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SYNTHESIS OF HIGH-CAPACITY IMMUNOAFFINITY SORBENTS WITH ORIENTED IMMOBILIZED IMMUNOGLOBULINS OR THEIR $F_{ab'}$ FRAGMENTS FOR ISOLATION OF PROTEINS

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SUMMARY

Two methods for synthesizing high-capacity immunoaffinity sorbents on Sepharose and Separon HEMA E-1000 are described. The first is the oriented immobilization of monovalent immunoglobulin $F_{ab'}$ fragments on a maleimide derivative of Sepharose via the formation of a covalent bond between the SH group of the $F_{ab'}$ fragment at the C-terminus of the molecule and the maleimide covalently coupled to Sepharose. The second method is based on the oxidation of the immunoglobulin carbohydrate component, located in the F_c fragment, by periodate with subsequent immobilization of the derivatives on hydrazide derivatives of Sepharose or Separon. Sorbents for the isolation of monoclonal antibodies from the culture supernatants and the elongation factor EF-G from a crude extract of *Escherichia coli* cells were obtained. These sorbents are characterized by a high capacity, minimal non-specific sorption and high stability.

INTRODUCTION

Immunoaffinity sorbents are widely used in biochemical and immunological studies for the isolation and identification of various antigens, including peptide

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and protein hormones, different receptors, immunoglobulins (for a review see, e.g., ref. 1) and individual mRNAs from translating polysomes [2, 3]. Detailed descriptions of the principles and practice of these methods have been given [4–6].

A Sepharose 4B–cyanogen bromide-activated derivative or other activated derivatives (for example, epoxy- or CH-activated derivatives) are usually used as supports to synthesize immunosorbents. In all instances the covalent coupling of the protein to a carrier proceeds through the interaction of its amino groups with the active groups of the carrier. Immunoaffinity sorbents have a low capacity in relation to the antigen, mainly owing to the partial inactivation of immunoglobulins during immobilization and to the shielding of the antigen-binding sites resulting from random immobilization of immunoglobulin (Ig) molecules on the insoluble support.

To eliminate these drawbacks, two approaches can be used. It is known that the main product of IgG hydrolysis by pepsin is $F_{(ab')_2}$ fragments [7, 8], retaining the antigen-binding capability. After reduction of the S–S bridge between monovalent $F_{ab'}$ fragments a thiol group is formed in the C-terminal region of the fragments, via which the monovalent $F_{ab'}$ fragments can be coupled with the insoluble carrier. This approach has been used to immobilize $F_{ab'}$ fragments on liposomes [9], small monolayer vesicles [10] and thiolated cellulose [11] through the formation of an S–S bridge between the $F_{ab'}$ fragments and the support. Although the coupling reaction is highly efficient, the sorbent is not stable under reducing conditions, which can be created even by thiol groups of the proteins present in the mixture to be separated. Stable sorbents can be achieved by using a maleimide group covalently cross-linked to an insoluble carrier interacting highly specifically with the protein thiol groups, forming a stable covalent bond. This approach has been used, in particular, for bonding the $F_{ab'}$ fragments to phospholipid derivatives of maleimide [12].

The other method of oriented immobilization consists in the following. It is known that almost all immunoglobulins contain carbohydrate residues bound to a constant region of their heavy chains. On mild chemical or enzymic oxidation, the carbohydrate residues generate aldehyde groups which can easily form stable bonds with hydrazide-containing reagents [13]. However, the presence of aldehyde groups in the protein can lead to intermolecular polypeptide chain cross-linking. This limitation can be eliminated by choosing appropriate conditions for the reaction.

In this paper we report conditions for the synthesis of immunosorbents based on Sepharose 4B and Separon HEMA E-1000 and on immunoglobulins G and also conditions for their application to the isolation of proteins.

EXPERIMENTAL

Reagents

Sodium acetate and sodium cyanoborohydride were purchased from Merck (Darmstdt, F.R.G.), sodium metaperiodate, adipic acid dihydrazide, cyanogen bromide, dithiothreitol and N-maleonyl-β-alanine from Fluka (Buchs, Switzerland), glycerol, sodium borohydride and Tween 20 from Sigma (St. Louis, MO,

U.S.A.), imidazole from Serva (Heidelberg, F.R.G.), and hydrazine hydrate, glycine, disodium phosphate, Tris, sodium tetraborate and boric acid from Soyuzkhimreaktiv (Moscow, U.S.S.R.). Sephadex G-25, G-150 and G-200, Sepharose 4B, protein A–Sepharose 4B and AH-Sepharose 4B were purchased from Pharmacia (Uppsala, Sweden), Separon HEMA E-1000 from Laboratory Instruments (Prague, Czechoslovakia) and PM-10 membranes were obtained from Amicon (Lexington, MA, U.S.A.).

Preparation of rabbit antibodies to mouse immunoglobulins

Rabbits were immunized by an electrophoretically homogeneous mouse IgG preparation prepared as described by Steinbuch and Audran [14], with additional purification by Sephadex G-200 gel chromatography. The mouse IgG preparations were immobilized on Separon HEMA E-1000 according to Taylor [15]. The rabbit antiserum to mouse immunoglobulins was diluted two-fold with 0.1 *M* sodium phosphate (pH 7.2)–0.5 *M* sodium chloride–0.05% Tween 20 and applied on a column (8×1 cm I.D., 7 mg IgG per ml support) with immobilized mouse IgG. The column was washed with ten volumes of 0.02 *M* phosphate buffer containing 0.5 *M* sodium chloride and 0.05% Tween 20, and the antibodies were eluted with 0.1 *M* glycine hydrochloride–0.2 *M* sodium chloride buffer (pH 2.2).

Oxidation of immunoglobulins by sodium metaperiodate [16]

A one tenth volume of 0.1 *M* sodium metaperiodate was added to the IgG solution (1–2 mg/ml) in 0.1 *M* sodium acetate buffer (pH 5.5), and the mixture was incubated for 20 min at 0°C. The reaction was stopped by addition of glycerol to a concentration of 0.015 *M*, and the mixture was additionally incubated for 5 min at 0°C. The reaction mixture was layered on a Sephadex G-25 column (30×1.6 cm I.D.) equilibrated with 0.1 *M* sodium acetate–0.5 *M* sodium chloride (pH 4.8), and the oxidized immunoglobulins were eluted with the same buffer.

Preparation of monovalent $F_{ab'}$ fragments of rabbit IgG

Rabbit antibiodies to mouse IgG in 0.1 *M* sodium acetate (pH 4.5) (2 mg/ml) were treated with pepsin (the enzyme/substrate ratio was 1:40) for 7 h at 37°C. Then the pH of the incubation mixture was raised to 7.5 by addition of 1 *M* Tris solution. The solution was centrifuged (27 000 *g*, 30 min) to remove aggregates and layered on a protein A–Sepharose 4B column to remove undigested IgG. The eluate was pooled and concentrated on a PM-10 membrane and applied to a Sephadex G-150 column (90×1.6 cm I.D.) equilibrated with 0.1 *M* sodium borate–0.2 *M* sodium chloride (pH 8). The $F_{(ab')_2}$ fragments eluted from the column were pooled and concentrated on a PM-10 membrane 5–10 mg/ml, transferred to a Sephadex G-25 (30×1.6 cm I.D.) column in 0.1 *M* sodium acetate–0.001 *M* EDTA buffer (pH 5.0) and reduced with dithiothreitol (0.02 *M*) for 1 h. The dithiothreitol was removed on a Sephadex G-25 column (30×1.6 cm I.D.) equilibrated with 0.1 *M* sodium acetate–0.5 *M* sodium chloride–0.001 *M* EDTA (pH 5.0) (buffer A).

Synthesis of amino, hydrazine and hydrazide derivatives of Separon HEMA E-1000

The synthesis was carried out according to a slightly modified version of the technique described in ref. 17.

Separon HEMA E-1000 (5 g) was incubated with 20 ml of concentrated ammonia solution for 1.5 h at 40°C. After completion of the reaction, the amino Separon was washed with 1 *M* sodium chloride solution and water.

Separon HEMA E-1000 (5 g) was incubated with 20 ml of hydrazine hydrate containing 2% sodium borohydride for 1.5 h at 40°C. After completion of the reaction, the hydrazino Separon was washed thoroughly with 1 *M* sodium chloride solution and water.

Separon HEMA E-1000 (5 g) was incubated with 20 ml of saturated adipic acid dihydrazide solution in 0.1 *M* sodium carbonate solution for 1.5 h at 40°C. After completion of the reaction, the hydrazido Separon was washed thoroughly with 1 *M* sodium chloride solution and water. The unreacted epoxy groups were inactivated by addition of one volume of 2% sodium borohydride solution [18].

Derivatives of Sepharose 4B

The hydrazide derivative of Sepharose 4B was obtained by treating cyanogen bromide-activated Sepharose 4B wiht adipic acid dihydrazide [19]. The maleimide derivative of Sepharose 4B was obtained as described in ref. 20. The sorbent contained 8 μmol of maleimide groups per ml of gel.

Immobilization of oxidized immunoglobulins on hydrazide derivatives of different supports

Oxidized IgG in 0.1 *M* sodium acetate–0.5 *M* sodium chloride buffer (pH 4.8) was incubated with the hydrazide derivative of the insoluble carrrier (5–10 mg IgG per ml of the carrier), preliminarily equilibrated with the same buffer, for 48 h at 4°C with stirring. The amount of unbound IgG was determined by absorption at 280 nm or by the method of Bradford [21]. The unbound IgG was washed off with two to three volumes of 0.1 *M* sodium acetate–0.5 *M* sodium chloride buffer (pH 4.8), then cyanoborohydride was added to a concentration of 0.1 *M* and the mixture was incubated for 24 h at 4°C with stirring [22]. After completion of the reaction, the gel was equilibrated with the initial buffer for chromatography.

Immobilization of $F_{ab'}$ fragments on the maleimide derivative of Sepharose 4B

$F_{ab'}$ fragments in buffer A (0.1 *M* sodium acetate–0.5 *M* sodium chloride–0.001 *M* EDTA, pH 5.0) were incubated and stirred for 24 h at 4°C with the maleimide derivative of Sepharose 4B, equilibrated with the same buffer (5–25 mg of $F_{ab'}$ fragments per ml of the carrier). After incubation the amount of the immobilized $F_{ab'}$ fragments was determined by measuring the change in the buffer absorption at 280 nm, at $A_{280}^{1\%} = 14.0$ for the $F_{ab'}$ fragments. The unbound $F_{ab'}$ fragments were washed off with buffer A and the sorbent was equilibrated with the starting buffer for chromatography. The unreacted maleimide groups were blocked using 0.01 *M* 2-mercaptoethanol solution.

Mouse immunoglobulins (IgG). These were labelled with ^{125}I using iodo-gene (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril) according to ref. 23.

Crude extract of Escherichia coli. This was prepared as described elsewhere [24].

Monoclonal antibodies to elongation factor EF-G. These were prepaped according to Kohler and Milstein [25].

Clone 37G2, not adapted to growth in ascite tumour, was cultivated in a Dulbecco's modified eagle medium (DMEM) containing 20% foetal calf serum. The cultivating media were clarified by centrifugation (800 *g*, 10 min) and the protein was precipitated with ammonium sulphate (50% saturation). The precipitate was collected by centrifugation (27 000 *g*, 30 min), dissolved in water (one tenth of the initial volume) and the solution was dialysed against 100 volumes of 0.02 *M* sodium phosphate–0.5 *M* sodium chloride buffer (pH 7.2). This solution was applied to a $F_{ab'}$-maleimide Sepharose 4B column (4×1 cm I.D.) equilibrated with the same buffer. The column was washed sequentially with five volumes of 0.02 *M* sodium phosphate buffer (pH 7.2) containing 0.5, 1.5 and 0.15 *M* sodium chloride. Monoclonal antibodies were eluted with 0.1 *M* glycine hydrochloride–0.2 *M* sodium chloride buffer (pH 2.2). Immediately after elution the eluate was neutralized with 2 *M* Tris–HCl buffer (pH 8.6) and dialysed against 0.02 *M* sodium phosphate–0.15 *M* sodium chloride buffer (pH 7.2).

Kinetics of binding oxidized immunoglobulins to amino, hydrazine and hydrazide derivatives of insoluble carriers

A 0.5-ml volume of a corresponding carrier in 0.1 *M* sodium acetate–0.5 *M* sodium chloride buffer (pH 4.8) was incubated with 2 ml of the oxidized IgG solution (0.96 mg/ml) in the same buffer. To determine the protein concentration, 0.1-ml aliquots were collected at definite intervals.

Determination of immunosorbent capacity

A 0.1-ml volume of the immunoaffinity sorbent was incubated with 0.3 ml of mouse IgG solution (^{125}I-labelled, specific activity 185 cpm/μg, concentration 2.66 mg/ml) in 0.02 *M* sodium phosphate–0.5 *M* sodium chloride buffer (pH 7.2) for 1 h at 20°C. After incubation the sorbent was washed three times with 0.5-ml portions of 0.02 *M* sodium phosphate–0.15 *M* sodium chloride buffer (pH 7.2) and the coupled protein was eluted with three 0.5-ml volumes of 0.1 *M* glycine·hydrochloride–0.2 *M* sodium chloride buffer (pH 2.2). The amount of the protein in the eluate was determined by counting the radioactivity.

Isolation of elongation factor EF-G from the crude extract of E. coli. This was done on the immunoaffinity sorbent prepared by immobilization of oxidized periodate of monoclonal IgG 37G2 on the hydrazide derivative of Sepharose 4B as described above, with the exception that all the buffers contained 0.005 *M* 2-mercaptoethanol.

RESULTS

Attachment of $F_{ab'}$ fragments to the maleimide derivative of Sepharose 4B

The maleimide derivative of Sepharose was prepared by the interaction of N-maleonyl-β-alanine with AH-Sepharose 4B in the presence of N-ethoxycarbonyl-

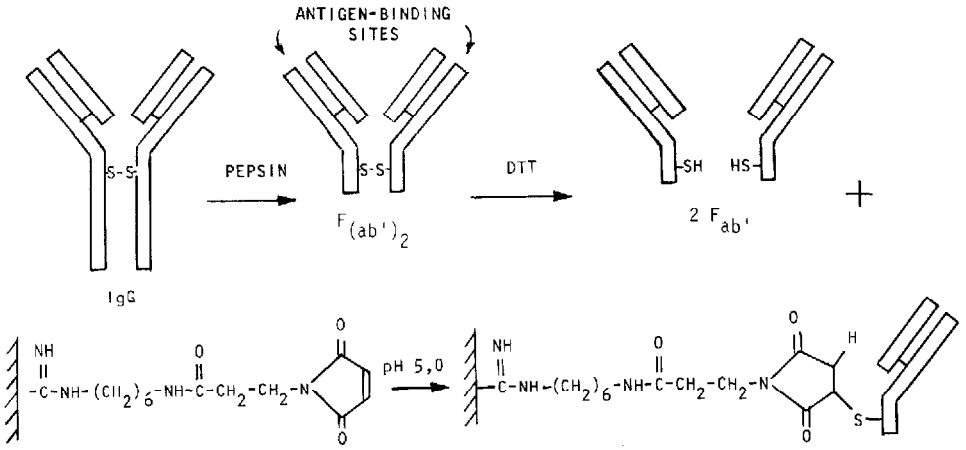

Fig. 1. Scheme of the preparation of immunoaffinity sorbents based on $F_{ab'}$ fragments and the maleimide derivative of Sepharose 4B. DTT = dithiothreitol.

2-ethoxy-1,2-dihydroquinoline (EEDQ). The reaction proceeded in anhydrous ethanol, using a five fold excess of N-maleonyl-β-alanine over the amino groups in the support, until the positive reaction to amino groups disappeared (interaction with trinitrobenzenesulphonic acid). As a result, the sorbent obtained contained 8 μmol of maleimide groups per ml of gel, as shown by titration of an added excess of 2-mercaptoethanol with Ellman's reagent.

The immunoaffinity sorbent was obtained according to the scheme presented in Fig. 1. Monovalent $F_{ab'}$ fragments, prepared as described above, contained 0.85–0.9 SH groups per molecule and, immediately after removal of dithiothreitol, were applied to the reaction with the maleimide derivative of Sepharose in buffer A (pH 5.0) in argon for 24 h at 4°C. After the reaction and thorough washing, the sorbent obtained contained 1.7 mg of $F_{ab'}$ fragments per ml gel (according to amino acid analysis).

The thioether bond formed between the $F_{ab'}$ fragments and the support proved to be fairly stable. After incubation of the sorbent with dithiothreitol (5.0 m*M*, pH 7.2, 24 h), no cleavage of $F_{ab'}$ fragments was observed. To determine the capacity of the sorbents obtained and the stability of the adsorption properties, a series of experiments on the sorption and desorption of antigen (mouse IgG) were carried out. The results are given in Table I. The capacity of the sorbent was on average 0.55 mol of antigen per mole of coupled $F_{ab'}$ fragments, the sorbent capacity remaining almost the same during a five-fold sorption–desorption.

The sorbent obtained was used for isolation of monoclonal IgG from the culture supernatant. The results of chromatography and SDS polyacrylamide gel electrophoresis analysis are presented in Figs. 2 and 3, respectively.

Attachment of oxidized immunoglobulins to hydrazide derivatives of insoluble carriers

To synthesize an immunoaffinity sorbent we chose several gels with the structures shown in Fig. 4. Amino Separon was selected as a negative control to dem-

TABLE I

SORPTION PROPERTIES OF IMMUNOAFFINITY SORBENTS

Experiment No.	Eluted antigen (mouse IgG)					
	A*		B**		C***	
	Amount (μg)	Capacity (mol/mol)	Amount (μg)	Capacity (mol/mol)	Amount (μg)	Capacity (mol/mol)
1	200 ± 20	0.51	172 ± 20	0.68	329 ± 25	1.98
2	290 ± 5	0.57	290 ± 4	1.14	220 ± 20	1.32
3	285 ± 7	0.56	255 ± 5	1.00	250 ± 12	1.51
4	280 ± 5	0.55	270 ± 5	1.06	270 ± 5	1.63
5	273 ± 8	0.54	265 ± 5	1.04	260 ± 7	1.57

*(A) Maleimide derivative of Sepharose; 100 μl of gel contain 170 μg of rabbit anti-mouse $F_{ab'}$ fragments.
**(B) Hydrazide derivative of Separon; 100 μl of gel contain 254 μg of rabbit anti-mouse IgG.
***(C) Hydrazide derivative of Sepharose; 100 μl of gel contain 166 μg of rabbit anti-mouse IgG.

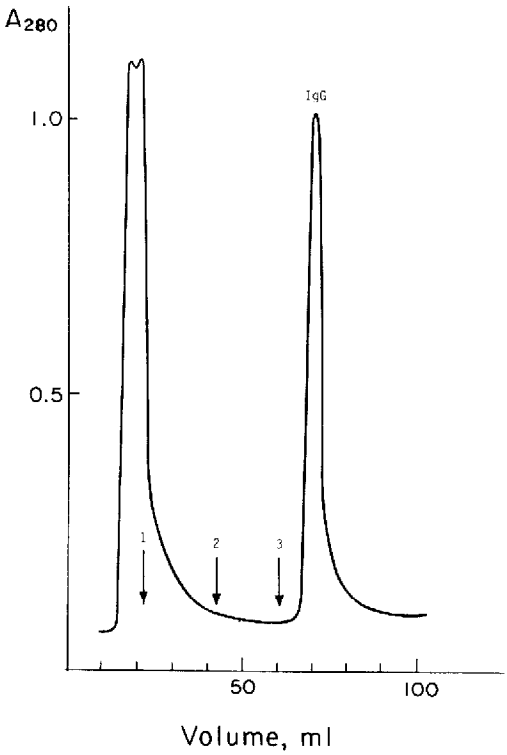

Fig. 2. Isolation of monoclonal IgG on an affinity sorbent with immobilized $F_{ab'}$ fragments. Column, 3.5 × 1 cm I.D., equilibrated with 0.02 *M* sodium phosphate–0.5 *M* sodium chloride buffer (pH 7.2); elution rate, 15 ml/h. Arrows show the change of elution buffers: buffer containing (1) 1.5 *M* sodium chloride, (2) 0.15 *M* sodium chloride, (3) 0.1 *M* glycine hydrochloride–0.2 *M* sodium chloride (pH 2.2).

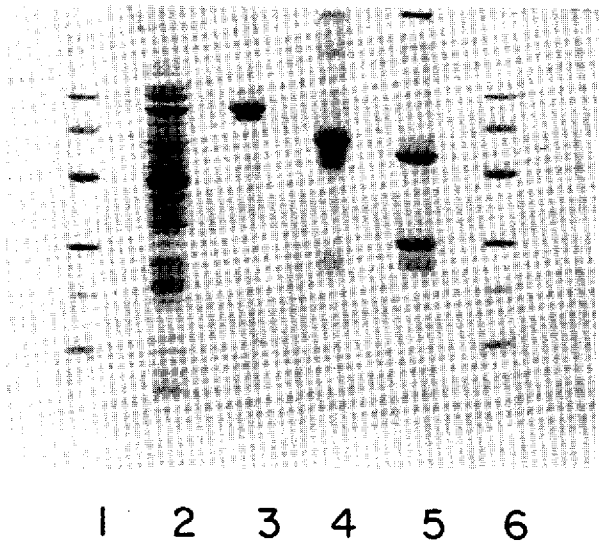

Fig. 3. Results of electrophoresis in polyacrylamide gel in the presence of SDS. Lanes 1 and 6, mixture of standard proteins (phosphorylase b, MW 97 500; bovine serum albumin, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; soybean trypsin inhibitor, 20 100; α-lactalbumin, 14 400); lane 2, crude extract of *E. coli* cells; lane 3, elongation factor EF-G eluted from the immunoaffinity column; lane 4, culture medium; lane 5, monoclonal IgG eluted from the immunoaffinity column.

onstrate that the coupling proceeds specifically via the formation of hydrazones. It is known that aldehyde groups, especially at alkaline pH, interact readily with amino groups, forming Schiff bases. This process leads, in particular, to intermolecular cross-links of polypeptide chains accompanied by the formation of protein oligomers. However, in the process of attachment to the insoluble support in

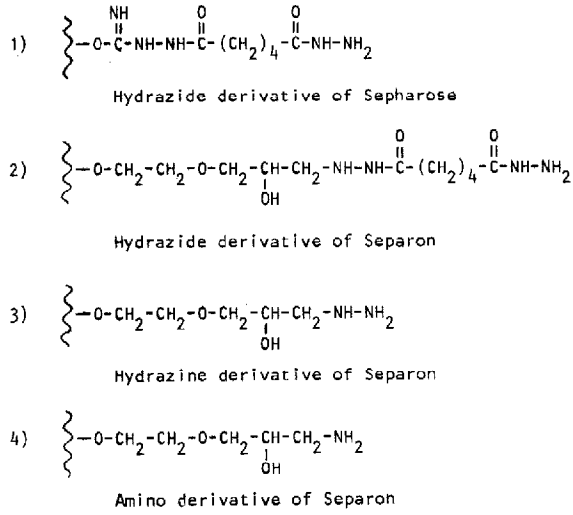

Fig. 4. Structure of functional groups in insoluble supports used for attachment of oxidized immunoglobulins.

TABLE II

KINETICS OF BINDING OF OXIDIZED SODIUM METAPERIODATE OF RABBIT IMMUNOGLOBULIN BY AMINO, HYDRAZINE AND HYDRAZIDE SORBENTS

Incubation time (min)	Amino Separon		Hydrazine Separon		Hydrazide Separon	
	mg protein per ml sorbent	Bound IgG (%)	mg protein per ml sorbent	Bound IgG (%)	mg protein per ml sorbent	Bound IgG (%)
15	0.1	2.6	0.56	14.5	0.16	4.1
45	0.26	6.7	1.36	35.2	0.66	17.1
120	0.46	11.9	2.00	51.8	1.26	32.6
360	0.56	14.5	2.16	56.0	1.46	37.8
30 h	0.6	15.5	2.76	71.5	2.76	71.5

acidic conditions (pH 4.8), this side-reaction is slower and the formation of hydrazone proceeds with a high yield. The kinetics of the attachment of an oxidized immunoglobulin are given in Table II. With amino Separon, approximately 15% of oxidized IgG is coupled during 30 h. With hydrazide and hydrazine Separons, the yield of coupled IgG during the same period exceeds 70%. After incubation of monoclonal antibody 37G2 with the hydrazide derivative of Sepharose for 60 h the binding proceeds almost quantitatively (data not shown).

To study the sorption parameters of the obtained affinity sorbents with the coupled rabbit antibodies to mouse immunoglobulins, 100-μl aliquots of the sorbents were subjected to a series of sorption–desorption cycles. The results are summarized in Table I. An immunoaffinity sorbent based on the hydrazide derivative of Separon binds about 1 mol of antigen per mole of an immobilized antibody, and a sorbent based on the hydrazide derivative of Sepharose binds, on average, 1.6 mol/mol.

Isolation of elongation factor EF-G

To isolate the elongation factor EF-G, an immunosorbent was obtained according to the technique described above for the immobilization of rabbit anti-mouse IgG on a hydrazide derivative of Sepharose. *E. coli* cells were disrupted by grinding with aluminium oxide. After centrifugation, ammonium sulphate was added to the supernatant to 50% saturation. The pellet was centrifuged and discarded and the ammonium sulphate concentration in the supernatant was increased to 70%. The precipitate was separated by centrifugation, dissolved in the starting buffer for chromatography and the solution obtained was applied to a column with immobilized monoclonal antibodies 37G2. After washing the column, the sorbed protein was eluted with glycine buffer (pH 2.2). The fractions were immediately neutralized to pH 7.5 with 1 *M* Tris–HCl. By this procedure 5.6 mg of homogeneous EF-G were separated from 10 g of cells. The results of chromatography and electrophoresis of the obtained protein in polyacrylamide gel in the presence of SDS are given in Figs. 5 and 3, respectively. The electrophoresis results show that the isolated protein is mixed with a smaller-molecular-weight component, an EF-G fragment, formed as a result of partial proteinolysis

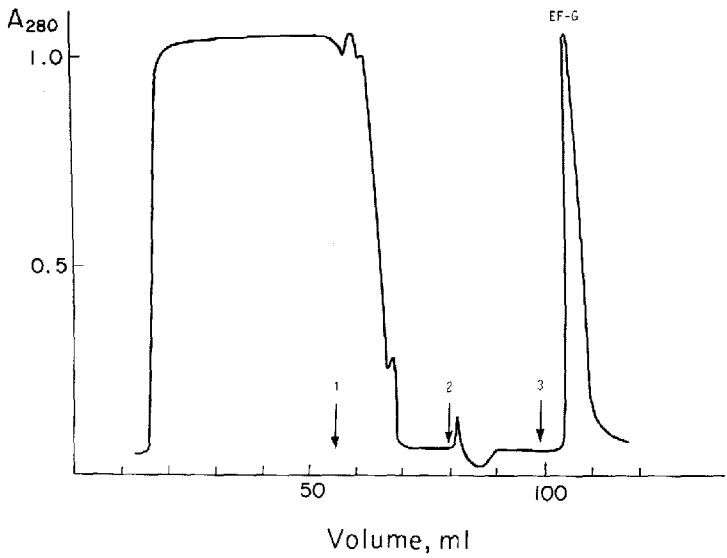

Fig. 5. Isolation of elongation factor EF-G from crude extract of *E. coli* cells on the sorbent with immobilized oxidized IgG on the hydrazide derivative of Sepharose. Column, 3.5×1 cm I.D., equilibrated with 0.02 *M* sodium phosphate–0.5 *M* sodium chloride–0.005 *M* 2-mercaptoethanol buffer (pH 7.2); elution rate, 15 ml/h. Arrows show the change of elution buffers: buffer containing (1) 1.5 *M* sodium chloride, (2) 0.15 *M* sodium chloride, (3) 0.1 *M* glycine hydrochloride–0.2 *M* sodium chloride–0.005 *M* 2-mercaptoethanol (pH 2.2).

during separation. The fragment retains all the immunological properties of the intact protein. A description of EF-G fragment formation is given in ref. 24.

DISCUSSION

Monoclonal antibodies, now obtainable in large amounts, have opened up new possibilities for their wide application in various fields, including the development of efficient methods for isolating proteins and other antigens that are not routinely available. We have developed a method of coupling immunoglobulins or their $F_{ab'}$ fragments ensuring high specificity and leading to the formation of a stable linkage between the antibody and the insoluble carrier. Here the immobilized molecules are coupled in a strictly oriented manner, providing accessibility of the antigen-binding sites without their inactivation. The presence of a long "spacer" between the functional groups, interacting chemically with the immobilized protein and the insoluble carrier, significantly decreases steric restrictions and maintains the high efficiency of antibody coupling. The maleimide and hydrazide groups retain their high specific reactivity in relation to immobilized proteins in aqueous solutions at pH 4.5–6.5.

A comparison of the sorption properties of sorbents with immobilized IgG and $F_{ab'}$ fragments shows that they are similar to each other in both capacity and stability. However, the method of immobilization of intact IgG is simpler, as the prolonged stage of obtaining $F_{ab'}$ fragments is eliminated. Nevertheless, in a num-

ber of instances it is more advantageous to use $F_{ab'}$ fragments for the preparation of immunosorbents. For example, if an antigen is isolated from mixtures containing components that interact with the F_c part of the immunoglobulin, this leads to a significant decrease in the specificity of the sorbent. If IgM immunoglobulins are available, it is also more expedient to immobilize their $F_{ab\mu}$ fragments, as an IgM molecule is too large and it is impossible to orient it on the support. $F_{ab\mu}$ fragments have a carbohydrate residue in the C-terminal part which, after oxidation with periodate, permits effective immobilization.

The high efficiency of the sorbents obtained is demonstrated with the example of the isolation of elongation factor EF-G from a very complicated mixture, such as a crude cell extract.

In conclusion, the suggested methods of preparing highly efficient immunosorbents drastically simplify and accelerate the process of the isolation of homogeneous preparations of many proteins not routinely obtainable at present.

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