Molecular analysis of maize cystatin expression as fusion product in *Escherichia coli*

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Abstract Nowadays, plant cysteine proteinase inhibitors “namely phytocystatins” have attracted researchers towards the identification of their molecular structures and novel physiological functions. Their important roles in plant developmental processes and different stress responses have been well known. In spite of advances in the understanding of phytocystatins, we lack enough data concerning their heterologous expression especially in the forms of fusion products that are most important whether for biochemical, pharmacological or clinical studies. The present work describes an easy method of expression, purification and functional characterization in *Escherichia coli* of maize cystatin as a part of maltose-binding fusion protein. Assessments revealed that upon expression of fused product the total antioxidation status of the induced recombinant cells is increased. This result leads to question ‘Is there any parallel functional correlation between anti-proteolytic and anti-oxidative systems?’ However, the present research will open a gate for the new studies regarding the putative communicative roles of these systems that may be existing in the biological world.

Keywords Antioxidation · Cystatin · *E. coli* · Expression · Maize · Phytocystatins

Introduction

Cystatins are proteinaceous reversible inhibitors executing specific inhibitory role on enzymatic activity of cysteine

proteinases (such as papain and cathepsin H) (Otto and Schirmeister 1997). They have been earlier identified in mammalian systems, and have been later on described in plants including rice (Abe et al. 1992), soybean (Botella et al. 1996), sorghum (Li et al. 1996), apple (Ryan et al. 1998), potato (Annadana et al. 2003), barley (Martinez et al. 2005a), Celosia (Gholizadeh et al. 2005), maize (Massonneau et al. 2005), sugarcane (Gianotti et al. 2006), alfalfa (Rivard et al. 2007), and tobacco (Zhang et al. 2009). Generally, cystatins are categorized into four following super-families: I) stefins, II) cystatins, III) kininogens and IV) phytocystatins in which stefins cover low molecular weight proteins (about 11 kD) without disulfide bonds; cystatins include proteins with molecular mass of about 13–24 kD having two disulfide bonds; kininogens consist of large glycosylated proteins with molecular weight ranging from 60 to 120 kD including three repeat domains similar to cystatins; and finally phytocystatins (plant cystatins) that have acquired the features of group I, II protein families in wide ranging molecular weights (5–87 kD) (Barrett et al. 1986; Rawling and Barrett 1990; Bateman et al. 2002).

Similar to all members of the cystatin super-families, phytocystatins have three conserved regions which interact with cysteine proteinase molecules. They include G residue at their N-terminus, “QxVxG” and W residue at C-terminus. Furthermore, phytocystatins diverge from non-plant types by a plant-specific sequence; [LVI]-[AGT]-[RKE]-[FY]-[AS]-[VI]-x-[EDQV]-[HYFQ]-N in their N-terminal of α -helix region (Turk and Bode 1991; Margis et al. 1998).

Various physiological functions have been attributed to phytocystatins that are mostly described in regulation of endogenous proteinases during seed maturation and germination (Arai et al. 2002; Martinez et al. 2005b; Hong et al. 2007), participating in defense mechanism against attack by insects and nematodes that generally contain cysteine proteinases in their guts (Irie et al. 1996; Urwin et al. 1997; Pernas et al. 1998;

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Rahbé et al. 2003), inhibition of viral infections (Gholizadeh et al. 2005; Campos et al. 1999; Cipriani et al. 2000; Shah and Bano 2009) and modulating programmed cell death (PCD) during plant development, senescence and HR responses (Belenghi et al. 2003; Solomon et al. 1999). Moreover, the most recent findings signify the involvement of abiotic environmental stresses such as drought and salt in phytocystatins induction (Gaddour et al. 2001; Corre-Menguy et al. 2002; Diop et al. 2004; Petya et al. 2007; Girard et al. 2007). Despite these recent extensive studies on phytocystatins, our knowledge regarding their expression as recombinant or fusion products is still poor (Gianotti et al. 2006; Sugawara et al. 2002; Oliva et al. 2004).

Recently, during maize large-scale cDNA sequencing project, a putative novel multidomain cystatin expressed sequence has been characterized from mixed tissues and released to databases (GeneBank accession number EU961198). However, there is no report regarding its expression in the individual tissues. Herein, we not only report the molecular cloning of maize multicystatin from leaf tissues, but also as a part of our biochemical and functional studies, we generated an over-expression construct to produce maize cystatin as a maltose-binding fusion protein in *Escherichia coli* cells for the first time.

To identify the consequence biochemical effects of maize cystatin over-production in *E. coli* cells, we compared the total antioxidation status of the recombinant cells expressing cystatin molecule with that of non-recombinants. Since, both cystatins and antioxidants are known as the common regulators of almost all the developmental processes and environmental stress responses in all living organisms (Arai et al. 2002; Gaddour et al. 2001; Kumar et al. 1999; Solomon et al. 1999; Halliwell 2006; Shao et al. 2007), therefore their parallel functional cooperation was speculated.

Considering cystatins as anti-proteolytic enzymes, our investigation results may provide a basis for the future studies concerning the functional correlations between anti-proteolytic and antioxidative systems that may be existing in the biological world.

Materials and methods

Materials The seeds of *Zea mays* L. were provided by Dr B. Baghban Kohnhrouz (Laboratory of Plant Genetic Engineering, Dept. Plant Breeding and Biotechnology of Tabriz University). Trizol reagent used for total RNA extraction was purchased from GIBCO BRL, USA (Cat. No.15596-013). The mRNA purification kit was from QIAGEN, USA (Cat. No.70022). Chemicals used for the cDNA synthesis were provided in cDNA synthesis kit, Promega, USA (Cat. No. C4360). pGEM-T Easy vector system I (Cat. No. A1360) was used in PCR product cloning. Restriction

enzymes were purchased from Promega (Madison, WI, USA) except *Eco*RI and *Bam*HI which were purchased from CinnaGen Company. *E. coli* strain TB1 and pMALc2X vector were supplied with protein fusion and purification system kit (Cat. No. E8000S; NEW ENGLAND, Biolab) and were used for bacterial transformation, recombinant construction and protein expression studies. *Taq* DNA polymerase, PCR buffer, dNTPs, and $MgCl_2$ for PCR amplification were obtained from CinnaGen. Fermentas DNA Extraction Kit (Cat. No. K0513) was used for the recovery and purification of the PCR product from the agarose gel. All the other analytical and molecular biology grade chemicals were purchased from Merck AG (Darmstadt, Germany) or Sigma (St. Louis, MO, USA).

mRNA purification and cDNA synthesis Total cellular RNA was isolated from maize leaves at early vegetative stage, using Trizol reagent. About 0.2 g of leaf material was well ground in liquid N_2 and Trizol (2 ml) for homogenization and powdering at room temperature (RT). Chloroform (200 μ l) was added to the mixture, then mixed for 15 s, kept on ice for 5 min and centrifuged at 13000x g for 15 min. The upper phase was transferred into the other tube and RNA was precipitated using equal volume of isopropanol. The pellet was washed in 1 ml of 75 % ethanol, dried at RT and dissolved in 30 μ l RNase-free water. The integrity of the RNA was tested on 1 % non-denaturing agarose gel using TBE running buffer. Poly (A⁺) RNA was purified from total RNA using purification kit and then double-stranded cDNA was synthesized according to the Promega cDNA synthesis kit guideline.

Specific cloning of maize cystatin The maize cystatin cDNA was amplified using a specific primer pair (forward primer: 5'-TTATTGAATTCTCCTCCACTACCAGAGCA-3' and reverse primer: 5'-ATATAGGATCCAGTTCCTGGCTGCTCGACT-3'). In order to do the directional cloning of the PCR-amplified fragment in an *E. coli* expression vector, *Eco*RI and *Bam*HI restriction sites were included at the 5' end of each primer. The PCR reaction was carried out by mixing the following components in a 0.5 ml PCR tube; primers, 100 pmol (1 μ l each); cDNA, 50 ng (1 μ l); PCR reaction 10x buffer, 2.5 μ l; 10 mM dNTP, 1 μ l; H_2O , 17.5 μ l and *Taq* DNA polymerase, 1 μ l. The reaction mixture was processed in a thermocycler (Techneh, Germany) under the following cycling program: denaturation at 94 °C for 1 min, annealing at 58 °C for 2 min, and extension at 72 °C for 2 min. Afterwards, PCR product was cloned in pGEM-T Easy vector system I and transformed to *E. coli* strain DH5 α transformants were selected on plates in the presence of X-Gal by blue/white screening method. A single recombinant colony was taken for plasmid DNA extraction and separation on 0.8 % agarose gel. The purified plasmid was processed for sequencing of the insert DNA at Microsynth DNA sequencing center, Switzerland.

Expression of maize cystatin as fusion protein in *E. coli* - After *EcoRI*—*Bam*HI treated PCR recovery, cDNA fragment was ligated into the *EcoRI*—*Bam*HI linearized pMALc2X expression vector and then transferred into competent *E. coli* TB1 cells via heat shock procedure. Competent cells were prepared according to the general calcium chloride wash protocols. The transformed cells were plated on LB medium (supplemented with Amp and X-gal) at 37 °C and a recombinant clone was selected for further gene expression studies.

Extraction and purification of fused cystatin Transformed cells were cultured in 500 ml of rich broth (Trypton+Yeast extract+NaCl)/glucose/Ampicillin. In order to induce the fused protein expression, IPTG was added in final concentration of 0.3 mM and incubated for 8 h at 37 °C. The cellular pellet was collected by centrifugation at (4,000x g for 10 min) and was dissolved in 25 ml of extraction buffer (20 mM Tris-Cl, 200 mM NaCl, 1 mM EDTA, 1 mM azide and 10 mM BME (β -mercaptoethanol); pH 8.1). The cells were frozen in the same buffer at -20 °C overnight and were then sonicated in short pulses of 15 s. Then, sample was centrifuged (10,000x g at 4 °C for 20 min) and the supernatant was used as crude extract to run in affinity column chromatography for fusion protein purification. Chromatography column was set with maltose binding protein (MBP)—specific amylose resin. The fusion protein was eluted out of the column by column buffer (extraction buffer plus 10 mM maltose) and analyzed for homogeneity by 10 % SDS-PAGE (Laemmli 1970).

In vitro inhibitory test for fused protein Papain and BANA (β -N-benzoyl-DL-arginine β -naphthylamide hydrochloride) substrates were used to assess the inhibitory activity of the prepared fusion protein (Misaka et al. 1996). A solution containing 0.1 ml of assay buffer (0.5 M sodium phosphate and 10 mM EDTA, pH 6.0), 2-mercaptoethanol (0.1 ml of 50 mM), 0.1 ml of papain solution (25 μ g) and 0.2 ml of test solution (containing 5, 10, 15, 20, 25, 30 and 35 μ l of purified fused cystatin) was incubated at 37 °C for 10 min. The reaction was started by the addition of BANA (0.2 ml of 1 mM incubated at 37 °C for 20 min) and then stopped with the addition of HCl/ethanol (1 ml of 2 % (v/v)) and 1 ml of 0.06 % p-dimethyl aminocinnam aldehyde/ethanol. The reaction mixture was measured at 540 nm and was compared with control sample containing lysate of non-recombinant *E. coli* cells.

Detection of stability between fused cystatin and papain complex About 0.25 μ g of purified fused cystatin protein was incubated with 0.2 μ g papain at 37 °C for 15 min in lysis buffer, pH 8.0 as described by Gianotti (Gianotti et al. 2006). The reaction was stopped by the addition of equal volume of electrophoresis sample buffer (4 % SDS, 100 mM Tris-HCl, pH 6.8, 20 % glycerol and 0.2 % bromophenol blue). Samples were run on SDS-PAGE and then transferred

onto nitrocellulose membrane for the detection of protein complexes.

Western-blot analysis In order to confirm the expression of recombinant product as well as formation of complexes between fused cystatin and papain enzyme, western-blot analysis was carried out using antibody against maltose-binding protein supplied in the kit. Initially, purified fused product was separated on SDS-PAGE (10 %) and then transferred onto nitrocellulose membrane using blotting buffer (0.025 M Tris-Cl pH 8.3, 0.192 M glycine and 20 % ethanol (Belenghi et al. 2003). Subsequently, the blot was kept in TBS-BSA buffer (0.02 M Tris-Cl, pH 7.5, 0.5 M NaCl, 1 % BSA) overnight at 4 °C and then incubated with specific antibody (anti-MBP) at 1:500 dilution in the same buffer at 37 °C for 1 h. After washing with TBS-T (TBS+0.05 % Tween 20), the membrane was incubated with alkaline phosphate conjugated goat-rabbit antibody (GAR^{XAP}) at 1: 20,000 dilution in TBS-BSA buffer for 1 h. After extensive washing, the signal band was visualized using a substrate solution containing nitro blue tetrazolium chloride (NBT, 0.33 mg/ml) and 5-bromo-1-chloro-3-indolyl phosphate (BCIP, 0.165 mg/ml) in Tris-Cl buffer (0.1 M pH=0.5) containing NaCl (0.1 M) and MgCl₂ (5 mM).

Antioxidant activity test for recombinant cell Total antioxidant activity of bacterial cells was determined using ferric reducing antioxidant power (FRAP) test (Benzie and Strain 1996). Bacterial cell culture and FRAP reagent (1:3) were mixed and incubated at 37 °C for 4 min. The assessment was carried out spectrophotometrically at A₅₉₃ and then antioxidation potential was determined against the standard curve of ferrous sulphate (Fe^{II}, 100–1,000 μ M). Ascorbic acid (100 μ M) and BSA were used as standard antioxidant and negative control sample, respectively.

FRAP values were calculated from the following formula:

$$\text{FRAP value } (\mu\text{mol Fe}^{\text{II}}\text{I}^{-1}) = (A_{593} \text{ of test sample} / A_{593} \text{ of standard}) \\ \times \text{FRAP value } (\mu\text{mol Fe}^{\text{II}}\text{I}^{-1}) \text{ of standard}$$

Three experimental samples including induced and non-induced recombinants along with non-recombinants were considered for test assay. All samples were analyzed in triplicate and the data were presented as mean value.

Results and discussion

Cloning and characterization of putative maize cystatin cDNA

Our understanding regarding the biochemical and physiological functions of plant cystatins is not full (Belenghi et al. 2003; Solomon et al. 1999; Li et al. 2006; Martinez et al.

Table 1 Theoretical physico-chemical characteristics of cystatins isolated from *Maize* and *Celosia*

	Maize	Celosia
Number of amino acids	251	117
Molecular weight	27.477	13.367
Isoelectric point	6.11	5.46
Amino acid composition	A: 14.3 %; R: 4.4 %; N: 2.8 %; D: 4.0 %; C: 0.4 %; Q: 3.2 %; E: 10.0 %; G: 6.8 %; H: 5.2 %; I: 2.0 %; L: 9.6 %; K: 7.2 %; M: 1.2 %; F: 5.6 %; P: 4.0 %; S: 5.2 %; T: 4.0 %; W: 1.2 %; Y: 0.8 %; V: 8.4 %	A: 11.1 %; R: 3.4 %; N: 9.4 %; D: 4.3 %; C: 0.9 %; Q: 3.4 %; E: 10.3 %; G: 2.6 %; H: 0.9 %; I: 4.3 %; L: 7.7 %; K: 9.4 %; M: 0.9 %; F: 5.1 %; P: 5.1 %; S: 6.8 %; T: 2.6 %; W: 2.6 %; Y: 3.4 %; V: 6.0 %
Instability index	32.80, stable	46.56, unstable
Aliphatic index	83.67	75.13

Data were calculated using compute tools at “<http://www.expasy.ch/tools>”

2009). To date, determining the natural targets and diverse biological functions of plant cystatins are of modern scientific challenges. Recently, the existence of multiple structural and functional forms of phytocystatins have been proposed by evaluation of their gene expression patterns in some plants such as *Arabidopsis*, barley, and tobacco in diverse conditions (Massonneau et al. 2005; Martinez et al. 2005a, b; Beyene et al. 2006).

In the present work, to identify the new cDNA clones encoding putative cystatin, we surveyed plants genome databases and found a maize putative cystatin cDNA clone “233392” which has been recently identified during large scale cDNA sequencing project (under GenBank accession number of EU961198). This clone has been isolated from the mixed tissues of maize plant; therefore its expression profile needed to be individually identified in different

organs. We amplified this novel cystatin from the leaf cDNA population at the early vegetative stage. In order to do directional cloning and further expression analysis of the amplified cDNA, *EcoRI* and *BamHI* restriction sites were inserted at its 5' and 3' ends, respectively.

Agarose gel electrophoresis of the PCR product and the nucleotide sequence of the amplified maize cDNA are not shown. Sequence alignment analysis of the isolated cDNA against clone 233392 was completely identical (results not presented). The size of the amplified sequence was consistent to the expected size of “774 bp”.

Fig. 1 SDS-PAGE and Western analysis of the expressed fused product: **a** crude protein was extracted from induced *E. coli* lysate (strain TB1) and electrophoresed on 10 % polyacrylamide gel. About 76 kD fused product was detected on the gel. FP: fusion protein; Lane 1. recombinant clone after induction with IPTG; lane 2. recombinant clone before induction with IPTG; lane 3. transformed cells containing vector alone after induction with IPTG and lane 4. protein molecular weight marker. **b** induced crude protein sample from *E. coli* cells carrying recombinant vector was subjected to western blot analysis. A signal (S) corresponding to fused product was detected on blot paper

Fig. 2 Assessment of cystatin activity in purified fusion product. The inhibitory activity of the solution containing fused cystatin was tested spectrophotometrically by measuring its absorbance at 540 nm. The figure show inhibition of papain activity by purified fused protein (closed stars). The control samples included: (open squares: solution without fusion product; closed circles: solution containing lysate of *E. coli* cells carrying vector alone)

Fig. 3 Interactions of papain with non-fused and fused maize cystatin. The three dimensional structures of papain, non-fused cystatin and MBP-fused cystatin were predicted by discovery studio proteomic software. Using the same software the interactions between papain and its inhibitor in the forms of non-fused and MBP-fused were also predicted and presented

A number of the theoretical physico-chemical characteristics of the predicted protein were calculated using “<http://www.expasy.ch/tools>” tools (Table 1) and compared to that of *Celosia* (Gholizadeh et al. 2005). The results revealed that maize cystatin molecule is more stable than *Celosia* cystatin.

Heterologous expression and purification of maize fused cystatin

Maize cystatin cDNA was expressed in *E. coli* cells as a part of maltose-binding fusion protein. The coding sequence of maize cystatin was directionally cloned downstream to *mal* gene (encoding maltose-binding protein) in pMALc2X expression vector (both treated with *EcoRI* and *BamHI*). Protein fusion tags were chosen for this work because of their

approved efficiency and their minimal effects on the bioactivity of the expressed fused proteins (Kellerman and Ferenci 1982; Duplay et al. 1984). In our previous work, it was proved that the presence of MBP fragment does not impair the inhibitory activity of *Celosia cristata* cystatin (Celocystatin) in vitro (Gholizadeh et al. 2005).

Expression of maize Mal:Cystatin fusion protein in TB1 *E. coli* cells was induced by IPTG and then analyzed in the soluble fraction of the induced bacterial culture by SDS-PAGE (Fig. 1a). Western-blot analysis using an antibody against MBP, furthermore confirmed the expression of about 76 kD fusion product (Fig. 1b). However, we expected a molecular weight of about 72 kD consisting of MBP (42.482), the product of 260 amino acid residues of cDNA and six amino acid residues from the expression vector. But the size of the detected product was found to be 4 kD larger.

Fig. 4 Detection of stable complex formation between fused cystatin and papain. For detection of complex formation, papain and cystatin mixture were subjected to non-reducing SDS-PAGE and western blot assay, respectively. Position of the expressed fused cystatin is shown on the figure. No additional signal corresponding to complex was detected on the blot paper. Lane 1. purified fused protein before incubation with papain; Lane 2. Purified fused protein after incubation with papain; Lane 3. Western signal related to lane 1; lane 4. Western signal related to lane 2

According to the former reports, it has been suggested that atypical migration of cystatin fusion proteins on SDS-PAGE in *Celosia cristata* and *Brassica campestris* is due to the structural features of fused proteins (Gholizadeh et al. 2005; Misaka et al. 1996).

Purification of the fused protein was performed by single step process using maltose-binding affinity chromatography. The purification yield was spectrophotometrically detected to be about 39 mg/l of bacterial cell culture. This high purification yield may confirm the excellency of pMALc2X expression and purification system for the over-production of maize cystatin in bacterial cells.

Fig. 5 Assessment of the antioxidant status of recombinant bacteria. Using FRAP test, the antioxidant capacity of the recombinant induced cells were analyzed. 1: non induced non-recombinant bacteria; 2: induced non-recombinant bacteria; 3: non-induced recombinant bacteria and 4: induced recombinant bacteria. As the result shows, the antioxidant ability of the induced recombinant cells is significantly ($p \leq 0.05$) increased as compared to the control samples

Table 2 Antioxidant activity of purified fused cystatin

	FRAP value ($\mu\text{mol Fe}^{\text{II}} \text{L}^{-1}$)	Relative activity
Ascorbic acid	118 ± 1.8	1.0
BSA	ND	ND
FP	ND	ND

Values are mean $\pm$ SD; n=3; ND: Not Detected

In vitro inhibitory activity of fused protein

Inhibitory activity of the expressed fused product was tested on papain as a model cysteine proteinase from *Carica papaya*. The activity of papain was analyzed spectrophotometrically at 540 nm as described formerly. Results showed that there is an inverse relationship between fusion product concentration and papain activity (Fig. 2). Papain activity decreases while fused protein concentration increases. No changes were observed in the absorbance of control solutions (one without fusion product and the second containing *E. coli* cell lysate carrying vector alone). This result proves that expressed fused protein is act as cystatin. Therefore, it can be concluded that the correct conformation of fused product remains intact during the expression and purification procedure and the prepared protein can be suitable for further structural or functional studies. Using discovery studio proteomics analysis software (<http://accelrys.com/products/discovery-studio/protein-modeling-sequence-analysis.html>) the schematic three dimensional structures of papain, maize cystatin and MBP-fused maize cystatin as well as their stable interactions have been computed. The results confirmed that the molecular interaction between papain and non-fused cystatin is similar to its interaction with MBP-fused cystatin (Fig. 3).

Stability of fused protein and papain complex

One of the reversible enzyme inhibition mechanisms is based on the covalent binding of enzyme and the special inhibitor. However, there are reports concerning the incapability of some cystatin molecules in stable interaction creation with papain (Machleidt et al. 1989; Bobek et al. 1993). In some cases, stable complex formation between fused cystatins and papain has been reported (Gianotti et al. 2006). In the present work, the non-reducing SDS-PAGE electrophoresis method was performed to identify the stability of the maize fused cystatin and papain complex. The SDS-PAGE product was then transferred to a nitrocellulose membrane and probed with an antibody against MBP. The results of Western blot assay showed the presence of only one signal on the blot paper. Comparison of blot result with that of SDS-PAGE gel confirmed that western signal corresponds to the expressed maize fused cystatin (Fig. 4). Since no additional signal was observed on the blot paper, therefore the possibility of stable complex formation

between maize fused cystatin and papain was ruled out. These results confirm the characteristic of a new plant cystatin “named CaneCPI-3” identified from sugarcane. In contrast, purified recombinant CaneCPI-2 has been reported to form stable complex with papain enzyme (Gianotti et al. 2006).

Changes in the antioxidation status of the recombinant cells

Our experiment results demonstrated that there is no significant difference between the growth patterns of bacteria under IPTG induced and non-induced conditions (data not presented). Therefore, it was deduced that fusion protein has no inhibitory effects on recombinant bacterial cell growth. Interestingly, analysis of the antioxidant status of the induced and non-induced cells using FRAP test revealed that during the induction period, the total antioxidative status of the recombinant cells is increased as compared to non-recombinant or non-induced cells (Fig. 5). On the other hand, non induced and induced non-recombinant bacteria show the similar pattern of antioxidative status. This indicates that the elevation in the bacterial antioxidation is not because of maltose-binding protein.

Since the purified fused product did not show the antioxidant activity in vitro (Table 2), it was predicted that the changes in the antioxidative status of the induced recombinant cells may be an indirect metabolic adaptation process in order to cope with the heterologous plant cystatin expression or activity in *E. coli* cells. However, antioxidative response can be as a result of over-production of every MBP-fused protein in induced *E. coli* cells and therefore it can be considered as a generalized response. Therefore, it remains to be explained whether whether antioxidation response is a response of general over-expression or is a specific response to cystatin over-expression.

Besides this, more clarity is needed to show how the fused cystatin alters the antioxidative ability of the bacterial cells. Further investigations are needed to shed light on the possible crosstalk and interplays between proteolytic and oxidation systems that may be existing in the recombinant cells.

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