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Purification of monoclonal antibodies from ascitic fluid using preparative electrophoresis

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Abstract

Four monoclonal antibodies (including Ig subclasses, G1, G2a and G2b) were purified from murine ascitic fluid by a preparative electrophoresis system using a charge- and size-based strategy. Most of the smaller contaminating proteins were removed at pH 8.3 when the ascitic fluid was passed through a cartridge containing a separating membrane with a pore size of M_r 100 000. After this single step, the immunoglobulin heavy and light chains were the only significant bands present when analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. A second step, involving electrophoresis at pH 6.4–7.5 depending on the antibody can be used to remove residual contaminants. For each of the antibodies tested, the recovery of activity at each step was over 80%. As this technology is directly scalable, purification of antibodies by the method described here could be considered a cost effective alternative to protein A chromatography.

Keywords: Preparative chromatography; Monoclonal antibodies; Immunoglobulins

1. Introduction

The processing of complex biological solutions is a major bottleneck in the biotechnology industry and there is a demand for cost-effective technologies for the purification of naturally occurring or recombinant proteins [1,2]. This is particularly true for monoclonal antibodies, where there has been an on-going search for simple generic methods of purification. Monoclonal antibodies have had an increasing number of research, therapeutic and diagnostic applications since the initial discovery by Kohler and Milstein [3,4]. The difficulties in bioprocessing have meant that recoveries of monoclonal antibodies in existing purification schemes are rarely reported but are often in the range of 10–70% [5–7]. Although new processes have become increasingly effective in terms of yield and recovery [8] they often utilise harsh pH or ionic strength conditions for elution which may not always be compatible with maintaining maximal biological activity.

The aim of this project was to evaluate the preparative electrophoresis technology (Gradiflow) for the purification of several different monoclonal antibodies. The large size and characteristic isoelectric point (p*I*) of mouse antibodies make them an ideal target for the technology.

2. Experimental

2.1. Antibodies

The antibodies used in this study were all gener-

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Table 1
Recovery as determined by ELISA

Antibody	Isotype	p*I*	Recovery	
			%	mg/ml antibody
1	IgG1	pH 7.3–7.5	94[a]	8.9
2	IgG2a	pH 6.8–7.7	73[b]	8.1
3	IgG2b	pH 6.6–6.9	79[b]	10.8
4	IgG1	pH 6.8–7.0	71[b]	10.0

[a] Denotes one-step purification.
[b] Denotes a two-step purification.

ated by conventional procedures [14] and supplied to Gradipore by Agen Biomedical, Brisbane, Australia, as murine ascitic fluids. Table 1 contains the properties of the target monoclonal antibodies.

2.2. Gradiflow technology

The separating cartridge of the Gradiflow [9–11] contains a set of polyacrylamide-based restriction and separating membranes to enable the separation of macromolecules on the basis of size and/or charge (see Fig. 1). A range of cartridges is available with M_r cut-offs ranging from 25 000 to 1 000 000. The ability to fractionate proteins over a range of pH and the use of membranes of different pore sizes enables any target protein to be separated by virtue of its size or isoelectric point.

The Model LM1000 (Gradipore, Sydney, Australia) (Fig. 2) contains peristaltic pumps, peltier coolers and power supply. It is controlled by a personal computer under a Windows 95 and Lab View format. Alternatively, a manually configured instrument is also available which can operate with conventional peristaltic pumps and power supply [10–13].

2.3. Modes of separation

2.3.1. Size-based separation (Fig. 1a)

For size separation a pH is selected at which all proteins have the same charge, in this case negative. Hence all of the proteins from the mixture circulating in the ''upstream'' compartment will try to migrate into the ''downstream'' compartment. If a membrane of restrictive pore size is selected, for example the M_r 100 000 used in this paper, molecules larger than

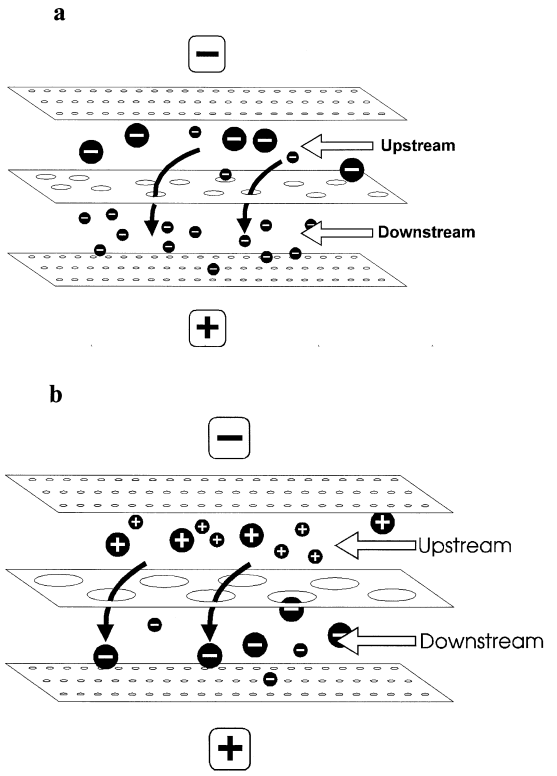

Fig. 1. (a) Size-based separation – this is the first step in which major contaminants are removed downstream from ascites fluid placed upstream. (b) Charge-based separation – this is the second step to remove any residual contaminants if requiring antibodies of higher purity.

M_r 100 000 (such as the target antibodies) will be unable to transfer across the membrane and remain upstream. As essentially all proteins in mouse ascitic fluid have p*I* values less than pH 7.7, pH 8.3 was selected for size separation in this paper. Under these conditions most of the ascitic fluid proteins are transferred ''downstream'' leaving behind the M_r 160 000 antibody molecules.

2.3.2. Charge-based separation (Fig. 1b)

For charge-based separation, a pH is selected between the isoelectric points of two proteins such that one protein will have a positive charge and the other a negative charge. In this example the protein mixture continuously circulates in the ''upstream'' compartment. When the current is applied, the negatively charged protein migrates through the mem-

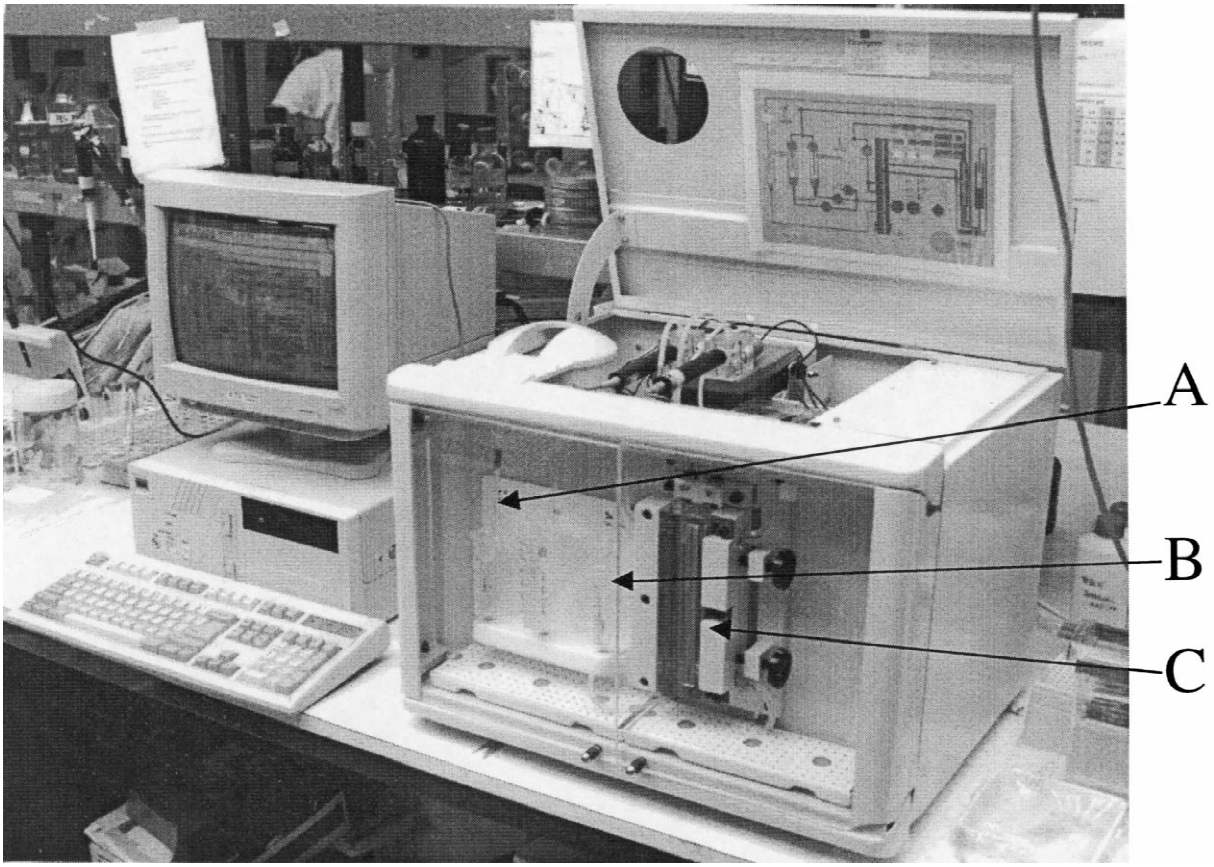

Fig. 2. The Gradiflow LM 1000. A is the upstream, B is the downstream and C is the separation cartridge.

brane to the ''downstream'' compartment. Continuous circulation of the upstream and downstream will allow complete separation of the two proteins.

The vast majority of non-antibody proteins in murine ascitic fluid have isoelectric points below pH 6.5 (Fig. 3) so that at a pH above pH 6.5, these proteins are negatively charged, and will migrate downstream leaving behind the antibody which normally has a p*I* above pH 6.6. For charge separations, a membrane with a largest pore size is usually employed (M_r $1{\cdot}10^6$) to allow for maximum transport across the membrane.

2.4. Purification of monoclonal antibodies

Each sample of ascitic fluid (0.5–2 ml) was diluted with at least three volumes of a buffer containing 40 m*M* Tris–borate, 1 m*M* EDTA, pH 8.3. Firstly, a size separation of each sample was carried out in this buffer for 30–40 min at 200 V with a M_r cut-off 100 000 separating membrane. Under these conditions, albumin and other impurities rapidly migrated across the membrane leaving behind the purified antibody upstream.

For higher purity, a second run was selected at a pH close to the p*I* of each specific antibody using a M_r cut-off $1{\cdot}10^6$ membrane. For example 40 m*M* Tris buffer can be adjusted to the required pH with acetic acid. The run time was 40 min at 200 V. The remaining impurities migrated through the membrane while the antibody remained upstream. By taking 50-μl aliquots of upstream and downstream at 10-min intervals during the first and second run, the purity of the target antibodies was then determined by denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The

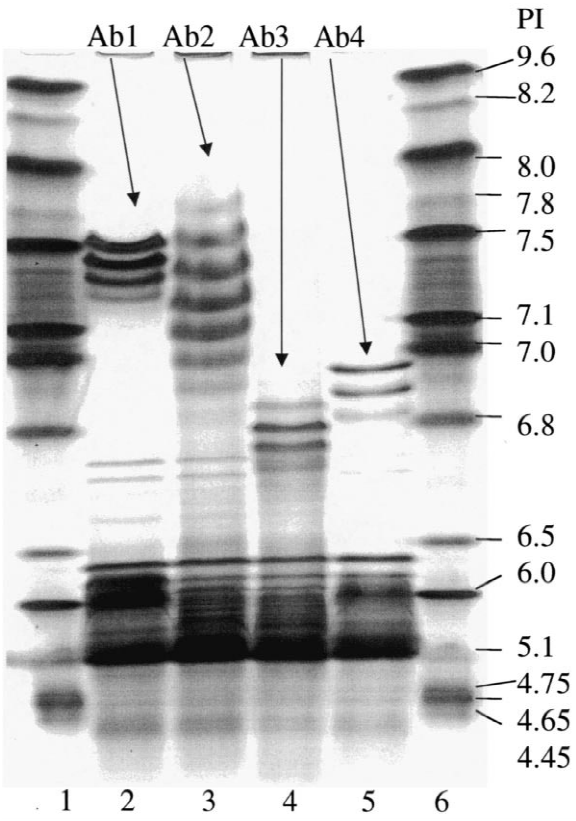

Fig. 3. IEF of the murine ascites fluids. Lanes 1 and 6 are Novex IEF markers, lanes 2–5 contain antibodies 1–4 (Table 1). Ab= Antibody; PI=isoelectric point.

percent recovery was determined by enzyme immunoassay (EIA) after the runs were completed.

The upstream and downstream were harvested after 40 min. For maximum recovery of antibody, a small amount (7 ml) of running buffer was pumped into the upstream and downstream for a minute at the end of the separation process with the current reversed. After the current reversal was switched off, the upstream and downstream were allowed to circulate for another minute before the upstream wash was harvested and combined with the initial antibody harvested. An additional 10–15% of antibody can be recovered in this washing process.

2.5. Isoelectric focusing

The p*I* values of the antibodies were determined by running an isoelectric focusing (IEF) gel using a Novex (San Diego, CA, USA) IEF gel apparatus as described by the manufacturer. Briefly, the running conditions involved a run time of 1 h at 100 V, 1 h at 200 V and 500 V for 30 min. The IEF gel was fixed with a solution of 12% (w/v) trichloroacetic acid (Sigma Product No. T8657) with 3.5% (w/v) 5-sulfosalicylic acid (Sigma Product No. S-3147) in deionised water for 30 min before staining with Gradipure Coomassie Blue.

2.6. Determination of antibody purity

Samples were analysed by SDS–PAGE on Gradipore 4–20%T SDS gels [T=(g acrylamide+g *N,N′*-methylenebisacrylamide)/100 ml solution]. The changes to sample purity with time were determined by comparing the protein bands upstream and downstream at different times.

2.7. Determination of antibody recovery

2.7.1. Protein concentration

Protein levels in the upstream and downstream were determined by measuring the ultraviolet absorption at 280 nm. A 1 mg/ml solution of mouse monoclonal antibodies was assumed to have an absorbance of 1.2 AU.

2.7.2. Enzyme immunoassay

The antibody activity was determined by a two-site EIA using either antigen or unlabelled sheep anti-mouse immunoglobulin as the capturing component. The microplate was first coated with either 50 μl of antigen (10 μg/ml) or rabbit anti-mouse immunoglobulins (10 μg/ml) Dako (Carpinteria, USA) in phosphate-buffered saline (PBS) pH 7.4 for 1 h at room temperature.

Excess antigen was removed by inverting and tapping the plate and the plate was washed three times with PBS containing 0.1% Tween 20 (PBS/T). Next, 50 μl of a suitable dilution in PBS/T of the monoclonal fraction under test was added and the incubation was allowed to proceed for an hour at room temperature. After removal of unbound antibody by washing, bound antibody was labelled by the addition of a 1/1000 dilution of horseradish peroxidase (HRPO)-labelled rabbit anti-mouse antibody and incubated for another hour. Finally, the

bound enzyme was detected after further washing by the addition of substrate and stop solutions [15].

3. Results and discussion

3.1. Isoelectric focusing

IEF is a technique that enables proteins to be characterised by their p*I* values which can be used to determine the best conditions for a charge based separation. An IEF gel of the starting material (Fig. 3) shows that each ascitic fluid has a unique IEF pattern with the major difference being the position of the multiple bands of target antibodies.

The IEF gel indicated a range of p*I* values from 6.6 to 7.7 for the four different antibodies derived from ascitic fluid samples. The p*I* values of the antibodies are listed in Table 1. The variety of isoforms provides plausible reason for the low recoveries from conventional ion-exchange protocols for antibodies as the charge heterogeneity could cause multiple broad peaks and tailing effects.

3.2. Purification of mouse antibodies

3.2.1. Size separation

Size exclusion was chosen as the first step when IEF (Fig. 1) revealed a wide variation in the isoelectric charge of individual antibodies. A membrane with a M_r cut-off of 100 000 was selected, as this pore size should retain the M_r 160 000 antibody, yet allow the rapid passage of smaller protein molecules. A pH of 8.3 was chosen so that the majority of murine ascitic fluid proteins have net negative charges at this pH.

A time course for the purification of antibody 4 is depicted in Fig. 4. Similar results were obtained for the other three antibodies (not shown). After 20 min, the most significant bands in the sample stream are the characteristic heavy and light chains of the monoclonal antibody. Acceptable purity was achieved after 30 min (lane 5 in Fig. 4) without substantial improvement at 40 min. Lower-molecular-mass proteins, with the most abundant being mouse serum albumin, rapidly pass through the M_r 100 000 membrane, leaving behind the antibody upstream. The downstream was harvested every 10 min and showed decreasing amounts of protein in each subsequent harvest (lanes 7–9). As expected most of the impurities are removed from the antibody in the first 10 min (lane 7), with large amounts of albumin present in the two initial downstream harvests that were collected at 10 min and 20 min. Albumin has disappeared in the upstream after 10 min. We interpret this finding, as residual albumin found downstream in the 20-min sample being in transit within the separation membrane when the 10-min sample was taken.

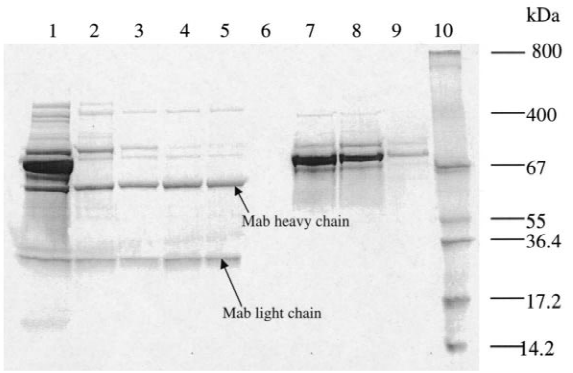

Fig. 4. SDS–PAGE of the purification of antibody 4. Lane 1 is upstream at 0 min, lanes 2–5 upstream after 10, 20, 30 and 40 min. Lanes 6–9 show downstream at 0, 10, 20 and 40 mins. Lane 10 contains SDS markers. Mab=Monoclonal antibodies; kDa=kilodaltons.

We chose these conditions for initial separation with a view to trying to select a universal first step that would allow the purification of any antibody in good yield with a high degree of purity. The Tris–borate buffer was selected because it has proven to be a useful buffer for separations under native conditions in many other applications [9–13]. The voltage was selected on the basis of the required speed of the separation with the aim of completing the process in less than 30 min. For most applications the higher the voltage the more rapid the purification.

Some antibodies are thought to be labile at room temperature and there are many reports that temperature can affect protein folding with lower temperatures increasing protein solubility. In this application, as the objective was to obtain pure antibodies in their native state, the separation process was carried out with Peltier coolers regulating at 7°C.

3.2.2. Charge separation

A higher degree of purity for each antibody can be achieved in a charged-based second step carried out at a pH near the p*I* of each antibody. As the isoelectric points vary, this meant a different pH was selected for each antibody. During the separations using either size or charge, there was no evidence that the solubility of each target antibody was affected by a pH higher than its p*I* or near its p*I*. The solubility of the proteins remained excellent with these operating conditions and choice of buffers used. Each different antibody solution was adjusted with acetic acid to the pH that was close to the p*I* prior to a charge separation. The membrane used for the charge separation was a M_r $1 \cdot 10^6$ pore size to increase the speed of removal of the contaminating proteins. Under these conditions the monoclonal antibody, now uncharged remained upstream and the contaminating negatively charged proteins migrated downstream. SDS–PAGE (Fig. 5) indicated that a high degree of purity was achieved for all antibodies using this second step. The relatively high p*I* of antibody 1 allows this antibody to be purified in a single step at pH 8.3 using a M_r $1 \cdot 10^6$ pore size membrane (instead of 100 000). We expected this antibody to migrate through the M_r cut-off $1 \cdot 10^6$ membrane because it does have a small negative charge at this pH but for short runs of less than an hour, it remained upstream. Indeed with longer separation times (1–2 h) some migration downstream was noted.

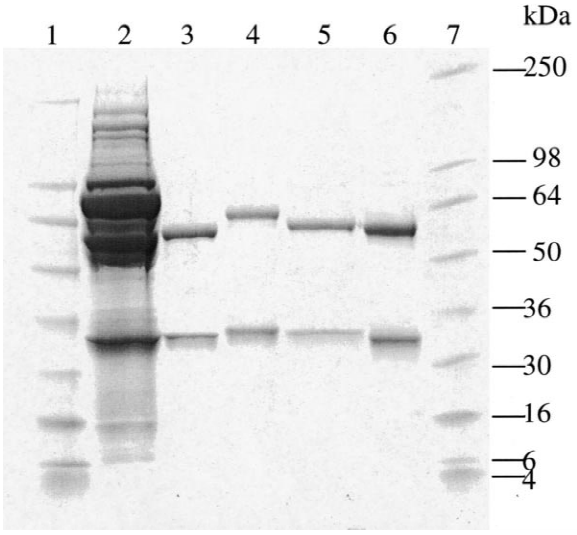

Fig. 5. SDS–PAGE of antibodies after the optional second step, involving the use of a pH close to the p*I* of each antibody. Lanes 1 and 7 contain SDS–PAGE molecular mass markers. For contrast, the original starting material for antibody numbered 1 is placed in lane 2 while lanes 3–6 are the four purified antibodies.

3.3. Recovery

The recovery of the monoclonal antibodies was determined by comparing the final biological activity of the antibody present upstream, downstream and in the washes with the starting material (Table 1). Although recoveries for antibodies 2, 3 and 4 (using two-stage purifications) were less than for antibody 1 (Table 1), quantities of >8 mg of each antibody were recovered per ml of ascitic fluid. This seems higher than the expected yields for other purification procedures [5–7,16,17]. These higher activities recovered may be due to a combination of the mild buffers used, the relatively short distance required for separation to occur within the separation cartridge and the short purification times.

The protocol developed takes advantage of the large size (M_r 160 000) of the monoclonal antibodies relative to other proteins in ascitic fluid and their relatively high p*I* values compared to other proteins in murine ascitic fluid. It remains to be established whether different antibodies or other isotypes, e.g., IgA, IgM or IgG3 can be purified with this general procedure, but these early results suggest that further investigation is warranted. The simple protocol described here may be less complex than conventional chromatographic purification techniques where more variables need to be considered [20–22,25]. However recent developments including those leading to the production of better resins [23] can make the chromatographic technique less iterative and time consuming [24].

The immunoglobulin fraction is usually about 2–25% of the total protein in ascitic fluids. The high content of lipid present in ascitic fluid is known to reduce the life of fractionation columns [18,19]. In our experiments, we were able to achieve excellent purification and recovery without the need to delipidise or pretreat the samples [26,27].

Although only small amounts (10–20 mg) of each target antibody were purified in this preliminary

work, linear scale up from the 15 cm^2 membranes (used here) to 200 cm^2 has been accomplished previously for haemoglobin/albumin separations [28]. Preliminary scale up from 2 ml to 10 ml of mouse ascites fluid on the 15 cm^2 cartridges showed incomplete removal of contaminants, indicating that a longer processing time or a larger separation cartridge may be required for larger quantities of proteins.

In conclusion, we have described a rapid and simple protocol for purifying antibodies from ascitic fluid with high yields. It will be interesting to see whether these conditions can be extended to a wider range of antibodies and whether scaled up versions of the technology can reproduce the high yields and convenience of the small-scale instrument.

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