Polish Journal of Veterinary Sciences Vol. 19, No. 1 (2016), 41–48

DOI 10.1515/pjvs-2016-0006

Original article

Prokaryotic expression, purification and antigenicity analysis of African swine fever virus pK205R protein

Xulong Wu[1], Lu Xiao[1,*], Bin Peng[1], Yin Wang[1,2], Zexiao Yang[1,2], Xueping Yao[1,2], Ling Hu[1], Xingyu Lin[1]

[1] College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, Sichuan, 611130, China
[2] Key Laboratory of Animal Disease and Human Health of Sichuan Province, Chengdu, Sichuan, 611130, China

Abstract

African swine fever is an acute, febrile and highly virulent porcine disease causing serious economic losses worldwide. The pK205R protein of the African swine fever virus (ASFV) is largely expressed in the early stages of infection, which has given the *K205R* gene extensive attention. In this study, the ASFV *K205R* was cloned and expressed in *Escherichia coli* BL21 (DE3). Expression of histidine-tagged pK205R with a molecular mass of 44 kDa was determined by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. Optimisation of culture conditions allowed induction of the recombinant protein with 0.4 mM Isopropyl β-D-thiogalactoside (IPTG) at 37°C for 2 h. The protein existed in cellular supernatant and was purified using a Ni-NTA resin column. The purified protein was used to immunize rabbits four times to enable the production of polyclonal antibodies, and the antiserum titre was detected by ELISA. The results showed that the purified pK205R can react with ASFV positive serum specifically by Western blotting. The pK205R had high antigenicity, which indicated that pK205R could be used as an antigen for detection of ASFV-specific antibodies in ELISA testing, and the recombinant protein could contribute to further research of the action and structure of pK205R.

Key words: indirect ELISA, pK205R, polyclonal antibody, Western blotting

Introduction

African swine fever (ASF) is an acute and highly virulent porcine disease, which results in high mortality in swine causing serious economic losses worldwide. The virus causes a haemorrhagic in domestic pigs, but persistently infects the natural hosts (warthogs and bushpigs) as well as soft ticks of the Ornithodoros genus with no signs of disease (Burrage 2013, Oganesyan et al. 2013). The clinical symptoms of ASF are very similar to classical swine fever, and the two diseases normally have to be distinguished by laboratory diagnosis (Ronish et al. 2011). African swine fever virus (ASFV) can be detected in the swine's tonsils, mandibular lymph nodes and other organs, and will spread throughout the body with subse-

Correspondence to: Yin Wang, e-mail: yaanwangyin@tom.com, tel: +86 1320 835 98 88
* These authors contributed equally

quent viremia (Gomez-Villamandos et al. 2013). To date, vaccines for ASF have not provided effective protection (Paton and Taylor 2011).

African swine fever virus is a double-stranded DNA virus which replicates in the cytoplasm of infected cells and is the only member of the *Asfarviridae* family (Sanchez et al. 2013, Takamatsu et al. 2013). Montgomery first described ASF in Kenya in 1921 (Montgomery 1921). The virus was next reported in Portugal, France, Belgium and other European countries in the 1980s (Wieland et al. 2011, Sampoli Benitez et al. 2013, Uttenthal et al. 2013). Currently, ASFV has crossed the Atlantic Ocean and outbreaks have been reported on some Caribbean islands, including Cuba and the Dominican Republic (de Glanville et al. 2014). The recent outbreak of ASFV outside of Africa was in Georgia at the beginning of 2007, and it has since spread to Azerbaijan and Russia (Diaz et al. 2012, Uttenthal et al. 2013). To date, there has been no recorded occurrence of ASF in China, but in the context of globalisation, with the development of import and export trade and frequent movement of personnel, ASF may soon extend to China.

So far, the biological function of ASFV pK205R is not completely clear, but immunofluorescence techniques showed that pK205R is increasingly expressed in Vero cells (Gutierrez-Castaneda et al. 2008, Cubillos et al. 2013), and pK205R can be detected dispersing in the cytoplasm through fluorescent microscopy 4 h after artificial infection of Vero cells (Cubillos et al. 2013). Then pK205R is gathered near the cell nucleus, which suggested that pK205R participates in the assembly of virions (Gutierrez-Castaneda et al. 2008). In addition, the pK205R protein antibody appears early in the infection, which contributes to early detection and treatment. In this study we described the construction of an expression plasmid, expressed it in *Escherichia coli*, and analysed the antigenicity after purification. Afterwards, ELISA and Western blot analysis demonstrated that the recombinant protein exhibited high antigenicity.

Materials and Methods

Plasmids, bacterial strains and animals

The *K205R* whole gene sequence was synthesised by Invitrogen (Shanghai, China), with reference to NCBI (GenBank: NC_001659.1). *Escherichia coli* DH5h and BL-21 (DE3) were used as host strains and were saved at the Animal Quarantine Laboratory, Sichuan Agricultural University, China. The expression vector pET-32a was also preserved by the Animal Quarantine Laboratory. The pMD18-T Simple Vector was purchased from TaKaRa BIO Inc., Dalian, China. The experimental rabbits were housed and fed at the laboratory at Sichuan Agricultural University, China, and were euthanised before the end of the study with permission.

Cloning the *K205R* gene and construction of the expression plasmid

A pair of primers were designed as shown below to amplify the *K205R* gene*; Bgl* II and *Not* I restriction sites were incorporated in the sense primer and antisense primer, respectively. The sense primer P1 was 5'-AGATCTATGGTTGAGCCACGCGA-3' (*Bgl* II restriction sites), and the antisense primer P2 was 5'-GCGGCCGCCTTCTTCATCATCTCTT-3' (*Not* I restriction sites). Polymerase chain reaction was carried out as follows: initial denaturation at 94°C for 5 min, followed by 30 consecutive cycles of denaturing at 94°C for 45 s, annealing at 60°C for 45 s, synthesising at 72°C for 1 min, and the final elongation at 72°C for 10 min. The PCR product was purified using the TaKaRa MiniBEST Agarose Gel DNA Extraction Kit (TaKaRa BIO Inc., Dalian, China). The purified DNA was ligated into the pMD18-T Simple Vector using T4 DNA ligase (TaKaRa), and the ligated constructs were transformed into *E. coli* DH5a cells cultured under ampicillin (100 μg/ml). The recombinant plasmid pMD18T-K205R construction was confirmed by DNA sequencing (Life Technology Inc, Shanghai, China).

The recombinant plasmid pMD18-T-K205R and pET-32a were both digested with *Bgl* II and *Not* I (TaKaRa BIO). The digested products were ligated into expression vector pET-32a at *Bgl* II/ *Not* I sites, and the pET32a-K205R were transformed into *E. coli* BL21 (DE3). A positive colony was selected from an LB agar plate containing 100 μg/ml of ampicillin.

Optimisation of expression conditions and identification of the protein expression form

Logarithmic phase *E. coli* BL21 (DE3) were induced in different concentrations of Isopropyl β-D-thiogalactoside (IPTG, 0 mM, 0.2 mM, 0.4 mM, 0.6 mM and 0.8 mM) and the samples were collected at different time-points (0 h, 2 h, 4 h, 6 h and 8 h) after the addition of IPTG. The centrifuged cells were mixed with loading buffer and heated for 5 min at 100°C after induction, and then analysed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

To identify the existing form of the protein (soluble protein or inclusion bodies), the induced products were harvested by centrifugation at 12,000 g for 10 min after ultrasonic disintegration on ice. The clear supernatant and the pellet were collected and analysed by SDS-PAGE followed by Coomassie Brilliant Blue (CBB, R-250) staining.

Expression and purification of the protein

The protein expression was induced with the optimal concentration of IPTG and time. Negative control cultures containing the empty pET-32a vector were processed in parallel. To obtain the recombinant protein, the host cells were collected from LB broth by centrifugation at 5000 rpm for 20 min, the cells were then re-suspended in deionised water, centrifuged at 10000 rpm for 10 min after ultrasonic disintegration on ice, and the clear supernatant was collected. The recombinant protein was purified with Ni-nitrilotriacetate (NTA) resin (Qiagen, CA, USA), according to the manufacturer's procedure, and the eluted fractions were analysed by 12% SDS-PAGE.

Production of polyclonal antibodies against the recombinant protein

The purified protein was used for producing antibodies in Chinese domestic rabbits. Pre-immunised rabbit serum was used as a negative control. The rabbits were first immunised subcutaneously using 500 μg recombinant protein in Freund's complete adjuvant. Three booster immunisations were performed with 500 μg recombinant protein each in incomplete Freund's adjuvant at 1 wk intervals, and the antiserum was collected after the last booster.

Western blotting

The pK205R protein was tested by Western blotting. The purified recombinant protein was analysed on 12% SDS-PAGE and electro-transferred onto a nitrocellulose membrane. The membrane was blocked with 1% bovine serum albumin (BSA, Sigma, MO, USA) at 30°C for 2 h, and then incubated with standard positive serum of ASFV from swine (ID.VET, Montpellier, France) at a dilution of 1:100 for 2 h at 30°C. The membrane was washed three times by PBS-T (0.05% Tween 20 in PBS), and then incubated in rabbit anti-swine HRP-IgG (Sigma) for 1 h at 30°C. The empty pET-32a vector cultures were used as negative controls in parallel. The immunoreactive protein was visualised using the Western blot analysis system.

Antiserum titre determination by ELISA

Antibody titre was measured using indirect ELISA (Hu et al. 2015, Shenyang et al. 2007). The ELISA plates were coated with purified recombinant protein (1:160 dilution, approximately 6.5 μg/ml, 100 μl/well) at 37°C for 1 h, then 4°C overnight, and washed three times with PBS-T (0.05% Tween 20 in PBS). Each well of the unbound area was blocked with 1% BSA for 1 h at 37°C and washed as described above. Different dilutions (1:1,000 to 1:1,024,000) of 100 μl polyclonal antibody against pK205R were then added and the plates were incubated for 1 h at 37°C. Pre-immunised rabbit serum was used as a negative control. The plates were washed four times and incubated with 100 μl horseradish peroxidase-conjugated (HRP) goat anti-rabbit IgG (dilution 1:4000, Sigma) for 1 h at 37°C. The o-phenylenediamine (OPD) substrate solution (100 μl/well) was added after four rounds of washing, and the wells were incubated for 15 min at 37°C in the dark. Coloration was stopped with 1 M of H_2SO_4 and the absorbance was measured at 450 nm using Microplate Reader (Bio-Rad, Model 680, USA).

Results

Construction of the prokaryotic expression vector pET-32a-K205R

The ASFV *K205R* gene was amplified and a 631 bp fragment was separated via 1.2% agarose gel electrophoresis (Fig. 1A). The purified *K205R* gene was cloned in *E. coli* cells (DH5α). Subsequently, the expression plasmid pET-32a-K205R was digested with the restriction enzymes *Bgl* II and *Not* I. Agarose gel electrophoresis results showed that two component fragments, at approximately 631 bp and 5900 bp, were in good agreement with the predicted sizes (Fig. 1B). The constructed pET-32a-K205R was verified by DNA sequencing.

Conditions optimal for protein expression

Small-scale optimisation assays were carried out to identify the optimal culture conditions. Using different concentrations of IPTG (0 mM, 0.2 mM, 0.4 mM, 0.6 mM and 0.8 mM) the cells were induced at 37°C for 6 h, and the expression capacity was

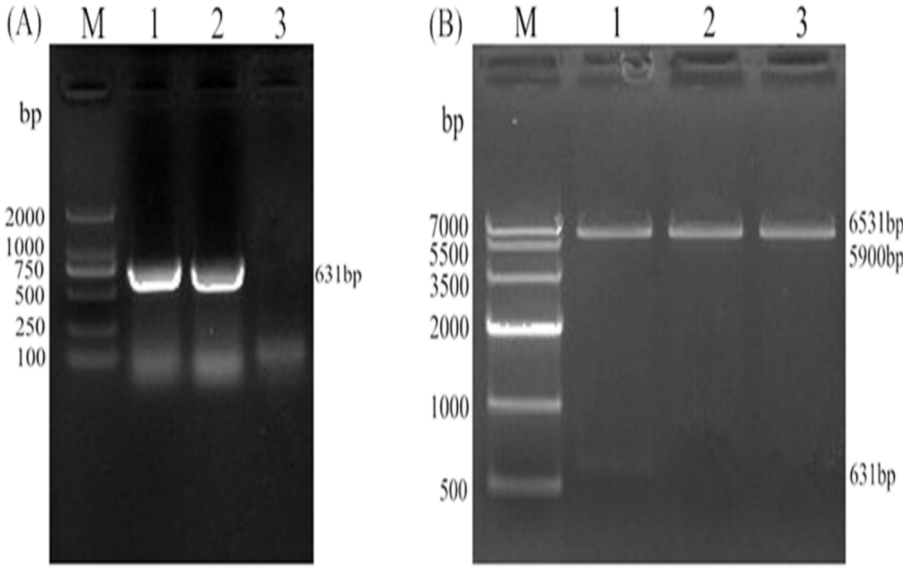

Fig. 1. Amplification of K205R gene and digestion of recombinant plasmid pET-32a-K205R construction. (A) Lanes 1-2: PCR products of ASFV K205R gene; lane 3: negative control; Lane M: DNA Marker. (B) Lane 1: Plasmid pET-32a-K205R was digested with *Bgl* II and *Not* I; lane 2: Plasmid pET-32a-K205R was digested with *Bgl* II; lane 3: Plasmid pET-32a-K205R was digested with *Not* I. Lane M: DNA Marker.

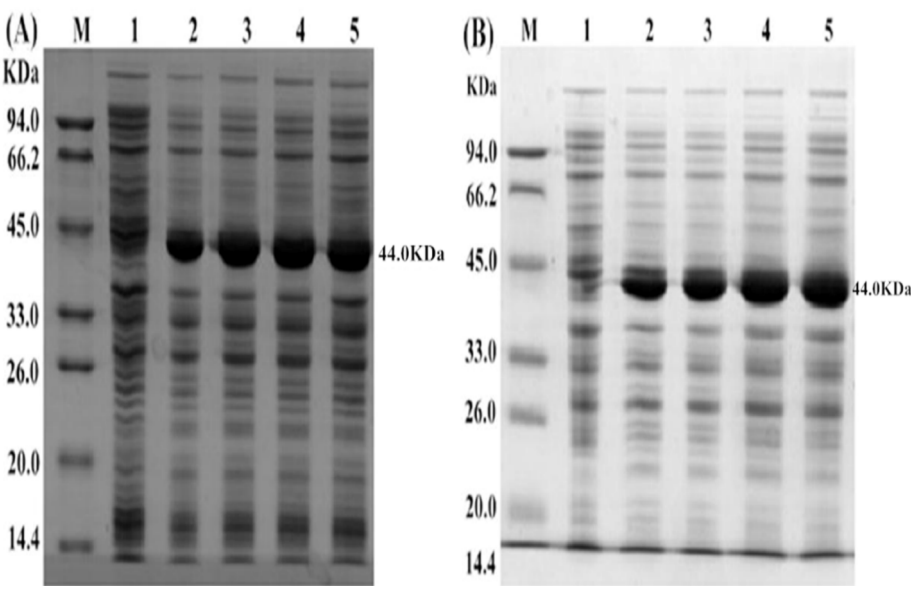

Fig. 2. Optimization of expression conditions. (A) Lanes 1-5: Concentration of IPTG was 0 mM, 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM, respectively. (B) Lanes 1-5: Cells were induced for 0 h, 2 h, 4 h, 6 h and 8 h by IPTG. Lane M: Protein Marker.

analysed by SDS-PAGE. Little difference was observed between the 0.4 to 0.8 mM concentrations of IPTG and the negative control did not react (Fig. 2A). In addition, 0.4 mM IPTG induced the cells at 37°C for different lengths of times (0 h, 2 h, 4 h, 6 h and 8 h). Analysis with SDS-PAGE showed the expression protein was sufficient after 2 h at 37°C (Fig. 2B). Therefore, a 0.4 mM concentration of IPTG and induction for 2 h were considered to be the optimal conditions for pET-32a-K205R expression. Furthermore, the cell supernatant and cell pellet were analysed after sonication, and the protein was found to be mainly distributed in the supernatant.

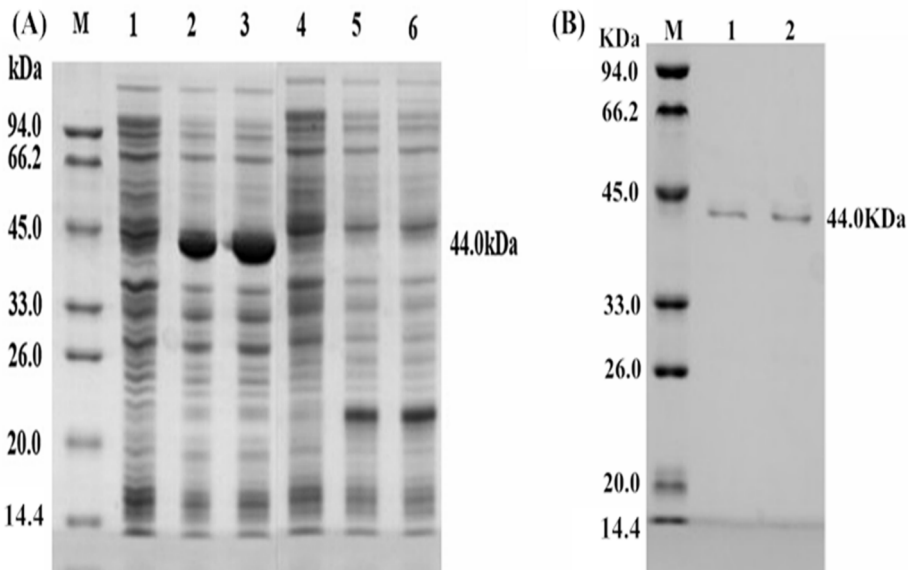

Fig. 3. Expression and purification of the fusion protein were analyzed by SDS-PAGE. (A) Lane 1: uninduced cells; lane 2: cells were induced for 2 h; lane 3: cells were induced for 4 h; lane 4: uninduced empty vector pET-32a; lanes 5-6: empty vector pET-32a was induced. (B) SDS-PAGE analysis of purified protein (lanes 1-2). Lane M: Protein Marker.

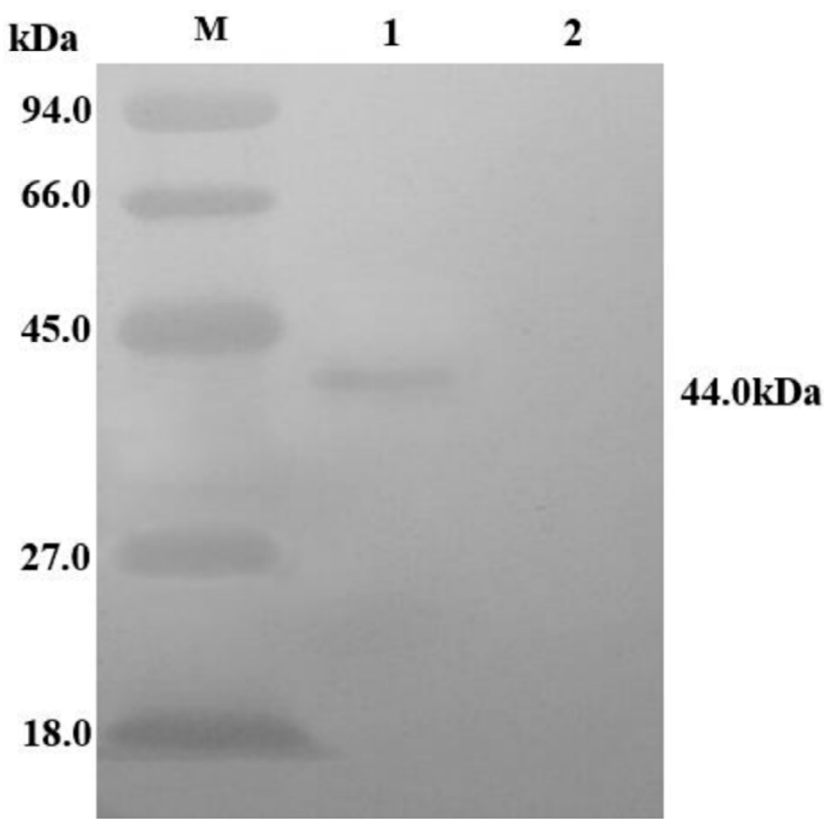

Fig. 4. Western blotting. Lane M: Pre-stained protein molecular weight marker. Lanes 1-2: Expression of purified protein was incubated with positive and negative porcine serum against ASFV, respectively.

Expression and purification of the pK205R protein

The expression of recombinant protein was induced at 37°C for 2 h with 0.4 mM IPTG. The protein was approximately 44 kDa (Fig. 3A). The expression cells were sonicated on ice with an ultrasonic disintegrator (Ningbo XinYi Co, Ltd, China) until it turned clear. The supernatant was loaded onto a pre-equilibrated Ni-NTA affinity column followed by elution. The purified protein had an expected molecular mass of 44 kDa (Fig. 3B).

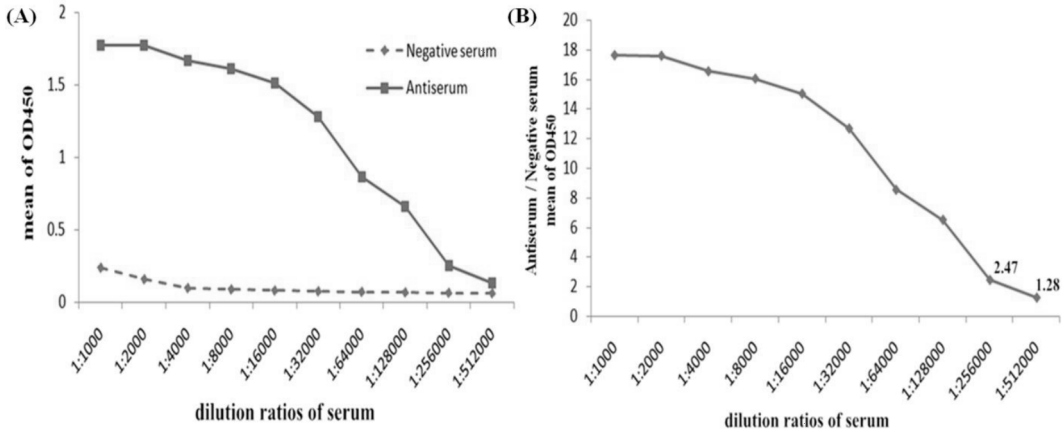

Fig. 5. Titer determination of polyclonal antibodies by ELISA. (A) Absorbance values at 450 nm of rabbit serum taken after the final immunization, and dilution at different ratios. (B) The ratio of anti-serum absorbance values at 450 nm compared with negative serum.

Western blotting

The purified protein was used as the antigen, ASF standard positive serum was used as the antibody and rabbit anti-swine HRP-IgG was used as the second antibody. Western blotting analysis showed that the recombinant protein band was 44.0 kDa (Fig. 4). The result indicated that the pK205R protein can react with ASF standard positive serum specifically.

Antiserum titre determination by ELISA

Rabbits were immunised with purified recombinant protein to generate the polyclonal antibody, and the final titre of antiserum was detected by ELISA. Prior to the first immunisation, blood samples were taken for the negative control. The mean OD_{450nm} of the negative control was 0.1008, and the mean OD_{450nm} of the negative control plus double standard deviation (SD) was 0.2124. As shown in Figure 5, the dilution ratio of serum ranged from 1:1000 to 1:512,000. The absorbance value ratio of antiserum to negative serum was 2.47 (1:256,000) and 1.28 (1:512,000) (Fig. 5). Therefore, the antiserum titre was determined to be 1:256,000.

Discussion

African swine fever is a contagious viral disease which can cause up to 100% mortality among domestic pigs, leading to serious socio-economic consequences (Atuhaire et al. 2013). There are few studies on ASFV *K205R*. The *K205R* gene is a conserved and specific gene of ASFV that appears in early infection and it amplifies the fastest (Gutierrez-Castaneda et al. 2008); these features have made it a focus of ASFV research. In addition, pK205R can be expressed in early infection, which makes it a powerful and available indicator for rapid detection of ASFV.

We chose an *E. coli* BL21 expression system to express the pK205R protein. This is a rapid and economic assay and it can complete a fairly comprehensive analysis in a relatively short time. The BL21 strain is protease-deficient and designed for high-level protein expression from T7 RNA polymerase-based expression systems (Zhang et al. 2003). It has the advantage of being naturally lacking the Lon protease and is engineered to be deficient for the OmpT protease (Baneyx and Mujacic 2004, Pacheco et al. 2012). The Lon and OmpT proteases found in other *E. coli* expression hosts may interfere with the isolation of intact recombinant protein. In this study, we selected BL21 (DE3) as the host bacterial strain in which the recombinant protein was over-expressed.

Small-scale test expressions are widely used as a predictive tool to determine the suitable IPTG concentration and induction time for large-scale growth. The expression level tended to be constant when the IPTG concentration was 0.4 mM and the induction time was over 2 h at 37°C. The cells were inducted, and the clear supernatant and pellet were analysed by SDS-PAGE; the results showed that the soluble protein existed in the clear supernatant. The constructed recombinant plasmid pET-32a-K205R carrying 6-His-tagged protein was conducive to the purification of protein (Liu et al. 2011). The protein purification system is based on Ni-NTA resins for recombinant protein carrying a small affinity tag consisting of six consecutive histidine residues. The high affinity of the Ni-NTA resins for 6-His-tagged protein is due to both the specificity of the interaction between histidine residues and immobilised nickel ions and to the strength with which these ions are held to the NTA

resin (Kirk 2014). Purified protein was used as the antigen to prepare the antiserum by immunising rabbits. The antiserum titre was detected by ELISA and Western blot analysis which showed that the pK205R protein had high antigenicity. This indicated that the pK205R protein could be used as an antigen for detection of ASFV-specific antibody in ELISA testing, and the recombinant protein could contribute to vaccine research of African swine fever.

Conclusion

In conclusion, pK205R had a high level of expression in *E. coli* BL21 under optimal expression conditions, and purified protein was obtained based on Ni-NTA resin and plasmids carrying 6-His-tagged protein. Enzyme-linked immunosorbent assay and Western blot analysis indicated that the pK205R protein had high antigenicity. In summary, we obtained a highly purified protein which will enable further investigation into the mechanism of pK205R action and structure. In addition, the recombinant protein could be used as an antigen to develop ELISA detection technology for ASFV. Although there has been no recorded occurrence of ASF in China, this method holds great potential for preventing ASFV spread into China.

Acknowledgements

We gratefully acknowledge financial support for this work from the planning subject of „the twelfth five-year-plan" in national science and technology for rural development in China (2013BAD12B04).

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