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Note

Separation of protected hydrophobic oligopeptides by normal-phase high-pressure liquid chromatography

FRED NAIDER, ROBERT SIPZNER and ALVIN S. STEINFELD

College of Staten Island, City University of New York, 50 Bay Street, Staten Island, N.Y. 10301 (U.S.A.)

and

JEFFREY M. BECKER

University of Tennessee, Department of Microbiology, Knoxville, Tenn. 37916 (U.S.A.)

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Protected hydrophobic oligopeptides have been used as models for investigating the conformational behavior of proteins[1]. In solution-phase synthesis of peptides, protected hydrophobic intermediates are often purified prior to continuing the chain elongation. Thin-layer chromatography is generally used as one method with which to evaluate the purity of these compounds. However, this technique has limited value when separating isomeric oligomers that differ only in the position of a single residue. This study was initiated to determine whether normal-phase high-performance liquid chromatography (HPLC) techniques could be applied to the resolution of structurally similar hydrophobic oligopeptides and to investigate the relationship of residue location and side-chain lipophilicity to retention behavior.

EXPERIMENTAL

The HPLC apparatus used was a Waters chromatograph (Waters Assoc., Milford, Mass., U.S.A.) equipped with a Model 6000M solvent delivery system, a U6K injector and a Model 450 variable wavelength UV detector operating at 220 nm. A 30 cm × 3.9 mm I.D. μPorasil silica column, also supplied by Waters Assoc., was used for all experiments.

Generally, the amount of sample injected was 5–20 μg dissolved in 5–25 μl of dichloromethane. All samples were eluted isocratically with cyclohexane–isopropanol.

Solvents were spectranalyzed grade and were purchased from Fisher Scientific (Springfield, N.J., U.S.A.).

Peptides were synthesized by the mixed anhydride procedure using isobutylchloroformate and N-methylmorpholine, in tetrahydrofuran as described previously[2–4]. All amino acid residues were of the L-configuration.

RESULTS AND DISCUSSION

We examined a mixture of *tert.*-butoxycarbonyl (Boc)-X-Met-OCH_3 peptides

(X = Met, Val, Ala, Pro and Gly) in order to determine the relationship of side-chain lipophilicity and retention behavior (Fig. 1). A sample containing 5 μg of each dipeptide in a total volume of 5 μl was injected onto a μPorasil column and eluted with isopropanol–cyclohexane (4:96). The retention times of individual dipeptides increased as a function of the X-residue in the order Gly > Pro > Ala > Met > Val. A scale of side-chain lipophilicity relative to H (glycine) predicts that the above residues increase in hydrophobicity in the order Val > Met > Pro > Ala > Gly[5]. This scale shows the same relative hydrophobicity of amino acids as scales derived from partition coefficients of amino acids in an octanol–water system[6] or obtained from measurements of hydrophobicity based upon the free energy of transfer of amino acids from water to dioxane or ethanol[7]. The retention behavior of the Boc-X-Met-OCH_3 peptides correlates well with the side-chain hydrophobicities of the component amino acid residues with the single exception of Boc-Pro-Met-OCH_3, which would be expected to behave like Boc-Met_2-OCH_3[5]. Although proline contains three methylenes that would be expected to contribute to its lipophilic character, proline-containing protected peptides unlike those containing alanine or methionine have relatively high solubility in water[8]. Thus it is not surprising that the properties of proline-containing peptides resemble those of more hydrophilic compounds on a polar silica support.

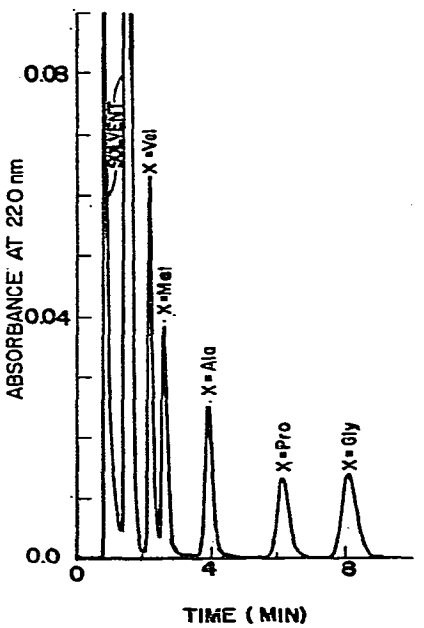

Fig. 1. Isocratic separation of Boc-X-Met-OCH_3 dipeptides on a 30 cm × 3.9 mm I.D. μPorasil column using isopropanol–cyclohexane (4:96), a flow-rate of 2 ml/min and ambient temperature.

A mixture of Boc-Met-X-OCH_3 (X = Met, Ala, Pro and Gly) peptides also showed a correlation of retention behavior and side-chain hydrophobicity (Table I). This correlation was further substantiated with two series of trimeric peptides, Boc-Met_2-X-OCH_3 (X = Met, Ala and Gly) and Boc-X-Met_2-OCH_3 (X = Val, Met, Ala and Gly) (Fig. 2 and Table I). These results suggest that the order of peptide retention

may be estimated from the sum of hydrophobicities of constituent amino acid residues. This observation has also been made with free peptides on non-polar stationary phases[9] and is similar to an approach used to estimate peptide retention on paper chromatograms[10].

TABLE I

RETENTION TIMES OF PEPTIDES ON μPORASIL

Peptides	*Retention time (min)* *	*Mobile phase***
Boc-Ala-Met-OCH_3	3.8	A
Boc-Met-Ala-OCH_3	3.2	A
Boc-Gly-Met-OCH_3	8.4	A
Boc-Met-Gly-OCH_3	6.2	A
Boc-Met-Met-OCH_3	2.6	A
Boc-Pro-Met-OCH_3	6.2	A
Boc-Met-Pro-OCH_3	4.8	A
Boc-Val-Met-OCH_3	2.2	A
Boc-Val-Met_2-OCH_3	3.3	A
Boc-Met_3-OCH_3	4.4	A
Boc-Gly-Met_2OCH_3	13.6	A
Boc-Ala-Met_2-OCH_3	6.9	A
Boc-Met-Ala-Met-OCH_3	9.0	A
Boc-Met_2-Ala-OCH_3	6.3	A
Boc-Met_2-Gly-OCH_3	13.1	A
Boc-Met_5-Ala-OCH_3	7.3**	B
Boc-Ala-Met_5-OCH_3	8.4**	B
Boc-Met-Ala-Met_4-OCH_3	11.1**	B
Boc-Val-Met-OCH_3	1.9	C
Boc-Val-Met_2-OCH_3	2.3	C
Boc-Val-Met_3-OCH_3	3.2	C
Boc-Val-Met_4-OCH_3	4.8	C
Boc-Val-Met_5-OCH_3	9.0	C

* Flow-rate 2.0 ml/min except where indicated.

** Flow-rate 1.0 ml/min.

*** Eluents: A = isopropanol–cyclohexane (4:96); B = isopropanol–cyclohexane (18:82); C = isopropanol–cyclohexane (7.5:92.5).

To examine the relationship between residue location and retention behavior, we chromatographed (Fig. 3) mixtures of Boc-X-Met-OCH_3 and Boc-Met-X-OCH_3 peptides (X = Ala, Gly or Pro) and observed in each case that when the less hydrophobic amino acid residue was at the carboxyl terminal position, the retention time was shorter than when the same amino acid was located at the amine terminal position. This trend was also observed for three isomeric tripeptides Boc-Met-Met-Ala-OCH_3, Boc-Met-Ala-Met-OCH_3, and Boc-Ala-Met-Met-OCH_3 (Fig. 4). A mixture containing 10–15 μg of each peptide dissolved in dichloromethane (total volume 15 μl) was eluted from the μPorasil column isocratically with isopropanol–cyclohexane (4:96). The peptides were separated with retention times of 6.3, 9.0 and 6.9 min, respectively. Similarly, a mixture of isomeric hexapeptides Boc-Ala-Met_5-OCH_3, Boc-Met_5-Ala-OCH_3 and Boc-Met-Ala-Met_4-OCH_3 (5 μg/peptide) in dichloromethane (5 μl), was eluted isocratically with isopropanol–cyclohexane (18:82) with retention times of 8.4, 7.3 and 11.1 min, respectively (Fig. 5). Thus, mixtures of

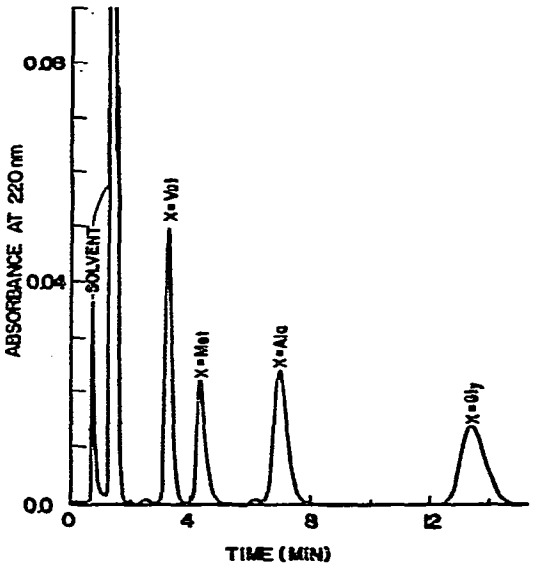

Fig. 2. Separation of Boc-X-Met_2-OCH_3 tripeptides. Chromatographic conditions as in Fig. 1.

relatively large peptides (molecular weight $\approx$ 858) differing only in the location of one amino acid residue and most likely residing in similar preferred conformational states can be separated using normal-phase HPLC. Furthermore, there is an apparent relationship between the position of the amino acid residues and the retention behavior of the peptide. In all cases examined similar peptides containing primarily

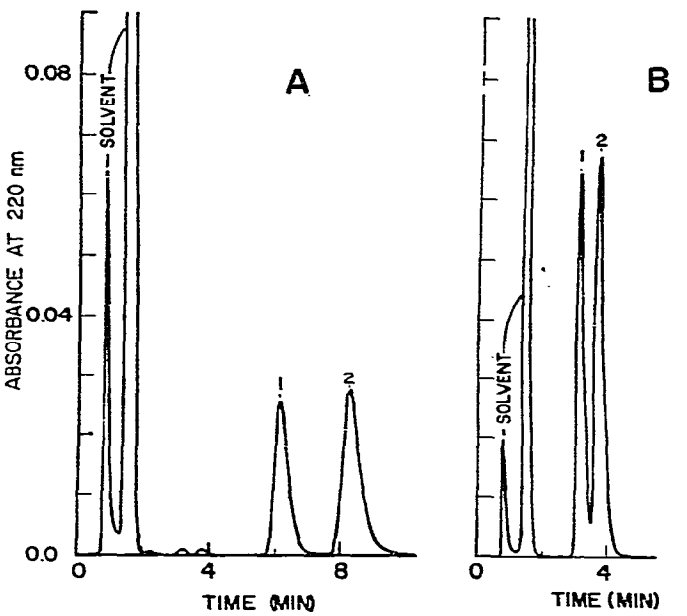

Fig. 3. Separation of isomeric dipeptides Boc-Met-X-OCH_3 and Boc-X-Met-OCH_3. Chromatographic conditions as in Fig. 1. (A), 1 = Boc-Met-Gly-OCH_3; 2 = Boc-Gly-Met-OCH_3. (B), 1 = Boc-Met-Ala-OCH_3; 2 = Boc-Ala-Met-OCH_3.

methionine and one alanyl residue are eluted more slowly when alanine is at the amine terminus than when it is at the carboxyl terminus. When the alanine is at an interior position of a tripeptide or hexapeptide, the longest retention times are observed. It appears that for the peptides examined and under our experimental conditions, strongest interactions between peptide and stationary phase occur for residues at amine terminal and interior positions, as compared with those at the carboxyl terminus.

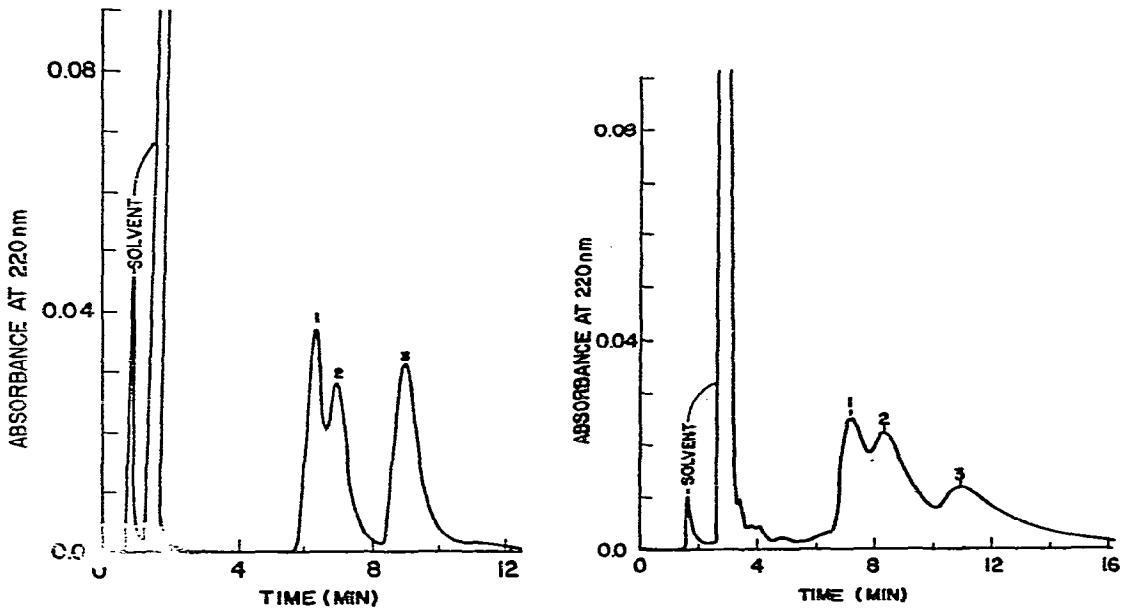

Fig. 4. Separation of isomeric tripeptides. Chromatographic conditions as in Fig. 1. 1 = Boc-Met_2-Ala-OCH_3; 2 = Boc-Ala-Met_2-OCH_3; 3 = Boc-Met-Ala-Met-OCH_3.

Fig. 5. Isocratic separation of isomeric hexapeptides Boc-Met_5-Ala-OCH_3 (1), Boc-Ala-Met_5-OCH_3 (2) and Boc-Met-Ala-Met_4-OCH_3 (3) on a 30 cm × 3.9 mm I.D. μPorasil column using isopropanol–cyclohexane (18:82), a flow-rate of 1 ml/min and ambient temperature.

To further test the resolving power of μPorasil for hydrophobic peptides, we attempted to separate the homologous series Boc-Val-Met_n-OCH_3 (n = 1–6). This series can be separated isocratically with isopropanol–cyclohexane (7.5:92.5) up to n = 5 (Table I). Under these conditions Boc-Val-Met_6-OCH_3 does not elute from the column. A mobile phase of the polarity necessary to elute this heptamer caused the lower homologues to coalesce. It thus appears that gradient elution would be necessary to separate the entire series. Nevertheless, it is apparent that normal-phase HPLC can be quite valuable in determining the dispersity of protected oligopeptides composed primarily of hydrophobic amino acids.

CONCLUSION

Protected hydrophobic di- and tripeptides differing only in one amino acid constituent, can be separated on μPorasil columns using isocratic elution techniques.

Retention behaviors of these peptides correlate with the hydrophobicities of the component amino acid residues with the single exception of proline-containing peptides. Isomeric di-, tri- and hexapeptides of identical composition but different sequence can be separated by the same techniques. Retention times of peptides containing primarily methionine and one other amino acid residue were influenced by the position of the "guest" amino acid residue. Thus methionine peptides containing either a single alanyl, glycyl, or prolyl residue at the carboxyl terminal positions showed shorter retention times than isomers with a corresponding residue located at the amine terminal. Peptides containing the corresponding residue in an internal location had the longest retention times. Thus solute binding with the solid support in this system appears to favor the internal peptide position and the amine terminus.

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