BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 498–502 (1997)
ARTICLE NO. RC977318

Detection of Myelin Basic Protein Isoforms by Organic Concentration

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Received July 22, 1997

An effective technique was developed, which allowed rapid isolation of highly pure myelin basic protein (MBP) including its distinct isoforms. The procedure employs homogenization of central nervous system (CNS) tissue in chloroform, which specifically extracts MBP. Subsequently, methanol was used to convert the protein susceptible to quantitative transfer into the acidic aqueous phase. MBP was purified from bovine, chicken, fish, human, guinea-pig, mouse, rabbit, rat, and swine brains. Analysis on SDS-PAGE and immunoblotting using polyclonal MBP-specific serum recognized proteins corresponding to the sizes of previously identified MBP isoforms of 21.5, 18.5, 17.2, and 14.2 kDa and three predicted isoforms of 20.2, 16.0, and 13 kDa. The MBP obtained was readily soluble in water and possessed the capacity to induce experimental autoimmune encephalomyelitis in susceptible mice. The protein was also suitable for use as a substrate for protein kinases.

Key Words: **myelin basic protein; purification; isoforms; organic extraction; EAE.**

Myelin basic protein (MBP) is one of the major components of the CNS myelin. Thus it is one of the candidate autoantigens in autoimmune disorders of the human CNS such as multiple sclerosis (1). MBP plays an important role in forming and maintaining the myelin structure by attracting oligodendrocytic membranes via protein-lipid interactions (1). MBP has a high net positive charge and binds electrostatically to negatively charged lipids such as phosphadityl choline and phosphadityl serine (1,2).

MBP exists as several different isoforms resulting from alternative splicing of the primary mRNA transcript (3-6). For mouse MBP (mMBP), mRNAs for altogether eight isoforms 13.0, 14.2, 16.0, 17.2-1, 17.2-2, 18.5, 20.2, and 21.5 kDa have been identified (3-6), four of which 14.2, 17.2, 18.5, and 21.5 have been detected at the protein level (7,8). For human MBP (hMBP) mRNAs for 21.5, 20.2, 18.5 and 17.2 kDa isoforms have been reported (9,10).

Several methods for the purification of MBP have been established. Usually lipids from brain tissue are first removed by organic extraction with subsequent separation of MBP from tissue remains using aqueous buffers (11-14). Extraction with the help of detergents has also been reported (15). All these methods involve multiple extraction and separation steps which makes them time consuming and expose MBP to proteolytic degradation by neutral and acidic proteases (16,17).

We describe here an effective organic extraction strategy which allows rapid simultaneous isolation of several MBP isoforms in stabile, water-soluble form. The protein product was effective in inducing EAE and could be phosphorylated by kinases. The method allowed also immunological identification of previously predicted MBP isoforms.

MATERIALS AND METHODS

Preparation of MBP-specific immune serum. A New-Zealand White female rabbit was immunized with guinea-pig MBP (gpMBP) purified by the method of Eylar et al. (12). Primary immunization was done by injecting 3 mg of protein in CFA intra muscularly. Two boosters in IFA were given, 3 and 6 weeks after the primary immunization. The serum antibody titer was determined by ELISA and the rabbit was bled 8 weeks after immunization. No signs of

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Abbreviations used: bMBP, bovine MBP; chMBP, chicken MBP; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; ECL, enhanced chemiluminescence; fMBP, fish MBP; gpMBP, guinea-pig MBP; hMBP, human MBP; MBP, myelin basic protein; mMBP, mouse MBP; M, molecular mass; MS, multiple sclerosis; rMBP, ratMBP; rbMBP, rabbit MBP; sMBP, swine MBP.

0006-291X/97 $25.00

paralysis were observed in the rabbit during the immunization. The serum did not crossreact with other cellular proteins.

Extraction of MBP. MBP was purified as follows: Frozen mouse brain tissue (whole brains, 3.08g) was homogenized at RT in 15 ml chloroform (Merck, Germany) with a Sorvall Omni-Mixer 17220 homogenizer using four 30 second bursts at full speed, intervened by 30 second pauses to avoid heating. The organic phase was separated from the tissue debris and aqueous phase by centrifugation at RT with 4500 × g, 5 min, using a Sorvall SA-600 rotor. Chloroform was collected and the aqueous phase discarded, followed by re-extraction of the tissue debris with 7.5 ml chloroform. The organic phase was separated as above. The pooled chloroform fraction (18.2 ml) was briefly washed with 4.5 ml water by vortexing and centrifugation as above, whereafter to chloroform, 9 ml methanol was added and the mixture was vortexed for 30 seconds. To this, 4.5 ml water containing 0.12 ml 1M HCl was added, and the mixture was vortexed keeping the pH at 2. At stabile pH the acidic aqueous phase was separated from the chloroform phase by centrifugation as above. Total 9 ml of acidic aqueous phase was recovered, concentrated to 3 ml by Büchi Rotavapor vacuum concentrator, gel-filtrated using a Pharmacia PD-10 column and lyophilized. The total yield of MBP was 4.3 mg according to Bradford analysis (18). Desalted material ready for lyophilization could be obtained within two hours.

SDS-PAGE and immunoblotting. Protein concentration was measured by the Bradford method (18) (Bio-Rad Laboratories, U.S.A.). MBP samples were mixed with reducing Laemmli sample buffer and heated for 5 minutes at 95°C prior to gel loading. Samples were run on 13,5% minigels (19). Proteins were visualised by Coomassie-staining (Coomassie brilliant blue G250, Merck, Germany).

For immunodetection the proteins were transferred from SDS-PAGE gels onto nitrocellulose (Sleicher & Schuell, Germany) with a Hoefer Semi-Phor TE70 semi-dry electrotransfer apparatus according to the manufacturer's instructions (42 mA, 30 min). Filters were blocked with PBS containing 0,4% Tween-20 (PBST) and 10% dry milk overnight at +4°C on an orbital shaker. The filters were incubated for one hour at RT on the orbital shaker in the presence of MBP-specific rabbit serum diluted 1:6000 with PBST containing 3% dry milk, washed three times with PBST and incubated for one hour at RT together with a polyclonal peroxidase-conjugated goat-anti-rabbit antibody (Chappel, Cooper Biomedical Inc., U.S.A.). Immunoblots were developed using the enhanced chemiluminescence kit from Amersham according to the manufacturer's instructions exposing on a Dupont Cronex 4 X-ray film.

Matrix-assisted laser desorption mass spectrometry of MBP. The molecular masses of the present isoforms were determined by a matrix-assisted laser desorption mass spectrometer (Lasermat, Finnigan-MAT Ltd., U.S.A.). Hen egg lysozyme (Merck, F.R.G.) was used as an external standard. Samples (0.5 μl) containing 100 - 1000 picomoles of protein were mixed with an equal volume of the matrix substance sinapinic acid (Aldrich, Germany) and the mixtures were dried onto the sample carriers for analysis. The molecular weights were determined as average from 10 sequential measurements.

RESULTS AND DISCUSSION

The novel extraction method described in this communication for purification of MBP from the CNS tissue of different animal species is extremely quick and easy. No complicated laboratory equipment is required and the method can easily be scaled to samples of greatly varying size. All protein visible in Coomassie staining reacted with MBP-specific antiserum (Figs. 1,2). The total MBP yields for mammalian CNS tissue varied from 1.4 mg/g (frozen brain) to 5.5 mg/g (frozen spinal cord). Generally, fresh tissue yielded higher amounts of MBP than frozen tissue. The yield of fish MBP (fMBP) from frozen brain of burbot (*Lota lota*) was significantly lower, 0.45 mg/g tissue. The obtained protein appeared as white powder and was readily soluble in water or aqueous buffers and did not contain co-purified protease activity as no degradation could be demonstrated upon prolonged incubation at +37°C in PBS (data not shown). The intactivity of the mMBP and human MBP (hMBP) was ascertained by laser-assisted mass spectrometry. The major form of mMBP resulted in an M value of 14.226 kDa, which is close to M = 14.210 kDa deduced from the mRNA. Two additional signals with M = 12.246 kDa and M = 18.587 kDa were obtained the higher corresponding to the deduced 18.5 kDa isoform (6) and the lower perhaps to a low molecular weight polypeptide seen in the MBP preparates slightly below the 14 kDa band (Fig. 1, lanes 4 and 5). The major peak of hMBP exhibited M = 18.626 kDa, which is close to M = 18.487 kDa deduced from the mRNA (9,10).

The efficancy of the method is demonstrated in Fig. 1, where also MBP isoforms previously predicted by mRNA analysis could be demonstrated in the purified samples. Pure MBP seemed to form multimeric complexes and distinct isoforms could be seen in dublicates, triplicates and quadruplicates forming similar patterns as monomeric forms (Fig. 1). Monomeric MBP showed typical anomalic retained migration on the gel (20), but the multimers seemed to run corresponding to their deduced molecular weights (Figs. 1 and 2). This may be due to different conformation and/or reduced positive net charge as the multimers are probably held together by negatively charged lipid moieties (1,2). Several reports describe detection of large molecular weight MBP polypeptides (21,22), but as on the other hand, MBP possesses a strong intrinsic property to form aggregates (Figures 1 and 2), the interpretation of these results is difficult.

MBP obtained with the organic extraction from brain tissue of bovine (bMBP), human (hMBP), swine, rabbit (rbMBP), chicken (chMBP), guinea-pig, rat (rMBP), mouse and burbot (*Lota lota*) (fMBP) were run on SDS-PAGE and developed by Coomassie-staining or immunodetection. In the immunoblot a band corresponding to the 21.5 kDa isoform was visible in each species except fish (Fig. 2), detected in the human and chicken samples only after prolonged ECL exposure. This suggests that the 21.5 kDa isoform detected in myelinating and remyelinating human CNS (10,23) is present also in mature human CNS. Detection of 21.5 kDa isoform in chicken brain is in contrast to previous analysis by Kerlero de Rosbo et al. (24).

Immunoreactivity at gel position corresponding to a 20.2 kDa MBP polypepdite was visible in bMBP, sMBP, rbMBP, gpMBP, rMBP (Fig. 2C, lanes 1,2,4,5,7 and 8, respectively) and mMBP (Fig. 1 lane 5), but not in chMBP or fMBP (Fig. 2C, lanes 6 and 10, respectively).

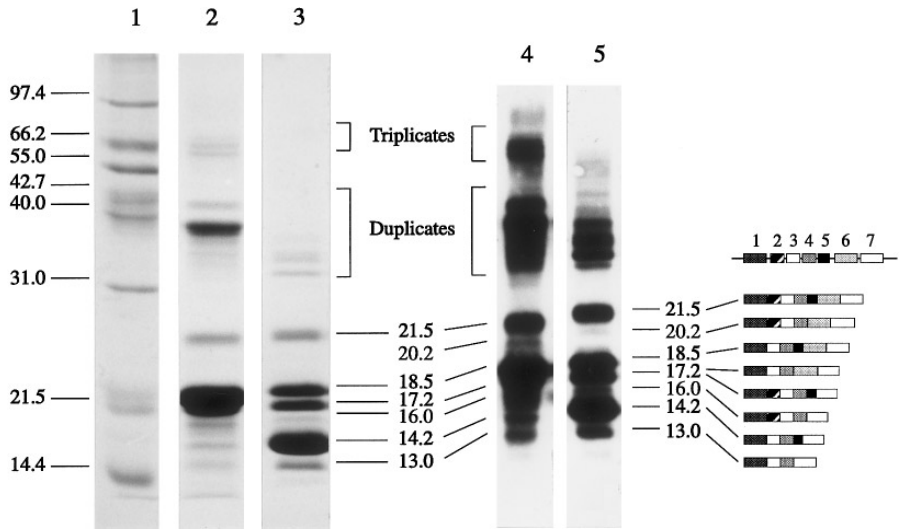

FIG. 1. Aggregates in mouse and swine MBP samples. SDS-PAGE (13.5%) and immunoblot mMBP and sMBP demonstrating aggregate formation. Lane 1, Promega mid-range molecular weight marker. The molecular weights of MBP isoforms are indicated (middle). Lanes 1 - 3, Coomassie staining, lanes 4 and 5, immunodetection using polyclonal rabbit serum specific for MBP. Lanes 2 and 4, sMBP 10μg; lanes 3 and 5, mMBP 11μg. The isoform pattern appears on the gel as duplicate and triplicate in both samples. For the swine sample, also a quadruplicate is visible (lane 4). This was also detected for the mouse sample after prolonged exposure (not shown). The monomeric MBP polypeptides migrate slower on SDS-PAGE gels than should be expected on the basis of their deduced molecular weights (20, see text). The MBP multimers are strongly immunoreactive as they offer vicinal epitopes to the divalent antibody. The exonic elements in mRNAs corresponding to the distinct isoforms are drawn according to Nakajima et al. (6) and Mathisen et al. (25).

In the human sample, the putative 20.2 kDa form was very close to the 18.5 kDa band (Fig. 2 C, lane 3). A corresponding mRNA in human CNS has been previously identified (9). The results may indicate that the 20.2 kDa isoform has a specific function only in the CNS of mammals.

The 18.5 , 17.2, and 16 kDa MBP isoforms were present in all species except fish (Fig. 2). In hMBP and rbMBP samples, an approximately 15 kDa protein was seen in Coomassie-staining and reacted with the antiserum (Fig. 2 A and B, lanes 3 and 5). However, no such polypeptide has been predicted previously, thus is not known at present whether this is a true isoform or derives by postranslational modifications from other forms.

The 14.2 kDa band representing the major isoform of mMBP and rMBP was present in all species, and in fish it appeared as two closely migrating proteins being the only immunoreactive form (Fig. 2, lane 10). The presence of a mRNA encoding an approximately 13 kDa isoform has recently been reported in mouse CNS tissue (25). Indeed, a protein of corresponding size was recognized by the MBP-specific antiserum both in mMBP and rMBP and also in other mammalian species after prolonged exposure (Figs. 2B and C). In the fMBP, a 13 kDa protein, clearly visible in coomassie staining, was not recognized by the gpMBP-specific antiserum (Fig. 2A, lane 10). The presence of a 16, 14.2 and 13.0 kDa MBP (Fig. 2, lane 3) in human brain has not been predicted previously. Thus these proteins represent novel isoforms in addidion to the known 21.5, 18.5 and 17.2 kDa proteins (9,10). In addition, a 10 kDa polypeptide became visible in the immunoblot after extended exposure in bMBP, hMBP, mMBP and rbMBP (Fig. 2C). However, presence of corresponding mRNA has not been reported.

The size of the immunoreactive polypeptides corresponded well to the predicted molecular weight values based on mRNA analysis (6,25). We have proved that MBP polypeptides obtained with partial thrombin digestion (26) are immunoreactive (not shown), but no as small peptides were obtained by the extraction. In addition, the smallest polypeptides in the purified preparates seemed to form multimers similar to the larger isoforms (Fig. 1) suggesting a natural structure.

The purified MBP was readily soluble in water making it suitable for various enzymatic analysies, e. g. protein kinase assays (27,28), and immunological purposes. Purified samples of mMBP and sMBP were shown to function as a substrate for MAP kinase activity induced in rat cerebellar granule cells *in vitro* by brain-derived neurotropic factor when analysed by the method of Courtney et al. (28). Phosphorylation could be demonstrated for the major isoforms (14.2, 17.2, 18.5 and 21.5 kDa for mMBP, 18.5 and 21.5 kDa for sMBP) and their apparent dimers (data not shown). Mouse MBP purified by this method was shown to induce severe EAE in 4/4 susceptible SJL OlaHsd mice (not shown). The protein is especially suited for T cell stimulation experiments in autoimmunity studies as all

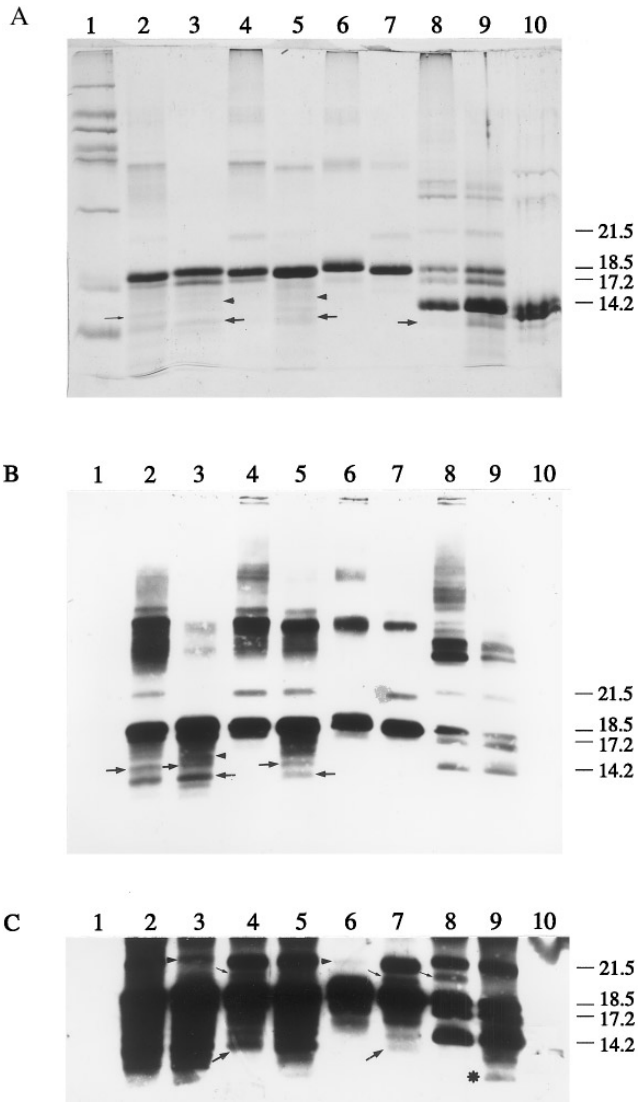

FIG. 2. MBP isoforms from various animal species. Coomassie-stained 13.5 % SDS-PAGE (A) and immunoblots employing exposure times of 10 sec (B) or 5 min (C) on ECL reaction. Lane 1, Promega mid-range molecular weight marker; lane 2, bMBP (4.8μg); lane 3, hMBP (4.3μg); lane 4, sMBP (5.2μg); lane 5, rbMBP (4,9μg); lane 6, chMBP (4.9μg), lane 7, gpMBP (4.8μg); lane 8, rMBP (5.9μg); lane 9, mMBP (5.4μg), lane 10, fMBP (*Lota lota*, 4.8μg). For immunodetection (B and C), half of the protein amounts used in A were run. Note that the relative mobility of MBPs is reduced compared to standard proteins of similar size (20). A) The presence of the 13.0 kDa isoform is indicated with short arrow (lanes 3, 5, 8). Arrowheads point a potential 15.0 kDa form in human and rabbit samples (lanes 3, 5). The appearance of a band corresponding to 14.2 kDa isoform is indicated for bovine sample by thin arrow (lane 2). B) To the left pointing arrows indicate the position of a potential 13.0 kDa form. To the right pointing arrows indicate 14.2 kDa isoform in the bovine, human and rabbit samples. The immunoreactivity of the putative 15.0 kDa isoform in hMBP is demonstrated by arrowhead. C) The presence of 21.5 kDa isoform in the human and chicken samples is demonstrated by arrowhead. Appearance of 13.0 kDa isoform is shown for swine and guinea-pig samples by short arrows. Thin arrows point to 20.2 kDa isoform where clearly visible. Appearance of immunoreactivity at approximately 10 kDa region is marked with asterisk for the mouse sample (lane 9). The immunodetection of fMBP was weak and mainly fMBP aggregates became visible.

known MBP isoforms seem to coextract and minor isofroms are thought to contain important pathogenic epitopes (29).

The central phenomenon behind the efficiency of this method is the almost complete removal of MBP from the CNS tissue into chloroform. The subsequent phase transfer from chloroform into acidic water was strictly dependent on methanol treatment. We assume that methanol removed MBP-associated lipids thus uncovering crucial protein domains to allow water-solubilizing conformational alterations by the acidic milieau as MBP could not be transferred to neutral water from chloroform/methanol. Once solublized in water, the protein could not be transferred back to chloroform/methanol. Interestingly, during the phase transfer, as long as the pH of the aqueous phase was increasing, more MBP continued to be transferred by adding dilute HCl. We observed that HCl could not be replaced by e. g. NaCl indicating the importance of the proton. The remaining debris could be used as starting material for isolation of proteolipoprotein by the method of Skalidis et al. (30).

ACKNOWLEDGMENTS

The authors thank Harry Kujari for helpful discussions and Petri Kulmala for providing rat brains. We also thank Tiina Matinlinna for excellent technical assistance. This work was supported by SITRA, the Sigrid Juselius Foundation, the Turku University Foundation, the Åbo Akademi signal transduction program, and the North-Karelian Fund of Finnish Cultural Foundation.

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