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Enhanced Expression of Chicken Cystatin as a Thioredoxin Fusion Form in *Escherichia coli* AD494(DE3)pLysS and Its Effect on the Prevention of Surimi Gel Softening

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The DNA encoding chicken lung cystatin was ligated into a thioredoxin−pET 23a+ expression vector and transformed into *Escherichia coli* AD494(DE3)pLysS. A high level of soluble recombinant thioredoxin−cystatin (trx−cystatin) was expressed in the cytoplasm of the *E. coli* transformant. As compared with recombinant cystatin (trx-free), a 38.7% increase of inhibitory activity in the soluble fraction was achieved by introducing the trx fusion protein. Trx−cystatin was purified to electrophoretical homogeneity by 3 min of heating at 90 °C and Sephacryl S-100 chromatography. The molecular mass of trx−cystatin was 29 kDa, which was the expected size based on its composition of recombinant trx (16 kDa) and chicken cystatin (13 kDa). The purified trx−cystatin behaved as a thermally stable and papain-like proteinase inhibitor comparable to either recombinant or natural chicken cystatins. The inhibitor could inhibit the gel softening of mackerel surimi.

KEYWORDS: Chicken cystatin; cysteine proteinase inhibitor; thioredoxin; pET 23a+; *E. coli* AD494-(DE3)pLysS; overexpression

INTRODUCTION

Surimi (a Japanese commercial name for minced fish) is a refined form of mechanically deboned fish meat; it has unique functionality including gel-forming ability and water- and oil-binding properties. These characteristics make it a valuable ingredient or base component for a broad range of food products. However, proteolytic disintegration of surimi gels is characterized by high activity at temperatures near 50 °C and by rapid and severe degradation of myofibrillar proteins, particularly myosin (*1*). This disintegration has detrimental effects on surimi quality, which substantially lowers the gel strength and elasticity (*2*−*4*). Cystatin, a cysteine proteinase inhibitor, could inhibit the autolysis or gel softening of mackerel (*5*).

The cystatin superfamily comprises a number of cysteine proteinase inhibitors that are widely distributed in vertebrate and plant tissues (*6*−*12*). It has been subdivided into three individual families on the basis of their size and structure. Members of families I and II are also respectively recognized as stefin and cystatin families. Family III is larger glycoproteins (M_r = 60−120 kDa), previously known as kininogen existing in blood plasma (*8*). Inhibitors of families I and II are structurally related but differ in certain aspects. The stefin family has a polypeptide of ∼100 residues without disulfide bridges, whereas the cystatin family is somewhat longer, ∼120 residues, and has two disulfide bridges (*8*). The best characterized members of the cystatins are chicken cystatin (*13*−*16*), human cystatin C (*17*−*21*), and rat cystatin S (*22*). These inhibitors inactivate lysosomal cysteine proteinases such as cathepsins B, H, and L, as well as several structurally similar plant proteinases such as papain and actinidin, by forming a tight equimolar complex. The formed complex has a very low dissociation constant of 20 nM−10 fM that effectively blocks the active site of proteinases (*13*, *17*, *18*, *20*, *23*−*26*). They are thus considered to be the physiological regulators for cysteine proteinases (*23*, *24*, *26*, *27*). According to the crystal structures of chicken cystatin and cystatin−papain complex, the papain has three contact regions acting as binding sites to form complementary wedge-shaped edges on the inhibitor. These wedge-shaped edges insert swiftly into the active-site cleft of papain with minimal conformational change (*28*, *29*).

The chicken-originated cystatin contains two disulfide bonds that link the residues Cys71−Cys81 and Cys95−Cys115 (*30*). The Cys71−Cys81 bond links a small segment of α-helical structure to the main β-sheet of protein, and the Cys95−Cys115 bond joins the two carboxy-terminal strands of this sheet (*28*). Reduction of the disulfide bonds of chicken cystatin leads to a drastic loss of the inhibitory activity (*30*). According to Bjork and Ylinenjarvi (*31*), selective reduction of the Cys95−Cys115 bond induced a conformational change and subsequently decreased the inhibitory activity of chicken cystatin. These phenomena suggest that the disulfide bonds may play an important role in the folding of molecular structure and inhibitory activity of chicken cystatin.

The low yield and complicated procedures in the purification of cystatin from plants or animal tissues make the running cost

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higher and the procedure more time-consuming (*11*). Therefore, large-scale production of cystatin by biotechniques is highly demanded. Although many cDNAs encoding cystatins and their variants have been cloned and expressed in *Escherichia coli* (*22*, *32*−*36*), most of these recombinant cystatins were found to be inert and/or insoluble inclusion bodies. Therefore, further treatments, such as dialysis by using urea, SDS, guanidine, or organic solvents, are necessary to get the soluble and correctly refolded structure of cystatin (*22*, *34*−*36*). These procedures would substantially increase the running cost and, consequently, limit their applications. In this study we have successfully constructed the thioredoxin−pET 23a+ expression vector encoding the chicken cystatin and transformed it into *E. coli* AD494(DE3)pLysS. The recombinant thioredoxin−chicken cystatin (trx−cystatin) was overexpressed as a soluble form in the cytoplasm of the transformant and purified to electrophoretical homogeneity simply by heating and Sephacryl S-100 HR chromatography.

MATERIALS AND METHODS

Materials. Total RNA isolation reagent (TriZol reagent), ELONGASE enzyme mix (proofreading DNA polymerase), T4 DNA ligase, reverse transcriptase (SuperScript II RT), LB media, X-Gal, protein ladder (protein marker, 10 kDa), and all of the primers for PCR reaction were the products of Life Technologies Inc. (Gaithersburg, MD). *Taq* DNA polymerase (AmpliTaq Gold) was purchased from Perkin-Elmer Inc. (Norwalk, CT). Restriction enzymes, pGEM-T easy vector, and DNA purification system were obtained from Promega Co. (Madison, WI), whereas the pET 23a+ expression vector was the product of Novagen Inc. (Madison, WI). The pTrxFus vector and *E. coli* Top 10F′ were purchased from Invitrogen Inc. (Carlsbad, CA), and the PCR purification kit was from Qiagen GmbH (Hilden, Germany). Agarose gel DNA extraction kit and RNase (from bovine pancreas) were obtained form Boehringer Mannheim GmbH (Mannheim, Germany). Isopropyl β-D-thiogalactopyranoside (IPTG), ampicillin, kanamycin, and papain (2× crystallized) were purchased from Sigma Chemical Co. (St. Louis, MO). Benzyloxycarbonyl-phenylalanylarginine-7-(4-methyl)-coumarylamide (Z-Phe-Arg-MCA) was the product of Peptide Institute (Osaka, Japan).

Purification of Cathepsins and Papain. Mackerel cathepsins B and L were purified from the dorsal muscle acetone powder according to the methods of Lee et al. (*37*) and Jiang et al. (*38*). Papain (2× crystallized, Sigma Chemical Co.) used in this study was further purified according to the method of Machleidt et al. (*39*).

Molecular Cloning with pGEM-T Vector. Total RNA was isolated from chicken lung according to the TriZol Reagent instruction manual. Standard molecular cloning techniques were performed according to the methods of Sambrook et al. (*40*). Chicken lung cDNA was synthesized by reverse transcription from total RNA using oligo-dT as primer. Polymerase chain reaction (PCR) was used to amplify the DNA of the chicken lung cystatin with 25 cycles, which was initiated by 30 s of denaturation at 95 °C, 30 s of annealing at 58 °C, 30 s of extension at 70 °C, and a final extension at 70 °C for 10 min in a DNA thermal cycler (Perkin-Elmer, GeneAmp PCR system 2400).

Oligonucleotides with sequences 5′-*GGTACC*TAGCGAGGACCGCTCCCGGCTCCTGGG (with *Kpn*I restriction cutting site, in italics), based on the nucleotide residues 128−153 of chicken cystatin (underscored), and 5′-*CTCGAG***TTA**CYGGCACTTGCTTTCCAGCAGTTT (with *Xho*I restriction cutting site, in italics; stop codon in boldface), based on the nucleotide residues 452−478 of chicken cystatin (underscored) (*41*), were used as primers for PCR. After being amplified, the PCR product was ligated into pGEM-T easy vector and transformed into cloning host, *E. coli* Top 10F′, according to the method of Hanahan and Meselson (*42*). *E. coli* transformant was screened by blue-white selection, PCR confirmation, and DNA sequencing using T7 and SP6 as sequencing primers.

Construction of Trx—Cystatin—pET 23a+ Expression Vector. To construct the trx−cystatin−pET 23a+ vector, the DNAs of thioredoxin (trx) and chicken lung cystatin were ligated into the pET 23a+ vector. The DNA fragment of trx released from the pTrxFus vector by using *Nde*I/*Bam*HI was first ligated into the pET 23a+ expression vector by using T4 DNA ligase. The chicken lung cystatin DNA was then inserted into the trx−pET 23a+ expression vector between the *Kpn*I and *Xho*I restriction enzyme cutting sites. After ligation of the DNA fragment and plasmid, the resulting plasmid was transformed into an expression host, *E. coli* AD494(DE3)pLysS.

Transformation and Screening of *E. coli* AD494(DE3)pLysS Transformant. The trx−cystatin−pET 23a+ was transformed into *E. coli* AD494(DE3)pLysS using a heat-shock procedure; the pET 23a+ vector and pLysS plasmid carry ampicillin and chloramphenicol resistance genes. In addition, *E. coli* AD494(DE3)pLysS is a kanamycin-resistant strain. The *E. coli* AD494(DE2)pLysS transformant carrying pET 23a+ can thus be screened using antibiotic-resistant plates (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, and 15 g of agar/L of distilled water, containing 100 μg of ampicillin, 15 μg of kanamycin, and 34 μg of chloramphenicol/mL). The selective single colony was further confirmed by PCR using cystatin primers to ensure the transformants with correct trx−cystatin−pET 23a+.

Cultivation of *E. coli* Transformant and Isolation of Recombinant Trx—Cystatin. *E. coli* AD494(DE3)pLysS transformant carrying trx−cystatin−pET 23a+ was cultivated in 10 mL of broth (10 mg of tryptone, 5 mg of yeast extract, 10 mg of NaCl, 100 μg of ampicillin, and 15 μg of kanamycin in 1 mL of distilled water) in a 50 mL flask at 37 °C overnight using an orbital shaking incubator (200 rpm, model S300R, Firstek Scientific, Taiwan). One milliliter of the activated culture was transferred into 50 mL of fresh broth in a 250 mL flask. During incubation, the OD_{600} (absorbance at 600 nm) was measured. While the OD_{600} reached 0.6, IPTG was added to a final concentration of 1 mM to induce the synthesis of recombinant trx−cystatin. After 4 h of incubation, the *E. coli* transformant cells were harvested by 30 min of centrifugation at 4000*g* and then suspended in 20 mL of 20 mM Tris-HCl buffer containing 0.5 mM sodium azide (pH 7.5, buffer A). The cells were sonicated under 240 W for 10 s and a pause for 20 s, which was performed in an ice bath 360 times using a sonicator XL 2020 system (HEAT Systems Inc., Farmingdale, NY).

The crude recombinant trx−cystatin was obtained from the soluble fraction of sonicated sample by 30 min of centrifugation at 5000*g*.

Purification of Trx—Cystatin and Recombinant 13 kDa Cystatin. The soluble recombinant trx−cystatin in buffer A was heated in a 90 °C water bath for 3 min and immediately cooled in ice water for 30 min. The heat-labile contaminant proteins were removed by 30 min of centrifugation at 20000*g*. The supernatant was used as crude trx−cystatin for evaluating its effect on surimi gels and further purification. After the supernatant was filtered through a 0.45 μm sterilized membrane (Gelman Sciences, Ann Arbor, MI), 1 mL of filtrate was applied onto a Sephacryl S-100 HR column (2.6 × 90 cm) and eluted with buffer A at room temperature (flow rate = 1 mL/min; collection = 10 mL/fraction). Fractions with papain inhibitory activity were pooled and concentrated by Amicon ultrafiltration with a YM 10 membrane (cutoff = 10 kDa; Amicon Co., Denvers, MA). The purified trx−cystatin was cleaved into recombinant trx and cystatin by enterokinase (Novagen Inc.). The reaction was performed in a 20 mM Tris-HCl buffer (pH 7.4) containing 50 mM NaCl and 2 mM $CaCl_2$ at 25 °C for 24 h. Separated 13 kDa recombinant chicken cystatin was further purified by FPLC Superdex 75 chromatography.

Protein Concentration. Protein concentration was determined by using the dye-binding method (*43*). Bovine serum albumin was used as a standard protein.

Sodium Dodecyl Sulfate—Polyacrylamide Gel Electrophoresis (SDS-PAGE). Purified trx-fused and recombinant cystatins in dissociating buffer (62.5 mM Tris-HCl buffer, pH 6.8, containing 5% β-mercaptoethanol, 3% SDS, and 0.002% bromophenol blue) were heated at 100 °C for 5 min. The M_r of cystatin was determined by 15% SDS-PAGE according to the method of Laemmli (*44*). Protein ladders with an M_r of 10 kDa (Life Technologies, Inc.) were used as protein marker.

Assay of Enzyme Inhibitory Activity. Cathepsins B (EC 3.4.22.1; 0.1 nmol) and L (EC 3.4.22.15; 0.088 nmol) and papain (EC 3.4.22.2; 0.05 nmol) were used as proteinases for the assessment of inhibition.

The concentrations of these cysteine proteinases were determined by active-site titration with E-64 as described by Barrett and Kirschke (*45*) and regulated to obtain a final fluorescent reading below 1000 in the control (without inhibitor) in a fluorescence spectrophotometer (model F-2000, Hitachi, Tokyo, Japan). The proteinase inhibitory activity of cystatin was assayed indirectly by measuring the residual proteinase activities using a fluorescence substrate of Z-Phe-Arg-MCA. Enzyme mixture (0.75 mL) comprised 5 μL of enzyme, 0.25 mL of 0.4 M sodium phosphate buffer (pH 6.0, containing 8 mM cysteine and 4 mM EDTA), 0.295 mL of deionized−distilled water, and 0.20 mL of serial concentrations of trx−cystatin or cystatin. Reactions were started with the addition of 0.25 mL of 40 μM Z-Phe-Arg-MCA solution (diluted in 0.1% Brij 35) and stopped by adding 1.0 mL blocking reagent (100 mM sodium acetate buffer, pH 4.3, containing 0.1 M sodium monochloroacetate) after 10 min of incubation at 40 °C. The amount of liberated aminomethylcoumarin was determined by a spectrofluorometer at an excitation of 350 nm and an emission of 460 nm. One unit of inhibitory activity was defined as the amount of cystatin that can inhibit 1 unit of the proteolytic activity, whereas 1 unit of proteolytic activity was defined as the amount of proteinase that can hydrolyze Z-Phe-Arg-MCA and release 1 μmol of aminomethylcoumarin within 1 min at 40 °C.

Thermal Stability. Purified chicken trx−cystatin in 0.2 M sodium phosphate buffer (pH 6.0) containing 2 mM EDTA, 0.1 M NaCl, and 0.5 mM NaN_3 was incubated at 30−100 °C for 30 min. After incubations, samples were cooled in ice water for 30 min and the residual activity was determined as described as above.

Effects of Trx—Cystatin on Mackerel Surimi Gel. Mackerel surimi was prepared using live mackerel dorsal muscle. After deboning, the mince was first washed with 4 volumes of chilled 0.4% $NaHCO_3$ solution, chilled water, and finally 0.3% NaCl for 5 min each time. The resulting mince was dehydrated to a moisture content of 78% by 15 min of centrifugation at 1500*g* and then mixed with 4% sucrose, 4% sorbitol, and 0.2% polyphosphate (a mixture of 50% sodium tripolyphosphate and 50% potassium pyrophosphate). The NaCl-free surimi was packaged in polyethylene bags (2.0 kg/bag) and stored at −40 °C until use. For evaluating the effect of trx−cystatin on the gel properties of mackerel surimi, frozen mackerel surimi was thawed to −3 °C in a refrigerator and mixed with 2.3, 4.5, and 9.0 units of crude trx−cystatin, which were about 1-, 2-, and 4-fold the inhibitory activity corresponding to the endogenous catheptic activity of 100 g of surimi. After grinding with 2.5% NaCl for 30 min, 3% potato starch was uniformly mixed with the ground surimi and then stuffed into a polyvinylethylene chloride tube (3.0 cm). All surimi gels were set in a 50 °C water bath for 30 min to investigate the effect of trx−cystatin on prevention of gel softening. After being heated at 90 °C for 30 min, the resulting gels were cooled in ice water for 30 min and stored in a refrigerator overnight. The gel properties were then examined by punch test using a rheometer with a 5 mm ball-type plunger. Ten determinations of breaking force (grams) and deformation (centimeters) were collected for each treatment. The change in protein patterns of surimi gels was observed using a 10% SDS-PAGE as described above.

RESULTS AND DISCUSSION

Molecular Cloning. For enhancing the expression of soluble chicken cystatin, the thioredoxin (*trx*) gene was inserted into 5′ flanking of cystatin open reading frame, which was expected to obtain more expression of the trx fusion form cystatin in bacterial host. Davis et al. (*46*) had indicated that trx is a good fusion protein for expressing the soluble form of protein in *E. coli* and excreting into the cytoplasmic space of host cell. After reverse transcription and primer PCR of the chicken lung DNA pool, a 354 bp cystatin fragment (**Figure 1**) was ligated into trx−pET 23a+ plasmid. The trx−pET 23a+ carries a strong T7 promoter for target gene transcription and is a powerful expression vector. The recombinant trx−cystatin−pET 23a+ plasmid (**Figures 2** and **3**) was then transformed into *E. coli* AD494(DE3)pLysS, which was a thioredoxin reductase mutant strain and can express the proteins with accurate disulfide bonds.

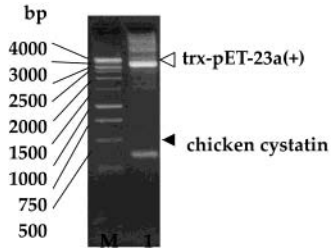

Figure 1. Analysis of trx–pET 23a+ plasmid and chicken cystatin in a 1.5% agarose gel: (lane M) 1 kb DNA marker; (lane 1) trx–pET 23a+ plasmid and mature cystatin DNA fragment. The cystatin was reverse transcription from chicken lung total mRNA and primers PCR with ELONGASE enzyme mix. A PCR product corresponding to cystatin size was inserted into the trx–pET 23a+ plasmid.

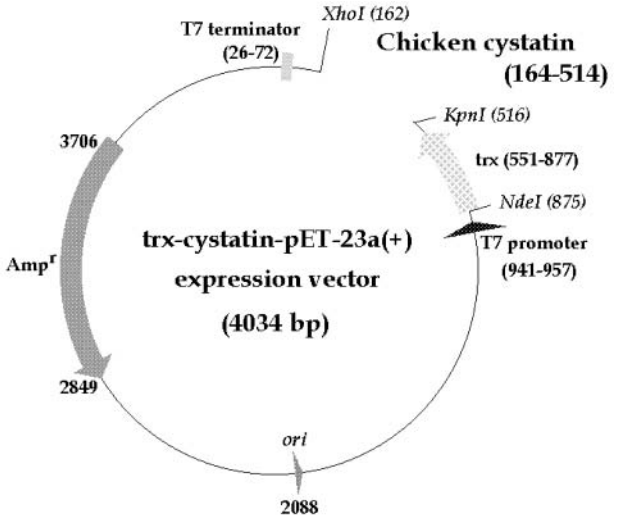

Figure 2. Construction of trx–cystatin–pET 23a+ with ampicillin resistance (Amp^r).

This mutant is considered to have high potential for the production of properly folded active proteins (*47*, *48*). In addition, the DE3 and pLysS in *E. coli* AD494(DE3)pLysS are λDE3 lysogen and pLysS plasmid, respectively. The former carries a chromosomal copy of the T7 RNA polymerase gene under the control of the *lacUV5* promoter, whereas the latter encodes a natural inhibitor of the T7 RNA polymerase. This T7 lysozyme can suppress the basal expression of T7 RNA polymerase prior to the induction and, consequently, stabilize the recombinant plasmid encoding the target protein, which may decrease the growth and viability of the host cell.

After IPTG induction, a high level of soluble recombinant chicken trx−cystatin was expressed as a major component in the cytoplasm of *E. coli* AD494(DE3)pLysS transformant (**Figure 4**, lane 1). From a comparison of the expression of trx−cystatin in *E. coli* with that of recombinant chicken cystatin (*49*), higher inhibitory activity and crude proteins of trx−cystatin were observed (trx−cystatin, 1306.3 units/L and 558.3 mg/L, respectively; recombinant chicken cystatin, 942.1 units/L and 537.2 mg/L of culture broth, respectively). Comparison with the data obtained from our previous study (*49*) shows increases of 38.7% inhibitory activity and 3.9% protein were observed in *E. coli* by introducing the trx fusion protein into the N terminus of the chicken cystatin. These data suggest that trx could enhance the production and increase the solubility of fused chicken cystatin. In the *E. coli* AD494(DE3) expression system, the trx fusion protein serves as a major physiological reductant of bacteria and then transfers into the oxidative cytoplasm, which

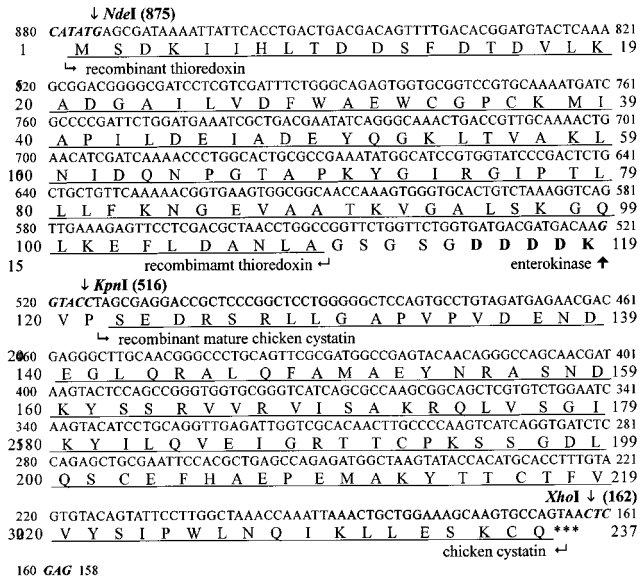

Figure 3. Construction of the sequence of trx–cystatin fusion protein. The composition of trx–cystatin nucleotide and its corresponding amino acid sequences are presented. The nucleotide sequence (158–880) is numbered by the pET 23a+ convention, and the numbers 1–237 indicate the trx–cystatin amino acid sequence. Stop codon is labeled by an asterisk (∗). The DNA region used for designing the sense and anti-sense are shaded. The restriction enzyme recognition fragment is in italic type, and the cutting sites and enterokinase cleavage site are indicated by arrows.

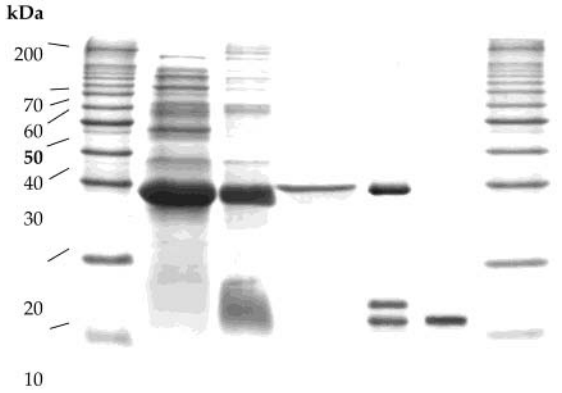

Figure 4. Purification of trx–cystatin from *E. coli* AD494(DE3)pLysS transformant cell lysate: (lane M) protein marker of 10 kDa ladder; (lane 1) cell lysate of *E. coli* transformant; (lane 2) crude trx–cystatin after 3 min of heat treatment at 90 °C; (lane 3) purified trx–cystatin after Sephacryl S-100 HR; (lane 4) protein profile after cleavage by enterokinase; (lane 5) purified 13 kDa recombinant chicken cystatin after FPLC Superdex 75 chromatography.

was deficient in thioredoxin reductase. The formation of disulfide bonds mostly occurred in cytoplasm without subsequent reduction and thus gave an appropriate folding of disulfide bonds in trx−cystatin (*50*).

Purification. Recombinant trx−cystatin existing in transformant cell lysate (**Figure 4**, lane 1) was purified to electrophoretical homogeneity by 3 min of heating at 90 °C (**Figure 4**, lane 2) and Sephacryl S-100 HR chromatography (**Figure 4**, lanes 2 and 3; **Figure 5**). Because the cystatin is a thermally stable inhibitor (*49*), a heat treatment was employed in this study, which excluded ∼40% contaminant proteins (**Table 1**). There was still ∼86% inhibitory activity left after heat treatment (**Table 1**). As shown in **Figure 4**, the trx−cystatin had an M_r of ∼29 kDa, which was the expected size, and consisted of 16

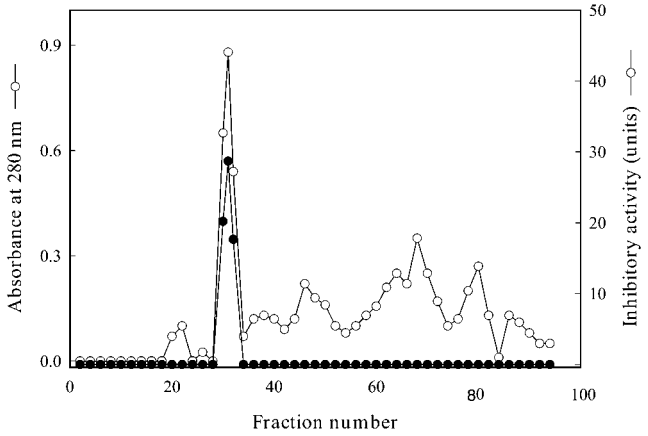

Figure 5. Chromatography of chicken trx–cystatin on Sephacryl S-100 HR. The column (2.6 × 90 cm) was pre-equilibrated with buffer A. Crude trx–cystatin (1 mL) was applied and eluted with the same buffer at a flow rate of 1 mL/min at room temperature, collection = 10 mL/fraction.

Table 1. Summary of the Purification of Trx–Chicken Cystatin from *E. coli* AD494(DE3)pLysS Transformant

procedure	total protein (mg)	total act. (units)	specific act. (units/mg)	yield (%)	purifn (fold)
cell lysate	558.3[a]	1306.3	2.34	100.0	1.0
heating (90 °C/3 min)	312.6	1127.0	3.61	86.3	1.5
Sephacryl S-100 HR	21.3	845.3	39.69	64.7	17.0
FPLC Superdex 75 (recombinant 13 kDa cystatin)[b]	10.1	685.2	67.84	52.5	29.0

[a] Starting volume of culture broth was 1000 mL. [b] Purified trx–cystatin was hydrolyzed by 10 units of enterokinase in 20 mM Tris-HCl buffer (pH 7.4) containing 50 mM NaCl and 2 mM $CaCl_2$ at 25 °C for 24 h and cleaved into recombinant trx and cystatin. Separated recombinant 13 kDa chicken cystatin was further purified by FPLC Superdex 75 chromatography.

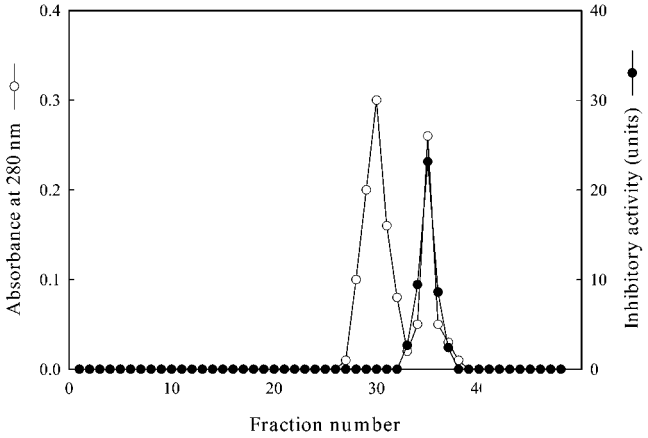

Figure 6. Chromatography of enterokinase cleavage trx–cystatin on FPLC Superdex 75. The separated recombinant cystatin (13 kDa) was eluted with buffer A at a flow rate of 0.5 mL/min, collection = 0.5 mL/fraction.

kDa of trx and 13 kDa of chicken cystatin (**Figure 3**). The trx−cystatin was hydrolyzed using enterokinase (**Figure 4**, lane 4), which can specifically cut the peptide bond after its recognition site, DDDDK (**Figure 3**, 115−119). The hydrolyzed trx−cystatin was further chromatographed on FPLC Superdex 75 (**Figure 6**). A pure 13 kDa protein (**Figure 4**, lane 5), corresponding to the recombinant cystatin that had been previously cloned (*49*), was obtained. As indicated in **Table 1**, 21.3 mg and 845.3 units of chicken trx−cystatin were obtained after Sephacryl S-100 HR chromatography. The recoveries of

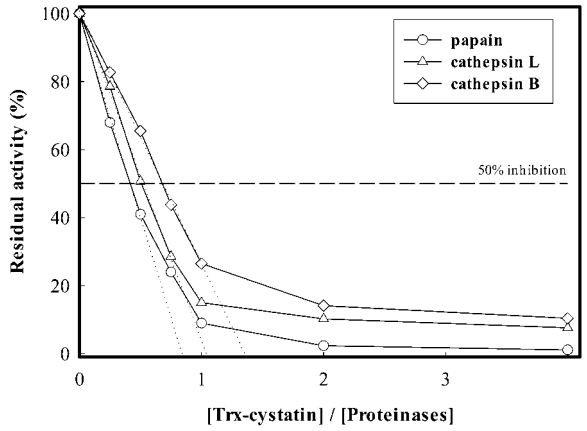

Figure 7. Inhibition profiles of chicken trx–cystatin against papain and mackerel cathepsins B and L. The [trx–cystatin]/[proteinases] was molar ratio.

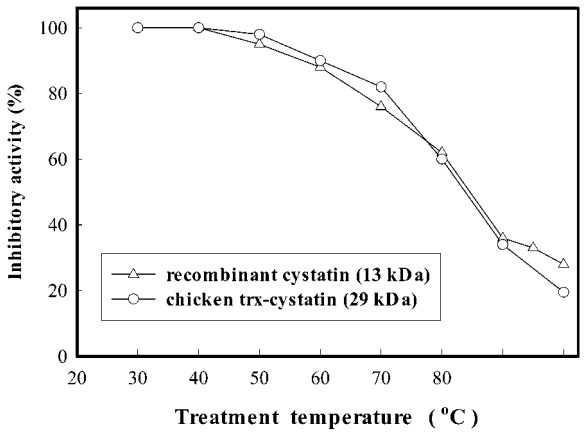

Figure 8. Thermal stability of chicken trx–cystatin (29 kDa) and recombinant cystatin (13 kDa).

chicken trx-fused and recombinant cystatins (the present study) were about 1.75- and 1.42-fold those of recombinant chicken cystatin (*49*). Also, the specific activity of trx-free cystatin, 67.84 units/mg, was 1.75-fold that (38.66 units/mg) of recombinant cystatin in a previous study (*49*). This increase might be due to the trx fusion protein in vivo, which acts as a covalently linked "chaperon" and thus improves the structure−function relationship of recombinant cystatin. Furthermore, it could also stabilize the recombinant plasmid and avoid accumulation of mutations during the production of foreign protein in bacteria (*51*). Therefore, in *E. coli* AD494(DE3)pLysS cytoplasm, the trx fusion protein enhanced the expression of soluble trx−cystatin with more correct folding of protein structure and higher specific activity. This phenomenon suggests that it would be a good system for obtaining the purified cystatin and, consequently, makes the use of recombinant inhibitor more possible in food processing.

Inhibition of Trx—Cystatin on Cysteine Proteinases. As shown in **Figure 7**, the inhibition of trx−cystatin on cysteine proteinases, such as papain and purified mackerel cathepsins B and L, was similar to those of recombinant chicken cystatin reported in our previous study (*49*). The inhibition of trx−cystatin against the proteinases used in this study was dose dependent when the ratio of trx−cystatin/proteinases was <1. No significant increase in the inhibition ability was observed at a ratio >1. This result, 1 molecule of trx−cystatin binding to 1 molecule of papain-like proteinases, coincides with that of the native cystatin family (*6*, *17*, *28*, *52*, *53*). Accordingly, the inhibition of trx−cystatin was mainly attributed to the function of cystatin, not to the trx fusion protein. Separation of trx and cystatin was observed within 1 h of reaction of trx−cystatin with papain at 37 °C (data not shown). From the data obtained, the recombinant cystatin is still active against papain-like proteinases when it is added in trx−cystatin form. In addition to titration, the apparent inhibition constants ($K_{i,app}$) of trx−cystatin binding to the papain-like proteinases were calculated according to the tight-binding inhibition (*54*). The $K_{i,app}$ values for interacting with papain and cathepsins B and L obtained from **Figure 7** were 20.8, 68.4, and 46.1 nM, respectively. For the inhibitory ability, trx−cystatin inhibited those cysteine proteinases in following order: papain > mackerel cathepsin L > mackerel cathepsin B, which was similar to that of recombinant cystatin (*49*).

Thermal Stability. After 30 min of incubation at various temperatures, the inhibitory activity of trx−cystatin was gradually decreased from 30 to 100 °C (**Figure 8**). The thermal stability of trx−cystatin was similar to that of 13 kDa recombinant chicken cystatin (**Figure 8**). There was ∼80% activity left after 30 min of incubation at 70 °C. However, the inhibitory activity of both trx−cystatin and 13 kDa recombinant cystatin decreased rapidly when the temperature was >70 °C. According to the data obtained, the thermal stability of trx−cystatin was comparable to that of recombinant cystatin (*49*).

Table 2. Effect of Chicken Trx–Cystatin on the Prevention of Gel Softening of Mackerel Surimi[a]

chicken trx–cystatin (units/100 g of surimi)	breaking force[b] (g)	deformation[b] (mm)	gel strength[b] (g·cm)
0	325.3a	7.9a	255.9a
2.3	416.8b	9.1b	377.1b
4.5	413.7b	8.6b	356.7b
9.0	432.0b	8.6b	371.7b

[a] Surimi gels with/without trx–cystatin were set at 50 °C for 30 min and finally heated at 90 °C for 30 min. The resulting gels were chilled in ice water for 30 min and stored in a 5 °C refrigerator overnight prior to the punch test. [b] Values are the means of 10 determinations; values bearing unlike subscripts in the same column differ significantly ($p < 0.05$).

Effects of Crude Trx—Cystatin on Mackerel Surimi Gel. As indicated in **Table 2**, the breaking force and deformation of control (inhibitor-free) of the surimi gel were 325.3 g and 7.9 mm, respectively. However, when 2.3 units of trx−cystatin/100 g (equivalent to the inhibitory activity corresponding to the endogenous catheptic cysteine proteinases) was added, the breaking force and deformation of surimi gel increased to 416.8 g and 9.1 mm, respectively. The gel softening of 50 °C set gels was obviously prevented by the addition of 2.3 units of trx−cystatin/100 g. No significant increase was observed even though higher amounts of trx−cystatin was added. This phenomenon further confirmed the equal molar ratio of trx−cystatin binding to catheptic cysteine proteinases as discussed in **Figure 7**.

Obvious degradation in myosin heavy chain (MHC) of control gel (**Figure 9**, lane 1) was observed; however, almost no degradation in MHC occurred in samples with various amounts of trx−cystatin (**Figure 9**, lanes 2−4). This result was consistent with that of the punch test. These data suggest that the gel softening of mackerel surimi is due to the proteolysis of endogenous catheptic cysteine proteinases (such as cathepsins B, L, and L-like) and further confirm the conclusions in our previous studies (*2*, *5*, *55*).

In conclusion, the *E. coli* AD494(DE3)pLysS expression system had a better production of the soluble form of trx−cystatin than recombinant cystatin that had been cloned previously (*49*). The trx−cystatin could be purified through a simple

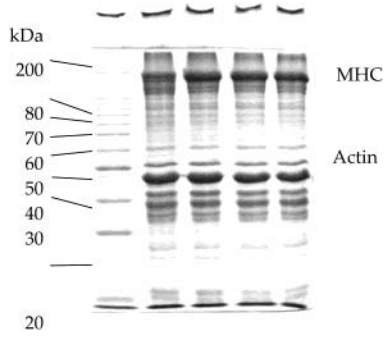

Figure 9. SDS-PAGE pattern of mackerel surimi gels set at 50 °C for 30 min: (lane M) protein marker of 10 kDa ladder; (lane 1) control (inhibitor-free) gel; (lane 2) with 2.3 units of trx–cystatin; (lane 3) with 4.5 units of trx–cystatin; (lane 4) with 9.0 units of trx–cystatin. MHC = myosin heavy chain.

procedure involving heat treatment and Sephacryl S-100 HR chromatography. Despite the existence of a trx fusion protein, the characteristics of trx−cystatin were still comparable to those of recombinant chicken cystatin. According to the data obtained (**Table 2**; **Figure 9**), trx−cystatin could be directly used to prevent gel softening of surimi without removal of trx from trx−cystatin. Higher expression in *E. coli* and a simple isolation procedure of trx−cystatin make this expression system more applicable for the production of trx−cystatin and use in seafood processing.

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