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Expression of Pseudorabies Virus Glycoproteins gB, gC and gD using Insect Expression System

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Abstract

Porcine pseudorabies virus (PRV) causes the Aujeszky's disease (AD) which is economically important disease in the swine industry worldwide. Killed or live vaccines have been used to control this disease, but their efficacy and side effects remain problems to be solved. To solve these problems, in this study, production of recombinant PRV glycoprotein gB, gC and gD was investigated in insect expression system. Glycoprotein gB, gC and gD are regarded as the major immunogenic antigens in PRV. Abundant production and immunogenicity of glycoprotein gB, gC and gD were confirmed by SDS-PAGE and Western blot analysis, respectively. Optimal infection dose and time were also determined for the production of each recombinant PRV glycoprotein. Confirmation of glycosylation of recombinant gB, gC and gD suggested their usefulness as antigens for the development of diagnosis kit or vaccines for Aujeszky's disease.

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Introduction

The epidemic Aujeszky's disease (AD) is an economically important disease of pigs and caused by pseudorabies virus (PRV). To control this disease, several attempts using killed or live virus vaccine and hyper-immune serum have been introduced, however, their efficacy and side effects remain problems to be solved (Kluge *et al*., 1999; Mettenleiter, 2000; Mulder *et al*., 1997). As safe and effective alternatives for it, the development of DNA or subunit vaccines from immunogenic proteins has been suggested (van Rooij *et al*., 2000; Hong *et al*., 2002; Shams, 2005; Yoon *et al*., 2006).

The integument of PRV virion is surrounded by the envelope, a lipid membrane containing several viral glycoproteins (Mettenleiter, 2000). These viral glycoproteins are important for interactions between viral particles and host cells and act as major targets for the immune responses of the infected animals. Among them, glycoproteins gB, gC and gD are regarded as the major antigens and play an important role in neutralizing antibody production (Mettenleiter, 1994; Mettenleiter, 1996). Therefore, several studies have been performed to develop these glycoproteins as subunit vaccines, but their expression level was not satisfied because PRV glycoproteins have transmembrane domain (Katzen *et al*., 2009; Koo *et al*., 2011). Poor production of these proteins was shown in previous our study (Yun *et al*., 2011), the expression level of PRV glycoproteins in Escherichia coli was not satisfactory.

In this study, therefore, we tried to express PRV glycoproteins

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by using baculovirus expression system (BES). The BES is widely used for the production of many useful recombinant proteins for the subunit vaccines and the generation of virus-like particles for use as vaccines (Beljelarskaya, 2002; Gromadzka *et al*., 2006; Lee *et al*., 2015; Matassov *et al*., 2007; Park *et al*., 2016). To provide the antigens for subunit vaccine, glycoproteins gB, gC and gD were produced using BES in insect cells and their glycosylation was analyzed.

Materials and Methods

Virus and insect cell

The Spodoptera frugiperda continuous cell line IPLBSF-21(Sf21 cells) was maintained at 27 ℃ in SF900 II serum-free medium (Gibco, USA). The Autographa californica nucleopolyhedrovirus (AcNPV-C6) was used as control in this study. Routine cell culture maintenance and virus production procedures were performed according to the published procedure (O'Reilly *et al*., 1992).

Generation of recombinant viruses

The coding gene for glycoprotein gB, gC and gD from previously constructed plasmids (Yun *et al*., 2011) were cloned into pBacPAK9 (Clontech Laboratories, Inc, USA), respectively. Construction of recombinant transfer vectors was confirmed by sequencing and restriction enzyme digestion analysis.

Recombinant AcNPVs expressing PRV glycoproteins under the control of polyhedrin promoter were generated by co-transfection with each recombinant transfer vector and a defective viral genome, bAcGOZA DNA (Je *et al*., 2001). Transfection was performed using Cellfectin II™ (Invitrogen, USA) reagent according to the manufacturer's instructions, and the recombinant viruses were purified and propagated in Sf21 cells as described previously (O'Reilly *et al*., 1992).

Preparation of protein samples

Sf21 cells were infected with virus in a 60-mm diameter dish (1×10^6 cells) at a multiplicity of infection (MOI) of 5. At 3 days after inoculation, the culture supernatant was harvested and washed with PBS, and then used to prepare SDS-PAGE and Western blotting samples. To determine optimal MOI, cells were infected with each virus at 1, 5, 10 MOI, respectively, and then protein samples were prepared at 3 days post-infection (p.i.). Time course analysis for the optimal production of protein was performed from 1 to 6 days p.i. using cells infected with each recombinant virus at 5 MOI.

SDS-PAGE and Western blot analysis

The prepared protein samples were mixed with sample buffer, boiled for 5 min and subjected to 10% SDS-PAGE gel. For Coomassie stains, gels were washed with deionized water and stained with BioSafe Coomassie (Bio-Rad, USA). For Western blot analysis, the proteins on SDS-PAGE were transferred to a nitrocellulose membrane (Pall Corp., 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 porcine anti-PRV (1:100) antibodies (Choongang Vaccine Lab., Korea) in TBST buffer for 1 h and washed. Subsequently, the membrane was incubated with 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, Korea).

Glycosylation analysis

Glycosylation of recombinant protein was determined whether it is glycoprotein or not using Gelcode Glycoprotein Staining Kit (ThermoFisher Scientific, USA). For linked site confirmation, the infected medium was replaced with fresh serum-free insect cell culture medium including 5ug/ml tunicamycin. The harvested cells were immunoblotted by the methods previously described for protein analysis using SDS-PAGE and Western blot analysis.

Results and Discussion

Expression of PRV glycoproteins

Recombinant viruses were generated to express PRV glycoproteins under the control of the AcNPV polyhedrin promoter using recombinant transfer vectors (Fig. 1). Generated

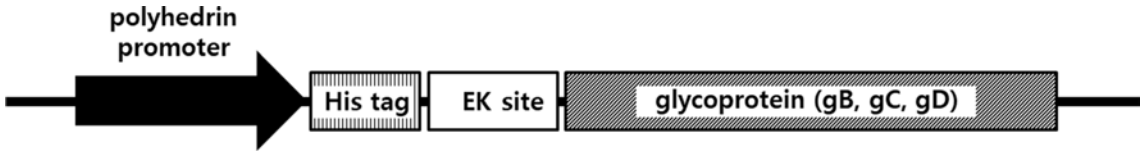

Fig. 1. Schematic representation of recombinant transfer vector pB9-gB, pB9-gC and pB9-gD for PRV glycoprotein gB, gC and gD genes, respectively..

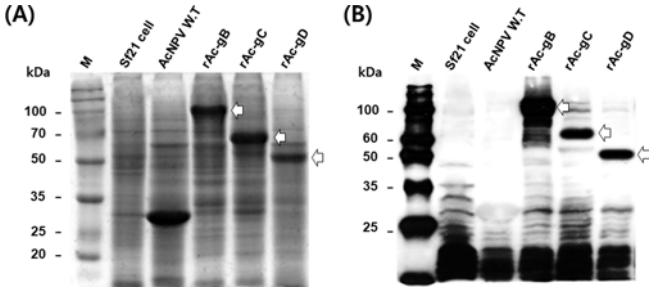

Fig. 2. Expression of PRV glycoproteins in Sf21 cells. Recombinant virus infected cells were harvested at 5 days post-infection, washed once with PBS, and lysed in lysis buffer. Proteins were separated on a 10% SDS-PAGE (A), transferred to nitrocellulose membranes for Western blot analysis using anti-PRV polyclonal antibody (B). Recombinant proteins are indicated with arrows

recombinant viruses were named as rAc-gB, rAc-gC and rAc-gD to express glycoprotein gB, gC and gD, respectively. To analyze the expression of the recombinant protein, Sf21 cells were infected with each recombinant virus and harvested at 5 days p.i. Recombinant glycoproteins were detected at approximately 100 kDa, 60 kDa and 50 kDa by rAc-gB, rAc-gC and rAc-gD, respectively, with the predicted protein sizes (Fig. 2A). These results were confirmed by Western blot analysis using anti-PRV antibody (Fig. 2B). Production of recombinant PRV glycoproteins was more abundant in baculovirus expression system than that of our previous study using E. coli expression system (Yun *et al*., 2011). This suggested that the production of PRV glycoproteins is more suitable in insect cells than E. coli. Among the PRV glycoproteins, gB, gC, and gD are regarded as being most important for the development of the antiviral humoral and cellular immune response (Mettenleiter, 1996; Mukamoto *et al*., 1991). Although several attempts for the production of PRV glycoproteins were conducted, however, they were unsuccessful (Hink *et al*., 1991; Prud'homme *et al*., 1997; Grabowska *et al*., 2009). Therefore, our results may provide a base for the further study to develop subunit vaccine for PRV using these glycoproteins.

As the result of successful production of glycoproteins, the

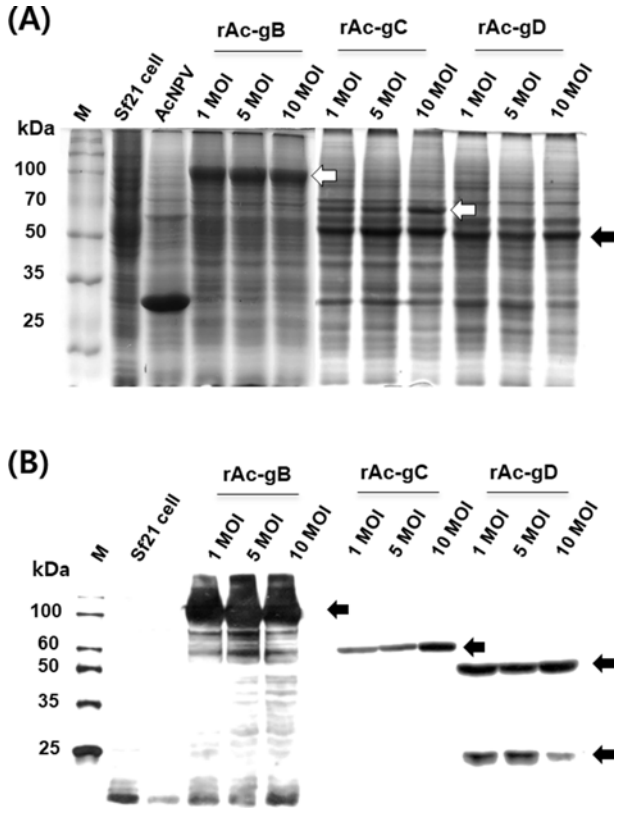

Fig. 3. Analysis of optimal multiplicity of infection (MOI) for PRV glycoprotein production in Sf21 cells. Protein samples were prepared from cells infected with virus at 3 days post-infection. Proteins were separated on a 10% SDS-PAGE (A), transferred to nitrocellulose membranes for Western blot analysis using anti-PRV polyclonal antibody (B). Recombinant proteins are indicated with arrows.

optimal virus MOI and post infection times were determined by the various MOI (Fig. 3) and harvesting times (Fig. 4). The yield of recombinant glycoprotein gB and gD was not differentiated by MOI, but gC was most produced at 10 MOI (Fig. 3). The production of recombinant protein was most abundant at 4 days p.i. for gD and 5 days p.i. for both gB and gC (Fig. 4). Besides, the reduced size of recombinant gD was detected even if it was a little amount. This result indicated that recombinant gD is not stable in insect cells.

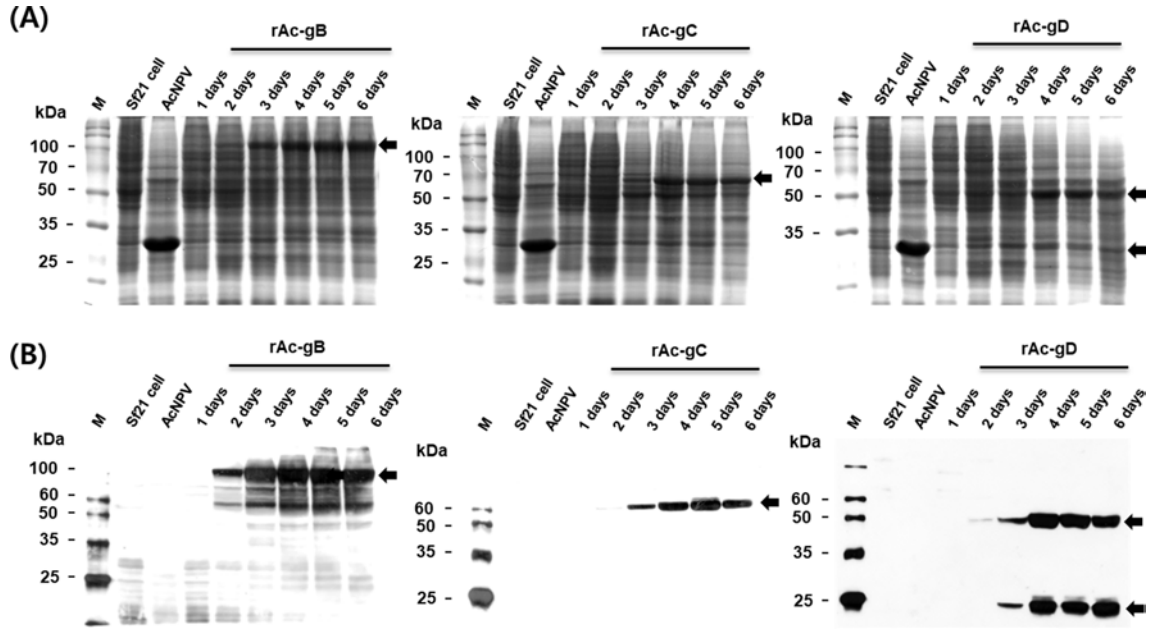

Fig. 4. Time course analysis of PRV glycoprotein production in Sf21 cells. Sf21 cells were infected with recombinant virus at 5 MOI for various times (1~6 days). Proteins were separated on a 10% SDS-PAGE (A) transferred to nitrocellulose membranes for Western blot analysis using anti-PRV polyclonal antibody (B). Recombinant proteins are indicated with arrows.

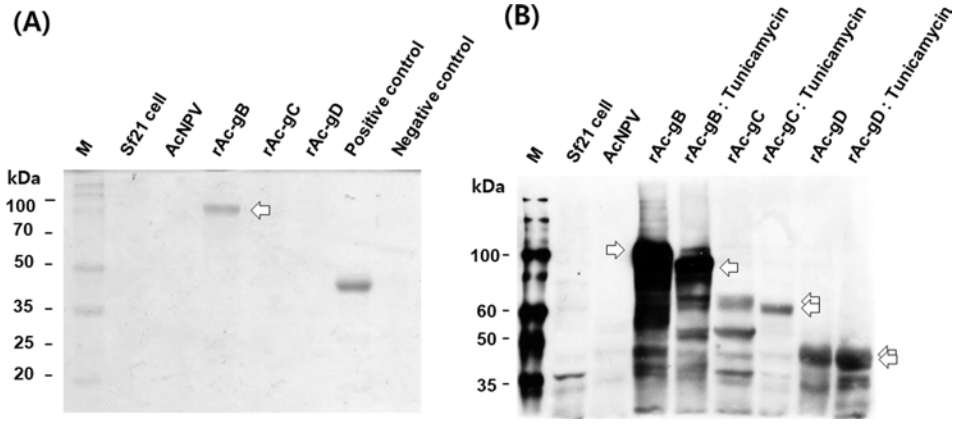

Fig. 5. Glycosylation confirmation of recombinant proteins produced in Sf21 cells. Cells were infected with recombinant viruses at 5 MOI for 4 days. Glycosylation was determined by staining method using glycoprotein staining kit (A) and glycosylation inhibitor method using tunicamycin (B). Positive and negative controls were provided from manufacturer.

Glycosylation analysis

Glycosylation process of glycoprotein is important because it may give the activity of protein. Therefore, we determined whether recombinant gB, gC and gD produced in insect cells are glycosylated. Glycosylation was determined using glycoprotein staining method and treatment of glycosylation inhibitor (Fig. 5). Glycosylation of recombinant gB was confirmed by both methods. However, glycosylation of recombinant gC and gD was confirmed only by treatment of glycosylation inhibitor. The size of recombinant proteins was reduced slightly by treatment of tunicamycin (Fig. 5B). Although glycosylation of recombinant gC and gD was not confirmed by staining method, these results suggested that all recombinant proteins produced in insect cells are glycosylated successfully.

Abundant production and glycosylation of recombinant PRV glycoproteins produced in insect cells suggest the usefulness of

these proteins as antigens for the development of diagnosis kit or vaccines for Aujeszky's disease.

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