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Expression of porcine circovirus type 2 capsid protein fused with partial polyhedrin using baculovirus

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Abstract

Porcine circovirus type 2 (PCV2) is an important infectious swine virus causing postweaning multisystemic wasting syndrome (PMWS). PCV2 capsid protein, encoded by ORF2 has type-specific epitopes, is very immunogenic, and is associated with the induction of neutralizing antibodies. For the efficient production of capsid protein, recombinant *Autographa californica* nucleopolyhedroviruses were generated to express ORF2 fused with two forms of a partial polyhedrin. Recombinant capsid protein was produced successfully with the partial polyhedrin fusion form and the yield was high, as was shown by SDS-PAGE. Production of recombinant capsid proteins in insect cells was confirmed by Western blot analysis using anti-His monoclonal antibody, anti-ORF2 monoclonal antibody, and anti-PCV2 porcine serum. Fusion expression with amino acids 19 to 110 of the polyhedrin increased the production of recombinant capsid protein, but fusion with amino acids 32 to 85 did not. Additionally, PCV2 capsid protein is a glycoprotein; however, the glycosylation of recombinant protein was not observed. The results of an Enzyme-linked immunosorbent assay (ELISA) showed that recombinant capsid proteins could be utilized as antigens for fast, large-scale diagnosis of PCV2-infected pigs. Our results suggest that the fusion expression of partial polyhedrin is able to increase the production of recombinant PCV2 capsid protein in insect cells.

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Introduction

Porcine circovirus type 1 and type 2 (PCV1 and PCV2) are very small (about 17 nm), spherical, non-enveloped viruses with icosahedral symmetry (Allan and Ellis, 2000). PCVs have circular, single-stranded DNA genomes of approximately 1700 nucleotides, encoding two major open reading frames, ORF1 and ORF2. ORF1 is predicted to encode a replicase protein that is essential for replication of the viral DNA (Nawagitgul *et al*., 2000). ORF2 encodes the capsid protein, which plays a key role in virus particle assembly. Although PCV1 persists in pig populations, the presence of PCV1 has not been associated with any recognized clinical signs or lesions. In contrast, PCV2 has been implicated as the major causative agent of postweaning multisystemic wasting syndrome (PMWS), a disease that is characterized by severe immunosuppressive effects in the porcine

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host (Meehan *et al*., 1998). Since PMWS was first identified in 1991(Allan *et al*., 1998; Clark, 1997; Meehan *et al*., 1998), it has become an economically important disease (Allan and Ellis, 2000; Trundova and Celer, 2007). The capsid protein encoded by ORF2 is associated with the induction of neutralizing antibodies (Mahe *et al*., 2000; Pogranichnyy *et al*., 2000). Therefore, it acts as a major target for the immune responses of the infected animals (Liu *et al*., 2004). To control PCV2 infection, a subunit vaccine generated using the capsid protein of PCV2 has been developed and is widely used (Fan *et al*., 2007).

Since the mid 1980s, insect baculoviruses have been utilized as expression vectors of foreign genes (Choi *et al*., 1999; Matsuo *et al*., 2006). The baculovirus expression vector system (BEVS) originated from basic studies investigating the use of baculoviruses for bio-control purposes. Now, BEVS is used routinely for the production of recombinant proteins for a variety of purposes ranging from structural studies to the development of vaccines (Blanchard *et al*., 2003). This system can produce authentically processed proteins at high yields due to the insect cells' capacity for signal peptide recognition and secretion; assembly of oligomeric proteins, proper post-translational modifications, such as glycosylation; and phosphorylation and disulfide bond formation (Liu *et al*., 2008). Baculovirus produces novel occlusion bodies called polyhedra, which are produced at high levels during the very late phase of virus infection. Polyhedrin is major protein formed polyhedra and it must be transported into the nucleus in large amounts, where occluded virus particles are assembled into polyhedral (Blissard and Rohrmann, 1990). The nuclear localization signal in polyhedrin was reported to be the amino acid sequence KRKK, located between positions 32 and 35 in the polypeptide chain (Jarvis *et al*., 1991). Additionally, the amino acids 19-110 region is required for the supramolecular assembly of polyhedrin into occlusion-like particles in the nucleus (Jarvis *et al*., 1991). Recently, we demonstrated that the production of foreign proteins fused with various partial polyhedrin could enhance the production of foreign proteins significantly (Bae *et al*., 2013). Among various fused polyhedrin regions, fusions with amino acids 19–110 were able to localize the foreign proteins in the nucleus and enhance the expression level most highly. In addition, the fusion of amino acids 32–85 showed not only enhanced protein production but also intact production of foreign protein without a partial polyhedrin.

Previously, we reported that the BEVS is sufficient to production of PCV2 capsid protein (Lee *et al*., 2012). In this study, we generated recombinant baculoviruses to express the fusion form of PCV2 capsid protein with two domains between amino acids 19 and 110 or 32 and 85 of polyhedrin, respectively, to enhance the production of capsid protein. The effects of the partial polyhedrin fusion were investigated through yield, glycosylation, and immunogenic properties of the recombinant protein.

Materials and Methods

Cells and viruses

The *Spodoptera frugiperda* continuous cell line IPLBSF-21(Sf21 cells) was maintained at 27°C in SF900 II serum-free medium (Gibco, USA). The *Autographa californica* multiple nucleocapsid nucleopolyhedrovirus (AcMNPV-C6) and PCV2 KSY-1 strain (GenBank accession number AF454546) were used in this study. Routine cell culture maintenance and virus production procedures were performed according to a published procedure (King and Possee, 1992).

Construction of transfer vector

The previously constructed pB9-PCV2ORF2 (Lee *et al*., 2012) containing ORF2 gene was used in this study. The nu-

Table 1. Primers used for amplification and sequencing in this study

Name of primer[a]	Primer sequence[b]
KDEL-R	**CTCGAGTTA**<u>CAGTTCATCTTT</u>GTGGTGGTGGTGGTGGTG
Polh 19-F	**GAATTC***ATA*ATGAAGTACTACAAAAATTTAGGTG
Polh 110-R	**AGATCT**GGTAACAATGGGGAAGCTGTCTTC
Polh 32-F (P1)	**GAATTC***ATA*ATGAAGCGCAAGAAGCACTTC
Polh 85T-R (P2)	ACGCCTCCTTGGATACGTCATGGTAAGCTTCATCGTGTCGG
Polh 85T-F (P3)	ACGATGAAGCTTACCATGACGTATCCAAGGAGG

[a]For each truncation construct, the forward primer (F) is in the forward orientation relative to the coding strand, and the reverse primer (R) is in the reverse orientation relative to the coding strand.
[b]Restriction enzyme site shown in bold; Kozak consensus translation initiation sequence shown in italics. The endoplasmic reticulum retention signal sequence, KDEL, is underlined.

cleotide sequence encoding polyhedrin amino acids 19 to 110 was amplified from genomic DNA of AcMNPV using the PCR primer set Polh 19-F/Polh 110-R (Table 1) and then cloned into pB9-PCV2ORF2 between the *Eco*R I and *Bgl* II sites, resulting in p19-110-PCV2ORF2 for rAc-19-110-PCV2ORF2. Specific deletions within polyhedrin fragments 32–85 was generated in p19-110-PCV2ORF2 by PCR and Splicing by Overlap Extension (SOE) as described previously (Warrens *et al*., 1997). Briefly, two nested primers were designed to be complementary and they carried two segments corresponding to flanking sequences upstream and downstream of the region to be mutated. The nested PCR reactions utilized the PCR primer sets Polh 85T-R (P2) and Polh 85T-F (P3). Each of the primers was used in reactions with regions upstream or downstream of the outside primers, Polh 32-F (P1) or PCV2ORF2-H6-R (P4). The products were then mixed, denatured, and used as the template in a third PCR amplification using only the outside primers (P1, P4). The final PCR products were cloned into the intermediate plasmid, and then the partial polyhedrin with the PCV2ORF2 fragment was cloned into pBacPAK9, resulting in p32-85T-PCV2ORF2. The endoplasmic reticulum retention signal sequence, KDEL, with PCV2ORF2 was amplified from pB9-PCV2ORF2, p19-110-PCV2ORF2, and p32-85T-PCV2ORF2 using the PCR primers PCV2ORF2-F, Polh 19-F, Polh 32-F, and KDEL-R and cloning into pBacPAK9, resulting in pB9-PCV2ORF2-KDEL, p19-110-PCV2ORF2-KDEL, and p32-110-PCV2ORF2-KDEL for rAc-PCV2ORF2-KDEL, rAc-19-110-PCV2ORF2-KDEL, and rAc-32-110-PCV2ORF2-KDEL, respectively.

Generation of recombinant baculoviruses

Recombinant AcMNPVs expressing various encoded fragments under the control of the polyhedrin promoter were generated by co-transfection with each transfer plasmid and a defective viral genome, bAcGOZA DNA (Je *at al*., 2001). Transfection was performed using Cellfectin II™ reagent (Invitrogen, USA) 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).

SDS-PAGE and Western blot analysis

The cell lysate was prepared by incubating the cells with RIPA buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 5% glycerol, 0.1% Triton X-100) containing a protease inhibitor cocktail (Sigma-Aldrich, USA) for 30 min on ice followed by sonication; then, the lysate was mixed with protein sample buffer and boiled. The protein samples were subjected to 12% SDS-PAGE and then transferred to a PVDF membrane. The membranes were blocked in 5% milk in Tris-buffered saline containing 0.05% Tween 20 and probed with each of the following antibodies: His-tag monoclonal antibody (Cell Signaling, USA) and PCV2-ORF2 monoclonal antibody (JENO Biotech, Korea). The membrane was then incubated with horseradish peroxidase-coupled anti-mouse IgG antibody (Cell Signaling, USA), and the bound antibodies were detected using an enhanced chemiluminescence system (Merck Millipore, Germany) according to the manufacturer's instructions.

Glycosylation analysis

Glycosylation of the recombinant protein was determined using Gelcode Glycoprotein Staining Kit (Pierce Biotechnology, USA). For linked site confirmation, the infected medium was replaced with fresh serum-free insect cell culture medium including 5 μg/mL tunicamycin or a final concentration of 3 mM Benzyl-N-acetyl-α-D-galactosaminide (BAG). The harvested cells were immunoblotted by the methods previously described for protein analysis using SDS-PAGE and Western blot analysis.

ELISA analysis

The rAc-PCV2ORF2, rAc-19-110-PCV2ORF2, and rAc-32-110-PCV2ORF2 protein samples were subjected to 12% SDS-PAGE. After running the gel, a strip 7 mm in width, encompassing proteins of the apparent molecular weights of 28, 40, 35 kDa were excised and proteins were extracted using the EzWay PAG Protein Elution Kit (KOMAbio-tech, Korea). Each eluted protein was diluted in coating buffer (3.7 g bisodium carbonate, 0.64 g sodium carbonate, pH 9.6) at ratios of 1:4, 1:2 and 1:1, respectively. After 3 h of incubation at 4 °C, plates were washed three times with TBS-T (6.06g Tris Base, 8.7g NaCl, pH 7.2, 0.05% Tween 20), blocked for 2 h at 37 ˚C with a blocking solution containing TBS-T and 2% bovine serum albumin (BSA), and then incubated with anti-PCV2 ORF2 monoclonal antibody. After washing three times with TBS-T, the plates were incubated with 100 μL of diluted secondary antibody, anti-mouse-IgG antibodies con-

jugated to horseradish peroxidase (Cell Signaling, USA), for 2 h at 37 °C. Reaction products were washed with TBS-T and 50 μL of tetramethylbenzidine (TMB) (Sigma-Aldrich, USA) was added to each well. After incubation for 15 min at room temperature in the dark, the reaction was stopped by adding 50 μL of stop solution. The absorbance of each well was measured with a spectrophotometer at 450 nm.

Results and Discussion

Enhanced production of recombinant capsid protein

Recombinant viruses were generated to express partial polyhedrin fused to the N-terminus of the PCV2 ORF2 under

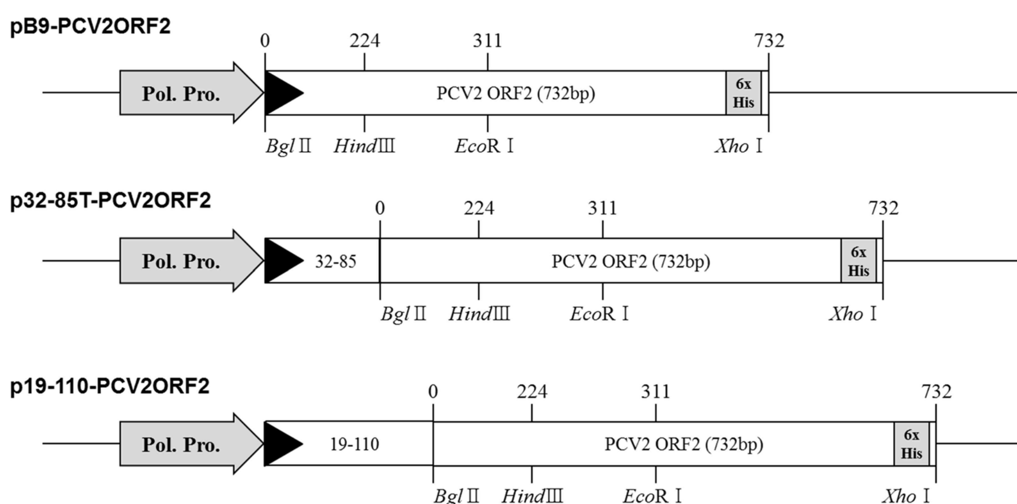

Fig. 1. Schematic representation of recombinant transfer vectors pPCV2ORF2, p19-110-PCV2ORF2. and p32-85T-PCV2ORF2.
The recombinant viruses were generated using these vectors and named rAcPCV2ORF2, rAc-19-110-PCV2ORF2, and rAc-32-85T-PCV2ORF2, respectively.

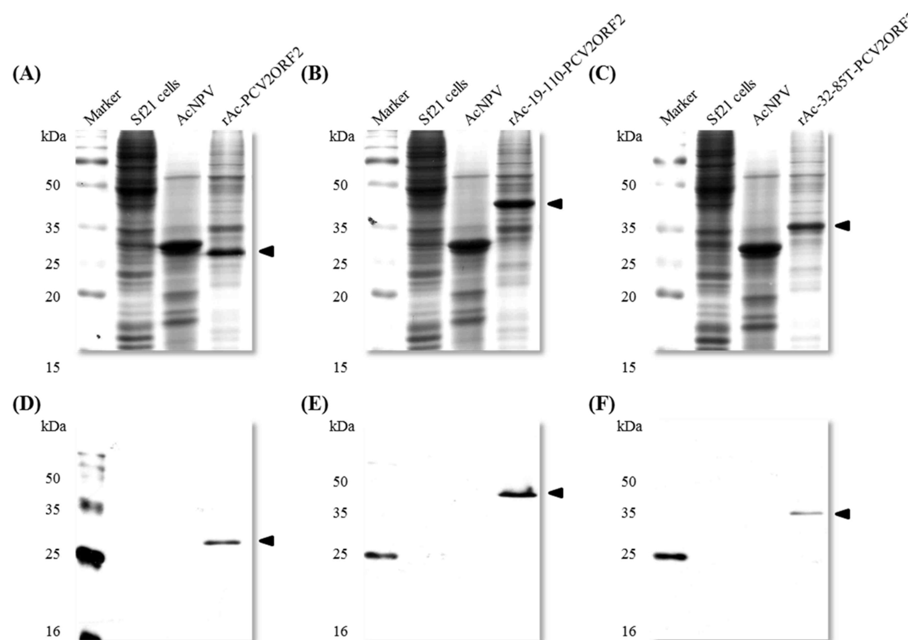

Fig. 2. Expression of the recombinant capsid proteins in Sf21 cells.
Proteins were separated by 12% SDS-PAGE (A, B, C) and transferred to nitrocellulose membranes for Western blot analysis using anti-His monoclonal antibody (D, E, F). Recombinant capsid proteins are indicated with arrowheads.

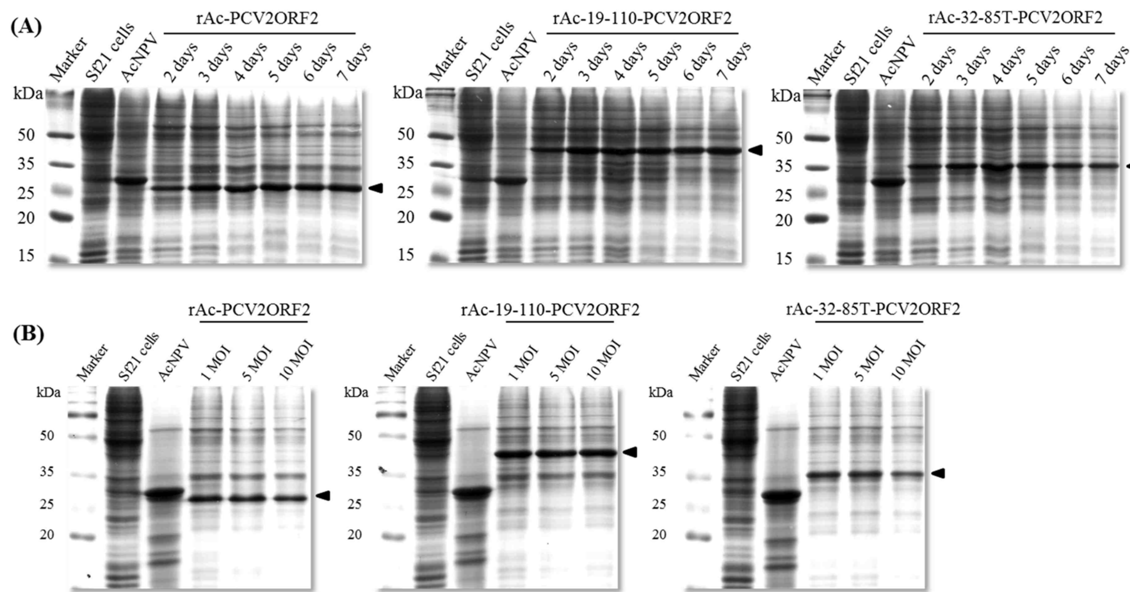

Fig. 3. Time course and optimal viral titer analysis for the production of recombinant capsid proteins in Sf21 cells.
The cells were infected with recombinant virus at various times (A) and at various viral titers (B). Each protein sample was separated by 12% SDS-PAGE. Recombinant proteins are indicated with arrowheads.

the control of the AcMNPV polyhedrin promoter (Fig. 1). To analyze the expression of the recombinant protein, Sf21 cells were infected with recombinant viruses and harvested at 4 d post infection. Recombinant capsid proteins were detected at approximately 28 kDa, 40 kDa, and 35 kDa by rAc-PCV2ORF2, rAc-19-110-PCV2ORF2, and rAc-32-85T-PCV2ORF2, respectively (Fig. 2). These sizes corresponded to the predicted protein sizes with the fused partial polyhedrin. However, cleavage of the recombinant protein when fused with polyhedrin 32–85 was not observed. This result suggested that the effect of partial polyhedrin fusion to the target protein may vary according to the target protein (Bae *et al*., 2013). The optimal multiplicity of infection (MOI) of virus and post infection time were determined by comparing various MOIs and harvesting times (Fig. 3). Our results showed that an MOI of 5 and harvest time at 4 d were optimal for the production of recombinant proteins.

As the yield of all recombinant capsid proteins was very high, the production of each recombinant protein was compared to the others with the same conditions of infection (Fig. 4). Fusion expression with partial polyhedrin 19–110 increased the production of recombinant capsid protein, but fusion with 32–85 could not increase production above that of the non-fused recombinant protein. Also, recombinant capsid proteins were detected with anti-PCV2 ORF2 monoclonal antibody or anti-PCV2 porcine serum, as well as anti-His monoclonal antibody.

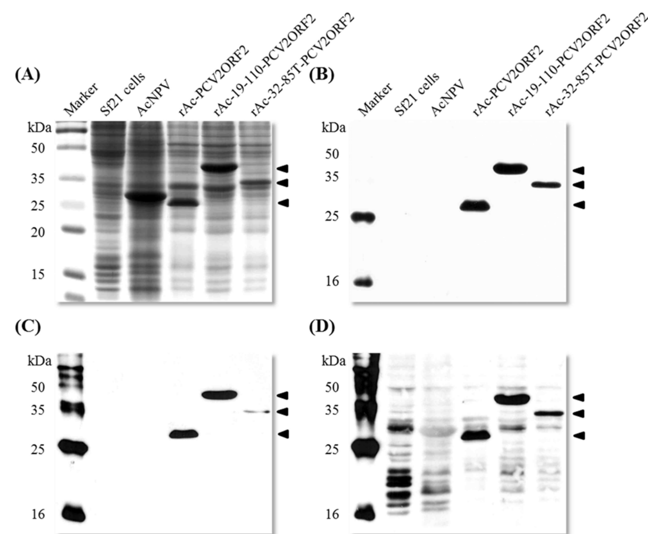

Fig. 4. Comparison of recombinant capsid protein expressions in Sf21 cells.
Sf21 cells were infected (MOI = 5) with recombinant viruses and were maintained for 4 days. Protein sample were separated by 12% SDS-PAGE (A), and transferred to nitrocellulose membranes for Western blot analysis using anti-His monoclonal antibody (B), anti-PCV2 ORF2 monoclonal antibody (C), and anti-PCV2 porcine serum (D). Recombinant proteins are indicated with arrowheads.

Although fusion of polyhedrin 19–110 increased the yield of recombinant PCV2 ORF2, it was lower than that of enhanced green fluorescent protein (EGFP) (Bae *et al*., 2013). These results corresponded to previous results that showed that fusion

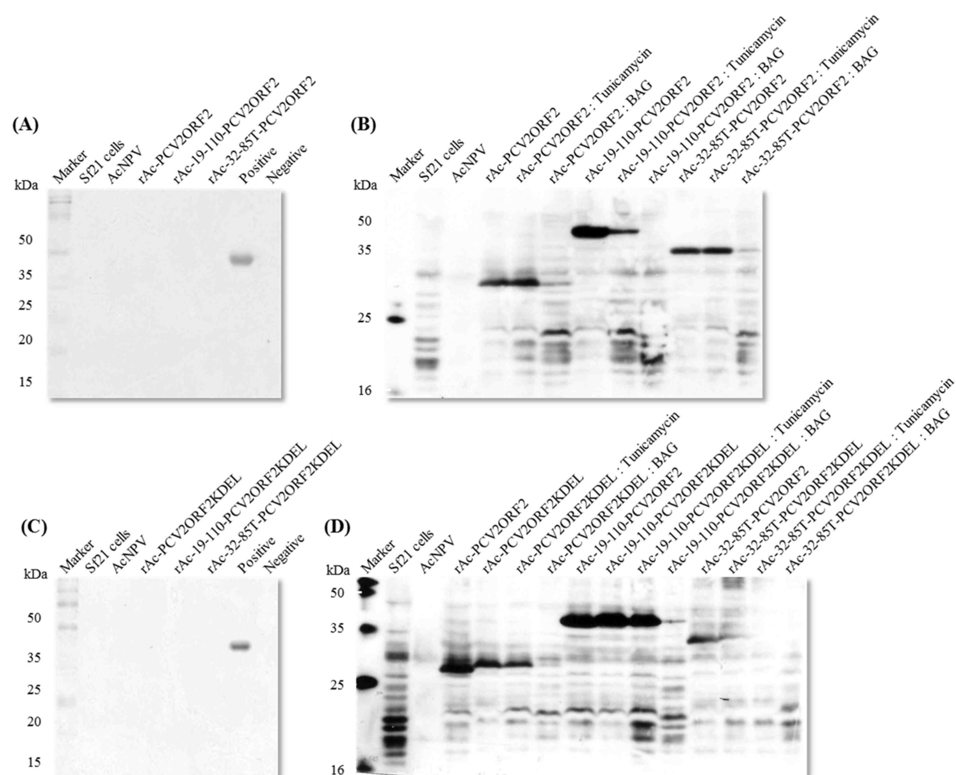

Fig. 5. Evaluation of the glycosylation of recombinant capsid proteins expressed in Sf21 cells. Sf21 cells were infected (MOI = 5) with recombinant viruses that were non-fused (A, B) and fused with ER retention signal (C, D) and were maintained for 4 d. Glycosylation was determined using a glycoprotein staining kit (A, C) and a glycosylation inhibitor method using tunicamycin and Benzyl-N-acetyl-α-D-galactosaminide (BAG) (B, D). (A) 1, rAc-PCV2ORF2; 2, rAc-19-110-PCV2ORF2; 3, rAc-32-85T-PCV2ORF2; 4, positive control; 5, negative control. (B) 1, rAc-PCV2ORF2; 2, rAc-19-110-PCV2ORF2; 3, rAc-32-85T-PCV2ORF2;

expression of the partial polyhedrin increase the production of foreign target proteins and that the fusion effects vary with the target protein. The detection of recombinant PCV2 ORF2 by not only monoclonal antibody but also anti-PCV2 porcine serum indicated that the fusion of the partial polyhedrin did not influence the antigenicity of recombinant PCV2 capsid protein and that it would be useful to the development of diagnostic tools and vaccines.

Glycosylation and immunogenicity analysis

In our previous report, we did not observe the glycosylation of recombinant PCV2 capsid proteins in insect cells (Lee *et al*., 2012). The glycosylation process is important in the post-translational modification of proteins because it may confer activity to the proteins. Therefore, we investigated this again using the polyhedrin fused-capsid protein produced in Sf21 cells. Additionally, to improve glycosylation in insect cells, recombinant capsid proteins were expressed by fusion with

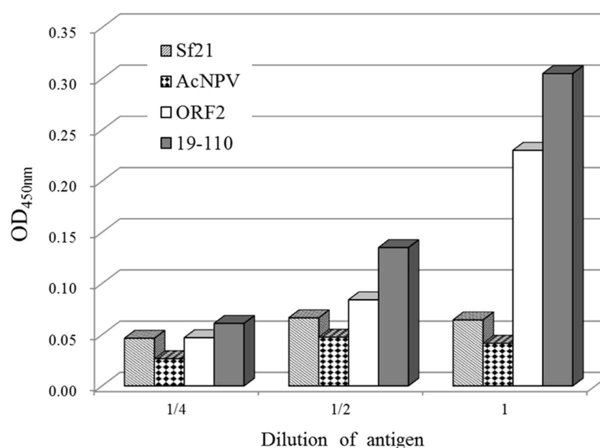

Fig. 6. Immunogenicity analysis of recombinant capsid proteins by ELISA. Antigen was prepared from gel-eluted recombinant capsid proteins and coated directly using a series dilution method.

KDEL (Lys-Asp-Glu-Leu), an amino acid sequence that is known to be the endoplasmic reticulum retention signal of AcMNPV chitinase (Hodgson *et al*., 2011). Glycosylation was determined using a glycoprotein staining method and treatment

of glycosylation inhibitors (Fig. 5). Unfortunately, glycosylation was not observed to any recombinant proteins as we had seen in the previous study. PCV2 ORF2 is known to have an N-linked glycosylation site (Hamel *et al*., 1998), but there is no study that has demonstrated this glycosylation. There are two possible reasons for our results: first, the insect cell system is not suitable for post-translational modification of PCV2 ORF2; and second, although PCV2 ORF2 possesses at least one glycosylation site from putative amino acids, it does not proceed towards glycosylation. However, glycosylation of PCV2 ORF2 may be not important for antigenicity, because the recombinant capsid proteins reacted with both ORF2 antibody and anti-PCV2 porcine serum. Thus, we evaluated the usefulness of recombinant proteins as antigens for diagnostics.

The immunogenicity of recombinant capsid proteins as diagnostic antigens was analyzed by an ELISA method with direct coating of antigens. The antigen levels were inversely proportional to the antigen dilution (Fig. 6). These levels were highest using the recombinant capsid protein fused with polyhedrin 19–110. This result suggested that partial polyhedrin fusion expression of PCV2 ORF2 can enhance not only the yield of capsid protein but also its immunogenicity.

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