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Retention behaviour of a template-assembled synthetic protein and its amphiphilic building blocks on reversed-phase columns

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ABSTRACT

The retention behaviour of a six-helix bundle template-assembled synthetic protein (TASP) molecule and its amphiphilic building blocks was investigated. The TASP consists of a circular template, *cyclo*(1–12)$[KG]_6$, and six identical potentially α-helical peptides of the sequence KLALKLALKALKLALKLA. As an α-helix, this peptide is amphiphilic along the axis of its helix. Based on this sequence, the retention times of a set of acetylated peptides containing from seven to twenty amino acids on a Nucleosil C_{18} column were compared with another set of peptides with the same amino acid composition but a non-amphiphilic structure. Peptide elution was effected with linear trifluoroacetic acid (TFA)–water to TFA–acetonitrile gradients. The difference in retention times increased with peptide length; the 9-mers eluted at the same time, but there was a difference of 3.5 min for the 13-mers and 22.3 min for the 20-mer, indicating the induction of secondary structure on binding to the stationary phase. The same pair of 20-mers on Vydac C_{18}, C_4 and biphenyl columns gave differences in retention times of 23.2, 16.7 and 12.3 min, respectively. The TASP molecule was irreversibly adsorbed to C_{18} stationary phases, whereas it was eluted from C_4 and biphenyl columns as a single sharp peak. Several side-products resulting from the synthesis of the TASP molecule were identified by matrix-assisted laser desorption ionization mass spectroscopy. A comparison of the retention times of these side-products and the results of pre-column denaturation experiments indicated that the tertiary structure of the TASP molecule is maintained on binding to biphenyl and C_4 columns.

INTRODUCTION

The *de novo* design of proteins with a predetermined three-dimensional structure is impeded by the limited knowledge of the rules governing protein folding. A promising approach to overcome this problem is the concept of template-assembled synthetic proteins (TASP) [1]. This approach combines the prediction of secondary structure with the power of synthetic chemistry: TASP molecules are built by coupling peptides with potentially amphiphilic secondary structures to a template. The self-association of the building blocks leads to compact structures.

The TASP molecule under investigation was designed to form a six-helix bundle with the lysine side-chains exposed to the exterior. The inner surface of the hollow bundle is lined with alanines forming a hydrophobic cavity of about 0.7 nm diameter.

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The TASP molecule displays several features which make it prone to irreversible binding on hydrocarboneous matrices. First, the molecule has an unusually high content of hydrophobic residues. The membrane protein bacteriorhodopsin [2], for example, contains 60% hydrophobic amino acids, whereas the TASP molecule described here contains 67%. Secondly, the highly basic TASP molecule has 36 amino groups but lacks carboxy groups. Interactions of the amino groups with surface silanol groups of the stationary phase could lead to irreversible adsorption, but they can be suppressed by end-capping and by using trifluoroacetic acid (TFA) in the mobile phase to prevent ionization of the remaining silanols [3].

Finally, as has been reported elsewhere [4–8], the binding of potentially amphiphilic helices to C_8 and C_{18} matrices induces a secondary structure and leads to a considerably stronger retention. As the TASP molecule contains six potentially amphiphilic helices, strong adsorption is expected.

The tertiary structures of most proteins are stabilized by hydrophobic interactions. On binding to a reversed-phase (RP) matrix, these interactions were ruptured. Various reports on the disrupton of the tertiary structure on binding to RP columns exist. Mant *et al.* [9] investigated the denaturation of nineteen proteins on C_{18}, C_8, C_4 and phenyl columns. On C_{18}, C_8 and C_4 sorbents, all the proteins were denatured. On the phenyl column, all the proteins except lysozyme and avidin were denatured, whereas the latter showed only partial unfolding. Similar findings were reported by Ingraham *et al.* [10], for the rigidity of the 129-amino acid lysozyme with four intrachain cystine cross-links. A comprehensive study of the RP chromatographic behaviour of proteins in different unfolded states has been made by Lin and Karger [11]. By using only mildly destabilizing conditions, such as low temperature (4°C), a C_4-bonded stationary phase and propan-1-ol as an organic modifier, a number of proteins yielded two well separated peaks corresponding to the folded and an unfolded conformation. In addition, they distinguished between different states of denaturation and found that the degree of unfolding was as follows: folded $\ll$ RP surface-unfolded $<$ pre-column urea-unfolded $<$ pre-column reduced and urea-unfolded.

There are no reports of the induction of an α-helical conformation on binding to biphenyl columns. In this work, the retention times of the potentially amphiphilic twenty-amino acid α-helix acLAKLALKLALKALKLALKLA on C_4, C_{18} and biphenyl columns was compared with the non-amphiphilic helical sequence acLAKALKLKLALALLAKLKLA, where the charged side-chains are distributed all around the helix cylinder. The helix-inducing effect was investigated as a function of peptide length using the same peptide and shorter fragments thereof. In addition, the retention behaviour of the TASP molecule was studied using C_{18}, C_4 and biphenyl columns. It was investigated whether the tertiary structure of the molecule is disrupted on binding to the stationary phase.

EXPERIMENTAL

High-performance liquid chromatography

A Spectra Physics SP 8700 solvent delivery system was used coupled to a Shimadzu UV-120-02 spectrophotometer and a Shimadzu C-R1B integrator–printer. The columns used were Vydac (The Separations Group, Hesperia, CA, USA) 214TP 5415 C_4, 218TP 5415 C_{18} and 219TP 5415 biphenyl (15 cm × 4.6 mm I.D., 5 μm particle size, 30 nm pore size, from Macherey-Nagel, Düren, Germany). The flow-rate was 1 ml/min. Eluent A was 0.1% TFA; eluent B was acetonitrile + 0.1% TFA. The absorbance units full scale (a.u.f.s.) values were between 0.2 and 1. All chromatograms were obtained at room temperature.

Peptide identification

The peptides were identified by directly analyzing the collected peak fractions with a laboratory-built laser desorption ionization mass spectrometer [12]. Typically, 5 μl of 10^{-5} *M* analyte solution were mixed with 5 μl of 10^{-1} *M* sinapinic acid and 1 μl of this mixture was applied to the probe tip.

Reagents

All reagents were of at least analytical-reagent grade. Acetonitrile (spectroscopy grade) and guanidine hydrochloride (GuHCl) were from Fluka (Buchs, Switzerland) and sodium dodecyl sulphate (SDS) from Behring Diagnostics (La Jolla, CA,

USA). The water was of MilliQ quality (Millipore, Bedford, MA, USA). The eluents were degassed by continuous sparging with helium.

Peptides

The design, synthesis and purification of the TASP molecule and its building blocks has been described in detail elsewhere [13]. Briefly, the potentially α-helical 20-mers and their shorter derivatives from four to nineteen amino acids were synthesized by solid-phase peptide synthesis using standard protocols for 9-fluorenylmethyloxycarbonyl protection of the N-terminus. After each coupling step, an aliquot of resin was removed to obtain the shorter fragments.

The TASP molecule was assembled in solution by coupling the protected potentially amphiphilic 18-mers via their free C-termini to the six ε-amino groups of the template. For reasons of simplification, the potentially amphiphilic α-helical peptide KLALKLALKALKLALKLA is denoted HELIX (H), the TASP molecule TH_6 and the incomplete coupling products TH_3, TH_4 and TH_5.

Pre-column denaturation

Unless otherwise stated, 10 μl of oligopeptide in 95% TFA (containing 10 μg peptide/μl) were mixed with 90 μl of 6 *M* GuHCl solution of 2% SDS solution and heated to 96°C for 10 min. After cooling to room temperature, 50 μl were injected onto the column.

RESULTS AND DISCUSSION

Retention behaviour of amphiphilic building blocks

Induction of secondary structure as a function of peptide length. Two sets of peptides from seven to twenty residues in length with the same amino acid composition, but differing in their sequence, were constructed; they are listed in Table I. The sequence of the first set gives an amphiphilic structure on α-helix formation, whereas the sequence of the second set is non-amphiphilic (see helical wheel diagrams in Fig. 1).

The peptides were chromatographed on a 5 μm, 10 nm Nucleosil C_{18} column (25 cm × 4 mm I.D.). Eluent A was 0.1% TFA; eluent B was 0.1% TFA in acetonitrile. A linear gradient from 0 to 80% B in 80 min was used. The retention times (t_R) are listed in Table I. The 22.3 min difference in retention time between the 20-mers decreases gradually with shorter sequences. The 9-mers elute at the same time. The retention times as a function of peptide length are shown in Fig. 2; only the sequences with strongly hydrophobic N- and C-termini were considered. Fig. 2 implies that the minimum length for secondary structure effects is between 9 and 13 residues. Thus, the high accuracy in the prediction of retention times for peptides up to sixteen residues reported by Guo *et al.* [14] cannot be obtained with the amphiphilic set of peptides. In this set, as shown by Houghten and DeGraw [15], the sequence domains must be taken into account.

TABLE I

RETENTION TIMES OF AMPHIPHILIC AND NON-AMPHIPHILIC SEQUENCES

Sequences with strongly hydrophobic residues at either end are printed in bold. The peptides were chromatographed on a 5 μm, 10 nm Nucleosil C_{18} column (25 cm × 4 mm I.D.). Eluent A was 0.1% TFA and eluent B 0.1% TFA in acetonitrile. A linear gradient from 0 to 80% B in 80 min was used.

Residue	Amphiphilic sequence	t_R (min)	Non-amphiphilic sequence	t_R (min)
20	**acLAKLALKLALKALKLALKLA**	**69.1**	**acLAKALKLKLALALLAKLKLA**	**46.8**
19	acAKLALKLALKALKLALKLA	60.1	acAKALKLKLALALLAKLKLA	48.2
18	acKLALKLALKALKLALKLA	57.8	acKALKLKLALALLAKLKLA	48.7
17	**acLALKLALKALKLALKLA**	**62.1**	**acALKLKLALALLAKLKLA**	**47.8**
15	**acLKLALKALKLALKLA**	**56.7**	**acKLKLALALLAKLKLA**	**47.5**
13	**acLALKALKLALKLA**	**49.8**	**acKLALALLAKLKLA**	**46.3**
9	**acALKLALKLA**	**41.2**	**acALLAKLKLA**	**41.2**
8	**acLKLALKLA**	**40.7**	**acLLAKLKLA**	**40.5**
7	acKLALKLA	33.4	acLAKLKLA	35.0

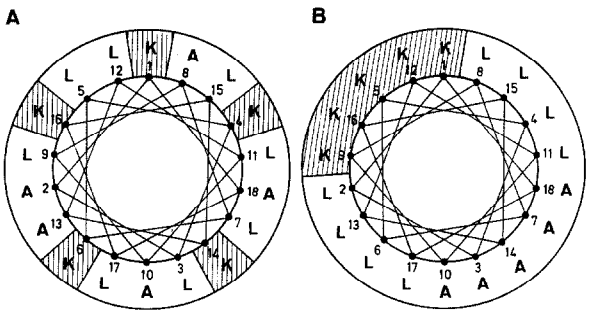

Fig. 1. Helical wheel diagrams of (A) the non-amphiphilic and (B) the amphiphilic 18-mer of Table I, looking from the C-terminal end (residue 18) down the helix axis.

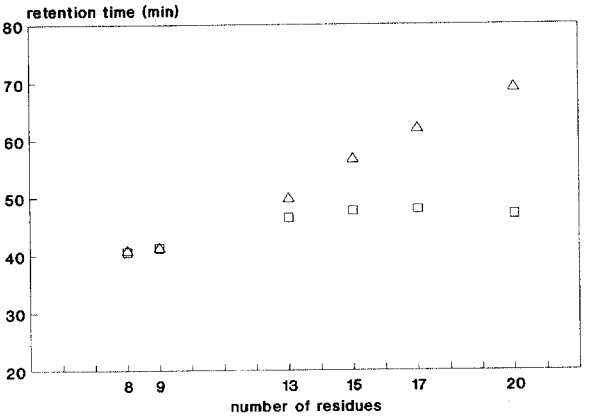

Fig. 2. Retention times of amphiphilic and non-amphiphilic peptides as a function of length. (△) Potentially amphiphilic; (□) non-amphiphilic peptide. Sequences and conditions as in Table I.

Induction of secondary structure as a function of the matrix. The retention times of the 20-mers in Table I were evaluated using C_{18}, C_4 and biphenyl columns of the same dimensions. The results are given in Table II and Fig. 3. The biphenyl stationary phase shows the weakest retention, whereas the C_{18} column exhibits the strongest interaction. As expected, the amphiphilic peptide is retained more strongly.

The results may suggest that C_{18} ligands have the strongest and biphenyl ligands the weakest effect on the induction of secondary structure. However, when the 4- to 20-mers were compared on C_{18} and biphenyl columns, there was no significant difference between short peptides without secondary structure effects and the longer sequences with putative ligand-induced conformational stabilization (Fig. 4). Note that the columns used for the two sets of separations have different dimensions. It seems that the interaction with biphenyl-bonded phases is in general weaker.

TABLE II

RETENTION TIMES OF THE AMPHIPHILIC AND NON-AMPHIPHILIC 20-MER

Sequences are given in Table I. The peptides were chromatographed on Vydac 5 μm, 30 nm columns (15 cm × 4.6 mm I.D.). Eluent A was 0.1% TFA; eluent B 0.1% TFA in acetonitrile. A linear gradient from 0 to 80% B in 80 min was used.

Matrix	t_R(min)		Δt_R
	Non-amphiphilic 20-mer	Amphiphilic 20-mer	
C_{18}	42.9	66.1	23.2
C_4	41.3	58.0	16.7
Biphenyl	37.5	49.8	12.3

Retention behaviour of TASP

TASP on C_{18}, C_4 and biphenyl columns. Fig. 5 shows the chromatograms of TASP (TH_6) and its building block peptide KLALKLALKALKLALKLA (denoted as HELIX) on C_{18}, C_4 and biphenyl columns. As predicted, TASP interacts strongly with the C_{18} matrix, leading to a small broad peak indicating denaturation and partly irreversible binding. In contrast, on the C_4 and biphenyl columns, the TASP molecule elutes much earlier and as a sharp peak. This result raises the question of whether the tertiary structure of the TASP molecule, in which predominantly lysine side-chains are exposed at the surface, is maintained on binding to C_4 and biphenyl phases.

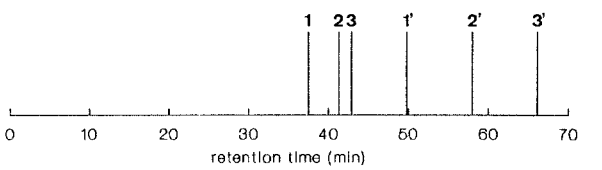

Fig. 3. Effect of matrix on retention time. 1, 2, 3 = Non-amphiphilic 20-mer peptide on biphenyl, C_4 and C_{18} columns, respectively; 1', 2', 3' = amphiphilic 20-mer peptide on biphenyl, C_4 and C_{18} columns, respectively. Sequences are given in Table I; conditions as in Table II.

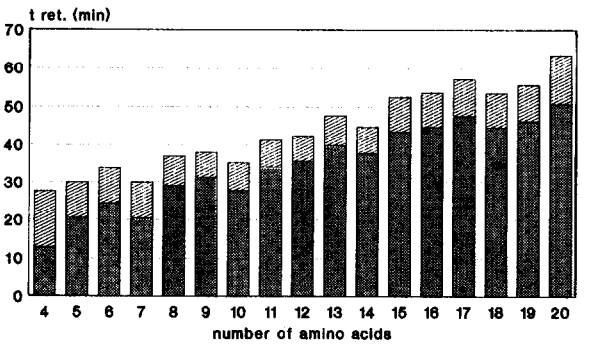

Fig. 4. Retention times of acetylated peptides a from four to twenty residues on C_{18} and biphenyl columns. Dotted areas, retention time on biphenyl column; hatched areas, retention time on C_{18} column minus retention time on biphenyl column. The sequences of the peptides are listed in Table I, or can be derived from this table. Columns: 5 μm, 10 nm Nucleosil C_{18} (250 cm × 4 mm I.D.) and 5 μm, 30 nm Vydac biphenyl 15 cm × 4.6 mm I.D.).

Conformation of TASP on RP matrices. It is generally known that most proteins are denatured on binding to a RP matrix [16]. For the TASP molecule, however, there were hints that this is not so.

First, as can be seen in Fig. 5, the elution times of TASP and HELIX are very similar. On the biphenyl column, for example, the retention time of HELIX was 15.0 min, whereas the TASP molecule eluted at 18.4 min. As has been reported for peptide oligomers, there is a linear relationship between the natural logarithm of the number [17] or the molecular mass [16] of monomers and the retention time. Based on this assumption, regression analysis was performed using t_R values of the 11- to 20-mers on the biphenyl column (data not shown). Under the conditions where the single HELIX elutes at 15.0 min and TASP at 18.4 min, it was estimated that a di-HELIX would elute at 21.9 min and a hexa-HELIX at 34.3 min. Hence, despite the rough estimation, it seems unlikely that the TASP molecule interacts with the phenyl matrix by fully exposing the hydrophobic faces of the six HELICES to it.

A second indication that the tertiary structure is maintained on binding to biphenyl columns is the retention behaviour of the intermediate condensation product TH_4 and TH_5. Interestingly, they co-eluted from the biphenyl columns with TH_6 in the same peak, although C_4 columns could partially separate them.

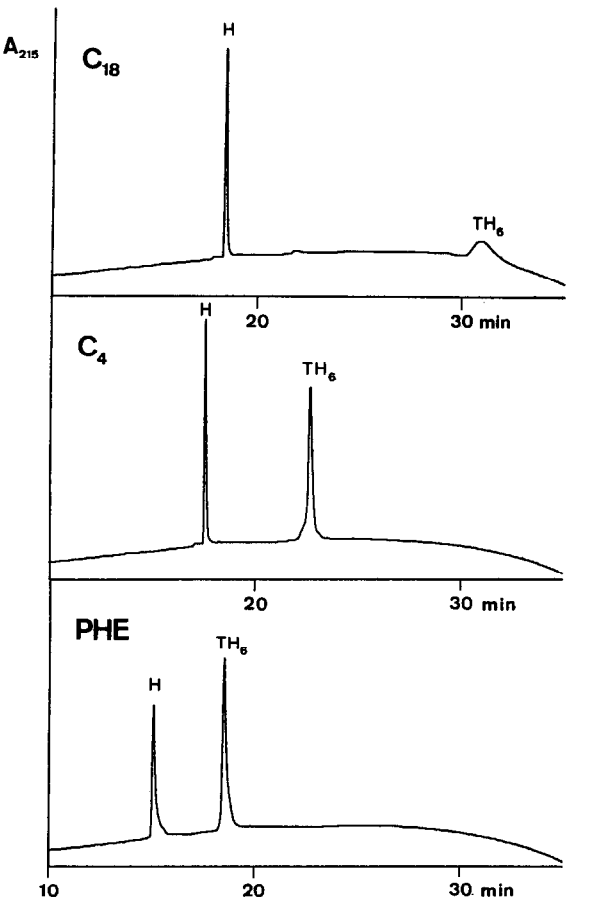

Fig. 5. Elution profiles of HELIX (H) and TASP (TH_6) on C_{18}, C_4 and biphenyl columns. Injection, 60 μl; a.u.f.s., 0.5; columns, Vydac 5 μm 30 nm 15 cm × 4.6 mm I.D.; solvent A, 0.1% TFA; solvent B, 0.1% TFA in acetonitrile. Linear gradient from 10 to 90% B in 30 min.

A fragment condensation with ≈5% imcompletely protected HELIX fragments and the identification of side-products by matrix-assisted laser desorption ionization mass spectroscopy gave further information. During the reaction, a small amount of di-HELICES, which were also partly condensed on the template, leading to TH_7 appeared as side-products. Fig. 6 shows the laser desorption ionization mass spectrum of the reaction mixture. In Table III, the experimentally determined masses are compared with the calculated masses.

The biphenyl chromatogram of the reaction mix-

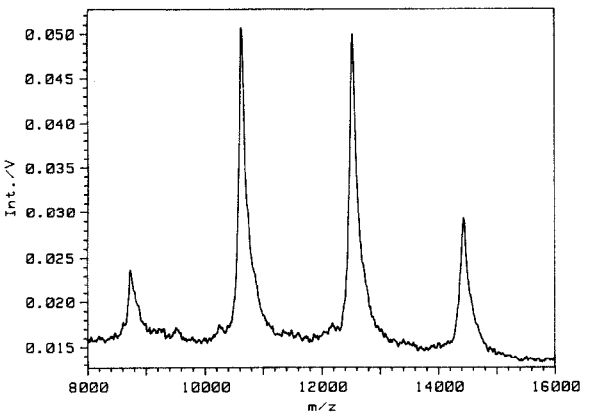

Fig. 6. Laser desorption ionization mass spectrum (measured in positive-ion mode) of TH_x, the products from coupling HELIX (H) to the template (T). For mass assignments, see Table III.

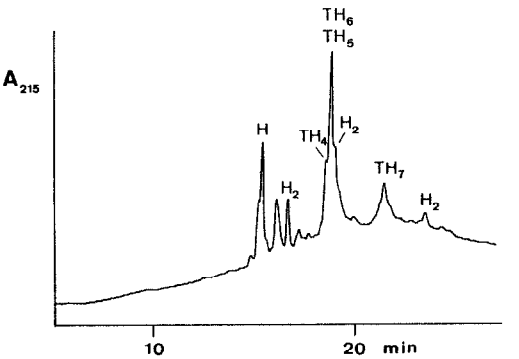

Fig. 7. Elution profile of crude product from a condensation fragment with incompletely protected HELIX. (H) HELIX; (H_2) di-HELIX; (TH_4, TH_5) incomplete TASP molecules; (TH_6) TASP molecule; (TH_7) TASP with an additional HELIX. Column, Vydac biphenyl 5 μm, 30 nm, 15 cm × 4.6 mm I.D. Solvent A, 0.1% TFA; solvent B, 0.1% TFA in acetonitrile. Linear gradient from 10 to 90% B in 30 min.

ture (Fig. 7) shows that three different di-HELICES (H_2) appear. As a HELIX contains five ε-amino groups, the three peaks may be ascribed to the three possible isomers. As has been reported by Buettner and Houghten [7], amphiphilic helices are bound to the RP stationary phase by exposing their hydrophobic side to the matrix. As with the HELIX, the di-HELICES are also assumed to expose their hydrophobic sides to the matrix. Since the putative hydrophobic contact areas are different for the three di-HELIX isomers, they are eluted as three distinct peaks. Another by-product is TH_7, a TASP molecule with an additional HELIX coupled to an ε-amino group of one of the six regular HELICES. The main peak contains TH_4, TH_5 and TH_6. Two details deserve closer examination: (1) H_2 elutes later than TH_x; (2) although TH_4, TH_5 and TH_6 elute at the same time, TH_7 the TASP molecule with an extra HELIX, is considerably more retained. From these characteristics it can be assumed that TH_4, TH_5 and TH_6 bind in their folded form as parallel helix bundles with most of the hydrophobic side-chains exposed to the interior. In contrast, the surplus HELIX in TH_7 is not integrated in the parallel α-helix bundle and therefore seems to be able to expose its hydrophobic face to the matrix, leading to the stronger retention of TH_7.

TABLE III

ASSIGNMENT OF MASS FOR THE PEPTIDES IN THE REACTION MIXTURE (SEE ALSO FIG. 6)

Peptide	Calculated molecular mass	*m/z*	*δ*
H	1919.6	1921.4	1.8
H_2	3821.2	3825.1	4.0
TH_4	8717.7	8729.9	12.2
TH_5	10619.2	10632.4	13.2
TH_6	12520.8	12532.0	11.2
TH_7	14422.4	14433.0	10.6

Pre-column denaturation of HELIX and TASP

Lin and Karger [11] distinguished between RP surface-unfolded, urea-unfolded and reduced-unfolded forms of proteins. Pre-column urea-unfolding was achieved by heating the protein in 8 *M* urea to 90°C for 2–5 min and cooling before injection. Similarly, the reaction mixture used here (dissolved in 95% TFA and containing H, H_2, TH_3, TH_4, TH_5 TH_6 and TH_7) was mixed with a nine-fold excess of 6 *M* GuHCl (v/v). No disrupture of the structure could be observed after 15 min at room temperature prior to injection onto a biphenyl column. Therefore, the samples were stored for 10 min at 96°C. Fig. 8 shows the chromatograms of pure HELIX with and without 6 *M* GuHCl and heat treatment. It demonstrates that this harsh treatment has only a minor effect.

Pre-column denaturation of the reaction mixture

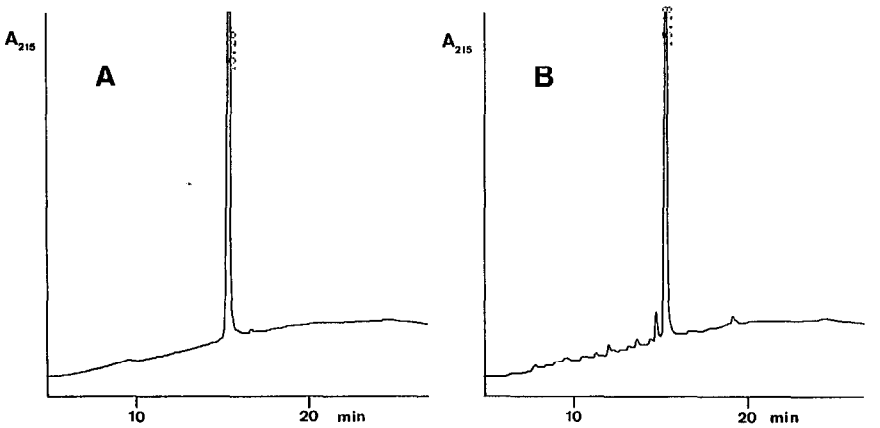

Fig. 8. Pre-column denaturation of HELIX with 6 *M* GuHCl and heat. (A) HELIX before, (B) HELIX after treatment. Column, Vydac biphenyl 5 μm, 30 nm, 15 cm × 4.6 mm I.D. Solvent A, 0.1% TFA; solvent B, 0.1% TFA in acetonitrile. Linear gradient from 10 to 90% B in 30 min.

shown in Fig. 7 indicates the breakdown of the tertiary structure followed by irreversible adsorption, whereas secondary structures of HELIX and the three H_2 isomers seem more resistant (Fig. 9). SDS is obviously not able to dissociate the tertiary structure, as can be seen in Fig. 10, which shows the chromatogram after treatment with 2% SDS and heat. This suggests that SDS renders the peptides more hydrophobic, but leaves the secondary and tertiary structures intact.

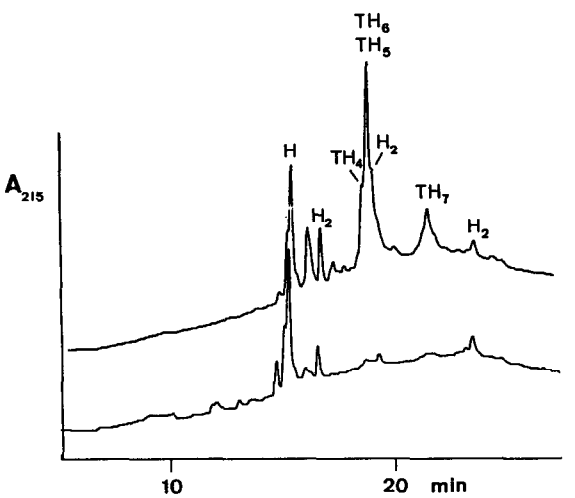

Fig. 9. Pre-column denaturation of the reaction mixture from Fig. 7 with GuHCl and heat. Upper profile, before denaturation. Column, Vydac biphenyl 5 μm, 30 nm, 15 cm × 4.6 mm I.D. Solvent A, 0.1% TFA; solvent B, 0.1% TFA in acetonitrile. Linear gradient from 10 to 90% B in 30 min.

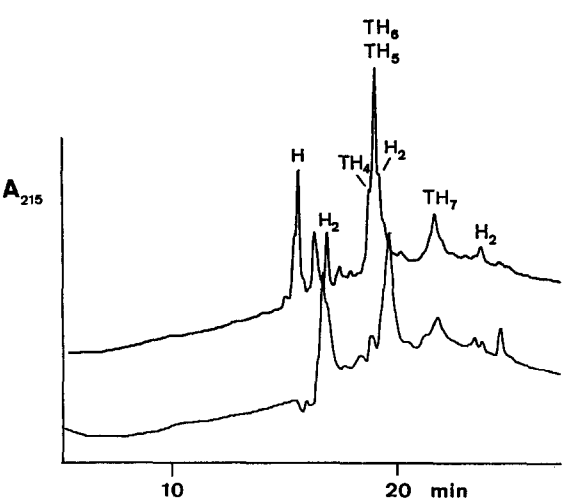

Fig. 10. Chromatogram of the reaction mixture from Fig. 7 after treatment with 2% SDS and heat. Upper profile, before treatment. Column, Vydac biphenyl 5 μm, 30 nm, 15 cm × 4.6 mm I.D. Solvent A, 0.1% TFA; solvent B, 0.1% TFA in acetonitrile. Linear gradient from 10 to 90% B in 30 min.

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