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Enhanced expression of the structural protein of porcine reproductive and respiratory syndrome virus (PRRSV) by SUMO fusion

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Abstract

The major structural proteins of porcine reproductive and respiratory syndrome virus (PRRSV) are derived from ORFs 4, 5, and 6. They have been considered very important to arouse the humoral and cellular immune responses against PRRSV infection and proposed to be the excellent candidate proteins in the design of PRRS bioengineering vaccine. However, the PRRSV structural proteins are produced in low levels in the infected cells because it forms insoluble protein and possesses several transmembrane regions. To overcome this problem, we fused the ORF4, ORF5, and ORF6 with SUMO (small ubiquitin-related modifier). The resulting fusion protein SUMO-ORF4, -ORF5, and -ORF6 were highly expressed in Bm5 cells. The level of protein expression using the *Bombyx mori* larvae was higher than that using Bm5 cells. In addition, fusion to SUMOstar, which is not processed by native SUMO proteases, significantly enhanced protein expression levels compared to SUMO fusion. This study demonstrated that SUMO or SUMOstar, when fused with PRRSV structural proteins, was able to promote its soluble expression. This may be a better method to produce PRRSV structural proteins for vaccine development.

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Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is the causative agent of the most important infectious disease affecting the porcine herds worldwide. It is an enveloped virus with a single-stranded positive sense linear RNA genome of approximately 15 kb. PRRSV belongs to the family *Arteriviridae*, genus *Arterivirus,* together with three other viruses; equine arteritis virus, lactate dehydrogenase elevating virus, and simian haemorrhagic fever virus (Conzelmann *et al*., 1993). There are three major structural proteins in PRRSV, glycoprotein 4 (GP4), glycoprotein 5 (GP5), and matrix protein (M), which are encoded by ORF4, 5, and 6, respectively. The predominant arteriviral envelope proteins are major glycoprotein GP5 and the unglycosylated M; the other membrane proteins occur only in minor quantities in the viral envelope. The 25-27 kDa GP5 protein is the most abundant envelope glycoprotein and a major inducer for neutralizing antibodies. The 18-19 kDa M protein is a membrane protein without N-terminal signal sequence and has been considered associating with the development of strong cellular immunity (Bautista and Molitor, 1997). Monoclonal antibodies specific for GP4 with in vitro neutralizing capacity have been

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described (Muller *et al*., 2001).

SUMO (small ubiquitin-related modifier) is an ubiquitin-related protein that functions by covalent attachment to other proteins. SUMO and its associated enzymes are present in all eukaryotes and are highly conserved from yeast to humans (Jentsch and Pyrowolakis, 2000; Melchior, 2000; Muller *et al*., 2001). Sumoylation as a reversible post-translational modification process has been shown to be involved in many cellular processes including nuclear-cytosolic transport, apoptosis, protein activation, protein stability, stress response, and cell cycle progression (Johnson, 2004; Zhou *et al*., 2005). In recent years, SUMO has become an effective biotechnological tool as a fusion system to enhance soluble expression of proteins and decrease proteolytic degradation, which could not be achieved by traditional expression systems (Butt *et al*., 2005; Sun *et al*., 2008). SUMO is then post-translationally and enzymatically cleaved from the desired protein by SUMO C-terminal hydrolases-isopeptidases (Butt *et al*., 2005). SUMO proteases remove SUMO from proteins by cleaving the C-termini of SUMO (-GGATY) in yeast to the mature form (-GG) or deconjugating it from lysine side chains (Li and Hochstrasser, 1999; 2000). Proteins such as SARS virus protein (Zuo *et al*., 2005), MMP13 (Marblestone *et al*., 2006), EGF (Su *et al*., 2006), metallothionein (Huang *et al*., 2009), and KGF2 (Wu *et al*., 2009) have been successfully expressed and purified using this fusion strategy. Recently, Liu *et al*. (2008) have created an R64T, R71E double mutant of yeast SUMO (smt3), termed SUMOstar, which is not recognized by native desumoylases (Liu *et al*., 2008). Fusion to SUMOstar enhanced protein expression levels at least 4-fold compared to either the native or 6xHis-tagged proteins.

Because the PRRSV structural proteins are produced in low levels in the infected cells, a recombinant baculovirus has been often used to express these proteins. Recently, we expressed CA (Korean PRRSV strain) structural proteins using bApGOZA system in Sf21 cells; however, the expression level was very low (Koo *et al*., 2008). In the present study, we report the high-level expression of PRRSV structural protein by synergistic effect of SUMO fusion and baculovirus expression system in Bm5 cells and *Bombyx mori* larvae.

Materials and Methods

Cells and viruses

The CA strain (KCTC 11131BP) was isolated from the sera of field pigs at the acute stage of PRRSV infection in 2006. The virus was passaged twice on sub-confluent monolayers of MARC-145 cells in minimum essential medium supplemented with 10% fetal bovine serum (Gibco BRL, Paisley, UK), nonessential amino acids, sodium pyruvate, and antibiotics in 5% CO_2 at 37°C. The virus was collected when 70% of the cells showed cytopathology. The supernatant was stored at -80°C as the viral stock. Bm5 cells were cultured at 27°C in TC-100 insect medium (WelGENE, Daegu, Korea) supplemented with 5% fetal bovine serum.

Animals

The larvae of the silkworm, *B. mori*, were F1 hybrid Baekok-Jam supplied by the Department of Agricultural Biology, National Institute of Agricultural Science and Technology, Korea. Silkworms were reared on an artificial diet at 25°C, 65±5% relative humidity, and a 16 h light: 8 h dark photoperiod.

RT-PCR

Viral RNA was extracted from infected cell cultures with Viral Gene-spin™ kit (iNtRON Biotechnology, Seoul, Korea) as recommended by the manufacturer. The viral RNA was used as a template for cDNA synthesis using RNA LA PCR Kit (TaKaRa, Shiga, Japan). Primers were based on the sequence of our previously sequenced PRRSV CA strain(Koo *et al*., 2008) PCR amplification was performed using AccuPower PCR Premix (Bioneer Co., Daejeon, Korea). The PCR products were isolated in 1% agarose gel and purified with a gel extraction kit (CosmoGenetech, Seoul, Korea).

Construction of transfer vector

The purified RT-PCR products were cloned into the T&A cloning vector (RBC Bioscience, Taiwan), and the plasmids were named pTRSV-ORF4, -ORF5, and -ORF6, respectively. They were digested with *Sac* I and *Pst* I, and cloned into pBmKSK4 (Koo *et al*., 2014), to get the recombinant transfer plasmid pBmRSV-ORFs 4 to 6. For construction of transfer vector with fusion tags, the SUMO fragment containing a *Bgl* II site at the 5' end and a *Sac* I site at the 3' end was amplified by PCR from

the pHismax-SUMO1 (kindly provided to us by Prof. Chung, Seoul National University). The amplified fragment was ligated into the pBmRSV-ORFs 4 to 6 digested with *Bgl* II and *Sac* I to create the new expression vector, pBmRSV-ORFs 4S to 6S. Using pI-SUMOstar (plasmid containing SUMOstar fragment) (LifeSensors Inc., PA, USA) as template, the SUMOstar fragment containing restriction enzyme site was generated. The plasmids were named pBmRSV-ORF5S*. The PCR primers used were 5'-GAAGATCTATGGGGGGTTCTCATCATCATC-3' and 5'-CGAGCTCACCCCCCGTTTGTTCCTGATAA-3' for SUMO, and 5'-GGAATTCATGGGTCATCACCATCATCATCAC-3' and 5'-CGAGCTCACCTCCAATCTGTTCTCTGTGAG-3' for SUMOstar. Authenticity of the inserted fragment was confirmed by restriction enzyme analysis and automated DNA sequencing.

Generation of recombinant baculoviruses

Bm5 cells were co-transfected with a mixture of purified transfer vector each containing a PRRSV gene, bBpGOZA DNA (Je *et al*., 2001), and Cellfectin (Invitrogen, CA, USA), according to the manufacturer's instruction. Briefly, 100 ng of bBpGOZA DNA and 500 ng of transfer vector DNA were mixed in a polystrene tube. Five microliters of Cellfectin was gently mixed with the DNA solution, and the mixture was incubated at room temperature for 1 h. The Cellfectin-DNA complex mixture was added dropwise to the medium covering the cells while the dish was gently swirled. After incubating at 27°C for 4 h, media were removed, and the cells were washed twice with fresh medium. Five days after adding the Cellfectin-DNA complex mixture to the cells, the medium containing viruses released by the transfected cells was transferred to a sterile container and stored at 4°C. A standard plaque assay procedure was used to obtain titers of the recombinant viruses. The purified recombinant virus was propagated in Bm5 cells. The titer was expressed as plaque forming units (pfu) per milliliter according to standard methods (O'Reilly *et al*., 1992).

Preparation of samples

Bm5 cells were infected with wild type BmNPV or recombinant virus in a 60-mm diameter dish (2×10^6 cells) at a multiplicity of infection (MOI) of 5. At 3 d after inoculation, the culture supernatant was harvested and the infected cells washed with PBS, and then used to prepare SDS-PAGE sample. Individual silkworm larvae on the first day of the 5th instar were injected with 1×10^5 pfu of wild type BmNPV or recombinant virus. At 3 to 5 d post-injection, the hemolymph and fat body were collected by cutting a caudal leg and dissection, respectively. A few crystals of phenylthiourea were added to the tubes to prevent melanization. The fat body was homogenized in 10 volumes of lysis buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 5% Glycerol, 0.1% Triton X-100 containing protein inhibitor cocktail (Sigma-Aldrich, USA)) for 3 min and incubated in an ice bath for 30 min. The homogenate was centrifuged at 13,000 rpm for 10 min. The supernatant contained the total protein extract. The hemolymph was centrifuged at 10,000×g for 10 min to remove hemocytes and cell debris, and the supernatant was stored at -70°C until further use.

Purification of 6xHis-tagged proteins

Lysis buffer was added to the fat body of *B. mori* larvae and homogenized in a sonicator. Purifications were performed using Ni-NTA spin columns (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. Ni-NTA eluates were trichloroacetic acid-precipitated, resuspended in Laemmli loading buffer, and analyzed by SDS-PAGE.

SDS-PAGE and western blot analysis

The prepared protein samples from cells and larvae were mixed with sample buffer, boiled for 5 min, and subjected to 12% SDS-PAGE gel. For Coomassie stains, gels were washed with deionized water and stained with BioSafe Coomassie. For Western blot analysis, the proteins were transferred to a nitrocellulose membrane (Pall Corp., NY, USA). After blotting, the membrane was blocked by incubation in 5% (w/v) non-fat dry milk in TBST buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 h with gentle agitation. The blot was incubated with 6xHis tag (1:1000) or porcine anti-PRRSV (1:100) antibodies (Choongang Vaccine Lab., Daejeon, Korea) in TBST for 1 h and washed. Subsequently, the membrane was incubated with anti-mouse and anti-pig IgG horseradish peroxidase conjugate for 30 min at room temperature. After repeated washing, the immunoreactive bands were visualized using the ECL Western Blotting Detection System (Elpis Biotech, Daejeon, Korea).

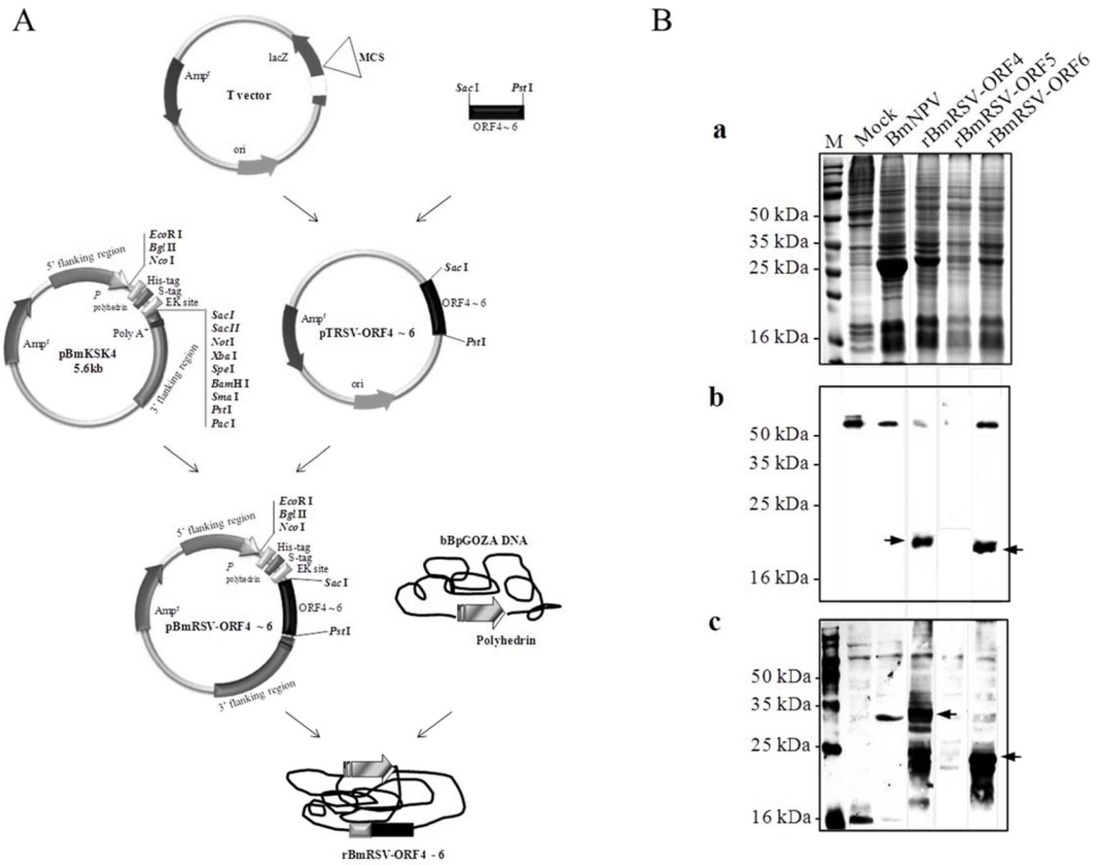

Fig. 1. Construction of the recombinant viruses (A) and expression of the PRRSV structural protein in Bm5 cells (B). The cells were infected at an MOI of 5 pfu per cell with the wild type BmNPV or rBmRSV-ORF4, rBmRSV-ORF5, and rBmRSV-ORF6 and harvested at 3 d post-infection. Proteins were separated on a 12% SDS-PAGE (a), transferred to nitrocellulose membranes for Western blot analysis and reacted with anti-6xHis tag (b) and porcine anti-PRRSV antibodies (c). M, protein size marker; Mock, mock-infected cells and BmNPV, wild type BmNPV. The recombinant proteins are indicated with arrows.

Results and Discussion

Expression of the PRRSV structural proteins in Bm5 cells

To generate the recombinant baculoviruses, Bm5 cells were co-transfected with a mixture of bBpGOZA and pBmRSV-ORFs 4 to 6 as described in Materials and methods. The recombinant viruses were called rBmRSV-ORF4, rBmRSV-ORF5, and rBmRSV-ORF6 (Fig. 1A). Bm5 cells were infected at a MOI of 5 with each recombinant baculovirus and incubated at 27°C until harvesting of the cultures. Expression of the recombinant protein in Bm5 cells was analyzed by SDS-PAGE and Western blotting. A negative control consisting of Bm5 cells infected with wild-type BmNPV did not show any reactivity, as expected. The detection of GP4 (ORF4) and M (ORF6) protein band was difficult on SDS-PAGE analysis, but was sufficient to produce detectable signals on Western blot analysis with 6xHis tag or porcine anti-PRRSV antibodies (Fig. 1B). For GP4, several bands were observed that would correspond to non-glycosylated and glycosylated and different glycosylated forms, respectively. A possible explanation for the various patterns of PRRSV proteins expression might be due to incomplete glycosylation in insect cells. However, we could not detect any GP5 (ORF5) signal, even under Western blot analysis. This result could be explained by the high hydrophobicity of this protein, which contains several transmembrane domains (Indik *et al*., 2000).

Enhanced expression of the PRRSV structural proteins by SUMO fusion in Bm5 cells

In order to enhance the protein expression, we constructed a

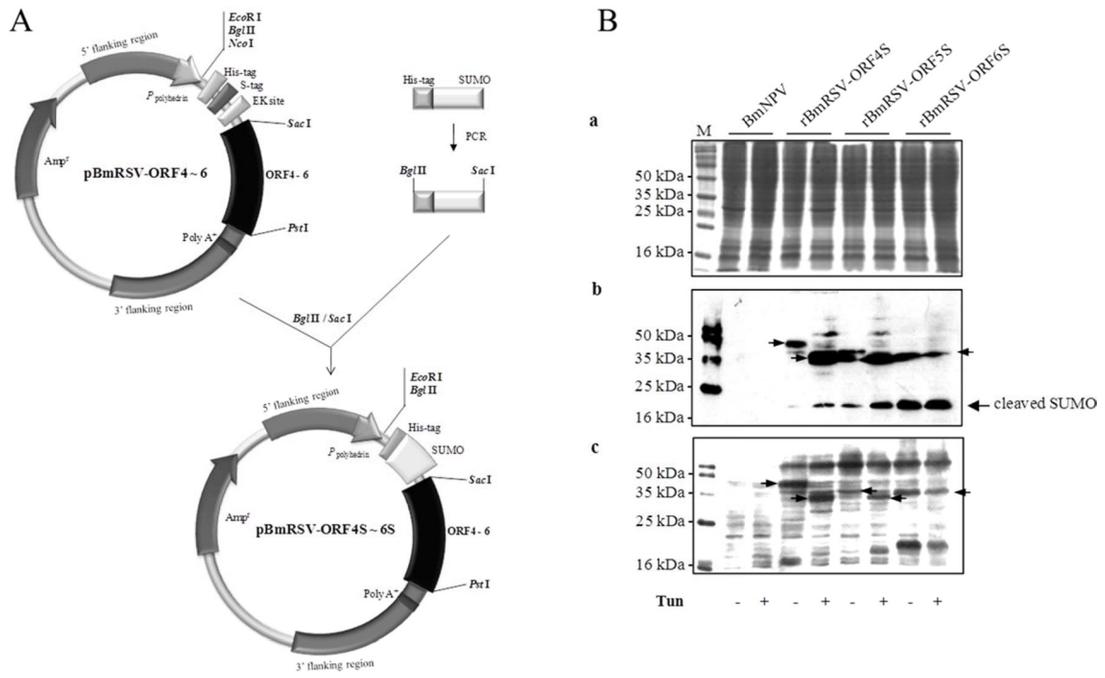

Fig. 2. Construction of the expression plasmid with SUMO fusion (A) and expression of the PRRSV structural protein in Bm5 cells (B). The cells were infected in the absence or presence of 5 μg/mL of tunicamycin with the wild type BmNPV or rBmRSV-ORF4S, rBmRSV-ORF5S, and rBmRSV-ORF6S and harvested at 3 d post-infection. Proteins were separated on a 12% SDS-PAGE (a), transferred to nitrocellulose membranes for Western blot analysis and reacted with 6xHis tag (b) and porcine anti-PRRSV antibodies (c). M, protein size marker and BmNPV, wild type BmNPV. The recombinant proteins and the cleaved SUMO proteins are indicated with arrows.

plasmid for the expression of ORF4, ORF5, and ORF6 fused to SUMO, respectively (Fig. 2A). There was a sequence of 6xHis tag for detection and purification fused upstream of the SUMO-ORF4, -ORF5, and -ORF6. The new viruses were called rBmRSV-ORF4S, rBmRSV-ORF5S, and rBmRSV-ORF6S. As shown in Fig. 2B (a), the detection of three major protein bands was also difficult on SDS-PAGE analysis; however, ORF5 enhanced the level of expression dramatically, as compared to the un-fused control (Fig. 2B and 1B). Previous reports demonstrated that SUMO fusions may increase the expression of recombinant proteins and enhance the solubility of partially insoluble proteins (Malakhov *et al*., 2004). However, expression efficiency may be affected by molecular weight, amino acid constitution, and the function of proteins, etc. Our results are consistent with the previous finding for SUMO function. A predominant protein at 45 kDa, 37 kDa, and 34 kDa, corresponding to the expected molecular size of SUMO-ORF4, -ORF5, and -ORF6, was observed respectively. Must be point out that SUMO is about 10 kDa but runs on an SDS-PAGE gel as 20 kDa. Cleaved SUMO was detected by Western blotting using anti-6xHis tag antibody, and each native protein was detected by Western blotting using porcine anti-PRRSV antibody. This observation suggests that the fusion protein is rapidly cleaved by the endogenous SUMO proteases in insect cells. The tertiary structure of the SUMO protein is specifically recognized and cleaved by SUMO protease. In addition, the glycosylation of expressed GP4 and GP5 in Bm5 cells was shown by the treatment of tunicamycin, an N-glycosylation inhibitor (Fig. 2B (b) and 2B (c)). M protein is unglycosylated membrane protein (Meulenberg *et al*., 1997).

Expression and purification of the PRRSV structural proteins in *B. mori* larvae

Insect cells infected with recombinant baculovirus have been used for the high-level expression of various eukaryotic gene products (Hummel *et al*., 1992; Kato *et al*., 2004; Kost *et al*., 2005; Lesley, 2001) because these insect cells are capable of a similar posttranslational modification to that occurring in mammalian cells. An alternative baculovirus expression system involves the use of silkworms and enables the production of recombinant proteins in silkworm larvae

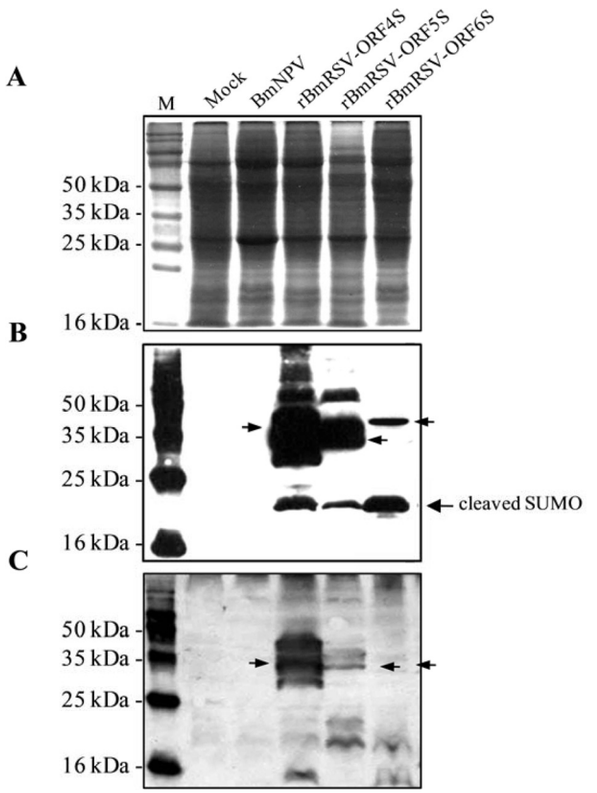

Fig. 3. Expression of the SUMO fusion protein in the fat body of *B. mori* larvae. Individual silkworm larvae on the first day of the 5th instar were injected with the wild type BmNPV or rBmRSV-ORF4S, rBmRSV-ORF5S, and rBmRSV-ORF6S. At 3 to 5 d post-injection, the fat bodies were collected by dissection and homogenized in lysis buffer. Proteins were separated on a 12% SDS-PAGE (A), transferred to nitrocellulose membranes for Western blot analysis and reacted with 6xHis tag (B) and porcine anti-PRRSV antibodies (C). M, protein size marker; Mock, mock-infected cells and BmNPV, wild type BmNPV. The recombinant proteins are indicated with arrows.

or pupae. Expression of the fusion protein in the fat body of rBmRSV-ORF4S, rBmRSV-ORF5S, and rBmRSV-ORF6S-injected *B. mori* larvae was detected via Western blot (Fig. 3). The molecular weight of each recombinant protein band was consistent with results obtained from Bm5 cells. No band was observed from wild type BmNPV-injected *B. mori* larvae. The fusion proteins were mostly expressed in fat body, but were few detected in the hemolymph of *B. mori* larvae (data not shown). The fat body is a very important organ for protein synthesis in the silkworm; the fat body is also the major site for virus proliferation. In most cases of expression of heterologous proteins in silkworm larvae, the foreign proteins are secreted into the hemolymph, and this facilitates the rapid extraction of proteins (Wu *et al*., 2001). In our experiment,

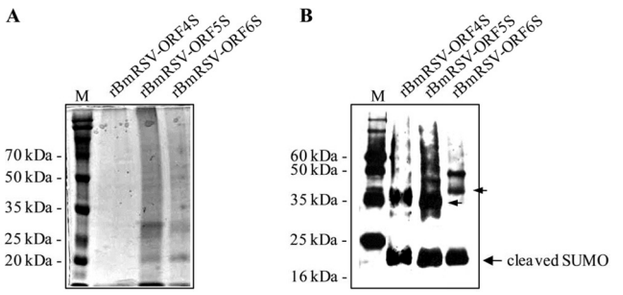

Fig. 4. Purification of the SUMO fusion protein in the fat body of *B. mori* larvae. 6xHis-tagged fusion protein was purified from rBmRSV-ORF4S, rBmRSV-ORF5S, and rBmRSV-ORF6S-injected *B. mori* larvae using the Ni-NTA spin kit under denaturing conditions. Proteins were separated on a 12% SDS-PAGE (A), transferred to nitrocellulose membranes for Western blot analysis and reacted with 6xHis tag (B). M, protein size marker. The recombinant proteins are indicated with arrows.

however, only a small amount of recombinant protein was detected in the hemolymph. These results indicated that the PRRSV structural protein may be associated with fat body cells rather than being secreted into the hemolymph and this is supported by the presence of transmembrane domain within each protein. Fig. 4 shows detection of the fusion proteins under denaturing conditions using the Ni-NTA column.

Expression of GP5 by SUMOstar fusion in *B. mori* larvae

A fusion tag that is not only a tool for detection and purification, but also enhances expression and/or solubility would greatly facilitate both structure/function studies and therapeutic protein production. In eukaryotic expression systems, however, the SUMO tag could be cleaved by endogenous desumoylase. In order to prevent SUMO cleavage and to obtain one band of target protein, we adapted an alternative SUMO-derived tag, designated SUMOstar, which is not processed by native SUMO proteases (Liu *et al*., 2008). First, we tried to fuse the SUMOstar and ORF5 of three major structural proteins because ORF5 is the most immunogenic proteins of the PRRSV. Surprisingly, as shown in Fig. 5A, SUMOstar-ORF5 fusion protein was first detected by SDS-PAGE. The results show that SUMOstar system makes significant impact in difficult-to-express proteins as previously reported.

In recent years, silkworm has become an ideal multicellular eukaryotic model system for basic research and practical

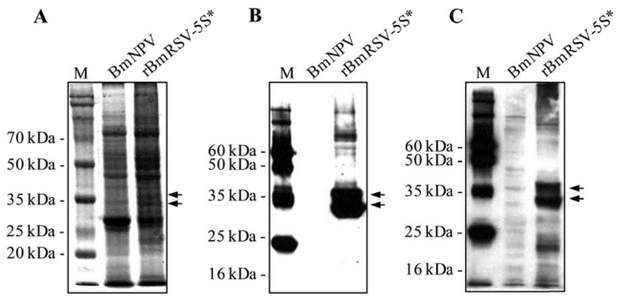

Fig. 5. Expression of the SUMOstar fusion protein in the fat body of *B. mori* larvae. Individual silkworm larvae on the first day of the 5th instar were injected with the wild type BmNPV or rBmRSV-ORF5S*. At 3 to 5 d post-injection, the fat bodies were collected by dissection and homogenized in lysis buffer. Proteins were separated on a 12% SDS-PAGE (A), transferred to nitrocellulose membranes for Western blot analysis and reacted with 6xHis tag (B) and porcine anti-PRRSV antibodies (C). M, protein size marker and BmNPV, wild type BmNPV. The recombinant proteins are indicated with arrows.

purpose. Silkworm, as a "biofactory" for producing targeted recombinant protein, has advantages such as low feeding cost, high production, easy purification, and high safety for biohazard. The SUMO or SUMOstar fusion system using silkworm would be very attractive for future expression of target proteins.

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References

Bautista EM, Molitor TW (1997) T cell responses to the structural polypeptides of porcine reproductive and respiratory syndrome virus. Arch Virol 144, 117-134.

Butt TR, Edavettal SC, Hall JP, Mattern MR (2005) SUMO fusion technology for difficult-to-express proteins. Protein Expr Purif 43, 1-9.

Conzelmann KK, Visser N, van Woensel P, Thiel HJ (1993) Molecular characterization of porcine reproductive and respiratory syndrome virus, a member of the arterivirus group. Virology, 193, 329-339.

Huang Y, Su Z, Li Y, Zhang Q, Cui L, Su Y, Ding C, Zhang M, Feng C, Tan Y (2009) Expression and Purification of glutathione transferase-small ubiquitin-related modifier-metallothionein fusion protein and its neuronal and hepatic protection against D-galactose-induced oxidative damage in mouse model. J Pharmacol Exp Ther 329, 469-478.

Hummel KB, Erdman DD, Heath J, Bellini WJ (1992) Baculovirus expression of the nucleoprotein gene of measles virus and utility of the recombinant protein in diagnostic enzyme immunoassays. J Clin Microbiol 30, 2874-2880.

Indik S, Valícek L, Klein D, Klánová J (2000) Variations in the major envelope glycoprotein GP5 of Czech strains of porcine reproductive and respiratory syndrome virus. J Gen Virol 81, 2497-2502.

Je YH, Chang JH, Kim MH, Roh JY, Jin BR, O'Reilly DR (2001) The use of defective *Bombyx mori* nucleopolyhedrovirus genomes maintained in *Escherichia coli* for the rapid generation of occlusion-postitive and occlusion-negative expression vectors. Biotechnol Lett 23, 1809-1817.

Jentsch S, Pyrowolakis G (2000) Ubiquitin and its kin: how close are the family ties? Trends Cell Biol 10, 335-342.

Johnson ES (2004) Protein modification by SUMO. Annu Rev Biochem 73, 355-382.

Kato T, Murata T, Usui T, Park EY (2004) Improvement of the production of GFP_{uv}-β1,3-*N*-acetylglucosaminyltransferase 2 fusion protein using a molecular chaperone-assisted insect-cell-based expression system. Biotechnol Bioeng 88, 424-433.

Koo HN, Bae SM, Choi JB, Shin TY, Yun BNR, Choi JY, Lee KS, Roh JY, Je YH, Jin BR, Yoo SS, Kim JS, Kim YI, Yoon IJ, Woo SD (2011) Characterization and expression of the pseudorabies virus (NYJ strain) glycoproteins in *Bombyx mori* cells and larvae. J Asia-Pacific Entomol 14, 107-117.

Koo HN, Oh JM, Lee JK, Choi JY, Lee KS, Roh JY, Je YH, Jin BR, Yoo SS, Kim JS, Kim YI, Yoon IJ, Woo SD (2008) Molecular characterization of ORFs 2 to 7 of Korean porcine reproductive and respiratory syndrome virus (CA) and its protein expression by recombinant baculoviruses. J Microbiol 46, 709-719.

Kost TA, Condreay JP, Jarvis DL (2005) Baculovirus as versatile vectors for protein expression in insect and mammalian cells. Nat Biotechnol 23, 567-575.

Lesley SA (2001) High-throughput proteomics: protein expression and purification in the postgenomic world. Protein Expr Purif 22, 159-164.

Li SJ, Hochstrasser M (1999) A new protease required for cell-cycle progression in yeast. Nature 398, 246-251.

Li SJ, Hochstrasser M (2000) The yeast ULP2 (SMT4) gene encodes a novel protease specific for the ubiquitin-like Smt3 protein. Mol Cell

Biol 20, 2367-2377.

Liu L, Spurrier J, Butt TR, Strickler JE (2008) Enhanced protein expression in the baculovirus/insect cell system using engineered SUMO fusions. Protein Expr Purif 62, 21-28.

Malakhov MP, Malakhova OA, Drinker M, Weeks S, Butt TR (2004) SUMO fusion and SUMO-specific proteases for efficient expression and puriWcation of proteins. J Struct Funct Genom 5, 75-86.

Marblestone JG, Edavettal SC, Lim Y, Lim P, Zuo X, Butt TR (2006) Comparison of SUMO fusion technology with traditional gene fusion systems: enhanced expression and solubility with SUMO. Protein Sci 15, 182-189.

Melchior F (2000) SUMO-nonclassical ubiquitin. Annu Rev Cell Dev Biol 16, 591-626.

Meulenberg JJ, Petersen den Besten A, de Kluyver E, van Nieuwstadt A, Wensvoort G, Moormann RJ (1997) Molecular characterization of Lelystad virus. Vet Microbiol 55, 197-202.

Meulenberg JJ, van Nieuwstadt AP, Essen-Zandbergen A, Langeveld JP (1997) Posttranslational processing and identification of a neutralization domain of the GP4 protein encoded by ORF4 of Lelystad virus. J Virol 71, 6061-6067.

Muller S, Hoege C, Pyrowolakis G, Jentsch S (2001) SUMO, ubiquitin's mysterious cousin. Nat Rev Mol Cell Biol 2, 202-210.

O'Reilly DR, Miller LK, Luckow VA (1992) Baculovirus expression vectors: a laboratory manual. WH Freeman & Co, New York.

Su Z, Huang Y, Zhou Q, Wu Z, Wu X, Zheng Q, Ding C, Li X (2006) High-level expression and purification of human epidermal growth factor with SUMO fusion in *Escherichia coli*. Protein Pept Lett 13, 785-792.

Sun Z, Xia Z, Bi F, Liu JN (2008) Expression and purification of human urodilatin by small ubiquitin-related modifier fusion in *Escherichia coli.* Appl Microbiol Biotechnol 78, 495-502.

Wu X, Kamei K, Sato H, Sato SI, Takano R, Ichida M, Mori H, Hara S (2001) High-level expression of human acidic fibroblast growth factor and basic fibroblast growth factor in silkworm (*Bombyx mori* L.) using recombinant baculovirus. Protein Expr Purif 21, 192-200.

Wu X, Nie C, Huang Z, Nie Y, Yan Q, Xiao Y, Su Z, Huang Y, Xiao J, Zeng Y (2009) Expression and purification of human keratinocyte growth factor 2 by fusion with SUMO. Mol Biotechnol 42, 68-74.

Zhou F, Xue Y, Lu H, Chen G, Yao X (2005) A genome-wide analysis of sumoylation-related biological processes and functions in human nucleus. FEBS Lett 579, 3369-3375.

Zuo X, Mattern MR, Tan R, Li S, Hall J, Sterner DE, Shoo J, Tran H, Lim P, Sarafianos SG (2005) Expression and purification of SARS coronavirus proteins using SUMO-fusions. Protein Expr Purif 42, 100-110.