DIFFERENTIAL EXPRESSION OF A CYSTEINE PROTEINASE AND CYSTATIN PAIR AS SIDE-BY-SIDE FUSION FORMS IN *ESCHERICHIA COLI*

A. GHOLIZADEH

Research Institute for Fundamental Sciences (RIFS), University of Tabriz, Iran
E-mail: aghz_bioch@yahoo.co.in

As a basic study, the fusion expressions of two functionally related proteins were described. The side-by-side fusion construction, expression, purification and functional characterization of Arabidopsis papain-like cysteine proteinase (CP) and cysteine proteinase inhibitor (CPI) were successfully carried out by using an Escherichia coli expression system without affecting the recombinant bacterial growth. The purification products of two different fused constructs designated as «R1: H_2N-maltose binding protein-CPI-CP-COOH and R2: H_2N-maltose binding protein-CP-CPI-COOH» showed inverse enzymatic/inhibitory activities, in vitro. Analysis of the constructs by using computational tools revealed that the arrangement of CP/CPI pair in fusion forms might be the important criteria for proper tertiary organization, structural folding and functional property. The overall results showed that the C-terminally located molecule could be the active folded structure in each fusion construct. The achievements of the present work may be utilized in a specific protein engineering application such as manufacturing the novel switchable expression systems in the future.

Keywords: *Arabidopsis, Cysteine proteinase, Cystatin, Fusion protein, Papain.*

Introduction. Cysteine proteases are known as a major class of proteolytic enzymes found in different organisms including viruses, bacteria, yeasts, protozoa, helminthes, insects, mammals and plants. They hydrolyze various proteins and play important role in degradation and turnover of a wide range of intracellular proteins. Based on the kind of organism or its organ, CP enzymes share in total cellular proteolysis between 30 to 90 % [1]. Plant CP are classified according to their structural and evolutional relationships into different families including caspases, papain-like proteases, calpains, streptopains, clostripains, calcium-dependent proteinases, ubiquitin C-terminal hydrolases and ubiquitin-specific proteinases [2]. However, the largest one of these families is most commonly exemplified by papain, a well described plant enzyme isolated from the latex of *Carica papaya* fruit [3].

In *Arabidopsis thaliana* plant papain-type CP themselves are classified into eight groups including: 1) senescence-induced, 2) stress-induced, 3) aleurain type, 4) cathepsin-b like, 5) bromelain-like, 6) KDEL type, 7) telo sequences, and 8) actinidain-like [4]. They are generally synthesized as small preproproteins of about 40–50 kDa that are proteolytically processed to mature active enzymes with molecular sizes of about 22–35 kDa [5].

Usually, the cells or the whole organisms use different strategies to protect themselves against undesired proteolysis. One of the common strategies is the control of proteolytic activity by inhibition process. It has been well understood that the CP-based proteolysis is regulated by the activity poised between the CP and CPI «the proteinaceous inhibitors referred as cystatins» [6, 7]. Cystatins constitute the largest and well characterized group of natural CP inhibitors in plant system [8]. These inhibitors have been known to bind near the protease active site, obstruct the substrate access, but not directly interfere with the catalytic site of enzyme molecule [9]. It has also been understood that cystatins mostly direct their inhibitory roles against the papain-type superfamily members. A survey of papain-type CP and CPI in plants has shown that the functionality of both families of proteins might be as a result of a coevolutionary process [10].

Using gene expression technologies, plant CP and CPI have been suggested to be contributed in many biological processes in a regulative manner. They have been found to be mostly involved in protein turnovers, PCD (programmed cell death) and degradation of proteins as biochemical response to several internal developmental shifting and external stressful environments. Tissues wounding, elevated temperatures, drought stress, salinity and various diseases have been reported to alter the gene expression patterns of CP and CPI [11, 12]. The activities of CP and CPI genes have been shown to be regulated in senescing and developing plants as well as in germinating seeds [13]. Recently, by using the bimolecular fluorescent complementation data

analysis, it has been reported that the functional interactions between CP and CPI molecules determines their mutual participation in specific biological pathways including anabolic and catabolic processes, different signaling routs and responding to various biotic and abiotic environmental cues throughout the plant life [10].

To date, several CP and CPI have been produced in various heterologous expression systems. Using the popular *Escherichia coli* expression systems, CP of *Carica papaya* [14], *Homo sapiens* [15], *Leishmania mexicana* [16], *Porphyromonas gingivalis* [17], *Periserrula leucophryna* [18] *Plasmodium knowlesi* [19], *Zea mays* [20], and CPI of *Celosia cristata* [21], *Saccharum officinarum* [22], *Hordeum vulgare* [23], *Zea mays* [24] have been expressed and purified as either active or inactive products. In all these reports, the expression and molecular characterization of CP or CPI proteins have been carried out, independently. On the other hand, they have not been studied together either as fused partners or non-fused coexpressed forms in any heterologous system, so far. Coexpression or simultaneous expression of two or more genes is the most popular technique used for the study of different protein-protein interactions. However, no attention has been given to the gene fusion technologies for the interactive functional studies, so far. Usually, gene fusions result in fusion proteins having the combined properties of the original gene products. These fusion forms have now many different applications in widespread areas of biotechnology [25, 26]. Despite this, they have not been used as a basic tool and model to study the interactive structures and functions. Therefore, we predicted that the use of fusion technology may be as a different approach to study the protein-protein interactions.

In this study, considering the possible interactive and functional fusion forms of a CP/CPI pair, we describe the cloning and side by side fusion construction and expression of *Arabidopsis* papain-like cysteine proteinase (GenBank accession no. At3G54940) and cystatin (GenBank accession no. AF411786) in *E. coli* cells. To analyze the functionality of the constructs, the fused proteins were purified and their enzymatic/inhibitory activities were assessed *in vitro*. The obtained experimental data were simultaneously analyzed and discussed by using the bioinformatic tools and comparative structural analysis to help understand about the tertiary organizations and the relative roles of a cysteine proteinase and its inhibitor in differential fusion forms.

Materials and methods. *Materials. Arabidopsis thaliana* plants provided by Dr. B. Baghbankohnehrouz (Genetic Engineering Lab, Plant Breeding and Biotechnology Department, Tabriz University, Iran) and grown under normal laboratory conditions. Trizol reagent (Cat. no. RN7713C; RNX™; Fermentas) was used for total RNA extraction. mRNA purification was carried out by using mRNA mini prep kit (Cat. no. 70022; QIAGEN). Acess-Quick™ RT-PCR System (Cat. no. A1701; Promega) was provided for reverse-transcription PCR reaction. pGEM-T easy plasmid vector used for the cloning of RT-PCR end product was from Promega. For purification of the DNA fragments from the agarose gel DNA Extraction Kit (Cat. no. K0513; Fermentas) was used. *E. coli* strain DH5α was from laboratory stock. Bacterial transformation was carried out by using *E. coli* strain TB1 provided in protein fusion and purification kit (Cat. No. E8000S; NEW ENGLAND, BioLab). Plasmid pMALc2X vector for recombinant protein expression studies were supplied in protein fusion and purification kit. Restriction enzymes were from CinnaGen. All the other chemicals were of molecular biology grade.

Cloning of CP and CPI coding regions. To clone CP/CPI cDNAs from the leaf tissues of *Arabidopsis* test plant, reverse-transcription PCR method was performed. For total RNA extraction, 0.2 g of test material was finely powdered by using liquid N_2 and homogenized in 2 mL of Trizol reagent. It was followed by addition of 200 μL chloroform, mixing for 15 sec, incubation on ice for 5 min and centrifugation at 13.000× g for 15 min. RNA in the upper phase was precipitated by the equal volume of isopropanol. The pellet was washed using 75 % ethanol and dissolved in 30 μL RNase-free water. Purification of poly(A^+) RNA population from total RNA was done by using oligo dT-columns according to the protocol of kit manufacturers. To perform the RT-PCR reactions, the specific primers were synthesized on the basis of previously reported CP and CPI sequences (GenBank accession nos. At3G54940 and AF411786, respectively) from *Arabidopsis* plant. For the designing of primer sequences, Primer3 software at http://www.primer-3plus.com/web_0.4.0/input.htm was used. In order to directionally clone the amplified fragments in the

expression vector, the specific restriction sites were included at the 5′ ends of each primer pair:

R1 CPI	F: 5′aggagaattcgaaaatggcggatcaacaag3′
	R: 5′gatatctagaacatcgtgatggtggttgaa3′
R2 CPI	F: 5′aggaggatccgaaaatggcggatcaacaag3′
	R: 5′gatagtcgacacatcgtgatggtggttgaa3′
R1/R2 CP	F: 5′caactctagaaagacctcaccatacgccaa3′
	R: 5′gttgggatccggaaacttgggtggctactg3′

The reactions of RT-PCR were performed according to the protocol of one-step AcessQuick™ RT-PCR system. For each RT-PCR reaction, a mixture of test mRNA sample (0.5 μg) with 25 μL of Master.

Mix and 1 μL of primer pair was prepared. The mixtures were then adjusted to a final volume of 50 μL, incubated at 45 °C for 45 min and finally subjected to PCR cycling program:

R1/R2 CPI	Denaturation	93 °C/30 sec
	Annealing	55 °C/2 min
	Extension	72 °C/1 min
	Final extension	72 °C/10 min
R1/R2 CP	Denaturation	93 °C/1 min
	Annealing	56 °C/1.5 min
	Extension	72 °C/2 min
	Final extension	72 °C/10 min

The amplification end products were separated on 1 % agarose gel and extracted using DNA gel extraction kit. The extracted fragments were cloned in pGEM-T easy PCR product cloning vector and transformed to *E. coli* strain DH5α [27]. Transformed bacteria were separately grown on IPTG/X-gal containing plate and a single white colony was selected from each one. The selected clones were processed for recombinant plasmid extraction using the previously reported alkaline lysis method [28]. Each cloned fragment was partially sequenced at DNA Sequencing Center of Microsynth, Switzerland.

Construction of CP/CPI fusion forms. The purified CP and CPI fragments from agarose gel were restricted by *Eco*RI, *Bam*HI, *Xab*I, and *Sal*I restriction enzymes and then ligated to each other in a directional manner using T4 DNA ligase. Two different linear fusion constructs «designated as H_2N–CP–CPI–COOH and H_2N–CPI–CP–COOH» were prepared, run and extracted from the gel material to proceed it for the next expression studies (Fig. 1).

Expression of fusion constructs in E. coli. Two set of pMALc2X expression vector were separately linearized at *Eco*RI – *Bam*HI and *Xab*I – *Sal*I restriction sites within their multiple cloning regions. The purified CP/CPI fused fragments from the agarose gel were directionally ligated into the already linearized expression vectors according to the protocol of kit manufacturers. The recombinant vectors were separately transferred to TB1 competent cells. The transformed bacterial cells were plated on the medium supplemented with Amp and X-gal at 37 °C overnight, and a white clone was selected from each plate for the next studies.

Assessment of recombinant bacterial growth. The effects of the expressed fused products on the rates and patterns of the recombinant bacterial growth were determined, spectrophotometrically. To do this, the growth of bacterial cells carrying CP–CPI and CPI–CP fusion constructs was assessed by the measurement of the cell culture OD at A_{600} at time intervals of 1 h. The overall experimental period of 8 h was considered for the growth assessments. The growth curves of each recombinant clone were plotted and compared to the non-recombinant cell culture as control sample.

Purification of fused CP/CPI products. For the extraction of fusion proteins, the recombinant cells were grown in 500 mL of rich broth medium supplemented with glucose and Amp. The expression of fused product was induced by the addition of IPTG (0.3 mM final concentration) for an incubation period of 8 h. The cells were then harvested and precipitated by centrifugation at 4,000× g for 10 min, followed by dissolving the cell pellet in 25 mL of extraction buffer consisted of 20 mM Tris-Cl, 200 mM NaCl, 1 mM EDTA, 1 mM sodium azide, and 10 mM BME. For sonication purpose, the harvested bacterial cells were frozen in the same extraction buffer at −20 °C and then they were sonicated in short pulses of 15 sec. The sonicated sample was finally centrifuged at 10.000× g at 4 °C for 20 min, and the obtained clear supernatant was used as crude protein extract of test recombinant bacterial cells.

The purification processes of the fused proteins were separately carried out from the crude extracts by using the single-step affinity column chromatography. For this purpose, columns having the dimensions of 2.5 cm × 45 cm were packed with amylase resins that were specific for the maltose-binding protein as a constant fusion partner of test products.

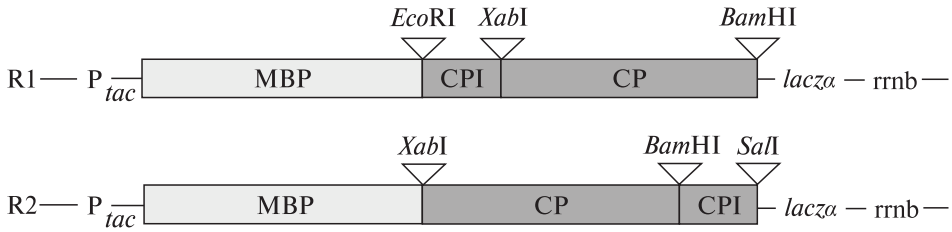

Fig. 1. Construction of fused products. The RT-PCR end products containing CP/CPI cDNAs were directionally cloned in pMALc2X expression vector. The schematic cloned maps of fused R1 (MBP–CPI–CP) and R2 (MBP–CP–CPI) constructs were presented

The bound MBP-fused CP/CPI pairs were separately eluted out from the amylase column by using the specific elution buffer consisted of the crude protein extraction buffer plus 10 mM maltose. The homogeneities of the eluted products were analyzed by separating them on 10 % polyacrylamide gel using SDS-PAGE [29].

Assessment of enzymatic/inhibitory activities. The eluted fusion proteins from amylase columns were subjected to cysteine proteinase/inhibitory activity test using BANA as substrate [30]. Solutions containing 0.1 mL of reaction buffer (consisted of 0.5 M sodium phosphate and 10 mM of EDTA, pH 6.0), 0.1 mL of 50 mM BME, and 0.3 mL (30 μg) of fusion proteins were separately incubated at 37 °C for 10 min. The reactions were then started by 0.2 ml of 1 mM BANA addition and incubation at 37 °C for 20 min. The stopping of the reactions were carried out with 1 ml of 2 % (v/v) HCl/ethanol and 1 ml of 0.06 % DMACA/ethanol. The relative proteinase activities of the test samples containing R1 and R2 products were separately assessed by measuring their absorbance at 540 nm presented as percentage data on the graph.

Computational analysis. The sequence analyzing BLAST (basic local alignment search tool; http://www.ncbi.nlm.blast.com) was performed for the nucleotide and deduced amino acid sequence analysis of the isolated cDNA fragments. The existence of the SP (signal peptide) in the protein sequences was predicted by SignalP software at http://www.expasy.ch/. Protein tertiary structural predictions were done using threading method by Discovery studio and Phyre v 2.0 servers. Solvent accessibilities and Electrostatic potentials of the fused constructs were predicted by SAS method using Discovery studio server.

Results. *Selection of a CP/CPI pair.* A CP/CPI pair was selected by a survey of cysteine proteinase and cystatin gene/protein sequences from *Arabidopsis thaliana* database. The CP/CPI pair (accession no: At3G54940 and AF411786, respectively) was selected according to their potent functional interactive structures computed by very sensitive threading method (Fig. 2, *a*). Using molecular docking analysis data, the interaction energy between the test CP and CPI molecules was predicted to be 19559.2 kcal/ mol.

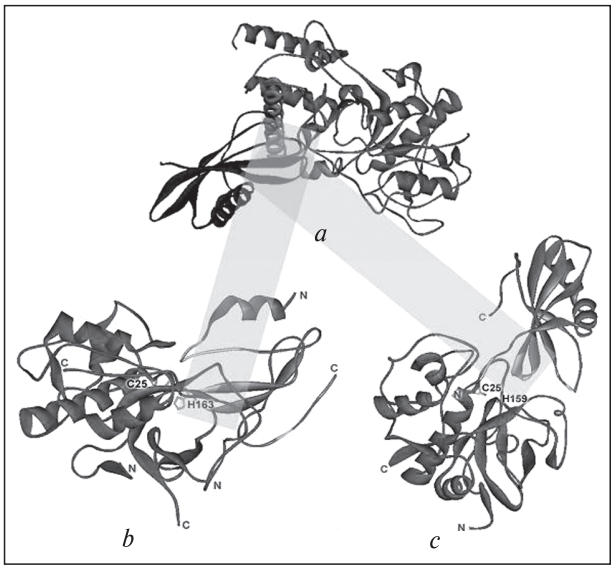

Fig. 2. Interaction mode of CP/CPI: *a* – detection of molecular interaction between test CP and CPI; *b* – comparison of test CP/CPI pair with Cathepsin-p41 interaction mode; *c* – comparison of test CP/CPI pair with papain-stefin B interaction mode

Comparison of the predicted interactive CP/CPI pair with the previously reported ribbon drawn inhibitory complexes between proteinaceous inhibitor molecules and different cysteine proteinases

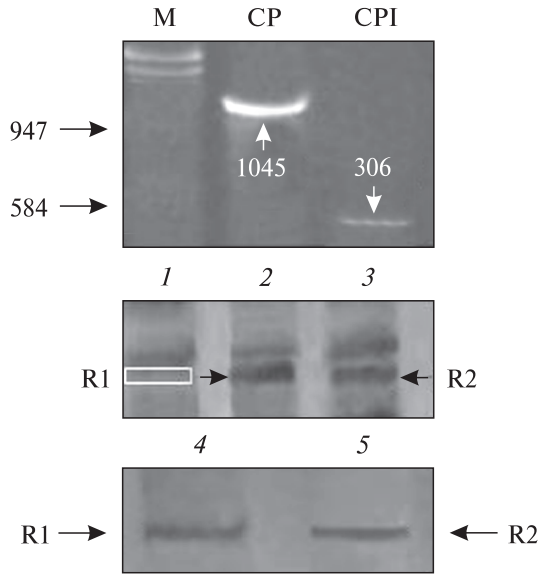

Fig. 3. Cloning and expression analysis of fused products. Upper – agarose gel analysis of RT-PCR end products; Middle – SDS-PAGE analysis of non-fused vector carrying bacteria (*1*), crude protein extract of bacterial cells harboring the R1 construct (*2*), crude protein extract of bacterial cells harboring the R2 construct (*3*); Lower – purified CP activities of R1 (*1*) and R2 (*2*), SDS-PAGE analysis of purified R1 (*4*) and R2 (*5*)

including Procathepsin L, zSpe B, Caspase 8-p35, Papain-Stefin B, Cathepsin L-p41, Caspase 3-BIR2 domain, Staphopain B and Staphostatin B [31] revealed that the selected *Arabidopsis* CP/CPI pair interaction mode is similar to papain-stefin B and cathepsin-p41 intermolecular complexes with their inhibitors (Fig. 2, *b*, *c*). Besides this, our comparative structural studies also revealed that the predicted interactive model for the test CP/CPI pair is similar to the already suggested exositic-type of interaction mode in plant system of papain/cystatin pair.

Molecular cloning and constructing of fused CPI-CP/CP-CPI. RT-PCR based cloning of CP/CPI transcripts was carried by using different types of primer pairs containing the specific restrictions sites for directional cloning. For this purpose, CP and CPI transcripts were amplified using one and two primer pairs, respectively (Fig. 3). Papain-type proteases are generally synthesized as inactive precursors and then produced as mature active enzymes after a proteolytic removal of pro and signal sequences. Therefore, to clone the mature sequence of the test CP, the presence of the pro and signal sequences were predicted by using online signal P-4.1 server. A signal peptide comprising of 22 amino acids along with a cleavage site between positions 22 and 23 were detected on the test proteinase molecule (computed graph not presented). Accordingly, the amplification of test CP was carried out by using a specific 5' primer sequence designed based on the predicted mature sequence.

The cloned sequences after site-specific restrictions with *Eco*RI, *Xab*I, *Bam*HI and *Sal*I enzymes were proceeded for the fusion construction of CP/CPI pairs. The restricted CP and CPI fragments were ligated in two different forms designated as «H_2N-MBP-CPI-CP-COOH and H_2N-MBP-CP-CPI-COOH». The first represents the fusion of inhibitor molecule at the N-terminal end of the proteinase, while the second represents the fusion of inhibitor on the C-terminal side of the proteinase. The ligated products were then directionally and separately cloned into pMALc2X *E. coli* expression vector in which gene of interest is C-terminally fused to the maltose-binding protein gene, *mal*E. Therefore, the final designated MBP-fused maps of CP/CPI were named as R1 and R2 (Fig. 1).

Differential functional expression of CPI/CP/CP-CPI constructs in E. coli. The MBP-tagged fusion expression of the R1 and R2 products in TB1 strain of *E. coli* cells was induced by adding IPTG to the culture medium and subsequently the soluble protein extracts of the recombinant bacteria were separately prepared and analyzed by SDS-PAGE (Fig. 3). Gel analysis confirmed the presence of the expressed products with the similar molecular weights of about 80 kDa that were consistent to the calculated sizes for the expected products. The expressed R1 and R2 fusion proteins were purified by single step maltose-affinity chromatography with the purification yields of about 34 and 29 mg/l of bacterial cell cultures, respectively. The purification yield of R1 product was slightly higher than R2.

To determine the effects of expressed fused products on the rates and patterns of bacterial growth, the optical densities of the bacterial cultures were measured at 600 nm during the incubation period of 8 h. The results revealed that growth rates and patterns of the R1 and R2 carrying recombinant bacteria are similar during the induction period. On the other hand, these bacteria do not show differential growth patterns during the experimental period (Fig. 4).

To test the functionality of the fused products *in vitro*, their proteinase activities were separately exam-

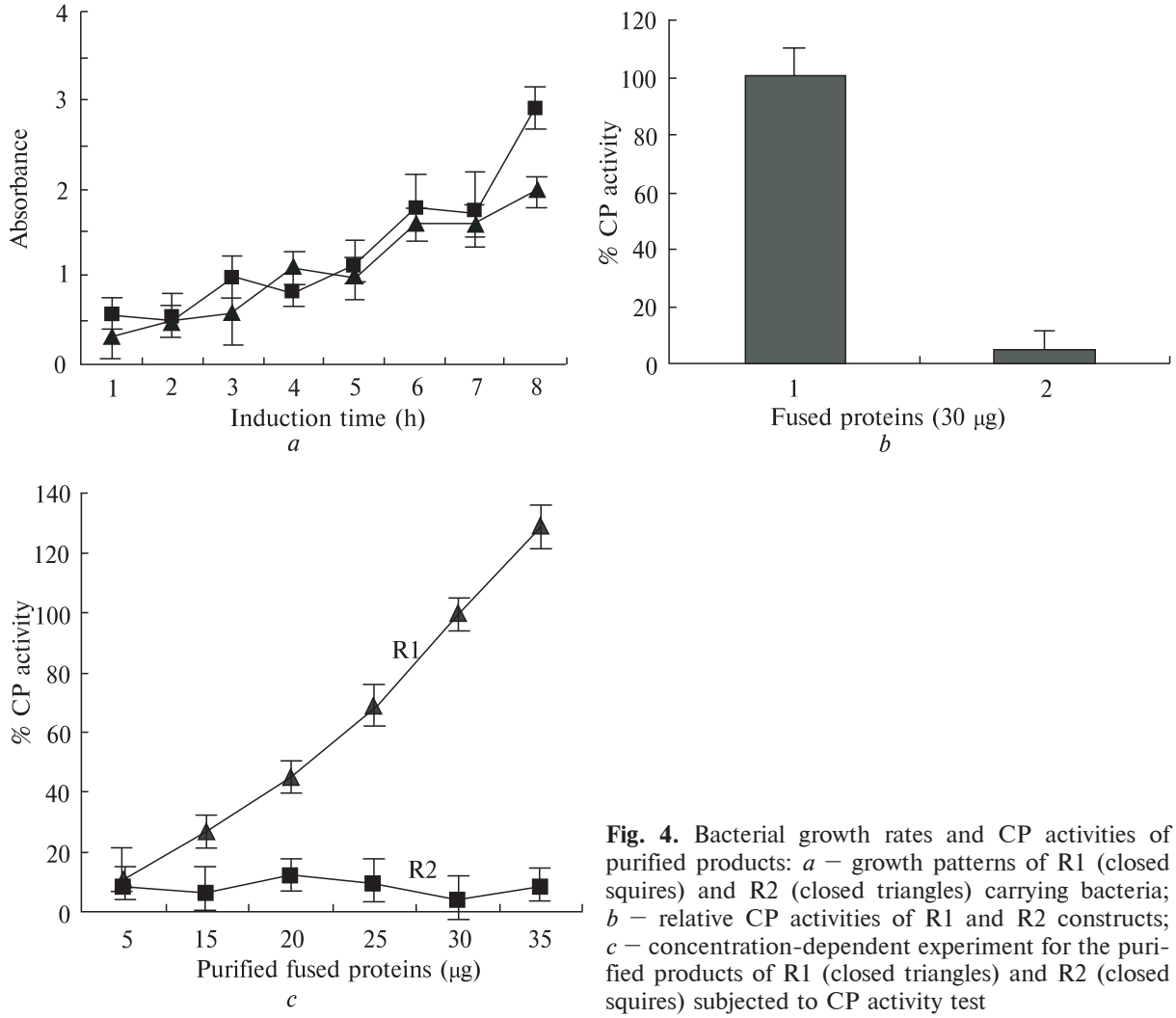

Fig. 4. Bacterial growth rates and CP activities of purified products: *a* – growth patterns of R1 (closed squires) and R2 (closed triangles) carrying bacteria; *b* – relative CP activities of R1 and R2 constructs; *c* – concentration-dependent experiment for the purified products of R1 (closed triangles) and R2 (closed squires) subjected to CP activity test

ined towards BANA synthetic substrate as described in materials and methods. The results indicated that proteinase activity of the purified fused CP is about 95 % decreased in R2 product as compared to R1. On the other hand, the fused CPI molecule exhibits about 95 % inhibitory activities towards CP in R2, but not in R1 (Fig. 4).

Concentration-dependent experiment showed a steady increase in CP activity as the concentration of purified R1 product increased between 5 to 35 units (Fig. 4). In contrast, no CP activity was considerably detected in the samples containing different concentrations of purified R2 product.

Computational characterization of CPI/CP/CP-CPI constructs. Parallel to the experimental approaches, the computational methods were also performed. Analysis of the predicted three dimensional structures of R1 and R2 constructs and comparison of CP/CPI molecular interactions in fused and non-fused forms by discovery studio server revealed that inter-molecular interactions of CP/CPI partners in R2 fusion form is more similar to non-fused type in which CPI inhibitory activity is predicted towards CP enzyme (Fig. 5). Besides this, R1 and R2 solvent accessibilities as well as their electrostatic potentials were predicted and compared (Fig. 5). Computed data showed that R2 construct has less solvent accessibility (−13624.68 against −13443.098), but more electrostatic potential (−13862.6841 against −14113.181) in compare to R1. The computational characterization results of R1 and R2 constructs were summarized and presented in Table.

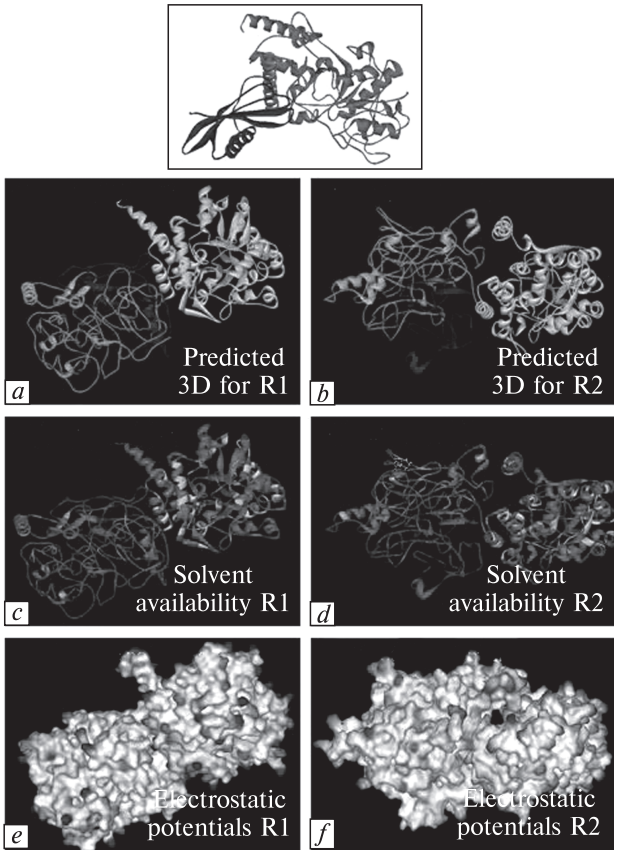

Fig. 5. Computational prediction of CP/CPI molecular interactions: *a*, *b*, *c*, *d* — molecular interaction of non-fused CP/CPI pair using discovery studio server; *e*, *f* — predicted three dimensional structures, solvent accessibilities and electrostatic potentials for R1 (*e*) and R2 (*f*) using discovery studio server

For further structural organization characterization, the three dimensional structures of R1 and R2 constructs were predicted by a different online protein structure prediction server, Phyre 2. Similar to the discovery studio outputs, the Phyre 2 results also confirmed that the structural organization of R2 is more similar to that of non-fused interactive form of CP/CPI pair in which CP enzymatic activity is predicted to be inhibited by CPI (Fig. 6).

Discussion. In the present study, we aimed to investigate the side-by-side fusion expression of a CP/CPI pair in an *E. coli* expression system. Previously, various CP or CPI proteins from different sources had been separately expressed and characterized using different *E. coli* expression systems [15, 18–21, 24]. However, as a different type of research, we conducted our experiments to clone and express CP and CPI proteins together as side by side fusion partners. We thought that the fusion expression of functionally related proteins such as CP and CPI may enhance our information on interactive structures. To examine this, the most popular interactive structure of *Arabidopsis* papain-type proteinase and inhibitor molecules was selected as experimental material. The main target of plant cysteine proteinases, papain-like subfamily, is the most thoroughly studied among plant CP enzymes. The papain-like proteinases consists of two interacting subdomains that delimitating the active site cleft at the surface where substrates can be bound [3]. Among various proteinase inhibitors, cystatins constitute the largest natural cysteine proteinase inhibitors directed against the papain superfamily members. They are known as exosite binding inhibitors that bind adjacent to the papain-type protease active site, and exert their inhibitory activity by obstructing the access of substrate, but not through the direct interaction with the enzyme catalytic site [31]. In agreement with the exositic interaction mode of papain/cystatin pairs, was the present predicted interactive structure of test CP/CPI pair from *Arabidopsis* plant (Fig. 2).

Computational characterization of R1 and R2 constructs (Force field CHARMm)

Model	Initial Potential Energy	Potential Energy	Van der Waals Energy	Electrostatic Energy	Initial RMS Gradient	Final RMS Gradient	Solvation Energy (Polar contri-bution), kT
	kcal/mol				kcal/mol · Angstrom		
R1	546675.86522	−15971.9429	−6532.9428	−14113.181	12916.880	0.76071	−13443.098
R2	442069.85122	−15570.2351	−6375.0249	−13862.6841	9740.3945	0.97086	−13624.68

R1/R2 Minimization Criteria: CONJUG> Minimization exiting with number of steps limit (200) exceeded. Total area/energy for R1 and R2: 35485.27 and 36980.w91.

The use of MBP tags for the expression of various plant cystatin molecules had been originally reported from our laboratory [22–24]. These tags had been found to minimally affect the inhibitory activity of the fused cystatins. Despite this, there was no report to our knowledge concerning the heterologous expression of cysteine proteinases with the use of MBP tags, as yet. As a first attempt, the heterologous expression of a cysteine proteinase along with its inhibitor molecule was tested in the fused form by using MBP carrying *E. coli* vector. Using MBP-based vector, we successfully produced R1 and R2 constructs as soluble products in *E. coli* cells. Previously, by using different types of *E. coli* expressing systems, the recombinant forms of cysteine proteases or theirs inhibitors had been mostly produced as insoluble inclusion bodies in bacterial cells [18, 22, 32, 33]. Rarely, the soluble non-toxic expressions of cysteine proteinases/cysteine proteinase inhibitors had also been recently reported in bacterial cell environments [20, 33]. In this regard, our present results revealed that growth rates and patterns of the R1 (H_2N-MBP-CPI-CP-COOH) and R2 (H_2N-MBP-CP-CPI-COOH) containing recombinant bacteria were similar during the induction period, indicating the non-toxic effects of the fused products for the recombinant bacterial cells. This might be due to the generation of non-correct conformations of CP enzymes as fused forms, or it could be due to CP enzymes inhibitions by the fused CPI inhibitor molecules. Alternatively, the key proteins of recombinant bacteria might not be degraded by the correctly folded CP enzymes generated as fused forms in cellular environments.

To speculate the role of each partner in the expressed R1 and R2 products, we tried to identify their structural organizations using computational methods and generate information regarding the relative function of each partner as well as the role that they may perform, *in vivo*. The computed results showed that the fusion of CPI molecule at the N-terminal end of the CP molecule (R1 construct) may affect the correct conformation of inhibitor, thereby no inhibitory activity towards CP enzyme is detected in R1 product. On the other hand, the fusion of CPI at the C-terminal part of the CP molecule may not affect its folding, so that the inhibition of CP enzymatic activity is considerably detected in R2 product. These computational results are so interesting and not only highlight the position-dependent folding of CPI in two different constructs, but also confirm the active structure of CPI in R2 rather than R1.

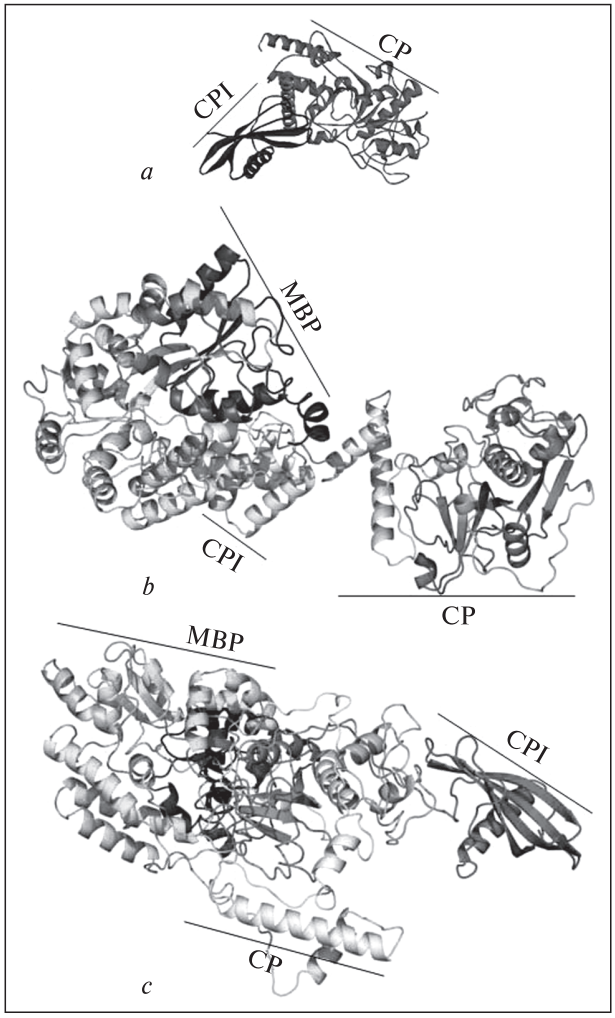

Fig. 6. Prediction of R1 and R2 organizations: *a* – interaction mode of non-fused CP/CPI pair; *b* – interaction mode of R1 product, *c* – interaction mode of R2 product predicted by phyre 2 server

According to the computational data, it is speculated that the normal growth of bacteria harboring the R2 construct most likely might be due to CP enzyme inhibition. While, the reason for the normal growth of bacteria harboring the R1 construct could be due to the non-degradation of cellular key proteins by R1-type fused CP, *in vivo*. It is generally believed that the rate of protein degradation in bacterial cells is increased by the starvation of a required nutrient, or that the expressed proteins

may be detrimental to cell growth. Thereby, one can assume that the expressed R1-type CP might become active after long time bacterial incubation by the starvation of media requirements.

Conclusion. This study, for the first ever time report, presented the fusion construction of two functionally related proteins is presented. A plant-type cysteine proteinase and cystatin pair was considered as test molecules. The parallel experimental and computational analysis data provided important clues about their interactive structures and functions as fused partners. The achievements of the present work may provide further information about the determinants of molecular folding and functional interactions and may be used in a specific protein engineering application in the future. The organizations and the functionalities of each fused partner in R1 and R2 constructs are predicted to be influenced by several internal or external factors. Therefore, it is proposed that the fused forms of functionally related proteins may be used as biotechnological tools to make the novel switchable expression systems. As a specific case, manufacturing a biological system expressing the fused forms of CP/CPI pair to control the relative activities of proteolytic compounds is recommended as one of the best examples in this research area.

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ДИФФЕРЕНЦИАЛЬНАЯ ЭКСПРЕССИЯ ПАРЫ ЦИСТЕИНПРОТЕИНАЗА – ЦИСТАТИН КАК ФОРМ СЛИЯНИЯ «БОК-О-БОК» У *E. COLI*

A. Gholizadeh

С использованием экспрессионной системы *E. coli* успешно создана слитая конструкция бок-о-бок, проведены экспрессия, очистка и дана функциональная характеристика папаиноподобной цистеинпротеиназы и ингибитора цистеинпротеиназы арабидопсиса без нарушения роста рекомбинантных бактерий. Очищенные продукты двух различных слитых конструкций, обозначенные как "R1: H_2N-maltose binding protein-CPI-CP-COOH and R_2: H_2N-maltose binding protein-CP-CPI-COOH", показали обратные ферментативные/ингибирующие активности *in vitro*. Анализ конструкций с использованием компьютерных методов показал, что расположение пары CP/CPI в слитых конструкциях является важным критерием правильной третичной организации, структурного фолдинга и функциональных свойств. С-терминально расположенные молекулы могут быть активными структурами в каждой слитой конструкции. Результаты работы можно использовать в белковой инженерии для создания новых переключаемых экспрессионных систем.

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ABBREVIATIONS:

IPTG:	isopropyl β-D-thiogalactopyranoside
BME:	ß-mercaptoethanol
MBP:	maltose-binding protein
BANA:	b-N-benzoyl-DL-arginine b-napthylamide hydrochloride
DMACA:	p-dimethylaminocinnamaldehyde