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Affinity chromatographic purification of immunoglobulin M antibodies utilizing immobilized mannan binding protein

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ABSTRACT

A method is described for the rapid and efficient affinity chromatographic purification of murine monoclonal immunoglobulin M (IgM) which utilizes immobilized rabbit mannan binding protein (MBP). This solid-phase matrix is shown to bind IgM-class antibodies from a variety of species. Conditions reported show a binding capacity of IgM from murine ascites of nearly 1 mg/ml of immobilized MBP support. The prepared gel is shown to possess an ability to bind not only mouse IgM, but also human and bovine IgM, although with a lesser affinity. The matrix can be regenerated and reused at least ten times without any apparent loss of binding capacity or specificity. Mouse monoclonal IgM purified from ascites fluid using this method is greater than 95% pure as shown by high-performance liquid chromatography analysis.

INTRODUCTION

The medical and commercial importance of antibodies has resulted in a variety of techniques designed to isolate and purify antibodies of a particular class. Immobilized protein A [1], protein G [2], and protein A/G [3] have been very useful tools for the affinity purification of immunoglobulin (Ig) G, and immobilized jacalin has been shown to possess binding properties for human IgA as well as IgD [4]. To date, little has been available for the efficient affinity isolation of IgM, even though it is another extremely valuable class of immunoglobulin. For example, in the production of monoclonal antibodies, IgM may be the only class of antibody response produced by the host against a certain antigen.

Traditionally, purification of IgM from sera, ascites, etc., has been accomplished by an initial precipitation [5,6] followed by fractionation using gel filtration chromatography, DEAE-cellulose ion-exchange chromatography [7,8], or zone electro-

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phoresis [9,10]. These methods are very time consuming, extremely labor intensive, and may damage the isolated product.

Recently, a single-step affinity chromatographic method has been described [11] for the purification of IgM based upon a temperature dependent interaction of immobilized complement protein C1q with IgM. The method is rapid, simple, and convenient compared to the methods previously available for IgM purification. However, as recognized by Nethery *et al.* [11], and confirmed in our laboratories, C1q binds not only to IgM-class antibodies, but also to IgG-class antibodies. Although the reported binding constant of C1q to IgG antibodies is 18 times weaker than the binding constant to IgM antibodies, the result of this binding ability of C1q to IgG is that preparations of IgM based upon an immobilized C1q affinity purification may be contaminated with various and sporadic levels of IgG which would need to be removed by other methods. In our observations, cell culture fluids and commercial preparations of monoclonal IgM ascites contain moderate quantities of IgG which may bind to immobilized C1q during purification. An additional problem with C1q chromatography is that the purified ligand is very difficult to obtain in large quantities.

Mannan binding protein (MBP) is a protein of little known biological function which derives its name from the observation that it binds to mannan, a complex carbohydrate produced by a variety of yeast organisms. MBP has a relative molecular mass (M_r) of about 650 000 consisting of approximately 18 identical subunits of roughly 31 000 M_r each. In addition, MBP possesses a gross structure remarkably homologous with that of C1q which also has 18 polypeptide subunits of 24 000–28 000 M_r each yielding a combined M_r of approximately 460 000 [12].

Immobilized MBP was prepared in our laboratory with the hope that this affinity support might be utilized for the purification of IgM class antibodies. From this research, a method has been developed for the rapid and efficient purification of murine monoclonal IgM using a simple one-step chromatographic procedure. This method is based upon both the calcium and 4°C-dependent binding of IgM to the MBP support, and the room temperature-dependent elution of the protein from the gel in the presence of calcium-binding ethylenediaminetetraacetic acid (EDTA). This purification procedure differs from that of immobilized C1q in that C1q affinity chromatography of IgM is based upon binding at 5°C in the presence of EDTA, and elution at room temperature in the presence of EDTA and /or potassium iodide. MBP affinity chromatography also differs from C1q affinity chromatography in that immobilized MBP possesses extremely low binding affinities towards various species of IgG. It is also shown that bovine and human IgM will bind to immobilized MBP, although the binding capacity is reduced as compared to that of murine IgM from ascites. Of additional significance is the finding that human polyclonal IgM can be isolated from sera in a single run with a purity of approximately 70% as seen by high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Materials

Basic buffer salts, Coomassie Brilliant Blue, Coomassie protein assay reagent, cyanogen bromide, human IgM (whole molecule), and mouse IgG (whole molecule) were obtained from Pierce (Rockford, IL, USA).

Bovine IgG (lyophilized, essentially salt-free), bovine IgM, EDTA, human IgG (lyophilized, essentially salt-free), monoclonal mouse IgM, kappa, ascites (MOPC 104E, product No. M 2521) and yeast mannan (*ex Saccharomyces cerevisiae*, product No. M 7504) were obtained from Sigma (St. Louis, MO, USA). Rabbit serum (trace hemolyzed, nonsterile) was obtained from Pel-Freeze (Rogers, AK, USA). Sepharose 4B and 6B were obtained from Pharmacia-LKB (Piscataway, NJ, USA). Dialysis tubing (10 mm, 12 000–14 000 M_r cutoff) was obtained from Spectrum Labs. (Los Angeles, CA, USA). Centricon 30 centrifugal concentrators were purchased from Amicon (Danvers, MA, USA).

Liquid chromatographic analysis was performed on a Waters 650 protein purification liquid chromatograph (Milford, MA, USA) using a Pharmacia Superose 6 HR 10/30 column (300 × 10 mm) with IgM elution buffer (see below) (0.2 μm filtered and degassed) as the mobile phase over 40 min with a flow-rate of 0.5 ml/min. Absorbance readings at 280 nm were used for detection.

Polyacrylamide gel electrophoresis was performed using a Novex (Encinitas, CA, USA) Mini-Cell system with a precast 12% acrylamide tris/glycine gel under reducing conditions at a constant voltage of 125 V. The gel was fixed and stained using a Coomassie Brilliant Blue stain. High- and low-molecular-mass markers were purchased from Bio-Rad Labs. (Richmond, CA, USA).

Deionized water was purified and filtered by a five-bowl Milli-Q water system (Millipore, Bedford, MA, USA) and used throughout, including all buffer preparations.

Preparation of immobilized mannan for isolation of MBP

Note: The preparation of immobilized mannan and its subsequent use for the isolation of MBP from rabbit serum is based on a method described by Kozutsumi *et al.* [13].

Sepharose 6B (1000 ml) was washed on a sintered glass Buchner funnel with 16 l of water, suction dried to a moist cake, transferred to a 4-l capacity beaker, and then suspended in 1000 ml of water with the aid of an overhead stirrer. The gel slurry was activated in a fume hood by portion-wise addition of 200 g of solid cyanogen bromide over a 20-min period while simultaneously maintaining the temperature at approximately 20°C (by addition of crushed ice directly into the stirred slurry) and maintaining the pH of the slurry at approximately 10.5–11.0 by dropwise addition of concentrated sodium hydroxide solution [14]. The cyanogen bromide-activated Sepharose was washed on a sintered glass Buchner funnel with 16 l of an ice-cold 0.1 *M* sodium hydrogencarbonate solution, then suction dried to a moist cake. The activated Sepharose beads were added to a solution of 17.8 g of yeast mannan dissolved in 1000 ml of 0.1 *M* sodium hydrogencarbonate. The reaction mixture was allowed to stir at room temperature overnight. The next day, the Sepharose beads were washed with 9 l of water and then suspended in 1.6 l of 1.0 *M* ethanolamine solution, pH 9.0, for 1 h at room temperature to block remaining active sites on the bead surface. Following an additional 9-l wash with water, 750 ml of immobilized mannan were packed into a glass column (60 × 4 cm) for use in the isolation of mannan binding protein.

Preparation of mannan binding protein (MBP)

Note: All operations were carried out at 4°C unless indicated otherwise.

A 10-l volume of rabbit serum was thawed and mixed with 10 l of mixing buffer (2.5 *M* NaCl, 20 m*M* imidazole, 40 m*M* $CaCl_2$, pH 7.8) and stirred for 30 min. The diluted rabbit serum was applied to the immobilized mannan column (750 ml gel) which had been equilibrated with loading buffer (1.25 *M* NaCl, 10 m*M* imidazole, 20 m*M* $CaCl_2$, pH 7.8). After the sample application, the column was washed with boading buffer until the absorbance at 280 nm of the column effluent was less than 0.05 (8 l of loading buffer were required). Bound MBP was eluted with elution buffer (10 m*M* imidazole, 1.25 *M* NaCl, 2 m*M* EDTA, pH 7.8) by collecting 125-ml fractions. Fractions 4, 5 and 6 were pooled and dialyzed against a 0.1 *M* sodium hydrogencarbonate buffer, pH 8.5 (10 l, 2 changes) for 2 days. The total absorbance at 280 nm of the MBP solution was 388.08. The purified MBP solution was used for coupling to cyanogen bromide-activated Sepharose 4B (as described below).

Coupling of mannan binding protein to cyanogen bromide activated Sepharose 4B

Sepharose 4B (110 ml) was activated with 22 g of cyanogen bromide at 20°C at pH 11.0 as described previously. The activated Sepharose 4B was washed with 2 l of ice-cold water followed by 1 l of 0.1 *M* sodium bicarbonate buffer, pH 8.5. The activated Sepharose 4B was suction dried to a moist cake and added to the MBP solution from above. The gel suspension was stirred at 4°C overnight. The reaction mixture was filtered and washed with 200 ml of a 1.0 *M* NaCl solution followed by 200 ml of water. The total absorbance at 280 nm of the filtrate was read and determined to be 37.86. Therefore, the amount coupled by difference was 348.20 (3.17 absorbance units per ml of gel).

The excess reactive groups on the immobilized MBP matrix were blocked as follows: the gel was suction dried to a moist cake and added to 100 ml of 1.0 *M* ethanolamine solution, pH 9.0. The gel suspension was stirred at room temperature for 1 h. The immobilized MBP was slowly and successively washed with 2 l each of water, 1.0 *M* NaCl, water, 10 m*M* tris(hydroxymethyl)aminomethane (Tris) + 1.25 *M* NaCl + 2 m*M* EDTA (pH 7.4), and water.

The immobilized MBP was stored in a 0.05% sodium azide solution at 4°C.

Test for coupling of the MBP to the gel

This in-process check is a quick and sensitive qualitative method for determining the efficiency of the final washing steps of the prepared gel. In a glass test tube, 0.2 ml of the immobilized MBP suspension were mixed with 2 ml of Coomassie protein assay reagent. The gel was allowed to settle at the bottom of the test tube. The settled gel gave a characteristic blue color of Coomassie-stained protein, while the supernatant remained colorless signifying that unbound protein was not present.

Preparation of monoclonal mouse IgM ascites fluid for purification

Five vials of monoclonal mouse IgM ascites (*ca.* 5 mg IgM per vial) were each reconstituted with 1.0 ml of water, and the contents were transferred to dialysis tubing. Each vial was washed with an additional 0.5 ml of water to remove residual products; the contents were transferred to the dialysis tubing as above. The reconstituted material was dialyzed overnight at 4°C against 5 l of a 10 m*M* Tris, 1.25 *M* sodium chloride, 0.02% sodium azide buffer, pH 7.4, in order to remove phosphate ions from the sample which will precipitate with calcium ions within the IgM binding buffer. After dialysis, the sample was removed and diluted 1:1 with IgM binding buffer (10 m*M* Tris, 1.25 *M* NaCl, 20 m*M* $CaCl_2$, 0.02% NaN_3, pH 7.4). The sample was maintained at 4°C.

Regeneration capabilities of a 5-ml immobilized MBP column

Note: All equilibration and washing steps were conducted at 4°C, and all elution steps were performed at ambient room temperature.

Immobilized MBP gel (5 ml) was packed into a disposable polypropylene column (Pierce), and the packed column was equilibrated with 20 ml of IgM binding buffer (4°C). Mouse IgM ascites solution (1.5 ml) prepared as described above was applied to the column. After the sample entered the gel bed, 1.5 ml of IgM binding buffer was applied to the column to ensure complete loading of the sample into the gel. The column was allowed to incubate for 30 min at 4°C and then was washed with 14 × 3 ml aliquots of IgM binding buffer. After washing, the column was removed from the cold room and allowed to incubate at room temperature for 1 h. After acclimation, the column was eluted with 15 × 3 ml aliquots of IgM elution buffer (10 m*M* Tris, 1.25 *M* NaCl, 2 m*M* EDTA, 0.02% NaN_3, pH 7.4). Absorbance of all the fractions was measured at 280 nm. Peak eluted fractions ($A_{280\,nm} > 0.020$) were pooled and concentration of IgM was determined using an $\varepsilon_{280\,nm}$ value of 1.18 mg^{-1} ml cm^{-1} [15].

The regenerability and reusability of the immobilized MBP column was tested by repeating the chromatography nine additional times using the same column as above, washing the column with 18 ml of a 0.05% NaN_3 solution between runs. Pooled eluted fractions from the initial and the final purifications, along with the starting material and peak break through fraction from the initial purification, were analyzed by HPLC. In addition, a portion of the pooled eluted fractions from the initial purification were concentrated, using a Centricon 30 (M_r cutoff = 30 000), in order that polyacrylamide gel electrophoresis under reducing conditions could be run on the sample.

Linearity of application of monoclonal mouse IgM ascites to immobilized MBP

Note: All equilibration and washing steps were conducted at 4°C, and all elution steps were performed at ambient room temperature.

Two 5-ml immobilized MBP columns were packed, and each was equilibrated with 20 ml of IgM binding buffer. To one column were applied 1.5 ml of the prepared monoclonal mouse IgM solution used above, and an additional 1.5 ml of IgM binding buffer was applied to the same column to ensure complete loading of the sample into the gel. To the other column were applied 3.0 ml of the prepared monoclonal mouse IgM solution used above. The columns were incubated at 4°C for 30 min and then each column was washed with 14 × 3 ml aliquots of IgM binding buffer. The columns were then removed from the cold room and allowed to incubate at room temperature for 1 h. Each column was then eluted with 15 × 3 ml aliquots of IgM elution buffer. Absorbance of all the fractions was measured at 280 nm. Peak eluted fractions ($A_{280\,nm} > 0.020$) were pooled and concentration of IgM was determined using an $\varepsilon_{280\,nm}$ value of 1.18 mg^{-1} ml

cm^{-1}. These pooled fractions were analyzed for purity by HPLC.

Binding of bovine and human IgM to immobilized MBP

Note: All equilibration and washing steps were conducted at 4°C, and all elution steps were performed at ambient room temperature.

Bovine IgM (2 ml of a 1.1 mg/ml solution) was dialyzed overnight at 4°C against 1 l of a 10 m*M* Tris, 1.25 *M* sodium chloride, 0.02% sodium azide buffer, pH 7.4. After dialysis, the sample was made 1:1 with IgM binding buffer and maintained at 4°C. In addition, human IgM (486 μl of a 4.4 mg/ml solution) was made 1:1 with IgM binding buffer and maintained at 4°C. Two 5-ml immobilized MBP columns were packed and each equilibrated with 20 ml of IgM binding buffer. To one column were applied 3.0 ml of the prepared bovine IgM solution. To the other column were applied the prepared human IgM sample followed by 2.0 ml of IgM binding buffer to ensure complete loading of the sample into the gel. The columns were allowed to incubate at 4°C for 30 min, then each was washed with 11 × 3 ml aliquots of IgM binding buffer. The columns were then removed from the cold room and allowed to incubate at room temperature for 1 h. Each column was then eluted with 12 × 3 ml aliquots of IgM elution buffer. Absorbance of all the fractions was measured at 280 nm. Concentration of IgM peak eluted fractions ($A_{280\,nm} > 0.020$) was determined using an $\varepsilon_{280\,nm}$ value of 1.18 mg^{-1} ml cm^{-1}. The peak break through fraction and peak eluted fraction from each of the IgM purifications were analyzed for purity by HPLC.

Binding of human IgM from serum to immobilized MBP

Note: All equilibration and washing steps were conducted at 4°C, and all elution steps were performed at ambient room temperature.

A freshly obtained sample of human blood (*ca.* 9 ml) was allowed to clot, was centrifuged, and the serum (*ca.* 3.5 ml) was pipetted off the top of the centrifuged clot. The serum was dialyzed overnight at 4°C against 4 l of a 10 m*M* Tris, 1.25 *M* sodium chloride, 0.02% sodium azide buffer, pH 7.4. The following morning, the sample was removed and diluted 1:1 with IgM binding buffer (total volume = *ca.* 7 ml). Immobilized MBP (5 ml gel) was packed into a column and equilibrated with 20 ml of IgM binding buffer. The dialyzed and diluted human serum sample (3 ml) was applied to the column. The column was allowed to incubate at 4°C for 30 min and then was washed with 14 × 3 ml aliquots of IgM binding buffer. The column was then removed from the cold room and allowed to incubate at room temperature for 1 h. The column was then eluted with 15 × 3 ml aliquots of IgM elution buffer. Absorbance of all the fractions was measured at 280 nm. Peak eluted fractions ($A_{280\,nm} > 0.050$) were pooled and concentration of IgM was determined using an $\varepsilon_{280\,nm}$ value of 1.18 mg^{-1} ml cm^{-1}. The starting material and pooled peak eluted fractions were analyzed for purity by HPLC.

Binding of various species of IgG to immobilized MBP

Note: All equilibration and washing steps were conducted at 4°C, and all elution steps were performed at ambient room temperature.

Bovine and human IgG (10 mg each) were dissolved in 1.5 ml of IgM binding buffer. In addition, 10 mg (714 μl) of mouse IgG was made 1:1 with IgM binding buffer. The antibody solutions were maintained at 4°C. Three 5-ml immobilized MBP columns were packed and each was equilibrated with 20 ml of IgM binding buffer. To each column was applied an individual IgG sample from above, followed by an additional 1.5 ml of IgM

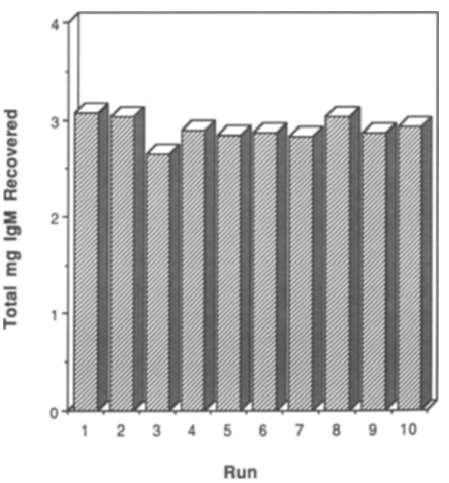

Fig. 1. Total monoclonal mouse IgM purified from ascites using a 5-ml immobilized MBP column over ten repeated purifications. Mean = 2.90 ± 0.124; variance = 0.015.

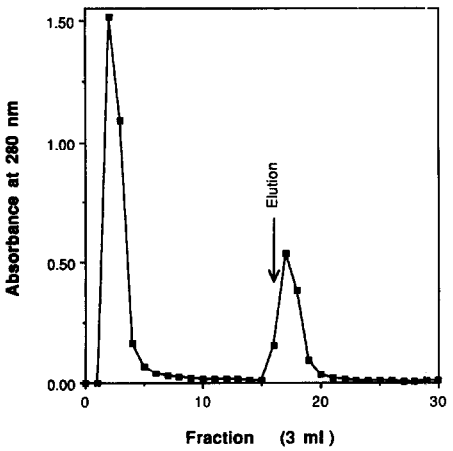

Fig. 2. A typical chromatographic profile of the purification of monoclonal mouse IgM from ascites using immobilized MBP.

binding buffer to ensure complete loading of the samples within the gel. The columns were incubated at 4°C for 30 min, then each was washed with 17 × 3 ml aliquots of IgM binding buffer. The columns were then removed from the cold room and allowed to incubate at room temperature for 1 h. Each column was then eluted with 8 × 3 ml aliquots of IgM elution buffer. Absorbance of all the fractions was measured at 280 nm. Concentration of IgG peak eluted fractions ($A_{280\,nm} > 0.010$) was determined using an $\varepsilon_{280\,nm}$ value of 1.44 mg^{-1} ml cm^{-1} (determined in our laboratory).

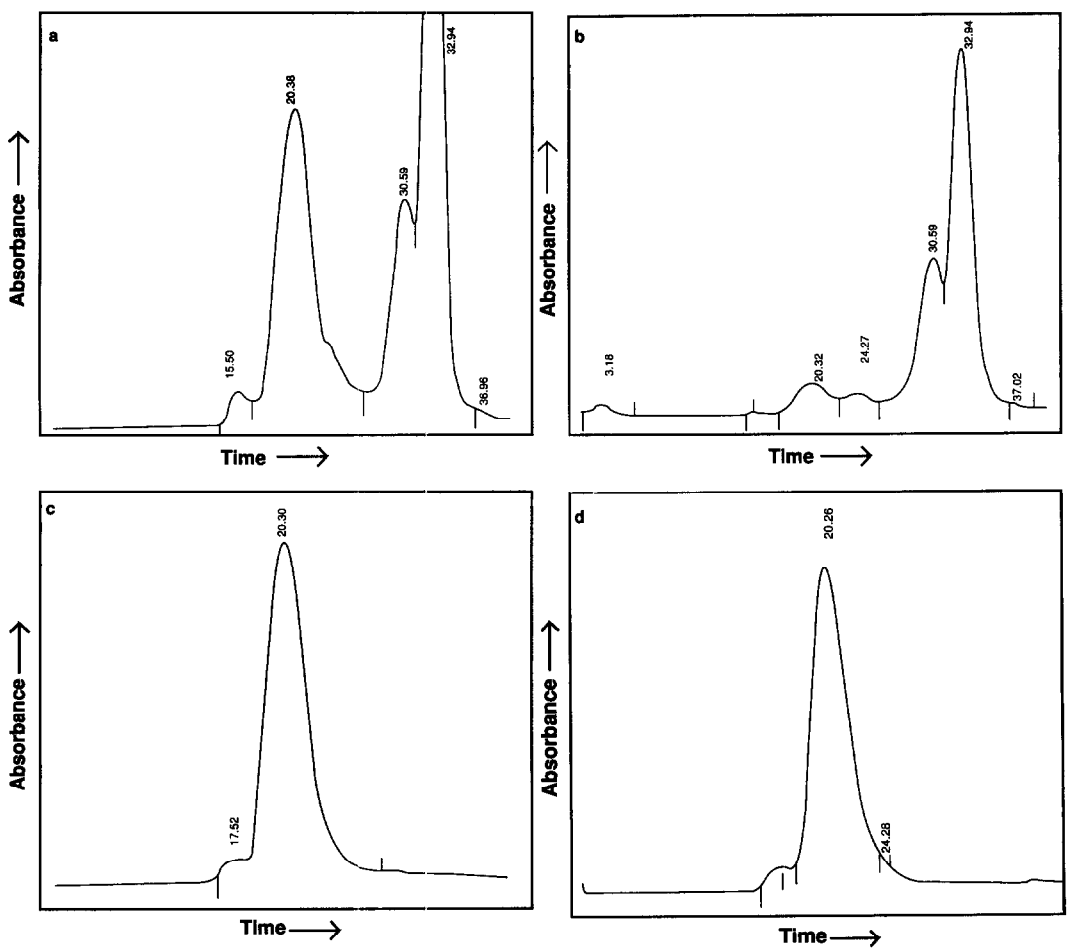

Fig. 3. HPLC analysis of (a) starting material, (b) peak break through fraction from run 1, (c) pooled eluted fractions of run 1 and (d) pooled eluted fractions of run 10 from repeated monoclonal mouse IgM ascites purifications using a 5-ml immobilized MBP column. Numbers at peaks are retention times in min: absorbance measured at 280 nm.

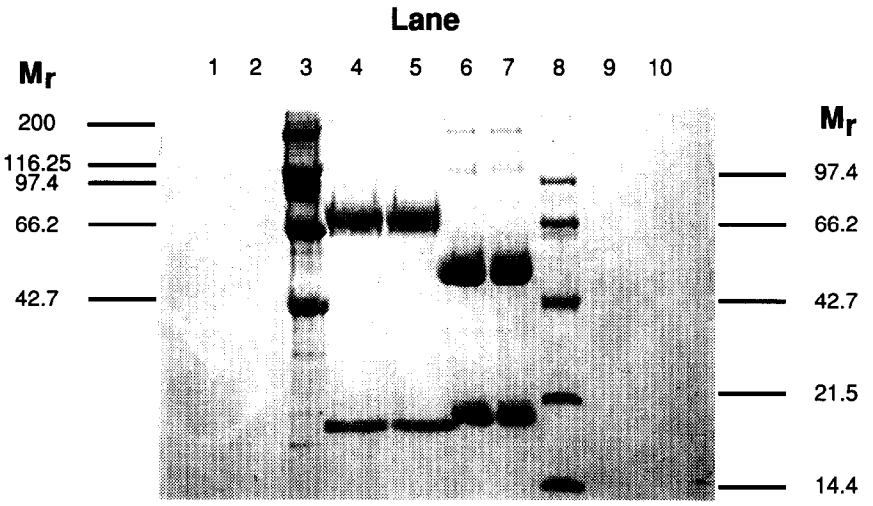

Fig. 4. Electrophoretic analysis under reducing conditions of pooled and concentrated eluted fractions of run 1 from repeated monoclonal mouse IgM ascites purifications using immobilized MBP. Staining was performed with Coomassie Brilliant Blue. Lanes: 1 and 2 = blank; 3 = high-molecular-mass protein standards; 4 and 5 = pooled and concentrated eluted fractions from IgM purification; 6 and 7 = human IgG standard; 8 = low-molecular-mass protein standards; 9 and 10 = blank. $M_r \cdot 10^3$.

RESULTS

Regeneration capabilities of a 5-ml immobilized MBP column

Our data show no apparent decrease in binding capacity of a 5-ml immobilized MBP column over ten repeated purifications using the same amount of the same starting material throughout. The average recovery was 2.90 ± 0.124 mg (variance = 0.015) of monoclonal mouse IgM for each purification.

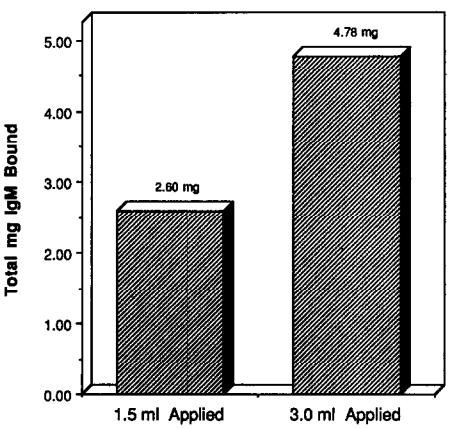

Fig. 5. Total monoclonal mouse IgM purified from 1.5 ml and 3.0 ml of ascites applied to 5-ml immobilized MBP columns.

This can be seen in a graph of the results shown in Fig. 1. Alternate coupling chemistries to what has been used here did not show signs of this stability. Dramatic decreases in total IgM binding (greater than 50%) at this identical loading level were seen after only a few regenerations when these alternate methods were used (data not shown). A typical chromatographic profile for the purification of monoclonal mouse IgM from ascites can be seen in Fig. 2.

HPLC analysis of the starting material shows that the IgM was the major protein component of the ascites fluid (40.3%) (Fig. 3a), and analysis of the peak break through fraction shows little sign of IgM (7.2%) (Fig. 3b) while the remaining peak areas were, more or less, the same as in the starting material. This shows that IgM is being selectivity purified from the ascites mixture with a good degree of efficiency. In addition, HPLC analysis of pooled eluted fractions from purification runs 1 and 10 indicates an eluted IgM product which is at least 95% pure (Fig. 3c and d). This shows that the binding remains specific for IgM throughout the ten repeated purifications. Polyacrylamide gel electrophoretic analysis, under reducing conditions, of the concentrated pooled fractions from run 1 support the HPLC results (Fig. 4). The band pattern is exact

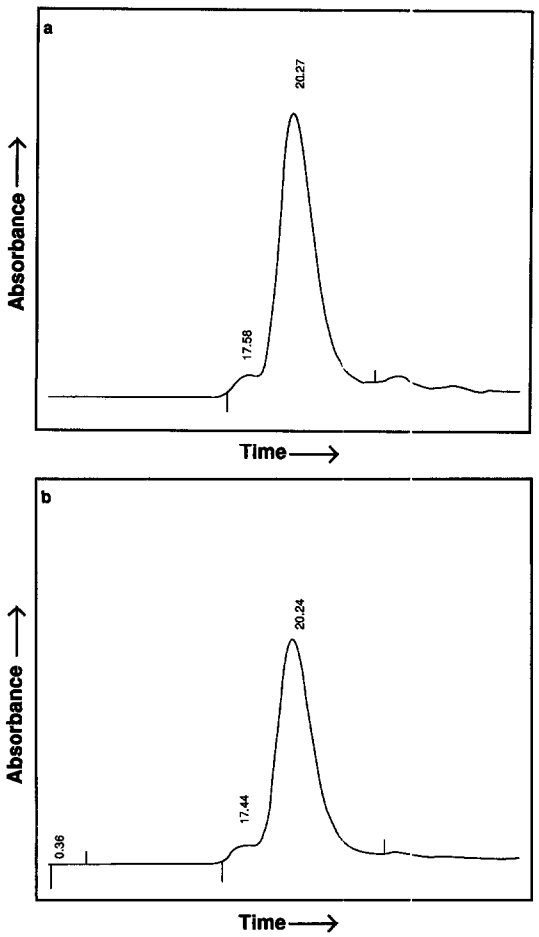

Fig. 6. HPLC analysis of pooled eluted fractions from (a) 1.5 ml, and (b) 3.0 ml applications of monoclonal mouse IgM ascites to 5-ml immobilized MBP columns. Numbers at peaks are retention times in min; absorbance measured at 280 nm.

in nature to the pattern produced by mouse IgM under reducing conditions (heavy and light chains), and is distinctly different from IgG under the same conditions. Also noted is that no other major bands are visible in the lanes containing IgM indicating a high degree of purity.

Linearity of application of monoclonal mouse IgM ascites to immobilized MBP

The data show that there is not a true linear binding response to the application of 1.5 ml and 3.0 ml of the prepared monoclonal mouse IgM ascites (Fig. 5). For a true linear response, it would be expected that the amount of IgM bound to the

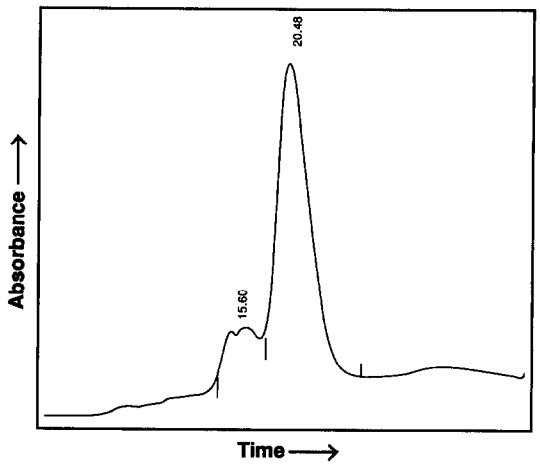

Fig. 7. HPLC analysis of the peak eluted fractions from the purification of human IgM using a 5 ml immobilized MBP column. Numbers at peaks indicate retention times in min; absorbance measured at 280 nm.

column when 3.0 ml of sample is applied would be twice the amount bound when 1.5 ml of sample is applied. However, there is only an 83.3% increase (4.78 mg as compared to 2.60 mg) in binding when twice the amount of starting material is applied. These results suggest that a 5-ml immobilized MBP column has a maximum binding capacity approaching 4.8 mg of monoclonal mouse IgM, or a capacity of roughly 0.96 mg of monoclonal mouse IgM per ml of gel. The actual binding capacity per ml of gel has yet to be determined.

HPLC analysis of pooled eluted fractions from the two different applications indicates an eluted IgM product which is at least 95% pure (Fig. 6a and b). This signifies a retention in purification specificity even though the amount of applied sample has been doubled.

Binding of bovine and human IgM to immobilized MBP

Our results demonstrate that bovine and human IgM can also be isolated using immobilized MBP, although the degree of binding with each of these two species appears significantly lower than that of monoclonal mouse IgM. Of the 2.2 mg of bovine IgM and the 2.14 mg of human IgM applied, 313 μg and 661 μg, respectively bound to the matrix (data not shown). Results show an excellent recovery

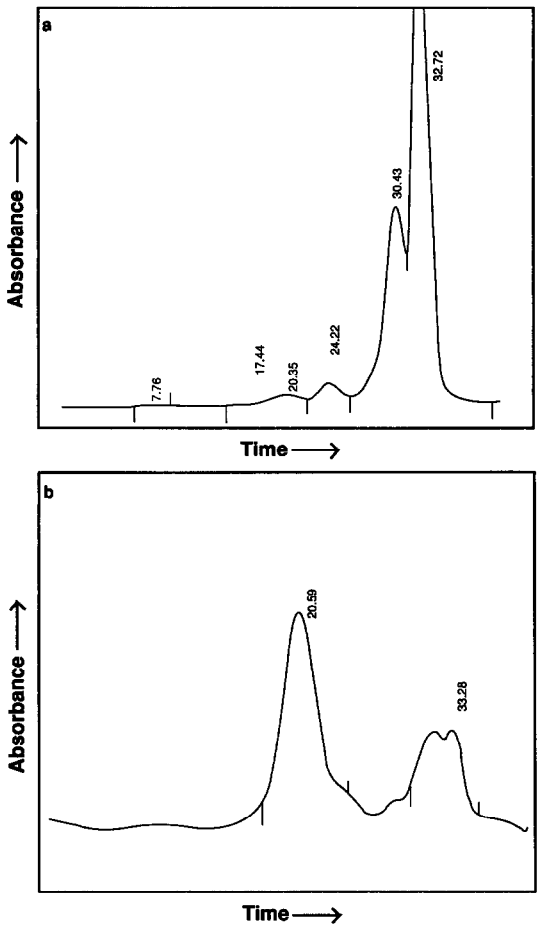

Fig. 8. HPLC analysis of (a) the starting material and (b) the pooled peak eluted fractions from the purification of human IgM from serum using a 5-ml immobilized MBP column. Numbers at peaks indicate retention times in min; absorbance measured at 280 nm.

(94% for bovine, 107% for human) of all protein signifying a low degree of non-specific protein binding to the support. The peak eluted fraction from the human IgM purification is 85.7% pure by HPLC analysis (Fig. 7), and the peak eluted fraction from the bovine IgM isolation is essentially 100% pure (a single peak with a retention time of 20.83 min; data not shown). This shows that immobilized MBP will bind at least two other species of IgM, even though the efficiency of binding is reduced as compared to that of monoclonal mouse IgM.

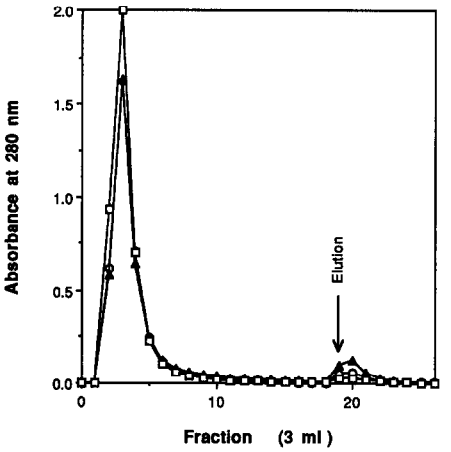

Fig. 9. Chromatographic profile of the attempted binding of 10 mg each of bovine (○), human (▲) and mouse (□) IgG to individual 5-ml immobilized MBP columns.

Binding of human IgM from serum to immobilized MBP

Our data show that approximately 1.2 mg of protein bound to a 5-ml immobilized MBP column when 3.0 ml of prepared human serum was applied to it. HPLC analysis of the starting material and pooled break through fractions shows that IgM is bound to the immobilized MBP column (Fig. 8a and b). The eluted product is 69.4% IgM as shown by HPLC analysis, whereas IgM comprises only 2.5% of the total protein in the starting material.

Binding of various species of IgG to immobilized MBP

From the chromatographic profile of the purifications of bovine, human, and mouse IgG (Fig. 9), it can be seen that immobilized MBP possesses a very low binding affinity towards IgG class antibodies. Of the 10 mg of each species of IgG applied, only 331 μg of bovine IgG, 637 μg of human IgG and 165 μg of mouse IgG bound to their respective columns. Therefore, the binding affinity of IgG by immobilized MBP is minimal as seen by the extremely small amount of protein eluted (1.6% to 6.7%) when large amounts of various sources of IgG are applied to the matrix.

DISCUSSION

This paper describes an affinity purification method for immonoglobulin M class antibodies

based upon the interactions of IgM with immobilized rabbit mannan binding protein. Of major importance has been the results showing the differences between MBP and C1q affinity chromatography. Whereas purification of IgM with immobilized C1q requires binding at 5°C in the presence of EDTA, and elution at room temperature in the presence of EDTA and/or potassium iodide, immobilized MBP purification of IgM requires binding at 4°C in the presence of calcium, and the elution at room temperature in the presence of EDTA. Another difference between the two purification methods is that immobilized MBP possesses minimal binding affinities towards a variety of species of IgG which cannot be shown with immobilized C1q. Only a single immobilized MBP chromatographic procedure is needed to obtain at least 95% pure monoclonal murine IgM from ascites. When human serum is purified with immobilized MBP, the eluted product is not as pure as that seen with ascites (*ca.* 70% pure); however, the matrix demonstrates an ability to bind very small amounts of IgM from a highly complex and concentrated mixture of other proteins.

It is speculated that the binding of IgM to immobilized MBP is based upon the interaction of the carbohydrate moieties of the IgM heavy chains with the MBP molecule. This idea is based on the fact that MBP is isolated by its interaction with immobilized mannan, a polymer of mannose. Since it is generally known that the Fc5μ region of the heavy chains of IgM is highly glycosylated, this speculation would seem logical.

Of additional importance is the finding that immobilized MBP will bind other species and sources of IgM antibodies. This may be a result of the general highly glycosylated nature of IgM class antibodies. While the research conducted here did not investigate the binding characteristics of all species of IgM antibodies, it can be inferred from the data presented that many other species of IgM may bind to this matrix, although with a lesser affinity.

Of practical significance is the finding that immobilized MBP is regenerable for at least ten purification runs with no apparent loss of binding capacity. These results suggest a stable linkage of the ligand to the gel which does not interfere with antibody binding sites. In conjunction, data suggest a moderately high binding capacity of 1 mg of monoclonal mouse IgM per ml of immobilized MBP.

In summary, we conclude that immobilized MBP, prepared as described, is a useful matrix for the affinity purification of monoclonal IgM class antibodies from mouse ascites. The gel possesses binding properties towards human and bovine IgM, and may bind other species of IgM not yet tested. The matrix also demonstrates a higher degree of selective binding of IgM over IgG than immobilized C1q and yields eluted products which are of high purity as shown by HPLC and polyacrylamide gel electrophoresis analysis.

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