

# **Identification of Possible Infectious Bursal Disease Virus Receptors**

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## PREFACE

The experimental work described in this dissertation was carried out in the School for Molecular and Cellular Biosciences, Department of Biochemistry, University of Natal, Pietermaritzburg from January 1998 to December 1999 under the supervision of Dr Theresa H. T. Coetzer and co-supervision of Dr Roger F. Horner. Most of the amino-acid sequencing work was carried out at the Max Planck Institute for Biochemistry, Martinsried, Germany as a guest of Reini Mentele and Dr Ennes Auerswald, Ludwig-Maximilians University, Munich, Germany. These studies represent original work by the author and have not been submitted in any other form to another university. Where use has been made of the work of others, it has been duly acknowledged in the text.



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## ABSTRACT

Infectious bursal disease virus (IBDV) is a chicken pathogen that infects the bursa of Fabricius, an organ involved in the development of the immune system in chickens. Infection by the virus leads to destruction of the bursa and immunosuppression. Infection by virulent strains may result in mortality. Current methods to combat the virus involve the use of vaccines. These are usually a mixture of live attenuated and oil inactivated virus. Variant strains of the virus are able to escape the vaccine-generated antibodies. In addition, the vaccines result in damage to the bursa. Identification of a receptor for IBDV could result in the development of either treatment for the virus or superior vaccines by interfering with the attachment of the virus to host cells.

Several methods for identifying IBDV binding proteins from the membranes of cells from the bursa of Fabricius were examined. Affinity chromatography of IBDV binding proteins with a matrix consisting of IBDV cross-linked to Sepharose 4B allowed separation of a number of virus binding proteins. In contrast, virus overlay protein blot assay (VOPBA) and reversible cross-linking with 2-iminothiolane proved less conclusive.

Predominant proteins in the affinity-separated fraction were of 40 and 32 kDa. These were further examined by N-terminal amino acid sequencing of the whole protein and N-terminal sequencing of peptides produced by endoproteinase Lys-C digestion of the protein respectively. The 40 kDa protein showed homology with human synovial stimulatory protein involved in the formation of autoantibodies in rheumatoid arthritis. Virus was also shown to bind to a 440 kDa protein complex. This 440 kDa protein complex appeared to consist primarily of a 40 kDa protein when examined by reducing Tris-Tricine SDS-PAGE. Analysis of bursal membrane proteins by Western blots using sera from rheumatoid arthritis patients revealed interactions between several IBDV proteins and the antibodies from rheumatoid arthritis patients. Using serum from one of the five patients showed a strong interaction at approximately 80 kDa and a weaker interaction at approximately 40 kDa. This may indicate an immune

reaction between a chicken homolog of the synovial stimulatory protein and antibodies in rheumatoid arthritis sera.

The 32 kDa protein showed homology to a *Pseudomonas fluorescens* protein. A section of this sequence was amplified by PCR from chicken DNA and RT-PCR from chicken RNA using degenerate primers constructed from the established N-terminal amino acid sequences and chicken codon usage tables. The fragment produced upon amplification from chicken DNA and RNA did not correspond to the predicted size of 177 bp. In contrast, when the RT-PCR product was heated and snap cooled before examination by agarose gel electrophoresis, the product consisted of two fragments, one of approximately 400 bp in size and one of approximately 200 bp in size.

The establishment of the 40 and 32 kDa chicken bursal membrane proteins as possible receptors for the virus could allow for the development of vaccines and/or treatment strategies for the virus. Treatment strategies or vaccines would be based on blocking of the interaction between IBDV and chicken host cells. Peptide mimics of the epitopes involved in such interactions could possibly achieve this.

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## ABBREVIATIONS

A <sub>280</sub>	absorbance at 280 nm
Ab	antibody
ABTS	2,2'-azinobis[3-ethyl-2,3-dihydrobenzthiazole-6-sulfonate]
AIDS	acquired immunodeficiency syndrome
BCIP	5-bromo-4-chloro-3-indolyl phosphate
Bis-acrylamide	<i>N,N'</i> -methylenebisacrylamide
bp	base pairs
BSA	bovine serum albumin
BSA-TBS	bovine serum albumin in Tris-buffered saline
c	concentration
C-terminal	carboxy terminal
CAPS	3-[cyclohexylamino]-1-propanesulfonic acid
cDNA	complementary deoxyribonucleic acid
dATP	deoxy adenosine tri-phosphate
dCTP	deoxy cytosine tri-phosphate
dGTP	deoxy guanine tri-phosphate
dNTPs	deoxy nucleotide tri-phosphates
dTTP	deoxy thymidine tri-phosphate
dist. H <sub>2</sub> O	distilled water
DEPC	diethylpyrocarbonate
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E	extinction coefficient
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
g	relative centrifugal force
HEPES	<i>N</i> -2-hydroxyethylpiperazine- <i>N</i> -2-ethanesulfonic acid
HIV	human immunodeficiency virus
HPLC	high-performance liquid chromatography

HRPO	horse radish peroxidase
IBD	infectious bursal disease
IBDV	infectious bursal disease virus
IPNV	infectious pancreatic necrosis virus
IgY	egg-yolk immunoglobulin
ARVL	Allerton Regional Veterinary Laboratory
kDa	kilo-Dalton
M <sub>r</sub>	molecular mass
mRNA	messenger RNA
N-terminus	amino terminus
NBT	nitroblue tetrazolium
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulfonylfluoride
PTC	phenylthiocarbamoyl
PTH	phenylthiohydantoin
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SPF	specific pathogen free
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide-gel electrophoresis
TBS	Tris-buffered saline
TE	Tris, EDTA
TEMED	<i>N,N,N',N'</i> -tetramethylethylenediamine
TEN	Tris, EDTA and NaCl
Tricine	<i>N</i> -[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
VOPBA	virus overlay protein blot assay
VP	viral protein



## CHAPTER 1

### INTRODUCTION

#### 1.1 History of IBDV

Infectious bursal disease (IBD) was first recognised as a distinct disease entity in 1962, and at that time was designated “avian nephrosis”. The virus was then isolated and identified as the infectious bursal agent (Winterfield *et al.*, 1972). Hitchner first used the term IBD in 1971 (Hitchner, 1971). The disease was also termed Gumboro disease, since the first outbreaks were reported from a small town called Gumboro in the state of Delaware, USA. A second serotype of the virus was reported later (McFerran *et al.*, 1980). Two distinct IBDV serotypes exist, serotype I contains viruses that are pathogenic to chickens, whereas serotype II viruses infect chickens and turkeys, but these infections are of unknown clinical significance (Cummings *et al.*, 1986).

In the USA studies indicated the presence of serotype II in US poultry flocks (Luckert, 1986). Increased mortality and morbidity in broiler flocks from the Delmarva area in the USA led to a search for possible causal infectious agents. A virus strain was isolated from broiler chicks that had bursal lesions in spite of the presence of high maternal antibody levels (Saif, 1984). This strain (MD) was shown to be antigenically different from strains isolated prior to that time and was designated a variant, while viruses isolated before that time were called classic viruses. Four variant viruses were isolated from SPF chickens that were vaccinated with classic vaccines and allowed to co-mingle with naturally infected broilers (Rosenberger and Cloud, 1986). It was then shown with monoclonal antibodies that a major antigenic shift had occurred in serotype 1 (Snyder *et al.*, 1992).

Serotype 1 strains are now classified into four groups: classical virulent strains, attenuated strains, antigenic variant strains and very virulent strains. Classical virulent strains of the virus cause bursal inflammation and severe lymphoid necrosis in infected chickens, resulting in immunodeficiency and moderate mortality.

Antigenic variant strains are recognised by escape from cross-neutralisation by antisera against classical strains. Chickens affected by the variant strains are characterised by severe atrophy of the bursa without the inflammation associated with classical strains (Vakharia *et al.*, 1994). Attenuated strains are generated by cell culture adaption of the virus: these do not cause disease in chickens and are used as live vaccines (Cursiefen *et al.*, 1979; Becht, 1994). Very virulent strains can break through very high levels of maternal antibody and cause high mortality. These strains cause lesions typical of IBDV and are antigenically similar to the classical strains (Cao *et al.*, 1998).

## **1.2 Classification of infectious bursal disease virus**

Infectious bursal disease virus (IBDV) belongs to a group of viruses known as birnaviruses. Infectious bursal disease is a highly contagious viral disease of young chickens, which is characterised by the destruction of lymphoid cells in the bursa of Fabricius. Since the virus targets actively dividing B-lymphocytes (Müller, 1986; Burkhardt and Müller, 1987), other lymphoid organs are also affected but to a lesser degree (Cheville, 1967). Among lymphoid cells isolated from the bursa of Fabricius, only a fraction can be infected *in vitro*. In contrast, thin sections of the bursa of Fabricius show that the *in vivo* situation is rather different as viral antigen can be detected in virtually all cells (Becht, 1994). In a flock between 3 and 6 weeks of age, that is fully susceptible, the clinical disease is responsible for losses due to impaired growth and death and excessive condemnation of carcasses because of skeletal muscle haemorrhages (Luckert and Hitchner, 1984). Birds less than 3 weeks of age have a subclinical infection, (Hitchner, 1971), which results in microscopic lesions in the bursa of Fabricius (Winterfield *et al.*, 1972) and immunosuppression (Allan *et al.*, 1984).

## **1.3 Economic importance of the virus**

Immunosuppression is the most important effect of the disease as it results in susceptibility to other chicken diseases, such as Newcastle disease, Marek's disease and infectious bronchitis. The virus targets actively dividing B-lymphocytes.

IBDV is a very stable virus and can persist in poultry houses after thorough cleaning and disinfection (Luckert and Hitchner, 1984). It is more resistant than reoviruses to heat, ultraviolet irradiation and photodynamic inactivation (Petek *et al.*, 1973) and is resistant to ether and chloroform. It is inactivated at pH 12.0 but unaffected at pH 2.0 and is unaffected by exposure for 1 h at 30°C to 0.5% phenol and 0.125% thiomersal (Benton *et al.*, 1967). Exposure to 1% phenol or 1% cresol for 1h inactivates the virus (Cho and Edgar, 1969) and virus infectivity is markedly reduced by exposure to 0.5% formalin for 6 h (Benton *et al.*, 1967) or to 1% formalin for 1 h (Cho and Edgar, 1969).

IBDV therefore is a difficult pathogen to control and it is even more difficult to completely rid the poultry industry of the virus. The disease is normally controlled by the combined use of live virus and inactivated oil emulsion vaccines containing serotype I IBDV. It has been shown that vaccination results in an increase in average bird weight, a decrease in mortality and therefore an increased net income, (McIlroy *et al.*, 1992). Thus vaccination or other means of control for IBDV is important in economical terms.

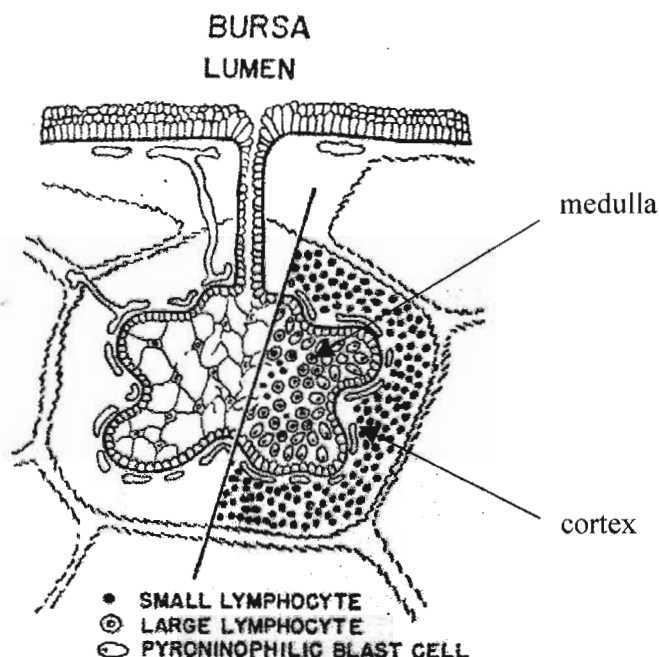
#### 1.4 Progression of the disease

Immunofluorescence studies of early IBDV propagation *in vivo* has shown that 4-5 h after peroral infection, initial virus replication takes place in gut-associated macrophages and lymphoid cells, particularly of the caecum. From there the virus is spread via the portal vein and main bloodstream to various organs and tissues. In the bursa of Fabricius, also infected by the same route, the virus finds optimum conditions and large numbers of permissive cells and replicates massively and rapidly. At 11 h post infection viral antigen is found in large amounts in the bursa of Fabricius but immunofluorescence attributed to IBDV is not detectable in other organs. The rapid multiplication in the bursa of Fabricius induces severe necrosis of bursal cells and results in the spread of large amounts of virus in the host. Grave clinical symptoms, marked lesions in other organs and high mortality are the consequences (Müller *et al.*, 1979).

The bursa increases in size after 4 to 5 days of infection as a result of oedema and hyperaemia (Cheville, 1967). The organ may have a gelatinous appearance during this period and may contain a white necrotic core in the centre of the organ. After this the bursa will begin to atrophy until it weighs approximately one third of its original weight by the eighth day post infection. The gelatinous transudate disappears as the bursa atrophies. Histological lesions in the bursa occur in conjunction with necrosis of the lymphocytes in the medullary area of the bursal follicles. The lymphocytes are soon replaced by heterophils, debris, and reticuloendothelial cells. All of the follicles may be affected by 3-4 days post infection. Cystic cavities and fibroplasia of the interfollicular connective tissue develop as inflammation decreases (Luckert and Hitchner, 1984). The rapid destruction of the bursa may be in part attributed to the stimulation of apoptosis in cells surrounding those infected with IBDV (Tanimura and Sharma, 1998). Cells in the bursa normally undergo apoptosis during maturation of the bird as the organ decreases in size but the number of apoptotic cells in the bursa is increased during infection with IBDV (Ratcliffe *et al*, 1996).

### 1.5 The bursa of Fabricius

The bursa of Fabricius is one of two primary or 'central' lymphoid organs in birds, the other being the thymus. These organs resemble one another in respect to their endodermal origin, embryonic development, lymphoid content and postnatal involution. Although immune reactions do not normally occur in these structures, their presence in neonatal life is essential if a normal population of immunologically competent lymphoid cells is to develop. The bursa is composed of lymphoid follicles whose medulla and cortex is separated by a single layer of undifferentiated endodermal epithelial cells, which give rise to the blast cells of the lymphocyte and plasmacyte series (Fig. 1). Neonatal bursectomy results in the inhibition of circulating antibody, while a similar procedure in young adulthood has little effect. It is proposed that lymphocytes in the peripheral tissue may be thymus-dependent, while larger blast cells and plasmacytes may be bursa-dependent. This implies a distinct cellular dissociation of the formulation of circulating antibody and the immunologic reactions termed 'cellular immunity' (Cheville, 1967).

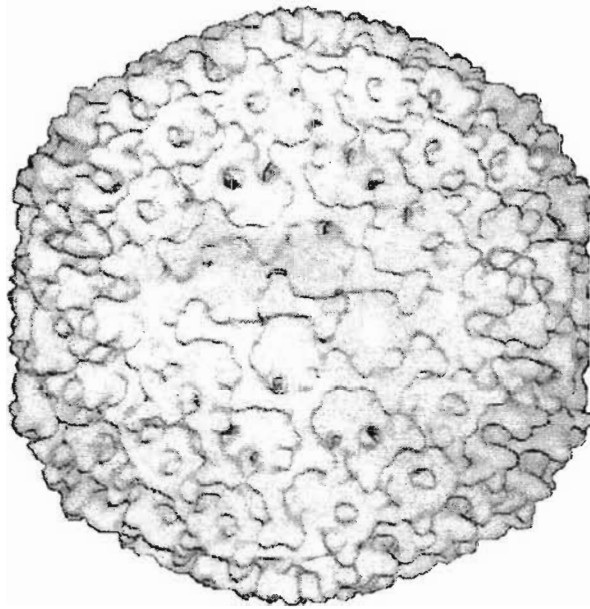


**Fig. 1.1 Diagram of anatomical arrangement of lymphoid and reticular tissue of the bursa of Fabricius.** Epithelium separating bursal medulla and cortex is continuous with epithelium of bursal lumen and gives rise to medullary lymphocytes (Cheville, 1967).

## 1.6 Biochemistry of the virus

IBDV is a member of the genus *Avibirnavirus* of the family *Birnaviridae* (Brown, 1986; Kibenge *et al.*, 1997). The generic name describes animal viruses with two segments of double stranded RNA (dsRNA; Dobos, 1979). The genus includes infectious pancreatic necrosis virus (IPNV) of fish, tellina virus and oyster virus of bivalve molluscs, and drosophila X virus of the fruit fly (*Drosophila melanogaster*). These viruses are non-enveloped, have a single capsid structure of icosahedral symmetry and a diameter of 58-60 nm (Dobos 1979). In IBDV two major viral proteins are found (VP2, 40 kDa; VP3, 32 kDa), the minor proteins comprise the large protein VP1 (90 kDa), the putative RNA-dependent RNA polymerase and VP4 (28-29 kDa), the viral protease (Becht, 1994). VP1 has been found to interact directly with the terminal ends of the double stranded viral RNA, leading to the formation of closed circles of dsRNA (Becht, 1994). Co-expression of VP1 with the IBDV polyprotein in mammalian cells has shown that VP1 forms complexes with VP3 leading to efficient encapsidation into virus like particles, and is likely a key step for the morphogenesis

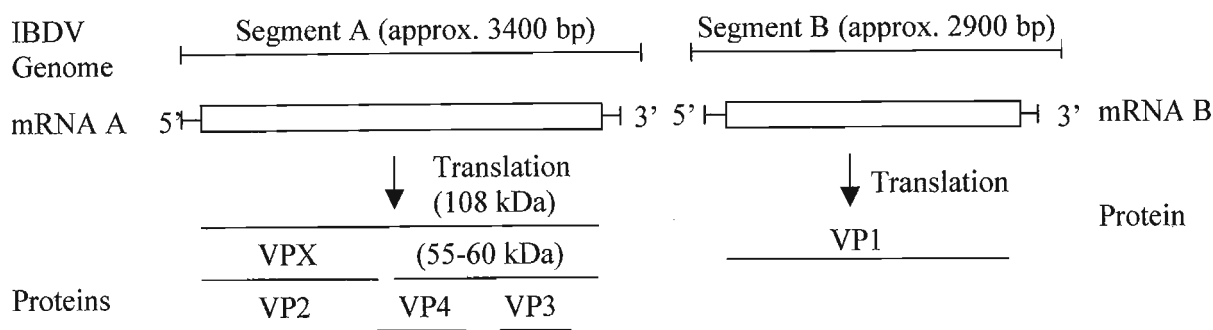
of IBDV particles. It is thought that the VP3 molecule may serve as an anchor between VP2 and VP1 (Lombardo *et al.*, 1999). None of the structural proteins of infectious bursal disease virus is appreciably glycosylated (Müller and Becht, 1982). A 3-D map of the structure of IBDV (Fig. 2) has been determined to 2 nm resolution using electron microscopy on unstained frozen hydrated specimens of the virus. The virus is predicted to have T13 symmetry, with trimers of VP2 facing the outside of the virus particle while VP3 faces inward (Böttcher *et al.*, 1997).



**Fig. 1.2** Three-dimensional structure of a frozen hydrated IBDV particle at 2 nm resolution (Böttcher *et al.*, 1997).

### 1.7 The genome of infectious bursal disease virus

The arrangement of the IBDV genome has been determined by analysis of virus recombinants obtained through genetic re-assortment (MacDonald and Dobos, 1981), and of protein products of *in vitro* translation (Mertens and Dobos, 1981; Nagy and Dobos, 1984; Azad *et al.*, 1985; Nagy *et al.*, 1987). The genomic organisation of IBDV is illustrated in Fig. 1.3.



**Fig. 1.3 Genomic organisation of IBDV and translation to polypeptides followed by processing.**

The open reading frame in mRNA A is indicated by double lined bars and proteolytic processing of the primary translation product is also illustrated. The viral polypeptides designated by their names (or  $M_r$ ) are indicated by single lines (Kibenge *et al.*, 1988).

The terminology of MacDonald and Dobos (1981) is used to refer to the individual genomic segments. The smaller genomic segment (segment B) of IBDV codes for a single polypeptide of 90 kDa (VP1).

The larger genomic segment (segment A) encodes four other polypeptides of  $M_r$  52 kDa (VPX), 40-41 kDa (VP2), 32 kDa (VP3), 28 kDa (VP4), (Müller and Becht, 1982; Azad *et al.*, 1985). Translated products of segment A from IBDV strain 002/73 amount to 169 kDa, yet it encodes proteins of 125 kDa, which, suggests a precursor product relationship between some of the translation products (Azad *et al.*, 1985). The amino acid sequences of VPX and VP2 of strain Cu-1 are very similar as shown by peptide mapping but the sequences of the other proteins are completely different (Müller and Becht, 1982; Dobos, 1979). In addition both VP2 and VPX react with the same monoclonal antibody on Western blots (Fahey *et al.*, 1985a; Becht *et al.*, 1988). This suggests that VPX is a precursor molecule to VP2. A 55-60 kDa polypeptide has similarly been found on Western blots of solubilized viral proteins of IBDV strain 002/73, developed with chicken immune sera raised against fusion proteins from recombinant *Escherichia coli* colonies containing VP3-encoding cDNA inserts (Azad *et al.*, 1986). A second open reading frame has been found for segment A which codes for a polypeptide with a calculated molecular mass of 16.5 kDa and an apparent molecular mass of 21 kDa (Mundt *et al.*, 1995). This 16.5 kDa protein is not found in the virion and thus has been designated non-structural (NS) protein VP5 (Böttcher *et*

*et al.*, 1997). VP5 has also been established as a virulence-determining factor, and a mutant virus that does not express this protein does not cause bursal lesions. This will be important in the generation of live attenuated vaccines for IBDV (Yao *et al.*, 1998).

It has been predicted that IBDV precursor proteins are cleaved by the putative viral protease VP4 (Hudson *et al.*, 1986). Deletion studies of segment A of viral cDNA have shown that VP4 contributes to the processing of a precursor polyprotein of VP2, VP3 and VP4 (Azad *et al.*, 1987; Duncan *et al.*, 1987; Jagadish *et al.*, 1988). Further deletion studies and expression of cDNA clones *in vitro* with rabbit reticulocyte lysates in a coupled transcription-translation system and in the Sindbis virus expression system (with BHK-21 and Vero cell cultures) have shown that VPX maturation to VP2 does not involve cellular proteases, and that the viral protease VP4 is responsible for processing of the polyprotein (Kibenge *et al.*, 1997). The cleavage sites for the processing of the polyprotein into the various viral polypeptides have been established by mutational analysis as being within amino acid residues <sup>511</sup>LAA<sup>513</sup> and <sup>754</sup>MAA<sup>756</sup> (Sánchez and Rodriguez, 1999).

### 1.8 Immunology of infectious bursal disease virus

Much work has been done on IBDV in terms of its immunological characteristics and its genetic sequence. This work includes cloning and sequencing of the entire IBDV genome (Azad *et al.*, 1985; Hudson *et al.*, 1986), followed by construction of primers for the amplification of the A segment of the IBDV genome using polymerase chain reaction (PCR) (Vakharia *et al.*, 1992) and comparison of the A segment sequences of various strains (Kibenge *et al.*, 1990; Bayliss *et al.*, 1990; Heine *et al.*, 1991; Brown *et al.*, 1994; Vakharia *et al.*, 1994; Brown and Skinner, 1996). Immunological characterisation has involved the use of monoclonal antibodies to define the epidemiology of IBDV in the United States (Snyder *et al.*, 1992). A region of variation in the sequence of VP2 seems to account for the variation in antigenicity of the virus (Bayliss *et al.*, 1990). Single amino acid changes in this region of the sequence have been proved to be sufficient to alter the neutralising properties of monoclonal antibodies (Schnitzler *et al.*, 1993).



IBDVs share common group antigens that may be detected by agar gel precipitation, the fluorescent antibody test and ELISA (Kibenge *et al.*, 1988). Both capsid proteins (VP2 and VP3) contain epitopes that are responsible for IBDV group antigenicity (Becht *et al.*, 1988). Western blotting of viral polypeptides and neutralisation of infectivity *in vitro* as well as protection of young chickens with an antibody to the 32 kDa viral polypeptide seemed to show that the 32 kDa protein (VP3) is a major protective immunogen in the host (Fahey *et al.*, 1985a; b).

Other research has shown that the other major structural protein, VP2 of approximately 40 kDa, is also a protective immunogen. For example, virus-neutralising antibodies produced against VP2 were able to passively protect chickens (Fahey *et al.*, 1989). Later work appears to show that neutralising antibodies to VP2 recognise a conformational epitope, while neutralising antibodies to VP3 recognise a non-conformational epitope and this is only very weakly recognised under non-denaturing conditions. This may explain why VP2 was not recognised as the major host protective antigen as Western blots are performed under denaturing conditions. Thus anti-IBDV antibodies strongly recognise VP3 but not VP2 (Azad *et al.*, 1987). There are at least three virus neutralising epitopes on VP2, one of which is strictly serotype specific, while VP3 contains only one neutralising epitope that neutralises virus infections with low titre (Darteil *et al.*, 1995; Becht *et al.*, 1988).

The possibility that VP3 may only be found in the interior of the virus capsid may account for the lack of neutralising antibodies to VP3 (Lombardo *et al.*, 1999). Several recombinant vaccines of VP2 protein have also been produced in *Saccharomyces cerevisiae*, fowlpox and virus herpesvirus (Fahey *et al.*, 1991; Bayliss *et al.*, 1991; Darteil *et al.*, 1995). These are all able to protect chickens from a lethal infection. The fact that these vaccines are recombinant vaccines means that variant strains can be countered by using the variant sequence in the recombinant. However, the process of producing a new recombinant vaccine to counter variant strains will still be a costly one, especially when taking into account the high dosages required for protection. Damage to the bursa of Fabricius still results upon challenge with IBDV in the case of the recombinant fowlpox virus vaccine (Bayliss *et al.*, 1991). The effect on the bursa of Fabricius after challenge with IBDV for the vaccine expressed in the yeast expression system was not measured.

The immune response of chickens to IBDV is paradoxical in that it results in stimulation of very high levels of antibody to the virus itself (Skeeles *et al.*, 1979) but simultaneous immunosuppression against many other antigens occurs (Allan *et al.*, 1972; Faragher *et al.*, 1974). An immune response to IBDV in surgically bursectomised chickens has been described, indicating that immunity develops at least in part in the spleen and other lymphoid organs (Käuffer and Weiss, 1980). Immunosuppression results directly from damage to the bursa.

### 1.9 Tropism of the virus

Little work appears to have been done on the specificity of tropism of the virus. To date it appears that the cellular target for IBDV has not been determined and neither has the viral antigen responsible for binding to the cellular targeting factor been described. Some work has been done on the process of infection in lymphoid cells, as well as establishment of a method of cultivating IBDV in cell culture. It has been found that replication of IBDV occurs primarily in populations of proliferating lymphoid cells (Müller, 1986). This was determined by enrichment of virus producing cells in gradients of bovine serum albumin and mitogenic stimulation of cells with fetal calf serum or an extract of bursae from susceptible chickens. Müller (1986) showed that immunoglobulin expression is not correlated with susceptibility to the virus and this was demonstrated by rosetting techniques and separation through a cell sorter. However, other work seems to indicate that IBDV binds and replicates preferentially in IgM-bearing B-lymphocytes (Luckert and Saif, 1991; Ogawa *et al.*, 1998). Additionally the binding of IBDV to the chicken B lymphoblastoid cell line, LSCC-BK3, was affected by treatment of cells with proteases and N-glycosylation inhibitors, suggesting that the attachment of IBDV is through a receptor of N-glycosylated protein (Ogawa *et al.*, 1998).

Infectious bursal disease virus has also been shown to change the potassium current properties of chicken embryo fibroblasts, it is thought that this effect may result from attachment and/or penetration, and may delay the apoptotic process in chicken embryo fibroblasts after IBDV infection (Repp *et al.*, 1998). IBDV has been adapted to replicate and produce cytopathic effects in primary cell cultures including chicken

bursal lymphoid cells, chicken embryo kidney and chicken embryo fibroblast cells (Luckert and Davis (1974), a single passage of virus sample in vitro is sufficient to reduce pathogenic properties of the isolate (Becht, 1994). This may be due to the formation of incomplete particles with lower densities, having stain penetration, irregular shape and poor assembly. The particles also contain unusual amounts of dsRNA and a different polypeptide pattern with an abundance of VP2 and a lack of the VP1, VP3 and VP4 (Müller and Becht, 1982). More recent evidence has shown that the restriction of replication of IBDV to replication in B-lymphocytes is not determined by the presence of serotype specific receptors. Receptor sites have been identified by virus overlay protein blot assay (VOPBA) for both pathogenic serotype 1 virus and for apathogenic serotype 2 virus. Common to both serotypes appears to be receptor proteins of molecular masses 40 and 46 kDa (Nieper and Müller, 1996). It has recently been reported that site directed mutagenesis of residues 279 and 284 of VP2 allow the virus to grow in chicken embryo fibroblasts (Lim *et al.*, 1999). This may indicate that these residues are involved in attachment if VP2 does not have any function other than attachment. Possibly the receptor for IBDV on chicken embryo fibroblasts is different to that found on B-lymphocytes.

#### **1.10 Viral attachment and penetration**

The process of viral infection is normally a highly specific process whereby the virus targets specific cells in a specific organ or organs (Wiley and Skehel, 1987). This tropism of the virus is in part mediated by an essential requirement for the virus to penetrate the host cell membrane and release its genome following which infection and replication can continue (Paulson, 1985). The virus thus requires a target molecule on the surface of the cell to which it can bind. The specificity of this targeting allows the virus to reach and infect cells rapidly enough so that the hosts' immune system cannot respond in time to defeat the infection. If virus were to infect simply by passive uptake of virus by cells, the host would easily be able to combat the infection (Lentz, 1990).

The target molecule and viral protein, which binds to the target molecule, varies between viruses. The target molecule can be any type of molecule presented on the host cell surface including various sugars, lipids and proteins or a combination of

these. The specificity of viral infection, which allows rapid infection of specific organs by the virus, provides an area where the virus infection process may be combated (Lentz, 1990). It has been described as the Achilles heel of infectious viruses and as such offers a target for antiviral agents (Lentz, 1988). Thus the receptor and VAP of IBDV are not only of interest for purely scientific reasons but their elucidation may provide a means for producing antiviral agents.

Isolation of virus receptors has proved extremely difficult because viruses can adhere non-specifically through electrostatic interactions to many materials including inert substances, so it is often difficult to distinguish between specific and non-specific binding (Tardieu *et al.*, 1982). Small particles may be taken up by fluid phase or constitutive endocytosis, and it is possible that some virus particles may be taken up by this mechanism (Steinman *et al.*, 1983). Specific virus receptors may be present in low quantities on the cell surface or may be labile, making identification difficult. Some viruses also appear to use more than one receptor and the interaction between virus and receptor may be mediated by another molecule, further complicating matters (Lentz, 1990).

## **1.11 Determination of viral receptors**

### **1.11.1 The human immunodeficiency virus (HIV) receptor**

Possibly the best defined virus receptor is CD4, the T-cell receptor molecule for HIV. The receptor for HIV was defined first by examining which cells were permissive for virus infection. These cells expressed CD4 (Klatzman *et al.*, 1984a; Gartner *et al.*, 1986; Tschachler *et al.*, 1987; Maddon *et al.*, 1986; Funke *et al.*, 1987) and monoclonal antibodies directed against CD4 blocked virus binding, syncytium formation and infection. (Dagleish *et al.*, 1984; Klatzmann *et al.*, 1984b; McDougal *et al.*, 1986a). Conversely, HIV blocks binding of anti-CD4 monoclonal antibodies to CD4 (McDougal *et al.*, 1986b). Immunoprecipitation of infected cells with anti-HIV antibodies precipitated a complex of the virus envelope glycoprotein (gp120) and the CD4 molecule (McDougal *et al.*, 1986a). Perhaps the most conclusive evidence that CD4 might be the receptor for HIV, came when it was shown that human cells lacking

the CD4 molecule and are resistant to infection become permissive to HIV after transfection of those cells with a cDNA clone encoding the CD4 molecule (Maddon *et al.*, 1986). The tertiary structure of CD4 is required for gp120 binding (Ibegbu *et al.*, 1989). The binding site of gp120 on CD4 has been further defined by using truncated derivatives of CD4 (Trauneker *et al.*, 1989; Berger *et al.*, 1988; Deen *et al.*, 1988; Arthos *et al.*, 1989). Initially the binding region was narrowed down to the first 106 amino acids of CD4 and then further refined to residues 31-57. This region is homologous to the second complementarity-determining region (CDR2) of immunoglobulin light chains. These studies strongly indicate that the CD4 molecule is part of the receptor site for HIV. However, other evidence indicates that the situation is more complicated. In cells exposed to HIV, binding of antibodies to HLA-DR block HIV binding. These findings suggest HLA-DR is involved in the receptor site for HIV (Mann *et al.*, 1988). HIV can infect glioma and rhabdomyosarcoma cell lines, some of which do not express surface CD4 molecules (Clapham *et al.*, 1989). Neither soluble CD4 or anti-CD4 antibodies inhibit infection of these cells. Thus CD4 does not seem to be required for infection of these cells (Lentz, 1990).

It has since been established that HIV utilises several chemokine receptors (CKR) as co-receptors for entry into the host cell. The CKR-3 and CKR-5 receptors have been shown to facilitate infection by primary HIV-1 isolates (Choe *et al.*, 1996), and a dual-tropic HIV-1 isolate uses fusin and  $\beta$ -chemokine receptors CKR-5, CKR-3 and CKR-2b as fusion cofactors (Doranz *et al.*, 1996). The fusin cDNA has been cloned and is predicted to be G-protein-coupled with seven transmembrane segments (Feng *et al.*, 1996). Fusin, however, does not promote entry of macrophage-tropic viruses, which are believed to be the key pathogenic strains *in vivo*. The principal co-receptor for macrophage-tropic viruses appears to be CKR-5 (Deng *et al.*, 1996; Alkhatib *et al.*, 1996). These results helped to elucidate several phenomena observed in the past decade including entry of HIV into cells lacking cell surface CD4 expression. Thus the complexity of the molecular mechanisms governing virus entry are demonstrated.

### 1.11.2 Other viral receptors and viral attachment proteins

To date several other viral receptors and viral attachment proteins (VAPs) have been characterised, as reviewed by Lentz (1990). A variety of different methods have been used to elucidate what the viral attachment proteins or viral receptors are.

The major receptor for the human rhinovirus is the intracellular adhesion molecule, ICAM-1 (Staunton *et al.*, 1989; Greve *et al.*, 1989). Staunton *et al.* (1989) used simian virus 40 transformed *Cercopithecus aethiops* (COS) cells transfected with the rhinovirus receptor and anti-ICAM-1 monoclonal antibody inhibition of virus binding and infection of cells *in vitro* (Staunton *et al.*, 1989). Greve *et al.* (1989) used anti-idiotypic monoclonal antibodies generated using viral epitopes, which inhibited virus infection in human cervical adenocarcinoma cells. They also purified a 95 kDa cell surface protein by lectin and monoclonal antibody affinity chromatography. The purified receptor showed sequence homology with ICAM-1, and a cDNA clone obtained from mouse transfectants expressing the rhinovirus receptor had essentially the same sequence as ICAM-1 (Greve *et al.*, 1989).

The mammalian reovirus type 3 cell surface receptor was isolated and characterised using anti-idiotypic anti-receptor antibodies (Co *et al.*, 1985a). The receptor was isolated using immunoprecipitation and characterised by two-dimensional electrophoresis. Following this, the receptor was further characterised and was found to have structural features similar to mammalian  $\beta$ -adrenergic and reovirus type 3 receptors. This similarity was noted after purified  $\beta$ -adrenergic receptor was immunoprecipitable with anti-reovirus receptor antibody and identical molecular masses and isoelectric points were found. Trypsin digests of the receptors were also found to be indistinguishable and purified reovirus receptor bound the  $\beta$ -antagonist [ $^{125}$ I]iodohydroxybenzylpindolol and this binding was blocked by the  $\beta$ -agonist isoproterenol (Co *et al.*, 1985b).

Identification of viral proteins responsible for binding is often performed using infection inhibition assays as with the La Crosse virus glycoproteins (Ludwig *et al.*,

1991). Here purified glycoproteins from the La Crosse virus were used to determine which glycoprotein was responsible for the attachment of virus to vertebrate and mosquito cell lines as well as mosquito midguts.

A cellular protein from a continuous mosquito cell line (C6/36) that appears to play a role in the attachment of Venezuelan equine encephalitis virus to these cells has been identified. Incubation of C6/36 cells with monoclonal antibodies directed against the cell surface polypeptide interfered with viral attachment. The molecular mass of the protein was determined to be 32 kDa using a protein blot-virus-binding assay. Further characterisation of the protein indicated a laminin binding domain due to its ability to bind directly with laminin as well as its immunologic cross-reactivity with the high affinity human laminin receptor (Ludwig *et al.*, 1996).

A 135 kDa protein of bovine herpesvirus that is released into infected cell cultures has been shown to be involved in viral attachment to Madin Darby bovine kidney cells. This protein is also present in the purified virus particle. The involvement of the protein in the attachment of the virus was established by adsorbing the 135 kDa protein to Madin Darby bovine kidney cells and showing that monoclonal antibodies against the protein inhibited adsorption of the protein to the cell membrane (Dubisson *et al.*, 1992).

A 40 to 42 kDa attachment polypeptide for canine parvovirus in A72 cells has been established in a canine tumor cell line (A72). Lectin interaction between glycoproteins was first suggested when neuraminidase or sialic acid specific lectin treatment of cells reduced virus binding. Similarly protease treatment of cells resulted in reduced virus binding. The protein responsible for binding was characterised with VOPBA. Virus bound to a 40 to 42 kDa protein from membrane fractions of A72 cells. The binding was specific since increasing amounts of unlabelled virions competitively inhibited binding of radiolabelled virions in a dose dependent manner. A polypeptide of similar molecular mass was immunoprecipitated from radiolabelled octyl glucoside extracts of A72 cells using purified virions, virion specific antiserum and protein A (Basak *et al.*, 1994).

The cellular receptor for poliovirus has been determined through transformation of mouse cells with human DNA and production of mouse cell lines susceptible to poliovirus. The transformed human gene was identified by its linkage to a human Alu repetitive sequence. The human RNA was then isolated by northern hybridisation analysis using a probe obtained by restriction analysis of the transformed human DNA. A 3.3 kb RNA was isolated and cDNA clones produced. Analysis of this clone shows that it encodes a 43-45 kDa receptor that has sequence homology with members of the immunoglobulin superfamily (Mendelsohn *et al.*, 1989). Using similar techniques but transforming human cells with mouse DNA, a putative receptor for the murine ecotropic retrovirus has been established (Albritton *et al.*, 1989).

Heparan sulfate appears to mediate the attachment of bovine herpesvirus 4 to the cell surface of Georgia bovine kidney cells. This interaction was demonstrated through competition between virus and heparin for attachment. Soluble heparin inhibits binding of virus before attachment and can displace virus after adsorption has occurred. Cells genetically or enzymatically rendered deficient in heparan sulfate are significantly resistant to infection but enzymatic removal of chondroitin sulfates A, B and C did not reduce binding of virus. Finally virus binds to immobilised heparin and elutes in the presence of soluble heparin in affinity chromatography (Vanderplasschen *et al.*, 1993).

A biochemical study on the KB-cell receptor for adenovirus was performed using three different approaches, i.e. affinity chromatography, immunoadsorption and cross-linking with a cleavable bifunctional reagent. The first system used an affinity gel consisting of adenovirus fibre linked to Sepharose by an intermediate bis(aminopropyl)amine arm, the amino groups of the adenovirus being preserved by prior citraconylation. The second system consisted of complete penton capsomere attached to anti-(penton base) antibody and cross-linked to polyacrylamide particles with glutaraldehyde. In this system, the penton fibre was appropriately projected outwards as in the virus. Both methods allowed separation from a KB-cell membrane extract fibre and penton-fibre binding material that inhibited virus attachment. The third method of cross-linking involved the use of a cleavable di-imidoester called 2-iminothiolane. The cross-linked complexes were isolated by sucrose density gradient



centrifugation. Polypeptides of 78, 42 and 34 kDa were common to the three selection systems (Hennache and Boulanger, 1977).

### 1.13 Methodologies for establishment of the IBDV receptor

To establish the receptor for IBDV, a variety of approaches, as described above, can be taken. Some of these are quite complicated and require some knowledge about what the receptor may be in order to be effective. The more complicated methods either involve generation of transformed cell lines that allow viral attachment/infection or use anti-idiotypic monoclonal antibodies that recognise the receptor and are generated against viral epitopes. This implies a pre-requisite of information about the receptor, e.g. a cDNA clone is available that is known to permit infection in transformed cells or information about epitopes on the VAP involved in attachment is known. Since very little information is available in the case of IBDV, a more basic approach has to be taken until more information is generated about both the receptor and the VAP. Sequence information of VP2 the proposed VAP (Bottcher *et al.*, 1997) is available but the region of binding has not been narrowed down and generation of anti-peptide antibodies would have to encompass large regions of the viral protein. The production of monoclonal anti-idiotypic antibodies from these anti-peptide antibodies would exponentially increase the cost and difficulty in establishing antibodies that inhibit viral infection.

Isolation of proteins involved in attachment may help in establishing antibodies that inhibit viral infection, as sequence information can be quite readily obtained from such isolated protein, and as such would remove the step of anti-idiotypic antibody production. To simplify the task, information on IBDV that has already been established can be used. The major capsid proteins of IBDV have been found to be non-glycosylated (Müller and Becht, 1982) and this implies a lectin interaction is unlikely and a direct protein-protein or protein-molecule interaction is more likely to take place. It is possible that the interaction may be similar to that, occurring between HIV gp120 and CD4, especially since IBDV also targets lymphocytes (B-lymphocytes rather than T-lymphocytes) and results in suppression of the immune system. Interactions such as the haemagglutinin sialic acid interaction of Newcastle disease virus (Suzuki *et al.*, 1985) are also possible as the receptor for IBDV has been

determined as glycosylated protein (Ogawa *et al.*, 1998). However, specific polypeptides of 40 and 46 kDa have been determined as possible receptors for IBDV (Nieper and Müller, 1996). Thus, a general carbohydrate residue as a receptor for IBDV is unlikely.

Since IBDV replicates only in actively dividing B-lymphocytes, examining cellular expression in B-lymphocytes may give a clue as to what the target protein is. The inconsistencies that have been found in the immunology of IBDV make the task more difficult. However, it now appears that VP3 is unlikely to be involved in attachment and VP2 is the most likely candidate as the VAP (Böttcher *et al.*, 1997).

In order to identify possible IBDV binding proteins in bursal membranes, a number of the methods discussed above were used. These include virus overlay protein blot assays to determine if binding between IBDV and specific polypeptides on the cell membranes of chicken bursal host cells can be established, thus identifying possible receptors. It may be possible to isolate virus receptor candidates by using 2-iminothiolane to reversibly cross-link the virus to its receptor, and isolate a virus receptor complex, which can be examined for non-viral polypeptides that are possible receptors. The results of these studies will be discussed in Chapter 3.

Affinity chromatography separation of IBDV binding proteins from host bursal cell membranes and protein sequencing of these virus-binding proteins will be discussed in Chapter 4. Further characterisation of some of the affinity-separated proteins by comparison with established protein sequences is included. Western blotting, with sera from rheumatoid arthritis patients, which may contain autoantibodies to synovial stimulatory protein (Hain *et al.*, 1996), was also used to investigate possible further homology between an affinity separated IBDV binding protein and the synovial stimulatory protein.

## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1 Materials

Most of the chemicals used in this study were from Sigma (St Louis, USA), Merck (Darmstadt, Germany) or Roche (Mannheim, Germany) and were of the highest purity available. Protein  $M_r$  standards were from Pharmacia LKB Biotechnology (Lund, Sweden). 2,2'-Azinobis[3-ethyl-2,3-dihydrobenzthiazole-6-sulphonate] (ABTS), Bovine serum albumin (BSA), phenylmethanesulfonylfluoride (PMSF), 5-bromo-4-chloro-3-indolylphosphate (BCIP), nitro blue tetrazolium (NBT), 100 bp DNA ladder, lambda phage DNA, *Hind*III restriction enzyme and RNase inhibitor were from Roche (Mannheim, Germany). Murine reverse transcriptase and *Taq* polymerase were from Promega (Madison, USA). Sepharose 4B, dialysis tubing (12 kDa cut-off), cesium chloride, deoxy nucleotide tri-phosphates (dNTPs), Ficoll, Freund's incomplete adjuvant, 2-iminothiolane, pepstatin A, rabbit anti-human IgG and rabbit anti-chicken IgG secondary alkaline phosphatase conjugated antibodies were from Sigma (St Louis, USA).

Oligonucleotide primers for PCR were synthesised by the University of Cape Town, Biochemistry Department synthetic laboratory.

Infectious bursal disease virus infected bursal material and specific pathogen free bursal material were obtained from Allerton Regional Veterinary Laboratory (ARVL). The infected bursal material was obtained from field specimens examined by Dr R. Horner and established as being infected with IBDV through post mortem investigation.

Sera from patients with confirmed rheumatoid arthritis were obtained from Professor R. Mody of the University of Natal, Medical Department.

Distilled water (dist.  $H_2O$ ) was obtained with a Milli-RO<sup>®</sup> 15 Water Purification System (Millipore, Marlboro, USA) and deionised water was obtained with a Milli-Q

plus Ultra-Pure Water System (Millipore, Marlboro, USA), and had a minimum resistivity of 18 M $\Omega$ .cm.

## **2.2 Bradford Dye-Binding assay**

The Bradford dye-binding assay provides a simple, rapid, efficient and inexpensive technique for protein quantification (Bradford, 1976; Read and Northcote, 1981) and was used in most cases for estimation of protein concentrations in this study. Serva Blue G dye binding to protein results in a change in absorbance maximum of the dye from 465 nm (cationic red form) to 595 nm (anionic blue form). The extinction coefficient of the blue dye protein complex is far greater than that of the free dye at 595 nm. This increases the sensitivity in protein quantification (Bradford, 1976).

The assay has a major advantage over other assays in that there is relatively little interference from other components at concentrations less than 1% (Bradford, 1976). The effects of Tris, acetic acid, sucrose, 2-mercaptoethanol, glycerol, ethylene diamine tetraacetic acid (EDTA), Triton X-100 and sodium dodecyl sulfate (SDS) can be eliminated with the use of an appropriate blank. However, at concentrations greater than 1%, particularly in the case of detergents, interference is too great and the compound must be removed or reduced to a level below 1% (Bradford, 1976).

The Bradford dye-binding assay does have a major disadvantage in that wide variation in amount of dye binding occurs between different proteins, requiring the use of a particular protein standard for each particular application. Minimisation of this variation was achieved by Read and Northcote (1981) by modifying the acid/alcohol ratios and increasing the dye concentration, thereby increasing the sensitivity but maintaining the ease and simplicity of the original assay. The modified assay is linear up to 25  $\mu$ g of protein (Splittgerber and Sohl, 1989).

Three Bradford dye binding protein assays have been established: a macro assay with a working range of 5-25  $\mu$ g of protein, a micro-assay with a range of 1-5  $\mu$ g of protein, and a microtitre plate assay which is accurate in the range of 0.2 – 1  $\mu$ g of protein. The micro assay was employed in the present study.

### 2.2.1 Materials

Dye reagent. Serva blue G dye (50 mg) was dissolved in a mixture of 88% (v/v) phosphoric acid (50 ml) and 99.5% ethanol (23.5 ml). The solution was made up to 500 ml with dist. H<sub>2</sub>O and stirred for 30 min at room temperature (RT) on a magnetic stirrer. The resulting solution was filtered through Whatman No. 1 filter paper and stored in an amber coloured bottle for up to 6 months. Visual checks were made for precipitation prior to use. If precipitation was observed, the reagent was filtered and re-calibrated.

Standard protein solution. Ovalbumin (1 mg) was dissolved in dist. H<sub>2</sub>O (1 ml). This solution was diluted in dist. H<sub>2</sub>O to 100 µg/ml for the assay.

### 2.2.2 Method

Assays for the calibration curve were carried out in quintuplicate at 5 concentrations of ovalbumin. Ovalbumin standard (10 - 50 µl of a 100 µg/ml working solution i.e. 1-5 µg protein) was diluted to a final volume of 50 µl with dist. H<sub>2</sub>O in polyethylene microfuge tubes. Dye reagent 950 µl was added and the solution immediately mixed by inversion. The colour was allowed to develop for 2 min after mixing and the A<sub>595</sub> was read in 1 ml plastic microcuvettes. The colour was stable for up to 1 h, so accurate timing was not necessary (Bradford, 1976). Concentrated samples were diluted in buffer, and dye reagent was added as before. Buffer alone added to dye reagent, served as the blank. The cuvettes were cleaned after use with 70% (v/v) ethanol and rinsed thoroughly with dist. H<sub>2</sub>O as this was found to be less damaging to the plastic microcuvettes than 25% (v/v) sodium hypochlorite. Linear regression analysis of the data yielded the following equation, allowing protein concentration of the samples to be calculated:

$$\text{Protein concentration } (\mu\text{g} / 50 \mu\text{l}) = \frac{A_{595} - 6.2 \times 10^{-3}}{0.0372}$$

## 2.3 Concentration of samples

Dilute protein samples obtained from affinity chromatography, placed in  $M_r$  12 000 cut-off dialysis bags, were routinely concentrated by dialysis against polyethylene glycol (PEG) ( $M_r$  20 000) as opposed to concentration against sucrose. Concentration against PEG at 4°C is found to be a rapid and effective method of concentrating protein samples without denaturing protein components and avoiding the contamination of the sample with sucrose. The PEG is of too large a molecular mass to pass into the dialysis membrane whereas salt ions move freely in and out and thus proteins in low salt buffers need not be dialysed to remove salt after concentration with PEG. The principle of concentration by dialysis involves the selective removal of water and other small molecules from the dialysis bag to the initially dry polymer along a concentration gradient. Care must be taken not to over dialyse as the equilibrium point in dialysis against PEG is often such that the sample will be virtually completely dehydrated. In cases where further concentration was necessary and where protein conformation need not be maintained, i.e. for reducing SDS-PAGE analysis, SDS-KCl precipitation was used. This involves the complexing of protein molecules with negatively charged SDS molecules and precipitation of these protein-SDS complexes with KCl. Up to 50 fold concentration of protein samples can be achieved in this way. However, the method is not suitable for non-reducing SDS-PAGE analysis, possibly because it results in overly high salt concentrations in the sample.

### 2.3.1 Dialysis against PEG

The protein sample was placed in a section of dialysis tubing (12 000  $M_r$  cut-off), the ends of which were sealed with dialysis clips. The filled tube was surrounded with a layer of PEG in a plastic tray and kept at 4°C. Once the sample had been sufficiently concentrated (usually 10-20 fold in 2-4 h). The bag was rinsed in dist.  $H_2O$  and the sample squeezed out.

### 2.3.2 SDS/KCl Precipitation

#### 2.3.2.1 Materials

5% (m/v) SDS. SDS (0.5 g) was dissolved in 10 ml of dist. H<sub>2</sub>O.

3M KCl. KCl (2.42 g) was dissolved in 10 ml of dist. H<sub>2</sub>O.

#### 2.3.2.2 Method

5% SDS (10  $\mu$ l) was added to the sample (100  $\mu$ l) in a 1.5 ml polyethylene microfuge tube. The solution was mixed by inverting the tube and 3 M KCl (10  $\mu$ l) was added. The mixture was again inverted and centrifuged (12 000  $\times$  g, 2 min, RT), The supernatant was discarded and the precipitate was dissolved in reducing treatment buffer (20  $\mu$ l; Section 2.3.3).

### 2.4 Tris-Tricine SDS-PAGE and PAGE

Electrophoresis is an analytical technique based on the migration of charged molecules in an electric field through a molecular sieving gel. While this aspect is used in both PAGE and Tris-Tricine SDS-PAGE, Tris-Tricine SDS-PAGE allows for relatively accurate determination of the molecular mass of a protein sample. The anionic detergent, SDS, binds tightly to most proteins in a ratio of about 1.4 g of detergent to 1 g of protein, thereby converting them from globular native amphoteric proteins into highly negatively charged rod like complexes, the length of which varies with the molecular mass of the protein (Reynolds and Tanford, 1970). In PAGE the effects of size, shape and conformation on migration of the complex cannot be determined and the estimate of molecular mass is thus inaccurate. A further modification of the SDS-PAGE technique is the use of reducing agents, such as 2-mercaptoethanol to reduce intra and intermolecular disulfide bonds of a protein moiety. This reduces the protein moiety down to its primary structure, i.e. a chain or chains of amino acids or polypeptide and facilitates binding of SDS to the polypeptide in disulfide bond regions. The effect of secondary and tertiary structure on migration

is thus eliminated. Thus migration of standard proteins of known molecular mass alongside the proteins of unknown molecular mass can be used to estimate the size of the unknown proteins (Neville, 1970).

For individual proteins in a sample to be well resolved, the origin of migration must be a tightly focussed band. A discontinuous buffer system was thus developed that made use of two different gel porosities and two different buffer systems (Ornstein, 1964; Davis, 1964). In the top layer a higher porosity gel is used which is called the stacking gel. The stacking gel has very little effect on the migration of proteins. The buffer in the sample and stacking gel is Tris-HCl (pH 6.8), while the running gel buffer is Tris-HCl (pH 8.8) and the electrode buffer Tris-glycine (pH 8.3). At this pH the ions in the sample will be stacked in order of mobility when in an electric field. The glycine ions at this pH have a small negative charge and thus are poorly mobile. On the other hand the chloride ions are highly mobile. The proteins themselves fall somewhere in between the mobilities of the chloride and glycine ions and are thus focused into a sharp band. Upon reaching the running gel, which is of a lower porosity, the mobilities of the ions change due to the increase in the pH of the running gel buffer. The glycine ions thus become more mobile and overtake the proteins in their migration. This leaves the proteins to separate out according to their mass, since they obtain a negative charge from SDS coating, which is dependent on the mass of the protein. The proteins are further separated due to the sieving effect of the gel, the smaller the protein the less it is hindered in its migration to the anode.

A modification of the system described by Laemmli (1970) replaces the less mobile glycine ion with the more mobile Tricine ion (Tris-Tricine buffer, pH 8.9) in the upper cathodic chamber Tris-Tricine buffer (pH 8.9) (Schägger and von Jagow, 1987). The separating and stacking gel buffers as well as the sample and lower anodic chamber buffers are all at pH-values in the range of 8.25-8.45. The proteins are focused in the stacking gel under an electric current between the leading chloride ion and trailing Tricine ion. Due to the increased pH used in this system and the greater mobility of the Tricine ion, smaller SDS-protein micelles (especially in the range of 5–20 kDa) are better resolved as the Tricine ion migrates closer to the chloride ion than would glycine.



Since PAGE does not utilise the effects of SDS, the estimation of molecular mass is inaccurate. However, the absence of SDS and reducing agents allows the separation of non disulfide complexes formed between proteins by electrostatic interactions. This is useful as some proteins are inactive when present only in monomer form. PAGE is performed using the reagents described by Laemmli (1970) with the exclusion of SDS. The gel is cast using an acrylamide monomer concentration gradient usually between 5 and 10%.

Polyacrylamide is a synthetic polymer of acrylamide which forms an even network when cross-linked by *N*, *N*'-methylenebisacrylamide. The cross-linking is initiated by *N*, *N*', *N*'', *N*''-tetramethylethylenediamine (TEMED), which catalyses the formation of free radicals from ammonium persulfate, which in turn initiate polymerisation (Hames and Rickwood, 1981). The exclusion of atmospheric oxygen by the water overlay allows the acrylamide to polymerise.

#### **2.4.1 Materials**

#### **2.4.2 Materials for PAGE**

Solution A: Monomer Solution [30% (m/v) acrylamide, 2.7% (m/v) Bis-acrylamide]. Acrylamide (73 g) and Bis-acrylamide (2 g) were dissolved and made up to 250 ml with dist. H<sub>2</sub>O and stored in an amber coloured bottle at 4°C.

Solution B: 4 × Running Gel Buffer (1.5 M Tris-HCl, pH 8.8). Tris (45.37 g) was dissolved in approximately 200 ml of dist. H<sub>2</sub>O, adjusted to pH 8.8 with HCl and made up to 250 ml.

Solution C: 4 × Stacking Gel Buffer (500 mM Tris-HCL, pH 6.8). Tris (3 g) was dissolved in 40 ml dist. H<sub>2</sub>O, adjusted with HCl to pH 6.8 and made up to 50 ml. This buffer was made up weekly, because, as a result of its poor buffering capacity at 2.1 pH units below its pKa at 4°C (Pharmacia products catalogue), pH drift can lead to anomalous running patterns under PAGE.

Solutions A, B and C were filtered through Whatman No. 1 filter paper before use.

Solution E: Initiator [10% (m/v) ammonium persulfate]. Ammonium persulfate (0.1 g) was made up to 1 ml just before use.

Solution F: Tank buffer (250 mM Tris-HCl, 192 mM glycine, pH 8.3). Tris (15 g) and glycine (72 g) were dissolved and made up to 5 l.

**Table 2.1 Reagents for two PAGE gels for the Bio-Rad Mini-Protein II® electrophoresis cell.**

Reagent	Running gel (%)		Stacking gel (%)
	10.0	5.0	4.0
A (ml)	5	2.5	0.94
B (ml)	3.75	3.75	1.75
C (ml)	0	0	0
E (μl)	75	75	35
Dist. H <sub>2</sub> O (ml)	6.15	8.65	4
TEMED (μl)	7.5	7.5	4.3

#### 2.4.3 Materials for Tris-Tricine SDS-PAGE

Anode buffer (0.2 M Tris-HCl, pH 8.9). Tris (48.44 g) was dissolved in 1.8 l of dist. H<sub>2</sub>O and adjusted to pH 8.9 with HCl and made up to 2 l.

Cathode buffer [0.1 M Tris-HCl, 0.1 M Tricine, 0.1% (m/v) SDS, pH 8.25]. Tris (24.22 g), Tricine (35.8 g) and 10% (m/v) SDS (10 ml) were mixed in 2 l of dist. H<sub>2</sub>O and adjusted to pH 8.25 with HCl if necessary.

Gel buffer [3.0 M Tris-HCl, 0.3% (m/v) SDS, pH 8.45]. Tris (72.7 g) and 10% SDS (6 ml), were dissolved in 150 ml dist. H<sub>2</sub>O, adjusted to pH 8.45 with HCl and the volume made up to 200 ml. The buffer was filtered through Whatman No. 1 filter paper and stored at 4°C.

Monomer solution (49.5%T, 3%C). Acrylamide (48 g) and N, N'-methylenebis-acrylamide (3 g) were dissolved in 100 ml of deionised H<sub>2</sub>O. The solution was filtered through Whatman No. 1 filter paper and stored in an amber coloured glass bottle at 4°C.

10% (m/v) Ammonium persulfate. Ammonium persulfate (0.1 g) was dissolved in dist. H<sub>2</sub>O (1 ml) just before use.

Non-reducing treatment buffer [0.75 M Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, pH 8.45]. Gel buffer (2.5 ml), 10% (m/v) SDS (4 ml) and glycerol (2 ml) were made up to 10 ml with dist. H<sub>2</sub>O. Aliquots (1 ml) were stored frozen for up to 3 months.

Reducing treatment buffer [0.75 M Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 8.45]. Prepared in the same way as non-reducing treatment buffer except for the inclusion of 10% (v/v) 2-mercaptoethanol (1 ml).

Molecular mass markers. Standard markers for molecular mass determination were: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and  $\alpha$ -lactalbumin (14.4 kDa). Lyophilised markers were reconstituted in non-reducing treatment buffer (100  $\mu$ l) and heated for 5 min for Coomassie staining or Western blot analysis. For silver staining the reconstituted markers were diluted 1:50 in non-reducing treatment buffer, and stored at 4°C.

**Table 2.2 Reagents for two Tris-Tricine gels for the Bio-Rad Mini-PROTEAN II® electrophoresis cell.**

Reagent	Separating gel (10%)	Stacking gel (4%)
Dist. H <sub>2</sub> O (ml)	7	4
Monomer (ml)	3	0.5
Gel buffer (ml)	5	1.5
Ammonium Persulfate (μl)	50	30
TEMED (μl)	7.5	15

#### 2.4.4 Method

For SDS-PAGE and PAGE, the Bio-Rad Mini-PROTEAN II® electrophoresis cell was assembled as described in the manufacturers' manual. Before use, the glass plates, spacers, combs and casting stand gaskets were washed with soap and water and cleaned with alcohol. The two glass plates (inner plate 7.3 × 10.2 cm, outer plate 8.3 × 10.2 cm) were positioned in the clamp assembly, separated by 1.5 mm polyethylene spacers. Removable silicone gaskets ensure that acrylamide solution does not leak from the sandwich assembly.

The separating gel was poured into the space between the glass plates through the use of a 10 ml syringe with a needle attached. The gel was poured to a depth of 3 cm from the top of the plate and overlaid with dist. H<sub>2</sub>O, also using a 10 ml syringe, to prevent atmospheric oxygen from inhibiting polymerisation.

For the formation of a gradient gel for native PAGE, the highest monomer concentration was placed in the first chamber of a Biorad gradient maker with an equal volume of the lower concentration of monomer placed in the other chamber. The requisite amounts of initiator and ammonium persulfate were added. The tap separating the chambers was opened and the tap closing off the gradient maker from the tubing attached to a peristaltic pump opened. The sample in the first container of the gradient maker was stirred rapidly while the solutions in the gradient maker were drawn through the apparatus using the peristaltic pump at maximum speed. The

piping from the exit of the pump was placed in-between the two glass plates of the casting apparatus and the gradient allowed to fill up to the required level between the two plates.

Formation of an interface between the gel and the overlay of water, (after approximately 1 h) indicated that the stacking gel had set. The layer of water was removed by inversion of the casting stand. Stacking gel solution was poured on top of the running gel layer and a 10 well comb inserted to form the sample wells. The comb was removed after the stacking gel had set (approximately 30 min) and the wells washed with dist. H<sub>2</sub>O. The gel holders were removed from the casting stand and inserted into the U-shaped chamber containing the electrodes. This forms a cathodic chamber at the top of the gel, which is sealed from contact with the lower anodic chamber by the silicone gaskets.

SDS containing cathode buffer (or tank buffer with no SDS in the case of PAGE) was poured into the cathode chamber to above the level of the glass plates and in contact with the gel. The apparatus was checked for leaks and if none were present, the samples and molecular mass markers were loaded into the wells using either a Hamilton™ syringe or a micropipette with gel loading tips. Samples were prepared with equal volumes of reducing or non-reducing treatment buffer and boiled for 2 min. In the case of PAGE, samples were prepared with equal volumes of tank buffer containing 20% glycerol and not boiled. Bromophenol blue (1-3 µl), which migrates with the buffer front, was added to each sample prior to loading to monitor the progress of electrophoresis. Anode buffer (or tank buffer with no SDS in the case of PAGE) was introduced into the lower chamber and the apparatus connected to a power pack. The voltage was set at 80 V (maximum current) until the bromophenol blue had migrated through the stacking gel. The voltage was then increased to 100 V for the remainder of the electrophoretic run. For PAGE the current was set to 18 mA per gel for the electrophoresis period. Electrophoresis was performed until the bromophenol blue dye marker was at the edge of the bottom of the separating gel. The gels were removed and placed in the appropriate staining solution, fixative or prepared for Western blot analysis.

## 2.5 Coomassie blue R-250 protein stain

SDS-PAGE gels, with at least 1 µg of protein per band, were stained with Coomassie blue R-250 stain. This method provides a simple way of viewing proteins separated by SDS-PAGE.

### 2.5.1 Materials

Stain stock solution [1% (m/v) Coomassie blue R-250]. Coomassie blue R-250 (1 g) was dissolved in dist. H<sub>2</sub>O (100 ml) and filtered through Whatman No. 1 filter paper.

Staining solution [0.125% (m/v) Coomassie blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid]. Stain stock (62.5 ml) was mixed with methanol (250 ml) and acetic acid (50 ml) and made up to 500 ml with dist. H<sub>2</sub>O.

Destaining solution I [50% (v/v) methanol, 10% (v/v) acetic acid]. Methanol (500 ml) and acetic acid (100 ml) were made up to 1 l with dist. H<sub>2</sub>O.

Destaining solution II [7% (v/v) methanol, 5% (v/v) acetic acid]. Methanol (70 ml) and acetic acid (50 ml) were made up to 1 l with dist. H<sub>2</sub>O.

### 2.5.2 Method

Gels were removed from the electrophoresis unit, placed in staining solution for 4 h, rinsed briefly in dist. H<sub>2</sub>O, placed in destain solution I overnight or for 4 h, and in destain II until the background had cleared completely. Gels were stored in polyethylene zip-seal bags and kept well hydrated until photographed. The gels were stable for long periods in this state.

## 2.6 Silver staining of proteins in polyacrylamide gels

In cases where small amounts of protein, separated on SDS-PAGE or PAGE needed to be visualised, a higher degree of sensitivity than that obtained with Coomassie

brilliant blue R-250 staining was achieved with the improved silver staining method of Blum *et al* (1987). This method utilises two chemical properties of thiosulfate: image enhancement by pre-treatment of fixed gels and formation of soluble silver complexes which prevents non-specific background staining during image development.

### 2.6.1 Materials

Fixative. [50% MeOH; 12% AcOH; 0.05% (v/v) 37% (m/v) HCOH]. Methanol (50 ml), acetic acid (12 ml) and formaldehyde (50  $\mu$ l) were made up to 100 ml with deionised H<sub>2</sub>O.

Washing solution 1. [50% (v/v) ethanol]. 50 ml ethanol was made up to 100 ml with deionised H<sub>2</sub>O.

Washing solution 2. [50% (v/v) methanol]. 50 ml methanol was made up to 100 ml with deionised H<sub>2</sub>O.

Pre-treatment solution. [0.02% (m/v) Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>]. Sodium thiosulfate (Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>·5H<sub>2</sub>O) (20 mg) was dissolved in 100 ml deionised H<sub>2</sub>O.

Impregnation solution. [0.2% (m/v) AgNO<sub>3</sub>; 0.075% (v/v) 37% (v/v) HCOH]. Silver nitrate, (0.2 g) and (37% m/v) formaldehyde (75  $\mu$ l) were dissolved in 200 ml deionised H<sub>2</sub>O.

Developing solution. [6% Na<sub>2</sub>CO<sub>3</sub>; 0.05% (v/v) 37% (v/v) HCOH; 0.0004% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>]. Sodium carbonate (6 g), 37% (m/v) formaldehyde (50  $\mu$ l) and pre-treatment solution (2 ml) were dissolved in 100 ml deionised H<sub>2</sub>O.

Stopping solution. [50% (v/v) MeOH; 12% (v/v) AcOH]. Methanol (50 ml) and acetic acid (12 ml) were made up to 100 ml with deionised H<sub>2</sub>O.

### 2.6.2 Method

All steps were performed on an orbital shaker (50 rpm, RT) and in scrupulously clean glass containers to minimise background staining. The gel was soaked in fixing solution for at least 1 h or overnight, washed in washing solution 1 ( $3 \times 20$  min). The gel was soaked in pre-treatment solution (1 min), washed in distilled or deionised water ( $3 \times 20$  s), and placed in impregnation solution (25 min), washed in distilled water ( $2 \times 20$  s) and placed in developing solution. As soon as the first bands became visible, the gel was washed in distilled water ( $2 \times 2$  min), and placed in stop solution (10 min). Finally the gel was washed in washing solution 2 and stored in an airtight container at 4°C.

## 2.7 Immunochemical techniques

### 2.7.1 Generation and isolation of antibodies

Antibodies were raised in chickens following inoculation with antigen emulsified with an adjuvant. The adjuvant enhances an immune response by providing a depot from where the antigen is slowly released. It also stimulates cellular responses such as those provided by macrophages. The presentation of the antigen on the surface of the macrophages increases the efficiency of their presentation to the lymphocytes and hence facilitates prolonged and improved antigen exposure to the immune system of the experimental animal (Roitt, 1991). Generally the adjuvant used in the initial inoculation is Freund's complete adjuvant which contains killed *Mycobacterium tuberculosis* cells and mineral oil. The immunogen and adjuvant are mixed by trituration to form a stable water-in-oil emulsion for inoculation. Subsequent inoculations use the immunogen emulsified with Freund's incomplete adjuvant, which consists only of mineral oil, to further stimulate the B-cell clone or clones, which produce antibodies specific to the inoculated immunogen (Roitt, 1991).

Antibodies were isolated using the water soluble polymer, polyethylene glycol  $M_r$  6000. IgG can be purified from serum and IgY from egg yolk using PEG precipitation (Polson *et al.*, 1964; 1985). PEG is a non-denaturing precipitating agent



which operates on a steric exclusion mechanism whereby proteins are concentrated in the extrapolymer space, eventually exceeding their solubility limit (Ingham, 1990). The concentration of the polymer required to precipitate a protein is a function of the net charge on a molecule, which is determined by the pH of the surrounding medium (Polson *et al.*, 1964).

The protocol used to purify IgG from chicken serum was the same as that for rabbit serum described by Polson *et al.* (1964), while a similar procedure (Polson *et al.*, 1980; 1985) was followed for the isolation of IgY from egg yolks.

#### 2.7.1.1 Materials

Borate-Buffered Saline [0.02% (m/v)  $\text{NaN}_3$ , pH 8.6]. Boric acid (2.16 g),  $\text{NaN}_3$  (0.2 g), NaCl (2.19 g), NaOH (0.7 g) and 37% (v/v) HCl (0.62 ml) were dissolved in 950 ml of dist.  $\text{H}_2\text{O}$ , adjusted to pH 8.6 with NaOH and made up to 1 l.

100 mM Na-phosphate buffer [0.02% (m/v)  $\text{NaN}_3$ , pH 7.6].  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  (13.8 g) and  $\text{NaN}_3$  (0.2 g) were dissolved in 950 ml of dist.  $\text{H}_2\text{O}$ , adjusted to pH 7.6 using NaOH, and made up to 1 l.

#### 2.7.1.2 Method for the isolation of IgG from rabbit serum

Blood was drawn from chickens into vacuum tubes by cardiac puncture, and centrifuged ( $1\,000 \times g$ , 10 min,  $4^\circ\text{C}$ ). The supernatant serum was mixed with 2 volumes of borate buffered saline. Solid PEG (6 kDa) was added to the diluted serum to a concentration of 14% (m/v), dissolved with constant gentle stirring, and the resulting solution centrifuged ( $12\,000 \times g$ , 10 min, RT). The pellet was dissolved in the original serum volume, using 100 mM Na-phosphate buffer, pH 7.6. PEG was again added to a final concentration of 14% (m/v), dissolved by stirring, and the solution centrifuged ( $12\,000 \times g$ , 10 min, RT). The pellet was redissolved in half the original serum volume using 100 mM Na-phosphate buffer, pH 7.6, containing 60% (v/v) glycerol. IgG fractions were stored at  $-20^\circ\text{C}$ .

To determine the concentration of IgG, the absorbance of a 1/40 dilution of a purified IgG fraction in 100 mM Na-phosphate buffer, pH 7.6, was read at 280 nm in 1 cm quartz cuvettes against a Na-phosphate buffer blank. The protein concentration was calculated using the equation:

$$A = E \cdot l \cdot c$$

Where A = absorbance at 280 nm; E = extinction coefficient of IgG = 1.43 mg/ml/cm (Hudson and Hay, 1980); l = light path length and c = IgG concentration.

### 2.7.1.3 Method for the isolation of IgY from chicken egg yolks

Egg yolks were separated from the egg white and washed carefully under running water to remove all traces of albumin. The yolk sac was punctured and the yolk volume measured in a measuring cylinder. Two volumes of 100 mM Na-phosphate buffer, pH 7.6, were added and mixed in thoroughly. Solid PEG (6 kDa) was added to 3.5% (m/v) and dissolved by gentle stirring. The precipitated vitellin fraction was removed by centrifugation ( $4\,420 \times g$ , 30 min, RT), and the supernatant filtered through absorbent cotton wool to remove the lipid fraction. The PEG concentration was increased to 12% [i.e. 8.5% (m/v) was added], the solution was mixed thoroughly and centrifuged ( $12\,000 \times g$ , 10 min, RT). The supernatant was discarded and the pellet was dissolved in 100 mM Na-phosphate buffer, pH 7.6, in a volume equal to that obtained after filtration. PEG was added to 12% (m/v), dissolved by gentle stirring and the precipitate harvested by centrifugation ( $12\,000 \times g$ , 10 min, RT). The supernatant was discarded and the final antibody pellet was dissolved in 1/6 of the original egg yolk volume, using 100 mM Na-phosphate buffer, pH 7.6, and stored at 4°C. The concentration of IgY was determined in the same manner as that for IgG, using an extinction coefficient ( $E_{280}^{1\text{ mg/ml}}$ ) of 1.25 (Coetzer, 1985).

### 2.7.2 Enzyme-linked immunosorbent assay (ELISA)

All immunoassays are based on the principle of strong binding between antigen and primary antibody. Antigens or antibodies bound to a solid phase can thus be used to capture the opposite partner. If an enzyme or other sensitively detectable label is chemically conjugated to the probe molecule, a measure of the concentration of the component (antigen or antibody) can be established. This is the basis for the enzyme-linked immunosorbent assay (ELISA), first introduced by Engvall and Perlmann (1971). The ELISA is a quantitative technique, which complements the qualitative data provided by Western blotting.

Indirect, non-competitive, solid phase ELISA is routinely used to determine the titre and specificity of an antibody. This type of ELISA offers several advantages to other types, the antigen does not need to be labelled and therefore does not necessarily have to be pure, also, a single labelled antibody may be used to test a number of antibodies raised against specific antigens as long as all the primary antibodies were produced in the same animal (Clark and Engvall, 1980). The antigen is adsorbed to the sides of wells of a polystyrene microtitre plate by non-specific hydrophobic interactions. The primary antibody is allowed to interact directly with the adsorbed antigen and form immune complexes. The amount of interaction between primary antibody and antigen is determined using an enzyme labelled secondary antibody, which recognises the primary antibody and thus the antibody-antigen complex. Thus the concentration of the primary antibody, which is proportional to the amount of interaction, can be determined. The detection system used in the present study utilised rabbit anti-chicken IgY antibodies coupled to horse radish peroxidase (HRPO) by the method of Hudson and Hay (1980). The enzyme HRPO has a high specificity for the hydrogen acceptor  $H_2O_2$ , which together catalyse the oxidation of ABTS (2,2'-azino-di-(3-ethyl)-benzthiozoline sulfonic acid), a hydrogen donor, to yield a soluble blue-green coloured complex which may be measured spectrophotometrically at 405 nm (Clark and Engvall, 1980). In the present study, ELISA was used to monitor the titre of chicken anti-IBDV antibodies. Plots of  $A_{405}$  versus log antibody concentration were constructed. The titre of the antibodies was taken as the highest dilution at which the primary antibody response was greater than that of a pre-immune antibody control sample.

### 2.7.2.1 Materials

Phosphate buffered saline (PBS), pH 7.4. NaCl (8 g), KCl (0.2 g),  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  (1.15 g) and  $\text{KH}_2\text{PO}_4$  (0.2 g) were dissolved in 1 l of dist.  $\text{H}_2\text{O}$ .

Antigen fix [70% (v/v) methanol]. Methanol (70 ml) was diluted to 100 ml with dist.  $\text{H}_2\text{O}$ .

0.5% (m/v) Bovine serum albumin-PBS (BSA-PBS). BSA (0.5 g) was dissolved in 100 ml PBS.

0.1 % (v/v) PBS-Tween. Tween-20 (1 ml) was diluted to 1 l with PBS.

0.15 M Citrate-phosphate buffer, pH 5.0. A solution of citric acid. $\text{H}_2\text{O}$  (21 g/l) was titrated with a solution of  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  (35.6 g/l) to pH 5.0.

Substrate solution [0.05% (m/v) ABTS and 0.0015% (v/v)  $\text{H}_2\text{O}_2$  in citrate phosphate buffer]. ABTS (7.5 mg) and  $\text{H}_2\text{O}_2$  (7.5  $\mu\text{l}$ ) were dissolved in citrate phosphate buffer, pH 5.0 (15 ml) for each ELISA plate.

Stopping buffer [citrate-phosphate buffer, 0.1% (m/v)  $\text{NaN}_3$ ].  $\text{NaN}_3$  (0.1 g) was dissolved in 100 ml citrate-phosphate buffer.

### 2.7.2.2 Method

Wells of microtitre plates (Nunc Immuno maxisorp F96 plates) were coated with antigen (150  $\mu\text{l}$ , overnight at  $4^\circ\text{C}$ ) at a predetermined concentration (0.1  $\mu\text{g}/\text{ml}$ ) in PBS. Non specific binding of antibody was prevented by blocking the wells with 0.5% (m/v) BSA-PBS (200  $\mu\text{l}$ , 1h,  $37^\circ\text{C}$ ), followed by washing of the wells 3 times with 0.1% (v/v) PBS-Tween. The same washing step was repeated after each subsequent incubation step with antibodies. Serial doubling dilutions of the primary antibody in 0.5% BSA-PBS (1 mg/ml) was added to individual wells (100  $\mu\text{l}$ ) and

incubated (2 h at 37°C). The secondary antibody-HRPO conjugate (120 µl) was incubated (1 h at 37°C) at a suitable dilution (1:300). Finally ABTS/H<sub>2</sub>O<sub>2</sub> substrate (150 µl) was added and incubated in the dark for optimal colour development. The enzyme reaction was terminated after 25 min by the addition of NaN<sub>3</sub>-citrate-phosphate buffer (50 µl), and the absorbance read at 405 nm in a Bio-Tek EL 307 ELISA plate reader. Titration curves were constructed from the spectrophotometric data.

### 2.7.3 Western Blotting

This technique was used to identify proteins immobilised on nitro-cellulose, a matrix with a high binding affinity for proteins (approximately 80-100 µg/cm<sup>2</sup>; De Maio, 1994). The immobilised proteins are characterised using specific antibodies as probes. Alternatively Western blotting may be used for the qualitative analysis of antibodies produced against purified proteins.

The method used in this study was essentially that of Towbin *et al.* (1979), with minor modifications. Methanol was included in the buffer to enhance hydrophobic binding of protein-SDS complexes to the nitro-cellulose membrane (Goodenham, 1984). Unoccupied binding sites on the membrane are blocked with non-fat milk and antigens are allowed to react with primary antibody. Primary antibody is detected with a enzyme-labelled secondary antibody detection system. The detection system comprises a secondary antibody directed against the primary antibody, labelled with an enzyme such as alkaline phosphatase, which catalyses a reaction with a precipitating substrate (e.g. BCIP/NBT) leading to the formation of a coloured band identifying the protein of interest.

In the present study Western blotting was employed to identify IBDV antigens in purified virus preparations and to characterise antibodies produced in chickens against whole virus.

### 2.7.3.1 Materials

Blotting buffer. Tris (9.08 g) and glycine (43.2 g) were dissolved in 1.4 l of dist. H<sub>2</sub>O. Methanol (600 ml) and, 10% SDS (2 ml) was added prior to use.

Tris buffered saline (TBS) (20m mM Tris, 200 mM NaCl, pH 7.4). Tris (2.42 g) and NaCl (11.69 g) were dissolved in 950 ml of dist. H<sub>2</sub>O, adjusted to pH 7.4 with HCl and made up to 1 l.

1% (m/v) Ponceau S. Ponceau S (0.1 g) was dissolved in 1% (v/v) glacial acetic acid (100 ml).

Blocking solution [5% (m/v) non-fat milk powder]. Low fat milk powder (5 g) was dissolved in TBS (100 ml).

Alkaline phosphatase linked secondary antibodies. Alkaline phosphatase linked rabbit anti-chicken IgG antibodies were obtained from Sigma (St. Louis, USA).

Substrate buffer (0.05 M Tris; 0.05 M MgCl<sub>2</sub>, pH 9.5). Tris 0.6 g and MgCl<sub>2</sub> (0.5 ml of a 1 M solution) were dissolved in 80 ml of dist. H<sub>2</sub>O, and adjusted to pH 9.5 with HCl and the volume made up to 100 ml.

BCIP/NBT substrate solution. 5-bromo-4-chloro-3-indolylphosphate (BCIP) (15 mg) and nitroblue tetrazolium (NBT) (30 mg) were dissolved in 1 ml 50% DMF. The dissolved substrates were added to substrate buffer (100 ml) and thoroughly mixed.

### 2.7.3.2 Method

Following SDS-PAGE with duplicate gels, one gel was stained to show the total protein pattern, while the other was used for blotting. Nitro-cellulose was cut to a suitable size and, to avoid entrapment of air, carefully floated onto blotting buffer before being totally immersed. The immersed nitro-cellulose was sandwiched with the gel lying squarely on top of it, between three pieces of Whatman No. 4 filter paper

and two pieces of scotchbright foam, also totally immersed in blotting buffer. The sandwich was placed into a Western blotting apparatus filled with blotting buffer. The apparatus was attached to a refrigerated circulator and kept at 10°C. Buffer in the apparatus was stirred using a magnetic stirrer bar during operation to ensure even distribution of heat. The apparatus was connected to a power supply, with the nitro-cellulose on the anodal side, and blotting was performed for 16 h at 30 V with unlimited current. After 16 h, the sandwich was removed and the filter paper was peeled off the gel. The gel was carefully removed and stained to assess the efficiency of the blotting, which was usually high.

The nitro-cellulose sheet was removed from the filter paper and blocked with blocking solution in TBS for 1 h, washed in TBS (3 × 5 min) and incubated for 2 h with primary antibody diluted in 0.5% BSA-TBS, washed in TBS (3 × 5 min) and incubated in alkaline phosphatase-linked secondary antibody in 0.5% BSA-TBS for 1 h and again washed in TBS (3 × 5 min). The membrane was immersed in substrate solution and reacted in the dark until bands were clearly evident against a lightly stained background. Finally the nitro-cellulose was removed from substrate, washed in dist. H<sub>2</sub>O and dried between two sheets of filter paper to ensure good preservation of the bands before photography.

## **2.8 Molecular biology techniques**

### **2.8.1 Agarose gel electrophoresis of DNA**

Agarose gel electrophoresis of DNA is a simple technique for resolving fragments of DNA from a few hundred base pairs to several thousand base pairs in length. The technique relies on the ability of the anode to attract the negatively charged DNA molecule through a sieving matrix of agarose. Agarose forms a complex reticular network when dissolved and cooled, thus forming the sieving matrix of the gel. The DNA is thus separated on the basis of the length of the molecule and once separated the DNA fragments can be easily visualised with the aid of ethidium bromide, which intercalates between the base pairs and fluoresces more once intercalated. The intercalation of ethidium bromide does increase the size of the DNA fragment and

retards the migration of the fragments by up to 15% if it is included in the electrolyte buffer of the gel. DNA of different sizes can be optimally separated by varying the agarose concentration of the gel (Sambrook *et al.*, 1989)

### 2.8.1.1 Materials

50 × Tris-acetate stock solution TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). Tris (242 g) was dissolved in 800 ml dist. H<sub>2</sub>O, glacial acetic acid (57.1 ml) was added and the buffer was made up to 900 ml with dist. H<sub>2</sub>O. EDTA (18.612 g) was dissolved in 90 ml dist. H<sub>2</sub>O, the pH was adjusted to 8.0 with glacial acetic acid and the solution was made up to 100 ml with dist. H<sub>2</sub>O. Finally, the Tris buffer and the EDTA solution were combined and autoclaved (121°C, 15 min).

TAE working solution. 50 × TAE buffer (20 ml) was diluted to 1 l with dist. H<sub>2</sub>O.

1% (m/v) agarose. Agarose (0.4 g) was dissolved in TAE working solution (40 ml) in an Erlenmeyer flask, with the neck loosely plugged with cotton wool. The solution was heated in a microwave oven with intermittent mixing to ensure that all the grains of agarose were dissolved in the minimum time required.

Gel-loading buffer [1 mM EDTA, 0.5% (m/v) bromophenol blue, 50% (v/v) glycerol]. EDTA (0.007 g) and bromophenol blue (0.1 g) were dissolved in dist. H<sub>2</sub>O (10 ml), and glycerol (10 ml) was added and thoroughly mixed in.

1% (m/v) Ethidium bromide. Ethidium bromide (0.2 g) was dissolved in dist. H<sub>2</sub>O (20 ml) and stored in an aluminium foil wrapped bottle.

Tris-EDTA buffer (TE buffer; 10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Tris (0.121 g) and EDTA (0.037 g) were dissolved in 95 ml dist. H<sub>2</sub>O, adjusted to pH 8.0 with HCl and made up to 100 ml.

HindIII buffer (10 mM Tris-HCl, 5 mM magnesium chloride, 100 mM sodium chloride, 1 mM 2-mercaptoethanol, pH 8.0). Tris (0.012 g), MgCl<sub>2</sub> (0.005 g) and



NaCl (0.058 g) were dissolved in 9.5 ml deionised H<sub>2</sub>O and adjusted to pH 8.0 with HCl. 2-mercaptoethanol (698 µl) was added and the buffer was made up to 10 ml with deionised H<sub>2</sub>O.

*Hind*III-cut lambda DNA standard markers. Lambda DNA (160 µl of a 0.25 µg/ml stock solution) was digested overnight at 37°C with *Hind*III (10 µl of a 10 U/µl stock solution) in a mixture containing *Hind*III reaction buffer and made up to a final volume of 200 µl with TE buffer.

#### 2.8.1.2 Method

The open ends of a clean, dry perspex casting tray (100 × 66 mm) were sealed with masking tape to form a mould for gelling of the agarose on a horizontal surface. The dissolved agarose solution was cooled to 60°C and ethidium bromide (1 µl) was added and mixed in thoroughly. A perspex 12 well comb was positioned in the casting tray and the warm agarose solution was poured carefully into the mould, ensuring that no air bubbles were formed. After the gel had set completely (15 – 20 min, RT), the masking tape was removed and the casting tray positioned in the electrophoresis tank. TAE working solution was added to cover the gel to a depth of approximately 1 mm, and ethidium bromide (5 µl) was added and mixed in to ensure even distribution with the buffer. Gel-loading buffer (1 µl) was added to the DNA samples prior to loading to increase the density of the DNA sample and monitor the progress of electrophoresis. Electrophoresis was carried out at 100 V (maximum current) for 1.0 - 1.2 h. On completion of the electrophoretic run, the casting tray was removed from the electrophoresis tank and the gel examined in an ultraviolet trans-illuminator, prior to photographing through a red filter with Polaroid® type 667 black and white instant film. The concentration of the DNA was determined by visual comparison with *Hind*III generated lambda DNA fragments of known size and concentration (Sambrook *et al.*, 1989).

### 2.8.2 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a technique that has allowed great advancement in the field of molecular biology. The technique works on the principle that polymerisation of DNA can occur from available 3' hydroxyl groups of DNA. Pre-requisites for polymerisation to occur are the available 3' hydroxyl group (supplied by an oligonucleotide primer), a polymerase enzyme, a template and free deoxyribonucleic acid bases (dATP, dCTP, dGTP, dTTP). If a small section of the sequence of DNA is known, an oligonucleotide primer can be generated complementary to the template sequence. This small sequence of DNA or oligonucleotide will bind to the template and polymerisation can proceed via the 3' hydroxyl of the primer. The complementary strand of DNA can also be used as a template for polymerisation in the same way but proceeding in the opposite direction. Thus two primers are required, the upstream or forward primer, which initiates polymerisation downstream or forwards, while the downstream or reverse primer initiates polymerisation upstream or reverse. Repeated cycles of denaturation of the double stranded DNA template, annealing of the oligonucleotide primers to the target sequence and polymerisation of the copy strands thus leads to amplification of the target sequence located between two primers. The newly formed strands are complementary to one another and provide a second double stranded template from which polymerisation can occur. However, denaturation of the DNA strands and annealing of the primers must take place between each cycle of polymerisation and this requires an increase in temperature, which most polymerases cannot withstand, thus fresh polymerase has to be added after each cycle. The breakthrough that allowed PCR to become the valuable tool it is, came when the polymerase from *Thermus aquaticus* (*Taq* polymerase), which is heat stable, was isolated. This allowed for repeated steps of denaturation, annealing and polymerisation with the same enzyme.

As an adjunct to PCR, a reverse transcription polymerase chain reaction (RT-PCR) has been developed. This allows RNA to be amplified into copy DNA (cDNA) by first making a DNA/RNA hybrid of the RNA target molecule using another enzyme called reverse transcriptase, which is usually isolated from Moloney murine leukemia virus (MMLV). The reverse transcriptase enzyme also requires a 3' hydroxyl for

polymerisation to occur and this is supplied by a primer. Amplification can then be performed as usual from the copied DNA strand of the DNA/RNA hybrid using forward and reverse primers (Sambrook *et al.*, 1989).

### 2.8.2.1 Materials

5 × reverse transcriptase reaction buffer [250 mM Tris-HCl, 375 M KCl, 15 mM MgCl<sub>2</sub>, 50 mM DTT, pH 8.3]. Tris (0.3 g), KCl (0.28 g), MgCl<sub>2</sub>·6H<sub>2</sub>O (0.03 g) and DTT (0.077 g) were dissolved in 9.5 ml dist. H<sub>2</sub>O and titrated to pH 8.3 with HCl. The solution was made up to 10 ml, aliquoted into 1 ml fractions and stored at −20°C.

10 × PCR amplification buffer [160 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 670 mM Tris-HCl (pH 8.8 at 25°C), 0.1% (v/v) Tween-20]. Tris (0.8 g) was dissolved in deionised H<sub>2</sub>O (9.5 ml) and adjusted to pH 8.8 with HCl. (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (0.2 g) was dissolved in this solution, Tween-20 (10 µl) was added and the solution made up to 10 ml with deionised H<sub>2</sub>O. The resulting mixture was aliquoted into 1 ml fractions and stored at −20°C.

100 mM Lithium chloride buffer, pH 7.0. Lithium chloride (0.42 g) was dissolved in 80 ml deionised H<sub>2</sub>O and titrated to pH 7.0 with HCl and made up to 100 ml.

dNTP stock solutions [100 mM of each dATP, dCTP, dGTP, dTTP]. dATP (49.1 mg), dCTP (46.7 mg), dGTP (50.7 mg) and dTTP (50.7 mg) were individually dissolved in 100 mM lithium chloride solutions, pH 7.0 (1 ml)

dNTP working solution for reverse transcription [10 mM of each dATP, dCTP, dGTP and dTTP]. dATP, dCTP, dGTP and dTTP stock solution (10 µl of each) were combined and diluted to 100 µl with deionised H<sub>2</sub>O.

dNTP working solution for polymerase chain reaction [2.5 mM each of dATP, dCTP, dGTP and dTTP]. dATP, dCTP, dGTP and dTTP stock solutions (2.5 µl of each) were combined and diluted to 100 µl with deionised H<sub>2</sub>O.

Primer 1 (reverse primer) stock solution (See Section 4.11.1). (3100 pmoles/ $\mu$ l).

Primer 2 (forward primer) stock solution (See Section 4.11.1). (4200 pmoles/ $\mu$ l).

Primer 1 (reverse primer) working solution (100 pmol/ $\mu$ l). Primer stock solution (1  $\mu$ l) containing 3100 pmoles was added to 30  $\mu$ l deionised H<sub>2</sub>O.

Primer 2 (forward primer) working solution (100 pmol/ $\mu$ l). Primer stock solution (1  $\mu$ l) containing 4200 pmoles was added to 41  $\mu$ l deionised H<sub>2</sub>O

Placental RNase inhibitor (20 units/ $\mu$ l), Roche (Mannheim, Germany).

25 mM MgCl<sub>2</sub>. MgCl<sub>2</sub>.6H<sub>2</sub>O (0.05 g) was dissolved in deionised H<sub>2</sub>O (10 ml), aliquoted into 1 ml fractions and stored at -20°C.

DEPC treated deionised H<sub>2</sub>O [0.1% (v/v) DEPC]. Diethylpyrocarbonate (DEPC, 0.1 ml) was added to 100 ml deionised H<sub>2</sub>O and the solution autoclaved at 121°C for 15 min.

Murine reverse transcriptase (200 units/ $\mu$ l), Promega (Madison, USA).

Taq polymerase (5 units/ $\mu$ l), Promega (Madison, USA).

### **2.8.2.2 Method**

For the generation of cDNA as a template for PCR, a reaction was set up in a total volume of 20  $\mu$ l. To a sterile PCR tube, 5  $\times$  reverse transcriptase reaction buffer (4  $\mu$ l), dNTPs (2  $\mu$ l), 100 pmoles reverse primer (1  $\mu$ l), placental RNase inhibitor (1  $\mu$ l), 1  $\mu$ g mRNA (1  $\mu$ l), 25 mM MgCl<sub>2</sub> (2  $\mu$ l), DEPC treated deionised H<sub>2</sub>O (8  $\mu$ l) and finally 200 units reverse transcriptase (1  $\mu$ l) were added. The reaction was incubated for 30 min at 37°C following which the reverse transcriptase was

inactivated by heating to 95°C for 5 min. To the PCR tube, amplification buffer (10 µl) and dNTPs (8 µl) (each at a concentration of 2.5 mM) forward primer (1 µl), reverse primer (1 µl), MgCl<sub>2</sub> (6 µl), deionised H<sub>2</sub>O (54 µl) and *Taq* polymerase (0.5 µl) were added. For the amplification using genomic DNA, an extra 19 µl of deionised H<sub>2</sub>O was added, as well as 1 µl of genomic DNA (1 µg) (Sambrook *et al.*, 1989). Amplification was carried out in a Perkin Elmer GeneAmp 2400 thermocycler (See Table 4.2 for times and temperatures). PCR products were analysed on a 1% (m/v) agarose gel for amplification using genomic DNA as a template and on a 1.8% (m/v) gel for the analysis of products from the RT-PCR reaction.

## CHAPTER 3

### PRELIMINARY INVESTIGATION OF IBDV BINDING PROTEINS

#### 3.1 Introduction

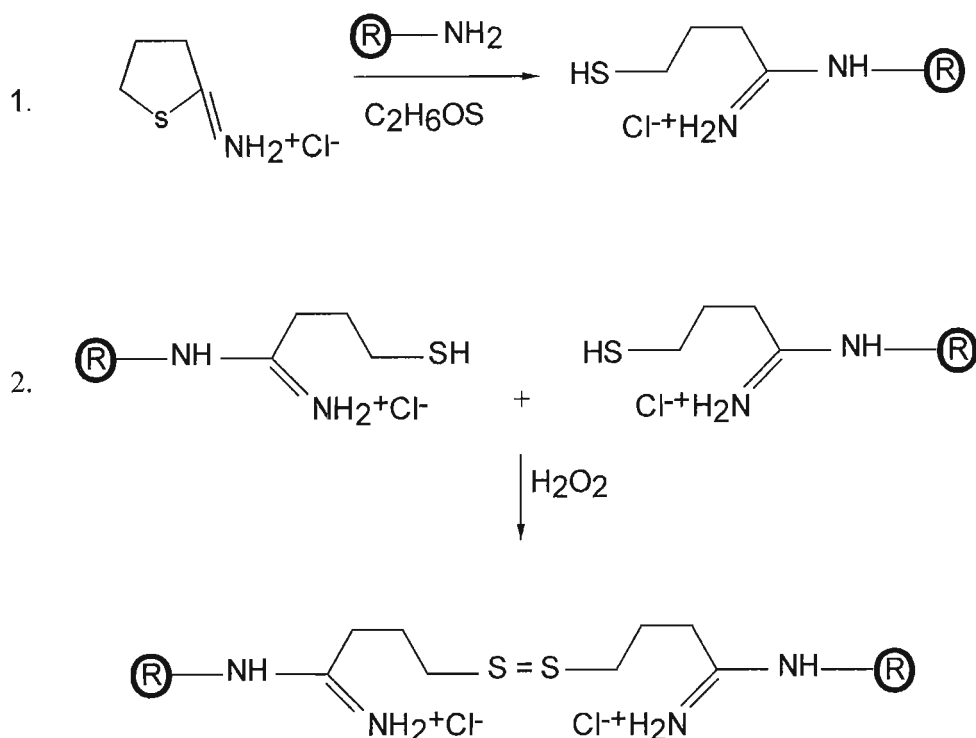
The objective of the study reported in this chapter was the identification of possible receptor proteins for IBDV that may be present on host cell membranes. Until now, no receptor for IBDV had been conclusively identified. However, possible receptor proteins of 40 and 46 kDa, were identified by Nieper and Müller in 1996, using a modified Western blot technique. No attempt has been made to purify or characterise these proteins. Since infection of the bursa of chickens by IBDV results in the production of large amounts of virions, infected tissue represents a good source of virus particles that are relatively easy to purify. Production of IBDV by cell culture not only results in a much lower production of virus but adaption of the virus to cell culture requires several passages, which cause the virus to lose virulence (Becht, 1994). It is one of the mysteries of the virus that in cell culture only a small proportion of actively dividing B lymphocytes are infected, while immune histology of infected bursal tissue shows viral antigen present in almost all cells (Cursiefen *et al.*, 1979; Tsai and Saif, 1992; Becht, 1994). Infected bursal tissue from field isolates was thus chosen as a source of viral particles. Recent field outbreaks of the disease provided large amounts of infected organs from post mortem examinations. Thus, using virus purified from infected tissue seems to represent the most natural state of the virus and use of cells or membranes from whole, uninfected tissue represents the most natural state of the organ prior to viral infection.

Virus can be purified with relative ease from infected tissue by homogenisation of the tissue and subsequent centrifugation to remove most of the cellular debris. The supernatant containing large quantities of virus can be ultracentrifuged over a sucrose cushion, thus removing most of the less dense impurities. Further purification of the virus can be achieved by centrifugation over cesium chloride, which forms a density gradient upon centrifugation, the virus thus equilibrating at its isopycnic point in the gradient (Azad *et al.*, 1985; Böttcher *et al.*, 1997).

Membranes of host cells can also be prepared from tissue by ultracentrifugation techniques. This relies on the lysis of cells and removal of the nucleus and other cell debris by relatively low speed centrifugation, followed by centrifugation over a sucrose cushion and collection of the membrane pellicle at the interface between the sucrose cushion and the supernatant (Nieper and Müller, 1998).

Virus receptors have been identified for a number of different viruses, and the methods for establishing these receptors have varied widely. Often something is known about the virus-host cell interaction that may give clues to what the receptor may be. One of the most conclusive ways to elucidate a receptor is to either knock out or stimulate production of a receptor molecule in the host cells at the gene level and determine the effect this has on infectivity of the virus, as was done with the HIV receptor, CD4 (Maddon *et al.*, 1986). However, when working with a virtually blind approach, the task becomes a little more difficult.

Two basic methods were used in initial attempts to identify virus-binding proteins. These methods included the use of 2-iminothiolane, to reversibly cross-link virus to cells, and the virus overlay protein blot assay (VOPBA). The first technique requires the virus to bind to the outer membrane of whole cells, presumably through the virus receptor on the host cell. The virus is then cross-linked to the reduced receptor molecules by 2-iminothiolane and the membranes of the cells disrupted with detergent. Virus, with attached receptor, is then purified by density gradient centrifugation. The receptor molecule is detached from the virus by reduction with mercaptoethanol. This method was attempted because it most closely resembled the natural state of viral attachment and would hopefully give more accurate results as to what proteins are involved in virus attachment. Fig. 3.1 shows the basic reaction mechanism for cross-linking two protein moieties with 2-iminothiolane.



**Fig. 3.1 Diagrammatic representation of the chemical coupling of proteins by 2-iminothiolane.** 1) Chemical coupling of 2-iminothiolane to the amino group of a polypeptide. 2) Oxidation of the sulphydryl groups of the 2-iminothiolane to form a disulfide bridge between 2 polypeptides (King *et al.*, 1978).

The second method of detection of virus binding proteins uses a modified Western blot technique. Proteins obtained from membrane fractions of host cells are subjected to SDS-PAGE and electro-blotted onto nitro-cellulose. Virus is overlaid onto the blot and binding of the virus detected either by autoradiography, if the virus is radioactively labelled, or by antibodies to the virus.

### 3.2 Purification of virus from infected bursal tissue

#### 3.2.1 Materials

Homogenisation buffer (0.02 M Tris-HCl pH 7.8). Tris (2.42) g was dissolved in 950 ml of dist. H<sub>2</sub>O, adjusted to pH 7.8 and made up to 1 l.



Dialysis buffer [0.01 M Tris-HCl pH 7.8]. Tris (1.21 g) was dissolved in 950 ml of dist. H<sub>2</sub>O, adjusted to pH 7.8 and made up to 1 l.

40% (m/v) sucrose. Sucrose (40 g) was dissolved in a minimum of homogenisation buffer and made up to 100 ml with homogenisation buffer.

Cesium chloride gradients. Two solutions were prepared by gradually dissolving cesium chloride in homogenisation buffer until the density reached 1.37 g/cm<sup>3</sup> and 1.27 g/cm<sup>3</sup> respectively. The density of the solutions was estimated by withdrawing 1-ml fractions of the solution and determining the mass on a balance. If the solution was not dense enough, more cesium chloride was added and if the solution was found to be too dense, more homogenisation buffer was added. Upon centrifugation, cesium chloride solutions automatically form a density gradient spanning the density of the original solution.

### 3.2.2 Method

Bursal tissue was homogenised in an equal volume of homogenisation buffer using a Potter homogeniser. The homogenate was centrifuged (17 000 × g, 15 min, 4°C) and two volumes of the supernatant layered over 1 volume of 40% (m/v) sucrose and centrifuged in a Beckman SW 40 rotor (70 000 × g, 2.5 h, 4°C) (Azad *et al.*, 1985). The virus pellet was resuspended in homogenisation buffer and layered over cesium chloride in homogenisation buffer (mean starting density 1.37 g/cm<sup>3</sup>), and centrifuged in a Beckman SW 40 rotor (70 000 × g, 6 h, 4°C). The pellicle of virus obtained was resuspended in homogenisation buffer and dialysed overnight against dialysis buffer for removal of cesium chloride. The dialysed suspension was layered over cesium chloride in homogenisation buffer (mean starting density 1.27 g/cm<sup>3</sup>) and centrifuged in a Beckman SW 40 rotor (70 000 × g, 6 h, 4°C). The pellicle was resuspended in homogenisation buffer and dialysed overnight against 0.01 M Tris-HCl, pH 7.8 (Böttcher *et al.*, 1997). SDS-PAGE and Western blot analysis of viral proteins were performed as described in Sections 2.4.3 and 2.7.3, respectively. Approximately 5 µg (for silver staining) and 50 µg (for Coomassie staining) of virus suspension was solubilised in 20 µl of reducing treatment buffer, or reducing treatment buffer

containing 3 M urea, and analysed by SDS-PAGE. Silver staining and Coomassie staining were performed as described in Sections 2.6 and 2.5. Viral antigens were probed with 1/2 000 dilutions of both chicken anti-IBDV serum and chicken anti-IBDV IgY, as described in Section 2.7.3.

### **3.3 Transmission electron microscopy of partially purified IBDV**

The contents of the partially purified virus fraction, obtained after the initial ultracentrifugation over 40% (m/v) sucrose, was examined by electron microscopy to check for the presence of viral particles.

#### **3.3.1 Materials**

Washing Buffer. [0.05 M cacodylate buffer, pH 7.10]. dimethylarsinic acid-sodium salt (sodium cacodylate, 8 g) was dissolved in 950 ml dist. H<sub>2</sub>O, adjusted to pH 7.1 with NaOH and the buffer was made up to 1 l.

2% (m/v) uranyl acetate. Uranyl acetate (2 g) was dissolved in washing buffer (100 ml).

#### **3.3.2 Method**

A 1/1 000 dilution of virus particles was made in dist. H<sub>2</sub>O and 10 µl of the diluted virus suspension placed on a formvar-coated, copper, transmission electron microscopy grid. The grid was incubated for 2 min with the virus suspension and excess suspension was removed with a small section of filter paper. The grid was negatively stained by floating it upside down for 30 s on a drop of uranyl acetate placed on a wax-filled petri dish. The excess uranyl acetate was removed with filter paper and the grid washed in a drop of washing buffer for 1 min, the excess washing buffer was removed with a piece of filter paper, and the grid viewed under a transmission electron microscope. Photographs were taken at a magnification of 105 000 ×.

### 3.4 Production of anti-IBDV antibodies in chickens

#### 3.4.1 Method

Antibodies against whole virus were raised in White Leghorn chickens by immunising laying hens in the breast muscle with 50 µg of purified virus emulsified in a 1:1 ratio with Freund's incomplete adjuvant. Further inoculations were administered in the same manner, with the same amount of virus, bi-weekly for a total of 6 weeks. The progress of the chickens' response to the viral immunogens was followed by ELISA analysis (Section 2.7.2) of antibody purified from eggs laid in the week of inoculation (Section 2.7.1).

### 3.5 Purification of membranes from bursal tissue

Chicken bursas were obtained fresh from Allerton Veterinary Regional Laboratory and stored at -80°C. Membranes were prepared from bursal tissue using the method of Nieper and Müller (1998).

#### 3.5.1 Materials

Washing buffer (0.025 M *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), 0.154 M NaCl, 0.5 mM MgCl<sub>2</sub>, pH 7.4). HEPES (0.6 g), NaCl (0.9 g) and 50 µl of a 1 M MgCl<sub>2</sub> solution were dissolved in 80 ml of dist. H<sub>2</sub>O adjusted to pH 7.4 with NaOH and the buffer made up to 100 ml.

Homogenisation buffer (0.025 M HEPES, 0.03 M NaCl, 0.5 mM MgCl<sub>2</sub>, 1 mM DTT, 1 mM PMSF, 1 µM pepstatin A, pH 8.0). HEPES (0.6 g), NaCl (0.175 g) and 50 µl of a 1 M MgCl<sub>2</sub> solution were dissolved in 80 ml of dist. H<sub>2</sub>O, adjusted to pH 8.0 with NaOH and made up to 100 ml. [Just before use, DTT (15 mg), PMSF (17 mg in 100 µl of DMSO) and pepstatin A (65 µg, 13 µl of a 5 mg/ml solution in DMSO) were added.

Restoration buffer [0.025 M HEPES, 0.6 M NaCl, 0.5 mM MgCl<sub>2</sub>, pH 8.0]. HEPES (0.6 g), NaCl (3.5 g) and 50 µl of a 1 M MgCl<sub>2</sub> solution were dissolved in 80 ml of dist. H<sub>2</sub>O adjusted to pH 8.0 with NaOH and made up to 100 ml.

25% (m/v) sucrose solution. Sucrose (12.5 g) was dissolved in a minimum of homogenisation buffer and then diluted to 50 ml with homogenisation buffer.

### 3.5.2 Method

Bursal tissue was minced carefully with scissors and washed three times with washing buffer. Cells were resuspended in a three-fold w/v excess of ice-cold homogenisation buffer and kept on ice for 10 min and homogenised with 30 to 40 strokes of a Dounce homogeniser. Isotonic conditions were restored by addition of restoration buffer, i.e. the NaCl concentration was raised to 0.154 M. The volume of restoration buffer added was calculated according to the following equation (Nieper and Müller, 1998):

$$y = \frac{124x}{446}$$

where x = the initial volume of the homogenised solution (ml) and y is the required volume of restoration buffer (ml).

EDTA was added to a concentration of 1 mM to the homogenate and centrifuged (5 000 × g, 10 min, 4°C). Two parts of the supernatant were layered over 1 part 25% (m/v) sucrose in homogenisation buffer and centrifuged in a Beckman SW 40 rotor (100 000 × g, 1 h, 4°C). Plasma membranes were collected from the interface, resuspended in washing buffer, recentrifuged (100 000 × g, 30 min, 4°C) and resuspended in washing buffer (Nieper and Müller, 1998).

### 3.6 Reversible cross-linking of virus to receptor molecules with 2-iminothiolane

Chicken bursas were obtained fresh from Allerton Veterinary Regional Laboratory and kept on ice before beginning the procedure.

#### 3.6.1 Materials

HEPES magnesium chloride sucrose (HMS) buffer [0.025 M Sucrose, 0.05 M HEPES, 0.05 M  $\text{MgCl}_2$ , pH 7.5]. HEPES (1.2 g), sucrose (0.86 g) and  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (1 g) were dissolved in approximately 90 ml dist.  $\text{H}_2\text{O}$ , adjusted to pH 7.5 with NaOH and made up to 100 ml.

Ficoll in HMS. Ficoll was gradually dissolved in HMS buffer until a starting density of  $1.079 \text{ g/cm}^3$  was obtained. During the dissolving procedure, 1 ml samples of the solution were withdrawn and weighed. If the density was calculated to be less than  $1.079 \text{ g/cm}^3$ , more Ficoll was added: more HMS was added if the desired density had been exceeded.

#### 3.6.2 Method

Tissue was carefully minced with scissors and suspended in HMS buffer. Coarse material was allowed to settle for five min. Three parts cell suspension were layered over 1 part Ficoll in HMS, with a starting density of  $1.079 \text{ g/cm}^3$ , and centrifuged ( $600 \times g$ , 30 min,  $12^\circ\text{C}$ ). Cells were washed twice with HMS ( $2\,000 \times g$ , 10 min,  $4^\circ\text{C}$ ) and resuspended in 50 ml of HMS. Virus suspension was added and adsorbed for 75 min at  $0^\circ\text{C}$ , with stirring. The cells were centrifuged ( $2\,000 \times g$ , 10 min,  $4^\circ\text{C}$ ) and suspended in 10 ml HMS, containing 1.5 mM 2-mercaptoethanol and 5 mg/ml 2-iminothiolane, and incubated for 30 min at  $0^\circ\text{C}$ . Cells were centrifuged ( $2\,000 \times g$ , 10 min,  $4^\circ\text{C}$ ) and resuspended in 10 ml HMS, containing 100 mM  $\text{H}_2\text{O}_2$ , and incubated at  $0^\circ\text{C}$  for 90 min. Cells were again centrifuged ( $2\,000 \times g$ , 10 min,  $4^\circ\text{C}$ ) and resuspended in 10 ml HMS, containing 0.25% (v/v) Triton X-100, and incubated for 1 h at  $0^\circ\text{C}$ . Lysed cells were centrifuged ( $5600 \times g$ , 10 min,  $4^\circ\text{C}$ ) and the supernatant layered over 40% (m/v) sucrose and centrifuged ( $76\,000 \times g$ , 2 h,  $4^\circ\text{C}$ ).

The pellet was resuspended in HMS. Samples were boiled in 0.0312 M Tris-HCl containing 5% (v/v) mercaptoethanol, 2% (m/v) SDS and 3 M urea, for 2 min before analysis by SDS-PAGE.

### **3.7 Virus overlay protein blot assay (VOPBA)**

The virus overlay protein blot assay (VOPBA) is an established method to identify proteins, which bind virus in free solution (Basak *et al.*, 1994; Nieper and Müller, 1996). The procedure is similar to that used in a regular Western blot, i.e. membrane polypeptides are blotted onto nitro-cellulose after reducing SDS-PAGE and polypeptides that bind virus are detected by overlaying the blot with virus particles and detecting the bound particles using appropriate methods. This is either performed with anti-viral antibodies or if the virus is radiolabelled, by autoradiography. The technique relies on the fact that a possible receptor maintains its virus-binding epitopes after SDS-PAGE and Western blotting.

#### **3.7.1 Materials**

As per Sections 2.4.3 and 2.7.3.

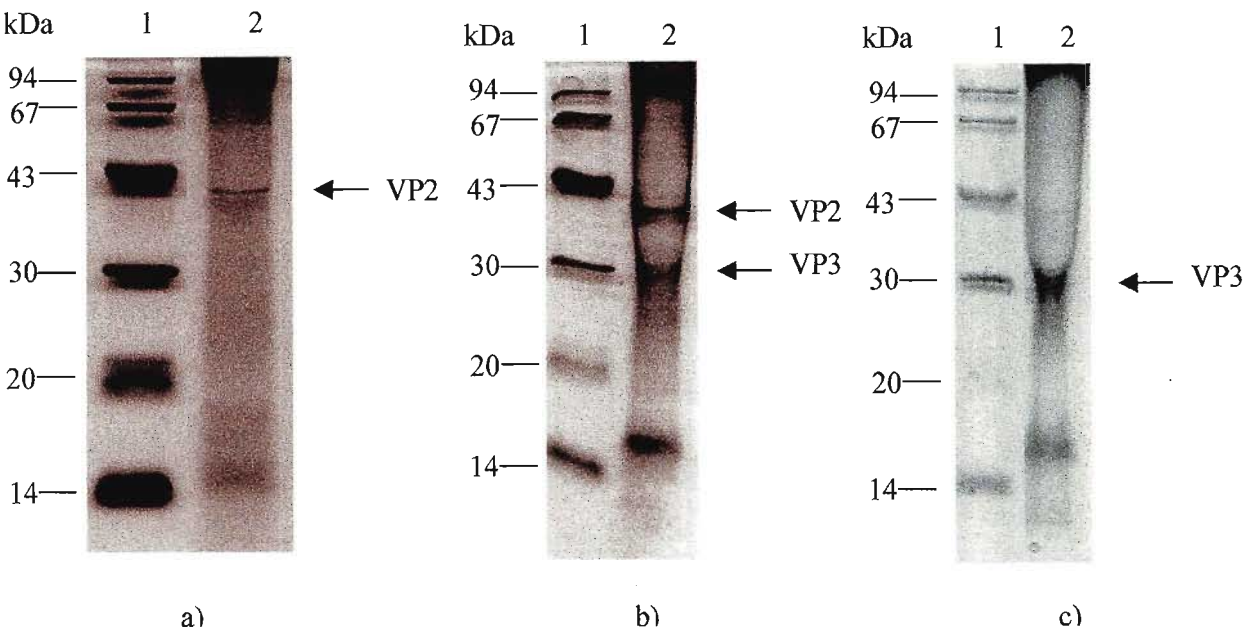
#### **3.7.2 Method**

Approximately 25 µg of bursal membrane protein were separated on a 7.5% SDS-PAGE gel and electro-blotted onto nitro-cellulose (Section 2.7.3). The blot was incubated in a 1/200 dilution of virus in TBS (prepared as described in Section 2.7.3) and incubated for 2 h at room temperature. Excess virus was washed off with TBS (3 × 5 min) and the blot incubated for 2 h in a 1/200 dilution of chicken anti-IBDV serum in BSA-TBS. The blot was washed with TBS (3 × 5 min), incubated in a 1/20 000 dilution of alkaline phosphatase conjugated rabbit anti-chicken antibody for 1 h and binding of the enzyme labelled detection antibody was detected with BCIP/NBT substrate, which is converted to an insoluble precipitate by alkaline phosphatase (Section 2.7.3).

3.8 Results and discussion

3.8.1 Virus protein profiles

SDS-PAGE of viral particles showed a relatively homogenous virus preparation (Fig. 3.2), with the major protein band identifiable as the 40 kDa VP2 structural protein. According to Becht, (1994) the two major structural proteins (VP2 and VP3)



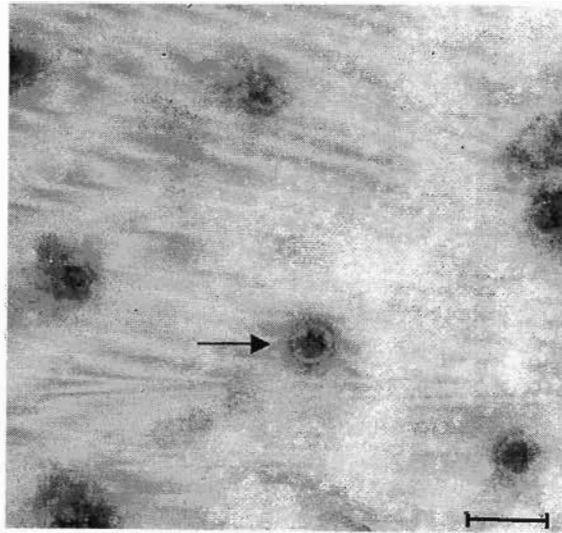
**Fig. 3.2 Reducing SDS-PAGE of viral proteins.** a) Approximately 5  $\mu$ g of virus was boiled in 20  $\mu$ l of reducing treatment buffer for 2 min and loaded in a well of a 10% Tris-Tricine SDS-PAGE gel and silver stained. b) Approximately 5  $\mu$ g of virus was boiled for 10 min in 20  $\mu$ l reducing treatment buffer containing 3 M urea, loaded onto a 10% Tris-Tricine SDS-PAGE gel and silver stained. c) Approximately 40  $\mu$ g of virus was boiled for 10 min in 50  $\mu$ l of reducing treatment buffer, loaded onto 10% Tris-Tricine SDS-PAGE gel and stained with Coomassie blue R250. Lane 1, molecular mass markers, phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and  $\alpha$  lactalbumin (14 kDa); lane 2, virus preparation.

are present in almost equal amounts. However, in virus preparations obtained through the methods described above, VP2 seems to be the predominate protein present in reduced, denatured samples of the virus. However, the visibility of the different viral proteins seems to differ when different stains and/or amounts of virus are examined

on reducing SDS-PAGE. The use of 3 M urea in the viral sample also seems to affect the visibility of the different viral proteins in the gel; a more denaturing solution may be required to fully break up the virus particles and give a truer representation of the quantities of the viral proteins.

### 3.8.2 Transmission electron microscopy of virus particles

Transmission electron microscopy of viral particles showed typical icosahedral capsid shapes of approximately 60 nm, with a darkly stained interior and clearer margin. These probably represent incomplete viral particles with a bouyant density of 1.29 g/cm<sup>3</sup>, which show stain penetration (Müller and Becht, 1982). However, other particles were also present showing little staining, and these probably represent the particles with a buoyant density of 1.33 g/cm<sup>3</sup>. The incomplete particles are usually found in virus preparations grown on chicken embryo fibroblasts (Müller and Becht, 1982). However, these may represent particles that were not fully formed in infected cells in the bursa, or may have been damaged in some way by the purification process. On a gross morphological scale the virus particles thus appear similar to those previously reported.

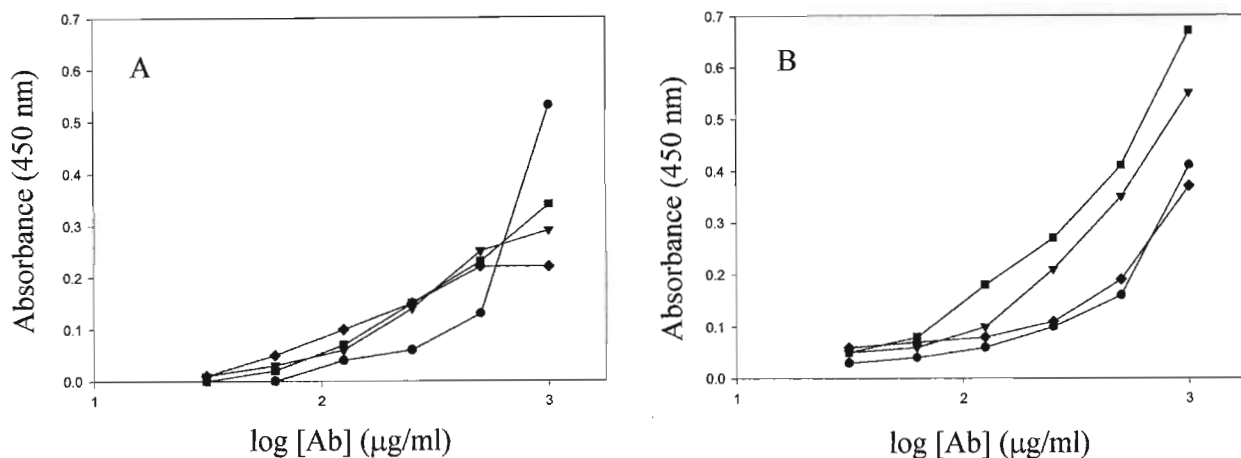


**Fig 3.3** Electron micrograph of negatively stained infectious bursal disease virus particles (virus particle indicated by arrow; bar represents 100 nm).



### **3.8.3 Determination of the titre of polyclonal anti-IBDV antibodies**

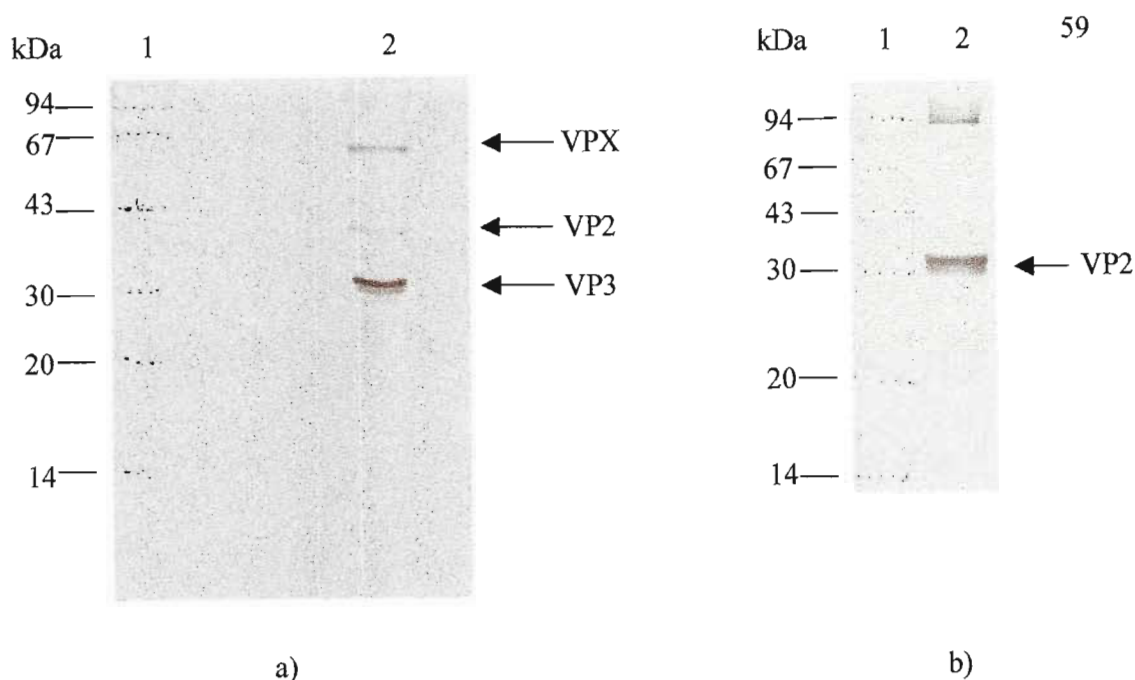
Analysis of anti-IBDV chicken IgY by ELISA showed that the immunisation schedule was successful, raising antibody levels above background. However, the chickens used to generate antibodies were laying hens obtained from Ukulinga research farm and these had been immunised with IBDV at an early age, thus background levels of anti-IBDV antibody in the pre-immune IgY were quite high. The antibody titres seem to peak at about 4 weeks and then drop back to levels present in the pre-immune serum. Thus the boosting of the immune response seems to be rapid and short-lived, again probably due to the presence of high levels of antibodies to the virus in the immune system of the bird, before inoculation. The bird's immune system is primed and ready to deal with the presence of viral antigen and does not need to raise its antibody levels much to deal with viral antigen. It was also found that in order to have accurate results in the assay, the viral proteins needed to be reduced and denatured with reducing treatment buffer before coating. This was due to a non-specific reaction seen with the whole virus and the enzyme labelled detection antibody, resulting in inaccurate estimation of antibody titre. Antibodies obtained at week 4 in the inoculation schedule, which showed the highest titre, were therefore used in other experiments involving anti-IBDV antibodies, such as Western blots.



**Fig. 3.4 Monitoring of antibody production of anti-IBDV chicken IgY by ELISA.** Microtitre plates were coated with reduced denatured IBDV (1 μg/ml) and incubated with serial doubling dilutions of pre-immune IgY (—●—); IgY from week 2 (—◆—); 4 (—■—) and 6 (—▼—) after the start of the immunisation programme (A, Bird 1; B, bird 2). Binding of antibodies was visualised by incubation with HRPO-linked detection antibodies followed by ABTS/H<sub>2</sub>O<sub>2</sub> substrate (section 2.7.2). Each point is the mean absorbance at 405 nm of duplicate samples.

### 3.8.4 Immunochemical analysis of viral proteins

Western blot analysis of the viral proteins with anti-IBDV chicken IgY and anti-IBDV chicken sera shows the major antigen recognised as being VP3, of approximately 30 kDa. This is presumably due to the conformation independent epitopes present on VP3 being recognised, due to the reducing, denaturing conditions of a typical Western blot. It is interesting to note that while both anti-IBDV IgY and chicken serum antibodies recognised VP3 as the major antigen, only VP3 was recognised in blots with serum (Fig 3.5), while other viral proteins (VP2 and VPX) were recognised in blots with anti-IBDV IgY. The protein profile in the Western blot probed with anti-IBDV chicken IgY (Fig. 3.5 a) thus looks quite different from the profile obtained in Fig. 3.5 (b). This may explain the high level of protection afforded by maternal antibody in baby chickens, due to the high titre of virus specific antibodies present in the IgY fraction of eggs.

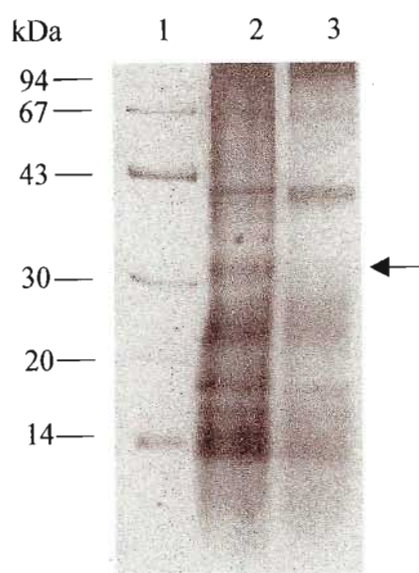


**Fig 3.5 Western blots of IBDV proteins.** Approximately 5  $\mu$ g viral proteins were separated on a 10 % reducing SDS-PAGE gel and electroblotted onto nitro-cellulose. Positions of molecular mass markers indicated (lane 1, as per Fig. 3.2) following transient Ponceau S staining. Separated viral proteins (lane 2) were probed with a 1/2000 dilution of anti-IBDV chicken IgY (a) or a 1/2000 dilution of anti-IBDV chicken serum (b). Binding of antibodies was visualised by incubation with alkaline phosphatase-linked detection antibodies followed by incubation with BCIP/NBT substrate (Section 2.7.3).

### 3.8.5 Reversible cross-linking of virus to possible receptor proteins with 2-iminothiolane

The protein profiles of virus cross-linked to possible receptor proteins and normal virus profiles were compared (Fig. 3.6). While on the whole the profiles appear to be similar, a band of approximately 32 kDa appears in the cross-linked sample (Fig 3.6, lane 2). This may represent a novel IBDV binding protein but the virus also contains a protein of this molecular mass (VP3) and it cannot be established for certain that it is not the virus protein. As shown in Fig. 3.2 VP3 is not always clearly visible in protein profiles of the virus but it is definitely present as shown by Western blots (Fig 3.5). The appearance of this band at 32 kDa may therefore be due to reaction induced by the cross-linking protocol, such as the reduction with 2-mercaptoethanol or the oxidation with  $H_2O_2$ . The band may represent a novel virus binding protein but Western blots cannot distinguish between the molecular mass of the virus protein and

the possible host receptor due to their similarity in size. Due to the inconclusive nature of the method, bursal membrane proteins were analysed for IBDV binding proteins using VOPBA.



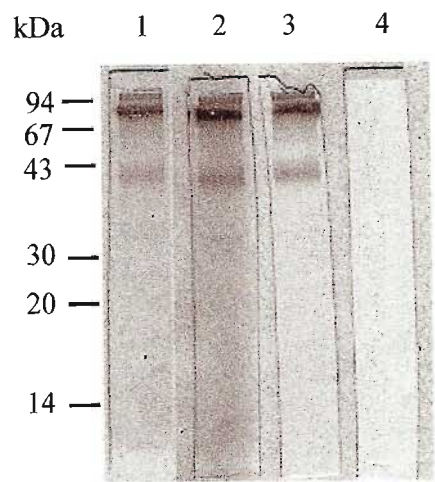
**Fig. 3.6 Reducing SDS-PAGE of 2-iminothiolane cross-linked virus and normal virus samples.**

Lane 1, molecular mass markers (as per Fig. 3.2); lane 2, cross-linked virus; lane 3, normal virus. Arrow indicates protein of approximately 32 kDa visible in cross-linked virus sample.

### 3.8.6 Virus overlay protein blot assay

The virus overlay protein blot assay (VOPBA) was used in an attempt to establish the presence of possible virus receptor polypeptides in membrane fractions from host cells. Antibodies detected protein bands at approximately 40 and 80 kDa, however these bands were also detected in incubation controls where no primary antibody and no virus were included in the incubations (Fig. 3.7). The technique has its limitations in that it assumes that the viral interaction with the polypeptide is non-conformational, which is not necessarily a good assumption. The virus overlay blot shown in Fig. 3.7 indicates that viral receptor polypeptides cannot readily be identified. One of the major problems with the assay was the interaction of the enzyme labelled detection antibody (rabbit anti-chicken IgY alkaline phosphatase conjugate) with polypeptides in the membrane fraction of chicken host cells. There may be an interaction with IgG present on the surface of B cells present in the bursa of Fabricius, shown by controls that contain no virus or no primary antibody. This indicates that the enzyme labelled

detection antibody could have bound directly to proteins on the bursal membrane rather than through the virus-primary antibody tier. Since the VOPBA technique could not establish specific IBDV binding proteins in the bursal membrane fraction, affinity chromatography using a column consisting of IBDV cross-linked to Sepharose 4B (discussed in Chapter 4) was performed.



**Fig. 3.7 Virus overlay protein blot assay of bursal cell membranes.** Lane 1, VOPBA; lane 2, no virus incubation control; lane 3, no primary anti-IBDV antibody incubation control; lane 4, no enzyme labelled detection antibody control.

**3.7 Conclusions**

Both virus-receptor cross-linking experiment and VOPBA results were inconclusive. This was probably due to the large number of variables that were present in these experiments. VOPBA has the problem of interactions between the enzyme labelled detection antibody and the chicken bursal membrane proteins. The cross-linking experiment was done with only partially purified viral particles. However, a 33 kDa band did seem to appear in the cross linked samples, although this may have been due to the differential visibility of viral protein on SDS-PAGE as shown in Fig. 3.2. Possibly the experiment would have been more successful had radiolabelled virus particles or monoclonal antibodies been used as detection agents, as performed by other researchers. This would reduce the number of levels of tiered binding and reduce the possibility of secondary reactions. However, the effect of using virus artificially grown on chicken embryo fibroblasts compared to naturally occurring

virus needs to be considered. Since IBDV does not grow normally on chicken embryo fibroblasts and needs to be passaged several times before it does begin to grow, one cannot regard IBDV grown in cell culture as the natural agent of IBD. This has been shown by mutation of residues in VP2 of very virulent virus, allowing for growth on chicken embryo fibroblasts (Lim *et al.*, 1999). Thus experiments performed with IBDV grown in cell culture or chicken embryo fibroblasts may not reflect the true events in the course of IBD. In this regard a more holistic approach to the identification of a receptor, or at least IBDV binding proteins, was adopted. It was considered that the use of membranes prepared from whole organs as opposed to cultured cells, as well as wild type virus prepared from field isolates of infected bursal material, may negate or reduce the possibility of artefactual data.

The techniques above did not conclusively identify IBDV-binding proteins and, in the case of the VOPBA, relied on IBDV binding to reduced-and-denatured chicken bursal membrane proteins. A different approach for identifying such IBDV binding proteins seemed to be required. In this regard, affinity chromatography appeared to be the most appropriate tool for identifying such proteins. It is also a much cheaper method than other methods described in Chapter 1, and does not require information about the receptor to be known before it can be implemented.

In Chapter 4, the affinity chromatography separation of IBDV binding proteins using a column of IBDV linked to Sepharose 4B will be discussed. Proteins separated using this method are further examined using N-terminal amino acid sequencing, a modified VOPBA and Western blotting.

## CHAPTER 4

### ISOLATION AND CHARACTERISATION OF IBDV BINDING PROTEINS

#### 4.1 Introduction

Receptor polypeptides of 40 and 46 kDa, from the membrane fraction of chicken embryo fibroblasts, have been identified by VOPBA as possible receptors for IBDV (Nieper and Müller, 1996). However these proteins have not been further characterized. The experiments described in this chapter involve the purification of virus binding proteins from proteins in the cell membrane fraction of bursal cells. The method used for purification was affinity chromatography using a virus-affinity column. Affinity chromatography is a useful method as virus-binding proteins can be separated from other non-binding proteins, thus achieving a fairly high level of purification (Hennache and Boulanger, 1977). Since the receptor for IBDV is likely to be a protein molecule (Ogawa *et al.*, 1998), affinity chromatography should enable isolation of possible receptor polypeptides. The major advantage of this method over those used in the previous chapter is the fact that it is a preparative method and not merely analytical. Thus other techniques, such as further purification or protein sequencing can be applied to the protein.

As stated in the previous chapter, a method whereby virus-binding protein can be identified in a state closest to the *in vivo* state is desirable. While affinity chromatography separation of virus binding proteins from bursal cell membranes does not use living cells and, therefore, does not represent the natural state of infection, it does represent a state where the membrane proteins are not denatured and should thus maintain their conformation. The latter may be very important for interaction with the virus ligand, as is the case of CD4 binding to gp120 of HIV (Ibegbu *et al.*, 1989). Other methods for the identification of virus binding proteins involve interaction between whole virus and reduced denatured polypeptides (e.g. the VOPBA). These may not represent the natural interaction of virus with host cell membrane proteins and may even lead to false interactions where virus would not normally interact with a specific protein (Tardieu *et al.*, 1982).

Isolation of virus binding proteins allows further information to be obtained regarding the protein of interest. Microsequencing of proteins is now an established technique and when combined with the information available in protein sequence databanks, may give large amounts of information regarding the function and specific nature of a protein. With the sequence information, one can also determine a possible DNA sequence using the codon usage tables of a particular species. Once the DNA sequence has been determined, oligonucleotide sequences for cDNA synthesis and polymerase chain reaction (PCR) can be established and used for these purposes. While determination of the DNA sequence from the protein sequence is not directly possible due to the degeneracy of the DNA code (for each amino acid there are several possible codons), degenerate primers can be developed which encompass the different possibilities for each amino acid residue (Sambrook *et al.*, 1989).

In this chapter, affinity chromatography was used for separation of IBDV binding proteins in the membrane fraction of bursal cells. A column consisting of Sepharose 4B coupled to IBDV was used for these purposes. IBDV binding proteins were further examined by N-terminal amino acid sequencing and the sequences compared to known sequences of other proteins. Cross reactivity between bursal cell membrane proteins and human proteins involved in rheumatoid arthritis that form autoantibodies, was investigated by Western blotting, using sera obtained from patients with rheumatoid arthritis. Finally, amplification of a section of the DNA sequence of one of the IBDV binding proteins, using PCR will be discussed.

#### **4.2 Separation of IBDV-binding proteins from bursal cell membranes**

IBDV-binding proteins from the membrane fraction of bursal cells can be separated by adsorption of the bursal membrane proteins to an affinity column consisting of IBDV coupled to Sepharose 4B, prepared by the method of March *et al.* (1974).



#### 4.2.1 Materials

2 M sodium carbonate.  $\text{Na}_2\text{CO}_3$  (21.19 g) was dissolved in dist.  $\text{H}_2\text{O}$  and made up to 100 ml.

Wash A (200 mM  $\text{NaHCO}_3$ , pH 9.6).  $\text{NaHCO}_3$  (8.4 g) was dissolved in approximately 450 ml of dist.  $\text{H}_2\text{O}$ , titrated to pH 9.6 with  $\text{NaOH}$ , and made up to 500 ml.

Wash B (100 mM  $\text{NaHCO}_3$ , pH 9.2).  $\text{NaHCO}_3$  (0.84 g) was dissolved in approximately 180 ml of dist.  $\text{H}_2\text{O}$ , titrated to pH 9.6 with  $\text{NaOH}$ , and made up to 200 ml.

Coupling buffer (100 mM  $\text{NaHCO}_3$ , 500 mM  $\text{NaCl}$ , pH 8.3).  $\text{NaHCO}_3$  (4.2 g) and  $\text{NaCl}$  (14.61 g) were dissolved in approximately 450 ml dist.  $\text{H}_2\text{O}$ , titrated to pH 8.3 with  $\text{NaOH}$ , and made up to 500 ml.

Blocking agent (1 M ethanolamine-HCl, pH 8.0). Ethanolamine (6.06 ml) was diluted with 80 ml dist.  $\text{H}_2\text{O}$ , titrated to pH 8.0 with  $\text{HCl}$  and made up to 100 ml.

Wash C (100 mM sodium acetate, 500 mM  $\text{NaCl}$ , pH 4.0). Acetic acid (5.72 ml) and  $\text{NaCl}$  (14.61 g) were dissolved in 450 ml dist.  $\text{H}_2\text{O}$ , titrated to pH 4.0 with  $\text{NaOH}$  and made up to 500 ml.

Chromatography buffer [10 mM Tris-HCl, 50 mM  $\text{NaCl}$ , 0.1% (m/v) Na-deoxycholate, 0.02% (m/v)  $\text{NaN}_3$ , pH 8.0]. Tris (1.21 g),  $\text{NaCl}$  (2.92 g), Na-deoxycholate (1 g) and  $\text{NaN}_3$  (0.2 g) were dissolved in 950 ml dist.  $\text{H}_2\text{O}$  and titrated to pH 8.0 with  $\text{HCl}$  and made up to 1 l.

#### 4.2.2 Method

Sepharose 4B (5 ml packed gel) was washed in a Büchner funnel, on a Whatman No.1 filter paper disc, with 150 ml dist.  $\text{H}_2\text{O}$ , transferred to a small beaker, allowed to settle

and the supernatant removed. Dist. H<sub>2</sub>O (5 ml) and 2 M Na<sub>2</sub>CO<sub>3</sub> (10 ml) were added to the gel and the mixture placed on ice, in a fume hood, and mixed gently until the temperature decreased to 4°C. The rate of stirring was increased and 1 M CNBr solution in acetone was added as rapidly as possible.

The gel mixture was mixed for 75 min, transferred to a Büchner funnel and washed with dist. H<sub>2</sub>O (100 ml), wash A (100 ml) and wash B (100 ml). During washing, the gel was stirred gently with a glass rod to prevent it from 'caking' during filtration, especially in the initial washing step as the gel tends to become sticky in the final stages of activation, unless the activating agent is quickly and uniformly removed. The activated gel was washed with excess coupling buffer, transferred to a glass bottle and allowed to settle. Excess coupling buffer was removed, and IBDV in coupling buffer (10 mg) added to the suspension and mixed end-over-end for 14 h at 4 °C.

The gel was allowed to settle and the excess coupling buffer, containing any unbound IBDV was removed and kept. The A<sub>280</sub> value of the excess coupling buffer and the initial value of the virus in coupling buffer were compared and an estimation of coupling efficiency was made. Blocking agent (10 ml) was added to the remaining gel and the slurry was mixed, end-over-end (2 h, RT). The gel was washed on a Büchner funnel, alternatively with coupling buffer and low pH buffer (wash C), to ensure that no free ligands remained ionically linked to the gel. The gel was finally washed and suspended in chromatography buffer.

The gel was packed into a column (6 cm × 0.5 cm i.d. = 4.7 ml) and washed with chromatography buffer (10 column volumes). Membranes (as described in Section 3.3; approximately 10 mg) were solubilised in chromatography buffer (10 ml) containing 0.5% (m/v) Na-deoxycholate, by stirring for 30 min at 4°C. The solubilised membranes were passed entirely through the column once and cycled through the column overnight at 4°C. Unbound proteins were eluted from the column with chromatography buffer. Raising the salt concentration stepwise to 150 mM followed by 600 mM first eluted loosely bound proteins, followed by tightly bound proteins; fractions (approximately 2 ml) were collected (Hennache and Boulanger, 1977). The peak corresponding to the fractions eluted with 600 mM NaCl was pooled

and dialysed overnight against chromatography buffer without Na-deoxycholate and concentrated by dialysis against PEG 20 000 (Section 2.3.1). The concentrated fraction was further concentrated with SDS-KCl precipitation (Section 2.3.2) before analysis by reducing Tris-Tricine SDS-PAGE (Section 2.4). Proteins from the pooled 600 mM NaCl fraction were electro-blotted onto nitro-cellulose and probed with a 1/1 000 dilution of chicken anti-IBDV IgY, to ensure that the eluted protein was of bursal membrane origin and viral proteins had not eluted from the column.

### 4.3 Copper staining of SDS-PAGE

To ensure that proteins were pure for N-terminal amino acid sequencing, the protein of interest needed to be isolated from the other proteins in the fraction that bound to the IBDV-affinity column. To this end the proteins were electrophoresed on a reducing SDS-PAGE gel (Section 2.4) and stained using the copper staining method of Lee *et al.* (1987). This is a 5-minute stain that yields negatively stained gels, in which protein shows as clear bands against a semi-opaque, light-blue background. The copper-stained protein was excised from the gel and destained. Normally protein would be electro-eluted from the gel slice before further treatment could take place. However, it was found that the protein in the excised band could be directly electrophoresed for a second time on a second gel, by placing the excised band directly into the well of the second gel. This reduced losses that would normally be encountered in electro-elution.

#### 4.3.1 Materials

Staining solution [0.3 M  $\text{CuCl}_2$ ].  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$  (5.11 g) was dissolved in dist.  $\text{H}_2\text{O}$  (100 ml).

Destaining solution [0.25 M Tris, 0.25 M EDTA, pH 9.0]. Tris (3 g) and EDTA (9.3 g) were dissolved in approximately 80 ml dist.  $\text{H}_2\text{O}$ , titrated to pH 9.0 with NaOH and made up to 100 ml.

### 4.3.2 Method

After electrophoresis, gels were dipped in dist. H<sub>2</sub>O for several seconds, and immersed in one smooth motion into 0.3 M CuCl<sub>2</sub>, and incubated in this solution (5 min, RT) with gentle agitation and washed for 2-3 min in dist. H<sub>2</sub>O. The band of interest was excised using a clean scalpel blade and incubated in destain solution (3 × 10 min), after which the protein band was ready for electrophoresis on a second gel.

### 4.4 N-terminal sequence analysis

Identification of the N-terminal sequence of a protein is useful in the characterisation of its structural and functional domains, especially with the aid of the large number of sequence databanks available today. The sequence is determined by repeated cycles of the Edman degradation reaction (Matsudaira, 1987). Each degradation cycle consists of three steps, coupling, cleavage and conversion. Coupling involves the conversion of the  $\alpha$ -amino group of the N-terminus of the polypeptide with phenyl isothiocyanate under basic conditions to generate a phenylthiocarbamoyl (PTC) polypeptide. In the second step the PTC-N-terminal residue is cleaved from the polypeptide by either gaseous or liquid tri-fluoroacetic acid, liberating an anilinothiazoline (ATZ)-amino acid derivative of the original N-terminal residue. This unstable derivative is finally converted to the more stable phenylthiohydantoin (PTH)-amino acid. Identification of these derivatives is facilitated through a combination of ultraviolet (UV) absorbance and HPLC, with a sensitivity of 1-10 pmol (Matsudaira, 1987). The new N-terminal residue is now available for another Edmann degradation cycle. This procedure is commonly performed by automated gas-phase sequencers for the simple and efficient analysis of proteins that have been separated by SDS-PAGE and electro-blotted onto polyvinylidene difluoride (PVDF) membranes (Matsudaira, 1987). The resulting N-terminal sequence may be compared with existing sequences in protein databases, permitting the identification and classification of the protein of interest (Lottspeich, 1994).

A number of post-translational modifications of protein molecules may interfere with the sequencing process, the most common of which is the N-terminal blocking of a

protein or peptide. Approximately 50% of all naturally occurring proteins are N-terminally modified by an acetyl, formyl or pyroglutamic acid group (Lottspeich *et al.*, 1994). These blocked proteins cannot be sequenced by conventional Edman degradation steps, and thus require prior enzymatic or chemical cleavage. Additionally, proteins may be modified during preparation or purification. Tris-Tricine SDS-PAGE gels were therefore polymerised four days in advance to ensure that all reactive peroxide radicals were depleted. In addition the free radical scavenger, thioglycollic acid, was included in the electro-transfer buffer to reduce the possibility of N-terminal blockage (Legendre *et al.*, 1993).

In the present study, N-terminal sequence analysis was applied to a major IBDV binding protein of approximately 40 kDa, isolated from bursal cell membranes using the IBDV-affinity column described in Section 4.3. As a possible receptor protein, this protein was of interest as a potential target for interfering with virus attachment and/or penetration. Therapies may be developed to combat viral infection by targeting the interaction between the virus and receptor molecules, and stopping the infection process before the virus has entered the host cell (Lentz, 1990).

#### 4.4.1 Materials

Electro-transfer buffer [10 mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS), 10% (v/v) methanol, 0.1 mM thioglycollic acid, pH 11.0]. CAPS (2.213 g) was dissolved in 850 ml dist. H<sub>2</sub>O, methanol (100 ml) and thioglycollic acid ( $\rho = 1.33 \text{ g/cm}^3$ ; 7  $\mu\text{l}$ ) were added. The pH was adjusted to 11.0 with NaOH, and made up to 1 l.

Destain solution [30% (v/v) methanol, 10% (v/v) acetic acid]. Methanol (30 ml) and acetic acid (10 ml) were diluted to 100 ml.

Stain solution [0.1% (m/v) amido black in destain solution]. Amido black (0.1 g) was dissolved in destain solution (100 ml).

#### **4.4.2 Method**

IBDV binding proteins were prepared as described in Section 4.1. Tris-Tricine SDS-PAGE separating gels were prepared as described in Section 2.4 three days in advance, while the stacking gel was prepared one day in advance. Protein sample (approximately 1  $\mu$ g) obtained from a copper stained gel as described in Section 4.2.1 was excised using a clean scalpel blade and placed directly into a well in the stacking gel and electrophoresed at 80 V (maximum current) for 15 min. At this point a small trace of copper chloride present in the gel slice had migrated through the stacking gel, indicating that the protein had eluted from the gel slice and begun its migration through the running gel. The voltage was increased to 100 V for the remainder of the electrophoretic run. Following electrophoretic separation, the protein was electro-transferred (as per Section 2.7.2) to PVDF membranes pretreated in 100% (v/v) methanol (15 s), deionised H<sub>2</sub>O (5 min) and stored in electro-transfer buffer. On completion of electro-transfer (0.2 A, 2 h), the PVDF membrane was washed in deionised H<sub>2</sub>O (5 min) and immersed in stain solution (2 min). The membrane was destained (10 min) and the protein of interest (approximately 40 kDa) excised with a clean sharp scalpel blade and rinsed in deionised H<sub>2</sub>O (2  $\times$  5 min), air dried and transferred to a 1.5 ml microfuge tube for storage at -20°C. N-terminal sequencing was undertaken on an automated Perkin Elmer Applied Biosystems Procise 491 Instrument at the Molecular Biology Unit, University of Natal (Pietermaritzburg), South Africa.

#### **4.5 Detection of the presence of high molecular mass protein complexes in affinity chromatography fractions using native PAGE**

Fractions were analysed by native PAGE to check for the presence of high molecular mass protein complexes in the chromatography peak that eluted with 600 mM NaCl from the IBDV affinity column.

#### 4.5.1 Method

Approximately 10 µg of protein was loaded onto a native PAGE gel (as described in Section 2.4.1.) and electrophoresed at 18 mA per gel with unlimited voltage, until the bromophenol blue had migrated off the gel. Gels were silver stained (as described in Section 2.6). The procedure was repeated with larger amounts of protein (25 µg) and copper stained as described in Section 4.3. The high molecular mass complexes were excised from the gel, destained and subjected to SDS-PAGE by placing the excised band directly into the well of a Tris-Tricine SDS-PAGE gel. The protein in the excised band was electrophoresed at 80 V with maximum current, until the bromophenol blue from the molecular mass markers had migrated into the running gel, following which the voltage was increased to 100 V for the remainder of the electrophoresis run. The gel silver stained as described in Section 2.6.

#### 4.6 VOPBA of high molecular mass protein complexes present in affinity chromatography fractions

To determine if IBDV bound specifically to a band present in the high molecular mass bands shown to be present in the affinity chromatography fraction, the proteins were subjected to PAGE (as described in Section 4.5.1), electro-blotted onto nitro-cellulose, using the method of Matsudaira (1987), and analysed by VOPBA.

##### 4.6.1 Materials

Electro-transfer buffer (As per Section 4.4.1 but without thioglycollic acid).

Phosphate buffered saline-Tween-20, pH 7.4 (PBS-Tween). NaCl (8 g), KCl (0.2 g),  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  (1.15 g),  $\text{KH}_2\text{PO}_4$  (0.2 g) and Tween-20 (500 µl) were dissolved in dist.  $\text{H}_2\text{O}$  and made up to 1 l.

Blocking solution [5% (m/v) non-fat milk in PBS-Tween]. Non-fat milk powder (5 g) was dissolved in 100 ml PBS-Tween.

Incubation solution [1% (m/v) non fat milk in PBS-Tween]. Non-fat milk powder (1 g) was dissolved in 100 ml PBS-Tween.

Alkaline phosphatase-linked secondary antibodies. Alkaline phosphatase linked rabbit anti-chicken IgG antibodies were from Sigma (St. Louis, USA).

Substrate buffer (as described in Section 4.7.2).

BCIP/NBT substrate solution (as described in Section 4.7.2).

#### **4.6.2 Method**

Approximately 10 µg of protein was separated on a native PAGE gradient gel ranging from 5 – 10% acrylamide and electro-blotted onto nitro-cellulose (Matsudaira, 1987), with the exclusion of thioglycollic acid, which was unnecessary. The blots were stained with 1% (m/v) Ponceau S in 1% (v/v) acetic acid, and destained in dist. H<sub>2</sub>O containing a drop of 500 mM NaOH. The nitro-cellulose blots were incubated in blocking solution (1 h) and washed with PBS-Tween (3 × 5 min), incubated with virus particles in incubation solution (2 h, RT) and washed in PBS-Tween (3 × 5 min). The blots were further incubated in primary anti-IBDV chicken serum diluted in incubation solution (2 h, RT), washed with PBS-Tween (3 × 5 min) and incubated with secondary rabbit anti-chicken IgY coupled to alkaline phosphatase diluted in incubation solution (1 h, RT). The blots were finally washed in PBS-Tween (3 × 5 min) and binding detected using BCIP/NBT substrate solution.

#### **4.7 Preparation of endoproteinase Lys-C digests of gel immobilised bursal membrane protein**

For sequence analysis of protein separated on SDS-PAGE gels, a method has been developed whereby the protein can be digested in the polyacrylamide gel by an endoproteinase and the resulting peptides eluted from the gel (Eckerskorn and Lottspeich, 1990). Peptides separated by HPLC can be N-terminally sequenced, as would a whole protein. More information is usually obtained in this way than for N-



terminal sequencing of the whole protein, unless the peptide sequenced is the N-terminal peptide, due to the fact that the endoproteinase cleaves the protein at specific residues (Eckerskorn and Lottspeich, 1989). Thus the residue preceding the N-terminus of the peptide is usually known, i.e. in the case of endoproteinase Lys-C the preceding residue would be lysine. For the current study the 32 kDa protein was subjected to endoproteinase Lys-C digestion for N-terminal analysis of peptide sequences. This section of the study was carried out at the Max Planck Institute for Biochemistry, Martinsreid, Germany.

#### 4.7.1 Materials

Endoproteinase Lys-C buffer [100 mM Tris-HCl, 2 mM EDTA, pH 8.3]. Tris (6.05 g) and EDTA (0.37 g) were dissolved in approximately 450 ml dist. H<sub>2</sub>O and titrated to pH 8.3 with HCl and made up to 500 ml.

10% (v/v) formic acid. Formic acid 10 ml was made up to 100 ml with dist. H<sub>2</sub>O.

Acetonitrile (HPLC grade).

#### 4.7.2 Method

The protein band of interest in the gel slice was washed with two changes of acetonitrile followed by two changes of endoproteinase Lys-C digestion buffer in a new 1.5 ml microfuge tube, with vortexing during the washing process. Approximately 100 µl acetonitrile or endoproteinase Lys-C digestion buffer was used per 0.25 cm<sup>2</sup> of polyacrylamide gel. This process was repeated four times. The gel slice was washed with 3 changes of acetonitrile and suspended in 100 µl of endoproteinase Lys-C digestion buffer. Endoproteinase Lys-C (1 µg) was added and the sample incubated overnight at 37°C. Acetonitrile (100 µl) was added directly to the incubated sample, vortexed and the supernatant transferred to a new 1.5 ml microfuge tube. Formic acid [10% (v/v), 100 µl] was added to the gel slice and vortexed. The alternate washing with acetonitrile and formic acid was repeated 4 to 6 times and the supernatants combined in the new tube. The liquid was removed from

the microfuge tube containing the combined supernatants by rotary evaporation. Formic acid [10 % (v/v), 100  $\mu$ l] was added to the dried sample, which was vortexed and applied to a Jupiter C<sub>18</sub> column (150 mm  $\times$  1 mm, Phenomenex). The peptides were separated by reverse phase HPLC using a 0-60% (v/v) acetonitrile gradient in dist. H<sub>2</sub>O (Eckerskorn and Lottspeich, 1989). Small sharp peaks in the middle of the gradient, corresponding to separated peptides, were collected and spotted onto a glass fibre filter pretreated with 5  $\mu$ l polybrene. The membrane was dried and sequenced using a 492cLc protein sequencer (Applied Biosystems, Perkin Elmer, Langen).

#### **4.8 Construction of primers from the *Gallus gallus* codon usage table**

The sequence data obtained from the internal peptides resulting from endoproteinase Lys-C digestion allowed the construction of primers for cDNA synthesis and consequent polymerase chain reaction amplification of a segment of the cDNA sequence of the protein. The codon usage tables for many organisms can be obtained from the internet and they are represented as the accumulated codon usage as a percent of the total found for identified sequences of *Gallus gallus* (Table 4.1). This information is thus a useful source for the construction of primers for cDNA synthesis from the RNA of host cells.

#### **4.9 Purification of total RNA**

Total RNA was purified from frozen bursal material stored at -80°C using the RNeasy (Qiagen, Hilden, Germany) purification kit. The kit includes ready-made buffers and mini-spin columns for simple extraction of RNA from a number of sources. Extraction of total RNA is achieved by selective adsorption of RNA to a silica-gel-based membrane. A specialised high salt buffer system allows up to 100  $\mu$ g of RNA longer than 200 bases to bind to the RNeasy silica-gel membrane. Tissue is lysed and homogenised in the presence of a highly denaturing guanidine isothiocyanate (GITC)-containing buffer, which immediately inactivates RNases to ensure isolation of intact RNA. Ethanol is applied to provide the appropriate binding conditions, and the sample is applied to an RNeasy spin column where total RNA binds to the membrane and contaminants are efficiently washed away. High quality RNA can be eluted with

as little as 30  $\mu$ l of water. The RNeasy mini procedure isolates RNA molecules longer than 200 nucleotides. Small RNAs will not bind quantitatively under the conditions used. The RNeasy procedure thus enriches for longer RNA molecules.

**Table 4.1** Distribution of codon usage in *Gallus gallus*. Figures are represented as a percentage occurrence for each particular amino acid. Obtained from <http://www.dna.affrc.go.jp/~nakamura/codon.html>

Phe	UUU	58.6	Ser	UCU	16.8	Tyr	UAU	36.4	Cys	UGU	36.9
	UUC	41.4		UCC	21.9		UAC	63.3		UGC	63.1
Leu	UUA	6.3		UCA	13.7	Stp	UAA	33.0	Stp	UGA	46.0
	UUG	12.8		UCG	7.3	Stp	UAG	21.0	Trp	UGG	100
Leu	CUU	12.1	Pro	CCU	25.2	His	CAU	36.5	Arg	CGU	10.2
	CUC	18.7		CCC	33.6		CAC	63.5		CGC	22.0
	CUA	6.0		CCA	26.6	Gln	CAA	26.0		CGA	9.1
	CUG	44.1		CCG	14.6		CAG	74.0		CGG	17.6
Ile	AUU	33.5	Thr	ACU	23.3	Asn	AAU	39.6	Ser	AGU	12.8
	AUC	50.6		ACC	33.7		AAC	60.4		AGC	27.5
	AUA	16.0		ACA	28.5	Lys	AAA	41.4	Arg	AGA	20.5
Met	AUG	100		ACG	14.5		AAG	58.6		AGG	20.5
Val	GUU	19.3	Ala	GCU	28.4	Asp	GAU	48.0	Gly	GGU	16.6
	GUC	23.1		GCC	34.6		GAC	52.0		GGC	32.5
	GUA	11.4		GCA	24.3	Glu	GAA	40.7		GGA	25.6
	GUG	46.1		GCG	12.7		GAG	59.3		GGG	25.2

#### 4.9.1 Materials

Ethanol [70% (v/v)]. Absolute ethanol (700  $\mu$ l) was made up to 1 ml with sterile deionised H<sub>2</sub>O in a sterile 1.5 ml microfuge tube.

Buffer RLT (lysing buffer) – provided in RNeasy kit (Qiagen, Hilden, Germany).

Buffer RW1 (washing buffer) - provided in RNeasy kit (Qiagen, Hilden, Germany).

Buffer RPE (elution buffer) - provided in RNeasy kit (Qiagen, Hilden, Germany).

#### 4.9.2 Method

Frozen bursal material (approximately 30 mg) stored at  $-80^{\circ}\text{C}$  was weighed into a chilled sterile 1.5 ml microfuge tube. To 1 ml buffer RLT, 10  $\mu$ l of 2-mercaptoethanol was added. The tissue was simultaneously disrupted and homogenised in 600  $\mu$ l of buffer RLT containing mercaptoethanol, using a rotor stator homogeniser. The lysate was centrifuged in a microfuge (12 000 rpm, 3 min, RT) and the supernatant transferred to a new 1.5 ml microfuge tube. 70% (v/v) ethanol (600  $\mu$ l) was added to the cleared lysate and mixed well by pipetting. A sample (700  $\mu$ l) of the mixture was applied to an RNeasy mini spin column placed in a 2 ml collection tube. The column was centrifuged in a microfuge (10 000 rpm, 15 sec, RT), the flow through discarded and the remainder of the sample added to the column and centrifuged (10 000 rpm, 15 sec, RT). The flow through was again discarded and 700  $\mu$ l buffer RW1 pipetted onto the RNeasy column and centrifuged (10 000 rpm, 15 sec, RT). The flow through and collection tube were discarded and the column transferred to a new 2 ml collection tube. Buffer RPE, to which ethanol had previously been added (500  $\mu$ l), was pipetted onto the column and centrifuged (10 000 rpm, 15 sec, RT). The flow through was discarded and a further 500  $\mu$ l buffer RPE was pipetted onto the column and centrifuged (12 000 rpm, 2 min, RT). The column was transferred to a 1.5 ml collection tube and 30-50  $\mu$ l of RNase free water pipetted directly onto the membrane. The column was centrifuged (10 000 rpm,

1 min, RT) and the process repeated if the expected yield of RNA was higher than 30 µg.

#### **4.10 Purification of DNA from chicken bursal material**

DNA was purified from chicken bursal material using the procedure of Douglas *et al.* (1992), with minor modifications. The modifications involved the dispersion of the bursal tissue into a particulate state more suitable for the rest of the procedure.

##### **4.10.1 Materials**

TEN buffer [10 mM Tris-HCl, 2 mM EDTA, 400 mM NaCl, pH 8.2]. Tris (1.21 g), EDTA (0.74 g) and NaCl (23.37 g) were dissolved in approximately 900 ml of dist. H<sub>2</sub>O, titrated to pH 8.2 with NaOH and made up to 1 l.

TE buffer [10 mM Tris-HCl, 1 mM EDTA, pH 8.0]. Tris 1.21 (1 g) and EDTA (0.37 g) were dissolved in approximately 900 ml dist. H<sub>2</sub>O, titrated to pH 8.0 with HCl and made up to 1 l.

10% (m/v) SDS. SDS (1 g) was dissolved in 10 ml dist. H<sub>2</sub>O.

7.5 M ammonium acetate buffer, pH 7.5. Ammonium acetate (57.81 g) was dissolved in 90 ml dist. H<sub>2</sub>O, titrated to pH 7.5 with HCl and made up to 100 ml.

Saturated NaCl. NaCl was dissolved in boiling dist. H<sub>2</sub>O until the solution was saturated.

##### **4.10.2 Method**

Bursal material (approximately 30 mg) was scraped from a frozen whole bursa with a new scalpel blade and placed in a 1.5 ml microfuge tube. TEN buffer (1 ml) was placed in the tube and the scrapings triturated through a 20-gauge needle using a sterile syringe. The dispersed tissue was allowed to settle and the supernatant

transferred to a new 1.5 ml microfuge tube and centrifuged ( $2\,000 \times g$ , 10 min, RT). Following this, the procedure of Douglas *et al.* (1992), was followed. The pellet was vortexed in 900  $\mu$ l of TEN buffer, followed by the addition of 100  $\mu$ l of 10% (m/v) SDS and incubated overnight at 55°C in a shaking waterbath. Saturated NaCl (250  $\mu$ l) was added to the sample and the tube shaken vigorously. The protein precipitate was removed by centrifugation ( $2\,000 \times g$ , 10 min, RT). Two volumes of 99.5% (v/v) ethanol were added to the supernatant, the sample stored at -20°C for 1 h, centrifuged ( $12\,000 \times g$ , 10 min, 4°C) and the pellet washed once with 70% (v/v) ethanol. The pellet was redissolved in TE buffer (100  $\mu$ l) at 55°C for 2 h in a shaking water bath. The DNA was reprecipitated with 0.5 vol of 7.5 M ammonium acetate, pH 7.5, and two volumes of 99.5% (v/v) ethanol and centrifuged ( $12\,000 \times g$ , 10 min, 4°C). The pellet was washed with 70% (v/v) ethanol and dissolved in TE buffer at 55°C (2 h).

#### **4.11 Reverse transcription polymerase chain reaction (RT-PCR) and direct PCR from chicken genomic DNA**

##### **4.11.1 Materials**

As described in Section 2.8.2.1

Primer 1 (5' GTA (TG)CC CAC (AG)GC (TG)GT GAT 3' - reverse primer).

Primer 2 (5' AAC CTG GC(CT) GA(CT) TT(CT) ATG 3' - forward primer).

##### **4.11.2 Method**

As described in Section 2.8.2.2

Amplification was carried out, using the times and temperatures described in Table 4.2, in a Perkin Elmer GeneAmp 2400 thermocycler. PCR products were analysed on a 1% (m/v) agarose gel for amplification using genomic DNA as a template and on a 1.8% (m/v) gel for the analysis of products from the RT-PCR reaction (as described in

Section 2.8.1). For examination of the presence of tertiary or quaternary structure in RT-PCR products, products were heated to 95°C for five min and snap-cooled in a -20°C ice bath containing 80% crushed ice and 20% NaCl by volume and examined by agarose gel electrophoresis.

**Table 4.2 Times and temperatures for denaturation, annealing and polymerisation cycles in PCR and RT-PCR using 8-fold degenerate primers for amplification from chicken DNA and RNA.**

Cycle	Denaturation (at 94°C)	Annealing (at 50°C)	Polymerisation (at 72°C)
First Cycle	5 min	1 min	2 min
Subsequent cycles	30 s	1 min	2 min
Last cycle	30 s	1 min	2 min

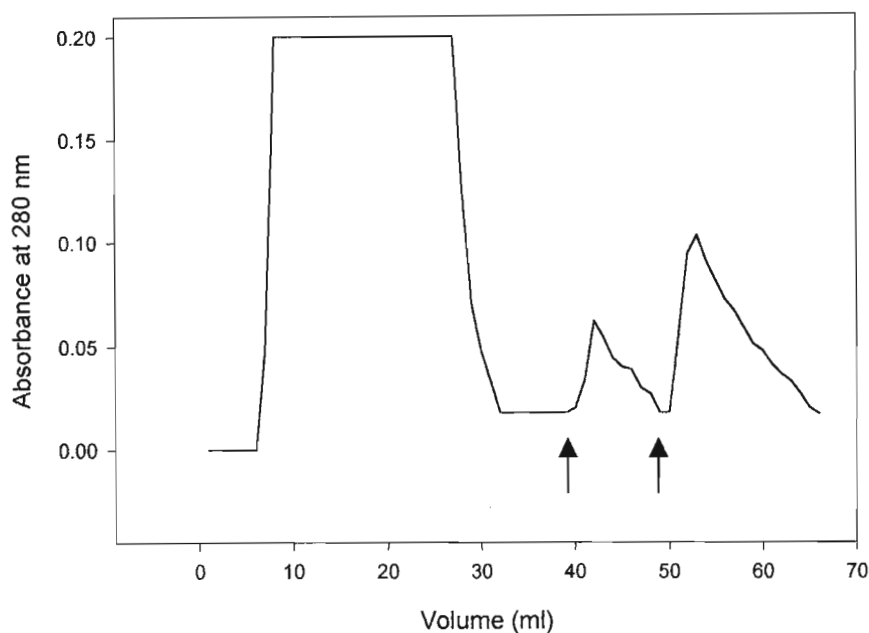
#### 4.12 Western blot analysis of bursal membrane fractions

Proteins in the membrane fraction of bursal cells were examined for an immune reaction with human rheumatoid arthritis sera to see if any reaction between autoantibodies present in some rheumatoid arthritis sera would react with chicken proteins homologous to human proteins involved in rheumatoid arthritis. Approximately 10 µg of protein was separated by reducing SDS-PAGE (Section 2.4) and electro-blotted onto nitro-cellulose. Western blotting of membrane fractions was performed according to Section 2.7.3. Blots were incubated in a 1/100 dilution of sera from 3 patients with rheumatoid arthritis (obtained from Professor R. Mody of University of Natal, Durban, Medical school) in blocking solution. Binding of primary antibodies was detected using a 1/13 000 dilution of rabbit anti-human IgG alkaline phosphatase detection antibody in blocking solution.

### 4.13 Results and discussion

#### 4.13.1 Affinity chromatography of bursal cell membranes on a IBDV-Sephacrose 4B column

The efficiency of coupling of IBDV to Sepharose 4B was calculated to be approximately 90% although this may be a slight overestimation, as excess virus may also have settled with the gel when the excess coupling buffer was removed from the settled gel. The elution profile of a typical chromatography run, with the IBDV-Sephacrose 4B column (Fig. 4.1) shows small peaks eluted with 150 mM and 600 mM NaCl.



**Fig 4.1** Elution profile of IBDV binding proteins from an IBDV-Sephacrose 4B affinity column.

Column: 6 cm × 0.5 cm i.d. (bed volume 4.7 ml); buffer, 10 mM Tris-HCl, 50 mM NaCl, 0.1% (m/v) Na-deoxycholate, pH 8.0. Arrows indicate the stepwise increase of NaCl concentration from 50 to 150 mM and from 150 to 600 mM.

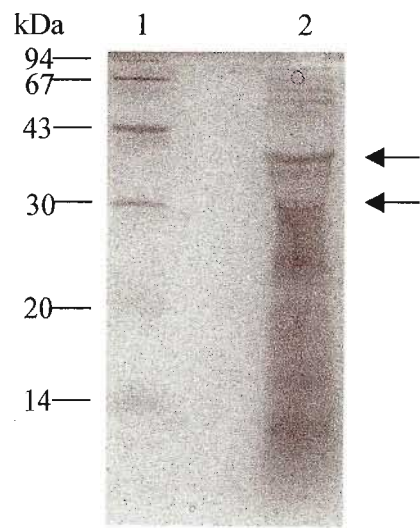
A column of unmodified Sepharose 4B, of the same dimensions, as the IBDV-Sephacrose 4B affinity column, was prepared as a control in case non-specific binding of protein to the Sepharose 4B occurred. Only a very small amount of protein bound to the column and eluted with the 150 mM NaCl but no protein was eluted with 600



mM NaCl, suggesting that the protein that elutes with 600 mM NaCl from the IBDV-affinity column specifically bound to IBDV.

**4.13.2 Tris-Tricine SDS-PAGE analysis of IBDV-affinity chromatography fractions**

The peak obtained from the IBDV-affinity column by elution with 600 mM NaCl was examined by Tris-Tricine SDS-PAGE (Section 2.4). Protein eluted with 150 mM NaCl was discarded as such weak binding probably represents non-specific binding of protein to the immobilised virus and/or the Sepharose 4B matrix. Tris-Tricine SDS-PAGE analysis (Section 2.4) of the protein eluted with 600 mM NaCl from the IBDV-4B affinity column showed a protein banding pattern in which a number of IBDV binding proteins could be distinguished (Fig. 4.2). The major proteins binding to the affinity matrix, appeared to be a protein with an approximate molecular mass of 40 kDa, as well as a pair of proteins of approximately 31-33 kDa. The 40 kDa protein was subjected to N-terminal sequencing, while the 32 kDa protein was digested by endoproteinase Lys-C and the resulting peptides sequenced.



**Fig. 4.2 Tris-Tricine SDS-PAGE analysis of fraction eluted from IBDV-affinity column with 600 mM NaCl.** Lane 1, molecular mass markers (as per Fig 3.2); lane 2, Affinity chromatography fraction. Arrows indicate major protein species at approximately 40 kDa and 32 kDa.

#### 4.13.3 N-terminal sequencing of a 40 kDa IBDV binding protein

Due to the relatively high concentration of the 40 kDa protein in the fraction of IBDV binding proteins eluted with 600 mM NaCl, it was decided that the protein band would be sufficient for N-terminal sequencing. The molecular mass of the protein also corresponds to that of one of the proteins suggested by Nieper and Müller (1996) to be a receptor for IBDV, and thus represents a good choice for N-terminal sequencing and identifying as a possible receptor. The 40 kDa protein was thus excised from a copper stained gel (Section 4.3), re-electrophoresed (Section 2.4) and electro-blotted onto PVDF using the method of Matsudaira *et al* (1987) (Section 4.4). The N-terminal sequence obtained (Section 4.4) is shown in Fig. 4.3. When compared to sequence databases, a 100 % homology was found with a *Homo sapiens* protein, involved in T-cell stimulation in rheumatoid arthritis, called synovial stimulatory protein (Hain *et al.*, 1996; Bläss *et al.*, 1999). Synovial stimulatory protein is suspected to be a trimer of three 70 kDa fragments, totalling 205 kDa. The N-terminal sequence corresponds to the N-terminal sequence of one of the tryptic peptides of the synovial stimulatory protein, which also has a  $M_r$  of 40 kDa. Thus the protein present in the affinity chromatography fractions may represent a cleavage product formed by endogenous proteases co-purifying with the membranes from bursal cells.

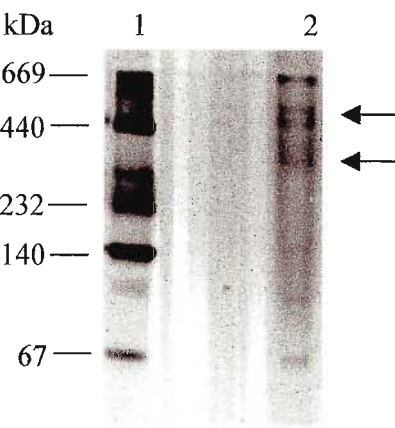
It is interesting to note that the synovial stimulatory protein is involved in T-cell activation as this may have some relevance in the progression of IBD. Binding of the virus to such a protein may either prevent or stimulate T-cell activation and thus have significant implications in the progression of the disease. Since the virus is known to primarily infect actively dividing B-lymphocytes, the protein may also be a B-cell homologue that is involved in B-cell activation and thus would be present in actively dividing B-cells. However, further information regarding the protein is needed before one can establish whether it is a possible receptor. The full sequence of the protein would help to establish if it is actually a homologue of the synovial stimulatory protein. How the protein is associated with the bursal membrane and whether it extends towards the extracellular space or the cytosol would also help to confirm the possibility of a receptor molecule.

- a) D-I-N-G-G-G-A-T-L-P
- b) D-I-N-G-G-G-A-T-L-P-X-P

**Fig. 4.3 Homology between the N-terminally sequenced 40 kDa chicken bursal membrane protein and human synovial stimulatory protein.** a) Sequence of N-terminal ten amino acid residues of the 40 kDa protein from IBDV-affinity chromatography and b) a 40 kDa tryptic peptide obtained from the human synovial stimulatory protein (Hain *et al*, 1996). X represents residues for which no information is available.

**4.13.4 Demonstration of protein complexes in IBDV-affinity chromatography fractions using native PAGE**

Several high molecular mass proteins that are probably protein complexes of smaller proteins appear to be present in the sample eluted from the IBDV-affinity column (Fig. 4.4). There are two high molecular mass proteins of about 440 kDa and one slightly smaller complex of about 350 kDa.

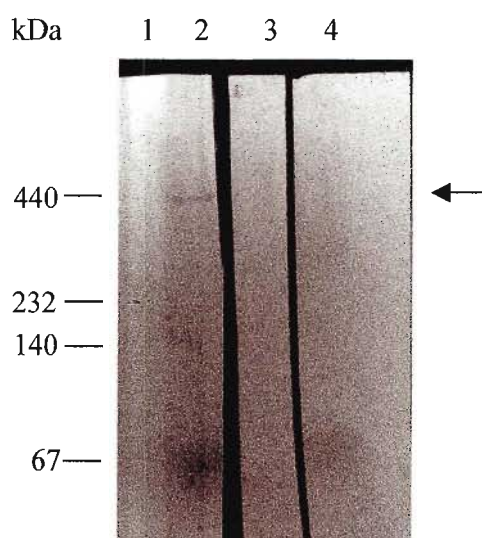


**Fig. 4.4 Demonstration of high molecular mass proteins in the fraction eluted with 600 mM NaCl from IBDV-affinity column.** Lane 1, molecular mass markers, thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase 232 kDa; lactate dehydrogenase, 140 kDa and albumin, 67 kDa; lane 2, affinity chromatography fraction (~10 µg). Arrows indicate the high molecular mass proteins complexes of 440 and ~350 kDa

#### 4.13.5 VOPBA of high molecular mass proteins present in IBDV-affinity chromatography fraction

The high molecular mass proteins present in the IBDV-affinity chromatography fraction were electro-blotted onto nitro-cellulose after native PAGE using CAPS buffer, which does not contain SDS and potentially maintains the complex in a stable state during the blotting procedure. The electro-blotted complexes were tested for virus binding using VOPBA.

The VOPBA (Fig. 4.5) of the protein complex present in the bursal membrane sample shows an apparent interaction between the virus and a high molecular mass protein of approximately 440 kDa (lane 2).

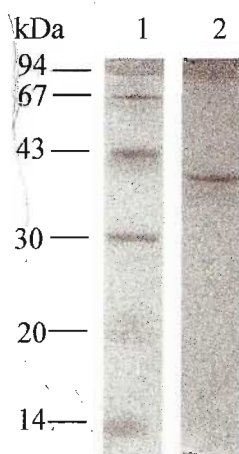


**Fig. 4.5 Virus overlay protein blot assay of protein complexes present in IBDV-affinity chromatography fraction.** Lane 1, molecular mass markers (as in Fig. 4.4), lane 2, virus overlay blot with 1/100 dilution of virus and primary anti-IBDV antibody, lane 3, no primary antibody control, lane 4, no virus control. Arrow indicates reactive band at approximately 440 kDa (lane 2).

No interaction was seen between this complex and the primary and secondary antibodies where either primary anti-IBDV antibody (Fig. 4.5 lane 3) or virus (fig. 4.5 lane 4) was omitted. This is in contrast to the VOPBA performed with reduced,

denatured protein separated by reducing SDS-PAGE, which showed no specific interaction between the virus and membrane proteins (Fig. 3.7, lane 1).

An immune reaction can be seen in the region of 67 kDa in the lanes where virus was omitted (Fig. 4.5, lane 4) and in the lane where nothing was omitted (Fig. 4.5, lane 2), however, this reaction is not seen in the lane where primary anti-IBDV antibody was omitted (Fig. 4.5, lane 3). The reaction seen in the lanes 2 and 4 is therefore presumably an immune reaction between the primary antibody and a protein at 67 kDa. The interaction between virus and the 440 kDa protein complex was also visible at higher dilutions of virus and primary antibody but these interactions were not clearly visible after photography. Thus conformation appears to be of importance in the interaction between the virus and possible receptors. The virus binding protein complex of 440 kDa appears to be composed primarily of a 40 kDa protein when excised from a copper stained gel and examined by Tris-Tricine SDS-PAGE (Fig.4.6).



**Fig. 4.6 SDS-PAGE analysis of the 440 kDa virus binding complex demonstrated by VOPBA.**

Lane 1, molecular mass markers (as per Fig 3.2); lane 2, reduced denatured virus binding complex at approximately 40 kDa.

#### **4.13.6 Internal sequencing of endoproteinase Lys-C digestion products of a 32 kDa IBDV binding protein**

As the 40 kDa band present in the IBDV binding affinity chromatography fraction was not the only major band present (Section 4.13.2), it was decided that the 32 kDa band should also be sequenced. The 32 kDa band was therefore excised from a

Coomassie stained reducing SDS-PAGE gel and subjected to endoproteinase Lys-C digestion. The peptides produced were N-terminally sequenced. A surprisingly high degree of sequence homology was found with the root adhesin from *Pseudomonas fluorescens* (De Mot *et al.*, 1992). Three peptides produced by endoproteinase Lys-C digestion were sequenced and their homology to the bacterial protein is shown in Fig 4.7.

The bacterial protein from *P. fluorescens* also shows homology to another protein from *P. aeruginosa*. The *P. aeruginosa* protein is of the porin class of proteins involved in membrane ion transport (De Mot *et al.*, 1992). This is interesting, as it has been noted that the binding of IBDV to chicken embryo fibroblast membranes changes the potassium current properties of the infected cell (Repp *et al.*, 1998). Thus binding of IBDV to the 32 kDa protein may be the cause of this effect. Although it was thought to be highly unusual to find such a high degree of sequence homology between proteins from such evolutionary distant species, the same was true for the 40 kDa chicken bursal protein which shows 100% homology to a human protein. It was therefore decided to determine if a section of the DNA sequence of the 32 kDa protein could be amplified from chicken RNA using RT-PCR or from chicken DNA using PCR through the construction of degenerate primers from the codon usage tables for the *Gallus gallus* species.

```

      5      10      15      20      25      30      35      40      45      50
                                KXIEDG
MKLKNTLGFAIGSIIAATSGATAQGGAVEGELFYKKQYNDSVKHIEDG
YNPGASIGYF
FNPGARIGYFLTDDLNLNSYDKTNHTRSNDGTGSQKIGGDTSSLTAQYH

FGQAGVDSLRPYVEGGFGHQSRGNVKADGHSGRDQSTLAIAGAGVKYYFT

NNVYARAGVEADYALDNGKWDYSALVGLGVNFGGNAGAAAPAPTAPAPE
                                NLADFMAQYPATN
PTPEPEPVAQVVRVELDVKFDFDKSVVKPNSYGDVKNLADFMAQYPATNV
                                XXXXXXRITAVG
EVAGHTSIGPDAYNQKLSQRRADRVKQVLVKDGVAPSRITAVGYGESRPV

ADNATEAGRAVNRRVEASVEAQAQ

```

**Fig 4.7** Sequence of the root adhesin from *Pseudomonas fluorescens* and internal peptide sequences of the 32 kDa chicken bursal membrane protein. Amino acid numbering is for that of the root adhesin (De Mot *et al.*, 1992). Sequences in bold are those obtained from digestion of the 32 kDa bursal protein. X, represents residues for which no information is available, underlined sequences represent mismatches.

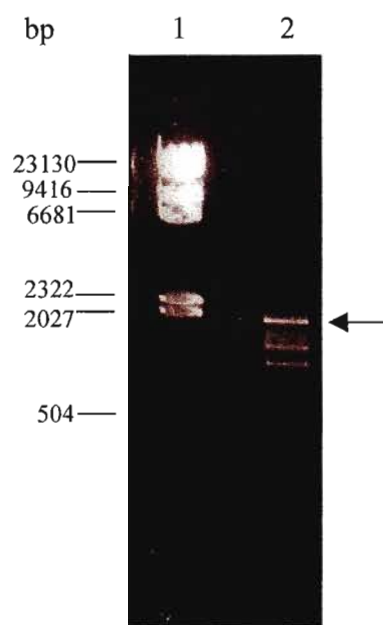
#### 4.13.7 Construction of degenerate primers followed by RT-PCR and PCR from genomic chicken DNA

Degenerate primers of 18 bases were constructed using Table 4.1. These primers are shown in Fig. 4.8. The forward primer corresponds to the N-terminal six amino acid residues from the peptide sequence homologous to amino acid residues 237-249 of the *P. fluorescens* root adhesin. The reverse primer corresponds to 5 of the 6 residues homologous to amino acid residues 289-293 of the *P. fluorescens* root adhesin. Due to the fact that the codon usage for arginine residues (residue 288) is very degenerate, and because of the high degree of homology found elsewhere in the protein, it was decided that residue 294 (Y) would be used for the 6<sup>th</sup> amino acid to construct the reverse primer. From this it can be seen that the expected amplification product should be 177 bp in length, corresponding to 59 amino acid residues.

Forward primer 5' AAC CTG GC(CT) GA(CT) TT(CT) ATG 3'  
Reverse primer 5' GTA (TG)CC CAC (AG)GC (TG)GT GAT 3'

**Fig 4.8 Eight-fold degenerate primers constructed from the peptide sequences obtained from endoproteinase Lys-C digestion of the 33 kDa bursal protein.** Degenerate residues are shown in brackets.

Amplification was observed using these primers for both amplification from chicken DNA and RNA by RT-PCR. The product did not correspond exactly with the bp size that would be expected if the *P. fluorescens* sequence was amplified. For the amplification from DNA, several products were seen with the major band appearing at 2 000 bp (Fig. 4.9).

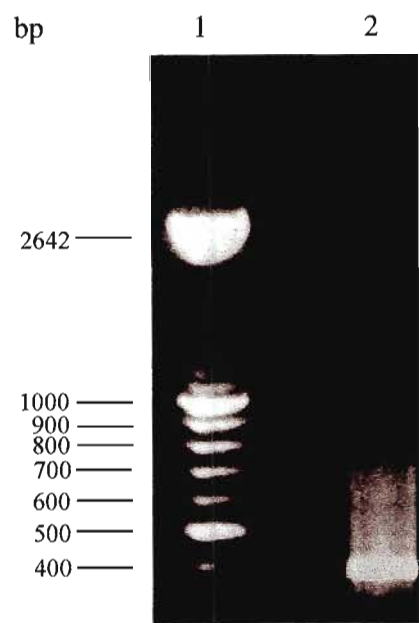


**Fig. 4.9 Agarose gel showing products from PCR of chicken genomic DNA using 8-fold degenerate primers.** Lane 1, molecular mass markers (*Hind*III cut lambda DNA); lane 2, PCR product. The major band is at approximately 2 000 bp (arrow).

It is possible that the amplification from DNA could encompass the amplification of an intron, which would increase the size of the expected reaction product considerably (Sambrook *et al.*, 1989). The amplification from RNA should result in the expected product of 177 bp but only if the RNA had been fully processed. Unfortunately since the RNA used was total RNA, this could include both unprocessed and processed

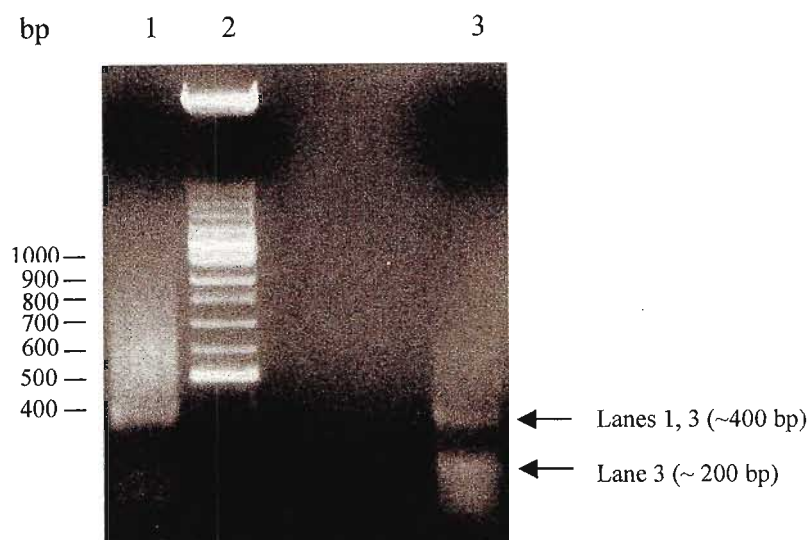


RNA. This may account for the unexpected size of the fragment generated during RT-PCR. The amplified fragment (Fig 4.10) appears at a size of approximately 400 bp.



**Fig. 4.10 PCR products from the RT-PCR of total chicken RNA using 8-fold degenerate primers.** Lane 1, molecular mass markers (100 bp ladder); Lane 2, RT-PCR reaction product.

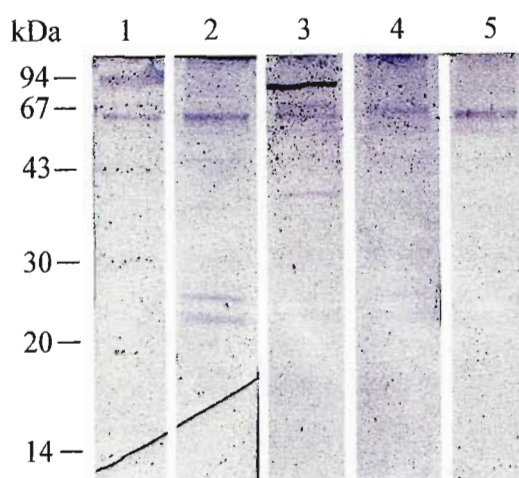
Later examination of the RT-PCR products with heating to 95°C and snap cooling before loading onto a 1.8% (m/v) agarose gel yielded a slightly different picture (Fig. 4.11), with the product dissociating into two bands (lane 3), one of the expected size (~ 200 bp) and one approximately double the size (~ 400 bp; lanes 1,3). This could possibly suggest dimer formation between two copies of the amplified DNA. This would require annealing between the ends of each copy i.e. a region where base pairing between the two ends can occur. No such region appears between the primers so annealing would have to occur in the region that exists between the primer sequences.



**Fig. 4.11 Examination of PCR products from the RT-PCR of total chicken RNA using 8 fold degenerate primers following heating and snap cooling.** Lane 1, PCR reaction product before snap cooling; lane 2, 100 bp ladder; lane 3, PCR reaction product after snap cooling.

#### **4.13.8 Immune reactions between human rheumatoid arthritis sera and chicken bursal membrane proteins**

It was shown previously that synovial stimulatory protein is an auto-antigen in humans and that antibodies to synovial stimulatory protein are present in some rheumatoid arthritis patients' sera (Hain *et al.*, 1996). Due to the high homology found between the major 40 kDa band observed in affinity chromatography fractions of chicken bursal membranes and human synovial stimulatory protein, it was hypothesised that further characterisation of the 40 kDa chicken protein might be possible through Western blot analysis of membrane proteins with sera from patients with rheumatoid arthritis. In this regard membrane fractions of bursal cells were tested to determine if any membrane proteins reacted with rheumatoid sera from human patients. Several rheumatoid arthritis patients' sera were tested using standard Western blotting techniques (Section 2.7.3; Fig 4.12).



**Fig. 4.12 Western blot analysis of chicken bursal membrane proteins with human rheumatoid sera.** Lane 1 molecular mass markers (as per Fig. 3.2); lanes 2 - 5 approximately 10  $\mu$ g of membrane protein was separated by SDS-PAGE and electro-blotted onto nitro-cellulose; lanes 2, 3, 5, bursal membrane proteins reactive to sera from patients with rheumatoid arthritis were detected using a 1/100 dilution of rheumatoid arthritis sera from 3 patients; lane 4, 1/100 dilution of non rheumatoid arthritis sera control. Primary antibody binding was detected by a 1/13 000 dilution of rabbit anti-human IgG alkaline phosphatase labelled antibody. Binding of enzyme labelled antibody was detected using BCIP/NBT substrate (Section 2.7.3.1).

Quite surprisingly several reactive bands (Fig. 4.12) were seen when compared to a non-rheumatoid arthritis serum control (lane 4). The strength of reaction and molecular mass of the reactive bands seen varied between patients. However, a similar pattern was observed between 3 patients with bands in the region 24-30 kDa (lanes 2, 3 and 5) and the non rheumatoid arthritis serum control (lane 4). A very reactive band was seen at a molecular mass of about 90 kDa (lane 3), although it is difficult to determine the molecular mass of higher  $M_r$  proteins separated using a 10% Tris-Tricine SDS-PAGE gel. This lane also contains a band at approximately 40 kDa, which the others do not. The serum used in lane 3 possibly contains autoantibodies to a synovial stimulatory protein homologue, which corresponds to a reaction with a 40 kDa breakdown product. The reaction with the non-immune control serum (lane 4) shows little specific reaction when compared to other lanes.

#### 4.14 Conclusions

The construction of an IBDV-affinity column and its application to separate IBDV-binding proteins from bursal cell membrane proteins seems to be a useful approach. Although several proteins were separated using this method, the presence of prominent proteins within the affinity-purified fraction indicates that these proteins are probably of interest. The separated proteins are not only possible receptors but may also be involved in other stages of viral infection. The 40 kDa synovial stimulatory protein homologue found within affinity-chromatography fractions is of particular interest due to the involvement of the human protein in activation of T-cells.

Binding of the virus to the high molecular mass bursal protein complex, which seems to consist primarily of a 40 kDa protein, suggests an interaction between the virus and the 40 kDa protein. This interaction seems to require non-denaturing conditions, highlighting the importance of such variables in virus protein interactions. Although inconclusive, the possibility of an immune reaction between human autoantibodies to synovial stimulatory protein and chicken bursal cell membrane proteins may also indicate further homology between the 40 kDa chicken protein and human synovial stimulatory protein. Binding of IBDV to this protein may be of great relevance to the progression of the disease but further sequence information needs to be obtained for this protein before any conclusions about its role in chickens may be made.

The sequence information obtained for the 32 kDa protein is also of interest because of the possibility of its involvement as a porin in bursal membranes. This is due to the fact that IBDV has been shown to cause changes in the potassium current properties of infected cells, and binding of IBDV to the 32 kDa protein could be linked to this. PCR and RT-PCR of a section of the sequence of the 32 kDa protein seems to show that such sequences are present within the chicken genome, lessening doubts as to whether a bacterial contaminant may have been present in the affinity chromatography fractions. These results are put into context of the current knowledge of IBDV receptors and other aspects of the disease in the following chapter.

## CHAPTER 5

### GENERAL DISCUSSION

Infectious bursal disease virus is a widespread chicken pathogen that infects the bursa of Fabricius. In so doing the virus causes damage to the chicken's bursa of Fabricius and this leads to immuno-suppression and susceptibility to other diseases. Vaccines (usually a mixture of live attenuated and oil inactivated virus) are available but these are not 100% effective and outbreaks of the disease occur regularly. Very little is known about the virus life cycle and the receptor for IBDV on bursal cell membranes. Treatment strategies or vaccines could be generated from information about the receptor. Treatment based on blocking the interaction between virus and receptor molecules could be envisioned. These treatments could possibly take the form of a peptide mimic of epitopes from the receptor or the viral attachment protein. More effective recombinant or peptide vaccines could also be generated from the information regarding the epitopes from the virus attachment or receptor proteins.

The aim of this study was to determine if possible receptors for IBDV could be identified on host cell membranes from the bursa of Fabricius. Several methods for the identification of receptors were examined and affinity chromatography emerged as the most successful. Virus overlay protein blot assay analysis of virus binding proteins did not successfully identify possible receptors when using reduced and denatured membrane protein samples (Chapter 3). Similarly reversibly cross-linking the virus to possible receptors did not offer much insight into possible receptors due to the confusion in molecular mass between viral and non-viral proteins (Chapter 3). However, using an IBDV-affinity column allowed the separation of a number of IBDV binding proteins from bursal membranes (Chapter 4).

Two of these binding proteins, of 40 and 32 kDa, were chosen as possible receptors due to the amount of protein present in the sample in comparison to the other proteins isolated, which were present in much lower concentrations. The two proteins were further examined by analysis of their amino acid sequences. The information that is presented here about these two proteins represents an important step towards the

elucidation of a possible viral receptor for IBDV, although much follow-up work is still required. However, it does offer some clues as to what the receptor may be, especially in conjunction with what other researchers have previously found in this context. It also offers avenues towards work that can be done in attempting to identify further receptors for the virus.

The peptide sequences of the proteins determined in Chapter 4, except that homologous to synovial stimulatory protein (Hain *et al.*, 1996), show high homology to bacterial proteins. Although it may seem unlikely that these would be present in the cells of the bursa of Fabricius, one must take into account that there are many unidentified genes that are in the chicken genome and the proteins identified may correspond to *Gallus gallus* genes as opposed to those of bacterial proteins. The presence of a protein that is homologous to the human synovial stimulatory protein also seems to indicate the diversity of proteins present within the chicken genome, giving further support to the idea that the proteins may be of chicken origin. Another factor to consider is that amplification was seen in both PCR and RT-PCR (Chapter 4) with primers constructed using the *Gallus gallus* codon usage tables (<http://www.dna.affrc.go.jp/~nakamura/codon.html>). The difference in sizes between the PCR products obtained from PCR and RT-PCR may also suggest the possibility of the presence of an intron, which would only be present in eukaryotic DNA (Sambrook *et al.*, 1989) and this would mean the proteins sequenced are most likely of chicken origin.

Another question that is posed by the sequence information is the nature of the bacterium to which the proteins are homologous. The bacterium in question is from *Pseudomonas fluorescens* (De Mot *et al.*, 1992), which is a plant root bacterium. It is doubtful whether a population of such a bacteria would be present within the bursa of Fabricius. However, the possibility does exist that a bacterial population exists within the bursa that is sufficiently large to allow extraction of bacterial membrane proteins from bursal tissue. The number of bacterial cells would therefore have to be quite high as the number of chicken cells in a single bursa would far outweigh any minor contaminant bacterial population and bacterial proteins would therefore be present in undetectable amounts when compared to chicken proteins. It is highly unlikely that bacterial proteins would have originated from chromatography buffers, as these

contained 0.02 % (m/v) sodium azide. If such a bacterial population does exist within the bursa, this may also be of interest as the presence of the bacteria may influence the health of a population of chickens. The fact that the bacterial proteins bind to IBDV would also be of interest as the proteins identified may have binding regions that are similar to the actual receptor.

Some of the routes that may be followed using the information generated in Chapter 4 include the generation of anti-peptide antibodies to peptides from the determined protein sequences. These anti-peptide antibodies may then be tested as vaccines in protection of chickens against IBDV infection. Monoclonal antibodies could also be generated and tested in a cell culture system to test for blocking of IBDV binding. Another route that could be taken is to establish whether transient expression of one of the proteins identified in a cell culture system allows for infection by IBDV. This would be a more conclusive step in identifying the protein of interest as a receptor.

The immune reaction seen between bursal membrane proteins and sera from patients with rheumatoid arthritis (Hain *et al.*, 1996) is also of interest. While initially performed to determine if further homology existed between the 40 kDa affinity purified protein and human synovial stimulatory protein, the reaction seen could also be useful in diagnosing rheumatoid arthritis in human patients. If further patients are tested and a clear distinction can be made between patients with rheumatoid arthritis and those without, the immune reaction could be adapted to a diagnostic test. This would allow early treatment of rheumatoid arthritis and could give insight to the causes of the disease by looking at the specific proteins that react with the sera from rheumatoid arthritis patients.

Since the virus appears capable of infecting a number of different cell types within the bursa and also lymphocytes in other areas of the chicken such as the thymus and spleen (Cheville, 1967), it may be that there is a general receptor for the virus but specific events post infection are required for the growth of the virus within the different cell types. The virus possibly elicits some type of stimulation response in the bursa causing cells to actively divide, a condition which appears to be required for virus growth (Müller, 1986; Burkhardt and Müller, 1987), and thus allowing the virus

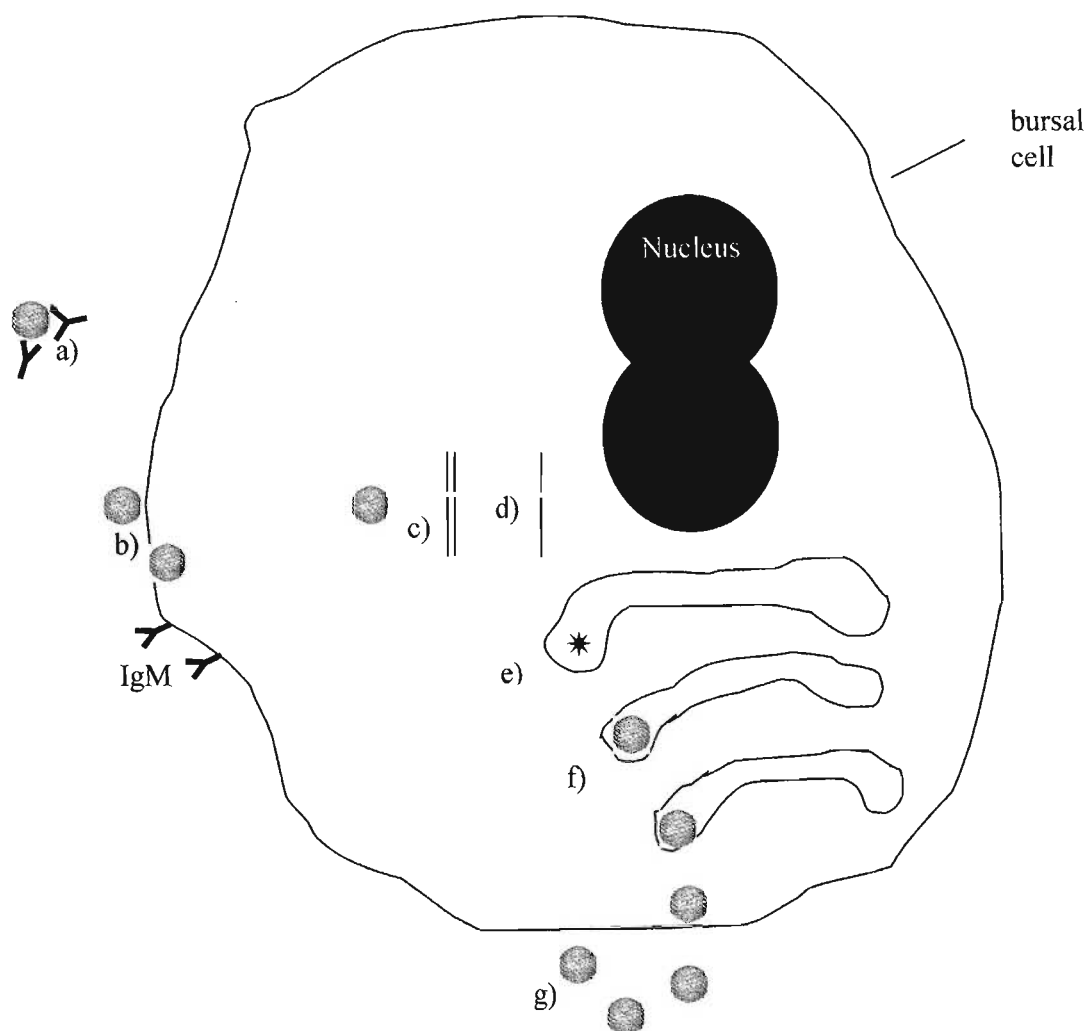
to infect virtually every cell in the bursa. The binding of the virus to the synovial stimulatory like protein may indeed cause this effect.

The recent advances in vaccine design mean that the virus will probably pose little threat to the poultry industry in the future. The vaccines that are produced today are either a mixture of oil emulsified, inactivated virus and live, non-pathogenic virus, or they may be a viral protein expressed in another virus genome. However, these vaccines may not be able to deal with the very virulent virus that escapes neutralisation by mutation (Cao *et al.*, 1998). The viral expression system of Lim *et al.* (1999) may be able to deal with the escape variant viruses by obtaining the variant sequence of that virus and creating a non-pathogenic virus vaccine with the mutated variant sequence. This will allow the production of antibodies specific to the variant virus. However, this would only be effective after the event of an outbreak of disease.

The information available on IBDV, although growing at a rapid rate, is still quite limited in comparison to viruses of other genera. As an overview of the IBDV infection process, a summary of some of the basic known events in IBDV infection is shown in Fig. 5.1. Virus may be neutralised by antibodies before attachment to the bursal cell membrane receptor (Fig. 5.1 a). Neutralisation is mainly due to conformational epitopes on VP2 and possibly to a single conformational epitope on VP3 (Becht, 1994). Virus that is not neutralised attaches to actively dividing B-lymphocytes bearing cell surface IgM (Fig. 5.1 b; Müller, 1986; Burkhardt and Müller, 1987; Luckert and Saif, 1991; Ogawa *et al.*, 1998). Attachment may occur through the 40 and 46 kDa polypeptides described by Nieper and Müller (1996) or through some other N-glycosylated polypeptide or protein complex (Ogawa *et al.*, 1998). During attachment and/or penetration, the potassium current properties of the infected cell are changed. This may be linked to apoptosis in IBDV infected cells (Repp *et al.*, 1998).

Once inside the cell, virus either uncoats or remains coated (Fig 5.1 c). It has been shown that with IPNV transcription can occur without uncoating. In either case, both segment A and segment B of the viral double stranded RNA genome are transcribed to mRNA by the putative RNA dependent RNA polymerase (VP1) present within the viral particle (Fig. 5.1 b; Becht, 1994). It is unknown where this takes place in the





**Fig. 5.1 Summary of the events that take place during infection of bursal cells with IBDV.**

a) Free virus (●) is neutralised before attachment to bursal cell membrane receptors by antibodies (Y). b) Virus attaches to actively dividing B-lymphocytes bearing cell surface IgM. c) Virus either uncoats or remains coated. d) mRNA (|) is produced from the double stranded viral RNA genome (||). Viral polypeptides are translated from segment B (VP1) and from segment A (VPX-VP3-VP4) as a polypeptide. e) The viral polypeptide is autocatalytically cleaved by the viral protease VP4 to produce VPX, VP3 and VP4. f) The viral polypeptides are assembled (\*) into complete viral particles in the Golgi (∞). g) Large numbers of viral particles are released.

host cell. Viral polypeptides are translated from segment B (VP1) and from segment A (VPX-VP3-VP4) as a polypeptide (Becht, 1994). In some strains of the virus, VP5 is produced from an alternate open reading frame (Mundt *et al.*, 1995), which is involved in the pathogenicity of the virus (Yao *et al.*, 1998). The viral polypeptide is

autocatalytically cleaved by the viral protease VP4 (Kibenge *et al.*, 1997) at LAA and MAA cleavage sites (Sánchez and Rodríguez, 1999) to produce VPX, VP3 and VP4 (Fig. 5.1 e). VPX is further cleaved to produce VP2, also a function of VP4 (Kibenge *et al.*, 1997). However, VPX is also incorporated into some viral particles (Müller and Becht, 1982). The viral polypeptides are assembled into complete viral particles (Fig. 5.1 f). VP1 appears to be important for the assembly process as it interacts with VP3 and leads to its efficient encapsidation (Lombardo *et al.*, 1999). However, this interaction is not essential for formation of virus particles as virus-like particles also form when only the polyprotein is expressed and not VP1 (Fernández-Arias, 1998). Large numbers of viral particles are then released (Fig. 5.1 g). Viral particles may be released before programmed cell death (Tanimura and Sharma, 1998) or expelled during necrosis (Becht, 1994).

There is a lack of information regarding both the receptor for IBDV and the events that take place in the cell, post infection, such as replication. This lack of information is probably due to the comparatively recent discovery and classification of the infectious agent of IBD, and also the lack of emphasis placed on the virus in comparison to other viruses (e.g. HIV). The elucidation of the receptor for IBDV may provide insight for the prevention of virus infection or possible treatments for the disease.

Blocking of the interaction of IBDV with the host cell will prevent further spread of the disease throughout the bursa. The establishment of the 40 and 46 kDa viral polypeptides as possible receptors for virus in chicken embryo fibroblasts by Nieper and Müller (1996) gives information about IBDV receptors for these cells. However, IBDV requires the mutation of residues 279 and 284 of VP2 to grow on chicken embryo fibroblasts (Lim *et al.*, 1999). Since VP2 is most likely involved in attachment of virus to the receptor (Böttcher *et al.*, 1997), the polypeptides established by VOPBA (Nieper and Müller, 1996) might not be the ones that are involved in the attachment of virus to cells in the bursa of Fabricius, especially if the only function of VP2 is attachment. The value of the VOPBA technique when used in conjunction with reducing SDS-PAGE is questionable, as conformation is quite likely to be involved in attachment of the virus to a receptor if a direct protein-protein

interaction is involved, as seen in the case of the HIV receptor, CD4 (Ibegbu *et al.*, 1989).

There is also the question of the virus antigen being present in most of the cells of an infected bursa, while the number of B-cells infected *in vitro* is far lower. This suggests that there is some mechanism whereby the virus infects virtually every cell in the bursa. Transport to other cells possibly occurs by motile cells that become infected (Burkhardt and Müller, 1987) but the virus would still require a receptor molecule to enter and infect other bursal cells.

During the experiments described, conditions were kept as close to the natural state as possible at all times, since cell culture generated virus is not identical to that found in nature, a cell culture system was not established. The establishment of a cell culture system using chicken B-lymphocytes as opposed to chicken embryo fibroblasts could assist with the identification of a receptor as these cells are the natural hosts for the virus (Müller, 1986; Burkhardt and Müller, 1987; Luckert and Saif, 1991; Ogawa *et al.*, 1998) and their use would represent a state closer to the *in vivo* situation.

The information presented in this study could provide the framework for identifying IBDV receptors on the natural host cells of the virus. Once the proteins are identified as receptors for the virus on chicken bursal cells, various avenues of research could be taken. These include the development of more effective vaccines using epitopes from either the receptor or viral attachment protein or possible therapies for the disease by interfering with the virus receptor interaction.

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