Journal of Chromatography, 570 (1991) 29–41
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5981

Study of the separation of mouse monoclonal antibodies by pseudobioaffinity chromatography using matrix-linked histidine and histamine

A. EL-KAK and M. A. VIJAYALAKSHMI*

Laboratoire de Technologie des Séparations, Université de Technologie de Compiègne, B. P. 649, 60206 Compiègne Cedex (France)

(First received February 4th, 1991; revised manuscript received April 29th, 1991)

ABSTRACT

The selective retention of proteins on matrix-linked histidine has been shown to depend on chromatographic conditions: pH, temperature and ionic strength. An extension of this study to separate mouse monoclonal antibodies on histidyl-Sepharose is presented here; the roles of different functional groups such as imidazole, primary amine and carboxyl groups are elucidated by using histamine-Sepharose and histidine linked via the carboxyl group of the α-amino acid. We separated two monoclonal antibodies, immunoglobulin G_1 (IgG_1) from a culture supernatant and IgG_{2b} from ascites fluid precipitated with 50% ammonium sulphate. The pseudoselective retention of monoclonal IgG_1 on the three different matrices and IgG_{2b} on histidyl-aminohexyl-Sepharose was achieved at pH 7.4. The purity of the final monoclonal antibody preparation determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis under reducing conditions proved the separation of the monoclonal antibodies (IgG_1, IgG_{2b}) from other contaminating proteins such as albumin and transferrin. Quantitation of the mouse monoclonal antibodies was carried out using enzyme-linked immunosorbent assay.

INTRODUCTION

The advent of the Köhler–Milstein method in 1975 for the biosynthesis of monoclonal antibodies (McAbs) [1] opened the door to important developments in immunology and many fields of biology [2] and technology. Monoclonal antibodies can be used as reagents in immunodiagnostics [3], imaging [4] and tumour therapeutics [5] or as purification tools [6].

McAbs have traditionally been produced in mouse ascites fluid. This method is not practical for the production of large amounts because a large number of animals are required and the risk of contamination of the final product is high. McAbs can also be obtained from hybridoma culture supernatants. The cells are grown in a culture medium containing fetal calf serum (FCS), which provides the necessary growth factors. While the addition of FCS increases the amount of contaminants in the medium, the purification of the McAbs is further hindered by their low concentration in the culture supernatants. Now various methods are

0378-4347/91/$03.50

available for the purification of McAbs, including gel permeation [7], ion-exchange [8], hydroxyapatite [9] and affinity chromatograpy [10]. Each of these methods has advantages and disadvantages. For example, immunoaffinity methods have the advantage of high specificity, but the obvious limitation of this technique is the requirement for relatively large amounts of purified antigen which may not be readily obtainable. Furthermore, affinity antibodies may be very difficult to dissociate once bound, and the conditions of elution can lead to loss of antigens and activity of antibodies.

Ion-exchange chromatography is probably the most commonly employed method for the purification of immunoglobulins (Ig) (monoclonal or polyclonal), but anion-exchange supports have low selectivity and certain immunoglobulins are not stable at low ionic strength and then precipitate on DEAE columns.

α-Amino acids, such as arginine, tryptophan and lysine, have been coupled to Sepharose for the purification of fibronectin, cellulose and plasminogen [11–13]. Histidine can also be used as ligand to adsorb proteins [14].

Histidine has many properties which makes it unique among the amino acids: weak hydrophobicity, weak charge transfer ability, nucleophilic attack due to its imidazole ring and a wide range of pK_a values [15,16]. Several proteins and peptides have been purified using histidine ligand affinity chromatography [17,18]. The versatile nature of histidine as a general ligand for the pseudobiospecific purification of many biological molecules has been demonstrated [17].

In this paper we describe the purification of mouse McAbs IgG_1 from culture supernatant and IgG_{2b} from ascites fluid using histidyl-Sepharose.

EXPERIMENTAL

Materials

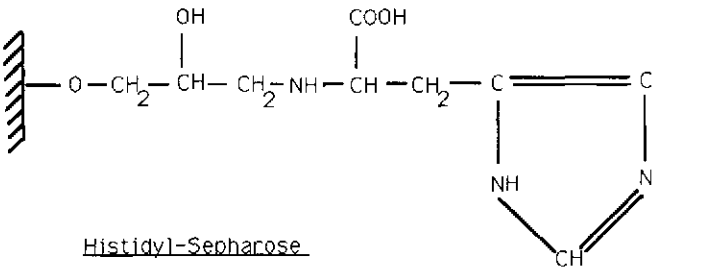
Aminohexyl-Sepharose and Sepharose 4B were obtained from Pharmacia (Uppsala, Sweden). Carbodiimide, L-histidine and histamine were obtained from Sigma (St. Louis, MO, USA). Epichlorhydrin and sodium borohydride were purchased from Merck (Darmstadt, Germany).

Preparation of histidyl-Sepharose and histamine-Sepharose

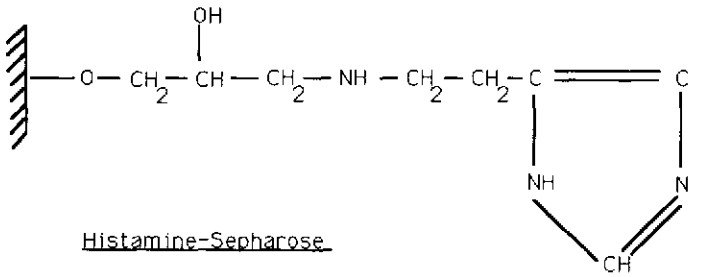
We adopted the experimental protocol mentioned in ref. 12. The gel was prepared by introducing active oxiran groups onto Sepharose 4B containing hydroxyl groups. Then the active oxiran ring was opened and coupled to the primary amine group of the histidine and histamine. The proposed structure of the adsorbent is illustrated in Fig. 1.

Preparation of histidyl-aminohexyl-Sepharose

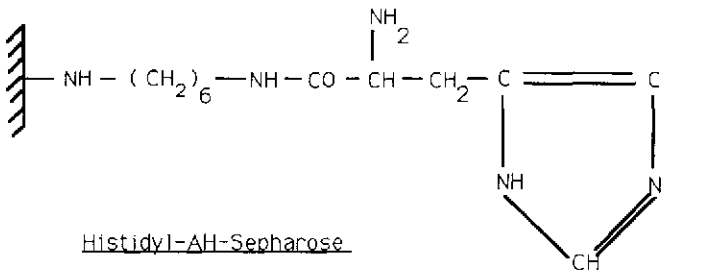
The gel was prepared by coupling histidine to aminohexyl-Sepharose by carbodiimide at pH 4.5–6 with lateral stirring for 16 h at room temperature, as described by the manufacturer.



Histidyl-Sepharose



Histamine-Sepharose



Histidyl-AH-Sepharose

Fig. 1. Structures of histidyl-Sepharose 4B, histamine-Sepharose 4B and histidyl-aminohexyl-Sepharose 4B.

Preparation of antibodies from cell culture supernatant

Hybridoma cell lines (VO 208) were grown in RPMI 1640 medium + 10% FCS. A 200-ml aliquot of culture supernatant was centrifuged at 1500 *g* for 10 min to remove cells, then filtered through a 0.45-μm filter.

Antibody was then precipitated with 50% ammonium sulphate at 4°C for 1 h with gentle stirring. The material was then centrifuged at 10 000 *g* for 15 min; the precipitate was dissolved in 25 m*M* Tris–HCl buffer (pH 7.4) and dialysed for 18 h against three 2-l changes of the same buffer.

Preparation of monoclonal antibodies from ascites fluid

Mouse ascites fluid (4 mg/ml) containing mouse McAbs (IgG_{2b}) against *Clostridium tyrobutyricum* was centrifuged at 1500 *g* for 10 min to remove cells, then filtered through a 0.45-μm filter. A 9-ml aliquot of mouse ascites fluid was diluted (1:1) in 25 m*M* Tris–HCl buffer (pH 7.4). The diluted ascites fluid was then precipitated with 50% ammonium sulphate at 4°C for 1 h with gentle stirring. The material was then centrifuged at 10 000 *g* for 15 min, and the precipitate was dissolved in 25 m*M* Tris–HCl buffer (pH 7.4) and dialysed against three 2-l changes of the same buffer.

Chromatography

Purification was performed using a column (6.3 cm × 1.0 cm I.D.) containing the gel prepared above, connected to a peristaltic pump (Gilson Medical Electronics, Villier-le-Bel, France). The outlet of the column was connected to a UV detector (UVICORD 2238 SII; LKB, Stockholm, Sweden) and to a fraction collector (REDIRAC 2112) (LKB). All chromatographic procedures were carried out at 4°C at a flow-rate of 1.5 cm/h. A 3-ml aliquot of the extract precipitated with a solution of 50% (w/w) ammonium sulphate was injected into the column and equilibrated with 25 m*M* Tris–HCl buffer (pH 7.4). Elution was performed at the same flow-rate with 25 m*M* Tris–HCl buffer (pH 7.4) containing increasing amounts of sodium chloride (0.1–0.4 *M*).

The absorbance of the eluate was measured at 280 nm. Fractions of 3 ml were collected. After each experiment the column was washed with three column volumes of a 0.05 *M* sodium hydroxide solution followed by washing with water and finally with the equilibrating buffer 25 m*M* Tris-HCl at pH 7.4.

IgG determination

Determination of antibody was performed by solid-phase enzyme-linked immunosorbent assay (ELISA) using microtitre plates coated with anti-mouse IgG (H + L) (Biosys, Compiègne, France). Polyclonal mouse IgG (5 mg/ml) (Biosys) was used as a standard protein.

A 100-μl aliquot of samples or standard was added to the wells and incubated at 37°C for 1 h. Plates were then washed four times with 0.1% Tween and 0.15 *M* sodium chloride in 0.01 *M* phosphate buffer with incubation of plates for 1–2 min at 37°C between each washing. Alkaline phosphatase-labelled anti-mouse IgG (100 μl) diluted 1:4000 in phosphate buffer containing 0.15 *M* sodium chloride, 0.1% Tween and 0.3% bovine serum albumin was added and the plates were further incubated for 30 min at 37°C. Plates were again washed four times with the same buffer, described above: 100 μl of reaction substrate were added containing 0.36% *p*-nitrophenylphosphate (PNPP) (Sigma) in 1 *M* diethanolamine buffer (pH 9.8). The reaction was terminated with 100 μl of 2 *M* dipotassium hydrogenphosphate and the absorbance was measured at 410 nm.

Protein quantitation and analysis

Protein concentrations were determined according to Bradford [19], and spectrophotometrically at 280 nm using an absorptivity of 14 for a 1% solution of IgG at 280 nm. The amount of ligand coupled to each gel was detemined by nitrogen estimation using the Kjeldahl method [20].

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The purity of the proteins separated by affinity chromatography was analysed by SDS-PAGE as described by Laemmli [21] using a 10% (w/w) gel. Samples diluted with water were applied under reducing conditions. The gel was stained with Coomassie Brillant Blue. The following proteins (Pharmacia) were used as standards: phosphorylase b, M_r 94 000 D; albumin, M_r 67 000 D; ovalbumin, M_r 43 000 D; carbonic anhydrase, M_r 30 000 D; trypsin inhibitor, M_r 20 100 D; and α-lactabumin, M_r 14 400 D.

RESULTS

Three different adsorbents, two coupled with histidine and the third coupled with histamine, were compared for the purification of mouse McAbs from the culture supernatant.

Histidyl-Sepharose

The result of purification on histidyl-Sepharose 4B by SDS-PAGE is shown in Fig. 2. The first large peak in the chromatogram contained IgG and several unbound proteins, transferrin and albumin (Fig. 3, lane 3). Peak 2, eluted with 25 m*M* Tris–HCl + 0.2 *M* sodium chloride (Fig. 3, lane 2), contained IgG, albumin and transferrin. The recovery of IgG and protein is shown in Table I.

Histamine-Sepharose

The result of purification on histamine-Sepharose is shown in Fig. 4. The first peak in the chromatogram contained traces of IgG and albumin as shown by SDS-PAGE (Fig. 5, lane 2). The second peak, eluted with 25 m*M* Tris–HCl + 0.2 *M* sodium chloride (pH 7.4), revealed that the McAbs were well separated from transferrin and albumin (Fig. 5, lane 4). The total recovery of protein and IgG is shown in Table I.

Histidyl-aminohexyl-Sepharose

The elution profile on histidyl-aminohexyl-Sepharose (Fig. 6) and SDS-PAGE shows that transferrin and albumin were separated in peaks 1, 3 and 4 (Fig. 7A, lane 3; Fig. 7B, lanes 2 and 3). The IgG was eluted in peak 2 after elution with 25 m*M* Tris–HCl (pH 7.4) containing 0.2 *M* sodium chloride (Fig. 7A, lane 4). The yield of purification and recovery of IgG are shown in Table II.

Mouse monoclonal antibodies separated only on histamine-Sepharose and

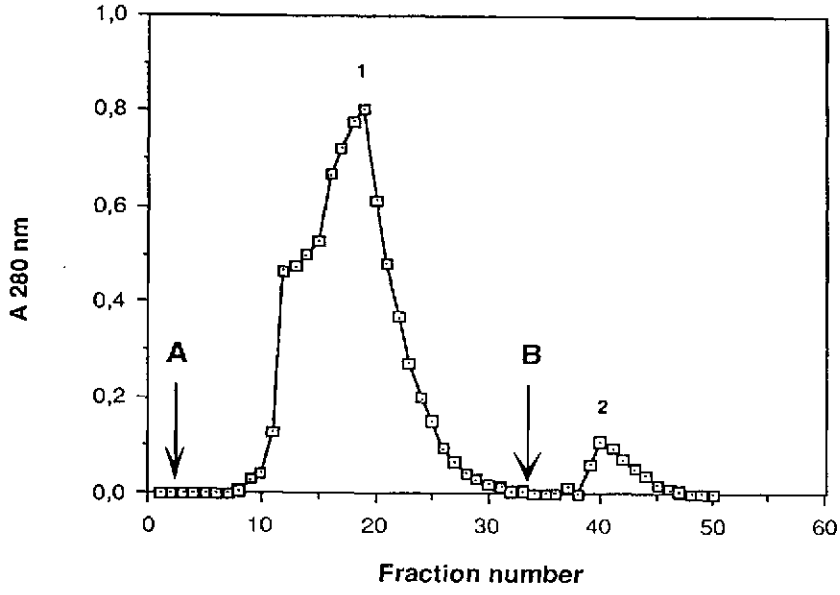


Fig. 2. Purification of mouse monoclonal antibodies (IgG_1) from culture supernatant on histidyl-Sepharose 4B. Conditions: (A) 0.025 *M* Tris–HCl buffer (pH 7.4); (B) 0.025 *M* Tris HCl (pH 7.4) + 0.2 *M* sodium chloride; injection, 3 ml of the extract precipitated with 50% ammonium sulphate; flow-rate, 1.5 cm/h; temperature, 4°C; volume of fraction, 3.0 ml.

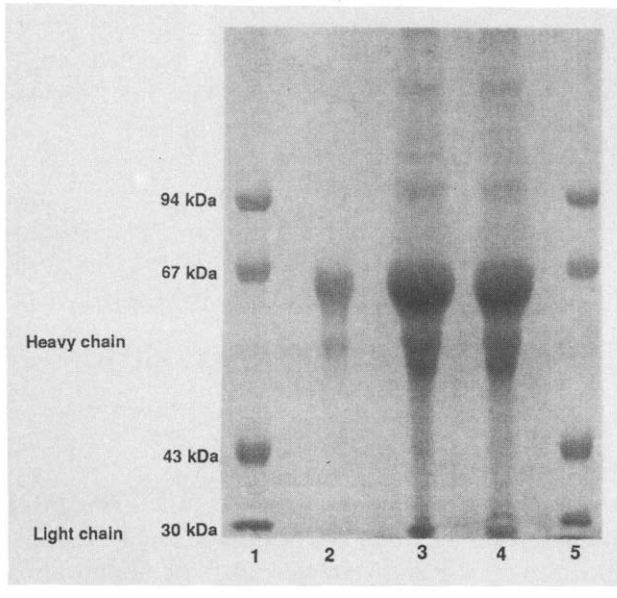


Fig. 3. SDS-PAGE of a fraction from separation of mouse monoclonal antibodies on histidyl-Sepharose. Samples: 1 = molecular mass markers; 2 = peak 2 from histidyl-Sepharose; 3 = peak 1 from histidyl-Sepharose; 4 = cell culture supernatant "start material"; 5 = molecular mass markers.

TABLE I

PURIFICATION OF MOUSE McAbs (IgG_1) FROM CULTURE SUPERNATANT ON HISTIDYL-SEPHAROSE AND HISTAMINE-SEPHAROSE AT 4°C

Fraction	Protein (mg)		Antibody (μg)		Specific activity (μg McAbs/mg)		Yield of mouse McAbs IgG_1 (%)		Purification factor	
	Peak 1	Peak 2	Peak 1	Peak 2	Peak 1	Peak 2	Peak 1	Peak 2	Peak 1	Peak 2
Cell culture supernatant (start material)	113		678		6		100		1	
After precipitation with ammonium sulphate 50%, dialysis and ultrafiltration	12		678		56		100		9.3	
Histidyl-Sepharose	9.2	1.23	380	250	41	204	57	37	6.8	34
Histamine-Sepharose	9.7	1.3	25	585	2.9	266	4	87	0.4	44

histidyl-aminohexyl-Sepharose. In terms of purity and recovery, the chromatographic results obtained from histamine-Sepharose and histidyl-aminohexyl-Sepharose are similar. Histidyl-Sepharose gave poorer results.

McAbs (IgG_1) were easily separated on both histamine-Sepharose and histidyl-aminohexyl-Sepharose under the same optimal conditions at 4°C with 25 m*M* Tris–HCl (pH 7.4) and eluted with the same buffer containing 0.2 *M* sodium chloride. The 87% (585 μg) antibody recovery was calculated from 3 ml of the extract precipitated with 50% ammonium sulphate containing (672 μg) antibody

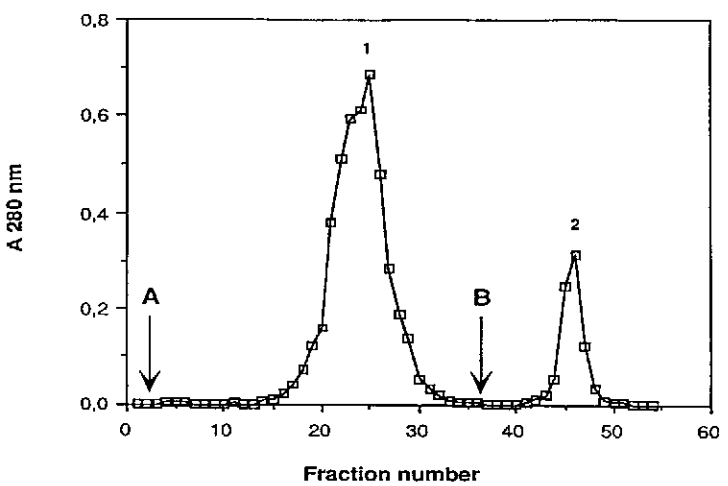


Fig. 4. Purification of mouse monoclonal antibodies (IgG_1) from culture supernatant on histamine-Sepharose 4B. Conditions: (A) 0.025 *M* Tris–HCl buffer (pH 7.4); (B) 0.025 *M* Tris HCl (pH 7.4) + 0.2 *M* sodium chloride; injection, 3 ml of the extract precipitated with 50% ammonium sulphate; flow-rate, 1.5 cm/h; temperature, 4°C; volume of fraction, 3.0 ml.

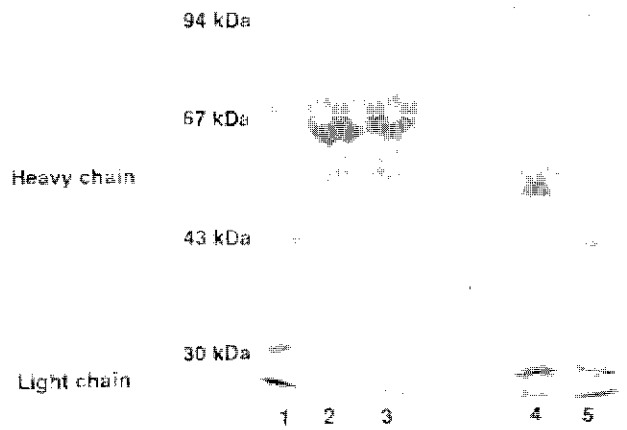


Fig. 5. SDS-PAGE of fractions from separations of mouse monoclonal antibodies on histamine-Sepharose. Samples: 1 = molecular mass markers; 2 = peak 1 from histamine-Sepharose; 3 = fetal calf serum (FCS); 4 = peak 2 from histamine-Sepharose; 5 = molecular mass markers.

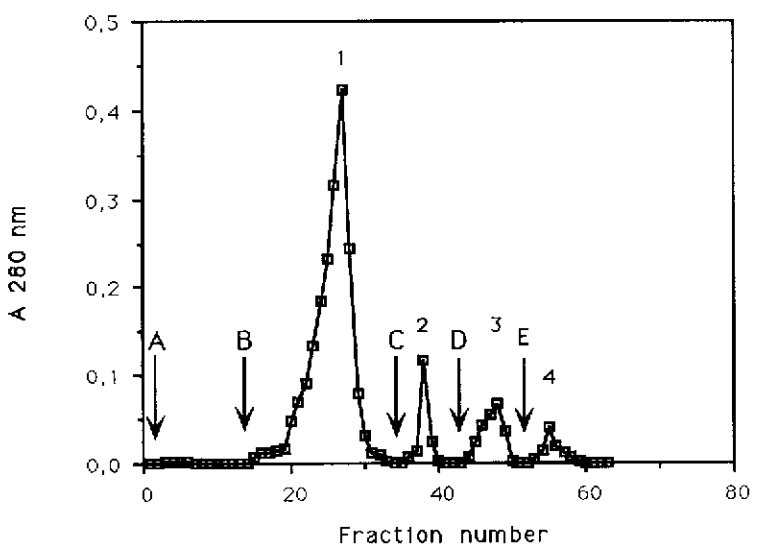


Fig. 6. Purification of mouse monoclonal antibodies (IgG_1) from culture supernatant on histidyl-aminohexyl-Sepharose. Conditions: (A) 0.025 *M* Tris–HCl (pH 7.4); (B) 0.025 *M* Tris–HCl (pH 7.4) + 0.1 *M* sodium chloride; (C) 0.025 *M* Tris HCl (pH 7.4) + 0.2 *M* sodium chloride; (D) 0.025 *M* Tris HCl (pH 7.4) + 0.3 *M* sodium chloride; (E) 0.025 *M* Tris–HCl (pH 7.4) + 0.4 *M* sodium chloride; injection, 3 ml of the extract precipitated with 50% ammonium sulphate; flow-rate, 1.5 cm/h; temperature, 4°C; volume of fraction, 3.0 ml.

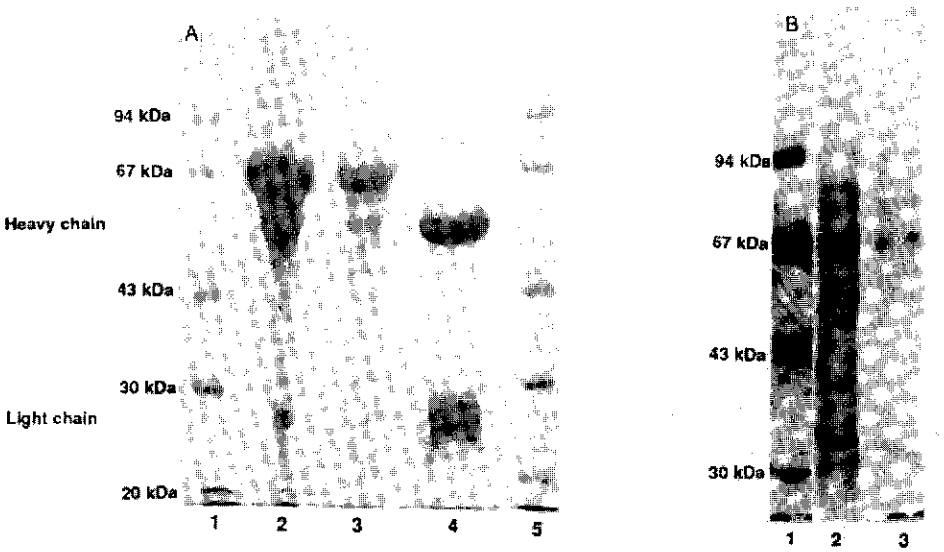


Fig. 7. (A) SDS-PAGE of fractions from separations of mouse monoclonal antibodies on histidyl-aminohexyl-Sepharose. Samples: 1 = molecular mass markers; 2 = cell culture supernatant "start material"; 3 = peak 1 from histidyl-aminohexyl-Sepharose; 4 = peak 2 from histidyl-aminohexyl-Sepharose; 5 = molecular mass markers. (B) SDS-PAGE of a fraction from separations of mouse monoclonal antibodies on histidyl-aminohexyl-Sepharose. Samples: 1 = molecular mass markers; 2 = peak 3 from histidyl-aminohexyl-Sepharose; 3 = peak 4 from histidyl-aminohexyl-Sepharose.

TABLE II

PURIFICATION OF MOUSE McAbs (IgG_1) FROM CULTURE SUPERNATANT ON HISTIDYL-AMINOHEXYL-SEPHAROSE 4B AT 4°C

Fraction	Protein (mg)	Antibody (μg)	Specific activity (μg McAbs/mg)	Yield of mouse McAbs IgG_1 (%)	Purification factor
Cell culture supernatant (start material)	113	678	6	100	1.0
After precipitation with ammonium sulphate 50%, dialysis and ultrafiltration	12	678	56	100	9.3
Peak 1	6.8	0	0	0	0
Peak 2	1.8	603	335	89	56
Peak 3	1.1	11	10	1.6	2.0
Peak 4	0.7	0	0	0	0

TABLE III

COMPARATIVE EFFICIENCIES OF THE DIFFERENTS GELS USING FOR MOUSE McAbs (IgG_1) PURIFICATION

Gel using	Purification factor	Yield (%)	Capacity of mouse McAbs (IgG_1) per ml of gel (μg)
Histidyl-Sepharose 4B	34	37	50
Histamine-Sepharose 4B	44	87	117
Histidyl-aminohexyl-Sepharose 4B	56	90	123

injected through the column (in the case of histamine) and 90% (603 μg) antibody recovery in the case of histidyl-aminohexyl-Sepharose (Tables I and II).

Thus in the case of histidyl-Sepharose it was difficult to separate McAbs without cross-contamination, and the recovery was less in comparison with histamine-Sepharose and histidyl-aminohexyl-Sepharose. Table III shows the efficiencies and capacities of the different gels used for mouse McAbs (IgG_1) purification.

Specificity

The best retention of McAbs (IgG_1) from culture supernatant was observed using histidyl-aminohexyl-Sepharose at 4°C with 25 m*M* Tris–HCl (pH 7.4). In

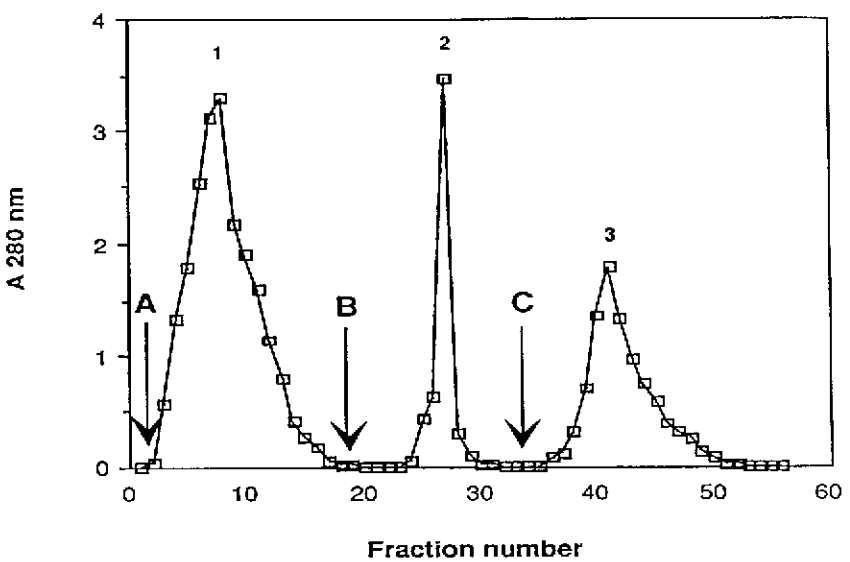


Fig. 8. Purification of mouse monoclonal antibodies (IgG_{2b}) from ascites fluid on histidyl-aminohexyl-Sepharose. Conditions: (A) 0.025 *M* Tris–HCl buffer (pH 74.); (B) 0.025 *M* Tris–HCl (pH 7.4) + 0.1 *M* sodium chloride; (C) 0.025 *M* Tris HCl (pH 7.4) + 0.2 *M* sodium chloride.

TABLE IV

PURIFICATION OF MOUSE McAbs (IgG_{2b}) FROM ASCITES FLUID ON HISTIDYL-AMINOHEXYL-SEPHAROSE 4B AT 4°C

Fraction	Protein (mg)	Antibody (mg)	Specific activity (mg McAbs/mg)	Yield of mouse McAbs IgG_{2b} (%)	Purification factor
Ascites fluid (start material)	225.0	20.0	0.088	100	1.0
After precipitation with ammonium sulphate 50%, dialysis and ultrafiltration	69.0	20.0	0.290	100	3.3
Peak 1	38.2	0.8	0.020	4	0.23
Peak 2	19.1	18.8	0.984	94	11.2
Peak 3	10.6	0	0	0	0

order to test the specificity of this sorbent toward other subclasses of mouse McAb, the same sorbent was used to isolate McAbs (IgG_{2b}) from ascites fluid. The result of purification of McAbs (IgG_{2b}) on histidyl-aminohexyl-Sepharose (Fig. 8 and Table IV) shows the separation of IgG_{2b} from other impurities, such as transferrin and albumin. The McAbs were separated as a pure fraction on SDS-PAGE (Fig. 9, lane 4) after elution with 25 m*M* Tris–HCl (pH 7.4) contain-

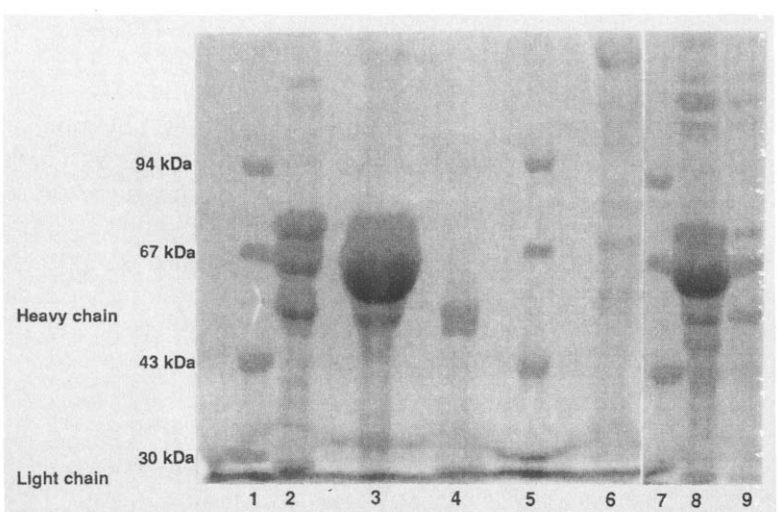


Fig. 9. SDS-PAGE of fractions from separations of mouse monoclonal antibodies (IgG_{2b}) on histidyl-aminohexyl-Sepharose. Samples: 1 = molecular mass markers; 2 = ascites fluid "start material"; 3 = peak 1 from histidyl-aminohexyl-Sepharose; 4 = peak 2 from histidyl-aminohexyl-Sepharose; 5 = molecular mass markers; 6 = peak 3 from histidyl-aminohexyl-Sepharose; 7 = molecular mass markers; 8 = peak 1 from aminohexyl-Sepharose; 9 = peak 2 from aminohexyl-Sepharose [after elution with 25 m*M* Tris–HCl buffer (pH 7.4) containing 0.2 *M* sodium chloride].

ing 0.2 *M* sodium chloride. A 2-ml aliquot of the extract precipitated with 50% ammonium sulphate containing 20 mg of antibody was injected into the column. The antibody recovery achieved was 94% (18.8 mg) (Table IV). This result proves that this sorbent can also be used to separate McAbs (IgG_{2b}) from ascites fluid. It is important to indicate that under this optimal condition for adsorption and elution of the antibodies the capacity of the column is 3.2 mg of antibody per millilitre of gel.

DISCUSSION

The technique of pseudobiospecific affinity chromatography using matrix-linked histidine has been applied previously to the purification of several proteins and peptides [17,18].

The adsorption mechanism is based on multiple interactions such as electrostatic, hydrophobic, charge transfer and possibility of nucleophilic attack due to the imidazole ring. This study has demonstrated the successful application of pseudobiospecific affinity chromatography in the purification of monoclonal antibodies. We have shown that the coupling method chosen and the chemical modification of functional groups of the histidine ligand are very important parameters.

Histidine contains a COOH group, an NH_2 group and an imidazole ring, all of which can contribute to the retention mechanism of proteins. Purification of McAbs (IgG_1) on histidyl-Sepharose was shown to give low recovery and poor separation of McAbs. In order to increase the efficiency of the adsorbent and to understand the role of the COOH group of histidine in the adsorption of protein, histamine-Sepharose affinity chromatography was tested for the retention of McAbs. The results shown in Fig. 4 revealed the separation of McAbs with high purity (>90%) and 87% recovery. These data indicate both the important role of the imidazole ring in the adsorption of the protein and the repulsion of the protein by the COOH group of the histidine. In order to overcome this repulsion and to maintain the non-toxic nature of the histidine ligand, for the purification of McAbs, we coupled the histidine to aminohexyl-Sepharose through the COOH

TABLE V

COMPARATIVE EFFICIENCIES AND CAPACITIES OF HISTIDYL-AMINOHEXYL-SEPHAROSE AND AMINOHEXYL-SEPHAROSE FOR MOUSE McAbs (IgG_{2b}) PURIFICATION

Gel using	Yield (%)	Capacity of mouse McAbs (IgG_{2b}) per ml of gel (mg)
Histidyl-aminohexyl-Sepharose	94	3.82
Aminohexyl-Sepharose	2	0.05

group; the column was used to purify McAbs from culture supernatant (IgG_1) and from ascites fluid (IgG_{2b}). McAbs were separated with high purity and recoveries of 90% for IgG_1 and 94% for IgG_{2b}. Chromatography on aminohexyl-Sepharose without histidine was used in order to determine whether the adsorption of McAbs is due to aminohexyl-Sepharose or to histidine. The data from SDS-PAGE (Fig. 9) and Table V prove that in the case of histidyl-aminohexyl-Sepharose this adsorption its due to histidine. From these results, we have found that pseudobiospecific affinity chromatography using histidine as a ligand is a successful method for purification of McAbs. The elution of the protein can be achieved under mild conditions by using sodium chloride gradients.

REFERENCES

1 G. Köhler and C. Milstein, *Nature*, 256 (1975) 495.
2 F. Shimizu, R. Wang and S. J. Varga, *Clin. Chem.*, 29 (1983) 1245.
3 D. E. Yelton and M. D. Scharff, *Ann. Rev. Biochem.*, 50 (1981) 657.
4 S. M. Larson, J. P. Brown and K. E. Hellston, *J. Nucl. Med.*, 24 (1983) 123.
5 J. Ritz and S. F. Schlossman, *Blood*, 59 (1982) 1.
6 G. J. Calton, *Methods Enzymol.*, 104 (1984) 381.
7 J. P. Bouvet, R. Pires and A. Pillot, *J. Immunol. Meth.*, 66 (1984) 299.
8 C. Bruck, D. Portelle, C. Glineur and A. Pillot, *J. Immunol. Meth.*, 53 (1982) 313.
9 B. Pavlu, V. Johanson, C. Nyghlen and A. Wichman, *J. Chromatogr.*, 444 (1986) 153.
10 P. J. Prowse, P. L. Ey and C. P. Jenkins, *Immunochemistry*, 15 (1978) 429.
11 M. Vuento and A. Vaheri, *Biochem. J.*, 183 (1979) 331.
12 M. A. Vijayalakshmi and J. Porath, *J. Chromatogr.*, 177 (1979) 201.
13 L. Suamri, F. Spitz and L. Arzadon, *J. Biol. Chem.*, 251 (1976) 3693.
14 M. A. Vijayalakshmi, *Trends Biotechnol.*, 7 (1989) 71.
15 G. M. Blackburn, H. L. H. Dodds and D. J. Shire, in R. M. S. Semellie (Editor), *Chemical Reactivity and Biological Role of Functional Groups in Enzymes (Biochemical Society Symposia*, Vol. 31), Academic Press, London, 1970, p. 81.
16 W. P. Jenks, in R. M. S. Semellie (Editor), *Chemical Reactivity and Biological Role of Functional Groups in Enzymes (Biochemical Society Symposia*, Vol. 31), Academic Press, 1970, p. 259.
17 S. Kanoun, L. Amourache, S. Krishnan and M. A. Vijayalakshmi, *J. Chromatogr.*, 376 (1986) 259.
18 A. Akoum, M. A. Vijayalakshmi, P. Cardon, B. Fournet, M. Sigot and J. F. Guespin-Michel, *Enzyme Microb. Technol.*, 9 (1987) 426.
19 M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
20 S. Hjlmarsson and R. Akesson, *Int. Lab.*, (1983) 70.
21 U. K. Laemmli, *Nature*, 227 (1970) 680.