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Rapid method based on reversed-phase high-performance liquid chromatography for purification of human myelin basic protein and its thrombic and endoproteinase Lys-C peptides

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SUMMARY

Reversed-phase high-performance liquid chromatography was applied to isolate myelin basic protein from human brain, followed by separation of proteolytic peptides thereof on the same chromatographic system. Brain tissue was delipidated under conditions that keep copurifying proteases inactive. The crude brain protein fraction was applied directly to a C_4 column The homogeneous protein obtained in this way was digested with thrombin and endoproteinase Lys-C in order to produce short defined myelin basic protein peptides. The purified peptides were used to determine the antigen fine specificity of myelin basic protein recognizing T lymphocyte lines isolated from multiple sclerosis patients.

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INTRODUCTION

Several lines of evidence suggest that multiple sclerosis (MS) is immune-mediated and that T lymphocytes (Tc) reacting against myelin components participate directly in the formation of MS lesions [1]. The myelin antigens that are important in MS have not been defined. Most attention has been focused on myelin basic protein (MBP), the dominant encephalitogen in all mammalian species examined so far [2]. Tc respond only to antigens that have been proteolytically processed by antigen-presenting cells (APCs) and bound to major histocompatibility complex (MHC) molecules that are expressed on the surface of these cells [3,4]. The encephalitogenic determinants of MBP vary in different animal species and between strains, depending on the presenting MHC class II molecules and the T cell receptors (TcR) that recognize the MBP-peptide–MHC complex [5–8]. In humans the MBP epitopes recognized by Tc have not been determined in detail to date [9,10].

Several approaches have been described for the purification of MBP and proteolytic peptides thereof from brain tissue of different animal species and humans [11–13]. The major form of human MBP (hMBP) has a relative molecular mass of 18 500, whereas additional minor hMBP variants (17 200 and 21 500) have been reported based on the molecular cloning of corresponding cDNAs [14]. Investigations on the role of MBP in MS would be greatly facilitated by the development of rapid and efficient methods for the isolation and purification of the native MBP molecule and of defined peptide fragments covering its whole sequence. The purification procedures described so far involve time-consuming precipitation and column chromatography steps [11–13]. Therefore, and in order to minimize the exposure of the protein preparation to co-purifying proteases [15,16], we developed a rapid isolation method for hMBP yielding sufficient amounts for Tc stimulation assays. Instead of pH-dependent or ammonium sulphate precipitations and ion-exchange chromatography [13], we subjected the crude delipidated protein fraction to preparative reversed-phase high-performance liquid chromatography (HPLC) with volatile buffers. This single purification step resulted in homogeneous hMBP. In addition this novel approach allows the preparation of proteolytic peptide fragments of MBP in two days in amounts sufficient for Tc stimulation tests from 10 g of frozen brain tissue. Our experiments have shown that the peptides produced in this manner are suitable for determining the fine specificity of human Tc lines recognizing hMBP.

Using the thrombic and Lys-C cleavage fragments we are currently investigating the immunodominant epitopes of hMBP, which are presented in association with distinct human major histocompatibility complex (HLA) class II molecules [17].

EXPERIMENTAL

Extraction of the MBP-containing protein fraction from brain tissue (delipidation)

Lipid extraction of brain tissue was carried out following the method of Eylar et al. [13] with essential time-saving modifications: 10 g of frozen (−80°C, 7 h post-mortem) human brain tissue were wrapped in aluminium foil and crushed with a hammer under liquid nitrogen to form a fine powder. The tissue powder was poured into a homogenizer (Bachhofer, Reutlingen, F.R.G.) containing 25 ml of methanol precooled to −30°C. The tissue was homogenized for 2 min in 15-s bursts followed by 15-s pauses to prevent warming. All extractions were performed in a cold room (4°C). After addition of 50 ml of chloroform (−30°C) the same homogenization process was repeated. The suspension was then transferred to 50-ml Falcon tubes. To each tube 5 ml of water, preadjusted to pH 2.1 with hydrochloric acid, were added to facilitate phase separation. The tubes were centrifuged at 200 *g* for 1 min, and the lower chloroform-containing phase was discarded. Then 1 g of Celite 545 (Fluka, Neu-Ulm, F.R.G.) was added to the methanol–water phase in a glass beaker. After stirring for 10 min the suspension was filtered through Whatman 41 filter paper (No. 1441185, obtained from Bender und Hobein, Munich, F.R.G.), and subsequently the filter cake was washed with 200 ml of ice-cold acetone to remove residual chloroform. The Celite–protein cake was resuspended in 40 ml of ice-cold water (pH 2.1). Whilst stirring for 3 h at 4°C the pH of the suspension was kept at 2.0–2.2 by addition of 0.1 *M* hydrochloric acid to prevent proteolytic degradation of hMBP [15,16]. Finally the suspension was centrifuged in 50-ml Falcon tubes for 10 min at 3300 *g*. The clear supernatants were pooled and concentrated to 5 ml on an Amicon concentrator.

Chromatography

HPLC was performed using a Beckman (Munich, F.R.G.) liquid chromatograph equipped with pump 126, variable-wavelength detector 167 and a Tandon PC system. For analytical work, a Vydac C_4 column (Nucleosil 300-5 C_4, 250 mm × 4 mm I.D.; Macherey-Nagel, Düren, F.R.G.) was used. Up to 10 mg of sample were chromatographed on a Vydac semi-preparative C_4 column (214 TP, 250 mm × 10 mm I.D.; obtained from Alltech, Unterhaching, F.R.G.), with properties almost identical with those of the analytical column. Solvent A was 0.1% (v/v) trifluoroacetic acid (Merck-Schuchardt, Hohenbrunn/Munich, F.R.G.) in water. Solvent B was 0.09% (v/v) trifluoroacetic acid in acetonitrile (both Merck, Darmstadt, F.R.G.). Solvents were always freshly prepared. The composition of the mobile phase was adjusted using a pump programme with pure solvent A from the A pump and pure solvent B from the B pump.

Proteolytic digestion of MBP and peptide analysis

Partial thrombic digestion of hMBP and more extensive thrombic digestion of its C-terminal half [amino acid (AA) residues 98–170][a] was carried out as described by Law et al. [18]. Endoproteinase Lys-C (Boehringer, Mannheim, F.R.G.) digestion was performed using the thrombic N-terminal fragment (AA residues 1–97) of hMBP. The digestion was carried out at 37 °C in 0.1 *M* Tris–HCl (pH 8.6) for 8 h. The peptide concentration was 0.35 mg/ml and 1.5 U Lys-C per mg peptide were used.

For AA analysis peptides were hydrolysed in constantly boiling hydrochloric acid for 22 h at 110 °C. AA analyses were carried out with an AA-analysator LC 6000 E (Biotronik, Munich, F.R.G.), using ninhydrin post-column derivatization. Protein concentrations were determined by the method described by Bradford [19] using a standard protein assay (Biorad, Munich, F.R.G.). Polyacrylamide (PAA)–urea gel electrophoresis followed the method of Swank and Munkres [20]. A low-molecular-mass protein mixture was used as standard. For some experiments aprotinin (bovine, 6500) or insulin, B chain (bovine, 3400) purchased from Pierce (Faust, Cologne, F.R.G.), were added as a molecular mass marker.

T cell lines

hMBP-specific Tc lines were derived from the peripheral blood of MS patients. The method of isolation and the determination of the antigen specificity and of the HLA restriction of the Tc lines are described elsewhere [17]. Briefly, $2 \cdot 10^4$ Tc were coincubated with $5 \cdot 10^4$ HLA-DR2-transfected L-cells as APCs in microtiter plates. hMBP was added to a final concentration of 3 μg/ml; the proteolytic fragments were added to approximately equimolar concentrations. Antigen-specific Tc proliferation was determined by measuring the [^{3}H]thymidine incorporation. Results (mean $\pm$ S.D.) (cpm) represent triplicate measurements.

RESULTS AND DISCUSSION

Previously, reversed-phase HPLC has been used to separate tryptic and synthetically obtained peptides of hMBP [23,24]. The purification procedure described here enables us to isolate hMBP in one step from all other components of human brain protein extract. It allows the rapid preparation of hMBP and proteolytic fragments thereof in two days in amounts sufficient for immunological studies, starting with 1–10 g of frozen brain tissue. Undesirable proteolytic degradation of hMBP by copurifying protease [15,16] was minimized by rapid processing of the material at a low pH range during delipidation, adsorption and desorption steps as well as during HPLC.

[a]For AA sequence see ref. 14.

HPLC purification of MBP from brain tissue

We modified the delipidation step described in ref. 13, resulting in a more rapid and protective purification of crude human brain proteins (see Experimental). Total protein yield from 10 g of wet tissue after lipid extraction was 109.7 mg.

Instead of conventional column chromatography and precipitations [11–13] we applied reversed-phase HPLC. A linear acetonitrile gradient was used to elute the protein components from a reversed-phase C_4 column (Fig. 1). Fig. 2 shows the purity of the hMBP peak after this chromatographic step. Interestingly, the main 18 500 hMBP fraction migrated slightly above the 21 500 standard in the gel system used. Loading of a high amount of purified hMBP onto the gel resulted in two weak additional bands above and below the 18 500 main MBP band (not shown), most likely corresponding to the 17 200 and 21 500 isoforms of hMBP [14]. No MBP band was detectable in the other major protein peaks (not shown). The volatile buffers used allowed direct lyophilization of the fractions, after which they were ready for proteolytic digestion. The yield of purified hMBP was 16.5 mg, which is more than 15% of total crude brain protein.

HPLC of thrombic- and endoproteinase Lys-C-cleaved hMBP peptides

MBP has several Arg–X bonds with different susceptibilities to thrombin cleavage. Under limiting conditions cleavage occurs preferentially at the Arg-97–Thr-98 bond [18][a]. The two fragments resulting from the partial thrombic

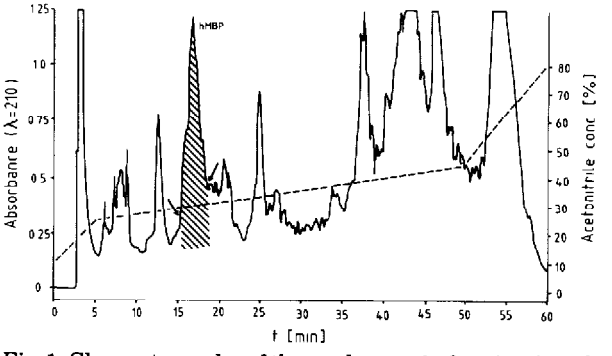

Fig. 1. Chromatography of the crude protein fraction from human brain. The gradient programme was: 0–5 min, 10–25% acetonitrile; 5–50 min, 25–45% acetonitrile; 50–60 min, 45–80% acetonitrile; 60–65 min, 80–10% acetonitrile. Zero marks the injection time of the sample (ca. 5 mg in 500 μl) and the beginning of the programme. The column was a Vydac 214 TP C_4 (5 μm, 250 mm × 10 mm I.D.). The runs were carried out at room temperature, and the flow-rate was 5.0 ml/min. Arrows indicate the collected hMBP fraction.

[a]Cleavage at Arg-97–Thr-98 of hMBP corresponds to the thrombic cleavage site Arg-95–Thr-96 of rabbit MBP, as described in ref. 18.

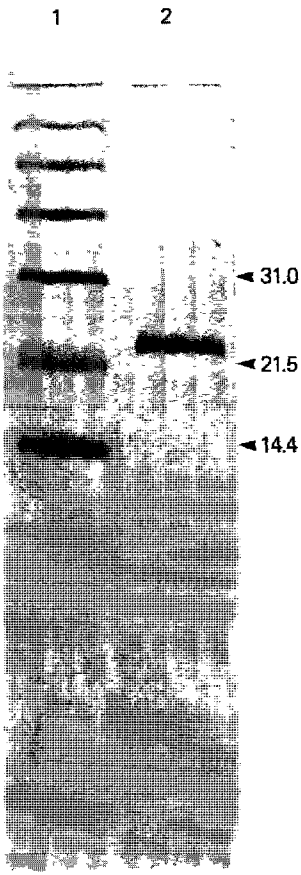

Fig. 2. Gel electrophoresis of purified hMBP. 2 μg of the collected and lyophilized hMBP peak were applied to a 10% SDS-PAA gel [20], and the gel as run for 5 h at 50 V. Lane 1, molecular mass markers; lane 2, hMBP.

cleavage were separated on the same column that was used for the isolation of hMBP (Fig. 3) They could be identified according to their size (Fig. 4) and their AA composition (not shown). The N-terminal part corresponds to AA 1–97 and the C-terminus to AA 98–170. The HPLC fractions were lyophilized and used after solubilization in water. The purity of the peptides was checked on a 10% PAA–urea gel (Fig. 4).

The C-terminal peptide from the hMBP/partial thrombic digestion was then subjected to a more extensive cleavage with thrombin (see Experimental), which resulted in two main peptides designated C1 and C2. The elution pattern of these peptides is depicted in Fig. 5. Fig. 6 shows the relative masses of the

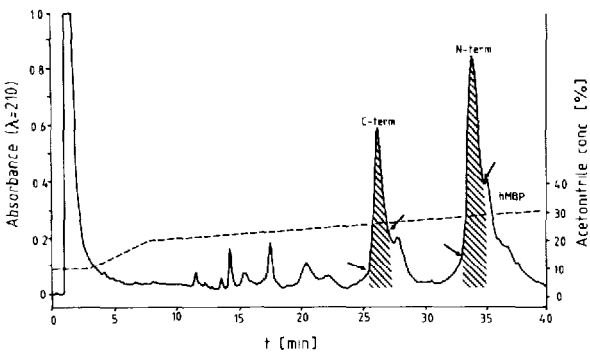

Fig. 3. Separation of partial thrombic peptides from hMBP The gradient programme was: 0–3 min, 10% acetonitrile; 3–8 min, 10–20% acetonitrile; 8–53 min, 20–35% acetonitrile; 53–63 min, 35–80% acetonitrile; 63–68 min, 80–10% acetonitrile. Zero marks the injection time of the sample (ca. 2 mg of hMBP/partial thrombic digest in 500 μl of digestion buffer containing 10% formic acid) and the beginning of the programme. The column was a Vydac 214 TP C_4 (5 μm, 250 mm × 10 mm I.D.). The runs were carried out at room temperature, and the flow-rate was 5.0 ml/min. Arrows indicate the collected peaks of the partial thrombic digest of hMBP.

C1 and C2 peptides analysed on a 15% PAA–urea gel. The AA analysis proved that the C1 peptide spanned AA 98–130 and the C2 peptide AA 131–170 (not shown). These peptides were used to narrow down the immunogenic epitopes present on the C peptide (see Tc stimulation). More prolonged thrombic digestion resulted in an additional cleavage at Arg-159–Asp-160 (not shown).

Partial cleavage of hMBP by thrombin under limiting conditions enabled us rapidly to prepare sufficient amounts of the N-terminal fragment (AA 1–97). Further digestion of this fragment by endoproteinase Lys-C resulted in small defined peptide fragments, one of them carrying a structurally and immunologically interesting Phe–Phe epitope that is lost during digestions with other proteases such as cathepsin D or pepsin [21,22]. Three main peptides were obtained (Fig. 7) and were designated N1, N2 and N3, corresponding to the AA sequences 14–53, 59–75 and 76–91, respectively, as deduced from the AA composition (not shown). The peptide fractions were lyophilized and solubilized in phosphate-buffered saline (PBS). They could be used to localize directly the MBP epitopes that are potentially recognized by MBP-specific Tc (see below).

T-cell stimulation experiments

The set of proteolytic fragments of hMBP described above was used to localize epitopes recognized by Tc lines derived from the peripheral blood of HLA-typed MS patients. These lines were coincubated with HLA-DR2-transfected L-cells and with hMBP or its proteolytic peptides as stimulating antigens. The antigen-specific proliferation of two Tc lines obtained from one MS

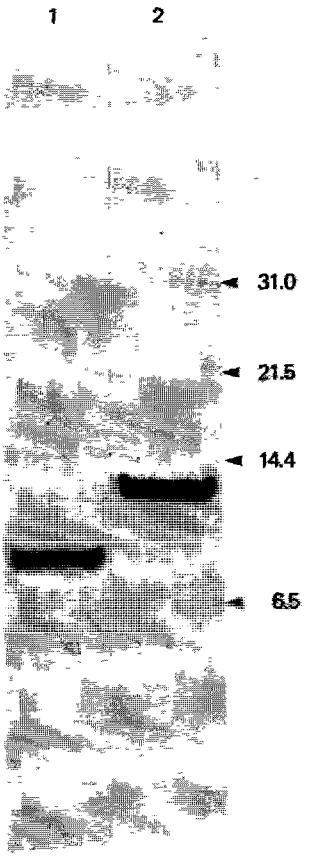

Fig. 4. Gel electrophoresis of purified peptides from the partial thrombic digest of hMBP: 2 μg of the collected and lyophilized hMBP C- and N-termini were applied to a 10% SDS-PAA gel [20] and the gel was run for 5 h at 50 V. Lane 1, hMBP partial thrombic C fragment; lane 2, partial thrombic N fragment.

patient is shown in Fig. 8. Tc line PS BP1 recognizes the thrombic fragment C1 (AA 98–130) (Fig. 8a), whereas Tc line PS BP7 responds to the Lys-C-produced fragments N3 (AA 76–91 of hMBP) (Fig. 8b). Slight contamination of C1 peptide by the C2 fraction (and vice versa) was observed, but could be compensated by using dilution series in the Tc stimulation studies (not shown).

The HPLC method described here allows the rapid purification of hMBP from a complex protein mixture and the separation of hMBP peptides ranging from 16 to 98 AA residues in size. In addition, our method may be useful for analysing physiological fluids that potentially contain MBP or MBP peptides. The proteolytic hMBP fragments described here have been used successfully to define the antigen fine-specificity of human Tc-recognizing hMBP (Fig. 8

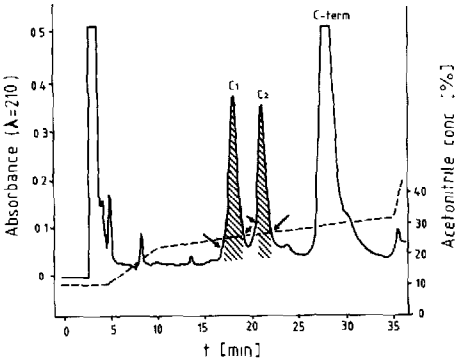

Fig 5. Separation of peptides from an extensive thrombic digest of the hMBP C-terminus (AA 98–170). The gradient programme was: 0–5 min, 10% acetonitrile; 5–10 min, 10–22.5% acetonitrile; 10–35 min, 22.5–32.5% acetonitrile; 35–40 min 32 5–80% acetonitrile; 40–45 min, 80–10% acetonitrile. Zero marks the injection time of the sample (ca. 500 μg in 50 μl of digestion buffer containing 10% formic acid) and the beginning of the programme. A Vydac C_4 column (Nucleosil 300-5 C_4, 250 mm×4 mm I.D.) was used. HPLC was carried out at room temperature, and the flow-rate was 1.0 ml/min. Arrows indicate the collected peptide fractions.

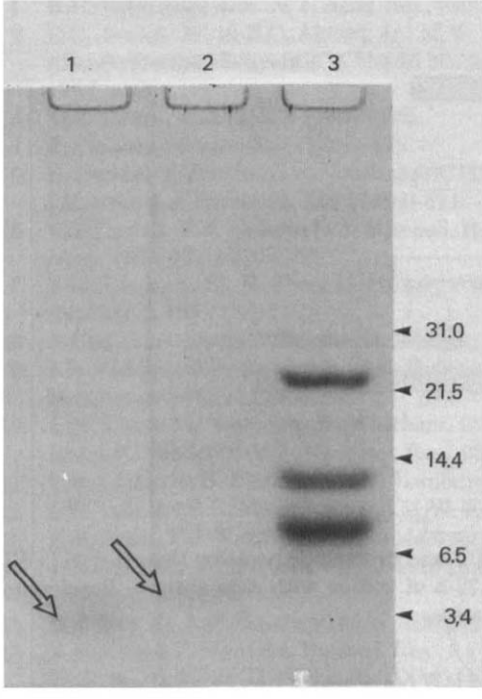

Fig. 6. SDS-PAA gel electrophoresis of purified peptides obtained by extensive thrombic digestion of the hMBP C-terminus: 2 μg of the collected and lyophilized peak fractions were applied to a 10% SDS-PAA gel [20] and the gel was run for 10 h at 25 V. Lane 1, C1 fragment; lane 2, C2 fragment; lane 3, hMBP plus its partial thrombic C and N fragments

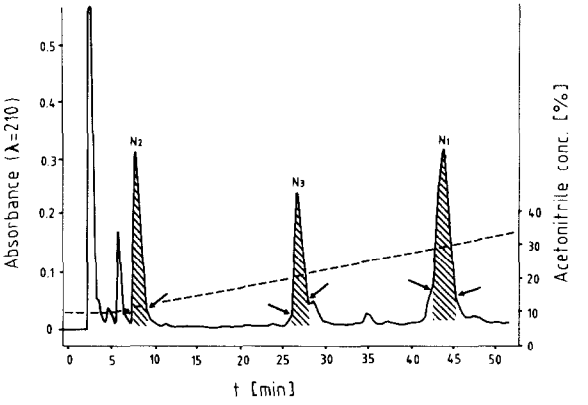

Fig. 7. Separation of Lys-C-produced peptides of hMBP fragment 1-97. The gradient programme was: 0-5 min, 10% acetonitrile; 5-55 min, 10-35% acetonitrile; 55-65 min, 35-80% acetonitrile; 65-70 min, 80-10% acetonitrile. Zero marks the injection of the sample (ca. 150 μg in 100 μl of digestion buffer containing 10% formic acid) and the beginning of the programme. A Vydac C_4 column (Nucleosil 300-5 C_4, 250 mm×4 mm I.D.) was used. HPLC was carried out at room temperature, and the flow-rate was 1.0 ml/min. Arrows indicate the collected peptide fractions.

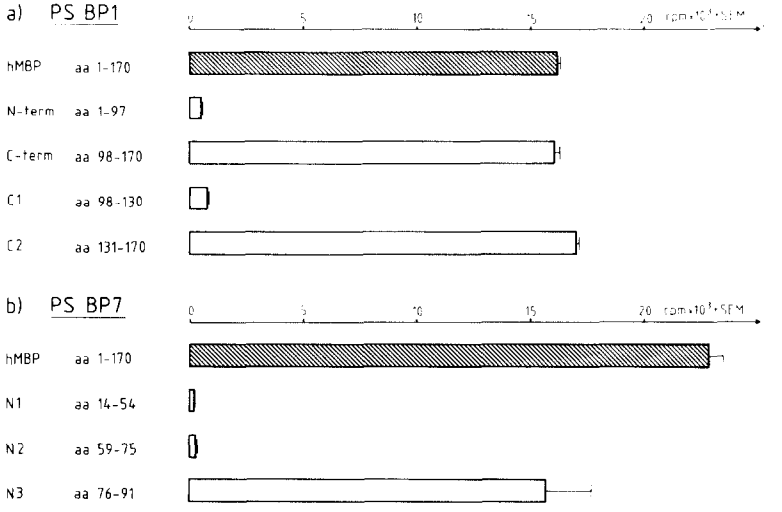

Fig. 8. Proliferative responses of Tc lines to hMBP and proteolytic peptides thereof. [^{3}H]TdR incorporation was quantified for each line after 72 h of culture with each antigen. Results are expressed as cpm ± S.D. from triplicate measurements.

and ref. 17). Using a set of proteolytic hMBP fragments has crucial advantages over direct screening with synthetic peptides. First, it allows the stepwise narrowing down of Tc epitopes on hMBP, starting with whole MBP molecules and ending with thrombic and endoproteinase Lys-C peptides. Furthermore, this approach conserves post-translational modifications of hMBP [25,26].

This may be more important than commonly assumed. For example, most MBP-specific, encephalitogenic Tc clones derived from the mouse PL/J strain recognize the critical epitopes 1–9 or 1–11 solely in an acetylated state [27,28].

The definition of MBP epitopes recognized by autoreactive T lymphocytes may contribute to a better understanding of the pathogenesis of inflammatory demyelinating diseases in humans.

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