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Factors affecting the specific activity of immobilized antibodies and their biologically active fragments

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ABSTRACT

Factors affecting the specific activity of immobilized antibodies and their biologically active fragments were studied with goat anti-mouse and goat anti-human immunoglobulin G. Antibodies were immobilized on HW 65 polymeric support matrix activated with carbonyldiimidazole, hydrazide and iodoacetic acid. The most significant factors influencing the specific activity of stochastic coupling of antibodies are multisite attachment, multiple orientations and steric hindrance imposed by crowding of antibody and the size of the antigen. In oriented immobilization the specific activity is affected only by steric hindrance. The specific activity of immunosorbents prepared by immobilization of F(ab') fragments can be improved to almost 100% by limiting the amount of protein immobilization and the size of the antigen. The present study shows the protocols for optimizing immobilized antibody performance.

INTRODUCTION

The ability to elicit an immune response to an antigen, and more particularly the advent of monoclonal antibody technology, has led to the widespread application of immobilized antibodies for analytical purposes. Antibodies covalently immobilized on a solid support matrix have been found to be useful for the immunoaffinity purification of enzymes [1–5], receptors [6–8] and extraction of substances from plasma [9–11]. Immobilized antibodies have been used to prepare enzyme reactors in the determination of substrates [12–14] and to prepare immunoreactors for flow injection immunoassays [15–17].

There are several methods available for immobilization of an antibody on a support matrix. The most popular coupling method has been the cyanogen bromide (CNBr) activation of agarose beads, followed by the direct attachment of antibody molecules or the attachment of antibody via an N-hydroxysuccinimide ester [18]. Other methods used include the following reagents: periodate [19], 1,1'-carbonyldiimidazole (CDI) [20], tresyl chloride [21], 2-fluoro-1-methylpyridinium toluene-4-sulfonate (FMP) [22] or epichlorohydrin [23]. The reagents acti-

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vate the support matrix, and this step is followed by direct or indirect attachment of the antibody.

Generally antibodies are covalently coupled to the solid supports through ε-amino groups. This method of coupling has several inherent problems associated with it. Since the antibody molecule has many lysine groups present (60–80 out of about 1350 residues), multi-site attachment is unavoidable. For the same reason multiple orientations frequently occur during immobilization, and as a consequence of both these processes the antibody loses most of its activity [18]. Some orientations of the antibody molecule may result in an active antibody that physically cannot bind an antigen, while other orientations of the antibody molecule may result in an inactive antibody due to a covalent bond at or near the antigen binding site. There are, moreover, four N-terminal amino groups on the antibody which are, by virtue of their lower pK_a, even more reactive than the ε-amino groups. The effects of attachment through these groups, which are in the binding site, would be even greater were it not for the overwhelming excess of ε-amino groups present. Random coupling results in an immunosorbent with low specific activity, generally in the order of 1–30% of the theoretical binding efficiency [24,25]. Multi-site attachment is further enhanced due to the high ratio of activated support groups to reactive antibody functional groups typically employed in coupling reactions.

The characteristics of the support matrix of an immunosorbent can have an important influence on the performance of solid-phase immunological reactions. Desirable matrix characteristics are: (1) loose, porous structure; (2) structurally stable spherical beads of uniform porosity and size; (3) easily derivatized; (4) polar but uncharged surface; and (5) mechanically and chemically stable [26–28]. The linkage between the support matrix and the immobilized antibody has a significant effect on the properties of the resulting immunosorbent. The linkage depends on the method of matrix activation and the functional group of the antibody used for coupling. Despite its popularity, CNBr activation has several drawbacks. For example, coupling of amines to activated polysaccharides introduces N-substituted isourea bonds that are not completely stable, particularly in the presence of nucleophiles [29]. The isoureas are positively charged at physiological pH (pK_a = 9.4), and as a result such immunosorbents exhibit ion-exchange properties that can give rise to non-specific interactions. In contrast, the reaction of CDI- and N-hydroxysuccinimide-activated support matrices with the amino groups give stable N-alkylcarbamate (urethane) linkages, devoid of additional charged groups over the wide pH range (*ca.* pH 2–10) normally used in affinity chromatography [20]. Similarly the tresyl- and FMP-activated supports give stable non-ionic linkages with the coupled proteins.

The two most important parameters used to characterize an immunosorbent are the coupling efficiency and the specific activity. The coupling efficiency (%) is defined as the ratio of the moles of antibody coupled to the moles of antibody offered. The specific activity is defined as the moles of antigen bound per mole of

antibody coupled expressed as a percentage. From either an analytical or a commercial standpoint, the usefulness of an immunosorbent will be determined by the values of these two parameters.

Studies have reported that the amount of antibody immobilized on a support matrix will affect the specific activity of the resulting immunosorbent. Immobilization of polyclonal antibodies to estradiol on CNBr-activated Sepharose 4B at different protein/matrix mass ratios resulted in a loss of immunoreactivity from 10 to 95% as the mass ratio was increased [30]. Similar experiments using constant amounts of goat anti-human albumin antibodies and a fixed volume of Sepharose 4B, activated to different levels by CNBr, have shown immunosorbents with 21–54% specific activity with decreasing levels of activation [31]. When the level of activation was constant and the amount of protein available for coupling was decreased, the specific activity increased from 30 to 42% [31]. In a comparative study of Sepharose and Cellulose as supports for immobilization of antibody and antigen, it was found that above 3–4 mg of antibody per gram of Sepharose, additional bound immunoglobulin G (IgG) was inactive [32]. A similar effect was observed for polyclonal antibodies to bovine serum albumin [33]. In contrast, the steric hindrance due to high antibody density did not appear to be a significant problem when monoclonal antibodies to asparagine synthetase were immobilized on Affi Gel 10. The observed difference in antigen binding efficiency between the highest (13.5 mg antibody per ml of gel) and the lowest (0.6 mg antibody per ml of gel) concentrations was less than 5% [34]. According to these authors the most important factor influencing the antigen binding capacity is the extent to which each antibody molecule is coupled to the resin. Cuatrecasas [18] and Goding [35] have also suggested that the extent of coupling (multi-point attachment) is an important factor influencing the antigen binding capacity of immunosorbents.

The pH of the coupling reaction is an important factor to be considered in covalent immobilization of antibodies to activated support matrices. Many reported procedures recommend the coupling of antibody to activated support matrix at pH 8–9.5 [36,37]. However, it has been found that sheep anti-porcine insulin antibodies retained the insulin binding activity when coupled at pH 6.5, but lost almost all the binding capacity when coupled at pH 9.5 to CNBr-activated agarose [18]. The conclusion of this study was that at high pH, multi-point attachment was favored, and as a result the antibody lost the tertiary structure necessary for optimum antigen-antibody interactions. In contrast to these findings, Pfeiffer *et al.* [34] have reported that the antigen binding capacity of monoclonal antibodies to asparagine synthetase has been partially retained when immobilized at high pH (*ca.* 8.7).

In order to eliminate some of the problems associated with random (stochastic) coupling of antibodies, two approaches have been used to immobilize the antibody or its fragment with well defined orientations. In one approach F(ab') fragments of antibodies have been immobilized on support matrices. The mono-

valent F(ab') fragments are obtained by the reduction of the inter heavy-chain disulfide bonds, in the hinge region of the $F(ab')_2$ fragment of the antibody [38,39]. It is known that the F(ab') fragments retain immunoreactivity [38,39]. The reduction of the disulfide bonds introduces one or more thiol groups in the C-terminal region of the F(ab') fragment, depending on the subisotype and the host species of the antibody. Therefore the F(ab') fragments can be immobilized on an appropriately activated support matrix through these thiol groups. Since the number of thiol groups is limited and they are located distal to the antigen binding site, the immunosorbents prepared by this method of immobilization would be expected to exhibit high specific activity. This approach has been used to immobilize F(ab') fragments on liposomes [40], small monolayer vesicles [41] and thiolated cellulose [42]. Immobilization in these cases was accomplished through the formation of a disulfide bridge between the support and the F(ab') fragment. Even though the coupling reaction is highly efficient, the immunosorbents prepared by this method are not stable under reducing conditions. However, stable immunosorbents could be prepared by using activated support matrices possessing maleimide or iodoacetamide functionalities. Both these groups react specifically with thiol groups forming a stable covalent thioether bond. This approach has been used to prepare immunosorbents with moderately high specific activity (*ca.* 57%) by immobilizing rabbit anti-mouse F(ab') fragments [43].

An alternative method of oriented coupling is based on the immobilization of the antibody through the carbohydrate moiety in the Fc region. The carbohydrate moiety is in a domain of the antibody spatially separated from the antigen binding sites and is not involved in antigen binding. Therefore this method of coupling would be expected to give immunosorbents with high specific activity. Immobilization is carried out by the oxidation of the antibody using periodate to generate aldehydes which can then be condensed with support matrices containing amino groups to produce a Schiff base. The Schiff base is subsequently stabilized by reduction with cyanoborohydride. An alternative to the use of amines is to use hydrazide-containing supports which on reaction with aldehydes produce stable hydrazone bonds. Studies have shown that the immunosorbents prepared by oriented coupling using hydrazide supports have higher specific activity (*ca.* 54%) compared to those prepared by random coupling using Affi Gel 10 (*ca.* 18%) [44]. Another study has reported that rabbit anti-human IgG antibodies immobilized on hydrazide gel have a specific activity of 29% compared to 6% for the same antibodies immobilized on activated carboxyl gel [24].

The ideal immunosorbent would possess very high specific activity, stability and minimum non-specific interactions. Since there are many factors which influence the properties of the immunosorbent, careful consideration must be given to each of them to optimize the conditions to obtain an immunosorbent with ideal properties.

The purpose of the present study is to make a systematic comparison of the covalent coupling methods taking into account the support matrix, coupling

chemistry and the nature of the immobilized protein. Previous reports have dealt with specific systems under specific reaction conditions, but it is extremely difficult to make comparisons between coupling modes developed from widely divergent protocols. In the present study the same antibody or its biologically active fragments are coupled to the same support derivatized with different reagents. The purpose of this approach is to examine the questions of controlled orientation, multi-site attachment and antibody loading on the biological activity of the resulting immunosorbents.

EXPERIMENTAL

Preparation of iodoacetamide gel

A 10-ml sample of Reacti-Gel HW 65 (CDI-activated synthetic polymer, exclusion limit for proteins $5 \cdot 10^6$ daltons, particle size 30–60 μm) (Pierce, Rockford, IL, USA) was transferred to a sintered-glass funnel and washed according to the manufacturer's instructions to remove the acetone. Immediately prior to use, the gel was washed with 0.1 *M* carbonate buffer, pH 9.0, drained and rapidly transferred to 20 ml of 0.5 *M* ethylenediamine, the pH of which was adjusted to 9.75 with 5 *M* HCl. The resulting gel slurry was mixed end over end for 8 h at room temperature and then transferred to a sintered-glass funnel to wash off the unreacted material. The gel was washed with 0.1 *M* phosphate buffer (PB) pH 7.4 until the washings were free from amines as confirmed by the absence of reaction with 2,4,6-trinitrophenylsulfonic acid (Sigma, St. Louis, MO, USA). Then the gel was drained and transferred to 20 ml of a solution, composed of 0.1 *M* iodoacetic acid (Sigma), 0.1 *M* 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Sigma) and 50 m*M* N-hydroxysulfosuccinimide (Pierce). This mixture was tumbled end over end for 24 h at room temperature. The gel was then washed as described above with 200 ml of 0.1 *M* PB, pH 7.4, to remove all the unbound materials and stored in the same buffer at 4°C until use. The number of amino groups introduced on to the gel was determined as described by Wilchek *et al.* [45] and was found to be approximately 40–50 μmol/ml of gel. The number of iodoacetyl groups on the gel was determined as described under *Determination of the activity of iodoacetamide gel.*

Preparation of hydrazide gel

Reacti-Gel HW 65 (10 ml) was equilibrated with 0.1 *M* PB, pH 7.4, as described above. Then a 20-ml solution of 0.5 *M* adipic dihydrazide (Aldrich, Milwaukee, WI, USA), pH 7.0 (prepared in 0.1 PB, pH 7.4), was added to the gel and tumbled end over end for 6 h at room temperature. The gel was then transferred to a sintered-glass funnel and washed with 0.1 *M* PB, pH 7.4, until the eluent was free of adipic dihydrazide as confirmed by the absence of reaction with 2,4,6-trinitrophenylsulfonic acid. The gel was then stored in the same buffer at 4°C until use. The number of hydrazide groups on the gel was determined as described by Wilchek *et al.* [45] and was found to be approximately 30–40 μmol/ml of gel.

Preparation of F(ab')

Affinity-purified goat anti-mouse and goat anti-human $F(ab')_2$ (*ca.* 8 mg/ml) were dialyzed against 0.1 *M* PB (pH 6.0, 1 m*M* EDTA) for 16 h. The $F(ab')_2$ was then reduced by the addition of 50 μl of 0.2 *M* 2-mercaptoethylamine (MEA) to 1 ml of each $F(ab')_2$ solution. The reaction mixtures were incubated at 37°C for 90 min. Resulting F(ab') was then separated from excess reducing agent by passing the reaction mixture through a Sephadex G-25 column (30 cm × 1 cm) equilibrated with Tris buffer (50 m*M* Tris-aminomethane, 1 m*M* EDTA, pH adjusted to 8.5 with 5 *M* HCl) at 1.0 ml/min. After separation, the concentration of F(ab') was determined from the absorbance at 280 nm, assuming a value of 14.2 for $A^{1\%}_{280}$.

Immobilization of F(ab') on activated iodoacetamide gel

Coupling of F(ab') was carried out in the Tris buffer (50 m*M* Tris-aminomethane, 1 m*M* EDTA, pH adjusted to 8.5 with 5 *M* HCl) for 6 h at 4°C. Both goat anti-mouse and goat anti-human F(ab') were used to prepare the immunosorbents. A 4.0-ml aliquot of F(ab') solution was used with approximately 0.5 ml of the gel. In order to obtain various antibody F(ab') densities on the support matrix, different amounts of F(ab') were reacted with a fixed volume of the gel. The procedure described below was used to determine the specific activity of the resulting immunosorbents. The amount of F(ab') coupled and the volume of the resulting immunosorbents were also determined as described below. Protocols for washing and handling the gel are described below under *Effect of the coupling reaction pH on immobilization of IgG on CDI-activated HW 65 gel.*

Oxidation of IgG

Affinity-purified goat anti-mouse IgG and goat anti-human IgG were dialyzed overnight at 4°C against acetate buffer (0.1 *M* sodium acetate–acetic acid, 0.15 *M* NaCl, 0.01% NaN_3, pH 5.5). The antibodies were then oxidized by the addition of 50 and 82.5 μl of 0.2 *M* sodium metaperiodate to 1 ml of goat anti-human IgG (13.1 mg/ml) and 1.65 ml of goat anti-mouse IgG (15.7 mg/ml), respectively. Oxidation was carried out at room temperature in the dark for 30 min. The oxidized antibodies were then passed through a Sephadex G-25 column (30 cm × 1 cm) equilibrated with the acetate buffer at 1 ml/min to remove the excess periodate and non-protein-associated aldehydes. After separation, the concentration of oxidized antibodies was determined from the absorbance at 280 nm, assuming a value of 14.2 for $A^{1\%}_{280}$. The immunological activity of the oxidized antibodies was tested after blocking the aldehyde groups with 0.1 *M* Tris buffer, pH 8.5, and then passing the solutions through affinity columns prepared by immobilization of the respective antigen, human IgG and mouse IgG. It was found that the antibodies retain more than 99% of their immunological activity after the oxidation.

Immobilization of oxidized IgG

Coupling of oxidized IgG was carried out in the acetate buffer (0.1 *M* sodium acetate-acetic acid, 0.15 *M* NaCl, 0.01% NaN_3, pH 5.5) for 6 h at room temperature. A solution of 4.0 ml of oxidized IgG (affinity-purified goat anti-human IgG or goat anti-mouse IgG) was used with approximately 0.5 ml of the gel. In order to obtain different antibody densities on the support matrix, different amounts of oxidized IgG were reacted with a fixed volume of the gel. Amounts of oxidized IgG coupled, the specific activity and the volume of each immunosorbent were determined using the procedures described below. Protocols for washing and handling the gel are described in the next section.

Effect of the coupling reaction pH on immobilization of IgG on CDI-activated HW 65 gel (reacti-Gel HW 65)

Immobilization of IgG was carried out in one of the following four coupling buffers: 0.1 *M* PB, pH 6.0, 7.0 and 8.0, or 0.1 *M* carbonate buffer, pH 9.0. In each case, the activated support matrix Reacti-Gel HW 65 (Pierce) was washed and equilibrated with the buffer according to the manufacturer's instructions. Approximately 0.5–1.0 ml aliquots of the gel equilibrated with the coupling buffer were placed in glass test tubes (Fisher Scientific, Pittsburgh, PA, USA) and centrifuged at 500 *g* for 5 min. Then the residual supernatants were carefully removed by aspiration. A 2.0-ml solution of affinity-purified goat anti-mouse IgG (anti-serum prepared by immunizing goats with mouse IgG) (*ca.* 5 mg/ml) or goat anti-human IgG (anti-serum from Pel-Freeze Biologicals, Rogers, AR, USA; purified in laboratory) (*ca.* 5 mg/ml) dialyzed against the appropriate coupling buffer was added to each tube containing the respective gel. The resulting gel slurry was tumbled end over end for 18 h at 4°C and transferred to a disposable chromatography column (Pierce). The columns were washed alternatively with 5 × 1 ml each of acetate buffer (0.1 *M*, pH 4.5) and borate buffer (0.1 *M*, pH 8.5), then with 1.0 ml of PB (0.1 *M*, pH 2.2) and finally with 2.0 ml of PB (0.1 *M*, pH 7.4) to remove the unbound proteins. The washings were collected, pooled and assayed for the protein content using the BCA Protein Assay method (Pierce). The amount of protein coupled to the gel was determined by taking the difference between the amount of protein added and the amount of protein in the washings after the coupling reaction. For convenience, this method will be referred to as the "method of difference". The columns were treated with 0.2 *M* Tris–HCl, pH 9.0, for an additional 4 h at room temperature to block any unreacted active groups. Finally, the columns were washed with PB (0.1 *M*, pH 7.4) and stored in the same buffer until use.

Determination of the volume of immunosorbent

The volume of each immunosorbent was measured by carefully transferring the gel into a calibrated 10-ml disposable pipette (Fisher Scientific). The end of the pipette was plugged with glass wool to retain the gel. Residual buffer in the gel

slurry was removed by applying 1.3 bar nitrogen pressure on the top of the pipette. The volume was read directly from the calibrated scale. Alternatively the volume of the immunosorbent was determined from the geometric dimensions of the column.

Study of the kinetics of immobilization of IgG

Immobilization was carried out either in 0.1 *M* PB, pH 6.0, or in 0.1 *M* carbonate buffer, pH 9.0. Coupling of the antibody (affinity-purified goat anti-mouse or goat anti-human IgG) to the support matrix (Reacti-Gel HW 65) was carried out essentially as described above. After a specified coupling time, 50 μl of supernatant were withdrawn from each tube by centrifuging the tubes at 500 *g* for 5 min. This 50-μl sample was then added to 450 μl of 0.1 *M* PB, pH 7.4, and assayed for the protein content by the BCA Protein Assay method. Samples were withdrawn at times 0.5, 1, 2, 9, 12 and 18 h from the start of the coupling reaction. The amount of protein coupled after a specified coupling time was determined by the method of difference.

Effect of coupling time on the specific activity of the immunosorbent

Coupling of each antibody (affinity-purified goat anti-mouse IgG or goat anti-human IgG) to Reacti-Gel HW 65 was carried out in two different coupling buffers (0.1 *M* PB, pH 6.0, and 0.1 *M* carbonate, pH 9.0) as described above. The coupling reaction was terminated after 0.5, 2.0 and 18 h by the addition of 0.5 ml of 2 *M* ethylenediamine (Fisher Scientific) (pH adjusted to 9.75) to each reaction mixture and mixing for an additional 6 h. The slurries were then transferred to disposable chromatography columns and washed as described above. The volume of the immunosorbents and the amount of antibody coupled were also determined as described above.

Determination of the specific activity of the immunosorbents

In order to determine the specific activity of the immunosorbents the following procedure was used. First, immunosorbents packed in disposable chromatography columns were washed with 1.0 ml of 0.1 *M* PB, pH 2.2, and reequilibrated with 0.1 *M* PB, pH 7.4. Then 1.0-ml aliquots of either affinity-purified mouse IgG (mouse IgG from American Qualex, La Mirada, CA, USA; purified in laboratory) (10 mg/ml) or human IgG (Gammastan, Cutter Biologicals, Division of Miles, West Haven, CT, USA) (10 mg/ml) γ-globulin fraction free of glycine was applied to the respective immunosorbent and allowed to pass through the columns until the immunosorbent was completely covered with antigen solution. Then the columns were capped and allowed to equilibrate for 1 h at room temperature. Next, the columns were washed with 15 ml of 0.1 *M* PB, pH 7.4, to remove all the unbound proteins as confirmed by the absence of absorbance at 280 nm. The bound antigens were eluted with 1.0 ml of 0.1 *M* PB, pH 2.2, followed by 4.0 ml of 0.1 *M* PB, pH 7.4, and the final volumes of the eluates were adjusted to a known

volume with the latter buffer. The amount of antigen bound to each immunosorbent was determined by measuring the absorbance of the respective eluates at 280 nm. The volumes of the immunosorbents were determined as described above. In order to establish that the antibody columns were saturated, pilot experiments were carried out by varying the concentration of the antigen solution and the incubation time. The antigen concentration of 10 mg/ml and the incubation time of 1 h were found to be the optimum conditions.

Determination of the activity of iodoacetamide gel

A 2-ml solution of 0.1 *M* MEA in Tris buffer (50 m*M* Tris-hydroxymethylaminomethane, 1 m*M* EDTA, 0.01% NaN_3, pH adjusted to 8.5 with 5 *M* HCl) was coupled to approximately 0.5 ml of iodoacetamide gel as described above. After 6 h of coupling at room temperature the gel slurry was carefully transferred to a disposable column and washed with 15 ml of the coupling buffer to remove the unreacted material. The amount of MEA coupled was determined by the method of difference. Quantitation of MEA was carried out as described elsewhere, using, 4,4'-dithiopyridine (Aldrich) as the assay reagent [46]. Standard solutions of MEA for the calibration curve were prepared in the Tris buffer. The volume of the gel was determined by the two methods described above. The number of iodoacetyl groups on the gel was approximately 20–30 μmol/ml of gel.

RESULTS AND DISCUSSION

In the preparation of immunosorbents the pH of the coupling reaction has been shown to be an important factor affecting the coupling efficiency [18] and the specific activity of immunosorbents [18,47]. In order to investigate the effect of the pH on immobilization of antibodies to Reacti-Gel HW 65, experiments were carried out with affinity-purified goat anti-mouse IgG and goat anti-human IgG. The results are shown in Fig. 1. As can be seen, the coupling efficiencies of the two antibodies after 18 h of reaction at 4°C were around 85–95% over the entire pH range (pH 6–9) under investigation. Coupling of antibody to Reacti-Gel HW 65 is mainly accomplished through the ε-amino groups (pK_a = 10.3) of the antibody. Therefore, one would expect to see a low coupling efficiency at a lower pH (*ca.* 6–7) compared to that at a higher pH (*ca.* 8–9). But the overall coupling efficiency depends on two competing events: rate of coupling of the antibody and the rate of hydrolysis of the active groups on the support matrix. The results of the experiment suggest that the antibody can be coupled to Reacti-Gel HW 65 over a wide pH range (*ca.* 6-9) with a high coupling efficiency (*ca.* > 85%). Similar observations have been made by Pfeiffer *et al.* [34] and Matson and Little [47] for random immobilization of monoclonal antibodies to CNBr-activated agarose and Affi-Gel 10 (N-hydroxysuccinimide), respectively. The observed results could be due to the presence of 60–80 amino groups on the surface of the antibody molecule and the availability of a large excess of reactive groups on the support

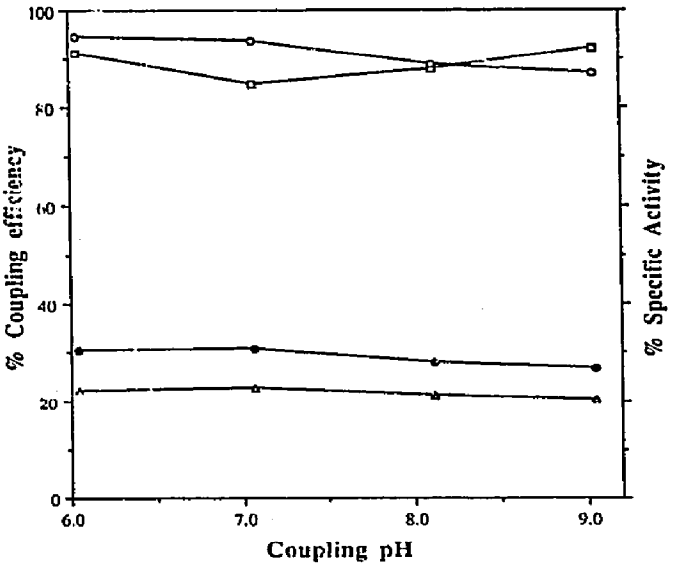

Fig. 1. Effect of pH on the immobilization of antibody on Reacti-Gel HW 65. Affinity-purified goat anti-mouse and goat anti-human IgG were coupled to CDI-activated HW 65 for 18 h in different coupling buffers (pH 6–9) as described in text. The specific activity of the resulting immunosorbents was determined under equilibrium conditions using mouse IgG and human IgG as antigens. Coupling efficiency: (□) goat anti-mouse IgG; (○) goat anti-human IgG. Specific activity: (●) goat anti-mouse IgG; (△) goat anti-human IgG.

matrix. These would compete with one another to give a high overall coupling efficiency over a wide pH range.

As depicted in Fig. 1, the specific activity of the immunosorbents did not change significantly with the pH of the coupling buffer. The specific activity of immobilized goat anti-mouse IgG varied from 30% at pH 6.0 to 27% at pH 9.0. The corresponding values for immobilized goat anti-human IgG were 22–20%. Pfeiffer *et al.* [34] have also reported that the pH of the coupling buffer has no significant effect on the specific activity of the resulting immunosorbents prepared by random coupling of monoclonal antibodies. In contrast it has been reported that immunosorbents prepared by stochastic coupling of antibodies at high pH (*ca.* 8.5) have lost almost 100% of their specific activity [18,47]. Even though the specific activity of immunosorbents was not sensitive to the pH of the coupling buffer the observed values were low (*ca.* 20–30%). This could be due to either multi-site attachment and multiple orientations or steric hindrance as a result of crowding of the antibody. The size of the antigen can also impose steric hindrance which, in turn, would give low specific activity.

In order to illustrate the hypothesis that the multi-site attachment causes a decrease in the specific activity, the effect of the coupling time on the specific activity of the immunosorbents was investigated. The results of these experiments are shown in Table I for goat anti-mouse IgG. As shown, the specific activity of the goat anti-mouse immunosorbents decreased from 37 to 31% as the time of

TABLE I

EFFECT OF COUPLING TIME ON THE SPECIFIC ACTIVITY OF IMMOBILIZED GOAT ANTI-MOUSE IgG

Affinity-purified goat anti-mouse IgG was coupled to CDI-activated HW 65 at two different pH values. Experimental details are given in text.

Experiment No.	Coupling time (h)	Antibody coupled (mg/ml of gel)	Volume of gel (ml)	Antigen bound (mg)	Specific activity[a] (%)
1[b]	0.5	11.22	0.49	1.64	37
2[b]	2.0	12.20	0.35	1.57	37
3[b]	18.0	13.87	0.33	1.40	31
4[c]	0.5	12.72	0.31	1.46	37
5[c]	2.0	13.13	0.35	1.47	32
6[c]	18.0	14.51	0.29	1.13	27

[a] Defined in text (average of three determinations).
[b] 0.1 *M* PB, pH 6.04.
[c] 0.1 *M* carbonate, pH 9.05.

coupling increased from 0.5 to 18 h. When the coupling was carried out at pH 9.0, the specific activity decreased from 37 to 27% for the same coupling times. Similar results were observed for the goat anti-human immunosorbents. The specific activity varied from 29 to 22% at pH 6.0 and from 27 to 20% at pH 9.0 as the time increased from 0.5 to 18 h. It is evident from the results that the specific activity of the immunosorbents decreases as the extent of the coupling reaction increases. Hence, the results of the experiment support the above stated hypothesis that the multi-site attachment causes a decrease in the specific activity of the immunosorbents as the extent of the coupling reaction increases. However, the decrease in specific activity (*ca.* 7–10%) with the increase in coupling time is very small, compared to the loss of activity (*ca.* 63–73%) during the first 0.5 h of the coupling reaction.

Since it was not possible to explain why the immunosorbents have lost nearly 63–73% of their specific activity even after 0.5 h of coupling, the kinetics of the coupling reaction were studied. Coupling of antibodies to Reacti-Gel HW 65 was carried out at 4°C in pH 6.0 and 9.0 coupling buffers. The results shown in Fig. 2 illustrate that the efficiency of the coupling is independent of the pH of the coupling buffer. In the first 0.5 h of the reaction, about 70% of the antibody was coupled to the support matrix. During the rest of the time the coupling efficiency increased slowly and reached an upper value of 90–95% after 18 h. Immediately after an antibody molecule is coupled to the support matrix, it can undergo multi-site attachment, because of the proximity of the molecule to the surface of the gel and the availability of excess reactive groups on the support matrix as well as on the antibody molecule. Further the rate of the multi-site attachment would

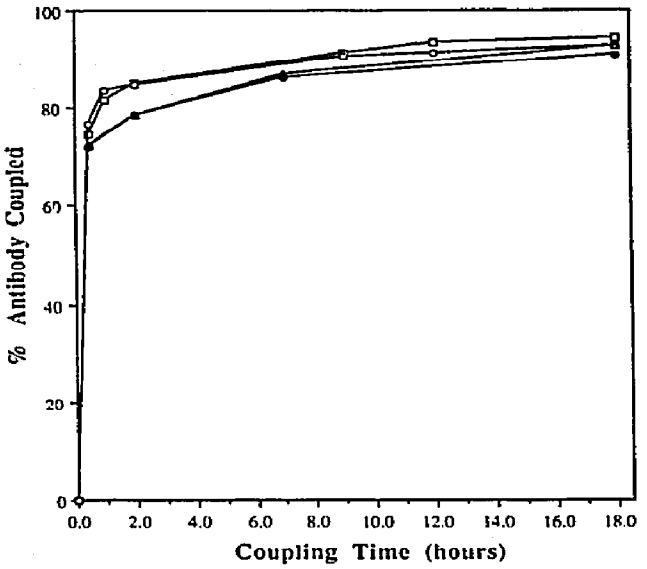

Fig. 2. Time course of immobilization of antibody to Reacti-Gel HW 65. Goat anti-mouse and goat anti-human IgG were coupled to CDI-activated HW 65 in two different coupling buffers (pH 6.0 0.1 *M* PB and pH 9.0 0.1 *M* carbonate buffer) for 18 h. Samples were withdrawn from the reaction mixture at specified times to determine the extent of coupling. Details are given in text. Goat anti-mouse IgG: (□) pH 6.0; (○) pH 9.0. Goat anti-human IgG: (△) pH 6.0; (●) pH 9.0.

be highest in the beginning of the reaction because of the high concentrations of the reactive groups. Since about 70% of the antibody was coupled to the support matrix in the first 0.5 h of the reaction it is reasonable to assume that the multi-site attachment would be critical and unavoidable even in this time frame. Thus the multi-site attachment and multiple orientations are the main reasons for the observed low specific activity of the immunosorbents (*ca.* 37% for goat anti-mouse IgG and 27–29% for goat anti-human IgG) prepared by coupling of antibodies for 0.5 h. Apart from this, crowding of the antibody can also be a significant factor affecting the specific activity.

Immobilization of monovalent F(ab') fragments of an antibody through the hinge region sulfhydryl groups theoretically should produce immunosorbent with high specific activity. The method of immobilization does not permit multi-site attachment. It further results in defined orientation of the F(ab') fragment leaving the antigen binding site unobstructed. However, the immobilization of goat anti-mouse F(ab') fragments on iodoacetamide-activated HW 65 gel has produced immunosorbents with low specific activity (*ca.* 26–30%). The F(ab') density (amount of F(ab') per ml of gel) on the support matrix of these immunosorbents was approximately 7–10 mg/ml of gel. This observation has led us to hypothesize that steric hindrance due to the crowding of the immobilized F(ab') fragment causes a loss of specific activity of the immunosorbents. In order to illustrate this hypothesis the effect of the F(ab') density on the specific activity of the immunosorbents was investigated. The coupling efficiency of the immunosorbents prepared by the immobilization of goat anti-human F(ab') was 50–67%. According

to the results, when 0.2 μmol of goat anti-human F(ab') was offered per ml of the gel only 0.1 μmol was coupled indicating the use of 0.33% of the active groups on the gel (30 μmol of iodoacetyl groups per ml of gel) for coupling. The fraction of active groups available for coupling is the same as that calculated for coupling of ovalbumin (M_r 43 000) to hydrazide-activated cellulose beads [48]. When a large molecule like F(ab') with only one reactive SH group is coupled to the gel it may mask a large number of active iodoacetyl groups on the surface of the gel without utilizing them for coupling. Likewise the coupling of more than one F(ab') molecule may block the accessibility of a large number of active groups within the pores of the gel. The observed low value of the number of active groups available for coupling is due to these two reasons. According to the results the coupling efficiency increases from 50 to 67% as the amount of the F(ab') decreases from 5.0 to 0.94 mg. This suggests that the amounts of F(ab') initially offered to the gel exceeded the number of available iodoacetamide groups. However, the coupling efficiencies observed for the coupling of F(ab') to iodoacetamide gel were lower than the values reported for the coupling of either reduced human IgG or human F(ab') (*ca.* 90%) [49]. The reported high efficiency could be due to the availability of more than one sulfhydryl group for the coupling of the reduced human IgG or F(ab') as compared to only one in F(ab') of goat IgG. The same authors have done experiments with reduced rabbit anti-human serum albumin IgG which has one sulfhydryl group, but the coupling efficiencies were not reported making a valid comparison impossible. The specific activity of the immunosorbents prepared with different (goat anti-human) F(ab') densities are shown in Table II. As can be seen, the specific activity of the immunosorbents dramatically increases from 25 to 42% as the density of F(ab') decreases from 4.54 to 1.14 mg/ml of gel. Similar results were observed for the goat anti-mouse F(ab') immunosorbents. The specific activity of the immunosorbents increases from 37 to 78% as the density dcecreases from 3.98 to 1.05 mg/ml of gel. These observations clearly support the above hypothesis that the steric hindrance due to the crowding of the F(ab') fragments causes a decrease in specific activity of the immunosorbents. This has been observed for the immobilization of intact antibodies on different support matrices [30,31,47,50–52]. To the best of our knowledge, there have been no previous reports on the effect of the F(ab') density on the specific activity. Yet the specific activity of the F(ab') immunosorbents could not be improved to a value closer to 100%. This could be due to the large size of the antigen (mouse IgG or human IgG) which would impose steric hindrance even at very low F(ab') densities.

Immobilization of oxidized IgG on hydrazide HW 65 gel also should give high specific activity because the method of coupling involves oriented immobilizaton. It has been reported that an immunoaffinity column prepared by immobilization of rabbit anti-ovalbumin has a specific activity of 85% [48]. The immobilization is accomplished through the aldehyde groups created by oxidation of the carbohydrate moiety in the Fc region. Thus the site of immobilization is distal from the

TABLE II

EFFECT OF F(ab') DENSITY ON THE SPECIFIC ACTIVITY OF IMMOBILIZED GOAT ANTI-HUMAN F(ab')

Goat anti-human F(ab') was coupled to iodoacetamide-activated HW 65 at pH 8.5 in 50 m*M* Tris–HCl buffer. Specific activity was determined under equilibrium conditions with human IgG as the antigen. Experimental details are given in text.

Density of F(ab') (mg/ml of gel)	Volume of gel (ml)	Amount of antigen bound (mg)	Specific activity[a] (%)
4.54	0.55	2.20	25
3.43	0.55	1.81	28
1.42	0.55	1.08	40
1.14	0.55	0.92	42

[a] Defined in the text (average of three determinations): M_r of goat anti-human F(ab') 45 000 and human IgG 160 000.

antigen binding site. On the contrary, the immobilization of antibodies (goat anti-human and goat anti-mouse) by this method gave low specific activity (*ca.* 30%). The antibody density was approximately 6–7 mg/ml of gel. Cress and Ngo [24] have also reported specific activity of 16–30% for a similar antibody–antigen system. These observations have suggested that the steric hindrance due to crowding of the antibody would be the reason for the low specific activity. In order to investigate this phenomenon, experiments were carried out with different antibody densities. Coupling efficiencies observed for oxidized goat anti-mouse IgG and oxidized goat anti-human IgG were between 47 and 70%. The coupling efficiency again increases with decreasing amounts of oxidized IgG. This observation tends to suggest that the amounts of oxidized IgG offered to hydrazide gel were closer to the amount required to saturate the gel. The coupling efficiencies are in agreement with those reported by others [24,48]. The specific activity of the immunosorbents are shown in Table III for immobilized goat anti-human IgG. As depicted in Table III the specific activity has increased from 23 to 47% as the density decreases from 3.97 to 0.75 mg/ml. Similarly for goat anti-mouse IgG the specific activity has increased from 29 to 51% when the antibody density decreased from 6.48 to 1.79 mg/ml. These observations lead to the conclusion that the density of antibody on the support matrix is a dominant factor in determining the specific activity of the immunosorbent. Nevertheless, the specific activity does not reach a value close to 100% even at very low antibody densities. This could be due to the steric hindrance imposed by the size of the antigen (*i.e.* IgG, M_r 160 000). The effect of the size of the antigen on the specific activity of immunosorbents is evident from the decrease in the specific activity from 85% for relatively smaller antigen (*i.e.* ovalbumin, M_r 43 000) to 37% for a larger antigen (*i.e.* Concanavalin A, M_r 102 000) [48].

TABLE III

EFFECT OF ANTIBODY DENSITY ON THE SPECIFIC ACTIVITY OF IMMOBILIZED OXIDIZED GOAT ANTI-HUMAN IgG

Oxidized goat anti-human IgG was coupled to hydrazide-activated HW 65 at pH 5.5 in 0.1 *M* acetate buffer. Specific activity was determined under equilibrium conditions with human IgG as the antigen. Experimental details are given in text.

Antibody density (mg/ml)	Volume of gel (ml)	Amount of antigen bound (mg)	Specific activity[a] (%)
3.97	0.88	0.81	23
2.68	0.88	0.61	26
0.75	0.88	0.31	47

[a] Defined in the text (average of three determinations); M_r of oxidized goat anti-human IgG and human IgG 160 000.

In order to illustrate the effect of steric hindrance imposed by the size of the antigen experiments were carried out with human F(ab') (M_r 60 000) as the antigen for immunosorbents prepared from goat anti-human F(ab'). As shown in Table IV the specific activity of the immunosorbents has increased from 39 to 100% when the density decreased from 4.54 to 1.14 mg/ml of gel. This clearly confirms that the site-directed immobilization of F(ab') does not result in loss of immunological activity (specific activity 100% for 1.14 mg/ml F(ab') density). The observed loss of specific activity at high densities is either due to steric hindrance caused by crowding of the F(ab') itself or to the size of the antigen.

A study of the effect of IgG density on specific activity of immunosorbents

TABLE IV

SPECIFIC ACTIVITY OF IMMOBILIZED GOAT ANTI-HUMAN F(ab') IMMUNOSORBENTS WITH A SMALLER ANTIGEN: EFFECT OF F(ab') DENSITY

Goat anti-human F(ab') coupled to iodoacetamide-activated HW 65 at pH 8.5 in 50 m*M* Tris-HCl buffer. Specific activity was determined under equilibrium conditions using human F(ab') as the antigen. Details are given in text.

Density of F(ab') (mg/ml of gel)	Volume of gel (ml)	Amount of antigen bound (mg)	Specific activity[a] (%)
4.54	0.55	1.26	39
3.43	0.55	1.10	45
1.42	0.55	0.87	86
1.14	0.55	0.82	100

[a] Defined in the text (average of three determinations); M_r of goat anti-human F(ab') 46 000 and human F(ab') 60 000.

TABLE V

EFFECT OF IgG DENSITY ON THE SPECIFIC ACTIVITY OF IMMOBILIZED GOAT ANTI-HUMAN IgG

Goat anti-human IgG was coupled to CDI-activated HW 65 at pH 6.0 in 0.1 *M* PB for 0.5 h at different antibody concentrations. Specific activity was determined under equilibrium conditions using human F(ab') as the antigen. Details are given in text.

Density of IgG (mg/ml of gel)	Volume of gel (ml)	Amount of antigen bound (mg)	Specific activity[a] (%)
11.18	0.60	1.43	21
5.26	0.60	0.66	21
3.50	0.60	0.39	19
1.70	0.60	0.19	18

[a] Defined in the text (average of three determinations); M_r of goat anti-human IgG 160 000 and human F(ab') 60 000.

prepared by random coupling of antibodies for 0.5 h has shown that the specific activity has not increased as expected when the density of IgG decreased (Table V, specific activity *ca.* 20% for all densities studied.The observed behavior can be explained by taking into account the effect of three factors that govern the specific activity: multi-site attachment, multiple orientation and steric hindrance. As the antibody density increases the effect of multi-site attachment and multiple orientation decreases, but steric hindrance increases. Conversely, the effect of multiple orientations and multi-site attachment is dominant at low antibody densities. Therefore, it is reasonable to assume that multiple orientation, multi-site attachment and steric hindrance due to crowding are the dominant factors in random immobilization. This is in agreement with the work of others [18,47].

From the present study it is possible to conclude that three factors are of importance for the preparation of immunosorbents with high quality. They are multi-site attachment, multiple orientations and steric hindrance as a result of antibody density and the size of the antigen. In the preparation of immunosorbents by random coupling of antibodies all three of these factors govern the specific activity of the resulting immunosorbent. Therefore, depending on the availability of reagents and the cost of the support matrix, care must be taken to achieve the highest specific activity by maneuvering the above three factors. In the site-directed immobilization the main factor that governs the specific activity is the crowding of the antibody. The multi-site attachment is either absent or less significant. Therefore, this method is less complicated and thus easier to optimize. Depending on the size of the antigen and the density of F(ab') one can achieve almost 100% specific activity by the site-directed immobilization of antibodies.

The support material upon which the antibody or antibody fragments have been immobilized is porous with a nominal pore size of 1000 Å. Most of the

activated groups will be on the interior of the support, a bead about 30–60 μm in diameter. According to the crystal structure of IgG [53,54] the largest dimension should be 240 Å and therefore it should not be possible for one or even two molecules to block access to the interior of the bead where binding sites would be abundant. However, the pores are not regular in size or shape so such blockage is definitely possible. Further studies are being carried out to establish whether such dramatic loading and antigen size effects would be observed on an essentially planar support.

It is often not necessary or desirable to achieve high capacity through high specific activity if the immunosorbent is used for affinity purification. If the capacity is too high, the antigen on elution will be too concentrated, will exceed its solubility and will precipitate in the immunoaffinity column. The present work, however, indicates clearly that by proper design of coupling reactions it is possible to achieve higher specific activity so that less antibody is required to prepare a column of a desired capacity. This may be important in cases where limited amounts of antibody are available. Finally it must be emphasized that caution must be exercised in generalizing the behavior of immobilized antibodies from just two examples. The present work indicates what is possible and suggests protocols for optimizing immobilized antibody performance.

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