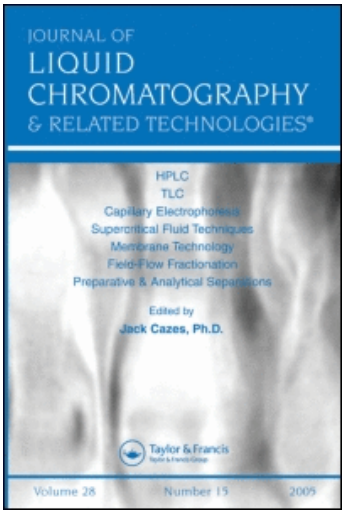

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LIQUID CHROMATOGRAPHIC STUDIES OF MEMORY EFFECTS OF SILICA IMMOBILIZED BOVINE SERUM ALBUMIN: I. INFLUENCE OF METHANOL ON SOLUTE RETENTION

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ABSTRACT

Silica immobilized bovine serum albumin (BSA) has been synthesized and studied chromatographically using D,L-tryptophan and L-Kynurenine. Site specific and background interactions have been measured as a function of temperature and treatment with methanol. The results indicate that solvent entrapment in the interior hydrophobic region of the protein may lead to small changes in conformation and/or dynamics which influence the site specific binding of the protein and hence changes in chromatographic retention. The entrapped solvents appear to be thermodynamically and kinetically stable such that their influence on the protein persists at elevated temperatures and over hundreds of column volumes of aqueous buffer eluent.

INTRODUCTION

Although bovine serum albumin (BSA) is a very stable protein which is held together by 17 disulfide bridges, its tertiary structure is relatively dynamic and can undergo rapid changes between one of five conformational states as a function of pH.[1] The indigenous binding of BSA as a function of tertiary structure has been the subject of numerous investigations and has been reviewed extensively.[1,2]

The first reported work discussing the enantiomeric selectivity of BSA for D- and L-tryptophan was in 1958.[3] The ability of the protein to selectively bind the L-isomer over the D-isomer has been attributed to both charge interactions and hydrophobic interactions which occur near tyrosine-411 in domain III.[4] This same site will bind related degradation products such as L-kynurenine and other structurally similar compounds like L-thyroxin.

The application of immobilized bovine serum albumin as a chromatographic packing has been demonstrated often. In most cases, separations have been carried out using aqueous buffer eluents in order to take advantage of the protein's indigenous binding. Allenmark and co-workers were the first to resolve the enantiomers of N-aroylamino acids on a silica immobilized BSA support.[5] Subsequently, these same investigators,[6-13] as well as others,[14-22] have separated various chiral compounds using either chemically or physically immobilized BSA or fragments of the protein.

Although the emphasis of most liquid chromatographic studies have been application oriented, mechanistic questions also have been considered. The chromatographic characteristics of immobilized BSA supports have been studied as a function of pH and ionic strength of the mobile phase as well as when small amounts of organic and amphophilic modifiers are added to the mobile phase. Very subtle modifications in the eluent conditions often lead to dramatic changes in chromatographic properties of the support.

Because of this, reproducible separations are difficult to obtain between laboratories and suggested models to explain solute-solvent-protein-surface interactions often are marred by inconsistencies. For example, Aubel and Rogers[15] have noted that pretreatment of the BSA-surface with simple aliphatic alcohols, tetrahydrofuran, or acetonitrile increases solute retention and enhances enantiomeric resolution for racemic mixtures of N-benzoyl-D,L-valine and of N-benzoyl-D,L-phenylalanine. They have attributed this behavior to two possible mechanisms, the removal of impurities from the bound protein or conformational changes in the protein's tertiary structure.

The first mechanism was suggested as the favored and major contributor. Whereas, Allenmark and co-workers have reported opposite findings[10] to those of Aubel and Rogers.[15] However, in this latter investigation, the experimental results appear to be flawed because the control protein column, which was supposedly not treated with organic modifier, was actually exposed to hexane which contained small amounts (i.e., 1-6%) of 2-propanol as a modifier and then rinsed with acetone prior to use.

The literature contains other examples of differing opinions or inconsistencies in experimental observations and theoretical interpretations about immobilized bovine serum albumin as well as other immobilized proteins. Much of the confusion can be attributed to gross over simplifications of very complex problems in protein chemistry/dynamics and solution equilibria when immobilized proteins are used as chromatographic packings.

Differences in the: 1) chemistry used for immobilization, 2) matrix material, 3) amount of bound protein, 3) packing and preconditioning procedures, 4) binding sites, and 5) eluent conditions are often minimized, not recognized, or ignored. Thus, the need for more controlled and systematic studies, which address fundamental mechanistic questions about solute-solvent-protein-surface interactions, is clearly evident.

In a previous investigation,[22] the influence of temperature and pH on the native binding of silica immobilized BSA was studied using D- and L-tryptophan as probe solutes. Because the protein has a binding site for the L-isomer and not the D-isomer, it was possible to measure simultaneously both the background and site specific binding.

In the case of D-tryptophan, plots of the natural logarithm of the chromatographic capacity factor vs reciprocal temperature in K were linear as expected. However, similar plots for L-tryptophan were curved with a maximum in binding (i.e., largest k') at 20-22 °C. This behavior, which is not explainable by a simple retention mechanism, was attributed to a possible phenomenological change in the bound protein.

In an effort to help clarify and to extend the above observations,[22] the current study was undertaken. The thermal dependencies of the binding of L-tryptophan as well as its oxidative degradation product L-kynurenine have been used to investigate solvent memory effects of silica immobilized BSA first exposed to the packing solvents, 2-propanol/methanol, and later to binary mixtures of phosphate buffer and methanol.

EXPERIMENTAL

Materials

The 3-aminopropyltriethoxysilane was from Huls America (Piscataway, NJ, USA), the LiChrospher SI-300 silica from EM Separations (Gibbstown, NJ, USA) and the 25% solution of glutaric dialdehyde from the Aldrich Chemical Company (Milwaukee, WI, USA). Sodium cyanoborohydride, D- and L-tryptophan, potassium chloride, and dibasic sodium phosphate were purchased from the Sigma Chemical Company (St. Louis, MO, USA). L-kynurenine was obtained from ICN Biochemicals (Cleveland, OH, USA). The solvents were either HPLC grade (methanol and 2-propanol) or reagent grade (toluene and 85% phosphoric acid) and were purchased from Fisher Scientific (Pittsburgh, PA, USA). The deionized water was prepared in-house using a Millipore (El Paso, TX, USA) MilliQ reagent water system.

Synthesis

Five grams of silica were mixed with deionized water, the water removed, and the material dried for 12 hr at 110 °C. The silica and 200 mL of water-saturated toluene were placed in a specially designed reaction flask equipped a sintered glass filter and bubbling ports, and the contents were stirred for 3 hr using a stream of dry nitrogen. Subsequently, 100 mL of the toluene were removed, 20 mL of 3-aminopropyltriethoxysilane added and the mixture was refluxed overnight. The toluene was drawn off via suction, the modified silica was washed four times by refluxing it with 100 mL portions of water-saturated toluene for 1 hr, and then dried under nitrogen.

The BSA was immobilized to the above derivatized aminopropyl-silica as follows.[22-24] A 5% solution of glutaric dialdehyde (115 mL), which was buffered to pH 7.0 with 50 mM Na_2HPO_4, and 2.3 g of $NaCNBH_3$ were added to a 300 mL reaction flask and the contents stirred with nitrogen until the borohydride had dissolved completely. The aminopropyl-silica (4.5 g) was added slowly and the walls of the reaction vessel were rinsed with 115 mL of pH 7.0 phosphate buffer. The reaction was carried out for 3 hr while stirring with a stream of dry nitrogen. The silica was allowed to settle and the supernatant removed via suction.

The resulting activated support was washed with ten 100 mL portions of deionized water, dried with a stream of nitrogen, and 3.2 g of it and 72 mL of phosphate buffer were added to a 250 mL round bottom flask. The contents of the flask were gently stirred while adding 90 mg of $NaCNBH_3$. Subsequently, 60 mL of

a pH 7.0 buffered solution of BSA (4.0 mg/mL) were added in drop-wise fashion using a separatory funnel. The funnel was rinsed with 11 mL of buffer and another 90 mg of $NaCNBH_3$ were added directly to the reaction flask while stirring the mixture. The protein coupling reaction was allowed to proceed for 20 hr at room temperature. The BSA modified silica was transferred to a 50 mL conical tube, centrifuged at 500 G, and the supernatant was decanted off and saved for later analysis. The resulting silica was washed with 80 mL of 0.2 M KCl buffered to pH 7.0 with 50 mM Na_2HPO_4 followed by 120 mL of deionized water. The washing supernatants were combined with the initial protein reaction supernatant and the UV absorbance measured at 279 nm.

An initial estimate of the amount of BSA coupled to the surface was determined by comparing the absorbance of the original reaction solution with the final collection of supernatants. A value of 52 mg of protein/g of silica was obtained. Later, more quantitative procedures, microelemental analyses of carbon, nitrogen and sulfur, (Huffman Laboratories), were performed on the immobilized support. These measurements indicated a coverage of 59 mg of the bound protein/g of silica.

Column Packing

The 2.1 mm i.d. x 150 mm stainless steel column was slurry packed in upward fashion by the following procedure. Approximately 0.5 g of the BSA modified silica was added gradually to 30 mL of 2-propanol which was contained in a dynamic packing apparatus. The apparatus was sealed, pressurized to 6000 psi using a Haskel (Burbank, CA, USA) model DST-52 pump and methanol as the carrier solvent.

Equipment

The liquid chromatographic system consisted of a Spectra-Physics (San Jose, CA, USA) model SP8810 precision isocratic pump, model Focus UV detector, and model Chromjet integrator. Samples were injected using a Rheodyne (Berkeley, CA, USA) model 7125 valve with a 20 μl loop.

The column temperature was controlled by placing it in a Fisher Scientific (Pittsburgh, PA, USA) model Isotemp 9500 refrigerated circulator bath and the flow rate was monitored with a Phase Separation, LTD (Queensberry, Clwyd, UK) model FLOSOA1 flow meter connected to the detector outlet.

Liquid Chromatographic Measurements

The solute (D-tryptophan, L-tryptophan and L-kynurenine) solutions were made fresh daily at a concentration of 0.001 mg/mL in deionized water. The buffer which was used both by itself as a mobile phase and in combination with methanol (i.e., 2, 4, & 6% alcohol/buffer V/V) as mobile phases were prepared by adjusting the pH of a 50 mM solution of Na_2HPO_4 to 7.4 using phosphoric acid. Immediately following packing, the column was conditioned overnight with the pH 7.4 buffer at a flow rate of 0.5 mL/min. Further conditioning was carried out at each temperature studied to allow the column to attain thermal equilibrium. Usually this corresponded to at least 30 minutes or longer. After conditioning, the retention times for the three solutes were measured from approximately 0.5 °C to 35 °C at approximately 4-5 degree intervals. Solutes were injected at least twice at each temperature studied. Once reaching the maximum temperature, the column was rapidly cooled to ambient temperature.

This same evaluation procedure was used for the other mobile phases studied. Complete thermal curves as described above were generated by using different mobile phases in the following sequential order: 1) 100% buffer, 2) buffer with 2% methanol, 3) 100% buffer, 4) buffer with 2% methanol, 5) 100% buffer, 6) buffer with 4% methanol, 7) 100% buffer, 8) buffer with 4% methanol, 9) 100% buffer, 10) buffer with 6% methanol, 11) 100% buffer, 12) buffer with 6% methanol, 13) 100% buffer.

Following these sets of experiments, the column was rinsed with deionized water and then the original packing solvents (2-propanol/methanol). Subsequently, the column was reconditioned by using the original procedure that followed packing and a final set of thermal measurements carried out.

RESULTS AND DISCUSSION

The immobilized bovine serum albumin support was prepared via initially derivatizing LiChrospher SI-300 silica with aminopropyl groups and then linking the protein to the surface with glutaric dialdehyde.[23,24] This same procedure has been used previously to modify ICN-300 silica,[22] however, based on the elemental analysis of carbon, nitrogen and sulfur, the surface coverage of the material prepared in the current work was approximately 40% greater (i.e., 59 mg of protein/g of silica vs 42 mg/g). Subsequently, the influence of temperature on the specific binding of L-tryptophan and L-kynurenine was studied chromatographically. Similar studies also were carried out for D-tryptophan which served as a background control since there is not a specific binding site in BSA for this latter isomer.

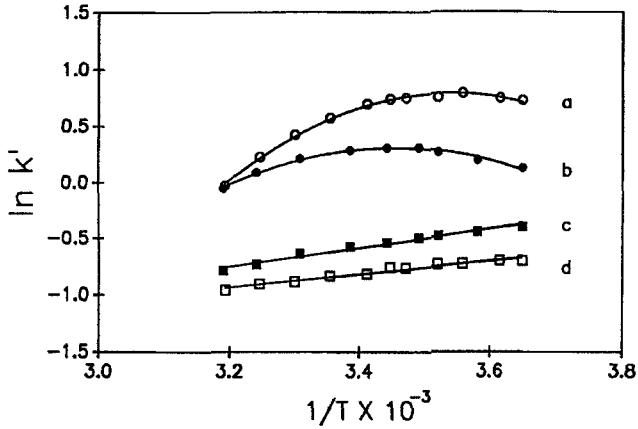

Figure 1. Influence of temperature on the binding properties of silica immobilized BSA. Solutes: Squares) D-tryptophan and Circles) L-tryptophan; Mobile phase: 50mM pH 7.4 phosphate buffer. Silica used for immobilization: Open symbols) LiChrospher-300; Closed symbols) ICN-300.

Plots of the natural logarithm of k' vs 1/T in K for both of the L-isomers were similar in shape to those obtained with the modified ICN-300 silica except they were offset to a lower temperature. This is illustrated in Figure 1 for L-tryptophan chromatographed using a 50 mM phosphate pH 7.4 buffer. Curve **a**, which is from the Merck derivatized silica, reaches a maximum in binding (largest k') at approximately 10 °C and curve **b**, which is from the ICN-300 immobilized BSA,[22] reaches a maximum in binding at approximately 20 °C. The initial increase in k' with increasing temperature at lower temperatures is thermodynamically inconsistent with a simple retention mechanism and has been attributed previously to a phenomenological change in the immobilized protein which increases either the number of specific binding sites or the strength of binding.[22] In the case of D-tryptophan, which does not specifically bind to BSA, plots of ln k' vs 1/T decreased linearly as a function of temperature (curves **c** and **d** for the ICN and Merck derivatized silica, respectively).

A further comparison of the L-isomer data obtained on the two BSA modified silicas (Figure 1) clearly demonstrates a difference in the specific binding characteristics of the two packings in region III where the L-tryptophan site is located. This may be due to either: 1) variations in the support material, 2) the amount of BSA bonded to the surface, or 3) both. In order to help clarify the reason for these differences, additional work is planned where various silica types, levels of coverage, and solutes which bind at other sites in the protein will be studied.

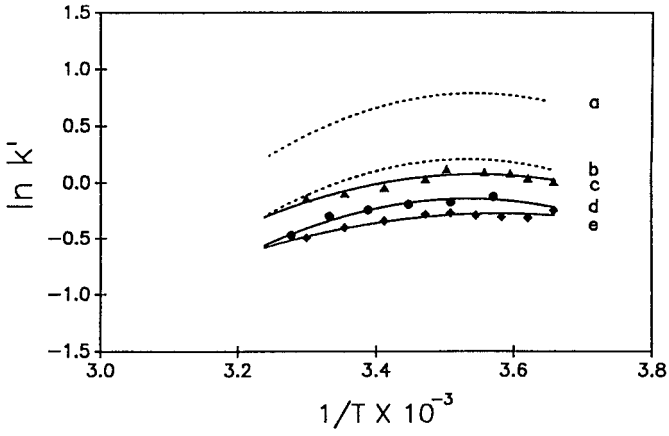

Figure 2. Influence of methanol on the binding of L-tryptophan by silica immobilized BSA. Mobile phase: 50mM pH 7.4 phosphate buffer a) immediately following packing, b) following exposure of column to the three different binary mobile phases containing methanol (i.e., those shown in curves **c-e**; c-e) with 2, 4, and 6% methanol added, respectively.

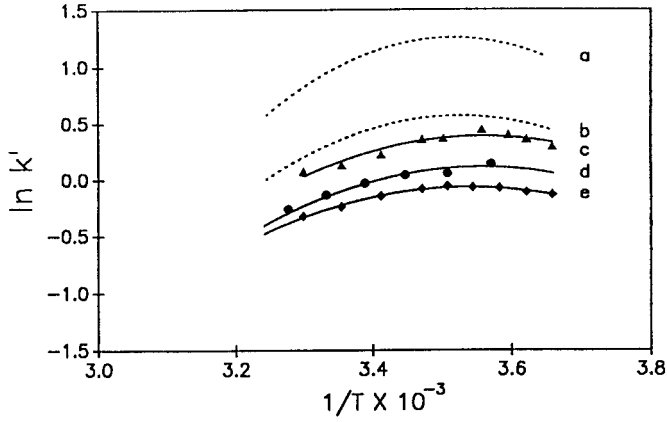

Figure 3. Influence of methanol on the binding of L-kynurinine by silica immobilized BSA. Mobile phase: same as Fig. 2.

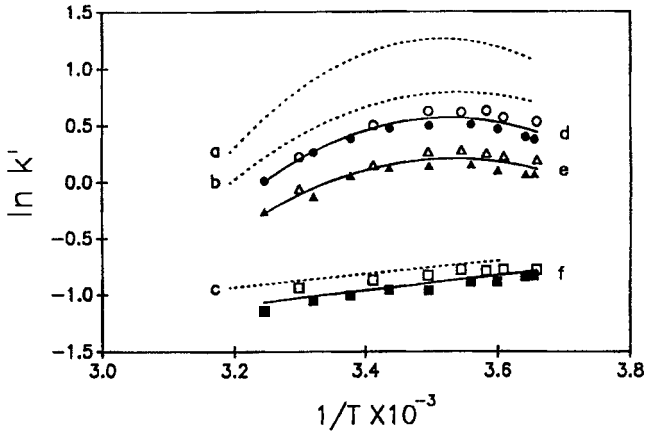

Figure 4. Influence of temperature on retention. Solutes: a & d) L-kynurenine b & e) L-tryptophan, c & f) D-tryptophan; Mobile Phase: 50mM pH 7.4 phosphate buffer; Evaluation: a-c) initial data, and d-f) post exposure to binary mobile phases containing methanol.

Following the initial thermal profile studies described above and which were carried out using 50mM Na_2HPO_4 pH 7.4 buffer as the eluent (Figure 1), a series of similar curves were generated by cycling between binary mobile phases consisting of the 50mM Na_2HPO_4 pH 7.4 buffer and small additions of methanol (2, 4, & 6%) and the original buffer. Each of the three methanol containing eluents was examined twice. Representative data from these studies are illustrated in Figures 2-4. Shown respectively in Figures 2 & 3 are the thermal curves as a function of varying additions of methanol for L-tryptophan and for L-kynurenine which also binds at the same site in the protein. The initial addition of 2% methanol resulted in the largest decrease (i.e., by a factor of approximately two) in the k' values for both the L-tryptophan and L-kynurenine (Curves **a** & **c** in Figures 2 & 3). When the column was reevaluated using the original buffer, the surface recovered only partially as illustrated by the curves **b** in Figures 2 & 3.

The excellent reproducibility of this partial recovery is demonstrated by the data summarized in Figure 4. The dotted curves, **a** & **b**, are second order regression fits of the initial data obtained using only the 50 mM phosphate buffer as the mobile phase for L-tryptophan and L-kynurenine, respectively. Whereas, solid curves **d** & **e** are the second order regression fits of the data obtained after the column had been exposed to 2% methanol (unfilled symbols) and after it had been cycled between all of the mixed mobile phases and buffer twice which represented a total of 9 complete thermal studies separating the two data sets (filled symbols). Also shown in Figure 4 for comparative purposes are the D-tryptophan data.

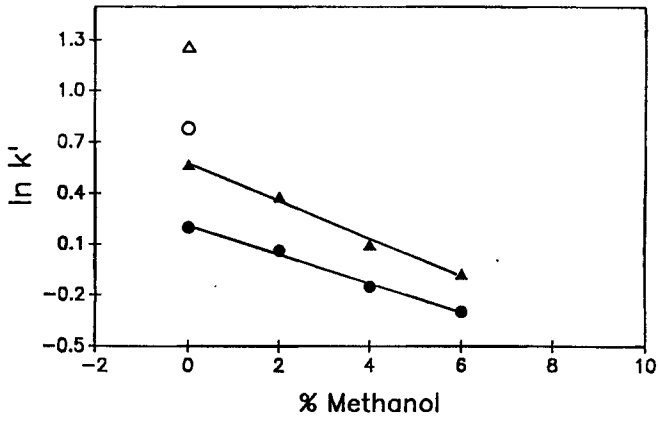

Figure 5. Retention as a function of methanol in the mobile phase. Temperature: 10 °C; Mobile phase: 50mM pH 7.4 phosphate buffer with 0-6% methanol added; Solutes: Triangles) L-kynurenine, Circles) L-tryptophan; Symbols: Unfilled) initial data following packing, filled) after exposure to 2% methanol.

A comparison of the reevaluation buffer data (curves **b** in Figures 2 & 3 and curves **d** & **e** in Figure 4), the 2% methanol data and the remaining curves for 4% methanol (curves **d** in Figure 2 & 3) and 6% methanol (curves **e** in Figure 2 & 3) show an approximate incremental drop in k' with addition of modifier. This relationship is illustrated further in Figure 5 for the maximum binding temperature, approximately 10 °C. Excluding the initial large decrease in k' (unfilled symbols in Figure 5), there is a linear relationship between the percentage of methanol in the mobile phase and ln k' (filled symbols in Figure 5). The regression coefficients for the two fits (solid lines) are both better than 0.99. The data in Figure 5 illustrate two important observations: 1) that the immobilized BSA can be alternated to a stable and reproducible form, and 2) that small additions of modifier, at least methanol, can be used to control retention in a predictable manner.

At this stage in the current work, it was believed that the above observations might be explained by one of two possible mechanisms, either loss of protein from the surface or a change in the protein's conformation which lead to reduced binding. In order to test these ideas and to better understand the changes which occurred in the immobilized BSA column (i.e., as noted by the large drop initially in retention) following its exposure to an eluent buffer containing small percentages of methanol, the column was reconditioned using the original packing solvents. In order to do this, the column first was washed overnight with deionized water at 0.5mL/min to eliminate residual buffer and then exposed to 2-propanol/methanol to simulate

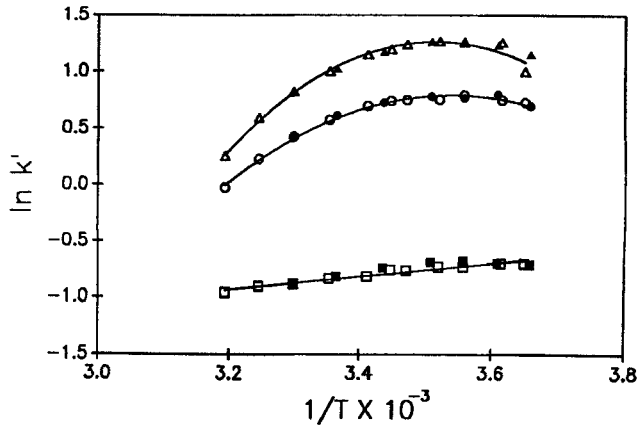

Figure 6. Protein recovery following treatment with 2-propanol/methanol. Mobile phase: 50 mM pH 7.4 phosphate buffer; Solutes: Triangles) L-kynurenine, Circles) L-tryptophan, and Squares) D-tryptophan; Curves: Unfilled symbols) initial set of data following packing, Filled symbols) last set of data following reconditioning with packing solvents.

packing conditions. Subsequently, the column was rinsed with 50 mM pH 7.4 phosphate buffer at a flow rate of 0.25 mL/min for 60 hours. Just prior to carrying out the final set of thermal experiments the column was further conditioned with the buffer mobile phase at 4 °C and a flow rate of 0.5 mL/min for one hour. Figure 6 shows the results of this experiment.

The data sets obtained in this latter experiment and the initial evaluation experiment which immediately followed packing are statistically identical for the three solutes and suggest that the initial change in the protein following exposure to the methanol-buffer mobile phase was not due to protein loss but to a stable yet switchable modification of the protein's structure.

CONCLUSION

The current work demonstrates that the binding properties of silica immobilized bovine albumin serum can be controllably altered using alcohol treatment. It is believed this may be due in part to solvent entrapment (i.e., in the current case, either 2-propanol and/or methanol) in the interior hydrophobic regions of the protein which leads to differences in binding at least at the L-tryptophan site which is located in region III near tyrosine-411. In the case of initial exposure of the immobilized BSA to mixtures of 2-propanol/methanol, binding was enhanced in the

aqueous buffer used as the mobile phase over that observed following exposure of the protein to binary mixtures of buffer and methanol. Such memory effects potentially may be useful in controlling the chromatographic behavior of immobilized BSA packing. Work is now in progress to explore these ideas.

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REFERENCES

1. T. Peters, Jr., in **Advances in Protein Chemistry**, Vol. 37, Ed. by C. B. Anfinsen, J. T. Edsall, F. M. Richards, Academic Press, New York, 1985, Vol. 37, p. 161.

2. V. M. Rosenoer, M. Oratz, M. A. Rothchild, **Albumin Structure, Function, and Uses**, Pergamon Press, New York, 1976.

3. R. H.McMenamy, J. L. Oncley, J. Biol. Chem., **233**, 1436 (1958).

4. L. Moravek, M. A. Saber, B. Meloun, Collect. Czech. Chem. Commun., **44**, 1657 (1979).

5. S. Allenmark, B. Bomgren, J. Chromatogr., **264**, 63 (1983).

6. S. Allenmark, B. Bomgren, H. Boren, J. Chromatogr., **316**, 617 (1984).

7. S. Allenmark, S. Anderson, J. Chromatogr., **351**, 231 (1986).

8. S. Allenmark, J. Liq. Chromatogr., **9**(2/3), 425 (1986).

9. S. Allenmark, S. Andersson, J. Bojarski, J. Chromatogr., **436**, 479 (1988).

10. S. G. Allenmark, **Chromatographic Enantioseparation Methods and Applications**, Halsted Press div. of John Wiley & Son, New York. 1988.

11. R. A. Thompson, S. Andersson, S. Allenmark, J. Chromatogr., **465**, 263 (1989).

12. S. Andersson, S. Allenmark, J. Liq. Chromatogr., **12**(3), 345 (1989).

13. S. Andersson, S. Allenmark, P. Erlandsson, S. Nilsson, J. Chromatogr., **498**, 81 (1990).

14. M. T. Aubel, L. B. Rogers, J. Chromatogr., **392**, 415 (1987).

15. M. T. Aubel, L. B. Rogers, J. Chromatogr., **408**, 99 (1987).

16. G. Gubitz, Chromatographia, **30**(9/10), 555 (1990).

17. I. W. Wainer, Trends Anal. Chem., **6**(5), 125 (1987).

18. S. Jacobson, S. Golshan-Shirazi, G. Guiochon, J. Am. Chem. Soc., **112**, 6492 (1990).

19. S. Jacobson, S. Golshan-Shirazi, G. Guiochon, Chromatographia, **31**(7/8), 323 (1991).

20. P. Erlandsson, S. Nilsson, J. Chromatogr., **482**, 35 (1989).

21. Z. Simek, R. Vespalec, J. High Res. Chromatogr., **12**, 61 (1989).

22. R. K. Gilpin, R. K., S. B. Ehtesham, R. B. Gregory, Anal. Chem., **63**, 2825 (1991).

23. H. H. Weetal, Methods Enzymol., **XLIV**, 134 (1976).

24. N. Jentoft, D. G. Dearborn, J. Biol. Chem., **254**(11), 4359 (1979).

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