Preparation of Solid-Phase Immunosorbents by Coupling Human Serum Proteins to Cyanogen Bromide-Activated Agarose

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The method of preparing solid-phase immunosorbents by covalently attaching proteins from whole human serum to cyanogen bromide-activated agarose has been investigated to determine optimum concentrations of cyanogen bromide and protein, and the optimum pH for the maximum attachment of proteins from serum. Two systems in which the above immunosorbents have proved useful are described: the removal of antibodies to normal serum proteins from anti-hepatitis B serum and the removal of light chain antibodies from anti-human immunoglobulin M serum.

The preparation of monospecific antiserum to antigens purified from human serum may require an absorption step to remove antibodies to contaminants in the purified antigen. Solid-phase immunosorbents have many advantages over those in solution in that they eliminate the possibility of excess antigen or soluble antigen-antibody complexes remaining in the antiserum, and the immunosorbent can potentially be regenerated and used repeatedly.

Cyanogen bromide activation has been employed to covalently couple various proteins to beaded agarose (2, 6). Agarose immunosorbents have been successfully employed for the purification of antigens and antibodies (3). In this study we investigated the parameters for linking human serum proteins to cyanogen bromide-activated agarose and the efficacy of the immunosorbents thus prepared. By employing appropriate conditions of pH and protein concentration, it was possible to completely couple all immunoelectrophoretically detectable proteins from whole serum, except albumin, to agarose. Albumin is less readily attached to agarose than other serum proteins such as immunoglobulins.

**MATERIALS AND METHODS**

**Chemicals.** Bio Gel A15m beaded agarose, 200/400 mesh, from Bio-Rad Laboratories, Richmond, Calif., was employed for the coupling of proteins. Cyanogen bromide was obtained from Eastman Organic Chemicals, Rochester, N.Y. Crystalline bovine albumin was purchased from Calbiochem, San Diego, Calif. All other chemicals were reagent grade.

**Serum proteins.** Normal human serum (NHS) or plasma was dialyzed at least 16 h against at least 25 volumes of the desired buffer and then concentrated by dialysis versus polyethylene glycol 20,000 (Union Carbide, Baltimore, Md.) or diluted to the desired protein concentration with the same buffer. Protein was determined by the method of Lowry and co-workers (4).

**Coupling to cyanogen bromide-activated agarose.** Procedures for preparation and activation of agarose were those described by Cuatrecasas (2). The settled bed volume (SBV) of agarose was equal to the volume of protein solution. After addition of the packed cake of activated agarose to the protein solution, the mixture was stirred for at least 30 min at 4°C and allowed to stand for at least 16 h at 4°C. The protein-agarose was washed with PBS (0.01 M phosphate, pH 7.2, 0.15 M NaCl, 3 × 10⁻⁴ M KCl, 3 × 10⁻⁴ M MgCl₂) until the absorbancy at 280 nm of the wash fluid was zero.

**Goat anti-human IgM serum.** Antiserum was obtained from goats which had been immunized with immunoglobulin M (IgM) purified from the serum of a patient with Waldenström's macroglobulinemia (J.D. Sipe and F.V. Schaefer, unpublished results). It contained antibodies to immunoglobulin light chains as well as to μ heavy chains.

**Goat anti-HB-Ag serum.** Unabsorbed antiserum to the hepatitis B antigen (HB-Ag), subtype AD, was kindly provided by H. E. Bond.

**Goat anti-human serum and anti-human albumin.** Goat anti-human serum and anti-human albumin were purchased from Hyland Laboratories, Costa Mesa, Calif.

**Immunoelectrophoresis.** Approximately 10 μl of serum or serum fraction were subjected to electrophoresis in a 1-mm layer of 1% agar (Oxoid I.D. Agar tablets, Oxoid Ltd., London) in 0.1 M borate-tris(hy-
dioxymethyl)aminomethane buffer, pH 8.6, containing 0.01 M NaCl and 0.02% sodium azide. The applied voltage was 150 V and for each 1- by 3-inch slide (about 2.5 by 7.6 cm) the time of electrophoresis was 15 min, and the current drawn was 12 mA. For each 3.25- by 4-inch slide (about 8.3 by 10.2 cm) the time of electrophoresis was 25 min, and the current drawn was 33 mA.

Approximately 100 μl of antisem was added to a trough spaced 2 mm from the edge of the antigen well, and diffusion was allowed to take place in a moist chamber at room temperature for 24 to 72 h.

**Agar gel diffusion.** Slides were coated with a 1-mm layer of 1% agarose (Marine Colloids, Inc., Springfield, N.J.) in 0.025 M barbital buffer (pH 8.6) containing 0.02% sodium azide. A 20-μliter amount of antigen and antibody were added to wells spaced 3-mm apart. Diffusion was allowed to take place at room temperature for 24 h in a moist chamber.

**RESULTS**

**Optimum ratio of cyanogen bromide to agarose.** When agarose was activated by cyanogen bromide at concentrations ranging from 5 to 500 mg/ml of settled bed volume of agarose, the amount of protein coupled at pH 11 reached a plateau at 240 mg of CNBr/ml of agarose. The amount of protein linked was 30 mg/ml of agarose (Fig. 1).

**Capacity of cyanogen bromide-activated agarose.** Cyanogen bromide-activated agarose was mixed with equal volumes of human plasma proteins at pH 11 ranging in concentration from 7 to 97 mg/ml. The maximum amount of protein linked was approximately 36 mg/ml of agarose (Fig. 2).

**Effect of pH on coupling of serum proteins to cyanogen bromide-activated agarose.** When normal human serum or plasma at concentrations, ranging from approximately 3 to approximately 30 mg/ml and pH values ranging from 5 to 11, was reacted with cyanogen bromide-activated (240 mg/ml) agarose, coupling was found to vary both with protein concentration and with pH. At higher protein concentrations (15–30 mg/ml) and acid pH only proteins in the immunoglobulin region were linked, whereas at lower concentrations (3–15 mg/ml) all proteins but those in the albumin region were completely linked.

The immunoelectrophoretic patterns of serum at two different dilutions, 6 and 24 mg/ml, pH values ranging from 5 to 11, and before and after coupling are shown in Fig. 3. Since the activated agarose was added to the protein solutions as a packed cake, there was almost no dilution upon linkage. Furthermore, 70% of the unattached protein was removed from the newly prepared immunosorbent by filtration, and 20% was removed by washing it with one SBV of PBS. The remaining 10% of unattached protein was removed by washing with several SBV of PBS or one or two of 3 M NaCl.

From the immunoelectrophoretic patterns of the filtrates after linkage, it can be seen that at the higher protein concentration (24 mg/ml) (Fig. 3C) proteins in the β, α, and albumin region remain at all pH values. Any dissimilarity in the pH 5 and 6 patterns is due to the terminal position of the pH 5 sample. When the two samples were subjected to electrophoresis in equivalent positions, the quantitative differ-
Fig. 3. Effect of pH and protein concentration on the qualitative linkage of human plasma proteins to activated agarose. A, Immunoelectrophoresis of normal human plasma diluted to $A_{500} = 20$ and dialyzed vs. top to bottom: 0.1 M phosphate (pH 5), 0.1 M phosphate (pH 6), 0.1 M phosphate (pH 7), 0.1 M phosphate (pH 8), 0.1 M carbonate (pH 9), 0.1 M carbonate (pH 10), and 0.1 M carbonate (pH 11). B, Immunoelectrophoresis of normal human plasma diluted to $A_{500} = 5$ and dialyzed as in A. C, Immunoelectrophoresis of proteins remaining in solution after linkage of preparations described in A to agarose activated with cyanogen bromide at 240 mg/ml. D, Immunoelectrophoresis of proteins remaining after linkage of normal human plasma dialyzed as described in B above.

ences were not observed. There are five arcs remaining at pH 5 and 6, six at pH 7, and four at pH's 8, 9, 10, and 11. Quantitatively, the total amount of protein linked was affected more by protein concentration than by pH (J.D. Sipe and F.V. Schaefer, unpublished observations).

At the lower protein concentration (6 mg/ml)
(Fig. 3D), traces of proteins in the alpha region remain at pH 7 and 8 as well as the albumin observed at all pH values. The presence of albumin in the samples of unattached proteins was confirmed by immunodiffusion of the samples versus goat anti-human albumin serum.

Albumin can be attached to agarose, but much less efficiently than immunoglobulins. When bovine albumin, 20 mg/ml, in 0.1 M carbonate (pH 11) was reacted with an equal volume of cyanogen bromide-activated (240 mg/ml) agarose, 42% of it was linked, as compared with 95 to 100% for goat immunoglobulins at the same concentration in 0.01 M phosphate, pH 8. Under the same conditions, bovine albumin at 5 mg/ml was 33% linked and human immunoglobulins at 5 mg/ml were 98% linked.

The differences in mobility of the unlinked proteins at pH 8 and 10 have consistently been observed, and we have no explanation for these observations.

Absorption of goat anti-HB-Ag serum. A 2-ml SBV of immunosorbent was prepared by coupling 2 ml of NHS, which had been dialyzed against 0.1 M carbonate buffer (pH 10) and diluted to a protein concentration of 25 mg/ml, to 2 ml of cyanogen bromide-activated (240 mg/ml) agarose. Goat anti-HB-Ag serum which contained antibodies to at least three NHS proteins was absorbed by passage through a column of immunosorbent at a flow rate of 0.2 to 0.3 ml/min. All contaminating antibodies detectable by agar gel diffusion were completely absorbed without any reduction in anti-HB-Ag titer (Fig. 4). Analysis of the absorbed antiserum by counterelectrophoresis (PhorSure, E. R. Squibb & Sons, Inc.) failed to detect any antibodies to NHS dialyzed serially from 1:2 to 1:4096 after a second absorption, although minor amounts were detectable after the first step.

The 2 ml of immunosorbent described in the preceding paragraph had capacity to absorb 2.5 ml of antiserum. Upon treatment with 0.1 M glycine-hydrochloride, pH 2.5, followed by equilibration with PBS, it had capacity to absorb 2.5 ml of antisemur. Similarly prepared immunosorbents have been regenerated at least five times with little or no loss of absorptive capacity. It might be mentioned that an absorbent prepared at pH 11 had similar absorptive and regenerative capacity.

Preparation of goat anti-human IgM (μ-chain specific) serum. To prepare an immunosorbent for removal of light chain antibodies from goat anti-human IgM serum, IgM was completely removed from NHS by passage through an anti-μ chain affinity column. (The antiserum from which this immunosorbent was prepared was obtained by absorption of an antisemur containing antibodies to both light and μ chains with IgG-agarose.) The NHS less IgM (NHS-IgM) was dialyzed against 0.1 M carbonate buffer (pH 11) and diluted to a protein concentration of 15 mg/ml. A 10-ml amount of immunosorbent was prepared from 10 ml of diluted NHS-IgM and a 10-ml SBV of cyanogen-bromide-activated (240 mg/ml) agarose. This immunosorbent had capacity to absorb 20 ml of anti-human IgM serum (Fig. 5) and could re-absorb 23 ml after regeneration with 0.1 M glycine-hydrochloride, pH 2.5. The small increase in capacity after regeneration is an example of

Fig. 4. Goat anti-HB-Ag serum after absorption with (top to bottom): A, human serum agarose, B, horse serum agarose, and C, unmodified agarose. The middle row of wells contains in each case from left to right: absorbed serum and serial dilutions of it from 1:2 to 1:128. The top row of wells contains HB-Ag-negative serum and the bottom row HB-Ag-positive serum.

Fig. 5. Immunelectrophoretic analysis of absorbed goat anti-human IgM serum. The top trough contains antiserum from the 17th ml fraction from an immunosorbent column which had 20-ml capacity, the center trough contains antiserum from the 7th ml fraction from the same column, and the bottom trough contains unabsorbed serum. Both antigen wells contain human plasma.
the slight fluctuations in column capacity that we have observed. Perhaps this is due to small variations in flow rate or ambient temperature. Similarly prepared immunosorbents have been regenerated at least five times. The absorbed antiserum was generally about 25 to 50% lower in titer with respect to anti-IgM activity than was the unabsorbed serum. The drop in titer was attributed to removal of light chain antibodies from the antiserum.

**Covalent nature of protein attachment to agarose.** The absorbed antisera were shown to be devoid of human protein when tested by agar gel diffusion versus anti-human serum (Fig. 6). No desorption of protein from the freshly prepared, or regenerated, PBS-washed immunosorbents was observed upon treatment with 4 M urea or 3 M NaCl.

**Immunological specificity of serum protein agarose immunosorbents.** When 2 ml of goat anti-HB-Ag serum was absorbed with an immunosorbent prepared from 2 ml of horse serum dialyzed against 0.1 M carbonate buffer (pH 11) and diluted to a protein concentration of 22 mg/ml, the contaminant antibodies remained (Fig. 4B). The anti-HB-Ag titer was also unaffected. When the same volume of antiserum was reacted with untreated agarose no reduction of either contaminant or anti-HB-Ag titer was observed (Fig. 4C).

**DISCUSSION**

The maximum amount of protein which can be attached to cyanogen bromide-activated agarose appears to be approximately 35 mg/ml SBV (Fig. 1 and 2).

The unprotonated free amino group is involved in the coupling of proteins to activated agarose (2). We have been able to link from serum all proteins except albumin by increasing pH and decreasing protein concentration. Apparently immunoglobulins are linked more readily than more anionic proteins. Linkage of proteins migrating between the immunoglobulins and albumin is facilitated by carrying out the linkage at pH 11 and diluting the serum to less than 15 mg of protein per ml (Fig. 3).

Cuatrecasas (2) reported a sharp decrease in the amount of alanine coupled to cyanogen bromide-activated (250 mg/ml) Sepharose at pH 11.5 versus pH 10.5. The concentration of the amino acid was 0.015 M. We do not know whether our success with coupling at pH 11 is due to a higher concentration of amino acid residues available in protein solutions of concentration from 3 to 97 mg/ml and/or preferred coupling with other amino acid residues at this pH.

We have not rigorously compared the efficacy of absorbents prepared at the various pH values. Complete linkage of all serum proteins except albumin is greater at pH 11, and absorbents thus prepared have acceptable capacities.

All of the attached serum proteins seem to be covalently bonded to agarose as they are not removed by strong dissociating agents. Similar findings have been reported for immunoglobulins attached to agarose (5). Furthermore, no human proteins can be detected immunologically in absorbed antisera (Fig. 6).

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