Journal of Chromatography, 604 (1992) 47–53
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 197

Rapid displacement chromatography of melittin on micropellicular octadecyl-silica

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ABSTRACT

Rapid high-performance liquid chromatographic analysis and displacement purification of melittin and its variants were carried out by reversed-phase chromatography. High speed of separation was achieved by the use of columns packed with a micropellicular stationary phase consisting of a thin C_{18} hydrocarbonaceous layer on the surface of 2-μm fluid-impervious silica microspheres at elevated temperature. In the case of melittin from bee venom or its synthetic variants the plots of the logarithmic retention factor against acetonitrile concentration in the eluent were straight lines whereas the van 't Hoff plots in the temperature range from 20 to 80°C were non-linear. Purification of melittins by displacement was carried out with benzyldimethylhexadecyl ammonium chloride as the displacer. In a 20-min displacement run at 40°C about 5 mg of highly pure melittin were isolated from 10 mg of synthetic mixture by using a 105 × 4.6 mm column. The results demonstrate that columns packed with micropellicular sorbents not only facilitate rapid high-performance liquid chromatographic analysis but are also suitable for fast peptide purification with high recovery.

INTRODUCTION

Melittin, a polypeptide consisting of 26 amino acids is the main component of honey bee venom. It binds strongly to natural and synthetic phospholipid bilayers and has served as a model peptide in numerous biochemical and pharmacological studies [1–4]. Its physico-chemical properties are well established. The amino acid sequence of melittin is as follows

NH_2–G–I–G–A–V–L–K–V–L–T–T–G–L–
1 12
P–A–L–I–S–W–I–K–R–K–R–Q–Q–COOH
14 26

In a separate study, two structural variants of melittin have been synthesized to investigate their structure and interaction with biological membranes in comparison to those of melittin proper [5]. These two peptides are G12A and P14A melittins in which alanine is substituted for glycine in the 12th position and for proline in the 14th position, respectively.

The need for highly pure melittin and its variants prompted us to investigate the potential of reversed-phase chromatography in the displacement mode for their purification. Whereas reversed-phase chromatography in the elution mode has widely been used for analysis and purification of peptides by high-performance liquid chromatography (HPLC) [6,7], the merits of displacement for preparative-scale purification have only recently been recognized [8–14]. Introduction of micropellicular sorbents that offer higher separation speed and product recovery than conventional porous stationary phases has further advanced the chromatography of biopolymers [15–18]. Therefore, columns packed with micropellicular stationary phases were employed for both the rapid HPLC analysis and displacement purification of melittins. In order to facilitate the analysis of fractions obtained in preparative chromatography, a rapid HPLC method was developed for the rapid assay of melittin by appropriate modifications of the instrument as described below in the Experimental section.

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0021-9673/92/$05.00

EXPERIMENTAL

Materials

Reagent-grade trifluoroacetic acid (TFA), formic acid (88%) and HPLC-grade acetonitrile (ACN) were obtained from Baker (Phillipsburgh, NJ, USA). Benzyldimethylhexadecyl ammonium chloride was from Fluka (Rokonkoma, NY, USA). Bee venom, melittin, apamin, and phospholipase A_2 were purchased from Sigma (St. Louis, MO, USA). The two melittin variants P14A and G12A were gifts from Dr. C. Dempsy. Hy-Tach columns (30 × 4.6 and 105 × 4.6 mm), packed with silica-based C_{18} micropellicular sorbents of 2-μm particle diameter were from Glycotech (Hamden, CT, USA). Eluents were prepared with deionized water from NanoPure unit (Barnstead, Boston, MA, USA), filtered through 0.45-μm membranes and degassed by sparging with helium.

Instruments

The liquid chromatograph consisted of a Series 4 pump, Model LC-85B variable-wavelength detector, Model LCI-100 printer plotter and Model 7500 laboratory computer (Perkin-Elmer, Norwalk, CT, USA). Before entering the column the eluent passed through a 500 × 0.25 mm heat exchanger coil and a Model 7125 sample injector (Rheodyne, Cotati, CA, USA) that were placed in a Model DL-8 constant-temperature bath (Haake-Buchler, Saddlebrook, NJ, USA). A 3.0-ml sample loop was used in displacement experiments. Some of the analytical separations were obtained with Model 1090 liquid chromatograph equipped with column compartment, diode array detector and Model 79994A analytical work station (Hewlett-Packard, Avondale, PA, USA).

Methods

Analytical separations. HPLC analysis of bee venom and synthetic melittins was carried out with short columns (30 × 4.6 mm) packed with micropellicular octadecyl silica. Elevated temperature and relatively high flow-rates were employed for the analysis by gradient elution with increasing ACN concentration in aqueous TFA as described previously for other peptide mixtures [15–17]. The actual gradient profiles were determined by tracer technique as described earlier [16].

Displacement chromatography. Instrumentation and methodology for displacement chromatography were similar to those described elsewhere [8,11,13]. The column (105 × 4.6 mm) was first equilibrated with a solution of 0.1% (v/v) TFA in water that served as a carrier. Thereafter, a solution of the sample mixture (feed) in the carrier was introduced into the column, and the separation was carried out with the displacer; 25 m*M* benzyldimethylhexadecyl ammonium chloride in 10% (v/v) aqueous ACN containing 0.1% (v/v) TFA. The "light" and "heavy" impurities shown on displacement chromatograms refer to those components of the sample that are retained less or more than the key product, *i.e.*, melittin or its analogue, respectively, as shown by the analytical separation of individual samples (Fig. 1). Fractions of the column effluent were collected at 30- and 15-s intervals for displacement experiments carried out at 23 and 40°C, respectively. A 5-μl aliquot from each fraction was mixed with 95 μl of 0.1% TFA in water and 20 μl from the diluted sample were analyzed by rapid HPLC. Quantitative measurements were made by the peak area and a calibration curve was prepared using melittin from bee venom as the reference. This preparation (Sigma Lot No. 116F-4011) was 73% (w/w) pure, according to the supplier and this value was used for the calibration curve. In the case of impurities present in the crude samples, it was assumed that these compounds have the same extinction coefficient as melittin.

Column regeneration. After emergence of the displacer front, the column was washed first with 3 ml of 50% (v/v) formic acid in 2-propanol then with 3 ml of 95% (v/v) aqueous ACN containing 0.1% (v/v) TFA. Subsequently, the column was reequilibrated with 6 ml of the carrier. Regeneration of the column was carried out at 80°C and at a flow-rate of 3 ml/min. In most cases the entire process was completed in less than 5 min.

RESULTS AND DISCUSSION

Analysis of bee venom and synthetic melittins

Bee venom is a complex biological material and contains besides melittin, which comprises nearly 50% of the total dry weight, proteins such as phospholipase A_2, hyaluronidase as well as other peptides such as apamin, minimine and mast cell de-

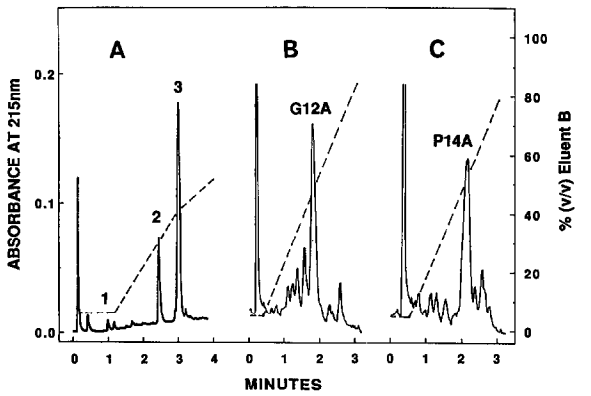

Fig. 1. Analytical chromatograms of samples containing (A) bee venom, (B) G12A melittin and (C) P14A melittin from synthetic mixtures. Column, Hy-Tach micropellicular C_{18} silica, 30 × 4.6 mm; eluent A, 0.1% (v/v) TFA in water; eluent B, 0.1% (v/v) TFA and 95% (v/v) ACN in water; flow-rate, 1 ml/min; temperature, 23°C. Elution: (A) linear increase of eluent B from 10 to 60% in 3 min and from 60 to 80% in 2 min; (B and C) linear gradient of eluent B from 5 to 80% in 3 min. Bee venom components identified were apamin (1), phospholipase A_2 (2) and melittin (3). Dotted lines represent the actual gradient profile of eluent B during the separation.

granulating (MCD) peptide. Fig. 1A shows an analytical chromatogram of bee venom obtained by rapid HPLC with a short column containing C_{18} micropellicular stationary phase. As seen the separation of bee venom constituents is brought about in 3 min. Similarly, samples of synthetic G12A and P14A melittins were also analyzed rapidly using the same column but different gradient as illustrated by the chromatograms in Fig. 1B and C. The relative hydrophobicity increased in the order, melittin < G12A < P14A. The weight percentage of melittin in bee venom and those of G12A and P14A melittins in the synthetic mixtures were found to be 54, 28 and 30%, respectively, as measured by the peak area in the analytical chromatograms.

Effect of mobile phase composition and temperature on the retention behavior of melittins

Crystallographic studied [1] suggest that the melittin molecule contains two α-helices comprising the amino acid residues 1–10 and 13–26, which are

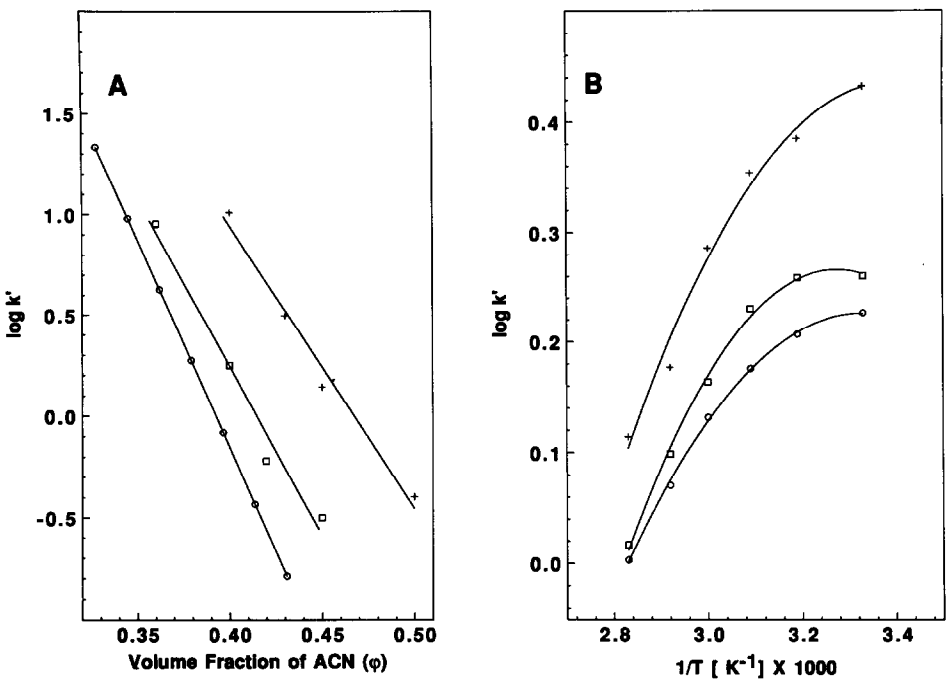

Fig. 2. (A). Effect of ACN concentration on the retention of melittins. Hy-Tach micropellicular C_{18} silica column, 105 × 4.6 mm; isocratic elution with aqueous eluents containing 0.1% (v/v) TFA and various concentrations of ACN; flow-rate 0.2 ml/min; temperature, 23°C. Samples were 2 μg each of melittin (○), G12A (□) and P14A (+) melittins. (B). Van 't Hoff plots for the retention of melittins. Hy-Tach micropellicular C_{18} silica column, 105 × 4.6 mm, isocratic elution with aqueous eluent containing 0.1% (v/v) TFA with 36% (v/v) ACN for both melittin (○) and G12A melittin (□) and with 38% (v/v) ACN for P14A melittin (+). k' = Retention factor; T = temperature.

linked together by residues 11 and 12, to give the molecular shape of a bent rod. One side of each helix contains 10 hydrophobic residues and a group of 6 polar amino acids is clustered at the opposite end. High-resolution NMR studies [2] have shown that at acidic pH and low ionic strength, melittin exists predominantly as an extended monomer and the residues 5–9 and 14–19 in the helices are maintained in highly structured order. This conformer is in equilibrium with a low abundant form of melittin due to *cis–trans* isomerism of the L-13–P-14 peptide bond.

Plots of logarithmic retention factor for melittin and its two synthetic analogues against ACN concentration in the eluent are depicted in Fig. 2A. Melittin elutes at about 40% of eluent B (organic modifier) and the substitution of Ala by Gly or Pro at positions 12 and 14, respectively, results in increasingly stronger binding of the peptide to the hydrophobic chromatographic surface. The latter peptides eluted nearly at 43% and 50% of eluent B, respectively. The data suggest that melittin proper and P14A melittin have the smallest and the largest hydrophobic contact area upon binding to the stationary phase, respectively. It is noted that the plots of log k' *versus* φ are non-parallel straight lines suggesting reversal of selectivity for the three peptides in a range of organic concentration lower than that shown for the data in Fig. 2A.

The retention behavior of melittin and the two synthetic analogues was further investigated by measuring the effect of temperature and the resulting van 't Hoff plots are shown in Fig. 2B. The composition of the eluent used to measure the retention of melittin and G12A melittin was 36% (v/v) aqueous ACN containing 0.1% TFA. As P14A melittin was found to be the most hydrophobic among the three melittin variants and it was eluted with 38% (v/v) aqueous ACN containing 0.1% TFA. In the absence of conformational changes of the eluite or structural changes in the stationary phase, the van 't Hoff plots are usually straight lines in reversed-phase chromatography [19]. As seen in Fig. 2B, the van 't Hoff plots for melittin and the two synthetic variants are curved suggesting changes in eluite conformation and concomitantly in the binding mechanism upon varying the temperature. Further investigations are needed to elucidate physico-chemical aspects of changing temperature on the chromatographic retention behavior of such relatively complex molecules as melittins.

Displacement chromatography of melittins

So far, displacement chromatography in the laboratory has been carried out exclusively by HPLC columns packed with conventional porous sorbents that have relatively large accessible surface area required in the chromatography of small molecules. Recent studies with micropellicular sorbents from this and other laboratories have revealed that with stationary phase particles which consist of fluid-impervious micropherical support coated with a thin retentive layer, analytical speed and column efficiency can be enhanced, particularly for separation of large molecules. Although the chromatographic surface area in columns packed with micropellicular stationary phases is relatively low, the enhanced efficiency, column stability and increased sample recovery makes them useful also for micropreparative isolation of proteinaceous compounds [15–18, 20–22]. The analysis of fractions collected during the displacement experiment was carried out with micropellicular C_{18} columns (30 × 4.6 mm) operated at 80°C. Under these conditions melittin containing samples were analyzed in less than a minute and a typical chromatogram is shown in Fig. 3.

Displacement chromatography on 105 × 4.6 mm columns yielded several milligrams of pure melittin from bee venom or melittin variants from synthetic mixtures. The feed was dissolved in 0.1% (v/v)

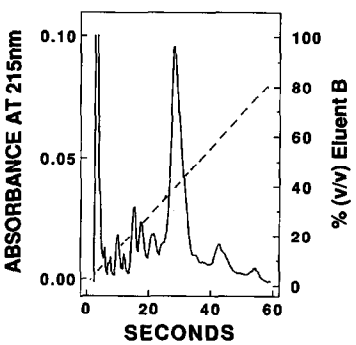

Fig. 3. Rapid HPLC analysis of melittin. Hy-Tach micropellicular C_{18} silica column, 30 × 4.6 mm; eluents as in Fig. 1; linear gradient from 0 to 80% B in 1.0 min, flow-rate, 3.0 ml/min, temperature 80°C. Sample: 5 μg melittin from Sigma. Dotted lines represent the actual gradient profile of eluent B.

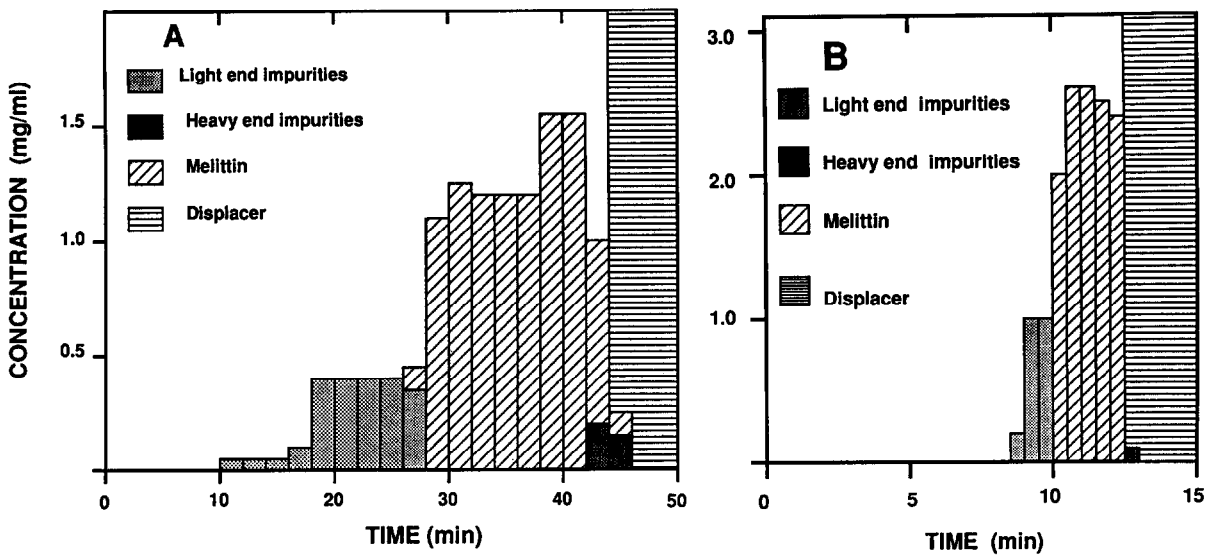

Fig. 4. Displacement chromatogram of crude synthetic P14A melittin at 23°C (A) and 40°C (B). Column, Hy-Tach micropellicular C_{18} silica, 105 × 4.6 mm, carrier, 0.1% (v/v) TFA in water; displacer, 25 m*M* benzyldimethylhexadecyl ammonium chloride in 10% (v/v) aqueous ACN containing 0.1% TFA; flow-rate, 0.2 ml/min (A) and 0.6 ml/min (B); feed: 10 mg of P14A melittin in 1.5 ml. Fractions were collected at 30 and 15 s at 23°C and 40°C, respectively. Aliquots of 5 μl of each fraction were mixed with 95 μl of 0.1% TFA and 20-μl aliquots of the diluted samples were analyzed by the procedure described in Fig. 3. The concentration of melittin was calculated on the basis of peak area and from the calibration curve (not shown) obtained at 215 nm using Sigma melittin as the reference.

aqueous TFA which was used also as the carrier to facilitate strong binding of the sample components to the chromatographic surface during loading on the the column. The displacer was 25 m*M* benzyldimethylhexadecyl ammonium chloride in aqueous solution containing 10% (v/v) ACN and 0.1% (v/v) TFA. This compound has been used successfully as the displacer for the separation of various peptides in our laboratory [10]. A displacement chromatogram of 10 mg of synthetic P14A melittin obtained at 23°C and at a flow-rate of 0.2 ml/min is shown in Fig. 4A. Under these conditions the breakthrough of the displacer front occurred in 45 min and the bands of product and the impurities show some overlap.

In order to speed up the separation the column temperature was increased to 40°C so that the viscosity of the mobile phase was reduced and the favorable effect of elevated temperature on both the diffusivity and sorption kinetics could be exploited for anhancement of separation efficiency. The displacement of the crude P14A melittin at 40°C and at flow-rate of 0.6 ml/min is illustrated by the chromatogram in Fig. 4B. Comparison of chromatograms obtained at 23°C (Fig. 4A) and 40°C (Fig 4B) shows that the efficiency of separation improved considerably and the time of separation decreased significantly with respect to the results obtained at 23°C and at 0.2 ml/min. The complete cycle including separation by displacement chromatography and column regeneration was completed in about

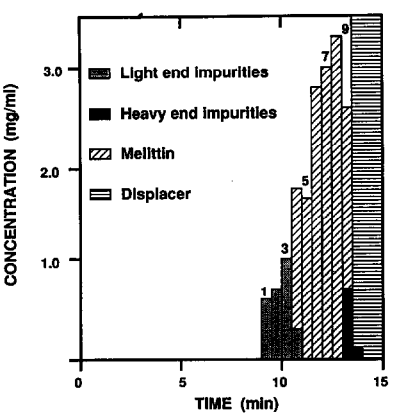

Fig. 5. Displacement chromatogram of bee venom. Conditions as in Fig. 4B except the feed was 10 mg of bee venom.

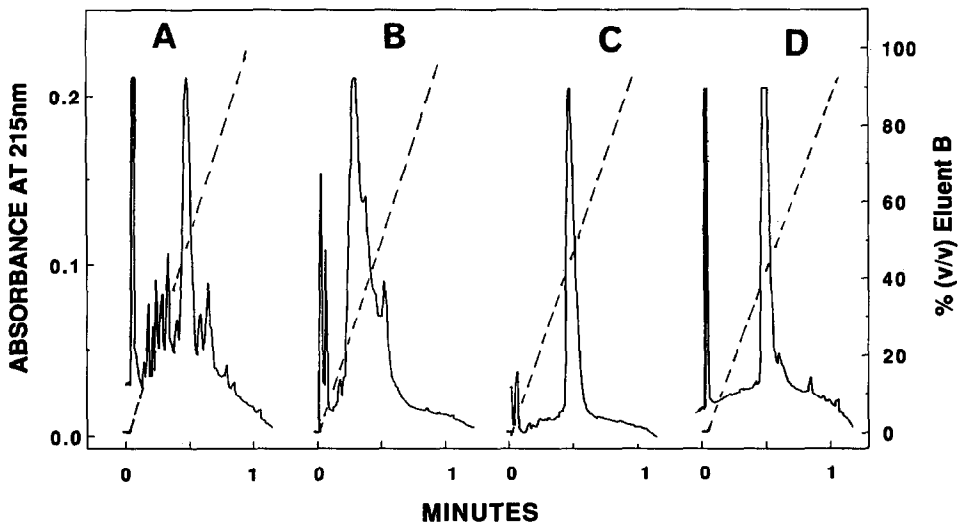

Fig. 6. Monitoring of the column effluent during displacement chromatography of bee venom. Chromatograms are shown for the analysis of fractions corresponding to (A) the feed, (B) impurities (fraction 3), (C) melittin (fraction 7) and (D) heavy-end impurities (fraction 9) as shown by the displacement diagram in Fig. 5. Dottes lines represent the actual gradient profile of eluent B.

20 min. Similar results were obtained for isolation of melittin from bee venom as shown in Fig. 5. The versatility of the rapid HPLC method is illustrated by good-resolution and high-speed analysis of the column effluent during the displacement chromatography of bee venom. The results of analysis of the feed and fractions containing the light-end impurities, melittin and heavy-end impurities are depicted in Fig. 6. The fractions of melittin collected between 11.5 and 13 min and free from other peptide components as determined by rapid HPLC were pooled. Total yield of purified melittin was 5.23 mg and the purity of the pooled sample was 99.1% as determined from the area under the peak by reversed-phase chromatography and from a calibration curve prepared from Sigma melittin as reference. It is noted that the elution pattern of the primary component in the displacement chromatogram varied resulting in steps in the bar diagram as depicted in Figs. 4 and 5. This could be due to incomplete development of the displacement train that can be improved by using longer columns. Under these conditions the bar heights are expected in to increase in sample concentration.

CONCLUSIONS

Displacement chromatography with micropellicular sorbents offers significant advantages for rapid purification of proteins and peptides on a micropreparative scale. The advantages of this approach are particularly pronounced when the column is operated at elevated temperature and at relatively high flow-rates as illustrated by the purification of melittin and its variants in less tan 15 min. Regeneration of the column, a generally time consuming step with traditional porous packings, required less than 5 min. Columns packed with micropellicular stationary phases are eminently suitable also for rapid analysis of fractions of the column effluent in preparative chromatography and offer the potential for process monitoring by HPLC.

ACKNOWLEDGEMENTS

The authors thank Dr. C. Dempsy for synthetic melittin variants. This work supported by Grants No. GM 20993 from National Institute of Health, US Public Health Service and No. BCS-9014119 from National Science Foundation.

REFERENCES

1 T. C. Terwilliger and D. Eisenberg, *J. Biol. Chem.*, 257 (1982) 6016.
2 J. Lauterwein, L. R. Brown and K. Wütrich, *Biochim. Biophys. Acta*, 622 (1980) 219.

3 A. W. Bernheimer and B. Rudy, *Biochim. Biophys. Acta*, 864 (1986) 123.
4 W. F. DeGrado, F. J. Kézdi and E. T. Keiser, *J. Am. Chem. Soc.*, 103 (1981) 679.
5 C. Dempsy and D. Engelman, Department of Molecular Biology and Biophysics, Yale University, New Haven, CT, personal communication.
6 W. R. Melander and Cs. Horváth, in Cs. Horváth (Editor), *High-Performance Liquid Chromatography —Advances and Perspectives*, Vol. 2, Academic Press, New York, 1980, pp. 114–319.
7 J. Frenz, W. S. Hancock, W. J. Henzel and Cs. Horváth, in K. M. Gooding and F. E. Regnier (Editors), *HPLC of Biological Macromolecules —Methods and Applications*, Marcel Dekker, New York, 1990, pp. 145–177.
8 J. Frenz and Cs. Horváth, in Cs. Horváth (Editor), *High-Performance Liquid Chromatography —Advances and Perspectives*, Vol. 5, Academic Press, New York, pp. 211–314.
9 H. Kalász and Cs. Horváth, *J. Chromatogr.*, 215 (1981) 295.
10 G. Viscomi, S. Lande and Cs. Horváth, *J. Chromatogr.*, 440 (1988) 157.
11 S. Cramer and Cs. Horváth, *Prep. Chromatogr.*, 1 (1988) 29.
12 Gy. Vigh, Z. Varga-Purchony, G. Szepesi and M. Gazdag, *J. Chromatogr.*, 386 (1987) 353.
13 A. Liao, Z. El Rassi, D. M. LeMaster and Cs. Horváth, *Chromatographia*, 24 (1987) 881.
14 A. Liao and Cs. Horváth, *Ann. N.Y. Acad. Sci.*, 589 (1990) 182.
15 K. Kalghatgi and Cs. Horváth, *J. Chromatogr.*, 398 (1987) 335.
16 K. Kalghatgi and Cs. Horváth, *J. Chromatogr.*, 443 (1988) 343.
17 Y. F. Maa and Cs. Horváth, *J. Chromatogr.*, 445 (1988) 71.
18 Y. F. Maa, S-C. Lin, Cs. Horváth, U-C. Yang and D. M. Crothers, *J. Chromatogr.*, 508 (1990) 61.
19 W. Melander, D. E. Campbell and Cs. Horváth, *J. Chromatogr.*, 158 (1978) 215.
20 K. Kalghatgi, *J. Chromatogr.*, 499 (1990) 267.
21 A. L. Lee, A. W. Liao and Cs. Horváth, *J. Chromatogr.*, 443 (1988) 31.
22 K. Kalghatgi and Cs. Horváth, in Cs. Horváth and J.G. Nickely (Editors), *Analytical Biotechnology —Capillary Electrophoresis and Chromatogrpahy (ACS Symposium Series*, No. 434), American Chemical Society, Washington, DC, 1990, pp. 162–180.