IMPROVED METHODS FOR
PRODUCTION AND CHARACTERISATION OF
JEMBRANA DISEASE VIRUS PROTEINS

This thesis is presented for the degree of
Doctor of Philosophy of Murdoch University

Judhi Rachmat

2010
DECLARATION

I declare that this is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution

......................................
Judhi Rachmat
Abstract

Jembrana disease is an acute disease of Bali cattle (Bos javanicus) in Indonesia caused by Jembrana disease virus (JDV), a lentivirus most closely related to Bovine immunodeficiency virus. Control of the disease in Bali cattle, which are important to the economy of Indonesia, is dependent on the continued availability of protein antigens for immunosurveillance procedures that have been developed. Further investigation is also required to characterise of the proteins of JDV and to provide methods of producing commercial quantities of recombinant proteins for vaccine manufacture.

A problem with the large scale production of viral proteins using recombinant technology was that the proteins have been mainly produced as insoluble products within inclusion bodies in bacterial cells and in that insoluble format they were unsuitable for use as antigens. A method for solubilisation of the insoluble proteins was developed that involved solubilisation of the inclusion bodies with low concentrations of urea in an alkaline solution and the method could be performed easily and at low cost without any detectable loss of antigenicity. The solubilised protein was successfully renatured without the formation of aggregates by dilution of the urea in the presence of the reducing agent dithiothreitol. The method would be suitable for use during the large scale production of recombinant viral proteins for vaccine manufacture.

To provide an additional reagent for diagnosis of the disease, mice were immunised with the recombinant capsid (CA) protein of JDV and a hybridoma was produced that secreted monoclonal antibodies reactive with the CA protein. This monoclonal antibody was effectively used in an immunoperoxidase assay to demonstrate virus in tissues. As an alternative to this technology, recombinant antibody fragments, scFv, reactive with the CA protein of JDV, were also produced by phage display technology. These scFv were expressed as soluble products in the periplasmic space of transfected host bacterial cells. The scFv reacted specifically with the CA protein in western immunoblots and although further optimisation of the methods of production of this scFv are required, the reagents developed can be for expression of the antibody when required, without the need for maintaining liquid nitrogen storage facilities that are necessary for storage of hybridomas.

The size and nature of the glycosylation of the envelope proteins SU and TM of JDV harvested from infected cattle was determined. Two proteins of 75 and 60 kDa were initially identified in SDS-PAGE as the SU and TM, respectively. They were initially
identified using a specific glycoprotein stain and then matrix-assisted laser
desorption/ionisation-time of flight (MALDI-TOF) mass spectrometric (MS) analysis
of the protein sequence. Further investigation on the unmatched mass value from
MALDI-TOF/MS data suggested that post-translational glycosylation occurred on N-
linked glycosylation sites of the JDV-SU and on O-linked glycosylation sites of the
JDV-TM. This is the first report of the characteristics of the envelope proteins of JDV
and their identification will facilitate further studies of the nature of the immune
response to JDV infection using immunoblotting procedures.
Acknowledgements

There are many people to thank. Without the help and understanding from the people around me, the way to this thesis would have been impossible.

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This page would not be complete without mentioning the great love and support I have received from my parents, my brothers and my sisters. My debt to them is truly without bound and is one that can never be repaid. I can only offer them all the thanks and love that a son and a brother can give. Finally, this work is also dedicated to my beautiful daughter, Anindya Aaqila Nurjannah Raisyaputri. May God bless my daughter with wonderful life.
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List of abbreviations

Viruses

BIV : Bovine immunodeficiency virus
BHV : Bovine herpesvirus
BPV : Bovine papillomavirus
CAEV : Caprine arthritis-encephalitis virus
EIAV : Equine infectious anaemia virus
FIV : Feline immunodeficiency virus
HIV : Human immunodeficiency virus
JDV : Jembrana disease virus
MVV : Maedi-visna virus
SIV : Simian immunodeficiency virus

Reagents

Amp : ampicillin
DAB : 3,3' diaminobenzidine tetrahydrochloride
DMEM : Dulbecco’s modified Eagle’s medium
DMSO : dimethyl sulfoxide
DTT : dithiothreitol
EDTA : ethylenediamine tetra-acetic acid
FCS : foetal calf serum
HAT : hypoxanthine-aminopterin-thymidine
HT : hypoxanthine-thymidine
IPTG : isopropylthiogalactoside
LB : Luria-Bertani
PBS : phosphate buffered saline
PEG : polyethylene glycol
PMSF : phenylmethylsulfonyl fluoride
HRP : horseradish peroxidase
SDS : sodium dodecylsulfate
TBS : tris-buffered saline
TEMED : N,N,N',N'-tetramethylene-ethylenediamine
Tris : tris (hydroxymethyl) aminoethane

Other

aa : amino acid
Ag : antigen
bp : base pairs
CA : capsid
CDR : complementary determining region
cfu : colony forming units
DNA : deoxyribonucleic acid
dpi : day post inoculation
ELISA : enzyme-linked immunosorbent assay
Fab : fragment antibody
GST : glutathione transferase
HRP : horseradish peroxidase
IN : integrase
ISH : in situ hybridisation
LTR : long terminal repeat
MA : matrix
MAb : monoclonal antibody
MALDI-TOF : matrix-assisted laser desorption/ionisation – time of flight
MS : mass spectrometry
NC : nucleocapsid
PAGE : polyacrylamide gel electrophoresis
PCR : polymerase chain reactions
PR : protease
RNA : ribonucleic acid
RT : reverse transcriptase
RT-PCR : reverse transcription-PCR
scFv : single chain variable fragment
SDS : sodium dodecyl sulfate
SU : surface unit
TM : transmembrane glycoprotein
VH : heavy chain variable domain
VL : light chain variable domain
Chapter 1

General introduction

Cattle in Indonesia, and especially Bali cattle (*Bos javanicus*), contribute to poverty reduction by improving efficacy in integrated farming systems. They are used as draught animals for rice production, for meat production, for grazing on land otherwise not used, and via manure contribute to soil fertility. The utilisation of cattle as draft power enables smallholder farmers to double food production from their available land. While Bali cattle have a number of advantages under Indonesian conditions, and have been widely distributed throughout Indonesia, they have disadvantages. A major disadvantage is their unique susceptibility to Jembrana disease and this disease is therefore a major threat to the success of the various Bali cattle distribution programs and consequently to the attempts to increase food production in Indonesia.

The discovery that Jembrana disease is caused by *Jembrana disease virus* (JDV) a lentivirus most closely related to *Bovine immunodeficiency virus* has led to the development of a variety of diagnostic reagents and a tissue-derived vaccine that in turn have led to better control of the disease. Molecular biological methods offer extensive promise for improvement in the initial diagnostic reagents and vaccines that were developed. The aims of the research reported in this thesis were to improve the methods developed previously for the production of recombinant proteins for antigens and for vaccine production, the development of monoclonal antibody (MAb) reagents for diagnostic techniques, and further characterisation of the envelope proteins of JDV that would enable enhanced diagnostic methods. As a background to these investigations, a review of Jembrana disease and JDV, and a review of literature related to the production of recombinant proteins, MAb and post-translational glycosylation of viral proteins was undertaken and is incorporated in Chapter 2.

Recombinant proteins of JDV have been produced for many years with a bacterial expression system but a major problem during their production by this system has been their production as insoluble proteins within inclusion bodies, and there has been minimal production of soluble protein, resulting in poor yields of protein. To increase yields, a method of solubilisation of the insoluble recombinant surface unit (SU) and capsid (CA) proteins of JDV was investigated, which would potentially increase the yield of these and other proteins produced by this expression system.
and enable their use as antigens in immunological assays and as experimental vaccines. The results of this investigation are reported in Chapter 3.

A MAb against the CA protein of JDV has been produced previously and this has provided extremely useful reagents for the development of diagnostic assays for the detection of JDV. Unfortunately, maintenance of the hybridomas in Indonesia has been difficult due to lapses in the supply of liquid nitrogen and the hybridoma was lost. It was also hypothesised that additional MAbs against the envelope glycoproteins (SU and TM) could provide a means of identifying the size of the glycosylated SU and TM glycoproteins of JDV in SDS-PAGE gels. Attempts were therefore made to produce additional MAb by conventional hybridoma technology and these results are reported in Chapter 4. Recent developments in combinatorial antibody libraries combined with the display of functional antibody fragments at the tips of filamentous phage, known as phage display system, allows direct selection of highly specific MAbs from naive combinatorial antibody libraries. It was hypothesised that this technology would lead to the selection of stable clones that could be used to express MAbs in a bacterial expression system and overcome problems of long-term storage of hybridomas. Attempts were therefore made to produce recombinant antibodies against the SU and CA protein of JDV using the Tomlinson I and J libraries, and these investigations are described in Chapter 5.

Successful production of MAbs against the CA proteins was achieved but was not successful against the envelope proteins of JDV. Therefore, to identify these proteins in native JDV preparations, an attempt was made to characterise 2 glycosylated proteins present in JDV by mass spectrometric analysis and these results are described in Chapter 6. Additional analysis of the mass spectrometric data for 2 glycosylated proteins of 75 and 60 kDa, that were identified as SU and TM, were undertaken to determine the nature of their polysaccharide moieties and these results are also reported in Chapter 6.

A general discussion of the research results reported in the thesis and recommendations for future research related to these results are presented in Chapter 7.
Chapter 2

Review of the literature

This Chapter contains a review of the literature relevant to the research undertaken and reported in this thesis, which concerns the development of reagents for the diagnosis and control of Jembrana disease and further characterisation of the envelope glycoproteins of the bovine lentivirus, *Jembrana disease virus* (JDV). This review is arranged into several sections: an initial section providing background information on the family *Retroviridae* (retroviruses); a section on the bovine lentiviruses including JDV and *Bovine immunodeficiency virus* (BIV); a section describing technical aspects of the production of soluble recombinant viral proteins; a section describing the technical aspects of the production of monoclonal antibody using conventional and recombinant techniques.

2.1 Characteristics of the *Retroviridae*

The family *Retroviridae* comprises a large group of viruses that have been detected in many vertebrate species. They have shared structural and genomic features and similar modes of reproduction but vary considerably in the type of disease with which they are associated.

All retroviruses have a genome consisting of 2 identical strands of single-stranded RNA enclosed within a protein coat (capsid) which is again enclosed by a lipid envelope. The viral envelope is formed from the plasma membrane of the cell as the virus is released from the cell and it contains virus-encoded glycoproteins important in attachment and penetration of the virus into the cell (Temin & Mizutani, 1970).

The retroviruses are unique in that their replication requires a reversal of the normal flow of genetic information. In all living organisms and many viruses, genetic information is stored as DNA and later transcribed into RNA, which serves as a template for protein synthesis. In contrast, retroviruses store their genetic information as RNA and they also contain the unique enzyme, reverse transcriptase (RT) that catalyses the reverse transcription of the RNA genome into DNA. All retroviruses contain 3 major open reading frames designated, from the 5'– to 3'-ends of the genome, *gag*, *pol* and *env* genes that are translated as polyproteins:
gag encoding the core proteins, which include the matrix (MA), the capsid (CA) and the nucleocapsid (NC), pol encoding the enzymatic proteins RT, integrase (IN) and protease (PR), and env encoding the envelope surface unit (SU) and transmembrane (TM) glycoproteins. The SU contains the determinants that interact with the host cell receptor and coreceptor, while TM not only anchors the SU/TM complex in the membrane but also contains domains that are critical for catalysing the membrane fusion reaction between viral and host lipid bilayers during virus entry. Human immunodeficiency virus (HIV) is one of the members of this family and a detailed understanding of the molecular biology of HIV is available in the review by Wang et al. (2000).

The replication of retroviruses is divisible into early and late phases (Turner & Summers, 1999) as depicted in Figure 2.1. Retroviruses recognise potential host cells through specific interactions between a host cell receptor and the viral envelope membrane glycoprotein SU. The main receptor for HIV-1 and HIV-2 is CD4 on T lymphocytes whilst the main co-receptors are the α-chemokine CXCR4 and the β-chemokine CCR5 (Dragic et al., 1996). After binding, fusion of the viral envelope with the cellular membrane occurs directly at the cell surface releasing the virus core into the cytoplasm of the cell (early phase). In the late phase of replication, the viral genome is reverse transcribed, via the action of RT, into double-stranded DNA and integrated into the genome of the host cell where it resides permanently as the "provirus". Viral protein is expressed from spliced messenger RNA transcripts of the proviral DNA and some such as SU and TM require post-translational glycosylation; these glycoproteins are incorporated into regions within the plasma membrane where the viral RNA genome is packed into capsids that bud from the membrane along with the viral envelope proteins. The cell-free particles undergo a further maturation process before they are capable of productive infection in appropriate target cells.
Figure 2.1. Schematic diagram illustrating the features of the retrovirus replication cycle. Following recognition of a specific cellular receptor by the viral envelope glycoprotein and adsorption of the virion to the cell surface, the viral core is released into the cell cytoplasm. The viral RNA is uncoated and reverse transcribed into a double-stranded DNA. The integrated provirus uses a combination of viral and cellular transcription factors to replicate new copies of its RNA genome. Some of these viral RNAs are exported to the cytoplasm to serve as new viral genomes, however a percentage of the viral RNA is spliced into smaller mRNA species that are translated by the host cell ribosomal machinery. The viral regulatory proteins Tat and Rev tightly control transcription and transport of viral mRNA to the cell cytoplasm and both proteins are essential for retrovirus replication. Figure from Turner & Summers (1999).
**Taxonomy**

The retroviruses are subdivided into 7 genera: *Alpharetrovirus*, *Betaretrovirus*, *Gammaretrovirus*, *Deltaretrovirus*, *Epsilonretrovirus*, *Spumavirus* and *Lentivirus* (Table 2.1). Previously, these viruses were grouped according to the nature of the diseases they produced and their electron microscopic appearance within infected cells and the core in the mature virus: concentric nucleocapsids were attributed to alpharetroviruses, gammaretroviruses, deltaretroviruses and spumaviruses; a rod or truncated cone-shaped core was characteristic of lentiviruses and betaretroviruses.

Table 2.1. Genera in the family *Retroviridae* and their principal hosts and associated disease.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Type species</th>
<th>Hosts</th>
<th>Genome of type species</th>
<th>Pathological features of disease</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alpharetrovirus</strong></td>
<td>Avian leukosis virus (ALV)</td>
<td>Avian</td>
<td>7200 bp</td>
<td>Oncogenic</td>
<td>(Venugopal, 1999)</td>
</tr>
<tr>
<td><strong>Betaretrovirus</strong></td>
<td>Mouse mammary tumor virus (MMTV)</td>
<td>Mammalian</td>
<td>8805 bp</td>
<td>Oncogenic</td>
<td>(Cardiff &amp; Wellings, 1999)</td>
</tr>
<tr>
<td><strong>Gammaretrovirus</strong></td>
<td>Murine leukemia virus (MLV)</td>
<td>Mammalian</td>
<td>8256 bp</td>
<td>Oncogenic</td>
<td>(Chesterman et al., 1966)</td>
</tr>
<tr>
<td><strong>Deltaretrovirus</strong></td>
<td>Bovine leukemia virus (BLV)</td>
<td>Mammalian</td>
<td>8000 bp</td>
<td>Oncogenic</td>
<td>(Van der Maaten et al., 1982, Willems et al., 2000)</td>
</tr>
<tr>
<td><strong>Epsilonretrovirus</strong></td>
<td>Walleye dermal sarcoma virus (WDSV)</td>
<td>Fish</td>
<td>12700 bp</td>
<td>Oncogenic</td>
<td>(Holzschu et al., 2003, Holzschu et al., 1995, Zhang et al., 1996)</td>
</tr>
<tr>
<td><strong>Lentivirus</strong></td>
<td>Human immunodeficiency virus (HIV)</td>
<td>Humans</td>
<td>9869 bp</td>
<td>Lymphocyte-tropic associated with immunosuppression, Macrophage-tropic strains associated with arthritis, pneumonia, encephalitis, anaemia</td>
<td>(Weber, 1989)</td>
</tr>
<tr>
<td><strong>Spumavirus</strong></td>
<td>Human spumavirus</td>
<td>Simians</td>
<td>13246 bp</td>
<td>Subclinical infections</td>
<td>(Kupiec et al., 1991)</td>
</tr>
</tbody>
</table>
Pathogenicity

The retroviruses may also be divided into 3 groups according to their pattern of pathogenicity: the oncogenic retroviruses or oncoviruses, many of which are associated with leukaemias and sarcomas; the spumaviruses that do not seem to be associated with any disease; lentiviruses, many of which are associated with immunodeficiencies, but some of which are associated with neurological lesions, some with arthritis and some with pneumonia (Weiss, 1996).

Most retroviruses are transmitted laterally but the oncoviruses or cancer causing viruses may also be transmitted vertically by integration of the provirus into the genome of germ cells (Mims, 1981). The proviral form of these oncoviruses can be associated with transformation of the host cells into cells that have a tumour producing potential (Maeda et al., 2008).

Retroviruses characteristically persist in infected animals for the life of the animal (Wells & Poiesz, 1990). Some produce clinical disease only after prolonged incubation periods, some produce intermittent clinical disease associated with reactivation of virus and expression of clinical disease after periods of latency (Meiering & Linial, 2002). During the periods of latency the virus may be detectable but it is often difficult to detect because it is present at only low levels, necessitating indirect means such as the detection of antibody for the recognition of virus infection.

In the period of clinical latency following HIV infection, there is still detectable virus in the peripheral blood; it was suggested that this virus is derived from infected CD4+ lymphoblasts that have reverted to a resting memory state (Marcello, 2006). In FIV infection, it was suggested that CD4(+)CD25(-) cells provide latent viral reservoirs for FIV infection and CD4(+)CD25(+) represent ideal candidates for a productive FIV infection (Joshi et al., 2005a, Joshi et al., 2005b, Joshi et al., 2004). In Equine infectious anaemia virus (EIAV) infections, the proviral DNA was present in tissues regardless of disease status, particularly in macrophages that are the primary cellular reservoir and site of viral replication of EIAV (Oaks et al., 1998).

Latency is not unique to retrovirus infections and is also seen in other virus types such as herpesviruses. However, the mechanism of latency or persistence of the virus in these groups differs from that in retroviruses. Herpesviruses persist between periods of clinical disease in lymphoid cells or in ganglionic neurons of the peripheral nervous system. For example, Bovine herpes virus type-1 (BHV-1) localises and persists in ganglionic neurons of the peripheral nervous system.
(Jones et al., 2006) and in germinal centres of pharyngeal tonsil (Winkler et al., 2000). The virus within these sites exhibits limited replication: nested reverse transcription-PCR (RT-PCR) on latently infected cattle showed a few cells contained latency-related transcripts but not other immediate-early, and late transcripts (Winkler et al., 2000). Latency has also been detected in the circulating lymphocyte of Bovine papillomavirus-1 infected cattle (Campo et al., 1994) and in neoplastic and non-neoplastic tissues of horses (Carr et al., 2001). Reactivation of viral expression after periods of latency is often indicated by the appearance of infectious virus at the site of the initial infection in an immune host.

Lentiviruses

The lentiviruses can be divided into 4 groups (Table 2.2) on the basis of the host with which they have evolved: primate (including the 2 human virus types, HIV-1 and HIV-2, and multiple Simian immunodeficiency virus [SIV] types), equine (EIAV), feline (Feline immunodeficiency [FIV]), ruminant lentiviruses including bovine (including BIV and JDV), and small ruminant lentiviruses infecting sheep (Maedi-visna virus [MVV]) and goats (Caprine arthritis encephalitis virus [CAEV] (Clements & Zink, 1996).

Lentiviruses, like other retroviruses, contain the obligatory major open reading frames gag, pol and env. The gag open reading frame encodes a precursor polyprotein (Gag) that is cleaved to form the non-glycosylated structural proteins MA, CA and NC. The NC is associated with the viral RNA genome and required for packaging RNA into the virion (Coffin, 1979, Coffin, 1992). NC is also essential for the 2 obligatory strand transfers during viral DNA synthesis via promotion of primer binding site homo-dimer (Egele et al., 2004, Huthoff & Berkhout, 2001). The CA forms the core of the virion, it is the most immunodominant viral protein (Coffin, 1979, Coffin, 1992) and the conserved C-terminus of the CA is important in virion assembly and release (Melamed et al., 2004).

The pol open reading frame encodes 3 enzymatic proteins, RT, IN and PR that are utilised during the replication process. RT is essential for the transcription of the viral RNA genome to a dsDNA intermediate or provirus (Temin, 1993). IN is an essential protein involved in incorporation of the provirus DNA into the host cell genome, and PR is vital for the cleavage of the viral polyproteins into individual subunit proteins during virus replication (Coffin, 1979, Coffin, 1992).

Table 2.2. Principle distinguishing characteristic of viruses in the genus Lentivirus
<table>
<thead>
<tr>
<th>Group</th>
<th>Species</th>
<th>Host</th>
<th>Genome size/accessory genes</th>
<th>Tropism/disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Simian immunodeficiency virus</em> (SIV)</td>
<td>Non-human primates</td>
<td>8816 bp</td>
<td>Primarily lymphocyte-tropic/acquired immunodeficiency syndrome</td>
<td>(Ringler et al., 1988, Stephens et al., 1997)</td>
</tr>
<tr>
<td>Ruminant group</td>
<td><em>Caprine arthritis-encephalitis virus</em> (CAEV)</td>
<td>Goat</td>
<td>9065 bp</td>
<td>Macrophage-tropic/arthritis, encephalitis and pneumonia</td>
<td>(Olsen, 2001)</td>
</tr>
<tr>
<td></td>
<td><em>Maedi-visna virus</em></td>
<td>Sheep</td>
<td>9203 bp</td>
<td>monocyte/macrophage-tropic/pneumonia, arthritis and mastitis</td>
<td>(Sargan et al., 1991)</td>
</tr>
<tr>
<td></td>
<td><em>Bovine immunodeficiency virus</em> (BIV)</td>
<td>Cattle</td>
<td>8482 bp</td>
<td>Lymphocyte and macrophage-tropic/possible immunosuppression</td>
<td>(Gonda et al., 1994)</td>
</tr>
<tr>
<td></td>
<td><em>Jembrana disease virus</em> (JDV)</td>
<td>Cattle</td>
<td>7732 bp</td>
<td>Lymphocyte-tropic/acute disease affecting</td>
<td>(Wilcox et al., 1995)</td>
</tr>
<tr>
<td>Feline group</td>
<td><em>Feline immunodeficiency virus</em> (FIV)</td>
<td>Domestic and large cats</td>
<td>9891 bp</td>
<td>Primarily lymphocyte-tropic/acquired immunodeficiency syndrome</td>
<td>(Parodi et al., 1994, Pecon-Slattery et al., 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- lacks gene encoding Vif, but contains gene S2</td>
<td></td>
</tr>
</tbody>
</table>

In lentiviruses, the polyprotein Env is translated and subsequently glycosylated in the endoplasmic reticulum where it also oligomerises (Chan et al., 1997). In the golgi, the attached oligosaccharides are processed and the glycoprotein is proteolytically cleaved into 2 subunits, the SU and TM, by a cellular PC6 protease of
the subtilisin-like pro-protein convertase family. The glycoprotein complex is subsequently transported to the plasma membrane via the secretory pathway (Miranda et al., 2002). The TM subunit anchors the SU subunit to the viral surface via a disulphide bond and together they form a homotrimer, which is situated on the surface of viral particles. The external SU subunit determines receptor specificity while the TM subunit is responsible for the fusogenic process during entry (Eckert & Kim, 2001).

The lentiviruses possess unique properties that differentiate them from other retroviruses, including their ability to infect both dividing and non-dividing cells (Lewis et al., 1992, Weinberg et al., 1991) and by their possession of additional accessory genes important in their replication. In addition to the major open reading frames, lentiviruses have 2 regulatory genes and up to 4 accessory genes, the number varying with the species of lentivirus, making them the most complex members of the retroviruses. The regulatory and accessory genes are located mainly between the pol open reading frame and the 3' end of the genome, and may include genes designated tat, rev, vif, tmx, vpu, vpr, vpx, vpw, vpy, S2 and orfA. They collectively regulate viral transcription, translation, and aspects of viral pathogenicity (Cullen, 1991).

The primate lentiviruses are the most complex of all retroviruses, possessing at least 6 accessory genes including tat, rev, vif, vpr, vpx or vpu and nef (Tristem et al., 1992). All lentiviruses possess tat and rev, which are expressed from multiply spliced transcripts, encode trans-acting regulatory proteins, and are essential for viral replication (Dorn et al., 1990, Gonda, 1992, Narayan, 1990). Most lentiviruses also possess vif, which is expressed from a singly spliced transcript, and encodes a virion-associated protein also essential for viral replication (Schrofelbauer et al., 2004, Volsky et al., 1995).

2.2  Bovine lentiviruses

2.2.1 Jembrana disease virus

History

Jembrana disease is an economically important infectious disease of Bali cattle that emerged for the first time in the latter months of 1964 in the Jembrana district of Bali, Indonesia (Pranoto & Pudjiastono, 1967). This outbreak spread throughout the island over the ensuing 12 months and then the prevalence waned, perhaps a
consequence of herd immunity, and it was not reported again for several years. Second and the third outbreaks of Jembrana disease were subsequently detected that included one in the Tabanan district in 1972 and one in the Karangasem district in 1981 (Hardjosworo & Budiarso, 1973, Putra et al., 1983). Despite attempts to implement quarantine measures to prevent the movement of cattle from Bali, the disease has subsequently spread to some other Indonesian islands: Lampung province in South Sumatra (Soeharsono & Darmadi, 1976); the Banyuwangi district of East Java (Tranggono, 1988); the Sawahlunto district of West Sumatra (Tembok, 1992); in the 1990s in South Kalimantan and then to West and East Kalimantan (Hartaningsih, personal communication). The initial occurrence of the disease in these areas was associated with high mortality rates and has since become endemic with lower mortality rates (Soeharsono, 1997).

Clinical features of Jembrana disease

The disease can be readily transmitted to naïve cattle by the inoculation of tissues, including blood and lymphoid tissues, from affected cattle; high titres of infectious virus are present in the plasma and spleen of affected animals during the course of the acute disease (Soeharsono et al., 1990). Experimental transmission studies have confirmed field observations that the disease is an acute (transient) febrile condition with characteristic clinical findings during the acute disease process including anorexia, lethargy, fever, erosions of the oral mucous membranes and enlargement of superficial lymph nodes. Other clinical signs less consistently observed were hypersalivation, a nasal discharge, diarrhoea with blood in the faeces and pallor of the mucous membranes (Soeharsono et al., 1990, Soesanto et al., 1990). In experimentally infected cattle housed indoors the case fatality rate associated with infection by the Tabanan/87 strain was 17% (Soesanto et al., 1990). Recovered cattle are viraemic for at least 2 years after infection, possibly for the life of the animal, and are therefore an important potential source of infection for other cattle. However, in animals that recover there are no reports of the recurrence of disease suggesting that these animals develop a solid immunity to infection.

Marked haematological changes are detected in affected Bali cattle: leukopenia as a result of a lymphopenia, eosinopenia and neutropenia, thrombocytopenia, anaemia, increased blood urea concentrations and diminished total plasma protein levels; these changes occur principally during the febrile period (Soesanto et al., 1990). Gross pathological changes include vascular damage such as mild exudates
and haemorrhages, but the most striking changes are lymphadenopathy and splenomegaly. Lymphoid tissues of all organs, particularly in the enlarged lymph nodes and spleen, feature proliferating lymphoblastoid cells predominantly throughout parafollicular (T-cell) areas, and atrophy of follicles (B-cell areas). A proliferative lymphoid infiltrate is also found in the parenchyma of most organs, particularly the liver and kidneys and an infiltrate containing proliferative macrophage-like cells is found in the lungs (Dharma, 1997).

Jembrana disease virus appears to have a particular affinity for Bali cattle and it is only in this species that severe lesions and case fatalities appear to occur (Soeharsono et al., 1995a). Other cattle types and buffalo, however, can be infected experimentally and become infected under field conditions. *Bos taurus*, *Bos indicus* and crossbred Bali (*Bos javanicus x Bos indicus*) cattle and buffalo develop disease and a persistent viraemia (Soeharsono et al., 1995a). The clinical changes and lesions that occur in these cattle types are consistent with those observed in Bali cattle, but they are much milder and would be more difficult to detect under field conditions (Wilcox et al., 1995).

Although only limited studies of the disease in *Bos taurus* have been conducted, the studies have indicated that the effects of infection in *Bos taurus* are less severe than in *Bos javanicus*. Infection of Friesian cattle induced an acute disease after a short incubation period of about 4 days. Clinical signs were fever and concurrent lymphadenopathy. Haematological changes included leukopenia as a result of lymphopenia, neutropenia and thrombocytopenia (Soeharsono et al., 1995a). Increased blood urea concentration and signs of anaemia consistently detected in Bali cattle were not detected in Friesian cattle. Histological lesions consistent with a mild form of Jembrana disease in Bali cattle were detected in some infected animals. However, while there was follicular atrophy until 5 weeks after infection in the spleen and lymph nodes of Bali cattle there was a marked follicular response in the spleen and lymph nodes of one Friesian animal in the immediate post-febrile period (Soeharsono et al., 1995a). Friesian cattle experimentally infected with JDV had no parafollicular proliferation of mononuclear cells in intestinal lymphoid tissue or haemorrhagic lesions (Soeharsono et al., 1995a).

Since the first outbreak of Jembrana disease in 1964, there have been several hypotheses regarding the cause of the disease. The condition was initially considered to be caused by rinderpest virus (Adiwinata, 1968, Pranoto & Pudjiastono, 1967). Another theory was subsequently developed that the disease was caused by a rickettsia-like agent (Hardjosworo & Budiarso, 1973) and this
persisted until the disease was demonstrated to be caused by a retrovirus (Wilcox et al., 1992) and subsequent sequence analysis confirmed it was a lentivirus, most closely related to BIV (Chadwick et al., 1995b, Lu et al., 2002a).

**Genome of JDV**

The genome of the Tabanan/87 strain of JDV, the only strain that has been completely sequenced, is 7732 bp. A schematic representation of the genome of JDV is shown in Figure 2.2 and the molecular characteristics of the JDV proteins (Chadwick et al., 1995b) are shown in Table 2.3.

<table>
<thead>
<tr>
<th>Coding area gene product</th>
<th>Protein</th>
<th>Number of amino acid residues</th>
<th>Predicted Mr (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gag</td>
<td>Matrix (MA)</td>
<td>125</td>
<td>14.3</td>
</tr>
<tr>
<td></td>
<td>Capsid (CA)</td>
<td>226</td>
<td>25.3</td>
</tr>
<tr>
<td></td>
<td>Nucleocapsid (NC)</td>
<td>85</td>
<td>9.2</td>
</tr>
<tr>
<td>Gag/Pol Precursor</td>
<td></td>
<td>1432</td>
<td>163</td>
</tr>
<tr>
<td>Pol precursor</td>
<td></td>
<td>1027</td>
<td>118</td>
</tr>
<tr>
<td>Env</td>
<td>Surface unit (SU)</td>
<td>422</td>
<td>47.8</td>
</tr>
<tr>
<td></td>
<td>Transmembrane (TM)</td>
<td>359</td>
<td>41.1</td>
</tr>
<tr>
<td>Vif</td>
<td></td>
<td>197</td>
<td>22.9</td>
</tr>
<tr>
<td>Tat</td>
<td></td>
<td>97</td>
<td>10.7</td>
</tr>
<tr>
<td>Rev</td>
<td></td>
<td>213</td>
<td>23.8</td>
</tr>
<tr>
<td>Tmx</td>
<td></td>
<td>164</td>
<td>18.5</td>
</tr>
</tbody>
</table>

The accessory genes of the bovine lentiviruses are less complex than that of the primate lentiviruses. JDV contains 4 small putative accessory genes designated vif, tat, rev and tmx that correspond to the accessory genes detected in BIV and are assumed to have similar functions in both viruses (Chadwick et al., 1995a). While the functions of the HIV-1 accessory genes have been extensively investigated and reviewed (Cullen & Greene, 1990), in both bovine viruses their functions remain largely uncharacterised. It is known that JDV Tat is very potent and can strongly activate not only its own long terminal repeat (LTR) but also the HIV LTR. In
contrast, HIV Tat cannot reciprocally activate the JDV LTR (Chen et al., 1999) and JDV Tat can functionally substitute for HIV Tat (Chen et al., 2000). Like HIV Tat, JDV Tat transactivates the HIV LTR at least partially in a TAR-dependent manner. However, the sequence in the loop region of TAR was not as critical for the function of JDV Tat as it was for HIV Tat (Chen et al., 2000). The BIV vif (Oberste & Gonda, 1992) like the HIV-1 vif (Goncalves et al., 1996) facilitates the infectivity and spread of virus.

![JDV Complete Genome](image1.png)

**Figure 2.2.** The JDV genome resembles that of BIV and HIV type 1 with the typical 5’ to 3’ gag, pol and env gene organisation. It contains also the regulatory protein encoding genes between or overlapping the pol and env reading frames. From Burkala (2001).

**Immunological reagents for the detection of bovine lentiviruses**

There are no reports of the successful replication of JDV in cell culture and methods of preparation of JDV proteins for antigens have been reliant on the extraction of proteins from infected cattle tissues or the use of recombinant DNA techniques. The JDV CA with 226 amino acid (aa) residues is a small and highly basic protein with an estimated Mr of 25.3 kDa that is abundantly expressed, as detected by SDS-PAGE and western immunoblotting techniques, in infected cells and can be
harvested from virus present in the plasma of infected animals during the acute febrile phase of the disease (Kertayadnya et al., 1993).

The development of antigens able to differentiate BIV and JDV has been problematic. The expression of antigenic and immunogenic JDV CA and TM proteins as fusion proteins to the glutathione-s-transferase (GST) in *Escherichia coli* has been reported previously (Burkala et al., 1998). Both recombinant proteins reacted in western blots with JDV and BIV antisera. The Gag protein of JDV has been cloned (Desport et al., 2005) as a series of overlapping fragments and when analysed by western immunoblotting using JDV and BIV hyperimmune sera, the MA and CA were recognised by both sera and the NC did not react with either of the sera. This analysis suggested that the N-terminal domain of Gag might contain more antigenic epitopes than the C-terminal domain.

Monoclonal antibodies (MAbs) against JDV have been produced by immunisation of mice with whole virus (Kertayadnya et al., 1993) and with recombinant JDV CA (Desport et al., 2005). Characterisation of these MAbs by determining their reactivity with different truncated Gag proteins of JDV by western immunoblotting (Desport et al., 2005) demonstrated that the antibodies produced from recombinant CA reacted with different epitopes compared with those produced by immunisation of mice with the whole virus. A BIV MAb that recognised epitopes specific to BIV was generated (Zheng et al., 2001). This MAb, designated 10H1, was produced using a recombinant fusion protein containing the CA of BIV. Based on the immunoreactivity of the BIV CA, 3 domains of antigenic importance were identified on the N-terminus of the protein and these domains were thought to be BIV-specific (Lu et al., 2002a, Zheng et al., 2001). Unfortunately, this region was recognised by JDV-positive sera (Desport et al., 2005).

**Biological and physiochemical properties of JDV**

The buoyant density of JDV purified from the plasma of infected animas was 1.15 g/ml in sucrose gradients (Kertayadnya et al., 1993); the diameter of particles at this density in the gradients was determined by electron microscopy to be between 96 and 124 nm (Kertayadnya et al., 1993). The virus in plasma derived from infected animals rapidly decline in infectivity at 4°C but was stable at –70°C (Kertayadnya et al., 1993).
The population of Bali cattle represents 19% of the total cattle population of Indonesia (Talib et al., 2002). The total population of Bali cattle in 2000 in the 5 major regions has been estimated as 718,000 in South Sulawesi, 443,000 in Nusa Tenggara Timur, 377,000 in Nusa Tenggara Barat, 529,000 in Bali and 255,000 in Lampung (Talib et al., 2002). There are other provinces with growing population such as in Southeast Sulawesi and East Kalimantan (Talib et al., 2002). The unique susceptibility of Bali cattle to both sheep-associated malignant catarrhal fever and also to Jembrana disease is of concern (Talib et al., 2002).

Although precise reporting of cases of Jembrana disease is not undertaken in Indonesia, it was estimated that there were about 2,000 cases of Jembrana disease in Indonesia between 1989 and 1992 (Soeharsono, 1997). However, due to the difficulty of recognising and diagnosing the disease even by trained staff, and as milder non-fatal cases are unlikely to be reported, this is likely to be a gross underestimate. It is only when there are high case fatality rates in association with outbreaks that collection of data is likely. In 2000, 168 cases were reported, 331 cases were reported in 2002 (Peternakan, 2002) and 116 cases were reported in 3 provinces in 2003: 95 in Bengkulu, 2 in Lampung and 19 in South Sumatra. The most recent cases have been identified in the Long Ikis district of East Kalimantan (Hartaningsih et al., 2005) (Figure 2.3).
Transmission of Jembrana disease

The mode of transmission of Jembrana disease under field conditions is not known but certain assumptions have been made based on the level of virus in blood at various stages of the disease process. During the acute disease the titre of infectious virus in peripheral blood is about $10^8$ per mL, and virus can also be detected in secretions. During the acute disease, transmission probably occurs by 2 methods: through direct transmission of virus in secretions between cattle in close contact, and through mechanical transmission of virus in the blood by haematophagous insects (Soeharsono et al., 1995b). However, mechanical transmission of JDV by arthropods, as seems likely, has not been responsible for extensive spread of Jembrana disease from endemic to adjacent areas. For example, the disease has not spread from Bali island to the adjacent islands of Nusa Penida and Lombok since the disease initially occurred in Bali in 1964 (Wilcox, 1997). Recovered Bali cattle are persistently viraemic but the titre of virus in blood by 60 days after recovery from the acute disease was only about 10 infectious doses per mL, virus could not be detected in secretions during this phase, and mechanical transmission of these low levels of virus by haematophagous
insects was considered unlikely (Soeharsono et al., 1995b). Persistent infections in recovered animals are a potential source of infection but how the virus is transmitted from these animals is unknown.

It has been revealed through epidemiological studies that the risk of transfer was intimately related to farming practices, and that an important risk factor was from contact transmission associated with JDV present in secretions, including saliva and possibly urine, and in lactating animals also in milk, during the acute febrile phase of the disease (Soeharsono et al., 1995b).

**Diagnosis of JDV infection**

The diagnosis of Jembrana disease has traditionally been based on clinical signs and the presence of characteristic pathological lesions in those animals that died, although diagnosis was difficult and diagnosis of the disease by these methods was often made only reluctantly. Hence the disease in East Java was initially referred to as Banuwangi disease, and in Lampung province in Sumatra it was referred to as Lampung disease (Soeharsono & Temadja, 1997). The development of an enzyme-linked immunosorbent assay (ELISA) and western immunoblotting assays for the detection of antibody to JDV in infected cattle (Hartaningsih et al., 1994) using a whole viral antigen prepared from the plasma of infected cattle enabled the distribution of the virus within Indonesia to be determined and consequent diagnosis of the disease in infected areas made with greater confidence. The use of a recombinant JDV CA antigen (Burkala et al., 1998) removed reliance on the use of whole virus antigens prepared from infected cattle. ELISA and western immunoblotting assays demonstrated that development of antibody against JDV in recently infected cattle was delayed (Hartaningsih et al., 1994, Wareing et al., 1999).

A major problem with the JDV serological assays that have been developed is that they cannot distinguish between antibody to JDV and BIV (Desport et al., 2005). There is a report that it is possible to differentiate antibody to BIV and JDV (Barboni et al., 2001) but attempts to confirm this were unsuccessful (Desport et al., 2005). A further problem, investigated in Chapter 6 (this thesis), is that the size and other characteristics of the glycosylated envelope proteins have not been determined and these glycoproteins have not been identifiable in western immunoblots (Hartaningsih et al., 1994).
**In situ** hybridisation (ISH) is commonly used to detect the presence of virus in tissues. Chadwick et al. (1998) used ISH with a digoxigenin (DIG)-labelled riboprobe to detect viral RNA of JDV in formalin-fixed paraffin-embedded tissue sections, concluding that JDV-infected cells were present in many tissues including spleen, lymph nodes, lungs, bone marrow, liver and kidney.

The use of a quantitative PCR for JDV RNA detection in plasma samples has also been reported (Stewart et al., 2005). The assay had a detection limit of $4.2 \times 10^4$ JDV genome copies per mL of plasma in experimentally infected cattle.

A monoclonal antibody developed against the CA of JDV (Kertayadnya et al., 1993) has been used for the development of specific immunoperoxidase assays on frozen and formaldehyde-fixed tissues.

### 2.2.2 Bovine immunodeficiency virus

BIV was first isolated in cell cultures from a dairy cow in Louisiana that had lymphocytosis, lymphadenopathy, neuropathy, and progressive emaciation (Van der Maaten et al., 1972). Based on serological evidence, the virus has since been reported from cattle in several countries (Amborski et al., 1989, Forman et al., 1992, McNab et al., 1994). Although the virus in these other countries appears to be non-pathogenic, the genetic relationship between the original BIV isolate and the virus in these other countries has not been determined.

BIV resembles HIV and other lentiviruses in its structural, genetic, antigenic and biological properties (Gonda et al., 1987). The mature virions of BIV are bar-shaped and 120-130 nm in diameter. The BIV genome contains the obligatory retrovirus structural genes in the order *gag*, *pol* and *env*, flanked on the 5' and 3' ends by a LTR. The core protein of the virus is encoded by *gag*, which produces a 53 kDa precursor Gag protein that is further processed into MA (p17), CA (p26) and NC (p15) (Battles et al., 1992, Rasmussen et al., 1990) and 3 small proteins, p2L, p3 and p2 (Tobin et al., 1994) in the mature virus (Gonda et al., 1994).

The BIV complete nucleotide sequence consists of 8,482 nucleotides (Garvey et al., 1990) while JDV has been reported to contain 7,732 nucleotides (Chadwick et al., 1995b). Apart from the nucleotide number, the difference between the 2 bovine lentiviruses consisted of small deletions and insertions located throughout the genomes (Chadwick et al., 1995a).
2.3 Potential Jembrana disease vaccines

Effective vaccines against a multitude of viral diseases have been produced by a range of methods including whole, live attenuated or inactivated pathogens, although the development of effective vaccines against lentiviruses has been difficult. Control of Jembrana disease within Indonesia will most likely require the development of not only efficacious vaccines but also vaccines of low cost.

Attenuated viral vaccines are effective in stimulating both humoral and cellular immune responses (Young & Ross, 2003). They are usually attenuated in their pathogenicity so that they still have ability to replicate but without causing overt disease. Disadvantages of this type of vaccine are that they can potentially revert to a virulent strain causing disease and they can be transmitted between individuals. Modification of the antigenic properties of live attenuated FIV vaccines improved the protection against FIV infection (Broche-Pierre et al., 2005). The close antigenic relationship between the non-pathogenic BIV and the pathogenic JDV (Desport et al., 2005) suggests that there may be some competitive interaction or cross-protective immunity between these 2 viruses and prior infection of cattle with BIV might protect against subsequent JDV infection.

Inactivated vaccines are safer than live attenuated vaccines because they cannot replicate in the host, although they are frequently less effective in inducing protective immunity (Chalmers, 2006, Ellis, 1999). Recently, a vaccine composed of inactivated FIV was used and induced high titres of antibody to the Env proteins (Hosie et al., 2005). A whole virus tissue-derived inactivated JDV vaccine has been reported to induce a protective immunity against subsequent challenge with JDV but this vaccine has several potential disadvantages including high cost that it was inactivated with detergent and might therefore be contaminated with adventitious infectious agents, and it was not amenable to commercial production methods (Hartaningsih et al., 2001).

During the last decade, recombinant subunit vaccines have emerged as a promising vaccine technology. A subunit vaccine can be produced in the form of synthetic peptides (Audran et al., 2005, Lopez et al., 2001), recombinant proteins or gene fragments (DNA or RNA) encoding the protein immunogens (Wang et al., 2006). By using a small and defined part of a pathogen and producing that subunit in a non-pathogenic host, the safety of vaccines will increase.

A potential target for a JDV vaccine is the envelope glycoproteins, and in a subsequent section of this review the literature examining methods by which such
glycosylated proteins might be produced using recombinant DNA technology is reviewed.

2.3.1 Recombinant protein vaccines

The first recombinant subunit vaccine to be produced was licensed in 1986 when the *Hepatitis B virus* surface antigen (HbsAg) was successfully expressed in yeast and this then replaced the plasma-derived hepatitis B vaccine used previously (Valenzuela et al., 1982). This vaccine was initially tested and produced protective antibodies in vaccinated chimpanzees (McAleer et al., 1984).

Recombinant DNA technology for the production of proteins potentially enables a large amount of high value protein, which is often in limited supply due to its low natural availability, to be produced. Several host systems are available for production of recombinant proteins: eukaryotic cells such as yeasts, filamentous fungi, insect cells, plants, mammalian cells and prokaryotes including bacteria such as *Escherichia coli*, *Bacillus* and *Staphylococcus sp*. Each host system has its advantages and disadvantages. The amount and quality of the produced recombinant proteins are influenced by factors such as gene copy number, transcription and translation efficiency, mRNA stability, stability and solubility of the proteins as well as post-translational modification (Liljeqvist & Stahl, 1999).

The choice of expression system to be used will often depend on the characteristics of the protein required. The heterologous host used may affect the immunogenicity and protective efficacy of the protein. In practice, the choice is a combination of the ease of growing the host, immunogenic and antigenic characteristics of the protein, and yield and ease of purification of the protein. Several issues such as the gene construct, solubility of the protein expressed and the nature of the fusion tag that is often incorporated into the design of the plasmid, all have to be considered (Hockney, 1994).

*Escherichia coli* has long been the primary prokaryotic host for heterologous protein expression, and there is a lot of information available about the use of this bacterium for the production of many different proteins. It has been successfully utilised to produce many functional human proteins such as human growth hormone (Singh & Panda, 2005), proinsulin (Winter et al., 2001), interferon-gamma (Khalilzadeh et al., 2003, Khalilzadeh et al., 2004) and antibody fragments (Santala & Lamminmaki, 2004). The advantages of *E. coli* include its relatively rapid growth, its utilisation of inexpensive cultural techniques, its ease of transformation and its
ease of maintenance. Nevertheless, the use of *E. coli* also has major disadvantages: it is unable to perform post-translational modifications such as glycosylation, phosphorylation and disulfide bond formation, modifications which occur in eukaryotic cells; the expression and accumulation of a recombinant protein in *E. coli* frequently causes the formation of insoluble protein aggregates (Clark, 2001) and this raises issues concerning methods of subsequent solubilisation of the expressed protein.

Expression in yeast such as *Saccharomyces cerevisiae* or *Pichia pastoris* offers advantages but also disadvantages in comparison to bacterial systems. Many recombinant proteins have been produced on a large scale using *S. cerevisiae*, including human serum albumin (Kang et al., 2000, Okabayashi et al., 1991), the HbcAg (Chen et al., 2004, Yoshida et al., 1991), insulin and hirudin (Mendoza-Vega et al., 1994, Vai et al., 2000). Like *E. coli*, these unicellular eukaryotes grow rapidly in relatively inexpensive and simple media and they are easy to transform and maintain. Unlike *E. coli*, yeasts are eukaryotic and therefore express and process proteins in a similar way to higher eukaryotes (Cereghino et al., 2002, Cereghino & Cregg, 1999). The secretory pathway of yeasts closely resembles that of the mammalian cells, thus they are capable of many posttranslational modifications, although they are not capable of complex modifications such as prolylhydroxylation and amidation, and the glycosylation of proteins in yeast can differ from that of higher eukaryotes (Sudbery, 1996).

The baculovirus expression system is commonly used to express heterologous proteins in insect cells (Kost & Condreay, 1999). Since insect cells are eukaryotic, proteins expressed will be post-translationally modified in a manner similar to that of mammalian cells (Kost & Condreay, 2002, Miller, 1993). Insect cells can be grown as suspension cultures, which enable the use of large scale bioreactors for easier production scale-up. Another significant advantage of insect cells is that unlike mammalian cells, they can be grown in medium that does not need to be maintained in CO₂ incubators. The most commonly used baculovirus system utilised Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) (Jones & Morikawa, 1996, Lu et al., 2002b) in a cell line derived from lepidopteran *Spodoptera frugiperda* ovarian (Sf9) cells.

Mammalian cell systems may be the only way to produce appropriately processed and active recombinant proteins. Gene transfer into mammalian cells may be performed either by infection with a virus carrying the recombinant gene of interest (Makrides, 1999) or by direct transfer of plasmid DNA (Geisse & Kocher, 1999).
Recombinant vaccinia virus vectors have been successfully used for the expression of recombinant genes (Moss, 1996). Since vaccinia is infectious to humans, safety aspects must be taken into considerations. Problems with vaccinia systems include their cytopathic nature and dependence on efficient transfection rates (Moss, 1996).

**Post-translational modification of viral glycoproteins**

Post-translational modification is a common phenomenon in eukaryotic cells that is associated with the chemical modification of one or more aa in a protein chain. There are a number of modifications that may occur at the post-translational stage. Glycosylation is the most necessary of these modifications as it is important in secretion, antigenicity and clearance of glycoproteins (Jenkins et al., 1996).

Glycosylation during post-translational processing requires the addition of carbohydrate structures, forming glycoproteins. The carbohydrate moieties of glycoproteins, called oligosaccharides or glycans, are composed of individual sugar residues or monosaccharides (Dell & Morris, 2001). Protein glycosylation is important for many cellular processes including cell interactions, protein interactions and protein folding. It is especially vital for the proper positioning and function of surface receptors; glycosylation of the envelope proteins was shown to be a key factor in the ability of HIV to evade recognition by the immune system (Rudd et al., 2001). The presence of N-linked carbohydrates on the FIV Env is important for the interaction of the virus and receptor (Willett et al., 2008). This is similar with both HIV and SIV. Mutations in the variable V1/V2 region of HIV SU affect the interaction between HIV SU and its receptor and co-receptor and alter the antigenicity of the envelope glycoprotein (Kolchinsky et al., 2001, Srivastava et al., 2003).

There are 2 main classes of glycoproteins, N-linked and O-linked, based on the site of attachment of the carbohydrate to the polypeptide chain (Medzihradszky, 2005, Peter-Katalinic, 2005). N-linked glycans are attached to the protein only at the aa sequences NXS or NXT and are covalently linked to the asparagine (N) residue. In contrast, O-linked glycans are linked to any serine (S) or threonine (T) in the polypeptide chain. The role of N-linked and O-linked carbohydrates in the function of the viral envelope glycoproteins is different but poorly understood (Medzihradszky, 2005).

The glycans are monosaccharide residues and while there are approximately 20 monosaccharide residues (Kobata, 1992), 5 types are common (Table 2.4). Glycans consist of one or more monosaccharide residues bonded by glycosidic bonds to
form polysaccharide chains. The glycan structure can be linear or branched depending on the synthesis pathways (Marquardt & Denecke, 2003), making them vary in terms of size, structure and composition. The glycans found on mammalian N-linked glycoproteins have a common core composition of Glc3Man9GlcNAc2 (Dell & Morris, 2001).

Table 2.4. The monosaccharides and additional residues found in glycoproteins. Data sourced from Kobata (1992).

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>Monoisotopic mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexose (Hex)</td>
<td></td>
</tr>
<tr>
<td>Glucose (Glc), Galactose (Gal), Mannose (Man)</td>
<td>162.0528</td>
</tr>
<tr>
<td>N-acetylhexosamine (HexNAc)</td>
<td></td>
</tr>
<tr>
<td>N-acetylgalactosamine (GalNAc), N-acetylglicosamine (GlcNAc)</td>
<td>203.0794</td>
</tr>
<tr>
<td>Deoxyhexose (DeoxyHex)</td>
<td></td>
</tr>
<tr>
<td>Fucose (Fuc), Rhamnose (Rha)</td>
<td>146.0579</td>
</tr>
<tr>
<td>Pentose (Pent)</td>
<td></td>
</tr>
<tr>
<td>Arabinose (Ara), Xylose (Xyl)</td>
<td>132.0423</td>
</tr>
<tr>
<td>Hexuronic acid (HexA)</td>
<td></td>
</tr>
<tr>
<td>Glucuronic acid (GlcA)</td>
<td>176.0321</td>
</tr>
<tr>
<td>Acetyl (Ac)</td>
<td>42.0106</td>
</tr>
<tr>
<td>N-glycolylneuraminic acid (NeuGc)</td>
<td></td>
</tr>
<tr>
<td>N-acetylneuraminic acid (NeuAc)</td>
<td>291.0954</td>
</tr>
<tr>
<td>Phosphate</td>
<td>79.9663</td>
</tr>
<tr>
<td>Sulfate</td>
<td>79.9568</td>
</tr>
<tr>
<td>2-Keto-3-deoxynonulosonic acid (KDN)</td>
<td>250.0689</td>
</tr>
<tr>
<td>Methyl (Me)</td>
<td>14.0157</td>
</tr>
</tbody>
</table>

Depending on the nature of the oligosaccharide chain, the glycan can be classified as oligomannose, complex or hybrid. Oligomannose glycans contain only mannose residues, whereas complex glycans have varied composition and a variable number of antennae stemming from the core. Hybrid types have the characteristic of both complex and oligomannose glycans (Dell & Morris, 2001). The structures of O-linked glycans are less defined than that of the N-linked glycans. O-linked glycans
are composed of 7 different cores, 4 of them are found in mammalian glycoproteins, with varied structures and compositions (Dell & Morris, 2001).

The analysis of protein glycosylation remains difficult due to the complexity of the process and the heterogeneity of the structures that are formed. This heterogeneity arises from the variation in the use of the glycosylation sites on the protein and the variations in the monosaccharides used. The complexity is increased further as different cells, tissues, organs and organisms exhibit different glycosylation patterns (Rudd et al., 2001). Therefore, analysis of glycoproteins needs multiple analytical methods and requires several analytical steps and a large amount of sample that can make it a very expensive process.

There are a few publicly available glycoprotein analysis tools. GlycoSuiteDB is one of the few glycoprotein databases available on the internet and it has only been available since 2001 (Cooper et al., 2001b). It is a database that collates information on glycoproteins from the scientific literature only, and consequently there are only a small number of entries (Cooper et al., 2003).

Another publicly available database is O-Glycbase maintained by the Centre for Biological Sequencing from the Technical University of Denmark. It is a database of O-linked glycoproteins only (Gupta et al., 1999). O-Glycbase is coordinated with NetOGLyc, a tool for predicting O-linked glycosylation sites (Julenius et al., 2005).

Both GlycoSuiteDB and O-Glycbase are extensively cross-linked to various nucleotide and protein databases.

GlycoMod is another tool that predicts possible oligosaccharide structures from their experimentally determined masses (Cooper et al., 2001a).

The identification and characterisation of post-translational modifications has become achievable with improvements in mass spectrometry (MS). MS was formerly useful in organic chemistry for the analysis of low molecular weight molecules but with new methods of ionization of proteins and peptides, MS now permits analysis of proteins (Domon & Aebersold, 2006), high molecular weight carbohydrates (Mutenda & Matthiesen, 2006), and nucleotides (Banoub et al., 2005, Mikelova & Schram, 1997) on a routine basis. MS has become the most powerful technique to determine the mass of a biomolecules and has several advantages over techniques like gel filtration or SDS-PAGE, principally the high accuracy, high sensitivity, and speed (Domon & Aebersold, 2006). The major uses of MS for protein analysis were reviewed by (Mann & Pandey, 2001).
Two desorption techniques, fast atom bombardment (FAB) (Dell et al., 1981) and plasma desorption (PD) (Maugh, 1985) initiated the development of new strategies for protein characterization by mass spectrometry. Although these two methods were successful, the breakthrough came with the development of matrix-assisted laser desorption/ionization (MALDI) (Hillenkamp et al., 1991) and electrospray ionisation (ESI) (Andersen et al., 1996, Gaskell, 1997, Ho et al., 2003). The techniques are fast, simple, and accurate, and have made mass spectrometric analysis of biomolecules a routine analytical activity. MALDI has been coupled to many different mass analysers but is mostly coupled to a time-of-flight (TOF) analyser.

For the localization of modifications and mutations or for identification by peptide mass fingerprinting, the protein needs to be fragmented into smaller peptides. The use of in-gel digestion (Huynh et al., 2009, Jimenez et al., 2001) is a good alternative for the generation of peptide fragments of a membrane protein. An in-gel approach has several advantages. First, only few materials are needed. Second, the purity of sample is not critically important because SDS-PAGE separates the protein of interest from contaminants. Third, the membrane protein is partially unfolded in SDS, providing better accessibility for the protease, whereas the denaturant is removed by washing the excised gel. Several examples have been published in which the identification of a modification was successful using a specific isolation procedure without having a complete peptide map (Lennon & Walsh, 1999, Qin & Chait, 1997, Tsur et al., 2005).

Peptide mass fingerprinting is an identification method first described by James and Yates (James et al., 1993, Yates et al., 1993). The peptide mass fingerprinting method is based on the idea to divide a whole protein into smaller fragments to measure the mass of each fragment and to then use the resulting list of masses, ie. a fingerprint, to identify the protein. The analysed protein is then compared with a database of proteins, and for characterisation, ie. to detect which variant of an identified protein was analysed or if the protein was modified by post-translational modification (Cottrell, 1994, Wise et al., 1997). The peptide mass fingerprinting method is also widely used to identify and characterise unknown samples (Cottrell, 1994, Wilkins et al., 1997).
Selection of appropriate gene constructs for expression of proteins

Selecting a minimal region of the protein required to elicit a strong immune response could reduce the length of the gene to be inserted into the expression vector and in some cases the gene needs to be truncated for the purpose of facilitating expression (Johne et al., 2004). Promoter sequences may also be altered to make production more efficient, especially in the case of toxic proteins (Chevalet et al., 2000). The secretion of expressed protein can be intracellular or directed into the medium or periplasm; secretion of the protein into the growth medium may protect the protein from cytoplasmic proteases and it can simplify the purification process. Extracellular production is often preferred to express proteins containing transmembrane regions (Sorensen & Mortensen, 2005) or that have a tendency to aggregate (Sorensen et al., 2004) as intracellular expression may lead to the formation of inclusion bodies, which requires solubilisation and refolding to obtain a soluble and active form.

Formation of recombinant proteins within inclusion bodies

Production of recombinant proteins by bacterial cells is usually the most convenient and cost-effective method of recombinant protein production and *E. coli* has become the most extensively used bacterial host. It is a genetically and physiologically well-characterised organism that grows rapidly and requires simple medium but its use is also affected by 2 problems: overproduction of recombinant proteins leading to the formation of inclusion bodies, and protein degradation (Baneyx & Mujacic, 2004, Cabrita & Bottomley, 2004, Markossian & Kurganov, 2004). Foreign proteins expressed in *E. coli* can contain regions that are recognised by specific host proteases, leading to cleavage and sometimes to subsequent degradation (Baneyx & Georgiou, 1990, Rozkov & Enfors, 2004). Inclusion bodies are very dense particulate amorphous structures (Bowden et al., 1991) that almost exclusively contain over-expressed proteins (Cabanne et al., 2005, Carrio et al., 1998, Razeghifard, 2004, Rinas & Bailey, 1992, Sakono et al., 2004, Valax & Georgiou, 1993). Formation of inclusion bodies can be beneficial if the protein product is susceptible to host proteases or is toxic to the cells in its active form; in this case, inclusion bodies are advantageous and strategies for increasing inclusion body formation have been developed. Although recombinant proteins with secondary structure and native-like conformations have been found in inclusion
bodies (Khan et al., 1998, Oberg et al., 1994) the proteins aggregated in inclusion bodies are normally inactive, unfolded or folded incorrectly and need refolding in the functional active format (Buchner & Rudolph, 1991, Misawa & Kumagai, 1999). The presence of high concentrations of the protein in inclusion bodies, however, up to 95% of the mass of the inclusion body, can simplify downstream processing (Han et al., 2004, Lilie et al., 1998). Inclusion bodies can be easily removed from host cell proteins and debris by low speed or gradient centrifugation (Haelewyn & De Ley, 1995, Taylor et al., 1986) or an expanded bed adsorption (Cabanne et al., 2005).

Inclusion bodies form not only with expression of heterologous recombinant proteins but also from homologous proteins (Rudolph & Lilie, 1996), indicating that the formation is not specific for “foreign proteins”. The formation of Inclusion bodies is also not specific for \textit{E. coli} since they have been found also in \textit{Bacillus subtilis} (Wang et al., 1989), in yeast such as \textit{Saccharomyces cerevisiae} (Binder et al., 1991) and in mammalian cells (Broido et al., 1991). They can also form from proteins like ß-lactamase secreted into the periplasm (Chalmers et al., 1990).

Reasons given for the formation of inclusion bodies include the heterologous nature of the protein (especially for proteins that require post-translational modifications), high protein synthesis rates, proteins with high hydrophobicity that aggregate intermolecularly as a result of non-covalent association, and a lack of available chaperones (Mukhopadhyay, 1997). Point mutations which change the hydrophobicity of a protein can alter the stability and solubility of the protein (Luck et al., 1992) and change considerably the amount of protein deposited in inclusion bodies (Wetzel et al., 1991).

To produce a product from inclusion bodies that is similar to the native form, a renaturation strategy is required (Clark, 2001, Rudolph & Lilie, 1996). General strategies for recovering native proteins from inclusion bodies normally involve 3 steps: (1) isolation of Inclusion bodies including washing several times to remove undesired co-precipitation proteins, (2) solubilisation of inclusion bodies in a strong denaturant such as urea or guanidinium chloride to break intermolecular interactions, and (3) renaturation of solubilised proteins by dialysis or dilution to remove the denaturant. For renaturation of proteins containing disulfide bonds, the supplementation of redox systems to the renaturation buffer is needed (Creighton, 1986). These redox systems provide the appropriate redox potential to allow formation and reshuffling of disulfide bonds.

This strategy to prepare soluble proteins from inclusion bodies is possible only for small proteins. Large multi-domain proteins normally fail to fold properly, often
leading to kinetically trapped intermediates that aggregate, even in diluted solutions and at low temperature (Jaenicke & Bohm, 1998). Moreover, the folding process in vitro can take a long time, up to several days. Due to competition between aggregation and folding in vitro (Kiefhaber et al., 1991), the protein concentrations must be kept low. Thus, to get a given amount of soluble protein, large volumes of buffers are needed. The yield of protein refolding can be improved by coupling denatured proteins to a matrix (Stempfer et al., 1996), adding low molecular weight additives (Rudolph & Lilie, 1996), genetically engineering hydrophobic patches (Wetzel, 1994), shifting the temperature rapidly (Betts & King, 1998), or adding molecular chaperones (reviewed by (Thomas & Baneyx, 1997)). However, the yields are still likely to be modest, especially for large multi-domain proteins. Moreover, refolding in vitro is an expensive and time consuming process that sometimes is not easy to scale up. To simplify the downstream processing, maximisation of the yield of soluble active product during production in vivo is an attractive alternative.

Tag protein fusions

The tags on most fusion proteins are commonly used to optimise the production and purification procedures of the target protein but they also can be used for detection and immobilisation purposes (Nilsson et al., 1997). The fusion tag can improve the solubility (Nallamsetty & Waugh, 2006) or proteolytic stability (Murby et al., 1991, Murby et al., 1996) of the overall protein. There are many well-defined affinity tags that have been described such as glutathione-S-transferase (GST), polyhistidine (His), maltose-binding protein (MBP) and these have been reviewed by (Terpe, 2003). No single affinity tag is ideal for all expression and purification systems.

2.4 Production of monoclonal and recombinant antibody

The use of virus-specific antibodies, and in particular the use of MAb, has been of considerable value in the development of various assay systems for the detection of microbial antigens and diagnosis of disease.

Monoclonal antibodies

Antibodies are composed of immunoglobulins that are produced by plasma cells. There are 5 isotypes of immunoglobulins, namely IgG, IgM, IgA, IgD and IgE. Once
a B-lymphocyte has been stimulated by foreign antigen to differentiate into antibody-producing plasma cells, they may produce only one single type of antibody molecule, binding to a particular site (epitope) on the antigen. The clonal selection of individual antibody producing B cells and continued propagation of the cloned cells is the basis of producing MAb.

Attempts to culture single antibody-producing cells in vitro were met with limited success until Kohler and Milstein (1975) developed a technique for the reliable long-term production of cloned antibody producing lymphocytes. Their methods enabled the production of individual antibodies of invariant specificity and selectivity, and the immortalisation of the antibody-producing cells, ensuring a virtually infinite supply (Kohler & Milstein, 1975). The procedure for the production of MAb involves immunisation of a mouse or a rat, fusion of the plasma cells from the immunised mouse or rat with myeloma cells to create hybridomas, then cloning the fused cells and harvesting the antibody product.

**Structure and function of antibody molecules**

A schema of a complete antibody molecule is illustrated in Figure 2.4. The basic dimeric antibody structure comprises 2 heavy chains (V\textsubscript{H}) of about 440 aa and 2 light chains (V\textsubscript{L}) of about 220 aa residues. These chains fold into 3 domains. Two of the domains (Fab domains) are identical and form the arms of the Y-shaped molecule. One light chain associates with the amino-terminal region of one heavy chain to form an antibody-binding site. The third domain (Fc) forming the base of the Y shape is folded together by the carboxy-terminal regions of the 2 heavy chains and is responsible for aspects of the immune response. The 4 polypeptide chains are held together by disulfide (S-S) bridges and non covalent bonds. The light chain can be divided into 2 regions, variable (V) and constant (C) region. The heavy chain contains one variable and 3 constant regions. Within the variable regions, 4 framework regions (F) and 3 hypervariable regions (CDR) can be discriminated. These CDRs form the majority of contact residues for the binding of the antibody to the antigen (Padlan, 1994).

**Recombinant antibodies**

Current molecular techniques enable the construction of recombinant antibody fragments that have antigen-binding properties. The general strategy taken is to isolate the mRNA from mouse splenocytes or MAb-producing hybridoma cells and to reverse transcribe the immunoglobulin encoding mRNA with a single primer, which binds either the heavy or light chain antibody gene near the beginning of the constant domain.

Reverse transcription of the mRNA results in the production of immunoglobulin variable region cDNA. The cDNA is then amplified by PCR with the original 3’ constant region primers and the 5’ leader signal primers, binding upstream from the variable region. The immunoglobulin heavy or light chain cDNA amplified by PCR can be cloned into a plasmid or phagemid vector. Once the immunoglobulin genes
are cloned, they can be sequenced and produced in large scale by simple *E. coli* fermentation methods (Buchner & Rudolph, 1991, Su et al., 2003).

**Single chain variable fragment (scFv) antibody**

ScFv is a product resulting from the development of the current biotechnology and antibody engineering. The 2 antigen binding variable regions of the heavy (VH) and light chain (VL) are artificially connected by a linker peptide, designated as single-chain variable fragment or single chain antibody (Bird et al., 1988, Orlandi et al., 1989). This resulting scFv facilitates the equal expression of both variable fragments in heterologous micro-organisms, mammalian cells and plants (Schirrmann et al., 2008).

The antigen-binding site is made up of the variable domains of light and heavy chains of a MAb. The smallest portion containing an antigen binding site is the variable fragment (Fv) of an antibody. The Fv fragment has the full intrinsic antigen binding affinity of one binding site similar to the binding pattern of the whole parental antibody (Takemura et al., 2000). The relative affinity of VH and VL fragments for each other depends on the particular sequence of the antibody. Low affinity may result in dissociation of the Fv fragment into its components (Horne et al., 1982).

To stabilise the association of the recombinant Fv fragments, they are joined with a short peptide linker and expressed as a single polypeptide chain; linker peptides, of 12-25 aa do not normally disturb the proper folding of the VH and VL domains (Bird et al., 1988, Huston et al., 1988). The most frequently used linker for scFv antibodies is (Gly4Ser)3 (a 15 aa peptide of 12 glycines and 3 serines) that bridges the ~4.5 nm gap between the C-terminus of one domain and the N-terminus of the other and has a flexible structure with enhanced mobility (Freund et al., 1993, Huston et al., 1988). This construction facilitates chain pairing and minimises refolding and aggregation encountered when the 2 chains are expressed individually. Comparison of the unlinked Fv fragment of the antibody McPC603 with the corresponding scFv containing a linker (Gly3Ser)4 (VH-linker-VL constructs) has shown no perturbation of the folding of the variable domains by the linker (Freund et al., 1993).

There are different forms of scFv; a soluble scFv antibody is the most popular form as it shares all the advantages of the MAb (Blazek et al., 2004). The scFv genes can also be fused to gene3 protein (g3p) of the phage to form recombinant phage display scFv antibody and leads to the expression of scFv on the surface of the
phage. An advantage of this form of scFv is that the supernatant of the phage-infected bacterial culture can be directly used in ELISA (Xu et al., 2004). This is useful for screening specific scFv to target antigens from a scFv library through multiple rounds of selection. However, this kind of scFv may exhibit some cross-reactivity (McElhiney et al., 2002).

Dimeric or mini-antibody is another form of scFv. Such molecules preserve the bivalency of native antibody molecules, which is a very effective means of increasing the functional affinity (avidity) to the surface of polymeric antigens (Kortt et al., 2001, Muller et al., 1998). ScFv fragments can be linked by a small modular dimerisation domain in the form of one or 2 amphipathic helices. These mini-antibodies assemble in dimeric form in *E. coli* and the avidity of the best of them is indistinguishable from a native antibody (Kortt et al., 2001).

Biotinylated antibodies are commonly used reagents in research and molecular diagnostics. The traditional approach to biotinylate antibodies is to conjugate a chemically active biotin to scFv antibody. This genetically biotinylated scFv enables the production of the antibody conjugate in bacteria, which could decrease costs of ELISA reagents (Grimm et al., 2004, Santala & Lamminmaki, 2004).

The small scFv fragments are considered promising for medical and biological applications because of superior tissue penetration, absence of side reactions involving the constant domains, as well as easy engineering of fusion proteins, such as scFv-coupled toxins, the creation of multivalent or bi-specific proteins (Hudson & Kortt, 1999, Leath et al., 2004).

**Fab antibody fragments**

The construction of Fab fragments (50 kDa) is similar to that of scFv (30 kDa). The difference is that both variable and constant regions of light chain and heavy chain still remain in Fab (Figure 2.4). Therefore, a molecule of recombinant Fab fragments has the complete antigen binding site and structure of a natural antibody, which may result in a relatively higher avidity than that of the same scFv. The thermodynamic stability of the corresponding Fab fragments is also higher than that of scFv (Rothlisberger et al., 2005). This is due to the 2 interchain disulphide bonds typically present in the variable domains (\(V_H\) and \(V_L\)) and additional disulphide bonds in the constant domains (\(C_H1\) and \(C_L\)). Although Fab fragments have these advantages, they are usually produced in the periplasmic space of *E. coli* at lower functional yields and do not have the same advantages of small size, especially in multivalent...
formats (Skerra & Pluckthun, 1988). Production of functional antibody Fab fragments in an oxidising bacterial cytoplasm has overcome those limitations in terms of yield, folding and functionality and is economic, rapid and efficient (Venturi et al., 2002). A number of Fab fragments have been developed and used in immunotherapy and diagnostics (Zhang et al., 2001) and have found some applications in clinical toxicology (Flanagan & Jones, 2004).

**Generation of scFv antibodies**

Established hybridoma clones are a rich source for generating scFvs. The variable domains of the antibodies expressed by the hybridoma cells can be derived relatively easily, and cloned into the scFv format. However, a major disadvantage is that not all scFv derived from existing antibodies are active. Another disadvantage is the non-human origin of most hybridoma clones, which may render the derived scFvs immunogenic (Blazek & Celer, 2003, Blazek et al., 2004).

ScFvs can also be selected from large naïve scFv repertoires or smaller “immunised” repertoires. The techniques and methods for such selections include phage display that is widely used due to its simplicity and ability to be adapted to many specific conditions, including selection on whole cells and tissues (Hust & Dubel, 2005) and the system has been extensively reviewed (Kotz et al., 2004, Pini & Bracci, 2000), ribosome display that has the capacity to screen libraries of greater size as well as facilitating diversity and efficient antibody maturation (He et al., 2004, Lee et al., 2004b), bacterial display (Bessette et al., 2004, Su et al., 2003) and yeast display (Boder & Wittrup, 2000, Feldhaus & Siegel, 2004, Powers et al., 2001, Siegel et al., 2004) that have several advantages over the phage system including use of flow-cytometry and sorting techniques to enable finer affinity discrimination of selected antibodies. Other techniques are periplasmic expression cytometric sorting (PECS) (Chen et al., 2001) and potentially some 2-hybrid systems to screen for intracellular antibodies.

**Display on phage particles**

The first paper describing phage display (Smith, 1985) described display of a fragment of the EcoRI enzyme in the central region of pIII, and showed that the recombinant phage could be enriched in a phage pool by panning the pool on antibodies recognising EcoRI. The display of an anti-lysozyme scFv on the surface
of filamentous phage particles by fusion of the antibody variable genes to one of the phage coat proteins paved the way for the generation of antibody repertoires on phage (McCafferty et al., 1990).

Over the years, most of the phage coat proteins have been tested for display of antibody fragments, with pIII as the most preferred choice (Ding et al., 2005, Gupta et al., 2001, Han et al., 2003, Yang & Shiuan, 2003). The scFv genes are inserted directly upstream of the gene for pIII, which results in fusion proteins upon expression. The phage particles produced from a phage vector are homogeneous in their content of pIII, whereas the phage particles produced from a phagemid vector in combination with a helper phage have pIII from both the phagemid (fusion proteins) and from the helper phage (non-fusion). The phage vectors offer the advantage that all phage particles display scFvs and therefore enable a more efficient selection. In contrast, the phage particles produced from phagemid systems are monovalent. On average the phage particles displaying a fusion protein display only one, whereas the vast majority do not display any (O’Connell et al., 2002); this is a clear advantage when high affinity is desired. The other advantage of phagemid vectors is their relative high transformation efficiency allowing the generation of libraries with higher diversity and the accurate measurement of the antibody library size (Hong et al., 2004).

Antibody repertories can be divided into categories based on the source used for their generation (Hoogenboom & Chames, 2000) (Figure 2.5). Natural antibody repertoires can be created from B-lymphocyte mRNA obtained from either immunised or non-immunised donors. The “immune” repertoires (Duggan et al., 2001, Okamoto et al., 2004) will be biased by the immune response of the donor and will therefore be enriched for antibodies directed against the antigen used for immunisation. In addition, several of the antibody genes may contain somatic hypermutations, and therefore encode high affinity antibodies (Hoet et al., 2005). However, there are several drawbacks: immunisation may not result in an immune response, toxic antigens may kill the donor, tolerance may limit or prevent an immune response toward self-antigens, ethical concerns may hinder active immunisation of humans, and the applicability range of a repertoire is limited to selections against the immunising antigen. In contrast, naïve repertoires (reviewed by (Winter et al., 1994)) can be applied in the selections against a wide range of targets, including those that cannot be used in immunisations. The caveats are the diversity requirement and the potential bias by the donors’ immunoglobulin repertoire.
To extend the repertoire diversity, “synthetic” repertoires have been generated in which parts of the natural antibodies are randomised (Sidhu et al., 2004). The classic approach is to exchange the CDR3 regions, which are normally created by the recombination of the V(D)J gene segments, with synthetic oligonucleotides. This strategy can be further extended to cover the other CDR regions (Fellouse et al., 2004). The synthetic repertoires are not biased, and can generally be applied in selections against any antigen, including self-antigens and toxic antigens. The synthetic repertoires may contain high fractions of non-active or unfolded antibodies (Rubinstein et al., 2003).

**Morphology and life cycle of the filamentous bacteriophage Ff**

The filamentous bacteriophage Ff is a rod-shaped particle (Figure 2.6) about 900 nm in length and 6.5 nm in diameter. It can be propagated by infection of *E. coli* that contain the F conjugative plasmid. The genome of the Ff bacteriophage is a covalently closed single-stranded DNA molecule (ssDNA) of about 6,400 nucleotides, encoding 11 proteins (Table 2.5) that can be grouped according to their
functions in the phage morphology or to their roles in the phage lifecycle (Russel, 1991, Russel et al., 1997).

Table 2.5. F-specific filamentous phage genes/proteins and properties. Data sourced from Russel et al. (1991).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Size (aa)</th>
<th>Function</th>
<th>Location</th>
<th>Used for display?</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>I</td>
<td>348</td>
<td>Assembly</td>
<td>Inner membrane</td>
<td></td>
</tr>
<tr>
<td>XI</td>
<td></td>
<td>108</td>
<td>Assembly</td>
<td>Inner membrane</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>II</td>
<td>409</td>
<td>Replication (nickase)</td>
<td>Cytoplasm</td>
<td></td>
</tr>
<tr>
<td>X</td>
<td></td>
<td>111</td>
<td>Replication</td>
<td>Cytoplasm</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>III</td>
<td>406*</td>
<td>Virion component</td>
<td>Virion tip (end)</td>
<td>Yes (N-terminus)</td>
</tr>
<tr>
<td>IV</td>
<td>IV</td>
<td>405*</td>
<td>Assembly (exit channel)</td>
<td>Outer membrane</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>V</td>
<td>87</td>
<td>Replication (ssDNA bp)</td>
<td>Cytoplasm</td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>VI</td>
<td>112</td>
<td>Virion component</td>
<td>Virion tip (end)</td>
<td>Yes (C-terminus)</td>
</tr>
<tr>
<td>VII</td>
<td>VII</td>
<td>33</td>
<td>Virion component</td>
<td>Virion tip (start)</td>
<td>Yes (N-terminus)</td>
</tr>
<tr>
<td>VIII</td>
<td>VIII</td>
<td>50</td>
<td>Virion component</td>
<td>Virion filament</td>
<td>Yes (N- and C-termini)</td>
</tr>
<tr>
<td>IX</td>
<td>IX</td>
<td>32</td>
<td>Virion component</td>
<td>Virion tip (start)</td>
<td>Yes (N-terminus)</td>
</tr>
</tbody>
</table>

a: Mature protein without signal sequence.

The phage capsid consists of 5 different proteins of which pVIII (50 aa) is the most abundant. pIII, pVI, pVII and pIX (406, 112, 33 and 32 aa, respectively) are all present in a few copies at the ends. The actual wall of the rod-like phage particle is composed of pVIII (2,700-2,800 copies). All the capsid proteins have hydrophobic membrane spanning domains and reside in the inner membrane until they are assembled into the mature phage particle (Willats, 2002) (Figure 2.6).

The infection process is initiated when the phage particle binds to the first of the 2 bacterial receptors needed for infection, the F-pilus, via an interaction with domain N2 of the pIII (Karlsson et al., 2003) as shown in Figure 2.7. Subsequently, domain N1 of pIII can interact with the second bacterial receptor, TolA, and complete the infection (Click & Webster, 1997, Deng & Perham, 2002, Deprez et al., 2002, Lubkowski et al., 1999, Nilsson et al., 2000).
Figure 2.6. Schematic illustration of M13 bacteriophage particle showing the organisation of the 5 coat proteins. The approximate number of copies of each M13 coat protein is indicated. The model was sourced from Willats (2002).

Three proteins encoded by the phage genome are involved in the replication of phage DNA. pII (410 aa) binds to the double-stranded replicative-form of the phage genome and introduces a specific cleavage on the (+) strand at the origin of replication. Upon completion of the rolling circle replication, the ssDNA is circularised by pII, and a stabilising complex is formed with pV (87 aa). pX (111 aa) is the product of an internal initiation of gene II at methionine 300, and probably controls DNA replication (Horiuchi, 1997).

The last 3 proteins encoded by the genome are required for the assembly of the phage coat. The pI (348 aa), pXI (108 aa) (Haigh & Webster, 1999) and pIV (405[+21] aa) (Marciano et al., 2001) proteins form a channel in the bacterial membrane and are required for the phage assembly and release. It has been speculated that this channel may set the restrictions on maximum size of proteins that can be displayed on phage (Marvin, 1998, Russel, 1991, Russel et al., 1997, Stassen et al., 1994).
Figure 2.7. Schematic illustration of the bacteriophage infection process via the 2 E. coli receptors needed: Pilus and TolA. Pilus is usually involved in the conjugal transfer of DNA between E. coli. It consists of a protein tube assembled by polymerisation of pilin subunits from the bacterial inner membrane. The pilin subunits are encoded by the traA operon on the F conjugate plasmid (Manchak et al., 2002). Upon interaction with N2 of pIII, pilus retracts and N1 of pIII can interact with the TolA leading to completion of infection. The model was sourced from Karlsson et al. (2003).
Chapter 3
Solubilisation and purification of insoluble recombinant JDV-ΔSU present in inclusion bodies

Summary
Methods were developed to express, solubilise and refold a truncated recombinant surface unit (ΔSU) protein of Jembrana disease virus that was expressed as an insoluble product in *Escherichia coli*. The protein was engineered with a glutathione-s-transferase (GST) tag to facilitate purification and was truncated by deletion of the N-terminal region of the protein to eliminate the hydrophobic transmembrane domain and cytoplasmic tail of the full-length protein but this truncation of the protein failed to increase the solubility of the protein expressed in *E. coli* and it was produced as an insoluble product within inclusion bodies. To purify the insoluble protein present within inclusion bodies, the inclusion bodies were partially purified from lysed bacterial cells by an initial washing step to separate the inclusions from cellular lipids and membranes. The effect of different buffers on the solubilisation of ΔSU inclusion bodies was examined and a denaturing buffer of 2 M Tris-HCl, pH 12, containing 2 M urea and 10 mM DTT was selected as an optimal solubilising buffer. The solubilised denatured proteins were then refolded by slowly diluting the protein solution with 10 volumes of refolding buffer (20 mM Tris pH 8.0 containing 0.1% CHAPS, 10 mM DTT and 1 mM PMSF). Final purification of the ΔSU was achieved by affinity column purification of the tagged protein with glutathione-Sepharose 4B resin (BioRad) and cleavage of the GST tag with PreScission™ protease (GE Healthcare). The purified solubilised ΔSU protein could be considered for use as a vaccine protein or antigen for serological tests.
Introduction

Recombinant proteins are widely used in medicine for production of vaccines and therapeutic proteins for human (Green & Gaston, 2006) and animal diseases (Weerasinghe et al., 2006). They are also used as antigens for immunological tests (Zhang et al., 2006).

Production of any recombinant protein in a heterologous host cell or organism is a complex multi-step process and is often problematic. Protein expression levels can be low or the purified protein may be insoluble or unstable. To generate large amounts of recombinant proteins, optimal conditions must be determined which require selection of an appropriate expression vector and expression host, and the ability of the fusion partner to facilitate purification of the expressed protein.

Glutathione S-transferase (GST) fusion vectors with expression in an *Escherichia coli* vector provide high transformation efficiency, simplicity and low cost of production, and are frequently chosen and used for recombinant protein production and purification (Terpe, 2006). GST fusion vectors provide GST fusion proteins that can be purified by binding to immobilised glutathione followed by competitive elution of the immobilised protein with reduced glutathione (Smith & Johnson, 1988). However, a significant problem with GST fusion proteins is that they are often expressed as insoluble proteins that are difficult to purify by standard procedures (Hollenbach et al., 1999, Iwata et al., 2000, Mercado-Pimentel et al., 2002, Terpe, 2003, Wang et al., 1997).

Recombinant JDV capsid (CA) and transmembrane (TM) proteins have been expressed in *Escherichia coli* as a fusion protein with GST (Burkala et al., 1998). The soluble fraction of the expressed proteins was purified using affinity chromatography with reduced glutathione and then used as an antigen to develop serological tests for JDV (Burkala et al., 1998). The majority of the expressed protein, however, was present as an insoluble product and yields would have been markedly enhanced if the insoluble product had been solubilised. An effort to increase the solubility of the expressed JDV fusion proteins by truncation of the protein still resulted in a considerable proportion of the expressed protein being present as an insoluble product within inclusion bodies (Burkala et al., 1998). This Chapter reports the development of a simple process for solubilisation and purification of the insoluble JDV recombinant ΔSU protein present in inclusion bodies that considerably enhanced the yield of soluble protein.
Material and methods

Protein expression

The map of the expression cassette for a fusion of the surface unit protein is shown in Figure 3.1. *E. coli* DH1 (F-, endA1, hsdR17 (rK-, mK+), supE44, thi-1, λ−, recA1, gyrA96, relA1) transformed with the pGEX-6P-1 plasmid containing JDV nucleotides 5556 – 6463 (numbering as described by (Chadwick et al., 1995b)) was grown overnight with shaking 225 rpm in 5 ml of Luria-Bertani (LB) medium supplemented with 100 µg of ampicillin per ml as a selection agent at 37°C. The overnight culture was diluted 1:40 in 200 ml of fresh LB-ampicillin medium, grown again at 37°C to an OD_{600} of 0.6 – 0.8, when pre-induction samples were taken as controls and protein production was induced with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 4 h at 37°C. During incubation, post-induction samples were taken every 1 h. Samples were then centrifuged (4,000 g for 10 min at 4°C) and cell pellets were resuspended in reducing SDS-PAGE loading buffer prior to electrophoresis.

Figure 3.1. Map of vector pGEX-6P-1 used to produce recombinant ΔSU in *E coli* (from Amersham website http://www4.amershambiosciences.com/pdfs/970004M2-01.pdf).
After 4 h of induction, the bacteria were harvested by centrifugation (4000 g for 10 min at 4°C) and the bacterial pellets were suspended in 10 ml of ice cold phosphate-buffered saline (PBS; 2 mM KCl, 140 mM NaCl, 10 mM K$_2$HPO$_4$, 2 mM KH$_2$PO$_4$, pH 7.4). Lysis of the bacterial cells was achieved by the addition of lysozyme (1 mg/ml final concentration) and incubation on ice for 15 min with occasional mixing, followed by freeze-thawing using liquid nitrogen, which resulted in a dramatic increase in viscosity. The viscosity was then reduced by treatment with benzonase (Novogen) (10 µg/ml final concentration) in the presence of 10 mM MgCl$_2$. The soluble and insoluble fractions of the *E. coli* lysate were separated by centrifugation (10,000 g for 20 min at 4°C). The supernatant fractions were collected and stored at –20°C for further analysis and the residual pellets were used for solubilisation.

**Solubilisation of insoluble proteins in inclusion bodies**

The pelleted inclusion bodies were washed 3 times with washing buffer (50 mM Tris-HCl buffer pH 8.0, containing 5 mM EDTA and 1% Triton X-100) by centrifugation (10,000 g for 15 min) after each wash step. The pelleted material was then washed once with distilled water to remove salt and detergent and centrifuged again (10,000 g for 30 min). The pelleted material was then resuspended in 50 mM Tris-HCl buffer at pH 8.0.

To determine the effect of different solubilisation buffers, 100 µl volumes of resuspended washed inclusion bodies were placed in microcentrifuge tubes and centrifuged (4,000 g for 10 min at 4°C). The supernatant was discarded and 1 ml of the various solubilisation buffers was added to each of the pellets. The suspensions were vortexed and left for 1 h at room temperature. The solubilisation buffers tested included 100 mM Tris-HCl buffer with or without additional 2% urea at pH 7 to pH 13 and other solubilisation buffers listed in Table 3.1. The relative soluble protein concentration of the suspensions was determined by centrifugation (10,000 g for 10 min) of the suspension, followed by filtration of the supernatant through a 0.45 µm filter and then determination of the relative protein concentrations by measuring the absorbance of the suspensions at 280 nm ($A_{280}$), where $A_{280} = 1.00$ is assumed to correspond to 1 mg of protein per ml (Lowry et al., 1951).
Table 3.1. Buffers tested for solubilisation of insoluble JDV ΔSU within inclusion bodies.

<table>
<thead>
<tr>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. 2 M Tris-HCl pH 12</td>
</tr>
<tr>
<td>B. 2 M Tris-HCl pH 12, plus 2 M urea and 10 mM DTT</td>
</tr>
<tr>
<td>C. 2 M Tris-HCl pH 12, plus 10 mM DTT</td>
</tr>
<tr>
<td>D. 50 mM Tris-HCl pH 8.5, plus 1% Sarkosyl</td>
</tr>
<tr>
<td>E. 50 mM Tris-HCl pH 8.5 plus 8 M urea</td>
</tr>
<tr>
<td>F. 50 mM Tris-HCl pH 8.5 plus 6 M guanidine-SCN</td>
</tr>
<tr>
<td>G. 50 mM Tris-HCl pH 8.5 plus 1% SDS</td>
</tr>
<tr>
<td>H. 100 mM Tris-HCl buffer pH 12.5, plus 2 M urea and 10 mM DTT</td>
</tr>
</tbody>
</table>

**Refolding of solubilised protein**

The solubilised ΔSU-GST was refolded by diluting the protein solution slowly with 10 volumes of refolding buffer (20 mM Tris pH 8.0 containing 0.1% CHAPS, 10 mM DTT and 1 mM phenyl methyl sulfonyl fluoride, a serine protease inhibitor [PMSF]) followed by storage overnight at 4°C.

**Cleavage of GST-JDV ΔSU**

After refolding, the fusion protein was applied to a disposable gravity column (Bio-Rad) containing 2 ml of glutathione-Sepharose 4B resin (Amersham Biosciences) equilibrated previously with binding buffer (PBS, pH 7.4) at 4°C. The column matrix was washed twice with the binding buffer and then that buffer was substituted with cleavage buffer (50 mM Tris HCl, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 8.0) to pre-equilibrate the column, altering the buffer composition required for efficient cleavage by PreScission™ protease (GE Healthcare). PreScission protease™ (2 U/100 μg of ΔSU-GST protein) was diluted in cleavage buffer and injected onto the column. Following the injection, the column was placed in a closed flow status at 4°C overnight. An elution buffer containing reduced glutathione (50 mM Tris-HCl and 10 mM reduced glutathione, pH 8.0) was then applied in a one-step gradient (100%) to elute the GST and PreScission™ protease. A series of 1 ml eluates were collected and stored on ice, then the proteins were precipitated with acetone and
ethanol, and stored at -20°C until subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

**SDS-polyacrylamide gel electrophoresis**

Fractions collected during the expression and purification procedures were subjected to SDS-PAGE in a Mini Protean III cell (Bio-Rad) for 1 h at 200 V by using a 4% stacking gel and a 12.5% polyacrylamide separation gel as described by Laemmli (1970). The samples were heated at 95°C for 10 min before electrophoresis. After electrophoresis, gels were stained overnight on a rocking platform at room temperature in a solution containing 0.1% (w/v) Coomassie brilliant blue R-250 (Bio-Rad) in 40% methanol, 50% water and 10% acetic acid. Excess dye was removed by multiple washing in 40% methanol, 50% water and 10% acetic acid.

**Results**

**Expression and solubilisation of inclusion body protein**

The recombinant fusion protein ΔSU-GST of ~65 kDa (ΔSU at ~40 kDa plus GST at ~26 kDa) was detected after induction of the cultures with IPTG and was present in the insoluble fraction of the cell lysate, presumably within inclusion bodies. The proteins present within the centrifuged and washed pelleted material are shown in Figure 3.2 (lane 2). This material contained not only ΔSU-GST but also other cellular proteins.
Figure 3.2. Coomassie brilliant blue stained SDS-PAGE of fractions collected during solubilisation, refolding, and cleavage of GST from to purify recombinant ΔSU-GST fusion protein. A: lane 1, MW markers; lane 2, pellet after cell lysis and washing; lane 3 and 4, supernatant and pellet after solubilisation in buffer B (2 M Tris pH 12 supplemented with 2 M urea and 10 mM DTT); lane 5, supernatant after refolding in refolding buffer (20 mM Tris pH 8.0 containing 0.1% CHAPS, 10 mM DTT and 1 mM PMSF); lane 6, 7 and 8, serial eluates from a glutathione Sepharose 4B column loaded with refolded protein (as illustrated in lane 5) and eluted with binding buffer. B: lane 1, MW markers in kDa; lanes 2-9, serial eluates from 10 mM reduced glutathione column containing bound ΔSU-GST fusion protein after treatment with PreScission™ protease.

The solubilisation of the insoluble ΔSU-GST product within the inclusion bodies was pH dependent in 100 mM Tris-HCl buffer. The solubilisation increased as the pH increased, increasing particularly from pH 10 to a maximum at pH 13. The addition of 2% urea to the 100 mM Tris-HCl buffer did not significantly affect the solubilisation (Figure 3.3).
Comparison of the solubilisation of the washed inclusion bodies and other insoluble cell products with the buffers shown in Table 3.1 is shown in Figure 3.4. The greatest solubilisation was achieved with 50 mM Tris-HCl buffer at pH 8.5 containing 6 M guanidine (Buffer F), followed by 2 M Tris-HCl at pH 12 containing 2 M urea and 10 mM DTT (Buffer B). An example of the product solubilised in 2 M Tris-HCl at pH 12 containing 2 M urea and 10 mM DTT is shown in Figure 3.2A, lane 2.
Figure 3.4. Relative yield of soluble recombinant ΔSU-GST protein consequent of treatment of washed inclusion bodies with various solubilising buffers, determined by measurement of the A_{280}. A, 2 M Tris-HCl pH 12; B, 2 M Tris-HCl pH 12 plus 2 M urea and 10mM DTT; C, 2 M Tris-HCl pH 12 plus 10 mM DTT; D, 50 mM Tris-HCl pH 8.5 plus 1% sarkosyl; E, 50 mM Tris-HCl pH 8.5 plus 8 M urea; F, 50 mM Tris-HCl pH 8.5 plus 6 M guanidine; G, 50 mM Tris-HCl pH 8.5 plus 1% SDS; H, 100 mM Tris-HCl pH 12.5 plus 2 M urea and 10 mM DTT.

As it was deemed that 6M guanidine could not be used for preparation of vaccine material (see Discussion), further solubilisation was conducted with Buffer B (2M Tris pH 12, 2 M urea and 10 mM DTT). SDS-PAGE of the supernatant and the non-solubilised pellet resulted in weak bands due to a diluted protein samples used. Some impurities from the host cell proteins were detected (Figure 3.2A, lane 3 and 4). The supernatant was then refolded overnight (Figure 3.2A lane 5) and applied directly to a glutathione Sepharose 4B column. Unattached material was eluted with binding buffer (PBS, pH 7.4) as shown in Figure 3.2A, lane 6). The buffer applied to the column was then changed to the cleavage buffer which provided more stringent wash conditions to release the ΔSU-GST fusion proteins, and PreScission™ protease to cleaves the GST-tag. After incubation period in a closed flow situation, the bound ΔSU-GST was eluted as 2 separate proteins of 40 kDa (ΔSU) and 26 kDa (GST) as shown in Figure 3.2B.

**Discussion**

The expression of recombinant JDV ΔSU-GST in *E. coli* resulted in mostly insoluble protein. Protein insolubility may be caused by a variety of factors including the need
for general molecular chaperones, cofactors, or protein partners for proper folding (Luo & Hua, 1998, Sun et al., 2005). The properties of proteins, such as the hydrophobic and hydrophilic regions of the aa in the protein sequences govern the protein structure and folding. In Figure 3.5 the distribution pattern along the aa sequences of full length JDV SU shows a highly hydrophobic region in the N-terminal region. This hydrophobic N-terminal end may be the decisive factor that contributes to the formation of full length-SU inclusion bodies. The proteins are more predisposed to aggregation due to increased intermolecular interactions of regions of the folding polypeptide chain. The truncation of the SU (ΔSU ) protein in the current study was designed to eliminate the hydrophobic region near the N-terminus of the full-length protein but this still did not prevent expression in an insoluble form.

Figure 3.5. Hydrophobicity analysis of the N-terminus of the 422 aa SU protein of JDV. The profile was determined using the Kyte and Doolittle scale; hydrophilic residues have a negative score (Kyte & Doolittle, 1982).

The formation of protein aggregates within cells is a general cellular response to the presence of mis-folded proteins when the proteins are expressed in a concentration exceeding the degrading capacity of the cells (Markossian & Kurganov, 2004). The expression of recombinant proteins within inclusion bodies in *E. coli* is affected by the specific growth conditions including growth temperature and time of induction with inducers, such as IPTG (Kang et al., 2007, Vera et al., 2007, Yeo et al., 2009)
but it is difficult to prevent their formation (Peternel et al., 2008). When present, it is often necessary to convert the insoluble recombinant proteins and possibly improperly folded protein into a soluble and correctly folded product to obtain a product that is biologically active (Singh & Panda, 2005). Purification of the inclusion bodies containing the protein of interest can assist in the removal of contaminating cell debris and host cell proteins from the final recombinant protein; impure inclusion body preparations result in a less effective refolding and necessitate further purification steps (Clark, 2001).

In this current study, advantage was taken of the presence of the expressed protein in an insoluble form in inclusion bodies to separate the inclusion bodies from bacterial cell proteins, resulting in partial purification of the expressed recombinant protein. This was easily achieved following disruption of the host cells by washing the pelleted inclusion bodies with 50 mM Tris buffer (pH 8) containing 1% Triton X-100 to remove lipid and *E. coli* membrane-associated proteins. The success of this initial step was illustrated by the presence of a high proportion of ΔSU protein, relative to other proteins, in the solubilized inclusion body preparations (Figure 3.2A, lane 2).

A number of methods and strategies have been used previously for converting inclusion body proteins into their soluble form (reviewed by Fischer, 1994, and Misawa & Kumagai, 1999). The suitability and use of each method has varied. Some have resulted in very low yields and some are incompatible with protein purification procedures that require the protein to remain in solution as they may result in re-aggregation and precipitation of the protein after removal of the solubilising agent (Goldberg et al., 1991, Sunitha et al., 2000). The various methods used have included high concentration of chaotropic agents such as urea (Cabanne et al., 2005) and guanidine hydrochloride (Tsumoto et al., 2003b), the use of detergents such as sodium dodecyl sulphate (SDS) (Lechtzier et al., 2002) and sarkosyl (Mercado-Pimentel et al., 2002, Zhuo et al., 2005) with reducing agents like dithiothreitol (DTT).

The need to consider the end use of the protein during the selection of the solubilising agents is illustrated by 6M guanidine-hydrochloride. This is used extensively to regenerate active proteins from inclusion bodies, but is not acceptable for proteins to be employed as vaccines since guanidine may be deleterious to the immunogenicity of the protein, and during attempts to remove the guanidine from the protein, either by dialysis (Tsumoto et al., 2003a) or ion-exchange chromatography (Ejima et al., 1999), significant re-aggregation of the
protein may occur. Meanwhile, urea has been generally favoured over the use of guanidine, providing for better refolding and improved yields (Wang et al., 2005a) and an improved immune response (Rothel et al., 1997). Detergents such as SDS are highly effective denaturing agents but bind tenaciously to the denatured proteins, making its complete removal from the protein problematic (Arakawa et al., 1994).

The method chosen for solubilisation of the insoluble SU protein involved the use of 2 M urea in 2 M Tris buffer pH 12, with the addition of 10 mM DTT. This provided considerable greater solubilisation of the insoluble proteins in inclusion bodies than other buffers investigated, with the exception of 6 M guanidine (see Figure 3.4, buffer B). Urea at a concentration of 2 M was less than the 8 M concentration that has been used in many previous studies (Tsumoto et al., 2003a, Villaverde & Carrio, 2003). The solubilisation of inclusion bodies expressed in E. coli by alkaline solutions has been previously reported (Jin et al., 1994, Lavallee et al., 1993, Suttnar et al., 1994). In the current study a high pH appeared to have a crucial role in destabilizing the aggregated proteins in the inclusion bodies. The addition of 10 mM DTT, a thiol containing reducing agent, allowed reduction of inter-chain disulfide bonds by thiol-sulfide exchange and increased solubilisation of the aggregated protein. The use of DTT has the advantage that it does not have to be removed prior to initiation of the refolding.

The molecular basis for the denaturation of proteins by urea remains unknown, despite its widespread use. Urea may act directly, by binding to the protein, or indirectly, by altering the solvent environment. Most versions of the direct interaction model (Auton et al., 2007, Dotsch et al., 1995, Zou et al., 1998) postulate that urea binds to and then stabilizes the denatured protein, thereby favouring unfolding (Liu et al., 1997). Alternatively, urea acts indirectly by altering the solvent environment, thereby mitigating the hydrophobic effect and facilitating the exposure of residues in the hydrophobic core (Courtenay et al., 2001, Tobi et al., 2003). The mechanism of urea-promoted unfolding may depend on the urea concentration (Timasheff & Xie, 2003).

A decrease in the concentration of Tris-HCl buffer from 2 M in the solubilisation buffer to 20 mM in the refolding buffer, in the presence of 0.1% CHAPS and 10 mM DTT, maintained the protein in a soluble format without aggregation. This was fortunate as aggregation is a phenomenon common to many refolded proteins (Bondos & Bicknell, 2003). The addition of the zwitterionic detergent CHAPS to the refolding buffer may have contributed to the continued solubility of the ΔSU protein;
this is one of a series of useful kosmotropes (salts that stabilize protein structures), chaotropes (salts that destabilize protein structures), aa, sugars, and detergents that can contribute to maintaining proteins in a soluble state (Bondos & Bicknell, 2003).

An attempt was made to cleave the GST tag from the ΔSU protein in the current study. Sometimes, it is necessary to remove the tag since it can cause unwanted immunological responses, inactivate the fused target protein or influence the quality of binding properties (Buning et al., 1996, Nilsson et al., 1997). Since GST is a relatively large tag (26 kDa), it is preferable to remove it to determine whether the truncated SU protein is soluble and properly folded. While several methods have been described for site-specific cleavage treatment of the fusion partner, enzymatic methods are preferred due to their specificity and milder reaction conditions required. The GST vector system (pGEX-6P-1) used in this study allows for purification of the GST fusion protein and subsequent cleavage of GST from the protein using the PreScission protease, which specifically recognizes the aa sequence Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro, cleaving between the Gln and Gly residues (Cordingley et al., 1990). Upon removal of the GST-tag with protease, SDS-PAGE identified 2 bands (~40 kDa and ~26 kDa), correlated with the ΔSU and GST proteins. Further testing of the eluted ΔSU is required as a possible complication associated with the use of reduced glutathione for elution is that it can affect target proteins containing disulfides (Sassenfeld, 1990) and the proteases that cleave after a basic residue such as thrombin and factor Xa, sometimes produce non-specific and unwanted products (Jenny et al., 2003).
Chapter 4
Development of monoclonal antibody for the detection of recombinant JDV proteins

Summary
This chapter reports the attempted generation of hybridomas producing monoclonal antibodies (MAbs) against the recombinant capsid (CA), truncated SU (ΔSU) and Tat proteins of JDV using hybridoma technology. BALB/c mice were immunised 4 times subcutaneously at 2-week intervals with the proteins excised from SDS-PAGE gels. Hybridomas secreting monoclonal antibodies against the proteins were developed by fusion of the spleen cells of immunised mouse with SN0 myeloma cells and detection of antibody secretion by western immunoblotting. Antibody secreting hybridomas were cloned by limiting dilution and the isotypes of the ΔSU and Tat MAb were determined as IgM and the CA MAb as IgG. A CA MAb designated LD1 was shown to be an effective reagent for use in a capture ELISA for quantitation of viral antigen and for an immunoperoxidase assay for the detection of viral antigen in tissue sections.
Introduction

Following the discovery of hybridoma technology (Kohler & Milstein, 1975) and the consequent development of monoclonal antibody (MAb) production technology, the use of MAbs has been extremely useful in medical and biological research, enabling a multitude of tasks such as the detection of viral antigens (Keuser et al., 2004, Oldoni et al., 2004), tissue and blood typing (Lee et al., 2004a, Noda et al., 2002), the identification of hormones (Fortunati & Frairia, 2003) and the identification of tumour antigens (Nustad et al., 2004).

A range of MAbs against JDV proteins would be extremely valuable for diagnostic purposes and particularly to aid in the identification of individual viral proteins. One technique that has been successfully used for the detection of JDV in tissues of cattle is immunoperoxidase staining, valuable as it can be conducted using fixed tissue sections. For immunoperoxidase staining, monoclonal antibodies produced previously against the 26 kDa CA protein (Kertayadnya et al., 1993) from mice immunised with whole viral preparations, have been used but unfortunately the hybridomas producing these MAbs were lost due to the difficulty of maintaining liquid nitrogen supplies in Indonesia. Monoclonal antibodies would also be extremely useful reagents for the characterisation of JDV proteins. Although the size of the native non-glycosylated proteins can be predicted and therefore identified with confidence in western immunoblotted viral preparations, the glycosylated native SU and TM proteins have not been characterised with regard to size. Monoclonal antibodies produced against recombinant SU and TM proteins would be extremely useful for characterisation of the native viral envelope glycoproteins and would then have application for improved diagnostic methodology. The MAbs would also have application during the development of Jembrana disease vaccines incorporating individual recombinant viral proteins.

This Chapter reports the production of MAb against the JDV-CA protein that was used for the development of an antigen capture ELISA and for immunoperoxidase staining for the detection of JDV antigen in tissues. It also describes the production of MAb against recombinant ΔSU and Tat fusion proteins to assist in the further characterisation of these proteins.
Material and methods

Preparation of antigens for immunisation of mice and serological tests

GST-fusion proteins

Recombinant glutathione-S-transferase (GST) JDV CA, ΔSU and Tat proteins were expressed from JDV\textsubscript{Tab/87} genes ligated into the pGEX-6-P system (Pharmacia). The JDV CA constructs were prepared as previously described (Burkala et al., 1998) and contained the full-length JDV \textit{capsid} gene. JDV Tat was prepared as previously described from exon 1 of the \textit{tat} gene (Setiyaningsih, 2006). JDV ΔSU was prepared as previously described in Chapter 3. All proteins were purified using the methods described in Chapter 3 except that GST was not cleaved from the final purified fusion protein (as these studies were undertaken prior to the studies reported in Chapter 3). A summary of the size of the proteins is shown in Table 4.1.

Table 4.1. Recombinant fusion proteins produced and their determined molecular weight.

<table>
<thead>
<tr>
<th>Fusion protein</th>
<th>Molecular weight (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA-GST</td>
<td>~ 50</td>
</tr>
<tr>
<td>CA-biotin</td>
<td>~ 37</td>
</tr>
<tr>
<td>ΔSU-GST</td>
<td>~ 65</td>
</tr>
<tr>
<td>ΔSU-biotin</td>
<td>~ 53</td>
</tr>
<tr>
<td>Tat-GST</td>
<td>~ 38</td>
</tr>
<tr>
<td>Tat-biotin</td>
<td>~ 27</td>
</tr>
<tr>
<td>GST</td>
<td>~ 26</td>
</tr>
</tbody>
</table>

GST protein

Preparation of GST for use as a control reagent was as previously described by Ditcham (2007). \textit{Escherichia coli} transformed with the pGEX plasmid with no insert were grown in 2YT supplemented with ampicillin (100 µg/ml) and induced with IPTG (final concentration 0.1 mM) at a culture \textit{OD}_{600} of 0.6. After 4 h of further growth, the bacteria were harvested by centrifugation (18,000 g, 3 min), washed in PBS by centrifugation and resuspended in 50 µl of lysis buffer/ml of original culture, and lysed by freezing and thawing. After clarification of the lysate by centrifugation, the
supernatant was passed 3 times through a column of Glutathione-Sepharose resin (0.2 ml volume, equilibrated with PBS). Bound and soluble GST was displaced with 10 column volumes of 30 mM glutathione in PBS.

Biotinylated fusion proteins

Biotinylated fusion proteins for immunisation of mice that would be recognised only by antibody to the JDV protein and not the GST fusion tag, were produced as previously described by Ditcham (2007). The same genes cloned into a pGEX-6P expression vector for the production of GST fusion proteins were ligated into the PinPoint Xa-1 T-tailed vector (Promega) for the expression of biotinylated proteins in *E. coli*. Briefly, the recombinant plasmids were transformed by heat-shock into JM109 competent *E. coli*. The transformed cells were then plated onto LB agar plates supplemented with 100 µg/ml ampicillin and incubated (37°C, overnight). The colonies were screened for transformants with inserts in the correct orientation by directional PCR with standard thermocycling conditions and a Tm of 50°C. Positive clones were verified by sequencing. Protein expression was induced by the addition of IPTG to a final concentration of 0.1 mM in LB broth, supplemented with 100 µg/ml ampicillin and biotin at a concentration of 2 µM. The expression of the proteins was monitored by SDS-PAGE and western immunoblotting using an alkaline phosphatase labelled streptavidin probe and western Blue chromogenic substrate (Promega).

For immunisation of mice, fusion proteins were first separated by SDS-PAGE (30% acrylamide, 0.3% bisacrylamide) and extracted from gels as described by Prussack (1989), with slight modification. Briefly, after SDS-PAGE, gels were placed in ice-cold 0.25 M KCl for 20 min to identify the protein bands. The bands of the appropriate size were then excised and eluted from the gel slices in 700 µl of elution buffer (0.01% SDS, 20 mM Tris-HCl pH 7.9, 1 mM CaCl₂) overnight at 37°C. The eluate was then centrifuged and unbound SDS was precipitated by the addition of KCl to a final concentration of 50 mM on ice for 10 min. The mixtures were centrifuged (10,000 g for 5 min) and the supernatant containing recombinant proteins were stored at -20°C until required.
Production of fragments of Gag protein

Biotin tagged truncated gag fusion proteins as shown schematically in Figure 4.1 were produced in *E. coli* JM109 following the procedure described by (Desport et al., 2005). Briefly, early log phase cultures were induced by the addition of IPTG and recombinant fusion proteins were harvested by pelleting the cultures by centrifugation. Pelleted cells were resuspended in cold lysis buffer, sonicated and centrifuged again. The biotinylated proteins were purified in batch by mixing the cell lysates with equilibrated Steptavidin Softlink resin (Promega). The bound proteins were washed with cold lysis buffer and then eluted from the resin by the addition of 5mM biotin in cold lysis buffer. Total and purified protein concentrations were determined using an assay based on the Bradford method (BioRad) with bovine serum albumin standards.

![Figure 4.1](image-url)

Figure 4.1. Schematic representation of primer combinations used to amplify JDV gag sequences for cloning in-frame into Pinpoint Xa-1 for protein expression. The picture sourced from Desport et al. (2005).

Generation of MAb-producing hybridomas

**Immunisation of mice**

A 500 µl volume of each biotinylated CA, ΔSU, and Tat recombinant protein extracted from SDS-PAGE gels was homogenised with an equal volume of incomplete Freund’s adjuvant (Sigma) and 200 µl of the suspension was injected subcutaneously into 8-week old female BALB/c mice. Further injections with freshly prepared emulsions were given at 2-week intervals. Four days after the fourth injection the mice were bled, the blood was allowed to clot at 4°C, the serum was removed from the clot following centrifugation (10,000 g for 10 min) and specific
antibody was detected by ELISA. If antibody was detected, the mice required for fusions were re-injected with a further 200 µl of freshly prepared protein emulsion and used for fusions 4-7 days later.

**Preparation of spleen cells**

When required for fusions, immunised mice were euthanised and the heart was punctured to obtain about 300 µl of blood, which was subsequently examined for antibody. In a laminar flow hood, the skin of the mouse was swabbed with 70% alcohol, the abdominal skin cut and resected, and the peritoneal cavity was opened and the spleen was removed and placed in a plastic 50 mm cell culture dish (Nunc) containing 10 ml Dulbecco’s modified Eagle’s medium (DMEM). The spleen was cut into several pieces and disrupted using small gauge needles on sterile 10 ml disposable syringes. After removal of the connective tissue and other large pieces of cellular debris, the cell suspension containing an estimated 10⁸ splenocytes was placed in a centrifuge tube. An aliquot of the suspension was stained with 0.4% trypan blue and counted in a haemocytometer to ensure at least 90% viability. The spleen suspension was gently centrifuged (500 g for 5 min) to pellet the cells and the cells were then resuspended in 10 ml of DMEM and incubated at 37°C for 15 min. For the preparation of feeder cells (splenocytes), spleens from normal BALB/c mice were processed as described above.

**Preparation of myeloma cells**

Two weeks prior to fusion, a vial of NS0 myeloma cells was recovered from liquid nitrogen and thawed in a 37°C water bath. The thawed cells were immediately transferred into 10 ml of DMEM (serum free) before centrifugation (500 g for 5 min) to gently pellet the cells. The cells were resuspended in growth medium (DMEM supplemented with 10% FCS, 0.58 mg/ml L-glutamine, 0.22 mg/ml sodium pyruvate, 1 ml/L fungizone, 200 IU/ml penicillin and 200 µg/ml streptomycin). The cells were then distributed into culture flasks and incubated in a 5% CO₂-in-air atmosphere at 37°C. To ensure the cells were in active growth phase for cell fusion; the cells were subcultured daily for 3 days prior to fusion. The concentration of viable cells at the time of fusion was determined by counting the cells with a haemocytometer following staining with 0.4% trypan blue.
Fusions

Cell fusions were performed by techniques similar to those described by Chan & Mitchison (1982). The mouse spleen cell suspension (above) containing an estimated 10^8 cells was mixed with the NS0 cell suspension (above) containing an estimated 2 x 10^7 cells in a 50 ml round bottom sterile centrifuge tube and gently centrifuged (100 g for 5 min). The supernatant was discarded and the cell pellets were disrupted by gently tapping the side of the tube. One ml of 43% PEG (MW. 1300-1600; Sigma) solution was added drop-wise over a 1 min period, at the same time shaking the tube in water bath at 37°C. The cell suspension was then incubated at 37°C for 1 min, then 1 ml of serum-free DMEM was added over a 1 min period, and the suspension was shaken gently for a further 1 min. Subsequently, 10 ml of serum-free DMEM was added over 1 min. The fused cells were gently centrifuged (100 g for 5 min) and the cell pellet gently resuspended in 50 ml of DMEM-HAT medium (DMEM supplemented with 20% FCS, 100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine, 0.58 mg/ml glutamine, 0.22 mg/ml sodium pyruvate, 200 IU/ml penicillin and 200 μg/ml streptomycin). An estimated 10^6 splenocytes from another freshly killed normal mouse were added to the cell suspension as feeder cells, mixed gently, and distributed into 96-well flat bottomed cell culture microplates (Nunc) in 100 μl/well volumes (resulting in about 5 plates per fusion). Trays were placed in a 5% CO₂ incubator at 37°C.

Four days after fusion, an additional 2 drops of HAT medium containing 10^6 feeder cells (spleen cells from a normal mouse) was added to each well with a sterile Pasteur pipette, followed 2 days later by a further 2 drops of feeder cells in HT medium (HAT medium without aminopterin). After 7 days, plates were examined daily for evidence of hybridoma formation. Ten to 15 days after fusion, the medium from wells containing hybridomas was screened by western immunoblotting for antigen-specific antibodies.

Cloning hybridoma cells by limiting dilution

To assure monoclonality, antibody-producing hybridoma clones were subcloned 3 times by a limiting dilution method. The cells in antibody-positive wells were diluted in 50 μl of cell suspension and mixed with 50 μl of DMEM-HT. The cells were serially diluted (2-fold) in DMEM-HT in wells of a 96-well plate. Wells were supplemented with an equal volume of feeder spleen cells and any positive wells containing single hybridomas were checked for antibody production by western
immunoblotting. Any positive wells containing single hybridomas were re-cloned by the same method. Cloned hybridomas were expanded in number by first cultivating them in 24-well and then in 6-well plates (Nunc) and finally in 25 cm$^2$ culture flasks (Nunc).

**Storage and recovery of hybridoma cells**

Hybridomas were stored in liquid nitrogen when not required. Approximately $10^6$ cells from a 25 cm$^2$ flask were centrifuged (500 g for 5 min) and then resuspended in a mixture of 90% (v/v) FCS and 10% dimethylsulfoxide (DMSO). The cells were pipetted into 2 ml cryogenic vials and frozen slowly overnight by placing them in an insulated container at -80°C, and they were then transferred into liquid nitrogen. When required, the frozen cells were removed from the liquid nitrogen and thawed rapidly in a 37°C water bath, and then immediately transferred aseptically into 10 ml of DMEM before centrifugation (500 g for 5 min) to gently pellet the cells. The pelleted cells were resuspended in growth medium (DMEM-HAT) supplemented with feeder spleen cells. The cells were then distributed into wells and cultured in a 5% CO$_2$ incubator at 37°C.

**Western immunoblot analysis for antibodies**

Fifteen days after fusion, the supernatants of the wells that contained large hybridomas (that covered minimal 50% of the bottom of the wells) were screened for protein-specific antibody by western immunoblotting using GST-ΔSU, CA and Tat proteins produced using the pGEX expression system. The proteins were fractionated by SDS-PAGE and then transferred to nitrocellulose membranes overnight at 4°C. Non-specific binding of antibodies to the membrane was blocked by incubating the membranes in TBS (200 mM NaCl, 50 mM Tris-HCl, pH 8.0) containing 0.05% (v/v) Tween-20 (TBST) and 5% (w/v) commercial skim milk powder for 1 h at room temperature. After washing 3 times with PBST (PBS containing 0.05% Tween-20), membranes were incubated with 100 µl of the medium (diluted 1:10) from wells containing hybridomas for 3 h at room temperature. The membranes were washed again 3 times with TBST and then incubated with 1:2,000 dilution of anti-mouse IgG horseradish peroxidase (HRP) conjugate for 1 h at room temperature. After 3 additional washings, HRP colour development reagent (Bio-Rad) was added until the colour developed. The reaction was stopped by washing the membranes in deionised water.
**Determination of Ig isotype**

The Ig subclass of MAb was determined with a Mouse Typer Sub-Isotyping Kit (BioRad) according to the manufacturer’s instructions. One hundred µl of antigen (GST-ΔSU, GST-Tat and GST–CA) diluted in a carbonate buffer (pH 9.6) was added to each well of the 96-well cell culture microplates. After incubation overnight at 4°C, the solution was discarded and the plate was washed 4 times with PBST (PBS containing 0.05% Tween-20). The wells were dried by tapping them hard onto tissue paper. Two hundred µl of blocking solution (5% skim milk powder in PBS) was then added to all the wells and the plates incubated at room temperature for 1 h. The blocking solution was then discarded, the wells were washed and dried as before, and 100 µl of hybridoma supernatant was added (ΔSU and Tat supernatants were diluted 1:10 and CA supernatants were diluted 1:250 in PBST) and incubated for 1 h at room temperature. The wells were washed and dried again and 100 µl of the appropriate rabbit anti-mouse panel reagent was added for 1 h at room temperature. The wells were then washed and dried and 100 µl of goat anti-rabbit horseradish peroxidase conjugate was added and incubated for 1 h. After washing the plate, 100 µl of ABTS substrate solution was added and incubated for 15 min at room temperature when the colour development reaction was stopped by the addition of 100 µl of 2% oxalic acid.

**ELISA for detection of antibody**

To obtain an optimum combination of CA antigen and CA MAb for ELISA, a checkerboard titration of antigen and antibody was performed by indirect ELISA using a Jgag6 antigen (a full length capsid protein; Figure 4.1) at concentrations ranging from 8.8 to 87.5 ng/well and a MAb dilution ranging from 1:100 and 1:3,000. The optimum combination was selected on the basis of the antigen: MAb combination producing the highest absorbance above 0.5.

The 96-well cell culture microplates were coated with 100 µl/well of 2-fold dilutions of Jgag6 protein in a carbonate buffer (pH 9.6) and allowed to stand at 4°C overnight. The plates were then washed 4 times with PBST to remove the non-adsorbed antigen. Sites not coated with the proteins were blocked with 200 µl/well of 5% (w/v) skim milk in PBST. After incubation at room temperature for 1 h, the plates were washed and dried as described previously. Then 100 µl/well of 2-fold dilutions of MAb (diluted with PBST) was added to the plate, which was then reacted at room temperature for 1 h and washed and dried. One hundred µl/well of
goat anti-mouse IgG conjugated to horseradish peroxidase diluted 1:4,000 with PBST was added to each well and the plates were incubated at room temperature for 1 h. The plates were then washed and 100 µl/well of a substrate solution (TMB; tetramethylbenzidine, Bio-Rad) was added to each well. After colour development at room temperature, the reaction was stopped by the addition of 100 µl/well of 2% oxalic acid and the absorbance read at a wavelength of 405 nm.

**Antigen capture ELISA for quantitation of viral antigen**

An antigen capture ELISA was performed as previously described (Stewart et al., 2005). Briefly, 96-well flat bottom cell culture microplates were coated with either a 1:200 dilution of the produced MAb or with a 1:1,000 dilution of the previously produced BC10 CA MAb (Kertayadnya et al., 1993) in 100 µl carbonate buffer pH 9.6 per well, then incubated at room temperature for 1 h. The microplates were washed 3 times with PBST and blocked with 200 µl of blocking solution (2% casein and 1-5% skimmed milk powder in PBST) and incubated at room temperature for 1 h. After the plates were washed 3 times with PBST, 100 µl of Jgag6 protein (100 ng/ml) in PBST containing 1% skim milk powder was added. The plates were again washed 3 times with PBST and 100 µl of a 1:4,000 dilution of polyclonal rabbit anti-JDV CA (from J. Brownlie) was added. After another 3 washes with PBST, 100 µl of goat anti-rabbit HRP conjugate (ICN, Australia) (1:4,000) was added to each well and incubated at room temperature for another 1 h. After another wash with PBST, 100 µl of TMB colour development reagent was added to each well, the microplates were incubated at room temperature until colour development and the absorbance of the colour reaction was determined at 450 nm.

**Immunoperoxidase staining of virus in fixed tissue sections**

The CA MAb was analysed by immunoperoxidase staining of formaldehyde-fixed paraffin-embedded issue sections from JDV-infected and control animals. The antigen retrieval was performed by microwave treatment of 6 µM tissue sections for 2 x 4 min treatments at high wattage (805 watt), followed by 2 x 4 min treatments at low wattage (230 watt). The sections were then left to cool in distilled water. The tissue sections were rinsed in PBS, blocked in 3% H2O2 for 5 min to remove endogenous peroxidases and then rinsed again before incubation for 1.5 h at room temperature with anti-CA (MAb LD1 or MAb BC10) diluted 1:50, 1:100 and 1:200 in PBS. The sections were then washed in PBS before the addition of secondary
antibody (HRP-conjugated goat anti-mouse IgG) (EnVision) for 30 min at room temperature. After washing, the bound antibodies were visualised as a brown insoluble precipitate by the addition of 3,3’-diaminobenzidine (DAB; DAKO) solution for 5-10 min at room temperature. The slides were counterstained with Harris haematoxylin and dehydrated before they were mounted with DePex (BDH Chemicals).

**Results**

**Production of MAb-secreting hybridomas**

BALB/c mice immunised 4 times with biotinylated ΔSU, CA and Tat extracted from acrylamide gels developed serum antibody that was detectable by western blot utilising the homologous GST-fused protein as an antigen.

One week after fusion, hybridoma cell growth was visible microscopically in the 96-well microplates and at about day 14, the number of hybridoma cells increased and grew large enough to where they occupied one half or more of the surface of each well (Figure 4.2).

![Figure 4.2. Microscopic image of hybridoma cells 14 days after fusion. Magnification X400.](image)

From the mice immunised with the biotin-tagged ΔSU, western immunoblotting detected antibody production in 68 wells containing hybridomas from 5 plates. Many
of the these supernatants reacted with more than one band, and to achieve mono-
specificity, the hybridoma cells from all the positive wells were then cloned by
limiting dilution and after 3 cloning procedures, 7 of the 68 hybridomas produced
MAb reactive with a single band of about 65 kDa in western immunoblots (Figure
4.3). The MAbs were all of IgM subclass and had kappa light chains.

Figure 4.3. The supernatant from hybridoma-containing wells which reacted with
recombinant ΔSU-GST proteins in western immunoblots. After cloning, 7 of the
hybridomas produced MAb that reacted to a ~65 kDa protein only (positive lanes
indicated by arrows). The control molecular weight markers and their size are
shown on the left.

Fusion of spleen cells obtained from BALB/c mice immunised with biotinylated-CA
recombinant proteins resulted in 74 wells containing hybridomas, which detected a
mixture of antibodies by western immunoblotting. After cloning by a series of 3
limiting dilutions, the supernatant from one hybridoma was selected that reacted
with one protein only of ~ 50 kDa (Figure 4.4). Cells from one well were expanded in
25 cm² culture flasks. This cloned hybridoma was selected for further analysis and
designated as LD1.
Figure 4.4. The supernatant from hybridoma-containing wells was screened against recombinant CA-GST fusion proteins by western immunoblotting. The positive hybridomas were cloned 3 times by a limiting dilution method and the supernatant from several clones that reacted with one protein only of ~ 50 kDa. The control molecular weight markers and their size are shown on the left.

Following fusion of spleen cells obtained from BALB/c mice immunised with biotinylated-Tat recombinant proteins, 34 wells containing hybridomas were produced but only one was detected that contained antibody against Tat. This positive hybridoma was re-cloned 3 times (Figure 4.5). The MAb against recombinant Tat protein was of IgM isotype and possessed kappa light chains.

Figure 4.5. The supernatant from hybridoma containing wells was screened against recombinant Tat-GST fusion protein by western immunoblotting. The positive wells were cloned by a limiting dilution method and one of the cloned hybridomas produced antibody reactive with the recombinant protein. The gel depicts the reactivity of multiple wells containing the same cloned hybridoma. The control molecular weight markers and their size are shown on the left.
Determination of optimal antibody and antigen dilution

A checkerboard titration was conducted to determine the optimal working dilution of the LD1 CA MAb and the optimal concentration of Jgag6 protein for maximum sensitivity. The optimum conditions were selected on the basis of the highest absorbance above 0.5. The optimal antigen concentration was 87.5 ng/well of Jgag6 and the optimal LD1 CA MAb dilution was 1:500 (Figure 4.6).

![Graph showing absorbance vs. Jgag6 concentration for different MAb dilutions](image)

**Figure 4.6.** A checkerboard titration to determine the reactivity of various dilutions of the LD1 CA MAb with varying dilutions of the Jgag6 protein by ELISA.

Analysis of reactivity of LD1 with different proteins

The MAb LD1 CA was reacted in western immunoblots against ΔSU, CA and Tat fusion protein products that were produced (Figure 4.7). The MAb LD1 reacted with CA-GST and CA-His (a gift from William Ditcham) but not with the ΔSU and Tat proteins.
The CA MAb LD1 was reacted in western immunoblots against the 9 overlapping fragments of Gag proteins (Figure 4.1) and the results are shown in Figures 4.8 and 4.9. The MAb LD1 recognised Jgag2, Jgag3, Jgag4, Jgag5, Jgag6, and Jgag11 but not Jgag1 (MA protein) and Jgag 7 confirming its specific reactivity to the CA protein.

This reactive epitope for this MAb was a region of CA that was encoded by a region of the JDV genome between nucleotides 604 and 810. The reactivity of LD1 monoclonal antibody was different to the hyperimmune sera when tested against the same truncated Gag proteins (Figure 4.9); the hyperimmune serum did not recognise the Jgag11 product whereas it was recognised by the MAb LD1, indicating a difference in reactivity between native and recombinant CA protein epitopes.
Figure 4.8. Schematic diagram of recombinant Gag fragments expressed in E. coli and reacted in western immunobLOTS with truncated proteins encompassing regions of the MA, CA and NC. The top (multi-coloured) bar represents the full length of the region covered by the individual peptides that were produced. Numbers below the bar show nucleotide numbers of the regions of gag encoding the truncated fragments Jgag1 to Jgag13. Those fragments shown in red were reactive to the LD1 anti-CA MAb, those in black were non-reactive. The schematic was adapted from Desport et al. (2005).

Figure 4.9. Reactivity of different truncated Gag proteins detected by hyperimmune sera (A) and anti-CA LD1 (B). Lane 1, Jgag1; lane 2, Jgag2; lane 3, Jgag4; lane 4, Jgag4; lane 5, Jgag5; lane 6, Jgag6; lane 7, Jgag7; lane 8, Jgag11; lane 9, Jgag13.

Comparison LD1 and BC10

To determine if the MAb LD1 was suitable for the antigen capture ELISA to quantitate virus, the reactivity of MAb LD1 was compared with the MAb BC10; as whole virus was unavailable for this, the Jgag6 peptide was used instead. The MAb
LD1 (at 1:200 dilution) was able to detect 1,750 ng/ml Jgag6 whereas the MAb BC10 (at 1:1,000 dilution) detected 875 ng/ml Jgag6 (Figure 4.10). Evaluation with different concentration of blocking solutions showed that the results were not significantly different, and 5% skim milk powder in PBS was arbitrarily selected for use in all serological assays.

Figure 4.10. Comparison of antigen capture ELISA with MAb BC10 at 1:1,000 dilution (top) and MAb LD1 at a 1:200 dilution using Jgag6 protein as antigen and with various blocking agents including casein and skim milk (SM) powder (bottom).
Immunoperoxidase staining of tissue sections

The IHC procedure was conducted with spleen tissue from uninfected control cattle and from cattle on the 2nd day of fever after experimental infection with JDV_{Tab/87}. The staining pattern was observed with 1:50, 1:100, and 1:200 dilutions of the CA MAb LD1. Granular brown staining in the cytoplasm was detected with all dilutions; however, the 1:200 dilution resulted in less background staining than the other dilutions. Comparison of the CA MAb LD1 with the MAb CA BC10 for immunoperoxidase staining of virus in formaldehyde-fixed paraffin-embedded sections showed that while both were effective reagents for the detection of virus antigen, the MAb BC10 provided more intense brown staining of tissues than did the MAb LD1 (Figure 4.11).

Figure 4.11. Immunoperoxidase staining using MAb CA LD1 (B) and MAb CA BC10 (C) of formaldehyde-fixed paraffin-embedded spleen tissue section from uninfected cattle (A) and JDV-infected cattle (B and C). Magnification X20.

Discussion

Only one MAb was produced against each of the 3 proteins that were selected. While further investigation may have resulted in a greater range of MAbs covering multiple epitopes, the aim of this project was to quickly produce a single MAb
against each of the 3 proteins, that could then be used to identify each of the proteins and expand the range of diagnostic reagents available for Jembrana disease. As the immunising protein was in a denatured state it was not expected that MAb would be produced against all epitopes on native proteins.

As the proteins used for this study were fusion proteins, to facilitate the process of selection of specific antibodies the fusion tag on the immunising protein was different to the fusion protein used as antigen in the serological test used for screening for MAbs. western immunoblotting was used as a screening assay and while it was possibly less sensitive than ELISA, it had the advantage of identifying the reactivity of the antibodies with proteins of the appropriate size. Additionally, certain antigens that bind to ELISA plates may also not be accessible for the antibodies due to steric hindrance (Mohammad & Esen, 1989). It would have been appropriate to test the MAbs against native protein antigens but these were not available.

The anti-CA MAb LD1 was shown to react specifically with the CA as it reacted to the CA-specific construct Jgag11. Desport et al. (2005) suggested that the Jgag11 region must contain a reactive epitope since it was recognised by the hyperimmune JDV sera and to a lesser extent hyperimmune BIV sera. In the previous investigation reported by Desport et al. (2005) the CA-specific MAb BC10 was reactive with the Jgag13 construct as was the MAb LD1. However, some CA-specific MAbs investigated by Desport et al. (2005), which included BC1 and BC10 that were produced by Kertayadnya et al. (1993), did not react with Jgag11 whereas the MAb produced in this study (LD1) reacted with Jgag11 indicating it was different to the other MAbs previously available for JDV research (Kertayadnya et al., 1993).

The potential application of the MAbs produced, particularly the anti-CA MAb LD1, was illustrated by its ability to be used as an alternative to MAb BC10 for the quantitation of virus (Stewart et al., 2005) and the staining of JDV in paraffin-embedded tissue sections. This was facilitated as this MAb was of IgG type. The MAbs against the SU and Tat proteins also have the potential to provide diagnostic and research reagents for the detection and characterisation of these 2 proteins but unfortunately, these MAbs were of IgM type and this will limit their usefulness; most commercially available labelled secondary immunoglobulins are directed against IgG.
Chapter 5

Production of single chain fragment antibody (scFv) against recombinant ΔSU and CA proteins of *Jembrana disease virus*

**Summary**

Single chain fragment antibody (scFv) that specifically bound to recombinant truncated SU (ΔSU)-GST fusion protein and capsid (CA) proteins from Tomlinson I and J phage display antibody libraries were produced. Two different methods of panning, a colony lift assay (CLA) and microtiter plate technique, were used to screen and identify the scFv: the CLA was used to detect scFv against the less soluble ΔSU protein and the microplate technique was used for the more soluble CA proteins. The phage-positive clones from both methods were transformed into *E. coli* HB2151 and the expression of soluble scFv was induced by the addition of IPTG. The scFv were then identified by ELISA. Comparison of scFv binding affinities assessed by indirect and capture ELISA tests indicated that the scFv produced against the SU was reactive not with the ΔSU component but the GST component of the ΔSU-GST fusion protein. The microplate panning generated colonies reactive with a truncated CA protein (Jgag6) and recombinant CA-Tat polyproteins. Four Jgag6 clones with the highest affinity were expressed as soluble scFv in the cell periplasmic area.
Introduction

The technology for the large scale production of highly specific antibody reagents has developed significantly with recent advances in recombinant antibody technology that may overcome many of the problems associated with the production of monoclonal antibodies (MAb) by conventional hybridoma technology (see Chapter 4). Phage display antibody technology provides an *in vitro* selection technique in which antibody fragments are genetically fused to the coat protein of a filamentous bacteriophage, resulting in display of antibody fragments on the exterior surface of the phage virion. The DNA encoding the fusion product resides within the virion. Once cloned, it is possible to improve the affinity and specificity of antigen binding by mimicking somatic hypermutation during an immune response (Gram et al., 1992). The method was introduced for the first time by Smith (1985) who fused a portion of the gene encoding the EcoRI endonuclease to the minor CA protein pIII present on the surface of bacteriophage M13. For reviews of the technique see Hoogenboom et al. (1998) and Winter et al. (1994).

The use of phage display technology has many applications, including the isolation of antigen-specific antibody fragments either as fragment-antigen-binding (Fab) or single-chain antibody fragments (scFv). Recent reports of these applications include the production of scFv against the SARS-associated coronavirus (Liu et al., 2005b), human fibrin clots (Yan et al., 2004) and the capsid (CA) protein and transmembrane (TM) glycoprotein (gp46) of *Maedi-visna virus* (Blazek et al., 2004, Celer et al., 2003). ScFvs have also been expressed intracellularly to inhibit the function of intracellular proteins (Lobato & Rabbitts, 2004). These intracellular antibodies or intrabodies have been applied to engineer a human MAb F240, which recognised the disulfide loop-bonded immunodominant epitope of HIV-1 gp41 (Liu et al., 2005a) and human MAb F102, which recognised the conserved CD4-binding region of HIV-1 gp120 (Wang et al., 2005b). These results suggested that the constructed scFv F240 and scFv F102 may inhibit viral replication and may be useful for gene therapy. Other research has demonstrated that these intrabodies can be directed to multiple HIV-1 target proteins that are present in cells, including the major CA p24 protein (de Haard et al., 1998), Gag p17 protein (Tewari et al., 1998), Vif (Goncalves et al., 2002) and Vpr (Krichevsky et al., 2003).

There are different methods of panning to isolate high affinity scFv against a wide variety of target antigens. Immunotube-based selection has resulted in production of scFV with high affinity for the target antigen but it requires a high volume of purified
antigen (Mi et al., 2005). Similar techniques have been described that use microplates or tagged antigens that can be isolated using magnetic beads (Siegel et al., 1997). However, those techniques may not be suitable for antigens with poor solubility or that tend to aggregate. An improved method for phage display panning was developed employing whole cells that produced specific phage after several rounds of panning (Radosevic et al., 2003, Siva et al., 2008, Sui et al., 2004).

Storage of hybridomas for conventional MAb production has always been a problem within Indonesia because of difficulties in maintaining liquid nitrogen storage facilities due to supply problems. To overcome the limitation represented by the storage of hybridomas producing MAb against JDV proteins prepared by conventional methods as described in Chapter 4, antibody phage display was attempted to produce specific scFv directed towards the recombinant truncated SU (ΔSU) and CA proteins of JDV. Due to the nature of the recombinant proteins used in this study, 2 methods of panning were employed for each of the 2 proteins. For the insoluble ΔSU protein, a colony lift assay (CLA) was used. This method is based on the production of scFv by phage-infected bacteria plated on IPTG-containing agar plates (Siegel et al., 1997). The scFv diffuse through the bacterial membrane and bind to the antigen-coated membrane that is placed under the bacterial membrane. The scFv that bind to the antigen-coated membrane are detected using antibodies specific for the tag fused to the scFv. An alternative microplate technique was also used, where phages are absorbed onto ELISA plates coated with CA that are subsequently eluted with trypsin. The eluted phages are used to infect *E. coli* of the TG1 genotype to produce scFv-phages, followed by soluble scFv expression in *E. coli* of the HB2151 genotype. The synthetic phagemid library, the human single-fold scFv libraries I+J (Tomlinson I+J), used for the generation of scFv antibodies directed against recombinant JDV SU and CA is described.

**Material and methods**

**Tomlinson I and J phage antibody libraries**

Two phage-display antibody libraries generated at the MRC Centre for protein engineering (Cambridge, UK) were used. Both libraries were based on a single human framework for V_H(V3-23/DP-47 and J_H4b) and V_K(012/02/DPK9 and J_K1), with side chain diversity (either NKK or DVT encoded library J and I, respectively) incorporated at positions in the antigen binding site that make contact with antigen
and are highly diverse in the mature repertoire (Rubinstein et al., 2003). The vector used for the expression of scFv antibody fragments is depicted in Figure 5.1. Phagemid vector pIT2 contains a lac promoter and a pelB leader sequence upstream of the scFv insert, which is then followed by hexa-histidine and myc tags, an amber stop codon and the gene encoding the pIII phage coat protein. After helper phage KM13 infection, scFv up-stream of the amber stop codon is displayed on the phage particle with the use of a suppressor strain such as TG1. However, scFv with a His6-myc tag is secreted into the periplasmic space with the use of a non-suppressor strain such as HB2151.

Figure 5.1. Schematic representation of the scFv expression vectors: amp, ampicillin resistance encoding gene; colE1 ori, origin of DNA replication; pelB, signal peptide sequence of bacterial pectate lyase; myc tag, sequence encoding an epitope recognised by the MAb 9E10. Adapted from the Tomlinson I+J protocol, http://www.geneservice.co.uk/products/proteomic/scFv_tomlinsonIJ.jsp.

In conjunction with the phage display antibody library, the E. coli strain TG1 (K12, Δ(lac-pro), supE, thi, hsdS5/F′traD36, proA*B*, lacI*, lacZΔM15) used for the phage rescue and the non-suppressor E. coli strain HB2151 (K12, ara, Δ(lac-pro), thiI/F′proA*B*, lacI°ZΔM15) used for the preparation of single-chain Fv fragments were supplied by I. M. Tomlinson, Laboratory of Molecular Biology (MRC, Cambridge).
Preparation of phage library stocks

Tomlinson I and J libraries were delivered as glycerol stocks. One hundred µl of the *E. coli* (TG1 genotype) glycerol stock was inoculated into 200 ml of pre-warmed 2YT broth containing 1% glucose and 100 µg/ml of ampicillin and incubated with shaking at 37°C to an optical density at 600 nm (OD$_{600}$) of 0.4. KM13 helper phage (2 x 10$^{11}$ pfu) was added to 50 ml of each culture and incubated without shaking in a 37°C water bath for 30 min. The remaining 150 ml of each culture was incubated for a further 2 h with shaking at 37°C and used to make secondary bacterial stocks. After incubation, the phage-infected culture was centrifuged (3,000 g for 10 min) and the resulting pellet was resuspended in 100 ml of 2YT broth containing 100 µg/ml of ampicillin, 50 µg/ml of kanamycin and 0.1% glucose and re-incubated overnight at 30°C. The resulting culture was clarified by centrifugation (3,000 g for 30 min) and phage particles were concentrated from the supernatant by precipitation with 20 ml of PEG/NaCl (20% [w/v] polyethylene glycol 6,000 in 2.5 M NaCl) for 1 h on ice. The suspension was then centrifuged (3,000 g for 30 min), the supernatant was discarded and the pellet resuspended in 4 ml PBS and re-centrifuged (3,000 g for 30 min) and the supernatant containing phage was kept at 4°C for short term storage or at –70°C in PBS with 15% glycerol for longer storage.

To titrate the phage, 10-fold serial dilutions of the phage suspension were prepared in PBS. To 100 µl of each dilution, 900 µl of TG1 cells at an OD$_{600}$ of 0.4 was added and the tubes then incubated at 37°C in a water bath for 30 min. Ten µl of each dilution was then spotted on a 2YT agar plate containing 1% glucose and 100 µg/ml ampicillin.

Protein preparations

A ΔSU-GST protein was prepared as previously described (Chapter 3). A biotinylated CA fragment (Jgag6) was expressed and solubilised as described previously (Chapter 4). A recombinant CA-Tat fused polyprotein (Lewis, 2008) was kindly provided by Dr Joshua Lewis (this laboratory). Ubiquitin (Sigma) was used as a control antigen for assessment of the quality of the panning process.
Preparation of scFV by panning using CLA

The detection of ΔSU scFv was attempted using CLA; the principle of the assay utilised is demonstrated in Figure 5.2 (Radosevic et al., 2003).

Selection of ΔSU phage

Solubilised ΔSU was separated by SDS-PAGE and electro-transferred to a nitrocellulose membrane. The membrane was stained with Ponceau red and after washing, non-reactive sites on the membrane were blocked by immersing it in 5% skimmed milk powder in TBST for 1 h at 4°C. The membrane was washed with TBST and the band that corresponded to ΔSU was excised. The excised ΔSU protein was then incubated with ~10^{12} phage in 1 ml of 5% skimmed milk powder in
TBST at 4°C for overnight. After 3 washes with TBST the bound phages were eluted by adding 500 µl of trypsin-PBS (50 µl of 10 mg/ml trypsin stock in 450 µl PBS) for 10 min and propagated in fresh TG1 cells by adding 250 µl of the eluted phage particles to 1.75 ml of exponentially growing TG1 cells at an OD_{600} of 0.4 and incubating them for 30 min at 37°C in a water bath without shaking. A sample was removed and the phage titrated. The remaining TG1 culture was centrifuged (microcentrifuge for 5 min) to pellet the cells and the pellet resuspended in 50 µl of 2YT and plated on a 100 mm 2YT agar plate containing 100 µg/ml ampicillin and 1% glucose. The plates were then incubated overnight at 37°C. After overnight growth, 2 ml of 2YT medium was added to the plate and the bacteria were collected by scraping with a glass spreader. Fifty µl of the scraped bacteria suspension was added to 50 ml of 2YT containing 100 µg/ml ampicillin and 1% glucose and grown at 37°C while shaking until the OD_{600} was 0.4. Then 5 x 10^{10} helper phage was added to 10 ml of this culture and incubated at 37°C for 30 min without shaking. The bacteria were centrifuged (3,000 g for 10 min) and the pellet resuspended in 50 ml of 2YT medium containing 100 µg/ml ampicillin, 50 µg/ml kanamycin and 0.1% glucose and incubated with shaking at 30°C overnight. The overnight culture was centrifuged (15 min at 3,300 g at 4°C) and 40 ml of the phage-containing supernatant was transferred to a new tube and precipitated with 10 ml of ice-cold PEG/NaCl (20% polyethylene glycol 6,000, 2.5 M NaCl) for 1 h. The phage suspension was centrifuged (3,300 g for 30 min) and the pellet resuspended in 2 ml PBS and centrifuged (~11,000 g for 10 min in a micro centrifuge) to remove bacterial debris.

The phage selection protocol was repeated a further 3 times (a total of 4 times) and the enrichment of specific phages in each round of the selection was assessed by polyclonal phage ELISA.

**Coating ΔSU proteins onto nitrocellulose membranes**

Two ml of ΔSU-GST protein (100 µg/ml) was further diluted in 4 ml of PBS and poured onto the surface of a nitrocellulose membrane (pore size 0.45 µm; Bio-Rad) in a culture dish and left overnight at 37°C. The excess suspension was then removed and the ΔSU-coated membrane was rinsed with PBS. Non-reactive sites on the membrane were then blocked by immersing it in 10 ml of PBS containing 3% (w/v) skimmed milk powder for 1 h at room temperature. The blocked membrane was rinsed twice with PBS and then air-dried.
Panning for ΔSU scFv

The phage rescued from each round of selection (as described above) were used to infect *E. coli* HB2151 cells (non-suppressor strain) for the attempted expression of a soluble scFv antibody. The bacteria were grown in 2YT culture medium at 37°C until OD₆₀₀ of 0.4 was achieved. Two hundred ml of the culture was infected with 10 µl of phage from each round of panning (above) and then incubated at 37°C for 30 min without shaking. Serial 10-fold dilutions of the phage-infected HB2151 bacteria were plated on YTAG plates (2YT agar containing 100 µg/ml ampicillin and 1% glucose) and grown overnight at 37°C. The dilution which resulted in isolated bacterial colonies was used for the CLA (further referred to the original bacterial plate).

The nitrocellulose ΔSU-coated membrane (prepared as described above) was soaked in 2YT medium containing 100 µg/ml ampicillin and 1 mM IPTG and then placed, coated side upwards, onto the surface of an YTAI agar plate (2YT agar containing 100 µg/ml ampicillin and 1 mM IPTG). Another non-coated membrane was placed on top of the original bacterial plate (referred to as the master membrane). The master membrane was removed from the original bacterial plate and placed, colony side up, on the antigen-coated membrane on the YTAI agar plate. The YTAI agar plate was then incubated overnight at 30°C. Both membranes were marked simultaneously by stabbing them with a needle for orientation purposes.

The master membrane was removed and placed, colony side up, onto a fresh YTAG agar plate. The plate together with the master membrane was stored at 4°C and later used for picking selected clones. The ΔSU-coated membrane was removed from the YTAI plate, washed 3 times with 10 ml PBST (PBS containing 0.05% Tween-20) for 10 min with gentle shaking, blocked with PBS/3% dried skimmed milk powder for 1 h at room temperature and rinsed twice with PBS/0.05% Tween-20. The membrane was incubated with 10 ml mouse anti-His antibody (Sigma) (recognising the His-tag fused to scFv) diluted 1:2,000 in PBS/1% dried skimmed milk powder, for 1 h at room temperature. After incubation, the filter was washed 3 times with 10 ml PBST for 10 min each time, with shaking, followed by incubation with 10 ml of 1:3,000 dilution of goat anti-mouse Ig-HRP (ICN) in PBS/1% dried skimmed milk powder for 1 h at room temperature. The membrane was washed 3 times with 10 ml PBST for 10 min each time with gentle shaking and then rinsed twice with PBS. The membrane was then treated with 10 ml of HRP colour development solution (Bio-Rad) until spots were visible when the membrane was rinsed with water to stop the reaction. The spots from the ΔSU-coated
membrane were used to identify the corresponding colonies responsible for the production of specific scFv on the master membrane and the original plate.

**Preparation of soluble ΔSU scFv**

Individual colonies were picked from each positive agar plates and resuspended in 100 µl 2YT medium containing 100 µg/ml ampicillin and 1% glucose in 96-well microplates and incubated overnight at 37°C with shaking. The overnight cultures were then diluted 1:20 and added to wells of another 96-well microplate containing 200 µl fresh 2YT medium supplemented with 100 µg/ml ampicillin and 0.1% glucose and grown at 37°C with shaking until the OD<sub>600</sub> was about 0.9. IPTG was added to a final concentration of 1 mM and the cultures were incubated with shaking overnight at 28°C. Bacteria were pelleted by centrifugation (1,800 g at 4°C for 15 min) and 50 µl of the supernatant was used for ELISA.

**ELISA**

To evaluate the specificity of the scFv against the ΔSU protein, 2 kinds of ELISA were used: an indirect phage ELISA and a capture phage ELISA.

**Indirect ELISA.** ELISA microplates (Maxisorp, Nunc) were coated overnight at 4°C with 100µl/well of 10 µg/ml ΔSU-GST fusion protein and GST alone in 100 mM carbonate buffer (pH 9.6). After 3 washes with PBST, the plates were subsequently blocked with 200 µl/well of 5% skimmed milk powder in PBS for 2 h at room temperature. The plates were washed 3 times with PBST and then 75 µl/well of the scFv supernatants were added to the plates and they were incubated at room temperature for 2 h. PBS was used as negative control. After 3 washes with PBST, the plates were incubated for 1 h with 100 µl/well of a 1:5,000 dilution of HRP-conjugated protein L (Sigma) in PBS. The plates were washed a further 3 times with PBST and once with PBS before they were reacted with 50 µl/well of TMB (3,3',5,5'-tetramethylbenzidine) colour development reagent for 5-15 min at room temperature. The colour development was stopped with 50 µl/well of 1 M H<sub>2</sub>SO<sub>4</sub> and the absorbance was determined at 450 nm and 650 nm.

**Capture ELISA.** ELISA microplates (Maxisorp, Nunc) were coated overnight at 4°C with 100 µl/well of goat anti-GST antibody (diluted 1:5,000 in 100 mM carbonate buffer pH 9.6). After 3 washes with PBST the plates were subsequently blocked with 100 µl/well of goat anti-GST antibody (diluted 1:5,000 in 100 mM carbonate buffer
pH 9.6). After 3 washes with PBST the plates were subsequently blocked with 200 µl/well of 5% skimmed milk powder in PBS for 1 h at room temperature. The plates were then washed 3 times with PBST and 100 µl/well of 10 µg/ml ΔSU-GST or GST only were added to the wells and incubated for 1 h at room temperature, then PBS was added to blank wells as a negative control. After 3 washes with PBST, 75 µl/well of the scFv-containing supernatant was added to the plates for 2 h at room temperature. After washing as described above, the plates were further incubated for 1 h with 100 µl/well of a 1:5,000 dilution in PBS of HRP-conjugated protein L (Sigma). The plates were washed a further 3 times with PBST and once with PBS before the addition of 50 µl/well of substrate solution containing TMB for 5-15 minute at room temperature. The colour development was stopped with 50 µl/well of 1 M H₂SO₄ and the absorbance determined at 450 nm and 650 nm.

Preparation of CA scFv by panning with microplate method

Selection of CA scFv-phage

The panning procedure was conducted using 96-well microplates. One hundred µl of Jgag6 (10 µg/ml), CA-Tat polyprotein (10 µg/ml) or ubiquitin (100 µg/ml) was added to wells of a 96-well microplate (Nunc). The plates were incubated for 3 h at room temperature and then the non-reactive sites were blocked by the addition of with 2% skimmed milk powder in PBS. The plates were then washed 3 times with PBS and 100 µl of the supernatant containing the phage library stocks was added to each well. Plates were incubated at room temperature for 2 h and washed 10 times with PBST. To the wells containing bound phage, 100 µl of 10 mg/ml trypsin in PBS was added and incubated at room temperature for 10 min. The eluted phage were used to infect 1 ml of log-phase TG1 cells at an OD₆₀₀ of 0.4 in 2YT medium and grown at 37°C for 1 h. Ampicillin was added to a final concentration of 100 µg/ml and glucose to a final concentration of 1% (w/v). Helper phage (5x10¹⁰ cfu) was then added to the TG1 cell suspension and the culture was incubated at 37°C for 30 min then centrifuged (3,000 g for 10 min at room temperature) to pellet the bacteria. The bacterial cells were then resuspended in 1 ml 2YT containing 100 µg/ml ampicillin, 50 µg/ml kanamycin and 0.1% glucose and cultured overnight at 28°C. The phage panning protocol was repeated a further 3 times.
Preparation of scFv-phage for monoclonal ELISA

Serial dilutions (10^{-1}, 10^{-2}, 10^{-4} and 10^{-6}) of *E. coli* TG1 infected with eluted scFv-phages from each round of selection were plated on 2YT agar with 1% glucose and 100 µg/ml ampicillin to enable the selection of individual separated colonies. These individual colonies were picked and inoculated into individual wells of a microplate with 100 µl of 2YT, 1% glucose and 100 µg/ml ampicillin and cultured at 37°C overnight with shaking (250 rpm). Then, 2 µl of the overnight cultures from each well was transferred into a new plate with 200 µl of 2YT, 1% glucose and 100 µg/ml ampicillin per well and incubated for 2 h at 37°C with shaking. The remainder of the bacterial culture from the overnight plate was stored frozen after the addition of 15% glycerol. Next, 10^9 pfu of the helper phage KM13 was added per well and shaken for 60 min. This was centrifuged (1,800 g for 10 min) and samples of the supernatant and the pellet were cultured in 200 µl of 2YT, 50 µg/ml kanamycin and 100 µg/ml ampicillin and cultured at 28°C overnight with shaking. The overnight culture was centrifuged (1,800 g for 10 min) and 75 µl of the phage containing supernatant was used in monoclonal phage ELISA.

Production of soluble scFv

The selected scFv-phage from each round of selection was inoculated into the *E. coli* HB2151 non-suppressor strain to produce soluble scFv antibody. HB2151 cells were grown in 2YT media at 37°C until the OD_{600} was 0.4. Two hundred µl of this culture was infected with 10 µl of scFv-phage, incubated at 37°C for 30 min without shaking and plated on 2YT agar with 100 µg/ml ampicillin. From this plate, a single colony was picked and inoculated into 5 ml 2YT, 1% glucose and 100 µg/ml ampicillin, and cultured at 37°C overnight with shaking (250 rpm). The overnight cultures were then diluted 1:20 in fresh 2YT medium containing 100 µg/ml ampicillin and 0.1% glucose and grown at 37°C with shaking until the OD_{600} was 0.9. The culture was then induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG), to a final concentration of 1 mM. The culture was incubated at 28°C overnight with shaking (250 rpm). The cells were centrifuged (3,300 g for 30 min) and the soluble scFv were recovered from the pellet by osmotic shock. The pellet was resuspended in ice-cold osmotic shock buffer [20% sucrose, 200 mM Tris-HCl pH 7.6 and 1 mM EDTA] and incubated on ice for 5 min. The suspension was pelleted by centrifugation in microcentrifuge (14,000 g for 5 min): the supernatant was collected and stored (sucrose fraction), the pellet was resuspended in ice-cold...
water, incubated on ice for 5 min, centrifuged in a microcentrifuge (14,000 g, 5 min) and the resulting supernatant containing the periplasmic fraction was collected. All osmotic shock fractions were either stored at 4°C and analysed immediately by ELISA or frozen at -20°C and analysed later.

**Polyclonal and monoclonal scFv-phage ELISA**

Wells of a 96-well microplate were coated with 50 µg/ml of ubiquitin, 10 µg/ml of Jgag6 or 10 µg/ml of CA-Tat polyprotein diluted in carbonate buffer (pH 9.6), 100 µl per well, and left at 4°C overnight. The plate was washed with PBS and blocked with 200 µl of PBS/2% skimmed milk powder for 60 min at room temperature, washed with PBS, then 100 µl of scFv-phage (about 2x10^{12} pfu) of either amplified polyclonal (polyclonal ELISA) collected after each round of selection or MAb (monoclonal ELISA) in PBS/2% skimmed milk was added and incubated at room temperature for 60 min. PBS was used as negative control. Bound scFv-phage was detected with the monoclonal anti-M13gp8 conjugated to HRP (Pharmacia) diluted 1:5,000 in PBS, 100 µl per well, and incubated for 60 min at room temperature. After each incubation step, the microplates were washed 3 times with PBST and finally with PBS. The ELISA was developed with 100 µl ABTS (BioRad) per well, and the absorbance was read after 5 min incubation at 405 nm with a microplate reader (BioRad).

**SDS-PAGE and western blotting of soluble scFv**

SDS polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were performed as described previously (Chapter 3). Briefly, 20 µl of all fractions of scFv were separated by 12.5 % SDS-PAGE, and then transferred electrophoretically to nitrocellulose membrane. The membrane was blocked with PBST containing 5% skimmed milk powder for 1 h before washing with PBST. The membrane was then incubated with 1:5,000 diluted HRP-conjugated anti-His antibody in 10 ml of PBST containing 2% skimmed milk powder for 1 h at room temperature. Bound anti-His was detected by the addition of HRP colour development reagent (Bio-Rad).
Results

Screening for ΔSU scFv by CLA

The panning procedure with ΔSU-GST proteins bound to a nitrocellulose membrane was used to selectively capture ΔSU-positive recombinant phage from 2 phage antibody display libraries (I and J). During 4 panning cycles, the number of ΔSU-GST specific phage increased significantly from the third round (Figure 5.3).

Figure 5.3. ELISA results demonstrating enrichment of ΔSU-GST scFv during panning. Four rounds of panning were conducted against ΔSU-GST proteins immobilised on nitrocellulose membranes. The bound phage from Library I (blue bars) and Library J (violet bars) after each round of selections were eluted with trypsin and were titrated. An increase in number of eluted phages could be seen as the panning progressed.

HB2151 bacteria that were infected with phage recovered after the third and fourth panning rounds were plated on individual YTAG agar plates in different dilutions and screened by CLA using nitrocellulose membranes coated with ΔSU-GST fusion protein (Figure 5.2). This screening resulted in isolated colonies at a $10^{-4}$ dilution. These single bacterial colonies were tested for their ability to bind to ΔSU-GST immobilised on nitrocellulose membranes and a number of scFv-positive-colonies from both phage libraries were identified (Figure 5.4).
Figure 5.4. Screening of diffused scFv antibodies secreted by the bacterial colonies grown on master filter to the membrane coated with recombinant protein ΔSU-GST. The scFv-antibodies were detected by anti-His antibody. (A) Antibodies identified after third round of selection and (B) antibodies identified after the fourth round of selection.

Forty single colonies obtained using CLA from the third and the fourth panning round of libraries I and J were selected and subcultured on YTAG plates and then tested for the production of antibody by CLA. Multiple scFv-binding spots, corresponding to colonies, were visualised on the membrane and an example of the results obtained during the third selection round of Library J is shown in Figure 5.5. A few positive single colonies were also obtained using Library I.

Figure 5.5. Bacteria colonies expressing potential antibody fragments (scFv). Forty selected colonies were tested for antibody production by CLA. Antibody fragments that diffused from master filter (A) and bound to the ΔSU-GST coated filter (B) could be detected by an enzyme colorimetric reaction. The corresponding positive colonies could be identified from the master filter (A).
Four positive colonies derived from the third round of panning using the J-library and 8 positive colonies from the fourth round of panning (4 using the J-library and 4 using the I-library) that showed a strong band on western immunoblotting, were further subcultured and re-screened by CLA for positive scFv antibody reactions and the results are shown in Table 5.1. Numerous positive colonies were identified although 2 of the 12 parental clones produced a failed to react when retested after subculture.

Table 5.1. Results of screening of parental clones with colony lift assay (CLA).

<table>
<thead>
<tr>
<th>Plate</th>
<th>Number of colonies</th>
<th>Number of positive colonies determined by CLA</th>
<th>Percentage positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>3J-1</td>
<td>200</td>
<td>98</td>
<td>49</td>
</tr>
<tr>
<td>3J-1A</td>
<td>196</td>
<td>49</td>
<td>25</td>
</tr>
<tr>
<td>3J-1B</td>
<td>192</td>
<td>57</td>
<td>30</td>
</tr>
<tr>
<td>3J-9</td>
<td>140</td>
<td>43</td>
<td>31</td>
</tr>
<tr>
<td>4J-1</td>
<td>158</td>
<td>17</td>
<td>11</td>
</tr>
<tr>
<td>4J-2</td>
<td>154</td>
<td>Negative</td>
<td>N/A</td>
</tr>
<tr>
<td>4J-6</td>
<td>260</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>4J-7</td>
<td>132</td>
<td>Negative</td>
<td>N/A</td>
</tr>
<tr>
<td>4I-1</td>
<td>180</td>
<td>75</td>
<td>42</td>
</tr>
<tr>
<td>4I-2</td>
<td>284</td>
<td>43</td>
<td>15</td>
</tr>
<tr>
<td>4I-3</td>
<td>115</td>
<td>24</td>
<td>21</td>
</tr>
<tr>
<td>4I-4</td>
<td>240</td>
<td>24</td>
<td>10</td>
</tr>
</tbody>
</table>

Five isolated colonies from each positive plate were subcultured and 10 colonies from each subculture (total of 50) were used to express soluble scFv antibody, determined by indirect and capture ELISA against ΔSU-GST and GST proteins. Most of the 50 colonies produced scFv that reacted with both ΔSU-GST and GST by both ELISA methods (Figure 5.6) but scFv produced by 2 colonies (referred to as clones 32 and 34) showed a relatively stronger affinity to antigen. However, there was a strong reactivity to both the ΔSU-GST and GST indicating that the selected scFv had affinity to the GST and not ΔSU protein.
Figure 5.6. Results of indirect (A) and capture (B) ELISA of scFv from individual colonies against ΔSU-GST and GST proteins. The scFv were produced from individual colonies after detection by CLA. Fifty colonies from the 10 positive plates were analysed by indirect and capture ELISA and the bound scFv was detected with protein L-HRP. The absorbance readings shown were the average of 3 replicate reactions.

Screening for CA scFv by microplate method

Four rounds of panning were undertaken on ubiquitin, Jgag6 and CA-Tat proteins bound to microplates. After each round of panning, the titre of the eluted phage was measured to monitor the efficiency of the selection process (Figure 5.7). After the third round, phage recovery increased almost 100-fold compared to the second round of panning, indicating the library was already enriched in each protein binder. However, during the fourth round the recovery decreased slightly in comparison to the third round.
Figure 5.7. The numbers of eluted scFv-phage after each of 4 rounds of selection that bound to ubiquitin (A), Jgag6 (B) and CA-Tat polyprotein (C). The titre was determined as cfu/ml with library I (brown bars) and the library J (green bars).

The increase in the number of protein-specific binders in the phage population was confirmed with polyclonal scFv-phage ELISA after each panning round. Higher absorbance values were obtained with each round of panning than in the previous round, indicating a progressive increase of the number of specific scFv-phage
binders in each panning round (Figure 5.8). The enrichment with Library J was greater than with Library I for all 3 proteins, including ubiquitin.

To identify individual specific scFv-phage binders, the eluted scFv-phage after the third round of selection were used for infection of TG1, which were then cultured to obtain single colonies. Twenty colonies from each library were selected and tested for their ability to bind to ubiquitin, Jgag6 and CA-Tat proteins with the monoclonal scFv-phage ELISA. When the value of absorbance of all colony samples for each binding protein was averaged, the average absorbance of scFv-phage clones for ubiquitin, Jgag6 and the CA-Tat polyprotein were 2.6049, 2.1529 and 1.4442 for library I and 2.4628, 2.3820 and 1.7622 for library J (Figure 5.9). Based on these average values, colonies producing absorbance values that exceeded the average absorbance were selected as potentially protein-specific scFv-phages (Table 5.2).
Figure 5.8. Results of polyclonal phage ELISA after each round of panning. The precipitated phages were reacted with ubiquitin (A), Jgag6 (B) or CA-Tat polyprotein (C) and detected with an anti-M13/HRP conjugated antibody at an absorbance of 405 nm. Library I (green bar) and library J (yellow bar).

Table 5.2. The number of clones (colonies) specific for each protein binder used.

<table>
<thead>
<tr>
<th></th>
<th>Ubiquitin</th>
<th>Jgag6</th>
<th>CA-Tat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Library I</td>
<td>15</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>Library J</td>
<td>14</td>
<td>15</td>
<td>12</td>
</tr>
</tbody>
</table>
Twenty selected clones reacted better to the truncated CA protein Jgag6 than the CA-Tat polyprotein (Figure 5.9) even though the CA-Tat polyprotein contained the full-length JDV CA, plus Tat encoded by exon 1 of the tat1 gene (Lewis, 2008).

Figure 5.9. The binding of 20 clones detected by a monoclonal phage ELISA to ubiquitin, Jgag6 and CA-Tat proteins. The 20 colonies (clones) from library I (top) and library J (bottom) were selected from the third round of panning.
In order to obtain specific scFv against the CA protein, 4 colonies of Jgag6 scFv-phage, 2 each from each library, designated as IF3, JG8, JB1 and ID6 were selected and used to infect non-suppressor strain *E. coli* HB2151 for expression of soluble scFv. The culture was grown overnight with shaking at 28°C and expression was induced with IPTG when the absorbance was 0.9. The distribution of the soluble scFv in the bacterial periplasm and in the culture supernatant was evaluated in 12.5% SDS-PAGE gels stained with Coomassie Blue and in western immunoblots reacted with anti-His antibody (Figure 5.10). western immunoblotting indicated that in the supernatant, the sucrose fraction, the periplasmic fraction, a distinct band of 30 kDa within the expected molecular weight range of soluble scFv was present and was of stronger reactivity in the sucrose and periplasmic fractions. The results indicated that most of soluble scFv was secreted into the periplasm but that a substantial amount of soluble scFv was also released into the medium (Figure 5.10). A major band of about 30 kDa corresponding to the monomeric form of the scFv was also detected in the pellet fraction of JB1 scFv by Coomassie Blue staining and western immunoblotting with anti-His antibody.
Figure 5.10. 12.5% SDS-PAGE (A and B) and western blot analysis (C and D) of scFv in the supernatant, sucrose fraction, periplasmic fraction and pellet of *E. coli* HB2151 after induction of expression with IPTG. The gels were stained with Coomassie Brilliant Blue and scFv were detected using His antibody. Lane 1, IF3 supernatant; lane 2, JG8 supernatant; lane 3, JB1 supernatant; lane 4, ID6 supernatant; lane 5, IF3 sucrose fraction; lane 6, JG8 sucrose fraction; lane 7, JB1 sucrose fraction; lane 8, ID6 sucrose fraction; lane 9, IF3 periplasmic fraction; lane 10, JG8 periplasmic fraction; lane 11, JB1 periplasmic fraction; lane 12, ID6 periplasmic fraction; lane 13, IF3 pellet; lane 14, JG8 pellet; lane 15, JB1 pellet; lane 16, ID6 pellet.

**Discussion**

Recombinant antibody can be produced in *E. coli* or other expression systems from cloned cDNA and due to the stability of the DNA constructs and ease of production compared with conventional monoclonal antibody technique, unlimited supplies of specific antibodies can be produced at a reasonable cost (Foord et al., 2007)
without the need for long term storage of hybridomas. The technique does have the disadvantage of producing only a monovalent product that may have a fast off-rate and poor retention time on the target (Cheng et al., 2005). In addition, there are challenges to overcome when isolating antibodies from the phage display library. Isolation of phage displayed antibodies is dependent on a number of parameters including the size of the library, phage preparation, conditions and time of incubation, blocking and washing during selection, and the method of elution of bound scFv (Chen et al., 2009).

In this current study, phage display technology was used to facilitate the selection of the scFv against recombinant ΔSU and CA proteins of JDV using the synthetic phagemid Tomlinson I and J libraries. Protein-specific scFv would have application in methods for the detection of protein either by ELISA or western immunoblotting and could have many applications in the development of diagnostic methods. This phage display technology has not been used previously to produce antibody against JDV proteins. The selection of the 2 JDV proteins ΔSU-GST and CA for which it was attempted was based on the premise that they would be useful proteins to explore as vaccines against Jembrana disease. They also have a role during the production and purification of these proteins and could be targets of immunological tests for the detection of virus during routine diagnosis.

Technical problems associated with the technology included problems associated with the selection of methods used for screening. Proteins have different tendencies of adsorption to nitrocellulose membrane and polystyrene. According to the manufacturer’s specification for the polystyrene microplate used in this work (Nunc MaxiSorp) the microplates have hydrophilic binding groups that attract hydrophilic regions on molecules. Since the recombinant CA proteins used were hydrophilic (soluble in water) they were likely to be well adsorbed onto the surface of the plate. In contrast, the solubilised ΔSU protein was more insoluble and probably has few hydrophilic aa residues exposed as a consequence it was likely to attach poorly to the surface of microplates whereas the presence of hydrophobic interactions and weak hydrogen bonds would allow its adsorption to the surface of nitrocellulose membranes (Ahmad et al., 2009). For that reason, in this study the microtitre plate method was used for panning and screening the phage antibody library using CA proteins and nitrocellulose membrane was used with the ΔSU protein. The nature of the solid binding surface, however, is not the only factor in selection of an appropriate matrix for protein binding, and the protein structure, size of a molecule,
charge, density and distribution in the protein molecule, and conformational rearrangements all must be considered (Kamyshny et al., 1999).

The production of scFv that reacted specifically with CA proteins appeared to be successful although additional studies will be required to optimise their expression and demonstrate their value for diagnostic purposes and in protein identification and purification. At least 4 clones were produced that expressed soluble scFv in *E. coli* and reacted with the Jgag6 ΔCA protein. Western immunoblotting results indicated the soluble scFv were expressed into the bacterial periplasm, under the culture conditions used, although some leakage of scFv fragments to the extracellular medium did occur. Antibody fragments expressed under the control of the *lac* promoter are known to be released to the extracellular medium after several hours of induction, even though signal sequences have directed them to the periplasm (Shibui & Nagahari, 1992, Takkinen et al., 1991). Control of leakage would be required to produce a single location for scFv fragments, which will improve downstream processing for recovery. One of the Jgag6-scFv clones, JB1, did not remain exclusively in the periplasmic space and was not released into the culture medium: a small proportion of the JB1-scFv was also present in the pellet as insoluble scFv. Possibly this may have been the result of a high concentration of scFv produced in the bacterial periplasmic space where the accumulation of scFv may then form insoluble aggregates. If this were the case, this insoluble scFv could be solubilised with urea and refolded (as described in Chapter 3) using a buffer containing aa, which destabilizes wrongly folded structures (Huston et al., 1994), or its solubility could be influenced by the mutation within the framework regions of the scFv (Duenas et al., 1995).

The expression of the scFv reactive with the JDV CA was limited and further optimisation of expression is required. It was, however, expressed into the periplasm which is preferred for large-scale preparation because this is of smaller volume than the extracellular fraction. Specific release of scFv using a technique such as osmotic shock, which would not rupture the inner cell membranes, could offer an attractive option for purification. If, however, it was instead chosen to promote extracellular scFv production, this might avoid the need for cell disruption.

Attempts to produce clones that would express scFv against the ΔSU protein with the phage display library were unsuccessful. The CLA method used in this experiment did result in the production of clones that expressed scFv that reacted with the ΔSU-GST but analysis of the results suggested they reacted against the GST component of the ΔSU-GST and not the ΔSU. The 26 kDa GST tag is 220 aa,
larger than other tags such as myc- or FLAG. This large size of GST may potentially influence the panning or may render it more immunogenic than the target protein ΔSU. Zhang et al. (2001) also described selection of false positives when GST fusion proteins were used for selection of antibodies specific to viral movement protein. A similar phenomenon was also reported by Murthy et al. (1999) during attempts to identify a consensus motif specific for the PDZ2 domain of a cytosolic protein tyrosine phosphatase by peptide phage display.

In conclusion, the results presented demonstrate successful production of a scFv with the capacity to bind to CA antigens of JDV. Further development of this scFv antibody could enable it to be used for immunodiagnosis of JDV infections and for identification of CA proteins during future attempts to produce Jembrana disease vaccines.
Chapter 6

Identification and preliminary analysis of JDV envelope glycoproteins by MALDI-TOF mass spectrometry

Summary

Matrix-assisted laser desorption/ionisation–time of flight (MALDI-TOF) mass spectrometry of the proteins of JDV derived from infected cattle was conducted to determine the size and potential glycosylation sites in the SU and TM glycoproteins. Virus extracted from the plasma of JDV-infected cattle was purified and subjected to SDS-PAGE and 2 proteins of 60 and 75 kDa were identified as potential glycosylated proteins by staining with a Gelcode® Glycoprotein Staining Kit (Pierce Biotechnology). MALDI-TOF/MS examination of 77 tryptic peptide fragments of the 75 kDa protein and 80 peptide fragments of the 60 kDa protein revealed significant identity of the aa sequence of these proteins to the predicted aa sequence of JDV SU and TM. Further analysis identified glycosylation sites in the fragments of the 2 proteins: 2 potential sites of N-linked glycosylation in the 75 kDa band and 3 fragments with 6 potential O-linked glycosylation sites in the 60 kDa protein. This study has confirmed the identity of the 75kDa and 60kDa as SU and TM glycoproteins, respectively.
Introduction

Although the predicted sizes of the non-glycosylated forms of the Env polyprotein and the SU and TM have been determined as 88.8, 47.8 and 41.1 kDa, respectively, and a number of potential glycosylation sites have been predicted from analysis of the sequence (Chadwick et al., 1995b), information about the size or the degree of glycosylation of the native forms of these proteins has not been reported.

Membrane glycoproteins are responsible for many important functional properties of not only eukaryotic cell surfaces but also virus-cell interactions (Parekh, 1991) and their post-translational modification by glycosylation is critical to their function. This glycosylation is a complex process varying in different cell types and cannot be easily predicted by simple rules. Two types of carbohydrate-protein linkages are known: O-linked and N-linked (Medzihradszky, 2005, Peter-Katalinic, 2005).

Although some characteristics of glycosylation can be obtained by metabolic labeling with radioactive saccharides or affinity chromatography with lectins such as concanavalin A (Bundy & Fenselau, 1999, Bundy & Fenselau, 2001), mass spectrometry (MS) offers several advantages and it has been used widely to characterise biomolecules such as carbohydrates (Siemiatkoski & Lyubarskaya, 2005), peptides and proteins (Stutz, 2005, Wysocki et al., 2005), DNA (Mauger et al., 2006), metabolites (Xu et al., 2006), phospholipids (Pulfer & Murphy, 2003) and also whole cells (Easterling et al., 1998). It provides a rapid method for identification of biomolecules based upon molecular weight and offers better resolution and accuracy than other techniques such as SDS-PAGE. In recent years, matrix-assisted layer desorption/ionisation (MALDI) MS has been used to characterise glycoproteins (Ayers et al., 2002, Barrientos et al., 2004, Kim et al., 2001, Serebryakova et al., 2006). MS procedures offer the opportunity for peptide mass fingerprinting and de novo sequencing. Peptide mass fingerprinting is a useful technique used to identify proteins whose aa sequences are known or have been predicted from DNA analysis of the encoding gene (Thiede et al., 2005). MALDI MS in combination with proteolytic assays has been used to identify the functional epitope on envelope glycoprotein gp41 of HIV-1 (Parker et al., 2001).

To provide information about the size of the native glycosylated proteins of JDV an attempt was made to identify the proteins present in native viral preparations obtained from the plasma of JDV-infected cattle by utilising matrix-assisted laser desorption/ionisation–time of flight (MALDI-TOF) MS. The data generated was also
used to identify glycosylation sites (both N- and O-linked) as well as the types of glycans attached to the glycosylated proteins.

Material and methods

Identification of potential glycoproteins in SDS-PAGE preparations of JDV

A plasma-derived preparation of JDV (30 µl) from JDV infected cattle was a kind gift from Dr. Nining Hartaningsih (Indonesia). The plasma was prepared as described by (Hartaningsih et al., 1994). Briefly, plasma collected from JDV-infected cattle and containing an estimated 10^8 infectious virions per mL of plasma was clarified by low speed centrifugation. The virus was then pelleted by centrifugation and the resuspended pellet subjected to sucrose gradient centrifugation. Visible bands in the sucrose gradient were harvested and resuspended in Tris-HCl buffer to 1% of the original plasma volume. The partially purified virus sample obtained was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Hartaningsih et al., 1994) but using a 4% stacking gel and 14% resolving gel. The protein bands were detected by staining with Coomassie Blue R20 in 40% methanol and 10% acetic acid or with a GelCode® Glycoprotein Staining Kit (Pierce Biotechnology) according to the manufacturer’s instructions. This glycoprotein detection system selectively stains glycoproteins in polyacrylamide gels using a modified Periodic Acid-Schiff (PAS) staining method (Zacharius et al., 1969). Staining of sugar moieties of the glycoproteins yields magenta bands with a light pink background. The Periodic Acid-Schiff reagent stains vicinal diol groups found mainly on peripheral sugars and sialic acids and is used as a general glycoprotein stain (Thornton et al., 1994).

Preparation of potential glycoproteins for MALDI-TOF MS analysis

Two protein bands of 75 kDa and 60 kDa visualised with both Coomassie Blue and the Glycoprotein Staining Kit were excised using a sterile scalpel blade and placed in separate Eppendorf tubes. Each gel segment was washed by covering it with 500 µl of PBS at 4°C for 30 min, then discarding the PBS and repeating the washing step. The gel pieces were then washed a further 2 times with 500 µl of 100 mM NH_4HCO_3 / acetonitrile (1:1, v/v) for 15 minute and dehydrated the gel in 200 µl of 100% acetonitrile. Digestion of the proteins was conducted by the addition of 200 µl of 50 mM NH_4HCO_3 containing 4 ng/µl of trypsin to fully immerse the gel pieces and
incubated for 30 min at room temperature before the tubes were incubated overnight at 37°C. The tubes were spun down for 10 s. The gels were washed with 200 µl of 25 mM NH₄HCO₃/acetonitrile and the peptides were extracted with 50 µl of 5% formic acid (HCOOH)/acetonitrile by sonicating the gel in a ultrasonic bath for 2 min and then incubated for 30 min at room temperature. The supernatant was transferred to a clean Eppendorf tube and dried in a speedvac. The dried peptides were reconstituted in 20 µl of 0.5% HCOOH in 65% methanol.

**MALDI-TOF MS analysis of tryptic peptides**

One µl of each peptide solution was mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid in 0.5% HCOOH/65% methanol, and dried at room temperature. The molecular masses of the tryptic peptides were then determined by MALDI-TOF MS (Voyager, Applied Biosystems) with the assistance of Proteomics International Inc. at Murdoch University. MALDI spectra were calibrated using a peptide mixture provided by the manufacturer. Monoisotopic peptide masses obtained from mass spectra were searched against the NCBI non-redundant protein database (NCBIinr) and MSDB databases using the MASCOT protein identification system (Matrix Science Ltd. London, UK, http://www.matrixscience.com). The same monoisotopic peptide masses were also subjected to FindPept (http://au.expasy.org/tools/findpept.html) and GlycoMod (http://au.expasy.org/tools/glycomod/) tools to predict the theoretical peptides and the glycosylation sites, respectively. The data was also compared to the predicted aa sequence of the JDV genomic sequence (GenBank accession no. U21603.1 gi: 733067) and the JDV Env precursor protein sequence (accession number gi: 733070). This Env precursor is assumed to be post-translationally processed to produce the SU and TM proteins (Chadwick et al., 1995b).

**Results**

**Identification of glycoproteins in SDS-PAGE gels**

When the native JDV proteins were separated by SDS-PAGE and stained with Coomassie Blue, 4 relatively abundant proteins with a MW of >41.1 kDa, the predicted size of the smallest non-glycosylated envelope protein (Chadwick et al., 1995), were identified with a MW of approximately ~250, ~75, ~60 and ~50 kDa (Figure 6.1). When stained with the GelCode® Glycoprotein Staining Kit (Pierce
Biotechnology), magenta bands with a light pink background indicated 3 of these proteins were glycosylated. The protein with a predicted mass of ~75 kDa was the most abundant while the 2 bands at ~250 kDa and ~60 kDa stained weakly.

Figure 6.1. Coomassie Blue (RHS) and GelCode® Glycoprotein Staining (LHS) of JDV proteins in 14% SDS-PAGE gels. The protein markers and their size are shown in the extreme LHS of the gel.

**MALDI-TOF/MS analysis**

Two potentially glycosylated protein bands of MW 75 and 60 kDa (Figure 6.1) were excised from the SDS-PAGE gel and subjected to in-gel digestion with trypsin, which cleaves after lysine and arginine residues, and the peptide digests were then analysed by MALDI-TOF MS. The m/z spectra of the mixture of peptides resulting from tryptic digestion of the 75 kDa proteins and the 60 kDa protein are shown in Figures 6.2 and 6.3.
Figure 6.2. Single MALDI-TOF/MS spectrum of the peptide mixture obtained from a tryptic digests of the 75 kDa protein. The labels associated with the peaks indicate the measured mass for the tryptic peptides. The ordinate axis represents the intensity of the detection signal of peptide detected by the MS at a specific mass to charge (m/z).

Figure 6.3. MALDI-TOF/MS spectra of a tryptic digests of 60 kDa protein excised from a preparative PAGE gel. The peak labels indicate the measured mass (m/z) for all the tryptic peptides that contribute to the whole protein.
The sequence of the monoisotopic peptide masses were examined for sequence similarity to proteins in the MSDB and NCBInr databases using the MASCOT search program. With this program, the protein hits were scored using a probability based Mowse (molecular weight search) score. The ion score is $-10 \times \log (P)$, where $P$ is the probability that the observed match is a random event. Individual ions that scored more than 51 indicated identity or extensive homology ($p<0.05$). The scores obtained ranged from 24 to 40, so that neither protein was identified as having a significant match to any protein in the MSDB database. However, the proteins with greatest identity to the 75 kDa peptides in the NCBInr database were envelope glycoproteins of HIV-1, Saimiriine herpesvirus-2 and the envelope glycoprotein of Caprine arthritis-encephalitis virus (Table 6.1). Proteins of greatest identity with the 60 kDa protein in the NCBInr database were of Porcine reproductive and respiratory syndrome virus, the envelope glycoprotein of HIV-1 and Hepatitis C virus (Table 6.2). The databases did not contain data for any bovine lentivirus protein.

Table 6.1. Comparison of peptides found in tryptic digests of the 75 kDa protein with proteins in the NCBInr database using the MASCOT search program. Only Mowse scores of more than 51 indicate identity or extensive homology.

<table>
<thead>
<tr>
<th>Mowse score</th>
<th>Protein</th>
<th>Virus</th>
<th>Molecular weight</th>
<th>Peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>Envelope glycoprotein gp120</td>
<td>HIV-1</td>
<td>826.42</td>
<td>QPVSKLR</td>
</tr>
<tr>
<td>28</td>
<td>Envelope glycoprotein gp120</td>
<td>HIV-1</td>
<td>1035.53</td>
<td>KLSDFQFGNK</td>
</tr>
<tr>
<td>26</td>
<td>Envelope glycoprotein gp120</td>
<td>HIV-1</td>
<td>939.41</td>
<td>KSINIGPGR</td>
</tr>
<tr>
<td>25</td>
<td>Glycoprotein 120</td>
<td>Saimiriine herpesvirus-2</td>
<td>1414.78</td>
<td>VITSSLQTSSSYK</td>
</tr>
<tr>
<td>24</td>
<td>Glycoprotein gp120</td>
<td>HIV-1</td>
<td>516.51</td>
<td>VGLTK</td>
</tr>
<tr>
<td>24</td>
<td>Surface glycoprotein gp120</td>
<td>Caprine arthritis-encephalitis virus</td>
<td>516.51</td>
<td>VIGTK</td>
</tr>
</tbody>
</table>
Table 6.2. Comparison of peptides found in tryptic digests of the 60 kDa protein with proteins in the NCBI nr database using the MASCOT search program. Only Mowse scores of more than 51 indicate identity or extensive homology.

<table>
<thead>
<tr>
<th>Mowse score</th>
<th>Protein name</th>
<th>Species</th>
<th>Molecular Weight</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>Polypeptide precursor NSP1 – NSP8</td>
<td><em>Porcine reproductive and respiratory syndrome virus</em></td>
<td>821.50</td>
<td>VAAEIYR</td>
</tr>
<tr>
<td>34</td>
<td>Envelope glycoprotein, V3-V5 region</td>
<td>HIV-1</td>
<td>913.50</td>
<td>DPITLPCR</td>
</tr>
<tr>
<td>29</td>
<td>Reverse transcriptase</td>
<td>HIV-1</td>
<td>1069.53</td>
<td>WGFYTPDGK</td>
</tr>
<tr>
<td>29</td>
<td>Polyprotein</td>
<td><em>Hepatitis C virus</em></td>
<td>989.54</td>
<td>TRVTGGSSAR</td>
</tr>
</tbody>
</table>

The peptide mass fingerprinting data obtained by MALDI-TOF MS were compared with the theoretical peptides calculated by Findpept software program. The FindPept program deduced the theoretical peptides that would be obtained by trypsin digestion of the aa sequence of the envelope proteins of JDV derived from the DNA sequence and then calculated the theoretical masses of the fragments. The 75 kDa peptide mass fingerprinting data generated by MALDI-TOF/MS resulted in masses for 77 peptides, 66 of which matched the theoretical peptide masses of JDV SU (aa 1-422) calculated by the FindPept program and 11 were unmatched and assumed to be glycosylated (Figure 6.4 and Table 6.3). The 60 kDa peptide mass fingerprinting data generated by MALDI-TOF/MS resulted in a mass for 94 peptides, 80 of which had high sequence identity to the deduced masses of the peptides of JDV TM (Env aa 423-781) calculated by the FindPept program and 14 were unmatched (Figure 6.5 and Table 6.4) and were assumed to be glycosylated.
Table 6.3. Comparison between the masses of experimentally observed with the theoretical masses and assignment of the tryptic peptide mass fingerprint MALDI-TOF/MS of the 75 kDa protein.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>m/z (detected)</th>
<th>m/z (calculated)</th>
<th>Residue position</th>
<th>Peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
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<tr>
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<td>1191.60</td>
<td>13-22</td>
<td>RGEKSTMRDL</td>
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<tr>
<td>P3</td>
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<td>1588.84</td>
<td>17-30</td>
<td>STMRDLLQRAVDKG</td>
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<tr>
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<td>2095.10</td>
<td>2094.11</td>
<td>25-42</td>
<td>RAVDKGHLTAREALDRWT</td>
</tr>
<tr>
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<td>2337.16</td>
<td>43-63</td>
<td>LEDHGEIHPWIILFCFAGAIG</td>
</tr>
<tr>
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<td>1737.95</td>
<td>59-76</td>
<td>AGAIGVIGWGRLGELNV</td>
</tr>
<tr>
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<td>2093.02</td>
<td>94-111</td>
<td>EAARNIDSLDWKWIRKVF</td>
</tr>
<tr>
<td>P8</td>
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<td>94-111</td>
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</tr>
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<tr>
<td>P10</td>
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<td>2397.12</td>
<td>152-172</td>
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<tr>
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<td>1037.51</td>
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<tr>
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<tr>
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<tr>
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</table>
Figure 6.4 Interpretation of the MALDI peptide mass fingerprint results of the 75 kDa protein. The arrows indicate the tryptic peptide fragment (P1 – P37) produced by MALDI-TOF/MS.
Table 6.4. Comparison of the masses of the experimentally produced peptides of the 60 kDa protein generated by MALDI-TOF/MS with the theoretical masses of the tryptic peptide mass fingerprint generated by FindPept software.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>m/z (detected)</th>
<th>m/z (calculated)</th>
<th>Residue position</th>
<th>Peptide sequence</th>
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<td>MTASVTAATL</td>
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<tr>
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</table>
Figure 6.5. Interpretation of the MALDI peptide mass fingerprint results of the 60 kDa protein. The arrows indicate the tryptic peptide fragment (P1 – P42) produced by MALDI-TOF/MS.

**Glycoprotein analysis**

The GlycoMod program was used to predict the mass of the glycopeptides and the numbers of glycosylation sites obtaining from the tryptic digest of the 75 and 60 kDa proteins. Analysis of the 11 unmatched masses of the 75 kDa protein predicted there was one glycosylated peptide of 2013.98 Da with 2 potential sites of N-linked
glycosylation at asparagine (N) residues at aa 285 and 301 (Table 6.5). Analysis of the 14 unmatched masses of the 60 kDa protein predicted there were 3 glycosylated peptides (1596.01 Da, 1802.11 Da and 1894.13 Da) and 6 potential sites of O-linked glycosylation at serine residues at aa positions 650, 668 and 707, and at threonine (T) residues at aa positions 653, 737 and 691 (Table 6.6).

Table 6.5. Prediction of unmatched masses of 75 kDa protein containing the motif 'N-X-S/T/C (X not P)'. Coloured (red) letters indicates the predicted position of glycosylated aa.

<table>
<thead>
<tr>
<th>Peptide mass (exp.observed)</th>
<th>Peptide sequence</th>
<th>Peptide mass (calc.)</th>
<th>Δm or predicted residual carbohydrate mass</th>
<th>Predicted carbohydrate side chain structure</th>
</tr>
</thead>
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<tr>
<td>2013.98</td>
<td>284-294 ENLSEGSAQVR</td>
<td>1188.57</td>
<td>825.41</td>
<td>1. (Hex)$_1$ (NeuAc)$_2$ (Sulph)$_1$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2. (Hex)$_1$ (NeuAc)$_2$ (Phos)$_1$</td>
</tr>
<tr>
<td></td>
<td>295-306 NCLDPINVTEPR</td>
<td>1369.67</td>
<td>644.31</td>
<td>(Hex)$_1$ (HexNAc)$_1$ (Deoxyhexose)$_1$ (Pent)$_1$</td>
</tr>
</tbody>
</table>

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Table 6.6. Predictions of unmatched masses of 60kDa protein containing S or T. Coloured (red) letters indicate the predicted position of glycosylated amino acids.

<table>
<thead>
<tr>
<th>Peptide mass (exp.observed)</th>
<th>Peptide sequence</th>
<th>Peptide mass (calc.)</th>
<th>Δm or predicted residual carbohydrate mass</th>
<th>Predicted carbohydrate side chain structure</th>
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</thead>
<tbody>
<tr>
<td>1596.01</td>
<td>650-655 SGDTPR</td>
<td>631.29</td>
<td>964.72</td>
<td>(Pent)$_5$ (Sulph)$_6$ (HexA)$_2$</td>
</tr>
<tr>
<td></td>
<td>736-742 GTGGDDR</td>
<td>676.28</td>
<td>919.73</td>
<td>(NeuGc)$_1$ (Pent)$_1$ (Sulph)$_6$</td>
</tr>
</tbody>
</table>
| 1802.11                     | 650-655 SGDTPR   | 631.29               | 1170.82                                     | 1. (Deoxyhexose)$_1$ (NeuAc)$_1$ (Sulph)$_6$ (HexA)$_2$
|                             |                  |                      |                                             | 2. (Hex)$_1$ (Deoxyhexose)$_1$ (Pent)$_1$ (Sulph)$_6$ (KDN)$_1$ (HexA)$_1$
|                             |                  |                      |                                             | 3. (Pent)$_2$ (Phos)$_6$ (KDN)$_1$ (HexA)$_1$ |
|                             | 667-671 LSEGK    | 532.29               | 1269.82                                     | 1. (Deoxyhexose)$_1$ (NeuGc)$_1$ (Sulph)$_6$ (HexA)$_2$
|                             |                  |                      |                                             | 2. (Hex)$_1$ (NeuGc)$_1$ (Phos)$_6$ (HexA)$_1$ |
|                             | 701-714 ILQPLS$^*$LVLLVR | 1589.06            | 213.05                                      | 1. (Pent)$_1$ (Sulph)$_1$
|                             |                  |                      |                                             | 2. (Pent)$_1$ (Phos)$_1$ |
|                             | 736-742 GTGGDDR  | 676.28               | 1125.83                                     | 1. (Hex)$_1$ (NeuGc)$_1$ (Sulph)$_6$ (HexA)$_2$
|                             |                  |                      |                                             | 2. (Hex)$_1$ (NeuGc)$_1$ (Phos)$_6$ (HexA)$_1$ |
| 1894.13                     | 650-655 SGDTPR   | 631.29               | 1262.84                                     | (HexNAc)$_1$ (NeuGc)$_1$ (Sulph)$_5$ (HexA)$_2$ |
|                             | 667-671 LSEGK    | 532.29               | 1361.84                                     | (Hex)$_2$ (NeuGc)$_1$ (Sulph)$_6$ (KDN)$_1$ |
|                             | 691-693 TLR      | 388.24               | 1505.89                                     | 1. (NeuAc)$_1$ (Pent)$_1$ (Sulph)$_6$ (KDN)$_1$ (HexA)$_2$
|                             |                  |                      |                                             | 2. (Deoxyhexose)$_2$ (NeuGc)$_1$ (Sulph)$_6$ (KDN)$_1$ (HexA)$_1$
|                             |                  |                      |                                             | 3. (Hex)$_1$ (Deoxyhexose)$_1$ (NeuAc)$_1$ (Sulph)$_6$ (KDN)$_1$ (HexA)$_1$
|                             |                  |                      |                                             | 4. (Hex)$_2$ (HexNAc)$_1$ (Deoxyhexose)$_1$ (Sulph)$_6$ (HexA)$_2$
|                             |                  |                      |                                             | 5. (Deoxyhexose)$_1$ (NeuGc)$_1$ (Pent)$_2$ (Sulph)$_6$ (HexA)$_1$
|                             |                  |                      |                                             | 6. (HexNAc)$_1$ (Deoxyhexose)$_1$ (Sulph)$_6$ (KDN)$_1$ (HexA)$_1$
|                             |                  |                      |                                             | 7. (Hex)$_1$ (NeuAc)$_1$ (Pent)$_3$ (Sulph)$_6$ (HexA)$_1$
|                             |                  |                      |                                             | 8. (HexNAc)$_1$ (Pent)$_3$ (Sulph)$_6$ (KDN)$_1$ (HexA)$_1$
|                             |                  |                      |                                             | 9. (NeuAc)$_1$ (Pent)$_1$ (Phos)$_6$ (KDN)$_1$ (HexA)$_2$ |

Carbohydrate symbols used are: Hex, hexose; Pent, Pentose; Sulph, sulphate; HexA, β-hexosaminidase A; NeuAc, N-acetylneuraminic acid; NAc, N-acetyl; NeuGc, N-glycolyneuraminic acid; KDN, 2-keto-3-deoxy-nonulosonic acid.
Discussion

The HIV envelope glycoproteins have a central role in the induction of the host immune response and are targets for protective immunity (Bukrinskaya, 2004, Chan & Kim, 1998, Gummuluru & Emerman, 2002) and it is assumed that this would also be true for JDV. Some preliminary study of the role of the JDV envelope proteins as potential vaccine immunogens has been undertaken utilizing recombinant SU and TM expressed in a bacterial expression system, and hence with non-glycosylated forms of these proteins (Ditcham, 2007), with minimal effect. Further work with these proteins as potential vaccines requires an improved understanding of the comparative structure of the non-glycosylated recombinant JDV proteins and the structure and glycosylation characteristics of the native proteins. While the size of the JDV Env polyprotein has been predicted (781 aa), this is considerably smaller than that of the other lentivirus envelope glycoproteins including the other bovine lentivirus BIV (904 aa) (Garvey et al., 1990), HIV (856 aa) (Martoglio et al., 1997), SIV (854 aa) (Fomsgaard et al., 1991), FIV (856 aa) (Olmsted et al., 1989) and EIAV (859 aa) (Rushlow et al., 1986) and the predicted structure of those envelope proteins is varied and it is possible that these differences could be even greater for JDV.

The use of peptide mass fingerprinting with the MALDI-TOF system, whereby the proteins were digested into smaller peptide fragments and then the resulting peptides were compared to those in protein databases, enabled identification of 2 native JDV proteins, one of 75 kDa and the other of 60 kDa, as the SU and TM proteins, respectively. Analysis of peptide mass fingerprinting data enabled 66 of 77 peptides of the 75 kDa protein to be matched to the JDV Env aa positions 1-422 (SU region) and 80 of 94 peptides of the 60 kDa protein matched to the JDV Env aa positions 423-781 (TM region). A search of the NCBInr database using Mascot software also revealed similarities of both proteins to other viral envelope glycoproteins, particularly lentiviral proteins, but there were no definitive matches as the scores obtained were less than 51. However, none of the other databases searched contained data for JDV proteins.

Further analyses of the 2 proteins using GelCode® Glycoprotein Staining Kit suggested that the 75 and the 60 kDa proteins were glycosylated and the glycosylation of these 2 proteins was confirmed by analysis of the MALDI-TOF data. One peptide fragment of the JDV 75 kDa SU was identified that had 2 potential sites of N-linked glycosylation. Three peptide fragments the 60 kDa TM protein were
identified with a total of 6 potential O-linked glycosylation sites. A lower number of potential glycosylation sites in the JDV Env than in other lentiviruses was predicted by examination of the sequence data (Chadwick et al., 1995b) and the low number of glycosylation sites identified by MALDI-TOF analysis was consistent with this analysis. The position and number of potential N- and O-glycosylation sites in the JDV 75kDa and 60 kDa proteins seems quite different to that in the envelope glycoprotein of the other lentiviruses. The envelope proteins of HIV-1 contain approximately 30-38 potential asparagine-linked (Asn-X-Ser/Thr) glycosylation sites (Ratner, 1992). HIV-1 Env gp120 contains 18 to 33 potential N-glycosylation sites (Korber et al., 2001) and is among the most heavily glycosylated envelope proteins of any retrovirus. HIV-1 has also 3-6 potential N-glycosylation sites in the TM protein, the number varying in different strains of the virus (Dedera et al., 1992). Potential O-glycosylation sites in different strains of HIV-1, HIV-2, and SIV were examined by Hansen et al. (1998) and demonstrated that SIV Env (gp110) contained more O-glycosylated (4–18 sites) than HIV-1 or HIV-2 (less than 5 sites). FIV has 20 potential N-linked glycosylation sites present in the deduced Env aa sequence (Olmsted et al., 1989).

The probable structures of the carbohydrate side chain at the glycosylation site predicted with the GlycoMod program used for the analysis were heterogeneous and possibly complex. It might be possible to use the molecular mass of carbohydrate side chain to search for their probable glycan structures, thus providing useful information towards the structural characterization of JDV glycoproteins. Other analytical methods such as complete cleavage of the oligosaccharide chains from the glycoproteins and mass spectrometry analysis of each purified oligosaccharide after separation of the carbohydrate mixture by high performance liquid chromatography would be required for the complete characterisation of the glycan structure.
Chapter 7

General discussion

Jembrana disease is a disease specific to the Indonesian archipelago and there are 2 major reasons for this: the geographic isolation of Indonesia and limited trade in live cattle from Indonesia to other countries, and the specificity of the disease for Bali cattle. One consequence of this is that research on the disease, especially studies involving the use of live animals, has been restricted to Indonesia and a small research group only. While our understanding of Jembrana disease and has improved markedly since the recognition that a lentivirus was the aetiological agent (Chadwick et al., 1995a), progress has been restricted by infrastructure and funding restraints.

Attempts to control the disease by limiting the movement of Bali cattle from Bali have slowed the spread of the disease from Bali to other islands but despite these restrictions it has also occurred on the islands of Java, Sumatra and Indonesian Borneo (Kalimantan). How this occurred is not fully understood and documented but it may well have, at least in the case of Java and Sumatra, involved the illegal movement of virus-infected Bali cattle from Bali. Future control of Jembrana disease will likely require more than quarantine, and will need the development of improved diagnostic and immunosurveillance procedures to monitor the distribution of the disease and provide rapid diagnosis, and the development of an effective vaccine.

To support the development of improved diagnostics and vaccines, an improved understanding of native JDV proteins and their production using recombinant techniques is required. The studies reported in this thesis have helped to improve our knowledge in this area. In this current study, only selected proteins were examined: the CA, the major and immunodominant protein of JDV that is likely to be a key protein in the development of diagnostics, and the 2 envelope proteins SU and TM involved in attachment and entry of virus into cells. There is remarkably little information available about the envelope glycoproteins of JDV, not even fundamental characteristics of the size of the native proteins and the degree and type of glycosylation. The studies reported in this thesis have added to our understanding of these glycoproteins and will facilitate their future use as antigens and vaccines.
For many years in this laboratory, recombinant JDV proteins and including CA and the envelope proteins SU and TM have been produced as fusions to GST in *E. coli* under the control of the *tac* promoter and used as antigens for ELISA and western blotting procedure for detection of antibody (Burkala et al., 1999, Burkala et al., 1998, Desport et al., 2005). Cloning and expression of recombinant protein in *E. coli* has been favoured because this bacterium has relatively simple genetics, a rapid growth rate and it is well characterised. The methodology needed for cloning and expression of proteins is relatively simple compared to many other expression systems, requiring only basic equipment, enabling it to be used in Indonesia. Most JDV recombinant proteins were expressed, however, in the form of insoluble proteins within inclusion bodies which has limited their use in serological assays and in experimental vaccines.

One of the aims of this thesis was to establish a simple method to solubilise the inclusion body proteins while retaining their antigenicity. The studies reported in Chapter 3 showed that SU-GST could be reliably and efficiently expressed in *E. coli* as inclusion bodies with good yields. The solubilisation of the SU-GST inclusion bodies was achieved with a combination of a low concentration of urea in an alkaline solution, with the addition of the reducing agent dithiothreitol (DTT) and the serine protease inhibitor phenylmethanesulfonyl fluoride, providing an acceptable yield of denatured protein for purification. The solubilised SU-GST protein was refolded by slow 10-fold dilution in 20 mM Tris pH 8.0 containing CHAPS and DTT and the refolded protein reacted in western immunoblots with bovine antisera. The technique developed for solubilisation of the proteins present in inclusion bodies is simple and the technology has been successfully used in Indonesia for the solubilisation not only of recombinant SU but also for the solubilisation of other JDV proteins present in inclusion bodies, including CA, Tat and TM. These solubilised proteins have been used successfully for the development of serological antigens and experimental vaccines (Ditcham, 2007, Lewis, 2008). The technique is a significant advance in the preparation of antigens and vaccines for JDV and removes the dependency on the need for expression of soluble proteins, which is always difficult using such bacterial expression systems.

The addition of the reducing agent DTT seemed to be important in the solubilisation process, probably because of the presence of disulfide bonds in the proteins. The SU protein of JDV contains 13 cysteine residues predicted to form 5 disulfide bonds (Chapter 6) and supposing that the aggregation of the protein is mainly caused by the creation of the intra- and interchain disulfide bridges, resulting in formation of
disulfide bonded aggregates. The addition of DTT probably prevented aggregation by inhibiting the formation of non-native disulfide bonds and dissolve disulfide bonded aggregates (Rothel et al., 1997).

During western immunoblotting procedures for the detection of antibody against JDV, reliance has been placed on the detection of the immunodominant 26 kDa CA protein as many of the other proteins that are observed with whole JDV preparations have not been identified (Hartaningsih et al., 1994, Kertayadnya et al., 1993). In Chapter 6, a study utilising a specific glycoprotein staining method and MALDI-TOF mass spectrometric analysis identified the 2 JDV envelope glycoproteins SU and TM in SDS-PAGE gels as being of 75 and 60 kDa, respectively. Identification of the size of these glycosylated proteins in SDS-PAGE gels will permit their use for better interpretation of western immunoblotting results with sera from JDV-infected cattle.

The MALDI-TOF mass spectrometric analysis of the glycosylated Env proteins SU and TM (Chapter 6) also provided information on the secondary glycan structure of these glycoproteins. MALDI-TOF mass spectrometry has proven to be a method of choice for glycoprotein analysis because it is one of the most sensitive methods for the analysis of glycoproteins (de Laurentiis et al., 2006). The GlycoMod method of determining the mass value from MALDI-TOF/MS data provided initial characterisation of the sugar moiety decorating at least 2 putative N-glycosylation site of the JDV-SU and 6 putative O-glycosylation sites of the JDV-TM.

To assist in the characterisation of JDV proteins and to provide methods for the long term production of MAbs in Indonesia, their production by conventional and recombinant techniques was investigated in Chapters 4 and 5 of this thesis. Monoclonal antibodies have been used widely for diagnostic purposes for many conditions and infections and for many years a JDV CA MAb BC10 (Kertayadnya et al., 1993) produced in Indonesia provided an invaluable reagent in immunoperoxidase assays for the detection of JDV in tissues and as a control reagent in western immunoblotting. Unfortunately, the hybridoma secreting this MAb was lost when liquid nitrogen supplies in which it was maintained were lost. During the current study, an alternative hybridoma was produced (Chapter 4) against the JDV CA which was of IgG2 isotype and this was identified as binding to the aa encoded by nucleotides 604-810 of the JDV capsid gene. This MAb was successfully used to identify JDV in formalin-fixed paraffin embedded tissue sections. Attempts were made to also produce a monoclonal against a recombinant SU but these attempts were unsuccessful. The attempts to produce a SU MAb
were undertaken prior to the identification of the whole virus SU and additional attempts using the glycosylated whole virus SU may have been worthwhile.

To overcome the problem of storage of hybridomas in liquid nitrogen in Indonesia, the use of recombinant antibody fragments was investigated as a means of providing a solution to the problem of long-term storage. These attempts to produce recombinant MAb (Chapter 5) utilised the phage display antibody method because the technique has been previously used for generating recombinant antibodies against the TM glycoprotein gp46 of Maedi visna virus (Blazek et al., 2004) and CA (Celer et al., 2003). Recombinant antibody fragments, scFv, with specificity for the CA protein of JDV were generated using the Tomlinson I and J phage display antibody libraries. Attempts were also made to produce scFv against a ΔSU with a GST fusion tag but the only scFv detected were against the GST component. Using an E. coli expression system, soluble scFv against the CA was expressed in the bacterial periplasm but it was produced in small quantities only. While clones are now available as a consequence of these studies that will allow the expression of CA scFv, further work is now required to optimise this system and obtain adequate stocks for further investigation, perhaps by modifying the bacterial growth conditions or modifying the method of expression. The success with the method shows that it does offer the potential for the production not only of CA scFv but also those against other viral proteins. The main difficulty and most time consuming part of attempts to produce scFv with the Tomlinson I and J libraries was the panning process. It is likely that with experience, greater efficiency of the phage technique could be obtained. Another problem was an apparent low affinity of the scFv to the JDV ΔSU and CA proteins and this is a noticeable characteristic of scFv isolated from this library (Kjaer et al., 2001, Wang et al., 2004) that can be improved by introducing diversity to the V genes of the original antibody. Diversity can be introduced by random mutations either by error-prone PCR (Meyer et al., 2002) or by applying bacterial mutator strains (Coia et al., 2001).
References


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