Soluble Expression, Purification and Characterization of the VP3 of the 1st Serotype Bluetongue Virus

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July 7, 2020

Abstract

Bluetongue (BT) is a non-contact infectious disease of domestic or wild ruminants caused by the Bluetongue virus (BTV). It is transmitted by Culicoides biting midges. BTV mainly infect sheep, and animals infected with BTV usually show clinical symptoms such as fever, mucosal edema, and ulcers. BTV virus is an icosahedral symmetric RNA virus with 27 different serotypes. BTV consists of 7 structural proteins (VP1-VP7) and 4 non-structural proteins (NS1, NS2, NS3/NS3a, NS4, NS5). The VP3 encoded by the L3 gene is relatively high conserved structural protein, and constitute the inner symmetric icosahedral core shell of virus particle. In this study, VP3 recombinant protein of bluetongue I virus was successfully expressed and purified by prokaryotic expression system. Moreover, to increase the amount of soluble protein, we used chaperone protein ptf16 and two fusion proteins NusA and TRX. The results show that chaperone protein can increase the solubility of VP3. Then the expression conditions were optimized and VP3 was purified by nickel affinity chromatography. The VP3 immunize Balb/c mice and the results show that the serum titer is 1:1.28×105. In the Dot-ELISA assay, we found recombinant protein VP3 react with the serum from immunized mice by inactivate BTV-1. The results of IFA showed that the antibody produced by immunized mice with recombinant protein VP3 could react with the VP3 protein expressed in 293T cell. In conclusion, we expressed the recombinant protein VP3 with the same conformation as eukaryotic expression system and had same immunogenicity with inactivated virus, which laid a foundation for further study on the structure and function of BTV.

Introduction

Bluetongue (BT) is a non-contagious, infectious, World Organisation for Animal Health (OIE) notifiable disease of ruminants caused by the Bluetongue virus (BTV, family Reoviridae, genus Orbivirus) and is spread by Culicoides spp. biting midges (Coetzee, Stokstad, Venter, Myrmel, & Van Vuuren, 2012; Kar, Ghosh, & Roy, 2004; Mokoena et al., 2019; Schulz et al., 2016). There are 27 distinct known BTV serotypes determined by the outer viral-capsid protein VP2 encoded by segment 2 of the dsRNA genome (Coetzee et al., 2012; Mokoena et al., 2019; Schulz et al., 2016). Furthermore, BTV infected sheep often show severe clinical signs, while cattle, goats and camelids are usually asymptomatic, although some clinical cases in cattle have been observed during the North European outbreak of BTV-8 (Backx, Heutink, van Rooij, & van Rijn, 2007; Caporale et al., 2014; Dal Pozzo, De Clercq, et al., 2009; Dal Pozzo, Saegerman, & Thiry, 2009; Eschbaumer et al., 2011). The BTV particle is a non-enveloped, complex virus of two capsids. The outer capsid is composed of VP2 and VP5. The inner capsid (named as "core") has two concentric layers in which the first layer is formed by VP3,and the other by VP7. Both VP3 and VP7 are highly conserved across all BTV serotypes (Kar et al., 2004; Roy, 2008; Stewart et al., 2012).

Commonly used BTV protein expression methods mainly include eukaryotic expression and prokaryotic expression. Xie (Xie et al., 2018) expressed the structural protein VP2 of BTV-25 in sf9 insect cells with

baculovirus, and Hassan (Hassan, Wirblich, Forzan, & Roy, 2001) used the baculovirus to express the structural protein VP5 in sf9 insect cells. Many studies express VP3 by inclusion bodies or truncated methods. Wang (Wang et al., 2013) expressed a truncated version of VP5 which lacked the first 41 a.a., because the expression level of full-length recombinant VP5 protein in *E.coli* BL21 was very low. The easiest, quickest, and cheapest technique in expression of proteins is the use of *E.coli* , that has been widely employed in industrial biotechnology for a long time (Hayat, Farahani, Golichenari, & Sahebkar, 2018). The high safety compared with that in other organisms, as well as need for cheap media and simple growth conditions (37°C) (Rosano & Ceccarelli, 2014).

Although great progress has been made in heterologous protein expression in *E. coli* , the expression of proteins with optimal solubility and appropriate structural and functional properties remains a problem (Hayat et al., 2018). Among various approaches to alleviate protein aggregation, it is widely recognized that the coexpression of molecular chaperones and fusion tag technology can assist with protein folding, which leads to an increased production of active protein (Lee, Kim, Jeong, & Lee, 2002; Stewart et al., 2012). In this study, the molecular chaperone pTf16 and two fusion proteins, TRX and NusA, were selected to co-express with the VP3 in order to increase the supernatant expression of the VP3 (Figure 1). The purpose of the study was to improve the solubility of the VP3, and then analyze the immunogenicity. To lay the foundation for further research on the structure and function of BTV protein VP3.

2. Materials and methods

2.1 Design and synthesis of primers and genes

The gene sequence of BTV-1 VP3 (GenBank accession No. MG255581.1) was codon-optimized for *E.coli* preference codons. The VP3 sequence primers of *EcoR I* and *Xho I* were designed in Primer 5.0: VP3-F: CGGAATTCATGGCCGCTCAGAACGAAC; VP3-R: CCGCTCGAGTTACACTGTTGGGGCAGCC, and synthesized by Shanghai Shenggong Biological Co., Ltd. The BTV-1 VP3 plasmid was used as a template for PCR and the recovery of the target gene.

The fusion protein NusA sequence conteined in the vector pET-43.1a was used as a template and the fusion protein primers of *Nde I* and*BamH I* were designed in Primer 5.0 (the thrombin sequence was inserted after the downstream primer cleavage site): NusA-F: GGAATTCCATATGATGAACAAAGAAATTTTGGCTGT; NusA-R: CGGGATCCCTGGTGCCACGCGGTTCTCGCTTCGTCACCGAACCAG.

2.2 Culture of bacteria and construction of expression vector

The pET-28a was selected and amplified in LB medium with kanamycin (50 μg/ml), and pET-43.1a, pTf16, and pET-32a were selected and amplified in LB medium with ampicillin (50 μg/mL). The strains were preserved in the Laboratory of Molecular Immunology at Zhengzhou University.

The NusA fusion protein was ligated to the pET-28a vector to construct a new vector pET-28a-NusA. The VP3 gene was ligated to the pET-28a-NusA, pET-28a, and pET-32a vectors, respectively. Thus far, we constructed 3 recombinant vectors: pET-28a-VP3, pET-28a-NusA-VP3 and pET-32a-VP3.

2.3 Protein expression identification and condition optimization

Competent cells of *E. coli* BL21 (DE3) were prepared according to the manufacturer's protocol (TaKaRa, China). Subsequently, 3 plasmids was transformed into the BL21(DE3) competent cells and the plasmid pET-28a-VP3 was transformed into pTf16-BL21. Then, 4 kinds of expressing bacteria were activated and expanded respectively. The pET-28a-VP3-pTf16-BL21 was added with 0.5 mg/ml L-arabinose during expansion culture. When the OD_{600} reached 0.6, 0.1 mM of isopropyl β-D-thiogalactoside (IPTG) was added to induce expression for 12 h at 25 . After the cells were harvested under centrifugation at 12000 rpm for

5 min at 4 , every 0.01 g pelleted bacteria were suspended in 150 μl Tris-HCl (25 mM Tris and 150 mM NaCl, adjust pH to 8.5 with HCl). Afterwards, the pelleted bacteria were lysed by sonication in an ice water bath. 200 μl of the crushed liquid was centrifuged at 12000 rpm for 15 min at 4 . The supernatant and the precipitate (resuspend the precipitate with 200 μl Tris-HCl) were separated and identified by SDS-PAGE and Western-blot.

The expression conditions of the supernatant with highest expression among four bacteria solutions were optimized with temperature (20 ,25 ,30 and 35) , IPTG concentration (0.1 mM,0.3 mM,0.5 mM and 0.7 mM) , L-arabinose (0.5 mg/ml,1.5 mg/ml,2.5 mg/ml and 3.5 mg/ml) and time (6 h,9 h,12 h and 15 h).

2.4 Purification of protein

The supernatant was collected after centrifugation at 12000 rpm for 15 min at 4 and filtered with a 0.45 μm filter. Then purified by Ni-NTA affinity chromatography. More specifically, the supernatant was combined with the Ni-NTA at 4 for 2 h, and then the binding buffer ((Tris-HCl, pH 8.5) equilibrated Ni-NTA column. After washing with 10 beds of washing buffer (Tris-HCl with 40 mM imidazole, pH 8.5), the recombinant VP3 was eluted with elution buffer (Tris-HCl with 80 mM imidazole, pH 8.5). The collected protein was dialyzed with Tris-HCl for 24 h (the Tris-HCl was changed every 3-4 h). The dialyzed protein was concentrated with silica beads for 2-4 h, when it was 1/2 or 1/3 of the original one in volume. Then we identified it by SDS-PAGE and used HRP Conjugated Anti-His Tag Mouse Monoclonal Antibodies (Ybbkine®Biotechnology Co., Ltd.) for Western-blot tests.

2.5 Mice immunization

Two 6-8 weeks old BALB/c mice were selected and each mouse was immunized with 30 μg of VP3. The protein and adjuvant are mixed at the ratio of 1:1 and emulsified before immunization. Freund's complete adjuvant was used for the first immunization on day 1, and Freund's incomplete adjuvant was used for the second immunization and third immunization on day 21 and 42, respectively. The blood was collected from tail vein on days 0 and 56 and stored at -20 . Moreover, we used 50 μg of inactivated BTV-1 virus mixed with adjuvant at the ratio of 1:1 and emulsified before immunization. One week after the third immunization, mouse blood was collected from tail vein and stored at -20 . The collected blood was used as positive control in Dot-ELISA test.

2.6 Indirect ELISA

The purified VP3 was mixed and diluted with carbonate buffer (pH 9.6), and then coated in a 96-well plate at 100 μl/well (placed at 4 for 1 hour). The buffer was discarded and washed 3 times with PBST. After that, 5% skim milk was added to the plate, 100 μl per well, and blocked at 37 for 2 hours. Then, the blocking solution in the plate was discard and the plate was washed 3 times with PBST. 200 μl of mouse serum was diluted with blocking solution to 1/1000 and added to the first well of the plate while 100 μl of blocking solution was added to the rest wells. After mixing, 100 μl of mixture in the first well was added to the second well. After mixing again, 100 μl of mixture in the second well was added to the third well and incubated at 37 for 1 hour. The primary antibody was discarded and washed 3 times with PBST. 100 μl of HRP- conjugated goat anti-mouse IgG was added to each well and incubated at 37 for 1 hour. The secondary antibody was discarded and wash 5 times with PBST. 100 μl of coloring solution was added to each well, and avoid light reaction for 5-10 min. Then, 100 μl of stop solution (2 mol/L H_2SO_4) was added to each well. When OD_{450} in well to be tested was 2.1 times great than OD_{450} in NC well, the corresponding antibody dilution factor is the antibody titer.

2.7 Dot-ELISA

First, a round hole of proper size was made on the NC membrane, and then it is soaked with double distilled water and dried. Added 1-2 μl sample into the circular hole. After being dried, the membrane was sealed with 5% skimmed milk (37 for 2 hours or 4 overnight). The blocking solution was discarded and washed 3 times with PBST, and the primary antibody (mouse anti-BTV-1 polyclonal sera) was incubated at 37 for 1 hour. After discarding the first antibodies, the plate was washed with PBST for 3 times, the second antibody (HRP-conjugated goat anti-mouse IgG) was incubated at 37 for 1 hour, and then washed with PBST for 3 times for AEC color reaction.

2.8 Indirect immunofluorescence assay (IFA)

The optimized VP3 gene was digested with *EcoR I* and *Xho I*and then cloned into pcDNA 3.1 to obtain the eukaryotic expression recombinant plasmid pcDNA 3.1-VP3. After the 293T cell line was recovered and subcultured with DMEM complete medium (10% serum and 90% DMEM), the cells were seeded at a density of 1.5×10^4cells/well in the 96-well plate. When the cells were 70% confluent, the plasmids pcDNA3.1-VP3 and pcDNA3.1 (BC) transient transfect into cells by transfection kit (jetPRIME® in vitro DNA & siRNA transfection reagent PROTOCOL). The cells were fixed with methanol at room temperature for 15 min, and washed third with PBS. Mouse anti-VP3 polyclonal sera was added to each well at 1:200 dilution, and incubated at 37 for 1 hour, and the plates were washed three times with PBS. Then, fluorescein isothiocyanate (FITC)-conjugated antirabbit IgG (Sigma-Aldrich) (1:1000 dilution) was added, before incubation at 37 for 1 hour, and five times washes with PBS. The cells were then observed under a fluorescent microscope.

3. Result

3.1 Expression of VP3

As shown in Fig. 2, four recombinant proteins expressed in *E coli*systerm including His-VP3, His-VP3-Tf16, His-VP3-NusA and TRX-His-VP3 were approximately 107 kDa, 107 kDa, 162 kDa, and 112 kDa, respectively. Compared with His-VP3,after the addition of fusion protein and molecular chaperone, the expression of VP3 in the supernatant increased. Taking into account,the fusion protein needs to be purified again after excised, resulting in loss of VP3. However, His-VP3-Tf would not have such a concern, so pET-28a-VP3-pTf16-BL21 (DE3) was chosen to optimize the expression conditions.

3.2 Optimization of expression conditions

The pET-28a-VP3-pTf16-BL21 was selected for the VP3 expression and a series of conditions optimization were carried out. There were 4 factors in the optimization, including temperature, IPTG concentration, L-arabinose concentration, and time. The results showed that VP3 expression was the highest in the supernatant when 0.3 mM of IPTG (Figure 3b) and 3.5 mg/ml of L-arabinose (Figure 3c) were used to induce expression at 20 (Figure 3a) for 12h (Figure 3d).

3.3 Purification and identification of VP3

VP3 eluted at the 100mM of imidazole concentration, and all fractions with high absorbance were collected and identified by SDS-PAGE and Western-blot after concentrate. The result showed that the purified VP3 has a single band, with a purity estimated to be over 75% (Figure 4a). Western blot analysis indicated that the VP3 reacted specifically with Anti-6xHis tag antibody (Solarbio, China) (Figure 4b). The concentration of VP3 following purification from BL21 (DE3) cells is 0.32 mg/ml.

3.4 Characterization of the VP3

The optimal coating concentration was 3.2 ng/μl determined by chessboard method. The titer of VP3 antibodies can reach 1:1.28×10^5 measured by ELISA method (Figure 5). The Dot-ELISA test showed that the purified VP3 could react with the serum of immunized mice with inactivated BTV-1 virus (Figure 6). IFA tests showed that the serum of immunized mice with VP3 expressed by *E. coli* could react to that expressed by 293T cells produce fluorescence (Figure. 7). It can be deduced that the VP3 expressed in this experiment is not much different in structure from the eukaryotic VP3.

4. Discussion

The recombinant proteins produced in *E.coli* often aggregate or degrade rapidly because of their inability to form correct tertiary structures due to anomalies in protein folding (Kim, Kweon, Lee, Park, & Seo, 2005; Nishihara, Kanemori, Yanagi, & Yura, 2000). In this study, we used the chaperone protein pTf16 to increase the expression of VP3 in supernatant and the results showed that the solubility of the protein was significantly improved. In fact, it has been demonstrated that chaperone over-production can slow the rate of protein expression in *E.coli* by sequestering nascent polypeptides for prolonged periods (Hu et al., 2007). The effect of the combination of the chaperone protein pTf16 with the recombinant vector is similar to the effect of the low temperature and reduced inducer concentration which slows down the production but increases the proportion of functional heterologous protein in the process of recombinant protein production (Hu et al., 2007; Shibui & Nagahari, 1992).

The most important chaperones in *E.coli* include pTf16, GrpE, GroEL, DnaK, DnaJ, and GroES. The molecular chaperone TF expressed by pTf16 was initially identified as a protein that binds to certain precursor proteins and facilitates their transport to membrane vesicles (Crooke & Wickner, 1987). Some studies have shown that TF may play a role in protein folding because it is related to nascent peptides and 50S ribosomes (Hartl & Hayer-Hartl, 2002; Hesterkamp, Hauser, Lütcke, & Bukau, 1996). In addition, TF is related to GroEL, which can enhance the binding of GroEL substrate and promote protein folding or degradation (Kandror, Sherman, Moerschell, & Goldberg, 1997; Kandror, Sherman, Rhode, & Goldberg, 1995). The effectiveness of these chaperones on protein folding, stability, and aggregation has also been demonstrated (Maeng, Nam, & Kim, 2011; Nishihara, Kanemori, Kitagawa, Yanagi, & Yura, 1998; Veisi et al., 2015). In addition to chaperone proteins, fusion tagging technology also facilitates the expression of soluble proteins in *E.coli* . Davis *et al* (Davis, Elisee, Newham, & Harrison, 1999). used the NusA solubilizing label to increase the solubility of bovine growth hormone by up to 90%, De (De Marco, Stier, Blandin, & de Marco, 2004) and his colleagues showed that the fusion protein purified by NusA fusion tag was more stable and higher in content than GST fusion tag.

When selecting the optimal expression vector, we found that the NusA fusion protein also affect the solubilization . Previous studies have reported that the NusA solubilizing tag can significantly improve the solubility of recombinant proteins in *E.coli* (De Marco et al., 2004; Zacharchenko, Barsukov, Rigden, Bennett, & Mayans, 2016). Considering the size of the fusion protein and the difficulty of subsequent purification, the chaperone pTf16 was finally selected for later experiments.

In addition, during protein purification, we found that the mounting effect of VP3 is not ideal. This may be due to the fact that VP3 is too large, which prevents the enrichment of VP3 on Ni-NTA, resulting in VP3 not fully binding with Ni-NTA. We then incubated the supernatant with the Ni-NTA for 2 h and reduced the flow rate during elution, which improved the VP3 bounding to Ni-NTA to a certain extent. The purity of the VP3 could reach 70% after purification, and it can produce certain immunogenicity. This study lays the foundation for further research on the structure and function of VP3 and other proteins of bluetongue virus. Apart from this, it provided materials for the preparation of BTV-1 VP3 monoclonal antibody.

In conclusion, we expressed four recombinant proteins in *E. coli,*including His-VP3, His-VP3-Tf, His-VP3-NusA and TRX-His-VP3. Compared with His-VP3 without molecular chaperone, His-VP3 with molecular

chaperone pTf16 increased its expression in the supernatant by about 30%. The VP3 purified by Ni-NTA has certain immunogenicity by Dot-ELISA and IFA test.

ACKNOWLEDGEMENTS

This work was supported by the National Key Research and Development Program (2017YFD0502304-4); '1125 talent gathering plan' project of Zhengzhou. We thank Dr. Yankai Liu for revising the grammatical errors and reorganizing this manuscript. Dr. Liu held a doctorate from Queen Mary University of London and now he is an associate professor of bioinformatics at Zhengzhou University.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

ETHICAL APPROVAL

Ethical statement is not applicable because sample collection has been gathered.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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