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Evaluation of the mode of binding of immunoglobulin to activated agarose

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ABSTRACT

Determining the orientation of the immobilization of proteins to solid-phase matrices is of critical importance in the development of systems that employ immobilized proteins. Among these are enzyme-linked immunoassays, immobilized enzymes and affinity chromatography matrices.

To determine the orientation of immunoglobulin G (IgG) on activated agaraoses, we coupled the immunoglobulin covalently to various activated matrices. The IgG was then cleaved with papain and the liberated fragments collected and analyzed using high-performance liquid chromatography. Only F_{ab} fragments could be detected regardless of the activation method used. This implies that IgG binds to these matrices predominantly via the F_c domain.

In order to develop a quantitative method of measuring the F_{ab} and F_c fragments, we compared the binding of IgG and its papain cleavage fragments to S-Zephyr columns and Mono-S columns. Comparison between these columns showed that IgG is bound more tightly to the S-Zephyr column and, in contrast, its retention on Q-Zephyr is less than on a comparable Mono-Q column. The resolution of IgG and its fragments was better in all cases on S-Zephyr than on Mono-S under the conditions employed.

INTRODUCTION

A variety of activated matrices have been developed for the coupling of proteins and other biologically active molecules to agarose. The most widely used and first developed matrix for protein immobilization was cyanogen bromide-activated agarose [1]. Unfortunately, cyanogen bromide is toxic, *per se*, it produces very volatile toxic gases during the activation procedure and is generally difficult to use despite several recent improvements in the method of activation, *e.g.* the use of triethylamine as the base. Activation with organic sulfonyl chlorides, such as *p*-toluenesulfonyl chloride (tosyl chloride) permit high yield of coupled products and very stable linkage between the immobilized protein and the support. The introduction of the tosyl etser can also be conveniently followed by UV spectroscopy, since the ester has a molar extinction coefficient at 261 nm of 480 M^{-1} cm^{-1} [2].

Other coupling methods employed commonly for protein immobilization include carbonyldiimidazole, epoxide, and fluoromethylpyridine activation methods. These are only a few of the many potential methods that can be employed, but are among the most often utilized. Carbonyldiimidazole produces an activated matrix which is relatively stable to hydrolysis but readily reacts with necleophilic residues on protein molecules [3]. Epoxide-activated agarose similarly provides a very stable linkage between the protein and the matrix, but in addition, the long chain bisoxirane provides an intrinsic spacer arm which separates the protein from the matrix. The resulting separation of the matrix and protein often results in higher activity of the immobilized biomolecule than that which is directly attached to the matrix [4].

Finally, one of the more recently developed reagents for activating agarose is 2-fluoro-1-methylpyridinium toluene-4-sulfonate (FMP). Fluoromethylpyridinium derivatives have been used to activate hydroxyl groups and, at the same time, provide ligands which guide the protein molecule to a particular orientation on the matrix [5]. Fluoro-

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methylpyridene also produces, as do tosyl esters and epoxides, stable secondary amine linkages between the matrix and the protein nucleophile.

Coupling of proteins to activated matrices, in each of the above methods, occurs through the available nucleophilic groups of the protein. It would be most advantageous to couple immunoglobulin G (IgG) to the matrix through a nucleophilic group on its F_c portion (see Fig. 1), thus permitting optimal availability of both antigen binding sites.

We have investigated IgG binding to Zephyr and Monobead-based anion and cation exchange supports and employed these supports in high-performance liquid chromatography (HPLC) for the rapid analysis of IgG and its proteolytic fragments. The S-Zephyr column was used to determine which portion (F_{ab} or F_c or both) of the IgG molecule was bound to five different 4% cross-linked activated agarose gels: cyanogen bromide, tosyl chloride, epoxide, FMP, and carbonyldiimidazole.

In developing the method utilized for separation of IgG and its various fragments, we examined several strong ion exchange resins that have been developed for HPLC purposes. The newest products in this area are the Zephyr materials produced by IBF-Biotechnics. These materials, in contrast to the well known Monobead-HPLC matrices, are available in bluk quantities, which should permit their facile use in large scale preparative applications, as well as analytical ones.

We have shown in previous work that S-Zephyr is a high-performance cation-exchange chromatography matrix with a load cxapacity for protein molecules twice that of Mono-S [6]. In terms of resolution, the performance of S-Zephyr and Mono-S columns are comparable under various conditions.

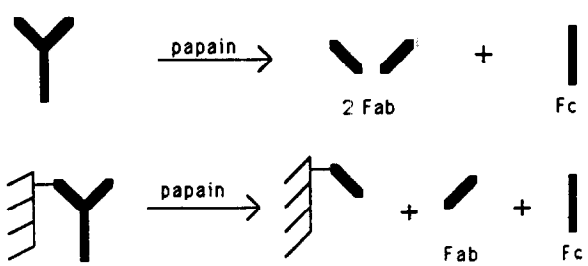

Fig. 1. Schematic depiction of immobilization and cleavage of an IgG molecule.

Even under high column loading, S-Zephyr is able to separate proteins in complex mixtures. This new column material will fill a nice in the range of available high-performance cation-exchange columns.

Anion-exchange chromatography has been used for many years in the purification of antibodies. However, conventional gravity-flow ion-exchange chromatography often requires hours to perform and can be limited by poor protein recovery. Purification of antibodies, however, can be done rapidly and efficiently using HPLC [7].

EXPERIMENTAL

Chemicals, materials and equipment

Cyanogen bromide, tosyl chloride, 4% cross-linked agarose activated to bind 4 mg and 10–12 mg of protein per ml gel, respectively, were obtained from Sigma (St. Louis, MO, USA). Likewise, epoxide and carbodiinidazole (CDI) activated 4% cross-linked agarose gels, activated at 30 μmol/ml, and 50 μmol/ml, respectively; goat IgG, crystalline papain, Tris and sodium acetate were also obtained from Sigma. FMP-activated agarose gel (4% cross-linked) (approx. 250 μmol/ml) was a gift of Bioprobe International (Tustin, CA, USA). Sodium azide was from Aldrich (Milwaukee, WI, USA) and immobilized papain and protein-G were from Pierce (Rockford, IL, USA). The Mono-S and Mono-Q HR 5/5 columns were purchased from Pharmacia. The 1-ml Q-Zephyr column and 1 ml S-Zephyr column were a gift from IBF-Biotechnics.

All water used was demineralized, glass-distilled and filtered through 0.2-μm nylon 66 filters (Schleicher and Schuell). Uniflo 0.2-μm syringe filter units were also from Schleicher and Schuell. Gradients were performed with an Isco 2360 analytical gradient programmer. The HPLC pump was a Waters 6000 HPLC pump. Absorption was measured with a Waters 450 UV variable-wavelength detector. Data collection from this spectrophotometer to a Tandy TRS 80 64K computer occurred via an A-bus and a Tandy multi-user interface.

Buffer solutions

The HPLC buffers that were used were 50 m*M* acetate (pH 4.0 and 5.0), 50 m*M* Tris (pH 8.0 and 9.0). Buffer solutions were adjusted with sodium hydroxide or hydrochloric acid until the desired pH

was reached. All buffers contained 0.2 g sodium azide per 1 buffer as a bacteriostat. High-ionic-strength buffers were prepared by dissolving sodium chloride in the standard buffers before adjusting the pH. All buffers were filtered through 0.2-μm nylon 66 filters.

Coupling procedures

Goat IgG was directly coupled to the activated matrices in a 0.2 *M* sodium bicarbonate buffer, pH 9.5, containing 0.5 *M* sodium chloride (coupling buffer). IgG was coupled to the FMP-activated matrix in the same buffer without added sodium chloride.

Typically, 3 ml of pre-swollen gel, washed with coupling buffer, was incubated with 5 mg IgG in the same buffer. During incubation the gel suspension was gently rotated on a Cole Parmer Roto Roque Model 7637 rotator for 48 h at room temperature (the CDI-activated gel was incubated and rotated at 4°C). Unbound or non-covalently bound IgG was removed from the matrices by washing with 5 column volumes each of 2 *M* sodium chloride and glass-distilled water until all unbound protein was eluted. Absorbance at 280 nm was measured for all fractions collected from the washing procedure and the amount of IgG bound was determined by difference. No free IgG was eluted in the final wash as shown by A_{280}. However, for FMP-activated agarose, the release of methylpyridone could be readily followed by measuring the absorbance at 297 nm (ε_{297}= 5900 M^{-1} cm^{-1}) of the elutate, after protein removal by ultrafiltration, and for the tosyl chloride-activated agarose at 261 nm (ε_{261}=480 $M^{-1}cm^{-1}$).

The agaroses were filtered and incubated with 4 ml 0.2 *M* Tris–HCl buffer, pH 9.5, for 2 days at room temperature. (The CDI-activated matrix was again incubated at 4°C). During incubation, the gel suspension was gently rotated. The gels were filtered and the immobilized IgG was immediately cleaved with papain.

Cleavage procedure for free IgG

Lyophilized IgG was cleaved with immobilized papain. Typically, 10 mg of IgG was dissolved in 1 ml digestion buffer (20 m*M* NaH_2PO_4, 20 m*M* cysteine–HCl, 10 m*M* EDTA pH 7.0). The Pierce immobilized papain slurry was mixed by gently shaking and 0.5 ml of the 50% immobilized papain slurry was added to a glass test tube. The slurry was washed twice by adding 4 ml of digestion buffer to the slurry and separating the gel from the buffer by centrifugation. The immobilized papain was then resuspended in 0.5 ml digestion buffer. The IgG solution was added to the tube and this mixture was incubated for 5 hours, or overnight, as indicated, in an incubator under continuous shaking (the mixture must remain homogeneous). After incubation, 1.5 ml of 10 m*M* Tris–HCl, pH 7.5, buffer was added and the fragments were separated from the immobilized papain by centrifugation.

The fragments were separated using a 2-ml protein-G column previously equilibrated with 5 gel volumes of binding buffer (50 m*M* acetate buffer pH 5.0). The sample containing the fragments was applied on the column. The non-bonding F_{ab} fragments were collected by washing the column with binding buffer (10 ml). The absorbance at 280 nm of every 2-ml fraction run through was measured on a Perkin-Elmer double-beam spectrophotometer. The F_c fragments and uncleaved IgG were eluted from the column using 50 m*M* glycine, pH 2.8. To neutralize the fractions collected, 0.5 ml of 0.1 *M* Tris–HCl pH 9, was added to each collection tube before the elution.

Cleavage procedure for immobilized IgG

The gels with immobilized IgG were washed with 5 column volumes of cleavage buffer consisting of 20 m*M* NaH_2PO_4, 50 m*M* sodium chloride, 10 m*M* cysteine, pH 7.2. This buffer was made just prior to the cleavage reaction to prevent oxidation of cysteine.

Crystalline papain was added to 3 ml cleavage buffer at a papain:IgG ratio of 1:100. This mixture was added to the filtered IgG-activated gels and incubated at 37°C for 4 h. During the incubation the gels were gently rotated.

The gels were filtered and the filtrates, which contained the liberated fragments, were collected. The gels were then washed with 3ml 50 m*M* acetate buffer, and the wash solution was added to the collected fragments. The solution containing the liberated fragments was then concentrated on an Centricon YM-10 filter in a IEC-Model HN-SII centrifuge (6000 *g*). The concentrated fragments were diluted in 50 m*M* acetate buffer pH 5.0 and chromato-

graphed on S-Zephyr as described below. When necessary, gel chromatography could be used to separate IgG fragments from whole IgG.

HPLC procedures

To determine the affinity of IgG to the HPLC chromatography columns, IgG was applied to a 1-ml column containing either S-Zephyr, Q-Zephyr, Mono-Q or Mono-S supports. A protein sample of 1 ml IgG/ml in the desired buffer was injected. All the columns were run at 1 ml/min. A 0.35-ml sample volume was injected at the beginning of the gradient. Usually the gradient consisted of 0–5 min of the desired buffer followed by 5–25 min of a linear (0–0.5 *M*) sodium chloride gradient in the same buffer. However, when a pH 4.0 buffer was used on S-Zephyr or Mono-S supports, the gradient consisted of 0–5 min of the desired buffer followed by 5–25 min linear (0–1.0 *M*) sodium chloride gradient in the same buffer. Protein elution was followed by the absorbance of the column effluent at 280 nm.

To determine the resolution of IgG and its proteolytic fragments, IgG fragments were created and separated as described in the cleavage procedure. The fragments were applied to S-Zephyr and Mono-S columns. The columns were run at 1ml/min and the same gradient was used as in the determination of IgG binding to the column materials. Protein elution was followed by the absorbance of the column effluent at 280 nm.

The liberated fragments were applied a S-Zephyr column, pH 5.0, to determine which portion, F_{ab} or F_c, IgG coupled to the activated agaroses. The column was eluted at 1ml/min and the gradient used was the same as that employed for chromatography of IgG. Protein elution was followed by the absorbance of the column effluent at 280 nm.

RESULTS AND DISCUSSION

Affinity of goat IgG for various HPLC chromatography martrices

The affinity of IgG to various HPLC ion-exchange supports was determined (Table I) and the affinity of IgG for cation exchangers was found to be higher than that for anion exchangers. In general, monoclonal antibodies have been found to bind weakly to cation exchangers and moderately to anion exchangers [8].

IgG was bound more tightly to S-Zephyr than to Mono-A Zephyr supports. This preferential binding of IgG on S-Zephyr can be explained be the fact that the charged group on Mono-S (sulfo-ethyl attached to a fairly rigid poly-divinylbenzene-styrene matrix) are less accessible than the ionic groups on S-Zephyr (sulphopropyl attached to cross-linked dextran-coated silica support). The sulphopropyl groups attached to dextran spacer arms may surround the protein molecule and the interaction of ionic exchange groups on all sides of the molecule (or in cavities on the molecule) is possible (see Fig. 2) [9].

The affinity of IgG to Q-Zephyr ion-exchange chromatography was found to be lower than for Mono-Q, which may be due to the fact that Mono-Q has the potential for multimodal interactions (both ion exchange and hydrophobic) due to its composition of hydrophobic polyvenyl benzene and its hydrophylic quatinary amine residues. Hydrophobic interactions, which are emphasized at high concentrations, tend to retain many protein molecules in fashion similar to that seen for Mono-Q under certain circumstances.

TABLE I

RETENTION TIME OF GOAT IgG ON HPLC COLUMNS AT VARIOUS pH VALUES

The HPLC buffers used were 50 m*M* acetate and 50 m*M* Tris. Usually, columns were run at 1ml/min and the elution gradient consisted of 0–5 min of desired buffer followed by 5–25 min linear (0–0.5 *M*) sodium chloride gradient in the same buffer. However, when a pH 4.0 buffer was used, the gradient consisted of 0–5 min of described buffer followed by 5–25 min linear (0–1.0 *M*) sodium chloride gradient in the same buffer.

Column	pH	Retention time (min)
S-Zephyr	4	18.0
	5	14.5
Mono-S	4	13.0
	5	9.3
Q-Zephyr	8	8.6
	9	9.5
Mono-Q	8	12.5
	9	13.6

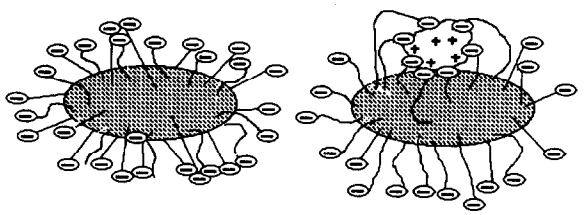

Fig. 2. Schematic depiction of protein binding to S-Zephyr support. (left) Zephyr particle without protein, showing free anionic "arms"; (right) protein on Zephyr particle surrounded by negatively charged "arms".

Chromatography of F_{ab} and F_c fragments

F_{ab} and F_c fragments were prepared by papain cleavage of IgG. The separated fragments were applied to S-Zephyr and Mono-S columns at various pH values (see Table II). The F_c fragment was held more tightly to S-Zephyr than to Mono-S, as seen by the various retention times, whereas F_{ab} fragments are not retained and appeared in the void volume of both columns. F_{ab} fragments have a low affinity for cation exchangers [10]. Our results suggest that the surface of F_{ab} fragments, at least for our polyclonal IgG system, may contain very few nucleophilic residues capable of coupling to the activated matrices.

Although the F_c fragments are retained longer on S-Zephyr than on Mono-S, the F_{ab} and F_c fragments on both columns are well separated. However, separation between the fragments and IgG is also important. A mixture containing IgG and both types of fragments was applied to S-Zephyr and Mono-S columns, and the resolution of IgG and its fragments was optimized (see Table III). Conversely, we were never able to obtain satisfactory separation of F_{ab}, F_c and whole IgG for either Q-Zephyr or Mono-Q matrices.

Separation of F_{ab} fragments from IgG, as well as of F_{ab} fragments from F_c fragments, is possible on both S-Zephyr and Mono-S columns, although resolution of IgG from F_c is considerably better on S-Zephyr supports than on Mono-S supports. As can be seen in Fig. 3, separation of IgG and F_c was poor on Mono-S under the conditions used, whereas the separation of IgG and its fragments on S-Zephyr was satisfactory under similar conditions.

As can also be seen in Fig. 3, the width of the peak tangents at the baseline are relatively high for both columns. To decrease the width of the peaks the chromatography was repeated using 50 m*M* acetate–acetonitrile buffer (90:10, v/v) (see Fig. 4). The width of the peak tangents for were determined for S-Zephyr (see Table IV). Addition of the 10% acetonitrile to the HPLC buffer resulted in a considerable decrease of the F_c peak width on S-Zephyr and the separation was not altered. On Mono-S, however, the separation performance decreased and the IgG and the F_c fragment could not be separated in the presence of the acetonitrile.

This shows that S-Zephyr is a very satisfactory matrix for the separation and quantification of IgG and its fragments.

TABLE II

RETENTION TIME OF F_{ab} AND F_c FRAGMENTS

F_c and F_{ab} fragments were applied on S-Zephyr and Mono-S. The HPLC buffers that were used were 50 m*M* acetate at the pH indicated. Usually columns were run at 1 ml/min, and the elution gradient consisted of 0–5 min of desired buffer followed by 5–25 min linear (0–0.5 *M*) sodium chloride gradient in the same buffer. However, when a pH 4.0 buffer was used, the gradient consisted of 0–5 min of desired buffer floowed by 5–25 min linear (0–0.5 *M*) sodium chloride gradient in the same buffer.

Column	pH	Retention time (min)	
		F_c fragments	F_{ab} fragments
S-Zephyr	4	13.0	1.6
support	5	11.2	1.8
Mono-S	4	10.9	1.7
support	5	8.4	1.5

TABLE III

RESOLUTION OF WHOLE IgG, F_{ab} AND F_c

A mixture of IgG and its fragments was applied to S-Zephyr and Mono-S, pH 5.0. The elution gradient consisted of 0–5 min of 50 m*M* acetate buffer followed by 5–25 min linear (0.05 *M*) sodium chloride gradient in the same buffer. Resolution is presented here as the ratio of retention times (t_r) for each of the proteins given.

Column	Mono-S	S-Zephyr
$IgG(t_{r1})/F_c(t_{r2})$	0.44	1.07
$IgG(t_{r1})/F_{ab}(t_{r2})$	2.09	4.80
$F_c(t_{r1})/F_{ab}(t_{r2})$	3.45	2.40

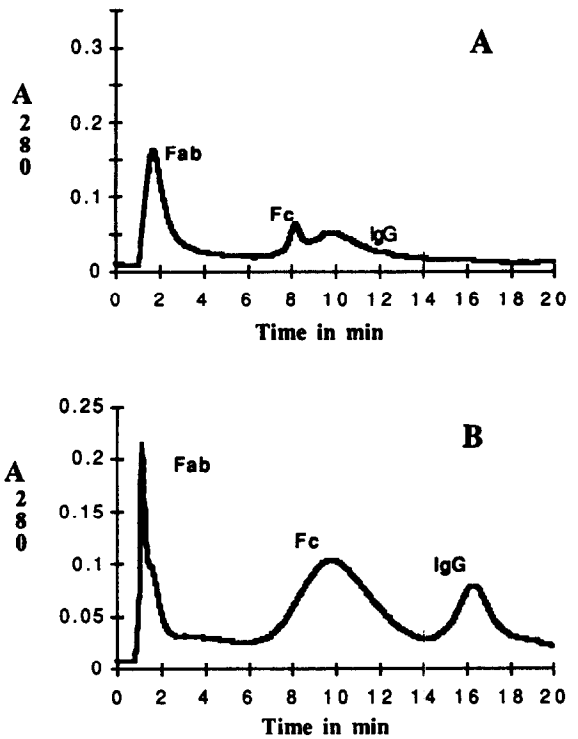

Fig. 3. Separation of a mixture of IgG and proteolytic fragments on (A) Mono-S and (B) S-Zephyr supports. The proteins were eluted from the column by a linear sodium chloride gradient. The gradient consisted of 0–5 min 50 m*M* acetate buffer followed by a linear (0–0.5 *M*) sodium chloride gradient in the same buffer.

Binding orientation of IgG

To determine whether or not IgG is coupled to different activated agaroses via its F_c and/or F_{ab} portion, IgG was coupled to agarose and the immobilized IgG was cleaved with papain. The liberated fragments were analyzed using S-Zephyr chromatography (see Fig. 5). Only F_{ab} fragments could be detected regardless which activation method was employed. However, in the sample of liberated fragments from immobilized IgG bound to cyanogen bromide activated agarose, a small amount of F_c fragments and uncleaved IgG could be detected. This may have been due to a small portion of the IgG which may have been ionically bound to the positively charged cyanogen bromide matrix.

The differences in concentration of IgG which could be immobilized on each of these different matrices is reflected in the different absorbances at 280 nm that are seen on HPLC of fragments obtained from various immobilized IgG preparations. What is important, however, is not the concentration of antibody bound to each of these matrices, but rather the presence or absence of F_{ab} fragments along with the F_c fragments which are readily seen.

Our study on the orientation of immobilization of IgG to agarose demonstrates that S-Zephyr chromatography provides the necessary tool for the separation of papain cleavage products from immobilized IgG. Other studies using electrophoresis as the method to separate F_{ab} and F_c have shown the same results as given here but with greater difficulty in potential quantitation of the amount of each fragment released.

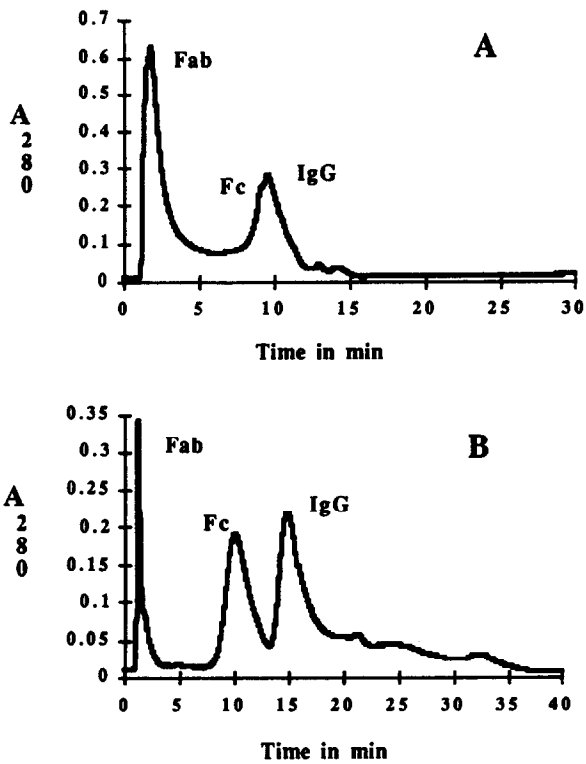

Fig. 4. Separation of a mixture of IgG and proteolytic fragments on (A) Mono-S and (B) S-Zephyr supports with acetonitrile added to the HPLC buffers. The proteins were eluted from the column by a linear sodium chloride gradient. The gradient consisted of 0–5 min 50 m*M* acetate 10% acetonitrile buffer followed by a linear (0–0.5 *M*) sodium chloride gradient in the same buffer.

TABLE IV

WIDTHS OF THE PEAKS TANGENTS

IgG and the F_c fragment were applied on S-Zephyr columns, pH 5.0. A 50 m*M* acetate buffer, pH 5.0, was used and the experiment was repated using the same buffer with 10% acetonitrile added. The column was run at 1 ml/min and the elution gradient consisted of 0–5 min buffer followed by 5–25 min linear (0–0.5 *M*) sodium chloride gradient in the same buffer. When buffer with added acetonitrile was used, the gradient consisted of 0–5 min buffer followed by 5–25 min linear (0–1.0 *M*) sodium chloride gradient in the same buffer.

Buffer	Width of the peaks tangents at the baseline (min)	
	F_c peak	IgG peak
No acetonitrile added	7.7	4.1
10% acetonitrile added	4.5	4.1

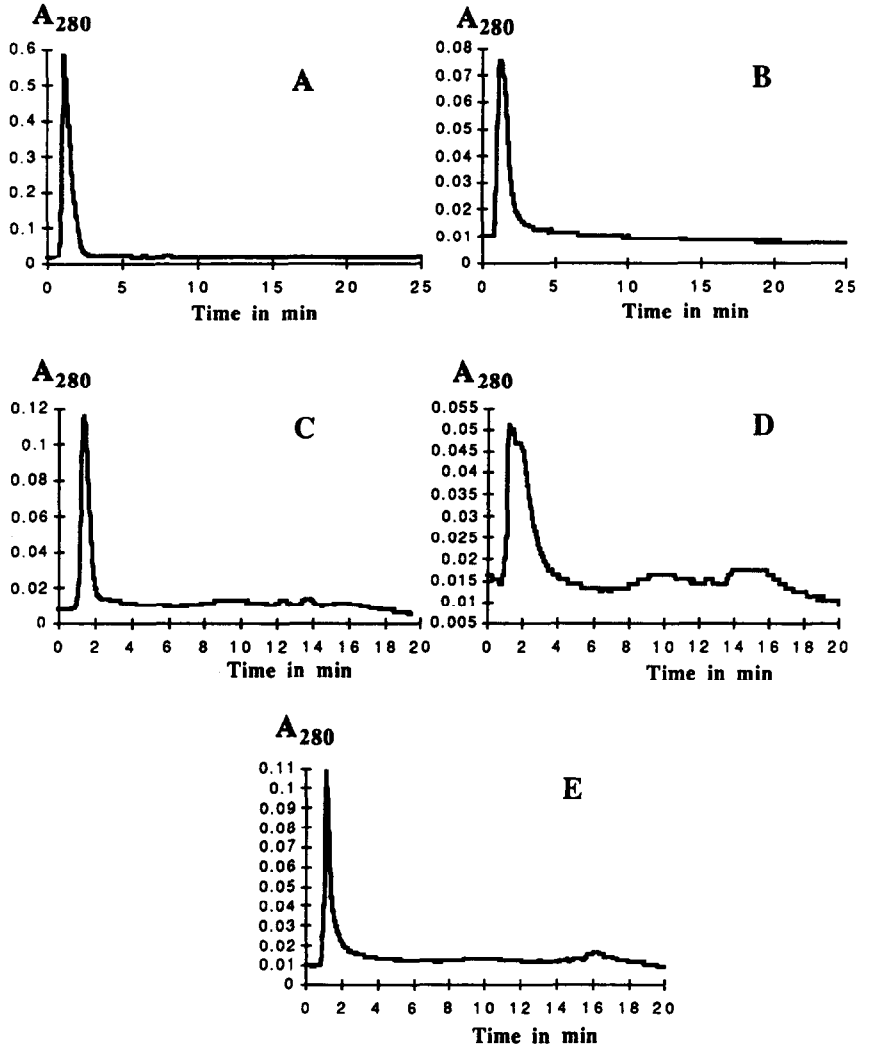

Fig. 5. Liberated fragments collected from papain cleavage of immobilized IgG, on (A) carbodiimidazole-activated, (B) tosyl chloride-activated, (C) FMP-activated, (D) cyanogen bromide-activated and (E) epoxide-activated agarose. The fragments were applied to an S-Zephyr column, pH 5.0, and eluted from the column by a linear sodium chloride gradient. The gradient consisted of 0–5 min wash with 50 m*M* acetate buffer followed by a linear (0–0.5 *M*) sodium chloride gradient in the same buffer.

There results, which suggest that nucleophilic binding to activated agarose of IgG occurs chiefly by the F_c portion, are somewhat unexpected. We had initially thought that a quantitative method would be necessary to demonstrate how many of each portion of the IgG molecules were immobilized by each of the two portions: F_{ab} or F_c. We now plan to investigate additional proteolytic digestion methods for treating the immobilized IgG, in order to confirm what the papain cleavage data indicates, namely that most IgG immobilization is by the F_c fragment. We also plan to extend our studies to immobilization of IgG via methods that are not dependent upon nucleophilic residues, such as diazotized aminophenyl agarose.

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