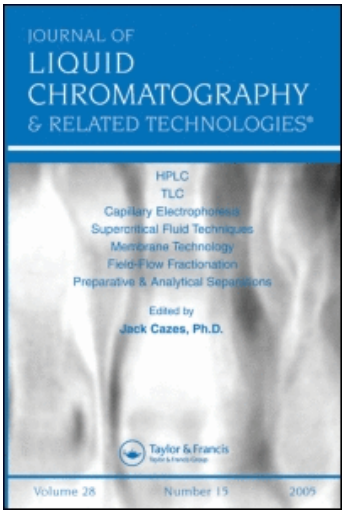

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Fast Affinity Chromatography Using Small Particle Silica-Based Packing Materials

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FAST AFFINITY CHROMATOGRAPHY USING SMALL PARTICLE SILICA-BASED PACKING MATERIALS

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Summary

Affinity chromatography is one of the most powerful techniques for the purification of biologically active proteins available (for review see [1]). The ability of this method to purify proteins is based on highly specific, selective or characteristic interactions with immobilized ligands. Several advantages over traditional soft gel affinity supports have been observed with the use of small particle silica based materials for high performance affinity chromatography. These include greatly improved mass transfer properties which

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allow separations that are not always practical in the low performance mode, greatly reduced equilibration and isolation times, high available ligand densities, small elution volumes, excellent recovery of very small quantities of protein and high dynamic capacities. The criteria for developing a general, derivatizable, high performance support for high performance affinity chromatography are discussed. The step-by-step examination of these criteria and experimental evidence for determining parameters such as ligand density, non-specific adsorption and column lifetime for such a system are described. Chromatographic results are shown for preparative separations of (i) receptor proteins, (ii) antibodies and (iii) active enzymes.

Introduction

Affinity chromatography has been extensively utilized for the purification of proteins [2]. Typically soft agarose supports with a variety of immobilization chemistries are utilized [3,4]. The chromatographic advantages of speed, resolution and recovery of purified biomolecules with fast or high performance affinity chromatography are obvious. While significant progress has been made in this area [4-6] application of the technique has been limited to those laboratories with the expertise to develop their own supports and adapt them for high performance use. We have sought to develop materials readily usable and available for affinity chromatography separations. For this purpose (preparative purification of proteins) a good generalized support requires; applicability to and compatibility with isolations involving either weak or strong affinity interactions; good capacity; a surface environment which preserves protein structure and activity; broad applicability to ligands or molecules of different sizes; the ability to tolerate a broad range of solvents and the rapid change in solvent composition necessary for efficient elution of proteins from a high capacity support; and good recovery of even very small amounts of protein.

High performance affinity chromatography imposes additional restraints on the support as the high solvent flow rates employed require a rigid, non-compressible matrix. The use of high flow rates also dictates a particle size which is small enough to allow good mass transfer properties. Pore structure and pore diameter will also influence the mass transfer properties of the support and must be considered. Finally, the derivatized support must be packed efficiently into a suitable column for chromatographic use. A generalized support designed for high performance affinity chromatography should also have all the positive attributes of a traditional affinity supports combined with the excellent mass transfer properties of a modern high performance chromatographic column. Additional desirable characteristics are; negligible ligand leakage resulting in good stability for the derivatized support; simple derivatization procedures with a broad range of ligand chemistries; derivatization of pre-packed columns; and high dynamic capacities (capacity under flow conditions).

In this study we have examined silica based wide pore (300A dia. average) materials with a bonded layer of hydroxyl and ether functions. Particle diameters were 7-10μ. Reactive sites for ligand attachment were epoxy moieties which react with nucleophiles such as primary amino, sulfhydryl or hydroxyl functions. The resulting chemical linkage is stable under chromatographic conditions compatible with silica based supports. Results of the use of these materials as a research tool for rapid purification and characterization of protein are discussed.

Materials and Methods

Bovine serum albumin (BSA), ovalbumin and concanavalin A were obtained from Sigma Chem. Co. (St. Louis, Mo.). Aminospiroperidol and iodospiroperidol were prepared by Dr. Carl Kaiser (SK&F - Medicinal Chemistry). All other reagents

were of the highest grade commercially available. All chromatographies were performed with a Beckman 344 chromatographic system.

Ligand Immobilization and Protein Chromatography

Columns (0.46 x 5 cm) containing 0.4-0.5g of 5μ, 10μ or 20μ activated epoxy supports were prepacked. The total available volume in each column was 0.5 ml. The level of active epoxide functions were 70μM/g. Ligand in each case was attached to the support by recirculating an appropriate solution through the column at 0.2-0.5 ml/min overnight. Afterwards excess ligand was removed by extensive washing with immobilization buffer or where appropriate organic solvent. Columns were equilibrated in chromatography buffer and samples applied to and eluted from the affinity columns as described in figure legends. These columns have been prepared on a commercial scale and are available from Beckman instruments as Ultraffinity™ EP columns.

Results

Dynamic Capacity

Studies used to determine dynamic capacity and stability of prepacked epoxide columns were based on the repeated isolation of the lectin Concanavalin A (Con A) on a 0.46 x 5cm column (10μ particle size) derivatized with the glycoprotein ovalbumin.

Buffer containing Con A (0.2 mg/ml) was pumped through the column at 1 ml/min until a 1% deflection of the absorbance at 280 nM was observed relative to a standard solution of 0.2 mg./ml Con A (Figure 1.) The 1% breakthrough capacity observed for the 0.46 cm diameter column at 1 ml/min was 10.2 mg of

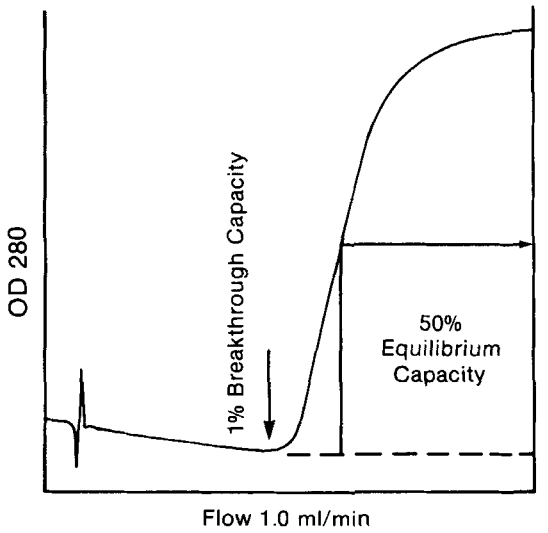

Fig. 1. Dynamic Capacity of 10ξ Particle columns Measured with Concanavalin A and Immobilized Ovalbumin

A prepacked column (0.46 x 5 cm) was derivatized with ovalbumin (200 mg) dissolved in 1M potassium phosphate buffer pH 7.0 (20 ml) by recycling through the column at 0.2 ml/min overnight. The derivatized column was washed with 0.1M phosphate buffer (pH 7) for 2 hours and then converted to loading buffer [Tris (.025M), NaCl (0.25M), $MnCl_2$ (0.5 mM, $CaCl_2$ (0.5 mM), pH 6.8].

Specificity for Concanavalin A (Con A) was demonstrated by injecting a mixture of Con A and bovine serum albumin (5 mg, 50 µl) into the column at a flow rate of 0.5 ml/min, after 4 ml had passed through the column elution buffer, (100 mM α-methyl mannoside in loading buffer) was introduced at 0.5 ml/min, elution of Con A free from BSA followed.

To measure dynamic capacities of the column a solution of Con A (.2 mg/ml) was passed through the column until unretained Con A was eluted to a 1% break through points). The column was allowed to further saturate before regeneration with α-methylmannoside.

Con A. The 50% equilibrium capacity was 12.5 mg at 1 ml/min. Since the column contains ~0.4 g of packing material, the dynamic capacity of this support for Con A at a linear velocity of 6 cm/min is, ~26 mg/g of support. The dynamic capacity of a similarly derivatized 1 x 10 cm column, at 1 ml/min (2 mg/ml) lectin solution, is 110 mg of Con A (28 mg/g of support).

Column dynamic capacities at various flow rates were also determined for the 5μ and 20μ diameter supports with similar pore size and epoxy surface. The plot in Figure 2A shows a comparison of these materials using the ovalbumin/Con A system. The data indicates little or no advantage for the 5μ diameter support over the 10μ but indicates a large drop in dynamic capacity for the 20μ material. When the data for the different particle sizes is normalized to reflect a constant pore volume for each of the particle sizes (Figure 2B), the dynamic capacity clearly increases as the particle diameter decreases. This result is expected if the kinetics of the affinity adsorption step are limited by diffusion at the flow rates examined. Since the adsorption step will often be diffusion limited most preparative affinity isolations will be improved by using particle sizes of 10μ or less.

Stability

The stability of the derivatized supports at 20°C was investigated by constantly pumping a 3M NaCl solution buffered to pH 7.0 through a 0.46 x 5 cm column derivatized with ovalbumin. The buffer was pumped at 1 ml/min for a total of 120 liters. The column dynamic capacity was determined at 15L intervals. THe resulting plot of column capacity vs total volume of buffer (Figure 3) indicates that the column remains functional throughout the test. The capacity after 120 liters of use was ~75 percent of the starting capacity. The column retained >95 percent of its capacity through ~35L of use. This would indicate that for isolations involving sample volumes of less

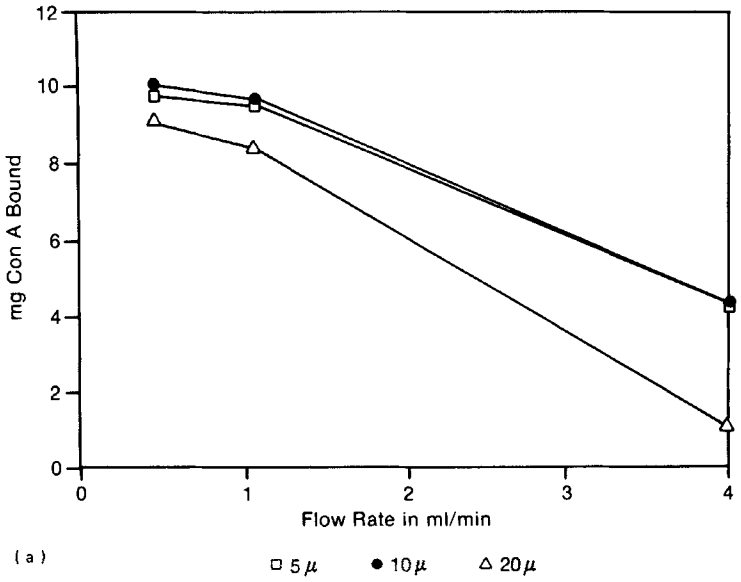

Fig. 2 Dynamic Capacity, Total Capacity and Particle Size

A) Epoxide coated silica particles of 5μ, 10μ and 20μ were examined for dynamic flow capacities as described in the text and in figure 1. Results obtained with these materials are shown as mg concanavalin A bound as a function of flow rate in ml/min. In B the data obtained in (A) was normalized to reflect a constant pore volume for each of the 5μ, 10μ and 20μ materials. As shown optimal results are obtained with particles of 10μ or smaller.

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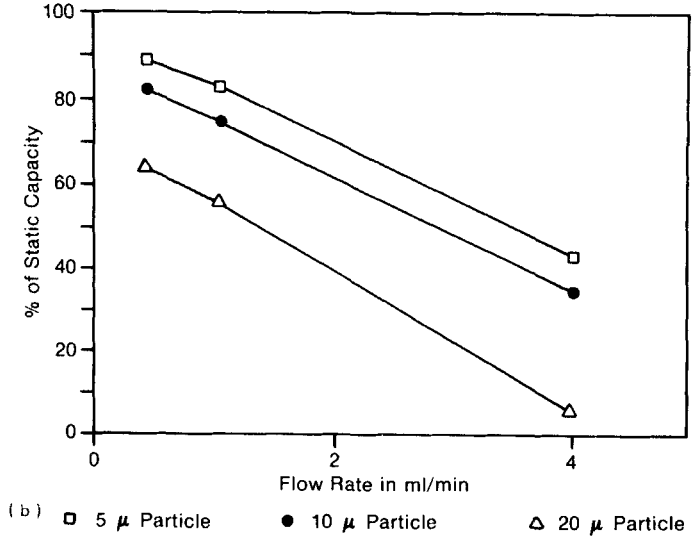

Fig. 2 (continued)

than 30 ml, over 1000 affinity isolations would be possible with no observable change in capacity.

Non-specific Adsorption

Adsorption mechanisms, other than biospecific interactions, generally arise from (i) the solid support; (ii) the spacer (if any) employed; (iii) the ligand itself; and (iv) the deactivating group (if any) employed.

Non-specific hydrophobic properties of the materials developed here were investigated by derivatizing a column with glycosamine. A sample of the protein BSA which should have no specific interaction with the ligand was chromatographed repeatedly. After each run, the mobile phase salt concentration was increased. The retention time of any eluted BSA was

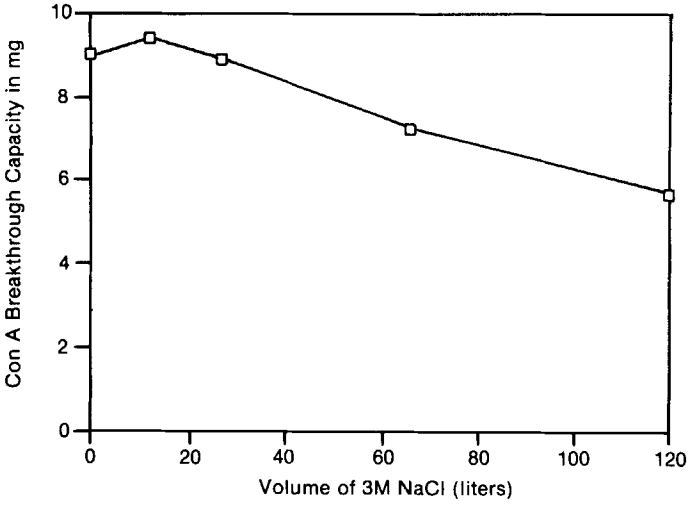

Fig. 3. Column Stability

In order to examine column stability an ovalbumin derivatized support (10μ) prepared as described in Fig. 1 was washed with extensive volumes of 3M NaCl in 50 mM tris-HCl buffer pH 7.0 at 1 ml/min. After various levels of washing, columns were checked for Con A binding capacity as described in the text. After 120 liters of washing the column retained 75 percent of its original capacity.

calculated for each run. The plot (Figure 4) indicates that for the hydrophobic protein BSA no retention occurs even at high salt concentrations (3M NaCl). The retention at low salt concentration is due to ionic interactions with the amine group of the glucosamine ligand.

Dynamic Overloading

The effect of particle size on isolations involving small sample volumes with very high concentrations of protein was also examined. Columns packed with

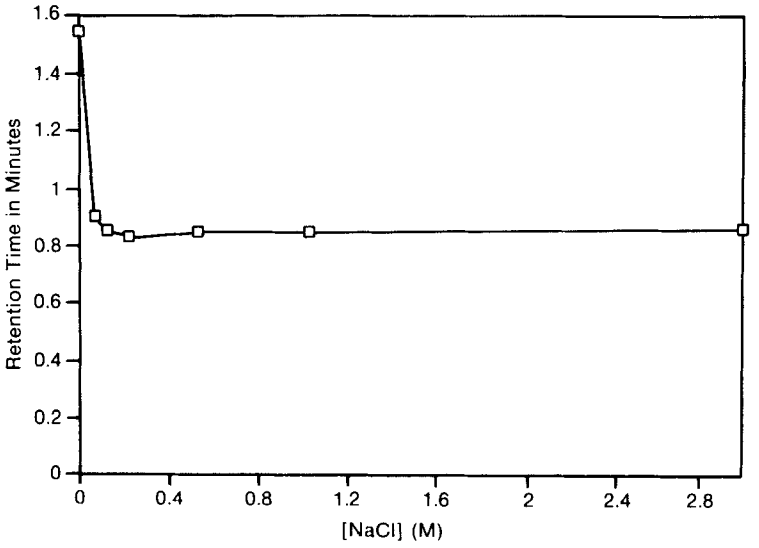

Fig. 4. Non-specific Adsorption and Salt Effects

To test supports for non-specific interactions columns were derivatized with glycosamine and bovine serum albumin applied to the column in the presence of varying concentrations of NaCl. As shown little interaction with the support occurred above concentrations of 50 mM NaCl.

5μ, 10μ, or 20μ epoxy supports were derivatized with BSA and used to isolate rabbit anti-BSA antibodies from high titer serum. A fixed volume of serum (200μl) containing 560μg (2.8 mg/ml) of anti-BSA specific IgG was injected at various flow rates for each particle size. The retained antibody was eluted from the column with low pH buffer (Figure 5A) and the recovery estimated by elution peak area at 280 nm. Purity was estimated by size exclusion HPLC using a Toyosoda TSK-3000SW column equilibrated with phosphate buffered saline.

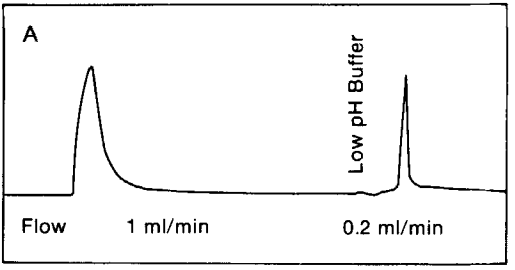

Fig. 5. Immunoaffinity Chromotography and Dynamic Capacities

The use of immobilized proteins was examined for purification of antibodies using BSA and anti-BSA antibodies. BSA was immobilized by recycling a 10 mg/ml solution through a 0.46 x 5 cm column at 0.2 ml/min. overnight. The derivatized column was washed with 0.1 M potassium phosphate pH 7 at 1 ml/min for one hour and converted to the loading buffer (0.02M phosphate, 0.2M NaCl, pH 7.0) for antibody isolation. Serum containing goat-anti BSA (20-5000µl) was injected into the column at 1 ml/min. Non-specific elution was with pH 2.4 buffer. Purity of the eluted peak was determined by size exclusion HPLC on a TSK 3000SW column.

In A is shown the chromatogram obtained on injection of 100µl of serum.

In B column dynamic capacities were determined as described in fig. 2. Values were estimated for 5µ, 10µ and 20µ packing materials.

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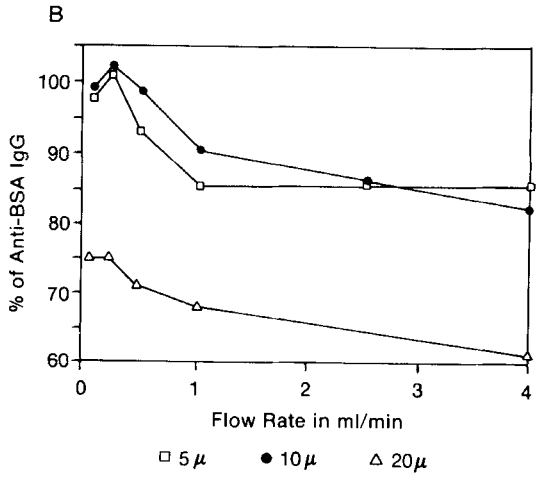

Fig. 5 (continued)

The resulting plots (Figure 5B) show the amount of antibody recovered from each injection as a function of flow rate. The 10μ diameter support recovered 100 ± 2% of the antibody at flow rates up to 0.5 m./min (3 cm/min). The 5μ support shows similar recoveries of antibody at lower flow rates (0.3 ml/min). Presumably the lower ligand concentration of the 5μ support was responsible for this effect. The 20μ support provides poor recoveries at the flow rates tested (>0.10ml/min, ~1cm/min). The static capacity of the saturated 10μ support was >10 mg of antibody.

Thus, even with static capacities which are much higher than required for binding the sample protein by affinity adsorption, dynamic overloading of the support will occur if sample concentrations are sufficiently high. The sample concentrations at which overloading will occur depends on the ligand concentration, the total volume of support, mass transfer limitations and the rate of adsorption for a given affinity interaction.

Isolation of Biologically Active Proteins By High Performance Affinity Chromotography

In order to examine the utility of these supports for high performance immunoaffinity chromotography rabbit immunoglobin was immobilized by recirculating a solution (0.7 mg/ml in 1M potassium phosphate buffer pH 7) through an epoxy column overnight at 0.2 ml/min. After washing to remove unbound protein, antisera containing goat anti-rabbit immunoglobin was injected onto the column and adsorbed antibody eluted with low pH buffer. To estimate purity eluted antibodies were chromotographed on a TSK-3000 SW size exclusion column. As shown in Figure 6 a substantial purification was observed. When antibodies were tested for activity on dot blot or Western probe assays little or no loss of activity was found to have occurred.

Enzymes and Receptor Proteins

Rapid isolation and purification of biologically active proteins using epoxide supports was explored for several significant proteins. Two examples are (i) mammalian acetylcholinesterase, from fetal bovine serum and (ii) dopamine receptor from bovine anterior pituitary membranes.

Acetylcholinesterase (AChE) is an important enzyme in neural transmission. Chemical characterization of this protein requires substantial amounts of purified enzyme. Recently, a method was developed for purifying large quantities of mammalian AChE from fetal bovine serum using procainamide affinity chromatography [8]. Adapting this affinity system to a high performance mode provides rapid purification of the enzyme (~90 minutes). To obtain similar levels of purification by soft gel techniques, affinity purification using a Sepharose 6B procainamide column (10 ml); size exclusion

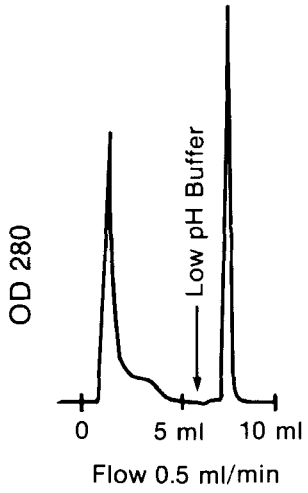

Fig. 6. Purification of Goat Anti Rabbit Immunoglobin

Rabbit immunoglobin was immobilized by recirculating a solution (0.7 mg/ml) through a 0.46 x 5 cm column overnight at 0.5 ml/min. The column was then washed extensively with 25 mM $NaPO_4$ pH 7.0 150 mM NaCl. Goat anti-rabbit immunoglobin antiserum was injected (2 ml) onto the column at 1 ml/min. and retained sample washed with 12 ml of PBS prior to elution with dilute HCl pH 2.0.

chromatography on Sepharose 6B (2.5 x 94 cm); pooling activity; and rechromatography on Sepharose 6B is required. The overall procedure requires several days to accomplish [9].

To determine appropriate chromatographic conditions, procainamide high performance affinity chromotography of fetal bovine AChE was first done on 0.46 x 5 cm columns. Partially purified AChE (0.1 to 1.0 mg protein) was applied and after washing with equilibration buffer and 0.2M phosphate pH 7.0

Table I. Binding of AchE To A Procainamide High Performance Affinity Column (0.46 cm x 5 cm As A Function of Flow Rate.

Flow Rate (ml/min)	% AChE Bound
1.0	0
0.5	1
0.2	48
0.15	95

A flow rate of 1 ml/min could be used to obtain 98% binding of applied AChE with a large procainamide column (1 cm x 10 cm).

buffer, enzyme was eluted with the selective AChE inhibitor: decamethonium. The amount of enzyme bound was found to be flow rate dependent (Table 1).

Larger columns (1 x 10 cm) were chosen for preparative scale purification. Approximately 15 ml of partially purified AChE(~100 U/mg) was applied to the column at a flow rate of 1 ml/min. The column was then washed with loading buffer, followed by 0.2M phosphate buffer. Bound enzyme was eluted with 100mM decamethonium. Fraction sizes were 5ml. As shown in Figure 7 the 1 cm diameter column bound >98% of the enzyme applied at a flow rate of 1 ml/min. The enzyme binding study on the 0.46 x 5 cm column (Table 1) suggests that higher flow rates can be used with little loss of enzyme. After dialysis to remove eluting ligand purified enzyme obtained in fractions 20 and 21 were found to possess specific activities of 2135 units/mg protein. Recovery of enzyme activity was 95%. Electrophoretically homogenous enzyme has a specific biological activity of 3300 units/mg. The additional 1.5 fold purification

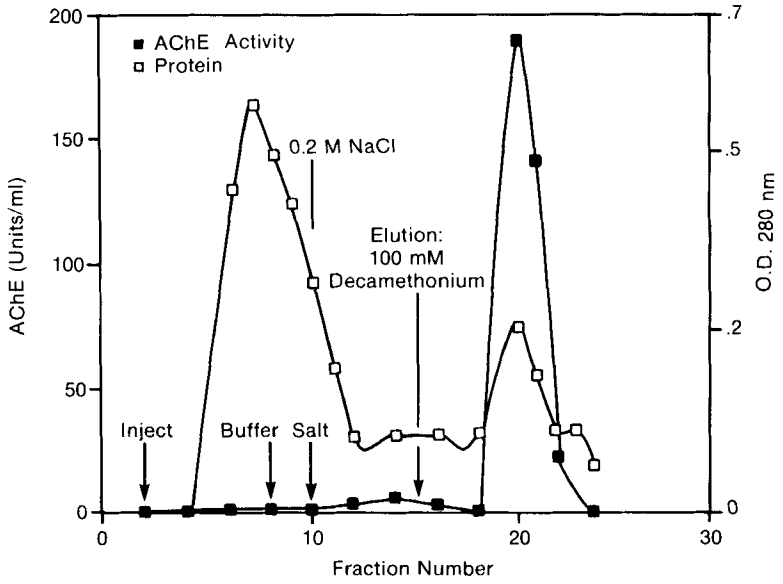

Fig. 7. High Performance Affinity Chromotography of Mammalian Acetylcholinesterase

Procainamide (Sigma) was attached to epoxy-activated columns by recirculating a 40 ml solution of 80μM procainamide in 1.0M potassium phosphate, pH 7.0, through the column at 0.2 ml/min overnight. Unbound ligand was removed by washing the column with potassium phosphate buffer (50 mM, pH 7.0) at 1.0 ml/min for 60 min. Partially purified AChE from fetal bovine serum was used. One Unit of activity is defined as the hydrolysis of 1μmol of substrate per min at 25°C. 100 mg protein was applied to a 0.46 x 5 cm procainamide column. After enzyme application, the column was washed with loading buffer and then with 0.2M phosphate buffer prior to elution with decamethonium. Enzyme activity was assayed after dialysis of each fraction as described in [9].

could be obtained by ion exchange chromatography although it is probable that homogenous enzyme could be obtained by high performance affinity chromotography with a modified washing procedure.

Dopamine Receptors

Dopamine receptors are plasma membrane bound proteins which recognize the neurotransmitter dopamine and subsequently initiate a physiological response. Much work has focused on the pharmacological characterization of these receptors and the demonstration of the regulation of the enzyme adenylate cyclase by a D_2 subclass of the dopamine receptor [11]. Purification these proteins first requires detergent solubilization from the membrane [12]. In this study the ligand aminospiroperidol was explored for use in high performance affinity chromotography. In figure 8 is shown the results obtained on chromotography of detergent extracts of bovine anterior pituitary plasma membranes. This model system is comparatively rich in dopamine receptors. As shown fractions were collected and assayed for receptor activity by assay for [^{3}H] spiroperidol binding. Spiroperidol is a specific dopamine receptor antagonist. A 20-fold purification was obtained from starting extract (0;29 pMol/mg protein to 5.7 pMol/mg protein). Considerable further purification is required to obtain homogenous material [12].

Discussion

Affinity chromotography, based on specific protein interactions with characteristic ligands, is one of the most powerful methods, available for the purification of proteins [1-7]. We have sought to develop a general, widely applicable support for use in high performance affinity chromotography. These supports have been prepared with a coating of epoxide functions attached to a spacer arm. The epoxide function was chosen because of its reactivity with

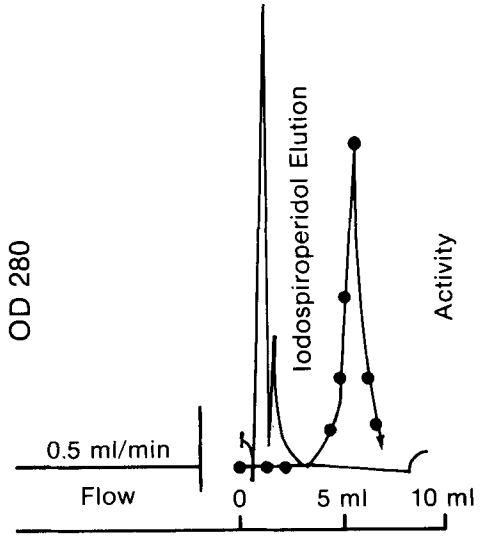

Fig. 8. High Performance Affinity Chromatography of Bovine Anterior Pituitary Plasma Membrane Dopamine Receptors

An epoxide column (0.46 x 5 cm) was derivatized with a solution of aminospiroperidol (10mg) in acetonitrile/water (20 ml, 98%). The derivatizing solution was recycled through the column at 0.2 ml/min at 35°C for 18 hours. The column was washed with acetonitrile/water followed by water. The column was then equilibrated with 0.2 percent digitonin 100mM NaCl, 10 mM Tris-HCl pH 7.0. Sample (1 ml of a 2 percent digitonin solubilized plasma membrane preparation) was injected onto the column at 0.5 ml/min. The starting material contained 900 fmol of activity in 3 mg of crude protein. The retained protein was eluted with 100 mM iodospiroperidol in 0.2 percent digitonin buffer pH 7.0. Fractions were desalted free of eluting ligand by Sephadex G-50 chromatography and analyzed for receptor activity, using [^{3}H] spiroperidol [12]. Bound from free ligand was separated by Sephadex G-50 chromatography. Radioactivity was determined by liquid scintillation counting using a Beckman LS 9800 counter.

amino, sulfhydryl, and hydroxyl functions, of proteins or compounds to be used as ligands. The supports were evaluated for stability, non-specific adsorption and maintenance of protein activity and recovery. Our results indicate that particle diameters of 10μ are optimal for most applications of high performance affinity chromotography. Larger particles had inferior dynamic properties and should be avoided for small column use.

The 10μ material utilized here showed no measurable ligand leakage and little non-specific interactions with proteins. Any non-specific interactions in purification protocols must be attributed to interactions with the immobilized ligand. The isolation of several active proteins was completed with high yields of activity. Enzyme (acetylcholinesterase) receptor protein (dopamine) and antibodies (anti-BSA, anti-rabbit IgG), were all isolated with good yields of activity.

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