Preparation and Characterization of Antigen and Antibody Adsorbents Covalently Coupled to an Inorganic Carrier

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Immuno-adsorbents were prepared by the use of an inorganic carrier. The proteins were covalently coupled to the carrier through a silane coupling agent. An immuno-adsorbent prepared with human γ -globulin was found to complex with specific antibodies. These antibodies could be recovered in high yield and purity from the adsorbent. Similarly, insolubilized antibodies to L-asparaginase were used to isolate and purify the enzyme from crude extracts. These insolubilized antibodies are extremely stable with time and may be reused many times.

The isolation and purification of antibodies are necessary steps in the study of antibody structure and synthesis. This is especially true where antibodies to specific antigens are concerned. Similarly, techniques that can be used to isolate and purify specific antibodies can also be employed for their detection. This area is of special interest in the field of diagnostic medicine and analytical biochemistry.

Campbell, Leuscher & Lerman (1951) found that, by covalently coupling antigens to insoluble polymers before allowing the antigen to react with antibodies in a serum sample, they were able to isolate and purify antibody that did not contain residual antigen.

Since the initial studies by Campbell *et al.* (1951) on 'immuno-adsorbents', several types of polymers have been employed as carriers for antigens. These include cellulose and its derivatives (Weliky & Weetall, 1965), polyaminopolystyrene, dextrans and poly(amino acids) (see Campbell & Weliky, 1968).

The present paper describes the preparation and characterization of two new types of immuno-adsorbents: one for isolation of antigen, the other for the isolation of antibodies. The results so far obtained with these materials indicate that bound antibodies retain their activity for extended periods of time and can be reused.

EXPERIMENTAL

Chromatographically purified 7S HGG* was purchased from Mann Research Laboratories Inc., New York, N.Y., U.S.A. Partially purified (30 i.u./mg) L-asparaginase was

obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Porous 96% silica glass, $78 \pm 5 \mu\text{m}$ pore size, was obtained from Corning Glass Works, Corning, N.Y., U.S.A. *Escherichia coli* B cells were purchased from Worthington Biochemical Corp.

Preparation of antisera. New Zealand white rabbits were immunized over a period of 4 months with HGG in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich., U.S.A.). The rabbits were bled 10 days after the last injection. The anti-L-asparaginase was prepared by immunizing rabbits similarly over a 6-month period. The rabbits were bled 10 days after the last injection.

Preparation of globulin fraction. The pooled sera to each antigen were fractionated at 50% saturation with $(\text{NH}_4)_2\text{SO}_4$ previously adjusted to pH 7.8.

Preparation of crude enzyme extract. *E. coli* B cells (10g) were homogenized in 19 vol. (v/w) of cold 0.5 M-tris-HCl buffer, pH 8.0, for 5 min. The homogenate was centrifuged at 16000g for 20 min and the supernatant collected.

L-Asparaginase assay. The assays were carried out at 37°C. To each tube was added 1.7 ml of 0.01 M-L-asparagine in 0.05 M-tris-HCl buffer, pH 8.6. To these tubes were added 0.3 ml, 0.2 ml or 0.1 ml of enzyme extract, and tris-HCl buffer was added to give a total volume of 2.0 ml in each case. The enzyme reaction was continued for 10 min. Reaction was stopped by the addition of 0.1 ml of 1.5 M-trichloroacetic acid. The tubes were left for 15 min at room temperature and then centrifuged for 5 min, and a 0.5 ml sample was taken from each. Each 0.5 ml sample was added to 1.0 ml of Nessler's reagent plus 6 ml of water. The colour was read spectrophotometrically at 480 nm. Production of NH_3 was determined and a standard curve prepared with $(\text{NH}_4)_2\text{SO}_4$. For these studies a unit of activity is defined as the production of $1 \mu\text{mol}$ of NH_3/min at 37°C.

Preparation of antigen-glass immuno-adsorbent. Porous 96% silica glass was first cleaned by heating for 3 h in 0.1 M- HNO_3 , dried in an oven at 90°C and finally heated at 700°C for 3 h under O_2 . A 4 g portion of the cleaned glass was treated with 10% (v/v) γ -aminopropyltriethoxysilane solution in toluene as previously described (Weetall &

* Abbreviations: HGG, human γ -globulin; BSA, bovine serum albumin.

Hersh, 1969; Weetall, 1969). The alkylaminosilane glass was converted into the arylamine derivative by reaction with *p*-nitrobenzoyl chloride followed by reduction of the nitro groups with $\text{Na}_2\text{S}_2\text{O}_4$. The glass was diazotized (Campbell, Garvey, Cramer & Sussdorf, 1964) and allowed to react with 0.25g of HGG. The immuno-adsorbent material was washed and stored at room temperature.

Isolation and purification of anti-HGG by a column technique. A column was prepared with 1g of HGG-glass and equilibrated in 1% NaCl solution. To the column was added 1.0ml of rabbit anti-HGG serum. The serum was passed through the column and washed out with 1% NaCl solution. The effluent was collected for antibody determination.

The antibodies complexed to the HGG-glass were eluted in 0.05M-glycine-HCl buffer, pH 2.3, and collected for antibody determination.

Determination of antibody activity. Antibody concentrations were determined by quantitative precipitin analysis as described by Kabat (1961), by using a cold 0.01M-borate-buffered saline, pH 8.0 (0.01M-sodium borate-buffered 1% NaCl, pH 8.0) for washes. Total protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Preparation of antibody-glass immunoadsorbent. A 7g portion of alkylaminosilane glass, prepared as described above, was treated for 3h with a 10% solution of thiophosgene in chloroform. The product was washed with chloroform and air-dried. This was added to 150mg of rabbit anti-HGG globulin or rabbit anti-L-asparaginase previously prepared by $(\text{NH}_4)_2\text{SO}_4$ precipitation (Kabat, 1961). The reactants were adjusted to pH 9.0 with solid NaHCO_3 and NaCO_3 and allowed to react for 4h at room temperature. The reaction was continued overnight at 5°C. The quantity of globulin coupled/g of glass was determined spectrophotometrically at 280nm by loss of protein from the solution. The final product was stored at 5°C in 0.01M-borate-buffered saline, pH 8.0.

Isolation of HGG on the antibody-glass immunoadsorbent by a batch technique. The antibody-glass derivative prepared with anti-HGG globulin was washed several times with successively 1% NaCl, 0.05M-glycine-HCl buffer, pH 2.3, and 0.1M-sodium phosphate buffer, pH 7.0. Then 3g of the washed preparation was added to 20ml of HGG dissolved in 0.01M-sodium phosphate buffer, pH 7.0, containing 4.2mg of protein. The preparation was stirred at room temperature and sampled at 5, 10 and 15min after addition of the antigen. The samples were filtered and the protein remaining in solution was determined spectrophotometrically at 280nm. The bound antigen was eluted with 0.05M-glycine-HCl buffer, pH 2.3.

The same experiment was repeated with glucose oxidase as a non-specific antigen.

Isolation and purification of HGG by a column technique. The antibody-glass derivative prepared with anti-HGG globulin had been stored for 40 days in 0.01M-borate-buffered saline, pH 8.0, at 6°C. A 2g portion was poured into a column and washed with 1% NaCl. To the column was added 0.60mg of HGG in 3ml of 1% NaCl. The effluent and the acid eluate obtained by elution with 1% NaCl and 0.05M-glycine-HCl-buffered 1% NaCl, pH 2.3, respectively were recovered and the HGG contents of both determined.

Maximum binding capacity of the antibody-glass column. For determination of maximum antigen capacity, the derivative used in the preceding section was tested. A 1% solution of HGG in 1% NaCl was passed through the column until the HGG concentration leaving the column was equal to that entering the column. The excess of HGG was washed out with 1% NaCl and the column was eluted with 0.05M-glycine-HCl buffer, pH 2.3.

The experiment was repeated with BSA as a non-specific antigen.

Separation of HGG from BSA by immuno-adsorbent. A solution of HGG-BSA (1:1, w/w) was prepared in 1% NaCl containing a total of 1.0mg of protein/ml. A total of 1ml was passed through a 2g column of antibody-glass. The column was washed out with 1% NaCl and eluted with 0.05M-glycine-HCl buffer, pH 2.3.

The eluate was concentrated and subjected to paper electrophoresis in 0.075M-sodium veronal buffer, pH 8.6, at 200V for 2h.

A similar experiment was carried out with ^{35}S -labelled HGG. To 1ml of a 1% solution of BSA was added 0.81mg of ^{35}S -labelled HGG (Campbell *et al.* 1964). The total mixture was passed through the column, which was then washed out with 1% NaCl and eluted with 0.05M-glycine-HCl buffer, pH 2.3. The radioactivities of effluent and eluate were measured with a Baird-Atomic gas-flow proportional counter.

Isolation of L-asparaginase from E. coli extract. A 2g column of coupled anti-L-asparaginase was prepared and washed consecutively with 0.05M-glycine-HCl buffer, pH 2.3, 0.1M-sodium phosphate buffer, pH 7.0, and 1% NaCl solution. The crude bacterial extract was then passed through the column and the effluent collected. The column was washed with 1% NaCl until no 280nm-adsorbing material was detectable in a 10mm flow-through cell.

The L-asparaginase was eluted with 0.05M-glycine-HCl buffer, pH 2.3, and immediately neutralized with dilute NaOH. The original extract, the effluent and the eluate were assayed for enzyme activity. Protein concentration was determined by the method of Lowry *et al.* (1951).

Immunological examination of the original antigen and crude bacterial extract. The anti-asparaginase was tested by the double-diffusion technique (Campbell *et al.* 1964) on 1% agar in 0.01M-sodium phosphate buffer, pH 7.0. The test antigens were immunizing antigen, *E. coli* extract and immuno-adsorbent-isolated asparaginase.

RESULTS

The quantity of protein covalently coupled to the immuno-adsorbents ranged from 14 to 18mg/g of glass in all experiments regardless of coupling technique. Storage of either antigen or antibody preparations for at least 90 days in 0.01M-borate-buffered saline, pH 8.0, did not affect activity.

HGG-glass immuno-adsorbent. The result of several antibody isolation experiments are given in Table 1. Efficiency ranged from 53.6% antibody retention at the highest loading to 100% antibody retention at the lowest loading. Antibody purity in all experiments exceeded 90%.

Table 1. *Isolation of rabbit anti-HGG on HGG-glass*

Antibody concentrations in serum, effluent and eluate were determined by quantitative precipitin analysis. Column efficiency represents the percentage of total antibody added that was complexed to the immuno-adsorbent. Antibody purity represents the percentage of protein recovered in the acid eluate that was precipitable with specific antigen. Experimental details are given in the text.

Antibody added (mg)	Antibody in effluent (mg)	Total antibody in eluate (mg)	Column efficiency (%)	Antibody purity (%)	Antibody recovery from column (%)
5.78	0	5.78	100	93.5	100
5.78	0	4.90	100	98.5	83
5.78	0.91	4.80	84.5	87.5	99
11.56	5.36	5.58	53.6	94.5	90

Table 2. *Complexing of HGG to 3.0g of antibody-glass immuno-adsorbent by a batch technique*

Experimental details are given in the text.

Time (min)	Antigen adsorbed (mg)	HGG adsorbed (%)
5	2.18	52
10	2.60	62
15	2.60	62

Anti-HGG-glass immuno-adsorbent. The results of the batch tests are shown in Table 2. A total of 4.2mg of HGG was added to the derivative and 1.20mg was recovered in the effluent, indicating that 3.00mg complexed to the immuno-adsorbent.

The antigen was eluted with 0.05M-glycine-HCl-buffered saline, pH 2.3 (0.05M-glycine-HCl-buffered 1% NaCl, pH 2.3). The total protein recovered was 2.92mg, suggesting that 97.3% of the complexed antigen was recovered.

The experiment was immediately repeated with the same antigen with similar results. However, this time only 2.0mg of antigen was adsorbed on the derivative. A 100% recovery was achieved on elution with 0.05M-glycine-HCl-buffered saline, pH 2.3. The maximum quantity of antigen was complexed within the first 5 min. Glucose oxidase, a non-specific antigen, was not picked up by the derivative.

The complexing of HGG by column technique was ascertained by adding 0.60mg of HGG to a 2g column. The results are given in Table 3. When 0.60mg of HGG was added to the column, the total amount was complexed to the bound antibodies. Overloading decreased the percentage of antigen complexed. Recovery of antigen on elution with 0.05M-glycine-HCl-buffered saline, pH 2.3 was 74–100% of the quantity complexed.

The maximum antigen capacity of 4.83mg of HGG was obtained from the 2g column. Maximum binding of a non-specific protein, in this case BSA, was determined. Only 0.70mg of BSA was recovered

on elution with 0.5M-glycine-HCl-buffered saline, pH 2.3. The remainder was recovered in the effluent.

Separation of BSA from HGG on the immuno-adsorbent was accomplished as described above. The acid eluate was subjected to electrophoresis. The results showed a slight trace of BSA present in the eluate. However, elution of the protein from the paper and spectrophotometric determination indicated that at least 80% of the total protein isolated was HGG. The derivative was stored at 6°C in 0.01M-borate-buffered saline, pH 8.0. Then 60 days later another separation experiment was carried out. In this experiment ³⁵S-labelled HGG was added. The results (Table 4) suggest that approx. 0.62mg of HGG complexed to the column. Of the HGG complexed, a total of 0.45mg could be eluted with acid, leaving 0.17mg remaining on the column. The initial HGG concentration added was 7.5%. The final HGG concentration after elution from the column was approx. 32%, a 4.3-fold increase.

Anti-L-asparaginase-glass. The increase in enzyme activity after one passage of crude extract through a 2g column is shown in Table 5. Increased activity ranged from 270% to 726%. This represents approx. 10% of the activity of the original immunizing antigen.

Immunological studies indicated that the anti-serum prepared with the L-asparaginase contained antibodies to at least three distinct antigens present in the *E. coli* extract, one major and two minor components. The major component was present in all antigen samples tested and was probably the enzyme. The two minor components were barely discernible in the immunizing antigen, but were strongly present in the extract and to a slightly smaller extent in the acid eluates.

The results observed with crude extract 3 in Table 5 were obtained 50 days after the experiment with crude extracts 1 and 2, indicating that the coupled antibodies are stable when stored at 6°C in 0.01M-borate-buffered saline, pH 8.0.

Table 3. *Isolation of HGG by a column technique on antibody-glass immunoadsorbent*

The recovery from the column represents the percentage of antigen complexed to the column that was recovered on elution. Experimental details are given in the text.

Antigen (mg)	Antigen complexed to column (mg)	Antigen eluted with acid (mg)	Recovery from column (%)
0.60	0.34	0.34	100
0.60	0.39	0.29	74
0.60	0.38	0.37	97
1.00*			

* Non-specific protein (glucose oxidase) passed through the column.

Table 4. *Separation of BSA and HGG on antibody-glass immunoadsorbent*

³⁵S-labelled HGG was used. Experimental details are given in the text.

Original antigen (c.p.m.)	Total protein added (mg)	Effluent		Eluate	
		(c.p.m.)	(mg)	(c.p.m.)	(mg)
13010	9.81	3119	8.0	7335	1.43
13010	9.81	3098	7.9	7350	1.50
13010	9.81	3352	8.4	7350	1.45

Table 5. *Purification of L-asparaginase*

Experimental details are given in the text.

Sample	Enzyme specific activity (units/mg)	Total activity (units)	Concn. of protein (mg/ml)	Activity increase (%)	Recovered activity (%)
Crude extract 1	0.28	214	6.00	—	—
Effluent	0.20	175	4.50	—	81.6
Eluate	2.00	13	0.32	726	6.1
Crude extract 2	0.122	162	9.20	—	—
Effluent	0.08	65	3.80	—	40
Eluate	0.32	7.5	0.50	270	5.0
Crude extract 3	0.36	266	5.4	—	—
Effluent	0.22	230	5.3	—	86.5
Eluate	2.50	30	0.30	700	11.3

CONCLUSIONS

Immunoadsorbents for the isolation of antibodies in general give low overall yields (Campbell & Weliky, 1968). Polyaminopolystyrenes give yields ranging from 27% to 45% of the total antibody exposed to the adsorbent. Recovery of antibodies complexed to these materials range from 30% to 50%. Cellulose derivatives, on the other hand, give higher yields and recoveries ranging from 80% to 100%. The cellulose derivatives, however, are difficult to use in columns because of such problems as swelling and packing due to pressure. In these laboratories all cellulose immunoadsorbent columns must be run under at least 15 lb/in² to obtain reasonable flow rates.

The inorganic carrier, when used for the isolation of antibodies, appears to match the percentage yields and overall recoveries of many types of organic derivatives, even though the total capacity of the glass immunoadsorbent is rather low compared with those of cellulose and polyaminopolystyrene derivatives (Campbell & Weliky, 1968). However, total recovery of complexed antibody and efficiency of the immunoadsorbent combined with the packing and flow characteristics make this material advantageous to use.

The antibody-glass derivatives were shown to complex and release specific antigen. They are stable over long periods of time when maintained in a buffered solution. Antibody specificity has been demonstrated by the enrichment of HGG from

an HGG-BSA mixture and by the purification of L-asparaginase.

The results of the HGG-adsorption experiments by a batch technique indicate several important points about this material: (a) the antigen-antibody complex-formation is complete within the first 5 min, since no additional antigen was adsorbed; this indicates that even molecules the size of globulin were able to diffuse quickly into the pores of the glass; (b) all the complexed antigen was recoverable by elution with acidified glycine buffer; this has been reported only a few times with antibody-type immunoadsorbents (Weliky & Weetall, 1965).

With the anti-L-asparaginase system the small increase in purity can be explained by the presence of contaminating antigens in the original immunizing antigen. The immunological findings indicate that at least three distinct antigens were present in the original immunizing antigen. Therefore, since antibodies to all these antigens were coupled to the carrier and these antigens were present in the crude extract, they were also recovered. This may also account for the low overall yield of activity units recovered from the extract. The employment of a

highly purified antigen should yield a greatly improved product of high enzymic activity with higher overall yield.

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