

Characterization of Recombinant Soyacystatin Expressed in *E.coli*

Özlem Akpınar¹

Haejung An²

¹Department of Food Engineering Gaziosmanpaşa University, Tasliciftlik 60250 Tokat Turkey

²School of Pharmacy, University of Southern California, 1985 Zonal Ave, PSC B4, Los Angeles, CA 90089

Abstract: Recombinant (r-) soyacystatin was characterized for their inhibitory activity against papain and compared to egg white cystatin. r-Soyacystatin expressed in *E. coli* was purified 4.33 fold as a recombinant protein with phenyl-Sepharose and DEAE. Egg white cystatin was purified by using affinity chromatography on CM-papain-Sepharose. The specific interaction of r-soyacystatin and papain was detected on isoelectric focusing gel. Papain and r-soyacystatin formed a complex and the complex was resolved in between pIs of cystatins and papain. Both cystatins showed high stability at the wide pH range (pH 4-10), and the thermal stability of soyacystatin was comparable at the temperature range (0-100 °C). The r-soyacystatin exhibited papain-like protease inhibition activity comparable to that of the egg white cystatin, which could inhibit papain.

Key words: soyacystatin, egg white cystatin, proteolytic activity, protein, purification

E. coli Tarafından Sentezlenen Recombinant Soyacystatinin Karakterizasyonu

Özet: Rekombinant (r-) soyasistatinin papaine olan inhibitörü aktivitesi karakterize edilerek yumurta beyazı sistatin ile karşılaştırılmıştır. *E.coli* de sentez edilen r-soyasistatin 4 phenyl-Sepharose ve DEAE kolonları rekombinant protein olarak 4.33 kat saflaştırılmıştır. İsoelektirik fokus jeli ile papain ve r-soyasistatin arasındaki spesifik interaksiyon tespit edilmiştir. Papain ve r-soyasistatinin bir kompleks oluşturduğu ve bu kompleksin pI si sistatin ve papain pI lar arasında çözündüğü tespit edilmiştir. Her iki sistatinde geniş bir pH aralığında (pH 4-10) stabilite göstermiş ve soyasistatinin termal stabiliteside yumurta beyazı sistatine benzer bulunmuştur test edilen sıcaklıklarda (0-100 °C). r-Soyasistatinin papaine karşı olan inhibitör aktivitesi yumurta beyazı sistatine benzer bulunmuştur.

Anahtar kelimeler: Soyasistatin, yumurta beyazı sistatin, proteolitik aktivite, protein, saflaştırma.

1. Introduction

Cystatins are potent inhibitors of cysteine proteinases found in animal and plant tissues and human biological fluids (Barret, 1981). They inhibit cysteine proteinases such as cathepsins B, H and L and several structurally similar plant proteinases such as papain and actinidin by making the reactive site of the enzyme inaccessible to substrates and to the thiol group reagents (Bjork et al., 1989, Nicklin and Barret, 1984). They form tight reversible complexes with the proteinases with the dissociation constants typically in the nanomolar ratio (Barret, 1981, Bjork et al., 1989).

Cystatin superfamily are grouped into four different families based on their occurrence, sequence and structure similarity. Cystatin family I, stefin, is known to have the smallest molecular weight of ~11 kDa. It has no intramolecular disulfide bonds and glycosylation. Cystatins family II exist in the secreted and tissues of mammalian and avian origin. It has a molecular weight of ~13 kDa

with 2 disulfide bridges (Barret, 1981). Cystatin family III, also called kininogens, has the largest molecular weight of 70,000 consisting of heavy and light chains and existing in mammalian blood (Gournaris et al., 1984). Cystatins family 4, were recently discovered and found in plants (Turk et al., 1997). They do not have a disulfide bond like family I. However, their amino acid sequence is closely related to cystatins of family II. Cystatin from a plant source, therefore, is classified as independent family referred to as "phytocystatin" (Abe et al., 1992, Turk et al., 1997). They have been identified in seeds, leaves, roots and fruit (Rele et al., 1980, Rodis and Hoff, 1984, Brzin et al., 1988, Olivia et al., 1988, Rowan et al., 1990, Hines et al., 1991, Lenarcic et al., 1992, Abe et al., 1994, Kimura et al., 1995, Song et al., 1995, Botella et al., 1996, Wu and Haard, 2000).

Phytocystatin shows a wide inhibition spectrum against cysteine proteinases from plant and animal origin. Abe et al. (1994)

reported that corn cystatin inhibited various cysteine proteinase, including cathepsins H and L and papain. It also weakly inhibits cathepsin B. Izquierdo-Pulido et al. (1994) reported that cystatin isolated from rice was inhibitory against heat activated arrowtooth flounder proteinase.

Recently, cystatins have received more attention for their potential role in protecting fish surimi proteins from proteolytic activities (Kang and Lanier, 1999, Tzeng et al., 2001, Jiang et al., 2002, Chen et al., 2002, Hsieh et al., 2002). Surimi is minced fish meat that has unique functionality such as gel forming ability, water and oil binding properties (Tzeng et al., 2001). These characteristics make surimi main ingredient for wide range of seafood analogs such as artificial crab. Alaska pollock has been the species mostly used for surimi manufacturing. Because of the maximized annual catch of Alaska pollock and its relatively higher price, some underutilized species have been used to produce surimi such as mackerel, arrowtooth flounder, hairtail, mackerel and Pacific whiting. However, these fish species suffer from high levels of endogenous protease activity which causes soft texture (An et al., 1996, Visessanguan et al., 2001). In the last few years, Pacific whiting has been successfully utilized in surimi production because of the large availability in the U.S. Northwest coast and the low price. On the other hand, Pacific whiting suffers from post-mortem softening as a result of hydrolysis of myofibrillar proteins by endogenous proteinase, after the death of the animal, becomes susceptible to autolysis by the endogenous muscle proteinases. The degradation of myofibrillar proteins causes adverse effects on surimi quality and lowers the gel strength (An et al., 1996). It was shown that cathepsin L was the major source of proteolytic activity in Pacific whiting surimi (An et al., 1994). In order to alleviate the proteolytic activities of the fish muscle, food grade protease inhibitors such as egg white, potato powder and bovine plasma protein (BPP) have been used in surimi production but their use has been limited due to their adverse effects on organoleptic properties of surimi. It was reported that specific cysteine proteinase inhibitors such as egg white cystatin

reduced the protease activity into a negligible level without causing noticeable sensory defects in surimi (An et al., 1994, Lee et al., 2000, Jiang et al., 2002).

The objectives of this study are to purify recombinant soyacystatin expressed in *E. coli*, characterize biochemical properties of recombinant soyacystatin and compare the inhibition efficiency of both of them against papain.

2. Materials and methods

2.1. Materials

Kanamycin, isopropyl β -D-thiogalactopyranoside (IPTG), papain, Sepharose 6B, Brij 35 (30% w/v), glycerol, N-benzoyl-L arginine-2-naphthylamide (BANA), L-trans-epoxysuccinyl leucylamido (4-guadino) butane (E-64), dimethyl sulfoxide (DMSO), β -mercaptoethanol (β ME), *p*-dimethylaminocinnamaldehyde, tricine, ammonium sulfate (AS), dithioerythritol, bovine serum albumin (BSA), low molecular weight standards including aprotinin (6,500), α -lactalbumin (14,200), trypsin inhibitor (20,000), trypsinogen (24,000), carbonic anhydrase (29,000), glyceraldehyde-3-phosphate dehydro genase (36,000), ovalbumin (45,000) and albumin (66,000), were purchased from Sigma Chem. Co. (St. Louis, MO). Iodoacetic acid was obtained from Calbiochem (San Diego, CA). Phenyl-Sepharose 6 fast flow, DEAE Sepharose fast flow, broad range of pI standards including trypsinogen (pI-9.3), lentil lectin-basic band (pI-8.65), lentil lectin-middle band (pI-8.45), lentil lectin-acidic band (pI-8.15), myoglobin-basic band (pI-7.35), myoglobin-acidic band (pI-6.85), human carbonic anhydrase B (pI-6.55), bovine carbonic anhydrase (pI-5.85), α -lactoglobulin A (pI-5.20), soybean trypsin inhibitor (pI-4.55) and amyloglucosidase (pI-3.50), were purchased from Pharmacia (Piscataway, NJ). Premade agarose gel for isoelectric focusing was purchased from FMC Corp. (Rockland, ME). Sodium caseinate was purchased from U.S. Biochemical Corp. (Cleveland, OH).

IPTG solution were prepared as 1 M stock solution in water and sterilized by filtration through 0.2 μ m sterile Acrodisc (Gelman Sciences, Ann Arbor, MI). The stock solutions

were stored at -20°C until used. The stock solution of synthetic substrates and E-64 were prepared in DMSO and stored at -20°C until used.

2.2. Purification of Soyacystatin

Cloned *E.coli* containing soyacystatin gene was donated by Dr. Hisashi Koiwa of Purdue University. The recombinant cells were grown in small scale in 5 mL LB broth with 50 $\mu\text{g/L}$ of kanamycin overnight at 37°C with vigorous shaking. The following day it was inoculated into a large media (250 mL LB broth with 50 $\mu\text{g/L}$ of kanamycin) and allowed to grow until OD_{600} reached to 0.6 (generally 3-4 hours after inoculation into a large culture). Finally, it was induced with 0.4 mM IPTG (final concentration) and incubated for 16 h at room temperature. The cells were harvested by centrifugation at $4,000\times g$ for 30 min using a Sorvall refrigerated centrifuge SS-34 rotor (DuPont Co., Newtown, CT).

r-Soyacystatin was purified by the method of Koiwa et al. (1998). Harvested cells were sonicated using Sonicor (Model UP-400) with ultrasonic probe (Copiague, NY), in 10 mL of 10-fold diluted McIlvaine's buffer (0.2 M sodium phosphate, 0.1 M sodium citrate, pH 7) in ice. Sonicated cell extract was used to purify the recombinant soyacystatin using phenyl-Sepharose and DEAE column with two step purification. Sonicated cell extract was loaded onto 2.5x25 cm phenyl-Sepharose column at 4°C and equilibrated with 20 mM potassium phosphate, pH 6, containing 20% saturated AS. Elution was initiated with 15% saturated AS in 20 mM potassium phosphate buffer pH 6. When A_{280} reading of the fraction started to decrease, the elution buffer was changed to 10% saturated AS in the same buffer.

Fractions were analyzed for protein concentration by measuring A_{280} value and the presence of cystatin band on SDS-Tricine PAGE. The fractions which had a visible cystatin band were combined. The sample was loaded in 2.5x25 cm DEAE column 4°C , equilibrated with 10 mM Tris, pH 8.8. After loading the sample, the column was washed with 10 mM Tris, pH 8.8, overnight and eluted with the linear gradient of 0-0.4 M NaCl in 10 mM Tris, pH 8.8. The fractions which had a

cystatin band on SDS-tricine PAGE were combined. The activity of combined fractions were analyzed for inhibitory activity as in section "inhibition assay against papain".

2.3. Purification of Egg White Cystatin

Egg white cystatin was purified according to Anastasi et al. (1983) by using CM-papain-Sepharose column from twelve eggs. CM-papain-Sepharose column was prepared according to the method of Axen and Ernback (1971). Papain (100 mg) was activated with 2 mM dithioerythritol and 1 mM disodium EDTA in 10 mL of 0.1 M sodium phosphate, pH 6, for 10 min at 20°C and allowed to react with 10 mM iodoacetic acid. After activating Sepharose resin with CNBr, the resin was washed with cold 500 mL of 0.1 M NaHCO_3 , pH 9.0. Activated papain solution was stirred with the Sepharose 6B overnight at room temperature for coupling. The resin was washed with 500 mL of 0.01 sodium acetate, pH 4.1, 400 mL of 0.1 M sodium phosphate, pH 7.6, containing 1 M NaCl; 200 mL of 0.1 M sodium phosphate, pH 7.6 containing 15 g/L glycine; 400 mL of 0.1 M sodium phosphate pH 7.6 containing 1 M NaCl; and finally 500 mL of 0.01 sodium acetate, pH 4.1.

Egg white cystatin was purified from twelve eggs according to Anastasi et al. (1983). The egg white was blended with equal volume of 0.25% (w/v) NaCl. The pH of the solution was adjusted to 6-6.5 with 5 M sodium formate buffer, pH 3. To remove ovomucin from the egg white the solution was centrifuged at $2,100\times g$ for 30 min. CM-papain-Sepharose, 25 mL, was equilibrated with 50 mM phosphate buffer, pH 6.5 containing 0.5 M NaCl and 0.1% Brij. The centrifuged egg white solution was stirred with the equilibrated CM-papain-Sepharose overnight at 4°C . The resin was washed with 50 mM phosphate buffer, pH 6.5, containing 0.5 M NaCl and 0.1% Brij until the A_{280} was less than 0.05. The CM-papain-Sepharose was packed into 2.5x25 cm column at room temperature and washed with 2 bed volumes of 50 mM phosphate buffer, pH 6.5, containing 0.5 M NaCl and 10% (v/v) glycerol. The bound protein was eluted with 50 mM phosphate buffer, pH 11.5, containing 0.5 M NaCl and 10% (v/v) glycerol at room

temperature. Fractions, 2 mL, showing inhibitory activity against papain were combined and the pH was adjusted to 7.4 with 5 M sodium formate buffer, pH 3.0.

2.4. Gel Electrophoresis

SDS-PAGE gels, 15%, were performed according to Laemmli (1970) and 16.5% tricine SDS-PAGE gel was performed according to Schagger and Jagow (1987). Since soyacystatin has a low molecular weight, Laemmli's SDS-PAGE system did not give good resolution; therefore, tricine SDS-PAGE was used. The samples were boiled for 5 min in the SDS-PAGE treatment buffer (1:1, v/v) and applied on 15% and 16.5% polyacrylamide gels. The gels were run under a constant voltage at 150 V, on ice, using Bio-Rad Mini-Protean II unit (Bio-Rad, Hercules, CA).

2.5. Protein Content

Soluble protein content was determined according to Lowry et al. (1951) using bovine serum albumin as a standard.

2.6. Isoelectric Focusing

Isoelectric focusing was performed in premade agarose gels (FMC Corp., Rockland, ME). The wicks were soaked in 1 M NaOH, as a catholyte, and 0.5 M acetic acid, as an anolyte. The samples were run under 25 W constant power with 1000 V limit using a Thin-Layer Isoelectric Focusing (Desaga Heidelberg). Protein bands were stained with 0.1% Coomassie Brilliant Blue R-250 for 30 min and destained in 25% (v/v) of ethanol and 9% (v/v) of acetic acid for 3 min. The destained gel was dried at 55°C for 30 min. To estimate the isoelectric point of the proteins a broad range pI standards were used.

2.7. Temperature Stability

Both purified egg white cystatin, 0.364 μ g, and soyacystatin, 0.266 μ g, were incubated in the range of 0-100 °C for 30 min and immediately cooled in ice. Residual activity of heat treated sample were analyzed for inhibitory activity against papain using BANA as a substrate.

2.8. pH Stability

Both purified egg white cystatin, 0.364 μ g, and soyacystatin, 0.266 μ g, were incubated with McIlvaine's buffer in the pH range of 4-10 at room temperature for 15 min and residual inhibitory activity was assayed against papain BANA as a substrate

2.9. Inhibition Assay Against Papain

Inhibitory activity of purified cystatins against papain was measured by the method of Abe et al. (1994) with slight modification. The concentration of this enzyme was determined by active site titration with E-64. The assay buffer was 0.25 M sodium phosphate, pH 6, containing 2.5 mM EDTA. Papain solution, 20 μ g/mL, was activated with 25 mM sodium phosphate, pH 7 containing 20 mM β -ME at 40°C for 10 min. The assay buffer, 0.2 mL, was mixed with 0.1 mL of the activated papain. After preincubation of the mixture with 0.2 mL of inhibitor at 40°C for 5 min, the reaction was started by adding 0.2 mL of BANA and incubated at 40°C for 10 min. The reaction was stopped by adding 1 mL of 2% (v/v) HCl in ethanol and the color was developed by adding 1 mL of 0.06% (w/v) *p*-dimethylaminocinnamaldehyde in ethanol. Reaction products were measured at 540 nm. A blank was prepared by substituting cystatin with water. The inhibitory activity was defined as a decreased amount of BANA-hydrolyzing activity per mL of inhibitor solution per hour. One "unit" of inhibitory activity (U) was defined as the changes in absorbance of 1.0 at 540 nm per h.

3. Results and Discussions

3.1. Purification of Recombinant Soyacystatin and Egg White Cystatin

r-Soya cystatin was purified from *E. coli* overexpressing BL21 (DE3) pETNM⁸⁻¹⁰³ gene and its properties were compared to egg white cystatin purified from egg white. The recombinant cells were grown LB broth. After IPTG induction, a high level of soluble r-soyacystatin was expressed as the major protein component in *E. coli* BL21 (DE3) pETNM⁸⁻¹⁰³ cells (Figure 1). The recombinant soyacystatin was purified to electrophoretic homogeneity by 20-10% saturated ammonium sulfate, phenyl-

Sepharose, and 0-0.4 M NaCl DEAE chromatograms. The purity of r-soyacystatin on each of the purification step is shown on SDS-PAGE (Figure 1). The molecular weight of r-soyacystatin was estimated to be approximately 11.2 kDa. As shown in Figure 1, the r-soyacystatins constituted a high percentage of the total cell protein. Approximately 19.95 mg of purified cystatin was obtained from 212.5 mg of proteins of *E.coli* cells with a specific activity of 15,341 U/mg. The purification used provided a simple purification protocol with a high yield of r-soyacystatin, which indicated a high potential for this protocol to be used in a commercial application.

Egg white cystatin was purified by affinity chromatography. For this study, CM-papain-Sepharose, which was effective in isolating cystatin from numerous egg white proteins, was used as the affinity media. By taking advantage of the instability of cysteine proteinase in alkaline conditions, the bound cystatin was eluted from CM-papain-Sepharose by increasing pH to 11.5. Egg white cystatin was purified from 12 pooled egg whites and the pure egg white cystatin is shown in Figure 2. Approximately 5.10 mg of purified cystatin was obtained from 29,700 mg of proteins of egg white proteins with a purification fold of 240.

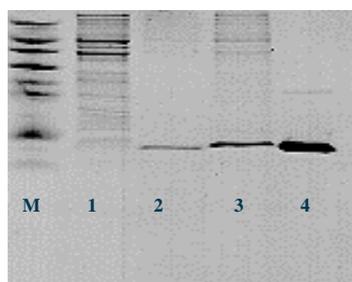


Figure 1. Various stage of purification of recombinant soyacystatin on SDS-tricine PAGE. (M) low molecular weight marker (1) 5 μ l of uninduced recombinant soyacystatin cell extract; (2) 5 μ g induced recombinant soyacystatin cell extract; (3) 5 μ g ammonium sulfate precipitated soyacystatin cell extract; (4) 5 μ g purified recombinant soyacystatin

3.2. Isoelectric Points of Cystatins and Papain

Isoelectric points of both cystatins and papain were determined by linear regression. Papain had an isoelectric point of 9.5 (Figure 2). This result is in agreement with the

isoelectric point of papain, 9.6 as reported by Sluyterman and Graff (1972). Egg white cystatin had two proteins with identical molecular weights. They were separated as two bands by isoelectric focusing with the pI's of 5.8 and 6.6. Soyacystatin also showed two isoelectric forms on the isoelectric focusing gel at pI 5.6 and 6.0. Brzin et al. (1990) reported that soybean cystatin showed three major bands at pI 5.3, 5.5, and 5.9 and two minor bands at 5.4 and 8.3. Our results are in agreement with the native isoelectric points of soyacystatin.

Cystatin and papain can form a complex resulting in changes in the isoelectric points (Anastasi et al., 1983). As seen in Figure 2, the complex was resolved between the papain and cystatin bands when papain and cystatin formed a complex. The pI of papain and egg white cystatin shifted to 8.82 and 9.20 as they formed a complex with each other. The complex between papain and soyacystatin was detected at pI 9.05 and 8.82 (Figure 2). Also, both cystatin complexes showed a weak band at 8.52.

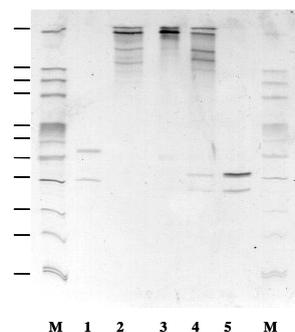


Figure 2. The complex formation of papain with soyacystatin and egg white cystatin on the IEF gel. (M) A broad range pI standards; (1) 1.04 μ g of egg white cystatin; (2) 5.6 μ g of papain with 1.04 μ g of egg white cystatin; (3) 5.6 μ g of papain; (4) 5.6 μ g of papain with 1.33 μ g of soyacystatin and (5) 1.33 μ g of soyacystatin; (M) A broad range pI standards.

3.3. Temperature and pH Stability

The temperature stability of r-soyacystatin was similar to that of egg white cystatin (Figure 3). After 30 min of incubation at 50°C or above, the inhibitory activity of r-soyacystatin was gradually decreased. There was 70% activity left after 30 min incubation at 50°C.

The effect of pH on cystatins stability was assessed by preincubating cystatin solution at arrange of pH values, then assaying the

inhibitory activity at the optimum pH. pH stability of both cystatins was tested by incubating them at different pH values. Both cystatins were relatively stable in the wide range of pH although they belong to different cystatin families (Figure 4). When the pH stability of egg white and soyacystatin was compared, soyacystatin seemed slightly more stable. However, a dramatic decline of the inhibitory activity at pH<4 was observed. As seen in Figure 4, 50 and 55% inhibitory activity remained in r-soyacystatin and egg white cystatin after 30 min incubation at pH 3.

Egg white cystatin is reported to be heat and pH stable protein (Fossum and Whitaker, 1968). The temperature and pH stability of r-soyacystatin was comparable to that of egg white cystatin. The most interesting characteristic in all four types of cystatin family is that they can survive the extreme pH and high temperature conditions which cause most proteins to denature (Brzin et al., 1983, Barrett et al., 1986, Izquierdo-Pulido et al., 1994, Tzeng et al., 2001).

3.4. Inhibitory Activity of Purified Cystatins

Cystatins form a reversible tight binding inhibitor papain-like proteinases (Nicklin and Barret 1984, Bjork et al., 1989). The inhibitory ability of r-soyacystatin was similar to that of egg white cystatin. When 2.00 μ g of papain was incubated with increasing concentration of pure r-soyacystatin and egg white cystatin, the linear concentration inhibition relationship were observed. The inhibitory activity of cystatins was dependent on dose when the ratio of cystatins/papain was smaller than 1. No significant increase in the inhibition ability was observed when the ratio of cystatins/papain was bigger than 1 (Figure 5). The results show that 1 molecule of r-soyacystatin binds to 1 molecule of papain coincides with that of native cystatin family (Abe et al., 1987, Bode et al., 1988, Arai et al., 1991, Abe et al., 1995, Tzeng et al., 2001, Jiang et al., 2002).

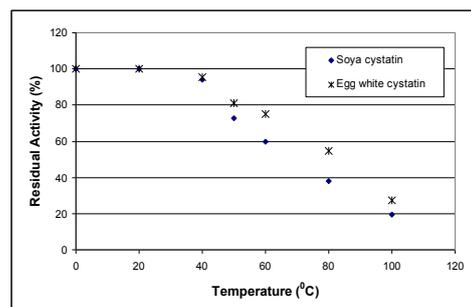


Figure 3. Thermal stability of soyacystatin and egg white cystatin. Egg white cystatin and soyacystatin were incubated in the temperature range of 0-100 °C and immediately cooled in ice prior inhibitory activity assay.

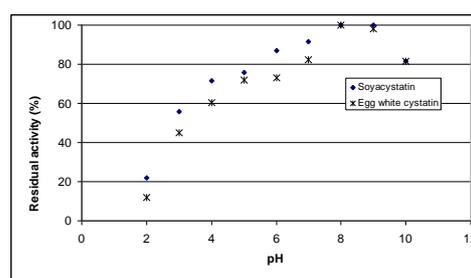


Figure 4. pH stability of soyacystatin and egg white cystatin. Egg white cystatin and soyacystatin were incubated in McIlvaine's buffer in the pH range of 4-10 at room temperature for 15 min prior to inhibitory activity assay.

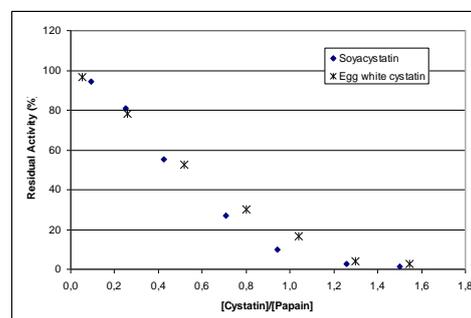


Figure 5: Inhibition profiles of r-soyacystatin and egg white cystatin against papain. The [cystatins]/[cysteine proteinase] was molar ratio.

4. Conclusion

Although cysteine proteinase inhibitors are widely found in nature, their level in natural sources is low and it is difficult and time-consuming to isolate cysteine proteinase inhibitors directly from natural sources. The more versatile approach to get large amounts of inhibitors is to produce these proteins into bacterial expression system. During the past few years many bioactive proteins had been expressed in bacteria by using recombinant DNA techniques.

Soyacystatin overexpressed in *E. coli* was easily recovered in a form than egg white cystatin. It revealed broad pH stability and

temperature tolerance and inhibition specificity similar to that of egg white cystatin. According to data obtained from this study, r-soyacystatin had biological and physical properties comparable to those of egg white cystatin. The data suggested that producing r-soyacystatin can be useful and economical for industrial application and accountable to control cysteine protease related softening in fish muscle.

Acknowledgment

This research was done in Oregon State University, Seafood Laboratory, Astoria, OR.

References

- Abe, K., Emori, Y., Kondo, H., Suzuki, K. and Arai, S. 1987. Molecular cloning of a cysteine proteinase inhibitor of rice (Oryzacystatin). *J. Biol. Chem.* 262, 16793-16797.
- Abe, M., Abe, K., Masaharu, K. and Arai, S. 1992. Corn kernel cysteine proteinase inhibitor as a novel cystatin superfamily member of plant origin. *Eur. J. Biochem.* 209, 933-937
- Abe, M., Abe, K., Iwabuchi, K., Domoto, C. and Arai, S. 1994. Corn cystatin I expressed in *Escherichia coli*: investigation of its inhibitory profile and occurrence in corn kernels. *J. Biochem.* 116, 488-492.
- Abe, M., Abe, K., Domoto, C. and Arai, S. 1995. Two distinct species of corn cystatin in corn kernels. *Biosci. Biotech. Biochem.* 59, 756-758.
- An, H., Weerasinghe, V., Seymour, T.A. and Morrissey, M.T. 1994. Cathepsin degradation of Pacific whiting surimi proteins. *J. Food Sci.* 5, 1013-1017
- An, H., Peters, Y.M., Seymour, T.A. 1996. Roles of endogenous enzyme in surimi gelation. *Trends in Food Sci. and Tech.* 7, 321-327.
- Anastasi, A., Brown, M.A., Kembhavi, A.A., Nicklin, M.J.H., Sayers, C.A., Sunter, D.C. and Barrett, A.J. 1983. Cystatin, a protein inhibitor of cysteine proteinases. *Biochem. J.* 211, 129-138.
- Arai, S., Watanabe, H., Kondo, H., Emori, Y. and Abe, K. 1991. Papain inhibitory activity of oryzacystatin, a rice seed cysteine proteinase inhibitor, depends on the central Gln-Val-Val-Ala-Gly region conserved among cystatin superfamily members. *J. Biochem.* 109, 294-298.
- Axen, R. and Ernback, S. 1971. Chemical fixation of enzymes to cyanogen halide activated polysaccharide carriers. *Eur. J. Biochem.* 18, 351-360.
- Barrett, A.J. 1981. Cystatin, the egg white inhibitor of cysteine proteinases. *Methods in Enzymol.* 80, 771-780.
- Barrett, A.J., Rawlings, N.D., Davies, M.E., Machleidt, W., Salvesan, G. and Turk, V. 1986. Cysteine proteinase inhibitors of cystatin superfamily. In: Barrett AJ, Salvesan G, editors. *Proteinase Inhibitors* The Netherlands Amsterdam: Elsevier Science Publishers B.V
- Bjork, I., Alriksson, E. and Ylinenjarvi, K. 1989. Kinetics binding of chicken cystatin to papain. *Biochemistry.* 28, 1568-1573.
- Bode, W., Engh, R., Musil, D.J., Thiele, U., Huber, R., Karshikow, A., Brzin, J., Kos, J. and Turk, V. 1988. The 2.0 Å X-ray crystal structure of chicken egg white cystatin and its possible mode of interaction with cysteine proteinases. *EMBO J.* 7, 2593-2599.
- Botella, M.A., Xu, Y., Prabha, T.N., Zhao, Y., Narasimhan, M.L., Wilson, K.A., Nielsen, S.S., Bressan, R.A. and Hasegawa, P.M. 1996. Differential expression of soybean cysteine proteinase inhibitor genes during development and in response to wounding and methyl jasmonate. *Plant Physiol.* 112, 1201-1210.
- Brzin, J., Kopitar, M., Turk, V. and Machleidt, W. 1983. Protein inhibitors of cysteine proteinases. Isolation and characterization of stefin, a cytosolic protein inhibitor of cysteine proteinase from polymorphonuclear granulocytes. *Hoppe-Seyler's Z Physiol. Chem.* 364, 1475-1480.
- Brzin, J., Popovic, T., Drobnic-Kosoroc, M., Kotnik, M. and Turk, V. 1988. Inhibitors of cysteine proteinases from potato. *Biol. Chem. Hoppe-Seyler.* 369, 233-238.
- Brzin, J., Ritonja, A., Popovic, T. and Turk, V. 1990. Low molecular mass protein inhibitors of cysteine proteinases from soybean. *Biol. Chem. Hoppe-Seyler* 371, 167-170.
- Chen, G., Tang, S., Chen, C. and Jiang, S. 2002. Overexpression of the soluble form of chicken cystatin in *Escherichia coli* and its purification. *J. Agric. Food Chem.* 48, 2602-2607.

- Fossum, K. and Whitaker, J.R. 1968. Ficin and papain inhibitor from chicken egg white. *Arch. Biochem. Biophys.* 125, 367-375.
- Gounaris, A.D., Brown, M.A. and Barrett, A.J. 1984. Human plasma α -cysteine proteinase inhibitor. Purification by affinity chromatography, characterization and isolation of an active fragment. *Biochem J.* 221, 445-452
- Hines, M.E., Osuala, C.I. and Nielsen, S.S. 1991. Isolation and partial characterization of soybean inhibitor of coleopteran digestive proteolytic activity. *J. Agric. Food Chem.* 39, 1515-1520.
- Hsieh, J.F., Tsai, G.J. and Jiang, S.T. 2002. Improvement of hairtail surimi gel properties by NADPH-sulfite reductase, recombinant cystatin, and microbial transglutaminase. *J. Food Sci.* 67, 3152-3158.
- Izquierdo-Pulido, M.L., Haard, T.A., Hing, J. and Haard, N.F. 1994. Oryzacystatin and other proteinase inhibitors in rice grain: potential use as a fish processing aid. *J. Agric. Food Chem.* 42, 616-622
- Jiang, S.T., Tzeng, S.S., Wu, W.T. and Chen, G.H. 2002. Enhanced expression of chicken cystatin as a thioredoxin fusion from in *Escherichia coli* AS494(DE3)pLysS and its effect on the prevention of surimi gel softening. *J. Agric. Food Chem.* 50, 3731-3737.
- Kang, I.S. and Lanier, T.C. 1999. Bovine plasma protein function in surimi gelation compared with cysteine protease inhibitors. *J. Food Sci.* 64, 842-846.
- Kimura, M., Ikeda, T., Fukumoto, D., Yamasaki, N. and Yonekura, M. 1995. Primary structure of a cysteine proteinase inhibitor from the fruit of avocado (*Persea americana Mill*). *Biosci. Biotech. Biochem.* 59, 2328-2329.
- Koiwa, H., Shade, R.E., Zhu-Salzman, K., Subramanian, L., Murdock, L.L., Nielsen, S.S., Bressan, R.A. and Hasegawa, P.M. 1998. Phage display selection can differentiate activity of soybean cystatins. *Plant J.* 13, 101-109.
- Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:860-865.
- Lee, J.J., Tzeng, S.S., Wu, J. and Jiang, S.T. 2000. Inhibition of thermal degradation of mackerel surimi by pig plasma protein and L-kininogen. *J. Food Sci.* 65, 1124-1129.
- Lenarcic, B., Ritonja, A., Turk, B., Dolenc, I. and Turk, V. 1992. Characterization and structure of pineapple stem inhibitor of cysteine proteinases. *Biol. Chem. Hoppe-Seyler.* 373, 459-464.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* 193, 256-946.
- Nicklin, M.J.H. and Barrett, A.J. 1984. Inhibition of cysteine proteinases and dipeptidyl peptidase I by egg white cystatin. *Biochem. J.* 223, 254-253.
- Olivia, M.L.V., Sampaio, M.U. and Sampaio, C.A.M. 1988. Purification and partial characterization of a thiol proteinase inhibitor from *Enterolobium contortisiliquum* beans. *Biol. Chem. Hoppe-Seyler.* 369, 229-232.
- Rele MV, Vartak HG, Jagannathan V. 1980. Proteinase inhibitors from *Vigna unguiculata* subsps *cylindrica*. I. Occurrence of thiol proteinase inhibitors in plants and purification from *Vigna unguiculata* subsps *cylindrica*. *Arch Biochem Biophys* 204:117-128.
- Rodis, P. and Hoff, J.E. 1984. Naturally occurring protein crystals in potato. Inhibitor of papain, chymopapain and ficin. *Plant Physiol.* 74, 907-911.
- Rowan, A.D., Brzin, J., Buttle, D.J. and Barrett, A.J. 1990. Inhibition of cysteine proteinases by a protein inhibitor from potato. *FEBS Lett.* 269, 328-330.
- Schagger, H. and Jagow, G.V. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 166, 368-379.
- Sluyterman, L.A. and De Graaf, M.J. 1972. The effects of salts upon the pH dependence of the activity of papain and succinyl-papain. *Biochim. Biophys. Acta.* 258, 554-561.
- Song, I., Taylor, M., Baker, K. and Bateman, Jr.R.C. 1995. Inhibition of cysteine proteinases by *Carica papaya* cystatin produced in *Escherichia coli*. *Gene.* 162, 221-224.
- Turk, B., Turk, V. and Turk, D. 1997. Structural and functional aspects of papain-like cysteine proteinases and their protein inhibitors. *Biol. Chem. Hoppe-Seyler.* 378, 141-150.
- Tzeng, S.S., Chen, G.H., Chung, Y.C. and Jiang, S.T. 2001. Expression of soluble form carp (*Cyprinus carpio*) ovarian cystatin in *Escherichia coli* and its purification. *J. Agric. Food Chem.* 49, 4224-4230.
- Wu, J. and Haard, N.F. 2000. Purification and characterization of a cystatin from the leaves of methyl jasmonate treated tomato plants. *Comp. Biochem. Physiol. Part (C)* 127, 209-220.
- Visessanguan, W., Menino, A.F., Kim, S.M. and An, H. 2001. Cathepsin L: Predominant heat-activated proteinase in Arrowtooth Flounder muscle. *J. Agric. Food Chem.* 49, 2633-2640.