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Magnetic Biospecific Affinity Adsorbents for Immunoglobulin and Enzyme Isolation

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Abstract

Magnetic biospecific affinity adsorbents for immunoglobulin and enzyme isolation have been prepared. They were obtained by a "post-magnetization" procedure involving a simple treatment of the various affinity gels with magnetic ferrofluid. The magnetic biospecific adsorbents tested include magnetic protein A–Sepharose for isolation of IgG antibodies, magnetic human serum albumin (HSA)–Sepharose for anti-HSA isolation, and magnetic 2′,5′-ADP for isolation of glucose-6-phosphate dehydrogenase from baker's yeast and hemolyzates of human red blood cells. For the latter enzyme, a 11,000-fold purification was achieved in one step.

Index Entries: Magnetic biospecific affinity adsorbents; biospecific affinity adsorbents, magnetic; affinity adsorbents, magnetic biospecific; adsorbents, magnetic biospecific affinity; immunoglobulin, isolation by magnetic affinity adsorbents; enzymes, isolation by magnetic affinity adsorbents; glucose-6-phosphate dehydrogenase, isolation by magnetic affinity adsorbents.

Introduction

A number of reports have been published during the last few years on magnetic adsorbents containing immobilized biomolecules. These studies include the use of

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such magnetic adsorbents in general-ligand and specific-ligand affinity chromatography (*1, 2*) and for enzyme immunoassay (*3*); in addition, magnetic material has been used for immobilized enzyme preparations (e.g., *1, 4*). Excellent reviews of the use of magnetic materials include those of reference *5* as well as the new references *6* (immobilized enzymes), *7* (immobilized living cells), and *8* (for drug targeting purposes).

The approach used by our group is to magnetize the adsorbent, which is already carrying the immobilized species, by treating it with a magnetic ferro-fluid (*1, 9*). This procedure has been termed "post-magnetization" to distinguish it from other approaches of "magnetization," such as entrapment of solid magnetic particles, e.g., iron oxide, in a polymer matrix (*2–4*).

In this paper, we describe the use of magnetic affinity supports prepared by post-magnetization for immunoglobulin purification and analysis, and also for the purification of glucose-6-phosphate dehydrogenase from human red blood cells.

Materials and Methods

Materials

Protein A-Sepharose CL-4B, CNBr-activated Sepharose 4B, 2′,5′-ADP-Sepharose 4B were obtained from Pharmacia (Uppsala, Sweden). Glucose-6-phosphate (disodium salt), 6-phosphogluconic acid (trisodium salt), $NADP^+$, and glucose-6-phosphate dehydrogenase from baker's yeast were supplied by Sigma (St. Louis, Mo., USA). Tri-Partigen® IgG immunodiffusion plates were purchased from Behring Institute (Marburg, W. Germany). Rabbit anti-human albumin antibodies were raised in rabbits by one of us (R. Mosbach). Magnetic ferrofluid (base, H_2O; magnetic saturation, 200 G; trademark AO5) was supplied by Ferrofluidics Corporation, 40 Simon Street, Nashua, New Hampshire, USA or Ferrox Ltd., Blackhorse House, 11 West Way, Botley, Oxford, England. Human serum albumin was purchased from the Behring Institute.

Assays

Glucose-6-phosphate dehydrogenase activity and the combined activity of glucose-6-phosphate and 6-phosphogluconate dehydrogenase were measured as described (*10*). Total IgG were measured using Tri-Partigen® immunodiffusion plates. Anti-human serum albumin antibodies were monitored by the passive hemagglutination test with human serum albumin coupled to sheep erythrocytes using glutaraldehyde (*11*) in microtiter plates. Protein was determined at 280 nm (1-cm light path).

Magnetization of Bioadsorbents

The bioadsorbents were magnetized and washed according to procedures given in (*1*); the ferrofluid used had a water base, which makes magnetization of the gels easy to carry out since the gels do not have to be pretreated with organic solvents.

Prior to magnetization, however, the gels must be washed and kept in distilled water before the ferrofluid is applied because incorporation of magnetic material into the gel is hindered by the presence of salts. In a typical experiment, 1 mL of settled Sepharose substituted with the affinity ligand, previously swollen in 0.1 *M* sodium phosphate buffer, pH 7.5, and washed with H_2O, was packed in a column (*1*). Subsequently, 6.5 mL of ferrofluid were pumped through the column and cycled for 4 h at a flow rate of 50 mL h^{-1} (usually at room temperature). After washing with 1 L of H_2O on a glass filter, the beads were washed with 200 mL of 1 *M* NaCl and 200 mL of the buffer. [In the study with 2′,5′-ADP Sepharose the original procedure involving treatment of the beads with albumin was followed (1)]. The dark brown gel beads were then ready for use. The ferrofluid solution left over can be reused for additional post-magnetization experiments.

The magnetic affinity gels after post-magnetization have a dark color—usually black or black–brown—and display excellent magnetic properties, for example, they are easily susceptible to commercial permanent magnets that have a pull of around 5 kg (*1*). Whether the small ferrite particles (~100 Å) of the ferrofluid solution are simply adsorbed to the gel, are precipitated out, or are "semi-entrapped" remains to be established. After magnetization and washing, some of the magnetic material leaked from the gels. For example, some leakage of magnetic material from protein A–Sepharose CL-4B and human serum albumin (HSA)-Sepharose–4B gels was observed when solutions of high protein concentrations and 0.1 *M* phosphate buffer, pH 7.0, or Tris-buffer, pH 7.5, containing 0.9 *M* NaCl were incubated with these preparations. In practice, this leakage of magnetic material from the gels should not be a problem since incubation conditions can be chosen where leakage is minimal. Furthermore, when leakage did take place from magnetic gels, it was more in the first step, i.e., in adsorption of the biomolecule by the magnetic affinity gel when a high protein concentration was present; there was generally no leakage during desorption of the purified material from the magnetic affinity gel. Leakage from the gel does not adversely affect the overall magnetic properties of the affinity adsorbents since they can be used repeatedly. Other magnetic gels, however, under the same experimental conditions, showed no leakage; e.g., 2′,5′-ADP-Sepharose 4B and con A-Sepharose (4B) (R. Mosbach, unpublished work).

Some gels have been used and stored for over 18 months, such as magnetic 2′,5′-ADP Sepharose and they have retained excellent magnetic and bioaffinity properties.

Procedures in the Use of Magnetic Bioadsorbents for the Isolation of Biomolecules

Magnetic Protein A–Sepharose CL 4B Magnetic gel (1 mL, settled volume) was washed extensively with 0.1 *M* Tris-HCl buffer, pH 7.5, containing 0.9% NaCl. It was suspended after washing in 2.5 mL of the same buffer and 0.5 mL of diluted human serum was added; the serum was diluted (1:2) with 0.9% NaCl. Incubation of the magnetic gel with the serum was allowed to take place for 1 h with intermit-

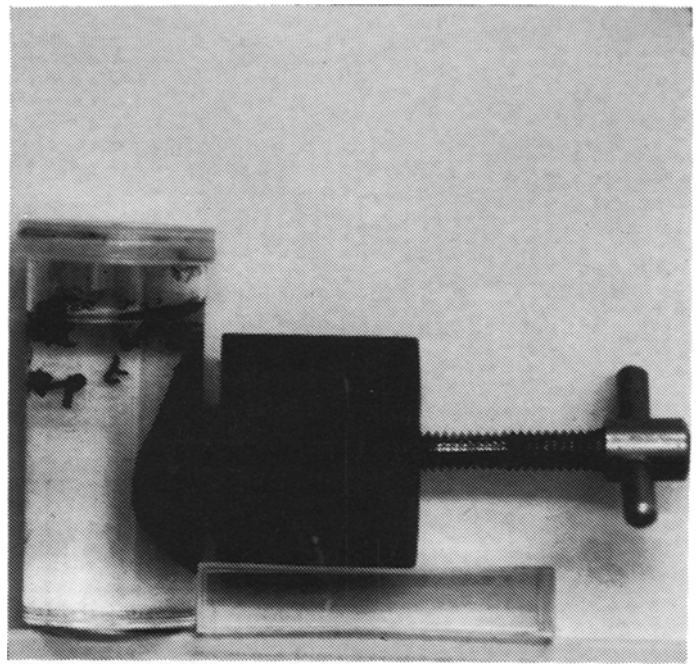

Fig. 1. Retention of a magnetic bioadsorbent with a permanent magnet.

tent agitation at 4°C. The magnetic gel was settled under the influence of a magnet placed on the outside of the reaction container (Fig. 1); and the supernatant was decanted (Fig. 2, batch 1). The gel was washed three times with the same Tris buffer (3 mL each time) for 15 min (batches 2–4). The protein was desorbed from the magnetic Protein A gel either with 3 mL of 0.1 M glycine–HCl buffer, pH 3.0, or with 1 *M* acetic acid (batches 5–7). IgG was measured as described under Assays.

Magnetic Human Serum Albumin–Sepharose 4B Human serum albumin (HSA, 20 mg in 3 mL of 0.1 *M* potassium phosphate buffer, pH 7.0, containing 0.5 *M* NaCl) was coupled to CNBr-activated Sepharose 4B; the gel (1 g dry weight) was re-swollen, washed, reacted with HSA, and subsequently treated with 1*M* ethanolamine (to block any unreacted groups) according to procedures essentially the same as those given in reference *12*. The HSA–Sepharose 4B gel was then washed with 0.1 *M* potassium phosphate, pH 7.0, containing 0.5*M* NaCl and with 0.1*M* sodium acetate buffer, pH 4.0, containing 0.5*M* NaCl. The gel was then magnetized and washed with distilled water as described under "Magnetization of Bioadsorbents" above.

In antibody binding experiments, the magnetic albumin-gel (1 mL, settled volume) was added to 2.5 mL of 0.1 *M* potassium phosphate buffer, pH 7.0, containing 0.5*M* NaCl and 0.5 mL of undiluted rabbit serum was then added. Incubation of the mixture was allowed to take place for 1 h at 4°C with intermittent shaking and the gel was washed subsequently three times with the phosphate buffer. Desorption of anti-HSA antibodies was effected by treating the magnetic gel with

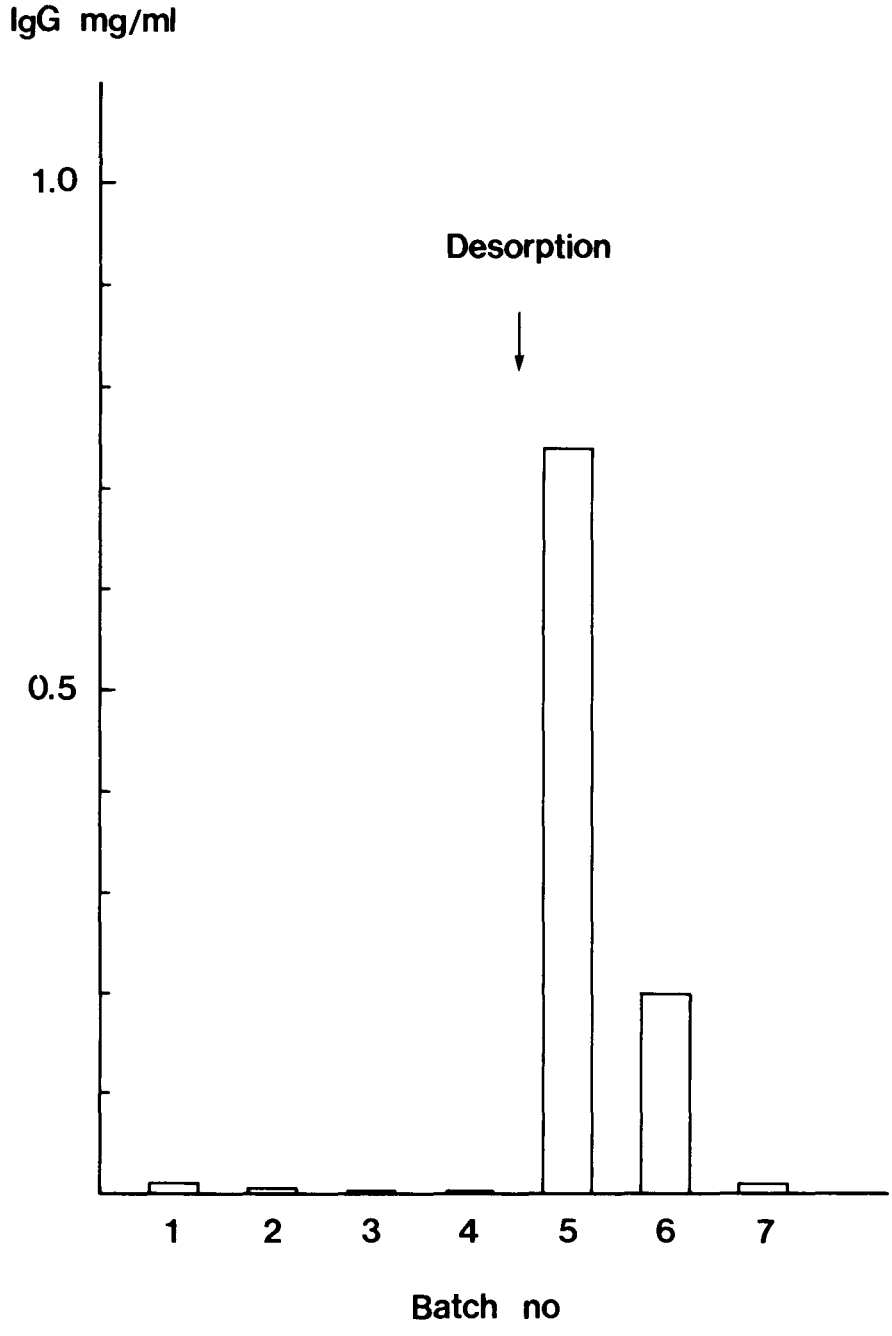

Fig. 2. Adsorbent: magnetic protein A–Sepharose CL 4B for purification of IgG antibodies. Eluent: glycine/HCl, pH 3.0 (0.1*M*) (see Materials and Methods); 1 mg/mL corresponds to 11.5 IU/mL. The values given refer to IgG found in the supernatant.

either 0.1*M* glycine–HCl buffer, pH 3.0, or 2*M* sodium thiocyanate for 30 min at 4° C. The batch numbers given in Fig. 3 correspond to those given in Fig. 2 with regard to the adsorption-washing-desorption sequence. Anti-HSA antibodies were measured by the method given under Assays.

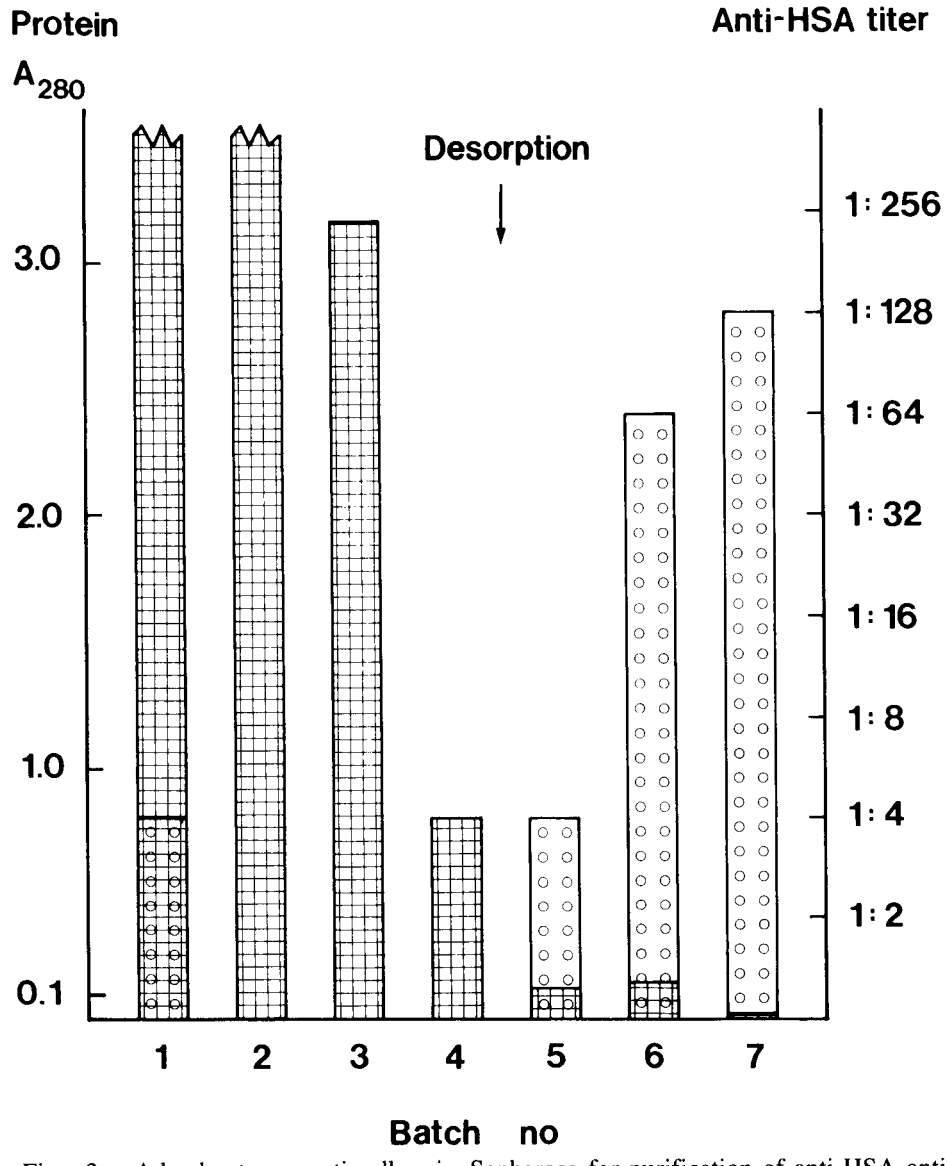

Fig. 3. Adsorbent: magnetic albumin–Sepharose for purification of anti-HSA antibodies. Eluent: glycine-HCl, pH 3.0 (0.1*M*) (see Materials and Methods). The titer is a measure of the specific antibody concentration. The values given refer to protein and titer found in the supernatant: # = protein, ○ = titer.

Magnetic 2′,5′-ADP Sepharose 4B In a preliminary study, magnetic 2′,5′-ADP Sepharose 4B (0.4 g) was added to a 2 mL solution (8 IU) of glucose-6-phosphate dehydrogenase from baker's yeast; the buffer used was 0.1*M* Tris/HCl, pH 7.6, containing 1 m*M* EDTA and 1 m*M* mercaptoethanol. This mixture was incubated for 30 min at 4° C with constant mixing. The gel was then retained by applying an external magnetic field and the "supernatant" poured off. The magnetic gel was then washed with 4 × 8 mL of the above buffer, each wash taking 15

min. The affinity-bound enzyme was eluted by incubation for 15 min with 8 mL of the same buffer containing 0.5 m*M* $NADP^+$.

Magnetic 2′5′-ADP Sepharose 4B was also used in the partial purification of glucose-6-phosphate dehydrogenase from human red blood cells. For this, hemolyzate was prepared as described in reference *13*. Magnetic affinity gel (1 g) was added to about 90 mL of fresh hemolyzate and allowed to incubate for 60 min at 4° C with constant mixing. After adsorption of the enzyme, the gel was retained by applying a magnetic field and washed extensively with 0.1*M* Tris/HCl buffer, pH 7.6, containing 1 m*M* EDTA and 1 m*M* mercaptoethanol. The enzyme was then eluted from the gel by incubation with 10 mL of the same buffer containing 0.5 m*M* $NADP^+$ for 30 min; enzyme recovery was usually around 60%.

Results and Discussion

(a) Magnetic Protein-A Sepharose CL 4B

Protein A from *Staphylococcus aureus* interacts with the complement binding region of IgG immunoglobulins from many species, and when immobilized on Sepharose CL 4B, protein A has been used for the isolation, purification, and analysis of IgG (e.g., *12*). A preparation of this immobilized protein A was magnetized by the post-magnetization procedure as described in Materials and Methods and after magnetizaion treatment the protein A gel still retained its bioaffinity.

This magnetic protein A gel was used for the isolation of IgG from human serum and, as can be seen from Fig. 2, when the gel was incubated with a serum solution, most of the IgG was bound by the magnetic gel; after three washes to remove loosely bound material, no trace of IgG could be found in the "supernatant". The bound IgG could then be desorbed from the magnetic protein A gel by incubation with either 0.1*M* glycine-HCl buffer, pH 3.0, or 1*M* acetic acid. It is worth noting that glycine-HCl buffer is more effective as an eluent for IgG antibodies than acetic acid since the recovery of the antibodies with glycine-HCl buffer was about twice that achieved with acetic acid.

(b) Magnetic Human Serum Albumin–Sepharose 4B

HSA-Sepharose 4B was magnetized and tested for its ability to bind anti-HSA antibodies from serum as described in Materials and Methods. The result of a typical experiment is shown in Fig. 3. After adsorption of the anti-HSA antibodies to the magnetic HSA-gel, the gel was washed and then bound protein was desorbed from the gel using either 0.1*M* glycine-HCl buffer, pH 3.0, or 2*M* sodium thiocyanate, which were equally effective in antibody desorption. As can be seen from Fig. 3, the HSA antibodies eluted from the gel had a much higher titer/protein ratio concentration than the original serum solution.

The fact that some antibody activity is left over in the supernatant after the adsorption step is probably caused by oversaturation of the gel with antibody (it should be added that no optimization of the HSA binding has been attempted at this

preliminary state of the investigation; likewise, the dissociation step could probably be more complete on longer incubation and/or using larger volumes of eluent). It can be concluded that the HSA gel after post-magnetization retains its bioaffinity and it can be used to advantage in purifying anti-HSA antibodies from serum.

(c) Magnetic 2′,5′-ADP Sepharose

2′,5′-ADP Sepharose is a biospecific adsorbent for $NADP^+$ dependent enzymes (*14*) and retains its enzyme binding ability after magnetization (*1*). In a model study we established that glucose-6-phosphate dehydrogenase from baker's yeast was adsorbed by magnetic 2′,5′-ADP Sepharose and could be quantitatively eluted from the magnetic gel after incubation with 0.5 m*M* $NADP^+$.

Magnetic 2′,5′-ADP Sepharose was subsequently used in the purification of glucose-6-phosphate dehydrogenase from hemolyzates of human red blood cells. The magnetic affinity gel was incubated with hemolyzate and the gel was then washed with 0.1*M* Tris-HCl buffer, pH 7.6, containing 1 m*M* EDTA and 1 m*M* mercaptoethanol; the enzyme was desorbed from the gel on incubation with 0.5 m*M* $NADP^+$ in the same buffer. The purification of the enzyme achieved by this one-batch process was 11,000-fold, which is comparable with what other workers have achieved when purifying this enzyme using unmagnetized affinity gels and similar washing procedures (*13, 15*). Morelli et al. (*16*) have used a column of 2′,5′-ADP Sepharose to purify native and variant forms of glucose-6-phosphate dehydrogenase from human red blood cells and they obtain an enzyme of high purity after subjecting the gel to a more elaborate washing procedure than we do. The application of such an elaborate washing procedure to magnetic 2′,5′-ADP Sepharose would probably give a more purified glucose-6-phosphate dehydrogenase preparation, and work is in progress to achieve this (T. Griffin, unpublished work).

This and other work (*1, 2, 9*) shows that magnetic affinity gels can be used in the rapid "pick-up" of enzymes from crude extracts and washing procedures can also be easily carried out. Such rapid pick-up of enzymes by magnetic affinity gels should be important in isolating enzymes that are either intrinsically unstable, susceptible to proteolytic degradation or that exist as labile variants, as in the case of glucose-6-phosphate dehydrogenase (*16*).

General Discussion

This paper demonstrates that a variety of gels containing immobilized biomolecules can be magnetized and retain their bioaffinity after post-magnetization treatment. Thus, in principle, magnetic affinity gels may find wide application since they allow the possibility of rapid purification and analysis of biomolecules. A magnetic gel can also be easily freed from particulate matter obviating the need for centrifugation or filtration steps. This is achieved by applying an external magnetic field to the vessel containing the magnetic affinity gel (*1*), which attracts and holds the gel on the side of the container and, consequently, the "supernatant" can be easily decanted or siphoned away; this cycle of washing

and decanting can be readily repeated. The fact that magnetic bioadsorbents can be applied in batch processes obviates the need for column chromatographic procedures, which is convenient in many practical applications. Furthermore, magnetic gels could be readily applied in the separation of biomolecules from highly viscous extracts that cannot be easily centrifuged, filtered, nor subjected to column processes.

Immunoglobulin purification and analysis using magnetic bioadsorbents has been shown in this communication. Magnetic protein A–Sepharose CL 4B should find application for the isolation of immunocomplexes involving IgG antibodies (and possibly for the purification of subclasses) that may have medical significance. In case the resolution using the batch procedure described here is not sufficient, the preparation obtained by this batch procedure could subsequently be further resolved by conventional column procedures for which the same magnetic support could be used.

Magnetic protein A-Sepharose CL 4B should find application since protein A is a general or group-specific ligand for IgG that interacts with the Fc part of IgG molecules. In contrast, the magnetic HSA-Sepharose 4B chosen here is a model for an immunospecific bioadsorbent and may be of interest both for purification and for rapid and convenient analysis of antibodies of clinical interest. By analogy, antibodies against compounds of interest in "environmental studies" could be coupled to agarose or similar supports, magnetized and then conveniently applied for the rapid analysis of such compounds.

Purification of horse liver alcohol dehydrogenase from crude extracts by magnetized 5′-AMP Sepharose has already been reported *(1)* and the isolation of glucose-6-phosphate dehydrogenase from red blood cells (described here) is another example of the usefulness of magnetic affinity gels for rapid enzyme isolation. We feel that the simple post-magnetization procedure described here is a useful alternative to the application of pre-magnetized polymers since it allows biospecific affinity adsorbents and other polymers to be made magnetic when required.

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