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TANDEM PURIFICATION OF MOUSE IgM MONOCLONAL ANTIBODIES PRODUCED *IN VITRO* USING 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 produced by cultivation of hybridomas *in vitro*. Hybridoma culture supernatants containing mouse IgM monoclonal antibodies were first chromatographed on an anion-exchange Mono Q column connected to a fast protein liquid chromatography system. This anion-exchange step offers the advantage of obtaining IgM antibodies in a concentrated form. 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 Brilliant Blue R 250 staining technique. Assessment of the immunoreactivity of isolated IgM monoclonal antibodies was performed by an enzyme linked immunosorbent assay using a solid phase adsorbed antigen against which IgM monoclonal antibodies were directed. The chromatographic procedures described allows the rapid isolation of mouse IgM monoclonal antibodies produced *in vitro* at a high degree of purity and in an immunoreactive state.

INTRODUCTION

The purification of IgM monoclonal antibodies usually requires multistep pro-

cedures 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 to denature IgM antibodies[2]. In addition, these techniques rarely avoid contamination with alpha-2 macroglobulin[1]. Recent developments in high-performance liquid chromatography have opened new possibilities in the isolation of IgG[6-9] and IgM monoclonal antibodies[9,10] from mouse ascitic fluids. However the usefulness of some methods[9] remains limited due to the contamination of ascitic fluids by host mouse immunoglobulins[10,11].

Because *in vitro* cultivation offers better possibilities of obtaining monoclonal antibodies free from irrelevant mouse immunoglobulins[11], we describe here a tandem chromatographic procedure to isolate rapidly mouse IgM monoclonal antibodies produced in vitro by hybridomas. Hybridoma culture supernatants containing IgM monoclonal antibodies were first chromatographed on a Mono Q anion-exchange column connected to a fast protein liquid chromatography (FPLC) system. The advantage of this anion-exchange step is that the IgM antibodies are obtained in a concentrated form. Eluted peaks from the Mono Q column 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 allows the rapid isolation of *in vitro* produced IgM monoclonal antibodies in a high degree of purity and 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[7,10].

Five hybridoma culture supernatants containing mouse IgM monoclonal antibodies were obtained from the following sources: (ESE 1 and ESE 7) were IgM monoclonal antibodies directed against endothelial cells isolated from human umbilical cord veins[12]; (ES 4, ES 9 and ES 15), specific for blood groups B, AB and A respectively, were obtained from BioScot (Edinburgh, U.K.).

The tandem chromatographic procedure was essentially as previously described[10]. Culture supernatants containing mouse IgM monoclonal antibodies were centrifuged at 700 *g* for 15 min prior to chromatographic separations on a anion-exchange Mono Q column.

Anion-exchange chromatography
Culture supernatants containing mouse IgM monoclonal antibodies (25 ml)

TABLE I

SEGMENTATION OF THE GRADIENT

Elution time (min)	Final buffer concentration (%)
1.5	0
8.5	28
13.5	35
20	100

were equilibrated in the starting buffer (25 ml) and then injected via a 50-ml Super-loop (335 × 30 mm) (Pharmacia) onto a Mono Q HR 5/5 anion-exchange column. The starting buffer (0%) was 20 mM L-histidine, pH 6.0, and the final buffer (100%) was 20 mM L-histidine, pH 6.0, containing 0.5 M sodium chloride. The 0–100% gradient, generated over 20 min at a flow-rate of 1 ml/min, was controlled by the liquid chromatography controller LCC-500 (Pharmacia) and segmented as shown in Table I.

Gel filtration chromatography

The 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 HR 10/30 column previously equilibrated with a low-ionic strength buffer containing 5 mM L-histidine, pH 6.0. The eluting buffer was 50 mM L-histidine, pH 6.0, containing 1.7 M sodium chloride. In some experiments a buffer containing 0.5 M sodium chloride in 50 mM L-histidine, pH 6.0, was used to equilibrate the Superose 6 column and to elute the IgM monoclonal antibodies.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Following the method of Mason and Williams[13], 100 μl of sample mixed with 100 μl of buffer containing 5% (w/v) sodium dodecyl sulphate (SDS), 25% (w/v) glycerol and 2 mM 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[14]. Gels were fixed in 40% methanol (v/v)–7% acetic acid (v/v) and then stained with Coomassie Brillant Blue R 250.

Enzyme linked immunosorbent assay

Mouse IgM antibodies present in peaks eluted from the Mono Q and the Superose 6 columns were detected by ELISA using a solid phase adsorbed antigen (endothelial cells) against which IgM monoclonal antibodies were directed.

A suspension of confluent endothelial cells was first diluted to a ratio of 1:4 (v/v) in Medium 199 (modified) supplemented with 20% (v/v) pooled human serum and 150 μg/ml of endothelial growth supplement (ECGS)[15]. This suspension was added (100 μl/well) to 96-well microtitre plates (Costar) and incubated 2 h at 37°C. Wells coated with human endothelial cells were then washed four times with 100 μl/well of Medium 199 (modified). After removing the washing medium, 100 μl/well of Medium 199 supplemented with 20% (v/v) foetal calf serum (FCS) and 150 μl/ml of ECGS were added to the wells and the endothelial cells were grown to confluent

density at 37°C. Once confluence was attained, contents of the wells were aspirated and the cells were rinsed 4 times with Medium 199. The cell monolayer was then fixed for 10 min at 4°C with 0.025% (v/v) glutaraldehyde containing 0.05% (v/v) Tween 20. After removing the fixative, 60 μl of all the eluted peaks from the Mono Q and Superose 6 columns were added to the wells and left for 1 h at 37°C. A negative control was performed by the use of Tris-buffered saline (pH 7.6) (TBS) instead of eluted peaks. A positive control was performed by including the culture supernatants containing mouse IgM antibodies. After incubation, the contents of the wells were aspirated and the cells rinsed 4 times with TBS containing 0.05% (v/v) Tween 20. A 60-μl volume of peroxidase conjugated rabbit anti-mouse immunoglobulin (diluted to 1/500) (Dakopatts) was added to each well for an additional 1 h incubation at 37°C. Contents of the wells were then aspirated and rinsed 4 times with TBS containing 0.05% (v/v) Tween 20 (100 μl/well). An amount of 20 mg of substrate (*o*-phenylenediamine) were dissolved in 20 ml phosphate citrate buffer (pH 5.5) and 20 ml distilled water containing 20 μl of a 30% (v/v) hydrogen peroxide solution. This substrate was added to each well (60 μl) and the enzyme reaction was allowed to proceed for 25 min in the dark at room temperature. The absorbance was read at 492 nm using a Titertek Multiskan MC photometer (Flow Laboratories).

RESULTS

Isolation of in vitro produced IgM monoclonal antibodies from hybridoma culture supernatants by FPLC anion-exchange chromatography

Using a small sample loading (200 μl) onto the Mono Q column, culture supernatants containing mouse IgM monoclonal antibody ESE 7 showed 3 major peaks eluting at 0.11, 0.15 and 0.22 *M* sodium chloride, respectively (Fig. 1). However,
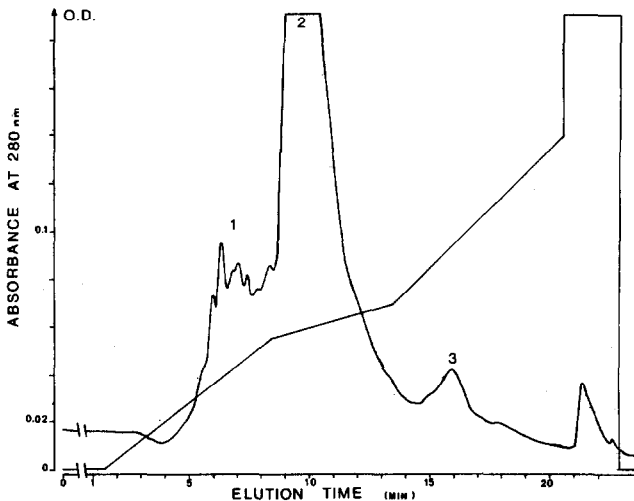

Fig. 1. Analytical separation of mouse IgM monoclonal antibody ESE 7 present in hybridoma culture supernatants using a Mono Q column connected to a FPLC system. Culture supernatants (0.2 ml in 5 ml of starting buffer) were injected into the column via a 50-ml Superloop. The gradient of 0–0.5 *M* NaCl in 20 m*M* L-Histidine (pH 6.0) was generated over 20 min at a flow-rate of 1 ml/min.

when the sample loading onto the Mono Q column was scaled up to 25 ml of culture supernatants, peaks 1 and 2 eluted at 0.08 and 0.12 M sodium chloride, respectively, whereas the salt concentration at which peak 3 eluted was unchanged (Fig. 2A). Similar elution patterns, under small and large sample loadings, were obtained with
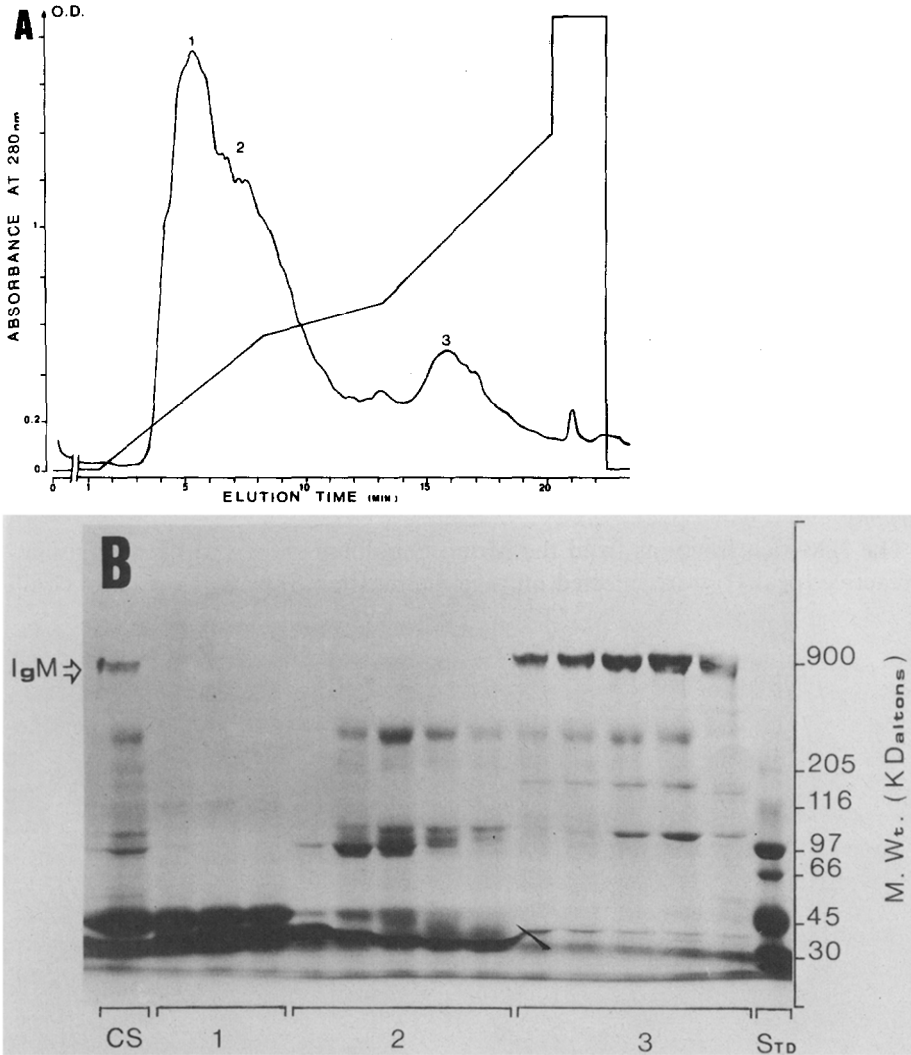

Fig. 2. (A) Preparative separation of mouse IgM monoclonal antibody ESE 7 present in hybridoma culture supernatants using a Mono Q column connected to a FPLC system. Culture supernatants (25 ml in 25 ml of starting buffer) were injected into the column via a 50-ml Superloop. The gradient of 0–0.5 M NaCl in 20 mM L-histidine (pH 6.0) was generated over 20 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. Lane CS contains the culture supernatant prior to chromatography on a Mono Q column. Lane Std contains molecular weight standards. Numbers below each lane correspond to peak numbers. IgM = Immunoglobulin M.

4 different culture supernatants containing IgM monoclonal antibodies (ESE1; ES 4; ES 9 and ES 15) (results not shown).

Eluted peak fractions were subjected to electrophoresis under non-reducing conditions on a 3–10% gradient SDS-polyacrylamide gel and the separated proteins were identified by Coomassie staining (Fig. 2B). In typical electrophoretic separations of eluted peaks, peak 1 contained 2 major protein bands at 30 and 40 kDa (fig. 2B, lane 1); peak 2 mainly contained protein bands at 95 and 300 kDa (Fig. 2B, lane 2). In addition to protein bands between 110 and 300 kDa, a 900 kDa protein band corresponding to the apparent molecular weight of IgM antibodies[2] was present in peak 3 (Fig. 2B, lane 3).

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

In order to assess the immunoreactivity of IgM monoclonal antibody ESE 7, eluted peaks from the Mono Q column were added to plastic wells coated with the antigen (human endothelial cells) against which this monoclonal antibody was directed. The strong immunoreactivity observed in peak 3 confirmed the presence of IgM antibody ESE 7 in this peak (Fig. 3). Similar results were obtained with another monoclonal antibody (ESE 1) directed against human endothelial cells (results not shown).

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 (peak 3) were injected on a gel permeation Superose 6 column equil-
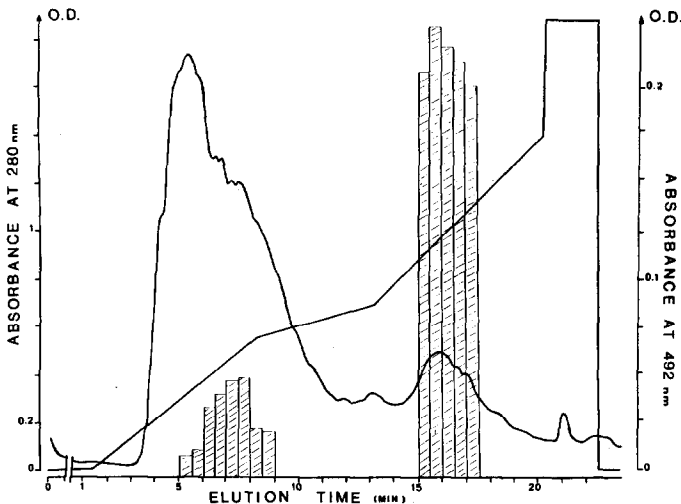

Fig. 3. Assessment of the immunoreactivity of isolated IgM monoclonal antibody ESE 7 using an ELISA. Eluted peak fractions of the Mono Q chromatographic profile were added to plastic wells previously coated with glutaraldehyde-fixed human endothelial cells, the antigen against which was directed the IgM antibody ESE 7. The hatched bars show the binding of the IgM monoclonal antibody to endothelial cells using peroxidase conjugated rabbit anti-mouse immunoglobulins. The absorbance was read at 492 nm.
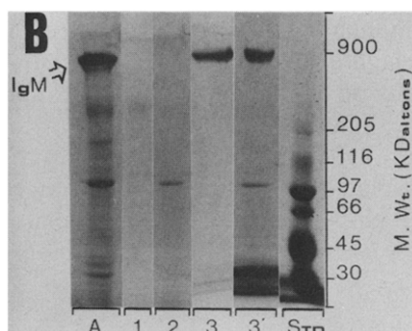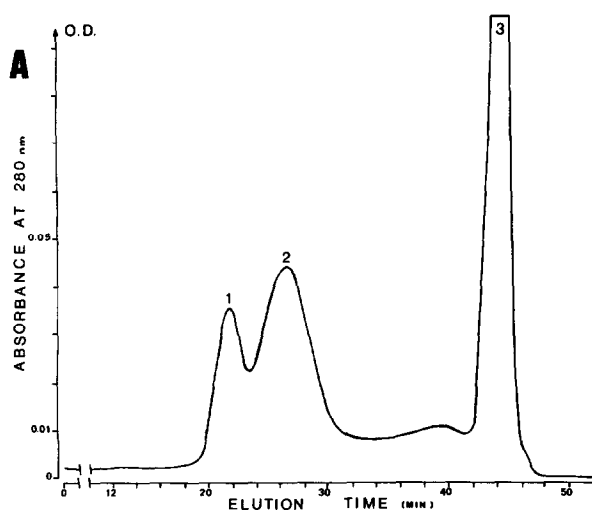
Fig. 4. (A) Gel permeation chromatography on a Superose 6 column of mouse IgM monoclonal antibody ESE 7 isolated from the Mono Q column. The IgM-rich fractions from the Mono Q column (peak 3) were concentrated by ultrafiltration (0.5–0.7 ml) and injected into the Superose 6 column at a flow-rate of 0.5 ml/min. The Superose 6 column was previously equilibrated in 5 mM L-histidine pH 6.0 and the IgM sample eluted from the column with a buffer containing 1.7 M NaCl in 50 mM L-histidine, pH 6.0. (B) Eluted peaks from the Superose 6 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. Lane A contains isolated IgM antibody from the Mono Q column. Lane Std corresponds to the molecular weight standards. Numbers below each lane correspond to peak numbers. IgM = Immunoglobulin M.

ibrated then eluted with low- and high-ionic strength buffers respectively (Fig. 4A). The eluted peaks were electrophoresed under non-reducing conditions on a 3–10% gradient SDS-polyacrylamide gel. Coomassie brillant blue staining of electrophoresed proteins showed that peaks 1 and 2 (Fig. 4A) contained protein bands at 300 and 110 kDa respectively (Fig. 4B; lanes 1 and 2) whereas IgM monoclonal antibodies were present in peak 3 (Fig. 4A) with a high degree of purity (Fig. 4B, lane 3). Contamination with a 110 kDa protein band sometimes occurred (Fig. 4B, lane 3′) but was easily removed by running the isolated IgM peak onto the Superose 6 column under normal ionic strength conditions (results not shown).

Similar results were obtained with 4 different culture supernatants containing IgM monoclonal antibodies (ESE 1; ES 4; ES 9 and ES 15) (results not shown). When chromatographed under normal ionic strength conditions, IgM monoclonal antibodies eluted just before peak 1 and tended to co-elute with the 300 kDa protein band (results not shown).

Assessment of the immunoreactivity of purified IgM monoclonal antibodies

The use of an enzyme linked immunosorbent assay with solid phase adsorbed antigen (human endothelial cells) indicated that the IgM monoclonal antibody ESE 7, present in peak 3, was strongly immunoreactive (Fig. 5). A slight immunoreactivity was also observed in peak 1 (Fig. 5). Similar results were obtained with another monoclonal antibody (ESE 1) directed against human endothelial cells (results not shown). Recovery of the IgM antibodies in peak 3, based on recovery of immunological activity, was about 60% of the activity originally present in culture supernatants.

DISCUSSION

This study demonstrates that mouse IgM monoclonal antibodies produced *in vitro* by hybridomas, are rapidly isolated with a high degree of purity using anion-exchange followed by gel filtration chromatography on a FPLC system. Purification of mouse IgM monoclonal antibodies using classical methods usually requires a preliminary precipitation step[1,3-5] known to denature IgM antibodies[2,10]. The results presented here indicate that pure IgM antibodies, isolated without prior precipitation, remain immunoreactive. Recent developments in high-performance liquid chromatography had already led to the isolation from mouse ascitic fluids of IgM monoclonal
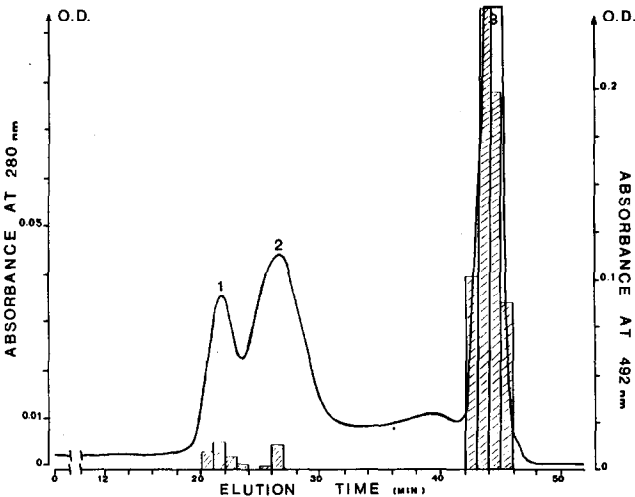

Fig. 5. Assessment of the immunoreactivity of purified monoclonal antibody ESE 7 using an ELISA. The ELISA procedure is similar to that described in the legend to Fig. 3. The hatched bars show the binding of the IgM antibody to endothelial cells using peroxidase conjugated rabbit antimouse immunoglobulins. The absorbance was read at 492 nm.

antibodies in a pure and immunoreactive state[9,10]. However the usefulness of some IgM purification methods[9] was limited due to contamination of ascitic fluids by host mouse immunoglobulins[10,11]. Instead of growing hybridomas *in vivo* as ascitic tumours, their cultivation *in vitro* was preferred in this study because it offered a better chance of obtaining IgM monoclonal antibodies free from irrelevant mouse immunoglobulins.

We have noticed that the salt concentrations at which the peaks eluted from the Mono Q column decreased with increased sample loadings. Such an effect indicates that culture supernatants contain proteins with a low net charge which are displaced by other proteins having a high net charge[16]. This causes proteins to be eluted from the Mono Q column more quickly. A similar effect was previously observed on a Mono Q column when purifying either mouse IgM antibodies from ascitic fluids[17] or Fab fragments from mouse IgG and human IgA antibodies[7,18]. Nevertheless, this loading effect improves the resolution of IgM antibodies from the Mono Q column since the elution time of the IgM peak was not affected compared to the others. In addition, this anion-exchange step offers the advantage of obtaining IgM antibodies from the Mono Q column in a concentrated form. Using a modification[10] of the gel filtration technique described by Bouvet *et al.*[19] as the second step of our tandem purification procedure, we obtained IgM monoclonal antibodies with a high level of purity. These mouse IgM antibodies were devoid of protease activity since long-term storage did not result in loss of antibody immunoreactivity. Bouvet *et al.*[19] reported that polyclonal IgM from human sera displayed a normal behaviour in a low-ionic strength buffer compared to monoclonal IgM antibodies. In this respect, the slight immunoreactivity observed in peak 1 (see Fig. 5) may reflect the presence of polyclonal IgM antibodies contained in the foetal calf serum added to the culture medium. In conclusion, the chromatographic procedure described in this study allows the rapid isolation of IgM monoclonal antibodies produced *in vitro* at a high degree of purity and in a immunoreactive state. Such a procedure can be adapted for large scale purification of IgM monoclonal antibodies.

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