Purification and Determination of Inhibitory Activity of Recombinant Soyacystatin Against Papain and Fish Protease

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Abstract: Recombinant (r-) soyacystatin was characterized for its inhibitory activity against papain and compared to egg white cystatin. r-Soyacystatin expressed in E. coli was purified with phenyl-Sepharose and DEAE 4.33 fold as a recombinant protein. Egg white cystatin was purified by using affinity chromatography on cm-papain-Sepharose. Inhibitory activity of r-soyacystatin was similar to that of egg white cystatin. The amount required to inhibit 50% activity of papain used in the assay, 2 µg, was 0.245 µg and 0.310 µg for soyacystatin and egg white cystatin, respectively. r-Soyacystatin inhibited 90% of autolytic activity in fish muscle.

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

Rekombinant Soyacystatininin Slaştırılması ve Papaine ve Balık Proteazlarına Karşı İnhibitör Aktivitesinin Saplanması

Özet: Rekombinant (r-) soyacystatininin papaine olan inhibitör aktivitesi karakterize edilerek yumurta beyazı sıstatinin ile karşılaştırılmıştır. E. coli de sentezlenen r-soyacystatin 4.33 kez saf rekombinant protein biçiminde phenyl-Sepharose ve DEAE kolonları ile saflaştırılmıştır. Yumurta beyazı sıstatin ise cm-papain-sepharose afinite kromatografisi kullanılarak saflaştırılmıştır. Araştırmada, 2 µg papainin %50 sini inhibe etmek için gerekten soyacystatin miktarı 0.245 µg ve yumurta beyazı sıstatin miktarı ise 0.310 µg dir. r-Soyacystatinin araştırma kullanılan balık kasındaki otolitik aktivitenin %90'ı inhibe etmiştir.

Anahtar kelimeler: Soyacystatin, yumurta beyazı sıstatin, proteolytik aktivite, protein, sallaştırma

1. Introduction

Cystatins are potent inhibitors of cysteine proteinases found in animal and plant tissues and human biological fluids (Barrett et al., 1986). They form tight reversible complexes with dissociation constants typically in the nanomolar ratio (Barrett et al., 1986, Bjork et al., 1989). They inhibit cysteine proteinases by making the reactive site of the enzyme inaccessible to substrates and to the thiol group reagents (Nicklin and Barrett 1984, Bjork et al., 1989). Cystatin superfamily are grouped into four different families based on their occurrence, sequence and structure similarity. Cystatin family I, stefin, are known to have the smallest molecular weight of ~11 kDa. It has no intramolecular disulfide bonds and glycosylation. Cystatins family II exists in the secrata and tissues of mamalian and avian origin. It has a molecular weight of ~13 kDa with 2 disulfide bridges (Barret, 1981). Cystatin family III, also called kininogens, have the largest molecular weight of 70,000 consisting of heavy and light chains and existing in mamalian 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., 1994; Turk et al., 1997).

Nowadays cystatins are started to receive enormous attention for their potential role in protecting fish proteins from proteinases attack (Chen et al., 2000, Kang and Lanier 1999, Tzeng et al., 2001, Hsieh et al., 2002, Jiang 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). Because of these characteristic

1 Master tezinden hazırlanmıştır.
surimi has been widely used as the main ingredient in 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 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). It has been shown that cysteine proteinase was the major source of proteolytic activity in these fishes (An et al., 1994a). Therefore it is important to control proteolytic activity during the processing into surimi. Otherwise high proteolytic activity causes adverse effect and lowers the gel strength in final product (An et al., 1996). Food grade protease inhibitors such as egg white, potato powder and 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 any sensory defects in surimi (An et al., 1994b, Lee et al., 2000, Jiang et al., 2002).

The objectives of this study are to purify recombinant soyacystatin expressed in E. coli, characterize inhibitory activity of recombinant soyacystatin against papain and compare the inhibition efficiency to that of purified egg white cystatin.

2. Materials and Methods

2.1. Materials

Kanamycin, isopropyl β-D-thiogalacto pyronoside (IPTG), papain, Sepharose 6B, Brij 35 (30% w/v), glycerol, N-benzoyl-L arginine-2-naphthylamide (BANA), L-trans-epoxyisuccinyl 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 dehydrogenase (36,000), ovalbumin (45,000) and albumin (66,000), were purchased from Sigma Chem. Co., St. Louis, MO. Phenyl-Sepharose fast flow and DEAE Sepharose fast flow were purchased from Pharmacia, Piscataway, NJ. Cloned E.coli containing soyacystatin gene were donated by Dr. Hisashi Koiwa of Purdue University.

IPTG solution were prepared as 1 M stock solution in water and sterilized by filtration through 0.2 μm sterile Acrodisc (Gelman Sciences, Ann Arbor, MI). The stock solutions were stored -20 °C until used. The stock solution of synthetic substrates and E-64 were prepared in DMSO and stored -20 °C until used.

2.2. Purification of r-soyacystatin

E.coli containing soyacystatin gene was isolated from single colony on agar plates by streaking. The recombinant cells were grown in small scale in 5 mL LB broth with 50 μg/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 μg/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 hr at room temperature. The cells were harvested by centrifugation at 4,000xg for 30 min using a Sorvall refrigerated centrifuge SS-34 rotor (DuPont Co., Newtown, CT).

Recombinant soyacystatin (r-soyacystatin) was purified by the method of Koiwa et al. (1998). Harvested cells were sonicated using Sonicer (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, until clear solution was obtained. Sonicated cell extract was centrifuged at 7,000xg for 10 min. The resulting supernatant was precipitated with 70% saturated AS. The precipitated protein was dissolved in 10 mL of 10 mM potassium phosphate, pH 6 and centrifuged at 7,000xg for
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10 min to remove the debris. To the supernatant, 20% saturated AS was added and centrifuged at 7,000×g for 10 min. The final supernatant was loaded onto phenyl-Sepharose column and equilibrated with 20 mM potassium phosphate, pH 6, containing 20% saturated AS. While the sample was eluted, the A280 value of each fraction was monitored. Elution was initiated with 15% saturated AS in 20 mM potassium phosphate buffer pH 6. When A280 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 A280 value and the presence of cystatin band on SDS-Tricine PAGE. The fractions which had a visible cystatin band were combined. Into the combined fractions, 80% saturated AS was added, stirred overnight at 4°C and centrifuged at 7,000×g for 10 min. The pellet was suspended in 10 mM Tris, pH 8.8 and dialyzed against 10 mM Tris, pH 8.8. The dialyzed sample was loaded in DEAE column 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. Combined fractions were dialyzed against 50 mM sodium phosphate, pH 6. The activity of combined fractions were analyzed for inhibitory activity as in section “inhibition assay against papain”.

2.3. Preparation of Cm-papain Sepharose

Cm-papain-Sepharose column was prepared according to the method of Axen and Emback (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. For activation of Sepharose resin, 5 mL water and 4 mL CNBr (from 25 mg/mL stock solution) were mixed with 100 mg of Sepharose 6B resin. The pH of the activated Sepharose resin was kept at 10-11 with 2 N NaOH for 8 min. After the pH treatment, the gel was washed with cold 500 mL of 0.1 M NaHCO3, 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 M 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.

2.4. Purification of Egg White Cystatin

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×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 A280 was less than 0.05. The cm-papain-Sepharose was packed into a column 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. 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.

2.5. Inhibition Assay Against Papain

Inhibitory activity of purified cystatins was measured by the method of Abe et al. (1994) with slight modification. The assay buffer was 0.25 M sodium phosphate, pH 6, containing 2.5 mM EDTA. Papain solution, 20 μg/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 hr.

2.6. Active Site Titration

E-64 stock solution (1 mM) was prepared in DMSO and diluted to a working concentration (1 μM) with water. The assay buffer (0.25 M sodium phosphate, pH 6 containing 2.5 mM EDTA) was mixed with 0.1 mL of papain solution in 25 mM sodium phosphate, pH 7 containing 20 mM β-ME. After preincubation of the mixture with 0.2 mL of inhibitor at 40 °C for 5 min, the inhibitory activity was measured against papain as described above.

2.7. 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).

2.8. Autolysis Assay

Autolysis assay was carried out according to Morrissey et al., (1993). Finely chopped Pacific whiting (3 g) with 3 mL inhibitor solution was incubated at 55 °C for 30 and 60 min for Pacific whiting fish fillet and Pacific whiting surimi, respectively. The autolytic reaction was stopped by adding 5% (w/v) cold TCA solution. To precipitate unhydrolyzed proteins, the solution mixture was incubated at 4 °C for 15 min and centrifuged at 6,100 × g for 15 min. The supernatant, which had TCA-soluble proteins was analyzed for oligopeptide contents with Lowry assay (Lowry et al., 1951), and the enzyme activity was expressed as nmols tyrosine released per minute. Sample blanks containing all components were kept on ice and used to correct for oligopeptide content originating from Pacific whiting. All treatments were run duplicate.

\[
\frac{\text{C}_{55}-\text{C}_0}{\text{(C}_{55}-\text{C}_0)} \times 100
\]

C_{55} = nmoles tyrosine/min without inhibitor at 55 °C
C_0 = nmoles tyrosine/min without inhibitor at 0 °C
I_{55} = nmoles tyrosine/min with inhibitor at 55 °C
I_0 = nmoles tyrosine/min with inhibitor at 0 °C

2.9. Protein Content

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

3. Results and Discussions

3.1. Purification of r-Soyacystatin

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 (Table 1). 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.
Table 1. Purification table of soyacystatin. The inhibitory activity was measured as decrease in BANA hydrolyzing activity. One unit of inhibitory activity was defined as the changes in absorbance of 1.0 at 540 nm per hour.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total Protein (mg)</th>
<th>Total Activity (U)</th>
<th>Specific Activity (U/mg)</th>
<th>Yield (%)</th>
<th>Fold purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli lysate&lt;sup&gt;d&lt;/sup&gt;</td>
<td>212.5</td>
<td>753219</td>
<td>3544.5</td>
<td>100</td>
<td>1.00</td>
</tr>
<tr>
<td>Ammonium sulfate precipitation</td>
<td>90.00</td>
<td>693600</td>
<td>7706.6</td>
<td>92.0</td>
<td>2.17</td>
</tr>
<tr>
<td>DEAE</td>
<td>19.95</td>
<td>299130</td>
<td>15341</td>
<td>39.7</td>
<td>4.33</td>
</tr>
</tbody>
</table>

<sup>d</sup>The starting volume was 500 ml. of cultivated broth

3.3. Inhibitory Activity of Purified Cystatins

Cystatins form a reversible tight binding inhibitor for papain-like proteases (Nicklin and Barret 1984, Bjork et al., 1989). The inhibitory ability of r-soyacystatin was comparable with egg white cystatin. The residual activity of papain with 0.400 μg of r-soyacystatin decreased dramatically to ~10%. The amount of purified soyacystatin and egg white cystatin (Figure 3) required to obtain 50% inhibition of papain was 0.245 μg and 0.310 μg, respectively. To obtain 90% inhibition of papain, the amount of soyacystatin and egg white cystatin was 0.400 and 0.550 μg, respectively.

E-64 is an extremely effective cysteine proteinase inhibitor. Absolute molarity of cysteine proteinases can be determined by stoichiometric titration with E-64 (Barrett and Kirschke, 1981). E-64 titration with papain allowed the calculation of active concentration of papain used in the assay. The inhibitory activity of cystatins was dose dependent 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 4). The results showed 1 molecule of r-soyacysatin binding to 1 molecule of papain coincided with that of native cystatin family (Bode et al., 1988, Arai et al., 1991, Abe et al., 1995, Tzeng et al., 2001, Jiang et al., 2002).

3.2. Purification of Egg White Cystatin

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.
3.4. Inhibition of Autolytic Activity in Fish Muscle

High levels of cysteine proteinase activity caused by cathepsin B, H, and L have been found in Pacific whiting and arrowtooth flounder (An et al., 1994, Wasson et al., 1992, Visessanguan et al., 2001). These cysteine proteinases have a strong potential to be active in postmortem muscle pH (5.5–6), degrade the myofibrillar proteins (Wisessanguan et al., 2001) and cause gel softening in surimi. Considering the high specific activity of E. coli cell lysate against papain (Table 1), E. coli cell lysate, soycystatin cell extract, was tested for its ability to prevent autolytic degradation of muscle proteins and the results were shown in Table 2. r-Soyacystatin cell extract inhibited 90% of autolytic activity in fish muscle. It has a high potential for use in improving seafood quality.

<table>
<thead>
<tr>
<th>Sample</th>
<th>TCA soluble peptides (nmol/mg)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pacific whiting fillet</td>
<td>4.11 ± 0.70</td>
<td>0.00</td>
</tr>
<tr>
<td>0.041% soycystatin cell extract</td>
<td>0.40 ± 0.17</td>
<td>90.0</td>
</tr>
<tr>
<td>0.1 mM E-64</td>
<td>0.13 ± 0.20</td>
<td>96.0</td>
</tr>
</tbody>
</table>

4. Conclusion

Although cysteine proteinase inhibitors are widely found in nature, their levels in natural sources are low. For example to obtain enough inhibitor for the characterization of stefin A (Brzin et al., 1983) or pig L-kinogen (Lee et al., 2000), a large amount of fresh blood is required. Likewise kilograms amount of rice seeds and liters amount of egg white yields only microgram of oryzacystatin (Abe et al., 1987) and egg white cystatin (Anastasi others 1983), respectively. In addition it is rather 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 in bacterial expression system. During the past few years many bioactive proteins has been expressed in bacteria by using recombinant DNA techniques. In this study, it was demonstrated that, soycystatin overexpressed in E. coli can be purified with high yield and purity. The r-soycystatin was easily recovered in a more concentrated form than egg white cystatin. It revealed inhibition specificity similar to that of egg white cystatin. It should be accountable to control cysteine protease related softening in fish muscle. The data suggested that producing r-soycystatin can be useful and economical for industrial application.

Acknowledgement

This research was done in Oregon State University, Seafood Laboratory, Astoria, OR. The author wishes to thank Dr. Haejung An for reviewing this paper.
References


