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# Immobilization of Protein A at high density on azlactone-functional polymeric beads and their use in affinity chromatography

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## ABSTRACT

This paper presents the results of the use of highly cross-linked, porous, hydrophilic copolymer beads with protein immobilized on their surface for affinity chromatography. Copolymer beads composed of vinyldimethyl azlactone (oxazolone) and methylene-bis-acrylamide in various ratios, with up to 3.5 mequiv./g azlactone functionality, will undergo nucleophilic attack by amines, as well as by thiols and alcohols. The ring-opening reaction of a nucleophile-containing ligand (*e.g.*, a protein) resulted in covalent attachment to the support. The reaction was rapid, half-complete in about 5 min, yielding proteins immobilized at very high densities, recombinant Protein A at 397 mg/g, and human immunoglobulin G at 225 mg/g. The reaction proceeded at significant levels from pH 4 to 9. There was a marked enhancement in the amount of protein coupled, its rate of reaction, and its biological activity when Protein A was made to react in the presence of high concentrations of sodium sulfate. Evaluation of affinity columns, prepared with Protein A immobilized at over 200 mg/g, gave molar ratios of immunoglobulin G to immobilized Protein A of 1:1 or greater. Up to 56 mg of immunoglobulin G was recovered per ml of column bed volume. The support combined high flow-rates with low back-pressures and no bed-volume changes upon changing mobile phases, including highly ionic aqueous solvents and ethanol.

## INTRODUCTION

There is an extensive literature documenting the coupling of proteins to matrices. Scouten[1] thoroughly reviewed ligand immobilization and affinity chromatography in a 1981 monograph. Much of the recent literature has been reviewed by Katchalski-

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Katzir[2], with special emphasis on immobilizing enzymes, and by Jervis[3], who focused on affinity chromatographic applications. The reviews summarized the numerous methods of immobilization and critically evaluated the advantages and disadvantages of each. Jervis[3] outlined several characteristics of an ideal activation/coupling chemistry which included: (1) rapid, efficient formation of a stable, uncharged, covalent bond under mild conditions with no side reactions; (2) easy blocking of residual activated groups with simple, hydrophilic, uncharged groups; and (3) use of inexpensive, non-toxic reagents in procedures which could be scaled up. A deficiency in at least one of these categories was exhibited by each method reviewed.

The synthesis and chemistry of azlactones (oxazolones), cyclic anhydrides of N-acylamino acids, have been subjects of an extensive recent review by Rao and Filler[4]. An important reaction is the ring-opening addition which occurs with various nucleophiles, $RNH_2$, RSH and ROH (Fig. 1). Reaction with a primary amine results in formation of an amide bond between the azlactone and the amine (*e.g.*, lysyl group on a protein) and a rearrangement such that the ligand is tethered to the polymer through two amide bonds, which should provide both separation of the ligand from the backbone of the polymer, and a linkage which is stable in most biological solutions. Three of the authors have reviewed the extensive literature of azlactones in polymers[5], and we have synthesized new polymeric beads of azlactones with acrylamides and other hydrophilic monomers and investigated them as potential supports for immobilizing biochemicals, especially proteins, with particular applications in the area of affinity separations[6–8].

We report here results which show that polymeric beads containing azlactone functionality have exceedingly high capacities for coupling proteins while retaining their biological activity. Several proteins have been coupled at greater levels than 100 mg of protein per g of polymeric bead. The coupling reaction is rapid, with more than half of the reaction occurring in the first 5 min, and efficient, with 60 to 100% of the protein being immobilized. Furthermore, these protein-derivatized beads, with a high density of an immobilized active ligand, *e.g.*, Protein A, are especially useful supports for high-performance liquid affinity chromatography. Protein A, immobilized at over 200 mg of protein per g of bead, maintained its ability to bind human immunoglobulin G (IgG) in a 1:1 molar ratio.

## EXPERIMENTAL

### *Materials*

Vinyldimethyl azlactone (VDM) was purchased from SNPE, (Princeton, NJ, U.S.A.). Methylene-bis-acrylamide (MBA) and all other organic reagents were obtained from Aldrich (Milwaukee, WI, U.S.A.). Recombinant Protein A (rProtA) was

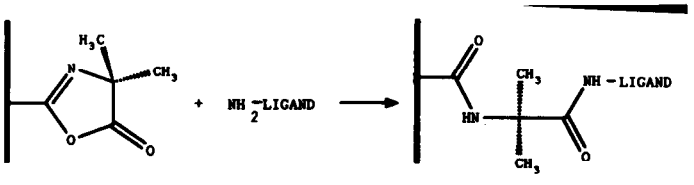


Fig. 1. The ring-opening reaction of dimethyl azlactone, as part of a polymer, with an amine.

purchased from Repligen (Cambridge, MA, U.S.A.). The best available grades of the other proteins were purchased from Sigma (St. Louis, MO, U.S.A.) Molecular weight standards were purchased as a kit prepared for electrophoretic use from Bio-Rad (Richmond, CA, U.S.A.). Na $^{125}$I was purchased from DuPont NEN (Billerica, MA, U.S.A.). Iodo-beads™ and bicinchoninic acid reagents were purchased from Pierce (Rockford, IL, U.S.A.). Electrophoresis gels were purchased from Pharmacia (Piscataway, NJ, U.S.A.).

*Polymer synthesis*

The polymeric beads were prepared by an inverse-phase polymerization process, as described by Rasmussen *et al.*[6]. Briefly, a water–dimethylformamide phase, containing the monomers, was suspended in a density-balanced heptane–carbon tetrachloride phase, using copoly[isooctylacrylate–acrylic acid] 90:10 (%, w/w) as a suspending agent. Binary polymers were prepared by ammonium persulfate–tetramethylethylene diamine-initiated polymerization with varying ratios of VDM and MBA. The given ratio of polymer (*e.g.*, 20:80 or 42:58) is understood to be the % (w/w) ratio of VDM to MBA monomers in the reaction. After polymerization, the beads were given sequential washes with acetone, water and acetone, dried to constant mass in a vacuum oven, and stored in a desiccator at ambient temperature until used. Table I lists the properties of the polymers used in this study, and Fig. 2 shows a scanning electron micrograph of a preparation of 42:58 beads.

Surface areas of beads were determined by the BET method of nitrogen adsorption. The mercury porosimetry method was used to determine pore sizes. Bead diameter measurements were made following hydration, using a Coulter counter Model TA2. Azlactone functionality was measured by complete reaction with an excess of ethanolamine (0.1 *M*), followed by back-titration with 0.1 *M* HCl with a Radiometer RTS822 Autotitrator.

*Protein iodination*

Proteins were radiolabeled with $^{125}$I by the chloramine-T reaction using Iodo-beads. The typical reaction contained 0.5 mg of protein (rProtA, human immunoglobulin or bovine serum albumin) and 100 $\mu$Ci of $^{125}$I-labeled sodium iodide in 100 m*M* sodium phosphate, 100 m*M* NaCl buffer (pH 7.5) and 2 Iodo-beads in a total volume of 500 $\mu$l. The reaction was terminated after 30–60 min by removal of the solution from the beads. Protein was separated from unreacted radioisotope on PD-10 col-

TABLE I

SUMMARY OF SOME PHYSICAL AND CHEMICAL PROPERTIES OF AZLACTONE POLYMERIC BEADS

| *Polymer* | *Azlactone functionality (mequiv./g)* | *Particle size ($\mu$m)* | *Pore size (Å)* | *Surface area ($m^2$/g)* |
|---|---|---|---|---|
| 20:80 | 1.12 | 54 | – | 221 |
| 30:70 | 1.54 | 72 | – | 170 |
| 42:58 | 2.06 | – | 555 | 186 |

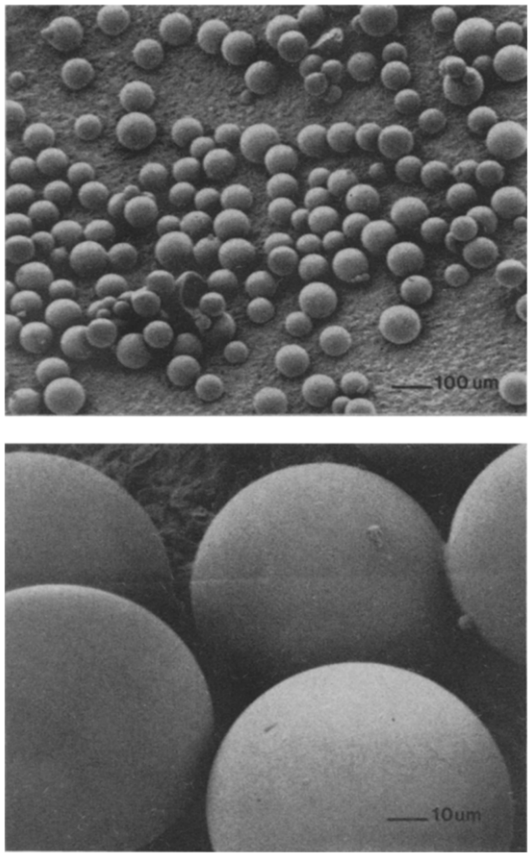


Fig. 2. Scanning-electron micrographs of 42:58 azlactone-functional beads. Dried, underivatized beads were prepared for electron microscopy by mounting them on a stage with conducting carbon cement, followed by sputter-coating with gold. The Model SX 30 scanning-electron microscope of International Scientific Instruments, (Pleasanton, CA, U.S.A.) was used. The magnification scale is indicated on each micrograph.

umns (Pharmacia). Typically 50% or more of the isotope was incorporated into the protein for a specific radioactivity of 0.10—0.15 $\mu$Ci/$\mu$g of protein. Radioiodinated protein solutions were stored frozen and used up to a month after preparation.

*Protein coupling*

The typical protein coupling reaction consisted of 10 mg of polymeric beads suspended in 200 $\mu$l of 25 m*M* sodium phosphate, 150 m*M* NaCl (pH 7.5) containing a variable amount of protein (20 $\mu$g to 5.0 mg). In some experiments up to 1.5 *M* $Na_2SO_4$ was substituted for the NaCl. The suspension was continuously rocked for the duration of the reaction. After the standard coupling time of 60 min, the reaction was terminated by the addition of the blocking reagent, 1.0 ml of 1.0 *M* ethanolamine in 25 m*M* sodium pyrophosphate, which had been titrated to pH 9.0 with HCl. After 5 min of continuous rocking, the sample was centrifuged, the supernatant solution

was removed, and fresh ethanolamine solution was added to continue the blocking of the residual azlactone functional groups. After 60 min of additional reaction, the beads were centrifuged and resuspended several times in the pH 7.5 phosphate–NaCl buffer. In time-course experiments protein coupling was allowed to proceed from 5 min to 24 h, with "zero" time points established by first blocking the azlactone functionality, as described above, then allowing reaction with the protein.

The amount of bound radiolabeled protein was determined in a Packard $\gamma$ scintillation counter (Model 5230). The specific radioactivity of the protein to be coupled was adjusted prior to each experiment by addition of unlabeled protein and ranged from 100 to 2000 cpm/$\mu$g of protein. After the initial determination of bound radiolabeled protein, the amount which was covalently linked to the polymeric beads was determined following incubation of the beads with 1.0% sodium dodecyl sulfate (SDS) for 4 h at 37°C with intermittent mixing, followed by centrifugation, removal of the supernate, and several additional wash cycles with SDS.

In those experiments performed without radiotracers, bound protein was determined by measuring both the amount bound to the beads and the residual protein in the supernatant solution by the bicinchoninic acid method[9].

In the pH profile experiments a ternary buffer of acetate, phosphate and pyrophosphate (50 m*M* each) was titrated to the final pH indicated (pH 4–9) with either HCl or NaOH. The lowest pH tested in the presence of sulfate was pH 4.5, since each protein began to precipitate in the presence of sulfate at pH 4.0. All other procedures were the same as those described above except that the reaction was allowed to proceed for 30 rather than 60 min.

*Activity of immobilized rProtA*

The biological activity of immobilized rProtA was determined by using excess amounts of radiolabeled human IgG. The amount of rProtA binding was determined with the radiolabeled rProtA, as described above. Unlabeled rProtA, coupled under identical conditions, was used to bind radiolabeled IgG. Amounts of 1.25 to 8.75 mg of radiolabeled IgG were added to 10 mg of 20:80 beads, containing 3–120 $\mu$g of rProtA and incubated at ambient temperature in 25 m*M* sodium phosphate, 25 m*M* sodium pyrophosphate buffer (pH 9.0), containing 1.0 *M* $(NH_4)_2SO_4$, for 60 min with continuous rocking. The beads were washed several times with the binding buffer, and the residual radioactivity was determined. The beads were then treated for 1 h with 1.0 *M* glycine (pH 2.0), washed several times, and this was followed by determination of residual radioactivity. To substantiate that the IgG was binding through the immobilized rProtA and not through residual azlactone functionality, beads were incubated with 1% SDS for 4 h at 37°C. In these experiments and the chromatographic experiments described below the stoichiometry of IgG:rProtA was calculated from the amount of radioactivity or absorbance at 280 nm eluted with the glycine treatment and 150 000 dalton for the molecular weight and 1.3 $cm^2$/mg for the extinction coefficient of human IgG and 45 000 dalton for rProtA.

*Affinity chromatography*

Immobilized rProtA for affinity chromatography was prepared by 60-min reactions of 250 mg of 20:80 beads with 1.25 to 25 mg of rProtA in a total volume of 5.0 ml of 25 m*M* sodium phosphate (pH 7.5), containing either 150 m*M* NaCl or 1.5 *M*

sodium sulfate. Residual azlactone functionality was inactivated by two treatments with ethanolamine, followed by washings with phosphate buffers and 1.0 *M* NaCl to remove adsorbed protein. Derivatized beads were stored at 4°C in 20% ethanol until used.

Chromatography was performed on a Pharmacia fast protein liquid chromatography (FPLC) system controlled by the FPLC Manager™ software (Pharmacia). Protein A affinity chromatography was performed with purified human IgG or human serum and either a 10 × 0.3 cm (0.70 ml) Omni column (Rainin, Woburn, MA, U.S.A.) or a 10.0 × 0.5 cm (2.0 ml) column (Pharmacia). IgG was dissolved in 25 m*M* sodium phosphate, 150 m*M* NaCl (pH 7.5). The protein concentration and flow-rate varied according to the experiment. Human serum was diluted with an equal volume of the phosphate buffer. All samples were filtered through a 0.2-$\mu$m filter immediately prior to injection into the column.

After the sample was loaded, the column was eluted first with the phosphate–150 m*M* NaCl buffer, followed by a step gradient to the same phosphate buffer with 1.0 *M* NaCl, to remove any non-specifically bound protein, except where otherwise indicated. After the column was returned to the low-chloride buffer the specifically bound IgG was eluted by a step gradient to 0.1 *M* glycine–2.0% acetic acid buffer (pH 2.2).

Recovery of protein from the column was determined by measurement of the absorbance at 280 nm of each fraction, since, at high protein concentrations (>2 mg/ml) the flow cell optical readings are unreliable. Purity of recovered IgG was monitored by electrophoretic analysis, performed on samples of each of the eluates from the Protein A affinity columns. The protein-containing fractions obtained from each elution of the affinity column were pooled and dialyzed against deionized water. Pools low in protein (high-salt elution) were lyophilized and redissolved in a smaller volume of water. All samples were prepared for electrophoresis by heating to 100°C for 5 min in SDS–mercaptoethanol reagent[10] and electrophoresed on Pharmacia 10–15% acrylamide gradient PhastGels™ at 250 V for 70 "accumulated V h" (about 25 min), using a PhastSystem electrophoresis instrument (Pharmacia). Gels were stained with 0.04% Coomassie Brilliant Blue in 10% acetic acid for 15 min and destained with methanol–acetic acid.

## RESULTS

### *Protein coupling*

An initial survey experiment in which rProtA was coupled to beads of varying azlactone content (bead formulations varied from 10:90 to 70:30) showed that the amount of protein coupled was not significantly dependent on the effective azlactone functionality between 1 and 3 mequiv./g (Fig. 3). The percentage of protein remaining bound after the SDS treatment, a measure of the amount of binding which was covalently coupled, ranged from 77 to 90%, suggesting a high degree of covalent coupling of rProtA.

In a second survey experiment, designed to measure the maximum binding capacity as a function of amount of available azlactone, we measured the concentration-dependency (0.1–10 mg/ml) of rProtA coupling to three beads: 20:80, 42:58, and 60:40. A plot of coupled protein *vs.* concentration (not shown) suggested a saturation

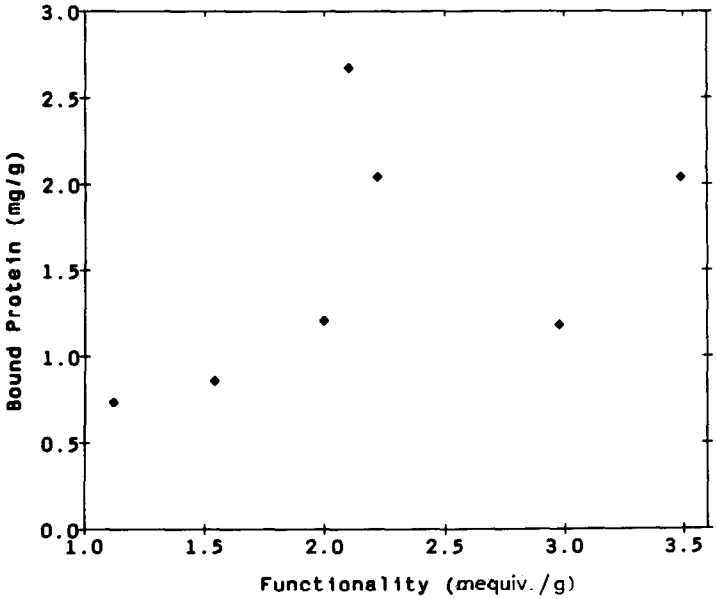


Fig. 3. The amount of rProtA coupled as a function of the titratable azlactone content. Azlactone functionality of the various VDM–MBA copolymers (10:90, 20:80, 30:70, 42:58, 60:40, and 70:30) was determined by alkali titration, as described in the text. Protein coupling was determined with radiolabeled rProtA (0.25 mg/ml) under the standard conditions described in the text, using the chloride-containing buffer.

effect, and a double reciprocal plot gave the following maximum protein coupling density estimates: 20:80, 248 mg protein per g of polymer; 42:58, 245; 60:40, 227. At the maximum observed protein couplings the percentages of residual bound protein following the SDS treatments were 99–100%, and the lowest value for the experiment was 91%. Based on these results, we concluded that there was little difference in the maximum amounts of protein which these beads could couple, and chose to use 20:80 and 30:70 beads for our subsequent studies, since greater azlactone density was not necessary for increased protein density.

We measured the coupling capacity of azlactone-functional beads for several proteins by varying the protein concentration (1–25 mg/ml) and allowing the reaction to progress for 60 min. rProtA and human IgG were coupled in 25 mM sodium phosphate buffer–150 m*M* NaCl (pH 7.5); however, since concanavalin A (Con A) tends to aggregate at this pH it was coupled using the same buffer at pH 6.0 (Fig. 4A). The efficiency of protein coupling (the percent of the available protein which was immobilized) was 6–38% for rProtA, 20–100% for IgG, and 52–100% for Con A. Although the efficiency of coupling decreased with increasing concentrations, the actual density of immobilized protein continued to increase. 91–99% (rProtA) and 60–65% (IgG) remained bound following the SDS treatment.

*Sulfate effect*

It has been previously reported that some activated polymeric supports will bind more protein under highly ionic coupling conditions[11–14]. The phenomenon has

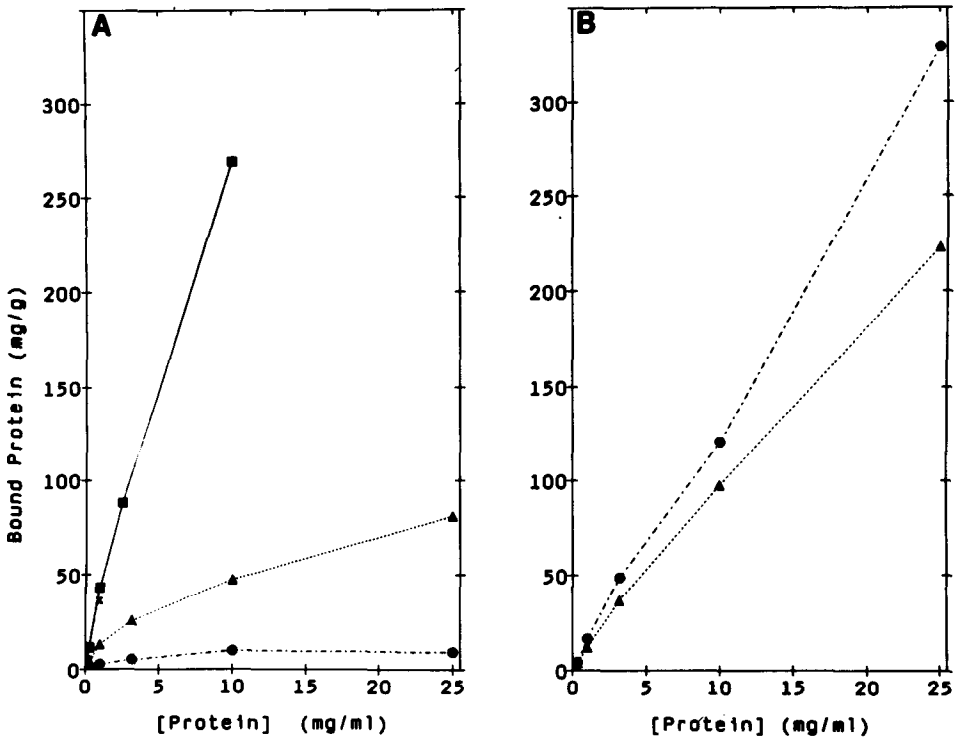


Fig. 4. The concentration dependency of the coupling of various proteins to azlactone-functional beads. 20:80 Beads were used, except for the Con A studies, which were with 30:70 beads. Protein concentrations varied from 0.1 to 25 mg/ml. Reaction conditions were the standard conditions described in the text in either 150 m*M* NaCl (A) or 1.5 *M* $Na_2SO_4$ (B), except that Con A was coupled at pH 6.0 rather than pH 7.5. ▲ = IgG; ● = rProtA; ■ = Con A. Note that the single Con A concentration (1.0 mg/ml) for which sulfate data were collected is indicated in A by ×.

been likened to the effect of high concentrations of sulfate or phosphate on the adsorption of protein on hydrophobic-interaction columns[14]. Fig. 5 depicts the effect of the concentration of sodium sulfate on the coupling density of rProtA. Coupling increases above 0.15 *M* sulfate and continues to increase up to the solubility limit, about 1.5 *M*. The effect is more apparent at 5 mg of rProtA/ml than at 0.25 mg/ml. There is also a significant effect of sulfate on the covalent coupling. In the absence of sulfate, the residual bound after the SDS treatment was 70–76%; in the presence of 1.5 *M* sulfate, 98% remained bound at 0.25 mg/ml rProtA and 100% at 5 mg/ml.

In the concentration-dependency experiments (Fig. 4B), IgG and rProtA showed enhanced coupling in the presence of sulfate, two-fold for IgG and more than ten-fold for rProtA. (It should be noted that sulfate had to be reduced to 0.75 *M* for IgG to prevent its precipitation.) Although the coupling densities are very high (225 mg/g for IgG and 325 mg/g for rProtA), from the shapes of the curves neither appears to have fully saturated the bead. In contrast to the sulfate enhancement of IgG and rProtA coupling, there was no effect on Con A. At 1.0 mg/ml of Con A 38.6 ± 1.8 mg of protein bound per g of polymer with sulfate and 42.0 ± 2.7 without sulfate. (Note

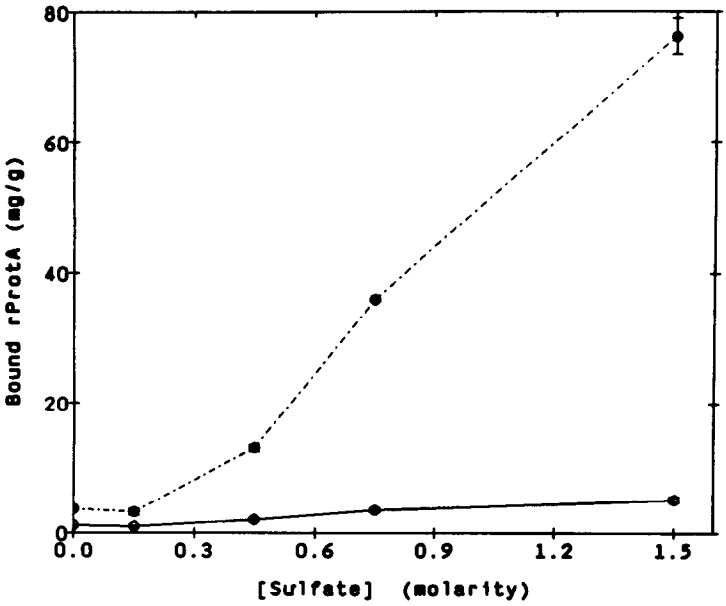


Fig. 5. The effect of sodium sulfate on the coupling of rProtA to azlactone-functional beads. 20:80 Beads and standard coupling conditions were used. Sulfate was varied as indicated from 0.0 to 1.5 *M*. In those experiments without sulfate the reaction medium contained 150 m*M* NaCl. Experiments performed at 5 mg/ml (●) and at 0.25 mg/ml (○) rProtA.

that the sulfate point is indicated by the × in Fig. 4A.) The coupling efficiencies for rProtA were 66–93% and 43–71% for IgG. In additional experiments we have observed that the enhanced binding of rProtA in 1.0 *M* sodium phosphate is 80% of that in 1.5 *M* sodium sulfate, while the effect of 3.0 *M* NaCl is only 8% of the sulfate effect.

In other experiments in the presence of sulfate (not shown) we have also immobilized trypsin, ovalbumin, and bovine serum albumin (BSA) at densities greater than 100 mg/g. Trypsin (102 mg/g) remained enzymically active, and ovalbumin (217 mg/g) was able to bind anti-ovalbumin antibodies[15].

*Immobilization time course*

The time courses for the coupling of rProtA and Con A to azlactone-functional beads (Fig. 6A) showed that the reaction is very rapid. More that 50% of the final binding density is achieved in the first 5 min. Fig. 6B shows the comparison of the time course for the binding of rProtA with and without sulfate. The 24-h coupling in the absence of sulfate (2.6 mg/ml) is less than 10% of the 5-min reaction in the presence of sulfate. In another time course at 25 mg/ml (not shown), 397 mg of rProtA bound per g of 20:80 polymer in 24 h. In this and all other cases studied, there was a slight increase in coupling by extending the reaction time to 24 h, but the immobilizations were essentially complete in 1-2 h.

*pH profile*

Since there was a marked pH effect on Con A coupling (Fig. 6A) which might

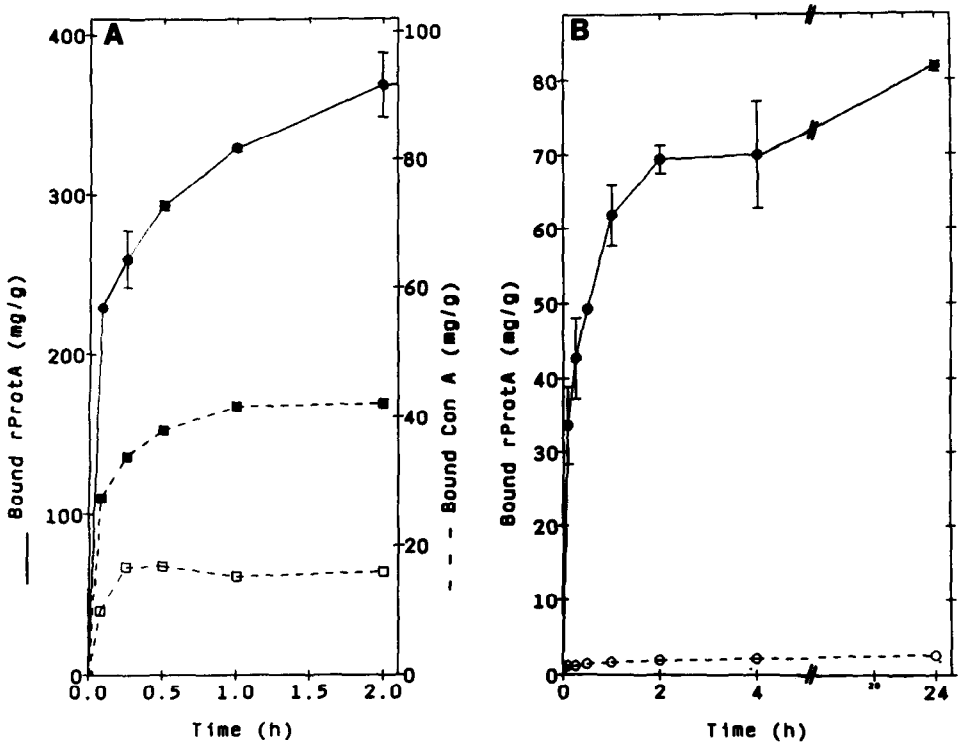


Fig. 6. The time course for the coupling of rProtA and Con A to azlactone-functional beads. (A) rProtA (●) (25 mg/ml) was coupled to 20:80 beads under the standard conditions in the presence of sulfate. Con A (1.0 mg/ml) was coupled to 30:70 beads at pH 6.0 (■) and pH 7.0 (□) in 150 m*M* NaCl. "Zero" time was determined by allowing the beads to react first with the 1.0 *M* ethanolamine blocking agent, followed by reaction with the protein, followed by another blocking step. Only the first 2 h of the 16-h course of the rProtA experiment are illustrated, since less than 10% increase was observed over the remainder of the experiment. (B) rProtA (5 mg/ml) was coupled as in (A) to 20:80 beads for 24 h in the presence of chloride (○) or sulfate (●).

be attributable to its known insolubility at high pH[16], we conducted a study of rProtA and BSA binding in the pH range 4–9. The shape of the pH profiles (Fig. 7) depends on whether sulfate is present. When it is present, the optimum is pH 8–9 with a steady, almost linear, increase in bound protein from pH 4.5–8.0. Without sulfate (*i.e.*, in 150 m*M* NaCl) the pH profile is characterized by a minimum at pH 5–7 with considerably greater activity at both the acid and alkali extremes of the tested range. 94% and 92% of rProtA and BSA, respectively, are resistant to solubilization by the SDS treatment at pH 4 (or 4.5), an effect independent of sulfate. Such high percentages indicate that the proteins are actually coupled to the support and not interacting ionically, as might be expected if the azlactone ring were opened by water hydrolysis to form a carboxyl group. Furthermore, these percentages are consistently 91% or higher across the pH range studied, if sulfate is present; however, in the absence of sulfate there is a minimum at pH 6.0 where the values are 73% for rProtA and 56% for BSA.

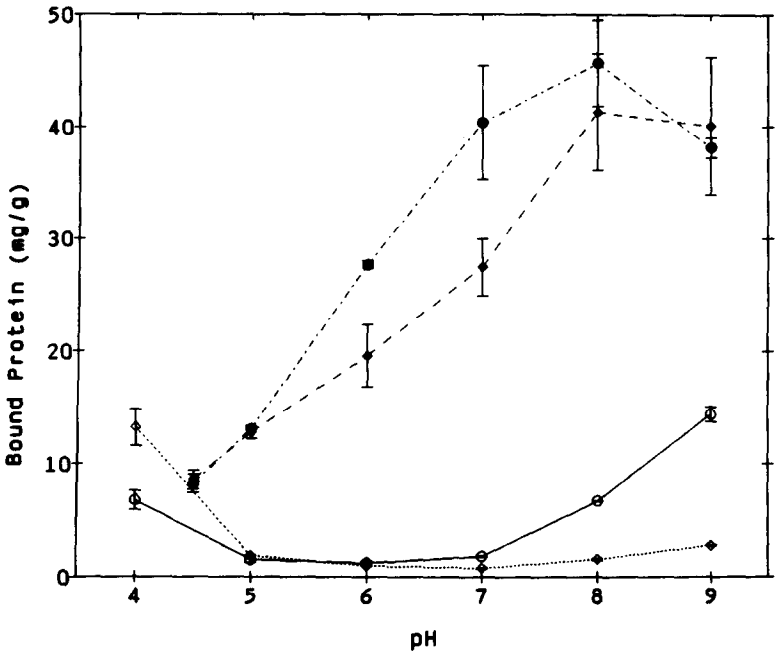


Fig. 7. The pH dependency for the reaction of rProtA and BSA in the presence of sulfate or chloride. The buffering agent in each experiment was 50 m*M* each of acetate, phosphate and pyrophosphate plus either 150 m*M* NaCl or 1.5 *M* $Na_2SO_4$, titrated to the designated pH. Protein concentrations were 2.5 mg/ml for rProtA (○) and BSA (◇). (The filled symbols indicate that sulfate was present.) The standard coupling conditions were followed with 20:80 beads, except that there was only a 30-min coupling reaction prior to the blocking step.

### *Activity of immobilized rProtA*

The activity of immobilized rProtA was first evaluated in small-scale "batch" experiments with radiolabeled human IgG. Molar ratios were calculated on the basis

TABLE II

ANTIBODY-BINDING ACTIVITY OF IMMOBILIZED rProtA

Derivatized beads with rProtA immobilized at the density indicated in the table were incubated with radiolabeled IgG sufficient to provide at least a two-fold molar excess of IgG over rProtA according to the procedures detailed in the text. The molar ratio was calculated on the basis of the amount of radioactivity specifically eluted by the acid step. Each value is an average of duplicates. The second value in each pair represents an identical experiment in which BSA was substituted for ethanolamine as the blocking agent after the rProtA coupling step.

| *rProtA density (mg/g)* | *Ratio eluted IgG–bound rProtA* |
|---|---|
| 2.7 | 0.5, 0.8 |
| 16.7 | 0.2, 0.2 |
| 40 | 0.5, 0.4 |
| 120 | 0.3, 0.5 |

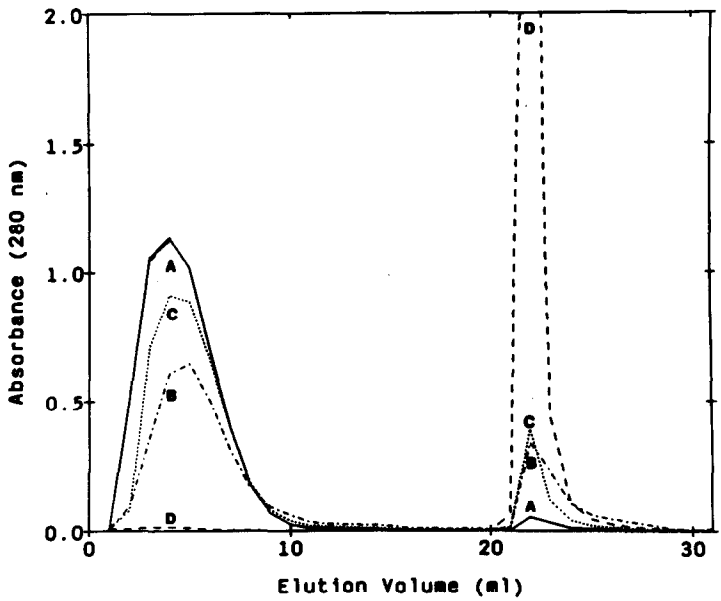


Fig. 8. The effect of coupling conditions on the efficiency of IgG binding to rProtA immobilized to azlactone beads. rProtA was coupled to 20:80 azlactone-functional beads with the standard conditions described in the text, using either 0.25 or 5.0 mg of rProtA/ml in the presence of either 150 m*M* NaCl or 1.5 *M* $Na_2SO_4$. The resulting rProtA densities are given in Table III. The beads were packed into 10.0 × 0.3 cm (0.70 ml) columns. Human IgG [4.5 ml of 1.0 mg/ml in 25 m*M* sodium phosphate, 150 m*M* NaCl (pH 7.5)] was injected into each column, and unbound protein was eluted with 14.5 ml of the same buffer. Antibody release was effected by eluting the column with 7.0 ml of 0.1 *M* glycine–2% (v/v) acetic acid (pH 2.2). The flow-rate for all steps was 0.5 ml/min, a linear flow of 7.1 cm/min. Fractions were collected, and the absorbance of each at 280 nm was determined and plotted as a function of elution volume. The amounts of IgG eluted from each column and the molar ratios of specifically bound and eluted IgG to coupled rProtA are listed in Table III.

of the amount of IgG which could be specifically eluted at 1.0 *M* glycine (pH 2.0). The modest levels of antibody binding which were obtained (Table II) might be expected when macromolecules must diffuse from a solution into the relatively small volume of a porous matrix in a limited amount of time.

*Affinity chromatography*

rProtA was immobilized to 20:80 beads under varying protein and salt concentrations to determine how its IgG-binding activity in an affinity column varies with the density of bound protein and with the presence of sulfate in the immobilizing medium. Affinity chromatograms of human IgG (Fig. 8) demonstrate that the amount of IgG recovered from the column is roughly proportional to the density of immobilized rProtA, even at a rProtA density (6 mg/ml) where the resulting IgG capacity is 26 mg/ml. However, on closer inspection, there is an improved correlation between high ratio of IgG to rProtA immobilized in the presence of sulfate ion in the coupling medium (Table III). The two preparations in chloride produced a molar ratio of 0.5, and the two in sulfate were 1.3. Reports in the scientific literature of such

TABLE III

EFFECT OF SULFATE AND rProtA DENSITY ON IgG RECOVERY FROM AN AZLACTONE AFFINITY COLUMN

rProtA was coupled to 20:80 beads, blocked, and packed into 0.70-ml columns as detailed in the text. For columns A and B 0.25 mg/ml rProtA were coupled to 250 mg of beads in a final volume of 5.0 ml. For C and D the protein concentration was 5.0 mg/ml, and other conditions were the same. A and C were used with chloride; B and D with sulfate. IgG (4.5 ml, 1.0 mg/ml) was injected into the column in 25 m*M* sodium phosphate–150 m*M* NaCl (pH 7.5), and eluted at 0.5 ml/min. Coupled densities were determined by radiometric experiments. Values of IgG bound are in units of mg IgG eluted from the column per ml of column bed volume. The IgG–rProtA ratio is a molar ratio calculated as described in the text. Values of replicate experiments are given for the sulfate-enhanced couplings.

| *Column* | *Sulfate present* | *rProtA density mg/ml* | *IgG bound mg/ml* | *Ratio Eluted IgG–bound rProtA* |
|---|---|---|---|---|
| A | | 0.072 | 0.12 | 0.49 |
| B | + | 0.25 | 0.92 | 1.11 |
| | | | 0.99 | 1.20 |
| | | | 1.22 | 1.47 |
| C | | 0.40 | 0.72 | 0.54 |
| D | + | 5.91 | 26.0 | 1.32 |
| | | | 26.4 | 1.34 |

molar ratios for immobilized Protein A have been rare, although manufacturers regularly give such information in their product literature. Muramatsu *et al.*[17] reported a ratio of 0.4; however, vendors (Genzyme and InFerGene, for example) generally indicate values of 0.8–1.4 for commercially available columns. Uhlen[18] has demonstrated that Protein A has five binding domains for IgG, all of which are independently functional; however, the maximum binding ratio observed in free solution was 2 mol of IgG per mol of Protein A.

Passage of 2.25 ml of human serum through the highest-density column (column D in Table III) resulted in the specific elution of 15 mg of human IgG (Fig. 9A), corresponding to a molar ratio of 1.1. SDS–polyacrylamide gel electrophoretic analysis (not shown) revealed that the heavy- and light-chain bands for the IgG completely disappeared from the flow-through fraction and appeared as clean bands in the acid-eluted fraction. Fig. 9A also shows the results of subjecting 3 mg of BSA to the same fractionation procedure; 99% of the albumin was recovered in the flow-through fraction and less than 1% in the acid-eluted fraction.

Fig. 9B presents results from similar experiments with a larger column (10 × 0.5 cm, 2 ml) containing beads derivatized to a higher rProtA density (214 mg/g, 16 mg/ml). As before, all of the IgG was removed from the human serum sample (compare Fig. 10, lane 2, injected serum sample, with lane 3, the flow-through fraction) and was recovered in the acid-eluted fraction (lane 6), where it appears as pure as the commercially available human IgG (lane 7). Despite the high density of rProtA, we observed none in the high-salt or acid-eluted fractions (compare lanes 4 and 5 with rProtA in lane 8). Lane 4 shows that albumin is probably the major non-specifically bound protein eluted by the high salt wash. After this experiment a control chroma-

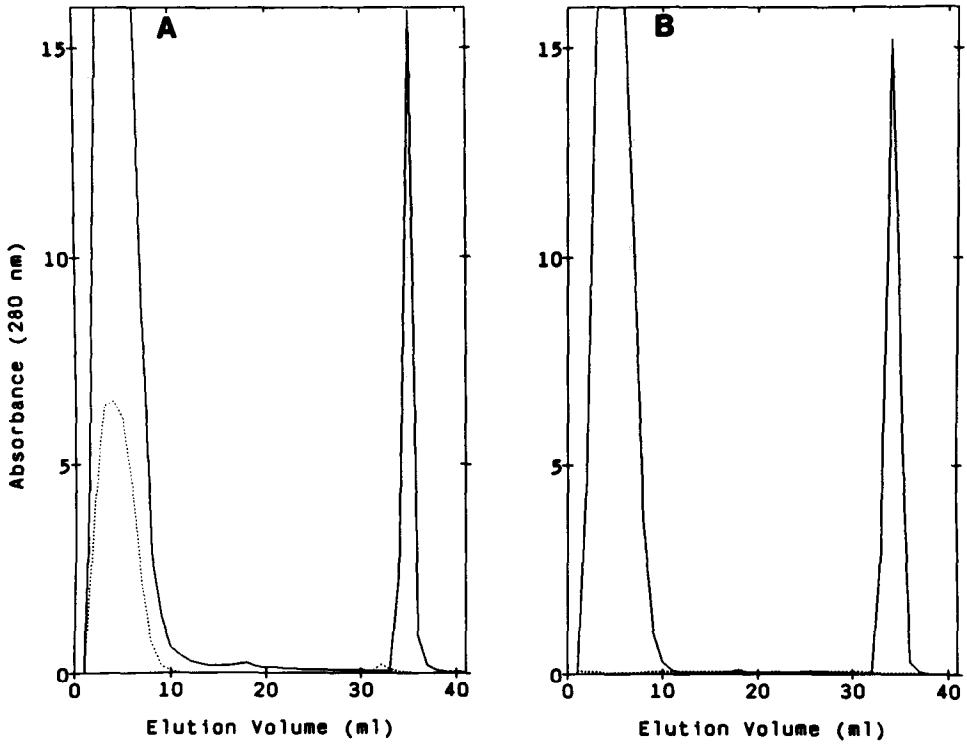


Fig. 9. Affinity purification of IgG from human serum on rProtA coupled to azlactone-functional beads. The chromatograms represented in A are from column D described in Fig. 8 and Table III. Those in B were obtained from a 10 × 0.5 cm (2.0 ml) column, packed with rProtA-azlactone beads, in which the rProtA density was 16 mg/ml. The derivative was prepared by allowing 250 mg of 20:80 beads to react with 84 mg of rProtA in 5.0 ml of sulfate-containing buffer for 60 min under the standard coupling conditions described in the text. In both A and B the solid line represents the elution profile following injection of 2.25 ml of human serum, diluted to 4.5 ml with elution buffer (25 m*M* sodium phosphate–150 m*M* NaCl, pH 7.5). The dashed line in A represents the elution profile following injection of 4.5 ml of BSA (0.7 mg/ml). The dashed line in B represents the elution profile of a "blank" chromatographic experiment immediately following the serum chromatogram (injecting 4.5 ml of buffer). Elution conditions for all columns were: 15 ml of the phosphate buffer, followed by 9.0 ml of the phosphate with 1.0 *M* NaCl (except for the BSA experiment), followed by return to the low-salt phosphate (7.0 ml), followed by 7.0 ml of the 0.1 *M* glycine–2% acetic acid buffer (pH 2.2), all at 0.5 ml/min. Fractions were collected and diluted as necessary to record absorbances at 280 nm.

togram was obtained by injecting starting buffer into the column, followed by the standard salt and acid steps in an attempt to find a "ghost" peak, *i.e.*, residual IgG which had remained bound from a previous experiment. The chromatogram (Fig. 9B) demonstrates that none was found. In subsequent experiments with this column (not shown) we were also unable to find a "ghost" peak after the column was saturated with specifically-bound IgG from which 112 mg of IgG was eluted.

Flow properties of azlactone-functional beads were examined in a 10 × 0.5 cm column in which the flow-rate was incrementally increased from 0.25 ml/min to 15 ml/min (the limit of the system). The increase in back pressure was a linear function of

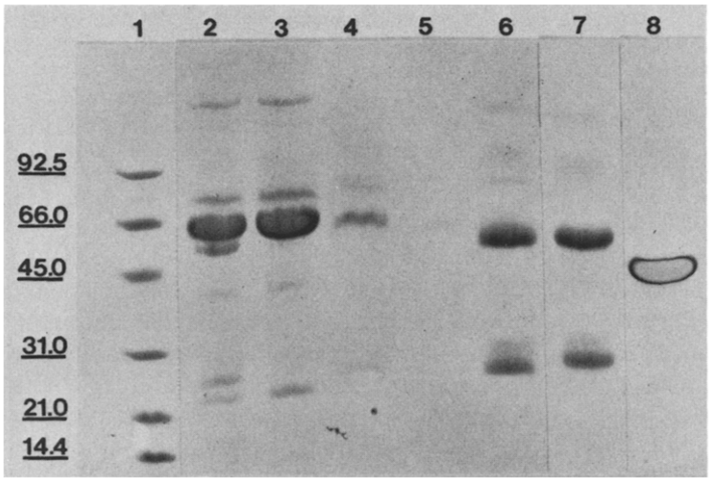


Fig. 10. Electrophoretic analysis of the chromatographic fractions from the rProtA-azlactone bead affinity column shown in Fig. 9B. The lanes contained the following samples (*ca.* 5 μg of protein per lane): 1 = molecular weight standards, given in kilodalton (phosphorylase *b*, 92:5; BSA, 66; ovalbumin, 45; carbonic anhydrase, 31; soybean trypsin inhibitor, 21; lysozyme, 14.4); 2 = unfractionated serum; 3 = flow-through fraction; 4 = fraction eluted at high salt concentration; 5 = fraction eluted with low-salt buffer between high-salt and acid elutions; 6 = acid-eluted fraction; 7 = human IgG; 8 = rProtA.

the flow-rate, *ca.* 0.1 MPa at 0.25 ml/min and 1.4 MPa (210 p.s.i.) at 15 ml/min. There was a slight compacting above 10 ml/min; however, this is understandable, since the column had been packed at 2 ml/min. At the highest flow-rate the linear flow-velocity was 76 cm/min. In another experiment at 7 cm/min linear flow, 100% of a 5-mg IgG sample was bound by the column used in Fig. 9A above, and 98% of it was recovered in the acid elution step.

Standard storage conditions for the column were 20% ethanol in water, 4°C. After 4 months' storage under these conditions or short-term storage (1–3 days) at higher ethanol concentrations (50, 70 and 100%) we observed no loss in biological activity. In addition, there were no solvent effects on the column bed volume.

## DISCUSSION

A wide variety of chemical reactions has been proposed for the coupling of proteins to solid supports, indicative of the general inadequacy of each in one or more important respects[1–3]. Among the most important requirements are speed of coupling, density of immobilized ligand, and retention of the biological activity of the immobilized protein. With these priorities in mind we have investigated the utility of the azlactone functionality for the immobilization of proteins.

Azlactone copolymer beads have a high capacity for immobilizing protein, and this capacity is largely independent of the degree of azlactone functionality within the range of 1–3 mequiv./g. Thus one may choose a formulation based primarily on considerations other than degree of functionality, *e.g.*, bead size, flow properties, and ease of preparation.

Protein coupling to azlactone-functional beads is very rapid, typically reaching half of the maximum in 5 min and completion in about 1 h. This compares very favorably with other forms of activated supports, such as oxirane (epoxide), cyanogen bromide, activated thiol, aldehyde and hydrazide, which have reaction times of 16–72 h[19–24]. Thus, azlactone supports could prove valuable in situations requiring very short coupling times.

The coupling of many of the proteins which we have investigated is enhanced in the presence of sulfate or phosphate ion. This effect was first noted in reference to oxirane-functional methacrylate copolymers[11–13]. Further investigations with an hydroxyethyl methacrylate polymer[14] suggested that the effect of sulfate (or other ion in the Hofmeister series) was similar to its effect in hydrophobic-interaction chromatography, where these ions are believed to promote a weak affinity between the more hydrophobic portions of the protein surface and the matrix by promoting the imminent precipitation of the protein[25]. In our investigations the effect was maximal at the solubility limit of sodium sulfate (about 1.5 *M*) and influenced both the amount of protein which was immobilized and the rate of immobilization. Unexpectedly, it also enhanced the resultant antibody-binding activity of immobilized rProtA, increasing the molar IgG–rProtA ratio from about 0.5 to 1.0–1.3.

The amounts of protein directly immobilized on supports which we have reported here (397 mg rProtA/g, 31 mg/ml) are higher than we have found reported elsewhere. For example, Taylor[19] reported an extensive study in which he coupled four proteins to nine supports at five pH values. The highest IgG density was 3 mg/g (only binding 25% of the protein offered for coupling) and only one-third of it was biologically active. His highest reported protein binding was 23 mg/g for alkaline phosphatase to cyanogen bromide-activated Sepharose™, only half of which was active.

In an extensive study of leakage problems associated with N-hydroxysuccinimide (NHS) ester-activated supports, Wilchek and Miron[26] show that the relatively high density yield of these supports (up to about 100 mg/g) is accompanied by very high leakage rates. They recommend a variation which greatly reduces the leakage rate but also reduces the density to about 20 mg/g and conclude that 'for very high capacity columns, alternative methods should be developed." The azlactone-functional beads we have studied couple protein at densities higher than those reported for NHS supports[26], and, to the limited extent which we have studied loss of ligand from the support, the leakage rate is considerably reduced compared to NHS supports.

The pH optimum, as measured in this study, is primarily a measure of the reactivity of the available nucleophiles. The reactivities in the presence of chloride at pH 6–9 are consistent with the free (unprotonated) ε-amine of lysine as the attacking nucleophile. The results in the presence of high concentrations of sulfate are consistent with the sulfate-induced perturbance of the apparent p*K* for the deprotonation of the ε-amine of lysine by at least one pH unit.

The reactivities in the acidic region are more difficult to interpret. A review of the literature revealed five reports of pH effects on azlactone reactivity[27–31], none of which could verify our observation. In the only study under acidic conditions, Baranowski *et al.*[27] reported on the reaction of a monomeric azlactone derivative of *p*-nitrobenzoyl valine with gelatin at pH 3, 7.4, and 10. On the acidic side they report-

ed a 20-fold *decrease* in reactivity (pH 7.4 to 3), in marked contrast to the 4- to 20-fold *increase* we observed (pH 7 to 4). Additionally, Chuaqui *et al.*[32], using a 2-phenyl azlactone, have shown that there is an acetic acid-catalyzed azlactone ring-opening reaction with various amino acids in $CCl_4$ resulting in the formation of an amide bond. They suggested that one of two possible cyclic intermediates involving proton transfers could account for the reaction. We speculate that Baranowski *et al.*[27] did not observe acid catalysis because the more strongly electron-withdrawing *p*-nitrophenyl group at the 2-position on the azlactone ring destabilized the proposed intermediates. This would not be expected with the 2-phenyl of Chuaqui or the polymer backbone we used.

*Affinity chromatography*

The affinity chromatography experiments demonstrate the utility of the high protein densities which we have reported. Even at the highest density of rProtA which we have tested for biological activity, 214 mg/g (16 mg/ml), the amount of IgG recovered (56 mg/ml) yields a ratio of 1.0. This unexpectedly high binding activity at such high ligand coupling densities is not only counter-intuitive, it is also opposite to the effect reported by Eveleigh and Levy[33], *i.e.*, continuously decreasing ratio as antibody coupling density increased from 1 to 22 mg/ml. Although they were coupling an antibody which is considerably larger than Protein A, the size of its final binding complex with albumin (218 000 dalton) is comparable to the rProtA-IgG complex we have used (195 000 dalton).

Despite the wide interest in antibody purification by Protein A, few studies are reported in the scientific literature of IgG yields from immobilized Protein A. A study by Lee *et al.*[34] details their experience with purifying a monoclonal antibody on Protein A–Sepharose. They routinely obtained 4–8 mg of antibody per ml of column bed. Other studies with immobilized Protein A reported recoveries of 1.8 mg IgG per ml[35] and 5 mg/ml[36]. An immobilized Protein G investigation reported a yield of 2–5 mg/ml[37]. Immobilization of Protein A onto an azlactone support, which has yielded as much as 56 mg of IgG per ml of column bed, is clearly a substantial improvement.

Such high biological activity at high density means that the sizes of affinity columns can be scaled down, providing a great time and cost advantage in large scale purification processes. In very-small-scale analytical uses, such as described by Janis and Regnier[38], a reduction in the volume of the affinity column means a shorter cycle time and greater efficiency.

This increase in capacity is obtained at no loss in the quality of the affinity separation. Electrophoresis shows that the IgG is depleted from the serum and that there is little or no contaminant in the recovered IgG fraction. Back-pressures are consistently low ($<0.3$ MPa), and, under the usual flow conditions, we have seen no changes in bed volume.

We have immobilized almost 400 mg of rProtA per gram of 20:80 bead. This means that the resultant graft polymer is over 25% protein. Chromatographic results with a preparation containing 214 mg rProtA per g of polymer indicated that it bound an additional 700 mg of IgG per g, so that the final protein mass approximated the mass of azlactone–acrylamide copolymer.

In his review, Jervis[3] outlined several characteristics of an ideal activation/coupling method. The results presented here support azlactone copolymer beads as

closely approximating his model. Azlactone reacts rapidly and efficiently to form stable, uncharged, amide linkages between the ligand and the polymeric backbone. If there are any hydrolytic side reactions which form carboxylates or other groups, they do not interfere in the affinity applications we have investigated. Excess sites are easily blocked with ethanolamine resulting in an uncharged, hydrophilic ligand. In addition, there is no need for toxic reagents in either the activation, the coupling, or the blocking steps. Finally, a great benefit of the ring-opening nature of the azlactone reaction which is especially useful for production-scale applications is that there are no by-products of the coupling reaction itself which would have to be removed.

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