Expression of Soluble Form Carp (*Cyprinus carpio*) Ovarian Cystatin in *Escherichia coli* and Its Purification

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A DNA encoding thioredoxin−mature carp ovarian cystatin (trx−cystatin) fusion protein was ligated into a pET-23a(+) expression vector and then transformed into *Escherichia coli* AD494(DE3) expression host. After induction by isopropyl β-D-thiogalactopyranoside, a high level of the soluble form of recombinant trx−cystatin was expressed in the cytoplasm of *E. coli*. The recombinant trx−cystatin could be purified by Ni^{2+}-NTA agarose affinity chromatography. The molecular mass (*M*) of the recombinant trx−cystatin was ∼28 kDa composed of recombinant thioredoxin (16 kDa) and recombinant mature carp ovarian cystatin (12 kDa). Both recombinant trx-fused and mature carp ovarian cystatins were stable at pH 6−11. No obvious decrease in activity was observed even after 5 min of incubation at 60 °C. They exhibited papain-like protease inhibition activity comparable to that of the mature carp ovarian cystatin, which could inhibit papain and mackerel cathepsins L and L-like, but not cathepsin B.

Keywords: *Carp; cystatins; protein; purification*

INTRODUCTION

The cystatin superfamily is constituted of various cysteine proteinase inhibitors, which can be subdivided into three families including stefin, cystatin, and kininogen according to their occurrence, sequence, and structure similarity (*1*). Stefin (family I) is the smallest inhibitor in the cystatin superfamily and has a molecular mass (*M*) of ∼11 kDa. This inhibitor has no intra-disulfide bonds and glycosylation. Cystatin (family II), existing in most secreta and tissues of mammalian and avian origin, is about 13000 with two disulfide bridges. However, that obtained from chicken was found with partially phosphorylated modification (*2*). Kininogen (family III) is the largest family with an *M* of 68−120 kDa consisting of heavy and light chains and mostly existing in mammalian blood (*1*, *3*). The related cystatins originating from plant seeds, for example, corn, rice, soybean, and sunflower, are more homologous to the family II cystatin in amino acid sequence, whereas the lack of two intra-disulfide bridges on these inhibitors is similar to stefin. They are, therefore, independently defined as a "phytocystatin" family (*4*−*9*).

Cystatin shows specific inhibitory action against several cysteine proteinases from animal tissues and organs such as cathepsins B, H and L, and from plants such as papain and actinidin with a competition and tight-binding interaction (*10*, *11*). Bode et al. (*12*) and Stubbs et al. (*13*) proposed the docking model interaction between papain and chicken cystatin or recombinant human stefin. They further identified the possible binding regions between papain and these inhibitors. Cystatin has a central well-conserved motif of 53-QVVAG-57 (number in chicken cystatin), an N terminus of 103-PW-104, and a C terminus of 9-GA-1, which can offer substrate-like binding edges to papain (*14*). Accordingly, it can hinder the cleft of the proteolysis area, which consequently inactivates the papain proteolysis (*14*). Arai et al. (*15*) further confirm the necessity of the central motif of the cystatin superfamily for inhibiting cysteine proteinases.

The low yield and complicated procedures in purification of cystatin from plants or animal tissues caused an increase in running cost and were time-consuming as well (*16*). Therefore, large-scale production of cystatin by biotechnological techniques is widely demanded. Although many cDNAs encoding cystatins and their variants have been cloned and expressed in an *Escherichia coli* expression system (*4*, *6*, *17*−*20*), most of these recombinant cystatins were found to be inert and/or insoluble inclusion bodies. Therefore, further treatments such as urea, organic solvents, or dialysis treatments are somewhat necessary to get solubilized and/or a correctly refolded structure of cystatin (*17*−*20*). These procedures would substantially increase the running cost and consequently limit their applications. In this study we have successfully constructed one DNA encoding trx−cystatin fusion protein and ligated the DNA into the pET-23a(+) expression vector. The recombinant trx−cystatin fusion protein has been overexpressed as a soluble form in the cytoplasm of *E. coli* AD494(DE3) and purified to electrophoretic homogeneity only by Ni^{2+}-NTA agarose affinity 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. (Nor-

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walk, CT). Restriction enzymes, pGEM-T easy vector, and a DNA purification system were obtained from Promega Co. (Madison, WI), whereas the pET-23(+) vector was the product of Novagen Inc. The pTrxFus vector and *Escherichia coli* Top 10F′ were purchased from Invitrogen Inc., 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). Benzyloxycarbonylarginylarginine-7-(4-methyl)coumarylamide (Z-Arg-Arg-MCA) was the product of Peptide Institute (Osaka, Japan). Live female carp (*Cyprinus carpio*) was obtained from a local fish market in Keelung during spawning season.

Purification of Cathpsins and Papain. Mackerel cathepsins B, L, and L-like were purified from the dorsal muscle acetone powder according to the methods of Lee et al. (*21*) and Jiang et al. (*22*). Papain used in this study was further purified according to the method of Machleidt et al. (*23*).

Molecular Cloning with the pGEM-T Vector. Total RNA was isolated from mature carp ovary according to the TriZol reagent instruction manual. Standard molecular cloning techniques were performed according to the guidelines of Sambrook et al. (*24*). Carp ovarian double-strand 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 mature carp ovarian cystatin with 25 cycles, which was initiated by 30 s of denaturation at 95 °C, 1 s of annealing at 58 °C, and 30 s of extension at 70 °C, and then by a 10 min DNA thermal cycler at 70 °C (Perkin-Elmer, GeneAmp PCR system 2400). Primers used in PCR were designed on the basis of the carp ovarian cystatin nucleotide sequence, 113−136 and 425−445 (underlined) (*25*): the forward primer with *Kpn*I recognition sequence (italic), 5′-*GGTACC*CACTGGGATTCCTGGAGGCCTTGTA-3′; and the reverse primer with *Xho*I recognition sequence (italic), 5′-*CTCGAG*CATGCAGGTGTT TTCAGTGAC-3′. After being amplified, the PCR product was ligated with pGEM-T easy vector and transformed into expression host, *E. coli* [Top 10F′: F′{lacI^{q}Tn10(Tet^R)}mcrAΔ(mrr-hsdRMS-mcrBC)ϕ80lacZ-ΔM15ΔlacX74deo RrecA1araD139Δ(ara-leu)7697galUgalKrpsL(StrR)endA1nupG] according to the method of Hanahan and Meselson (*26*). Transformed *E. coli* was screened by blue/white selection, *Kpn*I/*Xho*I digestion of plasmid 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 mature carp ovarian cystatin were seriatim ligated into the pET-23a(+) vector. The trx nucleotide fragment 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 mature carp ovarian cystatin DNA was then inserted into the trx−pET-23a(+) expression vector between *Kpn*I and *Xho*I restriction enzyme cutting sites. The constructed plasmid was then transformed into expression host, *E. coli* AD494 (DE3,Δara-leu7967ΔlacX74 ΔphoApvuIIpho RΔmalF3F′[lac+(lacI^q)pro]-trxB::Kan).

Cultivation of *E. coli* and Isolation of Recombinant Cystatin. *E. coli* AD494(DE3) with trx−cystatin−pET-23a-(+) was cultivated in 10 mL of LB broth (10 g of tryptone/L, 5 g of yeast extract/L, and 10 g of NaCl/L) containing 100 μg/mL ampicillin and 15 μg/mL kanamycin in a 50 mL flask at 37 °C overnight using a shaking incubator (200 rpm). One milliliter of the activated culture was inoculated into 50 mL of fresh LB broth containing 100 μg/mL ampicillin and 15 μg/mL kanamycin in a 250 mL flask. During incubation, the absorbance at 600 nm (OD_{600}) 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 cystatin. After 4 h of incubation, the transformed *E. coli* cells were harvested by 30 min of centrifugation at 4000*g* and suspended in a 20 mL of 20 mM Tris-HCl buffer (pH 7.9) containing 5 mM imidazole and 0.5 M NaCl (buffer A). The harvested cells were sonicated under 240 W for 10 s and then immersed in an ice bath for 20 s. Sonication was performed for 360 cycles using a sonicator XL 2020 system (HEAT Systems Inc., Farmingdale, NY).

The recombinant cystatin was isolated from the soluble fraction of the sonicated sample by 30 min centrifugation at 5000*g* for the further purification.

Purification of Recombinant Cystatin from *E. coli* AD494(DE3). The isolated soluble recombinant cystatin in buffer A was further centrifuged at 20000*g* for 30 min. After the supernatant was filtered through a 0.45 μm sterilized membrane (Gelman Sciences, Ann Arbor, MI), the filtrate was chromatographed on an Ni^{2+}-NTA agarose affinity column (2.6 × 2 cm), which was pre-equilibrated with buffer A. After being washed with 6 volumes of the same buffer, the recombinant cystatin was eluted with 2−3 volumes of buffer A and then dialyzed against 20 mM Tris-HCl buffer (pH 7.4) for enterokinase (Novagen Inc.) cleavage and characterization.

Purification of Wild-Type Carp Ovarian Cystatin. The ovary without eggs (average weight = 3.0 ± 0.5 g), obtained from carp during spawning, was homogenized with 30 mM sodium citrate buffer (pH 4.5, buffer B). After 30 min of centrifugation at 6000*g*, the supernatant was heated in a 70 °C water bath for 5 min and cooled immediately in ice water. The heated sample was further centrifuged at 10000*g* for 30 min and then eluted by CM Sepharose Fast Flow column (5.0 × 30 cm), which was pre-equilibrated with buffer B, using a linear gradient of 0−0.8 M NaCl in buffer B at a flow rate of 2.5 mL/min. Two milliliters per tube was collected.

Fractions with cystatin activity were pooled, concentrated, and dialyzed against 20 mM Tris-HCl buffer (pH 7.5) containing 0.01% sodium azide and 0.1 M sodium chloride. Five milliliters of the dialyzed sample was chromatographed on a Sephacryl S-200 column (2.6 × 85 cm), which was pre-equilibrated with the same buffer, at a flow rate of 0.5 mL/min. Five milliliters per tube was collected, and fractions with cystatin activity (tubes 74−78) was pooled and characterized.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Purified recombinant and wild-type cystatins in dissociating buffer (62.5 mM Tris-HCl buffer, pH 6.8, containing 3% SDS and 0.002% bromophenol blue) with and without 5% β-mercaptoethanol (β-Me) were heated at 100 °C for 5 min. The molecular mass (*M*) of cystatin was determined by 12.5% SDS-PAGE according to the method of Laemmli (*27*). Protein ladders with *M* of 10 kDa (Life Technologies, Inc.) were used as protein marker.

Protein Concentration. Protein concentration was determined by according to the dye-binding method (*28*). Bovine serum albumin was used as a standard protein.

pH and Thermal Stability. Cystatin in various buffers, pH 3.0−5.0 (50 mM sodium citrate buffer), 6.0−8.0 (50 mM sodium phosphate buffer), 8.0−10.0 (50 mM Clark and Lubs solutions), and 11.0−11.9 (50 mM disodium phosphate and sodium hydroxide solution), was incubated at 30 °C. After 30 min of incubation, an equal volume of 0.2 M sodium phosphate buffer (pH 7.0) was added, and the inhibitory activity was determined.

The recombinant or wild-type cystatin in 50 mM Tris-HCl buffer (pH 7.5) was incubated at 0, 25, 40, 50, 60, 70, 80, 90, or 100 °C for 5 min. After being cooled in ice water for 30 min, the remaining inhibitory activity was measured.

Assay of Enzyme Inhibitory Activity. Papain, bovine cathepsin B, and mackerel cathepsins B, L, and L-like (*27*, *28*) were used as proteinases for the inhibition assessment. The concentrations of these cysteine proteinases were determined by active-site titration with E−64 as described by Barrett et al. (*1*) and adjusted to obtain a final fluorescent density below 1000 in control (without inhibitor). The inhibitory activity of cystatin was assayed indirectly by measuring the residual proteinase activities using fluorescence substrate Z-Phe-Arg-MCA. These enzymes in 0.2 M sodium phosphate buffer (pH 6.0), containing 4 mM cysteine and 2 mM EDTA, with or without cystatin, were preincubated at 37 °C for 10 min. The enzyme mixture (0.5 mL) comprised 5 μL of enzyme, 0.25 mL of 0.4 M sodium phosphate buffer (pH 5.0 for

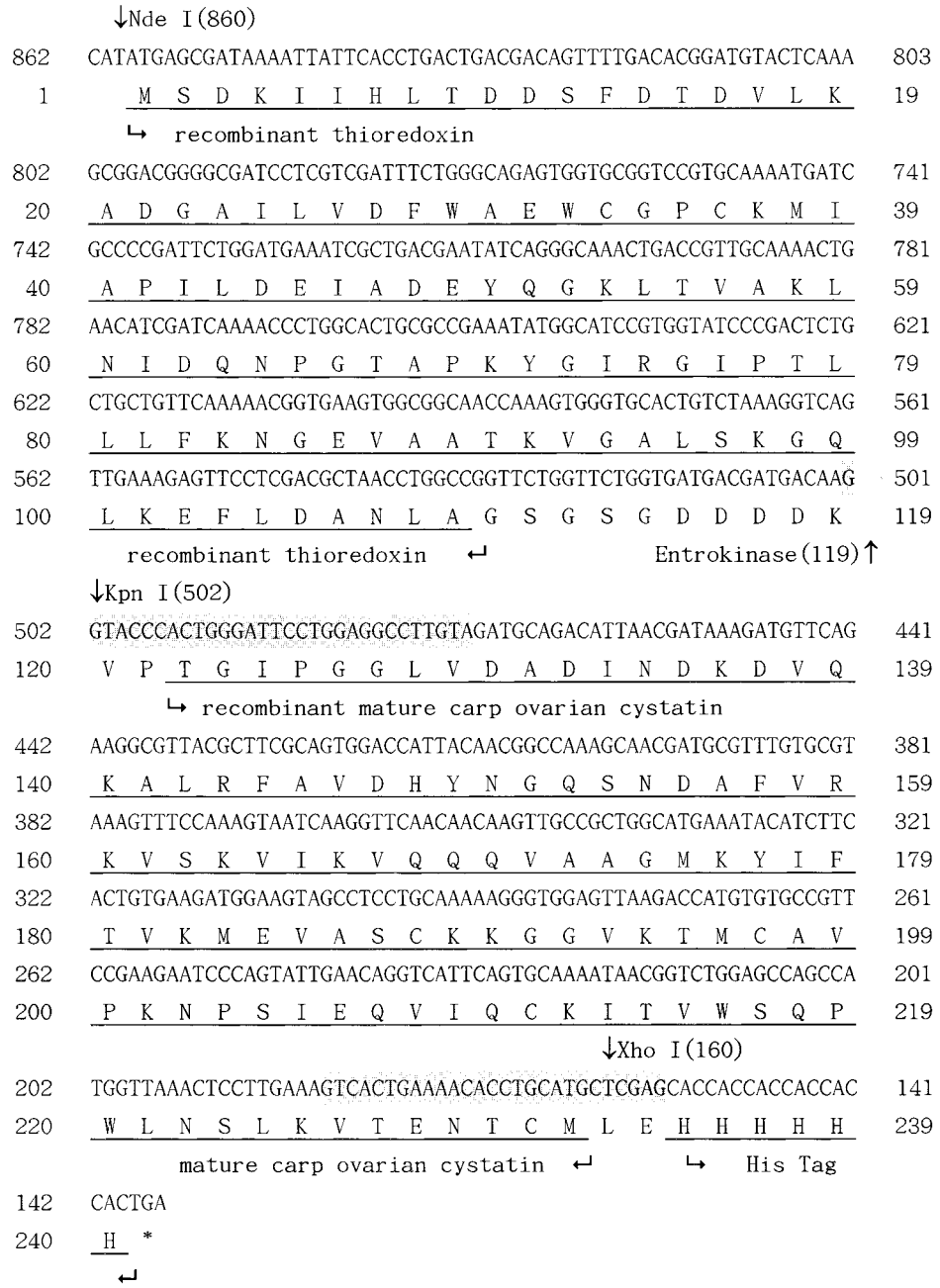

Figure 1. Construction of the trx−cystatin fusion protein. The composition of the trx−cystatin nucleotide sequence and its correspondent amino acid sequence are presented. The nucleotide sequence is numbered by the pET-23a(+) convention, and the number of amino acids indicated the trx−cystatin amino acid composition sequence. The stop codon is indicated by an asterisk (*). The DNA regions used for designing the sense and anti-sense are shadowed. The restriction enzyme cutting sites and the enterokinase cutting site were indicated by arrow.

cathepsin L, pH 5.5 for cathepsin L-like, and pH 6.0 for cathepsin B and papain) containing 8 mM cysteine and 4 mM EDTA, 0.295 mL of distilled water, and 0.20 mL of cystatin. The reaction was started by adding 0.25 mL of 40 μM Z-Phe-Arg-MCA solution and stopped by adding 1.0 mL of sodium acetate buffer containing 0.1 M sodium monochloroacetate (pH 4.3). The amount of liberated aminomethylcoumarin was determined by a spectrofluorometer at an excitation of 350 nm and emission of 460 nm. One unit of inhibitory activity was defined as the amount of cystatin that could inhibit one unit of the proteolytic activity, defined as the amount of proteinase that could hydrolyze Z-Phe-Arg-MCA and release 1 μmol of aminomethylcoumarin within 1 min at 40 °C.

RESULTS AND DISCUSSION

Molecular Cloning. According to Davis et al. (*29*), the fusion protein, thioredoxin (trx) was a good system for expressing the soluble form of protein in *E. coli* and easily secreted into cytoplasmic space of the host cell. Because the *E. coli* AD494(DE3) strain is a thioredoxin reductase mutant, it, therefore, enables the expression of the proteins with disulfide bonds and has high potential for the production of properly folded active proteins (*39*). The DE3 in *E. coli* AD494(DE3) strain is a lysogen of λDE3, which carries a chromosomal copy of the T7 RNA polymerase gene under control of the *lacUV5* promoter. On the other hand, the pET-23a(+) expression vector with a strong T7 promoter for a target gene is considered to be a powerful expression vector.

For overexpressing the soluble and active form of carp ovarian cystatin, the pET-23a(+) expression vector and the *E. coli* AD494(DE3) strain were, therefore, chosen as the expression system. A DNA encoding thioredoxin−

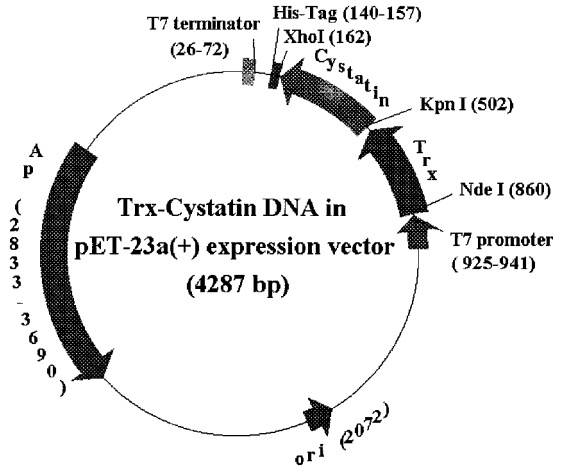

Figure 2. Construction of the pET-23a(+) expression vector.

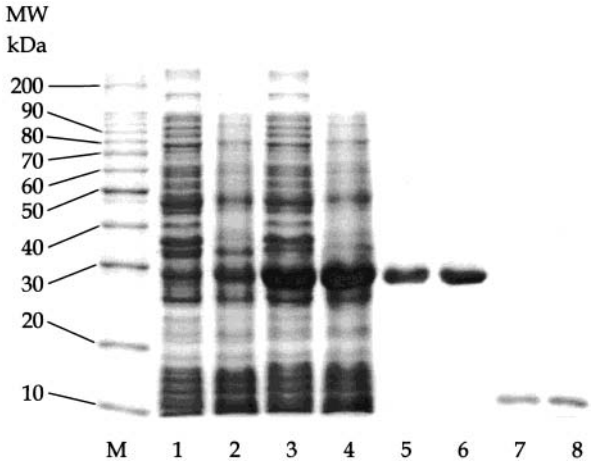

Figure 3. SDS-PAGE pattern of trx− and wild-type cystatins: M, 10 kDa protein marker; 1, precipitate of *E. coli* AD494(DE3) transformant; 2, supernatant fraction of *E. coli* AD494(DE3) transformant; 3, precipitate of IPTG induction *E. coli* AD494(DE3) transformant; 4, supernatant fraction of IPTG induction *E. coli* AD494(DE3) transformant; 5, trx−cystatin with β-Me; 6, trx−cystatin without β-Me; 7, wild-type cystatin with β-Me; 8, wild-type cystatin without β-Me.

mature carp ovarian cystatin was ligated into the pET-23a(+) expression vector (Figures 1 and 2) and then transformed into *E. coli* AD494(DE3). After IPTG induction, the *lac* repressor was released from the lac operator, a high level of the recombinant trx−cystatin protein was expressed in the cytoplasm of the transformed *E. coli* AD494(DE3), and about half of the trx−cystatin was observed in the soluble fraction of cell lysate (Figure 3, lane 4).

Purification. The trx−cystatin, tagged with an additional six histidine residues in the C-terminal of the protein, could be purified to electrophoretic homogeneity by using the Ni^{2+}-NTA agarose affinity chromatography (Figure 3, lanes 5 and 6), whereas the wild-type cystatin in carp ovary was purified by heating in a 70 °C water bath for 5 min, CM Sepharose Fast Flow, and Sephacryl S-200 chromatographs (Figure 3, lanes 7 and 8). According to SDS-PAGE, the trx−cystatin had an *M* of 28 kDa, which consisted of 16 kDa of trx and 12 kDa of recombinant mature carp ovarian cystatin. The thioredoxin portion was separated from the trx−cystatin protein after the treatment with enterokinase, and the remaining recombinant cystatin with six histidine residues in the C terminus of the protein was purified

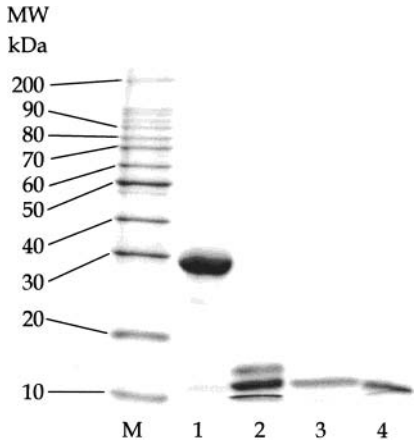

Figure 4. SDS-PAGE pattern of trx−cystatin treated with enterokinase cleavage: M, 10 kDa protein marker; 1, purified trx−cystatin; 2, trx−cystatin treated with enterokinase cleavage; 3, recombinant 12000 cystatin with β-Me; 4, recombinant 12000 cystatin without β-Me.

Table 1. Summary of the Purification of Recombinant Trx−Cystatin and Cystatin from *E. coli* AD494(DE3)

procedure	total protein (mg)	total act. (units)	specific act. (units/mg)	yield (%)	purifn fold
E. coli lysate[a]	514.4[a]	683.2	1.33	100	1.0
1st Ni^{2+}-NTA agarose (trx-cystatin)	20.1	370.7	18.44	54.3	13.9
2nd Ni^{2+}-NTA agarose (recombinant 12000 cystatin)[b]	8.9	352.1	39.56	51.5	29.7

[a] The starting volume was 500 mL of cultivated broth. [b] The purified trx-cystatin, from the first Ni^{2+}-NTA agarose affinity chromatography, was treated with enterokinase (10 units) cleavage in 20 mM Tris-HCl buffer (pH 7.4) containing 50 mM NaCl and 2 mM $CaCl_2$ at 23 °C for 24 h and then eluted through the second Ni^{2+}-NTA agarose affinity chromatography.

Table 2. Summary of the Purification of Wild-Type Cystatin from Carp Ovary

procedure	total protein (mg)	total act. (units)	specific act. (units/mg of protein)	yield (%)	purifn fold
carp ovary[a]	2606.8	473.8	0.18	100.0	1.0
heat treatment (70 °C, 5 min)	907.1	355.2	0.39	75.0	2.2
CM Sepharose FF	100.3	310.6	3.10	62.6	17.2
Sephacryl S-200 HR	4.3	137.1	31.88	28.9	177.1

[a] The average weight of the carp ovary without eggs was 3.0 ± 0.5 g.

by the Ni^{2+}-NTA agarose affinity chromatography. The purified recombinant cystatin had an *M* of 12 kDa (Figure 4, lane 2), which corresponds to that of wild-type cystatin (Figure 4, lanes 3 and 4). For the trx-fused, recombinant, and wild-type cystatins, 54.3, 51.5, and 28.9% of activity were recovered and 13.9, 29.7, and 177.1-fold purifications were achieved, respectively (Tables 1 and 2). According to SDS-PAGE, the *M* of purified trx-fused, recombinant, and wild-type cystatins were 28, 12, and 12 kDa, respectively (Figure 3, lanes 5 and 7; Figure 4, lane 3). These three forms of cystatins treated with and without β-Me revealed a single band on SDS-PAGE. Although the carp ovarian cystatin contains two disulfide bonds (*25*), the SDS-PAGE seemed to be not sufficiently sensitive to differentiate the *M* of those treated with or without β-Me (Figure 3, lanes 5−8). About 3.9% of trx-fused and 1.7% of recombinant cystatins of the total soluble protein were expressed in

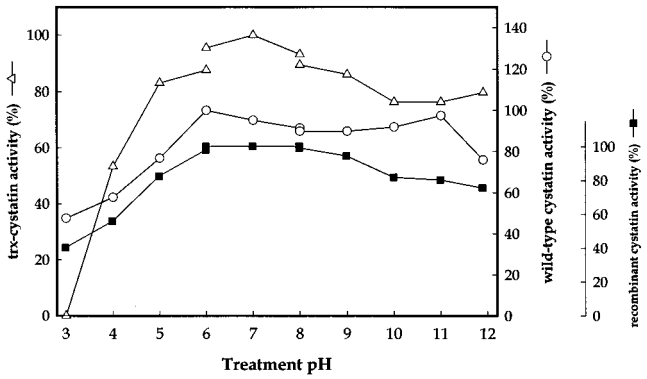

Figure 5. Comparison of the pH stability of trx− (△), wild-type (○), and recombinant (■) cystatins. Cystatins in pH buffers (50 mM citrate buffer, pH 3.0−6.0; 50 mM phosphate buffer, pH 6.0−8.0; 50 mM Clark and Lubs solution, pH 8.0−10.0; 50 mM Na_2HPO_4−NaOH buffer, pH 10.0−11.9) were incubated at 30 °C for 30 min, and then an equal volume of 0.2 M phosphate buffer (pH 7.0) was added. The residual inhibitory activity of cystatin was determined as described under Materials and Methods.

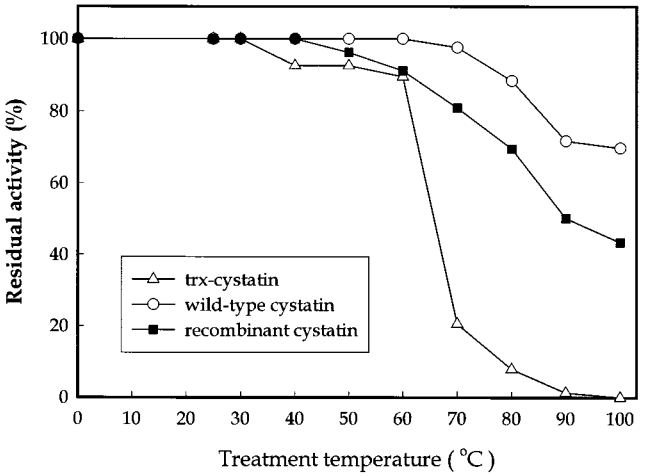

Figure 6. Comparison of the thermal stability of trx− (△), wild-type (○), and recombinant (■) cystatins. Cystatins in a 50 mM Tris-HCl buffer were incubated at 0, 25, 40, 50, 60, 70, 80, 90, and 100 °C for 5 min and then cooled in ice water for 30 min.

the cytoplasmic fraction of the *E. coli* transformant after IPTG induction and further purified (Table 1), whereas only 0.16% of cystatin was in carp ovary and egg (Table 2). This high level expression of the soluble form of trx−cystatin in the cytoplasm of *E. coli* AD494(DE3) and its rapid purification procedure give the expression system much more potential for use in food processing.

pH and Thermal Stability. Studies on the stability of cystatin from fish are still limited. As indicated in Figure 5, all of the purified trx-fused, wild-type, and recombinant (recombinant 12000 cystatin isolated from enterokinase cleavage of trx-cystatin) cystatins remained at almost constant high inhibitory activity in the pH range of 6.0−11.0. There was ∼70% of the original inhibitory activity left even after 30 min of incubation at pH 11.9 and 30 °C. However, a dramatic decline of the inhibitory activity at pH <6.0 was observed. As shown in Figure 5, trx−cystatin was almost inactivated, and only about 40 and 50% inhibitory activity remained in wild-type and recombinant cystatins, respectively, after 30 min of incubation at pH 3.0 and 30 °C. The stability of recombinant cystatin below pH 5.0 was significantly increased near the level

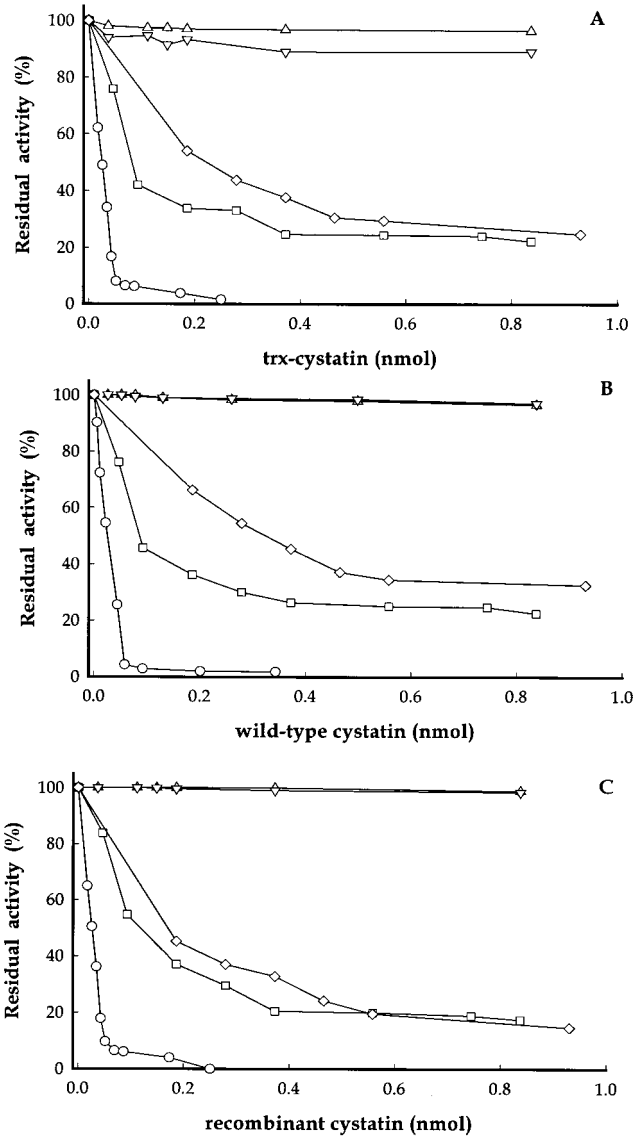

Figure 7. Inhibition of cysteine proteinases by trx− (A), wild-type (B), and recombinant (C) cystatins; 0.05 nmol of papain (○), 0.049 nmol of bovine cathepsin B (△), 0.01 nmol of mackerel cathepsin B (▽), 0.088 nmol of mackerel cathepsin L (□), and 0.075 nmol of mackerel cathepsin L-like (◇) proteinases in a 1.0 mL reaction mixture with various concentrations of cystatins were reacted at 40 °C for 10 min.

of wild-type cystatin after removal of fusion protein (Figure 5). However, the human spleen low molecular weight cysteine proteinase inhibitor was found to be stable at pH 2.0−10.0 (*30*). Although the human stefin was also found to be stable over a broad pH range, it was almost inactivated at pH 3.0 (*31*). Cystatins seemed to have broad pH range stability; however, the inhibitory activity was found to be labile in acidic pH (*32*).

No significant loss in inhibitory activity of all trx-fused, wild-type, and recombinant cystatins was observed after 5 min of incubation at 0−60 °C. The inhibitory activity of trx−cystatin decreased sharply to ∼20% when the temperature was >60 °C, whereas there was still >70% activity of the wild-type cystatin left at 80−100 °C (Figure 6). Although the recombinant cystatin revealed a better stability than trx−cystatin at 60−100 °C, it was slightly inferior to wild-type cystatin. Compared with the cystatins from land animals, such as pig plasma L-kininogen (*16*), human cystatin C (*33*),

and chicken cystatin (*34*), all of the trx-fused, wild-type, and recombinant cystatins were somewhat heat-labile protein inhibitors.

Inhibition of Cysteine Proteinase Cathepsins. The specificities of trx-fused and recombinant cystatins were almost similar to that of wild-type cystatin. The inhibitory activity of recombinant cystatin on papain and mackerel cathepsins L and L-like was almost similar to those of wild-type carp cystatin; the inhibitory activities against tested proteinases increased with the increase of added amount of cystatins (Figure 7). The 50% inhibition values of 0.05 nmol of papain by all trx-fused, recombinant, and wild-type cystatin were almost identical, ~0.025 nmol, indicating 1 mol of cystatin binding to 1 mol of papain. This was in agreement with that of rice cystatin (*35*). Although cystatin is a specific protein inhibitor for cysteine proteinases (*16*, *36*, *37*), neither recombinant form nor wild-type cystatin could inhibit mackerel and bovine cathepsins B (Figure 7). This might be because cathepsin B was found to simultaneously behave as a dipeptidyl carboxypeptidase and a papain-like endopeptidase. The former exopeptidase activity would affect the binding site of cathepsin B against cystatin (*38*). Accordingly, the proteinase inhibitory activity of recombinant 12 kDa cystatin behaved as wild-type cystatin. These data suggested that the thioredoxin was a suitable fusion protein in the *E. coli* expression of carp ovarian cystatin.

L-Kininogen, one of the natural cysteine proteinase inhibitors, had been found to be a good inhibitor of gel softening caused by cathepsins L and L-like in mackerel. However, the low yield of cystatin isolated from natural sources limited its application in the food industry. Therefore, high-level production of recombinant protein in bacteria was considered to be a major way to resolve this issue. In this study, the soluble recombinant cystatin was successfully cloned, overexpressed in *E. coli* AD494(DE3), purified through one simple affinity column chromatography, and characterized. The recombinant trx−cystatin protein was found to be a soluble and bioactive form in bacteria despite the existence of conjugated fusion protein in the cystatin molecule. It revealed broad pH stability, temperature tolerance within 60 °C, and inhibition specificity similar to that of wild-type cystatin. The data suggested that the recombinant cystatin may be a useful inhibitor for gel softening of fish mince, and its high level of soluble form expression and controllable production in transformed *E. coli* would make the use of recombinant cystatin more possible in surimi processing.

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