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Full Length Research Paper

Cloning, codon-optimized expression and homology modeling of structural protein VP1 from foot and mouth disease virus

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Structural protein VP1 of foot-and-mouth disease virus (FMDV) is the most frequently studied protein due to its significant roles in virus attachment, protective immunity, and serotype specificity. The coding sequence of VP1was amplified and then identified by polymerase chain reaction (PCR) and sequencing. To achieve high-level expression of VP1 protein, we optimized VP1 gene base on *Escherichia coli* preferred codons and synthesized the optimized gene. The synthetical gene was cloned into the fusion expression vector pET-28a and expressed in *E. coli* BL21(DE3). After induced with Isopropyl β-D-1-Thiogalactopyranoside (IPTG) and optimized the conditions of expression, the VP1 fusion protein was highly expressed and identified in inclusion bodies by SDS-PAGE and Western blotting. Based on the primary and secondary structure analysis of VP1, Three-dimensional structure of VP1 was developed by homology modeling methods. The validation of 3-D structure was done with the help of PROCHECK encompassing amino acid residues in the most favored region of almost all strains. Potential epitopes of VP1 was predicted with different methods. In this study, the VP1 protein was expressed in *E. coli* efficiently and highly purified VP1 was obtained, which laid a foundation of refolding and further study on activity of the protein. The VP1 model in the productive conformation can now be used for structure-based design purposes as well as structure-function relation of VP1 protein.

Key words: Foot-and-mouth disease virus, VP1 protein, codon optimization, homology modeling.

INTRODUCTION

Foot-and-mouth disease virus causes a highly contagious infectious disease foot-and-mouth disease (FMD) in cloven-hoofed animals (Xiao et al., 2007). This disease is widely epidemic all over the world and often results in considerable economic losses and dangers in the health of people. foot-and-mouth disease virus (FMDV) is the prototype member of the Aphthovirus genus of the family Picornaviridae. The virus exists in the form of seven different serotypes: A, O, C, Asia1, and South African Territories 1 (SAT1), SAT2, and SAT3, but a large number of subtypes have evolved within each serotype (Domingo et al., 2003). The capsid of FMDV is constituted of 60 copies each of the four proteins VP1, VP2, VP3 and VP4, in which VP1, VP2 and VP3 form the capsomeres, VP4 is inside of virus particle. The mutation rate of VP1 gene's nucleotide is the most highest in four structural protein (Knowles et al., 2003). The VP1 capsid protein is the dominating antigen, in which the prominent G-H loop, spanning residues 134-158, has been identified as the major immunogenic site for neutralizing antibodies (Collen et al., 1991). This is the main antigenic epitopes of FMDV and has formed the basis for the peptide approach to vaccination against FMD. In addition to this, the VP1 gene has become an ideal target for studies of genetic relationships among different isolates (Stram et

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al., 1995).

Antigenic variation within a serotype is a mechanism by which FMDV variants emerge to escape host immunity (Van Phan et al., 2010). Among the seven serotypes of FMDV, serotype A is antigenically one of the most divergent strains, which makes control by vaccination very difficult (Kitching, 2005). FMDV type A is classified globally into 10 major genotypes based on the VP1 gene sequence, with over 15% nucleotide divergence among the genotypes to date (Tosh et al., 2002). The effective expression of VP1 gene of FMDV type A is beneficial for not only the study of genetic variation stability of FMDV type A but also the study of epidemiology, the global tracing of virus movements, the clinical diagnosis and development of new vaccine. A high-level expression is needed for further research. Although many studies have been conducted on the expression of VP1, expression levels of genes has been low. This is because wild type VP1 genes sometimes can not effective expression in *Escherichia coli* due to differing codon usages between *E. coli* and original host. Therefore, an efficient and facile-to-use recombinant expression mode and system is required, for which *E. coli* appears to be the system of choice because of easy handling, fast growth, and high-level expression of proteins (Baneyx et al., 1999; Makrides, 1996). To overcome this problem, we used codon-optimized synthetic genes to expression the functional proteins after obtained wild type gene sequences. The versatility and efficiency of this approach was demonstrated already in literature (Wu et al., 2006; Ting et al., 2009; Rainer et al., 2008; Wang et al., 2010; Ji-Hoon et al., 2010; Rasheda et al., 2010).

The 3-D structure of VP1 protein of FMDV type A is unknown so far. Homology modelling is a reliable technique that can consistently predict the 3D structure of a protein with precision akin to one obtained at low-resolution by experimental means (Martin-Renom et al., 2000). This technique depends upon the alignment of a protein sequence of unknown structure (target) with that of a homologue of known structure (template) (Arnab et al., 2010). Since the study of 3D structure of a protein is helpful in recognizing the details of a protein, this method is increasingly becoming of widespread use in the field of bioinformatics (Paramsivasan et al., 2006). This work contributes to understanding of conformational and structure-function properties of VP1protein of FMDV type A.

MATERIALS AND METHODS

Virus and reagents

The FMDV A/F/72 strain was maintained in State Key Laboratory of Veterinary Etiological Biology. The RNeasy Mini Kit and DNA purification kit was obtained from QIAGEN (Germany). T4 DNA ligase, JM109 competent cells and pGEM-T easy cloning vector was obtained from Promega Corporation (Madison, Wisconsin, USA). Restriction endonucleases, polymerase, dNTP, IPTG and DNA and protein weight markers were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. (Dalian, China). The expression vector pET-28a, *E. coli* BL21 and Ni-NTA His Bind resin were obtained from Invitrogen corporation (California, USA). HRP-labeled goat anti- mouse serum was purchased from Sigma (St. Louis, Missouri, USA).

Reverse transcription-polymerase chain reaction (RT-PCR) and cloning of VP1 gene

Total RNA of FMDV was isolated from virus species of our laboratory. We designed and synthesized a(TaKaRa, Dalian, China) Pair of primers: Forward: 5′-CACAAATGTACAGGGATGGGT-3′and Reverse: 5′-GACATGTCCTCCTGCATCT-3′). The complete gene encoding region of the wild type FMDV VP1 protein was amplified by polymerase chain reaction (PCR) (The template was denatured at 95℃ for 5 min, followed by 30 PCR amplification cycles: 30 s at 94℃, 30 s at 55℃ and 72℃ for 1 min, and a final extension at 72℃ for 6 min) using of the pair of primers and then cloned into pGEM-T easy vector. Recombinant plasmids were transformed into *E. coli* strain JM109 for colony polymerase chain reaction (PCR) screening and sequencing.

Gene optimization, synthesis and plasmid construction

The genetic codons of wild type FMDV VP1 gene were optimized according to the web-based program, GENEOptimizer software from GENEART (http://www.geneart.com) (Puigbo et al., 2007). The optimized gene was synthesized and provided by Shanghai Sangon Biological Technology and Services Co, Ltd (ShangHai,China). The synthesized products were digested by BamHI and SalI, then cloning into the expression vector pET-28a to yield the recombinant plasmid pET-28a-VP1. The expression vector pET-28a carry on carry an N-terminal His-Tag. The inserted sequence was confirmed by PCR, restriction enzyme digestion, and DNA sequencing.

Protein expression and purification

The positive plasmid pET-28a-VP1 was transformed into *E. coli* BL21 (DE3) for the expression of the target protein. *E. coli* BL21(DE3) carrying the construct with the VP1 full-length optimized sequence was cultured at 37℃ in culture medium supplemented with kanamycin (50ug/ml) until the absorbance at 600 nm reached 0.5. The culture was induced by adding IPTG at a final concentration of 1 mM for 3 h at 37℃.

The interesting protein was purified by immobilized-metal affinity chromatography (IMAC) using polyhistidine tag. After the bacteria were harvested by centrifugation, the bacteria was resuspended in 50 ml PBS and then lysed by ultrasonication in an ice water bath. The suspension was then centrifuged at 12000 rpm for 20 min at 4℃, and the cell lysate were resuspended in buffer A (6M GuHCl, 50 mM NaH_2PO_4, 500 mM NaCl, pH 8.0). The protein was separated from contaminating proteins by Ni^{2+} chelate affinity chromatography. The supernate was loaded on columns at a flow rate of 1 ml/min after the columns pre-equilibrated with 1 ml buffer A. The protein bound with Ni-Agarose was eluted with a linear gradient of pH (8.0, 6.0 and 4.0) in buffer B (8M urea,50 mM NaH_2PO_4, 500 mM NaCl). The eluent was dialyzed with 300 ml PBS including 10% glycerol and were identified by SDS-PAGE. At the same time, the western blotting was used for checking immunogenicity of VP1 protein after optimal expression were varied or not compared with wild-type VP1 protein.

Homology modeling

The primary structure analysis of VP1 protein was obtained by use of ProtParam tool of ExPASy Proteomics Server (http://cn.expasy.org/cgi-bin/protparam). To be better evaluated, the possible secondary structures of VP1 protein were evaluated using the different web program. Secondary structures were predicted using the program PDBsum (http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/) and the Hierarchical Neural Network (HNN) (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_nn.html) (Arnab et al., 2010).

Homology modeling is a good method for routine structure determinations, which can provide remarkably good results if the sequence identity is high between the interesting protein to be modeled and the template protein (>75%) (Arnold et al., 2006). The first step in homology modeling technique requires recognition of the protein structures linked to the target sequence and the subsequently selection of templates (Centeno et al., 2005). An homologous template was selected on the protein database PDB (Protein Data Bank, http://www.rcsb.org/pdb/home/home.do) and then analysis of amino acid sequence showed that the optimal template protein codenamed 1ZBA1 in PDB was highly homologous (80.9%) with VP1 protein. The three-dimensional structures models of the FMDV VP1 protein were constructed by use of the program Swiss-PDB Viewer, SPDBV (http://www.expasy.ch/swissmod/SWISS-MODEL.html) (Schwede et al., 2003; Guex et al., 1997). The constructed model was evaluated by Ramachandran plot (http://nihserver.mbi.ucla.edu/SAVS/) (Ramachandran et al., 1963). Presence of pockets in the structure was predicted using CASTp server (http://sts.bioengr.uic.edu/castp/index.php) (Dundas et al., 2006).

Prediction of epitopes

In order to predict the B cell epitopes on VP1 proteins, the complete amino acid sequence of VP1was analyzed using the DNAStar Protean system. The surface properties of the structural proteins, such as hydrophilicity, flexibility, accessibility and antigenicity, were analyzed by the methods of Kyte–Doolittle, Karplus–Schulz, Emini and Jameson–Wolf, respectively (Kyte et al., 1982; Karplus et al., 1985; Emini et al., 1985; Jameson et al., 1988).

RESULTS

Cloning of VP1

A fragment of the gene encoding structural protein VP1 from FMDV A/F72 was amplified by PCR using specific primers. The agarose gel electrophoresis showed that the size of PCR product was about 750 bp, which coincide with the length of the expected (Figure 1).The 750 bp PCR product was cloned in pGEM-T easy vector and the recombinant plasmid was confirmed by restriction enzyme (EcoR I) digestion. The result of sequencing analysis showed that the VP1 region of the FMDV A/F/72 strain is 613 bp in length and codes for a polypeptide of 213 amino acids, including the complete open reading frame. Homology search confirmed that the sequence belongs to the coding region of the VP1 and has a high sequence similarity with several other VP1 genes of FMDV type A using the online BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

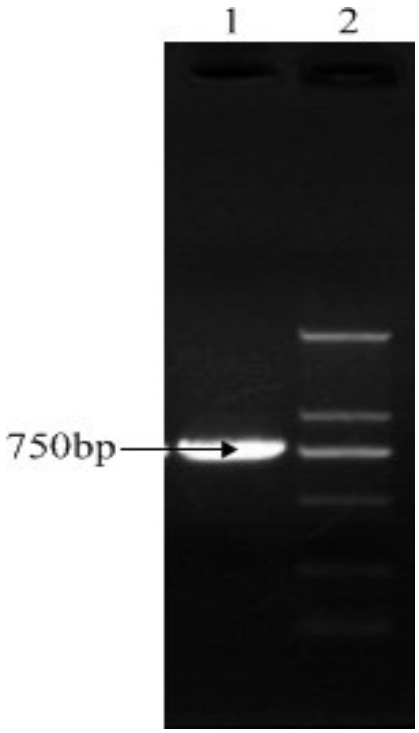

Figure 1. PCR products of the VP1gene, 1. DNA marker, 2. PCR products of the VP1gene.

Codon optimization and synthesis of VP1 gene

Without changing the amino acid sequence, we performed codon optimization for VP1 gene based on the field VP1 gene sequence using *E. coli* preferred codons. Similar to other organisms, *E. coli* uses only some of the 64 genetic codons. Those codons that organisms use most frequently are called optimal codons, while those that organisms rarely use are called rare or low-usage codons. For *E. coli,* low-usage codons include AGA, AGG, CGG, and CGA coding Arg; AUA coding Ile; GGA coding Gly; and CCC coding Pro (Ting et al., 2009). On that basis, a lot of work has been done in the following four areas, eliminating more than twelve repeated sequences, avoid excessive local content of GC or AT, cancel the potential secondary structure and clear away potential splicing sites. With those steps, the adaptive index of gene has greatly enhanced. The codon-optimized VP1 gene also obtained two restriction enzymes digestion site, BamHI and SalI. The result of sequencing showed that synthesized gene agrees with designed. The wild-type sequence and the codon-optimized sequence of the VP1 was translated into amino acids (Figure 2). After the sequence was confirmed to be correct, the target fragment and plasmid pET-28a were both digested with BamHI and SalI, and then ligated with T4 DNA ligase to yield the recombinant pET-28a-VP1-6his.

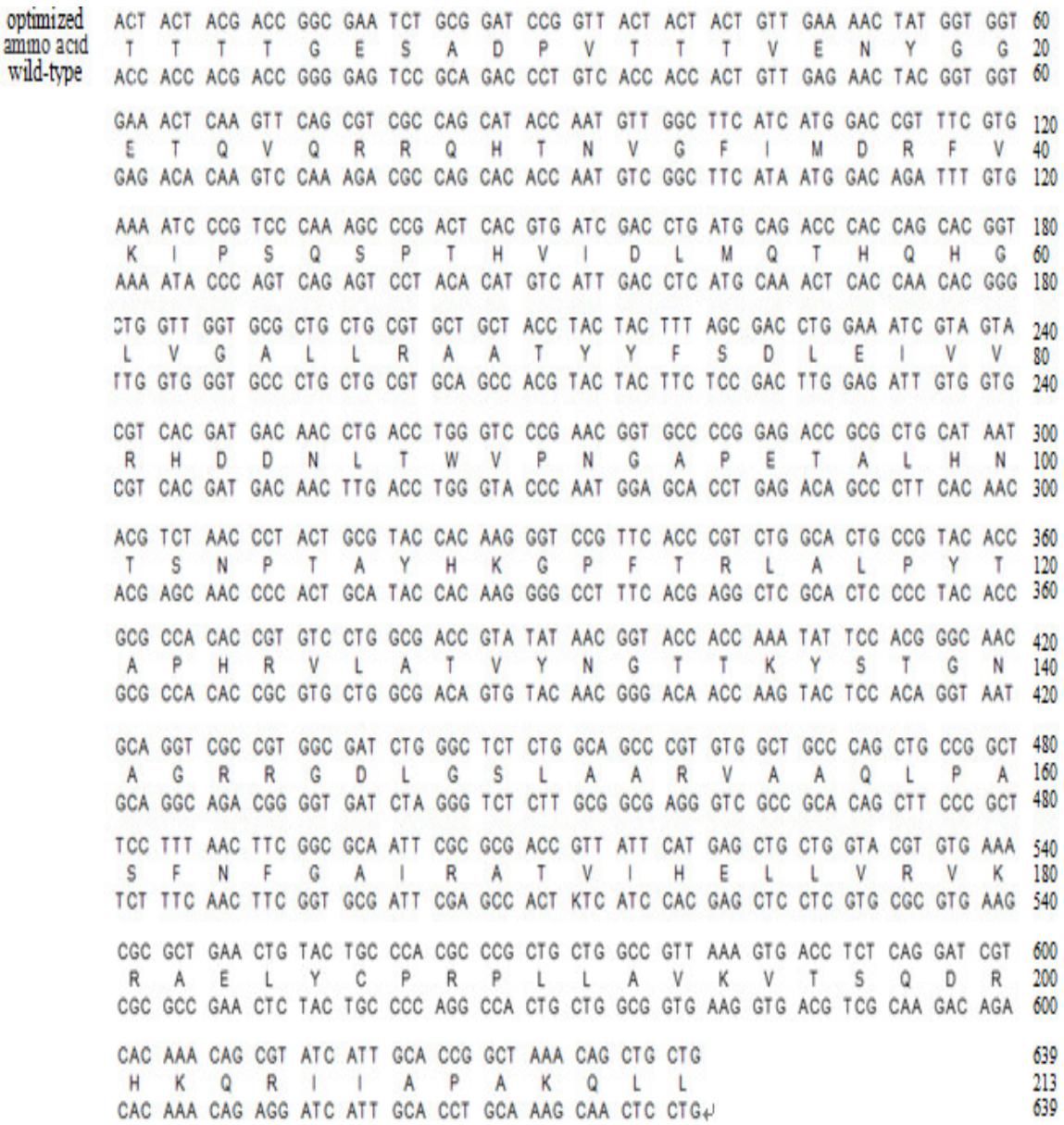

Figure 2. Nucleotide sequence of codon-optimized VP1gene and original VP1gene and their translational amino acid sequence.

Expression and purification of the codon-optimized VP1

The availably construct was transformed into BL21 (DE3) *E. coli*. This strain is a BL21 derivative designed to fit T7 expression system. The recombinant was expressed well in *E. coli* BL21 (DE3). We tested a series of expression conditions that differed in induction time, IPTG concentration, and induction temperature. After tested, conditions of protein expression are determined. The transformants grew fastest at 37℃.The optimum cell density for recombinant induction was reached at OD600 of 0.5 5to 0.6, and a time course and IPTG concentration study established that optimal protein expression occurred 5 h after induction with 1 mM IPTG. The molecular weight of the target protein was about 33 kDa, which was coincident with the theoretical value (Figure 3). SDS-PAGE of the purified VP1 protein showed that there was five bands on the gel and the abundant target protein appeared in the eluent of buffer B, pH 4.0.The recombinant bacterium produced high quantities of the fusion protein VP2, about 36% in total. According to my knowledge, this experiment is the first report of expression of fusion structural protein VP1 of FMDV

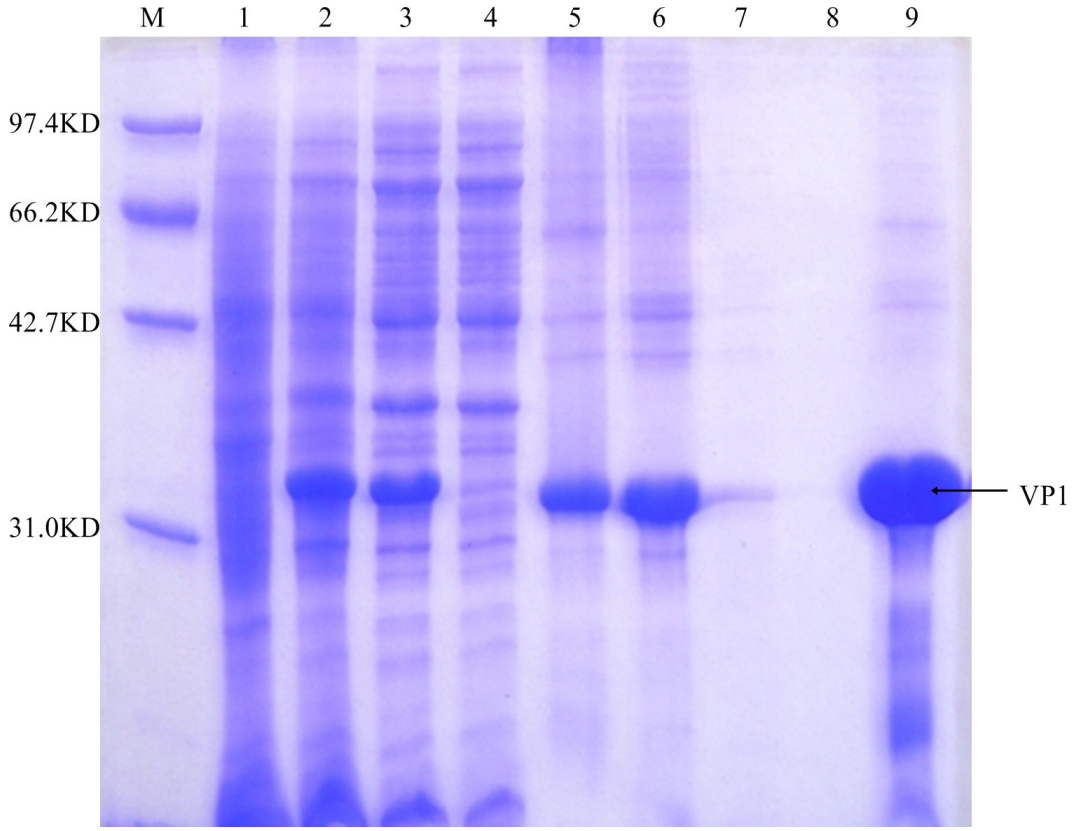

Figure 3. SDS-PAGE Results of VP1 protein. The results show that the structural protein VP1 after optimal expression has the size of 33 kDa, which is consistent with the expected size. 1. Uninduced control, 2. Inducing for 3 h, 3. Disintegrating liquid with ultrasonic, 4. Clarifying after disintegrating liquid with ultrasonic and centrifugation, 5. Purifying sample solution with Ni colum, dissolved inclusion bodies, 6. Purifying flowing liquid with Ni column, 7. Purifying Ni-Denature-8.0 wash solution with Ni column, 8. Purifying Ni-Denature-6.0 wash solution with Ni column, 9. Purifying Ni-Denature-4.0 euate with Ni column.

strain A/F72 in *E. coli.*

Western blot assay

In order to evaluate the antigenicity of the expressed fusion protein, the expressed samples were analyzed by 12% SDS–PAGE and then electrotransferred onto nitrocellulose membrane. In Western blot analysis, The FMDV positive mouse serum has cross-reacted with purified VP1 protein. The results of Western blot shows that the expressed fusion protein has strong immunogenicity.

Protein homology modeling and epitope

The analysis result of the primary structure analysis of VP1 protein indicated that its full length sequence contains 213 amino acid residues, the total molecular weight is 23.4 kD and theoretical isoelectric point (pI) is 9.83. The total number of negatively charged residues (Asp + Glu) and positively charged residues (Arg + Lys) are 15 and 23 respectively. The formula is $C_{1041}H_{1656}N_{308}O_{304}S_3$ and total number of atoms is 3312. The instability index (II) is computed to be 32.97.

The program PDBsum took the automatic algorithm: STRIDE (Frishman et al., 1995), which uses hydrogen bond energy and main chain dihedral angles to recognize helix, coils and strands. This arithmetic was used to predict the secondary structure of VP1. The wiring diagram of secondary structures of VP1 protein are shown in Figure 4. The result of the Hierarchical Neural Network method showed that the percentage of alpha helix, random coil, extended strand were 34.74, 15.96 and 49.30%, respectively.

The VP1 protein contains 213 amino acids, and results obtained with the protein data bank have shown that the template protein in the PDB (code:1ZBA1) has the highest sequence homology, at 80.9%. In this study, the model of VP1 was constructed using the automated homology modeling program Swiss-PDB Viewer, then energy minimization were performed to refined the model. The final total energy values for the VP1 models

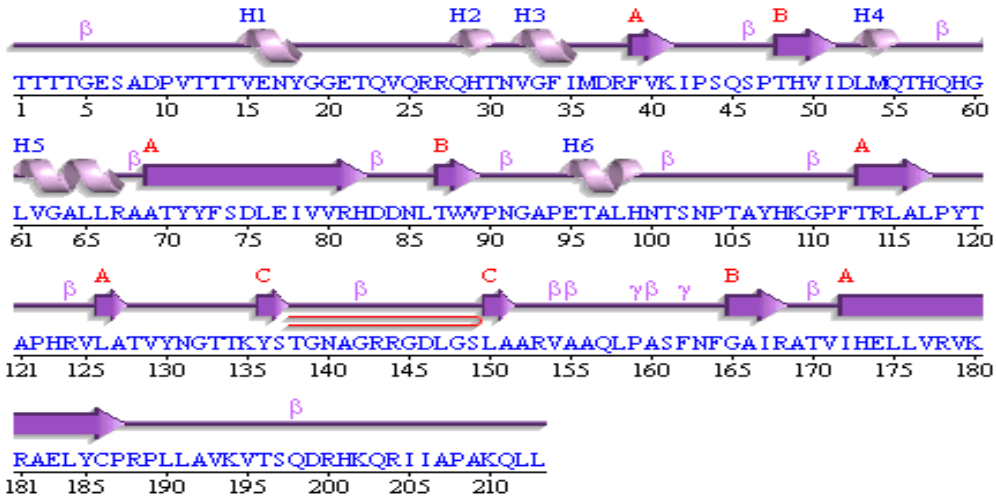

Figure 4. Secondary structure assignment of modeled VP1 protein.

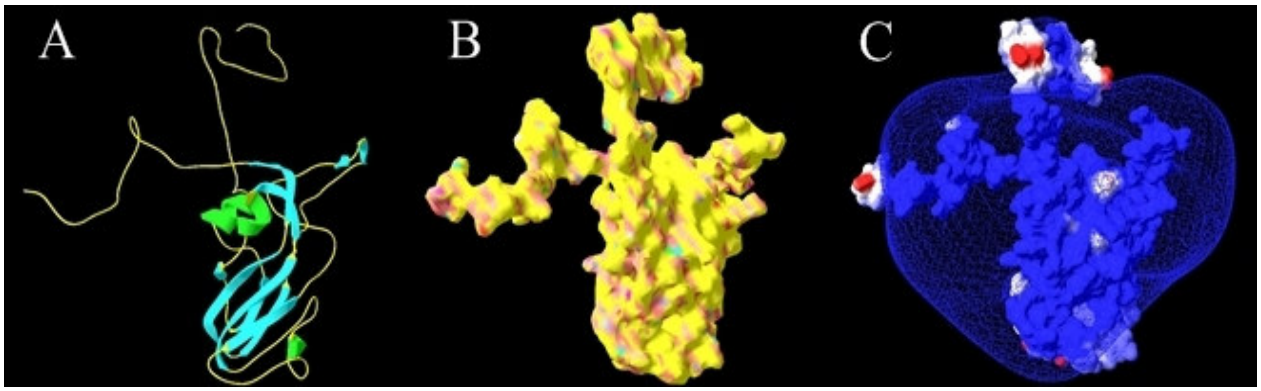

Figure 5. (A) Ribbon diagram of the 3D structures of the VP1 protein. The random coil, sheet, helix and turn are represented by different color respectively. (B) Molecular surface of VP1 protein. Electropositive, electronegative and neutral regions are in blue, red and white, respectively. (C) Mapping of electrostatic potentials on the molecular surface of VP1 protein.

were-5736.827 KJ/mol. The stable 3D structure, molecular surface of VP1 is displayed in Figures 5A and B. We also mapped the electrostatic potentials on the surface of VP1 protein (Figure 5 C). According to the structure character of the model, we found that the spatial structure of the protein VP1 have three typical β-sheet barrel structure and it is main existence mode of β-sheet in 3D structure. The inside and exposed β-sheet are comprised of Consecutive hydrophobic residues and repeating unit of hydrophilic-hydrophobic residues, respectively. The spatial structure of taking the β-sheet barrel as the main rules was appeared regular geometric construction. This structural feature will be propitious to assembling process of virus particles. In the space structure, there is also a small amount of alpha helix and the outside alpha helix there are a lot of hydrophilic amino acids. Hydrophilic alpha helix and β-sheet constitutes the hydrophilic surface of proteins molecular. In addition to this, plenty of random coil have increased surface flexible and antigenicity of protein in a large part.

To validate the homology modeled VP1 structure, a Ramachandran plot was drawn and the structure was analyzed by PROCHECK, a well known protein structure checking program. It was found that the phi/psi angles of 87.0% of residues fell in the most favored regions, 12% residues fell in the additional allowed regions, and 1.1% fell in generously allowed regions; none of the residues fell in the disallowed conformations (Figure 6).

Binding sites and active sites of proteins are often associated with structural pockets and cavities. CASTp server provides identification and measurements of surface accessible pockets as well as interior inaccessible cavities, for proteins and other molecules (Dundas et al., 2006). The result of CASTp server supplied shown that VP1 protein contains 23 pockets.

Variability, fragment mobility, and hydrophilicity are

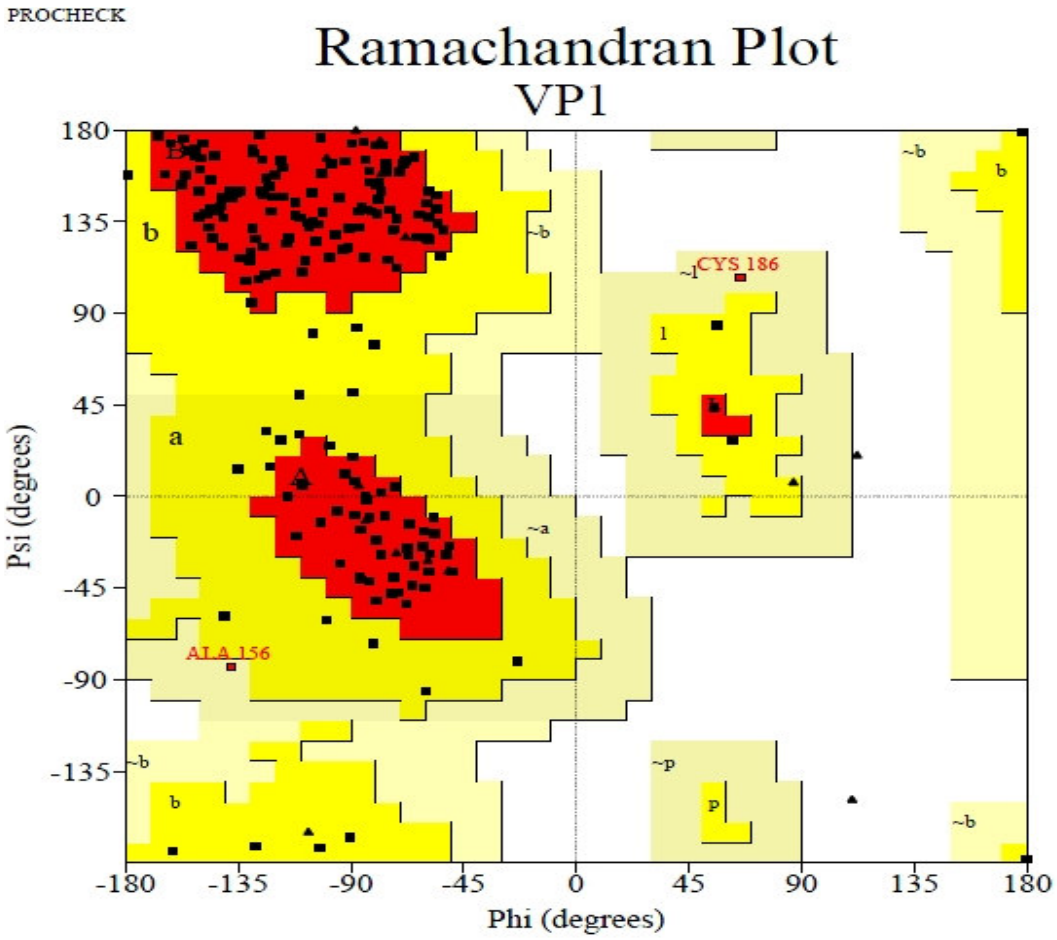

Figure 6. Ramachandran map of VP1 protein. The plot calculation on the three-dimensional (3D) model of VP1 protein was calculated with the PROCHECK program.

important features of antigenic epitopes. A flexibility plot, hydrophilicity plot, surface probability plot and antigenic index for the structural proteins were obtained by the methods of Karplus–Schulz, Kyte–Doolittle, Emini and Jameson–Wolf, respectively (Figure 7). Based on the results obtained with these methods and the secondary structure of VP1, the potential B cell epitopes on VP1 protein were predicted. The results showed that the potential epitopes include VP1a:22-32aa,VP1b:41-50aa, VP1c :94-105aa,VP1d :137-149aa,VP1e:196-205aa. The predicted epitopes to be displayed in the molecular surface of VP1 protein by Swiss-PDB Viewer (Figure 8).

DISCUSSION

FMDV is the etiological agent of an important disease of livestock (Sobrino et al., 2001). Developing safe and effective FMDV vaccine has become a research emphasis. It has been shown that FMDV structural protein VP1 carries critical epitopes which can induce the neutralizing antibodies. Furthermore, it is has been demonstrated that experimental animals immunized with VP1 protein can obtain protection against FMDV challenge (Brown, 1992). Production of VP1 in a series of expression systems has been performed in the search for a safe and effective FMDV vaccine (Nicole et al., 2010). The FMDV VP1 protein has been successfully expressed in different expression systems, but the expression level is lower. Recently, lots of researches have demonstrated that the target protein was highly expressed by codon optimization (Wang et al., 2010; Garmory et al., 2003). The preferences of synonymous codon are widespread in both prokaryotes and eukaryotes (Shaper, 1986). The research showed that the frequency of synonymous codon is differs in different species and has a significantly positive correlation between favorite codons and expression level (Ramakrishna et al., 2004; Swartz, 2001). Therefore, we can improve the expression level of gene by codon optimization. In this study, the well expression of VP1 protein is obtained through optimized VP1 gene using *E. coli* preferred codons.

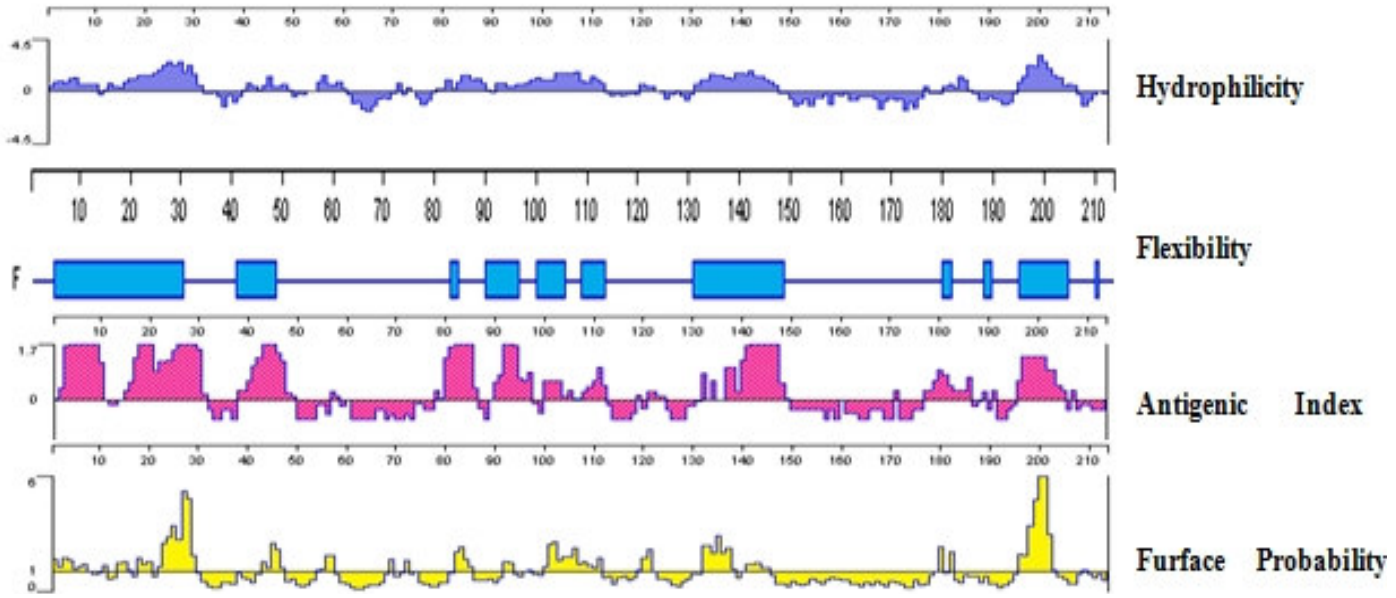

Figure 7. flexibility plot, hydrophilicity plot, surface probability plot and antigenicity index for structural protein VP1 of FMDV A/F72.

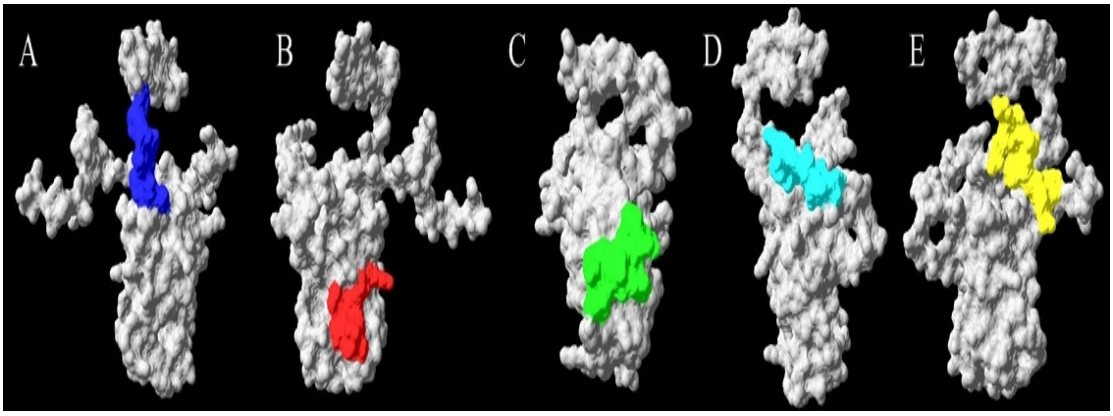

Figure 8. Putative exposed epitopes of VP1 protein mapped on molecular surfaces. Epitope regions of A (22-32aa), B (41-50aa), C (94-105), D (137-149aa) and E (196-205aa) are in blue, red, green, cyan and yellow.

It is widely known that *E. coli* is an important host organism for the production of foreign proteins. Because of its explicit genetic background, high level of expression, short reproductive cycle and resisting pollution, it has become one of the most widely used expression system (Grantham et al., 1980). But when using *E. coli*, we frequently encounter low-level or failed expression of foreign protein (Sinclair et al., 2002). In order to express the VP1 protein from FMDV strain A/F/72 other than traditional way of expressing, we optimized the coding sequence of VP1 according to the codon bias of *E. coli*. Rare codons and high GC contents can decrease the expressive efficiency, or even result in failed expression. Analysis of the original VP1 sequence shown that a portion of codons encoded by this gene are rare codons in *E. coli* and the GC content of the native gene is exorbitant. Therefore, to optimize the gene sequence for expression in *E. coli*, we took into account the codon bias of the host, and lowered the GC content of the VP1 gene. The result of expression revealed that the optimized gene was expressed in *E. coli* at a higher level. This result indicated that modification of protein sequence according to the codon bias of the expression host is an effective method to protein expression.

The pET system is the most powerful system yet developed for the cloning and expression of recombinant proteins in *E. coli*. The synthetical VP1 genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription and translation signals; expression is induced by providing a source of T7 RNA polymerase In

the *E. coli* (Saiz et al., 1991). The pET vectors also contain the cloning sites that encode a number of histidine tags, which facilitate detection and purification of the VP1 protein. By optimizing expression condition, the VP1 protein was accounted for 36%. In SDS-PAGE and Western blotting analyses, the specific band was identified with theoretical value. The integrity of opening reading frame was directly proved from this result. The biologically active VP1 protein was obtained through purification and renaturation as the His-tags are not affected protein secretion, folding and function.

The homology modeling method has not been used to generate the 3-D structure of structural protein VP1 from FMDV A/F72. In this study, the 3-D structure of VP1 satisfied on the basis of energy and mostly the amino acid resides within the most favored region in Ramachandran plot. The stereochemical spatial arrangement of VP1 was favored due to none of amino acid residues lying within the disallowed region. It would conclude that this structure fulfills the parameter provided for making a good model. The identification of viral B cell epitopes is important for understanding the antigenic structure and virus-antibody interactions at the molecular level, and is also useful in vaccine design. After secondary structure of VP1 protein were analyzed, the B cell epitopes were obtain through comprehensive analysis of four important features of antigenic epitopes. The predicted epitopes are similar to the findings of the previous study (Thomas et al., 1988). But, some suggestions put forth in the study are still at the theoretical stage, which still need testing in practice.

In this study, the VP1 protein of FMDV A/F72 was high-level expressed in *E. coli* using codon optimization. The recombinant VP1 could be produced and purified in adequate quantity to use as antigens in developing a convenient and economical diagnostic method and researching structure and function. The 3-D structure of VP1 possibly helps to better understand the nature and molecular structure. The predicted B cell epitope can be used as reference in future studies and peptides representing this site were promising candidates for a synthetic vaccine against FMD.

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