

Expression of porcine parvovirus VP2 gene requires codon optimized *E. coli* cells

Ting Qi · Shangjin Cui

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Abstract Porcine parvovirus (PPV) is a widespread infectious virus that causes serious reproductive diseases of swine and death of piglets. The gene coding for the capsid protein VP2 of PPV was amplified and inserted into the plasmid pET-32a (+), which was then used to transform *Escherichia coli* Rosetta, the capsid protein of PPV was fused to a polyhistidine tag, and the position of the affinity tag is in N-terminus. VP2 was expressed using different expression host bacteria, including *E. coli* BL21, and Rosetta, and different plasmid vectors, including pET-30a (+), pET-32a (+), and pGEX-6p-1. After selection, only the fusion protein inserted into pET-32a (+) was expressed well in *E. coli* Rosetta. The recombinant bacterium produced high quantities of the fusion protein VP2, about 8% in total. The expressed VP2 was antigenically similar to the native capsid protein according to a Western blot assay performed with polyclonal antibodies obtained from pigs vaccinated with PPV. A simple, easily commercialized procedure was used to purify this protein. This study provides a foundation for the application of VP2 protein in the clinical diagnosis of PPV and in the vaccination against PPV.

Keywords Expression · Porcine parvovirus · Purification · VP2 protein

Introduction

Porcine parvovirus (PPV) causes reproductive failure in pregnant sows. The reproductive failure is characterized by embryonic and foetal death, mummification, stillbirths, and delayed return to oestrus [1]. Although acute infection of postnatal, non-pregnant pigs is usually subclinical, PPV has also been linked to the occurrence of skin lesions in piglets [2], interstitial nephritis in slaughter aged pigs, and non-suppurative myocarditis in lactating piglets. More recently, PPV has gained importance as an agent with ability to increase the effects of porcine circovirus type 2 infection in the clinical course of postweaning multisystemic wasting syndrome (PMWS) [3, 4], a significant economical disease worldwide [5]. Because of its association with the above-mentioned clinical and pathological conditions, PPV is recognized as an economically important cause of reproductive failure, and vaccines containing inactivated PPV are marketed worldwide.

PPV is a small, non-enveloped, single-stranded, negative-sense DNA virus. Capsids of PPV are assembled from three viral proteins (VP1, VP2, and VP3). VP2 is the major capsid protein and consists of an eight-stranded antiparallel β -barrel motif with four large insertions between β -strands; the insertions (called loops) contain many B-cell epitopes [6]. Epitope-mapping experiments show that all of the epitopes generating neutralizing antibody are within VP2 [6, 7, 8].

Porcine parvovirus was initially described from sows with reproductive problems in Germany [9], but the virus has been subsequently found and studied in many parts of the world, including China, where it causes substantial economic loss. Although there is only one serotype of the virus, PPV can be classified into five clinical pathotypes (biotypes). The nonpathogenic NADL-2 strain, which is

T. Qi · S. Cui (✉)
Division of Swine Infectious Diseases, National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin 15001, China
e-mail: cuishangjin@yahoo.com.cn; cuishangjin@yahoo.cn

currently used as an attenuated vaccine, causes only limited viremia and does not cross the placental barrier in experimental infections [10]. NADL-8 and other virulent strains isolated from mummified and dead fetuses do cause a viremia and can cross the placenta and fatally infect fetuses [11]. A third group of virulent PPV strains, including the Kresse and IAF-A54 stains, has been associated with dermatitis [2] and, in contrast to the other virulent strains, kills immunocompetent fetuses [12]. The fourth group of PPV are the enteric strains, such as IAF-A83. Finally, a kind of PPV, which as yet lacks type strains, has been associated with PMWS and porcine respiratory disease complex (PRDC).

Porcine parvovirus cell or tissue-tropism determinants, host-range determinants, and determinants that confer hemagglutination properties have all been shown to be located in the capsid proteins [13]. The high degree of identity (97%) between the Kresse and NADL-2 PPV strains indicates that any difference in tropism must map to minor genomic differences. The NADL-2 and Kresse strains differ by five amino acids, all of which are located within the VP2-coding region [13].

Because VP2 is the main structural protein of PPV and constitutes most of the viral capsid, VP2 produced *in vitro* could self-assemble into virus-like particles [14]. These virus-like particles could then be used as a vaccine or as a diagnostic agent to detect the antibody produced by PPV infection or vaccination [15]. Similar set-up in the production and purification of VP2 VLPs using IMAC has been applied successfully for other parvovirus such as for B19 [16] and for infectious bursal disease virus [17]. In order to facilitate the use of VP2 for diagnosis and vaccination, the goal of this study was to find one better procedure to express VP2 *in vitro* and to purify this fusion protein.

Materials and methods

Materials

Strain 20-06 of PPV was isolated from a dead fetus delivered from a sow that was characterized by reproductive failure. The PPV-positive pig sera and *Escherichia coli* Rosetta were prepared and stored by our laboratory. Restriction endonucleases, polymerase, and DNA and protein weight markers were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. (Dalian, China). The plasmid pET-32a (+) was obtained from Novagen (Darmstadt, Germany). HRP-labeled anti-pig serum was purchased from Sigma (St. Louis, Missouri, USA). Ni-NTA His Bind resin was obtained from Invitrogen (Carlsbad, California, USA).

Prestained protein ladder was purchased from Fermentas International Inc. (Burlington, Canada).

Plasmid construction

Genomic DNA was extracted from the cell-cultured strain 20-06 of PPV using the classical phenol–chloroform extraction method and was used as a template to amplify the VP2 fragment by PCR. The sense strand primer (5'-TG AGGATCCATGAGTGAAAATGTGGAAC-3') includes a *Bam*HI restriction site (underlined), and the antisense strand primer (5'-CGCGTCGACTTCTAGTATAATTTTC TTG-3') includes a *Sal*I restriction site (underlined). The template was denatured at 95°C for 5 min, followed by 30 PCR amplification cycles (30 s at 94°C, 30 s at 30°C, and 72°C for 2 min) and a final extension at 72°C for 10 min.

The cloning strategy for constructing the recombinant plasmid is shown in Fig. 1. The PCR product and plasmid pET-32a(+) were both digested with *Bam*HI and *Sal*I, and then ligated with T4 DNA ligase to yield the construct. The construct was transformed into *E. coli*, and transformed bacteria were identified using both restriction enzyme digestion and PCR. Further confirmation of transformation was performed by sequencing.

Expression

Expression of the VP2 gene was carried out according to conventional protocol. In brief, *E. coli* Rosetta transformed with pET-VP2 was cultured at 37°C in culture medium supplemented with ampicillin (100 µg/ml). The transformed bacteria were induced by adding IPTG at a final concentration of 1 mM for 5 h at pH 7.0. After the cells

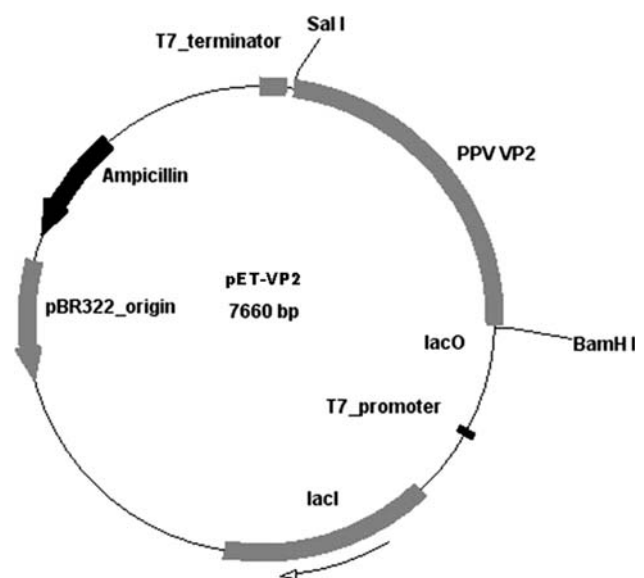


Fig. 1 The cloning strategy for constructing the recombinant plasmid

were harvested by centrifugation at $4,000\times g$ at 4°C for 20 min, the pellet was suspended in 10 ml buffer (20 mM Tris-HCl, pH 7.4, plus 200 mM NaCl) and then lysed by sonication in an ice water bath. The suspension was then centrifuged at $9,000\times g$ for 30 min at 4°C , and the pellet was kept on ice. The pellet was suspended in 10 ml buffer, 15 μl aliquots were centrifuged, and the pellet was resuspended in an equal volume of $2\times$ SDS loading buffer. The samples (plus a set of protein molecular weight standards in SDS-PAGE sample buffer) were subjected to SDS-PAGE and visualized using Coomassie Brilliant Blue, and then for analysis of the quantities of the fusion protein VP2.

Western blots

Western blots were performed according to standard procedure. Protein samples (suspension of the recombinant pET-VP2, pellet of the recombinant pET-32, protein of transformant with recombinant plasmid before induction) were separated by SDS-PAGE with 12% gel before electrophoretic transfer to a nitrocellulose membrane. Western transfer was carried out in cold transferring buffer (0.025 M Tris and 0.19 M glycine, 20% methanol). The nitrocellulose membrane was then blocked overnight at 4°C with 10% skimmed milk in TBST (Tris-buffered saline with 0.1% Tween 20, pH 8.0). The membrane was washed three times in 10 ml of TBST for 15 min each and then incubated with PPV-positive pig sera at 4°C for 60 min. The membrane was then washed and incubated for 60 min with horseradish peroxidase-conjugated rabbit anti-pig antibody. After further washing, immunoreactive proteins were visualized using DAB.

Purification

After induction by IPTG, the bacterial pellets were resuspended in guanidinium lysis buffer and shaken for 5–10 min at room temperature to ensure thorough cell lysis. The cell lysate was then sonicated on ice with 5-s pulses at high intensity and centrifuged at $3,000\times g$ for 15 min to pellet the cellular debris. The supernatant (10 ml) was mixed with 2 ml Ni-NTA resin in the column at 4°C and mixed slowly for 2 min. The resin was settled by gravity, and the supernatant was carefully removed by aspiration. The column was sequentially washed with 4 ml each of three wash buffers (in order, pH 7.8, 6.0, and 5.3). Each wash was repeated one more time. The fusion protein was eluted by 5-ml denaturing elution buffer (pH 4.0). The expression and purification protocol for His-tagged VP2 is shown in Fig. 2. Samples collected at different elution times, along with a set of protein molecular weight standards in SDS-PAGE sample buffer, were subjected to

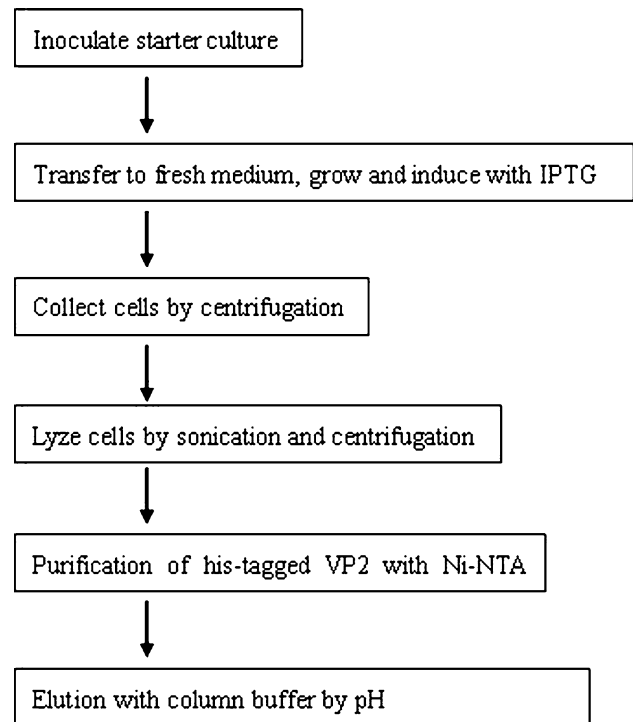


Fig. 2 Diagram of expression and purification protocol for pET-VP2

SDS-PAGE; the proteins were visualized using Coomassie Brilliant Blue.

Results and discussion

The High fidelity PrimeSTAR HS DNA polymerase and fewer than 30 amplification cycles were used to ensure accurate amplification of the VP2 gene. In addition, the cell-cultured virus was harvested 48 h after inoculation, when virus content in cells was high and when most viruses were present as mature virions. The final PCR result showed that the target band of VP2 gene was specific and bright (Fig. 3).

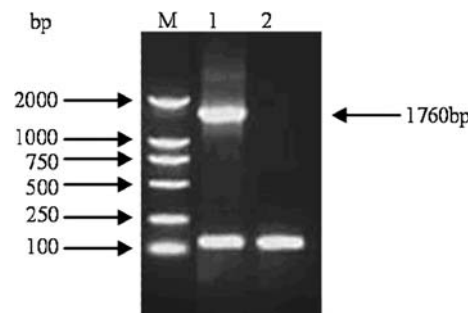


Fig. 3 Amplification of the whole sequence of VP2 gene. *M* DL Marker 2000, *1* the PCR product of VP2 gene, *2* negative control

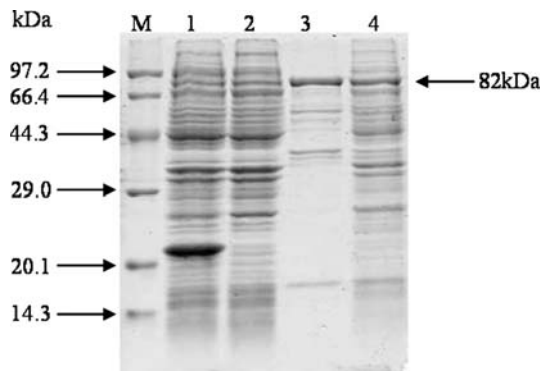


Fig. 5 SDS-PAGE analysis of recombinant proteins. *M* low molecular protein marker, *1* the whole bacterium of pET-32a(+) after induction, *2* the control of pET-VP2 before induction, *3* the pellet of induced pET-VP2 after sonication, *4* the supernatant of induced pET-VP2 after sonication

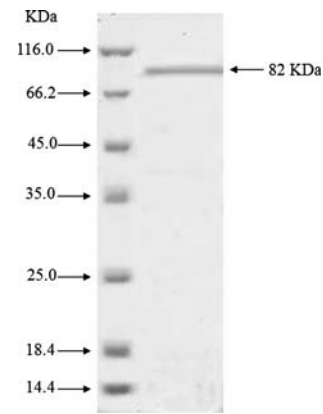


Fig. 7 SDS-PAGE analysis of the purified recombinant VP2 protein. Staining and destaining were done as described in the text

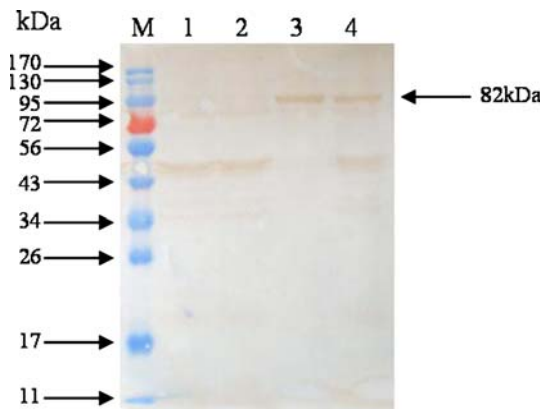


Fig. 6 Western blot analysis of recombinant proteins. *M* prestained protein ladder, *1* the whole bacterium of pET-32a(+) after induction, *2* the control of pET-VP2 before induction, *3* the pellet of induced pET-VP2 after sonication, *4* the supernatant of induced pET-VP2 after sonication. The first antibody is PPV-positive pig sera, the second antibody is horseradish peroxidase-conjugated rabbit anti-pig antibody

In order to confirm the identity of His-tagged VP2, the purified fusion protein was exposed to Western blot assay using PPV-positive pig sera. The polyclonal antibodies recognized His-tagged VP2, and the band has the appropriate molecular weight. Immunoblot of these membranes using anti-PPV antibodies showed that the fusion protein had epitopes derived from PPV (Fig. 6).

In order to purify the fusion protein (His-tagged VP2), we used Ni-NTA agarose. The results showed that the target protein could be conjugated to the resin. A single armed band was detected by SDS-PAGE (Fig. 7), Staining and destaining were done as described [20].

In order to confirm the identity of VP2 cut His-tag tail, the purified cut protein was exposed to Western blot assay using PPV-positive pig sera. The polyclonal antibodies

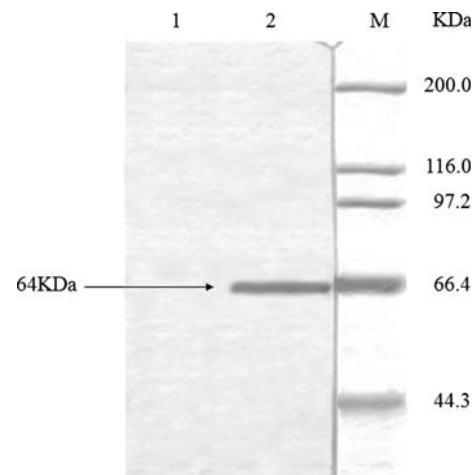


Fig. 8 Western blot analysis of Expressed VP2. *M* prestained protein ladder, *1* the control of pET-VP2 before induction, *2* the purified pET-VP2 cut the His-Tag. The first antibody is PPV-positive pig sera, the second antibody is horseradish peroxidase-conjugated rabbit anti-pig antibody

recognized cut VP2, and the band has the 64 kDa as molecular weight. The assay therefore provides evidence that the protein could be used as an efficient immunological reagent (Fig. 8).

At present, vaccines against PPV are produced by chemically inactivating isolated virus particles grown in primary cell cultures of porcine origin. The method is both labour intensive and costly, with the additional hazard of requiring the handling of large quantities of infectious virus [21]. Economic and safety considerations, as well as practical limitations associated with low yields of PPV particles from in vitro cultures, led us to the investigate recombinant sub-unit vaccines for PPV. The VP2 protein of PPV was shown to self-assemble into virus-like particles when expressed in insect cells by baculovirus infection [22]. In addition, the virus-like particles of PPV were found

to be highly immunogenic, and breeding sows were protected against reproductive failure in PPV challenge experiments [23]. Nonetheless, baculovirus-based systems for the production of recombinant proteins are still technically demanding, requiring sterile bioreactors that may be prohibitively costly for the production of vaccines for farm animals. Given that PPV causes serious economic losses for swine producers, development of safe, effective, and inexpensive methods for producing vaccines and diagnosing the disease is warranted.

In conclusion, we have established a procedure to produce the VP2 protein of PPV using plasmid pET-32a (+). After expression was optimized, a His-tagged VP2 was obtained. The fusion protein could be a useful antigen for detecting PPV.

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