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Fractionation of central nervous system myelin proteins by reversed-phase high-performance liquid chromatography

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Abstract

Chromatographic fractionation of central nervous system myelin proteins is hampered by their poor solubility in water and strong association with lipids. Moreover, several myelin membrane proteins undergo posttranslational acylation which further increases their hydrophobicity. Here, a method is described for a two-step delipidation and high-resolution fractionation by reversed-phase high-performance liquid chromatography of all myelin proteins. The elution was monitored of the two major protein components, *i.e.* myelin basic protein (MBP) and proteolipid protein (PLP), as well as of minor components, *viz.* myelin-associated glycoprotein (MAG) and myelin/oligodendrocyte glycoprotein (MOG). Whereas MBP and MOG elute as single sharp protein peaks upon chromatography, PLP and MAG are resolved into several different components. Following their delipidation and separation, all proteins including the hydrophobic transmembrane proteins can be transferred to fully aqueous solutions without detergents. The overall yield of myelin proteins obtained in this way exceeds 85%.

1. Introduction

The insulating, multilamellar myelin sheath that surrounds axons in the central nervous system (CNS) is crucial for adequate nerve function. Its degeneration may lead to severe impairment of sensory functions and to paralysis. About 20–25% of the CNS myelin mass is represented by a large number of different membrane-embedded proteins ranging from hydrophilic species such as myelin basic protein (MBP) to very hydrophobic transmembrane proteins like the myelin proteolipid proteins (PLP) [see for review ref. 1]. Detailed studies on myelination or demyelinating disorders such as multiple sclerosis [2] or the Guillain–Barré syndrome [3] require the availability of purified myelin proteins. Only in this way the structure as well as the physicochemical and immunological properties of such proteins can be evaluated.

In our studies on immune recognition of myelin proteins, we searched for an experimental approach of fractionating total myelin protein with high resolution and high yield in such a way that the myelin proteins could be transferred into completely aqueous solutions without detergents or organic solvents. In our hands, the application of previously described fractionation methods

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[4,5] led to incomplete delipidation of myelin proteins. As a consequence, protein solubility in aqueous solutions was limited and resolution of the different myelin proteins upon reversed-phase HPLC remained poor.

Here we describe a method that significantly improves resolution during HPLC by applying more extensive delipidation and a different mobile phase. It allows the transfer of myelin proteins into aqueous solution, even for the highly hydrophobic proteolipid proteins. Apart from its applicability in screening studies, this fractionation method may serve as a starting point for the purification of individual myelin proteins. The procedure described here has been found reproducible in the fractionation of CNS myelin proteins from bovine, guinea pig and human brain.

2. Experimental

2.1. Isolation, solubilization and delipidation of myelin

CNS myelin was isolated according to the procedure described by Norton and Poduslo [6]. Briefly, CNS white matter was homogenized in 0.32 *M* sucrose in a Dounce homogenizer. The suspension was layered over a 0.85 *M* solution of sucrose and centrifuged at 75 000 *g* for 30 min at 4°C (step 1). Myelin membranes at the interphase were pooled, collected by centrifugation and washed three times with de-ionized water by repeated centrifugation at 75 000 *g* for 15 min at 4°C (step 2). Subsequently, steps 1 and 2 were repeated twice and the collected myelin membranes were lyophilized and stored dry at −20°C.

The first delipidation step involves gel permeation over Sephadex LH-60 (Pharmacia LKB, Uppsala, Sweden) [5,7]. Samples of 320 mg lyophilized myelin membranes were dissolved in 8 ml 80% tetrahydrofuran (THF) (v/v) in water containing 0.1% trifluoroacetic acid (TFA) (v/v) immediately prior to application onto a Sephadex LH-60 column (35 × 2.5 cm I.D.) equilibrated in the sample solvent. Proteins and lipids were eluted at 0.25 ml/min and fractions of 3.7 ml were collected. The fractions that contained myelin proteins were identified by enzyme-linked immunosorbent assay (ELISA) using a PLP-specific monoclonal antibody following transfer of the protein contents to aqueous solution (see below and Fig. 1). For a second delipidation step, these protein-containing fractions were pooled and supplied with five volumes of ice-cold diethyl ether. After at least 2 h at −20°C (to optimize the separation between the ether and aqueous phases), the mixture was centrifuged at 4°C for 30 min at 3500 *g*. The lower aqueous phase together with the protein precipitate at the interphase was collected and lyophilized.

2.2. HPLC separation of total myelin proteins

Lyophilized and delipidated myelin proteins were dissolved in 2-chloroethanol containing 0.1% TFA (v/v) at a final protein concentration of 10 mg/ml. In order to allow protein retention by a reversed-phase matrix, the protein solution

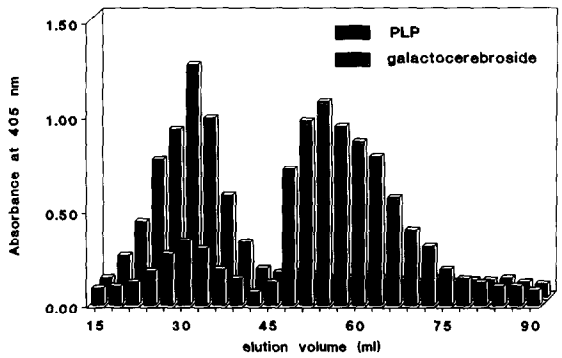

Fig. 1. Gel permeation chromatography of solubilized myelin over Sephadex LH-60. Proteins from bovine CNS myelin, solubilized in 80% THF/0.1% TFA, were separated from myelin phospholipids and glycolipids by gel permeation chromatography over Sephadex LH-60 and collected in fractions. Samples of each fraction were lyophilized and transferred to aqueous solution as described under Experimental for ELISA with monoclonal antibodies against PLP and galactocerebrosides, respectively. This assay revealed elution of significant amounts of low-molecular weight glycolipids in the void volume, together with PLP. For further details, see Experimental.

in 2-chloroethanol was subsequently diluted with at least five volumes of water containing 0.1% TFA (v/v). Alternatively, the solution of myelin proteins in 2-chloroethanol can be dialyzed in regenerated cellulose acetate membranes (Spectrum Medical Industries, Los Angeles, CA, USA) against water containing 0.1% TFA (v/v).

Samples of 10 mg of total myelin proteins were filtered through 0.22-μm filters and applied onto a semi-preparative reversed-phase column with a wide-pore C_3 matrix (5-μm Ultrapore C3, 250 × 10 mm I.D., Beckman Instruments, San Ramon, CA, USA). Proteins were eluted at a flow-rate of 2.5 ml/min with increasing amounts of solvent B [80% THF (v/v); 20% acetonitrile (v/v); 0.1% TFA (v/v)] in solvent A [0.1% TFA (v/v) in water] according to the following gradient: 0–30% B over the first 5 min and 30–100% B over the next 40 min. The eluate was monitored at 278 nm, collected in 1-min fractions and lyophilized. Lyophilized protein was dissolved in 2-chloroethanol and transferred to aqueous solution by dialysis in regenerated cellulose acetate membranes. It should be noted that aqueous solutions of myelin proteolipid proteins obtained in this way will form a precipitate upon freezing and thawing. This can be avoided by supplying glycerol to the protein solution to a final concentration of at least 10% (v/v).

2.3. Analysis of myelin proteins

The fractions collected after HPLC fractionation of total myelin protein were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and screened for recognition by monoclonal antibodies against MBP (Sigma, St. Louis, MO, USA), MAG (Boehringer Mannheim, Germany) [8], MOG [9] and PLP [10], respectively, by using standard techniques for ELISA. First, a 10% sample of all HPLC fractions was lyophilized, redissolved into 2-chloroethanol and extensively dialyzed against water in order to avoid interference of organic solvents with SDS-PAGE or ELISA. For each monoclonal antibody, the proportion of HPLC fractions coated onto microtiter plates (in PBS, pH 7.4) was adjusted in order to optimize the screening profiles. SDS-PAGE was performed with the Phast-system (Pharmacia LKB, Bromma, Sweden) according to the manufacturer's instructions. A fixed proportion of each fraction was used for SDS-PAGE analysis. It should be noted that the protein samples were not heated prior to application onto the gel in order to minimize aggregation of more hydrophobic proteins. Amino acid analysis of protein samples taken at various steps of the procedure was performed as described previously [11]. Detection of galactolipids in the eluate fractions upon gel permeation over Sephadex LH-60 was performed by standard ELISA using a galactocerebroside-specific monoclonal antibody [12].

3. Results and discussion

3.1. Delipidation of myelin proteins

In line with previous findings by Tettamanti *et al.* [13], Tandler and Fiszer de Plazas [14] and Diaz *et al.* [5], acidified 80% THF in water was found suitable for the complete solubilization of purified myelin membranes. In previous studies, delipidation of myelin proteins after solubilization in either 80% THF [5], mixtures of chloroform/methanol [7] or in 2-chloroethanol [4] was performed by gel permeation over Sephadex LH-60, a Sephadex derivative compatible with organic solvents. In this way, low-molecular mass lipids should be separated from the proteins that collectively elute in the void volume of the column. In our hands, however, resulting protein samples were never sufficiently delipidated to allow transfer to aqueous solvents without substantial loss due to precipitation. We examined the distribution over the gel permeation eluate fractions of proteolipid proteins, as markers of total myelin proteins, as well as of galactolipids, as markers of low-molecular mass phospholipids and glycolipids. Comparison of the respective elution profiles (Fig. 1) reveals that substantial amounts of galactolipids are collected in the void volume suggesting that they remain protein-associated. More extensive delipidation of myelin proteins, crucial to complete

solubility in aqueous solution, can be achieved by additional precipitation with diethyl ether following the Sephadex LH-60 gel permeation step. Essentially no protein loss occurs upon ether precipitation.

3.2. HPLC fractionation

In order to allow elution of extremely hydrophobic proteins such as myelin proteolipid proteins from reversed-phase matrices, organic solvents with high eluotropic properties like THF or 2-propanol are required. At the same time, viscosity of the organic modifier should be kept low to maintain peak sharpness and to avoid excessive back pressure in the HPLC system. We evaluated several eluents for their ability to produce high-resolution profiles upon reversed-phase HPLC of total myelin proteins and found optimal results when using a mixture of acidified THF and acetonitrile in a volume ratio of 4 to 1. By using this mixture as an eluent, resolution between individual protein components is substantially better than upon elution with THF alone [5] or with 2-propanol [4]. Fig. 2 illustrates the elution profile obtained. The use of a mixture of THF and acetonitrile as an eluent results in the resolution of different molecular species of PLP which cannot be achieved by previously described eluents.

Elution of myelin proteins was monitored by SDS-PAGE of the protein contents of each of the 50 fractions collected upon HPLC. Fig. 3 reveals the multitude of different protein species present in myelin and their elution along the gradient. Given our limited knowledge on the exact protein composition of CNS myelin, many of the protein bands visible in Fig. 3 remain unidentified as yet. In order to examine the behaviour of individual myelin proteins upon fractionation, four myelin proteins were selected for monitoring on the basis of the availability of monoclonal antibodies.

Thus, samples of the HPLC fractions were transferred to aqueous solution and assayed by ELISA using monoclonal antibodies to MBP, MAG, MOG and PLP, respectively, as representatives of both major and minor myelin proteins (Fig. 4). ELISA and SDS-PAGE monitoring of MBP and MOG reveals elution of these proteins as sharp peaks that are largely collected in a single fraction. The predominant 18.5 kDa form of MBP [15] is almost exclusively collected in fraction 9 while the two MOG bands at 27 and 53 kDa [16] appear only in fraction 24. SDS-PAGE also reveals the presence of a slightly larger form of MBP and small amounts of MBP-dimers that co-elute with MBP in fraction 9. It should be noted that very small amounts of MBP can be detected in a number of fractions following fraction 9. Comparison of SDS-PAGE and ELISA data suggests that this fraction represents less than 1% of the total amount of MBP. Upon re-chromatography in the same HPLC system, this small fraction of MBP persists in eluting later along the gradient than the major peak at fraction 9. We attribute this phenomenon to the persistence of small amounts of lipid-bound MBP [17], even under the denaturing conditions used.

Monitoring of MAG or PLP in the eluate fractions indicated their appearance in a substan-

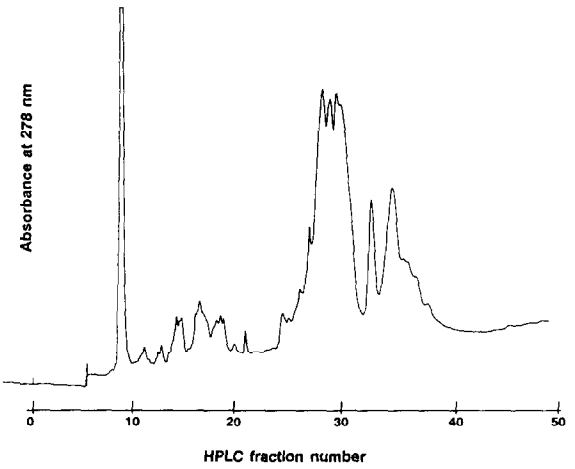

Fig. 2. Reversed-phase HPLC profile of total CNS myelin proteins. Following delipidation, myelin proteins were eluted from a wide-pore C_3 reversed-phase matrix by HPLC with increasing amounts of THF-acetonitrile (4:1, v/v) containing 0.1% TFA; the eluate was monitored at 278 nm. The major peak at fraction 9 represents MBP; the series of peaks between fraction 26 and 38 represent different molecular species of PLP that are resolved by HPLC (*cf.* Fig. 4). For further details, see Experimental.

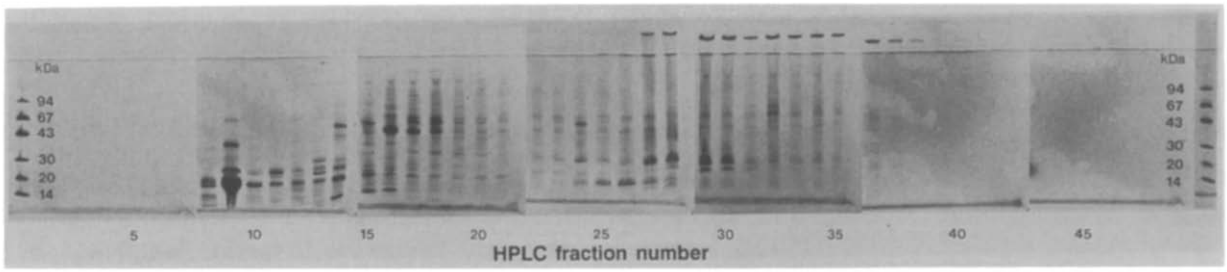

Fig. 3. SDS-PAGE analysis of myelin proteins after HPLC fractionation. Total CNS myelin proteins were fractionated by reversed-phase HPLC and a sample of each fraction was lyophilized, analyzed by SDS-PAGE and stained by coomassie brilliant blue. PLP is well-known to enter SDS-PAGE gels only with difficulty. Much of the PLP in fractions 27–37 can, therefore, only be seen precipitated at the site of application. For further details, see Experimental.

tial number of adjacent fractions. However, the designations MAG and PLP in fact indicate a variety of molecular species that share the same amino acid sequence but differ in length as well as in their extent of post-translational modifications. MAG includes a small 62 kDa as well as a larger 67 kDa form [18–20] while PLP may exist as a 30 kDa or a 25 kDa form (designated DM-20) [1,20]. These molecular species of MAG and PLP result from differential splicing of their respective primary transcripts, a phenomenon not uncommon among myelin proteins [1,19]. Different molecular forms have presently been identified for MBP, PLP, MAG and the myelin-associated enzyme 2′3′-cyclic nucleotide 3′-phosphohydrolase but also other myelin proteins may exist as different molecular species. Moreover, the different forms are substantially modified post-translationally. MAG has been reported to undergo extensive glycosylation as well as palmitylation [16] whereas PLP may contain up to six covalently linked long-chain fatty acids, mainly palmitic acid [19,21]. While glycosylation will not dramatically affect retention by reversed-phase matrices, acylation will. Thus, differentially modified molecular species of MAG and PLP may appear at slightly different positions in the HPLC profile. Consistent with this view is the much higher resolution in the UV absorption profile than in the ELISA profile. While successively eluting MAG and PLP species will produce separate UV absorption peaks, they may not necessarily be discriminated by a monoclonal antibody. The notion that individual myelin proteins may exist as sets of differentially modified molecular species may partially explain why also some other minor protein bands visible in the SDS-PAGE profile elute in more than two or three adjacent fractions.

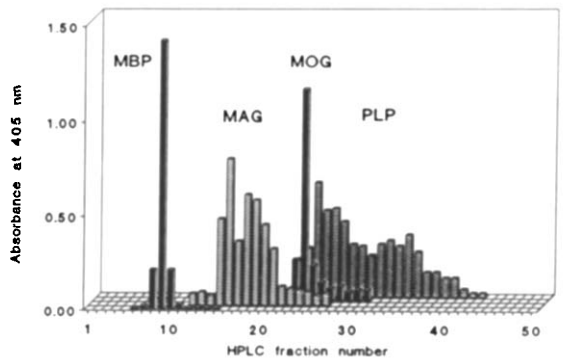

Fig. 4. ELISA of myelin proteins after HPLC fractionation. Total CNS myelin proteins were fractionated by reversed-phase HPLC and a sample of each fraction was lyophilized and transferred to aqueous solution according to Experimental. Using these solutions, ELISA was performed on each HPLC fraction under standard conditions with monoclonal antibodies against MBP, MAG, MOG, and PLP, respectively. For further details, see Experimental.

The yield of myelin protein following delipidation and reversed-phase HPLC fractionation was determined by amino acid analysis of samples taken at various steps along the procedure. By determining the yield for individual amino acids at each successive step, the overall yield of protein mass was found to exceed 85% (Table 1). The yields for proline and tyrosine as determined in the crude myelin sample deviated strongly from those found after delipidation. We attribute this variation to the presence of myelin

Table 1
Amino acid yields at succesive steps of the purification

Amino acid	Yield (nmol)			Final yield (%)
	Step 1	Step 2	Step 3	
Asx	22.18	19.43	18.35	83
Glx	22.28	19.96	18.80	84
Ser	29.51	28.12	26.02	88
Gly	31.55	29.04	27.64	88
His	9.04	8.23	7.52	83
Arg	14.38	13.43	13.07	91
Thr	25.81	23.88	22.87	89
Ala	28.45	26.53	25.72	90
Pro	9.42	12.48	11.87	126
Tyr	6.26	10.07	9.07	145
Val	18.17	17.28	16.68	92
Met	6.21	6.04	5.40	87
Ile	12.23	11.71	11.16	91
Leu	25.96	24.97	24.09	93
Phe	17.82	16.03	15.40	86
Lys	17.03	15.84	15.00	88

Values were determined upon acid hydrolysis of a fixed sample of the protein solutions obtained at various steps along the purification procedure. Step 1 is after solubilization and prior to any chromatographic manipulation; step 2 is after chromatography over Sephadex LH-60; step 3 is after passage over a reversed-phase HPLC column. Final yields are given as the percentage relative to the first sample taken.

lipids that interfere with the analytical method used. In line with previous data [1], amino acid analysis indicates that myelin proteins represent 20–25% of the dry weight of purified myelin membranes.

4. Conclusions

We have optimized a reproducible approach to high-resolution and high-yield separation of individual proteins from CNS myelin. Results shown in the present paper were obtained with bovine myelin but essentially identical results have been obtained with samples from human CNS myelin and guinea pig CNS myelin. Major as well as minor myelin proteins ranging from the highly hydrophilic MBP to the extremely hydrophobic PLPs can be separated in a single HPLC run. This fractionation method is useful in monitoring the protein composition of myelin as a function of development or disease. The fully delipidated state of all proteins following HPLC fractionation allows their solubilization into completely aqueous solutions. Thus, myelin proteins can be made available for a variety of further studies including *e.g.* immunological studies that would be precluded by the presence of organic solvents or detergents in protein samples.

5. Acknowledgements

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6. References

[1] M.B. Lees and S.W. Brostoff, in P. Morell (Editor), *Myelin*, Plenum Press, New York, NY, 1984, p. 197.
[2] N. Scolding, C. Linington and A. Compston, *Autoimmunity*, 4 (1989) 131.
[3] A. Khalili-Shirazi, R.A.C. Hughes, S.W. Brostoff, C. Linington and N. Gregson, *J. Neurol. Sci.*, 111 (1992) 200.
[4] O.A. Bizzozero, T.S. Odykirk, J.F. McGarry and M.B. Lees, *Anal. Biochem.*, 180 (1989) 59.
[5] R.S. Diaz, P. Regueiro, J. Monreal and C.J. Tandler, *J. Neurosci. Res.*, 29 (1991) 114.
[6] W.T. Norton and S.E. Poduslo, *J. Neurochem.*, 21 (1973) 749.
[7] O. Bizzozero, M. Besio-Moreno, J.M. Pasquini, E.F. Soto and C.J. Gomez, *J. Chromatogr.*, 227 (1982) 33.
[8] M. Poltorak, R. Sadoul, G. Keilhauer, C. Landa, Th. Fahrig and M. Schachner, *J. Cell Biol.*, 105 (1987) 1893.
[9] C. Linington, M. Webb and P.L. Woodhams, *J. Neuroimmunol.*, 6 (1984) 387.
[10] T. Yamamura, J.T. Konola, H. Wekerle and M.B. Lees, *J. Neurochem.*, 57 (1991) 1671.
[11] J.M. Van Noort, J. Boon, A.C.M. Van Der Drift, J.P.A. Wagenaar, A.M.H. Boots and C.J.P. Boog, *Eur. J. Immunol.*, 21 (1991) 1989.
[12] B. Ranscht, P.A. Clapshaw, J. Price, M. Noble and W. Seifert, *Proc. Natl. Acad. Sci. USA*, 79 (1982) 2709.
[13] G. Tettamanti, F. Bonali, S. Marchesini and V. Zambotti, *Biochim. Biophys. Acta*, 296 (1973) 160.

[14] C.J. Tandler and S. Fiszer de Plazas, *Life Sci.*, 17 (1975) 1407.
[15] R. Smith, *J. Neurochem.*, 59 (1992) 1589.
[16] J.-M. Matthieu and P. Amiguet, *Dev. Neurosci.*, 12 (1990) 293.
[17] P. Riccio, J.P. Rosenbusch and E. Quagliarello, *FEBS Lett.*, 177 (1984) 236.
[18] D.E. Frail and P.E. Braun, *J. Biol. Chem.*, 259 (1984) 14857.
[19] L. Pedraza, B.L. Frey, B.L., Hempstead, D.R. Colman and J.L. Salzer, *J. Neurosci. Res.*, 29 (1991) 141.
[20] K.-A. Nave, F.E. Bloom and R.J. Milner, *Proc. Natl. Acad. Sci. USA*, 84 (1987) 5665.
[21] Th. Weimbs and W. Stöffel, *Biochemistry*, 31 (1992) 12289.