Production of a Recombinant Major Inner Capsid Protein for Serological Detection of Epizootic Hemorrhagic Disease Virus

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Constructions of the major core protein, designated VP7, from epizootic hemorrhagic disease virus (EHDV) type 1 were made by amino- or carboxyl-terminal fusion of a six-histidine residue tag to the VP7-1 gene. The resulting fusion proteins were produced in a baculovirus expression system and purified by a rapid, one-step procedure using nickel-nitrilotriacetic acid technology. A high level of VP7-1 protein expression was detected with the N-terminal six-histidine tag fusion construct and was comparable to the level of expression observed with an untagged VP7-1 Bam construct. In contrast, the inclusion of a six-histidine tag at the C terminus adversely affected protein expression. The antigenicity of the N-terminal six-histidine tag EHDV VP7-1 product was identical to that observed with the native virus antigen and untagged EHDV VP7-1 recombinant protein, as determined by reactivity with EHDV specific antibodies in an enzyme-linked immunosorbent assay (ELISA) and Western blot. The high production and purity levels that can be attained for the N-terminal six-histidine tag VP7-1 protein and its reactivity with EHDV-specific sera in a competitive ELISA make it a suitable assay reagent.

Epizootic hemorrhagic disease virus (EHDV) is a member of the Orbivirus genus, one of six genera in the family Reoviridae (15). This virus causes disease in sheep, cattle, and wild ruminants and has important implications for the international livestock trade. At least eight serotypes of EHDV have been reported worldwide, but only two serotypes, designated EHDV-1 (14) and EHDV-2 (3), are known to be enzootic in the United States and Canada.

All EHDV serotypes share an antigen which enables their identification and differentiation from other orbiviruses, such as bluetongue virus (BTV) (4, 20). This group-specific antigen is specified by a protein (VP7) located on the inner coat of the virus particle, making it a suitable antigen for use in serological assays to specifically identify EHDV regardless of the serotype (16, 17). The most widely used serodiagnostic test for EHDV is an enzyme-linked immunosorbent assay (ELISA), which is used to determine which is most suitable for use as an assay reagent. The VP7-1 N-His product is further evaluated for its performance in an EHDV-specific c-ELISA.

ELISA and the presence of live virus, necessitating special handling requirements in the laboratory. To overcome some of these problems, Mecham and Wilson (9) cloned the gene encoding EHDV VP7 into baculovirus, and the recombinant protein was expressed in SF21 cultured insect cells. This recombinant protein was not purified from extraneous cell culture proteins prior to its use in a c-ELISA; therefore, an additional, antigen capture step had to be included to standardize the amount of VP7 protein on the microtiter plate.

In this report, an alternative approach is described to produce the EHDV VP7 protein in a highly purified form, enabling reagent characterization and quality control prior to its use in assays. Specifically, the gene encoding this protein was cloned to include a six-histidine tag at either the amino (VP7-1 N-His) or carboxyl (VP7-1 C-His) terminus into baculovirus and expressed in SF21 cultured insect cells. Data related to production, purification, and antigenicity are provided for the His-tagged and untagged VP7-1 proteins in an effort to determine which is most suitable for use as an assay reagent. The VP7-1 N-His protein is further evaluated for its performance in an EHDV-specific c-ELISA.

MATERIALS AND METHODS

Construction of a baculovirus transfer vector containing EHDV1 VP7 gene.

To construct VP7 genes, EHDV1 (Australian serotype 1) was propagated in BHK21 cells, and double-stranded RNA segment 7 was purified. The primers for cDNA synthesis and amplification of segment 7 were based on the published sequence for EHDV-1 (GenBank accession no. D10766). The reverse transcription-PCR-amplified BamHI fragments, containing VP7-1, were ligated with pCR2.1 vector (Invitrogen, Burlington, Ontario, Canada), and then the full-length VP7 gene was subcloned into transfer vector pBackPak1 N-His and pBackPaki C-His, as shown in Fig. 1B. Figure 1A shows the construction of the plasmid DNA used in this study and indicates the inserted foreign genes. All insertion sequences and reading frames were confirmed with an ABI 377 se-
quencer with a fluorescent dye terminator kit (Applied Biosystems, Streetsville, Ontario, Canada).

**Generation of recombinant baculovirus.** Recombinant viruses were generated by a previously described procedure (5, 6). Briefly, SF21 cells were cotransfected with linearized wild-type BacPAK6 viral DNA and recombinant transfer vector pBacPAK1 DNA to generate the recombinant baculovirus. Liposome-mediated gene transfer was employed with Lipofectin provided in the BacPAK Baculovirus Expression System kit (BD Biosciences Clontech, Mississauga, Ontario, Canada). A few viral plaques were picked, and recombinant viruses were verified by Western blot analysis. The isolated recombinant virus was purified by a consecutive plaque picking and used to produce a virus stock with a titer of 10^8 PFU/ml.

**Expression analysis and immunoblotting.** SF21 cells were infected with either wild-type AcNPV (Autographa californica nuclear polyhedrosis virus) or recombinant Ac-Bac-EHDV1-VP7 viruses at a multiplicity of infection of 5 PFU/cell and incubated at 27°C. After a predetermined incubation time, cells were harvested, and whole-cell lysates were analyzed with a discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) system with protein bands visualized by staining with Coomassie blue R-250. Western blot analysis was carried out by using anti-six-histidine antibody (QIAGEN, Mississauga, Ontario, Canada) or EHDV-1 VP7-specific monoclonal antibody (MAb) 18B2 (20) and the Amersham Biosciences Immune Blot system (7).

**Affinity purification of recombinant VP7-His.** A high-yield, homogeneous preparation of VP7-N-His was obtained by using the nickel-nitrilotriacetic acid (Ni-NTA) resin, according to the standard procedures described by the manufacturer (BD Biosciences Clontech, Mississauga, Ontario, Canada). Briefly, Ac-Bac-EHDV1-VP7-N-His-infected SF21 cell lysates were pelleted, and the supernatants were added to equilibrate Ni-NTA agarose in a 1:10 volume ratio. The bead slurry was then washed with 10 volumes of 50 mM Na-phosphate, 300 mM NaCl, 10% glycerol, 20 mM imidazole (pH 8.0). The VP7 protein was then eluted with 300 or 500 mM imidazole in 50 mM Na-phosphate, 300 mM NaCl, and 10% glycerol (pH 6.0).

**Purification of recombinant VP7 protein by ammonium sulfate precipitation.** Infected SF21 cell cultures were harvested 72 h postinfection, pelleted by low-speed centrifugation, washed with phosphate-buffered saline (PBS), and resuspended in 10 mM Tris-HCl (pH 7.5) containing 0.5% NP-40. Cell debris and nuclei were then removed by centrifugation at 2,700 × g for 10 min. A saturated
solution of ammonium sulfate, prepared in 100 mM Tris-HCl, pH 7.5, was added to the cytoplasmic cell extract to a final saturation of 20%. The precipitated protein was pelleted by centrifugation and resuspended in 10 mM Tris-HCl, pH 7.5. The insoluble material was removed by pelleting at 16,000 × g for 10 min. The extract of VP7 was stored at −70°C prior to analysis.

Enzyme-linked immunosorbent assay. The reactivity of VP7 with specific antibodies was evaluated by using a c-ELISA previously described by Zhou and Afshar (20). Briefly, the purified, recombinant VP7 proteins were coated on microtiter plates (Nunc-Immuno plate, Mississauga, Ontario, Canada) at 50 ng/well in a 100-μl volume. After an overnight incubation at 4°C, plates were washed three times with 0.01 M phosphate-buffered saline, pH 7.4, containing 0.05% Tween-20 (PBST) and then blocked for 1 h with 5% dry milk at room temperature. After being washed with PBST, the control and sample sera were placed in the plate at a final dilution of 1/5 in PBST containing 2.5% dry milk, followed by EHDV-1 VP7-specific MAb 18B2 at a 1:80 dilution. After incubation for 1 h at 37°C, the plates were further washed and incubated with secondary antibodies conjugated with horseradish peroxidase (Pierce, Brockville, Ontario, Canada) for another hour. The plates were washed and developed with a sub- strate solution (FAST o-phenylenediamine dihydrochloride; Sigma, St. Louis, Mo.). After 30 min, color development of the solution was measured as optical density at 450 nm (OD450). The results were calculated as the percent inhibition of MAb binding to the antigen by the sera (PI).

RESULTS

Construction and generation of recombinant baculovirus containing VP7. The entire coding sequence of EHDV-1 VP7 gene was cloned into the transfer vector pBacPAK1. Figure 1Ab and c shows the construction of the plasmid DNA used in this study and indicates the inserted foreign gene by N- and C-terminal fusion of a six-histidine residue tag to the VP7 gene. To fuse the VP7 gene to the six-histidine tag, the BamHI fragment containing the VP7 gene was inserted into the BamHI site of transfer vector pN-HIS or pC-HIS, as shown in Fig. 1B. Then, the resulting plasmids pBacPAK1-EHDV1-VP7-N-His and pBacPAK1-EHDV1-VP7-C-His were used to generate recombinant viruses. Specifically, these viruses were produced by cotransfection of SF21 cells with this vector and wild-type BacPAK6 viral DNA, as described in Materials and Methods. Recombinant viruses were purified by plaque assay and confirmed by PCR amplification and Western blotting (data not shown).

Expression of VP7 in SF 21 cell cultures. The EHDV VP7 gene constructs shown in Fig. 1 were expressed in SF21 cells under the control of a polyhedrin promoter. To evaluate VP7 protein expression, infected cells were harvested 3 days postinfection, whole-cell lysates were analyzed by 12% SDS-PAGE, and the gels were stained with Coomassie blue (Fig. 2A). A protein band with an approximate molecular mass of 39 kDa was observed in cells infected with recombinant baculoviruses representing the three different gene constructs (Fig. 2A, lanes 1 to 3). The corresponding protein was not present in cells infected with wild-type AcNPV or BacPAK6 or in mock-infected cells. Based on band intensity, it was evident that the cells infected by Ac-Bac-EHDV1-VP7-C-His (Fig. 2A, lane 2) exhibited a lower level of expression than cells infected with either recombinant virus Ac-Bac-EHDV1-VP7-N-His (Fig. 2A, lane 1) or Ac-Bac-EHDV1-VP7-Bam (Fig. 2A, lane 3).

The antigenicity and identity of the recombinant VP7 proteins was confirmed by Western blot reactivity with a VP7-1-specific MAb, 18B2 (Fig. 2B). The relative intensity of the reactive bands (Fig. 2B, lanes 1 to 3) was similar to that observed with Coomassie staining. His-specific antibody was employed to confirm the presence of the inserted six-histidine tag sequence for the VP7-1 N-His (Fig. 3, lane 1) and VP7-1 C-His (Fig. 3, lane 2) constructs and confirmed that VP7 was expressed as a fusion protein. As expected, the VP7 Bam construct was not detected by anti-His antibody (Fig. 3, lane 3). The relative intensity of the reactive bands was the same as that shown with both Coomassie staining and MAB 18B2 blotting.

Purification of native VP7 protein from SF21 cells infected with recombinant viruses. One-step purification of six-histidine-tagged VP7 proteins, VP7-1 N-His and VP7-1 C-His, was carried out by using Ni-NTA agarose (QIAGEN, Mississauga, Ontario, Canada) under native conditions as described in Materials and Methods. Figure 4 illustrates the high degree of purification which was achieved using this system by comparing a fractionated and Coomassie blue-stained, crude recombinant virus Ac-Bac-EHDV1-VP7-N-His-infected cell lysate (lane 1) with an Ni-NTA purified VP7-1 N-His preparation (lane 2). Although the level of purity for the His-tagged proteins was the same (data not shown), it was difficult to obtain enough VP7 C-His for routine purification, due to the low level of expression. The identity of the purified protein in both crude and purified samples was confirmed by Western blot reactivity with
EHDV VP7-specific MAb 18B2 (data not shown). Due to the absence of a His tag, purification of the VP7-Bam protein had to be carried out by an alternative method. Ammonium sulfate precipitation was selected, since it was potentially the least labor intensive, even though it was not a one-step method (see Materials and Methods for details). The results in Fig. 4, lane 3, show the presence of a major 39-kDa protein along with another, lower-molecular-mass protein and some minor contaminating proteins in the precipitated preparation. Western blot analysis of this preparation demonstrated MAb 18B2 reactivity with only the 39-kDa protein (data not shown).

**Evaluation of recombinant VP7 as a diagnostic reagent protein in competition ELISA.** The baculovirus-expressed VP7 N-His protein was evaluated as a diagnostic antigen for detection of EHDV-1-specific antibodies by a standard c-ELISA. Since there is a high serological cross-reactivity between BTV and EHD, BTV-specific sera were also included in this assay. To determine an optimal VP7 concentration for the c-ELISA, the recombinant VP7 protein was first titrated with a VP7 protein-based ELISA. Figure 5 shows that the purified VP7 N-His proteins could be effectively detected and quantitated by using the EHDV-specific MAb 18B2. As little as 25 ng/well of purified VP7 N-His protein gave an ELISA signal at least sixfold above the background reading, which had an $A_{450}$ of 0.1. Based on these results, it was determined that 50 ng/well would be used in the c-ELISA, since it resulted in an absorbance value of 1.0.

The results shown in Fig. 6 illustrate the ability of the VP7-specific MAb 18B2 to compete with sera collected from EHDV-1 (ED-19, ED-22, ED-24, and ED-59) and BTV (BT-720 and BT-533) experimentally infected cattle and field samples from cattle exposed to EHDV in Saskatoon, Quebec, and Alberta (SK-07, Qu-16, Qu-26, and AB-31). The specificity of the reaction was determined by the inhibition values exhibited with no sera (<1%) and a validated positive sera (98%). The results indicated that all the sera from EHDV experimentally infected and field-exposed animals strongly competed with MAb 18B2 for binding sites on the recombinant VP7 antigen, resulting in at least 50% inhibition, which was previously determined as the cutoff value for positive samples. In contrast, BTV-specific sera demonstrated c-ELISA inhibition values of 25% and 33%, considerably below the positive cutoff value. These results were reproducible and comparable to those obtained using the ammonium sulfate-purified VP7 Bam protein or highly purified native VP7 antigen preparations (data not shown).

**DISCUSSION**

The baculovirus system was selected for protein production due to the fact that recombinant EHDV VP7, produced in this...
system, was able to bind rabbit and bovine antibodies in an indirect ELISA (10) and bovine antibodies in a c-ELISA (9). In addition, this expression system has demonstrated the ability to yield large quantities of recombinant proteins that show biological properties similar to their native counterparts (18). The cDNA carrying the EHDV VP7-1 gene was successfully cloned in a baculovirus expression vector with a six-histidine tag at its N and C termini and also without a six-histidine tag. The advantage of producing His-tagged fusion proteins was that immobilized metal affinity chromatography could be used for their purification. This rapid, one-step process is amenable to the purification of proteins in their native state. A slightly more labor-intensive process, involving ammonium sulfate precipitation, was used for purification of the untagged VP7 protein. Even though successful expression of EHDV rVP7 in a baculovirus system has been previously reported (9, 10), this is the first report of a highly purified VP7 fusion protein that would have the advantage of being able to be accurately quantitated and monitored for stability. This type of standardization is extremely important to assay reproducibility and reliability. In addition, this type of reagent could lend itself to the development of precoated microtiter plates that could be stored long term under adverse conditions.

The evaluation of recombinant protein production levels indicated that the fusion protein containing the six-histidine tag at the N terminus was expressed at a level similar to that observed with the untagged protein and at a considerably higher level than that of the C-His fusion protein, suggesting that insertion into this location interfered with protein expression. A somewhat similar effect was described for a 33-kDa soluble subunit protein of a higher plant which carried a tail of six histidines at the C terminus, leading to a conformational change and correspondingly lower expression yields (13). Since protein production levels are an important selection criteria for an assay reagent, the VP7 C-His was considered unsuitable for extensive evaluation. Conversely, the efficient production of the untagged EHDV VP7 and VP7 N-His made these proteins suitable reagent candidates, as was found to be the case for other reovirus core proteins expressed in a baculovirus system (2, 11). Regardless of the protein construct, all three recombinant proteins were recognized by the EHDV-1 group-specific MAb 18B2, as illustrated by reactivity in Western blots and in a VP7 protein ELISA. This indicated that the presence and location of the His tag did not interfere with epitope binding. More importantly, these recombinant proteins were specifically recognized by polyclonal sera, derived from animals either experimentally infected with EHDV or naturally exposed to the virus, by using a c-ELISA. As reported by others (8, 16, 19), some heterologous cross-reactivity with BTV-specific sera was detected, but it was significantly less than the cutoff threshold of 50 PI. Therefore, the presence of a His tag did not influence the sensitivity or specificity of polyclonal antibody reactivity with these proteins. Also, binding of the recombinant His tag protein directly to the well of the microtiter plate did not appear to result in partial denaturation of the MAb binding epitope, as suggested by Mecham and Wilson (9). In fact, there was a high degree of correlation between the amount of antigen coated in a well and MAb binding, as indicated by OD readings in a direct ELISA.

In summary, the baculovirus-expressed EHDV VP7 N-His fusion protein could be produced in large quantities and easily purified, yielding a highly homogenous antigen preparation which was stable after numerous freeze/thaw cycles (data not shown). Such a preparation was successfully used in a c-ELISA, which is less labor intensive and more easily standardized than the antigen capture c-ELISA described by Mecham and Wilson (9). It can also be postulated that the unpurified recombinant VP7 preparation, used for the antigen capture c-ELISA, might be less stable than when the protein is in a highly purified state, due to the presence of extraneous proteins with enzymatic activity or the tendency for uncontrolled aggregation with extraneous proteins or even self aggregation. The latter phenomenon has been reported for other reovirus capsid proteins and may be related to the natural ability of these types of proteins to assemble into virus-like structures under suitable environmental conditions (12). Con-

FIG. 6. Performance of recombinant EHDV VP7 N-His in a competitive ELISA. EHDV-specific sera (ED-19, ED-24, ED-24, ED-59, SK-07, Qu-16, Qu-26, and AB-31) and BTV-specific sera (BT-720 and BT-533) were tested for their ability to compete with EHDV-1 VP7-specific MAb 18B2 and inhibit its binding to the EHDV VP7 N-His protein coated on microtiter plates. Competition was determined by PI of MAb binding to the antigen as a target control. The line bisecting the graph indicates 50 PI and was used as the cutoff point for scoring samples as positive or negative, as indicated on the right by an arrow.
siderations related to reagent quality control make the highly purified EHDV VP7 N-His protein uniquely suited for use in serodiagnostic assays by improving the reliability, reproducibility, and cost of existing assays that use partially purified native or recombinant VP7 preparations.

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REFERENCES