

CHAPTER 3

Insertion of AHSV VP2 Neutralising Epitope in the Modified VP7, VP7mt144, its Effect on VP7 Structural Features and Immunogenicity.

3.1. INTRODUCTION.

The use of VP7 particulate structures or trimers as a vaccine delivery system would depend on its ability to accommodate and efficiently present peptide or epitope inserts as well as the ability of these inserts to elicit an immune response. As shown in chapter 2, the insertion of six amino acids between amino acids 144 and 145 of AHSV-9 VP7 did not abolish the formation of crystals. The solubility was affected to some extent, with the size distribution of crystalline structures tending towards the smaller particulate structure. A greater proportion of protein also remained in the trimer form, not aggregating into particulate structures of any significant size. These observations, however, should not detract from the potential utility of VP7 as a presentation system for epitopes. Smaller particles as well as the trimers themselves may be more or equally efficient for peptide display.

VP7 crystals have been shown to generate a protective immune response in mice (Wade-Evans *et al*, 1997; Wade-Evans *et al.*, 1998). The modification of VP7 to create VP7mt144 and VP7mt144/200 would not be expected to affect this ability. The protective immune response should be enhanced by the insertion of identified neutralising epitopes at the cloning sites created in the modified proteins. The ability of the chosen epitope to generate an immune response would depend on various factors. Firstly, the insertion of the epitope into VP7 must not affect the structure or folding of VP7 such that the formation of trimers or crystal particulate structures is affected, depending on the choice of the presentation system. The epitope must be presented in such a way on the surface of the VP7 protein that it occurs in the native recognised form, therefore the epitopes must be sufficiently

exposed on the surface of the protein and the structure or presentation of the epitope must not be affected by the environment of and interactions with the VP7 protein.

With the aim of developing an improved vaccine against AHSV, an identified neutralising epitope of the VP2 protein was chosen to insert in the cloning site created at position 144 of VP7mt144. The major antigenic domain of VP2 contains many smaller fragments that elicit neutralising antibodies. A strong linear epitope was identified by Venter *et al.*, 2000, between amino acids 369 and 407 of AHSV-9 VP2. This was supported by the separate identification of a strong neutralising area between amino acids 379 and 413 of AHSV-3 VP2 (Bentley *et al.*, 2000) and amino acids 377 and 400 of AHSV-4 VP2 (Martinez-Torrecuadrada *et al.*, 2001). Based on this evidence, a sequence of 25 amino acids from amino acid 377 to 401 of AHSV-9 VP2 was chosen to insert at cloning site 144 of VP7mt144. The choice of a linear neutralising epitope should eliminate the possibility of the protein environment of VP7 influencing the natural presentation of the epitope, in comparison to a more structurally dependent epitope. The 25 amino acid sequence would provide a significant size insert into the VP7 protein to give some further indication to the size of peptide insertions that could be accommodated at site 144/145. If this insert abolishes the ability of the protein to associate into trimers and, in turn, to form crystals, a possible size limitation of inserts which could be accommodated at this particular site will have been established. If the insert does not abolish the ability of the protein to associate into trimers, the possibility that it may be used as an epitope display vehicle is retained despite possible influences on solubility and/or crystal formation.

The insertion of 25 amino acids at site 144/145 was studied, similarly to the modifications creating VP7mt144 and VP7mt144/200, in terms of its effect on VP7 structure, trimer formation and crystal formation, as this would affect the presentation of the inserted epitope to an immune system. Secondly, the immune response generated by the modified VP7 protein containing the chosen epitope was also investigated, as the intended aim of the study is to investigate the potential of the system to elicit a protective immune response.

3.2. MATERIALS AND METHODS.

3.2.1. Materials.

AHSV-9 L2 cDNA in pBS was obtained from Dr F. F. Maree. Primers used were supplied by GIBCO BRL. β -agarase was obtained from FMC BioProducts. The pMOS Blue cloning kit was obtained from Amersham Pharmacia Biotech. Other reagents used were obtained from suppliers described in section 2.2.1.

3.2.2. PCR Amplification of a Small Antigenic Region of VP2.

A primer pair was designed to amplify the region of amino acids 377–401 (nucleotides 1141-1215) of AHSV-9 VP2 protein. The forward primer (jv2f) contained an overhang of an *EcoRI* site with an additional GC tag and the reverse primer (jv2r) contained an overhang of a *XhoI* site with an additional GC tag. The sequence of the primers is indicated in the table 3.1. Two T_m values were calculated according to the formula applicable to primers greater than 18 nucleotides in length: $T_m = 81.5 + 16.6\log[Na^+] + 0.41(\%G+C) - 675/n$, where n is the number of nucleotides in the primer. T_{m1} applied to the first five cycles of amplification and T_{m2} applied to the second phase of amplification. The average of the T_m values of the two primers was calculated and adjusted appropriately. The annealing temperature was taken as 5°C lower than the appropriate T_m value.

Table 3.1. Primers used in PCR Amplification of VP2 Epitope.

<u>Primer name.</u>	<u>Directed position on VP2.</u>	<u>Sequence.</u>	<u>T_m values (°C)</u>
jv2f	Nucleotide 1141-1161	5' GCGAATTCGATCCAAATCATGATACATGG 3' <i>EcoRI</i>	$T_{m1} = 43.38$ $T_{m2} = 53.6$
jv2r	Nucleotide 1195-1215	5' GCCTCGAGATTCGCGCTCTGTTCTTTCTG 3' <i>XhoI</i>	$T_{m1} = 47.03$ $T_{m2} = 59.25$

Reactions were prepared as described in section 2.2, including a negative control of an identical reaction lacking pBS-L2 cDNA template. The PCR program used included:

- A 2 minute denaturation step at 94°C
- 5 cycles of denaturation at 94°C for 30 seconds, annealing at 45°C for 3 seconds, elongation at 72°C for 30 seconds
- 15 cycles of denaturation at 94°C for 30 seconds, annealing at 51°C for 30 seconds and elongation at 72°C for 30 seconds.

- 15 cycles of denaturation at 94°C for 30 seconds, annealing at 51°C for 30 seconds with an increase in temperature of 0.5°C per cycle and elongation at 72°C for 30 seconds.
- A cycle of 7 minutes elongation at 72°C
- Hold at 4°C indefinitely.

The PCR products of the reaction and negative control were analysed by electrophoresis on a 2% agarose gel (1XTAE), as described by methods in chapter 2 (2.2.3.2.), using Φ X174 as a marker.

3.2.3. Cloning of VP2 epitope into the Modified VP7mt144 Gene.

3.2.3.1. General.

Cloning of the VP2 epitope was achieved by methods described in chapter 2, with minor additions and variations. Where necessary, analysis of the VP2 epitope by agarose gel electrophoresis was carried out on a 2% agarose gel (1XTAE) as opposed to the standard 1% agarose gel. In ligation reactions (chapter 2, section 2.2.3.4) for the insertion of the VP2 epitope, the standard insert:vector ratio of 3:1 was increased to approximately 5:1.

3.2.3.2. pMOS Blue Cloning.

The TA method cloning using the pMOS Blue cloning kit was carried out according to instructions provided by the manufacturer, involving ligation and transformation steps. Competent MOSBlue cells provided by the manufacturer were used, as well as *E. coli* XL1Blue cells, which were made competent according to the CaCl₂ method described in section 2.2.3.4. Blue/white selection on ampicillin (100µg/ml) and tetracycline hydrochloride (12.5µg/ml) plates, supplemented with 10µl 2% IPTG and 50µl 2% X-gal, was used to distinguish recombinants. Recombinants were selected by methods described in chapter 2.

3.2.3.3. Isolation of VP2 Epitope by β -Agarase Digestion of Agarose.

The digestion products containing the VP2 epitope were separated by electrophoresis on a 1% Low Melting Point (LMP) agarose gel at a low voltage of 65V to prevent melting of the gel. Also, a short time of 15 minutes was used to run the small 80 base pair fragment a short distance so that it could be easily viewed for excision. The DNA was excised from the gel and agarose was digested by β -agarase according to the manufacturer's instructions (FMC BioProducts): the gel slice was equilibrated for 1 hour with 10X the volume of 1X β -agarase buffer (supplied by the manufacturer at a 50X concentration). The gel was melted at 70°C then cooled to 45°C for digestion by 1U β -

agarase. The solution was centrifuged in a microfuge for 5 minutes to remove any undigested agarose, which collected in the pellet. The DNA was precipitated by the addition of 0.5 X the existing volume of 7.5M AmAc and 2.5 X the volume of 100% ethanol with incubation at -20°C overnight. The DNA was pelleted by centrifugation and washed with 70% ethanol before resuspending in 15µl ddH₂O. Electrophoresis of a small sample on a 2% agarose gel was used to confirm the presence of DNA of the appropriate size.

3.2.4. Immune Response Investigations.

3.2.4.1. Preparation of Protein for Inoculation into Mice.

Protein was expressed on large-scale (chapter 2, 2.2.7.4) and processed by sucrose gradient centrifugation at 40000rpm for 20 hours (chapter 2, section 2.2.11). Gradients were performed in triplicate to ensure sufficient quantities of protein could be recovered. Fractions of all three gradients containing the trimer form of VP7mt144-VP2 were pooled. The total volume was diluted to 5ml with 100mM Tris-HCl pH=8, 50mM NaCl. The protein was collected by centrifugation in the SW50.1 rotor at 20 000rpm for 15hrs. The pelleted protein was resuspended in 100µl of sterile 1XPBS (pH=7.4). A 10µl sample of the collected protein was analysed by SDS-PAGE and the concentration was estimated by comparison to the size marker of known concentration. The concentration of sample was then adjusted to 400ng/µl with sterile 1XPBS.

3.2.4.2. Inoculation of Mice with VP7mt144-VP2.

Five Balb/c mice were inoculated with prepared samples of VP7mt144-VP2 trimers. At each time, each mouse was inoculated with 10µg of prepared protein, equivalent to a volume of 25µl. Three inoculations were given two weeks apart. At day 0, 25µl of protein (10µg) was inoculated with an equal volume of Freund's Complete Adjuvant (FCA). At day 14, 25µl of protein was inoculated with an equal volume of Freund's Incomplete Adjuvant (FIA). At day 28, 25µl of protein sample was again inoculated with an equal volume of Freund's Incomplete Adjuvant (FIA). Before each inoculation, fresh protein samples were prepared. Inoculations were administered to the mice subcutaneously.

Mice were bled on day 42 by cardiac puncture, recovering approximately 1ml from each mouse. Antiserum was prepared by low speed centrifugation (3000rpm) of the blood collected from each mouse. This separated red blood cells as a pellet from the plasma. The plasma was collected and aliquoted into smaller quantities of 200µl and stored at -70°C.

3.2.4.3. Western Blot Analysis of Immune Response.

3.2.4.3.1. Sample Preparation.

Samples of AHSV serotype 9-infected BHK (baby hamster kidney) cells were provided by Dr M. van Niekerk. Also provided were mock-infected BHK cells. Cells were provided on monolayers in 75cm³ tissue culture flasks and were harvested in the following ways. AHSV-9 infected cells were easily dislodged from the flask surface by gentle agitation. Uninfected cells were removed from the flask by trypsin digestion. The monolayer was rinsed with 1XPBS solution. 10ml of trypsin/versene solution (Highveld Biological) at 37°C was added to the monolayer and left at room temperature for 1 minute. The trypsin solution was discarded and the monolayer was incubated for a further 3 minutes at 37°C. Cells were then dislodged by gentle agitation in 5ml 1XPBS. Both infected and uninfected cells were collected by low speed centrifugation at 3000 rpm for 5 minutes. Cells were washed twice in 1XPBS and finally resuspended in a total volume of 200µl 1XPBS. Samples were stored at -20°C.

3.2.4.3.2. Western Blot.

Samples were prepared for and separated on a 12% polyacrylamide minigel (section 2.2.9.) run at 120V for 2 hours. Samples were blotted onto a nitrocellulose membrane (Hybond C) using the Biorad E.C. 140 Mini Blot Module in a glycine, Tris-based buffer (14.4g glycine; 3.03g Tris in 1 litre; pH=8.3). Blotting was run at 0.12Amp for 60 to 90 minutes. The membrane was rinsed in 1XPBS pH=7.4 for 5 minutes, before blocking with blocking solution (1XPBS; 1% milk powder) for 30 minutes. Primary antibody was diluted in blocking solution to a dilution of 1/100. The membrane was incubated overnight in primary antibody solution, at room temperature and with agitation. The membrane was then rinsed three times for 5 minutes in washing solution (1XPBS, 0.05% Tween 20) before incubation for 1 hour in secondary antibody. The secondary antibody, peroxidase-conjugated protein A (ICN), was diluted to a 1 in 1000 dilution with blocking solution. The membrane was then rinsed 3 times for 5 minutes in washing solution, followed by a single wash with 1XPBS for 5 minutes. The membrane was then incubated at room temperature in substrate for 30 minutes to 1 hour. The substrate for development was prepared in the following way: 60ng 4-chloro-1-naphthol was dissolved in 20ml ice-cold methanol, and 60µl hydrogen peroxide was added to 100ml 1XPBS. The two solutions were mixed just prior to use. The detection reaction was stopped by rinsing the membrane in dH₂O.

3.3. RESULTS.

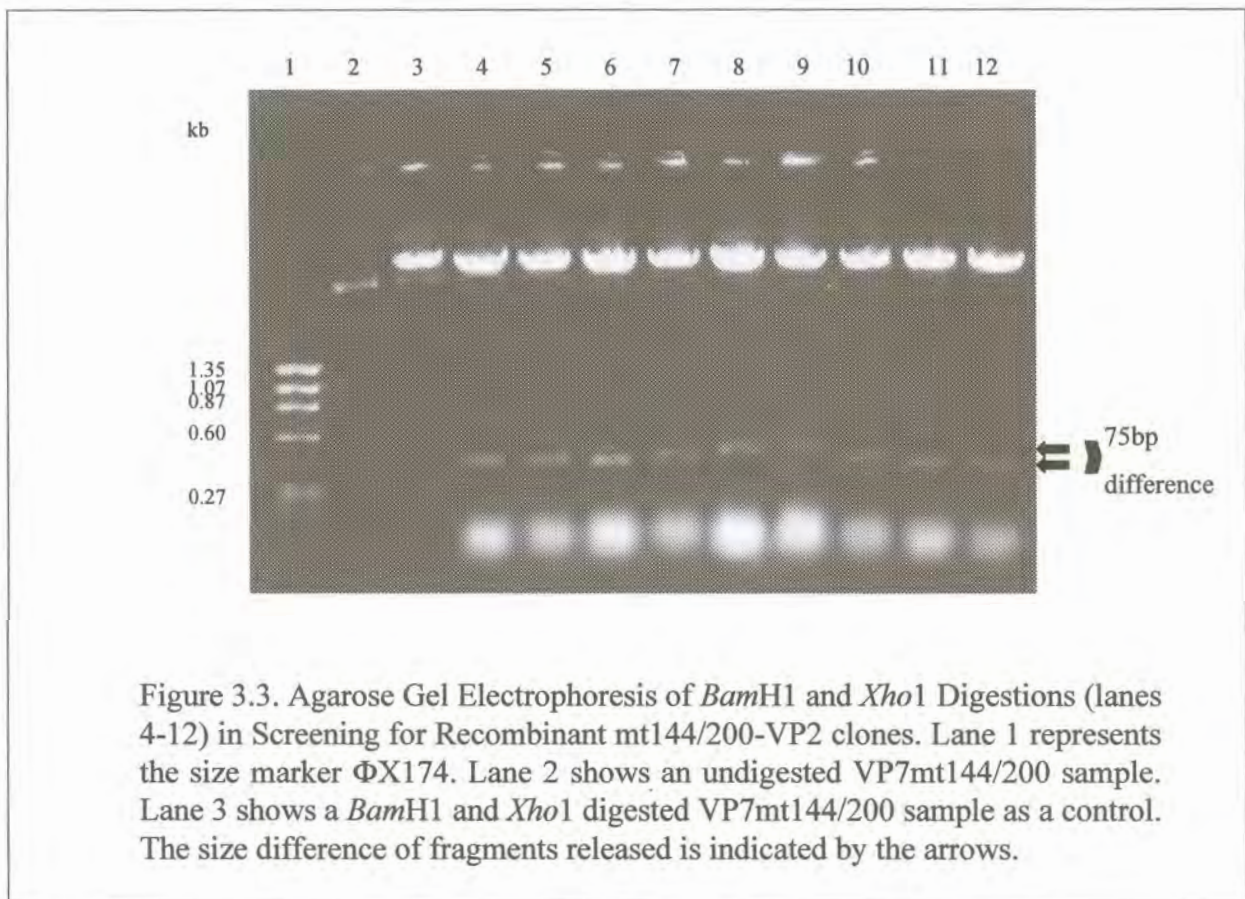
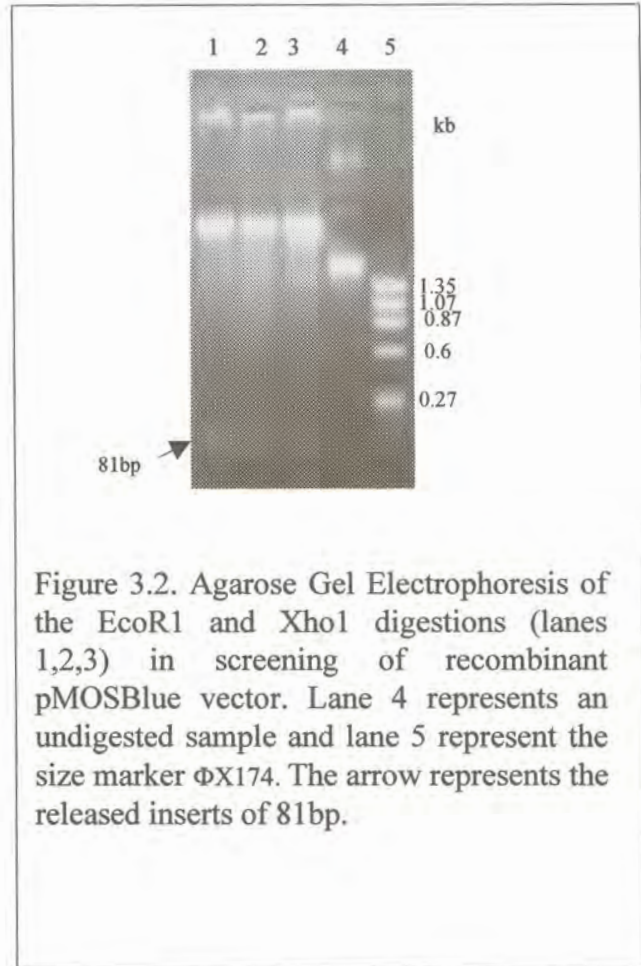
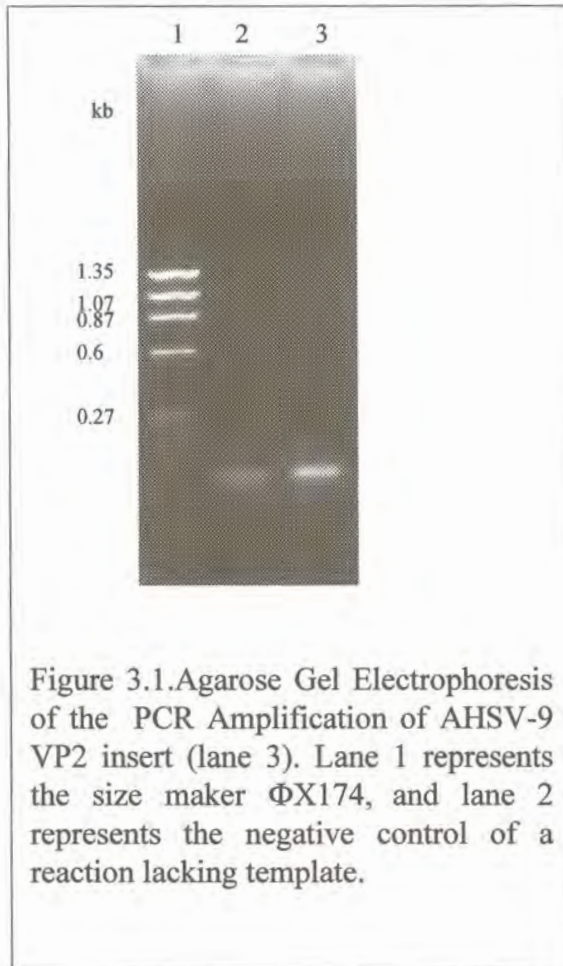
3.3.1. Construction of VP7mt144-VP2.

A sequence of 25 amino acids containing a suspected neutralising epitope of AHSV-9 VP2 was inserted into the cloning site created at position 144/145 of VP7mt144. PCR, using primers with restriction enzyme site overhangs, was used to amplify the segment of nucleotides 1141- 1215 (amino acids 377-401) of the cDNA clone of VP2 gene of AHSV-9. The forward primer was designed with an *EcoR1* overhang and the reverse with a *Xho1* overhang such that the amplified VP2 gene segment could be inserted between restriction enzyme sites *EcoR1* and *Xho1* at the 144/145 cloning site (section 3.2.2.; table 3.1.). The amplified DNA segment obtained was of the expected size of 81 base pairs. Figure 3.1 shows the PCR amplification of the AHSV-9 VP2 insert. The negative control shows a “fuzzy”, indistinct band of primer residues. The lane containing the amplified fragment shows a more intense band larger than the primer residues and which corresponds in size to the amplified segment.

Due to the small size of the amplified VP2 segment, and the corresponding difficulty in manipulation of this fragment, it was decided to first clone this segment into the pMOSBlue vector. This vector is specifically designed for the direct ligation of PCR-amplified products. Thus various problems associated with direct cloning of the segment into pFastBac-VP7mt144 could be avoided. These problems include the difficulty of digestion by restriction enzymes close to the ends of the PCR-amplified product, as well as the difficulty in purification of such a small fragment of DNA. The PCR-amplified VP2 segment was therefore directly ligated into pMOSBlue vector, after its retrieval by ethanol precipitation. *EcoR1* and *Xho1* digestion released a fragment corresponding to the expected size of 81 nucleotides, as can be seen in figure 3.2. The band appeared faint and fuzzy due to the particularly small size of the DNA fragment. Without the control of undigested sample, this band could be confused with contaminating RNA by virtue of its location and appearance in the gel. However, the uncut sample clearly indicated the absence of RNA and thus it could confidently be concluded to be the required fragment. This fragment then contained the appropriate sticky ends for cloning into VP7mt144 and VP7mt144/200.

Because of the small size of the fragment, the simple, commonly used methods for purification of DNA from a gel, including GENECLAN™ and the standard High Pure columns (Roche Diagnostics), could not be used to isolate and purify the fragment. The band of DNA was therefore excised from the gel and β -agarase (FMC Bioproducts) was used to dissolve the agarose of the gel (described in section 3.2.3.3). The fragment was then recovered by precipitation and cloned between the *EcoR1* and *Xho1* sites of VP7mt144 and VP7mt144/200. In screening, no recombinant VP7mt144-VP2 colonies were recovered on the first attempt. However, a double digestion by *BamH1* and *Xho1* showed a difference between the non-recombinant VP7mt144/200 and the recombinant VP7mt144/200-VP2. Non-recombinant VP7mt144/200 released a fragment of 375bp whereas the recombinant VP7mt144/200-VP2 released a fragment of 450bp. This difference of 75 base pairs could be distinguished on a 1% agarose gel, as shown in figure 3.3. Appropriate colonies were selected for further screening. A *Xho1* and *EcoR1* two-step double digest (not shown) confirmed the presence of the insert in selected colonies.

Due to the difficulties associated with the manipulation of the small fragment, it was decided not to repeat the failed attempt at the VP7mt144-VP2 cloning in the same way. The two modified genes, VP7mt144 and VP7mt144/200 are identical except for the presence of the cloning site at position 200/201 of VP7mt144/200. Therefore a recombinant VP7mt144-VP2 could be obtained by simply replacing a segment of VP7mt144, containing the cloning site but lacking the VP2 insert, with the corresponding segment of VP7mt144/200-VP2 containing the insert. This was achieved by *BamH1* and *Xho1* digestion of both VP7mt144 and VP7mt144/200-VP2. A two-step double digest was performed. Digestion was first carried out by *Xho1*, followed by *BamH1* with adjustment of the reaction volume and appropriate buffer. The smaller 5' segment released from VP7mt144/200-VP2 was cloned into the larger pFastBacVP7mt144 vector. Recombinant colonies were screened by the same *BamH1/Xho1* digestions used in their construction. Selected colonies of VP7mt144-VP2 were then screened for the correct insert by automated DNA sequencing.



3.3.2. Sequence Verification of VP7mt144-VP2.

Automated DNA sequencing of the selected clones confirmed the insertion of the 75 nucleotide segment of VP2 at site 144/145 of VP7mt144. PCR did not introduce any mutations in the VP2 sequence. The insertion also did not cause any shift in the reading frame and thus will be translated as a 25 amino acid insert of the VP7mt144 fusion protein. The sequence alignment of VP7mt144-VP2 to VP7mt144 is shown in figure 3.4. The insertion of the VP2 sequence is highlighted in red.

3.3.3. Physicochemical Properties and Structural Modeling of VP7mt144-VP2.

The effect of the insertion on the hydrophilicity profile of VP7mt144 was investigated (methods described in chapter 2, section 2.2.6). This is shown in figure 3.5. The VP2 insert was specifically chosen for the evidence of its role as a neutralising epitope (Venter *et al.*, 2000; Bentley *et al.*, 2000; Martínez-Torrecedrada *et al.*, 2001). In accordance with this property, it is a particularly hydrophilic sequence (Venter *et al.*, 2000). As can be seen in figure 3.5, the insert created a large hydrophilic area in the protein. With the rest of the protein being largely hydrophobic, the insertion of a relatively large, highly hydrophilic sequence may effect the folding of the protein from its original form. The folding of the protein was investigated by structural modelling, using the complete VP7 sequence of BTV-10 as a template (also refer to section 2.2.6). Figure 3.6. shows the results of the structural modelling. The green area is the bottom domain of α -helices and the blue area is the top domain of the β -sheets. The insert of VP2 sequence is indicated in yellow. It appears to loop out of the surface of the protein and does not appear to interfere with the folding of the protein into the α -helices and β -sheets. Thus, it is expected that the VP2 epitope will appear as a loop on the surface of the protein.

Figure 3.4. CLUSTAL X (1.81) Sequence Alignment of VP7mt144-VP2 to VP7mt144.

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mt144          GTTTAAATTTCGGTTAGGATGGACGCGATAGCAGCAAGAGCCTTGTCCGTTGTACGGGCAT 60
mt144vp2      GTTTAAATTTCGGTTAGGATGGACGCGATAGCAGCAAGAGCCTTGTCCGTTGTACGGGCAT
*****

mt144          GTGTCACAGTGACAGATGCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGA 120
mt144vp2      GTGTCACAGTGACAGATGCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGA
*****

mt144          TTGCAATCAATAGGTATAATGGTTTAAACAAATCATTCGGTATCGATGAGGCCACAAACCC 180
mt144vp2      TTGCAATCAATAGGTATAATGGTTTAAACAAATCATTCGGTATCGATGAGGCCACAAACCC
*****

mt144          AAGCAGAACGAAATGAAATGTTTTTATGTGTACTIONGATATGGTTTTAGCGGCGCTGAACG 240
mt144vp2      AAGCAGAACGAAATGAAATGTTTTTATGTGTACTIONGATATGGTTTTAGCGGCGCTGAACG
*****

mt144          TCCAAATTTGGGAATATTTACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTCG 300
mt144vp2      TCCAAATTTGGGAATATTTACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTCG
*****

mt144          CAACGACTGAAATTCATATAATGTTTCAGGCCATGAATGACATCGTTAGAATAACGGGTC 360
mt144vp2      CAACGACTGAAATTCATATAATGTTTCAGGCCATGAATGACATCGTTAGAATAACGGGTC
*****

mt144          AGATGCAAACATTCGGACCAAGCAAAGTGCAAACGGGGCTTATGCAGGAGCGGTTGAGG 420
mt144vp2      AGATGCAAACATTCGGACCAAGCAAAGTGCAAACGGGGCTTATGCAGGAGCGGTTGAGG
*****

mt144          TGCAACAATCTGGCAGATATTACGTACCGCCCGGGGAATTC-----
mt144vp2      TGCAACAATCTGGCAGATATTACGTACCGCCCGGGGAATTCGATCCAAATCATGATACAT 480
*****

mt144          -----CTCG
mt144vp2      GGAAAAACCATGTCAAAGAAATCAGAGAGAGGATGCAGAAAGAACAGAGCGCGAATCTCG 540
*****

mt144          AGCAAGGTCGAACGCGTGGTGGGTACATCAATTCAAATATTCGAGAAGTGTTGATGGATG 600
mt144vp2      AGCAAGGTCGAACGCGTGGTGGGTACATCAATTCAAATATTCGAGAAGTGTTGATGGATG
*****

mt144          CAGGTGCTGCGGGACAGGTCAATGCGCTGCTAGCCCCAAGGAGGGGGGACGCAGTCATGA 660
mt144vp2      CAGGTGCTGCGGGACAGGTCAATGCGCTGCTAGCCCCAAGGAGGGGGGACGCAGTCATGA
*****

mt144          TCTATTTTCGTTTGGAGACCGTTGCGTATATTTTGTGATCCTCAAGGTGCGTCACTTGAGA 720
mt144vp2      TCTATTTTCGTTTGGAGACCGTTGCGTATATTTTGTGATCCTCAAGGTGCGTCACTTGAGA
*****

mt144          GCGCTCCAGGAACTTTTGTCCCGTTGATGGAGTAAATGTTGCAGCTGGAGATGTCGTCCG 780
mt144vp2      GCGCTCCAGGAACTTTTGTCCCGTTGATGGAGTAAATGTTGCAGCTGGAGATGTCGTCCG
*****

mt144          CATGGAATACTATTGCACCAGTGAATGTTGGAAATCCTGGGGCACGCAGATCAATTTTAC 840
mt144vp2      CATGGAATACTATTGCACCAGTGAATGTTGGAAATCCTGGGGCACGCAGATCAATTTTAC
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mt144      AGTTTGAAGTGTTATGGTATACGTCCTTGGATAGATCGCTAGACACGGTTCGGGAATTGG
mt144vp2   AGTTTGAAGTGTTATGGTATACGTCCTTGGATAGATCGCTAGACACGGTTCGGGAATTGG 900
*****

mt144      CTCCAACGCTCACAAGATGTTATGCGTATGTCTCTCCCACTTGGCACGCATTACGCGCTG
mt144vp2   CTCCAACGCTCACAAGATGTTATGCGTATGTCTCTCCCACTTGGCACGCATTACGCGCTG 960
*****

mt144      TCATTTTTCAGCAGATGAATATGCAGCCTATTAATCCGCCGATTTTCCACCGACTGAAA
mt144vp2   TCATTTTTCAGCAGATGAATATGCAGCCTATTAATCCGCCGATTTTCCACCGACTGAAA1020
*****

mt144      GGAATGAAATTGTTGCGTATCTATTAGTAGCTTCTTTAGCTGATGTGTATGCGGCTTTGA
mt144vp2   GGAATGAAATTGTTGCGTATCTATTAGTAGCTTCTTTAGCTGATGTGTATGCGGCTTTGA1080
*****

mt144      GACCAGATTTCAGAATGAATGGTGTGTCGCGCCAGTAGGCCAGATTAACAGAGCTCTTG
mt144vp2   GACCAGATTTCAGAATGAATGGTGTGTCGCGCCAGTAGGCCAGATTAACAGAGCTCTTG1140
*****

mt144      TGCTAGCAGCCTACCACTAGTGGCTGCGGTGTTGCACGGTCACCGCTTTCATTAGTGTGCG
mt144vp2   TGCTAGCAGCCTACCACTAGTGGCTGCGGTGTTGCACGGTCACCGCTTTCATTAGTGTGCG1200
*****

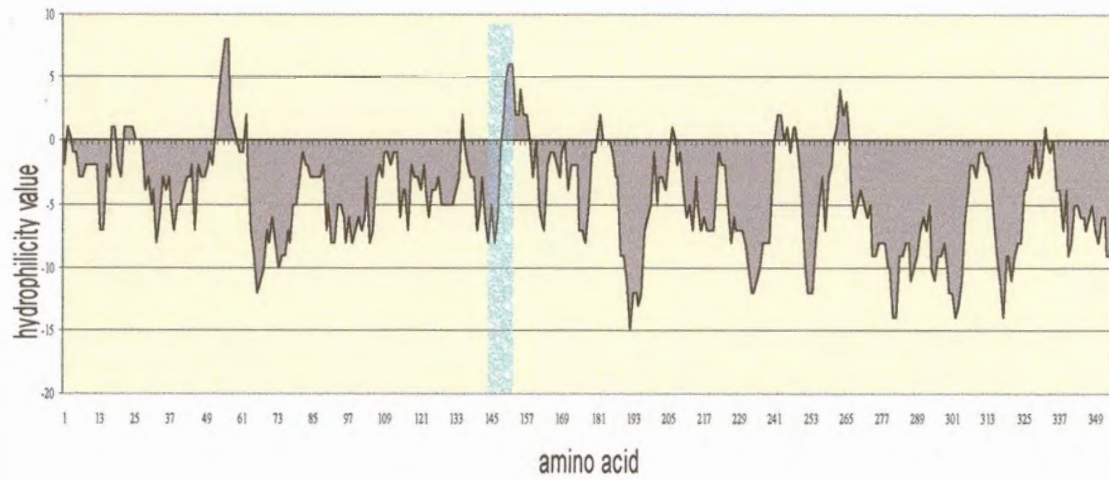
mt144      CGTCGGTTCTTATGCTGATAAAGTACGCATAAGTAATACGTCAATACCGAATACACTTAC
mt144vp2   CGTCGGTTCTTATGCTGATAAAGTACGCATAAGTAATACGTCAATACCGAATACACTTAC1260
*****

mt144      AGA
mt144vp2   AGA 1263
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(a)

VP7mt144 Hydrophilicity Plot



(b)

VP7mt144-VP2 Hydrophilicity Plot

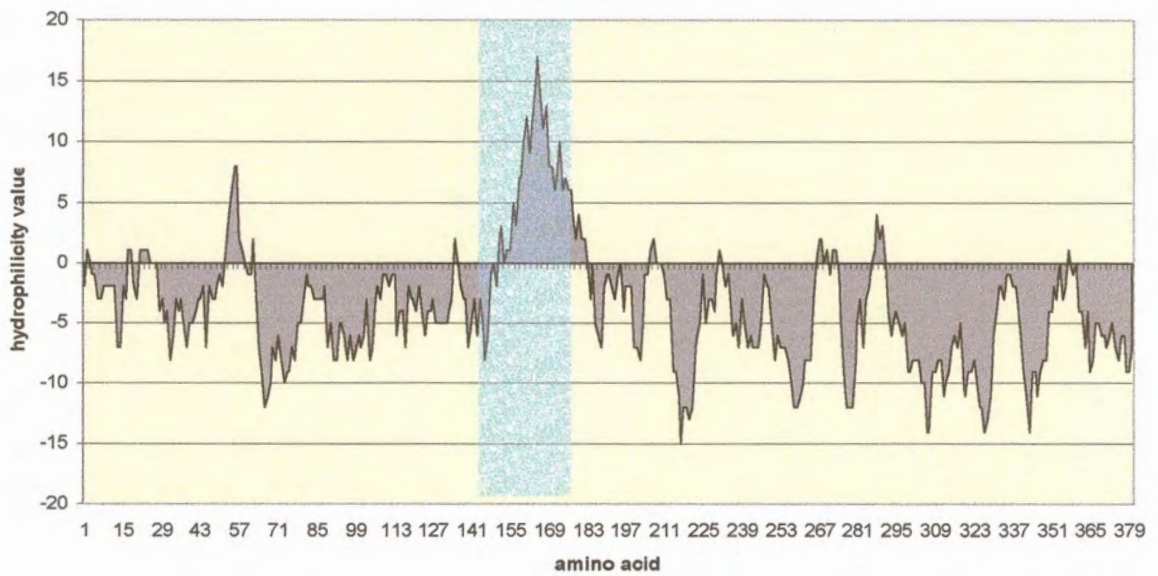


Figure 3.5. Hydrophilicity Plots of VP7mt144 (a) and VP7mt144-VP2 (b) according to the Hopp and Woods Predictive Method (Hopp and Woods, 1981; Hopp and Woods, 1983).

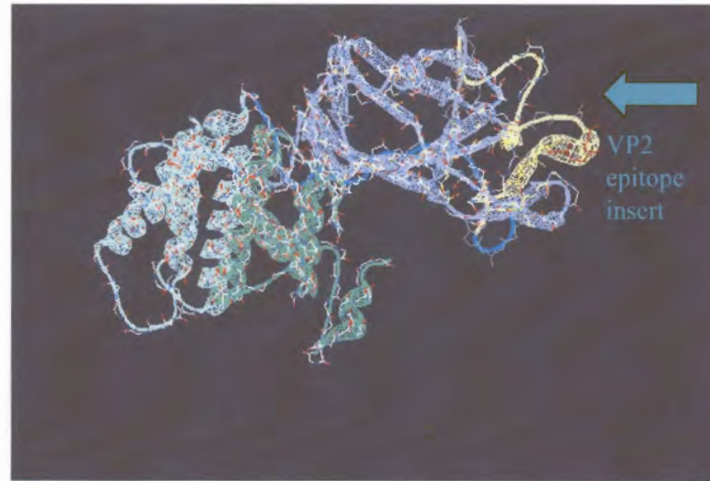


Figure 3.6. Structural Modelling of VP7mt144VP2. The bottom domain of α -helices is indicated in green. The top domain of β -sheets are indicated in blue. The VP2 insert is indicated in yellow.

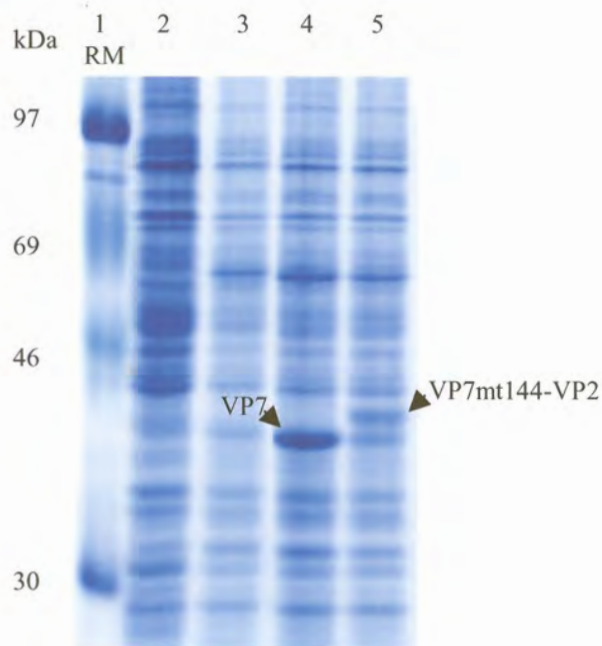


Figure 3.7 SDS-PAGE Analysis of Expression of VP7mt144-VP2. Lane 1 represents the protein size marker, lanes 2 and 3 show mock- and wild-type baculovirus-infected cell extract respectively. Lane 4 shows Bacmt144-infected cell extract. An arrow indicates the position of VP7mt144. Lane 5 shows Bacmt144-VP2-infected cell extract. An arrow indicates the position of VP7mt144-VP2.

3.3.4. Expression of VP7mt144-VP2.

The sequence analysis confirmed that the reading frame of VP7mt144-VP2 is intact and thus translation and expression of a full-length fusion protein is expected. The baculovirus expression system was used to express the modified protein, as described in chapter 2 (section 2.2.7). The recombinant baculovirus containing the modified gene was designated Bacmt144-VP2. Expression of the protein was analysed by SDS-PAGE (described in 2.2.8). The size difference of 25 amino acids in VP7mt144-VP2 compared to VP7mt144 would increase the size of the protein by approximately 3 kDa, which is clearly visible on an SDS-PAGE gel. As can be seen from figure 3.7, there is a significant size increase in VP7mt144-VP2 compared to VP7mt144. The level of expression of the protein is high, although does not appear to be as high as that of VP7mt144.

3.3.5. Light Microscope Observations.

The first indications of any particulate structures formed by the modified proteins can be seen in the recombinant baculovirus-infected cells under the light microscope. Similarly to VP7mt144 (section 2.3.5), cells infected with recombinant Bacmt144-VP2 baculovirus show distinct structures appearing as needle-like shapes in the two-dimensional field of vision of the light microscope. These structures are widespread, with the distribution being similar to that of VP7mt144. Often more than one structure is visible per cell. As for VP7mt144, fewer structures are visible in Bacmt144-VP2-infected cells compared to wild-type VP7-baculovirus infected cells. Light microscope observations are shown in figure 3.9., with photographs being taken at a 63X magnification.

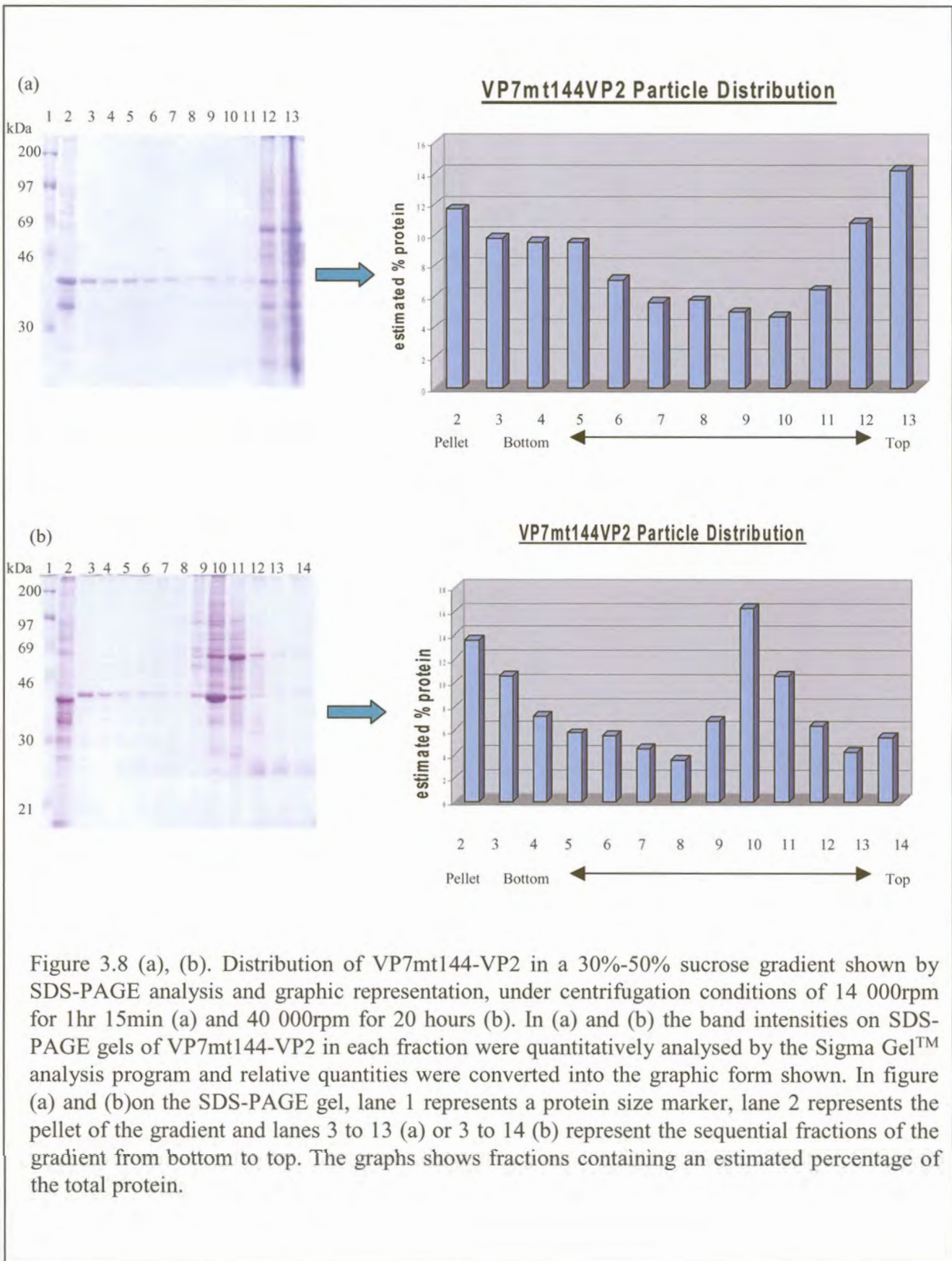
3.3.6. Solubility Studies of VP7mt144-VP2.

It was apparent under the light microscope that some form of particulate structure is still formed by the modified VP7mt144-VP2 proteins. The solubility study of VP7mt144-VP2 by sucrose gradient distribution gives more insight into the effect of the additional 25 amino acids on firstly trimer formation i.e. associations and interactions of individual proteins, and secondly on the particulate structure formation, involving interactions and associations of trimers.

The solubility and the particulate structure of VP7mt144-VP2 were investigated by its distribution in a discontinuous 30-50% sucrose gradient, as described by methods in chapter 2 (section 2.2.11). Figure 3.8. shows the distribution of VP7mt144-VP2 in the 30-50% sucrose gradient after centrifugation at 14000rpm for 1 hour 15 min (a) and increased conditions of 40000rpm for 20 hours (b). Both an SDS-PAGE analysis and a graphic representation of the distribution are shown. Under both centrifugation conditions, the distribution of VP7mt144-VP2 protein forms two peaks, clearly identified in the graphical representation. For the initial centrifugation conditions, at a lower speed and for a shorter time period, the peak occurring at the bottom of the gradient is shallow, with a significant proportion of protein found in the middle fractions. When one compares this to the distribution of VP7mt144 under the same conditions (figure 2.8.(b)), it appears that a slightly more uniform distribution of VP7mt144-VP2 protein occurs in fractions in the middle and towards the bottom of the gradient. This would indicate a greater distribution of varying sizes of particulate structures. The greatest proportion of particulate structures is still found in the pellet. Like VP7mt144, when centrifugation conditions were increased, the peak occurring in the top fractions shifted to lower down in the gradient. Similarly to VP7mt144, VP7mt144-VP2 sediments to a position in the gradient equivalent to proteins larger than itself, indicating that VP7mt144-VP2 occurring in this peak most likely exists in the form of trimers rather than a monomer of the protein.

3.3.7. Scanning Electron Microscopy Studies.

Samples of the VP7mt144-VP2 particulate structures were prepared in the same way as that of VP7mt144 and VP7mt144/200, described in chapter 2 (section 2.2.13). Structures observed under the S. E. M. varied in size and shape. The average length of structures was observed to be approximately 6 μ m. No distinct hexagonal structures characteristic of wild-type VP7 crystals were observed. Structures were distinctly angular but appeared more needle-shaped rather than the characteristic hexagonal-shape of wild-type VP7 crystals. Figure 3.10 shows structures observed under the S. E. M.



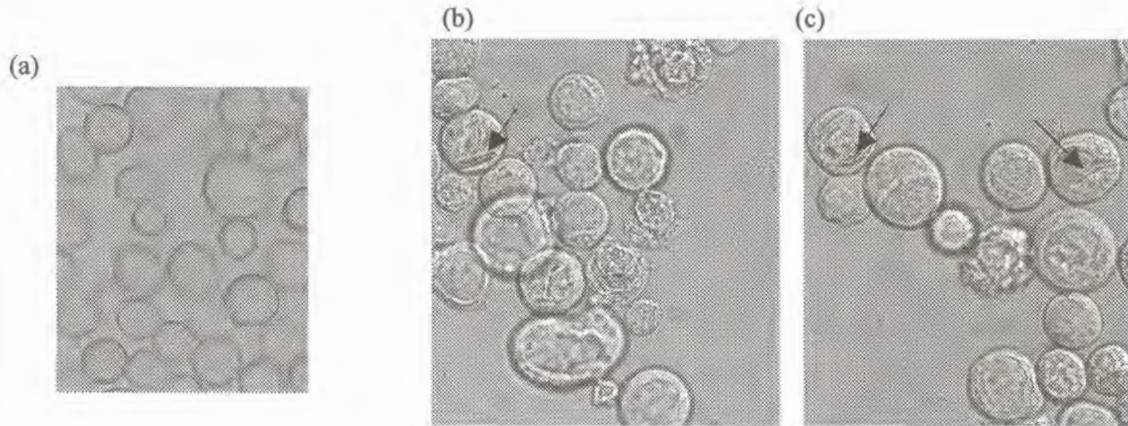


Figure 3.9 Light Microscope Observations of Recombinant VP7mt144VP2 baculovirus infected sf9 cells (b) and (c). The arrows indicate particulate structures visible within the cells, which are not visible in wild-type baculovirus infected cells (a). Photographs were taken at a 63X magnification.

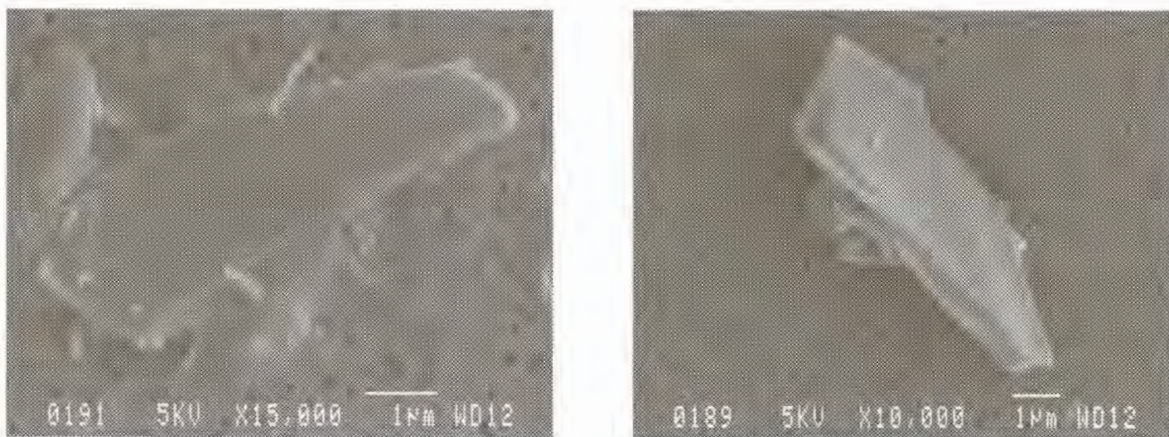


Figure 3.10. Scanning Electron Microscope Observations of VP7mt144VP2 Crystal Structures.

3.3.8. Immune Study of VP7mt144-VP2.

The ability of VP7mt144-VP2 to present the VP2 epitope to the immune system was studied by the ability of antiserum generated against the recombinant protein to detect and recognise native AHSV protein. As discussed previously, the particulate crystal structures as well as the constitutive trimers themselves may be suitable vehicles for the presentation of epitopes. Crystal particles of VP7 containing a different epitope were to be tested for their ability to generate an immune response by another colleague (Meyer, personal communication), and thus it was decided to test the constitutive trimers of VP7mt144-VP2 in this regard. This would provide a broader perspective on the whole in terms of the use of VP7 as an epitope delivery system.

The protein was prepared for inoculation as discussed in section 3.2.4.1. VP7mt144-VP2 was expressed on large-scale and harvested accordingly. Sucrose gradient centrifugation of cell lysate was used to separate trimers from the larger particulate structures of VP7mt144-VP2. Conditions chosen were those of a 30-50% sucrose gradient, spun at 40 000rpm for 20 hours. Fractions of the gradient identified to contain trimers were pooled. This would correspond to fractions 8, 9, and 10 shown in figure 3.8(b). The protein was collected from the sucrose solution by dilution of the sucrose and centrifugation to pellet the protein. The pellet was resuspended in sterile 1XPBS and adjusted to the appropriate concentration for inoculation into mice. This method of preparation of VP7mt144-VP2 produced a heterogenous mix of proteins and included significant proportions of baculovirus as well as sf9 cell proteins. If one looks at figure 3.8(b), one can see that the fractions of the gradient containing the VP7mt144-VP2 trimers also contain significant quantities of other proteins in comparison to other fractions containing the particulate structures of VP7mt144-VP2. This heterogenous mix might be expected to influence the presentation of the VP7mt144-VP2 trimers to the immune system of the mice.

The program for inoculation of mice is described in section 3.2.4.2. Three inoculations of 10µg of protein were given two weeks apart. The protein was prepared fresh each time to ensure minimal breakdown of the trimer structure in the sample. Serum was collected at day 42. The red blood cells were removed by centrifugation and the antiserum was stored at -70°C. The antiserum was tested for its ability to recognise native AHSV-9 proteins. This would give some indication to the antibodies generated against the protein used in

inoculation. With VP7 and VP2 sequences present in VP7mt144-VP2, the antiserum would be expected to recognise native VP7 in a Western blot, with the possibility of also recognising VP2 depending on how successfully the VP2 epitope is presented to the immune system.

AHSV-9- infected BHK cell extract (3.2.4.3.1) was separated on a 12% SDS-PAGE gel. Also included were an uninfected BHK cell extract as a negative control and a purified sample of VP7mt144-VP2 from Bacmt144-VP2-infected sf9 cells as a positive control. A western blot was performed as described in section 3.2.4.3.2. The prepared antiserum from the five mice was pooled and was used as the primary antibody at the higher end of the recommended concentration, at a dilution of 1/100. The result is shown in figure 3.11(a). No bands were visible in either mock-infected or AHSV-9 infected cell extracts. A single band was visible in the baculovirus-expressed VP7mt144-VP2 sample. This band corresponded to the size of VP7mt144-VP2. The response to the positive control indicated then that the antiserum was able to recognise the purified form of VP7mt144-VP2 but not the native AHSV-9 proteins expressed in BHK cells. The expression of these proteins in BHK cells was controlled by SDS-PAGE analysis of cell extract of AHSV-9-infected cells in comparison to that of uninfected BHK cells. A clear difference was visible in protein patterns on an SDS-PAGE gel. For confirmation of expression, a western blot analysis of the two samples was performed in which α -AHSV-6 antiserum was used as the primary antibody. VP7 of AHSV-6 shows cross-reactivity to VP7 of AHSV-9. The expression of AHSV-9 VP7 was confirmed by the appearance of a band corresponding to the expected size of VP7 (results not shown).

It was considered that the band detected by the antiserum might not be representative of VP7mt144-VP2, but rather another protein of similar size expressed in the baculovirus expression system. This would explain the presence of this band in cell extract of baculovirus origin and not the AHSV-9 infected BHK cells. A western blot analysis was thus performed directing the prepared antiserum to Bacmt144-VP2, Bacmt144, wild-type baculovirus and mock-infected sf9 cell extract. The result is shown in figure 3.11(b). Various proteins are detected in the Bacmt144-VP2-infected sample, which are also present in mock- or wild-type baculovirus-infected samples. A very faint band is visible in both Bacmt144-VP2- and Bacmt144-infected samples. It corresponds to the size of proteins VP7mt144-VP2 and the smaller VP7mt144 in the respective samples. Thus the antibodies

generated in the mice only weakly recognised the primary protein intended to be presented to the immune system in mice. The more dominant antibodies in the antiserum recognise wild-type baculovirus and sf9 insect cell proteins. This would affect a positive outcome of a western blot analysis.

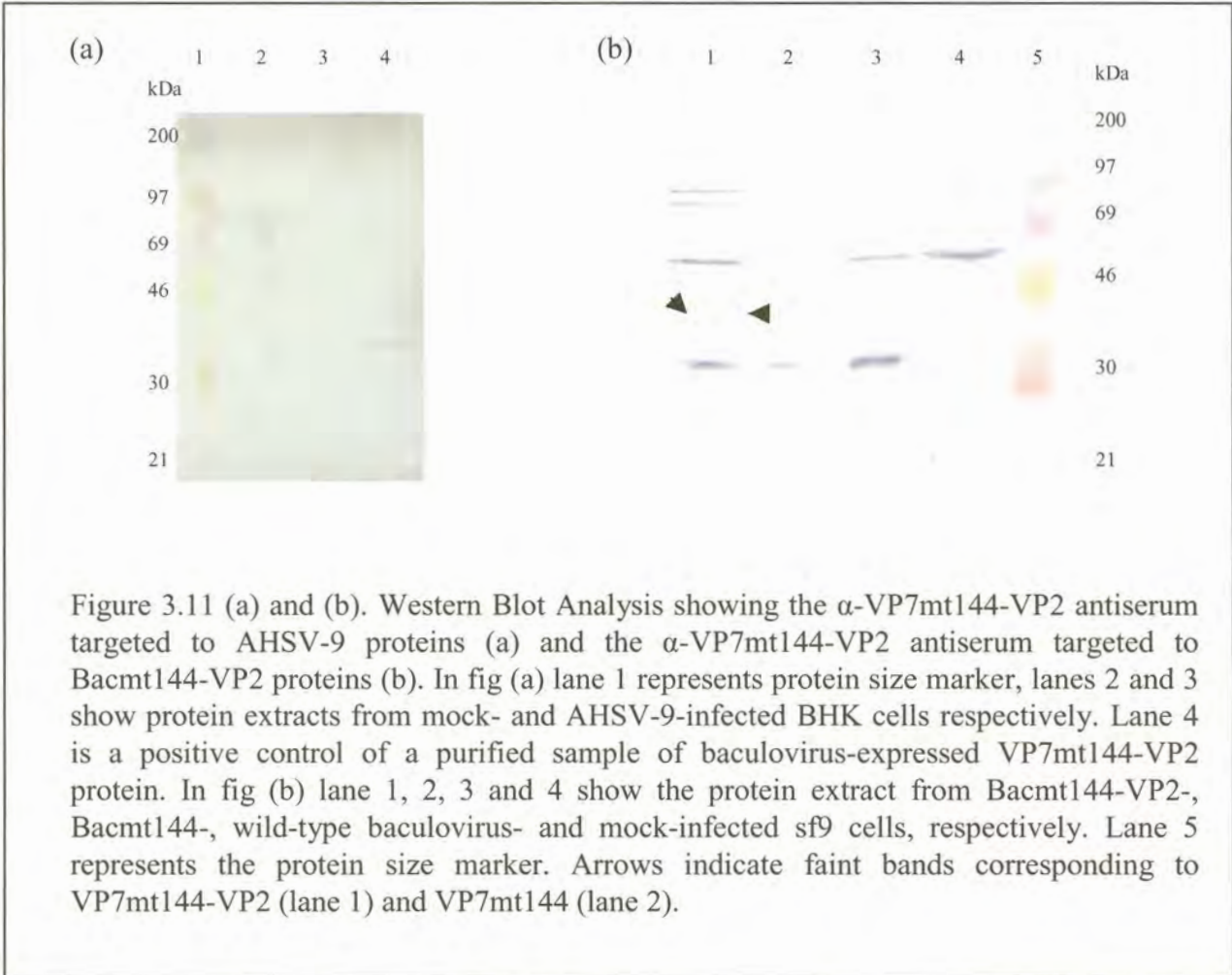
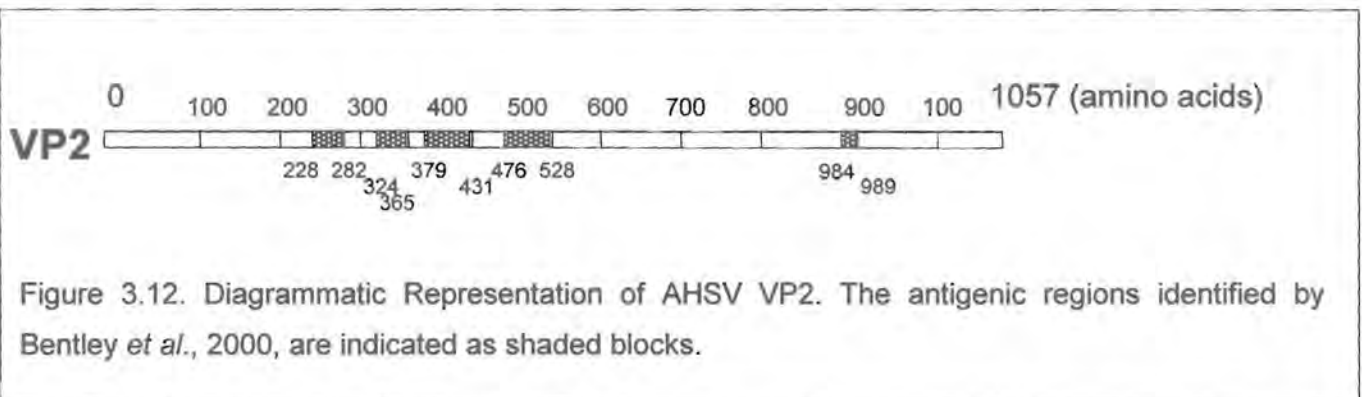


Figure 3.11 (a) and (b). Western Blot Analysis showing the α -VP7mt144-VP2 antiserum targeted to AHSV-9 proteins (a) and the α -VP7mt144-VP2 antiserum targeted to Bacmt144-VP2 proteins (b). In fig (a) lane 1 represents protein size marker, lanes 2 and 3 show protein extracts from mock- and AHSV-9-infected BHK cells respectively. Lane 4 is a positive control of a purified sample of baculovirus-expressed VP7mt144-VP2 protein. In fig (b) lane 1, 2, 3 and 4 show the protein extract from Bacmt144-VP2-, Bacmt144-, wild-type baculovirus- and mock-infected sf9 cells, respectively. Lane 5 represents the protein size marker. Arrows indicate faint bands corresponding to VP7mt144-VP2 (lane 1) and VP7mt144 (lane 2).

3.4. DISCUSSION.

In chapter 2, it was shown that the insertion of 6 amino acids at site 144/145 of VP7 did not abolish the formation of large particulate structures when expressed in insect cells. This supported the requirement for a robust nature of VP7 structures in the display of foreign epitopes. The direction of the investigation as described in this chapter was two-fold. Firstly, the extension of the size of possible inserts at this site, which would similarly not abolish the formation of particulate structures, was investigated. Secondly, the presentation of such an insert to an immune system to generate specific antibodies was investigated. Venter *et al.*, 2000, identified a strong linear epitope between amino acids 369 and 407 of AHSV-9 VP2. Bentley *et al.*, 2000, supported this region as having strong neutralising activity by the identification of residues 379-413 of AHSV-3 VP2 as containing neutralisation epitopes. Figure 3.12. shows antigenic regions of AHSV-3 VP2 identified by Bentley *et al.*, 2000. Furthermore, Martínez-Torrecuadrada *et al.*, 2001, identified a region of amino acids 377-400 of AHSV-4 VP2 which has the capability of inducing neutralising antibodies. A sequence of 25 amino acids from amino acids 377-401 of AHSV-9 VP2 was chosen for insertion into VP7mt144, and VP7mt144-VP2 was created. Similarly to chapter 2, the insertion was firstly investigated in terms of its effect on VP7 solubility and structure.



Structural modelling of VP7mt144-VP2 predicts the large hydrophilic insert of VP2 to occur as a large loop on the surface of the top domain of VP7. It does not appear to interfere greatly with the folding of VP7 and the top and bottom domains are distinctly intact. The structural modelling provides no indication to the effect of the insert on VP7 intermolecular interactions to form trimers and larger particulate structures, nor does it provide any

indication to how the VP2 epitope is presented in such associations. Light microscope observations show the presence of particulate structures of VP7mt144-VP2 in Bacmt144-VP2-infected cells. This indicates that the insert did not abolish the interactions of individual VP7 to form trimers and subsequently associate into larger particulate structures.

Smaller influences affecting the structural features of VP7 were studied by sedimentation analysis. This showed similar results to VP7mt144 in terms of its effect on redistribution of the size and proportion of particulate structures in comparison to VP7. Similarly to VP7mt144, a significant proportion of protein does not associate and accumulate into particulate structures, but rather remains associated in the trimer form. This is shown in figure 3.8 (b), with the strongest band of VP7mt144-VP2 occurring in fraction 9, a position in the gradient representing the sedimentation position of trimers. There is an observed difference in the size distribution of protein that does associate into particulate structures, with a more uniform distribution of particulate sizes occurring for VP7mt144-VP2 (figure 3.8(a)) compared to VP7mt144 (figure 2.8 (b)). The greater proportion of smaller particulate structures of VP7mt144-VP2 compared to VP7mt144 indicates, as described in chapter 2, weaker hydrophobic associations between the constitutive trimers in forming and stabilising larger particulate structures. The insertion of the 25 amino acid sequence therefore does not appear to influence the association of VP7mt144-VP2 into trimers, but does appear to influence the association of trimers into stable larger particulate crystal structures.

Sequences longer than 25 amino acids have been inserted into VP7 with similar conclusions being drawn. A 48 amino acid epitope of Hepatitis B virus (HBV) was incorporated into the amino terminus of BTV VP7. This did not abolish the trimer structure as the chimeric VP7 was incorporated into CLP's when co-expressed with VP3 and wild-type VP7. The insertion did, however, influence the interaction between trimers and VP3 in the core, indicated by the necessity for the presence of wild-type VP7 to stabilise interactions required for core formation (Belyaev and Roy, 1992). Larger inserts of 105 amino acids have been introduced to sites 177 and 200 of AHSV VP7, as reported by Maree (2000). Similarly, insertions did not affect the formation of trimers, as particulate structures were formed. However, trimer-trimer interactions were affected, as the structures were not of the characteristic shape and size of wild-type VP7 particles. A size limitation on inserts that do not disturb the formation of trimers has not been established.

As discussed previously, the observed changes in the size distribution of particulate structures does not detract from the potential of VP7 crystals to be used as an epitope display vehicle. Smaller particulate structures would provide a sufficient surface area for the display of multiple epitopes to the immune system. Larger particulate structures carrying epitopes may not be a viable option. The reduced stability of the larger structures due to weakened hydrophobic interactions between trimers may not permit the maintenance of the larger structure during the various stages of preparation and administration of a vaccine.

The results of the S.E.M. showed the particulate structures formed by VP7mt144-VP2 as not having the distinct hexagonal structure apparent for that of wild-type VP7, as well as VP7mt200 (Maree, 2000). This may be due to breakdown of the hexagonal shape during preparation of the samples for S.E.M. This is a feasible explanation, particularly when the stability of the larger particulate structures of VP7mt144-VP2 is questioned. When BTV VP7 crystals were grown by Basak *et al.*, 1992, different forms of crystal were obtained by trimeric associations. These included the hexagonal rod-shapes, rectangular plate-like shapes and diamond-shaped plates. Thus, it is possible for VP7 to associate into larger particulate structures taking different forms. It may be possible that the more needle-shaped structures observed for VP7mt144-VP2 are not a result of degradation of hexagonal crystals, but rather the primary form taken by the association of trimers of VP7mt144-VP2.

The structural studies of the effect of the 25 amino acid insert on VP7mt144 supported its potential as a scaffold for epitope presentation in terms of the physical structures it forms. The second important aspect for consideration is how inserted epitopes would be presented in these structures. Other particulate structures, such as the hepatitis B core antigen, have been successful in the presentation of epitopes to the immune system, inducing protective immune responses (Yon *et al.*, 1992; Schodel *et al.*, 1994; Milich *et al.*, 1995; Schodel *et al.*, 1996; Fehr *et al.*, 1998; Milich *et al.*, 2001). Whole virus particles have also shown potential as vaccine carrier systems, such as Japanese encephalitis virus (Konishi *et al.*, 1997; Hunt *et al.*, 2001), the murine leukemia virus (Kayman *et al.*, 1999) and the cowpea mosaic virus (Brennan *et al.*, 2001). Little is known of the potential of smaller composite structures such the VP7 trimer for use as an epitope display system.

The trimer structure of the VP7mt144-VP2 construct was specifically chosen to present to the immune system of mice. As discussed previously, in a similar investigation by a

colleague (Meyer, personal communication), particulate crystal structures were chosen as the vehicle for presentation of an epitope. Thus, the choice of trimers sought to provide a broader indication as to the potential of the various possible presentation systems of modified VP7. With indications of a reduced stability of particulate structures of VP7mt144-VP2, the trimers may provide an alternative system for epitope delivery. To investigate this, antibodies were generated against VP7mt144-VP2 trimers. Unfortunately, results were inconclusive. The western blot analysis of the prepared antiserum directed toward Bacmt144-VP2-infected cell extract showed only a faint detection of VP7mt144-VP2. When one considers the high level of expression of VP7mt144-VP2 in recombinant baculovirus-infected cells, the faint detection found in the western blot indicates a very poor immune response in the mice to VP7mt144-VP2. This may be due to the lack of purity of the sample used in inoculations. As described in section 3.3.8., when isolating the trimer form of the protein, a large proportion of the baculovirus and sf9 cell protein is simultaneously recovered. This contaminating protein must influence the effective presentation of the VP7mt144-VP2 protein as a whole to the immune system. The dominant antibodies in the antiserum are directed toward wild-type baculovirus and sf9 cell proteins (shown in figure 3.11.(b)). The fact that antiserum from five mice was pooled may also account to some extent for the poor detection of protein on the western blot. The variable immune responses from the five mice may have diluted α -VP7mt144-VP2 antibodies from better immune responses when the antiserum was pooled.

Another explanation for the weak immune response to VP7mt144-VP2 may be the form of the protein used in inoculation i.e. the trimer. The trimer may be a less effective form for exposure of the protein to the immune system, in comparison perhaps to larger crystal structures. Wade-Evans *et al.*, 1998, suggested that the conformation and assembly of VP7 into crystals was an important factor in the mechanism of the protection induced in mice, protecting against a heterologous serotype challenge. Results of a similar investigation, using modified VP7 crystals as the vehicle to present epitopes to the immune system (Meyer, personal communication), showed the antiserum to strongly detect VP7 constructs. Also, antiserum generated against VP7-NS3 crystals in rabbits was able to detect VP7 epitopes by western blot analysis (Meiring, 2001). Thus, the trimer structure may have been a significant factor in the weak response shown by the antiserum to VP7mt144-VP2.

Due to the poor immune response, the conditions used to test the ability of the antiserum to detect native AHSV-9 proteins produced by BHK cell infection did not produce a conclusive result. It cannot be deduced that the antiserum does not recognise VP7 or VP2 produced by AHSV-9 infection. The western blot analysis used was not of sufficient sensitivity to show informative, conclusive results. Another shortcoming of the western blot, which may be considered, is that it inherently involves a denaturing process. The conformation of the AHSV VP7 or VP2 protein is not preserved and thus antibodies would be required to recognise denatured epitopes in the SDS-PAGE gel. This may have been a factor in the negative result observed, although this has not played a role in the detection of α -VP7 antibody in previous experiments (Meyer, personal communication; Meiring, 2001).

More sensitive methods for the investigation of the antiserum would be required to determine if the VP2 epitope was efficiently presented in such a way to generate antibodies to the VP2 insert. Two methods with a greater sensitivity used in antibody detection are the ELISA and the virus neutralisation assay. In many cases the ELISA has been shown to be more sensitive and less cumbersome than a virus neutralisation assay which uses whole virus particles to detect the presence of neutralising antibodies (Paweska *et al.*, 1997; Wilson and Morgan, 1998; Pratelli *et al.*, 2002). However, in some cases ELISA does not detect antibodies in antiserum that tested positive by a virus neutralisation assay (Dixon and De Groot, 1992). This may be due to the fact that antibodies against different viral epitopes will react in the two different methods. With this in mind, and also considering the problem of insolubility of baculovirus-expressed VP2 (Du Plessis *et al.*, 1998) that would affect its preparation for use in an ELISA, the next approach for testing the neutralising immune response generated by the VP2 epitope would be the virus neutralisation assay. This is a more conclusive approach as it tests the ability of antiserum to recognise the native virus and would ultimately test the success of the trimeric construct in generating neutralising antibodies. The virus neutralisation assay will thus be a focus of future investigations.

CHAPTER 4

CONCLUDING REMARKS

VP7 particulate structures have shown potential as a vaccine delivery system. They are easily synthesised in large quantities by expression in recombinant baculovirus-infected cells (Chuma *et al.*, 1992; Maree *et al.*, 1998a; Maree *et al.*, 1998b). The crystal structure has been shown to be robust, with the insertion of foreign sequences at sites 177 and 200 not affecting the formation of crystals (Maree, 2000; Meiring, 2001). Furthermore, VP7 crystals have shown an inherent ability to generate a protective immune response in mice (Wade-Evans *et al.*, 1997; Wade-Evans *et al.*, 1998). These findings justified further investigation into the capacity of VP7 to display foreign peptides on its surface. To expand the range of sites available for the insertion of peptides from sites 177 and 200 (Maree, 2000), a cloning site between amino acid positions 144 and 145 was created. This was investigated in terms of its potential as a site for epitope display.

In summary of the findings of this study, the creation of a cloning site and resulting introduction of six amino acids at site 144/145 of VP7 did not abolish either the formation of crystal particulate structures or the trimer unit. The modification did affect the solubility of the protein. A relatively small increase in trimers, in proportion to the larger particulate structures, was noted. The change in solubility saw a more pronounced increase in proportion of smaller particulate structures in comparison to the relatively larger structures of wild-type VP7. The insertion of a larger sequence of 25 amino acids at this site similarly did not abolish the formation of crystal particulate structures or trimer associations. The observation of a wider distribution of smaller particulate structures of VP7mt144-VP2 presents indications that the insert may affect the stability of interactions in the formation of larger particulate structures. This may be a factor for consideration in the utility of the large crystal structure of VP7 as an antigen delivery system. In terms of addressing the size limits of epitopes able to be accommodated in VP7, the 25 amino acid insert does not significantly affect the structural features of VP7 to reject its potential function as an epitope delivery system.

The efficiency of the presentation of the chosen neutralising epitope of VP2 to the immune system at site 144/145 could not be determined in this study. Various factors may have influenced this outcome. The poor nature of the immune response to VP7mt144-VP2 as a whole, generating relatively low proportions of anti-VP7mt144-VP2 specific antibodies in the antiserum, was a strong contributing factor. This may be due to the inherent inefficiency of the individual trimer units of the protein to generate an immune response in comparison to the larger particulate structures. Also, the presence of relatively large proportions of contaminating baculovirus and sf9 cellular protein may have strongly influenced the effective presentation of VP7mt144-VP2 to the immune system. A second consideration regarding the inconclusive result is the analysis used for the detection of antibodies in the antiserum. The nature of the western blot method uses denatured protein for the capture of antibody in the antiserum. Although the VP2 insert was identified as a linear epitope (Venter *et al.*, 2000, Bentley *et al.*, 2000; Martinez-Torrecuadrada *et al.*, 2001), and thus its recognition should not be affected by the denaturation of the protein, recognition of VP7 may have been affected by its denaturation. A more sensitive and more accurate approach would be required to test the presence of neutralising antibodies in the antiserum. A virus neutralisation assay is a more conclusive approach as it tests the ability of antiserum to recognise the native virus, and would ultimately test the success of the trimeric construct in generating neutralising antibodies against the VP2 epitope. A positive result would indicate the efficient exposure of the VP2 epitope on the surface of VP7mt144-VP2. This is an essential criterion if site 144/145 is to be used as a site for the display of epitopes in the VP7 particulate structure vaccine system. Future study should be targeted at this aim.

Other future investigations should be directed down three main avenues:

- The flexibility of VP7 in accommodation of different size epitopes should be investigated. A size limit of epitopes, larger than 25 amino acids, should be established. Also the minimum size of epitopes for efficient recognition by the immune system should be established. This will depend on how well various sites present the epitopes to the immune system.
- The varying capabilities of the different cloning sites in modified VP7 in terms of accommodation and display of epitopes should be investigated. It would be expected that different sites would not have the same limitations in terms of size of inserts. The efficiency of the display of epitopes within the composite structures of VP7 would vary at different sites.

- Another relevant area of investigation would be to consider the efficiency in the generation of a suitable protective immune response by trimer units in comparison to the larger particulate crystal structures.

Further investigation in these areas will provide a strong foundation for the development of the potential of the VP7 vaccine delivery system.