

# Optimised Affinity Purification of Polyclonal Antibodies from Hyper Immunised Ovine Serum using a synthetic Protein A adsorbent, MAbsorbent® A2P

(Manuscript accepted 08 October 2004, *J Chromatogr B Analyt Technol Biomed Life Sci.*)

Anthony R. Newcombe<sup>1\*</sup>, Chrissie Cresswell<sup>1</sup>, Susannah Davies<sup>1</sup>, Keith Watson<sup>2</sup>, Guy Harris<sup>2</sup>, Kieran O'Donovan<sup>1</sup>, Richard Francis<sup>1</sup>

<sup>1</sup>Process Development Group, Protherics Ltd, Blaenwaun, Ffostrasol, Llandysul, Wales UK SA44 5JT <sup>2</sup>Technical Support Group, ProMetic BioSciences Ltd, 211 Cambridge Science Park Milton Road, Cambridge UK CB4 07A

\* To whom correspondence should be addressed. Tel: 44 (0)1239 851122. Fax: 44 (0)1239 858800, e-mail: tony.newcombe@protherics.com

**ABSTRACT:** Here we describe 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 ~27mg.mL<sup>-1</sup> 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.

## INTRODUCTION

Although affinity chromatography using Protein A derived from the cell wall of the micro-organism *Streptococcus aureus* and Protein G from *Streptococcus* as the affinity ligands have been widely used for the purification of monoclonal antibodies [1], 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 [2-5]. 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% crosslinked 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 [5]. This poster describes the optimum conditions for the isolation and purification of polyclonal antibodies from crude, hyper immunised 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).

## EXPERIMENTAL

### Factorial and adsorbent re-use experiments.

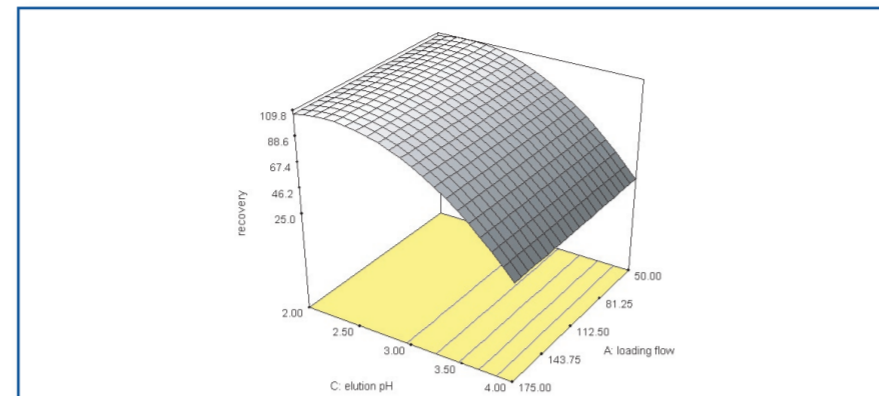
Factorial and adsorbent re-use experiments were undertaken using a 23.7mL (30.0cm bed height, 1.0cm diameter) MAbsorbent® A2P column (Tricorn 10/300 column, Amersham BioSciences UK, see figure legends for running buffers). For each factorial experiment, a new column was packed with unused matrix. Design 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 Eluted IgG was analysed using standard biochemical techniques.

## RESULTS AND DISCUSSION

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). 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 Protein G matrices evaluated under similar conditions (C. Cresswell & A.R. Newcombe, unpublished results).

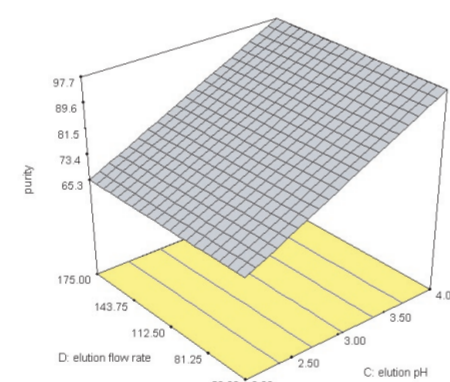
### Optimisation of IgG capture using MAbsorbent A2P.

In an attempt to determine optimum conditions for antibody recovery and purity using the MAbsorbent® A2P adsorbent, a fractional factorial experiment was designed. 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 3D models of the capture process. 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 (figure 1), but evaluation of the modelled data indicates a potential trade-off between purity and recovery when purifying IgG from ovine serum.



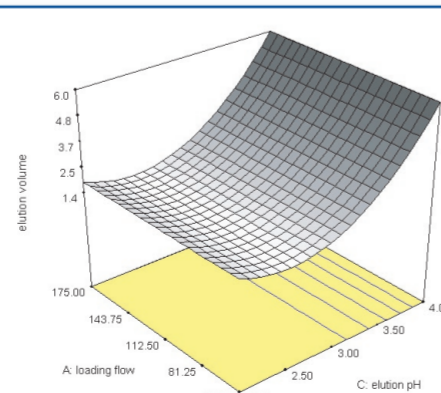
**Figure 1.** A three dimensional display showing the effect of elution pH and loading flow rate (cm.hr<sup>-1</sup>) on total ovine IgG recovery (%) using MAbsorbent® A2P chromatography. Data fitted to a quadratic model (R-Squared 0.964, Prob>F <0.0001) to show the curvature in the response. Recoveries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

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 (figure 2). Non reduced SDS PAGE analysis of IgG eluted from the column using 50mM sodium citrate pH 2.0 reveals the presence of a major contaminating band at ~55kDa, 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.



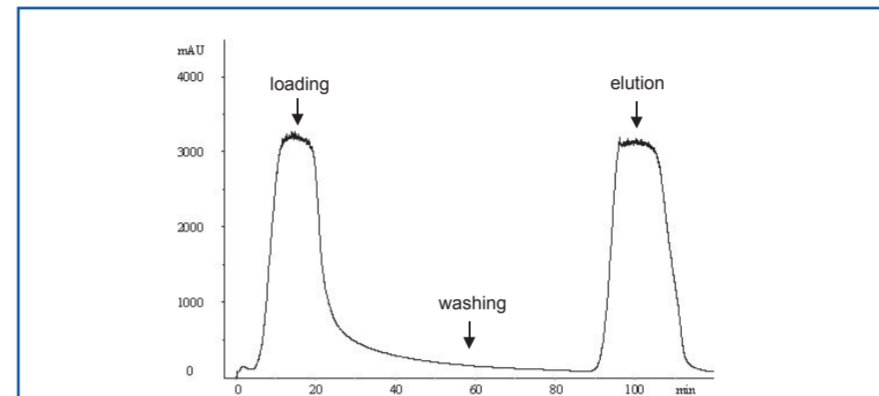
**Figure 2.** A three dimensional display showing the effect of elution pH and elution flow rate (cm.hr<sup>-1</sup>) on ovine IgG purity (%) using MAbsorbent® A2P chromatography. IgG purity was calculated using non-reduced SDS PAGE and scanning densitometry analysis. Data fitted to a linear model (R-Squared 0.737, Prob>F <0.0001).

In addition, as the elution pH increases above 3.0 the elution volume increases (figure 3). This is probably due to weak interactions with the polyclonal IgG isoforms and the ligand or base matrix during elution from the column.

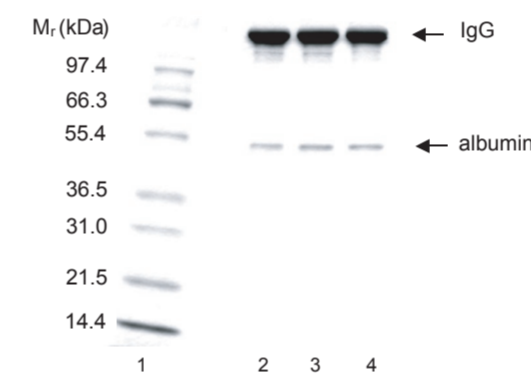


**Figure 3.** A three dimensional display showing the effect of elution pH and loading flow rate (cm.hr<sup>-1</sup>) on elution volume (shown as column volumes) using MAbsorbent® A2P chromatography. Data fitted to a quadratic model (R-Squared 0.949, Prob>F <0.0001) to show the curvature in the response.

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 (figures 4 & 5). Both IgG purity and recovery were consistent with predicted values.



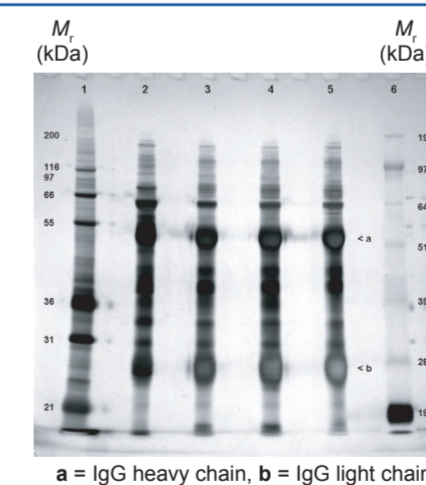
**Figure 4.** An 280nm chromatogram showing the purification of IgG from crude ovine serum using a MAbsorbent® A2P column using predicted optimum conditions. Column: Tricorn 10/300 column (Amersham BioSciences, UK). Sample: Hyper immunised ovine serum (0.2µm filtered), column loaded at 80% column capacity. Equilibration buffer: 25mM sodium phosphate pH 7.6, wash buffer: 25mM sodium phosphate, 100mM NaCl pH 7.6, Elution buffer: 50mM sodium citrate pH 3.0.



**Figure 5.** Non reduced SDS-PAGE analysis of IgG purified from crude ovine serum using a MAbsorbent® A2P column under optimised conditions. 1) Molecular weight Markers 2) eluted IgG, 83% purity 3) eluted IgG post neutralisation to pH 7.0 using 2M 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.

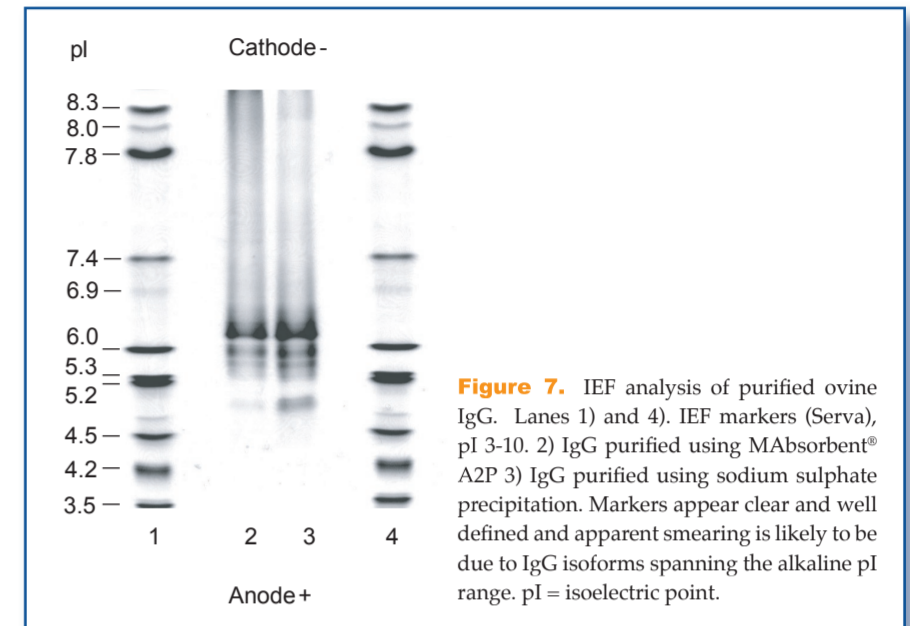
### Analysis of affinity purified IgG.

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 (figure 6).



**Figure 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) 2.0µg ovine serum, 3) 2.0µg IgG purified using MAbsorbent® A2P, pH neutralized using 2M Tris base, 4) 2.0µg IgG purified using MAbsorbent® A2P, pH neutralized using 1M NaOH, 5) 2.0µg 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).

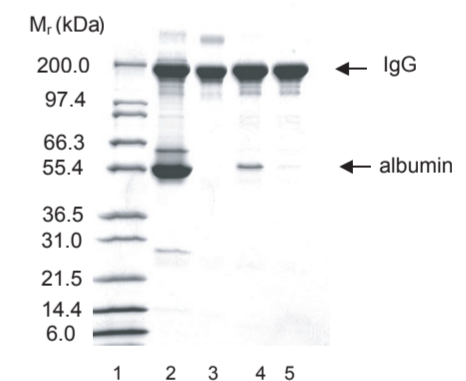
Analysis of purified IgG fractions by isoelectric focusing (IEF) reveals that polyclonal IgG is separated into more than 15 distinct protein bands with the majority of isoelectric points ranging from 4.5 – 6.5 (figure 7).



**Figure 7.** IEF analysis of purified ovine IgG. Lanes 1) and 4). IEF markers (Serva), pI 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 pI range. pI = isoelectric point.

### Removal of residual albumin

In an attempt to remove residual albumin from the MAbsorbent column prior to elution, 25mM caprylic acid was added to the post load wash buffer. The addition of caprylic acid to the wash buffer had little effect on the capacity of the matrix for ovine IgG, with capacities of 25mg.mL<sup>-1</sup> 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 (figure 8). Final IgG purities were comparable to those obtained with commercial Protein A and G matrices.



**Figure 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 25mM sodium phosphate, 25mM sodium caprylate, 100mM 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).

## CONCLUSIONS

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. 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.

### ACKNOWLEDGEMENTS

We thank Dr Tim Auton (Protherics Molecular Design Ltd), Dr Garnet Lewis (Strategic Protein Solutions, UK) and Dr Paul Nelson (Prism Training and Consultancy Ltd) for helpful discussions.

### REFERENCES

- [1] A.J. Racher, J.M. Tong, J. Bonnerjea (Authors), In Biotechnology Volume 5a: Recombinant Proteins. Wiley-VCH, Weinheim, Germany, 2003, p 247.
- [2] L. Guerrier, P. Giro, W. Schwartz, E. Boschetti, 2000. Bioseparation 9 (2000) 211.
- [3] A. Verdoliva, F. Pannone, M. Rossi, S. Catello, V. Manfredi, J Immunol Methods. 271(2002) 77.
- [4] S. Kabir, Immunol Invest. 31 (2002) 263.
- [5] R. Li, V. Dowd, D.J. Stewart, S.J. Burton, C.R. Lowe, Nat Biotechnol. 16 (1998) 190.