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ONE-STEP PROCEDURE FOR THE RAPID ISOLATION OF MOUSE MONOCLONAL ANTIBODIES AND THEIR ANTIGEN BINDING FRAGMENTS BY FAST PROTEIN LIQUID CHROMATOGRAPHY ON A MONO Q ANION-EXCHANGE COLUMN

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SUMMARY

A one-step chromatographic procedure was used to isolate rapidly mouse IgG monoclonal antibodies (mAbs) (IgG_1, IgG_{2a}, IgG_{2b}) contained in ascites fluids and Fab fragments contained in papain-treated mAb suspensions. Chromatographic separations were performed on an anion-exchange Mono Q column connected to a fast protein liquid chromatographic (FPLC) system. Detection of mAb or their antigen binding fragments (Fab) in eluted peaks was performed using sodium dodecyl sulphate–polyacrylamide gel electrophoresis together with a silver or a Coomassie Brillant Blue R 250 staining technique and solid phase radioimmunoassay with ^{125}I-labelled sheep anti-mouse antibodies directed against total immunoglobulins. Rapid assessment of the purity of isolated mAbs and their Fab fragments was performed by gel permeation chromatography on a TSK G 3000 SW column. Mouse mAbs and their Fab fragments were rapidly isolated (25 min), in a functionally active state, to a high degree of purity on the FPLC–Mono Q system compared to the time taken by other techniques.

INTRODUCTION

The extensive use of hybridoma technology has led to the adaptation or the development of several new methods for the isolation of murine immunoglobulins

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(IgGs) from culture or ascites fluids[1–4]. Precipitation with ammonium sulphate followed by DEAE-cellulose anion-exchange chromatography is extensively used for the isolation of mouse monoclonal antibodies (mAbs)[1,2]. However, this precipitation technique was shown, in some cases, to reduce significantly antibody activity[2,5]. Purification of mouse IgG mAb has been successfully performed using Protein A–Sepharose CL-4B affinity chromatography[3], but one subclass of mouse immunoglobulins (IgG_1) was reported to be bound only weakly and another (IgG_3) not at all[2,3]. Anion-exchange chromatography of mouse ascites fluids on DEAE-Affi-gel blue is as lengthy as the Protein A–Sepharose CL-4B technique and the isolated immunoglobulin fractions were shown to be contaminated with transferrin[2]. Very little information is currently available on the rapid isolation, from ascites fluids, of different mouse IgG subclasses under conditions where the purified antibody remains viable and free of protein contaminants. Recent developments in high-performance liquid chromatography together with anion-exchange columns have opened new possibilities in the separation of mAb from mouse ascites fluids[4,6].

The aim of this study was to develop a one-step chromatographic procedure to isolate rapidly from ascites fluids mouse mAb free from contaminating proteins and in a functionally active state. In addition, the same procedure was used to isolate antigen binding fragments (Fab) fragments from undigested IgG, papain and fragments of the C-terminal half of the heavy chain (Fc). Using a fast protein liquid chromatographic (FPLC) system, different subclasses (IgG_1, IgG_{2a}, IgG_{2b}) of mouse mAb or Fab fragments were isolated on an anion-exchange Mono Q column. Mouse mAb and the Fab fragments of IgG_1 antibodies were rapidly isolated (25 min), compared to the time taken by other techniques, in a high degree of purity using the FPLC–Mono Q system. Rapid assessment of the purity of isolated mAbs and their Fab fragments was performed by gel permeation chromatography on a TSK G 3000 SW column. The chromatographic procedure described provides a new method for the isolation of different subclasses of mouse mAbs and their Fab fragments.

EXPERIMENTAL

The FPLC separations were performed either on a Mono Q HR 5/5 prepacked anion-exchange column (50 × 5 mm) (Pharmacia, Uppsala, Sweden) or on a TSK G 3000 SW filtration column (600 × 7.5 mm) (LKB Instrument, Orsay, France). Materials and chemicals, unless stated otherwise, were as previously indicated[7]. Nine mouse ascites fluids or purified mouse monoclonal antibodies (mAbs) of different subclasses were obtained from the following sources: IgG_1 mAb [P2, P9, P10, P11, P12][5,8]; IgG_{2a} mAb [BL-4, P7] (a gift from Dr. J. Brochier), [CL-87] (a gift from P. Giraudon and Dr. F. Wild]; IgG_{2b} mAb [P8][9], [BL-15] (a gift from Dr. J. Brochier), [CL-15] (a gift from P. Giraudon and Dr. F. Wild). The preparation of Fab fragments from mouse IgG_1 mAb was as described by Hudson and Hay[10]. Briefly, digestion of IgG_1 mAb was performed at 37°C for 18 h with 1% (w/w) papain at pH 7.0 in the presence of both 0.01 *M* cystein and 0.002 *M* EDTA. The mixture of Fab and Fc fragments, purified mouse mAb and mouse ascites fluids was passed through 0.45-μm filters (Millipore), equilibrated in the starting buffer using a 50-ml Superloop (335 × 30 mm) (Pharmacia) and then injected via the Superloop on different columns connected to an FPLC apparatus. The following types of chromatography were used.

Anion-exchange chromatography. Mono Q HR 5/5 column. The experimental conditions for the purification of mouse IgG mAb were with a few modifications as described by Hill *et al.*[6]. The starting buffer was 0.02 *M* triethanolamine–HCl, pH 7.7 and the final buffer was 0.02 *M* triethanolamine–HCl, pH 7.7, containing 0.35 *M* sodium chloride. The gradient was generated over 25 min at a flow-rate of 1 ml/min. The isolation of Fab fragments from mouse IgG_1 mAb was performed using a linear 0.005–0.3 *M* phosphate gradient at pH 8.0. The gradient was generated over 25 min at a flow-rate of 1 ml/min.

Gel permeation chromatography. TSK G 3000 SW filtration column. Mouse IgG mAbs were equilibrated then purified using a mobile phase containing 0.05 *M* sodium acetate–HCl at pH 5.0. Flow-rate was 0.3 ml/min. High- and low-molecular-weight standards were from Boehringer-Mannheim (F.R.G.).

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Following the method of Mason and Williams[11], 50 µl of sample buffer containing 5% (w/v) sodium dodecyl sulphate (SDS), 25% (w/v) glycerol and 2% (w/v) dithiothreitol were heated at 100°C for 5 min and alkylated by addition of 20 µl of 1 *M* iodoacetamide. Reduced proteins were subjected to electrophoresis on a 10% SDS Laemmli gel[12]. Gels were fixed in 40% methanol–7% acetic acid and then stained either with Coomassie Brillant Blue R 250 or silver reagent[13]. Silver-stained gels bearing electrophoresed protein bands were scanned with a densitometer (PHI, Vernon). Peaks from densitometric tracings were cut out, weighed and expressed as a percentage of the total weight of peaks following the method of Toor *et al.*[14].

Solid phase radioimmunoassay. The solid phase radioimmunoassay (SPRIA) procedure used was similar to that described by Howard *et al.*[15], with minor modifications as published previously[7]. Mouse mAbs bound to polyvinyl chloride microtitre plates were identified using ^{125}I-labelled sheep anti-mouse antibodies directed against total immunoglobulins.

RESULTS

Separation of mouse monoclonal antibodies (mAb) from mouse ascites by FPLC anion-exchange chromatography

Mouse ascites fluids containing mAb P10 (IgG_1 subclass), separated on a Mono Q column, showed four major peaks which were eluted by a gradient of 0–0.35 *M* sodium chloride (Fig. 1A). Similar elution patterns were obtained with four different mouse ascites fluids containing IgG_1 mAb [P2, P9, P11 and P12] (results not shown). A SPRIA system using ^{125}I-labelled sheep anti-mouse antibodies directed against total immunoglobulins showed the presence of mAb in peak 2 (Fig. 1B). Eluted peak fractions were subjected to electrophoresis under reducing conditions on 10% SDS–polyacrylamide gels and the separated proteins were identified by staining with Coomassie brilliant blue. In typical electrophoretic separations of eluted peaks, transferrin was present in peak 1 which was eluted at 0.12 *M* sodium chloride, IgG_1 was present in peak 2 which was eluted between 0.154 and 0.18 *M* sodium chloride, albumin was present in peak 3 which was eluted at 0.245 *M* sodium chloride and peak 4 eluted at 0.3 *M* sodium chloride contained a mixture of high-molecular-weight proteins and albumin (Fig. 1C). The IgG_1 peak was slightly contaminated with transferrin. Densitometric tracings of silver-stained gels bearing electrophoresed protein

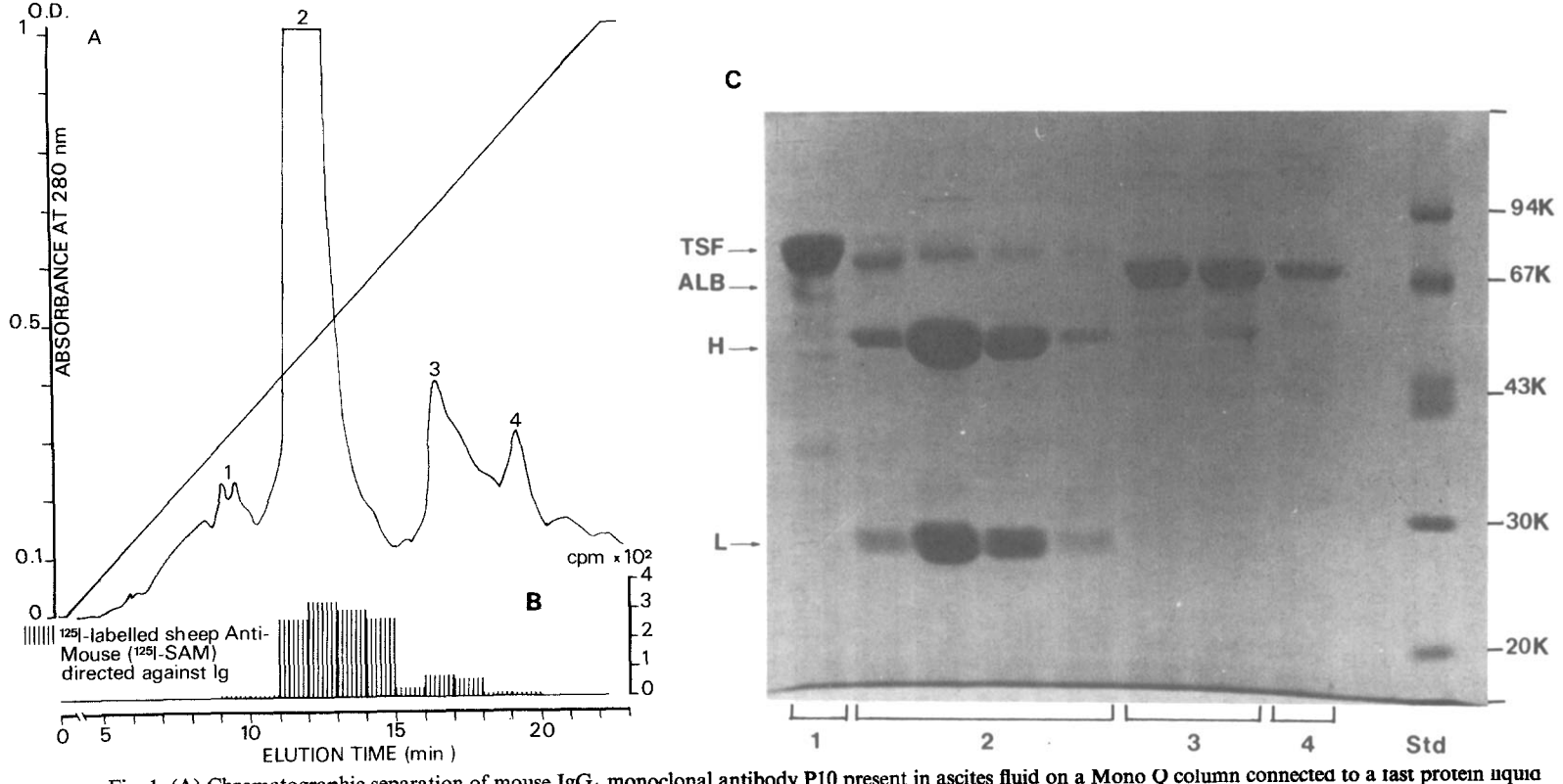

Fig. 1. (A) Chromatographic separation of mouse IgG_1 monoclonal antibody P10 present in ascites fluid on a Mono Q column connected to a fast protein liquid chromatographic (FPLC) system. Ascites fluid (3 mg of proteins per 15 ml) was injected into the column via a 50-ml Superloop. The gradient of 0–0.35 *M* NaCl in 20 m*M* triethanolamine buffer (pH 7.7) was generated over 25 min at a flow-rate of 1 ml/min. (B) Monoclonal antibodies (IgG_1) in each peak fraction of the Mono Q chromatographic profile were detected by solid phase radioimmunoassay (SPRIA) using ^{125}I-labelled sheep anti-mouse antibodies directed against total immunoglobulins. (C) Eluted peaks were treated with sodium dodecyl sulphate (SDS) and subjected to electrophoresis under reducing conditions on a 10% SDS–polyacrylamide gel. The gel was fixed and stained by Coomassie brilliant blue. Numbers below each lane correspond to peak numbers. TSF = Transferrin; ALB = albumin; H = heavy chain and L = light chains of IgG_1 monoclonal antibodies. The lane marked as Std contains low-molecular-weight standards.

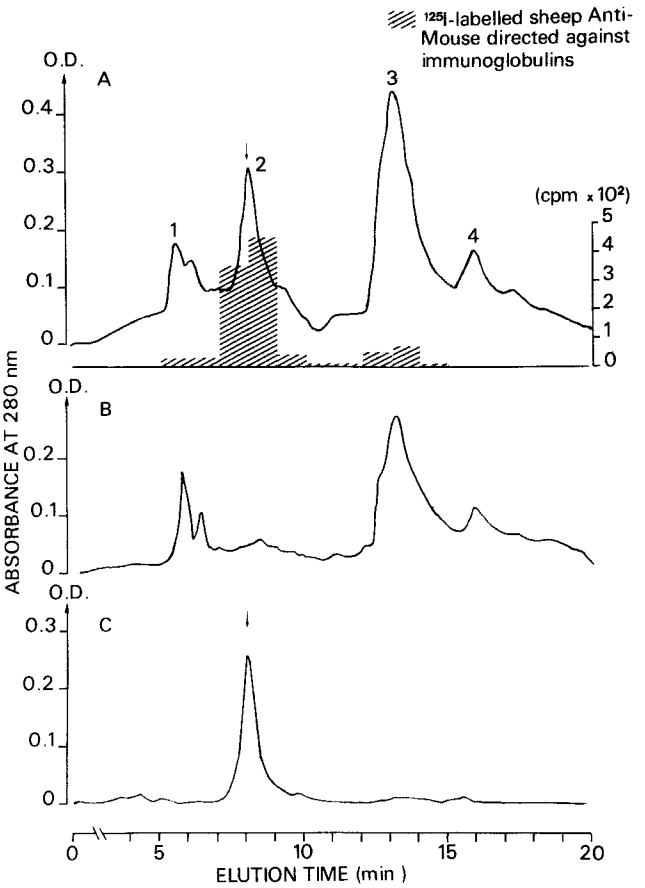

Fig. 2. Comparison of chromatographic profiles obtained on a Mono Q column: (A) mouse ascites fluid containing IgG_1 monoclonal antibody P2; (B) the flow-through material (containing no IgG_1) from a Protein A–Sepharose CL-4B column; (C) the isolated IgG_1 monoclonal antibody P2 obtained by affinity chromatography on Protein A–Sepharose CL-4B column. Monoclonal antibodies (IgG_1) in each peak fraction of the Mono Q chromatographic profile (A) were detected using ^{125}I-labelled sheep anti-mouse antibodies directed against total immunoglobulins. These results demonstrate that all of the mouse IgG_1 monoclonal antibodies present in the ascites fluid were bound to the Protein A–Sepharose CL-4B column and could be eluted with NaCl.

bands of isolated IgG_1 present in eluted peak 2 indicated that the transferrin contamination represented 7% of the total weight of peaks (results not shown). Monoclonal antibody P2 (IgG_1) from ascites fluid, isolated on a Protein A–Sepharose CL-4B column, when analyzed on the Mono Q column, showed one peak eluting at the same position as peak 2, which was previously shown to contain IgG_1 mAb (Fig. 2C). In addition, the Protein A–Sepharose CL-4B flow-through material of the ascites fluids, containing no mAb, when run on the Mono Q column showed the absence of the IgG_1 peak (Fig. 2B). Mono Q chromatographic profiles, SPRIA and SDS–polyacrylamide gel electrophoresis of mouse ascites fluids containing mAb of the IgG_{2a} subclass (P7, CL-87 and BL-4) were similar to those obtained for the isolation of

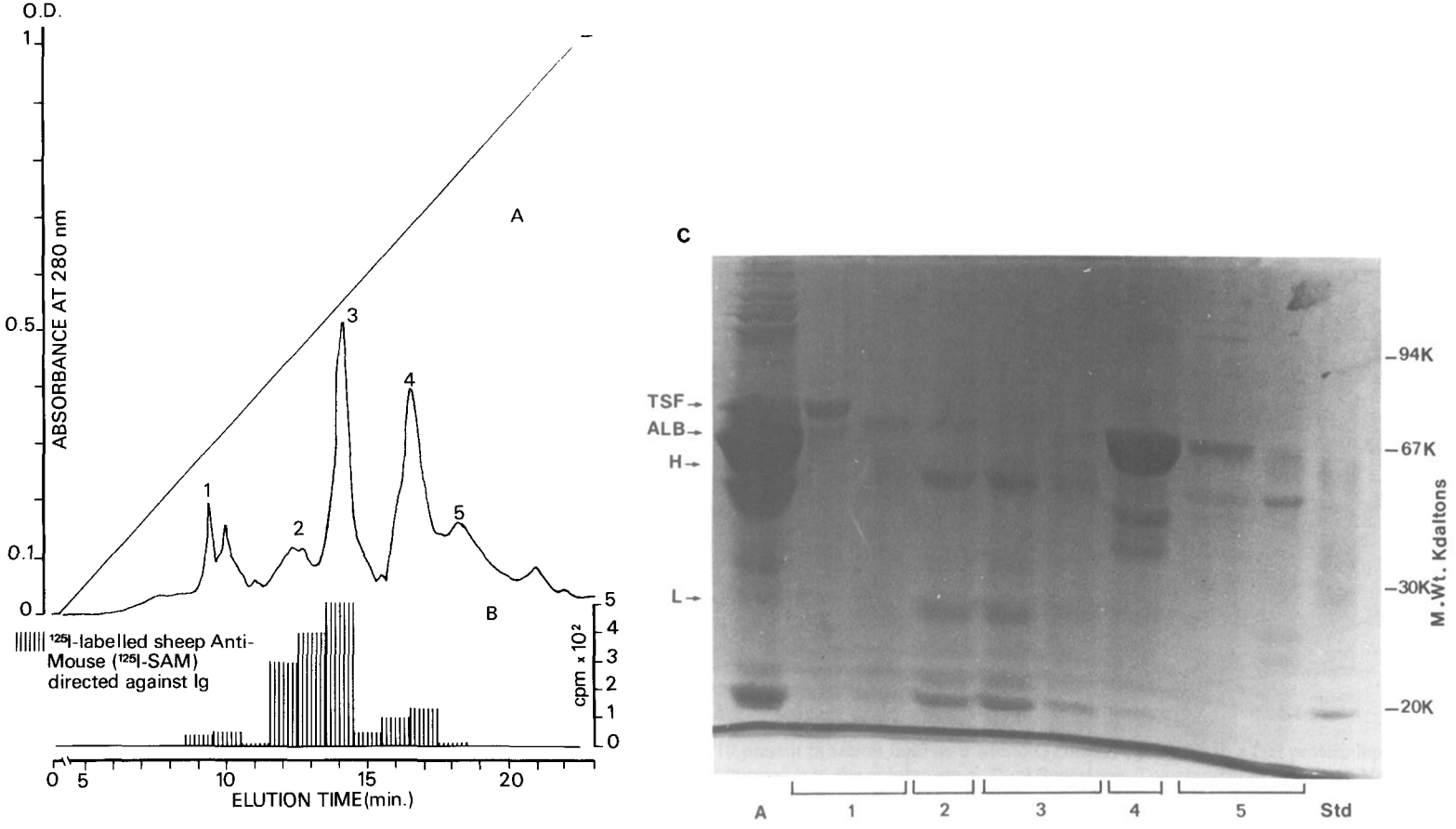

Fig. 3. (A) Chromatographic separation of mouse IgG_{2b} monoclonal antibody P8 present in ascites fluid on a Mono Q column connected to a FPLC system. Ascites fluid (1 mg of proteins per 15 ml) was injected into the column via a 50-ml Superloop. The gradient of 0–0.35 *M* NaCl in 20 m*M* triethanolamine buffer (pH 7.7) was generated over 25 min at a flow-rate of 1 ml/min. (B) monoclonal antibodies (IgG_{2b}) in each peak fraction of the Mono Q chromatographic profile detected by SPRIA using ^{125}I-labelled sheep anti-mouse antibodies directed against total immunoglobulins. (C) Eluted peaks were treated with SDS and subjected to electrophoresis under reducing conditions on a 10% SDS–polyacrylamide gel. The gel was fixed and stained with Coomassie Brilliant Blue R 250. Numbers below each lane correspond to peak numbers. TSF = Transferrin; ALB = albumin; H = heavy chains and L = light chains of IgG_{2b} monoclonal antibodies. The lane Std contains low-molecular-weight standards.

mouse IgG_1 mAb (results not shown). The Mono Q chromatographic profile of mouse ascites fluids containing mAb of the IgG_{2b} subclass (P8, CL-15 and BL-15) was different from that obtained for the isolation of mouse IgG_1 and IgG_{2a} mAb (Fig. 3A and results not shown). Mouse mAbs were observed by the SPRIA technique to elute in peaks 2 and 3 (Fig. 3B). Coomassie brilliant blue staining of electrophoresed eluted peaks showed that transferrin was present in peak 1, mAbs were present in peak 2 and to a much higher extent in peak 3 eluting at 0.2 *M* sodium chloride, and albumin was present in peaks 4 and 5 (Fig. 3C). The IgG-containing fractions were free of both transferrin and albumin but were contaminated with protein bands of about 20 kDa (Fig. 3C). When a mixture of purified mouse IgG_1 and IgG_{2b} mAb was analyzed on the Mono Q column, using a gradient of 0–0.35 *M* sodium chloride, the peak containing mouse IgG_1 mAb was eluted at 0.17 *M* sodium chloride whereas the peak containing IgG_{2b} mAb was eluted at 0.2 *M* sodium chloride (results not shown).

Assessment of the purity of eluted monoclonal antibody (mAb) by gel permeation chromatography

The Mono Q isolated mAb P7 (IgG_{2a} subclass) was separated on a TSK G 3000 SW gel permeation column (Fig. 4A). The eluted peaks were subjected to electrophoresis under reducing conditions on a 10% SDS–polyacrylamide gel. Coomassie brilliant blue staining of the electrophoresed proteins showed that peak 3 contained mouse IgG_{2a} mAb, whereas traces of transferrin and albumin were present in peak 4 (Fig. 4B). Similar results were obtained for Mono Q isolated mAb P10 (IgG_1 subclass) and P8 (IgG_{2b} subclass) (results not shown).

Purification of Fab fragments by FPLC anion-exchange chromatography

Mouse IgG_1 mAbs purified by the FPLC–Mono Q system were treated with papain as indicated in the Experimental. Use of the Mono Q column with a low protein load (1 mg) of digested mouse IgG_1 mAb resulted in two major peaks eluted by a gradient of 0.005 to 0.3 *M* phosphate (Fig. 5A). Papain when injected on the Mono Q column alone or with digested mAb failed to bind to the column (results not shown). Peaks eluted from the Mono Q column loaded with digested IgG_1 mAb were subjected to electrophoresis under reducing conditions on a 10% SDS–polyacrylamide gel (Fig. 5B). Silver-stained gels showed that the first peak contained a single protein band with an apparent molecular weight of 25 kDa (Fig. 5B, lane 1) and the second peak contained several low-molecular-weight protein bands with one predominating at about 30 kDa (Fig. 5B, lane 2). With larger amounts of papain-digested mouse IgG_1 mAb (8 mg) the first major peak was not retained on the column and required a second separation procedure using gel permeation chromatography to separate the 25-kDa protein from papain (results not shown).

DISCUSSION

Five mouse IgG_1, three IgG_{2a} and three IgG_{2b} monoclonal antibodies (mAbs) were isolated from ascites fluids in a single step with a high degree of purity. Similar results were obtained by Hill *et al.*[6] when purifying mouse IgG_1 mAb from ascites fluids on a Mono Q column. However, Burchiel *et al.*[4] using a Mono Q column

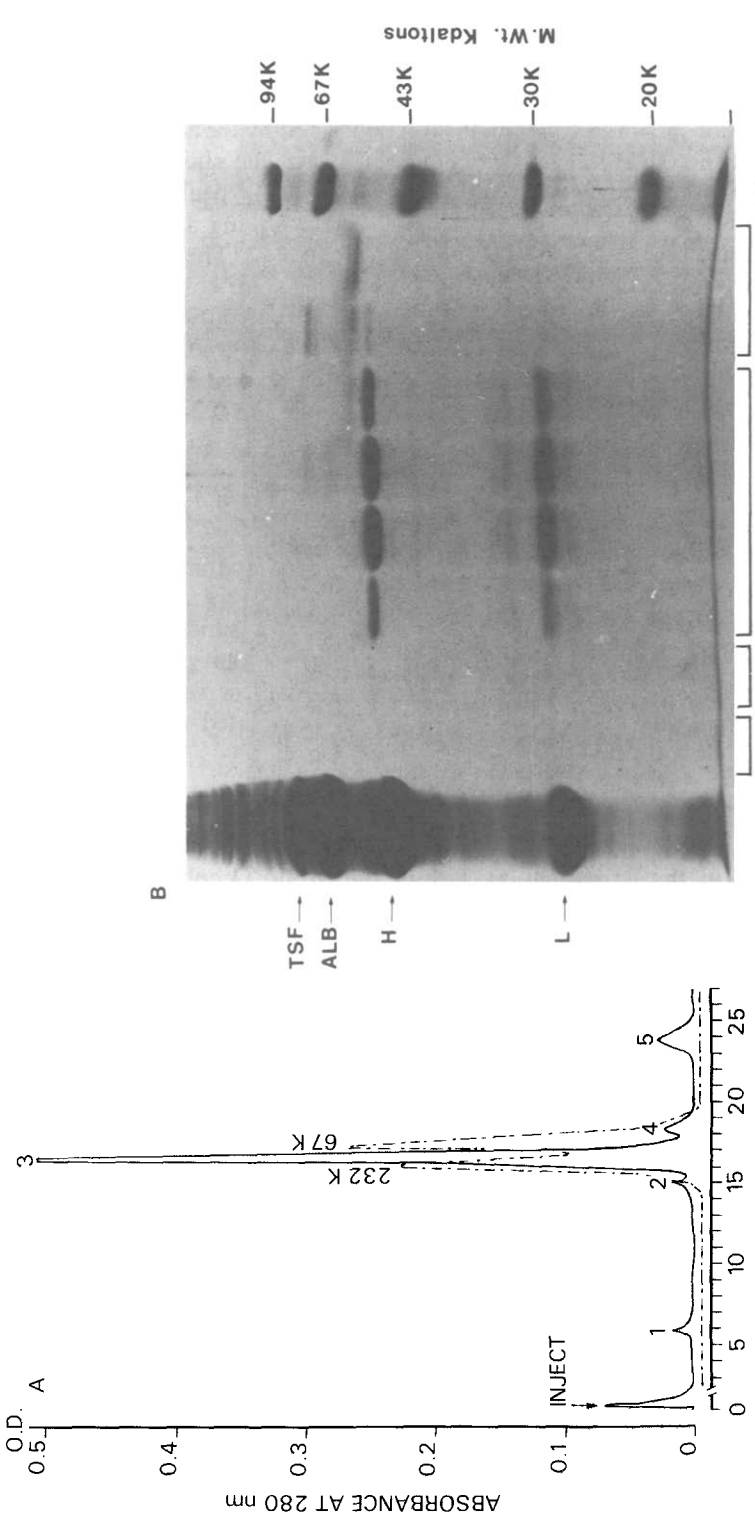

Fig. 4. (A) Gel permeation chromatography on a TSK G 3000 SW column of mouse IgG_{2a} monoclonal antibody P7 (——) isolated from the Mono Q column. The IgG_{2a}-containing fractions from the Mono Q column (0.3 mg of proteins per 0.5 ml) were equilibrated in 50 m*M* sodium acetate buffer (pH 5.0) and injected into the column at a flow-rate of 0.3 ml/min. A mixture of catalase (232 kilodaltons) and bovine serum albumin (67 kilodaltons) (– · –) was analyzed on the column under the same conditions as the IgG_{2a}-containing fractions. (B) Eluted peaks were treated with SDS and subjected to electrophoresis under reducing conditions on a 10% SDS–polyacrylamide gel. The gel was fixed and stained with Coomassie Brillant Blue R 250. Numbers below each lane correspond to peak numbers. TSF = Transferrin; ALB = albumin; H = heavy chains and L = light chains of IgG_{2a} monoclonal antibodies. The lane marked as Std contains low-molecular-weight standards.

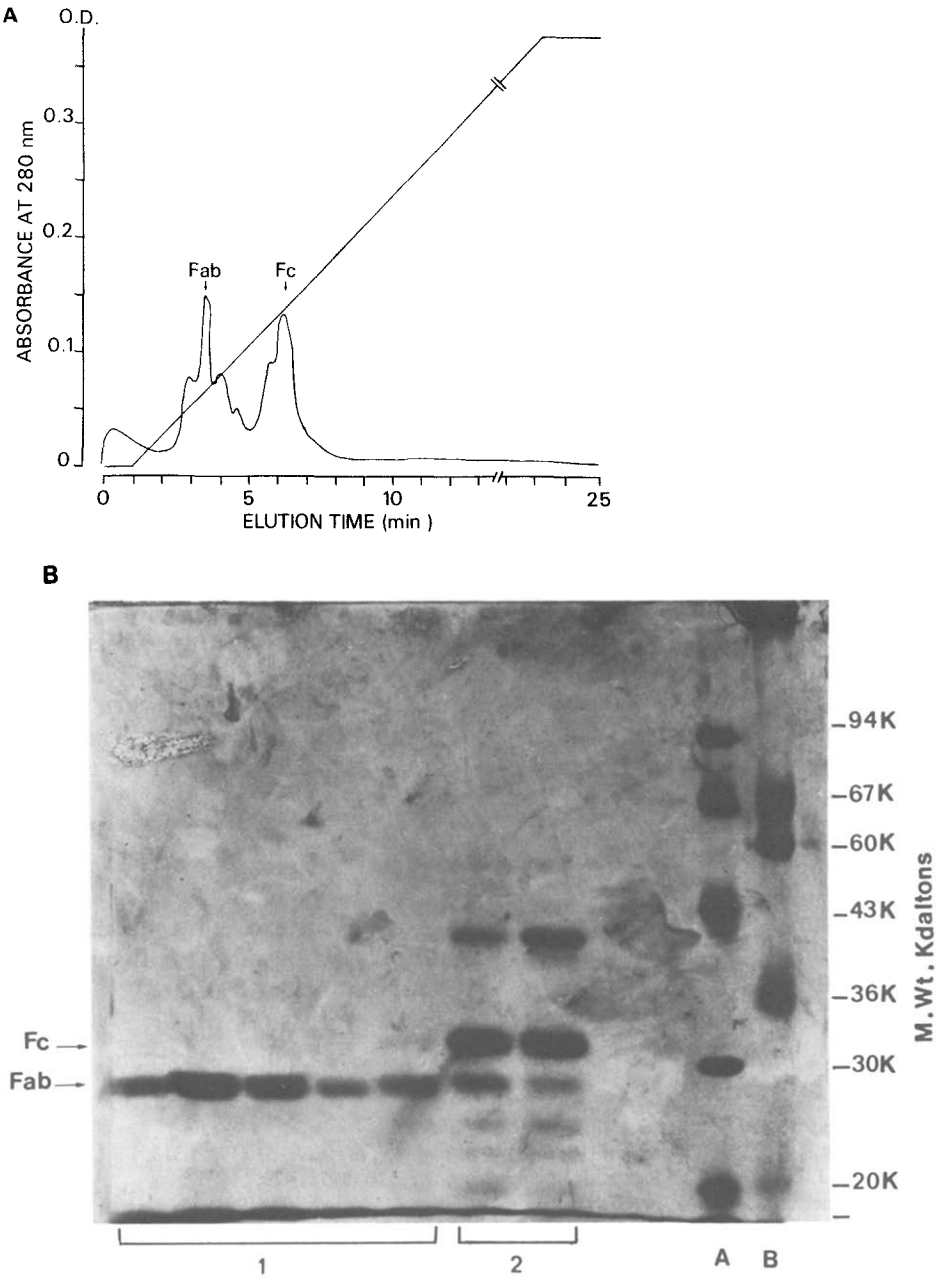

Fig. 5. (A) Chromatographic separation of mouse Fab fragments on a Mono Q column connected to a FPLC system. The papain-digested mouse IgG_1 monoclonal antibody P10 (1 mg of proteins per 0.5 ml) was directly injected into the column. The gradient of 0.005–0.3 *M* phosphate (pH 8.0) was generated over 25 min at a flow-rate of 1 ml/min. (B) Eluted peaks were treated with SDS and subjected to electrophoresis under reducing conditions on a 10% SDS–polyacrylamide gel. The gel was fixed and silver stained. Numbers below each lane correspond respectively to Fab and Fc peaks. Lanes A and B correspond respectively to low- and high-molecular-weight standards.

reported that transferrin was co-purified with mouse IgG_{2b} and IgG_3 mAb, necessitating a second separation by gel permeation chromatography. Burchiel *et al.*[4] used a linear gradient of 0 to 0.5 *M* sodium chloride established over a 20-min period, whereas in this study a linear gradient of 0 to 0.35 *M* sodium chloride was used over the same period of time. The higher salt gradient used by Burchiel *et al.*[4] may explain the greater amount of transferrin co-purified with mouse IgG_{2b} and IgG_3 mAbs. These mouse IgG mAbs purified on a Mono Q column were devoid of protease activity since long-term storage did not result in loss of antibody activity. We have compared the isolation of mouse IgG mAb by affinity chromatography on Protein A–Sepharose CL-4B columns with that obtained by anion-exchange chromatography on Mono Q columns and have found similar purities. The purification of mouse IgG mAb on a Mono Q column allowed the discrimination between mouse IgG_{2b} mAb and other subclasses (IgG_1 and IgG_{2a}). In addition, a mouse IgG_3 mAb [BL-11] (a gift from Dr. J. Brochier) showed a Mono Q chromatographic profile different from that obtained with mouse IgG_1, IgG_{2a} and IgG_{2b} mAbs[16]. Similarly Burchiel *et al.*[4] reported a different elution pattern between mouse IgG_{2b} and IgG_3 mAbs separated on a Mono Q column. This microheterogeneity may be the result of different carbohydrate contents[17]. When mouse IgG_1 mAb purified on the Mono Q column were digested with papain[10], two eluted peaks containing proteins with apparent molecular weights similar to those of mouse Fab and Fc fragments[18] were readily separated by the FPLC–Mono Q system. Depending on the protein load, it was observed in this study that Fab fragments were bound to the Mono Q column at low but not at high concentrations. Similar results were obtained by Biewenga *et al.*[19] when purifying Fab fragments from human IgA antibodies on a Mono Q column. The chromatographic procedure described in this study provides a new method for the isolation of mouse mAbs and their Fab fragments.

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