POLYACRYLAMIDE–PROTEIN IMMUNOADSORBENTS PREPARED WITH GLUTARALDEHYDE

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Received 10 April 1972

1. Introduction

The preparation of biologically active water-insoluble derivatives of antigens and antibodies using glutaraldehyde as the cross-linking agent has been reported [1]. The present paper describes a method where antigens and antibodies are coupled to glutaraldehyde activated beads of polyacrylamide gels [2]. The derivatives obtained were examined for their effectiveness in the use as immunoadsorbents. The results obtained proved that these derivatives behave as highly specific immunoadsorbents which allow the isolation of antigens and antibodies in high yields.

2. Experimental

2.1. *Materials and methods*

Crystallized human and bovine serum albumin and human and rabbit gamma globulin fraction II were purchased from Pentex (Kankakee, Ill., USA); horse radish peroxidase RZ 3 and cytochrome *c* were products of Sigma Co. (St. Louis, Mo., USA); bovine trypsin and chymotrypsin were purchased from Choay, France. 25% Aqueous solutions of glutaraldehyde was obtained from Schuchardt (München) and TAAB Laboratories, Reading, England, which were used without further purification.

Polyacrylamide beads Bio-Gel P of different classes and sizes were products of Calbiochem. Luzern, Switzerland.

The rabbit or sheep antisera employed in the present work were prepared as already described [1, 3].

The concentration of protein was determined by a modified biuret method [4]. The peroxidase content of different preparations was estimated from the extinction coefficient at 403 nm $E^{1\%}_{1\,cm} = 22$.

The antibody content of the different preparations was determined by the quantitative precipitation reaction of Heidelberger and Kendall [5].

Immunoelectrophoresis was performed according to the method described by Grabar and Williams [6] using gels of agarose at 0.8% concentration.

2.2. *Polyacrylamide–protein derivatives*

2.2.1. Preparation of activated (glutaraldehyde treated) polyacrylamide beads

Bio-Gel P 300 was allowed to hydrate for 24 hr in distilled water and was then washed several times with the same medium. To 100 ml of the hydrated gel, 500 ml of a 6% solution of glutaraldehyde in 0.1 M phosphate buffer pH 7 were added. The pH was adjusted if necessary. The suspension was incubated overnight at 37°. The gel was then washed 10 to 20 times with distilled water using 500 ml for each washing. At this state, the activated gel could be stored at +4° for at least a week without any appreciable loss of its capacity to bind proteins.

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Table 1
Coupling of protein on activated Bio-Gel P-300.*

Protein used for insolubilization	Quantity of Bio-Gel employed (ml)**	Quantity of protein added (mg)	Final concentration of protein in suspension (mg/ml)	Quantity of protein coupled (mg)	Quantity of protein coupled (mg/ml of beads)
Rabbit IgG	22	52	1.04	22	1
	8.5	24	1.2	13.2	1.5
Mouse IgG	10	15	0.75	15	1.5
Bovien serum albumin	24	60	1.2	36	1.5
	13	27	1.35	24	1.84
Cytochrome *c*	5	10	1	8	1.6
Trypsin	12	12	0.6	10.8	0.9
Peroxidase	5	10	0.7	1.43	0.28

* Same quantities of protein were coupled whether Bio-Gel P-300 100–200 mesh or minus 400 mesh were used.
** ml of gel after centrifugation at 3,000 *g* for 15 min.

2.2.2. Coupling of proteins to activated polyacrylamide beads

10 ml of 0.1 M phosphate buffer pH 7.4 containing 15 to 30 mg of protein were mixed with 10 ml of centrifuged (3,000 *g*, 10 min, 4°) activated beads and the suspension was allowed to rotate slowly at room temp. overnight or, in some experiments, for 48 hr at 4°. The suspension was centrifuged (3,000 *g*, 10 min, 4°), the supernatant removed and the sediment washed by successive decantations with buffered physiological saline until the optical density of the supernatant was less than 0.050 at 280 nm. The first supernatant and that of the two following washings were pooled and their protein content determined. The amount of protein conjugated on the gel particles was estimated from the difference between the initial amount of protein added and that found in the pooled solutions. In order to block any remaining free active aldehyde groups on the beads, the gel was suspended in an equal volume of 0.1 M lysine, pH 7.4 and left at room temp. for 18 hr. The gel particles were then exhaustively washed with buffered physiological saline followed by two washings with 50 ml of chilled glycine-HCl buffer 0.2 M pH 2.8 and one with 20 ml of 0.2 M K_2HPO_4. Washing with buffered physiological saline was then repeated until the optical density was 0 at 280 nm. The suspension was then stored at 4° in the presence of 0.01% sodium azide until used.

2.2.3. Use of the insoluble derivatives as immunoadsorbents

In order to employ the insoluble derivatives as immunoadsorbents the 10 ml of gel particles were mixed with an appropriate volume (5 to 20 ml) of whole immune serum. The suspension was gently stirred at room temp. for 1 hr, centrifuged (3,000 *g*, 10 min, 4°) and the supernatant was removed to estimate the amount of non-adsorbed antibodies. *All the subsequent operations were carried out at +4° C.* The gel particles were suspended in buffered physiological saline and washed by successive centrifugations until the supernatant had an optical density of less than 0.020 at 280 nm. Adsorbed antibodies were then eluted with two washings (20–30 ml each) of HCl-glycine 0.2 M pH 2.8 followed by one washing with HCl-glycine 0.2 M pH 2.2. The eluted antibodies were immediately neutralized with 1 M $K_2H PO_4$, filtered on a 0.45 μ Millipore filter and dialyzed against two changes (5 l each) of cold saline. In some experiments, 2 M and 5 M $MgCl_2$ were used as eluents [1, 7].

One can also use immunoadsorbents in column operation. Then, 1 vol of the gel immunoadsorbent was diluted with 2 vol of Bio-Gel P 30 and the suspension packed onto chromatography columns. Adsorption, washing and elution of antibodies were then carried out following precedures already described [1].

Table 2
Isolation of antibodies on Bio-Gel–antigen immunoadsorbents.*

Antigen	Quantity of insolubilized antigen (mg)	Antiserum used	Volume of antiserum added (ml)	Quantity of antibody added (mg)	Quantity of antibody adsorbed (mg)	Quantity of antibody eluted** (mg)	Antibody yield*** (%)
Rabbit IgG	22	Sheep no. 1	3	15	15	10.5	67
	80	Sheep no. 2	4	47	47	43	91.5
Human IgG	6	Rabbit	6.5	10	10.	9.1	91
Bovine serum albumin	27	Rabbit	6	24	24	17.8	74.2
Peroxidase	1.7	Sheep	2	8	8	7.7	96.2
Trypsin	2.1	Rabbit	4	3.2	3.2	3.1	96.7
Chymotrypsin	6.1	Rabbit	5	–	–	5.3	–

* Bio-Gel P-300 minus 400 mesh was used. The numbers represent mean values of two different experiments.
** Elution with 0.2 M glycine-HCl buffer pH 2.8.
*** The yield in antibody is calculated from adsorbed antibody vs. eluted protein.

Table 3
Isolation of antigens on Bio-Gel–antibody immunoadsorbents.*

Antibody used	Quantity of antibody insolubilized (mg)	Antigen added	Quantity of antigen added	Quantity of antigen fixed (mg)	Quantity of antigen eluted (mg)
Sheep anti-rabbit IgG	28.5	Rabbit IgG	10 mg	9.1	8.8**
Sheep anti-rabbit IgG	28.5	Whole rabbit serum	2 ml	Not determined	15.5**
Sheep anti-rabbit IgG γ-globulin fraction†	35	Whole rabbit serum	4 ml	Not determined	16**
Sheep anti-rat IgG	28.8	Whole rat serum	3 ml	Not determined	13.0**
Sheep anti-peroxidase	30.0	Peroxidase	8.5 mg	3.3	3.4***

* Bio-Gel P-300 minus 400 mesh was used. The numbers represent mean values of three different experiments.
** Elution with 0.2 M HCl-glycine buffer pH 2.8.
*** Elution with 5 M $MgCl_2$ pH 6.8.
† The γ-globulin fraction was prepared from sheep anti-rabbit IgG antiserum by ammonium sulfate precipitation. 270 mg of γ-globulin containing 35 mg of specific antibody were coupled to 180 ml of beads gel.

3. Results

Table 1 gives the conditions and efficiency of the coupling of several proteins to polyacrylamide-beads at different ratios of the two reagents. It appears from these results that the absolute amount of a given protein coupled to the particles is a function both of the protein concentration and the quantity of the activated beads. It occurs that the protein concentration is a limiting factor; above a certain

concentration of protein, agglutination of the particles takes place due to cross-linking of the beads by protein bridges. Under the conditions described in 2.2.2, 1–2 mg of proteins were coupled per ml of activated gel. Peroxidase, a protein possessing relatively few free amine groups [8], was the only exception encountered.

All the insoluble antigen derivatives of the present study were found to possess a high adsorbing capacity for homologous antibody. In all cases, the insolubilized antigen retained more than 70% of its initial antibody adsorbing capacity. In addition to Bio-Gel P-300, Bio-Gel P-100 and Bio-Gel P-60 (minus 400 mesh) were also tested and found to be equally effective. However, with the two latter preparations, less quantity of antigen was fixed per ml of activated gel.

The results obtained using various polyacrylamide antigen immunoadsorbents for the isolation of antibodies are included in table 2. It can be seen that recoveries of antibody from immunoadsorbents varied from 67 to 97%.

After concentration, the eluted proteins were subjected to immunoelectrophoresis. Such preparations were found to contain antibodies restricted to the IgG class and 60 to 100% of these were precipitating when examined by quantitative precipitation.

The immunoadsorbents were used repeatedly without noting appreciable loss in their binding capacity.

Pure antibodies and the γ-globulin fraction of immune sera were coupled to activated Bio-Gel P-300 minus 400 mesh and employed for the isolation of antigens. Table 3 shows that the antibody immunoadsorbents possess a high binding capacity for homologous antigen and permits its complete recovery. The proteins isolated from whole serum using the anti-IgG immunoadsorbents (see table 3) were concentrated to 10 mg/ml, submitted to immunoelectrophoresis and developed by anti-whole serum protein antisera. In all the preparations, only IgG immunoglobulins were detected.

To determine whether proteins were non-specifically adsorbed on the various immunoadsorbents, nonimmune normal sera or heterologous antisera were added in excess amounts. After incubation and washing, the immunoadsorbents were treated with glycine-HCl buffer at pH 2.2 in order to simulate protein elution. No proteins were isolated with any of the immunoadsorbents employed in the present work.

4. Discussion

Glutaraldehyde has been successfully employed for the preparation of water-insoluble protein polymers. Although these protein derivatives retained a substantial part of their biological activity and were successfully used as immunoadsorbents or in general for the isolation of specifically interacting constituents [1, 9], they consisted of a highly heterogeneous population of protein aggregates. In the present method, this shortcoming was overcome because proteins were linked to polyacrylamide particles of a standard size which gave rise to a homogeneous suspension of water-insoluble protein derivatives. The suspensions were particularly homogeneous when Bio-Gel P-300 minus 400 mesh was employed. Such homogeneous suspensions of antigen or antibody conjugates can be successfully employed in analytical or quantitative immunological methodology. Thus, for example, inhibition binding experiments with enzyme-labelled antigen can measure 20–200 ng of antigen using suspensions of homologous antibody prepared by the present method [10].

The insoluble derivatives retained more than 70% of their biological activity, i.e. antigen or antibody binding capacity. Furthermore, high enzyme activities (> 55%) were found in Bio-Gel enzyme conjugates prepared by the present procedure [2]. It appears, then, that the tertiary structure of a protein which is essential for its biological activity remains intact to a large extent by the present insolubilization procedure.

The insoluble derivatives as prepared by the present procedure and used for immunoadsorption offer several advantages: their antibody adsorbing capacity is particularly elevated and small amounts of antigen are sufficient to prepare an effective immunoadsorbent. Washing of the immunoadsorbent prior to the step of antibody elution is little time consuming and, in the majority of cases, the adsorbed antibody can be quantitatively recovered.

Nonspecific adsorptions are almost nonexistant and the immunoadsorbent can be re-used without

observing appreciable loss of its antibody binding capacity.

It seems to us that antibody-immunoadsorbents prepared by the present procedure are of special interest. They possess a high immunological specificity and can bind high quantities of antigen; furthermore, these can be quantitatively recuperated. Since approx. 10–15 mg of immunologically pure IgG can be isolated from whole serum (table 3) using 30 mg of insolubilized anti-IgG in one step, such immunoadsorbents might be particularly useful for the isolation of pure antigens in preparative quantities.

References

[1] S. Avrameas and T. Ternynck, Immunochemistry 6 (1969) 53.
[2] P.D. Weston and S. Avrameas, Biochim. Biophys. Res. Commun. 45 (1971) 1574.
[3] S. Avrameas, B. Taudou and T. Ternynck, Int. Arch. Allergy 40 (1971) 161.
[4] E.A. Kabat, in: Experimental Immunochemistry, 2nd ed., ed. Kabat and Mayer (Thomas, Springfield, Illinois, 1961).
[5] M. Heidelberger and F.E. Kendall, J. Exp. Med. 62 (1935) 697.
[6] P. Grabar and C.A. Williams, Jr., Biochim. Biophys. Acta 10 (1953) 193.
[7] T. Ternynck and S. Avrameas, Biochem. J. 125 (1971) 297.
[8] S. Avrameas and T. Ternynck, Immunochemistry 8 (1971) 1175.
[9] S. Avrameas and B. Guilbert, Biochimie 53 (1971) 603.
[10] S. Avrameas and B. Guilbert, Compt. Rend. Acad. Sci., Série D 273 (1971) 2705.