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Optimised affinity purification of polyclonal antibodies from hyper immunised ovine serum using a synthetic Protein A adsorbent, MAbsorbent® A2P

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Abstract

This report describes the applicability of a synthetic chromatography adsorbent for large-scale purification of polyclonal immunoglobulin G from hyper immunised ovine serum. Under optimised conditions, MAbsorbent® A2P was shown to bind ∼27 mg mL^{-1} of ovine immunoglobulin from undiluted serum, with eluted IgG purities of >95%, minor levels of albumin (∼1%) and undetectable levels of leached ligand in the purified preparations. The results presented here indicate that the optimised affinity capture of immunoglobulin from ovine serum using MAbsorbent® A2P is a feasible alternative to Protein A chromatography or sodium sulphate precipitation for the initial capture of antibodies from undiluted serum.

Keywords: Mimetic; Protein A; Chromatography; Affinity purification; Antibody; Serum

1. Introduction

Antibodies and antibody derivatives are currently thought to constitute 20% of biopharmaceutical products in development [1]. In an attempt to reduce upstream processing and feedstock costs, both polyclonal and monoclonal antibodies have been produced in animal serum [2], milk and eggs of transgenic animals [3] and transgenic plants [4,5]. In addition, there has been renewed interest in the use of polyclonal-derived therapeutics, and the expression of fully human polyclonal antibodies using transgenic animals has recently been reported [6]. As hyperimmunised animal serum contains a mixed population of antibodies against an entire range of epitopes, polyclonal antibodies are often considered more efficacious (especially for the treatment of acute illness and medical emergencies) as they can bind to multiple epitopes on the disease-causing agent. Affinity chromatography is potentially the most selective method for protein purification and is often included as an initial capture step of an antibody-derived therapeutic. Although affinity chromatography using Protein A derived from the cell wall of the micro-organism *Staphylococcus aureus* and Protein G from *Streptococcus* as the affinity ligands have been widely used for the purification of monoclonal antibodies [7], the high cost of these affinity adsorbents for commercial manufacture of therapeutic antibodies combined with the additional cost of expensive cleaning solutions has resulted in the recent development of a number of commercial alternatives to Protein A or G chromatography matrices [8–11]. Many of these chemical alternatives are applicable for downstream processing of immunoglobulin-derived biotherapeutics, including Fab fragments. Affinity adsorbents based on synthetic affinity ligands offer a number of advantages to compounds derived

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from biological sources, in particular robustness, relatively low cost, ease of sanitisation and potential lack of biological contamination. With increasing attention being paid to the economic aspects of biopharmaceutical production, the reuse of chromatography matrices is often essential for a cost effective process. Although the cost of Protein A or G matrix may be spread over many batches and issues concerning ligand leakage and reuse may be addressed with careful process design, the initial purchase of a Protein A or G column may be the single most expensive item in the list of materials required for commercial therapeutic antibody production. Protein A chromatography matrices may be over 15 times more expensive than many ion-exchangers and 5 times more expensive than synthetic affinity adsorbents and may account for >35% of the downstream material costs at large scale [12]. Although Protein A is a robust molecule that may be cleaned with relatively harsh cleaning conditions [13], the use of Protein A or G for a primary capture step of antibodies directly from a feedstock such as crude serum may not be commercially feasible at large scale due to the viscosity of the serum and relatively high concentration of antibody, other proteins (albumin, transferrin and other serum proteins) and non-protein impurities (lipids and often cryoprecipitates) that may remain non-specifically adsorbed to the column and impact the dynamic capacity and reuse of the column matrix. In addition, unlike many affinity matrices that may show IgG sub-class specificity, MAbsorbent® A2P binds all subclasses of IgG including IgG3, important for the purification of polyclonal-derived antibody therapeutics. The MAbsorbent® A2P ligand (Prometic BioSciences, UK) is composed of a di-substituted phenolic derivative of tri-chlorotriazine and is commercially available coupled to a 6% cross-linked agarose (Purabead®) base matrix. The ligand was discovered following screening of ProMetic's combinatorial ligand libraries for compounds active in binding IgG and is thought to mimic the structure of two key amino acid side chains of Protein A—Phe 132 and Tyr 133 that are found to play an important role in formation of the binary complex between Protein A and the Fc fragment of IgG [11]. This study describes the optimum conditions for the isolation and purification of polyclonal antibodies from crude, hyper immunized ovine serum using the synthetic Protein A ligand adsorbent, MAbsorbent® A2P and compares antibody quality and purity with IgG purified using sodium sulphate precipitation, currently used at production scale to manufacture the FDA approved biotherapeutic CroFab™ (Crotalidae Polyvalent Immune Fab) [2].

2. Experimental

2.1. Chromatographic separations

A frozen sample (5 L) of ovine serum from sheep hyper immunized with rattlesnake (*Crotalidae*) venom (Protherics Australasia Pty Ltd.) was thawed overnight and stored at 2–8 °C prior to use. To determine the concentration of antibody in hyper immunized ovine serum, a sample (10.0 mL) of serum was filtered through a 0.2 μm syringe filter (Sartolab® P20, Sartorius Ltd., UK) to remove insoluble debris and the soluble fraction left to equilibrate to room temperature. An aliquot of filtered serum (1.0 mL) was diluted with an equal volume of 25 mM sodium phosphate pH 7.6 (buffer A). The sample was loaded on to a 5.5 mL Protein G Sepharose 4 Fast flow (Amersham BioSciences, UK) C10/10 (7.0 cm bed height, 1.0 cm diameter) column at 100 cm h^{-1} equilibrated in buffer A. The column was washed to baseline (<0.05 AU) with buffer A and the antibody eluted using 100 mM glycine, HCl pH 2.5 and collected as a single fraction (to mimic the collection of IgG within a single bioprocess container at production scale). Fractionation of the product across the eluted peak has not been described in this report. However, this may help to demonstrate the selectivity of the adsorbent for IgG and albumin and is currently under investigation within this laboratory. The concentration of eluted antibody was determined using an absorption coefficient (1.0 mg mL^{-1}) at 280 nm of 1.5. Total antibody concentrations of 26–31 mg mL^{-1} serum were obtained. Antibody titres may be dependent on the immunization protocol used to generate specific polyclonal antibodies. Typical purities of >95% were observed as determined by Coomassie stained SDS–PAGE and scanning densitometry analysis. No detectable IgG band was observed in the flow through, which indicated that Protein G bound all ovine IgG isoforms under the conditions described.

Initial determination of IgG binding capacity of the MAbsorbent® A2P matrix was undertaken by packing a C10/10 column (Amersham BioSciences, UK) with 4.5 mL (5.7 cm bed height, 1.0 cm diameter) of MAbsorbent® A2P (ProMetic BioSciences). The column was cleaned and sanitized according to the manufacturer's instructions prior to use. A sample of serum was filtered through a 0.2 μm syringe filter (Sartolab® P20, Sartorius Ltd., UK) to remove debris and the soluble fraction left to equilibrate to room temperature. The column was equilibrated with 25 mM sodium phosphate pH 7.6 and overloaded with 60.0 mg of IgG per mL of matrix (8.9 mL serum, [IgG] 31.0 mg mL^{-1}) at 30 cm h^{-1}. No dilution or pH adjustment of the serum was made. The column was washed to baseline (<0.05 AU) with 25 mM sodium phosphate pH 7.6 and the bound protein eluted using 50 mM sodium citrate. The fractions were pooled, and the concentration of antibody determined using an absorption coefficient (1.0 mg mL^{-1}) at 280 nm of 1.5. Purities of approximately 76% were observed as determined by SDS–PAGE and scanning densitometry analysis.

2.2. SDS–PAGE analysis

Non-reduced SDS–PAGE electrophoresis was carried out using Novex® Tris–glycine pre-cast 4–20% polyacrylamide gels (Invitrogen Ltd., UK) and a discontinuous Tris–glycine buffering system. All samples were prepared by mixing and equal volume with two times SDS sample buffer (Invitrogen

Ltd., UK) and heating in a boiling water bath for 2 min. Gels were typically loaded with 5.0 μg protein per sample lane and electrophoresed at a constant voltage of 150 V. Protein bands were visualised by staining for a minimum of 60 min with 0.1% (w/v) Coomassie brilliant blue R250 (Sigma B-0149) and destained with 40% (v/v) methanol, 10% (v/v) glacial acetic acid. Band intensities were quantified by scanning densitometry analysis using ImageMaster Total Lab v2.01 (Amersham BioSciences, UK). The relative staining properties of ovine IgG and albumin has not been investigated and quantitative purities are therefore approximate. Silver staining (reduced SDS–PAGE analysis) was undertaken using a Novex® pre-cast 10% Bis–Tris polyacrylamide gel (Invitrogen Ltd., UK), which was electrophoresed for 35 min with at constant voltage of 100 V. All samples were diluted to 1.0 mg mL^{-1} total protein with H_2O. Fifty microliters of each diluted sample was mixed with 25 μL of Invitrogen four times sample buffer (Invitrogen Ltd., #NP0004), 10 μL of 10× reducing buffer (Invitrogen Ltd., # NP0007) and 15 μL of H_2O. The samples were then heated at 100 °C for 10 min in a dry heating block to fully reduce. Four microliters of each sample was then added to each well resulting in 2.0 μg of protein loaded per well. The SDS–PAGE gels were stained using SilverXpress® Silver Staining Kit (Invitrogen Ltd., UK) according to the manufacturer's instructions. After the gels were developed, the image was recorded using an Alpha Innotech FluorChem 8800 system and with subsequent annotation using the software AlphaEase® FC (Alpha Innotech Corporation).

2.3. Isoelectric focusing (IEF)

Isoelectric focusing (IEF) was undertaken using pre cast Novex p*I* 3–10 gradient gels (Invitrogen Ltd., UK) using an XCell SureLock™ mini electrophoresis unit (Invitrogen). Samples were dialysed overnight in 20 mM sodium phosphate with 10 mM NaCl, pH 7.6, diluted to working concentrations with H_2O then mixed with an equal volume of two times IEF sample buffer (Invitrogen) prior to loading. The gel was ran at a constant voltage for 1 h at a 100 V, 1 h at 200 V and 30 min at 500 V according to the manufacturer's instructions (Invitrogen). Protein bands were visualised by staining for >60 min with 0.1% (w/v) Coomassie brilliant blue R250 (Sigma B-0149) and destained with 40% (v/v) methanol, 10% (v/v) acetic acid.

2.4. Factorial and adsorbent re-use experiments

Factorial and adsorbent re-use experiments were undertaken using a 23.7 mL (30.0 cm bed height, 1.0 cm diameter) MAbsorbent® A2P column (Tricorn 10/300 column, Amersham BioSciences, UK, see figure legends for running buffers). For each factorial experiment, a new column was packed with unused matrix. Design Expert 6.06® (Stat-Ease Inc., USA) was used to model the process and predict optimum conditions to maximise purity, recovery and flow rates, and also minimise the wash volume, elution volume and total run time. The experimental design was augmented to include additional axial points (as a central composite design) to model curvature in the desired responses. The experimental data was fitted to models of increasing complexity. For each model the probability, standard deviation and R^2 values were assessed to determine the most suitable model for the response (data not shown). Residual albumin was removed from the MAbsorbent® A2P column with the incorporation of 25 mM caprylic acid to the wash buffer (sodium octanoate, Sigma C5038-100G). Absorbance measurements were obtained using a Cecil 2041 UV–vis spectrophotometer (Cecil Instruments Ltd., UK). Chromatographic experiments were undertaken using an ÄKTA Explorer 100 (Amersham BioSciences, UK).

2.5. HPLC analysis of MAbsorbent® A2P ligand leakage

Samples were adjusted to pH 3.0 with the addition of ∼17% (v/v) 1 M citric acid to prevent IgG antibody sequestering the ligand leakage product (Prometic). All of the standards and samples were filtered using a Centricon YM-30 centrifugal filter device (Millipore, UK) and assayed using a reverse phase HPLC (gradient elution) method. One hundred millilitres of standard/sample was loaded onto a Luna Phenyl–Hexyl column (40.0 mm × 4.4 mm, Phenomenex, OOB-4257-EO) with a Phenyl-Hexyl guard cartridge (Phenomenex, 4.0 mm × 3.0 mm, AJO-4351) at a flow rate of 0.5 mL min^{-1}. The retention time for the ligand leakage product was calculated at 5.1 min. The column was attached to and controlled via a Waters Alliance® HPLC system (Waters Ltd., UK), and peak analysis was carried out on the Waters Millennium® Chromatography manager 3.2 (Waters Ltd., UK). The two mobile phases were —Buffer A: 10 mM sodium phosphate, pH 7.0 and Buffer B: acetonitrile:water (80:20). The gradient elution protocol was as follows:

Flow (mL/min)	Time (min)	A (%)	B (%)
0.5	0	75	25
0.5	10	20	80
0.5	12	20	80
0.5	15	75	25

An A2P ligand leakage product standard curve was obtained in the range of 50.0 μg mL^{-1} and 25.0 ng mL^{-1}, in 75% 10 mM sodium phosphate pH 7.0, 25% acetonitrile:water (80:20) buffer. OH-ligand controls in sample buffer (10 mM citric acid, pH 2.5) and in the presence of ovine IgG antibody (5 mg mL^{-1}) were also analysed between concentrations 50.0 and 500.0 ng mL^{-1}. The limits of quantification and detection were 185 and 65 ng mL^{-1}, respectively (ProMetic BioSciences, internal report).

3. Results and discussion

Due to the high concentrations of antibody and impurities within crude ovine serum combined with technical difficulties determining accurate volumes loaded using a laboratory-scale column, the determination of dynamic binding capacity was assessed by overloading a MAbsorbent® A2P column with ovine serum containing a large excess of immunoglobulin. Although the IgG may preferentially bind to the matrix and overestimate the purity of eluted product under these conditions, this technique provided a rapid evaluation of the potential capacity of the matrix for ovine IgG. The binding capacity of MAbsorbent® A2P was approximately 27 mg of ovine polyclonal IgG per mL of adsorbent. The purity of the eluted IgG was approximately 80% as determined by SDS–PAGE and densitometry analysis, with the major contaminant showing an apparent molecular weight of 55 kDa, assumed to be ovine serum albumin (data not shown). Although minor impurities will contribute to the absorbance at 280 nm when determining antibody titre, these results suggest that MAbsorbent® A2P matrix can be successfully used to obtain high purity IgG from crude ovine serum using a single purification step. The binding capacity of the MAbsorbent® A2P for polyclonal ovine IgG was almost twice that of commercial Protein A and G matrices evaluated under similar conditions (Cresswell and Newcombe, unpublished results).

3.1. Optimisation of IgG capture using MAbsorbent® A2P

In an attempt to determine optimum conditions for antibody recovery and purity using the MAbsorbent® A2P adsorbent, a fractional factorial experiment was designed. A typical method of optimising processing conditions is to change one factor at a time until no further improvement in product quality is observed. However, this provides little information about interactions between individual factors, and often a 'true' optimum is not obtained. A factorial experimental design of 19 experiments was undertaken with all factors varied systematically. The analysis of resulting experimental data provides a strict mathematical framework for analysis using computer software, and Design Expert v6.06 (Stat-Ease Inc.) was used to analyse the generated data and predict complete three-dimensional (3D) models of the capture process (see Figs. 1–3). Examination of selected 3D curves suggests that flow rates up to 175 cm h^{-1} have little effect on the purity or recovery of eluted IgG. This indicates that a residence time of 10 min is likely to be sufficient to maintain the dynamic binding capacity of the matrix (loading in this case at 80% column capacity) and capture the polyclonal IgG under the conditions described. Increased back pressure was observed at flow rates greater than 175 cm h^{-1}, suggesting that the rigidity of the 6% agarose base matrix is the limiting factor affecting flow rates used for the purification of IgG from crude ovine serum. As expected, the elution pH was shown to be the most significant factor influencing product recovery, purity and elution volume. Design Expert 6.06 was used to predict the optimum conditions for the chromatography process and the modeled data suggested a maximum recovery of 95% ovine IgG with a purity of 85%. Unsurprisingly, the predicted optimum conditions for the process were similar to those recommended for use within the manufacturer's instructions. Predicted recoveries of greater than 95% may be obtained using the matrix (Fig. 1), but evaluation of the modelled data indicates a potential trade-off between purity and recovery when purifying

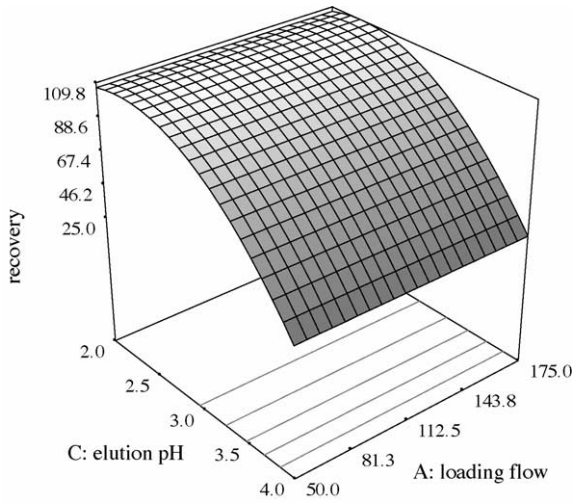

Fig. 1. A three-dimensional display showing the effect of elution pH and loading flow rate (cm h^{-1}) on total ovine IgG recovery (%) using MAbsorbent® A2P chromatography. Data fitted to a quadratic model (R^2 0.964, $P > F < 0.0001$) to show the curvature in the response. Recoveries of >100% are due to minor impurities, including other serum proteins contributing to the absorbance at 280 nm. The effect of this contribution will depend on both the concentration and extinction coefficients of the impurities present in eluted fraction.

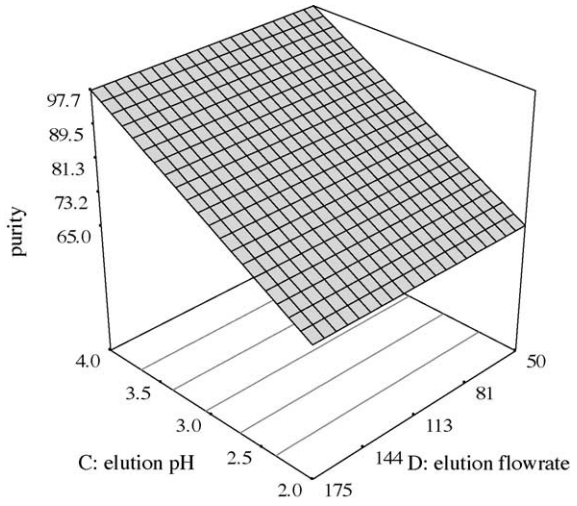

Fig. 2. A three-dimensional display showing the effect of elution pH and elution flow rate (cm h^{-1}) on ovine IgG purity (%) using MAbsorbent® A2P chromatography. IgG purity was calculated using non-reduced SDS–PAGE and scanning densitometry analysis. Data fitted to a linear model (R^2 0.737, $P > F < 0.0001$).

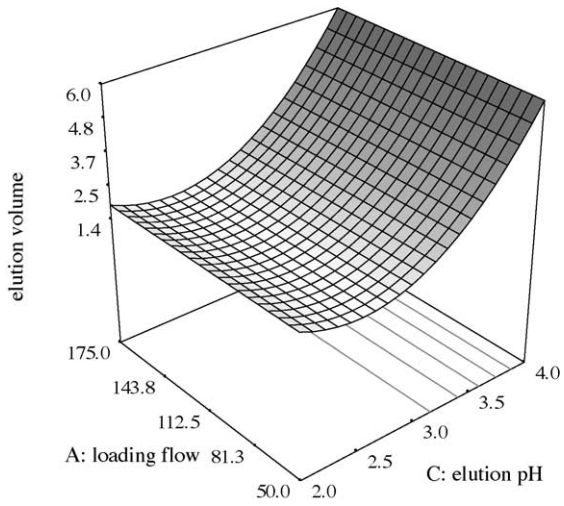

Fig. 3. A three-dimensional display showing the effect of elution pH and loading flow rate (cm h^{-1}) on elution volume (shown as column volumes) using MAbsorbent® A2P chromatography. Data fitted to a quadratic model (R^2 0.949, $P > F < 0.0001$) to show the curvature in the response.

IgG from ovine serum. Maximum recoveries (>95%) require an elution pH of less than 3.0. However, there appears to be a linear relationship between pH and eluted IgG purity, therefore as the pH of the elution buffer drops, the purity of the eluted IgG decreases (Fig. 2). Non-reduced SDS–PAGE analysis of IgG eluted from the column using 50 mM sodium citrate pH 2.0 reveals the presence of a major contaminating band at ~55 kDa, presumed to be serum albumin (data not shown). Although the majority of albumin remains in the unbound fraction and is washed through the column after loading, this suggests that a portion of ovine albumin may bind to the MAbsorbent® column. Recent reports suggest that several hydrophobic ligands bind to human serum albumin, including fatty acids, bilirubin, thyroxine and hemin [14,15]. It is possible that a modified fraction of ovine albumin may interact with the hydrophobic affinity ligand bound to the matrix. In addition, as the elution pH increases above 3.0 the elution volume increases (Fig. 3). This is probably due to weak interactions with the polyclonal IgG isoforms and the ligand or base matrix during elution from the column [16]. To assess whether the modeled data is consistent with the experimental results, ovine IgG was purified from ovine serum using a MAbsorbent® A2P column under the optimum conditions predicted by Design Expert 6.06 (Figs. 4 and 5). Both IgG purity and recovery were consistent with predicted values.

3.2. Analysis of affinity purified IgG

The aim of this study was to develop an optimised chromatographic method suitable for large-scale IgG capture using the synthetic Protein A adsorbent, MAbsorbent® A2P. It was hoped that such a method would provide at least comparable IgG purity to the current method of large-scale IgG purification (sodium sulphate precipitation) used for the

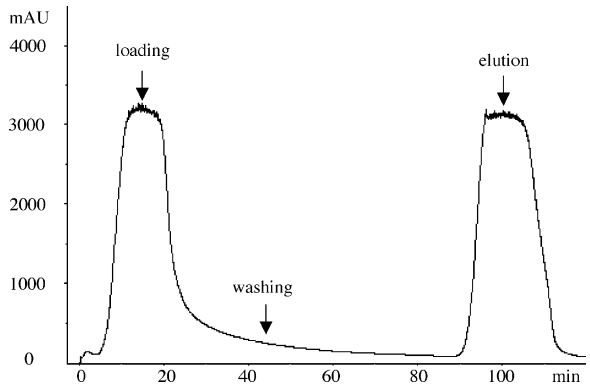

Fig. 4. An 280 nm chromatogram showing the purification of IgG from crude ovine serum using a MAbsorbent® A2P column using predicted optimum conditions. Column: Tricorn 10/300 column (Amersham BioSciences, UK). Sample: Hyper immunised ovine serum (0.2 μm filtered), column loaded at 80% column capacity. Equilibration buffer: 25 mM sodium phosphate pH 7.6, wash buffer: 25 mM sodium phosphate, 100 mM NaCl pH 7.6, elution buffer: 50 mM sodium citrate pH 3.0. Eluted IgG was collected as a single fraction (~1.8 column volumes) to ensure maximum IgG recovery and reflect the proposed collection of IgG within a single bioprocess container at production scale.

production of the FDA approved biotherapeutic, CroFab™. Analysis of the product by non-reduced SDS–PAGE purified under optimum conditions suggests a similar purity to IgG purified using sodium sulphate precipitation (Fig. 5). IgG purified under optimum conditions remained clear with no visible precipitation after neutralization to pH 7.0 with 2 M Tris base. No IgG appeared in the non-bound fraction, indicating that antibody capture was complete. Determination of total IgG recovery after affinity capture, pH neutralization and 0.2 μm filtration was approximately 90%. Analysis of monomer, dimer and aggregate levels in the IgG purified using MAbsorbent® A2P by size exclusion chromatography shows that monomer levels were similar to those observed in the IgG fraction purified by sodium sulphate precipitation (data not shown). Long-term stability studies are currently

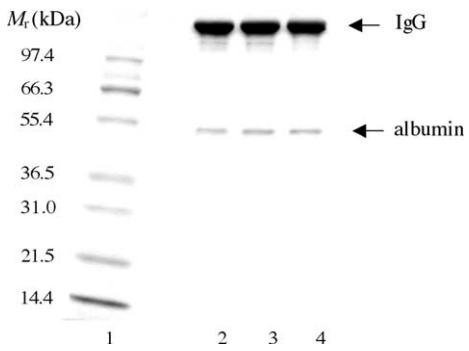

Fig. 5. Non-reduced SDS–PAGE analysis of IgG purified from crude ovine serum using a MAbsorbent® A2P column under optimised conditions. For further details see Section 2. (1) Molecular weight markers; (2) eluted IgG, 83% purity; (3) eluted IgG post-neutralisation to pH 7.0 using 2 M Tris base; (4) A sample of ovine IgG used to produce the FDA approved biotherapeutic, CroFab™ purified at manufacturing scale using sodium sulphate precipitation, 91% purity.

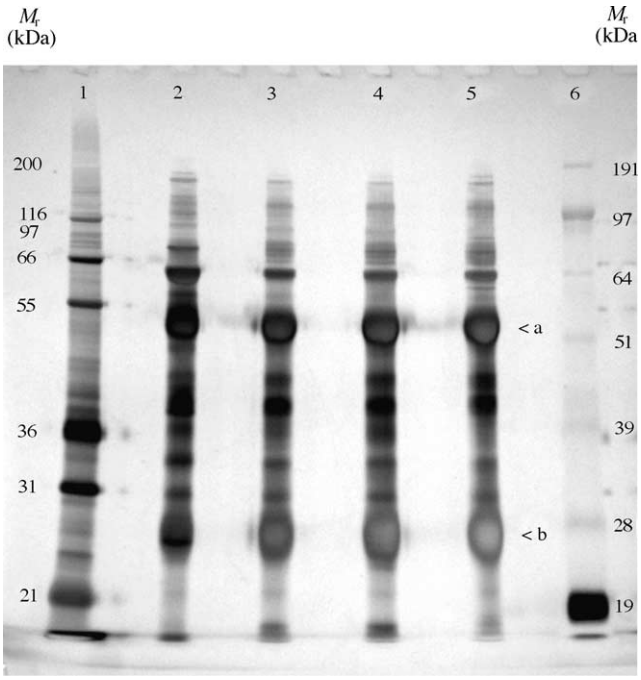

Fig. 6. Silver stained SDS–PAGE analysis of IgG under reducing conditions purified from ovine serum using MAbsorbent® A2P, and production scale sodium sulphate precipitation. (a) IgG heavy chain; (b) IgG light chain. (1) Molecular weight markers (Mark 12, Invitrogen); (2) 2.0 μg ovine serum; (3) 2.0 μg IgG purified using MAbsorbent® A2P, pH neutralized using 2 M Tris base; (4) 2.0 μg IgG purified using MAbsorbent® A2P, pH neutralized using 1 M NaOH; (5) 2.0 μg ovine IgG used to produce the FDA approved biotherapeutic, CroFab™ purified at manufacturing scale using sodium sulphate precipitation; (6) molecular weight markers (SeeBlue® Plus 2).

under investigation within this laboratory. Analysis of eluted IgG by silver stained SDS–PAGE indicated that IgG purified using MAbsorbent® A2P shows a comparable banding pattern to IgG purified by precipitation (Fig. 6). Although minor variations in band intensities were observed, a large number of bands were detected in all purified IgG samples reflecting the sensitivity of the staining method used. Analysis of purified IgG fractions by isoelectric focusing reveals that polyclonal IgG is separated into more than 15 distinct protein bands with the majority of isoelectric points ranging from 4.5 to 6.5 (Fig. 7). Comparable banding patterns are observed within both samples, suggesting that both techniques (sodium sulphate precipitation and MAbsorbent® A2P capture) purify a comparable range of IgG isoforms under the conditions described. Although increased intensity of a band with a p*I* of ∼5 is observed in the IgG purified by precipitation, it is possible that band intensities are variable and may be dependent on both non-specific and specific IgG levels within individual batches of serum. A comparable level of 'smearing' is also observed in both sample lanes. As both samples were dialysed into a buffer of low ionic strength and diluted with H_2O prior to analysis, apparent smearing is likely to represent minor levels of unresolved IgG isoforms spanning the alkaline p*I* range. Analysis of ligand leakage by HPLC also indicated that levels of ligand in the eluted IgG were

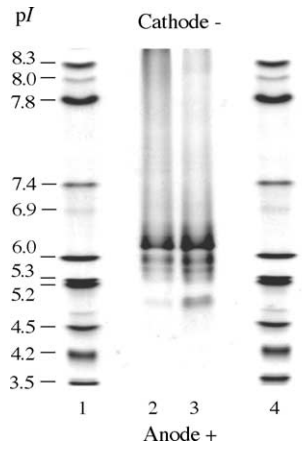

Fig. 7. IEF analysis of purified ovine IgG. Lanes (1) and (4). IEF markers (Serva), p*I* 3–10. (2) IgG purified using MAbsorbent® A2P. (3) IgG purified using sodium sulphate precipitation. Markers appear clear and well defined and apparent smearing is likely to be due to IgG isoforms spanning the alkaline p*I* range. p*I* = isoelectric point.

below the limits of detection (data not shown). Although the A2P ligand leakage product is non-toxic by maximum tolerated dose (MTD) testing in rats (after an intravenous dose of 0.6 mg kg^{-1} body weight) and non-mutagenic (Ames test negative) [17], detection and removal of any leached ligand is essential when manufacturing protein therapeutics.

3.3. Removal of residual albumin

In an attempt to remove residual albumin from the MAbsorbent® A2P column prior to elution, 25 mM caprylic acid was added to the post-load wash buffer. Caprylic acid has been used to remove albumin from hydrophobic charge induction chromatography adsorbents including MEP

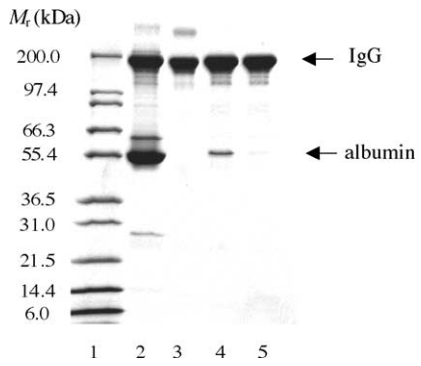

Fig. 8. Non-reduced SDS–PAGE analysis of ovine IgG purified from crude ovine serum using a MAbsorbent® A2P column. For further details see experimental methods. (1) Molecular weight markers (Mark 12, Invitrogen); (2) ovine serum; (3) sheep IgG, Sigma I-5131; (4) IgG purified by sodium sulphate precipitation used for production of the biotherapeutic CroFab™; (5) purified IgG using MAbsorbent® A2P (using optimised conditions with a post-load wash of 25 mM sodium phosphate, 25 mM sodium caprylate, 100 mM NaCl pH 7; (6) the addition of sodium caprylate to the post-load wash buffer removes the residual ovine albumin to <1% (as determined by scanning densitometry analysis).

HyperCel® (Ciphergen S.A, France), as a stabiliser for albumin solutions as well as a precipitation agent for immunoglobulins [18]. The addition of caprylic acid to the wash buffer had little effect on the capacity of the matrix for ovine IgG, with capacities of 25 mg mL^{-1} observed. The yield was also unaffected with >95% IgG recovered. However, improved IgG purities of >95% were consistently obtained with levels of albumin <1% in the eluted antibody fraction as determined by Coomassie stained SDS–PAGE (Fig. 8). Final IgG purities were comparable to those obtained with commercial Protein A and G matrices.

4. Conclusions

An important factor to consider when developing a purification process for a therapeutic antibody fragment is the suitability to operate the process under current good manufacturing practice (GMP) [19,20]. The process must be robust and tolerate minor variation in processing conditions, and therefore it is important to determine the key parameters that influence the performance of a process step. Although fractionation of polyclonal antibodies by precipitation using ethanol or salts such as sodium sulphate has been in practice for many years, large-scale precipitation may present technical difficulties such as poor reproducibility of the precipitation process and variable purity and recovery. The development of a robust and reproducible generic strategy for the purification of polyclonal antibodies is also often challenging due to the chemical diversity of the antibody species involved. Here, we have investigated the optimum conditions for the purification of ovine polyclonal immunoglobulin using a synthetic Protein A affinity adsorbent, MAbsorbent® A2P (ProMetic BioSciences) and assessed the suitability of the matrix as an alternative to traditional methods used for IgG capture. Although adsorbent reuse data has not been included in this report, previous studies have shown that reuse of the MAbsorbent® A2P chromatography matrix using hyperimmunised ovine serum with processing conditions recommended by the manufacturer shows no drop in IgG recovery or purity or detectable ligand leakage over 100 cycles (ProMetic BioSciences, internal report). Due to the chemical nature of the bound ligand it is feasible that the lifetime of the matrix may be extended further and the cost of the matrix may be depreciated over many batches of the antibody product. The results presented here indicate that MAbsorbent® A2P may provide a robust and economical alternative for large-scale purification of IgG for the production of antibody-derived biotherapeutics.

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References

[1] A.C. Roque, C.R. Lowe, M.A. Taipa, Biotechnol. Prog. 20 (2004) 639.
[2] CroFab, an antigen-binding fragment (Fab) antivenom of ovine origin, has been approved by the FDA for treatment of North American rattlesnake envenomation, Med. Lett. 1107 (2001) 55.
[3] Y. Echelard, H. Meade, Nat. Biotechnol. 20 (9) (2002) 881.
[4] E. Stoger, M. Sack, R. Fischer, P. Christou, Curr. Opin. Biotechnol. 13 (2002) 161.
[5] L.M. Houdebine, Curr. Opin. Biotechnol. 13 (2002) 625.
[6] J.M. Robl, P. Kasinathan, E. Sullivan, Y. Kuroiwa, K. Tomizuka, I. Ishida, Theriogenology 59 (2003) 107.
[7] A.J. Racher, J.M. Tong, J. Bonnerjea, Biotechnology. Recombinant Proteins, vol. 5a, Wiley-VCH, Weinheim, Germany, 2003, p. 247.
[8] L. Guerrier, P. Girot, W. Schwartz, E. Boschetti, Bioseparation 9 (2000) 211.
[9] A. Verdoliva, F. Pannone, M. Rossi, S. Catello, V. Manfredi, J. Immunol. Methods 271 (2002) 77.
[10] S. Kabir, Immunol. Invest. 31 (2002) 263.
[11] R. Li, V. Dowd, D.J. Stewart, S.J. Burton, C.R. Lowe, Nat. Biotechnol. 16 (1998) 190.
[12] D.K. Follman, R.L. Fahrner, J. Chromatogr. A 1024 (2004) 79–85.
[13] R.M. Baker, A.M. Brady, B.S. Cambridge, in: D.L. Pyle (Ed.), Separation for Biotechnology, vol. 3, Royal Society of Chemistry, Cambridge, UK, 1994, p. 427.
[14] P.A. Zunszain, P.A.J. Ghuman, T. Komatsu, E. Tsuchida, S. Curry, BMC Struct. Biol. 3 (2003) 6.
[15] S. Curry, S.P. Brick, N.P. Franks, Biochim. Biophys. Acta 1441 (1999) 131.
[16] D. Josić, Y.-P. Lim, Food Technol. Biotechnol. 39 (2001) 215.
[17] K. Mortelmans, E. Zeiger, Mutat. Res. 455 (2000) 29–60.
[18] M.C. dos Santos, M.R. D'Imperio Lima, G.C. Furtado, G.M. Colletto, T.L. Kipnis, W. Dias da Silva, Toxicon 27 (1989) 297.
[19] Medicines Control Agency, Rules and Guidance for Pharmaceutical Manufacturers and Distributors, TSO Press, Norwich, UK, 2002 (http://www.mhra.gov.uk).
[20] FDA Code of Federal Regulations (CFR), Title 21 Food and Drugs, National Archives and Records Administration, Office of the Federal Register, USA, 2004 (http://www.fda.gov).