THE PRODUCTION OF ANTIBODIES TO COUMARIN AND ITS MAJOR HUMAN METABOLITES

A thesis submitted for the degree of Ph.D.

by

Anthony Killard B.A. (Mod).

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School of Biological Sciences

Dublin City University

Dublin 9

Ireland

Under the supervision of

Prof. Richard O'Kennedy

To Mum, Dad, Jackie and Kieran.

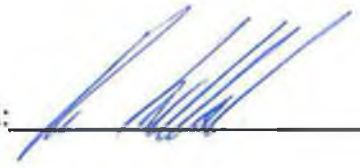
"An expert is a man who has made all the mistakes which can be made in a very narrow field."

Niels Henrik Bohr

"I think I must be an expert by now."

Anthony J. Killard

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D. is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my own work.

Signed: 

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PUBLICATIONS AND PRESENTATIONS

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ABSTRACT

Two strategies were applied to the production of antibodies to coumarin and its phase I metabolite, 7-hydroxycoumarin. One system was the production of Fab antibody fragments using the combinatorial phage display library, employing the phagemid vector, pComb3. Although a library of murine heavy and light chain genes was established following immunisations with coumarin and 7-hydroxycoumarin conjugates, preparation of the library in pComb3 was not possible due to associated problems with this vector.

An alternative antibody library system was obtained. This was the naive semi-synthetic human scFv library of Nissim *et al.* (1994). The production of antibodies to both coumarin and 7-hydroxycoumarin was investigated. Various production and isolation techniques were explored. Best results were obtained by isolating correctly folded scFv protein from the periplasm or culture supernatants of *E. coli*. In the case of coumarin, three soluble antibody clones were isolated and characterised. These clones showed the characteristics of poor affinity for free coumarin, but did bind well to coumarin-protein conjugates. Isolated anti-7-hydroxycoumarin clones also showed the same characteristics of no binding to free drug. It was theorised that, due to the structural simplicity of these molecules, affinity selection and screening is biased against the detection of clones with high affinity for free drug. This conclusion was in broad agreement with other findings (Danilova, 1994). Recommendations are made as to how the selection and screening procedures can be improved to isolate antibodies to such molecules.

Techniques for the *in vitro* production of the phase II metabolite 7-hydroxycoumarin-glucuronide were developed. These were accompanied by the development of analytical methods (capillary electrophoresis and HPLC) for the glucuronide. Purification methodologies using 7-hydroxycoumarin present in these metabolic mixtures and in urine were also developed. The coupling of 7-hydroxycoumarin-glucuronide to proteins for the production of antibodies was also explored. A 7-hydroxycoumarin-glucuronide-bovine serum albumin conjugate was successfully synthesised for use in further antibody production strategies.

ABBREVIATIONS AND SYMBOLS

α	Alpha - denoting anti, i.e. anti-7-hydroxycoumarin (α-7-hydroxycoumarin)
Ab	Antibody
Ag	Antigen
BSA	Bovine serum albumin
bp	base pairs
Cb	Carbenicillin
cDNA	copy (complementary) DNA
CDR	Complementarity determining region
CE	Capillary electrophoresis
CEA	Carcinoembryonic antigen
cfu	colony forming units
CIP	Calf intestinal phosphatase
CMV	Cytomegalovirus
DCM	Dichloromethane
DEAE	Diethyl-aminoethyl
DEPC	Diethyl-pyrocarbonate
DMSO	Dimethyl sulphoxide
dNTP	deoxynucleotidyl triphosphates
dsFv	disulphide Fv
DTT	Dithiothreitol
EBV	Epstein-Barr virus
EDC	1-ethyl-3(3-dimethylaminopropyl)carbodiimide
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
Fab	Antigen binding fragment
FCA	Freund's Complete Adjuvant
Fv	Variable fragment
Gdn	Guanidine
HAMA	Human anti-mouse antibody
HEL	Hen egg lysozyme
HEV	High endothelial venule
hGH	human growth hormone
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HSV	Herpes simplex virus
IFN	Interferon
IG	Intergenic region
Ig	Immunoglobulin
IPTG	Isopropyl-β-D-thiogalactopyranoside
IR	Infrared
ITAM	Immunoreceptor tyrosine-based activation motif
KLH	Keyhole limpet haemocyanin
Kn	Kanamycin
LOD	Limit of detection

LPS	Lipopolysaccharide
MeOH	Methanol
MHC	Major histocompatibility complex
mIg	membrane Ig
mRNA	messenger RNA
NHS	N-hydroxysuccinimide
OD	Optical density
7OHC	7-hydroxycoumarin
7OHCG	7-hydroxycoumarin-glucuronide
OPDA	*o*-phenylene diamine
Φ	Phi - denoting attachment of antibody to phage
PAGE	Polyacrylamide gel electrophoresis
PBL	Peripheral blood lymphocyte
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDA	Photodiode array
pfu	plaque forming unit
PMSF	Phenyl methyl sulphonylfluoride
PVDF	Polyvinylidene difluoride
RSV	Respiratory syncitial virus
RT	Room temperature
RU	Response unit
scFv	Single chain Fv
SDS	Sodium dodecyl sulphate
SPR	Surface plasmon resonance
Tc	Tetracycline
TCR	T cell receptor
tfu	titre forming unit
TGF	T cell growth factor
THG	Thyroglobulin
TLC	Thin layer chromatography
TNF	Tumour necrosis factor
Tris	Tris(hydroxymethyl)aminomethane
UDP	Uridine diphosphate
UDPGT	UDP-glucuronyl transferase
VSV	Varicella Zoster virus
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

1. INTRODUCTION

1.1 THE IMMUNE SYSTEM

The mammalian immune system is composed of a group of organs distributed throughout the organism called the lymphoid system. These structures give rise to, and harbour specialised cells which produce polypeptide signals called cytokines that control the immune response. These cells, associated proteins and cytokines orchestrate the surveillance, detection and removal of all foreign materials from the organism (Kuby, 1997; Roitt *et al.*, 1998).

1.1.1 The Lymphoid system

The lymphoid system is a collection of organs scattered throughout the body. The primary lymphoid organs comprise the bone marrow and the thymus. Bone marrow is the initial source of pluripotent haemopoietic stem cells which differentiate into lymphoid or myeloid progenitor cells. Lymphoid cells that mature in the bone marrow are called B cells. These develop the property of antibody production. Other lymphoid cells migrate to the thymus gland where they develop into various classes of T cell which possess the ability to recognise foreign antigenic epitopes via a surface immunoglobulin structure known as the T cell receptor (TCR). These cells are considered part of the adaptive immune response because they selectively recognise foreign structures in the body. Other cells of the immune system which develop from myeloid precursors go on to form phagocytic cells such as monocytes and macrophages as well as lytic cells such as neutrophils, basophils and eosinophils. These have no ability to differentiate between self and non-self, but respond non-specifically to damage and injury. These, in conjunction with soluble immune components such as complement and interferons comprise the innate immune system.

Many organs possess a secondary lymphoid function. The most important organ in this regard is the spleen. Also important are the lymph nodes which are scattered along the median line of the body and are connected to each other and the spleen via a network of lymphatic vessels. Although differing in structure, both organs act as sites at which T and B cells can interact with one another at high concentrations to bring about effective T

cell-dependent B cell activation. Other important secondary lymphoid organs are the mucosa-associated lymphoid tissues (MALT) present in the gastro-intestinal, urinogenital and respiratory tracts and the skin-associated lymphoid tissues (SALT). These all show similar structures as those found in the spleen and lymph nodes comprising areas of high B and T cell density. Lymphoid cells can circulate freely between the cardiovascular system and the lymphatic system either through the lymphoid organs themselves or across high endothelial venules (HEV).

1.1.2 The humoral immune response

Immune responses are normally initiated at a site of damage or injury, as a result of skin abrasions, for example, or tissue necrosis brought about by microbial infection. Polymorphonuclear cells are attracted to the site of damage via chemotaxis, either via complement C5a release by damaged cells or via the alternative complement cascade whereby bacteria bring about the release of complement C3. This infiltration is rapidly followed by other phagocytic cells that digest damaged tissues, necrotic cells and other foreign components such as bacteria. This infiltration of immune cells in a localised area leads to the symptoms of inflammation: heat (calor); pain (dolor); redness (rubor); and swelling (rigor). Opsonization is triggered by attachment of bacteria to non-specific receptors or via complement C3b receptors. Opsonization can also be carried out by other antigen presenting cells such as monocytes and macrophages. Normally for an effective adaptive response, both B and T cells must become stimulated via antigen presentation. T cell-independent activation of B cells, however, can be brought about by the cross-linking of surface Ig with a polyvalent antigen, or via a mitogenic antigen such as lipopolysaccharide (LPS), which cross-links surface Ig with a B cell mitogen receptor. The response brought about by T cell-independent antigens is poorer than T cell-dependent responses, resulting in limited class switching and affinity maturation, yielding predominantly low affinity IgM responses. Antigen presentation primarily takes place in the secondary lymphoid tissues, whether in the skin via Langerhan's cells or dendritic cells, in Peyer's patches in the gastrointestinal mucosa and other mucosal surfaces, or via circulating monocytes in the lymph nodes or spleen. These macrophages, or naive B cells - which recognise antigen via surface expressed mIgM and IgD - endocytose antigen.

Macrophages are attracted to the site of damage or infection by chemotactic factors such as complement components, blood clotting factors and cytokines. Large organisms and dead cells adhere to the surface of the macrophages and are phagocytosed. Naive B cells endocytose in response to the attachment of antigen to surface immunoglobulins. Once internalised, the process of phagocytosis takes place. Here, highly hydrolytic enzymes, peroxidases and lysozyme are secreted into the phagolysosome contaning the endocytosed antigen. Most of the antigen is then expelled from the cell by exocytosis. Some parts of the antigen such as short peptides are presented at the cell surface in association with class II MHC (Fig. 1.1). Antigen cross-linking of mIg on naive B cells also results in the first of the competence signals which drives the naive B cell from resting G_0 into G_1. Signal transduction is facilitated by the B cell receptor complex comprising mIg and two signal transducing Ig-α/Ig-β heterodimers. Immunoreceptor tyrosine-based activation motifs (ITAMs) present on the cytoplasmic side of Ig-α and Ig-β interact with members of the Src family of tyrosine kinases and Syk kinase, activating them. These phosphorylate tyrosine residues on the cytoplasmic tails of Ig-α/Ig-β, activating three pathways that bring about transcriptional modifications.

Although antigen presenting cells are involved in activation of T_H cells, B cells recognise and internalise antigen specifically and present antigen at concentrations of hundreds to thousands of times lower than other antigen presenting cells, and so are probably more important at lower antigen concentrations. The other cell types principally involved in antigen processing and presentation are dendritic cells and macrophages. All three cell types express MHC class II, but dendritic cells and B cells do so constitutively. Macrophages are activated by the phagocytosis of large antigens such as microorganisms, before MHC class II expression is induced.

T_H cells recognise antigen in the presence of the B cell or macrophage in association with MHC class II via their T cell receptor complex. When this occurs, the T and B cell form a complex. This causes the expression of CD40L on the surface of the activated T_H cell. CD40L interacts with the B cell surface glycoprotein CD40, which triggers the second B cell competence signal, which in turn triggers the B cell to express receptors for various cytokines. T_H cells also undergo internal rearrangements which result in the targeted

secretion of cytokines towards the B cell. T_H cells produce IL-2, IL-4 and IL-5 which drive the B cells into S phase, mitosis and B cell proliferation. These activated B cells are referred to as centroblasts. T_H cells form two distinct populations based on their cytokine secretion characteristics and are referred to as T_H1 and T_H2. T_H2 cells act most effectively as helper cells in B cell activation. T_H1 cells are primarily responsible for the activation of cytotoxic T cells.

Activated centroblasts now undergo a process of affinity maturation which involves somatic hypermutation and high affinity selection. Somatic mutation involves the introduction of point mutations, deletions and insertions in the rearranged VHJ segments of genes, most of which occur in the CDR regions. This mutation rate is estimated at 1 in 1000 base pairs per division and is 10^6 fold greater than normal mutation rates. This occurs late in the primary response. This mutation rate will bring about a single mutation in each antibody clone. Most mutations will result in decreased affinities. The few that are of higher affinity are positively selected by binding to antigen complexes on the surface of follicular dendritic cells. This interaction brings about a signal which induces up regulation of Bcl-2, so preventing apoptosis. Those that have decreased affinity and do not bind to antigen on follicular dendritic cells undergo apoptosis.

Many mutations are required to bring about the large increases in affinity seen in a mature immune response. Following a period of cellular rest, the centrocyte undergoes cycles of proliferation and mutation, followed by further rounds of positive selection via antigen binding on follicular dendritic cells.

Another important part of the differentiation of B cells is class switching. These events are also T_H cell-dependent and require the competence signals triggered by CD40/CD40L interactions, as well as cytokine induction. The proliferating, high affinity centrocyte will be triggered to form different isotypes as a result of the type of cytokine signalling it receives, which is, in turn, dictated by its local environment. B cells maturing in MALT will form IgA antibodies under the influence of TGF-β. B cells from non-mucosal lymphoid tissues such as the spleen, tonsils and lymph nodes will produce IgG subtypes

under the influence of IFN-γ or IL-4. Exposure to these various cytokines make certain switch sites on the C_H domains of genes accessible to recombinase enzymes.

High level soluble antibody production in plasma cells is triggered by IL-1 and CD23 secretion from the follicular dendritic cell. CD23 binds to CR2 on the plasma cell and switches off production of mIg with up-regulation of soluble antibody production. For memory B cell production, the centrocyte internalizes antigen and displays it in association with MHC class II to T_H cells, resulting in CD40/CD40L interactions necessary for memory cell formation. This high affinity antibody producing, fully differentiated cell circulates for long periods and is readily induced to form antibody-producing plasma cells following subsequent rounds of antigenic challenge.

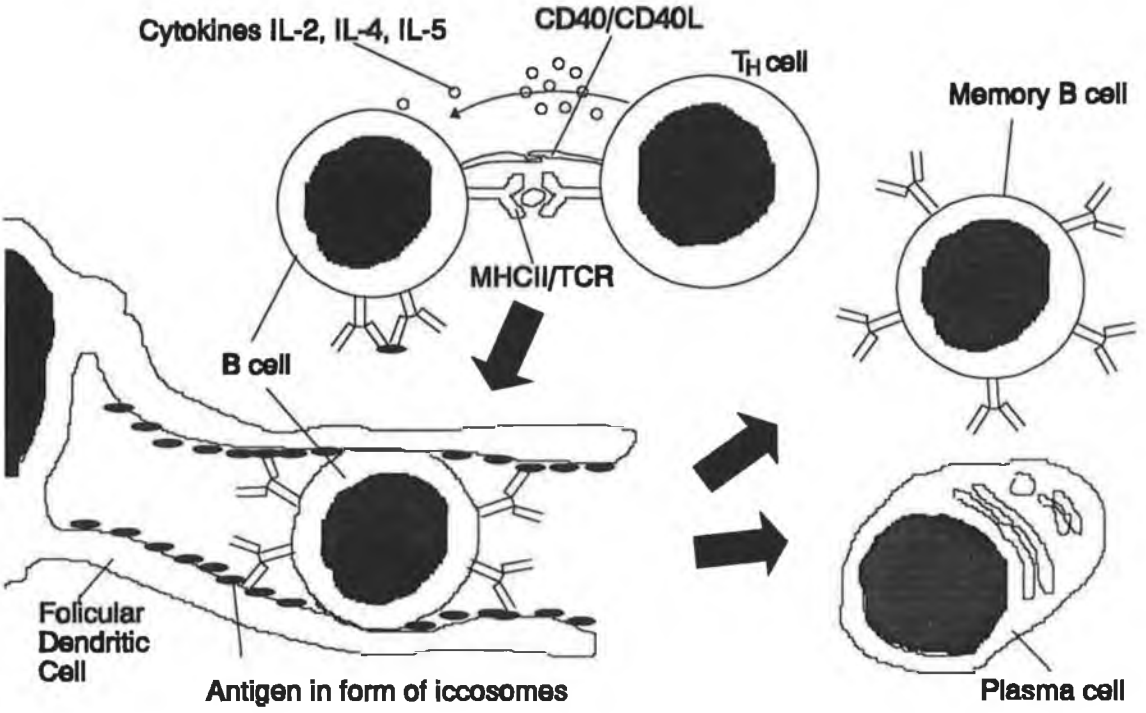


Figure 1.1. T_H cell-dependent B cell maturation in the humoral immune response. Antigen presentation via the B cell, in combination with surface cross-linking of antibodies at the B cell surface and interaction between the B and T_H cell via CD40/CD40L brings about the competence signals which trigger the B cell to enter the cell cycle. Expression of cytokine receptors by the B cell and production of cytokines by the T_H cell drives the proliferation of the B cell. The activated B cell (centroblast) producing antibody of good affinity interacts with antigen released from the surface of follicular dendritic cells in the form of immune complexes called iccosomes. Cytokine signals from the follicular dendritic cell drives the B cell into rapid division and somatic mutation. High affinity variants go on to interact with follicular dendritic cells, while low affinity B cells are apoptosed. After many rounds of affinity maturation, high affinity antibody producing B cells either become antibody-producing plasma cells or circulating memory B cells.

1.2 ANTIBODY STRUCTURE

Antibody molecules have come to symbolise the immense power of the mammalian immune system. Their power comes primarily from three characteristics; specificity, affinity and their ability to recruit effector functions. These characteristics are a result of their structure and ontogeny.

The structure of antibody molecules varies depending on their isotype, but are typified by the immunoglobulin G (IgG) molecule. (Roitt *et al.*, 1998) (Fig. 1.2). The basic four polypeptide chain model was proposed by Rodney Porter (Porter *et al.*, 1966). IgG consists of two identical heavy (H) and two identical light (L) chains. The H chain is some 450 amino acids (approximately 50 kDa) and the L chain is 212 amino acids (approximately 25 kDa) in length. The two H chains are held together by interchain disulphide linkages at the hinge region. The number and arrangement of these disulphide bonds varies between classes and subclasses (Edelmann and Poulik, 1961). L and H chains are also divided into distinct structural domains possessing intrachain disulphide bonds.

Antibodies are glycoproteins. All classes are glycosylated at the C_H2 domain and additional glycosylation occurs at the C_H1, hinge region and C_H2 domains, again depending on isotype. Glycosylation may also occur in the V region. The H chain constant domains are class-specific. IgG molecules possess three C domains. IgM has an additional constant domain which, in combination with a cross-linking component, the J chain, results in cross-linking between adjacent constant domains, yielding the pentameric IgM structure. IgA has only three C domains with a distinctive c-terminal octapeptide sequence. IgA can form dimers with the presence of a secretory piece and J chain component anchoring adjacent $C_\alpha2$ and $C_\alpha3$ domains. IgE has an additional C domain which results in the oblation of the hinge region. There is a single constant L chain domain which can be of two isotypes, either κ or λ.

Isotypic variation primarily results in changes in effector function of the various antibody types. The properties of antigenic recognition, specificity and affinity are as a result of the structure of the variable (V) domains present on both the L and H chains. Denoted by

their high sequence variability, these variable domains are further subdivided into framework regions and complementarity determining regions based on levels of sequence variability. The four framework (FR) regions possess relatively low levels of sequence variation. These flank the three complementarity determining regions (CDRs) which possess high levels of sequence variability, with most variability being found in the third CDR region, CDR3. The CDRs of both L and H chain form six hypervariable loop structures on a beta sheet framework resulting in a three dimensional structure that is the antigen binding site. It is the sum total of hydrophobic and electrostatic interactions, van der Waals forces and hydrogen bonding between the amino acid side chains of the CDR regions and the antigen molecule that results in the high affinity interaction between the two.

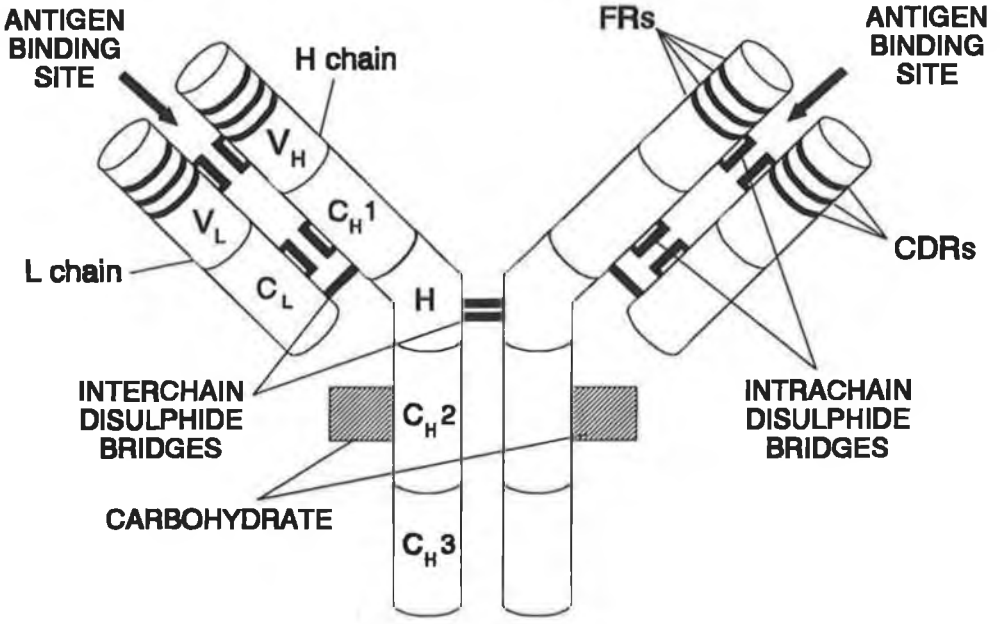


Figure 1.2. The immunoglobulin IgG1 molecule. The antibody is composed of four peptide chains: two identical light and two identical heavy chains. H chains possess five distinct domains, variable (V), three constant (C) and a hinge (H) region. Light chains possess a single V and C domain. H chains and adjacent L and H chains are held together by interchain disulphide bridges. Two of these connect H domains of IgG1. Intrachain disulphide bridges also exist in V and C regions, resulting in the folded globular structure characteristic of antibodies. Areas of high sequence variability termed complementarity determining regions (CDR) form six hypervariable loop structures on a series of sequence frameworks, known as framework regions (FR). This is known as the antigen binding site and is the site of antigen attachment to the antibody.

1.3 ANTIBODY CHAIN GENE RECOMBINATION

The variability present in the V regions of both L and H chains, and the differences in C region structure are a result of the genetic nature of antibody L and H chain germline genes (Tizard, 1995). Variable H chain regions are composed of sequences from three distinct loci, known as V, D and J regions. In L chain variable genes there are only V and J regions. The V region forms the 5' end of the variable region, the D sequence, a central region and the J region, the 3' end of the sequence. These genes are also found in this sequence on the germ line genes. V region sequences have evolved from common sequence ancestors to contribute to diversity. These V sequences have been grouped into families based on their 5' sequence similarity. Different groupings were originally proposed, based both on DNA and protein sequence data (Brodeur and Riblet, 1984; Dildrop, 1984).

Variability results primarily from the presence of multiple V, D and J region sequences on the germline gene, of which there are several hundred V_H, twelve D_H and four J_H segments. It is the recombination that occurs between these three regions that contributes further to variability. As a consequence of B cell maturation, recombination of VDJ (H chain) and VJ (L chain) sequences takes place. For a D region to become adjacent to a V region, an intervening sequence of other V and D regions is removed. This is done by a process of looping out. Here, intervening heptameric and nonameric complementary sequences form stem-loop structures and are removed (Fig. 1.3). This recombination is essentially random, with any V region recombining with any D and J region. The resulting VDJ or VJ sequence is now functional at the transcriptional level. Further diversity results from base shifts during recombination, somatic mutation and assortment between different H and L chain genes. Not surprisingly, the interface between V and D genes results in the CDR3 region in the V_H chain as a result of these factors contributing to diversity. Antibody class is also determined by recombination as a result of B cell ontogeny. Constant H region genes are found in sequence downstream from the variable region genes. These are in a specific order; μ, δ, γ, ε, and α. At the 5' end of each of these sequences, except δ, there is a switching sequence. In response to B cell maturation, C region genes are looped out at these switching sequences. IgM- and IgD-producing B cells

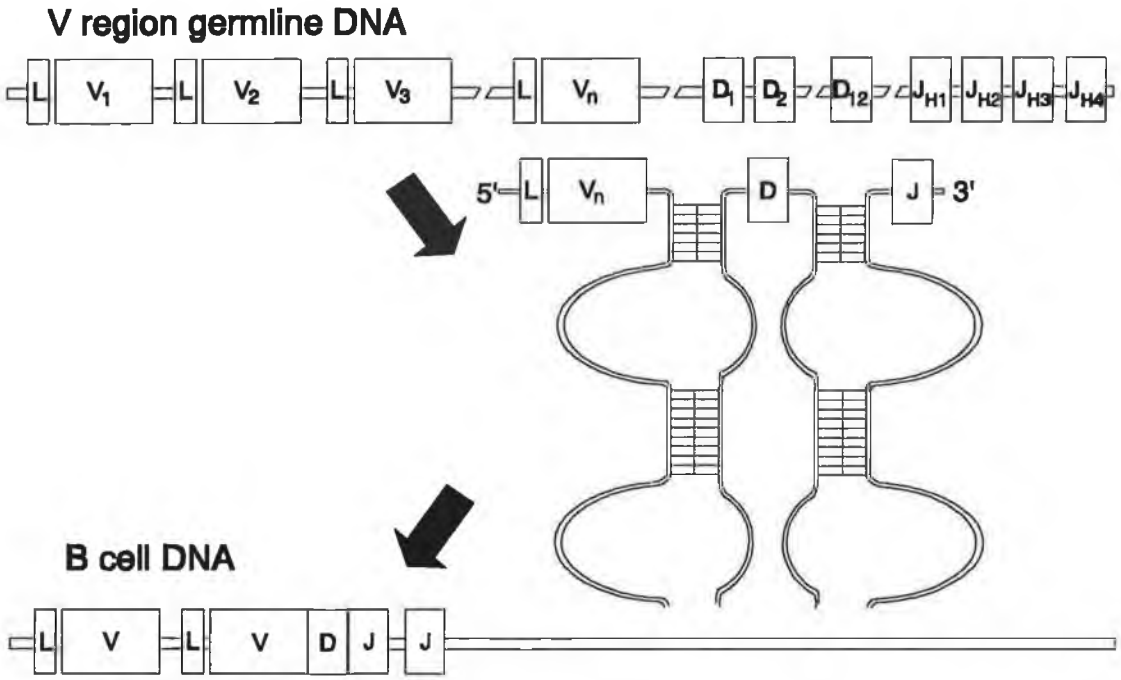


Figure 1.3. Antibody variable heavy chain gene recombination. Multiple V_H, V_D and V_J regions present on the variable heavy region of the antibody germline gene recombine with one another in an essentially random fashion by the formation of stem-loop structures of intervening sequences, created by areas of complementarity in distinctive heptameric and nonameric stretches. Recombinase enzymes remove the intervening stem-loop, introducing further variability due to variations at the site of cleavage. The adjacent VDJ region now forms a functional B cell variable heavy chain gene.

go on to produce IgG, IgA and IgE, depending on the individual environmental conditions to which that particular B cell is exposed. The resulting functional H chain transcripts now comprise VDJ and C region sequences in the case of H chains and VJC sequences in light chains, which result in the translation and assembly of intact immunoglobulin molecules.

1.4 MONOCLONAL ANTIBODY TECHNOLOGY

Prior to the advent of monoclonal antibody technology, antibody sera was mainly polyclonal in nature. These were made up of many antibody isotypes with various specificities to epitopes on the antigen of interest. Although such heterogeneous populations were useful in many serological applications, this heterogeneity also limited their potential in many areas. The development of monoclonal antibodies described by Kohler and Milstein in 1975 meant that a permanent source of homogeneous antibody with defined specificity and affinity could be produced. The homogeneity of the resulting monoclonal antibodies also allowed the elucidation of antibody structure by crystallographic techniques. The versatility of the antibody-antigen interaction has resulted in the use of monoclonal antibodies permeating almost every area of the biological sciences and beyond.

One area in which monoclonal antibodies have found widespread application is in the detection and therapy of neoplastic disease (von Mehren and Weiner, 1996; Bodey *et al.*, 1996; Karius and Marriott, 1997) where their properties of specificity and high affinity have led to them being described as 'magic bullets', with the ability to selectively target and destroy cancer cells. Many monoclonal antibodies have been developed with the aim of targeting tumour cells via tumour-specific or tumour-associated antigens. These cell surface markers assist in the differentiation between healthy and abnormal cells. In therapy, having selectively targeted the tumour, various means can be employed to destroy the cells. Antibodies alone can recruit natural effector functions. However, most monoclonal antibodies for clinical application are murine in origin, whose Fc portions do not effectively recruit human effector functions. Responses have been improved by the use

of mouse/human chimaeras which possess human Fc portions fused to murine variable regions to improve effector activity (Motmans *et al.*, 1996).

The enhancement of traditional radiotherapy and chemotherapy has been brought about by the use of monoclonal antibodies conjugated to radionuclides, toxins and other chemotherapeutic agents. Linkage of these components to an antibody increases their concentration at the site of action, resulting in reduced damage to healthy tissues while increasing the efficacy of the applied agent. Combining antibody and radionuclide also facilitates tumour imaging (Britton, 1996). Cancer detection and diagnosis are also facilitated by the use of monoclonal antibodies in immunocytochemistry (Beaty *et al.*, 1997).

Further improvements in the recruitment of cytotoxic cells for tumour destruction have been brought about by the linkage of two monoclonal antibodies to one another in a bispecific antibody. Here, one portion of the bispecific construct targets the tumour cell and the other targets cytotoxic cells via a suitable surface marker (Lindhofer *et al.*, 1996; Clark *et al.*, 1997). Prior to this, chemotherapeutic drugs had to be covalently linked to antibodies to target them effectively, which could affect the performance of the drug. Bispecific antibodies allow drug targeting without the requirement to conjugate the chemotherapeutic agent to the antibody.

In a further advance on combined antibody-chemotherapeutic treatment, antibodies are being coupled to enzymes which catalyse the breakdown of a non-toxic prodrug to a toxic component at the site of action in a system referred to as antibody-dependent enzyme prodrug therapy (ADEPT). A development of this technique utilises antibody catalysts to convert prodrug to drug. This catalytic antibody has been referred to as an 'abzyme' and the technique labelled antibody-dependent 'abzyme' prodrug therapy (ADAPT) (Wentworth *et al.*, 1996). The use of monoclonal antibodies is also widespread in other therapeutic fields, such as the control of sepsis (Wheeler and Bernard, 1996), treatment of viral disease and control of allergic responses (Lebecque *et al.*, 1997).

Although there is an abundance of *in vitro* and animal model data for the benefit of antibody-based reagents in the treatment of cancer, only limited numbers of treatments have proved successful enough to reach full scale human clinical trials.

The use of monoclonal antibodies as a therapeutic tool in humans has, however, been severely limited because the vast majority are murine in origin. The *in vivo* use of such antibodies in humans leads to a human anti-murine antibody (HAMA) response. This limits the number of times the antibody can be used in a single patient. The human response reduces the antibody serum half-life and can, in the worst cases, lead to anaphylaxis.

Attempts have been made to produce human monoclonal antibodies in the same manner as murine hybridomas. However, there are fewer suitable myeloma fusion partners of human origin, some of which secrete antibody fragments which results in mixed antibody populations. Also, immunization is difficult and normally relies upon accidental immunization via infection or disease. *In vitro* immunization protocols for human lymphocytes have improved in recent times but are still fraught with difficulties. The source of B lymphocytes is also a problem. Although tonsils and peripheral blood lymphocytes are quite readily available, they do not equal the spleen in terms of the quality and quantity of lymphocytes (Ohlin and Borrebaeck, 1996). Attempts have also been made to immortalize specific B lymphocytes using the Epstein-Barr Virus (EBV), again with only limited success (Kozbor and Roder, 1983). In an attempt to overcome these obstacles, various molecular genetic techniques have been applied to make murine antibodies more 'human' and so avoid anti-species responses when administered in man.

1.5 ANTIBODY ENGINEERING

Improved therapeutic and analytical applications of antibodies depended upon the development of techniques to genetically produce and manipulate antibody proteins through the field of study which is now broadly termed antibody engineering. Traditional molecular genetic techniques did not allow for the correct functional expression of whole antibody molecules. Most expression vectors are derived from plasmids that propagate

in *Escherichia coli* (*E. coli*), and although correct expression of recombined light (L) and heavy (H) chain genes is possible, glycosylation does not take place. This affects the serum half-life and other characteristics of the antibody molecule. Full glycosylation requires the use of eukaryotic expression systems (Wright and Morrison, 1997). Antibody fragments are, however, easily manipulated using the expression vectors available. Work has thus concentrated on those parts of the antibody involved in the antibody-antigen interaction, namely the Fab fragment and its subcomponents. Various portions of this region have been manipulated in numerous protocols. These include the whole Fab (antigen-binding fragment) region, $F(ab')_2$ (two antigen-binding fragments joined via the hinge region), Fd (V_H and C_H1 domains), Fv (variable fragment) and CDRs, the Fv region being the smallest fraction required to result in the complete antigen-antibody interaction (Fig. 1.4). It is believed that these small fragments may be more useful in many applications than whole antibody molecules as they possess improved tumour penetration characteristics, better pharmacokinetic properties (they are removed from the body more rapidly) and have reduced immunogenicity.

As stated, the absence of a reliable system to produce human antibodies was a significant problem for monoclonal antibody technology. The developing field of molecular genetics started to produce tools that could be applied to the problems encountered by those involved in antibody technology. An understanding of the processing of antibody messenger RNA (mRNA) (Schibler *et al.*, 1978) was fundamental to being able to manipulate these species *in vitro*. Early work also established the familial nature of antibody genes (Brodeur and Riblet, 1984; Dildrop, 1984). With the advent of the polymerase chain reaction (PCR), systems for amplifying specific DNA or RNA sequences soon became available. (Saiki *et al.*, 1985). It had been noted that large areas of antibody chain genes were conserved. Even in the variable regions, conserved sequences can be established when genes are divided into their respective families. These sequences proved ideal for the production of oligonucleotide primers that could flank the nucleic acid sequence of interest which could then be amplified and used for cloning (Orlandi *et al.*, 1989). In early work, systems for the expression of these amplified genes in prokaryotes were inadequate. Initial studies involved transfection of antibody genes into myeloma cells using viral vectors. For example, an Abelson murine leukaemia virus

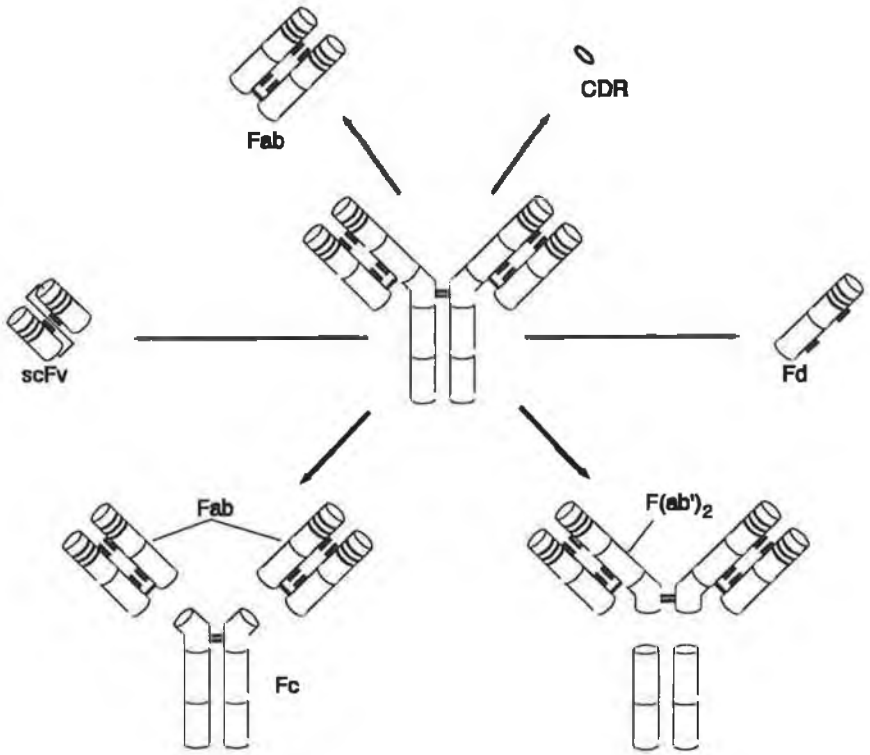


Figure 1.4. Fragments of the antibody molecule. Various fractions of the whole antibody molecule may be generated either through genetic or enzymatic and chemical manipulation. Digestion with enzymes such as papain and pepsin yield either Fab (antigen-binding fragment) or $F(ab')_2$ (two antigen binding fragments linked by a hinge region) fragments and Fc (crystalline fragment) regions. Antibody chain gene manipulation has resulted in whole Fab fragments, Fv (variable fragment) moieties, some stabilised using a synthetic linker (scFv - single chain Fv), V_H and C_H1 chains (Fd) or equivalent V_L chain sequences, as well as single CDR regions which have all been shown to be capable of binding antigen.

(AMuLV)-transformed lymphoid cell line resulted in the stable expression of a kappa (κ) L chain gene from a plasmid using an SV40 early promoter with selection via mycophenolic acid resistance (Rice and Baltimore, 1982). Full antibody production was restored in a non-L chain-secreting mutant hybridoma cell line when transfected with a trinitrophenyl-specific κ chain gene, again using the SV40 promoter and with selection using neomycin resistance (Ochi *et al.*, 1983). An $IgG_1\kappa_1$ antibody was also secreted intact in a similar system in a J558L myeloma cell line (Oi, *et al.*, 1983). In a later development, Brüggemann *et al.* (1989) used a human H chain gene DNA to produce a transgenic mouse. DNA rearrangements had been found to occur at a high frequency in the thymus and spleen when compared to other cells demonstrating that the cloned genes were under the same cellular control as the mouse's wild type antibodies. Brüggemann and Neuberger (1996) have developed this transgenic mouse to possess near complete human germline genes which undergo normal rearrangement and hypermutation. Such animals could be used to generate human monoclonal antibodies via mouse immunization.

Parallel improvements in the understanding of processing and transport systems in prokaryotes, especially *E. coli* gradually allowed the more efficient expression of foreign genes in prokaryotes using phage and plasmid vectors. The smallest antibody fragment required for complete antigen binding is the Fv fragment. Thus, for ease of cloning, much work concentrated on this portion. Skerra and Plückthun (1988) expressed the V_H and the V_L domains of the murine anti-phosphorylcholine IgA antibody McPC603 in *E. coli*. To target V_H and V_L chain genes to the periplasm, *ompA* and *phoA* leader sequences were used, respectively. Expression of the genes could be controlled via the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *lac* promoter/operator. This system did appear to result in functional Fv assembly. Skerra and Plückthun saw the translocation of protein to the *E. coli* periplasm as functionally equivalent to eukaryotic protein transport to the lumen of the endoplasmic reticulum. Others, however, had found the stable expression of Fv fragments a little more difficult (Glockshuber *et al.*, 1990). Fv fragments dissociated at low protein concentrations and were unstable under physiological conditions. This was because the interchain disulphide bonds present in the C_H1 and the C_L regions were missing. Various strategies have been explored to improve this situation. Three methods have been investigated. The first was glutaraldehyde cross-linking of V_H

and V_L while in the presence of antigen. The second method was the incorporation of cysteine residues into the V_H and V_L regions to yield a disulphide linkage (dsFv). The third method involved construction of a single chain fragment (scFv) using a 15 amino acid linker $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_3$. These systems did not improve yields of Fv but did improve the stability of the resulting product. It was established that the yield is determined by the correct folding of Fv and not V chain association. Brinkmann *et al.* (1993) also found a disulphide-stabilized Fv-toxin chimaera to be as stable as the amino acid-linked scFv.

The scFv is a popular means of producing stable Fv fragments as this does not require any additional steps once the V_H and V_L chain genes have been cloned into a vector with a linker present. It also avoids the necessity to insert cysteine residues into the V sequences which may affect the conformation of the binding site and also requires correct redox conditions for disulphide bond formation. The scFv fragment can also be purified in a single step. The presence of the linker may still, however, affect the conformation of the antigen-binding site and the function of the antibody molecule. Tang *et al.* (1996) demonstrated that randomisation of an 18 amino acid linker led to the selection of higher affinity scFvs and restored catalysis to a scFv version of a catalytic monoclonal antibody.

The ability to produce scFv fragments by recombinant methods has improved significantly in recent times and has allowed the system to move beyond *E. coli* as an expression host. Tavladoraki *et al.* (1993) successfully produced a transgenic artichoke that produces a scFv antibody directed against the artichoke mottled crinkle virus. The scFv is constitutively expressed and leads to reduced infection rates and a slower onset of disease. *Streptomyces* is used for the production of many extracellular components. It would be of more use than *E.coli* for antibody production as Gram negatives only secrete into the periplasm and require lysis of the outer membrane. *Streptomyces lividans* was used as a host for the extracellular production of V_H and V_L chains to hen egg-white lysozyme (HEL) (Ueda *et al.*, 1993) using suitable codon preferences. The cloned genes were placed in a *Streptomyces* multi-copy plasmid vector pIJ702 using the signal for the subtilisin inhibitor (SPssi), a major extracellular protein. Yields of 1 μg/ml of V chains resulted. In the absence of HEL, V_H and V_L dissociated. It is clear that this system would

have benefit greatly by scFv stabilisation. Chesnut *et al.* (1996) have developed systems which transiently express scFv antibody anchored to the eukaryotic cell surface which can be used to select cells expressing antibody from the rest of the cell population. The importance of eukaryotic expression is growing as the desire to produce whole, correctly expressed human antibodies continues. Persic *et al.* (1997) have developed several eukaryotic expression vectors that allow both transient and stable expression of either Fab fragments, or whole human antibodies, with specificity selected by insertion of V region genes. The use of *E.coli* cell-free translation systems was also exploited by Ryabova *et al.* (1997) which showed the requirement of protein disulphide isomerase and chaperones for correct disulphide bond formation and protein folding for improved levels of soluble scFv.

A potentially useful therapeutic scFv was produced by Savage *et al.* (1993). This murine scFv recognizes placental alkaline phosphatase (PLAP) which is expressed on many tumours and is used in the screening of ovarian and testicular cancer. The scFv was specific for PLAP and could be more useful in diagnosis if it were of human origin as this would minimise or totally remove the associated HAMA response.

Other fragments of the antibody molecule have been assessed for their possible uses. It was noted early in the study of antibody-antigen interactions that the H chain was the most important part of the antibody-antigen binding interaction and that the binding affinities of L chains are about 100-fold lower than H chains (Utsumi and Karush, 1964). However, there are instances where the light chain has been found to be dominant in the antibody-antigen interaction (Sun *et al.*, 1994). The use of the V_H/C_H1 (Fd) fragment provides a molecule with good binding activity without the difficulties associated with the expression of two antibody peptide chains (Kouki *et al.*, 1997). Other work has concentrated on larger fragments; the Fab or even $F(ab')_2$ portions of the antibody molecule. These fragments are still much smaller than the entire antibody, and so still possess advantages relating to tissue penetration and pharmacokinetics. These fragments can also be efficiently expressed in prokaryotic vectors and do not have the difficulties relating to stability experienced with Fv fragments. The absence of any interference in the structure of the antigen-binding site ensures that the original binding characteristics

of the antibody are retained. Many Fab antibodies have been cloned from whole antibody molecules. One such example is a human anti-rabies Fab antibody that was expressed in a bacteriophage lambda (λ) vector system using the *E. coli pelB* (pectate lyase) leader. The Fab was capable of binding to the rabies virus coat glycoprotein in a manner similar to whole antibody (Cheung *et al.*, 1992).

It is clear that, due to the ease in which they can be cloned and expressed in various hosts, and due to their improved characteristics, antibody fragments have a large role to play in various areas of therapy and diagnosis. Every useful fraction of the antibody molecule has been explored to assess its individual characteristics. Even the CDR region alone has now been explored as an antigen-binding tool (Levi *et al.*, 1993). In this system, the CDR-H3 region from a murine anti-HIV-1 V3 loop antibody was found to be capable of competing with whole antibody for the V3 loop. When the peptide was cyclized, it was also found to be capable of inhibiting HIV-1 replication and syncytium formation in infected cells. A single CDR is probably the limit in size reduction that useful antibody fragments can reach.

1.6 COMBINATORIAL PHAGE DISPLAY LIBRARIES

The development of the technique known as phage display - a system capable of the production of human antibody fragments - began back in 1985 when Smith (1985) demonstrated that a foreign peptide could be expressed on the surface of a virion by fusing it to a coat protein gene. The phage he used was a filamentous one, a member of the M13 family. These possess two distinct coat proteins, pIII and pVIII derived from corresponding coat protein genes *gIII* and *gVIII*, respectively. The pVIII coat protein is the major component expressing many thousands of copies. The pIII protein is a minor component of the virion coat expressed at only 5 copies per virion but with the important function of binding to the *E. coli* F pilus via its N terminus. Initial attempts were made to insert a peptide between the C and N termini of pIII to prevent disruption of terminal functions. This still severely reduced the infectivity and, thus, the efficiency of the virion. Parmley and Smith (1988) improved the methodology slightly by placing the inserted peptide at the very N terminus of the pIII protein. This interfered with function to a

lesser degree than central insertion but efficiency was still poor. Parmley and Smith also improved vector design by using a tetracycline resistance fd phage (fd-tet) that could propagate as a plasmid and did not need to reinfect to generate high titres. They also demonstrated that the phage particles could be affinity-purified in a process now known as panning. In their early version, phage was selected using biotinylated antibody to the foreign virion epitope attaching to a streptavidin-coated plate. Subsequent rounds of this enrichment procedure dilutes out weaker affinities and enriches higher affinity ligands.

Ward *et al.* (1989) were the first to use an M13 vector system to produce antibody chains in *E. coli*. To achieve this, mouse spleen V_H mRNA was amplified using PCR. These early attempts only assessed the ability to clone a single chain in vectors of poor efficiency.

Some early work concentrated on expressing antibody fragments using a λ vector as this phage was well understood and several suitable vectors were available. Huse *et al.* (1989) were the first to produce a combinatorial Fab antibody library using just such a vector. The λzapII vector allowed *in vitro* packaging of the cloned H and L chain genes following amplification using PCR. H and L chains were cloned separately in vectors λHc2 and λLc1, respectively, before being combined randomly. A library of 2.5 x 10^7 pfu was established, 1 x 10^6 of which were screened using plaque lifts - a far less efficient screening procedure than that allowed by filamentous phage display. Even so, 100 plaques were found that bound the KLH-*p*-nitrophenyl phosphonamidate antigen with affinities of 10-100 x 10^{-9} M.

Some workers persisted with the λ phage system. Persson *et al.* (1991) generated a combinatorial library to tetanus toxoid using human lymphocytes from peripheral blood (PBLs), 5 litres of which had undergone leukapheresis to produce 1 x 10^9 lymphocytes. They assessed the effect stimulation of the B cells with antigen *in vitro* prior to cloning and the effect of panning would have on generating greater yields of specific antibody. Unstimulated cells produced a library of 1 x 10^5, stimulated cells 6 x 10^5 demonstrating little improvement from antigen stimulation. This may be due to the inefficiency of *in vitro* stimulation.

As methods based on λ could only produce comparatively small libraries (10^6) and the screening procedures were poor, work progressed on systems based on filamentous phage display. Several groups looked at the ability of phage to display non-antibody proteins such as hormones and random peptides. Bass *et al.* (1990) improved the M13 system in several ways to efficiently display human growth hormone (hGH) as a pIII fusion. They introduced the M13 intragenic (IG) region into the M13 plasmid to produce a 'phagemid', an entity capable of propagating as both a plasmid (using *E. coli ori* and tetracycline resistance) or as a phage by becoming packaged via the IG region. The problem with the protein pIII being inactivated resulting in a phage incapable of F pilus infection was circumvented by the introduction of a helper phage M13K07. This phage possesses an in tact copy of *gIII*, but has a non-functional IG region and so cannot transcribe its genes unless in the presence of the phagemid. Thus, following cloning of the hormone gene, coinfection of phagemid and helper results in the production of phage particles expressing cloned and intact pIII and so are still capable of reinfecting *E. coli* efficiently. Virions expressing a single cloned pIII would be particularly desirable when cloning antibody genes as this would rule out avidity effects due to the presence of more than one antigen binding site per virion and would allow for the selection of high affinity antibodies amongst lower affinity molecules.

Several papers also reported similar phage display systems for the expression of random peptide sequences (generated by oligonucleotide synthesizers) on the surface of phage to identify important epitopes in antibody-antigen binding (Cwirla *et al.*, 1990; Devlin *et al.*, 1990; Scott and Smith, 1990).

The antibody Fv domain was first cloned and expressed in a filamentous phage by McCafferty *et al.* (1990) who used the V_H and V_L fragment genes from murine anti-lysozyme antibody and linked them via the $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_3$ linker to the *gIII* of fd phage.

Some attempts were made to exploit a similar technique based on the pVIII protein instead of pIII (Kang *et al.*, 1991a). This also used a helper phage designated VCSM13 and a phagemid vector pComb8. Still better than λ vectors in generating larger libraries,

this system makes it difficult to separate high affinity binders from lower affinity molecules due to avidity effects and has been superseded by the pIII system in most applications.

1.6.1 The pComb3 System

A single phagemid vector system capable of efficiently expressing intact Fab fragments in *E. coli* has now been established (Barbas *et al.*, 1991) (Fig. 1.5). This vector is known as phagemid pComb3 and contains a β-lactamase resistance gene (*bla*) and origin of replication for *E. coli* from plasmid pBluescript. It contains the IG region of the M13 phage and has directional cloning sites for PCR-amplified H and L chain genes. Both of these are placed downstream of *pelB* leader sequences for efficient *E. coli* periplasmic transport and the dicistronic message is under the control of the IPTG-inducible *lacZ* promoter. The H chain gene is placed C terminal to the *gIII* gene sequence where, upon expression it forms a fusion protein. This system has the potential to generate libraries that are capable of cloning the entire human immunological repertoire which is estimated to be in the region of 10^8 different specificities. With the helper phage VCSM13, the phagemid can propagate as a phage particle. By removing the *gIII* gene and in the absence of helper phage, it propagates as a plasmid and soluble Fab antibody fragments can be generated by induction with IPTG.

An outline of the pComb3 system is shown in Fig. 1.6. The first step in cloning the human immunological repertoire is the extraction of mRNA from approximately 10^8 B lymphocytes. This should theoretically contain the full repertoire of antigen specificities. Having done this, complementary DNA (cDNA) can be synthesized from the mRNA using oligo(dT) primers which complement the 5′ end of the RNA messenger and using reverse transcriptase to convert mRNA to its cDNA strand. This DNA is then used as the template for the PCR amplification of the antibody L and H chain genes. The 5′ ends of the C_H1 region and the C_L region are conserved and a single primer is required for each of these. Several primers are required to complement the 3′ ends of both the L and H chains as

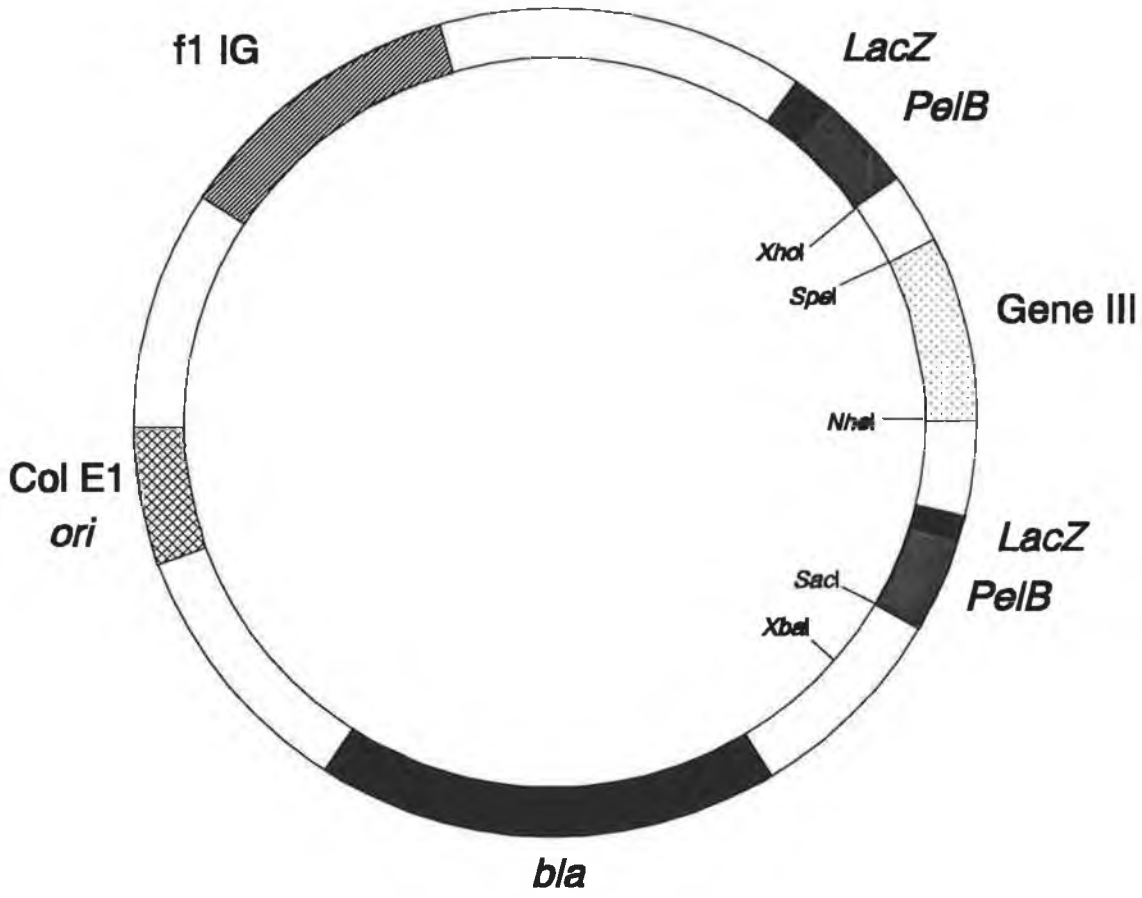


Figure 1.5. The pComb3 phagemid vector. Replication in E. coli is via the Col E1 ori. Resistance is via the β-lactamase resistance gene, bla. For phage packaging, pComb3 carries the intragenic region from f1 phage. Directional insertion of light and heavy chain genes is via SacI/XbaI and XhoI/SpeI sites, respectively, each of which is downstream from a LacZ promoter/operator and a pelB leader sequence. Downstream of the heavy chain gene insertion is gIII, for phage surface expression of the heavy/light chain Fab fragment.

these fall into specific groupings based on their DNA homologies. For PCR amplification, aliquots of cDNA are mixed with a single 5' and 3' primer of either the L or H chain which then undergoes thermal cycling to amplify these fragments. All the L chain aliquots and all the H chain aliquots are then pooled. The PCR primers are so designed that they also introduce restriction sites at the ends of the amplified L and H genes so they can be ligated into the vector. H chain genes are cloned into the vector first. The ends of the amplified sequences are cut with restriction enzymes and ligated into the phagemid. This is transformed into *E. coli* and propagated as a plasmid. The plasmid is repurified and then the L chain genes are inserted in a similar fashion. The phage is then used to coinfect *E. coli* along with the helper phage. The IG region on the phagemid allows the expression of phage genes, which assemble as a virion. The gene fusion of pIII and H chain is incorporated into the phage coat along with functionally correct pIII. Due to its non-functioning IG region, phage DNA cannot be packaged into the virion and so only phagemid is packaged. This results in an intact virion particle displaying wild type and recombinant pIII protein on its surface. During periplasmic transport, the L chain is able to reassociate with the H chain to form an intact Fab fragment expressed on the virion surface, while within the virion are the genes coding for the antibody L and H chains. The linkage of phenotype and genotype has been achieved. As *E. coli* is propagated, virions are secreted into the culture medium. When separated from whole cells by centrifugation, these can be used for panning. The antigen of interest is coated to a microtitre plate either chemically or as a protein conjugate. Phage is then added. Washing is used to remove those phage that are not expressing Fab capable of binding with antigen leaving the high affinity binders still attached (Fig. 1.7). These are then removed by more vigorous treatment and can then be propagated again in *E. coli*. Normally about three rounds of panning are performed to select Fabs of suitable affinities. Following purification of individual phage particles, soluble Fab antibody fragments can then be prepared by plasmid propagation of the phagemid and induction using IPTG. This generates H and L chains that are transported to the periplasm and can be recovered by lysis of the bacterial outer membrane or culture supernatant. The soluble antibody fragment can then be purified by affinity chromatography.

With the advent of a methodology powerful enough to potentially clone the complete immunological repertoire of humans, it has been used to generate human antibody fragments of clinical benefit. The system has also been used as a means of dissecting the immune response but not without some controversy over its ability to truly mimic a natural immune response. The generation of a phage display library must start with B lymphocytic material. For the generation of human antibodies, obtaining this material still remains the main difficulty. To generate murine antibodies, spleen cells are available. The most readily available source of B lymphocytes in humans is from peripheral blood. This has been used on several occasions to generate libraries (Persson *et al.*, 1991; Walker *et al.*, 1992). Peripheral blood has, however, only a low concentration of B lymphocytes (15-20% of cells) so large volumes of blood (approximately 0.5 l) are required to produce sufficient numbers of B cells to clone the entire repertoire. Various strategies have been applied to enriching the B cell concentration of peripheral blood. Most steps involve separation of the whole blood on a suitable gradient (Persson *et al.*, 1991). Also the blood contains only a small population of differentiated B cells reacting to recent antigenic challenge. Such a population could be greatly biased towards particular antigens making it extremely difficult to select antibody chains against the antigen of interest.

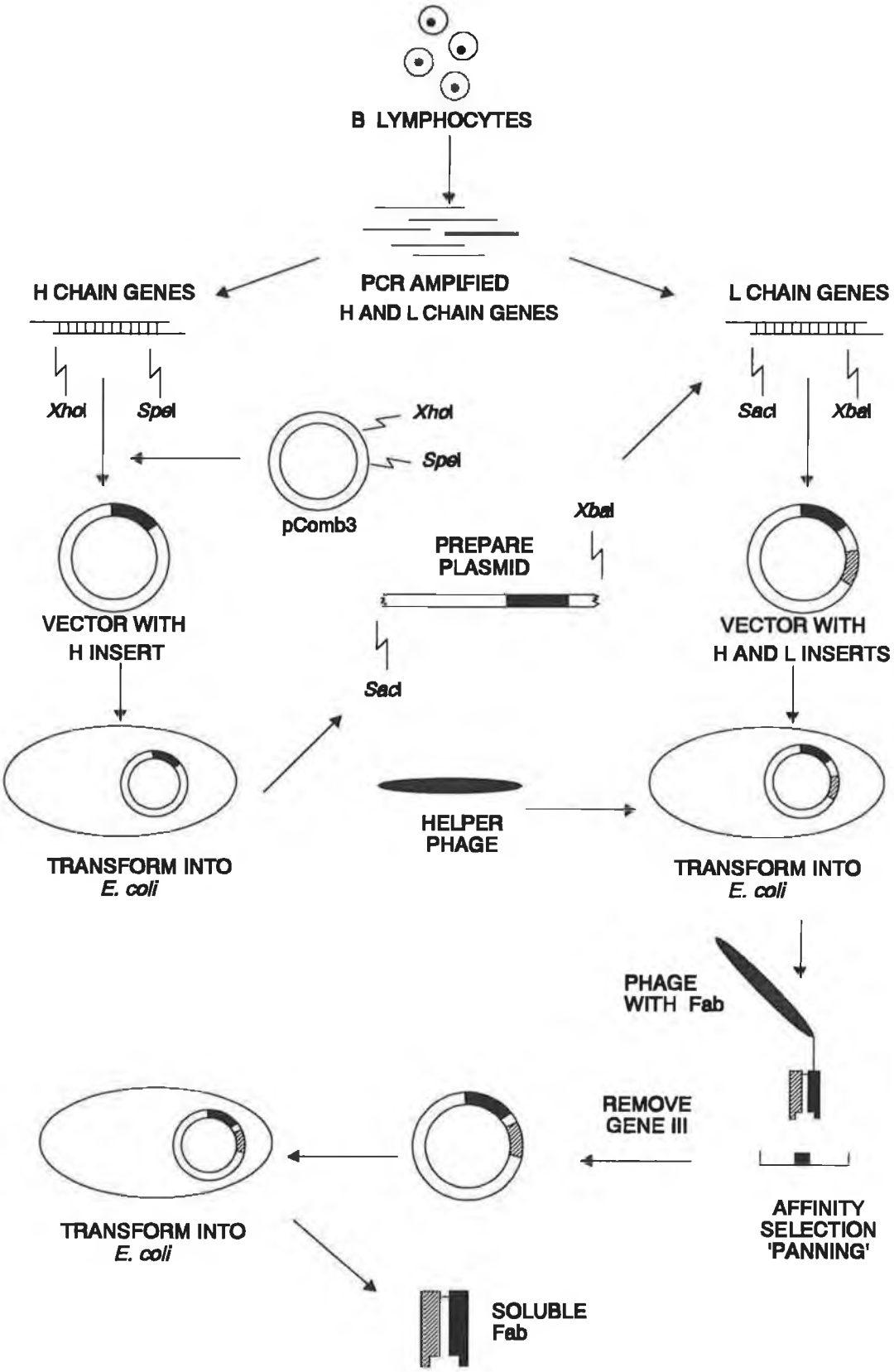

B LYMPHOCYTES
PCR AMPLIFIED
H AND L CHAIN GENES
H CHAIN GENES
L CHAIN GENES
XhoI
SpeI
SacI
XbaI
pComb3
PREPARE
PLASMID
VECTOR WITH
H INSERT
VECTOR WITH
H AND L INSERTS
HELPER
PHAGE
TRANSFORM INTO
E. coli
PHAGE
WITH Fab
REMOVE
GENE III
AFFINITY
SELECTION
'PANNING'
SOLUBLE
Fab

Figure 1.6. (previous page). Production of antibody Fab fragments using the pComb3 system. Antibody chain RNA is isolated from a suitable source, normally B lymphocytes. The RNA undergoes reverse transcription and PCR amplification using families of variable 5' primers and constant 3' primers, encoding restriction sites. Vector and PCR product are digested, ligated and transformed into E. coli. The heavy chain library is composed first and then recombined with light chains. Phage expression is brought about by infection with helper phage, and affinity selection takes place against the desired antigen immobilised to a solid support. When specific clones have been isolated, soluble antibody may be expressed by removing gIII and inducing high level expression from the LacZ promoter.

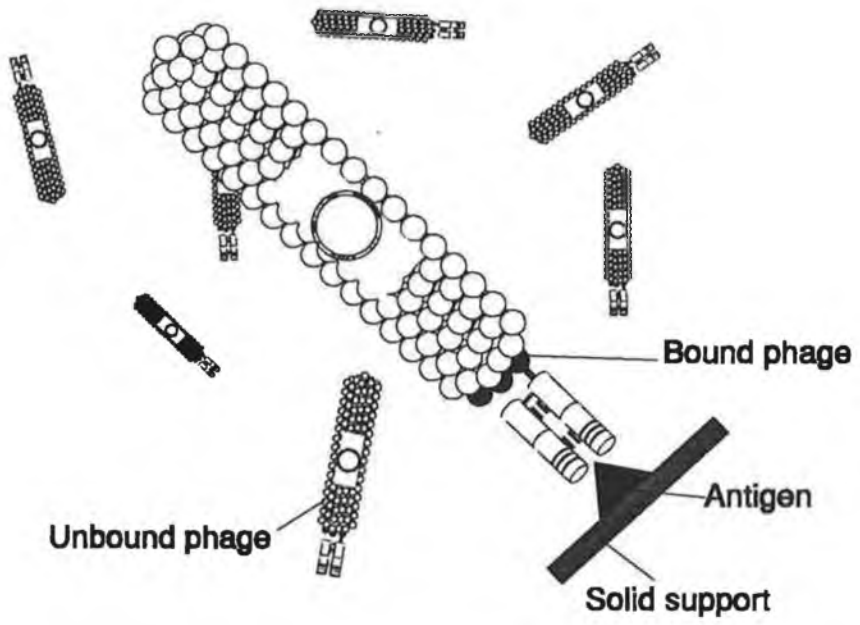


Figure 1.7. Affinity selection or panning. Polyclonal phage is allowed to bind to antigen on a solid support. Specific phage bind to the surface, and the rest of the material is removed by vigorous washing. Specific phage is then eluted from the antigen and this enriched material used to retransfect E. coli for screening or further rounds of affinity selection.

1.6.2 The Nissim Library

Nissim *et al.* (1994) produced a scFv phage display library of human origin from the peripheral blood lymphocytes of an unimmunised donor. To further enhance the diversity of the library, they inserted random nucleotide sequences from 4-12 residues in length at the CDRH3 site. This library also used a single L chain sequence (Vλ3), so diversity was only as a result of the H chain. This semi-synthetic human library was combined with libraries established by Hoogenboom and Winter (1992), resulting in a 'single-pot' library with very large diversity ($>10^8$). The Nissim scFv system is based on the phagemid vector pHEN1. This bears similarities to pComb3 in terms of resistance mechanism, M13 IG region, *E.coli ori*, as well as the presence of *lac*Z promoter/operators and *pel*B leader sequences. Significant differences exist at the site of antibody chain gene insertion. As the scFv is a single sequence, insertion at only one site is required, upstream from *pIII*. Soluble and phage surface expression are controlled by means of an amber mutation at the 3' of *pIII* which can either be suppressed or expressed depending on the *E.coli* host strain employed. Detection of the scFv is facilitated by the presence of a *c-myc* tag 5′ to the scFv.

The Nissim library offers the potential to produce human scFv antibodies against an antigen of interest without the need for prior *in vivo* immunisation, or the establishment of a combinatorial antibody library. Such a system exploits the concept that high affinity antibody species can be derived from so-called 'naive' repertoires if the diversity is great enough. The fact that the antibodies are of human origin is also an important consideration for their use in any form of *in vivo* therapy. The Nissim library has been shown to be capable of generating high affinity scFvs to antigens such as foreign or self proteins and various haptens.

1.6.3 Bacterial surface display

As well as phage and eukaryotic display systems, the bacterial cell surface has also been put forward as a means of displaying antibody libraries (Little *et al.*, 1993) with fusion to several cell surface components, e.g., lipoproteins, outer membrane proteins, secretory

proteins, as well as flagellae and fimbriae. Robert *et al.* (1996) used Staphylococcal species to assess their ability to display a viral coat protein via fusion to several surface components. Such a system would find it hard to match the numbers of phage particles that can be generated by bacteriophage infection, but might be adequate for the display of previously established monoclonal antibodies.

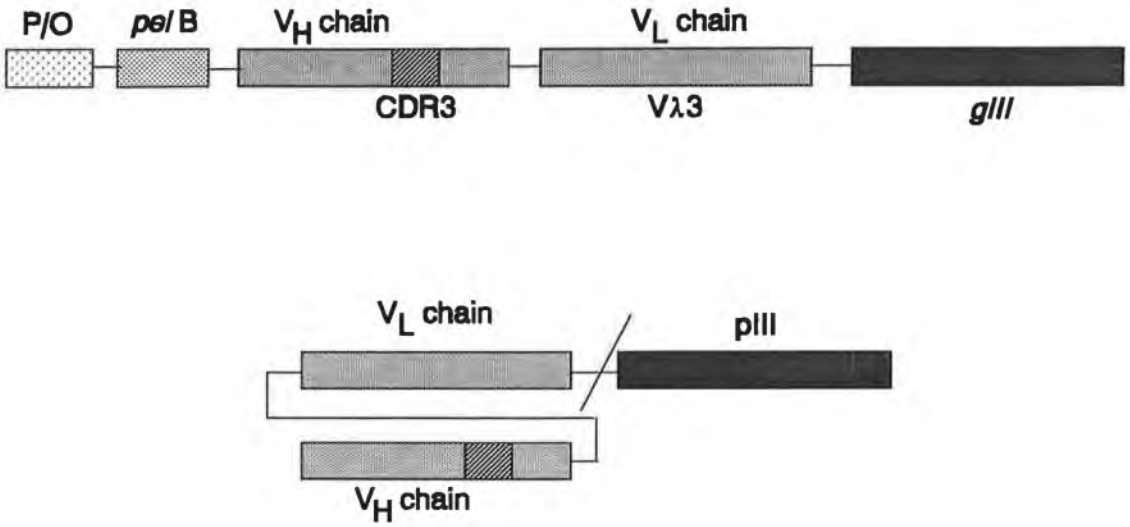


Figure 1.8. The Nissim library. The library is a semi-synthetic human scFv library. It consists of a phagemid backbone, with f1 IG region and bla resistance gene (not shown). Induction is via the lacZ promoter/operator (P/O) and secretion via the pelB leader sequence. The insert consists of a library of human variable heavy chain genes possessing a synthetic CDR3 region. The variable heavy chain is linked to a $V_\lambda 3$ variable light chain gene via a $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_3$ synthetic linker. Downstream of the variable light chain gene is an amber codon which allows production of scFv with or without pIII by expression in suppressor and non-suppressor strains of E. coli.

1.7 ANTIBODY CONJUGATES AND CHIMAERAS

Chimaeric antibodies can be formed by linkage of two antibody species, such as human and mouse. Antibodies have also been fused with proteins (toxins, enzymes, etc.) and solid supports (Fig. 1.9). Such constructs have a myriad of uses. The most obvious use that presents itself is in the area of antibody therapy of cancer. Examples of antibody conjugates in areas of imaging, radiotherapy, chemotherapy and toxin therapy are widespread (Reading *et al.*, 1989; Adair, 1992; LoBuglio and Saleh, 1992).

A bacterial toxin that has proven popular as an antibody conjugate component is the *Pseudomonas aeruginosa* exoenzyme S. A truncated protein derived from this known as PE40 lacks the binding domain of the toxin. This has been genetically fused to a scFv antibody directed against Tac, the p55 subunit of the human IL-2 receptor (Chaudhary *et al.*, 1989) and was found to be selectively cytotoxic to cells bearing the IL-2 receptor. Such a conjugate could have application in immunosuppressive therapy. The same toxin fragment was also linked to a scFv fragment constructed from the PR1 monoclonal antibody which targets normal prostate or adenocarcinoma prostate cells (Brinkmann *et al.*, 1993). Such a targeting system might be useful post-prostectomy to destroy remaining carcinoma cells.

A novel strategy using an antibody conjugate has been developed to remove blood clots (Holvoet *et al.*, 1992). Here, an scFv to fibrin is linked to a urokinase-type plasminogen activator. This activates the declotting cascade concentrating it to the area of the clot and was found to be 6-15 times more effective than plasminogen activator alone in an *in vitro* clotting system.

A metallothionein-antibody conjugate has also been established (Sawyer *et al.*, 1992) that could be used to deliver radioactive metal isotopes to tumours for therapy or imaging. A novel luminescent probe has been developed based on an antibody fusion with a photoprotein called apoaequorin (Casadei *et al.*, 1990). Such a construct was specifically targeted to hapten and exhibited Ca^{2+}-dependent bioluminescence.

As all the above examples demonstrate, antibody conjugates are extremely versatile and their uses are as widespread as there are examples of a need to target one substance to another.

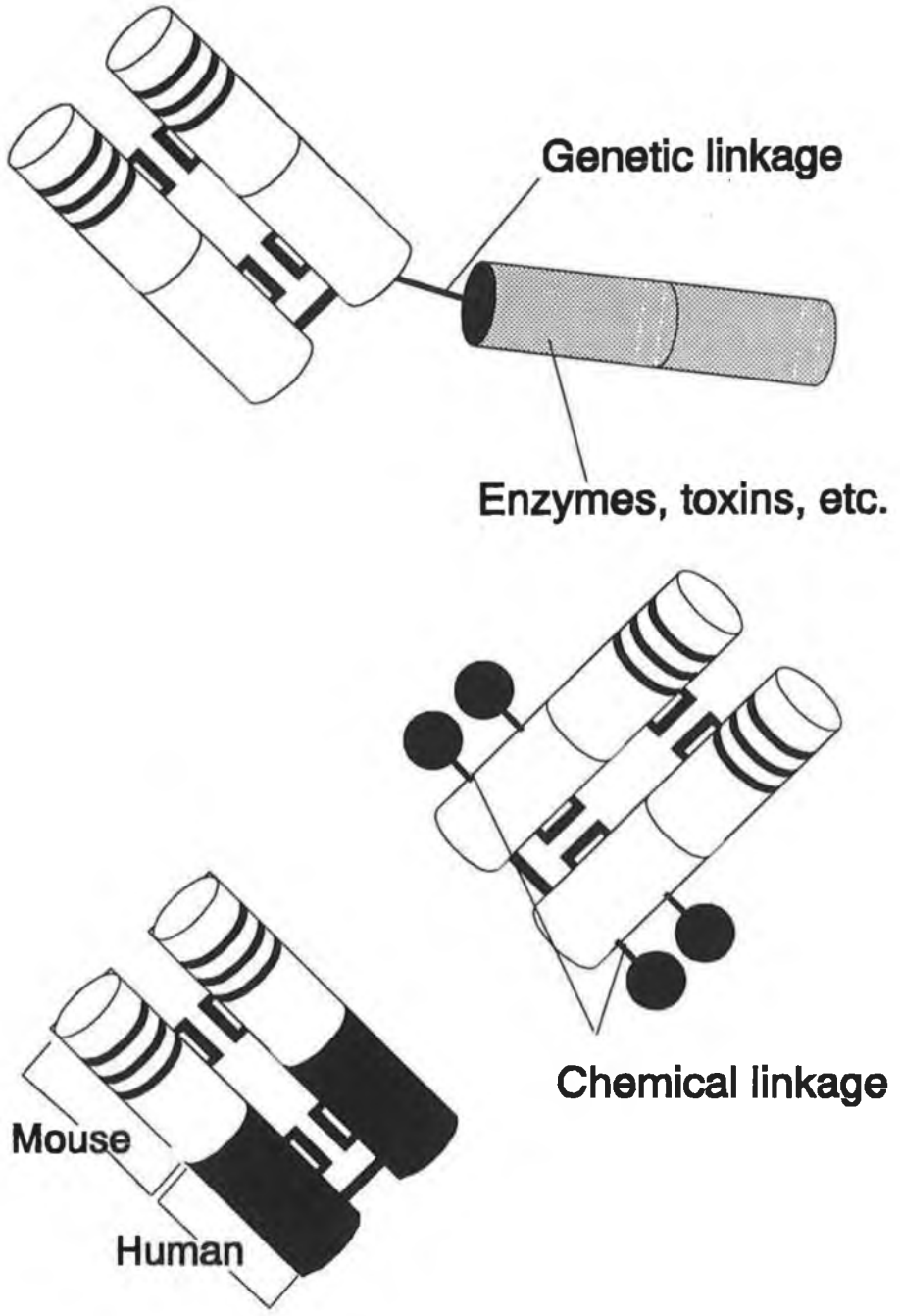


Figure 1.9. Antibody conjugates and chimaeras. Antibody conjugates may be produced by the joining of antibodies with proteins, enzymes and toxins by chemical or genetic methods. Chimaeras may be formed by the joining of variable regions from a mouse antibody with the constant regions of a human antibody.

1.8 HUMANISED ANTIBODIES

As has been stated, the production of human monoclonal antibodies has proved difficult. The relative potential of the applications that have been described above for therapies are drastically reduced because most of these are of murine or rat origin. In an attempt to find a path around the problems of human antibody production, the antibody engineer has turned to molecular genetic techniques.

It was postulated that most of the HAMA response was directed at the constant regions of the antibody molecule. Much of this part of the molecule can be discarded in some applications, e.g., removal of the Fc region. This cannot be done if antibody effector functions are required. Also, removal of Fc alone still leaves C_H1 and C_L regions. If the constant regions of a murine antibody could be replaced with those of a human antibody while retaining the variable regions for antigen binding, much of the HAMA response against the antibody would be lost. The body would see the molecule as less foreign. Also, human antibody effector functions are more efficient than murine functions when administered in man. Surfus *et al.* (1996) produced a human/mouse chimaera composed of mouse V regions from an anti-renal-cell carcinoma antibody fused to a human IgG1 antibody that was shown *in vitro* to enhance the antibody-dependent cellular cytotoxicity of renal cell carcinoma cells with IL-2 treated peripheral blood monocytes, showing possible *in vivo* therapeutic potential against renal cell carcinoma.

The resulting mouse/human chimaeras generated like that described above were better than completely murine antibodies with respect to immunogenicity. These chimaeras still carried long stretches of murine-specific sequences in the V regions, however, and so are still potentially immunogenic. It had long been established that most of the antigen binding of the antibody molecule was due to the three hypervariable regions, the CDRs present in the L and H chain V regions. The next stage in the 'humanization' process was to remove the murine CDRs and graft them into a human framework (FR) region. Such chimaeric antibodies have been termed 'humanized' antibodies. This approach appears to be proving popular. Early attempts used CDR sequences from established hybridomas like the B1-8 anti-4-hydroxy-3-nitrophenacetyl caproic acid antibody (Jones *et al.*, 1986).

This system dealt with the H chain CDRs only. This did not appear to bring about any reduction in hapten binding affinity in this instance. Fiorentini *et al.* (1997) produced a humanized Fab antibody using overlap PCR that recognises a surface marker over-exposed on most breast tumours. This was derived from a previously established murine monoclonal antibody, H23 with specificity to the Muc-1 peptide.

In a further example of the usefulness of humanized antibodies, blocking of CD11/CD18 on the surface of leucocytes using an antibody chimaera has been accomplished (Sims *et al.*, 1993). CD11/CD18 normally promotes the interaction of leucocytes with one another and with endothelial cells and may play a role in disorders such as adult respiratory distress syndrome and allograft rejection. Blocking these interactions without cell destruction may lead to treatments in these cases by preventing the associated tissue destruction characteristic of these disorders.

A diagnostic antibody already showing clinical benefits is a humanized version of the anti-placental alkaline phosphatase single chain antibody used in the detection of ovarian and testicular cancer (Savage *et al.*, 1993). Humanization will allow it to be administered a greater number of times in a single patient without the onset of a HAMA response.

With all these examples of clinically useful humanized antibodies, it might be assumed that there is little problem with them in terms of design and application. This is not the case. Problems with humanized antibodies stem from two incorrect assumptions. The first is that the FR regions do not have a role to play in the shaping of the antigen binding site. The second is that murine CDRs, when grafted onto a human FR will not be immunogenic in humans.

Taking the first assumption: although the CDRs play the most important role in the antibody's interaction with the antigen, the FR region does provide critical residues that place the CDRs in a correct conformation. Although good affinities may be obtained without recourse to the structure of the FR region, this is a hit and miss approach and may lead to unpredictable results. Several approaches have been taken for improving the affinity of humanized antibodies after CDR grafting. One system relies on the

introduction of FR amino acids adjacent to the CDRs in an iterative fashion (Co and Queen, 1991) until the binding affinity of the original antibody is restored. This is a thorough but time-consuming approach. A less thorough but less lengthy method is to test a suite of antibodies with different FR regions against the particular CDRs of interest (Kao and Sharon, 1993). Although not guaranteed to restore the full affinity of the parent antibody, it may considerably reduce the time taken to establish a useful chimaera of at least moderately high affinity. Molecular modelling of the antigen binding site is reducing the guess work involved in CDR grafting still further by allowing the identification of specific framework residues crucial to the restoration of full binding in a humanized antibody (Mateo *et al.*, 1997). They also found that residues selected by modelling restored original mouse sequences in the framework region. Baca *et al.* (1997) have used random mutagenesis of the framework regions, followed by selection of high affinity antibodies via phage display and have produced antibodies with affinities greatly in excess of the parent molecule.

The problem of the second assumption is, however, more difficult to circumvent. Roguska *et al.* (1996) have compared the use of CDR grafting to variable domain resurfacing in which only the crucial surface residues of the antigen-binding site are replaced, instead of whole CDR regions. This method could be shown to restore full binding of an antibody with the use of fewer murine residues than can be achieved with CDR grafting. The epitopes generated by CDR grafting onto a human framework will be many and varied. It is by no means certain whether the body will see these epitopes as foreign or not. If they resemble 'self' antigens then they will escape immunological attack. If they do not, however, the immune system may raise an anti-idiotypic response against them. Thus, to assess the fate of a particular humanized antibody in a particular patient would be impossible. For this reason, the holy grail of antibody engineering still remains the production of completely human antibody molecules.

1.9 AFFINITY MATURATION

It is accepted that one of the best sources of material for the generation of human libraries is bone marrow. Here, the whole spectrum of B cells should be present, awaiting triggering and maturation. Obtaining such material is not as simple as for blood, so, in obtaining the marrow, there should be some degree of certainty that the clones of interest can be derived from it. A debate has arisen as to whether combinatorial phage display is capable of selecting antibody fragments of the required specificity and affinity without prior stimulation of the immune system with the antigen in what has come to be known as a 'naive' library. (This is probably a misnomer as no immune system is ever truly naive.) Such a process is the ultimate aim of many interested in the generation of human monoclonal antibodies. There is a desire to be able to begin each round of selection by using the same library that should potentially have all combinations of specificity present, e.g., the Nissim library (Nissim *et al.*, 1994). From this it would then be possible to select high affinity binders. With this system, however, one encounters the problems of antibody affinity maturation.

In a natural immune response, the initial first line of antibody defence is via IgM antibodies. These are of relatively low affinity compared to their IgG siblings but their decavalency increases their potential. Individual B cells undergo a process of affinity maturation to convert a low affinity antibody to a high affinity one as a result of antigenic challenge *in vivo*. Such fine tuning involves somatic mutation and gene rearrangements from which high affinity molecules are selected and expanded. Without this maturation process, naive libraries will result in mainly low affinity antibodies. Many have concentrated on developing *in vitro* methods to further mimic antibody maturation. Further L and H chain recombination in a process called 'chain shuffling' has been used to improve affinities (Kang *et al.*, 1991b; Ohlin *et al.*, 1996). Most maturation processes involve the mutation of the V region followed by rounds of panning in an iterative process to select higher affinity molecules. To increase affinity to the V3 loop of the HIV gp120 coat protein, Thompson *et al.* (1996) used the sequential replacement of L and H chains in an scFv construct. L chain variations did not result in significant improvements in affinity, but H chain variations did, the mutations mapping to the CDR2 and CDR3

regions. The highly mutatable *mutD* strain of *E. coli* has a defective proofreading enzyme which results in increased rates of single base substitutions (Fowler *et al.*, 1986). Such a mutation system lacks specificity and would be an extremely inefficient way of generating mutations. Mutations would only rarely occur in the H and L chain genes. A more specific random mutagenic method is that of error-prone PCR (Gram *et al.*, 1992). Here, primers can be used to amplify the areas of concern, i.e., the V region, or more precisely the CDRs alone. By manipulating the conditions for PCR, the cycling can be induced to introduce errors. Such a technique has the potential to generate large numbers of mutations at the antigen-binding site. PCR is also employed in random oligonucleotide synthesis (Barbas *et al.*, 1992b), again targeting important areas such as the CDR region, with the ability to generate as many as 10^{20} sequence combinations.

The more precise method of site-directed mutagenesis has also been applied to the maturation of an antibody *in vitro* (Sharon, 1990). This demonstrated that most mutations leading to higher affinity were in the H chain and the majority of these were in CDR regions. This strengthens the idea that the H chain is the major component in the antigen binding interaction, the CDRs being of greatest importance. This would be useful for studying the effect individual amino acid changes may have on affinity leading to detailed information about the residues involved but would have limited use for generating large numbers of recombinants for affinity maturation and selection.

1.10 CATALYTIC ANTIBODIES

In chemical synthesis, rarely can the chemist match the ability of biological processes to produce a desired product in the enantiomeric form in which it is required. A case in point is the famous thalidomide drug controversy where one racemate suppressed nausea, the other caused teratogenesis in developing foetuses. Chemists also have to search long and hard for an organic or inorganic compound that will promote or catalyse a particular chemical reaction. Attempts have been made to turn to natural enzymes, but these have a tendency to be unwilling to catalyse reactions other than those for which they have been strictly evolved (Danishefsky, 1993). Consequently, chemists have turned their attention to nature's ability to synthesize vast combinations of peptides that interact with an antigen

in a specific way. In this manner, many millions of possible catalysts could be generated, screened and selected in a rapid process.

The area of catalytic antibodies has been extensively reviewed (Schultz *et al.*, 1990; Lerner *et al.*, 1991; Paul, 1996; Wade and Scanlan, 1997). For a chemical reaction to occur, a certain amount of free energy is required (entropy). The higher the entropic barrier, the less likely the reaction is to take place. Catalysts can lower this entropic barrier by increasing the stability of the reactive intermediate in the reaction. These chemical species are usually very short-lived. The concept of catalytic antibodies revolves around the theme that an antibody specific for this unstable intermediate could reduce the amount of free energy required for the reaction and, thus, accelerate it. Due to the short-lived nature and rarity of this intermediate, this brings about obvious difficulties in developing immunization protocols to raise a monoclonal antibody to such a substance as well as difficulties with screening. Attempts have been made to screen for catalytic antibodies raised against the substrate, but this approach has had limited success. Kohen *et al.* (1980) used an antibody to 2,4-dinitrophenyl hapten to enhance the ester hydrolysis of a dinitrophenyl aminohexanoxyl-coumarin conjugate. Although with only limited improvements in hydrolytic rates, it clearly demonstrated the potential of antibodies as catalysts.

Most work has concentrated on raising monoclonal antibodies to stable transition-state analogues of the unstable intermediate (Partridge *et al*, 1993). Antibodies raised using such analogues can increase reaction rates 10^3-fold over the uncatalysed reaction.

Initial attempts at the production of catalytic antibodies were based on chemical reactions that were already well understood. The Diels-Alder reaction is a very useful transformation that allows the production of complex natural compounds. It involves the concerted addition of a conjugated diene to an olefin resulting in a cyclohexene derivative. This reaction has a high entropic barrier but has a highly ordered bicyclic adduct intermediate. A suitable analogue to the intermediate was produced and was used to raise monoclonal antibodies (Hilver *et al.*, 1989). A single catalytic monoclonal antibody was derived that increased the bicyclisation of the two compounds. Meekel *et al.* (1996) also

showed that antibodies to transition state analogues for the Diels-Alder cycloadditions of trans- and cis- piperylenes to 4-nitroso-N-propylbenzamide were greater than 95% in favour of a single regioisomer.

Phosphoryl transfer reactions involving the amino acids threonine, tyrosine and serine are important in biological regulation and signalling and catalytic phosphorylation or dephosphorylation of these amino acids would be useful as an investigative tool. A first step to generating such catalytic antibodies is to produce antibodies capable of aryl phosphate monoester hydrolysis (Scanlan *et al.*, 1991; Tawfik *et al.*, 1997) as in the conversion of *p*-nitrophenyl phosphate to nitrophenol and phosphate. Monoclonal antibodies were raised against a suitable intermediate analogue, α-hydroxyphosphonate conjugated to keyhole limpet haemocyanin (KLH) via a diazo linkage or nitrobenzyl-phosphonate. Several antibodies isolated from both transition state analogues proved to be catalytic for the hydrolysis of *p*-nitrophenyl phosphate.

In their search for catalytic antibodies that catalyse acyl transfer reactions, Jandra *et al.* (1991) describe a technique called 'Bait and Switch'. This is concerned with inducing the inclusion of certain amino acids in the antigen binding pocket. Using charged groups on the analogue of the reactive intermediate that match those on the natural intermediate results in the inclusion of amino acids at the antigen binding site that possess a complementary charge. Such charge complementarity was found to bring about catalytic antibodies. When neutral analogues were used, catalysis was not induced (Kikuchi and Hilvert, 1996).

A further target for those investigating the uses of catalytic antibodies has been the peptide bond. Some peptide bonds are extremely stable. The Gly-Phe peptide bond has a half-life of about 7 years (turnover of $3 \times 10^{-9}.s^{-1}$). Iverson and Lerner (1989) have produced a metal-binding antibody that catalyses this reaction. The antibody was produced using a Co(III) triethylenetetraamine (trien)-Gly-Phe as antigen. The resulting metalloantibody was capable of binding cobalt and other metals and considerably increased the turnover of this reaction to $6 \times 10^{-4}.s^{-1}$.

The importance of metal-binding groups in enzymatic catalysis was further illustrated by Roben (1993) and Roben *et al.* (1993), who produced 'metalloantibodies' by combining light chains which were capable of binding metal ions with gp120-specific heavy chains, producing a Fab fragment with enhanced viral neutralisation capabilities, demonstrating a possible therapeutic use of catalytic antibodies in the treatment of HIV and AIDS. Blackburn and Deng (1993) have used the pComb3 system to derive metalloantibodies mimicking the action of zinc proteases.

In an attempt to deactivate proteins *in vivo*, catalytic antibodies have also been produced that partake in the two-step conversion of asparaginyl-glycyl to isoaspartyl-glycyl via an aspartyl-glycyl intermediate (Gibbs *et al.*, 1992). Wagner *et al.* (1996) have focused on the design of haptens for transition-state analogues of peptide cleavage sites to develop several catalytic antibodies to selectively target peptide bonds.

In a rather more unusual application, catalytic antibodies have been produced that are capable of the enzymatic degradation of cocaine to the non-narcotic ecgonine methyl ester and benzoic acid (Landry *et al.*, 1993). These were produced using phosphonate monoester transition state analogues and could be useful in the treatment of drug addiction.

The strength of catalytic antibody production relies on the efficiency with which the immunological repertoire can be exploited to yield as great a variety of antibody combinations as is possible. Cloning the immunological repertoire in *E. coli* can simplify the selection procedure greatly. Early work combining the search for catalytic antibodies with phage display expression systems in *E. coli* concentrated on the V_H chain gene alone (Sastry *et al.*, 1989) as it has been shown that the H chain still has significant binding affinities in the absence of the L chain (Utsumi and Karush, 1964). Chen *et al.* (1993) used both L and H chains to clone a Fab library to phosphonamidate in a λ vector. Three Fab antibodies were isolated that could hydrolyse a *p*-nitrophenyl substrate.

These examples clearly illustrate the ability of correctly selected antibody molecules to catalyse unfavourable reactions by stabilizing the reaction transition state. Catalytic

antibodies become a more powerful concept when combined with advances in combinatorial phage display libraries.

1.11 ENGINEERED ANTIBODIES IN THE TREATMENT OF DISEASE

Engineered antibody fragments are replacing monoclonal antibodies in many areas, most notably in cancer treatment and other forms of potential *in vivo* therapy, where developments such as antibody chimaerisation and humanisation, as well as other characteristics of antibodies, such as their size, tissue penetration and pharmacokinetic characteristics can be of greatest benefit. Hu *et al.* (1996) produced a disulphide-linked bivalent V_H-V_L-C_{H3} construct against the carcinoembryonic antigen (CEA), a popular tumour target that is found on colon neoplasms. These have been shown to be effective in targeting human colon cancer xenografts in athymic mice, allowing effective tumour imaging when labelled with ^{123}I. An scFv to the testicular and ovarian cancer marker, PLAP was derived from a monoclonal antibody (Deonarain *et al.*, 1997). However, problems with solubility and folding serve to illustrate some of the difficulties in converting established monoclonals to scFv, which can result in misfolding and insolubility.

Pereira *et al.* (1997) have exploited the full potential of combinatorial phage display technology to isolate a library of anti-melanoma Fab antibodies from the PBLs of a remissive melanoma patient treated with allogenic melanoma vaccinations. This resulted in Fab capable of binding to melanoma as well as some other tumour and normal cell types.

In a search for new therapies for viral disease, human combinatorial libraries are being greatly exploited. Research has concentrated on the most serious and chronic forms of viral disease such as AIDS (Burton *et al.*, 1991; Barbas *et al.*, 1992a), and hepatitis B (Zebedee *et al.*, 1992) and secondary viral infections closely related to HIV, respiratory syncytial virus (RSV) and herpes simplex virus (HSV) (Ping *et al.*, 1996; Barbas and Burton, 1996). Other viruses to receive serious attention have been influenza virus (Caton, 1990), cytomegalovirus (CMV) and varicella zoster virus (VSV) (Williamson *et al.*, 1993).

Many human high affinity antibodies have been generated using phage display technologies that show high affinity viral neutralisation properties that require clinical evaluation in the fight against AIDS, hepatitis and other viral diseases.

1.12 INTRACELLULAR IMMUNISATION

Another interesting application to emerge from the eukaryotic expression of antibody fragments is that of intracellular targeting. Here, the antibody molecule is produced inside a host cell using a suitable vector, probably viral. Intracellular production of the antibody could allow it to target within the cell. An IgM antibody to the 4-hydroxy-3-nitrophenacetyl hapten was targeted to the nucleus of a mammalian cell using the SV40 large T nuclear antigen as the L chain leader sequence (Biocca *et al.*, 1990). The plasmids for L and H chain genes were based on human cytomegalovirus (CMV) and the Drosophila heat shock protein hsp70, respectively, using a murine secretory piece and eukaryotic selectable marker genes *gpt* (guanine phosphoribosyl transferase) and *neo* (neomycin acetyl transferase). This system resulted in assembled and nuclear targeted molecules. Such a system could have great therapeutic potential for the destruction of intracellular pathogens such as viruses by producing antibody from within the cell where these organisms are normally hidden from the immune system. Such a system has been described for a scFv antibody against the gp120 coat protein of HIV-1 (Marasco *et al.*, 1993). The scFv was stably expressed and retained in the endoplasmic reticulum. It bound to HIV-1 gp120 inside the cell and inhibited processing of envelope precursor and syncytium formation. Thus, HIV-1 infectivity from these cells could be reduced and its spread to neighbouring cells slowed.

Intracellular targeting can also be used to block and deactivate oncoproteins with the potential to arrest malignant growth (Chen and Marasco, 1996). Wright *et al.* (1997) exploited this potential using an anti-erb-2 scFv antibody targeted to several breast carcinoma cell lines via recombinant adenovirus. This achieved high efficiency down-regulation of erb-2 and resulted in apoptosis in cell lines overexpressing the protein. Transfection of carcinoma cells with such antibodies may be extremely effective in arresting cancerous growth where erb-2 plays a regulatory function.

McMahon and O'Kennedy (1997) have developed a vector system for the intracellular expression of antibody libraries within eukaryotic cells. Clones conferring cytotoxic effects on the cell can be identified and selected from this population. Such intracellular cytotoxic antibodies may have many potential applications such as in therapies for cancer and viral infections such as HIV.

1.13 BISPECIFIC ANTIBODIES

Antibodies are bivalent (bind at two sites, and monospecific (bind a single species). This is for an important reason. Binding of the two Fab arms of the antibody molecule brings about conformational changes in the Fc portion. It is this signal that triggers the effector functions of the antibody molecule, e.g., complement activation or recognition by macrophages. Bivalency also results in the formation of immune complexes. For the antibody engineer, however, who tends only to be interested in the antibody-antigen interaction, such duplication sometimes seems wasteful and serves no additional purpose. It was envisaged that, rather than having a monospecific bivalent molecule, combining Fabs from two heterologous antibodies could result in a bispecific molecule capable of attaching to two different epitopes simultaneously. Such molecules have been termed 'bispecific' antibodies.

The production of bispecific antibodies has been reviewed (Nolan and O'Kennedy, 1990; Nolan and O'Kennedy, 1992). Production can be via either biological, chemical or genetic processes. Two distinct methods exist for biological production of monoclonal bispecific antibodies. The first involves the fusion of two hybridomas expressing antibodies of different idiotypes. Upon fusion, chromosomal mixing occurs and a further hybrid may result which produces H and L chains of both antibody types. This cell has been termed a quadroma because it descends from 4 original parents (two myelomas and two splenocytes). Because of this, yields of bispecific antibody can be low. Such cells also have poor chromosomal stability and may loose the ability to produce some chains.

A second method for the biological production of bispecific monoclonal antibodies uses the fusion of a hybridoma raised against one antigen with splenocytes from an animal

immunized with the second antigen. Such cells are called triomas as they are descended from three parent cells (one myeloma and two splenocytes). The quadroma system is more popular as it is easier to work with, although it does require unpredictable back selection procedures.

Chemical methods have also been developed for bispecific antibody production. Various strategies have been developed with varying degrees of success. An efficient and popular system involves the use of the cross-linking reagent *o*-phenylene dimaleimide, which, under conditions that prevent intersulphide bond formation between SH groups can be linked to one Fab type and then cross-linked to a second Fab when introduced. This system tends to produce yields of up to 50-70% bispecific antibody.

The nature of bispecific antibodies endows them with potential therapeutic efficacy (Nolan and O'Kennedy, 1990). Before bispecific antibodies, chemotherapeutic agents could only be targeted using antibodies via conjugation of the effector molecule directly to the antibody. Such conjugation could have detrimental effects on the antibody or effector substance by altering the therapeutic agent or the antigen-binding site. With bispecific antibodies, one Fab could be directed to the target, the other to the effector molecule. Such a system has several advantages over alternative therapies. As well as having no deleterious effects on the antibody or the drug this system also allows the bispecific antibody and the therapeutic agent to be administered separately. Administering bispecific antibody alone would allow it to target specific areas and non-specific interactions could be allowed to clear before administration of the therapeutic drug. The drug would be delivered more specifically and more safely to the target area.

Antibody engineering has facilitated the production of bispecific antibodies by genetic methods (Holliger and Winter, 1993; Pack *et al.*, 1992; Plückthun and Pack, 1997). Some researchers have found spontaneous assembly of bivalent scFv fragments (McGregor *et al.*, 1994). However, this is a result of dimerisation of non-adjacent L and H chain pairs and is not a reliable means of linking heterologous antibody fragments. Two strategies have exploited natural heterodimeric proteins or amphipathic helices with helix-turn-helix motifs (Pack and Plückthun, 1992). Both methodologies result in the production of stable

heterodimers. One popular heterodimeric protein pair are the leucine zippers, Jun and Fos (Kostelny *et al.*, 1992). The two proteins have a series of leucine residues down one side of an alpha helix which can interdigitate with each other forming a stable protein heterodimer. De Kruif and Logtenberg (1996) developed a bispecific antibody from scFv derived from the Nissim library (Nissim *et al.*, 1994) by mixing separately expressed scFv-Jun or scFv-Fos fusions following expression of the individual fusion products. Bispecific constructs have also been created from single chain Fvs by the fusing the V_H and V_L domains of two distinct scFvs and expressing them in a single host (Holliger *et al.*, 1993). This results in the association of the original V_H and V_L pairings, forming a bispecific 'diabody'. Other less successful linking methods have employed the direct linkage of two antibodies through another protein, e.g. cellulase (Mallender and Voss, 1994).

Many effector substances can be used and have been proposed for use in conjunction with bispecific antibodies. Bacterial and plant toxins are extremely potent cytotoxic agents. Nature has created them in a particular way. Most toxins are divided into two fragments or functional domains: the A (activity) fragment and B (binding) fragment. Only in combination can the toxin bring about its cytotoxic effects. The binding fragment mediates the cellular uptake of the A fragment normally either by mediated endocytosis or by forming a pore in the cell membrane. The A fragment then targets specific cell functions to bring about the destruction of the cell. Without the B moiety, the A chain is completely innocuous. Bispecific antibodies, however, also appear to mediate endocytosis of the A chain. This allows the toxin to be precisely targeted via an anti-tumour Fab arm present as one half of the antibody and the toxin held by the other Fab arm. Such a system has been applied using ricin A chain in prostatic carcinoma (Webb *et al.*, 1985).

Such bispecific antibody systems can also be used to retarget the cytotoxic functions of the immune system itself to tumours rather than using foreign toxins. Just such a bispecific antibody was produced with specificity for the c-*erb*B-2 protooncogene product and the FcγIII receptor (FcγRIII) (CD16) (Hsieh-Ma *et al.*, 1992). The c-*erb*B-2 protooncogene product is found on human breast cancer cells such as the SK-Br-3 cell

line. FcγRIII is found on mononuclear cells and stimulates cytotoxic activity. The bispecific antibody brought about significant lysis of SK-Br-3 cells in the presence of human monocytes. No significant lysis occurred with mixtures of the two parent antibodies demonstrating the bispecificity of the antibody was key in the cytolytic process. Bispecific $F(ab')_2$ fragments derived from the anti-c*erb*B-2 protooncogene anti-FcγRIII bispecific antibody were also as effective as whole antibody.

Clearly, the most popular bispecific construct for tumour therapy combines an anti-tumour marker antibody with an anti-CD3 antibody to bring about T cell lysis of the tumour. This has been done with the tumour antigen HER2 (Shalaby *et al.*, 1992), the multidrug-resistant glycoprotein (van Duk *et al.*, 1989) and uncharacterised antitumour antibodies (Weiner *et al.*, 1994). Other cytotoxic components of the immune system have also been redirected to tumours such as tumour necrosis factor α (TNFα) in combination with anti-CEA in human colon carcinoma Robert *et al.*, 1996). All these treatments show improved targeting of cytotoxic agents over monoclonal antibodies alone.

Some, not content with the bispecificity of antibodies took the system a step further and produced 'trispecific' antibodies composed of three heterologous Fab moieties (Tutt, *et al.*, 1991). $F(ab')_3$ molecules were assembled, all of which had an anti-CD37 arm (used to target to $CD37^+$ tumour cells) and an anti-CD3 arm (to target T cells). The third arm was to a series of cluster designation factors such as CD2, CD4 or CD8. Only trispecific antibodies containing anti-CD2 demonstrated strong cytotoxic targeting to $CD37^+$ tumour cells in the presence of resting T cells. Being able to bring about the targeting and stimulation of resting T cells to tumour cells gives such a trispecific molecule therapeutic potential. Tetraspecific moieties have also been put forward as viable constructs (Adair and Bright, 1993; Pack *et al.*, 1995), with the possibility of targeting more than one tumour marker for greater target specificity, or several cluster designation factors to more effectively recruit immune effector cells.

1.13.1 Bispecific antibody therapy in association with chemotherapeutic drugs

It is becoming clear that for more effective cancer therapies, several strategies must be combined. Antibodies directed to tumours alone are only poorly effective. Bispecific antibodies that target chemotherapeutic and other agents to tumour cells are more effective and reduced the side effects associated with such treatments. Such therapies can be further improved by using engineered antibody fragments from human origin and of smaller size to remove anti-species responses and result in improved pharmacodynamics of the antibody molecule. An engineered human bispecific antibody targeting chemotherapeutic drugs to tumour cells is a tool with great potential for therapeutic success. According to the presently available literature, however, most bispecific therapeutic reagents are still being derived from murine monoclonal antibodies with fusion systems based on quadroma or chemical linkage technology. Either work to date in this area is too young to yield results, has produced data too poor to publish, or is successful enough to warrant secrecy in the interests of patenting such therapies. Indeed, methodologies based on leucine zipper production of bispecific scFv and Fab antibodies have already been patented (Tso *et al.*, 1993; Curtis, 1992). This will limit, or at least slow the development and application of such systems until non-patented strategies can be developed.

1.14 COUMARIN

Coumarin is a ubiquitous secondary plant metabolite found in abundance in plants of the Rutaceae and Umbelliferaceae genus (Keating and O'Kennedy, 1997). In the past, it has had several industrial applications, mostly as a result of its odorous properties, such as a flavouring enhancer and odour stabiliser (Egan *et al.*, 1990). Coumarin has also been found to have several therapeutically useful applications, such as in the treatment of high protein lymphoedema (Casley-Smith and Casley-Smith, 1997), the treatment of burn injuries (Piller, 1997), and the treatment of chronic infections associated with immune suppression, e.g., brucellosis (Egan *et al.*, 1990). However, most research interest in coumarin and its metabolites has focused on their apparent immunomodulatory and anti-carcinogenic effects. To this end, most experimental cancer therapy using coumarin and its derivatives has been concentrated on neoplasias that were traditionally unresponsive

to existing treatments and would normally result in a high percentage of mortality. Three such cancers are prostate cancer (Mohler *et al.*, 1997), renal cell carcinoma (Ebbinghaus *et al.*, 1997) and malignant melanoma (Mohler *et al.*, 1997). The high mortality of these cancers is generally associated with their metastatic properties. Even following radical surgery in all three cases, either by nephrectomy, prostectomy, or skin grafting, the patients are at high risk of developing metastatic lesions which respond poorly to chemotherapeutic intervention or radiotherapy.

Coumarin and its metabolites (principally 7-hydroxycoumarin) have been found to have pleiotropic effects in such treatments (Seliger, 1997). They have been shown to have immunostimulatory properties, such as increasing the proteolytic activity of macrophages, interference with prostaglandin synthesis and their ability to stimulate cytokine production (Zlabinger, 1997), as well as having more direct effects on tumour cell growth and development (Seliger, 1997). Such properties have made the coumarins an important target for anti-cancer chemotherapy research.

Coumarin has other research applications such as its use as a measure of cytochrome P450 activity in liver samples for the monitoring of liver activity and liver damage (Cooke *et al.*, 1997). Coumarin metabolism via the cytochrome P450-dependent pathway can be induced in the presence of other xenobiotic agents such as phenobarbital, cocaine, carbon tetrachloride, caesium and cobalt chlorides and others (Pelkonen, *et al.*, 1997). Use of coumarins in cytochrome P450 assays provides a sensitive fluorimetric substrate. Coumarin metabolism itself has been used in conjunction with lignocaine metabolism as a measure of overall liver function with analysis of the phase I metabolites, 7-hydroxycoumarin and monoethylglycinexilyde in urine (Sotaniemi *et al.*, 1995).

1.14.1 The metabolism and pharmacokinetics of coumarin in man

With its widespread presence in the natural environment and occurrence in foodstuffs, as well as its potential *in vivo* therapeutic applications, the metabolism of coumarin in man and other species has been extensively studied (Pelkonen *et al.*, 1997). Coumarin is metabolised in man primarily via a cytochrome P450-dependent monooxygenase system,

CYP2A6 to the phase I metabolite, 7-hydroxycoumarin (Fig. 1.9). However, this is not the only pathway that exists in man and 3-hydroxylation may take place (Fentem and Fry, 1993). It is believed that at low coumarin concentrations, 7 hydroxylation is most important, but at high doses, the 3 hydroxylation pathway may predominate. The extent and rate of this phase I metabolism in humans is dependent on several factors such as genetics, tissue-type, sex, hormonal activity, chemical induction and the presence of liver disease and damage. This is important because 3-hydroxylation is linked to hepatotoxicity which takes place as a result of coumarin administration. Hepatotoxicity as a result of coumarin administration is rare (0.2-0.4%) (Cox *et al*, 1989). 3-hydroxylation is the predominant phase I pathway in the rat, where administration is linked to hepatotoxicity (Fentem and Fry, 1993). Although 7-hydroxylation is the predominant pathway in man differences in its metabolism due to the factors outlined above may vary the degree of 7-hydroxylation taking place. In the absence of this pathway, 3-hydroxylation may become a significant metabolic route, leading to hepatotoxic symptoms in such individuals (Van Iersel *et al., 1994)*. Phase I metabolism is rapid and extensive following first pass through the liver. Coumarin has a half-life of 1 h following peroral or intravenous administration and as little as 4% reaches the circulatory system following oral administration.

7-hydroxycoumarin is metabolised to the phase II metabolite 7-hydroxycoumarin-β-D-glucuronide via a UDP-glucuronyl transferase (Ritschel *et al.*, 1977) (Fig. 1.10). This highly hydrophilic metabolite is rapidly excreted in the urine. The other more minor secondary metabolic pathway is *o*-sulphation via sulphotransferase enzymes which may predominate if the glucuronidation pathway is blocked or absent (Clarke and Burchell, 1994). Due to the combination of rapid phase I and phase II metabolism, both coumarin and 7-hydroxycoumarin have low levels of bioavailability with near 100% excretion of a range of oral or intravenous doses taking place within 24 h.

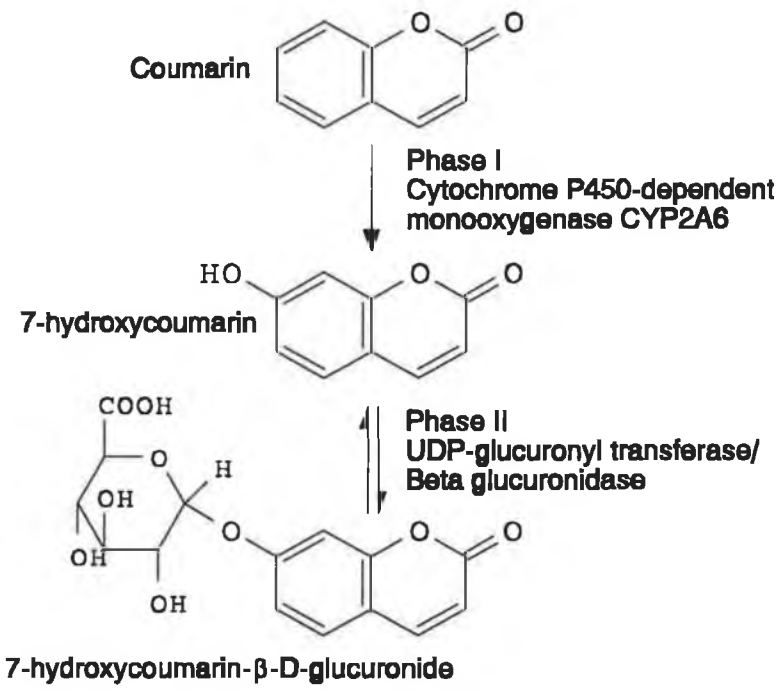


Figure 1.10. The major metabolic pathway of coumarin in man. Phase I metabolism is via a cytochrome P450-dependent monooxygenase liver enzyme which rapidly converts coumarin to 7-hydroxycoumarin. This is then metabolised to 7-hydroxycoumarin-β-D-glucuronide by an endoplasmic reticulum enzyme system, UDP-glucuronyl transferase, with the cosubstrate UDP-glucuronic acid.

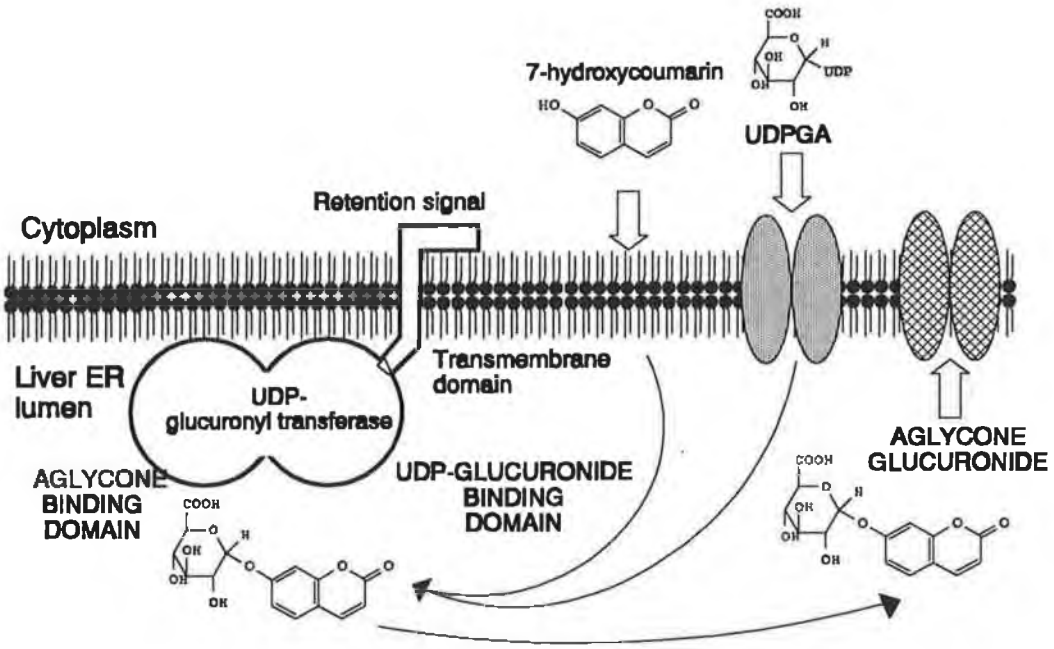


Figure 1.11. Phase II metabolism of coumarin via UDP-glucuronyl transferase. 7-hydroxycoumarin enters the lumen of the endoplasmic reticulum across the phospholipid bilayer. UDP glucuronic acid enters via a specific transporter. Conjugation takes place at the lumenal surface of the ER as the UDP-glucuronyl transferase is anchored in the membrane. The glucuronidated 7-hydroxycoumarin is transported via an aglycone glucuronide transporter for excretion.

1.14.2 Pharmacological role of coumarin and its metabolites

Due to its rapid metabolism to 7-hydroxycoumarin and its low bioavailability it has been suggested that coumarin is a prodrug. Although it has been shown to possess immunomodulatory and anti-proliferative effects on cells (Ebbinghaus *et al.*, 1997), its non-availability has led many to suspect that it may not play a significant pharmacological role (Weinmann, 1997). 7-hydroxycoumarin has been shown to have greater cytostatic properties than coumarin and it is suggested by many to be the active form of the drug (Seliger, 1997).

However, it could be equally well argued that, due to its rapid phase II metabolism, the bioavailability of 7-hydroxycoumarin is also quite low. Its ability to cross membranes via passive diffusion and enter cells is comparable to coumarin, so this does not offer 7-hydroxycoumarin an advantage over the parent molecule. Further investigation is still required to show the relative contribution of these components in the *in vivo* situation.

Glucuronidation is a major detoxification pathway for many xenobiotics. The increase in hydrophilicity normally leads to properties of decreased intracellular localisation due to the inability to traverse membranes and stick to other hydrophobic components such as lipids and proteins. It is thus mostly assumed that glucuronide metabolites do not play any pharmacologically significant roles. However, some glucuronides have been shown to have some pharmaceutical relevance, namely ibuprofen glucuronide (Niemeijer *et al.*, 1989), morphine glucuronide (Salem and Hope, 1997) and ferullic acid glucuronide (Ohta *et al.* 1997) and retinoid glucuronides (Abou-Issa *et al.*, 1997; Biesalski and Schaffer, 1997), and although no direct role for 7-hydroxycoumarin glucuronide has yet been shown to exist, it may exert its effect indirectly via a conjugation-deconjugation cycling mechanism (Casley-Smith and Casley-Smith, 1986, Kauffman, 1994) (Fig. 1.11). 7-hydroxycoumarin glucuronide may enter cells, possibly via a specific transport mechanism, where it becomes deconjugated intracellularly via β-glucuronidase and exerts its cytostatic or cytotoxic effects before being removed from the cells and reconjugated via the liver. Indeed, all body tissues have been shown to possess such β-glucuronidase activity (Bogan *et al.*, 1997). It is clear that

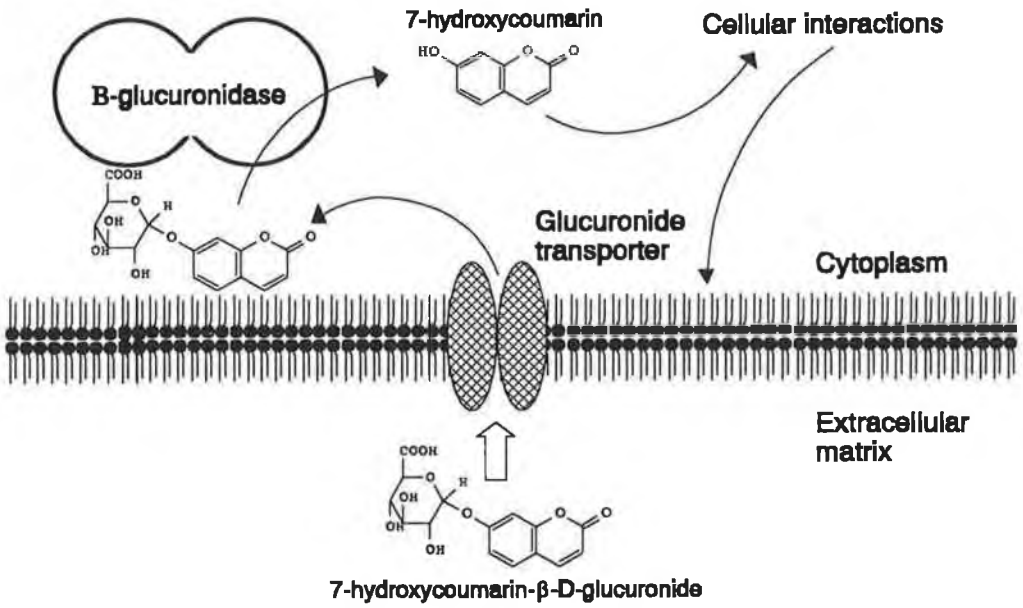


Figure 1.12. Proposed hypothetical cycling mechanism for 7-hydroxycoumarin-glucuronide. 7-hydroxycoumarin-glucuronide circulating in the system prior to excretion enters cells via a hypothetical glucuronide transport mechanism. On entering the cytoplasm, it is deconjugated by the ubiquitous β-glucuronidase enzyme, whereupon, the released 7-hydroxycoumarin exerts its pharmacological effects, before leaving the cell probably by passive diffusion.

further investigation of coumarin, 7-hydroxycoumarin and its glucuronide is still necessary for a full understanding of the mechanisms at work in their effects on cells. To study the use of coumarin as a chemotherapy, monitoring of its metabolism and elucidation of its mode of action, analytical methods must be available to monitor and measure these metabolites, both *in vitro* and *in vivo*, in various matrices, such as blood, urine, faeces, tissues and enzyme preparations.

1.14.3 The analysis of coumarin and its metabolites

1.14.3.1 Physical methods of coumarin analysis

As with most analyses, the techniques available to analyse coumarins has evolved with the technologies available (Bogan *et al.*, 1997). For qualitative analyses, early methodologies used paper chromatography (Swain, 1953; Berlingozzi, 1954), and then thin layer chromatography based on the fluorescent characteristics of coumarin and 7-hydroxycoumarin (Cholerton *et al.*, 1992). Quantitative methods applied have been spectrofluorimetry (Egan and O'Kennedy, 1993a), high performance liquid chromatography (with detection using UV absorbance and limits of detection for 7-hydroxycoumarin as low as 0.3 ng/ml (Bogan and O'Kennedy, 1996) and coumarin as low as 0.6 ng/ml) (Archer, 1988), gas chromatography/mass spectroscopy of coumarin (7.7 ng/ml LOD) (Hawthorne *et al.*, 1988) and capillary electrophoresis using UV detection with LOD of 1 μg/ml (Bogan *et al.*, 1996). Traditionally, 7-hydroxycoumarin glucuronide was measured indirectly via deconjugation of the glucuronide via either chemical or enzymatic treatment to 7-hydroxycoumarin which was then analysed by HPLC (Egan and O'Kennedy, 1992).

1.14.3.2 Immunoanalytical techniques for coumarin analysis

As an alternative to solid state physical methods of analysis, several immunoanalytical procedures have been developed for the analysis of coumarin and its metabolites. Although the immunoanalytical systems so far developed are not yet comparable with physical methods in terms of limit of detection and precision and accuracy, they still have

the potential to be significantly improved. They also offer other advantages such as not requiring large amounts of capital equipment. These can be prepared cheaply, easily and relatively quickly in most laboratories with only a limited amount of technical skill as compared to that required to run HPLC and CE systems. Several enzyme immunoassay strategies have been developed using polyclonal antibodies capable of detecting free coumarin and 7-hydroxycoumarin. These assays are heterogeneous competition ELISAs in which the antibody competes for bound protein-hapten conjugate and free drug. Best limits of detection have been achieved using alkaline phosphatase assay systems with LOD as low as 40 ng/ml (Egan, 1993; Egan and O'Kennedy, 1993; Bogan *et al.*, 1997).

A bispecific antibody was also developed to measure 7-hydroxycoumarin using polyclonal antibodies to 7-hydroxycoumarin and alkaline phosphatase in a chemically cross-linked construct (Reinartz *et al.*, 1996). This measured 7-hydroxycoumarin in the linear range of 0.1 to 1 μg/ml with a LOD of 30 ng/ml and illustrates the usefulness of a bispecific antibody in the immunoanalysis of coumarins.

Polyclonal anti-sera have also been used to measure 7-hydroxycoumarin concentrations using immunosensors. In these electroanalytical systems, protein-hapten conjugates of 7-hydroxycoumarin can be immobilised onto an electrode surface (Deasey *et al.*, 1994). Anti-7-hydroxycoumarin antibody labelled with HRP competes with the immobilised 7-hydroxycoumarin and free 7-hydroxycoumarin. After washing away unbound material, HRP-labelled anti-7-hydroxycoumarin antibodies are left bound to the electrode surface at a concentration inversely proportional to the quantity of free drug. In the presence of hydrogen peroxide, HRP becomes oxidised. This becomes reduced again at the expense of an electron from the mediator, hydroquinone, which itself becomes oxidised and is again reduced by drawing electrons from the electrode surface. Binding of antibody to the immobilised antigen thus results in an increase in the steady state current which can be measured amperometrically.

Another widely used immunosensor technology is bispecific interaction analysis using the Pharmacia Biosensor BIAcore™ system, based on the concept of surface plasmon resonance. Here, a narrow wedge of light is focused onto a fine gold surface at a range

of incident angles. The reflected light is detected by a series of photodetectors. At a certain critical angle known as the SPR angle, a component of the incident light is coupled through the gold film as an evanescent wave. The intensity of the reflected component at this critical angle is greatly reduced and can be measured by the detector. The angle at which SPR takes place, amongst other things is dependent on the mass of the gold film. Increasing the mass of the gold layer will change this SPR angle. Change in mass is proportional to change in SPR angle which is measured in response units. A reactive dextran matrix coupled to the sensor surface facilitates attachment of antibody or antigen components whos mass interaction may be monitored. This system has also been used in the design of a competitive immunoassay for 7-hydroxycoumarin (Bogan *et al.*, 1997). Here, 7-hydroxycoumarin-BSA was immobilised to the sensor surface via EDC/NHS coupling chemistry (Hermanson, 1997). A rabbit polyclonal anti-7-hydroxycoumarin antibody was mixed with a range of free 7-hydroxycoumarin concentrations and was found to be linear and quantifiable from 0.5 to 80 μg/ml. Sensor surfaces could be reused many times with surface regeneration in 5mM NaOH-20mM HCl-5 mM NaOH.

The bispecific antibody of Reinartz *et al.* (1996) was also evaluated using BIAcore™, which confirmed that the antibody was bispecific in nature.

1.14.4 Engineered antibodies for coumarin analysis

As is clear, many physical and immunological methods have been developed for the analysis of coumarin and its metabolites, principally 7-hydroxycoumarin. Immunoanalytical techniques have proved to be cheaper, and simpler, without the associated problems of sample preparation required for HPLC. The success of any immunoassay procedure relies predominantly on the quality of the antibodies being used.

Polyclonal antibody sera are cheap and relatively easy to prepare. However, it is the variation in the quality of polyclonal sera that is normally the problem in immunoassay development. Hybridoma production has given rise to the availability of homogeneous antibody preparations of fixed and measurable properties, but time and cost often limit

their use, and the outcome can often be a limited number of antibody species with unpredictable characteristics that may not be suitable for the analytical system in which it will be used.

Production and selection of antibodies through phage display libraries offers the potential of producing a broader range of monoclonal reagents than hybridoma technology in less time and with less cost. Their genetic nature means that they can also be further manipulated to improve their characteristics, either improving antibody affinity, or by the production of bispecific antibody reagents. If isolated from human origin, their potential clinical use is of great importance, along with their improved pharmacodynamics resulting from their smaller size. As bispecific reagents, novel analytical and therapeutic applications also become possible.

When applied to coumarin research, such benefits from antibody engineering are clear. The availability of reagents that recognise coumarin and its metabolites, 7-hydroxycoumarin and 7-hydroxycoumarin glucuronide would be very useful in their direct analysis without the need for sample preparation and deconjugation etc. As bispecific antibodies with both drug and enzyme specificities, more rapid immunochemical systems could be developed, either for enzyme immunoassays or for use in conjunction with immunosensor systems. Such reagents may also offer therapeutic potential. Bispecific antibodies utilising anti-tumour and anti-coumarin specificities may more effectively target these drugs to the site of action, combatting their low bioavailability. Development of an ADAPT system capable of 7OHCG deconjugaton might also show clinical benefit if conjugation-deconjugation cycling mechanisms are important in the pharmacology of these drugs. In all, recombinant antibodies in coumarin research have the prospect of delivering improved reagents with both analytical and clinical potential.

1.15 AIMS

The specific aims of this thesis were:

1. The development of combinatorial phage display systems for the production of antibodies that detect coumarin and 7-hydroxycoumarin, having the potential for the analysis of these drugs, either alone, or as bispecific antibodies, or in combination with other detection systems. Such bispecific antibodies also have the potential to be used as chemotherapeutic agents.

2. The study of 7-hydroxycoumarin glucuronide: its *in vitro* and *in vivo* metabolism, purification and development of drug conjugates for antibody production. Such antibodies could be applied to the development of immunoassays to 7-hydroxycoumarin-glucuronide to aid in the investigation of its relative contribution in the pharmacokinetics of coumarin and 7-hydroxycoumarin administration.

2. MATERIALS AND METHODS

2.1 MATERIALS

Unless otherwise stated, all standard laboratory reagents were purchased from Sigma Chemical Co., Poole, Dorset, England.

Solvents were purchased from Labscan, Unit T26, Stillorgan Industrial Park, Co. Dublin.

Restriction enzymes were purchased from Boehringer Mannheim UK Ltd, Bell Lane, GB-Lewes, East Sussex England.

Enzyme immunoassay microtitre plates (96 well) were purchased either from Nunc, PO Box 280-Kamstrup DK, Roskilde, Denmark, or Costar, 1 Alewife Ctr, Cambridge MA 02140, USA.

Antibodies were purchased either from Sigma Chemical Co. or Pierce and Warriner (UK Ltd, 44 Upper Northgate Street, Chester, Cheshire, England.

HPLC columns were supplied by Phenomenex, Melville House, Queens Avenue, Hurdsfield Industrial Estate, Macclesfield, Cheshire, SK10 2BW, England.

Molecular biology reagents were purchased from Promega Corporation (2800 Wood Hollow Road, Madison WI 53711-5399), Strategene, Qiagen Ltd (Unit 1, Tillingbourne Court, Dorking Business Park, Dorking, Surrey, RH4 1HJ, England), Gibco BRL Life Technologies Inc. (8400, Helgerman Court, Gaithersburg, MD 20877), Genosys Biotechnologies (Europe) (London Road, Pamisford, Cambridgeshire, CB2 4EF, England), or Molecular Biologische GmbH (Wagenstieg 5, D-37077, Göttingen, Germany) as stated.

Bacterial growth media was supplied by Oxoid, unless otherwise stated.

Cell lines were obtained from the European Collection of Animal Cell Cultures, Department of Animal Cell Resources, Centre for Applied Biology, Porton Down, Salisbury SP4 OJG, England.

BRIclone was supplied by Bioresearch Ireland Ltd, Dublin City University, Glasnevin, Dublin 9.

Coumarin, 7-hydroxycoumarin, and 7-hydroxycoumarin glucuronide were supplied by Schaper and Brummer, Salzgitter, Germany. 7-hydroxycoumarin-BSA and 7-hydroxycoumarin-THG conjugates were both gifts of Declan Bogan (Dublin City University), and coumarin-BSA and 3-acetylamino-7-acetocoumarin were supplied by Denise Egan (Dublin City University). Anti-7-hydroxycoumarin antibody was a gift of Gary Keating (Dublin City University).

The plasmid vector pComb3 was supplied by The Scripps Institute, Department of Immunology, 10666 North Torrey Pines Road, La Jolla, CA 92037, USA.

The Nissim library was supplied by The Medical Research Council, Centre for Protein Engineering, MRC Centre, Hills Road, Cambridge CB2 2QH, England.

The plasmid vector pJuFo was supplied by the Swiss Institute of Allergy and Asthma Research, Obere Strasse 22, CH-7270, Davos, Switzerland

2.2 EQUIPMENT

Standard spectrophotometric measurements were taken using a Shimadzu UV160A (Shimadzu Corp., 1 Nishinokyo-Kwabaracho, Nakagyo-Ku, Kyoto 604, Japan).

Ultracentrifugation was performed on a Beckman L8-70M ultracentrifuge (Beckman Instruments Inc., Bioindustrial Business Unit, Fullerton, CA 92634-3100, USA). High speed centrifugation was carried out on a Sorvall RC-5B (DuPont Instruments, Newton, CT, USA). Bench centrifugation was performed using a Heraeus Sepatech (Heraeus Instruments Inc., 111-A Corporate Blvd, South Plainfield, NJ 07080, USA) and centrifugation of 1.5 ml test tubes was performed using a Heraeus Biofuge 13.

All pH measurements were taken using a Jenway 3015 pH meter (Jenway Ltd, Gransmore Green, Felsted, Dunmow, Essex CM6 3LB, England.

Agarose gel electrophoresis and polyacrylamide gel electrophoresis were carried out using the Atto AE-6100 horizontal gel electrophoresis and AE-6450 vertical gel electrophoresis systems, respectively (Atto Corp., 2-3 Hongo 7-chome, Bunkyo-Kui, Tokyo 113, Japan).

Infrared analyses were performed using a Nicolet infrared spectrophotometer (The Nicolet Instrument Corp., 525-1 Verona Road, Madison, WI 53711, USA).

Freeze drying was performed using Hetosicc, Hetofrig and Hetovac (Heto Laboratory Equipment, A/S Gyderang 17-19 DK 3450, Allerod, Denmark.

High performance liquid chromatographic (HPLC) analysis was performed using either Beckman System Gold 507 autosampler, 126 pump, 166 UV detector and 168 photo diode array (PDA) detector, or Waters Corp. PDA detector (Waters Corp., 34 Maple Street, Milford, MA 01757, USA).

Analysis of microtitre plates was performed using the Titertek Multiscan Twinreader (Flow Laboratories Ltd., Woodcock Hill, Harefield Road, Rickmansworth, Hertfordshire, WD3 1PG, England).

Electrotransformations were performed using a BioRad Gene Pulser (BioRad Laboratories, BioRad House, Maylands Avenue, Hemel Hempstead, Hertfordshire HP2 7TD, England).

2.3 METHODS

2.3.1 General protocols

2.3.1.1 Culture media formulations

LB medium

For the growth of *E. coli* XL1-Blue

Tryptone	10 g/l
NaCl	10 g/l
Yeast extract	5 g/l
Bacteriological agar	1% (w/v)

SOC medium

For the recovery of cells following electrotransformation

Tryptone	20 g/l
Yeast extract	5 g/l
NaCl	0.5 g/l
KCl	2.5 mM
$MgCl_2$	20 mM
Glucose	20 mM
pH	7.0

Glucose and $MgCl_2$ added separately following autoclaving.

2xTY medium

For the growth of *E. coli* HB2151 and TG1

Tryptone	16g/l
Yeast extract	10 g/l
NaCl	5g/l

TYE medium

For the growth of HB2151 and TG1

Tryptone	10 g/l
Yeast extract	5 g/l
NaCl	8 g/l
Bacteriological agar	15 g/l

Terrific Broth

For the growth of *E. coli* XL1-Blue.

Prepared according to manufacturer's instructions with 0.8 % (v/v) glycerol.

Phosphate-buffered saline

All phosphate-buffered saline (PBS) used was according to Dulbecco 'A' formulation (Oxoid), as follows:

Sodium chloride	8 g/l
Potassium chloride	0.2 g/l
Disodium hydrogen phosphate	1.15 g/l
Potassium dihydrogen phosphate	0.2 g/l
pH	7.3

2.3.1.2 Standard molecular biology protocols

Standard molecular biology protocols were performed as described in Sambrook *et al.*, (1989).

2.3.1.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide separating gels from 10% to 20% (w/v) were prepared from 30% acrylamide (29.2% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide) in separating buffer from a 4X stock (1.5 M Tris-HCl, pH 8.8, 0.4% (w/v) SDS). Stacking gels were prepared at 5% (w/v) polyacrylamide in stacking gel buffer from a 4 X stock (0.5 M Tris-HCl, pH 6.8, 0.4% (w/v) SDS). Samples were prepared in 5 X sample buffer (60 mM Tris-HCl, pH 6.8, 25% (w/v) glycerol, 2% (w/v) SDS, 14.4 mM 2-mercaptoethanol, 0.1% (w/v) bromophenol blue) by boiling for 5 min. Samples were run on an Atto AE-6450 minigel gel until samples had reached the base of the stacking gel and then at 20 mA per gel until the dye front had reached the base of the gel. Gels were stained with Coomassie blue, or by silver staining, or were used for western blotting.

2.3.1.4 Coomassie blue staining

Gels were stained in Coomassie blue staining solution (0.2% (w/v) Coomassie blue R250 in 30:10:60 (v/v/v) methanol:acetic acid:water) for 30 min and destained overnight in destaining solution (10:7:53 (v/v/v) methanol:acetic acid:water).

2.3.1.5 Silver staining

Gels were fixed for 30 min at room temp. or overnight at 4°C in 40:10:60 (v/v/v) methanol:acetic acid:water. Protein was oxidised on 0.1% (w/v) potassium dichromate in 100 ml water acidified with 27 µl of 65% (w/v) nitric acid for 5 min while shaking. Following three 5 min washes in water, 0.2% (w/v) silver nitrate was added to the gel for 20 min under shaking, washed for 1 min with water and developed in 29.65% (w/v) sodium carbonate, 0.5% (v/v) formaldehyde, by adding and removing stain as desired. The staining was stopped using fixative solution.

2.3.1.6 Western blotting

Proteins were transferred from acrylamide gels to nitrocellulose using a BioRad semi-dry blotter in the same running buffer for 25 min at 15 V and blocked with blocking solution (5% (w/v) non-fat milk powder, 0.01% (v/v) antifoam A, 0.02% (w/v) sodium azide in PBS for 2 h at room temperature or overnight at 4°C. Intermediate, non-enzyme-labelled antibodies were added in blocking solution for 1.5 h, shaking at room temperature, followed by three 10 min washes in PBS. The nitrocellulose was washed with phosphate-free, azide-free blocking solution (150 mM NaCl, 50 mM Tris-HCl, pH 7.5) three times for 10 min. Alkaline phosphatase-labelled anti-mouse IgG antibody was added to the filter in 5% (w/v) fat-free milk powder in phosphate-free, azide-free blocking solution for 1.5 h, shaking at room temperature. Final washing was four 10 min washes in phosphate-free, azide-free blocking solution, prior to development with 5-bromo-4-chloro-3'-indolylphosphate (BCIP) and nitro blue tetrazolium chloride (NBT) reagents (Promega) in alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5) according to manufacturer's instructions. Colour development was stopped by the addition of 0.002M EDTA in PBS.

2.3.1.7 Dot blotting

Samples of 3 µl were dotted on to nitrocellulose filters, marked off in 0.9 cm x 0.9 cm squares and blotted as for western blots (section 2.3.1.6).

2.3.1.8 Bicinchoninic acid (BCA) assay

10 µl of protein sample were placed in wells of a 96 well microtitre plate (Nunc). 190 µl of BCA assay reagent (Pierce) (50 parts reagent A to 1 part reagent B) were added and incubated at 60°C for 1 h. The absorbance was read on a Titretek Twinreader Plus at 562 nm and compared to a set of protein standards from 0 to 2 mg/ml.

2.3.1.9 Production of anti-VCSM13 antibody in rabbit

VCSM13 phage was used to infect TG1 cells and phage particles isolated according to section 2.3.3.3. 0.5 ml of VCSM13 phage, at 1mg/ml, was mixed with 0.5 ml Freund's Complete Adjuvant and used to inoculate a New Zealand White rabbit at five sites i.d. Three similar boosts were performed in Freund's Incomplete adjuvant at 22, 42 and 124 days. The rabbit was sacrificed by exanguination and the serum prepared according to section 2.3.2.4. 40% (v/v) saturated ammonium sulphate was added to the serum and antibody allowed to precipitate. The precipitate was centrifuged at 4,000 x g for 10 min. and resuspended in PBS. Saturated ammonium sulphate was again added to 40% (v/v) saturation and antibody allowed to precipitate. This was again centrifuged at 4,000 x g for 10 min. The precipitate was finally redissolved in PBS and dialysed overnight at 4°C against 50 volumes of PBS. The sample was applied to a 1 ml protein G column equilibrated using PBS. The column was washed using 5 ml of PBS and the antibody was eluted in 0.1 M formate buffer, pH 2.8. One millilitre fractions were collected into 1.5 ml tubes containing 100 µl of 0.1 M Tris-HCl, pH 8.7. The fractions were analysed for the presence of antibody by measuring the absorbance at 280 nm. Fractions containing protein were pooled and dialysed against 50 volumes of PBS overnight at 4°C.

Rabbit serum was analysed five days subsequent to each immunisation by ELISA with 10 µg/ml VCSM13 phage and 1 in 10,000 horseradish peroxidase-conjugated anti-rabbit IgG antibody according to Campbell (1986).

2.3.1.10 Production and purification of anti-c-myc antibody from mycl-9E10

Mycl-9E10 hybridoma (ECACC) was cultured in Dulbecco's Modification of Eagle's Medium with 10% (v/v) foetal calf serum and 5% (v/v) BRIClone with 100 U/ml penicillin, 0.1 mg/ml streptomycin and 50 µg/ml gentamycin. Culture supernatants were precipitated with 40% (v/v) saturated ammonium sulphate, centrifuged at 2,500 x g for 30 min, resuspended in 10 mM phosphate buffer, pH 7.2 and dialysed against 50 volumes of the same buffer. Antibody was either used directly in enzyme immunoassays, or purified further by DEAE-cellulose chromatography. For DEAE-cellulose chromatography, a column was prepared. 4 g DEAE-cellulose were prepared in 10 mM phosphate buffer, pH 7.2 and used to pour a 15 ml column (3 cm x 2.5 cm diameter). Antibody was applied to the column in 1 ml fractions and washed with 10 column volumes of buffer. Antibody was eluted in 10 mM phosphate buffer, pH 7.2, with a linear gradient from 0 to 0.2 M NaCl. Fractions were collected, assayed, pooled and dialysed against 50 volumes of 10 mM phosphate buffer, pH 7.2.

The titre of anti-c-myc antibody was determined using chequerboard ELISA (Campbell, 1986) with doubling dilutions of antibody starting at 1 in 100 dilution, against doubling dilutions of horseradish peroxidase-labelled anti-mouse IgG beginning at 1 in 100 dilution with 1 in 100 anti-human IgG as control.

2.3.1.11 Standard enzyme immunoassay protocol using polyclonal antibodies

2.3.1.11.1 Non-competitive enzyme immunoassay

Protein-drug conjugates of appropriate type (coumarin-thyroglobulin, coumarin-bovine serum albulin, 7-hydroxycoumarin-bovine serum albumin, 7-hydroxycoumarin-ovalbumin) and concentration (up to 500 µg/ml) were coated onto immunoassay microtitre plate (Nunc) in 100 µl volumes for 2 h at room temperature. Wells were emptied and rinsed once with PBS, dried and blocked overnight with 1% (w/v) BSA at 4°C. The plates were washed once with PBS and dried. Dilutions of antibody were added to wells in 100 µl volumes and allowed to bind for 90 min at room temperature. Plates were washed three

times with PBS/Tween 20 (0.05 %, v/v) and once with PBS and tapped dry. Anti-species antibody conjugated to horseradish peroxidase, in PBS, was added to wells in 100 µl volumes and allowed to incubate at room temperature for 90 min. Plates were again washed with three time PBS/Tween and once with PBS. 0.04 mg/ml *o*-phenylenediamine in 50 mM sodium citrate, 0.15 M sodium phosphate buffer, pH 6.0 with 0.32 µl/ml H_2O_2 was added to each well and incubated at room temperature for 30 min. Substrate reactions were terminated by the addition of 50 µl 4 M H_2SO_4 per well. Absorbance was read at 492 nm.

2.3.1.11.2 Competitive enzyme immunoassay

Competitive enzyme immunoassays were performed as described in section 2.3.1.11.1, except that concentrations of free analyte (coumarin or 7-hydroxycoumarin) of up to 500 µg/ml were mixed with coumarin-protein or 7-hydroxycoumarin-protein conjugate as appropriate at a concentration predetermined by enzyme immunoassay and added to microtitre plate wells following coating and blocking of wells with drug-protein conjugate. Addition of primary and secondary antibodies was also as described in section 2.3.1.11.1.

2.3.1.12 Standard enzyme immunoassay protocol using single chain Fv antibodies

2.3.1.12.1 Non-competitive enzyme immunoassay

Protein-drug conjugates of appropriate type and concentration as described in section 2.3.1.11.1 were coated onto immunoassay microtitre plate (Nunc) in 100 µl aliquots for 2 h at room temperature. Wells were emptied and rinsed once with PBS, dried and blocked overnight at 4°C with 1% (w/v) BSA, ovalbumin or 5% (w/v) non-fat milk in PBS as appropriate. The plates were washed once with PBS and dried. Appropriate dilutions of scFv antibody in either 50 mM Tris, pH 7.4 or PBS were added to wells in 100 µl volumes and allowed to bind for 90 min at room temperature. (All washes and dilutions of anti-coumarin scFv clones took place in 50 mM Tris, pH 7.4 and all anti-7-hydroxycoumarin scFv clones took place in PBS. Wells were washed three times with either 50 mM Tris, pH 7.4 or PBS and 100 µl of a 1 in 400 dilution of anti-c-myc

antibody was added to each well for 90 min at room temperature. The wells were again washed and dried as before and 100 µl of a 1 in 5,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG antibody added to each well and incubated for 90 min at room temperature. Following washing, substrate was added as described in section 2.3.1.11.1.

2.3.1.12.2 Competitive enzyme immunoassay

Competitive enzyme immunoassay was performed as section 2.3.1.12.1, except that free analyte was mixed with antibody following coating and blocking of wells with drug-protein conjugate.

2.3.1.13. Agarose gel electrophoresis

Samples from 10 to 20 µl were prepared in 6x loading buffer (0.25% (w/v) bromophenol blue, 40% (w/v) sucrose). Samples were loaded into wells of agarose gels, typically between 0.8 and 1.2%. Gels were prepared using 50x TAE (242g/l Tris, 57.1 ml/l glacial acetic acid, 100 ml/l 0.5 M EDTA, pH 8.0) and SeaKem GTG agarose (FMC BioPoducts). Ethidium bromide was added to gels at 0.25 µg/ml. Samples were electrophoresed at 8 V/cm for 0.5 to 1.5 h.

2.3.2 Production of murine Fab antibody libraries to coumarin and 7-hydroxycoumarin

2.3.2.1 Production of a coumarin-thyroglobulin conjugate

Production of the coumarin-thyroglobulin (THG) conjugate was performed according to Egan (1993), and involved the production of 6-aminocoumarin diazonium salt from coumarin via the intermediates, 6-nitrocoumarin and 6-aminocoumarin. Briefly, 6-nitrocoumarin was prepared by adding 25 ml concentrated nitric acid to 25 g of coumarin dissolved in 50 ml concentrated sulphuric acid, mixed for one hour and precipitated on ice, and the precipitate filtered and dried at 37°C. The 6-nitrocoumarin was analysed by melting point, thin layer chromatography (TLC) (silica stationary phase, diethyl ether : petroleum ether (90:10 v/v) mobile phase, and infrared (IR) spectral analysis.

6-aminocoumarin was prepared by suspending 4 g of 6-nitrocoumarin in 100 ml hot distilled water, acidified with 4 ml concentrated hydrochloric acid, and reduced by the addition of 8 g reduced iron filings (May and Baker Ltd), maintained at 70°C with mixing for three hours and made alkaline by the addition of solid sodium hydrogen carbonate (pH 8). The hot mixture was filtered and precipitated on ice and dried at 37°C. Melting point, TLC and IR analyses were performed as above.

To conjugate 6-aminocoumarin to THG, 0.25g 6-aminocoumarin was mixed in 25 ml concentrated hydrochloric acid, followed by the addition of 2 ml distilled water, and cooling to 4°C. 2.5 ml of a cold 10% (w/v) sodium nitrite solution was added dropwise and mixed. This was then added dropwise to a solution of 0.2 g THG in 5 ml water, maintaining the pH at 7.0 with the addition of 0.5 M sodium hydroxide and stored at 4°C for two hours. The resulting coumarin-THG conjugate was dialysed extensively against distilled water at 4°C. A control reaction, containing no 6-aminocoumarin, was also performed with THG.

2.3.2.2 Characterisation of the coumarin-thyroglobulin conjugate

The resulting coumarin-THG conjugate was dissolved in dimethyl sulphoxide (DMSO):methanol:water (1:1:1) and analyzed spectrophotometrically. The conjugate was also analyzed by size exclusion chromatography using a Phenomenex SEC 4000 size exclusion column on a System Gold HPLC system using an ultra pure water mobile phase at a flow rate of 0.5 ml/min. Detection was via PDA. The THG control was analyzed on a Waters HPLC with PDA under identical conditions. The conjugate was also analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (section 2.3.1.3) on a 10% (w/v) reducing gel. The conjugate was also analyzed by enzyme-linked immunosorbent assay (ELISA) as follows: coumarin-THG was coated onto a microtitre plate (Nunc) at 50 µg/ml PBS, overnight at 4°C, washed with PBS, blocked with 10 µg/ml bovine serum albumin (BSA) in PBS. Detection of coumarin was via a rabbit anti-coumarin-BSA antibody (Egan, 1993), 1 in 100 dilution, and a horseradish peroxidase (HRP) conjugated anti-rabbit immunoglobulin G (IgG) (1 in 10,000). The ELISA format followed was that of Campbell (1986).

2.3.2.3 Spectrophotometric estimation of drug-protein coupling ratios

Spectrophotometric scans were taken of drug-protein conjugates of known concentration. Protein concentration was estimated at 280 nm as 1 AU = 0.741 mg/ml. A standard curve of drug concentration from 1 to 10 µg/ml was prepared and measured at 320 nm. The concentration of coumarin or 7-hydroxycoumarin in the drug-protein conjugates was calculated from the standard curve. These concentrations were converted to molarities and the molar ratios of drug to protein calculated.

2.3.2.4 Immunisation of mice with coumarin-thyroglobulin and 7-hydroxycoumarin-thyroglobulin

Balb/c mice were immunised with either coumarin-THG or 7-hydroxycoumarin-THG (7OHC-THG). Conjugates were prepared at 50µg in 50µl in sterile PBS. Initially, conjugate was mixed with 100 µl Freund's Complete Adjuvant (FCA). Mice were

immunised at several sites intradermally (i.d.). Mice were boosted after three weeks with 50µg of conjugate in 50µl sterile PBS and 100µl FCA intraperitoneally (i.p.). A final boost three weeks later was made using 250µg of immunogen in 250µl sterile PBS four days prior to the removal of the spleen. A control mouse was not immunised with immunogen. 0.2 ml blood samples were taken five days prior to each immunisation and serum prepared. Blood was allowed to clot for 3 h at room temperature (RT), and the clot allowed to shrink overnight at 4°C. The blood samples were centrifuged (Heraeus Biofuge 13) at 2,000 rpm for 30 minutes and the sera stored at -20°C.

Serum titre was analyzed by ELISA. Nunc microtitre plates were coated with doubling dilutions of either coumarin-bovine serum albumin (BSA) or 7OHC-BSA, starting at 50µg/ml in PBS and coated overnight at 4°C. Doubling dilutions of mouse sera, beginning at 1 in 100 were prepared in PBS, after blocking with BSA. Finally, HRP-conjugated anti-mouse IgG was added at 1 in 5,000 in PBS/BSA. A 1 in 100 dilution of serum from the control mouse was used as the control. Following o-phenylenediamine (OPDA) substrate addition and development, plates were read at 405 nm (Titretek Twinreader Multiscan) according to Campbell (1986).

2.3.2.5 Preparation of mouse spleen messenger RNA

Following immunisation, mice were sacrificed by exsanguination and blood serum stored as previously described. Mouse spleens were removed and transferred to a 5 ml chilled sterile 0.9% (w/v) NaCl solution. Messenger RNA (mRNA) was isolated from the spleens according to the method of Chomezynski and Saachi (1987) as follows. All glassware and reagents were prepared using diethyl-pyrocarbonate (DEPC)-treated ultrapure water and autoclaved at 121°C, 15 p.s.i. for 15 min. This was required to remove all contamination resulting from RNAse enzymes.

Spleens were placed in a glass hand homogeniser in 10 ml denaturing solution (4.0 M guanidine isothiocyanate, 0.25 M sodium citrate, pH 7.0, 0.1 M 2-mercaptoethanol) and gently homogenised. This was followed by the addition of 1 ml 2 M sodium acetate, pH 4.0 and mixed thoroughly, followed by 1 ml saturated phenol and 2 ml

chloroform/isoamyl alcohol (24:1), mixed vigorously for 10 seconds and placed on ice for 15 min.

The homogenate was transferred to a polypropylene tube and centrifuged at 10,000 x g for 20 min at 4°C. The upper layer was transferred to a fresh tube and mixed with an equal volume of isopropanol and incubated for 1 h at -20°C. The precipitated RNA was centrifuged at 10,000 x g for 20 min at 4°C and the pellet resuspended in 3 ml denaturing solution. The RNA was reprecipitated with 3 ml isopropanol at -20°C for 1 h and centrifuged at 10,000 x g for 20 min at 4°C. The pellet was washed in 70% (v/v) ethanol and dried under vacuum for 15 min. Finally, the pellet was resuspended in 3 ml sterile, ultrapure water.

2.3.2.6 Reverse transcription of mouse spleen mRNA

Reverse transcription was performed using the Promega PCR-related Reverse Transcription System. 10µl mRNA preparations were incubated with 5µg (10µl) oligo(dT) primer, and 25 U of rRNAsin ribonuclease inhibitor in a total volume of 40µl at 70°C for 10 min and then cooled on ice. This was added to a mixture of 50µl 10 X reverse transcription buffer, 50µl deoxynucleotide triphosphates (dNTPs) mix, (10 mM each dATP, dCTP, dGTP, dTTP), 100µl $MgCl_2$ (25 mM) and 6µl AMV reverse transcriptase (25 U/µl) in a total of 500µl, incubated at RT for 10 min, 42°C for 2 h and 90°C for 5 min. 1µl RNase H was added and incubated for 2 h at 37°C.

2.3.2.7 Amplification of antibody light and heavy chain genes using polymerase chain reaction

2.3.2.7.1 PCR Primers

PCR primers were obtained from the Genetics Department, Trinity College, Dublin. Primer stock concentrations were 0.2 mM. Primers used were as follows:

Variable Heavy Chain - 5′ Primers

V_HIB	5′-CAG GTG CAG CTC GAG GAG TCA GGA-3′
V_HIIB	5′-CAG GTC CAA CTC GAG CAG CCT GGG-3′
V_HIIIB	5′-GAG GTG AAG CTC GAG GAG TCT GGA-3′
V_HIIID	5′-GAA GTG ATG CTC GAG GAG TCT GGG-3′
V^HVA	5′-GAG GTT CAG CTC GAG CAG TCT GGA-3′

Variable Kappa Chain - 5′ Primers

KI	5′-GAC ATT GAG CTC ACT CAG TCT CCA-3′
KVI	5′-CAA ATT GAG CTC ACC CAG TCT CCA-3′

Constant Heavy Chain 1 - 3′ Primer

CG1	5′-GCA AGG ACT AGT ACC ACA ATC CCT-3′

Constant Kappa Chain - 3′ Primer

Ck1	5′-ACA CTC TCT AGA GTT GAA GCT CTT-3′

2.3.2.7.2 PCR reaction

A stock PCR reaction mixture was prepared as follows:

5µl each 10 mM dNTP stock (dATP, dCTP, dGTP, dTTP)
60µl 25 mM $MgCl_2$
100µl 10 X *Taq* polymerase reaction buffer (Promega)
Sterile ultrapure water to 1 ml

Standard PCR reaction mixture was as follows:

1µl mouse spleen cDNA (0.5-1µg)
1µl each primer stock (20 µM)
2.5 U (0.5 µl) *Taq* polymerase (Promega)
97µl stock PCR reaction mixture

PCR conditions varied depending on individual DNA and primer conditions (Innis and Gelfind, 1990), but typical thermal cycling conditions were as follows:

1. 95°C 5 min
2. 58°C 1.5 min
3. 72°C 1.5 min
4. 95°C 1 min

Steps 2-4 repeated 30 times

5. 58°C 2 min
6. 72°C 5 min

All ramping rates were at 2 s/°C.

2.3.2.7.3 Purification of PCR reaction products

PCR reaction products were precipitated with sodium acetate and isopropanol according to Sambrook, *et al.* (1989), resuspended in 20µl sterile ultrapure water and run on a 1.5% (w/v) agarose gel (SeaKem GTG, FMC BioProducts, 191 Thomaston Street, Rockland, ME 04841, USA) containing 0.5µg/ml ethidium bromide for 1 h at 80 V. The 700 base pair (bp) band was cut from the gel with a sterile scalpel and the DNA purified from the agarose using a Qiaex II purification System (Qiagen), according to manufacturer's instructions.

2.3.2.8 Insertion of antibody heavy or light chain genes into the pGEM-T plasmid vector

Gel purified light or heavy chain genes were ligated into the pGEM-T plasmid vector (Promega) as follows:

50 ng (1 µl) pGEM-T vector
2µl gel purified antibody chain genes
1µl 10 X reaction buffer
1µl ligase (Promega)
5µl sterile ultrapure water

Incubation was for 3 h at 15°C and overnight at 4°C.

2.3.2.9 Preparation of electrocompetent Escherichia coli XL1-Blue cells

Escherichia coli (*E. coli*) XL1-Blue (Stratagene) was streaked onto LB medium with 10µg/ml tetracycline (Tc) and grown for 24 h at 37°C. Single colonies were used to inoculate 10 ml of Terrific Broth (TB) (Sigma), with 2% (w/v) glucose and 10µg/ml Tc and grown overnight at 37°C, 200 rpm. The 10 ml was overnight culture was used to inoculate 1000 ml (2 x 500 ml) Terrific Broth, 2% glucose and 10 µg/ml Tc and grown at 37°C, 200 rpm to an optical density (OD) at 550 nm of 0.8 absorbance units (AU). The culture was chilled on ice for 15 min and centrifuged at 2,500 x g for 15 min at 4°C. The

media was thoroughly decanted and the cells resuspended in an equal volume of ice-cold 10% (v/v) glycerol in sterile ultrapure water, and centrifuged at 2,500 rpm for 15 min at 4°C. This was repeated using half a volume of 10% (v/v) glycerol (500 ml), and the cells were resuspended in a final volume of 20 ml 10% (v/v) glycerol. Cells were aliquoted in 300µl lots, snap frozen in liquid nitrogen and stored at -80°C.

2.3.2.10 Measurement of transformation efficiency of electrocompetent E. coli XL1-Blue cells

300µl of electrocompetent *E. coli* XL1-Blue cells were thawed on ice for 5 min. 2 µl of supercoiled pUC18 plasmid DNA (Stratagene) (100 ng/ml) was added, mixed gently by pipetting and allowed to incubate on ice for 1 min. Cells were pulsed in a BioRad Gene Pulser at 2.5 kV, 200 Ω, 25 µF in a 0.2 cm electroporation cuvette. 1 ml of prewarmed (37°C) SOC medium was added to the cells and pipetted gently, before transfer to a further 2 ml SOC medium and shaking at 200 rpm, 37°C for 1 h. Transformants were plated on LB agar supplemented with 10 µg/ml Tc and 100 µg/ml carbenicilin (Cb) and grown for 24 h at 37°C.

Electrocompetence was calculated as the number of positive transformants per microgram of pUC18 supercoiled plasmid DNA.

2.3.2.11 Transformation of E. coli XL1-Blue with pGEM-T plasmid vector containing light and heavy chain gene inserts

An appropriate volume of ligation mixture from 2.3.2.7 (2, 5 or 10 µl) was added to a 300µl aliquot of electrocompetent XL1-Blue cells and transformed according to section 2.3.2.10. Following 1 h incubation at 37°C, 200 rpm, the material was centrifuged at 2,500 x g, cells were resuspended in a minimal volume of liquid and spread on plates of LB containing 10 µg/ml Tc and 100 µg/ml Cb. Self-ligated pGEM-T vector was distinguished from insert ligation by the addition of 20 µl of 50 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (Promega) with 100 µl 100 mM isopropyl-β-D-galactoyranoside (IPTG) to plates of LB agar 30 minutes prior to use. Plates were incubated for 24 h at 37°C.

2.3.2.12 Isolation of light and heavy chain gene inserts from pGEM-T

Transformed *E. coli* XL1-Blue carrying light or heavy chain inserts were resuspended from LB agar plates in 1 ml sterile distilled water and transferred to 25 ml sterile vials (Sterilin). Cells were pelleted at 2,500 x g for 20 min and plasmid DNA minipreps carried out using Wizard miniprep (Promega) according to manufacturer's instructions. pGEM-T plasmid vector was digested with restriction enzymes, either for the removal of heavy or light chain gene inserts as follows (figures given for the digestion of 1 μg plasmid DNA in 10 μl reaction volumes):

Heavy Chain	Light Chain
10 U *Xho*I	10 U *Xba*I
3 U *Spe*I	5 U *Sac*I
1 μl 10 X Buffer H	1 μl 10 X Buffer A

Samples were incubated for 2 h at 37°C. The digested plasmid DNA was electrophoresed and the 700 bp band excised and purified according to section 2.3.2.7.3.

2.3.2.13 Preparation of pComb3 phagemid vector for insert ligation of antibody heavy chain genes

pComb3 (Scripps Institute) was transformed into *E. coli* XL1-Blue according to section 2.3.2.10. Individual colonies were used to inoculate TB with 2% glucose, 10 μg/ml Tc, 100 μg/ml Cb, and cultures grown overnight at 200 rpm, 37°C. Plasmid minipreps were prepared using Wizard Plus Minipreps (Promega). Plasmid DNA was digested as follows (figures given for the digestion of 1 μg of plasmid DNA in 10 μl reaction volumes):

Heavy chain	Light chain
10 U *Xho*I	10 U *Xba*I
3 U *Spe*I	5 U *Sac*I
1 μl 10 X Buffer	1 μl 10 X Buffer

Samples were incubated for 2 h at 37°C. The digested plasmid DNA was electrophoresed and the 700 bp band excised and purified according to section 2.3.2.7.3.

Ligation of heavy and light chain genes into pComb3 was performed using the Rapid DNA Ligation Kit (Boehringer Mannheim) according to manufacturer's instructions.

2.3.2.14 Transformation of E. coli XL1-Blue with pComb3 plasmid vector containing light and heavy chain gene inserts

Transformation was performed according to section 2.3.2.11.

2.3.3 Production of single chain Fv antibodies to coumarin and 7-hydroxycoumarin

2.3.3.1 The Nissim library

The human synthetic phage antibody single chain Fv (scFv) library (Nissim library) was obtained from the Medical Research Council, Cambridge (Nissim *et al.*, 1994). This consisted of the synthetic scFv library in *E. coli* TG1 cells. All bacterial stocks were maintained at -80°C as 10% glycerol stocks.

2.3.3.2 Growth of the Nissim Library

50 ml 2xTY with 100 µg/ml Cb was inoculated with 50 µl of library cells (approx. 10^8 cells). The culture was grown in a shaking incubator (at 200 rpm) at 37°C, until the OD at 600 nm had reached 0.5 AU. 10 µl VCSM13 helper phage (Stratagene) (10^{11} pfu/ml), were added to 10 ml of culture and incubated at 37°C, static for 30 min, centrifuged at 2,500 x g for 10 min and the cells transferred to 300 ml 2xTY with 100 µg/ml Cb and 25 µg/ml kanamycin (Kn) and incubated overnight at 30°C, 200 rpm.

2.3.3.3 Preparation of phagemid particles

The overnight culture from section 2.3.3.2 above was centrifuged at 5,000 x g for 10 min. The culture supernatant was transferred to 1/5th volume of PEG/NaCl (20% (w/v) polyethylene glycol 8000 in 2.5 M NaCl), mixed and incubated at 4°C for 1 h. This was centrifuged at 5,000 x g for 30 min (Heraeus Sepatech) and the pellet resuspended in 40 ml water. The phage particles were again precipitated with 1/5th volume of PEG/NaCl for 20 min at 4°C and centrifuged at 2,500 x g for 30 min. The supernatant was thoroughly removed and the pellet resuspended in 2.5 ml water, centrifuged at 2,500 x g for 10 min and the supernatant stored at -20°C.

Phage preparations were titred by adding 50 µl serial dilutions of phage preparation to 50 µl TG1 cells grown to an OD at 600 nm of 0.5 AU in 2xTY, 1% (w/v) glucose, incubated for 30 min at 37°C and plated on TYE with 100 µg/ml Cb. Phage titre was calculated as

titre-forming units (TFU) per ml.

2.3.3.4 Affinity selection of scFv antibodies using the Nissim library

16 wells of a Costar microtitre plate were coated with antigen at the appropriate concentration. For 7-hydroxycoumarin, 10 μg/ml 7-hydroxycoumarin-bovine serum albumin, for coumarin, 10 μg/ml coumarin-bovine serum albumin. All antigen was coated in PBS. An N-oxysuccinimide-activated binding plate (Nunc) was coated directly with 10 mg/ml 6-amino coumarin in PBS. All plates were coated overnight at 4°C. The plates were washed three times with PBS and blocked with 1% (w/v) BSA in PBS for 2 h at 37°C. Plates were then washed three times in PBS and 0.5 ml of library (approx. 10^{12} TFU) was placed in 3.5 ml 1% (w/v) bovine serum albumin in PBS and dispensed into 16 wells. The plates were shaken gently at RT for 30 min, followed by static incubation for 90 min.

Unbound phage was removed from wells by shaking the plate over a sink and the plates were washed 10 times with PBS-Tween (0.05 mg/ml Tween 20 in PBS) and 10 times with PBS.

250 μl of sterile elution buffer (1.6 ml 12 M HCl in 150 ml water, adjusted to pH 2.2 with solid glycine and made up to 250 ml) were added to each well and left for 10 min. Eluted phage was transferred to an equal volume of 2 M Tris-HCl pH 8.0 for neutralisation and stored at -20°C. Eluted, neutralised phage was used to transfect 10 ml cultures of TG1 (grown to an OD at 600 nm of 0.5 AU in 2xTY, 1% (w/v) glucose), for 30 min at 37°C. This was transferred to 100 ml of 2xTY, 1% (w/v) glucose and 100 μg/ml Cb overnight at 37°C. Cells were pelleted by centrifugation at 2,500 x g for 30 min and resuspended in 1 ml of 10% (v/v) glycerol and stored at -20°C. This material was used in repeated rounds of growth and selection as described (section 2.3.3.4).

Second and third affinity selection strategies were performed for the production of antibodies to 7-hydroxycoumarin. Three rounds of affinity selection were performed as above using 7OHC-BSA, 7OHC-OVA and 7OHC-BSA in rounds 1 to 3 of affinity

selection, respectively. The blocking solutions in rounds 1 to 3 were BSA, ovalbumin and non-fat milk respectively.

2.3.3.5 Affinity selection of antibodies using BIAcore™

Phage antibody particles from section 2.3.3.3 at a concentration of 5.5 x 10^9 tfu/ml were passed across a sensor chip surface immobilised with 7-hydroxycoumarin-BSA for 5 min at a flow rate of 10 µl/min. The sensor chip surface was washed with HEPES-0.5 M NaCl, pH 7.4 at 100 µl/min for 13.4 min. Glycine buffer, pH 2.2, was passed across the sensor surface at 10 µl/min for 20 min and the eluent was neutralised with an equal volume of 2 M Tris, pH 8.0. Aliquots of eluent were analysed by phage titre at various time periods and the phage counts performed according to section 2.3.3.3.

2.3.3.6 Screening of scFv antibodies by Phage ELISA

Glycerol stocks of affinity-selected scFv antibody were serially diluted in 2xTY and plated onto TYE with 100 μg/ml Cb and grown overnight at 37°C. Viable counts were determined as the number of cfu/ml of stock culture.

2.3.3.6.1 Preparation of clones for phage ELISA

Single colonies were selected from plates and used to inoculate individual wells of a 96 well cell culture dish containing 200 μl 2xTY, 1% (w/v) glucose and 100 μg/ml Cb. The plate was shaken (150 rpm) overnight at 37°C and used as a master plate to inoculate an identical plate. 30 μl of 60% (w/v) glycerol was added to each well of the master plate and stored at -20°C. The second plate was grown (shaken at 150 rpm) for 8 h at 37°C. 25 μl of 2xTY containing 1% (w/v) glucose, 100 μg/ml Cb and 10^9 VCSM13 helper phage per ml was added to each well and allowed to stand at 37°C for 30 min followed by shaking (150 rpm) for 1 h at 37°C. Plates were centrifuged at 2,000 x g for 10 min. The supernatant was discarded and replaced with a mixture of 200 μl 2xTY, 100 μg/ml Cb and 25 μg/ml Kn and grown at 150 rpm overnight at 30°C. The plates were centrifuged at 2,000 x g for 10 min and 75 μl of supernatant was used in the following phage ELISA. An anti-NIP clone (Nissim *et al.*, 1994) was used as a positive and negative control for the phage ELISA as outlined in section 2.3.3.6.2.

2.3.3.6.2 Phage ELISA

Enzyme immunoassay plates (Costar) were coated with 100 μl of either 10 μg/ml 7-hydroxycoumarin-bovine serum albumin, 7-hydroxycoumarin-OVA or 10 μg/ml coumarin-bovine serum albumin, as appropriate and left overnight at 4°C. A positive control well was coated with 10 μg/ml 3-nitro-4-hydroxy-iodophenylacetic acid (NIP)-bovine serum albumin in PBS. A negative control well was coated with 7-hydroxycoumarin-bovine serum albumin or coumarin-bovine serum albumin as appropriate. Plates were washed once with PBS. Plates were blocked with 100 μl 1% (w/v) bovine serum albumin in PBS

(or ovalbumin in the case of the second anti-7-hydroxycoumarin library) for 1 h at RT. The plate was washed three times with PBS-Tween. 75 µl supernatant and 25 µl 4% (w/v) bovine serum albumin (or ovalbumin) in PBS were added to each well for 2 h at RT. Plates were washed three times with PBS-Tween and incubated with 100 µl per well of 1 in 400 dilution of anti-M13 antibody (sheep or rabbit IgG, Genosys, Sigma or raised in rabbits (section 2.3.1.9)). This was followed by a 1 in 800 dilution of HRP-labelled anti-sheep or anti-rabbit IgG. This was incubated for 2 h at RT and plates were washed three times in PBS-Tween. 100 µl substrate (0.04 mg/ml *o*-phenylenediamine in 50 mM sodium citrate, 0.15 M sodium phosphate buffer, pH 6.0 with 0.32 µl/ml H_2O_2) was added to each well and incubated at RT for 30 min. Substrate reactions were stopped by addition of 50 µl of 4 M H_2SO_4 per well. Absorbance was read at 492 nm.

2.3.3.7 Transfer of pHEN1 to HB2151 for screening of soluble scFv antibody expression

Positive isolates from phage ELISA were identified and 100 µl of the phage supernatant was used to transfect 10 ml cultures of HB2151 grown in 2xTY with 1% (w/v) glucose and 100 µg/ml Cb. Transfection was performed according to section 2.3.3.2.

2.3.3.8 Screening of scFv isolates for soluble antibody expression

Glycerol stocks of HB2151 clones from section 2.3.3.7 were plated onto TYE with 100 µg/ml Cb, 1% (w/v) glucose in a 96 well tissue culture plate, and grown shaking (150 rpm) overnight at 37°C. This was used as a master plate to inoculate a second plate containing 200 µl 2xTY per well, 0.1% (w/v) glucose and 100 µg/ml Cb. This was grown, while shaking, until the absorbance was approximately 0.9 AU. 25 µl 2xTY with 100 µg/ml Cb and 9 mM IPTG were added and the plates grown for 16 h at 37°C and 150 rpm to induce soluble antibody production.

Enzyme immunoassay plates were coated with antigen as appropriate (see section 2.3.3.5.2), blocked and washed. 75 µl culture supernatant and 25 µl 4% (w/v) bovine

serum albumin in PBS were added to each well, mixed and incubated at RT for 90 min. Plates were washed three times with PBS-Tween and three times with PBS. 100 µl of a 1 in 400 dilution of anti-c-myc antibody in PBS was added to each well at RT for 90 min. The plates were washed again, three time with PBS-Tween and three times with PBS. 100 µl of a 1 in 100 dilution of a horseradish-peroxidase labelled anti-mouse IgG antibody was added and incubated for 90 min at RT. The plates were washed again as before and developed with *o*-phenylenediamine substrate as in section 2.3.3.5.2.

2.3.3.9 Analysis of the cellular distribution of scFv antibodies

Cultures of HB2151 selected for expression via phage ELISA were grown in 2xTY, 1% (w/v) glucose, 100 µg/ml Cb and induced as described in 2.3.3.8. Cell pellets were analysed for periplasmic expression of scFv by resuspending in 100 µl of PBS, 0.1% (w/v) phenyl methyl sulphonylfluoride (PMSF) and 1.5% (w/v) streptomycin sulphate. They were then subjected to three freeze-thaw cycles and centrifuged at 2,000 x g for 10 min (Heraeus Sepatech). Lysates were analysed by dot blotting (section 2.3.1.7).

2.3.3.10 Large scale scFv antibody production

E. coli HB2151 expressing appropriate antibody clones were inoculated into 5 ml cultures of 2xTY, 1% (w/v) glucose and 100 µg/ml Cb and shaken (200 rpm), overnight at 37°C. These were used to inoculate 500 ml cultures of the same media which were grown to an OD of 0.8 at 600 nm, centrifuged at 2,500 x g for 20 min at RT and resuspended in an equal volume of 2xTY, warmed to 30°C. The culture was again centrifuged at 2,500 x g for 2 min and resuspended in an equal volume of 2xTY, 1 mM IPTG and incubated at 200 rpm overnight at 30°C. Cells were harvested by centrifugation at 10,000 x g for 10 min. Antibody was isolated from culture supernatant, periplasm or intracellular compartments, as appropriate, and this is described in section 2.3.3.11.

2.3.3.11 Production of scFv antibody from intracellular compartments

The centrifuged bacterial pellet from above was resuspended in ice cold 50 mM Tris, pH 8.0, 1 mM EDTA, 100 mM KCl, 0.1 mM PMSF. Cells were disrupted, either by sonication or use of a beadbeater with 50% (v/v) 0.1 mm glass beads and three 1 min pulses followed by 1 min cooling time, on ice. The lysate was centrifuged at 25,000 x g for 20 min at 4°C and the pellet resuspended in either 7.5 M guanidine hydrochloride (Gdn HCl) or 8 M urea with gentle mixing for 2 h at RT. The denatured lysate was centrifuged at 25,000 x g for 20 min at 4°C and dialysed against several changes of 0.1 M Tris, 0.4 M arginine, 2 mM EDTA, pH 8.0 at 4°C over periods of time ranging from 2 days to 2 weeks. The refolded lysate was finally centrifuged at 25,000 x g for 20 min at 4°C.

2.3.3.12 Large scale phage antibody production

E. coli TG1 cells expressing appropriate antibody clones were prepared according to sections 2.3.3.2 and 2.3.3.3.

2.3.3.13 BIAcore™ kinetic analysis of anti-coumarin scFv antibody clones

A 5 min injection at 20 µl/min of scFv antibody at a concentration of 1 nM was passed over both a coumarin-BSA sensor chip surface (1,600 RU) and a BSA control surface (6,000 RU) in HEPES buffer, pH 7.4. After 10 min dissociation phase, the surface was regenerated using HEPES, 0.15 M NaCl, pH 7.4. Observed association and dissociation rates were calculated using BIAevaluation 2.1™ software (Pharmacia Biosensor).

2.3.4 The *in vitro* production of 7-hydroxycoumarin glucuronide

2.3.4.1 Preparation of porcine and bovine UDP-glucuronyl transferase

Porcine or bovine UDP-glucuronyl transferase (UDPGT) was prepared according to Tegtmeier *et al.* (1988). Fresh liver was cut into small pieces and washed in ice cold 0.9% (w/v) NaCl until the supernatant was clear. The supernatant was discarded and the liver was mixed with four times its mass (4 x) of 0.25 M sucrose (20%, w/v). The liver was homogenised (Ultra-Turrax) and centrifuged at 10,000 x g at 4°C for 15 min (Sorvall RC 5B). The collected supernatant was centrifuged at 190,000 x g, 4°C for 90 min (Beckman L8-70M). The resulting microsomal pellet was resuspended in 4 x the mass of ice cold 0.1 M Tris-HCl, pH 8.0, 0.1 mM dithiothreitol (DTT), 0.75% (w/v) sodium cholate, and shaken gently for 30 min. The homogenate was centrifuged at 190,000 x g for 1 h. Saturated ammonium sulphate to a final concentration of 40% (v/v) of saturation, was added gradually to the resulting clear supernatant while stirring. The resultant turbid solution was centrifuged at 10,000 x g at 4°C for 10 min, and saturated ammonium sulphate added to bring it to a final concentration of 70% (v/v) of saturation. The turbid solution was again centrifuged at 10,000 x g, for 10 min at 4°C and the precipitate resolubilised in 4 x its mass of 50 mM Tris-HCl, pH 7.4. It was dialysed against 50 volumes of the same buffer and stored at -20°C. Protein concentrations were measured using the BCA assay (section 2.3.1.8).

2.3.4.2 Initial analysis of the in vitro *production of 7-hydroxycoumarin-glucuronide by HPLC*

4 x 20 ml reaction mixtures, labelled A to D were prepared according to Table 2.1.

Table 2.1. Composition of reaction mixtures for the initial *in vitro* production of 7-hydroxycoumarin-glucuronide.

Component	A	B	C	D
UDP glucuronyl transferase	1.2 mg/ml	1.2 mg/ml	1.2 mg/ml	0 mg/ml
UDP glucuronic acid	2 mM	0 mM	2 mM	2 mM
Tris-HCl, pH 7.4	0.1 M	0.1 M	0.1 M	0.1 M
7-hydroxycoumarin	140 mM	140 mM	0 mM	140 mM
Ethanol	4% (v/v)	4% (v/v)	4% (v/v)	4% (v/v)
Saccharolactone	5 mM	5 mM	5 mM	5 mM
Magnesium chloride	5 mM	5 mM	5 mM	5 mM
Water	To 20 ml	To 20 ml	To 20 ml	To 20 ml

Sample A was the test sample, containing enzyme and both substrates, 7-hydroxycoumarin and UDP glucuronic acid. Sample B lacked UDP glucuronic acid, sample C lacked 7-hydroxycoumarin and sample D lacked UDP glucuronyl transferase. Saccharolactone was present as a β-glucuronidase inhibitor and magnesium ions was act as a cofactor for UDP glucuronyl transferase.

The samples were placed at 37°C revolving at 80 cycles per min, and 100 µl samples removed at various time intervals, precipitated with 20% (w/v) trichloroacetic acid and centrifuged at 13,000 rpm for 10 min at room temperature.

HPLC analysis conditions

HPLC analysis was performed on a System Gold chromatography station with System Gold software, using a Phenomenex Bondclone 10 C-18 reverse phase column (25 cm)

with a flow rate of 2 ml/min and a mobile phase of 700:300:2 water:methanol:acetic acid. Samples of 20 µl were injected with detection at 324 nm. Samples were compared against glucuronide standards from 0 to 100 µg/ml prepared in the same matrix.

2.3.4.3 Repeat analysis of the in vitro *production of 7-hydroxycoumarin-glucuronide by HPLC*

For the repeat analysis of the *in vitro* production of 7-hydroxycoumarin glucuronide, bovine UDP glucuronyl transferase was prepared according to section 2.3.4.1. A reaction mixture was prepared in triplicate according to Table 2.2.

Table 2.2. Composition of the reaction mixture for the repeat *in vitro* production of 7-hydroxycoumarin glucuronide.

Component	Concentration
7-hydroxycoumarin	0.77 mM
UDP glucuronyl transferase	1 mg/ml
Magnesium chloride	6.25 mM
Saccharolactone	6.25 mM
UDP glucuronic acid	1.25 mM
Ethanol	4% (v/v)
Tris-HCl, pH 7.4	125 mM
Water	To 4 ml

The reactions were carried out at 37°C in open 10 ml glass tubes. 200 µl samples were removed and 40 µl 20% (w/v) trichloroacetic acid added, mixed and centrifuged at 13,000 rpm for 10 min. Standards were also prepared (n=4) containing 0 to 1000 µg/ml 7-hydroxycoumarin glucuronide in the reaction components after treatment with trichloroacetic acid. 190 µl of sample was then added to 10 µl of a 1 mg/ml 4-hydroxycoumarin internal standard. These were analysed by HPLC.

HPLC conditions

Chromatography was performed on a System Gold chromatography station using System Gold software, with a Phenomenex Bondclone 10 C-18 column. This analysis used a gradient method to increase the retention time of 7-hydroxycoumarin-glucuronide, and allow for the simultaneous analysis of 7-hydroxycoumarin, 7-hydroxycoumarin-glucuronide and the internal standard 4-hydroxycoumarin. Solvent A was 950:50:2 water:methanol acetic acid (v/v/v). Solvent B was 100% methanol. The flow rate was 1 ml/min with detection at 320 nm. The gradient was as follows (all percentages are v/v):

0-14 min: Solvent A (100%) to Solvent A (50%)/Solvent B (50%)
14-22 min: Solvent A (50%)/Solvent B (50%)
22-23 min: Solvent A (50%)/Solvent B (50%) to Solvent A (100%)
23-32 min: Solvent A (100%)

7-hydroxycoumarin-glucronide concentration was calculated by calculating the peak height ratios of 7-hydroxycoumarin-glucuronide and the internal standard, 4-hydroxycoumarin.

2.3.4.4 Analysis of the in vitro *production of 7-hydroxycoumarin-glucuronide by capillary electrophoresis (CE)*

An enzymatic reaction mixture was prepared according to Table 2.2 to a total volume of 1.6 ml. Reactions were carried out in open 2.0 ml test tubes. Fractions were removed and analysed by CE. A standard curve of 7-hydroxycoumarin-glucuronide was prepared (n=3) in the same matrix as the reaction mixture, except that the enzyme solution was denatured prior to addition to the reaction mixture by boiling for 5 min.

CE separation conditions

Separation was carried out on a Beckman capillary electrophoresis instrument, P/ACE System 5500 using System Gold software according to the method developed by Bogan *et al.* (1996) for the analysis of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide. The capillary used was a 57 cm x 50 μm i.d. untreated silica capillary with a capillary-to-detector distance of 50 cm. The capillary was conditioned each day by 10 min rinses with 0.1 M hydrochloric acid, followed by 0.1 M sodium hydroxide, then deionised water and finally electrolyte buffer. Between each run, the capillary was rinsed for 0.8 min with 0.1 M NaOH, then a 2.5 min rinse with electrolyte solution. Samples were applied by 10 second pressurised injections at 0.5 p.s.i. Separation was achieved at 30 kV (rise time 0.2 min) at 25°C with detection at 320 nm. Typical running current was 170 μA.

2.3.5 Purification of 7-hydroxycoumarin-glucuronide

2.3.5.1 Chromatographic separation of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide

A series of chromatographic experiments were performed using a Lichroprep RP-18 reverse phase silica column, 15 cm x 1 cm i.d. prepared in methanol. Buffer flow was maintained via a peristaltic pump with flow rates in the order of 0.2 to 0.4 ml/min. Various mobile phases, pump speeds and fraction intervals were assessed for the effective separation of pure mixtures of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide. Fractions were analysed spectrophotometrically at 320 nm.

2.3.5.2 Chromatographic purification of 7-hydroxycoumarin-glucuronide from crude enzymatic reaction mixtures

Chromatographic conditions utilising a mobile phase of 50:50 methanol:water (v/v) was selected for the separation of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in crude enzymatic reaction mixtures derived from section 2.3.4, with flow rates typically in the order of 0.2 ml/min. Various sample volumes were assessed for their ability to be effectively separated under the given conditions. Relevant fractions of purified 7-hydroxycoumarin-glucuronide were analysed by HPLC according to section 2.3.4.2.

2.3.5.3 Chromatographic purification of 7-hydroxycoumarin-glucuronide from coumarin-treated urine

A healthy human volunteer was administered a 1 g p.o. dose of coumarin in 10 x 100 mg tablet doses subsequent to provision of a control urine sample. Urine from the treated volunteer was collected at 2 h. Control and treated urine samples were stored in aliquots at -20°C until use. Thawed samples were centrifuged and filtered through 0.45 μm sterile acetate filters.

Chromatographic separation of 100 μl aliquots of control urine, control urine spiked with

7-hydroxycoumarin-glucuronide and treated urine samples were performed as described in section 2.3.5.2.

2.3.5.4 Development of a methodology for the extraction of 7-hydroxycoumarin-glucuronide from aqueous solutions

4 ml of coumarin-treated urine was extracted with 2 ml of DCM:methanol at varying ratios from 10:90 to 95:5 (v/v) in 10 ml glass tubes. Tubes were rotated on a blood tube mixer for 30 min at room temperature. The organic phase was removed and extracted into a further 1 ml of 1 M KCl. The organic layer was dried and resuspended in 400 µl water and analysed spectrophotometrically at 320 nm.

2.3.5.5 Extraction of 7-hydroxycoumarin-glucuronide from enzymatic reaction mixtures and coumarin treated patient urine

Further extractions of either urine samples or enzymatic reaction mixtures were performed using a DCM:methanol ratio of 70:30 (v/v). Extracted samples underwent chromatography according to section 2.3.5.2.

2.3.5.6 Assessment of the thermal stability of 7-hydroxycoumarin-glucuronide at 60°C

A 1 mg/ml solution of purified 7-hydroxycoumarin-glucuronide was prepared in distilled water and maintained at 60°C in a water bath. Aliquots were taken and analysed by HPLC according to section 2.3.4.2. The 7-hydroxycoumarin-glucuronide peak area was monitored over time.

2.3.5.7 Assessment of the stability of 7-hydroxycoumarin-glucuronide to a range of different pH conditions

Samples of purified 7-hydroxycoumarin-glucuronide were prepared in aqueous solution over a range of pH values from 2 to 12.

2.3.5.8 In vitro enzymatic production of 3-amino-7-hydroxycoumarin-glucuronide

2.3.5.8.1 Preparation of 3-amino-7-hydroxycoumarin

3-amino-7-hydroxycoumarin was prepared from 3.5 g 3-acetylamino-7-acetocoumarin according to Bogan (1996). Briefly, 3.5 g 3-acetylamino-7-acetocoumarin was refluxed in 250 ml of 3 M hydrochloric acid for 30 min until all the solid present had reacted and turned orange. The pH was neutralised with solid $NaHCO_3$. The resultant yellow compound was filtered under vacuum, dissolved in a minimal volume of boiling water and an equal volume of methanol, added and the whole solution placed on ice. The recrystallised orange-yellow compound was filtered and tested by HPLC (section 2.3.4.2) employing photodiode array detection and infrared spectral analysis.

2.3.5.8.2 In vitro enzymatic production of 3-amino-7-hydroxycoumarin-glucuronide

0.1 g of 3-amino-7-hydroxycoumarin was dissolved in 1 ml water and placed in the reaction mixture described in Table 2.1. The mixture was incubated for 4 h at 37°C, precipitated with 1 ml 20% (w/v) trichloroacetic acid and centrifuged to precipitate protein. The sample was analysed by HPLC according to section 2.3.4.2 using photodiode array detection.

2.3.5.9 Production of a 7-hydroxycoumarin-glucuronide-bovine serum albumin conjugate

42 µmoles of 7-hydroxycoumarin-glucuronide (14.2 mg), 52 µmoles N-hydroxysuccinimide (1.87 mg) and 42 µmoles EDC (19.17 mg) were added to 270 mg bovine serum albumin (20:1 molar excess of 7-hydroxycoumarin-glucuronide to BSA) in 3 ml carbonate buffer, pH 9.6 and 1 ml dimethyl sulphoxide. The reaction mixture was stirred overnight at room temperature and dialysed against ultrapure water and freeze dried. The resulting conjugate was analysed by SDS-PAGE (see section 2.3.1.3) and HPLC size exclusion chromatography according to section 2.3.2.2 with photodiode array analysis. The reaction was also performed without 7-hydroxycoumarin-glucuronide.

Further examples of 7-hydroxycoumarin-glucuronide-bovine serum albumin conjugates were synthesised. A second conjugate was synthesised using borate buffer at pH 8.5 with a 200:1 molar excess of 7-hydroxycoumarin-glucuronide and a third was also prepared at this molar ratio. at pH 7.0

3. PRODUCTION OF MURINE Fab ANTIBODY LIBRARIES TO COUMARIN AND 7-HYDROXYCOUMARIN USING THE pComb3 SYSTEM

3.1 INTRODUCTION

3.1.1 Antibody phage display systems

Combinatorial antibody phage display technology, such as that developed by the Scripps Institute. La Jolla, San Diego, CA, has the potential to produce and screen large libraries of monoclonal antibodies to an antigen of interest. The pComb3 system utilises the technique of phage display, to mimic antibody presentation on the surface of B cells, with the linkage of genotype and phenotype resulting from the presence of only a single light and heavy antibody chain combination present on a phagemid particle within that phage. The enrichment technique of affinity selection, or panning, allows the selective amplification of phage particles carrying antibody specificities of interest from the rest of the population. Association of a single light/heavy chain gene combination with the correct protein expression is guaranteed by the characteristics of filamentous phage infection of *E. coli*, in which only a single phage particle can infect a cell, ensuring the correct linkage of phenotype and genotype (section 1.6.1).

3.1.2 Source and quality of material for library construction

Antibody chain genes have, in the past been isolated from several sources, notably from areas containing high B cell populations such as lymphoid tissues and blood. For the production of human antibody libraries, this has posed greater difficulties, as the best organ for B cell isolation in terms of quality and quantity of B cells is bone marrow. Although donation of bone marrow for such methodologies has been arranged, such procedures if performed routinely would be difficult to justify on ethical grounds. This would apply to other lymphoid tissues also. The second problem encountered with human monoclonal antibody production is lack of immunisation. Although naive, unimmunised libraries have been created and used effectively, it is still thought that the most reliable means of isolating high affinity light and heavy chain recombinations from such libraries is facilitated by immunisation. During this process, the events giving rise to large numbers of B cells expressing antibodies of high affinity occur. Placed in the context of antibody library production, such immunisations may result in the more reliable selection of high

affinity light and heavy chain recombinants. For the production of murine antibody libraries, the problems associated with immunisation are overcome. For the most effective library production from mice, immunisation would be recommended, followed by splenectomy, and isolation of B cell mRNA from this source.

3.1.3 Production of hapten-protein conjugates

For the development of an immune response to haptens such as coumarin and 7-hydroxycoumarin, the haptens must be coupled to a large, immunogenic component, normally a protein (see section 4.1.1). Traditionally, several proteins are used in this regard: BSA, THG, keyhole limpet haemocyanin (KLH), and ovalbumin (OVA). Each of these exhibit different characteristics, making them more or less suited to various applications. Traditionally, BSA has been reserved for use during screening because of its low cost, whereas THG is used for immunisations, having the advantage of creating slightly less soluble conjugates, which characteristically, provoke a better immune response (Hermanson, 1997).

3.1.4 T/A cloning vectors

T/A cloning vectors such as pGEM-T (Promega) have been developed for the simplification of the insertion of products from polymerase chain reaction into vectors. Polymerase chain reaction (PCR) of DNA fragments using *Taq* polymerase results in the characteristic 5′ adenylation of the amplified fragments. This characteristic has been employed for PCR fragment cloning using T/A cloning vectors (Promega). The pGEM-T vector is a modification of pGEM, which has been linearised and blunt ended followed by thymidylation of both 5′ blunt ends. Such vectors allow the direct insertion of PCR fragments without the need for restriction digestion of the PCR fragments prior to insertion. T/A cloning is generally less efficient than restriction/ligation using standard six-cutter restriction endonucleases, which result in four nucleotide overhangs. It does, however, circumvent problems associated with PCR fragment ligation in that effective restriction digestion at sites introduced close to both the 3′ and 5′ ends of the PCR fragment can not be easily validated due to the very small size change in the PCR

fragment, and the small size of the cut ends. Insertion into pGEM-T, followed by restriction digestion should yield a PCR fragment, which has now been correctly digested and should be ideal for further cloning.

3.2 RESULTS

3.2.1 Preparation of coumarin and 7-hydroxycoumarin protein conjugates

Diazo-coupled conjugates of coumarin and 7-hydroxycoumarin to BSA and 7-hydroxycoumarin coupled to THG were donated by Egan (1993). These conjugates had all been previously characterised by HPLC size exclusion chromatography and photodiode array analysis. Estimation of the coupling ratios of drug to protein were performed. Coumarin-BSA was estimated to possess approximately 12 moles coumarin per mole of BSA. This compared excellently with the estimates made by Egan (1993) of 12 molecules coumarin per molecule of BSA. 7-hydroxycoumarin-BSA was estimated to have approximately 3 moles of 7-hydroxycoumarin per mole of BSA.

BSA conjugates of coumarin and 7OHC were further analysed by ELISA using rabbit anti-coumarin and rabbit anti-7OHC antibodies (Egan, 1993). Both showed specific responses for the drug-protein conjugate alone, but not to protein (Figs. 3.1A and B)

3.2.2 Production of coumarin-thyroglobulin (coumarin-THG) conjugate

Coumarin-THG was prepared according to Egan (1993). This required the production of 6-nitro-coumarin (6-NO_2-coumarin), which was then converted to 6-amino-coumarin (6-NH_2-coumarin). These were analysed by infrared spectrophotometric, TLC and melting point analysis and identified as the correct species. The IR spectrum of 6-NO_2-coumarin (Fig. 3.2) shows a stretch at 1,736 cm^{-1} (-C=O), and 1,350 and 1,526 cm^{-1} (-NO_2 symmetrical and asymmetrical stretch). The IR spectrum of 6-NH_2-coumarin (Fig. 3.3) shows a stretch at 1,699 (-C=O) and 3,322 and 3,402 cm^{-1} (-NH_2 asymmetrical and symmetrical stretch, respectively)

6-amino-coumarin was coupled to THG according to Egan (1993). Following freeze drying, the resulting conjugate exhibited particularly poor solubility in water and other aqueous buffers as well as in organic solvents such as methanol, ethanol and DMSO. This lack of solubility would make it difficult to analyse by HPLC. Eventually, an effective

solute was found in the combined use of dimethyl sulphoxide (DMSO), methanol and water. C-THG was dissolved in 1:1:1 DMSO/MeOH/water (v/v/v), adding DMSO first, then MeOH and then water. A spectrophotometric scan showed characteristic peaks at 280 and 320 nm (Fig. 3.4), indicating the presence of coumarin and protein in the same sample. In HPLC analysis and photodiode array detection, two major peaks occurred, one at approximately 13 minutes - as expected for THG, which has a molecular weight of approximately 300 kDa (Fig. 3.5). The PDA scans of both these peaks give similar profiles characteristic of protein conjugated to coumarin (Fig. 3.6). This is manifest in the shoulder at approximately 320 nm. which is not present in THG alone.

Analysis of the coumarin-THG conjugate was also performed using ELISA, again using a rabbit anti-coumarin antibody (Egan. 1993). This also showed specific response for the antibody to the conjugate, and not to protein alone (Fig. 3.7).

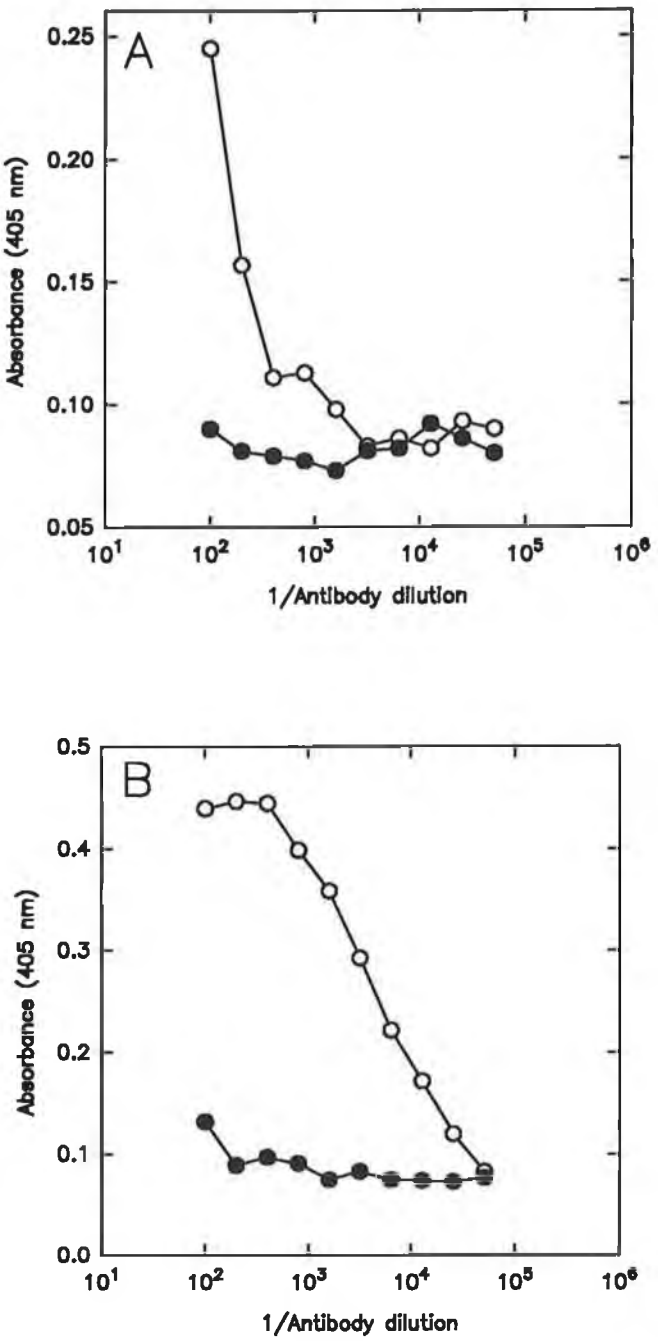


Figure. 3.1A and B. Analysis of coumarin-BSA and 7-hydroxycoumarin-BSA (7OHC-BSA) conjugates by ELISA. The response of the rabbit anti-coumarin antibody (A) was specific for the coumarin-BSA conjugate (50 µg/ml) (open circles), and did not recognise BSA alone (filled circles). The response of the rabbit anti-7OHC antibody (B) was specific for the 7OHC-BSA conjugate (50 µg/ml) (open circles), and did not recognise BSA alone (closed circles).

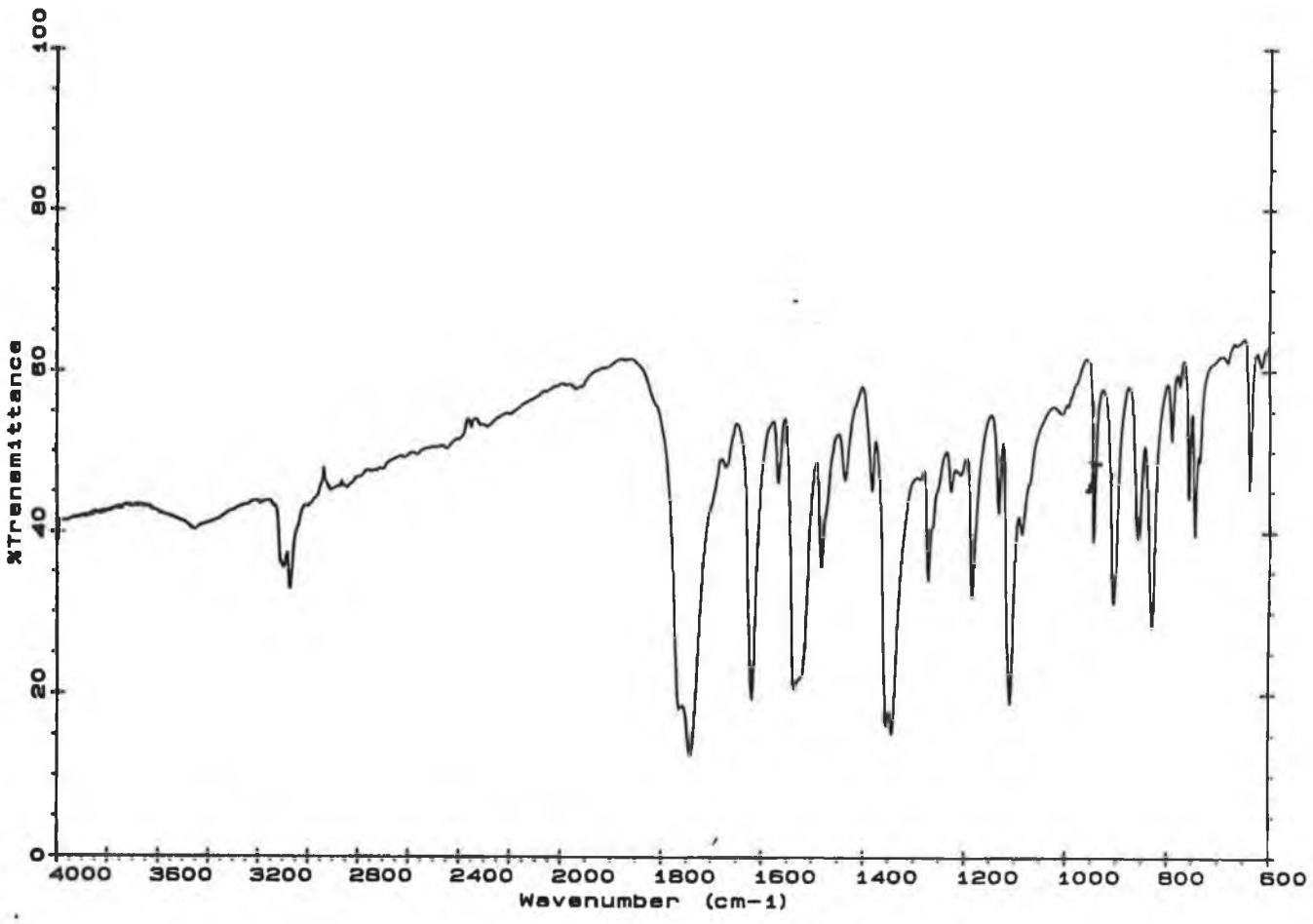


Figure 3.2. Infrared spectrum of 6-NO_2-coumarin from 600 to 4000 cm^{-1}. This shows a stretch at 1,736 cm^{-1} (-C=O), and 1,350 and 1,526 cm^{-1} (-NO_2 symmetrical and asymmetrical stretch), indicating the presence of an NO_2 present on the coumarin molecule.

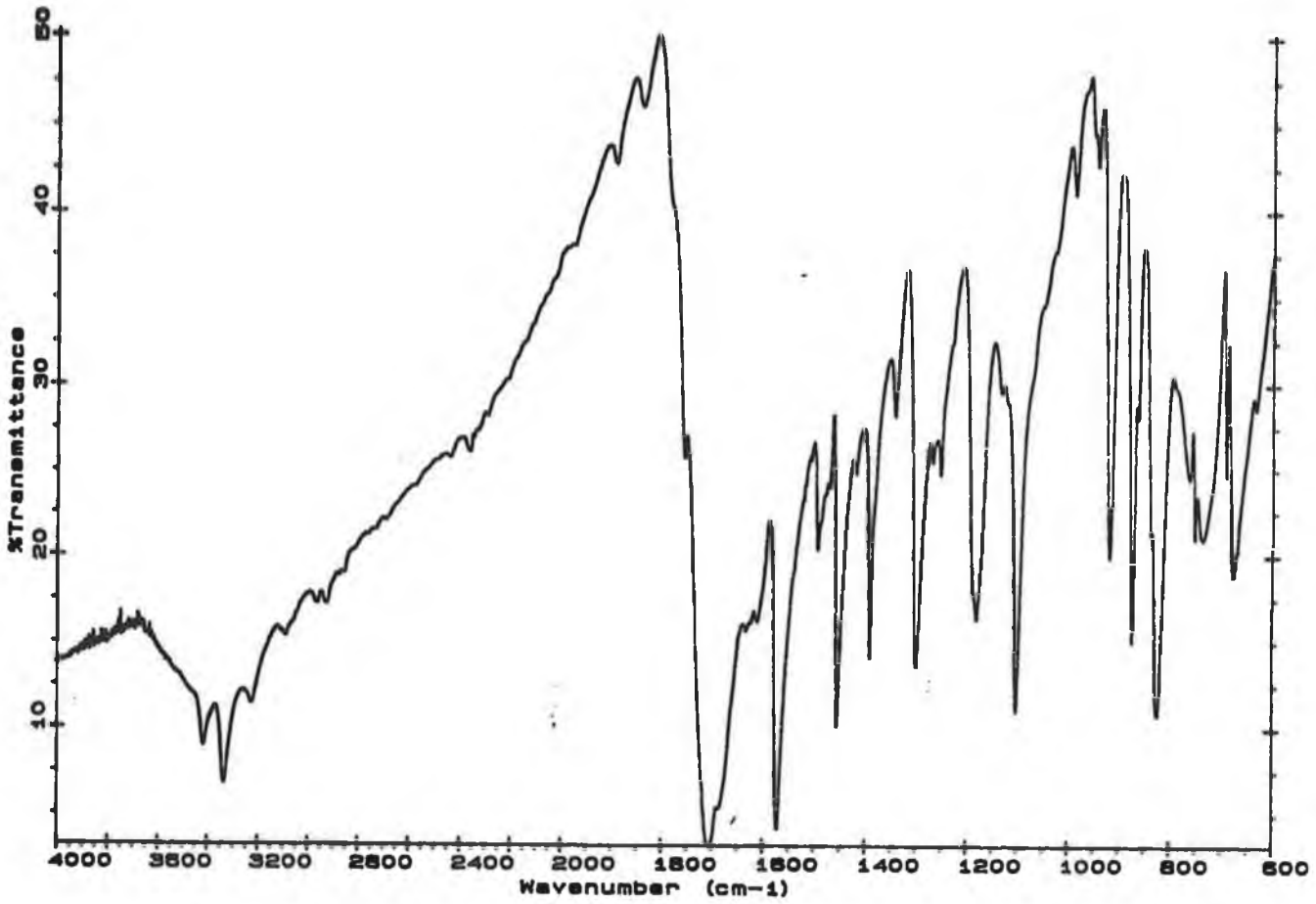


Figure 3.3. Infrared spectrum of 6-NH_2-coumarin from 600 to 4000 cm^{-1}. This shows a stretch at 1,699 (-C=O) and 3,322 and 3,402 cm^{-1} (-NH_2 asymmetrical and symmetrical stretch, respectively).

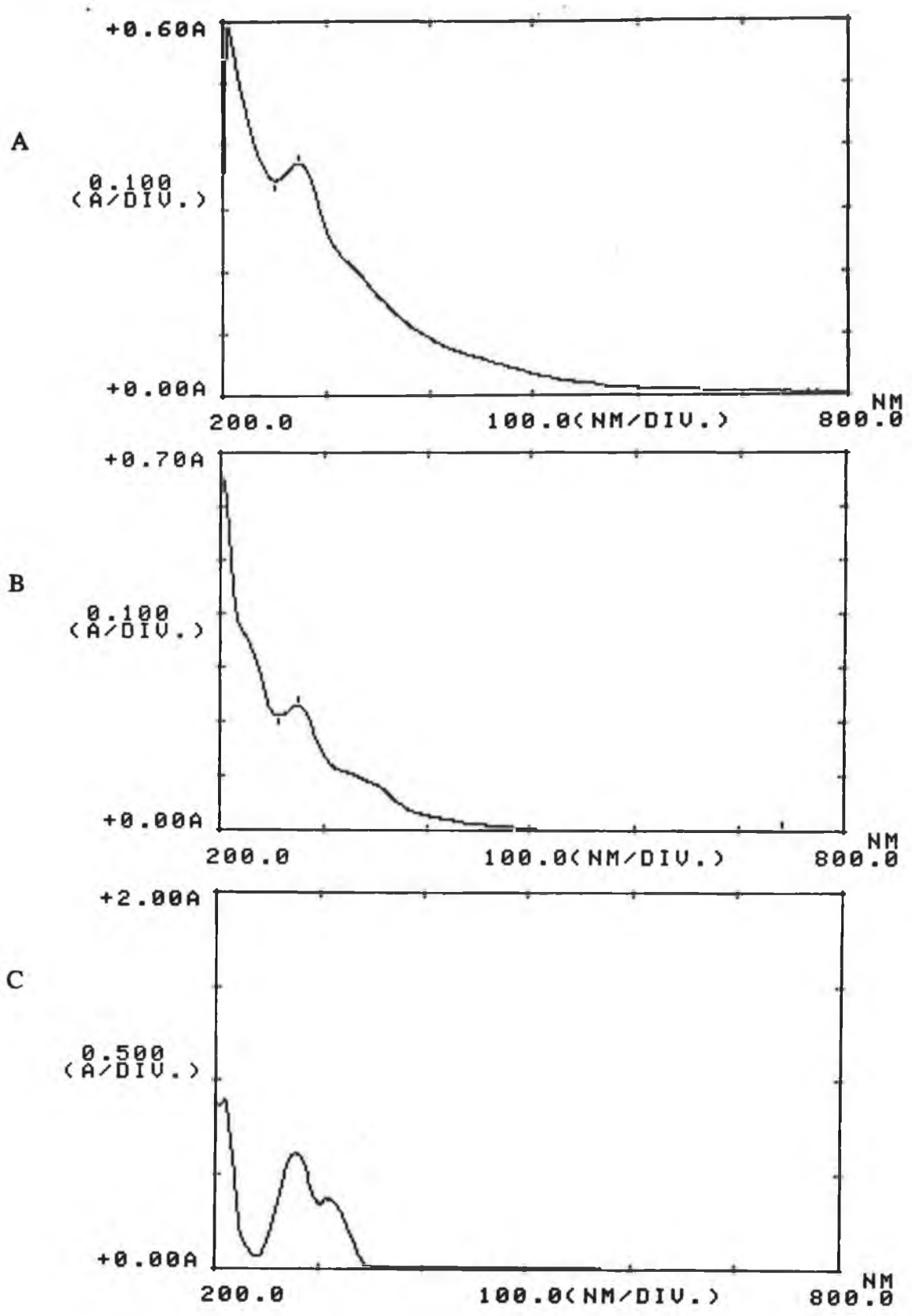


Figure 3.4. Spectrophotometric scan of thyroglobulin (THG) (A), coumarin-THG (B) and coumarin (C) from 400 to 200 nm. Comparison of the coumarin-THG with THG shows a distinct shoulder between 320 and 350 nm on the coumarin-THG sample that was not present on THG. This was indicative of the linkage of coumarin to THG.

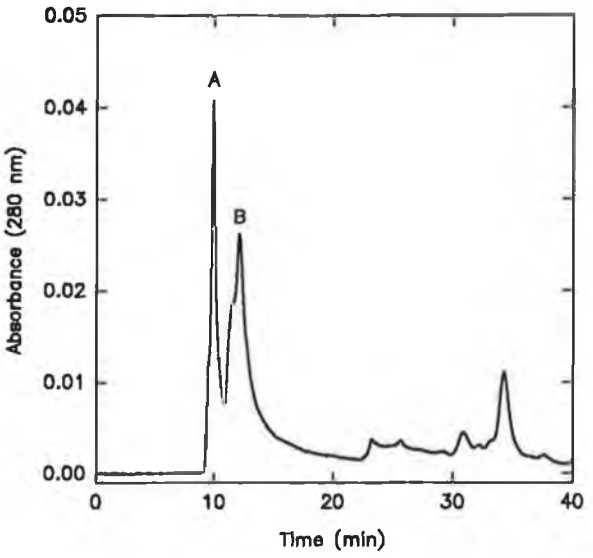


Figure 3.5. HPLC analysis of coumarin-thyroglobulin by size exclusion chromatography. The chromatogram shows predominant 280 nm peaks at 9.967 (peak A) and 12.167 (peak B) minutes. The retention time of 10 minutes corresponds to the retention of a large molecular weight protein such as thyroglobulin (300 kDa).

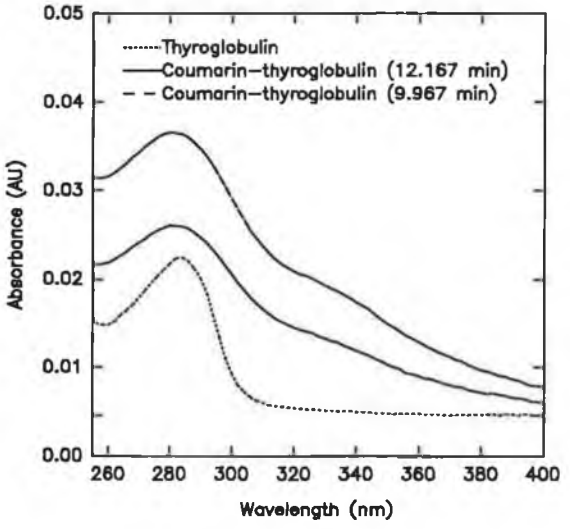


Figure 3.6. Photodiode array analysis of coumarin-THG. Protein peaks at 9.967 (peak A, Fig. 3.5) and 12.167 minutes (peak B, Fig. 3.5) show an additional absorbance shoulder at 320 nm. This was not present on the thyroglobulin control, indicating attachment of a coumarin-like molecule to the protein.

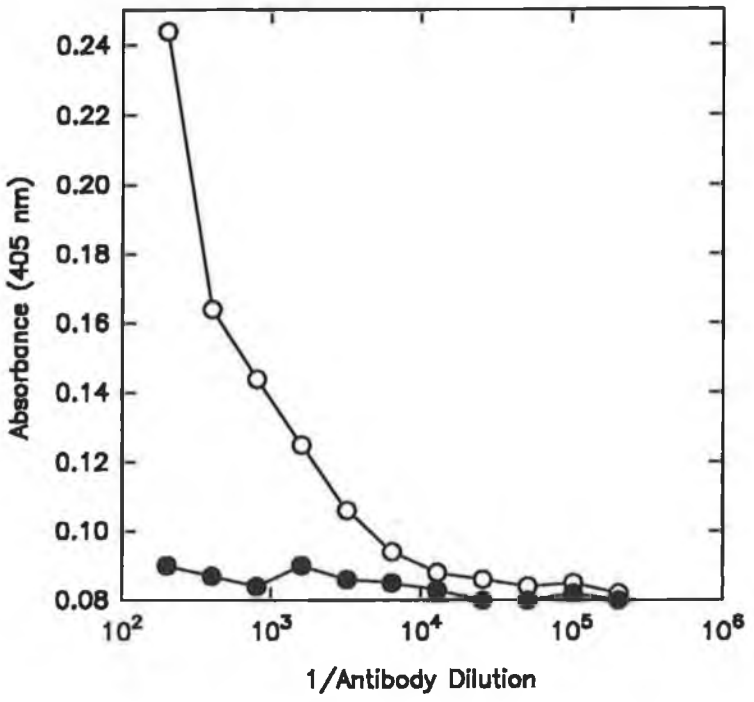


Figure 3.7. Analysis of coumarin-THG conjugate by ELISA with rabbit anti-coumarin antibody. The graph shows recognition of the coumarin-THG conjugate (50 µg/ml) (open circles), but not THG alone (filled circles).

3.2.3 Immunisation of mice with coumarin-THG and 7-hydroxycoumarin-THG

Titres of mouse serum were performed following initial and secondary immunization. Responses to coumarin and 7OHC were made using the appropriate BSA conjugate. The two mice immunised with coumarin-THG, mice 2 and 3, showed titres in excess of 1 in 10,000 following primary immunisation and 1 in 20,000 following secondary immunisation, as compared to a 1 in 100 dilution of control mouse serum (mouse 1) (Figs. 3.8A, B, C and D). Mice 4 and 5, immunised with 7OHC-THG showed titres in excess of 1 in 200 following secondary immunisation, as compared with a 1 in 100 dilution of control serum. These serum titrations were deemed high enough responses from which to prepare splenomic RNA following a final intravenous boost, as according to Campbell (1986).

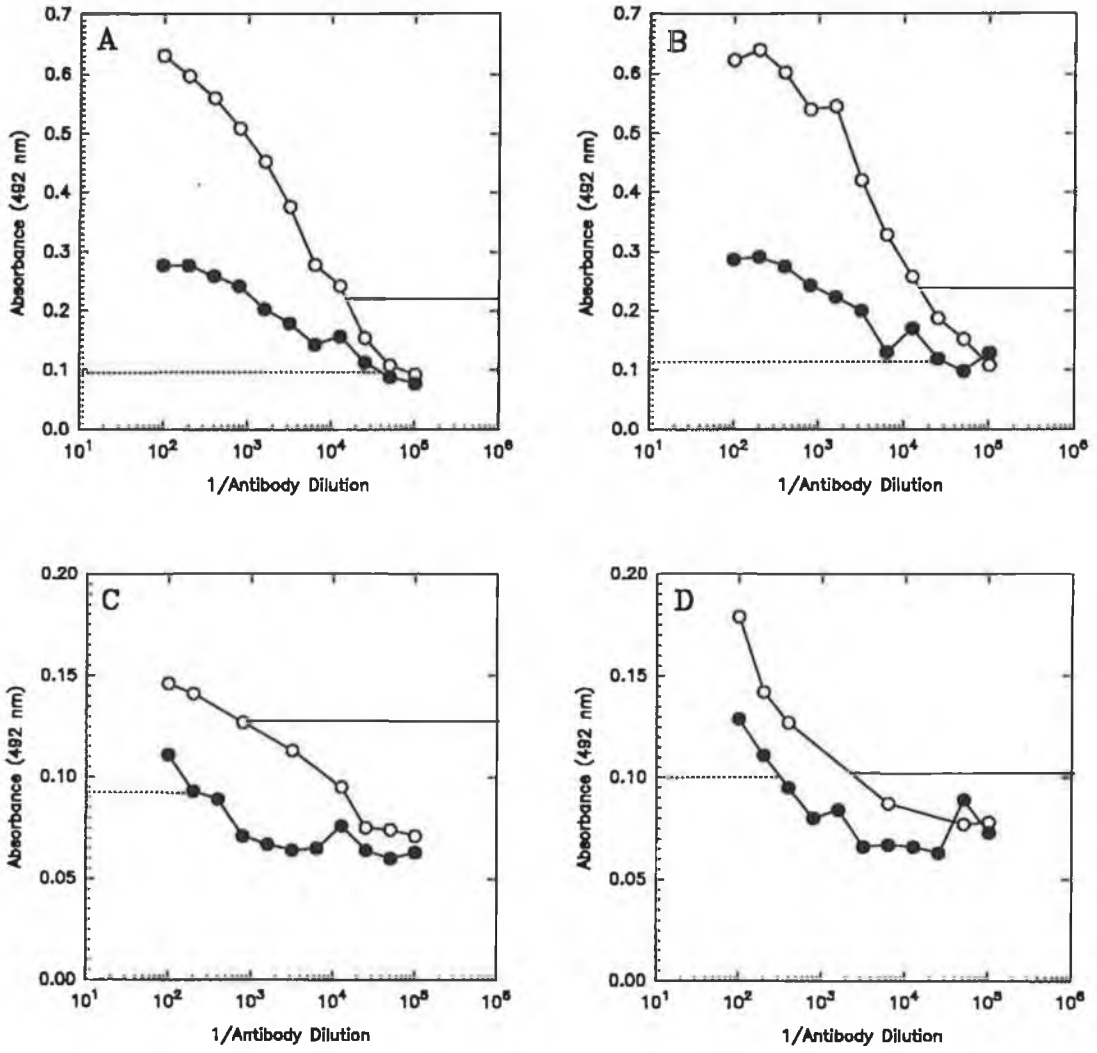


Figure 3.8 A, B, C and D. Mouse serum titres following primary (open circles) and secondary (closed circles) immunisation with either coumarin-THG (A and B), or 7OHC-THG (C and D). Both mice immunised with coumarin-THG gave similar titres of 1 in 12,800 as compared to 1 in 100 dilution of mouse control serum (solid line) following first immunisation and rising to 1 in 25,600 as compared to 1 in 100 dilution of mouse 1 control serum (dotted line) following secondary immunisation. In the case of mice 4 and 5 immunised with 7OHC-THG, titres following primary immunisation were 1 in 400 as compared to 1 in 100 control serum (solid line) and between 1 in 200 and 1 in 400 as compared to 1 in 100 control serum (dotted line) following secondary immunisation.

3.2.4 Preparation of splenomic RNA

Splenomic RNA was isolated from spleens from mouse 2 (coumarin-THG) and mouse 5 (7OHC-THG), yielding mRNA concentrations of 924 μg/ml and 720 μg/ml, respectively. Spleens from mice 3 and 4 were placed in storage at -80°C.

3.2.5 Selection of murine PCR primers based on homology plots

Homology plots of available murine 5′ heavy and light chain gene family sequences (Kabat and Wu, 1986) (Figure 3.9) were prepared (Figure 3.10). Primers were selected according to levels of sequence homology. A sequence already closely represented by a homolog was excluded, as mismatching during annealing may give rise to amplification of these species also. Sequences with no similar homologs were included. Using heavy chain primer sequences V_HIB, V_HIIB, V_HIIIB, V_HIIID and V_HVA resulted in at least 83% homology with primers. For light chains, inclusion of primers V_kI and V_kVI gave at least 83% homology with known sequences, except for V_kII with only 75% homology. The ability of primers to amplify non-specific sequences was not tested further. This rationale was merely chosen to reduce the numbers of primers required for library preparation.

Murine Heavy Chain 5′ Primers:

V_HIA	5′-GAG CTG CAG CTC GAG GAG TCA GGA-3′
V_HIB	5′-CAG GTG CAG CTG GAG GAG TCA GGA-3′
V_HIIA	5′-GAG GTC CAG CTC GAG CAG TCT GGA-3′
V_HIIB	5′-CAG GTC CAA CTC GAG CAG CCT GGG-3′
V_HIIC	5′-GAG GTT CAG CTC GAG CAG TCT GGG-3′
V_HIIIA	5′-GAG GTG AAG CTC GAG GAA TCT GGA-3′
V_HIIIB	5′-GAG GTG AAG CTC GAG GAG TCT GGA-3′
V_HIIIC	5′-GAA GTG AAG CTC GAG GAG TCT GGA-3′
V_HIIID	5′-GAA GTG ATG CTC GAG GAG TCT GGG-3′
V_HVA	5′-GAG GTT CAG CTC GAG CAG TCT GGA-3′

Murine Light Chain 5′ Primers

V_kI	5′-GAC ATT GAG CTC ACT CAG TCT CCA-3′
v_kII	5′-GAT GTT GAG CTC ACC CAA ACT CCA-3′
V_kIII	5′-GAC ATT GAG CTC ACC CAG TCT CCA-3′
V_kIV	5′-GAA AAT GAG CTC ACC CAG TCT CCA-3′
V_kV	5′-GAC ATC GAG CTC ACC CAG TCT CCA-3′
V_kVI	5′-CAA ATT GAG CTC ACC CAG TCT CCA-3′

Figure 3.9. Murine heavy chain and light chain gene family-specific 5′ PCR primers, incorporating the restriction sites (underlined) for XhoI and XbaI in the heavy and light chain genes, respectively.

Murine Variable Heavy Chain

	IA								
IA	100	**IB**							
IB	83	100	**IIA**						
IIA	83	79	100	**IIB**					
IIB	62.5	71	83	100	**IIC**				
IIC	75	75	92	71	100	**IIIA**			
IIIA	71	75	75	58	71	100	**IIIB**		
IIIB	79	71	75	58	71	83	100	**IIIC**	
IIIC	79	75	75	58	71	83	83	100	**IIID**
IIID	62.5	71	67	58	71	83	75	83	100	**VA**
VA	92	75	92	75	92	71	72	79	62.5	100

Murine Variable Light Chain

	kI					
kI	100	**kII**				
kII	75	100	**kIII**			
kIII	92	79	100	**kIV**		
kIV	79	67	87.5	100	**kV**	
kV	83	71	83	71	100	**kVI**
kVI	75	62.5	83	87.5	67	100

Figure 3.10. Homology plots of murine variable heavy and variable light chain gene family-specific 5′ primers, as described in Kabat and Wu (1986).

3.2.6 Murine PCR Primers

The physical characteristics of the murine PCR primers selected are shown in Table 3.1.

Table 3.1. Characteristics of murine antibody gene PCR primers.

Name	Absorbance (260 nm)	Concentration (mg/ml)	A_{260}/A_{280} ratio	kg/mole	Concentration (nM)
CGI	0.314	1.884	1.4004	7.353	0.26
V_HIB	0.234	1.404	1.6731	7.530	0.19
V_HIIB	0.352	2.112	1.4477	7.216	0.29
V_HIIIB	0.356	2.136	1.6953	7.585	0.28
V_HIIID	0.481	2.886	1.6230	7.576	0.38
V_HVA	0.216	1.296	1.5791	7.496	0.17
C_kI	0.385	2.310	1.5193	7.365	0.31
V_kI	0.281	1.686	1.4535	7.045	0.24
V_kVI	0.279	1.674	1.4628	7.304	0.23

3.2.7 PCR optimization

Using the standard PCR protocol (section 2.3.2.7), single amplified bands of approximately 700 bp, corresponding to amplified variable region genes were achieved using the following specific PCR conditions:

V_HIB/CG1	Annealing temp. 58°C
V_HIIIB/CG1	Annealing temp. 59°C
V_HIID/CG1	Annealing temp. 58°C, 10 μM concentration of PCR primers
V_HVA/CG1	Annealing temp. 58°C, 10 μM concentration of PCR primers
V_kI/C_k1	Annealing temp. 58°C
V_kII/C_k1	Annealing temp. 58°C

Primers V_HIIB/CG1 failed to amplify under a variety of conditions which involved the variation of annealing temperature, adjustment of $MgCl_2$ concentration and variation of primer concentration (Fig. 3.11), so was excluded from the group.

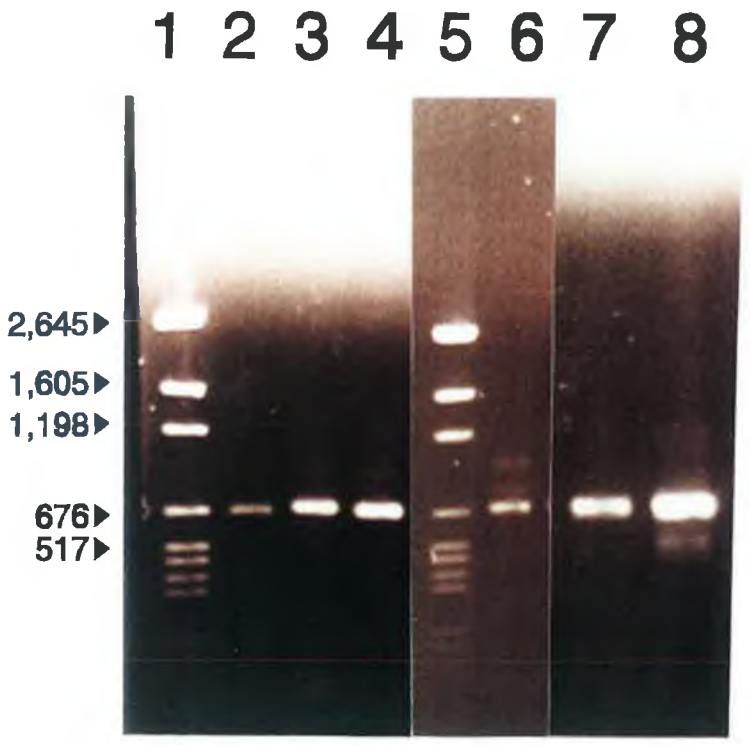


Figure 3.11. Optimisation of PCR conditions for the amplification of murine heavy and light chain genes from mouse 5 immunised with 7-hydroxycoumarin-THG. Lanes: 1,5: pGEM molecular weight markers (bp); 2: V_HIB; 3: V_HIIID; 4: V_HVA; 6: V_HIIIB; 7: V_κI; 8: V_κII. The gel shows specific amplification of 700 bp fragments using primer combinations CG1/V_HIB, CG1/V_HIIIB, CG1.V_HIIID, V_HVA, C_k1/V_kI and C_k1/V_kVI under the conditions described in sections 2. and 3. , but failed to amplify CG1/V_HIIB over a variety of conditions (not shown).

3.2.8 Preparation of pComb3 for heavy chain insert ligation

Plasmid pComb3 was digested with either *XhoI/SpeI* or *XbaI/Sac*I restriction enzymes for either heavy and light chain insertion, respectively. Digestion with *XbaI/Sac*I is shown in Fig. 3.12. This resulted in a predominant fragment of approximately 4,000 bp, as expected for a linear fragment of pComb3. No difference in molecular weight could be detected between single digested (*Xba*I) and double digested (*XbaI/Sac*I) pComb3. This was also the case for digestion for heavy chain insertion and digestion with *Xho*I and *Spe*I (data not shown).

Restriction-ligation controls were performed on pComb3 using *Xho*I and *XbaI/Spe*I (Fig. 3.13). Digestion with these two enzymes resulted in two distinct fragments of 3,073 and 956 bp. When the stuffer fragment was religated with the major fragment, significant ligation was observed, with the production of various multimers. When the fragments had been treated with calf intestinal phosphatase, however, there were only minor levels of ligation. This indicated both efficient ligation and dephosphorylation of plasmid DNA.

3.2.9 Measurement of the Electrocompetence of *Escherichia coli* XL1-Blue

Many batches of electrocompetent *E. coli* XL1-Blue were prepared with typical efficiencies of 2 x 10^8 transformants per microgramme plasmid DNA, using 100 pg supercoiled pUC18.

3.2.10 Insertion of mouse 5 heavy chain genes into pGEM-T

Test transformations of mouse 5 heavy chain genes ligated into *E. coli* showed 125 transformants/µl of ligation mixture. The pooling and concentration of ligation mixture resulted in the production of approximately 2 x10^3 transformants. Transformations were repeated until a library of approximately 4.75 x 10^5 heavy chain inserts was built up.

3.2.11 Insertion of mouse 5 variable light chain genes into pGEM-T

A variable light chain gene library in pGEM-T of approximately 3.2 x 10^5 light chain gene inserts was built up by pooling the results of several transformations. The plasmid library was analysed by restriction digestion (Fig. 3.14). Single digestion with *Xba*I yielded a single fragment of 3,700 bp and with *Xba*I and *Sac*I, two predominant fragments of 2,950 bp, 700 bp and a small 50 bp fragment.

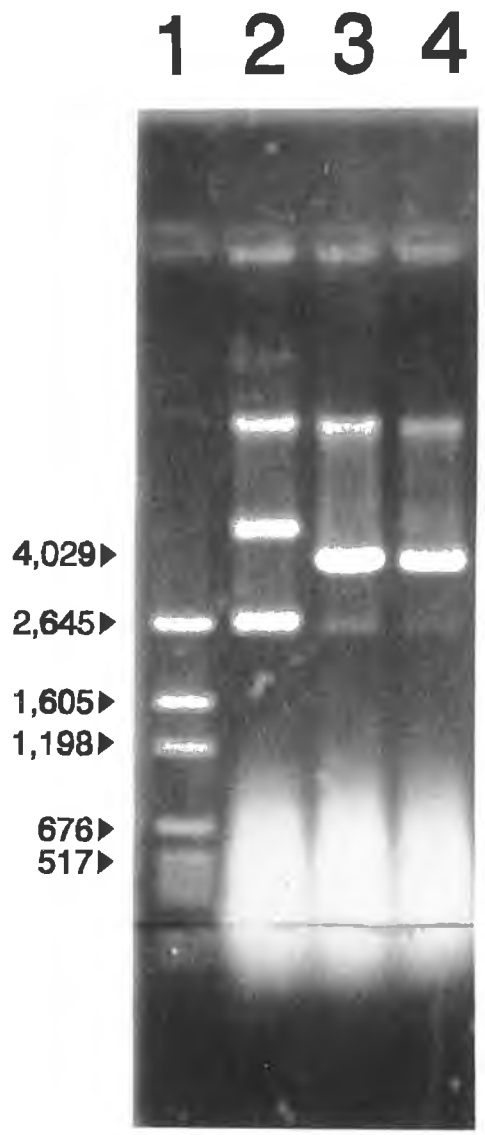


Figure 3.12. Restriction digestion analysis of pComb3. Lanes: 1: pGEM molecular weight markers (bp); 2: pComb3; 3: pComb3 XbaI digest; 4: pComb3 XbaI/SacI digest. pComb3 shows multiple bands indicating the presence of covalently closed circular and open circular DNA. Following either single or double digestion, an approximately 4,000 bp fragment resulted. No difference in molecular weight between single and double digest fragments could be detected.

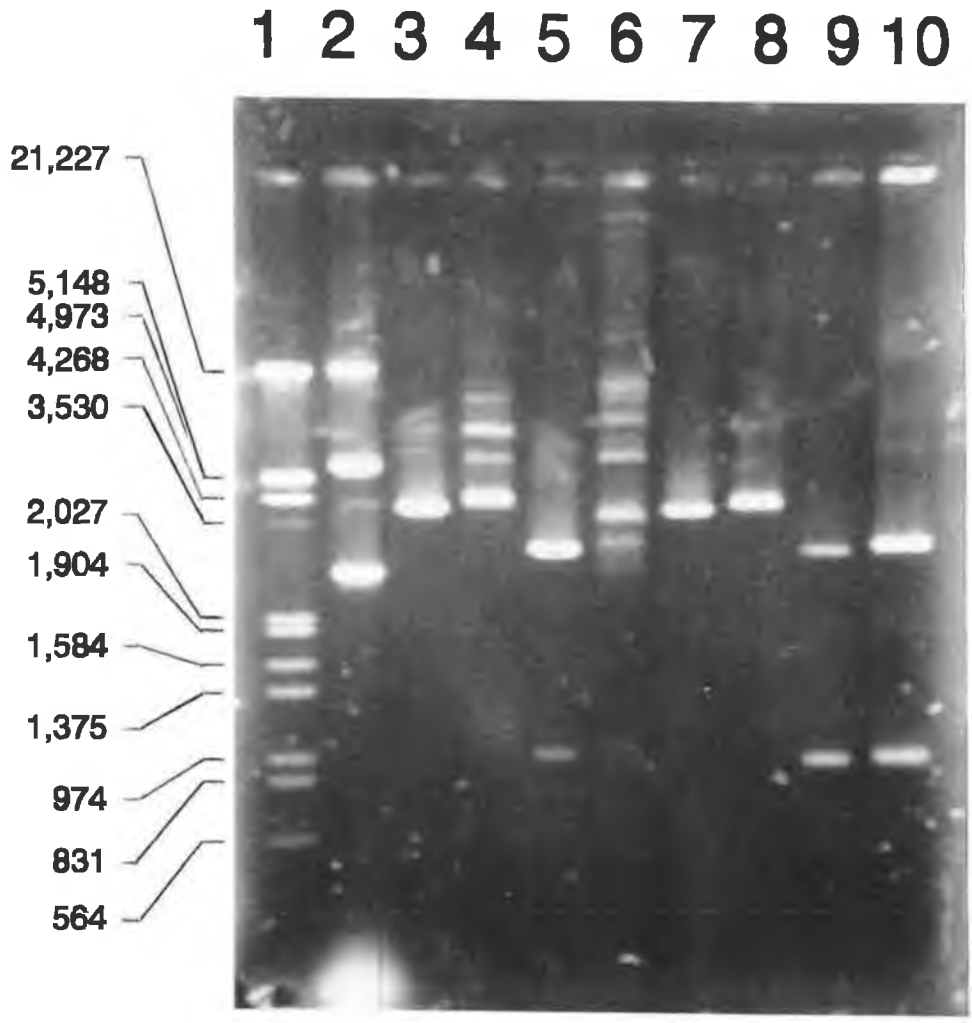


Figure 3.13. Plasmid pComb3 ligation and dephosphorylation controls. Lanes: 1 Lambda molecular weight markers (bp); 2: pComb3; 3-6: Not treated with calf intestinal phosphatase (CIP); 7-10: Treated with CIP; 3,7: pComb3 XhoI digest; 4,8: pComb3 XhoI digest following ligation; 5,9: pComb3 XbaI/SpeI digest; 6,10: pComb3 XbaI/SpeI digest following ligation.Digestion with the single enzyme XhoI resulted in a linear fragment of approximately 4,000 bp. Double digestion with XbaI and SpeI resulted in two fragments of 3,073 and 956 bp. Ligation of linearised XhoI-cut pComb3 resulted in several higher molecular weight bands, but following treatment with CIP, no ligation occurred. Ligation of the two double-digest fragments resulted in several higher molecular weight bands. Following treatment of both fragments with CIP, however, virtually no ligation took place.

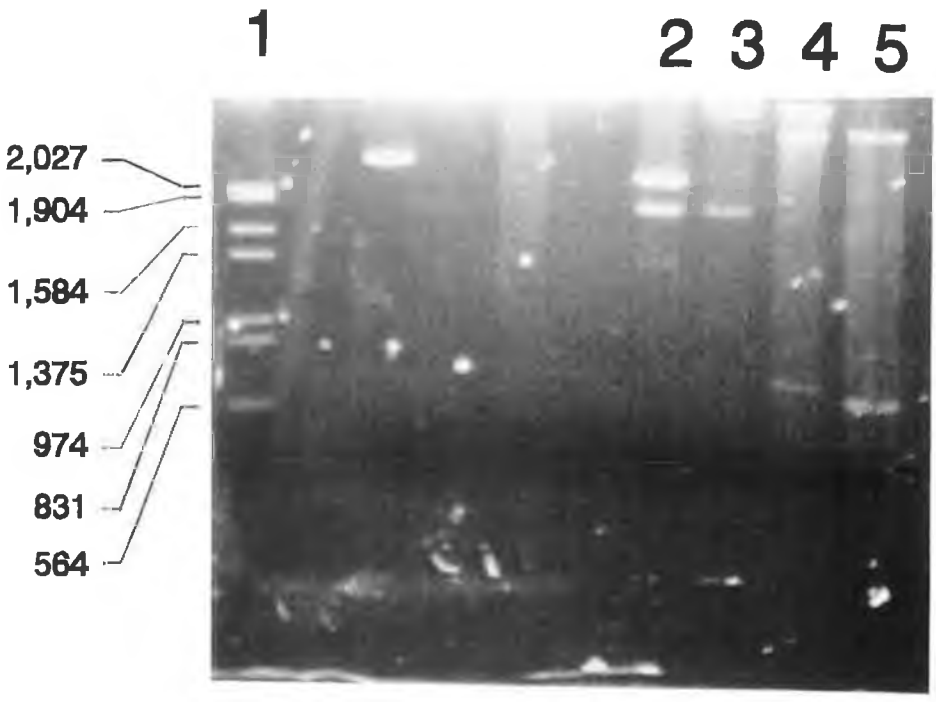


Figure 3.14. Restriction analysis of the mouse 5 light chain gene library in pGEM-T.Lanes: 1: Lambda molecular weight markers (bp); 2: pGEM-T:V_L; 3: pGEM-T:V_L XbaI digest; 4: pGEM-T:V_L SacI digest; 5: pGEM-T:V_L XbaI/SacI digest.Single digestion with XbaI resulted in a single digested band at approximately 3,700 bp. Digestion with SacI produced three bands of 2,950, 3,650 and 750 bp. Double digestion with XbaI and SacI resulted in bands of 2,950 and 700 bp.

3.3 DISCUSSION

3.3.1 Preparation of protein conjugates for immunisation

Production of an immunised library in the mouse begins with the preparation of a suitable antigen. Small haptens such as coumarin and 7-hydroxycoumarin are not immunogenic, and so require conjugation to a large protein, such as BSA or THG. Traditionally, the coupling methodology for coumarin and 7-hydroxycoumarin to these proteins was via diazo linkage between an amino group on the hapten and nitrogen or hydrogens on imidazole and phenolic ring structures such as those found on histidine and tyrosine residues respectively. Coumarin and 7-hydroxycoumarin possess no reactive side chains amenable for linkage, and so a linker must be chemically synthesized. The amino group is produced in a two step process. Initially, the benzene ring is nitrated at the 6 position, forming 6-nitro-coumarin. This is then reduced to 6-amino-coumarin, which is now amenable to linkage. The resulting aromatic amine can be converted to the highly reactive and unstable diazonium with sodium nitrite under acidic conditions at 0°C. The diazonium ion will now attack the aromatic rings on residues of tyrosine, histidine and tryptophan. Linkage via the diazonium bond produces distinctively coloured compounds. This results in distinctive visible spectral absorbances. Spectrophotometric analysis of the conjugate showed the presence of a shoulder between 320 and 350 nm. This is due to the presence of coumarin, which absorbs at 320 nm. No such shoulder is apparent with a sample of THG that has been through the same procedure in the absence of 6-NH_2-coumarin. To establish that the two moieties are linked to one another, chromatographic separation is desirable. Although thoroughly dialysed to remove small molecular weight components from the mixture, it is still good practice to characterise the conjugate by HPLC and photodiode array analysis. Separation by size exclusion chromatography resulted in peaks corresponding to a high molecular weight component with short retention time (9 to 12 min), which exhibited high absorbance at 280 nm, distinctive of proteins. Photodiode array scans show the shoulder between 320 and 350 nm as being concomitant with the protein peaks. The presence of two peaks may indicate some degradation in the protein sample or, possibly, different levels of conjugation.

3.3.2 Characterisation of coumarin-thyroglobulin (coumarin-THG)

Thyroglobulin tends to produce characteristically insoluble conjugates (Hermanson, 1997). This was also the case here. Insolubility was contributed to by the fact that coumarin is also extremely hydrophobic in nature. The conjugate proved very difficult to analyse due to this characteristic. Eventually, a solvent system was found to efficiently solubilise the conjugate, dissolving it sequentially in DMSO, methanol and water, which may have gradually released more hydrophobic interactions, allowing access to successively more hydrophillic ones. The presence of two peaks in the sample following PDA analysis is probably due to protein degradation over time. Initial PDA scans showed only a single peak, (data not shown). However, at this point, a solvent system was not available to produce large concentrations for effective analysis.

The insolubility of the conjugate was, however, advantageous during the immunisation procedure. Insoluble aggregates administered as immunogens elicit better immune responses than soluble antigens. This may be because they persist at the site of immunisation, prolonging immunological attack, rather than being rapidly cleared from the system. This may have contributed to the excellent immune response produced by mice immunised with coumarin-THG as evidenced by their titre, as compared to 7OHC-THG which was very soluble (Fig. 3.8). Another factor may have been the level of conjugation of the two conjugates used for immunisation.

3.3.3 Mouse serum antibody titres following immunisation

Although the titre produced by immunisation with 7OHC-THG was quite low, it must be measured against the control used (1 in 100 dilution of control serum) which is quite a stringent control, but is recommended by Campbell (1986) for the production of monoclonal antibodies. Titres in excess of 1 in 200 would typically be adequate for the setting up of a fusion, although titres in excess of 1 in 1,000 would be preferable.

3.3.4 PCR primer production

For effective library production, it is necessary to amplify a large number of heavy chain and light chain genes. Due to their structure, heavy and light chain genes have conserved 3′ sequences and variable 5′ sequences. However, the diversity of the 5′ variable region results from V gene segments, of which there are an estimated 300 to 1,000 on the mouse heavy chain germline genes and 300 on mouse κ light chain germline genes. These sequences have evolved from common sequence ancestors to generate antibody diversity. Consequently, they share large degrees of sequence homology. Homology at the 5′ end of these sequences has been analysed and used to divide sequences into families. These families allow the construction of sets of primers for PCR that are capable of amplifying groups of genes from these families.

The 5′ sequences of available antibody light and heavy chain genes (Kabat and Wu) were used to construct a set of family-specific primers (Fig. 3.9). These were modified for the insertion of restriction digestion sites. Due to the large group of primers this generates, a rationale was constructed to reduce the numbers of primers used. Primer sequences were further grouped in homology plots (Fig. 3.10). Here, the sequence similarity between the primers was assessed. Primers which were similar to a selected homolog were excluded from the group to be used, and those that had no very similar sequence homolog were included. In this way, a set of primers that had high sequence similarities to unused sequences was built up. Any misannealing during amplification may result in the amplification of non-specific sequences, without the need for large numbers of primers. This phenomenon was never actually tested, but could be assessed by amplifying antibodies with known sequences with non-identical, but similar 5′ primer sequences.

In the end, the selection of the heavy chain primers V_HIB, V_HIIB, V_HIIIB, V_HIIID and V_HVA yielded at least 83% homology with the other families. This meant that no more than 4 nucleotides differed between any two pairs of primers. Homology increased with the introduction of identical restriction sites into all primers. This strategy was also applied to light chain sequences which resulted in the choice of two primers V_kI and V_kVI which also resulted in at least 83% homology, except for V_kII to which they only had

75% homology.

The 3′ heavy chain constant region primer sequence selected was a $C_\gamma 1$ sequence. All heavy chains of a $C_\gamma 1$ subtype would be amplified. These tend to be produced in largest abundance, with high affinity. A kappa chain-specific constant 3′ primer was also selected for light chain amplification, as these comprise 95% of light chain subtype, compared to some 5% for lambda chains (Kuby, 1997).

3.3.5 PCR optimisation

PCR amplification was optimised, in most cases at standard $MgCl_2$ and primer concentrations, with slight variations in annealing temperature, around 58°C and 59°C. However, amplifications involving V_HIIID and V_HVA, required a reduction in primer concentration to 10 μM. Amplifications involving V_HIIB failed to produce single defined 700 bp bands over a range of PCR conditions.

3.3.6 PCR fragment purification

Several methods were assessed for the isolation and purification of PCR fragments from agarose gels such as phenol:chloroform cleanup (Sambrook *et al.*, 1989), Wizard kits (Promega), Spin-X columns (Costar), or without clean up (data not shown). However, the most reliable system was found to be Qiaex gel purification kits (Qiagen). These produced clean, reproducible preparations of high yield.

All attempts to clone digested heavy and light chain PCR fragments into pComb3 produced extremely low yields of transformants, or failed to work at all (data not shown). It was assumed that this failure was due to the inefficiency of the PCR fragments to digest. Inadequate overhangs can reduce the efficiency of restriction digestion. However, the design of these primers is similar to many others, with at least a 9 base overhang in the heavy chain primers (Roben, 1993). Several methodologies were also attempted to circumvent this problem. These involved concatamerisation of PCR fragments, followed by digestion, blunt end cloning (data not shown) and T/A cloning. Concatamerisation and

blunt end cloning failed to remedy the problem. Both techniques still depended on enzymatic modification of the PCR fragments. T/A cloning removes the need for modification of the PCR fragments following amplification and purification. Although with a potentially lower efficiency than cloning using six-cutter enzymes, greater than 10^3 transformants could be produced from a single ligation reaction. Repeated reactions could thus be used to establish a library of chains of high diversity. Some 10^4 heavy and 10^4 light chains would be sufficient to generate a library of good size (10^8). After several transformations, a heavy chain library of 4.75 x 10^5 transformants and a library of 3.2 x 10^5 light chains was produced, so large libraries are quite accessible via this methodology.

3.3.7 Construction of light and heavy chain gene libraries in pGEM-T

Insertion into pGEM-T also allowed the visualisation of correctly digested fragments, following double digestion with either *Xho*I and *Spe*I for heavy chains or *Xba*I/*Sac*I for light chains. These both yielded the expected 700 bp fragment, indicative of the inserted antibody chain gene. Bands produced in this way have also been efficiently restricted.

3.3.8 Heavy and light chain gene insertion into pComb3

Heavy chains derived from pGEM-T insertion were purified, and attempts were made to insert these into pComb3 (data not shown). However, these attempts also failed, or again, showed extremely low levels of transformation efficiency. It was concluded at this time that the limiting factor in the cloning procedure was not the PCR fragments, but the pComb3 vector. It had been assumed that efficient double digestion was taking place as these restriction sites are 57 bp and 12 bp apart for heavy and light chain insert sites, respectively. However, the stuffer fragment was never visualised by gel electrophoresis, and no differentiation could be made between single digested and double digested pComb3 on gels (Fig. 3.12). This is evidenced by the production of vectors based on modifications of pComb3 (Burioni *et al.,* 1997). They also suggest that efficient cutting of pComb3 is difficult in that overdigestion will reduce cloning efficiency and limiting digestion poses the risk of interfering in the cloning procedure. It is likely from these observations, and those seen by this author, that the failure of the cloning strategy was as

a result of the vector, either being inefficiently digested, or overdigested. The use of modified vectors, such as those proposed by Burioni *et al.* (1997) could alleviate this situation and bring about the production of large Fab antibody libraries to coumarin and 7-hydroxycoumarin.

4. THE PRODUCTION OF SINGLE CHAIN Fv (scFv) ANTIBODIES TO COUMARIN AND 7-HYDROXYCOUMARIN USING THE NISSIM LIBRARY

4.1 INTRODUCTION

The applications of coumarin in therapy and biochemistry have been discussed (Chapter 1). The usefulness of phage display libraries for the production of antibodies to coumarin has also been explored. Here, the isolation of antibodies to coumarin and 7-hydroxycoumarin from the Nissim phage display library is discussed. It explores the use of scFv material from culture supernatant, periplasmic lysates and inclusion bodies, as well as the use of scFv displayed on the phage surface as immunological reagents. It investigates the affinity purification of these molecules, to yield pure, high titre, stable reagents for subsequent analyses. The physicochemical characteristics of the resulting monoclonal scFv antibodies are also analysed, and their applicability in various analyses is assessed. Consequently, the applicability of the Nissim library for the isolation of antibody reagents to these molecules is evaluated.

4.1.1 Use of the Nissim library in the isolation of single chain scFv (scFv) antibodies

Alternatives to the production of large, immunised combinatorial phage display libraries such as the pComb3 system do exist. One such library system is the Nissim library (Nissim *et al.,* 1993). The concept behind the Nissim library was the creation of a naive human scFv library of very large size (10^8 clones) so as to make it highly probable that many scFv clones to any chosen antigen could be selected from it. Diversity was brought about using a library of variable heavy chain genes that had been mutagenised in the CDRH3 region, increasing diversity to 10^8. This library was further combined with previous scFv libraries (Hoogenboom and Winter, 1992) increasing the overall library size to much greater than 10^8.

4.1.2 Affinity selection strategies

Affinity selection can potentially be performed with antigen attached to a solid support. Matrices used have been in the form of immunoassay plates, immunoassay tubes and affinity columns (Kretzschmar *et al.*, 1995). With the use of immunoassay dishes and immunotubes, protein antigens are normally adsorbed. For the selection of antibodies to

peptides and haptens, these require linkage to a carrier protein to facilitate this adsorption. For this reason, optimal conjugate design is critical for efficient enrichment of phage antibodies to the hapten or peptide. Many conjugation chemistries are available for the coupling of haptens and peptides to carrier proteins, depending on their structure and the presence of suitable functional groups to facilitate linkage (Hermanson, 1997). Coumarin and 7-hydroxycoumarin lack such reactive functional groups, and must be derivatised in some way to make them amenable to conjugation (Egan, 1993). Aromatic ring conjugation can be performed by the formation of a diazonium derivative (Fig. 4.1). An active aromatic amine can react with the electron-rich nitrogens of the imidazole ring on histidine residues or *ortho* modification of the phenolic group of tyrosine residues. On BSA, there are 36 potential binding sites of this type. Efficient conjugate production involves the formation of conjugates with coupling ratios that maximise the number of haptens on their surface. This will encourage antigenic recognition of the hapten rather than the carrier protein and reduce interactions that rely on epitopes shared by hapten and carrier protein. High hapten coupling ratios, however, can have other practical difficulties. With many haptens, most notably hydrophobic molecules such as coumarin and 7-hydroxycoumarin, introduction of these to the surface of a protein may make it extremely insoluble, preventing its efficient use as an affinity ligand during affinity selection. It is likely that optimisation of conjugates is necessary for effective affinity selection strategies.

Another factor affecting the characteristics of affinity selection taking place on solid supports is the presence of a protein blocking agent. Heterogeneous protein mixtures such as non-fat milk are often used in such strategies. However, the presence of carrier protein free in solution may serve to remove interactions that take place between carrier protein and phage antibodies, resulting in enrichment of phage recognising hapten alone, rather than carrier protein epitopes.

An alternative strategy for selection of antibodies to haptens is the use of direct covalent attachment of activated hapten derivatives to solid supports. Many of the same coupling interactions available for the formation of hapten-carrier conjugates can be applied to solid supports. For example, N-oxysuccinimide is a highly active and unstable species that promotes the hydrolysis of amines with carboxyl groups. An amine such as

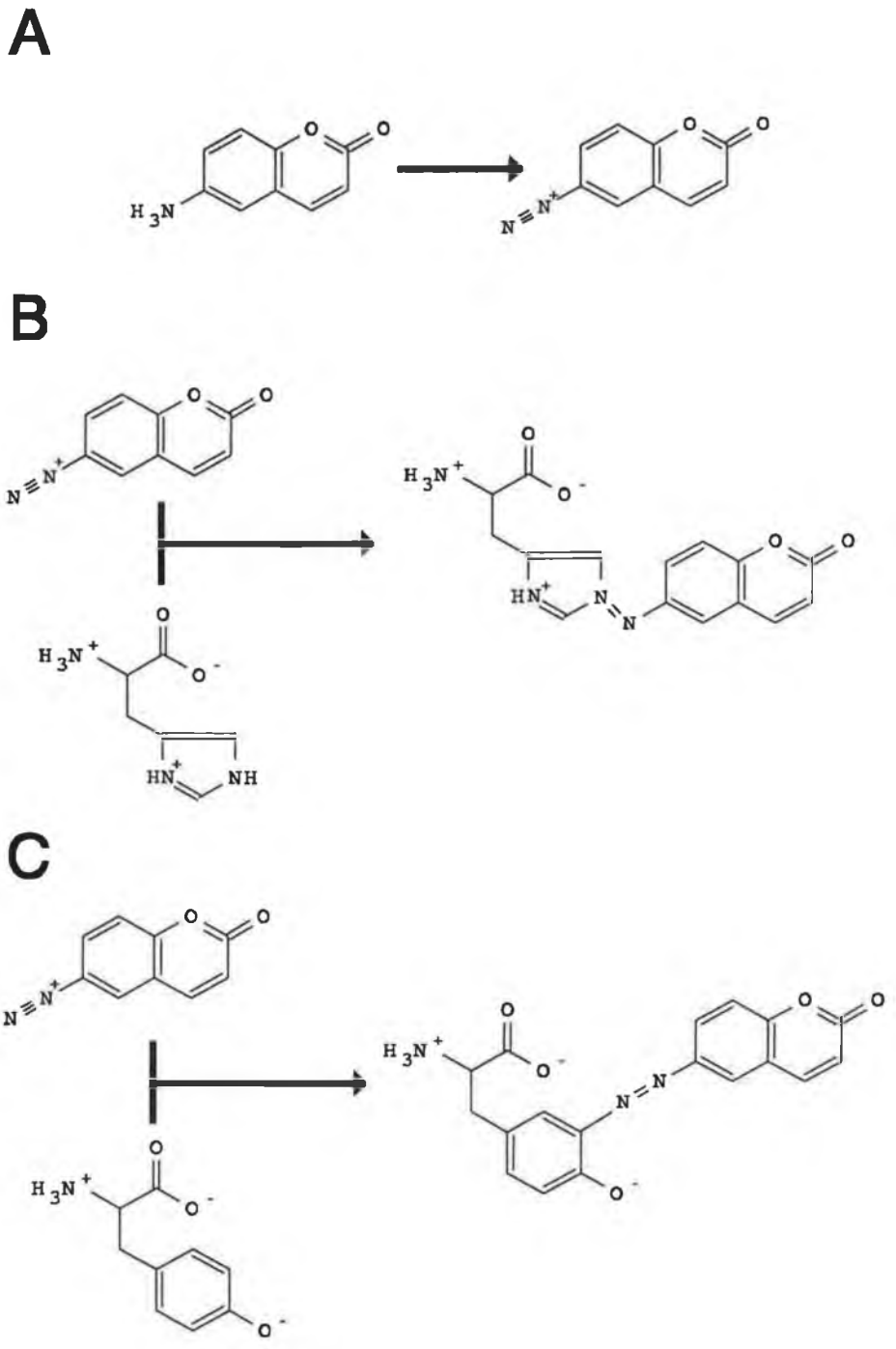


Figure 4.1. Diazonium coupling reaction of coumarin to histidine and tyrosine residues. A diazonium ion is formed by the reaction of 6-amino-coumarin with sodium nitrite under acidic conditions at 0°C (reaction A). This reacts principally with nitrogen on the imidazole ring of histidine residues (reaction B) or at higher pH with the phenolic side chain of tyrosine (reaction C).

6-NH_2-coumarin could thus be directly covalently linked to a solid surface. This strategy may oblate problems associated with the interaction of phage antibodies with the protein moiety of the hapten-carrier conjugate and avoid the need to produce and optimise the production of such conjugates.

4.1.3 Affinity selection of antibodies from libraries using BIAcore™

Selection and screening procedures using solid supports encourage the isolation of high affinity interactions over lower affinity ones (Kretzschmar *et al.*, 1995). In studies of antibody kinetics, it has been shown that the equilibrium affinity constant is mostly determined by the dissociation rate constant, k_d (Tijssen, 1985). Selection in this way encourages the isolation of antibodies with low dissociation rate constants. Such antibodies are optimal for subsequent use in ELISA and other immunoassay formats. However, antibodies are being increasingly used in immunosensor systems (Turner, 1997). These employ the detection of an antibody-antigen interaction by transduction to an electrical signal using a physical property such as surface plasmon resonance (Stenberg *et al.*, 1991) or electrochemistry (Ghindilis *et al.*, 1998). As well as having the potential for rapid, 'real-time' analysis, these systems also offer the possibility of the repeated use of the sensor surface. To accomplish this, the surface must be regenerable. That is, the antibody-antigen interaction that took place as a result of a previous analysis must be fully dissociated, leaving behind a re-usable sensor surface of either antigen or antibody, depending on the methodology employed. The ease of dissociation of the antibody-antigen interaction and, thus, the ease of regeneration of the sensor surface is primarily determined by the dissociation rate of the antibody used. Since most antibodies isolated via ELISA have low dissociation rates, they require more severe procedures to bring about full dissociation, making regeneration less efficient and more damaging to the sensor surface, reducing the number of regenerations possible.

As an alternative to enzyme immunoassay selection and screening procedures, some sensor devices, such as BIAcore™, facilitate using the sensor device to screen for clones with different dissociation rates, which would be of greater use subsequently in the same sensor and other devices (Malmborg *et al.*, 1996).

4.1.4 Induction of protein expression from phagemid pHEN1

As is also the case with pComb3, production of scFv antibody protein, and pIII protein from the pHEN1 phagemid is controlled by the *Lac*Z promoter/operator. *Lac*Z is a low level constitutive promoter that is repressed by glucose and greatly up-regulated by certain inducers such as lactose (Old and Primrose, 1980). Production of protein from pHEN1 is controlled by the incorporation of repressors and inducers in the growth medium. *E. coli* TG1 (pHEN1) and *E. coli* HB2151 (pHEN1) are normally cultured in 1% (w/v) glucose to repress constitutive expression when not required. High level induction is brought about by the removal of glucose from the medium, and replacement with a non-metabolisable inducer analogue of lactose, isopropyl-β-D-thiogalactopyranoside (IPTG). For scFv expression on the surface of phage, constitutive protein translation occurs in association with pIII. For soluble scFv protein production, large yields of protein are preferable, and induction with IPTG is used following the initial growth of the cultures to mid-log phase. Inducer concentration can vary but a concentration of 1 mM is typical (Buchner and Rudolph, 1991).

Growth conditions normally employed for high level scFv induction require a culture of mid-log phase, grown at 37°C, cultured in 1% (w/v) glucose to be transferred to a medium containing 1 mM IPTG and growth at the lower temperature of 25-30°C. This has also been found to be optimal for protein yield (Skerra *et al.*, 1991). Conditions of induction can affect the production and secretion characteristics of the proteins synthesised. Temperature, inducer concentration and growth phase may all affect the yield of protein. The age and condition of the cells may affect the release of antibody from the periplasm to the culture supernatant. Proteolytic degradation may also be a factor. Large scale protein production may also contribute to the overloading of protein production machinery and in the accumulation of protein as insoluble inclusion bodies.

4.1.5 Screening of antibody clones from phage display libraries

Screening of phage following affinity selection is performed using phage ELISA. This can be performed on polyclonal phage, showing the general level of enrichment occurring in

each round of selection, or on individual clones to identify antigen-binding recognition. Positive clones can be selected from the population and used for the production of soluble scFv antibody. In the Nissim library, soluble scFv production is achieved by the transfer of the pHEN1 scFv-containing phagemid from the amber suppressor *E. coli* strain TG1 to the non-suppressor *E. coli* HB2151. In the absence of helper phage and pIII, soluble scFv is produced (Hoogenboom *et al.*, 1991).

Screening of positive clones has also been performed on blotting membranes (Pharmacia Biotech, 1996). Individual *E. coli* clones expressing scFv (either as phage or soluble antibody) were blotted onto nitrocellulose containing antigen. Following blocking of the membrane, binding was detected with secondary antibodies. Positive clones could then be selected from the original culture plate.

It has been noted that the translation of exogenous protein in *E. coli* does not always result in the formation of correctly folded, soluble protein. Several factors are involved in the efficient folding and secretion of proteins from intracellular matrices. One factor is the presence of a leader or signal peptide at the C terminus of the protein (Cheung *et al.*, 1992). These characteristic sequences guide proteins across membranes, before being processed by enzymatic cleavage or resulting in the anchoring of the protein in a particular membrane. To facilitate secretion of scFv from the intracellular compartment of *E. coli*, upstream of the scFv is a leader sequence derived from the *E. coli* pectate lyase protein, pelB. Presence of the correct leader sequence is, however, no guarantee of transport. The ability of a protein to form a correctly folded, soluble molecule is sequence-dependant, and so difficult to predict. In eukaryotic cells, many chaperon proteins are present which guide the folding of proteins (Takagi *et al.*, 1988; Pelham, 1989). These mechanisms are not present in *E. coli* and so correct folding is solely dependant on the particular protein structure translated. It is thus likely that, while some clones may secrete into the periplasm and culture supernatant, others will not be capable of doing so. These may form misfolded, insoluble inclusion bodies within the *E. coli* cytoplasm (Buchner and Rudolph, 1991).

Analysis of positive scFv clones can be performed by ELISA. Here, culture supernatant

from scFv clones is analysed for the presence of scFv binding to antigen. In the Nissim library, recognition is via a c-myc peptide tag of approx. 1.5 kDa, that is recognised by mycl-9E10 anti-c-myc antibody. Direct detection of scFv can also be facilitated using blotting membranes such as nitrocellulose and PVDF (Towbin and Gordon, 1984; Stott, 1989). These do not indicate antigen binding activity, but merely the presence of scFv protein.

The presence of scFv material in the *E. coli* periplasm can also be detected by the gentle breaking of the outer membrane using freezing and thawing (Hughes *et al.*, 1969). Soluble, active scFv otherwise incapable of passage across the outer membrane can thus be detected.

4.1.6 Isolation and refolding of scFv antibody protein from insoluble inclusion bodies

As has been stated, many scFv clones fail to secrete antibody, either due to misfolding of protein upon translation, or due to the overloading of the cellular machinery. It may not always be possible to isolate clones that secrete antibody. Protocols have been developed for the isolation of soluble protein from intracellular compartments (Hughes *et al.* 1969). These require the lysis of the inner membrane, the denaturation and solubilisation of the protein inclusion bodies and the controlled refolding of the denatured protein to the active form. Lysis requires more severe treatment of the cells than outer membrane rupture, such as physical disruption techniques, like sonication, explosive decompression (X-press) or shearing against glass beads.

Denaturation is brought about by the use of high concentrations of guanidine hydrochloride or urea. This results in linearised peptide chains, removing inter- and intra-molecular interactions, with resulting protein solubility.

Refolding is performed by the gradual removal of denaturant from the protein environment, either by dialysis, or by the direct addition of a dilution buffer. Refolding begins from a point of low protein concentration. This dilute solution discourages intermolecular interactions during refolding. Gradual removal of denaturant allows

intramolecular interactions to come about. Hydrophobic residues will associate and predominate on internal surfaces, whereas hydrophilic interactions will occur with the aqueous matrix. Some protein will refold correctly. Yields of up to 40% (w/w) correctly folded material has been produced (Buchner and Rudolph, 1991). Yield can be further enhanced by the purification of scFv prior to refolding (section 4.1.7).

The level of refolded antibody has been improved by the incorporation of arginine at 0.4 M. This aids in the stabilisation of the protein during refolding. Refolding is a gradual process, taking up to 5 days to reach completion.

4.1.7 Affinity-purification of antibodies

For the production of high titre scFv material, without the presence of contaminants capable of the degradation of the sample, or causing problems associated with non-specific background interactions when used in assays, an efficient means of purification is required. Purification has been achieved using a protein A-scFv fusion protein system (Djojonegoro *et al.*, 1994). Many protein purification systems now used employ the highly efficient technique of metal chelate affinity chromatography (Skerra *et al.*, 1991; Vaughan *et al.*, 1996). This exploits the highly specific interaction that occurs between nickel ions and sequences of histidine residues. This can be used to purify scFv of any specificity, whether correctly folded or not. This can be particularly useful for the isolation of antibody from intracellular lysates. The Nissim library, however, does not incorporate the $(His)_6$ sequence tag necessary to do this. In the past, scFv from this library have been purified using anti-c-myc antibody (Kipriyanov and Little, 1997). This, however, was considered relatively inefficient. The traditional alternative is the use of antigen immobilised on a solid support in an affinity column (Hermanson *et al.*, 1992). In this way, scFv may be introduced to the column under conditions which promote specific interaction while allowing the removal of other non-specific proteins in the sample. Conditions are then found which result in the dissociation of antibody from antigen, without permanent effect on the antibody species.

4.1.8 Composition of affinity matrices

The range of affinity matrices available is broadly similar to the range of affinity ligands available for affinity selection. Carrier-hapten conjugates can be coupled to activated supports such as sepharose activated with CNBr (Pharmacia). Alternatively, haptens can be coupled directly via functional groups such as covalent linkage of 7-hydroxycoumarin-4-acetic acid to EAH-sepharose (Heiko Reinartz, Dublin City University, Personal Communication).

Traditionally, purification of polyclonal antibodies and many monoclonal antibodies using affinity columns containing antigen begins from a point of high ionic strength (0.5 M NaCl) to reduce non-specific background binding (Catty, 1989). Elution of antibody following sufficient washing is facilitated by the use of either high (11.5) or low (2.2) pH buffers that disrupt the antibody-antigen interaction, followed by immediate neutralisation of the purified species (Heiko Reinartz, Dublin City University, Personal Communication).

Monoclonal antibodies, however, exhibit specific individual characteristics that may differ significantly in a number of ways from the 'average' characteristics of a polyclonal antibody population (Catty, 1989). As a consequence, more individual antibody purification strategies may need to be developed to take into account this nature. This principally involves optimising the environmental conditions used for purification such as temperature, pH, ionic strength, use of ionic and non-ionic detergents, the presence of non-polar organic solvents, and the use of chaotropic agents. Any of these factors may affect the efficiency of the affinity purification as well as the quality of the antibody material subsequently derived.

4.1.9 Concentration of purified antibodies

Protein isolated from affinity purification is normally in dilute solution and requires concentration if it is to be used as an effective reagent. Several methods such as reverse osmosis, ultrafiltration and membrane filtration can be applied.

4.1.10 Immunoassay strategies for the measurement of drug-hapten concentrations

The quantitative analysis of drug haptens and other small molecules using antibodies is best facilitated by the heterogeneous competition immunoassay (Tijssen, 1985). Two competition strategies can be envisaged (Fig 4.2). In one scenario, antibody recognising the analyte is immobilised on a solid surface, such as the wells of an immunoassay plate. Enzyme-labelled analyte competes with free analyte present in standards and unknown test samples for binding to the antibody. The amount of enzyme label present is inversely proportional to the level of free analyte in the sample. This proportionality is log-linear over a range of analyte concentration which is usually determined by the antibody's affinity. Higher affinity antibodies yield more sensitive assays (Δsignal/Δconcentration) with lower limits of detection. This strategy requires the production of an enzyme-labelled conjugate of the analyte.

The second strategy involves competition of free antibody for bound analyte-protein conjugate and free analyte. The quantity of antibody binding to the surface is also inversely proportional to the level of free analyte over a specific concentration range. Detection this time is either via enzyme label directly attached to antibody, or the incorporation of intermediate antibody species to amplify the signal. Both strategies are equally acceptable. The latter, however, utilises hapten-protein conjugates that are normally synthesised as part of the antibody production strategy, and so requires no additional conjugates to be prepared.

The nature of competition immunoassays requires the optimisation of several parameters before it can perform effectively (Crowther, 1995). Since free analyte is competing with surface-bound analyte, the maximum concentration of free drug that can be measured is dependant on the amount of surface bound analyte. If only small concentrations of bound analyte are employed, antibody will saturate at a lower concentration, reducing the amount of free drug that can be bound. However, oversaturation of the surface will only result in wasted hapten-protein conjugate as excess amounts will not be adsorbed to the surface.

To compete effectively between bound and free antigen, antibody must be present in limiting concentrations. Otherwise, it may be capable of saturating both bound and free antigen with the result that no decrease in signal is obtained when free antigen is introduced. Experimentally, the dilution of antibody which yields 70% maximal binding to surface-bound antigen in the absence of free antigen has been used as an effective working dilution in such immunoassays (Crowther, 1995).

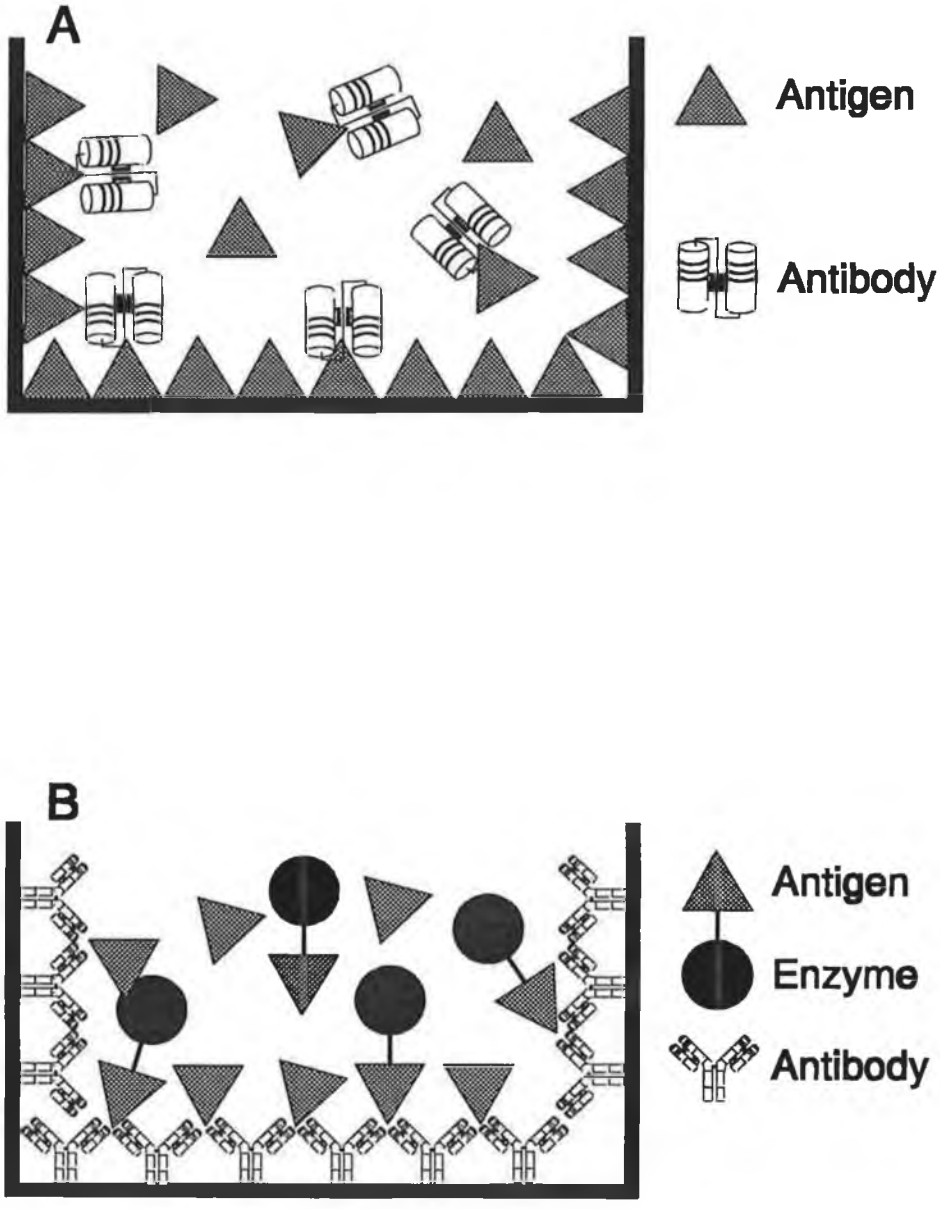


Figure 4.2. Competition ELISA strategies for the analysis of haptens. A. Antibody competes for free and bound antigen. B. Enzyme-labelled and unlabelled antigen compete for binding to surface-bound antibody. The enzyme is detected with a suitable substrate or amplified with secondary antibodies.

4.1.11 Antibody kinetics and equilibria

Antibody kinetic equilibria data may also be estimated using competition immunoassay strategies (Tijssen, 1985; Friguet *et al.*, 1985). This can be deduced from equilibrium equations of antibody-antigen binding. The equilibrium is defined by the equation:

$$Ab + Ag \underset{k_d}{\overset{k_a}{\rightleftharpoons}} Ab.Ag \qquad (1)$$

Where Ab is antibody and Ag is antigen and Ab.Ag is the antibody-antigen complex.

The on rate is defined by:
$k_a[Ab].[Ag]$ *in* $M^{-1}.s^{-1}$

The off rate is defined by:
$k_d[Ab.Ag]$ *in* s^{-1}

The association equilibrium constant, K_A, is defined by:

$$K_A = \frac{k_a}{k_d} = \frac{[Ab.Ag]}{[Ab] \,.\, [Ag]} \quad M^{-1} \qquad (2)$$

The dissociation equilibrium constant, K_D, is defined by:

$$K_D = \frac{1}{K_A} = \frac{k_d}{k_a} = \frac{[Ab] \,.\, [Ag]}{[Ab.Ag]} \quad M \qquad (3)$$

The association equilibrium constant can be found experimentally. For binding to monovalent hapten, the point at which 50% binding occurs:

$[Ab_{Unbound}] = [Ab_{Bound}]$

Since *[Ab] = [Ab.Ag]*, then:

$$K_A = \frac{k_a}{k_d} = \frac{1}{[Ag]} \quad M^{-1} \qquad (4)$$

This is performed experimentally according to Friguet *et al.* (1985). Antibody and a range of free antigen concentrations are allowed to reach equilibrium. At some antigen concentration, half of the antibody is bound at equilibrium. Samples from equilibration are then assayed against bound antigen to measure the quantity of unbound antibody. 50% binding can be calculated and the association equilibrium constant determined.

A non-equilibrated approximation of affinity measurement was used by Van Heyningen *et al.* (1983) and modified by Roben (1993). In this instance, [Ab] = [Ab.Ag] is estimated by competing antibody for free and surface-bound antigen. This assumes that at any given instant the rate constants for binding to free and surface-bound antigen are the same and that the amount of antibody not bound to either free or surface-bound antigen is negligible:

$$[Ab.Ag_{Free}] + [Ab.Ag_{Surface}] - [Ab_{Total}] \approx 0 \qquad (5)$$

In this instance, when:
$[Ab.Ag_{Free}] = [Ab.Ag_{Surface}]$

This approximates to:
[Ab] = [Ab.Ag]

thus yielding an estimate of the association equilibrium constant.

4.1.12 Analysis of antibody kinetics using BIAcore™

BIAcore is an analytical device based on the optical property of surface plasmon resonance (SPR). BIAcore acts as a mass balance. A wedge of light is focused to a point on the surface of a gold film. The intensity of the reflected wedge of light is analysed at a photodiode array detector. At an angle of incidence to the gold film, known as the surface plasmon resonance angle, a component of the incident light is propagated through the gold film as an evanescent wave. This excites delocalised surface electrons in the gold film known as plasmons, which absorb energy from the incident light. This leads to a decrease in the intensity of the reflected light at this angle. The angle at which surface plasmon resonance occurs is dependent on the characteristics of the gold film, including the refractive index of the opposite face of the gold film. Changes in the bulk refractive index at this side of the gold film will change the resonance angle. This change in bulk refractive index can be related to the mass bound to the gold film. The resonance angle is converted to an electrical signal which is expressed as response units (RU). Attachment of affinity ligands to the gold surface results in detectable mass changes. Coupling is normally performed using a dextran matrix attached to the gold film. This can be used to couple most species, which it maintains in solution. Rate constants for antibody-antigen interactions can be calculated using kinetic results arrising from BIAcore analysis (O'Shannessy and Winzor, 1996), based on the reaction scheme described in Equation 1 above.

4.2 PRODUCTION OF ANTI-COUMARIN ANTIBODIES FROM THE NISSIM LIBRARY

4.2.1 Phage ELISA of anti-coumarin phage antibodies

The phage ELISA for the anti-coumarin clones taken from the second and third rounds of affinity selection is shown in Fig. 4.3. Five clones out of 42 were positive after two rounds of affinity selection. This had risen to 12 out of 42 after three rounds. No positive clones to coumarin were detected prior to affinity selection (Fig. 4.4). These were designated α-C1 to α-C17 accordingly.

4.2.2 Phage ELISA of anti-coumarin phage antibodies raised against 6-amino-coumarin coupled via N-oxysuccinimide ester

The phage ELISA of clones screened following affinity selection against 6-NH_2-coumarin directly coupled to an N-oxysuccinimide (NOS) 96 well microtitre plate, and screened using coumarin-BSA is shown in Fig. 4.5. Four positive clones out of 94 could be identified. These were designated α-C(NOS)1 to α-C(NOS)4 accordingly.

4.2.3 Screening of anti-coumarin positive clones for soluble scFv antibody production

The results of dot blotting of individual anti-coumarin clones following affinity selection to either coumarin-BSA or 6-NH_2-coumarin are shown in Fig. 4.6. Out of the 21 positive anti-coumarin phage antibodies, only three showed significant scFv present in periplasmic lysates (Fig. 4.6a), and two of these showed antibody present in the culture supernatant (Fig. 4.6b). 27 clones were selected at random from the anti-coumarin library following three rounds of affinity selection. Several showed good secretion into the periplasm, with some extracellular expression also. The three anti-coumarin secretors were all from the anti-coumarin library selected using coumarin-BSA. None of the four clones selected on 6-NH_2-coumarin showed any secretion. The blot was repeated (Fig. 4.7).

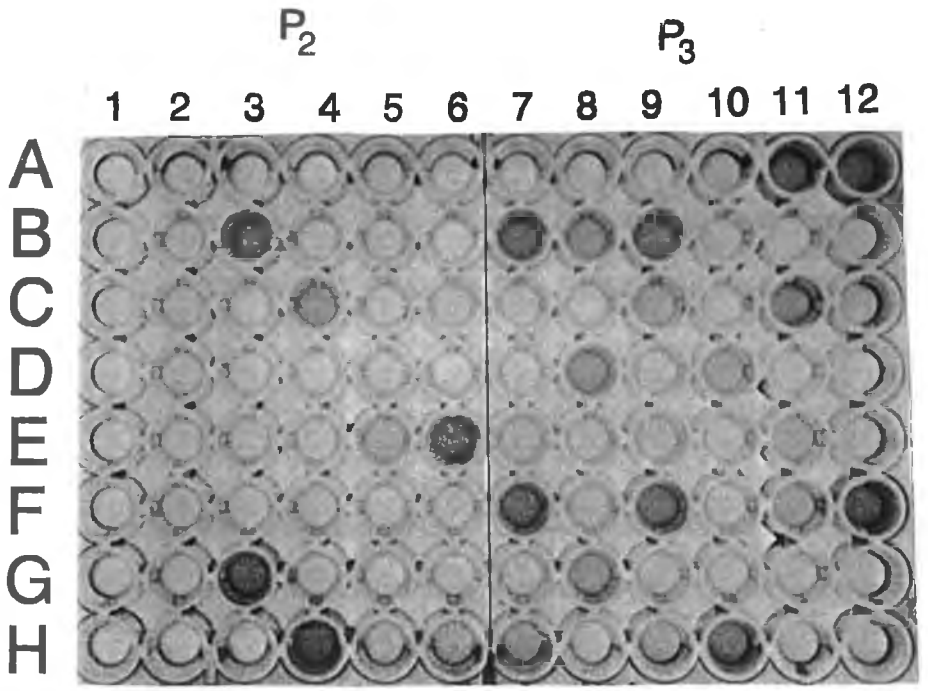


Figure 4.3. Phage ELISA analysis of anti-coumarin library following 2 (P_2) and 3 (P_3) rounds of affinity selection against coumarin-BSA. Clones from the second round of affinity selection showed 5 positives out of 42, and clones following three rounds of selection showed 12 good positives (with some weaker positives) from the same number of clones. Negative control: H6. Positive control: H7. The positive control failed to work, but positive wells could be clearly denoted without it.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.093	0.090	0.080	0.075	0.077	0.091	0.076	0.091	0.085	0.084	0.084	0.092
B	0.106	0.082	0.075	0.086	0.069	0.081	0.071	0.078	0.084	0.070	0.082	0.087
C	0.100	0.081	0.078	0.086	0.075	0.321	0.081	0.076	0.076	0.087	0.323	0.090
D	0.106	0.091	0.081	0.079	0.080	0.077	0.076	0.074	0.079	0.081	0.099	0.088
E	0.086	0.082	0.077	0.071	0.081	0.084	0.080	0.081	0.094	0.081	0.086	0.091
F	0.083	0.082	0.081	0.079	0.086	0.078	0.078	0.079	0.079	0.083	0.092	0.121
G	0.155	0.075	0.079	0.080	0.072	0.085	0.075	0.093	0.084	0.073	0.088	0.093
H	0.087	0.086	0.096	0.080	0.083	0.080	0.847	0.083	0.082	0.083	0.085	0.094

Figure 4.4. Phage ELISA data of 94 randomly selected clones from the original, unpanned Nissim library screened against coumarin-BSA. No clones were found to be positive in the 94 clones selected. Negative control (H6): α-NIP clone against coumarin-BSA. Positive control (H7): α-NIP clone against NIP-BSA.

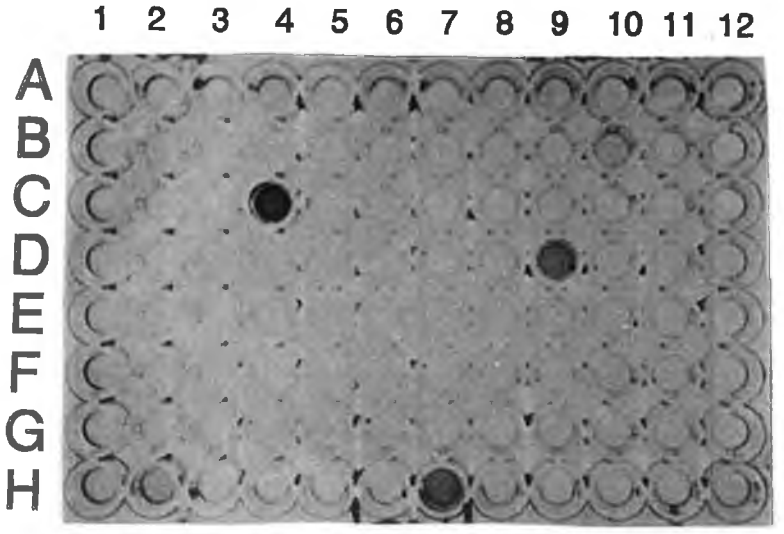


Figure 4.5. Phage ELISA of anti-coumarin clones selected using 6-NH_2-coumarin coupled via N-oxysuccinimide-activated microtitre plates. Four clones out of 94 were found to be positive to the coumarin-BSA conjugate used for screening. The four clones selected were B10 (α-C(NOS)1), C4 (α-C(NOS)2), D9 (α-C(NOS)3) and H2 (α-C(NOS)4). Negative control: H6. Positive control: H7.

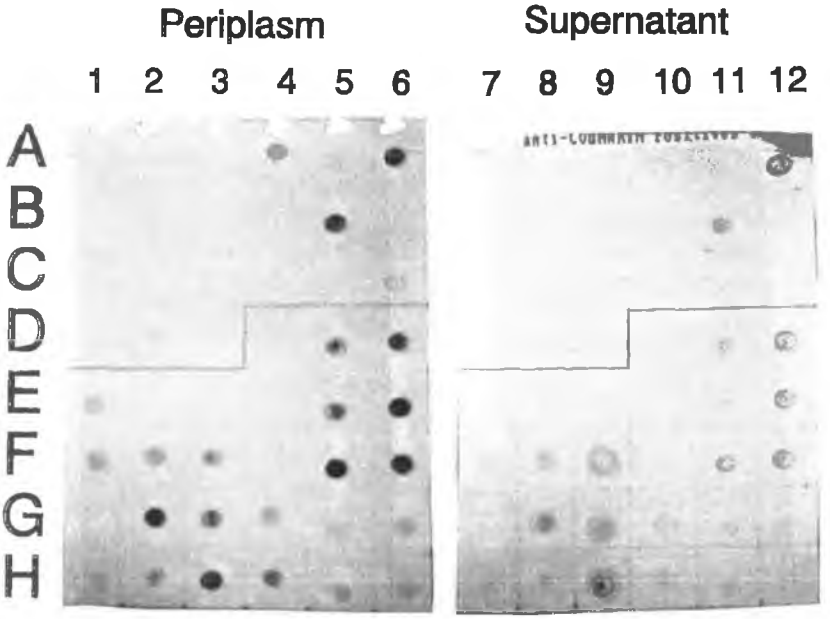


Figure 4.6. Dot blot analysis of periplasmic lysates (columns 1-6) and culture supernatants (columns 7-12) of 21 anti-coumarin clones selected on coumarin-BSA (Fig. 4.3) and 6-NH_2-coumarin (Fig. 4.5). The 21 anti-coumarin clones (A1-D3 and A7-D12)Three clones from selection on coumarin-BSA proved positive for periplasm containing scFv. Two of the clones that were positive for periplasmic secretion were also found to be positive for secretion into culture supernatant. None of the four clones selected with 6-NH_2-coumarin showed solubility in either periplasm or supernatant.

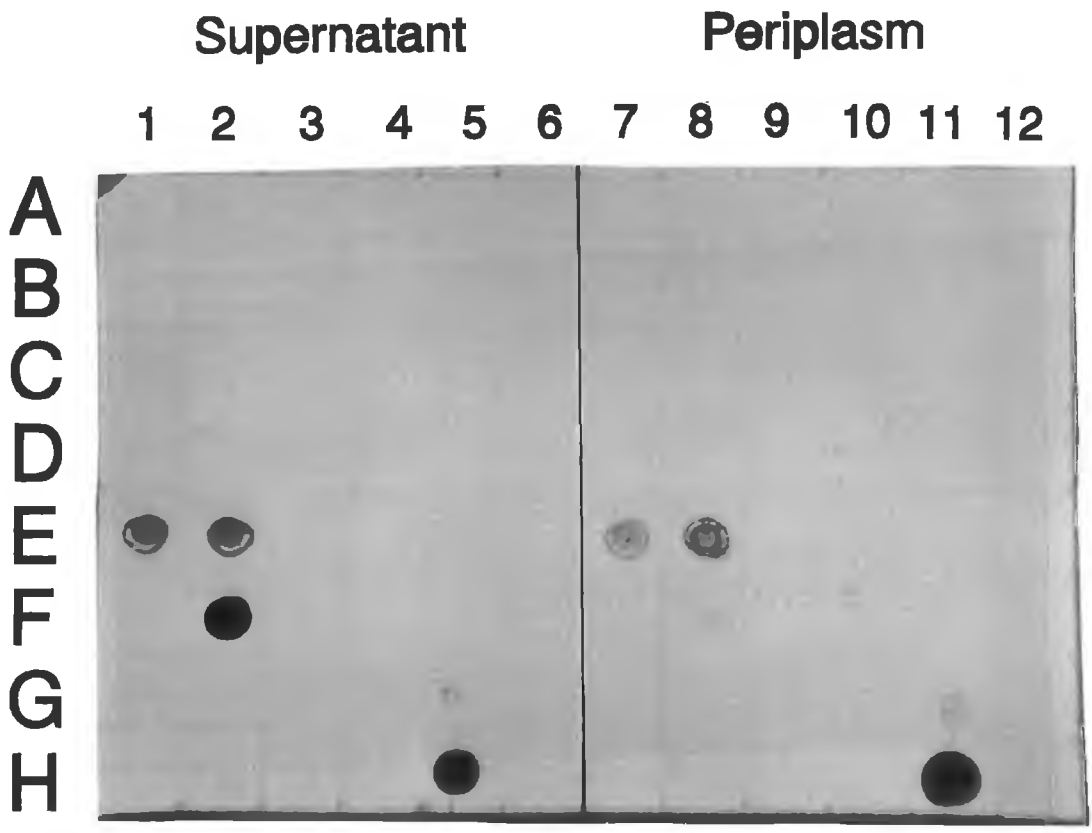


Figure 4.7. Repeat analysis of dot blots of 21 anti-coumarin clones from phage ELISA. Clones were numbered sequentially α-C1 to α-C21 in the order A1, B1, C1... for supernatants and A7, B7, C7... for periplasmic lysates, respectively. This confirmed the previous result of three clones secreting scFv, either into the periplasm or culture supernatant, or both. These were E1,E7 (α-C5), E2,E8 (α-C13) and F2,F8 (α-C14). Negative controls: G5 and G11. Positive controls H5 and H11.

This again showed the same three positive clones, designated α-C5, α-C13 and α-C14. All three showed extracellular secretion of scFv, particularly α-C14, but only α-C5 and α-C13 showed good levels of periplasmic scFv.

4.2.4 Optimisation of affinity purification of anti-coumarin scFv clones

4.2.4.1 Effect of high pH on α-C14 elution from coumarin-BSA

A 10 ml sample of culture supernatant from α-C14 was affinity purified on a coumarin-BSA column on a pH gradient from pH 7 to 12 (Fig. 4.8a). Dot blots of fractions showed six fractions to contain scFv antibody at pH between 8 and 10. This was repeated and positive fractions were obtained between pH 10.5 and 11.5 (Fig. 4.8b). A third gradient (Fig. 4.8c) showed a single positive fraction at pH 11.5. However, the signals achieved by dot blotting were extremely poor compared to controls, indicating small quantities of antibody binding to the coumarin-BSA column.

4.2.4.2 Batch purification of α-C5 and α-C13

5 ml volumes of periplasmic lysates of α-C5 and α-C13 were batch purified in 12.5 ml coumarin-BSA coupled to sepharose 4B which had been equilibrated with PBS. Washing was with 50 mM Tris/0.5 M NaCl, pH 8.5, and elution was with 50 mM Tris/0.5 M NaCl, pH 12, and concentration of the antibody by reverse osmosis. In both α-C5 and α-C13, purification of a 31 kDa scFv fragment did occur following elution and concentration (Fig. 4.9). However, these purified fractions were no longer detected when analysed by western blotting using the anti-c-myc antibody, mycl-9E10 (Fig. 4.10). Also present in unpurified fractions was a lower molecular weight fragment of approximately 20 kDa which recognised anti-c-myc antibody, but was subsequently lost upon purification, and was not present in purified fractions in SDS-PAGE analysis. This loss of recognition was also associated with loss of activity on ELISA (Figs. 4.11a and 4.11b). These clearly showed excellent responses from periplasmic lysates to coumarin-BSA, but not to BSA. However, following purification, nearly all activity had been lost.

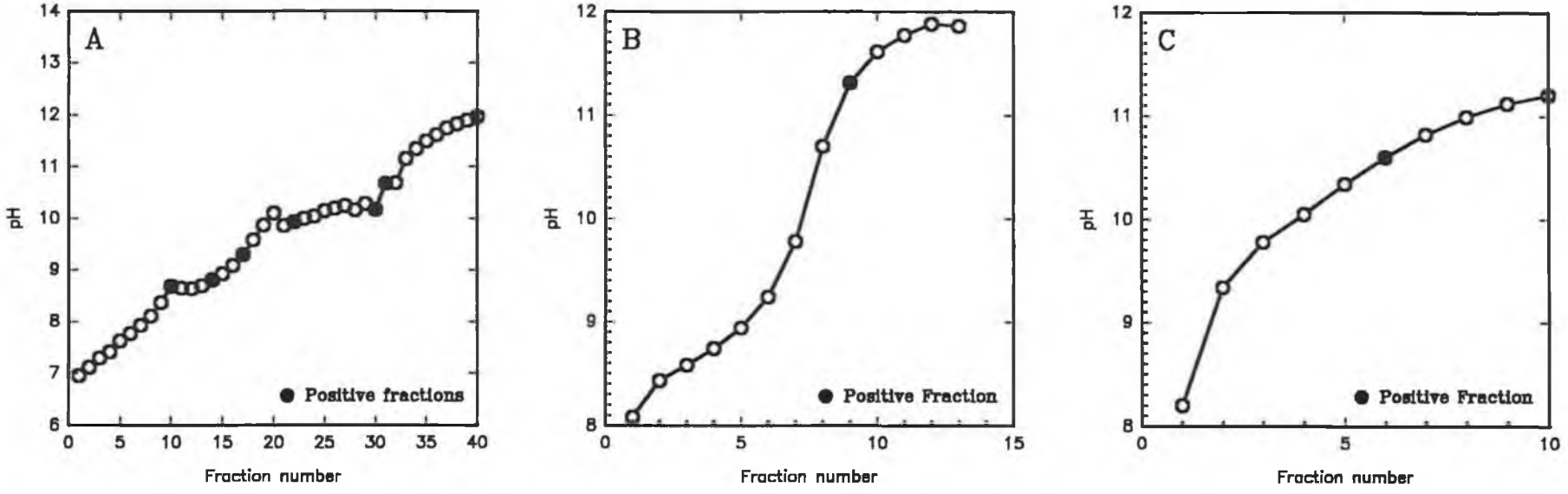


Figure 4.8A, B, and C. Purification of α-C14 using pH gradient elution. Analysis of fractions from affinity purification of α-C14 on a pH gradient from 7 to 12 showed most antibody eluting between pH 10.5 and 11.5. Successive improvement in the control of pH showed elution to occur between 10.5 and 11.5. Positive fractions containing antibody are shown in a shaded circle. 4.8A shows antibody in several fractions across a range of pH values. Why this was so is not clear. In 4.8B only a single fraction at pH 11.4 is positive. 4.8C. Again, only a single fraction proves positive at pH 10.5.

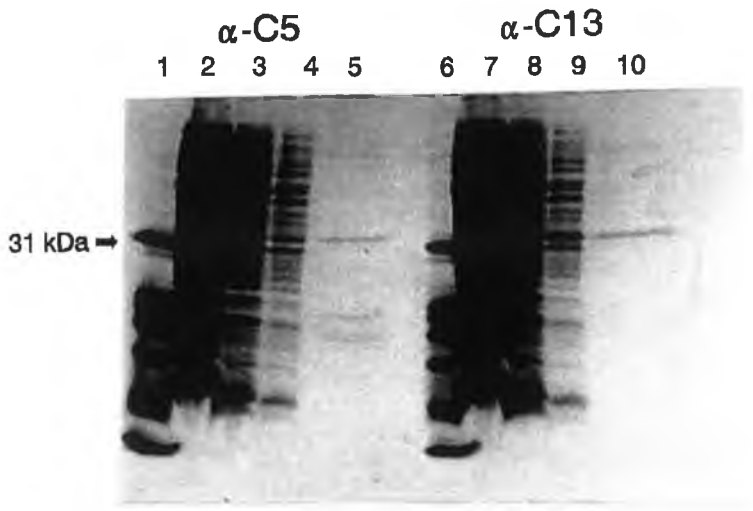


Figure 4.9. SDS-PAGE analysis of batch-purification of α-C5 and α-C13. Lanes: 1,6: Molecular weight markers; 2,7: Supernatant; 3,8: Periplasmic lysate; 4,9: Unbound fraction; 5,10: Affinity-purified fraction. In both cases, purification of a 31 kDa protein fragment can be seen following elution of scFv at pH 12. The concentrations of non-specific protein in supernatants and periplasmic lysates was very high and led to overloading of the gel.

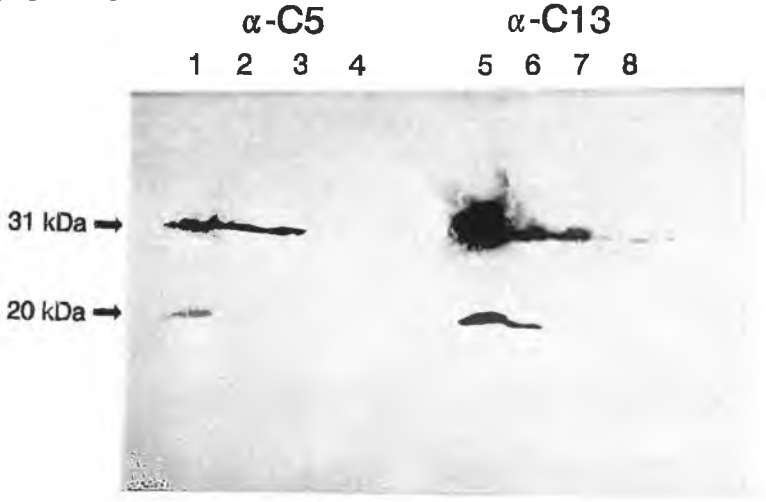


Figure 4.10. Western blot analysis of batch-purification samples of α-C5 and α-C13. Recognition was with α-c-myc antibody. Lanes: 1,5: Supernatant; 2,6: Periplasmic lysate; 3,7: Unbound fraction; 4,8: Affinity-purified fraction. In both cases, recognition of the 31 kDa scFv antibody is seen in periplasmic lysates, unbound fractions and column washings. However, only small responses are detected from the purified 31 kDa fraction.

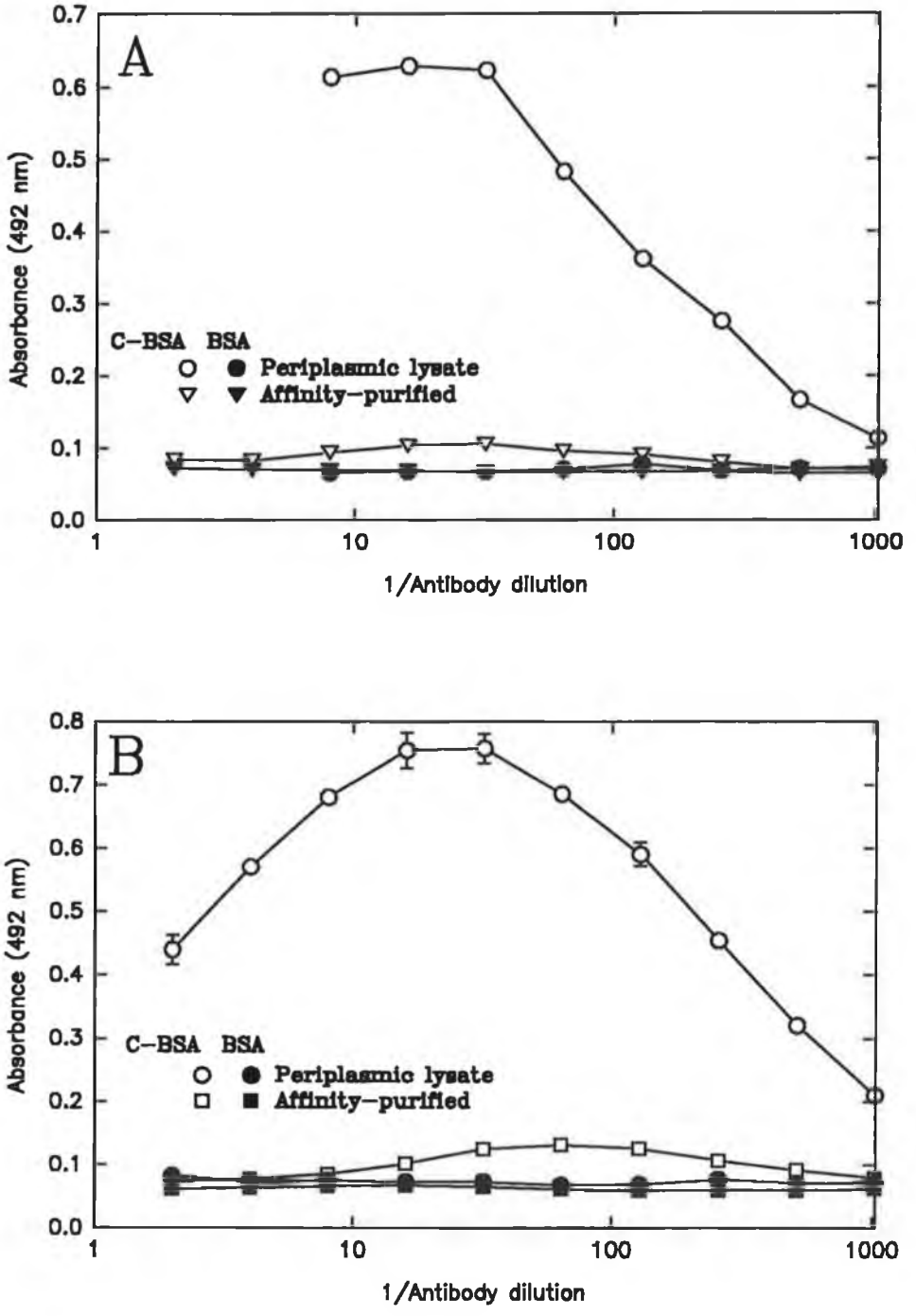


Figure 4.11A and B. ELISA analysis of periplasmic lysate and affinity-purified samples from the batch purification of α-C5 (A) and α-C13 (B). Periplasmic lysates of both α-C5 and α-C13 exhibited titres in excess of 1 in 1,000 to coumarin-BSA (C-BSA), with no response to BSA alone. Only marginal responses were produced using samples of α-C5 and α-C13 which had been affinity-purified samples.

4.2.4.3 Optimisation of α-C13 affinity purification

A 5 ml sample of α-C13 periplasmic lysate was dialysed with 10 mM Tris, pH 7.4, overnight at 4°C and affinity purified on a 12.5 ml coumarin-BSA column using three gradients:

1. Tris buffer gradient, pH 7.4, from 10 mM to 50 mM.
2. NaCl gradient in 50 mM Tris, pH 7.4, from 0 M NaCl to 0.5 M NaCl.
3. pH gradient in 50 mM Tris/0.5 M NaCl from pH 7.4 to 12.

10 x 10 ml fractions were collected on each of the three 100 ml gradients, and analysed by dot blotting (Fig. 4.12).

No antibody eluted as a result of increasing the ionic strength of the Tris buffer from 10 mM to 50 mM. However, significant amounts of antibody eluted as a result of increasing the NaCl concentration fro 0 to 0.5 M. Only negligible amounts of antibody then continued to elute in response to an increase in pH. Another significant difference from previous affinity purifications using high pH elution was that very little antibody was lost in the 'unbound' fraction of material when added to the column. The individual fractions from salt elution were analysed by western blotting (Figs. 4.13). Single purified 31 kDa bands were detected in samples 3 to 5. Analysis of these same fractions by dot blotting showed them to be detected by the anti-c-myc antibody (Fig. 4.12).

4.2.4.4 Comparison of phosphate-buffered saline (PBS) and Tris as buffers in ELISA of α-C5 scFv

An ELISA of α-C5 was carried out according to section 2.3.1.12.1, using either 50 MM Tris, pH 7.4, or PBS as wash and diluent reagents throughout the assay. The assay using Tris gave titres greatly in excess of 1 in 1,000, whereas use of PBS reduced this to below 1 in 1,000 (Fig. 4.14).

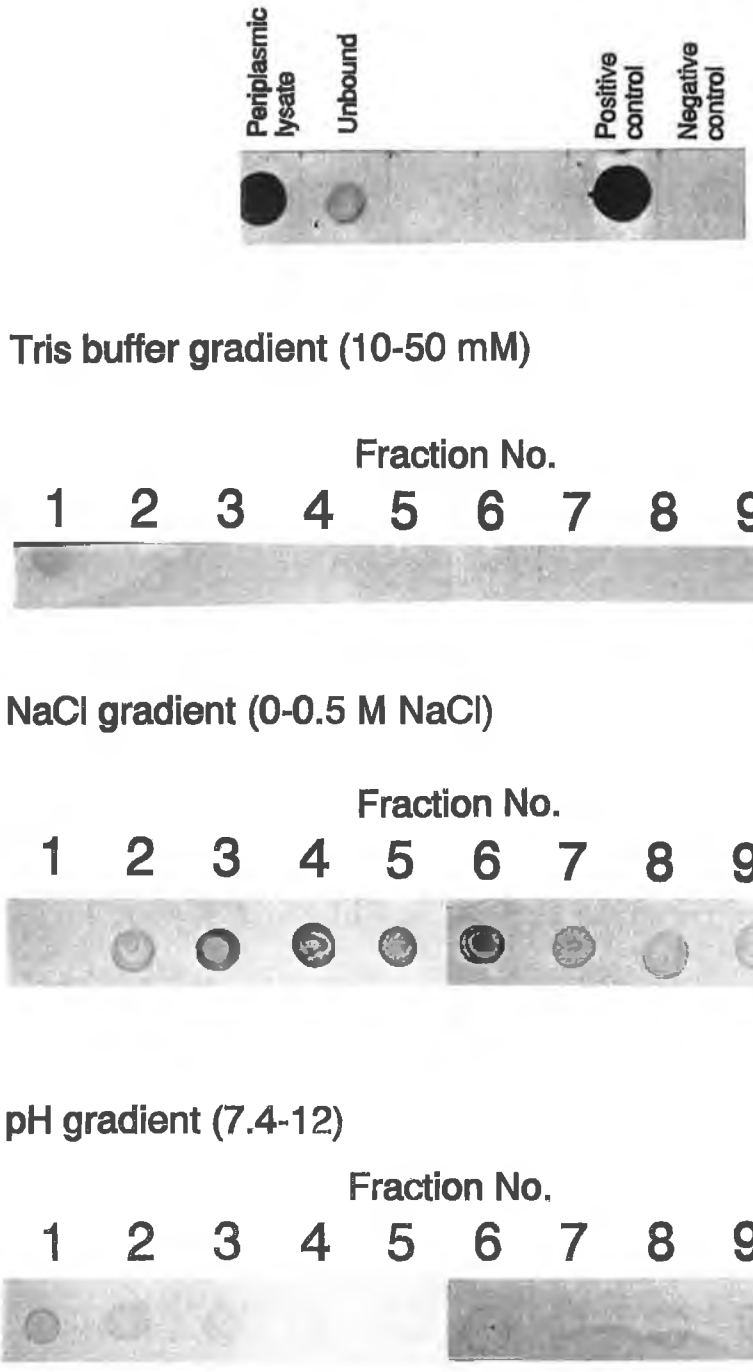


Figure 4.12. Sequential elution of α-C13 from a coumarin-BSA sepharose affinity column using Tris buffer, NaCl and pH gradients. Increasing Tris buffer concentration from 10 mM to 50 mM did not bring about scFv elution. Material in fraction 1 is the result of unbound material present on the column. Elution did occur in NaCl gradient in the range of 0 to 0.5 M NaCl, predominating in fractions 3 to 6. Negligible but detectable amounts of scFv were eluted in fractions from pH 7.4 to 12.

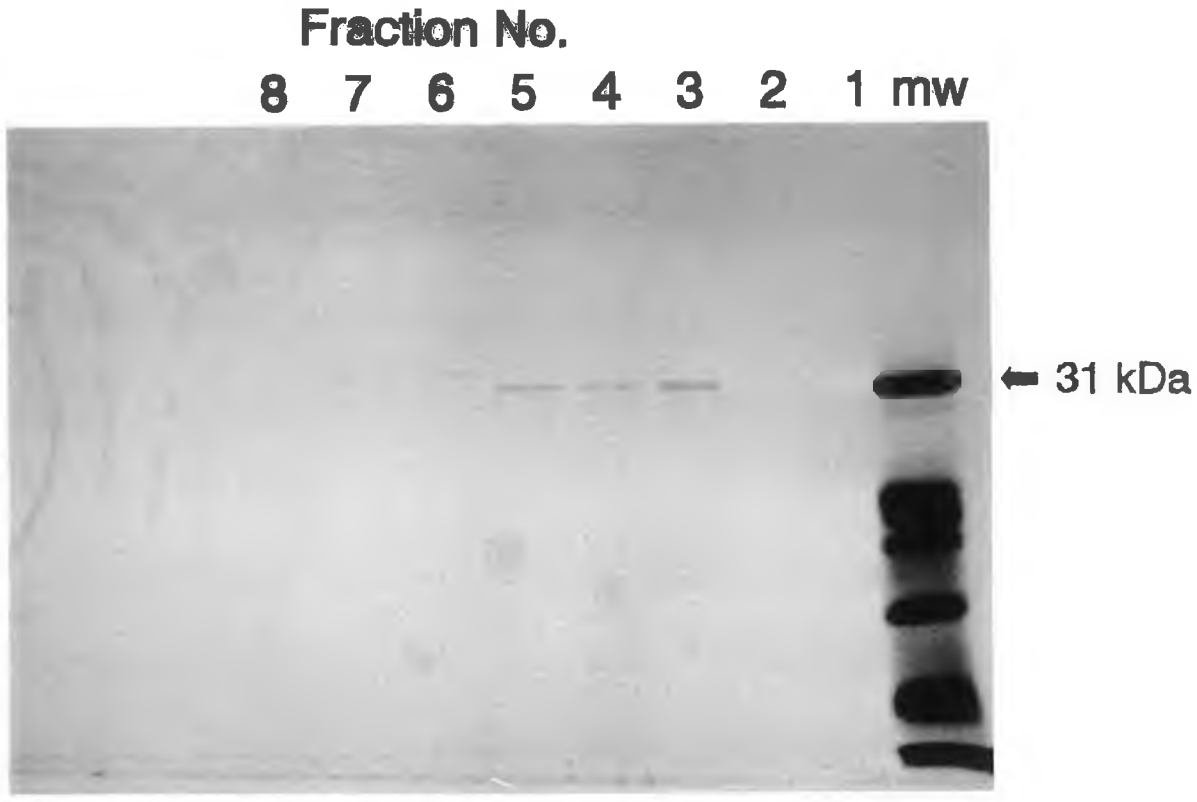


Figure 4.13. Western blot analysis of fractions from NaCl gradient elution of α-C13. Recognition of scFv was with α-c-myc antibody. This shows the presence of a 31 kDa protein fragment present in fractions 3 to 5.

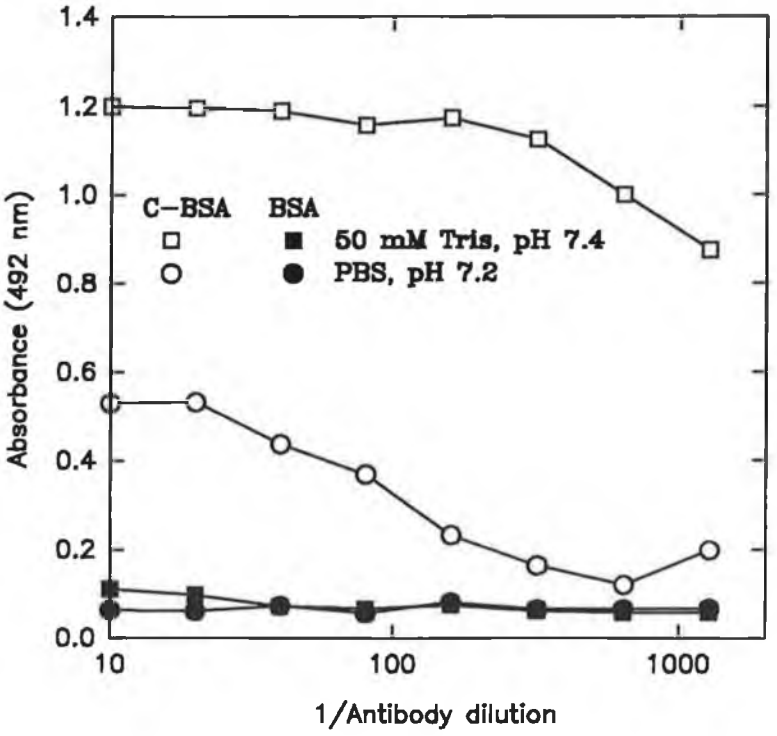


Figure 4.14. The titration of α-C5 antibody against coumarin-BSA (C-BSA) using either Tris buffer or PBS as wash buffers and diluent. Antibody diluted and washed with PBS, pH 7.2, greatly reduced scFv titre, as compared with the use of 50 mM Tris, pH 7.4, which yielded titres greatly in excess of 1 in 1,000. The absence of NaCl in the Tris buffer did not lead to an increase in the non-specific binding of scFv to BSA.

4.2.4.5 Purification of α-C5 and α-C13 using NaCl gradient

10 ml samples of periplasmic lysate of α-C5 and α-C13 in 50 mM Tris, pH 7.4 were purified on a 12.5 ml coumarin-BSA column using a 50 mM Tris, pH 7.4, 0 to 0.5 M NaCl gradient, collecting 10 x 10 ml fractions. Dot blots of purification fractions for α-C5 and α-C13 are shown in Figs. 4.15 and 4.16, respectively. Both showed elution of significant levels of antibody in fractions 3 to 6. Later fractions contained lower yields of scFv also. The fractions containing the greatest yields of antibody were pooled, concentrated and analysed by SDS-PAGE and western blotting. SDS-PAGE showed purification of two bands, one at approximately 31 kDa, and the other at approx. 29.5 kDa (Figs. 4.17 and 4.18). The lower molecular weight band was present in large amounts in both samples following concentration. In the α-C13 sample, the higher molecular weight component was entirely lost following concentration of the affinity-purified sample. The 31 kDa molecular weight fragment isolated from affinity purification and detected by SDS-PAGE was also detected on western blots which recognised the c-myc peptide tag (Fig. 4.18). However, loss of the 31 kDa fragment was associated with loss of recognition on western blots, the 29.5 kDa band showing no recognition by anti-c-myc. The loss of recognition on western blots was also accompanied by loss of activity in ELISA (Figs. 4.19 and 4.20). Periplasmic lysates yielded titres in excess of 1 in 1,000. However, nearly all activity was lost following affinity purification, α-C5 being marginally better than α-C13. The 20 kDa fragment recognising anti-c-myc antibody was again present in unpurified samples.

4.2.4.6 Purification of α-C14 using NaCl gradient

Culture supernatant from cultures of α-C14 were filtered through sterile 0.45 μm filters and 0.15% (w/v) streptomycin sulphate added. Supernatant was diluted 1 in 4 with 50 mM Tris, pH 7.4, and purified on a 12.5 ml coumarin-BSA column. Antibody was washed and eluted as in section 4.2.4.5 and samples analysed by dot blotting (Fig. 4.21). Although overall yields were poorer than for previous purifications of α-C5 and α-C13, antibody did elute into fractions 4 to 6 on the salt gradient. Again, however, when analysed by

4.22), significant activity was lost when the purified fractions were concentrated. This was again associated with loss of the western blot-active 31 kDa component and the appearance of the non-active 29.5 kDa component (Figs. 4.23 and 4.24).

4.2.4.7 Optimised purification of anti-coumarin scFv antibodies from culture supernatants and periplasmic lysates

Affinity purification of anti-coumarin clones was as previously described, with the additional incorporation of 1 mM PMSF into all buffers used in affinity purification and final samples. Culture supernatant from α-C14 production was concentrated 10-fold by ultrafiltration (Amicon) and dialysed into 50 mM Tris, pH 7.4. Purified antibody-containing fractions were pooled, dialysed into 50 mM Tris, pH 7.4 and concentrated on Centricon 10 units (Amicon). In all three cases, elution profiles were as previously described, with elution taking place in fractions 2 to 6 for α-C5 and α-C13, corresponding to 0.05 M to 0.15 M NaCl, and fractions 4 to 6 for α-C14, corresponding to 0.15 M NaCl (Fig. 4.25). All three samples showed elevated levels of the 31 kDa component over the 29.5 kDa fragment (Fig. 4.26). This also gave rise to titres in excess of 1 in 10,000 by ELISA (Figs. 4.27a, 4.27b and 4.27c), even after two weeks storage at 4°C. Typical yields of purified scFv were in the order of 1 mg scFv per litre of original culture.

4.2.4.8 Modification of scFv ELISA using anti-human F(ab')$_2$ antibody

The results of ELISA using a HRP-labelled anti-human F(ab')$_2$ antibody to detect scFv α-C5 and α-C13 are shown in Figs. 4.28a and 4.28b. Both periplasmic lysates showed decreased responses in association with decreases in antibody concentration. However, overall signals were typically very poor with absorbances no higher than 0.3 at 492 nm.

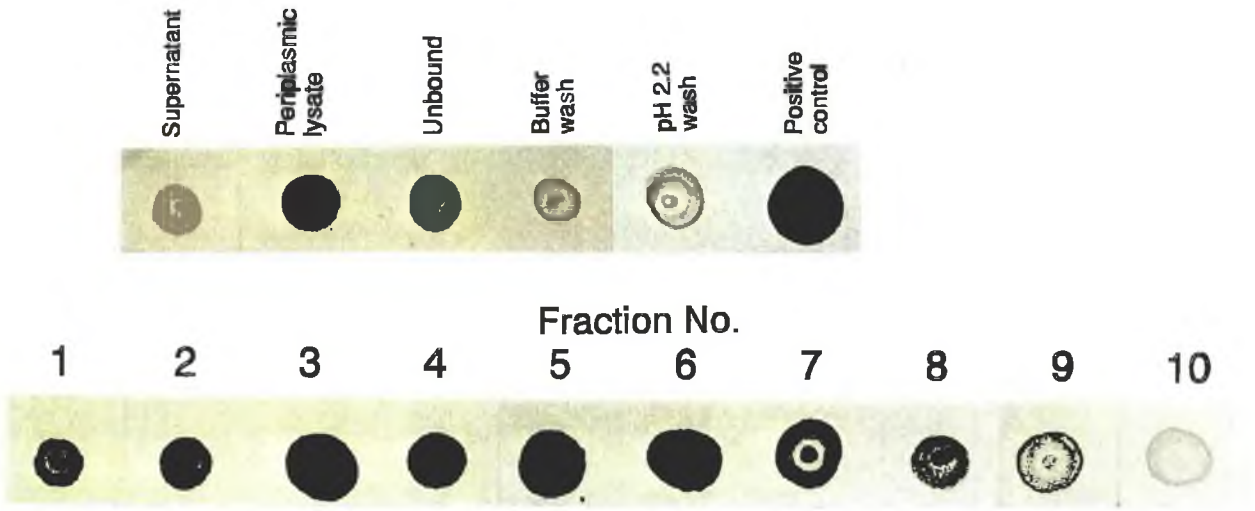


Figure 4.15. Dot blot analysis of fractions of α-C5 purified in an NaCl gradient. Culture supernatant, periplasmic lysate, unbound material from the column and column wash were all analysed as were fractions eluted in NaCl gradient from 0 to 0.5 NaCl (Fractions 1 to 10). This was followed by a wash in glycine buffer, pH 2.2. Highest levels of scFv were found in fractions 3 to 6. Lower concentrations of scFv was present in the supernatant, with a considerable amount in the periplasmic lysate. The unbound fraction was the material that passed through the column without binding. This contained appreciable amounts of scFv. Little antibody was present in the buffer wash prior to elution and also little eluted in glycine buffer, pH 2.2 which followed NaCl elution. Positive control:α-NIP.

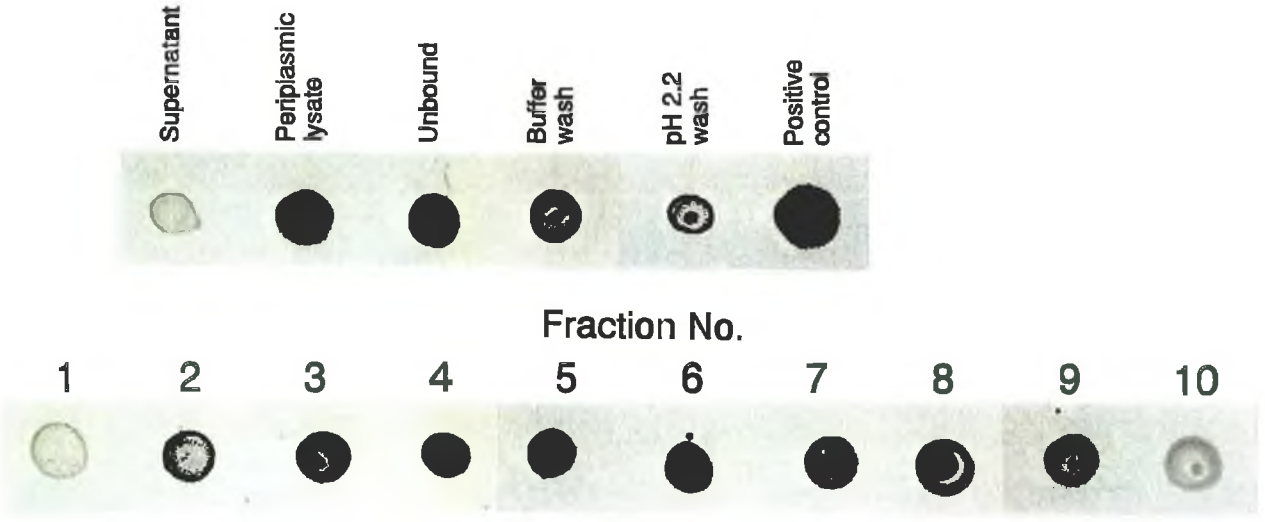


Figure 4.16. Dot blot analysis of fractions of α-C13 purified in an NaCl gradient. Highest levels of scFv were found in fractions 3 to 6. Quantities of scFv in supernatant, periplasm, unbound fraction, buffer and low pH washes was similar to that for α-C5 (Fig. 4.16). Positive control: α-NIP.

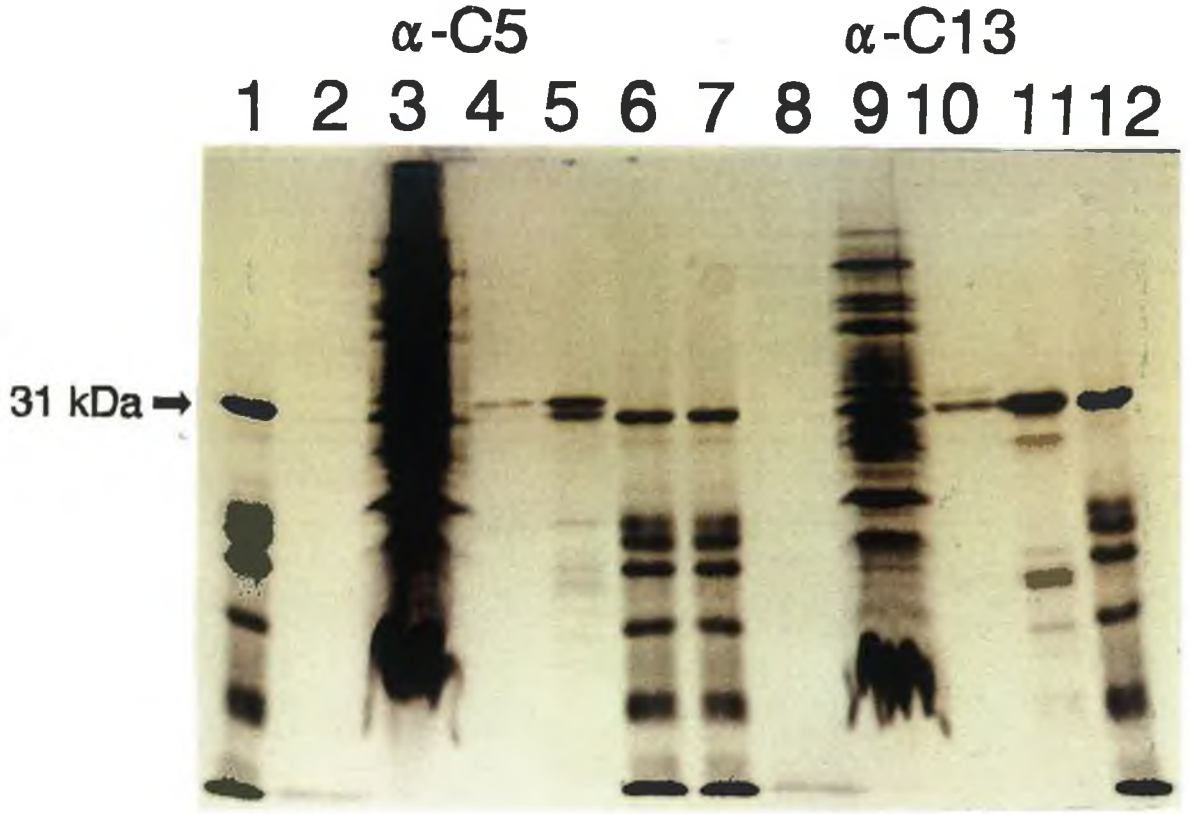


Figure 4.17. SDS-PAGE analysis of samples from the purification of α-C5 and α-C13 by NaCl elution from a coumarin-BSA sepharose affinity column. Lanes: 1,6,7,12: Molecular weight markers; 2,8: Supernatant; 3,9: Periplasmic lysate; 4,10: Affinity-purified fraction; 5,11: Affinity-purified and concentrated. Affinity purification brought about the enrichment of a 31 kDa protein fragment. This had degraded to a 29.5 kDa protein band following concentration. This was more significant in the α-C13 sample than in α-C5. Although enrichment of the scFv was significant, several other molecular weight species were still present following purification.

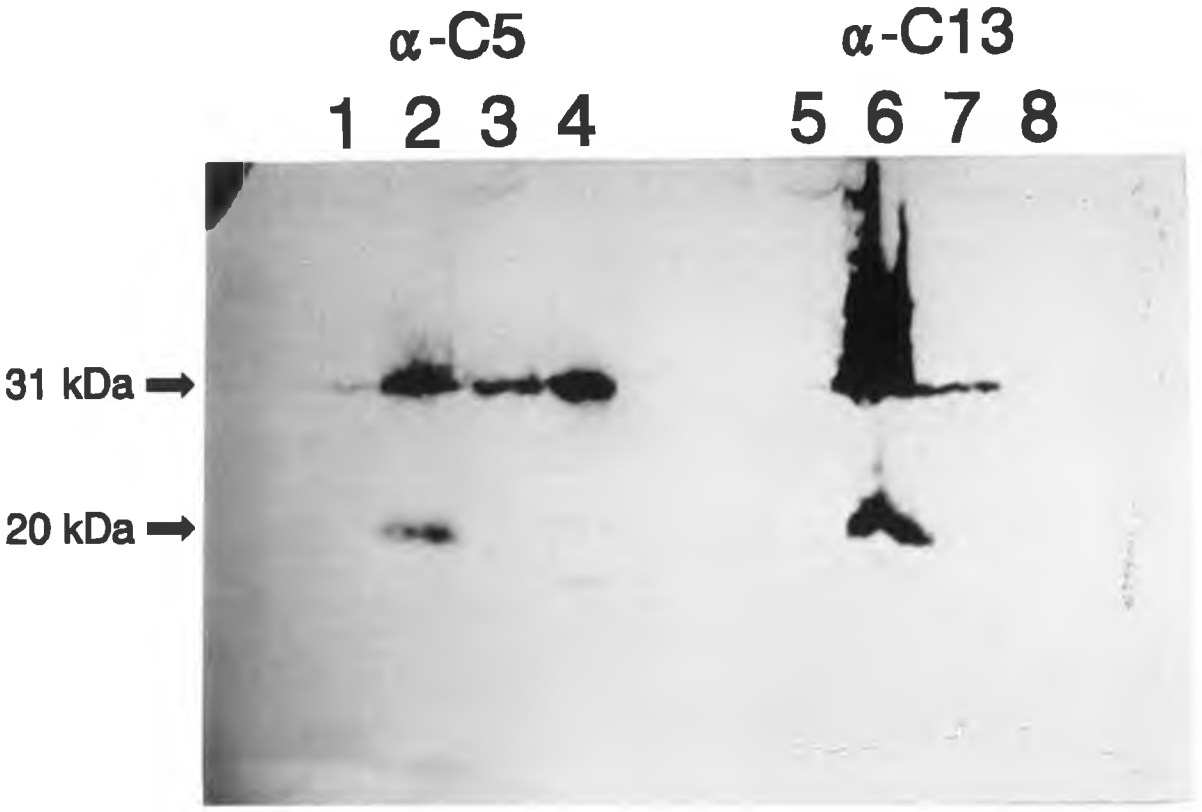


Figure 4.18. Western blot analysis of samples from the purification of α-C5 and α-C13 by NaCl elution from a coumarin-BSA sepharose affinity column. Lanes: 1,5: Supernatant; 2,6: Periplasmic lysate; 3,7: Affinity-purified; 4,8: Affinity-purified and concentrated. Recognition of 31 kDa components by the anti-c-myc antibody are clearly present in the periplasmic lysates and affinity-purified samples of both α-C5 and α-C13. Following concentration, the recognition of α-C13 is completely lost, associated with the complete loss of the 31 kDa band. However, the presence of significant levels of the α-C5 31 kDa component in the affinity-purified fraction has led to its recognition by anti-c-myc. High levels of protein in samples such as α-C13 periplasm led to non-specific binding and smearing.

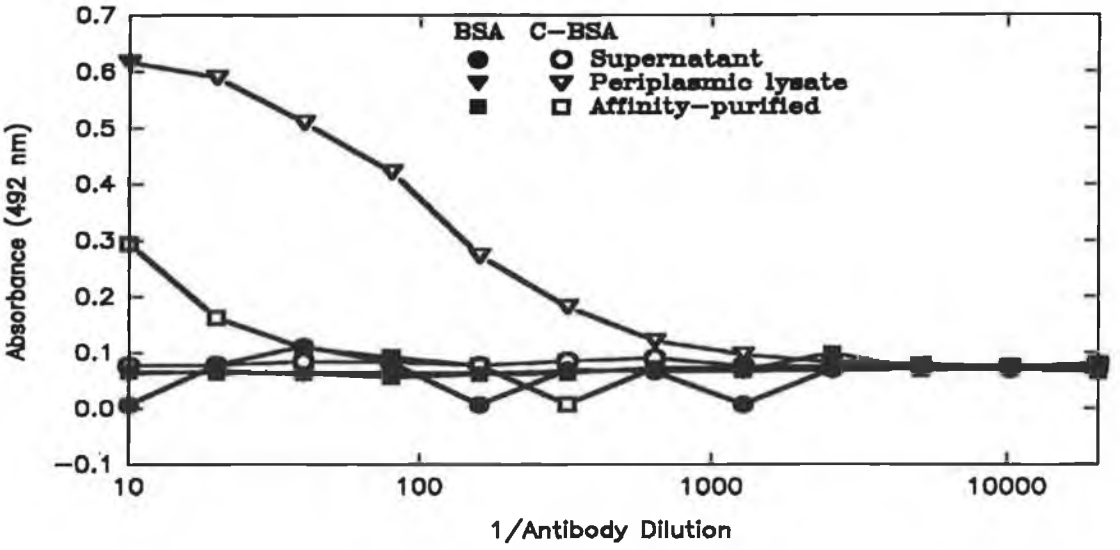


Figure 4.19. Analysis of samples from the NaCl gradient purification of α-C5 by ELISA. Titres in excess of 1 in 1,000 were achieved for periplasmic lysate to coumarin-BSA (C-BSA). This had dropped to less than 1 in 100 in the affinity-purified sample. Only a negligible signal was obtained from culture supernatants.

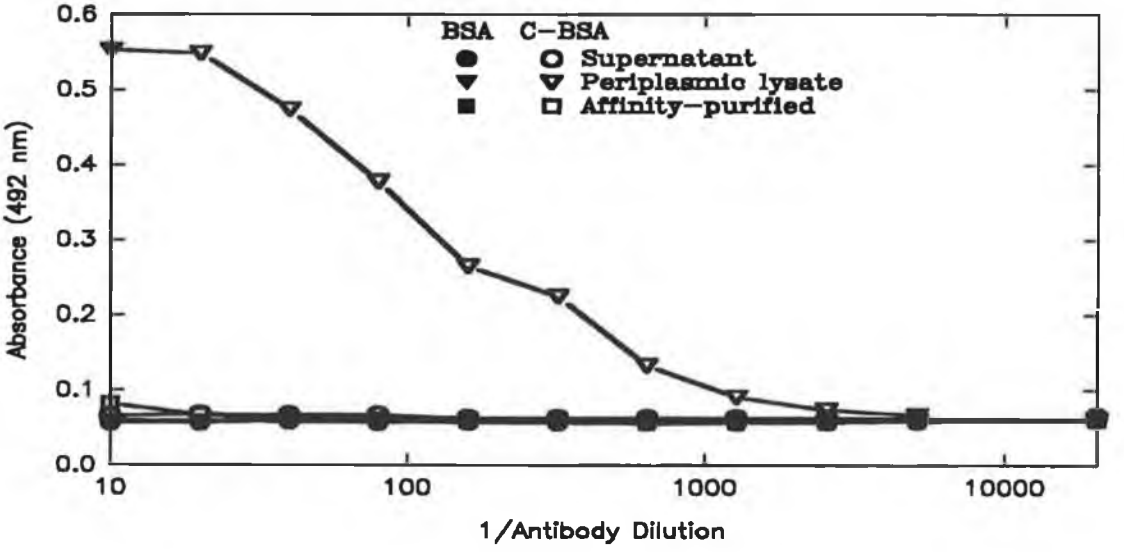


Figure 4.20. Analysis of samples from the NaCl gradient purification of α-C13 by ELISA. Titres in excess of 1 in 1,000 were achieved for periplasmic lysate to coumarin-BSA (C-BSA). Only negligible activity remained in the sample following affinity purification. Culture supernatant yielded a signal only marginally higher than background values.

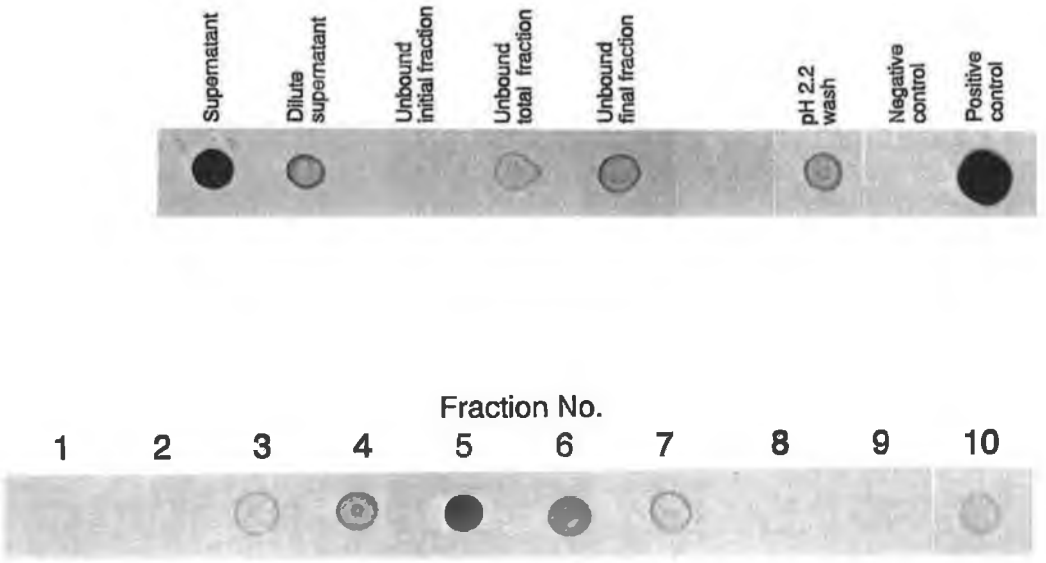


Figure 4.21. Dot blot analysis of α-C14 fractions purified by NaCl gradient elution on a coumarin-BSA sepharose affinity column. Antibody eluted primarily into fractions 4 to 6. Recognition of scFv was with α-c-myc antibody. Large concentrations of scFv were present in the culture supernatant, and the diluted culture supernatant used for application of sample to the affinity column. Fractions of sample after passage through the column showed little unbound material upon initial application of sample, but significantly more at the end of sample application. Some scFv also eluted in pH 2.2 buffer. Positive control:α-NIP. Negative control: HB2151.

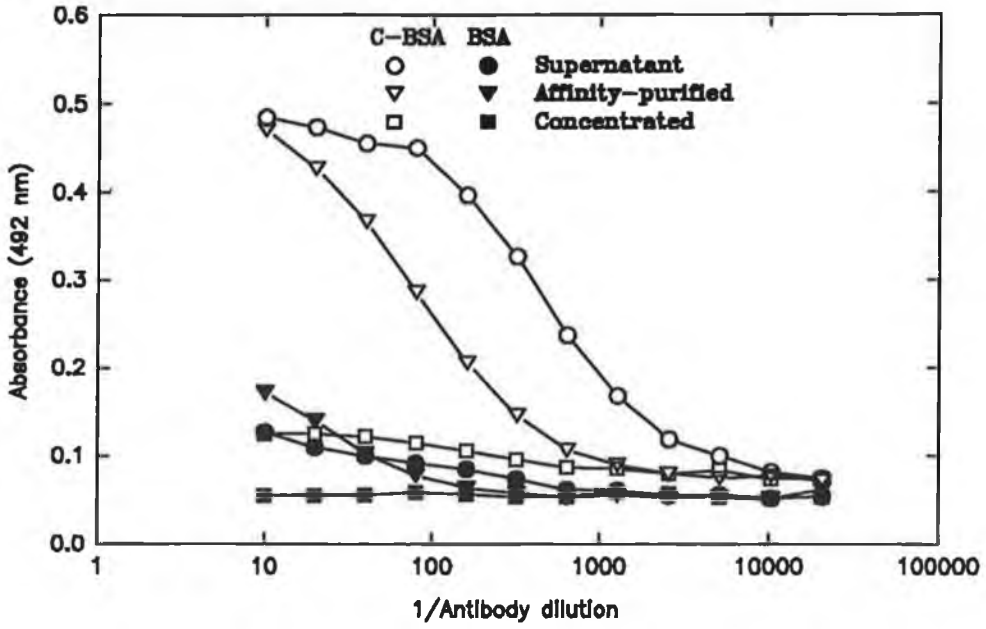


Figure 4.22. ELISA analysis of samples from α-C14 purification by NaCl gradient elution. Culture supernatant gave a titre in excess of 1 in 10,000 to coumarin-BSA (C-BSA). This had decreased to 1 in 1,000 following affinity purification. Signals were only marginally above baseline levels following concentration.

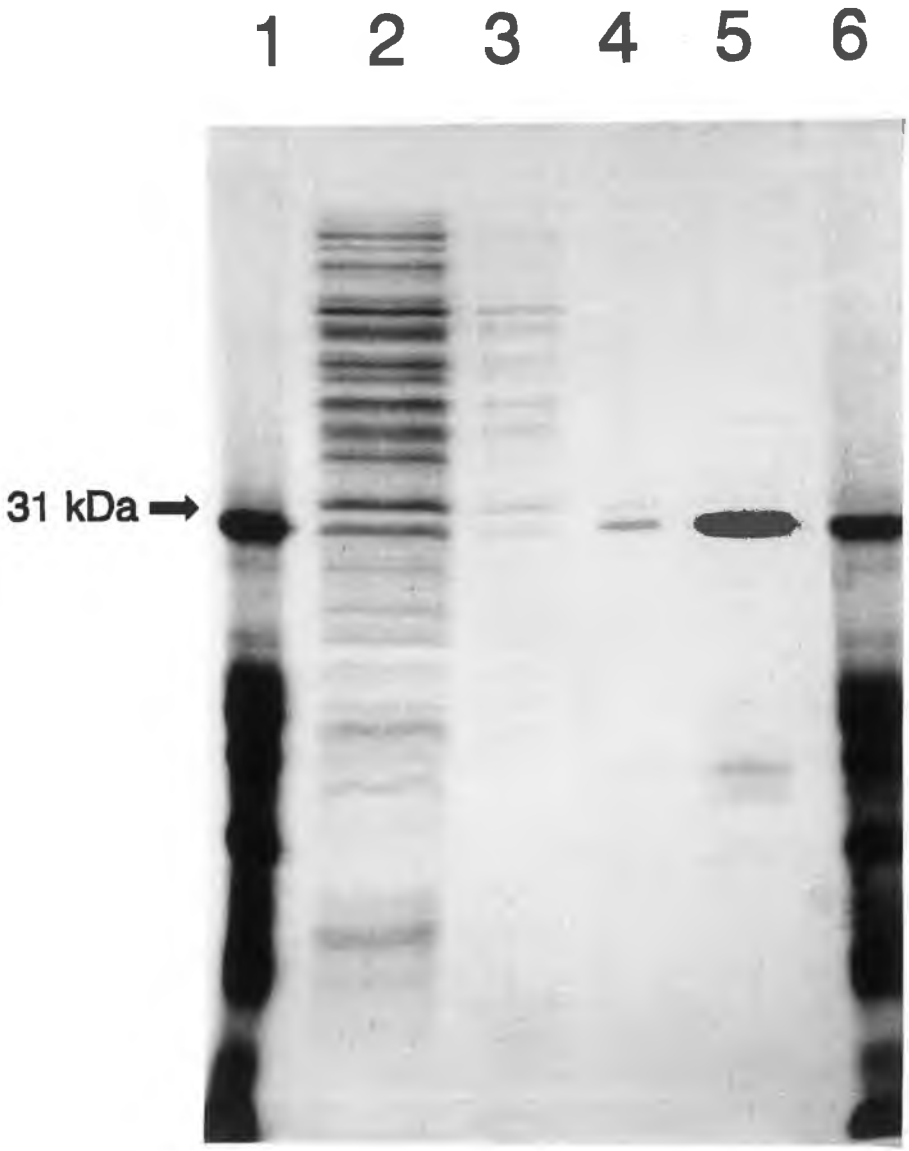


Figure 4.23. SDS-PAGE analysis of samples from the purification of α-C14 by NaCl gradient elution on a coumarin-BSA sepharose affinity column. Lanes: 1,6: Molecular weight markers; 2: Supernatant; 3: Unbound fraction; 4: Affinity-purified fraction; 5: Affinity-purified and concentrated. The presence of a 31 kDa and a 29.5 kDa fragment are both clearly visible in the affinity-purified sample, but only the 29.5 kDa component is visible following concentration of the scFv.

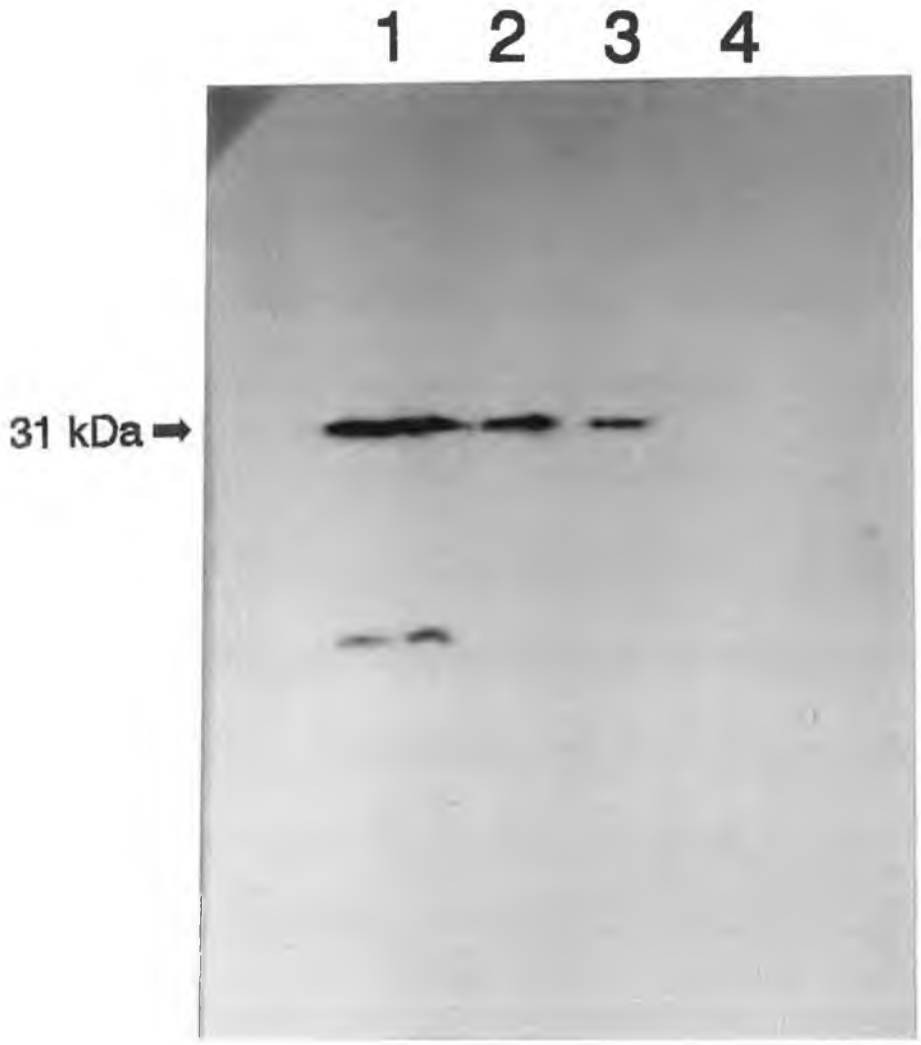


Figure 4.24. Western blot analysis of samples from the purification of α-C14 by NaCl gradient elution. ScFv was detected with α-c-myc antibody. Lanes: Supernatant; 2: Unbound fraction; 3: Affinity-purified fraction; 4: Affinity-purified and concentrated. Recognition of the 31 kDa scFv is apparent in supernatant, unbound material and affinity-purified samples, but has again been lost in the concentrated affinity-purified sample. This blot corresponds to the SDS-PAGE gel in Fig. 4.23.

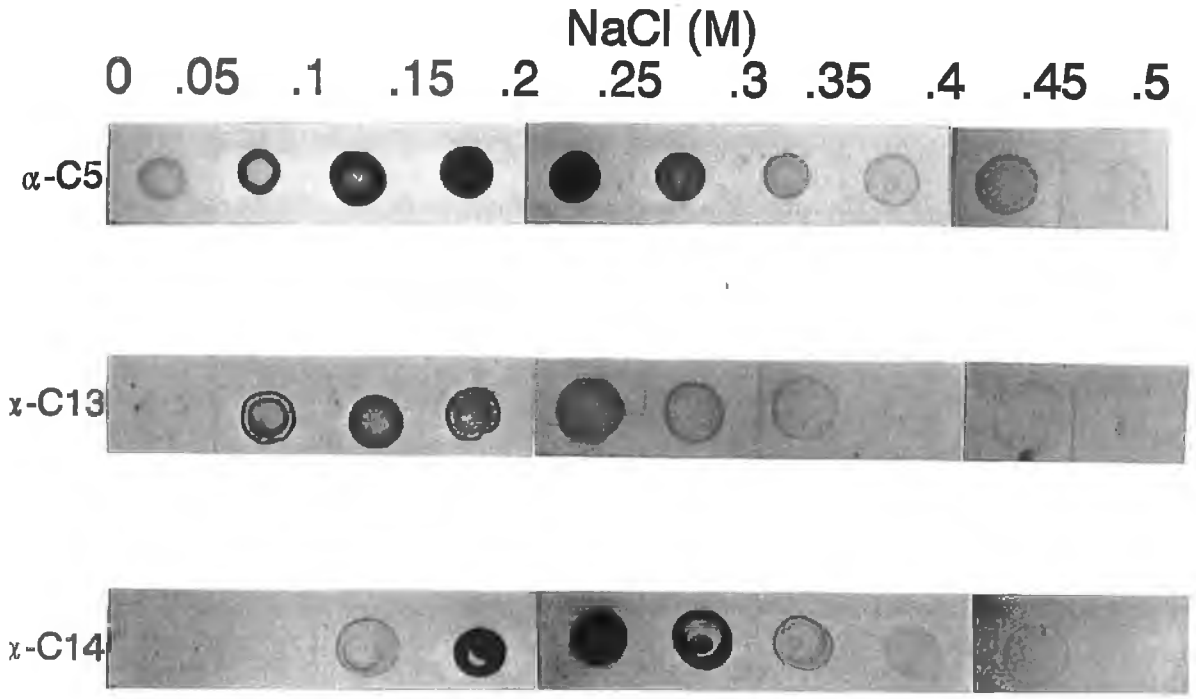


Figure 4.25. Dot blot analysis of Fractions from the affinity purification of α-C5, α-C13 and α-C14 by NaCl gradient elution on a coumarin-BSA sepharose affinity column, with the incorporation of PMSF. Recognition of scFv was with α-c-myc antibody. α-C5 and α-C13 show maximum scFv elution in fractions from 0.05 to 0.25 M NaCl and α-C14 shows maximum elution in fractions from 0.15 to 0.3 M NaCl.

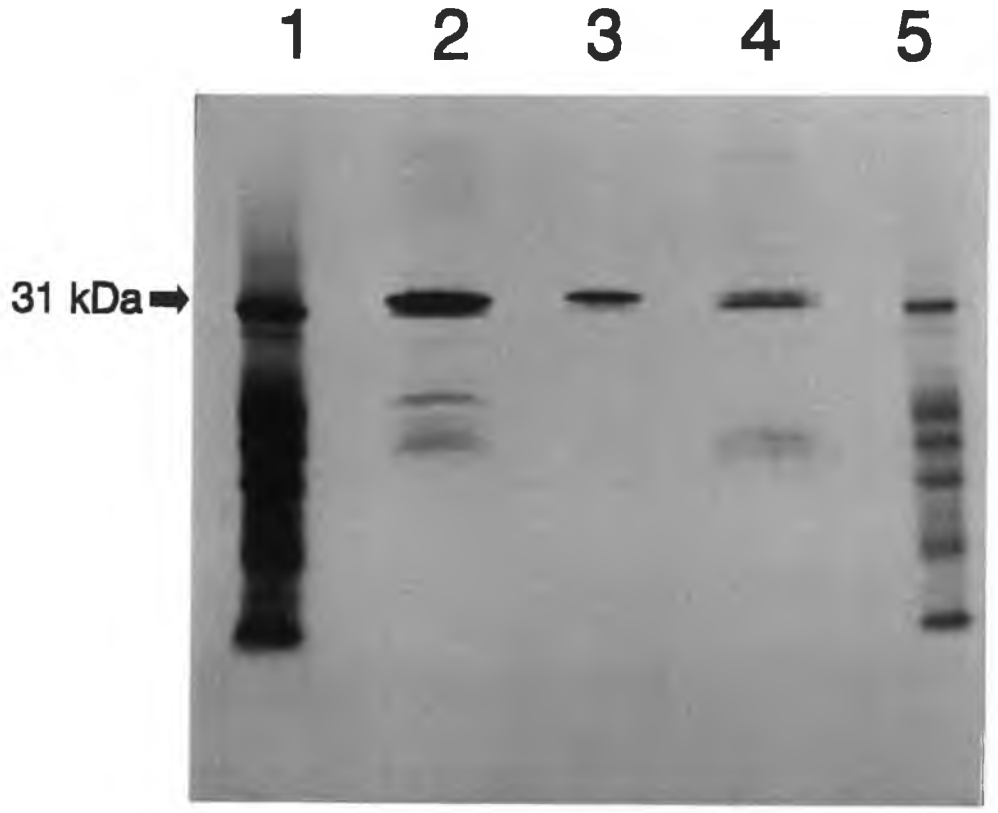


Figure 4.26. SDS-PAGE analysis of α-C5, α-C13 and α-C14 affinity-purified and concentrated using NaCl gradient in the presence of PMSF. Lanes: 1,5: Molecular weight markers; 2: α-C5; 3: α-C13; 4: α-C14. All three scFv preparations show the greatly improved yield of 31 kDa fragment over the 29.5 KDa component.

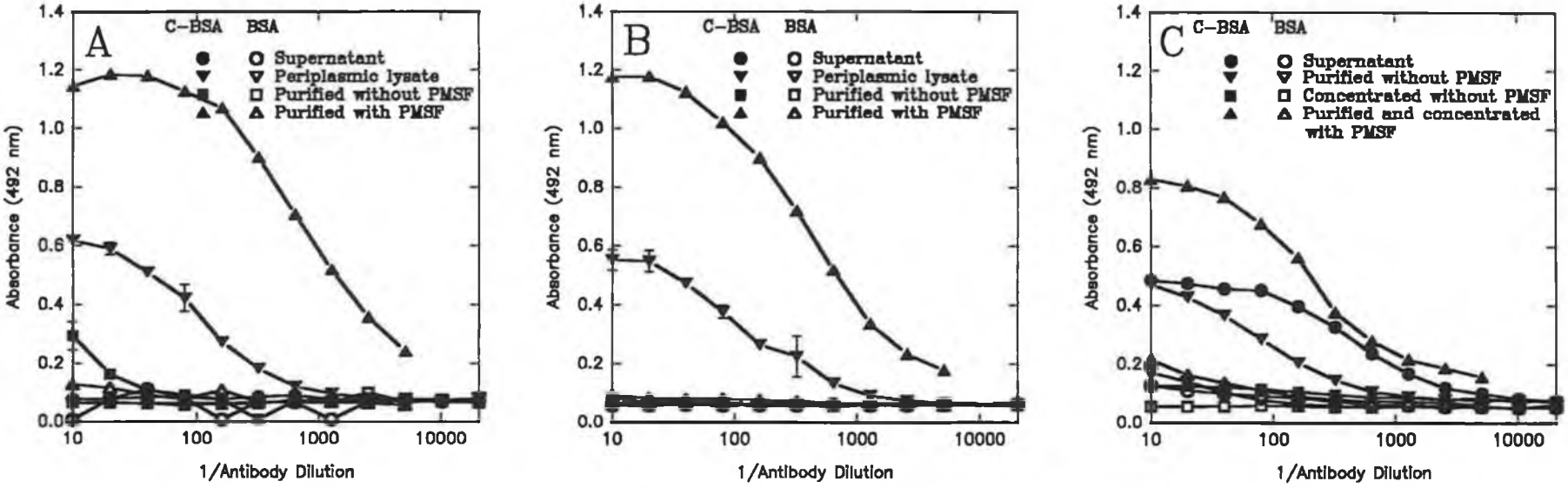


Figure 4.27. ELISA analysis of samples from the affinity purification of α-C5 (A), α-C13 (B) and α-C14 (C). Following affinity purification and concentration in the presence of PMSF, all three concentrated scFv samples yielded titres in the order of 1 in 10,000 to coumarin-BSA (C-BSA).

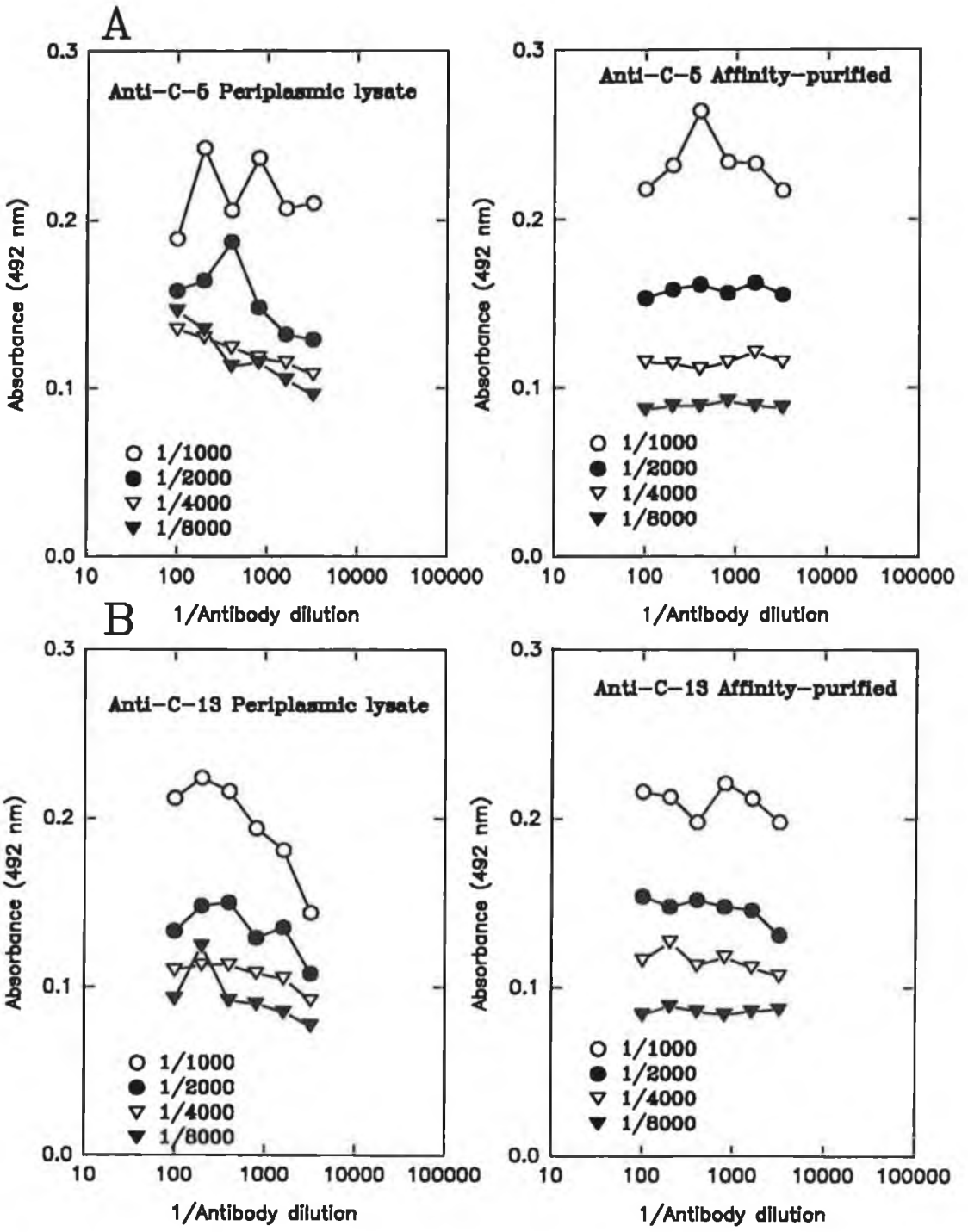


Fig. 4.28A and B. ELISA analysis of α-C5 (A) and α-C13 (B) scFv using HRP-labelled anti-human F(ab')$_2$ antibody. Response to decreases in antibody concentration were only achieved at high dilutions of conjugated antibody. However, signals were extremely low, the highest signals being less than 0.3 AU at 492 nm.

4.2.5 Characterisation of anti-coumarin scFv antibodies

4.2.5.1 Optimisation of competition ELISA for the detection of free coumarin

4.2.5.1a Selection of optimal antibody concentration

Optimal antibody concentration was calculated according to (Crowther, 1995) from the titres yielded in Figs. 4.27a, b and c. This was selected as 70% maximum binding, and corresponded to 1 in 400, 1 in 200 and 1 in 100 dilutions of α-C5, α-C13 and α-C14 antibodies, respectively.

4.2.5.1b Selection of optimal coumarin-BSA coating concentrations

The effect of coating concentration on signal for all three anti-coumarin scFvs is shown in Fig. 4.29. Levels above 150 μg/ml did not increase signals, so 150 μg/ml was selected as optimal coating concentration of coumarin-BSA.

4.2.5.1c Competition ELISA analysis of free coumarin using anti-coumarin scFv

The ability of the three anti-coumarin clones to compete between bound coumarin-BSA and free coumarin was assessed (Fig. 4.30). All three clones showed no recognition of free coumarin in the presence of 0.05% methanol, at coumarin concentrations ranging from 0.05 to 50 μg/ml. Competition ELISAs were repeated over a range of antibody dilutions (Figs. 4.31A, 4.32B and 4.32C), but these assays confirmed the original result that showed no recognition between the anti-coumarin scFv antibodies and free drug.

4.2.6 Assessment of the effect of methanol and NaCl concentration on competition ELISA with α-C5, α-C13 and α-C14

The effect of methanol concentrations up to 25%, and NaCl concentrations up to 0.5 M on the binding of α-C5, α-C13 and α-C14 to free coumarin were assessed at the 1 μg/ml free coumarin level (Figs. 4.32, 4.33 and 4.34). NaCl significantly reduced the binding of

all three scFv antibodies to surface coumarin-BSA. The presence of methanol appeared to enhance the interaction of antibody with coumarin-BSA significantly in the case of α-C14 (Fig. 4.34).

Data for different methanol concentrations were grouped, and student independent and paired t-tests performed, comparing the means of populations in the presence and absence of 1 μg/ml coumarin. In the cases of α-C5 and α-C13, a significant difference in binding to coumarin-BSA in the presence and absence of free coumarin was detected at the 0 and

Table. 4.2. Mean (X) and standard deviation (s) of populations from ELISA results investigating the effect of NaCl concentration on the binding of anti-coumarin scFvs in the presence and absence of free coumarin (n=5).

		NaCl concentration (M)					
		0	0.001	0.01	0.1	0.25	0.5
α-C5							
1 μg/ml coumarin	X	0.631	0.630	0.622	0.356	0.110	0.105
	s	0.012	0.007	0.045	0.039	0.010	0.005
No free coumarin	x	0.656	0.644	0.641	0.390	0.117	0.099
	s	0.012	0.007	0.006	0.026	0.007	0.006
α-C13							
1 μg/ml coumarin	X	0.703	0.702	0.705	0.610	0.250	0.222
	s	0.005	0.006	0.003	0.028	0.008	0.028
No free coumarin	X	0.715	0.719	0.713	0.628	0.261	0.218
	s	0.009	0.007	0.008	0.025	0.014	0.015
α-C14							
1 μg/ml coumarin	X	0.604	0.596	0.597	0.216	0.091	0.087
	s	0.031	0.037	0.026	0.040	0.004	0.007
No free coumarin	X	0.607	0.602	0.604	0.221	0.092	0.081
	s	0.024	0.026	0.029	0.058	0.005	0.002

0.001 M NaCl concentration levels (n=5). These were significant at the 95% confidence level (Table 4.2) and Figs. 4.35A, B and C. In most instances, the signal in the presence of 1 μg/ml free coumarin was lower than that with no free coumarin, but this was only statistically significant at the 0 and 0,001 M NaCl concentrations for α-C5 and α-C13.

4.2.7 The effect of NaCl concentration on anti-coumarin scFv binding to coumarin-BSA

The effect of NaCl concentration from 0 to 0.2 M on the binding of the three anti-coumarin-BSA clones is shown in Fig. 4.36. Maximum binding occurred at 0 M NaCl and this decreased rapidly to baseline levels when the NaCl concentration reached 0.2 M.

4.2.8 Competition of anti-coumarin-BSA scFv antibodies against coumarin-BSA

When normalised to a percentage of maximum binding, all three anti-coumarin-BSA scFv clones showed competition binding profiles to coumarin-BSA that were very similar to one another (Fig. 4.37). Relative affinities of the scFvs to coumarin-BSA were calculated as the molarity of free antigen which yielded 50% maximum absorbance, according to Roben (1993) and Tijssen (1985). This yielded relative affinities for the three clones of 3×10^7 M^{-1}, 4.5×10^7 M^{-1} and 2.5×10^7 M^{-1} for α-C5, α-C13 and α-C14, respectively. Since the coupling ratio of coumarin to BSA was 11.5:1, this would give relative affinities to the hapten of 3.45×10^8 M^{-1}, 5.18×10^8 M^{-1} and 2.88×10^8 M^{-1} for α-C5, α-C13 and α-C14 respectively. This resulted in an affinity ranking of α-C13>α-C5>α-C14.

4.2.9 Competition of α-C5 scFv with different coumarin-protein conjugates

The interaction of the α-C5 scFv clone with different coumarin-protein conjugates was assessed. Four conjugates were selected: three coumarin-BSA conjugates with different coupling ratios of coumarin to protein (11.5, 5.7 and 2.77 moles coumarin/mole BSA), and a coumarin-ovalbumin conjugate (7 moles coumarin/mole ovalbumin). These were used as competition reagents against bound coumarin-BSA (11.5 moles/mole) (Fig. 4.38).

All conjugates showed that they had been recognised by α-C5 scFv. In the case of the three coumarin-BSA conjugates, the level of recognition was proportional to the level of conjugation. α-C5 also recognised coumarin when attached to ovalbumin, giving a response similar to that of the coumarin-BSA (11.5 moles/mole) conjugate. However, ovalbumin is a smaller protein than BSA, being only approximately 44 kDa, this would equate to a coupling ratio of 10.5 moles coumarin/mole BSA, very similar to the 11.5 moles/mole conjugate.

4.2.10 Assessment of the multi-specificity of the anti-coumarin scFv clones to different coumarin derivative-BSA conjugates

Fig. 4.39 shows that the three anti-coumarin scFv antibodies bound specifically to coumarin-BSA, but not to 7-hydroxycoumarin-BSA or 7-hydroxycoumarin-glucuronide-BSA (see section 5). The level of binding to 7-hydroxycoumarin-BSA and 7-hydroxycoumarin-glucuronide-BSA was only fractionally higher than that for the control BSA.

4.2.11 Use of anti-coumarin-BSA scFv antibodies as western blot reagents

All three antibodies were used as western blot reagents on samples of coumarin-BSA, BSA, coumarin-OVA and OVA. However, due to their intolerance of NaCl, salt-containing buffers were substituted with 50 mM Tris, pH 7.4. The level of non-specific interaction between antibody and proteins on the surface of a membrane can be controlled by the incorporation of NaCl at different concentrations as well as detergents and the number of washing steps involved. Normally, a level is selected which reduces non-specific binding to a negligible level, while still giving good interaction with the specific protein. Low stringency buffers containing no NaCl resulted in high levels of non-specific background staining (results not shown).

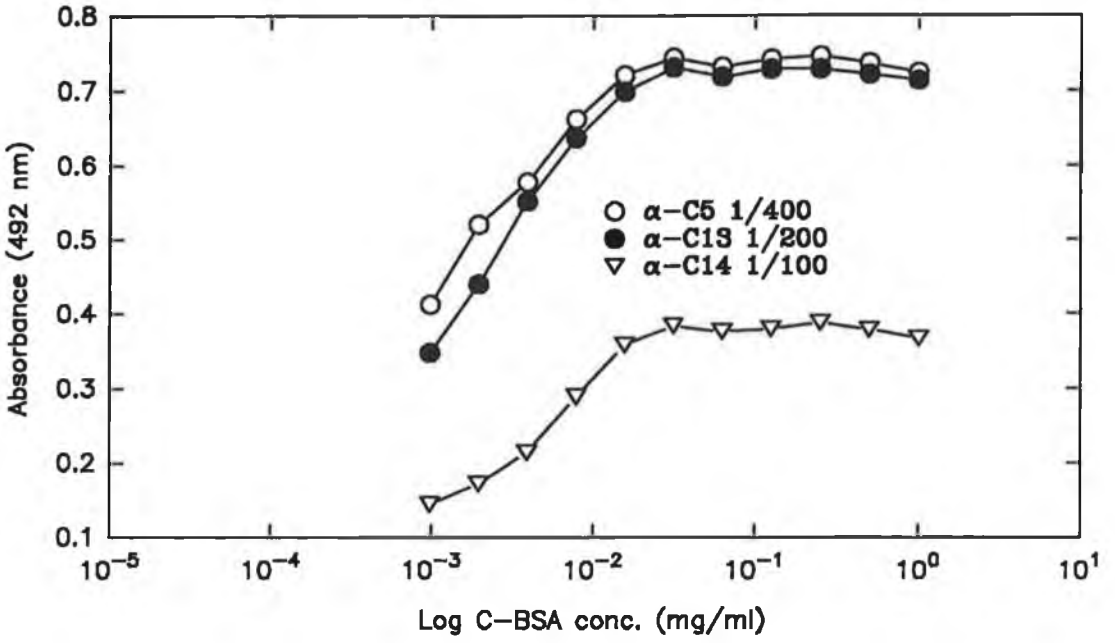


Figure 4.29. The effect of coumarin-BSA coating concentration on signal for the optimisation of competition ELISA in the analysis of coumarin. Increasing coating concentration above 150 µg/ml did not result insignificant increases in the amount of scFv bound. Optimal coumarin-BSA coating concentration for competition ELISA was estimated to be 150 µg/ml.

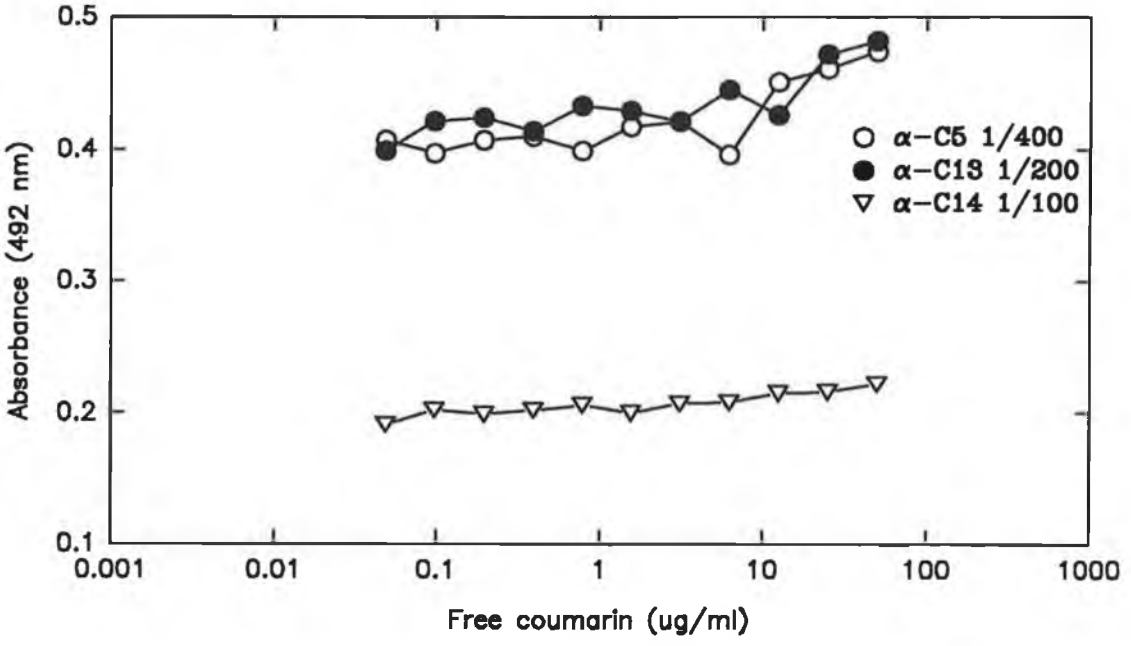


Figure 4.30. Competition ELISA of free coumarin (in 0.05 % methanol) against coumarin-BSA using α-C5, α-C13 and α-C14. No competition with free coumarin was apparent at concentrations of free drug from 0.05 to 50 µg/ml.

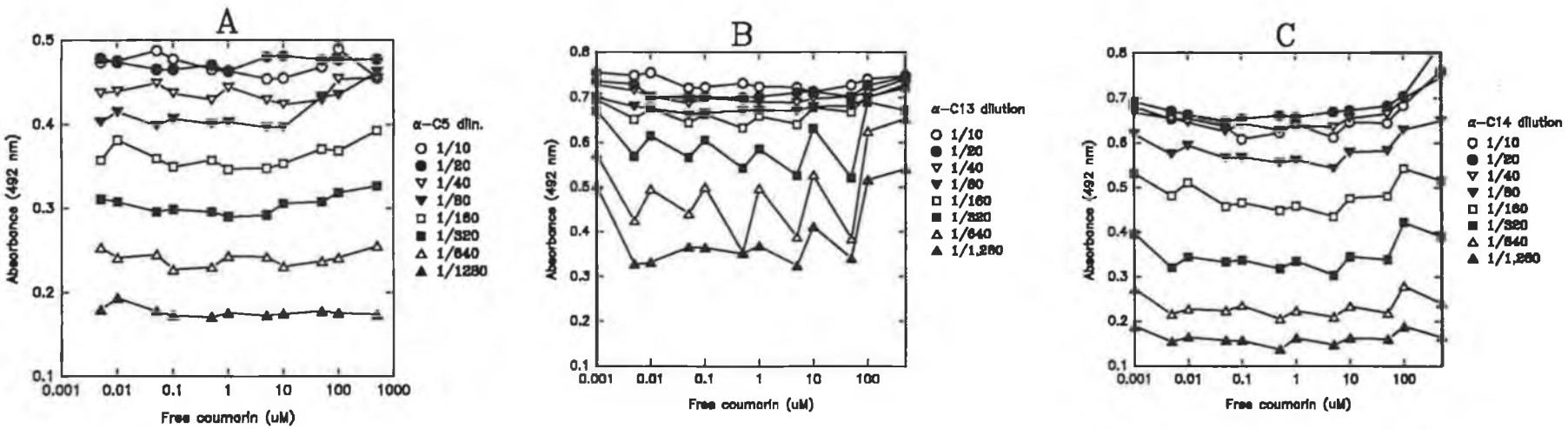


Figure 4.31A, B and C. Repeat analysis of competition ELISAs using α-C5, α-C13 and α-C14 for the detection of free coumarin using a range of scFv concentrations. No observable interaction between scFv and free coumarin was apparent at any of the drug concentrations or antibody dilutions analysed.

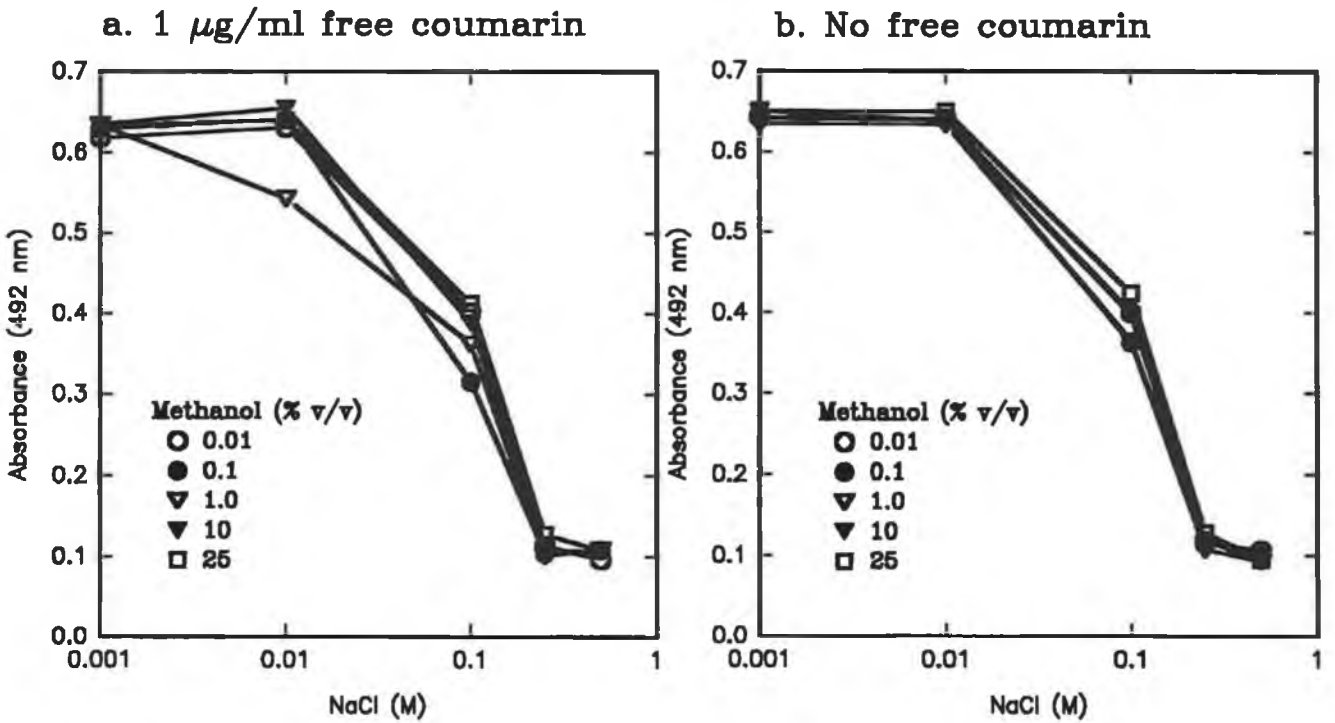


Figure 4.32. The effect of NaCl and methanol concentration on the binding of α-C5 in the presence of 0 and 1 μg/ml coumarin. Increasing NaCl concentration drastically reduced the binding interaction between α-C5 and coumarin-BSA. Methanol concentration had no significant effect between 0.01 and 25 % (v/v) (as found by paired and independent student t-tests). Significant reduction in the binding to coumarin-BSA occurred in the presence of 1 μg/ml coumarin at the 0 and 0.001 M NaCl levels. (Values for 0 M NaCl not shown due to logarithmic scale used. See Table 4.2).

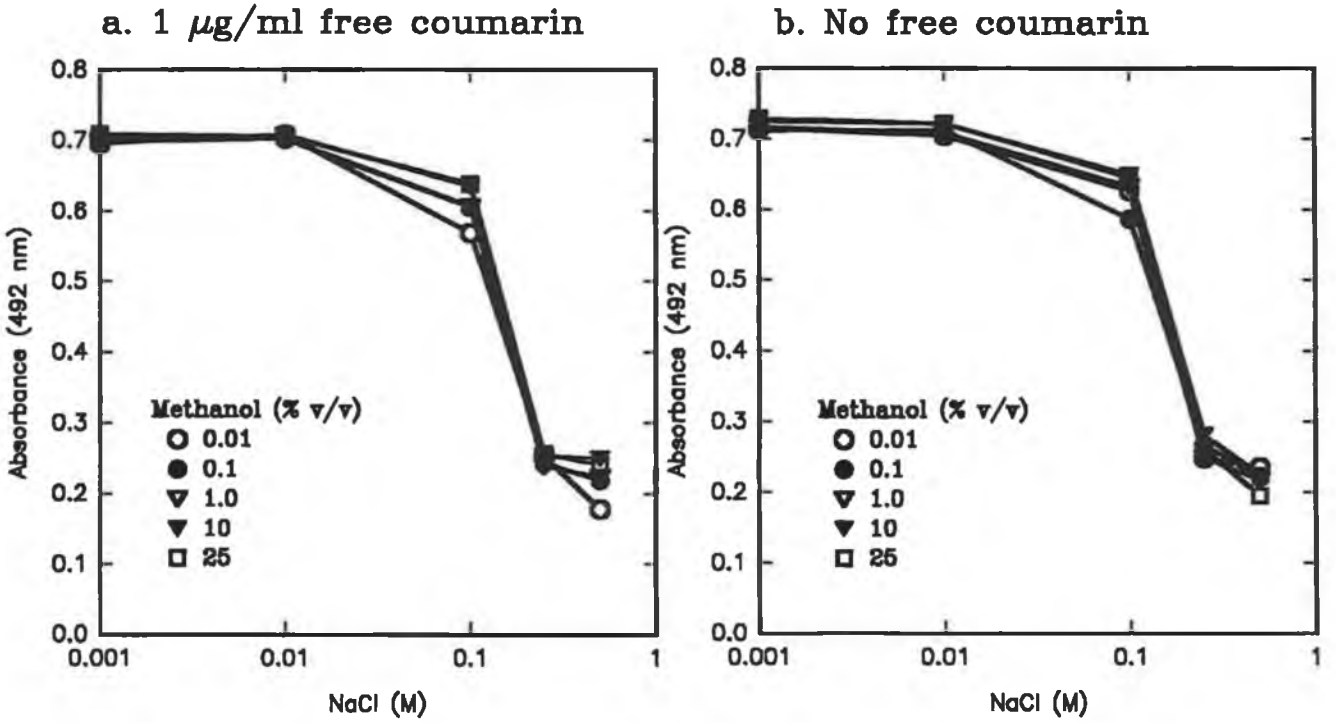


Figure 4.33. The effect of NaCl and methanol concentration on the binding of α-C13 in the presence of 0 and 1 μg/ml coumarin. Increasing NaCl concentration drastically reduced the binding interaction between α-C13 and coumarin-BSA. Methanol concentration had no significant effect between 0.01 and 25 %(v/v). Significant reduction in the binding to coumarin-BSA occurred in the presence of 1 μg/ml coumarin at the 0 and 0.001 NaCl levels.

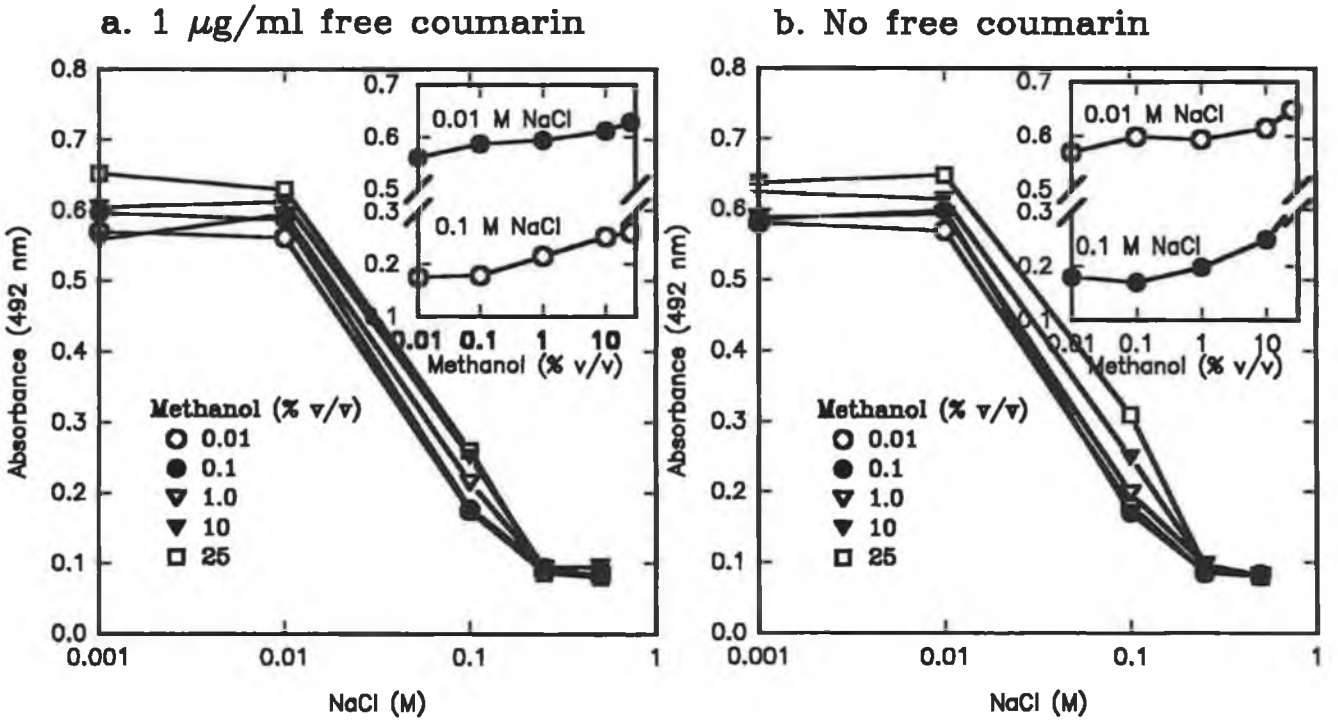


Figure 4.34. The effect of NaCl and methanol concentration on the binding of α-C14 in the presence of 0 and 1 μg/ml coumarin. Increasing NaCl concentration drastically reduced the binding interaction between α-C14 and coumarin-BSA. Methanol concentration had a significant effect, increasing the level of interaction between α-C14 and coumarin-BSA between 0.01 and 25 %(v/v), most significantly on samples containing 0.01 M and 0.1 M NaCl (see insert). No significant reduction in binding to coumarin-BSA was observed in the presence of 1μg/ml coumarin.

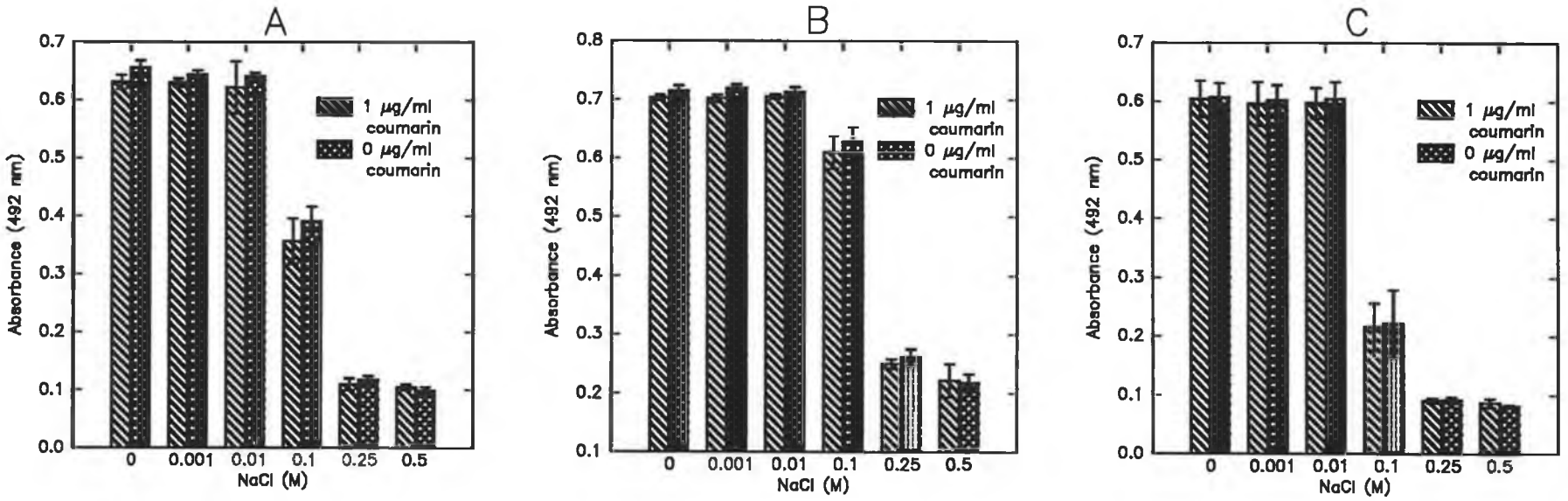


Figure 4.35A, B and C. Competition immunoassay of α-C5 (A), α-C13 (B) and α-C14 (C) against 1 µg/ml free coumarin at different NaCl concentrations. All three scFv clones show slightly lower absorbances in the presence of 1 µg/ml coumarin compared to 0 µg/ml coumarin at the 0, 0.001, 0.01 and 0.1 M NaCl concentrations. This was significant for α-C5 and α-C13 at the 0 and 0.001 M NaCl concentrations (as determined by independent and paired t-tests).

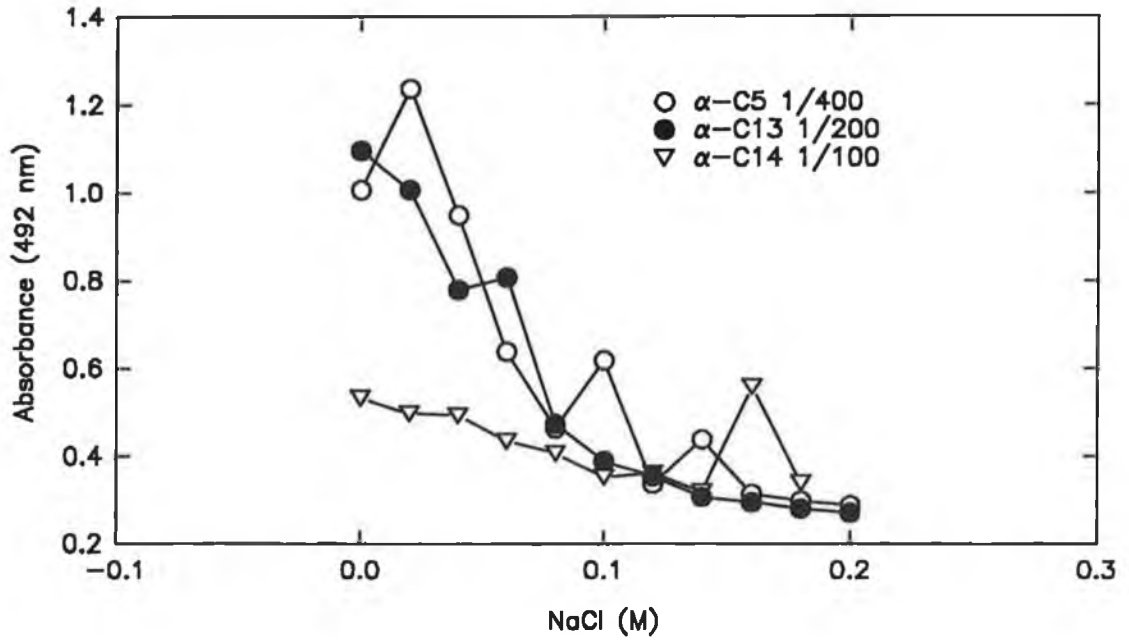


Figure 4.36. The effect of NaCl concentration on the binding of anti-coumarin-BSA scFvs to coumarin-BSA. All three scFvs show NaCl sensitivity from as low as 0.05 M. Baseline signals occur when NaCl concentration reaches 0.2 M.

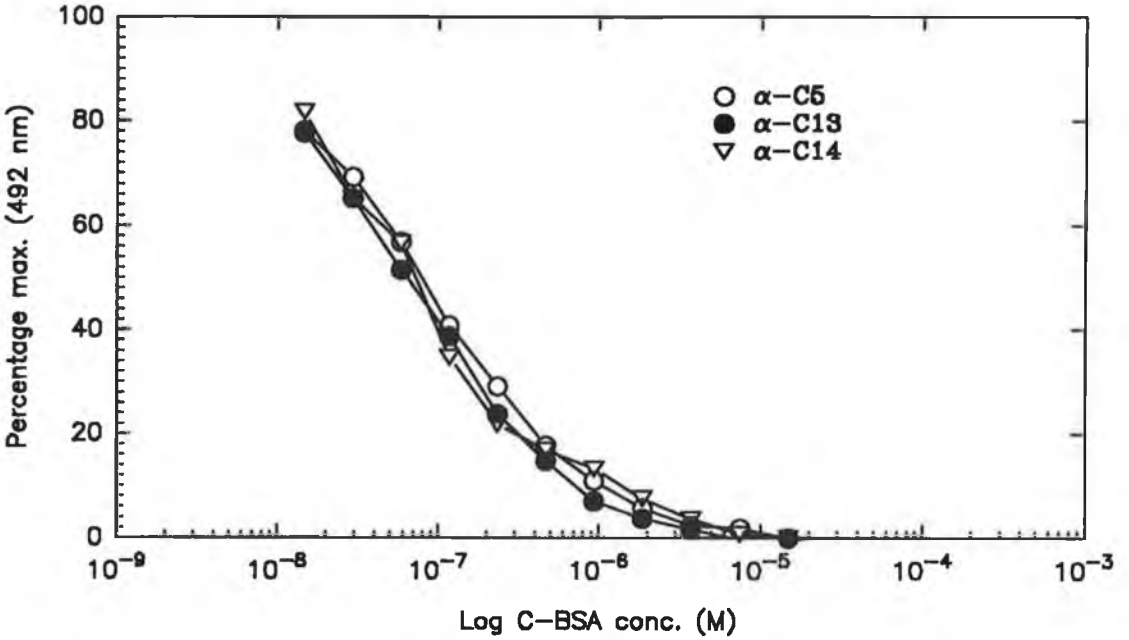


Figure 4.37. Relative affinities of α-C5, α-C13 and α-C14. All three scFvs show 50% binding with free coumarin-BSA at approx. 5 x 10^7 M^{-1} coumarin-BSA, which corresponds to a molarity of coumarin of approximately 5.75 x 10^8 M^{-1}.

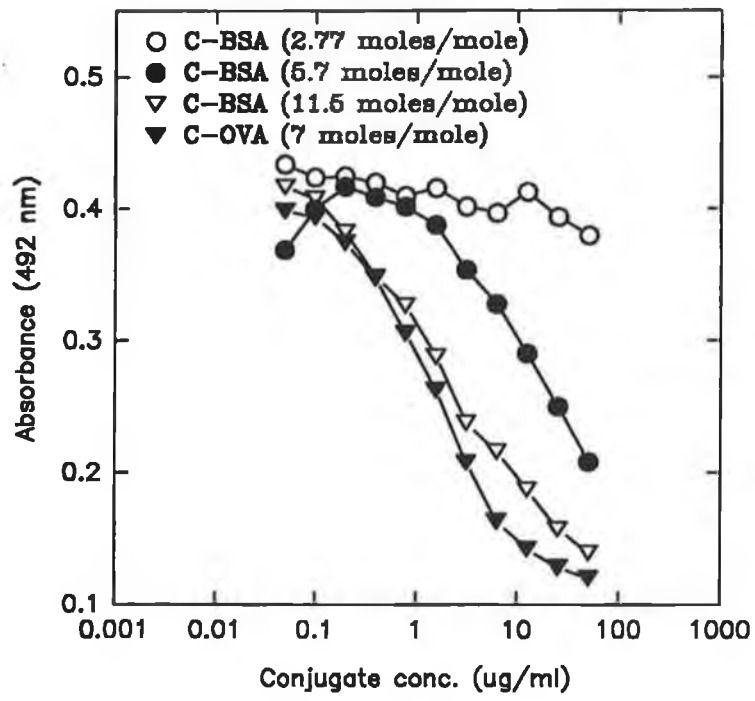


Figure. 4.38. Competition of α-C5 scFv for different coumarin-protein conjugates. Competition for coumarin-BSA (C-BSA) conjugates appears to be proportional to the level of conjugation of coumarin on the BSA carrier protein. α-C5 also recognised coumarin-ovalbumin (C-OVA) to the same degree as coumarin-BSA (11.5 moles coumarin/mole BSA). Due to the lower molecular weight of ovalbumin, this would be equivalent to a 10.5:1 coupling ratio on BSA.

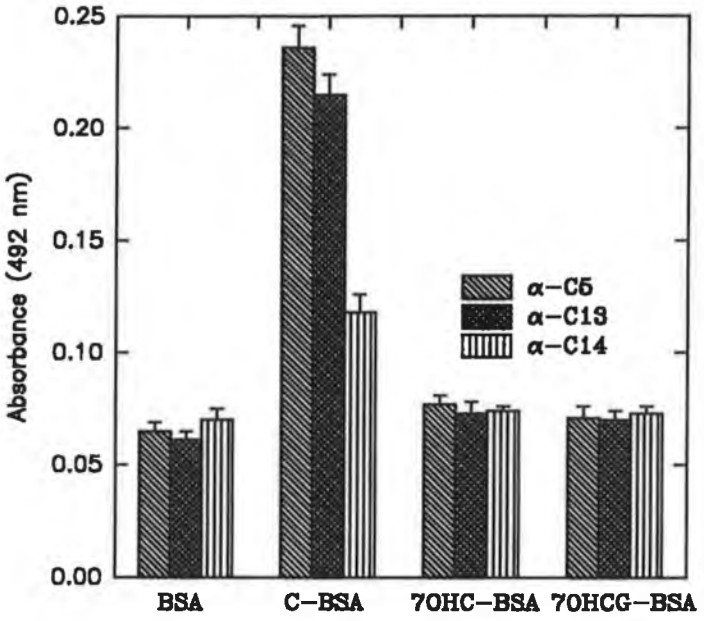


Figure 4.39. ELISA of anti-coumarin scFv antibodies α-C5, α-C13 and α-C14 to coumarin and its derivatives 7-hydroxycoumarin (7OHC) and 7-hydroxycoumarin-glucuronide (7OHCG) conjugated to bovine serum albumin (BSA). Antibody bound only to the coumarin-BSA conjugate and showed only background binding to 7OHC-BSA and 7OHCG-BSA as compared with the BSA control.

4.2.12 Characterisation and kinetic analysis of anti-coumarin-BSA scFv antibodies using BIAcore™

The BIAcore sensorgrams for the three anti-coumarin scFv clones are shown in Fig. 4.40. Antibodies were analysed at 1 mM concentrations on a surface of coumarin-BSA (2.77 moles/mole) of 1,600 response units (RU). Kinetic data was calculated for the three clones. Analysis showed that the three clones did not adhere perfectly to first order kinetics. However, all three clones gave affinity constants based on the observed on and off rates in the 10^9 M^{-1} range and were affinity ranked in the order α-C13>α-C5>α-C14. This affinity ranking corresponded with that found by competition immunoassay (Fig. 4.37). Controls using BSA coupled to the sensor chip surface (6,000 RU) did show some background binding of the scFv antibody to the BSA-dextran matrix (Fig. 4.41) of 31.6 RU, compared to 167.2 RU for the α-C5 clone.

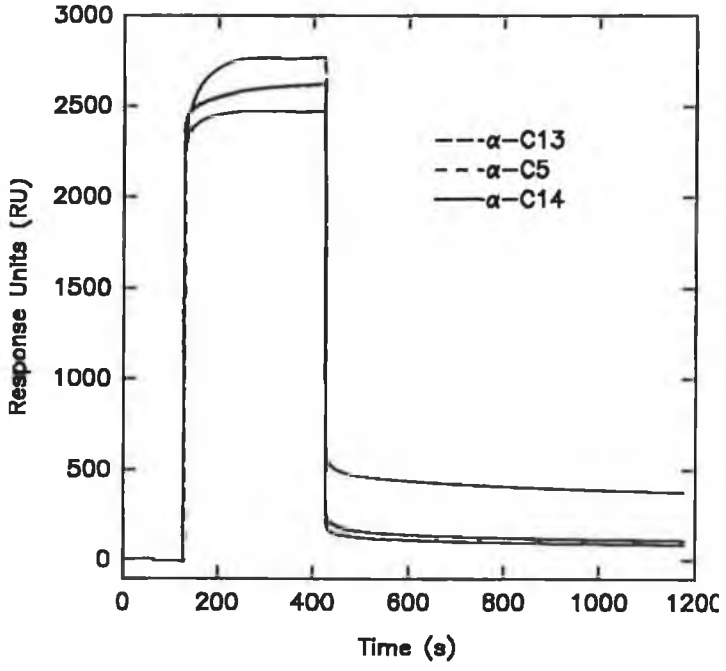


Figure 4.40. BIAcore sensorgrams of anti-coumarin clones α-C5, α-C13 and α-C14 to a coumarin-BSA surface coupled to dextran and yielding 1,600 response units (RU). All three clones show association and dissociation phases of the three clones. Kinetic data based on these sensorgrams yielded affinity constants in the range of 10^9 M^{-1}.

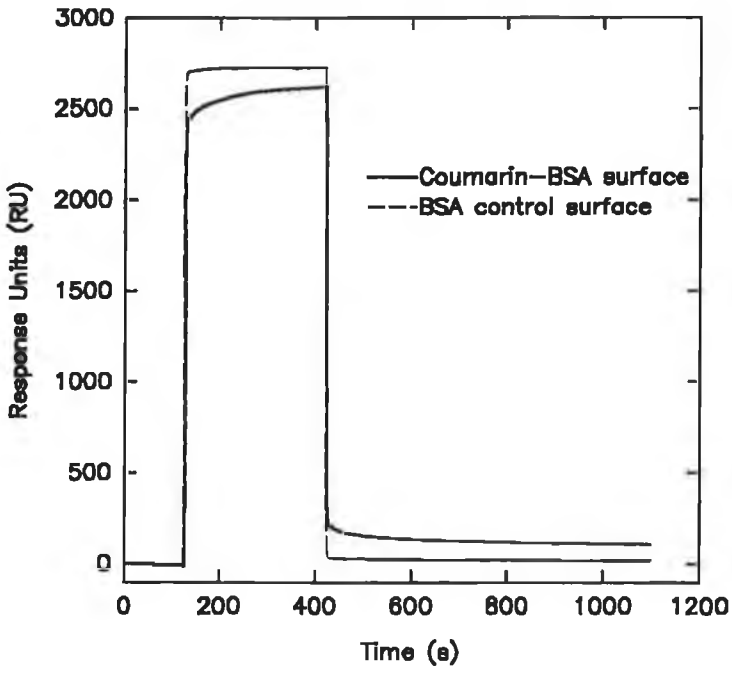


Figure 4.41. BIAcore sensorgrams of α-C5 scFv (1 mM) passed over a surface of coumarin-BSA (1,600 RU) linked to dextran and over bovine serum albumin (BSA) linked to dextran (6,000 RU). This showed some interaction of the scFv with the BSA-dextran surface (31. 6 RU) as compared to 167.2 RU over the coumarin-BSA surface.

4.3 PRODUCTION OF ANTI-7-HYDROXYCOUMARIN ANTIBODIES FROM THE NISSIM LIBRARY

4.3.1 Phage ELISA screening of first anti-7-hydroxycoumarin library

93 clones from the first anti-7-hydroxycoumarin library, following three rounds of affinity selection on 7OHC-BSA with BSA as blocking reagent, were screened on 7OHC-BSA and BSA (Fig. 4.42). This showed that all the clones which were positive to 7OHC-BSA were positive on the BSA control also. Positives were selected as those having values in excess of the positive control well, H7. This creates some discrepancies between the two plates. However, all clones which show values well in excess of the negative control on one plate, also did so on the other, indicating that these clones were also positive, if not reaching the positive control signal.

4.3.2 Phage ELISA analysis of second anti-7-hydroxycoumarin library

A second anti-7-hydroxycoumarin library was prepared with affinity selection using alternating 7OHC-protein conjugates and different blocking proteins (see section 2.3.3.4 and 2.3.3.6). Screening of 42 clones against 7OHC-OVA and OVA showed no response to the OVA control, while showing good enrichment of positive clones to 7OHC-OVA (Fig. 4.43).

A second phage ELISA of 96 clones was performed (Fig. 4.44). From this, 43 positive clones were selected and denoted α-7OHC1 to α-7OHC43 and used for further analysis.

4.3.3 Analysis of supernatant and periplasmic lysates of anti-7-hydroxycoumarin clones for the presence of scFv

Dot blot analysis of the periplasmic lysates and culture supernatants of the 43 positive anti-7-hydroxycoumarin clones is shown in Fig. 4.45. All 43 clones showed no secretion of antibody into periplasm or culture supernatant. Clones picked at random from the library showed secretion in 2 out of 5 clones. Since no individual clone exhibited better

secretion characteristics than another, the first twelve clones, α-7OHC1 to α-7OHC12 were selected for further studies on antibody refolding. α-7OHC1 to α-7OHC11 were analysed a second time and compared to controls and again showed poor scFv production. Clones α-7OHC6 to α-7OHC11 appeared slightly better, with α-7OHC10 and α-7OHC11 looking moderately better than the rest of the population.

4.3.4 Use of BIAcore for the affinity selection of anti-7-hydroxycoumarin clones from the Nissim library

To enrich for antibodies to 7-hydroxycoumarin-BSA, the phage antibodies of the original Nissim library were passed across a BIAcore chip surface immobilised with 7-hydroxycoumarin-BSA according to section 2.3.3.5. The phage counts eluting from the chip surface are shown in Fig. 4.46. A total of approximately 2.74×10^8 phage antibodies (tfu) from the original Nissim library were passed across the BIAcore chip surface. Phage antibody eluting in wash buffer fell to a level of approximately 5×10^2 tfu/ml. This level of phage elution was quite constant. Upon the addition of glycine buffer, however, specific elution of bound phage did occur. A total output of eluted phage of 1.26×10^4 tfu was produced. This represented an enrichment in excess of 1 in 20,000. No signal was produced by this binding on BIAcore sensorgrams (data not shown).

7-hydroxycoumarin-bovine serum albumin

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.729	0.545	0.713	0.214	0.163	0.409	0.679	0.690	0.686	0.714	0.266	0.555
B	0.688	0.581	0.682	0.142	0.677	0.643	0.115	0.620	0.244	0.650	0.134	0.669
C	0.640	0.338	0.679	0.630	0.655	0.572	0.580	0.505	0.122	0.125	0.395	0.708
D	0.581	0.690	0.220	0.621	0.285	0.646	0.675	0.182	0.182	0.577	0.615	0.682
E	0.090	0.672	0.248	0.610	0.670	0.681	0.639	0.264	0.119	0.656	0.447	0.174
F	0.717	0.709	0.418	0.663	0.219	0.599	0.116	0.172	0.660	0.577	0.690	0.690
G	0.330	0.161	0.680	0.695	0.674	0.708	0.379	0.669	0.180	0.666	0.633	0.718
H	0.622	0.436	0.687	0.173	0.691	0.471	0.662	0.433	0.102	0.686	0.705	0.702

Bovine serum albumin

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.583	0.529	0.613	0.135	0.114	0.196	0.540	0.676	0.591	0.632	0.131	0.387
B	0.561	0.569	0.623	0.137	0.530	0.443	0.086	0.591	0.134	0.557	0.099	0.580
C	0.590	0.268	0.660	0.558	0.622	0.513	0.572	0.466	0.085	0.078	0.236	0.613
D	0.509	0.629	0.113	0.620	0.178	0.560	0.614	0.120	0.114	0.514	0.519	0.614
E	0.071	0.626	0.179	0.519	0.642	0.602	0.550	0.142	0.075	0.564	0.309	0.641
F	0.661	0.664	0.367	0.608	0.176	0.452	0.099	0.134	0.605	0.472	0.634	0.652
G	0.191	0.121	0.658	0.636	0.636	0.608	0.213	0.644	0.106	0.613	0.577	0.597
H	0.586	0.258	0.644	0.111	0.626	0.235	0.535	0.174	0.081	0.656	0.655	0.647

Figure 4.42. Phage ELISA analysis of anti-7-hydroxycoumarin library clones on 7-hydroxycoumarin-BSA and BSA surfaces following three rounds of affinity selection. Both ELISAs show similar patterns of positives (shaded) and negatives (unshaded). Discrepancies only occur for wells that fall slightly below the positive control value, but are still positive readings. Positive clones are shaded. Positive control: H7. Negative control: H6.

7-hydroxycoumarin-ovalbumin Ovalbumin

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.348	0.516	0.416	0.294	0.277	0.516	0.083	0.122	0.108	0.077	0.078	0.136
B	0.431	0.504	0.407	0.265	0.456	0.375	0.099	0.112	0.106	0.076	0.107	0.103
C	0.314	0.377	0.356	0.293	0.292	0.365	0.076	0.096	0.089	0.081	0.077	0.097
D	0.437	0.315	0.518	0.436	0.477	0.409	0.100	0.082	0.146	0.116	0.150	0.129
E	0.559	0.506	0.475	0.485	0.466	0.402	0.173	0.126	0.128	0.129	0.133	0.124
F	0.487	0.511	0.463	0.461	0.459	0.417	0.136	0.132	0.121	0.114	0.115	0.115
G	0.382	0.277	0.323	0.354	0.273	0.390	0.091	0.084	0.093	0.107	0.073	0.124
H	0.382	0.569	0.330	0.365	0.474	0.494	0.796	0.158	0.077	0.090	0.119	0.069

Figure 4.43. Phage ELISA of anti-7-hydroxycoumarin library following three rounds of affinity selection on alternating protein conjugates of 7-hydroxycoumarin with different blocking proteins with screening on 7-hydroxycoumarin-ovalbumin (7OHC-OVA) and ovalbumin (OVA). All clones when screened on 7OHC-OVA showed elevated signals above background when compared to the same clones on OVA. None of the clones screened on OVA gave signals above the negative control (H6). Positive control: H7.

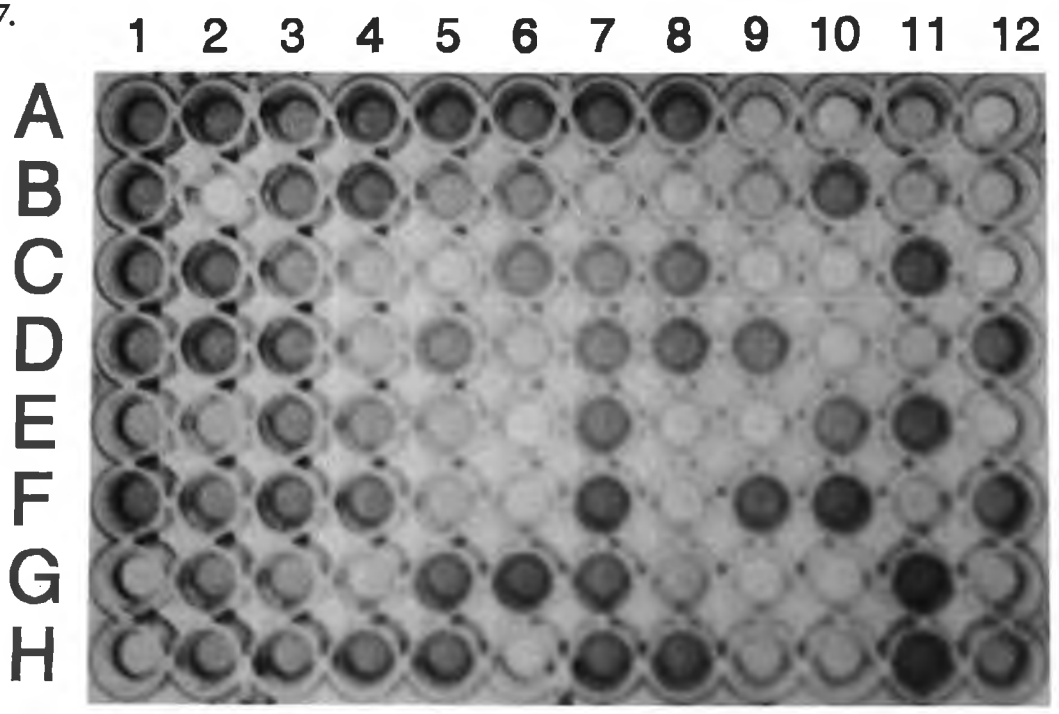


Figure 4.44. Phage ELISA analysis of anti-7-hydroxycoumarin library. 43 clones showing the highest responses were selected as positive from the phage ELISA. Positive control: H7. Negative control: H6.

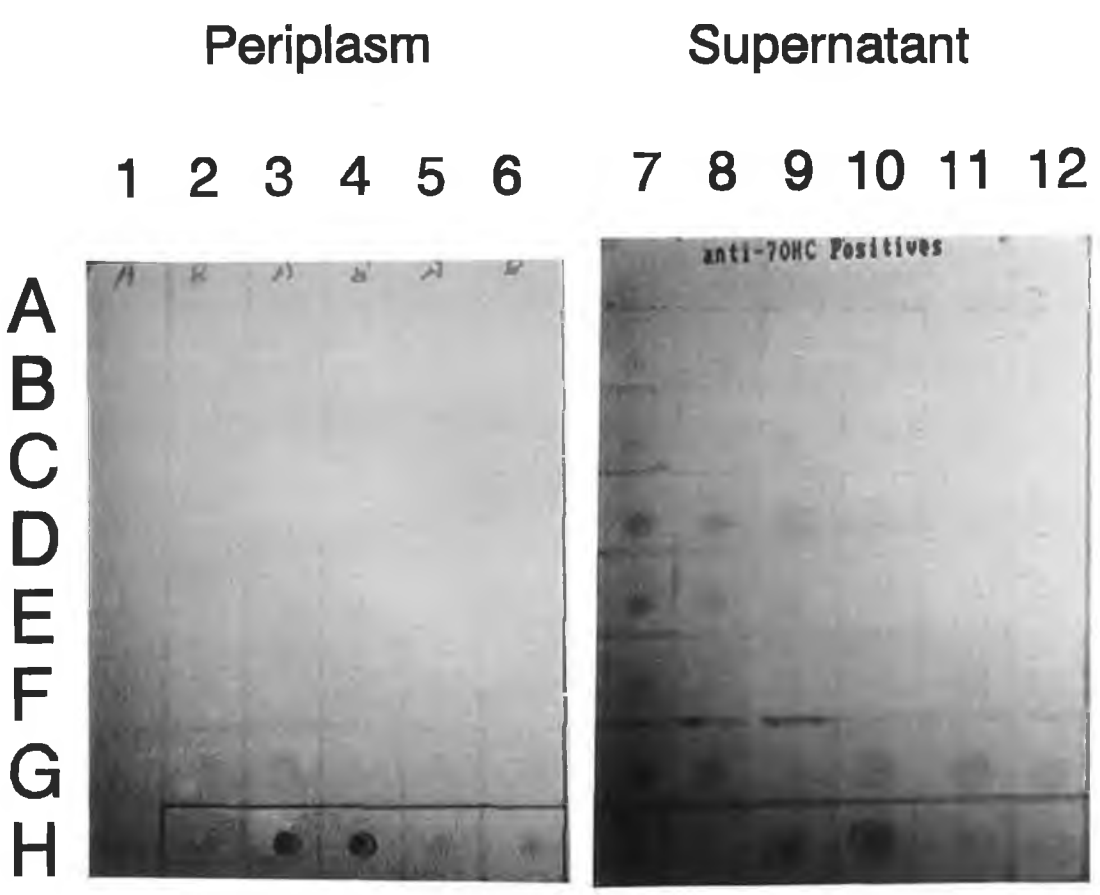


Figure 4.45. Dot blot analysis of supernatant and periplasmic lysates of anti-7-hydroxycoumarin positive clones from phage ELISA. The 43 positive clones (rows A to G and H1 and H7) were compared to five clones taken at random from the library (H2 to H6 and H8 to H12). All 43 clones failed to demonstrate any secretion into the periplasm or culture supernatant. The control clones tended to show higher levels of scFv production, especially H3 and H4.

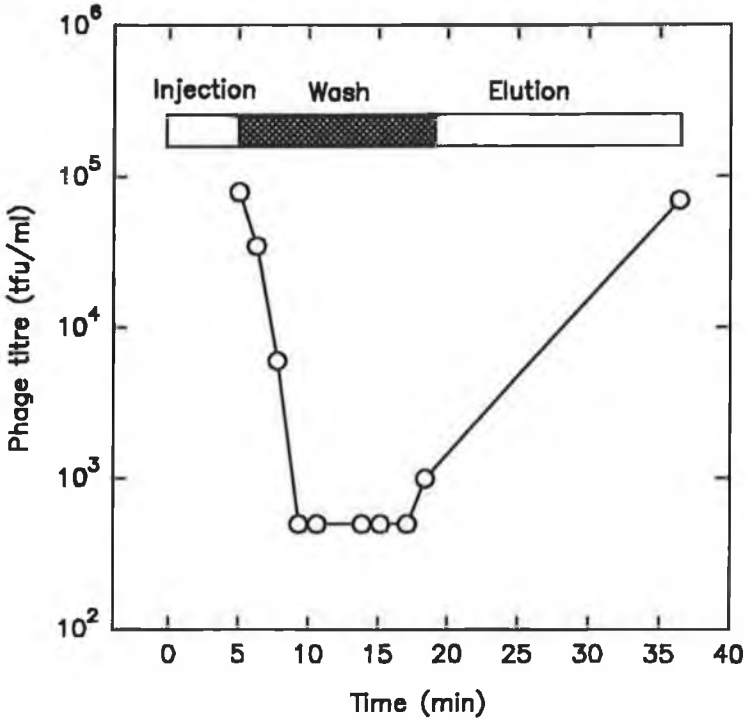


Figure 4.46. Use of BIAcore for the enrichment of anti-7-hydroxycoumarin positive clones from the Nissim library. Approximately 2.75 x 10^8 phage antibodies were injected onto the sensor ship immobilised with 7-hydroxycoumarin-BSA. The phage titres eluting from the sensor are shown. During washing with buffer, titres fell to 5 x 10^2 tfu/ml. When glycine elution buffer was applied to the sensor (pH 2.2), this titre increased to 7 x 10^4 tfu/ml. Over the 20 minute elution period, this equated to 1.26 x 10^4 phage.

4.3.5 Comparison of lysis methods in the isolation of scFv from intracellular compartments

The effect of periplasmic and intracellular lysis of clones α-7OHC10 and α-7OHC11 is shown in Fig. 4.47. This showed that freeze-thawing again released no scFv into the periplasm, but some antibody present in the intracellular compartment following sonication. Dot blot signals were also improved when antibody was denatured in 7.5 M guanidine hydrochloride.

Clones α-7OHC6 to α-7OHC8 were also sonicated and denatured in 8 M urea, which was also found to improve signals on dot blots, but still showed very low production of anti-7-hydroxycoumarin scFv antibody (Fig. 4.48).

4.3.6 Analysis of denatured and refolded anti-7-hydroxycoumarin clones

Clones α-7OHC6 to α-7OHC11 were denatured and refolded in arginine buffer for two days. Refolding greatly improved dot blot signals (Fig. 4.49). This was particularly evident in clones α-7OHC10 and α-7OHC11.

4.3.7 Induction and affinity purification of α-7OHC10

Analysis of the fractions from the affinity purification of a refolded intracellular lysate of α-7OHC10 is shown in Fig. 4.50. Antibody eluted from the 7-hydroxycoumarin column (7-hydroxycoumarin-4-acetic acid coupled to EAH-sepharose 4B) in glycine buffer, pH 2.2 in fractions 3 to 5. Following pooling of these fractions, they were found to have enriched for a 31 kDa protein fragment as seen by SDS-PAGE analysis (Fig. 4.51), as well as a lower molecular weight fragment, the molecular weight of which could not be accurately determined from the gel. Only the 31 kDa fragment was recognised by anti-c-myc antibody on western blots (Fig. 4.52). Analysis under reducing and non-reducing conditions indicated that α-7OHC10 was a single peptide. Recognition of the 31 kDa fragment only occurred on western blots following purification. Antibody may not have been present in sufficient quantities to yield a signal on western blots prior to purification.

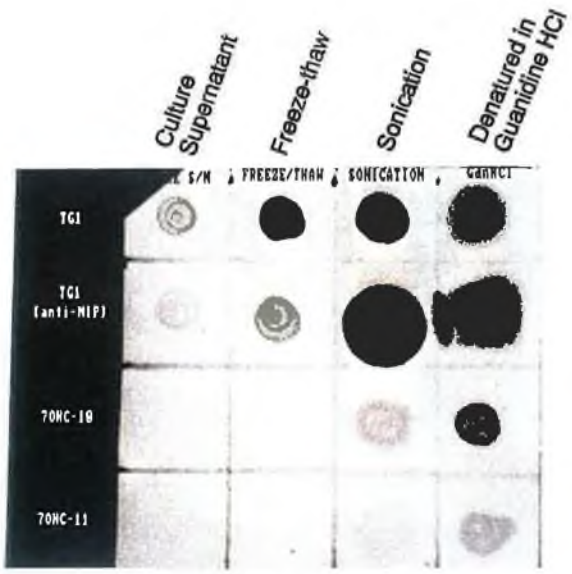


Figure 4.47. Dot blot analysis of lysis methods in the release of scFv from α-7OHC10 and α-7OHC11. Positive clone α-NIP showed good release of scFv following freeze-thawing and sonication, as well as into the culture supernatant. TG1 (pHEN1) also showed secretion of c-myc peptide. No antibody was present in the periplasmic lysate following freeze-thawing, but was present following sonication and denaturation. Denaturation of α-7OHC10 and α-7OHC11 significantly improved signals.

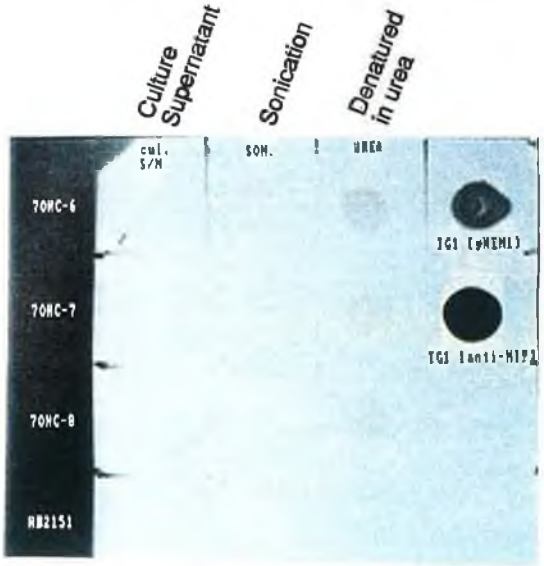


Figure 4.48. Dot blot analysis of the sonication and denaturation of α-7OHC6, α-7OHC7 and α-7OHC8 clones. Low yields of scFv were produced following sonication. Signals were again enhanced following denaturation in 8 M urea. The negative control, HB2151 showed no scFv production in all samples as expected. Positive assay controls: α-NIP and TG1 (pHEN1).

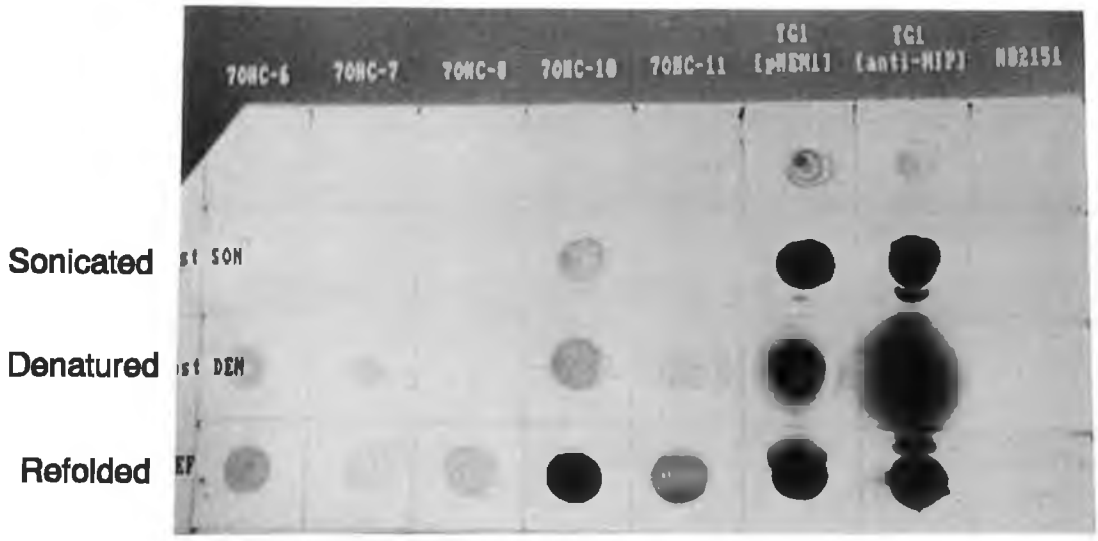


Figure 4.49. Dot blot analysis of anti-7-hydroxycoumarin scFv clones α-7OHC6 to α-7OHC11 (α-7OHC9 omitted). All five clones tested showed poor signals of scFv after sonication. This improved upon denaturation and was significantly better following refolding in arginine. α-7OHC10 was significantly better than the others and α-7OHC11 was moderately better. Positive clones TG1 (pHEN1) and TG1 (α-NIP) showed good levels of c-myc peptide and soluble scFv, respectively. Negative control HB2151 showed no scFv production as expected.

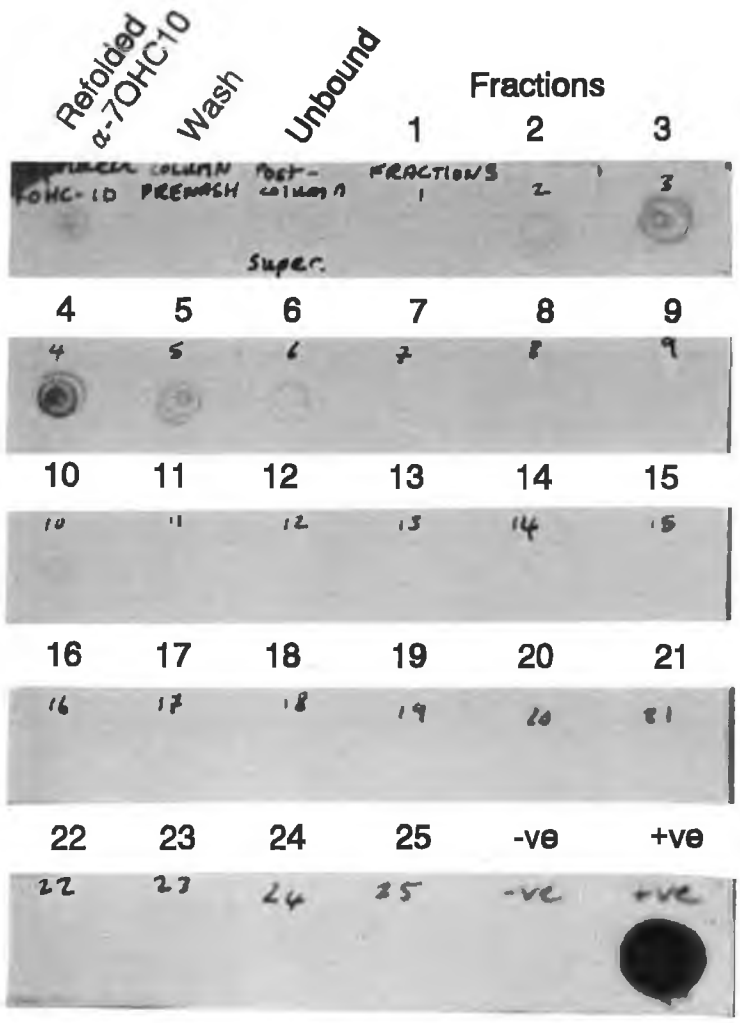


Figure 4.50. Dot blot analysis of fractions from the elution of α-7OHC10. ScFv eluted at pH 2.2 from the EAH-sepharose 7-hydroxycoumarin column primarily in fractions 3 to 5 with some material in fractions 2 and 6 also. Positive control: α-NIP. Negative control: HB2151.

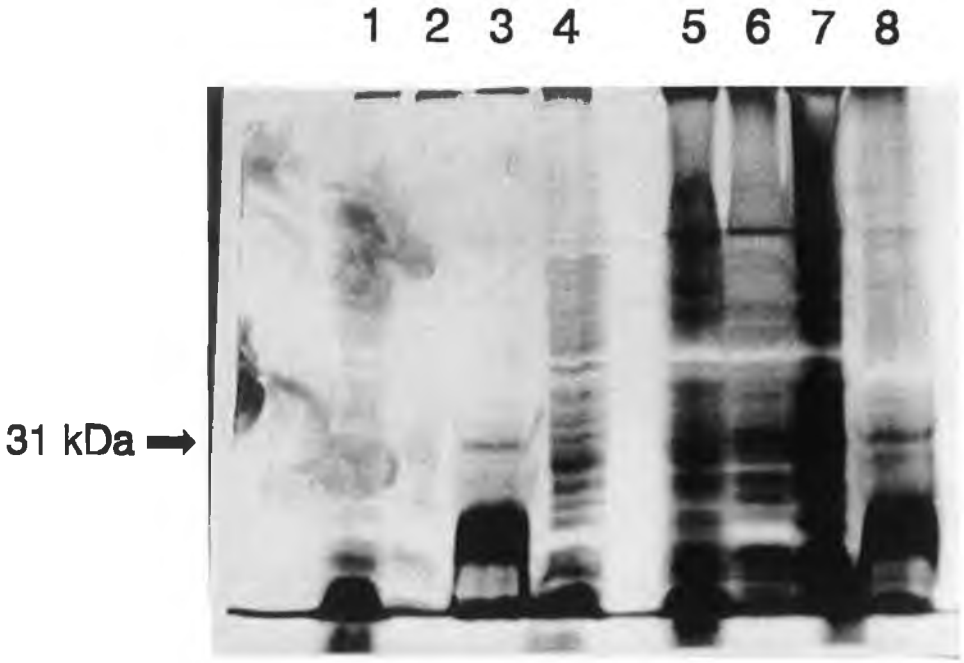


Figure 4.51. SDS-PAGE analysis of α-7OHC10 refolded scFv antibody before and after affinity purification. Lanes: 1-4: Reducing conditions; 5-8: Non-reducing conditions; 1,5: α-NIP (positive control); 2,6: HB2151 (negative control); 3,8: Purified α-7OHC10; 4,7: Unpurified α-7OHC10. Purification resulted in the enrichment of a 31 kDa fragment and a lower molecular weight band that was not recognised by western blotting (Fig. 4.52). Purification reduced the amount of non-specific protein in the sample. Analysis under reducing and non-reducing conditions showed that the 31 kDa protein was a single peptide fragment.

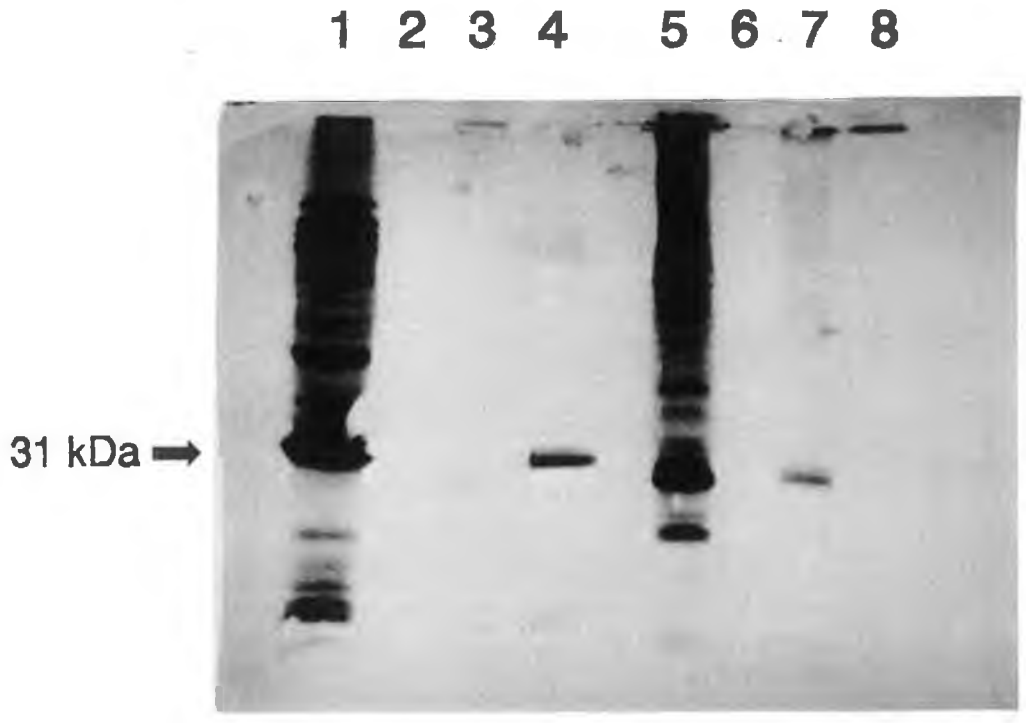


Figure 4.52. Western blot analysis of α-7OHC10 refolded scFv antibody before and after purification. Detection of scFv was with α-c-myc antibody. Lanes: 1-4: Non-reducing conditions; 5-8: Reducing conditions; 1,5: α-NIP; 2,6: HB2151; 3,8: Unpurified α-7OHC10; 4,7: Purified α-7OHC10. Purification led to the recognition of a 31 kDa fragment under both reducing and non-reducing conditions. No antibody could be detected in the unpurified samples.

4.3.8 ELISA of purified α-7OHC10

The ELISA of the purified α-7OHC10 scFv against 7OHC-BSA and BSA is shown in Fig. 4.53. The antibody showed no response to BSA, but did respond to 7OHC-BSA with a very poor signal which was proportional to the amount of 7OHC-BSA on the surface, with signals on the 6.25 μg/ml 7OHC-BSA wells still higher than controls at 1 in 20 dilution of scFv.

4.3.9 Affinity purification of α-7OHC9

Refolded α-7OHC9 was affinity purified on 7OHC-EAH-sepharose and analysed by dot blotting (Fig. 4.54). This showed α-7OHC9 to be present in fractions 1 to 4. A sizeable amount did not bind to the column and passed through in the unbound fraction. Dot blot analysis also showed that antibody did not elute at pH 3.5 (Fig. 4.55). SDS-PAGE also showed that although enrichment of a 31 kDa fragment occurred, poor levels of purification were achieved (Fig. 4.56). Western blotting showed the presence of the 31 kDa component in the refolded lysate material, the unbound fraction and the affinity-purified sample, detected by α-c-myc antibody. This was still present following concentration of α-7OHC9 (Fig. 4.57), which was also shown by dot blotting (Fig. 4.58).

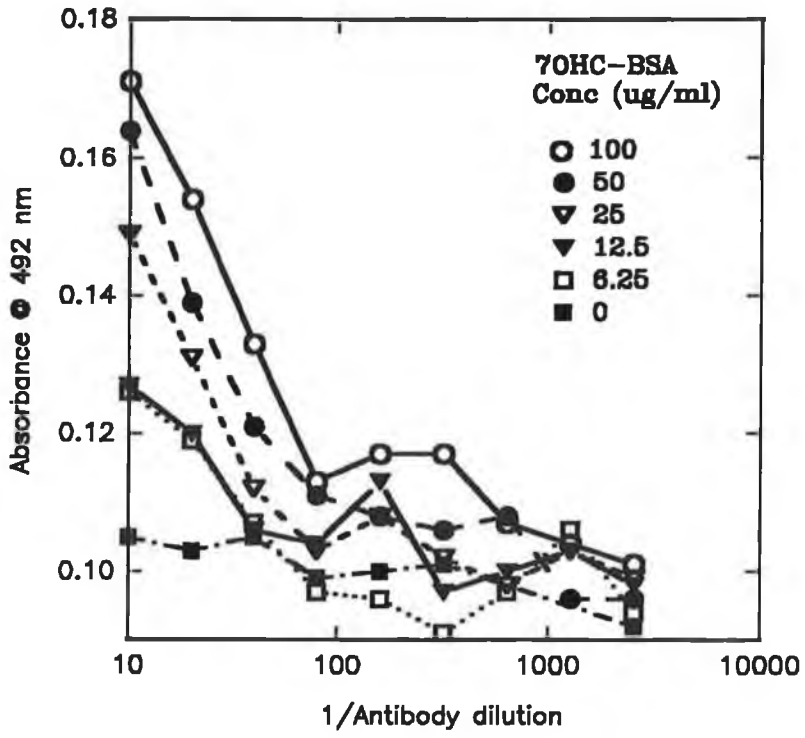


Figure 4.53. ELISA analysis of purified α-7OHC10 scFv. Antibody recognised 7-hydroxycoumarin-BSA (7OHC-BSA) but not BSA alone. Signals were very low, even at low dilutions (0.17 AU at 1 in 10), but gave titres in excess of 1 in 100.

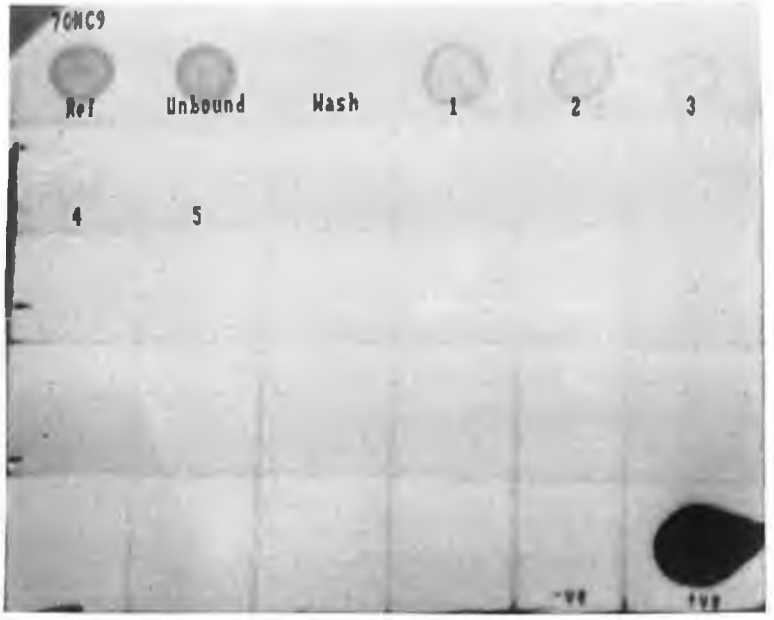


Figure 4.54. Dot blot analysis of fractions from the purification of α-7OHC9. Detection of scFv was with α-c-myc antibody. Positive control (+ve): α-NIP. Negative control (-ve): HB2151. A large proportion of the antibody did not bind to the column and passed through in the unbound fraction. Antibody eluted in fractions 1 to 4.

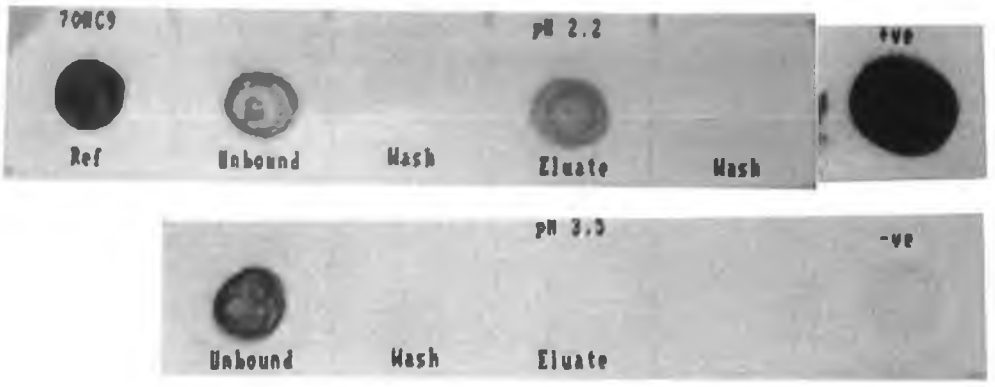


Figure 4.55. Dot blot analysis showing elution of α-7OHC9 in pH 2.2 and pH 3.5 buffers. Detection of scFv was with α-c-myc antibody. Positive control (+ve): α-NIP. Negative control (-ve): HB2151.ScFv eluted at pH 2.2, but not at pH 3.5.

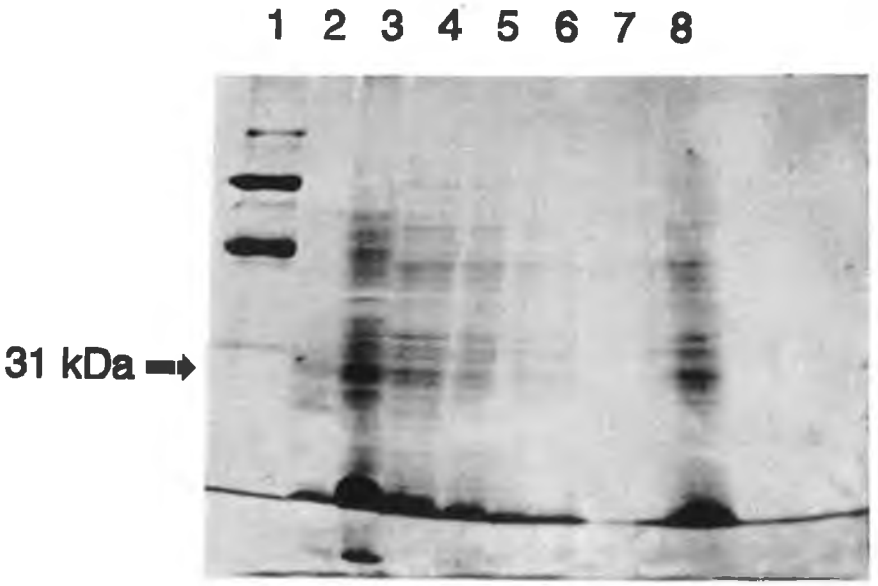


Figure 4.56. SDS-PAGE analysis of affinity-purified α-7OHC9. Lanes: 1: Molecular weight markers; 2: HB2151; 3: α-NIP; 4: Refolded α-7OHC9; 5: Unbound fraction; 6: Affinity-purified α-7OHC9; 7: Concentrated column washings; 8: Concentrated affinity-purified fraction. Enrichment of a 31 kDa protein fragment can be seen following concentration of the affinity-purified sample.

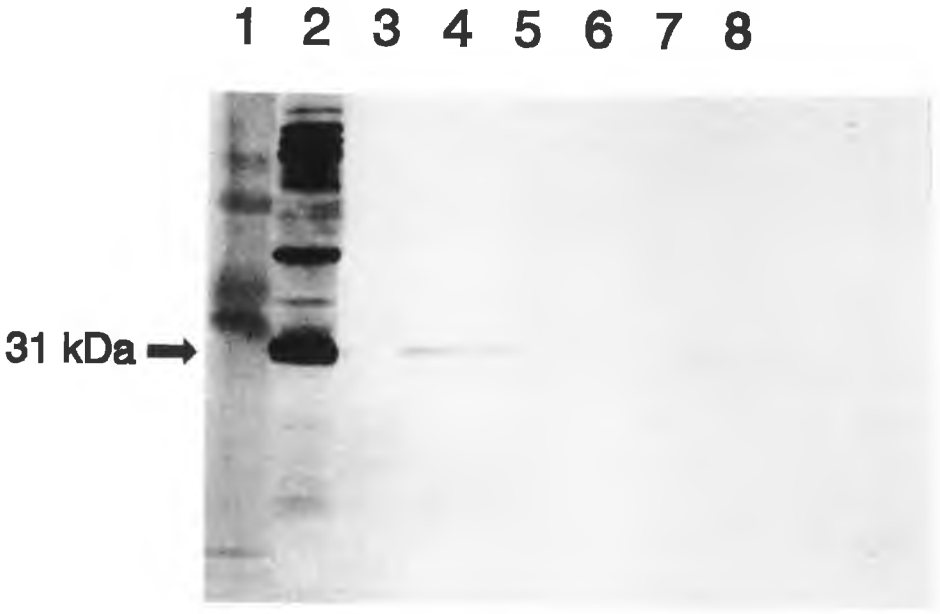


Figure 4.57. Western blot analysis of affinity-purified α-7OHC9. Detection of a 31 kDa fragment was seen in the refolded sample, the unbound fraction and in the concentrated affinity-purified sample, although the latter is extremely faint. No scFv was detected in column washings. Lanes: 1: Molecular weight markers; 2: α-NIP; 3: HB2151; 4: Refolded α-7OHC9; 5: Unbound fraction; 6: Affinity-purified α-7OHC9; 7: Concentrated column washings; 8: Concentrated affinity-purified α-7OHC9.

Figure 4.58. Dot blot analysis of affinity-purified α-7OHC9. ScFv material was detected in the unbound fraction and in the pooled affinity-purified fractions 1 to 4. The signal was significantly enhanced following concentration of the affinity purified fraction (α-7OHC9 conc.). No antibody was detected in concentrated column washings (conc. eluate). Negative control (-ve): HB2151. Positive control (+ve): α-NIP.

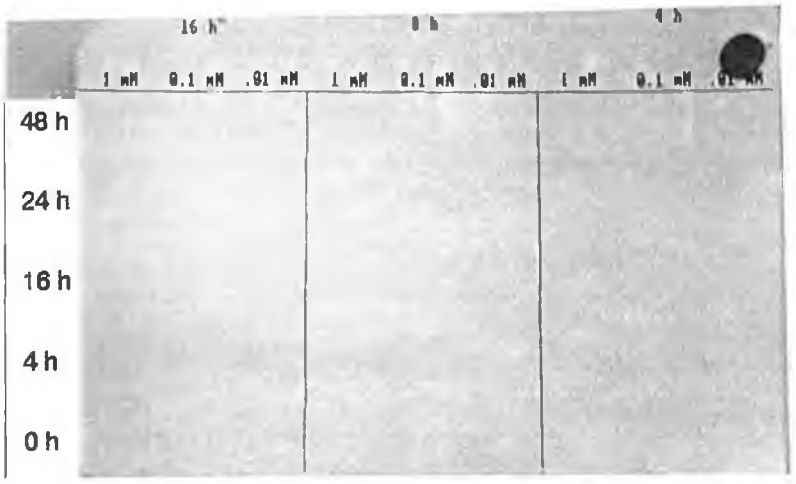
4.3.10 Induction optimisation of α-7OHC10

α-7OHC10 culture supernatant, periplasmic lysates and intracellular lysates were analysed by dot blotting for the presence of scFv following induction with IPTG (Fig. 4.59). During induction, several parameters were monitored. These were growth period prior to induction (4, 8 and 16 h), IPTG concentration (0.01, 0.1 and 1 mM) and growth period after induction. No scFv was present in culture supernatant or periplasmic lysate under any of the conditions tested. ScFv was present in most intracellular lysates, but no great differences in scFv yield were evident due to the changes in growth conditions. In general, 8 h growth pre-induction showed the overall highest levels of antibody, with 0.1 mM IPTG and 16 h post-induction growth being marginally better. Also, the 1 mM IPTG with 16 h pre-induction growth was very good at 0 and 4 h post-induction. These two scenarios were analysed a second time, this time also looking at the effect of growth temperature post-induction. This now showed 8 h growth pre-induction with 0.1 M IPTG and 2 h post-induction growth at 25°C to be optimal for scFv antibody yield (Fig. 4.60). However, further scale ups using these induction conditions failed to significantly increase scFv yield (data not shown).

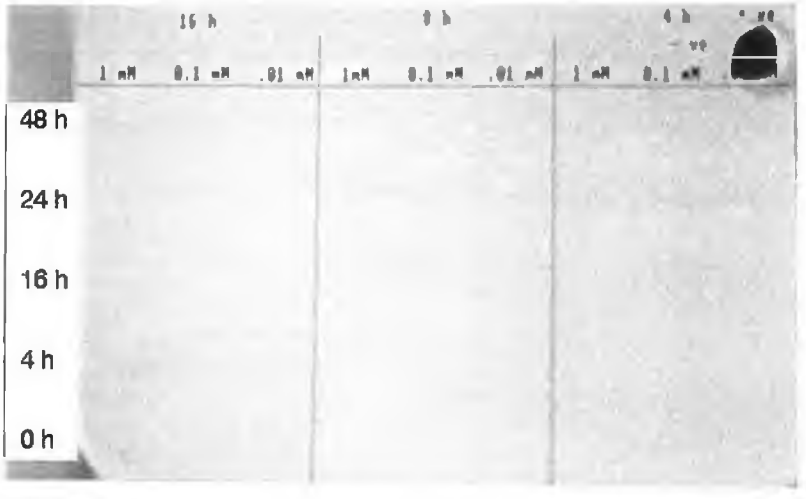
4.3.11 Precipitation of purified and concentrated scFv preparations

Following denaturation and refolding of α-7OHC9 and α-7OHC10, these were characterised by the precipitation of protein material following affinity purification and concentration. When analysed, no viable titres were obtained for α-7OHC9 (data not shown).

Culture supernatant



Periplasm



Intracellular lysate

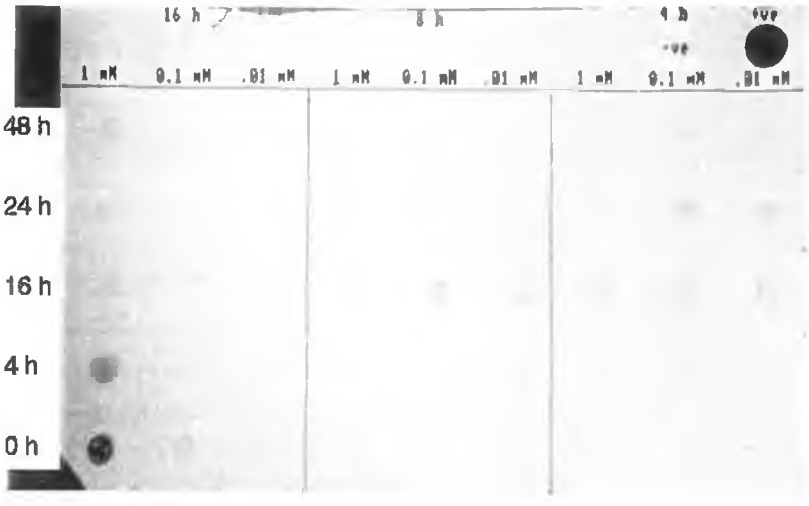


Figure 4.59 (previous page). Analysis of culture supernatants, periplasmic lysates and intracellular lysates of α-7OHC10 for the presence of scFv following induction under different conditions. All show pre-induction growth periods of 4, 8 and 16 h, IPTG inducer concentrations of 0.01, 0.1 and 1 mM IPTG and post-induction growth periods of 0 to 48 h at 30°C. No scFv was found in periplasm or supernatant, but was present in intracellular lysates, generally most significantly following 8 h pre-induction culture growth, but samples of 8 h pre-induction growth, 0.1 mM IPTG, 16 h post-induction growth and 16 h pre-induction growth, 1 mM IPTG, and 0 h post-induction growth showing slightly elevated levels above the others.

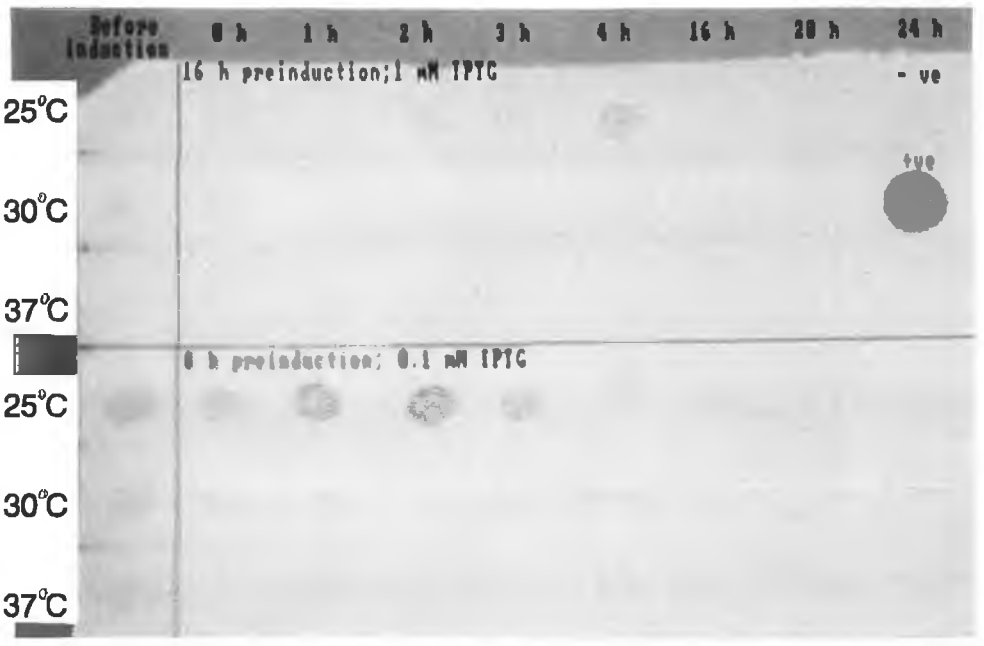


Figure 4.60. Dot blot analysis of the induction of α-7OHC10. Induction was performed, either with 16 h preincubation growth period and 1 mM IPTG, or 8 h preincubation growth and 0.1 mM IPTG, both with post-induction growth up to 24 h at 25, 30 and 37°C. Maximum yields of scFv from the 16 h pre-induction sample was at 4 h and 25°C. However, most scFv resulted from 8 h pre-incubation after 2 h, also at 25°C. Negative control: HB2151. Positive control: α-NIP.

4.3.12 Characterisation of anti-7-hydroxycoumarin phage antibodies

Six anti-7-hydroxycoumarin clones positive from affinity selection were prepared for the production of phage-expressed antibody (section 2.3.3.3). These phage antibody preparations were characterised by ELISA (Fig. 4.61). All six clones showed very similar profiles of recognition of 7OHC-BSA, but with high responses to BSA also. These were designated α-7OHCΦ to denote them as phage antibody clones, rather than soluble scFv antibodies.

4.3.13 Reselection, screening and isolation of α-7-hydroxycoumarin scFv clones

A further library of anti-7-hydroxycoumarin clones was prepared. Phage ELISA was performed using 7-hydroxycoumarin-OVA and OVA as control (Fig. 4.62). This showed that most clones bound to 7OHC-OVA, but not to ovalbumin alone. These clones were also tested against a 7OHC-BSA conjugate synthesised using EDC/NHS coupling chemistry (Keating, 1998) (Fig. 4.63). No response was seen to this conjugate. The clones were also tested for the presence of periplasmic or extracellular scFv production. Seven clones showed good levels of periplasmic scFv production (Fig. 4.64). These were in positions B2, B4, G4, A5, B5, D5 and H5, with B5 and D5 being the poorer secretors of the group. Nearly all the clones showed some level of extracellular expression, with C4, D5 and G4 being better than average (Fig. 4.64). Large scale periplasmic lysates of two clones, A5, and B4, were prepared and competition immunoassays performed using the periplasmic lysate. (Figs. 4.65 and 4.66). Both clones only showed poor responses on ELISA, but in both cases, competition with free drug could not be detected. In both instances, the presence of larger concentrations of 7-hydroxycoumarin increased the signal.

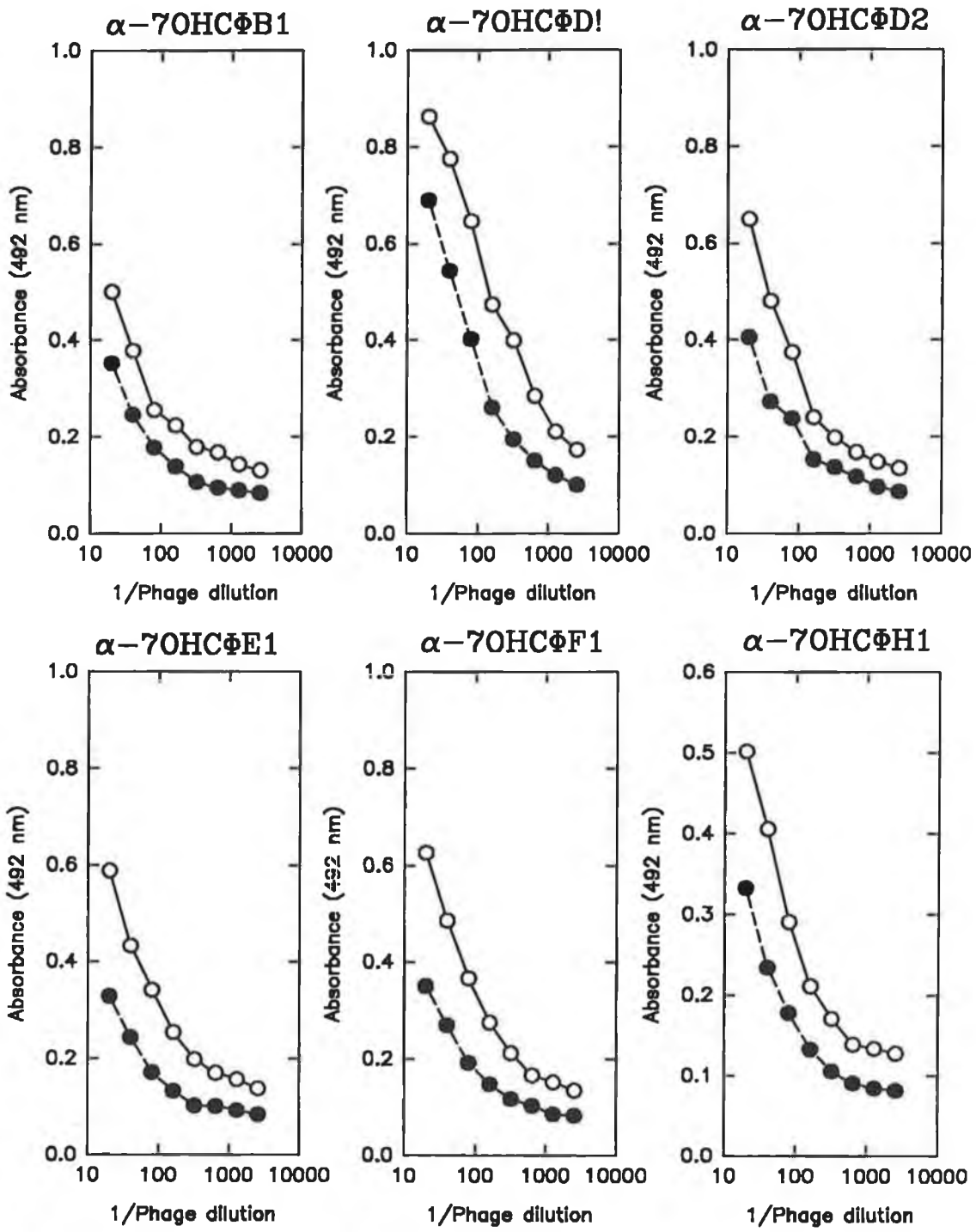


Figure 4.61. ELISA analysis of anti-7-hydroxycoumarin phage antibodies. Phage antibody clones were designated α-7OHCΦ to indicate attachment of the antibody to the phage particle. All six clones showed near identical titration profiles, with good responses to 7OHC-BSA, but were also characterised by unacceptably high background responses to BSA alone. Open circles: 7OHC-BSA; Filled circles: BSA.

	7-hydroxycoumarin-ovalbumin						Ovalbumin					
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.259	0.319	0.186	0.177	0.166	0.258	0.070	0.173	0.116	0.096	0.096	0.161
B	0.289	0.230	0.319	0.357	0.209	0.250	0.139	0.134	0.201	0.205	0.124	0.146
C	0.216	0.279	0.247	0.279	0.250	0.251	0.141	0.169	0.144	0.155	0.143	0.137
D	0.172	0.305	0.322	0.211	0.344	0.230	0.123	0.203	0.185	0.122	0.222	0.136
E	0.183	0.233	0.277	0.285	0.250	0.204	0.145	0.134	0.151	0.179	0.152	0.140
F	0.232	0.283	0.227	0.288	0.275	0.299	0.142	0.174	0.152	0.179	0.152	0.140
G	0.307	0.163	0.272	0.305	0.144	0.251	0.176	0.114	0.149	0.173	0.144	0.151
H	0.312	0.210	0.228	0.298	0.261	0.104	0.195	0.147	0.146	0.164	0.128	0.424

Figure 4.62. Phage ELISA analysis of anti-7-hydroxycoumarin library. 42 clones were analysed against 7-hydroxycoumarin-ovalbumin and ovalbumin. Clones did not bind to ovalbumin, but most bound to 7-hydroxycoumarin-ovalbumin. Positive control: H12. Negative control: H6.

	7-hydroxycoumarin-BSA (EDC/NHS)						BSA					
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.091	0.100	0.084	0.080	0.077	0.097	0.070	0.076	0.086	0.072	0.080	0.086
B	0.081	0.089	0.111	0.142	0.088	0.091	0.069	0.079	0.088	0.086	0.079	0.080
C	0.076	0.098	0.079	0.105	0.094	0.092	0.065	0.076	0.071	0.092	0.075	0.069
D	0.078	0.108	0.134	0.105	0.142	0.086	0.073	0.089	0.080	0.106	0.117	0.072
E	0.079	0.085	0.094	0.104	0.136	0.083	0.082	0.071	0.101	0.080	0.101	0.070
F	0.082	0.106	0.090	0.107	0.103	0.106	0.072	0.084	0.073	0.076	0.075	0.066
G	0.111	0.096	0.099	0.113	0.094	0.088	0.083	0.066	0.085	0.080	0.080	0.064
H	0.131	0.096	0.082	0.125	0.102	0.140	0.100	0.081	0.072	0.087	0.080	0.452

Figure 4.63. Phage ELISA analysis of anti-7-hydroxycoumarin library. Clones that were positive on 7-hydroxycoumarin-ovalbumin (Fig. 4.62) were not positive for the 7-hydroxycoumarin-bovine serum albumin (BSA) conjugate prepared by EDC/NHS coupling chemistry. Positive control: H12. Negative control: H6.

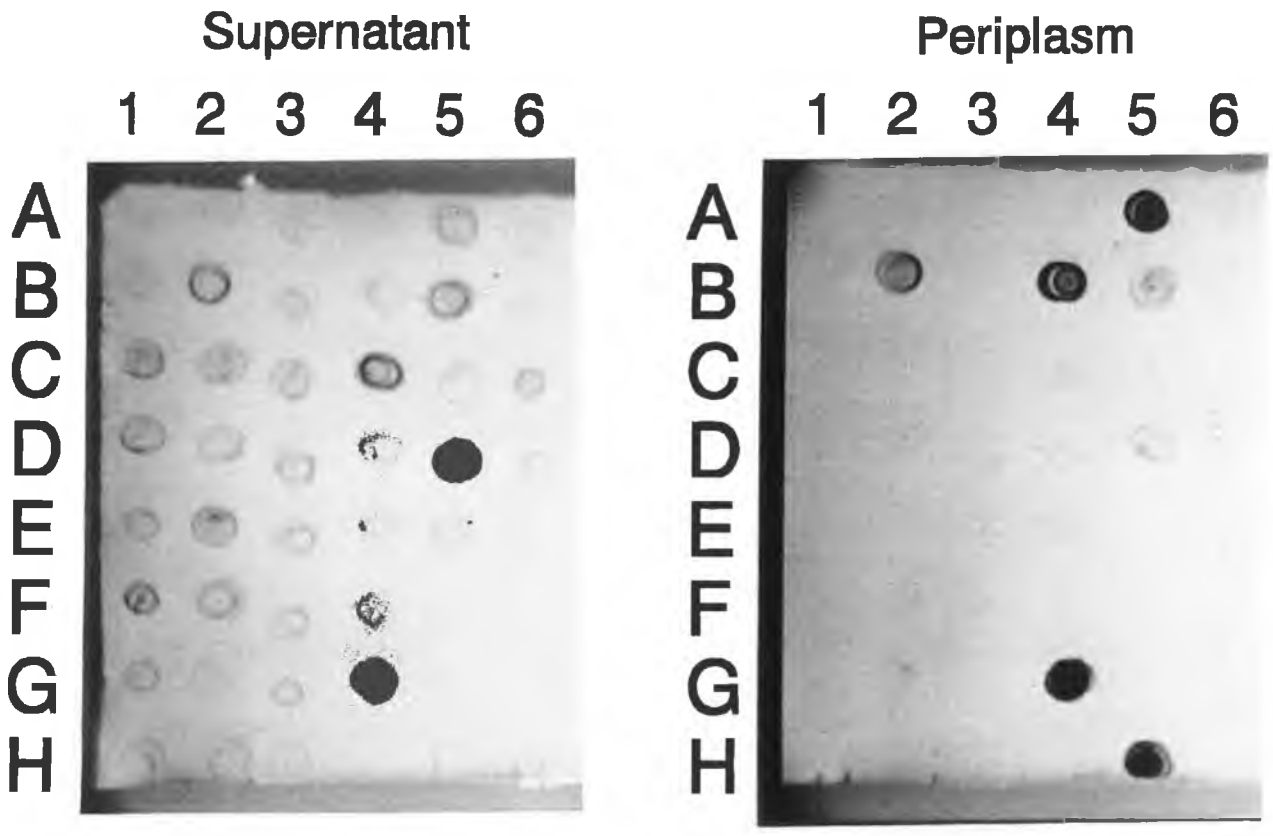


Figure 4.64. Dot blot analysis of supernatants and periplasmic lysates of 42 clones from the anti-7-hydroxycoumarin scFv library. Recognition of scFv was with α-c-myc antibody. All the culture supernatants showed some level of scFv secretion, with clones at positions D5 and G4 showing particularly good levels of secretion. In periplasmic lysates, several clones showed secretion of scFv, wit clones at positions B2, B4, G4, A5, B5, D5 and H5 with B5 and D5 being the poorest secretors.

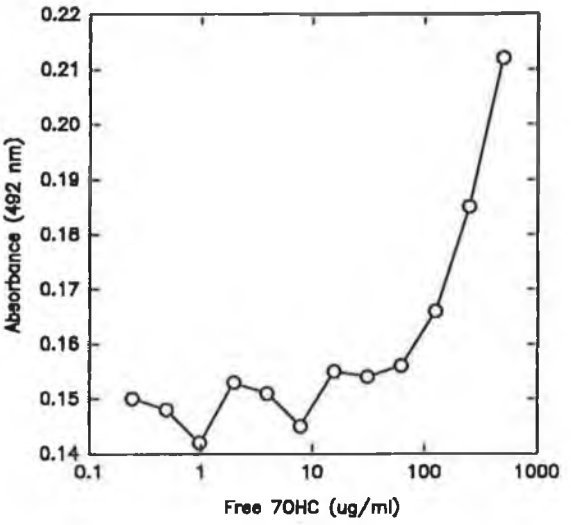


Figure 4.65. Competition enzyme immunoassay of α-7OHCA5 periplasmic lysate against free 7-hydroxycoumarin (7OHC) and 7-hydroxycoumarin-ovalbumin. No competition occurred. At high free 7-hydroxycoumarin concentrations, the signal was elevated, indicating enhanced binding to the bound conjugate.

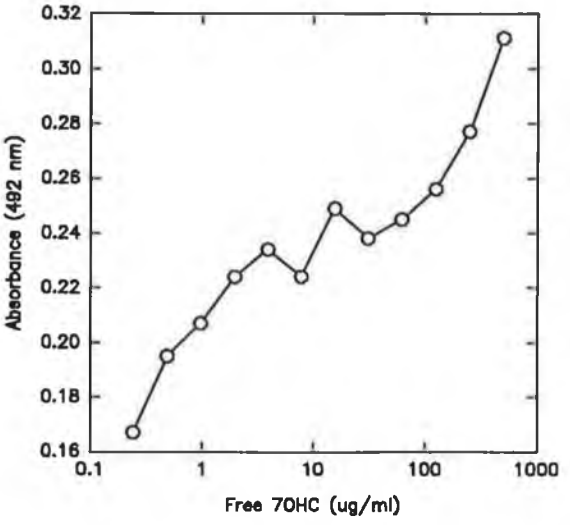


Figure 4.66. Competition enzyme immunoassay of α-7OHCB4 periplasmic lysate against free 7-hydroxycoumarin (7OHC) and 7-hydroxycoumarin-ovalbumin. As in the case of α-7OHCA5, no competition was noted, and signals were heightened at elevated 7-hydroxycoumarin concentrations.

4.4 PRODUCTION OF ANTI-COUMARIN scFv ANTIBODIES USING THE NISSIM LIBRARY

4.4.1 Selection of scFv antibodies to coumarin

Phage ELISA analysis of rounds of affinity selection clearly showed the library enriching for antibodies recognising the coumarin-BSA conjugate. No anti-coumarin clones were detected in the library prior to affinity selection. 12% of clones were positive after two rounds of selection and 28.5% after three rounds. Selection using 6-NH_2-coumarin coupled to plates was less efficient. However, since no detailed work was performed on optimising the coupling chemistry involved, enrichment may not have been as efficient as might be possible using direct coupling chemistries.

4.4.2 Screening of scFv antibodies to coumarin

Screening of positive scFv-producing clones can be performed using ELISA. This would simultaneously show the recognition of scFv for antigen, while also demonstrating extracellular expression if culture supernatant were used. However, many scFv clones positive by phage ELISA, prove negative when culture supernatant is analysed. Many clones may not be efficiently secreted due to particular protein structures that result in misfolding and intracellular aggregation. Others may secrete efficiently into the periplasm, but do not reach the extracellular matrix. Nissim *et al.* (1993) suggested that failure of detection using ELISA was due to a reduced sensitivity of the ELISA assay system as compared with phage ELISA. This is probably not the reason for their failure to detect scFv positive clones. Considering the problems associated with ELISA screening of clones, dot blotting was employed as a more reliable and sensitive technique for detecting scFv. Although dot blotting does not demonstrate interaction of the scFv with its antigen, recognition of antigen had already been shown to occur by phage ELISA.

Screening of clones was also extended to include periplasmic lysates of scFv-carrying *E.*

coli clones. Such lysates can yield concentrated scFv material (Roben, 1993) and do not have the liquid handling problems associated with large volumes of culture supernatant. They are thus, an important source of antibody material. Also, many clones that do not efficiently secrete into supernatants may secrete efficiently into the periplasmic space.

The inability of many scFv clones to secrete efficiently was borne out in section 4.1.3 where only three out of 21 clones showed efficient secretion, two of these into periplasm and supernatant (α-C5 and α-C13) and α-C14 solely into culture supernatant. Clones selected at random from the coumarin library after three rounds of affinity selection showed a variety of good and poor secretors with higher levels of expression than clones already selected as positive to coumarin. This may suggest that clones recognising coumarin excrete less efficiently from *E. coli* than clones not recognising the conjugate. The four clones screened as positive from selection using 6-NH_2-coumarin all failed to secrete. However, the fact that positive clones were detected using phage ELISA may be very important for the future production of anti-hapten antibodies without the requirement for drug-protein conjugates, as will be discussed in more detail.

Having selected three clones and elucidated their secretion characteristics, it was decided to treat α-C5 and α-C13 as periplasmic lysates and α-C14 as a culture supernatant for the purposes of antibody production.

4.4.3 Purification of scFv antibodies to coumarin

Initial attempts to affinity purify the anti-coumarin scFv α-C14 were carried out in a fashion typical of that used for polyclonal antibody purification and many monoclonal antibodies (Catty, 1989, Heiko Reinartz, Dublin City University, Personal Communication), employing high pH elution of antibody from a coumarin-BSA affinity column. Initially, extremely small quantities of scFv bound to the affinity column and following high pH elution, only small quantities of antibody were recovered. This did not improve following careful optimisation of pH conditions used for washing and elution. α-C5 and α-C13 were also tested under similar conditions, except this time using batch purification with periplasmic lysate being allowed to mix for a period of time with the

affinity matrix. This again failed to improve the yield of scFv. It also served to identify another characteristic of the affinity purification, namely that although a 31 kDa scFv component was purified which was detected by western blots, subsequent concentration resulted in its breakdown to a 29.5 kDa fragment that was no longer recognised by dot blotting, or detected by ELISA, suggesting the disappearance of the short c-myc peptide tag of approximately 1.5 kDa from the scFv molecule. A 20 kDa molecular weight fragment recognising anti-c-myc antibody, but incapable of binding to antigen was also formed. There appears to be two sites of significant proteolytic cleavage on the three scFv antibody clones (Fig. 4.67), which leads to the formation of the characteristic protein fragments as seen on western blots and SDS-PAGE gels. The 29.5 kDa fragment is purified by affinity chromatography, but is not detected by western blotting, whereas the 20 kDa fragment is detected by blotting, but because it lacks the major part of the V_H chain, is not affinity purified.

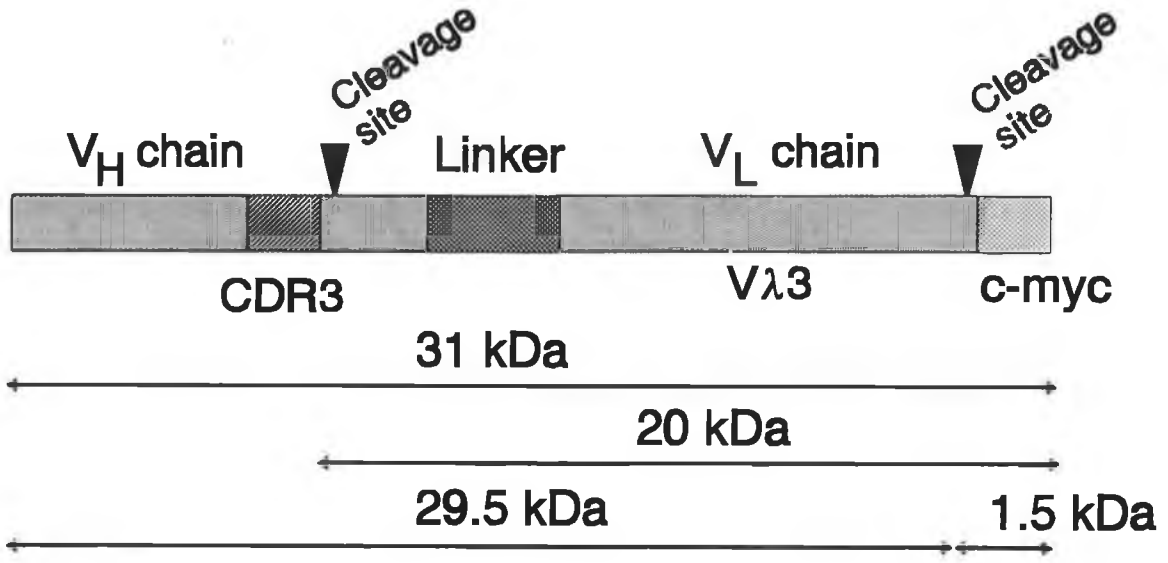


Figure 4.67. Major protein fragments produced by the proteolytic cleavage of the anti-coumarin antibodies α-C5, α-C13 and α-C14. Cleavage at two sites produces a 29.5 kDa fragment not detected by western blotting, but recognising antigen and a 20 kDa fragment detected by anti-c-myc antibody, but incapable of recognising antigen.

The ionic conditions of affinity purification were more thoroughly investigated due to early difficulties in the establishment of an effective affinity purification strategy. Periplasmic lysate of α-C13 was dialysed into 10 mM Tris, pH 7.4, prior to interaction with the affinity column, this time, with 10 mM Tris buffers used for equilibration instead of PBS. The effects of three gradients were investigated sequentially. Firstly, an increase in Tris buffer concentration from 10 to 50 mM showed no affect on the antibody-antigen interaction. Antibody did elute on the second gradient, however, when an increase in NaCl concentration from 0 to 0.5 M was employed. It was clear that large amounts of antibody had bound in the absence of, and eluted in the presence of approx. 0.15 M NaCl, a similar ionic strength as is found in PBS which contains a mixture of KCl and NaCl. Culture supernatants of 2xTY media contain approx. 0.85 M NaCl. These antibodies in the presence of PBS or 2xTY would be significantly inhibited from binding to the affinity matrix. The levels obtained following high pH elution were the result of small quantities of scFv remaining attached to the affinity column. This was further confirmed when α-C5 was assayed by ELISA using both PBS and Tris as buffers, resulting in greatly differing titres for the two systems. Subsequent purification and analysis of these scFvs was modified to allow for this salt intolerance and 50 mM Tris, pH 7.4, was used instead of PBS.

Subsequent purifications of anti-coumarin scFv were performed with an NaCl gradient between 0 and 0.5 M NaCl. This resulted in the efficient purification of the 31 kDa scFv fragment. However, degradation of the scFv, associated with loss of c-myc activity continued to be a problem. This was more significant when antibody was concentrated after affinity purification. It was suspected that degradation might be enzymatic due to the presence of protein contaminants from periplasm or culture supernatant. Such proteolysis should be inhibited by the presence of PMSF in lysis buffers. During affinity purification, however, protective PMSF may be removed during washing and elution. This may allow the reactivation of serine protease contaminants in the sample which coelute with the scFv. When concentrated, this activity may be greatly increased, bringing about an acceleration in the degradation of the antibody. If this was the case, then the inclusion of PMSF in wash buffers, elution buffers and purified fractions should reduce the level of degradation.

All three scFvs were repurified taking into account all the characteristics of these antibodies. This resulted in the more efficient enrichment of the active 31 kDa scFv component that was detected by western blotting and gave ELISA titres of 1 in 10,000 to coumarin-BSA with no detection of BSA alone. Antibody activity was also found to be stable upon storage at 4°C over several weeks at least.

The anti-c-myc antibody (mycl-9E10) used for recognition of scFv in ELISA and western blots would only be capable of detecting the 31 kDa fragment. An antibody recognising scFv, however, might recognise both 31 kDa and 29.5 kDa species, and so loss of the c-myc peptide tag during purification would not affect use of the antibody in assays. However, no anti-scFv antibodies were commercially available. The closest antibody recognising human scFv that was available was an anti-human $F(ab')_2$ antibody. This was used in conjunction with scFv in ELISA. Although a result was obtained, it was extremely poor. This is likely to be because only a fraction of the anti-human $F(ab')_2$ antibody is being used in scFv recognition, as there is only a single heavy and light chain variable region sequence. The synthetic nature of the CDRH3 region and the presence of a synthetic linker may all contribute to reduce the amount of anti-human $F(ab')_2$ antibody specific for the scFv to a very small proportion of the total polyclonal mixture, thus making it insensitive and non-viable in ELISA.

4.4.4 Characterisation of scFv antibodies to coumarin

To serve its purpose, an antibody must be applicable in the assay system for which it was developed. The three anti-coumarin clones isolated here were produced for the detection of the drug coumarin in a competition immunoassay, in which antibody would compete for the free analyte coumarin and the surface bound coumarin-BSA conjugate. For a competition assay to function effectively, several parameters must be optimised (Tijssen, 1985). Firstly, the scFv antibody concentration used in the assay must be optimised so as to make it limiting. If antibody is capable of binding to free and bound antigen in excess, this will not result in a measurable signal. Optimal antibody concentration was determined from titres of the three scFvs and taken at 70 % of maximum binding. At this level, they

will be limiting, but still provide enough antibody to bind antigen at a range of concentrations. Surface coumarin-BSA coating concentration was also maximised in this instance so as to effectively compete with free analyte and provide a high blank signal. Only as much antigen is used when increasing concentration does not result in an increase in signal. In the case of all three scFvs, this was found to be 150 μg/ml.

α-C5, α-C13 and α-C14 were tested in competition ELISA assays with free coumarin concentrations from 50 to 0.05 μg/ml. All three antibodies showed no detectable interaction with free drug at any of these concentrations. Coumarin was present in 0.05% methanol for efficient dissolution. Repetition of the assays varying antibody concentration again failed to show interaction with free drug.

The three anti-coumarin scFvs had displayed particular physicochemical characteristics. Significantly, they were capable of binding to the conjugate, coumarin-BSA. They did not, however, show any interaction with BSA alone. This suggested that interaction of antibody and antigen was absolutely dependent on the presence of the coumarin moiety. However, the lack of significant recognition of free drug indicated that interaction with the coumarin moiety alone was not capable of forming a high affinity antibody-antigen complex.

The NaCl sensitivity of the three scFv antibodies suggested that an ionic or electrostatic interaction between antigen and antibody was significantly important for the stabilisation of the antibody-antigen interaction. However, the extent of this type of interaction in the overall stabilisation of the complex must be limited due to this sensitivity. Coumarin is extremely hydrophobic and possesses no structures that would bring about electrostatic interactions with the scFv. Therefore, it must be concluded that this essential stabilising, but weak electrostatic force must be as a result of an interaction with the antibody and some component of the BSA carrier protein, probably a component of the amino acid by which coumarin is coupled, i.e. histidine or tyrosine. Complete stabilisation of the antibody-antigen interaction requires both hydrophobic interactions between scFv and electrostatic interactions between scFv and amino acid present on the parent protein. The three scFv antibodies thus probably principally recognise a combined hapten-protein

epitope of the coumarin-BSA conjugate.

The fact that coumarin was required to stabilise the antibody-antigen interaction suggested that there must be some affinity between the scFvs and free coumarin. Since the principle interactions between coumarin-BSA or coumarin and scFv appeared to be either electrostatic and/or hydrophobic in nature, then it might be suggested that adjustment of the ionic and polar environment in which the competition between coumarin-BSA and coumarin takes place, may enhance the interaction between free coumarin and scFv over coumarin-BSA and scFv. This was attempted by looking at the combined effects of NaCl and methanol concentration present during the antibody-antigen competition. However, increasing NaCl concentration only succeeded in destabilising coumarin-BSA-scFv interaction, without enhancing interaction with coumarin alone. Increasing methanol concentration also succeeded in only having a positive effect on the interaction of coumarin-BSA with scFv. This effect was only significant in the case of α-C14, but not α-C5 or α-C13. The effect of NaCl concentration on the interaction between scFv and coumarin-BSA was examined more closely and seemed to confirm earlier observations that antibody-antigen interaction was all but prevented at an NaCl concentration of 0.2 M. In the cases of α-C5 and α-C13, as the effect of methanol concentration was not significant, these data were used as replicates (n=5) from which to calculate means and standard deviations of absorbance for each NaCl concentration used. When populations in the presence and absence of coumarin were compared, significant differences were detected at the 0 and 0.001 M NaCl levels for α-C5 and α-C13, showing that binding to surface coumarin-BSA was reduced in the presence of 1 μg/ml coumarin in the case of α-C5 and α-C13. This was not so for α-C14. However, since there was greater standard deviation in this population due to the significant effect of methanol, the presence of an effect showing binding to free coumarin may have been lost.

Since the scFvs could not be effectively characterised using free coumarin to measure affinity, relative affinity measurements were made using coumarin-BSA conjugate. The method used was an approximation of the affinity equilibrium constant according to Van Heyningen *et al.* (1983) and Roben (1993). All three scFvs yielded very similar results,

$3 \times 10^7\ M^{-1}$, $4.5 \times 10^7\ M^{-1}$ and $2.5 \times 10^7\ M^{-1}$ for α-C5, α-C13 and α-C14, respectively. This figure represents moderate affinity, but was calculated based on a single mole of coumarin-BSA. With a multivalent hapten, the formula for K_A becomes:

$$K_A = \frac{k_a}{k_d} = \frac{1}{n\,[Ag]} \quad M^{-1}$$

where n is the valence of the antigen. Up to 11.5 scFv molecules may bind to a single coumarin-BSA molecule. If this were the case, relative affinities would be $3.5 \times 10^8\ M^{-1}$, $5.18 \times 10^8\ M^{-1}$ and $2.88 \times 10^8\ M^{-1}$ for α-C5, α-C13 and α-C14, respectively, under the conditions used.

Further confirmation of the characteristics of the three scFv antibodies resulted from their interaction with other coumarin-protein conjugates. The level of interaction between scFv and coumarin-BSA conjugates was directly related to the extent of drug conjugation on the BSA protein, again indicating the requirement for a shared coumarin-BSA epitope. Interaction with coumarin-OVA to the same extent as coumarin-BSA again suggested that the epitope did not depend on topographically proximal amino acid structures, but on the amino acid to which the coumarin molecule was linked, namely tyrosine and tryptophan. This could be further confirmed by the use of other coumarin-protein conjugates (coumarin-THG), enzymatic digests of coumarin-BSA and conjugates of coumarin-histidine and coumarin-tyrosine.

α-C5, α-C13 and α-C14 could not be effectively used for the analysis of free coumarin, but were capable of the characterisation of coumarin-protein conjugates by ELISA. Their use as reagents on western blots was, however, limited by their NaCl sensitivity. Blotting incorporates stringent washing steps using PBS and other salt buffers to prevent background staining. When these buffers were substituted with buffers suitable for the scFv interaction with coumarin-protein conjugates, the lack of stringency resulted in high background. Their use as reagents in blots is limited by this sensitivity, but may be optimised by careful control of NaCl concentration and the use of other agents such as non-polar detergents like Tween 20.

The multi-specificity of the three clones was also assessed using conjugates of coumarin, 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide coupled to BSA. This showed that the three antibodies were specific for the coumarin hapten and showed no significant specificity for the other haptens. This is not surprising as coumarin and 7-hydroxycoumarin were coupled in opposing orientations to the carrier protein, coumarin at the 6-position and 7-hydroxycoumarin at the 3-position (Fig. 4.68). When these are presented to the scFv, they are bound to present significantly different surfaces. This may have been less significant if the haptens had been free. 7-hydroxycoumarin-glucuronide possesses the intervening glucuronic acid molecule, and a stable interaction between the coumarin moiety and the amino acid to which it was linked was unlikely to take place.

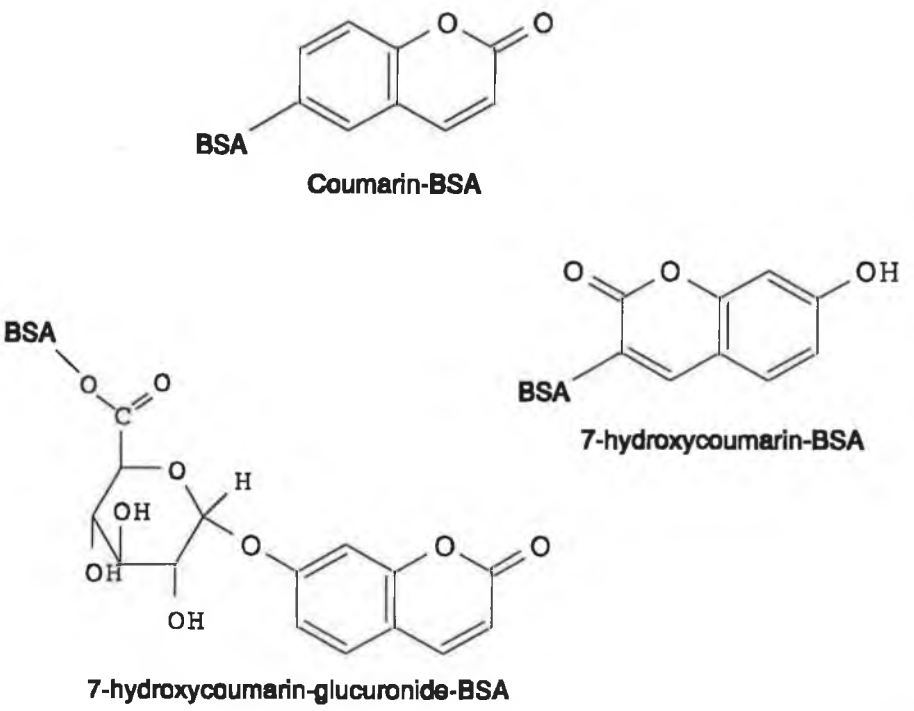


Figure 4.68. Orientation of coumarin, 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide on the carrier protein. Coumarin was coupled at the 6-position, 7-hydroxycoumarin at the 3-position and 7-hydroxycoumarin-glucuronide via the carboxyl group of the glucuronide moiety.

The three anti-coumarin scFv clones were also analysed by BIAcore. Kinetic analysis was performed on the three clones and the relative affinities for the three clones calculated.

Kinetic analysis showed that the three clones deviated from pseudo-first-order kinetics. This could occur for two main reasons (Nieba *et al.*, 1996; O'Shannnessy and Winzor, 1996). One is that binding is taking place with the matrix rather than the haptens present on the protein. This was demonstrated as occurring on the control surface. Since no binding to the BSA control occurred in ELISAs, it is probable that most of this interaction was a non-specific one between the antibody and the charged dextran matrix. This, coupled to the ionic sensitivity of these clones would suggest that some electrostatic interaction may occur between the dextran matrix and the scFv. Also, first order kinetics can be distorted by the presence of high concentrations of surface ligand. In this instance, rebinding may take place. Efforts were made to greatly reduce the amount of bound ligand (1,600 RU), but a lower concentration may have been more optimal.

Using the data from the observed on and off rates, all the clones had affinity measurements in the nanomolar range, with an affinity ranking of α-C13>α-C5>α-C14. These affinity measurement were strongly dependent on the matrix used for the measurement, ie., HEPES buffer without NaCl was used. The use of the standard phosphate-buffered saline buffer would have significantly reduced the affinity measurements. Indeed, 0.15 M NaCl was incorporated into the HEPES buffer to serve as a regeneration buffer. Such an easily regenerable sensor surface could have applications in sensor technology where an easily regenerable surface is an ideal characteristic of the assay system.

The calculated affinities were an order of magnitude greater than that found by competition enzyme immunoassay, which were calculated using equilibrium data. This may suggest a significant error in the kinetic data as generated by BIAcore analysis and demonstrates some of the possible inadequacies of the kinetic models used for such analyses. However, both studies yielded affinity rankings for the three clones in the order of α-C13>α-C5>α-C14.

4.4.5 Summary

The three anti-coumarin scFv antibodies α-C5, α-C13 and α-C14 all exhibited characteristics very similar to one another. All three possessed NaCl sensitivity and bound to coumarin-BSA with high affinity in the absence of NaCl, but to free coumarin with poor affinity. None of the antibodies bound to BSA alone. All three showed relative affinity measurements in the subnanomolar and nanomolar range by ELISA and BIAcore™ respectively. Major differences were observed in their secretion characteristics, α-C5 and α-C13 secreting into periplasm and supernatant in roughly equal quantities, whereas α-C14 secreted more efficiently into culture supernatant, bypassing the periplasm. This could lead to the suggestion that the structure of the antigen binding sites may be very similar, if not identical, then at least exhibiting certain critical polar and non-polar regions. α-C5 and α-C13 appear so alike that they may be derived from the same clone. α-C14 may possess sequence variations that do not affect antigen binding but aid in more efficient release of protein from the periplasm across the outer membrane. Nucleic acid sequence analysis of the three clones would confirm or refute this.

Screening of anti-coumarin antibodies using the Nissim library resulted in the isolation of three scFv antibodies that were capable of recognising coumarin with low affinity and a combined coumarin-BSA epitope with relatively high affinity. These also had a characteristic sensitivity to greater than 0.05 M NaCl which made them unsuitable as western blot reagents, but made them easily regenerable on the BIAcore sensor chip surface. This electrostatic interaction also resulted in some interaction with the BIAcore sensor chip dextran matrix, which contributed to non-linearity when kinetic parameters for the scFvs were measured.

4.5 PRODUCTION OF ANTI-7-HYDROXYCOUMARIN scFv ANTIBODIES USING THE NISSIM LIBRARY

4.5.1 Selection and screening of scFv antibodies to 7-hydroxycoumarin

Production of the anti-7-hydroxycoumarin scFv library began with affinity selection against a 7-hydroxycoumarin-BSA (7OHC-BSA) conjugate using BSA as blocking reagent and free in solution to remove non-specific BSA binders. In principle, this methodology should have been capable of the removal of anti-BSA clones from the phage population, while enriching for clones recognising 7-hydroxycoumarin only. Initially, however, this did not occur, and the library enriched for anti-BSA clones instead. It is clear that the use of soluble protein to remove reactivity with surface BSA, either as blocking reagent or as carrier protein is not sufficient. This may be as a result of the very large numbers of phage particles in the phage population used for affinity selection. If enough phage is present, binding of clones to free BSA and surface BSA may reach an equilibrium. Even following washing, many anti-BSA phage may be still adhering to the surface. Since the number of epitopes available to anti-BSA binders is greater than those to anti-7-hydroxycoumarin binders, and since the number of sites available to bind to BSA is far greater than to 7-hydroxycoumarin (the 7OHC-BSA conjugate used possessed 2.7:1 moles 7-hydroxycoumarin to BSA), then the numbers of anti-BSA clones enriched could greatly exceed the number of anti-7-hydroxycoumarin clones, even following a single round of affinity selection. With subsequent rounds of enrichment, this bias may only be exacerbated, until a point is reached where the population consists almost entirely of anti-BSA clones, making it impossible to screen anti-7-hydroxycoumarin clones.

The enrichment methodology was modified so as to avoid this enrichment to the carrier protein or blocking reagent. Changing the carrier protein and the blocking protein used on each round of affinity selection would ensure that enrichment of phage antibody to carrier protein or blocking protein on a previous round of selection would be removed in the following round. This was very successful, and all clones subsequently screened were not positive to the carrier protein or blocking reagent.

This phenomenon did not arise in the case of enrichment of anti-coumarin clones (section 4.2). What may have been crucial here was the level of conjugation of hapten to carrier protein. In the coumarin-BSA conjugate, the coupling ratio was 11.5:1 moles coumarin to BSA, whereas for 7OHC-BSA, this was only 2.7:1. Such a reduction in the number of hapten epitopes may bias affinity selection too much in favour of the carrier protein.

A high hapten:protein coupling ratio would be ideal for such affinity selection strategies. With peptides, approximately 10:1 molar ratio will effectively cover the surface of the protein, so revealing very few carrier protein epitopes for antibody binding (Andrew Williams, Cambridge Antibody Technology, personal communication). However, haptens such as coumarin and 7-hydroxycoumarin, having much lower molecular masses, would require much higher coupling ratios to achieve this near total occlusion of the carrier protein. Such an attempt would result in problems associated with the physical characteristics of such a conjugate. The hydrophobicity of these molecules would lead to an extremely insoluble conjugate that could not function as a useful affinity ligand (Hermanson, 1997). For effective selection of antibodies to 7-hydroxycoumarin, modification of the enrichment procedure in the way performed seems appropriate. Alternatively direct coupling of haptens to solid supports may also be another viable strategy. This was tested briefly for anti-coumarin antibodies which did appear to result in levels of enrichment. No optimisation of these methodologies was performed and significantly better levels of enrichment may be achievable.

As has been stated, not all clones positive by phage ELISA result in the secretion of scFv. Indeed, in most cases, this seems to be a relatively small proportion of the overall population. In the case of the 7-hydroxycoumarin screening, however, all 43 clones positive to 7OHC-BSA were found to be incapable of secretion. Clones taken at random from the population, i.e., not necessarily anti-7-hydroxycoumarin clones, showed greater levels of secretion. This phenomenon was also true of the coumarin library. It was initially suggested that such a result might be a consequence of the production of scFv molecules to hydrophobic haptens. The presence of hydrophobic residues in the scFv to bind to the hydrophobic hapten may result in difficulties associated with refolding where large areas of hydrophobicity are not normally exposed on the external surfaces of such globular

proteins. However, later rescreening found there to be populations of soluble anti-7-hydroxycoumarin clones, and that the earlier difficulties with insolubility might have been artefacts of the original affinity selection.

4.5.2 Isolation of scFv antibodies to 7-hydroxycoumarin from intracellular lysates

Prior to reselection and rescreening, however, the possibility of isolating scFv from intracellular lysates was investigated. In the conversion of whole monoclonal antibodies into antibody fragments for prokaryotic expression () it has been found that many of the resulting molecules are insoluble due to incorrect folding, so preventing secretion and resulting in intracellular inclusion bodies. The development of protocols to solubilise, denature and then refold these molecules into their correct conformation allowed the possible use of these molecules. Lysis of the inner membrane was normally performed by sonication or physical disruption techniques such as the use of a Beadbeater apparatus (Hughes *et al.*, 1969). Neither was significantly better than the other in terms of the yield of scFv released. In practical usage, however, sonication was the simpler of the techniques.

Following cell lysis, denaturation was performed in either 7.5 M guanidine hydrochloride or 8 M urea. Again, these performed equally well, and both resulted in elevated signals of scFv on blots. Denaturation may have resulted in the greater availability for recognition of the c-myc peptide by anti-c-myc antibody.

Efficient refolding is a lengthy procedure and can take up to 5 days (Buchner and Rudolph, 1991). Refolding also requires large concentrations of arginine which has been found to assist in the refolding of the antibody protein.

When applied to the anti-7-hydroxycoumarin clones α-7OHC9 and α-7OHC10, the refolding methodology did result in scFv that was capable of binding to 7-hydroxycoumarin coupled to EAH sepharose, indicating recognition of 7-hydroxycoumarin. Much scFv material did not attach, however. Actual yield could not be measured due to the presence of non-specific protein, but yields of up to 40% correctly

folded antibody have been achieved with these protocols (Buchner and Rudolph, 1991). Surprisingly, however, for affinity purification, much non-specific protein material was still present following extensive washing of the column prior to elution. Intracellular lysates contain very high concentrations of protein in comparison to periplasmic lysates and more extensive optimisation of washing and elution procedures would be required to improve the level of enrichment.

The main obstacle in the efficient use of scFv refolded from intracellular lysates was the initial yield of scFv from the cells. Yields of exogenous protein can be dramatically effected by the growth conditions of the culture such as growth temperature (Cabilly, 1989; Takagi *et al.*, 1988) and inducer concentration (Takagi *et al.*, 1988). For this reason, optimisation of the yield of scFv from α-7OHC10 was attempted by varying several factors: growth period prior to induction; inducer concentration; growth period post-induction and growth temperature post-induction. All these have been shown in the past to be factors important in determining antibody yield. In this instance, however, although small variations in yield could sometimes be noted when growth periods and inducer concentrations were varied in small scale tests, for full scale antibody production, yields were not seen to be visibly improved upon previous analyses. This poor yield may relate to the build up of these antibodies as inclusion bodies within the cell. Such deposits may inhibit the correct functioning of the cell, maybe even causing cell death before large yields of protein have built up.

Purified antibodies were also found to be prone to precipitation following concentration. This predisposition to precipitate may be connected with their inability to refold correctly during translation. This, in combination with an initial low yield of scFv, resulted in very low yields of correctly folded, soluble antibody. α-7OHC10 managed to show selective recognition of 7OHC-BSA over BSA on ELISA, but with extremely poor signals. This combination of characteristics meant that the production of scFv anti-7-hydroxycoumarin antibodies from intracellular lysates was not a viable option for the isolation of antibody material useful in immunoassays or other applications.

4.5.3 Production of phage antibodies to 7-hydroxycoumarin

If antibody could not be efficiently secreted, then antibody on the surface of phage - so-called 'phage antibodies'- might be used as an alternative source of antibody material. Secretion of scFv attached to pIII and assembled on the surface of the virion is assured, and large yields of phage antibody can be isolated. Six such clones were isolated and analysed by ELISA. Although supernatants from clones were shown to have no reactivity to the carrier protein, when used as concentrated phage stocks, a sizeable response to BSA was detected in all six clones, below the signal for 7OHC-BSA. The presence of large amounts of virion protein in these concentrated phage stocks may have resulted in significant degrees of non-specific interaction between virion and BSA, contributing to a high background and a very poor signal:noise ratio. In this form, such phage antibodies would have little use as affinity reagents. Application of these molecules may be found in a pVIII-type system (Kang *et al.*, 1991) for scFvs. Here, the copy number of scFv on each virion would be considerably increased. This would also in turn decrease non-specific interaction between phage and surface coatings, improving the signal:noise ratio of the assay and allowing the use of higher dilutions.

4.5.4. Affinity selection of scFv antibodies to 7-hydroxycoumarin using BIAcore

The potential of using BIAcore to select phage antibody clones to 7-hydroxycoumarin from the Nissim library was explored. Malmborg *et al.* (1996) had demonstrated that BIAcore could be used to select positive clones from a mixture of positive and negative phage clones. Malmborg *et al.* (1996) crucially demonstrated that below a specific phage titre of 3 x 10^8 tfu/ml, BIAcore was not capable of detecting a binding signal. Thus, it would be unlikely that any signal would result from a population of unselected phage such as the Nissim library used here. However, absence of a sensorgram signal did not mean that selection was not taking place. By monitoring phage counts, it was clear that elution of bound phage could be brought about after attachment to the BIAcore sensor chip surface. The level of enrichment was significant, yielding in excess of 1 in 20,000 enrichment of specific phage. This was well in excess of the 5-fold enrichment experienced by Malmborg and Borrebaeck (1995). However, since they began with a

mixture of 1:10 specific antibody and non-specific phage stock, the mixture was already significantly enriched and unlikely to result in large enrichment factors. Malmborg *et al.* (1996) also found that antibodies isolated in the early part of the elution phase had higher dissociation rate constants than those isolated later. It was demonstrated that specific phage could be selected from a large, diverse, unpanned antibody library in much the same way as has been achieved on solid supports. Such a selection procedure also has the potential to be used to select antibodies with high dissociation rates with the potential for use in regenerable immunosensor systems.

4.5.5 Optimised affinity selection and screening of scFv antibodies to 7-hydroxycoumarin

The affinity selection of antibodies to 7-hydroxycoumarin using the conjugates available was eventually achieved. This was done using rounds of affinity selection with different carrier proteins and different blocking reagents. In this way, two problems with the enrichment were eradicated. Firstly, the enrichment of antibodies to the blocking protein were prevented as a different one was used each time. Secondly, responses to the protein carrier were minimised also. This strategy did indeed result in clones that did not respond to the carrier protein alone, or the blocking solution. However, when tested against a conjugate of 7-hydroxycoumarin in which the drug was attached to the protein via a different linker at the 4-position rather than the 3-position, no response from the antibody occurred. Although some clones were now found to be secretors of soluble scFv, when tested against free 7-hydroxycoumarin, no competition was seen. A similar effect was noted as had occurred with anti-coumarin clones. That is, at higher free drug concentrations, the response to the bound conjugate was enhanced.

4.5.6 Characterisation of scFv antibodies to 7-hydroxycoumarin

Although further analysis of these clones was not performed, and they were only used in an unpurified form, they seemed to possess certain characteristics in common with the previously screened anti-coumarin scFv clones. Firstly, they did bind to the drug-protein conjugate to which they were raised, but not to the free drug or carrier protein alone.

They also showed that the increasing hydrophobicity of the test matrix due to the presence of high concentrations of 7-hydroxycoumarin, enhanced the interaction of the antibody with the drug-protein conjugate. This may indicate that an electrostatic interaction between the antibody and the antigen is a major stabilising influence. Since there are no polar or ionic components present on 7-hydroxycoumarin, it could also be concluded that a component of the protein provides this interaction. It might be concluded then, that the production of antibodies to coumarin and 7-hydroxycoumarin resulted in the selection of clones which did not bind to the free drug with high affinity , but selected antibodies to a drug-amino acid conjugate.

4.6 THEORIES AS TO WHY scFv ANTIBODY PRODUCTION STRATEGIES DID NOT RESULT IN ANTIBODIES WHICH SIGNIFICANTLY RECOGNISE FREE DRUG

Keating (1998) used hybridoma technology for the production of antibodies to 7-hydroxycoumarin. Similar results were encountered in which clones selected using 7-hydroxycoumarin conjugates coupled to proteins differing from that used for immunisation did not result in the selection of clones that were capable of binding free drug.

Danilova (1994) also obtained similar results in the isolation of hybridomas to small haptens such as digoxin and thyroxine. These exhibited all the characteristics of the anti-coumarin and anti-7-hydroxycoumarin clones described. That is, they bound thyroxine-BSA, but not BSA or free thyroxine with high affinity, but were capable of showing competition with the free thyroxine-BSA conjugate. Danilova concluded that these clones also bound to a combined epitope of the hapten-carrier and that this was not dependent on the carrier protein as antibody bound equally well to other protein-hapten conjugates, as did the anti-coumarin clones.

A mechanism can be proposed as to why, in the case of selection and screening using antibody scFv libraries, clones were selected that were not capable of binding to the free haptens, coumarin and 7-hydroxycoumarin, but bound to a combined epitope of the drug-hapten conjugate. This mechanism can also be extended to explain why this phenomenon

also occurs in the case of hybridoma screening.

In the selection of scFv to these haptens, selection was performed using, in one instance, the same coumarin-BSA conjugate throughout selection and screening. In the other case, selection used different conjugates, differing by their protein, but not their mode of attachment. In the case of coumarin, the coupling ratio was high, and this resulted in enrichment of clones to the drug-carrier conjugate, rather than to the protein alone. At lower coupling ratios, as was initially the case with 7-hydroxycoumarin-BSA, however, enrichment to the carrier came to predominate, and so coupling ratio is crucial in removing these effects. However, the alternative strategy of alternating the carrier protein and the blocking solution also circumvented this.

Affinity selection is performed using immobilised antigen on immunoassay plates. Such methodologies have the potential to select for clones with the highest affinities to the antigen. Such high affinities are based on the sum of attractive and repulsive forces, based on hydrophobic interactions, Van der Waals forces, ionic bonds and hydrogen bonding taking place between the antigen and the antibody. Since coumarin and 7-hydroxycoumarin are small, hydrophobic molecules, they would have a tendency to interact by a small number of hydrophobic forces alone. Such interaction may be of only low affinity. This molecule would occupy only a small pocket of the antigen binding site. Other interactions between the antibody and the antigen may provide higher affinity interactions. Such might involve interactions, not with the drug alone, but with parts of the protein, namely that part of the protein that is proximal to the hapten: the amino acid to which it is linked. Indeed, even a single electrostatic interaction can contribute significantly to the overall affinity of such an interaction (Tijssen, 1985). Enrichment of these clones over those that interact with hapten alone thus result in difficulties during screening anti-hapten antibodies from the enriched population. Again, during the screening process, which is an ELISA-based technique, these lower affinity binders are passed over for the higher affinity clones which yield the highest signals.

In the case of hybridoma technology, similar events may occur both *in vivo* and *in vitro* to bring about the same effects. In the antibody response following immunisation, affinity

maturation takes place, where high affinity clones are selected from lower affinity clones, which are apoptosed (Kuby, 1997). Indeed, it was shown by Reinartz (Dublin City University, Personal Communication) that only a small percentage of a polyclonal antibody response was found to be to the hapten, and the rest to components of the carrier protein. Already, the population of B cells may already be heavily biased in favour of high affinity clones not recognising the free hapten, but a hapten-carrier combination. Use of a hapten-protein conjugate during screening that just differs by the protein used is then not sufficient to prevent the selection of clones that recognise the hapten-amino acid epitope, as this is the same in both instances. The use of different protein carriers was again shown to be of no significance in the case of anti-7-hydroxycoumarin antibodies, as the resulting clones still recognised both conjugates.

It is clear, then, that the use of conjugates differing by the protein, but using the same coupling methodology are not sufficient for the selection of antibodies to these small haptens. Danilova's assertion - that the orientation of the hapten should be changed, along with the coupling methodology and the carrier, between, in the case of hybridomas, immunisation and screening, and in the case of scFv antibodies, during affinity selection and screening - is a valid one. Changing the orientation of the hapten and changing the coupling methodology would reduce the bias towards selecting and screening clones that relied on a certain interaction with the carrier, or, indeed, the linker. Also, antibodies would be selected that simultaneously bound different orientations of the same hapten, perhaps in different parts of the antigen binding site, which might significantly affect the resulting affinity.

Other viable alternatives for selection and screening are the use of direct covalent attachment of the haptens to solid phases. This would remove problems associated with the carrier protein, but may not remove problems associated with the linker, or the orientation of the hapten. Steric hindrance may also be a concern in this instance. Selection and screening using BIAcore may also be a viable alternative. Its ability to select low affinity binders may show that many of these are capable of interacting with the hapten and not the hapten-carrier combination. Another alternative would be the development of a screening method based on a competitive immunoassay in which

binding to free drug could be detected. Such work was attempted (data not shown), but was found not to be feasible. Several parameters of a competition assay require optimisation prior to the assay itself, such as optimum antibody, antigen and free analyte concentrations (Catty, 1989). Such analyses cannot be performed simultaneously on large numbers of clones as occurs with phage ELISA. This would further diminish the chances of detecting clones that were capable of binding to free antigen.

4.7 CONCLUSION

Antibody scFv fragments were screened from a naive library against coumarin and 7-hydroxycoumarin. The resulting clones were incapable of binding with high affinity to the free drug due mainly to the nature of the conjugates used to select and screen them. For the effective selection and screening of monoclonal antibodies to these haptens, either by genetic or hybridoma technology, the development of more sophisticated conjugates is desired. These conjugates should differ, not only by their carrier proteins, but also by their linker methodology and thus, the orientation of the hapten. It is felt that this will remove the problems encountered in these antibody production strategies.

5. 7-HYDROXYCOUMARIN-GLUCURONIDE: PRODUCTION, PURIFICATION, ANALYSIS AND CONJUGATION FOR THE PRODUCTION OF ANTIBODIES

5.1 INTRODUCTION

5.1.1 *In vitro* metabolic studies of 7-hydroxycoumarin

In vitro metabolic studies are useful for several reasons. They can be used to assess the relative contributions of various tissues and organs in the metabolism of a particular pharmaceutical component. The data gathered in this way can be compared to what is known of its metabolism in the *in vivo* situation. In the case of coumarin (Pelkonen *et al.*, 1997), there exists large interspecies differences in its metabolism. Such *in vitro* models can establish which organs are responsible for these variations in metabolism. Such variations may have significance for the toxicity or therapeutic effects of coumarin, 7-hydroxycoumarin and their metabolites.

In vitro studies of the human metabolism of coumarin have also been performed using tissue biopsies (Deasey *et al.*, 1995). This can yield important data as to the relative contribution of human organs in the metabolism of coumarin in man. Inter-individual differences in man may also have relevance for such factors as toxicity and therapy. Coumarin is also a useful model for the metabolism of a simple organic substrate.

The phase I metabolism of coumarin has been very well investigated (Pelkonen *et al.* 1997). However, for the reasons described in section 1.14, its phase II metabolism has been unjustifiably ignored. Although less important in terms of toxicity as phase I metabolism, phase II metabolism may play a significant role in the effectiveness of coumarin therapy. The development of *in vitro* metabolic methodologies and allied analytical techniques for 7-hydroxycoumarin-glucuronide are essential for such studies. Also, the development of suitable derivatives for the purpose of antibody production lead to the possibility of developing immunoanalytical tools to complement the solid state methods used. The production of 7-hydroxycoumarin-glucuronide also has the potential to be used in further pharmacological studies.

In the case of Tegtmeier (1988) and Brunner and Tegtmeier (1994), the purpose of their metabolic studies was the detoxification of chemical agents in an *in vitro* metabolic

system incorporating hollow fibre membrane technology. Glucuronidation is often correctly described as a major detoxification pathway for many xenobiotic substances. This has applications for environmantal clean up as well as the pharmacological applications described.

The metabolism of 7-hydroxycoumarin to its glucuronide has been described (section 1.14.1). It was assumed that the techniques used by Tegtmeier (1988) for the *in vitro* metabolism of lignins would be applicable to 7-hydroxycoumarin also. Here, the enzyme UDP-glucuronyl transferase was isolated from porcine liver. This was incubated with the necessary substrates: the aglycone and UDP-glucuronic acid. Other reaction components were also required. The presence of magnesium ions were necessary as an enzyme cofactor, Saccharolactone is an inhibitor of β-glucuronidase activity. This stops the reverse reaction of the removal of glucuronic acid from the glycone derivative, in our case, 7-hydroxycoumarin-glucuronide.

As well as the production of the glucuronide by *in vitro* means, analytical methods have to be applied to the study. Two methodologies were used: HPLC and CE.

5.1.2 The use of HPLC for the analysis of coumarin metabolism

In HPLC, the separation of components in a matrix is dependent on their partition in a mobile and a stationary phase (Fallon *et al.*, 1987). In the case of HPLC, the stationary phase is a narrow bore column solid phase, and the mobile phase is liquid. In the type of chromatography used here, called reverse phase chromatography, the solid phase is a hydrophobic material produced by the fusing of silica with long chain aliphatic hydrocarbon chains of 18 carbons in length (C18). Separation of relatively hydrophobic components is then brought about by adjusting, amongst other conditions, the composition of the mobile phase. A very hydrophobic mobile phase will encourage hydrophobic components to travel with it, whereas a less hydrophobic phase would cause these to be retarded on the hydrophobic stationary phase. In general, more hydrophobic phase will migrate more slowly than less hydrophobic components. Egan and O'Kennedy (1994) deveoloped HPLC conditions for the analysis of coumarin and 7-hydroxycoumarin. This

employed an isocratic mobile phase of methanol:water and acetic acid, with UV detection at 320 nm

Detection of the analytical species is normally performed by a single wavelength ultraviolet detector. For coumarin analysis, this is typically 320 nm. However, photodiode array analysis can also be applied to the separation of such components for the more detailed analysis that this technique yields.

5.1.3 Reverse phase chromatography

Reverse phase chromatographic separation can be applied to large scale chromatography in semi- and fully preparative separations. The principal is the same as that described above, except that only low pressures are used to perform the separation, and large column volumes are used to achieve larger yields.

5.1.4 Capillary electrophoresis (CE)

CE is a relatively modern analytical technique. It is a development of other electrophoretic systems such as gel electrophoresis (Beckman, 1991). Analytical mixtures are separated by the application of a high electrical potential to a sample in an ionic buffer in a narrow bore capillary. The fused silica capillary is negatively charged and attracts positively charged ions from the buffer. Cations in the buffer migrate towards the cathode, carrying water with them. This results in a net flow of buffer towards the cathode. Most solutes will migrate towards the cathode, regardless of their charge due to this electroosmotic flow. Dectection is via a detector window close to the cathodic terminal of the capillary. UV absorption is again the detection method most commonly employed. CE results in near instantaneous, high speed separations. This alters the requirements for sample clean up in certain analytical procedures, often simplifying the methodology. Also, unlike HPLC, it is capable of analysis in aqueous environments, so extractions with organic solvents is not required. Bogan *et al.* (1996) developed a CE separation strategy for the analysis of urine samples for the presence of 7-hydroxycoumarin-glucuronide.

5.1.5 Liquid/liquid extraction

Liquid extractions are commonly used for the isolation of organic hydrophobic components from aqueous mixtures. The imiscibility of organic solvents with water makes this possible. The extracted species partitions between the two phases, normally with much higher partition in the organic phase. The extraction of more water miscible solutes is more difficult, however, with such techniques and is normally far less efficient. Phases of various polarity can be constructed to attempt to partition the solute more efficiently into the organic phase. In this way, moderately hydrophobic components may be extracted into organic phases.

5.1.6 Conjugation strategies

The diazonium coupling methodology of haptens containing an aromatic amine have been described in section 4.1.2. The resulting conjugates are chemically robust. They do, however, require the chemical synthesis of the aromatic amine if one is not naturally available. This is performed under relatively severe chemical conditions. Such conditions may not be compatible with a compound such as 7-hydroxycoumarin-glucuronide. Alternatively, it might be conceived that an amine be introduced to 7-hydroxycoumarin prior to *in vitro* conjugation with glucuronic acid. In this way, production of an amino conjugate of 7-hydroxycoumarin-glucuronide might be possible.

Alternatively, coupling chemistries such as 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide / N-hydroxysuccinimide (EDC/NHS) could be applied to 7-hydroxycoumarin-glucuronide (Hermanson, 1997). This requires the presence of a carboxyl group, one of which is present on the glucuronic acid moiety of 7-hydroxycoumarin-glucuronide. This strategy tends to result in less stable conjugates, but is simple to perform. The carbodiimide, EDC can react with carboxyl groups, forming a very short-lived *o*-acylisourea intermediate. This is then capable of the interaction with primary amines, forming an amide bond. The reaction can be improved by the introduction of NHS. This results in the formation of a much more stable NHS ester

intermediate of the carboxyl-containing compound, which can then react with the primary amine (Fig. 5.1).

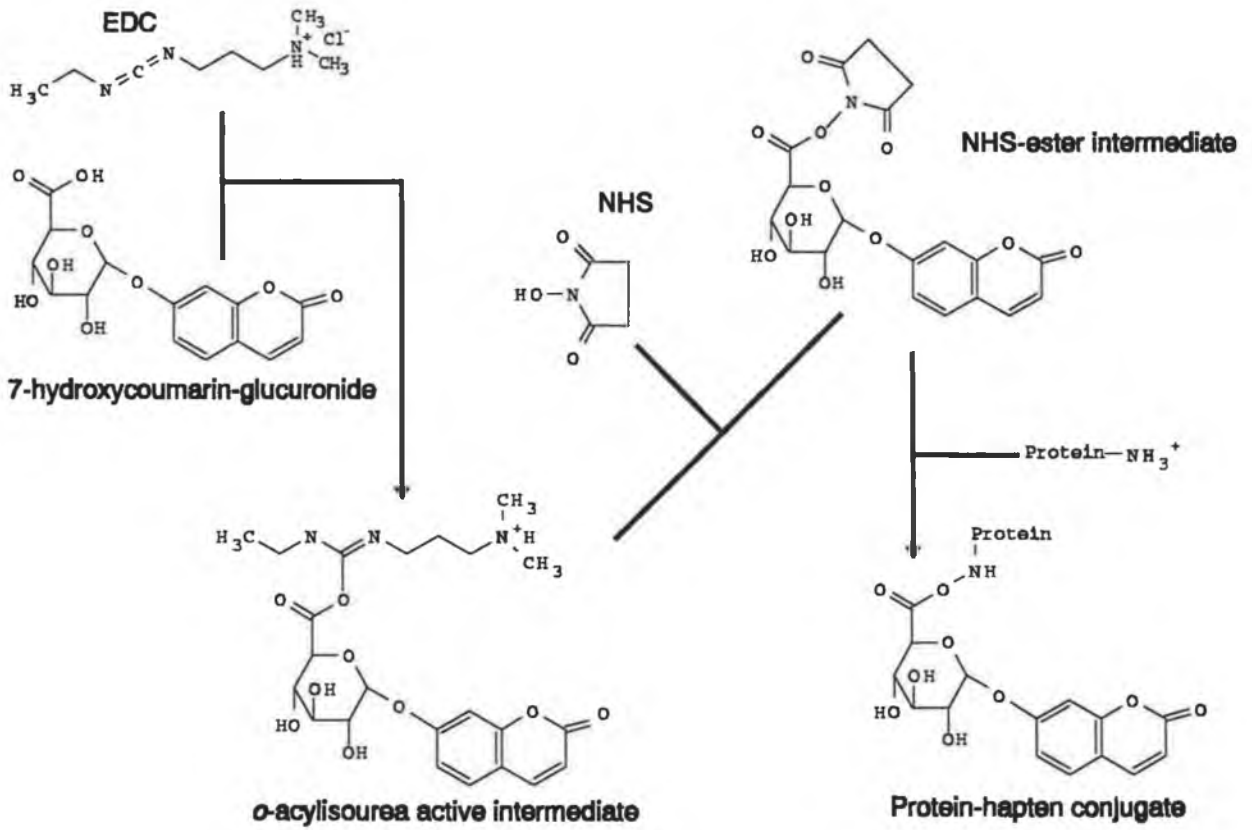


Figure 5.1. EDC/NHS reaction chemistry with 7-hydroxycoumarin-glucuronide. The carboxyl group of the glucuronic acid moiety of 7-hydroxycoumarin-glucuronide reacts with EDC to form the ubstable and short-lived o-acylisourea reactive intermediate. This can react directly with primary amines on proteins, but these are normally at a relatively low concentration in solution. Addition of NHS results in the production of higher concentration of more stable reactive intermediates which can react with primary amines over several hours.

5.2 The *in vitro* production of 7-hydroxycoumarin-glucuronide

5.2.1 Initial analysis of the in vitro production of 7-hydroxycoumarin glucuronide by HPLC

All the individual components of the reaction mixture were first analysed by HPLC under the conditions described (section 2.3.4.2). These components were shown not to have absorbances or retention times that would interfere with the analysis of 7-hydroxycoumarin-glucuronide (data not shown).

Figs 5.2 A to 5.2 D show chromatograms of all 4 reaction mixtures taken at 0 and 23 h. All chromatograms (except the samples lacking 7-hydroxycoumarin) show peaks at approximately 9 to 9.5 min, corresponding to 7-hydroxycoumarin, and a smaller peak at 11 min, corresponding to coumarin which is a contaminant of 7-hydroxycoumarin. Smaller peaks between 1.52 min and 2.5 min correspond to the other reaction components.

In sample A which contained all the reaction components, a peak between 2.98 and 3.26 min, corresponding to 7-hydroxycoumarin-glucuronide was apparent. This was not present in the other three reaction mixtures. The area of this peak increased over time (Fig. 5.2 A). Reaction mixture A was also spiked with 7-hydroxycoumarin-glucuronide, which led to a further increase in the peak at 3.26 min in the sample after 23 h incubation (data not shown).

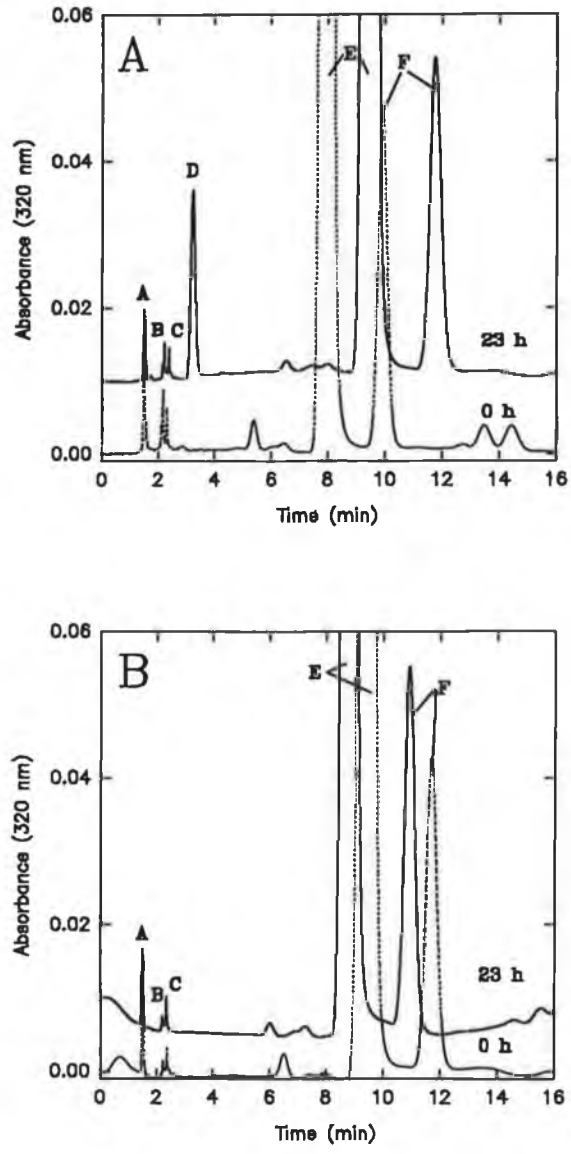


Fig. 5.2 A and B. HPLC chromatograms of enzymatic reaction mixtures from the initial analysis of <u>in vitro</u> 7-hydroxycoumarin production at 0 and 23 h. A contained all reaction components and B lacked the substrate UDP-glucuronide acid. All chromatograms show peaks A,B, and C, components of the reaction mixture, Tris, saccharolactone and magnesium chloride, respectively, and peaks E and F, the 7-hydroxycoumarin and coumarin contaminant, respectively. Both chromatograms showed shifts in the retention times of the components over time. In the case of A, the retention times increased marginally, and in B, decreased. Only in sample A was there seen the appearance of peak D, 7-hydroxycoumarin-glucuronide, which increased over time.

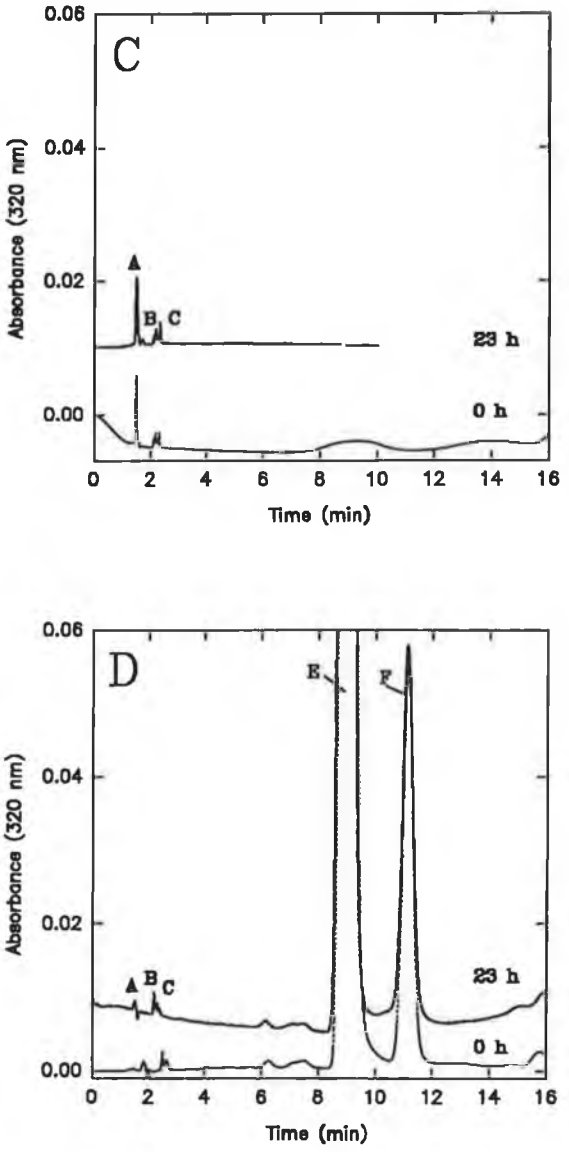


Fig. 5.2 C and D. HPLC chromatograms of enzymatic reaction mixtures from the initial analysis of <u>in vitro</u> 7-hydroxycoumarin production at 0 and 23 h. C lacked the substrate 7-hydroxycoumarin, and as a consequence, lacked peak E. Also, the coumarin contaminant (peak F) was not present. No production of 7-hydroxycoumarin-glucuronide was observed in either sample. In sample D, which lacked UDP-glucuronyl transferase enzyme, no shift in retention times was observed for the 7-hydroxycoumarin (peak E) and coumarin (peak F) peaks.

The rate of formation of 7-hydroxycoumarin-glucuronide in sample A is shown in Fig. 5.3. Production of 7-hydroxycoumarin-glucuronide had an initial rate of 7.93 x 10^{-2} μM/min, and a specific activity of 3.3 x 10^{-3} μM/min/mg protein. Approximately 14.4 μM 7-hydroxycoumarin-glucuronide had been formed after 23 h. This corresponded to a yield of 7-hydroxycoumarin-glucuronide of 0.29 μmoles or 0.097 μg. The level of 7-hydroxycoumarin-glucuronide production was calculated from a standard curve of 7-hydroxycoumarin-glucuronide concentration as shown in Fig. 5.4.

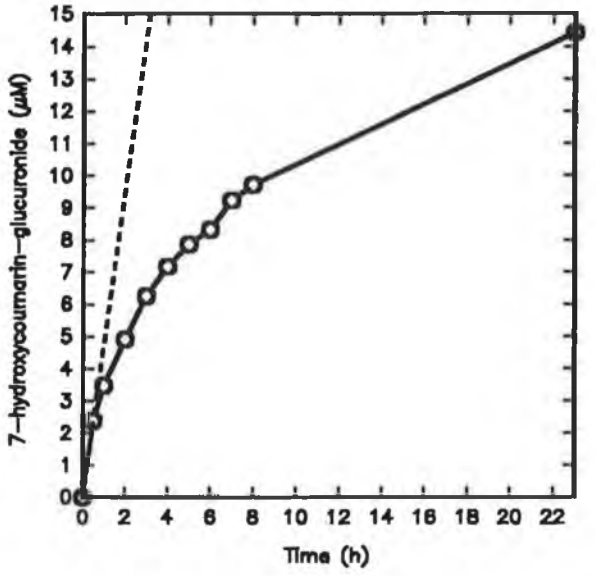


Figure 5.3. In vitro production of 7-hydroxycoumarin-glucuronide from the enzymatic conjugation of 7-hydroxycoumarin to glucuronic acid via porcine liver UDP glucuronyl transferase as analysed by HPLC. The initial rate of product formation was approximately 7.93 x 10^{-2} μM/min (r^2=0.99) (dashed line).

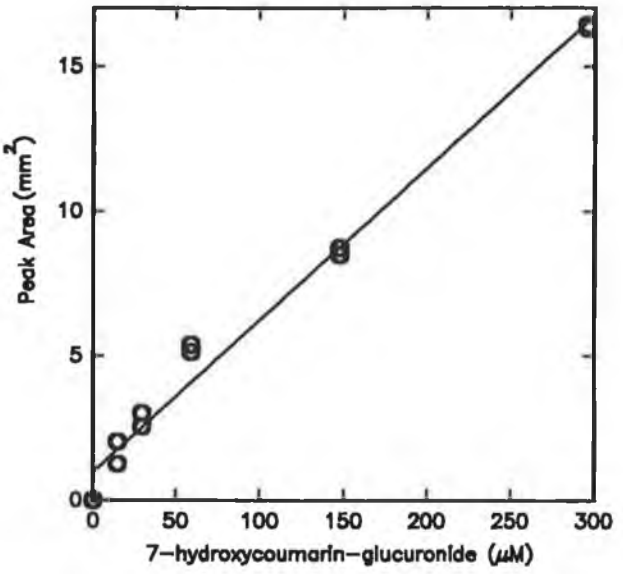


Figure 5.4. Standard curve of 7-hydroxycoumarin concentration prepared in enzymatic reaction mixture according to section vs. peak area as analysed by HPLC (n=2) (r^2=0.99).

5.2.2 Repeat analysis of the in vitro production of 7-hydroxycoumarin-glucuronide by HPLC

The *in vitro* production of 7-hydroxycoumarin-glucuronide was repeated using bovine UDP glucuronyl transferase, isolated according to section 2.3.4.1. The formulation of the reaction mixture was adjusted according to section 2.3.4.3, and HPLC analysis conditions were adjusted according to section.

Table 5.1 shows the mean peak absorbance ratio for the 7-hydroxycoumarin-glucuronide standards and their percentage relative standard deviation (n=4).

Table 5.1. Concentration of 7-hydroxycoumarin-glucuronide vs. the peak absorbance ratio for 7-hydroxycoumarin-glucuronide and 4-hydroxycoumarin.

7-hydroxycoumarin-glucuronide (μM)	**Peak absorbance ratio 7-hydroxycoumarin-glucuronide/4-hydroxycoumarin +/- standard deviation (n=4)**	**Standard Error (%)**
0	0	0
1.47	0.006+/- 0.0002	1.3
2.96	0.01+/- 0.0002	3.0
5.92	0.02+/- 0.0015	8.3
14.79	0.02+/- 0.0015	3.2
29.58	0.09+/- 0.003	3.2
59.15	0.19+/- 0.0069	3.7
147.89	0.46+/- 0.0099	2.2
236.62	0.73+/- 0.0407	5.6
295.77	0.88+/- 0.059	6.7

The reaction mixture was monitored over 150 min. Fig. 5.5 shows chromatograms for the reaction mixture at 0, 20 and 70 min. It was clear that an increase in the 7OHCG peak at 13.2 min (peak A) occurred over time. A decrease in the 7-hydroxycoumarin peak at 16.2 min (peak B) could also be observed over time.

Table 5.2 shows the mean 7-hydroxycoumarin-glucuronide concentration over time. This is illustrated in Fig. 5.6. It can again be seen that the reaction follows a typical enzyme reaction kinetic, and that the initial rate of reaction was found to be 4.127 μM/min and a specific activity of 1.03 μM/min/mg of protein. A yield of 1.35 μmoles (0.46 μg) of 7-hydroxycoumarin-glucuronide resulted after 150 min incubation. This also compared favourably with results determined by capillary electrophoresis (see section 5.2.3).

Table 5.2. Concentration of 7-hydroxycoumarin-glucuronide vs. time for the repeat production of 7-hydroxycoumarin-glucuronide by bovine UDP glucuronyl transferase.

Time (min)	**7-hydroxycoumarin-glucuronide (μM) +/- standard deviation (n=4)**	**Standard Error (%)**
0	0	0
10	47.1+/- 0.7	1.6
20	92.6+/- 5.5	5.9
30	122.4+/- 3.1	2.5
40	153.8+/- 3.1	2.0
50	177.8+/- 3.9	2.2
60	200.5+/- 5.5	2.7
70	233.1+/- 12.4	5.3
85	262.3+/- 4.7	1.7
90	280.4+/- 3.8	1.4
105	287.2+/- 3.0	1.1
120	302.6+/- 10.6	3.5
135	331.3+/- 0.6	2.0
150	336.7+/- 5.0	1.5

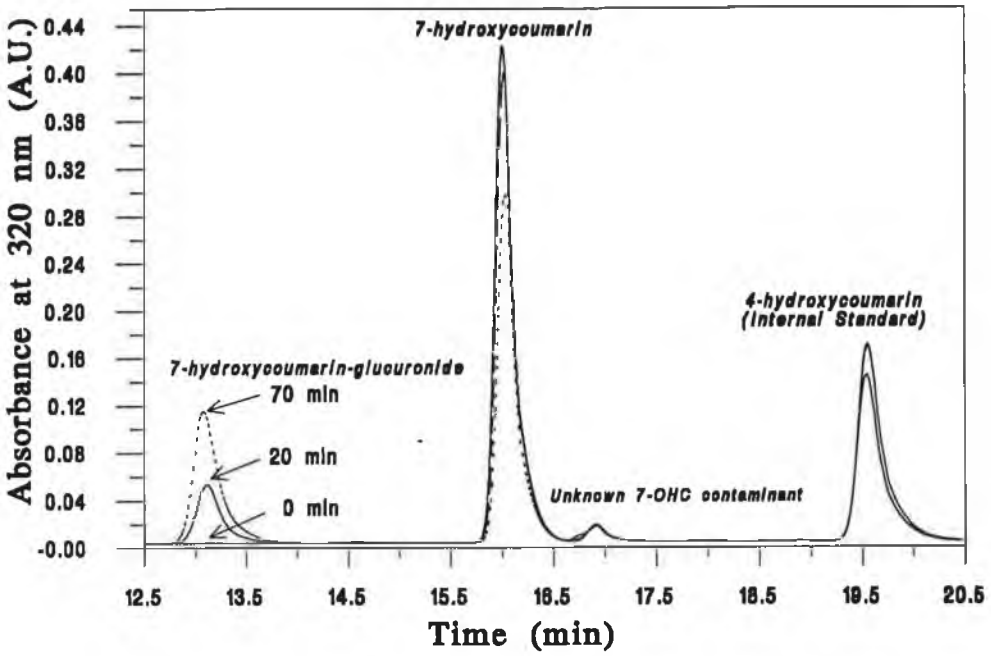


Figure 5.5. HPLC chromatograms of the repeat production of 7-hydroxycoumarin-glucuronide. Shown are offset chromatograms at 0, 20 and 70 min. This shows an increase in the 7-hydroxycoumarin-glucuronide peak at 13.2 min (peak A) over time. A decrease in the 7-hydroxycoumarin peak at 16.2 min (peak B) could also be observed over time.

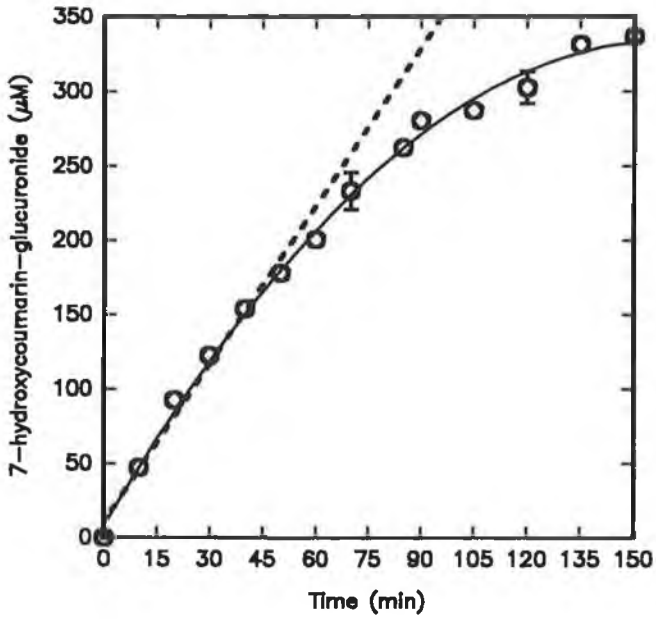


Figure 5.6. <u>In vitro</u> production of 7-hydroxycoumarin-glucuronide from the enzymatic conjugation of 7-hydroxycoumarin to glucuronic acid via bovine liver UDP glucuronyl transferase as analysed by HPLC. The initial rate of product formation was linear between 0 and 40 min and was estimated to be approximately 4.13 μM/min (r^2=0.995).

5.2.3 Analysis of the in vitro production of 7-hydroxycoumarin-glucuronide by capillary electrophoresis (CE)

Overlayed CE electropherograms of the metabolic solution for the production of 7-hydroxycoumarin-glucuronide are shown in Fig. 5.7. All electropherograms showed the presence of neutral compounds (A) at approximately 3.8 min, and magnesium chloride and 7-hydroxycoumarin peaks (B) at approximately 4.6 min. At 12, 47, and 82 min is the presence of a peak at 8.6 min, which corresponded to 7-hydroxycoumarin-glucuronide. This increased over time. The production of 7-hydroxycoumarin-glucuronide over time is shown in Table 5.3 and Fig. 5.8.

Table 5.3. Concentration of 7-hydroxycoumarin-glucuronide vs. time for the production of 7-hydroxycoumarin-glucuronide by bovine UDP glucuronyl transferase.

Time (min)	7-hydroxycoumarin-glucuronide (μM) +/- standard deviation (n=4)	Standard Error (%)
0	0	0
12	35.1 +/- 4.3	12.3
23	72.8 +/- 4.6	6.3
35	112.3 +/- 6.0	5.3
47	140.9 +/- 11.5	8.2
58	189.4 +/- 4.2	2.2
70	207.6 +/- 2.0	1.0
82	222.1 +/- 7.9	3.6

The initial rate of 7-hydroxycoumarin-glucuronide production in this instance was found to be 3.23 μM/min, with a specific activity of 2.02 μM/min/mg protein. A yield of 0.36 μmoles (0.12 μg) of 7-hydroxycoumarin-glucuronide was produced from this reaction mixture after only 82 min incubation.

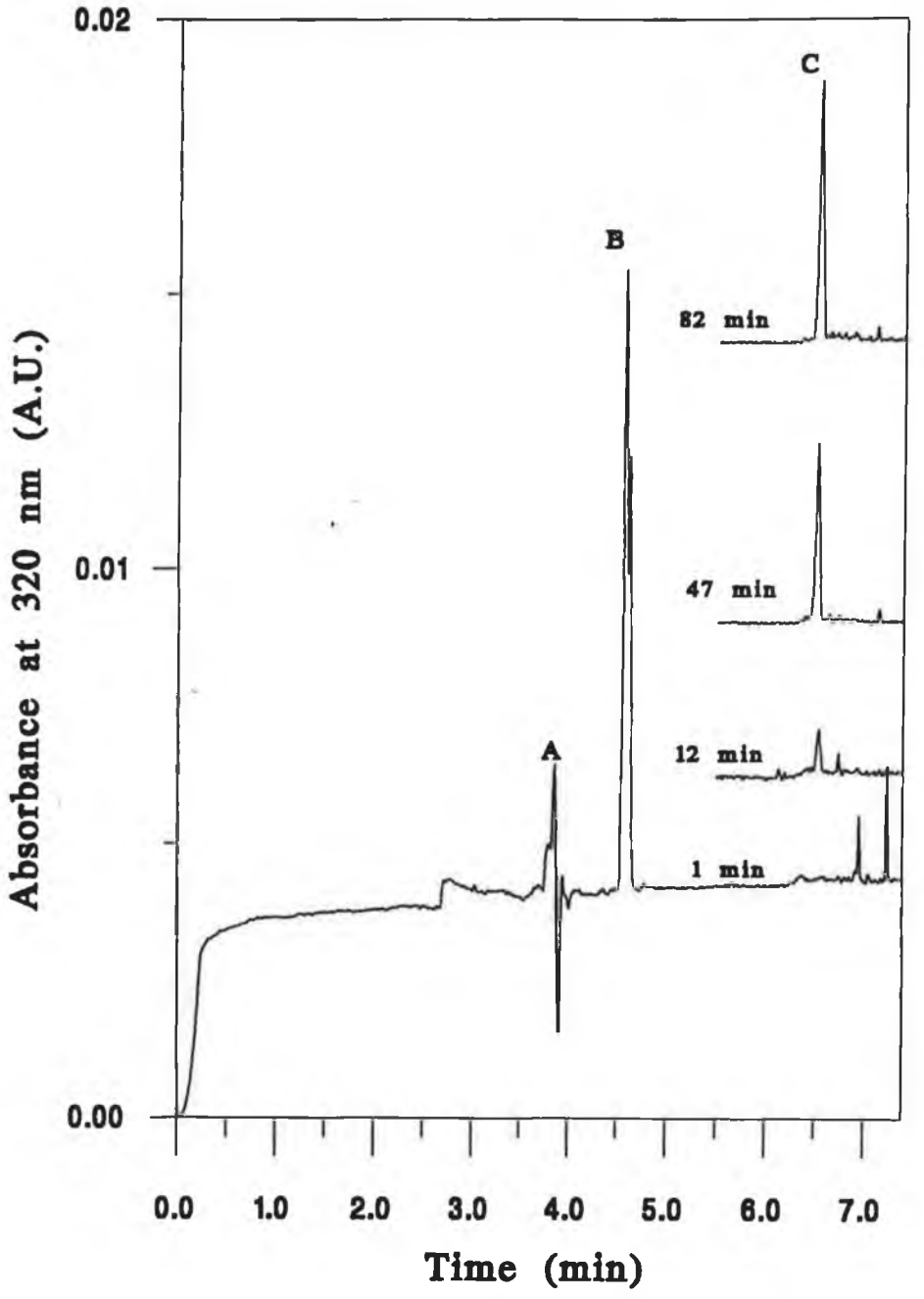


Fig. 5.7. CE electropherograms from the in vitro glucuronidation of 7-hydroxycoumarin to 7-hydroxycoumarin-glucuronide by bovine UDP-glucuronyl transferase. Electropherograms at 12, 47 and 82 min have been offset to show the increase in 7-hydroxycoumarin-glucuronide peak (C) at 6.5 min. All electropherograms showed typical peaks of neutral reaction components (peak A) and a magnesium chloride peak (B).

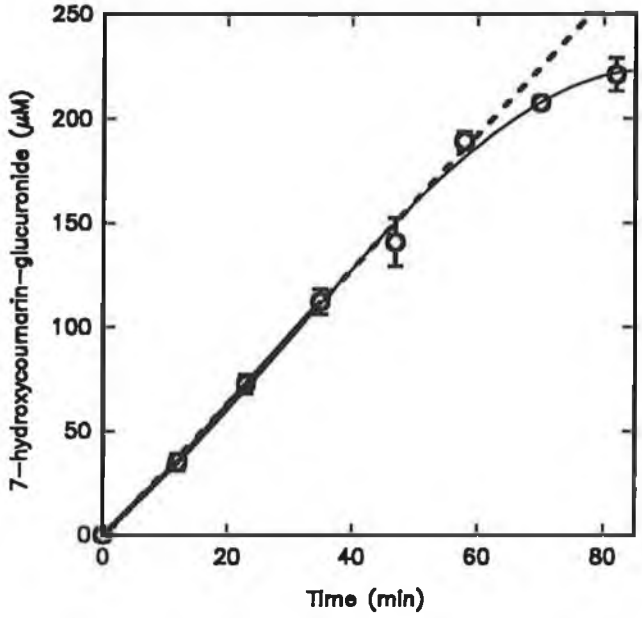


Figure 5.8. <u>In vitro</u> production of 7-hydroxycoumarin-glucuronide from the enzymatic conjugation of 7-hydroxycoumarin to glucuronic acid via bovine liver UDP glucuronyl transferase as analysed by CE. The initial rate of product formation was linear between 0 and 35 min and was estimated to be approximately 3.23 μM/min (r^2=0.995).

5.3 Purification of 7-hydroxycoumarin-glucuronide

5.3.1 Separation of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide by reverse phase column chromatography

Initially, pure samples of both 7-hydroxycoumarin (100 µl of 10 mg/ml) and 7-hydroxycoumarin-glucuronide (100 µl of 1 mg/ml) were analysed using a methanol mobile phase with flow rates of approximately 0.3 ml/min with fractions collected every 2.5 min (Figs. 5.9 A and B). These showed the elution of 7-hydroxycoumarin at between 4 and 8 ml and 7-hydroxycoumarin-glucuronide at about 3 to 6 ml, showing considerable peak overlap. A mobile phase of 700:300:2 water:methanol:acetic acid (v/v/v) resulted in 7-hydroxycoumarin eluting immediately from the column (fractions 1 to 5, and the 7-hydroxycoumarin at fractions 20 to 25 (Fig. 5.10). Using a simple combination of methanol and water and varying the ratio of the two, increasing quality of separation of both components could be achieved, with increasing separation occurring by increasing the ratio of water to methanol, so making the mobile phase more polar (Fig. 5.11 A, B and C.). Good separation was achieved when the ratio of water to methanol was in excess of 40:60 (v/v).

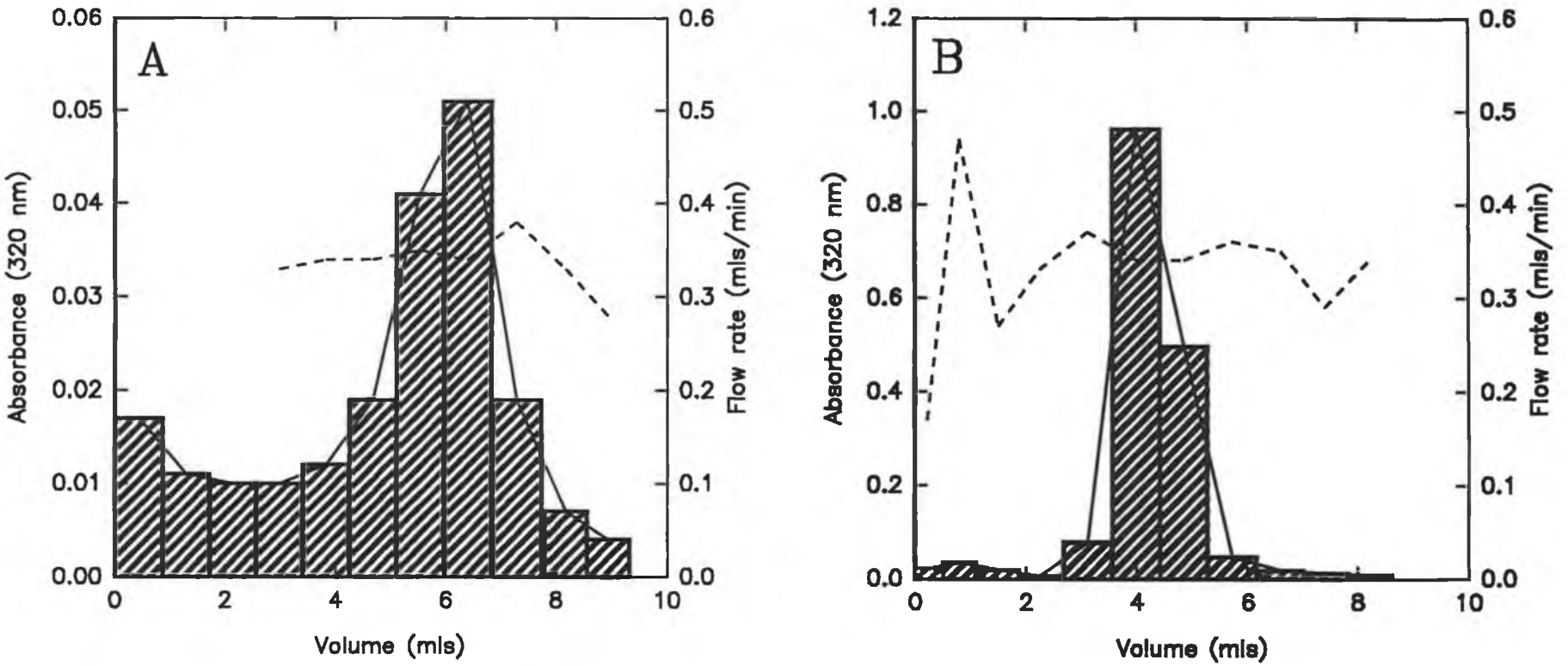


Figure 5.9 A and B. Elution of 100 µl of 10 mg/ml 7-hydroxycoumarin (A) and 100 µl 1 mg/ml 7-hydroxycoumarin-glucuronide (B) from a Lichroprep RP-18 reverse phase silica column in methanol mobile phase at approx. 0.3 ml/min (flow rate shown in dashed line). Fractions were collected over 2.5 min intervals and analysed at 320 nm (hatched box and solid line). 7-hydroxycoumarin eluted primarily in 4 to 8 mls and 7-hydroxycoumarin-glucuronide between 3 and 6 ml.

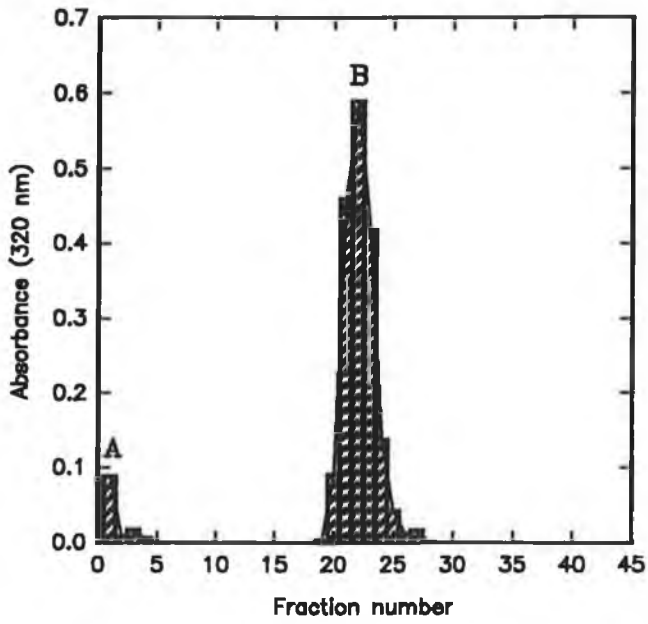


Figure 5.10. Elution of 100 µl 0.5 mg/ml 7-hydroxycoumarin/0.5 mg/ml 7-hydroxycoumarin-glucuronide from a Lichroprep RP-18 reverse phase silica column using a 700:300:2 water:methanol:acetic acid (v/v/v) mobile phase. Fractions were collected over 2.5 min (fractions 1 to 5), 5 min (fractions 7 to 8) and 10 min (fractions 9 to 45) intervals. Fractions were analysed spectrophotometrically at 320 nm (hatched box). 7-hydroxycoumarin-glucuronide eluted immediately from the column (peak A) in fractions 1 to 4 and 7-hydroxycoumarin eluted in fractions 20 to 27 (peak B).

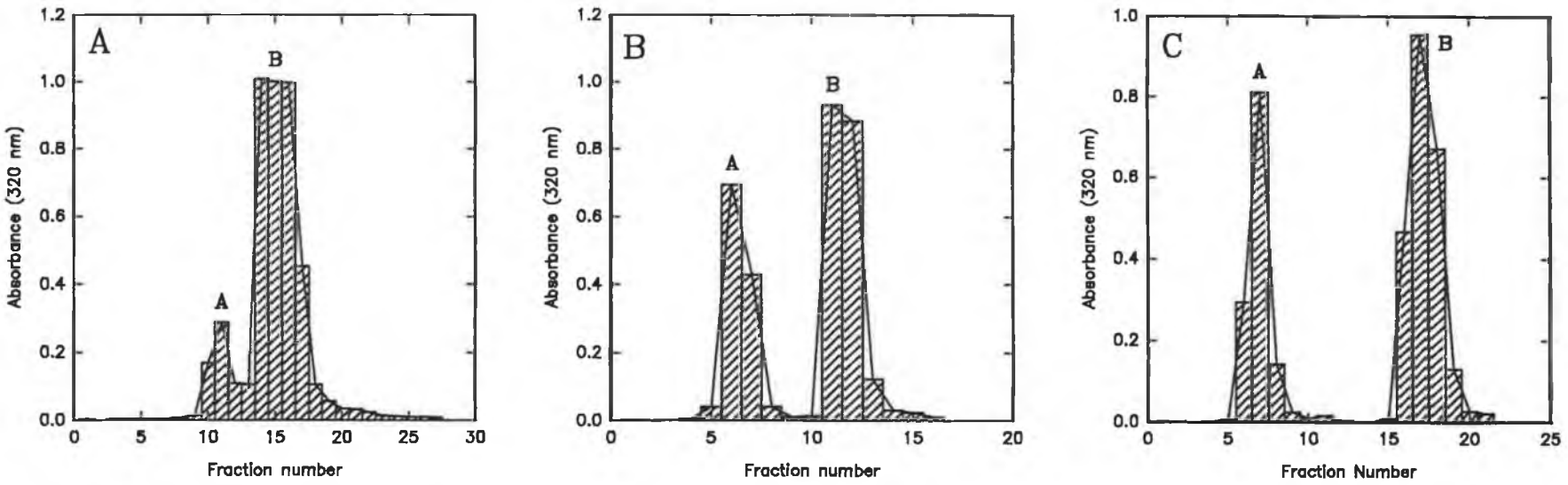
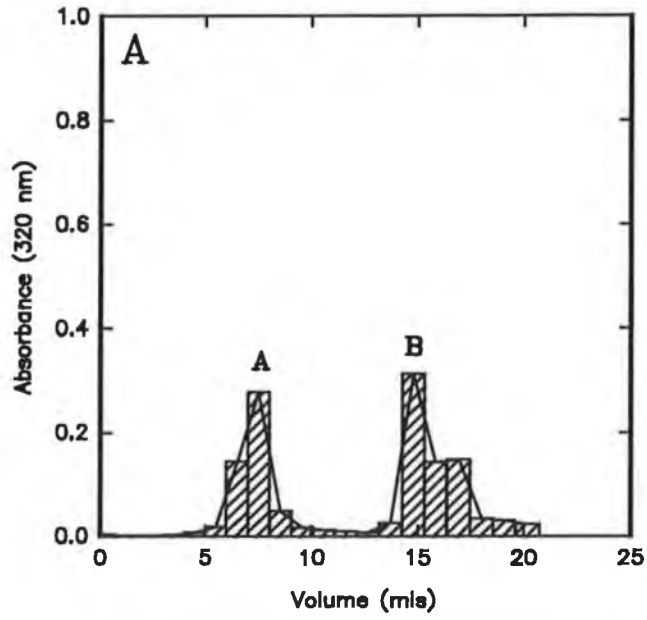


Figure 5.11 A, B and C. Separation of 100 µl 7-hydroxycoumarin/7-hydroxycoumarin-glucuronide (0.5 mg/ml/0.5 mg/ml) on a Lichroprep RP-18 reverse phase silica column in a mobile phase of 80:20 (A), 60:40 (B) and 50:50 (C) methanol:water (v/v). Flow rate was approximately 0.2 ml/min and fractions were collected over 2.5 min intervals and analysed spectrophotometrically at 320 nm. Separation of peaks of 7-hydroxycoumarin-glucuronide (peak A) and 7-hydroxycoumarin (peak B) increased with the increase in the ratio of water to methanol, with good separation being achieved at 50:50 methanol:water (v/v).

5.3.2 Purification of 7-hydroxycoumarin-glucuronide from crude enzymatic reaction mixtures by reverse phase column chromatography

Enzymatic reaction mixtures from the *in vitro* production of 7-hydroxycoumarin-glucuronide were applied to the reverse phase column according to the conditions described in section 2.3.5.2. Increasing volumes of reaction mixture were applied to the column from 100 to 400 µl (Figs. 5.12 A, B, C and D). All showed complete separation of the 7-hydroxycoumarin-glucuronide peaks from the 7-hydroxycoumarin peaks, up to a sample volume of 400 µl, although this did result in the peaks becoming less well resolved from one another.



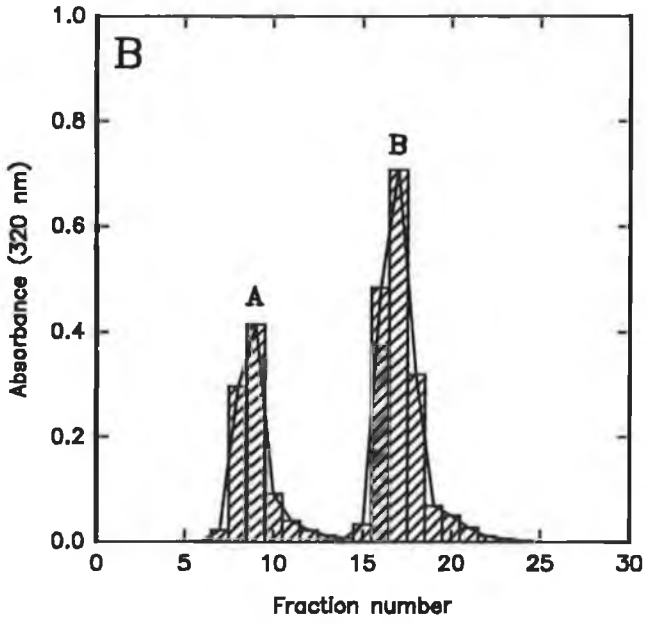


Figure 5.12 A and B. Purification of 7-hydroxycoumarin-glucuronide from 100 µl (A), 200 µl (B) of enzymatic reaction mixture on a Lichroprep RP-18 reverse phase silica column in a 50:50 methanol:water (v/v) mobile phase, with a flow rate of approximately 0.2 ml/min and fractions collected over 5 min intervals and spectrophotometric analysis at 320 nm. Separation of 7-hydroxycoumarin-glucuronide (peak A) and 7-hydroxycoumarin (peak B) was complete at 100 and 200 µl of sample.

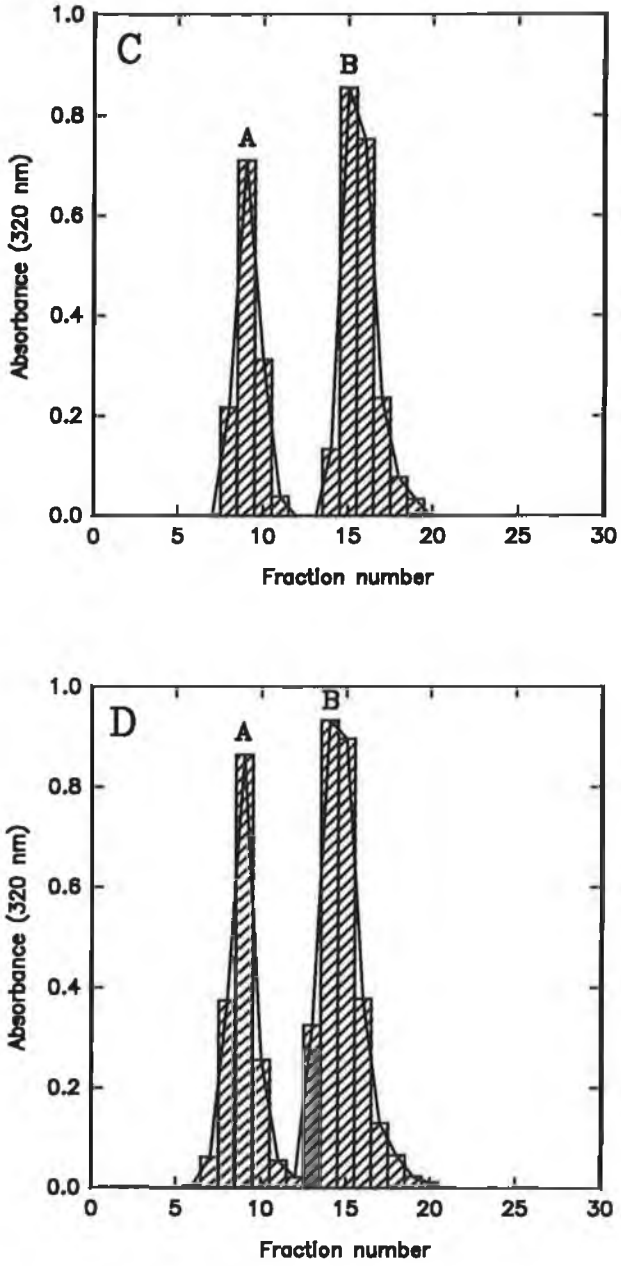


Figure 5.12 C and D. Purification of 7-hydroxycoumarin-glucuronide from 300 µl (C), 400 µl (D) of enzymatic reaction mixture on a Lichroprep RP-18 reverse phase silica column in a 50:50 methanol:water (v/v) mobile phase, with a flow rate of approximately 0.2 ml/min and fractions collected over 5 min intervals and spectrophotometric analysis at 320 nm. Separation of 7-hydroxycoumarin-glucuronide (peak A) and 7-hydroxycoumarin (peak B) was still complete at 300 and 400 µl of sample.

5.3.3 Purification of 7-hydroxycoumarin from coumarin treated patient urine using reverse phase column chromatography

100 µl samples of control urine, control urine spiked with 100 µg/ml 7-hydroxycoumarin-glucuronide and coumarin-treated volunteer urine were applied to the reverse phase column as described in section 2.3.5.2. (Fig. 5.13 A, B and C). All three samples showed the presence of glucuronides, as indicated by the large absorbance at 254 nm. This was present in all samples in elution volumes between 6 and 12 mls. The spiked sample showed an elevation in the 320 nm signal over the control urine. This 320 nm absorbance was much greater in the sample of coumarin-treated urine and is indicative of the presence of 7-hydroxycoumarin-glucuronide. No other significant peaks were noted in the urine samples tested.

5.3.4 Extraction of 7-hydroxycoumarin-glucuronide from urine in dichloromethane (DCM):methanol

The absorbance of the extracted samples of treated urine at 320 nm are shown in Fig. 5.14. This showed a clear increase in the 320 nm absorbance at a DCM:methanol ratio of 70:30 (v/v), indicating the highest extraction efficiency for 7-hydroxycoumarin-glucuronide from urine.

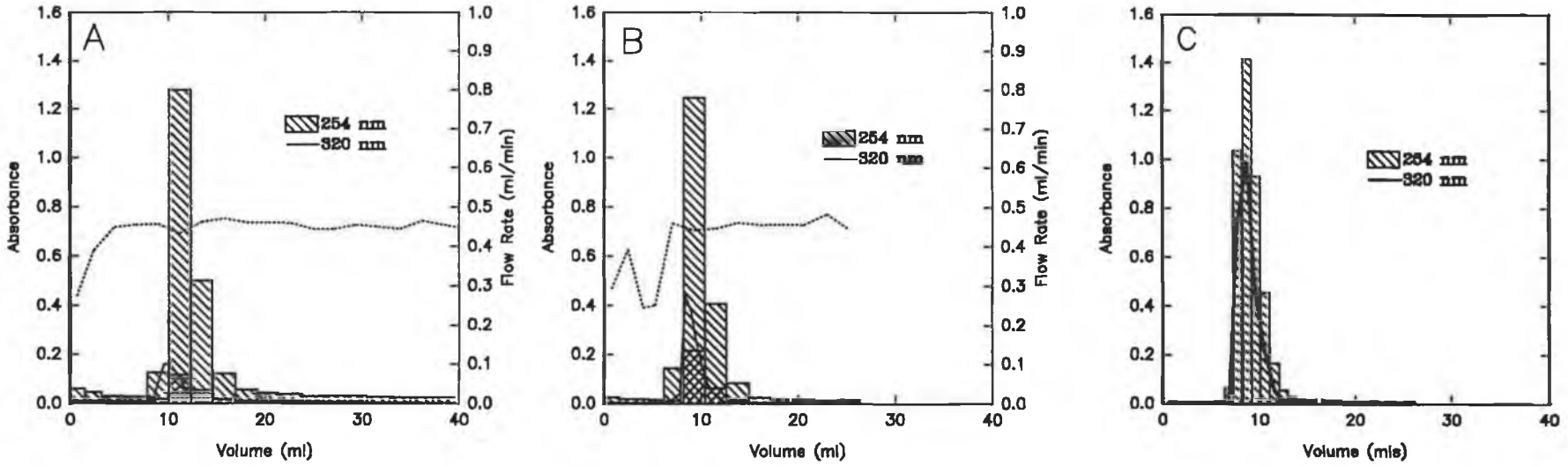


Figure 5.13 A, B and C. Purification of 7-hydroxycoumarin-glucuronide by reverse phase column chromatography from control (A), spiked (B) and treated (C) urine. Flow rate: (dotted line). A shows the presence of quantities of glucuronides in the urine, as observed by the absorbance at 254 nm (hatched bars). When spiked with 7-hydroxycoumarin-glucuronide (B), an increase in the 320 nm absorbance was observed at the same place in which the glucuronides were eluting (solid line). C clearly shows a further elevation in the ratio of 320/254 nm absorbance from the treated urine sample, indicating the presence of 7-hydroxycoumarin-glucuronide in association with other glucuronides in the urine.

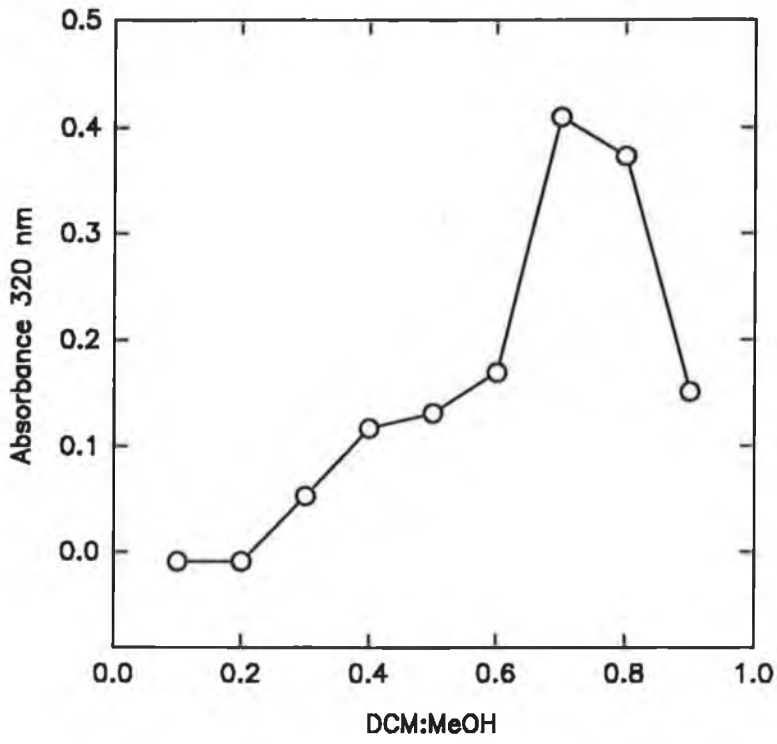


Figure 5.14. Extraction of 7-hydroxycoumarin-glucuronide into different proportions of DCM:methanol. Samples were analysed by HPLC. This shows that the greatest 320 nm absorbance was found in samples extracted with an approximate ratio of DCM:methanol of 70:30 (v/v).

5.3.5 Purification of 7-hydroxycoumarin-glucuronide from coumarin treated patient urine and enzymatic reaction mixtures by reverse phase chromatography following extraction in dichloromethane (DCM):methanol (70:30 v/v)

The chromatograms of the extracted enzymatic reaction mixtures are shown in Figs. 5.15 A and B. In both chromatograms, where 100 µl (5.15 A) and 400 µl (5.15 B) of extracted enzymatic sample were used, it would appear that significant concentration of 7-hydroxycoumarin taken place. However, the ratio of 7-hydroxycoumarin-glucuronide to 7-hydroxycoumarin had clearly decreased, indicating inefficient extraction of the conjugated 7-hydroxycoumarin. This methodology showed a decreased efficiency over the direct application of enzymatic reaction mixture to the column.

Chromatography of the extracted treated urine sample also showed a decrease in efficiency as compared with unextracted samples applied directly to the reverse phase column, indicating significant losses of 7-hydroxycoumarin-glucuronide during the extraction procedure (Fig. 5.16).

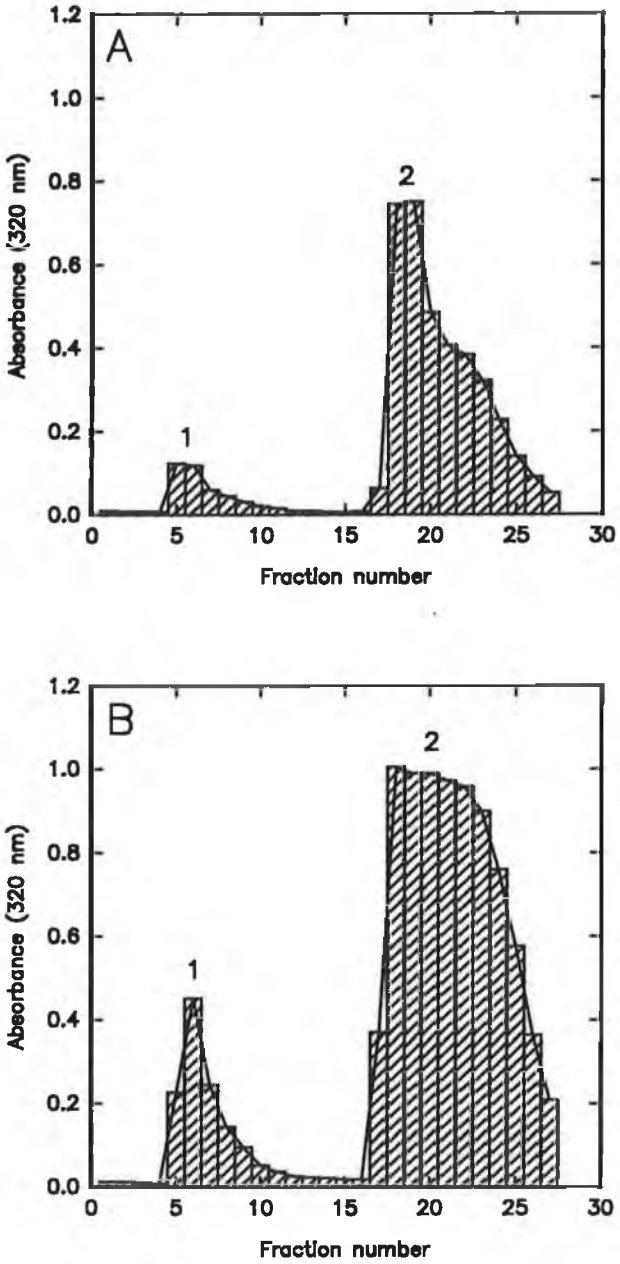


Figure 5.15 A and B. Purification of 7-hydroxycoumarin-glucuronide from crude enzymatic mixtures by reverse phase column chromatography following extraction in DCM:methanol. Application of both 100 µl of sample (A) and 400 µl (B) showed the separation of 7-hydroxycoumarin-glucuronide (peak 1) from 7-hydroxycoumarin (peak 2). However, both samples showed significant enrichment of the 7-hydroxycoumarin over its glucuronide, with no real increase in the concentration of 7-hydroxycoumarin-glucuronide as compared with previous, unextracted samples.

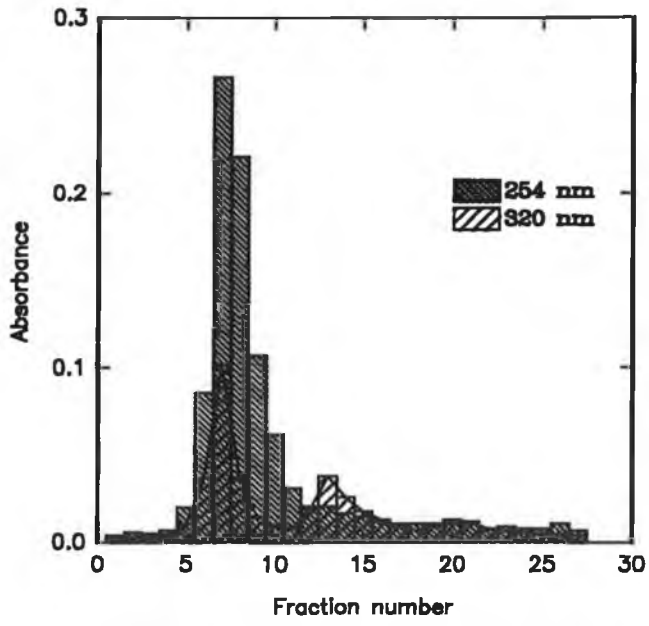


Figure 5.16. Chromatographic separation of extracted treated urine sample. This shows that 7-hydroxycoumarin-glucuronide predominated in the sample, but some 7-hydroxycoumarin had also become extracted and concentrated in the sample (peaking in fraction 13). The overall yield was also relatively low with maximum absorbances at 320 nm less than 0.3.

5.4 Characterisation of 7-hydroxycoumarin-glucuronide

5.4.1 Thermal stability of 7-hydroxycoumarin-glucuronide at 60°C

The peak area for samples of 7-hydroxycoumarin-glucuronide over time when incubated at 60°C is shown in Fig. 5.17. No apparent decrease in peak area was noted after 80 min incubation indicating good thermal stability of the compound at 60°C.

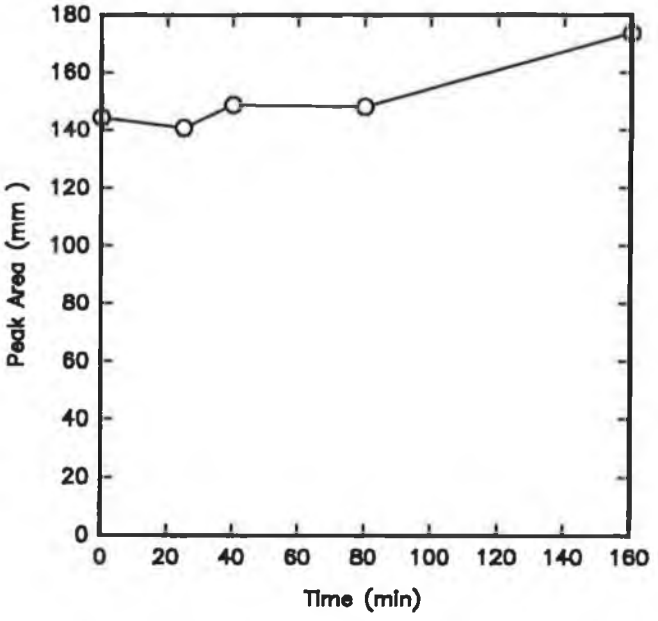


Figure 5.17. Analysis of 7-hydroxycoumarin-glucuronide by HPLC during incubation at 60°C over time. No reduction in 7-hydroxycoumarin-glucuronide peak size were observed over the 160 min incubation period.

5.4.2 Stability of 7-hydroxycoumarin-glucuronide with respect to pH

The peak height for samples of 7-hydroxycoumarin-glucuronide in aqueous solvent at different pH is shown in Fig. 5.18. Samples showed good stability at pH 7 and below, but began to decrease above pH 7 indicating instability in basic environments. However, degradation of the sample only became significant above pH 10.

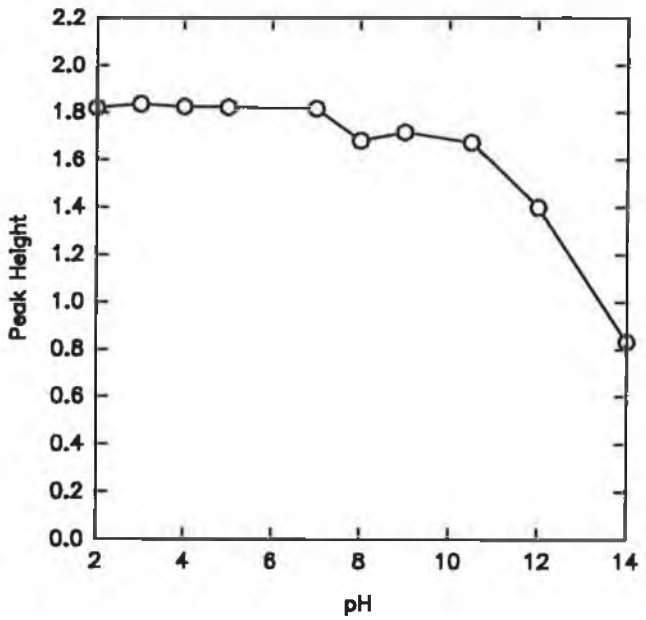


Figure 5.18. Analysis of 7-hydroxycoumarin-glucuronide by HPLC following preparation in different pH buffers. From pH 2 to 7, no reduction in peak is observed. However, above pH 7, significant decreases in the peak size of 7-hydroxycoumarin-glucuronide were observed. This became substantial at pH in excess of 10.

5.5 *In vitro* enzymatic production of 3-amino-7-hydroxycoumarin-glucuronide

Production of a 3-amino derivative of 7-hydroxycoumarin-glucuronide was investigated for its potential use in the conjugation of 7-hydroxycoumarin-glucuronide to proteins. This first required the production of 3-amino-7-hydroxycoumarin from the precursor 3-acetylamino-7-acetocoumarin, followed by the enzymatic glucuronidation of the 3-amino-7-hydroxycoumarin.

5.5.1 Production of 3-amino-7-hydroxycoumarin

The infrared spectrophotometric scan of 3-amino-7-hydroxycoumarin is shown in Fig. 5.19. This compares exactly with equivalent IR scans by Bogan (1996), indicating the production of 3-amino-7-hydroxycoumarin from the 3-acetylamino-7-acetocoumarin derivative. HPLC analysis shows the synthesised compound to be mostly 3-amino-7-hydroxycoumarin, with a small amount of unreacted 3-acetylamino-7-acetocoumarin (Fig. 5.20).

5.5.2 In vitro enzymatic production of 3-amino-7-hydroxycoumarin-glucuronide

Components in the production of 3-amino-7-hydroxycoumarin-glucuronide were analysed by HPLC according to section 2.3.4.2 using dual channel spectrophotometric monitoring at 320 and 254 nm as well as photodiode array analysis between 200 and 400 nm.

Fig. 5.21 shows the chromatogram and the photodiode array scan for 7-hydroxycoumarin-glucuronide. Worthy of note is the retention time of 3.33 min and the absorbance maxima at 320 nm, with a peak rising to 200 nm. When analysed by HPLC and PDA analysis, 3-acetylamino-7-acetocoumarin (R_t= 5.45 min) and 3-amino-7-hydroxycoumarin (R_t=4.32 min) both contained the coumarin absorbance maxima at 320 nm (Fig. 5.22). However, 3-acetylamino-7-acetocoumarin possessed a UV absorbance peak rising to 200 nm, whereas 3-amino-7-hydroxycoumarin showed a dip at 200 nm. When the enzymatic reaction mixture was analysed by HPLC, 5 distinct components could be identified. Two of these at 4.3 and 5.42 min could be clearly identified as the original components of the

mixture, 3-amino--hydroxycoumarin and 3-acetylamino-7-acetocoumarin (Fig. 5.23). Three new components, all with greater polarity than the parent compounds were also present. Two of them closely resembled one of the parental compounds with respect to their photodiode array scan, with the peak at 2.7 min resembling 3-amino-7-hydroxycoumarin and the peak at 2.9 min resembling 3-acetylamino-7-acetocoumarin. A third peak at 3.6 min also possessed the characteristic absorbance peak at 320 nm typical of coumarins. Its UV absorbance ridge, however, did not resemble the other components in the mixture. No further identification of these components was performed.

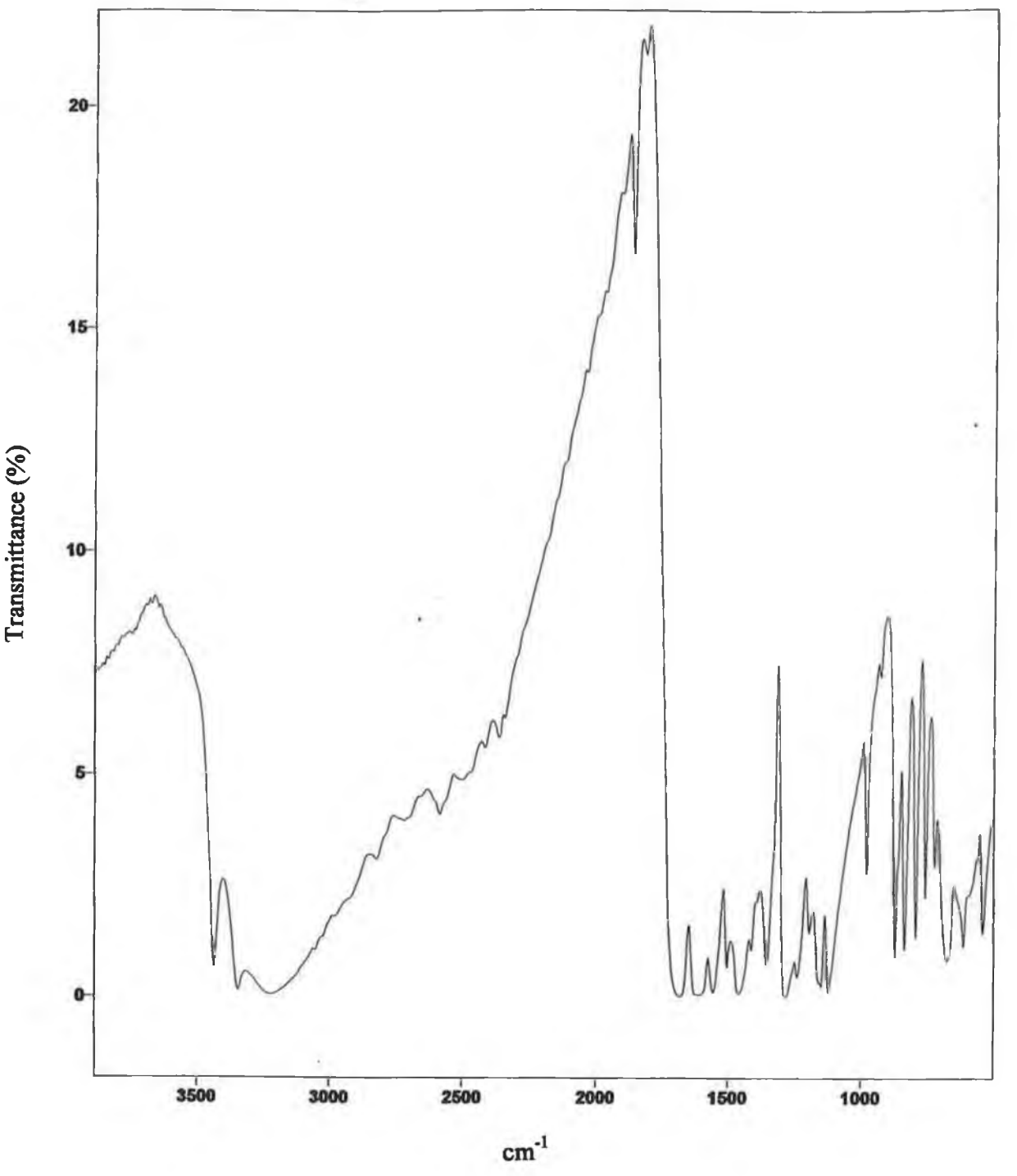


Figure 5.19. Infrared spectra of 3-amino-7-hydroxycoumarin. This shows a spectra typical of that expected for 3-amino-7-hydroxycoumarin, according to Bogan (1994).

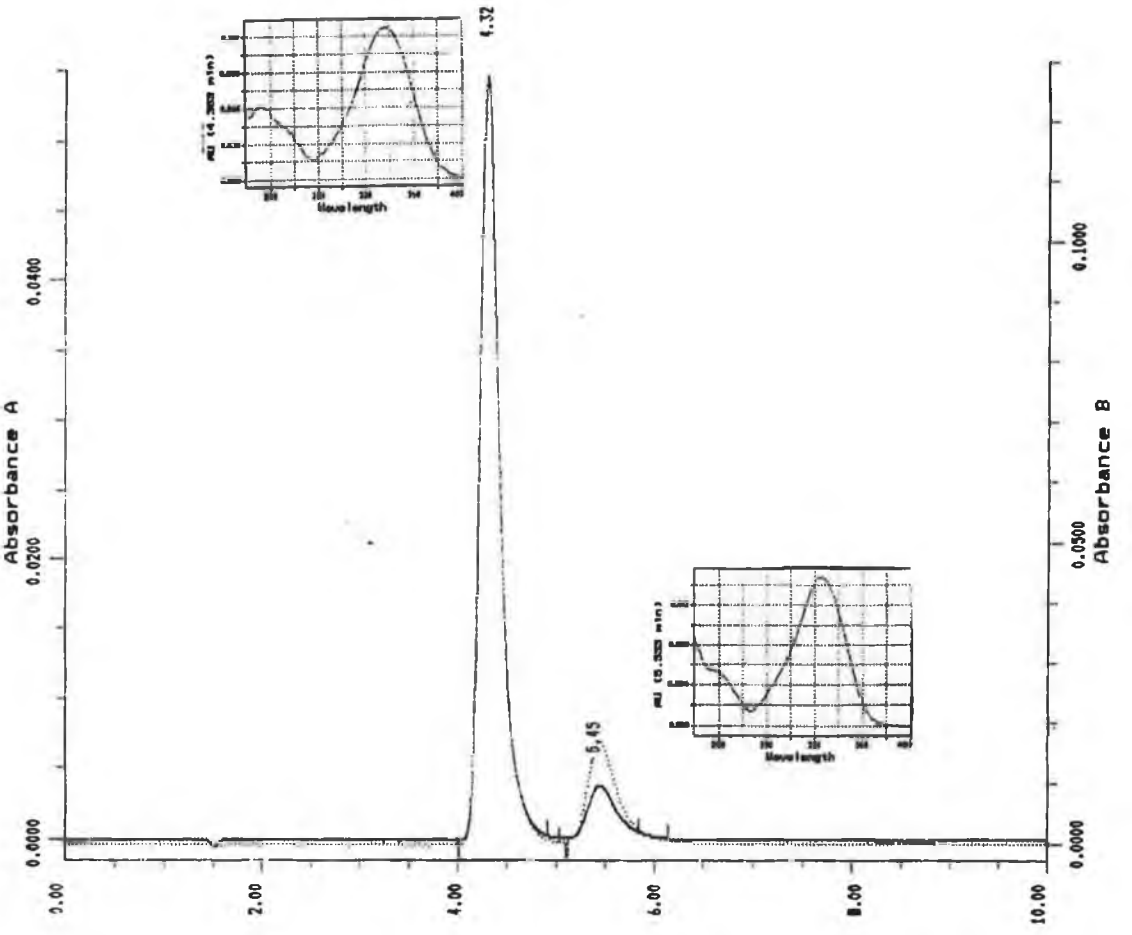


Figure 5.20. HPLC and photodiode array analysis of 3-amino-7-hydroxycoumarin. Absorbance at 254 nm (solid line). Absorbance at 320 nm (dotted line). Two peaks were present in the sample. The reaction product 3-amino-7-hydroxycoumarin at 4.32 min predominated in the sample with the presence of a smaller quantity of unreacted 3-acetylamino-7-acetocoumarin (5.45 min) also present. These showed typical PDA scans, both with absorbances at the 320 nm region, with one showing a rise in absorbance to 200 nm, the other showing a decrease in the same region.

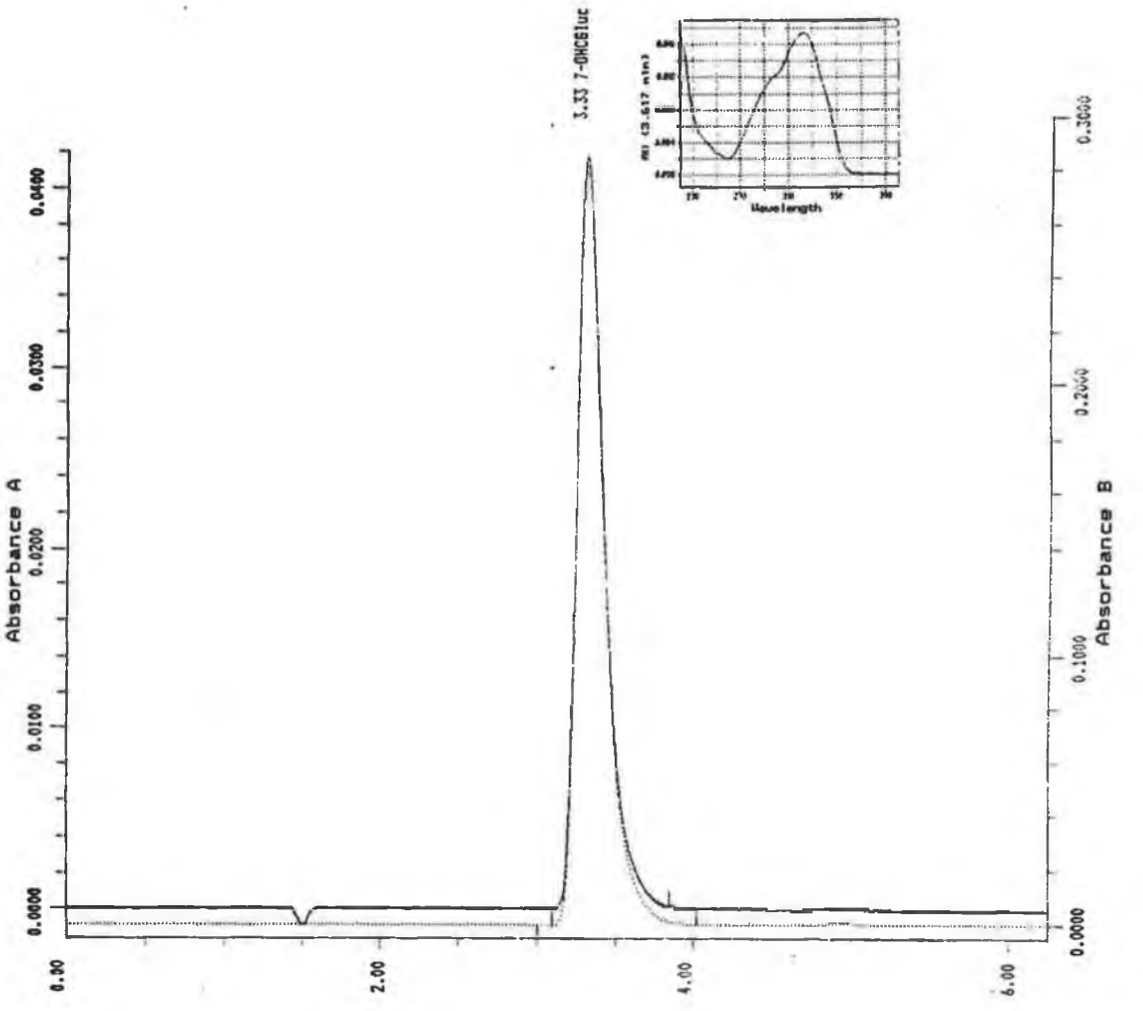


Figure 5.21. HPLC and photodiode array analysis of 7-hydroxycoumarin-glucuronide. Absorbance at 254 nm (solid line). Absorbance at 320 nm (dotted line). A single peak of 7-hydroxycoumarin-glucuronide was obtained at 3.33 min. This had the typical PDA scan as shown, with a peak at 320 nm, a ridge at 280 nm, and a rise up to 200 nm.

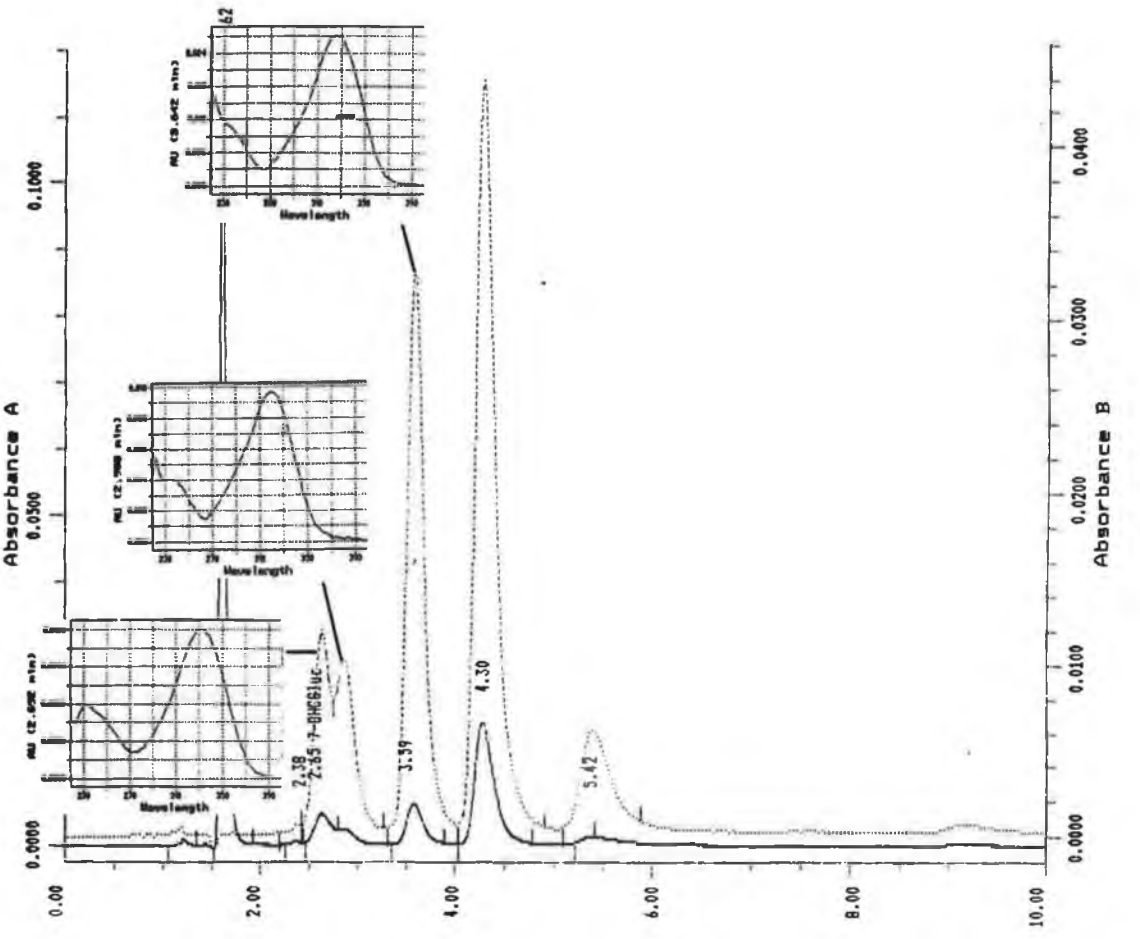


Figure 5.22. HPLC and photodiode array analysis of the <u>in vitro</u> metabolic production of 3-amino-7-hydroxycoumarin-glucuronide. Absorbance 254 nm (solid line). Absorbance at 320 nm (dashed line). Five components were present in the reaction mixture. Two at 4.20 and 5.42 min were the original reaction components, 3-amino-7-hydroxycoumarin and 3-acetylamino-7-acetocoumarin, respectively. Three new components, all showing decreased hydrophobicity were produced at 2.69, 2.99 and 3.59 min. The PDA scan of peak 2.69 min resembled 3-amino-7-hydroxycoumarin, whilst peak at 2.9 min resembled that of 3-acetyl-7-acetocoumarin. The peak at 3.59 min did not particularly resemble either of the starting components.

5.6 Production and analysis of a 7-hydroxycoumarin-glucuronide-bovine serum albumin conjugate prepared by EDC/NHS coupling chemistry

The 7-hydroxycoumarin-glucuronide-bovine serum albumin conjugate was analysed by SDS-PAGE (Fig. 5.23). This indicated the presence of a protein component of approximately 66 kDa, corresponding to bovine serum albumin. When analysed by size exclusion chromatography and photodiode array analysis (Fig. 5.24), this showed a predominant protein peak at 21.9 min. Concomitant with the protein absorbance maxima at approximately 280 nm was a second absorbance maxima at 320 nm indicating the presence of a coumarin moiety attached to the protein component. When compared to the spectrophotometric scan of bovine serum albumin alone, no such absorbance maximum could be seen at 320 nm.

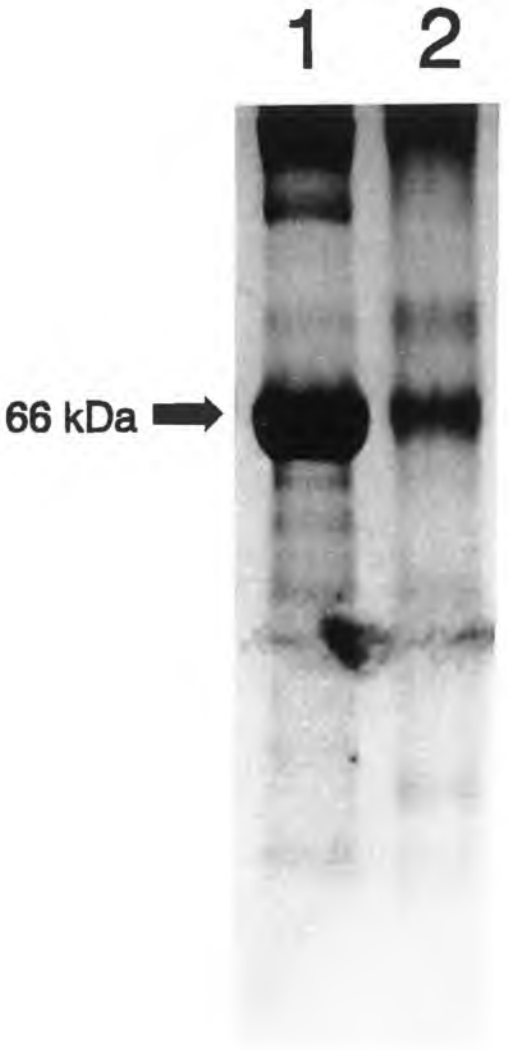


Figure 5.23. Analysis of bovine serum albumin and 7-hydroxycoumarin-bovine serum albumin conjugate by SDS-PAGE. Lanes: 1: bovine serum albumin; 2: 7-hydroxycoumarin-bovine serum albumin. The gel shows that both samples contained protein with a molecular weight of approximately 66 kDa, corresponding to the molecular weight of bovine serum albumin.

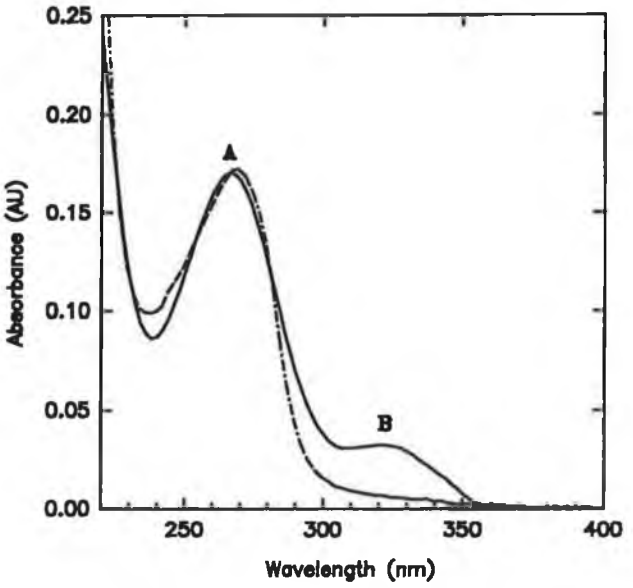


Figure 5.25. Photodiode array scans of 7-hydroxycoumarin-glucuronide-bovine serum albumin conjugate (solid line) and bovine serum albumin control (dot-dash line). Both samples exhibited the typical 260 nm absorbance peak indicating the presence of a protein (peak A). However, the 7-hydroxycoumarin-glucuronide-bovine serum albumin conjugate exhibited a second peak at 320 nm (peak B), indicating the presence of a coumarin-type molecule attached to the protein. This was not present in the bovine serum albumin control sample.

DISCUSSION

The purpose of studying the production, analysis, purification and conjugation of 7-hydroxycoumarin-glucuronide were outlined in section 1.14.

The study of the *in vitro* metabolism of 7-hydroxycoumarin-glucuronide served several purposes. The need for information on the pharmacokinetics of 7-hydroxycoumarin are important in its use as a therapy in several conditions such as high protein lymphoedema, brucellosis and experimental cancer therapies. Variations in phase II metabolism of the drug may relate to differences in the efficiency of the treatment regime. Inadequacies in phase II metabolism may also relate to sporadic cases of hepatotoxicity during such therapies. Studying individual differences in their ability to metabolise 7-hydroxycoumarin in liver samples such as liver biopsies may further confirm *in vivo* analyses, pointing to deficiencies in phase II metabolism in the liver. Such procedures have been used in the phase I metabolism of coumarin (Deasy, *et al.*, 1995). Interspecies studies have also served to elucidate the metabolic pathways of different organisms, leading to a fuller understanding of the development of these pathways. Studies of *in vitro* metabolism in different organs has also yielded information about the sites that are important in the drug's metabolism, and consequently its distribution in the organism. *In vitro* metabolism is also a source of the glycone for purification and use in other methodologies, such as in standards, and its application in the development of a conjugate for the production of antibodies to the glucuronide. Such antibodies would themselves be applied in various studies of phase II metabolism.

5.8 *In vitro* production of 7-hydroxycoumarin-glucuronide and analysis by HPLC

For the *in vitro* production and analysis of 7-hydroxycoumarin-glucuronide, an effective enzymatic reaction had to be developed as well as a method or methods of analysis of the reaction product formed. Tegtmeier *et al.* (1988) and Brunner and Tegtmeier (1984) had developed methods of *in vitro* glucuronidation using hollow fibre membrane systems for the biochemical detoxification of lypophilic compounds in which suitable reaction conditions for porcine UDP-glucuronyltransferases had been elucidated. Egan and

O'Kennedy (1994) had also developed HPLC conditions capable of the direct detection and resolution of 7-hydroxycoumarin and coumarin. These two methodologies were used as starting points for the analysis of the *in vitro* metabolism of 7-hydroxycoumarin by porcine UDP-glucuronyltransferase enzymes isolated from liver. HPLC analysis showed that the 7-hydroxycoumarin substrate and 7-hydroxycoumarin-glucuronide could be separated using this methodology. It also illustrated very clearly that all the reaction components (substrates and enzyme) were necessary to detect formation of the glucuronide product. Analysis of initial rates of product production (v_0) showed the rate to be very slow at 3.3×10^{-3} μM/min/mg under the initial reaction conditions used.

In subsequent experimentation, changes were made to both the enzymatic reaction mixture and the analytical methodology employed. With respect to the reaction mixture, UDP-glucuronyltransferase enzyme was isolated from bovine liver. The other notable alteration was the reduction of the 7-hydroxycoumarin substrate concentration. The analytical methodology also underwent some notable changes. The separation methodology used a gradient system. This was done primarily to increase the retention time of the glucuronide species, bringing it further from the void volume, while still giving good resolution of all components. Calculation of 7-hydroxycoumarin-glucuronide concentration was determined by ratio analysis against an internal standard, 4-hydroxycoumarin, which was also effectively resolved by the methodology applied. As a result v_0 of the bovine enzyme was found to be 4.13 μM/min (1.03 μM/min/mg protein). This was an increase in over 300-fold specific reaction rate over the initial reaction. It is not clear whether this is due primarily to the change in the source of liver enzyme, or due to substrate inhibition in the original reaction mixture due to the very high concentration of 7-hydroxycoumarin employed.

With respect to the HPLC methodologies used, no direct comparison can be made between the two methodologies with regard to precision, since the initial experiment was not performed with replicates. However, internal standardisation has been shown to be superior in terms of precision over direct measurements (Christian, 1986), so it might be assumed that this was also the case here. The standard errors of the standards used for analysis were all below 8% and typically below 5%, indicating acceptable precision. The

analytical methodology was, however, slower than the original chromatographic separation, requiring 25 min, as opposed to approximately 16 min for the original isocratic separation. However, due to sample stability and automation of sampling, this was not an inconvenience.

In terms of the overall yields of these methodologies for the production of 7-hydroxycoumrin-glucuronide, the initial methodology produced just less than 0.1 μg of conjugate in the 23 h reaction period, compared to nearly 0.5 μg for the second reaction within 2.5 h. Thus, the enzymatic methodologies employed were shown to be capable of yielding microgram quantities of 7-hydroxycoumarin-glucuronide.

5.9 *In vitro* production of 7-hydroxycoumarin-glucuronide and analysis by CE

The technique of capillary electrophoresis was applied to the analysis of the enzymatic reaction mixture. As has been discussed, CE offers rapid separations of reaction components based on their electrophoretic mobility in certain ionic solvents in high electrical potentials. A method had been developed by Bogan *et al.* (1996) for the analysis of 7-hydroxycoumarin-glucuronide in urine samples. This methodology was applied to the analysis of 7-hydroxycoumarin-glucuronide in crude enzymatic reaction mixtures. Crucially, treatment of the reaction mixtures for CE analysis with trichloroacetic acid was not appropriate and was not required. Changes in pH brought about by such treatments would significantly alter the separation characteristics of the assay. However, electrophoresis brings about instantaneous separation of the reaction components, at which point, the reaction ceases. To perform this analysis then, samples must be taken and analysed immediately. This is not a difficulty, since separation takes just 7.5 min, a periodicity quite convenient for regular sampling of the enzymatic reaction mixture. Use of this methodology for the analysis of enzymatic reaction mixtures demonstrated the resolution of 7-hydroxycoumarin-glucuronide from other reaction components.

The initial rate of reaction in the CE study was comparable to the HPLC study using bovine liver UDP-glucuronyltransferase, with a v_0 of 3.23 μM/min, but with a specific activity of 2.02 μM/min/mg protein, yielding only 0.12 μg of 7-hydroxycoumarin-

glucuronide in the 82 min incubation, with the rate of reaction dropping more rapidly than in the study which used HPLC. The reasons for these discrepancies could be several fold. Mostly, they might relate to the quality of the liver material used, its treatment and preparation and the yield of UDP-glucuronyltransferase enzyme protein in the sample. This does illustrate a very important consideration when extrapolating data from such experiments to the actual *in vivo* enzyme reaction rates of intact whole organs. It is probable that specific activities of *in vitro* prepared enzyme mixtures are lower than that in whole, in tact tissues. However, no significant studies comparing *in vivo* and *in vitro* metabolic rates have been performed to illustrate this.

The establishment of an *in vitro* metabolic reaction mixture for the conversion of 7-hydroxycoumarin to its glucuronide, accompanied by two reliable analytical methodologies by which to analyse the reaction products yielded a system that could be used to probe the phase II metabolic characteristics of different organs and species with respect to 7-hydroxycoumarin. These methodologies have been successfully exploited by Bogan *et al.* (1997) and Duffy *et al.* (1997) for the analysis of phase II metabolism of 7-hydroxycoumarin in rabbit tissues. It also resulted in a third source of 7-hydroxycoumarin-glucuronide, in addition to chemical synthesis and *in vivo* isolation from treated urine.

5.10 Purification of 7-hydroxycoumarin-glucuronide

Two metabolic sources of 7-hydroxycoumarin-glucuronide were available. Firstly, the enzymatic synthesis methodologies outlined in section 5.2, and secondly, 7-hydroxycoumarin-glucuronide from treated volunteer urine samples.

In the case of the enzymatic reaction mixtures, 7-hydroxycoumarin-glucuronide was in the presence of quantities of 7-hydroxycoumarin, coumarin and protein material. If 7-hydroxycoumarin-glucuronide from such a source was to have any application as a standard, or for the production of conjugates, it must first be separated from these other components.

Since 7-hydroxycoumarin-glucuronide was successfully separated from 7-hydroxycoumarin and coumarin using reverse phase chromatography by HPLC, it was assumed that such a reverse phase system might be applicable to the column chromatographic preparative-scale separation of these components also.

A reverse phase column with C-18 packing was prepared (section 2.3.5.1). Initially, pure samples of both 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide were applied to the column in a methanol mobile phase. These experiments helped to establish the separation methodologies applied later, such as pump speeds, flow rates, collection intervals etc. Initially, both samples were shown to elute within the first 10 ml of mobile phase, so rapid purification times could be established. However, in the mobile phase used, inadequate differences in retentions of the two components were found.

The conditions of the mobile phase in the initial isocratic HPLC separation were applied to the preparative column. In this case, 7-hydroxycoumarin-glucuronide and 7-hydroxycouamarin were seen to separate very well. However, 7-hydroxycoumarin-glucuronide eluted in the void volume and could not be successfully fractionated from the column. Under these conditions, the mobile phase was too polar to bring about adequate retardation of the water soluble glucuronide on the column. The coumarin contaminant of 7-hydroxycoumarin was not important in these separations because either its concentration was too small to be noted in chromatograms, or it coeluted with 7-hydroxycoumarin under the conditions applied.

A more simple mobile phase of methanol:water was applied to the separation. It was found that with higher methanol concentrations, 7-hydroxycoumarin-glucuronide was significantly retarded on the column. Decreases in methanol concentration further retarded 7-hydroxycoumarin on the column. At 50:50 methanol:water (v/v), good separation and retention of both components could be established. Good separation also occurred at 60:40 methanol:water (v/v).

Having established separations for pure mixtures of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide, the methodology was applied to the separation of crude

reaction mixtures. A range of volumes of the reaction mixture were applied to the column up to 400 µl, which brought about concomitant increases in the peaks of both 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide. The two compounds were still resolvable with 400 µl sample volumes.

The second readily available source of 7-hydroxycoumarin-glucuronide was the administration of coumarin to a healthy human volunteer and harvesting of the urine samples subsequent to this. Studies of the pharmacokinetics of coumarin metabolism in humans has shown its rapid excretion as 7-hydroxycoumarin-glucuronide in the urine, which takes place within 24 h, with maximum excretion approximately 2 h post-administration (Pelkonen *et al.*, 1997). Thus, urine from control and treated healthy volunteers was taken, the latter 2 h after administration of 1 g p.o. coumarin in 10 x 100 mg tablets. These were separated by the same analytical technique as the enzymatic mixtures. In this case, no 7-hydroxycoumarin peak was noted. In the control urine, a significant 254 nm absorbance peak was noted in the same position as would be found 7-hydroxycoumarin-glucuronide, indicating the presence of glucuronides, but with a low 320 nm absorbance, suggesting non-coumarin-derived glucuronides. When the control urine was spiked with 7-hydroxycoumarin-glucuronide, the 320 nm signal showed an increase at the same position as the 254 nm glucuronide peak. This suggested that natural glucuronides present in the urine were coeluting with 7-hydroxycoumarin-glucuronide. When treated urine was separated, a larger 254/320 nm peak also eluted at the same position as in the control urine sample. The higher 320/254 nm ratio also indicated that this was, indeed, the position at which 7-hydroxycoumarin-glucuronide was eluting.

As has been stated, many natural metabolic waste products are excreted as glucuronides, notably bilirubin and testosterone, and other hormones (Reid and Leppard, 1983). It is clear from this that the separation technique employed was not applicable for the direct separation of 7-hydroxycoumarin-glucuronide from treated urine samples because it does not separate glycone derivatives of different compounds.

Although simple to analyse directly, water-miscible glucuronide compounds are difficult to purify using liquid/liquid separation methodologies as they are not easily extracted with

organic solvents. The presence of the 7-hydroxycoumarin moiety would, however, endow them with some lipophilicity. An attempt was made to exploit this by the development of an extraction procedure for 7-hydroxycoumarin-glucuronide. This was based on an organic phase composed of dichloromethane (DCM) and methanol. These were chosen because the polar methanol would dissolve in the water-immiscible DCM, but could still dissolve quantities of 7-hydroxycoumarin-glucuronide. Various ratios of DCM:methanol were tested against pure samples of 7-hydroxycoumarin-glucuronide using equal volumes of the organic and inorganic mixtures. The presence of the glucuronide was analysed by the 320 nm absorbance of the dried, rehydrated samples. Maximum 320 nm readings were at a DCM:methanol ratio of 70:30 (v/v).

The extraction procedure was applied to the enzymatic reaction mixtures and urine samples containing 7-hydroxycoumarin glucuronide. As compared with unextracted enzyme mixtures, the yield of 7-hydroxycoumarin-glucuronide decreased significantly, whereas the level of 7-hydroxycoumarin in these samples was significantly increased. In urine, the yield of glucuronide in the sample had also decreased substantially, although the glucuronide that was present did show a high 320 nm absorbance. This may indicate that the extraction methodology was more efficient at extracting 7-hydroxycoumarin-glucuronide than other glucuronides present in the urine.

Overall, employment of an extraction methodology resulted in inefficient extraction of the 7-hydroxycoumarin-glucuronide, both from enzyme reaction mixtures and from samples. The availability of large volumes of urine, however, may allow for an improvement in the extraction methodology. Using large volumes of urine and small volumes of organic mixture may increase the partitioning of significant quantities of 7-hydroxycoumarin-glucuronide into the organic phase. This may also remove significant quantities of other glucuronides from the sample that do not possess lipophilic functional groups.

When extracted, separated samples from treated urine were analysed by HPLC, 7-hydroxycoumarin-glucuronide was shown to be present in significant amounts with only minimal contaminants present and was considered acceptable for use in further experimental procedures.

5.11 Physical analysis of 7-hydroxycoumarin-glucuronide

Some physical characteristics of the 7-hydroxycoumarin-glucuronide were analysed by HPLC. The molecule exhibited good thermal stability at 60°C. Of note was the sensitivity of the glucuronide to alkaline pH. Even mildly alkaline conditions brought about degradation of the conjugate, but this was significant above pH 10. This finding is substantially in accordance with other glucuronides which undergo rapid alkaline hydrolysis of the β-D ether linkage (Wilson *et al.*, 1983). Indeed, this lability is of importance when analysing such metabolites in urine. The composition and pH of the urine can vary substantially depending on dietary habits and the administration of other drugs such as antacids which make urine basic and may lead to deconjugation. Also, urine loses carbon dioxide upon standing and becomes alkaline. This results in the precipitation of salts such as inorganic phosphates and may also bring about deconjugation of 7-hydroxycoumarin-glucuronide and precipitation of insoluble 7-hydroxycoumarin leading to inaccurate results. This is important when data derived from such metabolic studies is related to the phase II inter-individual variability in humans and possible associated hepatotoxic effects (Bogan, 1996). Such analyses may benefit by being acidified upon collection.

5.12 Conjugate production

For the production of antibodies to small haptens - as has already been illustrated several times throughout this thesis - it is necessary to conjugate them to large immunogens such as proteins. As has also been demonstrated, coumarin and 7-hydroxycoumarin lack any functional groups that can be readily applied to a conjugation reaction. Production of amino derivatives of coumarin and 7-hydroxycoumarin have been used to produce conjugates for the purpose of antibody production (Egan, 1993; Bogan, 1996).

Of concern is the orientation of the hapten as displayed to the antigen-binding site. It might be thought that orientation of coumarin and 7-hydroxycoumarin in directions that leave the pyrone ring proximal to the antigen-binding site may bring about associated cross-reactivities with the resulting antibodies. For this reason, conjugation of coumarin

was at the 6 position and 7-hydroxycoumarin at the 3 position, resulting in opposing orientations (Egan, 1993; Bogan, 1996). This did yield antibodies that showed no cross-reactivity to either species.

The same concerns were initially applied to the conjugation of 7-hydroxycoumarin-glucuronide to proteins such as bovine serum albumin. The only functional group available for attachment with this molecule is the carboxyl group present on the glucuronic acid moiety. This coupling methodology of drug glucuronides has been successfully used (Abbott Laboratories, 1984). However, conjugation at the glucuronic acid moiety may orient the hapten with the 7-hydroxycoumarin pyrone ring proximal to the antigen-binding site, again leading to concerns of cross-reactivity. To circumvent this, glucuronide conjugates of 7-hydroxycoumarin with functional groups would be desirable such as 3-amino-7-hydroxycoumarin-glucuronide. In this way, the hapten could be oriented with its glucuronic acid component greatly exposed to the antibody, theoretically minimising cross reactivity.

Conjugate synthesis required the production of 3-amino-7-hydroxycoumarin from 3-acetylamino-7-acetocoumarin, previously synthesised by Bogan (1996). Infrared analysis of the product was in accordance with Bogan (1996). HPLC analysis, however, showed that the reaction was not complete and contaminants of 3-acetylamino-7-acetocoumarin remained. This two component mixture was applied to *in vitro* glucuronidation. Identification of the components by HPLC, coupled with photodiode array detection yielded 5 components in the reaction mixture. Two of these were the original components of the sample. Three new derivatives of significance were found in the mixture. These all had lower retention times, so greater polarity than the parent molecules. Two of these possessed photodiode array spectra similar to the two parental species and a third, which also appeared to be a coumarin derivative due to its absorbance at 320 nm, but otherwise, not significantly recognising the parental components.

It was presumed that glucuronidation of the parental components did take place in this mixture and that this resulted in the hydrophillic components produced. However, because no definitive identification of the components could be made without significant further

production, purification and anlysis by a methodology such as nuclear magnetic resonance, no further characterisation of these species was attempted. However, the potential clearly exists for the *in vitro* metabolic production of a glycone derivative of 3-amino-7-hydroxycoumarin.

Since pursuance of the production of a 3-amino- derivative of 7-hydroxycoumarin-glucuronide was found to be prohibitively complex, production of a conjugate of 7-hydroxycoumarin-glucuronide, coupled via the glucuronic acid moiety was pursued, with due regard given to the potential of such a methodology to result in antibodies that would be cross-reactive with either 7-hydroxycoumarin or coumarin.

The well established chemistry of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC)/N-hydroxysuccinimide (NHS) was shown to be applicable to the linkage of glucuronide conjugates to proteins (Abbott Laboratories, 1984). The method adopted for 7-hydroxycoumarin-glucuronide conjugation initially used a high pH of 9.6. Although awareness was given to the instability of 7-hydroxycoumarin-glucuronide at elevated pH, this was most pronounced above pH 10, and a high pH would ensure full dissociation of the carboxyl group of the glucuronic acid for full reactivity. When analysed by photodiode array and compared to controls, the approximately 66 kDa species was shown to possess a 320 nm absorbance peak, indicating attachment of a coumarin moiety to the protein. Since linkage could only be via the glucuronic acid, this suggested attachment of an in tact 7-hydroxycoumarin-glucuronide molecule to bovine serum albumin. This conjugate could be used in the production of antibodies to 7-hydroxycoumarin-glucuronide.

CONCLUSIONS

A method for the *in vitro* glucuronidation of 7-hydroxycoumarin using liver-isolated UDP-glucuronyltransferase was established. This also led to the development of two analytical techniques for the measurement of the enzymatic process based on HPLC and CE. Chromatographic techniques were also developed for the purification of 7-hydroxycoumarin from enzymatic reaction mixtures and urine.

Attempts were made to produce *in vitro* glucuronidated conjugates of 3-amino-7-hydroxycoumarin, but the complex mixtures produced meant that this was not a viable option. Instead, conjugation was performed via the carboxyl group of the glucuronide using EDC/NHS coupling chemistry. This resulted in stable 7-hydroxycoumarin-glucuronide-BSA conjugates with the potential to be used in the production of antibodies, either as polyclonal sera, or the selection and screening of clones from antibody phage display libraries.

6. OVERALL CONCLUSIONS

The purpose of this research was the development of genetic techniques for the production of antibodies to the experimental chemotherapeutic agent, coumarin, and its major human phase I metabolite, 7-hydroxycoumarin. Also, this research studied the phase II metabolism of 7-hydroxycoumarin to 7-hydroxycoumarin-glucuronide.

The first system for the production of antibody fragments to coumarin and 7-hydroxycoumarin to be evaluated was the pComb3 system for the production of Fab antibody fragments. The system required the production of light and heavy chain gene libraries from the splenocytes of immunised mice. These heavy and light chain gene libraries were successfully created in the cloning vector pGEM-T. However, all attempts to transfer these libraries to the phagemid vector pComb3 failed. Initially, it was thought that problems lay with restriction-digestion of the light and heavy chain genes. However, this was eventually shown not to be the case as these could be transferred to and from the pGEM-T vector. From my own results and the experiences of others (Burioni *et al.*, 1997), it became clear that difficulties in cloning were due to the characteristics of the vector, pComb3. Modified versions of this plasmid have been created, but time did not allow for their use in this instance.

As an alternative to the pComb3 vector system, work was developed using an alternative, naive human library, commonly referred to as the Nissim library (Nissim *et al.*, 1994). Here, a complete human antibody repertoire in the form of single chain scFv antibodies was present, along with increased diversity brought about by the presence of a random CDR sequence on the antibody heavy chain.

The library was evaluated at various stages for its ability to produce antibodies to coumarin and 7-hydroxycoumarin. It became evident that the conditions used for both affinity selection and screening would have a crucial impact on the quantity and type of antibody clones that would be selected from the library. This was particularly so for the small hydrophobic haptens that were being investigated, i.e., coumarin and 7-hydroxycoumarin. It became clear that the design of the hapten-protein conjugates required for selection and screening was of great significance. At low hapten coupling ratios, antibodies to the hapten were not sufficiently enriched over clones recognising the

carrier protein. At higher hapten coupling ratios, however, clones were successfully isolated that bound to the hapten-protein conjugate, but not to the carrier protein alone. Also, alternating the carrier protein used in affinity selection resulted in antibody clones with the same characteristics. Other methods of selection were also briefly explored, such as the use of haptens coupled directly to solid phases and use of BIAcore as an affinity selection system.

Detailed analysis of the clones derived from the affinity selection strategies, however, showed that these antibodies possessed only poor affinities to the hapten and bound predominantly to a combined protein-hapten epitope via a weak electrostatic interaction. Further characterisation suggested that this was most probably an interaction with the amino acid by which the hapten was attached to the parent protein. The structural simplicity and hydrophobic nature of these haptens militated against producing antibodies recognising free drug alone. To avoid this, conjugates utilising different conjugate chemistries would be desirable. In this way, selection would not be made predominantly against the type of linkage between hapten and carrier protein, but to the hapten alone. The other strategy of direct attachment of haptens may also be useful in this regard.

In vitro methods for the production of 7-hydroxycoumarin-glucuronide were developed using liver UDP-glucuronyl transferase from both bovine and porcine sources. These methods were accompanied by the development of methods for the analysis of 7-hydroxycoumarin-glucuronide using HPLC and CE. Methods were also developed for the purification of 7-hydroxycoumarin-glucuronide, from *in vitro* enzymatic synthesis, and from the urine of patients treated with coumarin. Finally, different means of producing protein conjugates of the glucuronide were investigated. An *in vitro* enzymatic synthesis route was found not to be viable due to the complexity of the reaction product. Linkage of the 7-hydroxycoumarin-glucuronide via the carboxyl group of the glucuronide residue using EDC/NHS chemistry was simpler and resulted in a higher yield of pure product, which could then be used for antibody production in either the ways previously discussed, or through the more traditional means of polyclonal and hybridoma production.

In summary, this work has contributed to the understanding of the mechanisms at work during affinity selection strategies to small, hydrophobic haptens, and has illustrated the special considerations required when working with such molecules. A significant contribution has also been made to the study of phase II metabolism of coumarin, by the development of *in vitro* metabolic methodologies and the analytical tools to accompany them. Also, the production of conjugates with which to produce antibodies to the phase II metabolite leaves possible further immunoanalytical research in this area.

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APPENDIX

Prize winning essays in Biochemistry

The Antibody Army: Friend or Foe?

Royal Irish Academy, National Committee for Biochemistry, Scince Writing Competition Winner, 1996.

Antibodies! Even my five year old nephew has heard of them: some alien superhero producing antibodies against a mutant virus created by an evil scientist to destroy the world...and all that on a children's cartoon. But what are they? They are protein molecules found in your bloodstream. Their function is to seek out foreign bodies such as viruses and bacteria and mark them for destruction. Hence the name 'anti-bodies'. The antibody army is a large one with an estimated one hundred million million million antibody molecules circulating in the body.

The shape of antibody molecules (also called immunoglobulins) is essential to the way they seek out alien invaders in the bloodstream. Their basic structure is often thought of in terms of the shape of the letter Y. The two arms of the Y are identical to one another. Each is able to attach to the foreign invader which is now called an antigen. The stem part of the Y interacts with the rest of the body's immune system, which results in the destruction of the foreign body.

Although all antibodies have the same basic structure, they can combine to form larger molecules that each perform slightly different jobs (Figure 1). The mainstay of the antibody army is the single Y molecule, called IgG (meaning immunoglobulin G). Another single Y molecule is called IgE. IgE have a special function in helping to combat parasitic infections. These have gained much attention because of their link with allergic responses, something we will return to later. IgA molecules are made up of two Ys linked tail to tail. These are important in protecting the external linings of the body, most importantly, the lungs and the alimentary canal. The biggest Ig molecule is IgM. Here, five Ys join in a ring structure. These are produced early on in the body's response to infection and so are weaker than the IgG molecules that are produced later. The presence of 10 places at which to attach the enemy invader helps to compensate for this weakness.

So how do these molecules seek out enemy invaders and go about having them destroyed, and how can they tell whether they are enemy invaders or not? Although the basic structure of each antibody is the same, the areas at the tips of the Y arms are different from one antibody to the next.

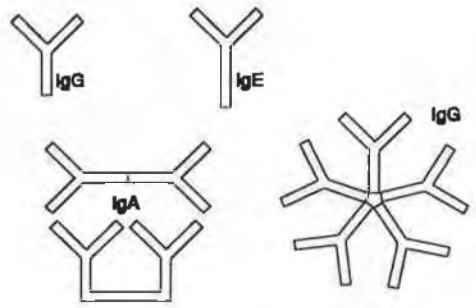

Figure 1. Antibody subclasses. IgG is the main antibody present in the body, accounting for 70-75%. IgA accounts for between 15 and 20%, and IgM makes up about 10%. IgE is only present in trace quantities.

Each antibody is produced by a different antibody-producing cell called a B cell. It is reckoned that the body can generate more than one hundred million different antibody molecules in this way. These will all attach themselves to slightly different chemical structures including those present in the body. Early in human development, before birth, the immune system is in a learning mode. Protected by the mother's immune system, and so being free of any foreign bodies, the only molecules present are that of the growing infant. These are called 'self' antigens. Many of the antibodies produced will attach to these self antigens. These antibodies are then removed from the immune system, or rather the B cells that produce them are removed. All that should remain are B cells that produce antibodies to molecules not already present in the body. This still leaves many millions of combinations that can seek out and bind to antigens present on viruses, bacteria and parasites. The immune system is now ready to take on all comers. However, it still has a lot to learn.

If the body becomes infected with, for example, a bacteria it has never encountered before, then somewhere in the immune system there is an antibody or even several antibodies that can bind to it. These could be spread out through the blood stream and lymphatic system. Lymph nodes are like busy market places for cells of the immune system. Here, many B cells can come into close contact with other immune cells, along with the invading bacteria. In this way, the bacteria has more chance

of coming into contact with B cells that produce antibodies which can attach to it. When this happens, the B cells producing these antibodies are triggered to multiply very rapidly and produce more antibody against the bacteria. So, often the first sign of infection can be the swelling of lymph nodes due to this extra activity. Most people are familiar with the swelling of their tonsils in response to a bacterial infection.

The antibodies produced at this early stage do not bind to the foreign body that strongly, so IgM molecules are used which can bind at 10 sites per molecule. In the meantime, the B cells undergo further changes that 'fine tune' the structure of the antibody so as to bind more and more strongly to the foreign antigen. This strength of antibody binding is called affinity. Eventually, the low affinity IgM molecules are replaced by high affinity IgG molecules. To become active, these IgG molecules must attach at both arms. This then allows the stem of the antibody to attach to other cells and molecules of the immune system. One important set of such molecules is called 'complement'. Binding of antibody to the bacteria causes a series of changes in the complement molecules, one result of which is the Membrane Attack Complex, where a hole is formed in the membrane of the bacteria, allowing its contents to spill out and be destroyed (Figure 2). Many other cells also attack foreign bodies such as cytotoxic T cells (Tc) which recognize antigen and natural killer (NK) cells which recognize the stem part of the antibody that is bound to antigen. These produce cocktails of enzymes that can also attack and break down bacteria and parasites. If the immune system wins the battle, all the foreign invaders are destroyed. Many of the B cells that produced the antibodies now go on to form memory cells. These are long-lived B cells that stay dormant, but are activated when the same invader tries to attack again. This is thankfully why, with most diseases, we only suffer them once.

Understanding the workings of the immune system allows us to go one step further and prevent the body from ever having to suffer a disease. This is the principle of vaccination. Here, the body is allowed to encounter the enemy on safer territory. In the case of viral immunizations, such as the measles, mumps and rubella viruses (MMR), the body is supplied either with virus that has been killed or virus that has been severely disabled, so making it incapable of causing disease. Antibodies are still produced against it and this results in

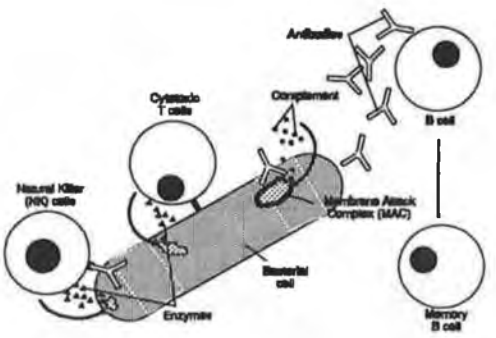

Figure 2. Antibody-mediated destruction of a bacterial cell. Antibodies attract proteins in the blood called complement. These form a ring structure in the membrane surrounding the bacterial cell causing its contents to leak out. It also attracts other cells to the bacteria which release enzymes that break down the bacteria. The B cells producing these antibodies can go on to form long-lived B cells.

subsequent resistance to disease.

So the antibody army keeps us alive from moment to moment. Its surveillance of our body for foreign invaders is constant and unceasing and goes on mostly without us noticing. The antibodies of our immune system are something we take for granted. The immune system is, however, extremely complex, and much of it remains undiscovered and poorly understood. Such complexity also leads to fallibility, and the immune system can fail to function in many ways. Many of these relate to how the antibody carries out its functions. In these instances, the antibody is guilty of treachery, turning on the body and inflicting damage.

Disease brought about by the failure of antibodies to function correctly can be broadly categorized into two groups between which there is considerable overlap. These are hypersensitivity and autoimmunity. Hypersensitivity refers to the misguided or exaggerated immune response, whereas autoimmunity refers to the attack by the immune system upon its own body.

The most common form of hypersensitivity is that of the allergic reaction. Most allergies are against substances in the environment that are of little or no harm, such as pollen, dust, penicillin and nut oils. The immune system, for reasons that are poorly understood, designates these as dangerous foreign invaders and mounts an immunological attack against them. In these cases the allergic response is

led by IgE antibodies. These are particularly powerful activators of immune cells called mast cells. These cells are stimulated to release extremely powerful and fast acting chemicals such as histamines, heparin, and prostaglandins. These bring about an immediate and extreme inflammatory response. In hay fever, the symptoms are runny nose, streaming eyes, itching, etc. This response is quite localized and not life threatening. Many people are also allergic to the dust mite, or rather its faeces. When these enter the lungs in airborne dust, an asthmatic response may result which inflames the airways, restricting breathing. This can, in the most extreme of circumstances, close the airways completely. The Asthma Society of Ireland estimate that a quarter of a million Irish people currently suffer with asthma. The incidence is much higher among children where 1 in 7 are thought to be affected, compared with 1 in 20 adult sufferers. Other more severe allergic responses are against antigens such as bee venom, certain nut oils and penicillin. Challenge with these antigens results in a systemic effect known as anaphylaxis. Here, mast cells are stimulated by IgE throughout the body. The most severe result of this is the loss of fluid from the blood stream into the surrounding tissues. This can result in the collapse of the circulation which may cause heart failure and death. An injection of adrenalin reverses most of these effects, along with a drip to restore blood volume.

The production of IgE antibodies is believed to be important in the battle against parasitic infections such as worms. Modern Western man is less prone to parasitic disease, and so this element of our immune system is less used. It is not yet clear why the body decides to make IgE antibodies to substances such as pollen, penicillin and nut oils, and not to other substances in the environment, or more importantly, why it makes them at all. Many believe that modern day environmental factors such as pollution and diet may be contributing to the allergic response. Whatever their cause, allergies are increasing in our modern world.

Another disease brought about by the misguided production of antibodies is haemolytic disease of the newborn. This can occur when a mother has a different blood group to her unborn infant. This occurs predominantly with an antigen found on red blood cells called Rhesus D. if the unborn infant has the Rhesus D antigen (Rh^+) and the mother does not (Rh^-), the mother will produce antibodies to the infant's Rhesus antigen. At the birth of a first child, some of the infant's red blood cells can be passed back into the mother's circulation. This is the time at which the mother's immune system produces anti-Rhesus antibodies. If the mother has subsequent Rh^+ babies, these anti-Rhesus IgG antibodies will cross the placenta and attack the growing infant's red blood cells, causing widespread symptoms, damaging the spleen and liver which becomes enlarged and results in jaundice. These symptoms can be prevented by the administration of an anti-D antibody to the mother following the birth of her first Rh^+ child. These antibodies clear up any of the infant's blood cells that may have entered her body and so a response is not mounted by the mother's immune system. This has greatly reduced the incidence of, and resulting death from this disease since the introduction of anti-D in the 1970s.

Autoimmunity can result in many diseases that attack various parts of the person's own body. Three such diseases are myasthenia gravis, rheumatoid arthritis and multiple sclerosis.

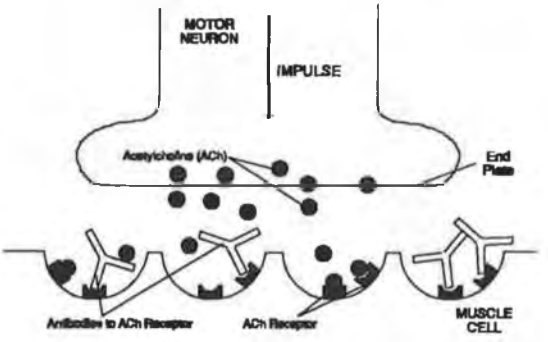

Figure 3. Myasthenia gravis. In a normal nerve impulse, the signal reaches the motor neuron end plate and causes the release of acetylcholine (ACh) which then crosses the synapse and binds to ACh receptors on the muscle. Autoantibodies associated with myasthenia gravis bind to the ACh receptors and prevent the binding of ACh. The impulse is weakened and this leads to decreased muscular movement.

Myasthenia gravis is a very rare disease which results in extreme muscular weakness. In this condition, 'self' antibodies (also called autoantibodies) are produced that cause the blocking of signals that are transmitted by nerve cells (neurons) from the nervous system to the muscles (Figure 3). To allow a muscle to move, an electrical impulse must pass from the nervous system (initially from the brain) to a muscle. To do this, it must travel from one neuron to the next. These

neurons have gaps between them called synapses. For the signal to pass from the end of one neuron to the start of the next, several types of transmitter chemical can be released from the neuron carrying the signal which travels across the synapse and stimulates the next neuron to trigger an impulse. One of these chemicals is called acetylcholine (ACh). In myasthenia gravis, antibodies are produced that bind to the receptors of ACh on the last nerve that connects to the muscle. Not as much ACh is allowed to reach the muscle, and this results in a weakened signal which impairs muscle movement.

Rheumatoid arthritis is a familiar disease to many. It results in the inflammation of the joints, often those in the hands, wrists and elbows. Other joints are also affected. This all results in limited joint movement, pain and joint deformation which can often be permanent. According to the Arthritis Foundation of Ireland, 1% of the Irish population are affected by rheumatoid arthritis, with women being three times more susceptible than men.

It is believed that rheumatoid arthritis is caused by an autoantibody of the IgM class. This antibody is, itself directed against an altered IgG antibody called rheumatoid factor. One theory suggests that an IgG antibody is initially produced to combat damage due to injury or infection at the joint. This IgG may itself become damaged as a result of the inflammatory response. This damaged IgG may then be seen by the immune system as foreign, whereupon, it produces an IgM antibody against it.

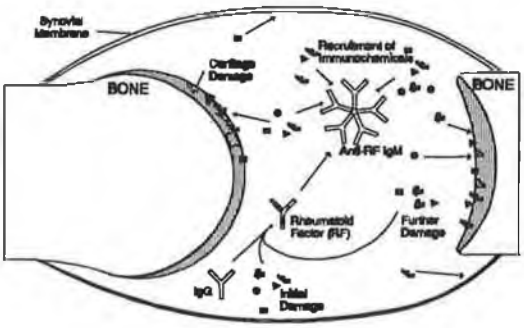

Figure 4. Rheumatoid arthritis. Initially, IgG present in the joints becomes damaged in some way. This rheumatoid factor (RF) causes the production of an IgM antibody against it. This IgM recruits immune chemicals to the region which damage surrounding tissue such as cartilage and the synovial membrane. They also further damage IgG producing more rheumatoid factor, and so producing more anti-RF IgM antibody.

This would lead to further damage due to inflammation and further modification of antibody (Figure 4). This cyclical pattern of inflammation leads to the long-term, progressively worsening symptoms that are characteristic of rheumatoid arthritis.

Multiple sclerosis (MS) is another disease that affects cells of the nervous system. This time, the cells affected are those of the central nervous system, namely the brain and the spinal cord. These areas are also made up of neurons. These neurons in particular have a protective coating called myelin. This acts like an insulator and helps the passage of electrical impulses along neurons. MS is caused by the production of autoantibodies to this

myelin layer. This results in its destruction and the impaired functioning of the nerves themselves when damaged myelin is replaced by scar tissue (Figure 5). Symptoms resulting from this are lack of muscular control, or loss of movement. The disease is slow but progressive. It is characterized by unpredictable periods of remission and exacerbation and has also been known to spontaneously clear up completely. MS is a very relevant Irish problem. Its prevalence is particularly high here, with approximately 1 in 1,000 suffers.

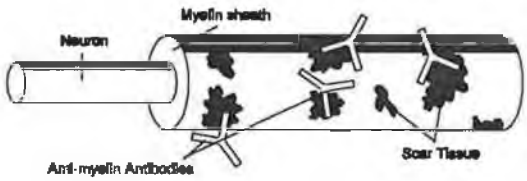

Figure 5. Multiple sclerosis. Autoantibodies are produced to the myelin sheath surrounding nerves of the central nervous system. These result in damage which is repaired by scar tissue. This damaged tissue impairs the quality of the myelin sheath and reduces the quality of the nerve impulses.

The cause of MS is still very unclear. Most now believe it is a result of a combination of factors. These may be a viral infection and an environmental factor. Genetic predisposition may also be involved. Many candidates for both the viral and environmental cause have been put forward. The most popular viral culprit at present is the

measles virus. In some instances, measles infection may bring about damage of myelin. This damaged myelin may then be seen by the immune system as foreign which then produces antibodies against it. These antibodies may cause the myelin further damage. Some believe that parts of the measles virus look like myelin, and so in producing antibodies against the virus, the same antibodies also cause damage to the myelin. The relationship between measles virus and MS is made hard to study because disease symptoms do not occur until many years after measles infection. Attempts to unequivocally link the two have not yet proved possible.

Antibody soldiers are very powerful and have at their disposal the considerable resources of the immune system. This gives them the strength to defend us from very many bacteria, viruses and parasites. They do, however, carry a double-edged sword, which, when turned upon the body can inflict considerable damage, and even kill. Research in immunology is some of the most exciting and fast moving in modern science. The untold complexity of the immune system ensures this will be a long task. As more becomes known, however, ways of directing the immune system to even greater good will be discovered. One such exciting area is the immunotherapy of many cancers. Further knowledge of the immune system will no doubt help us in the fight against many diseases. This knowledge will also show why the immune system sometimes turns on itself, causing what seems to be needless self destruction. In understanding this, even better cures for autoimmune diseases such as rheumatoid arthritis, myasthenia gravis and multiple sclerosis will inevitably follow. We wait.

It's Life Jim, but not as we Know it!

Royal Irish Academy, National Committee for Biochemistry, Science Writing Competition, Runner up, 1997.

> **With the discovery of viruses just over 100 years ago, and with the more recent discoveries of viroids (naked nucleic acids) and prions (the cause of BSE or mad cow disease), these biologically simple creations are challenging the traditional definitions of that elusive entity...LIFE!**

MERRING! No its not a Serbo-Croatian swear word, but an acronym used to teach the characteristics possessed by all living things: Movement, Excretion, Respiration, Reproduction, Irritability, Nutrition and Growth. All living things can move in some way, whether towards a stimulus such as food or light in plants, or by locomotion as in animals and humans. Excretion involves the removal of harmful waste products from an organism. Everyone is familiar with the waste product produced by yeast fermentation, alcohol. To produce energy with which to carry out the processes of life, all living things must perform respiration. This involves the release of energy from energy-rich molecules such as glucose. Although carbon-containing (organic) sources of energy are the most common, many microorganisms can use non-carbon (inorganic) sources such as sulphur bacteria in hot volcanic springs. To perpetuate themselves, all organisms must also possess the most important quality of life. Its ability to reproduce. This can be in the simple form of asexual reproduction from a single parent, or sexual reproduction with one offspring resulting from two parents. Both result in the perpetuation of the species and also introduce genetic diversity, resulting in its evolution. Irritability involves the sensing of, and response to the environment. In higher mammals such as humans, this is extremely complex and is controlled through our nervous system, but it is also present in extremely simple forms of life, for example when an amoeba responds to the presence of a harmful substance in its surroundings and move away from it. Nutrition is the intake of all the chemical substances required for the life processes of an organism which it is incapable of producing for itself. Nutritional complexity tends to increase with the complexity of the organism. Humans must take in large numbers of nutrients including carbon-containing compounds for energy and biosynthesis, the building blocks of life. Plants, however, require only carbon dioxide, oxygen, water, energy from the sun and several other inorganic components from which they synthesise all the materials with which they need to survive. Finally, a result of these combined life processes is an increase in the size and complexity of all living things, simply referred to as growth. Growth periods vary enormously from as little as 20 minutes for some bacteria to hundreds of years for some of our oldest trees.

However diverse, all forms of life on our planet have these seven traditionally defined characteristics. They have also all been found to carry out these life processes using the same fundamental building blocks. Cells. Alone, or in combination with other cells, all these functions can be performed.

The Cell - The Protein Factory

Just as diverse forms of life perform these same basic functions, so too all cell types, no matter how different, also carry out the same fundamental processes, the most important one of which is the production of proteins. Nearly all the functions of the cell are carried out using some protein or other. They are either structural or enzymes which direct and control all the reactions that take place within the cell. So important are they that the cell directs large amounts of resources to their production. In fact a quarter of the cell's dry mass is made up of the protein-making machinery, ribosomes. These are like individual self-contained assembly lines for proteins in the giant protein factory which is the cell (Figure 1). All the genetic information for the production of all these proteins is contained in the nucleus of the cell in the form of DNA (deoxyribonucleic acid). The DNA is like a permanent record of what the cell does and how it does it, and is extremely precious as it must be replicated faithfully to each generation. It is too precious to be used again and again to promote protein production directly. Much like an important piece of computer software, the DNA is used as the master copy for the production of working copies of the information. In a cell, these working copies for protein production are called RNA (ribonucleic acids), and though different from DNA in many ways, still encode the same information for protein production. Another reason for the presence of RNA is that DNA contains not only the information necessary for that cell, but also the information for every cell in the body. Obviously not all this information is required for our single cell, and only the information necessary is converted into RNA. The RNA molecules then travel

to the ribosome protein assembly line and the RNA is read into the ribosome like a punch tape. The information is read off the RNA in sequence, three nucleic acids at a time, each triplet encoding for a different amino acid to be joined to the growing chain. Eventually, the whole protein is synthesized, correctly folded and transported off to the part of the cell where it performs its function. All these proteins contribute to the performance of all our 'life' tasks: movement, excretion...etc. In this way a basic mechanism for the existence of life can be seen in this three step process of DNA to RNA to protein. However, nature has found ways of producing much simpler forms of reproductive structure that are clearly 'life', but do not conform to the basic cellular definition.

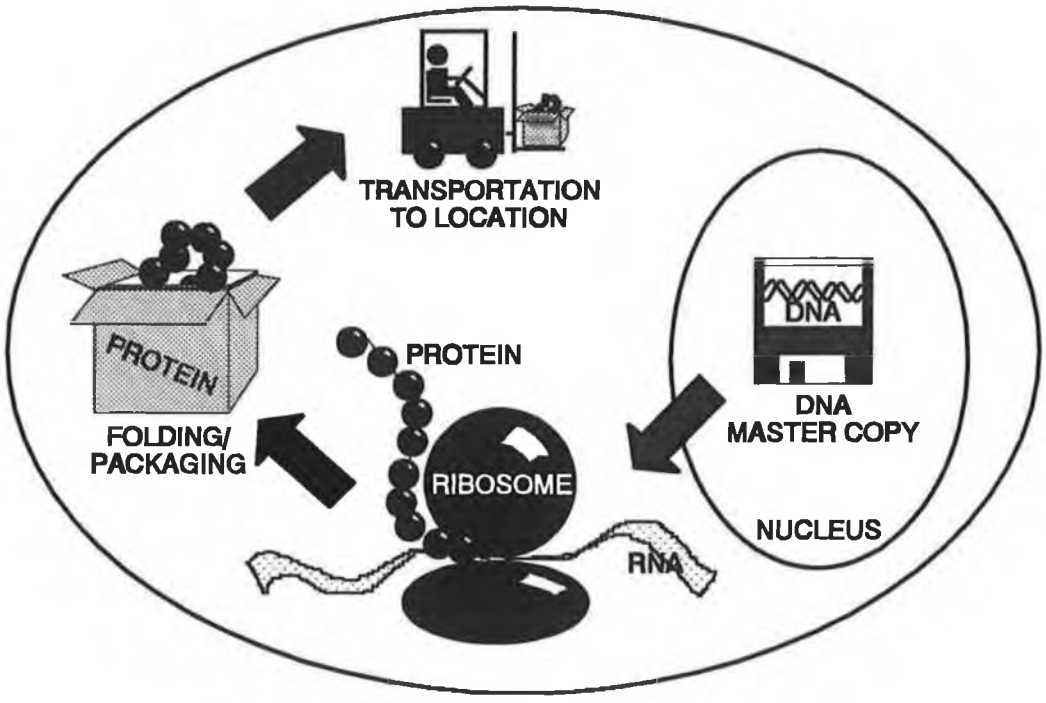

Figure 1. The Cell -The Protein Factory

DNA in the nucleus is transcribed to RNA which is read by ribosomes and converted into protein sequences. These proteins become folded and undergo further modifications and are transported to the region of the cell where they perform their function.

Viruses

Before the invention of the microscope, the cause of microbial disease remained unknown. All that could be shown was that certain tissues from infected individuals remained 'infectious' when brought into contact with healthy individuals, but when passed through fine clays, many such infectious tissues were rendered innocuous. Then with the invention of the light microscope, the cause of so many of these diseases (bacteria and other microorganisms) could now be seen directly.

It was a Russian scientist, Iwanowsky, who first observed that some infections still remained upon filtration. These smaller infectious agents he called viruses. And although most of the characteristics of viruses were defined indirectly, they were only visualized directly for the first time in 1942 with the invention of the electron microscope, the only device capable of visualizing these extremely small entities.

If viruses are not cells, what are they? They are basically composed of a nucleic acid (DNA or RNA), surrounded by a protein coat called a capsid. The capsid is composed mostly of repeating protein subunits which

tend to give viruses the most fascinating geometric shapes (Figure 2), but which protects the nucleic acid from environmental hazards such as ultraviolet radiation. Some viruses also surround themselves with a membrane called an envelope which they 'steal' from other cells. In fact everything a virus has, it has stolen from a cell.

Viruses carry out only one of our seven life functions; reproduction. When a virus comes into contact with some suitable uninfected host cells, (for example, influenza viruses in the air when someone sneezes for instance). These get carried to the cells of the lungs. Viruses have then developed clever ways of getting the cell to bring it inside. When this occurs, the cell is now doomed to become a slave to the viruses voracious reproductive appetite. The viral nucleic acid has been specially designed to hijack all the assembly line machinery of the cell to producing its own proteins and replicating its own nucleic acids. These then assemble into mature virus particles called virions. Eventually the cell becomes full to bursting with virus particles. The cell bursts (and dies), leaving the virions to go off and infect neighbouring cells or to be passed to other individuals.

Viruses have 'discovered', if you like, that they do not need all the machinery for reproducing themselves They can just borrow it (steal it?) from cells that do. This brings us closer to a definition of what constitutes life: that which can perpetuate itself. Many may argue with such a definition for various reasons, but all those things that we clearly agree are life exhibit this characteristic at least.

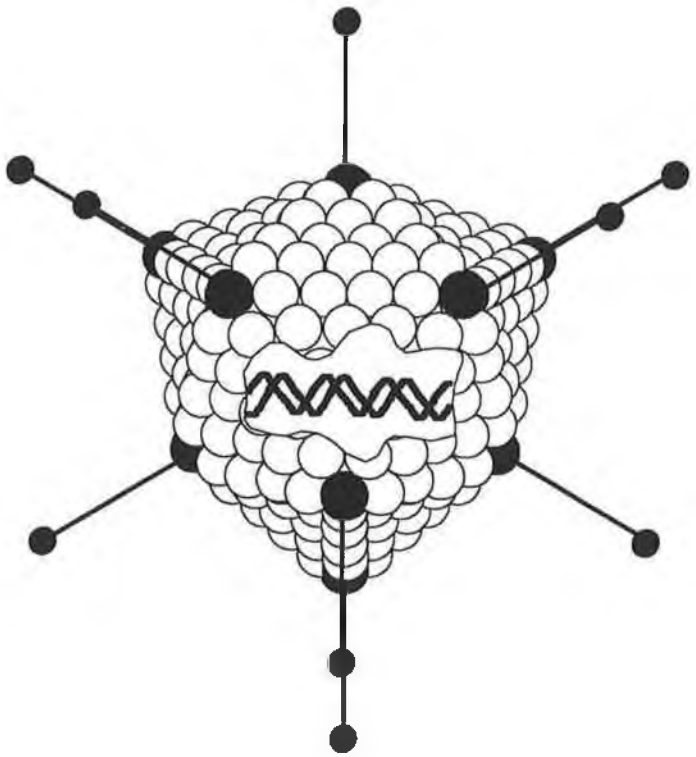

Figure 2. The Adenovirus

The adenovirus is composed of an icosahedral capsid encapsidating a DNA nucleic acid.

Viroids

Several plant diseases had been studied for which no infectious agent (fungal, bacterial or viral) could be identified. The only component that could be found in diseased extracts was RNA. After much study, it became clear that these plants were being infected by another new agent never seen before; a naked nucleic acid. This they called a viroid.

Viroid RNA is fascinating in that it alone is able to redirect the plant cell for its own reproduction. It is extremely resistant to the environment such as ultraviolet radiation and is at a lesser risk that animal viruses because of the absence of a plant immune system. The viroid RNAs are also inclined to bend and fold producing much secondary structure, which may allow them to behave like a protein and actually 'do things', rather than just get replicated. In fact, the RNA in cells also possess much secondary structure which is all involved in the control of protein production. The RNA from plant viroids is extremely similar to some of the plant's own nucleic acid. It is likely that the viroid 'evolved' to become separated from the plant RNA and developed the ability to be replicated by the plant's cells in much the same way as viruses are.

Now we see two very important elements to our new definition of life: reproduction and evolution. What is occurring is that as these viroids and viruses (and indeed cells) replicate, they do so quite faithfully, but not so faithfully as mistakes cannot happen. This leads to minor changes that may or may not be an improvement on the previous generation. Those changes that are an improvement - that is they help survival and further reproduction - may result in those individuals doing better than others in the population and so replicate more successfully. Evolution is just a result of the demand put on a faulty replication mechanism to improve.

Many have speculated that these RNA molecules could have been the first forms of life on our planet. Only they have been shown to possess the ability to carry information in the form of the genetic code, but also like proteins to 'do things', because of their secondary structure.

Viruses and viroids both fit our new definition. But is there anything else out there that doesn't conform to these rules? A group of diseases called the spongiform encephalopathies were thought for a time to be either the result of viruses or viroids, but no genetic material could ever be found associated with the disease. Their cause has only been seriously established in the last decade or so.

Prions

An isolated tribe were discovered in Papua New Guinea in 1957 who still practised cannibalism. It was also a male dominated society, and at meal times, the men would get all the good quality muscle tissue, but the women and children would be left with the offal and brains to eat. Accordingly, there was a high incidence of a disease in the women and children called Kuru. This is a neurological disorder, very similar to diseases in the Western world such as Creutzfeldt-Jacob's disease (CJD) and Gerstmann-Straussler syndrome (GSS). These neurodegenerative disorders are ultimately fatal. A similar disease in sheep, scrapie, has also resulted in the infection of cattle via dietary supplements which resulted in the cattle epidemic bovine spongiform encephalopathy (BSE) or mad cow disease. Spread of the disease to humans from meat containing infected offal has also been identified. The disease reached epidemic proportions in the United Kingdom, but due to strict control measures in Ireland, only isolated cases have occurred with no transmission to man having occurred here.

Out of any infectious disease this group of diseases has probably seen the most argument and debate as to their cause. Doctors who had studied patients with these diseases knew it to have a genetic component (nucleic acid), because diseases such as fatal familial insomnia (as the name suggests), were passed on in families. (Diseases such as CJD are familial and have also been contracted from infected growth hormone extract injected to treat dwarfism). Other scientists, however, could also demonstrate that these diseases were infectious and could be passed from one individual to the next, but that the infectious material did not contain any nucleic acids at all.

Following much intensive research, it has been found that the infectious component of this group of diseases is a protein, and for this reason they were termed prions (protein infectious agents). Prior to this, only agents carrying genetic information were shown to be capable of causing infectious disease. How was a protein able to bring about its own propagation, and what is the involvement of the genetic component that has been shown to exist?

I am sure much detail has still to be worked out, but the basic, underlying mechanism has now been elucidated sufficiently (Figure 3). The prion protein is a normal protein produced by cells in the brain. Its exact function has not been concluded yet. Genetic mutations in the DNA that codes for this protein have been shown to be linked with many of the cases of the disease that are passed down through families. These mutations bring about

an incorrect, misfolded version of the protein. Normally, such misshaped proteins would be destroyed, but in this instance, the changes in the protein's shape lead to several interesting characteristics. The protein becomes insoluble, which also renders it resistant to biochemical breakdown by protein-digesting enzymes called proteases. This insoluble, indigestible protein material builds up in areas of the brain forming crystalline structures which gradually damage brain tissue in small, discrete patches, giving the brain a sponge-like appearance, hence spongiform encephalopathies. This, however, is only half the story. If a healthy, uninfected individual ingests (or injects) some of this material into their body, the harmful protein resists any attempts at digestion and enters the body. When it reaches the brain, the 'bad' prion protein comes into contact with 'good' prion protein. The two proteins join together to form a complex in some way, which results in the bad prion protein changing the shape of the good protein. In a sense, bad prion protein has brought about its own replication. These two molecules of bad prion protein now come into contact with others, also resulting in their conversion. This process moves relentlessly on until enough damage accrues, causing death. No cure is yet available for these diseases, although drugs are available which slow their progression.

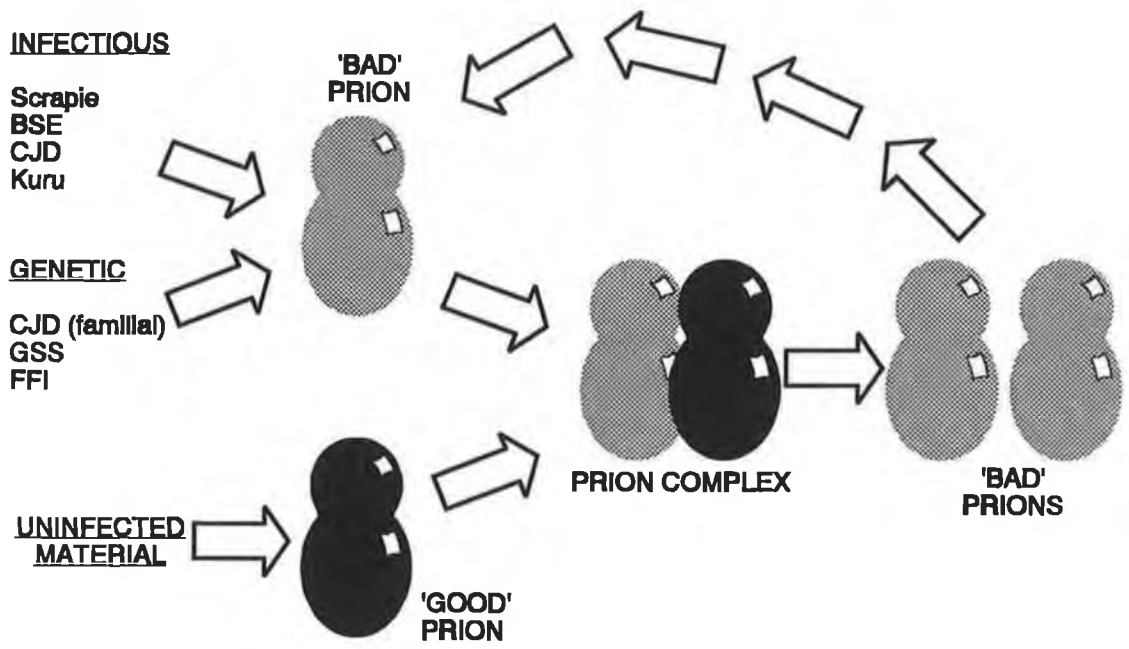

Figure 3.

Mechanism of Prion Replication

Misfolded 'bad' prion protein from either infectious or genetic sources comes into contact with correctly folded prion protein. Following complex formation, the correctly folded prion protein becomes misfolded. Now both proteins can go on to form complexes with correctly folded protein.

Does the prion fit our definition of life? Well it can reproduce itself using the components of a cell, just as viruses do. It does not, however, control its own evolution. This is selected for by genetic changes in a protein gene in the original 'host' individual, in whom such a mutation is of no evolutionary benefit. Once having left its original 'host', however, it does not appear to require an evolutionary mechanism by which to be sustained. But lacking the ability to mutate and change with the times, measures to control infection and genetic screening to reduce familial propagation of the disease may bring about their demise.

Thus, viruses and viroids do get safely into our definition of what is life. The prion is on shakier ground, however, but maybe if discovered on another planet, in another galaxy, we would hear that now familiar trekky phrase "It's life Jim, but not as we know it."