A new procedure for the isolation of the brain myelin basic protein in a lipid-bound form

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Myelin basic protein has been isolated from bovine brain using the nonionic detergent *n*-octyl-polydisperse oligooxyethylene. The purified basic protein contains large amounts of heterogeneous lipids.

Myelin *Basic protein* *Protein purification* *Lipid* *Protein structure* *Multiple sclerosis*

1. INTRODUCTION

The encephalitogenic myelin basic protein (MBP), a 18.4 kDa protein is the smallest of the central nervous system (CNS) myelin proteins, and accounts for about 30% of total myelin proteins [1-3].

The rationale for the purification, characterization and extensive study of the basic protein is its key role in the understanding [4], detection [5,6] and treatment [7] of the human disease multiple sclerosis. This protein was usually extracted from delipidated white matter with HCl at pH 1.6-3.0 and subsequently purified by chromatographic procedures either in the absence [8] or presence of urea [9]. Since the protein purified in this way is water-soluble, characterized by the absence of secondary structure [10-12] and bound lipids [13] and is probably denatured [2], we have little information on both its structure and function. Therefore, a new procedure for the purification of myelin basic protein from bovine brain is reported here. The protein purified in the nonionic detergent octyl-POE contains large amounts of bound lipids and it appears to have a secondary structure.

Abbreviations: MBP, myelin basic protein; octyl-POE, *n*-octyl-polydisperse oligooxyethylene

2. MATERIALS AND METHODS

Octyl-POE with an average of 5 oxyethylene units [14], and other reagents were of analytical grade. Hydroxyapatite was purchased from Bio-Rad, Ultrogel AcA 34 from LKB.

Myelin was prepared from bovine brain white matter as in [15]. Protein was determined with the Bio-Rad reagent using the microassay procedure and BSA as a standard. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a discontinuous system on 15% acrylamide gels [16]. Runs were carried out using 4.5% spacer gels. Prior to application to SDS-PAGE, protein samples were incubated with 1 volume of 2% SDS, 62.5 mM Tris-HCl, 10% glycerol, 5% Bromophenol blue (pH 8.8) for half an hour at room temperature. Following the run, the gels were stained for 2-3 h in 0.2% Coomassie blue R-250 and 0.05% Coomassie blue G-250 in ethanol:acetic acid:water (4:1:4, v/v), and finally destained with the same medium in the absence of Coomassie.

Phospholipid content was determined as in [17]. Phosphate, if present, removed by extensive dialysis. Cholesterol content was determined with the Beckman HPLC apparatus [18].

Thin-layer chromatography (TLC) was carried

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out on Merck silica gel 60 F_{254} 0.2 mm plates. Isolated basic protein (40–50 μl) was directly applied in octyl-POE as obtained after the last purification step. Myelin membrane and standards were solubilized in the same octyl-POE medium prior to application. The plates were developed for the separation of either phospholipids [19], glycolipids [19], or gangliosides [20]. Lipids were detected by different reagents: α-naphthol, iodine vapor, resorcinol, sulfuric acid potassium dichromate charring. The detergent was revealed with an ammonia/silver nitrate mixture (0.1 N $AgNO_3$:5 N NH_3, 1:1) by heating for 15–20 min at 120°C [21].

Electrophoretic blotting and immunological detection of the lipid binding basic protein were carried out as in [22]. The anti-MBP sera used for the immunoblotting technique were obtained according to [23] using as an antigen the basic protein prepared as in [9].

3. RESULTS

The isolation of the basic protein from bovine brain consists of extraction with the nonionic detergent octyl-POE, column chromatography on hydroxyapatite (HTP) and gel filtration. The procedure is outlined in table 1. Thawed myelin was homogenized in octyl-POE containing medium for solubilization. After 30 min incubation in ice, the suspension was centrifuged for 45 min at 40000 rpm in the 50.2 Beckman rotor. Purification of the protein was carried out at room temperature at pH 8.5.

The supernatant, containing mostly basic protein and proteolipid, was applied to a hydroxyapatite (HTP) column (0.9 ml HTP/ml extract) equilibrated with the low ionic strength eluant given in table 1. Most of the proteolipid remained bound to the column, whereas the basic protein was collected in the pass-through fractions between 0.8 and 2.3 column volumes. As an alternative, a batch procedure was used: following centrifugation of the suspended resin for 15 min at 12500 rpm in the JA-20 Beckman rotor, the basic protein was recovered in the supernatant within a very short time (30 min).

Nonadsorbed fractions were concentrated 10 times by ultrafiltration. During this step, part of the protein passed through the PM 10 membrane,

Table 1

FLOW SHEET OF THE PURIFICATION OF MYELIN BASIC PROTEIN

	MYELIN	357 MG PROTEIN	(100%)
1.	EXTRACTION WITH OCTYL-POE: 5 MG PROTEIN/ML IN 71 ML 1.1% OCTYL-POE, 420mM NaCl, 17mM TRIS-HCl, pH 8.5, 30 MIN IN ICE.		
	EXTRACT	253 MG PROTEIN/62 ML	(71%)
2.	NEGATIVE ADSORPTION CHROMATOGRAPHY ON HYDROXYAPATITE (HTP) (56 ML COLUMN), ELUENT: 1% OCTYL-POE, 10mM TRIS-HCl pH 8.5, ROOM TEMPERATURE, 140 MIN.		
	PASS-THROUGH FRACTIONS	92 MG PROTEIN/91 ML	(26%)
3.	ULTRAFILTRATION ON AMICON PM 10 MEMBRANE, ROOM TEMPERATURE.		
	CONCENTRATED HTP POOL	84 MG PROTEIN/8.8 ML	(24%)
4.	GEL FILTRATION ON ULTROGEL AcA 34 (244 ML COLUMN), ELUENT: 1% OCTYL-POE, 20 mM TRIS-NaPi, 100mM NaCl, pH 8.5, ROOM TEMPERATURE, 10 HOURS.		
	PURIFIED MBP POOL 31-36	46 MG PROTEIN/34 ML	(13%)
	STORED AT -25°C AS OBTAINED, OR AT -80°C AFTER EITHER LIOPHYLIZATION OR PRECIPITATION WITH AMMONIUM SULFATE TO 50% SATURATION.		

together with 2/3 of the phospholipids found in the nonadsorbed fractions. Using the YM5 Amicon membrane, no loss of protein was observed. The concentrated HTP pool, containing about 3% of myelin phospholipids, was then applied on an Ultrogel AcA 34 column (28 ml gel/ml applied sample) for the final purification of the basic protein.

The eluate profile is shown in fig.1. The basic protein is eluted between 0.70 and 0.83 column volumes. The contaminant proteolipid protein is eluted just before the basic protein.

Electrophoretic analysis in SDS of single fractions from the AcA 34 column shows the degree of purity of isolated basic protein (fig.1, inset, lane n). The smaller band which can be observed on the left (lanes e–m) just below the basic protein is a degradation product which appears after one day storage at room temperature. No other contaminants were detected.

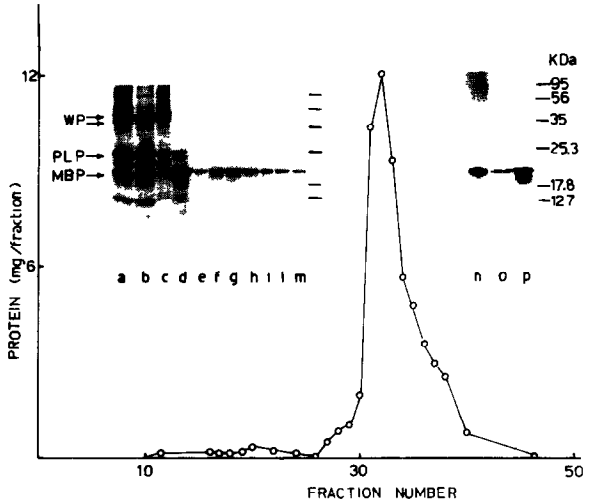

Fig.1. Gel filtration of the HTP pass-through fractions after concentration; 82.9 mg protein in 8.7 ml were applied to the AcA 34 column (2.4 × 54 cm); 5.6 ml fractions were collected at a flow rate of 26 ml/h. The phospholipid phosphorus peak (not shown) corresponded with the protein peak. The free phospholipids peak was not well detectable. V_o and V_t correspond to fractions 16 and 44, respectively. Other conditions as in table 1. The electropherograms of slab gels exhibit basic protein at different steps of purification, stained with Coomassie blue. From left to right, the samples are: (a) myelin proteins; (b) octyl-POE extract of myelin; (C) non-extracted material; (d) HTP pass-through fractions; (e–m) fractions 30–35 and 37, respectively, from the AcA 34 column, after 1 day; (n–p) purified basic protein, fraction 32, (n) as eluted from the column, (o) after lyophilization and two weeks storage at −25°C; (p) after 10 days at room temperature. The two major bands present in the higher M_r region are Wolfgram proteins (WP). The other major band which can be observed between the basic protein (lower major band) and the Wolfgram proteins is the proteolipid protein (PLP). The arrows (right) indicate the positions of protein standards. From top to bottom the standards are: phosphorylase *a*, catalase, glyceraldehyde-3-phosphate dehydrogenase, trypsinogen, myoglobin, cytochrome *c*. Conditions as described in section 2.

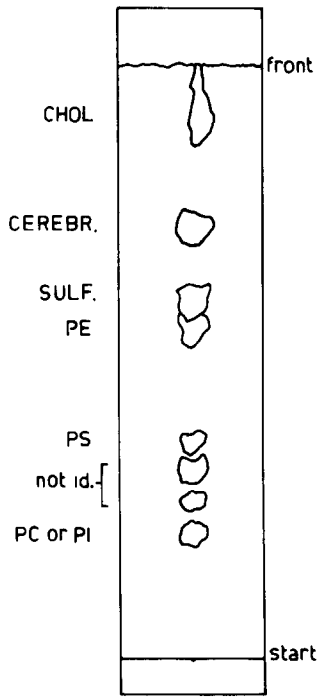

Fig.2. Lipids bound to purified myelin basic protein. One-dimensional TLC on silica gel plate using as eluting system: chloroform:acetone:methanol:acetic acid:water (40:20:10:10:5, v/v) for 180 min. Spots were detected by spraying the plates with α-naphthol reagent and heating at 100°C for approx. 5 min. The lipids are, from top to bottom: cholesterol (red); cerebrosides (bluish purple); sulfatides (bluish purple); phosphatidylethanolamine (PE) (yellow brown); phosphatidylserine (PS) (yellow brown); two unidentified spots (bluish purple), and phosphatidylcholine (PC) or phosphatidylinositole (PI) (yellow brown).

Purified basic protein contains high amounts of bound lipids: TLC (fig.2) shows the presence of cholesterol, cerebrosides, sulfatides, phosphatidylethanolamine and phosphatidylserine. Two spots could not be identified. The determination of phospholipid content by phosphate analysis gives as a result 1.23 μmol P_i (or 1.04 mg PL)/mg basic protein. The cholesterol content is 0.1 mg/basic protein.

The composition of amino acid is very similar to that described in [24]. The N-terminus appears to be blocked. The C-terminus is Ala-Arg, which differs from the expected sequence Ala-Arg-Arg. The identity of the lipid-binding basic protein with the lipid-free basic protein prepared in the conventional way [9] was confirmed by the immunoblotting technique using antibodies directed against the lipid-free basic protein (fig.3).

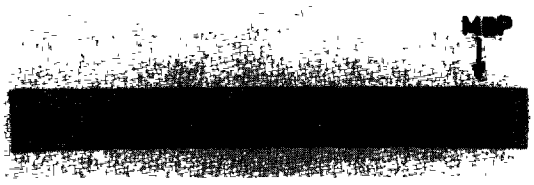

Fig.3. Detection of the purified lipid-binding basic protein (MBP) (40 μg) by immunoblotting. Electrophoresis was from left to right. The blot was incubated for 2 h at 37°C with the diluted (1:700) antibody and then treated with diluted (1:1000) anti-IgG peroxidase for 3 h at room temperature. Finally the blot was colored with diaminobenzidine. Other conditions as in [22]. On the right of the arrow a MBP fragment is also detected.

4. DISCUSSION

The most significant difference between the procedure described here for purification of the myelin basic protein and those previously reported [8,9] consists in the solubility properties of the resulting protein. Thus, the use of conventional methods [8,9] led to an apparently water-soluble protein which did not partition into lipid membranes [25]. Basic protein prepared by solubilization with deoxycholate, also does not contain lipids [26].

If prepared by our method, which requires the use of detergents [27], every protein molecule binds about 22 molecules of phospholipids and other lipids having a heterogeneous composition. The phospholipid content is 4-times higher on a weight basis (w/w) than for the ADP/ATP carrier [28] which is hydrophobic and was purified from mitochondria by a similar procedure [29].

In view of the localization of the myelin basic protein, which appears to play a role in maintaining the multilamellar structure of myelin sheath [30,31], it appears reasonable that such a protein should be membrane-bound. Our finding of a lipid-binding protein is more consistent with this function than a soluble protein such as obtained by conventional, and probably denaturing, procedures [8,9]. In preliminary experiments using circular dichroism, we find indeed that the protein obtained by our procedure contains a significant amount of β-structure whereas conventional preparations exhibited partial α-helical [26] and 'random' conformation [10–12]. A more detailed study of the properties of myelin basic protein is currently in progress.

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