IN VITRO STUDY OF THE CCMV CAPSID PROTEIN: CLONING, EXPRESSION, AND PURIFICATION

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In our current work we studied the expression of the Cowpea chlorotic mottle virus (CCMV) capsid protein in E. coli host cell. The CCMV proteins DNA was cloned into a vector containing a His-tag and a ubiquitin fusion partner. The gene was transformed in E. coli BL21(DE3) Rosetta cells and expressed. The expression process was optimized and shake flask expression was conducted on 37°C and 0.1 mM IPTG concentration. Cloning and expressing of CCMV capsid protein was successful, confirmed by sequencing results. The protein was purified with Ni-affinity chromatography.

Keywords: cloning, heterologous expression, CCMV virus capsid, affinity chromatography

1. Introduction

Virus-like particles (VLPs) are self-assembled protein structures with high similarity to the natural form, and with lack of genetic material. Empty virus capsids can encapsulate different molecules, bind targeting groups to a specific cell and they have numerous applications in virus research, medicine or drug discovery[1][2].

Cowpea chlorotic mottle virus (CCMV) is investigated for decades due to its ability to form fully functional empty capsids starting from monomers [3]. Studies for understanding the process of self-assembly in detail was carried *in silico*[4], *in vitro*[5] and *in vivo* [6] with significant results, however many questions remain open.

The CCMV is the member of the *Bromoviridae* virus family [7]. It contains icosahedral virus particles and four positive-sense, single stranded viral RNAs. The CCMV virus can have several polymorphic forms, depending on the chemical environment [8]. Changes in ionic strength, temperature, pH can affect

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the assembly of virus capsid. Decreasing the pH at high ionic strengths makes possible the *in vitro* formation of empty capsids. This phenomenon allows for studying how protein-protein and protein-RNA interactions affect the assembly.

The structure of CCMV coat protein has been determined by X-ray crystallography [9]. The quaternary structure (biological unit) presents 12 pentameric and 20 hexameric capsomers, with a triangulation number T=3 [10].
The CCMV capsid has an icosahedral topology and consist of 180 identical capsid proteins. The outer diameter of the capsid is 28 nm. The molecular weight of the capsid protein is 20 kDa [9].

Heterologous expression of the CCMV coat protein was studied in different systems. Zhao et al used E. coli host cells to express a full length and a deletion mutant form of the coat protein [5]. Producing several deletion mutants proved the importance of N- and C-terminal arms in the formation of the capsid. The C-terminal tail assures the stability of intercapsomer contacts while the N-termini stabilize the hexameric capsomers. In 2004, a yeast-based *Pichia pastoris* heterologous expression system was tested resulting a higher efficiency of expression [11]. Phelps et al introduced the *Pseudomonas fluorescens* expression system to produce coat proteins and VLPs with higher yields by bacterial fermentation [12].

The aim of our study is the expression of the CCMV capsid protein *in vitro* and study the dimerization of the protein and the self-assembly of the empty capsid. The protein will be expressed in *Escherichia coli* using a ubiquitous plasmid and purified for further investigations [13].
The designed gene is purchased from commercial agents and cloned into a *ubiquitin-pET 19b* based vector. The plasmid will be transformed in *E. coli BL21(DE3) Rosetta* host cell line for heterologous expression on small scale.
The built-in His-tag makes possible the purification of the proteins with batch nickel affinity chromatography and gel filtration column chromatography to achieve pure protein [14].

In our future work we will perform the digestion of the fusion protein with *ubiquitin C-terminal hydrolase 1* enzyme to extract the CCMV capsid protein and we will study the assembly of the CCMV capsid.

2. Methods

2.1. Bacterial transformation

pUC57 plasmid containing the CCMV proteins DNA was transformed into *E. coli Top10* cloning cell line. The protocol was adapted from Addgene [15]. 5 µL of DNA was mixed with 20 µL *E. coli Top10* competent cell and incubated on ice for 30 minutes. The tubes containing the mixture were kept on 42 °C for 60 secs to heat shock. After 2 minutes on ice 250 µL of LB was added and grown on

37 °C for 45 minutes in shaking incubator. Cultures were grown overnight on 37 °C.

2.2. Plasmid cloning by restriction enzyme digest (subcloning)

The mixture for digestion was prepared similarly for the PUC57 and pUBIQ: 2 µL B buffer, 1,5 µL SaCII, 1,5 µL BamHI and 5 µL UP with 5 µL PUC, respectively 10 µL pUBIQ in 20 µL final volume. After digestion both samples were purified by agarose gel electrophoresis. The vector (pUBIQ) and insert (PUC57) were extracted from the gel with *Thermo Scientific GeneJET Gel Extraction Kit*. During the ligation bonds are formed between the 5'-phosphate and 3'-hidroxyl ends of the DNAs, catalyzed by ligase enzymes. The ligation process was performed simultaneously for 2 hours on 25 °C and overnight on 16 °C.

The next step is the transformation of the new plasmids into a *E. coli Top10* competent cell. 50 µL competent cell with 5 µL plasmid was submitted to heat shock on 42°C for 60 sec then shaked on 37°C.

The desired colony of cells were isolated with Plasmid Miniprep Kit (Thermo Sc.).

A diagnostic digest was performed with SaCII and BamHI restriction endonucleases as described previously.

2.3. Shake flask test expression

The selected colonies were transformed into *E. coli BL21(DE3) Rosetta* host cell line. Cells were grown on LB agar plates, containing kanamycin and a well isolated colony was inoculated in 10 mL of LB medium with 30 µg/ml kanamycin and incubated for 4 hours at 37 °C in a shaking incubator.

The cell culture was transferred to 200 mL of LB medium and grown to OD_{600}=0.7. Expression was induced with 0.8 mM IPTG and incubated for 3 hours on 37 °C. Culture broth was centrifuged for 10 minutes on 20000 RPM and pellets were stored on -80 °C until further processing.

Cell pellets resulted from the expression were treated with 10 mL of lysis buffer (1X Phosphate Buffer Saline, 100 nM PMSF, 2 mM of DTT).

Bacterial cell suspensions for later use were stored at -80 °C. 600 µL of the liquid culture was mixed with 80% v/v sterilized glycerol to a 15% v/v final concentration. To revivify the cells 200 µL from the stock was introduced in 5 mL sterile LB media and incubated on 37 °C for 3 hours.

2.4. Protein expression optimization

To maximize the protein expression of the bacterial cells we planned an optimization process with changing the IPTG concentration and the temperature as shown in Table 1. The experiment was conducted parallel for the samples selected from digestion results.

Table 1

Optimization process as fcunction of IPTG concentration and temperature

IPTG concentration	Temperature	IPTG concentration	Temperature
0.1 mM	18 °C	0.1 mM	37 °C
0.5 mM	18 °C	0.5 mM	37 °C
1.0 mM	18 °C	1.0 mM	37 °C

Protein purification

4 mL of protein sample was loaded onto a HisTrap HP 5 mL column with Akta Purifier system were used. During the purification samples were collected based on the chromatogram and analyzed with SDS-PAGE. The protein contained a 10X His tag, therefore we purified the protein with nickel affinity chromatography. NTA-agarose beads were used on a column in a 1:10 NTA beads: protein ratio. The beads were introduced into the column and washed with 10 column volume (CV) washing buffer. Sample was added to the beads and rolled for 40 minutes on 4 °C. The flow through was saved and 1 mL washing buffer was added 8 times, followed by elution for 8 times. Fractions of 1 mL were saved and analyzed with SDS-PAGE.

3. Results

The gene of CCMV protein was cloned in pUC57 cloning vector with Xba1 and BamH1 restriction sites (Fig. 1).

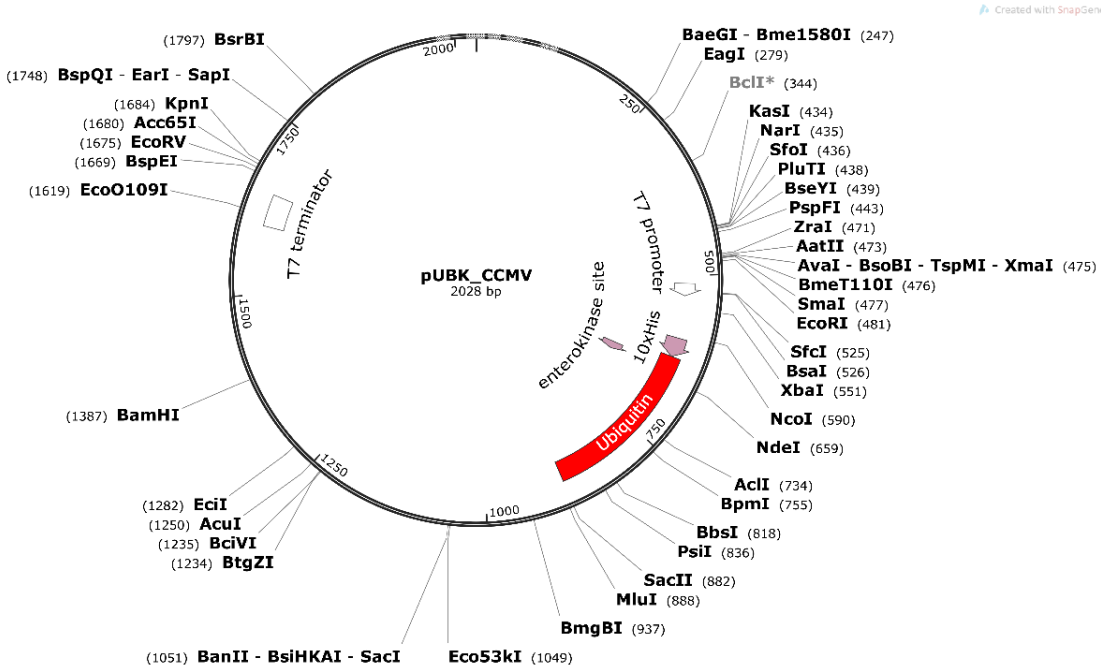

Fig. 1 Plasmid map for pUBIQ_CCMV

The gene was subcloned in a pUBIQ vector (pUBIQ_CCMV). The 10X His-tag makes possible the purification of the protein with nickel-affinity chromatography.

The plasmid contains an ubiquitin that assures the detection and increase the biological activity by affecting the solubility of the protein [16]. One main advantage of the ubiquitin is the fact that after the purification of the expressed protein the fusion partner can be removed using a hydrolase enzyme. Due to the strict recognition of the ubiquitin by the enzyme, the His-tag is also removed. Cloning of the plasmid containing the CCMV proteins DNA was followed by separation of the vector and the insert. After the digestion reaction and purification, the concentration of the vector (pUBIQ) was 23,869 µg/ml and for the insert (CCMV) 1.56 µg/ml. During the ligation reaction the vector and the insert prepared with digestion were combined.

Recombinant plasmids were identified with double digestion reactions, as described previously, in 13 identical mixtures (Fig. 2). Two samples (lane 9 and 10) were selected for further processing. To confirm the selection of the samples, gene sequencing was performed. Sample 1 (lane 9) showed an increased similarity to the theoretical one, thus this stock was used for expression.

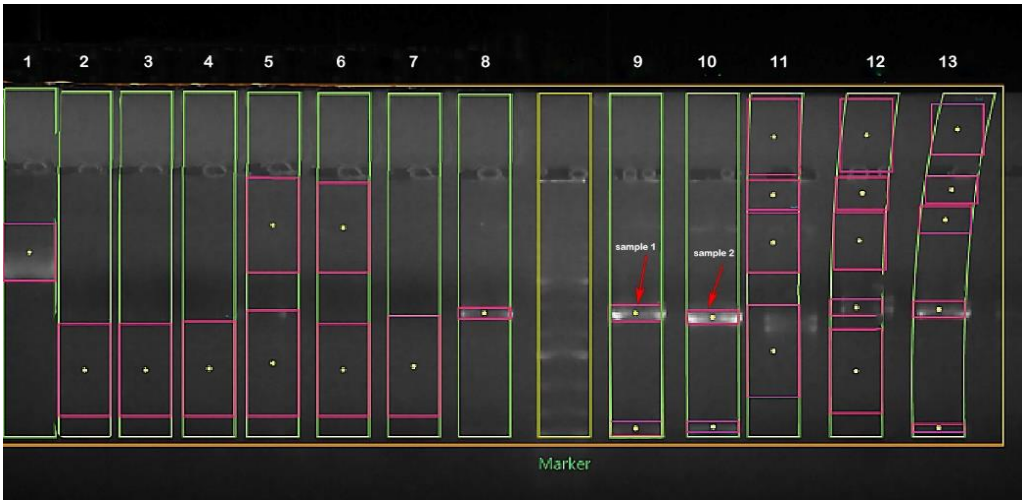

Fig. 2 Results of double digestions for the 13 samples

The selected cell stocks were used for protein expression. The shake flask test expression was successful in *Escherichia coli* BL21(DE3) host cell line, performed until OD_{600}=0.7. After shake flask test expression, we planned an optimization to maximize the expression yield. The SDS-PAGE results showed that the optimal conditions are 0.1 mM IPTG concentration and 37 °C incubation temperature (Fig. 3, lane 9). Expression of protein was repeated on 37 °C, induced with 0.1 mM IPTG and collected for purification.

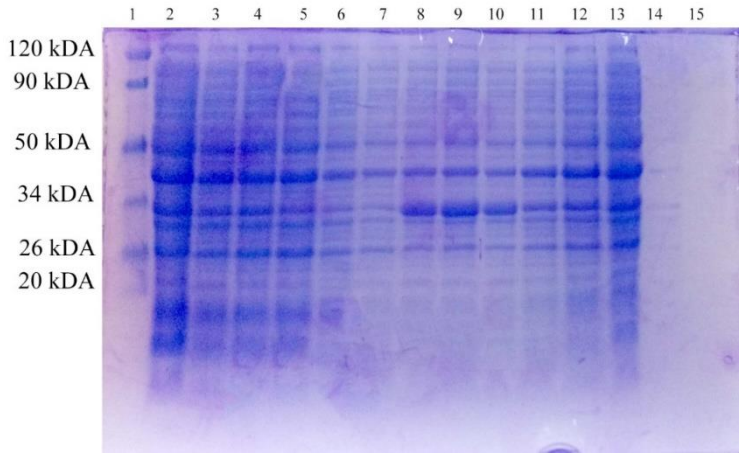

Fig. 3 SDS-PAGE for optimization of pUBIQ_CCMV expression

Fractions of 1,5 mL were saved and fractions belonging to the major peaks showed on the chromatogram (Fig. 4) were collected separately: green (CCMVI) and red (CCMVII). The fractions containing the proteins were dialyzed and stored for further analysis.

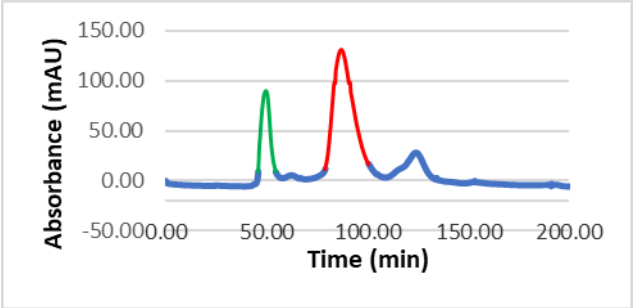

Fig. 4 Chromatogram for the purification of pUBIQ_CCMV protein expression

Results of the FPLC purification were verified by SDS-PAGE. The proteins have been separated properly; however other proteins were bound beside the His-tagged CCMV protein.

4. Conclusions

Cloning of the plasmid and expressing the pUBIQ_CCMV was successful, confirmed by sequencing results. Expression was optimized with a series of experiments with changing the expression temperature and the concentration of inducer. The protein was purified with Ni-affinity chromatography.

CCMV capsid protein can be produced with the plasmid construction presented, by heterologous expression. As this method is common and efficient, high yield of protein can be achieved.

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