ПРИКЛАДНАЯ БИОХИМИЯ И МИКРОБИОЛОГИЯ, 2013, том 49, № 4, с. 353–357

UDC 577.156

DIFFERENTIAL FUSION EXPRESSION AND PURIFICATION OF A CYSTATIN IN TWO DIFFERENT BACTERIAL STRAINS

© 2013 A. Gholizadeh

Research Institute for Fundamental Sciences (RIFS), University of Tabriz, Tabriz, Iran

e-mail: aghz_bioch@yahoo.co.in

Received October 1, 2012

To date, the identification of the novel multifunctional properties of cysteine proteinase inhibitors "known as cystatins" is the great of interests for molecular biologists. The efficient production, purification and correctly folded form of these proteins are the most important requirements for their any basic research. To the best of our knowledge, maltose-binding protein (MBP) fusion tags are being used to overcome the impediment to their heterologous recombinant expression in *Escherichia coli* as insoluble and bio-inactive inclusion bodies. In the present work, to evaluate the expression efficiency of a cystatin molecule in *E. coli* cells by using MBP tags, the expression of *Celosia* cystatin was studied in two different strains of this bacterium. The quantitative analysis results based on the one-step purification yield of the fused product showed the excellency of the *E. coli* TB1 strain in comparison to *E. coli* DH5α for the high-level production of active product.

DOI: 10.7868/S0555109913040053

Cysteine proteinase inhibitors (termed as cystatins) belong to a proteinaceous group of inhibitors that reversibly inhibit the enzymatic activity of cysteine proteinases [1]. They have been originally identified in mammalians, later on a number of putative cystatin sequences have been characterized from different plants [2–10]. The plant cystatins are generally named as "phytocystatins". Like all members of the cystatin superfamily, phytocystatins contain two conserved regions that interact with cysteine proteinase molecular structures. They include "G" residue at the N-terminus, "QxVxG" and "W" at the C-terminus. Moreover, phytocystatins differ from non-plant types due to the presence of a plant-specific sequence, [LVI]-[AGT]-[RKE]-[FY]-[AS]-[VI]-x-[EDQV]-[HYFQ]-N located in an N-terminal α-helix region [11, 12].

Various physiological and biological roles have been attributed for phytocystatins [13–18]. However, to increase the knowledge regarding the molecular structures and functions of these proteins, the efficient production and purification of their correctly folded three-dimensional structures are needed. These requirements are mostly achieved by the heterologous protein expression as recombinant or fusion products in bacterial cells. Among bacterial expression systems, *Escherichia coli* is the most popular one used for the production of foreign proteins in the form of recombinant products [19]. But, a major impediment to the production of recombinant proteins "included cystatins" in *E. coli* is their aggregation and formation of insoluble and biologically inactive inclusion bodies. Although, they can be converted into native forms, but this is a time consuming, labor-intensive, and so is a costly manner [20]. The best approach is the use of fusion tags that considerably enhance the solubility of the expressed products in the recombinant cells. Among the fusion tags, maltose-binding protein (MBP) tags have been generally considered to be the best fusion partner that not only provide the high level of protein expression, but also efficiently enhance the solubility, folding and stability of the fused proteins. The other attention to MBP tags is given to its rapid expression procedure and its easy and efficient one-step purification method that is done by maltose-affinity chromatography [20–22].

To date, very limited information is available concerning the heterologous expression of phytocystatins either in the forms of recombinant or fusion partners. The use of MBP tags for the expression of plant cystatins was originally and frequently reported from our laboratory [8, 23–25]. These tags were found to minimally affect the fused cystatins inhibitory activity against papain *in vitro*. Among the studied cystatins by our research team, *Celosia cristata* cystatin is the most characterized one that has been shown to be involved in the inhibition of TMV (tobacco mosaic virus)-induced programmed cell death in the host plant [8].

According to the protocol of MBP-based expression systems (New England Biolabs, UK), TB1 strain of *E. coli* is being used for the transformation process [26, 27]. In the present work, as a part of our expression studies with regard to *Celosia* cystatin, we were interested to examine the expression as well as the bioactivity of the same cystatin molecule in two different types of *E. coli* strains (including DH5α and TB1) at the same experimental conditions. The experiments were conducted to identify the *in vitro* activity of the MBP-fused product, the continuity of its expression

process in recombinant cells and the of growth patterns of the test bacterial cultures.

MATERIALS AND METHODS

Materials. DH5α strain of *E. coli* was obtained from Genetic Engineering labratory, Department of Plant Breeding and Biotechnology, University of Tabriz (Iran). *E. coli* strain TB1 and pMALc2X vector for bacterial transformation, recombinant vector construction, and protein expression studies were supplied in the protein fusion and purification system kit (New England Biolabs, UK). RNX^{TM} (-plus) kit (CinnaGen, Iran) was used for total RNA isolation. mRNA purification was done by mini prep mRNA purification kit (Qiagen, USA). RT-PCR reaction was carried out by AcessQuick^{TM} RT-PCR System (Promega, USA). Plasmid vector pGEM-T easy used for polymerase chain reaction (PCR) product cloning was from our laboratory stock. DNA Extraction Kit (Fermentas, USA) was used for the purification of the restricted fragment from the agarose gel. Restriction enzymes *Eco*RI and *Bam*HI used in the cloning procedure (CinnaGen, Iran). All the other chemicals used in this research were of molecular biology grade.

Cloning of cystatin cDNA. In order to clone cystatin cDNA from the leaf tissues of the *Celosia* plant, reverse transcriptase (RT)-PCR method was used (according to the protocol of RNX^{TM} kit). For total RNA isolation, about 0.2 g leaf material was fine powdered using liquid N_2, and 2.0 mL of RNX^{TM} reagent was added to homogenize the powder at room temperature (RT). Next, 200 µL of chloroform was added to the mixture, mixed for 15 s, incubated on ice for 5 min, and centrifuged at 13000 g for 15 min. The upper phase was transferred to another tube, and RNA was precipitated using an equal volume of isopropanol. The pellet was washed by 1 mL of 75% ethanol, dried at RT, and dissolved in 30 µL of RNase-free water. Poly (A^+) RNA was purified from the total RNA by using oligo dT-column according to the manufacturer's protocol.

For RT-PCR reaction, the specific primers were designed based on the already reported cystatin cDNA from *Celosia* plant (accession number: AJ535712) using Primer3 software at http://www.primer3plus.com/web_0.4.0/input.htm. The nucleotide sequences of the synthetic primers were as follows:

right primer:
5'TTCCGAATTCGCAAAAATGAGTTCC3';

left primer:
5'TTCAGGGATCCTTAGTTAGCAACGGC3'

The RT-PCR reaction was performed using one-step AcessQuick^{TM} RT-PCR System (Promega, USA). Aproximately 0.5 µg of each mRNA sample was mixed with 25 µL of master mix (2×) and 1 of µL primer set. The mixture was adjusted with nuclease-free water to a final volume of 50 µL. The reaction mixture was incubated at 45°C for 45 min and subjected to PCR cycling. Amplification was performed in Techneh type thermal cycler (Germany), with 25 cycles of 1 min denaturation at 93°C, 1.5 min annealing at 58°C, 2 min extension at 72°C, ending with 10 min of final extension at 72°C. The amplified products were then extracted from the agarose gel, and then cloned by using pGEM®-T Easy vector system [28]. The recombinant vector separately transformed to TB1 and DH5α strains of *E. coli*. Transformants were then spread on LB plates containing 100 µg/mL ampicillin, 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 40 µg/mL 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal) and incubated at 37°C.

A single recombinant colony was selected from the plate and processed for plasmid extraction using the alkaline lysis method [29]. The isolated plasmid was digested with *Eco*RI restriction enzyme and separated on 0.8% agarose gel. The cloned fragments were sequenced at the Microsynth DNA Sequencing Center (Switzerland).

The nucleotide sequence of the isolated cDNA was analyzed by using BLAST server at http://www.ncbi.nlm.blast.com/.

Expression of cystatin as fused product. The RT-PCR amplification product after agarose gel purification step was digested with *Eco*RI and *Bam*HI restriction enzymes, run on 1% agarose gel, extracted and purified from the gel, and ligated into the pMALc2X expression vector, which had already been linearized at the *Eco*RI and *Bam*HI sites within the multiple cloning region (Fig 1). The ligation mixture was separately transferred to competent *E. coli* TB1 and DH5α cells. For the preparation of competent cells, bacterial cells were grown in Luria Bertani (LB) media. When the OD_{600} reached 0.4, the cells were kept in ice for 15 min, centrifuged at 3500 g for 10 min at 4°C, and washed with 10 mL of 100 mM $CaCl_2$. Subsequently, they were centrifuged at 5000 g for 10 min, resuspended in 2 mL of chilled 50 mM $CaCl_2$, and kept in ice for 12 h. For the transformation of bacterial cells, the all ligation reaction product was added to 25 µL of competent cells, incubated on ice for 5 min, heated to 42°C for 2 min, and incubated at 37°C for 20 min after addition of 0.1 mL LB medium. The transformed cells were plated on LB medium (supplemented with Amp and X-gal) at 37°C, and a recombinant clone was selected for gene expression studies.

Extraction and purification of the expressed fusion protein. In order to extract the fusion protein, transformed cells were grown in 500 mL of rich broth/glucose/ampicillin medium (g/L): tryptone – 10, yeast extract – 5, NaCl – 5, glucose – 2 and sterile ampicillin in concentration 100 µg/mL. For the induction of fused protein expression, IPTG was added to a final concentration of 0.3 mM, and the mixture was incubated for 8 h at 37°C. The cells were harvested by centrifugation at 4000 g for 10 min, and the pellet was dissolved in 25 mL of protein extraction buffer containing 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1 mM EDTA, 1 mM sodium azide, and 10 mM β-mercaptoethanol.

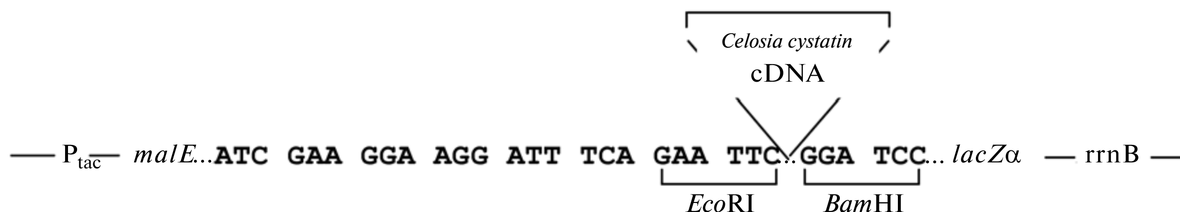

Fig. 1. The clone map of cystatin cDNA on pMALc2X expression vector. The coding region of the isolated cDNA was restricted with *Eco*RI and *Bam*HI restriction enzymes and inserted, with its correct reading frame, downstream of *malE* gene of *E. coli* that resulted in a fusion gene.

The cells were frozen in the same buffer at –20°C overnight and then sonicated with 3 pulses of 15 s. The sample was centrifuged at 10000 g at 4°C for 20 min, and the supernatant was used as crude extract.

The fusion protein was purified from the crude extract by affinity column chromatography. A column packed with amylase resin specific for the maltose-binding protein (MBP), which was a part of fused protein, was used. The fusion protein was eluted out from the column by using the column buffer (extraction buffer containing 10 mM maltose). The eluted product was analyzed for its homogeneity by SDS-PAGE using 10% polyacrylamide gel [30].

***In vitro* inhibitory test of fused protein.** The purified fusion proteins from two different *E. coli* strains were separately tested for their cysteine proteinase inhibitory activity using papain and β-N-benzoyl-DL-arginine β-napthylamide hydrochloride (BANA) as substrates [31]. For each test, a solution containing 0.1 mL of assay buffer (0.5 M sodium phosphate and 10 mM EDTA, pH 6.0), 0.1 mL of 50 mM 2-mercaptoethanol, 0.1 mL of papain solution (25 μg) and 0.2 mL of test solution containing 10 μL purified protein were incubated at 37°C for 10 min. The reaction was started by the addition of 0.2 mL of 1 mM BANA, incubated at 37°C for 20 min and then stopped with the addition of 1 mL of 2% (v/v) HCl/ethanol and 1 mL of 0.06% p-dimethylaminocinnamaldehyde/ethanol. The absorbance of the reaction mixture was measured at 540 nm.

Assessment of recombinant bacterial growth. To determine the effects of the expressed fused products on the growth of the recombinant bacteria, the growth of bacteria was assessed by measuring the OD_{600} of the cultures for 1 h time intervals. Bacterial growth curves were plotted for each test culture and compared to each other.

RESULTS AND DISCUSSION

Priming of cystatin transcript. *Celosia* cystatin transcript had been previously isolated from the leaf tissues by using a degenerate primer set and through the screening of the leaf cDNA library constructed on viral vector system λTriplEX2 [8]. In the present study, this cDNA was amplified from the leaf mRNA population by AcessQuick™ RT-PCR System using a specific primer pair designed based on the previously reported sequence.

Separation of the RT-PCR end product on the agarose gel and the analysis of the nucleotide sequence of the amplified cDNA revealed that the size and the sequence of the primed cDNA consisted of an open reading frame that was completely identical to the previously probed cystatin (Fig. 2; agarose gel photograph and the sequence alignment result not presented). The nucleotide and the correct cystatin open reading frame between *Eco*RI and *Bam*HI restriction sites are shown on the figure.

Differential expression and purification of cystatin. For the expression of *Celosia* cystatin cDNA, its coding region (as presented in Fig. 2) was inserted between *Eco*RI and *Bam*HI restriction sites of the expression vector pMALc2X, downstream of the maltose-binding protein sequence to yield fused *mbp*-celostatin. The recombinant vector was separately transformed into two different *E. coli* strains TB1 and DH5α using the same experimental materials and methods. The expression of the fused product was induced by IPTG and analyzed in the soluble fraction of the both test cultures. SDS-

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—————————————————————————————— ttcgcaaaa
——————————————————————————————— N  A  K
atgagttcctctaacaatcttgctggtggttggttcccagttgat
 M  S  S  S  N  N  L  A  G  G  W  F  P  V  D
cccaatagtccaaaaatccaaaagcttgctaggtgggctgttgac
 P  N  S  P  K  I  Q  K  L  A  R  W  A  V  D
gaggagaacaaaaaacctagtgcttataagttggagtacaaagga
 E  E  N  K  K  P  S  A  Y  K  L  E  Y  K  G
actttcaaagctgaagaacaaatagttgaagctaggaattcccgt
 T  F  K  A  E  E  Q  I  V  E  A  R  N  S  R
attagtcttgaggcagttcgagttcctttcgcagcaagtaataaa
 I  S  L  E  A  V  R  V  P  F  A  A  S  N  K
gaatggcacaagtatcaggcaatagtttatgaggatttgaacaac
 E  W  H  K  Y  Q  A  I  V  Y  E  D  L  N  N
aatttagagttgaaggagttcaagcctttgttgcaagccaatgat
 N  L  E  L  K  E  F  K  P  L  L  Q  A  N  D
gatgaatgtattgccgttgctaactaaggatcc ———————————
 D  E  C  I  A  V  A  N  * ————————————————
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Fig. 2. RT-PCR end product analysis of the primed cDNA. RT-PCR reaction was performed using one-step AcessQuick™ RT-PCR System using the specific primers designed based on the already reported sequence from *Celosia*. To confirm the amplification, the fragment was cloned and sequenced. The sequence result between *Eco*RI and *Bam*HI sites and the position of the primers are shown.

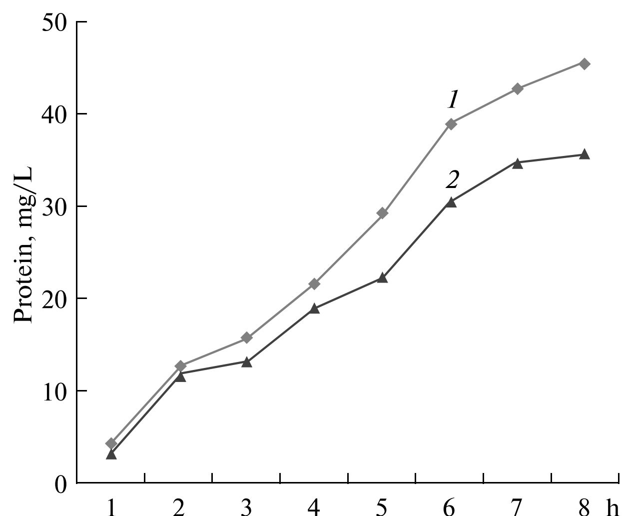

Fig. 3. Analysis of the fused protein expression. By using the same experimental procedures, the isolated cDNA was separately expressed in *E. coli* strains TB1 (*1*) and DH5α (*2*) in the presence of IPTG. The protein expression data represent the means of three replicates.

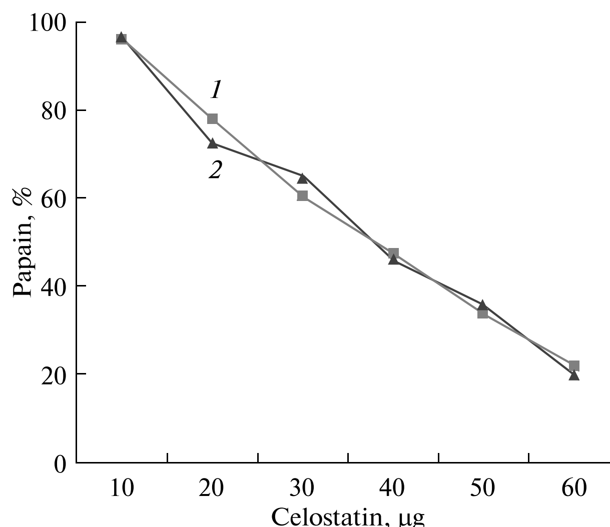

Fig. 4. Assesment of the inhibitory activities of fused protein. By using spectrophotometeric method, the inhibitory activities of the purified MBP-fused celostatin were estimated from *E. coli* strains TB1 (*1*) and DH5α (*2*).

PAGE analysis of the total soluble proteins confirmed the presence of the observable and detectable expressed product in both extracts (gel photograph not presented).

Purification of the fused cystatin by using single step MBP-affinity chromatography revealed the purification yields of about 46 and 37 mg/L of the *E. coli* TB1 and DH5α cell cultures, respectively (Fig. 3). The difference between the expression levels of fused cystatin was estimated to be about 10 units between the two cell types, 8 h after incubation. To evaluate the expression process during the induction period in each test culture, a time course experiment was performed. The results showed that the expression of the fused cystatin was linearly correlated with the time of the induction. This experiment result confirmed that the expression of the fused product was continuously induced in each of the tested strains. A continuous and linear correlation was observed between the expression process and the time of the induction in both strains of *E. coli*. Data analysis also revealed that the distance between the two trend lines had an increasing pattern, reached to the maximum after 8hrs incubation time (Fig. 3). In agreement with these results, the continuous expression patterns of MBP-fused cystatin molecules been previously reported [23, 24]. However, the increasing differential expression levels in two different bacterial strains are more interested result and it remains to be identified in detailed examinations.

Based on the obtained results, TB1 strain *of E. coli* is suitable for the conducting of fusion expression and purification studies of *Celosia* cystatin molecule, as compared to DH5α.

***In vitro* inhibitory activity of fused cystatin.** To identify the inhibitory activities of the expressed cystatin in both *E. coli* strains, the expressed product was separately purified from both strains and their inhibitory activities were examined. The results showed that papain activity decreases with the increasing concentrations of fused cystatin in both tested strains, sharing the same trend line (Fig. 4). These results confirmed that the inhibitory activity of the expressed cystatin not affected by its differential expression in two different cell types. On the other hand, MBP-fused product expresses in correct conformation in both strains.

The present results are consistent to our previous reports, in which it has been shown that MBP tags not affect the inhibitory activities of the fused cystatin molecules [8, 23–25].

Bacterial growth analysis. The growth rates of the both recombinant bacteria were evaluated by measuring OD_{600} for 1 h time intervals, under induced conditions. Comparison of the growth curves of both the test strains indicated that there are no significant differences in their growth (Fig. 5). This result also confirmed that the expression of *Celosia* cystatin protein has no inhibitory effect on the growth rates of the tested strains.

In the present study for the first ever time, the expression of *Celosia* cystatin was analyzed and compared between the two different strains of *E. coli*, TB1 and DH5α. The overall results showed that the expression levels of fused protein differ in tested strains. However, the expression patterns as well as the activities of the differentially expressed cystatin do not change during the expression period. A question rose as how the expression affected by DH5α cells. The continuous expression and the similarity of the growth curves suggested that the expression not affected by the induction of the recombinant cells, but it is contin-

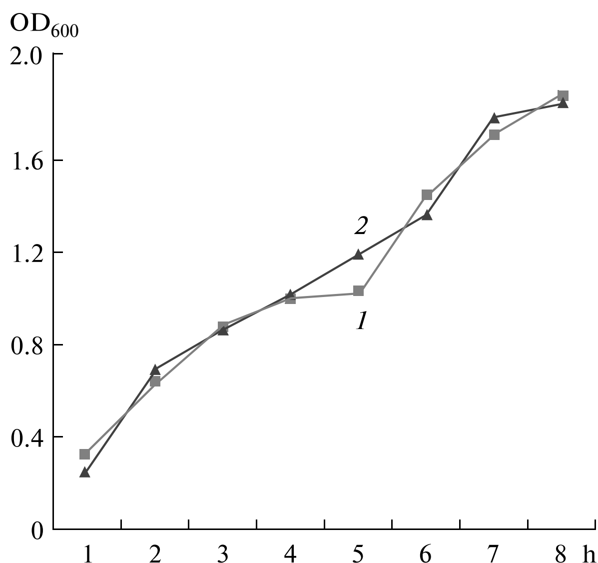

Fig. 5. Assesment of bacterial growth. Recombinant TB1 (*1*) and DH5α (*2*) cells were separately under the same experimental conditions and the growth of bacteria was assessed by measuring OD_{600}. OD values represent the means of three replicates.

uously regulated during the growth of the bacteria. However, this continuous regulation might be different in two *E. coli* strains, resulting in the different expression levels. Therefore, further studies are warranted to determine how the expression levels of the same fusion protein are altered in two different strains of *E. coli* cells when the same expression vector system and the same experimental conditions are used.

ACKNOWLEDGMENTS

The author of this paper is thankful to the Research Institute for Fundamental Sciences (University of Tabriz, Tabriz, Iran) for financial support.

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