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Expression of VP2 Gene Protein of Infectious Bursal Disease Virus Detected in Korea

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Abstract. The VP2 gene DNA (1.4 kb in approximate) of a very virulent infectious bursal disease virus (vvIBDV) Chinju strain detected in Chinju, Korea was cloned into the bacmid, a baculovirus shuttle vector, through transposition of the gene from initially cloned pFastBacHTa plasmid, a baculovirus expression vector, and was subsequently expressed in *Spodoptera frugiperda* (*Sf*) cells. Biological properties of the expressed VP2 subunit protein were characterized to aid in the development of genetically engineered diagnostic reagents and vaccines against the vvIVDV. When the VP2 DNA-recombinant bacmid was transfected and propagated in the *Sf* cells, the cells showed no occlusion formation, which is a positive evidence for the insertion of the VP2 DNA into the polyhedrin gene of the bacmid, whereas the occlusions were observed in the cells infected by the *Autographa californica* nuclear polyhedrosis virus, a wild baculovirus. The expression of VP2 DNA was identified by strong positive reaction in fluorescent antibody test using chicken anti-IBDV serum. The VP2 protein was determined as a polypeptide band with M_r of 48 kDa by the sodium dodecyl-polyacrylamide gel electrophoresis for the lysate of the *Sf* cells infected with the recombinant bacmid. The VP2 protein was successfully purified from the cell lysate by Ni-NTA affinity chromatography. The expressed VP2 subunit protein reacted specifically with chicken anti-IBDV serum in Western blotting.

Key words: expression, IBDV, VP2 protein

Introduction

Infectious bursal disease (IBD) is an immunosuppressive disease of young chicken characterized by severe depletion of B-lymphocytes in the bursa of Fabricius [1], which subsequently lead to immunodeficiency to other infections and vaccinations [2]. The etiological agent, infectious bursal disease virus (IBDV), belongs to the genus *Avibirnavirus* of the family *Birnaviridae* [3]. Genome of IBDV consists of two segments, A and B, of ds-RNA [4,5]. The segment A gene has two open reading frames (ORFs). The ORF2 encodes a 115 kDa precursor polyprotein which is cleaved into VP2, VP4 and VP3, while the ORF1 encodes a non-structural protein VP5 [6–9]. Among the proteins, VP2 is a structural protein containing the major antigenic epitope, which stimulates production of the neutralizing antibodies in the host [6,10]. The VP2 gene is, therefore, an important target for cloning and expression in the development of genetically engineered vaccines and diagnostic reagents.

Several expression systems of prokaryotic and eukaryotic vectors have been used for *in vitro* production of the subunit proteins from foreign genes. The prokaryotic expression is performed in bacterial vectors and hosts. These expression systems are useful in the production of proteins from cloned foreign genes, which have no posttranslational modifications such as acylation, glycosylation and phosphorylation. On the other hand, the eukaryotic

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expression system is fundamental for the production of glycosylated proteins or proteins which should be appropriately modified by posttranslational cleavages [11].

The IBDV genes have been cloned into vectors of *E. coli* [12], *S. cerevisiae* [13], fowlpox virus [14–16], herpesvirus [17], Marek's disease virus [18] and baculovirus [19–21] for expression. Vaccination of chicken with these expression products resulted in variable level of active or passive protection, except VP2 protein expressed in *E. coli*, a prokaryotic system, failed to promote neutralizing antibodies in chicken [12]. The VP2 proteins expressed in *S. cerevisiae* [13] and fowlpox virus [14,15] conferred passive protection of chicken against IBDV. Recently, baculovirus vector system has been widely used for the expression of IBDV genes in eukaryotic cells [19,21,22]. In the eukaryotic cells such as *Spodoptera frugiperda* (*Sf*), foreign proteins are expressed by replacing non-essential viral polyhedrin gene of baculovirus, which is highly expressed, with the foreign gene of interest. One of the advantages of the eukaryotic expression is that the posttranslational processes, such as folding, disulfide bonds and glycosylation, are performed accurately and allow full biological activities of the expressed proteins [13]. Vakharia et al. [21] constructed a recombinant baculovirus to express the polyprotein of segment A gene from an antigenic variant IBDV in *Sf* cells. The expressed polyprotein actively protected the chicken after immunization and was capable of passively protecting progeny when challenged with the antigenic variant and classical virulent IBDVs. Dybing and Jackwood [19] reported the expression of individual proteins of VP2, VP3, VP4 and complete polyprotein from an antigenic variant IBDV in the baculovirus system. When the protein-expressed *Sf*9 cell lysates were injected into chicken, the proteins conferred protection against challenges by the classical virulent and antigenic variant IBDVs. On the other hand, Wang et al. [22] reported the purification of baculovirus-expressed VP2 protein, which has five histidine residues at the C-terminus, by metal–ion affinity chromatography. The purified VP2 subunit protein showed ability of inducing neutralizing antibodies in chicken. Therefore, it was suggested that the VP2 subunit protein expressed from the vvIBDVs can maintain its biological properties after purification and provide specific protection of chicken against the vvIBDVs.

In the present study, the VP2 gene of a vvIBDV Chinju strain detected in Chinju, Korea was expressed in the baculovirus system, and the VP2 protein was purified and characterized for information in the development of genetically engineered diagnostic reagents and vaccines.

Materials and Methods

VP2 DNA

The IBDV segment A-1 DNA contained VP2 gene and cloned in *Eco*RI and *Sal*I sites of pTZ19R plasmid DNA, which was previously constructed from a vvIBDV Chinju strain detected in Chinju, Korea [23], was used for expression of VP2 gene in the present study. Polymerase chain reaction (PCR) was done to amplify VP2 DNA from the A-1 clone using sense primer having *Eco*RI site of GAATTC (5′CCGGAATTCATGACAAACCTGCAAGATCAAACCC3′) and antisense primer having *Sal*I site of GTCGAC and stop codon of TTA close to the *Sal*I site (5′GATCGTCGACTTACCTTAGGGCCCGGATTATGTCTTTG3′), which were based on the nucleotide sequence of VP2 gene (1356 bases) of the Chinju strain (GenBank AF508177) and CEF94 strain [24]. For the PCR mixture of 1 µl of A-1 DNA, 5 µl of 10× PCR buffer, 4 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP mixture, 1 µl each of 100 pM sense and antisense primers, 1 µl of *Taq* DNA polymerase (5 U/µl) (Perkin-Elmer, USA) and 36 µl of distilled water, the amplification was performed by thermocycler (Biometra, Germany) following the program of 94°C for 5 min and 30 cycles of 94°C for 1 min, 63°C for 1 min, 72°C for 1.5 min, and final extension at 72°C for 7 min.

Construction of Recombinant VP2 DNA Clone for Expression

Construction of recombinant VP2 DNA clone for protein expression was performed using Bac-to-Bac™ Baculovirus Expression kit reagents (Invitrogen, USA) by the manufacturer's suggestions. The amplified VP2 DNA was cloned into *Eco*RI and *Sal*I sites of pFastBacHTa plasmid DNA by ligation using T4 DNA ligase (Invitrogen). The recombinant plasmid DNA was transformed into competent *E. coli* DH5α cells by heat shock at 42°C for 45 s. After adding SOC medium (0.5% yeast extract, 2%

tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM $MgSO_4$, 20 mM glucose), the mixture was shaken for 1 h at 220 rpm, 37°C. The transformed cells were plated onto Luria Bertani (LB) agar (Invitrogen) containing ampicillin (50 μg/ml) and incubated at 37°C, overnight. Transformed colonies were cultured overnight in LB broth with ampicillin (50 μg/ml) by shaking at 220 rpm, at 37°C, and were subjected to DNA extraction by alkaline-lysis, restriction enzyme digestion and electrophoresis through 1% agarose gel for identification of recombinant DNA clones. To confirm whether the reading frame of the VP2 DNA in pFastBacHTa plasmid is in right orientation for protein expression, nucleotide sequencing was done using Dye Terminator Cycle Sequencing kit (Perkin-Elmer, USA) by the automatic sequencer (ABI prism 377, Advanced Biotechnologies, USA).

The VP2 DNA-recombinant pFastBacHTa plasmid DNA was purified and transformed into *E. coli* DH10Bac cells by heat shock for the site-specific transposition of the VP2 DNA from pFastBacHTa to a bacmid DNA, which is contained in the DH10Bac cells. After adding SOC medium and shaking for 4 h at 150 rpm, 37°C, the transformed cells were plated onto LB agar containing kanamycin (50 μg/ml), gentamicin (7 μg/ml), tetracycline (10 μg/ml), Bluo-gal (100 μg/ml) and isopropylthio-β-galactoside (40 μg/ml), and incubated at 37°C for 28 h. Transformed colonies were cultured overnight in LB broth with kanamycin (50 μg/ml), gentamicin (7 μg/ml) and tetracycline (10 μg/ml) by shaking at 250 rpm, at 37°C, and were subjected to DNA extraction by alkaline-lysis and purification. The VP2 DNA was identified from the recombinant bacmid by PCR using sense primer (5′CCCAGTCACGACGTTGTAAAACG3′) and antisense primer (5′AGCGGATAACAATTTCACACAGG3′) specific to M13/pUC vector, which are routinely used in gene manipulation. The recombinant VP2 DNA was also confirmed by nested PCR using sense primer having *Eco*RI site of GAATTC (5′GAATTCATGACAAACCTGCAAGATCAAACCC3′) and antisense primer having *Sal*I site of GTCGAC (5′GTCGACTTACCTTCAGGGCCCGGATTATGTCTTTG3′), which were specific to the VP2 gene of the Chinju strain.

Expression and Purification of VP2 Protein

The VP2 DNA-recombinant bacmid DNA was transfected into *Sf* High Five cells (*Sf* cells) (Invitrogen) and cultured in serum-free, Sf-900 II medium (Invitrogen) at 27°C followed by the Bac-to-Bac™ Baculovirus Expression kit manual (Invitrogen). Mock-infected *Sf* cells and *Sf* cells infected with the *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV), a wild baculovirus, were also cultured as control. Briefly, each 35-mm well of 6-well tissue culture plate (Nunc, USA) was seeded with 9×10^5 *Sf* cells in 2 ml of Sf-900 II medium containing penicillin (50 U/ml) and streptomycin (50 μg/ml), and the cells were attached at 27°C for 1 h. The recombinant bacmid DNA was mixed with cellfectin reagent (Invitrogen) and transfected into the *Sf* cells. The *Sf* cells were cultured with SF-900 II medium containing penicillin and streptomycin at 27°C for 72 h.

The *Sf* cells were harvested by centrifugation for 5 min at $500 \times g$ and sonicated three times for 10 s at 40% power using a microtip probe (Vibra Cell; Sonics and Materials, Inc., USA). The cell lysate was further centrifuged at $10{,}000 \times g$ for 10 min for separation of supernatant from the cell pellets and subjected to determination of the expressed protein. The harvested cells were also resuspended in lysis buffer (50 mM Tris–HCl, pH 8.5, 5 mM 2-mercaptoethanol, 100 mM KCl, 1 mM phenylmethyl-sulfonyl-fluoride, 1% Nonidet P-40), and the cell debris was removed by centrifugation at $10{,}000 \times g$ for 10 min. The expressed VP2 protein was purified from the supernatant by affinity column of Ni-NTA resin (Invitrogen) followed by the manufacturer's suggestion.

Determination of VP2 Protein

Cell lysate and Ni-NTA column-purified VP2 protein were used in determination of VP2 protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. For SDS-PAGE [25] analysis, cell lysate and purified VP2 protein were mixed with sample buffer (0.5 M Tris–HCl, pH 6.8, 10% glycerol, 5% SDS, 5% β-mercaptoethanol, 0.25% bromophenol blue). After boiling for 2 min at 100°C, samples were loaded in a 12% acrylamide-*bis*-acrylamide gel. After electrophoresis, the gel was stained with 0.1% Coomassie brilliant blue and de-stained for observation. Acrylamide gel containing separated polypeptide bands was blotted onto nitrocellulose membrane by electro-blotting system (Invitrogen) followed by the methods of

Laemmli [25]. The blotted proteins were blocked with tris-buffered saline (TBS) (20 mM tris, 500 mM NaCl, pH 7.4) containing 1.5% skim milk for 2 h at room temperature, and the membrane was washed three times with TBS. The membrane reacted with 1% BSA–TBS containing polyvalent chicken anti-IBDV serum for 2 h at room temperature and washed three times with TBS. Then, the membrane was reacted with goat anti-chicken IgG conjugated with horseradish peroxidase (KPL, USA), which was diluted to 1 : 2000 in 1% BSA–TBS. After washing three times with TBS, the membrane was reacted with a chromogen, 4-chloro-1-naphthol.

Fluorescent Antibody Test

To identify the VP2 protein expressed in *Sf* cells by Fluorescent antibody (FA) test, the cells were seeded onto cover-glass which contained in the wells of 6-well culture plate (Nunc) and inoculated with recombinant bacmid. Three days later, cell on the cover-glass were washed with cold PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, pH 7.2) and fixed with 1 : 1 solution of methanol : acetone for 10 min at −20°C. The cells were washed with cold PBS and reacted with polyvalent chicken anti-IBDV serum, which was diluted to 1 : 50 in PBS, for 1 h at 37°C. Cells were washed again and the bound antibodies were reacted with FITC-conjugated rabbit antiserum to chicken IgG (Cappel, USA), which was diluted to 1 : 500 in PBS, for 30 min at 37°C. Finally, cells were dehydrated with ethanol and mounted with glycerol, and observed by microscope.

Results

Construction of Recombinant VP2 DNA Clone

The VP2 DNA of approximately 1.4 kb, which was amplified from segment A-1 clone of the vvIBDV Chinju, was cloned into pFastBacHTa plasmid DNA, and the VP2 DNA was identified from the recombinant plasmid after digestion by *Eco*RI and *Sal*I (Fig. 1). In nucleotide sequencing, ATG start codon of the VP2 DNA was confirmed in right reading frame for expression at the end part of the cloning site of pFastBacHTa plasmid (Fig. 2). After transposition of the VP2 DNA from pFastBacHTa plasmid DNA to bacmid DNA, the VP2 DNA was identified in the bacmid by PCR using primers specific to M13/pUC vector. By the nested PCR using primers specific to the VP2 gene, the VP2 DNA of 1.4 kb was confirmed in the bacmid (Fig. 3).

Expression of VP2 Protein

When VP2 DNA-recombinant bacmid DNA was transfected and propagated in *Sf* cells, the cells showed no occlusion formation, which was a positive

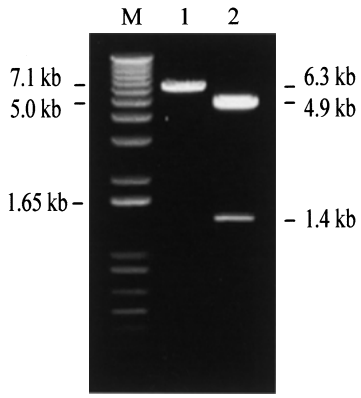

Fig. 1. Construction of recombinant DNA clone between VP2 gene of IBDV Chinju and pFastBacHTa plasmid DNA: 1, Recombinant DNA (6.3 kb) of VP2 DNA (1.4 kb) and pFastBacHTa (4.9 kb); 2, VP2 DNA identified from the recombinant DNA by digestion with *Eco*RI and *Sal*I; M, 1 kb plus ladder DNA marker (Invitrogen).

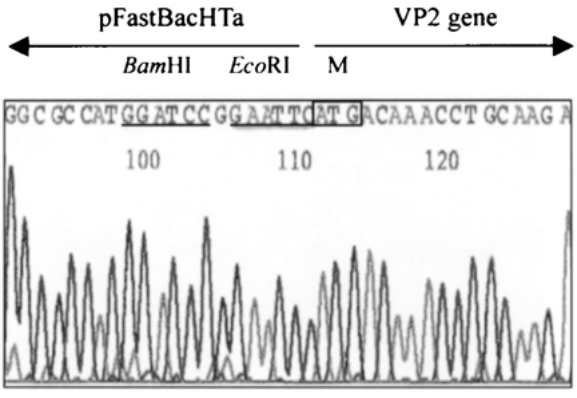

Fig. 2. Confirmation of reading frame of VP2 DNA in recombinant pFastBacHTa plasmid DNA. ATG codon for initiation of the VP2 protein expression was determined at the end part of multiple cloning site of the plasmid; M, methionine residue predicted from ATG codon of the VP2 DNA.

evidence for the insertion of VP2 DNA into the polyhedrin gene of the bacmid. Instead, the cellular shape was slightly altered to irregular and rough form. Whereas, *Sf* cells infected with wild *Ac*NPV exhibited occlusion formation by undamaged viral polyhedrin gene (Fig. 4). The *Sf* cells infected with the recombinant bacmid showed strong positive reaction in FA test using chicken anti-IBDV serum and FITC-conjugated rabbit anti-chicken IgG, whereas the mock-infected *Sf* cells had no specific FA reaction (Fig. 5).

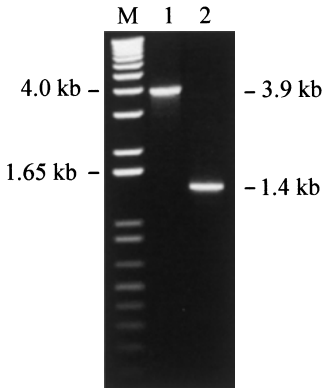

Fig. 3. VP2 DNA identified from recombinant bacmid DNA by PCR: 1, DNA of 3.9 kb consisting of part of bacmid and whole VP2 DNA amplified from the recombinant bacmid DNA by M13/pUC primers; 2, VP2 DNA of 1.4 kb amplified from the recombinant bacmid DNA by primers specific to VP2 DNA; M, 1 kb plus ladder DNA marker (Invitrogen).

In SDS-PAGE, lysate from *Sf* cells infected with the recombinant bacmid as well as supernatant liquid from the cell lysate revealed VP2 polypeptide band with M_r of 48 kDa approximately, whereas no polypeptide band corresponding to the VP2 protein was observed in mock-infected cells and *Ac*NPV-infected cells. The VP2 subunit protein was successfully purified by the Ni-NTA affinity chromatography. The VP2 polypeptide in the cell lysate and VP2 subunit protein purified by the affinity chromatography reacted specifically with chicken anti-IBDV serum by Western blotting (Fig. 6).

Discussion

Antigenic proteins of whole virion have been used as diagnostic reagents and immunogens for the control of viral diseases. However, the whole virus proteins sometimes exhibit low specificity in diagnostic tests and immune responses. Therefore, developmental efforts should be geared toward genetically engineered subunit proteins to overcome these problems.

In the present study, VP2 DNA of the vvIBDV Chinju in right reading frame for translation was cloned into the pFastBacHTa plasmid DNA. The VP2 DNA harbored in an expression cassette of the pFastBacHTa plasmid, which contains Tn7R and Tn7L attachment sequences, was transposed to the appropriate Tn7 attachment site on the bacmid DNA,

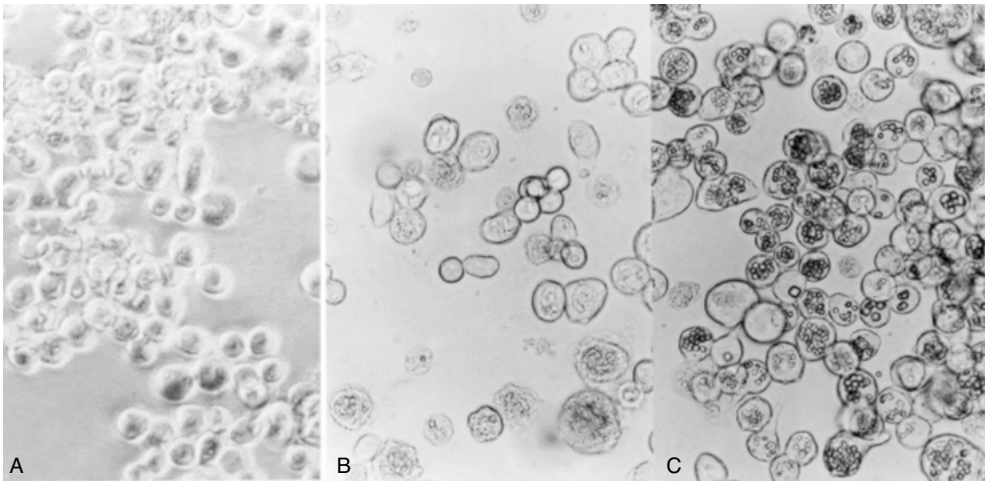

Fig. 4. Features of *Sf* cells infected with VP2 DNA-recombinant bacmid DNA showing no occlusion formation, which was formed in the cells infected with *Ac*NPV (baculovirus)-infected cells; A, mock-infected cells; B, recombinant bacmid-infected cells; C, *Ac*NPV-infected cells (×200).

a baculovirus shuttle vector for protein expression. The VP2 protein was successfully expressed in *Sf* cells when the recombinant bacmid was transfected into the *Sf* cells. There was no occlusion formation in the cells infected by the recombinant bacmid, because polyhedrin gene of the bacmid which is responsible for the occlusion formation (polyhedrosis) was unable to function by insertion of the VP2 DNA into the gene [26]. Expression of the VP2 protein was immediately identified from *Sf* cells by FA test. The VP2 protein band was further determined from lysate of the *Sf* cells as well as supernatant of the cell lysate by SDS-PAGE. The VP2 protein showed a polypeptide with M_r of 48 kDa which was similar to that of CEF94 strain [24], and reacted specifically with IBDV antiserum in Western blotting. By these findings, it was recognized that the VP2 subunit protein of the vvIBDV Chinju expressed by the bacmid and *Sf* cell system maintains its biological functions without deterioration of antigenic properties. The antigenic activity was also determined in the VP2 subunit protein purified from cell lysate by Ni-NTA affinity chromatography. Generally, eukaryotic system is preferred for the *in vitro* expression of foreign proteins, which require posttranslational modification similar to that of the naturally occurring proteins [11].

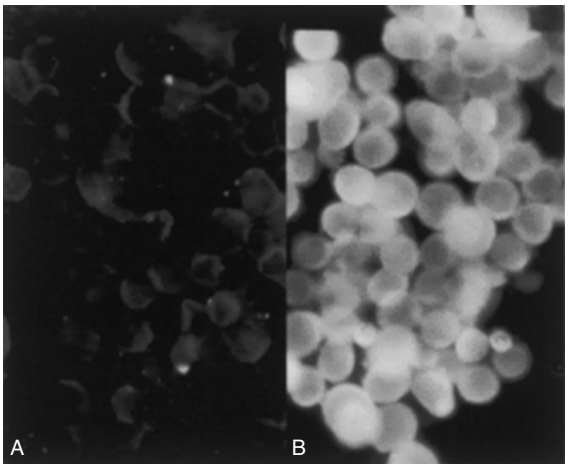

Fig. 5. Protein expression determined from VP2 DNA-recombinant bacmid DNA in *Sf* cells by fluorescent antibody test: A, mock-infected cells; B, recombinant bacmid-infected cells (×200).

Martinez et al. [27] expressed proteins of IBDV VPX (uncleavaged VP2) and VP3 in baculovirus system, and these proteins revealed immunogenicity similar to that of the native virus in eliciting neutralizing antibodies. The proteins also exerted antigenic activity to chicken sera in indirect ELISA. Vakharia et al. [21] expressed segment A polyprotein of an antigenic variant GLS by the baculovirus system, and the polyprotein showed protection of chicken and conferred maternal immunity to the antigenic variant and classical virulent IBDVs. Dybing and Jackwood [19]

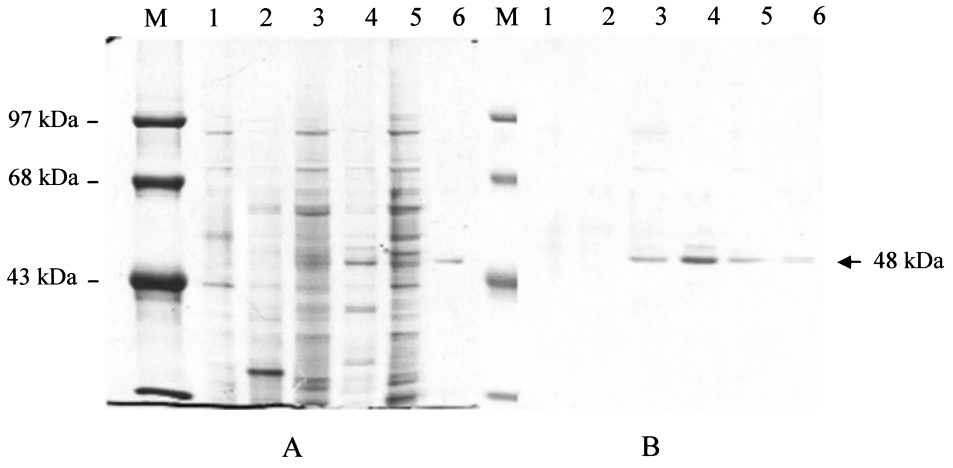

Fig. 6. Determination of VP2 protein (48 kDa) expressed from VP2 DNA-recombinant bacmid DNA in *Sf* cells by SDS-PAGE (A) and Western blotting with chicken anti-IBDV serum (B): 1, mock-infected cells; 2, *Ac*NPV-infected cells; 3, whole lysate from cells infected with the recombinant bacmid; 4, supernatant from lysate of cells infected with the recombinant bacmid; 5, pellet from lysate of cells infected with the recombinant bacmid; 6, VP2 protein purified from lysate of cells infected with the recombinant bacmid by Ni-NTA affinity chromatography.

also expressed individual or polyprotein of the VP2, VP3 and VP4 from an antigenic variant Md by the baculovirus system. There were protective activities of the expressed protein observed in chicken when challenged with the classical virulent or antigenic variant IBDVs. On the other hand, Phenix et al. [28] expressed VP2, VP4 and VP3 proteins in Semliki forest virus system, and the generated proteins reacted with IBDV-specific monoclonal antibodies. Also, the recombinant Semliki virus elicited specific neutralizing antibodies in chicken. Bayliss et al. [14] reported that the VP2 gene-recombinant fowlpox virus conferred passive protection of chicken against IBDV.

In conclusion, the VP2 subunit protein expressed from the vvIBDV Chinju in the present study can be used as diagnostic reagent and immunogen for the prevention and control of the vvIBDVs, especially for the indigenous strain of vvIBDV in Korea. Also, the VP2 subunit protein can be an alternative to avoid manipulating live virus in diagnostic tests and vaccine production.

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