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Full Length Research Paper

Construction and prokaryotic expression of the fusion gene PRRSV GP5 and *Mycobacterium bovis* Hsp70

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Porcine reproductive and respiratory syndrome (PRRS) is an economically important swine disease that has devastated the swine industry worldwide. Vaccination with live attenuated vaccine or inactivated vaccine is the main treatment to control PRRS. However, the disadvantages such as virulence resumption of the attenuated vaccine and low immunogenicity of the inactivated vaccine call for a more efficient and safer genetically engineered vaccine. In this study, the structural protein GP5 of the PRRS virus (PRRSV), one of the major protective antigens which stimulates a protective immune response was selected to develop a genetically engineered subunit vaccine. In order to promote the immune reaction of the host to GP5, heat shock protein 70 (Hsp70) was selected as immuno-adjuvant to enhance PRRSV GP5 immunogenicity. The Hsp70 gene was amplified by PCR from attenuated *Mycobacterium bovis*, and the PRRSV GP5 gene was amplified by RT-PCR from the total RNA of PRRSV SCQ strain which was isolated, identified and maintained by the Animal Biotechnological Center, Sichuan Agricultural University, China. The fusion expressing plasmid pET32-GP5-Hsp70 was constructed and expressed in *Escherichia coli* BL21. Ni^{2+}-chelating resin was used to purify the His-tagged fusion protein expressed under optimized expressing conditions. The rabbit anti-GP5-Hsp70 fusion protein antibody was made, and Western blot assay verified the successful expression of the fusion protein, making it possible for further investigation whether Hsp70 could improve the immunogenicity of the PRRSV GP5 subunit vaccine, or evaluating the immunogenicity of the GP5-Hsp70 subunit vaccine.

Key words: Porcine reproductive and respiratory syndrome virus (PRRSV) GP5 gene, *Mycobacterium bovis* Hsp70 gene, cloning, prokaryotic expression, identification.

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is caused by PRRS virus (PRRSV), a positive-sense single stranded enveloped RNA virus of the family *Arteriviridae* and the genus *Arterivirus* (Meulenberg 2000). PRRS has caused enormous financial losses to swine industry worldwide with clinical signs such as respiratory disease in growing pigs and reproductive failure in breeding swine (Tong et al., 2007). Especially, from 2006 till now, a highly pathogenic PRRSV spread widely in China and has given a serious problem to Chinese swine industry (Li et al., 2009).

PRRSV is subject to heterogenesis and always co-infects with other pathogens, such as *Steptococcus suis*, *Haemophilus parasuis* (*H. parasuis*), *Mycoplasma hyopneumoniae*, *Actinobaccillus pleuropneumoniae*, *Salmonella* spp., *Pasteurella multocida*, and swine influenza virus, which makes it more difficult to prevent and control (Groschup et al., 1993; Zeman et al., 1993). Vaccination is the most important way to prevent PRRS.

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Table 1. The primers of PRRSV GP5 gene and Hsp70 gene of *M. bovis*.

Gene Primer	Restriction enzyme
PRRSV GP5 (upper) P1 5'-AATGGATCCCTCGCCAGCAGCGAC-3'	*Bam*H Ⅰ
PRRSV GP5 (lower) P2 5'-CCGAAGCTT AGGACGACCCCATTG -3'	*Hin*d Ⅲ
Hsp70 (upper) P3 5'-AATAAGCTTACCATGGCTC GTGCG-3'	*Hin*d Ⅲ
Hsp70 (lower) P4 5'-AATCTCGAGTGCCATCCGGCTTTT-3'	*Xho*l Ⅰ

However, the traditional PRRSV vaccines such as modified live vaccine or killed vaccine showed low efficacy (Scortti et al., 2007; Okuda et al., 2008).

PRRSV genome is approximately 15 kb in size and consists of nine open reading frames (ORFs), with each ORF partially overlapping with consecutive ORF, and performs its own biological function, respectively (Conzelmann et al., 1993; Thiel et al., 1993). ORF5 is an important structural protein gene, encoding the major envelope glycoside protein GP5. GP5 is extensively considered to be the binding protein of the virus receptor, and is one of the most important antigen proteins eliciting antibody response in PRRSV infection, so the GP5 gene is a good candidate for developing genetically engineered subunit vaccines (Pirzadeh and Dea, 1998). However, the GP5 protein itself has poor immunogenicity (Foss et al., 2002).

Heat shock proteins (Hsps) are a group of highly conserved proteins, which are classified into Hsp90, Hsp70, Hsp60 and small Hsp families based on molecular size (Itoh et al., 1999). They play a significant role in protecting cells against various cellular stresses, such as heat, hypoxia, irradiation, oxidative stress and so on (Schroder et al., 2003; Atalay et al., 2009). They participate in antigen processing, presentation and T cell activation in immune response process, stimulate a specific cytotoxic T lymphocyte (CTL) response, and contribute to tumor immunity, infection immunity and the pathology mechanisms of autoimmune diseases (Koets et al., 1999; Wong, 1999; Atay et al., 2009; Tsan and Gao, 2009). They are involved in protein translocation, new protein assembly and the prevention of protein aggregation (Mokrushin et al. 2004). Hsps, expressed by prokaryotes and eukaryotes and their action as molecular chaperones have brought more focus on their important biological functions (Guerra et al., 2005; Jonak et al., 2009).

Among all Hsps, Hsp70 is the most conserved and widely studied. Hsp70 is mainly in the cytoplasm but also occurs in many cellular compartments (Itoh et al. 1999). There have been many reports on Hsp70 acting as potent immuno-adjuvant to enhance both innate and adaptive immune system (Prohaszka et al., 2002), and not only a good immuno-adjuvant but also a protective antigen (Li et al., 2009; Tsan and Gao, 2009). Therefore, Hsp70, performing as an immuno-adjuvant, gives hopes for development of an efficient and safe PRRSV genetic-engineering subunit vaccine (Ye et al., 2004).

The objective of this study was to construct a fusion vector with the PRRSV GP5 gene and the *Mycobacterium bovis* Hsp70 gene in pET-32a(+) plasmid, express the recombinant plasmid in *Escherichia coli* BL21, immunize the rabbit with the expressed fusion protein to prepare antiserum and detect the fusion protein to analyze its immunogenicity.

MATERIALS AND METHODS

Virus, bacteria and plasmid

The PRRSV SCQ strain was isolated, identified and maintained by the Animal Biotechnological Center, Sichuan Agricultural University, China. *E. coli* DH5α and BL21, pMD18-T Vector were purchased from TaKaRa Biotechnology Co. Ltd. (Dalian, China). The attenuated strain of *Mycobacterium bovis* (*M. bovis*) was obtained from Disease Control Center of Ya'an City, Sichuan province, China. pET-32a(+) plasmid was maintained by Animal Biotechnological Center, Sichuan Agricultural University, China.

Main reagents

Trizol® Reagent was purchased from GIBCO BRL Company. High Pure RNA Isolation Kit was the production of Boehringer Mannheim Corporation. Myco DNAout Kit was provided by TIANDZ GENE Company. 2×Taq PCR Master Mixture, Ligation Mixture, RT-Kit, DNA Marker III Restriction enzymes *BamH*I, *Hind* III and *Xhol* I were provided by TaKaRa Biotechnology Co. Ltd. (Dalian, China). Plasmid extraction kit was provided by Watson Biotechnologies, Inc.

Primer design and sequencing

Two pairs of primers were designed based on the ORF of Hsp70 gene of the *Mycobacterium tuberculosis* (GenBank ID:BX248335) and GP5 gene (GenBank ID:DQ379479) of the PRRSV SCQ Strain. The upper (P1) and lower primer (P2) of GP5 gene included *Bam*H Ⅰ and *Hind* III enzyme digestion sites, respectively. In order to fuse Hsp70 gene at the end of GP5 gene, the termination codon of GP5 gene was deleted in the lower primer. The upper (P3) and lower primer (P4) of Hsp70 gene included *Hind*III and *Xho*I restriction enzyme digestion sites, respectively (Table 1). Primer synthesis and the sequencing of the target genes were executed by Shanghai Invitrogen Biotech Company, China.

Amplification of target gene Hsp70 and construction of pMD18 -Hsp70 plasmid

The genome DNA of *M. bovis* was extracted using Myco DNAout Kit (TIANDZ GENE Company) as template. The full length of Hsp70

gene of *M. bovis* was amplified by PCR with P3/ P4 primers. PCR amplification of Hsp70 gene was carried out in a reaction mixture of 25 µl including 10.5 µl sterilized water, 12.5 µl 2×Taq PCR Master Mixture, 0.5 µl (20 µmol/l) of each primer (P3 and P4) and 1 µl template DNA. The thermal conditions and cycles were as follows: one cycle at 94°C for 5 min, followed by 30 cycles at 95°C for 1 min, 56°C for 40 s and 72°C for 2 min, and a final extension at 72°C for 10 min. The PCR product was inserted into pMD18-T vector to construct pMD18-Hsp70 plasmid. The pMD18-Hsp70 plasmid was transformed into DH5α host bacteria and extracted from the bacteria, then identified by PCR with primers P3/P4 and by double-digestion with restriction enzymes *Hind*III and *Xho*I, and also by sequencing of Hsp70 gene in the plasmid. The pMD18-Hsp70 plasmid was kept at -20°C for use.

Amplification of target gene PRRSV GP5 and construction of pMD18-GP5 plasmid

Virus RNA was extracted from PRRSV SCQ strain using Trizol® Reagant (GIBCO BRL Company) and purified with High Pure RNA Isolation Kit (Boehringer Mannheim Corporation). Reverse transcription (RT) was conducted with random primers using reverse transcriptase kit (TaKaRa Biotechnology Co. Ltd., Dalian, China), and PCR was carried out in a reaction mixture as above except for different primers (P1and P2) and cDNA template. The thermal conditions were as follows: one cycle at 94°C for 5 min, followed by 30 cycles at 95°C for 30 s, 55°C for 40 s and 72°C for 30 s, and a final extension at 72°C for 10 min.

PCR product was detected and purified. The recombinant pMD18-GP5 plasmid was identified by PCR with primers P1/P2, double-digestion with restriction enzymes *BamH*I and *Hind*III, and also by sequencing.

Construction of fusion expressed plasmid pET32-GP5-Hsp70

pMD18-GP5 and pET-32(α+) plasmids were both double-digested by *BamH*I and *Hind*III and purified. The GP5 gene was inserted into the pET-32(α+) plasmid and transformed into DH5α host bacteria. The transformed DH5α bacteria were cultured and the recombinant plasmid was identified by PCR and double-digestion. The recombinant plasmid was named as pET32-GP5, and kept at -20°C until use.

The pMD18-Hsp70 and pET32-GP5 plasmids were both double-digested with the restriction enzymes *Hind*III and *Xho*I and purified. A new recombinant plasmid, named pET32-GP5-Hsp70, was constructed by inserting Hsp70 gene into pET32-GP5 plasmid. The pET32-GP5-Hsp70 was digested with three pairs of restriction enzyme (BamHI /XhoII, BamHI/HindIII, Hind III/XhoII). PET32-GP5-Hsp70 plasmid was transformed into *E. coli* BL21 following the conventional procedure.

Expression, purification and analysis of fusion protein GP5-Hsp70

The fusion gene GP5-Hsp70 was expressed following the conventional protocol to obtain the fusion protein GP5-Hsp70. Briefly, an overnight culture of BL21 containing the recombinant plasmid pET-32-GP5-Hsp70 was refreshed with LB broth containing 50 mg/L ampicillin, when the culture grew to an optical density (OD) of 0.6 at 600 nm, the cells were induced with 1 mmol/L isopropyl-β-D-thiogalactoside (IPTG) for 4 h. Then, the cells were harvested by centrifugation at 13,000 rpm for 1 min at 4°C. The cell sediment was collected and resuspended with sterilized H_2O and equal volume of 2×SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) buffer, and SDS-PAGE was performed to detect the fusion protein. Different induction time, IPTG concentration and induction temperature were compared to study the optimal expressing conditions.

To detect the solubility of the fusion protein GP5-Hsp70, BL21 containing the recombinant plasmid pET32-GP5-Hsp70 was induced by IPTG following the optimal expressing conditions as optimized above. Cells were harvested by centrifugation at 13,000 rpm for 1 min at 4°C, resuspended in 20 mmol/l Tris-HCl (pH 8.0), antalzyme was added with the concentration of 1 mg into 1 ml BL21 solution, incubated at 37°C for 30 min, conserved at -20°C, frozen thawed after consolidation and repeated twice. The mixture was broken by ultrasonic wave on ice. The total lysate was divided into soluble and insoluble fractions by centrifugation at 13,000 rpm for 5 min at 4°C. The soluble and insoluble fractions were analyzed by 12% SDS-PAGE, and visualized using Coomassie Brilliant Blue.

Ni^{2+} chelating resin (200 µl), equilibrated with 5 ml of sterile deionized H_2O, 2 ml of 50 mmol/l Na_2SO_4, and 5 ml of PBS (pH 7.2), was used to purify protein from the expressing cells broken by ultrasonic wave with the conventional protocol mentioned above. The supernatant was passed through the column, followed by washing the column with 5 ml PBS (pH 7.2) and 5 ml wash buffer (20 mmol/l imidazole in PBS), respectively. The target protein was then eluted with 2 ml of elution buffer (200 mmol/l imidazole in PBS). The eluted fractions were detected by 12% SDS-PAGE.

Preparation of rabbit anti-GP5-Hsp70 antibody and Western blot analysis of protein GP5-Hsp70

The purified fusion protein GP5-Hsp70 was used to immunize white rabbits. All animal manipulations were approved by Sichuan Province Institutional Animal Care and Use Committee. The rabbits were immunized with 0.75 mg GP5-Hsp70 protein in complete Freund's adjuvant (Kothari et al., 2006; Liang et al., 2009). The booster immunization were performed weekly for 3 times after the first vaccination with 0.75 mg GP5-Hsp70 protein in incomplete Freund's adjuvant, then rabbit sera were collected at 10 days after the last immunization. The sera were tested by Western blot using the purified fusion protein GP5-Hsp70 as antigen (Done and Paton, 1995).

Western blot was carried out according to the standard procedure. Protein GP5-Hsp70 was separated by SDS-PAGE with 12% gel and electrophoretically transferred to a nitrocellulose membrane. The nitrocellulose membrane was then blocked overnight at 4°C with 10% skimmed milk in TBS (Tris buffered saline with 0.1%Tween 20, pH 8.0). After washing with TBS, the membrane was incubated for 60 min with the rabbit anti fusion protein GP5-Hsp70 antiserum at 4°C. The membrane was washed and incubated for 60 min with horseradish peroxidase-conjugated goat anti-rabbit antibody. After further washing, bands were visualized using diaminobenzidin (DAB) colour developing reagents.

RESULTS AND DISCUSSION

Construction of the pMD18-Hsp70 plasmid

The Hsp70 gene PCR fragment was approximately 2000 bp (Figure 1A), coinciding with its theoretical size. The results of pMD18-Hsp70 positive recombinant plasmid PCR amplification and double-digestion with restriction enzymes *Hind*III /*Xho*I are shown in Figure 1B. The sequence of PCR fragment was 98.6% identity with the sequence of *M. bovis* in the GenBank. The pMD18-Hsp70 plasmid was successfully constructed.

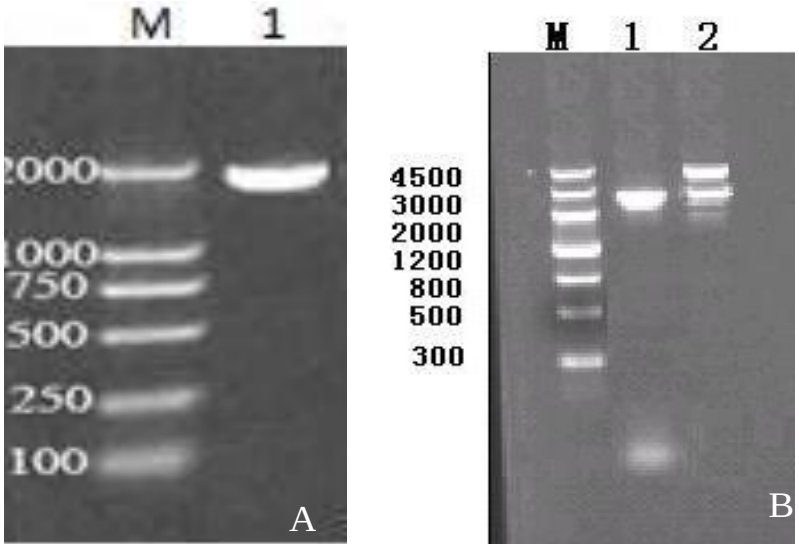

Figure 1. PCR amplification of *M. bovis* Hsp70 gene and identification of the recombination vector pMD18-Hsp70. **A.** Result of PCR amplification for *M. bovis* Hsp70 gene. Lane M, DNA marker 2,000; Lane 1, the amplified product of *M. bovis* Hsp70 (about 2,000 bp). B. Identification of the recombination vector pMD18-Hsp70 by PCR and restriction enzymes digestion. Lane M, DNA marker 4,500; lane 1, the amplified product of *M. bovis* Hsp70 (about 2,000bp) from the recombinant plasmid pMD18-Hsp70; lane 2, the recombinant plasmid pMD18-Hsp70 digested with *Hind*III and XhoI (the PCR products with about 2,000 bp and the pMD18-T vector of about 2,700 bp)

Construction of pMD18-GP5 plasmid

The acquired PRRSV SCQ GP5 gene was approximately 530 bp (Figure 2A), coinciding with its theoretical size. The pMD18-GP5 plasmid PCR amplified from recombinant bacteria with primer pairs P1/P2 showed a single objective product of about 530 bp in site (Figure 2B), and the pMD18-GP5 plasmid double-digested with restriction enzymes *Bam*HI and *Hind*III showed the objective band at 530 bp site as expected (Figure 2C). The sequence of GP5 PCR fragment was 99.7% identity with the sequence of PRRSV SCQ GP5 in the GenBank. The GP5 gene was successfully cloned into pMD18-T vector.

Construction of pET32-GP5-Hsp70

Double digested GP5 gene (*Bam*HI and *Hind* III) form pMD18-GP5 was inserted into the expressing plasmid pET-32a(+), named pET32-GP5. Double digested Hsp70 gene (*Hind*III and *Xho*l) from pMD18-Hsp70 was inserted into plasmid pET32-GP5. PCRs with two pairs of primers (P1 and P4, P3 and P4) and double digestion with three pairs of restriction enzymes *(Bam*HI and *Hind*III, *Bam*HI and *Xho*l, *Hind*III and *Xho*l) showed the successful construction of pET32-GP5-Hsp70 (Figure 3A and B). Sequence analysis also confirmed the expected GP5-Hsp70 sequence.

Expression of fusion protein GP5-Hsp70

The recombinant plasmid pET32-GP5-Hsp70 was expressed in host bacteria *E. coli* BL21. SDS-PAGE result showed that the molecular weight of the target His-tagged fusion protein was about 110 kDa (containing 6×histidine tag sequences), which was consistent with the theoretical value (Figure 4A). Several expression conditions were experimented, when the cell density for pET32-GP5-Hsp70 induction was at OD_{600} of 0.6, and the optimal protein expression occurred at 4 h post-induction with 0.5 mmol/l IPTG at 30°C (Figure 4B, C and D), and the fusion protein appeared mainly in the pellet of the induced pET32-GP5-Hsp70 transformed bacteria (Figure 4E).

Purification and Western blot assay of fusion protein GP5-Hsp70

Ni^{2+}-resin purified his-tagged GP5-Hsp70 protein was detected at 110 kD site by SDS-PAGE showing a single objective band (Figure 5A). The titer of rabbit anti-GP5-Hsp70 antibody was $1{:}2^5$ when the antibody was serial diluted and detected with agarose gel double diffusion method. When the purified fusion protein reacted with rabbit anti-GP5-Hsp70 antibody by Western blot, the antibody recognized the GP5-Hsp70 fusion protein at the site of appropriate molecular weight (Figure 5B).

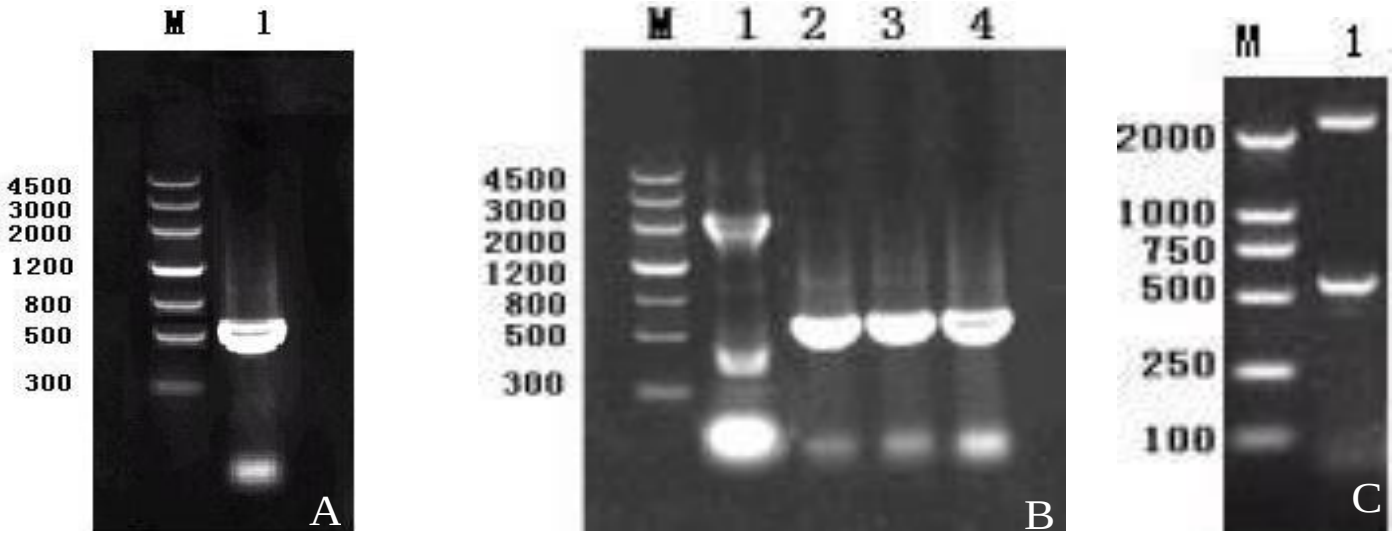

Figure 2. PCR amplification of PRRSV GP5 gene and identification of the recombination vector pMD18-Hsp70. **A.** Result of PCR amplification for PRRSV GP5 gene. Lane M, DNA marker 4,500; lane 1, the amplified product of PRRSV GP5 (about 530 bp); **B**. Identification of the recombinant bacteria by PCR. lane M, DNA marker 4,500; lane 1, the false amplified product of PRRSV GP5 from one selected recombinant bacteria; lanes 2, 3 and 4, the correct amplified product of PRRSV GP5 from three selected recombinant bacteria (the PCR products with about 530 bp); **C.** Identification of the recombination vector pMD18-GP5 by restriction enzymes digestion. Lane M, DNA marker 4,500; lane 1, the recombinant plasmid pMD18-GP5 was digested with *Bam*HI and *Hind*III (the PCR products with about 530 bp and the pMD18-T vector about 2,700 bp).

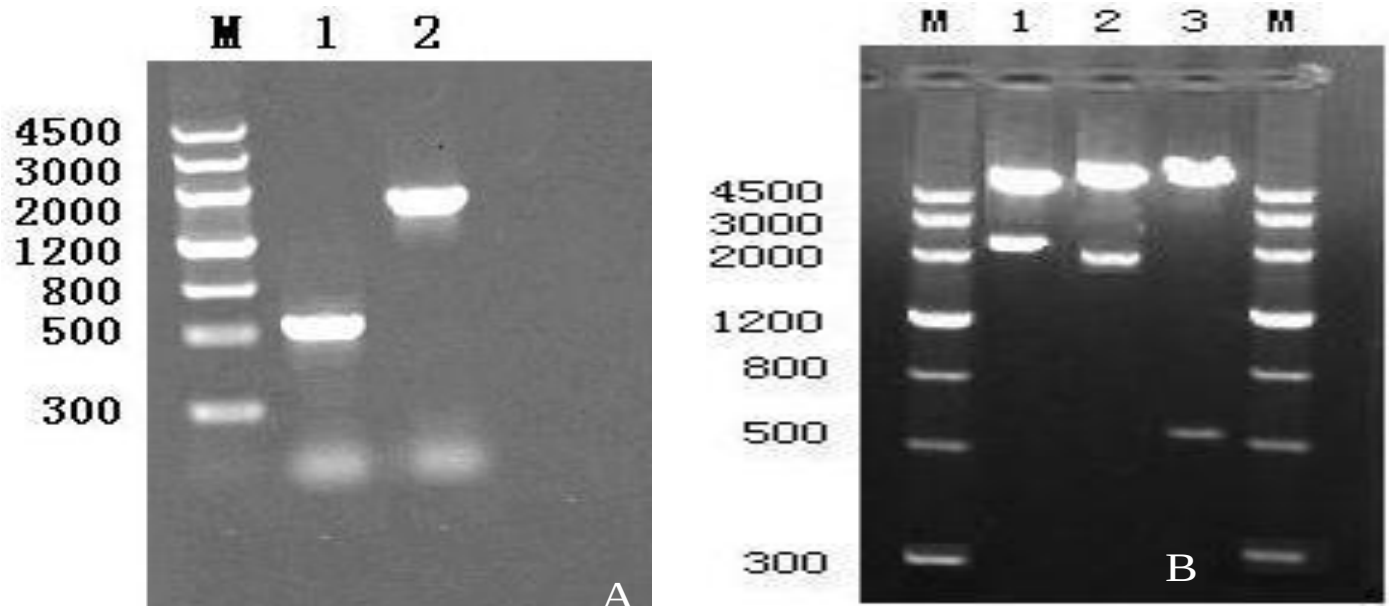

Figure 3. PCR amplification of PRRSV GP5 gene and *M. bovis* Hsp70 gene from the recombination vector pET32-GP5-Hsp70 plasmid, respectively, and identification of the recombination vector pET32-GP5-Hsp70 plasmid. **A.** Result of PCR amplification for PRRSV GP5 gene and *M. bovis* Hsp70 gene. Lane M, DNA marker 4,500; lane 1, the amplified product of PRRSV GP5 gene (about 530 bp); lane 2, the amplified product of *M. bovis* Hsp70 (about 2,000 bp). **B.** Identification of the recombination vector pET32-GP5-Hsp70 by restriction enzymes digestion. lane M, DNA marker 4,500; lane 1, the recombinant plasmid pET32-GP5-Hsp70 was digested with BamHI and Xhol (the PCR product of PRRSV GP5 gene plus *M. bovis* Hsp70 gene was about 2, 530 bp and the pET32(a)+ vector was about 4,700 bp); lane 2, the recombinant plasmid pET32-GP5-Hsp70 digested with *Hind*III and XhoI (the PCR product of *M. bovis* Hsp70 gene was about 2, 000 bp and the pET32(a)+ vector plus PRRSV GP5 gene was about 5, 230 bp); lane 3, the recombinant plasmid pET32-GP5-Hsp70 digested with BamHI and Hind III (the PCR product of PRRSV GP5 gene was about 530 bp and the pET32(a)+ vector plus *M. bovis* Hsp70 gene was about 6,700 bp) .

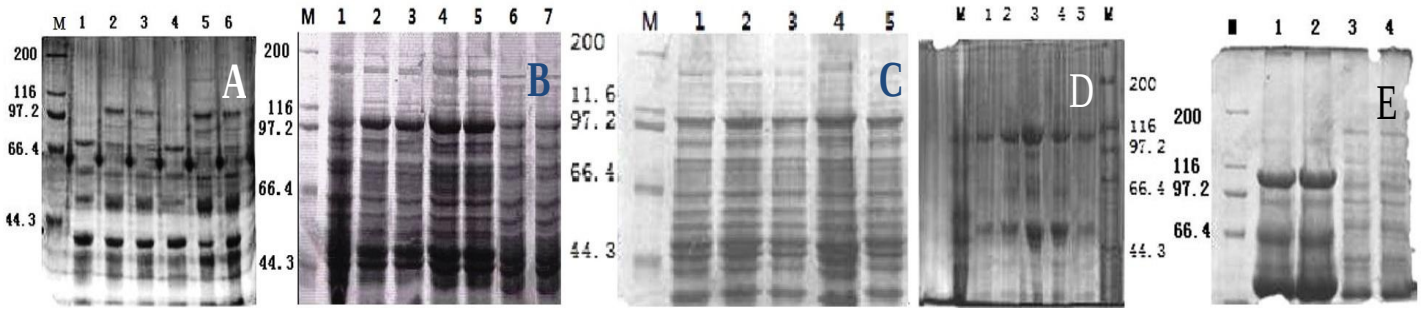

Figure 4. The optimization analysis of the expression conditions of the pET32a-GP5-Hsp70 protein in *E. coli* BL21. **A.** the pET32a-GP5-Hsp70 protein was expressed in *E. coli* BL21 host strain. M, Protein molecular weight marker; lanes 1 and 4, the pET32a-GP5-Hsp70 before induction; lanes 2, 3, 5 and 6, the pET32a-GP5-Hsp70 after induction by 0.5 mmol/l IPTG for 4 h in *E. coli* BL21 (about 110 kDa). B. Effect of different time of the pET32a-GP5-Hsp70 protein in BL21. M, Protein marker; lanes 1 to 6, the pET32a-GP5-Hsp70 protein was expressed respectively for 1, 2, 3, 4, 5 and 6 h after induction with 0.5 mmol/l IPTG. **C.** Production of recombinant plasmid pET32a-GP5-Hsp70 from BL21 in different IPTG concentrations. M, Protein marker; lanes 1 to 4, the pET32a-GP5-Hsp70 protein was expressed respectively after induction with 0.3, 0.4, 0.5 and 0.6 mmol/l IPTG. **D.** Effect of different temperature of the pET32a-GP5-Hsp70 protein in BL21. M: Protein marker; Lanes 1 to 5, the pET32a-GP5-Hsp70 protein after induction at 20, 25, 30, 37 and42°C; **E.** Solubility identification of the pET32a-GP5-Hsp70 protein; lane M, protein marker; lanes 1 and 2, sedimentation of induced transformed bacteria cracked by ultrasonic vibration showed that the pET32a-GP5-Hsp70 protein was expressed in the form of inclusion body; lanes 3 and 4, supernatants of induced transformed bacteria cracked by ultrasonic vibration without the pET32a-GP5-Hsp70 protein.

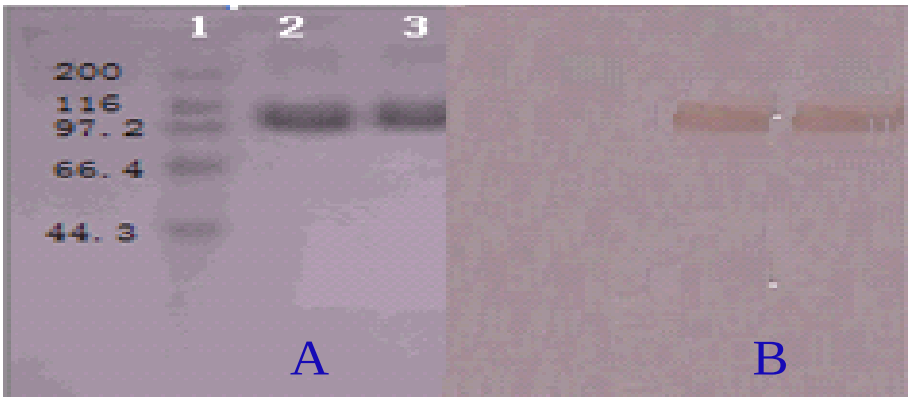

Figure 5. SDS-PAGE of the purified pET32a-GP5-Hsp70 protein and Western blotting analysis. **A.** The SDS-PAGE analysis of the purified pET32a-GP5-Hsp70 protein. Lane 1, protein marker; lanes 2 to 3, the recombinant protein was purified by the Ni^{2+}-NTA agarose gel. **B.** The immunogenicity of the recombinant pET32a-GP5-Hsp70 protein was analyzed by Western blotting with the rabbit anti-pET32a-GP5-Hsp70 serum. The recombinant protein pET32a-GP5-Hsp70 was recognized by the rabbit anti-pET32a-GP5-Hsp70 serum.

PRRS is difficult to be controlled. The best way to control PRRS is to apply effective vaccine. In this study, we successfully constructed and prokaryotic-expressed the fusion gene PRRSV GP5 and *M. bovis* Hsp70. The purified fusion protein was used to immunize the rabbit and produced specific antibody confirming the development of effective subunit vaccine of PRRS.

Poor immunogenicity and weak cross protection of PRRSV vaccines are the main issues in PRRSV immunezation. The utilization of vaccine adjuvants could improve vaccine immunogenicity (Royaee et al., 2004; Li et al., 2009). Hsp70 has attracted much interest for its potential in enhancing immunogenicity (Li et al., 2007; Zhang et al., 2007). In this study, we successfully fused the most important PRRSV antigen coding gene GP5 with Hsp70 gene from *M. bovis*, constructed and expressed fusion protein GP5-Hsp70. The efficiency of fusion protein GP5-Hsp70 in enhancing immunogenicity, cell mediated immunity and antibody responses should be further studied.

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