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SEPARATION OF HYDROPHOBIC PEPTIDE POLYMERS BY SIZE-EXCLUSION AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Four synthetic leucine polymers of 13, 22, 26 and 30 residues with the sequence Ac-$(Leu)_{10}$-$(Lys)_2$-Ala-amide and $(Lys)_2$-Gly-$(Leu)_n$-$(Lys)_2$-Ala-amide, where n = 16, 20 and 24, were used to determine the utility of reversed-phase chromatography in separating extremely hydrophobic peptides. Two mobile phases were examined, water–acetonitrile and water–2-propanol, both containing 0.1% trifluoroacetic acid (TFA). 2-Propanol was the preferred organic solvent, and the hydrophobic peptides (13, 22 and 26 residues) were resolved on the Altex Ultrapore C_3 column (300-Å pore size, 5-μm particle size, 2.9% carbon loading) in the 25–50% range of a linear AB gradient (A = 0.1% aq. TFA; B = 0.1% TFA in 2-propanol) increasing at 1% B/min. This procedure permits detection by absorbance at 210 nm, and the peptides can be recovered by evaporation or lyophilization. The 30 residue leucine polymer could not be eluted from the reversed-phase column. These peptides showed non-ideal behavior on size-exclusion chromatography in 0.1% aq. TFA containing 50% acetonitrile. However, this caused no problem in peptide purification. The resolution on a TSK G3000SW column was superior to that obtained on TSK G2000SW.

INTRODUCTION

We have been involved in the investigation of protein–lipid interactions which required the synthesis of extremely hydrophobic amphiphilic peptides to span the lipid bilayer[1]. Four synthetic leucine polymers of 13, 22, 26 and 30 residues of the sequence Ac-$(Leu)_{10}$-$(Lys)_2$-Ala-amide and $(Lys)_2$-Gly-$(leu)_n$-$(Lys)_2$-Ala-amide, where n = 16, 20 and 24, were prepared. Since it is unlikely that investigators would encounter peptides or proteins of higher hydrophobicity, we felt it appropriate to study the separation of these model hydrophobic peptides by reversed-phase and size-exclusion high-performance liquid chromatography.

High-performance liquid chromatography (HPLC), which has revolutionized the methodology for the separation of peptides and proteins, may be based on size-exclusion, reversed-phase, and ion-exchange chromatography (for recent reviews, see refs. 2–4). One of the major difficulties in reversed-phase HPLC is the purification of

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extremely hydrophobic peptides. The most popular solvent systems for separating peptides and proteins have been applied as linear gradients, ranging from 0.1% trifluoroacetic acid (TFA) in water to 0.1% TFA in acetonitrile or propanol. Three major problems are encounterd in devising a reversed-phase separation of hydrophobic peptides. The first is their limited solubility in aqueous media. Second, very hydrophobic peptides bind strongly to reversed-phase matrices and require high concentrations of organic solvents for elution. Under these conditions, the peptide may not be soluble and could precipitate[5]. Third, the peptides may be irreversibly adsorbed on the column support. Therefore, prior to reversed-phase chromatography an investigation of the solubility of hydrophobic peptides should be carried out. In our study we will compare acetonitrile and 2-propanol as eluents for reversed-phase chromatography (RPC) of these hydrophobic peptides.

The parameter having a major effect on the retention time under given mobile-phase conditions in RPC is the *n*-alkyl chain ligand density. In addition, it has also been reported that *n*-alkyl chain length can affect the retention time of components in RPC[6–11]. Though a chain length effect on the retention time of hydrophopic peptides has not been documented, it seemed appropriate to choose a support with short *n*-alkyl chain length and low ligand density for the separation of hydrophobic peptides in our study. It has been shown that for small peptides (8–36 residues) pore diameter had little effect on resolution[12] but for peptides (30–150 residues) the 300-Å pore matrices gave better resolution and recovery[2,3,13,16]. In addition, a particle size of 5 μm provides increased column efficiency (sharper peaks and increased resolution) compared to 10-μm matrices[17]. For these reasons, we have used a C_3 column with 300-Å pore size, 5-μm particle size and a carbon loading of 2.9%, which is low relative to most reversed-phase columns.

Due to the solubility properties of these hydrophobic peptides we have used 50% acetonitrile in 0.1% aq. TFA as the solvent system for size-exclusion chromatography. Regnier[2] evaluated many of the commercial size-exclusion columns and reported that the TSK G3000SW column had the greatest overall utility. We have evaluated both TSK G2000W and TSK G3000SW for their ability to resolve these small hydrophobic peptides. It has been reported previously that these columns can separate small peptides in the molecular weight range 900–8000 daltons, differing by only 740 daltons[12,18].

EXPERIMENTAL

Materials and methods

Unless otherwise stated, all chemicals and solvents were reagent grade: urea (Ultrapure, Canadian Scientific Products, London, Canada); TFA (Halocarbon Products, Hackensack, NJ, U.S.A.); acetonitrile (HPLC-grade, Fisher Scientific, Fairlawn, NJ, U.S.A.); 2-propanol (HPLC-grade, BDH, Toronto, Canada). Double distilled water was purified by passing it through a Milli-Q Water Purification System (Millipore Corporation, Bedford, MA, U.S.A.).

Routine amino acid analyses were performed on a Durrum D-500 amino acid analyser. Peptides were quantitated by amino acid analysis after hydrolysis with 6 *M* hydrochloric acid, containing 0.1% phenol, in evacuated, sealed tubes for 24–120 h at 110°C, by using the mean of the molar ratios of all accurately measurable amino

acids in the acid hydrolysate to calculate the concentration. It was shown that 5 days' hydrolysis times were required for the leucine polymers to ensure reproducibility of the amino acid analysis.

The percentage of organic solvent required for elution of the peptides was calculated as follows: The gradient hold-up volume was determined by using 10% eq. acetone in solvent B of an AB gradient. The system was equilibrated with A and the time from the start of the gradient to the observed off-scale change in absorbance at 270 nm was used to determine the hold-up volume. The solvent percentage at elution was calculated by subtracting this time from the peak elution time, then multiplying by the percentage B/min used in the linear gradient.

Peptide synthesis and purification

The synthetic peptide analogues (TM-15 and TM-36) of the sequence Ac-(Lys-Leu-Glu-Ala-Leu-Glu-Gly)$_n$-Lys-amide where n = 2 or 5 were synthesized and purified as described by Lau *et al.*[12]. The synthetic leucine polymers of 13, 22, 26 and 30 residues [Ac-(Leu)$_{10}$-(Lys)$_2$-Ala-amide; (Lys)$_2$-Gly-(Leu)$_n$-(Lys)$_2$-Ala-amide, where n = 16, 20 and 24] were synthesized as described by Davis *et al.*[1]. Standard solid-phase methodology was used to synthesize these peptides.

The crude leucine polymers (13, 22 and 26 residues) were purified by HPLC on an Altex Ultrapore RPSC C_3 reversed-phase column, 75 mm × 4.6 mm I.D., from Beckman (Palo Alto, CA, U.S.A.). The linear gradient for elution was established with solvents A and B. Solvent A consisted of 0.1% aq. TFA and solvent B consisted of 0.1% TFA in 2-propanol. The crude 13 residue peptide was dissolved in 80% A and 20% B, where solvent A contained 8 *M* urea. The sample was centrifuged at 12,800 *g* in an Eppendorf microcentrifuge (Model 5412) and aliquots of the supernatant were used for RPC. For purification of the 13 residue peptide the starting solvent of the gradient was 80% A and 20% B; the final concentration was 50% of each solvent. The gradient (1% B/min) was terminated after 30 min. The crude 22 and 26 residue peptides were dissolved in 70% A and 30% B where solvent A contained 8 *M* urea. The starting solvent of the gradient for purification of the 22 and 26 residue peptides was 70% A with 30% B; the final concentration was 50% of each solvent. The gradient (1% B/min) was terminated after 20 min. The flow-rates used were 1 ml/min, and the absorbance of the effluent was monitored at 210 nm. The 30 residue peptide could not be eluted from the reversed-phase column. The HPLC instrumentation consisted of a Spectra-Physics SP8700 solvent delivery system and SP8750 organizer module, combined with a Hewlett-Packard (Avondale, PA, U.S.A.) HP1040A HPLC detection system, HP3390A integrator, HP85 computer, HP9121 disc drive, and HP7470A plotter.

RESULTS AND DISCUSSION

Reversed-phase chromatography

Previously, we showed that five peptides 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, could be easily resolved on the C_3 column used in this study. In fact, a linear relationship between the natural logarithm of molecular weight and retention volume was observed[12]. The 36 residue peptide was reasonably hydrophobic, being eluted at an

TABLE I

AMINO ACID SEQUENCE AND MOLECULAR WEIGHT OF PEPTIDES SEPARATED BY REVERSED-PHASE AND SIZE-EXCLUSION HPLC

Peptide	*MW**	*Amino acid sequence*
TM-15	1667	Ac-(Lys-Leu-Glu-Ala-Leu-Glu-Gly)$_2$-Lys-amide
TM-36	3887	Ac-(Lys-Leu-Glu-Ala-Leu-Glu-Gly)$_5$-Lys-amide
13	1516	Ac-(Leu)$_{10}$-Lys-Lys-Ala-amide
22	2465	Lys-Lys-Gly-(Leu)$_{16}$-Lys-Lys-Ala-amide
26	2917	Lys-Lys-Gly-(Leu)$_{20}$-Lys-Lys-Ala-amide
30	3369	Lys-Lys-Gly-(Leu)$_{24}$-Lys-Lys-Ala-amide

* Monomeric molecular weight.

approximate acetonitrile concentration of 37%[12]. This peptide was included in a mixture along with the three leucine polymers of 13, 22 and 26 residues (Table I). Fig. 1 shows the separation of these peptides with an AC gradient which increased linearly at 2% C/min (C = 0.1% TFA in acetonitrile) from the starting solvent of 70% A and 30% C. The 26 residue leucine polymer was not eluted until approximately 73% acetonitrile. By comparison, Fig. 2 shows the separation of this mixture with an AB gradient which increased linearly at 1% B/min (B = 0.1% TFA in 2-propanol) from the starting composition of 75% A and 25% B. The 26 residue leucine polymer was eluted at a 2-propanol concentration of approximtely 40%. These results were in agreement with 2-propanol being a more effective solvent than acetonitrile in eluting peptides and proteins[3,19,20]. Hermodson and Mahoney[3] reported that the best resolution on RPC was usually obtained between 15% and 40% of the organic solvent in the gradient. This would suggest the use of 2-propanol rather than acetonitrile for the separation of hydrophobic peptides.

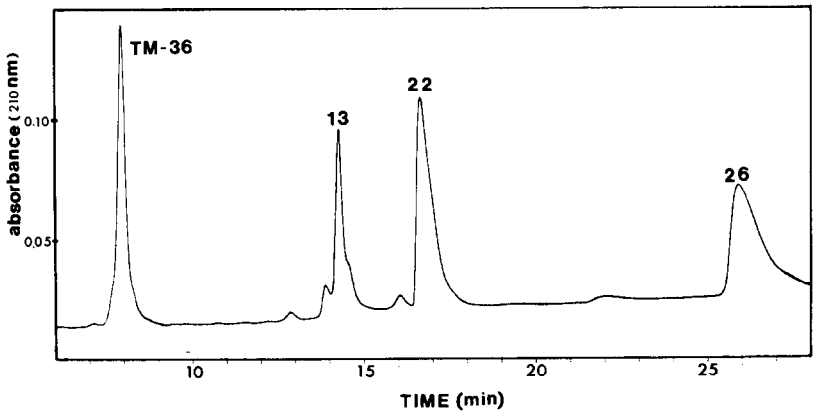

Fig. 1. Reversed-phase HPLC separation of four hydrophobic peptides with acetonitrile. TM-36 and the leucine polymers of 13, 22 and 26 residues contain 10, 10, 16 and 20 leucine residues, respectively. The sequence of each peptide is shown in Table I. An AC gradient was used which increased linearly at 2% C/min. Flow-rate 1 ml/min. Solvent A consisted of 0.1% aq.TFA and solvent C of 0.1% TFA in acetonitrile. The starting solvent was 70% A and 30% C. Column: Altex Ultrapore RPSC 75 × 4.6 mm I.D., *n*-alkyl chain length C_3, 5 μm particle size, 300-Å pore size, carbon loading 2.9%. The sample was dissolved in the starting solvent, where solvent A contained 8 *M* urea.

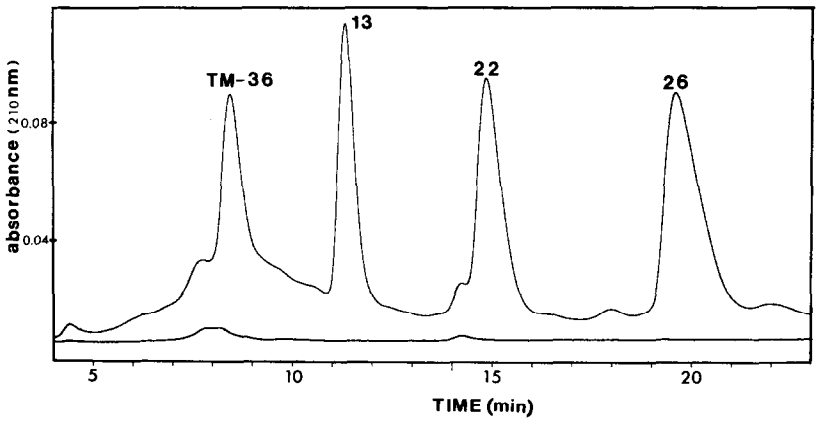

Fig. 2. Reversed-phase HPLC separation of four hydrophobic peptides with 2-propanol. TM-36 and the leucine polymers of 13, 22 and 26 residues contain 10, 10, 16 and 20 leucine residues, respectively. The sequence of each peptide is shown in Table I. An AB gradient was used which increased linearly at 1% B/min. Flow-rate 1 ml/min. Solvent A consisted of 0.1% aq. TFA and solvent B of 0.1% TFA in 2-propanol. The starting solvent was 75% A and 25% B. Column: Altex Ultrapore RPSC, 75 × 4.6 mm I.D., *n*-alkyl chain length C_3, 5 μm particle size, 300-Å pore size, carbon loading 2.9%. The sample was dissolved in the starting solvent, where solvent A contained 8 *M* urea.

Gerber *et al.*[21] and *Takagaki et al.*[22] have successfully separated hydrophobic peptides by RPC with a mobile phase consisting of formic acid, water and ethanol (AB gradient; 5% formic acid in water as solvent A and 5% formic acid in ethanol as solvent B). The samples were injected in 88% formic acid. This avoided problems of fragment solubility, provided the concentration of ethanol was sufficient at the beginning of the program (40%)[21]. The difficulty with this system is that peptide detection by absorbance is only possible at insensitive wavelengths (280 nm) due to the presence of formic acid. The detection of many peptides that do not contain any aromatic amino acids is impossible, as is the case of the hydrophobic peptides used in this study. To avoid these problems, we have used 0.1% TFA in both the aqueous and organic solvent to aid in peptide solubility. 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[2,3,12,19,23–25]. For many peptide mixtures, adequate concentrations for loading can be obtained by dissolution in the starting solvent itself. Otherwise, 6 *M* guanidine hydrochloride or 8 *M* urea may be used to facilitate sample dissolution in order to achieve reasonably high concentrations of sparingly soluble peptides[3]. In our experiments, we have routinely used 8 *M* urea–0.1% aq. TFA and the same percentage of the organic solvent as used in the starting solvent to dissolve the peptide sample. The urea always was eluted in the breakthrough peak of the column.

Monch and Dehnen[26] reported the use of 5% 2-methoxyethanol in both the aqueous (A = 0.05 *M* KH_2PO_4–2-methoxyethanol, 95:5, pH 2.0) and organic phase (B = 2-propanol–2-methoxyethanol, 95:5, adjusted to pH 2.0 with phosphoric acid) of an AB gradient. They suggested that the 2-methoxyethanol behaves like a surface-active agent, which, being adsorbed on the reversed-phase support, causes the proteins to be less strongly adsorbed and more readily eluted. We added 10% 2-

methoxyethanol to each solvent of our AC gradient (A = 0.1% aq. TFA–2-methoxyethanol, 90:10; C = 0.1% TFA in acetonitrile–2-methoxyethanol, 90:10). This solvent system had little effect on changing the retention time of the 22 residue leucine polymer. A small increase in retention time for this peptide was observed when this gradient system was compared with the same gradient without 2-methoxyethanol. These surface-active agents may have little effect on short-chain *n*-alkyl columns since they are unable to bind to the chains of the matrix and change its hydrophobicity. These effects may be restricted to matrices of longer chain lengths, like the octadecyl matrices.

Hearn *et al.*[27] have observed that polypeptides are adsorbed on alkylsilane columns at extremes of solvent polarity (aqueous buffer or high concentrations of organic solvent), while elution was achieved by intermediate concentrations of organic solvent. Tandy *et al.*[28] have used RPC to purify a very hydrophobic protein in organic solvents only. Although our peptides are completely soluble in 0.1% TFA in methanol or 0.1% TFA in acetonitrile, the above organic systems were inappropriate. For example, the 22 residue leucine polymer was eluted from the column in acetonitrile containing 0.1% TFA.

Power *et al.*[29] used a 0.05% TEA–TFA buffer system (triethylamine–trifluoroacetic acid) in both the aqueous and organic solvent and an AB gradient, where A consisted of 5% acetonitrile in 0.05% TEA–TFA and B was 0.05% TEA–TFA acetonitrile–1-propanol (1:1), to separate hydrophobic peptides. This solvent system was more effective in resolving the mixture than either acetonitrile or 1-propanol alone as the organic solvent. These results agree with our study in the use of acetonitrile and 2-propanol as organic solvents for separation of hydrophobic peptides on RPC.

Retention times will vary significantly with the age of the column. We have since observed that these peptides were retained much more tenaceously on a new column from the same manufacturer. These results would suggest that a reversed-phase column with an even lower ligand density would be more appropriate for hydrophobic peptides.

Size-exclusion chromatography

In size-exclusion chromatography ideal separations occur only when there are no ionic or hydrophobic interactions between the macromolecules and the matrix. 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 methods[2]. 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 0.1–0.2 *M* to overcome or minimize electrostatic effects[2]. Hydrophobic effects have been observed to result in deviations from the separation obtained by a pure size-exclusion process and ionic strengths above 0.6 *M* are not recommended[2]. It is known that hydrophobic interactions increase with increasing ionic strength of the medium[30].

Size-exclusion chromatography is used for two purposes, peptide–protein separation and/or molecular weight determinations. Of course, the non-ideal properties of size-exclusion columns can be advantageous in the separation of peptides and proteins. Rivier[31] included 15–30% acetonitrile in an aq. triethylammonium phos-

phate buffer as an organic modifier to decrease non-specific adsorption and increase overall solubility of the proteins. Their results showed a linear relationship between ln molecular weight and retention time in the molecular weight range 1000–44,000 daltons. Lau *et al.*[12] have used a TSK G3000SW column to resolve small peptides in the molecular weight range 900–4000 daltons with 0.1% aq. TFA–acetonitrile (1:1). Similarly, a linear relationship between ln molecular weight and retention time was obtained. This eluent is completely volatile, allowing sample recovery and UV detection at low wavelengths (210 nm). Rivier[31] commented that 0.1% aq. TFA–acetonitrile was inappropriate for size-exclusion chromatography of their peptide mixture. It should be noted that non-specific interactions may be more serious with one size-exclusion column than with another. With TSK G2000SW and TSK G3000SW columns used by Lau *et al.*[12,18] linear relationships were obtained between ln molecular weight and retention time for peptides in the range of 900–18,000 daltons in low ionic strength solvents with and without organic solvent [0.1% aq. TFA and 0.1% aq. TFA–acetonitrile or trifluoroethanol (1:1)] and high ionic strength solvents

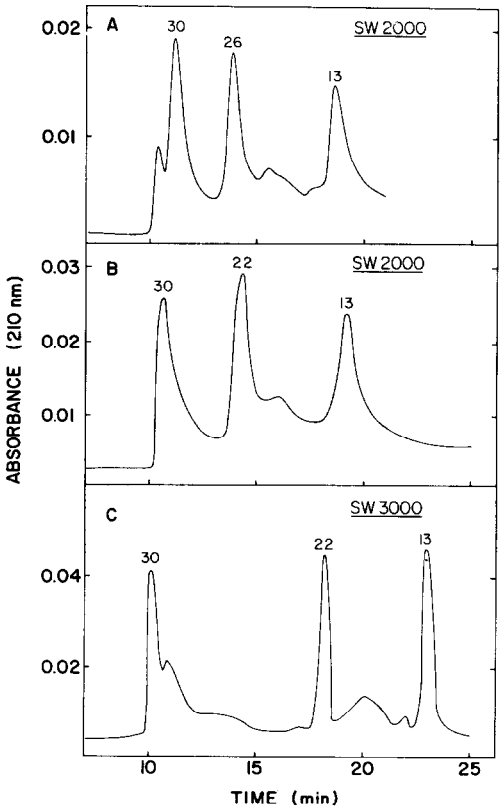

Fig. 3. Comparison of size-exclusion HPLC elution profiles of mixtures of hydrophobic peptides. The 13, 22, 26 and 30 residue leucine polymers of the sequence Ac-$(Leu)_{10}$-$(Lys)_2$-Ala-amide and $(Lys)_2$-Gly-$(Leu)_n$-$(Lys)_2$-Ala-amide, where n = 16, 20 and 24, were separated on TSK G2000SW (A and B) and TSK G3000SW columns (C), 600 × 7.5 mm I.D., with 60 × 7.5 mm I.D. guard columns (Toya Soda, Tokyo, Japan). The eluent was 0.1% aq. TFA and 0.1% TFA in acetonitrile (1:1). Flow-rate 1 ml/min.

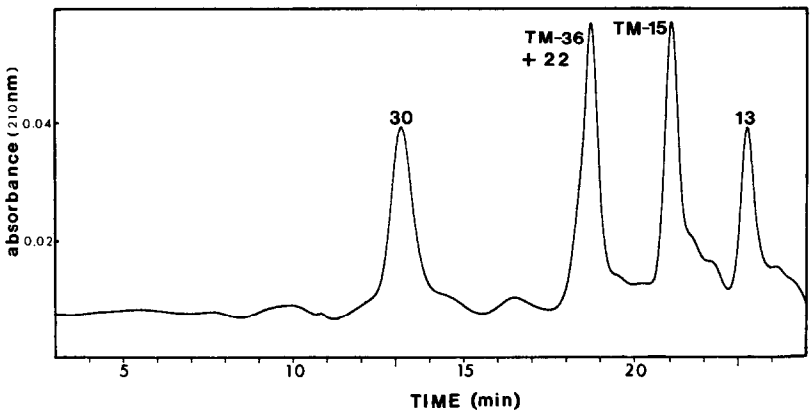

Fig. 4. Size-exclusion HPLC separation of three hydrophobic peptides of 13, 22 and 30 residues. Two peptide standards (TM-15 and TM-36) are included in the mixture. The sequence and molecular weight of each peptide is shown in Table I. Column: TSK G3000SW, 600 × 7.5 mm I.D., with guard column, 60 × 7.5 mm I.D., from Toya Soda (Tokyo, Japan). Flow-rate was 1 ml/min. The eluent was 0.1% aq. TFA and 0.1% TFA in acetonitrile (1:1).

with and without denaturant (1.1 *M* potassium chloride–0.05 *M* phosphate buffer, pH 7.0, in the presence and absence of 8 *M* urea). These results would suggest that the non-specific interactions with the support were minimal with the peptides tested.

Because the four leucine polymers used in this study are not water-soluble, 0.1% aq. TFA–acetonitrile (1:1) was chosen as the eluent for size-exclusion chromatography. Fig. 3 shows the separation of various mixtures of the hydrophobic peptides (Table I) on the TSK G2000SW and TSK G3000SW size-exclusion columns. The TSK G3000SW column provides better resolution and sharper peaks than the TSK G2000SW column for this peptide mixture. The results of Lau *et al.*[12] indicated that the 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 were monomeric and α-helical in 0.1% aq. TFA–acetonitrile (1:1), and gave a linear relationship between ln molecular weight and retention volume. We have shown by circular dichroism studies that all four of the leucine polymers (13, 22, 26 and 30 residues) are α-helical in this solvent system, in agreement with previous studies on the 30 residue peptide[1]. The synthetic two-stranded α-helical coiled-coils of TM-29 and TM-36, which are extremely stable to temperature and urea denaturation[18], are dissociated in 50% acetonitrile to their monomeric form. Though acetonitrile disrupts hydrophobic interactions, it stabilizes the α-helical conformation and behaves as a α-helix-inducing solvent[12]. In light of this evidence, the four leucine polymers are most likely monomeric in this solvent.

TM-15 and TM-36 were mixed with the three leucine polymers of 13, 22, and 30 residues to compare retention times on the TSK G3000SW column (Fig. 4). It is obvious that the leucine polymers were eluted in an anomalous manner. The 30 residue peptide (MW = 3369) was eluted considerably faster than TM-36 (MW = 3887). Similarly, TM-36 (MW = 3887) and the 22 residue leucine polymer (MW = 2465) was eluted in the identical position. TM-15 (MW = 1667) and the 13-residue leucine polymer (MW = 1516), though similar in molecular weight, were well re-

solved. These results can be explained by the non-ideal behavior of these extremely hydrophobic peptides during size-exclusion chromatography. The non-ideal behavior is unlikely to be due to electrostatic effects, since TM-36 and the three leucine polymers of 30, 26 and 22 residues all contain a similar number of positive charges at pH 2.0 (Table I). In addition, the low ionic strength and hydrophobicity of the eluent [0.1% aq. TFA–acetonitrile (1:1)] would tend to decrease the hydrophobic interaction with the support. Thus, the non-ideal behavior is most likely due to solvophobic effects.

CONCLUSIONS

We have demonstrated that extremely hydrophobic peptides (leucine polymers) of 13, 22 and 26 residues can be resolved by RPC on a C_3 column with a water–2-propanol mobile phase containing 0.1% TFA. The 30 residue leucine polymer could not be eluted from the reversed-phase column. These same peptides showed non-ideal behavior in size-exclusion chromatography with 0.1% aq. TFA–acetonitrile (1:1). However, excellent resolution was obtained on the TSK G3000SW column. Retention time values[32] greater than 3.9 were obtained for these peptides.

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