High-Level Production of Recombinant Chicken Cystatin by *Pichia pastoris* and Its Application in Mackerel Surimi

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A high level of the secreted form of recombinant chicken cystatin was expressed in *Pichia pastoris* X-33 by chromosomal integration of multiple copies of an expression cassette containing chicken cystatin under the control of glyceraldehyde-3-phosphate dehydrogenase promoter. The inhibition ability of the recombinant for papain-like proteinase was found to correspond to those of natural chicken cystatin. The recombinant cystatin substantially inhibited the proteolysis of myosin and gel softening, which consequently improved the gel properties of mackerel surimi.

Keywords: *Recombinant cystatin; mackerel surimi; Pichia pastoris; expression; cysteine proteinase inhibitor*

INTRODUCTION

Pichia pastoris, a methylotrophic yeast, is an efficient system for the production of recombinant proteins with high expression levels (*1*. *2*). The glyceraldehydes-3-phosphate dehydrogenase (GAP) promoter gene has been characterized and shown to constitutively express recombinant proteins to high levels in *P. pastoris* (*3*). The pGAPZαC vector uses the GAP promoter and *Saccharomyces cerevisiae* α-factor secretion signal to express recombinant proteins in *P. pastoris* and uses Zeocin as resistant agent. After transformation, the vector can be integrated into *P. pastoris* chromosome via homologous recombination between the GAP promoter locus of transformed vector and regions of homology within the genome (*1*). Such integrants can generate stable transformants and are extremely stable in the absence of selective pressure even when present as multiple copies.

The cystatin superfamily includes a number of cysteine proteinase inhibitors that are widely distributed in mammalian tissues and body fluids (*4*). Chicken cystatin is a small protein inhibitor (116 amino acids) of cysteine proteinases, including mammalian lysosomal cathepsins B, H, and L (*5*−*8*) and also the most well characterized member of the cystatin superfamily (*6*, *8*). It is a nonglycosylated protein with a molecular mass (*M*) of 13 kDa and two disulfide bonds. This cystatin is a reverse and tight-binding inhibitor of papain-like enzymes and is considered to contribute to the physiological control of proteinases (*9*−*12*). It has well-defined molecular structure of a relatively small size and great potential for application in food processing or as pharmaceutical aid.

To overexpress the chicken cystatin recombinant, cDNA encoding chicken lung cystatin was cloned into the pGAPZαC expression vector, using GAP as promoter and Zeocin as resistant agent, and transformed into *P. pastoris* X-33 expression host. A large quantity of active cystatin were expressed and secreted into the broth by α-factor preprosequence. The papain inhibition properties of the recombinant were found to correspond to those of the natural chicken cystatin.

A special concern with the cystatins is their potential role or use in protecting fish surimi proteins from proteinase attack. Cloning of chicken cystatin in pGAPZαC vector and expression in *Pichia* X-33 expression host were a wonderful arrangement for the expression of recombinant cystatin. The properly folded, active cystatin would be suitable for further studies or other applications. The purpose of this study was to develop a commercially useful source of cysteine proteinase inhibitor by constructing a recombinant *P. pastoris* X-33 strain that is integrated with chicken cystatin.

MATERIALS AND METHODS

Screening and Amplification of Cystatin cDNA from Chicken Lung mRNA. Total RNA from chicken lung was extracted using a Trizol RNA extraction kit (Gibco BRL products). The single-strain cDNA, produced from RT-PCR, was used as a template, and oligonucleotides with sequences 5′-CTCGAGAAAAGAGAGGCTGAAGCTAGCGAGGACCGCTCCCGGCTCCTGGG and 5′-TCTAGATTACTGGCACTTGCTTTCCAGCAGTTT, based on the open reading frame, nucleotide residues 128−478, of the chicken cystatin, were used as primers for PCR reactions. Restriction sites at the 5′ ends of the primers for *Xho*I and *Xba* I (underscored) were incorporated to facilitate subcloning of the product. Amplification was performed using proofreading polymerase (Gibco BRL products, catalog no. 10480-010) by PCR reaction for 35 cycles: denaturation, 94 °C for 30 s; annealing, 55 °C for 30 s; and extension, 68 °C for 50 s, in a DNA thermal cycler (Perkin-Elmer, GeneAmp PCR system 2400).

Construction of Cystatin Expression pGAPZαC Plasmid. The standard techniques of molecular cloning were performed essentially according to those reported by Sambrook et al. (*13*). The PCR product was first cloned into pGEM-T Easy vector (Promega) and then transformed into *Escherichia coli* Top 10 F′. After blue/white selection and midi-preparation, the correct plasmid was digested with *Nde*I and *Xho*I and finally ligated into pGAPZαC vector (Novagen).

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Transformation and Selection in *Pichia* X-33 Host. The pGAPZαC plasmid, ligated with cystatin, was digested with *Bgl*II (2 bp) in the GAP promoter region to linearize the vector and then transformed into *Pichia* X-33 by using the lithium chloride method (*14*). The colonies were selected by plating on YPDS agar plates (tryptone, 20 g/L; yeast extract, 10 g/L; dextrose, 20 g/L; sorbitol, 182.2 g/L; agar, 20 g/L) containing 100 μg/mL Zeocin. The yeast colony integrated by recombinant pGAPZαC-cystatin DNA with correct in-frame coding sequence into the *Pichia* genome was used for the protein expression.

Cultivation of *Pichia* X-33 and Isolation of Recombinant Cystatin. The *Pichia* X-33 strain integrated with recombinant pGAPZαC-cystatin DNA was cultivated in 5 mL of YPDS broth (tryptone, 20 g/L; yeast extract, 10 g/L; dextrose, 20 g/L; sorbitol, 182.2 g/L) containing 100 μg/mL Zeocin in a 50 mL flask at 30 °C using a shaking incubator (300 rpm) overnight. One milliliter of the resulted culture was inoculated into 50 mL of fresh YPD broth (tryptone, 20 g/L; yeast extract, 10 g/L; dextrose, 20 g/L) in a 250 mL flask and cultivated at 30 °C for 4−5 days in a shaking incubator (300 rpm).

After 10 min of centrifugation at 3000*g* to remove the *Pichia* cells, the supernatant was collected for further purification and characterization.

Sephacryl S-100 HR Column Chromatography. The recombinant cystatin was first precipitated by ammonium sulfate fractionation at 40−60% saturation and then dialyzed against 50 mM Tris-HCl buffer (buffer A, pH 8.0) containing 2 mM EDTA, 0.1 M NaCl, and 0.5 mM NaN_3 at 4 °C. The resulted sample was chromatographed on Sephacryl S-100 HR (2.6 × 100 cm), which was equilibrated with buffer A. The cystatin was then eluted using buffer A at room temperature. Ten milliliters per tube was collected at a flow rate of 1 mL/min.

Superdex 75 Column Chromatography. Fractions with proteinase inhibitory activity on Sephacryl S-100 HR chromatography were collected and concentrated to a minimal volume using Amicon ultrafiltration. The resulted sample was then applied onto a Superdex 75 column (1.0 × 30 cm), which was previously equilibrated with buffer A. The cystatin was then eluted by buffer A using an FPLC System with GP250 plus FPLC Controllers (Amersham Pharmacia Biotech. Inc., Piscataway, NJ) at room temperature. Two milliliters per tube was collected at a flow rate of 1 mL/min.

SDS-PAGE. SDS-PAGE analysis was performed according to the method of Laemmli (1970) using a mini-gel system (SE 260 vertical gel unit, Hoefer). The concentration of polyacrylamide gel was 15%. All samples were incubated with 1% β-Me at 50 °C for 30 min. After electrophoretic running, gels were fixed, stained, and destained according to the method of Neuhoff et al. (*16*). Carbonic anhydrase (31 kDa), soybean trypsin inhibitor (20.4 and 19.7 kDa), horse heart myoglobin (16.9 kDa), lysozyme (14.4 kDa), thyroglobulin (330 kDa), ferritin (220 kDa), albumin (67 kDa), catalase (60 kDa), lactate dehydrogenase (36 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.

Substrate SDS-PAGE for Activity Assay. The substrate SDS-PAGE was mainly according to the method of El-Shamei et al. (*17*). A 15% polyacrylamide gel containing 0.1% w/v casein was used for the activity assay. About 0.5−1.0 μg of sample protein was applied onto each well of the gels. After electrophoretic running, gels were prewashed with 2.5% Triton X-100 at least two times for 30 min to remove SDS and then incubated in 0.10 M phosphate buffer containing 2 mM cysteine, 1 mM EDTA, pH 6.0, and papain (0.01 mg/mL) at 40 °C for 150 min. Cystatin activity was stopped by staining solution, 0.01% Coomassie brilliant blue, 40% methanol, and 10% acetic acid. Active cystatin zones were visualized as intense blue bands against a clear background on the gel after destaining with 25% ethanol and 10% acetic acid.

Assay of Enzyme Inhibitory Sctivity. Cathepsin B (EC 3.4.22.1), cathepsin L (EC 3.4.22.15), and papain (EC 3.4.22.2) were used to assay the inhibitory activity of recombinant cystatin. The concentrations of these cysteine proteinases were determined by active-site titration with E-64 as described by Barrett et al. (*4*) and adjusted to obtain a final fluorescent density below 1000 in control (without inhibitor). The inhibitory activity of recombinant 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 wavelength of 350 nm and an emission wavelength 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.

Surimi Processing. Mackerel (*Scomber australasicus*, 400−500 g/fish) harvested from the Pacific Ocean was obtained from a local fish market in Keelung, northern Taiwan. After being headed, eviscerated, skinned, and deboned, mackerel mince was washed with 4 volumes (v/w) of chilled 0.4% $NaHCO_3$ solution for 5 min. The supernatant was decanted, and the sample was rinsed with chilled water and finally with 0.3% NaCl solution for 5 min each time. After being dewatered to a moisture content of 78% by 15 min of centrifugation (3000*g*), the sample was strained through a strainer with a mesh size of 3 mm to remove residual scales and pin bones. Finally, the resulted mince was further ground with 2.5% NaCl for 20 min and mixed uniformly with/without recombinant cystatin (10 units/g of mince). Ground mackerel surimi with or without recombinant was stuffed into polyvinylethylene chloride tubes (diameter = 2.5 cm) and incubated at 50 °C for 15, 30, 60, and 90 min to evaluate the inhibition ability on disintegration of surimi gel proteins. The resulting samples were then heated in a 100 °C water bath for 30 min and stored at 5 °C for 12 h. The gel properties were measured by punching test (model CR-200D rheometer, Sun Scientific Co., Ltd.; diameter of plunger = 5 mm) and SDS-PAGE [0.2 mg samples were dissolved in 0.8 mL of 62.5 mM Tris-HCl buffer (pH 6.8) containing 2.0% SDS, 8.0 M urea, and 5.0% β-Me and shaking-incubated at 30 °C for 24 h; concentration of polyacrylamide = 10%].

RESULTS

Amplification of Cystatin cDNA from Chicken Lung mRNA. A DNA fragment encoding chicken cystatin was amplified from chicken lung cystatin cDNA by PCR. One set of specific sense and antisense primers was designed based on 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 *Xho*I and *Xba*I sites of the pGAPZαC expression vector. The PCR amplified product was 350 bp in size (Figure 1).

Construction of Cystatin Expression pGAPZαC Expression Vector. To ensure the cystatin cDNA fragment in the 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 employed to construct the cystatin expression vector. The cDNA for chicken cystatin was then ligated with pGAPZαC expression vector in *Xho*I and *Xba*I

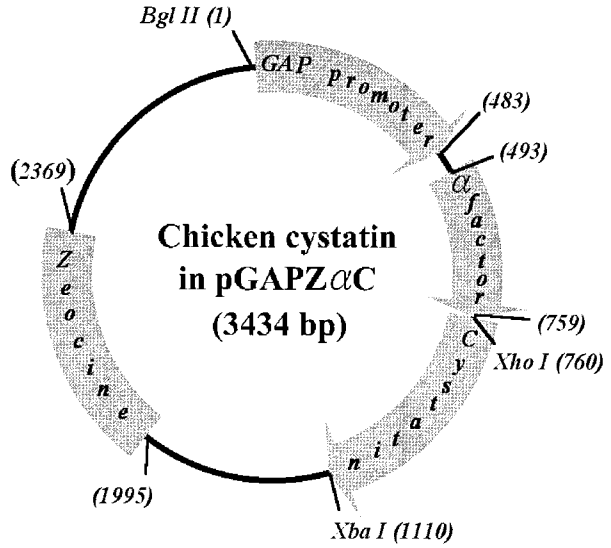

Figure 1. Construction of the cystatin-pGAPZαC expression vector.

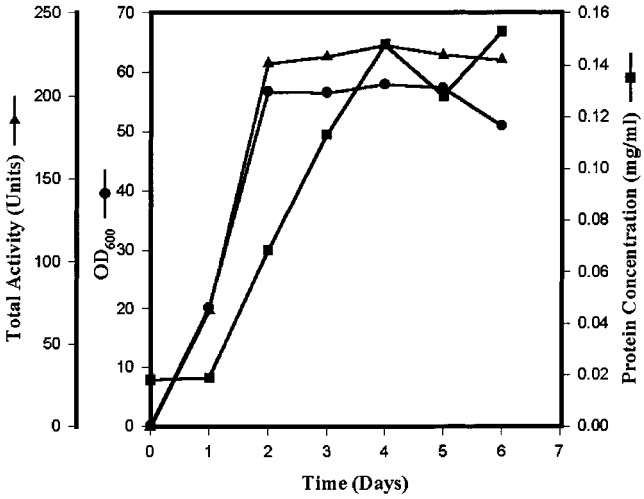

Figure 2. Growth curve of the recombinant *P. pastoris*.

restriction enzyme sites and introduced in-frame downstream of the α-factor in the pGAPZαC vector (Figure 1).

Expression and Purification of the Recombinant Chicken Lung Cystatin. After the pGAPZαC-chicken cystatin plasmid had been transformed into *P. pastoris* X-33 expression host, the expression vector was integrated into the genomic DNA due to the existence of the GAP promoter sequence. Because the chicken cystatin was under the control of the strong GAP promoter, a high level of the recombinant was expressed and secreted into broth by α-factor preprosequence during shaking cultivation (Figure 2). The highest level of cystatin activity (~6.33 units/mg) was observed after 2 days of shaking cultivation (Figure 2 or Table 1). Because no appreciable increase in cystatin activity was observed during the further cultivation, the 2 days of shaking cultivation was chosen in this study.

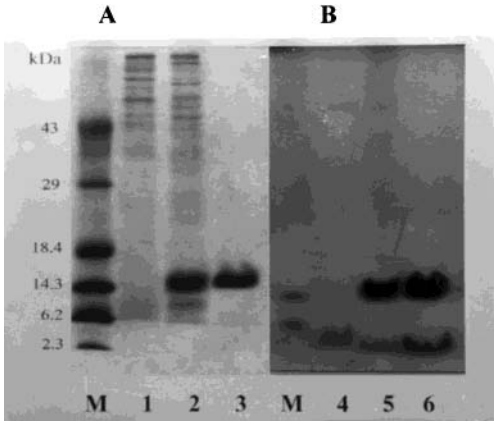

Figure 3. SDS-PAGE (A) and substrate SDS-PAGE (activity staining, B) of the recombinant chicken cystatin: lane M, low MW protein marker; lane 1, *P. pastoris* X-33 (nontransformed with pGAPZαC-cystatin expression vector); lane 2, crude recombinant cystatin secreted from *P. pastoris* X-33 (transformed with pGAPZαC-cystatin expression vector); lane 3, purified recombinant cystatin (treated with β-Me); lanes 4, 5, and 6, electrophorams of substrate (0.1% casein) SDS-PAGE, corresponding to 1, 2, and 3, on SDS-PAGE, respectively. Bands of the unhydrolyzed casein (Coomassie brilliant blue stained) indicate the existence of cystatin.

After 2 days of shaking cultivation, the *P. pastoris* cells were harvested by centrifugation at 4000*g* for 10 min. The supernatant, containing the secreted recombinant cystatin, was collected for the further purification and characterization and application experiments in mackerel surimi. The recombinant cystatin was purified to electrophoretical homogeneity by 40−60% saturated ammonium sulfate, Sephacryl S-100 HR, and Superdex 75 chromatographs (Figure 3A). The molecular mass (*M*) was estimated to be 14 kDa by SDS-PAGE (Figure 3A). The summary of the purification is shown in Table 1.

Inhibitory Activity. The inhibitory activity against papain was detected by substrate SDS-PAGE (Figure 3B). About 0.8 μg of supernatant, after removal of the *Pichia* cells, and 0.6 μg of the purified recombinant cystatin were electrophoretically running through a 0.1% casein−0.1% SDS−15% polyacrylamide gel, and then gels were incubated in a papain-containing solution to hydrolyze casein. The Coomassie brilliant blue stained unhydrolyzed casein band indicated the existence of papain inhibitor (Figure 3B), which corresponded to the recombinant cystatin appearing on SDS-PAGE (Figure 3A).

According to the titration curves of various papain-like proteinases by recombinant and wild-type chicken cystatins, **t**he recombinant chicken cystatin revealed almost the same inhibitory activity as wild type, which was purified from chicken egg white (Sigma Chemical Co., St. Louis, MO) against papain and cathepsins B and L.

Table 1. Purification of Recombinant Chicken Cystatin from *P. pastoris* X-33 Transformed with pGAPZαC-Cystatin Expression Vector

procedure	total protein (mg)	total activity (units)	specific activity (units/mg)	yield (%)	purification (fold)
Pichia cultivated broth	35.0[a]	221.4	6.33	100	1.0
ammonium sulfate 40−60%	22.6	183.2	8.11	82.7	1.3
Sephacryl S-100 HR	5.8	121.5	20.95	54.9	3.3
Superdex 75	2.5	100.48	40.19	45.4	6.3

[a] The starting volume was 500 mL of cultivated broth.

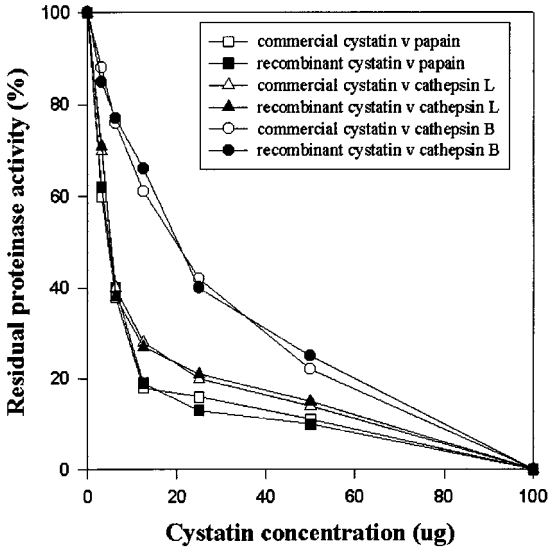

Figure 4. Comparison of the inhibition activities of recombinant chicken cystatin and commercial cystatin against papain-like proteinases (values in this figure are the means of three replicates).

Effect of Recombinant Cystatin on the Inhibition of Gel Softening (Disintegration) of Surimi during Incubation at 50 °C. As indicated in Figure 5, obvious degradation on myosin heavy chain and moderate degradation on actin of surimi gels preincubated at 50 °C for 15, 30, 60, and 90 min prior to the fixation (30 min of heating at 100 °C) were observed (Figure 5, lanes 2−5). However, only very minor degradation was obtained on samples with recombinant cystatin (Figure 5, lanes 6−9). To further investigate the effect of recombinant cystatin on gel softening of mackerel surimi, samples with/without recombinant cystatin were processed into surimi-based product. As shown in Table 2, the breaking force of samples without recombinant cystatin decreased from 1025*g* to 680−843*g*, whereas that of samples with recombinant cystatin decreased to 960−858*g* after 15−90 min of preincubation at 50 °C. Significant differences in breaking force, deformation (depth at the point of gel breaking), and gel strength between samples with and without recombinant cystatin added were observed ($p < 0.05$). On the basis of the sample without recombinant cystatin, improvements of about 80% in gel strength, 26% in breaking force, and 43% in deformation, which is an indicator of protein interaction, were obtained in samples with the addition of recombinant cystatin (10 units/g of mince) after 90 min of preincubation at 50 °C prior to the fixation (30 min of heating at 100 °C) (Table 2).

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 (*18*) or pig L-kininogen (*19*). Likewise, kilogram amounts of rice seeds yielded only micrograms of oryzacystatin (*20*). Accordingly, it is rather difficult and time-consuming to isolate cysteine proteinase inhibitors directly from the natural sources. The better method to get large amounts of inhibitors is to produce these proteins in recombinant bacterial 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 prokaryotic bacteria. The multi-disulfide proteins are, therefore, generally not folded correctly and, so, accumulated as insoluble forms in bacteria. The production of these recombinant proteins with properly folded structure and correct bioactivity usually needs solubilization of their inclusion body

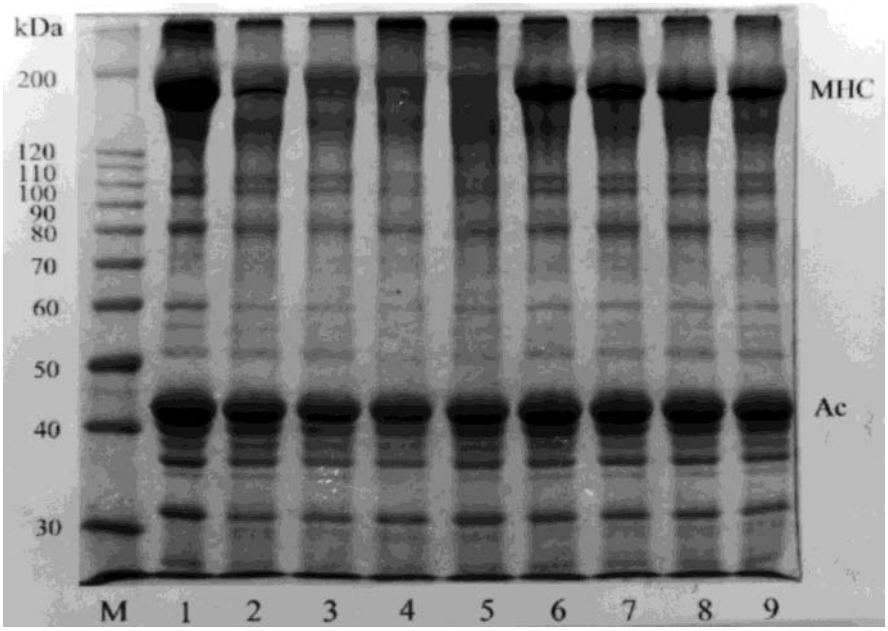

Figure 5. Effect of recombinant chicken cystatin on the inhibition of gel softening of mackerel surimi: lane M, protein marker; MHC, myosin heavy chain; Ac, actin; lane 1, heated immediately at 100 °C for 30 min; lanes 2, 3, 4, and 5, preincubated at 50 °C for 15, 30, 60, and 90 min, respectively, prior to heating at 100 °C for 30 min; lanes 6, 7, 8, and 9, ground with recombinant cystatin (10 units/g) and then preincubated at 50 °C for 15, 30, 60, and 90 min, respectively, prior to heating at 100 °C for 30 min. All samples were dissolved in 5 volumes of 62.5 mM Tris-HCl buffer (pH 6.8) containing 2.0% SDS, 8.0 M urea, and 5.0% β-Me and then incubated at 30 °C for 24 h.

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

treatment	incubation time at 50 °C (min)	breaking force (g)	deformation (mm)	gel strength (g·cm)
no inhibitor	0	1025 ± 36^{a}	12.5 ± 0.7^{b}	1281 ± 95^{a}
no inhibitor	15	843 ± 34^{c}	11.1 ± 0.8^{c}	936 ± 90^{b}
no inhibitor	30	731 ± 33^{d}	10.6 ± 0.5^{c}	775 ± 52^{c}
no inhibitor	60	720 ± 38^{d}	10.4 ± 0.5^{c}	749 ± 58^{c}
no inhibitor	90	680 ± 47^{e}	9.6 ± 0.8^{d}	653 ± 70^{d}
recombinant cystatin	0	930 ± 52^{b}	14.2 ± 1.4^{a}	1321 ± 133^{a}
recombinant cystatin	15	906 ± 47^{b}	14.3 ± 0.6^{a}	1296 ± 77^{a}
recombinant cystatin	30	913 ± 41^{b}	14.2 ± 0.3^{a}	1296 ± 70^{a}
recombinant cystatin	60	882 ± 40^{b}	13.5 ± 1.2^{a}	1191 ± 135^{a}
recombinant cystatin	90	858 ± 47^{bc}	13.7 ± 0.9^{a}	1175 ± 65^{a}

[a] Values are the means of 8−10 replicates, expressed as mean ± standard deviation. Values with unlike superscripts in the same column differ significantly ($p < 0.05$).

followed by refolding process. These procedures, however, would substantially increase the running cost and consequently limit their applications. To achieve a high level of expression with properly folded structure and correct bioactivity, we have successfully cloned the cDNA encoding chicken cystatin into the pET-23a(+) expression vector and then transformed it into *E. coli* AD494(DE3)pLysS expression host, which produced a high percentage of the soluble form of recombinant cystatin (*21*). However, the secreted form of recombinant cystatin would be much easier and more practical in industrial applications. In this study, the cDNA encoding chicken cystatin was cloned into the pGAPZαC expression vector and then transformed into *P. pastoris* X-33 expression host. As expected, a large quantity of active cystatin was expressed under control of the strong GAP promoter and secreted into the broth using the α-factor preprosequence (Figure 2). As shown in Figure 3 (lane 2), the recombinant cystatin constituted a high percentage of the total secreted protein in the culture broth of *P. pastoris*. 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 4). The inhibitory ability of both recombinant and wild-type cystatins against cathepsin B was weaker than that against cathepsin L and papain (Figure 4). This might be due to the greater inhibition constant (K_i) of cathepsin B than of cathepsin L and papain against both cystatins (*22*−*24*).

High levels of cysteine proteinase activities of cathepsins B, H, L, and L-like were observed in Pacific whiting and arrowtooth flounder (*25*−*27*), chum salmon during spawning migration (*28*), and mackerel (*29*−*31*). These proteases have been considered to be the main factors that caused gel softening of surimi. Accordingly, the presence of these proteinases found to be stable at 50−60 °C had become a crucial problem for surimi processing because the gel properties of surimi have been considered to be indicators for the cross-linking of myofibrillar and for the marketing price. According to our previous studies (*30*, *32*), cathepsins B and L had a high affinity for myosin and are very difficult to completely remove during surimi processing. There were still >80% activities of cathepsins B, L, and L-like left after the mincing and leaching processes and ∼68% left after the NaCl grinding process, which consequently caused gel softening during heating or setting. To retain high textural quality, the recombinant chicken cystatin was employed to inhibit the disintegration of mackerel surimi by cysteine proteinases. The degradation of protein gels of surimi was substantially inhibited (Figure 5), and the gel strength (breaking force × deformation) of surimi proteins was much improved (Table 2) by the addition of recombinant cystatin. The presence of recombinant cystatin (10 units/g of mince) resulted in a 40% improvement in deformation, which is considered to be an indicator of protein interactions (Table 2). The gel strength of mackerel surimi without recombinant cystatin was much lower than those with recombinant cystatin after 60 or 90 min of preincubation at 50 °C. No significant differences in gel strength and deformation were observed between samples with cystatin after 15−90 min of preincubation at 50 °C and those without cystatin and preincubation at 50 °C (Table 2). These results suggested that the recombinant cystatin could inhibit the gel softening of mackerel surimi caused by cathepsins and would have 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 wild-type cystatin, and the expression system developed in this study is useful and economical in terms of producing recombinant cystatin for industrial application.

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