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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

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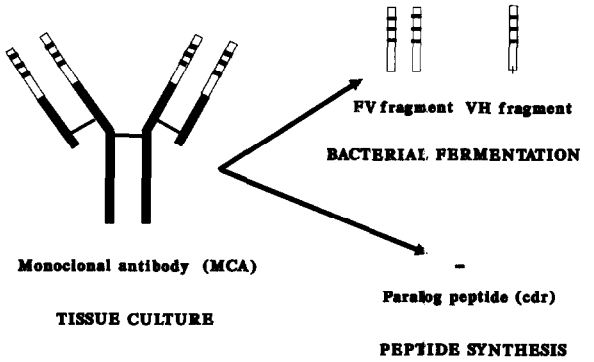


Fig. 1. Antibody fragments and their production. On the monoclonal antibody (MCA) the constant regions (C) are drawn in black, the variable regions (V) in white and the complementarity determining regions (cdrs) appear as three bands on the variable regions. Molecular masses: MCA = 150 000; FV fragment = 25 000; VH fragment = 12 500; paralog peptide = 1000–2000.

other antibody domains. In order of decreasing size, these antibody fragments have been described as FV fragments [10–12] (M_r 25 000), VH fragments or domain antibodies [13] (M_r 12 500) and "paralog" peptides [14] (M_r 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 differecnes) [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 TG1) 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* $NaHCO_3$ (BDH, Poole, UK)–0.5 *M* NaCl (BDH) (pH 8.3)] then immobilized on to *ca.* 1 g of cyanogenbromide-activated Sepharose 4B

(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 Ultraspec II spectrophotometer (Pharmacia).

Recovery of hen lysozyme from "spiked" horse serum

A 1-g amount of each immunoadsorbent was conditioned in phosphate-buffered saline [00.1 *M* Na_2HPO_4–NaH_2PO_4 (BDH)–0.15 *M* NaCl (pH 7)], then packed in a glass column (Pharmacia C10/20) to give column dimensions of 40 mm × 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 μg/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* $MgCl_2$ (BDH) (pH 7)], then dialysed into PBS. The purity of the recovered protein was determined by concentrating as required in a Speedvac concentrator (Stratech Scientific, London, UK), dialysing against 1 m*M* Tris (Sigma) (pH 8) and applying to a gel for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (see below). The chromatographic set-up consisted of a Multirac fraction collector (Pharmacia), a P1 peristaltic pump (Pharmacia), a Uvicord S monitor (Pharmacia) set at 280 nm and a flat-bed chart recorder (Pharmacia).

Separation of hen lysozyme from turkey lysozyme

Each column was loaded with a feedstock consisting of hen lysozyme at 25 μg/ml and turkey lysozyme (Sigma) at 50 μg/ml made up in PBS. This feedstock was applied until a stable breakthrough was reached; then the columns were washed back to the bascline with PBS.

Bound protein was recovered by eluting with desorption buffer and dialysing the peak 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 m*M* Tris–1 m*M* EDTA (Sigma) (pH 8)] 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 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* 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 β-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 m*M* Tris–1 m*M* 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

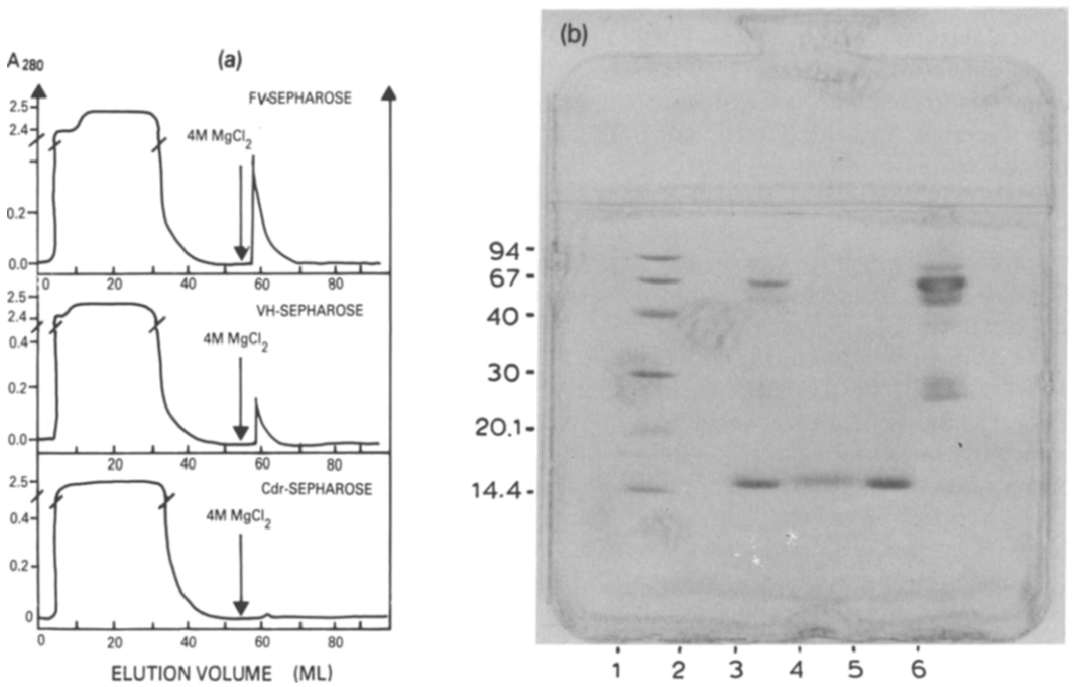
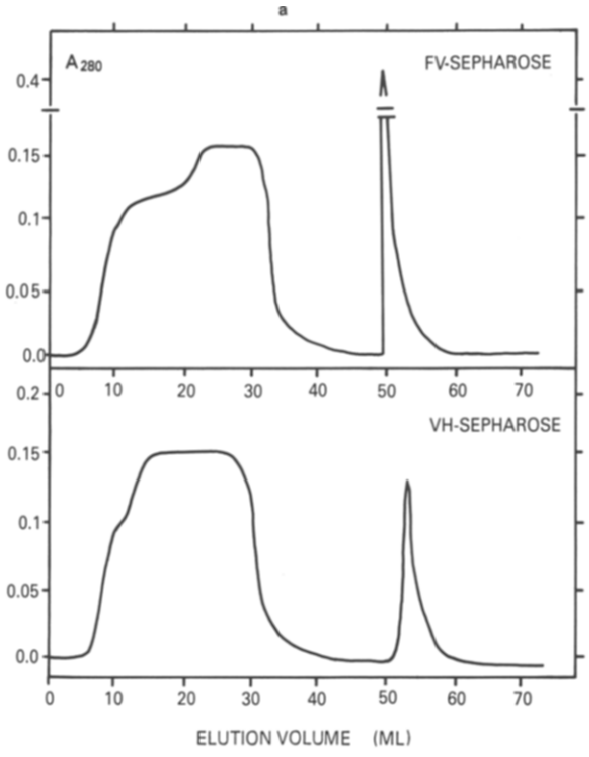


Fig. 2. (a) Recovery of hen-egg lysozyme from "spiked" serum using anti-lysozyme antibody fragments immobilized on cyanogen bromide-activated Sepharose 4B. The feedstock was made up and loaded in PBS. Bound lysozyme was desorbed by eluting with 4 *M* $MgCl_2$ and dialysed into PBS. (b) SDS-PAGE analysis 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).



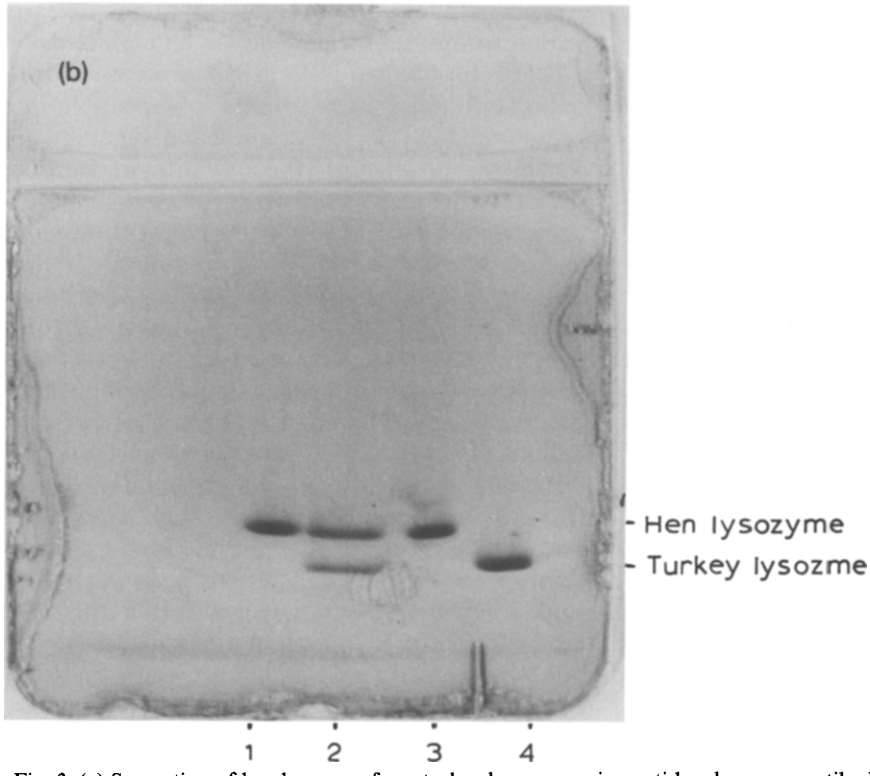


Fig. 3. (a) Separation of hen lysozyme from turkey lysozyme using anti-hen lysozyme antibody fragments immobilized on cyanogen bromide-activated Sepharose 4B. The feedstock was made up and loaded in PBS. Bound lysozyme was desorbed by eluting with 4 *M* $MgCl_2$ and dialysed into PBS. (b) Basic PAGE 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.

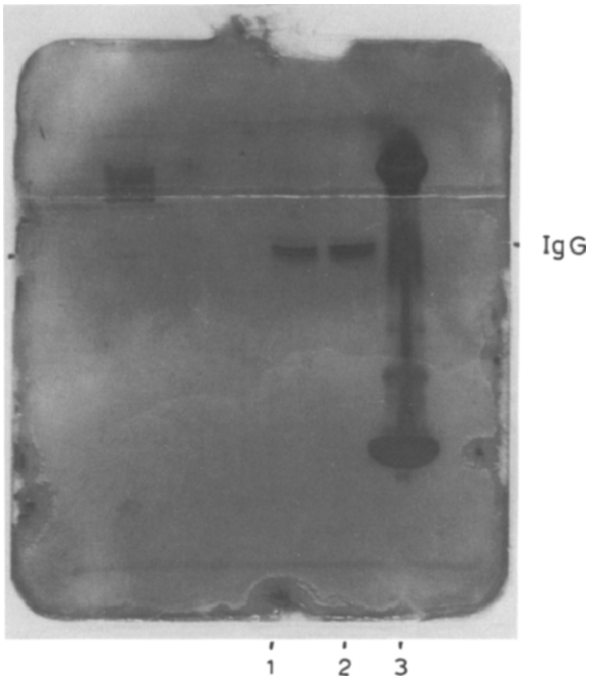


Fig. 4. Native PAGE analysis of rabbit anti-idiotype antibody recovered from rabbit serum with immobilized cdr peptide. Lanes: 1 = antibody recovered from column; 2 = rabbit antibody standard; 3 = feedstock (rabbit serum).

(Fig. 2a). However, the column was able to recover anti-idiotype antibody from serum as determined by native PAGE (Fig. 4). The recovered antibody was confirmed to have genuine anti-idiotype activity by enzyme-linked immunosorbent assay (ELISA) (results not shown).

DISCUSSION

We found that the FV column was specific for antigen and could recover antigen from spiked serum or from a closely related isoenzyme in a single step. FV fragments have a number of advantages over whole antibodies: they are cheaper to produce, they have more binding sites per milligram of protein and they are sufficiently small to be immobilized within the pores of chromatographic silica. We have discussed these advantages in more detail elsewhere [15]. We believe that the introduction of FV fragments presents an exciting opportunity to the technique of immunoaffinity chromatography.

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 immunoreagent currently available that binds antigen in a specific and predictable manner.

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