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Development and optimization of a single-step procedure using protein A affinity chromatography to isolate murine IgG_1 monoclonal antibodies from hybridoma supernatants

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ABSTRACT

Protein A affinity chromatography is a standard method of purifying murine monoclonal antibodies (mabs), primarily because it can be performed easily and achieves high-purity levels. Because of its high concentration capacity, it lends itself particularly well to the isolation of mabs from the supernatants of hybridoma cultures. Unfortunately, murine immunoglobulin (Ig) G_1 antibodies, a subclass which occurs frequently in the IgG mabs of mice, binds very poorly to protein A, leading to problems in this isolation procedure. For this reason an attempt was made to increase the effectiveness of protein A affinity chromatography in purifying mabs of this IgG subclass by optimizing the binding conditions. The influence of ionic strength, pH and temperature on the binding capacity of a protein A column was studied. The results show the significance of temperature in the binding of the murine IgG_1 mab tested to protein A. Further investigations were carried out to optimize the elution conditions and to study the contamination of mab preparations obtained with non-specific bovine protein A reactive Igs originating from culture medium supplement (10% foetal calf serum). An optimized, automatic single-step procedure to obtain highly purified murine IgG_1 mabs from hybridoma culture supernatants was developed.

INTRODUCTION

Parallel to the significance assumed by monoclonal antibodies (mabs) in almost all areas of medicine, biology and related disciplines, there has been an increase in the number of purification methods. This reflects the heterogeneity of this group of molecules and the various demands made on purity [1]. Today the amounts of mabs required may lie in the range of grams or even kilograms [2–4]. To produce amounts of this kind in laboratory animals, it is necessary to run a large-scale colony. This is not practicable and cannot be justified on ethical grounds [2,5–7]. For this reason large amounts of mabs are now produced in bioreactors [3,7,8]. In contrast, many research institutes and universities still produce small amounts of mabs in the "ascites mouse" [8]. However, even with laboratory-scale production, there is pressure from animal protection groups and some researchers to abandon the production of mabs in the peritoneal cavities of laboratory animals and to seek alternative sources. For this reason the production of mabs to be used in small- and medium-scale cell culture systems will assume increasing significance and therefore the development of purification methods is of particular interest.

The procedures used to purify proteins consist in various stages which are interspersed by intermediate conditioning steps such as concentration or the adjustment of pH and ionic strength [9]. However, the protocols often consist of an unnecessary number of stages [10]. The greater the number of purification steps required the lower the yield, hence single-step procedures are desirable. Such procedures are also less time-consuming and less labour-intensive.

The greatest drawback in isolating mabs from hybridoma culture supernatants is the large volume of the supernatants; compared with ascites, the concentration of the target molecule is lower by a factor of 100–1000 [11]. The concentrations which can be obtained by classical *in vitro* culture methods are between 10 and 100 μg/ml [12]. The major contami-

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nant in the hybridoma supernatants is water. This must be eliminated via reduction using filtration systems [3,8] or by the initial application of a purification method which has a high concentration capacity, such as cation-exchange or affinity chromatography [2,3,13]. A reduction in volume is required at the outset of the purification process as a large number of purification methods have only a very limited volume capacity.

The composition of the medium plays a significant role in the purification process [2–6,12,14,15]. The growth factors of mammalian cells are frequently not clearly defined and 10% volume of animal serum, usually foetal calf serum (FCS), is often added to the media as a source of growth factors. At concentrations most frequently used, the protein concentration in the supernatants is between 0.5 and 5 mg/ml, *i.e.* the concentration of the contaminating proteins may be greater than that of the mabs [12]. Therefore a single-step procedure for the isolation of mabs from hybridoma culture supernatants containing serum must be capable of both concentrating and isolating the mabs required.

Protein A affinity chromatography is a standard method of purifying murine immunoglobulin G (IgG) mabs. A prerequisite for its concentration capacity and thus for the effectiveness of the technique is that there is an effective binding affinity between the Fc fragment of the mab and the immobilized protein A of the column [16,17].

Most mabs are produced in mice, the most common being the IgG subclasses, 1, 2a, 2b and 3. The IgG_1 subclass occurs particularly frequently in the IgG mabs of mice [3,18]. However, although the affinity of the IgG subclasses 2a, 2b and 3 to bind with protein A is high, the purification of IgG_1 mabs from hybridoma culture supernatants by protein A affinity chromatography generally proves problematic. The reason for this is that the protein A affinity chromatography column has a very restricted binding capacity as a result of low affinity constants. This why many workers regard the purification of murine IgG_1 mabs by protein A affinity chromatography as ineffective and use alternative methods. However, compared with protein A affinity chromatography, the alternative methods have a number of drawbacks.

As a result of the frequent occurrence of the IgG_1 subclass among murine IgG mabs and the diverse advantages offered by protein A affinity chromatography, an attempt was made to increase the effectiveness of protein A affinity chromatography in purifying mabs of this IgG subclass by optimizing the binding conditions. An optimized, automatic single-step procedure was developed to obtain highly purified murine IgG_1 mabs from hybridoma culture supernatants.

EXPERIMENTAL

Monoclonal antibody DF4B7A6

The murine mab studied is designated as DF4B7A6 and was produced and characterized in the Institute of Veterinary Pathology of the Justus-Liebig-University (Giessen, Germany). In indirect enzyme-linked immunosorbent assay (ELISA) it recognizes the tumour-specific pyruvate kinase isoenzyme M_2 (M_2PK), but does not react with M_1PK from skeletal muscle, with pulmonary-specific M_2PK nor with L-PK taken from the liver [19]. DF4B7A6 belongs to the IgG_1 subclass; the light chains are of the kappa type.

Production and pretreatment of hybridoma supernatant

The hybridoma supernatants were produced with a commercially available cell culture system (Dynacell-Millipore, Eschborn, Germany). RPMI with added 10% FCS was used as the culture medium.

Immediately after harvesting, the supernatants were frozen at −30°C. Indirect ELISA was used to examine samples of the individual charges and to establish their anti-M_2PK titre. The charges with the highest anti-M_2PK titres were selected, pooled and mixed with 0.2 g/l sodium azide for the stemming of microbial growth. After sterile filtration through a 0.2-μm filter, the sample was split into portions and the supernatant frozen and stored for future use at −30°C. The protein content of the culture supernatant pooled was 5.1 mg/ml. The concentration of mabs was 51.6 μg/ml as determined by purifying a small volume of supernatant and estimating the protein concentration of the eluted mabs.

Immediately prior to chromatography, the supernatant was thawed in water at 38°C and degassed with a vacuum pump while being stirred gently with a magnetic stirrer for at least 30 min.

Equipment

A high-performance liquid chromatographic (HPLC) system (MAPS 700, Bio-Rad, Munich, Germany) equipped with two dual-piston pumps, a UV detector absorbing at 280 nm, a conductivity monitor, a programmable fraction collector and a HPLC gradient mixer was used. Each of the two dual-piston pumps could be connected alternately with two solvent reservoirs by a programmable motor valve. A personal computer was used to control the apparatus and to collect and process the data acquired. With the help of the standard HPLC software it was possible to load and wash the column automatically.

Although HPLC equipment was available, a traditional gel column was used for the chromatographic separation. Protein A Sepharose CL 4B (11 ml, Pharmacia, Freiburg, Germany) was used as the column material. The gel bed was 5.5 cm deep and the column was 1.6 cm I.D.

To assess the temperature dependence of the binding, the separations were conducted both at room temperature (about 22°C) and using a fitted cooling jacket. A thermostatically controlled waterbath was used to maintain the cooling water at a constant temperature of 2°C. A regulated tubular pump pumped the cooling water through the cooling jacket of the column and through a reverse-current cooler around the steel capillary leading to the column. This allowed the temperature inside the column to be reduced to 3.5°C.

Binding buffers

To investigate the effect of pH on the binding of the mabs onto the column, the binding capacity of the column was compared using buffers of various pH values. A 1.5 *M* glycine–sodium hydroxide buffer and a 1.5 *M* glycine–sodium hydroxide buffer with 3 *M* sodium chloride were used. A mix of one part binding buffer and two parts supernatants resulted in pH values given in Table I.

To clarify the effect of ionic strength on the binding between the mabs and the protein A of the column, binding buffers of various ionic strength were studied. The three buffers used had low (0.15 *M* glycine–sodium hydroxide), medium (1.5 *M* glycine–sodium hydroxide) and high (1.5 *M* glycine–sodium hydroxide plus 3 *M* sodium chloride) ionic strengths. Mixing the binding buffer in a ratio of 1:2 with the culture supernatant during loading and alternatively with phosphate-buffered saline (PBS) during equilibration and washing immediately prior to application to the column, gave a reduction in the saline concentration in the mix. A list of the binding buffers tested is given in Table I.

Chromatographic procedure

To optimize the binding of the mabs to the protein A of the column, the influence of pH, ionic strength and temperature during loading and washing on the binding capacity of the column was studied. Pooled supernatants (160 ml) were separated at three different ionic strengths, two different pH values and two different temperatures during loading and washing.

The column was first equilibrated for 40 min at a flow-rate of 1.5 ml/min, the mix consisting of two parts of PBS (pH 7.45) and one part of the respective binding buffer. This mixture was used to simulate closely the conditions present in the supernatant–binding buffer solution added later. The column was loaded continuously with a mix consisting

TABLE I

BINDING BUFFERS TESTED FOR THEIR CAPACITY TO ENHANCE THE BINDING OF MURINE IgG_1 MABS TO PROTEIN A

Values in parentheses indicate pH of buffer mixed with supernatant or PBS washing solution. ND. = not determined.

Binding buffer	pH I	pH II
1.5 *M* Glycine–sodium hydroxide plus 3 *M* sodium chloride	8.3 (8.2)	8.9 (8.7)
1.5 *M* Glycine–sodium hydroxide	8.3 (8.2)	8.9 (8.7)
0.15 *M* Glycine–sodium hydroxide	8.5 (8.2)	N.D.

of two parts supernatant and one part binding buffer. The HPLC preparative sample loop was not used to apply the supernatant–binding buffer mix. Instead, the supernatant and binding buffer were channelled along different lines, each to one of the two dual-piston pumps. The supernatant and the respective binding buffer were mixed immediately before being applied to the column in the HPLC gradient mixer. The flow-rate was kept constant throughout loading at a continuous rate of 1.2 ml/min. In this way, 160 ml of the pooled supernatant were applied to the column for each separation.

For the washing, the proportion of supernatant was again substituted by PBS (pH 7.45). After loading, the flow-rate was kept at 1.2 ml/min for the next 40 min until the UV detector signal had reached the baseline. It was then increased to 1.5 ml/min. During the 100-min washing procedure, the column was washed with 138 ml, *i.e.* with 12.5 times the volume of the column.

The mabs bound on the column were eluted with 100 m*M* citric acid, adjusted to pH 4.5 with 40% sodium hydroxide solution. Earlier experiments had shown that, under these conditions, a complete elution of the mabs could be achieved in an acceptable elution volume and that below this pH no additional protein peaks were visible on the chromatogram. To attain a greater concentration effect, the flow-rate was reduced to 0.5 ml/min while the elution was in progress.

The entire mab peak was collected without fractionation. The sample was immediately neutralized with a saturated sodium phosphate solution. Following elution, the column was regenerated with 100 m*M* citric acid adjusted to pH 2.8 with 40% sodium hydroxide solution to remove completely any small amounts of bound material. PBS (pH 7.45) with 0.05% sodium azide was used as a storage buffer for the column. Between separations the column was stored at 4°C.

Quantification of antibodies eluted

To compare the column capacity under various conditions, the amount of protein in the peak was calculated photometrically (Protein-Assay, Bio-Rad, Munich) and by integration of the chromatogram. The results were identical. If not indicated otherwise, values from the integration of the chromatogram are presented.

To determine the amount of mabs eluted using chromatogram integration, the mab peaks were integrated after their buffer gradients had been subtracted and this value was multiplied by a proportionality factor that had been established beforehand. The subtraction of the respective buffer gradient was necessary because, during elution, the change of buffers led to changes in the baseline. This made a correct evaluation of the chromatogram more difficult. For this reason, each of the binding buffers had a specific elution buffer gradient. This was achieved by beginning elution after the column had been equilibrated with a mix consisting of the binding buffer and PBS (pH 7.45) at a ratio of 1:2.

Determination of the specific activity of the mab in the post-column flow by indirect ELISA

Samples of post-column flow were taken automatically at 30 min intervals starting from the 20th minute after loading had commenced so that their anti-M_2PK activity could be determined later by indirect ELISA. This achieved with a programmable fraction collector and allowed the saturation kinetics of the column to be traced throughout the separations.

Investigations of contamination with non-specific bovine Ig

To determine the maximum contamination of the antibody preparations with bovine Ig contained in the FCS added to the culture media, 160 ml of unconditioned culture medium were applied to the column and, after elution, the amount of bovine antibodies bound on the column was determined. The 1.5 *M* glycine sodium hydroxide buffer (pH 8.3) was used as the binding buffer. Chromatography was performed at 3.5°C. This experiment was performed twice, with the FCS used to prepare the culture medium from different charges from the same manufacturer. The amount of protein in the eluted material was determined by the corresponding integral. The purity of the mab preparations was determined in a silver-stained gel after sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The samples were run on a 10% gel with a total monomer concentration of 10% under denaturating conditions.

RESULTS

Influence of pH on the binding capacity of the column

A comparison of the amount of protein eluted (*cf.* Table II) shows that raising the pH to 8.7, from 8.2, gives no improvement yield of mabs. In fact, the amount of protein in the separations performed at higher pH are slightly lower. Hence the influence of the pH in the pH span studied is only minor.

Influence of ionic strength on the binding capacity of the column

For the separations of 160 ml of supernatant with the cooled column the saline concentration did not significantly affect the mab yield (Table II). In contrast, when the separations were performed at room temperature, the mab yield increased as the saline concentration of the binding buffer increased. However, even when a binding buffer with the highest saline concentration (1.5 *M* glycine plus 3 *M* sodium chloride) was used, the yield was lower than that obtained using the cooled column. At room temperature, an increase in the glycine concentration and the addition of sodium chloride both increase the mab yield.

Influence of temperature on the binding capacity of the column

A decrease in the temperature to 3.5°C during loading and washing markedly increases the binding capacity (Table II). For separations with the cooled column, the other two parameters tested did not affect the yield. To estimate the effect of the ionic strength of the binding buffer on the mab yield during cooling, the volume of the supernatant was increased to 400 ml to saturate the column. These separations were performed only at a pH of 8.2 as no improvement in the yield is be expected when the pH value is raised to 8.7.

The results of these experiments show the great effect that temperature has on the binding of the mab to protein A. In contrast to separation at room temperature, the increase in post-column activity during the separations performed with the cooled column are slower. Therefore the corresponding activities measured in the post-column flow are achieved much later during loading (Fig. 1). Under the conditions tested, cooling the column leads to a stronger increase in the binding capacity than increasing the ionic strength. The curves for the three buffers tested are almost identical.

The protein values determined in the antibody peaks are almost identical in all three buffers tested (Table III). Therefore, with the cooled column, no further increase in column capacity can be achieved by increasing the saline concentration.

Performing the separations at 3.5°C resulted in a markedly improved efficiency of the purification procedure. A total of 19 mg of a murine IgG_1 mab in a 9.8-ml volume could be purified from 400 ml of supernatant without using a high salt concentration for binding. The concentration factor was 36.7. The

TABLE II

INFLUENCE OF pH AND IONIC STRENGTH OF THE BINDING BUFFER AND TEMPERATURE ON THE BINDING CAPACITY OF THE COLUMN

Volume of loaded hybridoma supernatant is 160 ml. N.D. = not determined.

Binding buffer	Antibody yield (mg)		Antibody yield (%)	
	pH 8.2	pH 8.7	pH 8.2	pH 8.7
Chromatography at room temperature				
0.15 *M* Glycine–sodium hydroxide	4.3	N.D.	51.8	N.D.
1.50 *M* Glycine–sodium hydroxide	5.6	4.9	68.2	59.3
1.50 *M* Glycine sodium hydroxide plus 3 *M* sodium chloride	6.4	6.2	77.6	75.2
Chromatography at 3.5°C				
0.15 *M* Glycine–sodium hydroxide	8.3	N.D.	100.1	N.D.
1.50 *M* Glycine–sodium hydroxide	8.3	8.2	100.1	99.4
1.50 *M* Glycine–sodium hydroxide plus 3 *M* sodium chloride	8.5	8.1	103.0	98.2

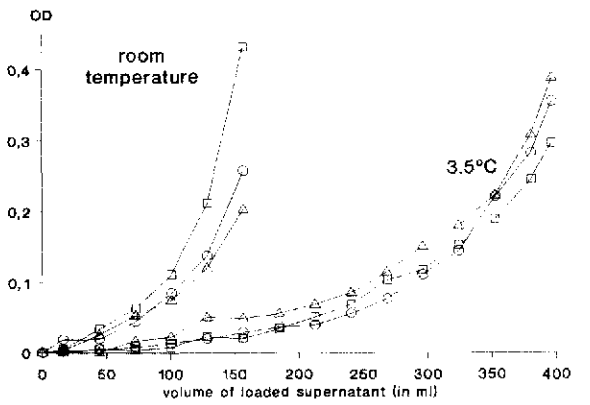

Fig. 1. Breakthrough curves as a function of ionic strength and temperature during loading and washing. Specific mab activity in the post-column flow during loading was measured by indirect ELISA. Curves of corresponding separations performed at room temperature and at 3.5°C have the same symbols. △ = 1.50 *M* glycine–sodium hydroxide plus 3 *M* sodium chloride; ○ = 1.5 *M* glycine–sodium hydroxide; □ = 0.15 *M* glycine–sodium hydroxide.

whole procedure takes about 12 h for equilibration, loading, washing and elution. The final concentration of mabs bound to the column after the throughput of 400 ml of supernatant was 1.73 mg/ml gel bed. The overall antibody yield under these conditions was more than 85%.

Optimization of elution conditions

The aim of an optimum elution procedure for mabs bound to the column is to obtain them in as concentrated a form as possible, under conditions that are as mild as possible. To optimize the elution conditions, the influence of temperature and the reversal of flow direction was investigated.

The curves given in Fig. 2 show the elution profiles of separations in which the column was loaded and washed under identical conditions. For peak A, cooling was maintained throughout the separation, including the elution. For peak B, the cooling apparatus was switched off after washing had been completed and the cooling jacket emptied. Peak C represents the elution profile of a separation in which the flow direction was reversed 20 min before washing was complete. At the start of the elution, the cooling was switched off and the cooling jacket emptied.

Although cooling throughout the elution only negligibly increases the retention time, the resulting peak is broader and flatter than the peak obtained without cooling. In addition, cooling throughout the elution produces peak tailing. This means that cooling not only increases the binding during loading, but also intensifies the weak interaction between the mab and protein A as the pH changes between binding and complete elution.

The reversal of the flow direction prior to elution significantly affects the form and position of the mab peak on the chromatogram. The emerging peak is sharper and appears earlier on the chromatogram than the peak seen without flow direction reversal.

Contamination with non-specific bovine Ig

When the column is loaded with 160 ml of culture medium, significant amounts of protein are bound (Fig. 3). The area under the chromatogram corre-

TABLE III

INFLUENCE OF IONIC STRENGTH OF THE BINDING BUFFER ON THE BINDING CAPACITY OF THE COLUMN

Volume of loaded hybridoma supernatant, 400 ml; pH of binding buffer–supernatant, 8.2; chromatography performed at 3.5°C.

Binding buffer	Antibody yield (mg)	Antibody yield (%)
0.15 *M* Glycine–sodium hydroxide	19.0	92.3
1.50 *M* Glycine–sodium hydroxide	18.0	87.2
1.50 *M* Glycine–sodium hydroxide plus 3 *M* sodium chloride	18.3[a]	81.3[a]

[a] In this separation a slight decrease of the flow-rate was noticed during the late elution procedure due to an air bubble in one of the inlet check valves of the pump head. The corresponding chromatogram could not therefore be used for quantitative purposes. The corresponding values from photometric estimation are presented.

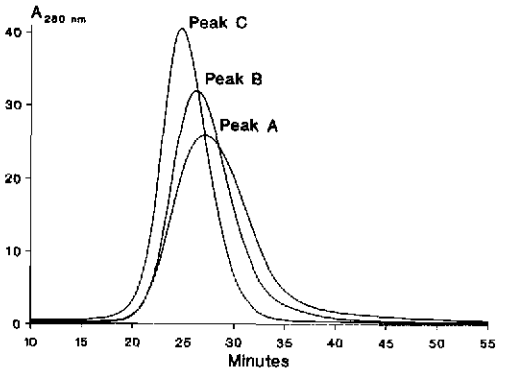

Fig. 2. Optimization of elution conditions. Peak A: elution at 3.5°C; retention time, 27.1 min; relative peak height (rph), 1.0; amount of bound protein, 8.3 mg; relative volume for collecting 95% of eluted Ig (vol. 95%), 1.6. Peak B: elution at room temperature; retention time, 26.6 min; rph, 1.3; amount of bound protein, 8.3 mg; vol. 95%, 1.3. Peak C: elution at room temperature; reversal of flow direction before elution; retention time, 24.8 min; rph, 1.7; amount of bound protein, 8.2 mg; vol. 95%, 1.0.

sponding to the amount of bovine Igs is about one sixth of the area of a comparable peak after the separation of an identical volume of culture supernatants. Two peaks can be seen which partially overlap (Fig. 4). A comparison of the chromatogram with the elution profile obtained after the column was loaded with an equally large volume of

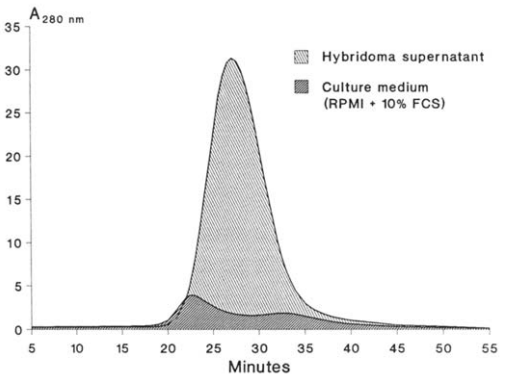

Fig. 3. Binding of non-specific bovine Ig originating from medium supplement (10% FCS) to the protein A column after loading of 160 ml of unconditioned culture medium. A corresponding peak from a separation of an equal volume of hybridoma supernatant performed under identical conditions (binding buffer 1.50 *M* glycine–sodium hydroxide; pH of binding buffer–supernatant mix, 8.2; chromatography performed at 3.5°C) is given for comparison.

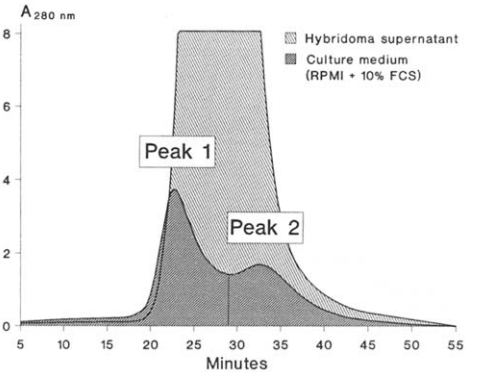

Fig. 4. Elution of non-specific bovine Ig. The chromatogram shows two overlapping peaks, suggesting the existence of two distinct Ig populations with different affinities towards protein A in FCS.

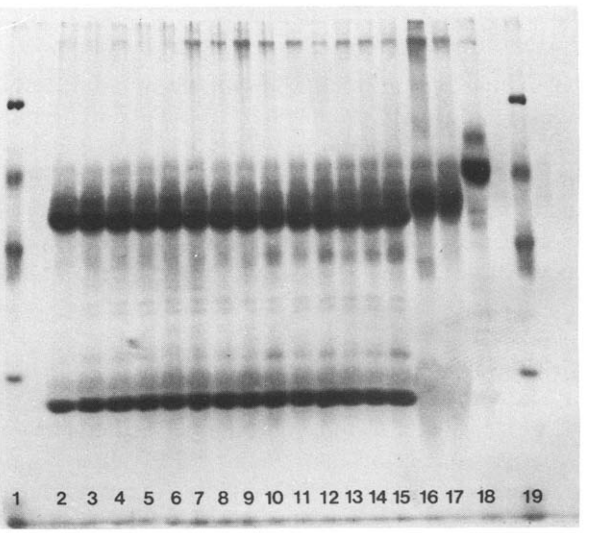

Fig. 5. Electrophoretic evaluation of mabs deriving from separations performed under different loading and washing conditions, non-specific bovine Ig originating from culture medium supplement (FCS) and crude supernatant. Lanes 1 and 19, molecular weight markers (94, 67, 43, 30 kDa); lanes 2–15, eluted mabs from separations of hybridoma culture supernatant performed under different conditions of ionic strength, pH, temperature and sample volume; lane 16, non-specific, protein A-reactive Ig from FCS (peak 1); lane 17, non-specific, protein A-reactive Ig from FCS (peak 2); lane 18, crude hybridoma culture supernatant. Purified mabs from different separations do not show significant differences with respect to contaminants and degree of contamination. Purified mabs are almost free of contamination with the exception of protein A-reactive bovine Ig, which can be seen as weak bands slightly above the light and heavy chains of murine mabs. Although eluting in two different peaks from the protein A column, non-specific bovine Ig from FCS cannot be distinguished by SDS-PAGE.

hybridoma supernatant under identical test conditions reveals that the first, taller peak for the bovine protein has a shorter retention time than the murine mabs. In contrast, the second, smaller peak has a longer retention time. No essential differences can be seen between the two media samples which were prepared using FCS from differing charges from the same manufacturer.

The visual evaluation of a silver-stained gel after SDS-PAGE reveals neither qualitative nor quantitative differences with respect to contaminants between the mab preparations obtained under differing conditions. The bovine Ig of both peaks obtained by chromatographic separation of unconditioned culture medium gives identical results on the silver-stained gel (Fig. 5). Compared with murine mabs, the heavy and light chains of the bovine protein A-reactive Ig have been shifted slightly towards heavier molecules. At each of the peaks obtained by purifying the hybridoma supernatants, an indistinct weak band is seen immediately above the light and heavy chains of the murine mabs. These bands represent the light and heavy chains of the contaminating bovine antibodies. With the exception of the Igs from the FCS supplement, there are no contaminants of any quantitative significance.

DISCUSSION

The aim of this work was to develop a simple, optimized single-step procedure for the preparative isolation of murine IgG_1 mabs. HPLC equipment was available which made it possible to perform the procedure almost entirely automatically, to store and process the data collected and to determine the amount of eluted mabs rapidly and smoothly. The supernatant was not applied with the integrated sample loop, but with one of the four buffer lines. This allowed the binding buffer to be mixed during loading via the gradient mixer in the ratio required. There are several advantages to this form of loading compared with the traditional sample loop method. The preparation of the raw material is reduced to a minimum; if the culture supernatants are sterily filtered and then frozen immediately after preparation, they only need to be thawed in water and degassed just before the chromatographic separation. The pH values and the saline concentration are fixed by mixing in binding buffer. If potentially denaturing conditions are required to achieve optimum binding, this form of loading allows the mabs to remain in a physiological environment until immediately before application to the column. One further advantage is that volumes of any size can be loaded continuously onto the column.

Binding occurring between the ligate and ligand is influenced by temperature, ionic strength, pH and the dielectric constants of the mobile phase [20]. The influence of the first three of these parameters on the binding strength of murine IgG_1 mabs to protein A was studied. It has been known for some time that the binding of murine IgG_1 to protein A is dependent on the pH [21]. In contrast to the other murine IgG subclasses, complete binding cannot be achieved if the pH is less than 8.0. Recommendations have been made that binding is performed at pH values considerably higher than 8.0 [18]. In this work, raising the pH value from 8.2 to 8.7 did not give an improvement in yield at any of the saline concentrations and temperatures tested.

Buffers with very high concentrations of saline are often recommended to strengthen the binding between protein A and murine IgG, in particular, IgG_1. Examples of these concentrations are 3 *M* sodium chloride plus 1.5 *M* glycine [22], 2 *M* sodium chloride plus 2 *M* glycine [23], 0.5 *M* phosphate [24] or 1 *M* Tris [18]. Improved binding is achieved by the intensification of hydrophobic interaction [17].

The work reported here confirmed the positive correlation between the saline concentration of the binding buffer and the binding capacity of the column. However, the increase in yield achieved with the highest saline concentrations tested (a mix of 1 *M* sodium chloride and 0.5 *M* glycine) was clearly lower than that from separations with the cooled column. In addition, applying such high concentrations of saline causes several problems. For example, parts of a liquid chromatographic system that are movable cannot be completely sealed. The result is that the mobile phase leaks between the moving and fixed parts, especially around the pump head pistons. The evaporation of the leaking mobile phase causes a crystalline residue to form, which causes wear and tear in these sections [25]. This problem is worse at high saline concentrations. Another problem is that at high saline concentrations there is a danger of aggregation formation and loss

of activity due to denaturing. The volumes that are then required mean that a lot of material is used.

Temperature has the most significant effect on the strength of binding. The strengthening of binding through cooling may occur as a result of the effects of two mechanisms. Low temperatures weaken hydrophobic interactions [17]. With a weakening of the intramolecular hydrophobic bonds, slight changes may be induced in conformation in either one or both binding partners which result in an overall increase in affinity. Alternatively, this effect could be achieved thermodynamically.

As the reaction that DF4B7A6 shows towards protein A is that of a "typical" murine IgG_1 mab, and since other workers [26] also showed that the binding of murine IgG_1 mab to protein A is sensitive to temperature, it can be assumed that despite the reported heterogeneity of this murine IgG subclass [27], binding to protein A depends greatly on temperature, at least in some mabs of the IgG_1 subclass.

Reversing the flow direction has already been described in the elution of mabs from an ion-exchanger column [2]. This procedure can also be used to advantage in affinity chromatography. The elution volume is smaller and the material obtained more concentrated. "Tailing", which is frequently lengthy, does not occur and the peak appears earlier on the chromatogram, *i.e.* the pH of the eluate obtained is higher and the volume required for neutralization is smaller. For these reasons flow reversal is greatly recommended with soft-gel columns that allow the flow to be reversed easily.

The use of medium supplements containing serum as the source of growth factors leads to the contamination of the hybridoma supernatants with unspecific bovine Ig. To reduce this contamination, FCS is most frequently used as it contains a relatively low level of Ig, which is often assumed to be negligible. However, FCS also contains a considerable concentration of non-specific Ig [16]. Of the bovine Igs it is only the IgG_2 subclass that possesses pronounced protein A reactivity. IgG_1 binds very poorly; no protein A reactivity is observed in the other bovine Ig classes [28].

According to previously reported information the protein A-reactive Ig contained in commercially-available FCS shows evidence of considerable fluctuations (between 0.03 and 0.26 mg/ml) between the individual charges. In the 10% supplement of FCS usually used, concentrations of non-specific bovine Ig were measured in culture media that correspond to the mab content. There is therefore a great potential risk of contamination with bovine protein A-reactive Ig for mabs purified with protein A from culture supernatants containing serum [16].

It is difficult to separate bovine IgG contained in FCS from murine IgG as a result of the conserved structure of the IgG. The most common methods of purifying mabs, such as ammonium sulphate precipitation, protein A affinity chromatography and ion-exchange chromatography, result in a "co-purifying" of non-specific Ig. When the concentration of bovine protein A-reactive Ig was determined in samples of differing murine mabs purified by protein A affinity chromatography of culture supernatants, contamination rates of up to 50% were found [16].

As is shown in the evaluation of the gels after SDS-PAGE and silver-staining, bovine protein A-reactive Ig is the only contaminant of quantitative significance. In the chromatographic separation of 160 ml of unconditioned medium, *i.e.* under conditions in which the binding sites on the column are available only to bovine Ig, the yield is about one sixth of the amount of protein obtained by purifying a corresponding volume of hybridoma culture supernatant. In the chromatographic separation of culture supernatant, a reduction in contaminants is expected, as a result of competition from superfluous murine mabs. Therefore, in the visual evaluation of samples of "purified" mabs on a silver-stained gel, in which it was possible to make a limited distinction between bovine and murine Ig, only relatively weak bands were seen at the position of the heavy and light chains of the bovine Ig. The actual rate of contamination is probably far below 17%, the maximum contamination rate possible. This degree of purity in the mabs obtained is adequate for most laboratory requirements.

It is therefore possible, with a minimum amount of effort and in a single-step procedure, to effectively purify and simultaneously concentrate murine IgG_1 mabs from culture supernatants using protein A affinity chromatography with a cooled column. As a result of the generally acknowledged longevity of protein A Sepharose, this procedure is economical, despite the relatively high initial outlay for the

column material. Relatively mild conditions are adequate for loading and elution and murine IgG_1 mabs can be obtained in a highly purified form using protein A affinity chromatography. This is important as a large proportion of murine mabs belongs to this subclass. The procedure developed is particularly well suited to laboratory-scale application and can also be applied to the purification of much larger volumes if correspondingly larger columns are used. Even on a laboratory scale, it is possible to obtain highly purified murine IgG mabs from mass cell cultures using a simple technique.

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