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PURIFICATION OF MONOCLONAL ANTIBODIES BY COMPLEX-DISPLACEMENT CHROMATOGRAPHY ON CM-CELLULOSE[a]

ANTHONY R. TORRES* and ELBERT A. PETERSON
Bio-Fractionations, 1725 S. State Highway 89–91, Logan, UT 84321 (U.S.A.)

SUMMARY

A single high-capacity chromatographic procedure was used to purify a monoclonal antibody from ascites fluid. A new displacement procedure involving a displacer protein complex is described. The scale-up from 1 ml to 450 ml of ascites sample was easily accomplished with this complex-displacement system. A monoclonal antibody recovery of 79% was measured for the largest-scale purification.

INTRODUCTION

There is an increasing demand for purified monoclonal antibodies. These highly specific antibodies[1] have found uses in many fields, including agriculture, biochemistry, medicine, immunology, microbiology, pharmaceutics and virology. In addition, larger amounts (g to kg/year) are required, as diagnostic and therapeutic applications expand. At the present time, chromatographic methods offer the best means of purification and there are several methods which work well for small-scale purifications[2–7]. However, the purification of large amounts of monoclonal antibodies remains a challenge[8]. The use of displacement chromatography seems advantageous in monoclonal antibody purification, since high resolution, high capacity and high recovery are important for expensive proteins.

The advantages of displacement chromatography in the separation of small molecules were pointed out by Tiselius[9] as early as 1943 and this approach has been pursued by Porath and Li[10] as well as other investigators[11] for protein separations. However, because of the lack of suitable spacing displacers, separations of complex protein mixtures were inadequate and the method was not considered useful. The first successful application of protein displacement chromatography was published by Peterson[12] in 1978 with the description of carboxymethyldextran (CM-D) displacers, both as final displacers and spacing displacers for anion-exchange columns. It was subsequently shown by Torres and Peterson[13] that the chromatographic behavior of model proteins in such a system matched that observed with small molecules in established displacement systems. This was an important observation, as it showed that these CM-Ds were classical displacers as described by Tiselius in the theory of

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displacement chromatography. The high resolution and high capacity of the CM-D displacement system were demonstrated with simple protein mixtures, such as the genetic variants of the very similar β-lactoglobulins A and B[14]. Chromatography was first performed on classical cellulosic ion exchangers and then on high-performance liquid chromatography (HPLC) columns under very similar conditions. The separations of 12.8 mg of the β-lactoglobulins A and B on a 150-μl HPLC anion-exchange column was the first demonstration of a separation of proteins by ion-exchange displacement chromatography by this column technology[15]. Horváth's laboratory has more recently repeated the separation of these two proteins on an HPLC column, using a natural anionic carbohydrate, chondroitin sulfate as a final displacer[16]. Their separation was accomplished without adding spacing displacers and relied on protein protein displacement. Although protein protein displacement can be useful, as discussed in the early years[17], spacing displacers will usually be necessary to separate proteins from complex biological mixtures.

The use of spacing CM-D displacers to isolate proteins from complex mixtures has been demonstrated with the separation of Gc-globulin from human serum[18], alkaline phosphatase from *E. coli* periplasm[19], and the resolution of an artificial mixture containing ovalbumin and other proteins[20]. The displacement separation of Gc-globulin represents a good model for a difficult separation, as serum contains well over a 100 proteins, and previous researchers have used up to 11 chromatographic steps to purify Gc-globulin[21]. The separation of *E. coli* alkaline phosphatase clearly shows that high-capacity separations can be achieved in displacement chromatography as the column was 80% saturated with protein sample before the displacer was applied.

In this paper, a new type of displacement chromatography is described. The same negatively charged CM-Ds used on anion-exchange columns were used as displacers with cation-exchange columns. However, the displacer in the latter case binds to the immobilized proteins and not the adsorbent. When an adequate amount of displacer has been bound to the protein, the protein is released from the column as a CM-D–protein complex. The single-step purification of mg to g amounts of a monoclonal antibody from mouse ascites fluid is described.

EXPERIMENTAL

Materials

Kontes (Vineland, NJ, U.S.A.) supplied the columns (5 cm × 2.5 cm I.D., 10 cm × 4.7 cm I.D., and 20 cm × 9 cm I.D.), column end-plates and mobile phase reservoirs. The smallest column (3 ml) was constructed from a 3-ml polypropylene syringe. The solutions, including the sample, for the last experiment (Fig. 4) were applied through a Kontes six-valve manifold and a Kontes Chromoflex LC pump. Flow-through pH and conductivity meters (Kontes) were used for selected experiments. Columns were packed with Whatman CM-52 (Clifton, NJ, U.S.A.). An Isco (Lincoln, NE, U.S.A.) UA-5 detector and recorder and an Isco Foxy fraction collector were used. Goat anti-mouse immunoglobulin G (IgG) (heavy and light chain) whole serum was obtained from Hyclone Lab. (Logan, UT, U.S.A.). The CM-D (reciprocal of the pellet volume, RPV = 25) was manufactured by Bio-Fractionations (Logan, UT, U.S.A.). An RPV of 25 indicates a high incorporation of carboxymethyl groups[14].

Sample preparation

Mouse ascites fluid containing an IgG_{2b} monoclonal antibody (4.6 mg/ml) was centrifuged at 500 *g* for 15 min to remove particulate matter. The ascites fluid was then filtered through Whatman No. 2 filter paper and finally through a 0.45-μm filter.

The ascites samples were diluted ten-fold with a 20 m*M* histidine · HCl, pH 5.7, buffer in order to decrease the normal saline to a level which would not interfere with the chromatographic process. For example, 1 l of 0.1 *M* histidine (free base) was added to a 500-ml ascites sample, then the mixture was adjusted to pH 5.7 with 1 *M* hydrochloric acid and diluted to 5 l with water.

Column chromatography

A 20 m*M* histidine · HCl buffer (pH 5.7, column buffer) was used to equilibrate the ion-exchange columns. The CM-D was used as a 1.5% solution in the same buffer. All buffers, displacers, and salt solutions were filtered through sterile 0.22-μm filters into sterile, autoclaved reservoirs for the last experiment (Fig. 4). This was done to decrease the chances of contaminating the purified antibody with endotoxin or bacteria. The flow-rate was set for 2–3 column volumes/h and fractions equal to *ca.* 1 column volume were collected. After each experiment, the column was washed with 1 column volume of 1.0 *M* sodium hydroxide, followed by several column volumes of water. The columns were equilibrated with 20 m*M* histidine · HCl (pH 5.7) when ready for reuse.

Gel electrophoresis

The Pharmacia (Piscataway, NJ, U.S.A.) PhastSystem with 10–15% gels and sodium dodecyl sulfate (SDS) buffer strips and the standard method from the PhastSystem manual were used to analyze the protein components. Samples were diluted with water to give an absorbance of *ca.* 1 at 280 nm. The diluted samples (10 μl) were mixed with 2 μ of 10% SDS and placed on a block at 95°C for 5 min. The microcentrifuge tubes were spun for 5 s in a microcentrifuge to move condensate to the bottom. The sample strip for application of 0.5 μl of each sample was used. The gel was stained with Coomassie Brilliant Blue R 350 (Pharmacia) according to the standard method in the PhastSystem manual.

Protein quantitation

The protein concentrations for the purified monoclonal antibodies were determined spectrophotometrically, using an extinction coefficient of 15 for a 1% solution at 280 nm with a 1-cm pathlength.

Nephelometry

A Beckman (Brea, CA, U.S.A.) Auto ICS rate nephelometer was used to measure the amount of mouse IgG in the chromatographic fractions (circles in Fig. 3). A 1: 16 dilution of goat anti-mouse IgG (heavy and light chain) whole serum (42 μl) was used with 10 μl of column fraction, following the Beckman instructions for manual operations. This instrument measures the rate of change of light scatter resulting from an immunoprecipitation reaction between antigen and antibody. Although this method can be used to determine concentrations, it was used in this project only to identify those fractions containing mouse IgG. The circles in Fig. 3 show the relative rate units

for each fraction. Background polyclonal antibodies present in ascites fluid could not be distinguished from the monoclonal antibody with this method as used.

CM-D determination

Small portions (50 μl) of the column fractions (Fig. 3) were allowed to react with anthrone[22] and then read at 625 nm to provide a measure of the CM-D carbohydrate concentration.

RESULTS AND DISCUSSION

A cation-exchange column was selected, because immunoglobulins are among the proteins most tightly bound to this type of sorbent and provides the highest capacity for purifying this class of proteins. Instead of a classical displacement system with positive displacers competing with the sorbed proteins for column sites, the same negatively charged displacers (CM-Ds) were employed that are used with anion-exchange columns. Under these conditions, the displacer is not sorbed by the negatively charged sorbent but forms complexes with proteins immobilized on the sorbent. When sufficient amounts of displacer are bound to the protein, the complex is released from the column. It was found that contaminating proteins could be removed as protein–CM-D complexes without removing the antibody. Transferrin was the critical contaminant, since it was present in relatively large concentrations and is the most difficult protein to separate from monoclonal antibodies by ion exchange[23]. After washing the unbound displacer from the column with column buffer, the monoclonal antibody was eluted with phosphate-buffered saline.

Figs. 1–4 show the separation of 1 ml, 8 ml, 65 ml and 450 ml of ascites fluid by complex-displacement chromatography on 3-ml, 20-ml, 160-ml, and 1200-ml CM-cellulose columns, respectively. The results in these four experiments were very similar and demonstrate the ease of scale-up. The first large peak in the chromatogram contained several unbound proteins, mainly albumin. Some of these had little or no

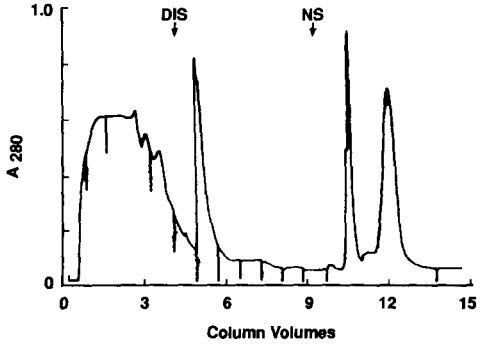

Fig. 1. Purification of a monoclonal antibody from 1 ml of ascites fluid. A 1-ml volume of ascites fluid was chromatographed on a 3-ml column of CM-cellulose. The column was equilibrated with 20 m*M* histidine · HCl (pH 5.7). The 1.5% displacer also contained 20 m*M* histidine · HCl (pH 5.7). The arrows show the application of the displacer and 0.15 *M* sodium chloride containing 10 m*M* sodium phosphate (pH 7.4). The column effluent was monitored at 280 nm at 2 a.u.f.s. DIS = Displacer; NS = phosphate buffered saline.

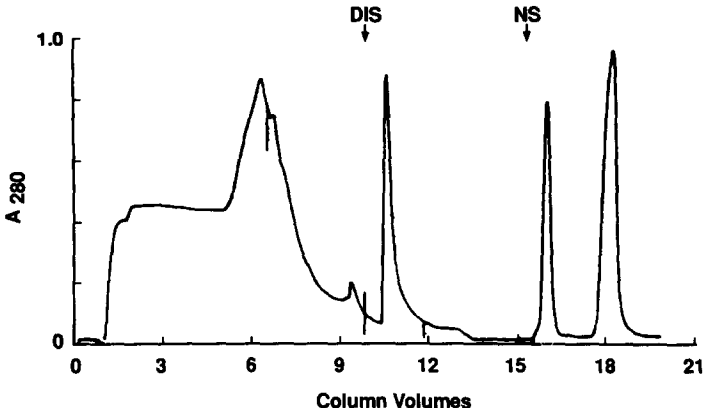

Fig. 2. Purification of a monoclonal antibody from 8 ml of ascites fluid. Conditions as for Fig. 1 except 8 ml of ascites fluid was chromatographed on a 20-ml column.

affinity for the sorbent under these conditions, but others were displaced by more tightly bound proteins that remained on the column.

The direct dilution of the normal saline (0.15 *M* sodium chloride) in ascites fluid with 9 volumes of 20 m*M* histidine · HCl (pH 5.7) allowed the binding of transferrin, the monoclonal antibody, and other proteins. It also obviated a dialysis or desalting step, which is often necessary before column chromatography. The samples were followed by column buffer (2–3 column volumes) and a 1.5% displacer (1–3.3 column volumes) in the same buffer. One column volume was found to be as effective as larger

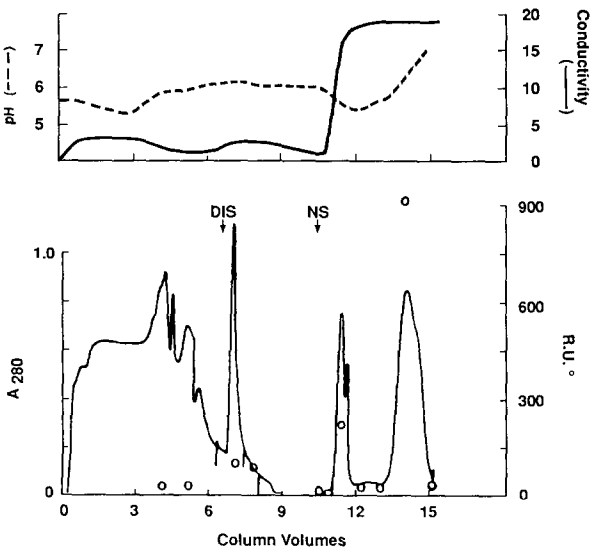

Fig. 3. Purification of a monoclonal antibody from 65 ml of ascites fluid. Conditions as for Fig. 1 except 65 ml of ascites fluid was separated on a 160-ml column. The circles show the rate units (R.U.) or relative IgG levels. Conductivity (in mS) and pH tracings are shown above.

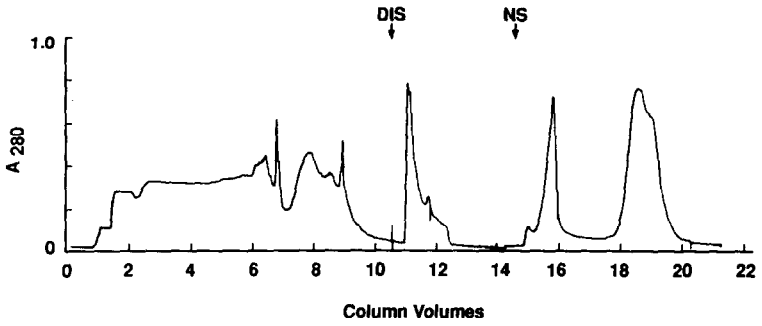

Fig. 4. Purification of a monoclonal antibody from 450 ml of ascites fluid. Conditions as for Fig. 1 except 450 ml of ascites fluid was separated on a 1200-ml column.

amounts. The displacer removed large amounts of transferrin and other proteins. The column was then washed with 2–3 column volumes of buffer to remove unbound displacer. This step was quite effective in removing displacer, as shown by a decrease in dextran concentrations from 7.1 mg/ml in fraction 8 to 0.035 mg/ml in fraction 12 (Fig. 3), as measured by the anthrone reaction. After this buffer wash, normal saline (0.15 *M* sodium chloride), containing 10 m*M* sodium phosphate (pH 7.4) was pumped into the column. The salt front eluted some transferrin and immunoglobulin. The last peak, containing the monoclonal antibody, was eluted when the effluent pH increased due to the phosphate buffer (Fig. 3). The pH increase followed about 2 column volumes behind the normal saline, as the dilute sodium phosphate buffer interacted with the sorbent and the remaining protein. This delayed pH increase was beneficial, as it allowed a more tightly bound transferrin (fraction 16, Fig. 4) to be separated from the monoclonal antibody. Transferrin is an iron-binding glycoprotein, which can contain one or more negatively charged sialic acids[24]. These negative charges as well as the amount of bound iron alter the isoelectric point and column affinity of transferrin.

Samples from the experiment shown in Fig. 4 were examined for purity by gel electrophoresis in Fig. 5. The gel photograph in Fig. 5 is representative of all the experiments. Lane 1 shows the proteins in the ascites fluid sample. Lanes 2 and 3 show the proteins present in the first peak (mainly albumin). Lane 4 contains the proteins in the second peak (fraction 12) after the displacer addition (mainly transferrin), lane 5 contains the proteins in the third peak (fraction 16) after the normal saline addition and the last three lanes show the purity of the monoclonal antibody from the last peak (fractions 18–20). A single chromatographic step thus resulted in a high-purity monoclonal antibody that was contained in normal saline with 10 m*M* sodium phosphate. A 79% antibody recovery was calculated from the ascites fluid used for Fig. 4: 1.63 g of purified monoclonal antibody from 2.07 g of sample.

The scale-up from 1 ml to 450 ml of ascites fluid was easily accomplished. Scale-up was limited by the amount of ascites fluid available and not by the chromatographic process. It should be possible to purify much larger amounts, if the mechanical stability of the sorbent in larger columns permits. Although there would be little advantage, this procedure should also work with HPLC cation-exchange columns.

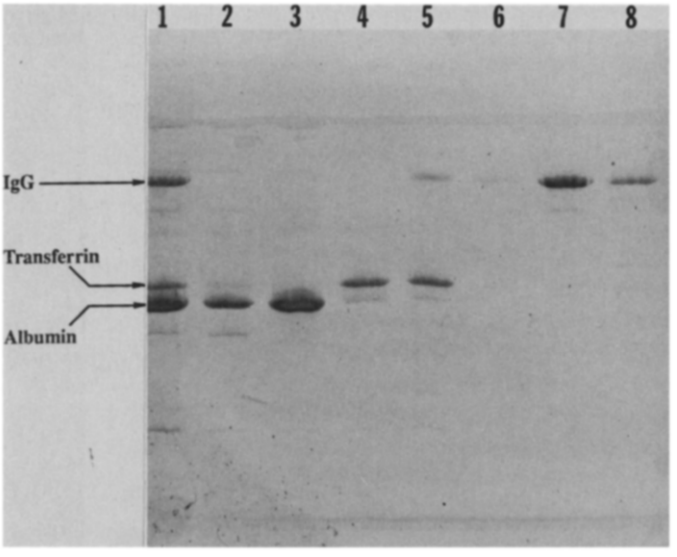

Fig. 5. Examination of column fraction by polyacrylamide gel electrophoresis. Fractions from Fig. 4 were subjected to electrophoresis in the presences of SDS on a 10–15% gel. Lane 1 shows the proteins in the ascites standard, lanes 2 and 3 show the proteins in the first peak (fractions 6 and 8) and lane 4 shows the proteins in the second peak (fraction 12) after the displacer addition (largely transferrin). Lane 5 shows the proteins in the peak (fraction 16) just after the addition of normal saline containing 10 m*M* sodium phosphate. Lanes 6–8 contain the purified monoclonal antibody (fractions 18—20).

The same chromatographic results have been observed for one IgG_3, three IgG_{2a}, and two IgG_{2b} isotypes from ascites fluid. However, two IgG_1 monoclonal antibodies could not be separated by this system, as they did not bind to the CM-cellulose adsorbent. IgM immunoglobulins have not been examined.

An IgG_{2a} monoclonal antibody against Pichinde virus (Arena virus family) was assayed for viral recognition before and after purification. The purified monoclonal antibody was undistinguishable by an indirect immunofluorescent assay from the unchromatographed one and the titer did not change[25]. Another purified monoclonal antibody against *Actinobacillus seminis* has been successfully used in an Eliza assay[26]. These immunoassay results are an indication that this complex-displacement chromatographic method is a mild procedure.

Mouse serum was chromatographed with this complex-displacement system to obtain an estimate of the percentage of the full range of antibodies that might be separated by this system. The wide variation in isoelectric points (p*I* 5.8–8.2) in polyclonal antibodies[27] indicates the potential range of isoelectric points that might be encountered in monoclonal antibodies. The results were very similar to those obtained with ascites fluid, with four peaks appearing. There was a large amount of polyclonal antibody in the same location as the monoclonal antibody in the above experiments (fractions 18–20, Fig. 4). However, nephelometric assays also showed the presences of IgG in the first two peaks. Using the distribution of serum polyclonal antibodies as a guide, we estimate that 60–80% of monoclonal antibodies can be separated by this system as described. Changes in buffers and pH should make it possible to separate other polyclonal or monoclonal antibodies that do not bind to

the sorbent under present conditions. The observations on serum antibodies also indicate that the low nephelometer rate unit readings in the albumin and transferrin peaks (Fig. 3) may be due to contaminating polyclonal antibodies present in ascites fluid. The same data indicates that about 60–80% of the host polyclonal antibody is in the monoclonal antibody peak.

The ion-exchange complex-displacement system described in this manuscript offers another method for monoclonal antibody purification. It should be useful for many purifications since it is a mild single step procedure with good recoveries. Standard ion-exchange elution chromatography offers several advantages over Protein A affinity chromatography for bulk monoclonal antibody purifications[28]. These advantages include the ability to routinely sanitize the columns with sodium hydroxide, mild conditions, less expensive and the ability to remove host polyclonal antibodies.

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