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COMPARISON OF REVERSED-PHASE AND WEAK ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR PEPTIDE SEPARATIONS

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SUMMARY

Weak anion-exchange and reversed-phase high-performance liquid chromatographic methods for peptide separations were compared using a tryptic digest of "rat small myelin basic protein". In these experiments, a number of tryptic peptides that were not resolved on the reversed-phase column could be separated on the weak anion-exchange column, and in other instances, as might be expected, reversed-phase chromatography provided better resolution of certain peptides than did the weak anion-exchange method. The results obtained strongly suggest that the combined use of these two methods of separation, which utilize different selectivities, can provide an excellent improvement in resolving power for a number of peptide separations.

INTRODUCTION

During the past decade, high-performance liquid chromatography (HPLC) has emerged as a powerful tool for peptide separations. The reversed-phase mode of HPLC (RP-HPLC) has become the most popular and broadly used technique for many separation problems in peptide chemistry[1–13]. Ion-exchange HPLC has also been employed for peptide separations, although to a lesser extent[14–18].

We recently reported a method for peptide separations using anion-exchange HPLC (AE-HPLC) on a weak AE bonded phase with mixtures of acetonitrile and triethylammonium acetate (TEAA) buffer as the eluent[19–23]. In the present work, we compare this method and a standard RP-HPLC method for peptide separations using a tryptic digest of "rat small myelin basic protein" (RSMBP) isolated from rat brain.

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EXPERIMENTAL

Apparatus

A Model 1084B liquid chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) equipped with a microprocessor, an automatic injector and a variable-wavelength detector was used. AE separations were carried out on a 30 × 0.4 cm MicroPak AX-10 column (Varian, Walnut Creek, CA, U.S.A.). A 15 × 0.46 cm Supelcosil LC-8-DB column (Supelco, Bellefonte, PA, U.S.A.) was used for RP separations. Specific conditions for all separations are given in the Figures.

Materials

RSMBP was a gift of Gladys Diebler and Marion Kies of the National Institutes of Health. Triethylamine was purchased from Eastman-Kodak (Rochester, NY, U.S.A.) and purified by distillation from phthalic anhydride. TEAA buffer solutions were prepared by titrating 0.01 *M* acetic acid solutions with triethylamine to pH 6.0. Glass-distilled acetonitrile was purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Trifluoroacetic acid (TFA) was obtained from Sigma (St. Louis, MO, U.S.A.). Water purified through a Millipore system (Millipore, Bedford, MA, U.S.A.) was used for all purposes. Aqueous solvents were filtered prior to use. Trypsin was purchased from Worthington Biochemicals (Freehold, NJ, U.S.A.).

Tryptic digestion

Two milligrams of RSMBP were incubated at 37°C for 24 h in 1 ml of 0.1 *M* ammonium bicarbonate buffer (pH 8.2) with a peptide–enzyme ratio of 50:1 (w/w). After incubation, the sample was freeze-dried and then taken up in 0.5 ml of water for injections.

Amino acid analysis

Eluted peptides were dried *in vacuo* and hydrolyzed with constant-boiling HCl in evacuated and sealed tubes at 110°C for 24 h. The hydrolyzates were analyzed on a Durrum D-500 amino acid analyzer.

RESULTS AND DISCUSSION

RSMBP contains 127 amino acid residues[24] and is expected to yield twenty-one peptide fragments and two arginine molecules upon digestion with trypsin. The tryptic digest of this protein was analyzed by weak AE-HPLC as previously described[19–23] and by RP-HPLC using a solvent system containing 0.1 % TFA in water and acetonitrile[12]. For peak assignments, in both systems, eluted peptides were collected and then subjected to amino acid analysis, and structural assignments were made by referring to tryptic peptides expected from the known sequence of RSMBP.

Fig. 1 shows the separation of the tryptic digest of RSMBP by weak AE-HPLC, where a resolution of sixteen peaks was observed. Amino acid sequences of the corresponding peptides and their positions in the total sequence are given in Table I. Peaks 7, 9, 11 and 13 each contain two peptides, which were not resolved from each other, whereas peaks 2–6, 8, 10, 12 and 14–16 correspond to single peptides. Peak 1 contains the two expected dipeptides and two free arginine molecules. Inspection of Table I shows that the identified peptides cover the total sequence of RSMBP.

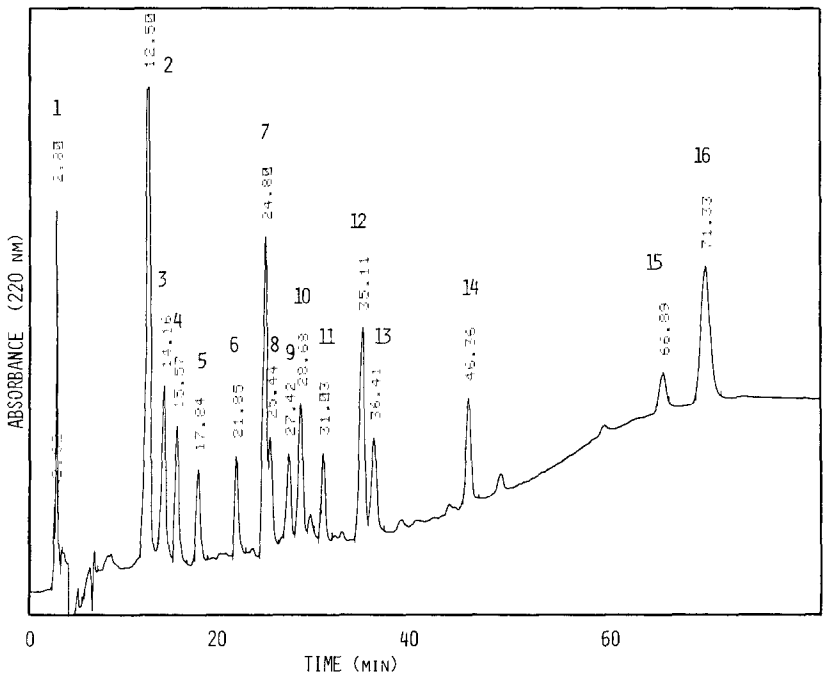


Fig. 1. Separation of a tryptic digest of RSMBP by weak AE-HPLC. Column: MicroPak AX-10 (10 μm), 30 × 0.4 cm. Temperature: 40°C. Eluents: A, acetonitrile; B, 0.01 *M* TEAA buffer (pH 6.0). Gradient program: linear starting from 23% B with a rate of 0.7% B/min for 40 min then 1% B/min to 100% B. Flow-rate: 1 ml/min. Peak identification and sequences are given in Table I. a.u.f.s. = 0.2 at 220 nm.

TABLE I

PEAK IDENTIFICATION AND SEQUENCES IN FIG. 1

Peak	*Sequence*	*Position in sequence*
1	His-Arg	32–33
	Gly-Arg	103–104
	2 Arg	54, 127
2	Phe-Ser-Trp-Gly-Gly-Arg	111–116
3	His-Gly-Phe-Leu-Pro-Arg	26–31
4	Asn-Ile-Val-Thr-Pro-Arg	89–94
5	Gly-Leu-Ser-Leu-Ser-Arg	105–110
6	Ser-Gly-Ser-Pro-Met-Ala-Arg	120–126
7	Arg-Pro-Ser-Gln-Arg	5–9
	Thr-Thr-His-Tyr-Gly-Ser-Leu-Pro-Gln-Lys	64–73
8	Ser-Gln-Arg	74–76
9	Ac-Ala-Ser-Gln-Lys	1–4
	Gly-Ala-Pro-Lys	50–53
10	Thr-Pro-Pro-Pro-Ser-Gln-Gly-Lys	95–102
11	His-Gly-Ser-Lys	10–13
	Gly-Ser-Gly-Lys	55–58
12	Tyr-Leu-Ala-Thr-Ala-Ser-Thr-Met-Asp-His-Ala-Arg	14–25
13	Phe-Phe-Ser-Gly-Asp-Arg	44–49
	Asp-Ser-Arg	117–119
14	Asp-Ser-His-Thr-Arg	59–63
15	Asp-Thr-Gly-Ile-Leu-Asp-Ser-Ile-Gly-Arg	34–43
16	Thr-Gln-Asp-Glu-Asn-Pro-Val-Val-His-Phe-Phe-Lys	77–88

Fig. 2 shows the separation by RP-HPLC of another aliquot of the same sample. An accumulation of several peaks near the void volume (designated as 1) and resolution of twelve peaks were observed. The amino acid sequences of the peptides and their positions in the total sequence are given in Table II. By deduction from the known sequence of RSMBP[24], the number of peptides accumulated near the void volume should total nine, including the two dipeptides, because peaks 2–13 contain the remaining twelve tryptic peptides contained in RSMBP (compare Tables I and II).

Comparison of the results obtained by the two methods applied here shows that all of the tryptic peptides, except for the two dipeptides, were strongly retained on the weak AE column, whereas some additional peptides had little or no retention on the RP column and were eluted without being resolved from one another. As a demonstration of the different selectivities of these two separation principles, peptides eluting near the void volume of the RP column (designated as peak 1 in Fig. 2) were collected and, after removal of the volatile solvent, injected onto the weak AE column. Six peaks were resolved (Fig. 3), and the amino acid sequences of the peptides contained in them are given in Table III. Peak I corresponds to the two dipeptides and two free arginine molecules. Peaks 3 and 4 each represent two peptides, which were not separated from each other, whereas peaks 2, 5 and 6 correspond to single peptides. This means that these peptides, except for the two dipeptides, were strongly retained on the weak AE column in contrast to the RP column, and three of them

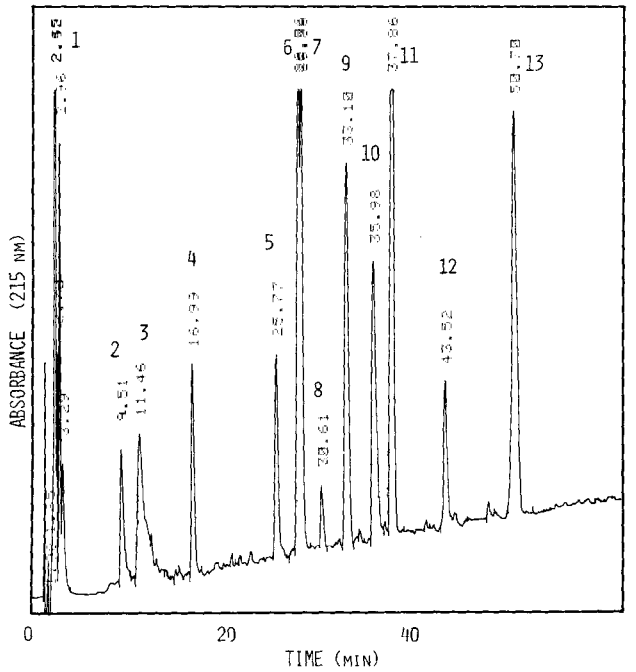


Fig. 2. Separation of a tryptic digest of RSMBP by RP-HPLC. Column: Supelcosil LC-8-DB (5 μm), 15 × 0.46 cm. Temperature: 30°C. Eluents: A, 0.1% TFA in water; B, 0.1% TFA in acetonitrile. Gradient program: linear starting from 0% B with a rate of 0.5% B/min. Flow-rate: 1.2 ml/min. Peak identification and sequences are given in Table II. a.u.f.s. = 0.4 at 215 nm.

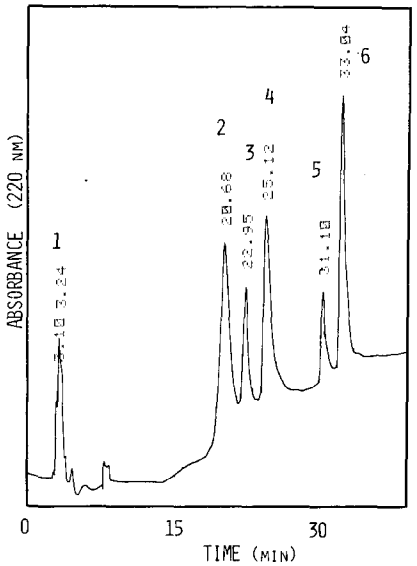
TABLE II

PEAK IDENTIFICATION AND SEQUENCES IN FIG. 2

Peak	*Sequence*	*Position in sequence*
1	Ac-Ala-Ser-Gly-Lys	1–4
	His-Gly-Ser-Lys	10–13
	His-Arg	32–33
	Gly-Ala-Pro-Lys	50–53
	2 Arg	54, 127
	Gly-Ser-Gly-Lys	55–58
	Asp-Ser-His-Thr-Arg	59–63
	Ser-Gln-Arg	74–76
	Gly-Arg	103–104
	Asp-Ser-Arg	117–119
2	Arg-Pro-Ser-Gln-Arg	5–9
3	Thr-Pro-Pro-Pro-Ser-Gln-Gly-Lys	95–102
4	Ser-Gly-Ser-Pro-Met-Ala-Arg	120–126
5	Asn-Ile-Val-Thr-Pro-Arg	89–94
6	Thr-Thr-His-Tyr-Gly-Ser-Leu-Pro-Gln-Lys	64–73
7	Phe-Phe-Ser-Gly-Asp-Arg	44–49
8	Gly-Leu-Ser-Leu-Ser-Arg	105–110
9	Tyr-Leu-Ala-Thr-Ala-Ser-Thr-Met-Asp-His-Ala-Arg	14–25
10	His-Gly-Phe-Leu-Pro-Arg	26–31
11	Phe-Ser-Trp-Gly-Gly-Arg	111–116
12	Asp-Thr-Gly-Ile-Leu-Asp-Ser-Ile-Gly-Arg	34–43
13	Thr-Gln-Asp-Glu-Asn-Pro-Val-Val-His-Phe-Phe-Lys	77–88

could be obtained in pure form. Another instance of the advantage of weak AE-HPLC over RP-HPLC in this particular application was the separation of two peptides by weak AE-HPLC (peaks 7 and 13 in Fig. 1) that were only slightly resolved on the RP column (peaks 6 and 7 in Fig. 2 and Table II). To illustrate this more clearly, the peptides from the RP column were collected and, after removal of the volatile solvent, injected onto the weak AE column. Fig. 4 shows their complete separation by weak AE-HPLC, and peaks 1 and 2 correspond, respectively, to the peptides represented by peaks 6 and 7 in Fig. 2. These two examples are also excellent demonstrations of the combined use of RP- and weak AE-HPLC methods for isolation of peptides, which were not resolved on the RP column (peaks 6 and 7 in Fig. 2).

In another instance of the combined use of the two methods, RP-HPLC could be used as a second separation step for four peptides which eluted as two pairs on the weak AE column (peaks 7 and 13 in Fig. 1 and Table I). As Fig. 2 shows, these two pairs were completely separated from each other on the RP column (peaks 2 and 6, and 1 and 7). However, in this case, the peptide of residues 117–119 was coeluted with other peptides in peak 1 and the peptides represented by peaks 6 and 7 were also coeluted (Fig. 2 and Table II); RP-HPLC would also function as a second separation step in this instance. Only the peptide of residues 5–9 could be obtained in pure form (peak 2 in Fig. 2) when the whole tryptic digest was injected onto the RP column. The peptide of residues 117–119 could also be isolated in pure form by first applying RP-HPLC to the tryptic mixture and then weak AE-HPLC (peak 5 in Fig. 3). Two pairs of peptides containing residues 1–4 and 50–53 (peak 9 in Fig. 1 and peak 1 in Fig. 2),



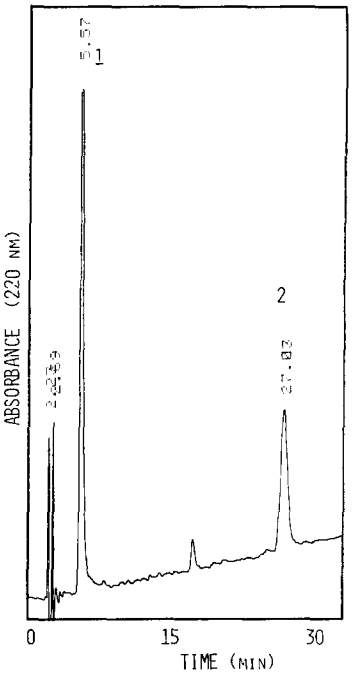


Fig. 3. Separation of peptides eluted from RP column (designated as 1 in Fig. 2) by weak AE-HPLC. Column details as in Fig. 1 except for temperature (50°C) and gradient program: isocratic with 26% B for 10 min then 1.5% B/min. Peak identification and sequences are given in Table III.

Fig. 4. Separation of peptides eluted from RP column (peaks 6 and 7 in Fig. 2) by weak AE-HPLC. Column details as in Fig. 1 except for gradient program: starting from 45% B with a rate of 0.5% B/min. Peaks 1 and 2 correspond to peaks 6 and 7 in Fig. 2, respectively. a.u.f.s. = 0.1 at 220 nm.

TABLE III

PEAK IDENTIFICATION AND SEQUENCES IN FIG. 3

Peak	*Sequence*	*Position in sequence*
1	His-Arg	32–33
	Gly-Arg	103 104
	2 Arg	54, 127
2	Ser-Gln-Arg	74–76
3	Ac-Ala-Ser-Gln-Lys	1–4
	Gly-Ala-Pro-Lys	50–53
4	His-Gly-Ser-Lys	10–13
	Gly-Ser-Gly-Lys	55–58
5	Asp-Ser-Arg	117–119
6	Asp-Ser-His-Thr-Arg	59–63

and 10–13 and 55–58 (peak 11 in Fig. 1 and peak 1 in Fig. 2), were the only pairs that could not be obtained in pure form by the combined use of RP-HPLC and weak AE-HPLC.

CONCLUSIONS

The results obtained clearly demonstrate the usefulness of the different selectivities of RP- and weak AE-HPLC methods for peptide separations. It is also apparent that the combined application of these two different separation principles can provide even more resolving power and thus yield a higher probability for the complete separation of a given mixture of peptides into its components.

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