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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].

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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* $NaHCO_3$–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 immunoadsorbent was conditioned in phosphate-buffered saline (PBS) and 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), made up in PBS (0.01 *M* Na_2HPO_4/NaH_2PO_4–0.15 *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* $MgCl_2$, 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 immunoelectron microscopy

Immunoadsorbent 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 × 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 immunoadsorbent 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 × 50 μg/ml) of lysozyme protein. This corresponds to 48 000 lysozyme units (16 mls × 3000 U/ml). The

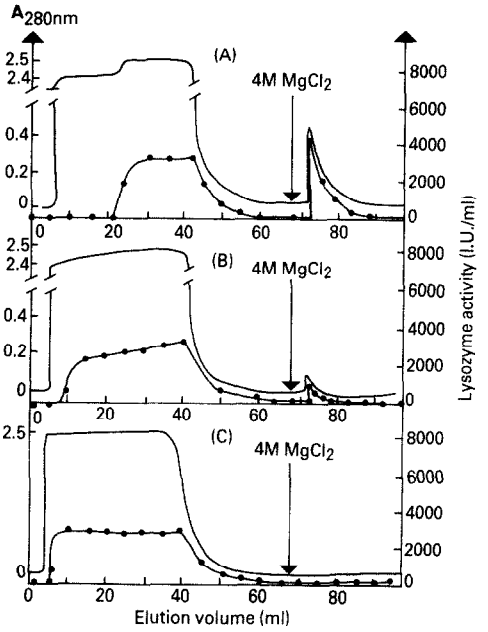

Fig. 1. (A) Recovery of hen-egg lysozyme from "spiked" serum using anti-lysozyme Fv fragments immobilised on Sorbsil C200 40/60. The feedstock was made up and loaded in PBS (0.01 *M* $NaHPO_4/NaH_2PO_4$–0.15 *M* NaCl, pH 7). Lysozyme was specifically eluted with 4 *M* $MgCl_2$ and dialysed into PBS. 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 immobilized 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 immobilized).

amount of lysozyme recovered in the 4 *M* $MgCl_2$ 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 acitivity 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 = (14.4/48) × 100 = 30%; and specific activity of immobilized Fv fragments = (0.8 × 25)/(3.4 × 14.3) × 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-labelled experiment was 78%. A summary of key data is given in Table I.

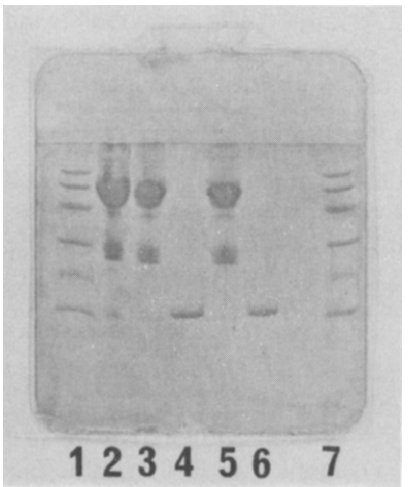

Fig. 2. SDS-PAGE of separation achieved with immobilized anti-lysozyme Fv fragments. Lanes: 1, 7 = Pharmacia low-molecular-weight standards (94 000, 67 000, 43 000, 30 000, 20 100, 14 400 dalton); 2, 3 = feedstock (5% horse serum "spiked" with 50 μg/ml of hen-egg lysozyme); 4 = lysozyme; 5 = column fall-through; 6 = lysozyme recovered from feedstock (4 *M* $MgCl_2$ fraction).

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 I

COMPARISON OF Fv FRAGMENTS AND WHOLE ANTIBODIES (MCA) IN IMMUNOAFFINITY CHROMATOGRAPHY

Affinity columns made with immobilized antibody fragments (Fv) and whole antibodies (MCA) were compared for their ability to recover antigen from spiked serum. Capacity for antigen was determined by breakthrough level (see Fig. 1); recovery of antigen protein was determined by following an FITC-labelled antigen.

Parameter	Fv fragment column	MCA column
Silica used (g)	1.0	1.0
Ligand used (mg)	4.0	4.0
Ligand coupled (mg)	3.4	2.0
Capacity for antigen (mg)	0.8	0.15
Antigen recovered (mg)	0.6	0.12
Recovery (%)	78	79

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 × 50 μg/ml). This correcponds to 9000 lysozyme units (3 ml × 3000 U/ml). The amount of lysozyme recovered in the 4 *M* $MgCL_2$ 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) × 100 = 80%; recovery of lysozyme activity = (3000/9000) × 100 = 33%; specific activity of immobilised antibodies = (0.15 × 75)/(2 × 14.3) × 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 baterial 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 fragment

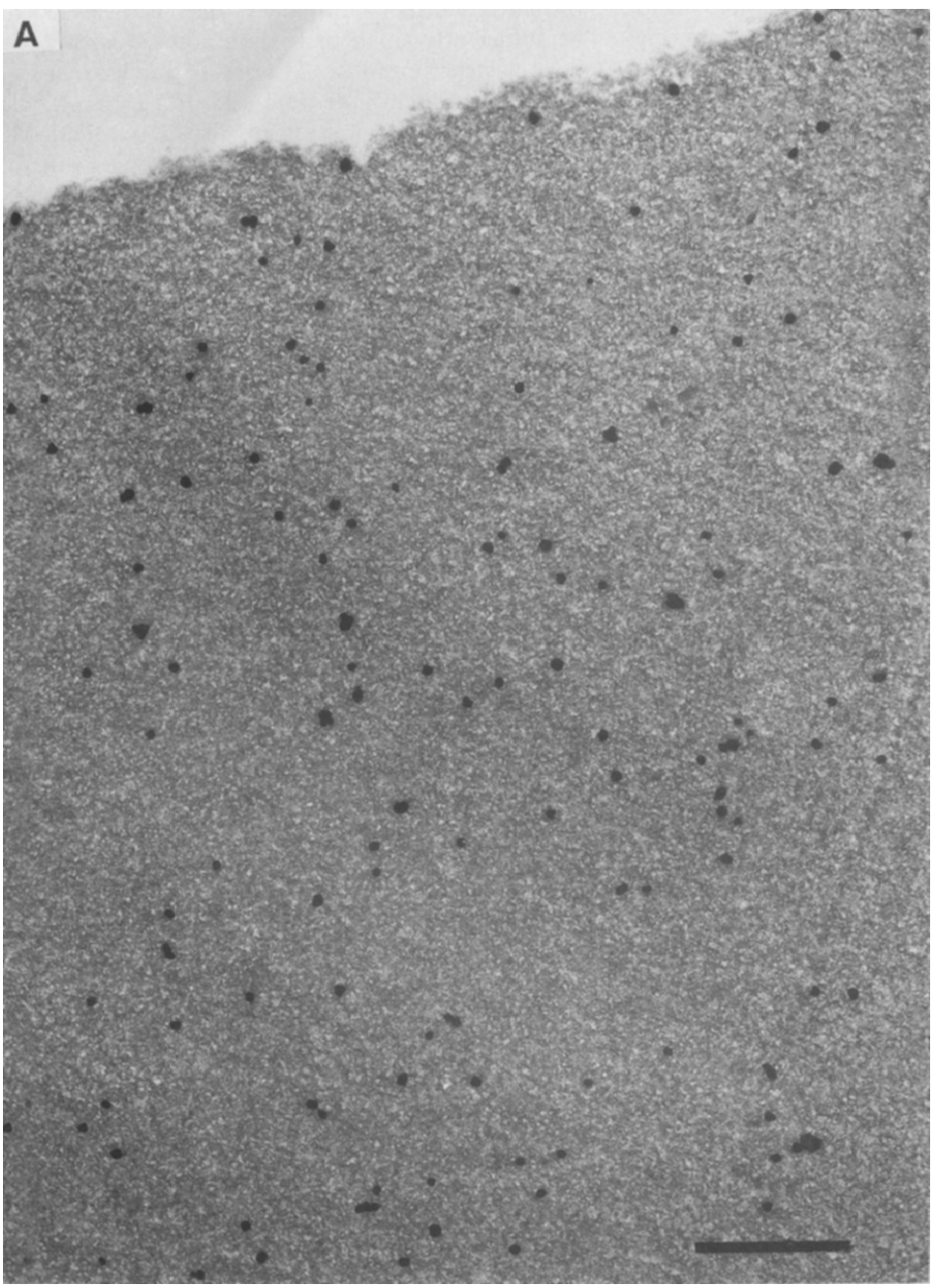

Fig. 3.

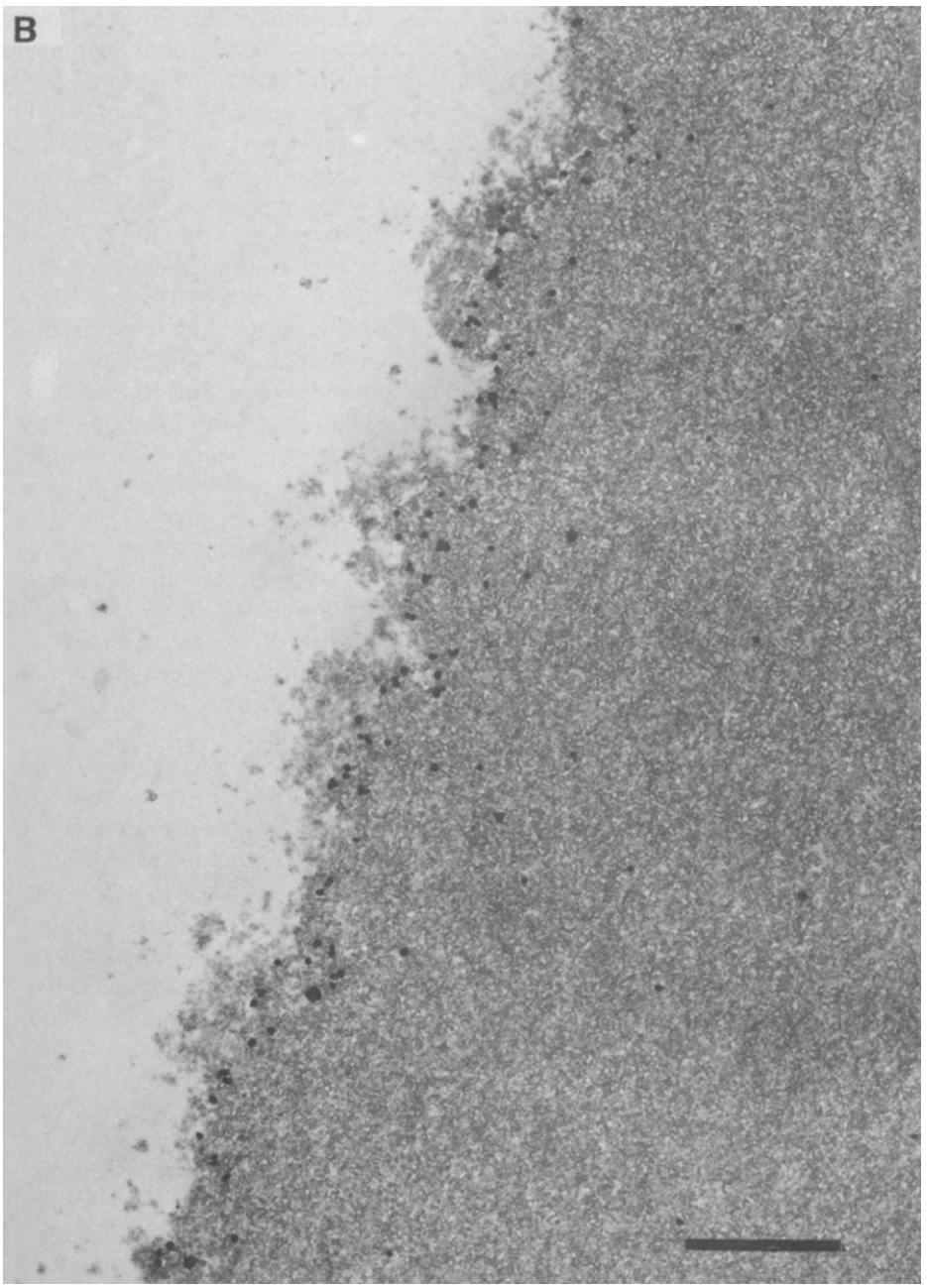

Fig. 3. (A) Immunoelectron micrograph of silica particle derivatized with anti-lysozyme Fv fragments. Particles were interacted with lysozyme, sectioned and then stained by labelling with rabbit anti-lysozyme followed by goat anti-rabbit–colloidal gold. Magnification 20 000 (bar = 1 μm). (B) Immunoelectron 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* $MgCl_2$) 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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