

Protein expression of the N-terminal region low MW glassfish egg protease inhibitor in *Escherichia coli*

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Abstract

The synthesized DNA of N-terminal region of low molecular weight glassfish egg protease inhibitor was cloned and expressed in Escherichia coli BL-21. Similarity of the synthesized DNA sequence before and after inserted was 78 %. The fusion protein Glutathion-S-transferase (GST)-protease inhibitor was purified by GSTrap FF affinity chromatography in which the molecular weight was 29 kDa. The GST-protease inhibitor was cleaved by trombin and was separated by Sephacryl HR-100 column chromatography. The specific inhibitory activity of the GST-protease inhibitor, protease inhibitor and natural inhibitor were 0.033, 7.117, and 19.70 Unit/mg, respectively.

Key words : protein expression, N-terminal region, glassfish protease inhibitor

Introduction

The cysteine protease inhibitors, cystatins, are subdivided into three subfamilies: stefins, cystatins, and kininogen based on their structural complexity. Stefin (family I) is the smallest inhibitor in the cystatin superfamily, which has a molecular weight of about 11 kDa and lacks of disulfide bonds. Human cystatin A and B (Ritonja *et al.*, 1985) are typical examples. Family II cystatin, which exists in most body fluid and tissues of mammalian and avian, is about 13 kDa with two disulfide bridges. Family II cystatin has been purified from chum salmon egg (Yamashita and Konagaya, 1991a). Kininogen (family III) is single chain glycoprotein containing three cystatin-like domains with molecular weight of 68 to 120 kDa (Barret *et al.*, 1986). The related inhibitor originated from plant seeds such as corn, rice, soybean, and

sunflower are more homologous to the cystatin family I in amino acid sequence, while the lack of 2 intra-disulfide bridges on that inhibitors is similar to the stefin (Abe *et al.*, 1987; Kondo *et al.*, 1990; Kouzuma *et al.*, 1996).

The interaction between proteases and their inhibitor was a target of intensive studying for the last two decades. Protease inhibitors have been purified from ovarian fluid carp (Tsai *et al.*, 1996), and egg and muscle of chum salmon (Yamashita and Konagaya, 1991ab), muscle of white croaker (Sangorin *et al.*, 2001), and Atlantic salmon and Arctic charr (Olenen *et al.*, 2003). Protease inhibitors in muscle of white croaker, hake, Argentine anchovy, castaneta, rough sea, and sea trout were investigated and compared (Borla *et al.*, 1998). The specific inhibitors of cysteine proteases are needed in preventing unwanted destructive proteolysis which can be used in therapy and research (Hernandez and Roush, 2002; Pol and Bjork, 2003), toxic for pest (Gruden *et al.*, 1998; Rogelj *et al.*, 2000), and food industry (Hamman *et al.*, 1990; Pandhere *et al.*, 2000).

Cystatin was cloned and studied at the molecular level; such as human cystatin C (Abrahamson *et al.*, 1987), human cystatin S (Isemura *et al.*, 1986), chicken cystatin (Colella *et al.*, 1989), mouse cystatin C (Solem *et al.*, 1990), and rat cystatin (Cole *et al.*, 1989). Protease inhibitors from plants were also cloned and studied (Ryan, 1990); such as in rice (Abe *et al.*, 1987), tobacco (Park *et al.*, 2000), leave of tomato cystatin (Jacinto *et al.*, 1998), and sugarcane cystatin (Soares-Costa *et al.*, 2002). Recently, cystatins have been cloned from rainbow trout (Li *et al.*, 1998), chum salmon (Yamashita and Konagaya, 1996), carp (Tsai *et al.*, 1996), and zebrafish (Gong *et al.*, 1997), and also from tentacle of jellyfish (Yang *et al.*, 2003).

Recombinant cystatin has been produced in bacteria, yeast, and mammalian cell expression system. Human cystatin C (Berti *et al.*, 1997), chicken cystatin (Auerswald *et al.*, 1989, 1993), rat cystatin S (Bedi *et al.*, 1998), and mouse cystatin C (Hakansson *et al.*, 1996) have been expressed successfully in *E. coli*. Recently, cystatins from fish were also expressed in

E. coli; rainbow trout cystatin C (Li *et al.*, 2000), and carp ovarian cystatin (Tzeng *et al.*, 2002). The total synthesis of the cystatin α gene was cloned and expressed successfully in *E. coli* (Katunuma *et al.*, 1988). Partial synthesized gene of rat salivary cystatin S was cloned and expressed in *E. coli* to produce its variant polypeptides including N-terminal polypeptide (Bedi *et al.*, 1998).

Recently the low molecular weight protease inhibitor was purified from ovarian glassfish (Ustadi *et al.*, 2005). To know the relationship between protein structure and inhibitory activity of the protease inhibitor, we cloned the synthesized DNA of its N-terminal part and established a bacterial system for expression of the protein.

Material and Methods

Material

Vector pGEX4T-1 (4969 bp), *E. coli* BL 21 (DE3), glutathione-Sepharose 4B, *EcoR* I, *Xho* I were purchased from Amersham-Pharmacia Biotech (Uppsala, Sweden). T4 DNA ligase was obtained from Novagen Inc. (Madison, WI, U.S.A.). Polymerase Chain Reaction (PCR) kit, the synthesized DNA, and oligonucleotides were purchased from Bioneer Inc. (Seoul, Korea). Most of other chemicals were purchased from Sigma Chemical (St. Louis, MO, U.S.A.).

Construction recombinant plasmid

The DNA encoded N-terminal amino acid region of glassfish egg protease inhibitor was synthesized by Bioneer Inc. (Seoul, Korea). The synthesized DNA sequence was 5'-CGGAATTCCACGCTAATAGGGTCATGCCTGATATGAACATGGATTATATGGATGCCCTCGAGCG-3' as a template for PCR. The synthesized DNA contained *EcoR* I and *Xho* I restriction enzyme site at 5' and 3' end, respectively. PCR was used to amplify the DNA of glassfish egg protease inhibitor with 30 cycles, which was initiated by 30 s of denaturation at 94 °C, 30 s of annealing at 60 °C, and then final extension step was at 72 °C for 2 min in a DNA

thermal cycles (Eppendorf personal PCR system). The forward primer was 5'-CGGAATTCCAC GCTAATAG-3' and the reverse primer was 5'-CGGCTCGAGGGCATCCAT-3'. After PCR reaction, PCR product and pGEX4T-1 plasmid were restricted by *EcoR* I and *Xho* I at 37 °C overnight and ligated at 16 °C for 2 hr. Ligated plasmid was transformed to *E. coli* XL-1-Blue by Inoue method (Sambrook and Russell, 2001). *E. coli* transformant was screened by antibiotic (50 µg/mL ampicillin) selection. Plasmid DNA was isolated from positive colonies and sequenced to check inserted DNA. The recombinant plasmid with correct in-frame coding sequence of inserted DNA was transformed into the host *E. coli* strain BL21 (DE3) for protein expression.

Expression of fusion protein

Positive colonies of *E. coli* strain BL21 in 30 mL LB broth containing 50 µg/mL ampicillin were used for protein expression. In brief, each bacterial colony was grown in 30 mL LB broth containing 50 µg/mL ampicillin until the optical density at 590 nm reached 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) (Bio Basic Inc., Canada) was then added to the final concentration of 1 mM. The cell was harvested by centrifugation of 1 mL culture at 1, 2, 3, 4, and 6 hr after inducing with IPTG. Before inducing, 1 mL culture was also harvested as 0 hr. The cell pellet was boiled in 50 µL of loading buffer for 10 min and electrophoresed on 12.5% polyacrylamide gel as described by Laemmli (1970).

Purification of recombinant protein

Recombinant fusion proteins were obtained from 250 mL cultures. The overnight culture of transformed *E. coli* (5 mL) was inoculated into 250 mL of fresh LB-Amp broth. The culture was incubated at 37 °C for 4 hr with vigorous shaking. When optical density of culture at 590 nm was reached 0.6, IPTG was added to final concentration of 1 mM in culture. Culture was further incubated for 5 h. The cells were harvested by centrifugation at 9000×g for 10 min at 4 °C and

incubated -20°C for 2 hr prior lysis. One gram of frozen cells was thawed, resuspended in 10 mL of phosphate buffer saline (PBS), and disrupted by sonication. Triton X-100 (20%) was added to a final concentration of 1%, mixed gently for 30 min, and then lysate was centrifuged at $15,000\times g$ for 20 min at 4°C . 8 mL supernatant was loaded into 1 mL GSTrap FF column (Amersham Biosciences Ltd., Uppsala, Sweden), equilibrated by PBS (pH 7.2) and eluted by elution buffer (50 mM Tris-HCl, pH 8.0 with 10 mM reduced glutathione). The purified fusion protein was assessed by SDS-PAGE stained by commassie brilliant blue. The fusion protein was finally dialysed against 10 mM Tris-HCl, pH 8.0 and degested with 100 U of thrombin at 37°C for 6 hr. After cleavage with thrombin, the protease inhibitor was purified by Sephacryl HR 100 (Amersham Biosciences Ltd., Uppsala, Sweden) column (2.6 x 100 cm), packed and equilibrated with 50 mM ammonium bicarbonate buffer, pH 7. The protein peak was assayed inhibitory activity against papain.

Inhibitory activity assay

According to the modified method of Borla *et al.* (1998), protease inhibitory activity was determined by measuring the inhibitory degree of papain activity using azocasein as the substrate. Two hundred μL of $1.7\ \mu\text{g}/\text{mL}$ inhibitor solution in buffer A was added to $100\ \mu\text{L}$ papain solution (0.1 U of activity) in buffer A. The inhibitor-papain mixture was incubated at 37°C for 5 min and then added to $250\ \mu\text{L}$ of $3.2\ \text{mg}/\text{mL}$ azocasein solution in buffer A. This mixture was incubated at 37°C for 30 min with its reaction stopped by adding $700\ \mu\text{L}$ of 20% trichloroacetic acid (TCA). The control was prepared by substituting $200\ \mu\text{L}$ inhibitor solution with $200\ \mu\text{L}$ buffer A. Blank was also prepared by adding $700\ \mu\text{L}$ of 20% TCA in advance before adding $250\ \mu\text{L}$ of substrate solution. Seven hundred and twenty μL supernatant obtained after the centrifugation at $10,000\times g$ for 5 min was added to $800\ \mu\text{L}$ of 1 N NaOH for developing color. Papain activity was expressed as the absorbance at 440 nm. One unit of protease activity was defined as the amount of enzyme that induced an increase of 1.0 absorbance unit at 440 nm

after incubation at 37 °C. Inhibitory activity was calculated by subtracting papain activity without inhibitor from that with inhibitor. One unit of inhibitory activity was defined as one unit decrease of papain activity.

Results and Discussions

Plasmid expression

The PCR product of synthesized DNA of N-terminal region glassfish egg protease inhibitor was inserted successfully in pGEX4T-1 and then cloned in *E. coli* XL-1-Blue by Inoue method (Sambrook and Russell, 2001). Agarose gel electrophoresis pattern of the isolated plasmid from several colonies is shown in Fig. 1. But after cutting at *EcoR* I and *Pst* I restriction site of pGEX4-1 (Fig. 4) and sequencing that part, there were just two colonies containing correct recombinant vector.

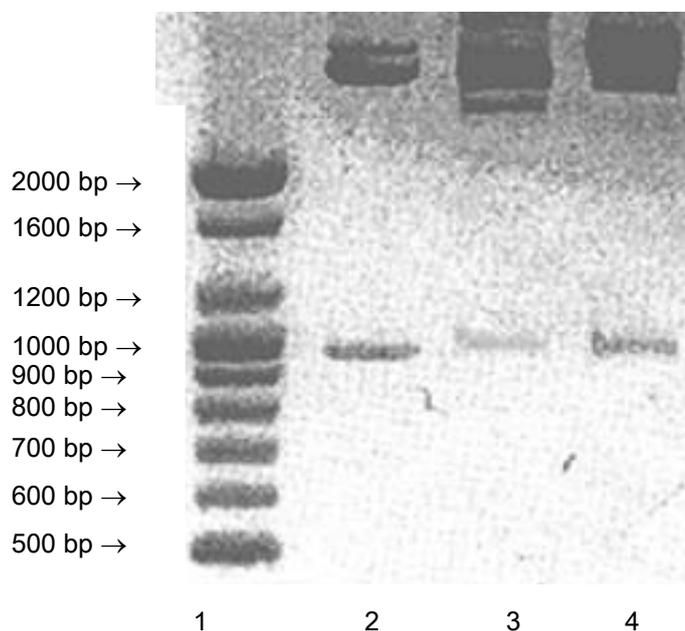


Fig. 1. Agarose gel electrophoresis pattern of pGEX4T-1 containing insert DNA cut at *EcoR* I and *Pst* I restriction sites. Lane 1, DNA marker (Bioneer D-1030); Lane 2, pGEX4T-1 without insert DNA; Lane 3 & 4, pGEX4T-1 containing insert DNA.

Protein expression

E. coli BL-21 mutant was grown in LB broth medium containing 50 $\mu\text{g}/\text{mL}$ and then the cells were harvested after Isopropyl- β -D-thiogalactopyranoside (IPTG) induction for 6 hr. Expression of the fusion protein GST-glassfish egg protease inhibitor was showed in Fig. 2. SDS-PAGE analysis showed the presence of a predominant induction band with molecular weight around 29 kDa.

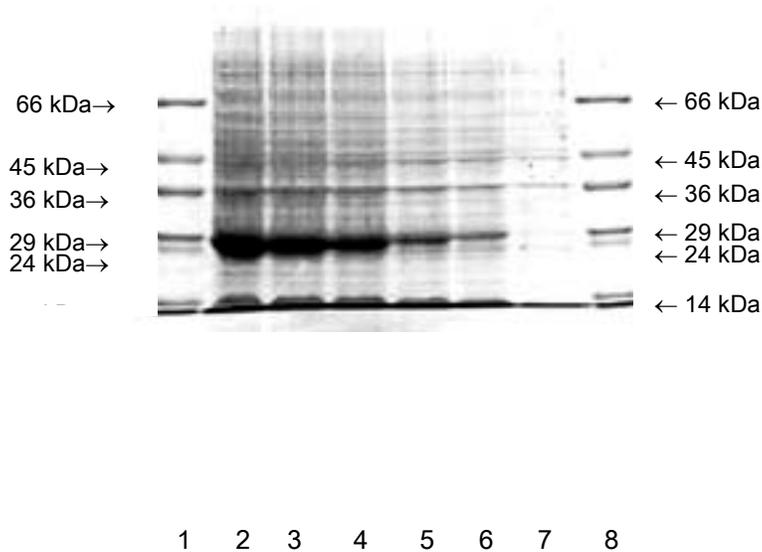


Fig. 2. SDS-PAGE pattern of fusion Glutathion-S-transferase (GST)-recombinant protein expression with *E. coli* B21 system. Lane 1 & 8, protein marker (Sigma MW-SDS-70L); Lane 2-6, after induction for 6, 4, 3, 2, and 1 hr.; Lane 7, 0 hr after induction

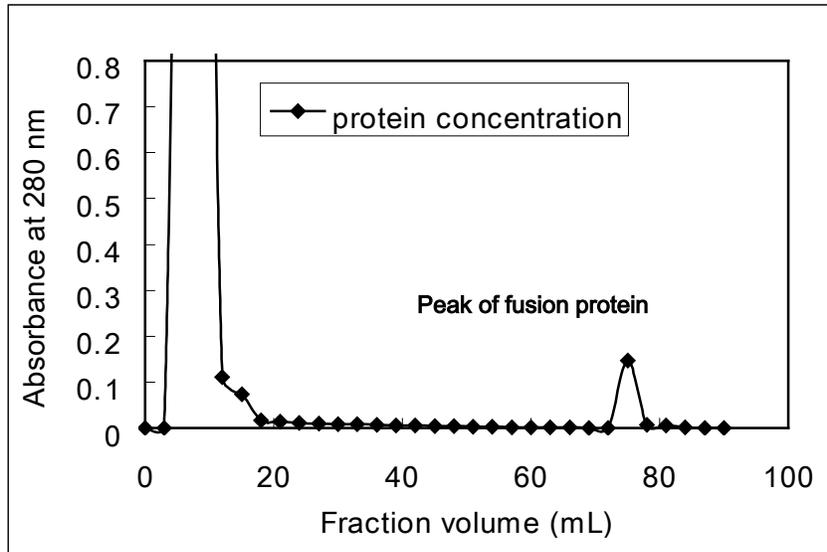
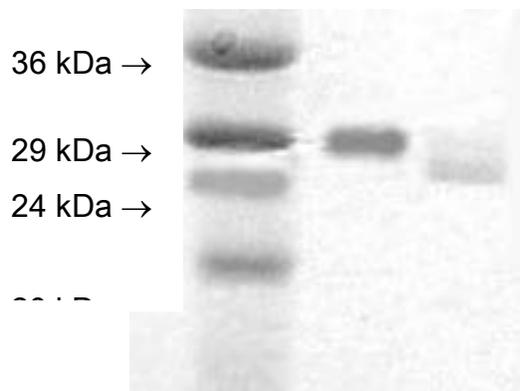


Fig. 3. GSTrap FF affinity chromatography pattern of GST-glassfish egg protease recombinant inhibitor.

GSTrap FF affinity chromatography pattern of fusion protein is shown in Figure 3. Small peak on SDS-PAGE analysis showed one protein band as purified GST- glassfish protease inhibitor with MW 29 kDa (Fig. 4).



1 2 3

Fig. 4. SDS-PAGE pattern of fusion GST-recombinant protein after and before cut with thrombin. Lane 1, Molecular size marker; lane 2, before cutting; lane 3, after cutting.

Purified GST- glassfish protease inhibitor was cleaved by thrombin. SDS-PAGE analysis showed one band as GST with MW 26 kDa, while protein band of recombinant protease inhibitor was not appeared (Fig. 4). After digestion, GST and recombinant protease inhibitor were separated by Sephacryl 100 HR chromatography (Fig.5). Specific inhibitory activity against of Peak II as a purified protease inhibitor was 7.117 U/mg (Table 1), while Peak I (GST) showed no inhibitory activity.

The fusion protein, Glutathion-S-Transferase (GST), was a good system for expressing soluble form of protein in *E. coli* and secreted into the cytoplasmic space of the host cell (Davis et al., 1999). Recombinant plasmid pGEX-4T-1 containing inserted DNA of N-terminal region of glassfish egg protease inhibitor was transformed to *E. coli* BL21 (DE3) cells. The BL21 genotype F⁻, *ompT*, *hsdS* (r_B^- , m_B^-) carries a chromosomal copy of the gene for T7 RNA polymerase under control of *LacUV5* promoter (Studier and Moffat, 1986). In addition, the pGEX-4T-1 expression vector with a *tac* promoter for chemical induced using IPTG and an internal *lac I^q* gene for use in any *E. coli* host is considered to be a powerful expression vector. After IPTG induction, the GST-glassfish egg protease inhibitor was over-expressed in the *E. coli* BL21.

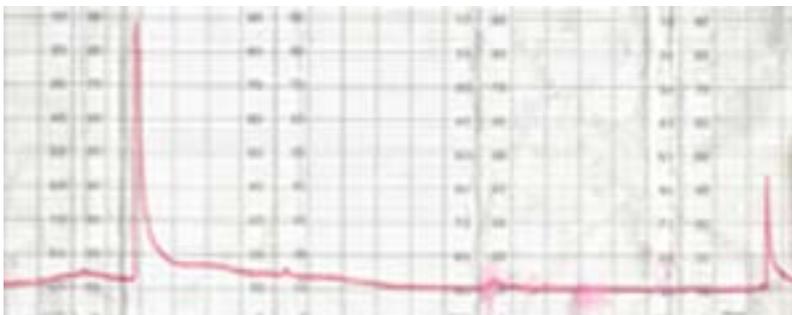


Fig. 9. Sephacryl HR-100 chromatography (Sephacryl HR100) of fusion GST (Peak I) and recombinant protein (Peak II) detect with GILSON 112 UV/VIS DETECTOR and record with BIO-RAD 1325 ECONO-RECORDER.

After purification of recombinant fusion GST-glassfish egg protease with GSTrap FF affinity chromatography, further cleaved of the fusion protein by thrombin and then separated by Sephacryl HR-100 gel permeation chromatography, 0.0125 mg of recombinant protease inhibitor was produced from 250 mL culture. One mg of recombinant peptide of several rat cystatin S was produced from 1 L culture, which was also expressed with pGEX-4T-2 vector and *E. coli* BL-21 (Bedi et al., 1998). Low production of recombinant glassfish egg protease might be caused by small density harvested cell. Dissolved oxygen and pH in the culture was not controlled, therefore culture of recombinant *E. coli* BL-21 might be not in optimal condition to produce maximum cell mass.

Table 1. Inhibitory activity of recombinant protease inhibitor against papain

Inhibitor	Total protein (mg)	Total activity (Unit)	Specific activity (Unit/mg)
GST-recombinant	3.573	0.10	0.033
Recombinant	0.0125	0.885	7.117
Natural	0.15	2.87	19.70

GST : Glutathione S-transferase (fusion protein)

Specific inhibitory activity of recombinant protease inhibitor, 7.117 U/mg, was lower than 19.70 U/mg specific inhibitory activity of natural protease inhibitor from glassfish egg due to loose of Met and Asn residues in deduced amino acids sequence. Bode et al. (1988) said that Met residue is one of the important residues in the active site of protease inhibitor.

Conclusions

Similarity of the synthesized DNA sequence before and after inserted was 78 %. The molecular weight of fusion protein Glutathion-S-transferase (GST)-protease inhibitor was 29 kDa. The

specific inhibitory activity of the GST-protease inhibitor, protease inhibitor and natural inhibitor were 0.033, 7.117, and 19.70 Unit/mg, respectively.

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