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INFLUENCE OF UREA ON THE HIGH-PERFORMANCE CATION-EXCHANGE CHROMATOGRAPHY OF HEN EGG WHITE LYSOZYME

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SUMMARY

The effects of urea on the high-performance cation-exchange chromatography of hen egg lysozyme are reported. The capacity factor, k', has been determined as a function of cation concentration with a polyaspartate column using the acetates of Na^+, K^+, Ca^{2+} and Mg^{2+}. Urea decreases lysozyme retention. Plots of log k' *vs.* log ionic strength show linear relationships. The slope of the plot describing the Ca^{2+} elution of lysozyme was the same in the presence of 5 *M* urea as in its absence.

In strong urea solutions and at elevated temperatures, lysozyme denaturation is evidenced by a marked decrease in k'. The temperature range for denaturation corresponded closely to that observed by intrinsic fluorescence and circular dichroism measurements. The potential utility and limitations of high-performance ion-exchange chromatography for studying protein denaturation are discussed.

INTRODUCTION

Recent advances in high-performance ion-exchange chromatography (HPIEC) have led to its development as an important method for biopolymer separations[1,2]. In many cases high-performance separations of proteins may be achieved 10–100 times faster than on conventional gel-type columns with equal or superior resolution[3]. Furthermore, in the high-performance mode it is generally easier to control precisely important separation variables (*i.e.* flow-rate, temperature, gradient formation, etc).

One problem commonly encountered in the liquid chromatographic analyses of multi-component protein mixtures is that of solubility. To deal with this problem, protein chemists have often employed urea as an effective protein solubilizing agent. Few systematic studies, however, of the effects of urea on ion-exchange retention parameters have been reported[4,5].

One drawback of using urea as a solubilizing agent is its well-known ability to denature proteins. In chromatographic applications where maintaining biological activity is necessary (*i.e.* isolation or preparative purification) urea denaturation presents an obvious limitation, although in some cases urea denaturation may be reversible. If biological activity is not of prime importance, however, the use of urea to obtain a soluble protein sample may be advantageous.

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The present study examines the effects of urea solutions on the retention characteristics of hen egg white lysozyme using cation HPIEC. The use of both nondenaturing and denaturing conditions on this retention are compared. Also, the use of chromatographic retention data to characterize denaturation is discussed.

EXPERIMENTAL

Materials

Crystalline hen egg white lysozyme (lot no. 7069) was purchased from Miles Laboratories (South Africa) and was used without further purification. Ultrapure urea was obtained from Schwarz/Mann (Spring Valley, NY, U.S.A.). All other reagents were ACS grade. High-purity water was used to make all samples and buffers and was prepared in-house with a water purification system designed to produce HPLC grade water by Mar Cor Medical Services (Harleysville, PA, U.S.A.).

Equipment

The chromatographic system employed consists of a Waters Assoc. M6000A pump (Milford, MA, U.S.A.), a Rheodyne Model 7125 injection valve equipped with a 20-μl sample loop (Cotati, CA, U.S.A.) followed by a 250 × 4.6 mm I.D. PolyCAT A WCX column purchased from Custom LC (Houston, TX, U.S.A.). According to the manufacturer's specifications, this column has a hydrophilic polyaspartate coating on a silica base which has a nominal pore size of 300 Å and a particle size of 7 μm[6]. The column was thermostated in a water jacket using a circulating water bath to maintain the temperature to ± 0.2°C. An 8-cm length of 0.009 in. I.D. 316 stainless-steel tubing placed prior to the column inlet was also heated to aid temperature equilibration. The column effluent was monitored for UV Absorbance at 280 nm using a Waters Assoc. Lambda-Max Model 480 LC Spectrophotometer in the 0.0–0.1 absorbance range. The signal from the detector was processed using a Hewlett-Packard 3390A Reporting-Integrator (Avondale, PA, U.S.A.).

Circular dichroic (CD) spectra were obtained in the far-UV (210–260 nm) with a Jasco Model CD-SP J-10 instrument. Measurements were made in a 0.10-mm path length quartz cell using a water-thermostated cell holder. Baselines were run with the cells and the buffers. The temperature in the cell was measured directly with YSI Model 42SC Tele-thermometer or equivalent to ± 0.5°C. Lysozyme concentrations of *ca.* 1 mg/ml were found to give suitable spectra.

Intrinsic fluorescence measurements were made on a Perkin-Elmer 650-10S Fluorescence Spectrophotometer (Norwalk, CT, U.S.A.) equipped with a thermostated cell holder. Temperature was measured in the 1 cm × 1 cm quartz sample cuvette to ± 0.5°C using a thermocouple. The fluorescence was observed by exciting the sample at 285 nm and recording the emission spectra from 280–400 nm. Spectra were acquired as the sample was heated from 25°C to 65°C. Lysozyme concentrations of *ca.* 50 μg/ml were found to give suitable spectra.

High-precision density measurements were made with a Sodev Densimeter Model 01D (Sherbrooke, Canada).

Buffers

Mobile phase buffers were prepared from the corresponding metal acetate

salts. As these salts are hydroscopic, buffer concentrations were determined using standard assay methods. For buffers containing sodium or potassium acetate, the density of a stock solution of the metal acetate was measured using a high-precision technique and the solution concentration was calculated from published concentration–density data[7]. These standardized solutions were then quantitatively diluted and the pH adjusted to give the appropriate buffers. For buffers of magnesium or calcium acetate, the final metal ion concentration was determined by titration with EDTA[8] after dilution and pH adjustment. All buffers were adjusted to pH 5.00 at room temperature with glacial acetic acid. To prepare mobile phase buffers containing calcium acetate and urea additive, a ten-fold concentrated stock solution of *ca.* 0.5 *M* calcium acetate was first prepared and its pH was adjusted to 4.85 with concentrated acetic acid. The calcium concentration of this stock was then determined by titration with EDTA. An aliquot of the stock solution was added to the appropriate amount of urea contained in a volumetric flask so that after dilution with water, the desired calcium and urea concentrations were obtained. It was found that a ten-fold dilution of the concentrated stock buffer would give a pH 5.0 when no urea was present. All solutions were filtered through a Millipore type HA (0.45 μm) filter and degassed by sparging with helium prior to use. Solutions containing urea were prepared fresh daily.

Chromatography

Chromatograms were obtained under isocratic conditions at a nominal flow-rate of 1 ml/min. Typical operating pressures were in the range of 500–1000 p.s.i. A 20-μl sample size was injected. After each change of mobile phase composition a period of *ca.* 20 min was allowed for equilibration. If only the temperature was changed, *ca.* 10 min was found to be adequate to achieve reproducible retention times. Retention times used to calculate the capacity factor, k', were measured to ± 0.01 min using a digital integrator. The observed solvent perturbation peak was taken to indicate the retention time of an unretained component. Measurements of k' made by replicate injections were found to be reproducible to better than $\pm 1\%$ in the k' range of 2–12.

Although the column was thermostated, mobile phase reservoirs were maintained at ambient temperature (21–24°C). As a test that under these conditions uniform temperature equilibration could be attained, the capacity factor was measured at different flow-rates. A temperature differential of 15°C between the solvent reservoir and column was selected and k' was measured at 1.0 ml/min and 0.5 ml/min. The k' measured at the two flow-rates was found to be in good agreement ($\pm 0.5\%$ at k' *ca.* 2).

It was our experience that several months of exposure of the column to the concentrated urea solutions did not have any noticeable effects on its performance or retention time reproducibility. Prolonged exposure of the column to temperatures above 60°C, however, did lead to column degradation, characterized by increased retention times and severe band broadening.

Sample preparation

Samples of lysozyme were prepared to contain *ca.* 2 mg/ml by dissolving the protein in each mobile phase. In some cases a small amount of water was added to

the sample to create a slight mobile phase–sample mismatch to facilitate the measurement of the time of the unretained component from the solvent perturbation. This was found not to effect the retention time of the protein component.

RESULTS

As a preliminary study, the effects of using various metal acetates on the retention of lysozyme were examined under isothermal, isocratic conditions. Plots of the capacity factor, k', against metal ion concentration are shown in Fig. 1. On a molar basis, divalent ions were generally found to be more effective than monovalent ions in eluting lysozyme (*i.e.* $Ca > Mg \gg K > Na$). Also, the change in k' per concentrations increment was greater for divalent ions than for monovalent ions as shown by the greater concavity of the divalent metal ion curves. The influence of selected cations on lysozyme retention using chloride salt buffers on a strong cation-exchange column has been previously reported[9]. Differences in experimental conditions, however, do not permit a direct comparison.

In Fig. 2 the retention data of Fig. 1 was replotted using the equation

$$\log k' = z \log a + \log c \quad (1)$$

where k' = capacity factor, a = ionic strength of the elution buffer, z = line slope, and c = constant, as a retention model. Based on an earlier theoretical treatment of Boardman and Partridge[10], Kopaciewicz *et al.*[9] have recently derived this as a retention model for protein HPIEC. Other authors have also suggested the use of an

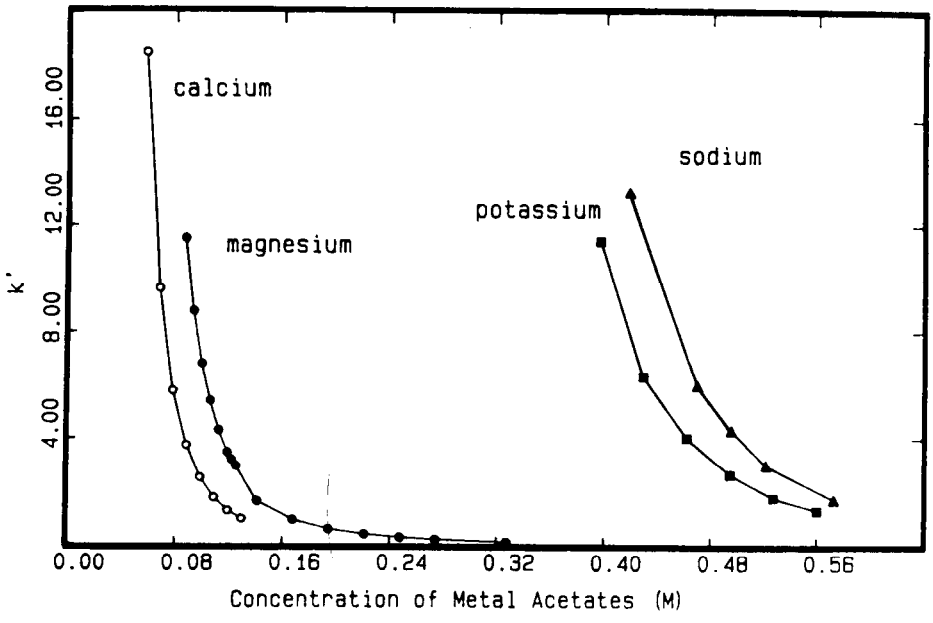

Fig. 1. Cation HPIEC retention characteristics of hen egg white lysozyme in various metal acetate buffers at pH 5.00 and 23.5°C. The capacity factor, k', is given by $k' = (t_R - t_0)/t_0$ where t_R is the retention time of lysozyme and t_0 is the retention time for an unretarded component.

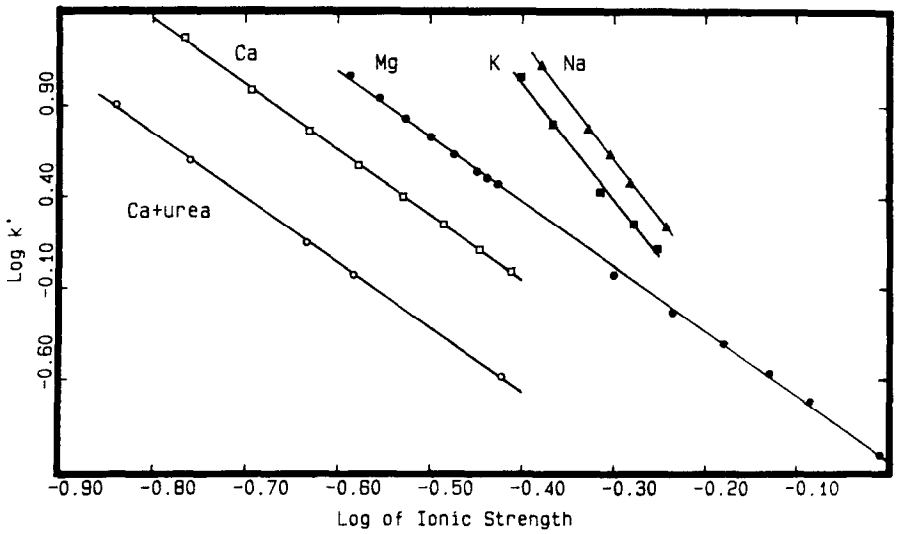

Fig. 2. Lysozyme retention data of Fig. 1 fit to an ion-exchange retention model (see eqn. 1). Data obtained using a calcium acetate buffer containing 5.0 *M* urea (pH 5.0 at 23.5°C) is also shown for comparison.

equation of this form[11,12]. Consistent with this simple model, linear plots were obtained. Similar linear correlations for other globular proteins have been reported[9]. It is interesting to note in Fig. 2 that in a calcium acetate buffer containing 5.0 *M* urea, the observed retention data still show a linear relationship and give a slope parallel to those of magnesium and calcium buffers containing no urea. Line parameters obtained using linear regression are given in Table I for comparison.

As lysozyme is known to be denatured by urea, studies were undertaken (1) to investigate the effects of urea on retention under non-denaturing elution conditions and (2) to examine the influence of denaturing conditions on retention. As a criterion to characterize denaturation, we chose to use far-UV CD spectral data as an independent reference method since the CD spectrum in this region is sensitive to changes in the secondary structure of the protein. Although other workers have used this

TABLE I

LINEAR PARAMETERS CHARACTERIZING THE HPIEC RETENTION OF LYSOZYME

Calculated from Fig. 2.

*Elution buffer**	*Slope***	*Intercept***
Sodium	−6.48 ± 0.22	−1.33 ± 0.07
Potassium	−6.32 ± 0.77	−1.51 ± 0.25
Magnesium	−3.55 ± 0.05	−1.04 ± 0.02
Calcium	−3.57 ± 0.03	−1.49 ± 0.02
Calcium plus 5.0 *M* urea	−3.54 ± 0.10	−2.09 ± 0.06

* Metal acetate–acetic acid buffers at pH 5.00.

** Error estimates are for the 90% confidence limit.

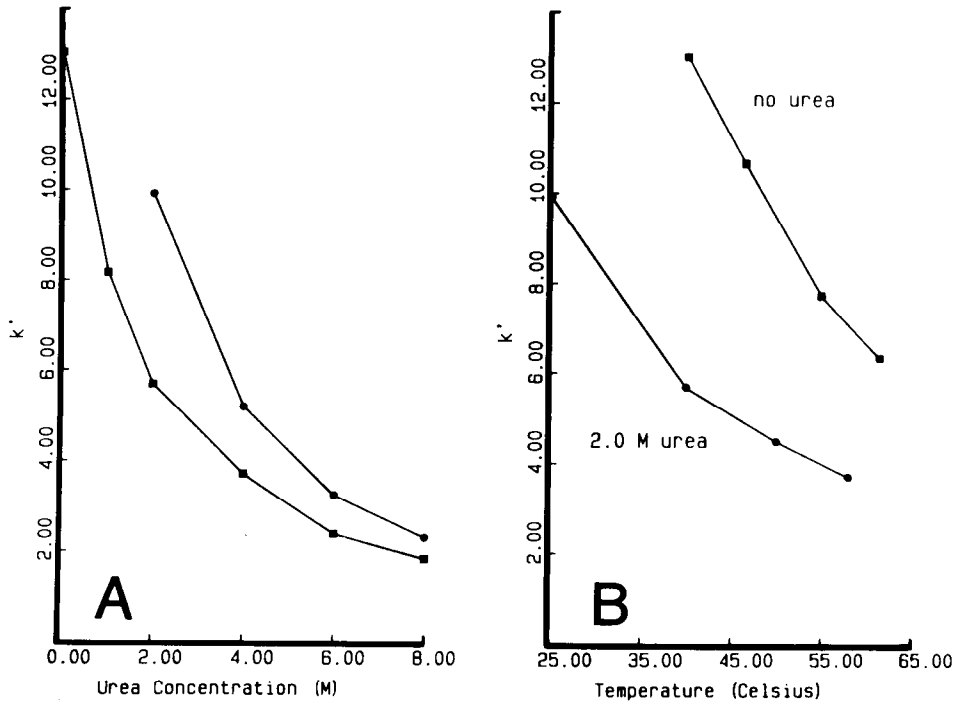

Fig. 3. Effect of urea on lysozyme retention under non-denaturing conditions. (A) Effect of urea concentration on retention at (●) 25°C and (■) 40°C. (B) Effect of temperature on retention (●) with and (■) without urea. In both (A) and (B) the calcium buffer concentration was 0.0502 *M*, the pH 5.00.

method to study lysozyme urea denaturation[12], we decided to make our own measurements to avoid any ambiguity related to experimental conditions.

The effects of urea on k' under non-denaturing conditions are presented in Fig. 3. Fig. 3a shows that k' decreases with increasing urea concentration both at 25 and 40°C. Fig. 3b shows that k' decreases with increasing temperature both in the presence and absence of urea. Qualitatively, no change in the far-UV CD spectra was observed even at 40°C in an 8*M* urea containing buffer. Similarly, no significant change in the far-UV CD spectra was observed at 65°C in the absence of urea.

In contrast to this, the influence of denaturing conditions on retention are depicted in Fig. 4. We observed (Fig. 4a) that a sharp drop in k' occurred over a small temperature range. The midpoint temperature of this transition, $T_{1/2}$, shifts to higher temperatures as the urea concentration is decreased (Table II). This transition was also observed in the absence of urea (Fig. 4b) but at much higher temperatures. Qualitatively, the regions of rapidly decreasing k' correlate with significant changes in the far-UV CD spectra under these conditions. Typical chromatograms obtained in 8 *M* urea in the urea thermal denaturation range are shown in Fig. 5.

Using the approach of Wells and Olson[13], the change in the intrinsic fluorescence spectra of lysozyme was measured in different concentrations of urea-buffer as a function of temperature. Upon heating, qualitatively a change in the spectra was evidenced by a shift in the emission spectra maximum from 343 nm to 355 nm with

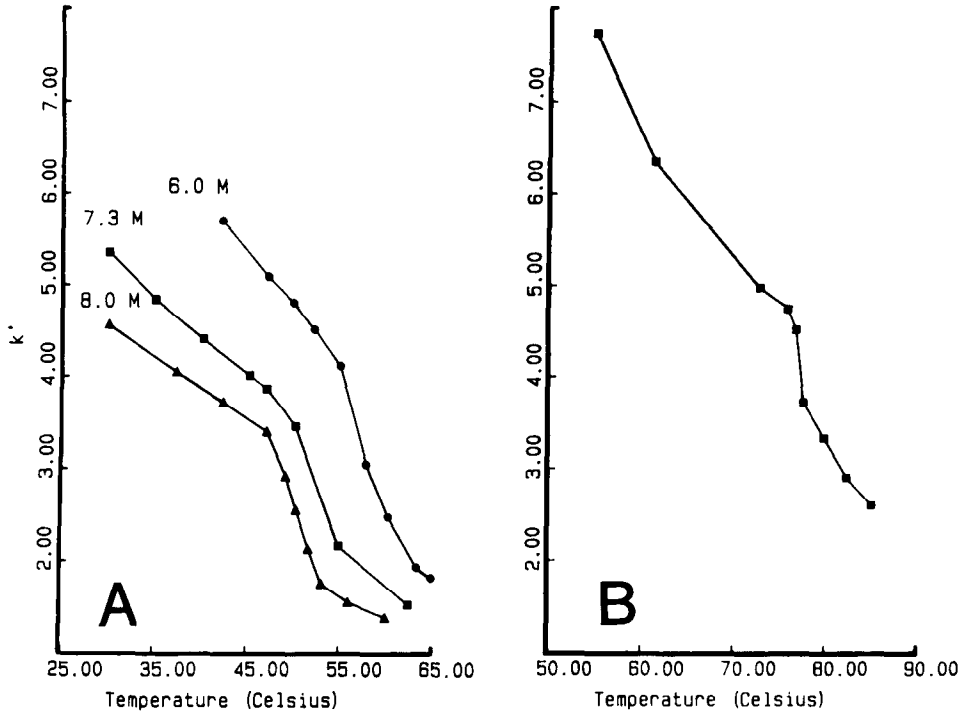

Fig. 4. Effect of denaturing conditions on lysozyme retention. (A) Urea thermal transition observed using different concentrations of urea. Calcium acetate concentration was 0.0401 *M*. (B) Thermal transition in the absence of urea. Calcium acetate concentration was 0.0502 *M*. The pH of the buffer was 5.00 in both (A) and (B).

about a 30% concomitant increase in intensity. From a plot of the fluorescence intensity at 348 nm against temperature, the midpoint temperature, $T_{1/2}$, of the urea temperature denaturation was determined. As shown in Table II the midpoint transition temperatures obtained from chromatographic retention data and fluorescence measurements are well correlated. It should be noted that after cooling to 25°C, the fluorescence spectra were not found to be entirely reversible. For example, a sample

TABLE II

COMPARISON OF THE MIDPOINT TEMPERATURES OF THE UREA THERMAL TRANSITION, $T_{1/2}$, DETERMINED USING CHROMATOGRAPHIC RETENTION DATA AND FLUORESCENCE

Urea (M)	$T_{1/2}$* *(°C)*	$T_{1/2}$** *(°C)*
8.0	50 ± 1	49 ± 1
7.3	52 ± 1	51 ± 1
6.0	58 ± 1	56 ± 1

* Determined graphically from the chromatographic retention data in Fig. 4a.
** Calculated from fluorescence data.

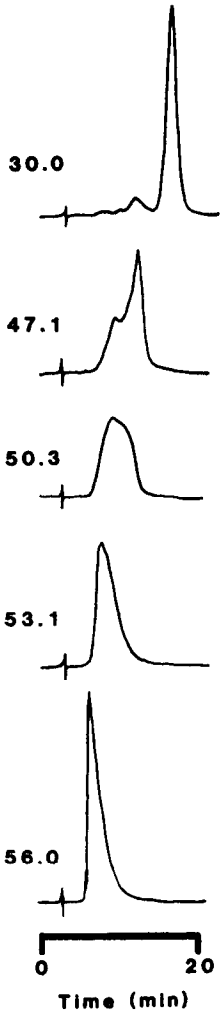

Fig. 5. Isocratic high-performance cation-exchange chromatograms of lysozyme in 8 *M* urea–calcium acetate buffer, pH 5.0, at selected temperatures. Note the decrease in peak height and apparent peak broadening through the temperature range of denaturation (47–54°C, see Fig. 4a).

that had been heated in 8 *M* urea–buffer gave a spectrum (after cooling) intermediate between so-called native and denatured forms. Samples heated in 6 *M* urea–buffer, however, gave a similar spectrum when cooled to that obtained prior to heating.

In a control experiment, lysozyme in a buffer containing 8 *M* urea was heated to 65°C for 15 min, cooled, and chromatographed at 25°C. It was found that a peak corresponding to that of native protein in a unheated control could still be easily observed. Other minor unidentified peaks, however, appeared at shorter retention times, indicating the formation of some non-reversible altered species which we suspect may be due to the carbamylation of lysozyme[14,15]. It is believed that these species may be involved in the observed non-reversibility of the fluorescence spectra.

DISCUSSION

The results of our studies with the globular protein, lysozyme, show that urea has a pronounced effect on the HPIEC retention behaviour. Generally, the presence of urea decreases lysozyme retention. This would be expected from its increase of the solvent dielectric constant. However, since additional solution variables are changed by the presence of urea (*i.e.* viscosity, ion solvation, etc.) a more detailed discussion of its effects are not presently addressed.

The abrupt drop in k' which we observed under progressively denaturing conditions is of particular interest to us. Based on evidence which we obtained using two independent equilibrium measurements, CD and fluorescence, we believe that this change in k' is caused by gross conformational changes which occur in the protein morphology during denaturation. This example of complex retention behavior highlights the importance of three-dimensional structural considerations in protein separations[16].

The correlation of the fluorescence and CD changes with chromatographic retention data has led to speculation as to the use of HPIEC data as a tool for studying protein denaturation. For example, one might take advantage of the chromatographic aspects of this method to test the relative stability of specific protein components in mixtures. However, the use of HPIEC to study and characterize denaturation is at this time regarded with caution. The possibility of surface denaturation occurring during binding and the kinetic aspects of the chromatography could present obstacles to clear-cut interpretations of the data.

Although lysozyme is a protein of special interest in our laboratory, its unusually high thermal stability[17] does present problems concerning (1) column stability at high temperatures and (2) possible urea decomposition. Further work with nonglobular and less stable proteins is currently in progress to determine if these results can be generalized.

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