ANALYSIS AND RECOVERY OF FV ANTIBODY FRAGMENTS PRODUCED IN ESHERICHIA COLI

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ABSTRACT

Cloned antibody binding sites (known as Fv antibody fragments) are attractive to the fine chemicals industry for several applications: in particular, as ligands in affinity chromatography. However, optimised systems for producing Fv fragments are not currently available. This thesis aims to establish new methods for the analysis and recovery of Fv antibody fragments that have been expressed in Escherichia coli cultures by recombinant DNA technology, thereby enabling improved control of the processes required for Fv production.

An important objective was to prepare and test generic analytical reagents that bind all Fv fragments regardless of their specificity. Tracer antibodies were raised against a common peptide motif and used to design a generally applicable immunoassay for analysing Fv protein in microbial culture. The immunoassay was used as a component part in a new analytical system that can determine total Fv and active Fv independently, thus providing the fermentation scientist with a complete picture of the immunoreactivity or "authenticity" of the Fv throughout the process. Using an Fv specific for human gonadotropin as an example, the immunoreactivity of the Fv in the fermenter was found to approach 100%.

A new process for recovering active Fv fragments from microbial cultures was designed and tested, using an Fv specific for estrone-3-glucuronide as an example. The new process was based on antigen affinity chromatography and used two antigen analogues: one as a column ligand, the other as a specific eluant. The recovery efficiency of active Fv was several-fold superior to that obtained with conventional methods.

Overall, the results show that the analysis and recovery of Fv fragments are related in analytical and production systems: both require the preparation and synthesis of reagents that can discriminate between Fv and non-specific biomass and between active Fv and inactive Fv.
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1. GENERAL INTRODUCTION

1.1 THE IMMUNOGLOBULIN MOLECULE AND HYBRIDOMA TECHNOLOGY.

1.1.1 Antibody Structure and function

Antibodies or immunoglobulins are glycoproteins with a molecular weight of approximately 150 kilo Daltons (kD). They comprise two identical heavy chains (H) of 50 kD each and two identical light chains (L) of 25kD each. The four chains are linked by disulphide bridges. Both heavy and light chains have variable regions at their N-termini and constant regions at their C-termini. Within the variable regions of each chain are three hypervariable regions (also known as complementarity determining regions or CDRs). The CDRs from one light chain and one heavy chain fold to form the antigen binding site of the antibody. There are therefore two (identical) binding sites on an antibody molecule - each comprising six different CDRs, the sequences of which determine the specificity of the antibody (Fig 1.1a).

Also see Day (1990) for a review of antibody structure and function.

In nature, free antibodies circulate in the blood and are a key component of the immune defence system of mammals and higher vertebrates. In brief: antibodies have a bifunctional mode of action - the antigen binding sites bind to the foreign component of an infectious agent and the constant region (also known as the effector region) participates in the recruitment of phagocytic cells to ingest the invading microbe or to stimulate cell cytosis via the complement system (Day, 1990). Mammals have an immune repertoire of approximately $10^6$-$10^7$ different specificities, and are therefore capable of raising an immune response to a wide range of foreign bodies. Antibody specificities which are required to cope with an acute infection are produced in abundance by clonal selection of the appropriate antibody producing B cells.
Fig 1.1: The structure of monoclonal antibodies (McAbs) and their fragments.

On the monoclonal antibody (McAb) the constant regions are drawn in black, the variable regions are in white, and the complementarity determining regions appear as three bands on the variable regions. See text for details.

Approximate molecular masses: McAb = 150,000; Fab = 50,000; Fv = 25,000; scFv = 27,000; $V_H = 12,500$; MRU = 1,000 - 2,000.
1.1.2 Hybridoma technology and monoclonal antibodies

The enormous repertoire of binding activities that is represented in a mammalian immune system was made accessible to biotechnologists by the advent of hybridoma technology (Kohler and Milstein, 1975). In brief, B-cells from a spleen (usually from a mouse) are fused with myeloma cells (from the same species) to produce so-called hybridoma cells. Hybridoma cells have the combined properties of their two parent cells: they secrete antibody and grow readily in culture. Hybridomas which secrete antibodies with a required specificity need to be selected from the huge number generated by a fusion. This is done by a process known as "screening" - where the supernatant fluids from a library of hybridomas are individually monitored for active antibody, typically by enzyme linked immunosorbent assay (ELISA) - see Goding (1980).

In order to maximise the chance of success, mammals (usually mice) are inoculated with the antigen of interest to drive the immune response towards selecting B-cells which secrete antibodies specific for said antigen. When these spleen cells are fused with myeloma cells there will be a preponderance of hybridomas producing antibodies with the required specificity.

Positive hybridoma cultures are then manipulated so that single cells are isolated and these can then be bulked up in culture so that all the cells have arisen from a single daughter cell and therefore all the antibodies produced in such a culture are of the same specificity. Antibody produced from a single cell line or "clone" as described above, are known as monoclonal antibodies.

Using this procedure it is relatively straightforward to produce milligram or gram quantities of pure antibody with a binding activity which is exquisitely specific for any protein, enzyme, or most organic molecules.

1.1.3 Applications of monoclonal antibodies (McAbs)

Industry exploited the opportunity presented by monoclonal antibodies. Examples of applications include: in vitro diagnostics (Clark, 1985; Harris, 1991); in vivo imaging
(Britton and Granowska, 1991); and affinity purification of biopharmaceuticals (Jack and Wade, 1987). The most significant impact of monoclonal antibodies has been in medical diagnostics where their high degree of specificity has made enormous improvements in product performance. For example, monoclonal antibodies have been raised which are specific for the pregnancy marker hormone - human chorionic gonadotropin (hCG) but do not cross-react with other very similar hormones found in the urine such as luteinizing hormone. These antibodies have been used to design a new generation of pregnancy tests suitable for use both in the home and the clinic (Porter et al, 1988).

1.1.4 Limitations of monoclonal antibodies
The general perception of opportunities for industrial application of immunoglobulins tends to be limited to in vitro diagnostics, in vivo imaging, therapy and affinity separations for bio-processing in the pharmaceutical industry. The projections of world market total product opportunities which may develop during this decade are of the order of £5 billion. For comparison, and to put this into its true perspective, it should be remembered that many individual pharmaceutical products approach this volume in their own right. Moreover, applications outside the clinical area are limited by the high cost of McAbs which are produced in expensive cell culture systems. Furthermore, this is a mature technology and there has been difficulty in achieving cost reductions below £1,000 per gram.

1.2 ANTIBODY ENGINEERING AND ANTIBODY FRAGMENTS
Recent advances in molecular biology have made it possible to 'design' many new biological reagents based on the structure of 'natural' antibodies. Important examples of antibody engineering are 'humanised antibodies', 'abzymes' and 'antibody fragments'. These are described in more detail below:-

1.2.1 Humanised monoclonal antibodies
Conventional monoclonal antibodies are produced in rodent cells, usually the mouse. This restricts their use for therapeutic applications such as the immunotherapy of cancer since the patient develops an anti-mouse immune response to the reagents.
This complication was identified 50 years ago when animal antisera were widely used to treat infectious diseases (Rather, 1943). A recent solution to this problem has been to make 'humanised' antibodies by 'grafting' the CDRs of mouse monoclonal antibodies specific for cancer cells onto human antibody constant regions. (Jones et al 1986; Verhoeyen et al 1988). These humanised antibodies have been found to be tolerated much better by patients as they are predominantly human and are therefore not recognised as 'foreign' by the immune system (Hale et al, 1988).

1.2.2 Abzymes or catalytic antibodies
Abzymes or catalytic antibodies are antibodies capable of catalysing a chemical reaction. They may be made by raising antibodies against transition state analogues. The activity of abzymes may be enhanced by transplanting catalytic motifs from 'natural' enzymes into the antibody binding site, using recombinant DNA techniques. Abzyme technology is a rapidly expanding field and several groups have reported the successful isolation of antibodies with a required enzymic activity. For a review see Schultz et al 1990.

1.2.3 Antibody fragments
In addition to producing new biological actives, antibody engineering is also being used to investigate ways of producing large supplies of immunoreagents at a reduced cost: by using recombinant DNA technology it has been possible to design genetic constructs which are readily expressed in microbial systems. [Microbial fermentation is potentially a much cheaper method of producing immunoreagents than the mammalian cell culture which is used to produce conventional McAbs. This is because bacteria, such as E.coli, grow much more quickly in culture than do hybridoma cells. Also bacterial growth media is much cheaper than mammalian cell culture media]. A notable success has been the production of "antibody fragments" such as Fab (Better et al 1988) or Fv (Skerra and Pluckthun, 1988) which possess the antigen binding site of an antibody but few or none of the other domains. Fv fragments have been produced in E.coli at levels up to 450 mg/litre (King et al, 1993). This is in stark contrast to conventional monoclonal antibodies which express poorly in E.coli (Boss and Ward, 1985).
In many biotechnological applications, the constant regions of antibodies are not required. As previously described, the 'natural' function of the constant region is to recruit phagocytic cells or to stimulate the complement system. Therefore, for applications other than therapy, the constant region is often surplus to requirements. Moreover, antibody fragments such as Fv or Fab may represent a source of reagents for biotechnological applications which have hitherto been excluded on economic grounds.

1.3 DESIGN OF ANTIBODY FRAGMENTS.

Many different antibody fragments have been described. They have been created by techniques in molecular biology - and to a lesser extent molecular modelling. A key objective has been to reduce fragments to a minimal size but still to retain their specificity and activity. It has been known for years that enzymatically produced fragments retained some activity; but it has taken recombinant DNA techniques to determine whether these fragments represented the ultimate binding reagent or whether it was possible to make still smaller fragments which carry a higher activity per unit weight.

1.3.1 Fabs

Fab fragments have a molecular weight of 50kD and represent the variable region and some constant region of an antibody (see Fig 1.1b, given earlier). They were first described by Porter (1959) who produced them by proteolytic digestion of whole antibodies. However, proteolytic digestion of antibodies is site-selective rather than site specific and proteolytically produced Fab contains a significant proportion of degraded, low molecular size peptides (Better et al, 1988). Recently, there have been reports of active Fab fragments being produced in E. coli by recombinant DNA techniques (Better et al, 1988; Pluckthun and Skerra, 1989). However, for reasons that are not clear, Fab fragments seem to be expressed less efficiently in E. coli than Fv or scFv (see below). Pluckthun (1992) suggests that the problem is with the periplasmic folding process, but it may be simply that the Fab fragment is just too large a foreign protein for E. coli to secrete efficiently.
1.3.2 Fv fragments

Fv fragments have a molecular weight of 25kD and comprise the variable region of one heavy chain (V\textsubscript{H}) and one light chain (V\textsubscript{L}) - fig 1.1c. At first glance, they would appear to be the smallest active antibody fragment permissible. They contain all six CDRs of a single binding site and the so-called framework regions between them.

Fv fragments were first produced by Hochman et al. who produced an Fv fragment proteolytically from an IgA (Hochman et al 1976). Fv fragments were first produced in mammalian culture by Reichmann et al (1988) and in \textit{E. coli} by Skerra and Pluckthun (1988).

1.3.3 Single-chain Fv fragments (scFv)

A perceived drawback of Fv design is that its two component chains are non-covalently associated and therefore susceptible to dissociation. This is in contrast to Fabs where the heavy and light chains are covalently linked by a disulphide bridge.

Consequently, there have been a variety of ingenious routes for improving the stability of the Fv structure. The most well-known example is to link the V\textsubscript{H} and V\textsubscript{L} by a short peptide chain which can be readily achieved by recombinant DNA techniques (Bird et al 1988). This design has become known as single-chain Fv or scFv - see Fig 1.1d. The linker has to be flexible, hydrophilic and uncharged. A commonly used linker is (GlySer\textsubscript{3})\textsubscript{4} - see Skerra et al, 1991. ScFv fragments have been shown to have improved thermostability at 37°C compared to an Fv of the same specificity (Glockshuber et al 1990); however, they also have some limitations. These limitations were elucidated by Berry (1992, unpublished data) whilst working at the Colworth laboratory, prior to registration for this thesis. A summary of the findings is given below. Firstly, scFv fragments were found to be less effectively produced in \textit{E. coli} culture than analogous Fv constructs. Secondly, prolonged storage of scFv fragments was found to result in extensive proteolysis of the peptide linker. This presumably results in Fv fragments with undesirable "tails" (i.e. the remnants of the digested linker). These tails could lead to problems such as non-specific binding. Thirdly, and most importantly, it was found that scFv fragments
were not particularly stable when subjected to some biophysical perturbations. For example, scFv anti-lysozyme was found to be completely inactivated by a series of freeze/thaw cycles. In contrast, the corresponding Fv survived the same process with very little activity loss. (see fig 1.2). This surprising result may be explained by considering the folding pathway of Fv with respect to the individual folding of \( V_h \) and \( V_l \) followed by their association (Hochman et al, 1976). Berry (1992, unpublished) hypothesised that the peptide linker connecting the C-terminus of \( V_h \) to the N-terminus of \( V_l \) may interfere with this folding pathway. Consequently, conventional Fv, although intrinsically less stable, can survive these harsh conditions by spontaneously refolding on return to a physiological environment.

Immunoglobulin fragments for industrial applications must be robust enough to cope with perturbations likely to denature them, such as: elevated temperatures, freeze/thaw, or extremes of pH. Consequently, many applications may find conventional Fv to have a more suitable structure than scFv.

1.3.4 \( V_h \) fragments or "Dabs".

\( V_h \) fragments were produced by Winter et al (1989) who described them as "Domain antibodies" or "Dabs". They have a molecular weight of 12.5kD and comprise the variable region of one heavy chain and therefore have just 3 CDRs of that antibody - see fig 1.1e - the remaining 3 are present on the light chain. It has been claimed that the \( V_h \) fragments can provide most of the binding efficiency of the Fv (Ward et al, 1989) on the grounds that most of the contacts between antigen (lysozyme in this case) and the antibody are located on the CDRs of the \( V_h \). It seems that in the case of this particular antibody, the \( V_h \) fragment can work as a functional fragment. However, since Ward's paper, only one other active \( V_h \) has been reported (Power et al 1992). The consensus that has emerged from all subsequent experience is that \( V_h \) alone does not usually embody sufficient of the antibody to retain a useful degree of specific binding (Staunton, 1990). Moreover, even when specific binding is observed, the reported affinity of \( V_h \) fragments for antigen can be 10-fold weaker than the parent monoclonal antibody. In contrast, Fv fragments have been found to have very similar affinities to the parent monoclonal antibody when comparative
Purified preparations of Fv anti-lysozyme and scFv anti-lysozyme were diluted in PBS to 1ug/ml and then sterile filtered. Each preparation was divided into three aliquots. One aliquot of each sample was used as a control and kept at 4°C until analysed; another aliquot was subjected to 3 cycles of freeze/thaw (freezing overnight at -20°C followed by thawing at room temperature); the third aliquot was subjected to 7 cycles of freeze/thaw. All three aliquots were analysed for their relative activity by making a range of dilutions in PBST and measuring signal in an ELISA.

(A) Fv anti-lysozyme (B) scFv anti-lysozyme
- control • 3 cycles of freeze/thaw ■ 7 cycles of freeze/thaw

(A) Signal in ELISA (A405nm)

Fv concentration (ug/ml)

(B) Signal in ELISA (A405nm)

scFv concentration (ug/ml)
studies have been made. (Ward et al. 1989).

Further disadvantages of V\textsubscript{H} fragments are that their expression in E. coli is very poor and that their solubility is low. Chothia et al. (1985) showed that in the native immunoglobulin molecule, the V\textsubscript{H} and V\textsubscript{L} domains bind each other by way of a mutual attraction between complementary hydrophobic patches on the two chains. It seems probable that the exposure of these patches on the V\textsubscript{H} fragment is at least partly responsible for the problems observed with expression and solubility.

1.3.5 Molecular recognition Units (MRUs)

The smallest antibody fragment described comprises a short peptide equivalent to a single CDR (fig 1.1f). Several terms have been adopted for them, "molecular recognition units", "paralog peptides", "CDR peptides". Despite the seeming improbability of obtaining a specific capture reagent with a short peptide - which can only achieve limited secondary structure - some successes have been achieved. For example Williams et al (1989) have isolated peptides which bind to the reovirus type 3 receptor.

An outstanding attraction of MRUs is that they are sufficiently short to be synthesised completely chemically by solid phase peptide synthesis (Merrifield, 1963). Consequently, there has been a particular interest in this area shown by pharmaceutical companies who often prefer chemical synthesis to biological synthesis for their actives. This is due in part to potential problems with contamination from toxins, viruses, and pyrogens for biologically produced actives.

However, despite the apparent success described above, other MRUs do not retain the specificity of their parent McAb - even when the 3D structure of the antibody is known and the MRU which makes most contact with antigen is synthesised (Berry and Davies, 1992).

A general picture seems to be emerging. It is sometimes possible to design MRUs which bind to receptor proteins - such as the reovirus type 3 receptor molecule.
They are therefore the passive partner in the interaction. It has so far proved difficult or impossible to design MRUs which actively capture "passive antigens". This point was illustrated by an MRU which could not bind the antigen against which the parent McAb was raised but did bind anti-idiotypic antibody (Berry and Davies, 1992).

1.3.6 Accessory functions 'tags' and "fusion tails".

Antibody fragments are often designed to carry short fusion 'tails', short amino acid sequences built onto the C- or N-terminus of $V_h$ or $V_l$. The C-terminus is usually chosen as the site at which to add the tail as this ensures that the tail is orientated away from the antigen binding site. (For more details and the three-dimensional structure of an Fv fragment, see chapter 3). Most of the tails described in the literature have been for the purposes of affinity purification or assay when they are often described as 'tags', and when tracer antibodies are raised against them they are known as 'epitope tags'. For example, the so-called "Flag™" tail which was designed purely as an antigenic structure, consists of 8 amino acids, the first four of which are recognised by a deliberately prepared antibody (Hopp et al 1988). The antibody was used to make an immunoaffinity column and was used in immunoassays as a detection reagent. This particular antibody only binds the "Flag™" sequence in the presence of calcium ions, so the immunoaffinity binding step is conducted in the presence of 0.3mM calcium chloride, while the elution step is in the presence of 2mM EDTA.

Another widely used antigenic tail is the myc tail (Ward et al, 1989). This is an eleven amino-acid tail from the myc oncogene product for which a monoclonal antibody (designated 9E10, Munro and Pelham, 1986) was already available. This tail is also used for assay and purification of Fv.

Perhaps the most well known non-antigen fusion tail is the polyhistidine tail (Hochuli et al, 1987; Arnold, 1991), which has been used successfully with immobilised metal affinity chromatography (IMAC). Reversible co-ordination complexes form between immobilised transition metal ions ($\text{Ni}^{2+}$, $\text{Zn}^{2+}$, $\text{Cu}^{2+}$ or $\text{Co}^{2+}$) and the imidazole...
group of histidine. This interaction is the basis for a simple, one-step, affinity purification procedure.

In addition to providing passive tags for purification and assay, it is sometimes necessary to design a fusion tail into the structure of an antibody fragment to confer an accessory function. Many potential applications will require antibody fragments to do more than just bind antigen: they may be required to adsorb to a surface, to be chemically linked to another molecule, or to be inserted into a liposome. This presents a problem: since antibody fragments have deliberately been engineered down to their minimal effective size (to allow expression in microbial systems,) they are now devoid of other domains which are used to confer accessory functions in conventional McAbs. In this context, a patent application has been filed describing the design and use of a number of fusion tails for different accessory functions (Davis et al. 1991).

Other fusion tails which have been designed to confer accessory function have been described in the literature. For example, a hydrophobic tail has been described by Laukannen et al (1993), which confers on the Fv an ability to insert into liposomes and other phospholipid membranes. The polypeptide utilised is identical to a 9-amino acid sequence of the *E. coli* major lipoprotein, which is normally acylated *in vivo* with two fatty acids. Thus the single chain Fv is expressed complete with the tail, to which the fatty acids are attached before secretion. When the complete molecule is produced in an *E. coli* host, the acylated tail causes the Fv to be anchored in the host cell membrane, so the product cannot be harvested without a membrane solubilisation step.

### 1.3.7 Modelling studies

The design of antibody fragments is sometimes aided by modelling studies, where it is possible to study the structure of proteins on a VDU screen in three dimensions.

Several antibody and antibody fragment structures have been solved and these may be accessed from the Brookhaven Protein Structure Database (established by
Bernstein et al, 1977, and continuously updated). Since the constant and framework regions adopt very similar structures from one antibody to the next (Rees et al, 1994) it only remains to model the structures of the CDRs. For most CDRs, very good models can be constructed from a knowledge of their size and amino acid sequence (Stanfield and Wilson, 1994).

It is also possible to make measurements of key distances in 3D. For example, to calculate the length required, and therefore how many amino acids needed, for a peptide linker to join a $V_H$ and a $V_L$ in a scFv construct. Similarly, when designing an antigenic tail or epitope tag it is possible to design one which is too short to reach the CDRs so as not to interfere with binding.

The combination of increasing computer power and an increasing database of known protein structures will ensure that modelling continues to be a valuable asset for the design of antibody fragments.

1.4 SYNTHESIS OF RECOMBINANT ANTIBODY FRAGMENTS:-- THE PROTEIN ENGINEERING CYCLE.

All of the antibody fragments described above, with the exception of MRUs, are most conveniently synthesised as recombinant proteins. MRUs are most conveniently synthesised by solid phase peptide synthesis (SPPS). A review of SPPS is outside the scope of this thesis; the reader is referred to an excellent text-book on the subject (Grant, 1992). The series of scientific activities which are required to produce a recombinant protein in a usable form has been described as the protein engineering "cycle". The "cycle" has been described with differing degrees of complexity and detail. The simplest description (Geisow, 1993) depicts the protein engineering "cycle" as a circle connecting three key activities: design, production and characterisation. A slightly more detailed description of the "cycle" is given in fig 1.3 which describes the protein engineering strategy used at the Colworth laboratory for producing Fv fragments in *E.coli*. In fig 1.3, the protein engineering "cycle" is depicted more like a flow diagram - with defined start and finish points. However,
Fig 1.3: The protein engineering strategy used at Colworth.

1. Characterise McAbs
2. Clone variable region genes from hybridomas
3. Assemble genes in E. coli expression system

Optimisation of Production

Recovery → Culture → Analysis → Recovery

SCALE-UP
unlike a flow diagram, cyclical elements of optimisation still feature. In particular, small amounts of purified Fv are needed to evaluate analysis systems, which in turn are required to optimise culture and recovery.

Key components of this protein engineering strategy and a brief description of the state-of-the-art are outlined below:-

1.4.1 Cloning Immunoglobulin genes

The key activity for the molecular biologist in the protein engineering cycle is the cloning of variable region genes from the hybridoma of interest. Once this has been achieved, it is relatively straightforward to: assemble them in vectors that express Fv; to add $C_L$ and $C_H$ constant region genes so that the product is an Fab; to include DNA which codes for a peptide linker so that the product is an scFv; or to add DNA that codes for a fusion tail.

Cloning immunoglobulin variable region genes is facilitated by taking advantage of the fact that framework regions are fairly well conserved from one antibody to the next. Therefore, DNA primers hybridising to the termini of framework 1 and framework 4 (the two extremities of the variable regions) can be used as generic primers.

Recently, there have been two important developments which have greatly speeded up the cloning of variable region genes. Firstly, the use of the polymerase chain reaction, or PCR, (Saiki et al., 1985) - where DNA is amplified by many orders of magnitude - typically in a 2 hour reaction. This provides sufficient DNA to clone straight into expression vectors. In brief, DNA primers are designed to hybridise to framework regions and also to incorporate restriction sites to facilitate cloning into expression vectors (Orlandi et al., 1989). There is no doubt that PCR has increased the speed of cloning but it is not without its problems. Foremost amongst these, is the problem of amplifying unwanted DNA sequences - either contaminants, from other experimenters in the laboratory, or so-called 'rogue sequences' (variable region DNA sequences which hybridoma cells transcribe but do not translate).
The second important innovation in cloning variable region genes is 'phage-display' technology (Barbas et al 1991; Clackson et al 1991). In brief, a library of genes is cloned and displayed at the surface of filamentous bacteriophage - thereby facilitating the isolation of antibody fragments of the desired activity and the genes encoding them. The technique undoubtedly has a powerful potential but its achilles heel is the tendency for phage to adhere non-specifically in detection systems - and thereby leading to the isolation of falsely positive phage.

1.4.2 Expression of antibody fragments in *Escherichia coli* cultures.

Expression of antibodies, or their fragments has been reported in several host organisms including filamentous fungi (Nyyssonen et al., 1993); higher plants (Hiatt and Ma, 1992); and even the silkworm (Reis et al., 1992). However, in the short and medium term, *Escherichia coli* will be the organism of choice due to the abundance of appropriate cloning and expression vectors. Furthermore, titres of 450 mg/litre have been reported for one Fv specificity in *E.coli* (King et al; 1993). However, titres of this level are the exception rather than the rule.

Despite these promising beginnings, production remains an empirical science, and each antibody fragment seems to be individual in its behaviour. For example, the Colworth group found that two Fv fragments with an extremely high level of sequence homology had radically different expression efficiencies (Table 1.1).

For each antibody fragment, a painstaking and labour-intensive optimisation of expression needs to be undertaken. The key parameters (including fermentation temperature, *E.coli* strain, plasmid type, growth media, induction time, harvest time) are systematically varied until near-optimal conditions are elucidated.

Another parameter relevant to expression was discovered by the Colworth group. It was found that a given clone bearing the gene encoding Fv anti-lysozyme could give two different colony types - 'small cream' colonies and 'large grey' colonies; the former gave good yields of Fv whilst large grey colonies gave very poor yields. The yield was very definitely affected by temperature of the culture system, with
Table 1.1. Fv yield in *E. coli* culture: dependence on Fv sequence.
The expression of an Fv specific for hen egg lysozyme (HEL) was compared with that of an Fv specific for placental alkaline phosphatase (PLAP). Although the two Fvs had very similar sequences and physical characteristics, their expression in *E. coli* was radically different when using identical culturing conditions (essentially those published by Ward et al., 1989 and with a culturing temperature of 37°C).

Yields are expressed as soluble Fv in the culture supernatant. This was assumed to be representative of the total yield of the culture since Fvs are not known to form insoluble aggregates. However, it should be noted that single-chain Fvs may also produce insoluble aggregates in the cytoplasm and periplasm (Somerville et al., 1994) in addition to any soluble protein detected in the supernatant.

The data in Table 1.1 were produced by the Colworth group.

<table>
<thead>
<tr>
<th>FEATURE</th>
<th>Fv anti-HEL</th>
<th>Fv anti-PLAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall identity</td>
<td>100%</td>
<td>81%</td>
</tr>
<tr>
<td>Framework identity</td>
<td>100%</td>
<td>94%</td>
</tr>
<tr>
<td>Overall charge</td>
<td>+3.2</td>
<td>+1.4</td>
</tr>
<tr>
<td>Hydrophobic AAs</td>
<td>61</td>
<td>67</td>
</tr>
<tr>
<td>Polar AAs</td>
<td>89</td>
<td>93</td>
</tr>
<tr>
<td>Expression vector</td>
<td>pUC19</td>
<td>pUC19</td>
</tr>
<tr>
<td>Host</td>
<td><em>E. coli</em></td>
<td><em>E. coli</em></td>
</tr>
<tr>
<td>Soluble Fv in culture supernatant</td>
<td>&gt;100 mg/litre</td>
<td>&lt;1 mg/litre</td>
</tr>
</tbody>
</table>
37°C being far superior to 25°C (Table 1.2). However, it is important to note that Fv anti-lysozyme may not be typical in this respect. Researchers working with different Fvs have reported improved yields by culturing at temperatures that are sub-optimal for growth i.e. below 37°C (Skerra et al., 1991; Anand et al., 1991). This phenomenon was explained by Somerville et al. (1994) who found that the expression of antibody fragments is toxic to the cell and results in reduced cell viability. The toxic effect is made worse by increased Fv synthesis at the higher temperatures. Therefore, culturing at reduced temperatures, such as 25°C can result in improved yields for some systems.

1.4.3 Analysis of expression in cultured media.

The optimisation of culture conditions requires a reliable experimental-base to analyse expression. The expression of conventional McAbs in culture can be analysed by established immunoassay systems for which mouse immunoglobulin standards are available. In contrast, reliable, quantitative immunoassay systems are not readily available for analysing antibody fragments in culture. Therefore, many research groups opt to record expression as recoverable antibody fragment - that protein retrieved from an antigen affinity column (King et al, 1993; Anthony et al, 1992; Riechmann et al, 1988). Indeed, this approach was used by the Colworth group to generate the data given in tables 1.1 and 1.2. However, there are many drawbacks with using antigen affinity chromatography in this way. Firstly, the technique cannot detect whether any inactive material is being produced which does not bind the column. Secondly, the technique is eminently unsuitable for assaying literally hundreds of samples which are generated in an optimisation study as described in the previous section. In brief, this is currently a weak link in the protein engineering cycle.

Analysis systems are also required to measure the efficiency of recovery protocols; and therefore they are an essential tool for recovery optimisation.

1.4.4 Recovery of antibody fragments from cultured media.

Fab fragments can be recovered from cultured media on a protein G column as they
The clone bearing the genes encoding Fv anti-lysozyme was found to give two colony types when streaked out on agar plates - "small cream" colonies and "large grey" colonies. Different colony types were found to give different yields of Fv when cultured in shake-flasks. Furthermore, the magnitude of this difference was temperature sensitive. The data in Table 1.2 were produced by the Colworth group; yields are given as milligrams of Fv protein per litre of culture.

<table>
<thead>
<tr>
<th>Colony type</th>
<th>Culturing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25°C</td>
</tr>
<tr>
<td>small cream</td>
<td>0.15 mg/L</td>
</tr>
<tr>
<td>large grey</td>
<td>&lt;0.1 mg/L</td>
</tr>
</tbody>
</table>
possess sufficient constant region to bind (Carter et al, 1992). However, since smaller fragments do not bind protein A or protein G in a predictable manner, alternative methods are required.

Fv antibody fragments are usually arranged to be produced in the supernatant of cultured media. This is achieved by prefixing $V_H$ and $V_L$ by leader sequences such as pel B (Lei et al, 1987) or omp A (Movva et al, 1980) which direct synthesis into the periplasm. It is then possible to optimise growth conditions so that cells gently lyse and release periplasmic contents into the medium. From the culture supernatant, a variety of techniques have been proposed and tried for recovering target Fv. The most widely used is antigen affinity chromatography (Riechmann et al 1988; Anthony et al 1992; King et al 1993). The outstanding advantage of using antigen affinity chromatography is that quality assurance is built into the process:- Fv recovered must be capable of binding target antigen and is therefore known to be active. However, a major limitation, is when the antigen is labile, prohibitively expensive, or in short supply.

Another published route is to tag the Fv with a polyhistidine tail and to recover by IMAC (Skerra et al 1991). However, a major limitation is that inactive as well as active Fv may be recovered. Also, there is a potential problem of other proteins with exposed histidines binding to the column. These would appear as contaminants in the Fv preparation.

1.5. PERFORMANCE OF ANTIBODY FRAGMENTS IN A MODEL APPLICATION.

Despite the early optimism which accompanied expression in microorganisms, it was important to determine whether these new antibody fragments maintained some or all of the attractive features of whole McAbs which had made them so successful in the biotechnology industry. Relevant features include:- specificity, affinity, robustness, and the ability to be immobilised on surfaces. Whether these new antibody fragments did indeed possess these features was systematically investigated by the use of a biorecovery system as a model application.
This investigation was carried out in the Colworth laboratory between 1990 and 1992. The author was the lead scientist in the investigation and the resulting publications are bound in at the back of this thesis. The results are outlined below in sub-sections 1.5.2 - 1.5.4.

1.5.1 Selection of Immunoaffinity chromatography (IAC) as a model application

Immunoaffinity chromatography (IAC) was a particularly pertinent application for testing the performance of antibody fragments. The technology is widely used as a research tool where exquisitely specific separations can be achieved in a single-step (Jack and Wade, 1987); however, it is very rarely used in industrial-scale separation processes (Jones 1991). A primary reason for this dichotomy has been the high cost of McAbs. Although other disadvantages such as instability and proteolysis of McAbs have also been cited, taken in the context of the practise of pharmaceutical companies using ion-exchange media once and then discarding, these other objections can be reduced to one of cost. It is therefore not surprising that both of the two reported production-scale applications of IAC have been for purifying high value biopharmaceuticals [factor VIII (Kaufman, 1989) and interferon (Secher, 1993)].

The potential of antibody fragments in IAC was systematically evaluated against a range of criteria. Fragments specific for hen egg lysozyme were used as these fragments were some of the first to become readily available to the scientific community. A model biorecovery system was designed in which the fragments were immobilised on chromatographic media and used to recover lysozyme from crude feedstocks. The results of this investigation are outlined below.

1.5.2 A comparison of Fvs with conventional monoclonals (see Berry et al. 1991.)

In the first study, it was found that Fv did indeed retain two key attributes of the parent McAb, specificity and affinity. As a result, affinity adsorbents comprising Fv could recover homogeneous lysozyme from crude feedstock in a single-step.

The model of recovering lysozyme from crude feedstocks using immobilised Fv anti-lysozyme was used for further studies to determine the relative merits of Fv
fragments and the larger McAb. This was investigated by immunostaining the captured antigen in immunoadsorbent silica particles and examination under the electron microscope. (Silica was chosen as it is a popular medium for analytical HPLC and also preparative-scale separations. This is due to its ability to withstand high flow-rates). It was found that silica derivatised with Fv could capture antigen throughout the internal porous structure of the particle, whereas silica derivatised with McAb could only capture antigen onto the surface of the particle. It was concluded that Fv fragments are sufficiently small to be immobilised within the pores of silica and still leave room for specific capture of antigen. In the model purification system this conferred an advantage of a 5-fold capacity increase in favour of immunoadsorbents comprising Fv.

1.5.3 A comparison of Fvs with other antibody fragments (see Berry and Davies, 1992)

In the second study, three different antibody fragments were compared; Fv, V_H and a molecular recognition unit or 'MRU'. It was found that the immunoadsorbent comprising Fv could recover homogeneous lysozyme from crude feedstock in a single-step, indicating that the Fv fragment had a high specificity and affinity for antigen: other researchers have reported it to be comparable to that of the parent McAb (Ward et al, 1989). The immunoadsorbent comprising V_H had problems with non-specific binding, indicating that the V_H fragment had reduced specificity for target antigen. The immunoadsorbent comprising MRU did not bind antigen at all, although it did bind anti-idiotype antibody very specifically. Therefore attention has since focused on Fvs, as they appear to be the smallest antibody fragments which bind antigen in a predictable manner. However, it is stressed that specific binding of antigen with MRUs has been achieved for some antibodies (Williams et al, 1989) and it is currently unclear why some MRUs are active and some are not.

1.5.4 Stability of Fv fragments (see Berry and Pierce, 1993).

In the third study, the potential for improving Fv stability by protein engineering was examined. As an example, the stability of Fv anti-lysozyme was compared with the equivalent single-chain Fv mentioned earlier. Automated equipment was designed
and used to run the model purification system of recovering lysozyme form crude feedstock for 100 cycles non-stop. Immunoadsorbents comprising scFv were extremely stable and maintained full starting capacity even after 100 cycles of loading and desorbing target antigen. This excellent stability was achieved by using a desorbing buffer of 4M MgCl₂. It was hypothesized that this high salt buffer promoted association between Vₙ and Vₙ (which is known to be primarily via interaction of hydrophobic patches). Thus inactivation of scFv - as described in subsection 1.3.3. - was avoided. Immunoadsorbents comprising conventional Fv were also remarkably stable; only 25% of starting capacity was lost in 100 cycles. The rationale for this outstanding stability is probably that the Fv structure is maintained during harsh elution conditions as a result of being immobilised.

1.5.5 Conclusions from immunoaffinity chromatography (IAC) model
The investigation in IAC had found that Fv anti-lysozyme (although not Vₙ or MRU) possessed the key attributes of specificity, affinity, robustness, and the ability to be immobilised on surfaces. [These findings have been used to support a patent application which claims immunoadsorbent materials comprising Fv fragments. A copy is bound in at the back of this thesis (Berry et al, 1991b)]. As all Fvs have a similar overall structure (by virtue of conserved framework regions) it seemed probable that other Fv specificities, once available, would also possess these attractive properties. This would make them suitable not only for IAC, but also for many other applications. Given this conclusion together with the relative merits of Fv over Fab and scFv (described in 1.3.1. and 1.3.3. respectively), the Colworth group decided to focus their future efforts on Fvs in preference to other fragment designs.

1.6 INDUSTRIALISATION OF ANTIBODY FRAGMENTS.
1.6.1 Cost considerations and hurdles to be overcome.
The critical question for industrial applications outside the fields which have been penetrated by conventional McAbs (i.e. diagnostics and therapy) is whether the costs of production can be brought down to the level currently achieved for mass produced enzymes. The two features of antibody fragment technology which make this
ultimate goal seem plausible are: firstly, that they can be expressed in microbial cultures (for which cheap growth media and fermentation scale-up are readily available); and secondly, that they represent a more focused binding reagent than conventional antibodies and therefore more binding activity is contained per unit protein, thus representing a saving in cellular metabolism in their synthesis.

However, exploitation of antibody fragment technology will require: reliable and rapid cloning of variable region genes, stable expression coupled with high yields, low cost recovery processes and, of critical importance, high-quality analysis systems which can reliably and accurately quantify the concentration and activity of antibody fragments. Analysis systems will have input at three key stages. Firstly, after cloning a new antibody specificity, analysis systems will be needed to confirm that the target protein can be expressed (initially at very low levels) and thereby to confirm that the correct genes have been successfully cloned. Secondly, during the optimisation of production, analysis systems will be needed to compare and rank the performance of different fermentation and purification protocols. This will enable lead options to be taken through to scale-up with a high degree of confidence. Thirdly, if and when scale-up has been successfully achieved, analysis systems will be required for process monitoring and for quality assurance.

In essence, all the components of the protein engineering cycle, given earlier in fig 1.3, will need to be optimised; all the current weaknesses pinpointed in 1.4 will need to be overcome. A few publications exist (Skerra et al, 1991; Anthony et al 1992) for a few favoured antibodies purporting to indicate that most or all of this is in hand, but in truth there is no generic technology and each of these areas is fraught with difficulty and disappointment.

Success adjudged against these criteria will only be achieved by a systematic programme of fundamental research targeted at each stage of the protein production. If this research is successful, the prize for industry will be a range of application opportunities for fine chemicals with specific binding activities. One of these opportunities, that of separation technology, is considered in more detail below.
1.6.2 Application opportunities in separation technology.

An important opportunity for the pharmaceutical industry is the separation of optical isomers. It has been estimated that more than 50% of new drugs coming onto the market are chiral and often only one of the optical isomers provides the desired function, while the other may be inactive or have completely different actions. A notorious example is thalidomide - one isomer effectively prevented nausea during pregnancy, the other isomer caused severe malformation in the limbs of developing foetuses (De Camp, 1989). Since the thalidomide case, product safety has taken a much higher priority and optically pure products are more likely to be approved by the FDA, even if the inactive isomer is thought to be harmless. Clearly, a chiral-specific synthesis is the preferable route to producing an optically pure product because this involves no wastage. However, many industrial chemical processes produce a 50/50 mixture of both chiral forms, and so the onus is on the pharmaceutical companies to separate out the active isomer. There are several chiral-specific separation media available, a well-known example of which is immobilised bovine albumin (Anderson et al, 1992). However, the mechanism of action of this media is poorly understood and separations are of unpredictable quality. In contrast, chiral separations of extremely reliable quality can be achieved by using immobilised antibodies which are specific for only one of a pair of optical isomers. This approach to chiral separations was first reported in the mid-1980s (Knox and Galfre, 1986), but, due to the high cost of conventional McAbs, the technology could never be cost-effective at large-scale. However, the onset of fragment technology could change the economics of antibody production, and it may soon become commercially viable to separate optical isomers by immunoaffinity chromatography.

Another opportunity in separation technology is the removal of chemical pollutants from the environment (Harris, 1991). Recent EEC legislation and guide-lines have placed increasing demands upon industry to clean-up their effluent streams. Legislative and safety requirements aim to ensure that organic pollutants are present at less than 0.5 - 1.0 ng/litre in drinking/river water and at less than 10-50 ng/litre in sewage and trade effluents. These target levels are far too low for precipitation methods to achieve successful removal of pollutants. Moreover, there is an urgent
requirement for a chelation technology which is effective at these molarities and for a chelation matrix which could be easily recycled. These are requirements which immobilised antibody fragments could fulfil.

Other examples of the potential use of immunoaffinity chromatography in industrial separations have been described in detail elsewhere. These include:- the recovery of recombinant proteins from fermenter feedstocks, especially when the target protein has been 'tagged' by a short peptide epitope (Sassenfield, 1990); the recovery of added value food ingredients from bovine milk (Kawakami et al, 1987); and the purification of industrial enzymes (Berry, 1991). All of these processes were extremely efficient at small-scale; but were not commercialised due to the drawbacks of conventional McAbs, as described previously. However, the economics of many of these processes will become much more favourable with the onset of Fv fragment technology.
2. OBJECTIVES AND SCOPE OF THIS THESIS.

2.1 AN OVERVIEW

This PhD project aimed to use leading-edge biochemistry to deliver benefits to the processes required for the production of Fv antibody fragments and thereby to make a focused contribution to the international effort that will be required to bring about the reduction in costs described in 1.6.1. More precisely, the project aimed to provide original solutions to the problems encountered during the analysis and recovery of Fv fragments that have been produced in *Esherichia coli*. Both of these embrace some common principles and may well be related in micro and macro systems. Particular attention was paid to the challenge of providing generic analysis and recovery systems that could process a family of Fv proteins, each with a different specificity.

The particular requirements to fulfil each sub-objective are considered in more detail below.

2.2 DESIGN AND EVALUATION OF NEW ANALYSIS SYSTEMS.

2.2.1 Generic tracer reagents

The expression of conventional monoclonal antibodies in culture can be analysed by immunoassays which use the generic binding proteins, protein A and protein G, or 'tracer antibodies' raised against the Fc region. Since none of these reagents interact with Fv, new binding reagents will be required.

A popular approach to tackling this problem is to tag the antibody fragment with short peptide epitopes for which tracer antibodies are readily available (Ward, 1989; Power et al, 1992). However, a potential disadvantage of this technique is that the presence of tags may be expected to have unpredictable effects on the expression, function, or toxicity of Fv fragments (Sassenfeld, 1990). Furthermore, these tags are thought to be susceptible to proteolytic cleavage (Ward et al, 1989) and so it may not be possible to design a quantitative analytical immunoassay with the tag system.
A novel approach would be to identify conserved motifs on Fv fragments - such as framework sequences - which could be used to raise generic tracer antibodies. An objective of this project was to take advantage of a side effect of the polymerase chain reaction (PCR). It was reasoned that Fv fragments whose genes have been cloned using 'generic' PCR oligonucleotide primers will carry an identical peptide 'motif' at their termini. (This concept is the subject of a patent application, Berry 1994, a copy of which is bound in at the back of this thesis). This project set out to test the feasibility of this approach, by raising rabbit antibodies against the C-terminal motif of the Vh chain and evaluating them for their utility as reagents for analysing Fv expression in *Escherichia coli* cultures.

2.2.2 Analysis systems which are compatible with culture supernatants.

Analysis systems need to function in culture supernatants, often with high biomass. This can be a problem when the analysis comprises an immunoassay since cell debris and other material can interfere with the specificity of binding at each stage of the assay - thus leading to inaccurate data. Therefore, a variety of different feedstocks needs to be analysed in the immunoassay system under investigation to determine the effect of non-specific biomass on the assay signal. The most thorough way of achieving this is to determine the signal that is generated in the immunoassay by a set of Fv standards made up in saline and then to repeat the exercise using the same set of standards but made up in a 'negative' culture supernatant.

This raises the question as to what a 'negative' culture should comprise. Initially, an obvious choice would be to use a supernatant which has been derived from the usual *E. coli* strain (JM109) and possessing the usual plasmid (pUC19), but containing no Fv genes. However, biochemical analysis found such a supernatant to be an inappropriate 'negative' - due to it containing very few *E. coli* proteins. In contrast, *E. coli* cultures which are actively producing Fvs contain much more protein - and therefore potential contaminants for interfering with immunoassays. This extra protein is due to cell lysis resulting from Fv accumulating in the periplasm. (Somerville et al., 1994).
What is required is either 'positive' supernatants which have then been totally depleted of Fv by antigen affinity chromatography or 'positive' supernatants containing Fv of a different specificity to the one under test - and therefore should not produce a signal in the assay.

It is inevitable that 'negative' supernatants will interfere with immunoassays to a certain extent. However, for assays to be quantitative, this interference should be minimal and preferably less than 10% of specific signal. Therefore, an important objective of this project was to estimate the extent to which analysis systems were susceptible to interference from non-specific biomass. Immunoassays which showed little or no interference could then be taken forward for analysing Fv in *E. coli* cultures. This validation of analysis systems has not been reported by other researchers in the field.

2.2.3 Analysis systems which measure Fv protein and Fv activity independently.

Most analysis systems which have been previously described measure binding activity units (Ward et al., 1989; Schmidt and Skerra 1993). Whilst this is probably the most critical parameter to measure - as it is the property required for applications - there are other parameters which should also be measured. The most important of these is Fv protein - whether active or not. If these two parameters are measured in parallel it gives the fermentation technologist a measurement of the % activity of his product:- there are many problems which could arise during fermentation, leading to the production of inactive Fv. Examples include: an over production of one of the polypeptide chains (*V*h and *V*l), poor association of *V*h and *V*l, incorrect folding of assembled Fv, or a deleterious mutation. It is possible - even likely - that different Fvs and different culturing conditions will lead to different specific activity or 'immunoreactivity' of the Fv that is produced in culture. Therefore, to optimise fully the culturing conditions, an analysis based on binding activity units alone is not sufficient. What is required is an independent measurement of Fv protein.

Fv protein (regardless of activity) has been determined in culture by immunoblotting techniques (Breitling et al, 1991). However, these investigators did not quantify their
Fv by cross-reference to a set of standards (see subsection 2.2.4) or compare Fv protein with active Fv. In contrast, this project set out to design an analysis system capable of measuring Fv protein and active Fv in parallel and in quantitative terms.

2.2.4 A method for producing a set of standards

A key objective of this project was to be able to establish the authenticity of an Fv preparation, in quantitative terms. For analysis systems to achieve this, signals generated by test samples need to be read against signal generated by a set of thoroughly characterised and quantified standards. Such Fv standards have not been reported by other researchers in the field. This project aimed to prepare and evaluate a set of standards according to the strategy given below:

The first requirement is to purify a stock preparation of Fv to homogeneity and then to quantify it for different parameters. Total protein can be conveniently measured by the absorbance at 280nm ($A_{280}$). This is a robust, reproducible, precise measurement. Furthermore, the extinction coefficient can be readily calculated from the sequence of the target Fvs - absorbance at 280nm is defined by the relative quantity of tryptophans, tyrosines, and phenylalanines (Wetlaufer 1962). Since genes encoding Fvs will have been sequenced as a matter of course, this is a straightforward objective. Once the stock preparation of Fv has been characterised in this way, it can be diluted to make a set of standards against which analytical procedures determining Fv protein can be cross-referenced.

The specific activity of an Fv preparation, or "immunoreactivity", can be measured and in contrast to enzymes, antibody molecules appear to be either completely active or completely inactive. This opinion has been strengthened by recent research using analytical biosensors: it was found that an antibody preparation of high concentration but low specific activity behaves exactly as though it were a preparation of fully active antibody but at a low concentration. (Gill and Hoare 1994). Therefore, immunoreactivities can be conveniently determined by passing a test sample down an antigen affinity column and determining the proportion which binds and is active - results can be expressed as a percentage. Once the stock preparation has been characterised in this way, it can be diluted to make a set of standards against which
analytical procedures determining Fv activity can be cross-referenced. Since the same stock preparation can also be characterised for Fv protein content, as described above, it should be possible to assign values to test samples in units of milligrams of active Fv.

The problem of standardising analytical procedures has been largely overlooked by other researchers, thereby making it difficult or impossible to compare the expression levels achieved in different laboratories. There is an urgent requirement for collaborating scientists to use thoroughly prepared and characterised standards so that these comparisons can be made. This project aimed to evaluate the above strategy as a generally-applicable approach for preparing such standards.

2.2.5 Elucidation of storage conditions for standards.
It is critically important that standards - once prepared - are stored in conditions which protect the properties against which they are measured. This is particularly relevant for activity standards. The timespans permissible for storage need to be determined -and fresh standards need to be prepared when these are exceeded. If this is not achieved, fermentation technologists will be generating progressively (and artificially) higher yields as the standards deteriorate in activity relative to Fv in the (fresh) culture. This project aimed to investigate the effect of different storage conditions on Fv activity and to make recommendations on which conditions to use for storing standards.

2.3 DESIGN AND EVALUATION OF A NEW RECOVERY SYSTEM.
2.3.1 Design of low affinity ligands for specific capture and mild release
This project aimed to design and test a new process for recovering Fv from E.coli cultures. It was considered that the new process should be based on the principle of antigen affinity chromatography so that immunoreactive Fv would be recovered. However, the new process should be free from some or all of the limitations of conventional antigen affinity chromatography.
One of the most severe limitations of conventional antigen affinity chromatography is that the affinities of some Fvs for antigen are so high that it is impossible to design elution buffers which release Fv without inactivating it. An approach to alleviate this problem would be to use antigen analogues - which recognise the Fv specifically but with reduced affinity because their molecular 'shape' resembles (but is not identical) to the target antigen.

An intriguing example for investigation would be to use Fv fragments specific for steroids where nature has provided a set of cross-reacting analogues (i.e. other steroids with similar structures) which bind with different affinities. It should be possible to synthesise specific, low affinity adsorbents, by immobilising one of the lower cross-reactants on an appropriate chromatography medium. Furthermore, due to the low molecular weight of steroids, it may be feasible to elute bound Fv from the column with a correspondingly higher affinity steroid analogue - thereby achieving specific adsorption and specific desorption - potentially all in mild conditions such as saline.

This project set out to investigate the feasibility of this idea using the example of an Fv which has a primary specificity for estrone-3-glucuronide and which cross-reacts with estriol-3-glucuronide and with estrone.

2.3.2 Identification of optimal desorption conditions by measuring the efficiency of the process.

The most critical step in the design of an immunoaffinity chromatography process - whether using immobilised antibody to purify antigen or immobilised antigen to purify antibody - is the choice of desorption buffer (Jack and Wade, 1987). Conventional desorption buffers, such as an extreme of pH, have been found to damage many target proteins. However, by a careful optimisation of the whole process, it is sometimes possible to identify desorption buffers which can efficiently release the target protein without inactivating it. A particularly elegant example (described by Tharaken et al., 1990) was to select an antibody which only binds to its target in the presence of magnesium ions. Desorption was achieved with the use
of citrate- or EDTA-containing buffer.

If mild desorption conditions can be identified, all the advantages of immunoaffinity chromatography: - sharp breakthroughs, single-step processes, exquisite specificity, make it a very attractive technique. Therefore, it is essential to carry out a thorough optimisation to determine preferential desorption conditions for the particular affinity process under investigation. This can only be done in combination with analytical techniques which can be used to determine the efficiency of the process. For example, the immunoassay systems designed to quantify Fv (as outlined in section 2.2) would be very useful tools for determining the % of Fv that could be recovered from an *E. coli* culture by antigen affinity chromatography. This procedure could be carried out for a number of different desorption conditions, thereby identifying which are optimal. A popular method for desorbing Fv fragments from antigen affinity columns is to use buffers with an extreme of pH (Ward et al., 1989; King et al., 1993). However, these researchers did not determine the % -efficiency for their recovery processes. Therefore, it was unclear whether an optimisation of the desorption buffers would be likely to be beneficial.

This project set out: to design a method for determining the % -efficiency of the recovery process described in 2.3.1; to use this method to identify the elution conditions that are optimal; and to compare the % -efficiency of this new recovery process with previously published protocols.
3. PREPARATION AND EVALUATION OF REAGENTS DESIGNED FOR ANALYSING FV FRAGMENTS IN CULTURE.

3.1 INTRODUCTION
Fv antibody fragments can be readily expressed in *E. coli*, however, a raft of underpinning technologies will be required to enable the reproducible supply of high-quality Fv batches. For example, analytical immunoassays or sensors will be required for controlling Fv production in microbial culture. Conventional monoclonal antibodies are routinely assayed by the generic binding proteins protein A and protein G but as these proteins do not bind Fvs (Derrick and Wigley, 1992), new binding reagents will be required. One approach is to tag the Fv fragments with peptide sequences for which binding reagents are already available. For example, Fvs have been tagged with epitopes - short amino acid sequences against which antibodies have been raised (Ward et al., 1989). A disadvantage with this approach is that the presence of tags may be expected to have unpredictable effects on the expression, function, or toxicity of Fv fragments (Sassenfeld, 1990). Furthermore, these tags are thought to be susceptible to proteolytic cleavage (Ward et al, 1989) and so it may not be possible to design a quantitative analytical assay with the tag system.

From the above, it will be clear that the design and preparation of generic binding reagents which are specific for untagged Fvs will greatly facilitate the analysis of antibody fragments in culture. An important first step towards designing generic binding reagents is the identification of motifs or epitopes present on all Fvs. It was reasoned that Fv fragments whose genes have been cloned by using the Polymerase Chain Reaction (PCR) would carry identical peptide motifs at the termini of their *V*<sub>H</sub> and *V*<sub>L</sub> chains (as determined by the PCR primers used); and that these motifs could provide an opportunity for designing generic binding reagents. [For a fuller description of this invention, refer to the patent application - Berry 1994 - bound in at the back of this thesis]. In the Colworth laboratory, monoclonal antibodies (of known specificity and function) are converted into Fv fragments so as to take advantage of the greatly improved expression systems for Fvs. In brief, *V*<sub>H</sub> and *V*<sub>L</sub>
genes are amplified from mRNA isolated from hybridoma cells using the PCR primers described by Orlandi et al. (1989). Cloned DNA is then assembled in the plasmid pSW1, described by Ward et al. (1989). The same (or very slightly modified) primers and the same plasmid are used for cloning all the Colworth Fvs (which consequently carry the same peptide motifs at their termini). These conserved motif sequences provide an opportunity for universal epitopes - antibodies raised against these sequences should bind all antibody fragments, derived from this process, if the motifs are sufficiently exposed to be available for immunochemical capture. The essential difference between this approach and epitope tagging (Munro and Pelham, 1986; Hopp et al., 1988) is that the PCR-determined motifs are very similar to the native sequence in the same region and are, therefore, unlikely to affect the properties of the Fv fragment.

Model 3D-structures of Fv fragments predict that the C-termini of $V_H$ and $V_L$ are superficial and distant from the binding site, whereas the N-termini are buried and close to the antigen binding site (Pluckthun and Pfitzinger, 1991; also see Fig 3.1). Therefore, the C-terminal motifs were the most attractive target epitopes for investigation. Attention was focused on the C-terminal motif of $V_H$ as it has the added advantage of being present on both dAbs and Fv; therefore, antibodies raised against this motif could be used to bind either of these two fragment designs. The objective of this study was to raise antibodies specific to the $V_H$ C-terminal motif (GQGTTVTVSS) and to assess their ability to act as generally applicable reagents for the analysis of Fv in microbial cultures. Most of the data in this chapter relates to preparation and evaluation of a rabbit polyclonal reagent. However, a mouse monoclonal antibody specific for the motif was also raised and a brief description of its properties is included.

3.2 METHODS

3.2.1 Preparation of immunogens and peptides carrying the motif sequence

Two classes of immunogen were used. Firstly, whole Fv protein [Fv anti-lysozyme, produced and purified according to the method of Ward et al (1989)]. This protein carries the motif sequence, and since the motif is thought to be exposed - see fig 3.1
Fig 3.1: 3D-model of Fv structure

Only the $\alpha$-carbon backbone is shown. The N termini of $V_H$ and $V_L$ are indicated as solid circles; C termini are arrowed. Coordinates$^1$ for mouse Fv anti-hen egg lysozyme$^2$ were obtained from the Protein Data Bank (Bernstein et al., 1977; Abola et al., 1987) at Brookhaven National Laboratory.

$^1$ Entry 1HFM

- it was a suitable candidate immunogen for raising a motif-specific response.

The second class of immunogen comprised derivatives of the motif which had been synthesised chemically by the method of Merrifield (1963). The peptide sequence KKGQGTTVTSS was synthesised, hereafter referred to as motl. Motl corresponds to the motif sequence with two additional lysines at its N-terminus. The addition of these lysines was found to improve the solubility of the peptide and to facilitate immobilisation. Motl was prepared as free peptide, as a multiple antigenic peptide, or "MAP", (Tam, 1988; McLean et al., 1991), and as a conjugate chemically linked to plant protein derivative (PPD). [All peptide synthesis and conjugation to PPD was undertaken as a customised order at Peptide and Protein Consultants, Exeter U.K.].

3.2.2 Inoculation of rabbits
Rabbits were inoculated sub-cutaneously with immunogens (either Fv protein or the MAP) at a concentration of 1 mg/ml in Freund's complete adjuvant, then boosted 30 days later with the same preparation in Freund's incomplete adjuvant. Test bleeds were taken on day 40 and were evaluated by ELISA for their response to motif-peptide and to Fv protein, as described below.

3.2.3 Evaluation of the immune response in rabbits by ELISA.
Solid-phases for enzyme linked immunosorbent assays (ELISAs) were prepared by sensitising microtitre plates with motl-PPD or Fv protein. As a negative control, plates were also sensitised with PPD only. Detailed protocols are set out below.

Motl-PPD (0.1 \( \mu \text{g/ml} \)), PPD alone (0.1 \( \mu \text{g/ml} \)) or Fv anti-lysozyme (1 \( \mu \text{g/ml} \)) were adsorbed onto microtitre plates overnight using sensitisation buffer (0.05M carbonate, pH10). Plates were washed with PBST [0.01M \( \text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4 \) - 0.15M NaCl, pH7 with 0.15% Tween 20 (Sigma)] and then incubated for 1 hour at room temperature with dilutions of test serum made up in PBST. Plates were then washed again with PBST before incubation with a 1/1000 dilution of goat anti-rabbit/alkaline phosphatase conjugate (Sigma). Plates were washed with PBST before development for approximately 30 minutes with substrate [para-nitrophenyl phosphate, pNPP,
3.2.4. Affinity purification of motif-specific antibodies from rabbit serum.

5mgs of the free peptide motl were immobilised onto 3ml wet volume of CNBr-activated Sepharose 4B (Pharmacia), according to manufacturer's instructions (also see Price et al., 1991). The motl-Sepharose was packed in a chromatography column (Pharmacia) and incorporated into a standard liquid chromatography set up (Pharmacia). Rabbit antisera were then loaded onto the column which was washed with PBS until the monitor had returned to baseline. Bound material was eluted with 4M MgCl₂ made up in distilled water. The eluted fraction was dialysed overnight into PBS. The immunospecificity of the affinity purified fraction was analysed by the ELISA systems described above in 3.2.3. Results were compared with those obtained for whole serum and the column fall through after making appropriate dilutions.

3.2.5 Evaluation of affinity purified rabbit anti-motif antibodies.

3.2.5.1. Direct ELISA.

Motif specific rabbit antibodies were tested for their ability to bind each of three Fv fragments [Fv anti-hen egg lysozyme, Fv anti-human chorionic gonadotropin (hCG), and Fv anti-glucose oxidase (G.Ox)] in a direct ELISA system. Each Fv preparation had been produced in shake-flasks [according to the method of Ward et al. (1989)] in E. coli. strain JM109; and then purified to homogeneity on antigen affinity columns [lysozyme (Sigma), hCG (Sigma), or G.Ox (Sigma) - as appropriate - immobilised on CNBr-activated Sepharose 4B (Pharmacia)]. Each Fv preparation was individually quantified by measuring its absorbance at 280nm and using extinction coefficients calculated individually for each Fv from sequence information (Gill and von Hippel, 1989).

Microtitre plates were sensitised by incubating with dilutions of Fv (from 5μg/ml to 5ng/ml made up in sensitisation buffer) for 1 hour at 37°C. Plates were then incubated for 1 hour at room temperature with a 1μg/ml solution of affinity purified
Fig 3.2  ELISA formats used for evaluation of the immune response in rabbits.

Solid phases for ELISA were prepared by sensitising microtitre plates with motl-PPD or Fv protein. As a negative control, plates were also sensitised with PPD only. See text for details.

Fig 3.2a: Measuring anti-motif response in serum.

| PPD-motif | PCA (serum) | anti-rabbit conjugate | A.P. | pNPP |

Fig 3.2b: Measuring anti-PPD response in serum.

| PPD | PCA (serum) | anti-rabbit conjugate | A.P. | pNPP |

Fig 3.2c: Measuring anti-Fv response in serum.

| Fv anti-lysozyme | PCA (serum) | anti-rabbit conjugate | A.P. | pNPP |
rabbit anti-motif antibody made up in PBST. The plates were then incubated for 1 hour at room temperature with a 1/1000 dilution of goat anti-rabbit/alkaline phosphatase conjugate (Sigma). Finally, the plates were developed for approximately 10 minutes with pNPP substrate which was made up to 1mg/ml in 1M diethanolamine buffer, pH 9.8. The plates were washed thoroughly with PBST between each incubation.

In order to assess the utility of this assay for monitoring Fv production, the first step was to examine for the prospect of interference by non-specific biomass. To this end, a typical fermenter culture of Fv anti-hCG (prepared as described in the next chapter, 4.2.2.2) was totally depleted of its activity by antigen affinity chromatography (again as described in the next chapter, 4.2.1.2). This sample was therefore representative of the total products of fermentation but without the Fv (hereafter described as a "negative fermenter culture"). To be effective, the assay had to represent this sample routinely as zero baseline. Furthermore, it should be possible to spike the negative culture with known amounts of Fv and obtain the same signal in the assay as for purified Fv. To test these principles, the negative fermenter culture was spiked to 50ug/ml (a realistic value for a typical fermenter culture - see next chapter, fig 4.5) and then diluted in sensitisation buffer for testing in the assay. Corresponding dilutions of the negative fermenter culture were also tested in the absence of Fv.

3.2.5.2 Capture ELISA
Motif-specific rabbit antibodies were tested for their ability to bind two Fv fragments (Fv anti-lysozyme and Fv anti-hCG) in an antigen capture ELISA. Microtitre plates were sensitised by incubating with a solution of appropriate antigen (made up in sensitisation buffer) overnight at 37°C. hCG was used at a concentration of 10ug/ml and lysozyme at a concentration of 1ug/ml (lysozyme had been found to give problems with non-specific binding at 10ug/ml). Sensitised plates were incubated with dilutions of Fv (from 10ug/ml to 0.15ug/ml made up in PBST). Plates were then incubated with a 1μg/ml solution of affinity purified rabbit anti-motif antibody in PBST, followed by a 1/1000 dilution of goat anti-rabbit/alkaline phosphatase conjugate (Sigma). Finally, the plates were developed for approximately 30 minutes
with pNPP substrate which was made up to 1mg/ml in 1M diethanolamine buffer, pH 9.8. The plates were washed thoroughly with PBST between each incubation.

As a control, the signal generated with the anti-motif antibody was compared with that generated by unpurified rabbit anti-Fv sera. Rabbit anti-(Fv anti-lysozyme) was prepared as described in 3.2.1. and 3.2.2. and used at a dilution of 1/1000 in PBST; rabbit anti-(Fv anti-hCG) serum was prepared by the same method and used at a dilution of 1/5000 in PBST. [In all other respects, the assay conditions used in these control experiments were as above].

3.2.5.3. Immunoblot
The utility of motif-specific antibodies was also evaluated in an immunoblot. Fv fragments were produced in shake-flasks according to the method of Ward et al. (1989); samples from these cultures were run out on polyacrylamide gel electrophoresis under denaturing conditions (using a Pharmacia homogeneous 20 Phastgel with Pharmacia SDS buffer strips). The gel was then electroblotted onto a 0.2μm nitrocellulose membrane (Schleicher & Schuell). The membrane was incubated with a solution of affinity purified rabbit anti-motif antibodies (2μg/ml in PBS with 0.05% Tween 20) for 1 hour at room temperature. The membrane was then incubated in a solution of conjugate [Sigma goat anti-rabbit/alkaline phosphatase diluted 1/2000 in PBS with 0.05% Tween 20] before incubating with substrate [BCIP/NBT (Promega)].

3.2.6. Generation of mouse monoclonal antibodies
Mice were inoculated with mot1-PPD and used to generate monoclonal antibodies specific for mot1 using standard techniques (Gani et al., 1987). Supernatant fluids from hybridoma cells were screened for antibody which bound to mot1 and cross-reacted with Fv anti-lysozyme. Screening assays comprised the solid phases described in fig 3.2 and an anti-mouse/alkaline phosphatase conjugate (Sigma).

One of these monoclonal antibodies, 4743.1 (which was isolated by M. Gani and T. Hunt) was selected for further study.
3.2.7. Evaluation of mouse monoclonal reagent

The mouse monoclonal was evaluated for its ability to bind three different Fvs in a direct ELISA system. Details were identical to those described for the rabbit antibody (in 3.2.5.1.) except that the conjugate was an anti-mouse/alkaline phosphatase reagent (Sigma) and that development time with pNPP was much longer (approximately 1 hour); the full experimental protocol is also included in the paragraph below.

The potential of using this direct ELISA as a generally applicable research tool for analysing and optimising Fv expression was evaluated. An Fv (Fv anti-Streptococcus) which had been recently-cloned and which did not express in the "standard" culture conditions published by Ward et al. (1989) was cultured in four different conditions in small shake-flasks:- 25°C in M9P medium (Ryan et al., 1989); 30°C in M9P medium; 25°C in M9P supplemented with yeast extract (Beta lab, 10g/L); and 30°C in M9P supplemented with yeast extract. Periplasmic lysates were made from the four cultures, according to the method of French et al. (1992). Duplicate samples of the four lysates were diluted 1/10 into 0.05M carbonate buffer, pH 10; filtered through a 0.2nm filter (Schleicher & Schuell); then applied to the wells of a microtitre plate (Sterelin), in duplicate, for 1 hour at 37°C. Plates were then incubated for 1 hour at room temperature with a 1ug/ml solution of 4743 monoclonal antibody made up in PBST. The plates were then incubated for 1 hour at room temperature with a 1/1000 dilution of goat anti-mouse/alkaline phosphatase conjugate (Sigma). Finally, the plates were developed for approximately 1 hour with pNPP substrate which was made up to 1mg/ml in 1M diethanolamine buffer, pH 9.8. The plates were washed thoroughly with PBST between each incubation.

3.3 RESULTS

3.3.1 Generation of anti-motif antibodies in the rabbit.

Rabbits which had been inoculated with the MAP developed an immune response against mot1. This antiserum was found to cross-react with Fv anti-lysozyme which had been adsorbed onto microtitre plates. (see Fig 3.3).

Rabbits inoculated with Fv anti-lysozyme developed an immune response against Fv
FIG 3.3: RESPONSE TO IMMUNOGENS IN RABBITS.

Fig 3.3a: Anti-motif response.
- • serum from rabbit inoculated with Fv anti-lysozyme
- ■ serum from rabbit inoculated with motif immunogen (MAP)
- ▲ normal rabbit serum

Fig 3.3b: Anti-Fv response.
- • serum from rabbit inoculated with Fv anti-lysozyme
- ■ serum from rabbit inoculated with motif immunogen (MAP)
- ▲ normal rabbit serum
anti-lysozyme. This antiserum was found to cross-react with motl which had been adsorbed onto microtitre plates. Perhaps surprisingly, this serum showed a stronger response to motl than the rabbit which had been inoculated with the MAP -see fig 3.3. Therefore, it represented a richer source of motif-specific antibody and was used as the feedstock in affinity purification as described below.

3.3.2. Affinity purification of motif-specific antibodies from rabbit serum and analysis of fractions
The affinity column motl-agarose was found to be able to recover motif-specific antibodies from serum. Approximately 1mg of antibody was recovered from 5mls of the rabbit anti-serum, as determined by O.D. 280 (also see fig 3.4 for chromatogram).

The affinity purified fraction was found to contain an anti-motif activity equal to that in the whole serum, an anti-Fv activity equal to 20-30% of that in the whole serum, and no anti-PPD activity. In contrast, the column fallthrough was found to contain a high anti-Fv activity and a low anti-motif activity which was not above the anti-PPD background. (see Fig 3.5).

3.3.3 Evaluation of rabbit anti-motif antibodies.

3.3.3.1 Direct ELISA.
The affinity purified rabbit anti-motif preparation was found to bind strongly to all three Fv fragments when the Fvs were adsorbed directly onto microtitre plates. All three Fvs produced a concentration-dependent signal and specific signal could be detected above base-line down to a concentration of about 50ng/ml (Fig 3.6). The important criterion was established that the negative fermenter culture did not interfere significantly with the signal:- in isolation it gave a good baseline; and when spiked with Fv, signal was very similar to that obtained with purified Fv. It was concluded that non-specific biomass would not register in the assay and therefore the assay was considered to be suitable for further development as a tool for monitoring Fv production in fermenters.
Fig 3.4: AFFINITY PURIFICATION OF MOTIF-SPECIFIC ANTIBODIES FROM ANTI-Fv SERUM (CHROMATOGRAM).

Rabbit anti-Fv serum was loaded onto a motif-agarose column. Bound antibodies were eluted with 4M MgCl₂.

PBS

O.D. 280

serum

4M MgCl₂

affinity purified fraction

Elution volume
Fig 3.5: AFFINITY PURIFICATION OF MOTIF-SPECIFIC ANTIBODIES FROM ANTI-FV SERUM (ANALYSIS OF FRACTIONS).

Fig 3.5a: Whole serum (before affinity purification).

Fig 3.5b: Affinity purified fraction.

Fig 3.5c: Fallthrough.
Fig 3.6: Evaluation of rabbit anti-motif antibodies in a direct ELISA.
Microtiter plates were sensitised by incubation with dilutions of Fv made up in carbonate buffer, pH10. Fv was traced with rabbit anti-motif antibodies as described in the Methods section. A negative fermenter culture was obtained by passing a typical culture through an antigen affinity column. It was then spiked with Fv at a level of 50μg/ml (a realistic value for a typical fermenter culture - see next chapter) and diluted in carbonate buffer and used to sensitise plates as above.
3.3.3.2 Capture ELISA.
When Fvs were captured onto solid-phase by their specific immunochemical binding to adsorbed antigen, Fv anti-lysozyme was recognised by the affinity purified rabbit anti-motif reagent; but the signal was very weak compared with that generated by the unpurified rabbit anti-(Fv anti-lysozyme) serum (see fig 3.7a).

Fv anti-hCG was not recognised by the affinity purified rabbit anti-motif reagent. In contrast, a very strong, specific signal was generated by the unpurified rabbit anti-(Fv anti-hCG) serum - confirming the Fv to be active (see fig 3.7b).

3.3.3.3 Immunoblot
Samples from shake-flask cultures probed with affinity purified motif-specific rabbit antibodies were found to give discrete bands close to the calculated molecular weights for Fv fragments and their component chains. Gels derived from cultures producing conventional Fv stained to give a single band at around 12.5kDa (corresponding to V\textsubscript{H} chain). Cultures producing single-chain Fv, scFv (Bird et al., 1988) stained to give a single band at around 26kDa (corresponding to Fv and linker). There was no cross-reaction from other \textit{E.coli} proteins in the culture supernatants, despite the presence of many non-specific (bacterial) proteins in these samples. See Fig. 3.8.

3.3.4 Generation and evaluation of mouse monoclonal antibodies
A motif-specific monoclonal antibody was isolated, 4743.1, which was found to bind to all three Fvs in direct ELISA - see fig 3.9. However, the signal in the direct ELISA was affected by the presence of a negative fermenter supernatant - therefore it would only be possible to use this reagent for semi-quantitative analysis. An analytical assay based on this system was found to be very useful for analysing the expression levels of a recently cloned Fv (Fv anti-\textit{Streptococcus}) in four different growth cultures. See Table 3.1.

3.4 DISCUSSION
An important first step towards designing generic binding reagents for analysing families of proteins is the identification of appropriate peptide motifs which are
Fig 3.7: Evaluation of rabbit antibodies in antigen capture ELISA.

a) Response with Fv anti-lysozyme.

Signal (O.D. 405nm).

Unpurified rabbit anti-Fv
- - -

Purified rabbit anti-motif
- - -

Fv anti-lysozyme (ug/ml).
Fig 3.7: Evaluation of rabbit antibodies in antigen capture ELISA.
b) Response with Fv anti-hCG.

Signal (O.D. 405nm).

Unpurified rabbit anti-Fv
- Purified rabbit anti-motif

Fv anti-hCG (ug/ml).
Fig 3.8: Evaluation of rabbit anti-motif antibodies for determining Fv in shake-flask cultures using immunoblotting.

Samples from shake-flasks were run out on SDS-PAGE gels, blotted onto nitrocellulose paper, and then probed with rabbit anti-motif antibodies as described in the Methods section. Bands were aligned with markers on equivalent gels which had been stained with silver (reagents were from Pharmacia).

Silver stained
Lane 1: Pharmacia low molecular weight markers.
Lane 2: Supernatant from Fv anti-lysozyme culture.

Immunoblotting
Lane 3: Supernatant from Fv anti-lysozyme culture.
Lane 4: Supernatant from single-chain Fv anti-lysozyme culture.
Fig 3.9: Evaluation of monoclonal anti-motif antibody in a direct ELISA.

**Signal A405**

<table>
<thead>
<tr>
<th>Fv concentration (ng/ml)</th>
<th>Supematant + Fv anti-hCG</th>
<th>Fv anti-hCG</th>
<th>Fv anti-lysozyme</th>
<th>Fv anti-G.Ox</th>
<th>Supematant only</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19.5</td>
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<td>39</td>
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<tr>
<td>2500</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>
Table 3.1: Use of a direct ELISA, comprising monoclonal anti-motif, for primary screen of the expression conditions for an Fv fragment.

The potential of using this direct ELISA as a generally applicable research tool for analysing and optimising Fv expression was evaluated. An Fv (Fv anti-Streptococcus) which had been recently-cloned and which did not express in the "standard" culture conditions published by Ward et al. (1989) was produced by four different culture conditions in small shake-flasks:- 25°C in M9P medium (Ryan et al., 1989); 30°C in M9P medium; 25°C in M9P supplemented with yeast extract (Beta lab, 10g/L); and 30°C in M9P supplemented with yeast extract. Periplasmic lysates were made from the four cultures by a method based on that of French et al. (1996). [Culture samples were centrifuged at 3,000 r.p.m. for 20 minutes at 25°C and the supernatants were removed. The cell pellets were resuspended in lysis buffer (20% sucrose in 200 mM tris buffer containing 1 mM EDTA and 500 μg/ml lysozyme, pH 7.5) and incubated at 25°C for 15 minutes. An equal volume of distilled water was added to each sample and the mixtures were incubated as before, then centrifuged at 13,000 r.p.m. for 5 minutes. The supernatants were filtered through 0.45μm syringe filter units.]

Duplicate samples of the four cultures were analysed for Fv content using the direct ELISA.

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>Incubation temperature</th>
<th>Signal in assay (A&lt;sub&gt;405&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M9P</td>
<td>25°C</td>
<td>0.81, 0.66</td>
</tr>
<tr>
<td>M9P</td>
<td>30°C</td>
<td>&lt; 0.02, &lt; 0.02</td>
</tr>
<tr>
<td>M9P + yeast extract</td>
<td>25°C</td>
<td>&lt; 0.02, &lt; 0.02</td>
</tr>
<tr>
<td>M9P + yeast extract</td>
<td>30°C</td>
<td>&lt; 0.02, &lt; 0.02</td>
</tr>
</tbody>
</table>
common to all members of the family. This problem was approached by taking advantage of a side effect of a technique which is widely used in gene cloning, namely the polymerase chain reaction (PCR). It was reasoned that families of proteins whose genes have been cloned using common PCR primers will carry identical motifs at their two termini. Furthermore, the C-terminal motifs of proteins are particularly attractive targets for capture, since they are often exposed as a consequence of the order in which proteins fold. A rabbit polyclonal antibody and a mouse monoclonal antibody were raised to the V\textsubscript{H} C-terminal motif \textit{(motl)} of Fv fragments. Both of these were found to bind three different Fv fragments (produced by the PCR route) when the Fvs were adsorbed onto the surface of microtitre plates.

Two analytical assays were designed in which Fv was directly and non-specifically adsorbed onto microtitre plates from whole test samples: one comprised affinity purified polyclonal anti-motif antibody, the other comprised monoclonal anti-motif antibody. These assays both have the outstanding advantage of giving signal with all Fvs (regardless of idiotype). The assay using polyclonal anti-motif antibody was found to be most suitable for quantitative analysis of Fv in culture because the signal it generated was not affected greatly by the presence of non-specific \textit{E. coli} biomass (i.e. the negative fermenter culture). The use of this assay to analyse Fv production throughout the course of a fermentation run is described in the next chapter. The assay using the monoclonal antibody was found to be less suitable for quantitative analysis of Fv in culture since its signal was affected by the presence of non-specific biomass. A possible explanation for this is that the monoclonal reagent may be expected to have a low affinity for motl. (Since motl is a mouse sequence, it is likely that motif-specific antibodies isolated from the mouse will have low affinities).

However, the attraction of the monoclonal reagent is that new batches can be supplied with a reproducible quality without the need for time-consuming peptide affinity chromatography or inoculation of more animals. Subsequently, the monoclonal reagent has been adopted in the Unilever laboratories for general use in the semi-quantitative analysis of Fv production in microbial culture. It is particularly useful when the expression levels of a recently cloned Fv are being analysed. This is because it is not possible to assemble elaborate assay systems (such as those
described in the next chapter which use polyclonal reagents raised against the particular Fv of interest) until at least 1-2mgs of that Fv have been produced and purified for an inoculation schedule. Since this initial production often requires some optimisation of culturing conditions (refer to table 3.1), anti-motif reagents which can be relied upon to bind the new Fv are extremely valuable analytical probes.

A disappointing finding was that the motif-specific antibodies did not bind Fv anti-lysozyme strongly (and did not bind Fv anti-hCG at all) when captured onto the surface of microtitre plates by antigen. A possible explanation for this is that the $V_H$ C-terminus is less available on Fv fragments when correctly folded than current models would suggest and that it is more available on some Fvs than others. This would imply that the motif-specific antibodies were able to bind to all Fvs strongly in the direct ELISA because the Fvs had been partially denatured during adsorption onto the microtitre plate, with the result that the motif was further exposed. Epitope exposure resulting from the adsorption of proteins onto microtitre plates has been reported by other investigators (Dierks et al., 1986; Holander and Katchalski-Katzir, 1986). Whatever the explanation, this specificity profile made it impossible to design an assay which could discriminate between active Fv and inactive Fv. An analysis of the levels of expression of Fv protein (whether active or not) has its uses: again a good example is when a recently cloned Fv is being investigated, the finding that the target protein is being synthesised and secreted is very encouraging information to have at the start of a project. However, for a thorough optimisation of the expression and production of a particular Fv, the analysis of Fv protein in isolation is of limited use. Therefore, further analytical assays which respond to active Fv need to be designed and tested. Data reported in this chapter would suggest that unpurified anti-sera raised against the particular Fv of interest would be useful reagents for this purpose. This topic will be addressed in the next chapter.

3.5 PUBLICATION

Most of the data presented in this chapter were published in the Journal of Immunological Methods [Berry et al. 167 (1994) 173-182]. A reprint of this paper is bound in at the back of this thesis.
4. INVESTIGATION OF Fv AUTHENTICITY IN FERMENTER CULTURES.

4.1 INTRODUCTION

In Chapter 3, a generic analytical immunoassay for determining Fv protein was developed. It is a very useful laboratory tool, especially for the rapid, semi-quantitative analysis of a large number of different Fv cultures that have been produced in small amounts. However, in order to optimise and scale-up the production of a selected Fv, a more complete analysis will be required. It will be imperative to determine not only the amount of Fv protein that is produced, under each set of conditions, but also its "authenticity". In other words, it needs to be investigated whether the protein produced in the fermenter is identical in terms of activity and functionality to a thoroughly characterised standard. Without this information, it will be impossible to assess the full impact of a change of growth medium, fermentation temperature, or host organism on the production process. Production of Fv which is not authentic may result from incorrect folding, dissociation of \( V_H \) and \( V_L \), from a mutation or (in the case of a eukaryotic expression system) from inappropriate glycosylation patterns. Since the scope of this thesis is limited to the investigation of \( E. coli \) cultures, there is not the problem of glycosylation to cope with, and the issue of authenticity reduces to one of determining that the target Fv has a high specific activity. [For antibodies and their fragments, specific activity is usually referred to as "immunoreactivity" (i.e. the proportion able to bind antigen) and individual molecules in a population are considered to be either completely active or completely inactive (Casey et al, 1995)].

An analysis system for investigating Fv authenticity requires a number of key components. Firstly, a standard preparation of the target Fv that has been characterised in terms of its purity, concentration, and immunoreactivity. Secondly, a pair of analytical immunoassays: one determining total Fv protein (whether active or not), the other determining active Fv only; both assays need to be able to operate in a fermenter culture without interference from non-specific biomass. Thirdly, a
method for storing the standard so that analytical data obtained from one fermenter run can be compared accurately and rigorously with data obtained from a previous run. In this chapter, these three aspects are investigated for an Fv specific for human chorionic gonadotropin (hCG) in order to establish a general approach for carrying out an analysis of Fv authenticity in fermenter cultures.

Of fundamental importance was the preparation of a small amount of Fv anti-hCG in shake flasks and to purify it by antigen affinity chromatography. This material was used as a standard preparation against which to reference the analytical immunoassays. Key criteria for characterising and quantifying the standard were: purity (as adjudged by SDS-PAGE); concentration (as measured by optical density at 280nm); immunoreactivity (as determined by analytical antigen affinity chromatography - Casey et al, 1995); and multimeric status (as determined by gel filtration chromatography). [The formation of multimers has been found to affect the signal in immunoassay - (McGregor et al, 1994) - therefore a standard without the presence of multimers would be preferable.]

It was possible to analyse total Fv protein in fermenter cultures by using the immunoassay, described in chapter 3, which comprised a rabbit anti-\(V_h\) motif reagent. This immunoassay had already been found to be fully quantitative for Fv anti-hCG and not to be subject to interference from non-specific biomass (refer to chapter 3 for details). However, the analysis of active Fv in fermenter cultures required a different immunoassay since the rabbit anti-motif reagent had been found not to bind Fv anti-hCG when complexed with antigen (refer to chapter 3). Therefore, a new assay was designed which comprised a rabbit polyclonal reagent that had been raised against the target Fv. This new assay would need to be evaluated to determine its working range and sensitivity to interference from non-specific biomass, using the principles elucidated in chapter 3. A further objective was to cross-reference the activity measured by this assay with the standard preparation of Fv anti-hCG. If the concentration and immunoreactivity of the standard are known, it should be possible to express the activity in a fermenter in terms of milligrams of active Fv per litre of culture. Finally, it was sought to produce the Fv anti-hCG in
a fermenter and to analyse both total Fv and active Fv across a time-course profile using the approach set out above. The ratio of these two figures could be used to estimate the immunoreactivity of the Fv as it was being produced in the fermenter.

The storage stability of the standard preparation was assessed by determining its activity after several month's storage at 4°C in sterile solution, at -20°C in frozen solution, and freeze-dried at room temperature. The optimal conditions would then be used to store the standard preparation.

4.2 METHODS
4.2.1 Establishing a standard preparation of Fv anti-hCG.

4.2.1.1 Production of Fv anti-hCG in shake flask culture.
Fv anti-hCG was produced by modifying the method of Ward et al (1989). The genes encoding Fv anti-hCG had been assembled on a pSW1 plasmid (refer to Ward et al, 1989) and then transformed into *E. coli* JM109. A 1% starter culture of this construct was inoculated into a culture medium of M9P (Ryan et al, 1989) supplemented with yeast extract (Beta lab 5g/litre). This culture was grown in a shaking incubator at 25°C for 17 hours before inducing with 0.5mM IPTG (isopropyl-beta-D-thiogalactopyranoside). Cultures were then grown for a further 24 hours.

4.2.1.2 Recovery and purification of Fv anti-hCG produced in shake-flasks.
Culture supernatants were clarified by microfiltration using a Flowgen minisette acrylic system (0.3μm screen omega cassette). Approx 1 litre of clarified supernatant was pumped directly onto an antigen affinity column at 4°C (hCG was immobilised onto CNBr - activated agarose at a concentration of 5mg/ml according to manufacturer's instructions). Columns were washed back to baseline with PBSA (phosphate buffered saline, pH7 with 0.1% azide); and then Fv was desorbed with 40mM glycine pH 2.5. (This desorption buffer had been optimised for this particular Fv. If too mild a pH is used, Fv is not desorbed; if too harsh, Fv dissociates resulting in precipitation of the V₅ chain). Desorbed Fv was collected directly into 1/10 volume 1M tris pH 8.5 (to neutralise the desorption buffer) and then immediately dialysed into PBSA. It was sterile-filtered and then kept at 4°C until
required.

4.2.1.3 Determination of purity, concentration, and immunoreactivity of recovered Fv anti-hCG.
The purity of the recovered Fv anti-hCG preparation was assessed by SDS-PAGE: a sample was run out on a Pharmacia homogeneous 20 Phastgel with Pharmacia SDS buffer strips; the gel was stained with silver-stain, according to manufacturer's instructions. Having found it to be of very high purity, the protein concentration of the Fv preparation was determined by measuring its optical density at 280nm and using an extinction coefficient of 1.6 (for a 1mg/ml solution) This coefficient was calculated from sequence information. [It is only possible to use this method for determining protein concentration if the preparation is very pure: any protein contaminants will also absorb at 280nm, leading to inaccurate data].

The immunoreactivity of the purified Fv preparation was determined by passing approximately 2mgs of Fv down an hCG affinity column, that had been equilibrated in PBS, washing with 0.5M NaCl (to remove any non-specifically bound protein) and then desorbing bound Fv with 50mM glycine, pH2.5. The immunoreactivity of the sample was determined by comparing the area of the desorbed 'peak' with the area of the fallthrough fraction. Areas were measured using the facility supplied with Frac-100 controller (Pharmacia).

4.2.1.4 Determination of multimeric status of purified Fv anti-hCG.
The multimeric status of Fv anti-hCG was analysed by gel filtration chromatography. A 1metre column (Pharmacia C25) was packed with S200 media (Pharmacia) and equilibrated with PBS (0.01M Na$_2$HPO$_4$ /NaH$_2$PO$_4$, 0.15M NaCl, pH 7). Approximately 1mg of Fv was applied to the column in 5mls of PBS. The pump speed was set at 1ml/minute. The elution profile of Fv was bench-marked against that of bovine albumin which is known to elute as two peaks: corresponding to albumin monomer (approx. 70kD) and dimer (approx. 140kD). This was done by applying 10mgs of albumin to the column and then 10mgs of albumin together with 1mg of Fv.
4.2.2 Analysis of total Fv and active Fv in fermenter cultures.

4.2.2.1 Design and testing of immunoassay for determining active Fv.

The immunosorbent surface used in the ELISA system was a specially designed peg, moulded in nylon, which dipped into the wells on standard flat-bottomed microtitre trays (Davis et al, 1983). The hCG (Sigma) was chemically linked onto the pegs using glutaraldehyde as described by Inman and Hornby (1972). Before use, the pegs were soaked in 0.5M EDTA (Sigma), pH8 and then blotted dry. This was to inactivate any alkaline phosphatase present in the hCG preparation. (Without this precaution, contaminatory alkaline phosphatase gives an unacceptably high background signal in the assay).

To generate a standard curve, the purified Fv anti-hCG preparation was diluted to give a series of doubling dilutions from 0.25 to 0.00195 μg/ml. The dilutions were made in PBST and then applied to Microtitre plates (Sterilin) in duplicate. The hCG pegs were applied to the wells (assembled on polystyrene bars so that a row of 12 pegs could be added at a time). The pegs were incubated in the Fv for 30 minutes at 25°C; then washed three times in tubs of distilled water and blotted dry.

The pegs were then placed in tracing reagent made up in PBST. This comprised a 1/1000 dilution of rabbit anti-Fv hCG serum (prepared as described earlier in 3.2.5.2); and a 1/50 dilution of non-specific sheep serum (to block any non-specific binding sites on the peg). The pegs were incubated in tracing reagent at 25°C for 30 minutes and then washed as before.

The pegs were then placed in a 1/1000 dilution of goat anti-rabbit IgG/alkaline phosphatase conjugate (Sigma) made up in PBST. The pegs were incubated in conjugate for 30 minutes at 25°C and then washed as before.

The pegs were then incubated for 30 minutes at 25°C in substrate buffer [1mg/ml pNPP (Sigma); 1M diethanolamine (Sigma); 1mM MgCl2 (BDH); pH9.8]. The pegs were then discarded, and the signal in the resulting solution was read at 410nm.
The sensitivity of the assay to non-specific biomass was investigated by measuring its response to a culture which had produced an Fv of another specificity (Fv anti-Streptococcus). This culture supernatant was 'spiked' to 10μg/ml with Fv anti-hCG to mimic a typical culture (many Fvs are expressed at approximately this concentration). The spiked supernatant was then diluted in PBST to produce a series of dilutions from 0.25 to 0.00195 μg/ml and then tested in the assay. In addition, the signal generated from the unspiked supernatant was determined by making a corresponding series of dilutions in PBST but without adding Fv anti-hCG.

4.2.2.2 Production of Fv anti-hCG in fermenter culture

Fv anti-hCG fragments were produced in 5 litre fermenters (LH 2000 series 1; 3 litre working volume) from E.coli JM 109, as described above. The growth medium was M9P (Ryan et al: 1989) supplemented with yeast extract (Beta lab: 10g/litre) and containing glycerol (30g/litre) instead of glucose as a carbon source. The growth temperature was 25°C and the pH was controlled by 6.8 by auto addition of 40% (w/v) NaOH. The impeller speed was 500rpm and the aeration rate was 0.1 v/v/m. The inoculum (1% v/v) was grown overnight on M9P medium supplemented with yeast extract (5g/litre) in a shaking incubator at 25°C. During the first 15 hours of fermentation, the impeller speed and air flow rate were gradually increased to maxima of 650 rpm and 0.3 v/v/m respectively.

Fv anti-hCG fragment production was induced by the addition of filter sterilised isopropyl-B-D-thiogalactopyranoside, IPTG (0.5M; 1ml/litre) during the late exponential growth phase (as indicated in figure 4.5).

4.2.2.3 Analysis protocols.

Samples (5mls) were removed from the fermenter at intervals of 11, 14½, 17, 35½, 38 and 40 hours for analysis. These samples were centrifuged for 15 minutes at 3,000 rpm and then filtered through an 0.2μm microfilter. Clarified samples were analysed by two immunoassays: one determining total Fv (whether active or not), the other determining active Fv only. Both assays were calibrated with the same standard, the Fv anti-hCG preparation described above in 4.2.1

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Total Fv was determined by using the motif assay described in chapter 3 (refer to 3.2.5.1 for details). Samples were diluted to 1/100 and 1/1000 in PBST and signal was read off against a standard curve which was generated from a series of doubling dilutions (2.5μg/ml to 9.8ng/ml) of the standardised Fv anti-hCG preparation.

The same samples were analysed for active Fv by using the ELISA described in this chapter (refer to 4.2.2.1 for details). Samples were diluted to 1/1,000, 1/10,000 and 1/100,000 and then read off against a standard curve that had been generated from a series of doubling dilutions (250ng/ml to 1.9 ng/ml) of the standardised Fv anti-hCG preparation. The concentrations for active Fv protein in the fermenter culture were corrected by multiplying by the % immunoreactivity that had been determined for the Fv anti-hCG standard.

4.2.3 Elucidation of optimal storage conditions for Fv anti-hCG standards.

Three different storage conditions were investigated: in solution and stored at 4°C; frozen and stored at -20°C; freeze-dried and stored at room temperature. For frozen samples and freeze-dried samples, it was important to establish the effect of the freezing and drying processes on activity before processed samples were laid down for long-term storage. Freeze-drying was carried out in distilled water as salts are often found to inactivate proteins during freeze-drying (Bagster, 1994). However, as many proteins are insoluble or poorly soluble in water, duplicate samples were prepared with an excess of bovine albumin in order to promote solubility and therefore to reduce activity losses until drying was complete. Protocols are given below:-

4.2.3.1 Investigation of the effect of freezing in and freezing drying from distilled water

Purified Fv anti-hCG was diluted to 1μg/ml in PBS. This was dialysed into water. Aliquots were frozen at -20°C and others frozen and then dried. The effect of freezing on activity was determined by thawing the frozen aliquots, making a series of dilutions in PBST and measuring signal in the ELISA. The signal was compared with the Fv in PBS which had not been dialysed or frozen. Likewise, the effect of
freeze drying on activity was determined by reconstituting in PBS, making a series of dilutions in PBST and measuring signal in ELISA.

4.2.3.2 Investigation of the effect of freezing in and freezing drying from distilled water containing bovine albumin.

Purified Fv anti-hCG was diluted to 1µg/ml in PBS and then spiked with bovine albumin (Sigma) at 5mg/ml. It was then dialysed into water. Samples of the dialysed preparation were frozen and freeze-dried. The effect of freezing and freeze-drying on Fv activity was determined as before.

4.2.3.3 Comparison of residual activity after storing Fv anti-hCG for 6 months in three different conditions: - Fridge, freezer, freeze dried.

Purified Fv anti-hCG was diluted to 1µg/ml. Aliquots were stored: at 4°C, sterile-filtered with 0.1% sodium azide as a bacteriostat; at -20°C, in frozen aqueous solution; and at room temperature in a desiccator after being freeze-dried in the presence of albumin as described above. The activity remaining after 6 months was determined by making a series of dilutions in PBST and measuring the signal in ELISA.

4.3 RESULTS
4.3.1 Determination of purity of Fv recovered from shake-flasks.

The recovered Fv anti-hCG preparation was found to be of very high purity by SDS-PAGE. See Figure 4.1. Furthermore, the V_H and V_L chains migrated to positions that were very close to their molecular weights as predicted by their aminoacid sequence (V_H = 13.5 kD; V_L = 11.9kD).

4.3.2 Determination of protein concentration and immunoreactivity of purified Fv anti-hCG

The purified Fv anti-hCG preparation had an optical density at 280nm of 0.32. Therefore it was assigned a concentration of 0.2mg/ml.
Fig 4.1: SDS-PAGE analysis of Fv anti-hCG recovered from shake-flask culture.

Lane 1: Pharmacia low molecular-weight markers.

Lane 2: Purified Fv anti-hCG.
The immunoreactivity of the purified Fv preparation was found to be 87% by passing it down an hCG affinity column and measuring the proportion which bound (and was therefore active). See Fig 4.2.

4.3.3 Determination of multimeric status of purified Fv by gel filtration chromatography
Fv anti-hCG eluted as a single peak that was consistent with the molecular weight of Fv monomer. See fig 4.3.

4.3.4 Performance of the immunoassay for active Fv.
The assay was found to have a working range from approximately 2ng/ml to 100ng/ml. Non-specific biomass was found not to interfere with the assay in that the assay curves with and without culture were identical. See Fig 4.4.

4.3.5 Analysis of expression of Fv anti-hCG in fermenter cultures
The fermenter reached a maximum concentration of approximately 40mg/L of Fv protein as adjudged by the motif assay and 50mg/L of active Fv as adjudged by the activity assay. The profiles for Fv build-up were very similar for both assays. When the figures for active Fv were corrected by 87% (the immunoreactivity of the Fv hCG standard - Fig 4.2) the profiles became even closer. See Fig 4.5.

4.3.6 Elucidation of storage conditions for Fv anti-hCG standards
Fv anti-hCG that had been dialysed into water and then frozen was found to have a significant loss of activity. Fv anti-hCG that had been dialysed into water and then freeze-dried also showed a large loss in activity (Fig 4.6a). However if Fv anti-hCG was stabilised with BSA at 5mg/ml, it was tolerant to both freezing and freeze drying (Fig 4.6b).

Standards that had been frozen and stored at -20°C had only a weak residual activity after 6 months. However, standards that had been kept at 4°C in sterile conditions or freeze-dried at room temperature both had a good residual activity after 6 months (Fig 4.6c).
Fig 4.2: Determination of immunoreactivity of Fv anti-hCG standard by analytical affinity chromatography.

Fv anti-hCG was loaded onto an hCG-agarose column in PBS. The column was washed with 0.5M NaCl before eluting bound Fv with 50mM glycine, pH 2.5.

\[
\text{Immunoreactivity} = \frac{\text{Active Fv}}{\text{Total Fv}} = \frac{\text{Peak}}{\text{Peak} + \text{Fallthrough}}.
\]

= 87% (by area of fractions).
Fig 4.3: Analysis of the multimeric status of the Fv anti-hCG standard.

The multimeric status of the purified Fv anti-hCG preparation was analysed by gel filtration chromatography. The elution profile of the Fv was benchmarked against that of bovine albumin which is known to elute as two peaks: corresponding to albumin monomer (approx 70kD) and dimer (approx 140kD).

It was found that the Fv eluted as a single peak that was consistent with the molecular weight of Fv monomer (approx 25kD).
Fig 4.4: Evaluation of immunoassay for active Fv
Working range and sensitivity to interference from
non-specific biomass.

A standard curve was generated by making a series of doubling dilutions of purified Fv anti-hCG in PBST. A "negative" fermenter culture i.e. a culture that had produced an Fv of a different specificity was spiked with Fv anti-hCG, so as to mimic a culture of Fv anti-hCG, and then diluted out in PBST to cover the working range of the assay. A corresponding series of dilutions of the unspiked negative culture was also made and tested in the assay as above.
Fig 4.5: Analysis of total Fv and active Fv in fermenter culture.

Fv fragments specific for hCG were produced in *E. coli* JM109. Cultures were induced with IPTG as indicated. Fv production was analysed at intervals by two different immunoassays. Total Fv protein (whether active or not) was determined by an immunoassay comprising the anti-motif reagent (for detailed protocol, refer to 3.2.5.1). Active Fv was determined by the immunoassay detailed in this chapter. In addition, the values for active Fv were corrected after taking into account the immunoreactivity of the Fv anti-hCG standard used.

![Graph showing concentration of Total Fv and Active Fv over fermentation time](image)
Fig 4.6: Stability of Fv anti-hCG standards
a) The short-term effect of freezing and freeze-drying in water.

Purified Fv anti-hCG was made up to 1μg/ml in PBS. This was dialysed into water. Samples were frozen and freeze-dried. The effect on activity was determined by making a range of dilutions in PBST and comparing signal in ELISA with an untreated control (i.e. Fv in PBS, not frozen).
Fig 4.6: Stability of Fv anti-hCG standards
b) The short-term effect of freezing and freeze-drying in the presence of albumin.

Purified Fv anti-hCG was made up to 1 ug/ml in PBS and then bovine albumin was added to a concentration of 5 mg/ml. The preparation was then dialysed into water. Samples of the dialysed preparation were frozen and freeze-dried. The effect on activity was determined by making a range of dilutions in PBST and comparing signal in ELISA with an untreated control (i.e. Fv in PBS, not frozen).
Fig 4.6: Stability of Fv anti-hCG standards

c) The effect of 6 months storage under different conditions.

Purified Fv anti-hCG was made up to 1μg/ml in PBS. Aliquots were stored: at 4°C, sterile-filtered with 0.1% azide as a bacteriostat; at -20°C in frozen solution; and at room temperature in a desiccator after being freeze-dried in the presence of albumin. The activities remaining after 6 months were compared by making a range of dilutions in PBST and measuring signal in the ELISA.
4.4 DISCUSSION

A subject of current importance for the biochemical engineer is to establish methods for determining the "authenticity" of a target protein produced in fermenter culture. In other words that a product produced in scaled-up culture is biochemically identical to a standard preparation or, at the very least, retains all of the desirable properties for which it was selected in the first place. For proteins produced in eukaryotic culture, the issue of authenticity becomes very complicated because it has been found that target proteins can be modified with different glycosylation patterns under different culture conditions (Goochee et al, 1990). However, for proteins produced in prokaryotic culture, there are less variables to cope with, and the issue of authenticity reduces to one of determining that the target protein has a high activity.

In the present studies, the immunoreactivity of an Fv produced in *E. coli* culture has been probed with a pair of immunoassays: one determining total Fv protein, the other active Fv only. Both assays were cross-referenced to the same standard preparation of known concentration and immunoreactivity and therefore data could be expressed as milligrams of total Fv / litre of culture and as milligrams of active Fv / litre of culture. The ratio of these two figures was used to estimate the immunoreactivity (or authenticity) of the Fv as it was produced in the fermenter.

A key stage in this investigation was the establishment of a standard in which there was a high degree of confidence. This standard was found to be very pure by SDS-PAGE. It was also found not to contain any multimeric species. This latter finding may have been anticipated as multimers have only been reported as a problem with scFv constructs; nevertheless it was an important confirmatory result. It was also important to determine the immunoreactivity of the standard. It would have been incorrect to assume that the standard was fully active simply because it had been purified by antigen affinity chromatography: although all Fv molecules purified by antigen affinity chromatography must have been active at the point of binding to the affinity adsorbent, it is quite possible that a significant proportion of them had been permanently inactivated on elution with pH 2.5 buffer. The extent of inactivation was determined by passing a sample of the purified Fv down the antigen column for a second time to see what proportion could still bind. In the event, it was found that
87% of the protein in the standard could still bind the antigen column. This was interpreted to mean that the standard was composed of 87% fully active Fv molecules and of 13% completely inactive Fv molecules. In other words it had an immunoreactivity of 87%.

It was found that the concentration of total Fv in the fermenter peaked at approximately 40mg/litre and that the concentrations of total Fv correlated very closely with active Fv right across the time-course profile. Therefore, it was deduced that the Fv produced in the fermenter approached an immunoreactivity of 100%. This very high immunoreactivity suggests that the component $V_H$ and $V_L$ chains associated very readily in culture and that folding to produce an active Fv was an efficient process. However, it is quite possible that the use of different antibody fragment specificities or different growth conditions would result in a lower immunoreactivity in the fermenter. This would seem particularly likely for scFv constructs which have been reported to have problems with folding (Somerville et al, 1994) and with the formation of aggregates (Whitlow et al, 1993).

In this investigation it was found that Fv anti-hCG was stable to freezing in and freeze-drying from distilled water containing albumin. It was also found that Fv anti-hCG still retained a high activity after six months when stored sterile-filtered at 4°C in the presence of 0.1% azide or when stored freeze-dried at room temperature. Perhaps surprisingly, significant activity loss was observed after six months storage in frozen aqueous solution at -20°C. It is not clear why this activity loss should occur after prolonged storage when no activity loss was observed after freezing for a short period. It is suggested that this result may be due to a deleterious affect of ice-crystals on the Fv structure, after long periods of storage at -20°C; furthermore, the storage stability of Fv in frozen solution may be better at -70°C and it is recommended that future studies should investigate this possibility.

4.5 CONCLUSION
The ability to determine protein authenticity directly in fermenter cultures would allow fermentation protocols to be optimised against the criterion of producing
authentic protein rather protein of an uncertain status. This chapter set out to establish an approach for estimating the authenticity of Fv fragments in fermenter culture. Almost inevitably, the approach needed some assumptions to be made: that individual Fv molecules are either completely active or completely inactive; and that the motif-specific assay recognises active Fv and all possible forms of inactive Fv. While there is good reason to believe that both assumptions approximate quite closely to the molecular behaviour in real systems, they are unlikely to be perfectly accurate in every eventuality. However, a start has been made: this chapter represents an early step towards determining protein authenticity in fermenter cultures. It seems likely that this research topic will continue to be high on the agenda of the biochemical engineer for some years to come.
5. A NEW PROCESS FOR RECOVERING ACTIVE FV FROM CULTURED MEDIA

5.1 INTRODUCTION

Chapters 3 and 4 investigated how to analyse Fv expression levels in *E. coli* cultures during the early stages of process optimisation and during scale-up. However, it is also critically important that Fv, thus produced, can be recovered from the culture in an active and usable form. The present chapter addresses this issue.

Conventional monoclonal antibodies may be conveniently recovered from cultured media using affinity adsorbents comprising protein A (Goding, 1980) or protein G (Akerstrom et al, 1985). Since neither of these reagents bind to Fv fragments (Derrick and Wigley, 1992) a different approach is required. A popular approach is to tag the Fv of interest with a polyhistidine tail and then to recover it from cultured media by immobilised metal affinity chromatography (IMAC). There has been a lot of research in this field (Skerra et al, 1991; Essen and Skerra, 1993). The technique has the advantage of being generic since it is relatively straightforward to graft a polyhistidine tail onto any Fv; however, there are also some disadvantages. These include: the potential for proteins other than Fv, but which have histidines exposed at their surface, to bind the column and therefore to contaminate the recovered Fv; and the technique's inability to discriminate readily between active and inactive Fv. [For many applications, it will be preferable only to have active Fv in a final product. For example, in the application opportunities discussed in Chapter 1 - such as separation technology - the presence of inactive Fv would at best result in reduced capacity and may result in deleterious effects such as non-specific binding.]

Another popular approach for recovering Fv from culture is antigen affinity chromatography (Riechmann et al, 1988; Anthony et al, 1992; King et al, 1993). This technique has the attraction of being very specific for active Fv in the binding-phase; however the operator is then left with the problem of how to release the Fv from the adsorbent. If too strong an eluant is used the Fv may be denatured, resulting in some inactive Fv in the recovered fraction. If too weak an eluant is used, the Fv will be
inefficiently released or not released at all. In the previous chapter, an eluant of pH 2.5 buffer was selected by trial and error; this resulted in the recovery of an Fv preparation with very good immunoreactivity (87%). Similarly, a leading group at Celltech elected to desorb one of their Fvs from an antigen affinity column with 100mM citric acid (which also has a pH of approximately 2.5); refer to King et al, 1993. However, it seems unlikely that it will be possible to find suitable buffers by trial and error for all Fvs, particularly high affinity Fvs. Furthermore, the problem will become much worse upon scale-up when the Fv will be exposed to the desorption buffer for longer due to the larger void-volume of process-scale columns. Clearly, a more rational design of eluants is required.

Some progress towards the rational design of eluants for antigen affinity chromatography was made by Anthony et al (1992) who purified an Fv specific for digoxin on an immobilised antigen analogue, ouabain. Anthony recovered bound Fv from the adsorbent with a 20mM solution of the same analogue. In this chapter, the potential of using immobilised antigen analogues was investigated in more detail. The example used was an Fv ("Fv 4155") which has a primary specificity for the hapten estrone-3-glucuronide (a urinary metabolite of estriol, a steroid hormone with a role in human fertility). This Fv was used because the monoclonal antibody from which it is derived is known to bind to two analogues of its hapten with progressively lower affinity (estriol-3-glucuronide and estrone). This offers the intriguing prospect of making an affinity column comprising immobilised estrone and eluting with a solution of estriol-3-glucuronide made up in a buffer of neutral pH. It was hypothesised that such an eluant might release Fv from the column very efficiently as estriol-3-glucuronide has a higher affinity for the Fv than the analogue on the column. Moreover, the eluant would not be expected to denature the Fv as can be the case with eluants comprising buffers of extreme pH. However, the proposed process would leave the purified Fv complexed with the second steroid, estriol-3-glucuronide. Therefore, an important objective of this study was to determine whether this Fv-complex could still function as an active species; for example, would it still be able to bind to native antigen in an immunoassay?
This chapter describes the synthesis of an affinity column comprising estrone and its use to recover Fv 4155 from *E.coli* culture supernatant. The amount of active Fv 4155 that could be recovered from the column by eluting with estriol-3-glucuronide was compared with the amount that could be recovered with "conventional" elution conditions (i.e. acidic buffer). Since this particular Fv can be produced with a myc tag (grafted onto the C-terminus of its V\_L chain) without any apparent deleterious effects on expression levels or activity, it was possible to monitor active Fv 4155 throughout the recovery process by using an ELISA comprising the myc-specific tracer antibody described by Ward et al. (1989). However, the "motif assay" (as described in chapters 3 and 4) could not be used to monitor total Fv throughout the process because the motif-specific antibodies do not recognise Fv 4155 in complex with estriol-3-glucuronide. Instead total protein was monitored using a colorimetric assay (Bradford, 1976). A further objective was to investigate the storage stability of the Fv recovered in complex with estriol-3-glucuronide. It was hypothesised that the Fv complex may have improved storage stability compared with native Fv as it is "locked-in" to an active configuration.

5.2 METHODS

5.2.1 Selection of antigen analogues

The fine specificity of the 4155 parent monoclonal antibody and its cross-reactivity profile with related steroids was worked out by a collaborating Colworth scientist, Dr. Coley, using the method published in Gani et al (1994). This was used to select a ligand and an eluant. In brief, the lowest affinity analogue (estrone) was chosen as ligand, and a mid-range affinity analogue (estriol-3-glucuronide) was chosen as an eluant. See Fig 5.1.

5.2.2 Synthesis of estrone-agarose affinity column

Estrone was coupled via its hydroxyl to a chromatography medium (Epoxy 6B Sepharose, Pharmacia). This product has a convenient 12-atom spacer arm. The coupling protocol was as detailed below:-

3 grams of epoxy 6B Sepharose [Pharmacia] was weighed out into 200ml glass
Fig 5.1: Steroid analogues used to purify Fv 4155

NATIVE ANTIGEN
Estrone-3-glucuronide
High affinity

COLUMN LIGAND
Estrone
Low affinity

ELUANT
Estriol-3-glucuronide
Medium affinity
beaker. 200mls of MilliQ water (i.e. water de-ionised by a Milli-Q purification system, Millipore, Watford, U.K.) was added to this and the gel was left to resuspend for 30 minutes.

4.3mg of estrone [Sigma] was weighed out into a sterile glass universal. This was dissolved in 5mls of HPLC grade dimethylformamide (DMF) [Aldrich]. The Sepharose was collected together in a P4 sinta glass using a vacuum pump and flask. (Care was taken not to dry the Sepharose out). It was then washed with a further 600mls of MilliQ water followed by 100mls of 85% DMF/15% MilliQ water. The Sepharose was carefully added to the estrone solution. Residual Sepharose in the sinta glass was washed out with additional 85% DMF in MilliQ. The volume in the universal was then increased to 25mls with 85% DMF in MilliQ.

7μl of 10M NaOH was added to the solution and the pH checked using a glass pH probe to confirm that the pH was 13+.

The glass universal was then placed in a 45°C incubator with slow rotation for at least 16 hours.

The Sepharose was again collected with a P4 sinta glass and washed with 200ml of 85% DMF in MilliQ, followed by 400mls of PBS and then equilibrated in PBS with 0.1% sodium azide as a bacteriostat.

The Sepharose was packed in a C16 column [Pharmacia] and stored at 4°C until required.

5.2.3 Expression of Fv 4155 in *E.coli* cultures

The genes encoding VH and VL of the monoclonal antibody 4155 were cloned from hybridoma cells by Lowe (a collaborating PhD student, Dundee University). The genes were assembled in a pUC19/E.coli expression system which was designed to produce Fv that was tagged on the C-terminus of its VL with the myc epitope (see Ward et al, 1989).

Cells were grown in shake-flasks for 5 hours at 37°C. The growth medium was a bactotryptone/yeast extract broth known as 2 x TY (Miller, 1972), supplemented with 1% glucose and 100 μg/ml ampicillin. The cells were pelleted by centrifuging at 4,000 r.p.m. for 30 minutes. The pellet was resuspended in 2 x TY with ampicillin.
but without glucose and left at room temperature for 1 hour. Fv synthesis was
induced by the addition of 0.5mM IPTG. Induced cells were incubated at 25°C, with
shaking, for 17 hours. After incubation, cells were pelleted by centrifugation at
10,000 r.p.m. at 4°C for 1 hour. The supernatant was clarified by passing through
an 0.2μm filter unit [Nalge (Europe) Ltd, Hereford, U.K.]

5.2.4. Recovery of Fv from culture by antigen analogue affinity chromatography.
5.2.4.1. Preliminary investigation of loading and elution conditions.
Clarified culture supernatant was applied to an estrone-agarose affinity adsorbent
packed in a C16 column (Pharmacia). The pump speed was 2mls/min. After loading,
the column was washed extensively with phosphate buffered saline (PBS) until the
absorbance monitor had returned to base-line. Bound Fv was eluted either with one
column volume of estriol-3-glucuronide (0.2mg/ml in PBS) or with one column
volume of 50mM glycine, pH 2.5. The desorption buffers were applied at 1ml/min.
The recovered fractions were immediately buffer-exchanged into PBS by desalting on
a PD10 column (Pharmacia) or by dialysis. [The PD10 column would be expected
to remove most of the excess (i.e. unbound) estriol-3-glucuronide but would not be
expected to remove estriol-3-glucuronide which had bound to the Fv]. Buffer-
exchanged fractions were analysed for Fv by SDS-PAGE as described in 5.2.5.

5.2.4.2 Quantitative analysis of recovery efficiency of Fv 4155: comparison of
different desorption protocols.
600mls of culture supernatant was produced as previously described. This feedstock
was used to compare the recovery efficiency obtained with two different desorption
protocols in a split-batch experiment, as set out below.

Half of the feedstock (300mls) was applied to a 9mls estrone-agarose affinity
adsorbent packed in a C16 column (Pharmacia). A sample of the fallthrough was
taken for frontal analysis every 100mls (designated fallthrough fraction A, B, and C
respectively) in order to check that the column capacity was not exceeded. Then the
column was washed with phosphate buffered saline (PBS) until the absorbance
recorder had returned to base-line. Bound Fv was desorbed with 20mls of 0.2mg/ml
estriol-3-glucuronide made up in PBS. Desorbed Fv was immediately buffer-exchanged into PBS on a 150mls column of G-25 M Sephadex (Pharmacia) packed in a C25 column (Pharmacia). The estrone-agarose column was re-equilibrated in PBS.

The second half of the feedstock (300mls) was then applied to the estrone-agarose adsorbent as before. This time bound Fv was desorbed with 20mls of 50mM glycine pH 2.5 buffer. Desorbed Fv was immediately buffer-exchanged into PBS as above. The buffer-exchanged fraction was confirmed to be pH 7 by analysis with pH indicator strips (Whatman). As this fraction was found not to contain much active Fv (see results, table 5.2), the estrone-agarose adsorbent was further eluted with 20mls of a denaturing buffer [4M guanidine hydrochloride (molecular biology grade, Sigma); 33.3% acetonitrile (hplc grade, BDH)] and the fraction thus recovered was dialysed into PBS. The column was re-equilibrated in PBS.

All fractions were taken for analysis for total protein and active Fv as described in 5.2.6 and 5.2.7

5.2.5 Determination of Fv purity by SDS-PAGE.
SDS-PAGE analyses of fractions isolated by affinity chromatography were carried out using the Pharmacia Phastsystem. Samples were boiled for 5 minutes in running buffer [10mM Tris-1mM EDTA (Sigma) (pH8)] with 2.5% SDS-5% β-mercaptoethanol (BDH) and 0.01% Bromophenol Blue (BDH) as a tracking dye, then run on a Pharmacia homogeneous 20 Phastgel with Pharmacia SDS buffer strips. The gel was stained using the silver staining protocol (Pharmacia).

5.2.6. Determination of total protein in feedstock and recovered fractions.
The total protein content in crude fractions (i.e. feedstock and fallthrough fractions) was determined using the Pierce BCA protein assay (Pierce, Rockford, Illinois, USA). 20ul of sample was incubated with 200ul of reagent in a microtitre plate for 30 minutes at 37°C, according to manufacturer's instructions. Then the absorbance was read at 570nm and converted to mg/ml protein using a standard curve generated
with bovine albumin. (As these samples contain a mixture of different proteins, a reference such as bovine albumin is appropriate).

The total protein content in pure fractions (i.e. fractions recovered from the affinity column was determined using the Coomassie Protein Plus assay (Pierce, Rockford, Illinois, USA) so as to achieve the sensitivity required. 100μl of sample was incubated with 100μl of reagent for 10 minutes at room temperature, according to manufacturer's instructions. Then the absorbance was read at 570nm and converted to μg/ml protein using a standard curve generated with a standard of Fv anti-hCG, prepared as described in Chapter 4. [For purified fractions, it is more accurate to use an Fv standard as different proteins can react differently to Coomassie stain, Bradford (1976)].

5.2.7 Determination of active Fv by ELISA.

Microtitre plates were sensitised with estrone-3-glucuronide by incubating wells with a 2μg/ml solution of an ovalbumin/estrone-3-glucuronide conjugate made up in carbonate buffer, pH 9.6. (The conjugate had been made according to the method of Gani et al, 1994). Sensitisation of the plates with conjugate took place overnight at 37°C.

Sensitised plates were aspirated and rinsed three times with PBST [Phosphate buffered saline containing 0.15% tween 20 (Sigma)]. Fv-containing samples were diluted in PBST and then applied to sensitised plates at a range of dilutions. The plate was incubated for 1 hour at room temperature.

Plates were washed with PBST as above and then incubated with a 2.6μg/ml solution of a monoclonal antibody with a specificity for the myc peptide (Munro and Pelham, 1986) made up in PBST. Plates were incubated for 1 hour at room temperature.

Plates were washed with PBST and then incubated with a 1/1000 dilution of antimouse/alkaline phosphatase conjugate (Zymed) for 1 hour at room temperature.

Plates were washed with PBST and then incubated with a 1mg/ml solution of paranitrophenolphosphate (Sigma) made up in substrate buffer (50mM diethylamine, pH 9.8, 1mM MgCl₂). Plates were incubated at room temperature until colour
development had occurred (usually about 1 hour).

The ELISA was standardised by assigning a concentration of 100 Units/ml of active Fv to the clarified *E.coli* culture and then making a series of dilutions of this feedstock in PBST. This dilution series was tested in the ELISA to generate a standard curve. Recovered fractions from chromatography experiments were analysed for active Fv by making appropriate dilutions in PBST so that their signal in the ELISA could be read off from a point in the middle region of the standard curve.

5.2.8 Determination of storage stability under different temperature conditions: comparison of Fv eluted with estriol-3-glucuronide and Fv eluted with pH 2.5 buffer.
The storage stability of two Fv preparations, as obtained by the two different elution conditions, was compared by storing aliquots of each at 4°C and at 37°C for 53 days. The preparation that had been eluted with estriol-3-glucuronide was diluted with PBS so that both preparations had an approximately equal concentration of active Fv at the start of the storage experiment. The risk of bacterial contamination was minimised by sterile-filtering each aliquot prior to storage and adding 0.1% sodium azide as a bacteriostat.

The retention of activity of these two preparations was compared by removing aliquots at intervals, making dilutions of the aliquots in PBST, and measuring signal in the ELISA. It was assumed that both Fv preparations would be fairly stable at 4°C and that any inactivation at 37°C could be measured relative to this base-line.
5.3 RESULTS

5.3.1 Elution profiles and SDS-PAGE analysis of peak fractions

Elution with pH 2.5 gave a sharp elution peak (see Fig 5.2). In contrast, elution with estriol-3-glucuronide gave a broad peak. This is partly because estriol-3-glucuronide absorbs at 280nm, the wavelength used for monitoring protein. However, Fv was found to be present in all four sub-fractions that were taken, indicating that the broad peak was also partly due to a slow desorption of the Fv (see Fig 5.3). Therefore it would seem to be important to elute with at least one column volume of antigen analogue. (In fact, the practise of eluting with two column volumes of antigen analogue was adopted for future work - see 5.2.4.2).

Both elution protocols produced pure Fv - the characteristic bands of V\textsubscript{H} and V\textsubscript{L}myc were clearly visible on SDS-PAGE, close to the molecular weights of 13.1 kD and 14.8 kD that were calculated from sequence information. For material which was eluted with estriol-3-glucuronide, there was also an additional band. It seems possible that it is due to a complex of estriol-3-glucuronide with one or other of the protein chains (see Fig 5.3).

5.3.2 Standardisation of the analytical immunoassay

The feedstock diluted out to produce a sigmoid curve against which the signal from fractions could be read off. Fig 5.4.

5.3.3 Determination of recovery efficiency of active Fv with ELISA.

The Fv preparation that had been eluted with estriol-3-glucuronide was found to contain approximately 90% of the active Fv present in the feedstock. In contrast, the Fv preparation that had been eluted with pH 2.5 buffer contained approximately 6% of the active Fv present in the feedstock. Furthermore, the Fv preparation eluted with pH 2.5 buffer had a poor specific activity. Elution with guanidine/acetonitrile buffer after the pH 2.5 buffer resulted in a further recovery of 14% of active Fv.

Full details are given in the recovery schedules laid out in tables 5.1 and 5.2.
Fig 5.2: Recovery of Fv 4155 from clarified *E. coli* culture

Clarified *E. coli* culture, containing Fv 4155, was loaded onto an estrone-agarose affinity adsorbent. After loading, the column was washed with PBS until the absorbance monitor had returned to base-line. Bound Fv was eluted with either a) 50mM glycine, pH 2.5 or b) 0.2mg/ml estriol-3-glucuronide made up in PBS.
Fig 5.3: SDS-PAGE analysis of chromatography fractions. Fv was produced in shake-flasks and recovered from culture supernatant on an estrone-agarose affinity adsorbent.

Lanes (Refer to chromatograms in fig 5.2).
1. Fv eluted with estriol-3-glucuronide (fractions 1 + 2, pooled).
2. Fraction (1+2) desalted on a PD10 column.
3. Fv eluted with estriol-3-glucuronide (fraction 3).
4. Fv eluted with estriol-3-glucuronide (fraction 4).
5. Fractions (3+4) desalted on a PD10 column.
6. Fv eluted with 50mM glycine, pH 2.5 (fraction P).
7. Fraction P, dialysed.
Fig 5.4: Standard curve for active Fv 4155

A concentration of 100 units / ml was assigned to the clarified *E. coli* culture, containing Fv 4155. A series of dilutions of this feedstock was made and tested in the ELISA to generate a standard curve.
Table 5.1: Recovery schedule for process comprising elution with antigen analogue.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume (mls)</th>
<th>Active Fv (Units /ml)</th>
<th>Total protein (mgs /ml)</th>
<th>Specific Activity (Units /mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feedstock</td>
<td>300</td>
<td>100</td>
<td>3.8</td>
<td>26.3</td>
</tr>
<tr>
<td>Fallthrough A</td>
<td>100</td>
<td>3</td>
<td>3.8</td>
<td>0.78</td>
</tr>
<tr>
<td>Fallthrough B</td>
<td>100</td>
<td>4</td>
<td>3.8</td>
<td>1.05</td>
</tr>
<tr>
<td>Fallthrough C</td>
<td>100</td>
<td>4</td>
<td>3.8</td>
<td>1.05</td>
</tr>
<tr>
<td>Washings</td>
<td>100</td>
<td>1</td>
<td>0.04</td>
<td>25</td>
</tr>
<tr>
<td>estriol-3-g fraction</td>
<td>28</td>
<td>1,000</td>
<td>0.05</td>
<td>20,000</td>
</tr>
</tbody>
</table>

Recovery efficiency for process comprising elution with an antigen analogue

Proportion of loaded Fv in recovered fraction =

$\frac{(28 \times 1,000)}{(300 \times 100)} \times 100\% = 93\%.$
Table 5.2: Recovery schedule for process comprising elution with conventional desorption buffers.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume (mls)</th>
<th>Active Fv (Units / ml)</th>
<th>Total protein (mgs / ml)</th>
<th>Specific Activity (Units / mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feedstock</td>
<td>300</td>
<td>100</td>
<td>3.8</td>
<td>26.3</td>
</tr>
<tr>
<td>Fallthrough A</td>
<td>100</td>
<td>4</td>
<td>3.8</td>
<td>1.05</td>
</tr>
<tr>
<td>Fallthrough B</td>
<td>100</td>
<td>5</td>
<td>3.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Fallthrough C</td>
<td>100</td>
<td>5</td>
<td>3.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Washings</td>
<td>100</td>
<td>0</td>
<td>0.04</td>
<td>0</td>
</tr>
<tr>
<td>pH 2.5 fraction</td>
<td>22</td>
<td>90</td>
<td>0.04</td>
<td>2,250</td>
</tr>
<tr>
<td>Guanidine fraction</td>
<td>36</td>
<td>120</td>
<td>0.015</td>
<td>8,000</td>
</tr>
</tbody>
</table>

Recovery efficiency for process comprising elution with conventional desorption buffers.

Proportion of loaded Fv recovered in pH 2.5 fraction =

\( \frac{(22 \times 90)}{(300 \times 100)} \times 100\% = 6.6\% \)

Proportion of loaded Fv recovered in guanidine fraction =

\( \frac{(36 \times 120)}{(300 \times 100)} \times 100\% = 14.4\%. \)
5.3.4 Storage stability of purified Fv preparations

The Fv eluted with estriol-3-glucuronide was found to be much more stable to prolonged storage at 37°C than the Fv eluted with pH 2.5 buffer. See Figs 5.5a, 5.5b, 5.5c, 5.5d and 5.5e.
Fig 5.5: Storage stability of purified Fv 4155 preparations
a: Starting activities at Day 0.
Fig 5.5: Storage stability of purified Fv 4155 preparations
b:- Remaining activities at Day3

Signal in ELISA (A 405nm)

- Analogue eluted/stored at 4°C
- Analogue eluted/stored at 37°C
- pH 2.5 eluted/stored at 4°C
- pH 2.5 eluted/stored at 37°C
Fig 5.5: Storage stability of purified Fv 4155 preparations

c: Remaining activities at Day7

Signal in ELISA (A405nm)

Dilution of sample

Analogue eluted/stored at 4°C
Analogue eluted/stored at 37°C
pH 2.5 eluted/stored at 4°C
pH 2.5 eluted/stored at 37°C
Fig 5.5: Storage stability of purified Fv 4155 preparations
d:- Remaining activities at Day 35

![Graph showing the storage stability of purified Fv 4155 preparations.](image)
Fig 5.5: Storage stability of purified Fv 4155 preparations

e:- Remaining activities at Day 53

Signal in ELISA (A405nm)

Analogue eluted/stored at 4°C
Analogue eluted/stored at 37°C
pH 2.5 eluted/stored at 4°C
pH 2.5 eluted/stored at 37°C

Dilution of sample

1/5 1/10 1/20 1/40 1/80 1/160 1/320
5.4 DISCUSSION

In this chapter, a new process for recovering Fv fragments from *E. coli* cultures was investigated: Fv was captured onto an affinity adsorbent comprising a low affinity antigen analogue and then desorbed with an eluant comprising a medium-affinity antigen analogue. An important part of this investigation was to compare the amount of active Fv recovered by the novel elution conditions with the amount of active Fv recovered by conventional elution conditions i.e. acidic buffer. To achieve a quantitative comparison, an analytical ELISA was used to determine the proportion of active Fv that could be recovered from culture by each method. The ELISA worked on the principle of capturing the Fv with antigen and then determining the amount of captured Fv by using a myc-specific tracer antibody (Munro and Pelham, 1986). Although there is some doubt as to the value of determining absolute concentrations of Fv using this system - due to the proteolytic loss of myc (Ward et al, 1989), the analysis was perfectly adequate for determining the proportion of Fv that could be recovered from culture. As analysis was carried out immediately after recovery, it is very unlikely that myc-specific proteolysis would have a significant effect on the data presented here. The ELISA was standardised against the feedstock (i.e. clarified *E. coli* culture supernatant) which was assigned a concentration of active Fv of 100 Units / ml. Such a standard is appropriate for an analysis set up to investigate the efficiency of a downstream recovery process, as the objective is to measure the amount of active Fv in recovered fractions relative to the feedstock. Thereafter efficiency can be expressed as a percentage. This is in contrast to chapter 4, where the analysis was set up to investigate primary production i.e. accumulation in the fermenter. In this instance, it is preferable that analyses are standardised in defined units (e.g. milligrams of active Fv per litre of culture); otherwise, expression levels cannot be readily compared with those obtained by other laboratories.

For the example in this investigation, it was found that the recovery efficiency of an affinity separation process comprising desorption with an antigen analogue was vastly superior to a process comprising desorption with a pH shock. Recovery of active Fv exceeded 90% when eluting with antigen analogue, compared with less than 10% when eluting with low pH buffer. The data suggest that there are two reasons for this
stark contrast. Firstly, the acidic buffer was not very effective at removing Fv from the column: a further 14% of the loaded Fv was recovered when the column was eluted with guanidine/acetonitrile buffer. Secondly, the Fv that was recovered with acidic buffer had a low specific activity, presumably due to permanent inactivation by the low pH. Whilst it seems unlikely that all Fvs would show such a pronounced sensitivity to acidic buffer (indeed the Fv described in the previous chapter could be eluted with pH 2.5 quite satisfactorily) the use of antigen analogues as specific desorbents in a mild background buffer - such as saline - would appear to be a generally attractive approach, particularly for large-scale columns where the exposure time to desorption buffer would be longer.

Another important finding was that the Fv-analogue complex was also more stable than the native Fv during prolonged storage. This is presumably because the analogue interacts with both $V_H$ and $V_L$, thereby having the effect of stabilising the Fv unit. It seems likely that antigen analogues would also protect Fv against other forms of inactivation - such as exposure to surfactants - by occupying the binding site and preserving it in its optimal conformation. A somewhat analogous approach has been used to stabilise enzymes with coenzyme analogues (Koch-Schmidt and Mosbach, 1977). However, this strategy has not previously been considered for stabilising Fvs, despite a lot of research on Fv stability. (Glockshuber et al, 1990).

In this investigation, an Fv specific for a steroid was used because nature has already provided a set of cross-reacting antigen analogues. Recent developments in molecular biology and biochemistry have brought the prospect of identifying antigen analogues even when suitable molecules do not exist in nature: "diversity libraries" have been designed and synthesised which contain literally millions of different molecules each with its own distinct molecular "shape". Many of these libraries contain peptide molecules (Scott and Smith, 1990; Lam et al, 1991) but libraries have also been prepared which contain nucleotides (Kenan et al, 1994) or carbohydrates (Kenan et al, 1994). From within these libraries it is possible to identify molecules which bind specifically to a receptor protein of interest, such as an antibody. Indeed, some antigen analogues have been identified which compete with the antigen for binding.
sites on the antibody but share no obvious homology. In this case, the analogues are usually referred to as "mimics". For example, a peptide has been identified which appears to mimic the shape of a carbohydrate antigen (Hoess et al, 1993). The technology is in its infancy and there are still some technical difficulties. Moreover, it is currently unclear whether all or just some antibody specificities will be amenable to this approach. Nevertheless, it seems highly likely that diversity libraries in general, and peptide libraries in particular, will become a useful source of ligands for affinity separations in the future (Baumbach and Hammond, 1992). Furthermore, once candidate peptide sequences have been identified, it is possible (at least in principle) to synthesise a range of peptide analogues with different binding affinities by making focused modifications to the sequence [see Pinilla et al. (1993)]. It is suggested that the discovery of such peptides would make it possible to design affinity separation processes featuring the benefits described in this chapter - including high recoveries and improved stability - for most Fvs regardless of their primary specificity.
6. GENERAL DISCUSSION AND CONCLUSIONS.

At the time of registration for this PhD (February 1993), the exciting advances in antibody fragment technology were lead by molecular biologists, particularly by Pluckthun and co-workers and by Winter and co-workers. These researchers and others pioneered methods for producing a number of differently designed antibody fragments by recombinant DNA technology. Of these molecular designs, the Fv antibody fragment appeared to be the most promising for exploitation in the Fine Chemicals Industry (refer to Chapter 1 for details). Since registration, advances in the field continue to be lead by molecular biologists. To summarise, there have been three important initiatives which are especially noteworthy. The first important initiative is the design and synthesis of bivalent and/or bispecific antibody fragments. There are several different methods for synthesising these constructs, however, the basic principle is to link two Fv fragments together. Such molecules could have the advantages of more substantive binding (in the case of a bivalent, monospecific reagent) or the ability to bind two target molecules together (in the case of a bispecific reagent). The reader is referred to Holliger et al., 1993; Pack et al., 1993; Kipriyanov et al., 1994; Whitlow et al., 1994; and Kipriyanov et al., 1995 for examples of this research. The second important initiative is the linking of antibody fragments to effector molecules. The appeal of this molecular design is that the antibody binding domain can target the effector molecule to a point of application so that the local concentration of the effector is enhanced. In this context, antibody fragments have been linked to enzymes (Ducancel et al., 1993; Verhoeven et al., 1995), to cytotoxins (Yang et al., 1995) and even to gene therapy delivery systems (Somia et al., 1995). The third important initiative is the preparation of antibody fragments that have been derived from the camel or, its near relative, the llama. This research has its routes in the finding that the camel naturally produces some peculiar immunoglobulin molecules that lack light chain (Hamers-Casterman et al., 1993). Furthermore, the sequence of the camel V_H chain was found to have some focused amino acid changes that make it more soluble than other known V_H domains. Therefore, it seems possible that V_H fragments derived from the camel may be exploitable as biochemical reagents without the drawbacks of the mouse V_H fragments referred to in Chapter 1. Indeed, preliminary research with camel V_H sequences has
been very promising (Muyldermans et al., 1994; Davies and Riechmann, 1995). Whereas it is very tempting to predict exciting applications both for Fv antibody fragments and for the more ambitious molecules described above, this vision will only become a reality if the Fine Chemicals Industry can produce them at a cost similar to that currently achieved for bulk enzymes. This will require inputs from other scientific disciplines in addition to molecular biology; principally, inputs will be required from biochemistry and from biochemical engineering. Moreover, it would seem most appropriate for these scientific disciplines to investigate the established molecular designs in the first instance. Therefore, this thesis maintained a focus on Fv antibody fragments despite the emergence of the more recent molecular designs that are described above.

From the biochemical engineering stand-point, the necessary reduction in costs requires that the entire process of antibody fragment production is considered for complete and optimal design. There are three key blocks of activity in this process: fermentation, analysis, and recovery. Of these, fermentation has recently been investigated and reviewed by a collaborating PhD student at UCL (Harrison, 1995). However, a detailed search of the recent literature resulted in no reports to the present date of quantitative analyses which have been rigorously standardised or of recovery schedules that have been thoroughly investigated and optimised in terms of their efficiency. Therefore, the present thesis aimed to investigate aspects of Fv antibody fragment production that are pertinent to analysis and recovery. The *Escherichia coli* expression system described by Ward et al. (1989) was used because of the relative ease at which different Fv specificities can be cloned and produced using this system. However, the general principles of analysis and recovery elucidated in this thesis are equally relevant to the other host organisms that are being investigated for expression of antibody fragments such as filamentous fungi (Nyyssonen et al., 1993), crop plants (Owen et al., 1992) and yeast (Ridder et al., 1995; Luo et al., 1995).

The starting point for this investigation was to design and synthesise generic tracer antibodies for use in general-purpose analytical systems that would recognise any Fv,
regardless of its specificity. Experiments reported in chapter 3 were concerned with raising tracer antibodies against a conserved peptide motif on the fourth framework region of the $V_H$ chain. It was found that the motif was exposed and available for capture on 3 out of 3 Fvs when they were immobilised directly onto a surface (such as a polystyrene microtitre plate). In contrast, the motif was either partially or totally obscured when the Fvs were complexed with immobilised antigen. This reactivity profile enabled the tracer antibodies to be used for a general-purpose analytical immunoassay for total Fv protein, but (disappointingly) not for active Fv. Nevertheless, a general-purpose analysis for Fv protein has its uses: either as part of a more complete analysis (as described in Chapter 4) or independently as a preliminary indicator of protein expression during the early stages of optimising an Fv production process. In this capacity, the monoclonal tracer antibody described in Chapter 3 is now in routine use in the Unilever laboratories in Colworth and in Vlaardingen. In future studies, it may be profitable to try to raise even better tracer reagents than those described in Chapter 3. Of particular value would be a monoclonal reagent which could recognise any Fv whether bound to antigen or not: such a reagent could be used for general-purpose analytical immunoassays for both total Fv and active Fv. To raise such a reagent, it would first be necessary to identify common epitopes on Fvs which remain exposed even when binding antigen. At the time of registering for this PhD, it was difficult to predict such epitopes with much chance of success because most published Fv structures were theoretical models (based on known structures of Fab fragments and whole antibodies). However, there are now several Fv structures in the Brookhaven Protein Structural Database [established by Bernstein et al. (1977) and continuously updated] that have been experimentally determined. Furthermore, as this is an active field of research (Phillips, 1996) the publication of more experimentally-derived Fv structures (with and without antigen complexed) can be expected soon. Therefore, it may well prove possible to identify common, exposed epitopes in the near future. Chapter 3 also established two important criteria for determining that an analytical system is not sensitive to interference from non-specific biomass: firstly, it should be ascertained that a representative negative fermenter feedstock produces a good base-line; and secondly, it should be ascertained that when this feedstock is "spiked" with Fv, a
signal is produced that is nearly identical to that obtained with the same concentration of purified Fv.

The tracer antibodies and analytical methods that had been developed from the research reported in chapter 3 were then used to analyse Fv production when scaling-up from shake-flask cultures to a 5 litre fermenter. Whilst scaling up a production process, it is very important to ensure that the target protein retains its "authenticity" i.e. that it retains the desirable properties for which it was selected in the first instance. For proteins expressed in *Escherichia coli*, there is not the problem of glycosylation to contend with and so authenticity can be determined by measuring the specific activity of the target protein. (Specific activity is often referred to as "immunoreactivity" for an antibody or Fv fragment). The issue of determining the immunoreactivity of Fv fragments in fermenter cultures was addressed in Chapter 4, using an Fv specific for human chorionic gonadotropin (hCG) as an example. Total Fv protein was determined by using the motif-specific immunoassay reported in Chapter 3; active Fv was determined by an antigen capture ELISA, using a rabbit reagent that was raised against the Fv of interest. The ratio of these two figures was designated immunoreactivity and expressed as a percentage. An important achievement of Chapter 4 was to establish an Fv anti-hCG standard preparation in which there was a high degree of confidence. The standard had been rigorously characterised and quantified in terms of its purity, immunoreactivity, and multimeric status. The immunoreactivity of the Fv anti-hCG in fermenter cultures was then measured relative to this standard. It was found that the immunoreactivity of this Fv was very high in fermenter culture, approximately 100%. However, it should be emphasised that this figure only relates to this particular Fv. Moreover, another research group (Somerville et al., 1994) working with a different antibody fragment system estimated that their cultures were producing protein with an immunoreactivity of only 5%. As yet, it is not possible to tell which of these figures will turn out to be the closest to that of an "average" producing culture. In either event, the methods described in Chapter 4 form a very thorough approach for making such immunoreactivity measurements, and the approach can be applied to any Fv of interest, regardless of its specificity, and in any host organism. In essence, the
approach entails measuring total Fv and active Fv independently and cross-referencing both measurements to a single standard that has been thoroughly characterised. A collaborating student at UCL (Davidson) is using this same approach to analyse Fv production in crop plants. Since registering for this PhD, there has been an initiative to use evanescent wave biosensors for monitoring Fv production in fermenter cultures (Harrison, 1995). This has the advantage of obtaining results in a few minutes rather than the few hours typically needed for an ELISA. In the future, it may be possible to use such instruments with two reporter cells: one measuring total Fv, the other measuring active Fv. Again the basic principles elucidated in this thesis, especially with respect to Fv standards, will be used.

Having investigated the analysis of Fv in fermenter cultures, attention was turned to recovery and these experiments are reported in Chapter 5. In many ways the requirements for recovery are similar to those for analysis only at macro rather than micro-scale: both systems require solid-phase supports that can discriminate between Fv and non-specific biomass and between active Fv and inactive Fv. [Although the Fv anti-hCG investigated in Chapter 4 was found to be fully active there is some evidence that other antibody fragments may produce a mixture of active and inactive conformers in culture (Somerville, 1994). Therefore the design of recovery systems that are selective for active product would be preferable]. Despite the similarities, there is also an important difference between analysis and recovery: in recovery, Fv has to be desorbed from the solid-phase after binding. This issue is central to the experiments described in Chapter 5. The strategy adopted was to design and test a new bioaffinity recovery process which used two antigen analogues: one as a column ligand, the other as a specific eluant. An Fv with a primary specificity for estrone-3-glucuronide (e3g) was used as an example because a set of suitably cross-reacting antigen analogues was readily available. Estrone (for which the Fv has a relatively low affinity) was used as the column ligand and a solution of estriol-3-glucuronide (for which the Fv has a mid-range affinity) was used as the eluant. The process was found to be very successful: a recovery efficiency of over 90% was obtained and Fv left in complex with estriol-3-glucuronide was found to have a several-fold improved
storage stability compared with native Fv. It is suggested that this recovery process
could also be applied to Fvs for which antigen analogues are not immediately
available (for example, Fvs raised against large antigens such as proteins or whole
cells). This could be done by synthesising peptide mimics of the appropriate epitope
on the large antigen. Price et al (1991) have reported the purification of a
conventional monoclonal antibody on an affinity absorbent comprising a peptide
epitope; and this work has recently been repeated for an Fv of the same specificity
(Berry, unpublished). Furthermore, it may be possible to generate a set of peptides
with different affinities for the Fv by making focused modifications to the primary
peptide sequence. [The reader is referred to the modification strategy published by
Pinilla et al (1993)]. Peptides with appropriate affinities could be selected from this
set and used as ligand and eluant, just as steroid analogues were used to purify Fv
anti-e3g in Chapter 5. A DTI-LINK project (lead by Dr M.R. Price at Nottingham
University) has recently been set up to investigate the feasibility of using peptide
analogues in this way to recover Fv fragments from crude feedstocks at large-scale.

In conclusion, this thesis describes a new experimental base and the preparation of
new immunoreagents that facilitate the analysis and recovery of authentic Fv antibody
fragments. The numerical findings of immunoreactivity and recovery only hold for
the particular Fvs described, however, the approach used to determine these
parameters is thought to be generally applicable to all Fvs.
APPENDIX 1: VECTORS USED BY THE COLWORTH GROUP
FOR EXPRESSION OF ANTIBODY FRAGMENTS.

Vectors used for expression of (a) Fv and (b) Fv tagged with the myc epitope.
VH and VL genes were assembled in pUC19 as described by Ward et al. (1989).
The Ward system uses the lac promoter and targets protein to the periplasm
by using the pel B leader.

Different V-genes were cloned from different hybridomas and would therefore
be non-identical in sequence except at their termini which are defined by the
PCR primers used to clone them. One of the termini, mot 1, was used as an
epitope for immunoanalysis of protein expression. (Refer to Chapters 3 and 4).
In Chapter 5, the myc tag was used as an epitope for immunoanalysis. This
differs from mot 1 in that it represents an extension from the V-gene and is
"foreign" in that it shares no homology with immunoglobulin sequences.
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Assay and purification of Fv fragments in fermenter cultures: design and evaluation of generic binding reagents


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Scope of the Journal

The JOURNAL OF IMMUNOLOGICAL METHODS is devoted to covering techniques for: (1) quantitating and detecting antibodies and/or antigens and haptenes based on antigen-antibody interactions; (2) fractionating and purifying immunoglobulins, lymphokines and other molecules of the immune system; (3) isolating antigens and other substances important in immunological processes; (4) labelling antigens and antibodies with radioactive and other markers; (5) localizing antigens and/or antibodies in tissues and cells, in vivo or in vitro; (6) detecting, enumerating and fractionating immunocompetent cells; (7) assaying for cellular immunity; (8) detecting cell-surface antigens by cell-cell interactions; (9) initiating immunity and unresponsiveness; (10) transplanting tissues; (11) studying items closely related to immunity such as complement, reticuloendothelial system and others. In addition the journal will publish articles on novel methods for analysing the organisation, structure and expression of genes for immunologically important molecules such as immunoglobulins, T cell receptors and accessory molecules involved in antigen recognition, processing and presentation. Articles on the molecular biological analysis of immunologically relevant receptor binding sites are also invited. Submitted manuscripts should describe new methods of broad applicability to immunology and not simply the application of an established method to a particular substance.

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Assay and purification of Fv fragments in fermenter cultures: design and evaluation of generic binding reagents


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Fv fragments whose genes have been cloned using common PCR primers carry identical peptide motifs at their termini. We have raised antibodies against the C-terminal motif of the VH chain GQG l'l VTVSS and evaluated their utility as reagents for the assay and purification of Fvs in fermenter culture. Three different Fvs were included in the investigation. We found that the motif was exposed and available for capture when Fv fragments were blotted onto nitrocellulose paper or adsorbed directly onto microtiter plates. In contrast, the motif was either partially or totally obscured when the Fv was complexed with immobilised antigen or when free in solution. This reactivity profile enabled us to develop a general-purpose assay for Fv protein, but not a general-purpose assay for monitoring active Fv. The apparent inaccessibility of the C-terminus of VH conflicts with currently held views on the three-dimensional structure of these molecules.

Keywords: Fv antibody fragment; Polymerase chain reaction; Peptide motif

Introduction

Advances in molecular biology have provided some new antibody fragments which possess the antigen-binding activity of a parent antibody but few, or none, of the other domains. For example, Fv fragments (Skerra and Pluckthun, 1988) comprise the variable region of the heavy chain (VH) with the variable region of the light chain (VL) and they have a molecular mass of 25 kDa. Domain antibodies or "dAbs" (Ward et al., 1989) consist of a single variable region – usually VH – and have a molecular mass of about 12.5 kDa. A particular attraction of Fv fragments is that they are readily expressed in E. coli and yields of up to 450 mg/l have been achieved (King et al., 1993). Important underpinning technology will be required in this field – for example immunoassays or sensors for controlling Fv production and affinity adsorbents for recovering Fv from cultured media. Conventional monoclonal antibodies are routinely assayed and purified by the generic binding proteins protein A and protein G but as these proteins do not bind Fvs (Derrick and Wigley, 1992), new binding reagents will be required. Attempts have been made to raise Fv-specific immunoreagents by inoculating rabbits with Fv protein. This approach has been successful in producing reagents suitable for the assay of individual Fv fragments (Berry and Pierce, 1993) but since Fvs (by definition) contain significant
amounts of variable sequence, new rabbit antibodies have to be generated for each Fv of interest and this is time-consuming.

To aid purification, Fvs may be encouraged to leak into the culture medium from which they can be purified on antigen columns (Anthony et al., 1992; King et al., 1993). This approach is, at best, costly and sometimes impossible (for example when the antigen is in limited supply or poorly defined). Another approach is to tag the Fv fragments with peptide sequences for which binding reagents are available. For example, Fvs have been tagged with histidine-rich tails to facilitate their purification on IMAC columns (Skerra et al., 1992). Fvs have also been tagged with epitopes – short amino acid sequences against which antibodies have been raised (Ward et al., 1989). A disadvantage with this approach is that the presence of tags may be expected to have unpredictable effects on the expression, function, or toxicity of Fv fragments (Sassenfeld, 1990).

From the above, it will be clear that the development of generic binding reagents which are specific for untagged Fvs will greatly facilitate the assay and purification of antibody fragments in culture. An important first step towards designing binding reagents is the identification of motifs or epitopes present on all Fvs. We reasoned that Fv fragments whose genes have been cloned by using the polymerase chain reaction (PCR) would carry identical peptide motifs at the termini of their VH and VL chains (as determined by the PCR primers used), and that these motifs could provide an opportunity for designing generic binding reagents. For example, in our laboratory, we run a programme in which monoclonal antibodies (of known specificity and function) are converted into Fv fragments so that we can take advantage of the greatly improved expression systems for Fvs. In brief, we amplify VH and VL genes from mRNA isolated from hybridoma cells using the PCR primers described by Orlandi et al. (1989).

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**Fig. 1.** Three-dimensional model of Fv structure. Only the a-carbon backbone is shown. The N-termini of VH and VL are indicated as solid circles; C-termini are arrowed. We used coordinates 1 for mouse Fv anti-hen egg lysozyme 2 obtained from the Protein Data Bank (Bernstein et al., 1977; Abola et al., 1987) at Brookhaven National Laboratory.

1 Entry 1HFM.
Cloned DNA is then assembled in the plasmid pSW1, described by Ward et al. (1989). We use the same primers (or very slightly modified primers) and the same plasmid for cloning all our Fvs (which consequently carry the same peptide motifs at their termini). These conserved motif sequences provide an opportunity for universal epitopes – antibodies raised against these sequences should bind all antibody fragments, derived from this process, if the motifs are sufficiently exposed to be available for immunochemical capture. The essential difference between this approach and epitope tagging (Munro and Pelham, 1986; Hopp et al., 1988) is that the PCR-determined motifs are very similar to the native sequence in the same region and are, therefore, unlikely to affect the properties of the Fv fragment.

Model three-dimensional structures of Fv fragments predict that the C-termini of VH and VL are superficial and distant from the binding site, whereas the N-termini are buried and close to the antigen binding site (Pluckthun and Pfitzinger, 1991; also see Fig. 1). Therefore, the C-terminal motifs were the most attractive target epitopes for investigation. We focused our attention on the C-terminal motif of VH as it has the added advantage of being present on both dAbs (i.e. VH) and Fv. In this study, we set out to raise polyclonal antibodies specific to the VH C-terminal motif (GQGTTVTVSS) by inoculating rabbits with this peptide linked to an appropriate carrier protein. We also attempted to raise polyclonal antibodies to this motif in its native conformation by inoculating rabbits with whole Fv protein and then selecting anti-motif antibodies by affinity purification on immobilised motif peptide. Both antibody preparations were evaluated for their ability to bind three different Fv fragments produced in E. coli and for their potential in general-purpose assays for monitoring Fv production in fermenters. An important feature of the investigation was to determine whether there were conditions under which the peptide motif would be exposed. If so, these conditions could be designed into the assay protocol. We went on to raise monoclonal antibodies to GQGTTVTVSS and to make motif-specific immunoadsorbents by immobilising them on agarose. The potential of these immunoadsorbents for recovering Fv from fermenter culture was investigated.

Methods

Preparation of immunogens carrying the motif sequence

The peptide sequence KKGQGTTVTVSS was synthesised by Protein and Peptide consultants (Exeter, UK). This sequence will hereafter be described as mot 1. Mot 1 corresponds to the motif sequence with two additional lysines at its N-terminus. The addition of these lysines was found to improve the solubility of the peptide and to facilitate immobilisation. Mot 1 was covalently linked to a carrier protein (PPD), also at Exeter.

Fv anti-lysozyme carrying the motif sequence (Ward et al., 1989) was used as an immunogen. It was purified to homogeneity by affinity chromatography on a lysozyme-Sepharose column (Ward et al., 1989).

Inoculation of rabbits and evaluation of immune response

Rabbits were inoculated sub-cutaneously with immunogens at a concentration of 1 mg/ml in Freund's complete adjuvant, then boosted 30 days later with the same preparation in Freund's incomplete adjuvant. Test bleeds were taken on day 40 and were evaluated by ELISA for their response to motif-peptide and to Fv protein. In brief, mot 1-PPD (0.1 μg/ml) or Fv anti-lysozyme (1 μg/ml) were adsorbed onto microtiter plates overnight using sensitisation buffer (0.05 M carbonate, pH 10). Plates were washed with PBST [0.01 M Na₂HPO₄/NaH₂PO₄ – 0.15 M NaCl, pH 7 with 0.15% Tween 20 (Sigma)] and then incubated for 1 h at room temperature with dilutions of test serum made up in PBST. Plates were then washed again with PBST before incubation with a 1/1000 dilution of goat anti-rabbit/alkaline phosphatase conjugate (Sigma). Plates were then washed with PBST before development for 30 minutes with substrate (pNPP at 1 mg/ml in 1 M diethanolamine buffer, pH 9.8). Normal rabbit serum was used as a negative control in these assays.
Affinity purification of motif-specific antibodies

5 mgs of the peptide mot 1 were immobilised onto 3 ml wet volume of CNBr-activated Sepharose 4B (Pharmacia), according to manufacturer's instructions (Price et al., 1991). The mot 1-Sepharose was packed in a chromatography column (Pharmacia) and incorporated into a standard liquid chromatography set up (Pharmacia). Rabbit antisera were then loaded onto the column which was washed with PBS until the monitor had returned to baseline. Bound material was eluted with 4 M MgCl₂ made up in distilled water. The eluted fraction was dialysed overnight into PBS. After dialysis, the affinity purified fraction was analysed by SDS-PAGE. The immunospecificity of the affinity purified fraction was analysed by the two ELISA systems described above (anti-motif, and anti-Fv). Results were compared with those obtained for whole serum and the column fall through after making appropriate dilutions.

Immunochemical characterisation of affinity-purified motif-specific antibodies

Motif-specific rabbit antibodies were tested for their ability to bind each of our three Fv fragments [Fv anti-lysozyme, Fv anti-human chorionic gonadotropin (hCG), and Fv anti-glucose oxidase (G.Ox)] in ELISA systems. Each Fv preparation had been purified to homogeneity on antigen affinity columns (lysozyme, hCG, or G.Ox – as appropriate – immobilised on CNBr-activated Sepharose 4B). Each Fv preparation was individually quantified by measuring its absorbance at 280 nm and using extinction coefficients calculated individually for each Fv from sequence information. Two assay formats were used – in the first, direct format, Fv was adsorbed directly onto the surface of microtiter plates and then traced with motif-specific antibodies; in the second, capture format, Fv was captured onto the surface of microtiter plates which had been sensitised with antigen and then traced as before. Each experimental protocol is given below.

Direct ELISA. Microtiter plates were sensitised by incubating with a solution of appropriate antigen (made up in sensitisation buffer) overnight at 37°C. A range of concentrations (0.1 μg/ml to 10 μg/ml) of antigen were investigated. Sensitised plates were incubated with dilutions of Fv made up in PBST. The plates were then incubated with a 1 μg/ml solution of affinity purified rabbit anti-motif antibody in PBST, followed by a 1/1000 dilution of goat anti-rabbit/alkaline phosphatase conjugate (Sigma). Finally, the plates were developed for 20 min with pNPP substrate which was made up to 1 mg/ml in 1 M diethanolamine buffer, pH 9.8. The plates were washed thoroughly with PBST between each incubation. Control plates were traced with rabbit polyclonal reagents raised against whole Fv protein (Fv anti-lysozyme, Fv anti-hCG, or Fv anti-G.Ox, as appropriate).

The suitability of these assays for monitoring Fv production in fermenters was evaluated by determining their sensitivity to a ‘negative fermenter culture’ (i.e. a typical fermenter culture from which all the Fv had been removed by antigen affinity chromatography (Anthony et al., 1992; King et al., 1993) but still containing non-specific E. coli proteins and media components).

Production of Fv fragments in fermenters

Fv anti-hCG fragments were produced in 5 l fermenters (LH 2000 series I; 3 l working volume) from E. coli JM109 carrying the fragment gene on a pSW1 plasmid (as described in the Introduction). The growth medium was M9P (Ryan et al., 1989) supplemented with yeast extract (Beta lab; 10 g/l) and containing glycerol (30 g/l) instead of glucose as a carbon source. The growth temperature was 25°C (unless otherwise indicated in the figure legends) and the pH was controlled at 6.8 by auto addition of 40% (w/v) NaOH. The im-
peller speed was 500 rpm and the aeration rate was 0.1 v/v/min. The inoculum (1% v/v) was grown overnight on M9P medium supplemented with yeast extract (5 g/l) in a shaking incubator at 25°C. During the first 15 h of fermentation, the impeller speed and air flow rate were gradually increased to maxima of 650 rpm and 0.3 v/v/min respectively.

Fv anti-hCG fragment production was induced by the addition of filter sterilised isopropyl-β-D-thiogalactopyranoside (IPTG) (0.5 M; 1 ml/l) during the late exponential growth phase (as indicated in figure legends). Samples (5 ml) were removed at known intervals for analysis and centrifuged for 15 min at 3000 rpm. The supernatant was then filtered through a 0.2 μm microfilter. Cells were lysed by a combination of osmotic shock and enzymatic treatment (French et al., 1992) and the lysates passed through a 0.2 μm microfilter. The resulting supernatant and lysate fractions were assayed using anti-motif antibodies as described below.

Fv anti-lysozyme was produced by a similar protocol except for the key difference that the fermentation temperature was 37°C (optimised).

Assay of Fv in fermenter cultures using anti-motif antibodies

**Immunoblot.** The utility of motif-specific antibodies for the assay of Fv fragments was evaluated by incorporating an affinity purified preparation in an immunoblot assay. In brief, samples from completed fermenter cultures were run on polyacrylamide gel electrophoresis under denaturing conditions (using a Pharmacia homogeneous 20 Phastgel with Pharmacia SDS buffer strips). The gel was then electroblotted onto a 0.2 μm nitrocellulose membrane (Schleicher & Schuell). The membrane was incubated with a solution of affinity purified rabbit anti-motif antibodies (2 μg/ml in PBS with 0.05% Tween 20) for 1 h at room temperature. The membrane was then incubated in a solution of conjugate [Sigma goat anti-rabbit/alkaline phosphatase diluted 1/2000 in PBS with 0.05% Tween 20] before incubating with substrate [BCIP/NBT (Promega)].

**ELISA.** The utility of motif-specific antibodies for the assay of Fv fragments was also evaluated by an ELISA in which test samples were adsorbed directly onto microtiter plates and then traced with anti-motif reagent (as described above). Samples were taken at intervals during the fermenter run, diluted as appropriate in sensitisation buffer, and then analysed as above. The assay was standardised with a set of standards (diluted from a stock solution of Fv anti-hCG) which had been purified to homogeneity on hCG-Sepharose and determined spectrophotometrically (using an extinction coefficient of A₂₈₀ = 1.6 as calculated from sequence information).

**Generation of mouse monoclonal antibodies and the preparation of motif-specific immunoadsorbents**

Mice were inoculated with mot 1-PPD and used to generate monoclonals specific for mot 1 using standard techniques (Gani et al., 1987) Hybridoma cells were screened for secreted monoclonals which bound to mot1 and cross-reacted with Fv anti-lysozyme, using assay systems analogous to those described above.

One of these monoclonal antibodies, 4743.1, was purified to homogeneity on protein A-Sepharose and then immobilised on CNBr-Sepharose (according to the manufacturer's instructions) to produce a motif-specific immunoadsorbent.

**Purification of Fv protein using motif-specific immunoadsorbent**

Culture supernatants containing Fv anti-hCG were passed down the immunoadsorbent; the binding of Fv was evaluated by determining Fv concentrations in the column fallthrough and comparing with the starting Fv concentration in the feedstock (using the ELISA described above). A range of loading conditions were evaluated including pH 4, pH 7, pH 10, 1 M NaCl, 50% ethylene glycol, and 0.05% SDS. In addition, the effect of other E. coli proteins and media components was evaluated by loading Fv in a weak feedstock of 1 mg/ml bovine albumin (Sigma) in PBS only.

**Results**

**Generation of anti-motif antibodies in the rabbit**

Rabbits which had been inoculated with mot 1-PPD developed an immune response against...
mot 1. This antiserum was found to cross-react with Fv anti-lysozyme which had been adsorbed onto microtiter plates. Motif-specific antibodies were purified from this antiserum to homogeneity in a single-step on mot 1-Sepharose. Typically about 1–2 mgs of motif-specific antibody could be recovered from 10 ml of serum.

Rabbits inoculated with Fv anti-lysozyme developed an immune response against Fv anti-lysozyme. This antiserum was found to cross-react with mot-1 which had been adsorbed onto microtiter plates. Motif-specific antibodies were purified to homogeneity from this antiserum using mot-1 agarose. Typically about 1–2 mg of motif-specific antibody could be recovered from 10 ml of serum.

**Immunochemical characterisation of anti-motif antibodies by ELISA**

Rabbit anti-motif antibodies which had been affinity purified on mot-1 agarose were found to behave identically regardless of whether the initial immunogen had been mot 1-PPD or Fv protein. Both antibody preparations bound strongly to all three of our Fv fragments when the Fv was adsorbed directly onto microtiter plates. All three Fvs gave a positive signal from wells sensitised with concentrations down to about 50 ng/ml (Fig. 2). Furthermore, the presence of negative fermenter culture did not interfere significantly with the signal, so this assay appeared to be suitable for further development as a tool for monitoring Fv production in fermenters. In contrast, when Fvs were captured onto solid-phase by their specific immunochemical binding to adsorbed antigen, only Fv anti-lysozyme was recognised by anti-motif reagent (Fig. 3). Furthermore, attempts to improve binding by using different buffer systems to give pH-shifts, mild denaturation etc, did not lead to recognition (results not shown). However, control plates traced with an appropriate anti-Fv reagent gave strong signal down to approximately 1 ng/ml Fv (data not shown). This confirmed that Fv was active and binding to antigen, but not being detected by our anti-motif reagent.

**Assay of Fv fragments in fermenter cultures using rabbit anti-motif antibodies**

**Immunoblot.** Samples from fermenter cultures probed with affinity-purified motif-specific rabbit antibodies were found to give discrete bands close to the calculated molecular weights for Fv fragments and their component chains. Gels derived from cultures producing conventional Fv stained to give a single band at around 12.5 kDa (corresponding to VH chain). Cultures
producing single-chain Fv, scFv (Bird et al., 1988) stained to give a single band at around 26 kDa (corresponding to Fv and linker). There was no cross-reaction from other E. coli proteins in either culture supernatants or cell lysates, despite the presence of many non-specific (bacterial) proteins in these samples. Some examples are shown in Figure 4.

**ELISA.** The assay based upon direct adsorption of the analyte (as utilised for Fig. 2) was used to determine a time-course profile of Fv production in fermenter cultures. From the profile, it was possible to select optimal fermentation conditions and to pinpoint optimal harvest times. For example, by optimising growth conditions we were able to direct Fv anti-hCG almost exclusively into the supernatant fraction (see Fig. 5): a distinct advantage since it makes downstream processing easier (see Discussion).

**Generation of mouse monoclonal antibodies and the preparation of motif-specific immunoadsorbents**

Hybridomas were isolated which secreted antibodies specific for mot 1. These monoclonals bound to Fv protein which was adsorbed onto microtiter plates, but it appeared that their affinity was lower than the rabbit anti-motif reagents, since signal developed less quickly under similar conditions (data not shown). Consequently these reagents were less attractive for assay development than the rabbit anti-motif antibodies.

An IgG monoclonal, 4743.1, was coupled to agarose at a level of 3 mg protein per ml wet gel.

**Purification of Fv anti-hCG using motif-specific immunoadsorbent**

The anti-motif immunoadsorbent comprising monoclonal 4743.1 was unable to recover Fv fragments from fermenter cultures under any of the running conditions investigated. However, in the
Fig. 6. Interaction of Fv fragments with motif-specific immunoadsorbent. Motif-specific monoclonal antibody was covalently coupled to agarose. A mixture of bovine albumin and Fv anti-hCG (albumin in a large excess) was passed through the immunoadsorbent in phosphate buffered saline, pH 7, containing 1 M sodium chloride. Fractions were taken for analysis at intervals. Total protein was determined by the BCA assay (Pierce UK) and Fv by direct ELISA (for details see Methods section).

presence of 1 M NaCl, the immunoadsorbent was able to bind Fv protein sufficiently to retard its progress relative to albumin. This interaction was concluded to be immunospecific since a blank column did not retard Fv under the same running conditions. An example is shown in Figure 6. The same effect was achieved in PBS if the Fv had been heated at 95°C for 5 min – to expose the motif – prior to loading onto the column.

Discussion

An important first step towards designing generic binding reagents for assaying and purifying families of proteins is the identification of appropriate peptide motifs which are common to all members of the family. We have approached this problem by taking advantage of a side effect of a technique which is widely used in gene cloning, namely the polymerase chain reaction (PCR). We reasoned that families of proteins whose genes have been cloned using common PCR primers will carry identical motifs at their two termini. Furthermore, the C-terminal motifs of proteins are particularly attractive targets for capture, since they are often exposed as a consequence of the order in which proteins fold. We raised polyclonal antibodies to the VH C-terminal motif (mot 1) of Fv fragments and found that these did indeed bind strongly to our Fv fragments (produced by the PCR route) when the Fvs were adsorbed onto the surface of microtitre plates or blotted onto nitrocellulose paper.

We have developed an immunoassay in which Fv is directly and non-specifically adsorbed onto microtiter plates from whole test samples. Adsorbed Fv is determined by a polyclonal antibody specific for mot 1. This assay has the outstanding advantage of giving a signal with all Fvs (regardless of idiotype) but has the two disadvantages of being unable to discriminate between active Fv and inactive Fv, or between Fv and VH (however, in practice with the Fvs used in this study we rarely found inactive Fv or free VH to be present in fermenter broths at levels above 10% of total antibody fragments – data not shown). Moreover, we have found this assay to be a particularly useful tool when a new Fv is being produced before more elaborate assays (to determine parameters such as immunoreactivity and subunit composition) have been developed. In this paper we have illustrated the use of the assay to optimise fermentation protocols for producing Fv anti-hCG. An important example was the selection of a fermentation protocol which directs Fv anti-hCG into the supernatant fraction (Fig. 5). This is a distinct advantage since it makes downstream processing easier (Anthony et al., 1992) particularly on a large scale. If Fv remains in the periplasm, it has to be released during processing. Procedures such as cell homogenisation release much cell debris (in addition to Fv) making recovery of active Fv difficult. Investigations with other Fvs in this assay suggested that the signal with each Fv was slightly different. This may be explained by considering which form of Fv is recognised. For example, it could be that the differences are due to the fact that some Fvs are more readily aggregated.
It was disappointing that anti-motif antibodies (mouse monoclonals) immobilised on Sepharose were unable to recover Fvs from fermenter broth. Although we were able to demonstrate some interaction in 1 M NaCl, it was too weak to be of practical value. The inability of anti-motif monoclonal antibodies to bind Fvs in this mode, despite their ready binding of Fvs adsorbed onto microtiter plates, may be explained by the likely conformation-shift of Fv during adsorption, with the result that the motif was further exposed. Epitope exposure resulting from the adsorption of proteins onto microtiter plates has been reported by other investigators (Dierks et al., 1986; Holander and Katchalski-Katzir, 1986). The view that neither our polyclonal or monoclonal anti-motif antibodies readily bind to fully-folded Fvs was supported by the finding that the signal in antigen capture ELISAs was also very weak for two out of three Fvs studied. Future work in designing generic binding reagents for purification should focus on smaller species such as affinity dyes (Jones, 1991; Lowe et al., 1992) or peptides [which can be screened from phage display libraries (Devlin et al., 1990; Scott and Smith, 1990) or designed from a knowledge of DNA sequence data to make so-called 'anti-sense' peptides (Chaiken, 1992; Scapol et al., 1992)]. These smaller reagents may have improved access to mot 1, which we conclude is not sufficiently exposed on fully-folded protein to be captured by large receptor proteins such as antibodies.

Current three-dimensional models of Fv fragments envisage a common framework structure with the complementary determining regions (CDRs) positioned close to the N-termini. The C-termini are superficial and distant from the antigen binding site (Pluckthun and Pfitzinger, 1991). We investigated whether the conformation depicted in these models for the VH C-terminus is sufficiently exposed to be available for capture by binding reagents such as antibodies. The immunochemical experiments produced some surprising results: motif-specific antibodies bound all our Fv fragments (but not all equally strongly) when they were adsorbed directly onto microtiter plates; they bound one out of three Fvs when captured onto the surface of microtiter plates by antigen; they bound none of our Fvs in free solution (in physiological strength saline). These findings are consistent with the view that the VH C-terminus is less available on Fv fragments in aqueous solution than current models would suggest and that the terminus is more available on some Fvs than others. We conclude that current models of Fv fragments (based on X-ray crystallography) may not exactly describe the structure of the proteins in solution and that the models may need to be refined to accommodate immunochemical data as it becomes available.

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Stability of immunoadsorbents comprising antibody fragments

Comparison of Fv fragments and single-chain Fv fragments

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ABSTRACT

Immunoadsorbents comprising Fv fragments specific for hen egg lysozyme were used to recover the enzyme from a 20-fold excess of bovine albumin. We designed automatic equipment to run this model purification system for 100 cycles non-stop and monitored the deterioration of the immunoadsorbents during the cycling procedure. Only minor losses (approximately 25%) in the immunoadsorbents' capacity were detected; this correlated well with ligand loss (measured by enzyme-linked immunosorbent assay) which was approximately 0.2% per cycle. A surprising finding was that the use of "single-chain" Fv fragments conferred only a minor advantage with respect to stability of the immunoadsorbents.

INTRODUCTION

A new generation of antibody fragments has brought an exciting opportunity to the technique of immunoaffinity chromatography. We have recently reported the use of Fv fragments as ligands [1,2]; these reagents are readily produced as recombinant proteins in E. coli [3,4]. Another group has reported the use of even smaller immunoreagents or "mini-antibodies" [5,6]; these reagents may be produced completely chemically by solid-phase peptide synthesis. The advantages of these antibody fragments compared with conventional monoclonal antibodies include: lower production costs, higher capacity for antigen on a weight for weight basis, better penetration in small-bore separation media. We have discussed these advantages in more detail elsewhere [1,2]; we have also discussed the relative merits of the different antibody fragments currently available [2]. We believe that the general availability of these new antibody fragments will broaden the use of immunoaffinity chromatography, particularly in industrial processes. However, to achieve this wider acceptability, affinity media comprising antibody fragments will need to be sufficiently stable to retain activity and specificity over many purification cycles.

The deterioration of affinity adsorbents during prolonged use may be caused by inactivation of the ligand, fouling, or ligand leakage. This deterioration has often been modelled by investigating one of these component causes in isolation; most work has been done on ligand leakage [7-10]. However, it is not always clear which of these route causes will contribute most to the deterioration of a particular adsorbent. Therefore, we took the view that the
most convincing evaluation of our Fv immunoadsorbents would be to measure their stability directly by monitoring changes in performance after repeated cycles of recovering target analyte from feedstock. Previous reporting of this type of data for the stability of affinity adsorbents has often been anecdotal [11-14]. In this study, we set out to evaluate the stability of affinity media comprising 4% agarose and Fv fragments specific for hen-egg lysozyme by monitoring the deterioration in the performance of these immunoadsorbents after being subjected to 100 cycles of a model purification system: the recovery of hen-egg lysozyme from a 20-fold excess of bovine albumin. The performance of the immunoadsorbents was measured against three criteria: capacity for target antigen, the sharpness of breakthrough upon reaching capacity, and the purity of product (i.e., lysozyme) recovered. We also determined ligand leakage (i.e., the presence of Fv in washings) by enzyme-linked immunosorbent assay (ELISA) and correlated these results with observed changes in capacity for antigen. This would allow us to estimate the relative contribution made by ligand leakage to the deterioration of immunoadsorbent performance and therefore also (by inference) the relative contribution made by inactivation of ligand.

A particular objective of this study was to compare and contrast the re-use potential of Fv antibody fragments with that of “single-chain” antibody fragments, or scFv. In Fv fragments, the two component chains of Fv (VH and VL) are held together, non-covalently, by three pairs of hydrophobic patches [15]; whereas in scFv fragments, VH and VL are also covalently linked by a short hydrophilic peptide chain [16,17]. For a fuller description of Fv structure and terminology see refs. 1 and 2]. Since Fv fragments are non-covalently associated, they may be expected to have poor re-use potential, especially after treatment with the harsh elution buffers typically used in immunoaffinity chromatography. These buffers are, of course, designed to disrupt the non-covalent interactions between antibodies and antigens and therefore they may also disrupt the, not dissimilar, non-covalent interaction between VH and VL. However, with scFv, even if VH and VL are temporarily dissociated on treatment with elution buffer, the linker peptide should keep VH and VL in close proximity thereby enabling reassembly when the column is re-equilibrated in a mild buffer (such as physiological strength saline, pH 7).

EXPERIMENTAL

Production of antibody fragments

A vector encoding the Fv fragment of a parent antibody specific for hen-egg lysozyme (the “D.1.3” antibody [18]) and tagged at the C-terminus of its VL with the myc peptide [19] was obtained from Dr. G. Winter (MRC, Cambridge, UK [20]). A vector encoding an analogous scFv fragment with a peptide linker sequence of (Gly–Gly–Gly–Gly–Ser)3 [17] and tagged at the C-terminus of its VL with the myc peptide was also obtained from Dr. G. Winter. (We have previously found that the myc peptide serves as a useful linking group for covalently coupling antibody fragments to solid phases without losing their binding activity [21,22].

The vectors were transformed into E.coli (strain JM109) and grown in cultured medium according to the method of Ward et al. [20]. Secreted Fv fragments were recovered from the medium by affinity chromatography on lysozyme–Sepharose [20]. Hen-egg lysozyme was obtained from Sigma (Poole, UK) and Sepharose from Pharmacia (Uppsala, Sweden).

Preparation of immunoadsorbents

An homogeneous preparation of antibody fragment (ca. 4 mg) at a concentration of 0.5 mg/ml was dialysed into coupling buffer [0.1 M NaHCO3 (BDH, Poole, UK)–0.5 M NaCl (BDH) (pH 8.3)] and immobilised on ca. 1 g of cyanogen bromide-activated Sepharose 4B (Pharmacia) according to that manufacturer’s instructions. One immunoadsorbent was made with Fv anti-lysozyme and one with scFv anti-lysozyme. Unreacted cyanogen bromide groups were blocked by washing overnight in 1 M ethanolamine pH 8 at 4°C according to manufacturer’s instructions. The efficiency of ligand coupling was determined by measuring the absorbance of the ligand solution at 280 nm before and after coupling. [The orientation of the ligands was checked by estimating the specific activity of the immobilized Fv (i.e., the % of Fv molecules in an active orientation/conformation). This was done by estimating the capacity of the immunoadsorbents for antigen and comparing with the amount of im-
mobilised Fv on a molar basis.] A "blank" column was also prepared in parallel for use as a negative control. This was 1 g of the same batch of activated Sepharose 4B which was subjected to the same coupling protocol but to which no immunoligand (i.e., Fv) was added.

Model purification system

A 1-g amount of each immunoabsorbent was conditioned in PBSA (0.01 M Na2HPO4/NaH2PO4–0.15 M NaCl, pH 7 with 0.1% sodium azide as a bacteriostat) and then packed in a glass column (Pharmacia C16). Each column was loaded with a feedstock of 1 mg/ml bovine albumin (Sigma) and 50 μg/ml hen-egg lysozyme made up in PBSA. (A 50 μg/ml solution of this lysozyme preparation had an activity of approximately 2550 I.U./ml) This feedstock was loaded until a stable breakthrough was reached; the columns were then washed back to baseline with PBSA. Flow-rates were kept at 150 ml/h throughout the experiments. Bound protein was recovered by eluting with desorption buffer (4 M MgCl2) and dialysing the peak into PBSA. The columns were then re-equilibrated in PBSA. Chromatograms were drawn by monitoring A280 on-line using a Uvicord monitor linked to a chart recorder (Pharmacia).

Testing of model purification system

Lysozyme activity was monitored across the chromatogram profile by assaying fractions using a suspension of Micrococcus (Sigma) according to that manufacturer's instructions. The 4 M MgCl2 fraction recovered from the immunoabsorbents was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Pharmacia homogeneous 20 Phastgel. (For a fuller description see ref. 2.)

We tested the specificity of our purification model by three control experiments. Firstly, a feedstock of 50 μg/ml lysozyme only was loaded onto the immunoabsorbents and eluted as above. Secondly, the albumin/lysozyme feedstock was loaded onto the blank column and eluted as above. Thirdly, a feedstock of 1 mg/ml bovine albumin and 50 μg/ml cytochrome c (Sigma) was loaded onto the immunoabsorbents and eluted as above.

Continuous cycling of purification model

We designed automated equipment to run our model purification system for 100 cycles non-stop. The automated equipment was custom-built by a local subsidiary of Lee Products (Westbrook, CT, USA). In brief, the feedstock and elution buffer (4 M MgCl2) were pumped onto the column from reservoirs as controlled by two miniature solenoid valves with zero dead volumes (Lee LFYA). These valves were placed on-line between the reservoir containing running buffer (PBSA) and the peristaltic pump so that when neither valve was activated PBSA pumped through the system. The timings of valve activation were pre-set by a controller comprising a microprocessor. At the end of a complete purification cycle another identical purification cycle was started automatically. Sufficient stocks of PBSA, feedstock and elution buffer were made up to last for 100 purification cycles. These were degassed and sterile filtered (Gelman 0.2 μm); all glassware had been sterilised by autoclaving.

A total of 100 purification cycles using the albumin/lysozyme feedstock was carried out on each immunoabsorbent and the blank column. The whole procedure was repeated for both Fv and scFv using freshly prepared immunoabsorbents. A total of 100 purification cycles using the albumin/cytochrome c feedstock was also carried out on the immunoabsorbents.

Analysis of immunoabsorbent deterioration

Chromatograms were drawn for all 100 purification cycles; these were compared for evidence of gradual changes in peak height or breakthrough shape. For cycle 1 and cycle 100, lysozyme activity was monitored across the chromatogram; these profiles were compared for evidence of immunoabsorbent deterioration. For cycle 1 and cycle 100, the purity of the lysozyme fraction recovered from the immunoabsorbents was analysed by SDS-PAGE. The findings were examined for evidence in immunoabsorbent deterioration.

Measuring ligand leakage

Ligand leakage (i.e., the presence of Fv or scFv in washings) was determined by ELISA. The solid phase used in the ELISA system was a specially designed nylon peg [23] which dipped into the wells of standard microtitre plates. Pegs were sensitised with
lysozyme at 50 µg/ml by coupling with glutaraldehyde [24]. Samples of washings from columns (200 µl) were added to the wells of a microtitre plate and wells plus pegs were incubated for 1 h at room temperature. The pegs were removed and washed with distilled water, then incubated for a further hour with 200 µl of a 1:5000 dilution of a rabbit serum specific for Fv anti-lysozyme [2]. The pegs were washed again and then incubated for 1 h with 200 µl of a 1:4000 dilution of conjugate [alkaline phosphatase-labelled goat anti-rabbit (Sigma)]. The pegs were washed again and then incubated with 200 µl of substrate solution (p-nitrophenyl phosphate, 2 mg/ml, in 1.0 M diethanolamine, pH 9.8) for approximately 30 min at room temperature. The pegs were then removed from the solution, and the optical density (at 410 nm) of the solutions was measured with an ELISA reader (Dynatech).

The assay for Fv and scFv was calibrated each time by drawing a standard curve with a carefully prepared and characterised set of Fv anti-lysozyme standards. Fv and scFv gave an equally strong signal in the assay. The assay was sensitive down to a level of 0.01 µg/ml (results not shown).

Since the assay works by capturing Fv with immobilised lysozyme, free lysozyme in solution interferes with the assay. Consequently, the assay could not be used directly to measure the presence of Fv across chromatogram profiles. Instead, we eluted immunoadsorbents in turn with each of the two buffers used in this study (PBSA and 4 M MgCl₂) in the absence of lysozyme and assayed the washings for Fv (in the case of 4 M MgCl₂ this was after dialysis into PBSA). We did this analysis immediately after cycle 1 and cycle 100 for both immunoadsorbents and then superimposed the data on the chromatogram profile: the assumption being that leakage during the previous cycle will have been similar.

RESULTS

Preparation of immunoadsorbents
The efficiency of ligand coupling was found to be between 80% and 90%. The specific activity of the immobilised ligand was found to be between 50% and 60%. Full details are given in Table I.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Fv</th>
<th>scFv</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligand used</td>
<td>4 mg</td>
<td>4 mg</td>
</tr>
<tr>
<td>Ligand immobilised</td>
<td>3.6 mg</td>
<td>3.2 mg</td>
</tr>
<tr>
<td>Antigen capacity</td>
<td>1.2 mg</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Specific activity</td>
<td>58%</td>
<td>55%</td>
</tr>
</tbody>
</table>

Testing of model purification system
When the albumin/lysozyme feedstock was loaded onto the immunoadsorbents, a breakthrough curve developed after about 20–25 ml which correlated with the first appearance of lysozyme activity in washings (Fig. 1A and C). The 4 M MgCl₂ peaks recovered from the immunoadsorbents were found to be homogeneous lysozyme (by analysis with SDS-PAGE). The peak recovered from the immunoadsorbent comprising Fv is shown in Fig. 2.

When the lysozyme (only) feedstock was loaded onto the immunoadsorbents, a breakthrough curve developed in the same position and the same amount of protein was recovered on elution with 4 M MgCl₂ (chromatogram not shown).

When the albumin/cytochrome c feedstock was loaded onto the immunoadsorbents, a breakthrough curve did not develop; no protein was eluted with 4 M MgCl₂ (chromatogram not shown).

Changes in capacity/activity of immunoadsorbents over 100 purification cycles
The immunoadsorbent comprising Fv developed a slightly shallower and earlier breakthrough as the cycling procedure progressed. Also, the peak eluted...
with 4 M MgCl$_2$ was about 25% smaller by cycle 100 (see Fig. 1A and B). For the immunoadsorbent comprising scFv, the breakthrough curve did not become shallower during the cycling procedure. Furthermore, the peak eluted with 4 M MgCl$_2$ was about 25% larger for cycle 100 than cycle 1 (see Fig. 1C and D). Table II details the capacity and recovery efficiency of each column at cycle 1 and cycle 100.

Results from the repeat experiments were essentially the same in all respects.

Changes in specificity of immunoadsorbents over 100 purification cycles

There was not a significant contamination of albumin in the lysozyme recovered from the immunoadsorbents even after 100 cycles (see Fig. 2). There was not a significant binding of protein from the albumin/lysozyme feedstock to the blank column even after 100 cycles (see Fig. 1F). There was not a significant binding of protein from the albumin/cytochrome c feedstock to the immunoadsorbents even after 100 cycles (results not shown).
Fig. 2. SDS-PAGE analysis of separation achieved with Fv Sepharose immunoadsorbent. Lanes: 1 = albumin-lysozyme feedstock; 2 = lysozyme recovered after 100 cycles; 3 = lysozyme recovered after 1 cycle; 4 = Fvmyc anti-lysozyme marker; 5 = lysozyme marker; 6 = Pharmacia low-molecular-weight standards (94 000, 67 000, 43 000, 30 000, 20 100, 14 400 dalton). (Fvmyc anti-lysozyme migrates to a single band due to the near identical molecular weights of its two component chains, V\textsubscript{H} and V\textsubscript{L} myc. The presence of both V\textsubscript{H} and V\textsubscript{L} myc was confirmed by Western blot analysis with V\textsubscript{H}-specific and myc-specific immunoreagents. Furthermore, myc can be selectively removed from Fvmyc by protease-activity with the result that 2 bands are detected by SDS-PAGE [27].)

**Ligand leakage**

Fv fragments were not detectable in washings when the immunoadsorbents were eluted with PBSA. In contrast, Fv was detectable in washings when the immunoadsorbents were eluted with 4 M MgCl\textsubscript{2}. Ligand leakage was highest as the MgCl\textsubscript{2} front passed through the immunoadsorbents and continued at measurable levels while MgCl\textsubscript{2} was being eluted. Ligand leakage returned to baseline when MgCl\textsubscript{2} was cleared from the immunoadsorbents by elution with PBSA (see Fig. 1A–D).

Ligand leakage was approximately twice as high from immunoadsorbents comprising Fv compared with immunoadsorbents comprising scFv. For both immunoadsorbents, ligand leakage had reduced significantly by cycle 100 (about two-fold) but was still readily detectable by the ELISA (see Fig. 1A–D).

In general ligand leakage was very low. The highest level of ligand detected in washings throughout the study was 0.4 \(\mu\)g/ml (for cycle 1 of the immunoadsorbent comprising Fv, see Fig. 1A). The lysozyme peak fraction from this cycle contained lysozyme at approximately 100 \(\mu\)g/ml; therefore leaked ligand in this fraction represents a contamination of 0.4%. At the other end of the spectrum, the lowest level of ligand leakage detected throughout the study was 0.1 \(\mu\)g/ml (for cycle 100 of the immunoadsorbent comprising scFv). In this case, leaked ligand represents a contamination of about 0.1% in the lysozyme peak fraction.

**DISCUSSION**

In this study, we used a model system of recovering hen lysozyme from a 20-fold excess of bovine albumin using immunoadsorbents comprising 4% agarose and Fv fragments specific for hen-egg lysozyme. We confirmed that binding of lysozyme to the

**TABLE II**

**CAPACITY AND RECOVERY EFFICIENCY OF IMMUNOADSORBENTS**

The amount of lysozyme bound was estimated from the position of breakthrough in Fig. 1 (for example the amount of lysozyme which bound the Fv immunoadsorbent during cycle 1 was approximately 24 ml x 2500 I.U. ml = 61 000 I.U.). The amount of lysozyme which eluted with 4 M MgCl\textsubscript{2} was determined by direct measurement of activity in this fraction after dialysis. (All results are expressed as I.U. • 1000). The recovery efficiency for each column was calculated as a% of these two figures.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Cycle</th>
<th>Lysozyme bound</th>
<th>Lysozyme eluted</th>
<th>Recovery efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fv</td>
<td>1</td>
<td>61</td>
<td>37</td>
<td>61</td>
</tr>
<tr>
<td>Fv</td>
<td>100</td>
<td>46</td>
<td>27</td>
<td>59</td>
</tr>
<tr>
<td>scFv</td>
<td>1</td>
<td>51</td>
<td>39</td>
<td>76</td>
</tr>
<tr>
<td>scFv</td>
<td>100</td>
<td>64</td>
<td>49</td>
<td>76</td>
</tr>
</tbody>
</table>
immunoadsorbents required specific interaction between Fv and its antigen by three control experiments. Firstly, lysozyme bound to the immunoadsorbents regardless of whether albumin was present or not. Secondly, lysozyme did not bind to a blank column (i.e. agarose with no Fv attached). Thirdly, cytochrome c (a protein of similar size and charge to lysozyme) did not bind the immunoadsorbents. Having validated our model, we used it as a tool for investigating the stability of the immunoadsorbents during repeated cycles of loading feedstock and eluting product.

We have found that immunoadsorbents comprising scFv can be re-used for 100 cycles without a significant loss of capacity for target antigen; in fact the scFv immunoadsorbent in our model system appeared to have an increased capacity (ca. 25%) for antigen after 100 cycles compared with the first cycle. This seemingly anomalous result may be explained as follows: we have previously measured the recovery of lysozyme protein from an Fv immunoadsorbent during a single purification cycle to be 75–80% [1]; therefore if the residual 20–25% of lysozyme protein were to accumulate on the immunoadsorbent cycle upon cycle, the immunoadsorbent would be irreversibly saturated after 5 cycles. However, since we have found that immunoadsorbents continue to bind and elute target antigen, even after 100 cycles, it is clear that this cumulative binding does not occur. Moreover, it is our opinion that during repeated cycling, there was a degree of randomness in the precise amount of lysozyme that was eluted at the end of each cycle and that a small proportion of the lysozyme eluted may have bound the column one or more cycles previously.

We also made the surprising finding that immunoadsorbents comprising “conventional” Fv ligands (i.e., with non-covalently associated Vh and Vl chains) are also remarkably stable to repeated cycling; the Fv immunoadsorbent in our model system did show a detectable capacity loss over 100 cycles but only about 25%. There may be several reasons for this unexpected stability. Firstly, the Fv derived from D.1.3 is known to have a very high binding constant (approximately 10^{15}/M) for association of its two component polypeptide chains, Vh and Vl [25]. This is significantly higher than for many other Fv fragments [25]. Secondly, the major mechanism for Vh and Vl association is the mutual attraction of three pairs of hydrophobic patches on the two chains [15]. Therefore, an elution buffer with high ionic strength (such as 4 M MgCl2) would be expected to promote association. Thirdly, Fv association may be stabilised by immobilisation, on agarose, possibly because of multi-site attachment. Whatever the reason, Fv remains intact over 100 cycles in our model system without the need for a peptide linker between Vh and Vl.

The amount of ligand leakage determined by ELISA correlated well with the observed loss of the immunoadsorbents’ capacity over 100 cycles. For example, the Fv immunoadsorbent was found to leak ligand at a concentration of 0.4 μg/ml on elution with 4 M MgCl2 during cycle 1; therefore an elution volume of 20 ml would remove 8 μg of Fv from the column (this represents 0.2% of the total Fv on the column). By cycle 100, ligand leakage had dropped to 0.2 μg/ml; therefore about 0.1% of the total Fv would be lost. Taking an average of these results, 100 cycles would be expected to result in a capacity loss of about 15% which would account for a large proportion of the 25% capacity loss measured experimentally. Another correlation between loss of immunoadsorbent capacity and ligand leakage was presented by the finding that Fv immunoadsorbents had a significantly higher capacity loss than scFv immunoadsorbents and that they were also found to leak more ligand by independent experiment. Taking these two correlations together, we conclude that ligand leakage made a major contribution to immunoadsorbent deterioration in this study.

A disappointing finding was that the efficiency of recovering active lysozyme from the Fv immunoadsorbents was in the order of 60%. Furthermore, it is unclear why this was lower than the recovery efficiency of the scFv immunoadsorbents which was in the order of 75%. However, we do not think that these results detract from our key findings about the stability of immobilised Fv and scFv.

Once the production of Fv fragments has been optimised in E. coli, they may be available from about US$ 30/g, a cost which has been achieved for other recombinant proteins in this organism. Since immunoadsorbents comprising Fv can be used for (at least) 100 cycles, the contributory cost of Fv fragments towards producing 1 g of target antigen would be in the order of 60 cents (assuming a target
antigen of similar molecular weight and a specific activity of 50% for immobilised Fv). This cost is very minor compared with labour costs and buffer costs [26]. Consequently, we conclude that the cost of immunoligand, often cited as a reason for not using immunoaffinity chromatography at process-scale, is no longer restrictive.

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Use of antibody fragments in immunoaffinity chromatography

Comparison of FV fragments, VH fragments and paralog peptides

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ABSTRACT

Some new antibody fragments have recently been described: FV fragments (M_r 25 000), VH fragments or “dAbs” (12 500) and paralog peptides (1000–2000). FV fragments, VH fragments and a paralog peptide that had been derived from a parent antibody with a specificity for hen lysozyme were produced. All three reagents were immobilized on Sepharose and evaluated for their ability to recover hen lysozyme from “spiked” serum and to separate hen lysozyme from turkey lysozyme. The FV column had excellent specificity for hen lysozyme, the VH column had significantly reduced specificity and the paralog peptide column did not bind lysozyme at all.

INTRODUCTION

Immunoaffinity chromatography is a high-resolution single-step technique that is simple to operate. However, owing to the prohibitive cost of monoclonal antibodies (MCA), the technique has hitherto been the preserve of the pharmaceutical industry where the high value of target proteins can offset the cost of preparing affinity media. Typical target proteins have been factor VIII [1–3], factor IX [4,5], interferon [6] and epidermal growth factor [7].

Conventional immunoaffinity chromatography uses whole MCA molecules as ligands. However, a brief consideration of the MCA structure makes it clear that much of the molecule is not required for specific binding to antigen, and is an unnecessary encumbrance in immunoaffinity chromatography. Typical monoclonal antibodies have a relative molecular mass (M_r) of 150 000 and consist of two identical heavy chains (M_r 50 000 each) and two identical light chains (M_r 25 000 each). Each chain has a variable region at its amino end, known as the variable light (VL) and variable heavy (VH) regions and a constant region at its carboxy end. The constant regions are responsible for natural effector functions such as binding to cell receptors and complement fixation; the variable regions are responsible for antigen binding. Both VL and VH regions each possess three hypervariable segments or complementarity-determining regions (cdrs). The cdrs from one VL and one VH fold together to form the antigen binding site; there are therefore two antigen binding sites on a conventional antibody (Fig. 1). The sequences of the cdrs are essentially unique for each MCA and this is the molecular basis for their individual specificity. (The structure and function of antibodies have been reviewed in detail elsewhere [8,9].)

Recently, some new antibody fragments have been prepared that possess the antigen-binding activity of a parent antibody but few or none of the
other antibody domains. In order of decreasing size, these antibody fragments have been described as FV fragments [10–12] (Mr 25 000), VH fragments or domain antibodies [13] (Mr 12 500) and “paralog” peptides [14] (Mr 1000–2000). These reagents present an exciting opportunity to the technique of immunoaffinity chromatography as they can be produced much more cheaply than MCA, which are typically produced in myeloma cells in expensive tissue culture media. In contrast, FVs and VHs may be cloned by recombinant DNA technology and produced in cheap bacterial media [13]; paralog peptides can be produced completely chemically by solid-phase peptide synthesis [14] (see Fig. 1).

We have recently shown that immobilized FV fragments can be used to recover target antigen from “spiked” serum [15] and have discussed the advantages of FV fragments over MCA in immunoaffinity chromatography [15]. In this paper, we set out to compare and contrast the utility of the three aforementioned immunoreagents, FV, VH and paralog peptides. We chose as a parent antibody an anti-hen lysozyme antibody “D.1.3”, since the three-dimensional structure of its complex with antigen has been solved [16] and plasmids encoding its FV and VH are available [13]. As a potential paralog peptide we chose the ten amino acid peptide sequence corresponding to the third complementarity determining region (cdr3) of the D.1.3 VH chain, as this sequence is known to make the most contact points with antigen [16]. We immobilized all three reagents on Sepharose and compared their ability to recover hen lysozyme from spiked serum and to separate hen lysozyme from turkey lysozyme (only seven amino acid differences) [16].

EXPERIMENTAL

Production of FV antibody fragments
A vector encoding the FV fragment of the D.1.3 antibody and tagged at its C-terminus with the “myc” peptide [17] was obtained from Dr. G. Winter (MRC, Cambridge, UK [13]). We have previously found that the myc peptide serves as a useful linking group for covalently coupling antibody fragments to solid phases without losing their binding activity [15,18]. The FV vector was transformed into Escherichia coli (strain BMH 71-18) and grown in cultured medium according to the method of Ward et al. [13]. Secreted FV fragments were recovered from the medium by affinity chromatography on lysozyme–Sepharose [13]. Hen-egg lysozyme was obtained from Sigma (Poole, UK) and Sepharose from Pharmacia (Uppsala, Sweden).

Production of VH antibody fragments
A vector encoding the VH fragment of the D.1.3 antibody and tagged at its C-terminus with the myc peptide was obtained from Dr. G. Winter [13]. The vector was transformed into E.coli (strain TGI) and antibody fragments were produced and purified as above.

Production of paralog peptide
The cdr3 of the VH domain (of the D.1.3 antibody) was produced synthetically by peptide synthesis and was obtained from Dr. N. Hutchinson (Babraham, Cambridge, UK). The peptide sequence was ARERDYRLDY with a free amino group at the N-terminus. Hereafter this peptide is designated cdr 3.

Preparation of immunoadsorbents
Three immunoadsorbents were made, one comprising FV fragments, one comprising VH fragments and one comprising our paralog peptide (cdr 3). Each immunoligand was made up in coupling buffer [0.1 M NaHCO3 (BDH, Poole, UK)–0.5 M NaCl (BDH) (pH 8.3)] then immobilized on to ca. 1 g of cyanogenbromide-activated Sepharose 4B.
USE OF ANTIBODY FRAGMENTS IN IMMUNOAFFINITY CHROMATOGRAPHY

(Pharmacia) according to the manufacturer's instructions.

FV fragments (one binding site on an \( M_r \) 25 000 protein) were coupled at a level of 1.6 mg of protein per gram of Sepharose. This would correspond to a loading of 5 mg/g for whole antibody (two binding sites on an \( M_r \) 150 000 protein). VH fragments (one binding site on an \( M_r \) 12 500 protein) were coupled at a level of 0.8 mg/g, which is the same molar level. The cdr 3 peptide was coupled at a level of 2 mg/g. This is a much higher molar level, but high coupling levels in molar terms have previously been found to be successful for paralog peptides [19]. Coupling efficiencies were found to be ca. 90% for all three ligands by measuring the absorbance of the ligand solution at 280 nm before and after coupling. Absorbance measurements were made with an Ultra spec II spectrophotometer (Pharmacia).

Recovery of hen lysozyme from “spiked” horse serum

A 1-g amount of each immunoadsortent was conditioned in phosphate-buffered saline [0.1 M \( \text{Na}_2\text{HPO}_4 \)-\( \text{NaH}_2\text{PO}_4 \) (BDH)–0.15 M \( \text{NaCl} \) (pH 7)], then packed in a glass column (Pharmacia C10/20 to give column dimensions of 40 mm x 10 mm I.D. Each column was loaded with a feedstock of 5% horse serum (Seralab, Crawley Down, UK), made up in PBS and spiked with hen-egg lysozyme to a final concentration of 50 \( \mu \)g/ml. This feedstock was loaded until a stable breakthrough was reached, then the columns were washed back to the baseline with PBS. Flow-rates were kept at 50 ml/h throughout the experiments.

Bound protein was recovered by eluting with desorption buffer [4 \( M \) \( \text{MgCl}_2 \) (BDH) (pH 7)], then dialysed into PBS. The purity of the recovered protein was determined by concentrating as above and applying to a basic PAGE gel (see below).

Recovery of anti-idiotype antibody from rabbit serum

The cdr3 column was loaded with 5 ml of serum from a rabbit that had been inoculated with FV fragments (from D.1.3) and was therefore expected to contain anti-idiotype antibodies [20]. The column was washed back to the baseline with PBS; bound protein was recovered by eluting with desorption buffer and dialysing into PBS. The purity of the recovered protein was determined by concentrating as above and applying to a native PAGE gel (see below).

SDS-PAGE

SDS-PAGE analysis was carried out using the Pharmacia Phast system. Samples were boiled for 5 min in running buffer [10 mM Tris–1 mM EDTA (Sigma) (pH 8)] with 2.5% SDS–5% \( \beta \)-mercaptoethanol (BDH) and 0.01% Bromophenol Blue (BDH) as a tracking dye, then run on a Pharmacia homogeneous 20 Phastgel with Pharmacia SDS buffer strips. The gel was stained using the Coomassie Blue (Pharmacia) staining technique.

Basic PAGE

Basic PAGE was carried out using the Pharmacia Phast system with a reverse polarity electrode assembly. Samples were prepared in acidic running buffer [0.112 M \( \text{acetate} \) (BDH)–0.112 \( M \) Tris (pH 6.24)] with 0.01% pyronin Y (Sigma) as a tracking dye; SDS was not included. The samples were then run on a Pharmacia homogeneous 20 Phastgel with acidic buffer strips prepared as follows: 2 g of agarose C (Pharmacia), 4.4 g of \( \beta \)-alanine (Sigma) and 4 ml of acetic acid (BDH) were added to 96 ml of distilled water and heated until the agarose had dissolved. The mixture was then allowed to cool to 70°C before pouring into casting moulds (empty buffer strip packages) and solidified by cooling to...
room temperature, the gel was stained using the Coomassie Blue staining technique.

Native PAGE

Native PAGE analysis was carried out using the Pharmacia Phast system. Samples were prepared in running buffer [10 mM Tris–1 mM EDTA (pH 8)] with 0.01% Bromophenol Blue as a tracking dye, then applied to an 8.25 gradient Phast gel with native buffer strips in place. After separation had taken place the gel was stained using the silver staining technique with silver nitrate obtained from BDH.

RESULTS

Performance of FV column

This column recovered hen lysozyme from spiked serum. The eluted peak was sharp (Fig. 2a) and the recovered lysozyme was homogeneous as determined by SDS-PAGE (Fig. 2b). This column could also separate hen lysozyme from turkey lysozyme. The breakthrough curve on reaching capacity was sharp and the eluted peak was sharp (Fig. 3a). The recovered hen lysozyme contained no contaminant turkey lysozyme as determined by basic PAGE (Fig. 3b).

Performance of VH column

This column recovered hen lysozyme from spiked serum. The eluted peak was sharp (Fig. 2a). However, the recovered lysozyme was slightly contaminated with serum proteins as determined by SDS-PAGE (Fig. 2b). Further, the size of the peak eluted with desorption buffer was ca. 50% smaller than that eluted from the FV column. The VH column was unable to separate hen lysozyme from turkey lysozyme; the eluted peak contained both isoenzymes as determined by basic PAGE (Fig. 3b).

Performance of cdr3 column

This column did not bind hen lysozyme and could not recover the enzyme from spiked serum.

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Fig. 2. (a) Recovery of hen-egg lysozyme from “spiked” serum using anti-lysozyme antibody fragments immobilized on cyanogen bromide-activated Sepharose 4B. The feedstock was made up and loaded in PBS. Bound lysozyme was desorbed by eluting with 4 M MgCl₂ and dialysed into PBS. (b) SDS-PAGE analysis of lysozyme fractions recovered from “spiked” serum with immobilized antibody fragments. Lanes: 1 = Pharmacia low-molecular mass markers; 2 = lysozyme fraction eluted from cdr-Sepharose; 3 = lysozyme fraction eluted from VH-Sepharose; 4 = lysozyme fraction eluted from FV-Sepharose; 5 = lysozyme standard; 6 = feedstock (5% horse serum “spiked” with 50 μg/ml hen-egg lysozyme).
Fig. 3. (a) Separation of hen lysozyme from turkey lysozyme using anti-hen lysozyme antibody fragments immobilized on cyanogen bromide-activated Sepharose 4B. The feedstock was made up and loaded in PBS. Bound lysozyme was desorbed by eluting with 4 M MgCl$_2$ and dialysed into PBS. (b) Basic PAGE analysis of hen lysozyme fractions separated from hen lysozyme/turkey lysozyme cocktail. Lanes: 1 = lysozyme eluted from FV-Sepharose; 2 = lysozyme fraction eluted from VH-Sepharose; 3 = hen lysozyme standard; 4 = turkey lysozyme standard.
DISCUSSION

We found that the VH column could remove antigen from spiked serum but there was some contamination of the recovered enzyme with serum proteins. This is possibly due to the exposure of some hydrophobic patches on the VH polypeptide which are buried in whole antibody [21] and FV fragments. The VH column was unable to separate hen lysozyme from turkey lysozyme. A rationale for this reduced specificity is that the FV fragment carries the same number of complementarity-determining regions (cdrs) as a whole antibody-binding site (i.e., six), but a VH fragment carries only three. Further, the peak eluted from the VH column was 50% smaller than that eluted from the FV column despite the fact that both columns contained the same number of antigen-binding sites. This suggests that VH is more readily inactivated than FV during immobilization. Additional disadvantages of VH in our hands include poor expression in laboratory culture (ca. 0.2 mg/l compared with 10 mg/l for FV) and poor stability in aqueous solution (a 0.5 mg/ml solution in PBS quickly precipitates when stored at 4°C).

The cdr column did not bind hen lysozyme. However, the binding of anti-idiotype antibody clearly demonstrated that the peptide was immobilized and available for binding to feedstock proteins. This itself is an interesting result and it is possible that immobilized cdr peptides represent a generally applicable route for purifying anti-idiotype antibodies. Nevertheless, it is clear that this particular peptide does not interact with antigen (i.e., it is unable to behave as a "paralog"), despite the fact that it was chosen for being the cdr which made the most contact points with antigen [16]. We were not surprised by this result because it seems unlikely that a short linear peptide could mimic the affinity and specificity of a full battery (i.e., six) cdrs arranged in optimum conformation. There have, however, been a few reported examples of linear peptides, derived from single cdrs, binding antigen [19,22–24]. Further, it is possible to improve the binding of cdr peptides or "paralog" peptides by incorporating some secondary structure by cysteine bridging [24]. We view this topic as an important emerging technology. For the time being, however, our conclusion is that the FV fragment is the smallest immunoagent currently available that binds antigen in a specific and predictable manner.
REFERENCES

Immobilization of Fv antibody fragments on porous silica and their utility in affinity chromatography

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ABSTRACT

Recent advances in molecular biology have allowed antibody binding domains to be cloned and expressed in Escherichia coli. The use of Fv antibody fragments as ligands in immunoaffinity chromatography is reported. Fv fragments specific for hen-egg lysozyme were immobilized on porous silica and used to recover antigen from spiked serum in a single step. Comparison with a conventional immunoadsorbent (whole antibodies immobilized on silica) showed the Fv-silica to have a fivefold superior capacity. Analysis of sectioned Fv-silica particles by immunoelectron microscopy indicated that captured antigen was evenly distributed throughout the internal porous structure of the particle.

INTRODUCTION

Immunoaffinity chromatography exploits the exquisite specificity of an antibody binding site. This means that very high resolution separations are achievable, typically in a single step. For example, the technique has been used to recover factor IX from serum fractions [1] and to separate different glycoforms of the same enzyme [2]. Another attraction is the enormous diversity of the immune system; it has been estimated to have the capacity for making $10^8$ different antibody specificities [3]. In practical terms, this means that it is possible to raise monoclonal antibodies (usually from the mouse) which are uniquely specific for any enzyme, serum protein, carbohydrate, cell, virus or small organic that is likely to be encountered.

Immunoaffinity chromatography is widely used for laboratory-scale preparative work (typically with 4% agarose as the base medium), but is rarely used either for analytical applications or for industrial-scale processes. Reasons for this include the high cost of monoclonal antibodies and their low capacity on a weight for weight basis (antibodies have a molecular weight of 150 000 dalton and two binding sites). Another disadvantage of antibodies being such large ligands is that they need wide-pore chromatographic media for their efficient immobilization, and even then their presence may significantly reduce the pore size available for antigen exchange [4]. This may lead to problems such as poor specific capacity or band spreading. These effects have been demonstrated for immunoadsorbents made with “wide-pore” silicas (nominal pore size 200–500 Å) [4]. Wide-pore silicas are a popular choice for the analytical- and preparative-scale high-performance liquid chromatography (HPLC) of proteins owing to their nearly ideal properties of high mechanical strength, well defined pore size, freedom from swelling effects, resistance to enzymatic degradation and very low non-specific adsorption. The only negative aspect of silica-based media, their inability to withstand extensive treatment with 0.5 $M$ sodium hydroxide solution, is no limitation for immunoaffinity chromatography where the proteinaceous ligand precludes the use of high-pH cleaning protocols. Moreover, there have been several recent reports describing the immobilization of antibodies and antibody fragments on silica for use in immunoaffinity chromatography [4–9].
Recent progress in molecular biology has made it possible to produce antibody fragments in *Escherichia coli* [10-14]. One of the best described species is the Fv antibody fragment. Fv fragments consist of the variable domains of the heavy and light chains of the parent antibody and have a molecular weight of 25,000 dalton [11]. They have been shown to have a similar or slightly lower affinity than the parent antibody [10,11]. Several ingenious features have been designed into Fv fragments by protein engineers to facilitate their production and recovery. For example, Fv fragments have been made with a signal peptide sequence (the "pel B" sequence [15]) so that they are secreted by the host bacterium into the growth medium [10]. Another example is the provision of a histidine-rich "tail" to facilitate recovery from growth medium by immobilized metal affinity chromatography (IMAC) [13].

Fv antibody fragments offer an exciting opportunity in immunoaffinity chromatography: they may be produced cheaply in cultured media, they have more binding sites per milligram of protein than whole antibodies and they are sufficiently small to be immobilized within the pores of rigid chromatographic media such as silica without significantly reducing the pore size.

In this paper, we describe the immobilization of Fv fragments, specific for hen-egg lysozyme, on silica particles with 200 Å pore size. We used this immunoadsorbent to recover lysozyme from "spiked" serum and compared its performance (in a packed column) with that of a traditional immunoadsorbent consisting of silica and whole antibody. Also, the efficiency with which individual immunoadsorbent particles captured target antigen was analysed by immunoelectron microscopy of ultra-thin sections.

**EXPERIMENTAL**

**Production of whole antibodies**

A hybridoma cell line which produces an antibody specific for hen-egg lysozyme, the "D.1.3 antibody" [16], was obtained from Dr. G. Winter (MRC, Cambridge, UK). The hybridoma was reproduced in mice. Purified monoclonal antibodies were recovered from ascites using protein-A Sepharose.

**Production of Fv antibody fragments**

A vector encoding the Fv fragment of the D.1.3 antibody and tagged at its C-terminus with the "myc" peptide [17] was obtained from Dr. G. Winter [10]. The vector was transformed into *E. coli* (strain BMH 71-18) and grown in cultured medium according to the method of Ward et al. [10]. Secreted Fv fragments were recovered from the medium by affinity chromatography on lysozyme-Sepharose [10].

**Preparation of tresylated silica**

Preparative-grade epoxy silica (Sorbsil 40/60 C200) was obtained from Crosfield Chemicals (Warrington, UK). This material has a particle size range of 40-60 μm and a nominal pore size of 200 Å. Epoxy-silica was hydrolysed to the diol by the method of Mohan et al. [4]. Diol-silica was dried and then reacted with tresyl chloride (Fluka, Buchs, Switzerland) by a method based on that of Nilsson and Mosbach [18]. The modification was that triethylamine (0.6 mol% of tresyl chloride) and 4-dimethylaminopyridine (0.6 mol% of tresyl chloride) were used in place of pyridine. The optimum level of tresylation was determined with respect to capacity for antigen (low-level tresylation resulted in poor coupling of Fv fragments; very high tresylation resulted in good coupling of Fv fragments but unacceptable inactivation of their binding sites; a compromise was required). The amount of tresyl groups on the optimally activated silica was measured by fluorine determination (oxygen combustion-ion chromatographic procedure), and this was useful in reproducing the same result when a new batch of diol-silica was used.

**Immobilization of immunoligands on tresylated silica**

Two immunoadsorbent materials were made, one consisting of Fv antibody fragments and the other whole monoclonal antibody (MCA).

Approximately 1 g of tresylated silica was washed with saline (0.15 M sodium chloride solution) and then with coupling buffer (0.1 M NaHCO3–0.5 M NaCl, pH 8.3). The washed silica was added to 4 ml of a ca. 1 mg/ml solution of the immunoligand (Fv or whole antibody) in coupling buffer. The slurry was rotated overnight at 4°C. The immunoadsorbent was blocked with blocking buffer (1 M ethanolamine–HCl, pH 8) and then washed three times...
with 0.1 M Tris buffer (pH 8). The amount of ligand immobilized was determined by measuring the ligand concentration before and after coupling to silica using the BCA protein assay.

**Running of immunoaffinity columns**

A 1-g amount of each immunoabsorbent was conditioned in phosphate-buffered saline (PBS) and then packed in a glass column (Pharmacia CL-10/20) to give column dimensions of 40 mm x 10 mm I.D. Each column was loaded with a feedstock of 5% horse serum (Seralab), made up in PBS (0.01 M Na2HPO4/NaH2PO4–0.15 M NaCl, pH 7) and spiked with hen-egg lysozyme (Sigma) to a final concentration of 50 μg/ml (ca. 3000 U/ml). This feedstock was loaded until a stable breakthrough was reached; the columns were then washed back to the baseline with PBS. Flow-rates were kept at 85 ml/h throughout the experiments.

Bound protein was recovered by eluting with desorption buffer (4 M MgCl2, pH 7) and dialysing the peak into PBS. The amount of recovered protein was determined by the BCA protein assay. Lysozyme activity was monitored across the chromatogram profile by assaying fractions using a suspension of Micrococcus (Sigma) according to the manufacturer's instructions. Non-specific binding was evaluated by passing the same feedstock down a “blank” column, which had been tresylated and blocked as above but no immunoligand was added.

The percentage recovery of lysozyme protein was also measured accurately by loading and eluting a known amount of FITC-labelled lysozyme [19,20] with spectrofluorimetric (Perkin-Elmer) detection.

**Analysis of lysozyme within particles by immuno-electron microscopy**

Immunoabsorbent particles were loaded with lysozyme by rotating slowly in a 1 mg/ml solution (in 0.1 M Tris buffer, pH 8) for 1 h at room temperature. They were then washed three times in Tris buffer. The washed particles were fixed in 1% paraformaldehyde-0.05% glutaraldehyde (made up in PBS, pH 7.6) for 2 h at 4°C. Following an overnight wash in PBS, the samples were dehydrated with 50, 70 and 90%, ethanol (15 min each) and absolute ethanol (2 x 30 min) and placed in several changes of hydrophilic resin: 3 parts LR Gold acrylic resin (London Resin, Woking, UK)–2 parts "low-acid grade" glycol methacrylate (Polysciences, Warrington, PA, USA)–0.1% benzoin ethyl ether (Polysciences). The samples were finally embedded in the above resin in gelatin capsules and polymerized at room temperature by illumination with an ultraviolet light source (360 nm).

Ultra-thin sections of the particles were collected on Formvar (2% in amyl acetate)-coated nickel grids and placed in 10-μl aliquots of 1% ovalbumin (Sigma) in PBS–5% normal goat serum, for 30 min at room temperature. The grids were then transferred to 10-μl aliquots of a polyclonal rabbit anti-lysozyme antibody (an in-house preparation) diluted 1:5 in PBS–5% normal goat serum–0.1% Tween 20 (Sigma) and incubated overnight at room temperature in a moist dish (the rabbit anti-lysozyme antibody had been found to be able to pair with the D.1.3 anti-lysozyme monoclonal antibody in a sandwich ELISA on microtitre plates; results not shown). Following a thorough wash with PBS, the grids were placed in 10 μl aliquots of goat anti-rabbit–colloidal gold (5 nm diameter) (Biocell), diluted 1:200 in PBS–1% ovalbumin–5% normal goat serum for 60 min at room temperature. The grids were then thoroughly washed in PBS, followed by distilled water. The colloidal gold staining was silver enhanced using a silver enhancer kit (Biocell) for 2–3 min at room temperature. The grids were examined using a transmission electron microscope without further counterstaining.

Control particles (where the immunoligand had been omitted) were stained and examined by the same protocol.

**RESULTS**

**Preparation and use of Fv-silica column**

Approximately 3.4 mg of Fv polypeptide were found to have been immobilized on 1.0 g of Sorbsil. This immunoabsorbent was used to recover lysozyme from spiked serum in a single step. The breakthrough curve for lysozyme was sharp (Fig. 1A). The recovered lysozyme was found to be homogeneous by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2). The capacity of the column for lysozyme (determined by the breakthrough point) was 0.8 mg (16 ml x 50 μg/ml) of lysozyme protein. This corresponds to 48 000 lysozyme units (16 mls x 3000 U/ml). The
Fig. 1. (A) Recovery of hen-egg lysozyme from “spiked” serum using anti-lysozyme Fv fragments immobilised on Sorbsil C200 40/60. The feedstock was made up and loaded in PBS (0.01 M NaHPO₄/NaH₂PO₄, 0.15 M NaCl, pH 7). Lysozyme was specifically eluted with 4 M MgCl₂ and dialysed into PBS. Total protein absorbance at 280 nm (top line) was monitored on-line; lysozyme activity (●) was monitored by assaying fractions. (B) Recovery of lysozyme from “spiked” serum using anti-lysozyme monoclonal antibodies immobilised on Sorbsil C200 40/60. (C) Interaction of “spiked” serum with a “blank” column (Sorbsil C200 40/60 which had been tresylated and blocked but on which no immunoligand had been immobilised).

amount of lysozyme recovered in the 4 M MgCl₂ fraction was 0.6 mg of lysozyme protein, as determined from the absorbance at 280 nm and the BCA protein assay (Pierce, Rockford, IL, USA). This fraction was determined to have a total activity of 14 400 U. This separation experiment was repeated twice and all the data agreed to within 5%. From these results, some parameters describing the performance of the immunoadsorbent were calculated as follows: recovery of lysozyme protein = (0.6/0.8) × 100 = 75%; recovery of lysozyme activity = (14,400/14,400) × 100 = 30%; and specific activity of immobilized Fv fragments = (0.8 × 25)/(3.4 × 14,300) × 100 = 41% (taking the molecular weight of lysozyme to be 14 300 dalton and the molecular weight of Fv to be 25 000 dalton). The recovery of lysozyme protein as determined by the FITC-labeled experiment was 78%. A summary of key data is given in Table I.

The “blank” column was found not to bind lysozyme or any serum proteins (Fig. 1C).

Preparation and use of MCA-silica column
Approximately 2 mg of monoclonal antibody (MCA) protein were found to have been immobi-

<table>
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<th>Parameter</th>
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IMMOBILIZATION OF Fv ANTIBODY FRAGMENTS

lized on 1.0 g of Sorbsil. This immunoadsorbent was used to recover lysozyme from spiked serum in a single step (Fig. 1B). There was a sharp breakthrough for lysozyme after reaching capacity and then a shallow “secondary breakthrough” (Fig. 1B). The capacity of the column for lysozyme (determined by the breakthrough point) was 0.15 mg (3 ml x 50 Ìg/ml). This corresponds to 9000 lysozyme units (3 ml x 3000 U/ml). The amount of lysozyme recovered in the 4 M MgCl₂ fraction was 0.12 mg of lysozyme protein, as determined by the absorbance at 280 nm and the BCA protein assay (Pierce). This fraction was determined to have a total activity of 3000 U. This separation experiment was repeated and all the data agreed to within 5%. From these results, some parameters describing the performance of the immunoadsorbent were calculated as follows: recovery of lysozyme protein = (0.12/0.15) x 100 = 80%; recovery of lysozyme activity = (3000/9000) x 100 = 33%; specific activity of immobilised antibodies = (0.15 x 75)/(2 x 14.3) x 100 = 39% (assuming antibodies to have a molecular weight of 150 000 dalton with two binding sites). The recovery of lysozyme protein as determined by the FITC-labelled experiment was 79%. A summary of key data is given in Table I.

Analysis of lysozyme within particles by immunoelectron microscopy

The immunoadsorbent particles consisting of Fv antibody fragments were found to have lysozyme bound to them evenly throughout their internal porous structure. A typical example is shown in Fig. 3A. The immunoadsorbent particles consisting of whole antibodies were found to have less lysozyme bound and in addition many of the particles were found to have most of their bound lysozyme at the surface of the particle. An example of this is shown in Fig. 3B. All negative control particles were found to give very low (or zero) background staining.

DISCUSSION

There are several reasons why Fv antibody fragments may be expected to be preferable to whole antibodies in immunoaffinity chromatography. First, we expect that Fv fragments will be cheaper to produce once their manufacture has been optimized; because they may be produced in E. coli in cheap bacterial media whereas whole antibodies are expressed in mammalian cells in expensive tissue culture media. Second, Fv fragments have an increased capacity for antigen on a weight for weight basis (one binding site on a 25 000 dalton protein as opposed to two binding sites on a 150 000 dalton protein). Third, Fv fragments may be expected to be sufficiently small to be immobilized within the porous structure of “wide-pore” silica without significantly reducing the pore size. For example, the silica used in this study (Sorbsil C200 40/60) is a typical preparative-scale chromatographic medium. With a pore size of 200 Å, there is scarcely room to accommodate whole antibody (molecular diameter = 150 Å [10]) and still leave room to engage and bind antigen specifically (molecular diameter of lysozyme = 40 Å [21]). In contrast, the immobilization of Fv fragments (molecular diameter ca. 50 Å) would not be expected to reduce the pore size so drastically. Although silicas with larger pore sizes are available, these media have the drawbacks of reduced surface area (and therefore reduced capacity for ligand) and reduced tensile strength (and therefore reduced resistance to high pressures) [22].

For the purification of very large antigens, it may still be necessary to resort to silica with pore sizes in excess of 200 Å [4]; however, whatever the size of the target antigen, it should be possible to use a correspondingly smaller pore silica (with associated advantages) if Fv ligands are used in preference to whole antibodies. In this study, we set out to test whether these perceived advantages could be manifested as demonstrable performance improvements in column chromatography.

We made an immunoadsorbent consisting of Fv fragments immobilized on Sorbsil C200 and another consisting of whole monoclonal antibodies (MCA) immobilized on the same medium. A direct comparison indicated both immunoadsorbents to be capable of recovering target antigen (hen-egg lysozyme) from spiked serum in a single step; however, the immunoadsorbent made with Fv fragments had a five fold superior capacity (0.8 mg compared with 0.15 mg) for antigen. One reason for this was that more Fv had been immobilized than MCA (3.4 mg compared with 2 mg). This was thought to be because the Fv fragments had improved access to the internal porous structure of the silica (and therefore had more tresylated sites to re-
act with). This explanation was supported by the finding that antigen distribution within the Fv particle was even (it seems likely that for a small antigen such as lysozyme, the antigen distribution will closely mirror the immunoligand distribution). Another contributory explanation for the superior capacity of the Fv column is the increased number of binding sites per milligram of protein (as discussed above).

The specific activity of immobilized Fv fragments...
Fig. 3. (A) Immunoelectron micrograph of silica particle derivatized with anti-lysozyme Fv fragments. Particles were interacted with lysozyme, sectioned and then stained by labelling with rabbit anti-lysozyme followed by goat anti-rabbit–colloidal gold. Magnification 20 000 (bar = 1 μm). (B) Immunoelectron micrograph of silica particle derivatized with anti-lysozyme monoclonal antibodies. Particles were interacted with lysozyme, sectioned and then stained by labelling with rabbit anti-lysozyme followed by goat anti-rabbit–colloidal gold. Magnification 20 000 (bar = 1 μm).
and immobilized MCA was ca. 40%. Similar figures have been published by others for immobilized antibodies [1,6] and immobilized “Fab” fragments [6] (made by proteolytic digestion of whole antibodies). We believe that the specific activity achievable for immobilized Fv fragments could be increased by improving their orientation by incorporating a short, lysine-rich peptide “tail” at their C-terminus. Such a tail could be readily incorporated by recombinant DNA technology. In this study, we used the “myc” peptide as a linking tail, which only has one lysine [10].

An important result was that the Fv-silica immunoadsorbent produced a sharp breakthrough curve for lysozyme and a sharp elution peak. Taken in conjunction with the finding that bound lysozyme was evenly distributed within the silica particles, this is compelling evidence that lysozyme had unrestricted access to binding sites within the internal porous structure of the media. The MCA-silica immunoadsorbent also produced a sharp breakthrough curve and a sharp elution peak. This is in keeping with the view that most of the lysozyme purified by this immunoadsorbent was bound and released from the surface of the particle where the kinetics are known to be fast [23]. Support for this view is given by the distribution of antigen in the electron micrograph (Fig. 3B). An interesting finding was that the MCA-silica immunoadsorbent produced a shallow “secondary breakthrough” (Fig. 1B). It is possible that this secondary breakthrough is the result of a gradual filling of sites, deep within the particles, which are poorly accessed by antigen due to size restrictions. The presence of a few internal binding sites which would fit this description is suggested by the electron micrograph (Fig. 3B).

The aim of this study was to evaluate the utility of Fv fragments as affinity ligands, and in particular to compare their performance with that of whole antibodies on porous silica (we chose silica because we believe the long-term potential for Fv ligands is in preparative-scale systems and therefore we wanted to do our pilot project on a chromatographic medium which can be readily scaled up). We used Fv fragments specific for hen-egg lysozyme as these were the first to become available to us, and evaluated their ability to recover target antigen from 5% serum (this system was intended to model the recovery of a recombinant protein from tissue culture media). Using this model recovery system, we have been able to demonstrate two clear advantages of using Fv fragments in place of whole antibody: a superior capacity for antigen, and an improved access of antigen to internal binding sites in the silica particles. The only disappointing result was a moderate recovery of lysozyme protein (75–80%) and a poor recovery of lysozyme activity (30–33%). The loss of activity was because the eluent used (4 M MgCl₂) inactivated lysozyme. A preliminary screening could not identify an elution buffer which improved the recovery, and our conclusion is that this particular antibody (and corresponding Fv fragment) had too high an affinity for the preparative affinity purification of enzymes. As hen-egg lysozyme has no medical or commercial interest, we do not propose to try to improve its recovery. Moreover, our model has served its purpose and future work should concentrate on isolating Fv fragments of an appropriate specificity and affinity to achieve commercially viable separations and evaluating the performance of Fv ligands in high-performance and/or preparative chromatographic conditions.

Some groups have recently tried to mimic the antibody binding site by making short peptides corresponding to a single complementarity determining region [24]. An analogous attempt to mimic the binding site of the D.1.3 antibody was not successful in our hands (results not shown) and in any event it seems unlikely that such a reagent can match the specificity of an Fv carrying a full battery (i.e., six) of complementarity-determining regions. It is our belief that for high-resolution separations (such as separating isoenzymes and different glycoforms of the same protein), the Fv represents the smallest functional fragment of the antibody which is currently available. Moreover, the combination of silica with Fv fragments may be used to make high-resolution separation media with excellent flow-rates. This concept offers excellent opportunities for the use of immunoaffinity chromatography in rapid analytical separations and scaled-up industrial processes.

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We thank Dr. Ian Chappell for critically reading the manuscript.
IMMOBILIZATION OF Fv ANTIBODY FRAGMENTS

REFERENCES

Polypeptide production.

The production of polypeptide by the steps of
(i) cloning a nucleic acid sequence by means of the polymeric chain reaction
(ii) transforming an organism with the cloned nucleic acid sequence
(iii) culturing the organism,
is characterised by purifying or assaying the polypeptide with antibody or other specific binding reagent having specific affinity to a sequence of amino acids coded by a 'primer' nucleic acid sequence utilised in the polymerase chain reaction.
This invention is concerned with the production of polypeptides by means of recombinant DNA technology. This is a subject of progressively growing commercial importance. In particular the invention is applicable to the production of polypeptides which are antibody fragments, although it is not confined to these polypeptides.

When a polypeptide is produced by means of recombinant DNA technology the necessary DNA sequence which codes for the desired polypeptide must first be obtained. This extra genetic material is then introduced into an organism (frequently the bacterial species E.coli) and the resulting transformed organism is then cultured so that it produces the desired polypeptide which is harvested from the culture.

When culturing the transformed organism it will often be necessary to assay for the desired polypeptide. For instance, this may be required during development work to optimise the growth conditions of a transformed microorganism when it will be desirable to assess the effect of a change in pH, or temperature or formulation of the growth medium. Later, during production an assay will be desirable to check production performance.

Assay techniques with the required sensitivity and specificity already exist. Two conventional techniques which are suitable are ELISA and Western Blot.

However, for these assay techniques, it is necessary to have available an antibody (or possibly an antibody fragment or other binding reagent) which exhibits specific binding affinity for the desired polypeptide. In the context of these assay techniques such an antibody may be referred to as a "tracer antibody".

Thus, in order to be able to assay for a desired polypeptide it is first necessary to obtain a supply of antibody (or fragments or other binding reagent) with specific binding affinity to the desired polypeptide.

An antibody or antibody fragment or other reagent with specific binding affinity to a desired polypeptide may also be required for purification of the peptide, for example by affinity chromatography. An antibody for this purpose may be referred to as a "capture antibody".

It will thus be appreciated that for every desired polypeptide there is likely to be a requirement for a binding reagent such as an antibody with specific binding affinity to the polypeptide. In many instances this will not be available and its provision will be an extra task in putting a desired polypeptide into production.

This requirement is particularly acute when the desired polypeptide is an antibody fragment which is wholly or largely formed by the variable region of an antibody. By definition these are highly variable and there is no pre-existing source of suitable antibodies.

One approach to this has been to transform an organism with fused genes so that it expresses the desired polypeptide fused onto an additional peptide against which an antibody is available. This additional peptide which may be referred to as a "tag" or "tail" can then be used in purification and/or assay. This is discussed by Sassenfeld in Tibtech 8, 88 (April 1990). Possible disadvantages arising from such tags are recognised.

The present inventors have now appreciated that antibodies to desired polypeptides can be obtained by taking advantage of a side effect of a well known technique used in gene cloning, namely the polymerase chain reaction (PCR). This technique "amplifies" the desired nucleotide sequence by preferentially replicating this sequence. The characteristics of the PCR technique will now be pointed out with reference to Figs. 1 to 6 of the accompanying drawings, which are diagrams illustrating DNA replication.

The procedure for gene amplification by the polymerase chain reaction starts with DNA (deoxynucleic acid) which includes a gene (a nucleotide sequence) coding for a desired polypeptide. Other nucleotide sequences are also present. For instance when it is intended to produce a fragment of a monoclonal antibody, the starting point for the polymerase chain reaction may be the genome of the hybridoma cells which produce the monoclonal antibody concerned. More usually, the starting DNA is cDNA artificially obtained by reverse transcription of mRNA recovered from hybridoma cells.

The polymerase chain reaction requires a pair of "primers" each of which is a relatively short nucleotide sequence able to anneal to a portion of the DNA strand adjoining a respective end of the desired gene. One primer is able to anneal with the sense strand at one end of the desired gene, the other with the antisense strand at the opposite end of the desired gene. Each primer used for the polymerase chain reaction may be a single oligonucleotide or may be a mixture of several oligonucleotides which are similar. Such a mixture of similar oligonucleotides may be referred to as a degenerate primer.

The polymerase chain reaction utilises a sequence of steps which are carried out in a repeated cycle. In a particularly convenient form, these steps are controlled by temperature. Initially a reaction mixture is prepared containing the starting genetic material, the two primers, monomeric nucleotides and DNA polymerase.

As illustrated in Fig. 1 the starting genetic material is double-stranded DNA including the desired gene (nucleotide sequence) with other nucleotide sequences at either side of this. S1 denotes the sense strand, with the desired nucleotide sequence designated NS; AS1 denotes the antisense strand with the (complementary) desired sequence NAS. In a first step this double-stranded DNA is denatured to single-stranded DNA by heating, for example to about 95°C for one minute.

Next it is cooled to a temperature at which annealing of DNA occurs, for example 30-65°C for about one minute. However, because this takes place in the presence of primer, some primer can anneal to the DNA ad-
The steps of denaturing, annealing and DNA synthesis are then repeated. The other primer P2 which is able to anneal to the antisense strand AS2 at a position adjoining the other end of the desired gene, as shown in Fig. 4. In the subsequent step of synthesis a new DNA strand is formed which extends from primer P2 as far as an end complementary to primer P1, but then cannot extend further. This strand is designated S3 in Fig. 5.

The sequence of denaturing, annealing and DNA synthesis is continued repeatedly. During subsequent steps primers can anneal to any of the DNA strands which are present including strands such as S3 which contains only the desired sequence plus terminal portions corresponding to the primers. This is illustrated by Fig. 6. The consequence of this is that during subsequent repetitions of the cycle the production of DNA which contains only the desired nucleotide sequence plus the terminal portions corresponding to the primers far outstrips the production of all other DNA. After a number of repetitions the DNA present in the mixture is almost entirely of this type.

The steps of the reaction could be brought about in other ways: for instance reagents could be added to denature DNA into single strands and later removed to allow annealing to occur. Primers might be added only when required rather than initially. So also might the reagents for DNA synthesis. It is possible that the procedure could be started directly from RNA. The essence of the technique is repeatedly carrying out a cycle of steps comprising:

- exposing a required nucleotide sequence in a nucleic acid strand,
- annealing a primer oligonucleotide adjacent an end of the required sequence, and
- synthesising a complementary nucleic acid strand extending from the primer.

These steps being carried out utilising a primer able to anneal to one nucleic acid strand adjacent to one end of the required sequence and a second primer able to anneal to the complementary nucleic acid strand adjacent the opposite end of the required sequence, thereby to produce clone strands of nucleic acid which are the required sequence with end portions determined by the two primers.

Subsequently such a clone sequence of nucleic acid is introduced into an organism as extra genetic material. This transformed organism is used to express the required polypeptide.

Usually the clone sequence will be produced as a double strand of nucleic acid, and is introduced into an organism in this double strand form.

The polymerase chain reaction has been known for a number of years and is described in the literature. References are Saiki et al., Science 230 1350-1354 (1985), Scharf et al., Science 233 1076 (1986) and Saiki et al., Science 239 487 (1988).

The considerable advantage arising from use of the polymerase chain reaction is that it provides a very high proportion of DNA containing little more than the required nucleotide sequence. This makes it highly likely that when DNA is incorporated into an organism the transformed organism contains the required DNA rather than some other, unwanted DNA.

If a clone sequence is incorporated intact into an organism which is used to express the polypeptide, the polypeptide will have terminal amino acid sequences coded by the PCR primers. These are referred to as 'motif' peptides and may or may not themselves contribute to the function of the polypeptide.

The primers which are used in the polymerase chain reaction must be capable of annealing to the original DNA at the required positions. However in order to do this it is not essential that they should be exactly complementary to the DNA at all positions, and they do not need to be unique to a single polypeptide coded by the nucleotide sequence between the primers.

Notably, it has been shown that in Fv antibody fragments both the heavy and light chains contain respective "framework regions" which have considerable homology from one antibody to another, especially within a species, together with tree hypervariable regions also known as complementarity determining regions (CDR). Amplification of antibody fragments by means of the polymerase chain reaction may be carried out using primers which correspond to portions of the framework regions flanking the hypervariable regions.

This was demonstrated by Orlandi, Winter et al in PNAS USA 86 3833 (1989) which discloses oligonucleotide primers able to anneal to regions at the ends of mouse nucleotide sequences coding for heavy chain variable domains and respectively kappa type light chain variable domains. As is there disclosed, these primers can be used for cloning light and heavy chain antibody fragments of mouse monoclonal antibodies independently of the binding specificity of the monoclonal antibodies concerned. These primers and some others are
A characteristic of using this technique to clone the genes for a number of polypeptides, e.g. a series of antibody fragments or other proteins with homologous terminal regions, is that all the polypeptides produced will have N-terminal and C-terminal amino acid sequences (motif peptides) which may not correspond precisely to natural amino acid sequences but are coded by the primers used (and are therefore the same). Although these motif peptides are not necessarily identical to the natural amino acid sequence coded by the starting gene, they will at least resemble it closely and therefore are not truly "foreign".

It is possible that the motif peptide at a terminal portion of a polypeptide will differ to a limited extent from the amino acid sequence coded by the copy of a nucleotide primer at the end of the clone sequence of nucleic acid.

This can happen because of the way in which the clone sequence is introduced into an organism. The primer sequence can incorporate a restriction site so that when the clone is incorporated into a vector such as a bacterial plasmid which is subsequently used to transform an organism, restriction enzyme can be used to cut away a terminal portion of the nucleic acid of the clone sequence. In this event, nucleotides from the vector will replace some or all of the nucleotides cut away from the clone sequence. These nucleotides from the vector are preferably prearranged to code for the same amino acids as the nucleotides from the clone which they replace, (or if not the same amino acids then a very similar sequence) in order that the motif peptide does not appear foreign but instead resembles a natural amino acid sequence. If the primer ends with an incomplete codon the vector should replace this with a complete codon.

Consequently, at least part of a motif peptide will be expressed from nucleotide sequence copied from a primer while part of a motif peptide may be expressed from nucleotide codons which are taken (wholly or partially) from elsewhere, but desirably do not substantially alter the amino acid sequence from that coded by the primer. In turn the primer has to resemble an existing nucleotide sequence in order to be able to anneal to a complementary sequence.

Broadly, the present invention arranges that a motif peptide will act as a target for a binding reagent. This reagent is then used in production of, or other work with, polypeptides which incorporate the motif peptide.

In a first aspect this invention provides a method of producing at least one polypeptide by culture of a genetically transformed organism comprising:

- cloning a required portion of a nucleic acid sequence by means of the polymerase chain reaction utilising a pair of oligonucleotide primers thereby to obtain a clone sequence with terminal portions defined by the primers,
- transforming an organism with a said clone sequence resulting from the polymerase chain reaction or a copy therefrom,
- culturing the transformed organism to express the polypeptide coded by the clone sequence,
- characterised by assaying or purifying the said polypeptide with a binding reagent displaying specific affinity towards an amino acid sequence in which there are at least three amino acids coded by a said primer.

A codon in the primer does of course code for both the identity of an amino acid and its position in a sequence.

A sequence of three amino acids is believed to be the minimum for specific affinity in binding and has been referred to as a "recognition kernel".

Usually, in this invention, the binding reagent will be intended to bind to a sequence of more than three amino acids but containing a sequence of at least three which are coded by the primer.

The requirement that the amino acid sequence contains a sequence of at least three amino acids coded by the primer allows for the possibility that the binding reagent is raised against an amino acid sequence which is slightly different from the sequence coded by the primer. Such a difference could be addition of one or more amino acids, especially when the primer has an incomplete terminal codon. It could be replacement of one or more amino acids by others.

If a degenerate primer is used to code the motif peptide, the binding reagents could be designed to have an affinity for a short region of at least three amino acids corresponding to a sequence of nucleotides which does not vary between the individual oligonucleotides in the degenerate primer.

Each primer used for the polymerase chain reaction may be a single oligonucleotide or, as mentioned earlier, may be a mixture of several oligonucleotides which are similar.

Preferably the binding reagent displays affinity towards an amino acid sequence including a sequence of four, five, six or more amino acids coded both as to identity and position by the primer.

It is likely that the binding reagent will display specific affinity towards an amino acid sequence of at least six amino acids, including the said sequence of at least three, preferably more, amino acids coded by the primer.
The overall sequence preferably contains at least six, possibly at least eight amino acids coded, both as to identity and position, by the primer. These amino acids may form a continuous sequence, or may be part of a sequence which includes some amino acids differing from whatever is coded by the primer. Preferably a majority of amino acids in a sequence to which the reagent displays affinity are coded by the primer. Possibly they will all be coded by the primer.

As explained above, a motif peptide may include amino acids expressed from nucleotide codons which did not originate from the primer. However, such nucleotides are desirably arranged not to give rise to any substantial difference in the expressed amino acid sequence, with the consequence that the resulting motif peptide will also be bound by the said binding reagent displaying affinity towards an amino acid sequence coded, at least partly, by the primer.

The binding reagent is preferably an antibody or an antibody fragment. Alternative binding reagents which could be used include antisense peptides, mimetic compounds resembling the binding site of an antibody, and affinity dyes.

Techniques for transforming an organism with foreign nucleic acid have become well known and this invention is not limited to any type of organism nor to any particular technique for effecting transformation. The nucleic acid sequence which is introduced may be a transcript from a clone made during the polymerase chain reaction rather than itself being a direct product of that reaction. It may be a complementary copy of inverse sense and, as mentioned above, a portion of the primer sequence may be removed before the remainder of the clone sequence is incorporated into the transformed organism.

As is well known, both strands of the DNA double helix represent coded information, but only one strand is utilised directly in protein synthesis. One of the two primers used in the polymerase chain reaction must be the primer for an antisense nucleic acid strand. It is therefore possible that an amino acid sequence to which there is specific affinity is a sequence expressed from part of a sense strand which is complementary to a primer rather than identical to a primer.

One advantage of this invention is that it avoids the introduction of a "foreign" amino acid sequence for use in assay or purification. This invention is especially valuable when applied to producing a plurality of different polypeptides with at least one primer being used for cloning all the respective nucleic acid strands. It then becomes possible to assay or purify the different polypeptides using similar antibody or antibody fragments with affinity for an amino acid sequence coded by the primer used in all the cloning steps. Thus, by utilising a binding reagent, e.g. antibody or fragment which binds to an amino acid sequence coded by the common primer, the same binding reagent can be utilised for more than one polypeptide. For each polypeptide this reagent can be sufficiently specific to bind the required polypeptide rather than anything else produced in the course of culturing the transformed organism but the reagent is not so specific as to be unique to a single polypeptide.

The invention is applicable to the production of polypeptides of a wide variety of types, so long as the original cloning of the nucleic acid is carried out by the polymerase chain reaction. However the invention is particularly applicable when the polypeptides come from a family of proteins with homologous terminal regions, so that primers which code for terminal regions can be used to clone a family of genes. An important example of such a family is immunoglobulin variable domains, when the primer can code for amino acid sequences resembling those in the framework region whereas the remainder of the polypeptide can include an antibody's hypervariable regions.

When the polypeptides are antibody variable domains and the primers code for amino acid sequences of the framework regions of those variable domains, it will be preferable to choose a C-terminal amino acid sequence to be recognised by binding reagent used in assay or purification. This C-terminal, unlike the N-terminal, is relatively exposed and remote from the binding site of the heavy chain variable domain. Consequently it is more accessible than the N-terminal, which will make it easier for the capture reagent to bind to the variable domain.

We have observed that for some Fv antibody fragments the accessibility of the C-terminal of the variable domain is somewhat affected by surrounding conditions, being enhanced by absorption onto a microtitre plate, or in the presence of a moderate electrolyte concentration such as 1M sodium chloride. These conditions appear to cause some modification to the conformation of the antibody fragment, without denaturing the protein.

The advantage of binding to the C-terminus of a polypeptide applies to both the heavy chain and light chain variable domains of antibodies. Moreover, a number of other proteins have a relatively exposed C-terminal portion so that the same advantages would be available.

When the polypeptides are antibody fragments it may be desirable to transform the same organism to express both a desired fragment of the light or heavy chain and a corresponding fragment of the other chain. The two fragments can be allowed to associate together spontaneously, before carrying out the step of assay or purification, which step can utilise a motif peptide of only one of the two associated chains.
In a second aspect the invention provides a kit of materials comprising an oligonucleotide primer for use in the polymerase chain reaction and a binding reagent displaying specific binding affinity towards an amino acid sequence in which there are at least three amino acids in sequence coded by the said primer.

In another aspect this invention provides an antibody, an antibody fragment or other binding reagent with specific binding affinity to one of certain amino acid sequences set out below.

When a reagent such as an antibody or antibody fragment binds to a polypeptide the binding is in accordance with an equilibrium reaction of the general form

\[ A + P \rightleftharpoons AP \]

where A denotes the reagent, P denotes the polypeptide and AP denotes the complex of reagent bound to the polypeptide. The concentrations are related by an expression of the form

\[ \frac{[AP]}{[A][P]} = K_a \]

Where \( K_a \) is a constant, referred to as the affinity constant. When there is specific binding affinity the value of \( K_a \) will generally be \( 10^4 \) or greater frequently substantially greater. For an antibody the value may be in the range \( 10^7 \) to \( 10^9 \). For an antisense peptide the value may be in the region of \( 10^5 \).

Primers for use in the polymerase chain reaction typically contain between 15 and 45 nucleotides so that they code for an amino acid sequence of 5 to 15 amino acids.

An antibody with specific binding affinity to a motif peptide (amino acid sequence) can be obtained by a procedure which starts with chemical synthesis of the motif amino acid sequence itself or something similar to it. Methods for the chemical synthesis of peptides containing as many as 15 amino acids (and indeed somewhat more) are well known.

The amino acid sequence is then covalently conjugated to a carrier protein. Techniques for effecting such covalent conjugation are also well known. Next a suitable host animal is immunised with the conjugate in order to generate an immune response. After an appropriate delay, serum is collected from the host animal.

Polyclonal antibodies with specific binding affinity to the amino acid sequence can then be extracted by affinity chromatography using a chromatography column on which the amino acid sequence has been immobilized. The resulting polyclonal antibodies will generally be satisfactory for use in this invention where work is being carried out on a laboratory scale. However, if desired, it would be possible to use monoclonal antibody generated by standard techniques or an antibody fragment derived from these. This will generally be desirable if work is on a production scale because it is inconvenient to provide polyclonal antibodies in large quantities.

As a possible alternative to chemical synthesis of the motif peptide, if the polymerase chain reaction has already been used in the production of a polypeptide, that polypeptide might itself be used for raising antibodies which include antibodies binding to a motif amino acid sequence coded, at least partly, by an oligonucleotide primer. (Antibodies which bind specifically to the motif can be recovered on an affinity column with the motif sequence immobilised thereon). These antibodies could then be used when producing another polypeptide in accordance with the invention, using the same two primers or at least one of them.

List of Drawings

Figures 1 to 6, already referred to above, are diagrams illustrating the polymerase chain reaction.

Figures 7 to 9 and 11 to 13 are graphs illustrating results obtained by assays referred to in the Examples below.

Figure 10 illustrates the fusion of two nucleotide sequences.

Examples

The invention will be further explained and illustrated by the following Examples which relate to the production of antibody fragments with variable regions cloned using the polymerase chain reaction as referred to above.

In these Examples materials are denoted by abbreviations as follows:

- **PBS** = Phosphate Buffered Saline (0.01M NaH₂PO₄/ Na₂HPO₄, yielding pH 7, together with 0.15M NaCl)
- **PBSA** = PBS + 0.1% by weight sodium azide
- **PBST** = PBS + 0.15% by weight Tween 20
- **PPD** = purified protein derivative, supplied by Statens Seruminstitut, Copenhagen
- **BCIP/NBT** = 5-bromo-4-chloro-3-indoyl phosphate and p-nitroblue tetrazolium chloride which is a substrate system for alkaline phosphatase and is supplied by Promega, Southampton
- **PNPP** = para-nitrophenyl phosphate, which is an alternative substrate system for alkaline phosphatase
Primers for amplifying heavy chain gene

The primer VH1 FOR 2 is disclosed in EP-A-368684. It has the sequence designated SEQ ID No 1 in the sequence listing at the end of this description.

A related primer which has two additional nucleotides at the 3' end is disclosed in EP-A-368684 as VH1 FOR. This is also disclosed in Orlandi, Winter at al PNAS USA 86 3383 (1989).

VH1 FOR 2 is referred to as a forward primer and is itself antisense. It was designed to anneal with the 3' end of the sense strand of sequences coding for mouse heavy chain variable domains. It codes (indirectly) for a C-terminal amino acid sequence (i.e. peptide) which is included, with its C-terminal on the right, in SEQ ID No 2 in the sequence listing and repeated separately as SEQ ID No 3. A sense strand, complementary to this primer codes directly for the same amino acid sequence. This sense strand is also included in SEQ ID No 2 and incorporates a Bst EII recognition site, as indicated in bold type with the cleavage site arrowed.

The primer VH1 BACK is disclosed in EP-A-368684 and in the Orlandi et al paper referred to above. It is in fact a mixture of very similar primers differing at certain nucleotides. It has the sequence designated SEQ ID No 4 in the sequence listing at the end of this description, where S is used to denote C or G, M to denote A or C, R to denote A or G and W to denote A or T. It is a sense strand and was designed to anneal with the 3' end of the antisense strand of any nucleotide sequences coding for mouse heavy chain variable domains. This primer incorporates as a Pst I recognition site whose position is indicated and has an incomplete codon at the 5' end. If this codon is completed appropriately the sequence codes for either of two N-terminal amino acid sequences shown as SEQ ID No 5 in which the alternative acids Glu and Gin are shown as Glx.

Primers for amplifying light chain gene

A suitable forward primer has the sequence designated SEQ ID No 7 in the sequence listing at the end of this description. This primer is itself antisense and is designed to anneal with the 3' end of the sense strand of sequences coding for mouse kappa-type light chain variable domains. The complementary sense strand is shown as SEQ ID No 8. It incorporates a Xho I recognition site as indicated.

A suitable back primer is VK1 BACK as shown in EP-A-368684.

Example 1

Raising of Antibodies

An amino acid sequence designated Mot1 and given in the sequence listing below as SEQ ID No 4 was synthesised chemically. As can be seen it consists of the sequence SEQ ID No 2 coded by the VH1 FOR 2 primer together with two additional lysines to enhance its solubility and facilitate eventual immobilization on an affinity chromatography column. This peptide, designated Mot1 was covalently conjugated onto the carrier protein PPD and the resulting conjugate, designated Mot1-PPD was used to inoculate rabbits.

Antibodies with specific binding affinity towards the motif peptide Mot1 were isolated by affinity purification as follows:

5mg of the peptide Mot1 was immobilised onto 3mls wet volume of CNBr-activated Sepharose 4B (Pharmacia) according to that manufacturers instructions. The resulting material was packed into a liquid chromatography column. Serum (5ml) from rabbits immunised as above was loaded onto the column which was then washed through with PBSA to remove unbound material (the fallthrough) after which the antibodies bound to the Mot1 peptide on the column were eluted with 4M aqueous MgCl2, then dialysed overnight into PBSA.

Analysis of the original serum and the affinity purified fraction by polyacrylamide gel electrophoresis showed that the procedure had isolated pure immunoglobulin.

Solutions of antibodies were tested for ability to bind to the Mot1 peptide by means of an ELISA assay, as follows.

A microtitre plate was incubated with a solution of Mot1-PPD conjugate (0.1µg/litre in carbonate buffer) at 37°C overnight, to allow the conjugate to become immobilised on the plate. Next the plate was washed and wells of the plate were exposed to test solution (containing the polyclonal antibodies derived as above) for 1
hour to allow binding to the immobilized peptide. The plate was then washed again and exposed to a solution of goat anti-rabbit antibodies conjugated to alkaline phosphatase (available from Sigma). These bind to the antibodies (which are of course rabbit antibodies) which have bound to the Mot1. Finally after a further washing the plate is exposed to PNPP substrate solution for alkaline phosphatase, which is converted by that enzyme to a coloured product. The optical density at 405nm is read after a period of about 30 minutes and is a measure of binding to the Mot1-PPD conjugate.

To check the specificity of the binding a similar assay was carried out in which the initial solution of Mot1-PPD conjugate was replaced with a solution of PPD itself so that this assay gives a measure of binding to PPD.

If there is more binding to Mot1-PPD conjugate than to PPD itself in these assays, they show that there is binding to the Mot1 peptide.

Serum from immunised rabbits was diluted to various extents and tested in these assays. The results are shown graphically in Figure 7 of the drawings in which the results of binding to Mot1-PPD are shown with circles and the results of binding to PPD alone are shown with triangles.

This shows that the serum contained antibodies which bind specifically to the Mot1 peptide as well as antibodies to PPD protein.

The affinity purified fraction was diluted and tested similarly. The results are shown graphically in Figure 8 of the drawings.

This shows that the affinity purification has successfully isolated antibodies which bind to the Mot1 peptide.

Example 2

Anti-lysozyme Fragments

In Example 5 of EP-A-368684, Winter et al describe the production of Fv antibody fragments from a known monoclonal anti-lysozyme antibody designated "D1.3".

The affinity purified antibodies from Example 1 were used in a capture ELISA assay of Fv anti-lysozyme fragments of this type. These fragments had the amino acid sequence set out as SEQ ID No 2 at the C-terminal end of the heavy chain.

Microtitre plates were incubated with 1μg/ml lysozyme carbonate buffer overnight at 37°C so that lysozyme is immobilised in the plate wells.

Next the plates were washed with PBST and wells of the plate were exposed for 1 hour at room temperature of about 20°C to a test solution containing the Fv fragments diluted in PBST, for these to bind to the lysozyme.

The plate was then washed again and exposed to a solution of the affinity purified antibodies, for these to bind to the motif sequence at the C-terminal of the heavy chain of the fragments.

After this the plate was washed again, and exposed to a solution of goat anti-rabbit antibodies conjugated to alkaline phosphatase (Sigma) followed by washing and exposure to PNPP substrate solution for the alkaline phosphatase.

The optical density at 405nm after about 30 minutes is a measure of binding to the plate. The binding is dependent on the presence of the Fv fragments and the optical density should therefore provide a measure of the concentration of these in the test solution.

It was found that this was indeed the case. A plot of optical density against concentration of Fv fragments in the test solution is shown as Figure 9. The results fit a sigmoid curve, as can be seen.

This demonstrates that antibodies raised against the twelve amino acid sequence Mot 1 had specific binding affinity to polypeptides incorporating the ten amino acid motif peptide.

The affinity purified antibodies from Example 1 were also used in a Western blot procedure for the detection of the Fv anti-lysozyme fragments, as follows.

A test sample containing the fragments was first subjected to polyacrylamide gel electrophoresis. The resulting gel was electrobotted onto a 0.2μm nitrocellulose membrane. This membrane was incubated successively with

(i) solution of the affinity purified antibodies (2μg/ml in PBSA + 0.05% Tween20),
(ii) solution of goat anti-rabbit alkaline phosphatase conjugate (Sigma: diluted 1 in 2000 in PBSA + 0.05% Tween20),
(iii) substrate for the alkaline phosphatase leading to development of colour where the alkaline phosphatase conjugate has been bound to the membrane.

This technique was demonstrated on a test sample which was supernatant from an E. coli culture expressing the Fv fragments. One lane of the electrophoresis was stained with silver stain and showed the presence of numerous bands corresponding to various E. coli proteins. A second lane was used for the Western blot
procedure described above and this showed a single band of colour at the molecular weight appropriate for 
$V_h$ fragments. (In the Fv fragments the $V_h$ and $V_l$ chains are not covalently linked and they separate during 
this analytical procedure). Hence the anti-motif antibodies were selectively detecting the Fv fragments in the 
presence of various other proteins.

Example 3

Anti-hormone Fragments

Fv antibody fragments were derived from mouse monoclonal antibodies with binding affinity to a human hor­
mone (hCG), by procedures similar to those described by Winter et al in EP-A-368684.

The nucleotide sequence coding for the heavy chain variable region was amplified by means of the PCR 
reaction using the primers VH1 FOR 2 and VH1 BACK already referred to above. The resulting cloned DNA 
sequence had end portions corresponding to the primers. It was introduced into a plasmid, in the course of 
which procedure the end portions of the clone strands of DNA were cut away with restriction enzyme at the 
Bst EII and Pst I sites. The plasmid provided nucleotide sequence which exactly replaced that between the 
Bst EII recognition site and the terminus of the clone strands (the 3' terminus of the sense strand, correspond­
ing to the C-terminus of the expressed polypeptide). Stop codons were added by the plasmid. The plasmid 
also provided nucleotide sequence which replaced that between the Pst I recognition site and the other ter­
minus of the clone strands of DNA (while eliminating some of the redundancy in the VH1 BACK primer and 
completing the incomplete codon at the 5' end of that primer).

In the same way the nucleotide sequence coding for the light chain variable region was amplified by means 
of the PCR reaction using the forward primer shown as SEQ ID No 8 and the VK1 BACK primer shown in EP- 
A-368684. The resulting cloned DNA sequence had end portions corresponding to these primers.

The cloned DNA sequence was also introduced into the plasmid referred to above. In the course of this 
the end portion from the Xho I recognition site was cut away by restriction enzyme and replaced with nucleotide 
sequence from the plasmid. This completed the codon at the 3' end of the sense strand and also added stop 
codons, as shown diagrammatically in Fig. 10. The nucleotide sequence from the plasmid is also shown as 
SEQ ID No 9. The motif peptide coded by the sequence after introduction into the plasmid is shown as SEQ 
ID No 10.

The constructed plasmid containing both cloned sequences of DNA was used to transform E.coli which 
then expressed light and heavy chains. These spontaneously associated with each other as Fv fragments with 
the motif peptide set out as SEQ ID No 3 at the C-terminus of the heavy chain and the motif set out as SEQ 
ID No 10 at the C-terminus of the light chain.

Samples taken from E. coli cultures expressing these fragments were subjected to cell lysing conditions 
and then tested by the Western blot procedure of the previous Example. A single band of colour was observed 
at the molecular weight for a $V_h$ fragment, denoting the presence of the desired Fv fragments.

This demonstrates that the anti-motif antibodies can be used in assaying a plurality of different polypep­
tides where these are derived via a cloning step using the same primer in the polymerase chain reaction.

The peptide shown as SEQ ID No 10 could be synthesised and used to raise antibodies, as in Example 
1. These antibodies could be used, as in Examples 2 and 3 to assay for expressed Fv fragments or for light 
chain variable domains.

Example 4

Affinity purified antibodies from Example 1 were used in a direct ELISA assay of three Fv fragments. These 
were the Fv anti-lysozyme fragments of Example 2, the Fv anti-hCG fragments of Example 3, and an Fv anti­
body fragment with binding agent affinity to glucose oxidase which was made by procedures similar to those 
set out in Example 3.

For each Fv fragment, microtitre plates were sensitised by incubating test solutions which were dilutions 
of Fv (made up in sensitisation buffer) for 1 hour at 37°C. Plates were then incubated for 1 hour at room tem­
perature with a 1ug/ml solution of affinity purified rabbit anti-motif antibody made up in PBST. The plates were 
then incubated for 1 hour at room temperature with a 1/1000 dilution of goat anti-rabbit antibodies conjugated 
to alkaline phosphatase (Sigma). Finally, plates were developed with pNPP substrate (Sigma). Plates were 
washed thoroughly with PBST between each incubation.

As in Examples 2 and 3, the optical density at 405 nm is a measure of binding to the plate, and hence a 
measure of the concentration of the Fv antibodies in the test solution.

A plot of optical density against concentration is shown as Figure 11. All three antibodies were detectable
by this assay at concentrations of 50 nanograms/ml and above.

Example 5

Rabbit antibodies produced as in Example 1 were used to monitor the production of Fv anti-hCG fragments in a fermenter. These fragments were expressed by transformed E.coli as in Example 3. The E.coli was in 5 litre fermenters. The growth medium was:

- Na₂HPO₄ 12.0 g/litre
- KH₂PO₄ 6.0 g/litre
- NaCl 0.5 g/litre
- NH₄Cl 5.0 g/litre
- L-proline 60 mg/litre
- glycerol 30 g/litre
- MgSO₄.6H₂O 0.49 g/litre
- CaCl₂.2H₂O 15 mg/litre
- Thiamine hydrochloride 1 mg/litre
- Yeast extract (Beta Labs) 10 g/litre

The growth temperature was 25°C or 30°C and the pH was controlled at 6.8 by auto addition of 40% (w/v) NaOH. The impeller speed was 500rpm and aeration rate was 0.1 v/v/m. The inoculum (1% v/v) was grown overnight on M9P medium supplemented with yeast extract (5g/liter) in a shaking incubator at 25°C. During the first 15 hours of fermentation, the impeller speed and air flow rate were gradually increased to maxima of 650 rpm and 0.3 v/v/m respectively.

Fv anti-hCG fragment production was induced by the addition of filter sterilised iso propyl-B-D-thiogalactopyranoside, IPTG (0.5M; 1 ml/liter) during late exponential growth phase (12-15 hours from inoculation). Samples (5mls) were removed at known intervals for analysis and centrifuged for 15 minutes @ 3,000 rpm. The supernatent was then filtered through a 0.2 μm microfilter. Cells were lysed by a combination of osmotic shock and enzymatic treatment and the lysates passed through a 0.2μm microfilter. The resulting supernatent and lysate fractions were assayed using anti-motif antibodies in a direct ELISA assay, as described in the previous example.

The assay was standardised with a set of standards - diluted from a stock solution of Fv anti-hCG - which had been purified to homogeneity on hCG-Sepharose and determined spectrophotometrically (using an extinction coefficient of A₂₈₀ = 1.9 as calculated from sequence information).

The results of these assays for fermentations at 25°C and 30°C are shown as Figures 12 and 13 respectively. Each figure is a plot of protein concentration against time. Protein in the culture supernatant is shown by squares, protein in the cell lysate is shown by circles.

As shown by Figure 12 compared with Figure 13, Fv anti-hCG was produced much more efficiently at 25°C (terminal yield was 62mg/litre) than at 30°C (terminal yield in was 15mg/litre). Furthermore, at the lower temperature, the Fv was found almost exclusively (90%+) in the supernatant (which makes recovery of Fv easier). In contrast, at the higher temperature most of the Fv was found to be in the lysate fraction.

Example 6

This example demonstrates the use of antibody to a motif peptide to recover Fv fragments incorporating the motif peptide from a feedstock which resembled a fermentor broth.

Rabbit anti-motif antibody was prepared and isolated by affinity chromatography as described in Example 1. This procedure was repeated until a stock of 6mgs of motif-specific antibody had been built up. This preparation was concentrated to 1mg/ml in an ultrafiltration cell (Amicon), fitted with a membrane with molecular-weight cut-off of 8,000 Daltons (Amicon YM8). The concentrated antibody preparation was mixed with an equal volume of a buffer solution chosen so that the mixture contained 0.1M NaHCO₃, 0.5M NaCl and was at pH 8.3 and coupled onto 2 grams of chemically-activated agarose (CNBr Sepharose 4B, Pharmacia) according to that manufacturer's instructions. Unreacted CNBr groups were blocked by washing with an excess of 0.1M tris, pH 8 according to Pharmacia's instructions.

The resulting motif-specific immunoadsorbent, prepared as above, was packed in a glass column (Pharmacia) and fitted to a standard liquid chromatography set-up (Pharmacia). 100mls of a feedstock comprising 6μg/ml Fv anti-lysozyme and 1mg/ml bovine albumin (Sigma) was loaded onto the column. This feedstock was representative of a fermentor broth. Unbound protein was removed by washing with PBS. Bound Fv was eluted by washing with aqueous 4M MgCl₂ and dialysed into PBS.
Example 7

A monoclonal anti-lysozyme antibody incorporated in its variable domain an amino acid sequence which was the same as that shown in SEQ ID No 2, except that valine at position 6 was replaced by leucine.

This anti-lysozyme antibody was bound to a microtitre plate. This was then exposed to a solution of the polyclonal antibody of Example 1. Binding of this polyclonal antibody to the anti-lysozyme antibody was demonstrated by the ELISA assay described in Example 1.

This demonstrates that the specific binding affinity was present despite one change in the amino acid sequence.

A further experiment confirmed that the antibody was binding to the relevant amino acid sequence rather than to some other site. Lysozyme was bound to a microtitre plate. Then the monoclonal anti-lysozyme antibody was allowed to bind to the lysozyme. The antibody of Example 1 did not bind to these anti-lysozyme complexes, because the whole variable domain of the antibody was blocked by the lysozyme.
SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
   (A) NAME: Unilever NV
   (B) STREET: Weena 455
   (C) CITY: Rotterdam
   (E) COUNTRY: The Netherlands
   (F) POSTAL CODE (ZIP): 3013 AL

   (A) NAME: Unilever plc
   (B) STREET: Unilever House, Blackfriars
   (C) CITY: London
   (E) COUNTRY: England
   (F) POSTAL CODE (ZIP): EC4P 4BQ

(ii) TITLE OF INVENTION: Polypeptide production.

(iii) NUMBER OF SEQUENCES: 10

(iv) COMPUTER READABLE FORM:
   (A) MEDIUM TYPE: Floppy disk
   (B) COMPUTER: IBM PC compatible
   (C) OPERATING SYSTEM: PC-DOS/MS-DOS
   (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:
   (A) APPLICATION NUMBER: GB 9216983.8
   (B) FILING DATE: 11-AUG-1992

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 32 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA primer

(iii) ANTI-SENSE: YES
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:
TGA GGA GAC GGT GAC CGT GTT CCC TTG GCC CC

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 3..32

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 17..23
(D) OTHER INFORMATION: /note= "Bst EII recognition site"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 17..18'
(D) OTHER INFORMATION: /note= "Bst EII cleavage site"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:
GG GCC CAA GGG ACC ACG GTC ACC GTC TCC TCA
Gly Gin Gly Thr Thr Val Thr Val Ser Ser

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Gly Gln Gly Thr Thr Val Thr Val Ser Ser
1    5     10

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA primer

(iii) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 9..14
(D) OTHER INFORMATION: /note = "Pst I recognition site"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 13..14
(D) OTHER INFORMATION: /note = "Pst I cleavage site"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AG GTS MAR CAG SAG TCW GG

22

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Gln Val Gln Leu Gln Glx Ser Gly

15

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: C-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Lys Lys Gly Gln Gly Thr Thr Val Thr Val Ser Ser

15

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA primer

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

G TTT GAT CTC GAG CTT GGT CCC

22
(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 22 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(iii) ANTI-SENSE: NO

(ix) FEATURE:
   (A) NAME/KEY: misc_feature
   (B) LOCATION: 10..15
   (D) OTHER INFORMATION: /note= "Xho I recognition site"

(ix) FEATURE:
   (A) NAME/KEY: misc_feature
   (B) LOCATION: 10..11
   (D) OTHER INFORMATION: /note= "Xho I cleavage site"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GGG ACC AAG CTC GAG ATC AAA C

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 21 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(iii) ANTI-SENSE: NO

(ix) FEATURE:
   (A) NAME/KEY: misc_feature
   (B) LOCATION: 1..2
   (D) OTHER INFORMATION: /note= "Xho I cleavage site"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CTC GAG ATC AAA CGG TAA TAA

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: C-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Gly Thr Lys Leu Glu Ile Lys Arg

Claims

1. A method of producing at least one polypeptide by culture of a genetically transformed organism comprising
   cloning a required portion of a nucleic acid sequence by means of the polymerase chain reaction
   utilising a pair of oligonucleotide primers thereby to obtain a clone sequence with terminal portions defined
   by the primers,
   transforming an organism with a said clone sequence resulting from the polymerase chain reaction
   or a copy therefrom,
   culturing the transformed organism to express the polypeptide coded by the clone sequence,
   characterised by assaying or purifying the said polypeptide with a binding reagent displaying specific affinity towards an amino acid sequence in which there are at least three amino acids in sequence coded by a said primer.

2. A method according to claim 1 applied to the production of a plurality of separate polypeptides, the method comprising
   a said step of cloning a respective portion of nucleic acid for each polypeptide, utilising a pair of
   oligonucleotide primers and the polymerase chain reaction, at least one of the primers being used for all
   of the said polypeptides,
   a said step of transforming an organism, for each polypeptide,
   a said step of culturing the respective organism to express each polypeptide, and
   assaying or purifying each polypeptide, using the same said binding reagent, which reagent displays affinity towards an amino acid sequence in which there are at least three amino acids in sequence coded by the primer used for all the polypeptides.

3. A method according to claim 1 or claim 2 wherein said amino acid sequence contains at least six amino acids.
4. A method according to claim 1 or claim 2 wherein said amino acid sequence contains at least six amino acids coded by the primer.

5. A method according to any one of the preceding claims wherein a primer used in the step of cloning is a mixture of oligonucleotides.

6. A method according to claim 2 wherein each polypeptide is an antibody variable domain.

7. A method according to claim 2 wherein each polypeptide is an antibody variable domain, and the said binding reagent displays affinity towards an amino acid sequence at the C-terminal thereof.

8. A method according to claim 7 wherein each polypeptide is an antibody heavy chain variable domain.

9. A method according to claim 7 wherein each polypeptide is an antibody light chain variable domain.

10. A method according to any one of the preceding claims wherein the said nucleic acid sequence codes for a fragment of one chain of an antibody, the organism is transformed with a nucleic acid sequence coding for a second chain of the antibody fragment as well as with the said nucleotide sequence, culturing the organism expresses both chains of the antibody fragment, and the two chains associate together before the said step of assaying or purifying.

11. A method according to any one of the preceding claims wherein the said binding reagent is an antibody or antibody fragment.

12. A method according to any one of the preceding claims wherein the said binding reagent has specific affinity to the amino acid sequence given herein in SEQ ID No 3 or the sequence given herein in SEQ ID No 10.

13. A kit comprising an oligonucleotide primer for use in the polymerase chain reaction and a reagent having specific binding affinity to an amino acid sequence in which there are at least three amino acids in sequence coded by the said primer.

14. A kit according to claim 13 wherein the primer corresponds to a nucleotide sequence coding for a framework region of an antibody variable domain.

15. A kit according to claim 14 wherein the primer is as shown in SEQ ID No 1 or SEQ ID No 7.

16. A binding reagent having specific binding affinity to the amino acid sequence given herein in SEQ ID No 3, or the sequence given herein in SEQ ID No 10.
Fig. 7.

WHOLE SERUM (BEFORE AFFINITY PURIFICATION)

O.D. 405 nm

0.6
0.4
0.2

SERUM DILUTION

○ ANTI-MOTIF ASSAY
△ ANTI-PPD ASSAY

Fig. 8.

AFFINITY PURIFIED FRACTION

O.D. 405 nm

0.6
0.4
0.2

SERUM DILUTION

○ ANTI-MOTIF ASSAY
△ ANTI-PPD ASSAY
Fig. 9.

![Graph showing O.D. 405 nm against Analyte Concentration (µg/ml)]

Analyte Concentration (µg/ml):
- 10
- 2.5
- 0.625
- 0.156

O.D. 405 nm:
- 1.0
- 0.8
- 0.6
- 0.4
- 0.2

Fig. 10.

3' CCC TGG TTC GAG CTC TAG TTT G 5' PCR primer (antisense)

5' GGG ACC AAG CTC GAG ATC AAA C 3' sense strand

↓ Xho I

...CTC GAG ATC AAA CGG TAA TAA plasmid

Gly Thr Lys Leu Glu Ile Lys Arg (stop) (stop)
Fig. 11.

- Fv ANTI-LCG
- Fv ANTI-LYSOZYME
- Fv ANTI-G.Ox

O.D. 405nm

Fv CONCENTRATION (ng/ml)
Fig. 12.

![Graph showing Fv Protein (mg/litre) vs Time (hours) for different conditions involving cell lysate and culture supernatant.]

- CELL LYSATE
- CULTURE SUPERNATANT

Fig. 13.

![Graph showing Fv Protein (mg/litre) vs Time (hours) for different conditions involving cell lysate and culture supernatant.]

- CELL LYSATE
- CULTURE SUPERNATANT
Immunoadsorbents.

Improved affinity purification media are provided by the use of small specific binding agents, especially Fv antibody fragments or single domain antibody fragments, immobilised on porous carriers having pore sizes in the range 30-1000 angstroms, preferably 30-300 angstroms. Silica is a preferred carrier. The small fragments are able to penetrate the pores and maximise the effective surface area of the carrier, and the microporous silica is sufficiently robust to be used at high pressure, so enabling the speed and/or throughput of a purification procedure to be increased.
REAGENTS

This invention relates to reagents having specific binding properties, and particularly to their use in immuno adsorption processes, especially immunoaffinity purification processes.

Natural antibodies, either polyclonal or monoclonal, have been used as specific binding reagents for a considerable time. When immobilised on solid phases they can be used in purification procedures.

Antibodies are large complex multi-chain proteinaceous structures. Although it has been appreciated for some while that substantial portions of these structures seem unrelated to the specific binding properties of the antibodies, the minimum portion necessary to provide adequate specific binding has been a matter of debate. It has already been shown that so-called Fv fragments, i.e., an antibody fragment essentially comprising only a single heavy-chain variable region and its corresponding light chain variable region, can exhibit specific binding activity. Very recently it has also been shown by Ward et al (Nature, 1989, Vol. 341, p544-546) that a single variable domain from an antibody ("Dab") can exhibit significant specific binding activity. The production of single variable domain antibodies (Dabs), as described by Ward et al, is also described in detail in EP 0368684 A1 (Medical Research Council) published on 16 May 1990. There is now some evidence that peptides much shorter than the Dab, so-called paralogs, can be designed to mimic antibody binding to some extent (Kauva et al, Biochromatography, 5, 1990, p22).

To be of practical use in immuno adsorption processes, specific binding activity alone is not sufficient. The specific binding agent must also be capable of being linked to a solid phase such as a carrier material in a column. Ideally, this linkage is achievable without any significant adverse effect on the specific binding activity. Such adverse effects can easily arise through chemical or conformational changes in the specific binding region, or simply by physical (steric) hindrance of access to the specific binding region. In the case of conventional specific binding reagents, particularly whole antibody molecules or large portions of such molecules such as Fab fragments, the specific binding region or regions comprise only a minor portion of the total molecule. The comparatively vast residual bulk of the molecule, which is apparently not directly involved in the specific binding activity, provides abundant scope for the existence of locations which can participate in chemical or physical linkage to solid phases. These regions can be relatively remote from the essential specific binding regions, such that the resulting linkages need not interfere with the specific binding activity.

However, in the case of a specific binding entity essentially comprising only one or more variable domains unassociated with any substantial portion of the originating antibody or antibodies, e.g. a Fv fragment or a single variable domain, the relative proportion of the molecule which participates in the essential specific binding activity is very much higher. Indeed, it might be expected that any attempt to link the small specific binding entity to a solid phase would entail a very high risk that the essential specific binding activity will be adversely affected.

In contrast, we have surprisingly found that it is possible to immobilise small specific binding agents on porous carrier materials. Indeed, not merely is this possible, but the resulting immuno adsorbent can have enhanced properties, particularly because the use of the small specific binding agent permits advantage to be taken of improved carrier materials. By means of the invention, it is possible to change immunoaffinity from a bench-scale laboratory technique into a technique that can be applied economically and efficiently on a scale appropriate for industrial recovery or purification of a wide range of commercially important materials.

An embodiment of the invention is an immuno adsorbent material comprising a specific binding agent having a molecular weight of not more than about 25,000, immobilised on a porous solid phase carrier material.

The invention particularly provides an immuno adsorbent material comprising a specific binding agent immobilised on a porous solid phase carrier material, wherein the specific binding agent comprises one or more variable domain proteins (VH and/or VL) unassociated with any substantial portion of originating antibody or antibodies. The specific binding agent can be a single variable domain protein (Dab), or a combination of variable domains, especially an Fv fragment. Fv fragments may be either 'natural' Fvs (where the V\textsubscript{H} and V\textsubscript{L} are held together by hydrophobic forces) or 'single-chain' Fvs (where the V\textsubscript{H} and V\textsubscript{L} are linked by a short peptide).

Conventional porous solid phase carrier materials can be used, such as agarose; polystyrene; controlled pore glass (CPG); cellulloses; dextrans; agarose-filled kieselguhr; and synthetic polymers and co-polymers such as the hydrophobic "PW" polymers manufactured by Tosoh, polytetrafluoroethylene (PTFE) that has been rendered hydrophobic, polymers of N-acryloyl-2-amino-2-hydroxyethyl-1,3-propane diol (optionally with other monomers), and co-polymers of 2-hydroxy methacrylate with ethylene dimethacrylate (HEMA). A particularly preferred carrier material is porous amorphous silica. These carrier materials may be particulate (e.g. beads or granules, generally used in extraction columns), or in sheet form, e.g. membranes or filters,
which can be flat, pleated, or hollow fibres or tubes.

Relatively incompressible carriers are preferred, especially silica. These have important advantages for use in industrial-scale chromatography because they can be packed in columns operable at substantially higher pressure than can be applied to softer carrier materials such as agarose. Moreover, silica, glass and synthetic polymers and copolymers such as "PW" polymers possess particularly appropriate densities for use in stirred tanks and fluidised beds. These media are also preferred for high speed analytical separation.

An important embodiment of the invention is an immunoadsorbent material comprising a specific binding agent immobilised on porous silica or the like, the specific binding agent comprising one or more variable domain proteins unassociated with any substantial portion of originating antibody or antibodies.

A particularly important embodiment of the invention is an immunoadsorbent material comprising a specific binding agent immobilised on a porous carrier material, such as silica, having a pore size of at least 30Æ but not greater than 1000Æ, wherein the specific binding agent comprises one or more variable domain proteins unassociated with any substantial portion of originating antibody or antibodies. Preferably, the carrier has a pore size of at least 60Æ. Preferably, the pore size is not greater than 500Æ, and more preferably not greater than 300Æ.

Another important aspect of the invention is the use of an immunoadsorbent material comprising an Fv or Dab fragment, on a porous carrier material selected from the group consisting of amorphous silica, controlled pore glass, and synthetic polymers and copolymers, wherein the carrier material has a nominal pore size in the range 30-300Æ, to enhance the speed and/or throughput of an affinity purification process.

For convenience, the invention will be particularly described in relation to the use of silica as the carrier material. The reader will appreciate, however, that the invention encompasses the use of other porous carrier materials having properties analogous to those of silica.

Chromatographic silicas generally have a nominal pore size in the range of 30-300 angstroms (Å). Silicas with pore sizes of 200-300 Å are available commercially, and recommended as wide-pore solid phases for use in traditional physical protein separations. These silicas may be derivatised with ligands to make functional high performance liquid chromatography (HPLC) media, such as anion-exchangers.

Silicas having pore sizes of 200-300 Å are still robust, and columns packed with such material can be operated at high pressure. However, such silicas are quite unsuitable as carriers for conventional immunoreagents (ie. whole antibody molecules or large antibody fragments such as Fab fragments) because although such large molecules may fit into the pores in the silica, the resulting immunoadsorbent material would be very inefficient because there would be much less room left within the pores for any antigen to enter and engage with the specific binding agent. (Mohan et al, in Separations for Biotechnology, ed. D L Pyle, 1990). If intact antibodies are used as an immunoadsorbent material on a silica support, the pore size of the silica needs to be substantially larger eg. in excess of 1000 Å. Silicas with a pore-size of 1000 Å have three disadvantages: they are more expensive to manufacture; they are less robust; and they have a lower surface area per unit volume (which in turn restricts the amount of ligand which can be immobilised). See Ritchie et al, Chromatography and Analysis, 1990.

**Pore size determination**

The nominal pore size of a carrier medium, such as amorphous silica, is often referred to in the art as the mean pore diameter, and expressed as a function of pore volume and surface area. In principle, pore volume and surface area can be determined by standard nitrogen absorption methods of Brunauer, Emmett and Teller (BET). The mean pore diameter is calculated from the Wheeler equation (MPD = (40000 × Pore volume)/Surface area). However, above a pore diameter of about 200 Å, the measurement of pore volume by nitrogen adsorption becomes less accurate; measurement of a water pore volume by titration of a dry sample until the onset of agglomeration will give a more accurate result.

For some media, the nitrogen adsorption method is unsuitable, for example, non-crosslinked agarose supports which could not withstand the drying step required for the nitrogen adsorption measurements. In this case, size exclusion chromatography can be used to estimate the mean pore diameter. Size exclusion chromatography is a well-known technique in which polymer standards are used to estimate the pore size. Very large polymers cannot enter any of the pores and are totally excluded. Between these extremes, polymers of intermediate size can enter a fraction of the pore volume and this fraction can be measured and expressed as Kd. Kd for excluded polymers is zero, and for totally included polymers is 1.

A standard system applicable to silicas involves chromatography of polystyrene standards with tetrahydrofuran as solvent. In this system, a polystyrene of molecular weight of about 34000 (log 10 MW = 4.5) would have the following approximate Kd values for the following silicas, 60 Å < 0.05; 200 Å 0.2-0.3; 500 Å 0.55-0.65; 1000 Å 0.75-0.85. Thus, silicas of pore size 1000 Å or less would have a Kd value of less than
0.85 for a polystyrene of molecular weight of 34 K daltons. It should be noted that because the Kd value is a function of the log of the molecular weight, small changes in the molecular weight of the 34 K standard (i.e., variation between 30 K and 40 K) would have little effect on the result.

Certain media cannot be used in tetrahydrofuran and in these cases a derivatised silica column can be calibrated against polystyrene and then used to calibrate the alternative media. For example, if a carbohydrate type support is to be evaluated, tetrahydrofuran would lead to dehydration but protein standards in aqueous buffer could be accommodated. In this case, a silica of appropriate pore size can be modified with diol groups by well known procedures, and this media calibrated with the polystyrene system. The solvent system can then be changed to one appropriate for chromatography. The protein standards are then run on the alternative test media and the relevant Kd values compared with the diol media. Such data will only be valid if no adsorption takes place on the column. Tests for adsorption and means of overcoming these by suitable modification of the solvent are well-known to those skilled in the art of gel permeation chromatography.

**Immobilisation**

There are many known protocols for immobilising proteins or polypeptides on chromatography media. Some of these may be used for immobilising single or multiple variable domain proteins. For example, diol silica may be activated by tresyl chloride and then coupled to a variable domain protein. Alternatively, epoxy-activated silica may be coated with a polymer such as polyethyleneimine (PEI) and the variable domain protein linked to the polymer coat by a bifunctional reagent such as glutaraldehyde. Procedures for linking proteins to other chromatography media based on agarose, polystyrene, control-pore glass and kieselguhr are also well established in the literature.

Although in practice we have found that a small specific-binding entity, such as an Fv, can sometimes be immobilised directly onto a solid phase without significant loss of activity, in some instances this may not be possible. An additional objective of the present invention is to facilitate the linking of such small specific binding entities to solid phases with even less risk of damage to their essential specific binding properties.

In another embodiment, the invention provides an immunoadsorbent material comprising a specific binding agent immobilised on a solid phase carrier material, wherein the specific binding agent comprises:

i) one or more variable domain proteins (VH and/or VL) unassociated with any substantial portion of originating antibody or antibodies; and

ii) a chemical group, preferably a peptide group, (hereinafter referred to as a linking group) which does not contribute to the essential specific binding properties but which can be coupled by chemical or other means to a solid phase carrier material without the essential specific binding activity of the agent being significantly affected. Preferably, the linking group comprises at least 5 amino acid residues. Preferably, the linking group does not comprise more than 20 amino acid residues.

An important embodiment of the invention is a single variable domain protein (Dab) attached to a proteinaceous 'tail' which acts as the linking group as defined above, the 'tail' being coupled to a chromatography medium having a pore size in the range 30-300 Å, preferably 60-300 Å without significant loss of specific binding activity.

The properties of the linking group can be chosen to suit the method of attachment most appropriate for the surface to be used. The linking group may be hydrophobic, hydrophilic or of mixed character. It can include potential sites for covalent linkage. Preferably, such a proteinaceous linking group will contain at least one, and more preferably a plurality, of amino acid residues, preferably cysteine, incorporating sulphhydril groups. Sulphhydril groups can act as chemical coupling agents for covalent attachment to chromatography media. This may be done using a bispecific reagent such as succinimidyl - maleimido-phe-nylbutyrate (SMPB). Alternatively, or in addition, the proteinaceous linking group contains at least one, and more preferably a plurality, of lysine residues which possess e-amino groups. The actual coupling can be achieved, for example, by means of conventional bifunctional chemical cross-linking agents. Preferably, such a chemical coupling agent is located at a site sufficiently remote from the variable domain sequence itself that any carrier which becomes coupled to the linking portion is held at a distance from the variable domain sequence. Indeed, the linking group can easily be designed so that the site of coupling orientates the specific binding region in an advantageous position remote from the carrier.

A further important embodiment of the invention is a variable domain provided with a hydrophobic 'tail' which enables the variable domain to be immobilised by non-covalent attachment onto a hydrophobic surface, e.g., porous plastics material, such as porous polystyrene. Silica derivatised with standard hydrophobic ligands, such as alkyl chains (e.g., C9 or C18) or phenyl groups, can easily take up hydrophobic tails of this type.

To provide a linking group with sufficient
hydrophobicity to achieve the purposes of the invention, the polypeptide chain comprising the linking group should contain a sufficient number (which may be as few as two, if the residues are adjacent) of amino acid residues selected from the group consisting of valine, leucine, iso-leucine, phenylalanine, tyrosine, tryptophan, proline and alanine. We have found that even if the majority of the amino acid residues in the polypeptide are other, relatively polar (and hence relatively hydrophilic), amino acid residues, the presence of merely a low proportion of residues from the above group can confer effective hydrophobicity on the polypeptide. The hydrophobic region or regions can be adjacent to regions of high charge density, i.e. the peptide claim is of mixed character, without the essential hydrophobicity of the linking group as a whole being lost.

A particularly preferred linking group comprises the “Myc” amino acid sequence:

GLU-GLN-LYS-LEU-ILE-SER-GLU-GLU-ASP-LEU-ASN

Since this group contains a lysine residue, it can also be used for covalent attachment onto surfaces.

The linking group will normally be attached at or near one end of a variable domain protein. Normally, the point of attachment will be the amino terminus of the peptide linking group. This is the left hand end of the sequences A and B as seen in Figure 2 of the accompanying drawings. Preferably, the variable domain protein(s) and the linking group have been produced together by expression in a genetically engineered recombinant mammalian or bacterial host, and comprised a proteinaceous tail on one end of the domain sequence. The linking group will comprise at least about 5 amino acid residues, to confer sufficient length to “distance” the variable domain from the surface or tracer to which it is linked.

If desired, a variable domain can be provided with a "natural" hydrophobic polypeptide tail eg. the transmembrane sequence from influenza virus. A phospholipid tail would be an alternative.

The invention also encompasses specific binding reagents composed of a plurality of variable domain proteins. These can be equivalent to natural Fv fragments, i.e. a heavy chain variable region with a light chain variable region, or they can comprise combinations of heavy chain or light chain variable region proteins. Such combinations are normally held together by relatively weak interactions. A linking group of the invention can be incorporated at or near one end of one of the variable region protein sequences, but more than one linking group, of the same or differing character, can be incorporated in the combination if desired. The individual variable domain proteins can be expressed separately during cloning. Generally they will combine naturally under mild conditions, which do not inhibit the weak interactions that can cause them to associate.

Advantages of the invention

Imunoaffinity purification is a technique which is extensively used as a research tool but has rarely been used in industrial-scale processes. The novel affinity media of the invention, with single or multiple variable domains as the biospecific ligand, will be more amenable to use in industrial-scale processes by virtue of the following advantages.

i) Reduced molecular weight of ligand

Single variable domain proteins (Dabs) typically have a molecular weight of about 12,000, and Fv fragments about 25,000, compared with approximately 150,000 for an intact antibody. The small proteins may be more easily accommodated in the small pores of rigid chromatography media such as silica. The use of rigid media facilitates scale-up from the laboratory bench to industrial plant. Chromatography silicas which are manufactured for protein purification typically have pores in the range 200 to 300 A. Single variable domains and Fv fragments can fit easily into such pores and free exchange of most antigens can still occur without steric hindrance. However, if the antigen is very large (such as a protein in excess of 150 kD) it may be advisable to use a silica of up to 500 A pore-size to be sure of allowing free antigen exchange. If the antigen is very small (such as a peptide or non-proteinaceous pharmaceutical product) it may be advisable to use silica with pores in the 30-200 A range. This would take advantage of the larger surface area of small-pore silica so that more immunoligand could be immobilised with a resulting increase in capacity per unit volume. Whatever the size of the target analyte, the use of a smaller immunoligand (a Dab or Fv in preference to a whole antibody) will allow a correspondingly smaller silica pore-size to be used. Small-pore silicas typically have the advantages of increased rigidity and a higher surface area.

ii) Reduced affinity of ligand

Some single or multiple variable domains have been found to have reduced affinity for antigen, compared with intact antibody. This may be used to advantage, enabling antigen to be desorbed from the affinity medium under milder conditions, eg. by the use of less harsh buffers than are typically required. This will have the two desirable effects of increasing column lifetime, and reducing risk of inactivating the target analyte (ie. antigen).

iii) Reduced cost of producing ligand

Single or multiple variable domain fragments may be produced at a lower unit cost (ie. cost per bin-
ring-site) than intact antibody for two reasons. First, there are expression systems available for expressing such fragments in bacteria. Since bacterial culture medium is cheaper than mammalian cell culture medium (typically used for production of intact antibodies by hybridoma cells) considerable savings may be made here. Secondly, since protein synthesis is very costly in terms of cell metabolism, a considerable advantage will be gained by the cell only making proteinaceous structures required for immunoadsorption (ie. binding domains) rather than whole antibodies.

Since Dabs and Fvs, produced by genetic engineering, should be cheaper to produce per binding-site than whole antibodies, immunoaffinity purification may now be used cost-effectively on a wider spectrum of target analytes. It is therefore economic to purify lower-value and/or smaller analytes than has been the practise hitherto.

iv) Reduced 'HAMA' response
In immunoaffinity purification, small amounts of the ligand have been shown to leak from the column during operation and appear as contaminant the ligand have been shown to leak from the column. In the case of a mouse antibody contaminant may be serious as a mouse antibody may produce an anti-mouse response in the patient - the so-called 'HAMA' response. It has been shown that the HAMA response is primarily directed against the Fc region of the mouse antibody and that variable domain fragments produce a diminished HAMA response. Therefore, any contaminating variable domain fragments in injectibles will be less serious than whole mouse antibody contaminants.

v) Reduced non-specific binding
It is desirable that there should be few, or indeed zero, potential sites for non-specific adsorption present on the immunoligand. By reducing the size of the immunoligand down to the minimum required for specific binding (ie. immobilising the binding domain only) specific binding will be maximised and non-specific binding minimised.

An immunoadsorbent material comprising a porous silica having a pore size in the range 30 to 300 A, preferably 60 to 300 A, loaded with a specific binding agent which is either a single variable domain (Dab) or an Fv fragment, therefore represents a very advantageous material. The silica carrier material can be manufactured relatively cheaply, and the resulting immunoadsorbent material is physically very robust and can be used in a wide variety of commercial scale immunoadsorption facilities.

The novel immunoadsorbent materials of the invention can be used to extract compounds containing a specific antigen from feedstocks such as fermentation broths, serum, milk whey, and blood.

Production of antibody fragments
The invention is not concerned in principle with novel ways of producing single domain antibody fragments, Fv fragments, or novel ways of producing combinations of such fragments with peptide tails. Fv and single domain fragments can be produced by classical enzyme digestion of intact conventional antibodies. See Hochman et al, Biochemistry, (1973) vol. 12, pages 1130-1135. More preferably, they are produced by genetic engineering, for example as described in Riechmann et al, J. Mol. Biol, (1988), vol. 203, pages 825-828 ; Skerra et al, Science, (1988), vol. 240, pages 1038-1040 ; and Ward et al (1989, supra). Ward et al (1989) disclose the production of an anti-lysozyme single domain antibody fragment having a "Myc" tail. This combination could be used in accordance with the present invention, but Ward et al only contemplate the use of the "Myc" tail as an epitope to assist them in their experimental identification and isolation of the anti-lysozyme Dab that they produced. Ward et al make no suggestion that the "Myc" tail might be ideal for immobilising the Dab on porous chromatographic media. As seen below, the procedure of Ward et al can readily be adapted to produce other "tails" on Dab fragments.

A method for the production of a variable domain fragment, and some illustrations of immunoadsorbent materials in accordance with the invention, are given below purely by way of example.

Drawings
The accompanying drawings show:
Figure 1: Three oligonucleotides useful in the preparation of single domain antibody reagents having linking groups.
Figure 2: Two linking group peptide sequences that can be produced by means of the oligonucleotides depicted in Figure 1.
Figures 3a-3c: Chromatograph profiles obtained in Example 2b.
Figures 4a-4c: Chromatograph profiles obtained in Example 2c.
Figure 5: SDS-PAGE results obtained in Examples 2c and 2d.
Figure 6: Chromatograph profile obtained in Example 3.
Figure 7a and 7b: Chromatograph profiles obtained in Example 4.
Figure 8: Chromatograph profile obtained in Example 5.
Example 1

a) Preparation of a vector containing the anti-lysozyme V\textsubscript{H} fragment D1.3 as a PstI - BstEII cassette.

The anti-lysozyme V\textsubscript{H} fragment D1.3 is excised as a PstI - BstEII fragment from the expression vector pSW1-VHD1.3-VKD1.3. This vector, and the other expression vector used in this example, pSW1-VHPOLY-TAG1, are described by Ward et al (1989). pSW1-VHPOLY-TAG1 is restricted with PstI and BstEII, and the anti-lysozyme PstI-BstEII V\textsubscript{H} fragment of D1.3 is ligated into the opened vector. This ligation creates an expression vector with the V\textsubscript{H} D1.3 fragment inserted and is essentially the same as the expression vector pSW1-VHD1.3-TAG1 (Ward et al.) but with the PstI and BstEII restriction sites incorporated. We can refer to this expression vector as pVHD1.3-TAG1.

b) Cloning of a linking group sequence downstream of the cloned V\textsubscript{H} gene in pVHD1.3-TAG1.

The replacement of TAG1 by a linking group sequence downstream of the V\textsubscript{H} gene is done by the technique of site directed mutagenesis with large oligonucleotides as described in Verhoeyen et al., Science (1988), 239, pages 1534-1536. Single stranded DNA template is prepared from mp19VHD1.3-TAG1. This is the HindIII-EcoRI fragment from pVHD1.3-TAG1, containing V\textsubscript{H} D1.3 and TAG1, cloned in the HindIII and EcoRI sites of mp19. Single stranded DNA obtained from this clone contains the coding strand of the V\textsubscript{H} D1.3-TAG1 sequence. A DNA oligonucleotide is hybridized to the template to serve as primer to polymerize a second DNA strand. This oligonucleotide contains the required linking group sequence flanked on either side by 12 bases homologous to the site of integration. The double stranded molecule is transformed in E.coli, where a certain proportion of the molecules is 'repaired' by incorporation of the activation sequence structure. The 12 flanking bases, homologous to the site of integration, are the last four codons of V\textsubscript{H} D1.3 and the two stop codons followed by six bases present in pVHD1.3-TAG1. The oligonucleotide replaces the TAG1 gene sequence with that of the linking group gene sequence.

The linking group can be of a hydrophylic, hydrophobic or mixed nature. Convenient restriction sites can be incorporated to facilitate manipulation of the DNA sequences.

Figure 1 of the accompanying drawings shows three oligonucleotide sequences I, II and III useful in the above procedure. Sequences I and II are alternative sequences for producing an identical hydrophobic linking group, and III can be used to produce a hydrophobic linking group.

Figure 2 shows the cDNA and amino acid sequences of two linking groups A and B. Linking group A is hydrophobic, and can be produced using either of oligonucleotides I and II. Linking group B is hydrophobic, and can be produced using oligonucleotides III.

Three plasmids derived in this manner, in which the linking group sequence structure contains 12 or 11 amino acids (n = 1), and designated pVHD1.3-ADI, pVHD1.3-ADII and pVHD1.3-ADIII, are produced using sequences I, II and III. These plasmids are expressed in E.coli (as in Ward et al.).

The V\textsubscript{H} fragments are checked for activity by ELISA, and for purity by SDS-PAGE.

V\textsubscript{H} and V\textsubscript{L} fragments, with and without "tails", and also Fv fragments, can be prepared readily using similar procedures following the teaching in the publications cited earlier.

Example 2: Immobilisation of an anti-lysozyme Fv on agarose and its use as an immunoadsorbent

a) Preparation of immunoadsorbent

2 mgs of anti-lysozyme Fv (with no linking group) at a concentration of approximately 400 μg/ml was dialysed against coupling buffer (0.1 M NaHCO\textsubscript{3} + 0.5 M NaCl pH8.3). Small-bore dialysis tubing was used (Spectrum 132580). CNBr-activated Sepharose 4 B (Pharmacia 17-0430-02) was swollen and washed in 1 mM HCl. 3 mls of swollen gel was added to the Fv preparation in a stoppered vessel. The mixture was gently rotated overnight at 4°C. The Sepharose was recovered by centrifugation and blocked by rotating overnight at 4°C with 1 M ethanolamine made up in coupling buffer. The immunoadsorbent was washed three times in Tris buffer (0.1 M Tris pH 8 + 0.1% azide) and packed in a glass column (Pharmacia 19-5002-01).

b) Recovery of lysozyme from a 10-fold excess of albumin

2 mgs of hen-egg lysozyme (Sigma L-6876) and 20 mgs of bovine albumin (Sigma A-7888) were made up in 20 mls of Tris buffer. This feedstock was loaded onto the immunoadsorbent which was then washed with Tris buffer. Bound protein was eluted with 4 M MCI. It is the point of breakthrough for lysozyme. It was found to be 0.5 mgs (see Fig. 3a). The extent of non-specific binding was determined by two control experiments :-
Control experiment 1

20 mgs of bovine albumin was made up in 20 mls of Tris buffer and applied to the immunoadsorbent. The immunoadsorbent was washed with Tris buffer; bound material was eluted with 4 M MgCl₂ made up in Tris buffer.

Control experiment 2

A 'blank' column was made by blocking 3 mls of swollen CNBr-activated Sepharose with 1 M ethanolamine made up in coupling buffer. The Fv immunoligand was not added. 20 mgs of lysozyme was made up in 20 mls of Tris buffer and applied to the column. The column was washed with Tris buffer; bound material was eluted with 4 M MgCl₂ made up in Tris buffer.

In both control experiments non-specific binding was minimal (see Figs. 3b and 3c respectively).

c) Recovery of lysozyme from 5% horse serum

1 mls of horse serum (Seralab S-0004a) was made up to 20 mls (ie. 5% serum) in Tris/Tween buffer (0.1 M Tris pH 8, 0.1% azide, 0.15% Tween 20 - Sigma P1379). This feedstock was spiked with 2 mgs of hen-egg lysozyme and passed through a 0.45 µm filter (Schleicher and Schuell 452100). 20 mls of spiked serum ('feedstock A') was loaded onto the immunoadsorbent which was then washed with Tris/Tween buffer. Bound material was eluted with 4 M MgCl₂ made up in Tris buffer.

The extent of non-specific binding was determined by two control experiments.

Control experiment 1

20 mls of unspiked serum (ie. no lysozyme added) was prepared as before. The unspiked serum was loaded onto the immunoadsorbent in Tris/Tween buffer and washing/elution conditions were repeated as before.

Control experiment 2

10 mls of spiked serum was loaded onto the 'blank' column (described in Example 3b) in Tris/Tween buffer and elution conditions were repeated as before.

In both control experiments non-specific binding was minimal (see Figs. 4b and 4c respectively).

d) Recovery of lysozyme from a mixture of proteins

4 mgs of each of the following proteins were added together, made up to 30 mls in Tris/Tween buffer and passed through an 0.45 µm microfilter:

- Bovine albumin (Sigma A-7888), Myoglobin (Sigma), Haemoglobin (an in-house preparation), Trypsin (Sigma T-8003), Lysozyme (Sigma L-6876), Transferrin (Sigma), Cytochrome c (Sigma C-7752) and Ovalbumin (Sigma).

This protein mixture ('feedstock B') was loaded onto the immunoadsorbent which was then washed with Tris/Tween buffer. Bound material was eluted with 4 M MgCl₂ made up in Tris buffer. The eluted fraction was found to be homogeneous lysozyme by analysis with SDS-PAGE after dialysing against Tris buffer (Fig. 5).

The loading of samples on the gel was as follows:

Lane 1 Feedstock B
Lane 2 Lysozyme recovered from feedstock B
Lane 3 Feedstock A
Lane 4 Lysozyme recovered from feedstock A
Lane 5 Lysozyme standard
Lane 6 Molecular weight markers

Example 3: Immobilisation of an anti-lysozyme Fv on porous silica and its use as an immunoadsorbent

a) Preparation of immunoadsorbent

6 mgs of anti-lysozyme Fv (with TAG1, ie. the "Myc" tail, as a linking group) at a concentration of approximately 240 µg/ml was dialysed against phosphate buffer (0.1 M NaH₂PO₄ pH7). The dialysis tubing was Spectrum (132580). Glutaraldehyde-activated silica with approximately 200 A pore-size (PREPSCALE Glutaraldehyde-P, J T Baker 7567-02) was washed in phosphate buffer. 3.5 mls of washed silica was added to the Fv preparation in a stoppered vessel. The mixture was slowly rotated, and sodium cyanoborohydride was added at 4°C in aliquots over a period of 5 hours to reach a final concentration of 0.1 M, and the mixture tumbled overnight at 4°C. The product (immunoadsorbent) was washed with phosphate buffer, followed by phosphate buffer containing 1 M sodium chloride. The immunoadsorbent was then washed and blocked with 0.2 M pH7 ethanolamine overnight at 4°C. The immunoadsorbent was equilibrated in Tris buffer and packed in a glass column (Pharmacia 19-5002-01).
b) Recovery of lysozyme from a 25-fold excess of Cytochrome c

A mixture of two proteins was made up to the following specifications in Tris buffer: hen-egg lysozyme (Sigma L-6876) @ 0.04 mg/ml and cytochrome c (Sigma C-7752) @ 1 mg/ml. 20 mls of this mixture was loaded onto the immunoadsorbent which was then washed with Tris buffer. Bound material was eluted with 4 M MgCl₂ made up in Tris buffer. The eluted fraction was dialysed against Tris buffer using Spectrum dialysis tubing (132580). A chromatograph profile was generated using an on-line spectrophotometer (LKB "UVICORD") set at 280 nm to detect both proteins.

The fate of lysozyme and Cytochrome c was determined by making specific measurements for these two proteins across the chromatograph profile (Fig. 6). Lysozyme was determined by measuring enzyme activity using a suspension of Micrococcus (Sigma M-3770). 2.5 ml of Micrococcus lysodeikticus suspension, containing 1.5 mg of M-3770 in 10 ml of 0.066 M potassium phosphate buffer, pH6.24 at 25°C, was pipetted into a quartz cuvette (1 cm light path). The absorbance at 450 nm of this suspension was between 0.6 and 0.7, measured using a LKB "ULTRASPEC" photometer. Lysozyme solution was added, and the decrease in absorbance at 450 nm monitored to determine the amount in Enzyme Units per ml. Cytochrome c was determined by measuring the optical density at 406 nm, the absorbance maximum for this protein.

It was found that the separation of lysozyme from Cytochrome c was total and that the breakthrough for lysozyme was sharp (Fig. 6). Since Cytochrome c and lysozyme are physically very similar (cytochrome c : M.W. = 12,300, pI = 10.5 ; lysozyme : M.W. = 14,500, pI = 11.0) their complete separation represents a high resolution event.

Example 3c: Immunoelectron microscopy of immunoadsorbent to show distribution of bound lysozyme

The immunoadsorbent described above was removed from the column. The immunoadsorbent was placed in a 1 mg/ml solution of lysozyme in Tris buffer. Excess lysozyme was removed by washing with Tris. Silica particles were fixed using 1% paraformaldehyde plus 0.05% glutaraldehyde in phosphate buffered saline for 2 hours at 4°C; then embedded in resin. Ultrathin sections (approximately 90 nm thick) were prepared on nickel grids.

Grids and sections were blocked with 1% ovalbumin plus 5% goat serum in phosphate buffered saline; then left overnight in a solution of rabbit anti-lysozyme antibody made up in 1% ovalbumin, 5% goat serum, 0.1% Tween 20 in phosphate buffered saline. Grids were then washed with phosphate buffered saline and incubated with goat anti-rabbit antibody conjugated to 5 nm colloidal gold (Biocell).

Electron microscopy showed the lysozyme to be evenly distributed throughout the silica particles. A negative control where the rabbit anti-lysozyme antibody was omitted proved to be blank. The enzyme was uniformly dispersed throughout the porous structure of the silica support, indicating that the Fv was located over the whole surface within the pores and that the enzyme had also become bound within the pores.

Example 4: Recovery of lysozyme from serum using an Fv-fragment immobilised on silica

a) Preparation of immunoadsorbent

Epoxy silica particles with pore-size of approximately 200 Å (C200, Crosfield Chemicals) were converted to the diol derivative by the method of Mohan et al (In Separations for Biotechnology ed D L Pyle, 1990). C200 Diol particles were tresylated as follows: 2 g of diol silica was stirred gently in 100 ml of dry acetone (BDH) containing 0.76 ml of dry triethylamine (Fluka) and 0.666 g of 4-dimethylaminopyridine (Fluka). 50 ml of a 2% solution of tresyl chloride (Fluka) made up in dry acetone was slowly dripped into the gently stirring silica over a period of two hours (keeping the temperature below 30°C). After a further hour, the contents were washed into a filter funnel and paper (Whatman No. 1) using ethanol (BDH). Further washing with ethanol was undertaken (5 x 30 mls), followed by washing with a : 1 ethanol/acetone mix (5 x 30 mls) and finally washing with acetone only (5 x 30 mls). The silica was then dried in a forced air (fan) oven overnight at 30°C.

0.9 g of tresylated C200 was washed with 100 mls of 1 M NaCl and then 100 mls of borate buffer (0.1 M Na₂ B₄ O₆/HC₁, pH8.5). The silica was then added to 14 mls of a solution of anti-lysozyme Fv (with no linking group) and rotated overnight at 4°C in a stoppered vessel. The Fv solution was approximately 350 µg/ml in PBS (0.01 M sodium phosphate, 0.15 M NaCl, pH7). The immunoadsorbent was recovered by centrifugation, approximately 1 mg of Fv protein was found to have been coupled by analysis with the BCA protein assay (Pierce). The immunoadsorbent was blocked by rotating overnight in 1 M ethanolamine made up in borate buffer. The immunoadsorbent was then washed three times in Tris buffer and packed in a glass column.
b) Recovery of lysozyme from 10% horse serum

20 mls of 10% horse serum (diluted in Tris buffer) was spiked with hen-egg lysozyme to a final concentration of 0.02 mg/ml. The spiked serum was passed through a 0.45 μm filter and loaded onto the immunoadsorbent, and eluted and analysed as in Example 3b. The eluted peak was found to be highly enriched for lysozyme (Fig. 7a).

To determine the extent of non-specific binding, a 'blank' column was made by blocking 0.2 g of tresylated C200 with 1 M ethanolamine made up in borate buffer. The Fv immunoligand was not added. 5 mls of 10% horse serum was spiked with hen-egg lysozyme to a final concentration of 0.1 mg/ml and applied to the blank column. The column was washed and eluted as before. Neither the lysozyme nor the serum bound to the column (Fig. 7b).

This demonstrates that the recovery of lysozyme is by virtue of specific interaction with the Fv immunoligand.

Example 5: Immobilisation of an anti-lysozyme Dab on porous silica and its use as an immunoadsorbent

a) Preparation of immunoadsorbent

1 g of glutaraldehyde-activated silica with approximately 200 A pore-size (PREPSCALE Glutaraldehyde-P, J T Baker 7567-02) was washed as in Example 3a. The washed silica was added to 10 mls of a Dab preparation. The Dab was an anti-lysozyme V<sub>H</sub> with the "myc" peptide as a linking group. The concentration of protein was approximately 70 μg/ml in PBS (0.01 M sodium phosphate, 0.15 M NaCl, pH7). Coupling was performed as in Example 3a.

b) Recovery of lysozyme from a 10-fold excess of cytochrome c

A mixture of two proteins was made up to the following specifications in Tris/Tween buffer (0.1 M Tris, pH8, 0.15% Tween) :- hen-egg lysozyme @ 0.01 mg/ml and cytochrome c @ 0.1 mg/ml. 20 mls of this mixture was loaded onto the immunoadsorbent, and eluted and analysed as in Example 3b. The fate of lysozyme and cytochrome c was determined as described in Example 3.

It was found that the separation of lysozyme from cytochrome c was total (Fig. 8). Since cytochrome c and lysozyme have very similar molecular weights and isoelectric points, their complete separation represents a high resolution event.

Claims

1. An immunoadsorbent material comprising a specific binding agent having a molecular weight of not more than about 25,000, immobilised on a porous solid phase carrier material.

2. An immunoadsorbent material comprising an Fv fragment or a Dab, immobilised on a porous solid phase carrier material.

3. An immunoadsorbent material according to claim 1 or claim 2, wherein the carrier material has a nominal pore size of less than 1000 A, preferably less than about 500 A, and more preferably less than about 300 A.

4. An immunoadsorbent material according to any one of claims 4 to 6, wherein the nominal pore size is at least about 30 A, preferably at least about 60 A.

5. An immunoadsorbent material according to any one of the preceding claims, wherein the carrier material is porous amorphous silica, controlled-pore glass, or a hydrophilic "PW" polymer.

6. An immunoadsorbent material comprising a specific binding agent immobilised on a solid phase carrier material, wherein the specific binding agent comprises:
   i) one or more variable domain proteins (VH and/or VL) unassociated with any substantial portion of originating antibody or antibodies; and
   ii) a chemical group, preferably a peptide group, which does not contribute to the essential specific binding properties but which can be coupled by chemical or other means to a solid phase carrier material without the essential specific binding activity of the agent being significantly affected.

7. An immunoadsorbent material according to any one of the preceding claims, wherein the specific binding agent, Fv fragment or Dab is coupled to the carrier material via a peptide linking group comprising at least 5 amino acid residues, but preferably not more than 20 amino acid residues.

8. An immunoadsorbent material according to claim 6 or claim 7, wherein the linking group is hydrophobic.

9. An immunoadsorbent material according to claim 7, wherein the linking group is a "Myc" tail.

10. Use of an immunoadsorbent material according.
to any one of the preceding claims in an affinity purification process.

11. Use of an immunoadsorbent material comprising an Fv or Dab fragment, on a porous carrier material selected from the group consisting of amorphous silica, controlled pore glass, and synthetic polymers and copolymers, wherein the carrier material has a nominal pore size in the range 30-300 Å, to enhance the speed and/or throughput of an affinity purification process.
I

homology Kpn1 EcoRI
5' TAG CCC TTA TTA CAG GTA CCC CTT ACC GGA ATT CCC (GCT ACC)n

BamHI homology
GGA TCC TGA GGA GAC GGT 3' n=0-5

II

homology Kpn1
5' TAG CCC TTA TTA CTT CAG GTA CCC CTT ACC GGA GTT CCC (GCT ACC)n

BamHI homology
GGA TCC TGA GGA GAC GGT 3' n = 0-5

III

homology Kpn1 SacII
5' TAG CCC TTA TTA GGG TAG CAA AAG CTT CGC (AAG TGC)n TAC CGC GGC

homology
TGA GGA GAC GGT 3' n = 0-5
A  
hydrophylic tail

Bam HI                 Kpn1

--------               --------
GGA TCC (GGT AGC)n    GGG AAC TCC GGT AAG GGG TAC CTG
Gly Ser Gly Ser Gly Asn Ser Gly Lys Gly Tyr Leu

n = 0-5

B  
hydrophobic tail

Sac II                 Hind III                 Kpn1

--------               --------               --------
GCC GCG GTA (GCA CTT)n GCG AAG CTT TTG GTA CCC
Ala Ala Val Ala Pro Ala Lys Leu Leu Val Pro

n = 0-5

Fig. 2
Fig 5.
<table>
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<tr>
<th>Category</th>
<th>Citation of document with indication, where appropriate, of relevant passages</th>
<th>Relevant to claim</th>
<th>CLASSIFICATION OF THE APPLICATION (Int. Cl.)</th>
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<tr>
<td>O,Y</td>
<td>NATURE, vol. 341, 12 October 1989, LONDON GB</td>
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<td>&quot;Binding activities of a repertoire of single immunoglobulin variable domains secreted from Escherichia coli.&quot;</td>
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<td>* abstract *</td>
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<td>CHROMATOGRAPHIA, vol. 27, no. 11, 1989, Braunschweig, Germany</td>
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<td>pages 569 - 573; T. HAYASHI et al.:</td>
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<td>&quot;HPLC analysis of human epidermal growth factor using immunoaffinity precolumn. I. Optimization of immunoaffinity column.&quot;</td>
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<td>WO-A-8909088 (TERRAPIN DIAGNOSTICS, INC.)</td>
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<td>* page 14, line 8 - page 16, line 25; claims *</td>
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<td>pages 1041 - 1043; A. KLAUSNER: &quot;Single chain antibodies become a reality.&quot;</td>
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The present search report has been drawn up for all claims.

Examiner

THE HAGUE

08 APRIL 1991

NOOJ F.J.M.

CATEGORY OF CITED DOCUMENTS

X: particularly relevant if taken alone
Y: particularly relevant if combined with another document of the same category
A: technological background
O: non-written disclosure
P: intermediate document
K: member of the same patent family, corresponding document
I: theory or principle underlying the invention
F: earlier patent document, but published on, or after the filing date
D: document cited in the application
L: document cited for other reasons

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