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Comparative study of thiophilic functionalised matrices for polyclonal F(ab′)$_2$ purification

Peter Kumpalume, Nigel K.H. Slater*

Department of Chemical Engineering, University of Cambridge, Pembroke Street, Cambridge CB2 3RA, UK

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Abstract

Thiophilic adsorbents have been developed using divinyl sulfone or epoxy activated Streamline™ quartz base matrix. Their capacity and selectivity for binding polyclonal F(ab′)$_2$ fragments generated by whole serum proteolysis was tested. Except for epoxy activated guanidine, all the adsorbents displayed high selectivity for F(ab′)$_2$ with dynamic binding capacities ranging from 3 to 10 mg/ml of adsorbent. Thiol immobilised ligands adsorbed more F(ab′)$_2$ and the recovery was equal to or more than that from amino immobilised ligands. All adsorbents showed good selectivity for IgG and the dynamic binding capacities were better than for F(ab′)$_2$.

Keywords: Stationary phases, CC; Immunoglobulin G; Polyclonal F(ab′)$_2$; Pyrimidine; Pyridine; Antibodies; Proteins

1. Introduction

Polyclonal F(ab′)$_2$'s are of current interest for use as antitoxins. Toxins have been traditionally treated with intact IgG's raised against a specific antigen. Consequently, purification methods have concentrated on producing an efficient adsorbent for IgG purification. However, IgG digestion products retain the antitoxic activity of IgG and it has long been recognised that they could be used for treating toxins [1]. Later Pope [2] showed that when sera are treated by proteases the product obtained is purer than that obtained using ammonium sulphate precipitation. Nevertheless, this did not trigger commercial production nor widespread use of enzyme treated sera. Two factors were advanced for this; the lack of control factors for the digestion, since uncontrolled digestion of sera leads to total digestion of the IgG molecule and hence loss of activity and the absence of a simple method for the estimation of antibody activity. In 1939, Pope [2] described a pepsin method for a controlled digestion of immune sera and this was followed by methods that could be used for the purification of the digest [3–5].

The digestion of IgG by pepsin produces F(ab′)$_2$ fragments with a molecular mass of about 100 kDa, together with smaller Fc fragments. In controlled digestion, antibody activity is retained in the F(ab′)$_2$ fragment. Since it is smaller than IgG, the therapeutic implications of using F(ab′)$_2$ fragments to treat toxins were immense since these molecules diffuse faster and hence can reduce death rates from snake envenomations significantly. The Fc fragment of IgG causes side effects hence must be removed before administration to patients.

Efficient separation of the Fc fragments from the active F(ab′)$_2$ fragments has remained a challenge. Earlier methods involved ammonium sulphate fractionation, heat treatment and combinations of the two [2–4]. It was observed that protein contaminants and Fc fragments could be eliminated by digesting the sera followed by heating, leaving the F(ab′)$_2$ fragments in solution. It has been claimed that ammonium sulphate fractionation, the traditional method for antitoxin production is time consuming, costly and produces a mixture of F(ab′)$_2$, and uncleaved IgG [6]. Against this background, a method was developed for IgG purification using caprylic acid [5]. In a comparative study of IgG purification using caprylic acid and ammonium sulphate fractionation [7], it was found that the caprylic acid method was better in terms of production times (8 days versus 15 days), yield (60% versus 49%), albumin contaminants (0.17 g/dl versus 0.53 g/dl) and turbidity (0.02 OD600 versus 0.03 OD600). Despite such advantages, there were no reports of the commercial use of caprylic acid to purify Fab or F(ab′)$_2$ until

* Corresponding author. Tel.: +44-1223-762953; fax: +44-1223-334796.
E-mail address: nigel_slater@cheng.cam.ac.uk (N.K.H. Slater).

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1994 [6]. In this method, the digest was heated to 56 °C for 15 min and centrifuged to remove fibrinogen. Caprylic acid was then added drop wise to a final concentration of 8.7% (v/v) with vigorous stirring for 30 min. This was followed by centrifugation and the filtrate was dialysed with buffer. When analysed by SDS-PAGE, the product ($F(ab')_2$) was homogeneous. Thus, this single step process was considered better than the traditional ammonium sulphate fractionation.

Chromatography has remained the most commonly employed method for the purification of IgG digests. Morimoto and Inouye [8], and Inouye and Morimoto [9] used hydrophobic adsorbents to purify $F(ab')_2$ fragments. In both cases it was shown by both SDS-PAGE and gel filtration that $F(ab')_2$ had been purified to homogeneity in a single step. Affinity purification of $F(ab')_2$ has remained relatively unchanged over the years. Proteins G and A are both unsuitable for $F(ab')_2$ purification as they bind to the Fc section of IgG. A common approach is to use the target antigen or a close chemical homologue of the antigen as a ligand. For antitoxins, the antigenic material is usually a complex protein mixture that is available in only milligram quantities. Anion exchange adsorbents are mainly used to bind contaminants whilst the $F(ab')_2$ passes through the column. Cation exchange matrices have been used rarely in the purification of $F(ab')_2$ although their potential had been shown [10]. Recently the authors have shown that cation exchange adsorbents may be used as viable alternatives in the manufacture of antitoxins [11].

Despite the many references available on the purification of IgG using thiophilic adsorbents, there are relatively few reports of the use of such adsorbents for the purification of $F(ab')_2$. Apart from the work by Yurov et al. [12] and ourselves [11] we are not aware of any other work on $F(ab')_2$ purification by thiophilic adsorbents. Of particular significance are the thiophilic adsorbents as they are highly selective for antibodies. They are also significantly cheaper than affinity adsorbents. These adsorbents meet our goal of developing a cheap but efficient adsorbent that can be incorporated in the manufacture of antitoxins.

The generic molecular structure of thiophilic adsorbents is $–SO_2–CH_2–CH_2–X–R$ [13] where X is an atom with a lone pair of electrons. Less electronegative atoms display greater thiophilicity than more electronegative ones (i.e. S > N ≫ O) [14,15]. It has been suggested that the interaction between the adsorbent and antibody is by electron donation–electron acceptance (from the lone pair on the ligand to the protein, and from the protein to the ligand through the d-orbitals of the S atom of divinyl sulfone) [14]. It has also been suggested that R must be a fully aromatic ring, a less aromatic ring or an aliphatic ring with at least two carbon atoms and no hydroxyl and amino groups attached to it [13]. Further work also showed that if R is a pyridine ring, the adsorbent is more selective for IgG than if R is a phenyl group [16]. Since thiophilic ligands adsorb both IgG and $F(ab')_2$ [11,12] it implies that the binding sites are in the Fab region. It is therefore expected that ligands that have pyridine or pyrimidine rings would show similar selectivity for $F(ab')_2$ as for IgG. On this basis, a range of thiophilic adsorbents were constructed that possess the ligands indicated in Fig. 1 and the adsorption and purification of $F(ab')_2$ fragments by this range of novel adsorbents was studied. Although some ligands such as 4-aminopyrimidine and 4-thiopyrimidine are readily available, they are very expensive and hence were not included in the experimental trials.

2. Materials and methods

All the ligands and reagents were purchased from Sigma–Aldrich, UK. Streamline™ quartz base matrix, a dense agarose coated bead made specifically for expanded bed chromatography, was purchased from Amersham Biosciences, UK (catalogue no: 17-5104-01). Whole serum from fluorescein hyper-immune sheep was provided by MicroPharm Ltd. (Newcastle, Emlyn, UK). All chromatographic experiments were conducted on an AKTA Explorer 100 supplied by Amersham Biosciences.

2.1. Preparation of adsorbents

The Streamline™ matrix was activated by the method of Porath [17] using both 1,4-butanediol diglycidyl ether (epoxy) and divinyl sulfone (hereafter, DVS). Typically, 10 ml of settled volume was washed exhaustively with water followed by 0.5 M Na_2CO_3, pH 11, and vacuum filtered. This was then re-suspended in 10 ml of 0.5 M Na_2CO_3. Five milliliters of divinyl sulfone was added and the reaction was allowed to proceed for about 20 h. Washing with water stopped the reaction.

Coupling of the ligands was based on a method by Oscarsson and Porath [18]. For amino-ligands (ligands immobilised via the amino group) the activated matrix was re-suspended in 20 ml of 0.1 M sodium phosphate, pH 7.0 containing 3 g of the amino-ligand. The mixture was left in an incubator at room temperature for 24 h after which it was washed exhaustively with water, ethanol and water again.

For thiol-ligands (ligands immobilised via the thiol group) 1.0 g of the ligand was dissolved in 40 ml of 0.1 M sodium phosphate buffer, pH 7.5. 1.5 g of $NaBH_4$ was added and the pH was adjusted to 6.8. N_2 was bubbled through for 10 min after which 10 g of the activated matrix was added. The tubes were sealed with Para film and transferred to an incubator. Reaction time was 3 h at room temperature. The beads were then washed exhaustively with water, ethanol and water.

For the guanidine ligand 20 ml of 0.1 M $NaHCO_3$ pH 8.5 containing 0.1 M guanidine hydrochloride was added to 10 g of beads. Reaction time was 24 h in an incubator shaker.

2.2. Whole serum digestion

Whole serum was diluted to twice its original volume with distilled water. The pH was then adjusted to 3.5 with

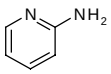
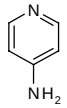
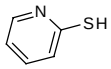
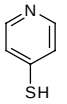
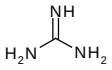
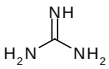
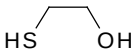
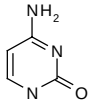
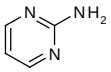
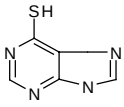
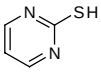
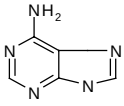
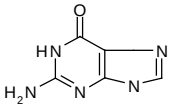
Ligand number	Ligand	Ligand name
(I)		2-aminopyridine
(II)		4-aminopyridine
(III)		2-thiopyridine
(IV)		4-thiopyridine
(V)		DVS Guanidine (activated with DVS)
(VI)		Epoxy Guanidine (activated with epoxy groups)
(VII)		2-mercaptoethanol (Scipac)
(VIII)		Cytosine
(IX)		2-aminopyrimidine
(X)		6-thiopurine
(XI)		2-thiopyrimidine
(XII)		Adenine (6-aminopurine)
(XIII)		Guanine

Fig. 1. A list of the adsorbents prepared and used in this study. All ligands were prepared using divinyl sulfone except for epoxy activated guanidine which was prepared using 1,4-butanediol diglycidyl ether.

1 M HCl and pepsin was added to a pepsin to total serum protein ratio (w/w) of 1:50. Samples were digested at 37 °C in an incubator shaker for typically 12 h, after which digestion was halted by raising the pH to 7.0 with saturated Tris base. The digest was assayed for fluorescein binding and by SDS-PAGE under non-reducing conditions.

2.3. Chromatography on AKTA explorer 100

Binding studies were conducted on an AKTA Explorer 100, an FPLC system capable of delivering up to 100 ml/min at a maximum pressure of 10 MPa. Two millilitres of each of the adsorbents were pack packed into a Pharmacia

C10/10 column with an adaptor. The adsorbents were then equilibrated with 10 ml of 10 mM sodium phosphate, pH 7 containing 1.2 M ammonium sulphate (buffer A). Twenty milliliters of serum digest at pH 7 containing 1.2 M ammonium sulphate was loaded at a flow rate of 1.0 ml/min. The column was then washed with 20 ml of buffer A. Elution was conducted with distilled water at a flow rate of 1.0 ml/min. Two milliliters fractions were collected during elution and the fractions containing the eluate were later pooled. For $F(ab')_2$ containing samples duplicate binding and elution data were obtained for adsorbents I, VI, VII and VIII and single binding and elution data for the remainder. For IgG containing samples duplicate binding and elution data were obtained for adsorbents IV, VI, VII and VIII and single binding and elution data for the remainder. Analysis of Variance (ANOVA) was conducted for each of the four complete sets of data.

2.4. Gel filtration

Analytical gel filtration chromatography was conducted using a Superose 12 10/30 HR column (Amersham Biosciences, UK). This high-resolution column separates proteins in the range 1–300 kDa on the basis of mass. The running elution buffer was 50 mM sodium phosphate, pH 7 containing 0.15 M NaCl to prevent ionic interactions. Flow rate was 0.5 ml/min. Highly concentrated samples were diluted before loading. Sample volume was 0.2 ml for $F(ab')_2$ samples and 0.1 ml for IgG samples.

2.5. Fluorescein binding and total protein assays

The concentration of fluorescein binding IgG or $F(ab')_2$ was determined by a fluorescence titration assay. Samples were diluted serially by a factor of 2 in test tubes, the final volume of the samples being 1.0 ml. 0.5 ml of 1000 nM fluorescein was added to the serially diluted samples, mixed and then incubated for 30 min at room temperature. The fluorescence from each sample was then measured on a Perkin-Elmer LS 50 fluorimeter (480 nm exc., 520 nm em.). Baseline fluorescence intensity was obtained from undiluted samples when maximum fluorescein binding led to quenching of the fluorescence signal. At high serum dilution little fluorescein binding occurred and a maximum fluorescence signal was observed. Thus, fluorescence intensity curves were obtained for each sample over a range of dilutions. The concentration of IgG or $F(ab')_2$ was determined at 50% fluorescence quenching when half of the fluorescein was bound. Total protein was determined by Bradford's method according to the suppliers (Sigma–Aldrich, UK) standard protocol.

2.6. Activation efficiency

This was performed to determine the number of moles of divinyl sulfone groups attached to the agarose on the functionalised Streamline^{TM} matrix. The method of Scoble and Scopes [19] was employed. Typically 1.0 g (wet weight i.e. vacuum filtered) of divinyl sulfone activated Streamline^{TM} matrix was washed exhaustively with distilled water. Two milliliters or 4.0 ml of 0.8 M mercaptoaectic acid in 0.1 M sodium carbonate pH 10.5 was added and the reaction was allowed to proceed for either 2 or 4 h. After this time, the beads were washed exhaustively with distilled water followed by 5.0 ml of 0.1 M HCl to protonate the acidic groups, then again by distilled water to remove the HCl. Five milliliters of 0.1 M NaCl was then added to the beads followed by titration with 0.01 M Tris base. Both the volume of Tris base added and the corresponding pH were recorded during the titration. Four different conditions were employed; 2 ml wet gel for 2 and 4 h, and 4 ml of gel for 2 and 4 h.

3. Results and discussion

3.1. Activation and ligand density

The vinyl groups on the divinyl sulfone activated Streamline^{TM} matrix readily react with nucleophiles. This reaction is likely to go to completion with small nucleophiles. For this reason mercaptoacetic acid was used. The stoichiometry is one to one and so is the reaction between the acid groups on the mercaptoacetic acid with a base such as Tris and hence the number of divinyl sulfone groups attached to the matrix can be estimated. The end point of this titration was at 28 μmol of Tris and hence the concentration of divinyl sulfone groups attached to the Streamline^{TM} agarose was 28 μmol/g of wet beads. This is equivalent to 200 μmol/g of dry beads.

3.2. Binding experiments with the adsorbents

An ideal adsorbent must have a significant binding capacity and selectivity for the target product as well as permitting the efficient elution of the product. This ensures that recovery is high and hence decreases production costs. Two variables were therefore used to compare the different thiophilic adsorbents; percent bound (the percent of total protein fed to the adsorbent that was bound) and percent eluted (percent of the bound protein that was eluted). During sample loading, the columns were deliberately overloaded so that the adsorbent would be saturated. The binding results for $F(ab')_2$ are summarised in Fig. 2.

There was a need to establish a threshold value to determine which adsorbents were practically viable. Since the columns were overloaded, a 20% adsorption and a 20% recovery of the bound protein were chosen. It can be seen from Fig. 2 that all of the ligands meet this requirement except for DVS activated guanidine, which binds more than 20% of the feed but elutes less than 20% of the bound protein. However, there are some variations in both the binding and elution results within the ligands. For

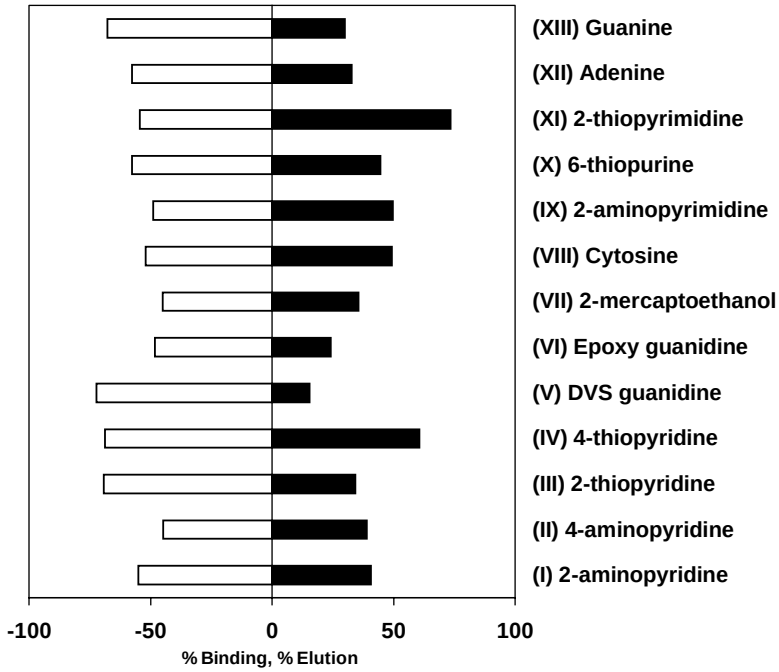


Fig. 2. $F(ab')_2$ purification on various adsorbents. Two milliliters of adsorbent were packed in a C10/10 column. After equilibration with 10 ml of 10 mM sodium phosphate containing 1.2 M ammonium sulphate, pH 7, 20 ml of serum digest was loaded. This was followed by washing with 20 ml of the equilibration buffer. Elution was done with 10 ml of distilled water. The flow-through and eluates were assayed for their protein concentration from which Fig. 2 was constructed. %Binding (□) denotes the percent of serum protein fed to the column that binds to the adsorbent. %Elution (■) denotes the percent of the bound serum protein that is recovered in the eluate. Flow rate was 1.0 ml/min. ANOVA indicates that binding and elution data are statistically different ($P < 0.05$) with a testing standard deviation of 4.4% for binding and 2.2% for elution.

those that are pyridine based, the thiol-ligands are better adsorbers than the amino-ligands (i.e. I and II compared with III and IV). Of these four adsorbents, 4-thiopyridine shows a much higher recovery than the rest. For those ligands that are pyrimidine based (VIII–XIII), there are some differences in both the binding and elution behaviour. Comparing 6-thiopurine and 6-aminopurine (adenine), both bind similar amounts of protein but recovery is better for 6-thiopurine. For 2-thiopyrimidine and 2-aminopyrimidine, it can be seen that binding is also similar (slightly better in the 2-thiopyrimidine) but recovery is significantly better in 2-thiopyrimidine. Thus, comparing derivatives with either amine or thiol groups on the same ring position (i.e. I compared with III, II compared with IV, X compared with XII and, IX compared with XI), it is observed that (a) thiol-ligands bind more than or similar amounts of protein to amino-ligands; (b) thiol-ligands have a better recovery than amino-ligands.

Comparing 2-aminopyridine with 2-aminopyrimidine (I compared with IX), and 2-thiopyridine with 2-thiopyrimidine (III compared with XI), shows that binding is slightly better in the pyridine ligands but recovery is better in the pyrimidine ligands than the pyridine based ligands. Thus, the more aromatic ligands bind $F(ab')_2$ more strongly than the less aromatic ones. The addition of the imidazole ring to cytosine (VII) to produce guanine (XIII) improved the binding but decreased the recovery.

3.3. Selectivity of the adsorbents

In the binding studies above, the discussion was based on the total protein adsorbed or eluted. This is because the feed to the adsorbents contained the target molecule ($F(ab')_2$), Fc digests and possibly other proteins that were not digested by pepsin. Analytical gel filtration chromatography was used to determine the purity of the eluted $F(ab')_2$ for each of the adsorbents. The results are given in Fig. 3, in which the gel filtration chromatograms of samples eluted from each of the adsorbents in Fig. 1 are shown. On curve XIV (the feed), three peaks of interest have been labelled as protein aggregate, $F(ab')_2$ and prominent Fc digest. The selectivity of these adsorbents for $F(ab')_2$ is rated in terms of the removal of both the protein aggregate and the Fc fragments. Fig. 3 shows that all of the adsorbents bind some of the molecules eluting in the Fc region albeit to different degrees. This molecule could be an Fc fragment or an undigested whole serum molecule as discussed in the IgG results. The least selective adsorbent is the epoxy activated guanidine agarose since it binds significant amounts of both the protein aggregate and the Fc fragments. Guanine, adenine, 6-thiopurine, cytosine, DVS activated guanidine and 4-thiopyridine bind negligible amounts of the protein aggregate hence are very selective. The other adsorbents, with the exception of epoxy activated guanidine, bind some protein aggregates. However, more than 90% of the total

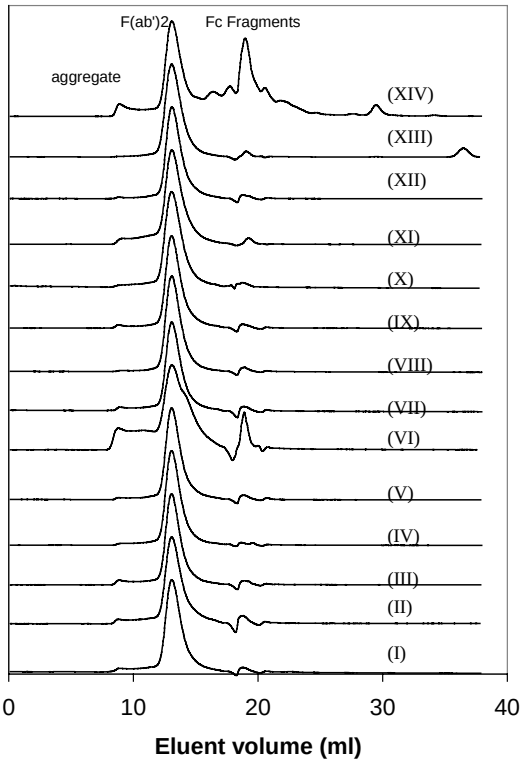


Fig. 3. Analytical gel filtration chromatograms of $F(ab')_2$ samples eluted from the adsorbents shown in Fig. 1, obtained using a Superose HR 10/30 column. Sample volume was 200 μl, eluent was 0.1 M sodium phosphate, pH 7.0 containing 0.15 M sodium chloride at a flow rate of 0.5 ml/min. Baselines of the chromatograms are offset vertically for presentational clarity. Curve XIV is the serum digest.

protein eluted by these adsorbents is $F(ab')_2$. There is no consistent molecular feature among the adsorbents that are highly selective for $F(ab')_2$. This makes it difficult to predict ligands that are likely to have a high selectivity for $F(ab')_2$.

The feed, flowthrough and the eluates from all the adsorbents were also analysed by SDS-PAGE. Fig. 4 shows an SDS-PAGE gel for 2-thiopyrimidine, and the results for other ligands were similar. As in Fig. 3, the $F(ab')_2$ eluate (lane 4) is free of gross contamination except for the low molecular mass Fc fragment contamination that eluted with 19 ml of eluent buffer in the analytical gel filtration chromatogram shown in Fig. 3 (curve XI). Thus, the SDS-PAGE results agree with the gel filtration results. In practice this low molecular weight contaminant is easily removed by diafiltration using a 50 000 NMWCO membrane. There was little differences in the specific activities of the eluted $F(ab')_2$ samples from different ligands.

3.4. Comparison with IgG purification

Apart from the other immunoglobulin classes (IgA, IgD, IgE and IgM), whole serum contains lipids, albumin, transferin, α-macroglobulin and other serum proteins as significant contaminants. In contrast, pepsin-digested serum contains $F(ab')_2$ and Fc fragments. Thus, the challenges faced in purifying IgG are different from those faced in purifying $F(ab')_2$.

The same adsorbents were loaded with whole serum in similar experimental conditions as those applied for $F(ab')_2$ purification with the exception that the volume of whole serum loaded was 10 ml. Fig. 5 is a summary of the protein binding studies and Fig. 6 shows the gel filtration results for each of the adsorbents.

As was the case with $F(ab')_2$, 20% binding and 20% elution was taken as a threshold value. It can be seen from Fig. 5 that three of the adsorbents, i.e. 6-thiopurine, DVS activated guanidine and guanine elute less than 20% of the bound protein and hence fail this requirement. Cytosine elutes more than 20% of bound protein but binds less

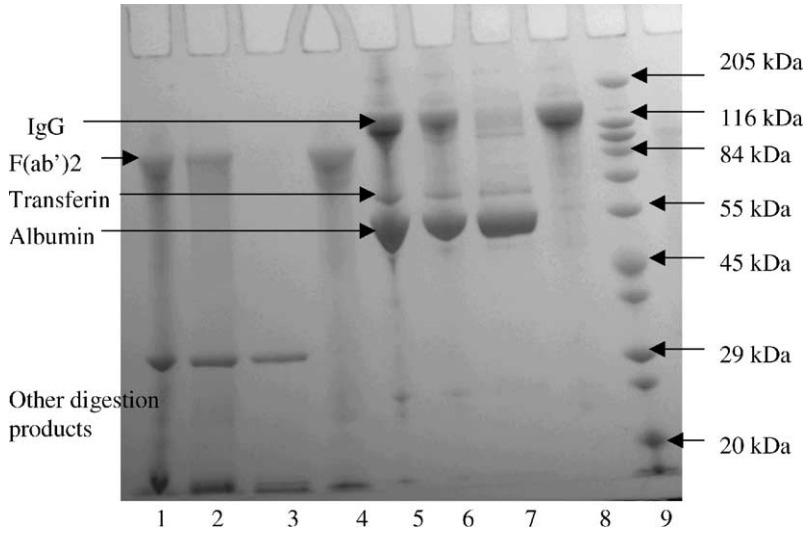


Fig. 4. SDS-PAGE of $F(ab')_2$ and IgG samples purified on 2-thiopyrimidine. The samples were run on a 10% NuPage gels commercially available from Invitrogen. Lanes 1 and 2: serum digest, lane 3: serum digest flowthrough, lane 4: serum digest eluate, lanes 5 and 6: whole serum, lane 7: whole serum flowthrough, lane 8: IgG eluate, lane 9: molecular weight marker (Sigma catalogue number M4038).

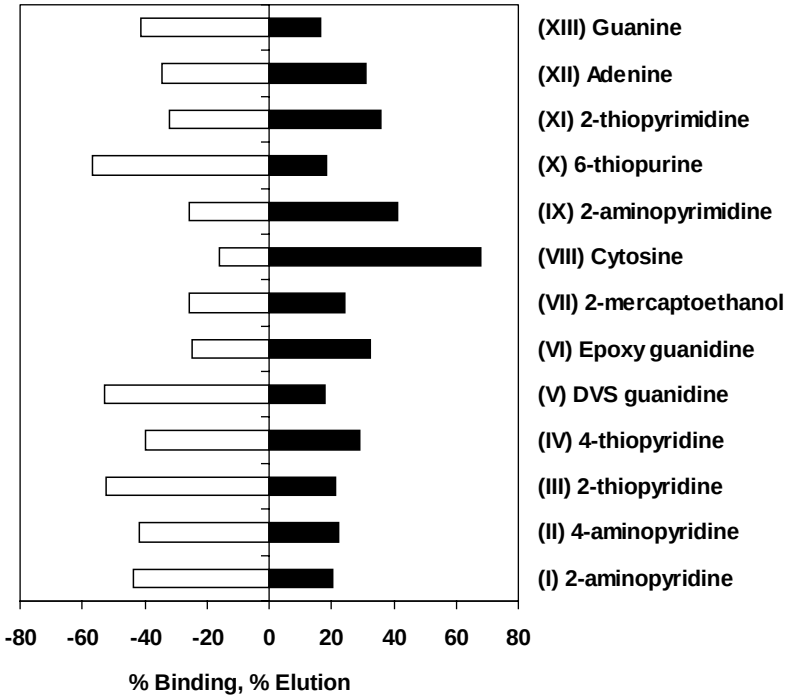


Fig. 5. IgG purification on various adsorbents. Two milliliters of adsorbent were packed in a C10/10 column. After equilibration with 10 ml of 10 mM sodium phosphate containing 1.2 M ammonium sulphate, pH 7, 10 ml of whole serum were loaded. This was followed by washing with 20 ml of the equilibration buffer. Elution was done with 10 ml of distilled water. The flowthrough and eluates were assayed for their protein concentration from which Fig. 5 was constructed. %Binding (□) denotes the percent of serum protein fed to the column that binds to the adsorbent. %Elution (■) denotes the percent of the bound serum protein that is recovered in the eluate. Flow rate was 1.0 ml/min. ANOVA indicates that binding and elution data are statistically different ($P < 0.05$) with a testing standard deviation of 1.4% for binding and 3.7% for elution.

than 20% of the load and hence also fails this requirement. Comparing 2-aminopyridine with 4-aminopyridine (I compared with II) shows that both binding and recovery are similar. A similar comparison between 2-thiopyridine and 4-thiopyridine shows that binding is better in 2-thiopyridine but recovery is better in 4-thiopyridine. Unlike the $F(ab')_2$ purification results, comparison of binding and recovery for the four pyridine ligands (I and II compared with III and IV) shows that the thiopyridines are better than or similar to the aminopyridines. In the pyrimidine ligands however (IX compared with XI), 2-thiopyrimidine has a slightly better binding but recovery is slightly better in 2-aminopyrimidine. Unlike the $F(ab')_2$ results, binding is better in 6-thiopurine but recovery is less than 6-aminopurine (adenine). Comparison of pyridine based and pyrimidine based ligands (I and III compared with IX and XI, respectively) shows a similar pattern as for $F(ab')_2$ results. Binding is better in the pyridine ligands whilst recovery is better in the pyrimidine based ligands. It was noted though that only 2-thiopyrimidine and 4-thiopyridine eluted as much $F(ab')_2$ as IgG. The rest of the adsorbents eluted more IgG than $F(ab')_2$.

In general, all adsorbents bind more IgG than $F(ab')_2$ as can be seen in Table 1, suggesting that the Fc region may play some role in the interaction between the antibody and the ligands. However, the molar concentration ratios (molar concentration IgG/molar concentration $F(ab')_2$) obtained from the elution results is nearly 1:1. This implies that favourable interaction is between the Fab region and the ligand as this kind of interaction is reversible.

3.5. Selectivity for IgG

The gel filtration results for IgG eluted samples are shown in Fig. 6. In curve XIV (feed), there are three noticeable peaks. These have been labelled as protein aggregate (could also be IgM), IgG and albumin. From this figure, the product from all of the adsorbents is free of albumin. This is characteristic of thiophilic adsorbents with pyridine or pyrimidine rings [16]. It can also be seen that some of the protein aggregates are retained by the adsorbents. There are two possibilities for this. It could be that the adsorbents are indeed binding the aggregate from the feed, which could happen if such aggregates have some hydrophilic characteristics. Alternatively, the aggregation might occur after the elution. Irrespective of mechanism, the aggregates are observed in both the serum digest and whole serum samples (curve XIV of Figs. 3 and 6). In both cases the aggregates elute with 8 ml of eluent (Figs. 3 and 6) and hence are of similar size. However, others have argued that aggregates occur at high pH. At low pH (pH < 6.0) this peak disappears, but was observed in gel filtration chromatograms run at pH 6.0, 7.0 and 8.0, indicating that their formation is indeed pH dependent [20]. This could be the case in this paper since the samples were run at neutral pH. In addition to the main peaks

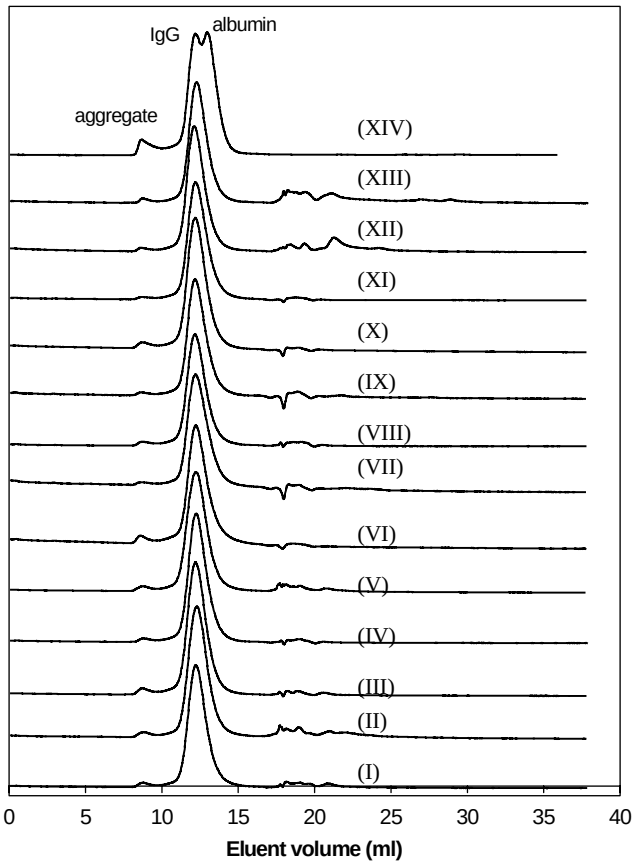


Fig. 6. Analytical gel filtration chromatograms of IgG samples eluted from the adsorbents shown in Fig. 1, obtained using a Superose HR 10/30 column. Sample volume was 200 μl, eluent was 0.1 M sodium phosphate, pH 7.0 containing 0.15 M sodium chloride at a flow rate of 0.5 ml/min. Baselines of the chromatograms are offset vertically for presentational clarity. Curve XIV is whole serum.

of whole serum, the eluates of some adsorbents, e.g. adenine and guanine (XII and XIII, respectively) show some proteins eluting at about 19 ml in Fig. 6. However, in curve XIV (whole serum) of Fig. 6, these proteins are not detected. Thus, these proteins may have been concentrated during the purification step. As mentioned in the $F(ab')_2$ section, there is a molecule eluting at 19 ml (as in the IgG results). It was suggested that this molecule in curves I–XIII of Fig. 3, could be an Fc fragment or an undigested protein. Since this molecule is found in both digested and undigested samples, it is more likely that it is a whole serum protein and that it is resistant to the proteolytic activity of pepsin. It is also hydrophilic as it binds to these thiophilic adsorbents.

The feed, flowthrough and the eluates were also analysed by SDS-PAGE. The results for one of the ligands, 2-thiopyrimidine, are shown in Fig. 4. The selectivity of this ligand for IgG needs no emphasis. Of the three dominant proteins (IgG, transferin and albumin), only IgG is detected in the eluate (lane 8, compare with Fig. 6, curve XI) leaving the albumin and transferin in the flowthrough (lane 7). Once again the SDS-PAGE results agree with the gel filtration results. The purification factor (specific activity of eluate/specific activity of feed) for this ligand was about 2.0. Although this may appear low, the SDS-PAGE results (lane 8 of Fig. 4) shows that the eluate is homogeneous. This low purification factor is due to the fact that IgG constitutes a large percentage of the total protein content.

3.6. The ideal adsorbent for $F(ab')_2$ or IgG purification

The choice of the ideal adsorbent for purification depends upon the intended use of the product. If, for example the purity of the end product were not important then the adsorbents with high binding capacities would be satisfactory.

Table 1
Showing dynamic capacities of the adsorbents inferred from elution data for both IgG and $F(ab')_2$

	$F(ab)_2$		IgG		Molar capacity ratio (μmol IgG/μmol $F(ab')_2$)
	Dynamic capacity (mg $F(ab')_2$/ml of bed)	Molar capacity (μmol $F(ab')_2$/ml of bed)	Dynamic capacity (mg IgG/ml of bed)	Molar capacity (μmol IgG/ml of bed)	
(I) 2-Aminopyridine	5.1	51.3	8.1	50.5	1.0
(II) 4-Aminopyridine	4.0	40.0	8.4	52.6	1.3
(III) 2-Thiopyridine	5.4	54.5	10.0	62.7	1.2
(IV) 4-Thiopyridine	9.6	95.6	10.6	66.1	0.7
(V) DVS guanidine	2.6	25.7	8.7	54.5	2.1
(VI) Epoxy guanidine	2.7	26.5	7.3	45.8	1.7
(VII) 2-Mercaptoethanol	3.7	36.8	5.6	35.3	1.0
(VIII) Cytosine	5.9	58.9	10.0	62.3	1.1
(IX) 2-Aminopyrimidine	5.6	55.9	9.7	60.4	1.1
(X) 6-Thiopurine	5.9	58.9	9.4	59.0	1.0
(XI) 2-Thiopyrimidine	9.2	91.7	10.4	65.0	0.7
(XII) Adenine	4.3	43.2	9.6	59.9	1.4
(XIII) Guanine	4.7	46.7	6.3	39.2	0.8

Twenty millilitres of serum digest or 10 ml of whole serum solutions containing 1.2 M ammonium sulphate were loaded on to 2.0 ml of the adsorbents at a flow rate of 1.0 ml/min. Unbound molecules were washed with 20 ml of 10 mM sodium phosphate pH 7.0 containing 1.2 M ammonium sulphate. Samples were eluted with 10 ml of distilled water. The eluates were assayed for their protein content from which Table 1 was compiled.

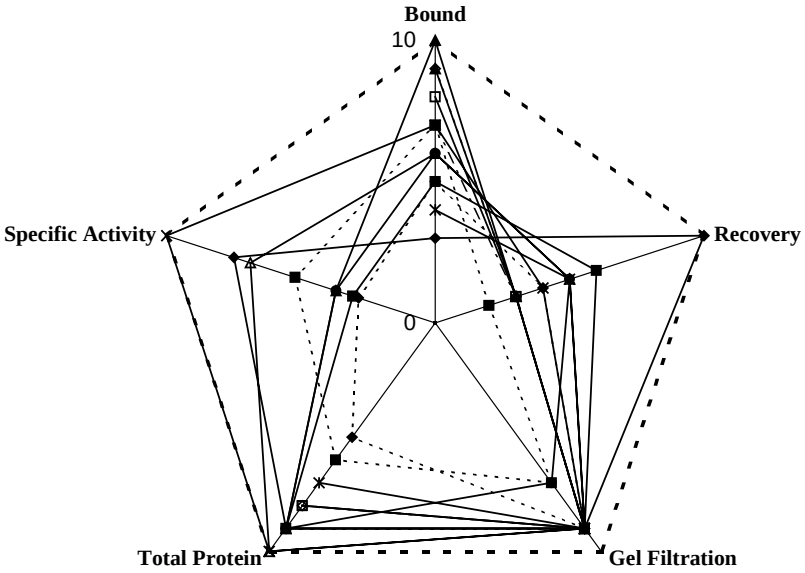


Fig. 7. A summary of the efficiency of the adsorbents on $F(ab')_2$ purification. Data from the binding, elution, gel filtration and fluorescein assays were normalised on a scale of 1–10 from which Fig. 7 was plotted. "Recovery" refers to the percentage of total protein bound that was recovered in the eluate. "Bound" refers to the percentage of total protein in the feed that was adsorbed by the column. "Total Protein" refers to the mass of total protein recovered in the process.

For polyclonal $F(ab')_2$ and IgG production though, purity is commonly more important than capacity. On the basis of these priorities, the adsorbents have been rated on a scale of 1–10 (where 10 is the best) on the basis of (1) their total protein binding capacities, (2) the total protein recovery, (3) the purity of eluted product (as measured by analytical gel filtration), (4) total product eluted and (5) specific activity, using data from the fluorescein binding assay. Figs. 7 and 8 are a visual representation of these ratings for $F(ab')_2$ and IgG, respectively. The adsorbents that are more efficient map the periphery of the plots and, as can be seen from both Figs. 7 and 8 such adsorbents have essentially good binding capacities, good yield and good selectivity for $F(ab')_2$. Comparing Fig. 7 with Fig. 8, it is noted that four of the more efficient adsorbents, i.e. cytosine, 2-thiopyrimidine, 4-thiopyridine and 2-aminopyrimidine are common to both. Two of the least efficient adsorbents namely guanine and divinyl sulfone activated guanidine are common to both Figs. 7 and 8.

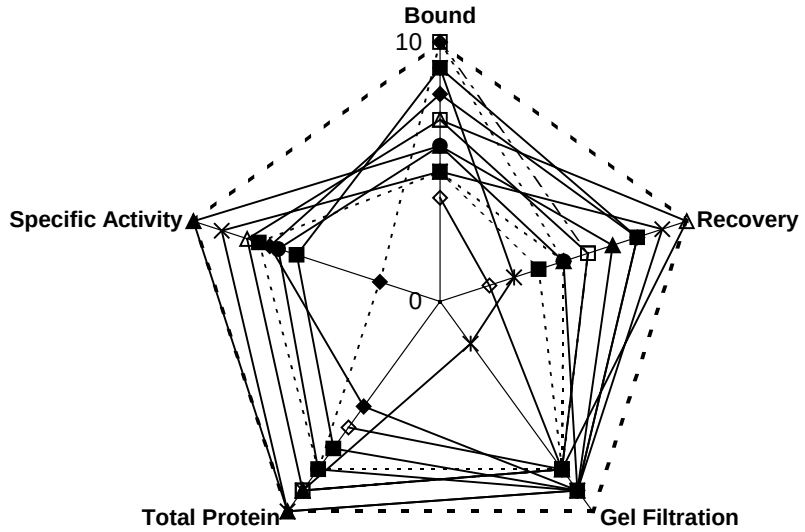


Fig. 8. A summary of the efficiency of the adsorbents on IgG purification. Data from the binding, elution, gel filtration and fluorescein assays were normalised on a scale of 1–10 from which Fig. 8 was plotted. "Recovery" refers to the percentage of total protein bound that was recovered in the eluate. "Bound" refers to the percentage of total protein in the feed that was adsorbed by the column. "Total Protein" refers to the mass of total protein recovered in the process.

Some of the ligands are highly soluble whilst others are sparingly soluble. All of the DNA base pairs and all ligands based on pyrimidine are sparingly soluble in water. As such saturated solutions were used for the coupling experiments. Thus, the density of ligands available for immobilisation would likely vary throughout the adsorbents. Although this could have had some impact on the capacity of the ligands, the readily soluble ones, i.e. guanidine, 2-aminopyridine, 2-thiopyridine, were by no means better adsorbents suggesting that solubility did not limit the capacity of these ligands.

4. Conclusion

The adsorbents that have been developed have good binding capacity and selectivity for both $F(ab')_2$ and IgG. The selection of these adsorbents was not just based on the likelihood that they would bind antitoxins, but also on how expensive it would be to prepare them.

It was observed that generally thiol-ligands bind more $F(ab')_2$ than amino-ligands. Similarly, recovery was better with the thiol-ligands, which implies that the interaction between the amino-ligands and the $F(ab')_2$ may be too strong to be eluted with distilled water. Pyrimidine based ligands bind as much $F(ab')_2$ as pyridine based ligands but the recovery in the former is better.

In IgG studies, three adsorbents namely 2-thiopyridine, DVS activated guanidine and 6-thiopurine show good binding capacity but their recovery is low. Cytosine which has a high recovery has a low binding capacity. There was a comparable binding characteristics between thiol- and amino-ligands but the thiol-ligands gave a better product yield. As was the case with $F(ab')_2$, pyrimidine based ligands bind less IgG than pyridine based ones but their recovery is better.

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