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Cloning and Codon-optimized Expression of Structural Protein Hypervariable Region of VP2 from Infectious Bursal Disease Virus

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ABSTRACT

Infectious bursal disease virus (IBDV) is the causative agent of Gumboro disease, an infectious disease of global economic importance in poultry. Structural protein VP2 of IBDV is the most frequently studied protein due to its significant roles in virus attachment, protective immunity, and serotype specificity. The objective of the present study was to improve the expression of hypervariable region of VP2 protein (hvVP2) in *Escherichia coli* (*E.coli*). The results showed that the hvVP2 was expressed in very low amount in *E.coli*. But, codon optimized hvVP2 protein showed significantly enhanced protein expression level. The coding sequence of hvVP2 was amplified and then identified by polymerase chain reaction (PCR) and sequencing. To achieve high-level expression of hvVP2 protein, we optimized hvVP2 gene base on *E. coli* preferred codons and synthesized the optimized gene. The synthetical gene was cloned into expression vector pET-26b and expressed in *E.coli* BL21 (DE3). After induction with Isopropyl-D-1-Thiogalactopyranoside (IPTG) and optimization the conditions of expression, the hvVP2 protein was relatively increased and identified by SDS-PAGE and Western blotting. Productive conformation can now be used for structure-based design purposes as well as structure-function relation of VP1 protein. It is suggested that the codon optimized hvVP2-His protein may be a useful option (but it is not enough) for developing diagnostic tests and immunization proposes.

1. Introduction

Infectious bursal disease virus (IBDV), the etiological agent of infectious disease or Gumboro disease, causes severe immune depression of B cell response in chickens by destruction of lymphocytes in bursa of fabricius (BF) (Wyeth et al., 1976). IBDV is a member of the Birnaviridae family and its genome consists of two segments of double-stranded RNA within a non-enveloped icosahedral capsid (Kibenge et al., 1988). Vaccines developed against the disease were effective for about 25 years. In 1987, a very virulent variant of the virus (vvIBDV) appeared and spread in many countries causing severe economic losses. The traditional vaccines were not effective because the new strain varied antigenetically and was more virulent (Brown et al,. 1994; Van Den Berg et al., 1991). In

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recent years, different researchers have used the recombinant technology to express structural proteins of IBDVs. VP2 protein is the major antigenic component that encodes for at least two epitopes which induce protective neutralizing antibodies (Fahey et al., 1989). For this reason, numerous studies have been performed to develop an alternative IBDV vaccine by expressing the VP2 protein in various expression systems such as *E. coli* (Azad et al., 1991; Rong et al., 2005), bacteriophages (Cao et al., 2005), yeast (Pitcovski et al., 2003), and plant (Wu et al., 2004). The *E. coli* expression system is known to be the fastest, easiest as well as an inexpensive technique for expression of usable amounts of recombinant protein. Recombinant VP2 expressed in *E. coli* react with a range of monoclonal antibodies (Azad et al., 1986; Jagadish et al., 1988). Epitope mapping studies of VP2 have shown at least two conformational epitopes on hypervariable region of the VP2 protein (hvVP2) between residues 206 to 350, which elicits virus neutralizing antibodies (Ismail et al., 1988; Vakharia et al., 1994). Hence, the VP2 protein is the most important candidate to produce vaccines and diagnostic tests (Martinez et al., 2000). The sequence of hvVP2 gene is used for most molecular phylogeny studies and serves to uniquely identify variant, classic, and very virulent IBDV strains (Brandt et al., 2001; Jackwood et al., 2007). Since the hvVP2 protein may be used for protective immunity against IBDV or diagnostic tests, the aim of this study was to increase expression of the hvVP2 protein in *E.coli* system**.**

2. Materials and Methods

2.1. RNA extraction

RNA was extracted from a vvIBDV has been previously isolated and characterized from East of Iran (Shamsara et al., 2006). Briefly, a sample was homogenized in RNase free water and extracted with phenol-chloroform. RNA was precipitated with isopropanol, dissolved in 25 µl of RNase free water and stored at -70˚C.

2.2. RT-PCR and gene cloning

In the present study, a set of primer pairs was designed according to 5́ and 3́ ends of hvVP2 as follow: 5́ GAC GGA TCC ATG ACA GCA GTG ACA GGC CC 3́ and 5́ GTT CTC GAG TCC TTT TGC CAC TCT TTC GT 3́. Two restriction enzyme sites, including *Bam*HI and *Xho*I, were added to facilitate cloning step (underlined). Two microgram extracted RNA was subjected to cDNA synthesis. RT-PCR was performed by ONE-STEP RT-PCR PreMix Kit (iNtRON, South Korea) according to manufacturer's protocol. After 30 minutes of incubation at 55 ˚C, the PCR was performed by pre-denaturation at 95 ˚C for 10 minutes, 30 cycles (94 ˚C for 30 s, 64 ˚C for 60 s, 72 ˚C for 60 s) and final extension at 72 ˚C for 10 minutes. The amplified segment was electrophorezed on a 1.0% agarose gel. The PCR product was ligated into pTZ57R-T vector (Fermentas), and transformed into *E.coli* DH5α cells. Cloning of hvVP2 gene was confirmed by restriction enzyme digestion and sequencing on the extracted plasmids from white colonies. Finally, the target sequence was cloned into pET26b plasmid by *Bam*HI and *Xho*I *restriction* enzymes.

2.3. Gene optimization, synthesis and plasmid construction

The genetic codons of wild type IBDV hvVP2 gene was optimized according to the web-based program, GENE Optimizer software from GENEART (http://www.geneart.com) (Puigbo et al., 2007). The optimized gene was synthesized and provided by Shaingene, China. The synthesized products were digested by *Bam*HI and *Xho*I, and a linker sequence coding for 16 amino acid residues $A(EAAAK)_3$ at the N-terminal end. Then it was cloned into the expression vector pET-26b to yield the recombinant plasmid pET-26b-hvVP2. The expression vector pET-26b carries a C-terminal His-Tag. The inserted sequence was confirmed by PCR, restriction enzyme digestion, and DNA sequencing.

2.4. Protein expression

The constructed plasmids were transformed into *E. coli* BL21 (DE3) and the expression was induced by adding IPTG at final concentration of 1 mM for 6 hours at 37 ˚C. The cells were harvested and then subjected for sonication in lysis buffer (10 mMTris-Cl pH 7.5, 1 mM EDTA). Both cell lysate supernatant and cell pellet were analyzed for the desired protein in SDS-PAGE and western blot.

2.5. SDS-PAGE and Western blotting

Analysis of the total protein extracted from *E. coli* transformed with pET26b-hvVP2 and codon optimized pET26b-hvVP2 plasmids were performed by 12% SDS-PAGE according to the method of laemmli (Laemmli, 1970). Proteins were transferred to a PVDF membrane using a semidry electro blotting system according to the manufacturer's instructions. The blots were blocked by 3% bovine gelatin in TBS buffer (20 mM Tris–HCl, 500 mM NaCl, pH 7.5) with gentle agitation for 1 hour. The membrane was incubated at room temperature with anti-His tag antibodies conjugated to horseradish peroxidase (HRP) (Serotec) at a dilution of 1:1000 for 2 hours. Then the membrane was washed for 10 minutes (three times) with TBST buffer (TBS buffer containing 0.05 – 0.1% Tween 20). The blots was developed by incubating in 10 ml TBS buffer containing 0.5 mg/ml 3, 3´-diaminobenzidine (DAB) substrate and 15 µl of 30% hydrogen peroxide.

3. Results and Discussion

3.1. The hvVP2 gene fragment

The hvVP2 gene fragment was amplified by RT-PCR using vvIBDV RNA as template and specific primers. The PCR product of hvVP2 gene fragment was verified by electrophoresis as an expected 555bp DNA fragment (Figure 1).

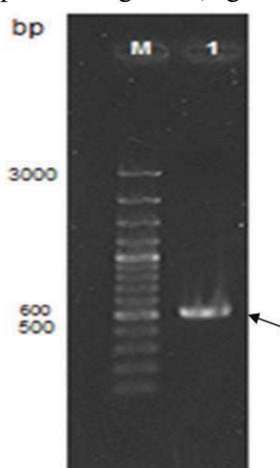

Figure 1. PCR product of amplified hvVP2, Lane M: molecular weight marker; lane 1: amplified hvVP2

3.2. The hvVP2 gene fragment cloning

In order to construct the recombinant plasmids, the PCR product of the hvVP2 gene fragment was inserted into pTZ57R/T vector. The recombinant plasmid, pTZ57R-hvVP2, was constructed and verified by restriction enzyme mapping and gene sequencing by M13 primers. At the next step, the hvVP2 gene fragment and codon optimized hvVP2 were subcloned into pET26b expression vector, and the recombinant plasmids were verified by restriction enzyme mapping.

3.3. Codon optimization and synthesis of VP1 gene

Without changing the amino acid sequence, we performed codon optimization for hvVP2 gene based on the field hvVP2 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). The codon-optimized hvVP2 gene also obtained two restriction enzymes digestion site, *Bam*HI and *Xho*I. The result of sequencing showed that the synthesized gene agrees with designed (Figure 2). After the confirmation of the sequence, the target fragment and plasmid pET-26b were both digested with *Bam*HI and *Xho*I, and then ligated with T4 DNA ligase to yield the recombinant p ET-26b-hvVP2-6his.

```
Query  9    TGACAGGCCCAGAGTCTACACCATAACTGCAGCCGATGATTACCAATTCTCATCACAGTA  68
Sbjct  75   TGACCGTCCGCGTGTTTACACCATCACCGCTGCTGACGACTACCAGTTCTCTTCTCAGTA  134

Query  69   CCAAGCAGGTGGGGTAACAATCACACTGTTCTCAGCTAATATCGATGCCATCACAAGCCT  128
Sbjct  135  CCAGGCTGGTGGTGTTACCATCACCCTGTTCTCTGCTAACATCGACGCTATCACCTCTCT  194

Query  129  CAGCATCGGGGGAGAACTCGTGTTTCAAACAAGCGTCCAAGGCCTTATACTGGGTGCTAC  188
Sbjct  195  GTCTATCGGTGGTGAACTGGTTTTCCAGACCTCTGTTCAGGGTCTGATCCTGGGTGCTAC  254

Query  189  CATCTACCTTATAGGCTTTGATGGGACCGCGGTAATCACCAGAGCTGTGGCCGCAGACAA  248
Sbjct  255  CATCTACCTGATCGGTTTCGACGGTACCGCTGTTATCACCCGTGCTGTTGCTGCTGACAA  314

Query  249  TGGGCTAACGGCCGGCACTGACAACCTTATGCCATTCAATATTGTGGTTCCAACCAGCGA  308
Sbjct  315  CGGTCTGACCGCTGGTACCGACAACCTGATGCCGTTCAACATCGTTGTTCCGACCTCTGA  374

Query  309  GATAACCCAGCCAATCACATCCATCAAACTGGAGATAGTGACCTCCAAAAGTGGTGGTCA  368
Sbjct  375  AATCACCCAGCCGATCACCTCTATCAAACTGGAAATCGTTACCTCTAAATCTGGTGGTCA  434

Query  369  GGCGGGGGATCAGATGTCATGGTCAGCAAGTGGGAGCCTAGCAGTGACGATCCACGGTGG  428
Sbjct  435  GGCTGGTGACCAGATGTCTTGGTCTGCTTCTGGTTCTCTGGCTGTTACCATCCACGGTGG  494

Query  429  CAACTATCCAGGGGCCCTCCGTCCAGTCACACTAGTAGCCTACGAAAGAGTGGCAAAAGG  488
Sbjct  495  TAACTACCCGGGTGCTCTGCGTCCGGTTACCCTGGTTGCTTACGAACGTGTTGCTAAAGG  554
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Figure 2. Nucleotide sequence of codon-optimized hvVP2 gene and original hvVP2 gene

3.4. Expression of the hvVP2 proteins

The availably constructs (pET26b-hvVP2 and codon optimized pET26b-hvVP2 plasmids) were 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 that the conditions of protein expression was determined. The transformants grew fast at 37° C. The optimum cell density for recombinant induction was reached at OD600 of 0.5 to 0.6, and a time course and IPTG concentration study established that optimal protein expression occurred 6 hours after induction with 1 mM IPTG. The molecular weight of the target protein was about 30 kDa, which was coincident with the theoretical value (Figure 3).

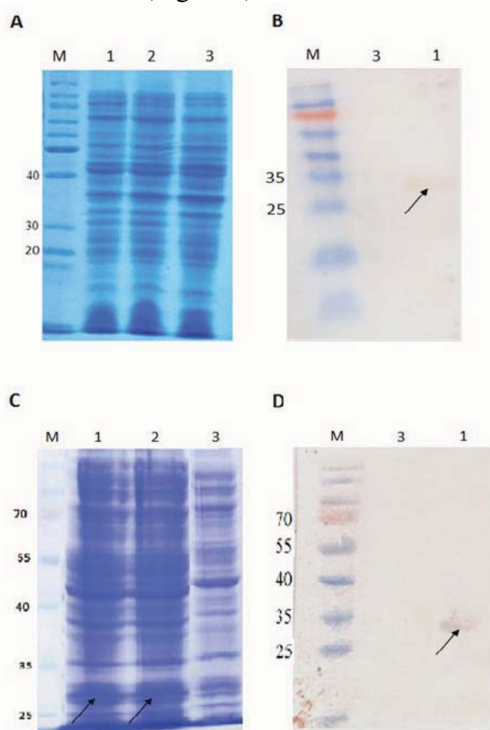

Figure 3. **A** and **B**, SDS-PAGE and western blot analysis of recombinant hvVP2; **C** and **D** codon optimized hvVP2. Lane M: molecular weight marker; lane 1: total protein extracted from *E.coli* harboring pET26b-hvVP2 induced with IPTG after 6 hours; lane 2: after 4 hours; lane 3: total protein extrated from *E. coli* harboring pET26b as a control after induction with IPTG after 6 hours. codon optimized hvVP2 expressed in a higher level than hvVP2 protein.

3.5 Western blot assay

In order to evaluate of the expressed protein, the expressed samples were analyzed by 12% SDS–PAGE and then transferred onto nitrocellulose membrane. In Western blot analysis, The His tag antibodies conjugated to HRP has cross-reacted with hVP2 protein (Figure 3).

According to our knowledge, this experiment is the first report of expression of structural protein hvVP2 of IBDV in the *E. coli*. Among the structural proteins, VP2 is a structural protein containing the major antigenic epitope, which stimulates production of the neutralizing antibodies in the host (Fahey et al., 1985; Hieine et al., 1991). The VP2 gene is, therefore an important target for cloning and expression in the development of genetically engineered vaccines and diagnostic reagent. This study represents the first report of expression of hvVP2 in the *E.coli*. Here we showed that hvVP2 is expressed in very low amount in *E.coli*. The use of codon optimized hvVP2 enhances considerably the amount of hvVP2 expression. However, this amount is not enough to produce the neutralizing antibodies in the host. In conclusion, we suggest that the codon optimized hvVP2-His protein may be a useful option (but it is not enough) for developing diagnostic tests and immunization proposes.

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