Journal of Chromatography, 500 (1990) 585–593
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 964

CARBOHYDRATES AS A TOOL FOR ORIENTED IMMOBILIZATION OF ANTIGENS AND ANTIBODIES

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(First received May 22nd, 1989; revised manuscript received September 5th, 1989)

SUMMARY

A biospecific sorbent for the isolation of ovalbumin antibodies was prepared by coupling of ovalbumin via its periodate-oxidized carbohydrate moiety to bead cellulose modified with adipic acid dihydrazide. The anti-ovalbumin IgG fraction isolated on this sorbent from immune rabbit serum contained only antibodies against protein determinants of ovalbumin. Thus, when these IgG were immobilized through their carbohydrate moieties to cellulose beads it became possible to prepare a biospecific sorbent for concanavalin A by oriented adsorption of ovalbumin. Ovalbumin was specifically adsorbed via its protein moiety and its carbohydrate part remained free for interaction with concanavalin A.

INTRODUCTION

Comparison of the yields and the times required for the purification of glutathione S-transferase (E.C. 2.5.1.18) by various methods illustrates the significant advantages of biospecific affinity chromatography[1]. However, when the immobilized affinity ligand is a protein, *e.g.*, antibody or enzyme, the low stability and capacity of the biospecific adsorbent may be a disadvantage. A solution to both these problems may lie in immobilization of glycoproteins through their carbohydrate moieties. Because the complementary binding sites of enzymes and antibodies are in their protein moieties, immobilization of these glycoproteins through their carbohydrate moieties is useful not only for stabilization of the protein conformation but also for better accessibility of the active sites.

The aim of this work was to prepare an efficient biospecific sorbent for concanavalin A (Con A). In order to obtain antibodies for the oriented immobilization of

0021-9673/90/$03.50

ovalbumin, by analogy with the preparation of polyclonal anti-chymotrypsin antibodies for oriented immobilization of chymotrypsin[2,3], we attached ovalbumin oxidized by periodate to the solid support modified with adipic acid dihydrazide. The ovalbumin immobilized in this manner was utilized to obtain an anti-ovalbumin fraction that reacted with the antigen determinants located in the protein part of ovalbumin only. This immunoglobulin fraction, after its immobilization, would bind ovalbumin via its protein moiety, while its carbohydrate part remained free for interaction with Con A. In this way, an efficient sorbent for the isolation of Con A was prepared by the biospecific sorption of ovalbumin to isolated anti-ovalbumin antibodies attached to bead cellulose, again through their carbohydrate moieties present in Fc fragments.

EXPERIMENTAL

Sephadex G-25 Fine, Mono Q HR5/5 and Superosa 12 HR 10/5 were obtained from Pharmacia (Uppsala, Sweden). Macroporous bead cellulose (exclusion limit for dextran 500 000, particle size 100–250 μm, water content 85%) was purchased from Severočeské chemické závody (Chemopetrol, Lovosice, Czechoslovakia). Adipic acid dihydrazide and Tween 20 were obtained from Sigma (St. Louis, MO, U. S. A.), sodium dodecyl sulphate (SDS) and sodium cyanoborohydride from Serva (Heidelberg, F.R.G.), ethylene glycol from UCB (Brussels, Belgium) and the remaining chemicals, of analytical-reagent grade, from Lachema (Brno, Czechoslovakia). X M50, YM 10 and YM 100 membranes from Amicon (Danvers, MA, U.S.A.) were used for ultrafiltration. Con A and Con A–Spheron 1000 (content of immobilized Con A, 0.4 μmol per gram of dry support) were kindly donated by Dr. K. Filka (Department of Biochemistry, Faculty of Natural Sciences, Charles University, Prague, Czechoslovakia) and crude ovalbumin by Dr. P. Gemeiner (Chemical Institute, Slovak Academy of Sciences, Bratislava, Czechoslovakia).

Isolation of ovalbumin (OA) on Con A–Spheron

A solution of crude OA (50 mg in 10 ml of 0.02 *M* Tris–HCl, pH 7.5, containing 1 m*M* $CaCl_2$, 1 m*M* $MnCl_2$ and 0.15 *M* NaCl) was applied to a column (17 × 3.5 cm I.D.) of Con A–Spheron 1000 equilibrated with the same buffer. After column washing with the above buffer, OA was eluted with 0.1 *M* borate buffer (pH 6.5). The flow-rate was 30 ml/h and the fraction volume 7 ml. The amount of OA eluted was 43 mg.

Oxidation of the carbohydrate moiety of ovalbumin

Ovalbumin (60 mg) isolated on the Con A–Spheron column was dissolved in 60 ml of 0.1 *M* acetate buffer (pH 5.5), then 6 ml of 0.1 *M* $NaIO_4$ were added and the reaction mixture was stirred in the dark for 20 min at 4°C. The reaction was stopped with 48 μl of ethylene glycol and the mixture stirred for a further 5 min. The low-molecular-weight components were removed on a Sephadex G-25 Fine column (30 × 3.5 cm I.D.) equilibrated with 0.1 *M* acetate buffer containing 0.5 *M* NaCl (pH 4.8). Ovalbumin oxidized in this way was then immobilized on activated bead cellulose.

Activation of bead cellulose with adipic acid dihydrazide

To 15 g of macroporous cellulose beads activated with 2,4,6-trichloro-1,3,5-triazine by the procedure of Kay and Crook[4] with a slight modification (content of reactive chlorine 15 μmol/ml), 151 mg of adipic acid dihydrazide in 15 ml of 0.05 *M* borate buffer (pH 9) were added with stirring. A pH of 9 was maintained for 4 h by addition of NaOH with stirring. The reaction product was washed in a column with five volumes of distilled water. The final content of dihydrazide was 12.7 μmol/ml (determined with 2,4,6-trinitrobenzenesulphonic acid by a modified method for the determination of free amino groups on a solid support[5]).

Immobilization of ovalbumin on bead cellulose modified with adipic acid dihydrazide

Oxidized OA (25 ml; see above and Table I) was added to 5 ml of cellulose activated with adipic acid dihydrazide and equilibrated with 0.1 *M* acetate buffer containing 0.5 *M* NaCl (pH 4.8). After 30 h of coupling with stirring at 4°C, sodium cyanoborohydride (35 mg) was added and the reaction proceeded for 6 h at the same temperature. The conjugate obtained was then washed on a sintered-glass filter with the above-mentioned 0.1 *M* acetate buffer containing 0.5 *M* NaCl (pH 4.8) up to the absence of protein in the eluate. As a control, ovalbumin oxidized with $NaIO_4$ was bound under the same conditions to non-activated bead cellulose. The amount of OA coupled was 1.8 mg/ml cellulose.

Preparation of rabbit antiserum against ovalbumin

The rabbit antiserum was prepared according to the optimized procedure described elsewhere[6]. A 2-ml volume of 0.7% ovalbumin and 2 ml of complete Freund adjuvant were mixed and used for the immunization of male rabbits according to the following scheme: 1 ml was injected intramuscularly at two sites, 1 ml subcutaneously at two sites, both doses being administered in 0.5-ml aliquots, and 1 ml was injected intracutaneously in 0.2-ml aliquots at five sites. The immunization was repeated after 14 and 110 days.

Preparation of immunoglobulins against ovalbumin (anti-OA-AB) by immunoaffinity chromatography on ovalbumin–cellulose

A 1-ml volume of rabbit antiserum was diluted with an equal volume of 0.1 *M* phosphate buffer containing 0.5 *M* NaCl and 0.05% Tween 20 (pH 7.2) and applied on a column (4 × 1 cm I.D.) with immobilized OA equilibrated with the same buffer. The antibodies were eluted with 0.1 *M* glycine–HCl containing 0.2 *M* NaCl (pH 2.2) at a flow-rate of 30 ml/h (see Fig. 1). The unbound fraction was rechromatographed on the same column. The pH of both eluted fractions was adjusted to 7.0 with 2 *M* Tris–HCl buffer (pH 8.6) and the fractions were combined.

Testing of isolated antibodies

The presence and quality of antibodies were tested by Ouchterlony double immunodiffusion[7] (Fig. 2A), immunoelectrophoresis[8] (Fig. 2B), by sodium dodecyl sulphate (SDS) electrophoresis according to Laemmli[9] using a 10–25% polyacrylamide gradient and focusing gel with 5% acrylamide, and by ion-exchange chromatography on a Mono Q column[10] (Fig. 3).

Immobilization of oxidized antibodies to bead cellulose modified with adipic acid dihydrazide

Oxidation of isolated antibodies and their immobilization were carried out by the same procedure as described for the oxidation and immobilization of ovalbumin.

Determination of amount of immobilized protein

The amount of immobilized protein was first determined from the difference in absorbance at 280 nm before and after the immobilization and then assessed from an amino acid analysis of the protein. Hydrolysis of the samples was performed in 6 *M* HCl for 20 h at 110°C and the amino acid composition of the hydrolysates was determined on an amino acid analyser[11,12].

Adsorption of ovalbumin on cellulose with attached anti-OA-AB and its use for immunoaffinity chromatography of Con A

Ovalbumin (0.5 mg in 0.5 ml of 0.02 *M* Tris–HCl buffer containing 1 m*M* $MnCl_2$, 1 m*M* $CaCl_2$ and 0.15 *M* NaCl, pH 7.5) was applied on the column (4 × 1 cm I.D.) of cellulose with immobilized antibodies against the protein moiety of ovalbumin at a flow-rate of 12 ml/h. The column was then washed with the same buffer up to removal of all unbound protein as tested by absorbance measurement at 280 nm. Then 0.5 mg of Con A in 0.5 ml of 0.02 *M* Tris–HCl buffer (pH 7.5) was applied to the column and the column was washed with the same buffer. Con A adsorbed to the immobilized ovalbumin was then eluted with 0.1 *M* borate buffer (pH 6.5); ovalbumin was eluted with 0.1 *M* glycine–HCl buffer (pH 2.2) containing 0.2 *M* NaCl (see Fig. 4). The eluted fractions were tested by Ouchterlony double immunodiffusion using antiserum against ovalbumin. The amounts of adsorbed and eluted Con A and ovalbumin were determined using spectrophotometric measurement at 280 nm.

Determination of molecular weights of proteins by gel chromatography

A Superose 12 HR 10/5 column was calibrated with standard proteins (lysozyme 14 300, β-lactalbumin 18 400, trypsinogen 24 000, pepsin 34 700, ovalbumin 43 000, bovine serum albumin 66 000, phosphorylase B 97 400 and β-galactosidase 116 000). The relative molecular weights of the Con A and ovalbumin were determined using 0.01 mg of each protein in 0.1 ml of sample.

RESULTS AND DISCUSSION

Purification of ovalbumin

Ovalbumin, as a glycoprotein, was purified by affinity chromatography on a column with immobilized Con A. The properties of the isolated ovalbumin were tested by several methods. By SDS electrophoresis, affinity-purified ovalbumin was shown to possess one fraction with molecular weight 43 000, while the crude preparation consisted of five distinct fractions. Immunoelectrophoresis supported the results achieved by SDS electrophoresis: the affinity-purified ovalbumin presented a single immunoprecipitation line with antiserum against the whole spectrum of hen egg-white proteins, in contrast to the crude preparation, which gave six immunoprecipitation lines. All of these results confirmed that the ovalbumin purified on immobilized Con A in the manner described was suitable for further experiments.

TABLE I

IMMOBILIZATION OF OXIDIZED OA ON BEAD CELLULOSE MODIFIED WITH ADIPIC ACID DIHYDRAZIDE

OA applied (mg/ml support)	*OA immobilized (mg/ml support)*	*Yield (%)*
3.5	1.8	56
5.5	1.7	32

Oriented immobilization of affinity-purified ovalbumin

The final objective of oriented immobilization of ovalbumin was to bind it exclusively by its carbohydrate moiety, leaving its protein portion free for interaction with antibodies. In this manner, an immunoaffinity sorbent for the isolation of antibodies reacting with the protein part of ovalbumin was prepared.

The purified ovalbumin, oxidized by periodate, was immobilized on bead cellulose activated with adipic acid dihydrazide. The results are given in Table I. The amount of bound ovalbumin was the same at two different concentrations of oxidized ovalbumin. Apparently, therefore, the support was saturated to capacity. The ovalbumin assumably bound to the support surface by its carbohydrate moiety, and only one carbohydrate domain was present in the ovalbumin molecule, being linked to Asp 292 of its protein component[13].

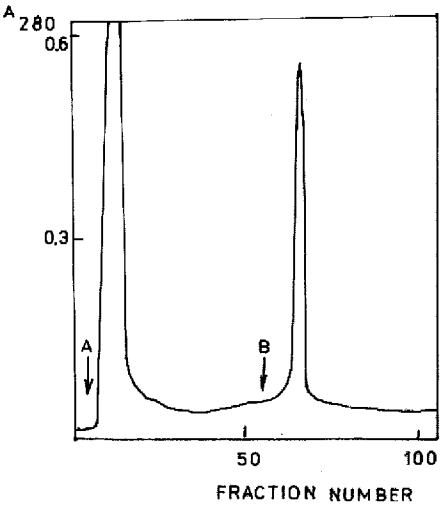

Fig. 1. Isolation of ovalbumin antibodies from rabbit antiserum by biospecific affinity chromatography on a column of ovalbumin immobilized by its carbohydrate moiety to the hydrazide derivative of cellulose. Rabbit antiserum was diluted twofold with 0.1 *M* sodium phosphate containing 0.5 *M* sodium chloride and 0.05% Tween 20 (pH 7.2) and applied to a column (4.0 × 1.0 cm I.D.) carrying immobilized ovalbumin. The column was washed with ten volumes of 0.02 *M* phosphate buffer containing 0.5 *M* sodium chloride and 0.05% Tween 20 (arrow A) and the antibodies were eluted with 0.1 *M* glycine hydrochloride and 0.2 *M* sodium chloride buffer (pH 2.2) (arrow B). The flow-rate was 0.5 ml/min.

TABLE II

ISOLATION OF ANTIBODIES AGAINST DETERMINANTS OF THE PROTEIN MOIETY OF OA BY IMMUNOAFFINITY CHROMATOGRAPHY

85 mg of rabbit antiserum against ovalbumin were applied to a column containing 1.8 mg of OA bound to 1 ml of cellulose.

IgG isolated (mg)	*Percentage of total serum proteins*	*Number of rechromatography runs*
1.94	2.2	4
1.88	2.1	4
1.70	2.0	2
1.04	1.2	2
1.46	1.7	2
1.37	1.6	2
0.86	1.0	2
1.28	1.5	2
1.25	1.5	2

Isolation of IgG with reactivity to the protein moiety of OA

The purpose of this experiment was to select the antibody fraction interacting solely with the protein part of ovalbumin from the large population of immunoglobulins present in rabbit polyclonal antiserum against ovalbumin purified by affinity chromatography.

This antiserum was applied to immunosorbent represented by ovalbumin covalently bound by its carbohydrate moiety to bead cellulose derivatized by adipic acid dihydrazide. The course of the affinity purification is shown in Fig. 1 and the reproducibility of the chromatographic separation is evaluated in Table II. The quality of the immunoglobulin fraction isolated was checked by ion-exchange chromatography on Mono Q using fast protein liquid chromatography (Fig. 3) and by testing the chromatographic fractions by double immunodiffusion and immunoelectrophoresis with bovine antiserum to rabbit immunoglobulins and rabbit antiserum to purified ovalbumin (Fig. 2A and B). This affinity-purified immunoglobulin fraction showed a single band in SDS electrophoresis.

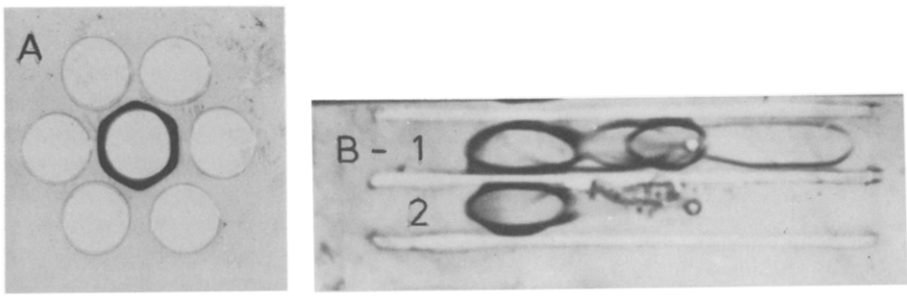

Fig. 2. Immunochemical characterization of ovalbumin antibodies to IgG purified on an ovalbumin–cellulose column. (A) Double radial immunodiffusion of purified ovalbumin antibodies (centre well) against ovalbumin (outer wells); (B) immunoelectrophoresis: whole rabbit antiserum (well 1) and isolated IgG (well 2), bovine antiserum against rabbit serum in channels.

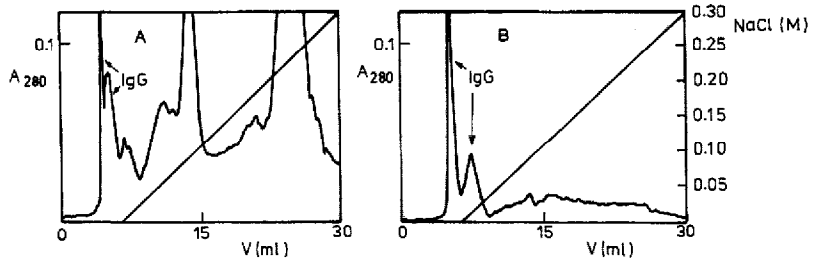

Fig. 3. Purity checking of isolated IgG fraction on a Mono Q column. (A) Original immune serum; (B) IgG fraction isolated by immunoaffinity chromatography. Mono Q HR 5/5 column, elution buffer 0.1 *M* Tris–HCl (pH 8.3). Gradient elution (0–0.5 *M* NaCl) was started after elution with 8 ml of the above buffer without NaCl. The flow-rate was 60 ml/h.

It was thus demonstrated that the above-described immunoaffinity chromatography makes it possible to isolate (immuno)active immunoglobulins by a single-step procedure.

Binding of antibodies to ovalbumin (oriented immobilized) on modified bead cellulose and its use for chromatography of ovalbumin and Con A

The immunoglobulin fraction containing antibodies only against the protein moiety of ovalbumin was oxidized and bound via its carbohydrate moiety (mostly present in the Fc fragment) to cellulose hydrazide. This affinity sorbent for oriented sorption of ovalbumin was employed for its immunoaffinity chromatography. It was shown (Table III) that ovalbumin can be bound and eluted repeatedly. The molar ratio between immobilized immunoglobulins and intercepted ovalbumin molecules

TABLE III

TWO-STEP CHROMATOGRAPHY OF OA AND CON A

Sorbent[a]	*OA applied (mg)*	*OA adsorbed (mg)*	*Con A applied (mg)*	*Con A adsorbed (mg)*	*Con A eluted (mg)*	*OA eluted (mg)*
A	0.500	0.138	0	0	0	0.130
	0.500	0.200	0.500	0.105	0.078	–
	0	0.200	0.500	0.136	0.142	–
	0	0.200	0.500	0.108	0.101	0.164
	0.500	0.145	0	0	0	0.150
	0	0	0.500	0.010	0	0
B	0.500	0.278	0	0	0	0.260
	0.500	0.256	0	0	0	0.263
	0.500	0.220	0	0	0	0.224
	0.500	0.240	0	0	0	0.236
	0.500	0.238	0	0	0	0.230
	0	0	0.500	0	0	0
	0.500	0.238	0.500	0.174	0.186	0.217
	0.500	0.230	0.500	0.190	0.172	0.228

[a] A, 0.40 mg or purified IgG; B, 0.45 mg of purified IgG bound to the column.

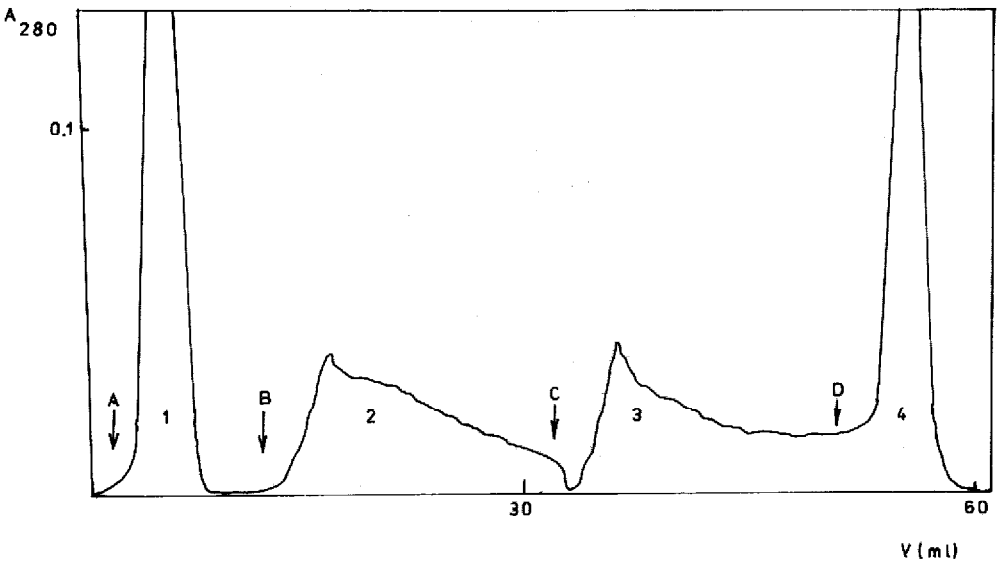

Fig. 4. Affinity chromatography of Con A on biospecifically adsorbed ovalbumin. Binding of (A) OA and (B) Con A: (A) OA (0.5 mg in 0.5 ml of 0.02 *M* Tris–HCl buffer containing 1 m*M* $MnCl_2$, 1 m*M* $CaCl_2$ and 0.15 *M* NaCl, pH 7.5) was applied to a column (4.0 × 1.0 cm I.D.) carrying immobilized antibodies against protein moiety of OA (at a flow-rate of 12 ml/h) and the column was washed with the same buffer (1, the excess of OA was eluted). (B) Con A (0.5 mg in 0.5 ml of 20 m*M* Tris–HCl buffer, pH 7.2) was then applied to a biospecifically adsorbed OA (2, the excess of Con A was eluted with the same buffer as above). Subsequent elution of (C) Con A and (D) OA: (C) bound Con A (3) was eluted with 0.1 *M* borate buffer (pH 6.5); (D) OA (4) was eluted with 0.1 *M* glycine–HCl buffer (pH 2.2).

was 1:1.7. Assuming the presence of two binding sites on each immobilized antibody molecule, we conclude that about 85% of column capacity was utilized. This yield is exceptionally high compared with common types of unoriented immobilization techniques where the usual IgG:OA ratio is about 1:0.7[14].

The following step served to verify the assumption that the bound ovalbumin was really oriented by its protein part towards the surface of the support and by its carbohydrate domain outwards where it was freely accessible to interaction with Con A, a substance specifically reacting with carbohydrates (Fig. 4). This assumption was fully confirmed. Con A could be repeatedly bound and eluted and thus the combined sorbent (cellulose–IgG–OA) with oriented ovalbumin bound to the solid support via immunointeraction of its protein part with covalently immobilized IgG could serve as a specific affinity sorbent for Con A purification.

The specificity of the Con A–OA interaction was also verified. It was shown that Con A was not adsorbed to the original support with immobilized immunoglobulins only. Because the free IgG reacts with Con A in solution[15], this finding may be considered as evidence that IgG is immobilized via its Fc fragments and its carbohydrate part is not accessible for interaction with Con A.

The molar ratio between interacting ovalbumin and concanavalin A was 1.7:0.63. Presumably the theoretical ratio of 1:1 cannot be achieved owing to steric hindrance (Con A is a much larger molecule than ovalbumin) and inaccessibility of

some IgG molecules bound in the pores of the carrier. In order to obtain the maximum loading of the column, excesses of OA and Con A were used in this experiment.

Finally, it may be concluded that the chosen IgG–OA–Con A model strongly suggests the applicability of the principle of oriented immobilization to the preparation of various sorbents suitable for the purification of different compounds in satisfactory yields and with good reproducibility. It may be expected that oriented immobilization will find utilization not only in separation processes, but also for analytical and research purposes.

CONCLUSION

Immobilization of ovalbumin by its carbohydrate moiety made it possible to isolate ovalbumin antibodies against determinants occurring in the protein moiety of ovalbumin. Ovalbumin adsorbed to these immobilized antibodies proved to be a highly biospecific sorbent for Con A. The molar ratio between immobilized ovalbumin antibodies and intercepted ovalbumin increased after their coupling by the carbohydrate moieties to cellulose modified with adipic acid dihydrazide. The described method could be useful for the immobilization of all glycoproteins containing active sites in protein moieties. The suitability of immobilization of antibodies through their carbohydrate moieties has also been demonstrated by Matson and Little[16], who maintain that oriented coupling of immunoglobulin G via the carbohydrate moiety may represent the best strategy for the preparation of immunosorbents. The same conclusion was reached by Ngo[17].

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