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Rapid purification and monitoring of immunoglobulin M from ascites by perfusion ion-exchange chromatography

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Abstract

A purification and on-line monitoring procedure for IgM was developed. Perfusion ion-exchange chromatography was used for rapid purification of IgM from ascites fluid and hybridoma supernatant. Crude ascites was directly loaded onto an ion exchanger. Due to the complexity of IgM, a two-step ion-exchange procedure had to be developed. This procedure involved a rapid cation-exchange chromatography capture step followed by further purification using anion-exchange chromatography. High linear velocities, in excess of 3500 cm/h, enabled separations to be performed under 5 min. Purity of the final product by SDS-PAGE was shown to be greater than 95%. Furthermore, the antibodies retained biological activity as measured by indirect immunofluorescence (IIF) and ELISA. The IgM peak was also monitored on-line using a novel *peak tracking* approach. This involved placing an antibody column (specific to the IgM) prior to the ion-exchange column and operating the ion-exchange column with and without the antibody column in-line. The missing peak that is identified by comparing the two chromatograms indicates where the IgM elutes.

Keywords: Immunoglobulins; Monoclonal antibodies

1. Introduction

Monoclonal antibodies have proven to be useful molecules for therapeutic and diagnostic applications. Purification of these molecules is a challenge due to the complexity of the molecule itself and the source from which it has to be purified. Furthermore, the purification protocol has to be such that it does not in any way alter or decrease the biological activity of the antibody.

In this paper we report on a rapid, dual column, procedure for purification of Mab 46.3, an IgM subclass murine antibody. Mab 46.3 has been identified as having a high specificity for colon carcinomas relative to the surrounding normal mucosa and is used as a marker for early detection of breast and colon cancer. A radioimmunoassay with ^{125}iodine-labeled IgM is used for detection of the cancer. Highly pure and biologically active antibody is therefore required for this assay.

Purification of IgM has proven to be very difficult due to the heterogeneity, size (M_r $900 \cdot 10^3$) and the unstable structure of the molecule. Several purification protocols have been described, most of which result in poor yields and immunoreactivity. Purification protocols in the literature have included precipitation followed by various forms of chromatography [1–3]. A non-chromatographic, *plasmapheresis* approach was developed by Tatum [4] and was shown to work quite effectively for purification of gram scale quantities of IgM. This approach utilized sequential precipitation of the IgM with ammonium

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sulfate and polyethylene glycol. Although precipitation does lead to enrichment of the sample, it is time consuming and can cause significant amounts of aggregation. Affinity purification has also been used in the past to purify IgM. Nethery et al. immobilized a complement protein C1q [5], Nevens et al. employed mannan binding proteins for affinity purification of IgM [6]. In spite of the relatively high specificity of these approaches, there is always some amount of cross reactivity, the mass capacity is low (typically less than 1–2 mg/ml of resin), the immobilized species can leach, the supports need to be re-generated frequently and they tend to be very costly. A recent article by Roggenbuck et al. demonstrated a two-step approach, HIC followed by SEC, for purifying IgM [7]. SEC was used as a polishing step to purify out the l chain dimer and traces of albumin that were found to co-elute during the HIC step. Bouvet and Piers [8] have used a single-step SEC approach for purifying IgM from the albumin. They used a high molarity buffer to increase the retention time of the IgM relative to the albumin via non-specific interactions. Though SEC can be effectively used to purify the IgM based upon large differences of its hydrodynamic size relative to that of the other impurities, it has potential limitations. IgM is known to precipitate in low salt and, as pointed out by the authors, there are recovery problems due to non-reversible adsorption to the SEC column. Also, the mass loadability on a SEC support is very low and the resolution is significantly affected by increasing the linear velocity of the mobile phase or increasing the mass of the sample loaded onto the column.

A two-step approach to purifying IgM from ascites fluid is presented. The separations were performed very rapidly by perfusion chromatography using Poros media. IgM could be eluted within 2–3 min by using a linear velocity in excess of 3000 cm/h.

2. Experimental

2.1. Reagents and chemicals

Mab 46.3 IgM isotype, raised against secreted colon cancer cells from hybridoma grown in mice as an ascitic tumor and cell culture hybridoma supernatant, was provided by Rhode Island hospital, Department of Medical Oncology. Trizma-base and Bis-Tris propane were from Sigma (St. Louis, MO). Mono and di-sodium phosphate and NaCl were from J.T. Baker (Phillisburg, NJ). Goat anti-mouse IgM antibody was purchased from Jackson Immunoresearch, (Westgrove, PA)

2.2. Chromatography

All the chromatograms were generated on a BioCAD-60 (PerSeptive Biosystems, Framingham, MA) equipped with a dual wavelength UV detector. The columns were as follows: Cation exchanger, 100 mm×4.6 mm I.D., 20 μm, POROS HS/M PEEK column; Anion exchanger, 100 mm×4.6 mm I.D., 20 μm, POROS HQ/M Peek column, and an aldehyde activated POROS, 30×2.1 mm. All the columns were from PerSeptive Biosystems.

2.3. Procedures

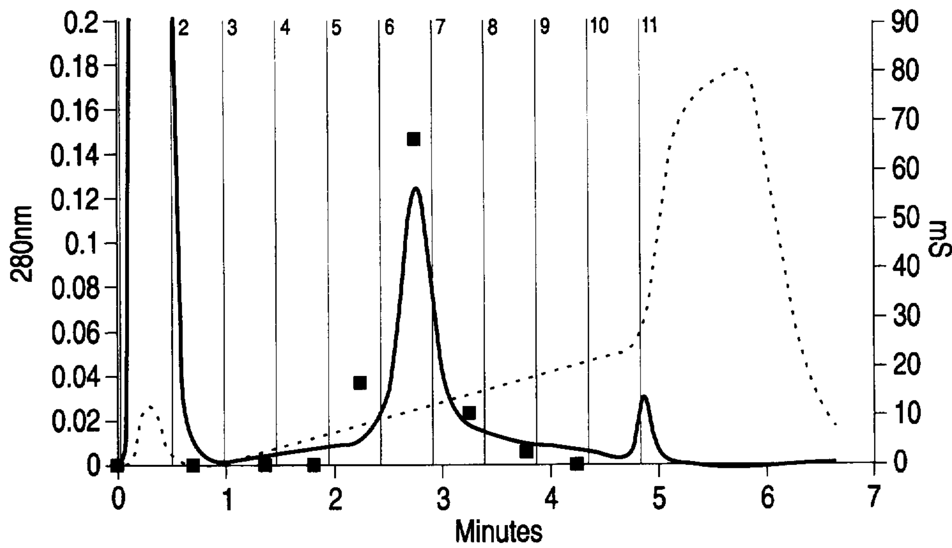
2.3.1. Chromatography

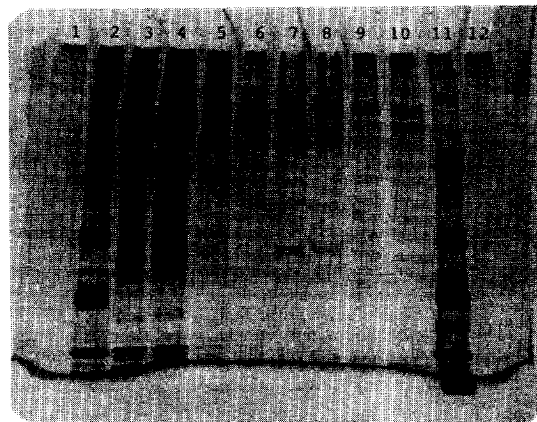
The mobile phase used for cation-exchange chromatography was A: 20 m*M* NaH_2PO_4, B: 20 m*M* Na_2HPO_4; C: deionized water; D: 3 *M* NaCl. The software in the system is capable of mixing appropriate amounts of the A, B and C buffers to generate the desired pH. pH 6.5, used for the separation, was optimized by running a *pH map* template in the BioCAD software. Crude ascites (2 ml, 1:5 dilution with PBS) was loaded directly onto a cation-exchange column. A gradient from 0–350 m*M* NaCl was run over 20 column volumes at flow-rate of 8.5 ml/min. Two ml fractions of the IgM peak were collected and based on the immunoactivity, determined by ELISA and gel, the fractions with substantial amounts of IgM were further purified by anion exchange.

For the anion-exchange chromatography, buffers A and B were replaced by Tris/Bis-Tris, pH 6 and 9 respectively. A dual, salt and pH, gradient form 200–350 m*M* NaCl and pH from 8–6 was run over 15 column volumes at 10 ml/min.

2.3.2. Construction of the Mab 46.3 and fluid production

The hybridoma secreting Mab 46.3 was constructed using immune spleen cells isolated from mouse immunized with antigens shed by the clone-D





SDS gel of the cation exchange fractions

Lane 1	blank	Lane 7	fraction 5
Lane 2	high molecular mass standards	Lane 8	fraction 6
		Lane 9	fraction 7
Lane 3	load	Lane 10	fraction 8
Lane 4	fraction 1	Lane 11	low molecular mass standards
Lane 5	fraction 2		
Lane 6	fraction 4	Lane 12	blank

Fig. 1. Cation-exchange chromatography as compared with SDS gel electrophoresis. (Top) Cation exchange of murine ascites on Poros HS/M 100×4.6 mm column. Gradient 0–350 m*M* NaCl over 20 column volumes, pH 6.5 with phosphate buffer; flow-rate: 8.5 ml/min.; sample: 2 ml of 1:5 diluted ascites. (Bottom) SDS-silver stained gels of the cation-exchange fractions.

of human colon carcinoma cell line (AATC, Rockville, MD). A female BALB/c mouse was immunized five times during a five month period with 100 μg of antigens. Immune spleen cells were fused with PAI myeloma cells. The reactivity of Mabs secreted by hybridomas growing in a selective medium was assessed by indirect immunofluorescence (IIF) analysis of acetone fixed frozen sections of normal colon

and primary colon carcinomas. Hybridomas displaying reactivity with primary colon carcinoma were cloned by limiting dilution and positive cultures were recloned in soft agar. The hybridoma cells were then injected into pristans primed BALB/C mice to produce ascitic fluid. The ascitic fluid was collected and clarified of cell debris by centrifugation.

2.3.3. Indirect immunoassay

Clone D of DLD-1 human colon cancer cells cytospins or frozen sections 4 to 6 μm thick were mounted on glass slides, acetone fixed and stained with Mab 46.3. A fluorescence labeled, goat anti-mouse IgM (Sigma), was used as the secondary antibody. Non-specific staining was assessed by examining tissue sections stained with culture supernatants from P3X63 Ag8 myeloma cells. Sections were examined using a Nikon Microphot FX equipped with epifluorescence.

2.3.4. ELISA

Cell lysate of DLD-1 human cancer colon cells, approximately 1 μg/well, was diluted in a bicarbonate buffer and immobilized on a 96 well Immulon-4 ELISA plate (Dynatech) as antigen. After blocking with 5% BSA, primary antibody (Mab 46.3) was incubated for 2 h at 37°C. Peroxidase-conjugated goat-anti mouse IgM (u-chain specific) antibody (TAGO immunologicals) was the secondary antibody. ABTS substrate (Zymed) dissolved in 0.1 *M* citrate buffer, pH 4.2 was added to visualize the color change. The absorbance was measured in a Microtek ELISA reader using a 405 nm filter. This

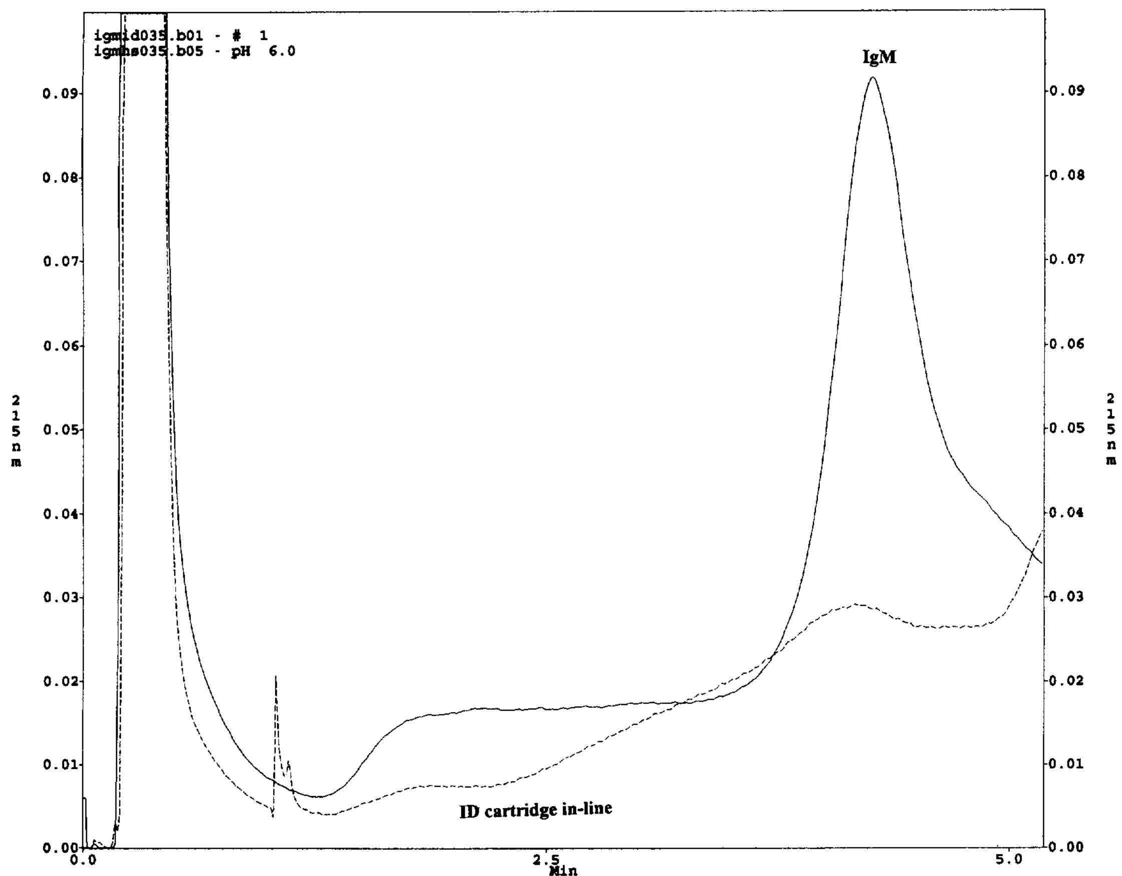
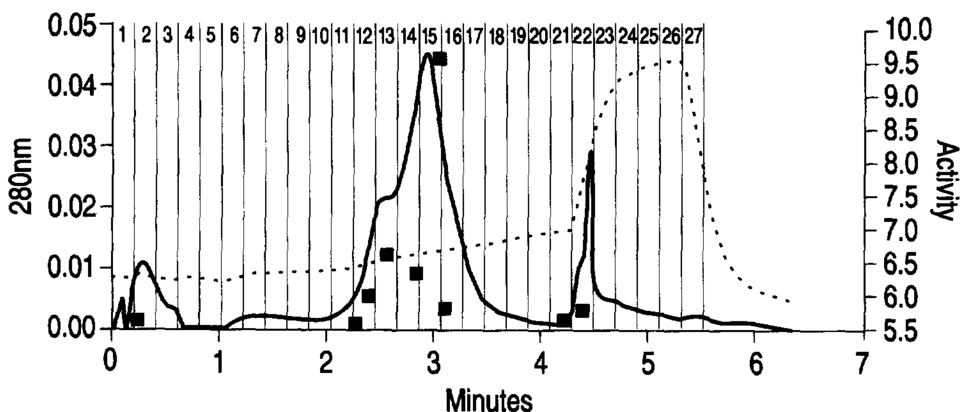


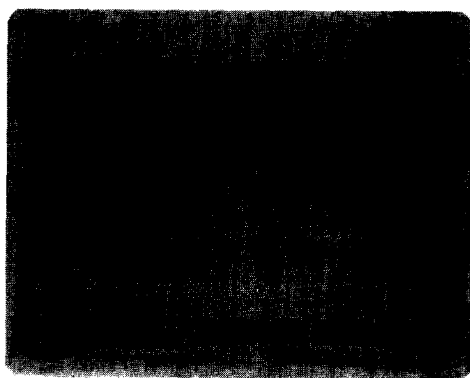
Fig. 2. Subtractive assay of IgM. Same conditions as in Fig. 1a.

procedure was used to analyze the HPLC fractions to determine the activity.

2.3.5. On-line, subtractive assay for IgM

The subtractive assay was performed by using a goat anti-mouse IgM immobilized antibody column placed upstream of the ion-exchange column. The goat anti-mouse IgM antibody was immobilized onto a aldehyde activated Poros column. The loading conditions have been outlined elsewhere [9]. Chromatograms were generated with and without the antibody column in-line. The two chromatograms were compared and the missing peak(s) were attributed to the antibody of interest.





SDS gel of anion exchange analysis of fractions 6 and 7 from cation exchange

Lane 1	high molecular mass standards	Lane 7	fraction 13
Lane 2	load	Lane 8	fraction 14
Lane 3	fraction2	Lane 9	fraction 15
Lane 4	fraction 3	Lane 10	fraction 16
Lane 5	fraction 7	Lane 11	fraction17
Lane 6	fraction 12	Lane 12	low molecular mass standards

Fig. 3. Analysis of fractions 6 and 7 from the cation-exchange step. (Top) Anion exchange of the of the fractions 6 and 7 from cation exchange. Column: HQ/M, 100×4.6 mm, 20 μm, gradient: 200–350 mM NaCl over 15 column volume, pH 8–6; sample volume: 2ml. (Bottom) SDS-silver stained gel of the anion-exchange analysis of fractions 6 and 7 from cation exchange. Fraction numbers indicated on top of Fig. 3a.

3. Results and discussion

One of the major goals in antibody purification is to recover the maximum amount of antibody with minimal loss in the biological activity. During chromatography there is a significant risk of labile molecules denaturing from extended contact time with the activated support used for separation. One way to minimize this would be to increase the linear velocity of the mobile phase which in turn would decrease the retention time of the molecule. However, with conventional porous supports, increasing linear velocity can have an adverse effect on the separation. Due to inefficient mass transfer in porous supports at higher linear velocities, there will be a significant amount of peak broadening and consequently a loss in resolution.

Perfusion chromatography has been shown in the past to be ideally suited for purification of large biomolecules [10–12]. The design of the pore facilitates rapid mass transfer with minimal band broadening. Since the diffusion rate of molecules to and from the pores is inversely proportional to the size of the molecule, inefficient mass transfer is a serious limitation for large molecules in conventional (non-perfusive, microporous) chromatography. Perfusive supports have large through-pores, which carry sample to and from the pores by convective flow, and short diffusive pores (<1 μm) which significantly reduce the time required for the sample to diffusive from the internal binding sites.

In the process of developing a method for purifying IgM we tried both, anion- and cation-exchange chromatography. The pH for each of the separations

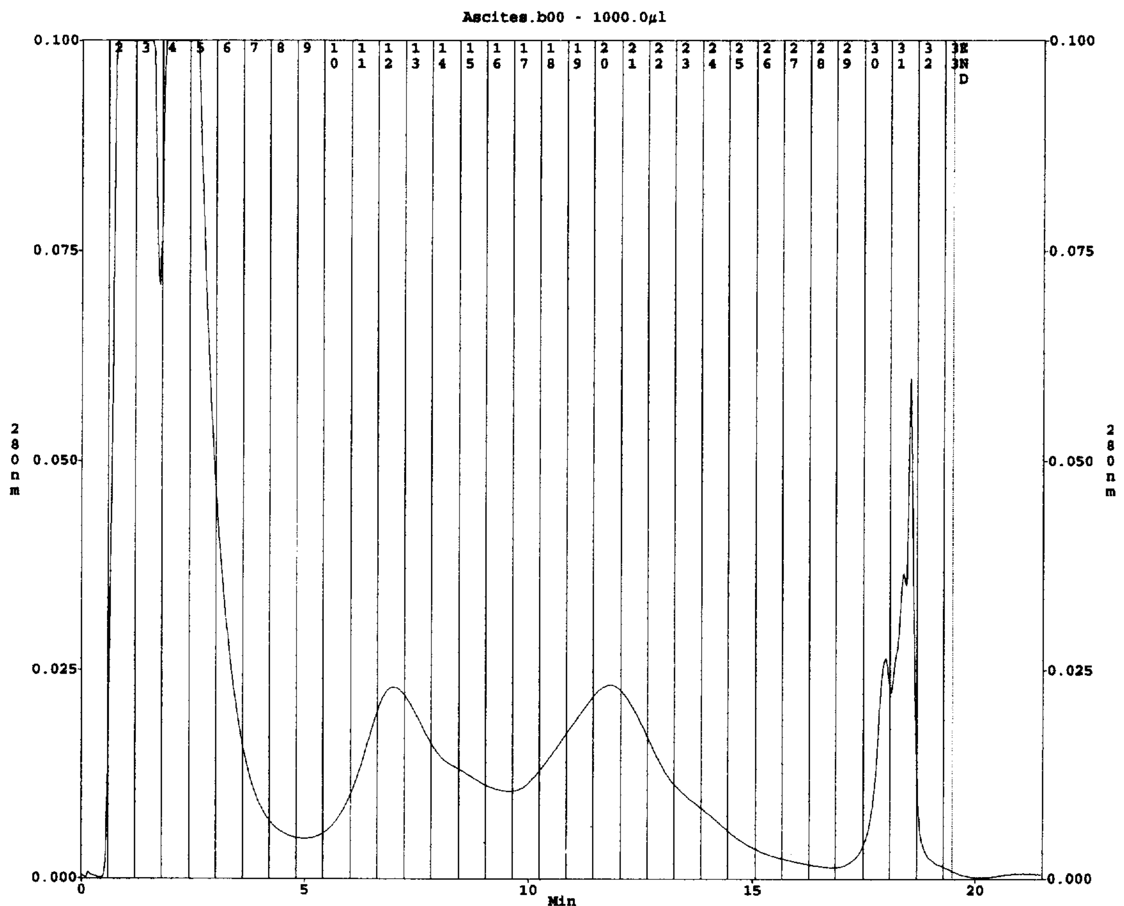
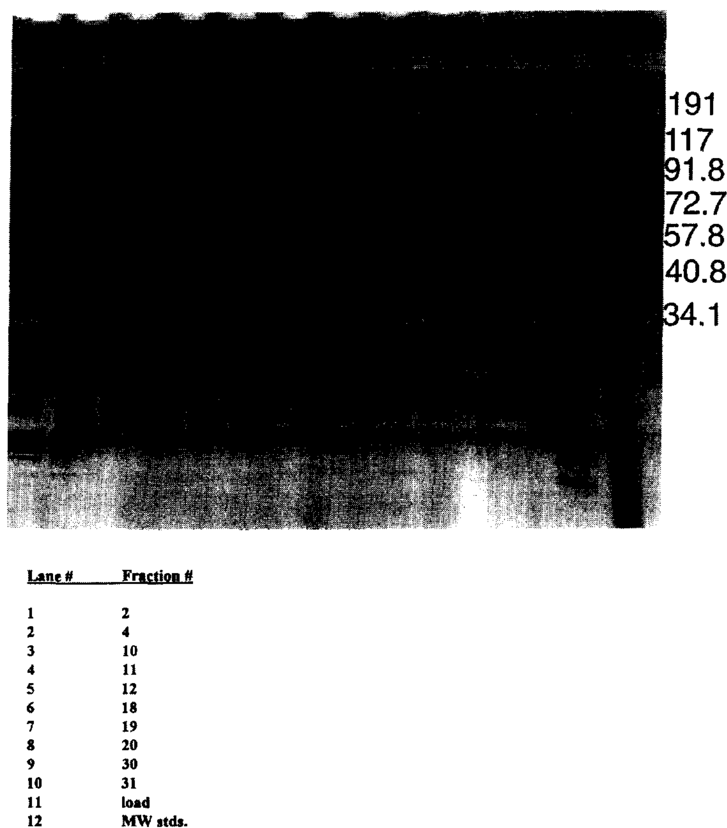


Fig. 4. (*continued on p. 169*)



Lane #	Fraction #
1	2
2	4
3	10
4	11
5	12
6	18
7	19
8	20
9	30
10	31
11	load
12	MW stds.

Fig. 4. Anion-exchange chromatography and SDS gel electrophoresis of the isolated fractions. (Top) Anion-exchange purification of the 1:5 diluted crude ascites. Same conditions as in Fig. 3a. (Bottom) SDS-silver stained fractions of the anion-exchange chromatogram. Fraction numbers indicated on top of Fig. 4a.

was optimized by using an automated *pH template* in the BioCAD software. Cation exchange proved to be the most efficient for isolating the IgM from ascites fluid. The peak of interest was well resolved from the sample matrix. Fig. 1a. shows the cation-exchange purification of IgM. A gel was run to determine the presence of other impurities within this peak. The gel, shown in Fig. 1b., revealed the existence of an additional impurity. Superimposed upon the chromatogram is an activity trace obtained by running an ELISA on the various fractions of the peak. The fractions with high levels of activity and relative purity (fractions 6 and 7) were collected for further purification. Fig. 2 shows the chromatograms from the subtractive immunoassay using a goat anti-mouse IgM antibody cartridge placed in-line with the cation-exchange column. The on-line monitoring is a quick and easy way of tracking the peak of interest especially in a more complex chromatogram and was used during the method development stages of the purification This eliminated the need for collecting fractions and running gels for every single chromatogram.

Fig. 3a shows the anion-exchange purification of fractions 6 and 7. Two mls of the total pool (4 mls of fraction 6 and 7) were injected onto an anion-exchange column and eluted using a combination of a salt and pH gradient from 200 to 350 m*M* NaCl and Tris/Tris-base, pH 8–6. The flow-rate was 10 ml/min. The anion-exchange purification (Fig. 3a.) shows the presence of an additional impurity which could not be resolved on the cation exchanger. ELISA indicated that the shoulder was an active form of the IgM and it is quite likely an isoform of

the antibody. Fig. 3b shows the silver stained SDS-PAGE gel of the various fractions collected from anion-exchange chromatography. The heavy and light chain of IgM is evident from the gels with minimal amounts of other impurities present. IIF microscopy data (not shown) indicated that the biological activity was retained during the purification process. A single step purification was also attempted using the anion-exchange conditions and the results are shown in Fig. 4a. An SDS gel (Fig. 4b) for the various fractions of the chromatogram indicated that the single-step purification was obviously not as efficient as the dual column approach. However, depending upon the purity requirements, IgM could be purified by a single-step procedure.

By utilizing high flow-rates, the two-step procedure could be completed within 10 min. After having identified the IgM in the cation-exchange chromatogram via a subtractive immunoassay, and having determined the activity by ELISA, subsequent purifications were done by directly loading the collected peak from the cation exchanger onto the anion exchanger. Since the IgM peak is well resolved from the impurities, this method can be easily scaled-up. Also, since the analysis time for the entire procedure is very short (less than 10 min. for the two steps) larger amounts of sample can be obtained by simply repeating the procedure several times. This will not be a practical approach for separations done on conventional supports since the run times are typically 30–40 min in duration and 60–70 min if equilibration is included.

4. Conclusion

In this study we have demonstrated a rapid and straightforward method for the purification of IgM. The rapid elution off the chromatographic support is ideal for purification of labile molecules such as IgM which can aggregate or can be denatured either in high salt or from extended contact with the chromatographic media. The strong anion- and cation-exchange supports used for this purification were *fimbriated* supports with the activated sites extending from the coated bead. The protein therefore does not come into contact with the base bead and this eliminates any type of non-specific interaction. In most conventional supports, denaturation and non-specific interaction is enhanced due to extended contact time with the surface.

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