CHROMSYMP. 501

EFFECTS OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SOLVENTS AND HYDROPHOBIC MATRICES ON THE SECONDARY AND QUATERNARY STRUCTURE OF A MODEL PROTEIN

REVERSED-PHASE AND SIZE-EXCLUSION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A series of five synthetic peptide polymers of 8, 15, 22, 29 and 36 residues with the sequence Ac-(Lys-Leu-Glu-Ala-Leu-Glu-Gly)$_n$-Lys-amide where n = 1–5 was used to examine protein denaturation during reversed-phase high-performance liquid chromatography (HPLC). Size-exclusion HPLC on a TSK G3000SW column was used to show that the 8-, 15- and 22-residue peptides are monomers in the starting solvent for reversed-phase chromatography, 0.1% aqueous trifluoroacetic acid (TFA) while the 29- and 36-residue peptides are dimers. These dimers have been shown to be extremely stable two-stranded α-helical coiled coils where the subunits are held together by hydrophobic interactions [S. Y. M. Lau, A. K. Taneja and R. S. Hodges, *J. Biol. Chem.*, (1984) in press]. In contrast, all five peptides are monomers in acetonitrile–0.1% aq. TFA (1:1). These five peptides were separated by reversed-phase chromatography with an increasing gradient of 0.7% acetonitrile per minute on three matrices of varying carbon loading, pore size, and alkyl chain length (C$_3$, C$_8$ and C$_{18}$). In all cases a linear relationship between the natural logarithm of the monomeric molecular weight and retention volume was obtained for these peptides, indicating that the 29- and 36-residue dimers had been dissociated on binding to the reversed-phase columns. These results strongly suggest that the vast majority of proteins are denatured upon binding to the hydrophobic matrix. This series of peptides, 900–8000 molecular weight range, was also used for two additional purposes; firstly, to evaluate the reversed-phase columns and, secondly, to evaluate the relationship of ln molecular weight *versus* retention volume on the TSK G3000SW column.

INTRODUCTION

High-performance liquid chromatography (HPLC) has revolutionized the methodology for the separation of peptides and proteins in the past five years and consists of size-exclusion chromatography (SEC), reversed-phase chromatography

(RPC) and ion-exchange chromatography (IEC) (for recent reviews, see refs. 1 and 2).

In reversed-phase chromatography the most commonly used solvent systems for the separation of peptides and proteins involve linear gradients, starting with water and increasing concentrations of organic solvent (methanol, acetonitrile or propanol). These solvent systems usually employ low concentrations of perfluorinated organic acids (trifluoroacetic acid) at a concentration of 0.05–0.1% (v/v) in both the water and the organic solvent.

Most reversed-phase matrices have relatively high hydrocarbon loadings, which cause a strong binding of the peptide or protein to the matrix, and the concentration of organic solvent is usually greater than 10% for peptide or protein elution. Since hydrophobic interactions play a major role in stabilizing the three-dimensional structure of a protein, it is reasonable to expect that the hydrophobicity of the matrix and/or non-polarity of the solvent system could denature a protein upon binding to or elution from the column. This may preclude the purification of multi-subunit proteins or of any proteins where a separation in the native conformation is desired. In addition, the hydrophobicity of a protein in its native conformation is dramatically different from its unfolded state, since the hydrophobic side-chains are buried during the folding process. It is of particular importance to avoid a situation where the folding state of the protein is changing during the separation procedure[2].

In order to clarify our understanding of denaturation during reversed-phase chromatography, we have used a model protein with a very stable quaternary structure to monitor this process. The synthetic peptide polymers of 29, 36 and 43 residues [Ac-(Lys-Leu-Glu-Ala-Leu-Glu-Gly)$_n$-Lys-amide, where $n = 4$–6] have been shown to form two-stranded α-helical coiled coils in benign medium, which are stabilized by hydrophobic interactions between the two chains[3–5]. The stability of these synthetic two-stranded α-helical coiled coils has been demonstrated by temperature and urea denaturation studies[3,4]. For example, tropomyosin, the most thoroughly studied two-stranded α-helical coiled coil is almost completely denatured in 6 M urea, whereas the 29-residue (TM-29) and 36-residue (TM-36) peptides in 6 M urea retain 22% and 70% of their helicity, respectively, as observed in benign medium[3]. Similarly, tropomyosin is completely denatured at 69°C, TM-29 and TM-36 retain 62% and 74% of their helicity at this temperature. Since the secondary and quaternary structure of these model proteins is so stble, a demonstration of complete denaturation on reversed-phase chromatography would then be representative of the situation for most proteins.

To investigate denaturation upon reversed-phase chromatography, we have used a mixture of five synthetic peptide polymers of 8, 15, 22, 29 and 36 residues (TM-8, TM-15, TM-22, TM-29 and TM-36) of the sequence Ac-(Lys-Leu-Glu-Ala-Leu-Glu-Gly)$_n$-Lys-amide (Table I). This mixture was separated on three reversed-phase columns of varying alkyl chain length, pore size and carbon loading. In addition, the conformation of the peptides was monitored by a combination of two methods. First, high-performance size-exclusion chromatography was carried out in various solvents to determine whether the peptides were monomeric or dimeric. Second, circular dichroism studies were performed in these same solvents to determine the helicity of the peptides.

TABLE I

PEPTIDE POLYMERS INVESTIGATED

Peptide	Monomeric molecular weight	Amino acid sequence
TM-8	927	Ac-(Lys-Leu-Glu-Ala-Leu-Glu-Gly)$_1$-Lys-amide
TM-15	1667	Ac-(Lys-Leu-Glu-Ala-Leu-Glu-Gly)$_2$-Lys-amide
TM-22	2407	Ac-(Lys-Leu-Glu-Ala-Leu-Glu-Gly)$_3$-Lys-amide
TM-29	3147	Ac-(Lys-Leu-Glu-Ala-Leu-Glu-Gly)$_4$-Lys-amide
TM-36	3887	Ac-(Lys-Leu-Glu-Ala-Leu-Glu-Gly)$_5$-Lys-amide

MATERIALS AND METHODS

Unless otherwise stated, all chemicals and solvents were reagent grade: urea (ultrapore, Canadian Scientific Products, London, Canada); trifluoroacetic acid (TFA) (Halocarbon Products, Hackensack, NJ, U.S.A.); trifluoroethanol (TFE) (Sigma, St. Louis, MO, U.S.A.); acetonitrile (HPLC grade, Fisher Scientific, Fairlawn, NJ, U.S.A.); 2-propanol (HPLC grade, BDH, Toronto, Canada); 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Aldrich, Milwaukee, WI, U.S.A.). Double-distilled water was purified by passing it through Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.).

Routine amino acid analyses were performed on a Durrum D-500 amino acid analyzer. Peptides were quantitated by amino acid analysis after hydrolysis with 6 M hydrochloric acid, containing 0.1% phenol, in evacuated, sealed tubes for 24 h at 110°C by using the mean of the molar ratios of all accurately measurable amino acids in the acid hydrolyzate to calculate the concentration.

Circular dichroism spectra were recorded on a JASCO-J5006 spectropolarimeter, attached to a JASCO DP-500N data processor. The instrument was routinely calibrated with an aqueous solution of recrystallized d-10-camphorsulfonic acid. Constant nitrogen flushing was employed. Ellipticity data were converted into conformation parameters by the procedures and equations described by Chen et al.[6]. The reproducibility of all spectra was within ± 3% for wavelengths greater than 205 nm.

Peptide synthesis and purification

The peptide analogues (TM-8, TM-15, TM-22, TM-29 and TM-36) were synthesized and purified as described by Lau et al.[3].

Reversed-phase and size-exclusion chromatography

The HPLC instrumentation consisted of a Spectra-Physics SP8700 solvent delivery system and SP8750 organizer module, combined with a Hewlett-Packard HP1040A detection system, HP3390A integrator, HP85 computer, HP9121 disc drive and HP7470A plotter.

The peptide mixture was separated on three reversed-phase columns, an Altex Ultrapore RPSC C-3 (Beckman, CA; 75 × 4.6 mm I.D.); a Whatman Partisil CCS/C$_8$ (Whatman, Clifton, NJ, U.S.A.; 250 × 4.6 mm I.D.); and a C$_{18}$ column, SynchroPak RP-P (Linden, IN, U.S.A.; 250 × 4.1 mm I.D.). The peptides were dissolved in 0.1%

TFA in water at pH 2.5. A gradient was constructed from 0.1% TFA in water (solvent A) and 0.05% TFA in acetonitrile (solvent B). The gradient program was as follows: linear gradient from 100% A to 85% A, 15% B at 5 min (3% B/min) followed by a linear gradient to 53% A, 47% B at 50 min (0.7% B/min). The flow-rate was 1 ml/min and the absorbance was recorded at 210 nm.

Size-exclusion chromatography was carried out on a TSK G3000SW column (600 × 7.5 mm I.D.) with a guard column (60 × 7.5 mm I.D.) (Toyo Soda, Tokyo, Japan). The flow-rate was 0.4 ml/min and the effluent was monitored by absorbance at 210 nm. The solvents used are described in the figure legends.

RESULTS AND DISCUSSION

The perfluorinated organic acid TFA was chosen for reversed-phase chromatography for the following reasons. Reversed-phase silica gel columns can contain surface silanols which act as weak acids that are ionized above pH 3.5–4. As a consequence, these weak acids are free to interact with basic compounds that are chromatographed on reversed-phase columns. The interaction can be overcome simply by suppressing the ionization of the surface silanols through the use of acidic mobile phases[1]. In addition, silica-based columns are more stable at low pH. TFA not only provides an acidic medium but is an excellent protein solubilizing agent, which is used routinely in solid-phase peptide synthesis to extract peptides and proteins from the resin support after cleavage. TFA is not only included in the aqueous solvent but also in the organic solvent at a concentration of 0.05–0.1% (v/v) to maximize peptide solubility in the organic solvent. TFA is completely volatile and can be used for UV detection at low wavelengths (210 nm) where the peptide bonds absorb strongly. The advantages of perfluorinated organic acids have been described previously[1,2,7-11]. Though most peptides are far more soluble at pH 1.5–3 than at high pH values, many proteins can be denatured in acidic solutions. Therefore, it was important to verify the conformation of our model proteins in the starting solvent, 0.1% aqueous TFA.

Of the three most commonly used organic solvents in RPC the order of effectiveness in eluting peptides and proteins has been found to be propanol > acetonitrile > methanol[2,9,12]. In general, acetonitrile is appropriate for most peptides and proteins, methanol being used for the very hydrophilic molecules and propanol for the more hydrophobic ones. The best resolution is usually achieved between 15% and 40% of the organic solvent in the gradient, and this should dictate the choice of solvents[2]. Considering the above, we have chosen acetonitrile as the organic solvent in these studies. The gradients were made by starting with 0.1% aq. TFA and eluting the peptides with increasing amounts of 0.05% TFA–acetonitrile. Aqueous solutions of alcohols are known to denature proteins, and the rate of denaturation increases with the increasing alkyl chain length of the alcohol. It was suggested that proteins in general do not differ greatly in their susceptibility to denaturation by alcohols[13]. Since the solvent systems used in RPC are protein denaturants, it was important to know the effect of these solvents on the quaternary and secondary structure of our model proteins.

Conformation studies by size-exclusion HPLC
It was shown previously that of the five synthetic peptides (TM-8, TM-15,

TM-22, TM-29 and TM-36) both TM-29 and TM-36 were two-stranded α-helical coiled coils in benign medium[3]. Since these two peptides have a defined secondary and quaternary structure which is representative of a native protein, they are ideal model proteins for studying denaturation during RPC. In this work the monomeric–dimeric structure of these five peptides was examined by size-exclusion HPLC on a TSK G3000SW column (Fig. 1). Three solvent systems were used: 0.1% aq. TFA (profile C), which represents the starting solvent for RPC; acetonitrile–0.1% aq. TFA (1:1) (profile B), which represents the upper limit of organic solvent generally used in RPC; and TFE–0.1% aq. TFA (1:1) (profile A). TFE was chosen because it is a non-interacting (inert) solvent with strong α-helix-inducing properties[14]. All five peptides (TM-8, TM-15, TM-22, TM-29 and TM-36) are monomers in the three solvents studied, except for TM-29 and TM-36, which are dimers in 0.1% aq. TFA (Figs. 1 and 2). This conclusion is based on the observation that the plots of ln
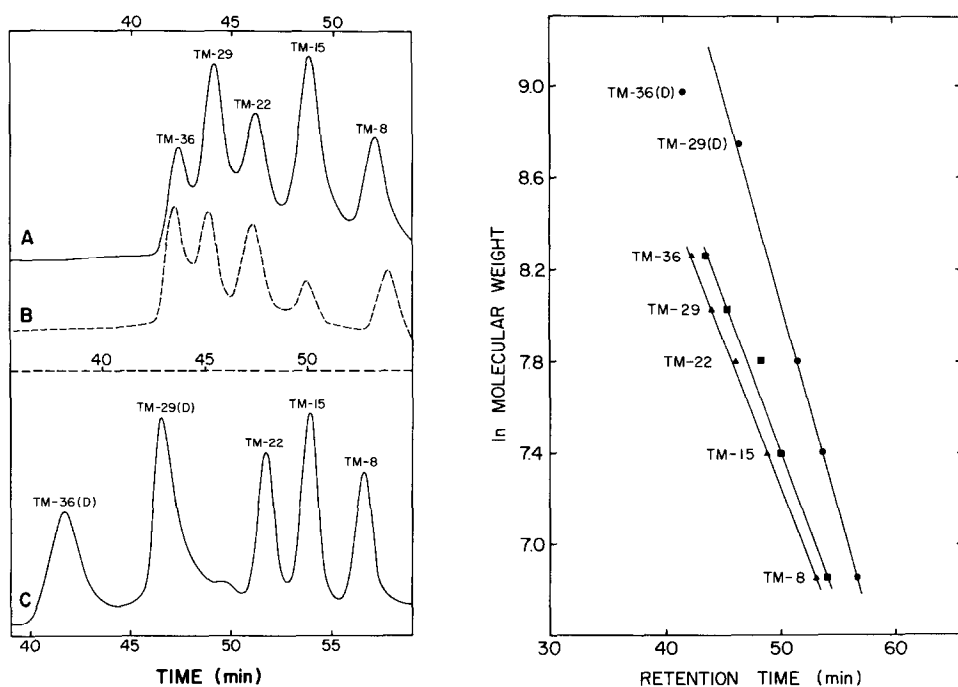

Fig. 1. Comparison of the size-exclusion HPLC elution profiles of a peptide mixture in three different solvent systems. The synthetic peptide mixture consisted of five peptides (TM-8, TM-15, TM-22, TM-29 and TM-36) of the sequence Ac-(Lys-Leu-Glu-Ala-Leu-Glu-Gly)$_n$-Lys-amide, where n = 1–5. Profile A (solid line): solvent, TFE–0.1% aq. TFA (1:1). Profile B (dotted line): solvent, acetonitrile–0.1% aq. TFA (1:1). Profile C (solid line): solvent, 0.1% aq. TFA. The time axes have been shifted to align the separations. The time axis for profile A is on the top; for profile B, it is represented by the dashed line in the center, and for profile C, it is on the bottom of the figure. Column: TSK G3000SW, 600 × 7.5 mm I.D. Sample volume: 1–5 μl of starting solvent. Flow-rate: 0.4 ml/min. The effluent was monitored at 210 nm. The symbol (D) denotes the dimeric form of the peptide.

Fig. 2. Plots of ln molecular weight *versus* retention time of the five peptides separated by size-exclusion HPLC in three different solvent systems (see Fig. 1, legend). The symbols are: ● = 0.1% aq. TFA, ▲ = TFE–0.1% aq. TFA (1:1), and ■ = acetonitrile–0.1% aq. TFA (1:1). The symbol (D) denotes the dimeric form of the peptide.

monomeric molecular weight versus retention time are linear for all five peptides in the solvent systems containing organic solvent (Fig. 2), whereas a linear plot could only be obtained for the five peptides in 0.1% aq. TFA when dimeric molecular weights were used for TM-29 and TM-36. Though an absolute retention time of a peptide was dependent on the solvent system, the differences in retention times between the monomeric peptides were very similar in the three solvent system used. This is shown in Fig. 1, where the time axes have been shifted to align the separations. These results agree with previous studies (3), where it was shown that in size-exclusion HPLC on TSK G2000SW all five peptides are monomers in 8 M urea–1.1 M KCl–0.05 M PO$_4$ buffer (pH 7.0) when compared to standards varying in molecular weight from 2000 to 18,000 daltons. In benign medium (1.1 M KCl–0.05 M PO$_4$ buffer, pH 7.0), TM-8, TM-15 and TM-22 are monomers while TM-29 and TM-36 are dimers. TM-29 and TM-36 were also shown to be dimers in this benign buffer by sedimentation equilibrium studies[3]. Both TM-29 and TM-36 were found to be completely α-helical in benign buffer with no further increase in helicity on the addition of TFE[3]. Similarly, in this study we have shown that TM-36 has a molar ellipticity of $[\theta]_{220}$ = −31,791° in 0.1% aq. TFA and that the addition of TFE and HFIP, both helix-inducing solvents, or acetonitrile do not increase the helicity. In fact, a small decrease in $[\theta]_{220}$ is observed with increasing concentrations of organic solvent (Fig. 3). In contrast, TM-15, which is a monomer in 0.1% aq. TFA with a very low molar ellipticity ($[\theta]_{220}$ = −4300°) increases its molar ellipticity substantially on the addition of organic solvents (Fig. 4). The two helix-inducing solvents HFIP and TFE show a maximum $[\theta]_{220}$ of −17,800° compared to −13,400° for acetonitrile. The rate of helix induction increases with the non-polarity of the solvent (Fig. 4). The results reported here agree with those reported for all five peptides in 1.1 M KCl–0.05 M PO$_4$ buffer (pH 7.0) with and without TFE[3]. It should be noted that the
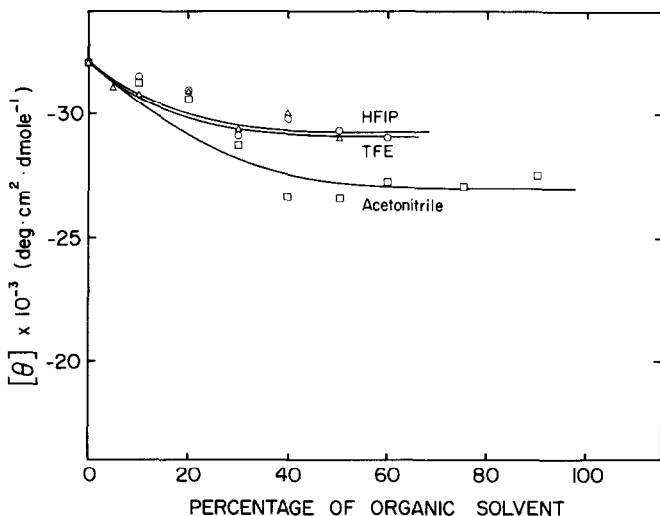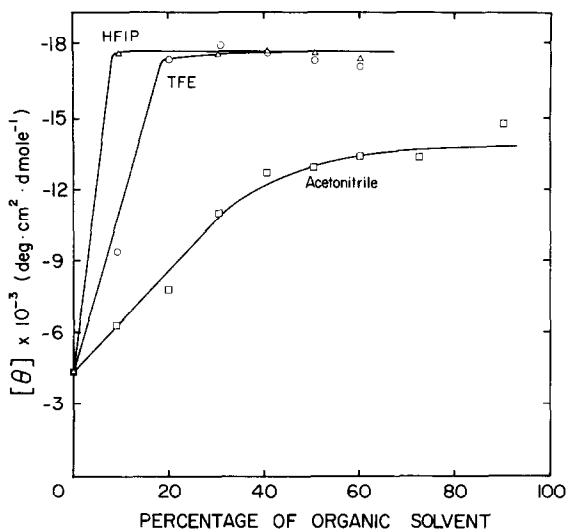

Fig. 3. Effect of three organic solvents on the molar ellipticity at 220 nm of the synthetic peptide TM-36 [Ac-(Lys-Leu-Glu-Ala-Leu-Glu-Gly)$_5$-Lys-amide]. The symbols are: △ = HFIP, ○ = TFE, and □ = acetonitrile.

Fig. 4. Effect of three organic solvents on the molar ellipticity at 220 nm of the synthetic peptide TM-15 [Ac-(Lys-Leu-Glu-Ala-Leu-Glu-Gly)$_2$-Lys-amide]. The symbols are: △ = HFIP, ○ = TFE, and □ = acetonitrile.

acidic pH of 0.1% aq. TFA (pH 2.5) does not denature two-stranded α-helical coiled coils. These molecules are exceptionally stable in acidic conditions[3,15,16]. Taken together, these results suggest that TM-29 and TM-36 are two-stranded α-helical coiled coils in the starting conditions used for RPC (0.1% aq. TFA) and the addition of organic solvent during RPC (0.05% TFA in acetonitrile) results in denaturation of the quaternary structure while stabilizing the secondary structure (α-helix) of the individual polypeptide chains. Disruption of the tertiary and quaternary structure of a protein in the non-polar medium of RPC is not surprising, when one considers that the major stabilizing forces are hydrophobic interactions. Since hydrogen bonds which stabilize the α-helix are exceedingly unstable' in the presence of water, one would expect that, as the non-polarity of the medium increases, the stability of the secondary structure (α-helix) in single-stranded polypeptides would increase[5,17].

    In size-exclusion chromatography ideal separations occur only when there are no ionic or hydrophobic interactions between the macromolecules and the support. Depending on the extent of surface partitioning, a mixture will be resolved by a pure size-exclusion process, by a mode dominated by surface partitioning, or by a combination of these modes[1]. Ion-exclusion effects have been observed on all commercial size-exclusion columns tested to date, and it has been recommended that they should be operated at ionic strengths greater than 0.1 to 0.2 M to overcome or minimize electrostatic effects[1]. On the other hand, hydrophobic effects have been observed to result in deviations from the separation obtained by purely a size-exclusion process, and ionic strengths above 0.6 M are not recommended. We chose 0.1% aq. TFA to evaluate the starting solvent for RPC. There are some advantages of this solvent in that the peptide or protein fractions can be lyophilized to remove the eluent and to give a salt-free sample. In benign media, our results were identical at both low (0.1% aq. TFA) and high ionic strength (1.1 M KCl–0.05 M PO$_4$ buffer, pH 7.0) for the

separation of these peptides[3]. As shown in Figs. 1 and 2, there are, however, some small changes in retention times of the peptides in the different solvents.

Regnier[1] evaluated many of the commercial size-exclusion columns and reported that the TSK G3000SW column had the greatest overall utility. We have evaluated this column at its lower molecular weight limit for resolving small peptides differing by only 740 daltons that ranged from 927 for TM-8 monomer to 7774 for the TM-36 dimer. It was also reported that good size-exclusion columns will resolve only solutes with approximately a two-fold difference in molecular weight[1]. The TSK G3000SW column was able to resolve completely TM-15 (molecular weight, MW 1667) and TM-22 (MW 2407) as well as TM-29 dimer (MW 6294) and TM-36 dimer (MW 7774), which have molecular weight ratios of 1.44 and 1.24, respectively (Fig. 1). The flow-rate in these experiments was 0.4 ml/min. The resolution can be further increased by reducing the flow-rate. The excellent separation between TM-29 and TM-36 dimers could also be aided by differences in their axial ratio (length/diameter). The two-stranded α-helical coiled coils are rod-like with an approximate diameter of 10 Å, and, as we increase the length of the polypeptide chain, the axial ratio will increase (TM-29 = 4.35 and TM-36 = 5.4). Size-exclusion chromatography is very sensitive to shape changes. For example, the two-stranded α-helical coiled-coil tropomyosin of molecular weight 66,000, gives an apparent molecular weight of 137,000 (ref. 4) with an axial ratio of 42.6, when compared with the calculation plot for globular standards. The TM-36 dimer shows the largest deviation from the linear plot in Fig. 2 which could be explained by this shape effect.

The TSK G3000SW column has been previously evaluated for the separation of proteins. A linear relationship between ln molecular weight and retention volume was obtained for 16 proteins, their molecular weights ranging from 480,000 (ferritin) to 6000 (insulin)[18,19]. In our study, we have shown that there is a linear relationship for peptides in the range 900 to 8000 daltons.

Reversed-phase HPLC

The synthetic peptide mixture consisting of five peptides, TM-8, TM-15, TM-22, TM-29 and TM-36, was resolved by RPC with a linear gradient of two solvents: solvent A consisted of 0.1% aq. TFA and solvent B of 0.05% TFA in acetonitrile (see Materials and methods). The same flow-rate (1 ml/min) and gradient was used to resolve the mixture on three *n*-alkyl matrices of varying chain length (C_3, C_8 and C_{18}). The elution profiles are shown in Fig. 5. The separation was almost identical on all three columns, but the C_{18} column provided the best overall resolution. The major difference in the three matrices was in the concentration of acetonitrile required for elution of the peptides: $C_8 > C_{18} > C_3$. Three points may be emphasized from these results (Fig. 5). First, the length of the column has little influence on the resolution[1,2]. In this experiment the C_3 column is only 7.5 cm, compared to the other two columns each of which is 25 cm in length. It has been shown that load capacity appears to increase in a non-linear manner with column length[1]. Thus, the longer columns would be more practical for preparative applications. Second, it has been reported that the most important parameter affecting the behavior of peptides in RPC appears to be pore size of the matrix. Matrices with 80–100 Å pore size do not provide optimum resolution and recovery of peptides larger than 30 residues. In general, for proteins and peptides (30–150 residues) the
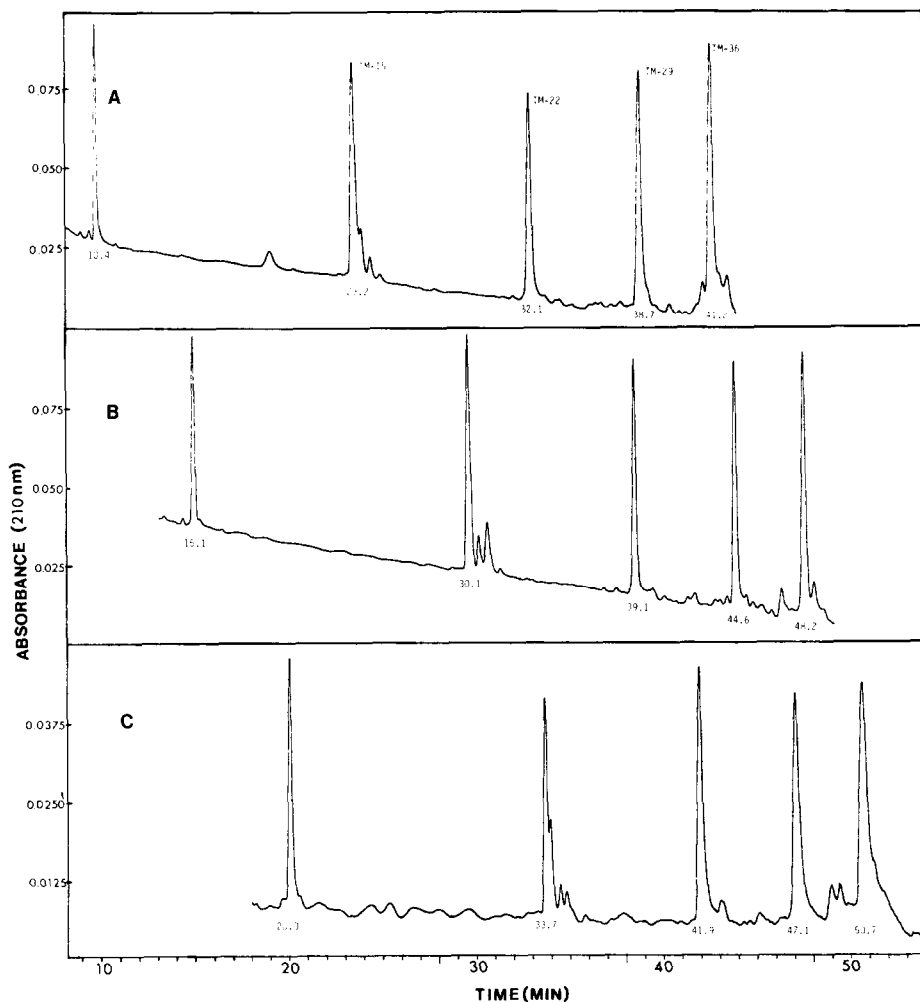
Fig. 5. Comparison of the reversed-phase HPLC elution profiles of a peptide mixture on three *n*-alkyl supports of varying chain length. The synthetic peptide mixture consisted of five peptides (TM-8, TM-15, TM-22, TM-29 and TM-36) of the sequence Ac-(Lys-Leu-Glu-Ala-Leu-Glu-Gly)$_n$-Lys-amide, where n = 1–5. A: C$_3$ column, Altex Ultrapore RPSC 75 × 4.6 mm I.D., 5 μm particle size, 300 Å pore size, carbon loading 2.9%. B: C$_{18}$ column, SynchroPak RP-P, 250 × 4.1 mm I.D., 6.5 μm particle size, 300 Å pore size, carbon loading 10%. C: C$_8$ column, Whatman Partisil 5 CCS/C$_8$ 250 × 4.6 mm I.D., 5 μm particle size, 60 Å pore size, carbon loading 9%. Conditions: AB gradient, solvent A consisted of 0.1% aq. TFA and solvent B of 0.05% TFA in acetonitrile. See Materials and methods for gradient details. Flow-rate 1 ml/min.

300 Å pore size matrices gave better resolution and recovery[1,2,20–23]. Lewis and De-Wald[24] have shown that the 300 Å matrix was superior to 100 Å matrices for the separation of larger proteins (50,000). For the small peptides used in this study, the molecular weight range from 900–4000 daltons (8–36 residues), pore diameter has little effect on resolution. The pore size of both the C$_3$ and C$_{18}$ columns is 300 Å compared to 60 Å for the C$_8$ column. These results suggest that the 300 Å pore size

is universally applicable to the separation of both small peptides and proteins. Third, the length of the n-alkyl chain and carbon loading had little influence on the resolution of the peptides used in this study. Of the three n-alkyl chain lengths examined, C_3, C_8 and C_{18} the C_3 contained a low carbon loading (2.9%) compared to the C_8 and C_{18} (9% and 10%, respectively). Many researchers have examined the role of the n-alkyl chain length in RPC by using various non-peptide organic molecules. In general, increased carbon content or chain length results in greater retention with a given mobile phase[25–30], and this agrees with the results of our study. Karch et al.[25] have recommended the use of C_{18} columns with high carbon content for difficult separations where the eluites have small relative retentions (hydrophilic). The increased retention on C_8 compared to the C_{18} support can be explained by the increased n-alkyl chain ligand density (ca. double for the C_8 column). Short n-alkyl chain length with low carbon loading would be ideal for use with very hydrophobic peptides[2]. The higher the carbon content, the greater the amount of a sample that can be separated without loss of resolution, which is important for preparative chromatography[25].

In this study the particle size of all three reversed-phase supports were similar, 5–6.5 μm. It has been shown that a particle size of 5 μm provides increased column efficiency (sharper peaks and increased resolution) compared to 10 μ matrices[31]. In general, the most applicable reversed-phase column still remains the C_{18} column with approximately 10% carbon loading and a particle size of 5 μm.

Effects of reversed-phase HPLC on protein denaturation

A plot of ln molecular weight *versus* retention time of the five synthetic peptides on the three reversed-phase columns is shown in Fig. 6. The results indicate that all five peptides are eluted from the columns as a function of their monomeric molecular weight. These results, in conjunction with the size-exclusion data, suggest that the
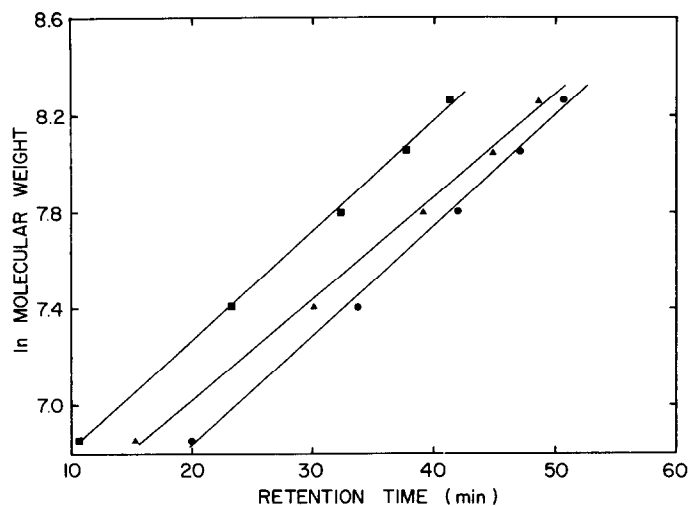

Fig. 6. Plots of ln molecular weight *versus* retention time of the five peptides separated by reversed-phase HPLC on three n-alkyl matrices of varying chain lengths (see Fig. 5, legend). ■ = C_3, ● = C_8, and ▲ = C_{18}.

quaternary structure of TM-29 and TM-36 is disrupted upon binding to the hydrophobic support. Both TM-29 and TM-36 are extremely stable dimers, as indicated by temperature and urea denaturation[3] in 0.1% aq. TFA, which is the starting solvent for RPC. Thus, it is the hydrophobic matrix that causes the disruption of the hydrophobic interactions between the two subunits of these peptides. This denaturation occurs even on the ultra-short (C[3]) matrices with low carbon loading. Although we have shown that the organic mobile phases used in RPC can cause denaturation and disruption of the dimer, the hydrophobicity of the matrix is the important factor. If it is desirable to separate proteins in their native conformation, the hydrophobicity of the matrix must be significantly reduced. Of course, the organic mobile phases would have to be replaced with non-denaturing solvent systems. Considerable progress has been made in this regard with the introduction of hydrophobic HPLC, which differs from reversed-phase HPLC in that little or no denaturation of proteins supposedly occurs[32–34]. In hydrophobic HPLC the hydrophobic groups are sparsely distributed on the matrix, and elution can be accomplished with decreasing salt gradients that are compatible with the native conformation of many proteins. It is known that hydrophobic interactions increase with increasing ionic strength of the medium[32] so the native molecules are bound at high ionic strength (*ca.* 2 *M*). The fact that enzymes can be bound during hydrophobic HPLC and recovered with full biological activity does not imply that denaturation or partial denaturation has not occurred. Proteins can rapidly renature on release from the support. It is well documented that enzymes can be purified on reversed-phase HPLC with recovery of biological activity, even though they must be denatured by both binding to the column and the mobile-phase solvents. Since TM-29 and TM-36 are stable two-stranded α-helical coiled coils in high-ionic-strength buffers[3,4] compatible with conditions used in hydrophobic HPLC, we feel that this series of model synthetic peptides would be ideal to study denaturation on these new matrices.

CONCLUSIONS

We have demonstrated that the hydrophobicity of reversed-phase columns results in the denaturation of proteins by disrupting the hydrophobic interactions stabilizing the native conformation. Denaturation occurs on binding to the matrix. Even the ultra-short (C[3]), 300 Å pore matrix with relatively low carbon loading (2.9%) does not prevent denaturation of these extremely stable synthetic two-stranded α-helical coiled coils. Though the organic solvents used in RPC do cause dissociation of the two subunits in this model protein, as demonstrated by size-exclusion chromatography, the primary cause of denaturation is the hydrophobicity of the matrix.

ACKNOWLEDGEMENTS

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