

Systematic Measurements of Peptide Adsorption in Hydrophobic Chromatography

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A systematic study has been made of peptide retention in reversed phase and hydrophobic interaction chromatography. Measurements have been made for a range of peptides under varied surface hydrophobicity, peptide structure, peptide charge, cosolvent concentration, temperature, pH, and concentration of added salt. The conditions and peptides have been organized so that, for at least three peptides, only a single variable is changed. These stepwise variations should provide benchmark data to validate molecular models and computer simulations that predict adsorption and retention in hydrophobic media from biochemical structure and a minimum of adjustable parameters.

Introduction

Hydrophobic interaction chromatography (HIC) and reversed phase chromatography (RPC) are valuable for protein purification but challenging to apply. HIC is commonly used for preparative separations,¹ while RPC is used mainly for analytical applications.² Preparative applications of both methods to proteins are limited by complex retention and selectivity behavior³ and the apparent effects of hydrophobic surfaces on protein stability.^{4–7} Consequently, there is a need for predictive tools to describe the effects of mobile and stationary phase variables on retention. While there are many hydrophobicity scales available for predicting peptide retention, these are empirically based and are built from gradient elution data under prescribed stationary and mobile phase conditions (e.g., refs 8 and 9). For models that attempt to describe the quantitative thermodynamics of such systems, the basic data should be collected under conditions of isocratic elution, where differences in adsorption free energies can be readily calculated. Furthermore, if models describing the effects of biomolecule structure as well as mobile and stationary phase variables are to be developed, isocratic elution data on a single set of molecules under carefully selected ranges of conditions are required for parametrization and validation. In particular, while there are abundant gradient elution RPC data on peptides available in the literature, there is not a comprehensive set of isocratic data that meets the above constraints.

We are developing a mesoscale computer simulation approach that predicts retention on hydrophobic chromatography media from three-dimensional structures of the polypeptides.^{10–12} This methodology incorporates an atomistic description of solutes with a lattice–dipole description of the solvent and uncharged surface regions. Because the model is physically based, many fewer parameters should be required than for empirical approaches. Furthermore, the method can be applied to a wider range of independent variables rather than only a prescribed set of conditions.

To parametrize and test our model, we have collected a set of isocratic peptide RPC elution data to allow comparison of

simulation and experiment in which only one variable is changed at a time while all other conditions are kept the same. For example, under the same mobile and chromatographic surface conditions, the retention of nine protected peptides of different composition were obtained on C₁₈ RPC particles. The protected peptides were selected as readily available peptides that span a range of hydrophobicity wide enough to produce significant variations in retention but narrow enough that the retention of multiple peptides could be measured under one isocratic condition. Subsequently, the effects of changing only the chromatographic surface were determined by measuring retention of some of the same peptides on C₄ RPC particles at the same solvent conditions. This approach was used to obtain the individual effects of changing temperature, cosolvent composition, pH, and salt concentration. Partially protected and unprotected versions of some of the same peptides were also included to determine the effect of changes in net charge. We found that peptide retention is such a strong function of these variables that not all peptides could be used for all conditions. Consequently, a series of peptides with varying hydrophobicity was used such that at least three peptides were used to establish the effects of each variable. We intend for these data to support the development of this and other models, including applications to protein adsorption on HIC as well as RPC media.

Materials and Methods

The peptides used are listed in Table 1. They were obtained from Sigma–Aldrich (St. Louis, MO) and used without further purification. Protecting groups at the N- and or C-termini, where present, are listed in Table 1. Peptides P3, U4, and U5 were numbered after their corresponding fully protected structures, F3, F4, and F5. Unprotected peptides U10 and U11 were numbered uniquely and in order of their relative chromatographic retention.

Chromatography was performed on an AKTA Explorer 10 chromatography system (GE Healthcare, Piscataway, NJ). Samples were prepared as 0.025 mg/mL solutions with 100 μ L injection volumes, and the flow rate was 1 mL/min. The RPC columns containing 15 μ m diameter alkyl-bonded silica particles (C₁₈ and C₄) with 200 Å pores were gifts from Novo Nordisk, A.S. (Gentofte, Denmark). Both columns were 4 mm in diameter and 25 cm in length. The phase ratios were measured using an unretained salt tracer. The average of several runs was 0.435

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Table 1. Synthetic Peptides Investigated

peptide number ^a	N-terminal protecting group ^b	sequence	C-terminal protecting group	CAS Registry Number
		Protected		
F1	Z	PLG	amide	14485-80-4
F2	N-(methoxysuccinyl)	AAPM	<i>p</i> -nitroanilide	70967-91-8
F3	N-(methoxysuccinyl)	AAPV	4-nitroanilide	70967-90-7
F4	Z	MG	ethyl ester	27482-82-2
F5	Z	VY	methyl ester	15149-72-1
F6	pGlu	FL	<i>p</i> -nitroanilide	85901-57-1
F7	Boc	AWMDF	amide	5534-95-2
F8	Z	GGL	<i>p</i> -nitroanilide	53046-98-3
F9	N-formyl	MLF	benzyl ester	70637-32-0
		Partially Protected		
P3	N-(methoxysuccinyl)	AAPV		107441-48-5
		Unprotected		
U4		MG		14486-03-4
U5		VY		3061-91-4
U10		LGTIPG		none
U11		LLY		20368-24-5

^aF = fully (both ends) protected, P = partial (single end) protection, and U = unprotected. ^bZ = benzyloxycarbonyl; Boc = *tert*-butyloxycarbonyl.

and 0.237 for the C₁₈ and C₄ columns, respectively. The columns were placed in a column oven (Mistral Spark, Sonntek, Inc., Upper Saddle River, NJ) to control temperature to within 1 °C for all experiments. The other mobile phase conditions were as specified in the text and captions below. Although the cosolvent compositions are expressed in volume fractions ϕ , the required amounts of acetonitrile and water were measured by mass to increase accuracy and reproducibility in preparation of the solutions. Trifluoroacetic acid (TFA) (0.0002 volume fraction) was included in all of the pH 2.0 solutions, and 25 mmol·L⁻¹ potassium phosphate was used as the buffer for pH 7.0 solutions.

In the chromatography experiments, the equilibrium adsorption constant was determined from peptide and inert tracer retention volumes, V_r and V_0 , respectively. Peptides were detected by UV absorbance at 215 nm. Sodium nitrate (20 g/L) was used as the inert tracer and was detected by UV absorbance at 310 nm.

Results and Discussion

Retention factors measured for the peptides in Table 1 were measured for a variety of stationary and mobile phase conditions. Retention factors were calculated according to

$$k' = \frac{V_r - V_0}{V_M} \quad (1)$$

where V_r is the peptide retention volume, V_0 is the retention volume of the inert salt tracer, and V_M is the mobile phase volume, which is V_0 with the extracolumn volume of tubing, fittings, etc. subtracted. Although free energies of adsorption were not calculated here, these retention factors could be related to equilibrium constants and free energy differences via

$$\Delta G^0 = -RT \ln(K) = -RT \ln\left(\frac{k'}{\phi_{\text{phase}}}\right) \quad (2)$$

where the phase ratio is estimated by

$$\phi_{\text{phase}} = \frac{V_{\text{col}} - V_M}{V_M} \quad (3)$$

where V_{col} is the total bed volume calculated from the dimensions.

Table 2. Retention Factor k' for Fully Protected Peptides at pH 2.0 in $\phi(\text{Acetonitrile}) = 0.30$ on C₁₈ Media at Four Temperatures

peptide	k' at $T/^\circ\text{C}$			
	10	25	35	45
F1		3.13		
F2		4.11		
F3	3.51	3.27	3.15	2.96
F4		7.82		
F5	17.9	11.6	9.76	8.32
F6		14.6		
F7		14.9		
F8	93.2	41.0	39.0	25.0
F9		66.4		

The fully protected (F) peptides were numbered according to their retention order on the C₁₈ media. The values and retention order for peptide F3 at 25 °C are slightly different from our previously reported values.¹¹ All of the data in Table 1 are based on extended measurements; two or more replicates were performed for nearly all the conditions, and averages are reported in Table 2. The average absolute deviation among replicate retention factors was 0.18. The average relative deviation was 2 %. This relative uncertainty was also representative for the other data shown in Tables 3, 4, and 5. As is generally expected, retention decreases with increasing temperature for the three peptides for which data were collected from 10 °C to 45 °C (peptides F3, F5, and F8). The variation of retention of the nine peptides does not directly correlate with peptide length or any other simple molecular property. However, the retention of the peptides at 25 °C has generally been predicted well by our method¹² and the effects of temperature described quantitatively.¹⁰

Table 3 shows average retention factors for the nine protected peptides (F1 to F9) as a function of pH, NaCl ionic strength, and acetonitrile concentration. As expected, the retention factors decrease with increasing cosolvent concentration. Because all of the peptides F1 to F9 were protected at both the carboxy and the amino termini, the only major expected effect of pH is a change in the ionization state of side chains. Indeed, the differences in retention factors between pH 2.0 and pH 7.0 are quite small except for peptide F7, which includes an aspartic acid residue that would be uncharged at pH 2.0 and has a net charge of -1 at pH 7.0. The drop in retention factor from 14.9 to almost zero is consistent with this change in charge. Notably, the changes in retention of the other peptides between the two pH values were quite small, with no apparent systematic trends. Given that the other peptides are not expected to change in charge between the two pH values, the insensitivity to pH for peptides F1 to F6 and F8 also indicates that the silanol groups of the substrate are well-end-capped in these materials. It also suggests that the results are likely to apply to similarly alkyl-bonded silicas of other commercial vendors. For increasing salt concentration with the C₁₈ column, the retention results for five peptides (F1, F4 to F7) at pH 2 and $\phi(\text{acetonitrile}) = 0.30$ showed changes in retention that are all greater than the average replicate error. Notably, the changes for this set were monotonic with ionic strength but not all were in the same direction. Peptides F1, F6, and F7 decreased in retention with increasing salt, while peptides F4 and F5 increased in retention with added salt. These trends did not correlate with apparent hydrophobicity as measured by retention.

Table 4 shows the retention factors for protected peptides F1 to F6 at 25 °C, with systematic changes in pH, acetonitrile concentration, and salt concentration. For the peptides investigated (F1 to F6), there were no significant changes in ionization of side chains. Consequently, it is not surprising that there were

Table 3. Effects of Salt, pH, and Acetonitrile Volume Fraction ϕ on Protected Peptide Retention Factors k' on C₁₈ Resin at 25 °C

peptide	k'							
	pH 2.0						pH 7.0	
	0 NaCl/mol·L ⁻¹			0.375 NaCl/mol·L ⁻¹	1 NaCl/mol·L ⁻¹	0 NaCl/mol·L ⁻¹		
	$\phi = 0.20$	$\phi = 0.25$	$\phi = 0.30$	$\phi = 0.30$	$\phi = 0.30$	$\phi = 0.20$	$\phi = 0.25$	$\phi = 0.30$
F1	23.4	7.82	3.13	2.68	2.39	23.0	7.66	2.87
F2			4.11				9.47	3.79
F3			3.27				7.26	3.00
F4			7.82	8.34	11.0	40.8	17.7	8.23
F5			11.6	12.2	16.8	82.2	31.5	12.0
F6			14.6	12.4	10.6			13.5
F7		55.0	14.9	14.0	11.0	21.5	3.08	0.78
F8			41.0					41.6
F9			66.4					

Table 4. Protected Peptide Retention Factors on C₄ Resin at 25 °C

peptide	k'							
	pH 2.0				pH 7.0			
	0 NaCl/mol·L ⁻¹		0.375 NaCl/mol·L ⁻¹	1 NaCl/mol·L ⁻¹	0 NaCl/mol·L ⁻¹			
	$\phi = 0.30$	$\phi = 0.30$	$\phi = 0.30$	$\phi = 0.30$	$\phi = 0.25$	$\phi = 0.20$	$\phi = 0.15$	
F1	2.26	2.08	1.83	2.24	5.72	13.5		
F2				2.71	6.85	17.6	64.1	
F3				2.27	5.71	14.6	53.7	
F4	4.75	5.46	7.89	4.70	9.93	20.0	50.3	
F5	6.80	7.85	11.29	6.77	16.2	41.2		
F6	8.51	8.11	7.94	8.26				

Table 5. Partially Protected and Unprotected Peptide Retention Factors on C₄ Resin at 25 °C, pH 2.0, as a Function of Acetonitrile Volume Fraction ϕ

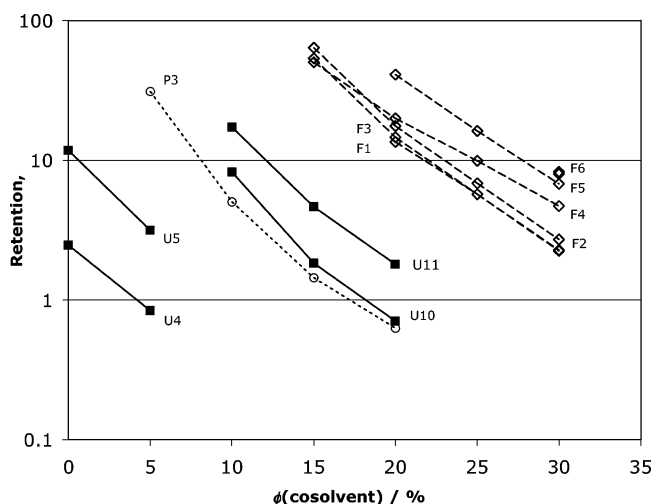
peptide	k'							
	0 NaCl/mol·L ⁻¹					0.375 NaCl/mol·L ⁻¹	1 NaCl/mol·L ⁻¹	
	$\phi = 0.00$	$\phi = 0.05$	$\phi = 0.10$	$\phi = 0.15$	$\phi = 0.20$	$\phi = 0.15$	$\phi = 0.15$	
P3		31.01	5.03	1.44	0.63	1.62	2.07	
U4	2.46	0.84						
U5	11.79	3.15						
U10			8.22	1.83	0.71	1.55	1.98	
U11			17.24	4.64	1.80	4.06	5.22	

only small changes in retention with pH at $\phi(\text{acetonitrile}) = 0.30$. The retention of five peptides, F1 to F5, were measured as a function of cosolvent concentration at pH 7.0 and found to increase in retention with decreasing cosolvent concentration. Four of the peptides were tested for salt concentration effects, with peptides F1 and F6 having weak decreasing trends in retention with salt concentration, while F4 and F5 had marked increases in retention with salt concentration. Again, these trends did not correlate with apparent hydrophobicity, as measured by retention in the absence of salt.

Ultimately, our goal is to investigate the retention of hydrophobic peptides and proteins on less hydrophobic HIC media under conditions with little or no cosolvent present but with varying salt concentration. A series of unprotected peptides was chosen to initiate steps in this direction. They were chosen to be partially or completely unprotected so that their charge would provide higher solubility in the absence of cosolvent as well as to reduce retention to measurable levels in low (or no) cosolvent. The retention factors of these peptides on the C₄ resin at pH 2.0 are shown in Table 5. Again, they follow the expected trend of decreasing retention with increasing cosolvent. This behavior is shown graphically in Figure 1.

Peptides P3, U4, and U5 were chosen because their protected versions are among our protected peptides. All three of these peptides had much lower retention than their fully protected counterparts. N-terminally protected peptide P3 had measurable, but much lower, retention than its fully protected counterpart F3. This is expected, as P3 would carry a fractional negative

charge at the unprotected carboxyl terminus with a pK in the vicinity of 2.0. In contrast, all the protected peptides have no functional groups with charges at pH 2.0. Peptides U4 and U5 show no measurable retention when the cosolvent volume fraction is higher than 0.05. These peptides are fully unprotected and would carry a positive charge at the N-terminus and a partial negative charge at the C-terminus. While the net charge is similar in magnitude but opposite in sign to P3, the retention is

**Figure 1. Retention as a function of cosolvent on C₄ media. Peptides are labeled as shown in Table 1.**

much less. This shows how simple charge arguments can explain the effects of added charge on retention; however, they are not reliable when treating the effects of combined positive and negative charges. These data with and without N- and C-terminal protecting groups will provide a valuable test of the ability of simulations to describe the effects of charge on retention. Figure 1 shows that the set of peptides spans a range of retention from volume fractions of 0.30 to 0.00. They were chosen to provide a test of the ability of models to predict effects of peptide composition and cosolvent concentration. Peptides U10 and U11 were included in the study to expand the set of unprotected, charged peptides with greater retention.

Notably, the trends in retention with increasing salt concentration are *not* monotonic for the three peptides P3, U10, and U11. Peptide P3 showed increments in retention factor monotonic with salt concentration, while peptides U10 and U11 exhibited a decrease in retention from 0 to 0.375 mol·L⁻¹ ionic strength and an increase in retention from (0.375 to 1.0) mol·L⁻¹. The main difference between these two unprotected peptides and the other unprotected species is the presence of the N- and partial C-terminal charge at pH 2.0. It is conceivable that the decrease in retention reflects salt effects on the solubility in solution where “salting in” occurs at moderate ionic strength, while “salting out” is found at high ionic strength. Such trends are commonly observed in solubilities of proteins and other hydrophobic solutes. We are aware of little reversed phase chromatography concerning such salt effects, and there seem to be no models that can predict such trends as a function of polypeptide structure.

Conclusions

We have investigated the retention of a set of protected and unprotected peptides as a function of cosolvent, pH, salt concentration, and stationary phase. While some of the general trends of cosolvent and temperature are as expected, the retention variations with salt concentration are not readily based on simple molecular concepts. Such complex and variable trends are important to investigate further for their connections to selectivity manipulation of peptide and protein separations by reversed phase and hydrophobic interaction chromatography. Furthermore, future modeling approaches that can describe such trends will be valuable for process development. The data

reported should facilitate crafting and validating such models for varying surfaces and peptides along with systematic changes in mobile and stationary phase conditions, solute charge, and salt concentration.

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Received for review July 30, 2005. Accepted November 13, 2005. This work was supported by the National Science Foundation (BES-0214183).

JE050303E