3 Results

3.1 PCR amplification of full length, 3’ truncated and 5’ truncated BFDV ORF C1.

PCR amplification of full length, 3’ truncated and 5’ truncated BFDV ORF C1 (Figure 3-1) was performed using optimized conditions, as described in Chapter 2, section 2.3. Three negative control reactions, 2 using DNA extract from BFDV negative psittacine birds and 1 water control was used. Amplification resulted in the desired 744, 330 and 649 bp fragments for full length (lane 2), 3’ truncated (lane 7) and 5’ truncated (lane 12) amplification, respectively. None of the control reactions resulted in amplification.

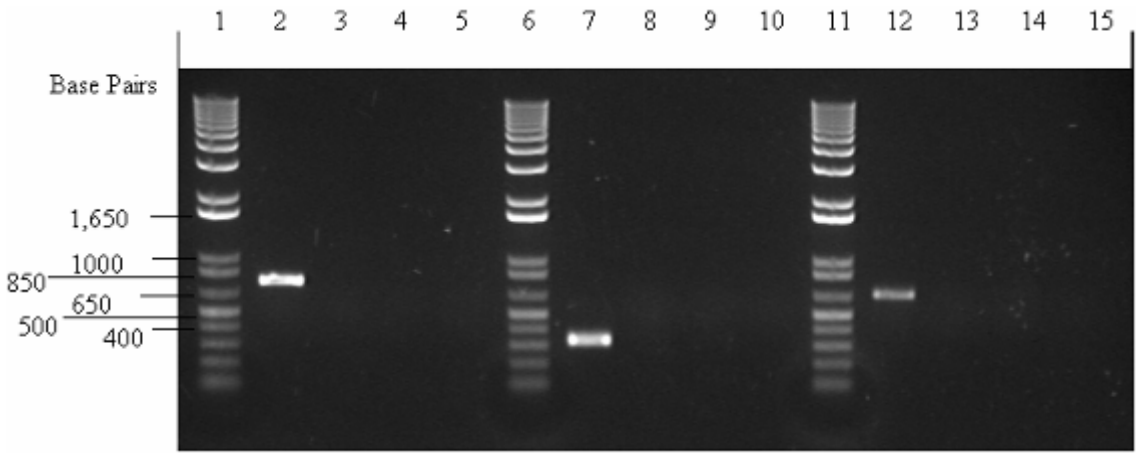

Figure 3-1. PCR amplification of BFDV full length, 3’ truncated and 5’ truncated ORF C1. PCR products were electrophoresed in a 1.0% agarose gel at 90V for 30 min. Lane 1: Invitrogen 1 Kb plus DNA ladder. Lane 2: Full length amplification of ORF C1. Lane 3: Control reaction using DNA extract from BFDV negative Psittaciforme. Lane 4: Control reaction using DNA extract from BFDV negative Psittaciforme. Lane 5: Water control. Lane 6: Invitrogen 1 Kb plus DNA ladder. Lane 7: 3’ truncated amplification of ORF C1. Lane 8: Control reaction using DNA extract from BFDV negative Psittaciforme. Lane 9: Control reaction using DNA extract from BFDV negative Psittaciforme. Lane 10: Water control. Lane 11: Invitrogen 1 Kb plus DNA ladder. Lane 12: 5’ truncated amplification of ORF C1. Lane 13: Control reaction using DNA extract from BFDV negative Psittaciforme. Lane 14: Control reaction using DNA extract from BFDV negative Psittaciforme. Lane 15: Water control.

3.2 Screening of Transformed *E. coli* JM109

3.2.1 PCR and restriction digest screening of transformants

Full length and 5' truncated transformants were screened by PCR using the primers Reverse 1 and Pinseq (Table 2-1). Thus full-length and 5' truncated colonies carrying the insert in the correct orientation were expected to give PCR product of 839 and 744 bp, respectively. The 3' truncated recombinants were screened by PCR with the primers Reverse 2 and Pinseq (Table 2-1) and expected to result in a fragment of 425 bp.

PCR screening detected both full length and 3' truncated recombinants. However, repeated PCR screening of 5' truncated colonies did not detect any positives. Full length and 3' truncated colonies found positive by PCR screening were further confirmed by restriction digest to have correctly ligated insert. These colonies were further sequenced by dye-terminator sequencing.

3.3 Dye-terminator Sequencing

All colonies found positive by both PCR and restriction digest were further sequenced with *Dye*-terminator sequencing to confirm whether the recombinants had correctly inserted amplicon in the correct reading frame. Both full length and 3' truncated recombinants carrying the insert in the correct orientation and reading frame were found (Figure 3-2 and Figure 3-3, respectively). These colonies were then inoculated in LB broth supplemented with ampicillin overnight to make glycerol stocks and to be used in protein expression experiments.

```
tca gct ggg atc cgg tac cga ttC ATG TGG GGC ACC TCT AAC TGC GCA TGC GCT
ser ala gly ile arg tyr arg phe met trp gly thr ser asn cys ala cys ala

GTA TGT TCA ATT CCG TCA GTT TGC CCC TAA CAA TCC CAG CAC TTA Aat cag atc tcc
val cys ser ile pro ser val cys pro OCH gln ser gln his leu asn gln ile leu
```

Figure 3-2. Results of dye-terminator sequencing of a full length recombinant BFDV ORF C1. Sequence of nt and a.a. are written 5' to 3'. In red is a portion of the vector sequence flanking the insert, in black are the 5' and 3' ends of insert.

```
cgg tac cga ttC ATG TGG GGC ACC TCT AAC TGC GCA TGC GCT ACA TTT CAG ATT
arg tyr arg phe met trp gly thr ser asn cys ala cys ala thr phe gln ile

ATT TTG AAG ATT ACC GAA TTA AGT TAG CTA AAA TGG AAA TGA GGC CCA atg aga tct
ile leu lys ile thr glu leu ser AMB leu lys trp lys OPA gly pro ser arg ser
```

Figure 3-3. Results of dye-terminator sequencing of a 3' truncated recombinant BFDV ORF C1. Sequence of nt and a.a. are written 5' to 3'. In red is a portion of the vector sequence flanking the insert, in black are the 5' and 3' ends of insert.

3.4 Optimisation of induction of full length and C-terminal truncated recombinant protein expression

3.4.1 Full length protein expression

Recombinant *E. coli* JM109 cells for expressing full length BFDV capsid protein were grown to stationary phase, diluted 1:20 in 2×YT broth and grown to OD_{600nm} 0.32, 0.62, 0.90 and 1.19, and induced with IPTG for 5.5 or 6.0 hrs with samples taken every 30 mins after induction. Cultures were then pelleted by centrifugation and cells lysed by freeze-thaw before separating soluble and insoluble protein fractions. The fractions were further electrophoresed in SDS-PAGE gels before transferring to nitrocellulose membranes and recombinant protein visualized by incubating the membrane with alkaline phosphatase conjugated streptavidin.

A full set of results from this optimisation experiment can be seen in Appendix A. Virtually all cell densities and lengths of induction, including non-induced cell culture (Appendix A, Figure A.1, lane 2), produced insoluble protein. An OD_{600nm} of 0.62 (Figure 3-4, below) with 1.5 and 2.5 hrs of induction (lanes 8 and 12, respectively) clearly gave the highest yield of insoluble protein. In addition, this cell density with the latter induction time gave the highest soluble protein yield (lane 11).

Figure 3-4. Western immunoblot of full length recombinant protein generated by inducing cell culture at OD_{600nm} 0.62. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lane 1: Soluble protein fraction before induction with IPTG. Lane 2: Insoluble protein fraction before induction with IPTG. Lanes 3, 5, 7, 9 and 11: Soluble protein fractions after 0.5, 1, 1.5, 2 and 2.5 hours of induction with IPTG, respectively. Lanes 4, 6, 8, 10 and 12: Insoluble protein fraction after 0.5, 1, 1.5, 2 and 2.5 hours of induction with IPTG, respectively.

3.4.2 C-terminal truncated protein expression

Recombinant *E. coli* JM109 cells for expressing C-terminal truncated BFDV capsid protein were grown to stationary phase, diluted 1:20 in 2×YT broth and grown to OD_{600nm} 0.316, 0.591, 0.940 and 1.174, and induced with IPTG for 5.5 hrs with samples taken every 30 mins after induction and then overnight. Cultures were then pelleted by centrifugation and cells lysed by freeze-thaw before dividing into soluble and insoluble protein fractions. The fractions were further electrophoresed in SDS-PAGE gels before

transferring to nitrocellulose membranes and recombinant protein visualized by incubating the membrane with alkaline phosphatase conjugated streptavidin.

A complete set of results for this optimisation experiment can be viewed in Appendix B. Virtually all cell densities and lengths of induction, including non-induced cell culture (Appendix B, Figure B.1, lane 2) produced insoluble protein. An OD_{600nm} of 0.940 (Figure 3-5, below) gave the highest yield of insoluble protein, with 3.5, 4.0, 4.5 and 5.0 hrs (lanes 2, 4, 6 and 8) of induction giving approximately the same yield. None of the cell densities or lengths of induction resulted in expression of soluble protein.

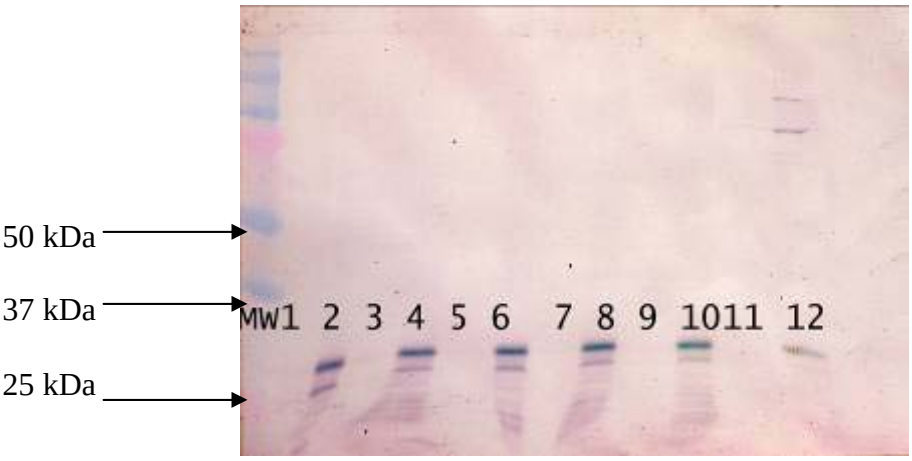

Figure 3-5. Western immunoblot of C-terminal truncated recombinant protein generated by inducing cell culture at OD_{600nm} 0.940. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lanes 1, 3, 5, 7, 9 and 11: Soluble protein fractions after 3.5, 4, 4.5, 5, 5.5 hours and overnight of induction with IPTG, respectively. Lanes 2, 4, 6, 8, 10 and 12: Insoluble protein fraction after 3.5, 4, 4.5, 5, 5.5 hours and overnight of induction with IPTG, respectively.

3.5 Purification of soluble full length recombinant BFDV protein

3.5.1 Full length soluble protein batch capture

Figure 3-6 illustrates the protein content of fractions taken during purification using the batch capture method. Lane 1 shows protein expression before induction and indicates, as was found in the optimisation of expression experiments, some leaky expression of recombinant protein. Crude lysate of induced cell culture (lane 2) featured a similar banding pattern to lane 1, however weaker. Electrophoresis of non-purified soluble fraction (lane 3) and the protein fraction that did not bind to the Softlink™ Soft Release Avidin Resin (lane 4) gave results almost identical to that of non-induced culture. The first wash (lane 5) removed some *E. coli* proteins, however a significant amount of the recombinant protein was also removed. The 2 subsequent washes (lanes 6 and 7) removed little *E. coli* proteins. Finally, protein elution (lanes 8, 9 and 10) released very little of the recombinant protein, and revealed the insufficiencies of this method for protein purification.

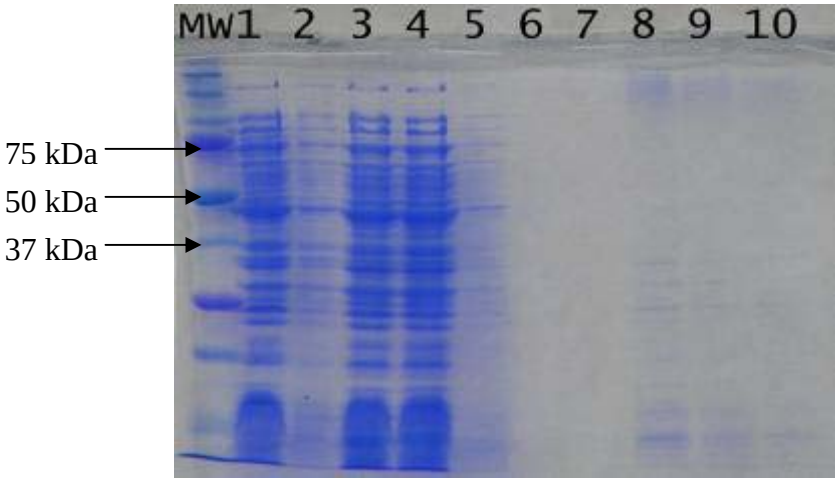

Figure 3-6. SDS-PAGE of full length soluble protein produced with cell culture OD_{600nm} 0.62 and 2.5 hrs of induction. Protein was purified using the batch capture method and the following fractions were separated by SDS-PAGE and visualized with Coomassie blue stain. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lane 1: Sample of non-induced culture. Lane 2: Non-purified sample after 2.5 hrs of induction. Lane 3: Non purified soluble fraction. Lane 4: Fraction that did not bind to Softlink™ Soft Release Avidin Resin. Lane 5: Wash 1. Lane 6: Wash 2. Lane 7: Wash 3. Lane 8: One hr elution. Lane 9: 5 hr elution. Lane 10: Overnight elution.

3.5.2 Full length soluble protein column capture

Figure 3-7 displays the protein content of samples taken during protein purification using the column capture method. The fraction of protein that did not bind to the avidin resin, seen in lane 1, was dense with proteins of various sizes including the size of the desired protein. The first wash (lane 2) removed some *E. coli* proteins and some of the recombinant protein. However, in both instances less protein was removed in this wash than in the equivalent in the batch capture method. The 2 subsequent washes (lanes 3 and 4) did not remove any protein. All 5 elutions (lanes 5, 6, 7, 8 and 9) released a small amount of correctly sized protein, however, as for the batch purification method; the elutions were not pure.

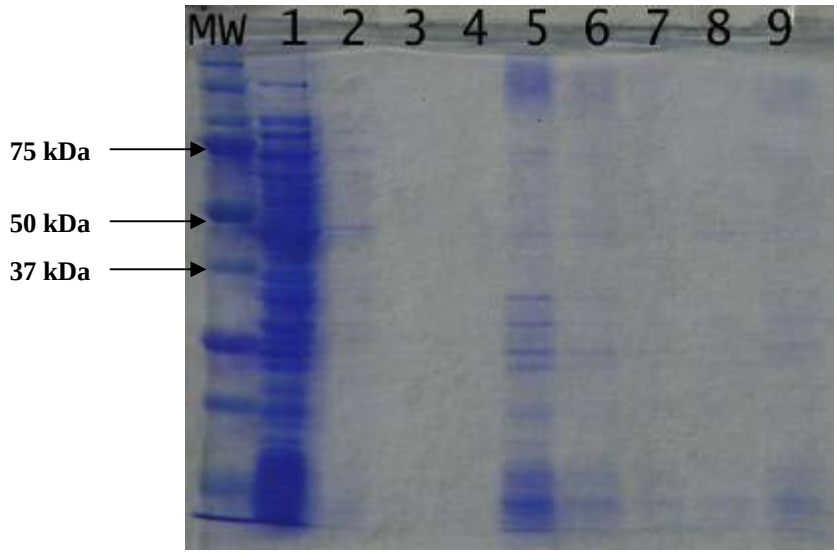

Figure 3-7. SDS-PAGE of full length soluble protein produced with cell culture OD_{600nm} 0.62 and 2.5 hrs of induction. Protein was attempted purified using the column capture method and the following fractions were separated by SDS-PAGE and visualized with Coomassie blue stain. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lane 1: Fraction that did not bind to Softlink™ Soft Release Avidin Resin. Lane 2: Wash 1. Lane 3: Wash 2. Lane 4: Wash 3. Lane 5: First elution. Lane 6: Second elution. Lane 7: Third elution. Lane 8: Fourth elution. Lane 9: Overnight elution.

3.6 Purification of insoluble full length recombinant BFDV protein

Insoluble full length IB protein was purified and solubilised using the Urea/DTT (Figure 3-8, lanes 1-5) and the B-PER method (Figure 3-8, lanes 6-10). In both methods, the resuspended pellet (Urea/DTT: lane 1 and B-PER: lane 6) was densely populated with proteins of varying molecular weight. Washing the pellet (lanes 2-4 and 7-9) removed some E. coli proteins and some protein at the molecular weight of the recombinant protein. The final products of both methods (lanes 5 and 10) were found to be highly impure.

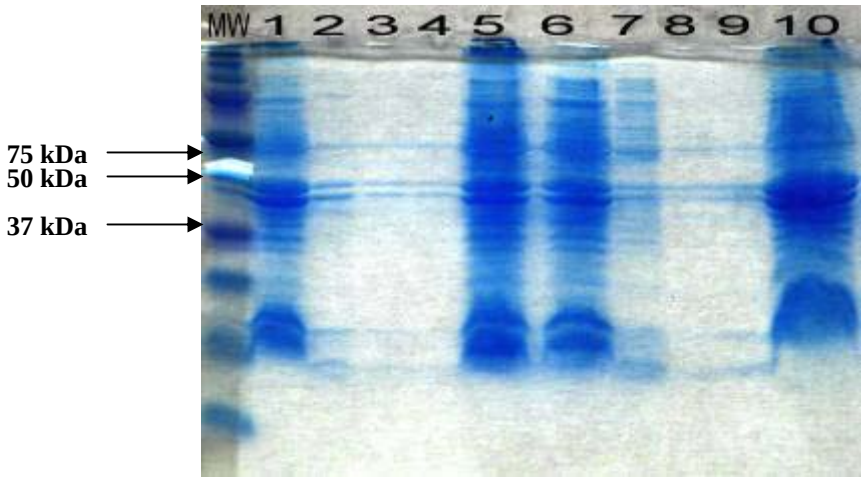

Figure 3-8. SDS-PAGE of full length inclusion-body protein produced with cell culture OD_{600nm} 0.62 and 2.5 hrs of induction. Protein was attempted purified and solubilised using the Urea/DTT (lanes 1-5) and B-PER methods (lanes 6-10). The fractions were visualized by SDS-PAGE and Coomassie blue staining. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lane 1: Sample of resuspended pellet for purification with the Urea/DTT method. Lane 2: First wash. Lane 3: Second wash. Lane 4: Third wash. Lane 5: Final product of inclusion-body protein purification and solubilization with the Urea/DTT method, Lane 6: Sample of resuspended pellet for purification with the B-PER method. Lane 7: First wash. Lane 8: Second wash. Lane 9: Third wash. Lane 10: Final product of IB protein purification and solubilization with the B-PER method.

3.7 Detection of full length recombinant protein with anti-BFDV-antibodies

To determine whether the recombinant protein could react with antibodies raised specifically against BFDV, the recombinant protein was electrophoresed in an SDS-PAGE gel, transferred to a nitrocellulose membrane and incubated with sera from a chicken inoculated with purified virus, non-inoculated chicken sera and sera from a naturally immune cockatoo. To visualize formed antigen-antibody complex, the membrane was further incubated with HRP conjugated anti-chicken-antibody or with goat-anti-cockatoo and then with HRP conjugated anti-goat-antibody. Alkaline phosphatase conjugated streptavidin was used as a positive control, to clearly distinguish antibody binding to the recombinant protein from antibody binding to other antigens, e.g. *E. coli* proteins. Results can be seen in Figure 3-9.

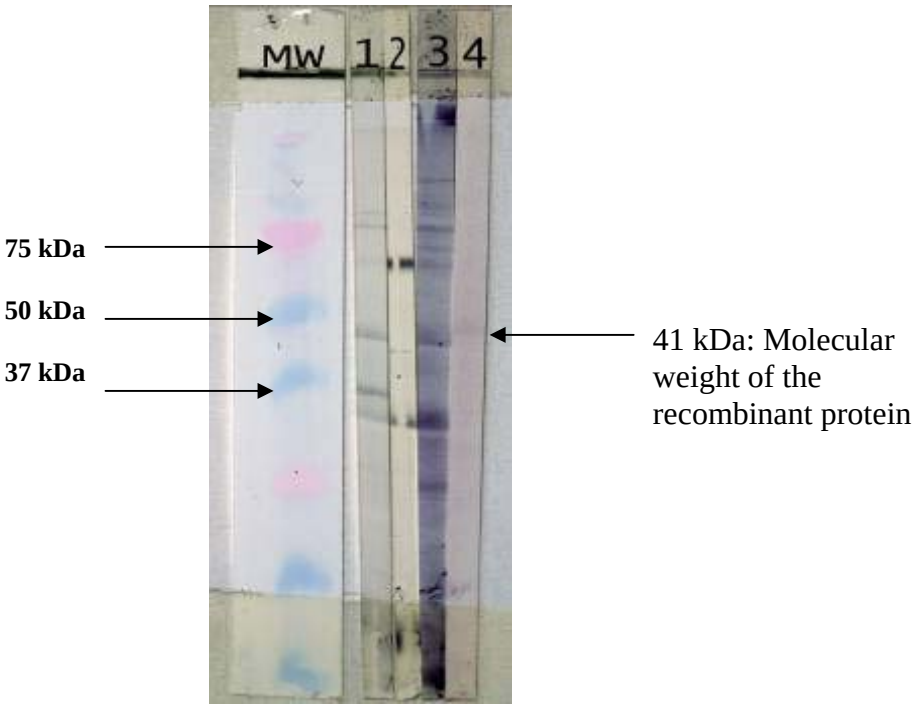

Figure 3-9. Western immunoblot of full length recombinant protein with the following antibodies. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lane 1: Chicken-anti-BFDV (1:200 dilution). Lane 2: Negative control; non challenged chicken sera (1:200 dilution). Lane 3: Sera from an immune cockatoo (1:200 dilution). Lane 4: Positive control; alkaline phosphatase conjugated streptavidin (1:2000 dilution).

Positive controls using streptavidin (lane 4) consistently detected the biotinylated recombinant protein located between 37 and 50 kDa. Both the BFDV challenged chicken sera (lane 1) and the non-challenged chicken sera (lane 2) resulted in several bands. However, the challenged chicken sera gave a band at the same molecular weight as the positive control (41 kDa), whereas the non-challenged chicken sera did not. Sera from an immune cockatoo also gave multiple bands, including one at desired molecular weight.

3.8 Detection of antibody-response to recombinant BFDV protein by western immunoblot, IHC and HI

Moderately high titres of non-specific autoagglutination were present in the pre and post inoculation sheep sera which interfered with the principles of the HI assay. Therefore western immunoblot and IHC was used to detect antibody response in sheep.

3.8.1 Western immunoblot detection of antibodies to full length recombinant protein in sheep sera

To determine whether the inoculated sheep had raised antibodies against the inoculum, Urea/DTT solubilised recombinant protein was electrophoresed in an SDS-PAGE gel, transferred to a nitrocellulose membrane and incubated with sera from both sheep pre inoculation, post inoculation and post boosting. Results can be seen in Figure 3-10.

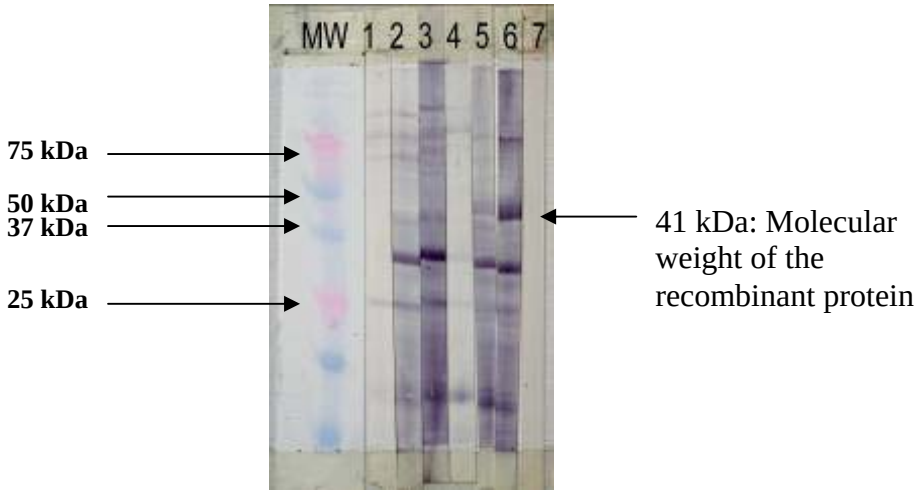

Figure 3-10. Western immunoblot of full length recombinant protein with sera from sheep pre inoculation, post inoculation and post boosting. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lane 1: Sheep 5 pre inoculation (1:200 dilution). Lane 2: Sheep 5 post inoculation (1:200 dilution). Lane 3: Sheep 5 post boosting (1:200 dilution). Lane 4: Sheep 761 pre inoculation (1:200 dilution). Lane 5: Sheep 761 post inoculation (1:200 dilution). Lane 6: Sheep 761 post boosting (1:200 dilution). Lane 7: Positive control; alkaline phosphatase conjugated streptavidin (1:2000 dilution).

None of the 2 sheep displayed antibody binding at the molecular weight of the recombinant protein (41 kDa) pre inoculation (Figure 3-10, lane 1 and 4). However, after inoculation (lane 2 and 5) both sheep produced antibodies that bound to the recombinant protein. This binding was increased after boosting (lanes 3 and 6).

3.8.2 IHC for detection of antibodies to native BDFV raised in sheep inoculated with recombinant BFDV capsid protein.

IHC was employed to determine whether inoculation of sheep with recombinant BFDV capsid protein would cause the sheep to raise antibodies that would recognise native BFDV. The results of IHC may be seen in Figure 3-11. Sera from sheep post (Figure 3-11A) and pre (Figure 3-11B) inoculation and a skin section from a chronically infected cockatoo were used for this procedure. As can be seen in Figure 3-11A, sera from inoculated sheep recognised BFDV inclusion bodies in the skin section (black arrow), whereas sera harvested from sheep pre inoculation did not react with the viral inclusions (Figure 3-11B).

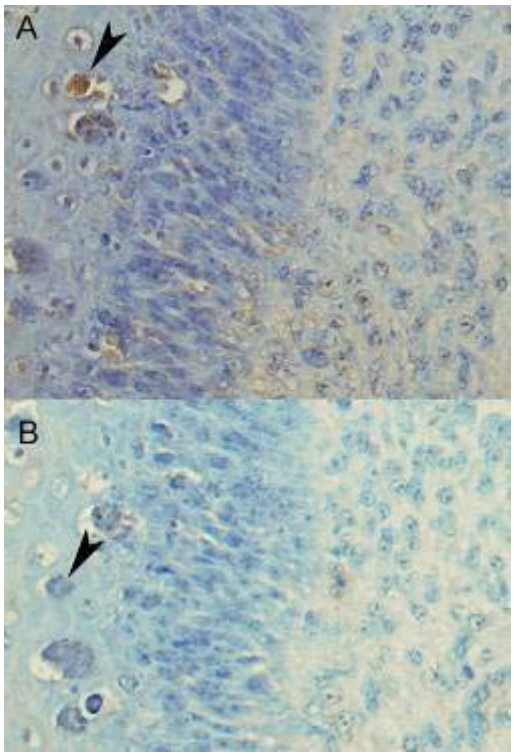

Figure 3-11. IHC using a section of skin from a chronically BFDV infected cockatoo. A) Skin section incubated with sera from inoculated sheep, arrow indicates location of a viral IB where an antigen-antibody complex formed has been visualised by binding of secondary antibody. B) Negative control. Skin section from chronically infected cockatoo incubated with sera from sheep pre inoculation. Arrow points to an example of a BFDV inclusion that was not recognised by the sera.

3.8.3 HI detection of antibodies to BFDV raised in psittacine birds vaccinated with recombinant BFDV capsid protein.

Twelve psittacine birds were vaccinated with the solubilised full length IB protein. Antibody response was determined by HI assay. An example of HI assay results may be viewed in Figure 3-12 and a full set of results can be seen in Table 3-1. Three birds (red tailed black cockatoo samueli, eastern long billed corella and galah-corella cross) displayed an increase in anti-BFDV-antibody titre. The red tailed black cockatoo had no antibody pre vaccination and its antibody titre has increased to 80 HI units (HIU) 11 days after vaccination. Boosting the animal resulted in an antibody titre of 160 HIU 32 days later. Similarly, the eastern long billed corella also showed no evidence of anti-BFDV-antibody before vaccination and the animal's antibody titre was at 20 HIU 11

days after vaccination. Unfortunately this bird was not available for further vaccine work, thus monitoring further increase in HIU was not possible. The third bird, a galah-corella cross, had an antibody status of 80 HIU before vaccination. Eleven days post vaccination; this bird had an increase in antibody titre to 160 HIU. Boosting this animal did not result in a further increase in antibody.

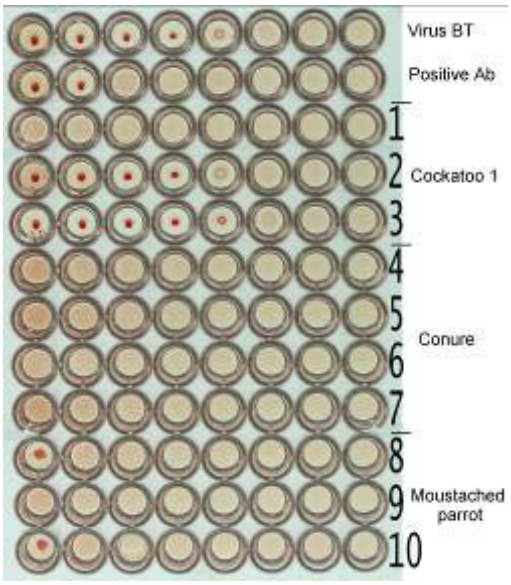

Figure 3-12. Example of microtitre plate showing results of HI assay of three birds vaccinated with solubilised full length recombinant IB protein. Rows 1, 2 and 3: Sera from the red tailed black cockatoo pre vaccination, 11 days post vaccination and 32 days post boosting, respectively. Rows 4, 5, 6 and 7: Sera from a conure pre vaccination, 11 days post vaccination, 32 days post vaccination and 46 days post vaccination, respectively. Rows 8, 9 and 10: Sera from vaccinated moustached parrot 1 pre vaccination, 11 days post vaccination and 14 days post boosting, respectively. Column 1 is a 1:20 dilution of sera. Subsequent columns are serially diluted 1:2 of the column to its left.

Table 3-1. Results of HI screening of experimentally vaccinated birds pre vaccination, post vaccination and post boosting. Blood was collected on the day of vaccination and a number of days (as labelled) after vaccinations and boosting. n/s: no sample taken that day. N/A: bird no longer available for experiment. “Auto-agg”: the HI result could not be considered positive or negative due to sera causing auto-agglutination. Days: refers to number of days post the last injection.

	Pre inoculation	**Post inoculation**						
Species	**HI result**	**Days**	**HI result**	**Days**	**Bosted Y/N**	**HI result**	**Days**	**HI result**
Redtailed black cockatoo Samueli	0	11	80	32	y	160	N/A	
Moustached parrot 1	0	11	0	32	y	n/s	14	0
Muostached parrot 2	Auto-agg	11	0	32	y	n/s	14	Auto-agg
Conure	0	11	0	32	n	0	46	0
Eastern long billed correla 1	0	11	20	N/A				
Derbyan	Auto-agg	11	Auto-agg	N/A				
Galah-Corella cross	80	11	160	32	y	160	N/A	
Sulphur crested cockatoo/Corella	80	11	40	46	n	80	N/A	
Eclectus parrot male	0	11	0	N/A				
Eclectus parrot female	0	11	Auto-agg	N/A				
Galah/Major Mitchell	160	14	160	N/A				
Eastern Long Billed Corella 2	160	14	Auto-agg	N/A				

4 Discussion

During this research project a biotinylated recombinant BFDV capsid protein, recognized by anti-BFDV-antibodies raised in chickens and cockatoos, was successfully expressed in a bacterial system. Two sheep and several psittacine birds were injected with the recombinant protein and their antibody response monitored by western immunoblot, IHC and HI. It was found that both sheep and birds inoculated with the recombinant protein induced the production of antibodies that specifically recognised native BFDV. These results show that the recombinant protein is antigenic and that native epitopes of the BFDV have been conserved. This recombinant protein has valuable potential future applications in immunisation of parrots, lorikeets and cockatoos and as antigen in ELISA and HI assays without the requirement of native virus preparation and as a tool for the production of monoclonal antibodies. In addition, this experiment has resulted in a large stock of sheep-anti-BFDV sera that can be used for consistent IHC, HI assays and western immunoblot. The capsid protein was the chosen element for the development of this vaccine as no other structural proteins have been found in association with BFDV.

4.1 PCR amplification of BFDV ORF C1 and screening of recombinant *E. coli* JM109.

PCR reactions were successfully optimised for amplification of full length, 3' truncated and 5' truncated BFDV ORF C1. Amplicons were further ligated into the pPinPoint™ Xa-1 T-vector and the recombinant plasmid successfully transformed into *E. coli* JM109. The full length and 3' truncated amplicons were drmined by PCR and restriction digest to be successfully ligated into the pPinPoint™ Xa-1 T-vector in the

correct orientation. Further screening by dye-terminator sequencing confirmed orientation of insert and desired reading frame.

Recombinant plasmids with correctly inserted 5’ truncated amplicon could not be detected during screening experiments. This may have been due to a number of factors. An insert must have been present in the vector and the vector must have been successfully transformed into the cell because these transformants were able to form colonies in the presence of ampicillin. Ampicillin resistance of *E. coli* JM109 is conferred only by the introduced vector. The linear pPinPoint™ Xa-1 T-vector has deoxythymidine overhangs at the 3’ ends and can therefore not recircularise in the absence of insert. Linear plasmids are unable transform into bacteria. Ergo the inability to detect 5’ truncated recombinants must have been due to incorrectly inserted amplicon and not absence of insert. Only a limited number of 5’ truncated recombinants were observed on the agar plates, none of these were found to contain correctly orientated insert. Because of the vector’s deoxythymidine overhangs at the 3’ ends and the deoxyadenine overhangs of the amplicon, the amplicon can only insert in 1 of 2 possible orientations. In light of this it might seem logical to assume that as much as 50% of the transformants could contain correctly inserted amplicon. Then why were no 5’ truncated recombinants detected during screening? The *E. coli* used in this experiment were determined to have leaky expression (see section 3.4 optimisation of protein expression) despite having the *lacI*^{q} allele which causes overexpression of the lac repressor and should therefore not be subject to leaky expression (Sambrook and Russel, 2001). Cells containing correctly inserted 5’ truncated insert may therefore have been expressing the protein while growing on the transformation plates and the expressed protein might impose growth limitations on the cell, resulting in minute colonies not detectable during viewing of plates. These colonies were therefore not picked and screened. This problem may be solved by trying a number of different *E. coli* strains for initial transformation.

This would allow for identification of clones with the right plasmid construct which could then be purified and then introduced into an appropriate strain of bacteria for protein expression. However, if the problem does not lie in growth inhibiting properties of basal recombinant protein expression, subcloning might be required for increasing occurrence of correctly oriented insert. Due to time constraints and the successful cloning of 2 amplification products, the full length and the 3' truncated, it was decided to focus on these, as protein expression of these would include all epitopes of the capsid protein that could be antigenic.

4.2 Optimisation of full length and C-terminal truncated protein expression

Induction of protein expression in *E. coli* JM109 under the control of IPTG was successful for both full length and C-terminal truncated recombinant protein. Rate of cell growth is believed to be an important influence on expression of foreign proteins in bacteria and other than optimal conditions can overload the bacterial protein synthetic apparatus, promoting IB formation. Therefore the density to which cells are grown before induction and after induction is important (reviewed in Sambrook and Russel (2001)). Because of this, optimisation of recombinant full length and C-terminal truncated protein expression was performed in order to obtain soluble protein that could be purified using the Promega PinPoint™ Xa protein purification system and to obtain the highest possible yield of recombinant protein. The length and time of induction was not found to be critical for the expression of recombinant protein in general, however did have an effect on yield of soluble recombinant protein. Optimal protein yield was found at OD_{600nm} 0.62 with 1.5 and 2.5 hours of induction for full length expressing cells and an OD_{600nm} of 0.940 with induction time between 3.5 and 5.0 hours for C-terminal truncated protein expressing cells. Full length soluble protein was obtained under specific conditions of cell density and length of induction, indicating the

importance of cell density and length of induction on expression of soluble protein. Soluble C-terminal truncated recombinant protein could not be obtained under the conditions examined. Trying a broader range of cell densities could possibly overcome this problem. Both full length and C-terminal truncated forms could be expressed in an insoluble form as IBs.

4.3 Purification of recombinant protein

The most immunogenic epitopes of the BFDV capsid protein have not been determined. However, the C-terminal truncated protein was designed on the basis of prediction of a major antigenic epitope, as seen in section 2.3, and could possibly result in a recombinant BFDV capsid protein more antigenic than the full length protein. Initial assessment of the antigenicity of the C-terminal truncated protein compared to the full length protein can in the future be performed using the sheep-anti-recombinant BFDV protein yielded in this project. However the aims of this project were not to characterise antigenic epitopes in the BFDV capsid protein and the recombinant full length protein would cover the complete set of epitopes. This, in addition to time consuming optimisation of full length protein purification, caused C-terminal truncated protein purification attempts to not be pursued.

4.3.1 Full length soluble protein

Expressed soluble full length protein was purified from crude lysate of recombinant expressing cells using the Promega PinPoint™ Xa purification system. Both batch capture and column capture were attempted in order to increase the binding of biotinylated recombinant protein to resin. The PinPoint™ Xa system uses Softlink™ Soft Release Avidin Resin for purification. This resin consists of resin beads with attached avidin molecules. Such avidin molecules are intended to bind to biotin - the

fusion tag used in this expression system, allowing for *E. coli* proteins and cellular debris to be removed by washing (Promega, 2001).

Separation of the different protein fractions collected during batch capture by SDS-PAGE revealed that the non-purified fraction and the non-bound fraction had an almost identical protein content including that of the recombinant protein. This indicates that the resin had low capacity to bind the biotinylated recombinant protein, despite the high affinity ($K_d \sim 10^{-15}$ M) avidin has for biotin (Nilson *et al.*, 1997). Further supporting this theory; the first wash released the recombinant protein in addition to *E. coli* proteins and the 2 subsequent washes did not reveal any protein. The 3 elutions released some protein; however, none of the bands corresponded to the molecular weight of the recombinant BFDV capsid protein, indicating that the resin retained non-biotinylated protein during wash steps, but not the biotinylated protein. Thus the protein of interest appears to have bound to the resin at very low level, and the portion of recombinant protein that may have bound was removed already in the first wash.

Purification attempts using the column capture method gave similar results, except a minute amount of the recombinant protein was observed in the elutions. The elutions from the resin were contaminated with *E. coli* proteins, but did have considerably less *E. coli* protein content than can be seen in non purified protein preparations. Thus the column method allowed for more binding between avidin and biotin than the batch method. The primary culture used in expression experiments was not supplemented with biotin, despite the direction of the manufacturer, because the presence of biotin in growth media was found to inhibit growth. The lack of biotin in primary culture would have caused the biotin ligase pathway to not be primed before the culture was diluted and recombinant protein expression induced. The result of this would be that fewer cells were able to perform biotinylation and therefore lowering the yield of expressed

biotinylated protein and further less protein would be available for binding to the avidin linked resin. Further, this would decrease the ability of biotinylated protein to compete with the non-specific binding activity of *E. coli* proteins, further explaining the high impurity of eluted protein. In addition, biotin not ligated to the recombinant protein as a result of not priming the biotin ligase pathway or a hight ratio of biotin to recombinant protein would result in left over biotin in the lysate. This free biotin would then compete with the recombinant protein for binding sites. Another factor that may affect the binding of the biotinylated protein to the resin is the folding of the protein as this may partially mask the biotin molecule thereby decreasing its ability to bind avidin.

Repeated attempts at using these 2 methods, both according to the directions provided by the manufacturer and several variants of these directions, were unsuccessful at producing entirely pure protein. It can be concluded however that the column capture method allows for more association between biotin and avidin than the batch method, however using this method for purifying recombinant BFDV capsid protein requires more optimisation in order to retain pure recombinant protein.

The reasons for inadequacies of this system when applied to the purification of recombinant BFDV capsid protein are not clear. However, they should be considered an effect of the properties of this recombinant protein and not a poorly designed purification system, due to successful use of this system for producing and purifying other proteins previously by this laboratory (unpublished data) and by others (Cress *et al.*, 1993).

4.3.2 Full length insoluble inclusion body protein

Insoluble IB protein was purified from crude lysate by low speed centrifugation and solubilised using the Urea/DTT and the B-PER methods. Purification by both methods

reduced the contaminating *E. coli* proteins but some were still evident in the protein preparation. The purified fraction was approximately 20 times more concentrated than the crude lysate, thus a large quantity of *E. coli* contaminants have been removed during the wash steps.

IBs are electron-dense structures that may be observed as refractile bodies under phase contrast microscopy. These structures appear as amorphous particles under electron microscopy and there have been no reports of regular structure (Chrunyk *et al.*, 1993). IBs were first observed in bacteria that globally missincorporated a.a. analogues into their proteins. Later, such aggregates have been observed in many recombinantly expressed proteins (Chrunyk *et al.*, 1993). Problems associated with protein solubility are often described in the literature; however no exact methods of solving these problems have been determined.

High level expression of cloned genes in *E. coli* may result in the protein forming IB aggregates. Several factors may contribute to the fate of a recombinant protein as an IB, including host cell, growth conditions and properties of the protein of interest. (Kane and Hartley, 1998) reviewed an extensive list of proteins originating from *E. coli*, *Bacillus* species, Cellulomonas, viral, mammalian and *S. cerevisiae* expressed in *E. coli* systems, and report that there seems to be no correlation between formation of inclusion bodies and promoter used, formation of disulphide bonds in the recombinant protein, molecular mass of the protein or the level of expression. Normal *E. coli* proteins expressed at high levels may also accumulate in the IB form, and therefore it cannot be assumed that IBs are simply a response to foreign proteins (Marston, 1986). This popular theory of ‘response to foreign protein’ is further contradicted by the fact that bacteria can package its own proteins into IBs, and no evidence has been reported for IB formation being an active process (Schein, 1989).

Several reasons have been postulated for why IBs occur. One of these is that proteins aggregate when they are expressed at a level that saturates the cell's degradation system. However, there are some proteins, including prochymosin which are also insoluble when produced at low levels. It does seem evident that IBs are not the sole product of precipitation, as solubilisation of recombinant protein IBs requires harsh chemical conditions, thus at some stage covalent, ionic or hydrophobic interactions must occur between the molecules in IBs (Marston, 1986). Schein (1988) expressed 3 different proteins in *E. coli* at 37°C and also at temperatures ranging between 23 and 30°C. When expressed at 37°C all 3 proteins were found as IB aggregates, however at temperatures between 23 and 30°C, 30-90% of the recombinant protein was found in the soluble form. These effects were observed for several different *E. coli* strains and plasmid constructs. The same results have been found with the P22 tailspike protein, diphtheria toxin, ricin A chain and several others (Schein, 1989). Thus temperature appears to be a major determinant of the solubility of foreign expressed proteins in *E. coli*. Expression of interferon-α2 in *E. coli* has shown that the percentage of insoluble protein does not correlate with the total amount of protein expressed (Schein, 1989). Others have demonstrated mutants that produce *less* inclusion IBs and mutants that produce *more* IBs than the wild type, indicating that a.a. sequence may influence the level of formation of IBs (Wetzel *et al.*, 1991; Chrunyk *et al.*, 1993).

Thus the exact mechanism of IB formation has not been elucidated. However, complications of IB occurrence may be overcome by mutations, altering temperature at which the protein is expressed and in some cases by decreasing the level of expression. For this experiment, attempting expression at lower temperatures would have been the next step in the effort to produce a higher soluble fraction of full length and C-terminal truncated protein. This was not performed due to limited time and the fact that expressed soluble protein was not considered Alfa Omega to big picture of this project.

IBs are dense and therefore sediment more rapidly than cellular debris during low speed centrifugation, a property that may be exploited for purification of IB protein (Marston, 1986). Centrifugation speeds ranging from 500 to 12,000 *g* have been reported for purification of inclusion bodies (Marston, 1986). In this experiment initial centrifugation of cell lysate was performed at 2,000 *g* for 7 min as described in section *2.8.2*. Considering the broad range of centrifugal speeds that have been reported for IB purification purposes, 2,000 *g* may not have been the optimal, and purification could possibly have been enhanced by either altering the speed or the centrifugation time, or a combination of both. Impurity of the final product may also be explained by the nature of IBs, because they commonly contain other components such as the 4 subunits of RNA polymerase, some of the bacterial outer membrane proteins (OmpC, OmpF and OmpA), 16S and 23S rRNA, and possibly circular and nicked plasmid DNA in addition to the recombinant protein (Schein, 1989).

4.4 Detection of full length recombinant protein with anti BFDV specific antibodies

Full length recombinant BFDV capsid protein was successfully recognised by sera from a naturally immune cockatoo and by sera from a chicken experimentally challenged with live BFDV. There was no reaction with naïve chicken sera, confirming that the antibody-antigen reaction seen with challenged chicken sera in fact was interaction between chicken-anti-BFDV-antibody and the recombinant protein.

These results show that specific anti-BFDV-antibodies recognise the recombinant protein even though the protein was solubilised in 2M Urea, thus the linear epitopes involved in the animal antibody response have been conserved in the recombinant protein.

4.5 Detection of antibody-response to recombinant BFDV protein in sheep and psittacine birds

The antibody response to inoculation with the recombinant protein was detected in sheep by western immunoblot and IHC and in psittacine birds by HI. The results for inoculated sheep will be discussed in sections 4.5.1 and 4.5.2 and results regarding vaccination of psittacine birds will be discussed in section 4.5.3.

4.5.1 Western immunoblot detection of antibodies to full length recombinant BFDV capsid protein in sheep

Western immunoblot with sera from the 2 sheep, both bled and inoculated at days 0, 21 and 44, successfully detected antibodies to the recombinant BFDV protein. These antibodies were not present before inoculation, but a strong signal was observed in sera from 21 days post inoculation. An even stronger signal was observed with sera collected 24 days post boosting (44 days post primary inoculation), showing as expected that boosting increases the antibody response. These results prove that the recombinant BFDV capsid protein induces an antibody response. Under optimal circumstances, this western immunoblot would have employed native virus in place of the recombinant protein, thereby determining whether these antibodies would recognise native virus. This was not possible due to the limited availability of purified virus, as the virus cannot be propagated in cell culture. However this uncertainty was clarified by IHC as discussed below.

4.5.2 IHC detection of antibodies to native BFDV raised in sheep inoculated with recombinant BFDV capsid protein

Screening inoculated sheep sera by IHC successfully demonstrated the ability of sera raised against the recombinant protein to recognise BFDV inclusions in skin sections

from a PBFD-affected cockatoo. Formation of antibody-antigen complexes was not detected with non-inoculated sheep sera, confirming that the antibody-antigen reaction observed with post inoculation sera was a result of sheep-anti-recombinant BFDV Protein-antibodies recognising native BFDV. This proves that inoculation with the recombinant protein induces an antibody response that also recognises native virus, giving the first evidence for the effectiveness of this bacterially expressed recombinant protein for use in vaccination to protect psittacine birds against BFDV.

4.5.3 HI detection of antibodies to native BFDV raised in psittacine birds vaccinated with recombinant BFDV capsid protein

HI successfully detected and quantified an increase in antibody titre towards native BFDV in 3 of 12 vaccinated birds. Two of these birds did not have a HI detectable anti-BFDV-antibody titre before vaccination. These results do not necessarily mean there was no antibody response to the vaccine in the remaining 10 birds. Some sera caused low levels of auto-agglutination when screened, interfering with the principles of the assay. Therefore the antibody titre of these sera could not be determined. The HI assay is not a sensitive technique, the first well in a row being a 1:20 dilution of sera. Therefore a low antibody titre would not be detected by HI. Inability to produce HI detectable antibody titres in some individuals may also be due to no previous exposure to the virus, thus a higher vaccine dose might be required to induce a HI detectable antibody response. Some birds became unavailable for boosting and/or bleeding at various times post vaccination (as indicated by “N/A” in results table). Therefore some birds may not have produced HI detectable antibody, as vaccination commonly requires more than one injection for inducing a high level antibody response at a high level and some birds may have increased their HIU status but were not detected because of lacking access to the animal.

A HI assay for the detection of psittacine-anti-BFDV-antibody has been optimised (Riddoch *et al.,* 1996) and has become the standard for detecting and quantifying antibody to BFDV in psittacine birds. This assay was successfully applied for quantification of antibody response in the experimental birds, however the assay was found insufficient for quantifying antibody response in experimental sheep, as the sheep sera caused low levels of auto-agglutination which interfered with the principles of the HI test.

4.6 Future directions

Before this recombinant protein can be used to vaccinate endangered species, further investigations are required as follows:

- Developing an efficient method to increase the volume of production of recombinant protein.
- Determining whether it will be necessary to include purification steps in the production of the recombinant protein.
- Vaccination trials to determine the minimal effective vaccine dose of recombinant protein that will induce a persistent effective immunity. This will include determining the number of doses required and the optimal time between doses.
    - Given the low sensitivity of HI for detecting circulating anti-BFDV antibodies in blood, the development of more sensitive serological assays (such as ELISA) will be required. The production of specific monoclonal antibodies will be a necessary element in the development of a serological assay.

  - A better understanding of the antigenic variation that exists among BFDV isolates from wild endangered psittacine bird species will also be required to ensure that any vaccine developed is efficacious against all antigenic “serotypes”.

- Additionally, experimentally vaccinated birds have to be challenged with live virus to determine whether vaccination confers immunity to BFDV infection. This will require the development of specific pathogen free flocks (SPF) of psittacine birds that can act as experimental models of infection.

- Determining the level of passively transferred immunity that occurs between parent (hen) and offspring via the egg yolk will facilitate protection of nestling birds.

- Developing novel techniques that can facilitate the safe and efficacious vaccination of free-living wild birds with minimum impact on individual birds and the environment should also be considered.

Future work required		
Action	Priority	Indicative cost estimate
Increasing production volume and purification of recombinant protein	Medium	$20-30,000
Development of more specific and sensitive antibody tests (such as ELISA) for BFDV antibody	High	$174,000
Production of monoclonal antibodies to BFDV (to facilitate the construction of sensitive and specific diagnostic assays)	High	$48,000
Improved understanding of the antigenic and genotypic variation of BFDV, particularly in endangered birds	Medium	$80,000
Develop an SPF flock that can be used for vaccination response and protection experiments	High	$170,000
Vaccination dose, challenge and effectiveness experiments	High	$180,000
Development of novel “minimum interference” techniques for administering vaccine to wild birds	Medium	$240,000
Environmental impact assessment of vaccination	High	$50,000

Conclusions

Expression of full length and C-terminal truncated BFDV capsid protein was successfully optimised. The full length protein was proven to be recognisable by sera raised against native virus in naturally infected psittacine birds, as well as experimentally infected chicken. Sera from a chicken that had not been challenged did not recognise the recombinant protein showing that the antibody-antigen complex formation seen with the challenged chicken sera was a result of anti-BFDV-antibodies recognising the recombinant protein. This shows that the antigenic epitopes of the native virus have been conserved in the recombinant protein. Further, the recombinant

protein was proven to be antigenic by inoculating sheep and detecting antibody response by western immunoblot. Pre inoculation sera did not display reactivity with the recombinant protein. The antisera raised in inoculated sheep was further proven by IHC to react with native BFDV, proving that inoculation with the recombinant protein induced an antibody response that recognises native virus. Finally, vaccination of psittacine birds with the recombinant protein resulted in an antibody response that can be detected by HI.

5 References

Almeida, M. S., Torloni, H., Lee-Ho, P., Vilar, M. M., Thaumaturgo, N., Simpson, A. J. and Tendler, M. (2003). Vaccination against Fasciola hepatica infection using a Schistosoma mansoni defined recombinant antigen, Sm14. Parasite Immunol, 25, 135-7.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nuc Acids Res, 25, 3389-3402.

Applied Biosystems (1995-1998). ABI Prism Primer Express, version 1.0

Arguello-Astorga, G., Herrera-Estrella, L. and Rivera-Bustamante, R. (1994). Experimental and theoretical definition of geminivirus origin of replication. Plant Mol Biol, 26, 553-6.

Ashby, E. (1907). The Emu, 6, 193-194.

Bassami, M. R., Berryman, D., Wilcox, G. E. and Raidal, S. R. (1998). Psittacine beak and feather disease virus nucleotide sequence analysis and its relationship to porcine circovirus, plant circoviruses, and chicken anaemia virus. Virol, 249, 453-9.

Bassami, M. R., Ypelaar, I., Berryman, D., Wilcox, G. E. and Raidal, S. R. (2001). Genetic diversity of beak and feather disease virus detected in psittacine species in Australia. Virol, 279, 392-400.

Chrunyk, B. A., Evans, J., Lillquist, J., Young, P. and Wetzel, R. (1993). Inclusion body formation and protein stability in sequence variants of interleukin-1 beta. J Biol Chem, 268, 18053-61.

Cobb, B. D. and Clarkson, J. M. (1994). A simple procedure for optimising the polymerase chain reaction (PCR) using modified Taguchi methods. Nuc Acids Res, 22, 3801-3805.

Coligan, J. E., Dunn, B. M., Ploegh, H. L., Speicher, D. W. and Wingfield, P. T. (Eds.) (2002) Curr Prot Protein Sci, John Wiley and Sons.

Cress, D., Shultz, J. and Breitlow, S. (1993) In Promega Notes Magazine, pp. 2-9.

Engel, B. (1992). Amplify for Analysing PCR Experiments. University of Wisconsin, USA.

Gerlach, H. (1994) In Avian Medicine: Principles and Application (Eds, Ritchie, B. W., Harrison, G. J. and Harrison, L. R.) Wingers Publishing, Lake Worth, Florida, pp. 894-903.

Gronenborn, B. (2004). Nanoviruses: genome organisation and protein function. Vet Microbiol, 98, 103-9.

Gutierrez, C., Ramirez-Parra, E., Mar Castellano, M., Sanz-Burgos, A. P., Luque, A. and Missich, R. (2004). Geminivirus DNA replication and cell cycle interactions. Vet Microbiol, 98, 111-9.

Hodgeman, T. C. (1988). A new superfamily of replicative proteins. Nat, 333, 22-23.

Jacobson, E. R., Clubb, S., Simpson, C., Walsh, M., Lothrop, C. D., Jr., Gaskin, J., Bauer, J., Hines, S., Kollias, G. V., Poulos, P. and et al. (1986). Feather and beak dystrophy and necrosis in cockatoos: clinicopathologic evaluations. J Am Vet Med Assoc, 189, 999-1005.

Jergens, A. E., Brown, T. P. and England, T. L. (1988). Psittacine beak and feather disease syndrome in a cockatoo. J Am Vet Med Assoc, 193, 1292-4.

Johne, R., Raue, R., Grund, C., Kaleta, E. F. and Muller, H. (2004). Recombinant expression of a truncated capsid protein of beak and feather disease virus and its application in serological tests. Avian Pathol, 33, 328-36.

Kane, J. F. and Hartley, D. L. (1998). Formation of recombinant protein inclusion bodies in Escherichia coli,. Trends Biotech, 6, 95-101.

Kock, N. D., Hangartner, P. U. and Lucke, V. (1993). Variation in clinical disease and species susceptibility to psittacine beak and feather disease in Zimbabwean lovebirds. Onderstepoort J Vet Res, 60, 159-61.

Latimer, K. S., Steffens, W. L., 3rd, Rakich, P. M., Ritchie, B. W., Niagro, F. D., Kircher, I. M. and Lukert, P. D. (1992). Cryptosporidiosis in four cockatoos with psittacine beak and feather disease. J Am Vet Med Assoc, 200, 707-10.

Law, R. H., Smooker, P. M., Irving, J. A., Piedrafita, D., Ponting, R., Kennedy, N. J., Whisstock, J. C., Pike, R. N. and Spithill, T. W. (2003). Cloning and expression of the major secreted cathepsin B-like protein from juvenile Fasciola hepatica and analysis of immunogenicity following liver fluke infection. Infect Immun, 71, 6921-32.

Liu, C., Ihara, T., Nunoya, T. and Ueda, S. (2004). Development of an ELISA based on the baculovirus-expressed capsid protein of porcine circovirus type 2 as antigen. J Vet Med Sci, 66, 237-42.

Liu, Q., Tikoo, S. K. and Babiuk, L. A. (2001). Nuclear localization of the ORF2 protein encoded by porcine circovirus type 2. Virol, 285, 91-9.

Marston, F. A. (1986). The purification of eukaryotic polypeptides synthesized in Escherichia coli. Biochem. J., 240, 1-12.

McOrist, S., Black, D. G., Pass, D. A., Scott, P. C. and Marshall, J. (1984). Beak and feather dystrophy in wild sulphur-crested cockatoos (Cacatua galerita). J Wildl Dis, 20, 120-4.

Meehan, B. M., Creelan, J. L., McNulty, M. S. and Todd, D. (1997). Sequence of porcine circovirus DNA: affinities with plant circoviruses. J Gen Virol, 78 (Pt 1), 221-7.

Nawagitgul, P., Morozov, I., Bolin, S. R., Harms, P. A., Sorden, S. D. and Paul, P. S. (2000). Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. J Gen Virol, 81, 2281-7.

Niagro, F. D., Forsthoefel, A. N., Lawther, R. P., Kamalanathan, L., Ritchie, B. W., Latimer, K. S. and Lukert, P. D. (1998). Beak and feather disease virus and porcine circovirus genomes: intermediates between the geminiviruses and plant circoviruses. Arch Virol, 143, 1723-44.

Nilson, J., Ståhl, S., Lundberg, J., Uhlèn, M. and Nygren, P. (1997). Affinity Fusion Strategies for Detection, Purification and Immobilization of Recombinant Proteins. Protein Expr and Purif, 11, 1-16.

Pass, D. A. and Perry, R. A. (1984). The pathology of psittacine beak and feather disease. Aust Vet J, 61, 69-74.

Pass, D. A. and Perry, R. A. (1985). Psittacine Beak and Feather Disease: An update. Aust Vet Practit, 15, 55-60.

Promega (2000). Technical Manual, No. 028. Madison, WI, USA, pp. 1-27.

Promega (2001). Technical Bulletin, No. 234. Madison, WI, USA.

Raidal, S. R. (1994). Studies on Psittacine Beak and Feather Disease, PhD thesis, University of Sydney, Sydney.

Raidal, S. R. (1995). Viral Skin Diseases of Birds. Sem Avian and Exotic Pet Med, 4, 72-82.

Raidal, S. R. and Cross, G. M. (1994a). Control By Vaccination of Psittacine Beak and Feather Disease in a Mixed Flock of Agapornis spp. Aust Vet Practit, 24, 178-180.

Raidal, S. R. and Cross, G. M. (1994b). The haemaglutination spectrum of psittacine beak and feather disease virus. Avian Pathol, 23, 621-630.

Raidal, S. R. and Cross, G. M. (1995). Acute Necrotizing Hepatitis Caused by Experimental Infection with Psittacine Beak and Feather Disease Virus. J Avian Med and Surg, 9, 36-40.

Raidal, S. R., Firth, G. A. and Cross, G. M. (1993a). Vaccination and challenge studies with psittacine beak and feather disease virus. Aust Vet J, 70, 437-41.

Raidal, S. R., McElnea, C. L. and Cross, G. M. (1993b). Seroprevalence of psittacine beak and feather disease in wild psittacine birds in New South Wales. Aust Vet J, 70, 137-9.

Raidal, S. R., Sabine, M. and Cross, G. M. (1993c). Laboratory diagnosis of psittacine beak and feather disease by haemagglutination and haemagglutination inhibition. Aust Vet J, 70, 133-7.

Ramis, A., Latimer, K. S., Niagro, F. D., Campagnoli, R. P., Ritchie, B. W. and Pesti, D. (1994). Diagnosis of psittacine beak and feather disease (PBFD) viral infection, avian polyomavirus infection, adenovirus infection and herpesvirus infection in psittacine tissues using DNA in situ hybridization. Avian Pathol, 23, 643-657.

Riddoch, P. A., Raidal, S. R. and Cross, G. M. (1996). Psittacine circovirus antibody detection and an update on the methods for diagnosis of psittacine beak and feather disease. Aust Vet Pract, 26, 134-&.

Ritchie, B. W., Niagro, F. D., Latimer, K. S., Steffens, W. L., Pesti, D., Ancona, J. and Lukert, P. D. (1991). Routes and prevalence of shedding of psittacine beak and feather disease virus. Am J Vet Res, 52, 1804-9.

Ritchie, B. W., Niagro, F. D., Lukert, P. D., Latimer, K. S., Steffens, W. L. and Pritchard, N. (1989a). A Review of Psittacine Beak and Feather Disease; Characteristics of the PGFD Virus. J Wildl Dis, 3, 143-149.

Ritchie, B. W., Niagro, F. D., Lukert, P. D., Steffens, W. L., 3rd and Latimer, K. S. (1989b). Characterization of a new virus from cockatoos with psittacine beak and feather disease. Virol, 171, 83-8.

Ritchie, P. A., Anderson, I. L. and Lambert, D. M. (2003). Evidence for specificity of psittacine beak and feather disease viruses among avian hosts. Virol, 306, 109-15.

Roitt, I., Brostoff, J. and Male, D. (2001) Immunol, Mosby, Edindurgh.

Sambrook, J. and Russel, D. W. (2001) Molecular Cloning a Laboratory Manual, Cold Spring Harbour Laboratory Press, New York.

Schein, C. H. (1989). Production of soluble recombinant proteins in bacteria. Biotech (N Y), 7, 1141-1149.

Schein, C. H. and Noteborn, M. H. (1988). Formation of soluble recombinant proteins in Escherichia coli is favored by lower growth temperature. Biotech (N Y), 6, 291-294.

Schoemaker, N. J., Dorrestein, G. M., Latimer, K. S., Lumeij, J. T., Kik, M. J., van der Hage, M. H. and Campagnoli, R. P. (2000). Severe leukopenia and liver necrosis in young African grey parrots (Psittacus erithacus erithacus) infected with psittacine circovirus. Avian Dis, 44, 470-8.

Sinnathamby, G., Renukaradhya, G. J., Rajasekhar, M., Nayak, R. and Shaila, M. S. (2001). Immune responses in goats to recombinant hemagglutinin-neuraminidase glycoprotein of Peste des petits ruminants virus: identification of a T cell determinant. Vacc, 19, 4816-23.

Studdert, M. J. (1993). Circoviridae: new viruses of pigs, parrots and chickens. Aust Vet J, 70, 121-122.

Tischer, I., Mields, W., Wolff, D., Vagt, M. and Griem, W. (1986). Studies on epidemiology and pathogenicity of porcine circovirus. Arch Virol, 91, 271-6.

Todd, D. (2000). Circoviruses: immunosuppressive threats to avian species: a review. Avian Pathol, 29, 373-394.

van Regelmortel, M. H. V., Fauquet, C. M., Bishop, D. H. L., Carstens, E. B., Estes, M. K., Lemon, S. M., Maniloff, J., Mayo, M. A., McGeoch, D. J., Pringle, C. R. and Wickner, R. B. (Eds.) (2000) Seventh report of the international committee on taxonomy of viruses, Academic Press.

Wetzel, R., Perry, L. J. and Veilleux, C. (1991). Mutations in human interferon gamma affecting inclusion body formation identified by a general immunochemical screen. Biotech (N Y), 9, 731-7.

Wylie, S. L. (1991). Studies on Psittacine Beak and Feather Disease, PhD thesis, Murdoch University, Perth.

Wylie, S. L. and Pass, D. A. (1987). Experimental reproduction of psittacine beak and feather disease/french moult. Avian Pathol, 16, 269-281.

Ypelaar, I., Bassami, M. R., Wilcox, G. E. and Raidal, S. R. (1999). A universal polymerase chain reaction for the detection of psittacine beak and feather disease virus. Vet Microbiol, 68, 141-8.

Appendix A. Optimisation of full length protein expression

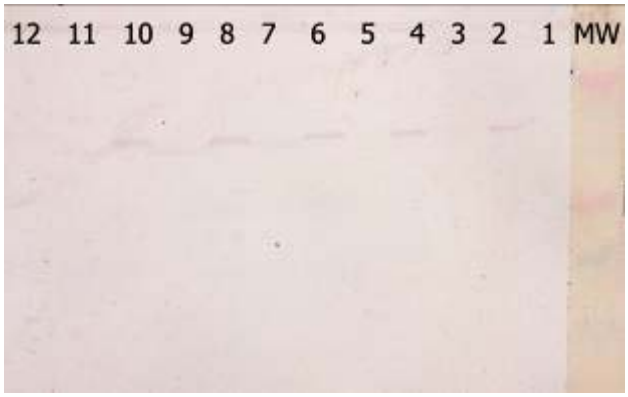

Figure A.1. Western immunoblot of full length recombinant protein generated by inducing cell culture at OD_{600nm} 0.32. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lane 1: Soluble protein fraction before induction with IPTG. Lane 2: Insoluble protein fraction before induction with IPTG. Lanes 3, 5, 7, 9 and 11: Soluble protein fractions after 1, 1.5, 2, 2.5 and 3 hours of induction with IPTG, respectively. Lanes 4, 6, 8, 10 and 12: Insoluble protein fraction after 1, 1.5, 2, 2.5 and 3 hours of induction with IPTG.

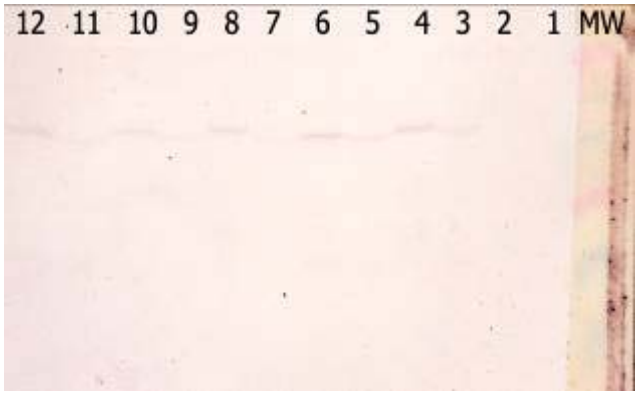

Figure A.2. Western immunoblot of full length recombinant protein generated by inducing cell culture at OD_{600nm} 0.32. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lanes 1, 3, 5, 7, 9 and 11: Soluble protein fractions after 3.5, 4, 4.5, 5, 5.5 and 6 hours of induction with IPTG, respectively. Lanes 2, 4, 6, 8, 10 and 12: Insoluble protein fraction after 3.5, 4, 4.5, 5, 5.5 and 6 hours of induction with IPTG.

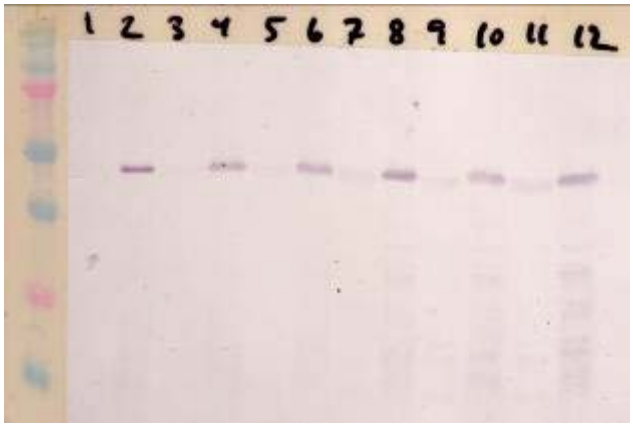

Figure A.3. Western immunoblot of full length recombinant protein generated by inducing cell culture at OD600nm 0.62. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lane 1: Soluble protein fraction before induction with IPTG. Lane 2: Insoluble protein fraction before induction with IPTG. Lanes 3, 5, 7, 9 and 11: Soluble protein fractions after 0.5, 1, 1.5, 2 and 2.5 hours of induction with IPTG, respectively. Lanes 4, 6, 8, 10 and 12: Insoluble protein fraction after 0.5, 1, 1.5, 2 and 2.5 hours of induction with IPTG.

Figure A.4. Western immunoblot of full length recombinant protein generated by inducing cell culture at OD600nm 0.62. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lanes 1, 3, 5, 7, 9 and 11: Soluble protein fractions after 3, 3.5, 4, 4.5, 5 and 5.5 hours of induction with IPTG, respectively. Lanes 2, 4, 6, 8, 10 and 12: Insoluble protein fraction after 3, 3.5, 4, 4.5, 5 and 5.5 hours of induction with IPTG.

Figure A.5. Western immunoblot of full length recombinant protein generated by inducing cell culture at OD600nm 0.90. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lanes 1, 3, 5, 7, 9, 11 and 13: Soluble protein fractions before induction, after 0.5, 1.5, 2, 2.5 and 3 hours of induction with IPTG, respectively. Lanes 2, 4, 6, 8, 10, 12 and 14: Insoluble protein fraction before induction, after 3.5, 4, 4.5, 5 and 5.5 hours of induction with IPTG.

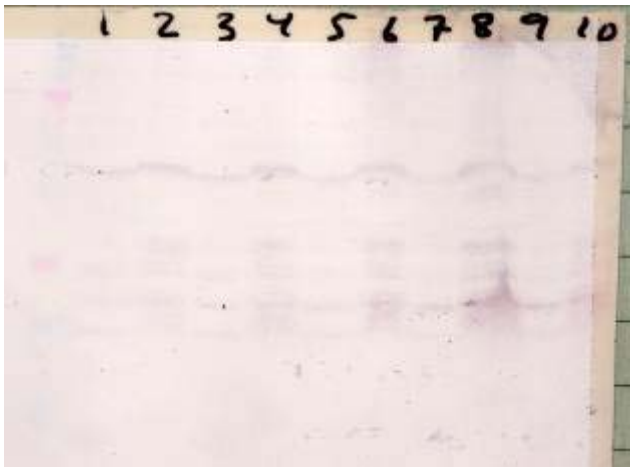

Figure A.6. Western immunoblot of full length recombinant protein generated by inducing cell culture at OD600nm 0.90. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lanes 1, 3, 5, 7, and 9: Soluble protein fractions after 3.5, 4, 4.5, 5 and 5.5 hours of induction with IPTG, respectively. Lanes 2, 4, 6, 8 and 10: Insoluble protein fraction after 3.5, 4, 4.5, 5 and 5.5 hours of induction with IPTG.

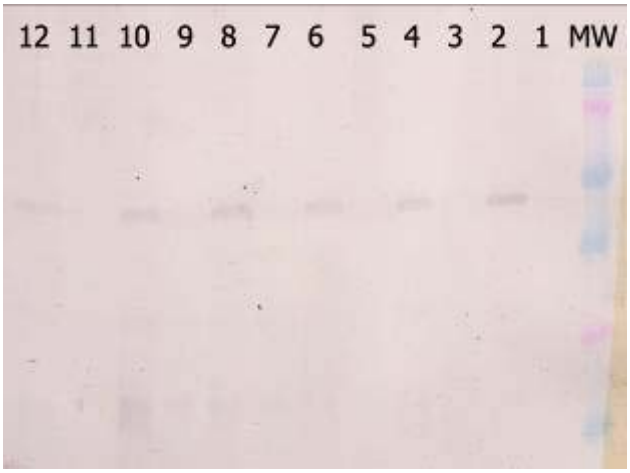

Figure A.7. Western immunoblot of full length recombinant protein generated by inducing cell culture at OD_{600nm} 1.19. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lane 1: Soluble protein fraction before induction with IPTG. Lane 2: Insoluble protein fraction before induction with IPTG. Lanes 3, 5, 7, 9 and 11: Soluble protein fractions after 0.5, 1, 1.5, 2 and 2.5 hours of induction with IPTG, respectively. Lanes 4, 6, 8, 10 and 12: Insoluble protein fraction after 0.5, 1, 1.5, 2 and 2.5 hours of induction with IPTG.

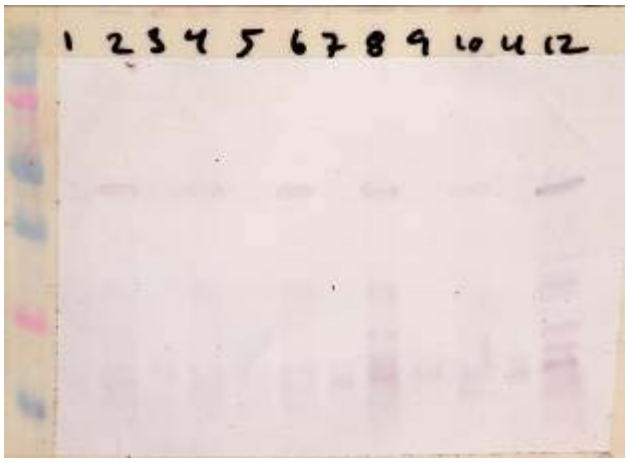

Figure A.8. Western immunoblot of full length recombinant protein generated by inducing cell culture at OD_{600nm} 1.19. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lanes 1, 3, 5, 7, 9 and 11: Soluble protein fractions after 3, 3.5, 4, 4.5, 5 and 5.5 hours of induction with IPTG, respectively. Lanes 2, 4, 6, 8, 10 and 12: Insoluble protein fraction after 3, 3.5, 4, 4.5, 5 and 5.5 hours of induction with IPTG.

Appendix B. Optimisation of C-terminal truncated protein expression

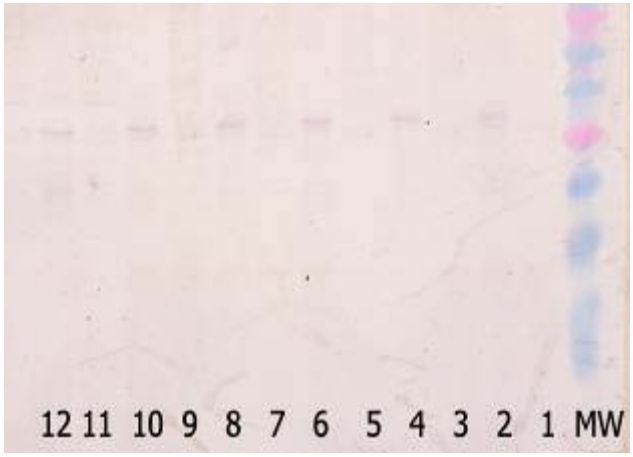

Figure B.1. Western immunoblot of C-terminal truncated recombinant protein generated by inducing cell culture at OD_{600nm} 0.316. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lane 1: Soluble protein fraction before induction with IPTG. Lane 2: Insoluble protein fraction before induction with IPTG. Lanes 3, 5, 7, 9, 11 and 13: Soluble protein fractions after 0.5, 1, 1.5, 2, 2.5 and 3 hours of induction with IPTG, respectively. Lanes 4, 6, 8, 10, 12 and 14: Insoluble protein fraction after 0.5, 1, 1.5, 2, 2.5 and 3 hours of induction with IPTG, respectively.

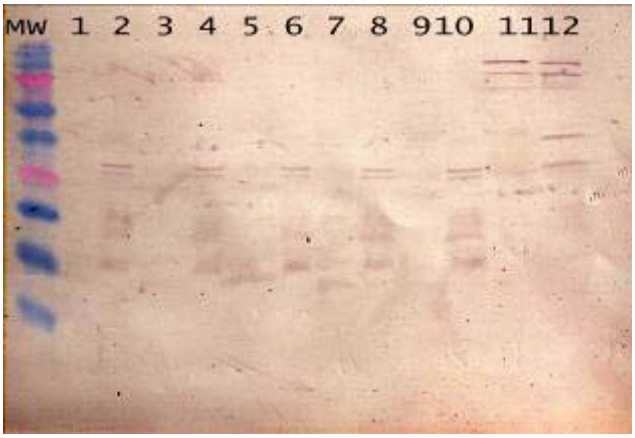

Figure B.2. Western immunoblot of C-terminal truncated recombinant protein generated by inducing cell culture at OD_{600nm} 0.316. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lanes 1, 3, 5, 7, 9 and 11: Soluble protein fractions after 3.5, 4, 4.5, 5, 5.5 hours and overnight of induction with IPTG, respectively. Lanes 4, 6, 8, 10 and 12: Insoluble protein fraction after 3.5, 4, 4.5, 5, 5.5 hours and overnight of induction with IPTG, respectively.

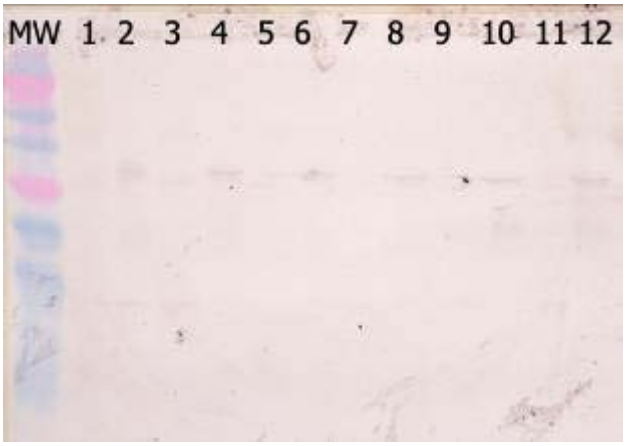

Figure B.3. Western immunoblot of C-terminal truncated recombinant protein generated by inducing cell culture at OD_{600nm} 0.591. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lane 1: Soluble protein fraction before induction with IPTG. Lane 2: Insoluble protein fraction before induction with IPTG. Lanes 3, 5, 7, 9, 11 and 13: Soluble protein fractions after 0.5, 1, 1.5, 2, 2.5 and 3 hours of induction with IPTG, respectively. Lanes 4, 6, 8, 10, 12 and 14: Insoluble protein fraction after 0.5, 1, 1.5, 2, 2.5 and 3 hours of induction with IPTG, respectively.

Figure B.4. Western immunoblot of C-terminal truncated recombinant protein generated by inducing cell culture at OD_{600nm} 0.591. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lanes 1, 3, 5, 7, 9 and 11: Soluble protein fractions after 3.5, 4, 4.5, 5, 5.5 hours and overnight of induction with IPTG, respectively. Lanes 2, 4, 6, 8, 10 and 12: Insoluble protein fraction after 3.5, 4, 4.5, 5, 5.5 hours and overnight of induction with IPTG, respectively.

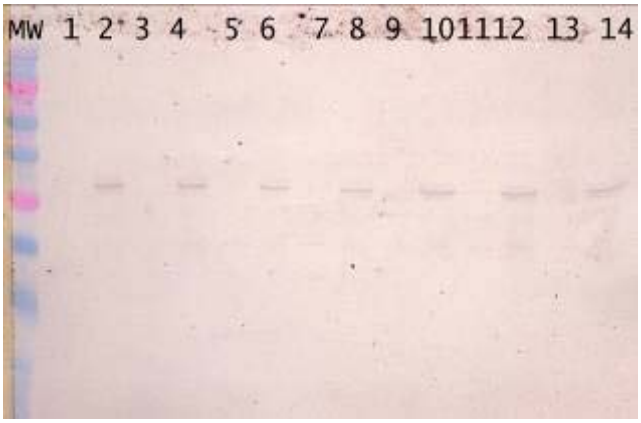

Figure B.5. Western immunoblot of C-terminal truncated recombinant protein generated by inducing cell culture at OD_{600nm} 0.940. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lane 1: Soluble protein fraction before induction with IPTG. Lane 2: Insoluble protein fraction before induction with IPTG. Lanes 3, 5, 7, 9, 11 and 13: Soluble protein fractions after 0.5, 1, 1.5, 2, 2.5 and 3 hours of induction with IPTG, respectively. Lanes 4, 6, 8, 10, 12 and 14: Insoluble protein fraction after 0.5, 1, 1.5, 2, 2.5 and 3 hours of induction with IPTG, respectively.

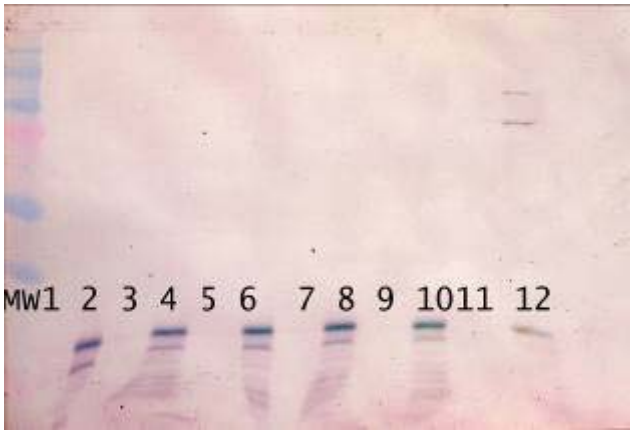

Figure B.6. Western immunoblot of C-terminal truncated recombinant protein generated by inducing cell culture at OD_{600nm} 0.940. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lanes 1, 3, 5, 7, 9 and 11: Soluble protein fractions after 3.5, 4, 4.5, 5, 5.5 hours and overnight of induction with IPTG, respectively. Lanes 2, 4, 6, 8, 10 and 12: Insoluble protein fraction after 3.5, 4, 4.5, 5, 5.5 hours and overnight of induction with IPTG, respectively.

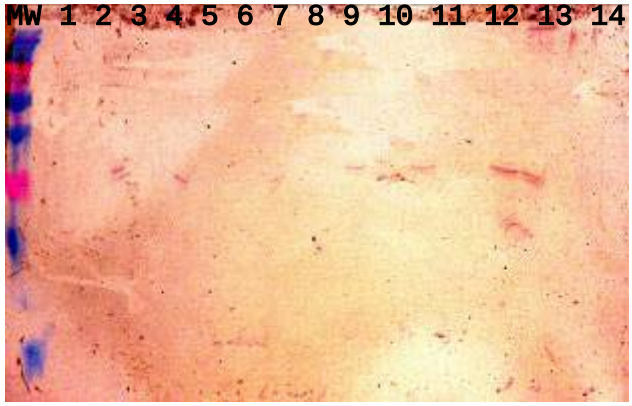

Figure B.7. Western immunoblot of C-terminal truncated recombinant protein generated by inducing cell culture at OD_{600nm} 1.174. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lane 1: Soluble protein fraction before induction with IPTG. Lane 2: Insoluble protein fraction before induction with IPTG. Lanes 3, 5, 7, 9, 11 and 13: Soluble protein fractions after 0.5, 1, 1.5, 2, 2.5 and 3 hours of induction with IPTG, respectively. Lanes 4, 6, 8, 10, 12 and 14: Insoluble protein fractions after 0.5, 1, 1.5, 2, 2.5 and 3 hours of induction with IPTG, respectively

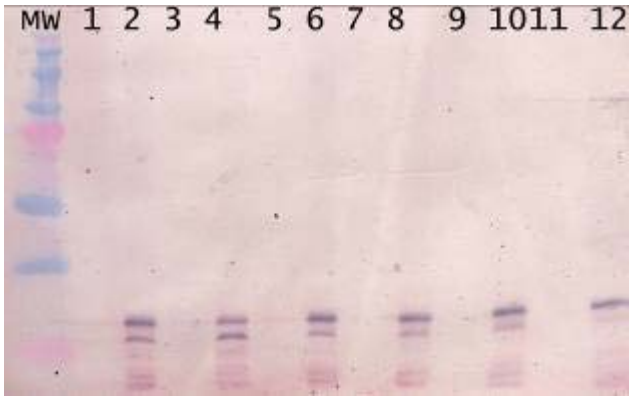

Figure B.8. Western immunoblot of C-terminal truncated recombinant protein generated by inducing cell culture at OD_{600nm} 1.174. Lane MW: Molecular weight marker; Precision Plus Protein Standards Dual colour (BioRad, Australia). Lanes 1, 3, 5, 7, 9 and 11: Soluble protein fractions after 3.5, 4, 4.5, 5, 5.5 hours and overnight of induction with IPTG, respectively. Lanes 2, 4, 6, 8, 10 and 12: Insoluble protein fractions after 3.5, 4, 4.5, 5, 5.5 hours and overnight of induction with IPTG, respectively.