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Purification of proteins on an epoxy-activated support by high-performance affinity chromatography

DOROTHY J. PHILLIPS*, BONNIE BELL-ALDEN, MARK CAVA[a], EDWARD R. GROVER and W. HARRY MANDEVILLE
Waters Division of Millipore, 34 Maple Street, Milford, MA 01757 (U.S.A.)
ROBERT MASTICO
University of Leeds, Leeds (U.K.)
WAYNE SAWLIVICH and GEORGE VELLA
Waters Division of Millipore, 34 Maple Street, Milford, MA 01757 (U.S.A.)
and
ANDREA WESTON
University of Rhode Island, Kingston, RI 02881 (U.S.A.)

ABSTRACT

The use of a rigid silica-based packing material with large particle and pore size, 37–55 μm and 500 Å pore, for affinity chromatography makes it possible to combine high selectivity with short analysis times. Both large and small molecules have been covalently bonded to the Protein-Pak™ Affinity Epoxy-Activated bulk packing for purification of glycoproteins, immunoglobulins, enzymes, lectins and other proteins. Recombinant protein A, GammaBind™ G, heparin, Cibacron Blue F3G-A, sulfanilamide, N-acetyl-D-glucosamine, concanavalin A and aminophenylboronic acid were covalently attached to the affinity packing for selective purification of proteins.

INTRODUCTION

Conventional chromatography of proteins relies on small differences in surface hydrophobicity (hydrophobic interaction and reversed-phase), surface charge (ion-exchange), and molecular size and shape (size exclusion) to separate target molecules that may be, at most, 0.1% of the dry weight of the starting material. However, these same proteins often have the characteristic ability to bind reversibly to other molecules as specific stable complexes. This ability has been utilized in affinity chromatography to purify proteins several thousand fold from complex mixtures in a single step [1].

Affinity chromatography was first used with cyanogen bromide activation on agarose supports [2]. This support is still widely used, but has disadvantages: ligand leakage, non-specific adsorption, matrix compressibility, and microbial attack.

[a] Present address: Zymark Corp., Hopkinton, MA 01448, U.S.A.

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Agarose is a soft gel and requires low flow-rates which result in long separation times, especially for large-scale purifications. Analysis times can be decreased by the use of rigid and non-adsorptive diol-bonded silicas adapted from size-exclusion chromatography [3]. Silica has certain advantages not possible with agarose: mechanical strength, well-defined and accessible pore volume, and resistance to microbial attack. These characteristics of silica along with the selectivity of covalently bound ligand result in a specific interaction and together have been combined to produce high-performance affinity chromatography (HPAC).

For the development of HPAC, the characteristics of the silica for purification of biopolymers were first defined. Silica used in high-performance liquid chromatography (HPLC) range in pore size from 60 to 4000 Å. Below 150 Å the performance of an HPAC column suffers because of poor solute diffusion and unavailability of the surface. Above 1000 Å the capacity is too low and the silica becomes friable. Therefore, pore sizes between 500 and 1000 Å are most commonly used. In conventional HPLC, small (<10 μm) particle sizes have been used to minimize band spreading and maximize resolution [4]. In HPAC, resolution depends on selectivity, not efficiency (plate count); thus, there are few advantages to high plate counts or small particle sizes in HPAC.

Immobilization reactions that form covalent bonds between the support and the ligand are required. A number of chemical reactions have been used including cyanogen bromide, active ester (N-hydroxysuccinimide), epoxide, tresyl chloride, carbonyldiimidazole, thiol and diazonium salts. Epoxy-activated packings have been developed because of the mild coupling conditions, simplicity of the reaction, the versatility of the epoxy functionality (immobilizes ligands by amino, thiol and hydroxyl groups), and the stability of the covalent bond with the ligand. The concentration of active groups is normally $\leqslant 5$ μmol/m^2 (about 300 μmol/g). For large proteins a monolayer is about 0.01 μmol/m^2. This coverage could lead to immobilization of a larger ligand by 100 bonds/molecule. While this would be a stable layer, the activity of the immobilized ligand may be low due to potential distortion of the three-dimensional structure required for activity. Turková *et al.* [5] reported that the affinity chromatographic efficiency of a specific adsorbent for porcine pepsin depended strongly on the concentration of the immobilized inhibitor. At low concentration (0.85 μmol of aminocaproyl-L-Phe-D-Phe-OCH_3/g dry gel), pepsin was eluted from the column as a sharp peak, whereas at high inhibitor concentration (155 μmol/g dry gel) several peaks of pepsin were noted in the chromatogram exhibiting the same proteolytic activity. The authors attributed the results to additional unspecific multipoint ligand–enzyme interactions with increasing ligand density on the support surface.

Each activation reaction has been used with a variety of lengths and types of spacer arms to assure that the immobilized ligand can be reached by the substance to be purified. Hydrophilic spacer arms (polyethylene glycol, for instance) are most favored. Lengths of spacer arms have varied from as few as 4 to as many as 50 atoms. Increasing the length of the spacer arm can reduce the capacity of the support. Therefore, the length and concentration of the spacer arm should be optimized for maximum activity.

A variety of immobilized ligands may be used in this technique. These molecules can be divided into general and specific ligands. General ligands include lectins such as concanavalin A, immunoglobulin-binding proteins, like Proteins A and G, and

triazine dyes, such as Cibacron Blue F3G-A. Specific ligands normally include antibodies directed against a particular antigen, and enzyme inhibitors or substrates. The most important characteristic of the immobilized ligand must be the ability to bind strongly to its specific biological compound but not so strongly that the conditions needed to release the protein also denature it.

The most common elution conditions involve introduction of a competing ligand, general conditions such as lowering the pH to less than 3, increasing the ionic strength of the buffer, or using a chaotropic buffer. Any of these methods may suffice depending on the hardiness of the immobilized ligand and the specific complex formed. HPAC, when used with the correct ligand and chromatography conditions, can result in the purest and, in some cases, most biologically active product of any method available.

A new silica-based affinity packing has been produced with the characteristics described above for use with biocompatible medium- and high-pressure LC instrumentation. The rigid silica-base has an average pore size of 500 Å and a particle size range of 37–55 μm. The Protein-Pak™ affinity epoxy-activated packing is manufactured by a polymerization process that encapsulates the silica matrix with a hydrophilic bonding layer containing the epoxide functionalities. The bonds formed between the silane with the epoxide functionalities and the silica are as stable as, if not more stable than, most other silica–silane interactions, minimizing any concern about epoxide leaching. Active sites of the silica are blocked by the hydrophilic bonding layer to produce low non-specific binding properties.

The ligands used to evaluate the performance of this new activated packing are listed in Table I. This report discusses the immobilization and affinity chromatography for N-acetyl-glucosamine, sulfanilamide, heparin and recombinant Protein A (rProtein A).

MATERIALS

Hemoglobin, carbonic anhydrase, antithrombin III, lysozyme, nitrophenyl acetate, glycine, wheat germ agglutinin, heparin, and sulfanilamide were purchased from Sigma (St. Louis, MO, U.S.A.). rProtein A was purchased from Repligen (Cambridge, MA, U.S.A.). Wheat germ meal was purchased from Hodgson (Gaines-

TABLE I

PROTEIN-PAK AFFINITY EPOXY-ACTIVATED PACKING COUPLINGS

Immobilized ligands	Applications
GammaBind™ G	Human immunoglobulin G (IgG)
rProtein A	Mouse IgG in serum
Sulfanilamide	Carbonic anhydrase
N-Acetyl-D-glucosamine	Wheat germ lectin
Heparin	Lysozyme, antithrombin III
Concanavalin A	Ribonuclease B, Horseradish peroxidase
Cibacron Blue	Bovine serum albumin
Aminophenylboronic acid	Uridine, adenosine, sorbitol

ville, MO, U.S.A.). The affinity packings used were the Waters Protein-Pak affinity epoxy-activated packing, epoxy-activated Sepharose from Pharmacia (Piscataway, NJ, U.S.A.) and BakerBond™ Prepscale™ glycidoxypropyl affinity matrix from J. T. Baker (Phillipsburg, NJ, U.S.A.). The Microcolumns and the advanced purification (AP) glass columns were from Waters, a Division of Millipore (Milford, MA, U.S.A.). Two HPLC systems were used. The Model 650 advanced protein purification system with the Model 484 tunable absorbance detector and the 745 data module was used. The second system was the Model 600E solvent delivery system with the 440 detector or 490 programmable multiwavelength detector. The HPLC systems were connected to a Foxy fraction collector and Waters Model 712 or 710 WISP autosampler. The Model 600E system was interfaced to the Waters 840 data and control station.

IMMOBILIZATION METHODS AND AFFINITY CHROMATOGRAPHY

Lectin isolation of N-acetylglucosamine affinity column

N-Acetylglucosamine coupling to epoxy-activated packing. Epoxy-activated silica (3 g, 154 μmol/g) was washed with water on glass filter followed by 0.1 *M* sodium hydroxide (20 ml each). N-Acetylglucosamine (200 mg) dissolved in 0.1 *M* sodium hydroxide was added to the silica. The mixture was heated at 45°C on a shaker for 18 h. The reaction mixture was centrifuged and the supernatant was discarded. The pellet was washed with water (2 ×), 0.05 *M* Tris chloride (pH 8) (2 ×), and 0.5 *M* sodium chloride (1 ×). The pellet was resuspended in 0.07 *M* sodium phosphate (pH 7.0) 0.15 *M* sodium chloride and poured into an AP1 (100 × 10 mm) glass column. The column was equilibrated with the same buffer.

Wheat germ preparation. Wheat germ meal (50 g) was stirred in hexane (150 ml) for 2 h. The mixture was filtered and the residue was air dried overnight. The residue was suspended in 0.05 *M* sodium acetate (pH 4.5) (250 ml). The protein was precipitated from solution by addition of solid ammonium sulfate to 40% saturation. The precipitate was collected by centrifugation and resuspended in acetate buffer. The solution was dialyzed against the same buffer overnight. The extract was centrifuged and the supernatant was applied to the N-acetyl-D-glucosamine (GlcNAc) column.

Sample application and elution. Wheat germ agglutinin (WGA) standard (10 μl, 5 mg/ml) in 0.07 *M* sodium phosphate (pH 7.0) with 0.15 *M* sodium chloride (buffer A) was injected at a flow-rate of 1 ml/min. At 7 min the eluent was switched to buffer B (buffer A + GlcNAc, 10 mg/ml) to elute bound protein. Crude wheat germ preparation (1.0 ml of 6.8 mg/ml) was applied and WGA was purified. WGA (1.2 mg/ml) was recovered with elution buffer.

Carbonic anhydrase isolation on the sulfanilamide column

Conditions for coupling sulfanilamide to epoxy-activated packing. Epoxy-activated packing (5 g, 155 μmol/g) was washed with water and methanol; the supernatants were decanted. Sulfanilamide (266 mg) dissolved in 3.6 ml of methanol was added to the washed silica in a capped glass test tube. The coupling was carried out on a rotating wheel for 24 h at 45°C. The supernatant was removed on a coarse-porosity sintered-glass funnel, and the packing was washed with 20 ml each of methanol (3 ×), methanol–water (1:1, v/v) (1 ×), water (1 ×), and 1 *M* sodium chloride (1 ×). All supernatants were saved for quantitation of sulfanilamide remaining in

solution by HPLC (amount bound determined by the difference between amount applied and amount in solution). The material was slurry-packed into an AP1 glass column and deactivated by recycling 100 ml of 1 *M* ethanolamine (pH 10) through the column at room temperature for 24 h.

Bovine hemolysate preparation. Bovine whole blood was centrifuged at 7700 *g* for 20 min to remove the red blood cells. The pellet (erythrocytes) was washed with cold 0.9% sodium chloride (3 ×) and centrifuged at 3000 *g* for 10 min after each wash. The pellet was washed with cold water (1:1, v/v) to lyse the cells and centrifuged at 3000 *g*. The supernatant (hemolysate) was used for chromatography and the pellet was discarded.

Sample application and elution. The hemolyzate (200 μl) was applied to the AP1-sulfanilamide affinity column. The column was washed with buffer C [100 m*M* Tris (pH 8.7) with 200 m*M* sodium sulphate] until the absorbance at 280 nm returned to baseline; the retained material was eluted with buffer D [50 m*M* Tris (pH 6.5) with 200 m*M* potassium thiocyanate]. The enzyme activity of the retained material (the peak fractions), carbonic anhydrase, was measured by esterase activity for conversion of nitrophenyl acetate to nitrophenol, and monitored by the increase in absorbance at 400 nm.

Other ligand couplings

Similar conditions were used for immobilizing heparin and rProtein A. The Protein-Pak affinity epoxy-activated packing was slurried in coupling buffer [buffer–packing, 3:1 (v/v)], allowed to settle and then the buffer was decanted. Heparin or rProtein A (3 mg/ml packing) were dissolved in the coupling buffer, 0.1 *M* sodium borate with 0.15 *M* sodium chloride at pH 8.5–9. After the ligand was added to the packing the coupling was carried out for three days at room temperature. Following coupling the packing was blocked with 1 *M* ethanolamine in 0.1 *M* sodium borate (pH 9.0) for 24 h. Excess ligand and blocking solution were removed by using 6 ml of solution per gram of packing for each wash: coupling buffer (1 ×); 1 *M* sodium chloride (4 ×), and 10 m*M* sodium phosphate (pH 7.4) with 150 m*M* sodium chloride (2 ×).

Determination of human IgG capacity

The coupling of epoxy-activated Sepharose 6B from Pharmacia and BakerBond Prepscale glycidoxypropyl affinity matrix (silica-based) were carried out in the Waters Microcolumn. The performance of the Waters Protein-Pak affinity epoxy-activated Microcolumn was compared to the other two packings. The volume of each packing used in the study was kept constant (silica: 1 g = 2 ml; agarose, 1 g = 3 ml). rProtein A (3 mg/ml) was immobilized on the three epoxy-activated packings at room temperature for 72 h in 0.1 *M* sodium borate (pH 8.3) with 0.15 *M* sodium chloride. The packings were then blocked with 1 *M* ethanolamine in the sodium borate buffer for 24 h. The amount of rProtein A coupled was determined by its extinction coefficient at 228 nm and/or by size exclusion chromatography on a Waters Protein-Pak 200SW column.

The human IgG (5–10 mg/ml) was applied to the rProtein A affinity packings after they were equilibrated in the binding buffer, 100 m*M* potassium phosphate (pH 7.4) with 0.15 *M* sodium chloride. After washing three times with the binding

buffer to remove unretained IgG, the bound IgG was eluted with 0.15 *M* sodium citrate (pH 2.2) with 0.15 *M* sodium chloride. The amount of human IgG was determined by its extinction coefficient, 1.4 A.U./(mg/ml), at 280 nm.

RESULTS AND DISCUSSION

Fig. 1 presents the chromatography for rProtein A coupled to the Protein-Pak affinity epoxy-activated packing in an analytical column (75 × 3.9 mm I.D.). Native Protein A is a 42 000 dalton polypeptide from *Staphylococcus aureus* and has five homologous sites for binding immunoglobulins through their Fc regions [6]. The rProtein A is used extensively for purification of immunoglobulins. Fig. 1 shows that human IgG was retained on the rProtein A affinity packing. When an IgG sample containing 2.6 mg in 100 μl was applied to the column, a small unretained breakthrough peak was observed, indicating that the column bound about 90% of the immunoglobulin.

Heparin is an acid mucopolysaccharide from animal tissue which prevents blood clotting. It contains an equal amount of D-glucosamine and D-glucuronic acid with O-

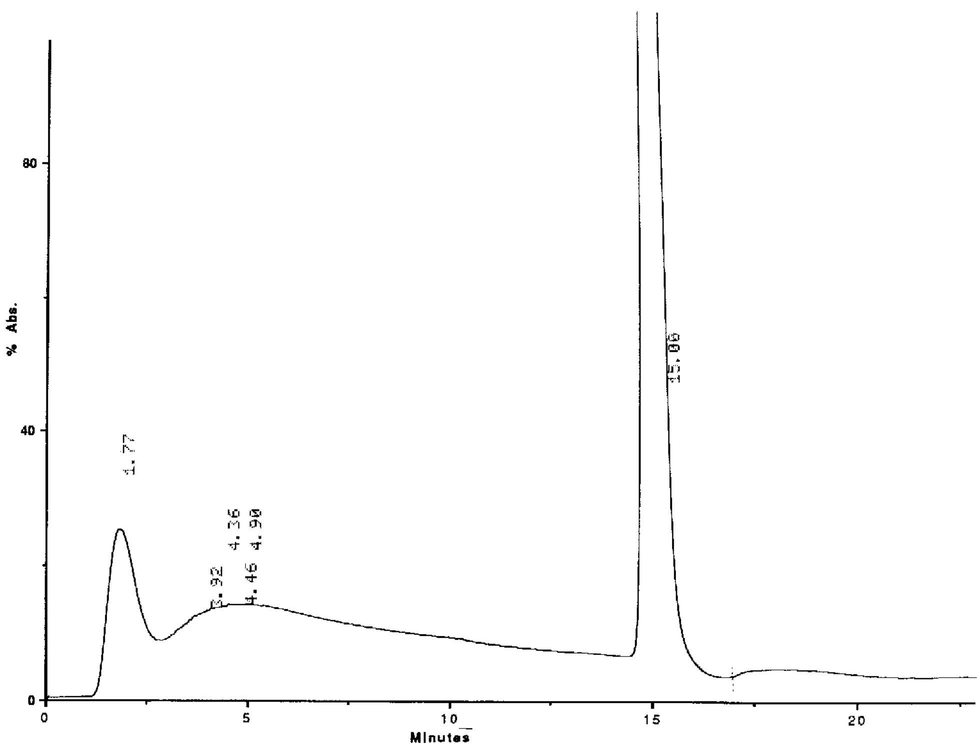

Fig. 1. Immobilized Protein A on Protein-Pak affinity epoxy-activated bulk packing for IgG isolation. Analytical column, 75 × 3.9 mm I.D.; buffer A, 10 m*M* sodium phosphate buffer (pH 7.4) with 150 m*M* sodium chloride; buffer B, 2% citric acid (pH 2.5) 150 m*M* sodium chloride; gradient and flow-rates, 0 to 10 min at 0.5 ml/min in buffer A, 10 to 14 min at 1 ml/min in buffer B and 14 to 22 min in buffer A at 1.5 ml/min for equilibration; detector, 280 nm. The sample was 2.6 mg human IgG (100-μl injection).

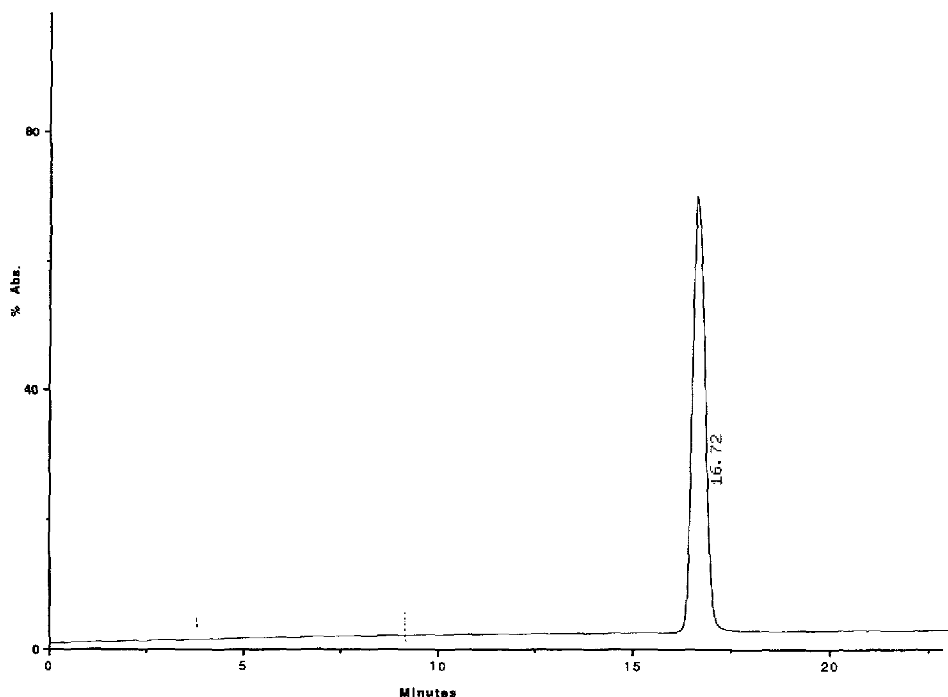

Fig. 2. Immobilized heparin for lysozyme isolation. Analytical column, 75 × 3.9 mm I.D.; buffer A, 10 m*M* Tris chloride (pH 7.5) with 10 m*M* sodium chloride; buffer B, 10 m*M* Tris chloride (pH 7.5) with 0.3 *M* sodium chloride; gradient and flow-rate, 0 to 10 min at 0.5 ml/min, 10 to 14 min at 1 ml/min in buffer B, 14 to 22 min in buffer A at 1.5 ml/min for equilibration; detector, 280 nm. The sample was a 10-μl injection of lysozyme, 200 μg.

and N-sulphate residues [7]. The heparin ligand is frequently used for the purification of antithrombin III, an inhibitor of thrombin [8]. The heparin affinity column bound lysozyme and antithrombin III as noted in Figs. 2 and 3, respectively. The heparin column had a capacity of about 2 mg lysozyme/ml based on protein breakthrough. Antithrombin III was retained on the column and is the third peak in Fig. 3. The peak at 14.96 min (peak 2) in Fig. 3 may be antithrombin II, which also binds to heparin but with less affinity than antithrombin III. Due to the significantly stronger affinity of heparin for antithrombin III than for lysozyme, a 10-fold higher salt concentration (3 *M versus* 0.3 *M* sodium chloride) was required to elute the antithrombin III.

Two applications involved the use of affinity chromatography to purify a selected material from a crude mixture. Carbonic anhydrase, a widely occurring zinc-containing enzyme, plays an important role in respiration, carbon dioxide transport, and other physiological processes [7]. Carbonic anhydrase is found in several places in the body including the red blood cells or erythrocytes [9]. Sulfanilamide, an inhibitor of carbonic anhydrase, was coupled to the packing at almost equal molar amount to the epoxy-activation level (155 μeq./g). In order to determine that the affinity column was functioning as expected, a mixture of hemoglobin and carbonic anhydrase standards was applied to the column; the earlier protein was unretained, whereas the enzyme was

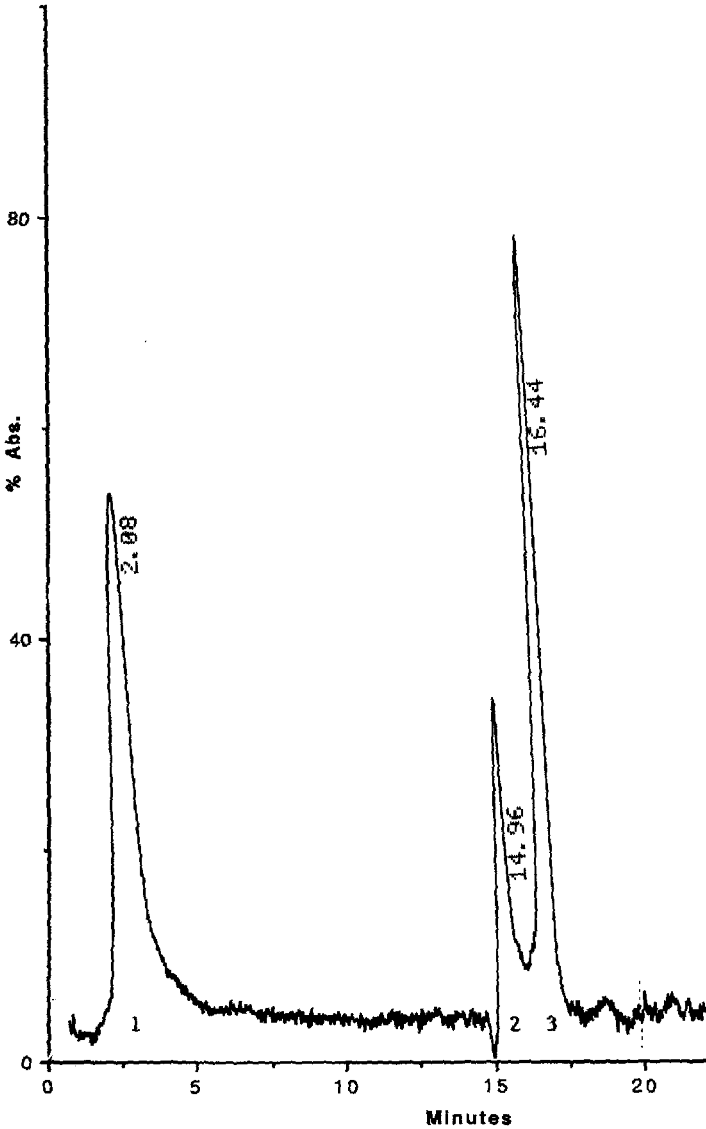

Fig. 3. Heparin coupled to Protein-Pak affinity epoxy-activated bulk packing for antithrombin III isolation. Analytical column, 75 × 3.9 mm I.D.; buffer A, 10 m*M* Tris chloride (pH 7.5) with 10 m*M* sodium chloride; buffer B, 10 m*M* Tris chloride (pH 7.5) with 3 *M* sodium chloride; gradient and flow-rates were 0 to 10 min at 0.5 ml/min, 10 to 14 min at 1 ml/min in buffer B, 14 to 22 min in buffer A at 1.5 ml/min for equilibration; detector, 280 nm. The sample was a 100-μl injection of antithrombin III, 29 μg. Peaks 1 and 3 are antithrombin III; peak 2 is unknown.

bound and released by a chaotropic agent, with 100% recovery, as shown in Fig. 4. The bovine hemolyzate was prepared and 200 μl of the preparation was applied to the column; the chromatogram is presented in Fig. 5. The second peak contained the esterase for conversion of nitrophenyl acetate to nitrophenol. Table II shows that of

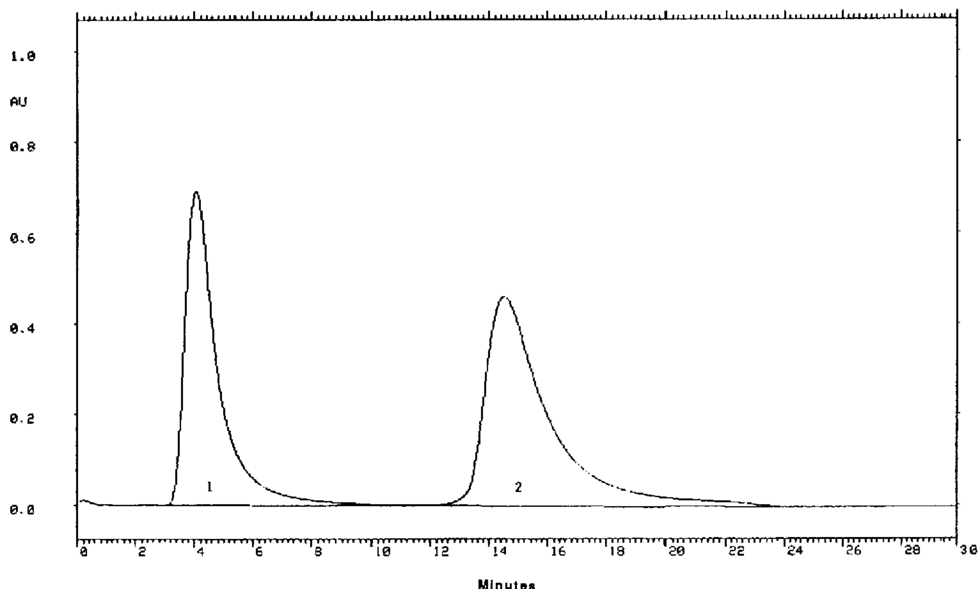

Fig. 4. Carbonic anhydrase isolation on sulfanilamide-coupled Protein-Pak affinity epoxy-activated bulk packing. AP1 glass column, 100 × 10 mm I.D.; buffer A, 100 m*M* Tris sulphate with 200 m*M* sodium sulphate (pH 8.7); buffer B, 200 m*M* potassium thiocyanate in 50 m*M* Tris sulphate (pH 6.5). Initial flow-rate, 1 ml/min; from 2 to 40 min, 2 ml/min. The chromatography was isocratic in buffer A for 7 min, followed by a step to 100% buffer B for 10 min; at 17 min the column was equilibrated in buffer A. The sample contained hemoglobin (1.5 mg, peak 1) and carbonic anhydrase (1.5 mg, peak 2). The recovered carbonic anhydrase had a specific activity of 13 units/mg for esterase activity for converting nitrophenyl acetate to nitrophenol.

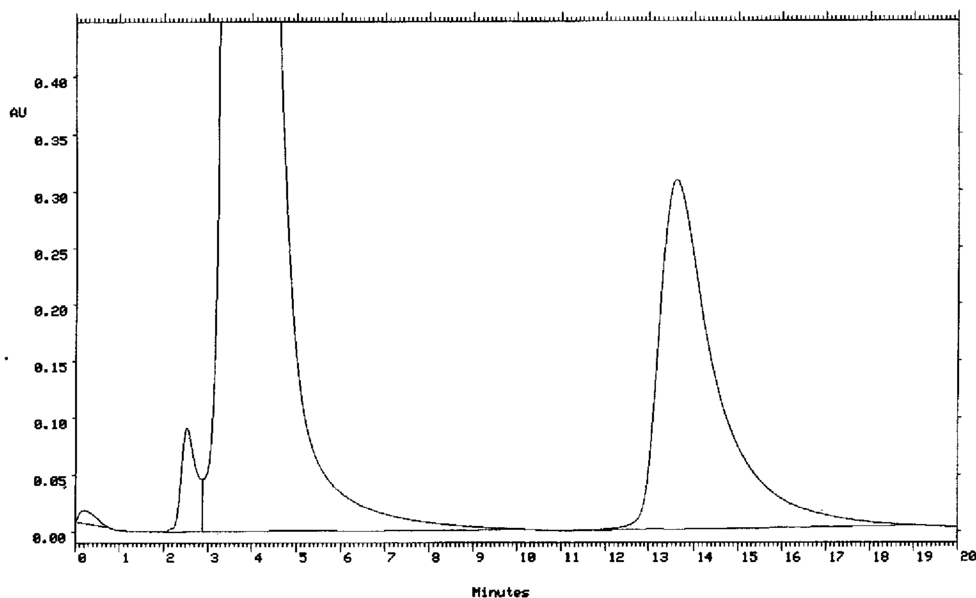

Fig. 5. Carbonic anhydrase purification from bovine hemolysate using sulfanilamide-coupled Protein-Pak affinity epoxy-activated bulk packing. The same as Fig. 4 except the sample was 200 μl hemolyzate from bovine erythrocytes. Peak 1 contains mostly hemoglobin (major component, 4.2 mg). Carbonic anhydrase (0.59 mg) is in peak 2 and was identified by its biological activity.

TABLE II

PURIFICATION OF CARBONIC ANHYDRASE FROM BOVINE HEMOLYZATE

The 200 μl of bovine hemolyzate (24 mg protein/ml) applied to the sulfanilamide-affinity column gave a retained peak (peak 2) with carbonic anhydrase activity. Peak 2 contained 12% of the protein applied and had a purification factor of 8.

	Protein concentration (mg/ml)	Total volume (ml)	Total protein (mg)	Total biological activity[a] (units)	Specific activity (units/mg)	Purification factor	% Recovery of biological activity
Crude	23.8	1.0	23.8	8.75	0.37	1	100
Affinity purified (peak 2)	0.06	10	0.59	1.75	2.96	8	100

[a] Carbonic anhydrase activity of the peak fractions was measured by converting nitrophenyl acetate to nitrophenol at pH 7.5.

the 4.8 mg applied, 0.59 mg was carbonic anhydrase and that there was an 8-fold purification of the enzyme.

The epoxy packing was able to bind covalently N-acetyl-D-glucosamine at pH 11, as had been reported for the epoxy-activated Sepharose 6B [10]. The mode of attachment of the ligand to the support is through one of the hydroxyl groups on the sugar; C-6 hydroxyl is unrestricted and kinetically favored over the C-4 and C-3

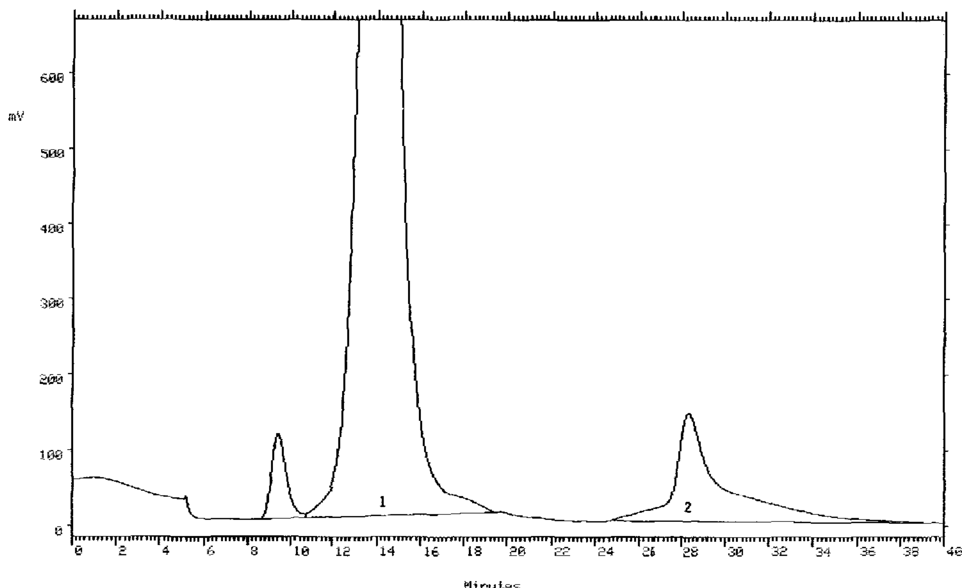

Fig. 6. Wheat germ lectin isolation using N-acetyl-D-glucosamine as ligand. API glass column, 100 × 10 mm I.D.; buffer A, 70 m*M* sodium phosphate (pH 7.0) with 150 m*M* sodium chloride; buffer B, buffer A with N-acetyl-D-glucosamine (10 mg/ml); gradient, 0 to 7 min at 1.0 ml/min in buffer A and a step at 7 min to buffer B (1 ml/min); detector, 280 nm. The sample was 1 ml of crude wheat germ preparation; 1.2 mg/ml of the lectin was eluted.

TABLE III

HUMAN IgG CAPACITY ON EPOXY-ACTIVATED PACKINGS WITH rPROTEIN A LIGAND

Recombinant Protein A was immobilized at 25°C for 72 h in 0.1 *M* sodium borate (pH 8.5) with 0.15 *M* sodium chloride. Human IgG binding buffer, 10 m*M* sodium phosphate (pH 7.4) with 0.15 *M* sodium chloride; and elution buffer, 0.1 *M* sodium citrate (pH 2.2) with 0.15 *M* sodium chloride.

	Capacity mg IgG/ml packing	% Recovery bound IgG	Ratio mg IgG/mg rProtein A ligand
Waters Protein-Pak Affinity Epoxy-Activated Microcolumn	17	86	8
Pharmacia epoxy-activated Sepharose 6B	11	63	5.5
Bakerbond Prepscale glycidoxypropyl affinity matrix	13	73	6.5

hydroxyl groups. The C-2 hydroxyl group is blocked and cannot react. Wheat germ lectin specifically binds N-acetyl-D-glucosamine and its polymers. The affinity packing in the AP1 glass column retained the wheat germ agglutinin standard, which was eluted by competitive binding of the free N-acetyl-D-glucosamine in the mobile phase at a concentration of 10 mg/ml. Elution by the free sugar has been found to be a more gentle method than the more common technique of using dilute acetic acid [10]. Wheat germ agglutinin was purified from the wheat germ meal preparation on the N-acetyl-D-glucosamine column, as presented in Fig. 6. An amount of 50 g of wheat germ meal yielded a crude preparation with 6.8 mg/ml of total protein. When the crude sample was applied to the affinity column, 1.2 mg/ml of wheat germ agglutinin was recovered with the elution buffer.

A study was conducted to compare the performance of the Protein-Pak affinity epoxy-activated packing with that of two other epoxy-activated materials, epoxy-activated Sepharose and Bakerbond Prepscale glycidoxypropyl affinity packings. The three packings coupled about the same amount of rProtein A, 0.7 mg/ml. The Protein-Pak affinity epoxy-activated Microcolumn showed a slightly higher human IgG capacity and % mass recovery than the Pharmacia and Baker columns, 17 mg human IgG/ml with 86% recovery *versus* 11 mg/ml with 63% and 13 mg/ml with 73% recovery, respectively. The data are summarized in Table III. rProtein A on the Waters affinity packing also performed better than a protein A column on the epoxy-activated silica from Alltech which had a human IgG capacity of 7 mg/g or 14 mg/ml [11].

CONCLUSIONS

Since affinity chromatography is the only chromatographic method in which specific molecules are isolated on the basis of their selective interaction with an immobilized ligand, there is increasing demand for immobilization of ligands via a variety of functional groups. Activated affinity packings such as Protein-Pak affinity epoxy-activated packing, which can covalently bond molecules with amino, hydroxyl or sulfhydryl groups, have broad applications for purification of proteins.

The applications shown here demonstrated that this epoxy-activated packing can bind small and large molecules through amines or hydroxyl groups. It was possible

to recover the proteins retained on the immobilized ligands with maintenance of biological activity. The rProtein A ligand performed better for human IgG isolation when immobilized on the Protein-Pak epoxy-activated Microcolumn than on either the Pharmacia or Baker affinity packing.

This more rigid packing material can withstand greater backpressures, thus allowing higher flow-rates and significantly shorter analysis times for large scale preparative chromatography. Most purifications were carried out on the derivatized Protein-Pak affinity epoxy-activated packing in less than 1 h. The results of these studies show the potential for preparative-scale separations that can be performed at high flow-rates, keeping analysis times significantly shorter than those for soft gels (for which the slower linear velocities must be used).

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