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THERMODYNAMICS OF α-LACTALBUMIN DENATURATION IN HYDROPHOBIC-INTERACTION CHROMATOGRAPHY AND STATIONARY PHASES COMPARISON

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SUMMARY

These studies present an evaluation of the role of surface hydrophobicity and temperature on the denaturation characteristics of α-lactalbumin under hydrophobic-interaction chromatography conditions, and a description of a model system to evaluate the role of chromatographic columns in the denaturation process of proteins.

The chromatographic characteristics of α-lactalbumin have been compared using commercially available poly(methyl); -(ethyl)- and -(propyl)aspartamide columns. These columns were designed for hydrophobic-interaction chromatography and represent a homologous series of stationary phases with increasing hydrophobic character. The elution profiles and retention times of α-lactalbumin have been also compared in different mobile phases and as a function of temperature. The chromatographic characteristics of the Beckman CAA-HIC column, another commercially available column for hydrophobic-interaction chromatography, have also been evaluated, permitting further insight into the effect of bonded phases on protein denaturation.

The retention time–temperature curves obtained are sigmoidal, characteristic of the classical transition curves of protein denaturation. Thermodynamic parameters of the denaturation of α-lactalbumin are calculated from the retention data and the calculated transition temperatures and free energies of denaturation are used for comparison of the different columns. The model system and the calculated thermodynamic values represent a useful method for the evaluation of such columns, and can provide an estimate of the contribution of the stationary and mobile phases to the protein denaturation process.

INTRODUCTION

Hydrophobic-interaction chromatography (HIC) is the last of the classical liquid chromatographic techniques to be converted into the high-performance

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mode[1–7]. Modern HIC has been developed based on earlier work with soft organic gels[8–11], and experience with protein reversed-phase chromatography[11–16]. HIC uses descending salt gradients, most frequently ammonium sulfate, to separate the analyte from a mildly hydrophobic stationary phase. A variety of silica-based stationary phases have been developed and are used routinely for protein separations. The popularity of HIC is due to the elution conditions, which usually provide excellent mass and activity recoveries for most proteins.

It has been shown in reversed-phase chromatography that multiple peaks[16,17], peak shape distortion[18] and changes in the retention volume of a pure protein result from conformational changes[18]. It has been also shown that the magnitude of these chromatographic alterations is related to the extent of unfolding of a particular protein[18–20]. In the case of papain, two widely separated peaks have been observed. The early eluting peak was identified as the native form and the late eluting peak as the denatured form of the enzyme[17]. Since unfolding modifies structure of the protein and consequently the complementary surface, *i.e.*, the attachment points between the protein and the stationary phase, the adsorption and retention of the protein is altered. This relationship between the surface of a protein and the stationary phase defines the mechanism of separation and selectivity in all adsorption chromatographies, such as reversed-phase liquid chromatography, HIC, electrostatic interaction chromatography (EIC), metal interaction chromatography (MIC) and affinity chromatography. These techniques can sometimes detect extremely intimate modifications of the complementary surface but might be blind for major alterations of proteins if they do not affect the complementary surface[19]. A study of the role of chromatographic parameters in conformational changes can provide insight into the phenomena involved in the separation of proteins[18,20,21]. Such studies serve as a model to study the interactions between proteins and solid surfaces, an interface where it is known that protein conformational changes can occur[22].

A kinetic model for the events occurring during protein chromatography has been suggested which involves two steps[20]. The first step is the kinetically rapid initial contact of the protein with the surface, while the second step, kinetically slow, includes all further conformational events occurring until elution. It has been shown that the major cause of protein denaturation under reversed-phase chromatographic conditions is adsorption onto the stationary phase, which eventually causes denaturation[20]. When the apparent hydrophobicity of the surface is altered by the adsorption of *n*-propanol[20] or of non-ionic detergents[23] prior to separation, a greater amount of native protein is recovered. These observations led to the development of non-ionic, mild stationary phases, specifically designed for HIC[2,6,7].

In HIC the high salt content of the starting mobile phase increases the surface tension of the mobile phase, and correspondingly, the solvent–stationary phase interfacial tension. The free energy of adsorption of the protein to the stationary phase is negative. Decreasing the salt concentration thus decreases the interfacial tension and permits the elution of the protein[20].

Many experimental parameters affect the interfacial tension and can change the elution characteristics of proteins. The type and concentration of salt and the pH of the mobile phases affect retention, resolution, selectivity and peak shape. The ligand density, charge characteristics and hydrophobicity are variable parameters of the stationary phase. In low-pressure chromatography, using chemically modified soft

gels, the length of the bonded alkyl chain has a tremendous effect on the retention and selectivity of the separation[11]. Similar behavior has been observed in high-performance HIC, using stationary phases prepared by the same bonding chemistry[7]. This allows the surface hydrophobicity to be modulated by attaching *n*-alkyl homologues with different chain length[7]. The retention of proteins under identical mobile phase conditions generally increases as the hydrophobicity of the stationary phase increases. This change in the retention time varies from protein to protein and may result in significant differences in resolution and selectivity.

The temperature is a very important parameter in the modulation of column selectivity. In protein chromatography, the column temperature might affect the mass and activity recovery of proteins by altering the conformational equilibria of proteins. It has been shown that the column temperature can have a major impact on the retention behavior of certain proteins under reversed-phase[13,14,16–18] and hydrophobic-interaction chromatographic conditions[24,25]. In the HIC separation of α-lactalbumin, peak distortion and non-linear changes in retention have been observed as a function of temperature[26,27]. These anomalies have been interpreted as the result of protein denaturation. In this communication, we explore the effect of temperature on these phenomena in context with the surface hydrophobicity using four different, commercially available HIC columns. The retention time–temperature curve is sigmoidal in those cases where denaturation occurs during the chromatography. These curves are similar to the transition curves of protein denaturation studied by spectroscopic techniques, and are analyzed by the same approach.

EXPERIMENTAL

Materials

HPLC-grade water was prepared by a Milli-Q water system (Millipore). Ammonium acetate, phosphate buffered saline (120 m*M* NaCl, 2.7 m*M* KCl in 10 m*M* phosphate buffer, pH 7.4), grade III ammonium sulfate, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), and calcium-depleted α-lactalbumin were purchased from Sigma (St. Louis, MO, U.S.A.). The α-lactalbumin was prepared fresh daily as a 2 mg/l solution in water.

Equipment

A Hewlett-Packard (HP) (Palo Alto, CA, U.S.A.) Model 1090 chromatograph equipped with a diode array detector, HP 85B personal computer, DPU multi-channel integrator, HP 9121 disc drives, and an HP 7470 A graphics plotter, was used. The column temperature was controlled by immersing the column in a thermostatted water bath (Lauda RC6, Brinkmann). To improve temperature equilibration of the mobile phase, a coiled tube was inserted in the mobile phase line and immersed in the water bath.

Stationary phases

Poly(methyl)-, -(ethyl)- and -(propyl) aspartamide columns (PolyLC), were obtained from the Nest Group (Southboro, MA, U.S.A.). The Spherogel-CAA-HIC was obtained from Beckman (San Ramon, CA, U.S.A.). The aspartamide columns were 250 × 4.6 mm, while the Beckman column was 100 × 4.6 mm.

Mobile phases

Weighed amounts of the salts were dissolved in a volumetric flask, and the pH was adjusted with either glacial acetic acid or ammonium hydroxide. The premixed phosphate buffered saline (PBS) was dissolved in HPLC-grade water according to the supplier's instructions. The HPLC-grade water was degassed prior to use, and all solvents were filtered through 0.45-μm Nylon 66 filter (Rainin, MA, U.S.A.).

The following mobile phase pairs were used:

(IA) 2 *M* ammonium sulfate, 0.05 *M* ammonium acetate (pH 6)
(IB) 0.05 *M* ammonium acetate (pH 6),
(IIA) 2 *M* ammonium sulfate, 0.05 *M* HEPES (pH 7.5)
(IIB) 0.05 *M* HEPES (pH 7.5),
(IIIA) 2 *M* ammonium sulfate, PBS (pH 7.4)
(IIIB) PBS (pH 7.4)

Gradient conditions

Two different gradient systems were used for the elution of α-lactalbumin: a 25- or a 5-min linear gradient, from 0 to 100% B solvent. The flow-rate was 1 ml/min and the elution was monitored at 280 nm.

RESULTS AND DISCUSSION

The effect of temperature on the retention time of α-lactalbumin using different HIC columns

The retention times of calcium-depleted α-lactalbumin at different temperatures are shown in Fig. 1 using the poly(alkyl)aspartamide columns. Chromatography was performed at pH 6.0 using ammonium acetate in both mobile phase A and B. A 20-min descending linear gradient of ammonium sulfate was used for protein elution. A marked change in the retention time as a function of the chromatographic temperature is evident in Fig. 1 from which a number of observations can be made. First, the retention of α-lactalbumin increases with increasing stationary phase hydrophobicity. Under identical mobile phase and temperature conditions α-lactalbumin elutes first from the methyl, then the ethyl, then the propyl column. This retention behavior is expected, since elution should occur at a well defined interfacial tension, which is ultimately determined by the given protein–stationary and mobile phase combination. Because the surface tension of these stationary phases decreases with increasing hydrophobicity while the surface tension changes of the mobile phase are identical, the elution order expected to be methyl > ethyl > propyl. Second, the shapes of the retention time–temperature curves at different for each column. Under these chromatographic conditions the retention time is a linear function of the temperature on the methyl column. A slightly non-linear dependence of the retention time can be observed on the ethyl column, while a clearly sigmoid elution curve is seen on the propyl column. The latter results has previously been observed for α-lactalbumin with other HIC columns.

Effect of mobile phase conditions on the elution profile of α-lactalbumin

Retention times identical to those observed with the acetate buffer, were obtained with all columns using 50 m*M* HEPES-based mobile phases at 7.5, indicating

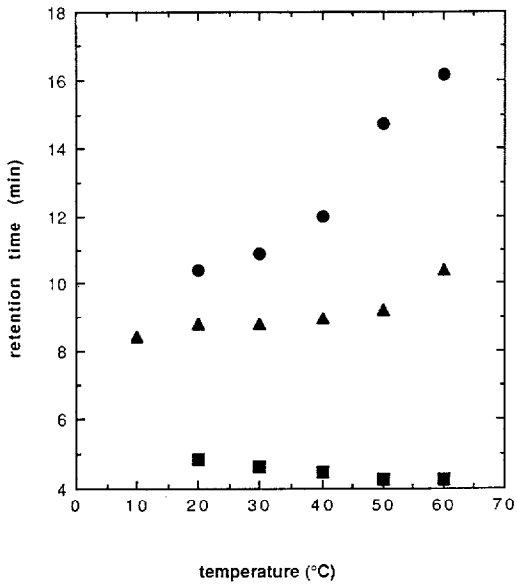


Fig. 1. Effect of surface hydrophobicity and temperature on the retention of α-lactalbumin on the poly(alkyl)aspartamide columns at pH 6.0 using the ammonium acetate-based HIC mobile phases: ■ = methyl; ▲ = ethyl; ● = propyl column. Further details on the chromatographic parameters are described in the Experimental section.

no differences between these two buffers, ionic strength and pH on the chromatograpic behavior of α-lactalbumin as a function of temperature. Next we investigated the effect of a physiological buffer system, PBS, on the chromatography of α-lactalbumin. The chromatograms of α-lactalbumin using PBS-based mobile phases at pH 7.4 are displayed in Fig. 2. At lower temperatures, α-lactalbumin elutes as a single sharp peak, which then starts to broaden as the temperature increases. At about 40°C, a new peak starts to emerge after the principal peak. At high temperatures, a single symmetrical peak, slightly broader than at low temperatures is observed. Previous studies identified the earlier eluting peak as the native conformation of α-lactalbumin, while the late eluting peak represents the denatured form[26]. The retention time shifts as a function of conformational change are in agreement with solution studies on the denaturation of α-lactalbumin. A significant increase in the Stokes radius of α-lactalbumin has been observed as denaturation progresses[28], which is accountable for increased complementary surface area, consequently longer retention time.

In Fig. 3A, the retention times of α-lactalbumin at different temperatures are displayed using a propyl column and PBS-based mobile phases. The change in the salt type and composition apparently changes the shape of the retention time–temperature curve. The curve has three characteristic segments: at low and high temperatures the retention time is a linear function of the ascending temperature and between those extremes, the retention sharply increases with increasing temperature. Similar curves were obtained using the methyl, ethyl and Beckman columns, illustrating the occurrence of a general trend.

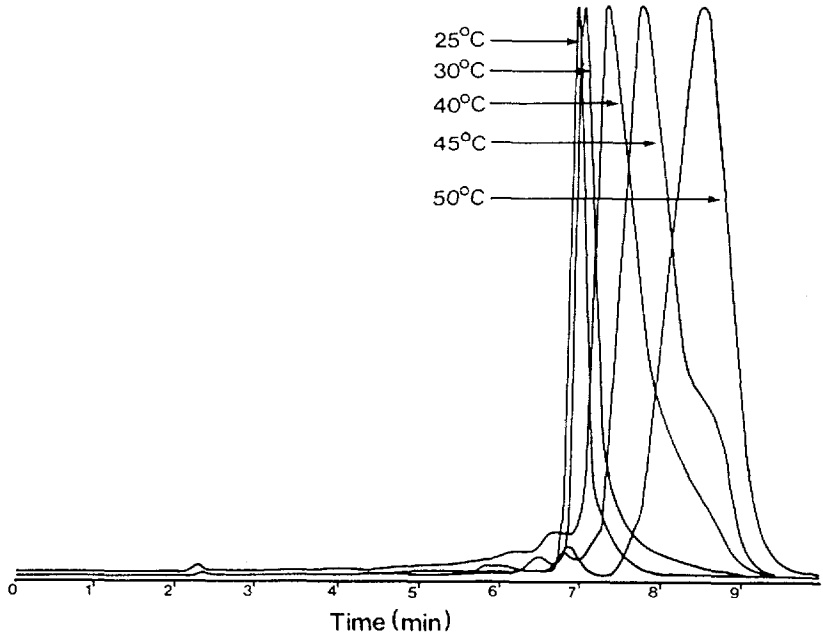


Fig. 2. Effect of temperature on the chromatograms of α-lactalbumin on the poly(propyl)aspartamide column at pH 7.4 using the PBS-based HIC mobile phases. The peak areas in the figure are not comparable because the chromatograms were normalized to better represent the characteristic change in the retention time and peak shape. Further details on the chromatographic parameters are described in the Experimental section.

These retention time–temperature curves resemble the classical thermal transition curves of α-lactalbumin denaturation obtained by spectroscopic techniques[29]. Similar transition curves have been obtained previously in reversed-phase chromatography following the temperature dependency of the peak height of ribonuclease A as a function of temperature[18]. Various parameters such as peak height, retention volume (time), peak width, peak symmetry, or the appropriate statistical moments can be used to indicate changes as a function of temperature. The appropriate parameter might differ in various applications and should be selected on the basis of its sensitivity to denaturation.

Analysis of the retention–temperature curves

Classical transition curves have been constructed for proteins in solution using spectroscopic data acquired by techniques such as UV, circular dichroism and fluorescence. The free energy of unfolding has been estimated from a two-state model, in which a native protein undergoes thermal unfolding to a denatured form.

Based on the obvious similarity between the classical transition curves and the one shown in Fig. 3A, we assumed a two-state denaturation model, and applied the classical methodology to quantitate the thermodynamic values of protein denaturation under HIC conditions.

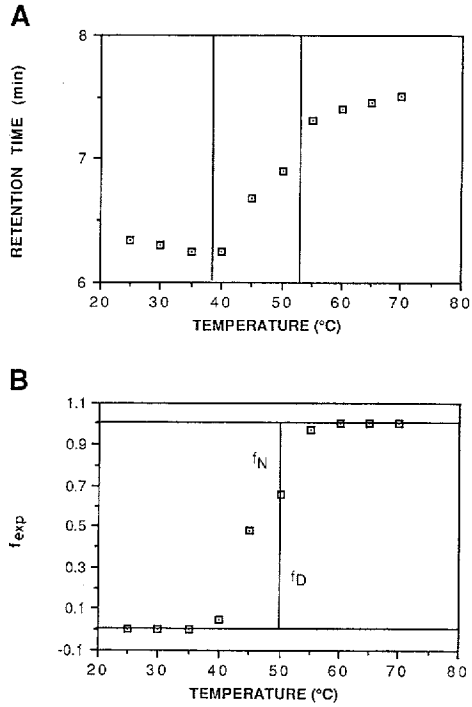
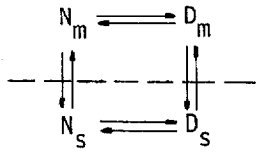


Fig. 3. (A) Effect of temperature on the retention time of α-lactalbumin on the poly(propy)aspartamide column at pH 7.4 using the PBS-based HIC mobile phases. Chromatographic conditions as in Fig. 2. (B) The transition curves of α-lactalbumin. The f_{exp} values were calculated, according the description in the text based on the data displayed in Fig. 3A.

In the present work the following simple model was used as a basis of further theoretical analysis.



where N_m and N_s are the native forms in the mobile phase (m) and on the surface (s) respectively, and D_m and D_s are the corresponding denatured forms. The early eluting peak, at any given temperature T, corresponds to N_s and the late eluting one to D_s. We assume that the concentration ratio of the two peaks is determined by the adsorption on the stationary phase, and the conformational effects of the mobile phase can be ignored as a first approximation. Accordingly, we will concentrate on the equilibria displayed in the lower part of the model as $N_s \rightleftarrows D_s$.

In general, the fraction of the unfolded population (f_{exp}) of α-lactalbumin can be calculated from chromatographic data as follows,

$$f_{exp} = \frac{t_{exp} - t_N}{t_D - t_N} \tag{1}$$

where t_{exp} is the measured retention time and t_N and t_D are the extrapolated retention times of the native and denatured components, respectively, at temperature T if denaturation would not occur. It is known that the retention time is a linear function of the column temperature in systems where the mechanism of the retention not changing with the temperature. The extrapolated retention times have only theoretical value without any physical explanation, as in the classical denaturation studies, and they serve as numbers for further calculations.

In eqn. 1, the only experimentally determined value is t_{exp}; t_N has been calculated using linear regression. The number of data points used for the regression procedure were chosen to optimize the regression coefficient. Once the parameters (A and B) of the fitted line have been obtained from the $t_{exp} = AT + B$ equation, the t_N value can be calculated. Similarly, the t_D value can be obtained at any given temperature, using linear regression on the t_{exp} data of the high-temperature section of the transition curve.

According to the two-state model, the fractions of the native (f_N) and denatured (f_D) components can be determined as $f_N = 1 - f_{exp}$, and $f_D = f_{exp}$. The equilibrium constant of denaturation (K_d) can be expressed as

$$K_d = \frac{f_D}{f_N} = \frac{f_{exp}}{1 - f_{exp}} \tag{2}$$

In Fig. 3B the f_{exp} values calculated from the data shown in Fig. 3A have been plotted as a function of the temperature.

Effect of stationary phase hydrophobicity on the transition temperature T_{tr}

To further investigate the contribution of the stationary phase on the denaturation process, the chromatography was repeated under identical elution conditions as described in Fig. 2, using the Poly(ethyl)aspartamide, the Poly(methyl)aspartamide and the Beckman CAA-HIC columns. The retention times have been converted to f_{exp} values and the transition curves are displayed in Fig. 4. The results show that the most significant retention changes occur over a 2–4°C temperature range for the poly-(methyl) and -(ethyl) columns and these portions of the curves are parallel. The transition range for the propyl and the Beckman phase is wide, covering an approximately 20°C temperature range. This broadening can be the result one of two major causes, which were neglected above. First, it is possible that the two-state denaturation model is not quite correct, in which case conformational intermediates can exist in the transition region but the resolution of the column does not allow the separation of those species[30,31]. Secondly, it is possible that the two-state model is correct, but conformational changes in the mobile phase cannot be excluded from the model.

Based on previous results, the second explanation appears correct[26]. Wavelength ratios of the upslope side of the α-lactalbumin peak differ from those of the apex and the downslope side. The values of the upslope side are similar to the values of the native protein[26]. It was shown in previous reversed-phase liquid chromatography studies[21,32,33], that conformational refolding in the mobile phase results in a broad peak. The native form elutes in the upslope side of the peak and the denatured form in

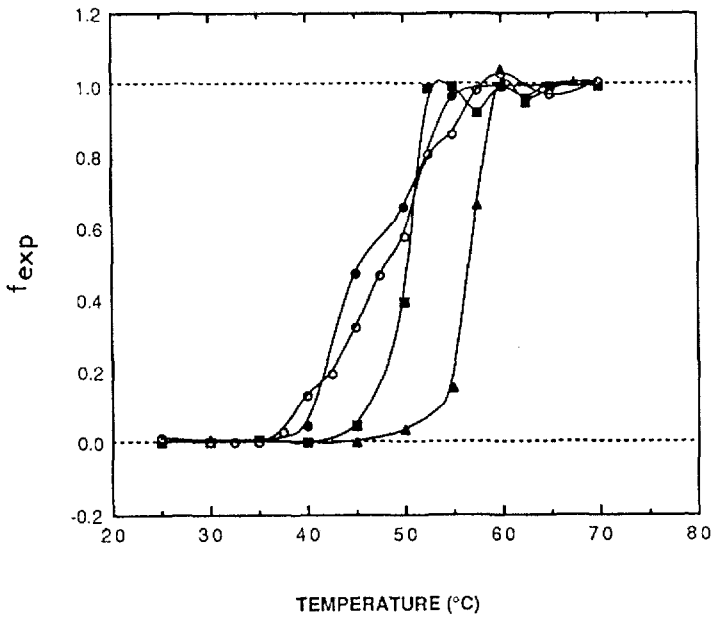


Fig. 4. Transition curves of α-lactalbumin on different HIC columns: ■ = methyl; ▲ = ethyl; ● = propyl; ○ = CAA-HIC column. Further details on the chromatographic parameters are described in the Experimental section.

the tail side of the peak. This behavior has been observed in RPLC of ribonuclease A (refs. 18 and 21) as well as in HIC of α-lactalbumin[26].

With respect to the column effects on the transition temperature of α-lactalbumin, the following observation can be made from Fig. 4. The transition temperature on the propyl column is *ca.* 46°C, on the methyl column *ca.* 50°C and on the ethyl column *ca.* 56°C. It is known that α-lactalbumin is a calcium-binding protein (binding constant K_f *ca.* 10^6–10^9)[34,35], and the removal of the Ca^{2+} ion destabilizes the structure[36]. The transition temperature decreases from about 58°C to 31°C upon removal of calcium[37]. Sodium and potassium ions also effect α-lactalbumin conformation as a function of their concentration[38]. Sodium chloride (200 m*M*) in Tris buffer at pH 8.0 has been shown to stabilize α-lactalbumin to thermal denaturation[38,39]. PBS contains 120 m*M* sodium chloride and 2.7 m*M* potassium chloride which might affect the conformation, but the ionic strength contribution of the high (2 *M*) ammonium sulfate should be dominating[40]. It is apparent that the high ammonium sulfate concentration significantly stabilizes the α-lactalbumin conformation.

The transition temperatures obtained from the different columns do not follow the order expected from surface hydrophobicity (propyl < ethyl < methyl). The negative effect of the surface hydrophobicity on the conformational stability is illustrated by the transition temperature difference obtained on the propyl and ethyl phases. The deviation, in case of the methyl column, from the order expected from homologues, might be related to differences in the coverage, the accessibility of the aspartamide groups, and/or steric hindrances.

The calculation of the free energy of denaturation (ΔG_{app})

The deduction of the f_{exp} values allows the calculation of the apparent free energy of transition, neglecting the effects of activity, by using the following equation

$$\Delta G_{app} = -RT \ln K_d = -RT \ln \frac{f_{exp}}{1 - f_{exp}} \quad (3)$$

where R and T correspond to the universal gas constant and the absolute temperature, respectively. The free energy of denaturation (ΔG_{app}) calculated from the experimental results is compared with literature data at 25°C and displayed in Table I. The higher free energy of denaturation in the presence of calcium ions clearly indicates its effect on the stabilization of α-lactalbumin. The calculated free energies of this work are in the same order of magnitude as of the cited literature data. The free energy of denaturation of α-lactalbumin for the propyl, methyl and CAA-HIC columns are similar to each other and to the values previously obtained for the calcium-depleted α-lactalbumin in solution[41]. The seemingly high ΔG_{app} value for the ethyl column suggests the most effective stabilization by the high-salt-concentration mobile phase. The values obtained are consistent with the assumption of the apoenzyme stabilization by sodium chloride and ammonium sulfate, and structural destabilization by the hydrophobic surface. These results also indicate the possibility of a mixed-mode mechanism of denaturation, involving electrostatic and hydrophobic forces. Further investigation is necessary to understand the observed anomalies which than will help to understand the phenomena involved in protein high-performance liquid chromatography.

CONCLUSIONS

The surface hydrophobicity is a crucial parameter in hydrophobic interaction chromatography of proteins. The astronomically large variety of proteins dictates the development of novel stationary phases, including ones with different hydrophobicities. The stationary phases synthesized for HIC applications are less hydrophobic than the classical reversed phases. Accordingly, one would expect less

TABLE I

FREE ENERGY OF DENATURATION OF α-LACTALBUMIN UNDER HIC CONDITIONS AT 25°C

	ΔG^* (kcal/mol)	ΔG_{app}** (kcal/mol)
No Ca^{2++}	3.76	
12 m*M* Ca^{2++}	8.86	
Propyl column		5.54
Ethyl column		10.94
Methyl column		5.14
CAA-HIC column		4.28

* Conditions: pH 7.0, data from ref. 41.

** Conditions as described in the experimental section.

surface-mediated denaturation. HIC apparently represent milder chromatographic conditions for proteins than reversed-phase chromatography and biological activity is usually recovered after separation. One of the main reasons for the popularity of HIC is the erroneous belief that denaturation cannot occur in HIC. However, it must be noted that protein denaturation is a complex phenomenon depending on numerous environmental parameters such as pH, temperature, ionic strength, dipole of the solvents and the surface hydrophobicity. Certain proteins will unfold under HIC conditions if the other parameters permit.

Thermodynamic analysis of chromatographic data according to a two-state model permitted the quantitative comparison of different HIC columns. The hydrophobicity of the stationary phase affects the retention time and peak shape of proteins under hydrophobic interaction chromatographic conditions. Increasing surface hydrophobicity resulted in longer retention times applying identical mobile phases. Greater surface hydrophobicity in conjunction with high temperature favors denaturation conditions. High concentrations of ammonium sulfate, as expected, stabilize the tertiary structure of α-lactalbumin.

It has been shown that the selection of the column has a major impact on the separation, on the apparent efficiency of the column, on the denaturation and on the mass and/or activity recovery of α-lactalbumin. To understand the phenomena involved, a series of commercially available columns designed for HIC have been evaluated. Columns prepared by the same bonding chemistry and with systematically varied hydrophobicity are helpful to design and optimize separations. The thermodynamic method used for the α-lactalbumin model system may be useful for quantitative column comparison and for the evaluation of other chromatographic parameters, such as pH, column temperature and buffer type.

The primary goal of the present work was to provide a simple method to compare different columns under identical chromatographic conditions. The extension and detailed study to the quantitative characterization of surface hydrophobicity will be the subject of continuing work. Careful understanding of the role of column characteristics, such as bonding chemistry, coverage, surface area, and the weight of the stationary phase in the packed column would allow one to make quantitative evaluation of different stationary phases, and understand their roles on the retention mechanism and on the protein denaturation process.

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REFERENCES

1 J. L. Fausnaugh, E. Pfannkoch, S. Gupta and F. E. Regnier, *Anal. Biochem.*, 137 (1984) 464.
2 D. L. Gooding, M. N. Schmuck and K. M. Gooding, *J. Chromatogr.*, 296 (1984) 107.
3 J.-P. Chang, Z. El Rassi and Cs. Horváth, *J. Chromatogr.*, 319 (1985) 396.
4 S. C. Goheen and S. C. Engelhorn, *J. Chromatogr.*, 317 (1984) 55.
5 Y. Kato, T. Kitamura and T. Hashimoto, *J. Chromatogr.*, 292 (1984) 418.
6 N. T. Miller, B. Feibush and B. L. Karger, *J. Chromatogr.*, 316 (1984) 519.
7 A. J. Alpert, *J. Chromatogr.*, 359 (1986) 85.

8 J. Porath, L. Sundberg, N. Fornstedt and P. Ohesson, *Nature (London)*, 245 (1973) 465.
9 B. H. J. Hofstee, *Pure Appl. Chem.*, 51 (1979) 1537.
10 S. Hjertén, *Methods Biochem. Anal.*, 27 (1981) 89.
11 S. Shaltiel, *Methods Enzymol.*, 104 (1984) 69.
12 J. D. Pearson, N. T. Lin and F. E. Regnier, *Anal. Biochem.*, 124 (1982) 217.
13 S. Y. M. Lau, A. K. Taneja and R. S. Hodges, *J. Chromatogr.*, 317 (1984) 129.
14 A. J. Sadler, R. Micanovic, G. E. Katzenstein, R. V. Lewis and C. R. Middaugh, *J. Chromatogr.*, 317 (1984) 93.
15 K. A. Cohen, K. Schellenberg, K. Benedek, B. L. Karger, B. Grego and M. T. W. Hearn, *Anal. Biochem.*, 140 (1984) 223.
16 S. A. Cohen, S. Dong, K. Benedek and B. L. Karger, in I. M. Chaiken, M. Wilchek and I. Parikh (Editors), *Affinity Chromatography and Biological Recognition*, Academic Press, New York, 1984, p. 479.
17 S. A. Cohen, K. Benedek, S. Dong, Y. Tapuhi and B. L. Karger, *Anal. Chem.*, 56 (1984) 217.
18 S. A. Cohen, K. Benedek, Y. Tapuhi, J. C. Ford and B. L. Karger, *Anal. Biochem.*, 144 (1985) 275.
19 K. Benedek, B. Hughes, M. B. Seaman and J. Swadesh, *J. Chromatogr.*, 444 (1988) 191.
20 K. Benedek, S. Dong and B. L. Karger, *J. Chromatogr.*, 317 (1984) 227.
21 X. M. Lu, K. Benedek and B. L. Karger, *J. Chromatogr.*, 359 (1986) 19.
22 J. D. Andrade (Editor), *Surface and Interfacial Aspects of Biomedical Polymers*, Vol. 2, Protein Adsorption, Plenum Press, New York, 1985.
23 K. Benedek, in preparation.
24 P. Štrop, *J. Chromatogr.*, 294 (1984) 213.
25 J. L. Fausnaugh and F. E. Regnier, *J. Chromatogr.*, 359 (1986) 131.
26 S.-L. Wu, K. Benedek and B. L. Karger, *J. Chromatogr.*, 359 (1986) 3.
27 S.-L. Wu, A. Figueroa and B. L. Karger, *J. Chromatogr.*, 371 (1986) 3.
28 Y. Hiraoka, T. Segawa, K. Kuwajima, S. Sugai and N. Murai, *Biochem. Biophys. Res. Commun.*, 95 (1980) 1098.
29 Y. Hiraoka and S. Sugai, *Int. J. Peptide Protein Res.*, 23 (1984) 535.
30 M. J. Kronman, S. K. Sinha and K. Brew, *J. Biol. Chem.*, 256 (1981) 8582.
31 M. Mitani, Y. Harushima, K. Kuwajima, M. Ikeguchi and S. Sugai, *J. Biol. Chem.*, 261 (1986) 8824.
32 A. Permyakov, L. A. Morozova and E. A. Burstein, *Biophys. Chem.*, 21 (1985) 21.
33 Y. Hiraoka and S. Sugai, *Int. J. Peptide Protein Res.*, 26 (1985) 252.
34 P. L. Privalov, *Adv. Protein Chem.*, 33 (1979) 167.
35 W. R. Melander, H. J. Lin, J. Jacobson and Cs. Horváth, *J. Phys. Chem.*, 88 (1984) 4527.
36 W. R. Melander, H. J. Lin, J. Jacobson and Cs. Horváth, *J. Phys. Chem.*, 88 (1984) 4536.
37 K. Gast, D. Zirwer, H. Welfle, V. E. Bychkova and O. B. Ptitsyn, *Int. J. Biol. Macromol.*, 8 (1986) 231.
38 W. Pfeil, V. E. Bychkova and O. B. Ptitsyn, *FEBS Lett.*, 198 (1986) 287.
39 J.-J. Schaer, M. Milos and J. A. Cox, *FEBS Lett.*, 190 (1985) 77.
40 J. CL. Van Ceunebroeck, J. Krebs, I. Hanssens and F. Van Cauwelaert, *Biochem. Biophys. Res. Commun.*, 95 (1986) 604.
41 M. Ikeguchi, K. Kuwajima, M. Mitani and S. Sugai, *Biochemistry*, 25 (1986) 6965.