Recombinant BFDV capsid protein – the "I can't believe it's not butter" of viruses

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Introduction

Psittacine beak and feather disease (PBFD) remains one of the most common viral diseases of both wild and captive birds, even after over 20 years of research and many attempts to develop options for vaccination and treatment.

Many different researchers have attempted unsuccessfully to grow the virus in cell culture and a vaccine has even been produced, but is not a commercial reality due to the difficulty of producing enough antigen. Recombinant protein technology is widely used to study the structure and function of many proteins and these recombinant proteins are also gaining favour as alternatives to attenuated or inactivated vaccines.

We present here the production of a recombinant His-tagged Beak and Feather Disease Virus (BFDV) capsid fusion protein using a baculovirus expression system and subsequent evidence that this protein is morphologically and functionally similar to native virus particles.

Materials and Methods

All materials were part of the Bac-toBac baculovirus expression system, supplied by Invitrogen, Australia, unless otherwise specified.

Production of recombinant baculovirus
Insert generation – Viral DNA was extracted from feather samples using published methods (Ypelaar et al 1999) and the ORF C1 of BFDV was amplified using primers designed to amplify the entire ORF C1 of BFDV. Forward and reverse primers were based on the BFDV sequence AF080560 (GenBank), a sulphur-crested cockatoo isolate. Restriction sites for BamHI and EcoRI, respectively, were constructed into primers bacBFDVC1F and bacBFDVC1R so that the amplified PCR product could be ligated into the baculovirus expression vector.

Transformation of *E. coli* and preparation of Bacmid - After PCR amplification, both the PCR product and the pFastBacHT donor plasmid were digested with the restriction enzymes BamHI and EcoRI, then the digested PCR product was ligated into the vector and transformed into DH5α E. coli cells. Cells were grown on 2YT agar plates supplemented with ampicillin, then selected colonies were grown overnight in 2YT broth supplemented with ampicillin. Plasmid DNA was purified using a S.N.A.P. midiprep kit and sequenced to determine correct orientation of the insert using primers M13forward and M13 reverse.

Plasmid DNA containing the insert in the correct orientation was then transformed into DH10Bac E. coli cells and cultured on 2YT agar plates supplemented with kanamycin, gentamycin and tetracycline, plus IPTG and Bluo-Gal. White colonies were selected, grown in 2YT broth supplemented with kanamycin, gentamycin and tetracycline, then plasmid DNA was purified and analysed by PCR.

Transfection of Sf9 insect cells and production of recombinant baculovirus – Sf9 insect cells were grown in Sf-900 II serum-free medium supplemented with streptomycin, then recombinant bacmid DNA was transfected into the cells using Cellfectin Reagent in unsupplemented Grace's Medium. After culture for 72 hours, the cells were centrifuged and the supernatant collected. The titre of this P1 viral stock was then determined by viral plaque assay and viral stocks were amplified by transfection of Sf9 cells with P1 virus stock at an MOI of 0.2. The cells were grown for 72h, the cells pelleted by centrifugation and the supernatant collected. The process of plaque assay (to determine viral titre) and infection of cells (to generate recombinant virus stock) was continued until virus stocks with titre of between $1x10^6$ and $1x10^9$ were produced.

Production of recombinant BFDV capsid protein
Cell culture – Sf9 insect cells were transfected with recombinant baculovirus stocks at an MOI of 0.2, then grown for 5 days at 28C with shaking. Cells were pelleted by centrifugation at 1500rpm for 10mins and the pellets either processed immediately or stored at –80C.

Protein purification by Ni-NTA affinity purification – cell pellets were lysed by the addition of 5 volumes of lysis buffer (50mM Tris-HCl pH 8.5, 100mM KCl, 1mM PMSF, 4.9mM β-mercaptoethanol, 0.5U/mL DNAseI). The addition of the non-denaturing detergents Igepal 630 and Triton X-100 to the lysis solution was also trialled.

Cell were lysed by mixing on a vertical rotor for 30mins, then either sonicated 3 times or subjected to 4 cycles of freezing in liquid nitrogen and thawing at 40C. The crude lysate was centrifuged at 10 000rpm to pellet the cell debris and the supernatant collected. The supernatant was then mixed with pre-equilibrated Ni-NTA resin (Qiagen, Australia) overnight to bind the protein to the resin.

After the flow-through had been collected, the resin was washed 3 times with wash buffer (25mM NaH_2PO_4, 250mM NaCl, 5mM imidazole), then bound protein was removed from the resin with elution buffer (25mM NaH_2PO_4, 250mM NaCl, 300mM imidazole). Fractions from all steps, including a sample of the insoluble pellet, were analysed by SDS-PAGE and western blot, using mouse anti-histidine monoclonal antibody (Serotec, Australia) and goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma-Aldrich, Australia).

Protein purification by ultracentrifugation – cells were lysed as above, then the crude lysate centrifuged over a 40% (w/v in PBS) sucrose cushion at 28 000rpm for 2 hours at 4C in a Beckman Sw55Ti rotor (Beckman, England). The supernatant was then discarded and the sucrose cushion mixed with CsCl solution to a density of approximately 1.4g/ml. The sucrose/CsCl solution was then centrifuged at 28 000rpm for 18h at 4C in a Beckman Sw55Ti rotor (Beckman, England). 0.5mL aliquots were collected, dialysed in PBS overnight to remove the CsCl and samples from each fraction were analysed by SDS-PAGE and western blot, using mouse anti-histidine monoclonal antibody (Serotec, Australia) and goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma-Aldrich, Australia).

Protein concentration – protein-containing fractions, as identified by SDS-PAGE and western blot, were pooled and dialysed against a 20% (w/v in PBS) solution of polyethylene glycol, MW 20kDa (PEG 20K).

Quantification of protein fractions – serial 1:2 dilutions of a sample of the concentrated protein were made in PBS and the fractions run on an SDS-PAGE gel in conjunction with a set of standards of known concentration. The gel was then stained with Coomassie brilliant blue and analysed by densitometer (Perkin-Elmer, Australia).

Structural and functional analysis of recombinant protein
Immunogenicity – 4 chickens were injected with approximately 100ug each [check with Meredith] of the recombinant capsid protein, mixed with Freund's incomplete adjuvant, then boosted 2 weeks later. Blood was collected pre-inoculation, at each inoculation and 2 weeks post-inoculation and the sera collected for analysis by haemagglutination-inhibition (HI) to detect specific antibodies. Additional serum samples were collected 1 and 2 months after the second inoculation and analysed by HI.

Haemagglutination and haemagglutination-inhibition assays – 50ul of a sample of protein was analysed by haemagglutination assay (HA) as described by Raidal *et al.*, (1993). After the HA titre was determined, a 1:100 dilution of the stock protein sample was made, then 50ul of chicken anti-BFDV (80HIU/50ul) was diluted by serial 1:2 dilutions across a microtitre plate and incubated with 50ul per well of the 1:100 protein solution.

Structural analysis – 1ul of the concentrated protein stock was applied to a formvar-coated electron microscope grid, negatively stained with 1% phosphotungstic acid (PTA) and visualised by transmission electron microscopy.

Results

Production and purification of recombinant BFDV capsid protein
Protein purification – the recombinant capsid protein could not be purified by Ni-NTA affinity chromatography. The inclusion or exclusion of the various detergents made no difference to the affinity of the histidine-tagged protein for the Ni-NTA resin.

The recombinant protein was successfully purified by differential and isopycnic ultracentrifugation and concentration in 20% (w/v in PBS) PEG 20K successfully concentrated the protein solution 100-fold within 12 hours.

The recombinant fusion protein had a size of 36kDa and was successfully detected by western blotting using mouse anti-histidine monoclonal antibodies, as well as polyclonal chicken anti-BFDV IgY, rabbit anti-BFDV IgG, sheep anti-recombinant BFDV capsid expressed in E. coli (Bonne, 2004) and psittacine anti-BFDV IgY from a number of different psittacine species.

Structural and functional analysis of recombinant protein
Chickens inoculated with the recombinant protein developed HI titres by 2 weeks after the second inoculation. HI titres remained consistent at 80HIU/50ul for 2 months after the second inoculation.

Haemaggutination and haemagglutination-inhibition – the recombinant protein successfully agglutinated type A Galah erythrocytes and did not agglutinate type B Galah erythrocytes. Additionally, haemagglutination by the recombinant capsid protein was inhibited by chicken anti-

BFDV IgY. Transmission electron microscopy revealed virus-like particles of between 17.6 and 19nm in diameter.

Discussion

This represents the first report of a recombinant BFDV capsid protein with structural and functional characteristics similar to the native virus. BFDV capsid protein has been produced by other authors in E. coli cells, both in full length (Bonne, 2004) and truncated (Johne *et al.*, 2004)forms. Additionally, recombinant BFDV capsid protein has also been expressed by means of a baculovirus expression system (Heath *et al.*, 2006), but the conformation and functionality of the expressed protein was only partially investigated. Heath *et al* (2006) produced full length and 5' truncated forms of the capsid protein and have shown that BFDV, like PCV and the geminiviruses, has a nuclear localisation sequence (NLS) at the 5' terminus of the region coding for the capsid protein. This NLS codes for a DNA binding region within the capsid protein which, like the geminiviruses, likely acts as a transport mechanism for viral DNA.

Heath *et al* (2006) reported higher expression levels with the capsid protein which was 5' truncated to remove the NLS, which potentially makes it more attractive than the full-length expression system for production of vaccine antigen, however the morphology and functionality of the truncated protein has not yet been evaluated. In use as antigen in a vaccine, for example, it may be that antibodies directed against the NLS are necessary for the development of a protective immune response.

Recombinant technologies have provided us with new and exciting options for the production of protein to use in vaccines and diagnostic tests, such as ELISA. The next phase of our research will be to develop monoclonal antibodies against the recombinant protein and to evaluate their use in a range of diagnostic tests and to evaluate the potential use of the proteins as a vaccine antigen.

References

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