

**VP4: A Putative Protease Encoded by
Infectious Bursal Disease Virus**

by

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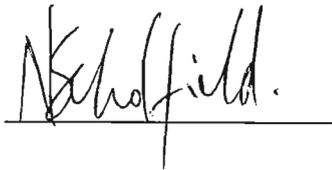
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Preface

The experimental work described in this thesis was carried out in the Department of Biochemistry, University of Natal, Pietermaritzburg, from January 1995 to January 1998 under the supervision of Dr Theresa H.T. Coetzer.

These studies represent original work by the author and have not been submitted in any other form to any other university. Where use has been made of the work of others, it has been duly acknowledged in the text.

A handwritten signature in black ink, reading "Nicola Gillian Scholfield", written over a horizontal line.

Nicola Gillian Scholfield

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Abstract

Infectious bursal disease virus (IBDV) causes an acute and highly contagious disease affecting young chickens, which is responsible for significant losses in the poultry industry world-wide. The virus specifically infects and destroys B-cell precursors within the bursa of Fabricius, an avian lymphoid organ, leading to immunosuppression. IBDV has a bi-segmented, double-stranded RNA genome. The larger segment encodes a 110-kDa precursor polyprotein, designated NH₂-VPX-VP4-VP3-COOH, in a single open reading frame. The autocatalytic processing of this precursor into mature proteins is a critical step in viral replication and VP4 is the putative protease responsible for this cleavage. This study concerns the development of a strategy to clone and express recombinant VP4 and describes the use of VP4 as a marker for rapid and effective detection of IBDV. VP4 cDNA was produced and amplified by optimisation of a reverse transcription coupled to the polymerase chain reaction (RT-PCR), providing a clear and sensitive assay. Anti-peptide antibodies were raised against a selected peptide from VP4 and were used to probe homogenates of infected bursae for the native protein to assess their potential for immunological detection. These antibody-related results are promising though inconclusive, due to the complex nature of the assayed sample. Amplified VP4 cDNA from KwaZulu-Natal strains of IBDV isolated from 1989 to 1997 was also examined by restriction fragment length polymorphism (RFLP) analysis to determine the relatedness of local IBDV to global strains. All KwaZulu-Natal samples produced identical patterns, which were most similar to one of ten international strains examined, namely, the British strain UK661. Samples infected with IBDV were also probed for VP4 activity. Double basic amino acid cleavage sites have been proposed for the putative protease and infected samples were assayed for activity against the fluorogenic peptide Cbz-Arg-Arg-AMC. Demonstrably higher activity was found in infected versus uninfected samples, although the origin of this activity is unclear. The findings in this study suggest that VP4 warrants further attention, both as a marker for infectious bursal disease, and as a novel viral protease.

Abbreviations

A ₆₀₀	absorbance at 600 nm
A ₄₅₀	absorbance at 450 nm
A ₂₈₀	absorbance at 280 nm
Ac	acetate
AEBSF	4-(2-aminoethyl)-benzenesulfonyl fluoride
AMC	7-amino-4-methylcoumarin
AMV-RT	avian myeloblastosis virus reverse transcriptase
AP	alkaline phosphatase
BCIP	5-bromo-4-chloro-3-indolyl phosphate
Bis	N,N'-methylenebisacrylamide
bp	base pair(s)
BSA	bovine serum albumin
ccc	covalently closed circular
Cbz	carbobenzoxy
cDNA	complementary deoxyribonucleic acid
CEF(s)	chicken embryo fibroblast(s)
C-terminus	carboxy terminus
ddH ₂ O	distilled, deionised water
DFP	di-isopropyl fluorophosphate
DHFR	dihydrofolate reductase
dH ₂ O	distilled water
DIG	digoxigenin
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
ds	double stranded
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTT	dithiothreitol
DXV	Drosophila X virus
E	extinction coefficient
ε	molar extinction coefficient
E-64	L- <i>trans</i> -epoxysuccinyl-leucylamido(4-guanidino)butane
EDTA	ethylenediaminetetra-acetic acid, disodium salt

EEO	electroendosmosis
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EtBr	ethidium bromide
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
HIV	human immunodeficiency virus
HRPO	horseradish peroxidase
IBD	infectious bursal disease
IBDV	infectious bursal disease virus
IPNV	infectious pancreatic necrosis virus
IPTG	isopropyl- β -D-thiogalactopyranoside
g	relative centrifugal force
IgG	immunoglobulin G
IgM	immunoglobulin M
IgY	egg-yolk immunoglobulin
kb	kilobase(s)
K_d	dissociation constant
kDa	kilodalton(s)
KZN	KwaZulu-Natal
LB	Luria-Bertani
mAb	monoclonal antibody
MBS	meta-maleimidobenzoyl N-hydroxysuccinimide ester
MCS	multiple cloning site
MMLV-RT	Moloney murine leukaemia virus reverse transcriptase
MOPS	4-morpholine-propanesulfonic acid
M_r	relative molecular mass
mRNA	messenger ribonucleic acid
NBT	nitro blue tetrazolium chloride
NRTB	non-reducing sample treatment buffer
NS	non-structural protein
NTA	nitrilotriacetic acid
N-terminus	amino terminus
oc	open circular
ON	overnight
ORF	open reading frame
ORF A1	large opening reading frame of IBDV segment A

ORF A2	small opening reading frame of IBDV segment A
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulfonyl fluoride
RdRp	RNA-dependent RNA polymerase
RE(s)	restriction enzyme(s)
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
RT	room temperature
RTB	reducing sample treatment buffer
RTn	reverse transcription
RTase	reverse transcriptase
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPF	specific pathogen free
ss	single stranded
SSC	saline sodium citrate
TBS	Tris-buffered saline
TEMED	<i>N,N,N',N'</i> -tetramethylethylenediamine
T_m	melting temperature
TMB	3,3',5,5'-tetramethylbenzidine
U	unit of enzyme activity
UV	ultraviolet
VLB	viral lysis buffer
VP	viral protein
VPg	genome-linked viral protein
vv	very virulent

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Chapter 1

Introduction

1.1 Poultry considerations

The South African poultry industry includes large-scale commercial operations and subsistence farming, each contributing significantly to national protein requirements. Poultry is, however, subject to a vast range of viral, bacterial and fungal diseases, and is particularly susceptible to disease under intensive farming methods. Even where these diseases do not result in outright mortality, they contribute to reduced growth of broilers, impaired egg production of layers and breeders and condemnation of carcasses. This is of great economic significance for the poultry industry, particularly because of the frequency of diseases and concomitant pathological changes. Post-mortem losses connected with disqualifying whole chickens, their parts, or particular organs from human consumption are of primary consideration, with the costs of evaluation of the meat also taking their toll. Diseases and pathological changes also impact negatively on the consumer appeal of meat, on its shelf life and nutritive value and pose a considerable epidemiological and epizootic threat. The development of relatively cheap and simple diagnostic techniques and cheaper, more effective vaccines that have pertinent local relevance are thus important goals to strive for, perhaps particularly so for developing countries.

This study is concerned with aspects of a globally significant poultry pathogen: infectious bursal disease virus, the aetiologic agent of infectious bursal disease (IBD). It is of significance that infectious bursal disease topped the most recent list of the most serious poultry diseases (Van der Sluis, 1999).

1.2. History of infectious bursal disease

1.2.1 Early investigations

Infectious bursal disease emerged as a clinical entity in 1957, in the form of a syndrome recognised by Albert Cosgrove on a broiler farm in the Gumboro area, southern Delaware, in the United States of America. The syndrome began to appear frequently throughout portions of Delaware, Maryland and Virginia, recurring successively in five or more flocks on the same farm. Cosgrove described the syndrome in a 1962 paper where it was termed 'avian nephrosis', as a consequence of gross changes noted in the kidney (Cosgrove, 1962).

The syndrome became known as Gumboro disease, and continued to spread rapidly, a fact correlated with the transportation for re-use of leftover feed from affected farms (Edgar and Cho, 1965). Edgar subsequently suggested the use of planned infection as a method of control and that chickens could be infected by exposure to contaminated litter or to chickens already exhibiting the disease (Edgar, 1966). Edgar also proposed that the syndrome be named 'infectious bursal disease', in recognition of bursal lesions associated with the disease (Edgar, 1966), and replacing the term 'avian nephrosis-nephritis'. Early attempts to isolate the aetiological agent were hampered by a lack of specific pathogen free (SPF) eggs and by insufficient knowledge of appropriate viral and serologic techniques. However, various *in vivo* characterisation studies sought understanding and identification of the infectious bursal agent: Helmboldt and Garner (1964) evaluated disease pathogenesis using infected tissue homogenates, Cheville (1967) examined the effects of the agent on bursal lymphoid cells and Cho and Edgar (1968) documented gross changes in the bursa and population shifts in circulating blood cells in experimentally infected chickens. Assessing the properties of the agent, Benton *et al.* (1967) and Cho and Edgar (1969) concluded that it must be a virus, resistant to extreme conditions of heat and pH and to a wide range of chemicals. These findings readily explained the previously identified role of contaminated litter, feed and equipment in the transmission of the disease.

The virus had an unusual nature, and it was not until reliable methods were developed to isolate the virus in embryonated eggs and adapt it to tissue culture that in-depth examinations were possible. In 1976, Nick, Cursiefen and Becht were able to describe its structural and growth characteristics in sufficient detail to allow their conclusion that the virus would not fit into any previously recognised taxonomic category. The appropriate category did not exist until 1979, when Dobos and co-workers proposed that five animal viruses, including infectious bursal disease virus be classified as the family *Birnaviridae*.

Hitchner (1970) accounted for some of the early difficulty in isolating the infectious agent in his investigations of embryonated eggs refractory to infection. Firstly, embryos were resistant to infection if their dams were immune; the effect of passive neutralising antibodies had not been considered. The immune status of breeders supplying the eggs had not been determined in early studies and SPF eggs were not readily available in the mid 1960's. This work led Hitchner to further examine the influence of maternal antibodies in chickens as well as embryos, and found that they protected chickens from bursal pathology and clinical disease up to three weeks of age (Hitchner, 1971). However, offspring of non-immune hens did not

present clinical IBD when infected during the first 21 days, a feature which agreed well with field observations. Subclinical IBD occurring prior to three weeks of age was not recognised until the role of IBDV in immunosuppression was elucidated by structured trials (Giambrone *et al.*, 1976), following studies reporting the influence of IBDV on vaccination against other infections (Cho, 1970; Faragher *et al.*, 1974; Wyeth, 1975).

The first IBDV vaccine was a bursal homogenate obtained from chickens infected with a field isolate (Edgar and Cho, 1973). This 'unattenuated vaccine' suppressed IBD mortality by planned infection and was a more precise delivery method than spreading virus-contaminated litter, but it did also contribute to immunosuppression and dissemination of field virus. A live attenuated vaccine was subsequently developed, based on mild isolates passaged in SPF eggs (Snedeker *et al.*, 1967). This vaccine, Bursa Vac[®], remains widely used on an international scale, both as a primer and in the control of so-called hypervirulent or 'very virulent' (vv) IBDV.

1.2.2 Distribution and evolution of IBDV

Between 1960 and 1964, IBD had affected most regions of the United States (Lasher and Davis, 1997) and reached Europe in the years from 1962 to 1971 (Faragher, 1972). From 1966 to 1974, the disease was identified in the Middle East, southern and western Africa, India, the Far East and Australia (van den Berg *et al.*, 2000). IBD is currently an international problem: 95% of the 65 countries responding to a 1995 survey conducted by the Office International des Epizooties declared cases of infection (Etteradossi, 1995), including New Zealand, which had been disease-free until 1993.

Infectious bursal disease is extremely contagious. In infected flocks, morbidity is high, with up to 100% serological conversion after infection, whilst mortality is variable. Although IBD mortality was under control by vaccination by 1976, the more subtle effects of immunosuppression and the economic impact of IBD were beginning to exert an influence, and fresh challenges were to be thrown up in the following decade. Since 1987, an increase in specific mortality has been described across the globe. Vaccination failures in America in the mid-1980's revealed new strains that differed antigenically from the classical virus and were responsible for up to 5% of specific mortality (Snyder *et al.*, 1988). These viruses did not cause characteristic clinical signs of infection, but had major immunosuppressive potential. They were termed 'variant' because they could cause infection in chicks with an antibody titre normally considered protective. Immunisation against the 13 variant strains and

their six sub-groups has required the development of specific vaccines (Giambrone and Glosser, 1990; Müller *et al.*, 1992).

In 1987, high mortality rates of 50% to 60% in laying hens and 25% to 30% in broilers were observed in Europe (Chettle *et al.*, 1989; van den Berg *et al.*, 1991) and subsequently in Japan (Nunoya *et al.*, 1992). These hypervirulent strains (vvIBDV) caused up to 100% mortality in SPF chickens. By 1989, the emergence of very virulent strains was being reported in Europe, Asia, and South Africa. In South Africa specifically, IBDV was considered to be of relatively low pathogenicity until mid 1989, when a very virulent strain appeared and spread rapidly throughout the country. The disease caused havoc in the poultry industry for over two years, having a very high specific mortality; up to 60% in layer pullets and up to 40% in broilers. The introduction of early multiple vaccinations did little to raise a timeous serological response in commercial broilers (Horner *et al.*, 1994). The vvIBDV strains are significantly more pathogenic than the classical strains and are also capable of causing infection in chicks with antibody titres normally considered protective (van den Berg and Meulemans, 1991). No antigenic mutations of vvIBDV have been detected, hence these viruses are considered to be pathotypic variants (van den Berg *et al.*, 1991). The only valid criterion for classifying such strains is virulence, based on mortality and bursal lesions caused in SPF chickens. Increases in virulence appear unrelated to antigenic variation, and research continues in the effort to determine specific virulence markers for vvIBDV, which have not yet been identified.

1.3 Infectious bursal disease virus

1.3.1 Classification of IBDV

IBDV is the prototype member of the avibirnavirus genus of the family *Birnaviridae* (Dobos *et al.*, 1979, 1995), which encompasses at least five animal viruses. The generic name describes the genetic complement of these viruses, where 'bi' signifies the double-strandedness (ds) as well as the bisegmented nature of the genome and 'rna' indicates the composition of the viral nucleic acid. Other *Birnaviridae* include the *Aquabirnaviridae*, namely infectious pancreatic necrosis virus (IPNV) of fish (mostly salmonids), tellina virus and oyster virus of bivalve molluscs and the *Entombirnaviridae*, namely *Drosophila* X virus (DXV) of the fruit fly, *Drosophila melanogaster* (Dobos *et al.*, 1995). A new member of the *Aquabirnaviridae*, blotched snakehead virus, was recently isolated from a fish cell line (Riji John and Richards, 1999). Morphological and physicochemical similarities between IBDV

and IPNV have been noted (Todd and McNulty, 1979), and it has been hypothesised that the initial outbreaks of IBDV arose from mutation of an aquabirnavirus (Lasher and Shane, 1994). However, the origins of IBDV remain unclear.

Two serotypes of IBDV have been identified, namely, serotype 1, which is pathogenic for chickens and serotype 2, which is apathogenic and has been isolated from chickens and turkeys (McFerran *et al.*, 1980). The two serotypes are differentiated *in vitro* by the absence of cross-neutralisation, and *in vivo* by the absence of cross-protection (Jackwood *et al.*, 1982; Ismail *et al.*, 1988). In addition to this serological classification, viral strains are also classified according to virulence. IBDV may thus be considered apathogenic, attenuated (vaccine strains), classical virulent, variant or hypervirulent/very virulent. Serotype 2 strains cause neither mortality nor bursal lesions and are consequently termed apathogenic or avirulent. With reference to serotype 1 strains, there is some inconsistency in descriptions of viral virulence. In particular, the term 'hypervirulent' has been used to describe vvIBDV from Europe and variant IBDV from the United States, although the latter have only a 5% specific mortality. In the extensive world trade environment, preservation of food safety and security is of extreme importance. It is significant to note that antigenic variant viruses have only been identified in North America (Jackwood and Sommers, 1999) and that very virulent strains of IBDV have been found in Europe, Asia, and South Africa. No vvIBDV strains have thus far been identified in the Americas or Australasia and it is certain that these countries will exercise great care to ensure this.

1.3.2 Host range, susceptibility factors and transmission

Only chickens (*Gallus gallus*) develop IBD after infection with serotype 1 viruses. However, signs of IBDV infection have been found in wild birds (Nawathe *et al.*, 1978; Motohiko *et al.*, 1998; Wilcox *et al.*, 1983), including Antarctic penguins (Gardner *et al.*, 1997) and ostriches (Cadman *et al.*, 1994). Wild flocks associating with birds farmed in commercial concerns could possibly act as reservoirs of infection and mutation of virus.

Young chickens aged between three and six weeks are maximally susceptible to IBDV infection. This corresponds to the period of maximum bursal development, during which acute clinical signs are observed. If dams are inoculated against the virus, excellent passive protection is provided by maternal antibodies against immunosuppression, bursal lesions and mortality. The half-life of such antibodies varies between three days (for broilers) and five days (for laying hens) (DeWit, 1999). Consequently, if antibody titre of chicks is known at

hatch, the precise time of maximum flock susceptibility can be determined, which is essential for effective vaccination (Lucio and Hitchner, 1979). Infections occurring prior to three weeks of age are normally subclinical and immunosuppressive (Winterfield *et al.*, 1972; Allan *et al.*, 1972), but clinical cases may be observed up to the age of 16 to 20 weeks (Okoye and Uzoukwu, 1981). Light strains of laying stock are more susceptible to disease than the heavy broiler strains (Bumstead *et al.*, 1993).

IBDV is transmitted horizontally (between individuals) and infection is acquired via the oral or respiratory route. Infected chickens excrete the virus in faeces as early as 48 h after infection and may transmit the disease by contact over a 16-day period (Winterfield *et al.*, 1972). The virus is transmitted by direct contact with excreting subjects, or by indirect contact with contaminated materials. Although no vectors have been specifically described, evidence exists to indicate the presence of IBDV in mosquitoes (Howie and Thorsen, 1981) and the virus has been isolated from lesser mealworms (McAllister *et al.*, 1995). IBDV is extremely resistant to the outside environment (Section 1.3.3.2), which enhances the potential for direct transmission. The virus can survive for four months in contaminated bedding and premises (Benton *et al.*, 1967) and up to 56 days in lesser mealworms (McAllister *et al.*, 1995). Without effective cleaning, disinfection and insect control, the resistance of the virus causes persistent contamination of infected farm areas.

1.3.3 Viral characteristics

1.3.3.1 Morphology

Electron microscopy of negatively stained IBDV has shown that the virus is a non-enveloped, single shelled particle with a diameter of 60 to 65 nm, with capsid architecture based on a skewed (right handed) T=13 icosahedral lattice (Özel and Gelderblom, 1985). Recent electron cryomicroscopy of unstained specimens combined with computer image processing has confirmed the T=13 lattice structure, with trimer clustering of subunits (Böttcher *et al.*, 1997). The relative arrangement of external trimers produces a honeycomb pattern on the surface of the virus, whereas the trimers on the internal surface are triangular or Y-shaped and pack more closely to form a nearly continuous shell (Böttcher *et al.*, 1997). The virion has no lipid content (Dobos *et al.*, 1995).

1.3.3.2 Physicochemical properties

The relative molecular mass (M_r) of a virion is about 67×10^6 ($\pm 13\%$) (Dobos *et al.*, 1979), and its buoyant density in CsCl is 1.33 g.ml^{-1} (Dobos *et al.*, 1995). IBDV has a sedimentation rate of 460S in sucrose gradients (Todd and McNulty, 1979).

Contributing significantly to the commercial importance of IBDV as a pathogen is its remarkable stability and its persistence in poultry houses even after thorough cleaning and disinfection (Lukert and Saif, 1991). It is more resistant than reoviruses (which also have a segmented dsRNA genome) to heat, ultraviolet radiation and photodynamic inactivation (Petek *et al.*, 1973) and is resistant to ether and chloroform. Cho and Edgar (1969) found that IBDV retained almost 100% infectivity after exposure to 60°C for 90 min. A recent study has shown that reduction of viable virus was $\sim 99\%$ after 1 min at 71°C , but that the time to completely inactivate IBDV in virally inoculated poultry products at 71°C and 74°C internal cooking temperatures was greater than 6 min (Mandeville *et al.*, 2000). It is highly pH-tolerant, stable at pH 2.0 and only inactivated at pH 12.0 and also resists exposure to thiomersal (0.125% m/v) (Benton *et al.*, 1967). The virus can be inactivated by exposure (1 h) to phenol (1% m/v) or cresol (1% m/v) (Cho and Edgar, 1969) and its infectivity is markedly reduced by 6-hour exposure to 0.5% (v/v) formaldehyde (Benton *et al.*, 1967) or by 1-hour exposure to 1% (v/v) formaldehyde (Cho and Edgar, 1969). In the context of tissue culture, IBDV replication in chicken embryo fibroblast (CEF) cells is highly sensitive to actinomycin D (Petek *et al.*, 1973; Müller and Becht, 1982).

1.3.3.3 Nucleic acid composition and properties

The dsRNA genome of serotype 1 IBDV consists of equimolar amounts of two segments of RNA, approximately 3.3 and 2.8 kilobases (kb), which are referred to as segments A and B respectively (Müller *et al.*, 1986; Kibenge *et al.*, 1990; Mundt and Müller, 1995). The M_r values of segments A and B are 2.2×10^6 and 1.9×10^6 respectively (Müller and Nitschke, 1987a). Segments A and B of serotype 2 viruses are both smaller than their serotype 1 counterparts, by about 70 and 20 base pairs (bp) respectively (Becht *et al.*, 1988).

Properties of IBDV RNA consistent with double-strandedness include: resistance to ribonuclease (RNase) degradation at high salt molarity (Spies *et al.*, 1987), a buoyant density of 1.62 g.ml^{-1} in Cs_2SO_4 gradients, a melting point of 95.5°C in the presence of RNase, a base composition reflecting the pairing of adenine and uracil as well as guanine and cytosine

(Müller *et al.*, 1979), precipitation from 4 M- but not 2 M- LiCl and staining green with acridine orange (Azad *et al.*, 1985).

The IBDV genome segment A encodes a 110-kDa polyprotein (VPX-VP4-VP3) in a large open reading frame (ORF), designated ORF A1 (Hudson *et al.*, 1986; Spies *et al.*, 1989). The polyprotein is cleaved autocatalytically to yield the individual viral proteins (VP), termed VPX (~48 kDa), VP4 (~28 kDa) and VP3 (~32 kDa) (Müller and Becht, 1982; Azad *et al.*, 1985, 1987; Hudson *et al.*, 1986). VPX (also known as pre-VP2, pVP2 or VP2a) undergoes a second, independent, and as yet undefined proteolytic processing step to yield VP2 (~40 kDa) (Kibenge *et al.*, 1997). Segment A also contains a second ORF (ORF A2), which precedes and partially overlaps the polyprotein coding sequence and encodes VP5 (~21 kDa) (Spies *et al.*, 1989; Mundt *et al.*, 1995). The smaller genome segment B contains a single ORF encoding VP1 (~90 kDa) (Morgan *et al.*, 1988). (See Figure 1-1 for a diagrammatic representation of these details of structure and processing). Synthetic transcripts of IBDV dsRNA derived from cloned cDNA can give rise to replicating virus in Vero cells and chicken embryo cells, as demonstrated by Mundt and Vakharia (1996).

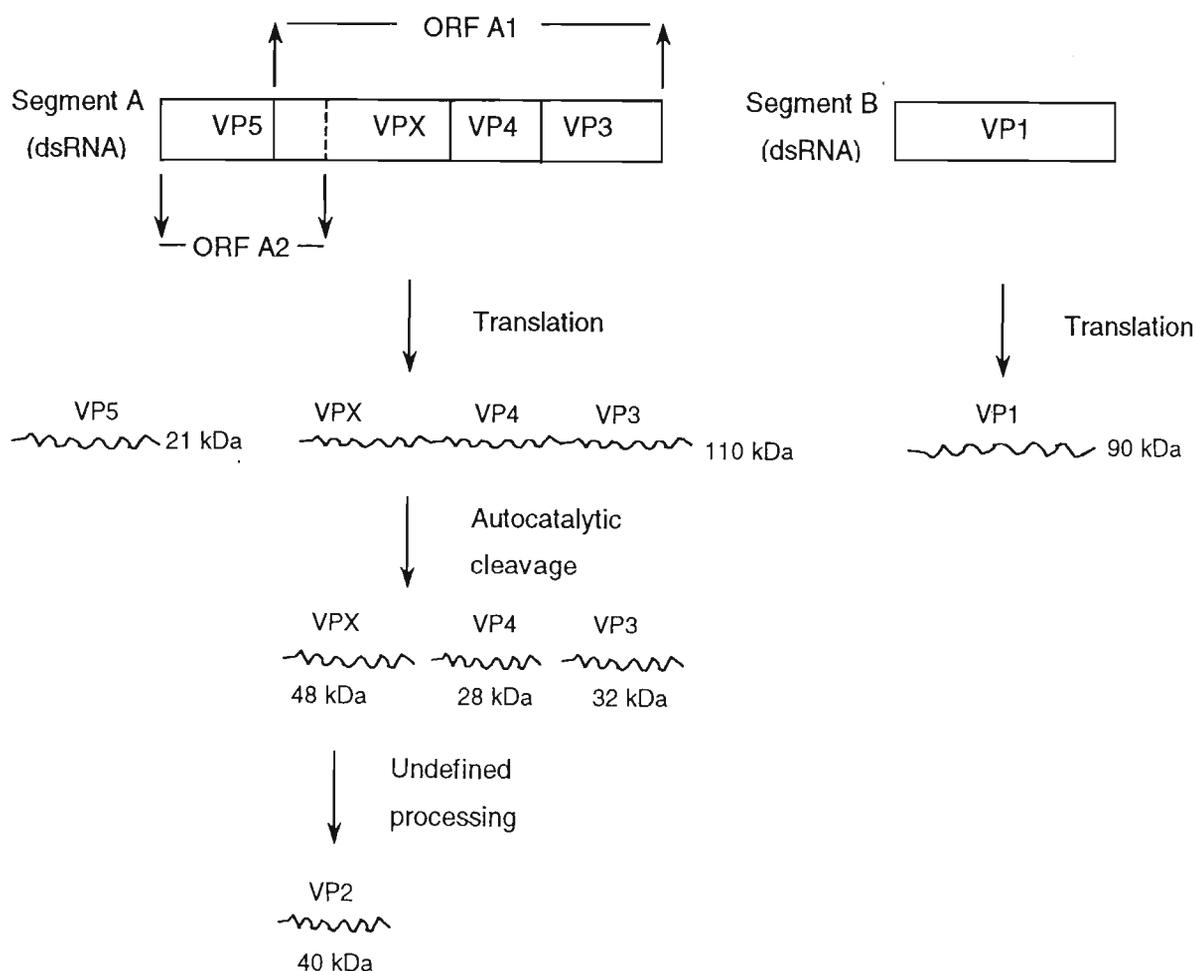


Figure 1-1. Schematic diagram of IBDV genome and production of viral proteins.

See text for details.

1.3.3.4 IBDV proteins

a). VP1, putative RNA polymerase.

Evidence strongly suggests that this protein is the RNA-dependent RNA polymerase (RdRp), which transcribes single-stranded (ss) RNA from viral dsRNA (Spies *et al.*, 1987; Morgan *et al.*, 1988; Koonin, 1992). This process is a prerequisite for replication of dsRNA viruses. The protein has been shown to exist in two size forms, namely 95 kDa and 90 kDa, which may have a precursor-product relationship (Müller and Becht, 1982; Jackwood *et al.*, 1984; Tacken *et al.*, 2000). This virion-associated enzyme has been reported for the birnaviruses IPNV and DXV (Mertens *et al.*, 1982; Dobos, 1995a; Bernard and Petitjean, 1978).

Lombardo *et al.* (1999) and Tacken *et al.* (2000) have recently shown a relatively strong and specific interaction between VP1 and VP3, and proposed that this interaction may have a bearing on the regulation of viral RNA synthesis, or be part of the replication apparatus. It is likely that VP1-VP3 complexes are present in mature virions since the timing of their release into the culture medium (Tacken *et al.*, 2000) is consistent with the release of extracellular progeny virus particles (Petek *et al.*, 1973). A role of this nature has been mooted for the interaction between RdRp and coat protein of the tobacco mottling vein virus (Hong *et al.*, 1995), and a similar interaction in alfalfa mosaic virus (Quadt *et al.*, 1991) has been shown to constitute coat protein inhibition of minus-strand synthesis by the RdRp. Alternatively, it has been suggested that the interaction may be concerned with viral assembly or encapsidation, as is the case for hepatitis B viruses, where an interaction between the viral polymerase and capsid protein is required for encapsidation of pre-genomic RNA (Ziermann and Ganem, 1996).

VP1 is present as free polypeptide and as a genome-linked protein (termed VPg) within virions, where it forms proteinaceous knobs tightly bound to the ends of both segments A and B (Müller and Nitschke, 1987b). Each genome segment is in fact circularised by these structures (Müller and Nitschke, 1987b). Xiang *et al.* (1998) have also reported an interaction between a VPg, which is covalently linked to the genomic RNA of poliovirus, and the viral polymerase. They suggest that a direct interaction between these molecules is involved in the mechanism of initiation of viral RNA synthesis, which may also be the case for the VPg and VP1 of IBDV. It has also been suggested that this process may involve two VP1 molecules in birnaviruses, one serving as a primer and the other for polymerase chain elongation (Dobos, 1995a).

b). VP2, capsid protein.

The existence of an intermediate processing product which is a precursor of VP2 has been documented and designated VPX, pVP2 or VP2a. Details of this processing have not been defined, but it does not involve cellular proteases (Kibenge *et al.*, 1997) and the efficiency of the proteolysis required appears to be dependent on the strain of IBDV (Becht, 1980; Müller and Becht, 1982). Since VP2 does not accumulate intracellularly, as the other viral proteins do, post-translational modification of VPX into VP2 probably occurs during or after virus assembly (Müller and Becht, 1982). Kibenge *et al.* (1999) have recently shown that processing of VPX to VP2 is not necessary for capsid assembly, and that it probably only occurs as a capsid maturation cleavage.

VP2 is a glycosylated structural protein that contains at least two to three closely linked antigenic sites capable of inducing virus-neutralising antibodies in chickens (Becht *et al.*, 1988; Fahey *et al.*, 1991; Öppling *et al.*, 1991a). This suggests that it is at least partially exposed to the outer surface of the capsid. It is the main host protective antigen (Azad *et al.*, 1987; Becht *et al.*, 1988; Fahey *et al.*, 1989). The immunodominant neutralising epitope is conformationally dependent and is located in the so-called 'hypervariable' region, a 145-amino acid section of VP2 between residues 206 and 350 (Azad *et al.*, 1987, Bayliss *et al.*, 1990). This region displays the greatest amount of amino acid sequence variation between IBDV serotypes 1 and 2 and among subtypes of serotype 1 (Bayliss *et al.*, 1990; Heine *et al.*, 1991; Vakharia *et al.*, 1994).

VP2 is also a potent apoptotic inducer; expression of VP2 triggers programmed cell death in a wide variety of mammalian cell lines (Fernández-Arias *et al.*, 1997). However, it remains to be established whether VP2 is the factor responsible for the virus-induced apoptosis of the bursa which accompanies IBDV infection.

c). VP3, capsid protein.

In addition to the 32 kDa form of VP3, two smaller forms are also often detected at ~30 kDa and ~27 kDa, clearly illustrated by the use of anti-VP3 monoclonal antibodies by Tacken *et al.* (2000). These forms are frequently seen in IBDV-infected cells but are usually ignored or confused with VP4. In IPNV, one such form has been designated VP3a (Dobos, 1995b).

This structural protein is the other constituent of the proteinaceous capsid of IBDV. It contains a very basic carboxy-terminal region that might interact with the packaged RNA and

is therefore expected to be on the inner surface of the capsid (Hudson *et al.*, 1986). VP3 is highly immunogenic and the initial antibody response following infection with live IBDV or injection with inactivated vaccine is directed against it (Fahey *et al.*, 1985). VP3 contains group-specific antigen sites (Becht *et al.*, 1988; Öppling *et al.*, 1991b), and minor neutralising epitopes (Jagadish and Azad, 1991; Whetzel and Jackwood, 1995) which inhibit virus attachment to susceptible cells (Reddy *et al.*, 1992).

d). VP4, putative protease.

VP4 has often been described as a minor virion component because it has been detected in purified virions prepared by a variety of methods (Kibenge *et al.*, 1988). However, Granzow *et al.*, (1997) used immunocytochemical techniques to show that VP4 may not be a constituent of mature virions, and that its presence in virion preparations is due to contaminating VP4-containing tubules. These tubules are present in purified IBDV virion preparations as well as in IBDV-infected cells, where they have intracytoplasmic and intranuclear locations. It should be noted that antibodies directed against chicken tubulin, VP2 and VP3 did not label the tubules at all. The function of the tubules is unclear, as is the mechanism for their intranuclear location; VP4 has no obvious nuclear localisation signal (Görlich and Mattaj, 1996). A possibility that presents itself is that these tubules may form a structure analogous to that of the multi-catalytic protease, although the cleavage it may be responsible for is unclear.

Hudson *et al.* (1986) first proposed that IBDV had monocistronic segments from which a precursor polyprotein was synthesised. Processing of this precursor into mature viral proteins even when synthesised as a fusion protein in *Escherichia coli* led these researchers to further propose that the polyprotein was proteolytically processed *in vivo*. They also suggested that the 28-kDa viral protein might have a role to play in this processing. From size estimates of the viral proteins, Hudson *et al.* (1986) estimated that the N-terminus of VP4 was within residues 441-542 and that of VP3 was within residues 710-740. Examination of the deduced amino acid sequences in these regions revealed dibasic Arg-Arg and Lys-Arg residues at sites 452/453 and 722/723 respectively. Proteolytic cleavage has been shown to occur commonly at pairs of dibasic residues during processing of certain peptide hormone precursors and plasma proteins (Douglas *et al.*, 1984; Schwartz, 1986), as well as yeast α -factor and killer toxin peptides (Julius *et al.*, 1984; Bathurst *et al.*, 1987). Hence these dibasic residues became proposed cleavage sites for the viral protease.

Hudson *et al.* (1986) had also noticed that within and close to the proposed protein boundaries, the sequence Ala-x-Ala-Ala-Ser (AxAAS, where x is any amino acid) was repeated three times between residues 483-503 and also appeared at residues 752-756. However, the cleavage potential of the AxAAS motif was unknown and no homology of this structure to any cleavage sites could be found in protein databases at the time. In IPNV, a sequence spanning the C-terminus of VP2 (Manning and Leong, 1990) can be aligned with the triple AxAAS motif (Heppell *et al.*, 1993) in the form of two well-conserved copies of the sequence Ala-Ala-Gly-Gly-Arg-Tyr.

Subsequent deletion-expression studies utilising bacterial expression of the IBDV polyprotein gene showed that the only deletion mutants affecting processing were those in which large N-terminal portions of VP4 were deleted (Azad *et al.*, 1987). Expanding these investigations, Jagadish *et al.* (1988) engineered site-specific deletions, insertions and base substitutions within the polyprotein gene to assess their effect on processing, all of which indicated the involvement of VP4. Jagadish *et al.* (1988) also directed mutagenesis to change the dibasic ⁷²²Lys-Arg⁷²³ pair to the non-basic ⁷²²Ile-Cys⁷²³ residues. Unfortunately, monoclonal antibodies (mAbs) directed against VP3 failed to detect any VP3 at all when the products were assessed, and thus the effect of this change on the VP4-VP3 junction was unknown. Results using mAbs specific for VP2, however, suggested that this change had in fact inhibited cleavage at the VP2-VP4 junction, perhaps by inducing a conformational change.

Brown and Skinner (1996) pointed out that significant homologies had still not been found between the VP4 sequence and that of any known protease (Koonin, 1992; Dougherty and Semler, 1993). However, as Koonin (1992) had alluded to 'marginal similarity to serine protease active centres', Brown and Skinner (1996) carefully examined and compared deduced amino acid sequences of VP4 and proposed that ⁵⁴⁶His, ⁵⁸⁹Asp and ⁶⁵²Ser were candidate IBDV residues for a serine protease catalytic triad. Spacing between the residues and sequences around the proposed active-site aspartate and serine residues are consistent with that observed in viral and cellular serine proteases (Chambers *et al.*, 1990) and components of the catalytic triad are found in IPNV in similar contexts and positions (Brown and Skinner, 1996). Recently, researchers in France have made further advances in the characterisation of VP4. Using site-directed mutagenesis, they have shown the indispensability of a serine-lysine catalytic dyad for VP4 activity in both IPNV (Ser⁶³³, Lys⁶⁷⁴; Petit *et al.*, 2000) and IBDV (Ser⁶⁵², Lys⁶⁹²; Lejal *et al.*, 2000). These two residues are conserved in the VP4 sequences of IPNV, all strains of IBDV and DXV (Lejal *et al.*,

2000). They propose that the active site lysine acts as a general base in the activation of the nucleophilic active site serine. Catalytic dyads of this nature have been characterised from prokaryotic serine proteases and hydrolases and from a yeast mitochondrial inner membrane protease (Lejal *et al.*, 2000).

Brown and Skinner (1996) also demonstrated that a motif (⁷⁰⁰GxxxxG) downstream of the putative active site in IBDV and IPNV showed conservation with the substrate binding sites of the serine proteases of tick-borne encephalitis virus and flavivirus and of trypsin. In VP4, however, the motif is much further removed from the putative active site (48 or 49 residues) than for the other proteases (12 to 17 residues) (Brown and Skinner, 1996). In continuing deletion-expression studies, removal of the putative substrate-binding site by deleting 36 amino acids (residues 686-722) from VP4 abolished cleavage of the VPX-VP4 junction (Kibenge *et al.*, 1997). This is in agreement with observations for IPNV where the non-structural protein (NS, IPNV term for VP4) active site for cleavage at the VPX-NS junction was reported to reside in the C-terminal 32-35 amino acid residues of NS (Duncan *et al.*, 1987; Magyar and Dobos, 1994). Proteolytic activity was also lost at the VP4-VP3 junction by deletion of 70 amino acids (residues 454-524) from the predicted VP4 N-terminus (Kibenge *et al.*, 1997). Previous work (Jagadish *et al.*, 1988) had suggested that amino acid residues 535-597 of the IBDV polyprotein were essential for cleavage at the VP4-VP3 junction, and Kibenge *et al.* (1997) extended these sequences even closer to the predicted VP4 N-terminus.

However, Sánchez and Rodríguez (1999) have recently carried out a systematic search for the proteolytic processing sites based on the analysis of protein products accumulated after transient expression of mutant forms of the polyprotein. The expression system utilised an inducible recombinant vaccinia virus able to infect mammalian cells (African green monkey kidney epithelial BSC-1 cells, Fernández-Arias *et al.*, 1998). They have shown by deletion and site-directed mutagenesis that in fact ⁵¹¹LAA⁵¹³ and ⁷⁵⁴MAA⁷⁵⁶ are the sites essential for the processing of the VPX-VP4 and VP4-VP3 precursors respectively, with the proposed scissile bond between the alanine residues. The latter has the AxAAS motif proposed as an alternative cleavage site (Hudson *et al.*, 1986; Brown and Skinner, 1996) and it was determined that the specificity of this cleavage is dictated by the conserved AA dipeptide. A second, minor processing site for the VPX-VP4 site lies within the 19-amino acid stretch containing the triple AxAAS sequence (residues 485-503). Further site-directed mutagenesis replacing both ⁴⁵²RR⁴⁵³ and ⁷²²KR⁷²³ with a neutral Ala-Gly peptide confirmed that these

dibasic pairs are not essential for polyprotein processing (Sánchez and Rodriguez, 1999). The recent research of Lejal *et al.* (2000) has defined the IBDV cleavage motif as (Thr/Ala)-x-Ala ↓ Ala-(Ser-Gly), which is not fully conserved for the cleavage sites of IPNV and DXV. It appears that although their cleavage site motifs have strong similarities, the VP4 proteases of birnaviruses are species-specific (Lejal *et al.*, 2000).

Hence, although a direct demonstration with purified protease is still lacking, various expression studies in *E. coli* (Jagadish *et al.*, 1988; Lejal *et al.*, 2000), yeast (Jagadish *et al.*, 1990), insect cells infected with baculovirus (Vakharia *et al.*, 1993) and mammalian BSC-1 cells infected with vaccinia virus (Fernández-Arias *et al.*, 1998; Sánchez and Rodriguez, 1999) all strongly indicate that proteolytic processing of the 110-kDa IBDV polyprotein is carried out by VP4, in what appears to be a co-translational event. VP4 is the focus of this investigation and will be discussed further in Section 1.5 in terms of its significance to this study.

This information on VP4 is correct at the time of writing, however, when the experimental parts of this investigation were planned and executed, not all of this information was available. Hence knowledge of the formation of tubules by VP4 and the precise location of active site and cleavage site residues may well have altered some of the experimental approaches taken.

e). VP5, function unknown.

The non-structural VP5 has only been detected in IBDV-infected cells, namely cultured CEFs and infected bursal tissue (Mundt *et al.*, 1995). VP5 proved non-essential for IBDV replication in cell culture (Mundt *et al.*, 1997), but appears to play a role in viral pathogenesis; a virus deficient in VP5 can replicate in the bursa of inoculated chickens but does not induce bursal lesions (Yao *et al.*, 1998). Deduced amino acid sequence reveals a highly basic, cysteine-rich nature for VP5 and conservation of these positions among strains (Mundt *et al.*, 1995). A 17-kDa protein that is probably virion-associated has also been found in IPNV (Havarstein *et al.*, 1990), but has not been correlated with IPNV ORF A2 and has limited homology with IBDV VP5, although the position of three cysteine residues is conserved. Koonin *et al.* (1991) proposed an RNA/DNA binding activity for the similarly sized cysteine-rich proteins of plant viruses, which are presumed to have a regulatory function during replication. Very recent research suggests that VP5 has an important role to play in

the release of virion progeny, accumulating in and disrupting the host cell plasma membrane and inducing cell lysis (Lombardo *et al.*, 2000).

f). General viral protein considerations.

Analysis of the protein composition of purified viral particles indicates that the virus comprises mainly VP2 (51%) and VP3 (40%), with a little VP4 (6%) (which may be an artefact, see Granzow *et al.*, 1997) and VP1 (3%) (Dobos *et al.*, 1979). VP5 does not appear to be a component of the IBDV virion (Mundt *et al.*, 1995). Evidence suggests that VP2 is at least partially exposed and that VP3 is associated with the RNA and thus has an internal location. Böttcher *et al.* (1997) have used this evidence to tentatively identify the proteins in their model. They proposed that the outer part of the capsid comprises VP2, and that the Y-shaped features on the internal capsid surface are formed by VP3, with the basic C-termini extending inwards to interact with the RNA. They suggested that material forming a rim around each five-fold axis on the inner surface might be VP4, but considering the interactions recently revealed between VP3 and VP1 (Lombardo *et al.*, 1999; Tacken *et al.*, 2000), it seems more likely that this protein is in fact VP1. The model of the virion proposed by Böttcher *et al.* (1997) thus predicts that there would be 780 copies of VP2, 600 copies of VP3 and 60 copies of VP1, numbers corresponding quite well with the observed composition. The total molecular mass expected for the capsid shell would be approximately 56 MDa ($780 \times 40 \text{ kDa} + 600 \times 32 \text{ kDa} + 60 \times 90 \text{ kDa}$) (adapted from Böttcher *et al.*, 1997), which is within the range of the estimated molecular mass.

The amino acid sequences specified by ORF A1 and ORF A2 of birnaviruses DXV, IPNV and IBDV have been compared for homology. Significant sequence homology between the three viruses is restricted to the amino (40%) and carboxy (25%) regions of VPX, a 21-residue domain near the carboxy terminal of VP3 (33%) and the product of ORF A2, VP5 (44%) (Chung *et al.*, 1996). Interestingly, the region encoding the putative viral protease that has the identical processing function in all three viruses shows little overall identity (16-18%) (Chung *et al.*, 1996).

1.3.3.5 Replication

The synthesis of virus-specific single- and double-stranded RNA by viruses with dsRNA genomes is mediated by virion-associated, RNA-dependent RNA polymerases. This has been demonstrated for reovirus (Shatkin, 1969), rotaviruses (Verwoerd *et al.*, 1972; Lewandowski *et al.*, 1969) and birnaviruses, IPNV (Mertens *et al.*, 1982), DXV (Bernard and Petitjean,

1978) and IBDV (Spies *et al.*, 1987). Genome-linked proteins have been demonstrated in these three birnaviruses (Revet and Delain, 1982; Perrson and Macdonald, 1982; Müller and Nitschke, 1987b), indicating that they replicate their nucleic acid by a semi-conservative strand displacement mechanism (Bernard, 1980; Mertens *et al.*, 1982; Spies *et al.*, 1987). This is in contrast to reovirus and cytoplasmic polyhedrosis virus which produce ssRNA conservatively (Joklik, 1974; Smith and Furuichi, 1980). However, Mundt and Vakharia (1996) have showed that the synthesis of minus strands must proceed on the plus strands, and that only the plus-strand RNAs of both genome segments were sufficient to initiate replication of dsRNA. These results are in agreement with general features of rotavirus and reovirus replication, where the plus-strand RNAs serve as a template for the synthesis of progeny minus-strands to yield dsRNA.

A single round of replication takes 16 to 20 hours and occurs in the cytoplasm. Transcription of viral RNA involves synthesis of two genome-length messenger RNA (mRNA) species, one for each genome segment, which lack 3'-poly A tails (Dobos *et al.*, 1995). The mRNA is translated by the cellular translation machinery, but host macromolecular synthesis is not inhibited (Dobos *et al.*, 1995). Peak rates of viral RNA and protein synthesis are reached from six to eight hours post-infection. The polyprotein is autocatalytically processed by VP4, probably co-translationally, to release structural proteins VP3 and VPX. Assorted protein-protein and protein-RNA interactions contribute to capsid and virion assembly and VPX is trimmed to mature VP2 during virus maturation. The details of IBDV replication are not, however, fully clarified.

1.4 Infectious bursal disease

1.4.1 Disease definition

Infectious bursal disease is a highly contagious viral disease of young chickens affecting the immune system. It is characterised by the destruction of lymphoid organs, in particular the bursa of Fabricius, where B-cells mature and differentiate. IBDV specifically targets immature B-cells and when not fatal, the resulting infection can cause severe immunosuppression (Dobos *et al.*, 1995).

1.4.2 Role of the healthy bursa of Fabricius

The bursa of Fabricius is named after its discoverer, the anatomist Hieronymus Fabricius (1621). It is a primary lymphoid organ unique to avian species. In 1956, Glick reported the significant chance discovery that early surgical bursectomy leads to a marked reduction in antibody synthesis, highlighting the role of the bursa in the development of humoral immune response. It is a round or oval sac about the size of an almond connected by a short stalk to the dorsal region of the cloaca at its junction with the colon (Payne, 1971). The bursa is seeded by lymphoid precursors between days eight to 14 of embryogenesis, which subsequently proliferate to form lymphoid follicles (Houssaint *et al.*, 1976). Each follicle contains about 10^5 lymphocytes that originated from only two to four precursor cells (Pink *et al.*, 1985). The bursa contains roughly 10^4 such follicles, a total of 10^9 cells. This massive production of B-cells continues in the immature chicken until the bursa undergoes regression at the onset of sexual maturity. The term 'B-cell' was in fact coined because of the central role of the bursa in avian B-cell development, and this specialised and secluded organ provided an excellent opportunity for their study (Funk and Thompson, 1996).

1.4.3 Disease symptoms

1.4.3.1 Clinical signs

The incubation period of IBD is very short, taking only two to three days. In acute cases, chickens are depressed, prostrate with ruffled feathers, dehydrated and suffer from watery diarrhoea (McFerran, 1993). Fatalities appear on the third day of infection, reach a peak by day four and then drop rapidly, with surviving chickens recovering to apparent health after five to seven days. Disease severity depends on age and breed sensitivity, strain virulence and degree of passive immunity (Lasher and Shane, 1994). Any initial infection on a given location is generally very acute, with high mortality if caused by a hypervirulent strain. If the virus persists and is transmitted to successive flocks, the clinical forms of the disease disappear earlier and are gradually replaced by subclinical forms (McFerran, 1993). However, acute outbreaks may still occur and infection may also not be apparent when the viral strain is of low virulence or if maternal antibodies are present (McFerran, 1993).

Clinical signs of IBD may vary considerably between one farm, region, country or continent and another. Van den Berg *et al.* (2000) summarise global clinical forms as follows:

- the classical form, as described since the early 1960's and caused by the classical virulent strains of IBDV. Specific mortality is relatively low and the disease is most often subclinical, occurring after a decline in the level of passive antibodies
- the immunosuppressive form, prevalent in the United States and caused by low pathogenicity strains of IBDV, as well as by variant strains, such as Delaware variant E and GLS. These strains partially resist neutralisation by antibodies against the classical viruses
- the acute form, first described in Europe and then in Asia and South Africa and caused by hypervirulent or 'very virulent' strains of IBDV (vvIBDV). This form is characterised by an acute progressive clinical disease, with markedly high specific mortality.

1.4.3.2 Pathology and lesions

Within 36 h of IBDV infection, the bursal medulla exhibits lymphocyte degeneration and necrosis. By 48 h, almost complete loss of lymphocytes occurs in the medulla, accompanied by blast cell mitosis, macrophagocytosis and plasmacytosis (Cheville, 1967; Käufer and Weiss, 1976). The increased weight of the bursa at this time is due to oedema, hyperaemia and the accumulation of heterophils (McFerran, 1993). The most severe cases are characterised by major infection of the mucous membrane and a serous transudate, giving the bursal surface a yellowish colour. The bursa may also exhibit petechiae and other haemorrhages (McFerran, 1993). By the fifth day, the bursa reverts to normal size and by the eighth day has atrophied to less than a third of normal size. Certain variant strains are reported to cause rapid bursal atrophy without an inflammatory phase (Lukert and Saif, 1991), while in acute forms of the disease caused by hypervirulent strains, macroscopic lesions may be observed in the other lymphoid organs (Inoue *et al.*, 1994).

Perinatal chicks infected with IBDV exhibit immunosuppression (Faragher *et al.*, 1972; Allan *et al.*, 1972; Giambrone *et al.*, 1977), as a result of the cytolytic infection of B-cells (Hirai *et al.*, 1979; Sivanandan and Maheswaran, 1980). Mortality and clinical signs of IBD have also been associated with immune complexes (Ley *et al.*, 1979; Skeeles *et al.*, 1979b), a depletion in circulating levels of haemolytic complement (Skeeles *et al.*, 1979a) and clotting

abnormalities (Skeeles *et al.*, 1980). It is likely that the latter give rise to the frequently observed haemorrhages in the pectoral and thigh muscles. A variety of studies have shown that infection with pathogenic serotype I strains of IBDV leads to the induction of apoptosis both *in vivo* and *in vitro* (Inoue, *et al.*, 1994; Tham and Moon, 1996; Vasconcelos and Lam, 1994; Vasconcelos and Lam, 1995). This death of infected B-cells may contribute to the immunosuppressive effect of IBDV infection (Vasconcelos and Lam, 1994). Recently, it has been proposed that IBDV may also have a direct immunosuppressive effect on T cells and their functions (Sharma *et al.*, 2000) and it appears part of this effect may be due to the induction of suppressor T-cells (Kim and Sharma, 2000).

Although the other lymphoid organs (thymus, spleen, caecal tonsils, Harderian glands, Peyer's patches and bone marrow) are affected, the principal target of IBDV is the bursa, where it destroys immature B-cells in active division (Cheville, 1967, van den Berg *et al.*, 2000). This is evidenced by the fact that bursectomised chickens can survive an otherwise fatal infection (Käufer and Weiss, 1980), and still mount significant antibody responses to virus; mature and committed lymphocytes escape in the periphery. Studies show that following oral inoculation, initial viral replication occurs in gut-associated lymphoid cells and that secondary replication in the bursa is responsible for high viral titres and mortality (Käufer and Weiss, 1980). However, wholly immature lymphocytes are resistant to IBDV infection as shown by the experiments of Beug *et al.*, (1981).

Müller (1986) showed that the bursa provides a microenvironment favouring active division of B-cells and Burkhardt and Müller (1987) further confirmed that actively dividing B-cells are the specific targets of IBDV, particularly those bearing immunoglobulin M (IgM) (Hirai and Calnek, 1979). The significance of cell divisions or perhaps just the S-phase of the cell cycle has been demonstrated for other viruses - the parvoviruses, for example (Siegl and Gautschi, 1973). Flow cytometry investigations of chicken B-lymphoblastoid cell lines suggest that the IBDV host range of cells is mainly controlled by the presence of a virus receptor composed of N-glycosylated protein. This protein is associated with the differentiation stage of B-cells characterised by the bearing of surface IgM (Ogawa *et al.*, 1998). This accounts for the paradoxical immune response to IBDV, in which immunosuppression co-exists with high IBDV antibody titres. The mature and competent lymphocytes expand as a result of stimulation by virus whereas the immature lymphocytes are destroyed.

Fatalities occur when chickens are infected at three to six weeks of age, when the bursa is maximally developed and the protective effect of maternal antibody, if present, is waning (Hitchner, 1971). These factors allow massive replication of virus in birds infected at this age. Classically, mortality peaks by the third day post infection but death may still occur over the next five to seven days giving a flock mortality of up to 15% (Parkhurst, 1964). In such chickens the clinical disease is additionally responsible for losses due to reduced weight gain and feed efficiency, as well as excessive condemnation of carcasses because of skeletal muscle haemorrhages (Lukert and Saif, 1991).

The destruction of immature B-cells in the bursa causes immunosuppression, which is more severe in younger birds (Faragher *et al.*, 1972). The greatest economic losses caused by IBDV are a result of immunosuppression, which impacts production and causes increased susceptibility to other diseases, as well as interfering with effective vaccination against Newcastle disease, Marek's disease and infectious bronchitis (Giambrone *et al.*, 1976; Okoye, 1984). The intensive methods employed in modern commercial concerns exacerbate these concerns and have further impact with respect to the importance of IBDV as a viral pathogen.

1.4.4 Disease diagnosis

1.4.4.1 Clinical and histological diagnosis

Clinical diagnosis is based on disease evolution (mortality peak followed by recovery in five to seven days), observation of symptoms and examination for specifically distinctive lesions, particularly in the bursa (Lukert and Saif, 1991). Diseases such as avian coccidiosis, Newcastle disease, stunting syndrome, chicken infectious anaemia, mycotoxicoses and nephropathogenic forms of infectious bronchitis can be clinically mistaken for IBD. In subclinical cases, an atrophy of the bursa may be confused with Marek's disease or infectious anaemia, and requires histological examination for differentiation (McFerran, 1993).

Histological diagnosis is based on detection of changes to the bursa as described in Section 1.4.3.1. The ability to cause lesions in lymphoid organs such as the thymus, spleen or bone marrow (Inoue *et al.*, 1999) is considered potentially diagnostic of vvIBDV. Although labour-intensive and requiring necessary experience, the advantage of histology-based diagnosis is that acute, chronic and subclinical forms can be distinguished.

1.4.4.2 Serological diagnosis

In areas where IBDV is endemic, most broiler flocks possess anti-IBDV antibodies which may be induced by pathogenic IBDV or by attenuated vaccines. Current serological tests cannot distinguish between these antibodies and are thus of little use for diagnosis. However, the quantification of anti-IBDV antibodies is important to 1), confirm the success of vaccination in laying hens, and 2), measure the titre of passive maternal antibodies in young chickens and thereby determine the appropriate date for vaccination (DeWit, 1999). Serology is likewise essential to confirm the disease-free status of SPF flocks.

The most commonly used quantitative tests are the detection of precipitating antibodies by gel immunodiffusion (Cullen and Wyeth, 1975), enzyme-linked immunosorbent assay (ELISA) (Marquardt *et al.*, 1980) and serum neutralisation tests in cell culture (Weisman and Hitchner, 1978). Agar gel immunodiffusion is the simplest and cheapest but least sensitive assay, and results are only obtained after a 48-h incubation. Variability in results may depend on the particular investigator, as well as the viral strain used as antigen (Wood *et al.*, 1984). Serum neutralisation tests have the disadvantage of needing specialised equipment and five days for incubation. These assays are much more sensitive than gel immunodiffusion and have better correlation with the level of protection of subjects tested (Weisman and Hitchner, 1978). The ELISA is the most rapid and sensitive method and presents the fewest variations due to the strain of viral antigen, although considerable variability can occur with certain commercial kits (Kreider *et al.*, 1991). Although the correlation between serum neutralisation and ELISA is high, ELISA does not detect low neutralising titres of residual maternal antibodies, which are sufficient to hamper vaccine administration. ELISAs which use a recombinant VP2 protein as the sole antigen may be better correlated with protection (Jackwood *et al.*, 1999).

1.4.4.3 Virus-based diagnosis

Infectious bursal disease virus may be detected in the bursa of Fabricius of chicks in the acute phase of infection, ideally within the first three days after the appearance of clinical signs. The virus may be identified by its proteins, or by its nucleic acids, as described below.

a). Detection of viral antigens.

IBDV antigens may be detected in thin sections of an infected bursa by direct and indirect immunofluorescence (Allan *et al.*, 1984), or by peroxidase staining in the bursal follicles. The specificity of these tests is enhanced by the use of mAbs. Bursal suspensions can be used in gel immunodiffusion assays which examine the reaction of the test material with a specific

antiserum or mAb (Snyder *et al.*, 1992), and may also be probed via agglutination tests. These may utilise latex beads or sheep red blood cells coated with anti-IBDV antibodies (Nakamura *et al.*, 1993; Nachimutu *et al.*, 1995).

Viral antigens in bursal suspensions may also be captured and detected by sandwich ELISA, using an anti-IBDV antibody followed by an adapted anti-species enzyme conjugate (Snyder *et al.*, 1988). The use of polyclonal serum for capture enhances the sensitivity of the test, and the use of mAbs in the capture or detection stages allows for more precise antigenic characterisation of the virus. Different batteries of mAbs enable a tentative identification of the United States variants (Snyder *et al.*, 1988) and vvIBDVs (Etteradossi *et al.*, 1997).

b). Detection of viral genome.

DNA probes labelled with ^{32}P (Kibenge, 1992), biotin (Jackwood *et al.*, 1990) or digoxigenin (Hatchcock and Giambrone, 1992) have been used on blots of infected tissues to detect the multiple virus strains of IBDV serotypes 1 and 2. No genomic probe enabling differentiation between variant viruses or vvIBDVs has yet been described, probably because of the high sequence homology among serotype 1 strains.

RT-PCR allows the detection of viral RNA in homogenates of infected organs or embryos and in cell culture, irrespective of viral viability. The choice of amplified zone is dependent on the objective, hence when the aim is to detect multiple strains of the virus, primers are selected from regions where the sequence is highly preserved (Wu *et al.*, 1997). When characterisation of the amplified fragment is to allow for identification of different IBDV strains, the central, so-called 'variable' section of VP2 is generally chosen (Liu *et al.*, 1994). The fragment may then be characterised by direct sequencing and analysis of the analogous amino acid sequence. The simultaneous presence of four amino acid residues (Ala²²², Ile²⁵⁶, Ile²⁹⁴, Ser²⁹⁹) is considered indicative of vvIBDV (Brown *et al.*, 1994; Cao *et al.*, 1998). In the study of genomic variations as a whole, RT-PCR with nucleotide sequence analysis is usually applied. Even though this method can obtain clearer genetic information, it is laborious, relatively expensive and not suitable for some microorganisms with large genomes using current sequencing techniques (Liu *et al.*, 1994). Davis and Boyle (1990) and Lee *et al.* (1992) adapted the RT-PCR test for detection of IBDV and Wu *et al.* (1992) used RT-PCR and sequencing to detect differences in IBDV isolates. Lin *et al.* (1993) were able to show differences between highly virulent and less virulent Japanese IBDV isolates using PCR and sequencing of the cDNA fragments.

The electrophoretic profile of the amplified fragment may also be examined after digestion with different restriction endonucleases (REs), which can be compared to detect any RFLPs (Jackwood and Nielsen, 1997; Liu *et al.*, 1994). The absence of restriction sites for enzymes *Bst*I and *Sty*I, located respectively at codons 222 and 253 of the VP2 gene, has been correlated with an atypical antigenicity, such as that found in the variant virus strains from the United States (Jackwood and Jackwood, 1994; Jackwood and Nielsen, 1997). Liu *et al.* (1994) have also showed that there are genetic variations among most IBDV isolates by RT-PCR-RFLP analysis. Determination of genomic variation is more sensitive and reproducible than the use of serum neutralisation tests for classification of viruses, hence RT-PCR followed by RFLP analysis offers additional information for classification of IBDV strains that are closely related (Liu *et al.*, 1994), or indeed that are widely dissimilar (Jackwood and Sommers, 1997). Locally relevant RT-PCR and RFLP tests continue to be developed for detection and differentiation of IBDV in clinical samples (Kataria *et al.*, 1998).

Virus isolation, electron microscopy, immunofluorescence, immunodiffusion, virus neutralisation, ELISA and monoclonal antibody assays have all been used for the diagnosis of IBD (Lukert and Saif, 1991). These techniques suffer several disadvantages; they are time-consuming, labour-intensive, expensive, non-specific or insensitive. And importantly, these techniques do not have the ability to detect low levels of IBDV antigens in tissues or in matter such as fowl litter. Conversely, PCR is a most suitable identifying technique which is highly sensitive and specific, and has been successfully used to detect a vast number of significant viruses, including human immunodeficiency virus (HIV) type 1 (Guatelli *et al.*, 1989), hepatitis B virus (Ulrich *et al.*, 1989) and numerous agricultural pathogens.

1.4.5 Disease prevention and control

1.4.5.1 Exclusion and eradication

The extreme resistance of IBDV to physical and chemical agents (Benton *et al.*, 1967) accounts for its persistence in the external environment despite disinfection, and as such, eradication seems unrealistic. Currently, prevention of IBD is a matter of strict hygiene standards and effective vaccination (Lasher and Shane, 1994). Sanitary precautions include 'all in/all out' farming methods, cleaning and disinfection of all equipment and premises, efficient fumigation and pest removal, and a rest period between depopulation and restocking. All bedding and dung must be eliminated and composted and under no circumstances should feed remaining from previous flocks be re-used (Lukert and Saif, 1991; McFerran, 1993).

1.4.5.2 Vaccination

In addition to strict hygiene controls, the success of any vaccination protocol depends on the choice of vaccine, and crucially, on the vaccination schedule, because of the influence of maternal antibodies and bursal development (Section 1.3.2). The existence of different pathotypes and the presence of antigenic variants must also be taken into account. Humoral immunity plays a decisive role in the protection of chickens against IBDV and a very close correlation exists between titres of neutralising antibodies and protection (Jackwood *et al.*, 1999; van den Berg *et al.*, 1991).

Currently, IBDV is controlled by the use of attenuated live virus vaccines and oil-emulsion inactivated virus vaccines containing serotype 1 IBDV (Jackwood *et al.*, 1999). Thornton (1977) outlined the still-valid principles governing the choice and use of these vaccines. The ideal vaccine must offer a balance between efficacy and innocuity; it must not cause disease or bursal lesions, must not be immunosuppressive or excreted and must confer long-lasting immunity even in chickens with high levels of maternal antibodies. Unfortunately, the ideal vaccine does not yet exist (McFerran, 1993).

a). Live virus vaccines.

These are very widely used and are prepared from viral strains attenuated by serial passage in embryonated eggs. Depending on the degree of attenuation, these cause histological lesions of varying severity in the bursae of SPF chickens and are classified accordingly as mild, intermediate or hot (van den Berg *et al.*, 2000). Mild strains are used mainly for vaccination of breeder flocks. This type of vaccine is very sensitive to interference by maternal antibodies and are administered when these have disappeared, between week four and week eight, depending on whether or not the dams were inoculated with an oil-emulsion vaccine before lay (Jackwood *et al.*, 1999).

Intermediate strains are used to vaccinate broilers and pullets (Mazariegos *et al.*, 1990) and are usually administered through drinking water, although nebulisation is also possible. They are also administered to chicks in breeder flocks at risk of challenge by highly pathogenic strains. Although these vaccines are also sensitive to maternal antibody, they may be administered to day-old chicks for protection of those with insufficient levels of specific antibody (Mazariegos *et al.*, 1990). Live IBDV vaccines are compatible with other avian disease vaccines, but hot strains may also provoke immunosuppression, exacerbate the pathogenicity of other immunosuppressive viruses (Marek's disease virus, chicken anaemia

virus) and jeopardise the immunisation of poultry against other diseases (Giambrone *et al.*, 1976). A vaccine for *in ovo* vaccination of embryos has recently been developed which avoids interference by maternal antibodies (Haddad *et al.*, 1997). The vaccine is a mixture of virus and specific antibody which is injected into 18-day-old embryos. Chicks hatched from these eggs are subsequently immunised throughout the growing period. However, this procedure is necessarily time-consuming, labour-intensive and expensive and does not have wide-spread use.

b). Inactivated vaccines.

Preparation of these vaccines involves inactivation of bursal homogenates of infected chicks or viral cultures on embryonated eggs or fibroblasts using formaldehyde. These vaccines are then presented as oil emulsions. Inactivated vaccines are used to produce high, uniform and persistent levels of antibody in dams that have been vaccinated with live virus or that have been naturally infected (Wyeth and Cullen, 1978). These vaccines are administered subcutaneously or intramuscularly at age 16 to 20 weeks, prior to lay. Progeny of dams vaccinated in this manner have protective antibodies until the age of about 30 days (van den Berg *et al.*, 1991; Wyeth and Chettle, 1990). The chicks are thus protected during the period of susceptibility against IBDV strains that only provoke immunosuppression. However, they remain vulnerable to highly pathogenic strains that cause high mortality at later stages (van den Berg *et al.*, 1991). Where there is no risk of vvIBDV infection, boosting of laying hens with an inactivated vaccine is fully justified. However, the duration and uniformity of the conferred immunity depends on the concentration and antigenic specificity of the vaccine virus.

c). Subunit vaccines.

Various vaccines based on purified VP2 (Fahey *et al.*, 1989; Macreadie *et al.*, 1990; Vakharia *et al.*, 1993) and recombinant live vaccines, generated by insertion of the VP2 coding region within the genome of heterologous viruses (Heine and Boyle, 1993; Darteil *et al.*, 1995; Pitcovski *et al.*, 1996; Tsukamoto *et al.*, 1999) have been described, and have varying efficacy in laboratory tests. It has been suggested that full immunological protection against IBDV requires the production of neutralising antibodies against several VP2 conformational epitopes (Müller *et al.*, 1992). For this reason, empty virus-like particles formed by expression of IBDV ORF A1 also hold promise as an effective vaccine (Fernández-Arias *et al.*, 1998). No commercial versions of subunit vaccines are currently available.

1.4.5.3 Alternative control strategies

Considering the commercial significance of IBD and the relative complexity of vaccination protocols, alternative methods to control IBDV are under investigation.

Recent studies indicate that ascorbic acid supplementation improves the humoral and cellular response to vaccination against IBDV and may also ameliorate the concomitant immunosuppression (Wu *et al.*, 2000). Supplementation with a vitamin E-selenium combination has also been shown to enhance immune responses in chickens infected with IBDV (Panda and Rao, 1994). Such relatively cheap and simple dietary therapies are surely worth further investigation, particularly for implementation in developing countries.

Different lines of blood-related poultry show highly variable susceptibility to experimental infection with the same strains of IBDV (Bumstead *et al.*, 1993). The results of crosses between resistant and susceptible lines show that resistance is a dominant hereditary characteristic. However, the genes responsible for resistance have not yet been identified and genetic selection for resistance has not yet been practised. Nevertheless, this is also an attractive avenue for exploration.

The generation of VP5-deficient IBDV may contribute to the development of live attenuated vaccines, since VP5 is not vital for replication and yet plays an important role in viral pathogenesis (Yao *et al.*, 1998). VP5 also appears to have a role in the release of virion progeny (Lombardo *et al.*, 2000). As enzymes, VP1 and VP4 are also attractive therapeutic targets and much precedent exists for the control of viral diseases via the control of their proteases in particular, as detailed in Section 1.5.2.

As a final point, no evidence exists of transmission of IBDV to humans (Pedersen *et al.*, 1990) and the disease thus has no direct impact on human health. However, the administration of high levels of antibiotics to combat secondary infection as a result of virus-induced immunosuppression constitutes a growing public health concern.

The remainder of this chapter moves away from general infectious bursal disease considerations and focuses on the putative IBDV protease, VP4, which is the central subject of this study. Section 1.5.1 provides the enzymatic background and Section 1.5.2 contextualises proteases in terms of viral enzymes, while Sections 1.5.3 and Section 1.6 detail the motivations and objectives driving this investigation of VP4.

1.5 Proteases

1.5.1 Protease classification and catalytic mechanisms

Proteolytic enzymes, also known as proteases or proteinases, are a diverse group of enzymes which hydrolyse the peptide bonds between the constituent amino acids of peptides and proteins. They occur in organisms from all five kingdoms, are encoded by numerous viruses, and have diverse and essential functions ranging from digestion to molecular processing and cellular regulation to fertilisation. Their role in pathologic processes ranging from arthritis and cancer metastasis to infection by viruses and disease-causing protozoan parasites has been the subject of intensive investigation (Roose and van Noorden, 1995).

Two groups of proteases are recognised, namely exo- and endopeptidases. The exopeptidases catalyse the sequential removal of amino acid residues from either the amino (N)- or carboxy (C)-termini of peptides and proteins, and are termed aminopeptidases and carboxypeptidases respectively. Endopeptidases, also known as proteinases, catalyse the cleavage of internal peptide bonds within a polypeptide. Proteolytic enzymes usually exhibit a certain degree of specificity, both with respect to catalytic conditions and substrate. The substrate specificity is often dictated by the identity of the amino acids on either side of the cleaved peptide bond, termed the scissile bond. These regions are bound by the substrate-binding pockets which comprise the active site of an enzyme. Regions of substrate on the N-terminal side of the scissile bond are designated $P_1, P_2, P_3, \dots P_n$, and those on the C-terminal side are designated $P_1', P_2', P_3', \dots P_n'$. The substrate-binding pockets on the enzyme into which these regions fit are similarly termed $S_1, S_2, S_3, \dots S_n$ and $S_1', S_2', S_3', \dots S_n'$ (Schechter and Berger, 1967).

Four mechanistic classes of proteases are recognised by the International Union of Biochemistry, and within these classes, six families of proteases are currently recognised (Table 1-1). Each family has a characteristic set of amino acids comprising the catalytic unit or active site of the protease and members of each family are thought to have evolved convergently from a common ancestor.

Table 1-1. The four currently recognised classes of proteases. (After Barrett, 1994).

Class	Representative protease	Active site residues	Diagnostic inhibitor ^a
Serine I (mammalian)	Chymotrypsin	Asp ¹⁰² , Ser ¹⁹⁵ , His ⁵⁷	DFP
Serine II (bacterial)	Subtilisin	Asp ³² , Ser ²²¹ , His ⁶⁴	DFP
Cysteine	Papain	Cys ²⁵ , His ¹⁵⁹ , Asp ¹⁵⁸	E-64
Aspartic	Penicillopepsin	Asp ³³ , Asp ²¹³	Pepstatin
Metallo I (mammalian)	Carboxypeptidase A	Zn, Glu ²⁷⁰ , Trp ²⁴⁸	EDTA;
Metallo II (bacterial)	Thermolysin	Zn, Glu ¹⁴³ , His ²³¹	1,10-phenanthroline

^a E-64, *L-trans*-epoxysuccinyl-leucylamido(4-guanidino)butane;

EDTA, ethylenediaminetetra-acetic acid;

DFP, di-isopropyl fluorophosphate

Serine and cysteine proteases form covalent enzyme-substrate complexes whereas the aspartic and metallo-proteases do not. However, the overall effect of protease activity, namely, scissile bond cleavage, is identical for all classes and the differences between catalytic mechanisms are fairly subtle. Nucleophilic attack on the carbonyl group requires a nucleophile, in the form of oxygen or sulfur, to approach the slightly electrophilic carbonyl carbon. This process is facilitated by general base catalysis removing a proton from the attacking nucleophile and some electrophilic influence on the carbonyl oxygen to increase the polarisation of the C=O bond. The precise functional groups performing these functions (nucleophilic attack, general base catalysis and electrophilic assistance) differ between the four protease classes. The tetrahedral intermediate formed after the initial nucleophilic attack requires breakdown by general acid catalysis to assist in the release of the amine. Again, the groups playing this role differ for each protease class (Neurath, 1989).

a). Serine proteases.

Michaelis complex formation between enzyme and substrate is facilitated by non-specific binding of S and S' residues to P and P' residues respectively. The close proximity of crucial active site residues allows Ser¹⁹⁵ (chymotrypsin numbering) to transfer a proton to the imidazole ring of His⁵⁷, a charge transfer which is facilitated and stabilised by the negative charge of Asp¹⁰². This system boosts the electronegativity of the oxygen of the active site Ser¹⁹⁵, and results in nucleophilic attack on the carbonyl carbon of the scissile bond. At this point, enzyme and substrate are in an unstable tetrahedral transition state, but the oxygen

anion is stabilised by hydrogen bonding by -NH- groups of amino acids in the so-called oxyanion hole (Gly¹⁹³, Ser¹⁹⁵). In this activated state, cleavage of the peptide bond occurs. The C-terminal portion of the substrate extracts the proton from His⁵⁷ to form a new terminal amino group and subsequently dissociates, while the N-terminal portion remains covalently attached to the enzyme via an acyl linkage. Deacylation of the acyl-enzyme intermediate is essentially a reversal of the above process, with a water molecule taking the place of the released substrate polypeptide. The water molecule transfers a proton to His⁵⁷ and the hydroxyl group makes a nucleophilic attack on the carbonyl carbon of the ester. This results in the formation of a second tetrahedral transition state and subsequent cleavage of the acyl bond. The substrate portion with newly formed carboxy terminus dissociates, and the proton is transferred from His⁵⁷ back to Ser¹⁹⁵ and the enzyme returns to ground state. Thus the catalysis proceeds by an intermediate acyl transfer from substrate, to enzyme via a covalent bond, to water. This is the common feature between serine proteases and other transferases in biology (Dunn, B.M., 1989).

b). Cysteine proteases.

This family bears great mechanistic similarity to the serine proteases, also proceeding by intermediate acyl transfer to water. In this case the attacking nucleophile is the sulfur atom of the active site Cys²⁵ (papain numbering). A histidine side chain (His¹⁵⁹) is again involved in a hydrogen acceptor/shuttle role, stabilised by Asp¹⁵⁸. Maintaining the analogy with serine proteases, the -NH- groups of Gln¹⁹ and Cys²⁵ provide the stabilising hydrogen bonds of the oxyanion hole.

c). Aspartic proteases.

These enzymes do not rely on nucleophilic attack for catalysis and do not form covalent intermediates with substrates. Catalysis is mediated by two aspartic acid side chains (Asp³³ and Asp²¹³, penicillopepsin numbering) which are in close geometric proximity. In the enzymatically active pH range (pH 2-3), one of these is ionised and the other is not. They share a hydrogen bond between two of their oxygens and, in the native form, are hydrogen bonded to a water molecule. After formation of the Michaelis complex, general acid-general base catalysis occurs with the attack of the hydrogen-bonded water molecule on the substrate carbonyl group. A tetrahedral intermediate is formed and the scissile bond is cleaved. The low pH optimum of aspartic proteases and the numerous hydrogen bonds within the active site cleft facilitate this catalytic mechanism. Breakdown of the tetrahedral intermediate yields

a non-covalent product complex containing both halves of the substrate. Dissociation of either half can follow to give an acyl product complex or an amino product complex, the final dissociation of which returns the enzyme to ground state.

d). Metallo-proteases.

Like aspartic proteases, these enzymes do not form covalent intermediates. This class of enzyme has no oxyanion hole component to effect catalysis on the carbonyl group of the scissile bond, but utilises co-ordination to a metal ion instead (Dunn, B.M., 1989). This metal is usually zinc, although other transition metals can substitute. In the native enzyme, the metal atom is tetrahedrally co-ordinated to a water molecule and three amino acid side chains (two histidines and a glutamic acid in carboxypeptidase A and thermolysin). The water molecule may be displaced by co-ordination to the substrate carbonyl, perhaps via a transition state, but is thought to remain in the active site. This water molecule is also hydrogen-bonded to a glutamic acid side chain (Glu²⁷⁰, carboxypeptidase numbering). The carboxyl group of Glu²⁷⁰ acts a general base, removing a proton from the water molecule and assisting its attack on the peptide carbonyl. The attack is further facilitated by the co-ordination of the substrate to the metal ion, which exerts a strong electrophilic attraction on the carbonyl oxygen. A proton must be transferred to the leaving nitrogen atom and could be derived from Glu²⁷⁰. Hence the glutamic acid acts as a 'shuttle', analogous to one of the catalytic groups in the aspartic proteases and the histidine in serine and cysteine proteases.

e). Inhibition of proteases.

As detailed above, serine and cysteine proteases have strongly nucleophilic amino acids within their catalytic sites. These are usually aligned with hydrogen bond acceptors to promote dissociation of the nucleophile in the approach to the transition state, thereby increasing the fraction in the hyper-reactive state. Effective inhibitors of these proteases will thus comprise molecules able to introduce electrophilic groups to chemically modify the nucleophile or general base, thereby rendering the catalytic apparatus inactive (Dunn, B.M., 1989). Aspartic and metallo-proteases do not rely on nucleophilic attack but rather upon general acid-general base catalysis of the attack of a water molecule. As the catalytic residues lack the aggressive nucleophilicity of serine and cysteine proteases, effective inhibition of these proteases relies more on secondary binding interactions along the active site cleft and on transition state analogues. In the case of the metallo-proteases, the metal ion can be exploited for inhibition by the introduction of functional groups, which lead to nearly irreversible chelation (Dunn, B.M., 1989). Specific inhibitors have proved very useful in the

determination of protease classes (Barrett, 1994) and are often referred to as diagnostic inhibitors, as detailed in Table 1-1.

1.5.2 Protease significance in viral infection

In all of the five kingdoms of living organisms, proteases are involved in diverse and significant physiological processes, from functional activation and inactivation of proteins by single proteolytic events to the complete dissolution of proteins to constituent amino acids. They are also encoded in the genetic material of many viruses. These protein envelopes surrounding a core of DNA or RNA are unique in their position between living and non-living matter and are classified within their own kingdom, the *Viridae*. Living organisms by definition respire and reproduce; viruses only reproduce, and cannot do so independently. They must invade a living host, and having gained entrance may exploit the host's cellular machinery for their own reproduction without any requirement for respiration. Such viral invasion and reproduction may have consequences ranging from the development of a fever blister (herpes simplex virus type I) to agricultural disaster (foot-and-mouth disease virus).

The replication strategy adopted by an invading virus is dependent on the nature of its genetic material. Positive-strand RNA viruses can utilise their RNA directly as the message, translating it as a single large polyprotein. Full expression of the various structural and non-structural proteins within the polyprotein requires controlled and directed proteolysis. Negative-strand RNA viruses and DNA viruses normally produce monocistronic messages and therefore do not require proteases for polyprotein cleavage, but may nevertheless rely on proteolysis for maturation. Hence proteases are key processors in viral replication, and it is in fact exceptional for a virus not to require a proteolytic event at some stage in this cycle (Dougherty and Semler, 1993; Kräusslich and Wimmer, 1988). One or more proteases are usually involved, and can be of host or viral origin.

Table 1-2 details a variety of proteases involved in viral replication, and is representative but not exhaustive. Putative classifications and presumed functions have been included. A number have been classified as 'trypsin-like cysteine' proteases, in contrast to classical papain-like cysteine proteases. These are enzymes which have a reaction mechanism similar to the serine protease trypsin, but with a cysteine instead of a serine as the nucleophile in the catalytic triad (Bazan and Fletterick, 1988; Gorbalenya *et al.*, 1989). Virus-encoded aspartic, serine, cysteine, metallo- (Liu *et al.*, 2000) and metal ion-dependent (de Francesco *et al.*, 1998) proteases have been identified.

Table 1-2. Proteases involved in viral replication. (After Kemp *et al.*, 1992).

Family	Genus	Virus	Genome	Protease ^a	Class ^b	Functions
Picornaviridae	Enterovirus	Poliovirus	RNA	3C and 2A	TL-Cys	Polyprotein processing and inhibition of host systems
	Rhinovirus	Rhinovirus	RNA	3C and 2A	TL-Cys	
	Aphovirus	Foot and mouth disease virus	RNA	? 3C	? TL-Cys	
Picornaviridae-like plant virus	Comovirus	Cow pea mosaic virus	RNA	24K	TL-Cys	Polyprotein processing
	Potyvirus	Tobacco etch virus	RNA	Nla	TL-Cys	Polyprotein processing
				HCPPro	PL-Cys	Autocatalytic release
				P1	Serine?	Autocatalytic release
Togaviridae	Alphavirus	Sindbis virus	RNA	nsP2	PL-Cys	Polyprotein processing
				capsid protein host cellular	Serine	Autocatalytic release
	Rubivirus	Rubella virus	RNA	NS	Metallo-	Envelope maturation
	Flavivirus	Yellow fever virus	RNA	NS3	Serine	Polyprotein processing
Retroviridae		HIV	RNA	p11 host cellular	Aspartic ?	Polyprotein processing Envelope maturation
Coronaviridae		Murine hepatitis virus	RNA	L-Pro	PL-Cys	Polyprotein processing
Birnaviridae		IBDV	RNA	VP4	Serine?	Autocatalytic release
		IPNV		NS	Serine?	Autocatalytic release
Orthomyxoviridae		Influenza virus	RNA	host cellular	?	Envelope maturation
Paramyxoviridae	Morbillivirus	Measles virus	RNA	host cellular	?	Envelope maturation
Adenoviridae	Mastadenovirus	Adenovirus	DNA	23K	Cysteine	Maturation
Myoviridae		T4 phage	DNA	Gp21	Serine	Maturation

^a Proteases are virally encoded except where indicated as host cellular

^b TL-Cys, trypsin-like cysteine protease; PL-Cys, papain-like cysteine protease, see text for details

Processing of viral precursor proteins has also been demonstrated in other viral systems not listed in Table 1-2, including cardioviruses (Palmenberg *et al.*, 1992), reoviruses (Zweerink and Joklik, 1970), caulimoviruses (Torruella *et al.*, 1989), tymoviruses (Morch *et al.*, 1982), orthopoxviruses (VanSlyke *et al.*, 1991) and herpesviruses (Welch *et al.*, 1991).

Not all viruses dependent on proteolysis encode their own proteases; many make use of those present in the host cell. Those that do, however, have some selective advantage in that the range of cells in which they may replicate is not limited by the host cell's protein processing specificity. The viral protease must also have evolved to avoid the diverse protease inhibitors and control mechanisms present in host cells, and as a consequence, are expected to be fairly unusual molecules. As more viral proteases are identified, it is apparent that many have primary structures unlike those of any non-viral proteases and have reaction mechanisms significantly different to those characterised from higher organisms. Naturally, these distinctive mechanisms provide an ideal 'weak link' to be exploited by the development of highly specific viral inhibitors (Kay and Dunn, 1989; Kemp *et al.*, 1992). Highly specific, non-toxic compounds can be identified using three-dimensional computer reconstruction models of proteases on the basis of primary sequence (Cohen *et al.*, 1991). With particular emphasis on the protease active sites, the models are screened as receptors (proteases) for ligand docking (inhibitors). This system is most attractive as less material is needed for computer modelling than for protein crystallography, and the computer screening programs speed up optimisation of an inhibitor enormously (Bugg *et al.*, 1993).

Research clearly indicates that manipulation of viral protease activity is an effective strategy in the control of viral disease. Ni *et al.* (1995) showed that the attenuation of a vaccine strain of encephalitis may be attributable to diminishing protease activity. A variety of synthetic inhibitors have been developed against the aspartic protease of HIV (Kempf *et al.*, 1991; Lam *et al.*, 1994) and synthetic peptides show promise against the protease of hepatitis C virus (Ingallinella *et al.*, 2000, Han *et al.*, 2000). Inhibition of the cysteine protease of foot-and-mouth disease virus has reduced viral yields *in vitro* (Kleina and Grubman, 1992) and a variety of inhibitors have been identified against the herpesvirus proteases (Waxman and Darke, 2000). Inhibitors of pancreatic and leukocyte elastase also proved effective in reducing *in vitro* viral yield in HeLa cells infected with poliovirus or rhinovirus respectively (Molla *et al.*, 1993) and there is further hope for a cure for the common cold in the form of mechanism-based inhibitors of the rhinovirus protease (Matthews *et al.*, 1999), one of which has entered clinical trials.

As detailed in Table 1-2, virus-encoded proteases appear to fulfil one of three possible functions during the replication cycle, namely, those of polyprotein cleavage, virion maturation and inactivation of host systems. The viral replication strategy leading to the production of polyproteins requires the co-evolution of mechanisms to allow its functional separation into different domains. Consequently, most viral proteases are involved in the processing of a high molecular mass polyprotein into functional gene products (Dougherty and Semler, 1993). In many cases, the most important event is the separation of structural from non-structural domains. However, proteolytic processing of non-structural polyproteins may not be merely for separation but for the activation of diverse functions. Proteolytic cleavage *in vivo* is an irreversible process and proteases do not have repair functions. Cleavage of a polyprotein may thus trigger irreversible changes in the properties of viral translation products by activating enzymes or by inducing conformational changes in structural proteins (Dougherty and Semler, 1993).

Proteolytic maturation occurs mainly in icosahedral or enveloped viruses. The maturation cleavages in the protein capsids of non-enveloped (or naked) virions generally take place after the capsid has been assembled and in most instances after the nucleic acid has been packaged. These cleavages are carried out by viral proteases and are often autocatalytic, the capsid protein doubling as its own maturation protease. In the case of enveloped viruses, maturation involves cleavage of envelope (glyco)proteins which are usually carried out by host proteases. In both instances, proteolytic maturation is essential for the development of infectious viral particles (Kemp *et al.*, 1992). Looking at the beginning of the infective cycle, it is also likely that proteolysis has a role to play in viral uncoating after entry into target cells.

In some cases, viral proteases facilitate viral replication in an indirect way by inhibition of host cell systems. Proteases of picornaviruses such as poliovirus are involved in the shutdown of host cell protein synthesis and HIV protease has been shown to cleave cytoskeletal proteins *in vitro*, perhaps indicating a role in release of virus from the cell (Kemp *et al.*, 1992). Viral proteases can also be involved in the processes by which cells are killed during lytic infections. Cell death can result from virus-induced cell-cell fusion to produce syncytia. Syncytia formation occurs in retroviral infections and in parainfluenza infections, where the host protease cleavage of the F protein has a central role (Kemp *et al.*, 1992).

Thus it is apparent that within the cycles of viral infection and replication, different types of proteolytic cleavage occur and all cleavage events for a given virus may not be the same.

This results in a post-translational regulation which represents a cascade of tightly controlled cleavage events (Dougherty and Semler, 1993). However, it should also be noted that viral proteases alone might not be sufficient to effect all cleavages; co-factors of both viral and cellular origin have been implicated along with the viral enzymes in several processes (Kräusslich and Wimmer, 1988). Much remains to be discovered about the complex role of these enzymes, with their unique specificities and mechanisms and accessory functions that may be distinct from protein cleavage.

1.5.3 Investigative appeal of the putative IBDV protease

As is apparent from Section 1.5.2, viral proteases are highly intriguing and vitally important enzymes in viral infection and replication. In addition, IBD is a disease of great commercial significance. Hence the possibility that IBDV may encode its own protease presents a number of compelling reasons to investigate its putative activity, namely:

- an enzyme of this nature is an ideal target for anti-viral agents, in the form of both inhibitors and antibodies, and in addition, a potential vaccine based on VP4 would have excellent local relevance;
- VP4 could likewise prove a specific and sensitive diagnostic protein, from the point of view of both antibody- and PCR-based assays; VP4 is highly conserved among IBDV strains and furthermore, GenBank and SwissProt searches did not uncover any significant protein homologies, other than with VP4 of other birnaviruses;
- a putative protease appears to be a suitable region to investigate for classification and identification of local strains and perhaps to understand the origin and dissemination of strains;
- as an enzyme possibly subjected to mutation, VP4 may hold clues to the observed increases in IBDV virulence, and may perhaps be artificially mutated to attenuate virulence as a potential vaccine strain;
- finally, characterisation of VP4 would increase the general understanding of viral enzymes and may lead to fresh protease insights.

1.6 Investigative objectives

As detailed in the preceding section, the investigation of the putative protease of IBDV holds much potential. Consequently, VP4 was the focus of this study, the objectives of which encompassed cloning and expression of recombinant VP4; using VP4 as a marker in nucleic acid-based and immunological assays for rapid and effective detection of IBDV; using VP4 sequence information to assess the relatedness of South African IBDV to global strains and examination of the enzymatic activity of VP4.

Evidence in the literature suggested that a recombinant protein route would yield sufficient quantities of VP4 for biochemical characterisation. Hence, strategies to clone and express IBDV ORF A1 in its entirety, yielding correctly processed VP4 and coat proteins, were investigated (Chapter 4). A reverse genetics system for the dsRNA virus also has the potential to facilitate understanding of the regulation of viral gene expression and pathogenesis, assisting the development of potential vaccines. It became necessary to later modify this objective to cloning and expression of VP4 alone, as explained in Chapter 4.

An RT-PCR procedure was developed to amplify the VP4 gene as a key step towards cloning (Chapter 4), which was also useful in the pursuit of a complimentary objective. This was the investigation of local strains of IBDV spanning years including those in which a very virulent outbreak had occurred. The RT-PCR was assessed as a locally relevant diagnostic assay for IBDV (Chapter 4) and amplified fragments were subjected to RFLP and phylogenetic analysis to examine the relationship between local and global strains of IBDV (Chapter 4).

Preliminary investigations of VP4 at an immunological and enzymatic level were also undertaken. Anti-peptide antibodies were raised in chickens and rabbits against an appropriate region of VP4 (Chapter 5). These antibodies were assessed for recognition of the peptide and of viral protein in crude fractions of infected and uninfected bursae (Chapter 5). The crude fractions were also assayed for proteolytic activity against a synthetic peptide substrate (Chapter 5). The effects of assorted inhibitors and the anti-peptide antibodies on this activity were also examined (Chapter 5).

Chapter 2

General materials and methods:

Molecular Biology

2.1 Introduction

Aspects of IBDV were examined at both the nucleic acid and the protein level. For convenience and simplicity, general materials and methods in molecular biology will be presented in this chapter while those for protein biochemistry follow in Chapter 3. Details of specific techniques within these broadly defined areas will be described in subsequent relevant sections.

2.2 Materials

Common laboratory reagents were from BDH (Poole, UK), Boehringer Mannheim (Mannheim, Germany), ICN (Aurora, USA) or Merck (Darmstadt, Germany) and were of analytical or higher purity. Distilled water (dH₂O) was obtained with a Milli-RO[®] 15 Water Purification System (Millipore, Marlboro, USA). Distilled, deionised water (ddH₂O) was obtained with a Milli-Q Plus Ultra-Pure Water System (Millipore, Marlboro, USA). The minimum resistivity of the ddH₂O was 18 MΩ.cm. Sterilisation of glassware, disposable plasticware, buffers and solutions took place either by autoclaving at 121°C, 15 psi for 20 min on liquid cycle or by filtration through 0.22 µm membranes. Sterile technique was employed for all manipulations of bacteria, virus-infected samples and nucleic acids. For convenience, the sources of specialised materials are listed below.

Bursal tissue infected with IBDV was obtained from Dr Roger Horner (Allerton Regional Veterinary Laboratory, Pietermaritzburg, South Africa). Agarose (type I-A: low EEO), acridine orange and rubidium chloride were from Sigma (St Louis, USA). Media components for bacterial culture were from Biolab (Midrand, SA). Oligonucleotide primers were synthesised by the Department of Virology, University of Natal Medical School (Durban, SA) or by Gibco BRL (Paisley, UK). Thin-walled PCR reaction tubes (0.2 ml) were from QSP (Petaluma, USA). Sterile, disposable plasticware for cell culture was from Corning/Costar (Cambridge, USA). [α -³²P]dCTP was from ICN Pharmaceuticals (Aurora, USA). Random hexamer primers, T4 DNA ligase and *E. coli* strain JM109 were from Promega (Madison, USA). Restriction enzymes were either from Boehringer Mannheim (Mannheim, Germany),

Promega (Madison, USA), Amersham (Cleveland, USA) or New England Biolabs (Beverly, USA). Moloney murine leukaemia virus reverse transcriptase (MMLV-RT) was from Gibco BRL (Paisley, UK) or Promega (Madison, USA). *Taq* DNA polymerase was from Promega (Madison, USA), Biorline (BIOTAQ™ DNA polymerase, London, UK) or TaKaRa Biomedicals (Ex Taq, Gennevilliers, France). Proteinase K, deoxyribonucleoside triphosphates (dNTPs), ampicillin, kanamycin, digoxigenin (DIG) DNA labelling and detection kit and 4-morpholine-propanesulfonic acid (MOPS) were from Boehringer Mannheim (Mannheim, Germany). Magna charge nylon membrane was from Micron Separations (MSI), (Westborough, USA). QIAexpress Type IV kit and QIAEX II gel extraction kit were both from QIAGEN GmbH (Hilden, Germany). NucleoSpin Extract 2-in-1 gel extraction kit was from Machery-Nagel (Düren, Germany). T7 Sequenase version 2.0 DNA sequencing kit was from Amersham (Cleveland, USA).

2.3 Culturing bacteria

This study required the culture of various strains of *E. coli* for a variety of purposes. General methods for the growth of *E. coli* supported by solid and liquid media are described below.

2.3.1 Reagents

Luria-Bertani (LB) medium [1% (m/v) tryptone, 0.5% (m/v) yeast extract, 86 mM NaCl, pH 7.0]. Tryptone (10 g), yeast extract (5 g) and NaCl (5 g) were dissolved in dH₂O (1 litre) and the solution sterilised by autoclaving.

LB agar [1.5% (m/v) bacteriological agar in LB medium]. Bacteriological agar (15 g) was added to LB medium (1 litre) and autoclaved. Heat of autoclaving is sufficient to completely dissolve the agar, which melts at 100°C and remains liquid down to about 44°C.

Ampicillin stock solution [100 mg.ml⁻¹ ampicillin]. Ampicillin (300 mg) was dissolved in ddH₂O (3 ml) and the solution sterilised by filtration. The solution was stored at -20°C in a light-tight container for no longer than 3 months. Stock ampicillin was added to media in a 1:1000 ratio to give a working concentration of 100 µg.ml⁻¹.

Kanamycin stock solution [25 mg.ml⁻¹ kanamycin]. Kanamycin (75 mg) was dissolved in ddH₂O (3 ml) and the solution sterilised by filtration. The solution was stored at -20°C in a light-tight container for no longer than 3 months. Stock kanamycin was added to media in a 1:1000 ratio to give a working concentration of 25 µg.ml⁻¹.

2.3.2 Procedure

LB agar was cooled to about 55°C, appropriate volumes of stock antibiotics added and plates poured (approximately 45 ml each) following standard laboratory procedure (Stanier *et al.*, 1987). A sterile toothpick was used to remove and streak out a trace of cells from vials stored at -70°C, and streaked plates were incubated at 37°C overnight (ON). A single colony was subsequently picked from plates to inoculate volumes of LB medium, containing appropriate antibiotics, as required. LB medium was cooled to about 50°C before addition of antibiotics. Plates with colonies were sealed with masking tape and stored at 4°C for use as convenient, but were not kept any longer than 10 days.

2.4 Transformation of *E. coli*

The term transformation broadly defines the uptake of any DNA molecule by any type of cell, regardless of whether the uptake results in any detectable change in the cell, or whether the cell involved is bacterial, fungal, animal or plant. For the purposes of this study, transformation will specifically refer to the uptake of plasmids by assorted strains of *E. coli*.

Most species of bacteria are able to take up DNA molecules from the medium in which they grow. Such DNA is often degraded, but may occasionally survive and replicate in the host cell. This occurs particularly when the DNA molecule is a plasmid with an origin of replication recognised by the host. In the current study, strains of *E. coli* were transformed in order to 1) propagate sufficient quantities of expression vectors, and 2) express genes subsequently cloned into these vectors. Transformation and cloning thus allow a large number of molecules (both DNA and protein) to be produced from limited starting material, which are in addition functionally purified by the process as a single clone contains a single form of vector.

Although some species of bacteria will take up limited amounts of DNA under normal circumstances, natural transformation is not a major process by which the majority of species obtain genetic information (Stanier *et al.*, 1987). Consequently, bacteria have to undergo

some form of physical and/or chemical treatment in order to enhance their ability to take up plasmids; such cells are then said to be competent.

Most methods for transformation are based on the observations of Mandel and Higa (1970), who showed that bacteria soaked in ice-cold solutions of calcium chloride and then briefly heated could be transfected with bacteriophage λ DNA. Rubidium chloride and potassium chloride are also effective salts in the treatment, though exactly why it works is not understood. Possibly the salt treatment causes precipitation of DNA on the cell surface, or it may render a change in the cell wall that improves DNA binding. Soaking in salt solution, however, affects only DNA binding: when DNA is added to competent cells it remains attached to the cell exterior. Movement of DNA into the cells is stimulated by briefly raising the temperature to 42°C, but again, the reason why this heat-shock is effective is unclear.

2.4.1 Transformation of competent JM109 cells

E. coli strain JM109 was used for propagation and storage of pQE plasmids. Preparation and transformation of competent cells was conducted according to the supplier's instructions.

2.4.1.1 Reagents

Trituration buffer [100 mM CaCl₂, 70 mM MgCl₂, 40 mM sodium acetate (NaAc), pH 5.5]. CaCl₂ (2.20 g), MgCl₂ (2.13 g) and NaAc (0.82 g) were dissolved in ddH₂O (100 ml), titrated to pH 5.5 with HCl and made up to 150 ml. The solution was made up fresh before use and sterilised by sequential filtration through 0.45 μ m and 0.22 μ m membranes.

Glycerol solution [80% (v/v)]. 99.5% (v/v) glycerol (8 ml) was diluted to 10 ml with ddH₂O and sterilised by autoclaving.

2.4.1.2 Procedure

Competence protocol. Antibiotics were not included at any stage during this process. Fresh cultures of JM109 cells were grown on LB agar plates from stocks stored at -70°C, as described in Section 2.3.2. A single colony was picked into 25 ml LB medium and incubated at 37°C ON with vigorous shaking. Using a 250 ml flask, LB medium (100 ml) was inoculated with cells from the ON culture (1 ml) and shaken at 37°C until the A₆₀₀ reached 0.52 (mid log phase, 3.5 h). The cells were chilled in an ice water bath (3 h) and collected by

centrifugation (2500×g, 20 min, 4°C). From this point on, care was taken to ensure that the cells were kept ice-cold.

The media were decanted and the pelleted cells gently resuspended in ice-cold trituration buffer (4 ml), diluted to 100 ml with the same buffer and incubated on ice (45 min). The cells were centrifuged (1800×g, 10 min, 4°C), the supernatant discarded and the pellet gently resuspended in ice-cold trituration buffer (10 ml). Glycerol solution (~1.9 ml) was added dropwise with gentle swirling to a final concentration of about 15% (v/v). Cells (200 µl) were aliquoted into cryovials, snap-frozen in liquid nitrogen and stored at -70°C.

Transformation protocol. Aliquots (200 µl) of competent cells were thawed on ice and dimethylsulfoxide (DMSO) (3 µl) added to each tube. DMSO has been empirically determined to improve transformation efficiency (Kushner, 1978). The solution was mixed briefly before addition of plasmid DNA (10-20 ng) and incubation on ice (30 min). Cells were heat shocked in a water bath at 42°C (90 sec), and cooled on ice (1 min). LB medium (2 ml) was added to each aliquot and the cells shaken gently at 37°C (1 h) to allow recovery. Cells were concentrated before plating by centrifugation in a microfuge [5 000 rpm, 1 min, room temperature (RT)], removal of the supernatant and resuspension in LB medium (200 µl). This concentrated cell suspension was plated on LB plates containing 100 µg.ml⁻¹ ampicillin and incubated at 37°C ON (Section 2.3.2).

2.4.2 Transformation of competent M15 [pREP4] cells

E. coli strain M15 containing the repressor plasmid pREP4 was used for production of recombinant proteins when transformed with pQE plasmids. Preparation and transformation of competent cells was conducted according to the supplier's instructions.

2.4.2.1 Reagents

Transformation buffer 1 (TFB1) [100 mM RbCl, 50 mM MnCl₂, 30 mM potassium acetate (KAc), 10 mM CaCl₂, 15% (v/v) glycerol, pH 5.8]. RbCl (1.2 g), MnCl₂ (0.99 g), KAc (0.29 g), CaCl₂ (0.15 g) and 99.5% (v/v) glycerol (15 ml) were dissolved in ddH₂O (70 ml) and titrated to pH 5.8 with HCl. The solution was made up to 100 ml and sterilised by sequential filtration through 0.45 µm and 0.22 µm membranes. TFB1 was made up fresh before use.

Transformation buffer 2 (TFB2) [10 mM MOPS, 10 mM RbCl, 75 mM CaCl₂, 15% (v/v) glycerol, pH 8.0]. MOPS (0.21 g), RbCl (0.12 g), CaCl₂ (1.1 g) and 99.5% (v/v) glycerol (15 ml) were dissolved in ddH₂O (70 ml) and titrated to pH 8.0 with NaOH. The solution was made up to 100 ml and sterilised by autoclaving. TFB2 was made up fresh before use.

Psi broth [LB medium, 4 mM MgSO₄, 10 mM KCl]. MgSO₄ (0.098 g) and KCl (0.075 g) were dissolved in LB medium (100 ml) and sterilised by autoclaving.

2.4.2.2 Procedure

Competence protocol. Fresh cultures of M15 [pREP4] cells were grown on LB agar plates (containing 25 µg.ml⁻¹ kanamycin) from stocks stored at -70°C, as described in Section 2.3.2. A single colony was picked into LB medium (1 ml) and incubated at 37°C with vigorous shaking until mid log phase was reached (2 h). Using a 250 ml flask, LB medium (100 ml) was inoculated with the entire pre-culture (1 ml) and shaken at 37°C until the A₆₀₀ reached 0.5 (4 h). The culture was centrifuged (385×g, 5 min, 4°C) and the supernatant discarded. From this point on, care was taken to ensure that the cells were kept ice-cold.

Pelleted cells were gently resuspended in ice-cold TFB1 (30 ml) and incubated on ice (1.5 h). The cells were centrifuged (385×g, 5 min, 4°C), the supernatant discarded and the pellet gently resuspended in ice-cold TFB2 (4 ml). Cells (500 µl) were aliquoted into cryovials, snap-frozen in liquid nitrogen and stored at -70°C.

Transformation protocol. Plasmid preparations (20 µl or less) were transferred into cold 2.5 ml cryovials and kept on ice. Aliquots (500 µl) of competent cells were thawed on ice and gently resuspended. Cell suspension (125 µl) was transferred to each vial of plasmid preparation, which was mixed carefully and incubated on ice (20 min). Cells were heat shocked in a water bath at 42°C (90 sec). Psi broth (500 µl) was added to each vial and the cells shaken gently at 37°C (1.5 h) to allow recovery. Subsequently, 50 µl and 450 µl aliquots were plated on LB plates containing kanamycin (25 µg.ml⁻¹) and ampicillin (100 µg.ml⁻¹) and were incubated at 37°C ON (Section 2.3.2). For a negative control, cells were transformed with Tris-EDTA (TE) (Section 2.8.2) (20 µl) and the entire transformation mix was plated on a single plate. For a positive control, cells were transformed with pQE-16 control plasmid (1 ng) in TE (20 µl) and dilutions of the transformation mix (1:10, 1:100, 1:500 in Psi broth)

plated out (100 μ l). The cells should yield 1×10^6 transformants per μ g of plasmid according to the QIAexpress specifications.

2.5 Isolation of plasmid DNA

Plasmids are particularly useful in molecular biology, and the development of recombinant DNA technology would not have been possible without them. These self-replicating closed circles of dsDNA, which occur naturally as dispensable genetic elements in bacteria, have been adapted and reconstructed to facilitate a vast array of procedures with relative simplicity (Stanier *et al.*, 1987).

If plasmids are to be used as cloning vehicles, as they were for the purposes of this investigation, it is essential that they be isolated from the large amount of bacterial chromosome DNA that is also present in the cells. A variety of methods exist for the extraction and purification of plasmids from bacteria, all of which exploit the physical differences of size and conformation between plasmid and bacterial DNA.

2.5.1 Plasmid mini-prep by alkaline lysis

The method used in this study is an adaptation of those of Birnboim and Doly (1979) and Ish-Horowitz and Burke (1981). In any purification of nucleic acids, whatever structure contains the genetic material needs to be ruptured in such a way that the nucleic acid is released without damage. Bacteria are suspended in an isosmotic solution of glucose, and treated with ethylenediaminetetra-acetic acid (EDTA), which removes magnesium ions essential for preserving the framework of the cell envelope. The resulting sphaeroplast is lysed by the addition of a detergent (sodium dodecyl sulphate, SDS), which removes lipid molecules and causes disruption of the cell membrane. This method minimises the physical forces required to liberate the plasmid from the pressurised cell interior.

If lysis occurs under alkaline conditions (pH 12.0–12.5), hydrogen bonding in non-supercoiled DNA (i.e. the bacterial chromosome) is broken, which causes the double helix to unwind and separate. Addition of acid (potassium acetate) at this stage leads to re-aggregation of the denatured strands into a tangled mass, which is easily pelleted by centrifugation, leaving purified plasmids in the supernatant. As an added advantage, the specific usage of SDS and acetate also renders protein and RNA insoluble, allowing these contaminants to be removed by the centrifugation step. Deproteinisation by phenol extraction

and nuclease treatment to remove RNA may thus not be necessary if this alkaline denaturation method is employed. For unknown reasons, however, omitting phenol extraction sometimes results in DNA which is resistant to cleavage by restriction enzymes; hence this step was always included in plasmid isolations.

2.5.1.1 Reagents

EDTA stock solution [100 mM Na₂-EDTA, pH 8.0]. Na₂-EDTA (9.31 g) was dissolved in ddH₂O (230 ml), titrated to pH 8.0 with NaOH, made up to 250 ml and sterilised by autoclaving.

500 mM Tris-HCl, pH 8.0. Tris base (60.55 g) was dissolved in ddH₂O (950 ml), titrated to pH 8.0 with HCl, made up to 1 litre and sterilised by autoclaving.

Sphaeroplast buffer [25 mM Tris-HCl, 10 mM Na₂-EDTA, 50 mM glucose, pH 8.0]. 500 mM Tris-HCl (5 ml), glucose (0.90 g) and 100 mM Na₂-EDTA (10 ml) were dissolved in ddH₂O (80 ml), titrated to pH 8.0 with HCl, made up to 100 ml and sterilised by autoclaving. The solution was made up freshly and cooled to 4°C before use.

SDS stock solution [10% (m/v) SDS]. SDS (10 g) was dissolved in a total volume of 100 ml dH₂O. This process required gentle heating.

Alkaline lysis solution [200 mM NaOH, 1% (m/v) SDS]. NaOH (0.80 g) and 10% (m/v) SDS (10 ml) were dissolved in a total volume of 100 ml ddH₂O and sterilised by autoclaving.

Neutralisation solution [5 M KAc, pH 4.8]. Potassium acetate (KAc, 29.45g) was dissolved in ddH₂O (60 ml), glacial acetic acid (11.5 ml) and ddH₂O (28.5 ml) were added and the solution sterilised by autoclaving before storage at 4°C. This solution is 3 M with respect to potassium and 5 M with respect to acetate.

Tris-saturated phenol [phenol equilibrated with 500 mM Tris-HCl, pH 8.0]. This was prepared as per Sambrook *et al.* (1989a). Before use, phenol must be equilibrated to a pH >7.8 because DNA will partition into the organic phase at acid pH. Phenol was removed from storage at -20°C, warmed to RT and melted at 68°C. Hydroxyquinoline was added to 0.1% (m/v), acting as an antioxidant, a partial RNase inhibitor and a weak chelator of metal ions (Kirby, 1956). Its yellow colour also helps to identify the organic phase. An equal

volume of 500 mM Tris-HCl, pH 8.0 was added and the mixture stirred on a magnetic stirrer (15 min) and allowed to settle. As much as possible of the upper aqueous phase was removed by aspiration, and the process was repeated with 100 mM Tris-HCl, pH 8.0 (5-fold dilution of a 500 mM solution) until the pH of the phenolic phase was >7.8 , as measured with pH indicator strips. When finally equilibrated, 0.1 volumes of 100 mM Tris-HCl, pH 8.0 was added and the phenol was stored at 4°C in an amber bottle.

Phenol:chloroform [Tris-saturated phenol:chloroform, 1:1 (v/v)]. Equal volumes of Tris-saturated phenol and chloroform were gently mixed together and stored at 4°C in an amber bottle.

Ethanol solution [70% (v/v)]. 99.5% (v/v) ethanol (140 ml) was diluted to 200 ml with ddH₂O and stored at 4°C.

2.5.1.2 Procedure

LB medium containing appropriate antibiotics (5 ml in a 25 ml glass bottle) was inoculated with a single transformed bacterial colony (Sections 2.4.1.2 and 2.4.2.2). The container was loosely capped and incubated at 37°C ON, with vigorous shaking. An aliquot of the ON culture (1.5 ml) was placed in a microfuge tube and centrifuged (10 000 rpm, 30 sec, RT) in a microfuge. The remainder of the ON culture was stored at 4°C for use as needed. Medium was removed from the pelleted bacteria by aspiration, leaving the cells as dry as possible.

The pellet was resuspended by vigorous vortexing in ice-cold sphaeroplast buffer (100 µl), ensuring that the cells were completely dispersed. The cells were maintained at RT (5 min) before the addition of freshly prepared alkaline lysis solution (200 µl). The solution was mixed gently by about five inversions, since no vortexing should occur at this stage. The cloudy bacterial suspension cleared visibly after the dispersal of the lysis solution and was incubated on ice (5 min).

Ice-cold neutralising solution (150 µl) was mixed into the viscous bacterial lysate by inversion or slow vortexing (10 sec), causing precipitation of contaminants. The tube was incubated on ice (5 min) before centrifugation (12000×g, 5 min, 4°C), after which the supernatant was transferred to a clean tube. An equal volume of phenol:chloroform was added and mixed in by vigorous vortexing. The solution was centrifuged (16 000 rpm, 2 min,

RT) in a microcentrifuge, and the upper aqueous phase (containing the plasmids) transferred to a fresh tube.

Ice-cold 99.5% (v/v) ethanol (2.5 volumes) was added and mixed in thoroughly. DNA was allowed to precipitate by incubation either at -70°C for a minimum of 5 min, or longer at -20°C , as convenient. Plasmid DNA was pelleted by centrifugation ($12\,000\times g$, 15 min, 0°C), the supernatant was removed and the pellet washed with ice-cold 70% (v/v) ethanol (1 ml). Plasmid DNA was stored under 70% (v/v) ethanol at -20°C until required.

2.5.2 Cracking procedure for plasmid size estimation

The cloning systems used in this study did not allow for simple selection of recombinants, by blue/white colour screening, for example. The easiest way to screen antibiotic-selected colonies for recombinants was to estimate the size of plasmid DNA without endonuclease digestion, using the cracking procedure based on the method of Barnes (1977). This rapid disruption technique yields enough plasmid DNA to load in a single lane of an agarose gel. The plasmid size can be visualised following electrophoresis, and should indicate whether or not it carries an insert.

2.5.2.1 Reagents

2 \times Cracking solution [200 mM NaOH, 0.5% (m/v) SDS, 20% (m/v) sucrose]. NaOH (0.4 g), sucrose (10 g) and 10% (m/v) SDS (2.5 ml, Section 2.5.1.1) were dissolved in ddH₂O (50 ml) and sterilised by autoclaving.

2.5.2.2 Procedure

A sterile toothpick was used to remove a portion of individual colonies from plates, which was smeared at the bottom of a microfuge tube containing 10 mM Na₂-EDTA, pH 8.0 (5 μl of 100 mM stock solution, Section 2.5.1.1, diluted to 50 μl with sterile ddH₂O). The cells were resuspended by vortexing after the addition of 2 \times cracking solution (50 μl), and incubated at 70°C (5 min). The mixture was allowed to cool to room temperature, gel loading buffer (2 μl , Section 2.6.5) was added and the solution was mixed by vortexing. After incubation on ice (5 min), bacterial debris was pelleted by centrifugation in a microfuge (12 000 rpm, 3 min, RT) and 25-50 μl of the supernatant run on a 0.7% (m/v) agarose gel (Section 2.6.6).

2.6 Agarose gel electrophoresis of nucleic acids

The separation and visualisation of nucleic acids is an essential analytical and very useful preparative method, which can yield results within a couple of hours. In this study, agarose gel electrophoresis was used to: visualise, monitor and confirm purification of viral dsRNA; visualise products and product sizes from plasmid purification, RT-PCR, ligation and RFLP analysis; and to purify products of RT-PCR and assorted endonuclease restrictions from their associated contaminants. In addition, samples were electrophoresed with known amounts of marker DNA, which allowed estimation of both the size and concentration of a sample band. Size is estimated in terms of length in bp.

2.6.1 Principles of agarose gel electrophoresis

Agarose is a high molecular mass polysaccharide isolated from agar or recovered directly from agar-bearing marine algae from the class Rhodophyta. This linear galactan hydrocolloid is an alternating 1,3-linked β -D-galactopyranose and 1,4-linked 3,6-anhydro- α -L-galactopyranose, but different agarose preparations vary significantly with respect to their physical and chemical properties (Allen and Budowle, 1994a). Type I-A: low EEO agarose (Sigma) has a low sulfate content, and thus low electroendosmosis (EEO) and also has sufficiently high gel strength to be suitable for all electrophoretic separations carried out in the current study.

Preparation of agarose gels simply involves melting the agarose in the presence of the required buffer and pouring the molten solution into a casting mould where it is allowed to solidify. The resultant gel has a macroreticular structure, the pore size of which is determined by the agarose concentration. Consequently, the effective separation of nucleic acids is also dependent on agarose concentration, as detailed in Table 2-1.

Table 2-1. Effective agarose gel concentrations for resolution of linear nucleic acids.

Agarose concentration (% m/v)	Size range of linear molecules effectively separated (bp)
0.5	1 000 – 30 000
0.7	800 – 12 000
1.0	500 – 10 000
1.2	400 – 7 000
1.5	200 – 3 000
2.0	50 – 2 000

The phosphate linkers of nucleic acids are negatively charged at the slightly alkaline pH at which electrophoresis is conducted, ensuring a uniform charge-to-mass ratio. When an electric field is applied across the gel, nucleic acids migrate towards the anode, tending to orient end-on (Aaij and Borst, 1972), and separating on the basis of size. The rate of migration is inversely proportional to the log of the number of base pairs (Helling *et al.*, 1974), larger molecules being slowed down by their increased frictional drag and by their inability to slip through the gel pores as efficiently as smaller molecules.

Conformation of the nucleic acid will also affect its migration: superhelical circular plasmid DNA usually migrates faster than its nicked circular or linear form (Thorne, 1967). The intercalating dye EtBr (EtBr) affects conformation by lengthening the molecules and making them more rigid, causing a decrease in mobility of approximately 15% (Sambrook *et al.*, 1989b).

2.6.2 EtBr staining and detection by UV fluorescence

In 1973, Sharp and co-workers introduced electrophoresis of DNA fragments through agarose gels in the presence of EtBr as a rapid, inexpensive assay for the cleavage products of restriction endonucleases (Sharp *et al.*, 1973). This ultraviolet (UV)-fluorescent dye has planar rings that associate with nucleic acids by intercalating between adjacent base pairs, causing partial unwinding of the double helix. Because EtBr associated with nucleic acid constitutes a higher fluorescent concentration than unbound dye, small amounts of nucleic acid can be detected in the presence of free EtBr in the gel. Double- and single-stranded

forms of both DNA and RNA may be detected with EtBr, although the dye has a lower affinity for single-stranded nucleic acids, which consequently fluoresce less brightly.

Brunk and Simpson (1977) improved this technique by determination of the optimum UV wavelength (302 nm) for detection of EtBr-DNA complexes. Radiation at this wavelength is absorbed by the dye and re-emitted at 590 nm, in the red-orange range of the visible spectrum. Excitation at 254 nm and 366 nm produce far less fluorescence, and in addition, the short-wave UV radiation causes photonicking and photobleaching within a very short time period.

2.6.3 Improving sensitivity with decreased dye concentration

Dutton *et al.* (1995) noted that fluorescence in Tris buffer varied as a function of the DNA:EtBr (m/m) ratio, with maximal fluorescence occurring at DNA:EtBr values between 0.5 and 3 (Figure 2-1).

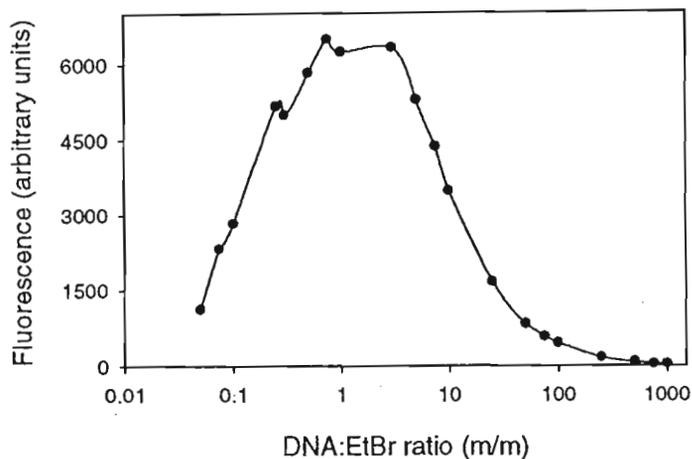


Figure 2-1. Graph illustrating the relationship between fluorescence and the DNA:EtBr (m/m) ratio.

The fluorescence (excitation wavelength, 360 nm; emission wavelength, 590 nm; arbitrary units) of 15 μg of DNA with varying amounts of EtBr was measured in a spectrofluorometric plate reader. (After Dutton *et al.*, 1995).

From Figure 2-1, it is clear that excess EtBr can be as much cause of poor fluorescent yields and low sensitivity as too little dye. Bearing this in mind, the range of DNA optimally stained in agarose gels of varying lane width was calculated (Table 2-2), using the standard EtBr working concentration of $0.5 \mu\text{g}\cdot\text{ml}^{-1}$ (Sambrook *et al.*, 1989c). These calculations revealed that $0.5 \mu\text{g}\cdot\text{ml}^{-1}$ was in fact too much EtBr for the quantity of DNA present per band. Further calculations (Table 2-2) determined that a working concentration of $0.27 \mu\text{g}\cdot\text{ml}^{-1}$ should give a higher fluorescent yield and hence greater sensitivity, and EtBr was used at this

concentration throughout this study. Since EtBr is moderately toxic and a powerful mutagen, almost halving the working concentration also has safety benefits.

Table 2-2. Range of DNA optimally stained by differing concentrations of EtBr.

No. of wells	Lane width (mm)	DNA per band optimally stained at	DNA per band optimally stained at
		0.5 $\mu\text{g}\cdot\text{ml}^{-1}$ EtBr (ng)	0.27 $\mu\text{g}\cdot\text{ml}^{-1}$ EtBr (ng)
16	2.5	1.7 – 9.4	0.9 – 5.1
12	4	2.6 – 15.0	1.4 – 8.1
8	6.5	4.1 – 24.4	2.2 – 13.2

Throughout this study, nucleic acid pellets (plasmid DNA, viral dsRNA, amplified DNA from RT-PCR) were resuspended after precipitation and collection by centrifugation. Considering the optimum amounts of DNA stained per band at 0.27 $\mu\text{g}\cdot\text{ml}^{-1}$ EtBr and that nucleic acids are more stable concentrated than dilute, an effort was made to resuspend nucleic acids in volumes of sterile ddH₂O giving a final concentration of approximately 10 $\text{ng}\cdot\mu\text{l}^{-1}$. This allowed small volumes of nucleic acid samples to be loaded on gels for optimal visualisation, and also ensured that samples were concentrated enough for downstream applications. Common pellet sizes and resuspension volumes are detailed in Table 2-3.

Table 2-3. Resuspension volumes for nucleic acid pellets.

Approximate size of pellet	Resuspension volume
	(μl)
not visible	3
visible speck (•)	5
pin head (♠)	10
match stick end (♣)	20
match head (♠)	50

2.6.4 Photography and estimation of band size and concentration

Nucleic acids were visualised by placing gels on a 300 Series Foto UV DNA Transilluminator (Fotodyne, New Berlin, USA) and exposing them to 300 nm UV radiation. Gels were photographed using a direct copy hood attached to a camera loaded with Polaroid 667 film. Consistently good photographs were obtained using an f-stop aperture of f/8, a shutter speed of 2 sec and a developing time of 30 sec.

The size and amount of nucleic acid in each band was estimated by reference to known amounts of molecular size markers electrophoresed simultaneously. *Hind* III-cut λ DNA was used as marker DNA, unless otherwise mentioned (Table 2-4). Smaller quantities of marker DNA (125-50 ng) electrophoresed best for gels with many wells (narrow lane width), while relatively more DNA (250-500 ng) was optimal for gels with fewer wells (wider lane width). Literature values for the sensitivity of EtBr staining vary from one to five ng, depending on photographic exposure time (Allen and Budowle, 1994b; Sambrook *et al.*, 1989d). In this study, 0.6 ng of the 564 bp-marker DNA could still be visually detected when run on a 16-well gel, demonstrating the higher sensitivity achieved using a lower concentration of dye.

Table 2-4. Stoichiometric distribution of mass among *Hind* III-cut λ DNA fragments.

Fragment size (bp)	Total λ DNA and amount per restriction fragment (ng)				
	500	250	125	100	50
23130	239.0	119.5	59.8	47.8	23.9
9416	97.0	48.5	24.3	19.4	9.7
6682	69.0	34.5	17.3	13.8	6.9
4361	45.0	22.5	11.3	9.0	4.5
2322	24.0	12.0	6.0	4.8	2.4
2027	21.0	10.5	5.3	4.2	2.1
564	6.0	3.0	1.5	1.2	0.6

2.6.5 Reagents

Hind III-cut λ DNA molecular size markers. Phage λ DNA (125 μg) was placed in a microfuge tube with a three-fold excess of *Hind* III (38U) and the supplier's *Hind* III 10 \times buffer (50 μl). The volume made up to 500 μl with ddH₂O. The tube was incubated at 37°C for 24 h to allow complete digestion, yielding a stock solution of molecular size markers of 250 ng. μl^{-1} . The solution was stored at 4°C where it remained stable for many months.

50 \times Tris-acetate buffer (TAE) [2 M Tris-acetate, 50 mM Na₂-EDTA, pH 8.0]. Tris base (242 g), glacial acetic acid (57.1 ml) and Na₂-EDTA (18.61 g) were dissolved in dH₂O (500 ml), titrated to pH 8.0 with HCl and made up to 1 litre. The stock solution was stored at RT and diluted 50-fold with dH₂O to yield a 1 \times working solution as needed. This tank buffer was re-used for electrophoresis a maximum of three times.

EtBr stock solution [10 mg. ml^{-1} EtBr]. EtBr (1 mg) was dissolved in dH₂O (100 ml) by stirring for several hours on a magnetic stirrer. The container was wrapped in aluminium foil to prevent photobleaching, and the solution was stored at RT. EtBr was used at a working concentration of 0.27 $\mu\text{g}.\text{ml}^{-1}$.

Gel loading buffer [0.25% (m/v) bromophenol blue, 0.25% (m/v) xylene cyanol FF, 1 mM Na₂-EDTA, pH 8.0, 50% (v/v) glycerol]. Bromophenol blue (50 mg) and xylene cyanol FF (50 mg) were dissolved in dH₂O (8 ml), 100 mM Na₂-EDTA (200 μl , Section 2.5.1.1) and 99.5% (v/v) glycerol (10 ml) were added and the volume made up to 20 ml. The solution was sterilised by autoclaving and stored at RT.

2.6.6 Procedure

For the purposes of this investigation, agarose gels were run in a MINNIE Submarine Agarose Gel Unit, model HE 33 (Hoefer, San Francisco, USA). The open ends of a casting tray were sealed with masking tape and the tray was placed on a level surface. A comb was positioned in the tray, ensuring that the teeth were perpendicular. Powdered agarose (mass appropriate for the desired gel concentration) was weighed out in a 100 ml Erlenmeyer flask and 1 \times TAE (37 ml) carefully added, rinsing down any agarose grains adhering to the sides of the flask.

The neck of the flask was stoppered with rolled-up laboratory paper towel and the slurry heated in a microwave oven with careful swirling at regular intervals, preventing boiling and

keeping all grains in the solution. This was continued until the agarose had completely dissolved, as evidenced by the clearing of the solution and disappearance of 'lenses' of partially dissolved agarose, which are easily checked for by holding the solution up to the light.

The molten agarose was cooled to about 50°C by rotating the flask under running tap water. EtBr (1 µl of 10 mg.ml⁻¹ stock solution) was added with swirling and the liquid gel poured rapidly and steadily into the prepared casting tray. Any air bubbles under or between the teeth of the comb were removed, and the gel was allowed to set completely at RT (15-20 min). Refrigeration of gels to hasten the setting process was avoided, as this appeared to result in skewed bands with poor definition. This effect is probably caused by uneven polymerisation.

The comb and masking tape were carefully removed and the casting tray placed in the electrophoresis tank. Sufficient 1×TAE (about 250 ml) was poured in to just cover the surface of the gel to a depth of about 1 mm. EtBr (6.8 µl of 10 mg.ml⁻¹ stock solution) was added to the tank buffer and dispersed with gentle agitation of the pod.

A small drop of gel loading buffer (about 0.5 µl) was mixed with nucleic acid samples and molecular size markers and the solution slowly pipetted into the submerged wells. Following electrophoresis [8 V.cm⁻¹ (100 V), unlimiting current, 1 h, RT] the gel was examined by UV light and photographed as required (Section 2.6.4). When information was needed rapidly, samples were electrophoresed at 16 V.cm⁻¹ (200 V) for 15 – 30 min. Bands obtained were not as straight, sharp or resolved as in the slower separation, but were still easily discernible.

2.7 Purification of DNA for downstream manipulations

In this study, purification of DNA was required for a number of downstream applications, which included removal of contaminants from PCR products before sequencing and ligation reactions, removal of stuffer fragments from restricted plasmids and desalting DNA that required double digestion. Various techniques were employed to achieve these more discriminating clean-up procedures, though all had disadvantages and were not entirely satisfactory as far as yield, simplicity, speed and reliability were concerned.

2.7.1 Extraction of DNA fractionated in agarose gels

Two commercial kits and the recovery method of and Tautz and Renz (1983) were used to extract DNA from agarose gel slices.

The QIAEX II gel extraction system (QIAGEN, Hilden, Germany) is based on solubilisation of agarose and selective adsorption of nucleic acids onto QIAEX II silica gel particles in the presence of a chaotropic salt. The salt leads to a reversible disruption of the hydrate shell of the nucleic acid, allowing adsorption. Contaminants are removed by washing the pelleted gel, and the nucleic acids are subsequently eluted in a low salt buffer. The NucleoSpin Extract 2-in-1 kit (Machery-Nagel, Düren, Germany) functions on very similar principles, except that a high-affinity membrane in a spin column binds nucleic acid in the presence of a chaotropic salt, rather than the silica gel. These kits were used according to the manufacturers' instructions, and although the procedures proved to be rapid, simple and organic solvent-free, both kits gave inconsistent yields, some of which were as poor as 5.6%.

Tautz and Renz (1983) found that a freeze-squeeze method for extraction of DNA from agarose gels could be optimised with respect to pH, ionic composition of elution buffers and the concentration of the gel. While rather tedious, this method proved to be the most reliable, with consistent yields of at least 50%. However, the method requires ethanol precipitation of the purified DNA and residual ethanol can cause difficulties with restriction.

2.7.1.1 Reagents

QIAEX II gel extraction kit (QIAGEN, Hilden, Germany). The QIAEX II silica gel particle suspension was used with the supplied buffers according to the manufacturer's instructions. DNA was eluted from the particles with ddH₂O.

NucleoSpin Extract 2-in-1 kit (Machery-Nagel, Düren, Germany). The NucleoSpin extract columns were used with the supplied buffers according to the manufacturer's instructions. DNA was eluted from the membrane with ddH₂O.

Equilibration buffer [300 mM NaAc, 1 mM Na₂-EDTA, pH 7.1]. NaAc (4.08 g) was dissolved in ddH₂O (90 ml), 100 mM Na₂-EDTA (1 ml, Section 2.5.1.1) was added, titrated to pH 7.1 with NaOH and made up to 100 ml.

MgCl₂ solution [1 M MgCl₂, 10% (v/v) acetic acid]. MgCl₂ (2.03 g) was dissolved in ddH₂O (7 ml), glacial acetic acid (1 ml) was added and the volume made up to 10 ml.

2.7.1.2 Procedure

DNA fragments were electrophoretically separated and stained as described (Section 2.6.6) but using 0.6% (m/v) agarose gels as the lower percentage gel has a higher elution efficiency (Tautz and Renz, 1983). Bands of interest were excised from the gel using a clean scalpel, minimising the amount of excess agarose, and keeping exposure to UV radiation as short as possible. The gel slices were processed according to the instructions of the gel extraction kit manufacturers or by the method of Tautz and Renz (1983) as presented below.

A gel slice (usually about 150 µl in volume) was placed in a microfuge tube with a volume of equilibration buffer at least 10 times that of the slice (usually about 1.5 ml). The intact slice was incubated in the dark at RT with gentle agitation for about 20 min, transferred to a clean microfuge tube and completely frozen in liquid nitrogen (20 min). On removal, the microfuge tube was immediately centrifuged (30 000×g, 15 min, RT) and the supernatant transferred to a clean tube.

MgCl₂ solution (¹/100 volume of supernatant) was added, followed by 99.5% (v/v) ice-cold ethanol (2.5 volumes) and incubation at -70°C (10 min) to precipitate the eluted DNA. The tubes were centrifuged (15 000×g, 15 min, 0°C) and the pelleted DNA washed with 70% (v/v) ethanol, dried briefly, dissolved in ddH₂O (10 µl) and centrifuged again (12 000×g, 1 min, RT) to bring down any residual agarose.

An aliquot (usually 1 µl) of the DNA purified by any of the above methods was run on a 1% (m/v) agarose gel with molecular size markers to estimate the concentration (Section 2.6.4), and the remainder stored at -20°C until required.

2.7.2 Direct recovery of DNA using spin columns

NucleoSpin Extract columns from the NucleoSpin Extract 2-in-1 kit (Machery-Nagel, Düren, Germany) were also used to purify PCR product directly from a PCR mix. The columns were used with the supplied buffers according to the manufacturer's instructions and an aliquot (usually 1 µl) of purified DNA was run on a 1% (m/v) agarose gel with molecular size markers to estimate the concentration (Section 2.6.4). The remainder of the purified PCR

product was stored at -20°C until required. The procedure was very rapid and simple but had a poor and inconsistent yield, the best achieved being 45%.

2.8 Reverse transcription polymerase chain reaction (RT-PCR)

The polymerase chain reaction has made far-reaching contributions to biochemistry and molecular biology since Saiki *et al.* designed the original protocols in 1985. The process has simplified existing technology and inspired the rapid development of new techniques. The number of journal articles covering PCR has amplified annually in an exponential fashion profoundly reminiscent of PCR itself! For the purposes of this investigation, PCR was preceded by a reverse transcription (RTn) step to produce dsDNA from viral dsRNA. Subsequently, the quantities of dsDNA resulting from the PCR step could be manipulated for sequencing, RFLP analysis and gene cloning and transcription of recombinant protein.

2.8.1 Principles of RT-PCR as applied to viral dsRNA

PCR allows the selective amplification of a chosen region of DNA without any need for cloning. It is fast, very sensitive, incredibly specific, high yield, exceptionally versatile and yet inherently simple; the reaction is driven by a DNA polymerase and by successive rounds of temperature cycling.

The heat-stable DNA polymerase requires a single-stranded DNA template, but is primed by a short double-stranded region. Hence, details of the sequences immediately flanking the region of DNA to be amplified are needed for the synthesis of two oligonucleotide primers. These are constructed such that each primer initiates the synthesis of the strand complementary to the one it binds to, and primers are thus distinguished as forward or reverse, or alternatively, upstream or downstream, or 5' or 3' (Newton and Graham, 1994).

The genetic material of interest in this study was viral dsRNA, which needed conversion to DNA using reverse transcriptase (RTase) before any PCR processes would be possible. The RTase also has a requirement for single-stranded template and can be primed using the same primers intended for PCR. While it is recognised that nested primers may give better results in terms of yield, a single pair of primers for both RTn and PCR was chosen for economy and to simplify the optimisation and procedure as a whole. Hence, following denaturation of the dsRNA, RTase was used to catalyse its transcription into double-stranded complementary DNA (cDNA).

Two RTase enzymes are commercially available: Moloney murine leukaemia virus RTase (MMLV-RT) and avian myeloblastosis virus RTase (AMV-RT). MMLV-RT has comparable 5'-3' polymerase activity to AMV-RT, but has a significantly lower RNase H activity and is thus the enzyme of choice when high numbers of full-length cDNA are required (Kotewicz *et al.*, 1988). However, the AMV-RT has a temperature optimum of 42°C and therefore transcribes RNAs rich in secondary structure more efficiently than the murine enzyme, which has optimal activity at 37°C (Sambrook *et al.*, 1988e). Knowing that dsRNA is a difficult template molecule (Section 4.6.2), I wanted to utilise the most effective aspects of both enzymes but was uncertain of continued MMLV-RT activity at 42°C. Discussion revealed that MMLV-RT in fact produced slightly *more* RT-PCR product when incubated at 42°C rather than 37°C (A. Hamel, Manitoba Government Department of Agriculture, Canada, personal communication). Hence, MMLV-RT was used throughout this study at the elevated temperature.

The template cDNA starts out double-stranded in the PCR reaction. Primers and nucleotides (in excess) and DNA polymerase are added in an appropriate buffer containing magnesium ions. The DNA duplex is heat-denatured, the solution cooled to a temperature where the primers will anneal but template will not re-anneal, and the temperature is adjusted to the optimum of the DNA polymerase, whereupon complementary strand synthesis begins. This temperature cycle allowing denaturation, primer annealing and strand extension to occur in sequence is repeated sequentially, resulting in the exponential accumulation of dsDNA from the targeted region (Sambrook *et al.*, 1989f). Initially, synthesis will go beyond the sequence complimentary to that of the other primer, but these longer sequences only accumulate in a linear fashion provided the amount of starting DNA is limiting. Amplification is not, however, an unlimited process: after 25-30 cycles of PCR, the amount of enzyme becomes limiting and target strand re-annealing competes with primer annealing (Newton and Graham, 1994).

PCR conditions should be optimised with respect to a number of factors for each application. These factors include amount of template; amount, melting temperature (T_m) and specific sequence of primers; concentration of dNTPs and magnesium ions and whatever downstream manipulations the amplified DNA may be intended for. However, there are ranges and guidelines which facilitate this optimisation process, a variety of which are summarised in Table 2-5 and Table 2-6 for PCR conditions and primer design respectively.

Table 2-5. Recommended ranges for PCR components and conditions.^a

Factor	Range	Comment
Template DNA	0.05-1 µg	Template to primer ratio should be such that primers do not become limiting and lead to poor yield.
Primers	0.1-0.5 µM	Higher primer concentrations can promote mispriming, non-specific background and formation of primer-dimers, all of which also lead to low yield.
dNTPs	20-200 µM	Should give optimal balance between yield, specificity and fidelity; low [dNTP] minimises mispriming and improves fidelity.
Mg ⁺² ions	0.5-2.5 mM over total [dNTP]	DNA polymerase requires free magnesium over that bound by template DNA, primers and dNTPs. [Mg ⁺²] may affect primer annealing, strand dissociation temperature, enzyme activity, formation of primer-dimers, specificity, and fidelity. Needs optimisation, though low [Mg ⁺²] often best.
PCR buffer	10-50 mM Tris-HCl, pH 8.3-8.8; up to 50 mM KCl, (NH ₄) ₂ SO ₄	Buffering needed to ensure optimal enzyme activity; salts included to facilitate primer annealing. Note that NaCl at 50 mM or KCl above 50 mM inhibits <i>Taq</i> polymerase activity.
Thermostable DNA polymerase	1-2.5 units (U)	Too much enzyme leads to non-specific background; too little to poor yield.
Denaturing time and temperature	30 sec, 95°C	Incomplete denaturation will cause PCR failure hence sufficient heating is essential, avoiding excess temperature exposure, which can lead to loss of enzyme activity.
Annealing time and temperature	30 sec, 5°C less than T _m ; 55-72°C ideal	Annealing should occur rapidly hence time allowed can be short. Stringent annealing temperatures maximise specificity.
Extension time and temperature	1 min per kb, 72°C	Optimal temperature and time sufficient to complete extension at an average rate of nucleotide incorporation.
Final extension time and temperature	2 min per kb, 72°C. Cool to 4°C.	Ensures that all amplified DNA is fully extended and fully double-stranded and that the product is cooled to an appropriately stable temperature.
Number of cycles	25-30	Dependent on amount of starting material, however too many cycles can increase non-specific background products as plateau effect occurs.

^a After Innis and Gelfand, 1990, Taylor, 1991a and Sambrook *et al.*, 1989g

Table 2-6. Guidelines for PCR primer design.^a

Recommendation	Comment
G+C content between primers should be similar and around 50-60%.	T _m of primers should be similar to facilitate symmetrical priming and extension, and should be sufficiently high to promote specificity of binding.
Primers should have minimal self-complementarity.	Prevents formation of secondary structures within primers thereby allowing effective binding.
Complementarity between primers should be low, particularly at 3' ends.	Prevents formation of primer-dimers and resulting competition for PCR components, 3' end is critical as DNA polymerase extends 5' to 3'.
3' end must have high complementarity with target sequence and any sequence mismatches should be at least 10-12 bp from 3' end.	Mismatched bases extend inefficiently; effective binding at 3' end is critical as DNA polymerase extends 5' to 3'.
Runs of 3 or more G/C at 3' end should be avoided.	Can promote mispriming at G- and C-rich sequences.
Restriction sites for cloning should be built in with high homology or added as a 5' tag, with sufficient clamping bp included.	Homology ensures effective primer binding and an unbound 5' tag in initial rounds of PCR quickly becomes incorporated in successive extensions as DNA polymerase extends 5' to 3'. Clamping bp ensure efficient restriction.
Internal region of target sequence should be checked for any restriction site to be used for cloning.	Prevents subsequent internal cleavage of amplified product when preparing ends for cloning.
Internal region of target sequence should be checked for any regions of high complementarity with primers.	Prevents mispriming at internal sites and subsequent generation of artefacts.
Length should be 25-30 bp.	Must be long enough for sufficient discrimination, in terms of both sequence and annealing temperature.

^a After Innis and Gelfand, 1990, Clackson *et al.*, 1991 and Sambrook *et al.*, 1989g

A number of specific RT-PCR protocols were devised and optimised in the course of this investigation and details of these will follow in subsequent chapters. The method outlined in Sections 2.8.2 and 2.8.3 is the optimised technique specifically covering the amplification of the VP4 gene from IBDV using the primers VP4f1 and VP4r1 (Section 4.4.1), and will subsequently be referred to as standard VP4 RT-PCR. The conditions for this standard method are summarised in Table 2-7 at the end of Section 2.8.3.

2.8.2 Reagents

Tris-EDTA solution (TE) [10 mM Tris-HCl, 1 mM Na₂-EDTA, pH 8.0]. 500 mM Tris-HCl (2 ml, Section 2.5.1.1) and 100 mM Na₂-EDTA (1 ml, Section 2.5.1.1) were diluted with ddH₂O (90 ml), titrated to pH 8.0 with HCl, made up to 100 ml and sterilised by autoclaving.

Primer stock solutions [30 µM and 10 µM forward or reverse primer]. Forward and reverse primers were obtained as lyophilised pellets from the supplier and resuspended in TE to give separate master stock solutions (300 µM), which were stored at -20°C. Aliquots of each master stock (10 µl and 5 µl) were diluted with TE (90 µl and 145 µl) to give stock solutions of 30 µM and 10 µM, used for RTn and PCR respectively. The working concentrations of primer in these reactions were 1.5 µM and 0.1 µM respectively.

dNTP stock solution [5 mM dNTPs]. An aliquot of each dNTP (10 µl) was taken from supplied stocks (100 mM lithium salts stored at -70°C) and placed in a microfuge tube. Sterile ddH₂O (160 µl) was added to bring the final concentration of dNTPs to 5 mM. The working concentrations of dNTPs in RTn and PCR reactions were 0.5 mM and 0.2 mM respectively.

5× RTase buffer [250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl₂, pH 8.3]. This buffer was supplied with the MMLV-RT (Gibco BRL, Paisley, UK).

20× dithiothreitol (DTT) stock solution [200 mM DTT]. DTT (31 mg) was dissolved in sterile ddH₂O (1 ml), dispensed into aliquots (10 µl each) and stored at -20°C. DTT was not subjected to multiple freeze-thaw cycles; aliquots were thawed just before use and unused portions discarded. DTT was used in the RTn reaction at a working concentration of 10 mM.

10× NH₄ buffer [160 mM (NH₄)₂SO₄, 670 mM Tris-HCl, 0.1% Tween[®]-20 (v/v), pH 8.8]. This buffer was supplied with the BIOTAQ™ DNA polymerase (Bioline, London, UK).

MgCl₂ stock solution [50 mM MgCl₂]. This stock solution was supplied with the BIOTAQ™ DNA polymerase (Bioline, London, UK). MgCl₂ was used in the PCR reaction at a working concentration of 1.5 mM.

2.8.3 Procedure

Denaturing viral dsRNA and annealing primers. Viral dsRNA (10 ng, 4.5 fmoles), 30 pmoles of each primer (1 µl of 30 µM stock solutions) and ddH₂O making up a total volume of 12.5 µl were placed in a 0.2 ml thin-walled PCR reaction tube and centrifuged briefly. Primers were present at 6667-fold molar excess over template. The tube was floated in a boiling water bath (97.5°C, 5 min) and immediately cooled in a bath of crushed ice and NaCl (3:1 m/m, -21°C) (Merck Tables). This procedure allows denaturation of the dsRNA and annealing of the short oligonucleotide primers without re-annealing of the template strands.

RTn reaction. A master mix of 5× RTase buffer (4 µl), DTT (1 µl of stock solution) and dNTPs (2 µl of stock solution) was prepared, the actual volume of each reagent being a multiple of the number of samples processed. An aliquot of this RTn mix (7 µl) was added to each sample tube, which was kept in the ice/NaCl bath until the addition of RTase (0.5 µl, 100 U of MMLV-RT), bringing the final volume to 20 µl. The sample tubes were centrifuged briefly in a microfuge (10 000 rpm, 1 sec) to coalesce droplets and tapped gently to fully disperse the MMLV-RT before incubation in a water bath (42°C, 1.5 h) to allow reverse transcription to occur. Any of this product not used immediately in a PCR reaction was stored at -20°C.

PCR reaction. A master mix of 10× NH₄ buffer (5 µl), MgCl₂ (1.5 µl of stock solution), dNTPs (2 µl of stock solution) and ddH₂O (34 µl) was prepared, the actual volume of each reagent being a multiple of the number of samples processed. An aliquot of this PCR mix (42.5 µl) was added to a fresh 0.2 ml thin-walled PCR reaction tube, followed by an aliquot of RTn reaction product (5 µl) and 10 pmoles of each primer (1 µl of 10 µM stock solutions). The tubes were centrifuged briefly and placed in a GeneAmp[®] PCR System 2400 thermocycler (Perkin Elmer, Norwalk, USA).

The thermocycler was programmed to allow a 'hot-start', constituting an initial denaturing step (94°C, 2 min), lowering the temperature to the annealing temperature (67°C, 40 sec) and holding this temperature while thermostable DNA polymerase was added to each tube (0.5 µl, 2.5 U of BIOTAQ™). This brought the final volume of the PCR reaction to 50 µl. The reaction was allowed to continue for a further 25 cycles (denature 94°C, 1 min; anneal 67°C, 1 min; extend 72°C, 1.5 min), followed by a final extension period (72°C, 5 min) to ensure that all molecules were fully double-stranded. Tubes were held at 4°C until they were removed from the thermocycler for analysis of the product and/or storage at -20°C.

Table 2-7. Summary of conditions for standard VP4 RT-PCR.

Reverse transcription reaction		
Component	Quantity/concentration	Procedure
viral dsRNA	10 ng	boil together 5 min, flash
primers	30 pmoles, 1.5 µM	cool in ice:NaCl (3:1 m/m)
dNTPs	0.5 mM	add to RNA-primer mix,
DTT	10 mM	incubate 42°C, 1.5 h
MMLV-RT	100 U	
Polymerase chain reaction		
Component	Quantity/concentration	Procedure
RTn product	5 µl	hot start:
10× NH ₄ buffer	5 µl	denature 94°C, 2 min
MgCl ₂	1.5 mM	cool to 67°C,
dNTPs	0.2 mM	hold 67°C, 40 sec
primers	10 pmoles, 0.2 µM	
		25 cycles:
		94°C, 1 min;
<i>Taq</i> polymerase	2.5 U	67°C, 1 min;
		72°C, 1.5 min
		final extend 72°C, 5 min

Chapter 3

General materials and methods:

Protein Biochemistry

3.1 Introduction

A variety of methods were used to examine the protein biochemistry of IBDV. General techniques are described in this chapter, while specific details of more specialised procedures will be described in subsequent relevant sections.

3.2 Materials

Common laboratory reagents were from BDH (Poole, UK), Boehringer Mannheim (Mannheim, Germany), ICN (Aurora, USA) or Merck (Darmstadt, Germany) and were of analytical or higher purity. Distilled water (dH₂O) was obtained with a Milli-RO[®] 15 Water Purification System (Millipore, Marlboro, USA). Distilled, deionised water (ddH₂O) was obtained with a Milli-Q Plus Ultra-Pure Water System (Millipore, Marlboro, USA). The minimum resistivity of the ddH₂O was 18 MΩ.cm. For convenience, the sources of specialised materials are listed below.

Bursal tissue infected with IBDV was obtained from Dr Roger Horner (Allerton Regional Veterinary Laboratory, Pietermaritzburg, SA). Protein molecular mass standards were from Pharmacia LKB Biotechnology (Lund, Sweden). Bovine serum albumin (BSA), 4-(2-aminoethyl)benzenesulfonylfluoride (AEBSF) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitroblue tetrazolium (NBT) substrate tablets were from Boehringer Mannheim (Mannheim, Germany). 3,3',5,5'-tetramethylbenzidine (TMB) tablets, casein, *L-trans*-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64), ovalbumin, pepstatin, 1,10-phenanthroline, Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) were from Sigma (St Louis, USA). Chiron Mimotopes (Victoria, Australia) synthesised a 15-amino acid peptide used for raising anti-peptide antibodies. Hybond[™]C hybridisation transfer membrane was from Amersham International (Buckinghamshire, UK). Nunc Immuno Maxisorp F96 multiwell plates and FluorNunc[®] 96-well fluorimetry plates were from Nunc Intermed (Roskilde, Denmark). The fluorogenic peptide substrate carbobenzoxy-Arg-Arg-7-amino-4-methylcoumarin (Cbz-Arg-Arg-AMC) was from Cambridge Research Biochemicals (Cambridge, UK).

3.3 Assaying for protein

The dye-binding method described by Bradford (1976) is a simple, sensitive and interference-resistant assay, and as such was routinely used to determine protein concentration. Microgram quantities of protein can be detected, even in the presence of metal ions and reducing agents. The assay is, however, affected by detergents such as Triton X-100 and SDS and by the presence of chaotropic agents such as urea. Friedenauer and Berlet (1989) found that Triton X-100 [0.008% (v/v)] and urea (0.16M) increased the sensitivity of the assay by an average of 33% and 14% respectively, whereas SDS [0.004% (m/v)] caused a 75% decrease in sensitivity.

The dye used in the assay binds to basic and aromatic amino acid residues at low pH, forming a blue complex that has an absorbance maximum at 595 nm (Compton and Jones, 1985). Sulfonic acid groups of the dye interact with these amino acids, resulting in its reduction from a cationic species (yellow) to an anionic form (blue). The content of these amino acid residues differs in different proteins, and hence a specific standard curve relating absorbance at 595 nm to concentration should be constructed for each protein. A modification by Read and Northcote (1981) largely overcomes this problem and was implemented in the assay used in this study. The modification replaces the original dye, Coomassie brilliant blue G-250, with Serva blue G and alters dye and phosphoric acid concentrations, causing a slight loss in sensitivity.

3.3.1 Reagents

Dye reagent. Serva Blue G dye (50 mg) was dissolved in 88% (v/v) phosphoric acid (50 ml) and added to 99.5% (v/v) ethanol (23.5 ml). The solution was made up to 500 ml with dH₂O, stirred (30 min), filtered through Whatman No. 1 filter paper and stored in an amber bottle in the dark at RT. Each batch of reagent was calibrated separately and inspected periodically for the presence of precipitate, which if present was removed by filtration and the reagent subsequently recalibrated.

Standard protein solution (1 mg.ml⁻¹ ovalbumin). Ovalbumin (10 mg) was dissolved in dH₂O (10 ml). This stock solution was diluted to 100 µg.ml⁻¹ for the assay and used immediately.

3.3.2 Procedure

Assays for the standard curve were carried out in quintuplicate at five incremental concentrations of ovalbumin. Ovalbumin working solution (10-50 μl of the 100 $\mu\text{g}\cdot\text{ml}^{-1}$ solution, i.e., 1-5 μg protein) was diluted to a final volume of 50 μl with dH_2O in a 1.5 ml polyethylene microfuge tube. Dye reagent (950 μl) was added and the solution mixed by inversion. After incubation at RT for 2 min, the absorbance at 595 nm was determined in a 1 ml plastic microcuvette, with dH_2O (50 μl) in dye reagent (950 μl) serving as a blank. Samples of unknown protein concentration were diluted to a total volume of 50 μl in buffer before the addition of 950 μl dye reagent. Buffer (50 μl) treated similarly served as a blank. Since the dye binds plastic to a degree, cuvettes were washed with detergent between readings. Protein concentration of unknown samples was calculated using an equation generated by linear regression analysis of the standard curve.

3.4 Tris-Tricine SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was introduced by Shapiro, Vinuela and Maizel in 1967 and has since become a popular method for the detection of heterogeneity and for the estimation of protein molecular mass. In this investigation, reducing and non-reducing SDS-PAGE were used to examine the protein constituents of fractionated bursal tissue and partially purified IBDV. In conjunction with western blotting (Section 3.11), SDS-PAGE was used to detect the putative viral protease (VP4) in bursal fractions and to evaluate the specificity of antibodies raised against a peptide sequence from VP4.

3.4.1 Principles of polyacrylamide gel electrophoresis

Polyacrylamide, being a microreticular gel, is able to impose steric resistance on proteins migrating under the influence of an electric field, so that large proteins will be retarded to a relatively greater extent than small molecules having the same charge to mass ratio. This steric resistance also helps to minimise diffusion and keep separated bands relatively sharp. The polyacrylamide gel is formed by the co-polymerisation of acrylamide with the cross-linking agent N,N' -methylenebisacrylamide (Bis). The polymerisation reaction may be initiated by ammonium persulfate and accelerated by N,N,N',N' -tetramethylethylenediamine (TEMED). The porosity of the gel is dependent on the relative amount of cross-linking agent to total acrylamide (%C) and on the total mass of acrylamide in the gel volume (%T). The %T determines the effective separation range of the gel.

The discontinuous electrophoresis method developed by Ornstein (1964) and Davis (1964) consists of a large pore stacking gel overlaid on a small pore separating/running gel. Initially, the gels contain Tris buffer with a counter ion of high mobility (the leading ion), while the electrode compartments contain Tris buffer with a counter ion having a relatively low, but pH-dependent, mobility (the trailing ion). The pH in the electrode compartments and the stacking gel is chosen so that the proteins of interest have a mobility intermediate between that of the fast and slow counterions.

The protein sample is loaded onto the stacking gel and upon application of the electric potential, a sharp interface forms between the leading and trailing ions, at which the proteins of intermediate mobility are concentrated in a tight stack, having been swept up in the downward migration of the ions. The large pore stacking gel stabilises the system from convective disturbance, but hardly impedes protein migration.

When the focused stack of proteins reaches the separating gel, a change in pH causes an increase in the mobility of the trailing ion. The leading ion/trailing ion interface thus increases its rate of migration, overtaking the protein bands and leaving them to separate in a uniform voltage gradient. The smaller pore structure of the separating gel now imposes a sieving effect on the migrating proteins and the separation achieved depends on the charge and size of the protein molecules, as well as on the concentration of the separating gel.

3.4.2 Effect of SDS

SDS is a detergent having a hydrophilic, negatively charged sulfonic acid "head" and a 12-carbon, aliphatic, hydrophobic "tail". SDS has the unique property of complexing with most proteins in the constant ratio of 1.4 g SDS per gram of protein (Reynolds and Tanford, 1970) to form characteristic rod-like structures. Secondary protein structure is thus disrupted, and oligomeric proteins will also disaggregate. The proportion of SDS present in these complexes is sufficient to mask the intrinsic charge on the protein and hence all such complexes have similar negative charge to mass ratios. When subjected to electrophoresis, SDS-bound proteins will separate solely on the basis of size, due to differences experienced in steric retardation. Migration is thus an inverse function of size and therefore also of relative molecular mass (M_r), providing the basis for the determination of molecular weights by comparison with migration of standard proteins of known M_r (Neville, 1971). SDS alone does not disrupt tertiary structure held in place by disulfide bonds and for this reason a reducing agent such as β -mercaptoethanol is included in reducing SDS-PAGE.

3.4.3 Significance of the Tris-Tricine system

Laemmli (1970) described a discontinuous stacking system using Tris and glycine for the preliminary concentration of dilute samples in SDS on slab gels. Schägger and von Jagow (1987) proposed the Tris-Tricine system as an alternative to this highly successful protocol, substituting Tricine for glycine as the trailing ion in the stacking gel, at a higher pH. This system is useful for separation of proteins from 5-100 kDa, provides better resolution of smaller proteins (5-20 kDa), can tolerate higher salt concentrations in protein samples and has a single gel buffer which remains stable for longer than that of the Laemmli system. Tris-Tricine SDS-PAGE was used routinely throughout this study.

3.4.4 Reagents

SDS solution [10% (m/v) SDS]. SDS (10 g) was dissolved in a total volume of 100 ml dH₂O. This process required gentle heating.

Monomer solution [48% (m/v) acrylamide, 3% (m/v) bisacrylamide]. Acrylamide (48 g) and bisacrylamide (3 g) were dissolved in dH₂O (50 ml), made up to 100 ml, filtered through Whatman No. 1 filter paper and stored in an amber bottle at RT.

Initiator [10% (m/v) ammonium persulfate]. Ammonium persulfate (0.1 g) was dissolved in dH₂O (1 ml), just before use.

Anode buffer [200 mM Tris-HCl, pH 8.9]. Tris base (24.22 g) was dissolved in dH₂O (900 ml), titrated to pH 8.9 with HCl and made up to 1 litre.

Cathode buffer [100 mM Tris-HCl, 100 mM Tricine, 0.1% (m/v) SDS, pH 8.25]. Tris base (12.2 g), Tricine (17.9 g) and 10% (m/v) SDS solution (10 ml) were dissolved in dH₂O (900 ml), titrated to pH 8.25 with HCl and made up to 1 litre.

Gel buffer [3 M Tris-HCl, 0.3% (m/v) SDS, pH 8.45]. Tris base (72.7 g) and 10% (m/v) SDS solution (6 ml) were dissolved in dH₂O (150 ml), titrated to pH 8.45 with HCl and made up to 200 ml.

Non-reducing sample treatment buffer (NRTB) [100 mM Tris-HCl, 1% (m/v) SDS, 20% (v/v) glycerol, pH 8.45]. Tris base (0.121 g), 10% (m/v) SDS solution (1 ml) and 99.5% (v/v) glycerol (2 ml) were dissolved in dH₂O (4 ml), titrated to pH 8.45 with HCl, made up to 10 ml, aliquoted and stored at -20°C.

Reducing sample treatment buffer (RTB) [100 mM Tris-HCl, 1% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, pH 8.45]. Tris base (0.121 g), 10% (m/v) SDS solution (1 ml), 99.5% (v/v) glycerol (2 ml) and β-mercaptoethanol (1 ml) were dissolved in dH₂O (4 ml), titrated to pH 8.45 with HCl, made up to 10 ml, aliquoted and stored at -20°C.

Tris-Tricine M_r standards [phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa) (each protein at 1 μg.μl⁻¹), in reducing or non-reducing sample treatment buffer. Lyophilised protein standards containing sucrose were obtained as a kit from Pharmacia (Lund, Sweden). The standards were reconstituted in either reducing or non-reducing sample treatment buffer from which glycerol had been omitted.

HNO₃, 20% (v/v) [36.5% (v/v) 55% HNO₃]. 55% (v/v) HNO₃ (36.5 ml) was diluted to 100 ml with dH₂O and stored in an amber bottle at RT.

3.4.5 Procedure

Gloves were worn during sample preparation and loading to prevent introduction of skin keratins into samples. These proteins appear as contaminant bands at approximately 68 kDa on reducing SDS-PAGE and may also react non-specifically with antibodies in western blots (Ochs, 1983; Shapiro, 1987).

Tris-Tricine SDS-PAGE was carried out as described by Schägger and von Jagow (1987) using a Bio-Rad Mini-Protean[®] II vertical slab electrophoresis unit assembled as described in the manufacturer's manual. If gels were intended for silver staining (Section 3.6), all glassware was soaked overnight in 20% (v/v) HNO₃ (Section 3.4.4). Stacking and separating gels were prepared as described in Table 3-1. Separating gels were cast and allowed to polymerise for 1 h. Stacking gels usually polymerised within 20 min. Gels were cast using 1.5 mm spacers and combs.

Table 3-1. Preparation of separating and stacking gels for Tris-Tricine SDS-PAGE.

Component	Separating Gel (10% T)	Stacking Gel (4% T)
Monomer	3.6 ml	0.5 ml
Gel buffer	6 ml	1.5 ml
ddH ₂ O	8.4 ml	4 ml
Initiator	60 µl	30 µl
TEMED	6 µl	12 µl
Total volume	18 ml	6 ml

Protein samples [containing at least 2 µg protein per band for Coomassie Blue staining (Section 3.5) or 100 ng per band for silver staining (Section 3.6)] were combined in a 1:1 ratio with the relevant sample treatment buffer, and boiled for 10 min. After cooling, bromophenol blue marker dye (1 µl) and the samples loaded into gel wells with a micropipette (disposable 25 µl tip). Gels were run at 80 V, current unlimiting, until the samples reached the separating gel, when the voltage was increased to 100 V.

3.5 Coomassie blue R-250 protein stain for polyacrylamide gels

Coomassie Brilliant Blue R-250 is a non-polar, sulfated triphenylamine dye used to detect at least 1 µg of protein per band on a gel (Dunn, M.J., 1989). This method allows simple and fairly rapid visualisation of proteins separated by SDS-PAGE.

3.5.1 Reagents

Stain stock solution [1% (m/v) Coomassie blue R-250]. Coomassie blue R-250 (1 g) was dissolved in dH₂O (100 ml) by magnetic stirring for 1 hr at RT and filtered through Whatman No. 1 filter paper.

Stain working 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 dH₂O.

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 litre with dH₂O.

Destaining solution II [5% (v/v) methanol, 7% (v/v) acetic acid]. Methanol (50 ml) and acetic acid (70 ml) were made up to 1 litre with dH₂O.

3.5.2 Procedure

After removal from the electrophoresis pod, gels were placed in stain working solution and agitated (4 h). Gels were removed from the stain, rinsed with dH₂O, immersed in destaining solution I (4-16 h) and subsequently left in destaining solution II until the background had fully cleared. Gels were stored hydrated in polyethylene zip-seal bags and were stable in this form until photography was convenient.

3.6 Silver stain for proteins in polyacrylamide gels

Silver staining of proteins is up to 100-fold more sensitive than the Coomassie blue R-250 stain (Section 3.5). This technique relies on the reduction of solubilised ionic silver to its insoluble metal, which may be achieved in strongly acidic or basic solution (Allen and Budowle, 1994c). The basic procedure, employing sodium carbonate or sodium hydroxide, is sensitive but incurs high background staining. The inclusion of a pre-incubation step with sodium thiosulfate (Blum *et al.*, 1987) considerably reduces background staining by preventing precipitation of insoluble silver complexes on the surface of the gel.

3.6.1 Reagents

Fixative [50% (v/v) methanol, 12% (v/v) acetic acid, 0.05% (v/v) 37% formaldehyde]. Methanol (100 ml), acetic acid (24 ml) and 37% (v/v) formaldehyde (100 µl) were made up to 200 ml with ddH₂O.

Wash solution [50% (v/v) EtOH]. 99.5% ethanol (100 ml) was made up to 200 ml with ddH₂O.

Pre-treatment solution [0.02% (m/v) sodium thiosulfate]. Na₂S₂O₃·5H₂O (40 mg) was dissolved in ddH₂O (200 ml).

Silver nitrate solution [0.2% (m/v) AgNO₃, 0.075% (v/v) 37% formaldehyde]. AgNO₃ (400 mg) was dissolved in ddH₂O (150 ml), 37% (v/v) formaldehyde (150 µl) was added and the solution made up to 200 ml with ddH₂O just before use.

Developing solution [6% (m/v) NaCO₃, 0.05% (v/v) 37% formaldehyde, 0.0004% (m/v) Na₂S₂O₃]. NaCO₃ (12 g) was dissolved in ddH₂O (150 ml), 37% (v/v) formaldehyde (100 µl) and Na₂S₂O₃.5H₂O (4 ml of thiosulfate solution above) were added, and the solution made up to 200 ml with ddH₂O just before use.

Stopping solution [50% (v/v) methanol, 12% (v/v) acetic acid]. Methanol (50 ml) and acetic acid (12 ml) were diluted to 100 ml with ddH₂O.

3.6.2 Procedure

Gloves were worn throughout the silver stain process to prevent the development of fingerprints (transferred keratins) on the gels. Using distilled, deionised water and HNO₃-treated glass containers (Section 3.4.5) throughout the procedure gave the best results in terms of band clarity and low background. Gels were removed from the electrophoresis pod and agitated in 50 ml of reagent for each step as follows:

- a) fixative (minimum 1 h, preferably ON)
- b) wash solution (3 × 25 min)
- c) pre-treatment solution (2 min)
- d) ddH₂O rinse (3 × 25 sec)
- e) silver nitrate solution (25 min)
- f) ddH₂O rinse (2 × 25 sec)
- g) developing solution (until bands were visible)
- h) ddH₂O rinse (until bands were sufficiently intense)
- i) stopping solution (at least 20 min).

Gels were stored hydrated in polythene zip-seal bags until photography was convenient.

3.7 Substrate SDS-PAGE

Co-polymerisation of a protein substrate (such as gelatin) with polyacrylamide can allow visualisation of proteases after electrophoretic separation, as described by Heussen and Dowdle (1980). Following electrophoresis, denaturing SDS is removed from proteins and gels by several washes with a non-ionic detergent, usually Triton X-100. This is believed to assist in renaturation of the protease. Gels are then incubated in appropriate assay buffers facilitating digestion of the incorporated substrate by immobilised proteases. Areas of proteolytic activity are thus evident as clear bands against a stained background and can often

be detected with silver-stain sensitivity. Protease molecular mass can be estimated, though not with particular accuracy, as incorporation of the protein substrate increases apparent molecular mass and does so heterogeneously.

3.7.1 Reagents

Protein solution [1% (m/v) gelatin in gel buffer]. Gelatin (0.1 g) was dissolved in gel buffer (10 ml, Section 3.4.4) with gentle heating, just before use.

Triton X-100, 2.5% (v/v). Triton X-100 (5 ml) was diluted to 200 ml with dH₂O.

Assay buffer [Phosphate buffered saline (PBS), pH 7.2]. NaCl (8 g), KCl (0.2 g), Na₂HPO₄·2H₂O (1.15 g) and KH₂PO₄ (0.2 g) were dissolved in dH₂O (950 ml) and made up to 1 litre.

0.1% (m/v) Amido black. Amido black (0.1 g) was dissolved in 30:10:60 (v/v/v) methanol:acetic acid:dH₂O (100 ml) and filtered through Whatman No. 1 filter paper.

3.7.2 Procedure

Separating gels were cast as per Section 3.4.5, except that 3 ml of gel buffer was replaced with 3 ml of protein substrate solution (Section 3.7.1), giving a final concentration of 0.16% (m/v) of protein in the separating gel. Stacking gels were cast as described in Section 3.4.5. To ensure that enzyme activity was maintained, samples were never boiled prior to electrophoresis. Following electrophoresis, gels were soaked in two changes of 2.5% (v/v) Triton X-100 over 1 h to remove SDS. Gels were then incubated in assay buffer (ON, 37°C) to allow digestion of the incorporated substrate, and were subsequently stained with amido black for 1 h. Gels were destained in several changes of methanol:acetic acid:dH₂O (30:10:60) until regions of digestion were visible as clear bands against a darkly stained background.

3.8 Isolation of chicken antibodies from egg yolks

Egg yolk immunoglobulin (IgY) was isolated from egg yolks by polyethylene glycol (PEG) precipitation, as described by the improved procedure of Polson *et al.* (1985). This simple, mild and efficient technique for fractionation of proteins within heterogeneous mixtures was introduced by Polson *et al.* in 1964. PEG is a neutral, water-soluble, high molecular mass

polymer, which causes precipitation of proteins via steric exclusion; proteins are concentrated in the extrapolymer space until they exceed their solubility limit and precipitate. The IgY isolation method is essentially a two-step procedure, with vitellin and fats removed in an initial precipitation step and antibodies purified in subsequent precipitation steps.

3.8.1 Reagents

100 mM sodium phosphate buffer, 0.02% (m/v) NaN₃, pH 7.6. NaH₂PO₄·2H₂O (15.60 g) and NaN₃ (0.2 g) were dissolved in dH₂O (950 ml), titrated to pH 7.6 with NaOH and made up to 1 litre.

3.8.2 Procedure

Eggs laid by chickens immunised with the same antigen were kept separate to determine whether individual responses differed. Egg yolks were separated from whites and gently rinsed under running water to remove all traces of albumin. The yolk membranes were pierced to allow the yolk volume to be collected and measured. Two volumes of sodium-phosphate buffer were added and mixed in thoroughly. Solid PEG (6 kDa) was added to 3.5% (m/v) and dissolved with gentle stirring. The precipitated vitellin fraction was removed by centrifugation (4 420×g, 30 min, RT) and the supernatant filtered through absorbent cotton wool to remove lipids.

The PEG concentration of the clear filtrate was then increased to 12% (m/v) by adding 8.5% (m/v) solid PEG, dissolved with gentle stirring. The solution was centrifuged (12 000×g, 10 min, RT), the supernatant discarded and the antibody pellet redissolved in a volume of sodium phosphate buffer equivalent to that obtained after filtration. PEG was again added to 12% (m/v) and dissolved with gentle stirring. The solution was centrifuged (12 000×g, 10 min, RT) and the supernatant discarded. To ensure gentle resuspension, the antibody pellet was dislodged from the walls of the centrifuge tube with a glass rod and left to slowly dissolve ON in sodium phosphate buffer ($\frac{1}{6}$ of the original yolk volume). The final antibody solution was centrifuged to remove any undissolved material (27 000×g, 10 min, RT) and stored at 4°C, the NaN₃ in the buffer acting as a preservative.

3.9 Isolation of rabbit antibodies from serum

A method similar to that used for IgY isolation from egg yolk was used for isolation of rabbit antibodies. PEG (6 kDa) was used to precipitate immunoglobulin G (IgG) from rabbit serum, using conditions optimised by Polson *et al.* (1964) in their studies on PEG precipitation of serum components.

3.9.1 Reagents

Borate buffered saline [35 mM boric acid, 37 mM NaCl, 17.5 mM NaOH, 8 mM HCl, pH 8.6]. Boric acid (2.16 g), NaCl (2.19 g), NaOH (0.7 g) and 37% (v/v) HCl (0.62 ml) were dissolved in dH₂O (950 ml), titrated to pH 8.6 and made up to 1 litre.

3.9.2 Procedure

One volume of serum was mixed with 2 volumes of borate buffered saline and solid PEG (6 kDa) added to 14% (m/v). After dissolving the PEG with gentle stirring, the mixture was centrifuged (12 000×g, 10 min, RT) and the supernatant discarded. The antibody-containing pellet was resuspended in the original serum volume, using sodium phosphate buffer (Section 3.8.1). PEG was added to 14% (m/v) as before, dissolved with stirring and the solution was centrifuged (12 000×g, 10 min, RT). The pellet was resuspended in 1/2 the original serum volume, using sodium phosphate buffer containing 60% (v/v) glycerol and the preparation stored at -20°C, the NaN₃ in the buffer acting as a preservative.

3.10 Determination of antibody concentration

The concentration of any pure protein solution can be calculated, provided the molar extinction coefficient (ϵ) and light-path length are known (Schultze and Heremans, 1966). The molar extinction coefficient of a compound is represented by the absorbance of a 1 M solution using a 1 cm light path (Dawson *et al.*, 1968). Alternatively, an extinction coefficient (E) describing the absorbance of a 1 mg.ml⁻¹ solution across a 1 cm light path may be used, and concentration (in mg.ml⁻¹) can thus be determined from the absorbance.

The absorbance at 280 nm of IgY and rabbit IgG solutions (diluted 1:40 in sodium phosphate buffer) was determined and the concentration of undiluted antibodies calculated. The extinction coefficients for 1 mg.ml⁻¹ IgY and IgG at 280 nm are 1.25 (mg.ml⁻¹)⁻¹.cm⁻¹ (Coetzer, 1985) and 1.43 (mg.ml⁻¹)⁻¹.cm⁻¹ (Hudson and Hay, 1980) respectively.

3.11 Enzyme-linked immunosorbent assays (ELISAs)

An enzyme immunoassay (EIA) is a quantitative immunological procedure in which the antigen-antibody reaction is monitored by measurements of enzymatic activity. Hence an EIA combines the specificity of antibodies with the sensitivity of enzymatic assays. When one immunoreactant is immobilised on a solid phase to facilitate separation from free antigen or antibody, the EIA is known as an ELISA (Engvall and Perlmann, 1971). Since partial denaturation of proteins usually accompanies adsorption to a solid phase, ELISAs complement western blotting, which gives a qualitative indication of antibody targeting of fully denatured proteins (Van Regenmortel, 1988a; 1988b). ELISAs can be structured to be competitive or non-competitive and in both of these systems, either antigen or antibody may be immobilised on the solid phase. In this study, non-competitive ELISAs with immobilised antigen were used to evaluate the progress of anti-peptide antibody production during immunisation procedures, and to detect the presence of the VP4 protein in crude bursal fractions. Hence details of this particular ELISA system are given below.

Briefly, the surfaces of wells of polystyrene microtitre plates are coated with antigen and the uncoated surfaces blocked with an unrelated protein before exposure to a solution of antigen-specific primary antibody. Residual antibody is washed away, and thus any antibody binding to antigen is quantified in subsequent steps. Non-ionic detergents such as Tween[®]-20 are included in the washing solution to ensure that non-specific protein interactions are minimised. Secondary antibodies, which are usually raised against the species IgG of the primary antibody, detect primary antibody in the next step. These secondary antibodies are conjugated to a reporter enzyme (Clarke and Engvall, 1981). More than one secondary antibody and hence more than one reporter enzyme may bind to each bound primary antibody, and thus the signal is amplified by this indirect detection method. Conjugated enzymes have been chosen to be easily assayable, and will usually catalyse the formation of a soluble coloured product from a colourless substrate, which can be quantified spectrophotometrically.

Secondary antibodies conjugated to horseradish peroxidase (HRPO) were used throughout this study. This enzyme catalyses the transfer of electrons from the substrate to peroxide, generating a coloured product (Kemeny and Chantler, 1988). A variety of soluble chromogenic substrates are available for HRPO, one of the most sensitive being 3,3',5,5'-tetramethylbenzidine (TMB), which was used in this study. TMB is more rapidly oxidised than other HRPO substrates, so colour development is faster. This increased

sensitivity can lead to an increase in non-specific background, so particular care was taken to wash plates thoroughly between steps and to stop colour development timeously.

3.11.1 Reagents

Phosphate buffered saline (PBS), pH 7.2. NaCl (8 g), KCl (0.2 g), Na₂HPO₄·2H₂O (1.15 g) and KH₂PO₄ (0.2 g) were dissolved in dH₂O (950 ml) and made up to 1 litre.

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

1% (m/v) casein in PBS (PBS-casein). Casein (1 g) was dissolved in PBS (100 ml).

Tween[®] in PBS, 0.1% (v/v) (PBS-Tween[®]). Tween[®]-20 (1 ml) was made up to 1 litre with PBS.

0.15 M citrate-phosphate buffer, pH 5.0. A 0.15 M solution of citric acid·H₂O (21 mg·ml⁻¹) was titrated with a 0.15 M solution of Na₂HPO₄·2H₂O (35.6 mg·ml⁻¹) to pH 5.0.

Substrate [0.05 M citrate-phosphate buffer, pH 5.0, 0.1 mg·ml⁻¹ TMB, 10% DMSO, 0.0015% H₂O₂]. One TMB tablet was dissolved in DMSO (1 ml) and diluted to 10 ml with citrate-phosphate buffer, pH 5.0 (3 ml), and dH₂O (6 ml). 30% (v/v) H₂O₂ (2 µl) was added just before use.

TMB stopping solution [2 M H₂SO₄]. H₂SO₄ (19.6 g) was diluted to 100 ml with dH₂O.

3.11.2 Procedure

The volume of reagents is given per well and applies throughout except where stated otherwise in subsequent relevant sections. PBS-BSA and PBS-casein are both blocking agents and were used as appropriate for particular applications. Nunc Immuno Maxisorp F96 multiwell plates were coated with antigen (150 µl, ON at 4°C) and any uncoated areas blocked with blocking agent (200 µl, 1 h at 37°C). Plates were washed with PBS-Tween[®] (3×) before appropriate dilutions of primary antibody in blocking agent were added (100 µl, 2 h at 37°C). Plates were washed with PBS-Tween[®] (3×) before addition of HRPO-linked secondary antibody (diluted with blocking agent, 120 µl, 1 h at 37°C) and washed again with

PBS-Tween® (3×) before addition of substrate solution (150 µl). Once colour had developed adequately (usually within 10-20 min), stop solution (50 µl) was added and the absorbance of each well read at 450 nm on an EL 312 Bio-Kinetics Reader (Bio-Tek Instruments).

3.12 Western blotting onto nitrocellulose membranes

Western blotting or immunoblotting involves the electrophoretic transfer of proteins to nitrocellulose and their subsequent detection on this surface; specific antibodies are used to identify protein antigens previously separated by electrophoresis. Nitrocellulose has a high protein-binding capacity and is thus the membrane commonly used for blotting proteins. In the present study, western blotting was used to detect fully denatured VP4 protein in crude bursal fractions.

The method used in this study was essentially that of Towbin *et al.* (1979), with minor modifications. Following SDS-PAGE, the anodal electrophoretic mobility of SDS-coated proteins is used to transfer them from the gel to the nitrocellulose by electroelution. Optimal transfer of proteins is obtained in low ionic strength buffers and with low electrical current. Sites on the nitrocellulose remaining unoccupied after transfer are blocked with a protein solution to prevent non-specific adherence of antibodies to the membrane. The blot is probed with antigen-specific antibodies (primary antibodies), and as for ELISA (Section 3.11), antigen-antibody complexes are detected with secondary antibodies directed against the primary antibody. These secondary antibodies are labelled with a reporter enzyme which catalyses a reaction leading to the precipitation of an insoluble coloured product at the site of the immune complex. This two-step detection method involving both a primary and a secondary antibody increases the number of reporter enzyme molecules present at the target, giving an amplified signal.

Secondary antibodies conjugated to alkaline phosphatase (AP) were used for western blots throughout this study, with the combination of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) as substrate. BCIP is hydrolysed by AP to form an intermediate that undergoes dimerisation to produce an indigo dye. The NBT is reduced to the NBT-formazan by the two reducing equivalents generated by the dimerisation. Together these substrates form an intense, insoluble black-purple stain on blots which does not fade with time, unlike insoluble substrates generated using an HRPO system (Moe and Kirkeby, 1982; Doria *et al.*, 1988). This reaction proceeds at a steady rate, allowing accurate control of the colour development and hence relative sensitivity. The AP system also has greater

sensitivity than those available for HRPO, and was thus the enzyme-conjugate of choice for this study.

3.12.1 Reagents

Blotting buffer [25 mM Tris-HCl, 192 mM glycine, 20% (v/v) methanol, 0.1% (m/v) SDS, pH 8.3]. Tris base (9.08 g) and glycine (43.2 g) were dissolved in 2 litres of dH₂O. Methanol (600 ml) and 10% (m/v) SDS (3 ml) were added and the solution made up to 3 litres. The pH varies between pH 8.1 and 8.5 depending on the quality of the reagents. The pH should not be adjusted, since this increases buffer conductivity, resulting in a higher initial current and decreased initial resistance. This buffer should only be used once, as it loses its ability to maintain a stable pH during transfer. SDS and methanol increase protein mobility, but may also reduce protein binding to nitrocellulose.

Tris-buffered saline (TBS) [25 mM Tris-HCl, 200 mM NaCl, pH 7.4]. Tris base (2.42 g) and NaCl (11.69 g) were dissolved in dH₂O (950 ml), titrated to pH 7.4 with HCl and made up to 1 litre.

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

Blocking reagent [5% (m/v) low fat milk powder]. Low fat milk powder (5 g) was dissolved in TBS (90 ml) and made up to 100 ml with TBS. A five-fold dilution was made of an aliquot of this solution (20 ml made up to 100 ml with TBS) to prepare 1% (m/v) low fat milk powder blocking reagent, which was used as an antibody diluent.

Substrate [100 mM Tris-HCl, 5 mM MgCl₂, 0.15 mg.ml⁻¹ BCIP, 0.3 mg.ml⁻¹ NBT]. Substrate was obtained as a ready-to-use tablet from Boehringer Mannheim. One tablet was dissolved in dH₂O (10 ml) and used immediately.

3.12.2 Procedure

Protein mixtures were resolved by Tris-Tricine SDS-PAGE (Section 3.4.5) and the gels rinsed with blotting buffer. Gloves were worn throughout to prevent development of keratin bands on the blot (Section 3.4.5). Nitrocellulose membrane was cut to the approximate size of a gel and pre-soaked in blotting buffer for about 5 min, as were pieces of Scotchbrite® foam and

Whatman no. 4 filter paper. The “sandwich cartridge” of a Hoefer TE series Transphor[®] electroblotting apparatus was submerged in chilled blotting buffer. A sandwich was assembled on one side of the cartridge as follows: one piece of Scotchbrite[®] foam, three pieces of Whatman no. 4 filter paper, nitrocellulose, gel, three pieces of Whatman no. 4 filter paper, one piece of Scotchbrite[®] foam. Care was taken not to trap air bubbles between the layers, especially between the nitrocellulose and the gel. The cartridge was closed and slotted into the blotting apparatus (pre-filled with blotting buffer) with the gel at the cathode and the nitrocellulose at the anode. The apparatus was maintained at 8°C by a refrigerated circulator, and the buffer stirred by magnetic stirrer to maintain uniform conductivity and temperature during transfer. Gels were either blotted for 2 h (200 mA, unlimiting volts) or ON (30 V, unlimiting current).

Following electrotransfer of proteins, the nitrocellulose was transiently stained with Ponceau S (30 sec) and rinsed with dH₂O to reveal the positions of M_r standards. These were marked with a pencil and the blot was totally destained by drop-wise addition of 500 mM NaOH to the dH₂O. Unoccupied protein binding sites were blocked by incubating the nitrocellulose in blocking reagent (1 h at RT). The blot was washed with TBS (3 × 5 min) and incubated with an appropriate dilution of primary antibody in blocking reagent (2 h at RT). The blot was again washed with TBS (3 × 5 min) and incubated with secondary antibody appropriately diluted with blocking reagent (1 h at RT). Following a final wash with TBS (3 × 5 min), substrate was added, and the colour-forming reaction allowed to proceed until dark bands were visible against a lightly stained background. Rinsing the blots thoroughly with dH₂O stopped the reaction, allowing blots to be dried between sheets of filter paper and stored until photography was convenient.

Chapter 4

Strategies for recombinant expression of VP4, the putative IBDV protease

4.1 Introduction

The details known about VP4 are tantalisingly few. Jagadish *et al.* (1988) demonstrated by site-directed mutagenesis that VP4 is involved in viral precursor polypeptide processing, Brown and Skinner (1996) identified a possible serine-protease motif for VP4 and Kibenge *et al.* (1997) extended the findings of Jagadish *et al.* (1988) and confirmed that the VP4 C- and N-termini are essential for proteolytic activity. VP4 is thus of great interest for further investigation, not only as an enzyme involved in viral polyprotein processing, but also as an IBDV marker which may have use in diagnostics and in classification. Microgram quantities of purified VP4 would aid such investigations greatly and developing strategies for the production of such amounts of enzyme was an important objective of this study.

With this objective in mind, different approaches were considered. Purifying VP4 directly from virus or infected material presented some difficulties. Firstly, IBDV particles from infected cell culture medium have been shown to contain mainly VP2 (51%) and VP3 (40%) and a little VP4 (6%) and VP1 (3%) (Dobos *et al.*, 1979), indicating that VP4 is only found in minute amounts in mature IBDV particles (Becht, 1980). Purification of sufficient quantities of VP4 from infected cells would thus require a significant quantity of source material. Although this may have been possible with cell culture, passage has been shown to cause attenuation of virulence (Snedeker *et al.*, 1967; Izawa *et al.*, 1978; Yamaguchi *et al.*, 1996), which would negate the objectives of examination of the outbreak strain(s). Classical virulent and very virulent (Brown *et al.*, 1994) strains cannot be propagated *in vitro* (McFerran *et al.*, 1980) and therefore SPF birds must be infected to generate quantities of virus, which requires time and resources beyond the scope of this project. The Allerton Regional Veterinary Laboratory (Pietermaritzburg) has an archive of infected material spanning the years of interest, but the samples obtained were a finite resource for purification of virus alone. In addition, many samples contained haemorrhagic tissue that may have released increased volumes of cellular serine proteases on homogenisation, making subsequent purification of small quantities of a possible serine protease of uncertain specificity increasingly difficult.

Heterologous expression of VP4 via recombinant DNA techniques appeared a feasible way to circumvent these difficulties. Considering the small size of the IBDV genome, RT-PCR

seemed the appropriate way to obtain material for cloning. This approach is efficient when compared to preparing a cDNA library and would additionally generate quantities of genetic material for the analysis described in the objectives of this study. Initially, the entire IBDV ORF A1 was to be amplified by RT-PCR, allowing examination of the sequence of the coat proteins VP2 and VP3 as well as VP4. Azad *et al.* (1987) showed that expression of the complete segment in *E. coli* results in the production of correctly processed VP3 and produces VP2 which is conformationally recognisable by virus-neutralising mAbs. This is encouraging and potentially useful with respect to the production of subunit vaccines. It was hoped that expression of the IBDV ORF A1 polyprotein would also allow autocatalytic cleavage and release VP4 in a fully active form, bearing in mind that the cleavage sites proposed by Jagadish *et al.* (1988) have not been conclusively confirmed. This approach promised to simplify the production of VP4 in an active form for enzyme characterisation.

In terms of selecting an expression system, the methylotrophic yeast *Pichia pastoris* has produced impressive results as a high-level heterologous gene expression system, generating grams per litre of a variety of intracellular and extracellular proteins, as detailed in Table 4-1 (see Cregg *et al.*, 1993 for a review). A number of enzymes are among the successfully expressed proteins, many of which exhibit significant activity (Tschopp *et al.*, 1987a, 1987b; Despreaux and Manning, 1993; Sun *et al.*, 1997). The potential for high-level expression is not the only feature that makes the *Pichia* system particularly attractive for the production of foreign protein. It also offers: 1), the simplicity and low expense typical of prokaryote manipulation with the advantages of eukaryote expression; 2), the option of secretory or cytosolic protein expression; 3), glycosylation which involves much shorter N-linked oligosaccharide chains than those of *Saccharomyces cerevisiae*; 4), control of expression by a strong and tightly regulated promoter and 5), looking to longer-term advantages, high-productivity fermentation technology has already been developed for *P. pastoris* (Wegner, 1983).

Table 4-1. Proteins successfully expressed in *P. pastoris*.

Protein	Expression levels (grams.litre ⁻¹)	Secreted/Intracellular and Mut Phenotype	Reference
Hepatitis B surface antigen	0.4	Intracellular, Mut ^S	Cregg <i>et al.</i> , 1987
β-galactosidase	^a	Intracellular, Mut ⁺	Tschopp <i>et al.</i> , 1987a
Invertase	2.3	Secreted, Mut ⁺	Tschopp <i>et al.</i> , 1987b
Bovine lysozyme c2	0.55	Secreted, Mut ⁺	Digan <i>et al.</i> , 1989
Streptokinase	0.08	Intracellular, ^b	Hagenson <i>et al.</i> , 1989
Tumour necrosis factor	10.0	Intracellular, Mut ^S	Sreekrishna <i>et al.</i> , 1989
Tetanus toxin fragment C	12.0	Intracellular, Mut ⁺ /Mut ^S	Clare <i>et al.</i> , 1991a
Mouse EGF ^c	0.45	Secreted, Mut ^S	Clare <i>et al.</i> , 1991b
Petussis antigen p69	3.0	Intracellular, Mut ^S	Romanos <i>et al.</i> , 1991
Aprotinin	0.8	Secreted, Mut ⁺ /Mut ^S	Vedvick <i>et al.</i> , 1991
Human serum albumin	4.0	Secreted, Mut ⁺ /Mut ^S	Barr <i>et al.</i> , 1992
Kunitz protease inhibitor	1.0	Secreted, ^c	Wagner <i>et al.</i> , 1992
HIV-1 gp120	1.25	Intracellular, Mut ⁺ /Mut ^S	Scorer <i>et al.</i> , 1993

^a β-galactosidase was expressed at 20000 U per mg total protein

^b Mut phenotype was not described

^c EGF: epidermal growth factor

Secretion of the heterologous protein is a major advantage with respect to purification. *P. pastoris* secretes very little native protein and the protein concentration of the minimal growth medium is very low, which means almost all protein subsequently found in the growth medium is recombinant and neatly separated from contaminating proteins (Barr *et al.*, 1992).

However, the nature of the viral polypeptide raised uncertainties as to its fate within the host expression system: would autodigestion occur, and if so, at which position and would the released VP4 have any activity? It was decided to investigate both the intracellular and the secreted route in order to maximise the chances for successful expression and correct processing of the viral polypeptide, even if processing occurred subsequent to purification. Additionally, the eukaryotic yeast system would possibly provide a better indication of what occurs in the bursa than a bacterial expression system.

However, full-length RT-PCR from the dsRNA template of IBDV ORF A1 had associated difficulties discovered after some experimentation. At this stage, it also became apparent that the *Pichia* system is quite subtle in its implementation (D. York, University of Natal Medical School, South Africa, personal communication). A decision was taken to focus the RT-PCR on VP4 alone and to find an appropriate system to use in *E. coli*, thereby eliminating variables and streamlining the recombinant expression.

The *QIAexpress* system (QIAGEN, Hilden, Germany) allows integrated expression and purification of recombinant protein from *E. coli*, utilising the properties of *QIAexpress* pQE vectors and a nickel-nitrilotriacetic acid (Ni-NTA) resin (Hochuli *et al.*, 1987). This resin is highly selective for proteins with an affinity tag of six consecutive histidine residues (6×His) (Hochuli *et al.*, 1988). With the potential for one-step purification of a pure recombinant protein ready for direct use in downstream procedures, this system was most attractive.

Immobilised metal chelate affinity chromatography was first used to purify proteins in 1975 (Porath *et al.*, 1975) and has subsequently become a widely used technique. The NTA ligand is particularly successful, occupying four of the six binding sites in the co-ordination sphere of the Ni²⁺ ion and leaving two sites free to interact with the 6×His tag. This allows NTA to bind the metal ions tightly under a variety of conditions not supported by alternative resins, which only have three sites available for chelation (Hochuli, 1989a). Because of this ion-binding stability, the NTA resin is able in turn to bind 6×His-tagged protein extremely tightly. This results in highly discriminating purification from contaminating proteins: from less than 1% to greater than 95% homogeneity (Janknecht *et al.*, 1991).

Tagged proteins bind to the resin with very high affinity ($K_d = 10^{-13}$) and their interaction does not require any functional protein structure, unlike that of purification systems which rely on antigen/antibody or enzyme/substrate interactions (Hochuli *et al.*, 1987). Consequently, Ni-NTA can be used to purify a variety of proteins, even those that are insoluble under non-denaturing conditions (Hochuli, 1989b), such as receptors, membrane proteins and proteins forming inclusion bodies. Elution, on the other hand, can be achieved under very mild conditions; using imidazole (100-200 mM) as a structural competitor or by lowering the pH to 5.9 and causing protonation of the histidines. Table 4-2 details the stability of the binding reaction to various reagents.

Table 4-2. Reagents compatible with the Ni-NTA-6×His interaction.^a

Reagent	Concentration
Guanidine HCl	6 M
Urea	8 M
β-mercaptoethanol	20 mM
Triton [®] X-100	2% (v/v)
Tween [®] 20	2% (v/v)
CHAPS	1% (w/v)
Glycerol	50% (v/v)
Ethanol	30% (v/v)
NaCl	2 M
MgCl ₂	4 M
CaCl ₂	5 mM
Imidazole	≤ 20 mM

^a After QIAGEN QIAexpress (1995)

The 6×His tag itself is very small (0.84 kDa) compared to other affinity tags that are full-length proteins, such as glutathione S-transferase (26 kDa) and maltose binding protein (40 kDa) (QIAGEN, 1995). As such, it rarely alters or contributes to immunogenicity. It also remains uncharged at physiological pH and generally does not affect structure, function, secretion or compartmentalisation of the attached protein. This has been examined for a wide variety of proteins, including enzymes (Döbeli *et al.*, 1990), transcription factors (Janknecht *et al.*, 1991), antigens (Stüber *et al.*, 1990, Takacs and Gerard, 1991) and membrane proteins (Bush *et al.*, 1991). There is even a precedent for the use of this system in the expression of an enzymatically active viral protease - that of human spumaretrovirus, as reported by Pfrepper *et al.* (1997). Consequently, removal of the tag by protease cleavage is seldom required.

The QIAexpress system additionally provides for the highly sensitive detection of recombinant 6×His-tagged proteins. In the case of the system's Type IV constructs, an additional Arg-Gly-Ser (RGS) sequence is encoded 5' to the 6×His tag and a monoclonal antibody has been prepared against the specific amino acid sequence RGS-(His)₄. This antibody is available from QIAGEN for a variety of useful screening and detection procedures, including screening of positive expression clones and immunohisto/cytochemistry.

This chapter describes purification of dsRNA from KwaZulu-Natal strains of IBDV (KZN IBDV) (Section 4.2) for the preparation of RT-PCR amplified cDNA to be cloned into both the *Pichia* and *E. coli* expression systems detailed above (Sections 4.3 and 4.4). The result of RFLP analysis conducted on KZN IBDV VP4 cDNA and comparison with that of other IBDV strains is also reported (Section 4.5).

4.2 Purification of virus and genetic material

Infected bursal material was obtained from samples collected and frozen by Allerton Regional Veterinary Laboratory. Diseased bursae of individual chickens were removed and coarsely minced with PBS before storage at -70°C in plastic cryovials. The methods used to extract virus from bursal tissue and subsequently isolate dsRNA from virus are adaptations of those of Azad *et al.* (1985) and Vakharia *et al.* (1992) and are summarised in Figure 4-1 at the end of Section 4.2.2. Uninfected bursal material was subjected to identical procedures to provide an appropriate negative control.

4.2.1 Reagents

Formaldehyde solution, 10% (v/v) [27% (v/v) 37% formaldehyde]. 37% (v/v) formaldehyde (54 ml) was measured into a glass reagent bottle, mixed with tap water (146 ml) and stored at RT.

2 \times saline sodium citrate (2 \times SSC) [300 mM NaCl, 30 mM Na-citrate, pH 7.0]. NaCl (17.53 g) and Na-citrate (8.82 g) were dissolved in dH₂O (900 ml), titrated to pH 7.0 with NaOH, made up to 1 litre and dispensed into aliquots (100 ml each), which were sterilised by autoclaving.

Sucrose solution, 40% (m/v). Sucrose (10 g) was dissolved in 2 \times SSC (15 ml), the volume made up to 25 ml and the solution sterilised by autoclaving.

Viral lysis buffer (VLB) [100 mM Tris-HCl, 150 mM NaCl, 12 mM EDTA, 1% (m/v) SDS, pH 7.5]. Tris base (3.03 g) and NaCl (2.19 g) were dissolved in dH₂O (150 ml) and 100 mM EDTA (30 ml, Section 2.5.1.1) and 10% (m/v) SDS (25 ml, Section 2.5.1.1) were added. The pH was titrated to 7.5 with HCl, the volume made up to 250 ml and the solution sterilised by autoclaving.

Proteinase K, 25 mg.ml⁻¹. Proteinase K from *Tritirachium album* (25 mg) was dissolved in sterile ddH₂O (1 ml), dispensed into aliquots (50 µl each) and stored at -20°C. Multiple freeze-thaw cycles were avoided.

Tris-saturated phenol [phenol equilibrated with 500 mM Tris-HCl, pH 7.0]. This was prepared as for Tris-saturated phenol, pH 8.0 (Section 2.5.1.1) except that the Tris buffers used were at pH 7.0.

8 M LiCl solution. LiCl (33.91 g) was dissolved in dH₂O (100 ml), dispensed into aliquots (25 ml each) and sterilised by autoclaving.

10 mM sodium phosphate buffer, pH 7.0. NaH₂PO₄.2H₂O (1.56 g) was dissolved in dH₂O (950 ml), titrated to pH 7.0 with NaOH and made up to 1 litre.

Acridine orange stock solution [50 mg.ml⁻¹ acridine orange]. Acridine orange (500 mg) was dissolved in 10 mM sodium phosphate buffer, pH 7.0 (10 ml) and stored at RT. Stock solution was diluted with dH₂O to give a working concentration of 30 µg.ml⁻¹.

4.2.2 Procedure

Aliquots of bursal material were partially thawed on ice before the addition of an equal volume of cold 2×SSC. These high salt conditions improve resistance of viral dsRNA to nuclease digestion (Spies *et al.*, 1987) and may also contribute to the stability of the viral protease. The sample was kept on ice and cut finely with sharp scissors to disrupt connective tissue. This mixture was homogenised (1 500 rpm, 5 min) over ice in a Potter S homogeniser (B. Braun, Melsungen, W. Germany). A centrifugation step (17 000×g, 15 min, 0°C) pelleted cellular debris, allowing the supernatant to be carefully layered onto a 40% (m/v) sucrose cushion before ultracentrifugation in a Beckman SW40 rotor (86 000×g, 2.5 h, 2°C).

The supernatant and sucrose were carefully removed and the virus-containing pellet drained thoroughly before it was resuspended in VLB (1 ml or 500 µl, depending on size). Gentle pipetting was necessary to disrupt the pellet sufficiently for resuspension. Proteinase K (500 µg.ml⁻¹) was added and the solution incubated at 37°C for 1 h to allow digestion to occur.

Following lysis, digested virus (500 μ l) was aliquoted into microfuge tubes and thoroughly mixed with half a volume of phenol (pH 7.0) and half a volume of chloroform. Tubes were centrifuged in a microfuge (15 000 rpm, 1 min, RT) and the aqueous phase removed to a clean tube. The organic phase was re-extracted with VLB (600 μ l) and the aqueous phases pooled. A second phenol:chloroform extraction was performed on this aqueous pool and nucleic acids were subsequently precipitated by the addition of ice-cold 99.5% (v/v) ethanol (2 volumes) and storage at -20°C for 2 h. Nucleic acids were pelleted by centrifugation (15 000 \times g, 15 min, 0°C) and resuspended in an appropriate volume of sterile ddH₂O (Table 2-3).

Double-stranded viral RNA was further purified from host nucleic acids using the fractionation procedure of Diaz-Ruiz and Kaper (1978), based on the differential solubility of nucleic acids in concentrated LiCl. High molecular mass ssRNA was precipitated from this solution by addition of 8M LiCl ($1/3$ volume, 2 M final concentration) and incubation at 0°C (8-18 h), and pelleted by centrifugation (15 000 \times g, 15 min, 4°C). This pellet was usually discarded. Double-stranded RNA was precipitated from the supernatant by further addition of 8M LiCl ($1/2$ volume, 4 M final concentration) and incubation at 0°C (8-18 h), and pelleted by centrifugation (15 000 \times g, 15 min, 4°C). The pellet was washed twice with 70% (v/v) alcohol (1 ml), resuspended in an appropriate volume of sterile ddH₂O (Table 2-3) and stored at -20°C . When convenient, aliquots of the nucleic acid fractions were run on agarose gels (Section 2.6.6) to assess purity and concentration. If the dsRNA samples were still contaminated, one or two more cycles of salt fractionation were performed.

To confirm the double-stranded nature of the genetic material, an aliquot of partially purified viral RNA was analysed by acridine orange staining following agarose gel electrophoresis. As described by McMaster and Carmichael (1977), acridine orange can either intercalate into double-stranded nucleic acids (green fluorescence at 530 nm), or bind electrostatically to phosphate groups of single-stranded molecules (red fluorescence at 640 nm). An agarose gel was prepared and electrophoresis performed as described in Section 2.6.6, but without the addition of EtBr to the agarose and the tank buffer. Following electrophoresis, the gel was incubated in the dark in acridine orange solution (30 $\mu\text{g}\cdot\text{ml}^{-1}$, 30 min) and destained in an enamel pan through a number of changes of phosphate buffer (Section 4.4.1). The enamel adsorbs acridine orange and thus reduces the time needed to remove background fluorescence. The gel was viewed on a UV transilluminator as described in Section 2.6.6.

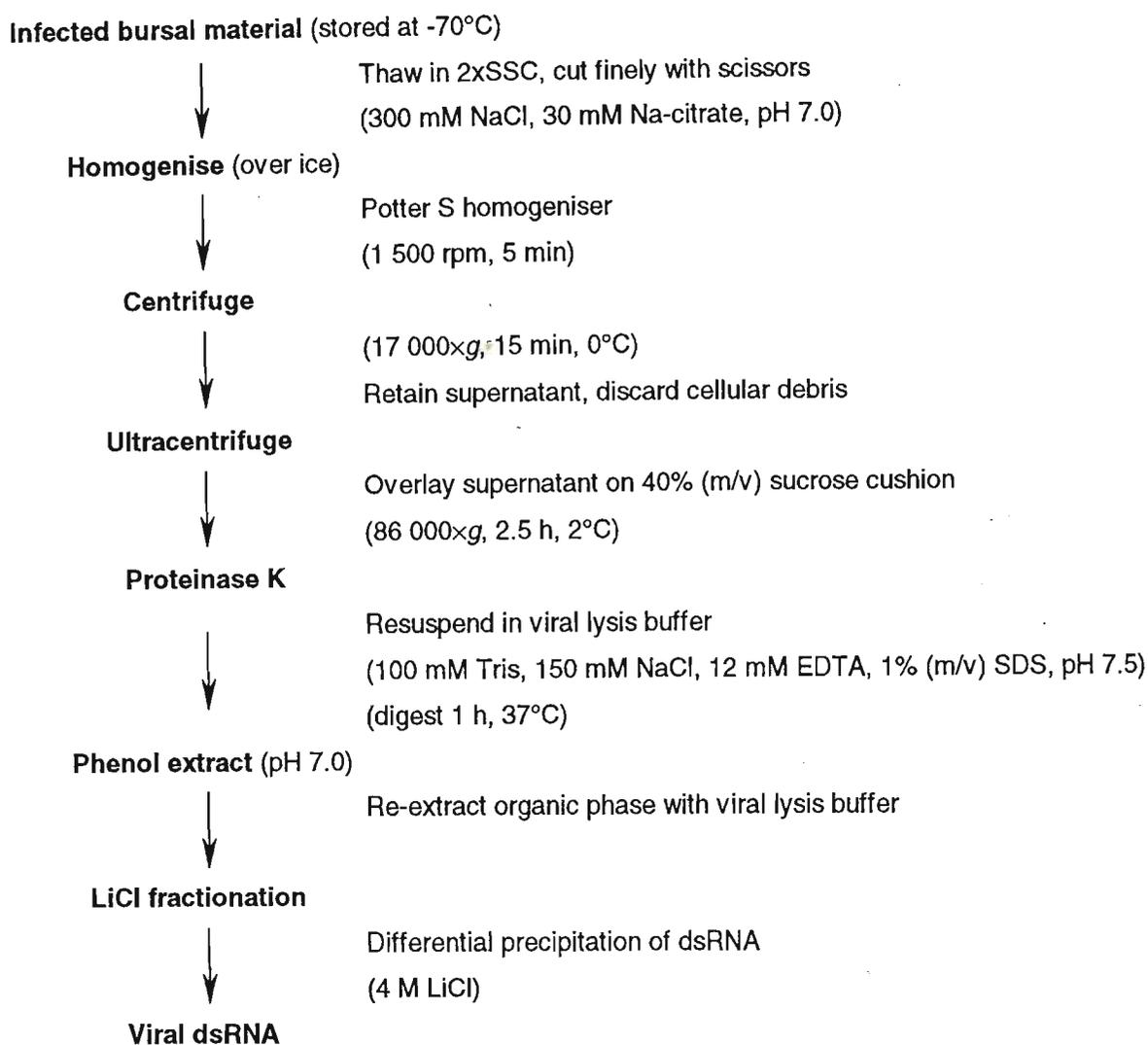


Figure 4-1. Flow diagram detailing the purification of viral dsRNA from infected bursal material.

4.3 Facilitating recombinant expression of VP4 in *P. pastoris*

A *Pichia* expression kit provided by Invitrogen® (San Diego, USA) was available for use in this study. This kit comes supplied with three vectors, which direct either intracellular (pHIL-D2) or secreted (pPIC9 and pHIL-S1) production of a cloned gene. The review by Cregg *et al.* (1993) shows that heterologous proteins have been produced with equal success via both intracellular and secreted expression in *P. pastoris*. Since there was no way to determine which of these would be most suitable for the viral polypeptide, I aimed to attempt both. The vectors pPIC9 and pHIL-S1 differ only in the nature of the secretion signal sequence, though the signal for the *S. cerevisiae* α -factor prepro-peptide (found in pPIC9) has been used with the most success (Scorer *et al.*, 1993, Cregg *et al.*, 1993). Consequently, pPIC9 was the vector chosen for the secreted expression of the viral polypeptide.

Briefly, heterologous expression is achieved as follows. Alcohol oxidase is the enzyme controlling the first step of methanol metabolism in *P. pastoris*. This enzyme has a poor affinity for the molecular oxygen required for oxidation and is consequently produced by the yeast in large quantities ($\geq 30\%$ of total soluble protein) to compensate (Cregg *et al.*, 1985). A tightly regulated promoter controls expression of the alcohol oxidase gene (*AOXI*) at the level of transcription and it is this promoter which has been isolated to drive heterologous expression in *P. pastoris*. (Ellis *et al.*, 1985).

The three expression vectors each contain the *AOXI* promoter and the 3' region of the *AOXI* gene, separated by a multiple cloning site (MCS), a transcription termination sequence and the *Pichia* wild-type *HIS4* gene. The gene of interest is cloned behind the promoter and the recombinant plasmid linearised before transformation of *his4* mutant (His^-) yeast sphaeroplasts. These linearised plasmids are able to generate stable transformants of *P. pastoris* by homologous recombination between common sequences in the plasmid and the *Pichia* genome (Cregg *et al.*, 1985; 1989). The result is gene insertion at either the *AOXI* locus or the *his4* locus, or gene replacement at the *AOXI* locus. In each case, the gene of interest and the wild-type *HIS4* gene are integrated in the genome, allowing His^+ transformants to be selected on histidine-deficient media.

However, the phenotype of *P. pastoris* transformed by *AOXI* gene replacement changes from Mut^+ (Methanol ututilisation plus) to Mut^s (Methanol ututilisation slow), since production of alcohol oxidase is greatly reduced by the loss of this gene (Koutz *et al.*, 1989). Table 4-1 shows that both Mut phenotypes have favoured the expression of a variety of proteins, hence transformants of both phenotypes should be selected. Control of the *AOXI* promoter is finally deregulated by derepression (growth on glycerol as opposed to glucose) and induction (growth on methanol), resulting in expression of the heterologous gene (Cregg *et al.*, 1989). The most efficient clone may be selected by monitoring the production of foreign protein using SDS-PAGE over a time course.

4.3.1 Primer design enabling use of Invitrogen® expression vectors

The intention was to use RT-PCR to isolate the sequence spanning IBDV ORF A1, and to simultaneously introduce elements allowing the amplified DNA to be cloned into and expressed from vectors used in the *Pichia* system. Consequently, these primers had additional requirements to satisfy while still considering the guidelines for primer design detailed in

Table 2-6. These vector-specific design elements are summarised in Figure 4-2 and are elaborated in the remainder of this section.

Forward primer SAf1 : 5' CAA GAA ITC GCA GCG ATG GCA AAC CTG 3'
 (27-mer; bases 117-143)
 52% G+C content; $T_m=71^\circ\text{C}$

Reverse primer SAR1 : 3' CTC CTG GAA CTC ACT CTT AAG ACC CTC 5'
 (27-mer; bases 3156-3182)
 52% G+C content; $T_m=71^\circ\text{C}$

Figure 4-2. Sequence of RT-PCR primers designed to clone IBDV ORF A1 into Invitrogen[®] vectors.

Coloured type depicts the following: **green**, *EcoR* I restriction cloning sites; **magenta**, clamping base pairs; **red**, start codon and stop codon of IBDV ORF A1; **turquoise**, bases constituting a Kozak consensus sequence. Bases which mismatch the target RNA are underlined. The bases are numbered according to Bayliss *et al.* (1990).

A search of the GenBank genetic sequence database and consultation of relevant literature yielded sequences and partial sequences of a variety of IBDV strains. The 5' and 3' sequences flanking the large ORFs of these strains were aligned and examined for sufficient conservation to ensure the best chance of primer binding to the as yet unsequenced KZN IBDV ORF A1. These regions appear to be highly conserved among strains of the virus found world-wide.

The IBDV ORF A1 sequence of the American IBDV variant termed GLS (Vakharia *et al.*, 1994) was checked for a variety of plasmid restriction sites. (This strain was chosen at random to be sufficiently representative for use as a model). These sites within the Invitrogen[®] vectors are necessary for their linearisation before transformation of yeast sphaeroplasts. It was determined that *Bst*B I and *Stu* I would be appropriate for linearisation of both expression vectors to eventually generate His⁺Mut^S and His⁺Mut⁺ phenotypes respectively. Neither of these restriction sites was present in IBDV ORF A1.

For simplicity and cost-effectiveness, a single set of primers was designed for use in both RTn and PCR, which would additionally ensure that the amplified product could be cloned into either pHIL-D2 or pPIC9. This meant that the same restriction site (*EcoR* I) had to be

incorporated into both primers, as it is the only site available for cloning in pHIL-D2. This is not ideal, both in terms of restricting complementarity between primers and in terms of cloning, since it does not allow forced orientation. However, the restriction site was added to the 5' ends where inter-primer complementarity is not so compromising and the versatility of the amplified product would provide concession for losing directional cloning. Before the primer designs were finalised, the ORF A1 sequence of the GLS variant was checked for the presence of *EcoR* I sites; none were found. A reference appendix (New England Biolabs, 1995) revealed that *EcoR* I cuts with 100% efficiency when the site is just 1 bp from the end of a DNA fragment, hence the clamps of three and six bp added to the 5' ends of the primers were considered adequate.

Correct initiation of translation of a cloned gene from pHIL-D2 requires an initiating ATG codon in a Kozak consensus sequence; that is, with a purine at position -3 and guanine at position +4 (Kozak, 1987). The first condition was already met within the viral sequence surrounding the initiating ATG of the large ORF, but the +4 guanine needed to be built into the forward primer. With respect to pPIC9 expression, it was essential that the foreign gene be cloned in frame with the signal sequence initiation codon. The restriction site was carefully placed to achieve this while at the same time ensuring that the untranslated leader remained as short as possible; this apparently favours *AOX1*-driven expression (Cregg *et al.*, 1989).

Once the sequence of the primers had been determined as described above, the ORF A1 sequence of the GLS IBDV variant was checked for any internal regions of significant homology with either primer. The local similarity (SIM) program (Huang and Miller, 1991) at the ExPASy website (<http://expasy.hcuge.ch/sprot/sim.html>) allowed appropriate comparisons to be made. The highest random homologies found were 56% and 59% for the 5' and 3' primers respectively, and the longest random contiguous homologies were five and eight bp respectively. These values were all sufficiently low to expect that the T_m of the primers at these random sites would be sufficiently reduced to prevent mispriming (Sambrook *et al.*, 1989h).

4.3.2 Attempting RT-PCR of IBDV ORF A1

The primers SAf1 and SAR1 described above were intended to amplify 3066 bp constituting ORF A1 of IBDV. The principle is as described in Sections 2.8 and 2.8.1. However, much optimisation of the PCR parameters had to be effected before even partial amplification was achieved. The ranges evaluated within these parameters are summarised in Table 4-4; the reagents and procedures described below are those which resulted in an attenuated amplification of ~1500 bp.

4.3.2.1 Reagents

Tris-EDTA solution (TE) [10 mM Tris-HCl, 1 mM EDTA, pH 8.0]. 500 mM Tris-HCl (2 ml, Section 2.5.1.1) and 100 mM EDTA (1 ml, Section 2.5.1.1) were diluted with ddH₂O (90 ml), titrated to pH 8.0 with HCl, made up to 100 ml and sterilised by autoclaving.

Primer stock solutions [30 µM and 10 µM forward or reverse primer]. Forward and reverse primers were obtained as lyophilised pellets from the supplier and resuspended in TE to give separate master stock solutions (300 µM), which were stored at -20°C. Aliquots of each master stock (10 µl) were diluted with TE (90 µl) to give a stock solution of 30 µM used for both RTn and PCR. The working concentrations of primer in these reactions were 1.5 µM and 0.1 µM respectively.

dNTP stock solution [5 mM dNTPs]. An aliquot of each dNTP (10 µl) was taken from supplied stocks (100 mM lithium salts stored at -70°C) and placed in a microfuge tube. Sterile ddH₂O (160 µl) was added to bring the final concentration of dNTPs to 5 mM. The working concentrations of dNTPs in the RTn and PCR reactions were 0.5 mM and 0.1 mM respectively.

5× RTase buffer [250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl₂, pH 8.3]. This buffer was supplied with the MMLV-RT (Gibco BRL, Paisley, UK).

20× DTT stock solution [200 mM DTT]. DTT (31 mg) was dissolved in sterile ddH₂O (1 ml), dispensed into aliquots (10 µl each) and stored at -20°C. DTT was not subjected to multiple freeze-thaw cycles; aliquots were thawed just before use and unused portions discarded. DTT was used in the RTn reaction at a working concentration of 10 mM.

10× PCR reaction buffer [500 mM KCl, 100 mM Tris-HCl, 1% Triton X-100 (v/v), pH 9.0].

This buffer was supplied with the *Taq* DNA polymerase (Promega, Madison, USA).

MgCl₂ stock solution [25 mM MgCl₂]. This stock solution was supplied with the *Taq* DNA polymerase (Promega, Madison, USA). MgCl₂ was used in the PCR reaction at a working concentration of 1.25 mM.

4.3.2.2 Procedure

Denaturing viral dsRNA and annealing primers. Viral dsRNA (10 ng, 4.5 fmoles, purified as described in Section 4.2), 30 pmoles of each primer (1 µl of 30 µM stock solutions) and ddH₂O making up a total volume of 12 µl were placed in a 0.2 ml thin-walled PCR reaction tube and centrifuged briefly. The tube was placed in a heating block (100°C, 5 min) and immediately cooled in a water bath (42°C, 5 min), or alternatively, boiled in a water bath (5 min) and immediately cooled in crushed ice, prior to the RTn reaction. This procedure allows denaturation of the viral dsRNA and annealing of the short oligonucleotide primers without re-annealing of the template strands.

RTn reaction. A master mix of 5× RTase buffer (4 µl), DTT (1 µl of stock solution) and dNTPs (2 µl of stock solution) was prepared, the actual volume of each reagent being a multiple of the number of samples processed. An aliquot of this RTn mix (7 µl) was added to each sample tube of denatured RNA, which was kept in the water bath until the addition of RTase (1 µl, 200 U of MMLV-RT), bringing the final volume to 20 µl. The sample tubes were centrifuged briefly in a microfuge (10 000 rpm, 1 sec) to coalesce droplets and tapped gently to fully disperse the MMLV-RT before incubation in a water bath (42°C, 1 h) to allow reverse transcription to occur. Any of this reaction mix not used immediately in a PCR reaction was stored at -20°C.

PCR reaction. A master mix of 10× PCR reaction buffer (5 µl), MgCl₂ (2.5 µl of stock solution), dNTPs (1 µl of stock solution) and ddH₂O (34 µl) was prepared, the actual volume of each reagent being a multiple of the number of samples processed. An aliquot of this PCR mix (42.5 µl) was added to a new 0.2 ml thin-walled PCR reaction tube, followed by an aliquot of RTn reaction product (5 µl) and 30 pmoles of each primer (1 µl of 30 µM stock solutions). The tubes were centrifuged briefly and placed in a GeneAmp[®] PCR System 2400 thermocycler (Perkin Elmer, Norwalk, USA).

The thermocycler was programmed to allow a 'hot-start', constituting an initial denaturing step (99.9°C, 5 min), lowering the temperature to the annealing temperature (71°C, 40 sec) and holding this temperature while thermostable DNA polymerase was added to each tube (0.5 µl, 2.5 U of *Taq* DNA polymerase). This brought the final volume of the PCR reaction to 50 µl. The reaction was allowed to continue for a further 25 cycles (denature 95°C, 1 min; anneal 71°C, 1 min; extend 72°C, 2 min), followed by a final extension period (72°C, 5 min) to ensure that all molecules were fully double-stranded. Tubes were held at 4°C until they were removed from the thermocycler for analysis of the product and/or storage at -20°C.

4.3.3 Dot-blot analysis of attenuated RT-PCR product

The homology of the ~1500 bp attenuated RT-PCR product and gel-purified IBDV ORF A1 was checked using a DIG DNA labelling and detection kit (Boehringer Mannheim). Labelling the RT-PCR product allowed its subsequent detection in an RNA/DNA dot-blot hybridisation assay and enabled an assessment of the homology between the attenuated product and the original viral RNA depending on the intensity of detection. The protocols followed were as supplied by the manufacturers.

Digoxigenin is a naturally occurring steroid isolated from plants of the *Digitalis* species. Covalently coupled to dUTP to form DIG-11-dUTP, it allows the labelling of nucleic acid probes, which can be detected following hybridisation using sensitive and non-radioactive techniques. In this particular application, DIG-11-dUTP was incorporated in a DNA probe (the ~1500 bp attenuated RT-PCR product) using random primed labelling catalysed by the Klenow enzyme. The DIG-labelled probe was hybridised to membrane-bound viral RNA (gel-purified IBDV ORF A1) using a dot blot method. The hybrid was subsequently detected via enzyme immunoassay using an anti-DIG antibody conjugated to alkaline phosphatase, which catalysed the formation of a visible product from the colourimetric substrates NBT and BCIP.

4.3.3.1 Reagents

a). Random primed DNA labelling.

10× Hexanucleotide mixture [1.56 mg.ml⁻¹ random hexanucleotides in buffered solution].

This was supplied in the Boehringer Mannheim kit.

10× dNTP labelling mixture [1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.35 mM DIG-11-dUTP, 0.65 mM dTTP]. This was supplied in the Boehringer Mannheim kit.

4 M LiCl solution. 8 M LiCl (5 ml, Section 4.2.1) was diluted with dH₂O (5 ml), dispensed into aliquots (1 ml each) and sterilised by autoclaving.

b). Quantification of DIG-labelling.

Labelled control DNA [DIG-labelled pBR328, random primed labelled, 20 ng.μl⁻¹ with approx. 5 ng.μl⁻¹ label]. This was supplied in the Boehringer Mannheim kit.

DNA dilution buffer [50 μg.ml⁻¹ herring sperm DNA in buffered solution]. This was supplied in the Boehringer Mannheim kit.

c). Hybridisation for RNA dot blotting.

20× saline sodium citrate (20× SSC) [3 M NaCl, 300 mM Na-citrate, pH 7.0]. NaCl (35.06 g) and Na-citrate (17.65 g) were dissolved in dH₂O (185 ml), titrated to pH 7.0 with NaOH, the volume made up to 200 ml and the solution sterilised by autoclaving.

RNA dilution buffer [dH₂O: 20× SSC: formaldehyde (5:3:2) (v/v)]. dH₂O (25 ml), 20× SSC (15 ml) and formaldehyde (10 ml) were mixed together in a sterile Pyrex[®] bottle (100 ml).

d). Immunological detection.

Tris-buffered saline (TBS). See Section 3.12.1.

Blocking stock solution [10% (m/v) blocking reagent in Tris buffer]. Blocking reagent (powdered casein digest supplied in the Boehringer Mannheim kit, 10 g) was dissolved in TBS (100 ml) with heat pulses in a microwave oven (3-4 min total) and the solution sterilised by autoclaving.

Hybridisation buffer [5x SSC, 0.1% (m/v) sodium-lauroylsarcosine, 7% (m/v) SDS, 2% (m/v) blocking reagent, 50% (v/v) formaldehyde]. 20x SSC (50 ml), sodium-lauroylsarcosine (0.2 g), SDS (14 g) and blocking stock solution (40 ml) were dissolved in 100 ml dH₂O with gentle heating and the volume made up to 200 ml with formaldehyde.

2x Wash solution [0.1% (m/v) SDS in 2x SSC]. SDS (0.1 g) was dissolved in 20x SSC (10 ml) and dH₂O (~80 ml) with gentle heating and the solution made up to 100 ml.

0.1x Wash solution [0.1% (m/v) SDS in 0.1x SSC]. SDS (0.1 g) was dissolved in 20x SSC (0.5 ml) and dH₂O (~90 ml) with gentle heating and the solution made up to 100 ml.

Antibody blocking solution [1% (m/v) blocking reagent in TBS]. Blocking stock solution (10 ml) was diluted in TBS (90 ml).

Detection buffer [100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl₂, pH 9.5]. Tris base (1.21 g) and NaCl (0.584 g) were dissolved in dH₂O (80 ml) and titrated to pH 9.5 with HCl. MgCl₂ (1.0 g) was dissolved in this solution and the volume was made up to 100 ml.

4.3.3.2 Procedure

All steps were carried out at room temperature and all on an orbital shaker with the exception of colour development.

a). Random primed DNA labelling.

An aliquot (100 ng) of the ~1500 bp attenuated RT-PCR product (Section 4.3.2) was diluted to a final volume of 15 µl with dH₂O. This template DNA was subsequently denatured by boiling (10 min in a water bath) and rapidly cooled on ice to maintain single-strandedness. Hexanucleotide mixture (2 µl), dNTP labelling mixture (2 µl) and Klenow enzyme (1 µl, 2 U) were added, the reaction mix was centrifuged briefly and incubated at 37°C ON. Labelling was terminated by the addition of 0.2 M EDTA (2 µl). The labelled DNA probe was precipitated with 4 M LiCl (2.5 µl), ice-cold ethanol (75 µl) and incubation at -70°C (1 h). The probe was pelleted by centrifugation (13 000×g, 15 min, 0°C) and washed with ice-cold 70% (v/v) ethanol (100 µl). The pellet was dried, resuspended in TE (Section 2.8.2; 50 µl) and stored at 4°C.

b). Quantification of DIG-labelling.

The efficiency of labelling was estimated using the supplied DIG-labelled control DNA and information supplied by the kit manufacturer (Boehringer Mannheim Biochemica, 1993). Consultation of a table correlating amount of template and length of reaction time revealed that approximately 260 ng of DIG-labelled DNA should have been obtained under the conditions described above.

Aliquots of the freshly synthesised RT-PCR product probe and labelled control DNA were thus diluted to a final concentration of $\sim 1 \text{ ng} \cdot \mu\text{l}^{-1}$ with DNA dilution buffer. These solutions were subjected to five further 10-fold serial dilutions with DNA dilution buffer. A series of these dilutions (1 μl of each, thus ranging from 100 pg - 0.01 pg) was spotted onto a piece of Magna charge nylon membrane, fixed by brief exposure (~ 5 sec) to UV light and visualised according to the protocol described below for immunological detection. The concentration of the DIG-labelled RT-PCR product was estimated at $3 \text{ pg} \cdot \mu\text{l}^{-1}$ by visual comparison of the spot intensities of the control and the experimental dilutions. This corresponds to 250 ng of labelled product (undiluted), synthesised from 100 ng of template DNA. The labelling efficiency was thus 1 in 2.5, which is within expected levels (Boehringer Mannheim Biochemica, 1993).

c). Hybridisation for RNA dot blotting.

Aliquots of gel-purified IBDV ORF A1 (Section 2.7.1) from 1990 and 1992 IBDV isolates and of *Hind* III-cut λ DNA (negative control) were diluted in RNA dilution buffer to a concentration of $1 \text{ ng} \cdot \mu\text{l}^{-1}$. These solutions were subjected to five further 10-fold serial dilutions with RNA dilution buffer and were boiled in a water bath (10 min) before rapid cooling on ice. The dilutions (1 μl of each) were spotted onto a piece of Magna charge nylon membrane and fixed by brief exposure (~ 5 sec) to UV light. The membrane was incubated in hybridisation buffer (1 h, 50°C). The ~ 1500 bp RT-PCR product probe was denatured by heating in a boiling water bath (10 min) and rapidly cooled on ice. Following dilution in hybridisation buffer to a final concentration of $20 \text{ ng} \cdot \text{ml}^{-1}$, the denatured probe was incubated with the membrane (ON, 50°C). The membrane was washed with $2\times$ wash solution (2×5 min, RT) and $0.1\times$ wash solution (2×15 min, 68°C) to remove unbound probe.

d). Immunological detection.

The membrane was equilibrated in TBS (1 min) and incubated in antibody blocking solution (1 h). After decanting the blocking solution, the membrane was incubated (30 min) with anti-DIG-AP antibody, previously diluted 1:5 000 in antibody blocking solution. The membrane was washed (2×15 min) in TBS to remove unbound antibody and equilibrated in detection buffer (2 min). NBT (45 μ l) and BCIP (35 μ l) (both solutions supplied in the Boehringer Mannheim kit) were mixed with detection buffer (10 ml) and the solution added to the membrane, which was incubated in the dark. Colour development was allowed to proceed until distinct spots were evident against a lightly stained background. The membrane was removed from the substrate solution, washed with TBS and dried between two sheets of filter paper to ensure preservation of results.

4.3.4 Functional assay of RTase by radioactive incorporation

A highly sensitive technique was needed to examine the product of the RTn reaction to investigate the attenuation of the RT-PCR amplification. This procedure was based on the functional assay for the RTase enzyme included with the manufacturer's instructions (Gibco BRL, Paisley, UK). Primers were used both together and separately to determine whether each was priming strand synthesis effectively, hence double- and single-stranded cDNA were expected from these reactions. The two denaturation techniques were also examined to highlight possible differences in efficiency. Two negative controls (without RNA and without enzyme) were included, but no positive control was available.

4.3.4.1 Reagents

$[(\alpha\text{-}^{32}\text{P})\text{dCTP}$ [$3000 \text{ Ci.mmol}^{-1}$, $10 \mu\text{Ci.}\mu\text{l}^{-1}$ in 5 mM Tris-HCl , $\text{pH } 7.4$]. The radionuclide was supplied by ICN Pharmaceuticals, Aurora, USA.

4.3.4.2 Procedure

Radionuclide incorporation. Various RTn reactions were set up as described in Section 4.3.2.2 and according to the following grid. The tracer [α - 32 P]dCTP (10 μ Ci per reaction) was included in the RTn master mix added to the denatured viral RNA-primer solution.

Sample number:	1	2	3	4	5	6	7	8
dsRNA (10 ng)	+	+	+	+	+	+	+	-
SAf1 forward primer (30 pmol)	+	-	+	-	+	+	+	+
SAr1 reverse primer (30 pmol)	-	+	-	+	+	+	+	+
denature: 100°C → 42°C	+	-	+	-	+	+	+	+
denature: boil → ice	-	+	-	+	+	+	+	+
MMLV-RT (200 U)	+	+	+	+	+	+	-	+

Electrophoresis and autoradiography. Each reaction mix (20 μ l) was loaded into a separate well of a 1% (m/v) agarose gel (18 wells, 200 ml volume) leaving a lane free between samples. The gel was prepared and samples electrophoresed as described in Section 2.6.6, except that EtBr was omitted and electrophoresis was conducted ON at RT [1.5 V.cm⁻¹ (30 V), current unlimiting]. The gel was removed from the electrophoresis apparatus, placed on a sheet of 3 MM Whatman filter paper and the gel surface covered with a film of ClingwrapTM. The gel was dried under vacuum (40 min, 60°C) and the vacuum maintained further (30 min) without heating. The ClingwrapTM was discarded after drying and the gel exposed to X-ray film (Fuji Super HR-G) in an X-ray cassette (24 h or 48 h, -70°C). Film was immersed in developing solution (Polycon A variable contrast, 4 min), rinsed (H₂O, 30 sec) and transferred to fixative solution (Perfix Hi-speed, > 2 min). The film was finally rinsed in running tap water (10 min) and hung up to dry before examination on a light box.

4.4 Facilitating recombinant expression of VP4 in *E. coli*

A decision was taken to attempt recombinant expression of VP4 alone, rather than IBDV ORF A1 in its entirety, and to do so from *E. coli* rather than *P. pastoris*. This was to avoid the difficulties encountered with production of a full-length RT-PCR product and to simplify expression as a whole. With this in mind, the QIAexpress system was selected to further streamline the process leading to purified recombinant protein, utilising the properties of QIAexpress pQE vectors and a nickel-nitrilotriacetic acid (Ni-NTA) resin (Hochuli *et al.*, 1987).

Figure 4-3 illustrates the elements present in pQE vectors, which belong to the pDS family of plasmids (Bujard *et al.*, 1987). The gene of interest is cloned into the polylinker site, and in the current study, behind the coding sequence for the 6×His tag for N-terminal placement. (The particular QIAExpress vectors resulting in this configuration are known as Type IV or pQE-3x constructs). Expression is extremely efficient and can only be prevented by high levels of *lac* repressor. To ensure high levels for tight regulation, the K-12 derived *E. coli* host strains M15[pREP4] and SG13009[pREP4] contain multiple copies of the plasmid pREP4, which expresses the *lac* repressor constitutively. Expression from the recombinant pQE plasmid is rapidly induced by the addition of sufficient isopropyl-β-D-thiogalactopyranoside (IPTG), a synthetic inducer which inactivates the repressor protein and clears the promoter, allowing transcription to occur (Pardee *et al.*, 1959). *E. coli* strains carrying the *lacI^q* mutation of the repressor gene are suitable for storage and propagation of the pQE plasmids, since the mutated promoter produces large quantities of the *lac* repressor (Müller-Hill *et al.*, 1968). Such strains are able to block expression without carrying the pREP4 plasmid and may be used as expression hosts although without such tight transcriptional regulation.

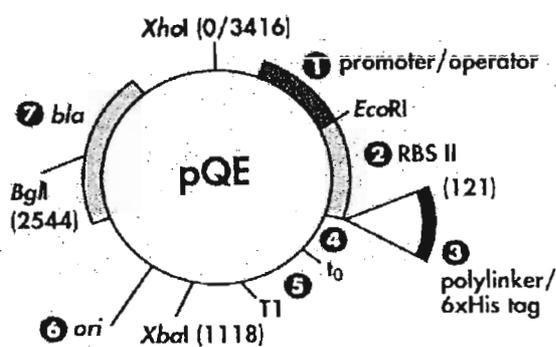


Figure 4-3. Features of and elements present in QIAexpress pQE Type IV vectors.

1) Optimised promoter/operator consisting of the phage T5 promoter and two *lac* operator sequences, which increase the probability of *lac* repressor binding and ensure effective repression of the powerful T5 promoter; 2) Synthetic ribosome binding site RBS II designed for optimal mRNA recognition and binding for efficient translation; 3) Coding sequence for 6×His 5' to polylinker cloning region (*Bam*H I, *Sph* I, *Sac* I, *Kpn* I, *Sma* I, *Xma* I, *Sal* I, *Pst* I, *Hind* III) to result in N-terminal placement of affinity tag; 4) Translational stop codons in all three reading frames for convenient preparation of constructs; 5) Two strong transcriptional terminators, *t₀* from phage lambda and T1 from the *rrnB* operon of *E. coli*, preventing read-through transcription and ensuring stability of the expression construct; 6) ColE1 origin of replication; 7) β-lactamase gene (*bla*), conferring ampicillin resistance allowing the selection of transformed *E. coli*. (After QIAGEN QIAexpress, 1995).

4.4.1 Primer design enabling use of QIAGEN expression vectors

The intention was to use RT-PCR to isolate the region spanning the likely coding sequence of the IBDV VP4 protein (Azad *et al.*, 1987; Jagadish *et al.*, 1988), and to simultaneously introduce elements allowing the amplified DNA to be cloned into, and expressed from, vectors used in the QIAexpress system. Consequently, these primers had additional requirements to satisfy while still considering the guidelines for primer design detailed in Table 2-6. These vector-specific design elements are summarised in Figure 4-4 and are elaborated in the remainder of this section.

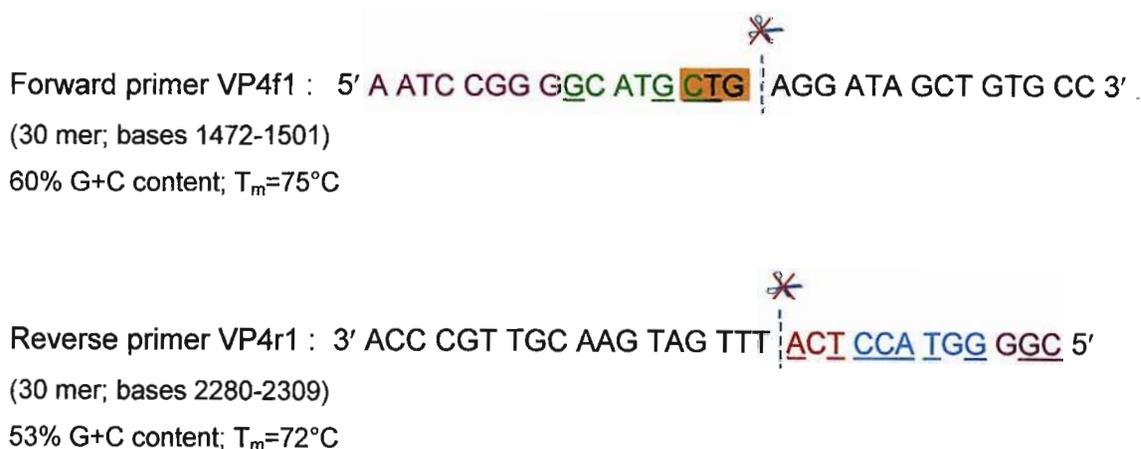


Figure 4-4. Sequence of RT-PCR primers designed to clone IBDV VP4 into QIAGEN vectors.

Coloured type depicts the following: **green**, *Sph* I restriction cloning site; **turquoise**, *Kpn* I restriction cloning site; **magenta**, clamping base pairs; ✂, indicates the mutation of possible protease cleavage sites between VP2/VP4 and VP4/VP3, **orange**, arginine codon (AGG) mutated to leucine codon (CUG); **red**, translational stop codon. Bases which mismatch the target RNA are underlined. The bases are numbered according to Bayliss *et al.*, (1990).

The sequence from a variety of different strains of IBDV (Section 4.3.1) between and surrounding the two possible cleavage sites flanking the IBDV VP4 protein was aligned and examined for sufficient conservation. This was to ensure the best chance of primer binding to these regions within the as yet unsequenced KZN IBDV segment A. The nucleotide sequence of VP4 is well conserved among globally-dispersed strains of the virus, and in fact at the amino acid level, the region is better conserved than the rest of segment A (Bayliss *et al.*, 1990).

The VP4 sequence of the GLS IBDV variant (Vakharia *et al.*, 1994) was searched for restriction sites constituting the polylinker of the pQE-3x plasmids. (This strain was chosen at random to be sufficiently representative for use as a model). Four of the eight sites were found within the VP4 sequence and were thus unavailable, while the remaining four were inspected for suitability in terms of cleavage efficiency and proximity to the 5' end of the polylinker. The latter consideration has bearing on the number of foreign amino acids added to the N-terminus of the recombinant protein.

The *Sph* I site was conveniently incorporated into the forward primer with only three base pair mismatches, maintaining homology and limiting the addition of non-viral amino acids downstream of the 6×His tag to five, as illustrated in Figure 4-5. According to a reference appendix (New England Biolabs, 1995), *Sph* I restriction is not particularly efficient close to the ends of DNA fragments, managing only 50% cleavage with four clamping base pairs after 20 h. Eight 5' clamping base pairs were included in the forward primer to ensure that the *Sph* I restriction was as efficient as possible. Having incorporated the *Sph* I site, the reading frame could be established and the appropriate pQE-3x vector (pQE-32) selected to ensure in-frame cloning with the translation initiation codon. Finally, the design of the forward primer included a mutation of the possible VP4 N-terminal cleavage site from Arg-Arg to Leu-Arg. This mutation was introduced to prevent the loss of the 6×His tag by trypsin-like protease cleavage at this point.

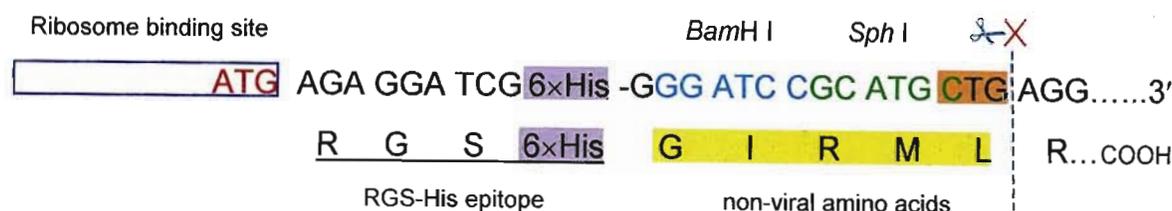


Figure 4-5. Expected sequence around the pQE-32 *Sph* I site after cloning of VP4 RT-PCR product.

The amino acid sequence corresponding to the N-terminus of recombinant VP4 is given under the relevant codons. **ATG**, translation initiation codon; ~~X~~, indicates the mutation of possible protease cleavage sites between VP2/VP4 and VP4/VP3; CTG, arginine codon (AGG) mutated to leucine codon (CUG).

Kpn I cleaves efficiently close to the end of DNA fragments, hence this site was incorporated into the reverse primer with three clamping base pairs known to allow >90% cleavage after 2 and 20 h (New England Biolabs, 1995). For simplicity, a translational stop codon was built in

to replace the second basic amino acid at the C-terminal cleavage site. This allowed the insertion of the cloning site without adding extra protein coding sequence to the 3' end of the RT-PCR product and without having to work the site into the target sequence with high homology. In addition, both possible protease cleavage sites were now mutated thus requiring no cleavage from any other source to release the recombinant VP4.

Once the sequence of the primers had been assembled as described above, the IBDV ORF A1 sequence of the GLS variant was checked for any internal regions of significant homology with either primer. The local similarity (SIM) program (Huang and Miller, 1991) at the ExPASy website (<http://expasy.hcuge.ch/sprot/sim.html>) allowed appropriate comparisons to be made. The highest random homologies found were 53% and 57% for the 5' and 3' primers respectively, and the longest random contiguous homologies were six and five bp respectively. These values were all sufficiently low to expect that the T_m of the primers at these random sites would be sufficiently reduced to prevent mispriming (Sambrook *et al.*, 1989h).

4.4.2 RT-PCR of VP4 and efforts to clone the amplicon

The primers VP4f1 and VP4r1 described in Section 4.4.1 were used in standard VP4 RT-PCR experiments as described in Sections 2.8.2 and 2.8.3 to amplify the 838 bp constituting the coding sequence of the putative protease. The fragment thus obtained was used for cloning into the QIAGEN expression vector pQE-32.

4.4.3 Reagents

The REs and T4 DNA ligase were supplied with appropriate 10× reaction buffers by the manufacturers (Amersham, Cleveland and Boehringer Mannheim, Mannheim respectively). *Sph* I has a markedly higher salt requirement (100 mM as opposed to zero NaCl) than *Kpn* I, necessitating separate digestions.

4.4.4 Procedure

The QIAGEN expression plasmids pQE-32 and pQE-16 were bulked up by transformation of competent JM109 cells (Section 2.4.1) with plasmids supplied in the QIAGEN Type IV kit. Sufficient quantities of pQE-32 and pQE-16 were subsequently purified from these transformed cells by alkaline lysis (Section 2.5.1). RT-PCR product was obtained via the standard VP4 RT-PCR described in Sections 2.8.2 and 2.8.3. Vector and insert were

subjected to a double digest by *Kpn* I and *Sph* I, and ligation was attempted following gel purification (Section 2.7.1) of the restricted products. A variety of permutations of the restriction and ligation procedures were tested as explained below. Numerous control reactions were conducted for both restriction and ligation and are detailed along with their results in Section 4.6.3.

Restriction reactions. The pQE-32 vector and RT-PCR product were purified by gel purification or with the use of spin columns (Sections 2.7.1 and 2.7.2). An appropriate amount of 10× RE reaction buffer was added to aliquots of these purified substrates in microfuge tubes. *Kpn* I and *Sph* I were added (0.5 µl of each, corresponding to 5 U and 4 U respectively). The tubes were incubated at 37°C (2-25 h) after careful mixing. Initially, the digests were conducted sequentially in the same tube in the order of salt concentration requirements; *Kpn* I followed by *Sph* I. In later attempts to improve ligation and/or cleavage efficiency, vector and insert were digested with *Sph* I and subsequently gel purified (Section 2.7.1) and subsequently digested with *Kpn* I.

Ligation reactions. Aliquots of gel purified (Section 2.7.1) vector and insert restricted with both *Kpn* I and *Sph* I were added to microfuge tubes in a variety of molar ratios (see Table 4-3). Appropriate amounts of ligase buffer and ddH₂O were added, followed by T4 DNA ligase (1 or 0.5 U). The solution was mixed gently and incubated at and between 4°C and RT for 16 or 40 h.

Table 4-3. Components and conditions varied for ligation reactions.

[Vector] (nM)	[Insert] (nM)	Molar ratio of vector to insert	Units of ligase	Reaction time	Reaction temperature
1.8-0.35	0.58-0.12	3:1			
1.8-0.35	1.4-0.36	1:1			
1.8-0.35	5.2-1	1:3	0.5 or 1	16 or 40 h	4-22°C
0.25-0.35	2.5-3.5	1:10			

Transformation of competent M15[pREP4] cells. This was performed as described in Section 2.4.2 with various ligation mixes, using TE as a negative control and pQE-16 (1 ng) as a positive control. Competent cells were also transformed with cut vector and self-ligated cut vector in order to monitor background. The plasmid DNA of selected colonies was screened using the cracking procedure detailed in Sections 2.5.1 and 2.5.2.

4.5 RFLP analysis of amplified VP4

RFLP analysis is an alternative to complete sequencing and a convenient technique for detecting genomic differences at specified locations. It has been used directly for detection of inherited diseases such as thalassaemias and haemophilia (reviewed by Martin, 1987) and has also found application in forensic science with the generation of 'DNA fingerprints' (Marx, 1988).

The procedure is based on the premise that mutations can create or destroy restriction sites within a genome, thereby causing changes in the restriction fragmentation pattern (Mathews and van Holde, 1990). Traditionally, Southern hybridisation was used to detect the fragments of interest among the great number generated by genomic digestion. However, the use of PCR has made it possible to amplify only the region of DNA around the restriction site(s) of interest, which can then be exposed to the relevant RE(s). In effect the DNA is screened before the analysis, rather than after, as is the case using Southern hybridisation. This confers a number of advantages, including a great saving in time, the ability to use many different tissues, significant reduction of the amount of DNA needed as starting material and elimination of the need to use radioactivity (Ivinson and Taylor, 1991).

Specific amplification of IBDV fragments by RT-PCR has been used to detect the virus in infected tissue (Davis and Boyle, 1990; Lee *et al.*, 1992; Wu *et al.*, 1992; 1997; Jackwood *et al.*, 1996; Lin, 1997) and restriction analysis of such RT-PCR fragments has implemented differentiation of IBDV virus strains, often with significant local relevance (Liu *et al.*, 1994; Lin *et al.*, 1993; Dormitorio *et al.*, 1997; Jackwood and Jackwood, 1994; 1997; Jackwood and Nielsen, 1997; Dybing and Jackwood, 1996; Kataria *et al.*, 1998; Cao *et al.*, 1998; Jackwood and Sommers, 1997; 1998; Tsai *et al.*, 1998). In RFLP analysis specifically, not only is the presence or absence of a restriction site determined, but the sizes of the resulting DNA fragments are measured and compared. In this study, IBDV samples collected from 1989-1997 (excluding 1996) from areas around KwaZulu-Natal were examined. (1996 was only excluded for reasons of immediate availability of samples at the time of experiment).

The VP4 region of viral RNA purified from these samples was amplified by RT-PCR and subjected to RFLP analysis using twenty different restriction enzymes as described below.

4.5.1 Reagents

The REs used in the RFLP analysis were: *Bam*H I, *Bcl* I, *Bgl* I, *Eco*R I, *Hinc* II, *Hind* III, *Ksp* I (*Sac* II), *Nde* I, *Pst* I, *Pvu* II, *Rsa* I, *Sac* I, *Sal* I, *Sau*3A I, *Sfu* I (*Nsp* V), *Sma* I, *Ssp* I, *Stu* I, *Xba* I and *Xho* I. The enzymes were supplied with appropriate 10× reaction buffers by the manufacturers [Boehringer Mannheim (Mannheim, Germany), Promega (Madison, USA), Amersham (Cleveland, USA) and New England Biolabs (Beverly, USA).]

4.5.2 Procedure

Purification of dsRNA. Double-stranded viral RNA was purified from samples across the eight years from 1989 to 1997 (excluding 1996) as detailed in Section 4.2.2. Aliquots (1 µl for each year) of dsRNA were run on an agarose gel to assess purity and estimate concentration (Section 2.6.6).

RT-PCR of VP4 region. Volumes of dsRNA (containing ~10 ng) were removed from each sample and subjected to standard RT-PCR as described in Section 2.8.3. An aliquot (2 µl) of each RT-PCR reaction was run on an agarose gel to estimate the size and concentration of amplified bands (Section 2.6.6).

Restriction enzyme analysis. Volumes of amplified DNA (containing ~10 ng) were removed from each RT-PCR sample and incubated directly with individual REs (5 U), 10× reaction buffer (1 µl, supplied by manufacturer) and ddH₂O in a final volume of 10 µl. Samples were incubated ON at the optimum temperature for each RE. All samples digested by a particular enzyme were run in parallel lanes on the same agarose gel and the sizes of all bands noted. Control digestions using phage λ DNA were conducted simultaneously to confirm that the REs were active under the conditions used.

4.6 Results and discussion

4.6.1 Purification of virus and genetic material

Partially purified virus was obtained as a finely textured, reddish-brown pellet following ultracentrifugation. Viral dsRNA isolated from such pellets and further purified by salt fractionation is illustrated in Figure 4-6. Aliquots of these samples were also run on a gel subsequently stained with acridine orange. A banding pattern identical to that shown in Figure 4-6 was obtained, but with the doublet of viral RNA and the *Hind* III-cut λ DNA size markers fluorescing green and the low molecular mass contaminants fluorescing red-orange. This is in contrast to the uniform orange fluorescence of the samples stained with EtBr. (These results are not shown).

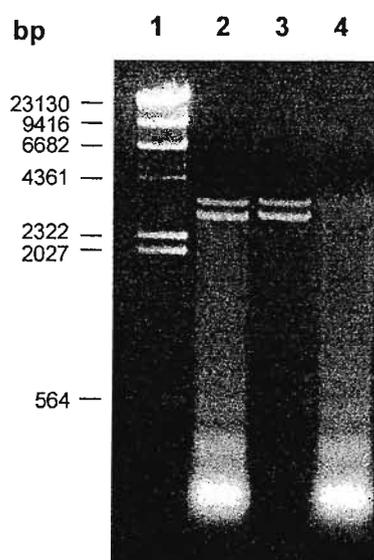


Figure 4-6. Purification of IBDV dsRNA from infected bursal tissue.

IBDV dsRNA was purified from infected bursal tissue as described in the text. Samples were run on an agarose gel (1% m/v) and stained with EtBr as follows: lane 1) *Hind* III-cut λ DNA markers (250 ng total); lane 2) total RNA; lane 3) RNA obtained from 4 M LiCl pellet; lane 4) RNA obtained from 4 M LiCl supernatant.

4.6.2 Facilitating recombinant expression of VP4 in *P. pastoris*

Full-length amplification of the IBDV ORF A1 (3066 bp) was not achieved despite wide variation of the reaction parameters, as detailed in Table 4-4. When partial amplification did occur, it was as a single consistent band of ~1500 bp, as illustrated in Figure 4-7. The concentration of the amplified fragment was $\sim 3 \text{ ng} \cdot \mu\text{l}^{-1}$, as estimated from this electrophoresis (Section 2.6.4).

Table 4-4. Conditions varied for optimisation of IBDV ORF A1 RT-PCR.

Reverse transcription reaction		
Component	Range of quantities/concentrations	Variations in procedure
viral dsRNA ^a	0.2-500 ng	<u>Denaturation:</u> 1) boiled together 5 or 10 min, cooled at 37°C, 42°C or in ice bath
primers ^b	SAf1/SAr1; 14-30 pmoles random hexamers; 50 pmoles	2) heated together (5 or 10) min to 100°C in heating block, cooled at 37°C, 42°C or in ice bath
dNTPs	0.5-1.8 mM	<u>cDNA synthesis:</u> added to RNA-primer mix, incubated at 37°C or 42°C for times ranging between 1 and 3 h
DTT	10 mM	
MMLV-RT ^c	100-200 U	
Polymerase chain reaction		
Component	Range of quantities/concentrations	Variations in procedure
RTn product	5-20 µl	<u>Hot start:</u> ^d denatured at 99.9°C for 5 min; cooled to 71°C/37°C ^e , held 71°C/37°C for 40 sec; proceeded to cycle 1
PCR buffer	5 µl	
MgCl ₂	0.625-2.5 mM	
dNTPs	0.05-0.2 mM	
primers	SAf1/SAr1; 0-30 pmoles	
<i>Taq</i> polymerase ^f	2.5 U	<u>Cycling parameters:</u> initially denatured at 99.9°C/95°C for 5 min, followed with 25-30 cycles; denatured at 95°C, 30 sec-1 min, annealed at 71°C/37°C, 1-1.5 min, extended at 72°C, 1-2.5 min, final extension at 72°C, 5 min

^a Viral dsRNA was isolated from a variety of bursal samples collected in 1990 and 1992.

^b Random hexamers were used alone or in combination with primers SAf1 and SAr1 (Section 4.3.1).

^c MMLV-RT was from Gibco BRL (Paisley, UK) or Promega (Madison, USA).

^d Hot starts were introduced to facilitate PCR optimisation.

^e A low-temperature anneal at 37°C was performed in contrast to the routine anneal at (T_m-5)°C.

^f *Taq* DNA polymerase was from Promega (Madison, USA), Bioline (BIOTAQ™ DNA polymerase, London, UK) or TaKaRa Biomedicals (Ex Taq, Gennevilliers, France).

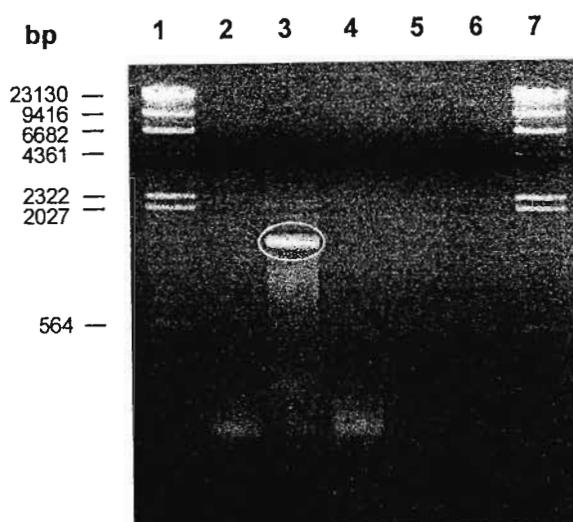


Figure 4-7. Partial amplification of IBDV ORF A1 by RT-PCR.

Double-stranded RNA was purified from IBDV and PCR amplification of IBDV ORF A1 attempted as described in the text, using primers SAf1 and SAr1. An aliquot (5 μ l) of each sample subjected to PCR was run on an agarose gel (1% m/v) and stained with EtBr: lane 2) no template control; lane 3) IBDV dsRNA (10 ng); lane 4) no RTase control; lane 5) no primers control; lane 6) no *Taq* control. Lanes 1) and 7) contain *Hind* III-cut λ DNA markers (250 ng). The band corresponding to an amplified fragment of ~1500 bp is circled.

The absence of amplification within the control tubes and the lack of any other product bands suggested that the ~1500 bp fragment was the result of an attenuated amplification of viral cDNA. This was partially confirmed by a diagnostic digest using *Nde* I, which resulted in a banding pattern consistent with the known 5' sequence of the IBDV ORF A1 (Figure 4-8). A further diagnostic digest using *Xba* I failed to produce any cleavage, which is consistent with 3' attenuation of the amplified fragment.

In addition, the ~1500 bp fragment was DIG-labelled and used in a dot-blot screen (Section 4.3.3) to probe gel-purified IBDV ORF A1 (Section 2.7.1), using phage λ DNA as a control. The probe hybridised readily to serial dilutions of viral RNA isolated from a variety of bursal samples from 1990 and 1992, and only bound high concentrations of phage λ DNA in a very weak, non-specific manner. These results are shown in Figure 4-9.

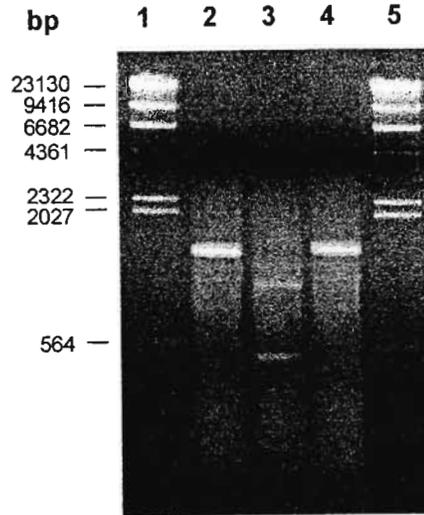


Figure 4-8. Diagnostic digest of partially amplified RT-PCR product.

Aliquots (5 μ l) of partially amplified fragment of IBDV ORF A1 (~1500 bp) were subjected to separate enzymatic digestions by *Nde* I and *Xba* I (1 U, 37 °C, 2 h) which have restriction sites in IBDV ORF A1 at base pairs 629 and 2825 respectively. Samples were run on an agarose gel (1% m/v) and stained with EtBr as follows: lane 2) uncut partially amplified IBDV ORF A1 (~1500 bp); lane 3) *Nde* I digest of the ~1500 bp fragment, yielding bands of ~ 988 and 512 bp; lane 4) *Xba* I digest of the ~1500 bp fragment, no digestion. Lanes 1) and 5) contain *Hind* III-cut λ DNA markers (250 ng).

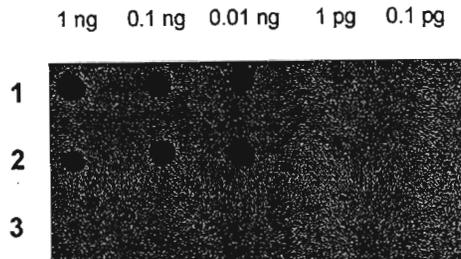


Figure 4-9. DIG-labelled dot-blot analysis of attenuated IBDV segment A RT-PCR product.

An aliquot (1 μ l) of a range of 10-fold serial dilutions of IBDV dsRNA from 1990 (row 1) and 1992 (row 2) as well as phage λ DNA (row 3) was dot blotted onto a positively charged nylon membrane and subsequently probed with DIG-labelled PCR-product (1 μ l of 20 μ g.ml⁻¹ solution) as described in the text. The amount of sample DNA per dot appears above the rows.

The persistent failure of RT-PCR despite variation of the reaction conditions suggested that the process was blocked at some initial step, either denaturation, primer binding or RTn. It is possible that the source DNA also contained some contaminant, since partial amplification was achieved using higher dilutions of source DNA. Dilution of the starting material may have diluted contaminants such as EDTA, detergents and phenol to levels where they were no longer inhibitory (Taylor, 1991b).

However, the most likely point of failure was resistance of the target dsRNA to denaturation. The RNA-RNA duplex is known to be the most stable nucleic acid hybrid (Bodkin and Knudson, 1985) and in addition, the IBDV genome is covalently bound at the 5' termini by a genome-circularising protein (Müller and Nitschke, 1987a). It is likely that this protein contributes further to the denaturation resistance of the genome caused by the high stability of the RNA-RNA double helix.

Other researchers in the field (Azad *et al.*, 1985; Davis and Boyle, 1990) have mentioned the difficulty involved in separating the two strands of viral RNA to allow reverse transcription. Although some (Azad *et al.*, 1985; Vakharia *et al.*, 1992) have opted for the use of the highly toxic reversible denaturant methylmercuric hydroxide (Bailey and Davidson, 1976), Davis and Boyle, (1990) described a simplified denaturation scheme that resulted in the amplification of a 150-bp segment. An adaptation of this method and the standard boiling followed by flash cooling in ice (Sambrook *et al.*, 1989i) were the denaturation techniques used in this study that resulted in partial amplification. These denaturations were conducted with both primers present, the aim being to promote rapid annealing of the short primers to ssRNA once the strands had separated while preventing rehybridisation of the RNA. It is possible that swamping the dsRNA with primer favours this reaction, which may additionally explain why at least partial amplification was achieved using lower rather than higher amounts of template.

No amount of modification of the reaction conditions could increase the length of the partially amplified fragment, and the specificity of the attenuated amplification was puzzling. If the reverse primer were mispriming at upstream sites of lower homology, a ladder pattern of many amplified fragments corresponding to the non-specific priming would be expected. Additionally, the correct length fragment produced from the high homology designated binding site should still have been present, and at a higher concentration than misprimed fragments. The primer designs were carefully re-checked and regions of internal homology re-examined. The reverse primer site of highest random homology (59%) is 1652 bp from the forward primer binding site (Section 4.3.1). It is possible that the dsRNA assumes some complex secondary or even tertiary structure that allows only partial strand separation under the denaturation conditions used. If this strand separation is halted and the correct binding site is unavailable, the reverse primer may have bound the site at 1652 bp and allowed the formation of a truncated cDNA strand. In the PCR itself, the T_m of the reverse primer at this

random site may have been just high enough to allow limited amplification of 1652 bp (~1500 bp). While perhaps possible, this sequence of events does seem highly unlikely.

At this point, it seemed appropriate to assess the cDNA produced by the RTn reaction and perhaps gain further insight into the failure of the RT-PCR. The entire volume (20 μ l) of a RTn reaction (Section 4.3.2.2) was subjected to agarose electrophoresis and EtBr staining, (Sections 2.6.1 and 2.6.2) but no bands other than those of template dsRNA were detected (results not shown). The reaction was examined more closely by a radioactive incorporation assay (Section 4.3.4.). Despite two attempts, using two different sources of MMLV-RT and allowing extensive development, no distinctive bands of any length appeared on the X-ray films (results not shown). Hence, whether or not a truncated cDNA was produced in the RTn reaction remained unclear, and the products of single primer reactions and possible differences in efficiency of denaturation remained unknown. A clearer autoradiograph may have resulted if the nucleic acid had been precipitated before loading the gel (Roth *et al.*, 1985), and if the exposure time had been increased. However, it was subsequently discovered that the X-ray film used had expired over two years previously, which may have contributed significantly to the absence of results.

Considering the sensitivity of DIG detection, it would perhaps have been useful to repeat the incorporation assay using DIG-11-dUTP as a tracer instead of the radionuclide, thereby simplifying the whole procedure. However, at this point the aims of the project were reassessed in terms of the difficulties inherent in obtaining a relatively long RT-PCR product from this particular target molecule. It was decided to narrow the scope of the investigation and to focus on the putative protease, VP4, which is encoded by 810 bp and deemed an achievable target length. Whilst making this decision, it became apparent that the *Pichia* system is quite subtle and difficult to get results from (D. York, University of Natal Medical School, South Africa, personal communication), even although the promised outcomes are so desirable. Thus, the decision was also made to attempt recombinant expression of VP4 from *E. coli*, thereby simplifying procedures and ideally producing meaningful results within a reasonable time period.

4.6.3 RT-PCR of VP4 and efforts to clone the amplicon

Reducing the length of the sequence intended for amplification allowed successful RT-PCR of the VP4 gene alone, as illustrated in Figure 4-10. The 838-bp amplification was distinct and clean and all negative controls, including a preparation of uninfected bursa, were clear. Other researchers who managed successful RT-PCR without the use of methylmercuric hydroxide (Davis and Boyle, 1990; Liu *et al.*, 1994; Jackwood and Jackwood, 1994) were all amplifying regions less than 700 bp. This appears to support the suggestion that the longer RT-PCR designed for cloning in *P. pastoris* failed because of insufficient denaturation over the entire ORF.

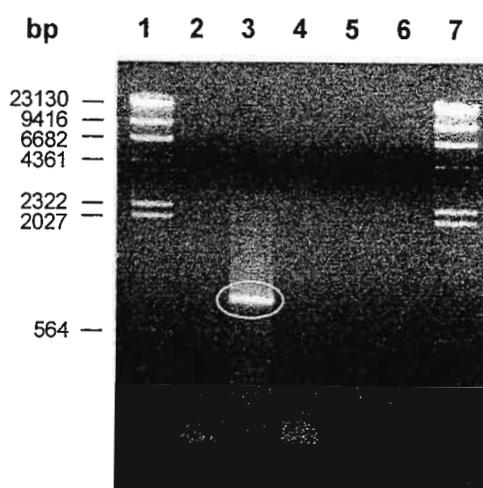


Figure 4-10. Amplification of the VP4 gene sequence from IBDV by RT-PCR.

Double-stranded RNA was purified from IBDV and PCR amplification of IBDV VP4 performed as described in the text, using primers VP4f1 and VP4r1. An aliquot (5 μ l) of each sample subjected to PCR was run on an agarose gel (1% m/v) and stained with EtBr as follows: lane 2) no template control; lane 3) IBDV dsRNA (10 ng); lane 4) no RTase control; lane 5) no primers control; lane 6) no *Taq* control. Lanes 1) and 7) contain *Hind* III-cut λ DNA markers (250 ng). The band corresponding to an amplified fragment of 838 bp is circled.

Unfortunately, all attempts to clone the 838 bp amplified fragment into the expression vector pQE-32 and obtain transformed M15[pREP4] cells were unsuccessful. Figure 4-11 is representative of results obtained from a number of ligation reactions, as outlined in Table 4-3 (Section 4.4.4).

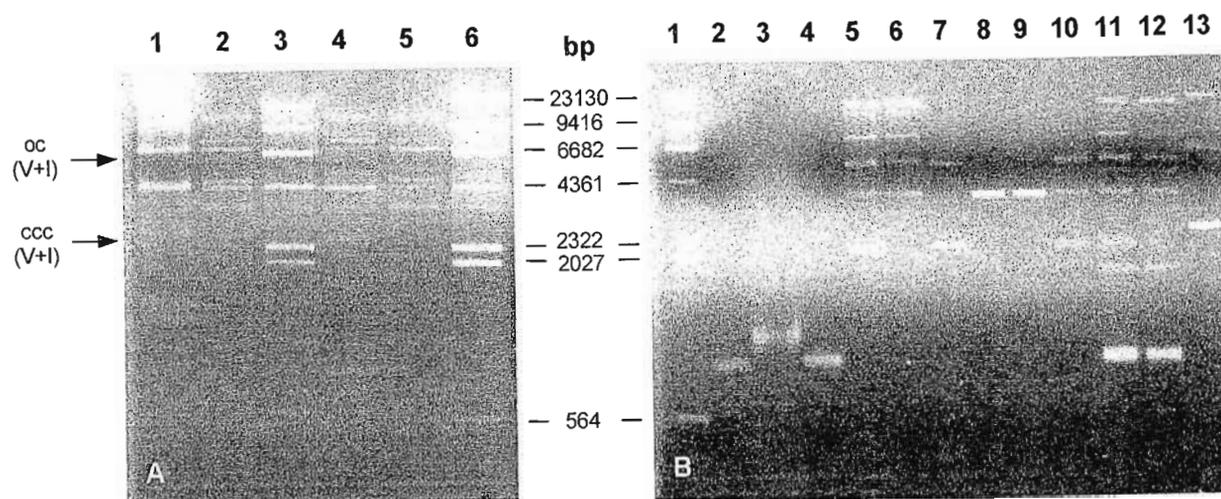


Figure 4-11. Analysis of ligation reactions involving pQE-32 and RT-PCR amplified VP4.

Ligation of RT-PCR amplified VP4 (referred to as 'insert') into the vector pQE-32 was attempted as described in the text, under a variety of conditions as summarised in Table 4-3. Gels A and B contain samples of ligation/control reactions conducted at 4°C and RT respectively. An aliquot (5 µl) of each sample/ligation mix was run on an agarose gel (1% m/v) and stained with EtBr as follows. Gel A: lanes 2), 4) and 5), ligation reactions with vector:insert ratios of 1:1, 1:3 and 3:1 respectively; lanes 3) and 6), *Hind* III-cut λ DNA markers (125 ng and 250 ng respectively); lane 1), *Hind* III-cut λ DNA markers (125 ng) religated under the same conditions as samples. Gel B: lane 1), *Hind* III-cut λ DNA markers (125 ng); lane 2), uncut insert; lane 3), uncut and self-ligated insert; lane 4), double-digested and self-ligated insert; lane 5), *Kpn* I then *Sph* I digested and self-ligated vector; lane 6), *Sph* I then *Kpn* I digested and self-ligated vector; lane 7), uncut vector; lane 8), *Kpn* I digested vector; lane 9), *Sph* I digested vector; lane 10), *Sph* I digested and self-ligated vector; lane 11), *Kpn* I then *Sph* I digested vector and insert ligated 1:10; lane 12), *Sph* I then *Kpn* I digested vector and insert ligated 1:10; lane 13), vector pQE-16. The labels oc (V+I) and ccc (V+I) indicate the positions of very faint bands corresponding to the open circular (oc) (lane 4) and covalently closed circular (ccc) (lane 2, lane 4) forms of ligated vector (V) and insert (I).

Table 4-5 summarises the results of ligation experiments such as those illustrated in Figure 4-11. Controls such as self-ligation of both vector and insert, including vector cut with a single RE and uncut insert and the confirmation of ligase activity by religation of *Hind* III cut λ DNA fragments were among these reactions. The ligations were assessed by electrophoresis to simplify the detection of recombinant molecules and to determine the prominent ligation permutations. Recombinant transformants were considerably harder to detect since the pQE vectors do not have blue/white selection requirements. A number of transformations were conducted and a sample of the resulting colonies screened, all of which contained host vector without insert. The results of various transformation reactions are presented in Table 4-6.

Table 4-5. Products of ligation reactions involving pQE-32 and RT-PCR amplified VP4.

Molecular size (bp)	Description of molecule
838	linear insert
1676	2 linear inserts
between 2027^a and 2322	ccc vector
slightly above 2322 , 2514?	ccc vector+insert, or 3 linear inserts
3462	linear vector
4300	linear vector+insert
moderately above 4361	oc vector
5976	3 inserts+1 vector (linear)
slightly below 6682	oc vector+insert
6924	2 linear vectors
7762, 8600	2 vectors+1 insert or +2 inserts (linear)
17310 or 18148	5 vectors, or 5 vectors+1 insert (linear)

^a Band sizes appearing in **bold** are *Hind* III-cut λ DNA markers

The exact position of circularised molecules is difficult to predict on an agarose gel, particularly so as they may also assume different conformations. Plasmids have three conformational forms which run on 1% (m/v) agarose gels in descending order as follows: the relaxed state in which one or both strands are nicked, also known as the open circular (oc) form; the linear form where both strands have been cut at a restriction site, and the supercoiled or covalently closed circular (ccc) conformation (Brown, 1990). The control plasmid pQE-16 (4032 bp) was useful as an additional size marker in this regard, as it is a pQE-3x type plasmid which contains the dihydrofolate reductase (DHFR) gene. Although the DHFR gene is 268bp shorter than the 838-bp VP4 gene generated by RT-PCR, the pQE-16 plasmid nevertheless gave an indication of how far the various forms of recombinant pQE32+VP4 would migrate.

Hence bands appearing in agarose gels such as those illustrated in Figure 4-11 were interpreted according to possible ligation permutations, including self-ligation, and the position of pQE16. In this manner, the identities of the bands were assigned as detailed in Table 4-5. However, despite numerous variations of the ligation procedure mentioned in Section 4.4.4, bands indicating successful circularised ligation of vector and insert (labelled oc (V+I) and ccc (V+I) in Figure 4-11A) were present only at the very limit of detection

(< 1 ng). In addition, the identity of ccc vector+insert was uncertain since the linear ligation of 3 inserts (2514 bp) would run very close to the expected position of the ccc molecule; slightly above the 2322 bp molecular size marker.

Nevertheless, very small quantities of ccc DNA can still generate numerous transformants - at the transformation efficiency of the control plasmid, each picogram of ccc DNA should give rise to a clone when plated. This is demonstrated by the appearance of colonies after transformation with cut vector; the vector remaining uncut is not visible on an agarose gel (Figure 4-11B, lanes 8 and 9), and yet there is sufficient present to result in transformed bacteria. It is therefore possible that colony blots probed with DIG-labelled RT-PCR product may have revealed the presence of colonies transformed with recombinant vector. However, comparable colony numbers were obtained under similar conditions from ligation mixes and religated cut vector (entries 1. and 6. in Table 4-6), which suggests that the contribution from recombinant plasmids was very small. It is likely that almost all the colonies obtained from transformation with the various ligation mixes contained either uncut vector or religated vector. This in turn suggests that the possible ccc V+I band in Figure 4-11A is more likely a concatemer of three linear inserts.

Table 4-6. Summary of results obtained from various transformations of competent M15[pREP4] cells.

Transforming agent	Description	Quantity of cells plated	Colony numbers	Results of screening ^a
1. ligation mixes	assorted	50 μ l	~100 to ~300	all contained pQE-32 only
		450 μ l	~1000 to ~3000	
2. sterile TE ^b	20 μ l	500 μ l	zero	
3. pQE-16	1 ng total	100 μ l, 1:10 ^c	~200	all contained pQE-16 only
		100 μ l, 1:100	~25	
		100 μ l, 1:500	~4	
4. cut pQE-32	5 ng total	50 μ l	~5	1% remain uncut
		450 μ l	~50	
5. self-ligated cut pQE-32	20 ng total, cut with single RE	50 μ l	~1400	all contained pQE-32 only
		450 μ l	confluent growth	
6. self-ligated cut pQE-32	20 ng total, cut with both REs	50 μ l	~100 to ~300	all contained pQE-32 only
		450 μ l	~1000 to ~3000	

^a 42 colonies were screened for each ligation mix (14 for each ratio), six colonies for pQE-16 and cut pQE-32 controls (two and three colonies off each plate respectively)

^b In another negative control, an LB-agar plate containing antibiotics was exposed to the lab atmosphere and incubated with the other plates; no colonies were detected

^c Transformation mixes were diluted with Psi broth to give the ratios indicated before the pQE-16 positive controls were plated

It therefore appears that failure to obtain transformed M15[pREP4] cells containing the VP4 gene was related to poor ligation efficiency and not failed transformation. The ligation experiments were designed specifically for high efficiency; cleavage with two non-compatible sticky-ended REs and removal of the stuffer fragments by electrophoresis are the most favourable conditions for effective ligation (Sambrook *et al.*, 1989j). Interpolation of a range of experimental insert concentrations (Table 4-4) onto a graph representing these conditions (Figure 4-12) reveals that significant quantities of monomeric circular chimaera were expected, even for the lowest concentration of insert used. In each case illustrated, the amount would have been easily visible on an agarose gel, as detailed in Table 4-7.

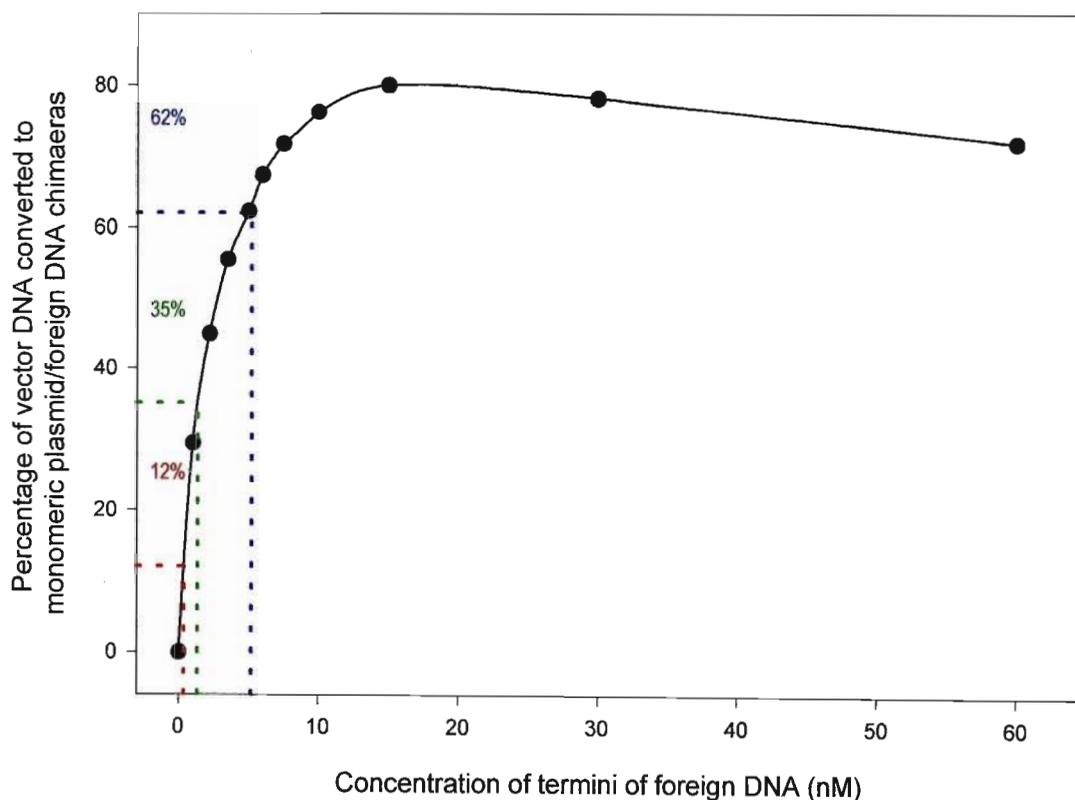


Figure 4-12. Efficiency of ligations forming monomeric circular chimaeras.

The ligation efficiency for a fixed amount of vector DNA ligated to increasing quantities of a foreign DNA insert with compatible termini is illustrated. The vector has been cleaved with two different restriction enzymes and the stuffer fragment removed by electrophoresis. The interpolations are from insert termini of concentrations 0.36 nM (—), 1.34 nM (—), and 5.2 nM (—) respectively, representing the range investigated in a variety of ligation reactions. (After Sambrook *et al.*, 1989j).

Table 4-7. Amount of monomeric circular chimaera expected from ligations.

Concentration of insert termini (nM)	Percentage of vector DNA converted	Corresponding amount of recombinant vector (ng)
5.2	62	12.4
1.34	35	7.0
0.36	12	2.4

The rate of ligation of DNA fragments is dependent on numerous factors: DNA concentration, concentration of compatible ends, size of fragments, whether the fragments are blunt or sticky ended, T_m of single-stranded sticky ends, reaction time, incubation temperature and ionic

composition of the solution (Hackett *et al.*, 1988). Under fairly standard reaction conditions, most of these factors are not an issue and can be fairly easily checked and/or varied, as was the case for the experiments under discussion: ratios of vector to insert were used and varied as is standard (Dugaiczky *et al.*, 1975); the T_m s of both *Kpn* I and *Sph* I sticky ends are 12°C, indicating that some cooling of the reaction is necessary; ligation time was varied widely, the ligase buffer was supplemented with fresh ATP and DTT and a fresh supply of ligase was tested. It thus seems apparent that the failure of the ligation is more likely attributable to the nature and quality of the restricted fragments.

Electrophoresis of vector restricted separately by the two REs revealed that each did cut the pQE-32 plasmid to completion within the limits of EtBr staining detection (Figure 4-11B, lanes 8 and 9). Confirming double digestion, however, was not possible on agarose gels as the stuffer fragment was only 8 bp long. Similarly, it was not possible to confirm the digestion of the two ends of the RT-PCR product. Steps were taken to maximise restriction efficiency, such as increasing both reaction time and amount of enzyme and extensive gel purification to remove inhibitory contaminants. The sequence of restriction in the double digest was also varied, namely *Kpn* I followed by *Sph* I and vice versa. However, self-ligation of vector continued to produce considerable concatemerisation and recircularisation (Figure 4-11B, lanes 5 and 6), indicating incomplete restriction. Interestingly, changing the order of digestion to *Sph* I followed by *Kpn* I eliminated the formation of ccc vector but not the oc form (Figure 4-11B, compare lanes 5 and 6 with lane 7). The reason for this is unclear, particularly considering DNA was gel purified between enzymatic reactions. Self-ligation of insert would only produce concatemers at 4°C when the insert was uncut (Figure 4-11A, lane 3) and likewise, any manner of ligation of vector and insert would only occur at this temperature (compare Figure 4-11A lanes 2, 4 and 5 with 4-11B, lanes 11 and 12). This suggests that some of the RT-PCR product remained blunt-ended despite the double digestion. Hence it appears that the restricted vector and insert molecules were a heterogeneous population, with very few molecules cut with both *Kpn* I and *Sph* I, some not restricted at all and some cut with either one or the other enzyme. These variations would allow the formation of ligated molecules fitting the sizes of bands obtained by agarose gel electrophoresis of assorted ligation mixes (Table 4-5).

Fuchs and Blakesly (1983) offer many explanations and trouble-shooting suggestions with respect to inefficient restrictions and a number of these were applied during the attempts at ligation and cloning. A positive control using phage λ DNA revealed that the REs were quite

capable of effecting double digestion of this DNA with just an hour for each digestion and no need for gel purification between digests. The implication is that the restriction inefficiencies were somehow intrinsic to the vector and insert DNA. This is unusual since the vector was supplied by reputable manufacturers and the insert DNA was readily cut at internal sites by an assortment of other REs (see Section 4.6.4 below). (A faint possibility remains that the supplied plasmids may have degenerated in some way due to prolonged (over two years) storage, albeit at -20°C . In all tested respects, however, the supplied plasmids appeared perfectly functional). *Taq* polymerase lacks proof-reading ability (Krawczak *et al.*, 1989), and while it is possible that mutations were introduced into the restriction site during PCR, it is most unlikely that these would occur consistently among numerous reactions. This could only be possible if the primer itself contained an error within the sequence of the restriction site, a situation which is also highly unlikely.

The common link between the two DNA sources is their gel purification. Having exhausted numerous other plausibilities, it is suggested that the gel purification steps did not perform as optimally as hoped with respect to quality as well as quantity, thereby allowing some carry-over of contaminants which hampered restriction and possibly affected conformation. A low-salt optimum isoschizomer (*Bbu* I) is available for *Sph* I; a simultaneous double digest substituting *Bbu* I for *Sph* I would eliminate one gel purification step and perhaps confirm these suspicions about the quality of gel purified DNA, as opposed to inefficiencies of the REs themselves. Gel purification appears central to the success of this particular cloning experiment, but the exceptionally poor yields (only about 8% after two purifications) and possible contamination of the DNA eventually forced a halt to these procedures. Attempts at ligation were thus reluctantly abandoned at this stage, although further analysis of the RT-PCR product remained in the form of RFLP analysis (Section 4.6.4) and investigation of the putative protease was also scheduled at the protein level (Chapter 5).

4.6.4 RFLP analysis of amplified VP4

Restriction sites within the cDNA sequence of VP4 from the GLS variant of IBDV (Vakharia *et al.*, 1992) were determined using Sequaid II™ v3.81 software (copyright 1991, D. Rhoads and D. Roufa, Kansas State University, Molecular Genetics Laboratory, Kansas, USA). After examination of the VP4 restriction map, REs were carefully chosen to ensure the generation of only two or three clearly separated restriction fragments. Twenty REs were selected from available stocks, ten of which had restriction sites within VP4, and ten of which did not. Amplified cDNA from samples across the eight years from 1989 to 1997 (excluding 1996)

was digested and analysed as described in Section 4.5.2. Figure 4-13 is representative of these digestions, while Figure 4-14 illustrates control reactions confirming the ability of the enzymes to restrict under the given reaction conditions.

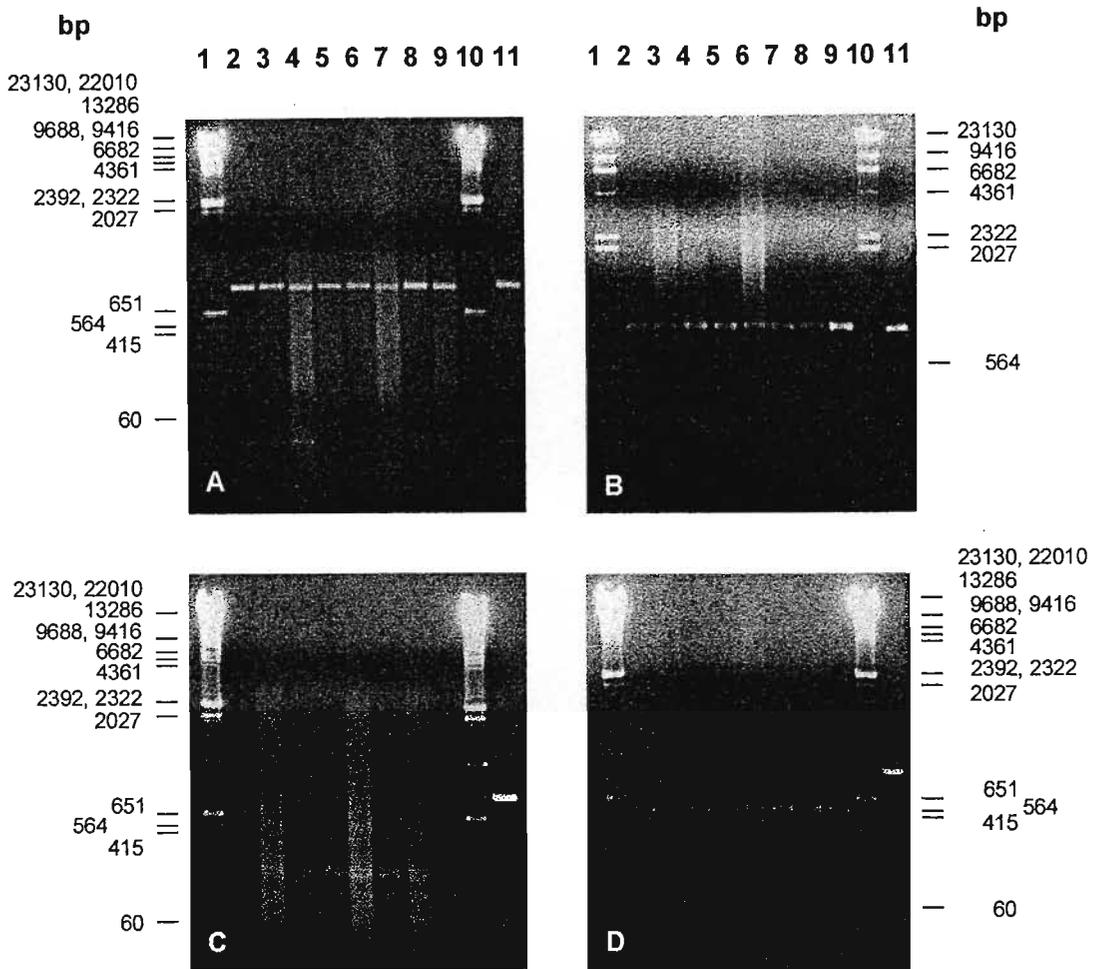


Figure 4-13. RFLP analysis of VP4 cDNA from KZN IBVDV from 1989-1997, excl. 1996.

The REs used in the analysis of RT-PCR amplified VP4 cDNA were: *Bam*H I, *Bcl* I, *Bgl* I, *Eco*R I, *Hinc* II, *Hind* III, *Ksp* I (*Sac* II), *Nde* I, *Pst* I, *Pvu* II, *Rsa* I, *Sac* I, *Sal* I, *Sau*3A I, *Sfu* I (*Nsp* V), *Sma* I, *Ssp* I, *Stu* I, *Xba* I and *Xho* I. All samples digested by a particular enzyme were run in parallel lanes on a single agarose gel (1.5% m/v, except B which is 1% m/v) and stained with EtBr. The four panels (A-D) are representative of the gels obtained for all 20 enzymes. A), *Hind* III digest, illustrating an RFLP; B), *Pst* I digest, a negative control; C), *Rsa* I digest, showing 322-, 298- and 200-bp fragments; D) *Sal* I digest, showing 575- and 246-bp fragments. Lanes 1 and 10, gels A, C and D contain *Hind* III- and *Bgl* II- cut λ DNA size markers (separate digests); lanes 1 and 10 of gel B contain *Hind* III- cut λ DNA size markers; lanes 2-9, digest of RT-PCR amplified cDNA from the VP4 region of KZN IBVDV from the years 1989 to 1997, excluding 1996 (10 ng of each); lane 11, uncut RT-PCR amplified VP4 cDNA (10 ng).

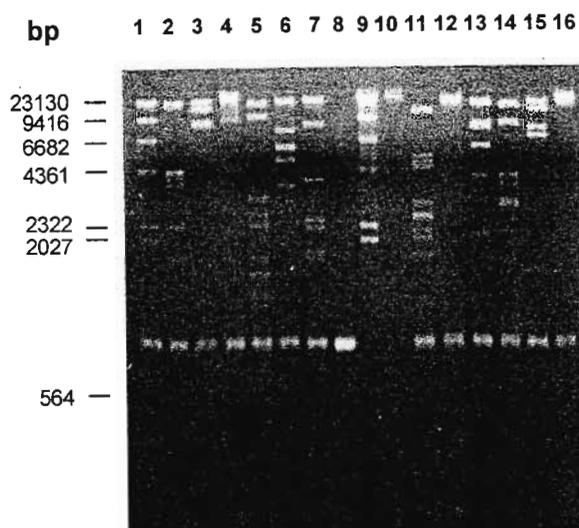


Figure 4-14. Digestion of phage λ DNA by REs which do not cut VP4 cDNA from KZN IBDV.

All REs which did not cut the VP4 cDNA from KZN IBDV were included in the control digestions using phage λ DNA as substrate. Each enzyme was incubated overnight with phage λ DNA (25 ng) mixed with RT-PCR amplified VP4 (10 ng) from KZN IBDV and the digests run on an agarose gel, 1% (m/v). Lanes: 1), *Hind* III; 2), *Pvu* II; 3), *Sma* I; 4), *Bcl* I; 5), *Bgl* I; 6), *EcoR* I; 7), *Nde* I; 8), uncut VP4 cDNA; 9), *Hind* III-cut λ DNA size markers; 10), uncut λ DNA; 11), *Pst* I; 12), *Sac* I; 13), *Sfu* I (*Nsp* V); 14), *Ssp* I; 15), *Stu* I; 16), *Xba* I.

The patterns and sizes of restriction fragments obtained in this manner were examined for comparison with the known restriction sites within the GLS sequence. Agarose gels displaying the digestion products revealed that 1), the VP4 sequence of KZN IBDV appears to be identical for all samples analysed and 2), this sequence lacks three restriction sites present in the sequence of the GLS strain, namely a *Hind* III site, a *Pvu* II site and a *Sma* I site. As indicated in Figure 4-14, all REs which did not cut RT-PCR amplified VP4 were still capable of restricting phage lambda DNA mixed with the VP4 cDNA. This eliminates the possibility that enzymes did not cut because of inhibition by the PCR buffer or PCR carry-over components such as nucleotides.

A wider perspective of the VP4 sequence of KZN IBDV can be gained by comparison of its restriction information with that of a variety of other strains. To do this, the SEQNET Sequence Retrieval System (SRS; <http://www.seqnet.dl.ac.uk/srs5>) was used to search the EMBL and GenBank databases for published sequences, which were subsequently analysed by the SRS restriction map application. The results are summarised in Table 4-8. The sequences used for comparison are listed as follows with relevant references and database

accession numbers. Serotype 1: GLS (Vakharia *et al.*, 1992; M97346), UK661 (Brown and Skinner, 1996; X92760), 52/70 (Bayliss *et al.*, 1990; D00869), STC (Kibenge *et al.*, 1990; D00499), PBG-98 (Bayliss *et al.*, 1990; D00868), Cu-1 (Spies *et al.*, 1989; X16107), 002-73 (Hudson *et al.*, 1986; X03993), GZ29112 (Law *et al.*, 1998; AF051837), Harbin (Hu and Zhang, 1998; AF092171). Serotype 2: OH (Kibenge *et al.*, 1991; M66722).

Table 4-8. Location of restriction sites in the VP4 sequence of a variety of IBDV strains.

RE	Site ^a	GLS	KZN	UK661	52/70	STC	PBG98	Cu-1	002-73	OH	GZ ^b	Harbin
<i>Bam</i> HI	1920	+	+	+	+	-	+	+	+	-	+	+
	1926	-	-	-	-	-	-	-	-	+	-	-
<i>Bcl</i> I	none	-	-	-	-	-	-	-	-	-	-	-
<i>Bgl</i> II	none	-	-	-	-	-	-	-	-	-	-	-
<i>Eco</i> RI	1732	-	-	-	-	+	-	-	+	+	-	-
<i>Hinc</i> II	1727	+	+	+	+	+	+	+	+	-	-	+
<i>Hind</i> III	2104	-	-	-	-	-	-	-	-	-	+	-
	2192	+	-	-	+	+	+	+	+	+	+	+
<i>Ksp</i> I	1760	+	+	+	+	+	+	-	+	-	+	+
<i>Nde</i> I	none	-	-	-	-	-	-	-	-	-	-	-
<i>Pst</i> I	none	-	-	-	-	-	-	-	-	-	-	-
<i>Pvu</i> II	1533	-	-	-	-	-	-	-	-	+	-	-
	1655	+	-	-	+	+	+	+	-	-	+	+
	1670	-	-	-	-	-	-	-	+	-	-	-
<i>Rsa</i> I	1504	-	-	-	-	-	-	-	-	+	-	-
	1680	+	+	+	+	+	+	+	+	+	+	+
	1755	-	-	-	+	+	+	+	-	-	-	+
	1978	+	+	+	+	+	+	+	-	-	+	+
	2112	-	-	-	-	-	-	-	-	+	-	-
	2204	-	-	-	-	-	-	-	-	+	-	-
<i>Sac</i> I	none	-	-	-	-	-	-	-	-	-	-	-
<i>Sal</i> I	1725	+	+	+	-	+	+	+	-	-	-	+
<i>Sau</i> 3AI	1897	-	-	+	-	-	-	-	-	-	-	-
	1920	+	+	+	+	-	+	+	+	-	+	+
	1926	-	-	-	-	-	-	-	-	+	-	-
	2060	-	-	-	-	+	-	+	-	-	-	+
<i>Sfu</i> I	1937	-	-	-	-	-	-	-	-	+	-	-
<i>Sma</i> I	1748	+	-	+	-	-	-	-	-	-	+	-
<i>Ssp</i> I	1869	-	-	-	-	+	-	-	-	-	-	-
<i>Stu</i> I	2129	-	-	-	-	-	-	-	-	+	-	-
<i>Xba</i> I	none	-	-	-	-	-	-	-	-	-	-	-
<i>Xho</i> I	1606	+	+	+	+	+	+	+	+	-	+	+
	1612	-	-	-	-	-	-	-	-	+	-	-
	1833	-	-	-	-	-	-	-	-	+	-	-
	1992	-	-	-	-	-	-	-	-	+	-	-

^a Location of restriction sites is numbered according to Bayliss *et al.* (1990)

^b GZ is strain GZ29112

What is most apparent from Table 4-8 is that the VP4 sequences of all 11 strains appearing there have unique restriction profiles. As expected, the profile of the serotype 2 strain, OH, is quite different to that of the other serotype 1 strains. With specific reference to KZN, the unique restriction profile of the VP4 sequence suggests that the strain is indeed a locally derived strain, although additional sequence information from all viral proteins is needed before this can be claimed with certainty. Two attempts were made to sequence the RT-PCR VP4 cDNA to confirm the results of RFLP analysis and perhaps find further regions of sequence unique to KZN VP4. Unfortunately, no sequence could be obtained; it appeared that the amount of template used was insufficient and the primer:template ratio too low.

The data presented in Table 4-8 were analysed using the phylogeny inference program PHYLIP v3.5c (<http://evolution.genetics.washington.edu/phylip.html>; J. Felsenstein, Department of Genetics, University of Washington, Seattle, Washington, USA). Specifically, the restriction sites maximum likelihood program RESTML (Felsenstein, 1992) was used to produce the graphical representations of these data (Figure 4-15). The model used is largely based on that of Smouse and Li (1987), incorporating the work of DeBry and Slade (1985).

From Figure 4-15, it appears that VP4 of the KZN strain is identical to that of UK661 within the constructs of the RESTML algorithm. This is an interesting result and perhaps holds important clues as to the origins of the KZN strain, although as mentioned above, the RFLP pattern of one viral protein is only a starting point with respect to such comparisons. In addition, it is likely that restriction site data will exaggerate both strain distance and proximity, being only a 'summary' of base sequence data, and this effect may be particularly apparent on translation to amino acid sequence data, due to the degeneracy of the triplet code.

Nevertheless, it is highly likely that KZN is closely related to UK661, especially since the pathology data links them as very virulent strains appearing in the late 1980's (Brown and Skinner, 1996). Figure 4-15 indicates that the vaccine strain PBG98 (Baxendale, 1976) is the nearest neighbour to KZN and UK661, a result which Brown and Skinner (1996) also determined in cluster analysis of the amino acid sequences of VP4 from UK661 and a number of other strains. Whether or not UK661 and KZN were derived from mutation of PBG98, a vaccine strain, is unknown, although an intriguing possibility. The KZN strain may in fact be derived from the UK661 strain, rather than each separately having a common ancestor. Because the vv strains of IBDV are so diverged from other serotype 1 viruses, Brown and Skinner (1996) indicate that the origins of vvIBDV remain unclear. They assume that the

source is likely to be some as yet unrecognised reservoir, either a geographically isolated population of poultry or a different avian species. The apparent identity of KZN strains isolated across the years from 1989 to 1997 coupled to their similarity to UK661 suggests that 1), the tests conducted here have not extended far back enough to 'catch' the transition to the very virulent phenotype, or that 2), the RFLP analysis is not detailed enough to pick up any variation. It is also entirely possible that this transition does not manifest itself within the VP4 sequence. Again, further and more detailed sequence analysis is needed as the RFLP patterns only begin to make intriguing suggestions about origins and consequences.

Another interesting feature of Figure 4-15 is the suggested relationship between divergence and virulence. Brown and Skinner's (1996) amino acid cluster analysis revealed that UK661 (a vv isolate) was the most diverged in VP4 among serotype 1 strains. However, their work illustrates that the serotype 2 strains OH and 23-82 are even further diverged and are non-pathogenic. In Figure 4-15, the attenuated strain GZ29112 also appears highly diverged. The implication is that while certain mutations either have no effect or perhaps even enhance the specificity or efficiency of the putative protease, further divergence has the opposite effect. This lends further support to the role of mutation of key processing enzymes in the determination of phenotype. Additional sequence information of the KZN strain is needed before such potentially far-reaching and useful correlations can be made. However, Brown and Skinner (1996) did identify two mutations within the amino acid sequence of UK661 VP4 which could potentially affect cleavage by the putative protease. These are the substitutions in the polyprotein at positions 651 (asparagine to serine) and 452 (isoleucine to leucine), which may influence a proposed active site serine and the postulated dibasic cleavage site between VP2 and VP4 respectively. Considering the likelihood that UK661 and KZN are closely related, it would be instructive to assess these particular regions (bases 2079 and 1482, numbered according to Bayliss, 1990) in the KZN sequence for mutation. Unfortunately, none of the enzymes appearing in Table 4-8 restrict at these sites, and the REs that do are frequent cutters and would create too many fragments for meaningful RFLP analysis. Such investigation will have to await nucleic acid or protein sequencing.

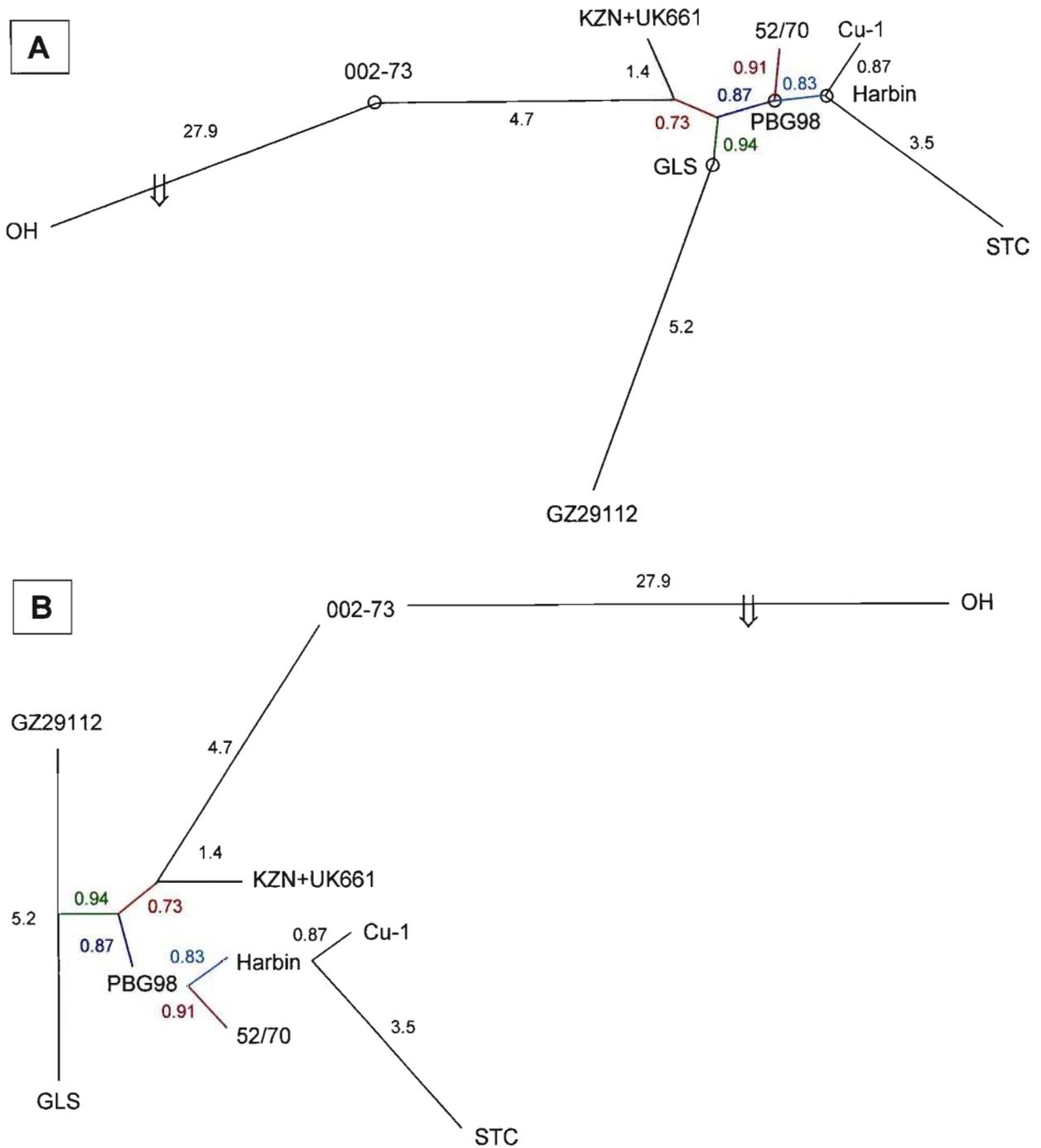


Figure 4-15. Phenograms displaying the relatedness of VP4 RFLP patterns among various IBDV strains.

Data analysed by the RESTML program were used to produce phenograms by the programs DRAWGRAM and DRAWTREE in the PHYLIP v3.5c package (J. Felsenstein, Department of Genetics, University of Washington, Seattle, Washington, USA). The relationship between RFLP patterns of the KZN isolate and those of serotype 1 strains GLS, UK661, 52/70, STC, PBG98, Cu-1, 002-73, GZ29112, Harbin and serotype 2 strain OH is displayed. (A) is the phenogram produced directly by DRAWTREE, (B) is the same data redrawn for simplification. Branch lengths are proportional to the expected percentage of nucleic acid substitutions based on the observed data. (These percentages appear above the corresponding branches, with percentages in colour relating to branches of corresponding colour). The symbol ⇓ indicates that the branch length shown for OH is not to scale.

4.7 Concluding remarks

Although successfully amplified by RT-PCR, the gene for the IBDV putative protease, VP4, was not cloned into a suitable expression vector. However, a strategy was established and should prove feasible with persistence regarding the ligation reaction and improved gel purifications of the amplified DNA.

The RT-PCR itself worked effectively and was specific and sensitive, being clearly negative for uninfected bursal tissue and effectively detecting 2.5 ng of viral dsRNA, although the limits of sensitivity were not explored. The method may be of use for local diagnosis of IBDV, especially coupled with RFLP analysis. The restriction analysis of the VP4 RT-PCR fragment revealed that the KZN strain appears to be unique and that VP4 remained unchanged from early 1989 to 1997. Any mutations possibly affecting virulence of KZN IBDV by affecting VP4 await further analysis. The RFLPs did show VP4 of the KZN strain to be most similar to a vv strain of IBDV from the United Kingdom, UK661, which has implications as to the origin of the KZN strain. In distinguishing these strains from other IBDV isolates and from each other, both lack *Hind* III and *Pvu* II sites at 2192 bp and 1655 bp respectively, and UK661 has a unique *Sau*3AI site at position 1897 bp.

Chapter 5

Preliminary immunological and enzymatic examination of IBDV VP4

5.1 Introduction

Obtaining purified VP4 directly from infected material has many inherent difficulties (outlined in Section 4-1), hence the decision to attempt recombinant synthesis of the putative protease. However, the location of VP4 within infected tissue, the status of its activity during the virus life cycle and the nature of its action *in vivo* were of great interest. The preliminary immunochemical and enzymatic investigations of VP4 within IBDV-infected bursal tissue, which are discussed in this chapter, were initiated to promote a fuller understanding of the role of VP4 in infection.

The highly discriminating affinity of antibodies for their antigens makes them extremely useful as agents of specific detection. This enables their use in sero-diagnostics and immunotherapy and makes them powerful research tools for such techniques as immunoaffinity purification, determination of immunological relatedness of proteins and detection of specific compounds by immunostaining such as occurs in western blots, ELISAs and immunocytochemistry.

Antibodies can be produced against a protein of interest by a variety of methods, resulting in antibodies with a variety of properties. The method chosen is thus dependent on experimental requirements. The most common method of raising antibodies is immunisation of an experimental animal with a whole protein, which contains many epitopes and stimulates numerous B-cell clones. This polyclonal response results in a population of antibodies with vastly heterogeneous specificities. A considerably more expensive and time-consuming procedure can produce a homogenous antibody population of known specificity from a single B-cell clone; these are the single-epitope specific monoclonal antibodies (mAbs) (Köhler and Milstein, 1975). Such clones are generated by fusing a single primed B-cell with an immortal myeloma to produce an immortal B-cell hybridoma. Their specificity can limit the application of these antibodies to a certain extent. Finally, an animal immunised with a single peptide from a target protein produces what are known as anti-peptide antibodies. In addition to recognising the free peptide, these antibodies may cross-react with the target protein, provided that some of the conformations adopted by the peptide in solution mimic those adopted in the corresponding region in the target protein. Since several B-cell clones are

stimulated by the peptide, the antibodies produced are polyclonal, but because they are all directed against the selected peptide, they have specificity more similar to mAbs (Lerner, 1984). This specificity arises from the fact that a sequence of 10 or more residues is likely to be unique to a particular protein. These antibodies are also versatile because they recognise the target sequence in many different conformations and all have different 'reading frames' as they bind to separate overlapping regions of the peptide. This increases the likelihood that the antiserum will recognise whole protein in both its native and denatured forms.

Anti-peptide antibodies thus have some of the advantages of both anti-protein and monoclonal antibodies. Additionally, they allow detection of a target protein that does not have to be purified itself. For the purposes of this study, a versatile antibody preparation was required to recognise the enzyme in its denatured form (for use in ELISAs, western blots and potential immunocytochemical labelling) and preferably also in its native form (to assess possible effects of the antibodies on enzyme activity). Since the VP4 amino acid sequence has been deduced, this appeared to be an ideal situation for the use of anti-peptide antibodies, allowing the production of VP4-specific antibodies without the associated difficulties of purifying VP4. It was hoped that the anti-peptide antibodies would in fact facilitate subsequent purification of VP4 from bursal homogenates by identifying VP4-rich fractions. In addition, anti-VP4 antibodies may also be useful in the characterisation of field isolates, as has been the case for anti-peptide antibodies which differentiate between NDV pathotypes (Hodder *et al.*, 1994).

Bursal homogenates were also deemed good targets for probing in terms of specific proteolytic enzyme activity and it was hoped that overlap would be found between the immunological and enzymatic screening. It was of interest to know whether VP4 remained active in the bursa and if so, what possible influence it might have on pathogenesis. As an initial step, gelatin-containing substrate gels were run to obtain an indication of the size of any proteolytic species. For subsequent simplicity and ease of data gathering, the synthetic peptide Cbz-Arg-Arg-AMC was selected for use as substrate (Barrett and Kirschke, 1981). The fluorogenic nature of the substrate allows simultaneous screening of numerous small volume samples in 96-well plates.

With respect to the Arg-Arg specificity of the substrate peptide, deletion-expression studies suggested that VP4 has a dibasic cleavage site (Azad *et al.*, 1987; Jagadish *et al.*, 1988) and Brown and Skinner (1996) proposed that it has a serine-like catalytic triad. Although this cleavage is synonymous with the endogenous serine proteases involved in the clotting cascade

and other cellular processes (Davie *et al.*, 1991), it was hoped that any elevation in activity in infected tissue would at least provide a starting point for investigation. It was also hoped that the anti-peptide antibodies against VP4 would affect activity and thereby provide some confirmation of its location. However, binding of anti-enzyme antibodies to enzymes reportedly has varied effects, with inhibition or enhancement of activity, as well as no alteration in enzyme activity being observed (Richmond, 1977). A wide range of inhibitors was also utilised in an attempt to identify protease type and garner hints at specificity.

This chapter describes how potentially useful anti-peptide antibodies were designed against a region of VP4 (Section 5.3.1), and how conjugated peptides were subsequently prepared (Section 5.3.2) and used in parallel with free peptide to raise antibodies in chickens and rabbits (Section 5.3.3). The resulting anti-peptide antibodies were evaluated by ELISAs and western blots (Section 5.3.4) and examined for possible effects on VP4 activity (Section 5.4.3). Bursal homogenates were partially fractionated (Section 5.2) to facilitate this immunochemical screening of the antibodies. These fractions were also assayed for enzyme activity using gelatin-containing substrate gels (Section 5.4.1) and the fluorogenic peptide substrate Cbz-Arg-Arg-AMC (Section 5.4.2) in the presence or absence of appropriate inhibitors (Section 5.4.3).

5.2 Partial purification of virus for analysis of proteins

Identification of the tissue location of VP4 was a relevant objective, not only to focus on its properties but also to consider any secondary role(s) it may have in pathogenesis related to its location. The distribution of VP4 within the infected bursa is additionally of interest in terms of vaccine design with VP4 as a potential target.

Bursal material was crudely fractionated to provide a framework for the immunochemical and enzymatic examination of VP4. This process was based on that employed to obtain a concentrated viral pellet for the purification of viral genetic material, as described in Section 4.2.2. The fractionations are summarised in Figure 5-1 at the end of Section 5.2.2. Uninfected bursal material was subjected to identical procedures to provide an appropriate negative control.

PEG precipitation of bursal homogenates was investigated as an alternative fractionation method as it had found specific application for the purification of a virus. Polson (1974) described the purification of influenza virus from the extraneous proteins of allantoic fluid by

precipitation with PEG (6 kDa) at concentrations of 1 to 4% (m/v). Precipitation of a virus or a protein from solution by the filamentous polymer PEG occurs via displacement from solution followed by dehydration to the point of over-saturation and precipitation (Polson, 1977). This is the same principle used to purify immunoglobulins, described in Sections 3.8 and 3.9. However, the system proved unsuitable for IBDV purification, probably because the bursal homogenate contained a great variety of proteins in concentrated form.

5.2.1 Reagents

Phosphate buffered saline (PBS), pH 7.2. As per Section 3.7.1.

Triton X-100, 1% (v/v) in PBS. Triton X-100 (1 ml) was diluted to 100 ml with PBS.

Sucrose solution, 40% (m/v). As per Section 4.2.1.

5.2.2 Procedure

Aliquots of bursal material were partially thawed on ice before the addition of an equal volume of cold PBS. The sample was kept on ice and cut finely with sharp scissors to disrupt connective tissue. This mixture was homogenised (1 500 rpm, 5 min) over ice in a Potter S homogeniser (B. Braun, Melsungen, W. Germany). A centrifugation step (17 000×g, 15 min, 0°C) pelleted cellular debris, allowing the supernatant to be removed. Aliquots of this initial supernatant were stored at -70°C and are referred to as fraction A. The pellet was resuspended in minimal Triton-X 100 solution (~2.5 ml, ON at 4°C) and centrifuged again (17 000×g, 15 min, 0°C). Triton-X 100 was used to solubilise any protease that may be membrane bound, with the reaction conducted at low temperature to promote hydrophobic interactions. Aliquots of this Triton-X 100 extract were removed and stored at -70°C and are referred to as fraction B.

The remaining fraction A supernatant was carefully layered onto a 40% (m/v) sucrose cushion and ultracentrifuged in a Beckman SW40 rotor (86 000×g, 2.5 h, 2°C). The resulting supernatant over the sucrose cushion appeared to have two components, a clearer upper phase and a more turbid lower phase. Aliquots of each of these phases were removed and stored at -70°C and are referred to as fractions C and D respectively. The remaining supernatant and sucrose were carefully removed and the virus-containing pellet drained thoroughly before

resuspension in PBS (500 μ l) and storage at -70°C . Gentle pipetting was necessary to disrupt the pellet sufficiently for resuspension. This fraction is referred to as fraction E.

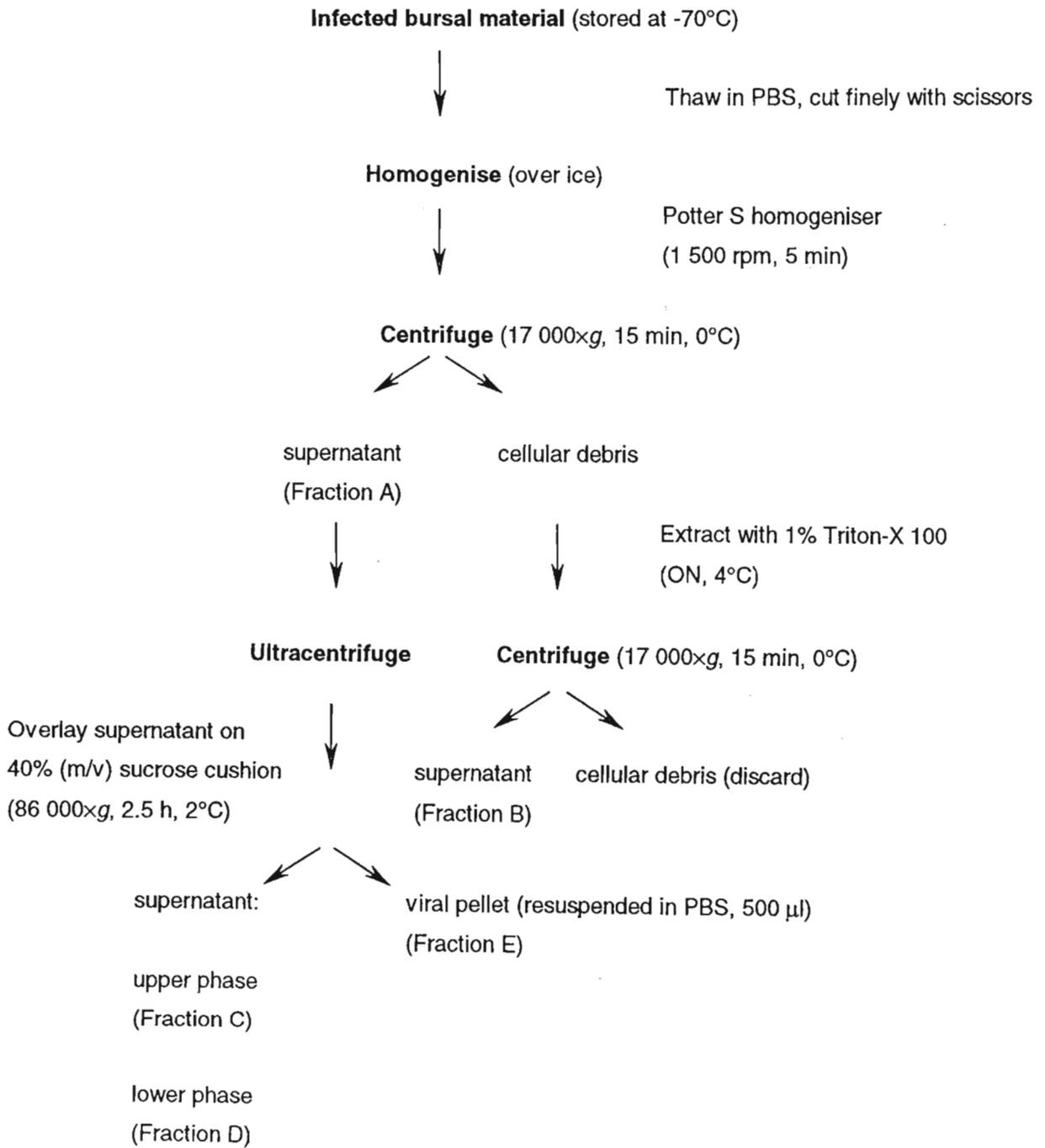


Figure 5-1. Flow diagram detailing partial fractionation of bursal material by sequential centrifugation.

5.3 Raising and evaluating anti-peptide antibodies against a peptide from VP4

Anti-peptide antibodies are versatile and yet specific and as such, extremely useful. Such antibodies were considered most suitable to further these investigations of VP4, and were a particularly appropriate alternative since purified VP4 was not available. Thus the decision was taken to produce anti-VP4 antibodies by immunising experimental animals with a suitable peptide from the putative protease. This peptide was selected with the aid of a computer software package, based on the parameters discussed in Section 5.3.1.

5.3.1 Selection of an immunogenic peptide from VP4

A number of sequence-related features influence the B-cell stimulatory potential of a peptide within an experimental animal. Parameters linked to surface availability such as hydrophilicity, accessibility and flexibility of short polypeptide segments have been correlated with the location of continuous epitopes in proteins (Cármenes *et al.*, 1989). Relative scales describing the contribution of each amino acid to such parameters have been derived and utilised in a variety of predictive algorithms to locate the position of epitopes using primary sequence data. This is particularly useful for the preparation of antibodies against proteins about which little is known except the corresponding nucleic acid sequence. A variety of these predictive methods are described briefly below.

Hopp and Woods (1981) and Kyte and Doolittle (1982) devised scales based on regions within proteins of hydrophilicity and hydropathy respectively. Hydrophilic side chains are usually located on the surface to maximise interactions with water, whereas hydrophobic residues are typically buried inside the protein core to minimise interactions with the polar environment. Stretches of hydrophilic residues are thus more likely to be surface-located and accessible to antibodies. In some cases, hydrophilic peaks within polypeptide chains have been shown to correspond with known protein epitopes (Hopp, 1986), although Hopp and Woods (1981) caution that “not all antigenic determinants are associated with high points of hydrophilicity and not all high points are associated with antigenic determinants”. Nevertheless, they maintain that one determinant is always located at the highest point of hydrophilicity.

However, the exclusion of water molecules in the binding of antibody and antigen is of great significance in the attraction between the two. This is partly because of the electrostatic component of the binding force, which is dramatically increased when water is excluded due

to the inverse contribution of the high dielectric constant of water (Roitt, 1991). Additionally, hydrophobic groups exclude water in their interactions and result in increased entropy; hydrophobic forces are consequently thought to contribute up to 50% of the total strength of the antibody-antigen bond (Roitt, 1991). It would thus appear that a potential peptide determinant is more likely to elicit an antibody response if it contains both hydrophilic and hydrophobic regions.

Surface residues are usually more mobile and flexible than internal residues and crystallographic measurement of this mobility has been used to assign atomic temperature factors (also known as B-factors, or Debye-Waller factors) to the various amino acid residues. Karplus and Schultz (1985) devised an epitope prediction method based on B-factors, which Van Regenmortel and Daney de Marcillac (1988) suggest is the most reliable of the presently available prediction methods. Flexibility of antigen contributes to binding affinity in three ways: 1) bond rotation exposing a buried hydrophobic side chain, 2) flexing of the α -carbon backbone to bring interacting residues into closer contact, and 3) lateral displacement of antigen residues to avoid electron cloud overlap with those of the antibody (Roitt, 1991).

Finally, a more empirical approach was utilised by Welling *et al.* (1985), who devised an antigenicity scale based on the percentage of each amino acid residue present in known antigenic determinants compared with the percentage of the amino acid residues in the average composition of a protein.

A software package, *Predict7*, (Cármenes *et al.*, 1989) was used to select a suitably antigenic peptide from VP4. The software implements a variety of these prediction algorithms and generates relative scales for any input protein sequence, which can then be displayed graphically (Figure 5-2). Although Rooman and Wodak (1988) have suggested that no particular predictive method can give more than 60% accuracy, the cumulative assessment of a number of such methods is useful for the identification of potential epitopes.

The VP4 sequence was thus analysed in terms of hydrophilicity (Hopp and Woods, 1981), surface probability or accessibility (Emeni *et al.*, 1985; Janin *et al.*, 1978), side chain flexibility (Karplus and Schultz, 1985) and antigenicity (Welling *et al.*, 1985). The sequence was also searched for N-glycosylation sites (Asn-X-Ser or Asn-X-Thr, where X is any residue) as these have additional antigenic potential (Hubbard and Ivatt, 1981). VP4 does not, however, contain any such N-glycosylation sites.

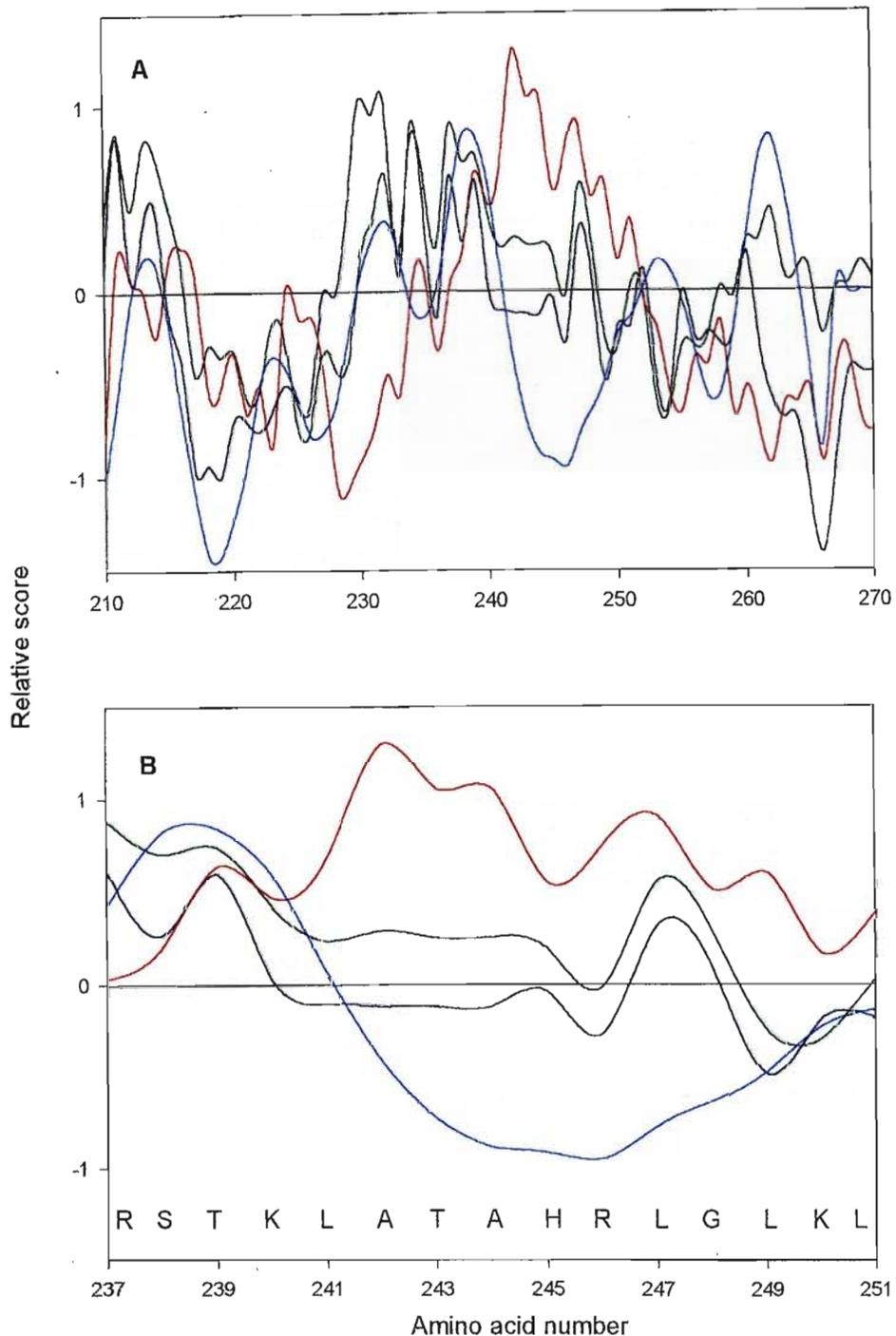


Figure 5-2. Epitope prediction profiles for regions of IBDV VP4.

Panel A shows epitope prediction profiles for residues 210 to 270 of VP4; Panel B is an expanded view of these plots for the peptide sequence selected for anti-peptide antibody production, VP4 residues 237-251. The amino acid residues comprising this region are detailed above the X-axis. The Y-axis is labelled with arbitrary units of hydrophilicity (—), accessibility (—), flexibility (—) and antigenicity (—), the latter three scales normalised with respect to the first. A software package, *Predict7*, (Cármenes *et al.*, 1989) was used to generate these data by implementing a variety of predictive algorithms, details of which appear in the text.

As indicated in Figure 5-2, VP4 residues 237-251 constitute a peptide with high immunogenic potential. The sequence is greater than 10 residues in length and contains a local peak of hydrophilicity, flexibility, accessibility and antigenicity, a high central plateau of antigenicity and hydrophobicity (by inference from low hydrophilicity) and another local peak of hydrophilicity, accessibility and antigenicity (shown from left to right on Figure 5-2B). The 15-residue peptide corresponds to residues 689 to 703 in the primary sequence of VP4 (Bayliss *et al.*, 1990), namely: RSTKLATAHRLGLKL. Some modifications to this sequence were made in the synthesis of the peptide. A C-terminal cysteine, synthesised as an amide, was added to allow thioether coupling to a carrier protein. This was to ensure high purity and better mimicry of the internal region of the protein and to prevent racemisation of the peptide mix, which can occur when the C-terminal is cysteine-acid (G. Tribbick, Chiron Mimotopes, Australia, personal communication). The N-terminal end was synthesised as a free amine, increasing solubility and mimicking an uncharged peptide bond, hopefully increasing cross-reactivity with whole VP4. The peptide (1767.2 Da) was synthesised by Chiron Mimotopes (Victoria, Australia).

5.3.2 Synthesis of conjugated peptides

Although peptides of 10 residues or longer are considered immunogenic by some authors (Harlow and Lane, 1988), peptides are usually coupled to carrier proteins before immunisation, increasing their immunogenicity up to 1000-fold (Mariani *et al.*, 1987). The selected VP4 peptide was conjugated to carrier proteins using the coupling reagent meta-maleimidobenzoyl N-hydroxysuccinimide ester (MBS) as described by Kitagawa and Aikawa (1976), using the modifications of Liu *et al.* (1979). This heterobifunctional reagent acylates amino groups via its active ester and also forms thioether bonds with sulfhydryl groups (Kitagawa and Aikawa, 1976). In this study, MBS was used to link peptides to carrier proteins by the acylation of amino groups on the carrier protein and subsequent formation of thioether bonds with the C-terminal cysteine of the peptide. Due to the heterobifunctional nature of MBS, conjugation must be carried out in two distinct steps, which serendipitously prevents the formation of carrier or peptide dimers. A 30-50% yield of conjugate is usually obtained using MBS (Muller, 1988). Glutaraldehyde conjugation was unsuitable for the peptide used in this study, because exclusive conjugation of the peptide to the carrier by the C-terminus would be prevented by the presence of the ϵ -amino group of the lysine residue in the peptide (Avrameas and Ternynck, 1969).

Ovalbumin was used as a carrier protein for immunisation of rabbits and rabbit albumin for chickens. These carriers were used instead of keyhole limpet haemocyanin, which is extremely immunogenic in experimental animals (Polson *et al.*, 1980) and elicits antibodies that have been found to react non-specifically in western blots (Coetzer *et al.*, 1991).

5.3.2.1 Reagents

50 mM sodium phosphate buffer, pH 7.0. $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (0.69 g) was dissolved in dH_2O (90 ml), titrated to pH 7.0 with NaOH and made up to 100 ml.

Ellman's reagent [10 mM 5,5'-dithiobis(2-nitrobenzoic acid), (DTNB) in 50 mM sodium phosphate buffer, pH 7.0, 10% (v/v) methanol]. DTNB (40 mg) was dissolved in methanol (100 μl) and diluted to 10 ml with sodium phosphate buffer.

16 mM MBS. MBS (5 mg) was dissolved in dimethylformamide (DMF, 1 ml). DMF must be amine-free and 'dry' to prevent undesired side reactions.

Reducing buffer [100 mM Tris-HCl, 1 mM EDTA, 0.02% (m/v) NaN_3 , pH 8.0]. Tris base (1.21 g), EDTA (0.037 g) and NaN_3 (0.02 g) were dissolved in dH_2O (90 ml), titrated to pH 8.0 with HCl and made up to 100 ml.

10 mM DTT. DTT (7.71 mg) was dissolved in reducing buffer (5 ml) just before use.

Chromatography buffer [100 mM sodium phosphate, 0.02% NaN_3 , pH 7.0]. $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (6.9 g) and NaN_3 (0.1 g) were dissolved in dH_2O (450 ml), titrated to pH 7.0 with NaOH and made up to 500 ml.

5.3.2.2 Procedure

Carrier protein (0.0925 μmol , either 4.16 mg ovalbumin or 6.10 mg rabbit albumin) was dissolved in phosphate buffer (2 ml). MBS (279 μl , 3.56 μmol , giving a 1:40 molar ratio of carrier protein:MBS) was slowly stirred into the carrier protein solution and acylation of the carrier allowed to proceed (RT, 30 min). Unreacted MBS was removed by chromatography on Sephadex G-25 (1.5 \times 11.5 cm column) pre-equilibrated in chromatography buffer at a flow rate of 10 $\text{cm} \cdot \text{h}^{-1}$. The A_{280} of the eluate was monitored and the activated carrier collected as the first peak. The subsequent peak, representing unreacted MBS, was discarded.

Peptide (6.54 mg, 3.7 μmol , giving a molar ratio of peptide to activated carrier of 40:1) was dissolved in reducing buffer (1 ml). DTT (1 ml) was added and reduction of the peptide allowed to proceed in an incubator (1 h, 37°C). Reduced peptide was separated from excess DTT by chromatography on Sephadex G-10 (1 \times 12 cm column) pre-equilibrated at RT in chromatography buffer at a flow rate of 10 $\text{cm}\cdot\text{h}^{-1}$. Fractions (500 μl) were collected manually in microfuge tubes and 10 μl of each fraction mixed with an equal volume of Ellman's reagent to construct the elution profile. This reagent produces a yellow product after reaction with reducing agents, with a light yellow colour marking the elution of the reduced peptide and an intensely yellow colour marking the elution of excess DTT.

The reduced peptide was immediately mixed with activated carrier and incubated for 3 h at RT. Carrier activation and peptide reduction should ideally proceed at the same time so that they are mixed immediately after preparation but, in practice, the time of elution of each is difficult to control exactly. Where necessary, the activated carrier, rather than the reduced peptide, was allowed to stand before the final reaction commenced, as Muller (1988) states that coupling yield is strongly dependent on the availability of sulfhydryl groups. Unbound peptide was removed by dialysis against four changes of sodium phosphate buffer over 16 h at 4°C.

Estimating the efficiency of coupling proved difficult. Kitagawa and Aikawa (1976) titrated the maleimide groups on the carrier using a sulfhydryl reagent (such as β -mercaptoethanol, which binds to MBS) and Ellman's reagent. By determining the maleimide content of the carrier before and after conjugation, the efficiency of both carrier activation and coupling were estimated. In the present study, irreproducible results were obtained using this method, so alternative methods of estimating coupling efficiency were investigated. The carrier-conjugated peptide (called the conjugated peptide throughout) and the free carrier were scanned between 185 and 900 nm, but no marked differences in their spectra were noted. However, all three species (free, unmodified carrier, MBS-modified carrier and MBS-modified carrier-peptide conjugate) were visible as sharp single bands on a silver stained SDS-PAGE gel (Figure 5-3). Increases in molecular mass between the species indicated successful MBS modification to the carrier and subsequent conjugation of the peptide.

5.3.3 Production of antibodies in chickens and rabbits

Because different species may respond differently to an immunogen (Harlow and Lane, 1988), both chickens and rabbits were immunised with the VP4 peptide. Chickens in particular are excellent animals for experimental antibody production, since large quantities of antibody are concentrated in the egg yolk to provide passive immunity for the developing chick (Polson *et al.*, 1980). These antibodies are termed IgY, where the 'Y' refers to their location in the egg yolk. Since antibodies produced throughout the immunisation protocol can be obtained by collecting the eggs, no bleeding of the chicken is necessary. Parallel experiments were conducted in chickens and rabbits with the free and conjugated peptide to assess relative immunogenicity.

5.3.3.1 Reagents

100 mM sodium phosphate buffer, pH 7.6. $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (1.56 g) was dissolved in dH_2O (75 ml), titrated to pH 7.6 with NaOH and made up to 100 ml.

5.3.3.2 Procedure

a). Chickens.

Two chickens were immunised with free peptide (250 μg each, dissolved in heat-sterilised sodium phosphate buffer) and two with peptide-rabbit albumin conjugate (about 200 μg peptide each). Since the exact efficiency of peptide-carrier coupling could not be calculated (see Section 5.2.2.2), 40% coupling efficiency was assumed (Muller, 1988) to calculate the required dose of conjugated peptide. The immunogen was triturated with an equal volume of adjuvant until a thick emulsion formed (Freund and McDermott, 1942). Because chickens respond unfavourably to repeated doses of Freund's complete adjuvant (T. Coetzer, University of Natal (Pietermaritzburg), South Africa, personal communication), the free peptide was administered alternately in Freund's complete and incomplete adjuvant (FCA and FIA respectively). Conjugated peptide was administered in FCA for the first immunisation and subsequently administered in FIA. Chickens were injected intramuscularly with inoculum (1 ml and 1.2 ml total for free and conjugated peptide respectively) at two to four sites on either side of the sternum at weeks zero, one, two, six and 10. Eggs were collected daily from the start of the immunisation protocol.

b). Rabbits.

Two young rabbits were immunised with free peptide (250 µg each, prepared as for chickens) and two with peptide-ovalbumin conjugate (about 200 µg each of peptide, calculated as for chickens). Immunogens were triturated with an equal volume of adjuvant until a thick emulsion formed. FCA was used for all immunisations with free peptide and for initial immunisations with conjugated peptide. FIA was used for subsequent immunisations with the conjugated peptide. Rabbits were injected subcutaneously with inoculum (1 ml and 1.2 ml total for free and conjugated peptide respectively) at two to four sites on either side of the spine (Chase, 1977) at weeks zero, two, six and 10.

Blood was collected five, eight and 12 weeks after the first immunisation. The area surrounding the central ear vein was shaved and cleaned with alcohol. A needle was carefully inserted into the ear vein and blood collected into dry glass test tubes. Clots were gently loosened from the walls of the collection tubes with a sealed Pasteur pipette and blood was stored for 16 h at 4°C to allow complete clotting. The serum was aspirated and centrifuged (1500×g, 10 min, RT) to remove any remaining erythrocytes.

5.3.4 Assessment of antibody production and reaction with bursal fractions

Following isolation of chicken (Section 3.8) and rabbit (Section 3.9) antibodies, ELISAs (Section 3.11) were used to monitor antibody production in experimental animals by titrating test antibodies against the free peptide, coated as antigen. The titre of each antibody is defined as the lowest antibody concentration that gives an absorbance value higher than the controls. The highest titre anti-peptide antibodies produced by each animal were identified for subsequent use in further immunochemical investigations. It should be noted that free peptide used as antigen can adopt many conformations, both in the experimental animal and in ELISAs. Only some of these mimic the natural conformations in the corresponding constrained region of the target protein. Hence of all antibodies elicited, only a proportion will be directed against peptide conformations naturally occurring in whole VP4.

Test material in the form of bursal homogenate spin fractions (Section 5.2.2) was probed with the high titre anti-peptide antibodies in further ELISAs and western blots. Electroblotting denatures the antigen to a larger extent than that which occurs in an ELISA, and consequently western blots indicate antibody recognition of fully denatured antigen. These antibodies were

also assessed for any effect on the enzymatic activity against a synthetic substrate, as detailed in Section 5.4.3.

5.3.4.1 Reagents

As per Sections 3.11.1 and 3.12.1.

5.3.4.2 Procedure

a). ELISAs monitoring progress of antibody titre.

The procedure followed is as detailed in Section 3.11.2 and the volume of reagent is given per well unless otherwise stated. For monitoring the production of anti-peptide antibodies in chickens and rabbits, free peptide was coated as antigen ($1 \mu\text{g}.\text{ml}^{-1}$ in PBS, $150 \mu\text{l}$, ON at 4°C) and remaining sites blocked with PBS-BSA ($200 \mu\text{l}$, 1 h at 37°C). Test antibodies were subsequently titrated (serial doubling dilutions from $500 \mu\text{g}.\text{ml}^{-1}$ to $\sim 1 \mu\text{g}.\text{ml}^{-1}$ in PBS-BSA, $100 \mu\text{l}$, 2 h at 37°C) and detected with HRPO-linked secondary antibody ($120 \mu\text{l}$ in PBS-BSA, 1 h at 37°C). TMB/ H_2O_2 ($150 \mu\text{l}$) was used as substrate and absorbance monitored at 450 nm after the addition of stop solution ($50 \mu\text{l}$).

b). ELISAs assessing recognition by anti-peptide antibodies in IBDV-infected bursal homogenate fractions.

The procedure followed is as detailed in Section 3.11.2 and the volume of reagent is given per well unless otherwise stated. Samples of five crude fractions of IBDV-infected bursal homogenate (Section 5.2.2) were coated as antigen (serial five-fold dilutions from $50 \mu\text{g}.\text{ml}^{-1}$ to $0.4 \mu\text{g}.\text{ml}^{-1}$ in PBS, $150 \mu\text{l}$, incubated ON at 4°C). Remaining sites were blocked with PBS-casein ($200 \mu\text{l}$, 1 h at 37°C). In four separate experiments, rabbit and chicken antibodies against free and conjugated peptide were applied ($10 \mu\text{g}.\text{ml}^{-1}$ in PBS-casein, $100 \mu\text{l}$, 2 h at 37°C) and detected with HRPO-linked secondary antibody ($120 \mu\text{l}$ in PBS-casein, 1 h at 37°C). TMB/ H_2O_2 ($150 \mu\text{l}$) was used as substrate and absorbance monitored at 450 nm after the addition of stop solution ($50 \mu\text{l}$).

c). Western blots assessing recognition by anti-peptide antibodies in IBDV-infected bursal homogenate fractions.

The procedure followed is as detailed in Section 3.12.2. Samples of five crude fractions of IBDV-infected and uninfected bursal homogenate were boiled with NRTB and subjected to SDS-PAGE (Section 3.4.5). The separated proteins were electroblotted (200 mA, 2 h) and the remaining sites on the nitrocellulose blocked with blocking reagent (1 h at RT). In four separate experiments, chicken and rabbit antibodies against free and conjugated peptide were applied (100 $\mu\text{g}\cdot\text{ml}^{-1}$, 3 h at RT), and detected by rabbit anti-IgY-AP or goat anti-rabbit-AP secondary antibodies (manufacturer's recommended dilutions, 1 h) and BCIP/NBT substrate.

5.4 Assaying bursal fractions for proteolytic activity

Once the infected bursal material was partially fractionated (Section 5.2.2), the search for VP4 by enzymatic as well as immunochemical means could begin. This entailed looking for digestion at specific molecular masses after running the fractions on gelatin substrate gels (Section 5.4.1) and screening fractions for activity against a fluorogenic synthetic substrate (Section 5.4.2). A variety of class-specific protease inhibitors and anti-peptide antibodies against VP4 were also applied (Section 5.4.3) to observe any effect on activity.

5.4.1 Activity in substrate gels

Substrate SDS-PAGE allows sensitive and convenient assessment of proteolytic activity by detection of the digestion of gelatin incorporated in polyacrylamide gels. Unboiled samples of infected and uninfected bursal homogenate fractions were subjected to non-reducing substrate SDS-PAGE, as described in Section 3.7. The amount of protein run for each sample (determined by microBradford assay, Section 3.3) was varied quite considerably, as detailed in Table 5-1. The resulting zymograms were examined after staining for evidence of gelatin digestion.

Table 5-1. Range of bursal homogenate proteins^a examined by substrate SDS-PAGE.

IBDV-infected bursal homogenate fractions					Uninfected bursal homogenate fractions				
A	B	C	D	E	A	B	C	D	E
		(μg)					(μg)		
131	208	302	216	14	85	164	146	56	8
116	104	151	108	7	43	82	73	28	4
12	10	14	10	8	12	10	14	10	8
6	5	7	5	4	6	5	7	5	4

^a Bursal homogenate fractions are as designated in Section 5.2.2;

A: initial supernatant after homogenisation of bursa

B: Triton-X 100 extract of initial cellular debris after homogenisation of bursa

C: upper phase of supernatant after ultracentrifugation of fraction A over 40% (m/v) sucrose

D: lower phase of supernatant after ultracentrifugation of fraction A over 40% (m/v) sucrose

E: resuspended viral pellet obtained after ultracentrifugation of fraction A over 40% (m/v) sucrose.

5.4.2 Activity against Cbz-Arg-Arg-AMC

Synthetic peptide substrates consist of single amino acids or short peptides (recognition sequences), which are covalently modified at their N- and C-termini. The N-terminus may be blocked with a stabilising agent, such as a benzoyl, carbobenzoxy or acetyl group. The C-terminus is usually coupled by an amide bond to a chromogenic or fluorogenic leaving group, such as 4-methoxy- β -naphthylamine, *para*-nitroaniline or 7-amino-4-methylcoumarin (John, 1992; Sarath *et al.*, 1989). The fluorogenic substrates have a low intrinsic fluorescence but hydrolysis of the amide bond by proteolytic cleavage releases a highly fluorescent leaving group, which facilitates assays of higher sensitivity than those obtainable with chromogenic leaving groups. In the case of cathepsin L, the lower limit of sensitivity using the substrate Cbz-Phe-Arg-AMC is 0.3 ng. The intensity of the fluorescence provides a direct indication of enzyme activity (Barrett and Kirschke, 1981).

The substrate Cbz-Arg-Arg-AMC was used in this investigation to identify potential VP4 activity within crude fractions of infected bursal homogenate (Section 5.2.2). The substrate target region was selected on the basis of deletion-expression studies which indicate that VP4 has dibasic specificity (Azad *et al.*, 1987; Jagadish *et al.*, 1988) and on sequence analysis which has provided evidence of a serine-like active site (Brown and Skinner, 1996). For the simultaneous assay of large numbers of samples across the bursal fractions for infected and uninfected tissue, samples were analysed in a 96-well microfluorimetry plate.

5.4.2.1 Reagents

Substrate stock solution (1 mM Cbz-Arg-Arg-AMC in DMSO). Cbz-R-R-AMC (1.1 mg) was dissolved in DMSO (1.5 ml) and stored at 4°C.

Substrate working solution [20 µM Cbz-Arg-Arg-AMC in 0.02% (v/v) DMSO]. Substrate stock solution (100 µl) was made up to 5 ml with dH₂O. This solution was stored at 4°C for no longer than 48 h. The substrate was used in assays at a final concentration of 5 µM.

Assay buffer (PBS, pH 7.2). See Section 3.7.1.

5.4.2.2 Procedure

All reagents were pre-equilibrated to 37°C. Samples of IBDV-infected and uninfected bursal homogenate fractions were diluted in assay buffer (75 µl total) and pre-incubated (4 min, 37°C) before the addition of substrate working solution (25 µl). Fluorescence was monitored continuously on a Hitachi F-2000 spectrofluorimeter (excitation wavelength, 370 nm, emission wavelength, 460 nm) for 210 min. Readings were taken at zero, five, 10, 15 and 30 min and thereafter every 30 min up to 210 min.

5.4.3 Inhibition of activity against Cbz-Arg-Arg-AMC

Although there are indications that VP4 may be a serine protease, testing the effect of a broad spectrum of inhibitors on the activity of a protease often permits its classification into one of the four currently recognised mechanistic protease classes (Neurath, 1989). Inhibition data may also provide insight into substrate specificity and as such are useful when examining an uncharacterised or even putative protease such as VP4. It is recognised that such inhibitor profiling can only be definitive for a purified enzyme, and also that cleavage of the fluorogenic peptide in such crude samples is not conclusively or exclusively proof of the presence of VP4. However, it was thought that such information would still be useful in these preliminary investigations, despite the crudeness of the protein fractions under study. The effect of anti-VP4 peptide antibodies on activity was thus investigated with interest and the hope of extracting some specificity despite the heterogeneous samples.

5.4.3.1 Reagents

See Section 5.4.2.1 for substrate solutions and assay buffer.

10× Inhibitor stock solutions. Inhibitor stock solutions were made up at 10× the concentrations used in the final assay mixture and stored at -75°C. AEBSF (24 mg, 100 mM) was dissolved in dH₂O (1 ml), while pepstatin A (6.9 mg, 10 mM), E-64 (3.6 mg, 10 mM) and 1,10-phenanthroline (19.8 mg, 100 mM) were each dissolved separately in 'dry' DMSO (1 ml).

10× Antibody stock solutions. Chicken and rabbit antibodies against free and conjugated VP4 peptide, which showed the most positive response in an ELISA, (Section 5.3.4) were identified and diluted with PBS yielding 5 mg.ml⁻¹ solutions of antibody. Non-immune chicken and rabbit antibodies were prepared similarly for use in control reactions.

5.4.3.2 Procedure

Enzyme activity against Cbz-Arg-Arg-AMC was assayed as described in Section 5.4.2, except that samples of IBDV-infected and uninfected bursal homogenate fractions were diluted in assay buffer (65 µl total) and pre-incubated (4 min, 37°C) with inhibitor or antibody solutions (10 µl) before the addition of substrate working solution (25 µl). The fluorescence was recorded at time zero and at 5 min intervals up to 30 min. Controls were included for each inhibitor solvent (i.e., dH₂O and DMSO) and for PBS to monitor any affect on enzyme activity. The inhibitors and uncleaved substrate were also assessed separately to ensure that neither contributed significantly to background fluorescence.

5.5 Results and discussion

5.5.1 Raising and evaluating anti-peptide antibodies

5.5.1.1 Synthesis of conjugated peptides

Chickens and rabbits were immunised with both free and carrier-conjugated VP4 peptide. Evidence of successful conjugation to rabbit albumin and ovalbumin (Section 5.3.2) was needed, however, before the animals were injected. As illustrated for rabbit albumin in Figure 5-3, all three species (free, unmodified carrier, MBS-modified carrier and MBS-modified carrier-peptide conjugate) were visible as sharp single bands on a silver-stained SDS-PAGE gel. Increases in molecular mass of ~300 Da and ~2 kDa between respective species indicated that successful MBS modification to the carrier and subsequent conjugation of the peptide had occurred. A similar effect was noted for the MBS modification of ovalbumin and conjugation to the VP4 peptide, although these results are not shown.

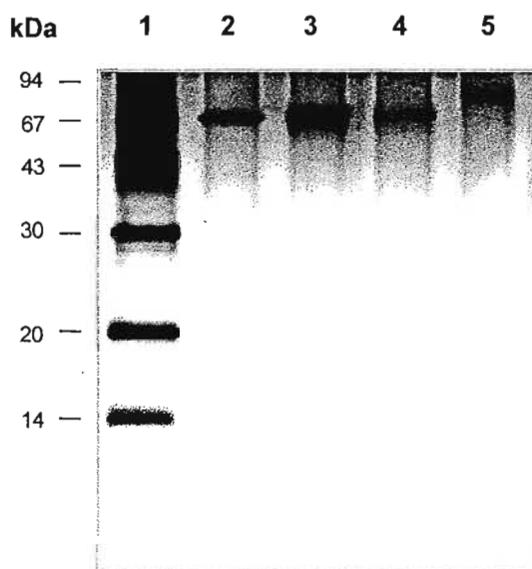


Figure 5-3. SDS-PAGE gel showing coupling of VP4 peptide to rabbit albumin.

Unmodified rabbit albumin (66 kDa, lane 2, lane 4), rabbit albumin-MBS (~ 66.3 kDa, lane 3) and rabbit albumin-MBS-peptide (~68.3 kDa, lane 5) were electrophoresed and silver stained as detailed in the text. Pharmacia M_r standards (lane 1) were used as described in Section 3.4.4.

5.5.1.2 Assessment of anti-peptide antibody production

ELISAs (Section 5.3.4) were used to monitor the antibody production in experimental animals by titrating test antibodies against free VP4 peptide, coated as antigen. Figures 5-4 and 5-5 illustrate the results of ELISAs using antibodies raised in chickens and rabbits respectively, against both free and conjugated peptide.

a). Response of chicken antibodies.

Antibodies raised in chickens against both free and rabbit albumin-conjugated VP4 peptide showed good recognition of free peptide from weeks six and eight post-immunisation, with week eight giving the highest signal and having a titre of below $2 \mu\text{g.ml}^{-1}$ in each case. (Figure 5-4, all panels). Even antibodies from week three post-immunisation were quite reasonable when compared to the non-immune preparation, except in the case of one chicken (F1) immunised with free peptide. Although the response to free peptide was slightly slower than the response to conjugated peptide, week eight responses at low antibody concentration demonstrate comparable immunogenicity (Figure 5-4, 'F' plots vs. 'C' plots), and in fact antibodies raised against free peptide appear slightly more sensitive (higher A_{450}).

Individual chickens immunised with the same antigen had slightly different responses: against free peptide, F1 showed more consistent titration of week six and week eight antibodies than F2, despite a slow start shown by week three antibodies; against conjugated peptide, C1 had the most sensitive antibodies of all chickens at high concentration (i.e., greater than $15 \mu\text{g.ml}^{-1}$), whereas C2 demonstrated a pro-zone effect. The pro-zone is usually an indication of high titre antibodies in great excess, participating in non-specific protein interactions and thereby causing steric hindrance to the binding of secondary antibodies. Both week six and week eight antibodies titrated well for C1 and C2, although the C1 antibodies were more sensitive at the functional concentration of $10 \mu\text{g.ml}^{-1}$.

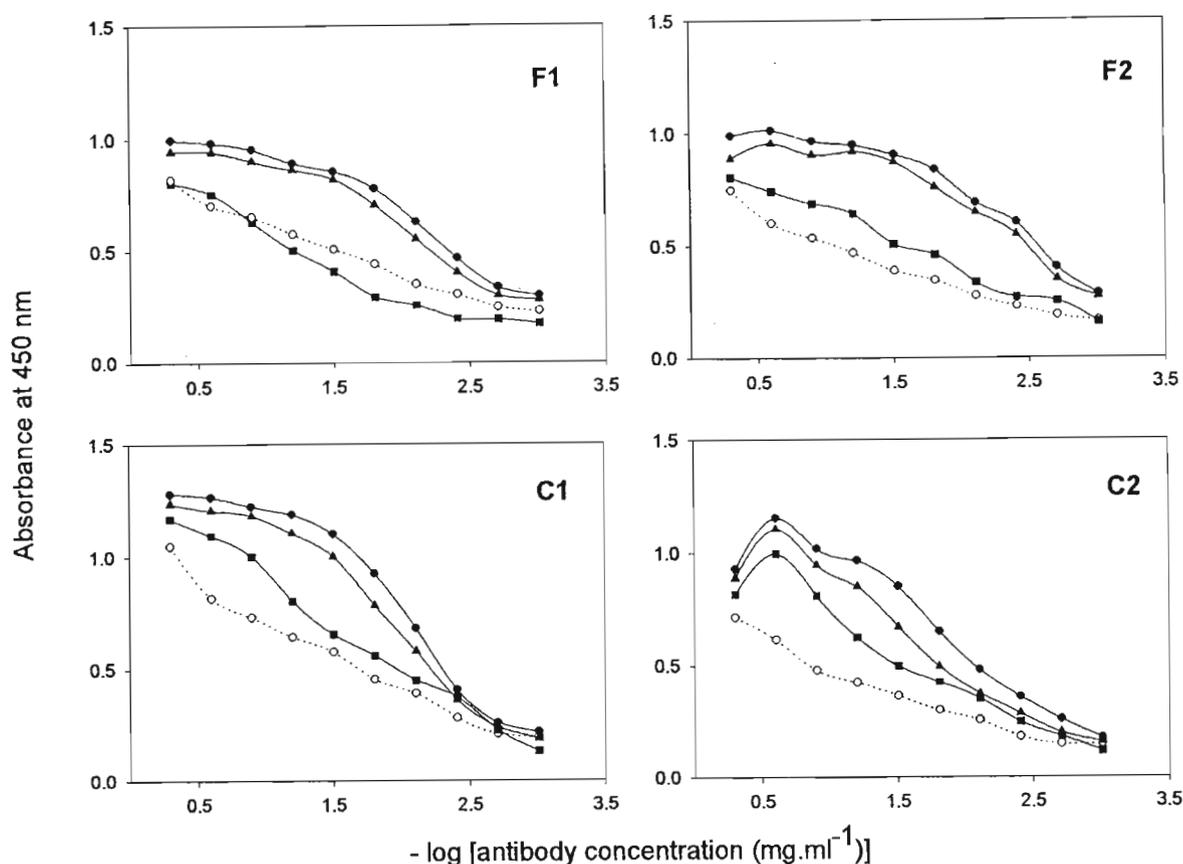


Figure 5-4. ELISAs monitoring titre of chicken antibodies produced against free and rabbit albumin-conjugated VP4 peptide at various weeks post-immunisation.

Peptide ($1 \mu\text{g}.\text{ml}^{-1}$) was coated for 16 h at 4°C . Antibody responses directed against the free (F) and conjugated (C) peptide are shown, with two chickens immunised with each antigen (labelled F1, F2 or C1, C2). Absorbance values at 450 nm represent the average of duplicate experiments. Non-immune antibodies (○) and antibodies from week three (■), week six (▲) and week eight (●) post-immunisation were titrated between 250 and $0.1 \mu\text{g}.\text{ml}^{-1}$ as doubling dilutions. Rabbit anti-chicken-HRPO was used as secondary antibody with TMB/ H_2O_2 as substrate.

b). Response of rabbit antibodies.

The four rabbits immunised with free and ovalbumin-conjugated VP4 peptide gave quite diverse antibody responses, although all exhibited slight pro-zone effects (Figure 5-5). With the exception of one rabbit immunised with free peptide (F2), the rabbits produced high titre antibodies by weeks five, eight and 12 post-immunisation, with week 12 giving the highest signal and having a titre of below $2 \mu\text{g}.\text{ml}^{-1}$. The response by F2 was insufficiently different from that of the non-immune reaction for these antibodies to be considered useful.

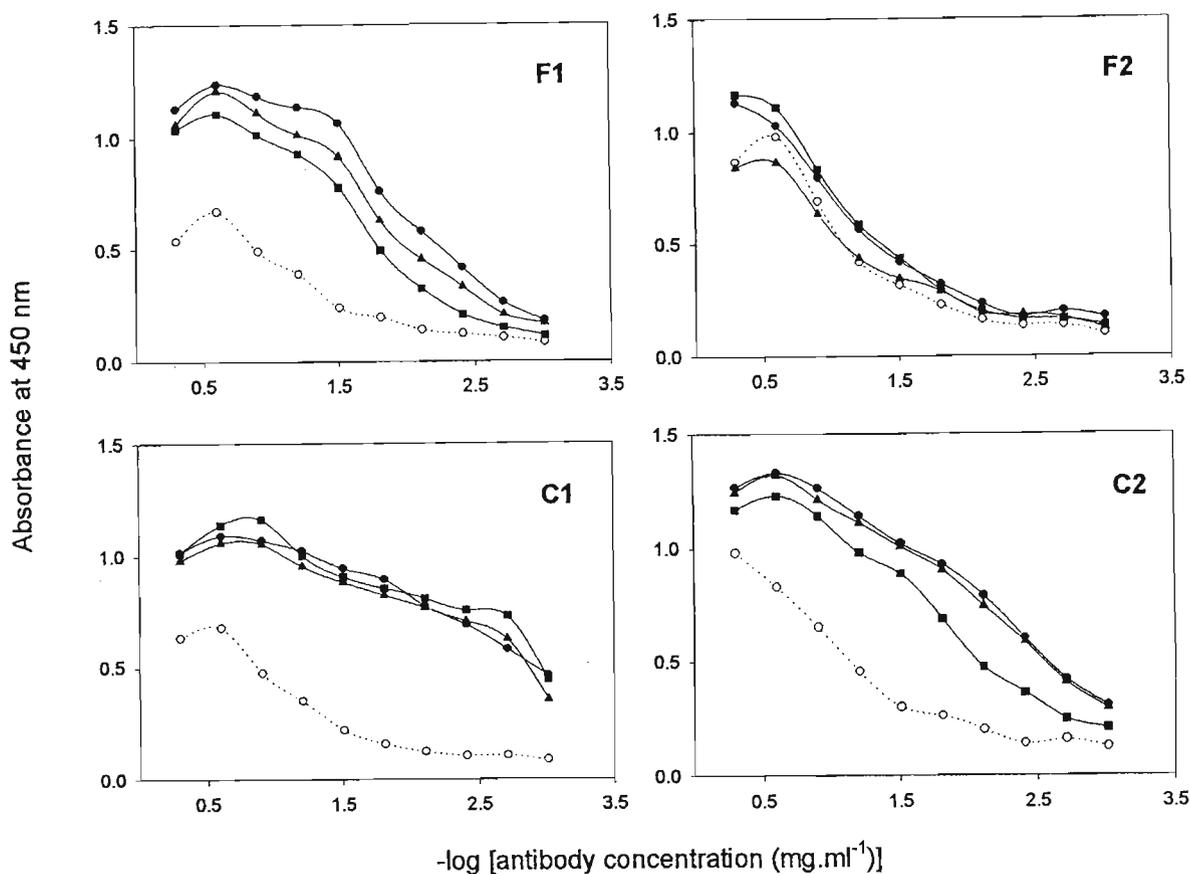


Figure 5-5. ELISAs monitoring titre of rabbit antibodies produced against free and ovalbumin-conjugated VP4 peptide at various weeks post-immunisation.

Peptide ($1 \mu\text{g}.\text{ml}^{-1}$) was coated for 16 h at 4°C . Antibody responses directed against the free (F) and conjugated (C) peptide are shown, with two rabbits immunised with each antigen (labelled F1, F2 or C1, C2). Absorbance values at 450 nm represent the average of duplicate experiments. Non-immune antibodies (o) and antibodies from week five (■), week eight (▲) and week 12 (●) post-immunisation were titrated between 250 and $0.1 \mu\text{g}.\text{antibody}.\text{ml}^{-1}$ as doubling dilutions. Sheep anti-rabbit-HRPO was used as secondary antibody with TMB/ H_2O_2 as substrate.

As with the chickens, the rabbit antibody response to free peptide was slightly slower than the response to conjugated peptide (Figure 5-5, 'F' plots vs. 'C' plots), and in this case, antibodies raised against the conjugated peptide appeared more sensitive. This is particularly noticeable at high antibody dilution where the A_{450} for week 12 anti-(conjugated peptide) antibodies is almost double that of anti-(free peptide) antibodies. Between the individual rabbits immunised with conjugated peptide, the response of C2 appears more consistent than that of C1, titrating smoothly and evenly down to $1 \mu\text{g}.\text{ml}^{-1}$.

Hence, high titre anti-VP4 peptide antibodies recognising free peptide in an ELISA were successfully raised in both chickens and rabbits. The selected peptide ultimately elicited antibodies of similar strength in both species irrespective of whether it was conjugated or not, although the animals were initially slower to respond to free peptide. Overall, production of anti-peptide antibodies was more consistent in chickens than in rabbits, with respect to smooth and even titration of antibody in ELISAs. This may be indicative of the specificity of antibody binding, which is investigated further in the following section (Section 5.5.1.3) with whole VP4 as the target within bursal homogenates.

5.5.1.3 Reaction of anti-peptide antibodies with IBDV-infected bursal fractions

a). ELISAs.

The anti-VP4 peptide antibody response in an ELISA to IBDV-infected bursal homogenate fractions is shown in Figure 5-6. It is apparent that the antibodies raised in rabbits failed to recognise any antigen within any of these fractions, as demonstrated by the almost flat curves very similar to the non-immune reaction and the no antigen control, with low A_{450} values for both anti-(free peptide) and anti-(conjugated peptide) rabbit antibodies. This is in contrast to their recognition of free peptide in an ELISA, illustrated in Figure 5-5. It is likely that the conformation assumed by the peptide within the rabbits does not match that of whole VP4 and/or that these particular amino acid residues may form a discontinuous epitope in the whole protein.

The chicken antibodies do appear to have bound to some form of antigen in all fractions, with responses well above that of the no antigen control. The response to fraction E appears particularly convincing as it titrates with the amount of protein present, and the reaction by the chicken anti-(free peptide) antibodies (red curve) is well above that of the non-immune reaction. The curves illustrating the response in fractions A to D are quite different to those of fraction E, although very similar to one another. However, the 'hill' shape of these curves is likely an experimental anomaly caused by the reddish-brown colour of these fractions, which would mask the yellow colour of the substrate and contribute to a low absorbance at 450 nm at the higher protein concentrations. This masking colour is reduced on dilution and the absorbance rises accordingly. Fraction E had no such colour to start with and does not exhibit the same low absorbance at high protein concentration.

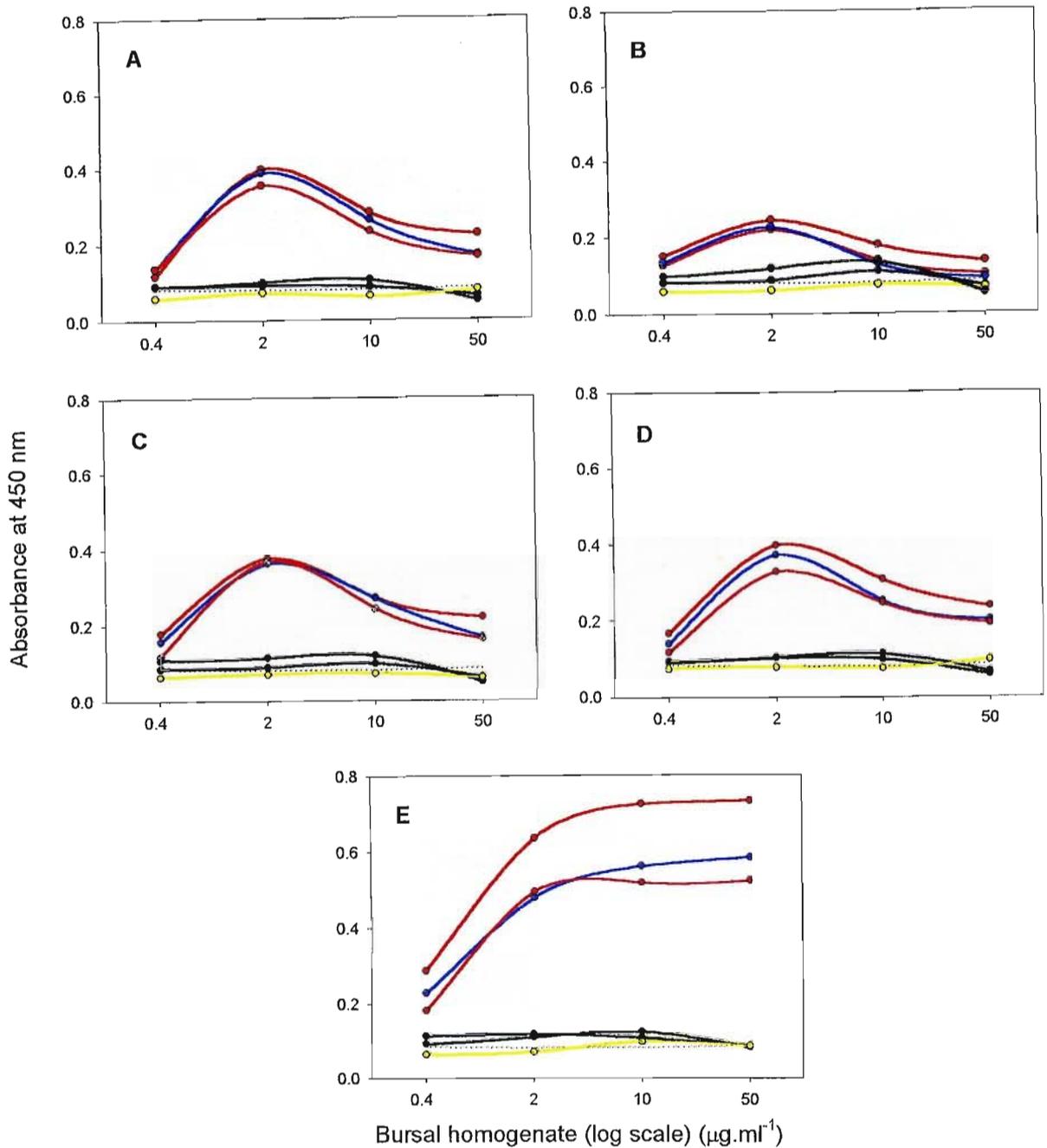


Figure 5-6. ELISAs showing recognition of IBDV-infected bursal homogenate fraction proteins by chicken and rabbit anti-VP4 peptide antibodies.

Serial five-fold dilutions (in PBS, from 50 $\mu\text{g.ml}^{-1}$) of IBDV-infected bursal homogenate fractions A-E (Figure 5-1, Section 5.2) were coated to wells of ELISA plates for 16 h at 4°C. A fixed concentration of test primary antibody (10 $\mu\text{g antibody.ml}^{-1}$) was applied and detected as described in Section 5.3.4. Absorbance values at 450 nm of a no antigen control (···), non-immune chicken antibodies (●), non-immune rabbit antibodies (●), anti-(free peptide) antibodies from chicken F2 (●) and rabbit F1 (●) and anti-(conjugated peptide) antibodies from chicken C1 (●) and rabbit C1 (●) represent the average of duplicate experiments. Rabbit anti-chicken-HRPO and sheep anti-rabbit-HRPO were used as secondary antibodies with TMB/H₂O₂ as substrate. Statistical analysis was not performed due to the preliminary nature of the experiment.

The chicken immune responses illustrated against fractions A to D are very similar to the non-immune reaction, yet both are considerably above that of the no antigen control. This is probably a reflection of the fact that IBDV is a commercially important virus. This follows because the non-immune IgY was prepared from supermarket eggs and it is highly likely that the laying chickens had some antibody protection against IBDV. These anti-IBDV antibodies in the non-immune IgY preparation may have readily reacted with IBDV present in the bursal homogenates. Clearly, a pre-immune serum would have been more appropriate, although the experimental chickens were not SPF, and unfortunately, had been immunised against IBDV when young. This was an experimental oversight, which should have been checked before immunisation with peptide and peptide conjugates. However, the non-immune antibodies against IBDV vaccine perhaps provide some indication of the background generated by the anti-IBDV vaccine antibodies of the experimental chickens. The chickens immunised with free and conjugated peptide would thus appear to have mounted only a slightly enhanced response (compared to the non-immune reaction) for fractions A to D. It is difficult, however, to speculate on the true A_{450} values at higher protein concentration because of the colour masking described in the previous paragraph. For all fractions the anti-(free peptide) antibodies performed slightly better than those raised against conjugated peptide and in the case of fraction E at least, the response was considerably enhanced in comparison to the non-immune reaction. Although such grading is only qualitative, it appears from Figure 5-6 that fraction E contains the largest quantity of anti-peptide antibody-responsive antigen, followed by fractions D, C and B with the overall bursal homogenate response (fraction A) most similar to that of fraction D.

The fact that the chicken anti-peptide antibody response was so similar to the non-immune reaction is interesting and yet complicating and a number of scenarios present themselves to explain this. It may be that neither anti-free nor anti-conjugated peptide antibodies recognise whole VP4 within the homogenate fractions and that it is merely an existing immune response to IBDV by the experimental animals which were not SPF chickens that is observed; or, that the anti-peptide antibodies are recognising whole VP4, but the effect cannot be fully appreciated in fractions A to D because of the colour masking at high protein concentration; or, that the anti-peptide antibodies are recognising whole VP4, and the antigen to which they are specifically responding is mostly concentrated in fraction E. In any event, the response of the IgY anti-(free peptide) antibodies to fraction E remains a positive one, although exactly what they are binding to is not certain due to the unpurified nature of these samples and the possibility of a non-specific immune response. One would, however, hope that the antibodies

are recognising whole VP4. If this is the case, the data presented in Figure 5-6 suggests that VP4 is not significantly membrane-bound (fraction B; Table 5-1) and that it appears to concentrate in the viral pellet fraction (fraction E) as opposed to the supernatants above the sucrose cushion (fractions C and D). This location was examined to some extent by western blots and enzyme activity assays, as described in the sections following.

b). Western blots.

IBDV-infected and uninfected bursal homogenate fractions were probed as western blots using the best of the anti-VP4 peptide antibody preparations, as identified by the screening ELISAs shown in Figures 5-4 and 5-5 and as used in ELISAs against IBDV-infected bursal homogenate fractions (Figure 5-6). A silver-stained SDS-PAGE gel (Figure 5-7) illustrates the protein content of the infected and uninfected bursal fractions and provides a reference for the western blots of identical gels (Figure 5-8).

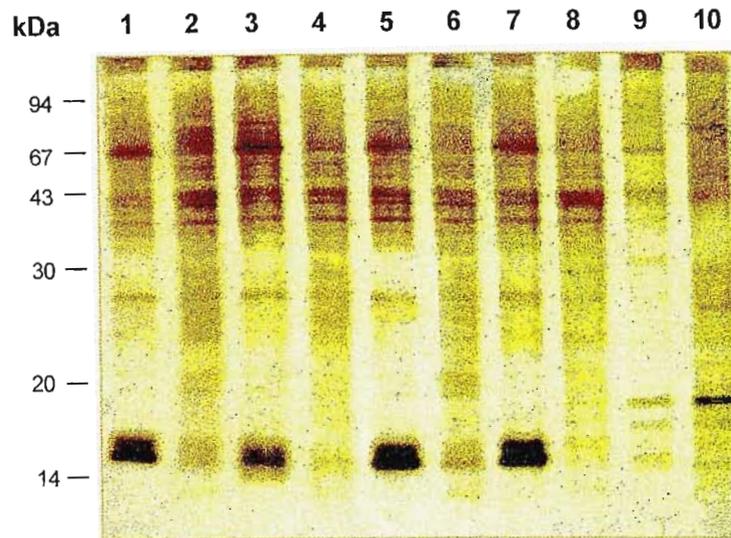


Figure 5-7. SDS-PAGE gel of IBDV-infected and uninfected bursal homogenate fractions.

IBDV-infected and uninfected bursal material was homogenised and partially fractionated by sequential centrifugation, and samples of fractions electrophoresed and silver stained as described in the text. The samples were run in adjacent lanes as an infected and uninfected pair for each fraction as follows: fraction A, (lanes 1 and 2) fraction B, (lanes 3 and 4), fraction C, (lanes 5 and 6) fraction D, (lanes 7 and 8), fraction E (lanes 9 and 10).

It is clear from Figure 5-7 that the bursal homogenate fractions A to D contain numerous identical protein species. This implies that the fractionation process employed was not a particularly effective one, and an examination of the quantity of viral dsRNA in each fraction may perhaps have been revealing in this regard, using the procedure described in Section 4.2.2. However, the fractionation was specifically tailored for the separation of virus from bursal material, and fraction E does appear reasonably different from fractions A through D, consistent with the ELISA results shown in Figure 5-6. It is also interesting to note clear differences in all fractions between the IBDV-infected and uninfected samples.

Table 5-2 details the sizes of bands appearing in the individual fractions and lists viral products of corresponding size, bearing in mind the difficulty of precise size estimation and the variety of viral protein size forms, as mentioned in Section 1.3.3.4. The identity of the low molecular weight species (~16 kDa) prominent in the infected fractions is unclear; no IBDV proteins of this size have been reported in the literature and prior investigators have made no mention of viral degradation products. Since the fractions were to be probed for proteolytic activity, protective protease inhibitors were not added to the initial homogenate and hence the general stability of proteins during the fractionation is uncertain. Numerous cellular proteases must be present in the homogenate (see Section 5.5.2) and may well have resulted in non-specific degradation, despite the low temperatures employed during the fractionation procedure. However, the distinct bands at low molecular mass suggest distinct proteins as opposed to a ladderred or smeared effect expected from non-specific proteolysis.

Table 5-2. Size and possible identification of protein bands appearing in SDS-PAGE gels of IBDV-infected and uninfected bursal homogenate fractions.

		Fraction										Potential identification
		A		B		C		D		E		
	I ^a	U ^b	I	U	I	U	I	U	I	U		
180 ^c	*		*	*	*	*	*	*	*	*	IgG; dimerised VP1?	
											110	
75					*	*	*	*			VP4 precursor polyprotein	
67	*		*	*	*	*	*	*	*		VPX+VP4?	
		55	*	*	*	*	*	*	*		serum albumin	
		47	*	*	*	*	*	*	*		VP4+VP3?	
42	*		*	*	*	*	*	*	*	*	pVP2	
40	*		*	*	*	*	*	*	*		VP2	
36	*		*	*	*	*	*	*	*			
35	*		*	*	*	*	*	*	*			
33			*		*		*				VP3	
									31		VP3	
								30		*	VP3	
27	*	*	*	*	*	*	*	*	*		VP3, VP4?	
											26	
											24	
						23	*	*			*	
		21				*						
		19				*						
									18	*		
									17		VP5	
											16.5	
16			*		*		*					
15	*		*		*	*			*			
											14	

^a 'I' denotes IBDV-infected fraction

^b 'U' denotes uninfected fraction

^c All protein molecular sizes are given in kDa and are estimations

* Denotes the same size band occurring in the marked fraction

Examining the IgY response in the western blots (Figures 5-8A and 5-8B), bands were detected at ~32 kDa in infected fractions A, B and E using antibodies produced against both free and conjugated VP4 peptide. A band was detected at ~27 kDa by chicken anti-(conjugated peptide) antibodies in all uninfected fractions. Firstly, this indicates that neither chicken produced anti-peptide antibodies that recognise denatured VP4, as no 28-kDa band is apparent. Secondly, the strong band at ~32 kDa in fraction E suggests that the IgY is in fact primed to recognise IBDV, as this corresponds to the M_r of VP3, the major IBDV antigen (Fahey *et al.*, 1985). The chickens used to raise antibodies were not SPF, had been

vaccinated against IBDV and may also have been exposed to sub-clinical levels of IBDV infection, as mentioned in Section 5.5.1.3. Obviously this is not ideal for this particular investigation and the use of non-vaccinated, SPF chickens would have been preferable. However, if this ~32 kDa band does indicate the presence of virus, the IgY is analytically useful insofar as providing an IBDV marker. In this case, besides its presence in fractions A and E, virus is also present in fraction B, perhaps implying some degree of membrane association.

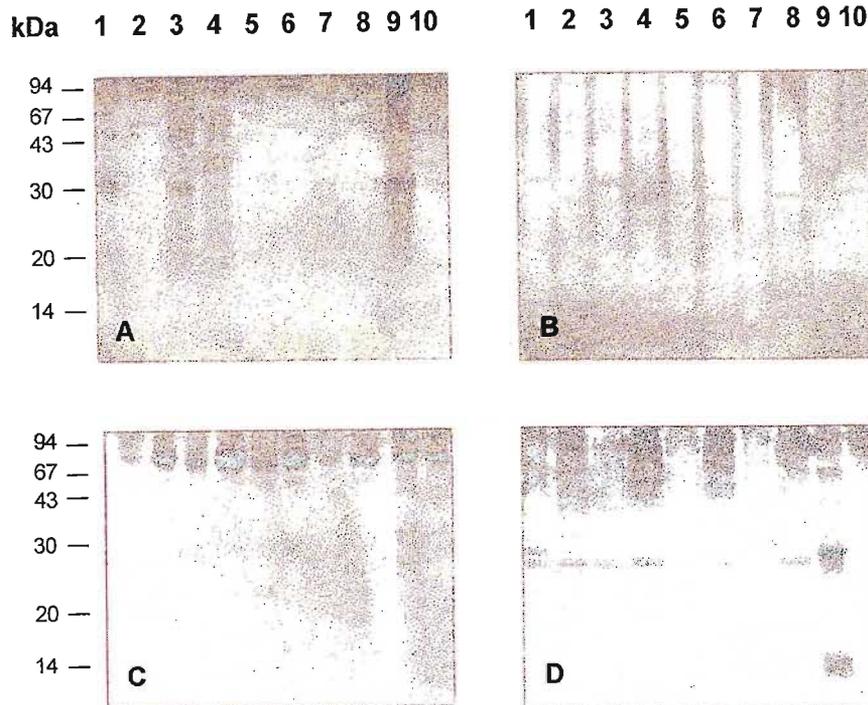


Figure 5-8. Western blots showing recognition of IBDV-infected and uninfected bursal homogenate fraction proteins by chicken and rabbit anti-VP4 peptide antibodies.

Samples of IBDV-infected and uninfected bursal homogenate fractions were boiled with NRTB before electrophoresis on four identical SDS-PAGE gels. Infected and uninfected samples of each fraction appear in adjacent pairs of lanes as for Figure 5-7. Separated proteins were electroblotted and remaining sites on the nitrocellulose blocked as detailed in the text. In four separate experiments, chicken and rabbit antibodies against free and conjugated peptide were applied ($100 \mu\text{g. ml}^{-1}$), and detected by rabbit anti-IgY-AP or goat anti-rabbit-AP secondary antibodies and BCIP/NBT substrate. Blots A and C: anti-(free peptide) antibodies from chicken F2 and rabbit F1; blots B and D: anti-(conjugated peptide) antibodies from chicken C1 and rabbit C1.

The exclusive presence of the ~27 kDa band in uninfected samples (Figure 5-8B) is not easily explained. It may be an indication of another disease altogether, in this case confined to the control chicken. Likewise, it is possible that the ~32 kDa band arises from a source unrelated

to IBDV. The lymphoid cells of the bursa are also the target cells for neoplastic transformation of the oncornavirus causing lymphoid leukosis (Cooper *et al.*, 1969).

Proteins are completely denatured after electroblotting and no renaturing process was carried out in these experiments. It is thus likely that there may be some differences between the ELISA and western blot responses. It must be noted that the colour development reactions for the western blots were allowed to proceed for an extended period and hence some non-specificity is expected.

In contrast to the negative response in ELISAs, the western blots of bursal homogenate fractions probed with rabbit antibodies show distinct evidence of binding (Figures 5-8C and 5-8D). However, in the case of the anti-free peptide antibodies (Figure 5-8C), the detection appears to be non-specific, with a band at ~75 kDa predominating in all fractions and most distinct in the uninfected fractions. The identity of this protein is unknown, as are those detected in fraction E at ~32 kDa and ~27 kDa. In this case, there is no reason why the rabbit antibodies could be detecting any viral proteins other than VP4. Rabbits are not subject to infection by any birnavirus.

The rabbit anti-conjugated peptide antibodies show the most promising results with respect to specificity (Figure 5-8D). A distinct band at ~28 kDa is detected in the infected fraction E and is also present in infected fraction A. If this is indeed VP4, this evidence would appear to contradict the understanding that VP4 is not packaged into the viral particles, as its presence is only detected here in the viral fraction and in the whole homogenate. However, it is certainly possible that its presence may have been removed from the remaining fractions by non-specific proteolytic activity. Also detected in this fraction are low molecular mass bands of ~14 and ~13.5 kDa of unknown identity and a band at ~60 kDa which may be unprocessed VP4+VP3. Interestingly, none of the bands detected in this fraction are actually visible in the corresponding silver stain, Figure 5-7. This may be an indication of the sensitivity of immunodetection techniques using high titre, highly specific antibodies. An unknown protein at ~27 kDa is again detected, in both infected and uninfected Fractions A and B and in uninfected fraction D.

Considering the western blots as a whole, it is likely that corresponding non-immune reactions would have been revealing, and in the case of the chicken antibodies, particularly so. An ideal assessment would be to use enhanced chemiluminescence for detection with anti-

peptide antibodies, which could be subsequently stripped and the blot re-probed with non-immune serum. It is likely this would confirm suspicions that the IgY is sensitised to IBDV. In this vein, it is intriguing that the prominent low molecular mass bands of ~15 and ~16 kDa, visible only in infected samples in the silver-stained gel (Figure 5-7), are not detected in any of the western blots. If the IgY does have some specificity for other IBDV proteins, this lack of detection supports the possibility that these proteins are of host origin and may be of physiological significance in IBDV infection.

5.5.2 Assaying bursal fractions for proteolytic activity

5.5.2.1 Activity in substrate gels

Although varying amounts of bursal homogenate protein were run on substrate gels (Table 5-1), areas of possible proteolytic activity were consistently indistinct and appeared as faint amorphous shapes at ~20 kDa and slightly lower, rather than distinct bands. These regions were most readily detected in fraction D, in both the infected and uninfected samples. Due to their indistinct nature, these results are not shown.

The sensitivity of a zymogram can be as good as a silver stain, detecting as little as 10 ng of active enzyme. However, these samples were only crude fractions of unconcentrated bursal homogenate and it is uncertain how much active enzyme any of them may contain among a multitude of other proteins. It must also be noted that these samples of bursal material had been stored at -70 °C for a number of years, with unknown effects on the stability of enzymes through the multi-step zymogram process. The assay conditions used were also very general and it is possible that a change in pH, ionic strength or buffer ion or the addition of cofactors such as calcium or a reducing agent are necessary for clearer results.

5.5.2.2 Activity against Cbz-Arg-Arg-AMC

Fluorogenic peptide substrates can detect less than a nanogram of enzyme, providing that the enzyme is purified and concentrated (Barrett and Kirschke, 1981). Nevertheless, it was hoped that this substrate would prove to be sufficiently sensitive to detect activity in these crude fractionations, with the fluorescence being an added advantage for the assay of such turbid preparations. Figure 5-9 illustrates the results of assays for activity against Cbz-Arg-Arg-AMC in IBDV-infected and uninfected bursal homogenate fractions.

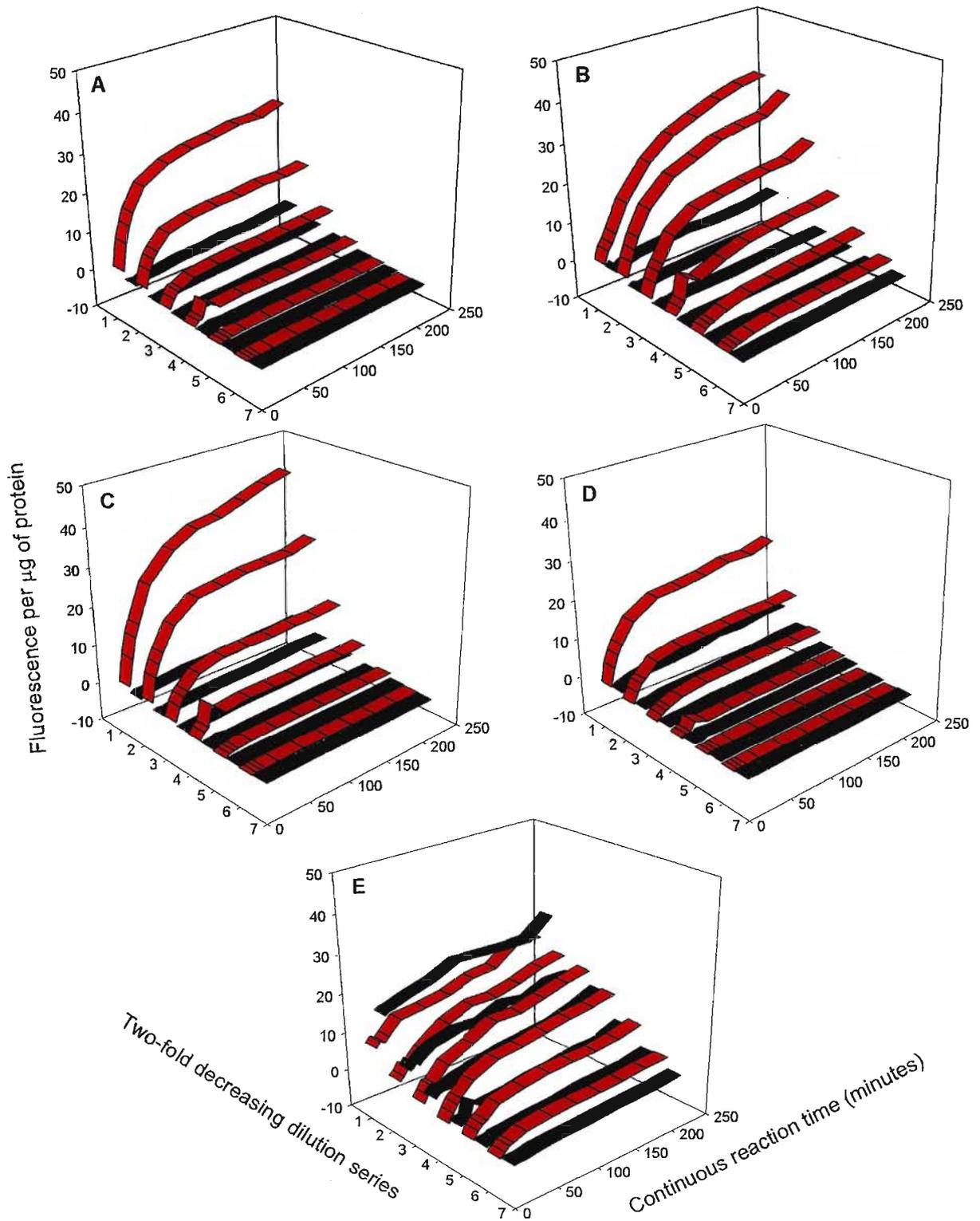


Figure 5-9. Continuous assays against Cbz-Arg-Arg-AMC conducted on a dilution series of IBDV-infected and uninfected bursal homogenate fractions.

Proteolytic cleavage of the fluorogenic peptide was assayed in IBDV-infected and uninfected bursal homogenate fractions A to E (Section 5.2), as described in Section 5.4.2. The figures are labelled with the letter of the corresponding fraction, infected samples (—), uninfected samples (—). Statistical analysis was not performed due to the preliminary nature of the experiment.

It is clear from Figure 5-9 that a titrateable enzyme activity with characteristic response over time exists against Cbz-Arg-Arg-AMC in IBDV-infected bursal homogenate fractions A, B, C and D. These curves (red) contrast with the virtually flat curves of the uninfected samples (green), indicating that this activity appears restricted to bursa infected with IBDV. Quantitatively, fractions B and C exhibited the highest amount of fluorescence per μg of protein, although this does not necessarily indicate the highest concentration of enzyme, as the effect of endogenous promoters/inhibitors is unknown. It is also highly likely that this activity is attributable to more than one enzyme. In fraction E, although there is an activity which does titrate, the form of the curves at higher concentration was uneven and the effect of dilution not as marked as for the other fractions. In addition, the uninfected samples exhibited curves of similar appearance and only drop below those of infected samples at higher dilutions. These responses may indicate the presence of more than one enzyme in fraction E with activity against the substrate, one of which is affected less by dilution than others and hence the improvement in consistent titration with dilution. Exactly which other enzymes may have concentrated with material that is allegedly a viral pellet (fraction E) is cause for speculation.

As a whole, Figure 5-9 clearly indicates a significantly elevated activity against Cbz-Arg-Arg-AMC in IBDV-infected versus uninfected fractions. However, this proteolytic response may arise from a number of sources, which are not mutually exclusive. At this stage, it is unclear whether the enzyme(s) present are IBDV proteins, a host reaction specific to IBDV infection, or a generalised reaction to infection.

The symptoms of IBDV infection pertaining to the bursa include severe oedema, necrosis and inflammation, and the virus replicates in macrophages and granulocytes as well as B-cells (Käufer and Weiss, 1976; Müller *et al.*, 1979), all of which indicate many opportunities for the release and/or up-regulation of host proteases. As detailed in Table 5-3, an array of endogenous proteases is very likely to be present in these crude fractions of IBDV-infected and uninfected bursal homogenates. Not every protease present will exhibit activity against Cbz-Arg-Arg-AMC, although many are serine proteases and would no doubt have some specificity for this substrate. The influence of the highly unrefined reaction conditions and presence/absence of endogenous inhibitors/activators cannot be gauged, but it is almost certain that host enzymes are making some contribution to the fluorescence recorded in these assays. It is perhaps relevant at this point to recall the result from western blots (Section 5.5.1.3 b) which most positively identified VP4; antibodies from rabbit C1 detected a 28-kDa

band in infected fraction E alone. This information in conjunction with the results of the fluorescent substrate assays described above may indicate that all activity against Cbz-Arg-Arg-AMC in fractions A through to D cannot be attributed to VP4, unless it is present at concentrations below the limit of western blot detection.

Table 5-3. Endogenous proteolytic enzymes which may exhibit activity against the substrate peptide Cbz-Arg-Arg-AMC.

Protease	Origin	M _r ^a (kDa)	Reference
Trypsin	mast cell granules	35, 37	Katunuma and Kido (1988)
Cathepsin B	lysosomes	24	Barrett (1973)
Plasmin	plasma	85	Wallen and Iwanaga (1968)
Kallikrein	plasma	52, 38, 33	Fujikawa <i>et al.</i> (1980)
Factor XIIa	plasma	28	Griffin and Cochrane (1976)
Factor Xa	plasma	45	Fujikawa and Davie (1976)
Thrombin	plasma	39, 28	Lundblad <i>et al.</i> (1976)

^a M_s are given as indicated by SDS-PAGE

5.5.2.3 Inhibition of activity against Cbz-Arg-Arg-AMC

Due to the fairly complex nature of these results, the effects of protease class-specific inhibitors and of chicken and rabbit anti-peptide antibodies are shown both as three-dimensional ribbon graphs and as tables of percentages. The graphs illustrate the trends and the tables demonstrate the quantitative differences between samples. The effects of these reagents on IBDV-infected and uninfected bursal homogenate fractions A to E (Section 5.2) are illustrated as follows: protease inhibitors, Table 5-4 and Figure 5-1; chicken antibodies, Table 5-5 and Figure 5-11 and rabbit antibodies, Table 5-5 and Figure 5-12.

a). Effects of protease class-specific inhibitors.

As illustrated by Figure 5-10, fractions A to D clearly show a number of consistent trends, the first of which is continued titration of enzyme activity in the presence of inhibitors in a time-dependent manner. Table 5-4 again indicates the marked increase in activity in IBDV-infected versus uninfected material, and for all four of these fractions demonstrates significant inhibition of activity by E-64 and 1,10-phenanthroline. These inhibitors were both applied as solutions in DMSO, and it must be noted that although this solvent did appear to have some

quenching effect on fluorescence, it was insufficient to account for the degree of inhibition demonstrated. Of the other two inhibitors, AEBSF appears to have some slight inhibitory affect, whereas the influence of pepstatin is negligible.

Fraction E presented somewhat differing results. Figure 5-10 reveals irregularly shaped plots over the time course for uninfected samples in particular, and illustrates higher fluorescence in uninfected versus infected samples. Considering the infected samples, Table 5-4 shows some inhibition by AEBSF comparable to that of the other fractions, while in contrast, the effect of E-64 is negligible. The effect of 1,10-phenanthroline is also reduced in comparison to fractions A through D, but appears to exhibit the most influence on the fraction E activity. Pepstatin A again has little apparent effect, and this is also the case for all the inhibitors when tested against uninfected samples.

Table 5-4. Effects of protease inhibitors on activity against Cbz-Arg-Arg-AMC in IBDV-infected and uninfected bursal homogenate fractions.

Inhibitor	Sample	% Activity per fraction of bursal homogenate ^a				
		A	B	C	D	E
No inhibitor	Infected	100	100	100	100	100
	Uninfected	30	13	16	33	126
AEBSF	Infected	58	60	69	74	71
	Uninfected	26	15	14	36	116
E-64	Infected	11	8	5	14	94
	Uninfected	27	14	11	27	118
Pepstatin A	Infected	82	84	90	98	78
	Uninfected	24	15	15	40	101
1,10-Phenanthroline	Infected	12	16	20	16	60
	Uninfected	20	10	12	33	107

^a Percentages are calculated for each sample as fluorescence per μg of protein after 30 min reaction time, relative to that of infected material without inhibitor

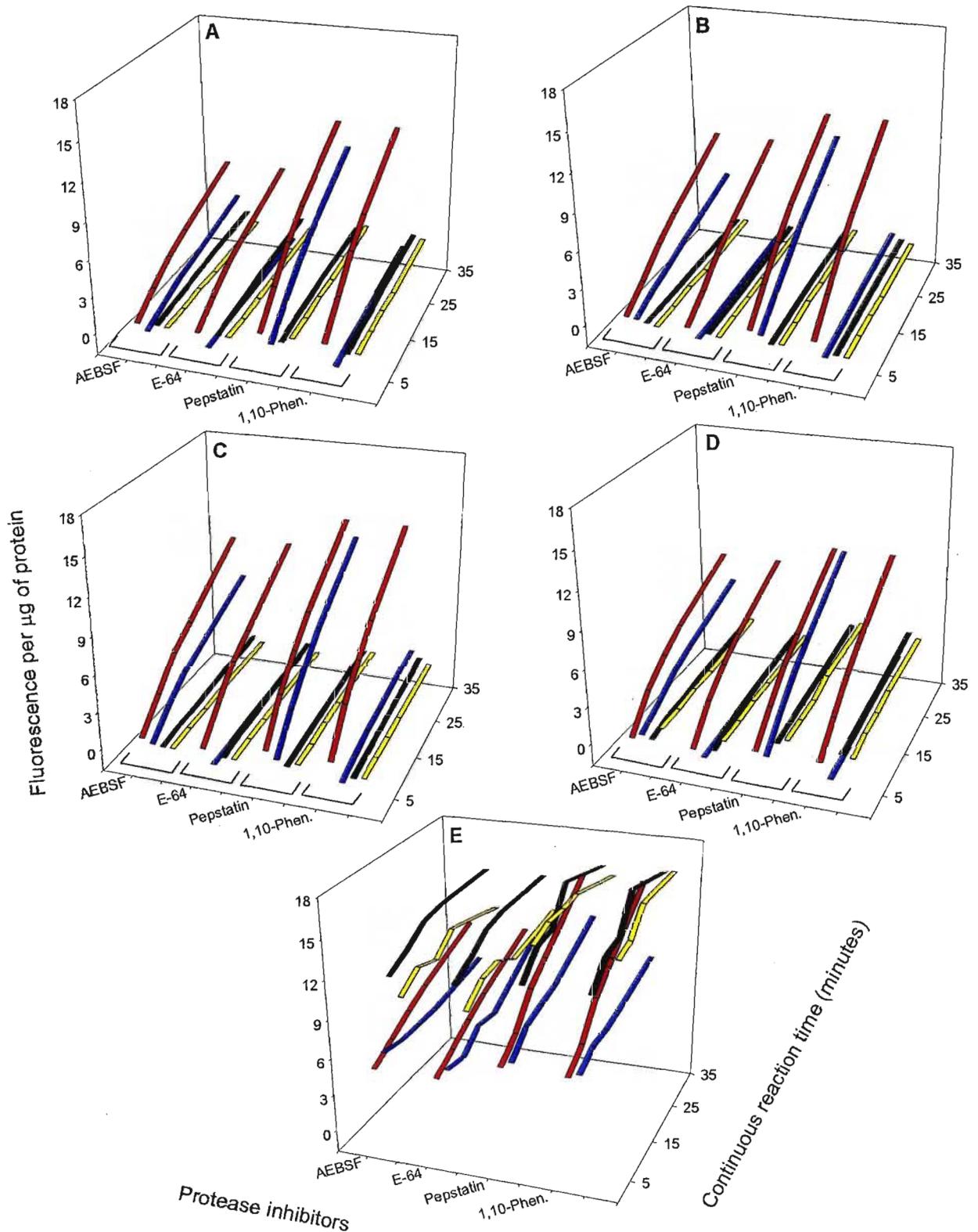


Figure 5-10. Inhibition by protease inhibitors of activity against Cbz-Arg-Arg-AMC in a dilution series of IBDV-infected and uninfected bursal homogenate fractions.

Inhibition of proteolytic cleavage of the fluorogenic peptide was assayed in IBDV-infected and uninfected bursal homogenate fractions A to E (Section 5.2) as described in Section 5.4.3. The panels are labelled with the letter of the corresponding fraction. The response over time of each fraction to AEBSF, E-64, pepstatin and 1,10-phenanthroline (1,10-Phen.) was monitored. The plots shown for each inhibitor are: infected without inhibitor (—), infected with inhibitor (—), uninfected without inhibitor (—) and uninfected with inhibitor (—). Statistical analysis was not performed due to the preliminary nature of the experiment.

As to the interpretation of these results, it is interesting that both E-64 and 1,10-phenanthroline reduced the activity in IBDV-infected samples of fractions A to D to less than that of uninfected samples. This suggests the presence of a calpain; a cysteine protease with a metal ion (Ca^{+2}) dependency (Powers and Harper, 1986). Since calpains are ubiquitous and constitutively expressed, it is likely that this activity has a bursal origin, although one would also expect appropriate amounts of the endogenous inhibitor calpastatin to already be exerting an inhibitory effect on these calpains. More importantly, small peptides are known to be poor substrates for calpain (Waxman, 1981), which questions the likelihood that the fluorogenic peptide would have been a suitable substrate. It is also most doubtful whether a calpain would cleave an Arg-Arg peptide. Cathepsin B, on the other hand, will cleave such a peptide and is also inhibited by E-64 and thus may be contributing largely to the observed activity. Chelating agents such as 1,10-phenanthroline are also known to sometimes affect serine proteases (Benyon and Salvesen, 1986), and as AEBSF also causes some inhibition, it is possible that a serine protease is making a contribution to the overall fluorescence as well. It should also be noted that although E-64 is considered a definitive cysteine protease inhibitor, it has been proved a most effective inhibitor of trypsin (Sreedharan et al., 1996). It is therefore possible that the inhibitory effect of E-64 may also have a serine protease component.

These points with respect to the presence of a serine protease are also applicable to fraction E, although the fact that no single inhibitor is able to significantly reduce the activity suggests the presence of more than one class of protease. If this is the case, it is interesting that the selected dipeptide can elicit this relatively non-specific response. The presence of a calpain is not likely in this fraction as E-64 has very little effect, although there is decreased fluorescence with the metalloprotease inhibitor, 1,10-phenanthroline. These comments pertain only to the IBDV-infected fraction E - the surprisingly high and virtually uninhibitable activity of the uninfected fraction E remains baffling and quite unexpected. The possibility remains, however, that increased amounts of protease in the infected fraction E may have increased general proteolysis, which in itself may have inactivated enzymes contributing specifically to the uninfected levels of activity against Cbz-Arg-Arg-AMC.

The evidence for multiple proteases could be examined further by the use of additional inhibitors and combinations of inhibitors. Leupeptin will inhibit both serine and cysteine proteases (Umezawa, 1976), as will peptide chloromethyl-ketones, although the selectivity of the latter can be made more discerning by choice of peptide (Salvesen and Nagase, 1989).

Other serine protease inhibitors such as di-isopropyl fluorophosphate (DFP) and phenylmethylsulfonyl fluoride (PMSF) could also be employed, although the toxicity, relatively low reaction rates and instability of DFP in aqueous solutions are limiting factors. Antithrombin III inhibits thrombin and Factor Xa and could be used to examine the contribution of the clotting cascade proteases, although it will also inhibit other trypsin-like proteases (Rosenberg and Damus, 1973). Peptide diazomethanes may also prove useful for detecting the presence of a cysteine protease (Salvesen and Nagase, 1989). The highly specific diazomethane inhibitor Cbz-Phe-Ala-CHN₂ will eliminate contributions by cathepsin B specifically (Barrett and Kirschke, 1981). The presence of a calpain could also be more closely investigated with the use of specific calpain inhibitors, the calpastatins (Murachi, 1983; Crawford et al., 1988) and by increasing the Ca⁺² concentration. In addition to these, the use of α_2 -macroglobulin (Barrett, 1981) may also help to 'distil out' activity against Cbz-Arg-Arg-AMC that could be attributable to VP4. (The putative high specificity of VP4 as an endoproteinase would hopefully allow it to escape the generalised endoproteinase inhibition of α_2 -macroglobulin).

Again, it must be noted that subjecting the crude samples of putative protease to an inhibitor series can serve only to give an indication of protease class. Extensive use of peptide substrates with purified protease and ultimately, amino acid sequencing of purified protease are needed to resolve the classification beyond doubt.

b). Effects of anti-VP4 peptide antibodies as inhibitors.

Considering trends in Figure 5-11, it is apparent that as in the ELISAs (Section 5.5.1.3), the difference between the effects of non-immune and immune IgY is insignificant for all homogenate fractions. This is further indicated by comparable percentage fluorescence in Table 5-5 for all three preparations of IgY (i.e., non-immune, anti-free VP4 peptide and anti-conjugated VP4 peptide) within each fraction. Comments made with respect to the reaction of non-immune antibodies in ELISAs are probably also applicable to this situation. However, although the effects of all three antibody preparations appear similar, there is still a marked difference between activity with and without the application of any such antibody. This is quite clear from Table 5-5. In this respect, IgY appears inhibitory for IBDV-infected samples in fractions A and D, has no effect on uninfected samples in fraction A and slightly boosts activity of these samples in fraction D. In fractions B and C, IgY is only slightly inhibitory for infected samples and appears to have no effect on uninfected samples. In fraction E, the uninfected samples again exhibit much higher activity against Cbz-Arg-Arg-AMC than the

infected samples, and the activity of both types of sample is boosted in the presence of all three IgY preparations.

Table 5-5. Effects of anti-VP4 peptide antibodies on activity against Cbz-Arg-Arg-AMC in IBDV-infected and uninfected bursal homogenate fractions.

Antibody ^a	Sample	% Activity per fraction of bursal homogenate ^b				
		A	B	C	D	E
No antibody	Infected	100	100	100	100	100
	Uninfected	41	17	19	45	181
Chicken NI	Infected	42	78	78	42	106
	Uninfected	32	22	17	43	203
Chicken α VP4 Free	Infected	37	71	72	43	124
	Uninfected	34	16	18	45	199
Chicken α VP4 Conj.	Infected	39	75	77	41	113
	Uninfected	35	15	17	46	221
Rabbit NI	Infected	115	95	95	71	395
	Uninfected	121	32	40	120	648
Rabbit α VP4 Free	Infected	75	86	90	73	411
	Uninfected	88	22	31	87	490
Rabbit α VP4 Conj.	Infected	87	101	101	70	386
	Uninfected	40	24	32	89	534

^a NI, non-immune; α , anti-; Free, free VP4 peptide; Conj., carrier-conjugated VP4 peptide

^b Percentages are calculated for each sample as fluorescence per μ g of protein after 30 min reaction time relative to that of infected material without antibody

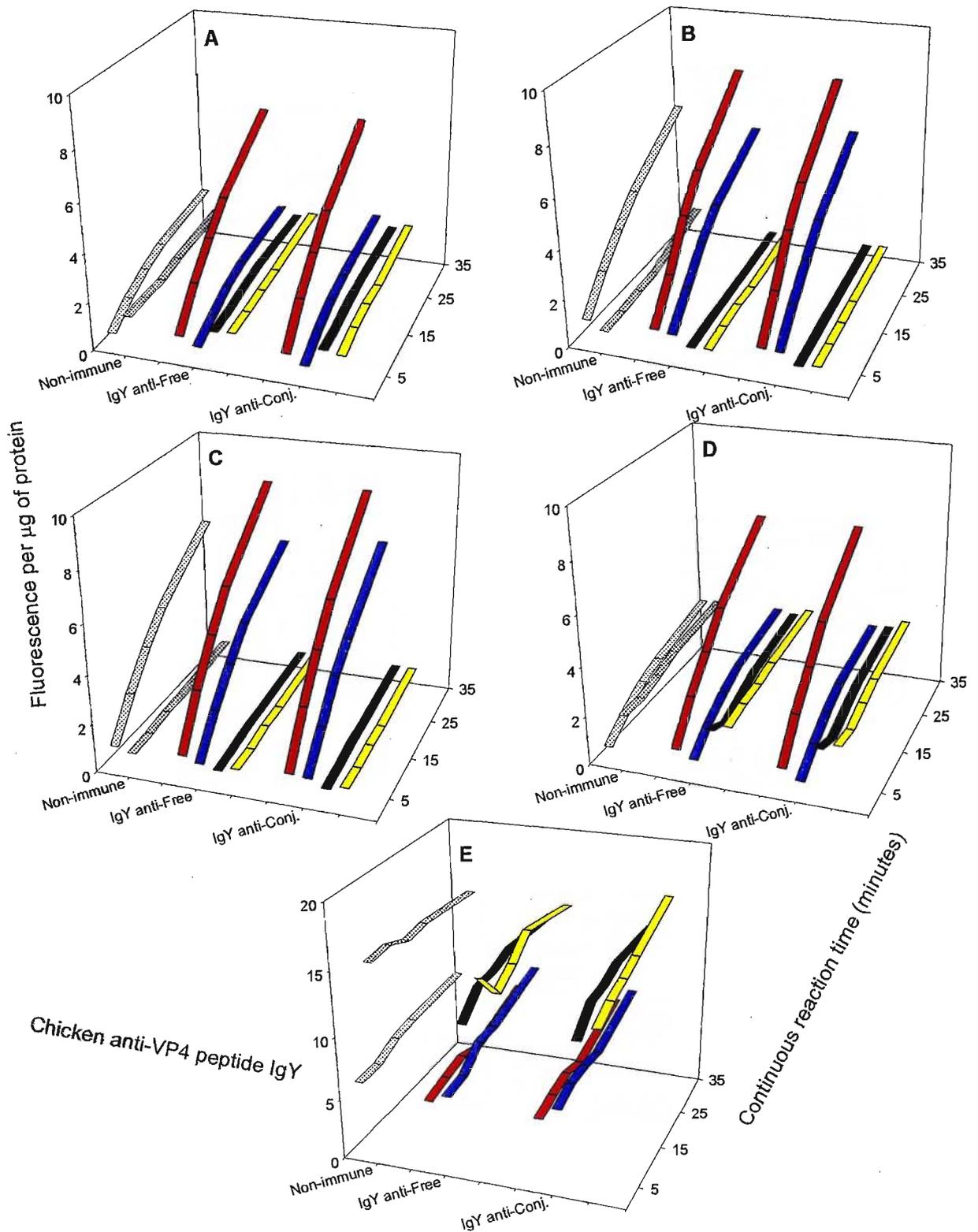


Figure 5-11. Inhibition by chicken anti-VP4 peptide antibodies of activity against Cbz-Arg-Arg-AMC in a dilution series of IBDV-infected and uninfected bursal homogenate fractions.

Inhibition of proteolytic cleavage of the fluorogenic peptide was assayed in IBDV infected and uninfected bursal homogenate fractions A through E (Section 5.2) as described in Section 5.4.3. The figures are labelled with the letter of the corresponding fraction. The response over time of each fraction to chicken non-immune, anti-free and anti-conjugated (anti-Conj.) VP4 peptide antibodies was monitored. The curves shown for each set of antibodies are: infected with non-immune antibody (light grey), uninfected with non-immune antibody (dark grey), infected without antibody (—), infected with antibody (—), uninfected without antibody (—) and uninfected with antibody (—). Note two-fold increase in range of scale of Y-axis for fraction E. Statistical analysis was not performed due to the preliminary nature of the experiment.

Similarly, there is no greatly appreciable difference between the effects of the three preparations of rabbit antibodies within each homogenate fraction, as illustrated by plots of similar shape (Figure 5-12) and comparable relative percentages reflected in Table 5-5. As with the IgY though, rabbit antibodies do have an effect on the activity of fractions against Cbz-Arg-Arg-AMC. Trends are again similar for fractions A and D and fractions B and C. Antibody has a slightly inhibitory effect against infected samples in A and D and in contrast, boosts the activity of uninfected samples. (However, the non-immune antibody boosts activity against infected fraction A, and anti-conjugate antibodies have little effect on uninfected fraction A). In fractions B and C, antibody is only slightly inhibitory for infected samples and marginally boosts that of uninfected samples. In fraction E, the uninfected samples again exhibit higher activity against Cbz-Arg-Arg-AMC than the infected samples, and the activity of both types of sample is massively boosted in the presence of all three antibody preparations, none more so that the activity of uninfected sample treated with non-immune antibodies.

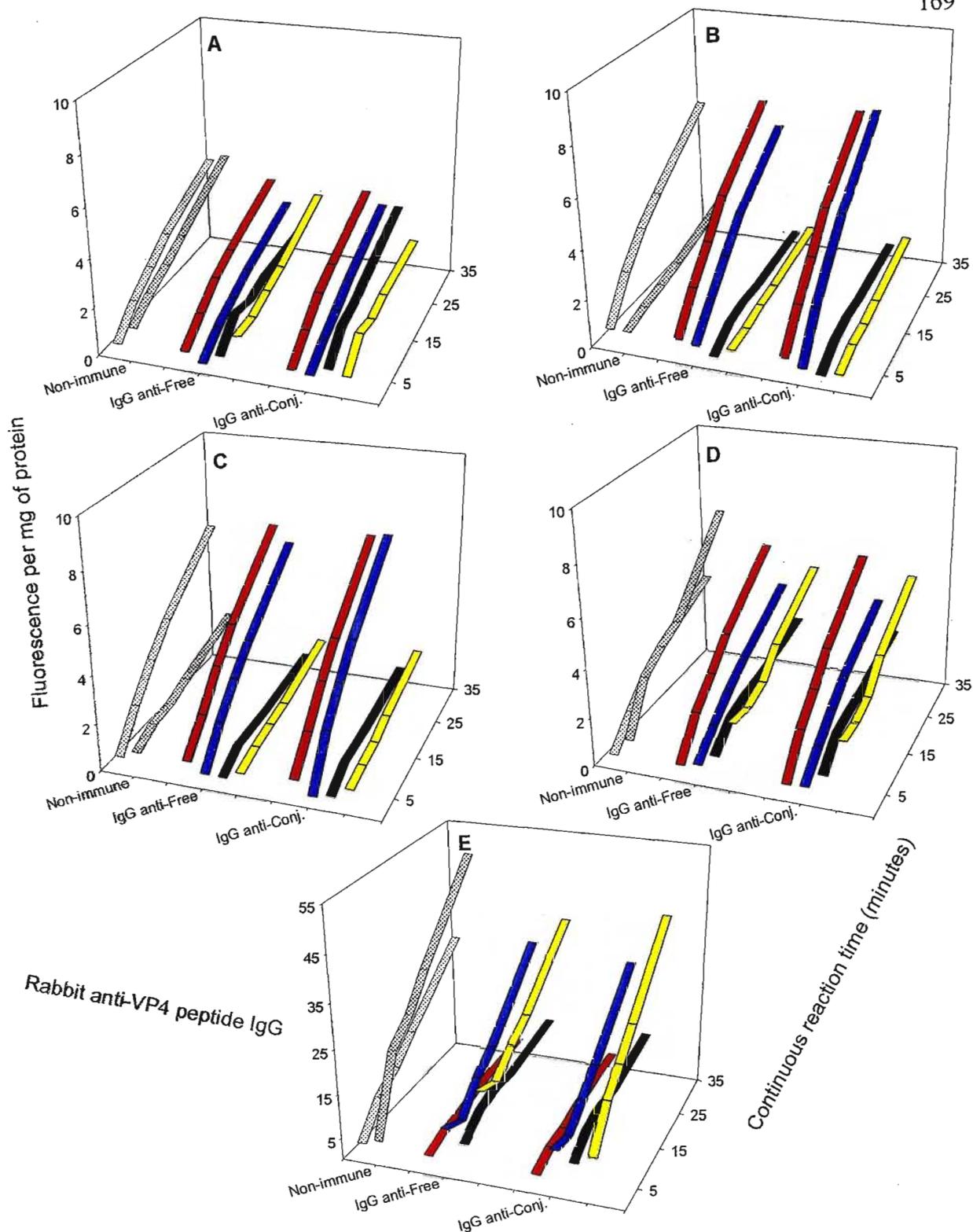


Figure 5-12. Inhibition by rabbit anti-VP4 peptide antibodies of activity against Cbz-Arg-Arg-AMC in a dilution series of IBDV-infected and uninfected bursal homogenate fractions.

Inhibition of proteolytic cleavage of the fluorogenic peptide was assayed in IBDV-infected and uninfected bursal homogenate fractions A through E (Section 5.2) as described in Section 5.4.3. The figures are labelled with the letter of the corresponding fraction. The response over time of each fraction to rabbit non-immune, anti-free and anti-conjugated (anti-Conj.) VP4 peptide antibodies was monitored. The curves shown for each set of antibodies are: infected with non-immune antibody (light grey), uninfected with non-immune antibody (dark grey), infected without antibody (—), infected with antibody (—), uninfected without antibody (—) and uninfected with antibody (—). Note five-fold increase in range of scale of Y-axis for fraction E. Statistical analysis was not performed due to the preliminary nature of the experiment.

The results of the investigations with antibodies described above are intriguing but difficult to interpret. As discussed with respect to the results of ELISAs and western blots (Section 5.5.1.3), specific identification of the proteins to which antibodies were binding is difficult. Thus, while clues should have been supplied by these preceding experiments, they are not able to shed much light on these results of the effect of antibodies on activity against Cbz-Arg-Arg-AMC. Some contradictions present themselves; ELISAs monitoring the antibody response to bursal fractions (Section 5.5.1.3) indicated a very poor response from rabbit antibodies, and yet these same antibody preparations clearly had some effect on activity against Cbz-Arg-Arg-AMC. A likely explanation is that these effects have nothing to do with VP4 and probably little to do with any manner of antibody binding at all. It is possible that factors in the antibody preparation carried over from serum are contributing to a catalytic cascade (such as that of the clotting cascade enzymes) and causing the particularly massive increases in activity seen for fraction E. Unfortunately, this explanation is not applicable to the similar effect seen with IgY in fraction E, as the possibility of contamination of these antibody preparations with serine proteases is remote. The significance of the western blot result (Section 5.5.1.3 b) which most positively identified VP4, namely the detection of a 28-kDa band by rabbit antibodies in infected fraction E alone, remains uncertain. Ultimately, in-depth speculation about the significance of the results presented in this section (5.5.2.3 b) is meaningless, although the indication is that similar experiments may well yield valuable results when employed against a purified enzyme and when the antibodies used do not contain a sub-set raised against an IBDV vaccine.

5.6 Concluding remarks

The VP4 peptide selected for the raising of anti-peptide antibodies fulfilled many of the appropriate criteria, and high titre anti-VP4 peptide antibodies recognising free peptide in an ELISA were successfully raised in both chickens and rabbits. The selected peptide ultimately elicited antibodies of similar strength in both species, irrespective of whether it was conjugated or not. Partial fractionation of bursal homogenate provided a useful starting point for obtaining the VP4 protein for subsequent analyses. However, this crude separation yields heterogeneous fractions, with apparent protein overlap between fractions which has complicated the interpretation of results. Anti-peptide antibodies raised in rabbits failed to recognise any antigen within any of these fractions in ELISAs, but did detect a band corresponding to the M_r of VP4 in western blots of infected fractions A and E. Antibodies raised in chickens had a positive ELISA response to fraction E in particular, and detected certain bands in western blots. However, non-specific immune responses are a distinct

possibility as the chickens were not SPF and had previously been immunised against IBDV. Better use should also have been made of non-immune reactions in western blots and these issues should be addressed in any further investigations. Thorough and meaningful assessment of these antibodies is naturally difficult without purified VP4.

Zymograms conducted on these fractions were unsuccessful, the fractions were perhaps too crude and the samples too old and it is not known whether the addition of factors such as calcium ions or reducing agents may have allowed the detection of proteolytic enzymes, which were certainly present as evidenced by activity against Cbz-Arg-Arg-AMC. A distinct and titrateable activity was detected in infected fractions, which was inhibitable by synthetic protease inhibitors and was affected by the application of anti-peptide antibodies. However, these results are far from significant and are more useful as starting points for a more rigorous investigation using purified VP4, either from virus-infected material or prepared as a recombinant enzyme.

Chapter 6

General Discussion

Infectious bursal disease has been a considerable problem within the global poultry industry for decades, and the recent waves of antigenic variant and hypervirulent forms of the causative virus have been the source of significant losses. The disease is highly contagious and is characterised by destruction of the lymphoid organs, most specifically the bursa of Fabricius, the site of B-cell maturation and differentiation. Direct losses are linked to specific mortality, but the indirect impact of infection is substantial, due to growth retardation and condemnation of carcasses and secondary infections caused by virus-induced immunosuppression. This study has been focused on a virally encoded protein, VP4, which is a putative protease. Viral proteases play a crucial role in the replication of many of viruses, and thus VP4 was an attractive target for investigation. Cloning and expression of recombinant VP4 and using VP4 as a marker in nucleic acid-based and immunological assays for rapid and effective detection of IBDV were objectives of this study, along with the assessment of the relatedness of KwaZulu-Natal IBDV to global strains and examination of the enzymatic activity of VP4.

As summarised in Table 6-1, many of these objectives were achieved, accompanied by a variety of useful technical outcomes. Despite concerted efforts, VP4 was neither cloned nor expressed and thus could not be subjected to the intended characterisation. The subsequent search for VP4 within infected bursal material was very preliminary, as a consequence of time restraints, and the putative protease remains elusive. However, much important groundwork has been laid and effective strategies have been developed to facilitate the eventual cloning and expression of VP4.

Table 6-1. Summary of practical outcomes of experimental work.

Feature	Section
Increased sensitivity to EtBr in agarose gels with decreased dye concentration	2.6.3
Adapted procedure for purification of viral dsRNA from infected bursae	4.6.1
Designed primers for RT-PCR of VP4	4.4.1
Performed DIG labelling of 1500 bp fragment of IBDV ORF A1	4.3.3
Optimised RT-PCR which works on local strain dsRNA without use of CH ₃ HgOH	2.8
Established strategy for cloning and expression of VP4	4.4
Performed RFLP and phylogenetic analysis of VP4 to reveal local strain uniqueness	4.6.4
Adapted crude fractionation procedure for bursal homogenates	5.2
Selected appropriate peptide from VP4 for raising of anti-peptide antibodies	5.3.1
Produced anti-VP4 peptide antibodies in chickens and rabbits	5.5.1.2
Determined binding of antibodies in infected samples using ELISAs, western blots	5.5.1.3
Examined activity against Cbz-Arg-Arg-AMC in infected versus uninfected samples	5.5.2.2
Examined inhibition of activity against Cbz-Arg-Arg-AMC by synthetic inhibitors	5.5.2.3
Observed effects of anti-VP4 peptide antibodies on cleavage of Cbz-Arg-Arg-AMC	5.5.2.3

6.1 Significance and implications of results

The most important of the achievements summarised in Table 6-1, namely, the development of an effective RT-PCR procedure, the RFLP and phylogenetic analysis, and the raising of specific anti-peptide antibodies are further discussed below.

6.1.1 RT-PCR of IBDV VP4

Before the development of an RT-PCR procedure was possible, the virus and its dsRNA had to be purified from frozen samples of infected bursae. An adaptation of two previously published methods (Azad *et al.*, 1985; Vakharia *et al.*, 1992) provided sufficient good quality material for subsequent investigation (Section 4.6.1).

The RT-PCR procedure (Section 2.8) works well and effectively detects 2.5 ng of purified viral dsRNA, without artefacts and without having to use the highly toxic denaturant CH₃HgOH. No PCR product was detected when using uninfected bursa as a negative control. The limits of detection were not determined and it remains to be seen how sensitive this particular RT-PCR can be made, however, it certainly has potential as a specific and effective means of detecting IBDV. In a related vein, one of the most useful features of the PCR is its ability to 'fish out' the target from unrefined samples. Davis and Boyle (1990) refined their

RT-PCR process to eliminate purification steps required to process impure bursal or faecal samples, from initial tissue handling through to final PCR product, by a rapid cell lysis and capsid digestion process. It would be most useful if the RT-PCR described in this study could be similarly optimised to provide rapid results from easily obtainable samples, such as fowl litter, for example.

With respect to specificity, the chicken is susceptible to a wide variety of infectious agents and a potential diagnostic method should be able to discriminate clearly among them. Wu *et al.* (1992) described an RT-PCR detecting IBDV with great specificity and sensitivity (2 femtograms of dsRNA), in which the primers used were also directed against VP4. Subjected to their procedure, the genomic nucleic acids of turkey haemorrhagic enteritis virus, infectious bronchitis virus, reovirus, *Salmonella enteritidis* and *E. coli* all failed to produce PCR products. The RT-PCR described in this study should be tested for similar specificity, possibly including avian lymphoid leukosis virus, Newcastle disease virus and *Haemophilus paragallinarum*, the former for its bursal location and the latter two for their relevance to KwaZulu-Natal. For most viral detection systems, the optimum sites for PCR primers are within sequences unique to the viral genome, which are also conserved among all isolates or strains of the virus. VP4 is highly conserved among IBDV strains, which is perhaps also an indication of its functional importance.

In terms of the failure to clone the RT-PCR product and express recombinant VP4, disappointment and experimental restriction accompanied the inability to achieve gel purification rapidly with high yield, despite the claims of assorted kit manufacturers. The difficulty experienced attempting to generate microgram quantities of purified cDNA was also a contributing factor in not obtaining the sequence of the PCR product. Numerous colleagues working in the field of molecular biology share this frustration and all await the advent of a truly efficient gel purification system. However, it is possible that restriction and ligation reactions prior to cloning should have been attempted without the extensive gel purifications outlined. In this case, sufficient material may have been available to overcome the restriction and ligation inefficiencies.

6.1.2 RFLP and phylogenetic analysis of amplified VP4

In order to assess the position of KZN IBDV in the global picture, sequence information was obtained from the PCR-amplified VP4 cDNA and compared with that of 10 other strains of IBDV (Section 4.6.4). This involved restriction of VP4 cDNA by 20 different restriction

enzymes and computer-aided 'restriction' of the known VP4 sequences of the other strains. This data was subjected to phylogenetic analysis with the help of a bio-informatics program, and resulted in the construction of a phenogram, illustrating strain relatedness (Figure 4-15). Mastering the details of this computer program to obtain this result deserves mention as a useful outcome of this study.

The restriction analysis of the PCR-amplified VP4 cDNA ultimately revealed that the KZN strain appears to be unique and that VP4 remains unchanged from early 1989 to 1997. The RFLPs showed VP4 of the KZN strain to be most similar to a vv strain of IBDV from the United Kingdom, UK661, and they are both in turn closest to the vaccine strain PBG98. In distinguishing the KZN and UK661 from other IBDV isolates and from each other, both lack *Hind* III (at 2192 bp) and *Pvu* II sites (at 1655 bp) and UK661 has a unique *Sau*3 AI site (at 1897 bp). The possibility that the KZN strain is derived from UK661 is intriguing, and if so has ramifications for increased vigilance in the poultry industry with respect to contamination control and international travel of personnel. The similarity of UK661 and the KZN strain to PBG98, an attenuated vaccine strain, is a point of interest and concern and requires further investigation.

Changes at the protein level may or may not have occurred as a result of the disappearance of the restriction sites mentioned above, due to the degeneracy of the triplet code. The amino acid residues that could be affected are at positions 687, 688 and 508, 509 respectively. Since UK661 shares the loss of these restriction sites, its amino acid sequence (Brown and Skinner, 1996) was checked for changes at the positions given. Positions 687 and 688 are in a region of VP4 to which no particular significance has been attached, whereas positions 508 and 509 are very close to a recently proposed (Sánchez and Rodriguez, 1999) N-terminal cleavage site for VP4. However, no changes are evident, suggesting conservative substitution. Although it is not certain that the base pair changes resulting in the loss of these restriction sites in UK661 and KZN are identical, it is likely.

It must be reiterated that the possibilities suggested by the RFLP analysis await confirmation and further clarity from sequencing of the PCR product. Additionally, *Taq* polymerase has no proof reading ability and as such, the PCR procedure is not infallible. The results of the restriction analysis are, however, convincing in their consistency across the eight different samples tested.

6.1.3 Immunological and enzyme activity assays

Aiming to provide a specific immunological detection method, anti-peptide antibodies were raised in chickens and rabbits against a peptide region of VP4. This involved analysis of the VP4 amino acid sequence and selection of a suitably antigenic peptide (Section 5.3.1). It was encouraging to determine by ELISA that the selected peptide did elicit a specific antibody response in both rabbits and chickens. These antibodies were subsequently used to probe samples of crudely fractionated IBDV-infected bursal material for whole VP4 using ELISAs and western blots (Section 5.5.1.3).

As regards any of the work dealing with the IBDV-infected and uninfected bursal homogenate fractions, it must be noted that these samples had been stored at -70°C for considerable time periods, ranging from six months to eight years. The effect of storage on the ability to purify virus from these samples and on the maintenance of viral protein integrity is uncertain, but may be responsible for a number of the unusual results obtained.

Probing bursal homogenates with chicken and rabbit anti-VP4 peptide antibodies using ELISAs and western blots appeared to indicate that VP4 is mainly found in the viral pellet fraction. The results obtained using chicken anti-VP4 peptide antibodies were probably skewed because the antibodies were raised in non-SPF chickens which were immunised against IBDV and which may also have experienced subclinical IBDV infection. Nevertheless, there are clear signs of specific binding to protein by the chicken and rabbit antibodies.

This suggests that VP4 is associated with the viral particle, which is interesting considering that VP4 is thought to be autocatalytic. As such, it should have remained in the host cell cytoplasm, as appears to be indicated by its presence in the tubules described by Granzow *et al.* (1997). It is possible that these tubular structures do in fact spin down in the same fraction as the viral particles, thereby contributing to a high VP4 presence in this fraction. A small amount of VP4 is known to be associated with viral particles, although its purpose for doing so is unknown and may just be an experimental artefact. However, molecular VP4 present in the cytoplasm of infected cells may have been long destroyed by cellular proteases and further immunocytochemical investigations would possibly help to illuminate the location of VP4 during infection. Naturally, it is difficult to assess the quality of the anti-VP4 peptide antibodies within such crude samples and without testing them with purified VP4.

All that can be stated with certainty with respect to the activity of bursal homogenate fractions against the synthetic peptide Cbz-Arg-Arg-AMC is that it was significantly higher in IBDV-infected versus uninfected bursal homogenate fractions. As discussed in Chapter 5, it is impossible to tell at this stage to what such activity is attributable. Further investigations of this nature using purified or recombinant VP4 and a battery of such peptide substrates would certainly prove most revealing.

6.2 Suggestions for ongoing investigation

This study has formed a solid foundation for the detailed investigation of VP4 and much useful information may be obtained by the expansion of these findings. Some possible directions for continued investigation are outlined below, and may lead to the development of locally relevant diagnostic techniques and vaccines, as well as new therapeutic agents.

6.2.1 RT-PCR and cloning of IBDV VP4

Establishing the sensitivity of the RT-PCR procedure in crude samples and determination of its discriminatory power by testing against other chicken pathogens have been identified as important goals. The rapid and effective method of Davis and Boyle (1990) and the virus-neutralising technique of Jackwood *et al.* (1996) could be most usefully employed as practical diagnostic procedures, and recent improvements in the amplification and cloning of the entire coding regions of segments A and B (Akin *et al.*, 1999) should be employed for examination of full-length polyprotein as was initially planned for this project.

The RT-PCR product needs to be sequenced; this fairly simple undertaking should yield a variety of useful data, perhaps including further insight into the origins of this particular vv strain of IBDV. It is also possible that sequencing may reveal reasons why the cloning of the amplified VP4 fragment has proved difficult. Cloning and expression of VP4 remain important objectives as outlined in the introduction.

6.2.2 Immunology and VP4

The ELISAs and western blots should be repeated with anti-VP4 peptide antibodies raised in SPF chickens along with non-immune serum controls and should perhaps be further optimised. Alternatively, affinity purification of the anti-VP4 peptide antibodies against immobilised peptide would provide a pool of highly specific antibodies separate from any endogenous anti-IBDV antibodies. It would seem that more extensive fractionation of the

bursal homogenate would aid the clarity of results obtainable using these procedures. The cellular location of VP4, what components it co-localises with and its role in the time course of infection are features of IBDV infection about which little is known and yet which would seem to be vital for viral viability. Immunocytochemistry using anti-VP4 antibodies to probe infected bursal samples would no doubt provide some clarity.

It is of interest whether antibodies against VP4 or VP4 peptides confer any protection against IBDV, and a challenge situation is envisaged using SPF birds immunised with assorted peptides from VP4 to ascertain this. B-cells tend to react with natively folded whole protein, while T-cells recognise short, unfolded peptide chains. Consequently, linear peptides such as these short, synthetic regions of VP4 can prime antigen-specific T-cells, thereby enabling the host to mount an effective response on subsequent exposure to natural infection (Roitt, 1991). Although peptide stimulation of T-cells is difficult to predict, the challenge situations described above may reveal further protective effects of peptide immunisation.

As stated by Kibenge *et al.* (1988), future immunisation strategies will need to develop protection against all pathogenic field strains of IBDV and avoid setting up selection pressures that may cause new variants to emerge. They suggest that recombinant live vector vaccines offer the best potential because they give the greatest flexibility and could be engineered to produce a broad immune response with less risk of inducing antigenic variants and would be economically viable for commercial poultry production. Considering the proposed role of VP4 in viral reproduction, the prospect of a recombinant virus with compromised VP4 activity appears promising. In the light of the increasing importance of food security, the need for safe, non-chemically saturating ways of continuing to supply good sources of protein should drive investigations of this nature.

6.2.3 VP4, the putative protease

Clearly the most important issue in this regard is definitive proof of proteolytic activity associated with VP4, which in turn depends on having a purified protease to examine. Hence the importance of viable recombinant expression of VP4, although purification from infected material is surely possible. Freshly infected material and the removal of viral particles from the interface of overlaid 40% and 60% sucrose cushions after ultracentrifugation (Fahey *et al.* 1985) may be useful starting points to facilitate purification. A careful examination of protein homology searches using databases such as SwissProt may reveal common motifs with other proteases, and as such provide an angle from which to investigate VP4 activity. Ongoing

investigations of the proteolytic activity of VP4 from the other birnaviruses may also yield useful comparative information.

Characterisation of any proteolytic activity that VP4 may exhibit would greatly assist the understanding of its mode of action and role in the infective process. Insights are sought with regard to the location of VP4 within infected tissue, the status of its activity during the virus life cycle and the nature of its action *in vivo*. It is of interest to determine if any VP4 activity exists to affect host proteins, for example, the numerous peptide hormones that contain a double arginine site. Viral proteases are a topic generating much research interest as a result of their significance to viral viability, and furthering the understanding of these enzymes could lead to better ways to control viral infection.

In a mammalian *in vitro* expression system, Sánchez and Rodriguez (1999) have recently shown that the ⁵¹¹LAA⁵¹³ and ⁷⁵⁴MAA⁷⁵⁶ sites are essential for the processing of the VPX-VP4 and VP4-VP3 precursors, respectively. The latter has the AxAAS motif proposed as an alternative cleavage site (Hudson et al., 1986). Sánchez and Rodriguez (1999) confirmed that the dibasic ⁴⁵²RR⁴⁵³ and ⁷²²KR⁷²³ sites are not the polyprotein processing sites, using further site-directed mutagenesis. These findings question the usefulness of the Cbz-Arg-Arg-AMC synthetic substrate in the current study, and underline the need for purification of VP4 which can subsequently be tested against a battery of peptide substrates. Alternatively, N- and C-terminal peptide sequencing of purified VP2, VP4 and VP3 may lead to clarity on the exact position of the proposed autocatalytic cleavage sites between these proteins.

6.2.4 General IBDV considerations

Much remains to be discovered about IBDV and the outbreak of a highly virulent strain in KwaZulu-Natal. It is likely that the virulence will be linked to changes in the coat proteins, as has been the case in other parts of the world. Investigation of the marked tropism of IBDV for the bursa is also an area of great interest and is being pursued in this laboratory with the hope of determining which molecules are involved in this binding. Bursectomised birds are protected from infection by IBDV and if mechanisms can be discovered to 'hide' the bursa from the virus, a similar protection may be conferred. Enzymes and proteins unique to viruses are characteristics to be exploited for control; in this regard, VP4, the RdRp and VP5 may well provide new and effective targets. With respect to the control of such a resilient

virus as IBDV, the role of wild birds, insect populations within poultry houses and biting insects such as mosquitoes in the transmission of the disease also warrants investigation.

On a final note, the crossover of pathogens from animals to humans is a reality and in all likelihood, an issue which will continue to demand attention in years to come. As recently as the end of 1997, a pathogenic strain of chicken influenza, H5N1, killed six and infected 18 people in Hong Kong. As a preventive measure, all poultry in affected areas which had not already succumbed to the disease were slaughtered - over a million chickens and 400 000 other birds. Understanding emerging diseases and the mechanisms of increases in virility is an important consideration in an increasingly intermingling global population needing to be sustained by increasingly intensive food production methods.

6.3 Concluding remarks

Marked increases in local virulence of IBDV are a global phenomenon and have resulted in significant losses to the poultry industry. As part of an attempt to understand this occurrence in KwaZulu-Natal, South Africa, the efforts of this study have focused on VP4, the putative IBDV protease. This work has further demonstrated the use of both PCR and RFLP analysis in the detection and identification of IBDV within infected bursal material. Specifically, RFLP analysis of VP4 revealed unique variations in the KZN strain and suggests a link with the UK very virulent strain, UK661 and the British vaccine strain PBG98. However, these variations were consistent within amplified DNA from 1989 to 1997 and thus offer no clues about the transition to increased virulence, nor the subsequent reversion to a controllable strain. Anti-VP4 peptide antibodies have potential promise, but require further investigation, as does the presence of an Arg-Arg specific proteolytic activity in infected bursae. The difficulty encountered in the interpretation of results from immunological and enzymatic examination of these crude protein fractions of bursa underlines the usefulness of the dsRNA detection and PCR for diagnostics and identification of IBDV. This study represents an encouraging initial investigation of the KZN strain of IBDV, which should be pursued further in the interests of locally relevant diagnostics, vaccines and control of a commercially important pathogen.

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