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CHARACTERIZATION OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC STATIONARY PHASES USING RIBONUCLEASE A

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SUMMARY

The protein ribonuclease A (RNase A) represents a good model protein for studying reversible conformational refolding during gradient elution. Work is described utilizing RNase A under gradient conditions to evaluate several different reversed-phase materials. Columns (10 cm × 4.6 mm I.D.) were packed with Partisil C_{18}, Vydac C_{18}, Nucleosil C_4, Nucleosil C_{18} and an adamantyl-modified Partisil silica. Measurements of the apparent first-order rate constant of refolding, as a function of temperature, are presented and compared for each stationary phase. Comparisons of peak shapes as functions of flow-rate and temperature are also discussed.

INTRODUCTION

This study follows up the work of Karger and co-workers in which the reversible conformational changes of RNase A in reversed-phase high-performance liquid chromatography (RP-HPLC) were reported[1] in addition to measurements of the first-order rate constant of refolding as a function of the gradient steepness (at a given column temperature) and as a function of temperature[2]. RNase A has been used as a model protein for studies of renaturation and denaturation processes in solution[3–11] and represents a good model protein for studying reversible conformational refolding during gradient elution.

In their work, Karger and co-workers[1,2] used an *n*-butyl-bonded stationary phase and a gradient mobile phase from 10 m*M* orthophosphoric acid (pH 2.2) to 1-propanol–10 m*M* orthophosphoric acid (45:55, v/v) at room temperature. They observed a broad band followed by a sharper overlapping peak and determined, from the absorbance ratio A_{288}/A_{254}, that the broad band at 25°C represented the native or folded state of RNase A and the later eluted, overlapping, sharp peak represented an unfolded or denatured state. They also observed changes in the band shape at 25°C as the flow-rate was varied while the gradient steepness was kept constant. The broad band was small at high flow-rates and increased in size relative to the late eluted peak as the flow-rate was decreased. These results and others[1] suggested that the dena-

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turated RNase A was renatured (refolded) in the mobile phase during its travel through the column.

By varying the mobile phase flow-rate while the gradient steepness was kept constant, Karger and co-workers[2] were able to determine the apparent first-order rate constants for refolding by measuring the change in the peak height of the denatured species (sharp, late-eluting peak). The rate constants were also determined as a function of temperature by varying the gradient steepness (to maintain a constant retention time) for each flow-rate[2].

Based on these earlier studies, we extended the refolding rate measurements to several different reversed-phase stationary phases. They included an adamantyl-modified silica phase, a C_4 phase and several C_{18} phases with varying pore sizes. We hoped that by comparing the refolding rates over a range of temperatures for each stationary phase and using our knowledge of the chromatographic behavior of RNase A we could better understand and characterize the properties of the reversed-phase packing materials tested in this study. As the refolding rates provide an indication of the degree of denaturation of a protein as a result of its interaction with a particular stationary phase surface, a classification of stationary phases based on this information could be utilized when developing strategies for the chromatography of certain proteins.

EXPERIMENTAL

Chemicals and materials

Bovine pancreatic ribonuclease A was purchased from Sigma (St. Louis, MO, U.S.A.) and was used as received. RNase A samples were prepared as 10, 20, and 40 mg/ml solutions in doubly distilled, deionized water.

All of the following chemicals were of analytical-reagent grade, HPLC grade or better, unless stated otherwise, from J. T. Baker (Phillipsburg, NJ, U.S.A.): acetone, acetonitrile, methanol, 1-propanol, 2-propanol, cyclohexanol, carbon tetrachloride and orthophosphoric acid.

Doubly distilled deionized water was used for preparing mobile phases A and B. Mobile phase A was 10 m*M* orthophosphoric acid (pH 2.2) and mobile phase B was 1-propanol–10 m*M* orthophosphoric acid (45:55, v/v). All mobile phases were filtered using a vacuum filtration apparatus and 0.45-μm nylon 66 membrane filters (Schleicher & Schüll, Keene, NH, U.S.A.) before being degassed by helium sparging prior to use.

The stationary phase materials were Nucleosil C_{18}, Nucleosil C_4, Partisil-10 ODS-3 (C_{18}), Vydac 218TPB10 (C_{18}) from Alltech (Deerfield, IL, U.S.A.) and some adamantyl-modified Partisil silica provided by Dr. J. Pesek (San Jose State University, San Jose, CA, U.S.A.). All the materials had a 300-Å pore size, except Partisil (85 Å) and the Nucleosil C_{18} (500 Å). The particle size of the materials was 10 μm, except Nucleosil C_{18} (7 μm).

Apparatus

The chromatographic system consisted of a Series 400 solvent-delivery system, an LC-235 diode-array UV detector and a GP-100 graphics printer (Perkin-Elmer, Norwalk, CT, U.S.A.) and an electric six-port injector with a 5-μl loop (Valco In-

struments, Houston, TX, U.S.A.). Peak areas were determined with a Spectra-Physics (San Jose, CA, U.S.A.) SP4270 integrator. The temperature difference between the mobile phase and the column was minimized by inserting a coiled tube, with a volume of approximately 3 ml, between the pump outlet and the injector and immersing both the tubing and the column in a temperature-controlled water-bath. Columns were packed using a Haskel pump and a laboratory-made slurry reservoir.

Procedures

The HPLC columns were packed using 10 cm × 0.46 cm I.D. stainless-steel tubing, Parker stainless-steel unions and 0.5-μm stainless-steel frits from Alltech. Columns were packed under constant pressure (6000 psi) with the empty column filled with carbon tetrachloride and using a slurry solvent of cyclohexanol–2-propanol (50:50, v/v) with methanol as the push solvent.

RESULTS AND DISCUSSION

Extension of previous work to a C_{18} phase

We extended some of the earlier work by Karger and co-workers with a C_4 LiChrospher SI 500 packing[1] to a C_{18} phase for comparison. The RP-HPLC gradient elution behavior of RNase A on Partisil-10 ODS-3 (C_{18}) at various temperatures is illustrated in Fig. 1 with 10 m*M* orthophosphoric acid (pH 2.2) as one mobile phase

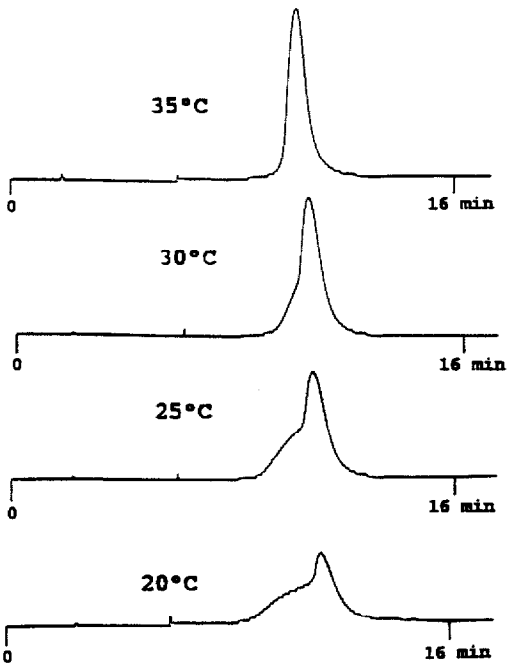

Fig. 1. Band profile of ribonuclease A as a function of temperature. Column: 10-μm Partisil C_{18}, 10 cm × 4.6 mm I.D. Eluents: solvent A, 10 m*M* H_3PO_4 (pH 2.2); solvent B, 1-propanol–10 m*M* H_3PO_4 (pH 2.2) (45:55, v/v); gradient, 0–85% B in 25 min, linear. Flow rate: 1 ml/min. Sample: 100 μg of RNase A.

(A) and 1-propanol–10 m*M* orthophosphoric acid (pH 2.2) (45:55, v/v) as the second mobile phase (B). This behavior was similar to that observed by Karger and co-workers on the C_4 phase[1]. As can be seen, at 20°C RNase A eluted as a broad shoulder followed by a sharper, overlapping peak. As the temperature increased, the shoulder gradually narrowed until it eventually disappeared at 35°C and RNase A eluted as a single sharp peak. It must also be mentioned that similar to Karger and co-workers' study[1], the RNase A mass recovery was observed to be greater than 95%, based on peak-area measurements, over this temperature range and under these conditions.

Except for the inherent delay time of the system, the use of an isocratic hold before starting the gradient for ensuring the absence of the native state on the bonded phase at temperatures above 20°C was found to be unnecessary in our work. This was demonstrated by the insignificant difference between peak profiles with and without isocratic holds of 30 min at both 20 and 25°C.

In Figure 2, the gradient elution behaviour of RNase A on the Partisil C_{18} packing is presented as a function of flow-rate at 15, 20 and 25°C. A linear gradient of 0–85% B over 25 min was run at a flow-rate of 1 ml/min, and this particular gradient

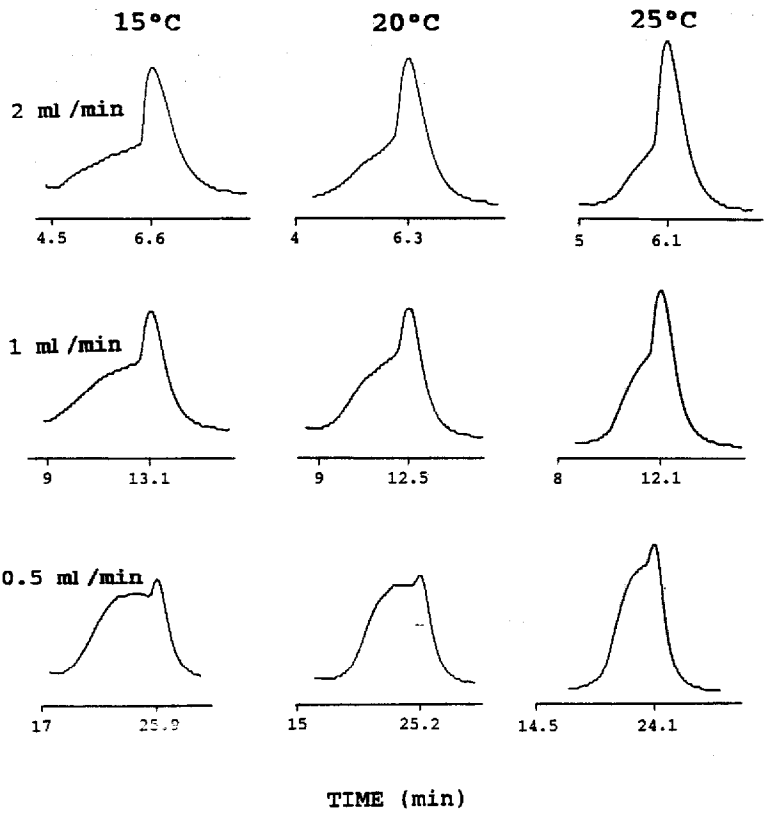

Fig. 2. Behavior of RNase on Partisil C_{18} as a function of flow-rate at several temperatures. Linear gradient conditions: at 2 ml/min, 0–85% B in 12.5 min; at 1 ml/min, 0–85% B in 25 min; at 0.5 ml/min, 0–85% B in 50 min. Other conditions as in Fig. 1.

steepness was maintained for the other flow-rates (*i.e.*, 0–85% B over 50 min at 0.5 ml/min). We noted the expected increase in the size of the initial shoulder (native state) and the corresponding decrease in the peak height of the later eluted, overlapping sharp peak (denatured state) as the temperature was decreased. Similarly, an increase in the native or refolded state and a decrease in the denatured or unfolded state was observed as the flow-rate was decreased. This seemed to support the refolding mechanism reported earlier by Karger and co-workers[1,2].

In that mechanism it is assumed that above 20°C only denatured RNase A is initially adsorbed on the surface of the stationary phase. When the mobile phase is sufficiently rich in solvent B to desorb the denatured RNase A from the stationary phase, some of the dissolved protein molecules will begin to refold to the native state. All of the RNase A in the mobile phase which refolds at 20°C or above should elute without readsorbing on the stationary phase, because the solvent strength desorption is stronger than that required to elute the native state. Conversely, some unfolding of the native state to the denatured state may also reoccur in the mobile phase at temperatures (30–40°C) were both the native and denatured states of RNase A are at equilibrium. Given this gradient behavior, the denatured RNase A should migrate down the column distributing between the mobile and stationary phases, which allows for the continued formation of native RNase A in the mobile phase[1,2].

This chromatographic behavior explains the resulting profile of two overlapping bands, consisting of an initial broad band or shoulder followed by a sharper eluting band. As the time the protein takes to migrate through the column varies inversely with the flow-rate, the protein will spend a longer time in the mobile phase at lower than at higher flow-rates, and the longer the protein remains in the mobile phase the more time it has to refold to the native state.

Fig. 3 represents the behavior of RNase A as a function of flow-rate at 11.5°C. It can be assumed that at this temperature all of RNase A was not adsorbed in a denatured state on the surface of the stationary phase[1,2]. Again, the increased presence of the native state was evident with the decreased flow-rate.

Adamantyl-modified Partisil silica

The chromatographic behavior of RNase A as a function of flow-rate at 20°C on an adamantyl-modified silica column is illustrated in Fig. 4. It was clear that at a flow-rate of 0.5 ml/min the amount of native RNase A relative to that of the denatured state was much greater on the adamantyl column than on the Partisil C_{18} columns.

Initial comparisons of the adamantyl and Partisil C_{18} stationary phases with the solutes phenol, aniline, pyridine and toluene indicated much more polar interactions on the adamantyl than on the C_{18} phase[12]. These results were probably due to more silanol interactions on the adamantyl than on the C_{18} phase because of a poor adamantyl phase coverage. Conversely, Yang and Gilpin[13] demonstrated an increased shielding effect by the rigid, ball-like structure of adamantane from underlying residual silanols. They observed this effect to be greater on the adamantyl phase than on other silica surfaces modified with linear or slightly branched chains. In our work, it was hoped that the bulky structure of the adamantyl group would be less likely to penetrate the structure of the protein than a more linear alkyl-bonded phase. By further decreasing another possible source of disruption to its conformation dur-

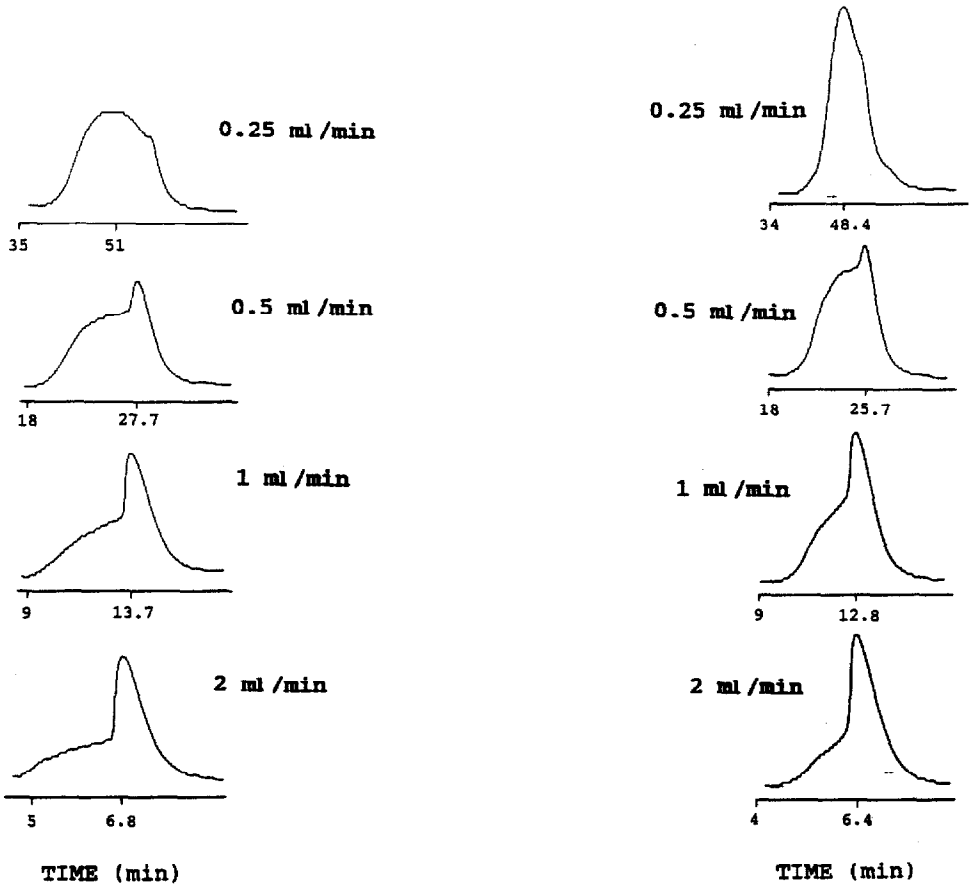

Fig. 3. Behavior of RNase A on Partisil C_{18} as a function of flow-rate at 11.5°C. Gradient: 0–85% B in 100 min at 0.25 ml/min, linear. Other conditions as in Fig. 2.

Fig. 4. Behavior of RNase A on adamantyl-modified silica as a function of flow-rate at 20°C. Sample: 200 μg of RNase A. Other conditions as in Figs. 2 and 3.

ing adsorption and elution, the protein might experience less denaturation. Based on the results of the study with small molecules[12] and the retention data in Fig. 5, it seemed possible that silanophilic interactions could be playing much more of a role in retention on the adamantyl than on the C_4 phases and certainly more than on the C_{18} columns.

Dependence of elution time on temperature and type of bonded phase

The plot in Fig. 5 demonstrates the dependence, at constant gradient steepness, of the retention time of the late-eluting peak (denatured state) with temperature. From these results, it can be seen that the retention increased as the temperature decreased for all the stationary phases tested. There also seemed to be a trend associated with the type of bonded phase, in that all of the C_{18} phases demonstrated relatively similar retention times, whereas both the C_4 and adamantyl phases showed increasingly greater retentions.

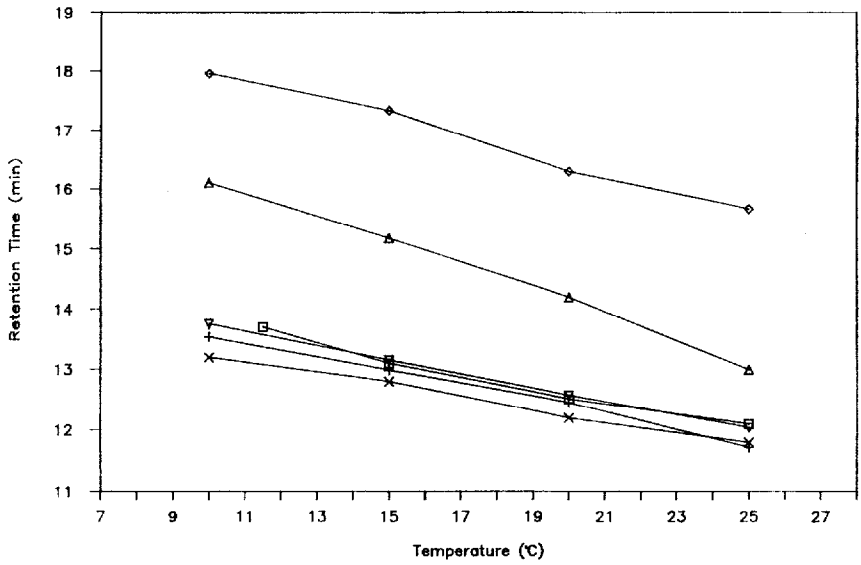

Fig. 5. Plots of the retention time of the late-eluting denatured peak of RNase A *vs.* temperature for several columns. Columns: □, Partisil C_{18}[1]; +, Partisil C_{18}[2]; ◇, adamantyl-modified Partisil silica; △, Nucleosil C_4; ▽, Nucleosil C_{18}; ×, Vydac C_{18}. Flow-rate: 1 ml/min. Other conditions as in Fig. 1.

Peak profiles of refolding RNase A

Fig. 6 provides a comparison of the peak profiles of RNase A on five different columns (adamantyl-modified Partisil silica, Partisil C_{18}, Vydac C_{18}, Nucleosil C_4 and Nucleosil C_{18} as a function of temperature at a flow-rate of 0.5 ml/min. At 20°C and below, the number of observed peaks differed for several columns. The fact that the profile on the adamantyl column differed greatly from the C_{18} columns was not surprising, but the significant difference between the Vydac C_{18} and the Partisil C_{18} (same pore size) was surprising. The possible relationship between these profiles and the refolding rate constants is discussed later.

Refolding rate constants

The apparent refolding rate constant (k_f) was determined by following the method used by Karger and co-workers[2], in which the height of the late-eluted peak (denatured state) was measured as a function of the mobile phase flow-rate while maintaining a constant gradient steepness. By plotting the logarithm of the peak height against the time RNase A spent in the mobile phase at various temperatures for each column, a linear dependence was demonstrated. The values of k_f were then determined from the slopes of the best straight lines through the plotted points.

If it is assumed that the native state of RNase A does not exist on the bonded phase surface above 20°C, then k_f represents the refolding kinetics in the mobile phase. This would hold true even if there were some refolding on the stationary phase, as long as it was negligible. However, the peak profiles at 20°C in Fig. 6 for the Vydac and the adamantyl columns were similar to the profiles observed by Karger and co-workers[2] on a C_4 column at significantly below 20°C (15°C). A third overlapping peak, which eluted in front of the two other bands, was assumed to represent native

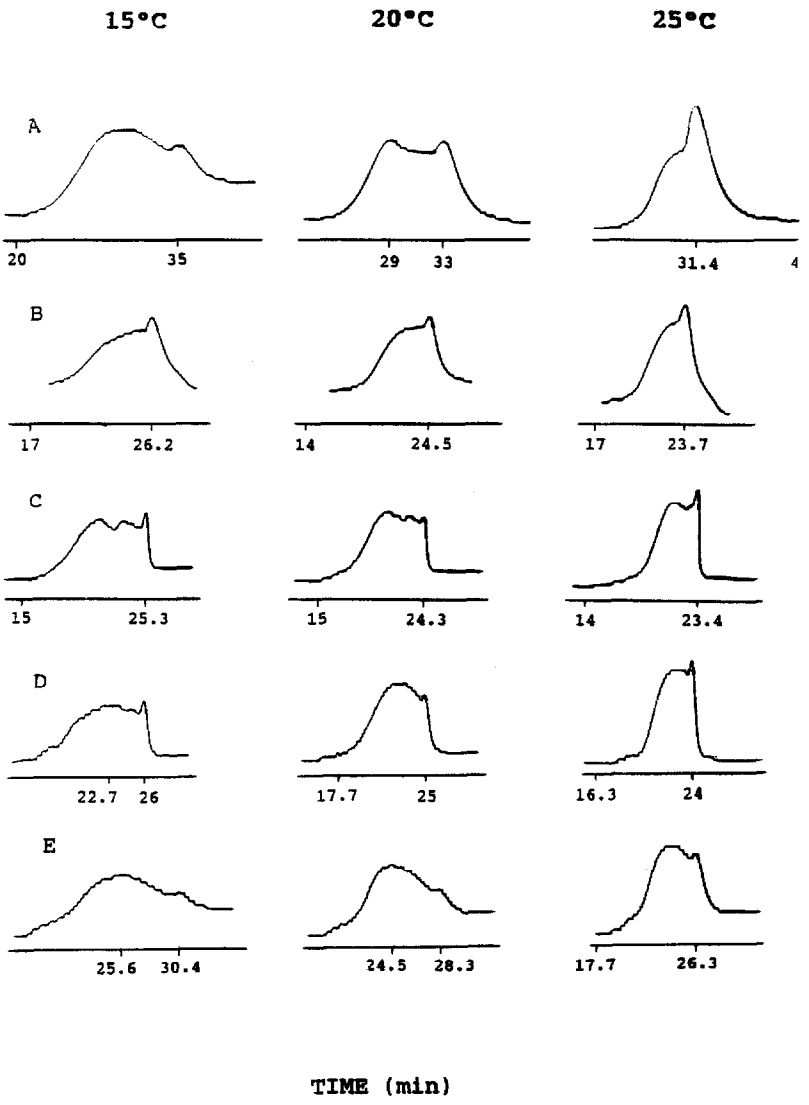

Fig. 6. Behavior of RNase A as a function of temperature on several reversed-phase columns at a flow-rate of 0.5 ml/min. Columns: (A) adamantyl-modified Partisil silica; (B) Partisil C_{18}; (C) Vydac C_{18}; (D) Nucleosil C_{18}; (E) Nucleosil C_4. Samples: 100 μg of RNase A (Partisil, Vydac, Nucleosil) or 200 μg RNase A (adamantyl silica). Gradient: 0–85% B in 50 min, linear. Other conditions as in Fig. 1.

RNase A which had not been denatured on the bonded phase. As some native RNase A will exist on the bonded phase at temperatures below 20°C, much of the native RNase A which eluted in the first of two (as opposed to three) overlapping peaks below 20°C was assumed not to have been denatured on the bonded phase either.

In Fig. 7, the refolding rate constant, k_f, is plotted against temperature for several reversed-phase columns. A constant gradient steepness was maintained for all of the columns and temperatures evaluated. For this reason, the retention time in the

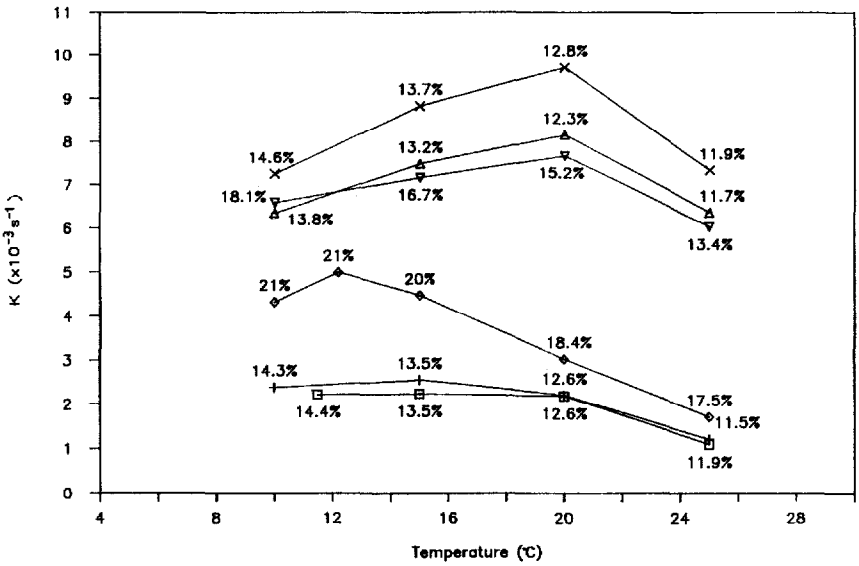

Fig. 7. Plots of the apparent refolding rate constant, k_f, *vs.* temperature for various reversed-phase columns. The percentage of 1-propanol on elution is also included for reference. Columns: □, Partisil C_{18}[1]; +, Partisil C_{18}[2]; ◇, adamantyl-modified Partisil silica; △, Vydac C_{18}; ×, Nucleosil C_{18}; ▽, Nucleosil C_4. See text for other details.

gradient system varied depending on the temperature and the particular column which were being evaluated. As our objective was to observe the effects of the different stationary phases on the overall elution behavior of RNase A (including k_f), this variability was not undesirable. It must be noted that the data in Karger and coworkers' work[2] were obtained under experimental conditions which varied the gradient steepness in such a way as to maintain a constant retention time for any given flow-rate at any temperature. When the same procedure was applied in our work, the k_f values increased only slightly from those listed in Fig. 7 for temperatures below 25°C. The percentage of 1-propanol in the elution of the denatured RNase A was also determined and is shown in Fig. 7. This is important as the percentage of 1-propanol present on elution, and therefore on desorption, will affect the ability of the protein to refold.

As was observed in Fig. 5, the consistency of our data was again well illustrated by the Partisil C_{18} columns in Figure 7. The agreement was very good, especially when considering the method used to determine the k_f values. The data indicated that the k_f values for the Partisil C_{18} columns were lower than those for the adamantyl column and much lower than those for the Vydac C_{18} and Nucleosil C_4 columns.

It was interesting that the differences between the Vydac and Partisil C_{18} stationary phases, indicated by the data in Figs. 6 and 7, were clearly not evident on comparing their retention times in Fig. 5. As discussed carlier, the data in Fig. 5 seem to illustrate the dependence of retention time on the type of bonded phase utilized. The fact that there was a difference between some of the peak profiles and k_f values in Figs. 6 and 7, respectively, could have been due to the different pore sizes of the stationary phase materials. The pore size of the Vydac C_{18} and Nucleosil C_4 silicas

were 300 Å compared with 85 Å for Partisil C_{18} and 500 Å for Nucleosil C_{18}. The structure of the adamantyl group combined with an overall poor coverage (greater silanophilic interactions) of the Partisil silica base could have contributed to its particular peak profiles and intermediate k_f values and also the longer retention times of RNase A.

The rate behavior of the 300 Å stationary phases (Vydac C_{18} and Nucleosil C_4) was similar to that of the C_4 packing (Vydac silica C_4, 300 Å) used in Karger and co-workers' work[2]. Further, as this type of rate behavior has been observed in refolding processes in solution for systems close to equilibrium[5], these results were not surprising.

CONCLUSIONS

The extension of some earlier work by Karger and co-workers[1,2] has resulted in both similar and additional information regarding the conformational refolding of RNase A in gradient elution RP-HPLC. The different elution profiles and the range of apparent refolding rate constants of RNase A observed in this study suggested some interesting characterizations for the stationary phases evaluated.

The trend indicated by the plotted values of the apparent refolding rate constants, k_f, seemed to illustrate more of a correspondence with the pore size of the stationary phases than with the actual bonded-phase material. This dependence also seemed to be supported by the different elution profiles of some of the stationary phases. However, this trend did not apply to the retention times of RNase A, which appeared to be more dependent on the type of bonded phase.

Further studies should include additional stationary phases of varying manufacturer, pore size and type. The use of another well defined reversible protein, of similar molecular weight if possible, would also be of interest. Future work might also include the use of on-line fluorescence spectroscopy in addition to the spectroscopic techniques reported previously[1,2] for further identification of the conformational states.

The information acquired from such work, combined with our present knowledge of the RP-HPLC behavior of RNase A, should assist in a better understanding of the chromatographic interactions between the stationary phase and the protein during adsorption and elution, thus providing another perspective from which the properties of reversed-phase stationary phases could be classified and then used when developing a strategy for the chromatography of certain proteins.

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