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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

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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 (V_H and V_L) are held together, non-covalently, by three pairs of hydrophobic patches [15]; whereas in scFv fragments, V_H and V_L 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 V_H and V_L. However, with scFv, even if V_H and V_L are temporarily dissociated on treatment with elution buffer, the linker peptide should keep V_H and V_L 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 "D1.3" antibody [18]) and tagged at the C-terminus of its V_L 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 V_L 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 loosing 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* $NaHCO_3$ (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 immunoadsorbent was conditioned in PBSA (0.01 *M* Na_2HPO_4/NaH_2PO_4–0.15 *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* $MgCl_2$) and dialysing the peak into PBSA. The columns were then re-equilibrated in PBSA. Chromatograms were drawn by monitoring A_{280} 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* $MgCl_2$ fraction recovered from the immunoadsorbents 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 immunoadsorbents 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 immunoadsorbents 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* $MgCl_2$) 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 toal of 100 purification cycles using the albumin/lysozyme feedstock was carried out on each immunoadsorbent and the blank column. The whole procedure was repeated for both Fv and scFv using freshly prepared immunoadsorbents. A total of 100 purification cycles using the albumin/cytochrome *c* feedstock was also carried out on the immunoadsorbents.

Analysis of immunoadsorbent 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 immunoadsorbent deterioration. For cycle 1 and cycle 100, the purity of the lysozyme fraction recovered from the immunoadsorbents was analysed by SDS-PAGE. The findings were examined for evidence in immunoadsorbent 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* $MgCl_2$) in the absence of lysozyme and assayed the washings for Fv (in the case of 4 *M* $MgCl_2$ 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 I

SPECIFIC ACTIVITY OF IMMOBILISED LIGANDS

The orientation of immobilised ligand was checked by determining the specific activity of Fv. This was done by comparing the immunoadsorbents' capacity for antigen with the amount of immobilised Fv on a molar basis (assuming molecular weights of 25 kilodalton for Fv and 14.4 kilodalton for lysozyme). The capacity for antigen was estimated from the position of breakthrough in Fig. 1 (for example, the capacity of the Fv-immunoadsorbent at cycle 1 was approximately 24 ml × 50 μg/ml = 1.2 mg). The amount of ligand immobilised was determined by measuring the absorbance of the ligand solution at 280 nm before and after coupling.

Parameters	Fv	scFv
Ligand used	4 mg	4 mg
Ligand immobilised	3.6 mg	3.2 mg
Antigen capacity	1.2 mg	1.0 mg
Specific activity	58%	55%

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* $MgCl_2$ 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* $MgCl_2$ (chromatogram not shown).

When the albumin/lysozyme feedstock was loaded onto the "blank" column, a breakthrough curve did not develop; no protein was eluted with 4 *M* $MgCl_2$ (see Fig. 1E).

When the albumin/cytochrome *c* feedstock was loaded onto the immunoadsorbents, a breakthrough curve did not develop; no protein was eluted with 4 *M* $MgCl_2$ (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

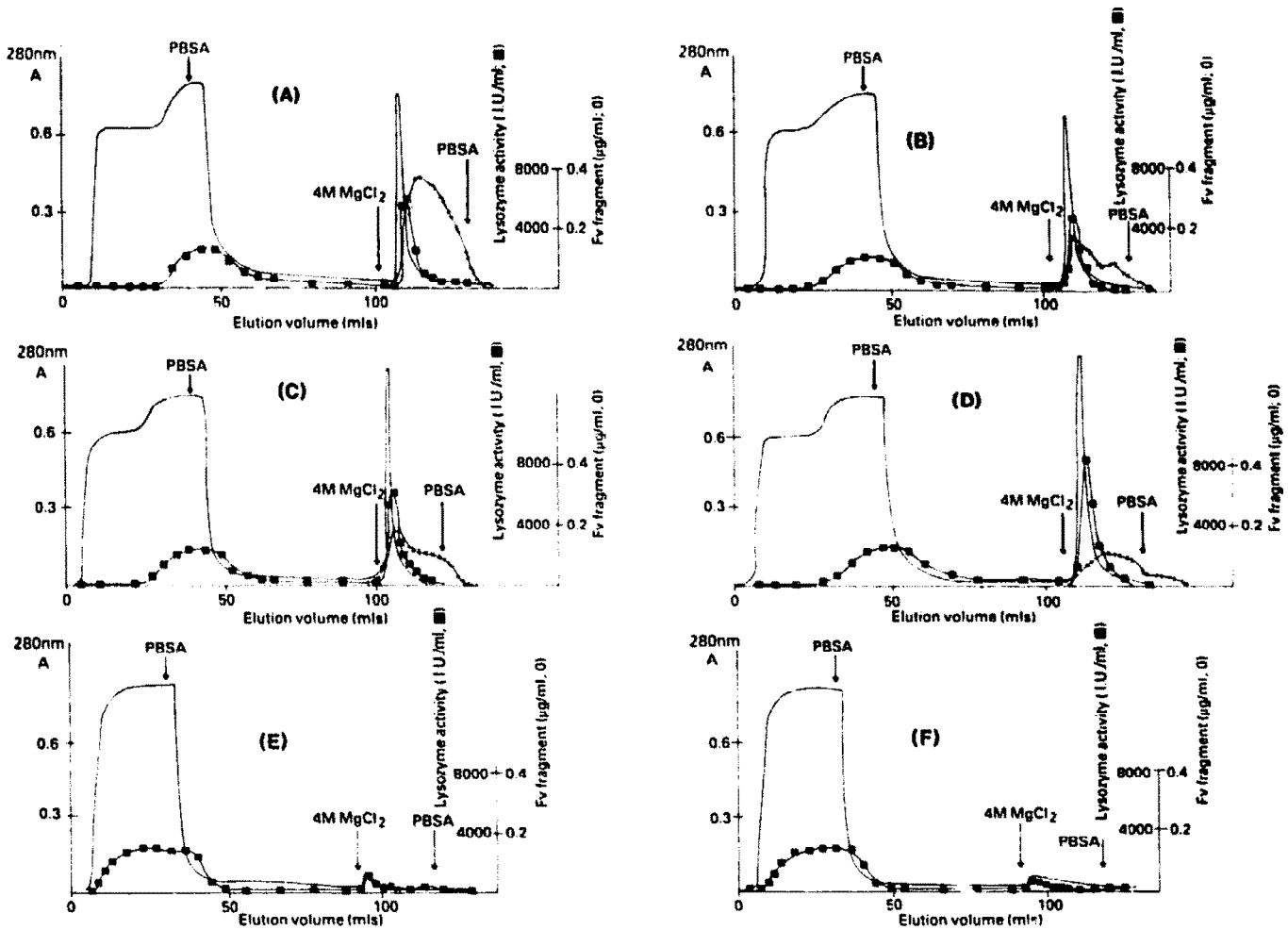

Fig. 1. Changes in immunoadsorbents' performance after repeated recovery of lysozyme from a 20-fold excess of albumin: comparison of chromatograms. (A) Fv Sepharose after 1 cycle. (B) Fv Sepharose after 100 cycles. (C) scFv Sepharose after 1 cycle (D) scFv Sepharose after 100 cycles. (E) "Blank" Sepharose (*i.e.*, no immunoligand coupled) after 1 cycle. (F) Blank Sepharose after 100 cycles. Absorbance at 280 nm (——) was measured on line; (■) lysozyme activity and (○) ligand (*i.e.*, Fv or scFv) leakage were monitored by assaying fractions.

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).

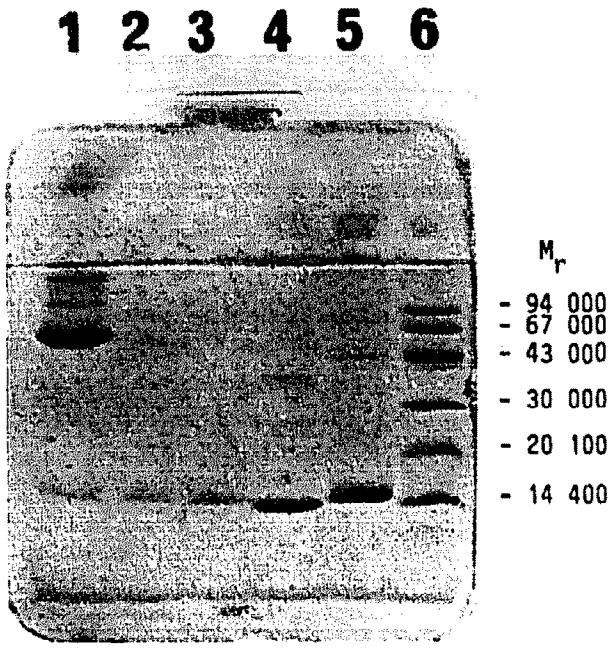

Fig. 2. SDS-PAGE analysis of separation achieved with Fv Sepharose immunoadsorbent. Lanes: 1 = albumin lysozyme feedstock; 2 = lysozyme recovered after 100 cycles; 3 = lysozyme recovered after 1 cycle; 4 = Fv*myc* anti-lysozyme marker; 5 = lysozyme marker; 6 = Pharmacia low-molecular-weight standards (94 000, 67 000, 43 000, 30 000, 20 100, 14 400 dalton). (Fv*myc* anti-lysozyme migrates to a single band due to the near identical molecular weights of its two component chains, V_H and V_L *myc*. The presence of both V_H and V_L*myc* was confirmed by Western blot analysis with V_H-specific and *myc*-specific immunoreagents. Furthermore, *myc* can be selectively removed from Fv*myc* by protease-activitiy 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* $MgCl_2$. Ligand leakage was highest as the $MgCl_2$ front passed through the immunoadsorbents and continued at measurable levels while $MgCl_2$ was being eluted. Ligand leakage returned to baseline when $MgCl_2$ 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 μg/ml (for cycle 1 of the immunoadsorbent comprising Fv, see Fig. 1A). The lysozyme peak fraction from this cycle contained lysozyme at approximately 100 μg/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 μg/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 × 2550 I.U. ml = 61 000 I.U.). The amount of lysozyme which eluted with 4 *M* $MgCl_2$ 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.

Ligand	Cycle	Lysozyme bound	Lysozyme eluted	Recovery efficiency (%)
Fv	1	61	37	61
Fv	100	46	27	59
scFv	1	51	39	76
scFv	100	64	49	76

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 V_H and V_L 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 $10^{10}/M$) for association of its two component polypeptide chains, V_H and V_L [25]. This is significantly higher than for many other Fv fragments [25]. Secondly, the major mechanism for V_H and V_L 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* $MgCl_2$) 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 V_H and V_L.

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* $MgCl_2$ 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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