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Processing of multimer FMD virus VP1-2A protein expressed in *E. coli* into monomers

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Expressions of several genes in bacteria were carried out by independent promoter. However, in case of eukaryotes ribosome skipping and introduction of IRES are employed as alternative to multiple translation initiation. Foot and mouth disease virus (FMDV) 2A peptide has been widely used for co-expression of multiple genes in eukaryotic, plant and mammalian systems. The 18 amino acid 2A peptide of FMDV facilitates efficient co-translational dissociation of the polyprotein into discrete protein products. To study the role of 2A in multimeric protein production a construct consisting of tandem repeat of 4 units of C- terminal VP1 linked through 2A sequence was made and expressed in *E. coli*. Along with tetramer protein, trimer, dimer and monomer proteins were produced. Stability studies showed that the tetramer protein was cleaved to smaller monomer on storage. The results provide scope for using FMDV 2A for expressing multiple genes under a single promoter in prokaryotes.

Keywords: Expression, Foot and mouth disease, Multigene, 2A Peptide, Proteolysis

Foot and mouth disease virus (FMDV), a picornavirus with an RNA genome encodes a single poly-protein which is cleaved in the host cell to produce different protein products[1]. A self processing activity which has been later identified as ribosomal skip has been reported in FMDV between the terminal glycine of the 2A and the initial proline of 2B. FMDV 2A protease is 18 amino acids long and when engineered as a fusion protein, has been shown to mediate cleavage both *in vitro* using eukaryotic cell free translation system[2] and *in vivo* in plants[3] and cultured mammalian cells[4]. This 2A sequence is compatible with different sub-cellular targeting signals and can be used to co-express up to four proteins[5]. In the present study application of 2A peptide in production of multicopy protein in bacterial system has been reported FMDV gene coding for immunoreactive, C- terminal portion of VP1 (270 bp) along with 2A was linked in tandem repeat of four (tetramer) and was expressed in *E. coli* cells. The hexahistidine tagged protein was purified by nickel affinity chromatography and studied for its stability upon storage.

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Materials and Methods

PCR Amplification and cloning of FMDV VP1-2A—Primers used for amplification of the sequences corresponding to C-terminal half of VP1 along with 2A have been reported elsewhere[6]. The forward primers were included with KpnI (along with His-tag), BamHI, EcoRI and HindIII, while the reverse primers carried BamHI, EcoRI, HindIII and NotI. The gene for FMDV- P1-2A of 'O' serotype available in the plasmid pBO-P1-2A in the lab was used as the template DNA amplication. The 270 bp C-terminal VP1 along with 54 bp of 2A and 6 bp of 2B of FMDV 'O' serotype were amplified using specific primers with different restriction sites (Fig. 1). The vector pET32a and fragment 1 was cut with KpnI and Bam HI and gel purified (Minielute gel extraction Kit, Quiagen). The digested fragments and linearised vector were ligated by T4 DNA ligase to yield a construct, which were transferred into *E. coli* DH5α cells in LB with 50 µg of ampicillin. Screenings of the recombinant colonies were carried out by PCR and restriction enzyme digestions. The recombinant plasmid releasing gene fragment of 330 bp upon digestion was used as vector for the cloning of second fragment. The above steps were repeated two more times to get plasmid carrying 4 copies of the gene linked in frame. The plasmid with tetrameric gene

construct was named pET1.3 (O4) and was bidirectionally sequenced to confirm the correct orientation and frame.

Expression and characterization of protein —Expression of protein was carried out in *E. coli* BL21 (DE3) pLysS cells which were made competent for transformation by treating with calcium chloride[7]. The cloned gene was induced for expression as per manufacturer's protocol with suitable modification. *E. coli*, BL21 (DE3) pLysS carrying the pET1.3 (O4) plasmid were grown in 8 ml LB medium for overnight in presence of 50 µg/ml ampicillin. Overnight culture 250 µl was inoculated to 25 ml fresh LB and grown till OD reached 0.9 to 1.0 at A540. The culture was centrifuged at 6000 g for 5 min and the cell pellet was resuspended in 25 ml of fresh LB. Cells were induced for expression with IPTG (isopropyl-beta-D-thiogalactopyranoside) 1m*M* in presence of rifampicin (200 µg/ml) for 1-4 hr at 30°C.The cells were pelleted at 10,000 g for 5 min and resuspended in 3 ml of TE containing 10 m*M* PMSF (phenyl methyl sulfonyl fluoride) and stored at -70°C till further analysis. The lysate was used as a source of expressed protein for purification and SDS-PAGE analysis.

For laboratory scale production of recombinant protein, 100 ml of bacterial culture was induced as described above. The protein was affinity purified from whole bacterial lysate using His+-Ni chelation agarose column (Sigma) under denaturing conditions, as per the manufacturer's protocol. An aliquot of 20 µl from each fraction was mixed with equal volume of 2X sample loading buffer and subjected to 12% SDS-PAGE analysis along side molecular weight marker as described by Laemmli[8]. The pooled protein fractions were concentrated by polyethylene glycol and dialyzed against PBS (Phosphate Buffered Saline) and stored at -70°C.

Results and Discussion

Tetramer of 270 bp gene coding for C-terminal half of VP1 along with C-terminal fusion of 2A-2B coded by 60 bp fragment has been constructed (Fig. 1). The recombinant plasmids were confirmed by release of insert upon RE digestion and nucleotide sequencing.

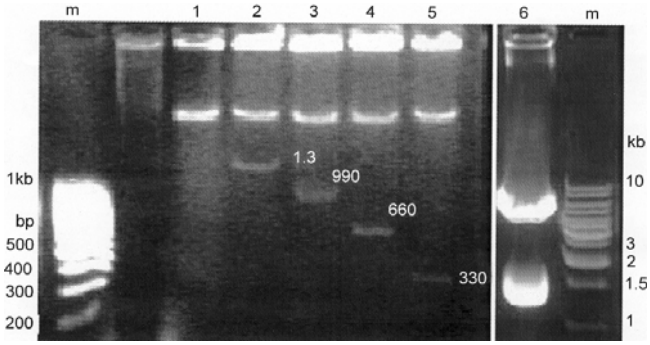

Fig. 2—Restriction digestion analysis of pET1.3(O4) clone DNA in 1.3% agarose gel: 2µg of pET1.3(O4) DNA was digested with KpnI and aliquot into 5 equal parts. Four parts were separately digested with NotI(Lane2), HindIII(Lane3), EcoRI(Lane4), BamHI(Lane5) showing release of tetramer 1.3 *k*b, trimer 990 bp, dimer 660 bp and monomer 330 bp; Lane 1 shows linearised pET1.3(O4) DNA with KpnI; Lane 6 shows pET1.3(O4) digested with KpnI and NotI releasing 1.3 *k*b tetramer insert; Left m– 100 bp marker. Right m– 1 *k*b marker.

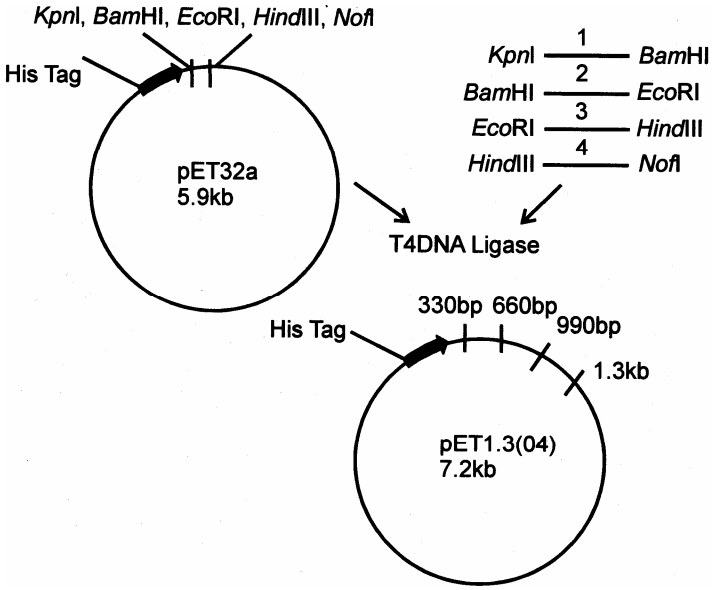

Fig. 1—Strategy of cloning of FMDV VP1 C-terminal half 270 bp along with 2A linking as tetramer in pET32A vector.

Double digestion of recombinant plasmid DNA with KpnI in combination with BamHI, EcoRI, HindIII and Not I have resulted in the release of 330, 660, 990 bp and 1.3 kb inserts (Fig. 2) respectively which correspond to monomer, dimer, trimer and tetramer of the VP1-2A. Sequence analysis of the cloned fragment showed that the insert was in frame with ATG of the vector, with His tag at 5′ end of vector and 2A peptides at C- terminus of each fragment.

Expression of the tetramer pET1.3 (O4) gene was induced by the addition of 1 m*M* IPTG. Upon SDS-PAGE analysis of the affinity purified proteins from the cloned bacterial lysate showed four protein bands of size 66, 54, 42, 30 kDa corresponding to the pET32a carrying 4, 3, 2 and 1 copy of the insert, respectively (Fig. 3). Each monomer of insert of size 330 (270+60) is expected to code for 110 amino acids giving rise to protein size of 48 kDa (12x4) in case of tetramer. However the observed size was 66 kDa owing to the presence of fusion tag of 18 kDa at N terminal end. The intensity of 66 kDa protein band was high when induction was carried for a period of 1 to 2 hr. However, on increasing the time period of induction, the concentration of 66 kDa protein was reduced and additional lower size protein bands were observed. The affinity purification of the 66 kDa protein was found possible if the induction was allowed for 1 to 2 hr. The quantity of expressed protein was found to be 5 mg/l. However, longer the duration of induction or storage of the induced lysate for a period of one week at -70°C had resulted in the purification of 30 kDa protein which may consist of 18 kDa fusion and 12 kDa monomer (Fig. 4). As reported in eukaryotic cells, it was presumed that the release of 30 kDa protein might be due to ribosomal skipping mechanism of 2A peptide[10]. Besides complete tetramer 66 kDa protein was also expressed along with trimer, dimer and monomer proteins. Also longer the induction period the concentration of tetramer protein was reduced with the simultaneous increase in the concentration of smaller proteins. This indicated that the protein was cleaved to monomers with N-terminal fusion tag 30 kDa, dimer 42 kDa and trimer 54 kDa. Since the His tag is at 5' end of protein it facilitated the affinity purification of proteins along with 18 kDa fusion tag. The cleaved fragment present downstream of first monomer did not carry the His tag, so protein of size 12 kDa, 24 kDa and 36 kDa could not be recovered by affinity column.

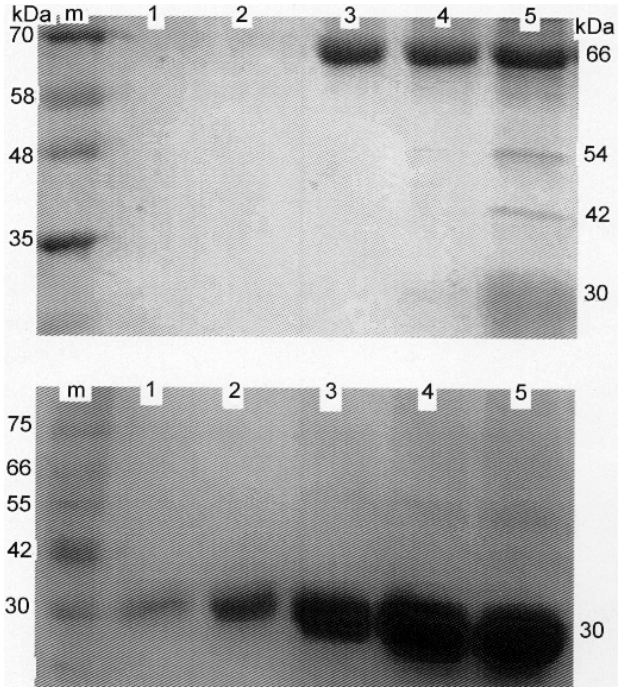

Fig. 3—SDS-PAGE analysis of affinity purified protein: pET1.3(O4)clone *E. coli* cells after induction lysed by freeze thawing and the cell lysate was affinity purified: (*A*) Affinity purified fractions from 1hr of induced cell lysate: Lane 1 and 2 initial fractions contain no protein; Lane 3 and 4 show protein band of tetramer 66 kDa; Lane 5 shows tetramer 66 kDa and lower intensity trimer 54 kDa, dimer 42 kDa and monomer 30 kDa; m molecular weight marker. (*B*)Affinity purified fractions from 4 hr of induced cell lysate: Lane 1 to 3 show fractions containing monomer 30 kDa protein; Lane 4 and 5 show monomer 30 kDa protein and low intensity of trimer 54 kDa and dimer 42 kDa protein band. *m* molecular weight marker.

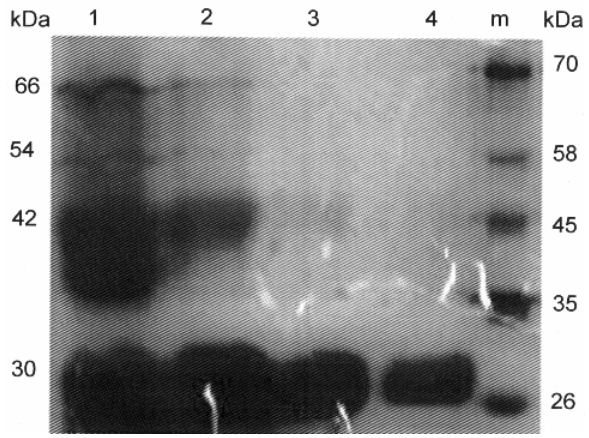

Fig. 4—SDS-PAGE analysis of stored protein at -70°C: Protein fraction from pET1.3(O4) *E.coli* cells 1hr induced and the protein was affinity purified dialysed against PBS(Lane 1- Before storing) (Lane 2-One week after storing); Protein fraction from pET1.3(O4) *E.coli* cells 4 hr induced and the protein was affinity purified dialysed against PBS (Lane.3-before storing); (Lane 4-one week after storing); *M*– molecular weight marker.

It has been reported that polyprotein N-terminal to 2A typically accumulates in excess over the C-terminal portion due to the self proteolysis activity of 2A at C terminal end[9]. Several latest reports confirm that cleavage after 2A is due to ribosomal skipping in eukaryotic cells and 2A has no proteolysis activity[10]. This study reports for first time 2A activity in prokaryotic, system not confirming skipping the co-expression or proteolysis which requires further investigation. The observed proteolysis activity of VP1-2A in prokaryotic cells clearly shows that there is scope of using this junction for expression of multiple genes under a single promoter.

Acknowledgement

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