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HIGH-PERFORMANCE LIQUID AFFINITY CHROMATOGRAPHIC SEPARATION OF MOUSE MONOCLONAL ANTIBODIES WITH PROTEIN A SILICA

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SUMMARY

Protein A, a bacterial cell wall protein found in *Staphylococcus aureus*, has been widely used for the analysis of immunoglobulins. By attaching protein A to a microparticulate silica support, a rapid and efficient chromatographic sorbent has been created for the separation of monoclonal antibodies. Examples are given of rapid separations (within 10 min) of murine monoclonal antibodies, belonging to various IgG subclasses and including IgG1. The monoclonal antibodies were isolated with a high purity and with 60–90% recovery of activity. The high-performance liquid affinity chromatography technique based on protein A provides a useful method for monitoring monoclonal antibodies in crude samples, such as ascites and cell culture supernatants.

INTRODUCTION

The purification and analysis of monoclonal antibodies has received considerable attention during the last years. Several chromatographic methods have been applied to the separation of monoclonal antibodies, including gel, ion-exchange, hydroxylapatite and affinity chromatography.

Protein A, a bacterial cell wall protein found in strains of *Staphylococcus aureus*, has been widely used as a reagent for immunoglobulins (Ig)[1]. It binds with high specificity to the Fc-region of Ig, mainly IgG, from various mammalian species[1]. When immobilized on a solid matrix, protein A can be used for the separation and purification of immunoglobulins[2] by affinity chromatography. Recently, affinity chromatography on immobilized protein A has been applied for the purification of monoclonal antibodies from mouse ascites and cell culture supernatants[3].

The purpose of this study was to explore the possibilities for the separation of mouse monoclonal antibodies from crude samples by high-performance liquid affinity chromatography (HPLAC) on protein A, bound to microparticulate silica.

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MATERIALS AND METHODS

Chromatography

The HPLC systems was a Varian LC 5500, equipped with a UV-200 detector (Varian, Walnut Creek, CA, U.S.A.), and a Shimadzu CR 3A integrator (Shimadzu, Kyoto, Japan).

A SelectiSpher-10 protein A column (5 × 0.5 cm) with a particle size of 10 μm and an average pore diameter of 500 Å (Pierce, Rockford, IL, U.S.A.) was used for all HPLAC experiments. All chromatographic procedures were carried out at room temperature (20–23°C) at a flow-rate of 2 ml/min with a typical pressure drop of 30 atm. After filtration (0.22 μm), crude samples were injected as such or mixed with the mobile phase. During chromatography, fractions were collected for subsequent immunochemical and electrophoretic analysis.

Immunochemical methods

All monoclonal antibodies used for this study were selected because of their isotype and were produced by standard procedures[4]. The antibody isotype was determined by double immunodiffusion[5] using rabbit anti-mouse IgG1, IgG2a, IgG2b, and IgG3 antisera (Miles, Elkhart, IN, U.S.A.).

The determination of the antibody titer was performed by solid-phase enzyme-linked immunosorbent assay (ELISA)[6], using the specific antigen coated on a microtiter plate. The antigens were for the IgG1 antibody, tissue plasminogen activator (TPA), and for the IgG2b antibody, gal-α-1-4 gal, coupled to bovine serum albumin[7]. The plates were incubated with the monoclonal antibody and subsequently with alkaline phosphatase labelled antimouse immunoglobulins (Dakopatts, Copenhagen, Denmark). The IgG2a antibody was directed to a cell surface antigen and quantitated by rat antimouse IgG2a, coated on the plate. After incubation with cell culture supernatant, bound antibody was determined by incubation with biotin-labelled rat antimouse antibody, followed by incubation with avidin-labelled peroxidase (Dakopatts). The IgG3 antibody was directed to blood group A and was determined by hemagglutination.

Electrophoretic analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli[8] in 6–22% gradient gels. In some cases, samples were reduced by adding 2-mercaptoethanol to the sample buffer to a final concentration of 2%. Gels were stained with Coomassie Brilliant Blue. The following proteins were used as standards: albumin (molecular weight, MW, 67 000), ovalbumin (MW 43 000), carbonic anhydrase (MW 30 000), trypsin inhibitor (MW 20 100) and α-lactalbumin (MW 14 400).

RESULTS AND DISCUSSION

During the last few years, most of the attention for immunoglobulin separations with protein A has shifted from studies of human immunoglobulins to applications of mouse monoclonal antibodies. The early work by Kronvall *et al.*[9] showed that protein A reacts with the four mouse subclasses of IgG (IgG1, IgG2a, IgG2b,

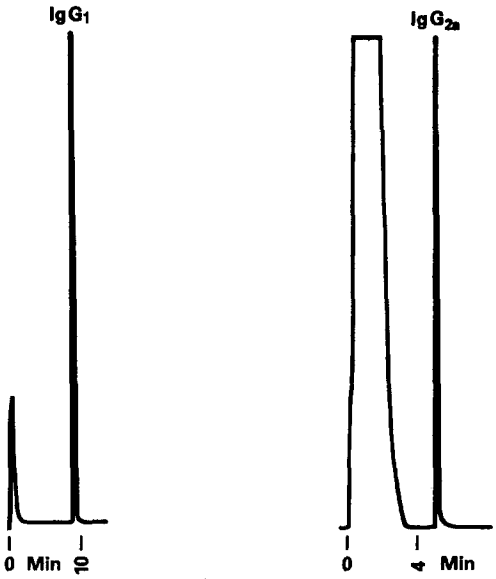

Fig. 1. Purification of monoclonal antibodies (IgG1) from the ammonium sulphate fraction of mouse ascites on SelectiSpher-10 protein A. Conditions: (A) 0.05 *M* glycine (pH 9.0) (0–9 min); (B) 0.05 *M* glycine (pH 2.3) (9–14 min); injection, 1 ml (diluted 1:20 with mobile phase); detection, 280 nm.

Fig. 2. Purification of mouse monoclonal antibodies (IgG2a) from cell culture supernatant on Selecti-Spher-10 protein A. Conditions: (A) 0.05 *M* glycine (pH 9.0) (0–5 min); (B) 0.05 *M* glycine (pH 2.3) (5–8 min); injection, 3.2 ml; detection, 280 nm.

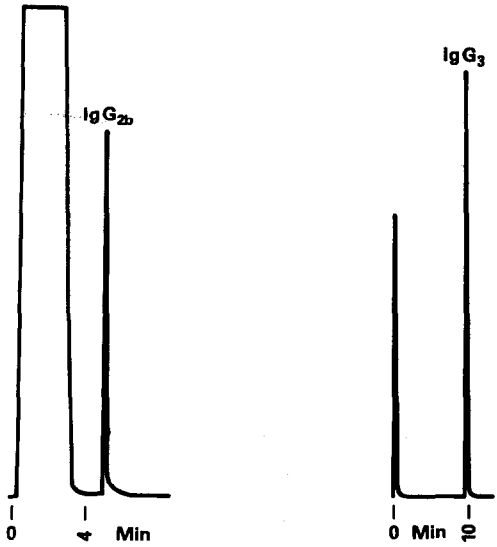

Fig. 3. Purification of mouse monoclonal antibodies (IgG2b) from cell culture supernatant on Selecti-Spher-10 protein A. Conditions: (A) 0.05 *M* glycine (pH 9.0) (0–5 min); (B) 0.05 *M* glycine (pH 2.3) (5–8 min); injection, 3.2 ml; detection, 280 nm.

Fig. 4. Purification of monoclonal antibodies (IgG3) from mouse ascites on SelectiSpher-10 protein A. Conditions: (A) 0.05 *M* glycine (pH 9.0) (0–9.5 min); (B) 0.05 *M* glycine (pH 2.3) (9.5–13 min); injection, 100 μl mouse ascites (diluted 1:1 with mobile phase); detection, 280 nm.

IgG3). Later it was shown[10,11] that the mouse subclasses of IgG can be isolated by protein A affinity chromatography.

Protein A, immobilized on various soft gels such as agarose, has been extensively used for the purification of immunoglobulins[2]. However, with the introduction of HPLC matrices in affinity chromatography[12,13] techniques were developed that combine the selectivity of conventional affinity chromatography with the speed, high resolution and sensitive detection of HPLC. In a recent paper[14], HPLAC on immobilised protein A was used for the separation of human subclasses of IgG. In the present study, we have explored the potential of the protein A HPLAC method for the separation of mouse monoclonal antibodies from crude samples. Figs. 1–4 show typical separations of various subclasses of mouse monoclonal antibodies from both ascites and cell culture supernatants. Highly efficient separations are achieved within 10 min. Sharp peaks, high yields and increased sensitivity were obtained, so that it should be possible to determine the amount of mouse monoclonal antibodies merely by measuring the profile of the absorbance at 280 nm. The estimated minimum detectable amount based on absorbance measurements is in the range of 0.5–1 μg/ml (data not shown).

Generally, the usefulness of protein A for separating mouse IgG1 has been limited by the poor binding of IgG1 to protein A under physiological conditions. Antibodies of the mouse IgG1 subclass show heterogenity in their binding to protein A[3]. However, by increasing the pH[10] and/or the ionic strength (up to 2 *M* sodium chloride), the affinity of mouse IgG1 to protein A can be enchanced considerably. By raising the pH to 9.0 (as shown in Fig. 1) we were able to bind the TPA-specific IgG1 efficiently to the SelectiSpher protein A column. The antibody activity in terms of its binding to the antigen (TPA) was followed by ELISA during chromatography. Activity was not present in the "dropthrough" fractions, but was only found in the fractions eluted at low pH. Quantitative estimates of activity cannot be given at this point, due to the non-quantitative nature of the ELISA procedure. Individual IgG1 monoclonal antibodies vary in the strength of their binding to protein A, even at pH 9. Nevertheless, at least in this case, the mouse IgG1 showed a strong affinity for protein A. This indicates the potential for using protein A for IgG1 purification. The use of high salt concentrations to favour hydrophobic interactions enhances the possibilities to IgG1, *e.g.* 2 *M* sodium chloride, 2 *M* glycine (pH 9.0) can be used for the separation of mouse IgG1 (data not shown).

A major objective in the purification of monoclonal antibodies is to achieve sufficient purity and recovery of the products. The separations of the mouse IgG2a and IgG2b monoclonal antibodies (Figs. 2 and 3, respectively) were examined in more detail. The antigen (IgG2b)/immunological (IgG2a) activity was followed during the chromatographic procedure by ELISA and the purity by SDS-PAGE (Fig. 5). As indicated in Fig. 5, we estimate the purity to be high (>90%). Contamination of inherent antibodies in the eluate is not expected, since the culture medium used in this case does not contain any significant amounts of immunoglobulins. As to activities, we found a substantial loss of activity by elution with glycine at low pH (*ca.* 40% of the original activity was lost). However, by minimizing the time of exposure at low pH and by elution with other buffers such as citrate (0.05 *M* citrate pH 2–3), the recovery of activity increased considerably (*ca.* 90% of the original activity). By carefully selecting chromatographic conditions the loss of activity can thus be avoid-

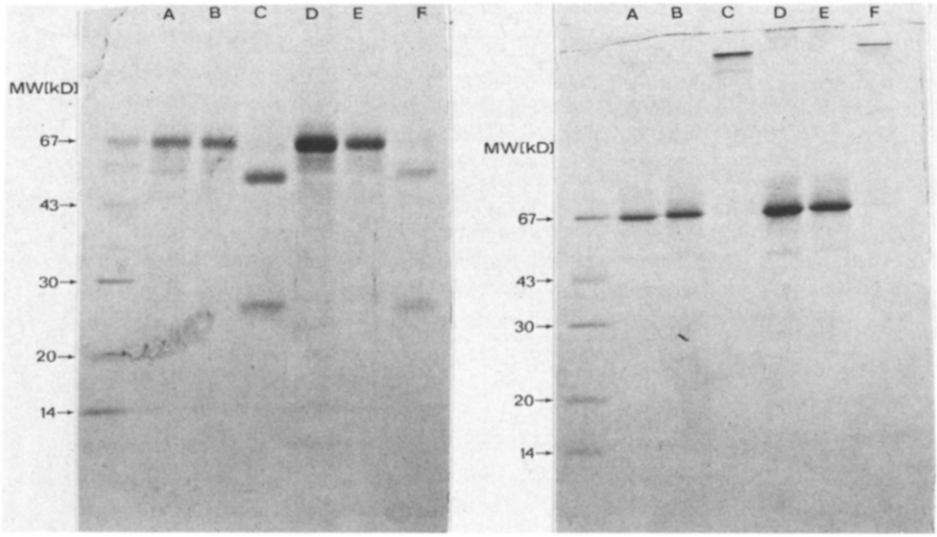

Fig. 5. SDS-PAGE of fractions from separations of mouse monoclonal antibodies on SelectiSpher-10 protein A. Left: reduced samples; right: non-reduced samples. A = IgG2a sample, B = IgG2a "dropthrough", C = IgG2a eluate, D = IgG2b sample, E = IgG2b "dropthrough", F = IgG2b eluate.

ed. Fig. 4 shows the separation of a subclass IgG3 antibody from mouse ascites. In this case, activity was also only found in fractions eluted at low pH, indicating a high affinity of mouse IgG3 for protein A.

The SelectiSpher-10 protein A column was used at least 100 times without loss in performance. Any loss of protein A during chromatography was undetectable by monitoring the absorbance at 280 nm and/or by SDS-PAGE. It was unnecessary to include any special washing steps during the long periods of operation. When not in use, the SelectiSpher column was stored in 0.1 *M* sodium phosphate (pH 7.0) containing 0.05% sodium azide at 4–6°C. The separation time can probably be shortened to 2–4 min. This means a throughput of samples in the range of 20 per hour. By a suitable automation of the analysis, simple handling of many samples can be achieved. The evaluation of new cell clones for monoclonal antibody production or for product control during cell cultivation may be facilitated.

In conclusion we feel that the use of the HPLAC method using protein A provides a significant advance in the study of monoclonal antibodies. The inherent speed and overall performance of HPLAC makes it attractive for quantitative analysis when combined with non-specific detection methods, such as spectrophotometry.

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