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Polymer-coated reversed-phase packings with controlled hydrophobic properties

II. Effect on the selectivity of peptide separations

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ABSTRACT

We have designed and synthesized novel reversed-phase packings of non-porous and porous polymethacrylate-coated silicas. By varying the hydrophobicity of the polymer coating, selective unfolding of polypeptides may be achieved, thus enabling manipulation of the chromatographic profile. This study characterizes these packings through their employment for separations of model synthetic peptides of defined secondary, tertiary and quaternary structure. Thus, the packings were applied to the reversed-phase separation of α-helical amphipathic and non-amphipathic peptides of the same amino acid composition but different sequences. In addition, selective unfolding of model two-stranded α-helical coiled-coil peptides was achieved with these packings. Through the observation of the chromatographic behaviour of these model peptides on the various polymethacrylate-coated silicas, we were able to confirm the potential of such tailored packings for separations of peptides and proteins.

INTRODUCTION

We have previously evaluated [1] the potential of a novel concept for protein separations by reversed-phase high-performance liquid chromatography (RP-HPLC), where selective unfolding of proteins is achieved by varying the hydrophobicity of the polymer coating on non-porous and porous polymethacrylate-coated silicas. This approach involves manipulation of the chromatographic pattern of a protein mixture containing proteins of different labilities. In addition, maintenance of a multi-protein complex, stabilized by non-covalent interactions, is also possible on such stationary phases.

The present study serves to extend the characterization of these polymer-coated packings through their employment for separations of model synthetic peptides. By observing the chromatographic behaviour of these peptides of defined secondary, tertiary and quaternary structure, we sought to gain further insight into the potential benefits of such packings for polypeptide separations by RP-HPLC.

EXPERIMENTAL

Materials

HPLC-grade water and acetonitrile were obtained from J. T. Baker (Philipsburg, NJ, USA) and HPLC-grade trifluoroacetic acid (TFA) from Pierce (Rockford, IL, USA). Dithiothreitol (DTT) was purchased from Schwarz-Mann Biotech (Cleveland, OH, USA). Potassium chloride and potassium dihydrogenorthophosphate were purchased from BDH (Toronto, Canada).

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Peptides

Peptides were synthesized (structures shown in Table I) on an Applied Biosystems (Foster City, CA, USA) peptide synthesizer, using the general procedure for solid-phase peptide synthesis described by Hodges and co-workers [2,3].

Apparatus

The HPLC instrument consisted of an HP1090 liquid chromatograph (Hewlett-Packard, Avondale, PA, USA) coupled to an HP1040A detection system, HP9000 Series 300 computer, HP9133 disc drive, HP2225A Thinkjet printer and HP7440A plotter.

Reversed-phase packings

Monospher (non-porous silica; 1.7-μm mean particle diamter) and LiChrospher (porous silica: 10 μm; 300-Å pore size) supports from E. Merck (Darmstadt, Germany) were coated with poly-2-hydroxyethylmethacrylate (P2HEMA), polyethylmethacrylate (PEMA) or octadecylmethacrylate-methylmethacrylate copolymer (POMA) as described in ref. 1. The packings were then packed into stainless-steel columns (36 × 4.6 mm I.D.; Bischoff, Leonberg, Germany) as decribed previously [1].

The relative hydrophobicities of these packings are P2HEMA < PEMA < POMA.

RESULTS AND DISCUSSION

Synthetic model peptides

The sequences of two series (G and L series) of synthetic model peptide polymers of 21, 28 and 35 residues employed in this study are shown in Table I. These two series of peptides have the same amino acid composition, but different sequences. Both series of peptides consist of an initial seven-residue sequence containing a cysteine residue [Lys-Cys-Ala-Glu-Gly-Glu-Leu (G series) or Lys-Cys-Ala-Glu-Leu-Glu-Gly (L series)], followed by a repeating seven residue (heptapeptide) repeat: [Lys-Leu-Glu-Ala-Gly-Glu-Leu] for the G series; [Lys-Leu-Glu-Ala-Leu-Glu-Gly] for the L series. All of the synthetic peptides in the present study were acetylated at the N-terminal and amidated at the C-terminal to rule out possible repulsive ionic interactions due to the presence of these end groups. Circular dichroism studies have demonstrated that both sets of peptides have a high potential to form α-helical structure in a non-polar environment, such as the hydrophobic stationary phase of a reversed-phase packing and the organic modifier employed in the mobile phase [4,5]. In Fig. 1, the amino acid sequences of the G and L series peptides are viewed as axial projections of α-helices in an helical wheel. The hydrophobic amino acid residues, *e.g.*, leucine residues, are segregated on one side of the α-helix in L series peptides to form amphipathic helices con-

TABLE I

SYNTHETIC PEPTIDES EMPLOYED IN THIS STUDY

Peptide[a]	Peptide sequence[b]
G21	Ac–Lys–Cys–Ala–Glu|Gly|Glu|Leu|[Lys–Leu|Glu|Ala|Gly|Glu|Leu|]$_3$–amide
L21	Ac–Lys–Cys–Ala–Glu|Leu|Glu|Gly|[Lys–Leu|Glu|Ala|Leu|Glu|Gly|]$_2$–amide
G28	Ac–Lys–Cys–Ala–Glu|Gly|Glu|Leu|[Lys–Leu|Glu|Ala|Gly|Glu|Leu|]$_3$–amide
L28	Ac–Lys–Cys–Ala–Glu|Leu|Glu|Gly|[Lys–Leu|Glu|Ala|Leu|Glu|Gly|]$_3$–amide
G35	Ac–Lys–Cys–Ala–Glu|Gly|Glu|Leu|[Lys–Leu–Glu–Ala|Gly|Glu|Leu|]$_4$ amide
L35	Ac–Lys–Cys–Ala–Glu|Leu|Glu|Gly|[Lys–Leu–Glu–Ala|Leu|Glu|Gly|]$_4$–amide

[a] The "G" and "L" series of peptide polymers represent series of, respectively, non-amphipathic and amphipathic α-helical peptides. For each peptide analogue, the letter represents the series to which it belongs and the number denotes the number of amino acid residues it contains.

[b] Ac = N^{α}-Acetyl; amide = C^{α}-amide. The residues (Gly and Leu) shown between the vertical lines are responsible for the sequence variation of peptides with the same amino acids composition.

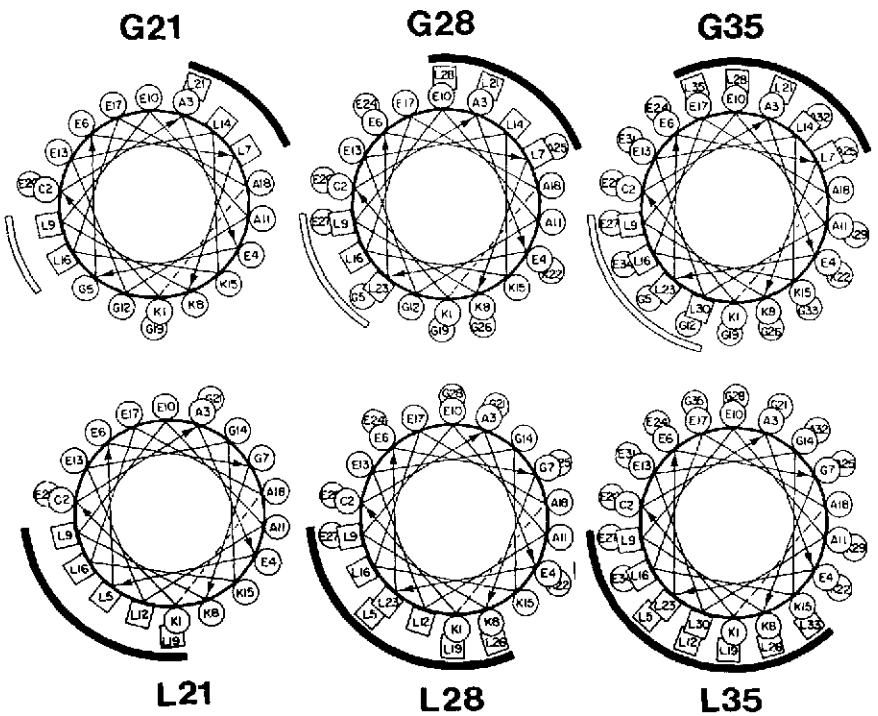

Fig. 1. The amino acid sequence for the G and L series peptides (Table I), represented as helical wheels. The perimeter of a wheel corresponds to the polypeptide backbone and the external circles and squares represent the individual amino acid side chains. The leucine residues are denoted by squares and all other side chains by circles. Since the α-helix has 3.6 residues per turn, adjacent side chains in the sequence are separated by 100° of arc on the wheel. Two hydrophobic surfaces are observed on opposing sides of the helix for the peptides in the G series as indicated by the solid and open bars. The peptides of the L series are amphipathic, containing one dominant hydrophobic surface on one side of the α-helix as indicated by the solid bars. The G and L series peptides are denoted by the letters G and L, respectively, followed by a number which denotes the number of residues in the polypeptide chain.

taining a preferred binding domain [4,5]. In contrast the hydrophobic residues of the G series peptides are more evenly distributed on opposite sides of the α-helix to form non-amphipathic helices.

The sequence about the cysteine residue of the L series peptides (Table I) is identical to that found in tropomyosin (Lys–Cys–Ala–Glu–Leu–Glu), the best understood example of a two-stranded α-helical coiled coil [6–9]. The amino acid sequence of the heptapeptide repeat (Lys–Leu–Glu–Ala–Leu–Glu–Gly) used on the remaining heptads of the L series peptides (Table I) was chosen based on the criteria described by Hodges *et al.* [10]. These criteria were based on the observation that tropomyosin and other two-stranded α-helical coiled-coil proteins were stabilized by hydrophobic residues at positions 2 and 5 of a repeating heptad sequence [(X–N–X–X–N–X–X)$_n$ where N is a non-polar residue]. In the present study, positions 2 and 5 of the repeating heptad of the L series peptides are occupied by Leu residues. Indeed, polymers of a similar heptad sequence, [Lys–Leu–Glu–Ser–Leu–Glu–Ser]$_n$ [10,11] verified the hypothesis of Hodges *et al.* [6] that positions 2 and 5 were responsible for the hydrophobic interactions stabilizing the two-stranded α-helical coiled coils.

The synthetic L-series peptides were deemed to represent an ideal model system for probing the chromatographic properties of the polymer-coated supports described in this study. A straightforward shifting of the residues in the heptad sequences of the amphipathic L series peptides produced the non-amphipathic G series peptides (Table I), enabling a comparison of the ability of the various packing to separate amphipathic and non-amphipathic peptides of the same composition but differ-

ent sequence. The presence of the cysteine residue at position 2 of the first heptad of the L series peptides allows a determination of the effect of varying packing hydrophobicity on the stability of coiled-coil peptides both in the absence and presence of a covalent disulphide bond linking the two polypeptide chains. Finally, the 35-residue L series peptide is small enough for easy synthesis of peptide analogues (and large enough to form a stable three-dimensional structure capable of tolerating sequence changes) [3], allowing an examination of the effect of varying hydrophobicity of the column packing on the stability of peptide analogues, where the one or more leucine residues in the critical 2 and 5 positions of the heptads (Table I) has been substituted with a less hydrophobic residue.

Separation of amphipathic and non-amphipathic peptides of the same amino acid composition but different sequence

Fig. 2 shows the elution profiles of respective pairs of the G and L series peptides on the three chromatographic packings. From a previous study by Hodges and co-workers [4,5], it would be expected that, if the peptides of both series were bound to the reversed-phase packings in their monomeric form, then the amphipathic L series peptides (containing a preferred binding domain) would be eluted later than their respective G series analogues. For the majority of the elution profiles shown in Fig. 2, this is indeed the case, with the POMA packing, particularly, exhibiting good selectivity in separating the monomeric peptide pairs in run times of less than 4 min. However, there is a very interesting selectivity shift for the 35-residue analogues on the P2HEMA packing, the most hydrophilic of the three employed. The elution of peptide L35, which is known to be a coiled-coil dimer in 0.1% aq. TFA [12,13], prior to G35 suggests that the hydrophobic domains of the amphipathic L35 peptide are not being exposed to the P2HEMA packing, *i.e.*, L35 is being maintained as a dimer on this column, even in the absence of a disulphide bond between the L35 monomers. Although Ingraham *et al.* [14] demonstrated that a similar 36-residue peptide was eluted primarily in a dimeric form from an hydrophobic

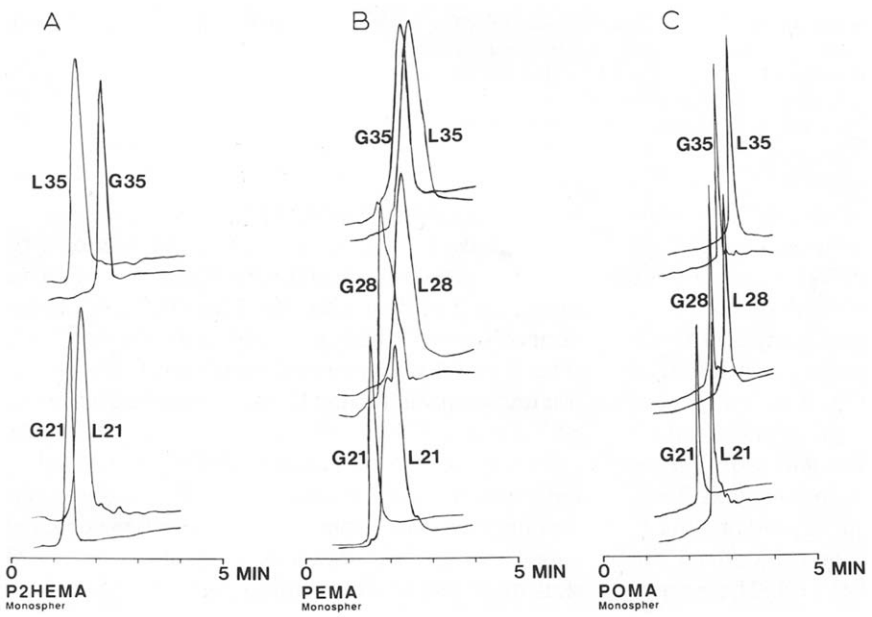

Fig. 2. Reversed-phase chromatography of the G and L series peptides (Table I). Pairs of peptides of the same composition and polypeptide chain length but different sequences were chromatographed on P2HEMA (A), PEMA (B) and POMA (C) packings. Conditions: linear A–B gradient elution (20% acetonitrile/min) at a flow-rate of 1 ml/min, where eluent A is 0.05% aqueous TFA and eluent B is 0.05% TFA in acetonitrile; temperature, 26°C. The peptides were dissolved in 0.05% aq. TFA containing 10 m*M* DTT prior to application to the columns to ensure that the peptides remained in their reduced form.

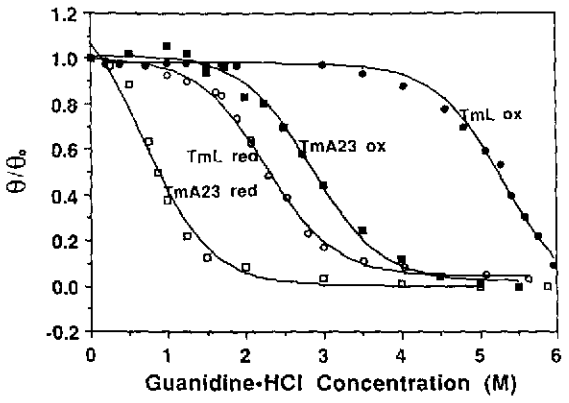

Fig. 3. Guanidine hydrochloride denaturation profiles of synthetic peptide analogues in 0.1 *M* KCl, 0.05 *M* orthophosphate buffer, pH 7. θ/θ_0 represents the ratio of the ellipticity at 220 nm at the indicated molarity of guanidine hydrochloride to the ellipticity without guanidine hydrochloride. TmL Red and TmL Ox refer to the reduced and oxidized forms of peptide L35 (Table I), respectively; TmA23 Red and TmA23 Ox refer to the reduced and oxidized forms of peptide TmA23, respectively, where the leucine at position 23 of TmL has been substituted by alanine.

interaction chromatography column operated in reversed-phase mode, the maintenance of this dimeric state required temperatures as low as 0°C. Even under these conditions, some breakdown of quaternary structure was evident. In contrast, no disruption of the quaternary structure of the 35-residue L series peptide was evident on the P2HEMA packing, even at room temperature. The elution of peptide L21 after its non-amphipathic analogue, G21, could be expected, since the amphipathic L21 is already monomeric in the starting solvent [12,13].

Selective unfolding of model two-stranded α-helical coiled-coil peptides by polymethacrylate-coated packings

The 35-residue L series peptide (now termed TmL) was chosen as the model polypeptide for further characterization of the polymer-coated packings. The effect of the packings on the chromatographic behaviour of the reduced (TmL Red) and oxidized, *i.e.*, disulphide-bridged (TmL Ox) forms of TmL was determined. In addition, an analogue of TmL, denoted TmA23, where the leucine residue at position 23 was substituted by an alanine residue, was also employed. In order to rationalize subsequent peptide chromatographic behaviour, it was necessary to establish, by non-chromatographic means, the relative stabilities of the dimeric forms of the reduced and oxidized forms of the TmL and TmA23 polypeptides.

Stability of coiled-coil dimers of TmL and TmA23. Fig. 3 shows the guanidine hydrochloride denaturation profiles of the oxidized and reduced forms of TmL and TmA23. In non-denaturing (benign) medium, both the reduced and oxidized forms of the two peptides are two-stranded α-helical coiled-coils. However, the effect of increasing concentrations of the denaturating guanidine hydrochloride on the dimers differs dramatically depending upon the stability of the α-helical coiled coils. Clearly, the oxidized, disulphide bridged forms of the TmL and TmA23 are more stable than their respective reduced forms, as evidenced by the higher concentrations of guanidine hydrochloride required to produce the same drop in ellipticity ratio for the oxidized coiled-coils compared to their reduced forms. The marked positive impact of a disulphide bridge to coiled-coil stability has been previously demonstrated by Hodges and co-workers [3,15]. Also clear is the decrease in coiled-coil stability resulting from the leucine to alanine substitution at position 23. Leucine 23 is at one of the key hydrophobic positions which serve to stabilize the coiled-coil structure (Table I), and similar destabilization effects have been reported by Hodges and co-workers [3,15–18], when leucine residues in the hydrophobic positions of the heptad repeats have been replaced by residues with less hydrophobic side-chains.

These results can be better understood by observing the computer-generated three dimensional structures of the coiled-coils (Fig. 4). The left structure of Fig. 4 shows the coiled-coil dimer of TmL. The hydrophobic (leucine) residues of the 3–4 repeat responsible for the formation and stabilization of the two-stranded α-helical coiled-coil are buried between the two polypeptide chains. Oxidation of the cysteine residues at position 2 of each 35-residue chain (TmL Ox), forms a 70-residue disulphide-linked polypeptide and helps to stabilize the coiled-coil structure. In the middle structure of Fig. 4, all side-chains have been removed from the TmL dimer except the leucine side-chains in the 3–4 repeat positions. It can be seen that the leucine side-chains intercalate to form a continuous hydrophobic surface along the inside of the coiled-coil. By compari-

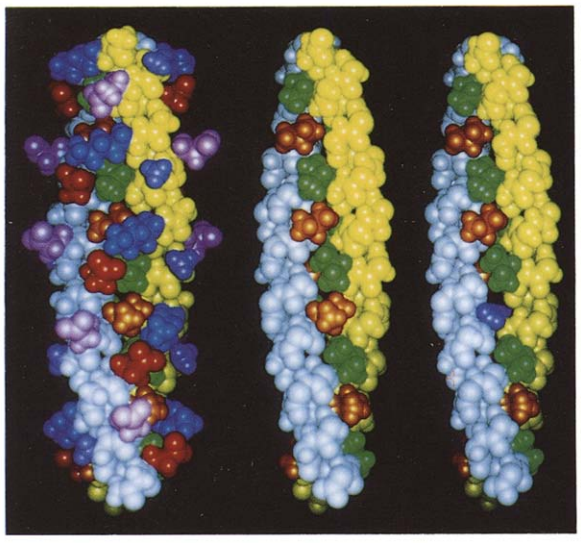

Fig. 4. Space-filling representations of two-stranded α-helical coiled-coils. Left: coiled-coil of TmL. Middle: coiled-coil of TmL with all side-chains removed except the leucine residues in the hydrophobic 3–4 repeat positions of both chains (positions 5, 9, 12, 16, 19, 23, 26, 30 and 33, starting from the top of the polypeptide chains). Right: coiled-coil of TmA23 with all side-chains removed except the leucine residues in the hydrophobic 3–4 repeat positions of both chains (see above); position 23 is now occupied by alanine. The main chain atoms of one α-helix are white and the other yellow. The side-chains are coloured as follows: left and middle coiled-coils of TmL, leucine residues are brown for the white polypeptide chain and green for the yellow chain; alanine residues are purple; glutamic acid residues are red or pink; lysine residues are blue; glycine residues are white; right coiled-coil of TmA23, as for TmL, except that alanine side-chains are now blue. This figure was derived by utilizing molecular dynamics and energy minimization with torsion angles (Φ, ψ) and inter-chain leucine–leucine residue backbone constraints.

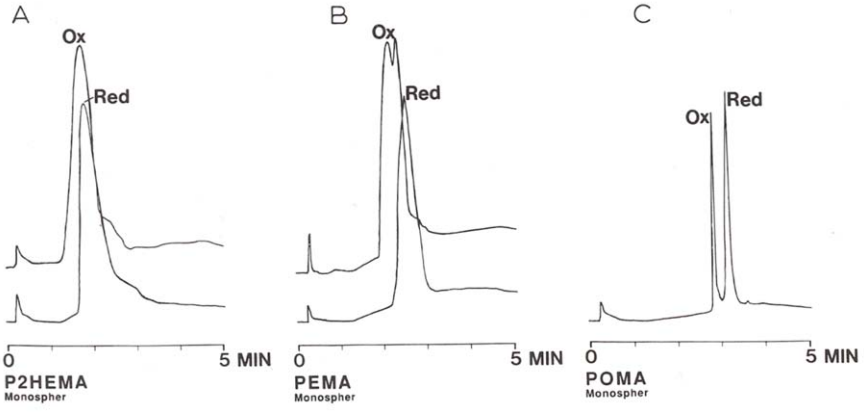

Fig. 5 Reversed-phase chromatography of synthetic model peptide TmL (L35 in Table I). The elution profiles shown are of the oxidized (Ox) and reduced (Red) forms of the peptide on P2HEMA (A), PEMA (B) and POMA (C) packings. Conditions as in Fig. 2. The reduced form of the peptide was obtained by dissolving it in 0.05% aq. TFA containing 10 m*M* DTT prior to application.

sion (Fig. 4, right structure), two leucine residues are replaced by two alanine residues in the centre of the coiled-coil of TmA23. There is now a hole through the centre of the coiled-coil above and below the two interacting alanine residues. Thus, not only are the hydrophobic interactions of lower magnitude in the TmA23 dimer, but water, which is excluded from interaction with hydrogen bonds of the α-helix near the hydrophobic core of TmL, is more accessible to those hydrogen bonds on TmA23. The net result is that both the oxidized and reduced TmA23 coiled-coils are less stable than their TmL counterparts, as has been shown by extensive characterization of the stability of these model peptides, determined by monitoring (by circular dichroism) the ellipticities of the peptides as a function of guanidine hydrochloride concentration [16–18]. Calculated values for the transition midpoint of guanidine hydrochloride concentration (at which the helical content of the peptide has been reduced by 50%) and the free energies of unfolding of the molecules clearly demonstrated the greater stability of the TmL dimer compared to the TmA23 dimer.

RP-HPLC of oxidized and reduced forms of TmL. From Fig. 5, the elution times of the oxidized and reduced forms of TmL are very similar on the least hydrophobic packing, P2HEMA. TmL Red has already been shown to be maintained as a dimer on this packing (Fig. 2, top left elution profile), indicating that both the reduced and oxidized polypeptides maintain their quaternary structure on P2HEMA. On the more hydrophobic PEMA packing, there is evidence that both the oxidized (note the doublet, suggesting conformational changes) and reduced forms of TmL are undergoing at least partial structural change. It would be expected that if the 70-residue disulphide-linked TmL (TmL Ox) was completely unfolded on the surface of the packing, allowing full accessibility of all the side-chains to interact with the stationary phase, then it would be eluted later than the monomers of its reduced form (TmL Red), based upon the increase in hydrophobicity. However, TmL Red is being eluted slightly later than TmL Ox on PEMA, suggesting that at least partial unfolding of the less stable (Fig. 3) TmL Red dimer has been effected. The unfolding of the TmL Red coiled-coil is even more pronounced on the most hydrophobic POMA packing, with baseline resolution of the disulphide-linked TmL and the TmL Red monomers. The observation that TmL Ox is still eluted before the reduced monomers even on the POMA packing is again suggestive of only partial (if any) unfolding on this packing, reflecting similar results by Hodges *et al.* [3] on a traditional C_8 column. This again reflects the stability conferred to the TmL coiled-coil by the presence of a disulphide bond (the guanidine hydrochloride concentration required to denature 50% of the α-helical structure $[G \cdot HCl]_{1/2}$, was 5.3 *M* compared to 2.3 *M* for TmL Red; Fig. 3).

RP-HPLC of reduced and oxidized forms of TmA23. Fig. 6 shows a similar elution pattern of the oxidized and reduced forms of TmA23 on the three

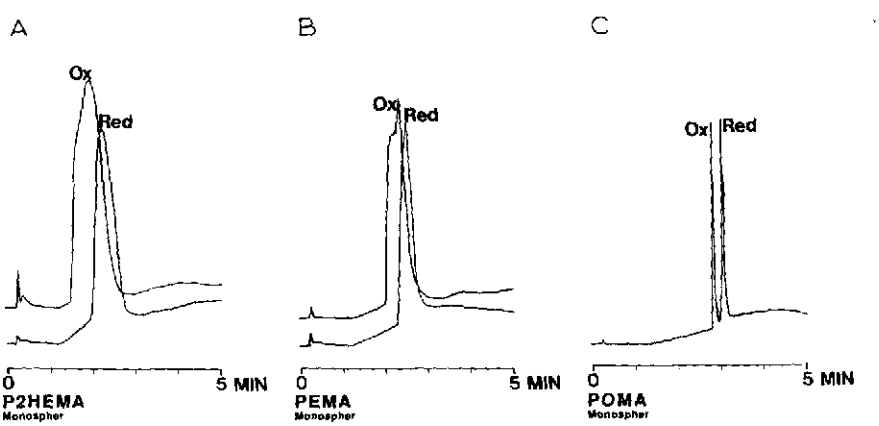

Fig. 6. Reversed-phase chromatography of synthetic model peptide TmA23 in its reduced and oxidized form. This peptide was obtained by substitution of alanine for leucine at position 23 of peptide TmL (L35 in Table I). Conditions as in Fig. 2. Other details as in Fig. 5.

packings as was observed for the respective forms of TmL (Fig. 5). The major difference lies in the distinctly later elution of TmA23 Red than TmA23 Ox on the P2HEMA packing. Thus, the reduced form of the alanine substituted dimer is undergoing at least partial breakdown of quaternary structure even on the least hydrophobic packing, reflecting the destabilizing effect of the leucine to alanine substitution (Figs. 3 and 4). Whether the oxidized TmA23 analogue is maintained as a full coiled-coil on this packing, or whether it undergoes partial unfolding, is difficult to ascertain, although some conformational change is apparent on the more hydrophobic PEMA packing, as was observed for TmL Ox (Fig. 5). The disulphide bridge clearly still confers considerable stability to even the destabilized (compared to TmL) TmA23 analogue, as evidenced by the later elution of the TmA23 Red monomers compared to TmA23 Ox on the most hydrophobic POMA packing.

Comparison of RP-HPLC at TmL and TmA23. Fig. 7 compares the elution behariour of pairs of

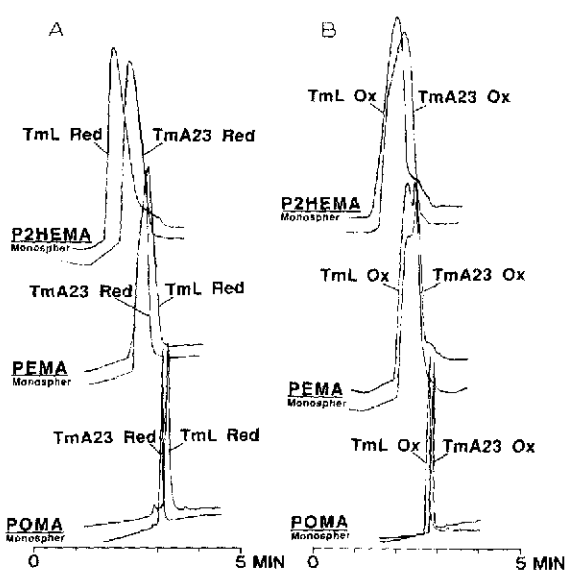

Fig. 7. Reversed-phase chromatography of synthetic model peptides on P2HEMA (top profiles), PEMA (middle profiles) and POMA (bottom profiles) packings. TmL and TmA23 refer, respectively, to peptide L35 (Table 1) and peptide L35 containing an alanine substitution (for leucine) at position 23. Ox and Red refer, respectively, to the oxidized (B) and reduced (A) forms of the peptides. The reduced forms of the peptides were obtained by dissolving them in 0.05% aq. TFA containing 10 m*M* DTT prior to application.

reduced (left profiles) and disulphide linked (right profiles) TmL and TmA23 peptides. It would be expected that, because of the greater hydrophobicity of a leucine side-chain compared to that of alanine, that full unfolding of TmL and TmA23 coiled-coils would result in later elution of TmL (oxidized or reduced) compared to the corresponding forms of the alanine substituted analogues. From Figs. 2 and 5, it is known that TmL Red is probably maintained in its native coiled-coil form on the P2HEMA packing; from Fig. 6, at least partial disruption of the coiled-coil of TmA23 Red was apparent. Hence, the later elution of TmA23 Red compared to TmL Red is not surprising, with the hydrophobic core of the former dimer being more fully exposed than that of TmL Red. The elution order is reversed on the most hydrophobic packing (POMA) as would be expected if the more hydrophobic core of TmL, compared to TmA23, was now able to interact more fully with the stationary phase. This result suggests that the reduced coiled-coils are completely unfolded on the POMA column. The co-elution of the peptide analogues on the PEMA packing represents intermediate conformational states of the polypeptides on a packing intermediate in hydrophobicity between P2HEMA and POMA.

The elution profiles of the oxidized analogues (Fig. 7B) serve as another illustration of the stabilizing effect of a disulphide bridge on both the TmL and TmA23 analogues. Considering the stability of the oxidized dimers of TmL (Fig. 5) and TmA23 (Fig. 6) observed previously on P2HEMA, the essential co-elution of TmL Ox and TmA23 Ox is not unexpected. The destabilizing effect of the leucine to alanine substitution is again apparent from the elution profile on the most hydrophobic packing (POMA) where TmA23 Ox is eluted after the more potentially hydrophobic TmL Ox. If disruption of the quaternary structure (if any) of both dimers had occurred to the same extent on POMA, then the more hydrophobic 70-residue TmL polypeptide would have been eluted after the 70-residue alanine-substituted analogue. This result agrees with the $[G \cdot HCl]_{1/2}$ values of 2.9 *M* and 5.3 *M* for TmA23 Ox and TmL Ox, respectively. The elution behaviour of the oxidized analogues on the PEMA packing again suggests intermediate conformational states of the disulphide-linked chains on the PEMA packing.

CONCLUSIONS

In this study, we have extended our original evaluation [1] of the potential of a novel concept for polypeptide separations by examining the effect of polymethacrylate-coated silicas on RP-HPLC of synthetic peptides of defined secondary, tertiary and quaternary structure. By selective unfolding of these model peptides, we have characterized further these packings of varying hydrophobicity, thus confirming the potential of such tailored packings for separation of peptides and proteins.

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