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Note

Separation and isolation of human myelin basic protein peptides by reversed-phase high-performance liquid chromatography in volatile buffers

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Myelin basic protein (MBP) is a protein of molecular weight 18 500 and approximately 170 residues[1,2]. MBP comprises 30% of central nervous system (CNS) myelin proteins and has been extensively studied because of its ability to induce experimental allergic encephalomyelitis[3]. MBP or its fragments enter cerebrospinal fluid of humans after damage to CNS myelin[4,5]. The mechanisms by which this antigenic material is cleared from the body are unclear but may have relevance to immune events in certain diseases such as multiple sclerosis.

The study of MBP and the catabolism of it and its peptides has necessitated development of a versatile high-performance liquid chromatographic (HPLC) method for the analysis of complex mixtures of these substances and for the isolation of products for further use or characterization. Large quantities of the purest possible MBP and MBP peptides were needed for the production of antibodies, for radioimmunoassay studies, and for use as enzyme substrates.

The solvents trifluoroacetic acid and acetonitrile are commonly used for reversed-phase chromatography of proteins and peptides[6–8]. They have been used previously to chromatograph MBP peptides[9,10]. The gradient-elution system described here proved to be an effective and efficient choice for a variety of separation problems. Furthermore, separation conditions devised using a reversed-phase analytical column were transferred directly to a semi-preparative column of the same type for isolation of large quantities of product.

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MATERIALS AND METHODS

Reagents

Trifluoroacetic acid, Sequenal grade, was purchased from Pierce (Rockford, IL, U.S.A.). Acetonitrile, UV grade, was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Organic-free water was prepared by UV irradiation of Milli-Q (Millipore-Continental Water Systems, Bedford, MA, U.S.A.) water in a Model 816 HPLC water reservoir (Photronix, Medway, MA, U.S.A.).

MBP and MBP peptides

MBP and MBP peptides 45–89* and 45–88 were prepared from human brain tissue as described previously[9,12]. The method for the separation and purification of human MBP peptides 45–88 and 45–89 by isocratic elution[9] was replaced by the gradient elution described in this report. The numbering of the amino acid residues is shown in Fig. 1.

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45                  50                  55                  60
PHE GLY GLY ASP ARG GLY ALA PRO LYS ARG GLY SER GLY LYS ASP SER HIS HIS PRO ALA

65                  70                  75                  80
ARG THR ALA HIS TYR GLY SER LEU PRO GLN LYS SER HIS GLY ARG THR GLN ASP GLU ASN

85          89
PRO VAL VAL HIS PHE
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Fig. 1. The amino acid sequence of human MBP 45–89 (ref. 9).

MBP peptide 44–69 was synthesized using a Beckman Automatic Peptide Synthesizer (Model 990) according to the method of Merrifield[14]. Residue 44, which is not shown in Fig. 1, is phenylalanine. MBP peptides 53–69, 69–81, and 80–89 were synthesized by Peninsula Labs. (San Carlos, CA, U.S.A.).

HPLC

A Waters (Millipore, Waters Products Division, Milford, MA, U.S.A.) liquid chromatograph equipped with two Model 6000A pumps, Model 660 solvent programmer, Model U6K injector, Model 450 variable-wavelength detector, and a Series B-5000 Omniscribe strip chart recorder (Houston Instrument, Austin, TX, U.S.A.). For analytical work, a Vydac 218 TP 54 RP column (C_{18}, 25 cm × 4.6 mm I.D.,

* Although the numbering of amino acid residues has been based previously on the bovine sequence[11], the numbering system followed in the present paper conforms with that presented for human MBP[2,9]. Thus, human MBP peptide 45–89 is from the same MBP region as bovine MBP peptide 43–88, the latter containing a residue at position 74 which is deleted in human MBP. Other human MBP peptides tested and their bovine counterparts are as follows: human peptide 45–88 or bovine 43–87, human peptide 44–69 or bovine 42–67, human peptide 53–69 or bovine 51–67, human peptide 69–81 or bovine 67–80, and human peptide 80–89 or bovine 79–88.

5-μm particles), obtained from The Separations Group (Hesperia, CA, U.S.A.) was used. Milligram quantities of sample were chromatographed on a Vydac semi-preparative column (201TP510, 25 × 1.0 cm I.D., 5-μm particles) with properties almost identical to those of the analytical column. A Waters guard column packed with Bondapak C_{18} Corasil was always used. In addition, for analytical runs a scavenger column, consisting of a Whatman guard column (Whatman, Clifton, NJ, U.S.A.) packed with Bondapak C_{18}/Corasil, was installed in the A-pump line between the reference valve assembly and a mixing T-connector from the B pump. The T-connector then directed flow to the pulse dampener on the A pump.

Solvent A was 0.1% (v/v) trifluoroacetic acid in water. Solvent B was 0.1% trifluoroacetic acid in acetonitrile–water (9:1). Solvents were prepared fresh daily and were filtered and degassed with Nylon-66 membrane filters (0.45 μm pore size) purchased from Rainin (Woburn, MA, U.S.A.). The composition of the mobile phase was adjusted using the solvent programmer with pure solvent A from the A pump and pure solvent B from the B pump.

Other methods

Amino acid sequencing from the amino terminus of the peptides was performed with a Beckman Sequencer, System 890M (Beckman Instruments, Palo Alto, CA, U.S.A.).

RESULTS

A mixture of human MBP and MBP peptides was separated successfully using a gradient-elution system of aqueous trifluoroacetic acid and acetonitrile. MBP and MBP peptides 69–81, 53–69, 44–69, 45–89, 45–88, and 80–89 were well resolved by a 20-min linear gradient of 10–50% B (Fig. 2).

The development of a method for purifying synthetic MBP peptide 44–69 is shown in Fig. 3. The major peak, demonstrated subsequently by sequence determination and amino acid analysis to be MBP peptide 44–69, was not retained by the column at initial conditions of 20% B (Fig. 3A). A second separation, beginning at 0% B and utilizing a 15-min linear gradient of 0–30% B, was performed. The peptide eluted at about 20% solvent B (Fig. 3B) and 25 min. In the third run, initial conditions were 12% B, the range of the gradient was narrowed to 12–24% B and the gradient slope was reduced. The peptide eluted at 20% B and 20 min (Fig. 3C). The fourth run, in which the initial conditions and the shape of the gradient were altered, resulted in a separation (Fig. 3D) deemed adequate for the collection of a large quantity of the major peak. At this point, the analytical column was replaced with the semi-preparative column so that 1.5 mg crude synthetic MBP peptide 44–69 could be chromatographed per run (Fig. 3E). The major peak was collected from a series of ten sample applications. Analysis of the freeze-dried product from the semi-preparative column by amino acid sequencing demonstrated that it was MBP peptide 44–69.

Retention time of the peptides varied less than 1% from run to run on the same day and from day to day.

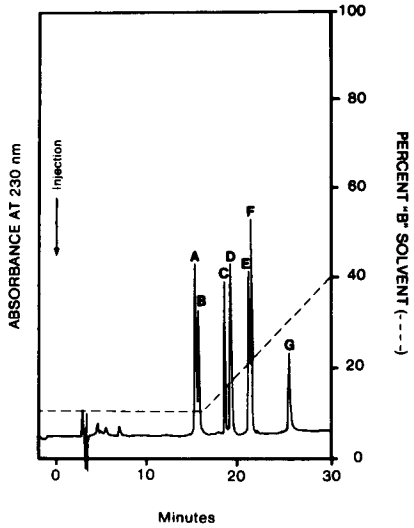

Fig. 2. Reversed-phase HPLC chromatogram of a mixture of MBP and six MBP peptides. Experimental conditions: initial conditions 10% solvent B for 6 min, 20-min linear gradient 10–50% solvent B; room temperature; 230 nm; 1.0 a.u.f.s.; chart speed 0.5 cm/min; flow-rate 1.0 ml/min; 25 cm × 4.6 mm I.D. Vydac C_{18} column. Peaks: A = 69–81; B = 53–69; C = 45–88; D = 44–69; E = 45–89; F = 80–89; and G = MBP. A sample containing 10 μg each of MBP and MBP peptides was applied to the column.

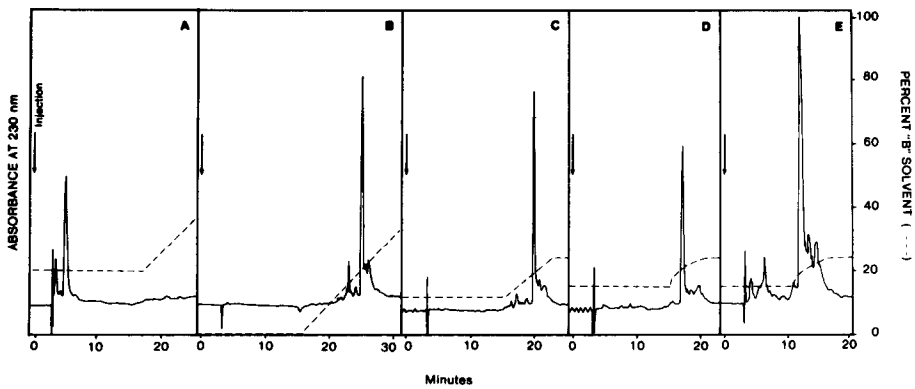

Fig. 3. Development of a method for purifying milligram quantities of synthetic MBP 44–69. Experimental conditions for panels A–D: 25 cm × 4.6 mm I.D. Vydac C_{18} column; flow-rate 1.0 ml/min; 230 nm; 0.4 a.u.f.s.; chart speed 0.5 cm/min; 20 μg sample. For panel E, 25 × 1.0 cm I.D. Vydac C_{18} column; flow-rate 4.7 ml/min; 230 nm; 2.0 a.u.f.s.; chart speed 0.5 cm/min; 1.5 mg sample. Elution systems: (A) 6 min initial conditions of 20% B and a 30-min linear gradient 20–80% B; (B) 6 min at initial conditions of 12% B and an 8-min linear gradient 12–24% B; (D,E) 6 min initial conditions of 15% B and a 6-min convex gradient 15–24% B (controller curve 5). All separations were done at room temperature (23°C).

DISCUSSION

Reversed-phase HPLC has been used previously to examine peptides derived from MBP[15,16]. These methods, however, utilized buffers which are not volatile and cannot be removed easily if the substances separated are to be subjected to further analysis.

MBP peptides were successfully resolved using reversed-phase HPLC with an isocratic elution system of volatile buffer[13], but the method was inadequate for the separation of complex mixtures of peptides. Recently, Kira *et al.*[10] utilized a gradient of acetonitrile in 0.1% trifluoroacetic acid at 34°C to separate a mixture of relatively small MBP peptides (2–9 amino acid residues). The method described here permits the separation at room temperature (23°C) of MBP from MBP peptides ranging in size from 10 to 45 amino acid residues.

A gradient-elution system composed of volatile solvents offered the opportunity to adjust several variables to attain the desired results. For each new application problem, an initial separation, using initial conditions of 20% B and a 30-min linear gradient of 20–80% B, was performed. Depending upon the complexity of the mixture, as few as three or as many as ten different chromatograms were assessed to optimize the separation. Variables were composition of the beginning and final solvents, slope of the gradient and the shape of the gradient.

This method has proved useful in the isolation of milligram quantities of MBP and some MBP peptides. It should also be useful in the analysis of products from enzymatic degradation of MBP and large peptides thereof. In addition, the method can potentially be used to analyze physiological fluids in which MBP or MBP peptides many appear.

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