Journal of Chromatography, 326 (1985) 433–442
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 581

ISOLATION OF TRYPTIC PEPTIDES OF MYELIN BASIC PROTEIN BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

GLADYS E. DEIBLER*, LISA F. BOYD, RUSSELL E. MARTENSON and MARIAN W. KIES
Section on Myelin Chemistry, Laboratory of Cerebral Metabolism, Building 36, Room 1A-05, National Institute of Mental Health, 9000 Rockville Pike, Bethesda, MD 20205 (U.S.A.)

SUMMARY

A reversed-phase high-performance liquid chromatography (HPLC) system was developed to obtain individual tryptic peptides of myelin basic protein (BP). Because of the similar charge and hydrophobicity of some of the tryptic peptides of the whole protein, several of these were not clearly separated by a single HPLC system. Therefore, the BP was first cleaved specifically between residues 97 and 98 with thrombin, and the two resulting fragments were separated by ion-exchange chromatography. When the thrombic fragments were digested with trypsin separately and subjected to HPLC, all of the peptides were satisfactorily separated. Elution times of all of the tryptic peptides of human BP were established. Differences among homologous peptides, derived from different mammalian BPs, were readily detected from their elution patterns inasmuch as a change in a single amino acid residue was usually sufficient to cause a shift in the retention time of the peptide. An amino acid difference detected by a peak shift could be confirmed by amino acid analysis. The technique has been used to isolate short peptides of rabbit, monkey, porcine, bovine, and human BP for sequence analysis.

INTRODUCTION

Characterization of tryptic digests by high-performance liquid chromatography (HPLC) has largely replaced paper chromatography and electrophoresis. The procedure is faster and more sensitive, and it can be used for positive identification of individual peptides when the latter are completely separated. Recently, HPLC was used successfully to compare tryptic peptides of the 14 kilodalton (kD) myelin basic protein (BP) from mouse with those of the corresponding BP from rat[1]. The elution patterns obtained from the two proteins were similar, but not all of the peptides were completely separated. Dizdaroglu and Krutzsch[2] have described a procedure which can be used to identify the known tryptic peptides derived from the 14 kD BP of rat, but they were forced to use two column fractionations, reversed-phase and weak anion-exchange, to identify all of the peptides.

The present paper describes a reversed-phase HPLC technique which can be used to analyze all of the tryptic peptides of the 18.5 kD BP molecule without any

problems of peak overlap. This is achieved by first subjecting the BP to limited thrombic digestion[3] so that two large fragments, Peptides 1–97 and 98–170, are formed. These peptides are easily separated by carboxymethylcellulose column chromatography[3]. When tryptic digests of these two large fragments are subjected to HPLC, the individual peptides can be recovered for analysis. In the present paper, human BP is used to illustrate the method. The procedure has also been applied successfully to porcine, bovine, rabbit, and monkey BPs[4,5].

EXPERIMENTAL

Materials

Acetonitrile was purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Sequanal-grade trifluoroacetic acid (TFA) was obtained in sealed 1-g ampules from Pierce (Rockford, IL, U.S.A.). The water was purified by glass distillation in a glass still, equipped with a UV light purchased from Kontes Scientific Glassware/Instruments (Vineland, NJ, U.S.A.). L-Tosylamido-2-phenylethylchloromethyl ketone-treated trypsin was purchased from Worthington Biochemical (Freehold, NJ, U.S.A.). Thrombin (E.C. 3.4.21.5) from human plasma was purchased from Sigma (St. Louis, MO, U.S.A.).

Methods

Thrombic digestion of human BP and purification of peptides. A 100-mg amount of human BP-component 1[6] was dissolved in 25 ml of 0.1 *M* ammonium acetate (pH 7.5), and incubated for 1.5 h at 25°C with 100 μl of thrombin (1 NIH unit/μl)[3]. The incubation mixture was adjusted to pH 3.0 with 1 *M* acetic acid and lyophilized. The lyophilized digest was dissolved in 0.01 *M* acetic acid–0.005 *M* sodium hydroxide (pH 4.7), and applied to a carboxymethylcellulose (Whatman CM-23) column (11 × 1.5 cm I.D.) (Whatman, Clifton, NJ, U.S.A.). The flow-rate was 20 ml/h, and the column eluate was monitored at 230 nm. The fragments were eluted with a 1-l sodium chloride gradient, 0.1 *M* to 0.5 *M*. The peak fractions were pooled, lyophilized, and desalted by gel chromatography on Sephadex G-25F with 0.01 *M* hydrochloric acid as the eluent. After lyophilization, each fraction was dissolved in 0.01 *M* hydrochloric acid and subjected to gel chromatography on Sephadex G-75.

Tryptic digestion. Human BP-component 1 and the thrombic fragments (1 mg each) were dissolved in 250 μl of 0.1 *M* ammonium hydrogen carbonate (pH 8.0), and incubated for 24 h at 37°C with L-tosylamido-2-phenylethylchloromethyl ketone-treated trypsin at an enzyme–substrate ratio of 1:50 (w/w). After lyophilization twice, the digests were stored at −20°C in a desiccator until analyzed.

HPLC analysis. The lyophilized tryptic digests were dissolved in 50 μl of 0.1% TFA, and 10 μl (100–200 μg) of digest were subjected to reversed-phase HPLC on a Varian liquid chromatograph, Model 5000, equipped with a Varichrom spectrophotometer (Varian, Walnut Creek, CA, U.S.A.). The eluate was monitored at 210 nm. A Beckman Ultrasphere-ODS column (250 × 4.6 mm I.D., 5-μm particle size) (Beckman, Berkley, CA, U.S.A.) with a guard column (20 × 4.6 mm I.D.), packed with the same material, was used for chromatography. The solvent system consisted of 0.1% TFA in water and 0.1% TFA in acetonitrile; the flow-rate was 1.0 ml/min. The temperature was usually 29°C, although in some experiments a temperature of 35°C was used.

The following program was used for chromatography of the digest of the N-terminal fragment (1–97): 0–5 min, 0% acetonitrile; 5–75 min, 0–25% acetonitrile; 75–105 min, 25–60% acetonitrile; 105–110 min, 60% acetonitrile; 110–115 min, 60–0% acetonitrile; and, finally, a 20-min equilibration with 0% acetonitrile before injection of the next sample. The same column system and flow-rate were used for chromatography of the tryptic peptides of the C-terminal fragment (98–170) except that the temperature was usually 35°C. The program for elution was as follows: 0–5 min, 0% acetonitrile; 5–20 min, 0–5% acetonitrile; 20–30 min, 5% acetonitrile; 30–130 min, 5–30% acetonitrile; 130–135 min, 30–60% acetonitrile; 135–140 min, 60% acetonitrile; 140–145 min, 60–0% acetonitrile; and, finally, a 20-min equilibration with 0% acetonitrile.

Amino acid analysis. All of the tryptic peptides from both thrombic fragments of human BP were collected, dried in a Savant Speed Vac Concentrator (Savant, Hicksville, NY, U.S.A.), and hydrolyzed in constant-boiling hydrochloric acid in an evacuated desiccator[7] at 110°C for 22 h. The same tube was used for collection and hydrolysis. Amino acid analysis was carried out with a Beckman 121MB amino acid analyzer, equipped with the 126 data system for quantitation.

RESULTS AND DISCUSSION

An example of the separation of tryptic peptides of Fragment 1–97 achieved

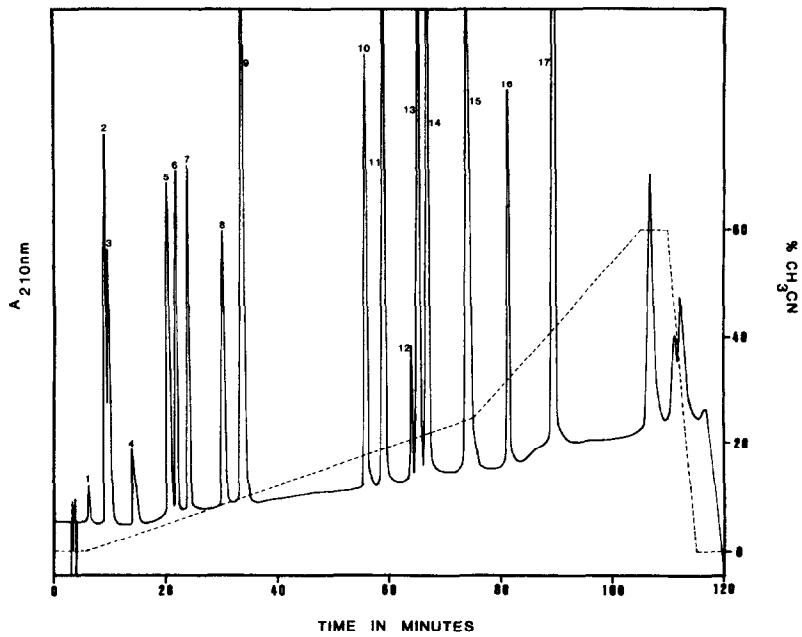

Fig. 1. Separation of tryptic peptides from human BP fragment 1–97. The gradient N-terminal program (dashed line) is described under Experimental. Zero time marks the injection of the sample (*ca.* 100 μg in 10 μl) and the beginning of the program. Column: Ultrasphere-ODS (5 μm), 250 × 4.6 mm I.D. Temperature: 29°C. Eluents: 0.1% TFA in water and 0.1% TFA in acetonitrile. Flow-rate: 1 ml/min. Peak identification and sequences are given in Table I. Absorbance units full scale = 0.64 at 210 nm.

TABLE I

PEAK IDENTIFICATION AND SEQUENCE OF TRYPTIC PEPTIDES OF HUMAN BP FRAGMENT 1–97

Peaks are shown in Fig. 1.

Peak No.	*Retention time (min)**	*Sequence***
1	6.15 ± 0.24	^{55}Gly-Ser-Gly-Lys^{58}
2	9.06 ± 0.29	^{10}His-Gly-Ser-Lys^{13}
3	9.67 ± 0.36	^{32}His-Arg^{33}
4	13.92 ± 1.02	^{54}Arg-Gly-Ser-Gly-Lys^{58}
5	20.34 ± 0.39	^{76}Ser-His-Gly-Arg^{79}
6	21.76 ± 0.18	^{1}Ala-Ser-Gln-Lys^{4}
7	23.92 ± 0.15	^{50}Gly-Ala-Pro-Lys^{53}
8	30.13 ± 0.07	^{5}Arg-Pro-Ser-Gln-Arg^{9}
9	33.56 ± 0.17	^{59}Asp-Ser-His-His-Pro-Ala-Arg^{65}
10	55.76 ± 0.26	^{92}Asn-Ile-Val-Thr-Pro-Arg^{97}
11	58.99 ± 0.31	^{66}Thr-Ala-His-Tyr-Gly-Ser-Leu-Pro-Gln-Lys^{75}
12	64.18 ± 0.30	^{14}Tyr-Leu-Ala-Thr-Ala-Ser-Thr-Met(O)-Asp-His-Ala-Arg^{25}
13	65.05 ± 0.12	^{44}Phe-Phe-Gly-Gly-Asp-Arg^{49}
14	66.61 ± 0.17	^{14}Tyr-Leu-Ala-Thr-Ala-Ser-Thr-Met-Asp-His-Ala-Arg^{25}
15	73.61 ± 0.11	^{26}His-Gly-Phe-Leu-Pro-Arg^{31}
16	81.04 ± 0.17	^{34}Asp-Thr-Gly-Ile-Leu-Asp-Ser-Ile-Gly-Arg^{43}
17	89.00 ± 0.14	^{80}Thr-Gln-Asp-Glu-Asn-Pro-Val-Val-His-Phe-Phe-Lys^{91}

* Average ± standard deviation (n = 3).

** Numbers above residues denote their position in the sequence[5,8,9].

at 29°C is shown in Fig. 1. Free arginine (residue 54), when present, was eluted isocratically at 0% acetonitrile just before peak 1. In order to resolve Peaks 2 and 3 and separate peaks 12, 13, and 14, a shallow gradient (0.36% acetonitrile/min) was used. When the gradient was increased to 1.00% acetonitrile/min, neither peaks 2 and 3 nor peaks 12, 13 and 14 were separated. If the temperature was raised to 35°C, peaks 2 and 3 overlapped to a greater extent than at 29°C (data not shown). There was no need to maintain the shallow gradient after peak 15. Therefore, at 75 min, the gradient was increased to 1.17% acetonitrile/min to hasten the elution of the most hydrophobic peptides (peaks 16 and 17).

All of the peaks were collected and identified by amino acid analysis (Table I), The peaks beyond number 17 (Fig. 1) consisted of UV-absorbing contaminants and are not listed. Although peaks 2 and 3 were not completely separated, their amino acid compositions clearly identified them. After the column was cleaned with 60%

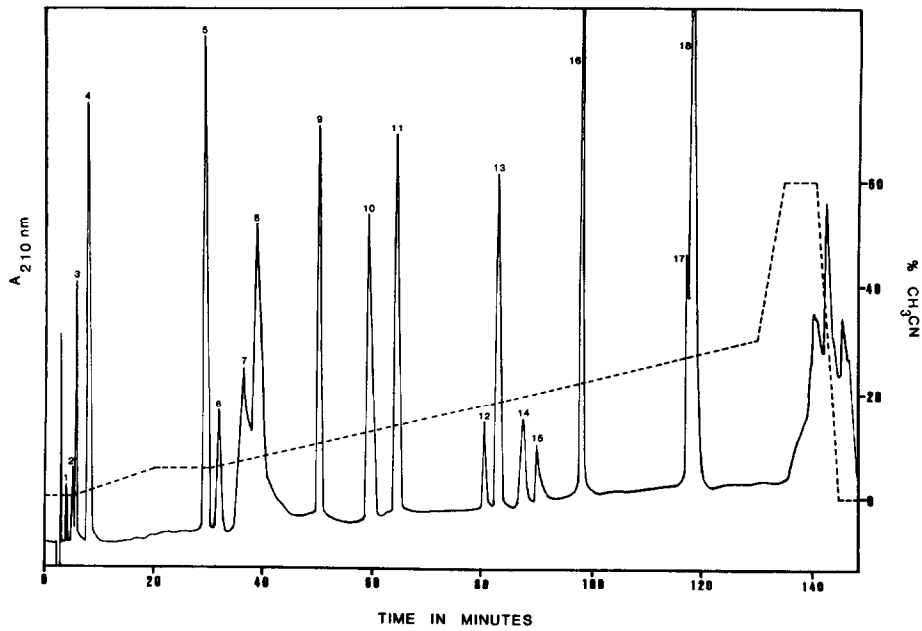

Fig. 2. Separation of the tryptic peptides (*ca.* 100 μg in 10 μl) from human BP fragment 98–170. The gradient C-terminal program is described under Experimental. The temperature was 35°C. Peak identification and sequences are given in Table II. Other details as in Fig. 1.

acetonitrile and reequilibrated at 0% acetonitrile, no peptides were eluted when a blank gradient was run. The recovery of tryptic peptides was 80–85% on the basis of the nmol of thrombic fragment digested with trypsin and the nmol of peptide recovered as determined by amino acid analysis. All of the tryptic peptides of fragment 1–97 were recovered.

An example of the separation of tryptic peptides of fragment 98–170 achieved at 35°C is shown in Fig. 2. After isocratic elution of peak 1 (Arg-170) at 0% acetonitrile, peaks 2, 3, and 4 were eluted with a gradient of 0.33% acetonitrile/min. Peak 5 was eluted isocratically at 5% acetonitrile, while peaks 6 to 18 were eluted with a shallow gradient (0.25% acetonitrile/min). The temperature was adjusted to 35°C for this fractionation because, at 29°C, peaks 6 and 7 were incompletely separated. Better separation of peaks 6 and 7 was obtained at 35°C. By changing the temperature it was possible to obtain either peak 6 or peak 8 completely separated, but overall, better separation was obtained at the higher temperature. Different gradients and/or isocratic elution at 29 or 35°C did not improve the resolution beyond that shown in Fig. 2.

One problem was the anomalous behavior of peak 7, which was not as sharp as the other peaks in the series. In attempting to explain this phenomenon we examined the elution pattern of some highly purified peak 7, which had been identified as Thr-Pro-Pro-Pro-Ser-Gln-Gly-Lys. Under the same conditions at 35°C, it also yielded a broad, unsymmetrical peak. When the front and back halves of the peak were collected separately and reapplied to the column, each peak had the original shape. We suspect that this behavior may have resulted from a relatively slow equilibrium

TABLE II

PEAK IDENTIFICATION AND SEQUENCE OF TRYPTIC PEPTIDES OF HUMAN BP FRAGMENT 98-170

Peaks are shown in Fig. 2.

Peak No.	*Retention time (min)**	*Sequence***
1	4.74 ± 0.13	170 Arg 106 107
2	6.10 ± 0.07	Gly-Arg 160 162
3	6.80 ± 0.08	Asp-Ser-Arg 136 139
4	8.17 ± 0.14	Ser-Ala-His-Lys 131 135
5	29.68 ± 0.29	Ala-Ser-Asp-Lys 156 159
6	32.09 ± 0.15	Leu-Gly-Gly-Arg 98 105
7	36.31 ± 0.29	Thr-Pro-Pro-Pro-Ser-Gln-Gly-Lys 140 142
8	38.59 ± 0.17	Gly-Phe-Lys 163 169
9	51.14 ± 0.12	Ser-Gly-Ser-Pro-Met-Ala-Arg 153 155
10	60.06 ± 0.23	Ile-Phe-Lys 143 153
11	65.17 ± 0.47	Gly-Val-Asp-Ala-Gln-Gly-Thr-Leu-Ser-Lys 108 113
12	80.59 ± 0.13	Gly-Leu-Ser-Leu-Ser-Arg 123 130
13	83.13 ± 0.20	Pro-Gly-Phe-Gly-Tyr-Gly-Gly-Arg 106 113
14	87.61 ± 0.22	Gly-MMArg-Gly-Leu-Ser-Leu-Ser-Arg 106 113
15	90.01 ± 0.35	Gly-DMArg-Gly-Leu-Ser-Leu-Ser-Arg 114 122
16	98.20 ± 0.39	Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg 114 130
17	117.69 ± 0.13	Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg-Pro-Gly-Phe-Gly-Tyr-Gly-Gly-Arg 114 130
18	118.19 ± 0.11	Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg-Pro-Gly-Phe-Gly-Tyr-Gly-Gly-Arg

* Average ± S.D. (n = 3).

** Numbers above residues denote their position in the sequence[8].

between conformers of the peptide having *cis*- and *trans*-configurations[10] of the N-terminal Thr-Pro peptide bond and possibly also *cis'*- and *trans'*-conformations[11] of the ψ-dihedral angle of Pro-101 at the Pro-Ser bond.

Identities of the peaks are given in Table II. It can be seen that the N^G-monomethylarginine (MMArg)- and N^G,N'^G-dimethylarginine (DMArg)-containing peptides (peaks 14 and 15) were completely separated by this procedure. Peaks 17 and 18 were found to have the same amino acid composition. Peak 17 may have contained a Glu, rather than a Gln, at position 121 as a result of deamidation. The peaks beyond number 18 did not contain amino acids, and a subsequent blank run showed no peptide-containing peaks. The recovery of tryptic peptides from fragment 98–170 was the same as that for fragment 1–97. All of the tryptic peptides of fragment 98–170 were recovered.

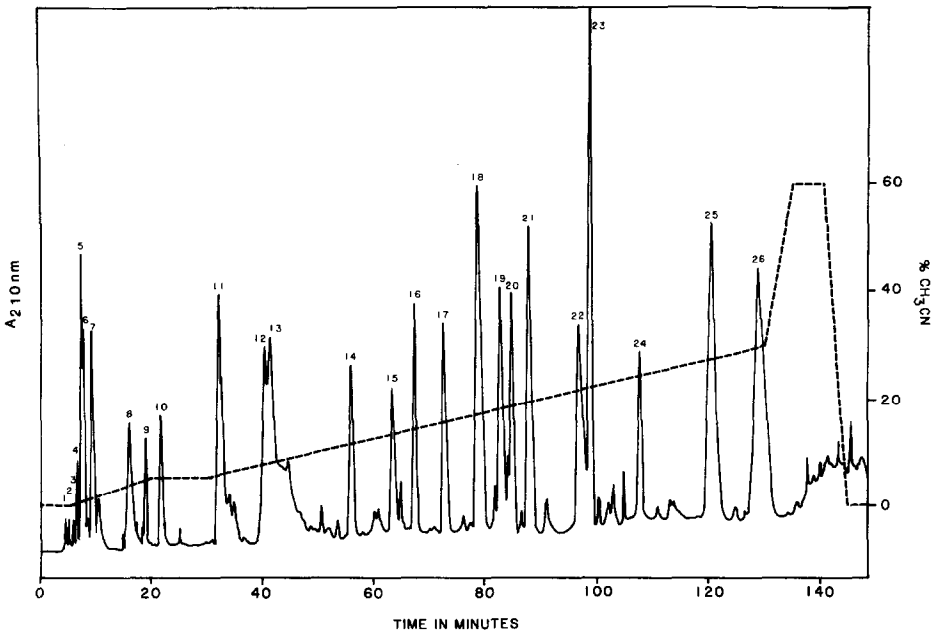

Fig. 3. Separation of tryptic peptides (*ca.* 100 μg in 10 μl) from whole human BP. The C-terminal program described under Experimental and shown in Fig. 2 was used. The temperature was 35°C. Peak identifications are given in Table III. Other details as in Fig. 1.

Fig. 3 and Table III show that when a tryptic digest of the whole human BP was subjected to HPLC at 35°C according to the procedure used to separate the peptides derived from fragment 98–170, most of the peptides were separated from one another. However, peaks 11, 12, 13 and 19 each contained two peptides, while peak 21 consisted of peptide 14–25 as well as the two forms of peptide 106–113, containing monomethyl- and dimethylarginine.

Tryptic peptides that have species-related differences in amino acid composition could be differentiated in many cases on the basis of retention times. Table IV compares the retention times of several homologous tryptic peptides, derived from thrombic or peptic fragments of BP in the course of our sequence investigations[4,5]. For these comparisons, both N-terminal and C-terminal programs were run at 35°C. For some sets of homologous peptides a clear relationship between retention time and hydrophobicity was shown. Replacement of Ala by Val in peptide 50–53 increased the retention time by 12 min, and removal of Phe from peptide 44–49 decreased the retention time by 22 min. No significant changes in retention time resulted from the replacement of Ala by Thr in peptide 66–75 and of Gly by Ser in peptide 59–65 (bovine, monkey) and in peptide 45–49 (monkey, bovine). However, a second replacement of Gly by Ser in peptide 45–49 (rabbit) resulted in a moderate (3.6 min) decrease in retention time. Comparison of the retention times of human and monkey peptides 59–65 suggests that Pro may be somewhat more hydrophobic than Ala, although the observed result could have been due to conformational differences. The relatively large decrease in retention time of peptide 140–142 that resulted from the substitution of Leu for Phe may indicate that Phe is more hydrophobic than Leu;

TABLE III

PEAK IDENTIFICATION AND SEQUENCE OF TRYPTIC PEPTIDES OF WHOLE HUMAN BP

HPLC peaks are shown in Fig. 3 and amino acid sequence are shown in Tables I and II.

Peak No.	*Retention time (min)*	*Peptide*	*Peak No.*	*Retention time (min)*	*Peptide*
1	5.5	170	14	55.8	163–169
2	6.1	55–58	15	63.2	153–155
3	6.2	107–107	16	67.1	143–152
4	6.7	160–162	17	72.3	92–97
5	7.4	10–13	18	78.3	66-75
6	7.7	32–33	19	82.4	44–49, 108–113
7	9.3	136–139	20	84.5	123–130
8	15.5	76–79	21	87.5	14–25, 106–113
9	18.9	1–4	22	96.5	26–31
10	21.6	50–53	23	98.4	114–122
11	32.0	5–9, 131–135	24	107.7	34–43
12	40.1	98–105, 156–159	25	120.1	114–130
13	41.2	59–65, 140–142	26	128.5	80–91

alternatively, it may indicate that Phe interacts in a more specific manner with the column matrix. Although both residues are hydrophobic, their relative positions vary among different hydrophobicity scales[13].

After a year of constant usage the Ultrasphere-ODS column utilized for the development of this separation of tryptic peptides started to deteriorate. Therefore, a new Ultrasphere-ODS column with a pre-column of the same material was utilized. Both columns had excellent efficiency, but slightly different selectivity. The first column separated peaks 2 and 3 and peaks 13 and 14 of fragment 1–97 better than the second. By lowering the temperature to 27°C and making the gradient more shallow (0.30% acetonitrile/min), resolution of all the tryptic peptides of fragment 1–97 was achieved on the second column with the N-terminal program described under Experimental. Also, good resolution of all the tryptic peptides of fragment 1–97 was obtained on the second column with the C-terminal program at 29°C. The difference in selectivity between the two columns was also noticed when the tryptic digest of fragment 98–170 was subjected to the C-terminal program at 35°C, as described under Experimental. However, all of the peptides could be identified even though the separation of peaks 10 and 11 was not as good on the second column.

The solvent system in this separation of tryptic peptides was chosen because it was completely volatile. The collection of the separated peptides, evaporation of the fractions, and hydrolysis for amino acid analysis could all be carried out in the same tube. Since the desired separation of the tryptic peptides was achieved, it was not necessary to try any other elution systems.

The results of the present study have shown that nearly all of the 31 peptides present in a tryptic digest of the 18.5 kD human BP can be separated by a single HPLC procedure. The separation is improved if the BP is first cleaved with thrombin and the two large fragments isolated prior to tryptic digestion. By slight changes in the gradient and the temperature, the individual peptides in each of these digests can be completely separated.

TABLE IV

COMPARISON OF HOMOLOGOUS BP TRYPTIC PEPTIDES

*Peptide**	*Retention time (min)***
76 79 Ser-( )-His-Gly-Arg (human, monkey)	15.6 ± 0.35 ($n=3$)
Ala-Gln-His-Gly-Arg (bovine)	22.6, 23.5
50 53 Gly-Ala-Pro-Lys (human, bovine, rabbit)	21.3 ± 0.39 ($n=5$)
Gly-Val-Pro-Lys (monkey)	33.5, 33.7
59 65 Asp-Ser-His-His-Pro-Ala-Arg (human)	33.0
Asp-Ser-His-His-Ala-Ala-Arg (monkey)	30.1. 29.5
Asp-Gly-His-His-Ala-Ala-Arg (bovine)	30.2, 30.9
Asp-( )-His-Ala-Ala-Arg (rabbit)	23.0, 23.2
66 75 Thr-Ala-His-Tyr-Gly-Ser-Leu-Pro-Gln-Lys (human, monkey)	58.2 ± 0.26 ($n=3$)
Thr-Thr-His-Tyr-Gly-Ser-Leu-Pro-Gln-Lys (bovine, rabbit)	57.6 ± 0.85 ($n=4$)
44 49 Phe-Phe-Gly-Gly-Asp-Arg (human)	62.0
Phe-Gly-Gly-Asp-Arg (monkey)	40.4, 39.3
Phe-Gly-Ser-Asp-Arg (bovine)	40.9, 39.5
Phe-Ser-Ser-Asp-Arg (rabbit)	36.8, 36.4
80 91 Thr-Gln-Asp-Glu-Asn-Pro-Val-Val-His-Phe-Phe-Lys (human)	88.1
Thr-Gln-Asp-Glu-Asn-Pro-Val-Val-His-Phe (monkey)	78.3, 78.2
76 89 Ser-( )-His-Gly-Arg-Pro-Gln-Asp-Glu-Asn-Pro-Val-Val-His-Phe (rabbit)	83.8, 84.3
Ala-Gln-His-Gly-Arg-Pro-Gln-Asp-Glu-Asn-Pro-Val-Val-His-Phe (bovine)	84.4, 86.0
140 142 Gly-Phe-Lys (human)	38.4, 39.0
Gly-Leu-Lys (porcine)	28.6, 28.5
143 152 Gly-Val-( )-Asp-Ala-Gln-Gly-Thr-Leu-Ser-Lys (human)	65.6, 64.8
Gly-Ala-Glu-Asp-Ala-Gln-Gly-Thr-Leu-Ser-Lys (porcine)	56.8, 55.8
114 122 Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg (human)	97.8, 98.3
Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys (porcine)	96.4, 96.3

* Numbering of residues in the bovine, monkey, rabbit and porcine peptides is based on an alignment with the human BP. Sequence data for the bovine, monkey, and rabbit peptides are from Shapira *et al.*[12] with corrections by Deibler *et al.*[5]. Porcine peptide data are from Kira *et al.*[4].

** Individual values or the average ± standard deviations are given.

ACKNOWLEDGEMENTS

We thank Mrs. Dory Feldman for her typing of the manuscript and Mr. J. D. Brown for the drawing of Fig. 3 and photography.

REFERENCES

1 C.-H. J. Chou, R. Shapira and R. B. Fritz, *J. Immunol.*, 130 (1983) 2183.
2 M. Dizdaroglu and H. C. Krutzsch, *J. Chromatogr.*, 264 (1983) 223.
3 M. J. Law, R. E. Martenson and G. E. Deibler, *J. Neurochem.*, 42 (1984) 559.
4 J. Kira, G. E. Deibler, H. C. Krutzsch and R. E. Martenson, *J. Neurochem.*, 44 (1985) 134.
5 G. E. Deibler, H. C. Krutzsch and R. E. Martenson, *J. Biol. Chem.*, 260 (1985) 472.
6 G. E. Deibler, L. F. Boyd and M. W. Kies, in E. C. Alvord, Jr., M. W. Kies and A. J. Suckling (Editors), *Experimental Allergic Encephalomyelitis: A Useful Model for Multiple Sclerosis*, Alan R. Liss, New York, 1984, p. 249.
7 W. J. Dreyer and E. Bynum, *Methods Enzymol.*, 11 (1967) 32.
8 P. R. Carnegie, *Biochem. J.*, 123 (1971) 57.
9 B. W. Gibson, R. D. Gillion, J. N. Whitaker and K. Biemann, *J. Biol. Chem.*, 259 (1984) 5028.
10 E. Benedetti, A. Bavoso, B. DiBlasio, V. Pavone, C. Pedone, C. Toniolo and G. M. Bonora, *Biopolymers*, 22 (1983) 305.
11 R. Nagaraj, Y. V. Venkatachalapathi and P. Balaram, *Int. J. Peptide Protein Res.*, 16 (1980) 291.
12 R. Shapira, S. McKneally, F. Chou and R. F. Kibler, *J. Biol. Chem.*, 246 (1971) 4630.
13 D. Eisenberg, *Ann. Rev. Biochem.*, 53 (1984) 595.