ПРИКЛАДНАЯ БИОХИМИЯ И МИКРОБИОЛОГИЯ, 2011, том 47, № 4, с. 408–414

UDC 547.96:576.8

OVER-EXPRESSION, PURIFICATION AND FUNCTIONAL CHARACTERIZATION OF *Celosia* ClpS AS A FUSED PROTEIN IN *Escherichia coli*

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Received November 16, 2010

A ClpS homologue from *Celosia cristata* was expressed as maltose-binding fusion protein under the control of strong inducible *tac* promoter of pMALc2X vector in TB1strain of *Escherichia coli*. SDS-PAGE analysis showed that fused ClpS is produced as about 63 kDa protein in recombinant bacteria. Expressed product was purified to homogeneity with a yield of about 31 mg/l of bacterial culture. The results indicated that heterologous expression of *Celosia* ClpS does not affect bacterial growth under different induced conditions. Total cellular antioxidant assessment results revealed that the induction of ClpS activates the bacterial antioxidative system. Since, the purified ClpS did not exhibit antioxidant activity in vitro, we speculated a functional corelation between bacterial protelolytic apparatus and its anti-oxidative system. This prediction may contribute to our better understanding of functional relationship between proteolytic and antioxidative systems in biological worlds in the future investigations.

In all living organisms, proteolysis is known as a major post-translational regulatory mechanism that is not only required for the quality control of proteins but also for the important regulatory cellular processes such as timing of the cell division cycle, heat shock response, different stress responses, adaptations to nutritional alterations, cell signaling mechanisms and developmental processes particularly in which programmed cell death is involved [1, 2]. The proteolytic machinery has been known to be carried out by several ATP-dependent protease-chaperone pairs that often belong to Clp (caseinolytic protease) protein family in all organisms investigated hitherto. Clp are serine proteases that are generally composed of an endopeptidase containing a protease domain and unfoldase containing the ATPase/chaperone domain [3, 4]. Protein degradation process in the cytosol of *Escherichia coli* is carried out by at least three ATP-dependent Clp known as ClpAP, ClpXP and ClpYQ in which Clp P and Y are endopeptidase, while Clp A, X and Q act as chaperones [5–8].

ClpS is a newly identified component of *E. coli* proteolytic system. It acts as a substrate modulator of the ClpAP machine through the binding to the N-terminal domain of the chaperone ClpA [9]. The crystal structure of ClpS in complex with the isolated N-terminal domain of ClpA in two different crystal forms at 2.3–3.3 Å has been identified in *E. coli* cells [10]. It has been very recently reported that ClpS binds directly to N-terminal destabilizing residues of target proteins through its substrate binding site distal to the ClpS-ClpA interface and mediates the N-end rule pathway in *E. coli* [11].

In compare to bacteria, there is limited information concerning the structures and mechanisms of function of proteolytic apparatus in plants. Clp-dependent protein degradation process in *Arabidopsis thaliana* is predicted to be performed by a single 350 kDa core complex, ClpP/R, in which ClpP is an endonuclease and ClpR is a regulator component [12]. After genome sequencing and annotation projects in plants, cyanobacterial-homologues of ClpS gene had been identified [13, 14]. For the first time, we reported the presence of an actinobacterial-type ClpS gene in *Celosia cristata* [15]. But how these ClpS interact with proteolytic system is still remained obscure in planta.

Stimulation of cellular redox system and production of oxidative compounds is one of the common cellular reactions in response to various environmental changes. This phenomenon is called as "oxidave burst" that is mostly leads to death of the cells [16]. To better cope with the environmental stimuli and inhibition of cell death, oxidative burst is often modulated by cellular antioxidative system [17, 18]

In the present research, the activation of cellular antioxidative system following the activation of proteolytic apparatus was predicted. To test this idea, an over-producing construct for the production of *Celosia* ClpS was generated in *E. coli* cells and the effect of its induction on the growth of recombinant bacteria and their antioxidative status were experimented. As the results of this research work, the molecular cloning, successful expression and purification of *C. cristata* ClpS in *E. coli* as a part of maltose-binding fusion protein and its effect on the activation of antioxidation system in recombinant bacteria are reported for the first time.

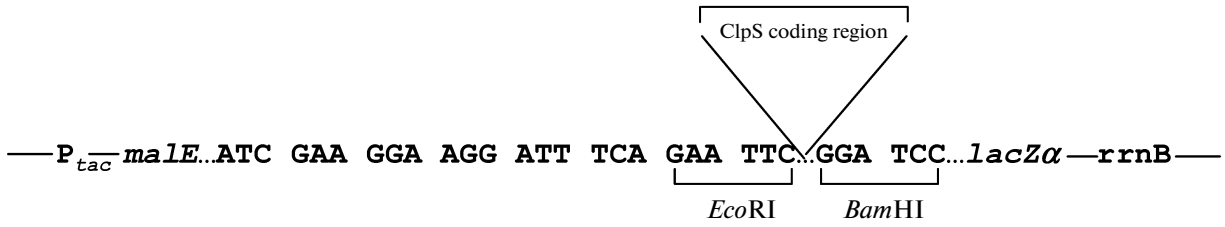

Fig. 1. The cloned map of the ClpS gene on pMALc2X expression vector. *Eco*RI and *Bam*HI digested ClpS coding region from *Celosia* plant was inserted, with its correct reading frame, downstream of *malE* gene of *E. coli* and resulted in an MBP-ClpS fusion gene.

MATERIAL AND METHODS

Materials. *Celosia cristata* seeds were obtained from the division of Biochemistry, IARI (New Delhi, India) and grown in growth house conditions. *E. coli* strain TB1 and pMALc2X vector for bacterial transformation, recombinant vector construction and protein expression studies were supplied in protein fusion and purification system kit (New England Biolabs, UK). Plasmid vector pGEM-T easy (Promega, USA). *Taq* DNA polymerase, buffer, dNTPs, and $MgCl_2$ were used for polymerase chain reactions (PCR) amplification (CinnaGen, Iran). DNA Extraction Kit (Fermentas, UK) was used for the purification of the PCR product from the agarose gel. Restriction enzymes *Eco*RI and *Bam*HI were used in cloning procedure (CinnaGen, Iran). All of the other chemicals used in this research work were of molecular biology grade.

Cloning of Celosia ClpS cDNA. Cloning of *Celosia* ClpS cDNA was carried out by standard PCR using the specific primers designed based on the ClpS cDNA sequence reported in our previous work [15]. The 30 base pairs synthetic primer sequences were:

right primer:
5'ATACGAATTCATGGCCGGAGCAGGATATAG3';

left primer:
5'ACTAACGGATCCGTGTCCAGCTGCAGGGTC3'.

To do the directional cloning of the PCR-amplified fragment in *E. coli* expression vector, *Eco*RI and *Bam*HI restriction sites were included at the 5' end of each primer. The cDNA sequence was amplified from *Celosia* complete leaf cDNA library constructed on λTriplEX2 vector as described already [15].

PCR reaction was carried out in a reaction volume of 20 μl containing 5x PCR buffer, 5 ng templete DNA, 1 pmol of each primer, 10 mM dNTP mix, 2 units *Taq* DNA polymerase. Amplification was performed in Techneh type thermal cycler (Germany), with 25 cycles of 1 min denaturation at 95°C, 2 min annealing at 57°C, and 2 min of extension at 72°C, ending with 10 min of incubation at 72°C. The amplified product was analyzed on 1% agarose gel using Tris-borate-EDTA (TBE) running buffer and sliced from gel, ligated into pGEM-T easy vector and transformed into DH5α *E. coli* cells. The positive recombinant clones were selected by blue-white screening method [19]. Plasmid DNA was then purified from recombinant bacterial cells [20] and proceeded for DNA sequencing at New Delhi University (South campus, India).

Expression of ClpS gene as fused product. The 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 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 transferred into competent *E. coli* TB1 cells. For preparation of competent cells, the cells were grown in LB media when the OD_{600} reached to 0.4, they 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 $CaCl_2$. Then after, 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 transformation of bacterial cells, the ligation reaction was mixed with 25 μl competent cells, incubated on ice for 5 min, heated to 42°C for 2 min and after addition of 0.1 ml LB medium, it was incubated at 37°C for 20 min. 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 expressed fusion protein. To extract the fusion protein, transformed cells were grown in 500 ml of rich broth/ glucose/Amp. For the induction of fused protein expression, the inducer molecule isopropyl-β-D-thio-galactoside (IPTG) was added to a final concentration of 0.3 mM and incubated for 8 h. The cells were harvested by centrifugation at 4000 g for 10 min and the pellet was dissolved in 25 ml of extraction buffer containing 20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM azide and 10 mM BME. The cells were frozen in the same buffer at −20°C over night and were then sonicated in short pulses of 15 s. Sample was centrifuged at 10000 g at 4°C for 20 min and the supernatant used as crude extract.

The fusion protein was purified from the crude extract by affinity column chromatography. It was done in a column packed with amylase resin specific for the maltose-binding protein (MBP), which was a part of fused protein.

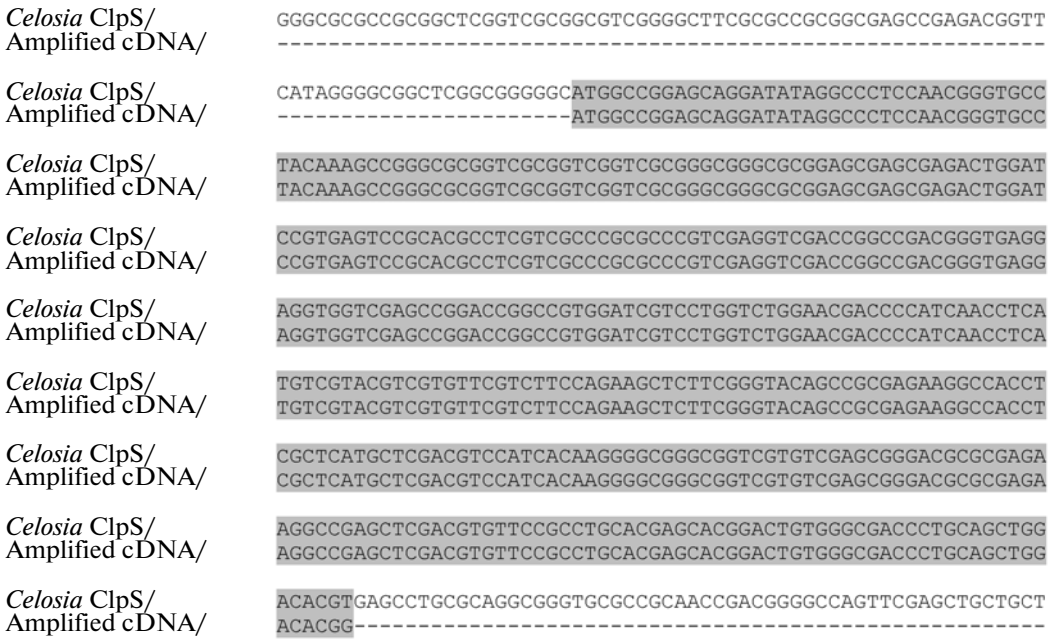

Fig. 2. Nucleotide sequence alignment between amplified cDNA and *Celosia* ClpS.

The fusion protein was eluted out from the column with column buffer (extraction buffer plus 10 mM maltose). The eluted product was analyzed for its homogeneity by SDS-PAGE electrophoresis using 10% polyacrylamide gel [21].

Assessment of recombinant bacterial growth. In order to find out the effects of expressed fused product on the growth of recombinant bacteria**,** the cells were separately induced at different concentrations of IPTG (0.1, 0.3, 0.5 and 1 mM) and the growth of bacteria was assessed by measuring the optical densities of the cultures at 600 nm for 1 h time intervals. Bacterial growth curves were drown for each induced test culture and compared to each other as well as to control non-induced culture.

Antioxidant activity test. The total antioxidant activity of the bacterial cells was determined using ferric reducing antioxidant power (FRAP) test [22]. To 1 ml of bacterial cell culture, 3 ml of FRAP reagent was added and the reaction mixture was incubated at 37°C for 4 min. The assessment was carried out spectrophotometerically at A_{593}. Antioxidant potential of samples was determined against the standard curve of ferrous sulphate (Fe^{2+}, 100–1000 μM). Ascorbic acid (100 μM) served as a standard antioxidant and BSA considered as negative control. FRAP values were calculated as follows: FRAP value (μmol Fe^{2+} ml^{-1}) = A_{593} of test sample/A_{593} of standard × × FRAP value (μmol Fe^{2+} ml^{-1}) of standard. Three experimental samples including non-recombinant, recombinant induced and non-induced were considered for test assay. The antioxidative FRAP test was similarly repeated for the purified fused ClpS in vitro and compared to those of ascorbic acid and BSA. All of the samples were analyzed in triplicate and the data were presented as mean values.

RESULTS

Cloning of Celosia ClpS cDNA. In the present research, *Celosia* ClpS was amplified by PCR using *Celosia* leaf cDNA library as template and a pair of specifically designed primers which created *Eco*RI and *Bam*HI restriction endonuclease recognition sequences at 5' and 3' ends of the ClpS coding region (Fig. 1). The amplification product was purified from agarose gel and was then cloned into pGEM-T easy vector for sequencing. Analysis of the sequence data confirmed that the amplified cDNA is completely identical to the already reported ClpS sequence from *Celosia* plant. The results of homology search between the nucleotide sequences of the PCR amplified cDNA and *Celosia* ClpS gene by CLASTAW (http// www.genome.ad.jp) has been demonstrated (Fig 2). The amplified ClpS cDNA was used for the expression studies in *E. coli* cells.

Expression of amplified ClpS in *E. coli* cells. *Celosia* ClpS coding region on pGEM-Teasy vector was restricted with *Eco*RI and *Bam*HI enzymes and was then cloned into *Eco*RI and *Bam*HI sites within the multiple cloning region of pMALc2X *E. coli* expression vector as shown in Fig. 1. Transformed TB1 cells harboring the recombinant plasmid were allowed to grow under the induced condition using 0.3 mM IPTG as inducer molecule. Total crude proteins of non-recombinant, recombinant induced and non-induced cultures were separately isolated and separated on 10% polyacrylamide gel by SDS-PAGE electrophoresis (Fig. 3). Analysis of the gel revealed that *Celosia*

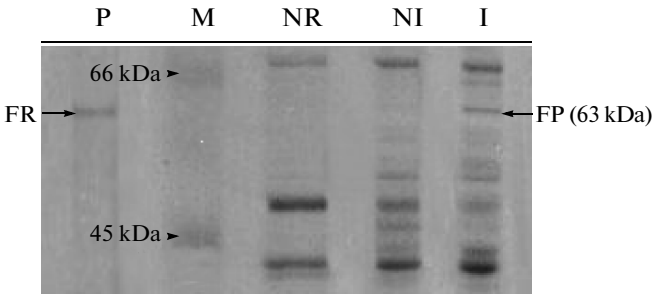

Fig. 3. SDS-PAGE analysis of the fused product expression. I – induced recombinant fused sample; NI – non-induced recombinant fused sample; NR – non-recombinant/non-fused sample; M – Sigma protein low molecular weight marker and P – purified fused protein (FP). The gel was stained with comassiee brilliant blue dye.

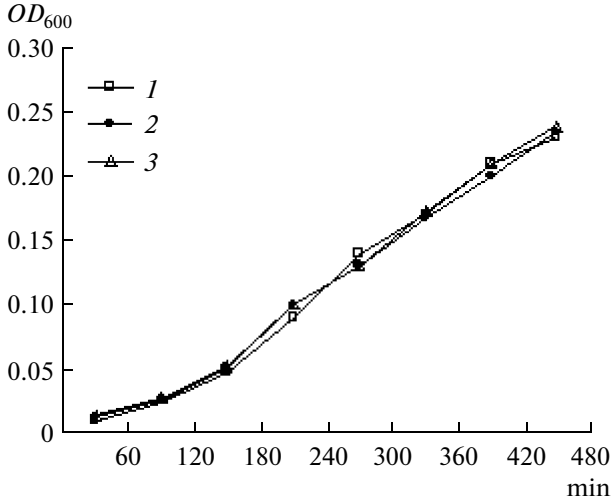

Fig. 4. Effect of IPTG dosage on bacterial growth: *1* – non-induced cells; *2* – induced with 0.1 mM of IPTG; *3* – induced with 1.0 mM of IPTG. The growth of recombinant bacterial cells were evaluated by measuring the OD_{600} values of the test cultures at 1 h time intervals for overall experimental period of 8 h.

ClpS protein is expressed as a part of maltose-binding protein fused product with a molecular weight of about 63 kDa (Fig. 3). As it has been shown in the photograph, this expressed product is not observed in non-recombinant or non-induced lines. The expressed product was purified to homogeneity by maltose binding affinity chromatography at the yield of 31 mg/l of induced bacterial culture. The purified protein sample was also analyzed by SDS-PAGE and compared with crude sample as shown in Fig. 3.

Effect of plant ClpS induction on bacterial growth. In order to determine the effect of inducer dosage on the growth of recombinant bacteria, an experiment was performed in which IPTG concentration was varied from 0.1 to 1.0 mM. Data corresponding to minimum (0.1 mM) and maximum (1 mM) concentrations of IPTG were presented on the graph (Fig. 4). Each test was carried out in two replicates and the results were presented as mean values. The growth of bacteria was spectrophotometerically assessed by measuring the OD_{600} values of each test culture at 1 h time intervals for overall 8 h time period. The growth curve of bacteria in each induced culture was also separately compared to non-induced control culture (data not shown). In overall, the growth curve of the cells exposed to IPTG induction not significantly differs from the curve obtained for non-induced culture. The results showed that increasing concentrations of inducer molecule have no inhibitory effects on bacterial cell growth. Recombinant bacteria exposed to different concentrations of IPTG exhibit the similar pattern in their growth curves. The OD_{600} values obtained for each comparable time points show no statistically different data ($P \leq 0.05$).

Changes in antioxidant status of recombinant cells. Antioxidant ability of the recombinant cells carrying fused ClpS was determined by FRAP method as described in materials and methods section. The FRAP values were calculated to be 0.085, 0.12 and 0.09 μmol Fe^{2+} ml^{-1} for non-recombinant (cells carrying MBP vector alone/non fused vector), recombinant (cells carrying MBP-ClpS vector/fused vector) induced (with 0.3 mM IPTG) and non-induced cultures, respectively (Fig. 5). As the results show, there is a significant difference between the recombinant induced sample and non-recombinant culture or between the induced and non-induced cultures in terms of their FRAP values. The values between the non-recombinant and non-induced samples are not statistically different ($P \leq 0.1$).

The antioxidant status of the recombinant cells exposed to different concentrations of IPTG (0.1, 0.3, 0.5 and 1 mM) were similarly assessed by FRAP test. The results showed that the total antioxidant capacity of the cells is increased with increasing concentrations of IPTG (Fig. 5b). The FRAP value of the induced culture reaches up to 0.16 at 1 mM IPTG used. Data revealed that the total antioxidant ability of the non-recombinant bacterial cells is remained constant at different dosages of IPTG used.

FRAP test assay was also performed to identify the antioxidant activity of the purified fused ClpS in vitro. In compare to antioxidant activity of 100 μM ascorbic acid (114.8 μmol ml^{-1}), the 100 μM of purified fused ClpS did not show antioxidant activity in vitro.

DISCUSSION

Cloning and expression of Celosia ClpS cDNA in *E. coli*. In our previous work, a complete ClpS cDNA clone was randomly isolated from *C. cristata* leaf expression library constructed on the λTriplEX2 vector at the vegetative growth stage. Sequence data analysis showed that *Celosia* ClpS is highly identical to the corresponding proteins from actinobacteria [15]. Therefore, it was for the first

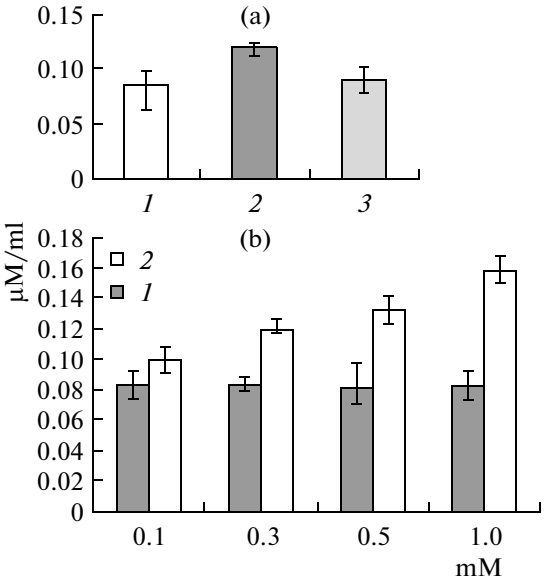

Fig. 5. Assessment of antioxidant activity.

(a) The antioxidant capacity of the recombinant induced cells were analysed in terms of μM Fe^{2+}/ml cell culture at three different cellular conditions: *1* – induced nonfused; *2* – induced fused and *3* – non-induced fused cells.

(b) Antioxidant ability of the recombinant (cells carrying fused MBP-ClpS vector, *1*) and non-recombinant cells (cells carrying MBP vector alone/non fused vector, *2*) were compared with increasing concentrations of IPTG.

time speculated on the presence in the plant system of two more distant homologues of ClpS proteins (including cyanobacterial and actinobacterial types), as had been already detected in bacteria [23]. In the present research, *Celosia* ClpS coding region was specifically amplified from *Celosia* leaf cDNA library taken from laboratory stock (Fig. 2). The amplification product was attempted to be expressed in *E. coli* cells.

Although, ClpS protein is a new component of the proteolytic machine that has been partially characterized in *E. coli*, but still now, very limited information is available concerning its structures and functions in eukaryotic system.

As a part of our functional studies, *Celosia* ClpS coding region was cloned into pMALc2X *E. coli* expression vector in which the strong "*tac*" promoter and "*male*" translation initiation signals are used to give high-level expression of cloned sequences under induction with IPTG [24, 25].

SDS-PAGE analysis revealed that ClpS protein is expressed as a part of maltose-binding protein fused product with a molecular weight of about 63 kDa, consisting of MBP (42.482 kDa), the product of 132 amino acid residues of ClpS cDNA and six amino acid residues derived from the expression vector (Fig. 3). The expressed product was purified to homogeneity using one step purification method by MBP affinity chromatography at the yield of 31 mg per liter of bacterial culture [26].

This purification yield indicates that *Celosia* ClpS is highly expressed in bacterial cells and it may have no toxic effects on *E. coli* cells. Despite this, whether *Celosia* fused ClpS is expressed as an active product in bacterial cells still needs to be investigated in future.

This work for the first time describes the heterologous expression of a ClpS gene from plant origin in bacterial cells, successfully. Thus far, there has been no reports concerning the corresponding recombinant or fused products in the literatures. As plants lack ClpA molecular chaperon to interact with their ClpS component, therefore it was already speculated that plant cyanobacterial-type ClpS proteins may interact with ClpC, known as a functional homologue of ClpA in plant system [9]. Newly, after finding an actinobacterial-type ClpS gene in *Celosia* plant, the presence of a different type of molecular chaperon or a novel target of ATP-dependent Clp protease has been predicted in plants [15]. Considering the successful production of plant actinobacterial-type ClpS in *E. coli* cells, its possible cooperation with *E. coli* ClpA chaperon is speculated in recombinant bacteria.

Effect of induction on bacterial growth and cellular antioxidant status. Our experimental results revealed that there is no significant difference between the growth patterns of bacteria exposed to minimum (0.1 mM) and maximum (1 mM) dosages of IPTG used (Fig. 4). The results also showed that the growth curve for cells exposed to IPTG induction not measurably differs from the curve obtained for non-induced culture. Therefore, it may be deduced that differentially expressed amount of plant ClpS fusion protein has no inhibitory effects on recombinant bacterial cell growth. But how *Celosia* ClpS function in bacterial cells, needs to be investigated.

One of the common responses of the cells to their environmental changes is the excessive formation of reactive oxygen species (ROS) that is normally balanced with the cellular antioxidative system to adapt the cells to the new conditions/environments [17, 18].

Based on this view, we predicted possible changes in the total antioxidative status of the recombinant induced cells carrying plant ClpS as a component of plant Clp-dependent proteolytic system. In biological cells, proteolysis and antioxidation processes are known as the natural targets of cell death phenomenon. In general, proteolysis elevations activate cell death metabolic pathways while the antioxidative lightening inhibits cell death. We predicted that cellular proteolytic and antioxidation systems may be functionally balanced in such a way that capable of preventing cell death process. Therefore, heterologous overproduction of a proteolytic component predicted to activate the antioxidation pathways in recombinant cells.

In order to test the antioxidant capacity of the recombinant bacterial cells over-producing fused plant ClpS, FRAP method was used. It is known as a simplest and re-

producible method for the assessment of total antioxidant abilities of various biological systems [22].

The results indicated that during the induction period, the total antioxidative status of the recombinant cells (cells carrying MBP-ClpS fused vector) is increased as compared to non-recombinant cells (cells carrying MBP vector alone /non fused vector), suggesting that MBP might not be involved in cellular antioxidative response (Fig. 5). The antioxidant capacity of the induced recombinant cells was also found to be increased with the increasing concentrations of the inducer molecule.

This result may have two explanations. Firstly, antioxidative response can be as a result of over-production of every fused protein in induced *E. coli* cells and therefore it can be considered as a generalized response to genetic recombination process. Secondly, the antioxidative changes may be due to the specific activity or the particular molecular interactions of the expressed fused ClpS in recombinant cells. So, it can be considered as a specialized response to plant ClpS production and function in bacterial cells. We suggest a functional balancing between bacterial proteolytic and antioxidation processes leads to cell death inhibition and so results in the normal growth of the recombinant cells upon induction. As the purified fused ClpS did not show the antioxidant activity in vitro, it is reliably predicted that the changes in the antioxidative status of the induced recombinant cells is a indirect metabolic adaptation process to cope with hetrologous plant ClpS activity/challenges in *E. coli* cells.

Despite these, more attempts will be needed to identify the molecular interactions of the expressed ClpS fused product in recombinant *E. coli* cells. More investigations also needed to find out the possible relations / interactive events between the proteolytic and antioxidative systems of the recombinant cells.

– *Celosia* ClpS can be successfully expressed as maltose-binding fused product in *E. coli* cells. It can be overproduced at different concentrations of IPTG inducer molecule in bacterial cells without affecting their normal growth. This will provide a successful way for future studies on molecular interactions of the fused ClpS in recombinant bacteria. The possible cooperation of fused *Celosia* plant ClpS with *E. coli* ClpA molecular chaperon is speculated.

– Over-expression of fused ClpS in *E. coli* cells leads to the significant increase in total antioxidant status of the induced cells.

– Further research on the mechanism of how fused ClpS changes the total antioxidative ability of the bacterial cells is recommended. It has to be finding out: (1) whether the antioxidative response is a generalized action that is commonly depended on genetic recombination process in *E. coli* cells; (2) whether the antioxidative changes in induced cells is a specialized response that is particularly depended on ClpS overproduction and activity in *E. coli* cells.

– We speculate that the alterations in the total cellular antioxidative ability can be an indirect metabolic adaptation process in response to hetrologous production of ClpS or its activity in recombinant induced cells. Our future attempt is to find out the functionality of the fused plant ClpS in *E. coli* cells and to investigate its possible molecular interactions with *E. coli* proteolytic and antioxidative systems.

ACKNOWLEDGMENTS

The authors of this paper are thankful the Research Institute for Fundamental Sciences (RIFS), University of Tabriz, Iran and Indian Agricultural Research Institute (IARI), New Delhi, India for their financial support of this work.

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