

STATIC AND DYNAMIC STUDIES OF PROTEIN
SORPTION BY POROUS ALKYL SILICA

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ABSTRACT

Porous alkylsilicas are important stationary phases (sorbents) for separating proteins by high performance liquid chromatography and have great potential for large scale isolations. We have employed batch experiments, frontal chromatography, and surface energy measurement techniques to study the factors which influence protein sorption to these materials. Proteins such as bovine serum albumin are sorbed irreversibly from phosphate buffer at pH 7 and pH 2. Addition of alcohol at sufficient concentration to reduce the surface tension of the solvent to less than 34 ergs/cm² effected desorption at pH 2 but not at pH 7. Proteins were desorbed by solutions of nonionic surfactant at pH 7. The chain-lengths of alkyl groups on the silica had little influence on sorption isotherms which, in the batch studies, were of the general Langmuir shape. The isotherms derived from the frontal experiments were indicative of two-stage desorption process.

INTRODUCTION

The sorption of proteins from aqueous solution to polymeric surfaces has been of interest primarily from the perspective of biocompatibility¹⁻³. However, the use of beds packed with porous silica based materials has become an important means for achieving the analytical and/or preparative separations of proteins⁴. One class of materials, called reversed-phase packings, are prepared

by the reaction of chloroalkylsilanes with surface silanol groups to form alkylsiloxysilica⁵. Spectral evidence⁶ suggests that the chemically bonded moieties are inhomogeneously distributed into high density regions where the moieties are less than 4 Å apart and low density regions where the distances exceed 8 Å. In either case, since the hydrodynamic Stokes diameters of many proteins of interest are greater than 50 Å, they may be viewed as interacting with a hydrocarbonaceous layer. This contrasts with alkylsubstituted carbohydrate-based matrices which contain many fewer alkylresidues where interactions occur with one or few surface sites⁷. We have demonstrated⁸ that at pH 2 proteins were desorbed from alkylsilicas by alcoholic-phosphate solutions, and that desorption was related to the surface tension of the desorbent, the surface energy of the protein, and the surface energy of the sorbent. Herein, we describe further the sorption of bovine serum albumin (BSA) to porous alkylsilicas.

MATERIALS AND METHODS

Batch sorption studies were conducted in 9 ml vials, fitted with teflon-lined screw caps, into which was weighed quantities of alkylsilica such that the ratio of sorbent to added solution was 25 mg per ml. The volume of added solution was varied to assure that sufficient liquid was available for concentration determination by multiple methods. The vials were shaken on a platform shaker (Eberbach Incorporated, Ann Arbor, MI, USA) operated at 80 oscillations/minute with an amplitude of ±2 cm. (Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.) Room temperature was controlled at 23 ± 1°C. Insulation was packed around the vials to dampen temperature variations due to drafts, air conditioner cycling, and heat generated by the shaker motor. Experiments were an hour in length except where noted. Sorbent was separated from solution (S₁) by filtration through a 0.45 micron pore GA-6 Metrical

filter (Gelman Instrument Company, Ann Arbor, MI, USA). Two controls were used in each determination. One consisted of BSA in solvent and subjected to all test conditions other than alkylsilica contact (C_1). The other was solvent contacted with sorbent (C_2). The latter corrected for trace amounts of impurities that might be extracted from the silica so that: $(\text{Response } S_1 - \text{Response } C_2) \cdot (\text{Conc. BSA initial}) / (\text{Response } C_1) = \text{Conc. BSA after contact}$. Material balance yielded the amount BSA sorbed per gram of alkylsilica. Duplicate controls and samples were used for each determination.

Three methods were used to measure S_1 and C_2 depending on the initial concentration of BSA. For concentrations between 0.1-1 mg/ml, differential absorbance of the protein containing solutions at 278 nm were determined using either a Beckman Model DB or Model DV-7 Spectrophotometer. Alternatively, the absorbance at 390 nm of the fluorescamine treated protein solutions was measured. The method for treating the solution was essentially that described in the literature⁹ except that the reagent (Floram, Roche Diagnostics, Nutley, NJ, USA) was dissolved in acetonitrile instead of dioxane and prepared fresh for each set of analyses. For assays of pH 2.1 protein solutions, buffer pH was adjusted to pH 11 so that the final pH was about 9. Below 0.1 mg/ml, concentrations were determined by measuring the relative fluorescence at 474 nm of the treated solutions excited at 390 nm using a Perkin-Elmer MPF-44E Spectrofluorometer. In all three methods the responses were linear over the concentration ranges for which they were used. At 0.1 mg/ml all three methods agreed to within $\pm 2\%$.

Bovine Serum Albumin (BSA) essentially fatty acid free was obtained from Sigma Chemical Company (St. Louis, MO, USA) and used without further purification.

Solvents were prepared with ACS reagent-grade potassium dihydrogen phosphate and 85% phosphoric acid, HPLC grade organic solvents, and water purified with a Continental (USA) ion-exchange-carbon cartridge circulating system. The pH of 0.05M KH_2PO_4

solutions was adjusted to 2.1 with 85% H_3PO_4 in the presence of the organic component. The nonionic surfactant used was an ethoxylated alcohol (Neodol 91-6) obtained from Shell (Houston, TX, USA). Sodium azide (0.02%) was added to aqueous solutions as a bacteriostat.

Frontal experiments were performed on a Model 8000 B (Spectra-Physics Corp., Santa Clara, CA, USA) liquid chromatograph equipped with a Spectroflow Monitor SF 770 (Schoeffel Inst., Westbrook, NJ, USA) set at 280 nm. The mixing chambers and injector of the chromatograph were bypassed to reduce lag time and backmixing between the pump and the column. BSA solution was pumped from one reservoir for the specified time then solvent pumped from another until return to original baseline was achieved. Flow rate was 1.0 ml/min. The column was a 25 cm X 4.6 mm octadecyl-dimethylsiloxysilica Supelcosil LC-18, 100 Å pore (Supelco, Bellefonte, PA, USA). The oven temperature was set at 25°C.

The recorder trace was digitized with a Hi-pad (Houston Instruments, Austin, TX, USA) and area slices computer calculated. The isotherm was then calculated as described by Gluckauf¹⁰, i.e. (slice of area between plateau and desired concentration of eluent/total area of trailing edge) (volume of eluent between column void volume and the plateau X concentration of sample solution) = amount desorbed. Material balance gives amount still sorbed. The amount of sorbent in the column was estimated to be 2.3 g (11).

The alkylsilicas (5 µm diam.) used in the batch studies were supplied graciously by Supelco, Incorporated and were comparable to materials that are packed into chromatographic columns. The chain lengths of alkyl groups were either 8 or 18 carbon atoms and the pore diameters either 100 Å or 500 Å. Typical reported silica surface areas were 170 m²/g for the 100 Å and 60 m²/g for the 500 Å material. Typical alkyl concentration was 3.3 µmoles/m².

RESULTS AND DISCUSSION

The results of batch studies of the sorption of BSA from

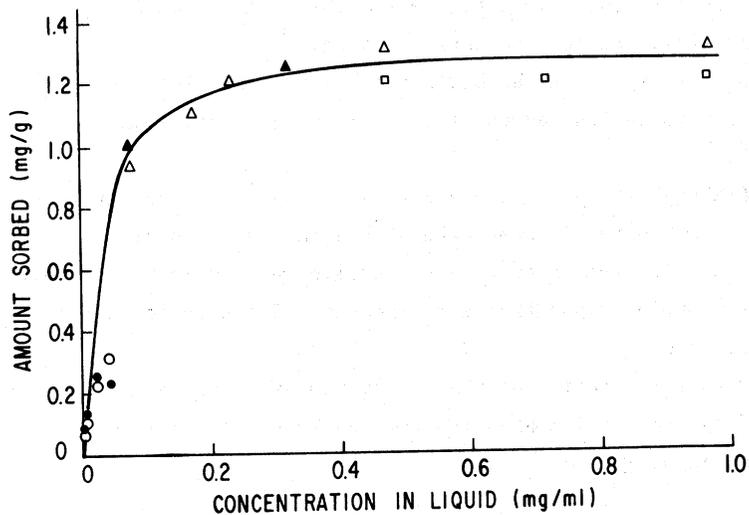


Fig. 1. Static sorption of bovine serum albumin onto alkylsilicas. Solvent: 2-propanol (40%)/0.05M phosphate, pH 2.1. (Δ) 500 Å pore, data using C₈ and C₁₈ alkyl groups superimposable; (Δ) 100 Å pore, data using C₈ and C₁₈ alkyl groups superimposable; (○) 100 Å pore, C₁₈; (○) 100 Å pore, C₈; (□) conc. in solvent determined by UV measurement, other data by fluorescence. T = 23 ± 1°C.

isopropanol(40%)-phosphate buffer solution (pH 2.1) are shown in Figure 1. Little, if any, difference in affinity for BSA is exhibited by the octyl- or octadecyl-dimethylsiloxysilicas. Since the surface energies of octane and octadecane do not differ greatly, this observation is consistent with the proposal made in the introduction that the protein may be viewed as interacting with a hydrocarbonaceous layer. Additional evidence for the comparability of the two sorbents is evidenced by the result obtained from gradient elution chromatography. When BSA was injected onto columns packed with the materials and the proportion of isopropanol varied linearly over a range of 30%-40%, desorption was observed at ~33%. As demonstrated earlier, this

corresponds to the composition where the surface tension of the solvent falls between the surface energy of the protein and of the sorbent, i.e., the Helmholtz energy of interaction (ΔF_{smp}) of protein with sorbent across a film of solvent passes through zero¹².

Although the protein has access to a greater percentage of surface in the 500 Å pore sorbent than in the 100 Å pore, the total surface is less than in the smaller pore sorbent, so that similar binding capacities are expected. The data shown reflect such behavior.

Data for sorption of BSA from a number of solvents was tested by numerical regression for adherence to the linear form of the Langmuir Equation:

$$\frac{C_M}{S} = \frac{K}{S_p} + \frac{C_M}{S_p};$$

where C_M = the final concentration of protein, S = mg of protein sorbed/g of sorbent, S_p = apparent capacity of the sorbent in mg protein/g, and K is the desorption constant. The coefficients of correlation were greater than 0.97 for all data sets except for the pH 7, 1% Neodol set which had a coefficient of 0.84. The standard errors for the line-slopes ($1/S_p$) were under 5%. The errors for the intercepts were much higher so that K should be considered accurate to no more than two significant figures.

Alcoholic solutions, at low pH were investigated because they are commonly used eluents for analytical separations of peptides and proteins. As predicted, a large change in K was observed over the range where ΔF_{smp} changes sign. $K \rightarrow 0$ for alcoholic solutions at pH 7 ($1 < \% < 40$) even though desorption at lower percentages might be anticipated because fewer apolar amino acid residues may be exposed than at the lower pH. The isotherm data demonstrate that desorption can be effected at pH 7 with solutions of nonionic surfactant at relatively high concentrations. Studies of the chromatographic properties of such systems are being

TABLE 1
Sorption¹ of BSA to Octyl- and Octadecyl- Silica.

Solvent	Apparent Binding Capacity (mg/g)	Desorption Constant (g/ml)X 10 ³
<u>pH 2.1</u>		
0.05M Phosphate(27) ²	3.2	5
30% 2-Propanol(29)	3.2	68
40% 2-Propanol(32)	1.9	130
0.01% Neodol(21)	2.6	11
1.0% Neodol(18)	2.6	110
<u>pH 7.0</u>		
0.05M Phosphate(8)	1.3	30
0.01% Neodol(13)	1.2	40
1.0% Neodol(22)	1.5	300

¹ 1 Hour contact time at 23 ± 1°C.

² Number of experiments in parentheses.

concluded and will be reported elsewhere¹³.

The apparent binding capacity in the absence of organic additive is much higher at pH 2 than pH 7. It has been shown recently¹⁴ that polystyrene random coils are sorbed in pores of such small size that they would be expected to exclude the molecule if the latter's hydrodynamic size is considered to be rigid. At low pH, BSA is much elongated and more flexible than at neutral pH¹⁵. Therefore it may have more access to the sorbent pores so that the amount sorbed/g is greater. At neither pH does the capacity as determined in these batch experiments approach the value calculated for a complete monolayer if the molecule is assumed to be an ellipsoid with axial dimensions of 34 X 142 Å. When similar silica-based materials are used in size-exclusion chromatography, they are observed to exclude BSA from about 90%

of the total pore volume¹⁰. If we assume, therefore, that the protein has access to ~10% of the available surface area, capacities of 200 mg/g and 38 mg/g are calculated depending on whether the molecules are viewed as having the long or short dimension lying on the surface. It is interesting to note that the observed capacities are much lower than the 2 mg/m² reported for the sorption of BSA onto hydrophobic homopolymer latices². Values of 1-2 mg/m² have been reported for both native and denatured BSA monolayers spread at the air/water interface¹⁷.

Somewhat different results are obtained when sorption of BSA is studied under dynamic conditions. In this approach, protein solution is pumped onto a column packed with alkylsilica (surface area 170 m²/g) until a plateau is reached. At the plateau, the concentration of protein in the column effluent is equal to its concentration at the inlet, which in the case shown is 1 mg/ml. Subsequently, solvent without protein is pumped. The effluent profiles for eluents containing 40% and 30% isopropanol are given in Figure 2. BSA breakthrough is near the column interstitial volume with the higher alcohol concentration, but occurs much later with the lower concentration. When BSA was dissolved in buffer containing no alcohol, 96 ml was pumped before protein appeared in the effluent (not shown) and the ascent to the plateau was extremely slow (>60 ml). To determine whether kinetic factors caused the asymmetry of the profiles, experiments were performed at two flow rates. The profiles (Figure 3) were superimposable indicating that these factors were not major contributors. These data suggest also that appreciable shearing of the macromolecule did not occur. More skewing of the edge eluting at the interstitial volume would be expected at the higher flow if this were so.

Sorption isotherms were calculated from the trailing edges of the profiles from the experiments with 30% and 40% isopropanol as solvents. The results, which are graphed in Figure 4, differ strikingly from those of the batch experiments. Bimodal lines described sorption at both solvent compositions. The slopes of the upper segments indicated capacities of 17 and 16 mg/g for the

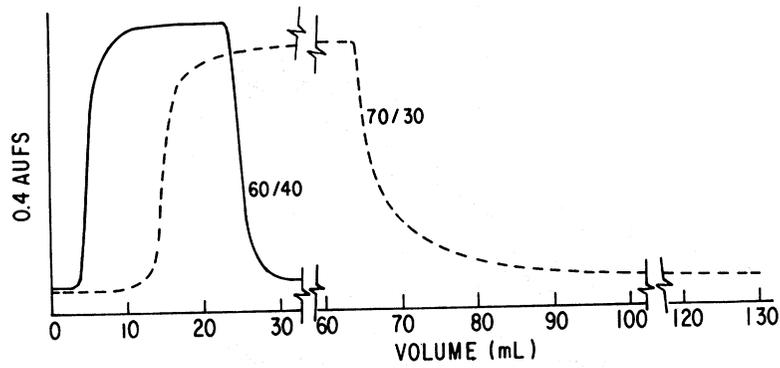


Fig. 2. Frontal profiles. Column: 25 cm long X 4.6 mm I.D. packed with Supelcosil-LC-18. Solvents: (—) 40% 2-propanol, pH 2.1; (---) 30% 2-propanol, pH 2.1. Solutions: as solvents, but containing 1 mg/ml BSA.

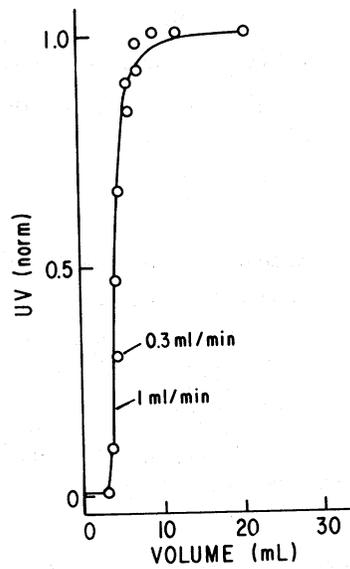


Fig. 3. Effect of flow rate (—) 1 ml/min; (O) 0.3 ml/min. Other conditions as in Figure 2.

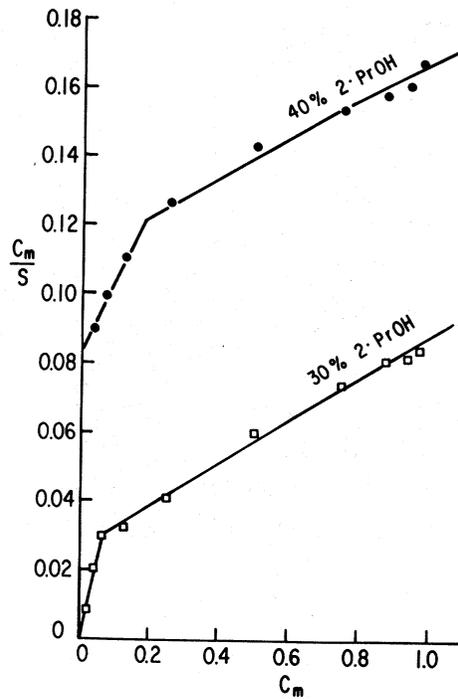


Fig. 4. Dynamic sorption of BSA. (O) 40% 2-propanol (pH 2.1); (X) 30% 2-propanol. C_M is conc. of BSA in effluent, S is mg sorbed per g alkylsilica.

greater and lesser alcohol contents. The capacities for the lower line-segments were 4.8 and 2.5 which are in the same order of magnitude as the capacities found in the batch experiments.

One possible explanation for the difference is that, under the gentle conditions of agitation used in the batch experiments to avoid particle fracture, a film of protein develops around the outermost particle surface which inhibits further solute migration into the particle.

Estimation of capacity from consideration of surface area of a spherical particle gives a value of the same order as observed in the batch experiments and as calculated from the lower line-

segments. Much longer exposure time should lead to greater sorption in the batch studies. Experiments to test this were inconclusive due to uncertainties produced by agitation of protein solution for days. Turbulent flow within the column could reduce the resistance to solute transfer leading to higher capacities. On the other hand, if increased capacity were due to greater migration into the pores, retarded desorption should result since the effective diffusivity of a protein in pores of comparable size is ~10% of the bulk diffusivity¹⁸. The results here show that the "extra" protein sorbed in the frontal experiments is more easily desorbed. A two-site sorption model may apply. Slightly enhanced penetration and sorption may occur in the dynamic system, then other protein molecules cluster around the former rather than complete monolayer coverage in the pore. The energetics of such systems have been described¹⁹.

CONCLUSIONS

Porous alkylsilicas are effective sorbents for proteins such as bovine serum albumin and have binding capacities as high as 50 mg/g.

Chainlength of the alkyl group has little influence on sorption of BSA.

Large quantities of alcohol or other organic modifiers are required for protein desorption at pH 2. Desorption is favored when the surface tension of the solvent falls between the surface energies of the protein and the sorbent.

Alcoholic buffers do not effect desorption at pH 7, but nonionic surfactants can desorb proteins at physiological pH.

Sorption isotherms obtained from batch studies could be expressed by the Langmuir Equation, but capacities were lower than expected for monolayer coverage.

Under the dynamic conditions of frontal chromatography, higher capacities were found and a two-stage desorption process observed, as evidenced by a bimodal isotherm.

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