Journal of Chromatography, 215 (1981) 255–261
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,866

PARTITION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF β-LIPOTROPIN AND SYNTHETIC β-ENDORPHIN ANALOGUES

DONALD YAMASHIRO and CHOH HAO LI*
Hormone Research Laboratory, 1088 HSW, University of California, San Francisco, CA 94143 (U.S.A.)

SUMMARY

Distribution constants of 14 acetylamino acid amides were measured in two solvent systems useful for partition chromatography of peptides. Free energies of transfer of side-chains, expressed by the chromatographic parameter ΔR_M, linearly correlated with calculated surface areas for amino acids with hydrocarbon side-chains. Values for a variety of side-chains linearly correlated with ΔR_M (side-chain) values previously measured for the 31-residue peptide β-endorphin. It was concluded that accessibility of the amino acid residues in β-endorphin to the solvent environment can explain its separation from some very closely related analogues. High-performace liquid chromatography was inadequate for effecting these separations.

INTRODUCTION

The separation of peptides based on differences in hydrophobicity can be effected by counter-current distribution (CCD), partition chromatography (PC) and adsorption chromatography. The introduction of Sephadex by Porath and Flodin[1] provided a support so well suited for PC of peptides[2,3] that it is capable of substituting for CCD[4]. Adsorption chromatography is an alternate means of separation based on hydrophobic differences. In recent times, the development of high-pressure systems has permitted use of supports with small particle diameters for rapid and efficient adsorption chromatography of peptides. Efforts are now being made to better understand, in a quantitative way, the relationship between chromatographic behavior and chemical structure of peptides in both partition[4–6] and adsorption chromatography[7].

The empirical correlation of partition coefficients with calculated surface areas of non-polar molecules has been frequently reported[8,9]. Chothia[10] has pointed out the linearity of the correlation between calculated surface areas of amino acid side-chains and the Tanford hydrophobicity scale. A linear correlation has been shown for the hydrophobicities of amino acid side-chains of β-endorphin (β-EP) in PC and the Tanford scale[5,6]. An examination of this relationship and its consequences on the PC of peptides is presented herein, together with a comparison with high-performance liquid chromatography (HPLC).

0021-9673/81/0000–0000/$02.50

EXPERIMENTAL

Theory

The mobility of a peptide in PC is most conveniently described by its R_f value, from which the R_M value[11] is calculated, thus: $R_M = \ln [(1 - R_f)/R_f]$. By the Martin hypothesis[12], the difference in R_M values of two peptides A and B is related to the free energy of transfer associated with the structural difference by $\Delta R_M = \Delta\mu°/RT$, with the relationship to partition coefficient being $\Delta R_M = \ln (K_A/K_B)$. In this work, transfer is assumed to occur from aqueous phase to organic phase and K is the concentration of solute in the organic phase divided by that in the aqueous phase. The term ΔR_M is used in this paper instead of free-energy values since it gives a convenient picture of the magnitude of these energies associated with various structural features of peptides. It is assumed for β-EP that ΔR_M (residue) = ΔR_M (side-chain) + ΔR_M (Gly), where the last term is obtained by PC of camel β-EP (β_c-EP) and Des-Gly^2-β_c-EP (see refs. 5 and 6).

Materials

Acetylamino acid amides were either synthesized[13] or obtained from Vega-Fox Biochemicals. β_c-Endorphin (see Fig. 1) and β_h-EP (human β-endorphin) were synthesized as described previously[14]. Omission analogues of β_c-EP were individually synthesized as detailed previously[6]. Ovine β-lipotropin (β_s-LPH) and its pyroglutamyl form (<Glu^1-β_s-LPH) were prepared from natural sources[15].

```
H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-
                    5                   10

  Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-
                   15                   20

   Ala-Ile-Ile-Lys-Asn-Ala-His-Lys-Lys-Gly-Gln-OH
                   25                   30
```

Fig. 1. Amino acid sequence of camel β-endorphin.

Sephadex G-50 fine was sieved on a No. 325 screen (U.S. standard series) to give a 20–44 μm fraction, which was packed in columns as an aqueous slurry. Agarose (Bio-Gel A-0.5m) was obtained as an aqueous slurry and used directly.

Partition experiments

A sample (*ca.* 20 mg) of acetylamino acid amide was stirred in a mixture of 50 ml of each phase of the solvent system for 20 min at 24°C. The phases were separated, and each was diluted with 2 volumes of water and lyophilized. For solvent system A (see below), repeated lyophilization was required to remove ammonium acetate (NH_4OAc). A final lyophilization from water or 10% acetic acid (5 ml) was performed in tared test-tubes. Residue weights were used to calculate K values.

Chromatography

Sephadex G-50 and agarose columns were first equilibrated with the lower phase of the solvent system and then with several volumes of mobile phase to achieve the most efficient conditions possible[4]. Solvent systems were: A, 1-butanol–pyridine–0.6 *M* NH_4OAc (5:3:10); B, 1-butanol–pyridine–0.6 *M* NH_4OAc (10:3:10).

HPLC of β-EP and analogues was performed on 25 or 50 μg samples in a 250 mm × 4.5 mm "reversed-phase" C_{18} coated silica gel support (Partisil 10 ODS-2, 10 μm) (Altex), with fluorescamine detection[16]. HPLC of β_s-LPH and $<Glu^1$-β_s-LPH was performed on 50 μg samples in a 250 mm × 4.6 mm C_8 coated support of porosity 330 Å (a gift from Drs. Sidney Udenfriend and Stanley Stein). Elution was performed at 0.1 cm/sec with a buffer of fixed composition (isocratic): 1 *M* pyridine–0.5 *M* acetic acid (85 ml) mixed with 1-propanol (15 ml).

RESULTS AND DISCUSSION

In previous work[17], the hydrophobicities of various amino acid residues in camel β-EP (Fig. 1) were measured and compared with values from the Tanford scale[5,6]; the latter values correspond to transfer from pure water to pure ethanol[18]. We decided to obtain values that are more relevant to actual conditions in PC. For this purpose, the distribution constants of acetylamino acid amides were measured in two typical solvent systems useful in the PC of peptides (Table I), one of which (system A) was used in the β-EP work. Amino acids with charge groups and cystine are not considered in this work. Values of ΔR_M were calculated as indicated in Ex-

TABLE I

VALUES OF ΔR_m FOR AMINO ACID SIDE-CHAINS (s.c.) IN ACETYLAMINO ACID AMIDES AND CAMEL β-ENDORPHIN

Amino acid residue	*Acetylamino acid amide*				*β_c-EP*	
	Solvent system A		*Solvent system B*		*Solvent system A*	
	K	*ΔR_m (s.c.)**	*K*	*ΔR_m (s.c.)**	*ΔR_m (s.c.)***	*ΔR_m (residue)***
Trp	7.57	−3.33	3.85	−2.48		
Phe	3.81	−2.64	2.55	−2.06	−1.42	−1.19 (4)
Tyr	3.06	−2.43	1.26	−1.36	−1.77	−1.54 (1)
Leu	2.47	−2.21	2.02	−1.83	−1.28	−1.05 (14)
Ile	1.88	−1.94	1.86	−1.75	−0.58	−0.35 (22)
Met	1.28	−1.56	1.05	−1.18	−0.87	−0.64 (5)
Val	1.10	−1.40	1.23	−1.34	−0.78	−0.55 (15)
Ala	0.394	−0.38	0.46	−0.35	−0.04	+0.19 (22)
Pro	0.332	−0.21	0.485	−0.41	−0.08	+0.15 (13)
Thr	0.321	−0.17	0.362	−0.11	−0.13	+0.08 (6), +0.12 (12)
Gly	0.270	0.00	0.324	0.00	0.00	+0.23 (2)
Ser	0.246	+0.10	0.279	+0.15	−0.09	+0.08 (7), +0.21 (10)
Gln	0.208	+0.26	0.280	+0.15	+0.06	+0.29 (11)
Asn	0.176	+0.43	0.235	+0.32	+0.29	+0.52 (20)

* Calculated from $\Delta R_m = \ln (K_A/K_B)$ where *A* is acetylglycinamide and *B* is any other acetylamino acid amide.

** Values taken from ref. 6. Numbers in parentheses refer to residue position in the β_c-EP sequence.

perimental. Those for strictly hydrocarbon side-chains were then plotted against side-chain areas reported by Shrake and Rupley[19] (Fig. 2A). The results for the two solvent systems indicate that free energies of transfer are linearly related to side-chain areas, even in the complex solvent systems commonly used in the purification of peptides. However, it may be noted that the slope of the line can vary from one solvent system to another. It is proposed that this is a measure of the discriminating power of a solvent system. From a practical standpoint, the solvent system used should be that with the highest possible discriminating power with the restriction that a favorable distribution constant for the peptide must also be obtained.

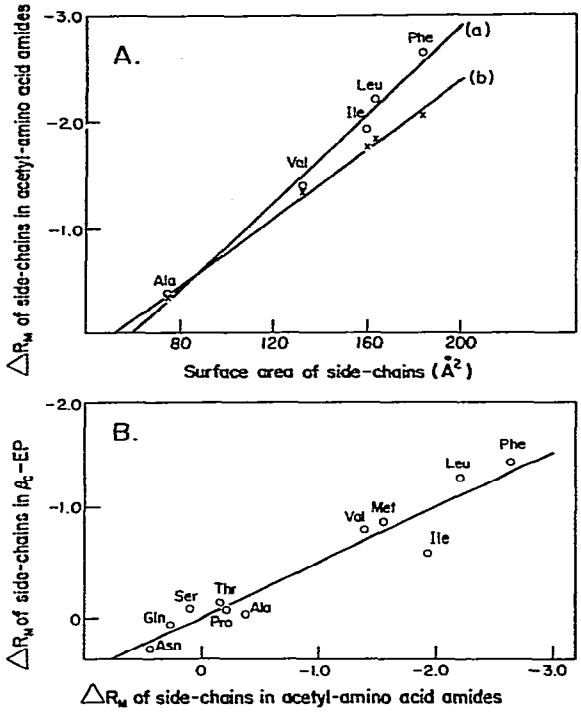

Fig. 2. A: Correlation of ΔR_m values of side-chains in acetylamino acid amides with their calculated surface areas: (a), in 1-butanol–pyridine–0.6 *M* NH_4OAc (5:3:10) (correlation coefficient, 0.993; slope, 0.0205), open circles; (b) in 1-butanol–acetic acid–water (4:1:5) (correlation coefficient, 0.998; slope, 0.0160), crosses. B: correlation of ΔR_m values of side-chains of amino acids in camel β-endorphin (β_c-EP) and acetyl-amino acid amides (correlation coefficient, 0.959; slope, 0.503).

The ΔR_M values obtained for amino acid side-chains in acetyl amides now gives a reference against which those previously measured for side-chains in β_c-EP can be compared (the latter are repeated in Table I). When one set is plotted against the other, for internal residues only, a linear correlation is observed (Fig. 2B). The degree of correlation would be better ($r = 0.990$, slope $= 0.55$) if Ile is excluded. Since Ile is adjacent to another Ile in the β_c-EP sequence, it is possible that this juxtaposition of side-chains known to cause steric hindrance in chemical reactions results in the solvent-accessible surface area of each residue being occluded by the other. The correlation indicated in Fig. 2B suggests that the separation of β_c-EP from its omission

analogues is due primarily to close-range interactions of solvent environment with individual residues in the peptide. However, the fact that the ΔR_M values for β_c-EP are less than those for the acetyl amides, as indicated by the slope of 0.50 in Fig. 2B, suggests that the solvent-accessible areas of side-chains on β_c-EP are being occluded by near or distant atoms in the peptide sequence. The extent to which this occlusion occurs will affect the capability of purifying large synthetic peptides.

The quantitative significance of ΔR_M measurements indicated in Table I is shown by the approximate equation for separation in PC: $R_s = (\sqrt{N}/4)(\Delta R_M)(1 - R_f)$, where R_s is the degree of resolution and N is the number of theoretical plates in the column. Since an R_s value of 0.75 can give useful separations and columns can routinely be operated at $N = 250$ and R_f of 0.2, an impurity must differ in R_M from the desired product by an absolute magnitude of at least 0.25 for separation to be possible. Examination of ΔR_M (residue) values of β_c-EP (Table I) shows that those residues most likely to show less than 0.25 in absolute magnitude are Ser, Thr, Pro, Ala and Gly. A peptide missing any one of these residues can be expected to be difficult to separate from the parent peptide. From a practical synthetic standpoint, it can be recommended that particular attention should be paid to the incorporation of these into a peptide chain.

The ΔR_M values of the amino acid residues just mentioned show a significant enough absolute magnitude (*ca.* 0.1 or greater) in β_c-EP that separation is conceivable. In the case of β_c-EP and Des-Gly^2-β_c-EP ($\Delta R_M = +0.23$), separation is effected on Sephadex G-50 in solvent system A with a column showing about 500 plates[5]. For the more demanding separations of β_c-EP from Des-Ser^7-β_c-EP ($\Delta R_M + 0.08$), β_c-EP from Des-Thr^{12}-β_c-EP ($\Delta R_M + 0.12$), and β_c-EP from Des-Ser^{10}-β_c-EP ($\Delta R_M + 0.21$), a solvent system with greater discriminating power and giving lower R_f values than system A could be employed. One such run was typical of all three [Fig. 3(a)]. Alternatively, solvent system A could be used to separate β_c-EP from Des-

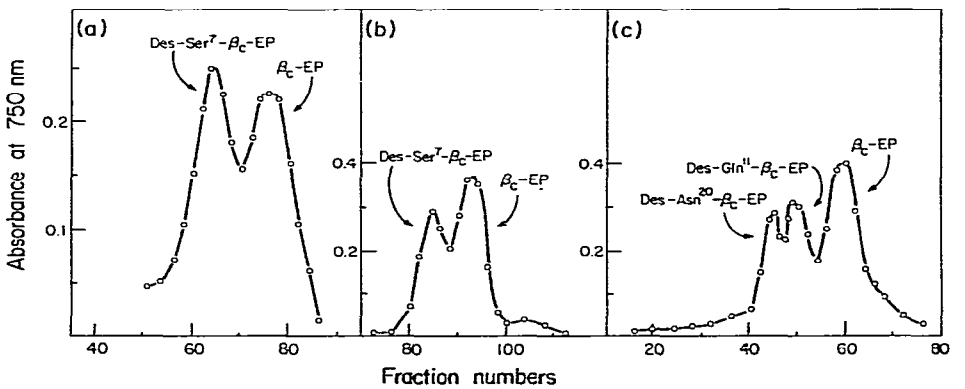

Fig. 3. Partition chromatography of peptide mixtures: (a) β_c-EP (2.8 mg) (R_f 0.144) and Des-Ser^7-β_c-EP (2.7 mg) (R_f 0.172) on a column (46 cm × 1.29 cm) of Sephadex G-50 in solvent system B (mobile phase, 25.5 ml; fraction, 2.31 ml; flow-rate, 3.7 ml/h); (b) β_c-EP (1.6 mg) (R_f 0.176) and Des-Ser^7-β_c-EP (1.0 mg) (R_f 0.192) on a column (42.5 × 1.0 cm) of agarose in solvent system A (mobile phase, 9.0 ml; fraction, 0.55 ml; flow-rate, 2.0 ml/h); (c) β_c-EP (3.8 mg) (R_f 0.36), Des-Gln^{11}-β_c-EP (1.9 mg) (R_f 0.43), and Des-Asn^{20}-β_c-EP (1.1 mg) (R_f 0.47) on a column (65 cm × 1.45 cm) of Sephadex G-50 in solvent system A (mobile phase, 32 ml; fraction, 1.54 ml; flow-rate, 7 ml/h). In all cases, fraction numbering started at time of sample application and detection was by the Folin–Lowry method.

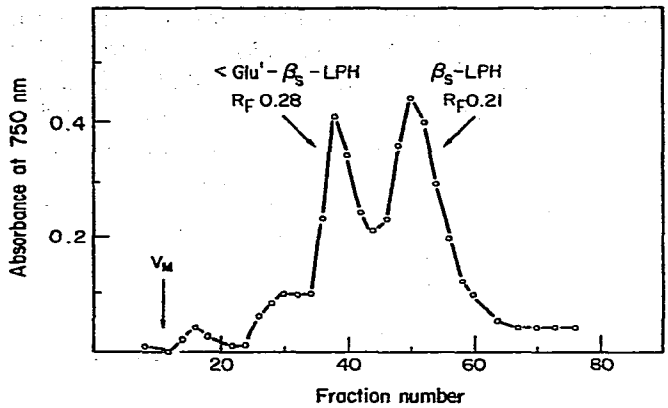

Fig. 4. Partition chromatography of crude natural β_s-LPH on agarose. Column, 20 cm × 1.05 cm; solvent system, 2-butanol–0.5 *N* acetic acid containing 0.5 *N* NaCl and 0.03 *N* trichloroacetic acid (8:11); sample, 10.4 mg; fraction, 0.40 ml; mobile phase, 4.35 ml; flow-rate, 1.8 ml/h; detection by Folin–Lowry method.

Ser^7-β_c-EP on an agarose support [Fig. 3 (b)]. This support is more powerful than a Sephadex-G-50 column of the same length. For example, the PC of human β-EP could be effected on a 40-cm column exhibiting about 1150 theoretical plates. It may be noted that a short agarose column (*ca.* 20 cm) is capable of separating the 91-residue ovine β-LPH from pyroglutamyl^1-β_s-LPH, a difference involving only N-terminal Glu and <Glu, respectively[15] (Fig. 4).

An examination on HPLC of β_c-EP and analogues involving omissions of residues that are not strongly hydrophobic was carried out. The separation of β_c-EP from both Des-Asn^{20}-β_c-EP and Des-Gln^{11}-β_c-EP is relatively easy by PC on Sephadex G-50 [Fig. 3(c)], but no separations occur in HPLC (Fig. 5). Similarly, separation of β_c-EP from Des-Gly^2-β_c-EP could not be effected. The very difficult cases involving

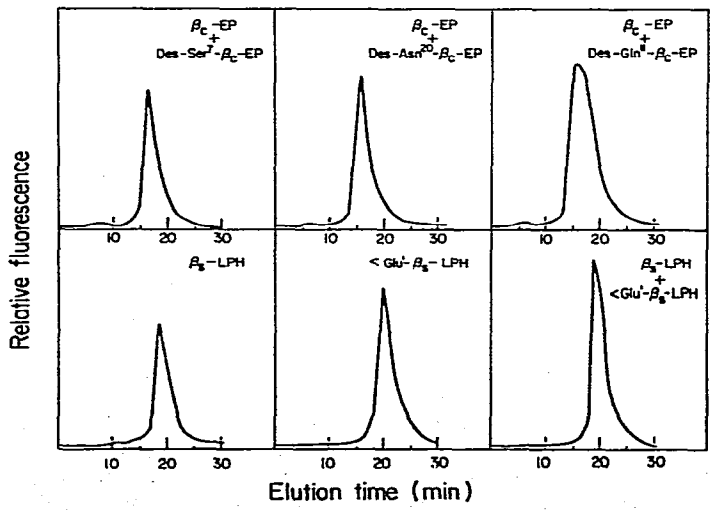

Fig. 5. HPLC of various peptides and their mixtures. Column dead time, 3 min; pre-column dead time, 4 min; sample applied at zero time.

separation of Des-Ser^7-β_c-EP, Des-Ser^{10}-β_c-EP and Des-Thr^{12}-β_c-EP from β_c-EP cannot be achieved in HPLC, as exemplified in one such run (Fig. 5, β_c-EP + Des-Ser^7-β_c-EP). Although β_s-LPH and <Glu^1-β_s-LPH can be effectively chromatographed on HPLC, they cannot be separated (Fig. 5). From these results we conclude that the amino acid residues that probably interact with the support in HPLC are those involving the bulky hydrophobic side-chains. Interaction with most of the other neutral residues may be too weak to afford effective "handles" for separation.

The advantage of a partition process appears to lie in the ability of solvent molecules to make a close approach to different parts of a molecule, particularly as the size of the peptide increases. The present study suggests that the solvent-accessible surface areas of individual amino acid side-chains in a linear peptide of moderate size, such as β-EP, is no less than about 50% of that observed for "naked" side-chains in acetylamino acid amides. This can account for the power of PC to separate peptides that differ in only a single amino acid residue.

ACKNOWLEDGEMENTS

We thank David Chung for HPLC experiments, and K. Hoey and W. F. Hain for technical assistance. This work was supported in part by grants from the National Institute of Health (GM-2907), National Institute of Mental Health (MH-30245) and the Hormone Research Foundation.

REFERENCES

1 J. Porath and P. Flodin, *Nature (London)*, 183 (1959) 1657.
2 D. Yamashiro, *Nature (London)*, 201 (1964) 76.
3 G. Schmidt-Kastner, *Naturwissenschaften*, 51 (1964) 38.
4 D. Yamashiro, in C. H. Li (Editor), *Hormonal Proteins and Peptides*, Vol. 9, Academic Press, New York, 1980, p. 25.
5 D. Yamashiro and J. Blake, in C. H. Li (Editor), *Versatility of Proteins*, Academic Press, New York, 1978, p. 81.
6 D. Yamashiro, *Int.J. Peptide Protein Res.*, 13 (1979) 5.
7 J. L. Meek, *Proc. Nat. Acad. Sci. U.S.*, 77 (1980) 1632.
8 R. B. Hermann, *J. Phys. Chem.*, 71 (1972) 2754.
9 J. A. Reynolds, D. B. Gilbert and C. Tanford, *Proc. Nat. Acad. Sci. U.S.*, 71 (1974) 2925.
10 C. Chothia, *Nature (London)*, 248 (1974) 338.
11 E. C. Bate-Smith and R. G. Westall, *Biochim. Biophys. Acta*, 4 (1950) 427.
12 A. J. P. Martin, *Biochem. Soc. Symp.*, 3 (1950) 4.
13 D. Yamashiro, *J. Org. Chem.*, 42 (1977) 523.
14 C. H. Li, D. Yamashiro, L-F. Tseng and H. H. Loh, *J. Med. Chem.*, 20 (1977) 325.
15 D. Yamashiro and C. H. Li, *Biochim. Biophys. Acta*, 451 (1976) 124.
16 M. Rubinstein, S. Stein and S. Udenfriend, *Proc. Nat. Acad. Sci. U.S.*, 74 (1977) 4969.
17 C. H. Li and D. Chung, *Proc. Nat. Acad. Sci. U.S.*, 73 (1976) 1145.
18 Y. Nozaki and C. Tanford, *J. Biol. Chem.*, 246 (1971) 2211.
19 A. Shrake and J. A. Rupley, *J. Mol. Biol.*, 79 (1973) 351.