Overexpression of the Soluble Form of Chicken Cystatin in *Escherichia coli* and Its Purification†

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A cDNA encoding chicken cystatin was cloned into the pET-23a(+) expression vector and then transformed into *Escherichia coli* AD494(DE3)pLysS expression host. An active soluble form of cystatin was expressed in the cytoplasm of *E. coli* induced by isopropyl β-D-thiogalactopyranoside. The recombinant chicken cystatin was purified to electrophoretic homogeneity by a simple and rapid method involving heat treatment and Sephacryl S-100 gel filtration chromatography. The recombinant cystatin behaved as a thermal-stable protein and exhibited papain-like protease inhibition activity comparable to the natural chicken cystatin.

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

INTRODUCTION

The cystatin superfamily includes a number of cysteine proteinase inhibitors that are widely distributed in vertebrate and plant tissues (Abe et al., 1987, 1992; Barrett et al., 1986; Brzin et al., 1983, 1990; Lee et al., 2000; Rowan et al., 1990). It is subdivided into three individual families, designated I−III, on the basis of molecular structure and size. The members of family I, the stefins, and family II, the cystatins, are small proteins (MW = 11−14 kDa), which are found mainly in tissues and secretions, whereas inhibitors of family III, the kininogens, are larger glycoproteins (MW = 60−120 kDa) existing in blood plasma (Barrett et al., 1986). The inhibitors of families I and II are structurally related but differ in certain aspects. The stefins (family I) are polypeptides of ∼100 residues without disulfide bridges, whereas the cystatins (family II) are somewhat longer, ∼120 residues, and have two disulfide bridges (Barrett et al., 1986). The best characterized members of the cystatins are chicken cystatin (Anastasi et al., 1983; Barrett, 1987; Fossum and Whitaker, 1968; Turk and Bode, 1991), human cystatin C (Abrahamson et al., 1987, 1991; Bjork et al., 1995; Lindahl et al., 1992a; Zerovnik et al., 1997), and rat cystatin S (Bedi et al., 1998). These inhibitors inactivate cysteine proteinases such as cathepsins B, H and L and several structurally similar plant proteinases such as papain and actinidin by forming tight equimolar complexes (K_d = 20 nM−10 fM) and consequently blocking the active site of the enzymes (Abrahamson et al., 1987, 1991; Anastasi et al., 1983; Bjork and Ylinenjarivi, 1990; Lindahl et al., 1988, 1992a,b; Nicklin and Barrett, 1984). They are considered to contribute to the physiological control of proteinases (Bjork and Ylinenjarivi, 1990; Lindahl et al., 1988; Machleidt et al., 1989; Nicklin and Barrett, 1984). The kinetics of these interactions are consistent with the complexes being formed by simple, reversible, bimolecular reactions with association rate constants approaching those of a diffusion-controlled rate (Bjork et al., 1989; Bjork and Ylinenjarivi, 1990; Lindahl et al., 1992b). This finding is in agreement with the crystal structures of chicken cystatin and the complex of family I (cystatin) with papain (Bode et al., 1988; Stubbs et al., 1990). These structures thus show that the proteinase binding site comprises three regions of polypeptide chain which form a wedge-shaped edge in the inhibitor. This wedge-shaped edge inserts into the active-site cleft of papain with minimal conformational changes of either protein.

The native chicken cystatin contains two disulfide bridges in the residues Cys71−Cys81 and Cys95−Cys115 (Grubb et al., 1984). 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 (Bode et al., 1988). Reduction of the disulfide bonds of chicken cystatin caused a drastic loss of the inhibitory activity (Grubb et al., 1984). Bjork and Ylinenjarvi (1992) reported that selective reduction of disulfide bridge Cys95−Cys115 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 molecular structure of chicken cystatin and its inhibitory activity.

Recently, many cDNAs encoding cystatins and their variants have been cloned and expressed in an *Escherichia coli* expression system. However, most of these recombinant cystatins were expressed in insoluble form of inclusion bodies such as rat salivary cystatin S (Bedi et al., 1998; Sharma et al., 1995), human cystatin C

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(Gerhartz et al., 1998), and human salivary cystatin SN (Bobek et al., 1994). To obtain active cystatin, it is, therefore, necessary to solubilize the insoluble inclusion body in urea or other solvents and to refold into proper structure by dialysis or other methods. These procedures would substantially increase the running cost and consequently limit their applications.

In this study we have successfully constructed a recombinant pET-23a(+) plasmid encoding chicken cystatin. The recombinant cystatin in soluble form with cysteine−proteinases inhibitory activity has been overexpressed in *E. coli* AD494(DE3)pLysS and purified to electrophoretic homogeneity by a simple and rapid procedure involving heat treatment and Sephacryl S-100 HR gel filtration chromatography.

MATERIALS AND METHODS

Materials. Cathepsins B and L were purified from mackerel dorsal muscle (Jiang et al., 1994; Lee et al., 1993). Papain (type III) was purchased from Sigma Chemical Co. (St. Louis, MO) and further purified according to the method of Machleidt et al. (1995). RNA extraction kit (TRIZOL reagent), reverse transcriptase (SuperScript II RT), *Taq* DNA polymerase, ELONGASE enzyme mix (proofreading DNA polymerase), T4 DNA ligase, LB media, X-Gal, and all of the primers for PCR reaction were the products of Life Technologies Inc. (Life Technologies Inc., Gaithersburg, MD). Protein marker and all restriction enzymes were obtained from Promega Inc. (Promega Inc., Madison, WI). Sephacryl S-100 HR resin was purchased from Pharmacia (Uppsala, Sweden). Isopropyl β-D-thiogalactopyranoside (IPTG), ampicillin, kanamycin, and chloramphenicol were the products of Sigma Chemical Co. Z-Phe-Arg-MCA was obtained from Wako (Peptide Institute, Inc., Japan). Competent cells of *E. coli* strain, Top 10 F′ (Invitrogen Inc., Carlsbad, CA) and AD494(DE3)pLysS (Novagen Inc., Madison, WI), were used for transformation. The pGEM-T Easy vector (Promega Inc.) and pET-23a(+) vector (Novagen Inc.) were used for the construction of cloning vector and expression vector, respectively.

The *E. coli* AD494(DE3)pLysS strain is a thioredoxin reductase (*trxB*) mutant; therefore, it enables the expression of the proteins with disulfide bonds and has high potential for the production of properly folded active proteins (Derman et al., 1993). The DE3 in *E. coli* AD494(DE3)pLysS strain is a lysogen of λDE3, which carries a chromosomal copy of T7 RNA polymerase gene under control of the *lacUV5* promoter. The pLysS plasmid encoded with T7 lysozyme, a natural inhibitor of T7 RNA polymerase, can suppress the basal expression of T7 RNA polymerase prior to the induction and thus stabilize the pET recombinant encoding target protein that may affect the cell growth and viability. On the other hand, the pET-23a(+) vector is considered to be a powerful expression vector. When the target gene is cloned into the pET-23a(+) vector, the expression is controlled by bacteriophage T7 transcription and translation signals and T7 RNA polymerase in the host cell.

Screening and Amplification of Cystatin cDNA from Chicken Lung mRNA. Total RNA from chicken lung was extracted using TRIZOL reagent. The cDNA, produced from RT-PCR, was used as template. The oligonucleotides with sequences 5′-*CATATG*AGCGAGGACCGCTCCCGGCTCCTGGG, based on the nucleotide residues 128−153 of chicken cystatin (underlined) (Colella et al., 1989), and 5′-*CTCGAG*TTACTGGCA-CTTGCTTTCCAGCAGTTT, based on the nucleotide residues 452−478 of chicken cystatin (underscored) (Colella et al., 1989), were used as primers for PCR reactions. Restriction sites at the 5′ ends of the primers for *Nde*I and *Xho*I (italic) were incorporated to facilitate subcloning of the product. Amplification was performed using proofreading DNA polymerase in 50 μL of 60 mM Tris-SO_4 (pH 9.1), containing 18 mM $(NH_4)_2SO_4$, 1.5 mM $MgSO_4$, 200 μM each dNTP, 400 nM each primer, and 25 pg of cDNA, by Polymerase Chain Reaction for 35 cycles; denaturation at 94 °C for 30 s; annealing at 55 °C for 30 s; and extension at 68 °C for 50 s in a DNA thermal cycler (Perkin-Elmer, GeneAmp PCR system 2400).

Construction of Cystatin Expression pET-23a(+) Vector. The standard techniques of molecular cloning were performed essentially according to the method of Sambrook et al. (1989). The PCR product was first cloned into pGEM-T Easy vector and then transformed into *E. coli* Top 10 F′ according to the method of Hanahan and Meselson (1980). Following blue/white selection and sequencing, the vector containing the correct in-frame chicken cystatin cDNA sequence was prepared by minipreparation and then digested with *Nde*I and *Xho*I. After agarose gel elution, the cDNA of interest was ligated by T4 DNA ligase with pET-23a(+) vector, which was predigested with *Nde*I and *Xho*I.

Transformation and Selection from *E. coli* AD494-(DE3)pLysS. The pET-23a(+) vector ligated with cystatin was transformed into *E. coli* AD494(DE3)pLysS. The *E. coli* AD494 is anti-kanamycin strain, and the pET-23a(+) vector and pLysS plasmid carry the antiresistance genes of ampicillin and chloramphenicol, respectively. pET-23a(+)-transformed AD494-(DE2)pLysS can, therefore, be selected by this resistance. After resistance selection by plating on LB agar plates (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 15 g/L agar) containing 100 μg/mL ampicillin, 15 μg/mL kanamycin, and 34 μg/mL chloramphenicol, and sequencing, a bacterial colony containing the recombinant plasmid, pET-23a(+)-cystatin, with correct in-frame coding sequence was used for the protein expression.

Cultivation of *E. coli* and Isolation of Recombinant Cystatin. The *E. coli* AD494(DE3)pLysS strain transformed with pET-23a(+)-cystatin plasmid was cultivated in 10 mL of LB broth (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) containing 100 μg/mL ampicillin, 15 μg/mL kanamycin, and 34 μg/mL chloramphenicol 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, 15 μg/mL kanamycin, and 34 μg/mL chloramphenicol in a 250 mL flask. During incubation at 37 °C using a shaking incubator (200 rpm), the absorbance at 600 nm (OD_{600}) was measured. When 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 cells were harvested by centrifugation at 4000*g*. The harvested *E. coli* cell was resuspended in 30 mL of 50 mM Tris-HCl buffer (pH 8.0, containing 2 mM EDTA, 0.1 M NaCl, and 0.5 mM NaN_3) and followed by sonication. The cells were sonicated under 240 W for 10 s and then stopped for 20 s. This sonication was performed for 360 cycles using a sonicator XL 2020 system (HEAT Systems Inc.).

After sonication, the recombinant cystatin was isolated from cytoplasm of *E. coli* AD494(DE3)pLysS by centrifugation at 5000*g* to remove the cell fragments. The resulting recombinant cystatin was collected for the further purification and characterization.

Purification of Recombinant Cystatin. The crude recombinant cystatin isolated from cytoplasm of *E. coli* AD494-(DE3)pLysS was heated at 90 °C for 3 min and then centrifuged at 100000*g* for 10 min to remove the heat-denatured proteins. The supernatant was applied onto a Sephacryl S-100 HR column (2.6 × 100 cm) and eluted using 50 mM Tris-HCl buffer (pH 8.0) containing 2 mM EDTA, 0.1 M NaCl, and 0.5 mM NaN_3 at room temperature. The flow rate was 1 mL/min, and the eluent was collected with 1 mL/tube. Fractions with proteinase inhibitory activity were collected and concentrated with ultrafiltration. The resulting sample was subjected to inhibitory activity and SDS−PAGE analyses.

SDS−PAGE. SDS−PAGE analysis was performed according to the method of Laemmli (1970) using a minigel system (SE 260 vertical gel unit, Hoefer). The concentration of polyacrylamide gel was 15%. After the samples with and without 1% β-mercaptoethanol were incubated at 50 °C for 30 min, all samples were subjected to SDS−PAGE analysis. After electrophoretic running, gels were fixed, stained, and destained

according to the method of Neuhoff et al. (1988). Carbonic anhydrase (31 kDa), soybean trypsin inhibitor (20.4 and 19.7 kDa), horse heart myoglobin (16.9 kDa), lysozyme (14.4 kDa), and myoglobin fragments (8.1, 6.2, and 2.5 kDa) were used as markers. The density of protein bands was measured by densitometer (Image Master VDS, Pharmacia) to determine the relative protein concentration.

Assay of Enzyme Inhibitory Activity. Cathepsin B (EC 3.4.22.1), cathepsin L (EC 3.4.22.15), and papain (EC 3.4.22.2) 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. (1986) 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 6.0) 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 an 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 nmol of aminomethylcoumarin within 1 min at 37 °C.

Assay of Cystatin Thermal Stability. Both the recombinant and commercial cystatins in 0.2 M sodium phosphate buffer (pH 6.0) containing 2 mM EDTA, 0.1 M NaCl, and 0.5 mM NaN_3 were incubated at various temperatures for 30 min. The remaining papain inhibition activity was measured as described above.

RESULTS

Amplification of Cystatin cDNA from Chicken Lung mRNA. A DNA fragment encoded chicken cystatin was amplified from the chicken lung cystatin cDNA by PCR. One set of specific sense and antisense primers was designed on the basis of the open reading frame sequence of chicken lung cystatin. All primers, incorporating one restriction site at the 5′ end, were designed so that the corresponding PCR products could be inserted between the *Nde*I and *Xho*I sites of the pET-23a(+) expression vector. The PCR amplified product was 363 bp in size.

Construction of Cystatin Expression pET-23a(+) Vector. To ensure the cystatin cDNA fragment in a correct reading frame, the PCR amplified fragment was first cloned into pGEM-T Easy cloning vector for screening and sequencing. The vector containing the correct in-frame cystatin cDNA sequence was used to construct the cystatin expression vector. The cDNA for chicken cystatin was ligated with pET-23a(+) expression vector in *Nde*I and *Xho*I restriction enzyme sites and introduced in frame downstream of the T7 promoter of the pET-23a(+) vector.

Expression and Purification of the Recombinant Chicken Lung Cystatin. Because the chicken lung cystatin was cloned in pET-23a(+) plasmid under control of the strong T7 promoter, expression is induced by providing a source of T7 RNA polymerase in the AD494(DE3)pLysS cell. High-level expression of the recombinant chicken cystatin was observed after 4 h of induction by IPTG (Figure 1).

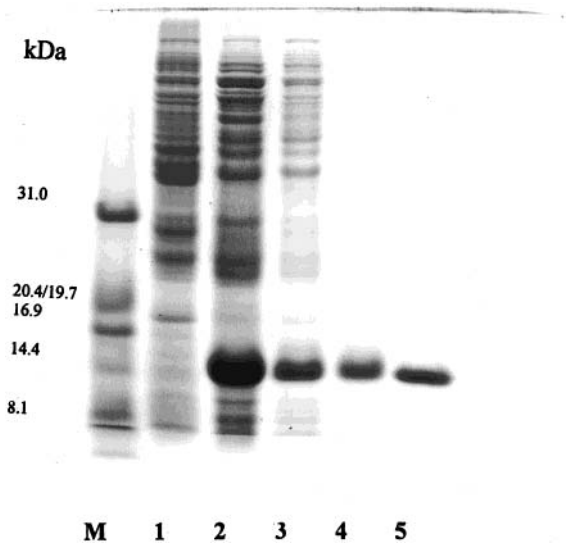

Figure 1. SDS-PAGE analysis of recombinant chicken cystatin expression: lane M, low M_w protein marker; lane 1, *E. coli* AD494(DE3)pLysS]nontransformed with pET-23a(+)-cystatin expression vector]; lane 2, crude recombinant cystatin extracted from *E. coli* cell [transformed with pET-23a(+)-cystatin expression vector]; lane 3, crude recombinant cystatin heated at 90 °C for 3 min and centrifuged at 100000*g* for 10 min to remove the heat-denatured proteins; lane 4, purified recombinant cystatin (treated with β-Me); lane 5, purified recombinant cystatin (without β-Me treatment).

After cultivation, induction, and harvest of the cystatin-transformed *E. coli* AD494(DE3)pLysS, the *E. coli* cells were disrupted by sonication. The crude recombinant cystatin was then heated at 90 °C for 3 min and centrifuged at 100000*g* for 10 min to remove the heat-denatured proteins. The recombinant cystatin was eluted at fractions 350−360 mL on a Sephacryl S-100 HR gel chromatography column. According to the SDS−PAGE analysis (Figure 1), the recombinant cystatin was purified to electrophoretic homogeneity and had a molecular weight of 14 kDa. The migration distance of the purified recombinant cystatin treated with β-mercaptoethanol (β-Me) was slightly less than that without β-Me treatment (Figure 1). This phenomenon suggested that the soluble form of recombinant chicken cystatin, produced from *E. coli* AD494(DE3)pLysS, contained an intramolecular disulfide bond.

Inhibitory Activity and Thermal Stability. As shown in Figure 2, the recombinant chicken cystatin and wild-type chicken cystatin purified from chicken egg white (obtained from Sigma Chemical Co.) had essentially the same inhibitory activity toward papain and cathepsins B and L. The recombinant chicken cystatin behaved as a thermal-stable protein comparable to the natural chicken cystatin. After 30 min of incubation at 100 °C, the recombinant cystatin retained 30% of its original activity (Figure 3).

DISCUSSION

Cysteine proteinase inhibitors are widely distributed in nature, but their levels are rather low. For example, a large amount of fresh blood was required to obtain enough inhibitor protein for the characterization of human stefin A (Brzin et al., 1983) or pig L-kininogen (Lee et al., 2000). Likewise, kilogram amounts of rice seeds yielded only a microgram of oryzacystatin (Abe et al., 1987). Accordingly, it is rather difficult and time-consuming to isolate cysteine proteinase inhibitors

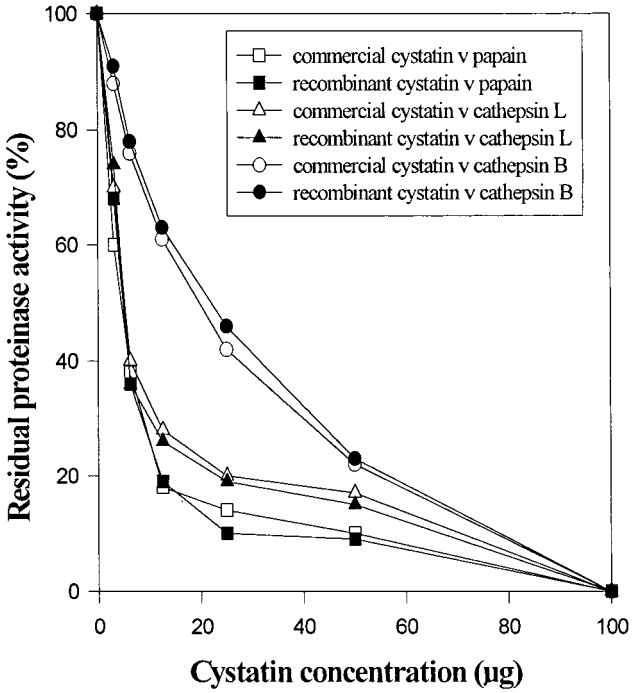

Figure 2. Inhibition of the papain-like proteinase activities by recombinant chicken cystatin and commercial cystatin (values in this figure are the means of three replicates).

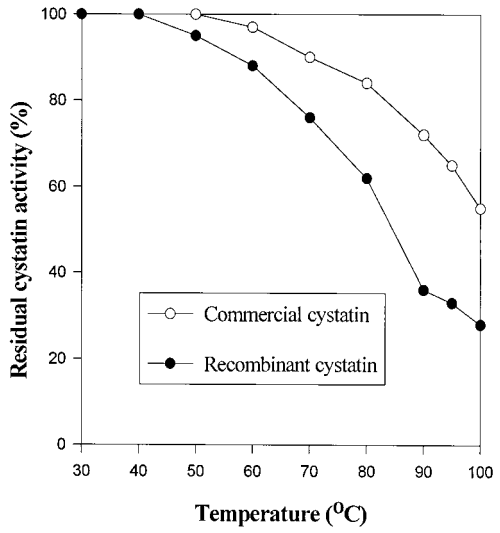

Figure 3. Thermal stability of recombinant chicken cystatin and commercial chicken cystatin incubated at different temperatures for 30 min (values in this figure are the means of three replicates).

directly from the natural sources. The better method to get large amounts of purified inhibitors is to produce these proteins in *E. coli* or other expression systems. As we know, during the past few years, many bioactive proteins have been expressed in bacteria by using the recombinant DNA techniques, rather than the traditional purification from natural sources. However, the disulfide bonds in native eukaryotic proteins were often not accurately expressed in bacteria. The multi-disulfide proteins are, therefore, generally not folded correctly and accumulate as insoluble forms in bacteria. The production of these recombinant proteins with properly folded structure and correct bioactivity usually requires solubilization of their inclusion body followed by refolding process. These procedures, however, would substantially increase the running cost and consequently limit their applications.

Table 1. Purification of Recombinant Chicken Cystatin from *E. coli* AD494(DE3)pLysS Transformed with pET-23a(+)-Cystatin Expression Vector

procedure	total protein (mg)	total act. (units)	sp act. (units/mg)	yield (%)	purifn (fold)
E. coli cell extraction	540.0[a]	962.8	1.78	100	1.0
90 °C, 3 min incubation	215.2	831.9	3.87	86.4	2.2
Sephacryl s-100 HR	12.5	483.2	38.66	50.2	21.7

[a] The starting volume was 1000 mL of cultivated broth.

Expression of several cysteine proteinase inhibitors including human stefin A, stefin B, rat stefin A, and oryzacystatin using bacterial expression system has been reported (Abe et al., 1988; Jerala et al., 1988; Kaji et al., 1989; Katunuma et al., 1988; Strauss et al., 1988; Thiele et al., 1988). However, a common issue in these studies was the low level of expression of recombinant cystatins, despite the use of a variety of vectors (Fong et al., 1989) and chemically synthesized genes, in which the codons were optimized for the bacteria (Fong et al., 1989; Kaji et al., 1990). To achieve high-level expression as well as a soluble form of recombinant cystatin, the cDNA encoding chicken cystatin was cloned into the pET-23a(+) expression vector and then transformed into an *E. coli* AD494(DE3)pLysS expression host.

As expected, the expression level of recombinant cystatin was quite high in the soluble form and no significant band of insoluble fraction (data not shown) was found in SDS−PAGE near 13 kDa. This phenomenon suggested that the recombinant cystatin was expressed mostly in soluble form. As shown in Figure 1, the recombinant cystatin constituted a high percentage of the total cell protein and was produced in soluble form in the cytoplasm of *E. coli* AD494(DE3)pLysS cells. SDS−PAGE analysis suggested that the recombinant cystatin contained disulfide bonds (Figure 1). The disulfide bonds were properly formed because the purified recombinant cystatin showed cysteine proteinase inhibitor activities comparable to that of the wild-type chicken cystatin (Figure 2). Because the cystatin is a heat-stable protein (Keilova and Tomasek, 1974), purification of recombinant cystatin was facilitated by heating at 90 °C for 3 min. This step removed ∼60% of the contaminant proteins while retaining 86% of the inhibitory activity (Table 1). The recombinant cystatin was then purified to electrophoretic homogeneity in high yield (∼50%) simply by Sephacryl S-100 HR gel filtration (Figure 1 and Table 1). Approximately 12.5 mg of purified cystatin could be obtained from 540 mg of proteins of *E. coli* cells (Table 1). This simple, rapid, and high-yield purification procedure greatly increases the potential of industrial application of this recombinant cystatin.

Chicken cystatin, purified from egg, was reported to be a heat-stable protein (Keilova and Tomasek, 1974) and a reversible, tight-binding inhibitor of papain-like proteinases (Bjork and Ylinenjarivi, 1990; Lindahl et al., 1988; Machleidt et al., 1989; Nicklin and Barrett, 1984). As shown in Figure 2, the residual activities of papain and cathepsin L with 25 μg of recombinant cystatin decreased dramatically to ∼20%, whereas that of cathepsin B with 25 μg of recombinant cystatin still had ∼40% left, compared to the control. The inhibitory ability of recombinant cystatin against cathepsin B appeared to be weaker than that against cathepsin L

Table 2. Effect of the Recombinant Cystatin on the Gel Property of Mackerel Surimi at pH 7.0

treatment	h at 55 °C	breaking force[a] (g)	deformation[a] (mm)	gel strength[a] (g·cm)
none	0	630 ± 15a	10.6 ± 0.8a	668a
none	2	426 ± 18c	8.2 ± 0.4b	349c
cathepsin B	2	399 ± 11c	7.9 ± 0.4b	315d
cathepsin L	2	405 ± 12c	8.1 ± 0.4b	365c
recombinant cystatin	2	577 ± 17b	9.5 ± 0.6a	548b
cathepsin B and cystatin	2	557 ± 21b	9.4 ± 0.5a	504b

[a] Values in the same column bearing unlike letters differ significantly ($p < 0.05$). Values in this table are the mean of five replicates.

and papain (Figure 2). The explanation for this is that the inhibition constant (K_i) of cathepsin B by wild-type cystatin is greater than those of cathepsin L and papain (Auerswald et al., 1992, 1995; Bjork et al., 1994). The inhibitory ability of the recombinant chicken cystatin against papain and cathepsins B and L appeared to be comparable with that of wild-type chicken cystatin (Figure 2). The thermostability of the recombinant cystatin was also comparable to that of the natural chicken cystatin. There was ~30% activity remaining after 30 min of incubation at 100 °C (Figure 3).

High levels of cysteine proteinase activity caused by cathepsins B, H, L, and L-like have been observed in Pacific whiting and arrowtooth flounder (An et al., 1994; Wasson et al., 1992a,b), chum salmon during spawning migration (Yamashita and Konagaya, 1990), and mackerel (Jiang and Chen, 1998; Jiang et al., 1997; Lee et al., 1993). These proteases have been considered to be the main factors that caused gel softening of surimi. The purified recombinant cystatin was added in the mackerel surimi to test its ability to prevent gel softening. As shown in Table 2, the gel strength of mackerel surimi without recombinant cystatin or with cathepsins B or L was much lower than those with recombinant cystatin after 2 h incubation at 55 °C. Although the recombinant cystatin could not completely inhibit the gel softening of mackerel surimi presumably due to some proteases other than the cysteine proteinases, it still has a high potential for use in improving seafood quality. According to the data obtained from this study, the recombinant chicken cystatin had biological and physical properties comparable to those of the wild-type cystatin, and the expression system and purification procedure developed in this study are useful and economical in terms of producing recombinant cystatin for industrial application.

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