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TANDEM PURIFICATION OF IgM MONOCLONAL ANTIBODIES FROM MOUSE ASCITES FLUIDS BY ANION-EXCHANGE AND GEL FAST PROTEIN LIQUID CHROMATOGRAPHY

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SUMMARY

A tandem chromatographic procedure was used to isolate rapidly mouse IgM monoclonal antibodies. Mouse ascites fluids containing IgM monoclonal antibodies were first chromatographed on an anion-exchange Mono Q column connected to a fast protein liquid chromatography system. The IgM-rich fractions from the Mono Q column were then injected on a gel filtration Superose 6 column equilibrated with a low-ionic strength buffer and eluted with a high-ionic strength buffer. Assessment of the purity of isolated IgM monoclonal antibodies was performed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis together with a Coomassie Brillant Blue R 250 staining technique. Assessment of the immunoreactivity of isolated IgM monoclonal antibodies was performed by a enzyme linked immunosorbent assay using a solid phase adsorbed antigen against which IgM monoclonal antibodies were directed. The chromatographic procedure described provides a new method for the rapid purification of mouse IgM monoclonal antibodies to a high degree of purity and in a immunoreactive state.

INTRODUCTION

The purification of IgM monoclonal antibodies usually requires multistep procedures involving DEAE-cellulose anion-exchange chromatography[1,2], Ultrogel Ac A34 or G-200 Sephadex gel filtration chromatography[1–3] and protamine sulphate-Sepharose or concanavalin A-Sepharose affinity chromatography[4,5]. Most of these purification procedures are combined with a preliminary precipitation step[1,3–5] known

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to denature IgM antibodies[2,6]. In addition, these techniques rarely avoid contamination with alpha-2 macroglobulin[1,6]. Direct isolation of IgM monoclonal antibodies from human pathological sera by G-200 Sephadex gel filtration chromatography was made possible by increasing the retention time of IgM antibodies onto the column with a low-ionic strength buffer[6]. However, classical gel filtration chromatography remains of limited use due to the length of time involved for each separation[1]. Very little information is currently available on the rapid purification of IgM monoclonal antibodies from mouse ascites fluids under conditions where the purified antibody remains immunoreactive and is free of protein contaminants. Recent developments in high-performance liquid chromatography have opened new possibilities in the isolation of mouse monoclonal antibodies[7–10] and their antigen binding fragments (Fab)[8].

We describe in this study a tandem chromatographic procedure to isolate rapidly, from mouse ascites fluids, IgM monoclonal antibodies free from contaminating proteins and in a immunoreactive state. Mouse ascites fluids containing IgM monoclonal antibodies were first chromatographed on a Mono Q anion-exchange column connected to a fast protein liquid chromatography (FPLC) system. Eluted peaks containing IgM monoclonal antibodies were then chromatographed on a gel filtration Superose 6 column equilibrated with a low-ionic strength buffer. Assessment of the purity of isolated IgM antibodies was performed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) together with a Coomassie staining technique. Assessment of the immunoreactivity of isolated IgM antibodies was performed by enzyme linked immunosorbent assay (ELISA) using the antigen against which IgM antibodies were directed. The chromatographic procedure described provides a new method for the rapid isolation of pure IgM antibodies in a immunoreactive state.

EXPERIMENTAL

The FPLC separations were performed on a Mono Q HR 5/5 prepacked anion-exchange column (50 × 5 mm) and a Superose 6 HR 10/30 prepacked gel filtration column (300 × 10 mm) (Pharmacia, Uppsala, Sweden). Materials and chemicals, unless stated otherwise, were as previously indicated[8]. Rabbit anti-mouse IgM was from Miles Labs., U.S.A.) and goat anti-rabbit IgG conjugated with alkaline phosphatase was from Miles-Yeda (Israel).

Seven mouse ascites fluids containing IgM monoclonal antibodies were obtained from the following sources: P13, P14, P15, P16, P17 were IgM monoclonal antibodies directed against a human blood platelet alpha-granule glycoprotein called thrombospondin[11]; MAS 037 C, directed against human fibronectin, was obtained from Sera-Lab; 633 A, specific for furunculosis fish disease (*Aeromonas salmonicida*), was obtained from BioScot (Edinburgh, U.K.). Mouse ascites fluids were passed through 0.45-μm filters (Millipore) prior to chromatographic separations on a anion-exchange Mono Q column.

Anion-exchange chromatography

Mono Q HR 5/5 column. Mouse ascites fluids containing IgM monoclonal antibodies (1–2 ml) were equilibrated in the starting buffer (20 ml) and then injected

via a 50 ml Superloop (335 × 30 mm) (Pharmacia) onto a Mono Q anion-exchange column. The starting buffer was 20 m*M* L-histidine, pH 6.0, and the final buffer was 20 m*M* L-histidine, pH 6.0, containing 0.5 *M* sodium chloride. The gradient was generated over 25 min at a flow-rate of 1 ml/min.

Gel filtration chromatography

Superose 6 HR 10/30 column. The method of Bouvet *et al.*[6] involving G-200 gel filtration chromatography with a low-ionic strength buffer was used with minor modifications and was adapted, in this study, to the FPLC system. IgM-rich fractions from the Mono Q column were concentrated by ultrafiltration (Amicon) (0.5–0.7 ml) and injected at a flow-rate of 0.5 ml/min onto a gel filtration Superose 6 column previously equilibrated with a low-ionic strength buffer containing 5 m*M* L-histidine, pH 6.0. The eluting buffer was 50 m*M* L-histidine, pH 6.0, containing 1.7 *M* sodium chloride. In some experiments a buffer containing 0.5 *M* sodium chloride in 50 m*M* L-histidine, pH 6.0, was used to equilibrate the Superose 6 column and to elute the IgM monoclonal antibodies.

SDS-PAGE

Following the method of Mason and Williams[12], 100 μl of sample mixed with 100 μl of buffer containing 5% (w/v) SDS, 25% (w/v) glycerol and 2 m*M* N-ethylmaleimide were heated at 100°C for 5 min. Unreduced proteins were subjected to electrophoresis on a 3–10% gradient polyacrylamide gel in the presence of SDS according to Laemmli[13]. Gels were fixed in 40% (v/v) methanol–7% (v/v) acetic acid and then stained with Coomassie Brillant Blue R 250.

ELISA

Mouse IgM antibodies present in peaks eluted from the Mono Q column were detected by an ELISA similar to that described by Beards and Bryden[14] with minor modifications. Briefly, 100 μl of purified antigen (human platelet thrombospondin) (5 μg/ml) diluted in phosphate–buffered saline (pH 7.4) was added to poly(vinylchloride) microtitre plates (Nunc) and incubated overnight at 4°C. Wells coated with thrombospondin were then washed three times with 250 μl/well of phosphate-buffered saline containing 0.05% Tween 20 (washing buffer). The last wash was left for 30 min at room temperature. After removing the washing buffer, 100 μl of all the eluted peaks were added to the wells and left for 1 h at 37°C. A negative control was performed by the use of phosphate-buffered saline instead of eluted peaks. A positive control was performed by including the mouse ascites fluids containing IgM antibodies. After the incubation, the content of the wells was aspirated and rinsed 3 times with the washing buffer. One hundred microliters of rabbit anti-mouse IgM (diluted to 1/300) were added to each well for an additional 1 h incubation at 37°C. After aspirating and rinsing 3 times with the washing buffer, 100 μl of goat anti-rabbit IgM conjugated with alkaline phosphatase (diluted to 1/3000) were added to each well and incubated for 1 h at 37°C. The content of the wells was then aspirated and rinsed 3 times with the washing buffer. *p*-Nitrophenylphosphate (1 *M*) dissolved in diethanolamine buffer was then added to each well (100 μl). The enzyme reaction was allowed to proceed for 1 h at 37°C and then stopped with 10 μl/well of 0.5 *M* sodium hydroxide. The absorbance was read at 405 nm using a Titertek Multiskan MC photometer (Flow Laboratories).

RESULTS

Isolation of IgM monoclonal antibodies from mouse ascites fluids by FPLC anion-exchange chromatography

Mouse ascites fluids containing IgM monoclonal antibody P13 showed 5 major peaks when separated on a Mono Q column with a gradient of 0–0.5 *M* sodium

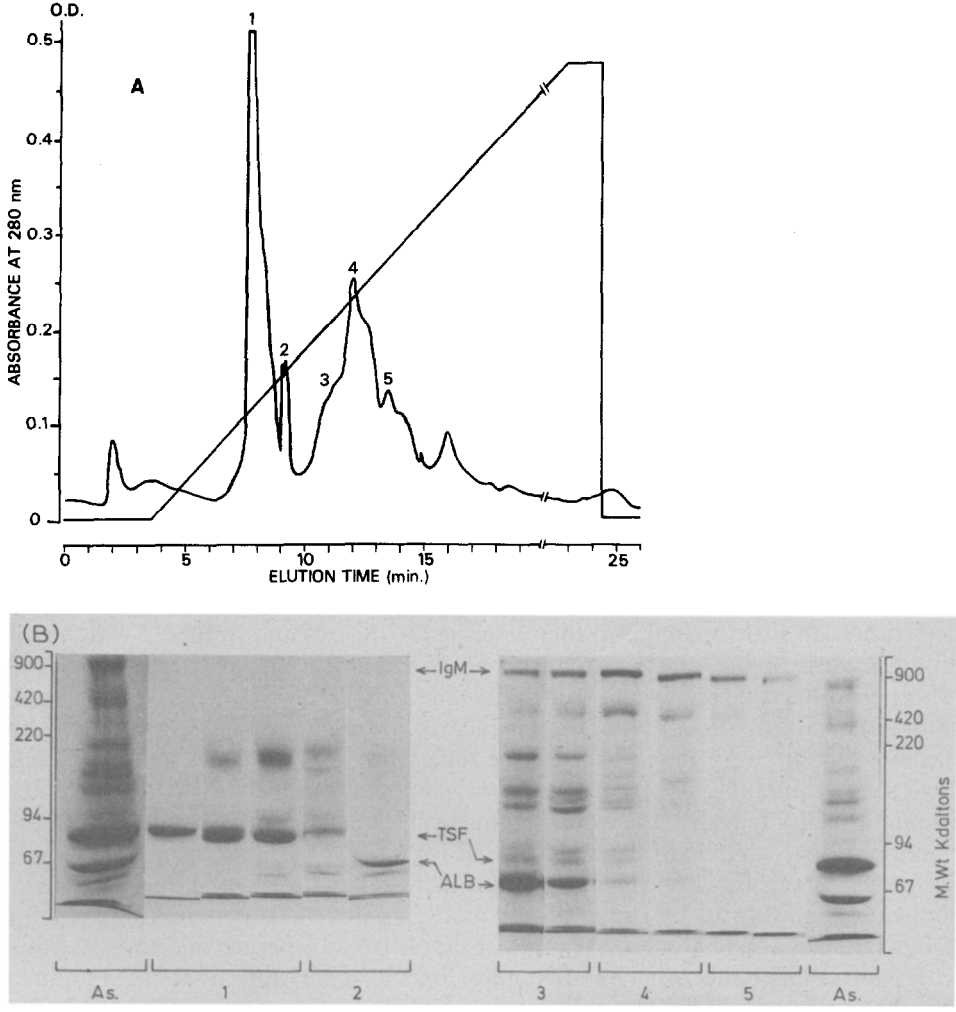

Fig. 1. (A) Chromatographic separation of mouse IgM monoclonal antibody P13 present in ascites fluid on a Mono Q column connected to a FPLC system. Ascites fluid (3 mg of protein per 15 ml) was injected into the column via a 50-ml Superloop. The gradient of 0–0.5 *M* NaCl in 20 m*M* L-histidine (pH 6.0) was generated over 25 min at a flow-rate of 1 ml/min. (B) Eluted peak fractions were treated with SDS and subjected to electrophoresis under non-reducing conditions on 3–10% gradient SDS-polyacrylamide gels. Gels were fixed and stained with Coomassie Brillant Blue R 250. Lanes As. contain mouse ascites fluid prior to the chromatography on a Mono Q column. Numbers below each lane correspond to peak numbers. Lanes 3 and 4 were loaded with 80–200 μg of eluted material whereas lane 5 was loaded with 30–60 μg. IgM = immunoglobulin M; TSF = transerrin; ALB = albumin.

chloride (Fig. 1A). Similar elution patterns were obtained with 6 different mouse ascites fluids containing IgM monoclonal antibodies (P14, P15, P16, P17, MAS 037 C and 633 A) (results not shown).

Eluted peak fractions were subjected to electrophoresis under non-reducing conditions on 3–10% gradient SDS-polyacrylamide gels and the separated proteins were identified by Coomassie staining (Fig. 1B). In typical electrophoretic separations of eluted peaks, peaks 1 and 2 which were eluted at 0.05 *M* and 0.08 *M* sodium chloride respectively, mainly contained transferrin and a 150 kDA protein band (Fig. 1B; lanes 1 and 2). In addition to protein bands between 80 and 420 kDa, albumin and a 900 kDa protein band corresponding to the apparent molecular weight of IgM antibodies[2] were present in peaks 3 and 4 which were eluted between 0.18 and 0.2 *M* sodium chloride (Fig. 1B; lanes 3 and 4). Only the IgM protein band and traces of a 420 kDa protein band corresponding to the molecular weight of α_2-macroglobulin[6] were present in peak 5 which was eluted at 0.25 *M* sodium chloride (Fig. 1B; lane 5). Using rocket imunoelectrophoresis, the recovery of IgM monoclonal antibodies from the Mono Q column was 80% of the amount of IgM antibodies originally present in the ascites fluid.

Assessment of the immunoreactivity of isolated IgM monoclonal antibodies by enzyme linked immunosorbent assay

In order to assess the immunoreactivity of IgM monoclonal antibody P13, eluted peaks from the Mono Q column were added to plastic wells coated with the

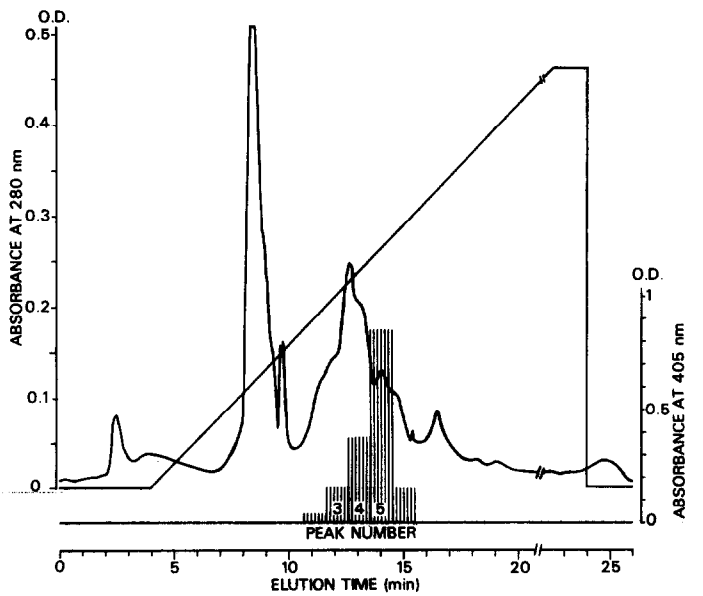

Fig. 2. Assessment of the immunoreactivity of isolated IgM monoclonal antibody P13 using an ELISA. Each eluted peak fractions of the Mono Q chromatographic profile were added to plastic wells coated with thrombospondin, the antigen against which was directed the IgM antibody P13. The upper part shows the chromatographic separation. The bottom part shows the binding of IgM monoclonal antibody P13 to adsorbed thrombospondin using rabbit anti-mouse IgM and goat anti-rabbit IgG conjugated with alkaline phosphate.

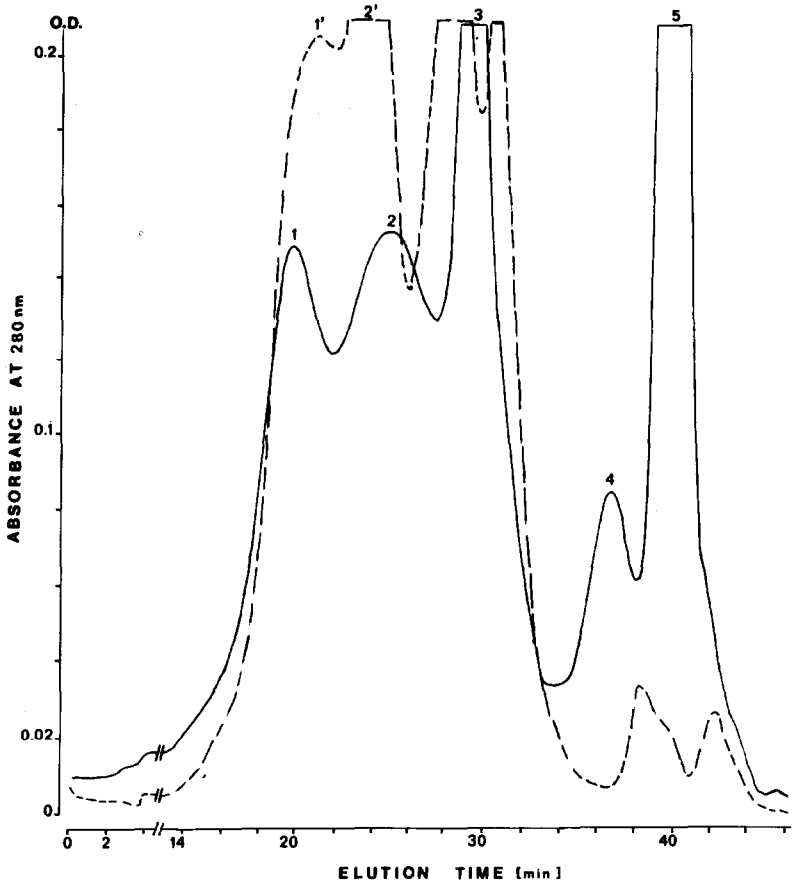

Fig. 3. Gel permeation chromatography on a Superose 6 column of mouse IgM monoclonal antibody P13 isolated from the Mono Q column. The IgM-rich fractions from the Mono Q column (peaks 4 and 5) were concentrated by ultrafiltration (0.5–0.7 ml) and injected into the column at a flow-rate of 0.5 ml/min. (———) Prior injection, the Superose 6 column was equilibrated in 5 m*M* L-histidine, pH 6.0. The IgM sample was then injected and eluted from the column with a buffer containing 1.7 *M* NaCl in 50 m*M* L-histidine, pH 6.0. (– – – –) Under normal ionic strength conditions, the IgM sample was injected into the Superose 6 column previously equilibrated with 50 m*M* L-histidine, pH 6.0, containing 0.5 *M* NaCl and eluted with the same buffer.

antigen (human platelet thrombospondin) against which monoclonal antibody P13 was directed. Results obtained confirmed the presence of IgM antibodies P13 in peaks 3, 4 and 5 and showed a strong anti-thrombospondin activity in peak 5 (Fig. 2). Similar results were obtained with four other mouse ascites fluids containing IgM antibodies (P14, P15, P16 and P17) directed against human platelet thrombospondin (results not shown). Recovery of the IgM antibodies in peak 5, based on recovery of immunological activity, was about 75% of the activity originally present in the ascites fluids.

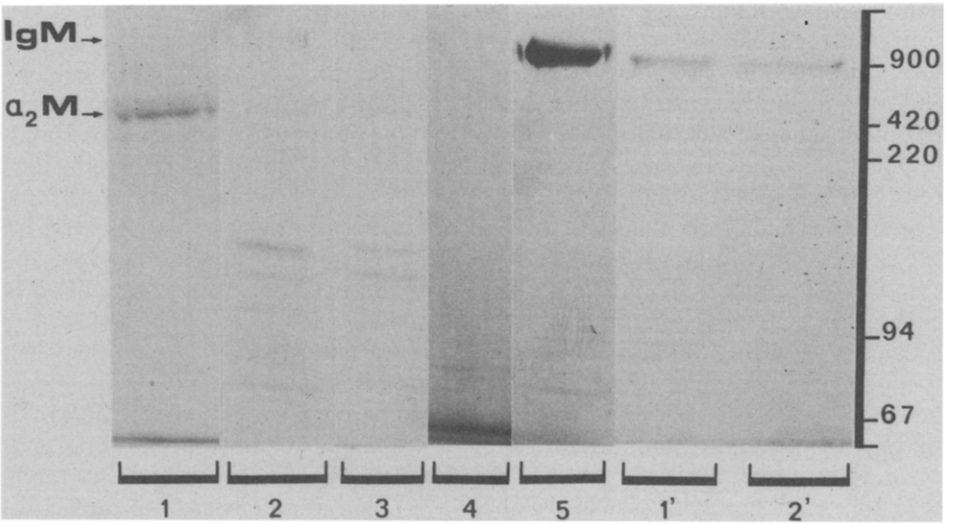

Fig. 4. Assessment of the purity of isolated IgM monoclonal antibodies by SDS-PAGE and Coomassie staining. The eluted peaks from the Superose 6 gel permeation column were treated with SDS and subjected to electrophoresis under non-reducing conditions on a 3–10% gradient SDS-polyacrylamide gel. The gel was fixed and stained with Coomassie Brillant Blue R 250. Numbers below each lane correspond to peak numbers. Lane 5 was loaded with about 100 μg of eluted material. IgM = immunoglobulin M; α_2M = α_2-macroglobulin.

Purification of isolated IgM monoclonal antibodies by FPLC gel permeation chromatography

The IgM-rich fractions from the Mono Q column shown to be strongly immunoreactive (peaks 4 and 5) were injected on a gel permeation Superose 6 column either equilibrated with a low-ionic strength buffer (Fig. 3A) or a regular buffer (Fig. 3B). The eluted peaks were subjected to electrophoresis under non-reducing conditions on a 3–10% gradient SDS-polyacrylamide gel. Coomassie brillant blue staining of electrophoresed proteins clearly showed that IgM monoclonal antibodies eluted in peak 5 (Fig. 3A) with a high degree of purity (Fig. 4; lane 5) when IgM-rich fractions were gel-filtered through a column equilibrated with a low-ionic strength buffer and eluted with a high-ionic strength buffer. On the other hand, IgM monoclonal antibodies eluted in peak 1′ (Fig. 3B) and tended to co-elute with α_2-macroglobulin as shown by SDS-PAGE (Fig. 4; lanes 1′ and 2′) when using normal ionic strength conditions of chromatography. The use of an enzyme linked immunosorbent assay with solid phase adsorbed antigen indicated that IgM monoclonal antibodies present in peaks 1′, 2′ and 5 were immunologically active (results not shown).

DISCUSSION

Results obtained in this study demonstrate that mouse IgM monoclonal antibodies are rapidly isolated from ascites fluids with a high degree of purity using anion-exchange followed by gel filtration chromatography on a FPLC system. Purification of mouse IgM monoclonal antibodies from ascites fluids using classical

methods usually requires a preliminary precipitation step[1,3–5] which has been shown to denature IgM monoclonal antibodies[2,6]. The results presented here indicate that pure IgM antibodies, isolated without prior precipitation, remain immunoreactive. While a tandem procedure has been employed in this study, it was observed during the anion-exchange step that most proteins from the mouse ascites fluids were found, like IgM antibodies, in more than one eluted peak. It is possible that interactions occur between IgM antibodies and other proteins, such as albumin and α_2-macroglobulin, to explain their elution at the same salt concentration. Similar observations were previously reported using the FPLC-Mono Q system[17,18]. Alternatively, the IgM antibodies in peak 3, eluting at a lower salt concentration than IgM antibodies contained in peaks 4 and 5, may represent inactive IgM monoclonal antibodies. The presence of inactive IgM monoclonal antibodies in peak 3 could explain the weak immunoreactivity obtained by ELISA. Stanker *et al.*[10] purifying mouse IgG_1 antibodies from ascites fluids by hydroxylapatite chromatography, also reported the elution of a inactive IgG_1 antibody at a slightly lower phosphate concentration than does the active IgG_1 antibody. Most of the classical IgM purification methods rarely avoid contamination with α_2-macroglobulin[1,6]. Using a modification of the gel filtration technique described by Bouvet *et al.*[6] as the second step of the tamdem purification of IgM antibodies, we shown in this study that purified mouse IgM monoclonal antibodies were free of contaminants including alpha α_2-macroglobulin. Furthermore, these mouse IgM antibodies were devoid of protease activity since long-term storage did not result in loss of antibody immunoreactivity. Recently, Stanker *et al.*[10] reported the purification of mouse IgG and IgM monoclonal antibodies from ascites fluids by hydroxyapatite chromatography with a 0.01–0.3 *M* phosphate gradient (pH 6.8). Under the chromatographic conditions used by the authors, both IgG and IgM antibodies eluted between 0.15 and 0.2 *M* sodium phosphate. Since ascites fluids are often contaminated with host mouse IgG antibodies[10,15], the isolation of mouse IgM monoclonal antibodies from ascites fluids by hydroxyapatite chromatography cannot avoid a contamination of IgM with non-specific mouse IgG antibodies. In this respect, the purification of mouse IgM antibodies from ascites fluids by hydroxyapatite chromatography remains possible only when Balb/c mice are immunosuppressed or irradiated to avoid the presence of host mouse immunoglobulins in ascites tumors. On the other hand, the isolation on a Mono Q column of mouse IgM monoclonal antibodies from ascites fluids allows their separation from non-specific mouse IgG antibodies since both immunoglobulin classes elute differently from the Mono Q anion-exchange column[16,19]. In this respect, the chromatographic procedure described in this study provides a new method for the rapid isolation (80 min) of mouse IgM monoclonal antibodies to a high degree of purity (greater than 90% pure) and in a functionally active state. Furthermore, this procedure can be adapted to the large scale purification of IgM monoclonal antibodies using preparative scale FPLC techniques.

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