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SEPARATION OF MYELIN BASIC PROTEIN PEPTIDE 43–88 AND ITS FRAGMENTS BY ANALYTIC AND PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

RICHARD D. GILLIOM*, JOHN N. WHITAKER* and JEROME M. SEYER

Neurology and Research Services of the Memphis Veterans Medical Center and the Departments of Neurology, Anatomy and Biochemistry of the University of Tennessee Center for the Health Sciences, Memphis, TN 38104 (U.S.A.)

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SUMMARY

Reversed-phase (RP) high-performance liquid chromatography (HPLC) using a solvent mixture of triethylammonium formate buffer and methanol permitted the rapid separation of myelin basic protein peptide 43–88 and a mixture of synthetic fragments from this peptide. The elution times for some of the peptides were markedly affected by minimal changes in the solvent mixture. Attempts to separate the same peptides by gel permeation HPLC resulted in poor resolution and an aberrant elution pattern unrelated to molecular size. With the use of the volatile triethylammonium formate buffer in the RP-HPLC, material could be more readily separated and easily recovered by freeze-drying. Analysis of the components separated by this system of RP-HPLC demonstrated that the preparation of normal human BP peptide 43–88 results in an admixture of peptides 43–87 and 43–88. This procedure of RP-HPLC should make it possible to analyze the degradation of myelin basic protein peptide 43–88 and to isolate the degradation products for characterization.

INTRODUCTION

Myelin basic protein (BP) is a protein of 18,500 daltons which comprises 30% of central nervous system myelin proteins[1]. It has been extensively studied because of its encephalitogenic property of inducing experimental allergic encephalomyelitis in animals[2,3]. BP-like antigens enter cerebrospinal fluid of humans after acute central nervous system myelin injury[4,5], and material cross-reactive with BP peptide 43–88 is the dominant antigenic form under such conditions[5,6]. In order to identify the catabolic steps for the clearance and catabolism of BP peptide 43–88[7,8], it has been necessary to establish a rapid method for the analysis and isolation of small peptides resulting from the degradation of BP peptide 43–88. Utilizing reversed-phase (RP) high-performance liquid chromatography (HPLC), an effective and efficient analyti-

* Present address: Chemistry Department, Southwestern At Memphis, Memphis, TN 38112, U.S.A.

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cal technique has been developed. The method described also permits the isolation and recovery of peptides for further characterization.

MATERIALS AND METHODS

Reagents

All organic solvents were distilled in glass, UV grade (Burdick & Jackson Labs., Muskegon, MI, U.S.A.). A heart cut, boiling point 89.0–89.1°C, of triethylamine (Fisher, C.P.) distilled through a 12-in. Vigreux column was employed. Formic acid (Baker, Analyzed Reagent) was used as supplied.

Basic protein peptides

Human BP peptide 43–88 was prepared as previously described[9]. Briefly, human BP was isolated by ion-exchange chromatography on carboxymethyl-cellulose from a pH 3 extract of postmortem human brain. BP was digested with bovine brain cathepsin D at pH 3.5, and BP peptide 43–88 was purified by a combination of carboxymethyl-cellulose chromatography and gel filtration on Sephadex G-50 superfine. The amino acid composition and amino-terminal amino acid sequence of this preparation have been previously described[10]. All other BP peptides were custom synthesized (Peninsula Laboratories, San Carlos, CA, U.S.A.) based on the reported sequence of Shapira *et al.*[11] for all except peptide 79–88 which followed the human BP sequence described by Carnegie and Moore[3]. The numbering of residues is based on the sequence of the 169 residues in bovine basic protein[12] as modified[13]. The synthesized peptides were:

51–67, Lys-Arg-Gly-Ser-Gly-Lys-Asp-Ser-His-His-Pro-Ala-Arg-Thr-Ala-His-Tyr
74–84, Lys-Ser-Gly-His-Arg-Thr-Gln-Asp-Glu-Asn-Pro
67–80, Tyr-Gly-Ser-Leu-Pro-Gln-Lys-Ser-Gly-His-Arg-Thr-Gln-Asp
79–84, Thr-Gln-Asp-Glu-Asn-Pro
81–86, Asp-Glu-Asn-Pro-Val-Val
83–88, Asn-Pro-Val-Val-His-Phe
79–88, Thr-Gln-Asp-Gln-Asp-Pro-Val-Val-His-Phe
83–95, Asn-Pro-Val-Val-His-Phe-Phe-Lys-Asn-Ile-Val-Thr-Pro

HPLC

A Waters Associates HPLC apparatus consisting of two Model 600A solvent delivery pumps, Model 660 solvent programmer, Model U6K injector, guard column packed with μBondapak C_{18}/Corasil, μBondapak C_{18} (particle diameter 10 μm) RP analytical stainless-steel column, 30 cm × 4 mm, and a Model 450 variable-wavelength detector was used. The analogue signal was recorded on a Houston recorder, measured at 210 nm with paper drive of 0.5 in./min.

Triethylammonium formate (TEAF) was prepared by titrating 0.04 *M* formic acid to pH 3.15 with distilled triethylamine. TEAF buffer was filtered and degassed with Millipore filters (pore diameter 0.45 μm, HAWP 04700; Millipore, Bedford, MA, U.S.A.). The organic solvents used were also filtered and degassed with Millipore filters (FHUP 04700, pore diameter 0.5 μm). Samples were dissolved in TEAF–

methanol (70:30) and eluted at a flow-rate of 1.5 ml/min, the composition of the mobile phase was adjusted using the solvent programmer, with pure TEAF from pump A and pure methanol from pump B. After HPLC separation into individual peaks, fractions were either disposed of by leaving the "waste–recycle–collect" valve on "waste" or were collected by switching the valve to "collect". The collected fractions were recovered by freeze-drying.

Other methods

Amino acid analysis was performed on samples hydrolyzed under an atmosphere in nitrogen in constant boiling 6 *N* hydrochloric acid at 108°C for 24 h. An automatic amino acid analyzer (Beckman) was utilized with a single column method[9,14]. No correction factors were used for losses of labile amino acids or for the incomplete release of valine. Amino acid sequencing from the amino-terminal was performed with a Beckman sequencer Model 890C as previously described[9].

RESULTS

A mixture of human BP peptide 43–88 and a group of synthesized human BP peptides (Table I) was successfully resolved using TEAF–methanol (70:30) (Fig. 1). The elution times of the peptides were very sensitive to mobile phase composition. As an example, TEAF–methanol (70:30) eluted peptide 43–88 in 3.88 min, but at a ratio of 75:25 it failed to elute in 20 min. At methanol concentrations above 40%, it eluted so near the void volume that no resolution was achieved. Similarly, peptide 83–95 failed to elute at a TEAF–methanol ratio of 70:30, eluted as a broad peak in 21.75 min using a ratio of 55:45, and emerged as a sharp peak in 3.5 min using a ratio of 40:60. The resolution of the fast eluting peptides 51–67, 74–84, and 67–80 could be improved by eluting with TEAF–methanol (80:20) (Fig. 2). Due to baseline drift, it was not possible to obtain satisfactory chromatograms with gradient elution with increasing amounts of methanol. Retention times were reproducible among determinations and with the same batch of TEAF. Retention times varied approximately 1%

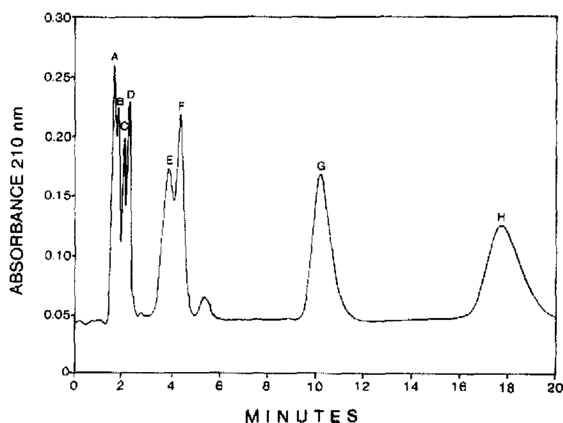

Fig. 1. RP-HPLC chromatogram of a mixture of eight peptides of human basic protein peptide 43–88. See Table I for peptide designation. Experimental conditions: mobile phase, TEAF–methanol (70:30); 1.5 ml/min; 210 nm; 0.4 a.u.f.s.; recorder paper speed, 0.5 in./min; 30 cm × 4 mm μBondapak C_{18}.

TABLE I

SYNTHETIC MIXTURE OF PEPTIDES USED FOR FIG. 1

Symbol	*Peptide*	*Injected (μg)*	*Retention time (min)*
A	51–67	1.64	1.63
B	74–84	1.56	1.75
C	67–80	1.33	2.00
D	79–84	4.32	2.25
E	43–88	11.12	3.88
F	81–86	5.39	4.32
G	83–88	4.55	10.12
H	79–88	3.67	17.50

among batches of TEAF prepared at different times. Chromatograms using premixed TEAF–methanol in the mobile phase were found to give retention times that increased from day to day unless the mixture was tightly stoppered overnight. To eliminate this problem TEAF and methanol were stored separately and mixed using the solvent programmer. Use of acetonitrile rather than methanol as the organic constituent of the mobile phase resulted in diminished resolution.

An assessment was made of the sensitivity gained by detection at 210 nm rather than at 225 nm, the wavelength used previously for column chromatography[9] for analysis and purification of BP peptides. Using peptide 79–88 as a model, RP-HPLC of 7.62 μg revealed a peak of 102 arbitrary units (peak height × peak width at half-height) when detected at 225 nm while a 9.11-μg sample gave a peak area of 261 arbitrary units when measured at 210 nm. Thus, an increased sensitivity of 2.1, $(261/102)/(0.11/7.62) = 2.1$, was gained by detection at 210 nm. Recordings made at 280 nm or 258 nm revealed minimal or no deviation from baseline with levels of peptides readily detected at 210 nm.

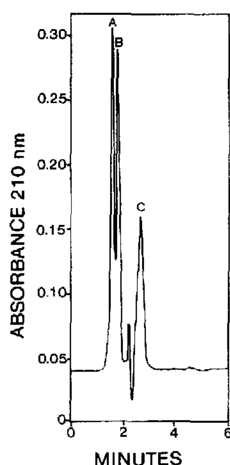

Fig. 2. RP-HPLC chromatogram of 27.3 μg of peptide 51–67 (A), 26.1 μg of peptide 74–84 (B), and 22.1 μg of peptide 67–80 (C). Experimental conditions as in Fig. 1 except mobile phase: TEAF–methanol (80:20).

Attempts were also made to utilize the Waters I-60 column, monitored at 225 nm, for the separation of human BP peptide 43–88 from its smaller fragments. Peptides 43–88 and 79–88 were tested. A solution of NH_4HCO_3, a chemical selected because of its usefulness in gel filtration chromatography of BP peptides[9], failed to elute BP peptide 43–88 from this column at a concentration of 0.04 *M*. At increased concentrations of 0.10–0.15 *M* NH_4HCO_3 this peptide could be eluted but in a broad peak with considerable "tailing". BP peptides 43–88 and 79–88 could be resolved on the I-60 column with this concentration range of NH_4HCO_3; however, peptide adsorption to the column was indicated by the fact that BP peptide 43–88 eluted after the smaller BP peptide 79–88. Increasing the NH_4HCO_3 concentration further to 0.2–0.5 *M* narrowed the peak and reduced the tailing of BP peptide 43–88 but changed the adsorption so that BP peptides 43–88 and 79–88 co-eluted. Another problem, probably related to adsorption, experienced with this column and the NH_4HCO_3 solution at a concentration of 0.1–0.5 *M* was that the areas of the peaks were dependent upon NH_4HCO_3 concentration when equal amounts of BP peptide 43–88 were injected. With the I-60 column and buffer solutions of 0.25 *M* K_3PO_4 titrated to pH of 4.0, 7.0 or 7.6 with H_3PO_4, BP peptide 79–88 but no BP peptide 43–88 was successfully eluted. BP peptide 43–88 was eluted from the I-60 column using 0.10–0.25 *M* K_3PO_4 but as one rather than two major peaks as observed using RP-HPLC, *vide infra*. Because of these unsatisfactory results and because the strongly basic character of these solvents risked collapse of the column packing, further efforts to use the I-60 protein column were abandoned.

The increased resolution and sensitivity afforded by the techniques for RP-HPLC described above are illustrated by the behavior of the preparation of BP peptide 43–88 previously believed to be pure[10]. Fig. 3a shows the presence of two major, designated as peaks I and II, and two minor components in the sample. Conventional chromatography and polyacrylamide gel electrophoresis showed only one component[10]. The early appearing peak I (Fig. 3a) presumably is obscured by the first four peptides (Fig. 1). Separation and collection of the two major components by RP-HPLC permitted their separation from each other (Fig. 3b and 3c). The small peak at 2.7 min remained with the second component even though it was carefully

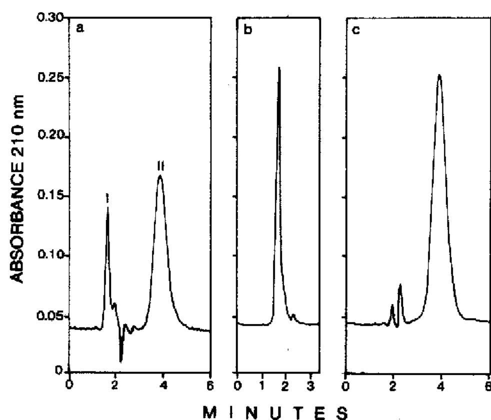

Fig. 3. RP-HPLC chromatograms of "pure" BP 43–88 (a), isolated peak I (b) and isolated peak II (c). Conditions the same as described in the legend for Fig. 1.

TABLE II

AMINO ACID ANALYSIS OF HUMAN BP PEPTIDE 43–88 COMPONENTS

Amino acid	*Expected**	*I***	*II*
Aspartic acid	4	3.5	3.6
Threonine	2	1.7	1.7
Serine	4	4.2	3.7
Glutamic acid	3	2.8	2.9
Proline	4	4.3	4.4
Glycine	7	6.6	6.7
Alanine	3	3.4	3.2
Valine	2	1.1	0.9
Leucine	1	1.0	1.1
Tyrosine	1	0.9	0.0
Lysine	3	3.1	2.9
Histidine	5	4.1	4.0
Arginine	4	4.2	4.0
Phenylalanine	2	0.8	1.9

* Numbers represent the number of residues based on the known sequence of human BP peptide 43–88[11].

** Roman numerals used in the column headings have the same designation as that used in Fig. 3 to designate HPLC peaks.

excluded from the collection. This small peak may represent an aggregate of the peptide. Amino acid analysis of the isolated two major components demonstrated that they differed by one phenylalanine residue (Table II). Since a phenylalanine residue is present at both the amino and carboxyl termini of human BP peptide 43–88[11], it was unclear which phenylalanine residue was absent in peak I (Fig. 3). This was resolved by amino acid sequencing which revealed an amino terminal sequence of Phe-Gly-Ser-Asp-Arg-Gly for peak I (Fig. 3, Table II). Thus, peak I and peak II (Fig. 3) are BP peptide 43–87 and BP peptide 43–88, respectively.

The amino acid analysis of peak B (Fig. 3) revealed nothing in the expected position of tyrosine eluting from the column used for amino acid analysis[14]. Instead, an "unknown" peak calculated to represent 0.5–0.6 residues per mole eluted just prior to lysine. The nature of this unexpected behavior of tyrosine is presently under study.

DISCUSSION

Two of the major characteristics of BP are its highly cationic total charge and its lack of significant intramolecular stabilization[1,3,15]. Because of its non-globular conformation, gel filtration of BP may demonstrate an apparent molecular weight several fold its monomeric molecular weight of 18,500[16]. The highly cationic features of BP promotes its adherence to surfaces[5,17]. Urea is included in the aqueous buffers used for ion exchange chromatography to decrease or prevent this adherence[18,19].

Although BP has been isolated by gel permeation HPLC[20], the present investigation has shown that the isolation of small peptides of BP still poses problems for the use of this method, particularly when recovery for further characterization is planned. Buffers initially selected for gel permeation HPLC were chosen for their

volatility for further characterization of peptides. The lack of correlation between elution times and molecular weights of the peptides is presumably related to the focal distribution of polar and non-polar amino acid residues which, depending on where cleavage of BP occurs, leads to the generation of peptides with a range of conformations and charge properties.

RP-HPLC has previously been utilized to examine peptides formed by digesting BP with trypsin[21]. Triethylammonium phosphate and a gradient of acetonitrile were used on a C_{18} column, but no attempts were reported to isolate and characterize the peaks separated by this method. In the present investigation BP peptide 43–88 and small fragments thereof were separated by RP-HPLC on a C_{18} column exposed to isocratic conditions of the volatile combination of 70% TEAF and 30% methanol. Similar conditions have been found effective in separating neuropeptides[22]. The system and conditions selected permitted both the analysis of small peptides of BP and their subsequent recovery and characterization. The resolution of the method was illustrated by the separation of two peptides from a preparation of human BP peptide 43–88 shown by polyacrylamide gel electrophoresis conducted at either pH 8.8 (ref. 9) or at pH 2.5 in the presence of urea[10] to be a single peptide species. Recovery of the two peptides and their characterization by amino acid analysis and partial amino acid sequencing demonstrated the two peptides to be peptide 43–87 and peptide 43–88. The possible modification of the tyrosine residue by the method of RP-HPLC awaits clarification.

The origin of peptide 43–87 is unknown. Since the human BP used for producing BP peptide 43–88 customarily contains an array of BP peptides[23], it is possible that a peptide whose carboxyl terminal was the histidine at position 87 existed in the human BP preparation. It is known that a lysosomal carboxypeptidase active at pH 5.5 can cleave the terminal phenylalanine from bovine BP peptide 43–88 (ref. 24) and that BP.peptide 43–87 is present in bovine brain delipidated in acetone[25]. The degradation of human BP by cathepsin D is the present study occurred at pH 3.5, and the cathepsin D used appears to be pure[9]. Also, the processing of brain tissue in this study involved delipidation with chloroform and methanol.

These studies illustrate the usefulness of RP-HPLC for separating peptides of a protein which has many problematic features for conventional chromatographic analysis. The procedure as determined in this investigation should allow the separation and identification of small fragments of BP. This procedural step would then permit the recognition of small peptides drived from enzymatic degradation of BP and large peptides thereof. Additionally, the procedure has the potential for the same approach to analyze body fluids in which BP or its peptides may appear.

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