Expression of Pseudorabies Virus (PRV) Glycoproteins gB, gC and gD using Bacterial Expression System

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The Pseudorabies (PR), also called Aujeszky's disease (AD), is an infectious viral disease caused by an alpha herpes virus and has domestic and wild pigs, as well as a wide range of domestic and wild animals, as the natural host. Pseudorabies virus (PRV) virions contain several envelope glycoproteins. Among them, gB, gC and gD are regarded as the major immunogenic proteins. We expressed these glycoproteins using the bacterial expression system and analyzed recombinant proteins. Expression of glycoproteins gC and gD were observed on SDS-PAGE or Western blot analysis, but gB was not. Optimal concentration of IPTG and inducing time were determined as 1.0 mM and 4 h, respectively, for the expression of both gC and gD in *E. coli*. A sodium dodecyl sulfate (SDS) was the most efficient detergent in solubilizing insoluble recombinant protein.

Key words: Pseudorabies virus, Glycoproteins gB, gC and gD, Bacterial expression system

Introduction

Pseudorabies or Aujeszky's disease (AD), caused by pseudorabies virus (PRV), is a serious illness of pigs and makes significant economic losses in the pig industry (Hong *et al.*, 2002). PRV virions contain several envelope glycoproteins which are important for interactions between viral particles and host cells and act as major targets for

the immune responses of the infected animals. Glycoproteins gB, gC and gD are regarded as the major immunogenic proteins and the antibodies induced by them can neutralize virus *in vitro* or *in vivo* (Mettenleiter, 1994; 1996). Attempts to control AD in swine include vaccination with killed or live virus vaccines and passive immunization with hyperimmune serum (Hsu *et al.*, 1984). And several immunogenic proteins use to development vaccines. However, PRV glycoproteins have transmembrane domain and the cell-based expression of these membrane proteins is difficult (Katzen *et al.*, 2009). Our recent report also indicated this fact, and the expression efficiency was not satisfied (Koo *et al.*, 2011).

Bacterial expression system is most popular and frequently used to produce recombinant proteins (Le *et al.*, 2009). *E. coli* can grow rapidly to high cell densities, and strains used for recombinant protein production have been genetically modified and high-level expression of recombinant proteins. Expression of the recombinant proteins depends on the various culture conditions and recombinant expression plasmids require a strong transcriptional promoter to control high-level gene expression. However, high-level expression of recombinant proteins in *E. coli* often precipitates into insoluble aggregates called inclusion bodies that are, in general, misfolded proteins and thus biologically inactive (Singh *et al.*, 2005; Rabhi-Essafi *et al.*, 2007). Insoluble proteins are devoid of biological activity and need solubilization, refolding and purification procedures to recover functionally bioactive product. In general, effective solubilization of insoluble proteins requires appropriate detergents or chaotropic agents. This often results in a very low recovery of the refolded protein (Song *et al.*, 2009).

In this study, we tried to express PRV glycoproteins using pET vector in *E. coli* and characterized recombinant proteins because there is little report about the expression of these in bacterial system. Furthermore, we determined

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Table 1. Primers used for amplification of the PRV genome

Primer	Nucleotide sequence ^a
gB-F	5' - <u>GGA TCC</u> ATG CCC GCT GGT GGC GGT CTT - 3'
gB-R	5' - <u>AAG CTT</u> CAG GGC GTC GGG GTC CTC GCT - 3'
gC-F	5' - <u>GAA TTC</u> ATG GCC TCG CTC GCG CGT GC - 3'
gC-R	5' - <u>AAG CTT</u> CGG CCC CGC CCG GCG GTA - 3'
gD-F	5' - <u>GGA TCC</u> ATG CTG CTC GCA GCG CTA TTG - 3'
gD-R	5' - <u>AAG CTT</u> CGG ACC G GG CTG CGC TTT TAG - 3'

^a Restriction sites are underlined.

the optimal solubilizing detergent for the expressed insoluble recombinant proteins through comparing several detergents.

Materials and Methods

Construction of the expression plasmid

The primers used for PCR amplification are listed in Table 1. The purified each PCR products were cloned into the T&A cloning vector and then digested with *Bam*HI (*Eco*RI) and *Hind*III restriction enzymes and inserted into the pET-21a(+) expression vector (Fig. 1). Expression plasmids were named pET-gB, -gC and -gD, respectively. Sequencing and the restriction enzyme digestion confirmed that the cloning was successful.

Expression of the PRV glycoproteins in *E. coli*

Escherichia coli BL21 (DE3) cells were transformed with pET-gB, -gC and -gD. A single colony from the transformants was added into LB medium containing ampicillin. This was cultured to an optical density (OD₆₀₀) of 0.6 and then 1.5 ml of the culture medium was added to 30 ml LB medium containing ampicillin and further incubated at 37°C up to an OD₆₀₀ of 1.0, then isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce protein expression. Cells were harvested by centrifugation at

10,000 g for 10 min and washed with PBS, resuspended in lysis buffer (50 mM Tris-HCl, 5% glycerol, 50 mM NaCl, pH 7–8). The resuspended cells were lysed by sonication and mixed with protein sample buffer and boiled. Protein samples were analyzed by SDS-PAGE and Western blotting.

RT-PCR

A total RNA was extracted from induced cell with Easy-spin™ Total RNA Extraction Kit (iNtRON Biotechnology, Korea) as recommended by the manufacturer. The RNA was used as a template for cDNA synthesis using Maxime RT Premix Kit (iNtRON Biotechnology, Korea). PCR amplification was performed using AccuPower PCR Premix (Bioneer Co., Korea). Primers used for PCR amplification are listed in Table 1. The amplified PCR products were separated by electrophoresis in 1.0% agarose gel.

Optimization of expression conditions

Different concentrations of IPTG (0.5, 1.0, 1.5, 2.0 and 2.5 mM) were added into the growing bacterial culture to determine the optimal concentration of IPTG for protein induction. As 1.0 mM IPTG was found to be the optimal concentration, it was added in bacterial culture and growth was analyzed for 24 h at 4 h interval (0, 4, 8, 12, 16, 20 and 24 h).

Solubilization of recombinant proteins

Various detergents such as Nonidet P-40 (NP-40), Triton X-100, Tween 20, Tween 80 and sodium dodecyl sulfate (SDS) were prepared. Cells were resuspended in lysis buffer, and then incubated in ice for 1 h with the detergents at 1% (w/v). After incubation, insoluble fractions were collected by centrifuged at 16,000 g for 10 min at 4°C. Total, soluble fractions (supernatant) and insoluble fractions (pellet) were analyzed by SDS-PAGE and Western blotting.

Results and Discussion

Expression of PRV glycoproteins

Expression of the PRV glycoproteins in *E. coli* was analyzed by SDS-PAGE, Western blotting and RT-PCR.

Fig. 1. Structure of the expression plasmid. Each genes were cloned between the *Bam*HI (*Eco*RI) and *Hind*III of pET-21a(+), downstream of the sequence coding the His-tag and expression plasmid under the control of the T7 promoter.

Fig. 2. Expression of glycoproteins gB, gC and gD in *E. coli*. The cells culture was induced with 1.0 mM IPTG and incubated for 8 h at 37°C. The cell pellets 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 and reacted with anti-His monoclonal antibody (B). Glycoproteins gC and gD are indicated with arrowheads.

Using Coomassie blue staining, detection was difficult except for the glycoprotein gC (Fig. 2A). To confirm the presence of expressed proteins, the total proteins were transferred to nitrocellulose and analyzed by immunoblotting using anti-His monoclonal antibody (Fig. 2B). The

Fig. 3. Agarose gel electrophoresis analysis of RNA extraction products (A) and RT-PCR products (B) of PRV glycoprotein genes. Lane 1, DNA maker; Lane 2, gB; Lane 3, gC; Lane 4, gD.

glycoproteins gC and gD were detected at approximately 55 and 45 kDa, respectively, with one band but gB was not on Western blot analysis. To elucidate the reason for none or low expression of target proteins, we checked whether target genes were transcribed to mRNA or not by RT-PCR. Extracted RNA from induced cells were confirmed by electrophoresis on 1.0% agarose gel (Fig. 3A), and then we used them for RT-PCR. The RT-PCR products of glycoproteins gB, gC and gD could be detected by agarose gel electrophoresis were approximately 2.7, 1.4 and 1.2 Kb, respectively, corresponding to the expected sizes (Fig. 3B). This may suggest that low or none expression of PRV glycoproteins are not associated to the transcription but to the translation process. Expression of recombinant proteins in *E. coli* is generally difficult when the codon use in the recombinant gene differs from the codon use in the host cells. Also, high-level expression is lead to accumulation as inclusion bodies in *E. coli*, insoluble proteins formed results as high-level expression of recombinant proteins. Insoluble proteins are difficult to detection because not solubilized in lysis buffer. In this case, solubilization of insoluble proteins requires appropriate chaotropic agents or detergents (Singh *et al.*, 2005). Therefore, in the case of low expression level, we are able to optimizing the codon usage in the gene to match *E. coli* codon bias or to solubilizing the recombinant proteins.

Optimal expression conditions

For the maximal expression of PRV glycoproteins, the

Fig. 4. IPTG concentration analysis of glycoprotein gC (A, B), gD (C, D) expression in *E. coli*. The cells culture was induced with various concentration of IPTG (0.5–2.5 mM) and incubated for 8 h at 37°C. Proteins were separated on a 10% SDS-PAGE (A, C), transferred to nitrocellulose membranes for Western blot analysis and reacted with anti-His monoclonal antibody (B, D). Glycoproteins gC and gD are indicated with arrowheads.

Fig. 5. Time course analysis of the glycoprotein gC (A, B), gD (C, D) expression in *E. coli*. The cells culture was induced with 1.0 mM and incubated for various times (0~24 hours). Proteins were separated on a 10% SDS-PAGE (A, C), transferred to nitrocellulose membranes for Western blot analysis and reacted with anti-His monoclonal antibody (B, D). Glycoproteins gC and gD are indicated with arrowheads.

Fig. 6. Effect of various detergents (gC, A-D; gD, E-H). Proteins were separated on a 10% SDS-PAGE (A, C, E, G), transferred to nitrocellulose membranes for Western blot analysis and reacted with anti-His monoclonal antibody (B, D, F, H).

optimal IPTG concentration and inducing time were determined. Although 1.0 mM concentration of IPTG appeared highest expression for both glycoproteins, the concentration of IPTG may be immaterial to the expression level (Fig. 4). Expression of glycoproteins gC and gD appeared to start at 4 h post-induction, no further increase after 4 h post-induction in our experiments (Fig. 5). The results assumed that PRV glycoproteins were not accumulated during the expression time and glycoproteins were degraded. It is known that recombinant protein synthesis in the pET system would be too rapid for protein expression (Semba *et al.*, 2008). As expected, we observed that PRV glycoproteins expressed rapidly. As a result, optimal inducing time was determined as 4 h for the expression of both gC and gD in *E. coli*.

Solubilizing detergent

The presence of PRV glycoproteins in both the soluble (supernatant) and insoluble fractions (pellet) was confirmed by SDS-PAGE and Western blotting. The PRV glycoproteins were present in insoluble fractions (Fig. 6). Expression of recombinant proteins in *E. coli* often results in accumulating then as insoluble aggregates *in vivo*. To avoid protein aggregation, *E. coli* is to lower the cultivation temperature to 15–20°C (Ong *et al.*, 2008). In general, insoluble proteins are solubilized by use of a high concentration of chaotropic agents or detergents (Singh *et al.*, 2005). Effective solubilization of insoluble proteins requires appropriate chaotropic agents or detergents. To solubilize insoluble proteins, we compared the efficacy of several detergents such as Nonidet P-40 (NP-40), TritonX-100, Tween20, Tween80 and SDS. As a result, except when the cell lysate was treated with SDS, PRV glycoproteins remained in the insoluble fraction (Fig. 6). It is known that SDS is helpful to the solubilization of insoluble protein and its removal required for the refolding of produced protein. However, the use of SDS for solubilization may helpful to the purification step because insoluble protein could not be purified.

When compared with other systems, the expression level of PRV glycoproteins in *E. coli* was not satisfactory, but more production of gC and easy and faster expression system have a value to more study. We are currently investigating the purification method of recombinant protein for the development of diagnosis kit or vaccines.

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