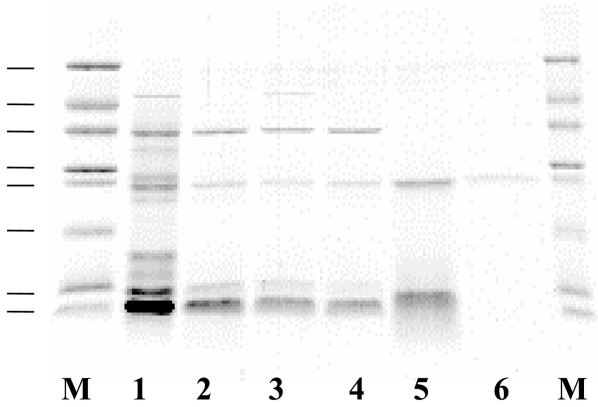

Figure 3. Various purification stages of cathepsin L purification as analyzed on 15% SDS-PAGE gel. (M) Low-molecular-weight markers; (1) 7.34 μg Pacific whiting crude muscle extract; (2) 7.4 μg crude muscle extract after heat treatment; (3) 3.155 μg dialysate of heat-treated crude muscle extract; (4) 4.31 μg ammonium sulfate fraction; (5) purified Pacific whiting cathepsin L, 6.85 μg PI and (6) 4 μg PII.

however, it had no effect on P-II. This result indicates that P-II is a pure enzyme. Approximately 29.6 mg of pure PII was obtained from 11 756 mg fish proteins with a 21-fold purificaion. These results agree with the work previously reported by Seymour *et al.* [36] who purified cathepsin L from Pacific whiting.

3.3 Inhibitory activity

When 1 μg of papain was incubated with inreasing concentrations of pure r-soyacystatin and egg white cystatin, linear concentration inhibition relationships were observed. According to he titration curves of papain by r-soyacystatin and egg white cystatin the required amount to reach 50% inhibition of papain was obtained with 0.208 μg and 0.304 μg, respectively (Fig. 4). As shown in Fig. 1, lane 2, the r-soyacystatin constituted a high percentage of the total secreted protein in the culture broth of *E. coli*. Considering the purification of r-soyacystatin which decreased the yield and increased the cost, *E. coli* cell lysate was directly tested for its inhibitory activity against papain. To achieve the same inhibitory activity as r-soyacystatin for titration of papain, the amount of *E. coli* cell lysate was 0.822 μg (Fig. 4). Although slightly more is required to obtain equivalent inhibitory effects, purification is not needed. Therefore, the recombinant inhibitor prepared as a lysate would be an economical alternative.

According to the titration curves of cathepsin L by r-soyacystatin and egg white cystatin, the same linear concentration inhibition relationship was observed when 0.1 μg cathepsin L was incubated with increasing concentrations of r-soyacystatin and egg white cystatin (Fig. 5). For 50%

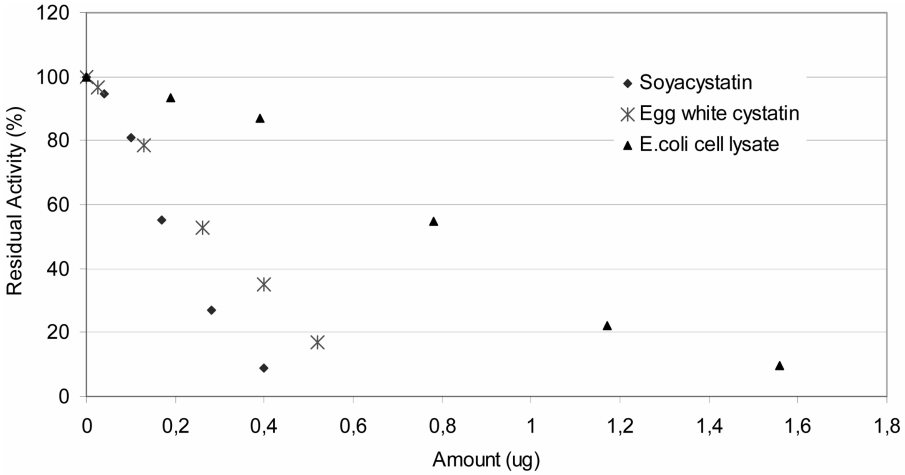

Figure 4. Inhibitory activity of cystatins against papain. Inhibitory activity of *E. coli* cell lysate was tested against 1 μg papain using BANA as a substrate at 40°C for 10 min (values in this figure are the means of three replicates).

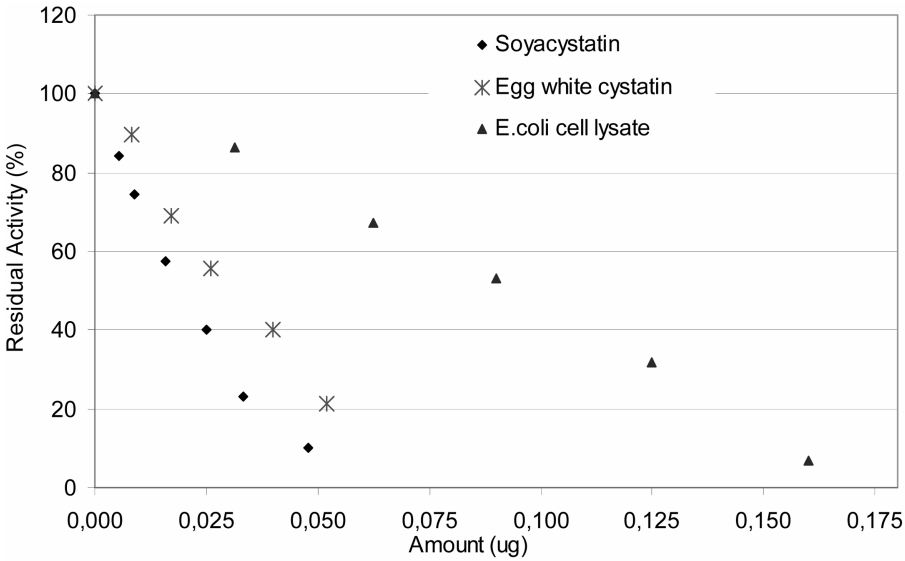

Figure 5. Inhibition of cathepsin L by cystatins. Cathepsin L was incubated with soyacystatin at 30°C for 5 min, and residual activity of cathepsin L was analyzed at 30°C for 10 min and pH 5.5 by using Z-Phe-Arg-Nmec (values in this figure are the means of three replicates).

inhibition of cathepsin L used in the assay, 0.0245 μg soyacystatin, 0.0326 μg egg white cystatin, or 0.0886 μg *E. coli* cell lysate (Fig. 5) was needed.

The inhibitory activity of cystatins was dose-dependent against papain and cathepsin L when the ratio of cystatins/cysteine protease was smaller than 1. There was no significant increase in the inhibition ability when the ratio of cystatins/cysteine protease was bigger than 1. The same results were obtained for cathepsin L (Figs. 6 and 7), indicating that one molecule of r-soyacysyatin binds to one molecule of cysteine protease. This was in aggreement with the findings for the native cystatin family [22, 23, 43, 44]. As shown in Figs. 6 and 7, the inhibitory ability of r-soyacystatin against papain and cathepsin L appeared to be comparable with that of egg white cystatin. Egg white cystatin was reported to be a reversible and tight binding inhibitor of papain-like proteinases [3]. r-Soyacystatin was reported to be a non-competitive inhibitor of papain [15]. It was found that it was more inhibitory and cysteine proteinase inhibitor and E-64 [33]. Figure 5 shows that r-soyacystatin was also a strong inhibitor of cathepsin L.

3.4 Inhibition of autolytic activity in surimi

High levels of cysteine proteinase activity due to cathepsin B, H, and L have been reported in Pacific whiting and arrowtooth flounder [28, 29]. These cysteine proteinases are highly active in *post mortem* muscle condition (pH 5.5 – 6) and thus can degrade the myofibrillar proteins [28] and cause gel degradation in surimi. After cooking Pacific whiting surimi for 30 min at 60°C, myosin heavy chain is completely lost [42]. It is known that myosin degradation is the cause of a loss of strength in surimi gels. Although cathepsin B is the most active cysteine proteinase in Pacific whiting fillets, cathepsin L is the major source of proteolytic degradation of Pacific whiting surimi since cathepsin B and

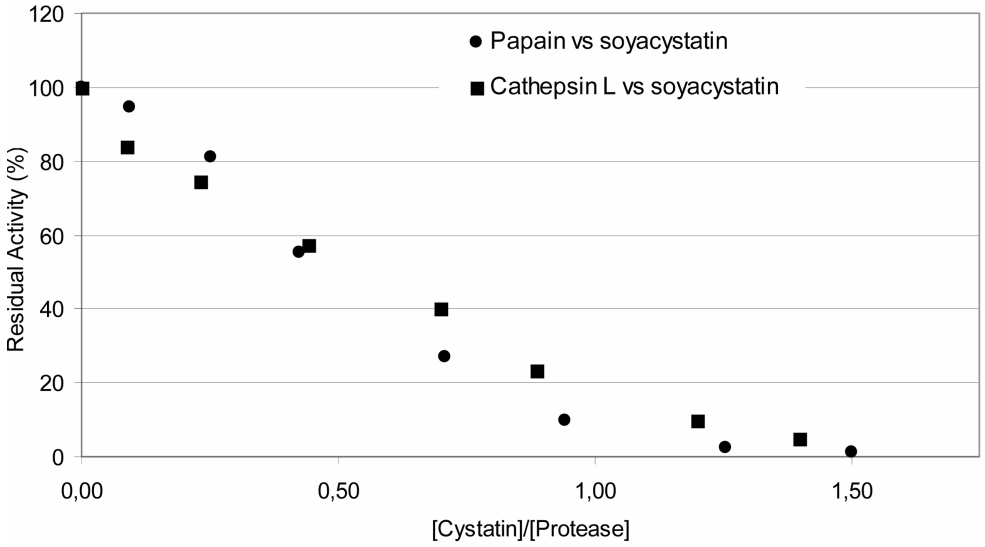

Figure 6. Inhibition profiles of r-soyacystatin against papain and cathepsin L. [Cystatins]/[cysteine proteinase] is the molar ratio (values in this figure are the means of three replicates).

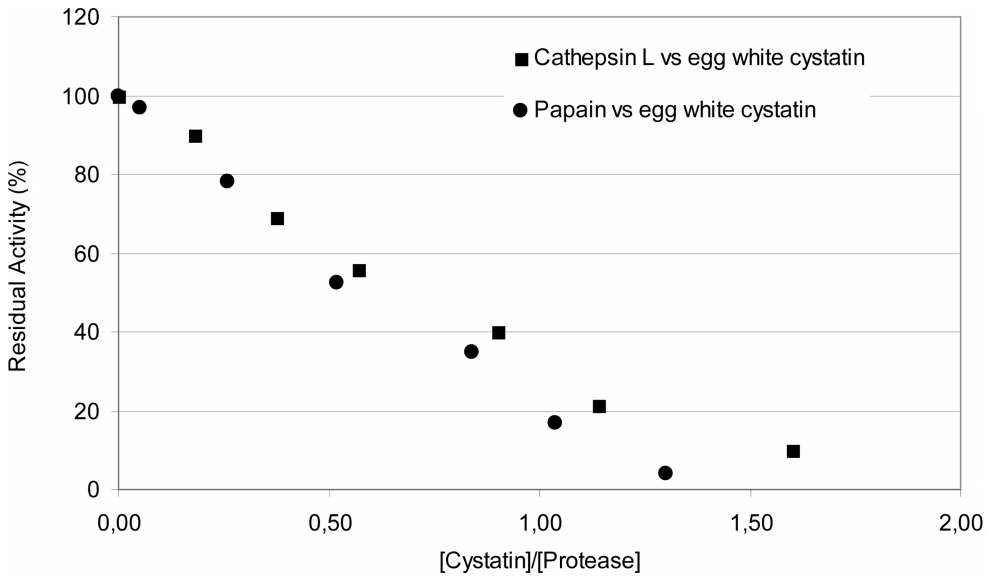

Figure 7. Inhibition profiles of egg white cystatin against papain and cathepsin L. [Cystatins]/[cysteine proteinase] is the molar ratio (values in this figure are the means of three replicates).

H and other proteinases are removed during the processing of surimi by washing but cathepsin L is not completely removed by the washing step [29]. Consequently, cathepsin L is the main proteinase present in surimi. To further investigate the effect of recombinant cystatin on preventing autolytic activity of Pacific whiting surimi, r-soyacystatin without purification (*E. coli* cell lysate) was tested with an autolytic activity assay using Pacific whiting surimi by incubating surimi samples with and without inhibitors at 55°C for 60 min. Previous reports showed that BPP is the most effective food grade inhibitor to control the protease in surimi [42]. E-64 was used to identify another inhibitory substance that would replace BPP in this application. Soyacystatin cell extract tested in the surimi autolytic assay at the 200-fold less concentration than BPP showed the same level of inhibitory activity with 1% w/w BPP in preventing the autolysis of Pacific whiting surimi tested in this assay. Increasing the soyacystatin concentration from 0.0052% (equal to 552 U inhibitory activity) to 0.041% (equal to 4349 U inhibitory activity) did not significantly increase the percent inhibition in the autolysis of surimi (Table 2).

r-Soyacystatin is speculated to be more effective than other forms of proteinase inhibitors, such as BPP, for surimi application due to its small molecular mass, which should facilitate easy diffusion into the muscle cells in surimi, resulting in more effective prevention of autolytic activity in surimi caused by cysteine proteinase. Pacific whiting, the most abundant fishery resource of the U.S. Northwest coast, has a limited market due to high proteolytic activity related to softening during cooking which might be utilized for surimi by this process.

Table 2. Autolytic activity of parasitized Pacific whiting fillet with and without addition of inhibitors

Sample	TCA-soluble peptides (nmol Tyr/min)	% Inhibition
Pacific whiting surimi	2.56 + 0.018	0
0.041% w/w soyacystatin cell extract	0.490 + 0.015	80.8
0.0052% w/w soyacystatin cell extract	0.625 + 0.043	75.6
0.1 mM E-64	0.415 + 0.037	83.8
1% BPP	0.624 + 0.024	75.6

4 Concluding remarks

According to the data obtained in this study r-soyacystatin had inhibitory properties comparable to those of egg white cystatin. r-Soyacystatin from *E. coli* inhibited the autolytic activity of Pacific whiting surimi caused by cathepsin L. To achive the same inhibitory level as soyacystatin, a higher amount of BPP was required. We demonstrated that adding r-soyacystatin from *E. coli* may be a feasible method to substitute BPP in surimi application.

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