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KINETICS OF UNFOLDING OF PROTEINS ON HYDROPHOBIC SURFACES IN REVERSED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

As a continuation of previous studies, we present in this paper measurements on the kinetics of denaturation of papain, soybean trypsin inhibitor and lysozyme on *n*-butyl-bonded silica gel surfaces used in reversed-phase liquid chromatography (RPLC). In all cases, native and denatured peaks widely separated from one another are observed. The rate constants for denaturation or unfolding are determined by the measurement of the peak area of the native protein as a function of the incubation time that the species spends on the bonded-phase surface. The results reveal that a slow denaturation step occurs with a half-life of *ca.* 15 min. In addition, studies of denaturation as a function of the amount of 1-propanol in the initial mobile phase suggest an additional unfolding step when the protein comes in contact with the bonded-phase surface. The extent of this latter step decreases as the concentration of 1-propanol increases, further suggesting that 1-propanol sorption on the bonded stationary phase may play a role in this behavior. Other studies are conducted with α-chymotrypsinogen, in which injection is made after the start of the gradient. The extent of denaturation is observed to be a function of the organic modifier employed. The results of this paper provide insight into the denaturation process in RPLC and suggest approaches to minimize this behavior.

INTRODUCTION

Reversed-phase liquid chromatography (HPLC) on hydrocarbonaceous-bonded silica gel packings is a widely adopted method for the separation of oligopeptides and proteins[1]. Most basic studies to-date have dealt with an examination of parameters that control retention and mass recovery (*e.g.*, mobile phase conditions[2,3], silica type[4] and gradient elution conditions[5]). Mechanistic studies of the retention process, particularly for proteins, have been less researched. This fact is understandable, given the complexity and diversity of biopolymers.

An examination of retention mechanisms and phenomena involved in the chromatographic process can be important for an understanding of protein RPLC. First, optimum separation conditions could be understood and predicted. Secondly, peak

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shape could be controlled to yield sharply eluted bands. Thirdly, it is known that adsorption on the nonpolar *n*-alkyl surface phase along with the harsh conditions required for elution can lead to denaturation of proteins[6]. An understanding of the retention process and the causes of denaturation could lead to improved methodologies that permit milder chromatographic conditions.

We have been studying the influence of denaturation on the resultant RPLC behavior of proteins. We have shown that both distorted peak shape[7] and multiple peaks[8,9] can be obtained, depending on whether the denaturation changes are reversible, with half-lives comparable to chromatographic elution. In the case of the acid-stable proteins, soybean trypsin inhibitor[8] and papain[9], studies have revealed that two or more peaks widely separated from one another can be obtained under certain chromatographic conditions. Analysis of individual collected bands have further shown that the first-eluted peaks are active and the last-eluted peak is inactive. The relative areas of these peaks were found to be a function of a variety of parameters, such as column temperature, pH and contact time of the protein with the adsorbed surface. Further studies revealed that irreversible denaturation occurred with protein adsorption on the bonded-phase surface.

In this paper, we present a continuation of this work with an examination of the kinetics of denaturation or unfolding of papain on a hydrophobic *n*-butyl-bonded phase surface as a function of mobile-phase composition and column temperature. The results suggest a rapid denaturation step upon protein contact with the surface, followed by a slow denaturation step for adsorbed active protein. Measurements are also presented for soybean trypsin inhibitor, lysozyme and α-chymotrypsinogen. Taken as a whole, the results of this work provide insight into the adsorption process and suggest improved approaches to reduce denaturation in the RPLC separation of proteins.

EXPERIMENTAL

Apparatus

The chromatographic system consisted of an SP 8000 liquid chromatograph and an SP 8310 fixed-wavelength (254 nm) UV detector (Spectra-Physics, Santa Clara, CA, U.S.A.). In some experiments a Spectro-Monitor III variable-wavelength detector (LDC, Riviera Beach, FL, U.S.A.) was utilized. Column temperature was controlled to ±0.2°C by immersing the injector, column and 3 m × 0.01 in. I.D. stainless-steel tubing in a thermostatted waterbath. Peak areas were integrated with an HP3380A integrator (Hewlett-Packard, Avondale, PA, U.S.A.). The column packing material, LiChrospher Si 500, was bonded with *n*-butyldimethylchlorosilane and end-capped with a mixture of hexamethyldisilazane and trimethylchlorosilane (4.1 μmol/m^2). In a number of the experiments, mobile phase A was 10 m*M* H_3PO_4 (pH 2.2) and mobile phase B was 1-propanol–water (45:55, v/v) with an overall H_3PO_4 concentration of 10 m*M*. In general, a linear gradient from 0 to 100% B in 15 min was used with a flow-rate of 1 ml/min. Other specific conditions are described in the text. Protein samples were made up in water (20 mg/ml). The difference-spectroscopic measurements were made with a Cary 14 UV spectrometer.

Materials

Silane reagents were purchased from Silar Laboratories (Scotia, NY, U.S.A.). LiChrospher Si 500 (10 μm) silica was from MCB Reagents (Gibbstown, NJ, U.S.A.). All proteins, papain Type IV, soybean trypsin inhibitor Type I-S, lysozyme Grade 1, and α-chymotrypsinogen were purchased from Sigma (St. Louis, MO, U.S.A.) and used as received. HPLC-grade water and organic solvents were from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) or J. T. Baker (Phillipsburg, NJ, U.S.A.). Reagent-grade acids were used without further purification.

RESULTS AND DISCUSSION

Experimental design

As noted above, we have previously shown[8,9] that under certain conditions, the RPLC of soybean trypsin inhibitor and papain can lead to several peaks, the first one(s) being active and a later-eluted one being denatured. The area of the denatured peak was found to grow at the expense of the native peak(s) as the column temperature was raised or the mobile-phase pH was lowered. Furthermore, reinjection of the first fraction(s) yielded both the first and second peaks, whereas reinjection of the second fraction produced only the second peak. Finally, both proteins were found to remain active in the solution in which they were injected into the column (mobile phase A in the gradient system). Based on these and other results, it was concluded that these proteins were irreversibly denatured on the *n*-alkyl-bonded phase surface under the mobile phase and column temperature conditions.

We have also previously shown that the extent of the irreversible denaturation is a function of the incubation time of the protein with the *n*-alkyl-bonded phase surface[9]. Correspondingly, injection of protein after the start of the gradient resulted in a decrease in the area of the denatured peak. In this paper we explore these phenomena in more detail by measuring the first-order kinetics of denaturation.

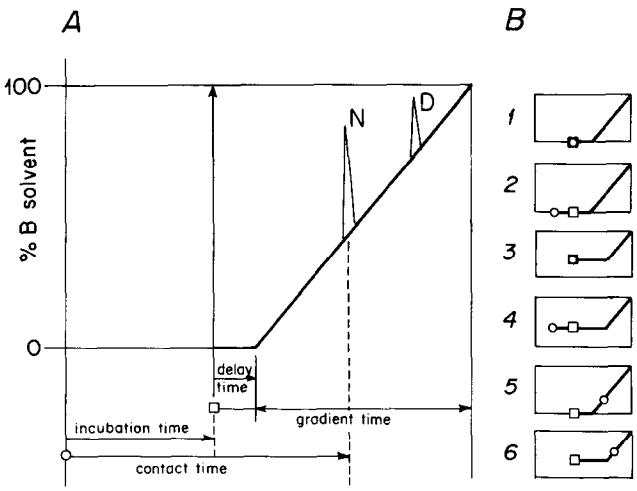

Fig. 1. Illustration of incubation experiments. (A) Definition of incubation, contact, delay and gradient times. (B) Illustration of various kinetic experiments of denaturation on the bonded phase surface. See text for details. ○, Injection time; □, start of the gradient.

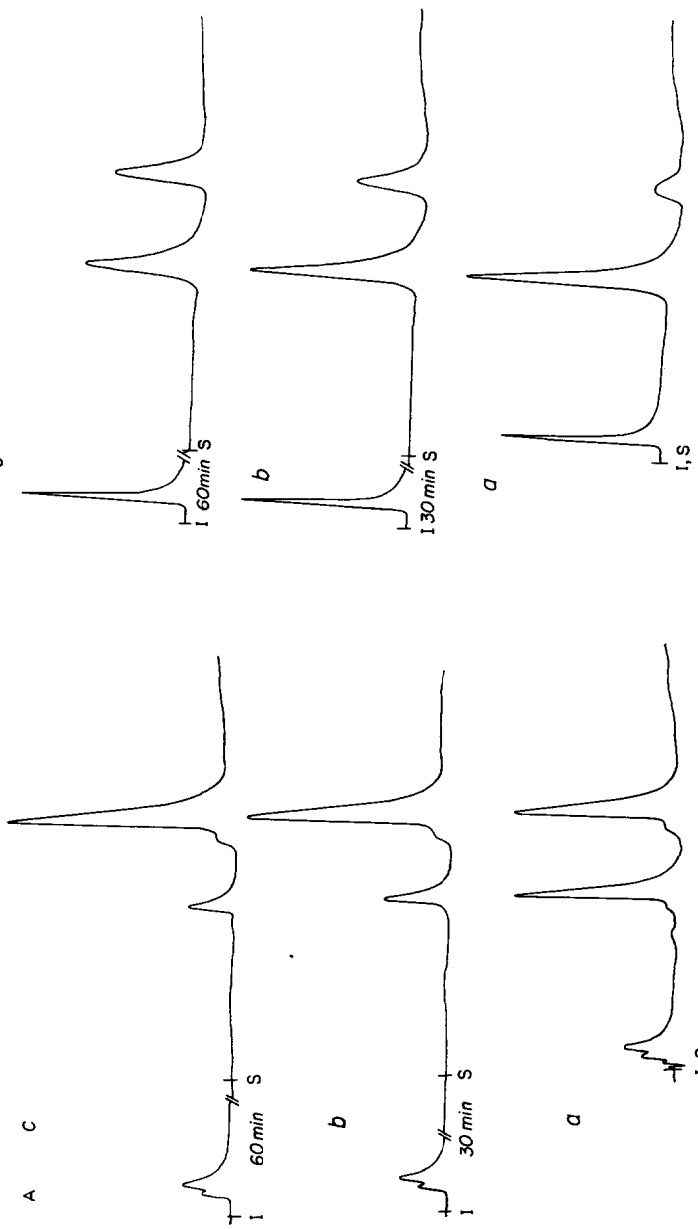

Fig. 2. Chromatographic behavior of papain as a function of on-column incubation time at mobile phase compositions of (A) 10 m*M* H_3PO_4 (pH 2.2), and (B) 1-propanol–water (5.4:95.6, v/v) in which the total H_3PO_4 concentration is 10 m*M*. Conditions: Column = C_4 bonded phase on 10 μm LiChrospher SI-500; mobile phase A = 10 m*M* H_3PO_4 (pH 2.2); mobile phase B = 1-propanol–water (45:55, v/v) in which the total H_3PO_4 concentration is 10 m*M*; gradient rate = 3% propanol/min, 15-min linear gradient; flow-rate = 1 ml/min; sample = 20 mg/ml papain in mobile phase A, 6 μl injected; detection at 280 nm; column temperature = 5°C. I = Injection time; S = start of gradient. Incubation times: a = 0 min; b = 30 min; c = 60 min.

Fig. 1 illustrates the general methodology involved in the kinetic measurements. In Fig. 1A the definition of the various times are presented. The gradient time is the time necessary to operate the linear gradient in 1-propanol concentration. A delay time (in this work = 4 min) occurs after the gradient is started at the pumping system until the mobile phase change reaches the column. This delay is a consequence of the need to displace 4 ml of mobile phase (at a flow-rate of 1 ml/min) between the pump and the head of the column. The contact time is defined as the time from injection until the appearance of the native protein band in the chromatogram. [Note that the "true" contact time of the native protein with the bonded surface would equal the above contact time minus the time required to elute an unretained component from the column (in this case 1 min)]. Finally, the incubation time equals the time from injection until the start of the gradient.

Fig. 1B provides a more detailed picture of the experiments undertaken. In plot 1 of this figure, the normal gradient with mobile phase A is shown, with injection and start of the gradient occurring simultaneously. The peak area of the native peak under this condition is taken as the zero time. In plot 2, the protein is injected and allowed to incubate on the column for a period of time after which the gradient is started and elution is permitted to occur. The area of the native protein peak is again measured, and the incubation time is taken from injection until the start of the gradient at the pump. It is to be noted that care was taken to remove 1-propanol from the bonded-phase surface upon recycling back to starting conditions for each incubation experiment[10].

By way of illustration, Fig. 2A shows the changes in peak area for the native and denatured peaks of papain as a function of incubation time in mobile phase A. At a column temperature of 5°C, it can clearly be seen that the first peak decreases and the second peak increases with incubation time.

Returning to Fig. 1B, plot 3 shows the experiment in which the starting mobile phase consists of 1-propanol–water (10 m*M* H_3PO_4 overall). In order to maintain the same contact time of protein with the bonded surface, relative to the experiment with mobile phase A (plot 1), an isocratic hold is established for the time that would normally be required to allow the gradient composition to reach the corresponding mobile phase composition. Note that the delay time is included in plots 1 and 3. Plot 4 shows the corresponding incubation experiment in which the mobile phase is a combination of mobile phase A and a given percentage of mobile phase B. The peak area of the active species is measured for given incubation time intervals and compared to that area for zero incubation time in plot 3.

Fig. 2B shows chromatograms of papain as a function of incubation time for a mobile phase consisting of 1-propanol–water (5.4:94.6, v/v), (10 m*M* H_3PO_4 overall), pH 2.2, T = 5°C. As in Fig. 2A, the denatured peak area grows at the expense of the native peak with increase in incubation time of the protein on the bonded phase surface. It is further interesting to note that for a given incubation time, more native peak is observed when 5.4% 1-propanol is included in the mobile phase than when only the 10 m*M* H_3PO_4 aqueous solution is employed. The point is reinforced in Fig. 3, which presents chromatograms of papain as a function of mobile phase composition at 5°C with constant contact time with the bonded-phase surface. Thus, the isocratic hold time increases from Fig. 3a to 3d with increasing concentration of 1-propanol.

Finally, returning to Fig. 1B, plot 5 illustrates the example of injection after the start of the gradient, and plot 6 is a similar example, except that the starting mobile phase contains 1-propanol. (If the rate of phase equilibration is fast, plots 5 and 6 will likely yield identical results.) Note that two parameters are simultaneously varied in this experiment —the mobile phase into which the protein is injected and the contact time with the surface. We shall deal with these examples later in the paper.

The rate constants for unfolding on the bonded phase surface can be determined in a simple fashion, based on the following analysis. Consider the example in which the incubation mobile phase is 10 mM H_3PO_4 (pH 2.2). In this case, we can assume that the rate of denaturation follows first-order kinetics, and for zero incu-

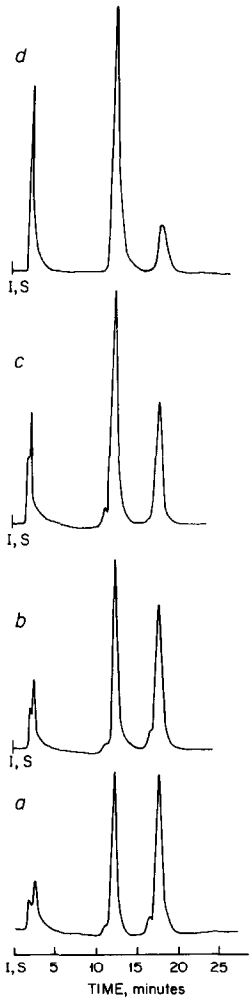

Fig. 3. Chromatographic behavior of papain with constant surface contact time as a function of the starting 1-propanol mobile phase concentration. a = Control, mobile phase A; b = 4% mobile phase B in A, *i.e.* 1.8% 1-propanol; c = 8% mobile phase B in A, *i.e.* 3.6% 1-propanol; d = 12% mobile phase B in A, *i.e.* 5.4% 1-propanol. Other conditions: see Fig. 2.

bation time, *i.e.* plot 1, Fig. 1B, we have

$$A = ae^{-k^*t_G} \quad (1)$$

where A = amount of native peak eluted from the column, a = amount of protein injected into the column (native under the injection conditions), t_G = time from injection until elution of the native peak and k^* = average rate constant for the various conditions involved in the gradient (delay time, linear gradient in 1-propanol until elution). In the incubation experiments k^*t_G can be assumed to be constant.

In plot 2, Fig. 1B, the incubation time is varied and an additional first-order kinetic equation can be written as

$$a = Ne^{-kt} \quad (2)$$

where N = injected material, k = rate constant and t = incubation time. At the end of the incubation time when the gradient is started, a quantity of native protein, a, is present. Substitution of eqn. 2 into eqn. 1 yields

$$A = Ne^{-kt}\, e^{-k^*t_G} \quad (3)$$

or

$$\ln A = (\ln N - k^*t_G) - kt \quad (4)$$

Since the terms in parentheses on the right hand side of eqn. 4 are constant, a plot of ln A *vs.* t will allow the calculation of k, the rate constant of unfolding on the bonded phase surface. Similar arguments can be used when 1-propanol is included in the mobile phase during incubation, *e.g.* plots 3 and 4, Fig. 1B.

As will be seen later, the results suggest a rapid denaturation step, prior to the slow step measured in this work. Since such a step will be common to both the zero time and the incubation times, any loss in the amount of the native peak due to this effect will be incorporated into the k^* value. The important point is that the rate constant for the slow unfolding step, k, can still be determined.

Papain

Based on the above analysis, a series of experiments were conducted in which the area of the native peak of papain was determined as a function of incubation time at various mobile phase compositions. Plots of the logarithm of the peak area (log A) of the native peak *vs.* incubation time were constructed for three mobile phase compositions (0%, 1.8%, and 2.7% (v/v) 1-propanol in 10 mM H_3PO_4, pH 2.2) at various temperatures. Typical results are shown in Fig. 4 in mobile phase A, *i.e.* 0% 1-propanol. Good linearity was observed from 0 to approximately 20 min incubation times, indicative of the expected first order kinetics.

Least-squares analysis of the linear plots permitted determination of first-order rate constants, and selected values are shown in Table I. The reproducibility of the rate constants from run to run was found to be ±4%. As expected, the rate constant for disappearance of the native peak increased with increasing column temperature. Analysis of the temperature dependence of the rate constants for mobile phase A (10 mM H_3PO_4) yielded an average activation energy of 17 kcal/mol.

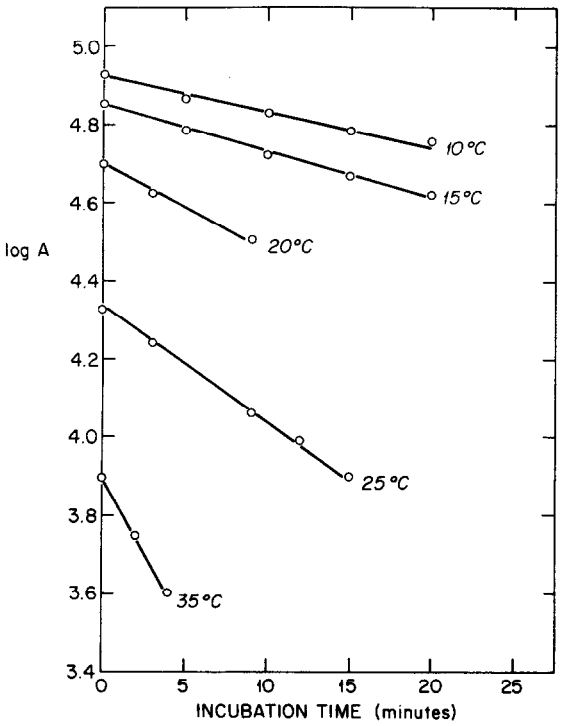

Fig. 4. First order kinetic plots of the rate of denaturation of papain at various column temperatures as a function of incubation time on the bonded phase surface. Log A = logarithmic area of the first (active) peak. Incubation solvent: 10 mM H_3PO_4 (pH 2.2). Other conditions: see Fig. 2.

It is interesting to note in Table I that at any given temperature the rate constant for disappearance of the native species is greater the more 1-propanol there is in the mobile phase. This result can be contrasted with Fig. 3, where it was shown that at constant contact time a greater amount of native papain was observed, the higher the 1-propanol concentration in the mobile phase. This comparison suggests that several mechanisms are involved in the disappearance of the native papain species. We shall return to this shortly.

We have already suggested that the disappearance of native papain is a conse-

TABLE I

RATE CONSTANTS FOR DENATURATION OF PAPAIN ON THE *n*-BUTYL-BONDED PHASE

Gradient conditions: see Fig. 2.

Incubation mobile phase	*$k \cdot 10^{-4}$ (sec^{-1}) at a column temperature (°C) of*		
	15	*20*	*35*
10 mM H_3PO_4 (pH 2.2)	4.6	8.8	29
1.8% 1-Propanol in mobile phase A	4.5	9.7	38
2.7% 1-Propanol in mobile phase A	6.8	11.9	–

quence of irreversible conformational unfolding (*i.e.* denaturation) on the bonded phase surface to yield a denatured peak which grows with incubation time. For this model to be valid, it is necessary to eliminate other factors which could potentially cause the loss of the native peak with incubation time. We will now consider these potential factors and argue against their significance in these experiments.

It is known that papain is a proteolytic enzyme[11], and consequently, a separate experiment was conducted to determine the extent of autoproteolysis in solution under the mobile phase conditions. A papain sample was allowed to stand in mobile phase A (10 m*M* H_3PO_4, pH 2.2) for 30 h at 10°C and was then injected into the RPLC column. No change in the peak pattern was observed over a freshly prepared sample. Hence, autoproteolysis in solution appears to be very slow under acid conditions, in agreement with that found by others[12]. If autoproteolysis were to occur, a series of peaks would be observed between the unretained species and the denatured peak, representing the papain fragments. As can be seen from Figs. 2 and 3, this was not found. Moreover, the peak area of the unretained peak remained relatively constant with incubation time and hence extra material did not build up in this peak. Thus, it can be concluded that the disappearance of the papain is not a result of autoproteolysis. Nevertheless, care was exercised to minimize autoproteolysis in the incubation experiments. Fresh samples of the enzyme were always prepared, and the incubation times were maintained under 20 min.

On another point, all mobile phases were carefully degassed with helium to remove oxygen (as well as to prevent gas bubble formation). Hence, oxidation of the native state is not expected to yield the results in Table I. Also, an experiment was conducted in which the injected native peak was allowed to be fully denatured on the bonded-phase surface, and incubation was continued for a period of 30 h in mobile phase A. The peak area of the denatured peak remained constant over this time period, indicating that mass recovery was not time dependent. A previous study[3] has shown that mass recovery was high for proteins under the gradient conditions of this paper. Therefore, it is reasonable to conclude that the disappearance of the native peak is not a result of a decrease in the mass of protein recovered from the column.

Returning to Table I, the rate constants reveal a slow unfolding process on the bonded phase surface (*e.g.* half-life at 20°C in mobile phase A *ca.* 15 min). (It can be noted that separate studies revealed that papain in solution was not denatured under any incubation conditions used in this work.) It is well known from adsorption studies that globular proteins can slowly unfold at the gas–liquid[13] and the liquid–solid[14] interfaces. Indeed, it has been suggested that hysteresis in the adsorption isotherm of phosphorylase b on *n*-butyl-Sepharose is a result of reorientation and conformation changes[15]. In this latter example, as well as the present one on *n*-butyl-silica, the driving force for unfolding is undoubtedly hydrophobic.

The rate constants in Table I measure a slow denaturation step. However, other effects appear to be operative as well, based on the results in Fig. 3. As we have noted, for the same contact time, the amount of eluted native papain is significantly higher the greater the concentration of 1-propanol in the mobile phase. The adsorption of 1-propanol on the stationary phase[16] is likely to play an important role in the results of Fig. 3. First, the overall change in the interfacial tension from the start to the end of the gradient will be less, the higher the initial concentration of 1-prop-

anol. Secondly, and more importantly, the increasing hydrophilicity of the surface with added 1-propanol will alter the adsorption behavior of papain in its initial contact with the surface. The initial driving force for unfolding and exposure of hidden hydrophobic amino acids for interaction with the stationary phase will clearly be less the more hydrophobic the phase. The results in Fig. 3 suggest that a rapid denaturation step takes place during the initial adsorption of papain on the surface and that this step is different from the one measured in Table I.

The decrease in the extent of denaturation with added organic modifier argues strongly for less hydrophobic surfaces than currently employed by RPLC. Obviously, one approach to achieving more hydrophilic surfaces is to adsorb polar agents on the bonded phase, such as nonionic detergents[17]. Alternatively, weakly hydrophobic chemically bonded surfaces could be used for separation[18–20].

The results in Fig. 3 suggest that when organic solvents are used as eluents for RPLC, it is better to have at least a small amount of organic solvent in the starting solvent of a gradient system, if the goal is to elute proteins in an active state. Such a strategy can also help in the linearization of a gradient[21] and reduce the time required to return to starting conditions[10].

The denaturation studies with papain also suggest that an important factor in unfolding is the contact time of the protein with the bonded-phase surface. Limiting the time a protein molecule spends in a column would seem to be one means by which to minimize denaturation. The composition of the mobile phase and the column temperature will influence the effect of contact time on unfolding. We will next turn to several proteins which have behavior comparable to that of papain and explore further the role of the mobile phase in denaturation.

Soybean trypsin inhibitor and lysozyme

As noted above, soybean trypsin inhibitor (STI) was observed to follow similar irreversible denaturation as papain on the alkyl bonded-phase surface[8]. Several active peaks were found, and each decreased with incubation on the bonded-phase surface. We measured the disappearance of the first active peak with incubation time and determined the first-order rate constant for denaturation. Table II presents the results for STI from 20 to 30°C, using 10 m*M* H_3PO_4 (pH 2.2) as the incubation mobile

TABLE II

RATE CONSTANTS AND ACTIVATION ENERGIES FOR THE DENATURATION OF LYSOZYME AND SOYBEAN TRYPSIN INHIBITOR ON THE *n*-BUTYL-BONDED PHASE

Incubation mobile phase: 10 m*M* H_3PO_4 (pH 2.2). Gradient conditions: see Fig. 2.

Temperature (°C)	$k \cdot 10^{-4}$ (sec^{-1})	
	Lysozyme	*STI*
10	2.6	–
15	4.4	–
20	8.6	18.5
25	15.0	42.0
30	32.0	81.0
ΔE_A (kcal/mol)	22.0	19.0

phase. It can be observed that at a given temperature the rate of unfolding is greater than twice that for papain (and lysozyme as well, to be discussed). However, the activation energy, 19 kcal/mol, is comparable to papain.

We next turned to another globular protein, lysozyme, which is known to unfold reversibly in solution[22]. Fig. 5 shows the two-peak profile for lysozyme at 5°C. It can be seen that, as in the case of papain and STI, the area of the first peak decreases with incubation time and the second peak correspondingly increases.

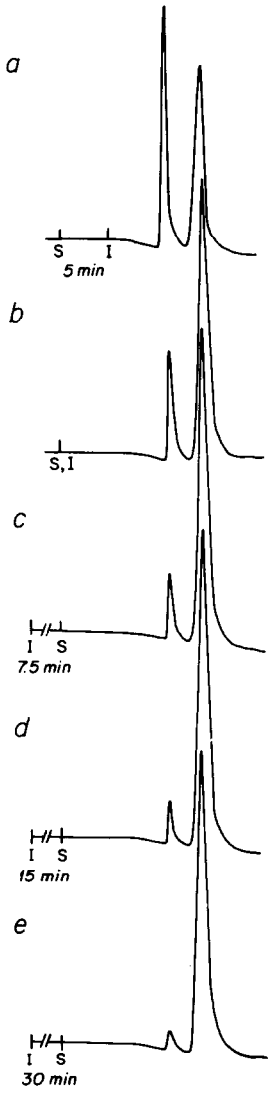

Fig. 5. Chromatographic behavior of lysozyme as a function of injection times relative to the start of the gradient. S = Start of gradient; I = injection time. Sample: 10 mg/ml lysozyme in mobile phase A, 5 μl injected. Other conditions: see Fig. 2. a = Injection 5 min after start of gradient. Incubation times: b = 0 min; c = 7.5 min; d = 15 min; e = 30 min.

The characterization of the first and second peaks were complicated by the known reversibility of the refolding process in solution. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis[23] of the collected fractions showed that both peaks possessed the same molecular weight. (This conclusion is reinforced by the reinjection experiments, see below.) Thus, it can be concluded that the two-peak pattern is not the result of impurities. Because of the reversibility in solution, it was not possible to determine the "true" activity of the collected fractions. As with ribonuclease A[7], we therefore compared by difference spectroscopy at 25°C the native state in mobile phase A with that in mobile phase B. It is known that in mobile phase B lysozyme will be denatured[24]. It was found that the absorbance ratio A_{280}/A_{254} was greater than unity for the denatured state relative to the native state in this experiment. We next determined in the chromatogram the absorbance ratio A_{280}/A_{254} for the first and second peaks of lysozyme. The ratio was 40% greater for the second peak, relative to the first.

Based on the above experiments and the subsequent incubation experiments, we conclude that the first peak is a native form of lysozyme and the second peak a denatured form. Interestingly, in contrast to papain, reinjection of the second fraction yielded the first and second peaks. Evidently, sufficient time was available for some of the denatured lysozyme to refold in solution. However, the rate of refolding was

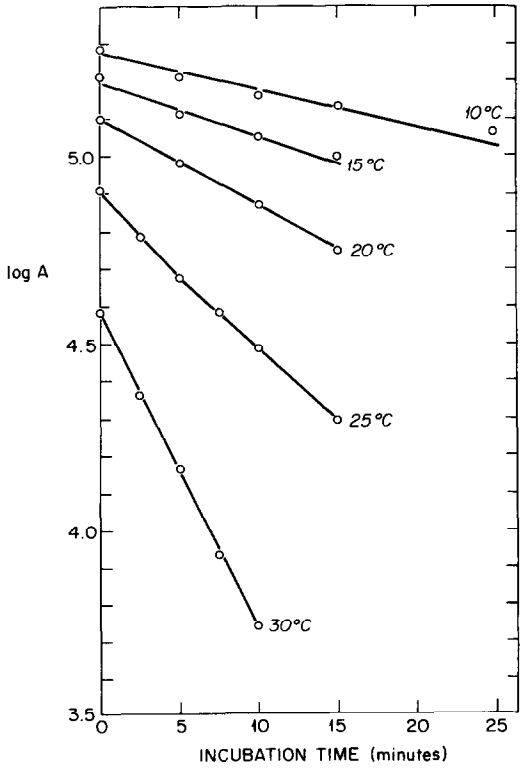

Fig. 6. First-order kinetic plots of the rate of denaturation of lysozyme at various column temperatures as a function of incubation time on the bonded phase surface. Log A = logarithmic area of the first (active) peak. Incubation solvent: 10 mM H_3PO_4 (pH 2.2). Other conditions: see Fig. 2.

slow enough on the column so that two peaks widely separated from one another were observed, rather than a single broad peak as in the case of ribonuclease. Thus, lysozyme appears to be irreversibly denatured under the chromatographic conditions. The role of kinetics in determining chromatographic behavior has recently been examined in detail[25].

The rate of unfolding for lysozyme on the bonded-phase surface was next determined in a manner similar to that for papain and STI. Fig. 6 shows plots of log *A vs.* incubation time at various temperatures in mobile phase A. Care was again taken to remove oxygen from the mobile phase by degassing in helium. Also, rate constants were determined for incubation times of 20 min or less.

Table II lists the rate constants for lysozyme in mobile phase A. Values comparable to papain are observed. Furthermore, the activation energy of 22 kcal/mol is comparable to that of STI and papain. It can be concluded that all three proteins provide a consistent picture of slow denaturation on bonded phase surface.

The influence of the initial concentration of 1-propanol on the extent of denaturation was observed to be similar for lysozyme as for papain. Thus, the ratios of areas of the first peak for lysozyme without incubation were found at 10°C to be 1.00:1.18:2.95 for 1-propanol concentrations in mobile phase A of 0:0.9%:1.8%, respectively. It is more than likely that the explanation for these results is similar to that in the case of papain. A rapid denaturation step occurs upon adsorption and the extent of the step is dependent on the amount of n-propanol sorbed onto the stationary phase.

In addition to the above experiments, we compared the rate of unfolding of lysozyme in 10 m*M* H_3PO_4 (pH 2.2) to that in 10 m*M* trifluoroacetic acid (TFA) (pH 2.2). TFA is widely used in RPLC[26], in part because of its ease of removal from collected proteins fractions. At 22°C, the first-order rate constant with H_3PO_4 was $10.5 \cdot 10^{-4}$ sec^{-1}, whereas that with TFA was $31.2 \cdot 10^{-4}$ sec^{-1}. Thus, denaturation was facilitated in TFA. The reason for the greater rate of unfolding in TFA relative to H_3PO_4 is likely related to the fact that TFA is a chaotropic agent and is known to destabilize the native state[27].

α-Chymotrypsinogen

When operated under normal gradient conditions, *i.e.* mobile phase A to mobile phase B (plot 1, Fig. 1B) only a single peak was observed for α-chymotrypsinogen, even at 5°C (see Fig. 7A). However, upon injection 9 min after the start of the gradient (plot 5, Fig. 1B) two peaks were found, as shown in Fig. 7B. As observed for papain[9], the ratio of areas of the first peak to that of the second depended on the point of injection. The later in the gradient that injection occurred the greater was the area of the first peak. Note also in Fig. 7B that the first peak was retained in the gradient even after the 9-min injection point. (The unretained peak was eluted in the same time as the small signal at approximately ten minutes in the chromatogram, see Fig. 7B.)

It is known that the unfolding of α-chymotrypsinogen in acid medium is reversible and relatively facile[28]. Thus, as in the case of lysozyme, it was not possible to collect the peaks and analyze for activity. However, collection and reinjection of both fractions did result in the appearance of both peaks. Based on analogous results with other studied proteins[8,9], it is suggested that the first peak is native and the second denatured.

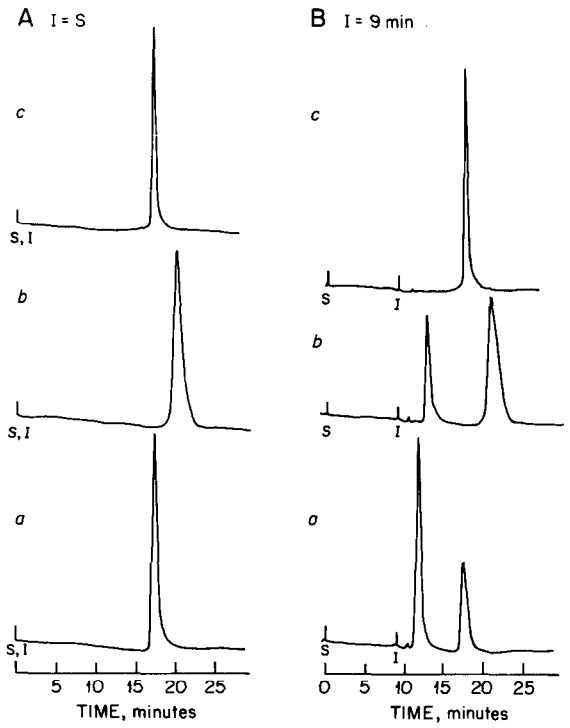

Fig. 7. Chromatographic behavior of α-chymotrypsinogen as a function of organic modifier and injection time. A = Injection at the start of the gradient. B = Injection 9 min after the start of the gradient. Mobile phases: (a) 1-propanol–water (45:55, v/v); (b) 2-propanol–water (45:55, v/v); (c) acetonitrile–water (80:20, v/v). The overall concentration of H_3PO_4 in each mobile phase is 10 m*M*. Other conditions, see Fig. 2.

In Fig. 7B, injection after the start of the gradient simultaneously varied the composition of the mobile phase into which the protein was injected and the contact time with the bonded-phase surface. Both factors decrease the extent of denaturation. (Note that α-chymotrypsinogen was native in the injected sample solution of mobile phase A.) Injection into a mobile phase containing 1-propanol reduced the rapid denaturation caused by initial protein adsorption on the bonded-phase surface. In addition, the incubation experiments showed that denaturation was reduced the smaller the contact time of the protein with the surface.

Based on the results in Fig. 7B, we next decided to vary the organic solvent and to observe the change in the two-peak behavior for α-chymotrypsinogen. We conducted the same experiment of injecting 9 min after the start of the gradient, using acetonitrile and 2-propanol, and the results are also shown in Fig. 7B. In the case of 2-propanol, two peaks were again observed; however, the relative amount of native to denatured was somewhat less than found for 1-propanol. In the case of acetonitrile, only a single peak was observed at a retention time corresponding to the denatured species.

In comparing 2-propanol with 1-propanol, it is interesting to note that the latter facilitates denaturation in solution more than the former[28], a trend opposite to that observed in the chromatographic experiments. The results in Fig. 7B can be

explained on the basis of the amount of organic solvent sorbed into the stationary phase. (Note that the contact time is roughly similar for the native state in these two cases.) While the molar concentration of 1-propanol and 2-propanol in the mobile phase at the 9-min injection point is similar (*ca.* 2 *M*), the slope of the surface tension *vs.* concentration ($d\gamma/dc$) curve at this concentration is much greater in the case of 1-propanol[29]. Since this slope controls the amount of adsorption (Gibbs equation), a greater amount of 1-propanol, relative to 2-propanol, will be adsorbed on the bonded phase, making the phase more hydrophilic. As a result, it can be anticipated that the extent of denaturation upon adsorption by the protein will be less for 1-propanol than 2-propanol, as observed in Fig. 7B.

In the case of acetonitrile, it is interesting to note that the mobile phase concentration of this modifier at the 9-min injection point is much higher (*ca.* 5 *M*) than for the alcohols. We have previously shown that a significantly larger amount of acetonitrile is required for protein elution than for 1-propanol[3]. The approximate surface tension of the mobile phase of acetonitrile at the injection point is 35 dyne/cm (ref. 29), which is comparable to the surface tension of the mobile phase at the point at which the native α-chymotrypsinogen is eluted by either alcohol. According to the solvophobic theory[30], we might expect that the native state protein injected into the acetonitrile mobile phase would not be adsorbed on the stationary phase to a significant extent and that the species would thus be eluted close to the unretained peak. The fact that only a denatured peak is observed (eluted at a surface tension of *ca.* 29 dyne/cm) suggests that α-chymotrypsinogen is very rapidly unfolded in the acetonitrile mobile phase or on the bonded phase. Thus, acetonitrile appears to be strongly denaturing under these conditions.

CONCLUSIONS

We have demonstrated the important role of kinetic processes of unfolding in the resultant chromatographic behavior of proteins in RPLC. It has been shown that the denaturant characteristics previously found for papain and STI apply to other proteins as well. Indeed, the phenomenon would appear to be a general one. As long as the refolding kinetics are sufficiently slow, relative to the chromatographic elution, two peaks separated from one another will be observed. If the half-life for refolding is comparable to chromatographic elution, then distorted peak shapes will result[7]. This behavior is typical of that found for kinetically controlled chemical conversions in chromatographic columns[25,31].

The denaturation observed in this work can be viewed in terms of a classical two-state model (native/denatured). While intermediates in the unfolding process in solution are known to occur for specific proteins[32], it is likely that the chromatographic procedure is not sufficiently fast to observe these intermediates except in unusual cases.

This study has provided insight into the RPLC separation of proteins. Two steps in the unfolding process have been proposed —one occurring rapidly when the protein contacts the stationary phase and a second with a half-life of *ca.* 15 min. With regard to the rapid denaturation step, we have seen that the type and concentration of organic modifier in the mobile phase plays a significant role in the extent of unfolding. 1-Propanol can cause the bonded phase surface to be less hydrophobic

(relative to 10 m*M* H_3PO_4 as mobile phase) by virtue of the adsorption of the organic modifier on that phase[16]. This effect appears to reduce the rapid denaturation step. As we have noted, the result argues for use in RPLC of starting mobile phase gradients containing 1-propanol to potentially reduce the extent of denaturation. Clearly, strongly adsorbed hydrophilic agents (*e.g.*, surfactants) could be useful in this regard, as well as hydrophilic bonded phases.

Our results have further shown that the popular mobile phase system used in the RPLC of proteins, 0.1% TFA with an acetonitrile gradient, is strongly denaturing. Evidently, many proteins are driven into an unfolded state in this system. A single, sharp peak of denatured species is frequently observed, and this may be one reason for the wide use of this mobile phase. With less denaturing conditions, multiple or broadened peaks may be observed, as in this paper; however, peaks with biological activity can be recovered[9].

It can be hypothesized that the rapid denaturation step involves adsorption of the protein in a specific orientation, other orientations being less likely to cause unfolding. It has been suggested in another context that because of the large size of proteins, adsorption energies can be significantly different from one orientation to another[33]. It is also possible that some surface heterogeneity in terms of adsorption sites also exists[14]. Associated with these effects is the hydrophobic driving force for the unfolding of the protein in order to increase the number of contact points between the biopolymer and the surface. This driving force will be reduced as 1-propanol is sorbed on the stationary phase.

With respect to the slow unfolding process, the results argue that the contact time of the protein with the bonded-phase surface should be kept to a minimum in order to maintain the native state as much as possible. It is possible that the mechanism of this slow step involves reorientation of protein on the bonded-phase surface which causes denaturation or, alternatively, diffusion of the biopolymer to active sites for unfolding. It is also worth mentioning that the measured activation energies of *ca.* 17–22 kcal/mol are comparable to those found for *cis–trans* isomerization around proline-containing peptides[34]. Indeed, this isomerization has been assumed to be a rate-determining step for unfolding (or refolding) in solution[35]. Whether this agreement in the chromatographically derived activation energies with those in solution is fortuitous will have to await future studies.

While the goal of this work has been to provide insight into the chromatographic process, it should also be noted that the methodology developed here may prove useful in studying protein adsorption on surfaces in general. In this approach, experiments can be conducted in dilute solutions and a direct measure of the amount of native and denatured protein can be obtained. An understanding of protein adsorption on surfaces can be important in diverse areas, *e.g.*, immobilized enzymes, clotting on surfaces, etc.

Finally, it needs to be emphasized that other factors can cause multiple peak behavior for proteins in RPLC besides conformational unfolding. For example, deglycosylation[36] can occur with an RPLC column. Microheterogeneity, aggregation, etc., as well as conformational unfolding, have also been suggested by others[37,38] to lead to multiple and/or broadened bands.

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