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# Activation of Trisacryl Gels with Chloroformates and Their Use for Affinity Chromatography and Protein Immobilization

T. MIRON AND M. WILCHEK*

*Department of Biophysics, Weizmann Institute of Science, Rehovot, Israel*

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## ABSTRACT

In this study we describe the activation with chloroformates of Trisacryl-GF-2000, a new synthetic gel support that is stable, hydrophilic, and contains large amounts of hydroxyl groups available for activation.

Of all the reagents tested, the activation with *N*-hydroxysuccinimide-chloroformate and *p*-nitrophenylchloroformate in organic solvents provides the best activation yield and subsequent coupling. When Trisacryl was activated in acetone with the chloroformates in the presence of 4-dimethylaminopyridine as base and catalyst, up to 30% of the hydroxyl groups, (i.e., 1/repeating unit) could be activated. Amino-containing ligands and proteins could be coupled to these carriers at pH 8 or higher. For better results in affinity-chromatographic applications, spacers of ϵ-amino caproic acid or diaminohexane were introduced. The efficacy of these columns was demonstrated by purification of enzymes, antibodies, and antigens. The performance of these new columns were compared with that of Sepharose columns activated in various ways. In every case, the properties of the Trisacryl support proved superior with particular reference to the purity of the product obtained.

**Index Entries:** Chloroformates; Trisacryl-GF-2000; synthetic gel-filtration support; affinity chromatography, carriers in; protein immo-

*Author to whom all correspondence and reprint requests should be addressed.

bilization; enzyme purification; antibody purification; antigen purification.

## INTRODUCTION

The major type of carriers used in affinity chromatography and for immobilization of proteins are polysaccharides or a combination of polysaccharide with acrylamides (*1*). One of the problems encountered with the columns when used in affinity chromatography is the nonspecific adsorption of compounds other than that to be purified. The nonspecific adsorption may originate from the chemistry used for coupling the ligand to the polymer, which may introduce ion-exchange or hydrophobic properties to the carrier (*2,3*). The softness and porosity of these carriers may also contribute to the nonspecific binding.

Recently, a synthetic gel-filtration support derived from copolymerization of *N*-acryloyl-2-amino-2-hydroxymethyl-1,3-propane diol (Trisacryl-GF-2000) was introduced (*4,5*). The support is claimed by the manufacturer to possess improved properties compared to other commercially available carriers. Among the advantages claimed for these gels are that they possess a well-defined structure, are resistant to medium pressures, and are comprised of relatively small particles with a narrow bead size distribution that allows high flow rates. They are also very hydrophilic and stable to low and high temperatures, as well as to organic solvents and denaturing agents. Another advantage of the synthetic polymer is that it is not biodegradable. The same properties are required from good affinity chromatography carriers (*6*) and this gel is therefore an excellent candidate for affinity chromatography studies and applications.

The problem with this gel is that even though it contains hydroxyl groups like the polysaccharides, they are not as reactive (nucleophilic) as the polysaccharides because of, perhaps, the higher pK of the hydroxyl groups. Many reagents are in use today for activating polysaccharides. In this study we tried many of these different reagents and found that the best activation procedure in our hands is that mediated by chloroformates, namely *p*-nitrophenylchloroformate and hydroxysuccinimidechloroformate (*7*). In the following discussion, we describe the activation of Trisacryl-GF-2000 with these reagents and their use in affinity chromatography and protein immobilization. A comparison with Sepharose activated by the same procedure is also presented.

## MATERIALS AND METHODS

### *Materials*

Trisacryl-GF-2000 was a gift from Dr. E. Boschetti; IBF, *p*-nitrophenylchloroformate, 1,6-diaminohexane, trinitrobenzene sulfonic acid

(TNBS), ε-aminocaproic acid, and triethylamine (TEA) were obtained from Fluka. Dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), *p*-nitrophenol, 4-dimethylaminopyridine (DMAP), 1-ethyl-3, 3(3-dimethylaminopropyl)carbodiimide (EDC), trypsin, α-chymotrypsin, bovine serum albumin (BSA), and avidin were obtained from Sigma. *D*-Tryptophan methyl ester, ε-dinitrophenyl (DNP) lysine, rabbit antiBSA serum, and goat antiDNP serum were acquired from Bio-Yeda (Rehovot). *N*-Hydroxysuccinimidechloroformate was synthesized as described by Gross and Bilk (*8*). The TEA and pyridine were dried on pellets of KOH. Acetone was dried on $K_2CO_3$. DNP–rabbit serum albumin (DNP–RSA) was from BIO–Yeda.

## Activation of Trisacryl-GF-2000 with Chloroformates

Trisacryl-GF-2000 was dehydrated with increasing concentrations of acetone (25, 50, 75, and 100%) and then washed with dry acetone. The dry polymer was resuspended in acetone containing the required amount of chloroformate. The suspension was cooled to 4°C with an ice bath and between 1.1 to 1.3 equivalent (to the chloroformate) of either TEA in pyridine or DMAP in acetone were added dropwise (via a separation funnel). The reaction mixture was stirred for 1 h at 4°C. The activated gel was washed with cold acetone, 5% acetic acid in dioxane, methanol, and isopropanol and stored at 4°C in isopropanol. To ensure complete removal of reagents, the methanol washings were checked for the presence of NHS or *p*-nitrophenol by diluting an aliquot with 1.0 mL of 0.25*N* $NH_4OH$ and reading the absorbance at 260 or 400 nm, respectively (*9*). A typical example for the activation procedure is as follows: 5 g of wet gel was dehydrated with acetone. The gel was suspended in 5.0 mL acetone, containing *p*-nitrophenylchloroformate or *N*-hydroxysucciminidechloroformate (300 μmol/mL) at 4°C. To the cold suspension was added dropwise 5.0 mL of the freshly prepared solution of 4-dimethylaminopyridine (330 μmol/mL) in acetone. The reaction mixdescribed above. Using the conditions exemplified, carrier containing 60 or 100 μmol/wet g of *p*-nitrophenol or hydroxysuccinimide were obtained, respectively.

## Determination of Active Groups on the Carrier

The amount of active groups present on the *p*-nitrophenyl derivate of Trisacryl was determined by basic hydrolysis of a weighed sample with 0.2*N* NaOH for 5 min at room temperature. The released *p*-nitrophenylate was assayed spectrophotometrically at 400 nm, $E = 17{,}000$. The amount of active groups present on the *N*-hydroxysuccinimide derivative of Trisacryl was assayed after basic hydrolysis in 0.2*M* $NH_4OH$ (5 min at room temperature). The released *N*-hydroxysuccinimide was assayed spectrophotometrically at 260 nm, $E = 10{,}000$ (*9*).

### Coupling of Protein and Amino-Containing Ligand to the Carbonate-Activated Carriers

Alkylamines, amino acids, or proteins (trypsin, DNP-RSA, α-chymotrypsin, BSA, monoclonal and polyclonal antibodies) were coupled to the activated Trisacryl. Coupling was performed either in phosphate buffer (0.1*M*), pH 7.5, or 0.1–0.2*N* $NaHCO_3$, with agitation at 4°C. When the NHS-activated Trisacryl was used, the reaction was stirred from 4 to 16 h at 4°C. When coupling was performed with *p*-nitrophenyl carbonate gels, the coupling was accomplished at pH 9.0 ($NaHCO_3$/NaOH) with longer periods of agitation (48 h, 4°C). The conjugated gel was then washed on a sintered-glass funnel with 0.1*N* $NaHCO_3$. Excess active groups were removed with 0.1*N* $NH_4OH$ in cases in which the immobilization of biologically active molecules (e.g., enzymes) were designed. If stable ligands were coupled, such as alkylamines or amino acids, the excess reactive groups were removed with 0.1*N* NaOH. The gel was then washed until neutrality with $H_2O$.

### Coupling of Diaminohexane

To a solution (5-mL) containing 0.2*M* diaminohexane, adjusted to pH 8.3 or 9.0 with HCl for coupling to *N*-hydroxysuccinimide or *p*-nitrophenyl activated Trisacryl, respectively. One gram of activated Trisacryl (after isopropanol washings) was added. The suspension was shaken overnight at 4°C, then washed with 20% DMF, $H_2O$, 0.1*N* NaOH, and $H_2O$. The amount of free amino groups (20 μmol/wet g) on the polymer was determined using trinitrobenzene sulfonic acid (*10*).

### Coupling of Protein to Activated Trisacryl-GF-2000

#### *Coupling of Trypsin and α-Chymotrypsin*

A stock solution (1.0 mL) of 5 mg/mL of trypsin in 1 m*M* HCl was added to activated Trisacryl (1.0 g) (after short washing with cold water to remove the isopropanol). Then, 2.0 mL of cold 0.2*N* $NaHCO_3$ was added and the suspension shaken overnight at 4°C. The conjugate was filtered and washed with 0.1*N* $NaHCO_3$. The first washing was collected separately to determine unbound trypsin. The conjugate was washed with 0.1*M* $NH_4OH$ and then $H_2O$. The conjugate was kept at 4°C in 0.1*N* phosphase buffer, pH 7.5. Assay of trypsin activity was done at pH 8.5, either at room temperature or at 40°C. The hydrolysis rate of the substrate benzoylarginine ethyl ester (BAEE) was recorded on a pH-stat (Radiometer, Copenhagen model TTTIC). Assay of α-chymotrypsin was done at room temperature at pH 8.0 with acetyl tyrosine ethyl ester as substrate. The reaction was also followed on a pH-stat.

### *Coupling of BSA*

A stock solution (2 mL) of BSA (5 mg/mL) in 0.1*N* $NaHCO_3$ was added to 1.0 g of activated Trisacryl. The suspension was shaken overnight at 4°C and treated as described above.

### *Determination of Bound Protein*

Assay of bound protein was perfomed either after total hydrolysis of the conjugate with 6*N* HCl at 110°C for 22 h or by determination of unbound protein based on absorption at 280 nm. This was accomplished either after dialysis to remove the leaving group *p*-nitrophenol or in case of *N*-hydroxysuccinimide activation, after dilution of the first unbound washing with HCl or acetic acid (*9*).

## *Preparation of Trisacryl-ϵ-Aminocaproic Acid Active Esters*

ϵ-Aminocaproic acid was coupled to the activated carbonate, derivatives of Trisacryl-GF-2000, as described for the amino analogs. the carboxyl-containing gel was further activated with *N*-hydroxysuccinimide or *p*-nitrophenol, as follows: Trisacryl-amino caproic acid was dehydrated with increasing concentrations of dioxane (25, 50, 75, and 100%). One gram of Trisacryl caproic acid was suspended in 3 mL of absolute dioxane containing 150 mg of NHS or 180 mg of *p*-nitrophenol, 270 mg DCC in 5 mL dioxane was added to the suspension. The reaction mixture was shaked overnight at 25°C, filtered, and washed with dioxane, methanol, and isopropanol. The activated esters were used for further coupling with amino-containing ligands. Before coupling of protein, the activated carrier was washed with cold water. Coupling of protein was performed at 0.1*N* $NaHCO_3$ or PBS for 16 h at 4°C, with shaking. Coupling of proteins to the carboxyl derivate of Trisacryl could also be accomplished directly in the presence of EDC. One gram of Trisacryl-amino caproic acid was mixed with 3 mL of protein solution. The pH was adjusted to 5.0 at room temperature. To the suspension was added (dropwise, over a 20-min span) a freshly prepared aqueous solution of EDC. A 25-fold excess of EDC (over the concentration of the carboxyl group of the Trisacryl) was used. The pH was kept between 5.0 and 6.0. The reaction mixture was shaken overnight at 4°C, then washed on a sintered-glass funnel with PBS.

## *Affinity Chromatography*

Antigens and antibodies were coupled to NHS activated Trisacryl-GF-2000. The NHS group was attached either directly to the column or through the ϵ-aminocaproic acid. The proteins to be purified

were applied in PBS, pH 7.4, and eluted by different means. The nonspecific adsorption was checked either by using nonactivated gel or the gel containing the spacer. Also, nonrelevant antigen and antibodies were applied.

## RESULTS

### Activation

Because of the improved properties of the Trisacryl-GF-2000 in gel filtration, which we have confirmed by passing several proteins and showing that they do not stick to the carrier, we decided to apply the same beads for affinity chromatography. Since this resin contains three hydroxyl groups/repeating unit of polymer, we decided to activate these groups using reagents that have previously been used to activate polysaccharides (*1*). Among the various activation methods used (e.g., CNBr, tosylchloride, carbonyldiimidazole, and chloroformates), the best results were obtained using the chloroformates, namely *p*-nitrophenylchloroformate or *N*-hydroxysuccimidechloroformate. The activation procedure, some of the derivatives prepared, and the binding of proteins is shown in Scheme 1.

In order to obtain good activation with the chloroformates, the reaction has to be done in an organic solvent in the presence of a base that serves both as a "scavenger" for the hydrochloric acid released and as a catalyst. The best results were obtained when the activation was performed in the presence of dimethylaminopyridine (DMAP). Up to 30% of the hydroxyl groups on the Trisacryl could be activated. When the reaction was performed in the presence of other bases, such as triethylamine, only about 10% of the hydroxyl groups were activated. In most of our experiments to be described, we used the amounts of reagents for activation as exemplified in the Method section. The swelling properties of the matrix did not change upon activation. The activated resin can be stored for prolonged periods of time of up to 1 yr, either as a dry powder or in isopropanol.

### Coupling

Coupling of proteins in aqueous solution at pH 8 proceeds with lower yield when compared with Sepharose activated in the same manner and containing the same levels of active groups (Table 1). In order to increase the yield of the coupled protein, a spacer of ε-aminocaproic acid was introduced. The amount of spacer that could be introduced under the described conditions reached usually about 20–30% of the available active groups. The spacer could be introduced either in aqueous media or in organic solvents. In order to bind proteins or other ligands, the carboxyl group on the Trisacryl was converted to the *N*-hydroxysuccimide ester or the *p*-nitrophenyl ester. About 2.5 mg/wet g of protein was cou-

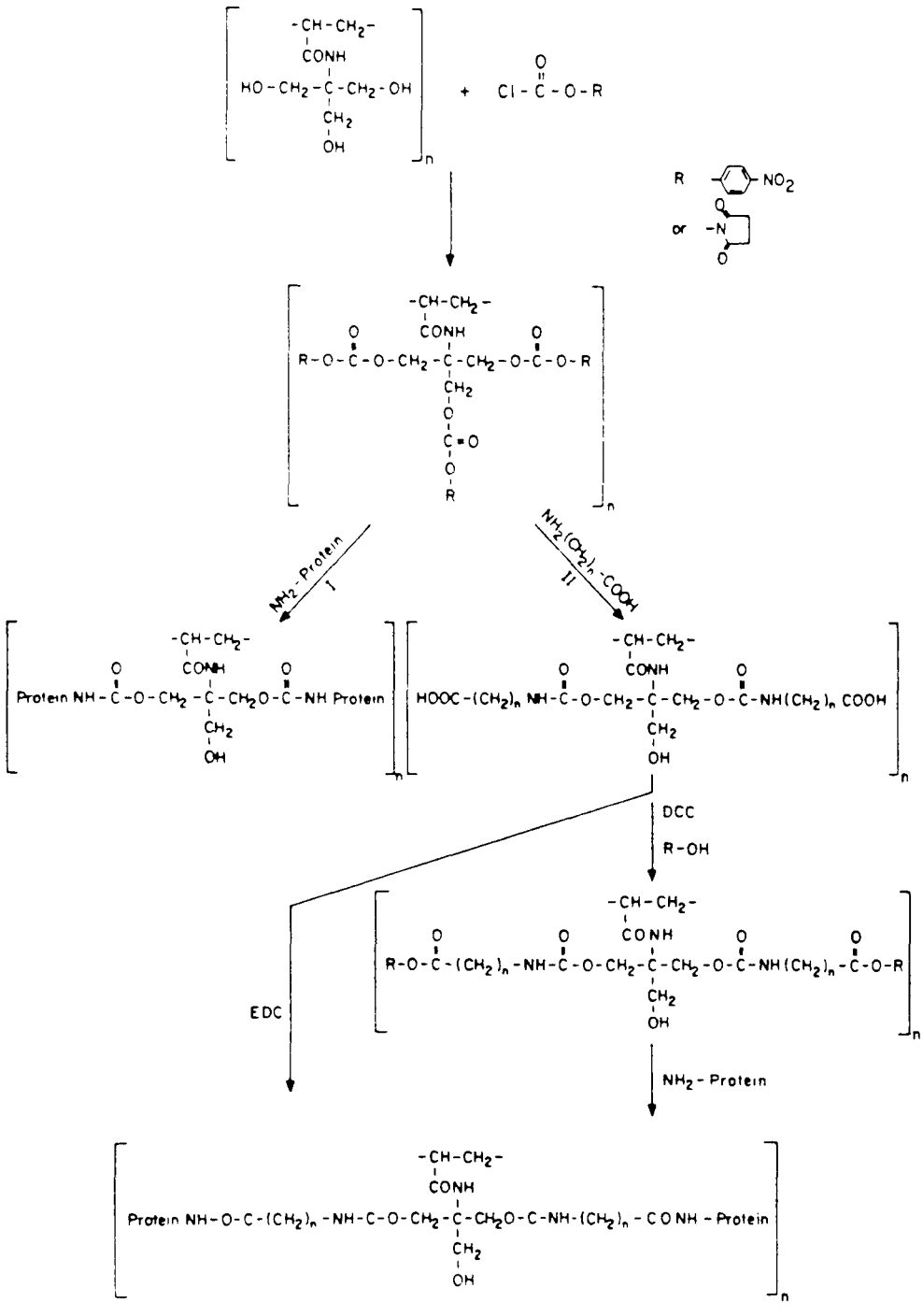


Fig. 1. Structure of activated Trisacryl and its coupling products.

pled. Alternatively, protein could also be bound directly to the carboxyl group via the use of carbodiimide.

TABLE 1
Binding Capacity of Proteins to Carbonate Carrier[a]

| Protein coupled | Carbonate activated | |
|---|---|---|
| | Cl-Sepharose 4B, mg protein/wet g | TA-GF-2000, mg protein/wet g |
| Avidin | 1.6 | 0.3 |
| Bovine serum albumin | 6–12 | 0.8–1.0 |
| Trypsin | 3–8 | 0.8–1.0 |

[a]*N*-Hydroxysuccinimide- or *p*-nitrophenyl-activated carriers.

## Affinity Chromatography

In order to demonstrate the utility and efficiency of Trisacryl-GF-2000 as an affinity chromatography matrix, we purified several proteins, including α-chymotrypsin and antibodies to BSA and DNP. In all the cases, the performance was checked either by the use of direct coupling or through the ε-aminocaproic acid side chain and compared with that of Sepharose under similar conditions.

## Purification of α-Chymotrypsin

α-Chymotrypsin was purified on a column containing D-tryptophan methyl ester (Table 2). Two milligrams of the enzyme were applied to 1 mL of the column in Tris-HCl 0.05*M*, pH 8.0, and eluted with acetic acid, followed by ethylene glycol. As can be seen from Table 2, α-chymotrypsin did not bind to the column of Trisacryl without a spacer, although the enzyme bound readily to the Trisacryl with the spacer.

TABLE 2
Purification of α-Chymotrypsin on Immobilized D-Tryptophan Methyl Ester[a]

| | Trisacryl-GF-2000 | | Cl-Sepharose 4B | |
|---|---|---|---|---|
| | -D-Tryp-OMe, 6 μmol/g | -ε-Amino-caproyl -D-Tryp-OMe, 14 μmol/g | -D-Tryp-OMe, 15 μmol/g | -ε-Amino-caproyl -D-Tryp-OMe, 36 μmol/g |
| 0.05*M* Tris, pH 8.0 | 100 | 40 | 40 | 42 |
| 0.2*M* Acetic acid | | 54 | | 40 |
| 50% Ethylene glycol, in 1*M* NaCl | | 6 | 60 | 18 |

[a]Two milligrams of α-Chymotrypsin (in 0.05*M* Tris, pH 8.0) were applied on 1 mL packed gel (in Pasteur pipet preequilibrate with 0.05*M* Tris, pH. 8.0). Unbound protein was removed with 0.05*M* Tris, pH 8.0. Bound protein was eluted with 0.2*M* acetic acid, followed by 50% ethylene glycol in 1*M* NaCl. Data represents percent of loaded protein.

α-Chymotrypsin bound to Sepharose with or without the spacer. The failure of the directly activated Trisacryl may be because of the lower amounts of D-tryptophan bound to the carrier or because of steric difficulties. The α-chymotrypsin could be eluted under milder conditions from the Trisacryl column, compared to the Sepharose column. The specific activity of the eluted protein from the Trisacryl column was increased by 25%. The unadsorbed protein was devoid of activity.

### *Purification of antibodies*

Since the majority of applications of affinity chromatography presently is in the purification of antibodies and antigens, we checked the efficiency of the Trisacryl in the purification of two antibodies; one against a high-molecular-weight antigen (BSA) and the other against a low-molecular-weight hapten (DNP). In all the cases it was compared to the same antigens and antibodies that were coupled to the Sepharose.

Antibodies to BSA were purified on BSA coupled to the carriers either via the spacer or directly (Table 3). The serum containing the antibody was applied to the column in PBS and washed with the same buffer. The capacity of the columns was determined to be about 2 mg of antibody/mL of column. Despite the fact that the amount of BSA that could be coupled to the Sepharose was about five times that coupled to Trisacryl, the capacity for adsorbing the corresponding antibody was the same. As can be seen from the elution profile (Table 3), milder conditions for elution were sufficient for the Trisacryl column. It is interesting that milder elution conditions were also required on Sepharose when the antigen was bound through a spacer containing carboxyl groups. Antibodies to DNP were purified on columns containiing ε-DNP-lysine coupled directly to the carriers activated with hydroxysuccinimide. No additional spacer was introduced since the lysine residue is already derivatized to a

TABLE 3
Purification of AntiBSA Antibodies on Immobilized-BSA[a]

| | Trisacryl-GF-2000 | | Cl-Sepharose 4B | |
|---|---|---|---|---|
| Elution with | -BSA, 1 mg/g | ε-Aminocaproyl -BSA, 2 mg BSA/g | -BSA, 6 mg/g | ε-Amino-caproyl, 3.4 mg BSA/g |
| 0.2*M* Gly.HCl, pH 2.7 | 80 | 100 | 51 | 87 |
| 0.25*N* $NH_4OH$ | 20 | | 26 | 13 |
| Guanidine HCl | | | 23 | |

[a]One milliliter of rabbit antiBSA serum was applied on 1 mL packed columns that were preequilibrate with PBS. Unbound proteins were washed off with PBS, bound antibodies were eluted with 0.2*M* glycine HCl buffer, pH 2.7, followed with 0.25*M* $NH_4OH$, and then 6*M* guanidine HCl. Protein concentrate was determined according to absorbance at 280 nm. The results represent the % of total eluted antiBSA.

spacer. The antibodies were applied in PBS and eluted as shown in Table 4. The capacity of the Trisacryl for antibody was about 2 mg and for Sepharose about 3 mg of antibody/mL of column. Again, milder elution conditions were required for the Trisacryl column.

## DISCUSSION

The general trend in affinity chromatography these days is in the direction of immunoaffinity chromatography, namely the use of immobilized antibodies (monoclonal and polyclonal) for the purification of the corresponding antigen, whether it be an enzyme, cytotoxin, hormone, or peptide (*1*). Because of the high affinity between antibodies and their respective antigens, more drastic elution conditions are required than those usually applied in affinity chromatography. The use of 6*M* guanidine, 3*M* KSCN, or 1*M* $NH_4OH$ are not rare for elution. The application of such harsh conditions requires very stable carriers and bonds.

The most prevalent carrier presently used is Sepharose, and the most prevalent reaction applied for coupling the ligands is the cyanogen bromide reaction (*12*). Neither Sepharose nor the isourea bond formed upon binding amine-containing ligands are completely stable, even when milder conditions than those described above are used (*13*). In addition, when peptides were coupled to Sepharose, they tended to leak from the column, and after total hydrolysis of the leaked peptides with HCl, the solution became dark brown, indicating the presence of carbohydrate residues (unpublished results). Furthermore, many researchers habitually block residual active groups with amines—a practice that inadvertently introduces other functional groups that may serve as sites for nonspecific adsorption (*3*). We also encountered all these problems during this study when applying Sepharose as a carrier. We observed many bands (including heavy and light chains) on the SDS gels of the eluted material from the antibody-Sepharose columns that had been activated by different means and procedures.

Because of these problems, we looked for different hydrophilic carriers. Recently we used Trisacryl-GF-2000, a new synthetic gel-filtration

TABLE 4
Purification of AntiDNP-Antibiodies on Immobilized DNP[a]

| Elution with | Trisacryl-GF-2000 -DNPL | Cl-Sepharose 4B -DNPL |
|---|---|---|
| 0.2*M* Gly·HCl, pH 2.7 | 62 | 0 |
| 0.25*M* $NH_4OH$ | 38 | 100 |

[a]One milliliter of goat antiDNP serum was applied on immobilized DNPL column, (1 mL packed in a Pasteur pipet preequilibrate with PBS). The Unbound protein was washed off with PBS, antiDNP antibodies were eluted with 0.2*M* glycine buffer, pH 2.7, followed 0.25*M* $NH_4OH$. The results are the % total eluted antibodies.

support, with excellent results. This support has many advantages over the existing supports, as described by the manufacturer and confirmed experimentally by us. Also, the support contains as many as three hydroxyl groups/repeating unit (this makes the polymer very hydrophilic), which enables activation using the same reagents developed for activation of polysaccharides. Of all the reagents checked, it was found that activation with chloroformates in organic solvent gave the best activation yields, whereas with other reagents there was no (CNBr) or marginal activation. Even though the resins were highly activated, coupling of protein proceeded with low-coupling yields, indicating that the proteins do not stick to the beads and that the reaction occurs only upon occasional collisions between the active groups on the beads with the protein (Table 1). In spite of this low yield of coupled protein, the resultant column could be used efficiently. To increase the coupling yield, a spacer was introduced between the matrix and the protein. The spacer used (the ϵ-amino caproic acid group) could be introduced in high yield. The carboxyl group introduced could be further activated with *N*-hydroxysuccinimide or *p*-nitrophenol esters, and in this study the resultant ester was used to couple protein in higher yield. It is interesting to note that even though the amount of protein coupled to the Trisacryl was usually lower than that coupled to Sepharose under the same conditions, the amount of antibody adsorbed to the antigen column was the same in both cases. This may be explained by the possibility that the antigen on the Trisacryl is bound to the outer surface of the beads and is more available to the antibodies.

Further support to this explanation is evident from the behavior of immobilized trypsin. Trypsin immobilized on Trisacryl shows the same activity profile v temperature as the native enzyme, whereas trypsin immobilized on Sepharose shows lower activity (unpublished results).

The carbamate bond formed upon coupling of amino-containing ligands to the activated carbonate column was completely stable and no leakage could be detected upon prolonged storage. In addition to better stability over that observed for polysaccharide columns, the Trisacryl columns are devoid of charged groups, thus eliminating much of the nonspecific adsorption resulting from electrostatic interactions. Mild base treatment completely removes excess reactive groups (which failed to participate in the initial coupling reaction with the active carbonate), and the original hydroxyl groups are regenerated, thus eliminating another source of nonspecific binding.

Since the activation is performed with *p*-nitrophenylchloroformate or *N*-hydroxysuccinimidechloroformate, the amount of activation can easily be determined spectrophotometically, as described earlier (*7*). In this, the efficiency and usefulness of the Trisacryl columns were demonstrated by their application for purification by affinity chromatography of several compounds, including antibodies and antigens, that had previously been purified by other methods. Our activated gels were also used

for the excellent purification of the as yet uncharacterized human cytotoxin (*14*). In the study of the human cytotoxin purification, a monoclonal antibody against the cytotoxin was coupled to the *N*-hydroxysuccinimide ester of Trisacryl ϵ-aminocaproic acid. A product containing a single band on SDS-gel electrophoresis was obtained from a crude extract of the cytotoxin. When Sepharose was used as the carrier, many bands were obtained (unpublished results). The question is whether the exceptionally good results obtained with the cytotoxin reflect a new generation of columns or whether this particular case is but a fluke. To answer this question, more work and further applications will have to be done.

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