Journal of Chromatography, 327 (1985) 213–219
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 560

PROTEIN A-COATED GLASS BEADS

UNIVERSAL SUPPORT MEDIUM FOR HIGH-PERFORMANCE IMMUNOAFFINITY CHROMATOGRAPHY

T. M. PHILLIPS*, W. D. QUEEN, N. S. MORE and A. M. THOMPSON
Immunochemistry Laboratory, George Washington University Medical Center, 2300 I Street, N.W., Washington, DC 20037 (U.S.A.)

SUMMARY

High-performance immunoaffinity chromatography (HPIC) is a technique for the fast isolation and quantitation of both antibodies and antigens. Protein A-coated glass beads provide a stable general immobilization support for most immunoglobulin G (IgG) antibodies. In conjunction with the modern expanding repertoire of monoclonal antibodies, HPIC can be applied to the quantitation and isolation of any biological material, in an active form.

INTRODUCTION

Affinity chromatography is a useful tool for isolating and concentrating small quantities of biological materials. The utility of the technique can be expanded by using the specificities of immobilized antibodies[1–3]. At present, most applications of immunoaffinity are confined to low-pressure systems which give specific isolations, but are not rapid. The reactive side chains of the low-pressure chromatographic media couple the antibody molecules in a random fashion and do not always orient the antigen receptors of the antibody in the right direction.

Protein A, which is a coat protein from Cowans Strain *Staphylococcus aureus*, has been shown to have receptors which bind to the tail or Fc portion of the immunoglobin G (IgG) molecule, therefore, anchoring the IgG with its antigen receptors facing into the mobile phase of the column[4,5]. Therefore, protein A makes an excellent immobilization medium for anchoring IgG molecules to chromatographic supports.

We have utilized a technique whereby protein A can be chemically linked to commercially available controlled pore glass beads via a carbonyl diimidazole derivative, present on the surface of the beads. Glass beads have the correct properties to withstand the pressures of high-performance chromatography, and therefore, are the perfect choice as a support for coating with protein A.

In this communication we will outline the general technology involved in producing protein A-coated glass beads, and the conditions for performing immunoaffinity isolation of both antibodies and antigens.

0021-9673/85/$03.30

THEORETICAL

Antibody–antigen reaction

The basis of immunoaffinity chromatography is the specificity of an immobilized antibody to recognize and capture its reactive antigen:

$$\text{immobilized antibody} + \text{antigen} \rightleftharpoons \text{antibody–antigen complex}$$

In this way, the antibody (in our work, either rabbit antihuman serum albumin or rabbit antihuman IgG) retains the antigen (human serum albumin or human IgG), while the rest of the non-reactive material is washed through the column. The antibody–antigen complex is held together by weak bonds, which can easily be broken by a wide variety of different agents, the most common of which are either acid or chaotropic ion solutions[6]. Introduction of such buffers via a gradient or buffer selection system changes the ionic parameters within the column mobile phase, which in turn breaks the bond by which the immobilized antibody binds the antigen. In this way, the antigen is released and is carried through the column by the mobile phase. Finally, the eluted antigen appears as a secondary peak which is measured by the column detector and collected, in a biologically active form, for further studies.

Protein A–antibody interaction

The protein A molecule has five IgG binding receptors, but when it is chemically immobilized, it has only two active receptors, which can bind to the Fc or tail portion of IgG molecules of most subclasses[7]. These IgG binding receptors react with areas on the constant region of the heavy chains of the IgG molecule and orient their antigen receptors away from the protein A molecule. In the immobilized state, this orientation would turn the IgG antigen receptors into the mobile phase of the column and give them the maximum availability to the antigen, which is being carried in the mobile phase. In this way, the IgG molecule would be placed in the optimal position for maximum efficiency of antigen binding. Fig. 1 demonstrates this interaction and its use in immunoaffinity chromatography.

EXPERIMENTAL

Materials

Glycophase controlled-pore glass beads (pore size 200 Å) which have carbonyl diimidazole reactive side-chains derivatized on their surfaces are commercially available from Pierce (Rockford, IL, U.S.A.). Protein A (Pharmacia, Piscataway, NJ, U.S.A.) was obtained as a lyophilized, pure product. Rabbit antibodies to both human serum albumin and human IgG, heavy-chain specific, were obtained as pure IgG fractions (Miles Biochemicals, Elkhart, IN, U.S.A.) and dialysed against phosphate-buffered saline (PBS, 0.01 *M* phosphate, pH 7.2) before coupling them to protein A. The carbodiimide, 1-cyclohexyl-3-(2-morpholinethyl)metho-*p*-toluene sulphonate was purchased from Pierce (Rockford, IL, U.S.A.) and used as the rabbit IgG–protein A cross-linker. All column fittings were obtained from Biorad Labs. (Rockville Centre, NY, U.S.A.). Human serum albumin and human IgG used in the examples were obtained from the serum and plasma of healthy volunteers. Purified

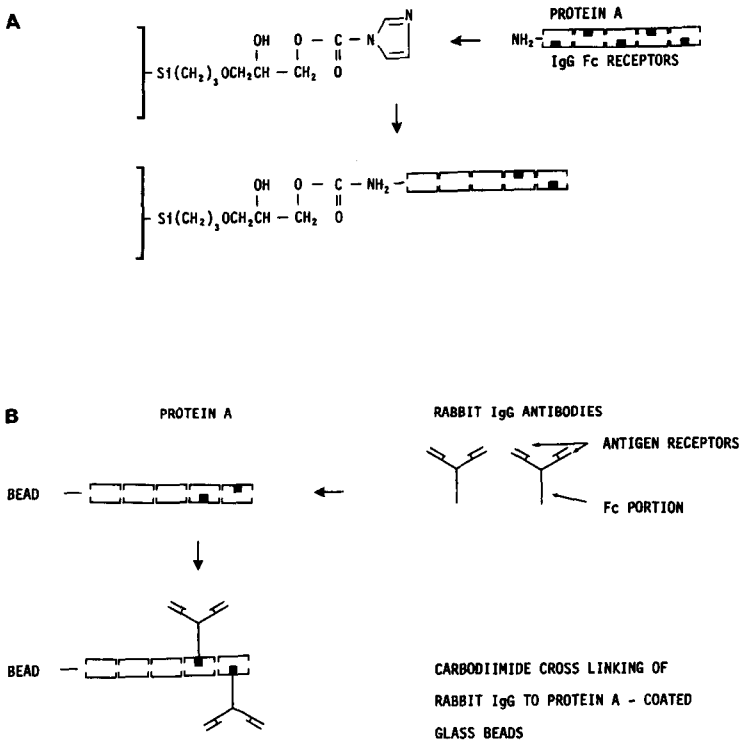

Fig. 1. Diagrammatic representation of the process for binding rabbit antibodies to protein A-coated glass beads. (A) Illustration of the chemistry used to couple protein A to the carbonyl diimidazole-derivatized glass beads. (B) Mechanism of IgG antibody binding to the Fc receptors on the Protein A-coated glass beads.

human serum albumin and human IgG standards were obtained from Sigma (St. Louis, MO, U.S.A.). These reagents were made up in PBS at concentrations of 100 mg/ml and 1.8 mg/ml, respectively. Agarose for immunoelectrophoresis was also obtained from Sigma.

Column construction

The glass beads were washed three times in 0.1 *M* hydrochloric acid and then extensively washed in doubly distilled water prior to the addition of 10 g of the derivatized beads to 5 mg of purified protein A, which had previously been reconstructed in 10 ml of 50 m*M* carbonate buffer (pH 9.0). The bead–protein A mixture was placed into a 15 ml capped tube and mixed for 18 h at 4°C on an overhead mixer. The beads were removed and throughly (ten times) washed in PBS by sedimentation and decantation.

Rabbit antibody to either human serum albumin or human IgG was coated onto the protein A-coated glass beads by adding 10 mg of the purified IgG fraction of the antibody to the 10-g batch of beads. This reaction was performed in 10 ml of PBS. The mixture was transferred to a clean 15-ml capped tube and mixed for 1 h at 4°C on the overhead mixer. Following the rabbit IgG binding to the protein A, all

unreacted protein A binding sites were saturated by further incubating the beads with 10 ml of normal rabbit IgG (1 mg/ml in PBS) for 30 min on the overhead mixer. The beads were sedimentation-washed five times in PBS and the bound rabbit IgG was cross-linked by adding 10 ml of a 10 m*M* solution of the carbodiimide to the sedimented bead pellet. The carbodiimide was dissolved in 50 m*M* carbonate buffer at pH 9. The mixture was again incubated for 1 h at 4°C on the overhead mixer.

After this final incubation, the beads were washed five times in PBS and slurry-packed into a 15 cm × 4.6 mm I.D. stainless-steel column at 1000 p.s.i.

Chromatography

The bead-packed column was installed into a Beckman 340 isocratic high-performance chromatography system (Beckman, Palo Alto, CA), equipped with an Autochrom Model III OPG/S solvent selector/gradient controller (Autochrom, Milford, MA, U.S.A.) a Model 160 Beckman UV detector, set at 280 nm, a Beckman 112 pump and a Shimadzu C-RIB peak integrator (Shimadzu Scientific Instruments, Columbia, MD, U.S.A.). The samples were injected by means of an Altex 210 injector port with a 100-μl sample loop. The column was isocratically developed in 0.9% sodium chloride, 0.1 *M* sodium acetate buffer (pH 6.5) for 20 min at a flow-rate of 0.5 ml/min. The column was jacketed and maintained at 4°C with a recycling ice bath, connected to the column jacket. Following the initial 20 min, during which the rabbit antibody–antigen reaction had taken place and the non-selected material had run through the column, an antigen elution phase was started. Either a pH gradient was automatically generated from 6.5 to 1.0 by the addition of 0.1 *M* hydrochloric acid to the initial buffer, over a 10-min period, or the solvent selector was automatically changed to a chaotropic elution buffer and that buffer was maintained for the same period of time. Table I gives some of the most popular elution buffers. Elution of the bound material was accomplished by maintaining the elution conditions for a further 20 min, before reverting back to the original running buffer to regenerate the column. Fractions of the affinity-isolated material were collected in 200-μl samples in a modified ISCO Cygnet fraction collector (ISCO, Lincoln, NB, U.S.A.). The purity of all the peaks were analyzed by immunoelectrophoresis in agarose gels, using rabbit anti-human serum albumin and rabbit anti-human IgG.

TABLE I

COMMONLY USED ELUTION BUFFERS FOR IMMUNOAFFINITY CHROMATOGRAPHY

Buffer	*Application*
Acid buffers	
0.33 *M* Citrate, pH 2.0	General protein elution
0.1 *M* Glycine, pH 1.5	General protein elution
0.1 *M* Tris–glycine, pH 1.0	General protein elution
0.9% Sodium chloride, 0.1 *M* sodium acetate buffered to pH 1.0 with 0.1 *M* hydrochloric acid	General protein elution
Chaotropic buffers	
2.5 *M* Sodium thiocyanate	Antibody elution
2.5 *M* Polyvinylpyrrolidone-iodine	Antibody elution
6–8 *M* Sodium chloride	Antibody elution

RESULTS AND DISCUSSION

At present, we have produced over 50 batches of protein A-coated glass beads and have found that the carbonyl diimidazole coupling reaction binds between 4.2–4.7 mg of protein A per 10-g batch of beads. Antibody coupling to the protein A was also found to be reasonably efficient, binding between 7–8 mg of rabbit IgG per 10-g batch of coated beads. The production of the protein A-coated glass beads is such that large batches can be produced at one time and stored in PBS to which 0.1% sodium azide has been added, or the beads may be lyophilized and stored dry. In the former case, we have observed that beads kept under such conditions are useable for up to one year if kept at 4°C. Packed columns kept at room temperature are useable for one to three weeks, depending on the type of antibody. Conventional rabbit IgG antibodies remain active for the longest time, while monoclonal mouse IgG has a short life of one week at room temperature. Under conditions where the column is maintained at a constant temperature of 4°C, all types of antibodies will remain active for several months. It was also found that running the columns at 4°C, gave well defined, sharper peaks. Generally, it was found that chaotropic ion elution preserved the immobilized antibody better than acid elution, the former being used for 40–50 column elutions and regenerations, while acid elutions shortened the column life to 20–25 runs.

In our experience, greater stabilization of the immobilized antibody is obtained by using carbodiimide cross linking of the rabbit antibodies to the protein A rather than the more conventional glutaraldehyde. There was also no detectable rabbit IgG elution in the carbodiimide prepared columns. Glutaraldehyde was found to reduce the efficiency of the immobilized antibody, giving antigen yields which were up to 25% less than those obtained with columns using the carbodiimide crosslinking.

Typical elution chromatograms, obtained with acid and chaotropic elution buffers are shown in Fig. 2. Fig. 2A is produced by passing 100 μl of whole human serum through a column containing immobilized rabbit antibody, directed against human serum albumin. The bound antigen in this chromatogram was eluted by acidification of the running buffer with 0.1 *M* hydrochloric acid. The chromatogram shows the primary peak, which is the non-albumin components of the sample, and the secondary peak arising at 12.0 ml, which is the eluted albumin. The recovery rate of the serum albumin was found to be low (75%) when using whole human serum but increased to a recovery of 87% when purified albumin was run through the column. This suggested that the levels of serum albumin were higher than the binding capacity of the immobilized antibody. The chromatogram shown in Fig. 2B demonstrates the isolation and recovery of human IgG, by chaotropic ion elution, from a 100-μl sample of whole human plasma. Again, the primary peak, which ranges from 2.5–10.5 ml is the non-IgG material being washed through the column. The secondary peak at 14 ml is the released human IgG. The recovery of the IgG was 94% which could not be improved, even when a purified IgG solution was passed through the column. Immunoelectrophoretic examination of the peaks produced in Fig. 2A and B demonstrated that there was no loss of rabbit antibody in any of the eluted peaks and that the secondary peaks in both chromatograms contained either pure human serum albumin or pure human IgG. However, in the case of Fig. 2A, the primary peak did contain 8% human serum albumin, which further suggests that the capacity of the column had been saturated.

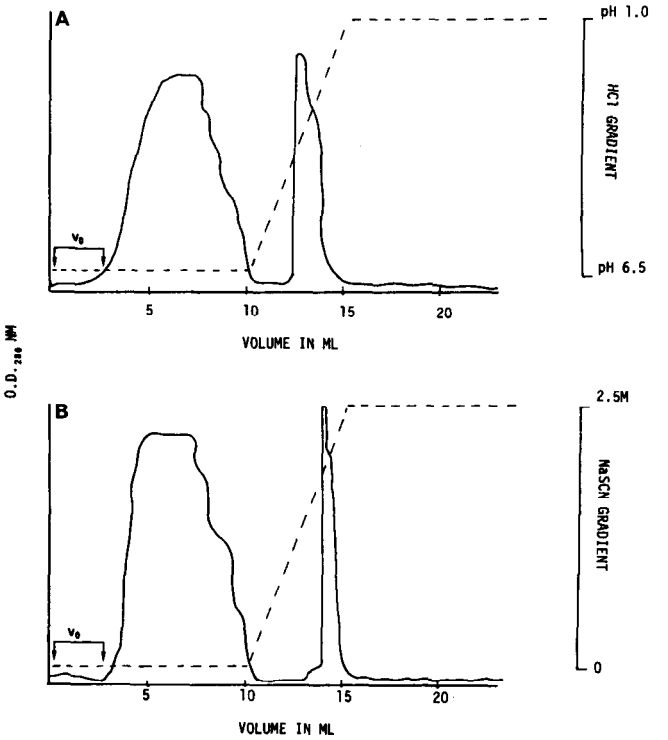

Fig. 2. Typical chromatograms produced by 15 cm × 4.6 mm I.D. HPIC columns run at 0.5 ml/min in 0.9% sodium chloride, 0.1 *M* sodium acetate buffer (pH 6.5) and monitored at $O.D._{280}$ nm with the detector set at 0.8 a.u.f.s. The columns were maintained at 4°C throughout the runs. The acid or chaotropic ion gradients are shown as dashed lines. (A) Isolation of human serum albumin from fresh human serum. The bound albumin being recovered in the second peak (12–15 ml) by acid elution. (B) Illustration of affinity isolation and recovery by chaotropic ion elution of IgG from fresh human plasma. The human IgG is recovered in the second peak which elutes at 14–15 ml. In both chromatograms, V_0 indicates the dead volume of the column.

High-performance immunoaffinity chromatography (HPIC) technology is applicable to the rapid measurement and isolation of any biological material, especially immunoglobulins in specialized body fluids, such as cerebral spinal fluid, tears, and synovial fluid. In each of these cases, the quantity of fluid available is minute and difficult to analyze. We have already reported the application of a high-performance affinity system to the quantitation of IgG and total protein–IgG ratios in cerebral spinal fluid[8] and have quantitated secretory IgA in human tear fluid by immunoaffinity chromatography[9]. The most important aspect of HPIC technology is that the technique not only measures the material under investigation but also recovers it in a biologically active form. This is useful in both clinical and research studies. The scope of the technique is greatly expanded by the ever-increasing repertoire of monoclonal antibodies, the majority of which are of a IgG subclass which can be bound by protein A[10]. This makes the technology available for the isolation of hormones and enzymes from either body fluids or cell extracts and makes it applicable on a semi-preparative scale to the isolation of shed or secreted materials from cell lines in

tissue culture. HPIC technology can also be applied to forensic sciences, where immobilized antidodies to such biological materials as blood group antigens would be helpful in isolating and identifying the presence of such materials in eluates of body secretions on clothing and other materials. In such cases, HPIC technology would provide rapid results, coupled with immunological specificity.

CONCLUSIONS

HPIC can be applied to the isolation and quantitation of any biological material, provided an antibody can be raised against the material. The use of controlled-pore glass beads with a carbonyl diimidazole-derivatized coating, to which protein A may be chemically bound, provides a simple yet universal support medium for the immobilization of most IgG antibodies. This support medium can withstand high pressures and may be used as an immunoaffinity packing in standard high-performance liquid chromatography (HPLC) columns. The packed columns may be used in any HPLC equipment and provide the power and specificity of immunological reactivity to the isolation of specific biological materials.

REFERENCES

1 W. H. Scouten, *Affinity Chromatography*, Wiley, New York, 1981. Ch. 10, p. 272.
2 H. H. Weetal, in M. L. Hair (Editor), *Chemistry of Biosurfaces*, Marcel Dekker, New York, 1972, p. 597.
3 R. Wallin and H. Prydz, *FEBS lett.*, 51 (1975) 191.
4 A. Forsgren and J. Sjoquist, *J. Immunol.*, 97 (1966) 822.
5 G. Kronvall and D. Frommel, *Immunochemistry*, 7 (1970) 124.
6 T. M. Phillips, J. S. MacDonald and M. G. Lewis, in B. Serrou and C. Rosenfeld (Editors), *Immune Complexes and Plasma Exchanges in Cancer Patients*, Elsevier/North Holland, Amsterdam, 1981, Ch. 1, p. 3.
7 L. Bjorck and G. Kronvall, *J. Immunol.*, 133 (1984) 964.
8 T. M. Phillips, N. S. More, W. D. Queen, T. V. Holohan, N. C. Kramer and A. M. Thompson, *J. Chromatogr.*, 317 (1984) 173.
9 T. M. Phillips, N. S. More and A. M. Thompson, *Ann. Clin. Sci.*, in press.
10 E. B. Myhre and G. Kronvall, in S. E. Holm and P. Christensen (Editors), *Basic Concepts of Streptococci and Streptococcal Diseases*, Redbook, Chertsey, 1981, p. 209.