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Isolation, Purification and Verification of Peripheral Nerve Myelin Derived from Bovine Cauda Equina

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Abstract

Background: Animal models of peripheral neuroinflammation are essential to understanding the pathogenesis of, and deducing new treatments for demyelinating polyneuropathies. This paper updates and describes adapted methods for the isolation, purification and verification of bovine peripheral nerve myelin used to reliably induce peripheral neuritis in susceptible mice.

Methods: Myelin was isolated via discontinuous sucrose and continuous cesium chloride density gradient ultracentrifugation from frozen adult bovine cauda equina. Negatively stained electron microscopy was employed to confirm myelin structure. Phase contrast and routine light microscopy, gravimetric analyses, gel electrophoresis and lens culinaris fluorescent western blotting, analytical colorimetric assays and indirect immunohistochemistry were performed to analyze whole myelin or its protein and lipid subcomponents. Myelin isolates were used to induce experimental autoimmune neuritis in 8-12 Weeks old female Swedish Jim Lambert/ Jackson mice.

Results: Negatively-stained electron microscopy demonstrated the two-dimensional repetitive lamella structure of myelin and a lack of organelle contamination. Lipids accounted for ~72% of the dry mass of bovine peripheral nerve myelin, while ~25% was protein/ proteolipid and <4% insoluble residue based on gravimetric analyses. Glycoproteins accounted for the majority of isolated myelin proteins, with myelin protein zero (~28 KDa) being the most prevalent protein. Phospholipids accounted for ~60% of the total dry weight of bovine peripheral nerve myelin. Surface expression of galactocerebroside and sulfatide, two major lipid subcomponents present in myelin, was detected by indirect fluorescent immunohistochemistry. Bovine peripheral nerve myelin reliably induced a severe macrophage-predominant demyelinating polyneuropathy with associated axonal loss in female mice, consistent with experimental autoimmune neuritis.

Conclusions: These updated and adapted methods using readily available resources can be applied to rapidly isolate, purify and verify peripheral nerve myelin from animal sources, and determine its protein and lipid composition prior to use in peripheral neuritis animal models.

Keywords: Colorimetric assays; Experimental autoimmune neuritis; Fluorescent western blot; Histology; Peripheral nerve myelin, Protocols

Abbreviations: BPNM: Bovine Peripheral Nerve Myelin; BSA: Bovine Serum Albumin; CNS: Central Nervous System; EAN: Experimental Autoimmune Neuritis; GBS: Guillain-Barré Syndrome; LFA-FITC: Fluoresceinated Lens Culinaris Agglutinin; NGS: Normal Goat Serum; MBP: Myelin Basic Protein; P0: Myelin Protein zero; PBS: Phosphate Buffered Saline; PFA: Paraformaldehyde; PMP-22: Peripheral Myelin Protein-22; PNS: Peripheral Nervous System; RT: Room Temperature; SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis; SJL/J: Swedish Jim Lambert/ Jackson; TBS: Tris-Buffered Saline; TEM: Transmission Electron Microscopy

Introduction

Myelin, the complex biological membrane that facilitates saltatory transmission of electrical impulses along axons, is derived from the differentiation of Schwann cell plasma membranes in the peripheral nervous system (PNS) and from oligodendrocytes in the central nervous system (CNS) [1]. Although similarities exist between PNS and CNS myelin, there are unique differences in molecular composition. Extensive information is known on myelin composition, synthesis and maintenance in mammalian and non-mammalian species [1]. The importance of myelin to PNS function is evidenced by the devastating effects of demyelination in acquired autoimmune neuropathies such as Guillain-Barré syndrome (GBS) and Chronic Inflammatory Demyelinating Polyradiculoneuropathy and toxic neuropathies, such as due to perhexiline [2].

Studies using native myelin remain important despite advances in peptide biosynthesis. This is the case for a severe murine experimental autoimmune neuritis (EAN) model of GBS [3-6]. Mice immunized with purified myelin proteins or peptide fractions are often resistant or develop very mild forms of the disease, making these models inadequate to study PNS inflammation and demyelination seen in GBS [7]. Similarities in neural-immune responses between mice and humans make it essential to work with reliable mouse PNS inflammation models to understand the pathogenesis of demyelinating polyneuropathies and find novel molecular therapeutic targets. Detailed protocols using modern equipment and consumables to isolate mammalian PNS myelin are difficult to find in the medical literature. We adapted

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or modified traditional protocols to isolate, purify and rapidly verify bovine PNS myelin and its protein and lipid components using readily available resources available to most general scientific laboratories. Myelin isolates were used to reliably induce EAN in susceptible female mice.

Methods

Isolation and purification of bovine peripheral nerve myelin from cauda equina

Bovine peripheral nerve myelin (BPNM) was isolated from frozen intrathecal cauda equina of United States Department of Agriculture-certified adult cows <30 months of age (Innovative Research, Novi, Michigan, U.S.A.), with modifications [8,9]. The thawed cauda equina was carefully dissected and ground to a fine powder in liquid nitrogen and weighed. A 5% weight/volume solution in 0.29 M sucrose (>99.9% pure; EMD Chemicals, Gibbstown, New Jersey, U.S.A.) in distilled water was homogenized three times at 25,000 revolutions per minute for at least 30 seconds on ice using a Polytron PT 1200E hand-held homogenizer (Brinkmann-Kinematica, Lucern, Switzerland). The homogenate was filtered through a 50 μm Nitex® nylon lab pak mesh (Sefar Filtration Inc., Depew, New York, U.S.A.) and kept overnight at 4°C.

A discontinuous sucrose gradient was generated by layering the filtered homogenate over a 0.85 M sucrose solution in distilled water into an Optiseal™ polyallomer ultracentrifuge tube (Beckman Coulter, Palo Alto, California, U.S.A.) in a 3:4 ratio. The gradient was centrifuged at 82,500 g for 45 minutes at 4°C in an Optima LE-80K Ultracentrifuge with Type 70 Ti fixed angle rotor (Beckman Coulter). The crude myelin was carefully collected from the sucrose interface using Luer Lok™ tip syringes attached to 15G BD Yale™ reusable hypodermic needles (BD Labware, Franklin Lakes, New Jersey, U.S.A.), and subjected to osmotic shock four times by resuspending 1:3 in distilled water, homogenizing and centrifuging at 82,500 g for 15 minutes at 4°C as described above. The supernatant (containing cellular fragments and debris) was completely removed and the myelin pellet completely aspirated. The partly purified myelin extracts were pooled and stored at 4°C overnight.

Further purification was performed using a continuous cesium chloride (99.999% pure; MP Biomedicals, Solon, Ohio, U.S.A.) gradient, generating a 0.3 M cesium chloride-myelin suspension in distilled water. A continuous 0.3-1.3 M cesium chloride gradient was generated (equal volume of 1.3 M cesium chloride in distilled water added to the bottom of a 0.3 M solution in Optiseal™ ultracentrifuge tubes, carefully titled to 45°, rotated 360° across their vertical axes then brought upright within 60 seconds). The 0.3 M cesium chloride-myelin suspension was layered over the 0.3-1.3 M continuous cesium chloride gradient (in a 1:5 ratio) and centrifuged at 82,500g for 30 minutes at 4°C. Purified myelin was collected and subjected to osmotic shock as described above. Purified myelin was lyophilized using a RC 10.22.TT centrifugal evaporator with an RCT 60 refrigerated cold trap (Jouan Inc., Winchester, Virginia, U.S.A.), weighed and stored at -20°C under anhydrous conditions using size 4 mesh indicating Drierite (W.A. Hammond Drierite Company, Xena, Ohio, U.S.A.).

Transmission electron microscopy (TEM)

Negatively-stained myelin images were acquired as previously described, with modifications [10]. Briefly, a 5 μL droplet of BPNM (100-200 μg/mL in deionized distilled water) was applied to a Formvar-coated, carbon stabilized 200 mesh copper grid for one minute. The droplet was then wicked away with Whatman® filter paper (No.1; Maidstone, England) and replaced immediately with a droplet of 1% aqueous potassium phosphotungstate for one minute, after which Whatman filter paper was again used to wick the solution away. The grid was allowed to air dry for a few minutes before viewing on the electron microscope. Photomicrographs were recorded on a JEOL 200CX electron microscope (Tokyo, Japan) operating at 80 KeV. All photomicrographs were digitized by scanning the negatives into an image editing system (Photoshop; Adobe Systems Inc., San Jose, CA, U.S.A.) at high resolution (16 bit grayscale, 600 line/inch; ScanMaker 8700 [Microtek USA, Carson, California, U.S.A.]). A carbon diffraction line grating replica with a known line density (1250 lines/mm) was similarly imaged, photographed, digitized and used as an internal reference scale when calculating scale bars.

Light and phase contrast microscopy

Light microscopy of BPNM smears was performed by adapting a protocol for visualizing intact myelin sheaths in peripheral nerves [11]. 40-50 μL of a 20 mg/mL suspension of lyophilized BPNM in deionized distilled water was layered evenly unto poly-l-lysine coated glass slides (VWR Scientific) and incubated for 10 minutes at 37°C to bind myelin. Smears were fixed with 100 μL of 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 at RT for 30 minutes in a fume hood. BPNM smears were post-fixed with 1% osmium tetroxide in 0.1 M phosphate buffer and stained with 0.1% toluidine blue in distilled water, washed, mounted, sealed with quick drying clear nail polish and viewed immediately using a Zeiss Axioskop Epifluorescent Microscope (San Diego, California, U.S.A.). Digital images were generated using a Zeiss Axiocam MRc 5 digital camera and processed with the Adobe Photoshop CS2 software program (Adobe Systems, San Jose, California, U.S.A.). For BPNM phase contrast microscopy, smears were prepared as described above and fixed with 100 μL of 4% paraformaldehyde (PFA) in 1X PBS for 5-10 minutes at 37°C. After washing three times with 100 μL of fresh 1X PBS for 5 minutes each, glass coverslips were mounted on the slide with a small drop of Vectashield® (Vector Laboratories, Burlingame, California, U.S.A.), sealed, viewed and imaged as described above.

Gravimetric analysis

BPNM fractionation into lipid and protein components for gravimetric analysis was performed by adapting previously published protocols [12,13]. 5-10 mg of lyophilized BPNM was weighed with a Sartorius CPA124S analytical balance (Goettingen, Germany) and mixed thoroughly with 4 volumes of distilled water. 20 volumes of 2:1 volume/volume chloroform ($CHCl_3$): methanol (CH_3OH) were added, mixed thoroughly at RT and filtered using pre-weighed Whatman® ashless analytical filter paper (No. 42; Maidstone, England). The insoluble residue was filtered three times with 20 volumes of 2:1 $CHCl_3$:CH_3OH and pooled for subsequent use. The residue was separately washed with the same volume of water, 100% ethanol and 100% ether on filter paper that was carefully dried in nitrogen gas (Alphagaz 1 N2 99.999% pure, Air Liquide, Houston, TX, U.S.A.) and weighed using an analytical balance to determine the amount of insoluble residue.

To separate lipids and proteolipid-protein fractions, the volume of the pooled filtrate in a single borosilicate glass tube was measured. A 0.2 volume of 0.05 M potassium chloride in deionized distilled water was added and mixed thoroughly to generate a single phase, and centrifuged with a Beckman Coulter Allegra X-12R Centrifuge (Fullerton, California, U.S.A.) in a swinging bucket rotor at 2400 revolutions

per minute for 20 minutes at RT. The lower phase was collected and saved. The tubes were washed twice with small amounts of upper phase solvent ($CHCl_3$:CH_3OH:H_2O in ratio 3:48:47) and centrifuged as above to completely remove the lower phase. The total volume of the lower phase was measured, 100% CH_3OH was added (contains $CHCl_3$: CH_3OH: H_2O in ratio 86:14:1) to restore 2:1 $CHCl_3$:CH_3OH ratio and dried under nitrogen stream at 60°C.

Four volumes of distilled water and 20 volumes of 2:1 $CHCl_3$:CH_3OH were added to the dried substance, resuspended, and centrifuged at 2400 revolutions per minute for 20 minutes at RT as above. The lipid containing supernatant was collected and transferred to a clean, previously weighed glass tube, leaving the precipitate (containing proteolipid-protein and neutral solvent insoluble lipid) behind. The process was repeated at least 6 times until there was no visible precipitate. The precipitate was pooled in a single previously weighed glass tube and resuspended in at least 20 volumes of 2:1 $CHCl_3$:CH_3OH. The suspension was centrifuged as above, and heated at 65°C for 20-30 minutes to extract neutral solvent insoluble lipid from the pellet (maximal constriction of the pellet being indicative of complete extraction). The cooled supernatant was transferred to the lipid containing supernatant, dried completely to constant weight under nitrogen stream, then in vacuo with Drierite and paraffin oil placed in a Nalgene Nunc vacuum dessicator (Thermo Fisher Scientific, Rochester, New York, U.S.A.). Similarly, the precipitate was dried under nitrogen stream and in vacuo under Drierite to constant weight, yielding the amount of the proteolipid-protein fraction.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

BPNM was partially delipidated by treatment with ether-ethanol (3:2, volume/volume), adapting a previously published protocol [8]. Briefly, 5 mg of lyophilized BPNM suspended in the solvent (final concentration of 1 mg/mL) was mixed vigorously. The protein pellet was collected by centrifugation at 2500 g for 3 minutes at RT in a Beckman Coulter Allegra X-12R Centrifuge, repeating 5 times. The final pellet was dried under nitrogen stream at RT, resuspended in deionized distilled water and homogenized using a Branson sonifer 250 sonicator (VWR Scientific). Protein concentration was determined by absorbance at 280 nm with a BMG Labtech FluoStar Optima microplate reader (Durham, North Carolina, U.S.A.), using known concentrations of bovine serum albumin (BSA; Innovative research) to generate standard curves. Protein samples were stored at -80°C prior to electrophoresis.

The Mini-Protean III apparatus (Bio-Rad Laboratories, Richmond, California, U.S.A.) was used for gel electrophoresis, according to the manufacturer's instructions, with 15% polyacrylamide slab and stacking gels. Denatured 50 µg aliquots of BPNM protein, 15µg bovine brain myelin basic protein (MBP; 50% pure, Sigma-Aldrich) and BSA standards (1.0-25.0 µg) were used for electrophoresis. The Precision Plus Kaleidoscope molecular weight marker (Bio-Rad Laboratories) was used for molecular weight determination. BPNM protein subfractions were identified based on expected molecular weight. Bovine brain MBP was used as a control to verify that the isolated myelin was not of central origin. Electrophoresis was run for 50-60 minutes at 175 V.

Following electrophoresis, the gel was carefully washed and stained with 0.1% weight/volume Coomassie brilliant blue G (Sigma-Aldrich) mixed with 25% methanol (volume/ volume) and 5% glacial acetic acid in deionized distilled water for 1 hour at RT in a Sci-ERA

orbital shaker (Bellco Glass Inc., Vineland, New Jersey, U.S.A.). Digital images were obtained using an AlphaImager® HP gel documentation system (Cell Biosciences, Santa Clara, California, U.S.A.) attached to a Sony ICX267AL 1.39 Megapixel CCD camera and processed using the AlphaView and Adobe Photoshop CS2 software programs. BPNM protein subfractions were quantified by spot densitometry, using known quantities of BSA to generate standard curves. The percentage of each subfraction relative to the total amount of myelin protein quantified on the gel was determined.

Lens culinaris agglutinin fluorescent western blot

Glycoprotein detection was performed using fluoresceinated lens culinaris agglutinin (LCA-FITC; Vector Laboratories, Burlingame, California, U.S.A.) following SDS-PAGE, as described [14] with modifications. LCA is a plant lectin that specifically binds to glycoprotein α-mannose residues. Protein subfractions were transferred from the gel to polyvinylidene difluoride membranes using a Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell apparatus, according to the manufacturer's instructions. Membranes were blocked for 30 minutes at RT with 3% BSA in Tris-buffered saline (TBS). 10 µg/mL LCA-FITC in 1% BSA and 0.05% Tween-20 in TBS was applied to the membranes for 1 hour at RT in the dark in an orbital shaker. Following three washes in 0.5% Tween-20 in 1X phosphate buffered saline (PBS), the membrane was excited at 365 nm, visualized and imaged with the AlphaImager® HP system (Cell Biosciences, Santa Clara, California, U.S.A.) using appropriate filters. BSA standards acted as negative glycoprotein controls.

Indirect fluorescent immunohistochemistry

Monogalactosylsphingolipids are a major component of myelin, a feature that differentiates this from other biological membranes [1]. BPNM was layered on poly-l-lysine coated glass slides, fixed with 4% PFA, washed three times with 100 µL of fresh 1X PBS for 5 minutes per wash, and blocked with 100 µL of 1% normal goat serum (NGS) in 1X PBS for 60 minutes at RT in a humid hybridization bath chamber (VWR Scientific). Without washing, slides were incubated with 100 µL of freshly prepared 5 µg/mL mouse anti-cow galactocerebroside IgG_3 antibody (Millipore Corporation, Billerica, Massachusetts, U.S.A.) diluted in 1% NGS in 1X PBS for 60 minutes at RT. Following three washes, slides were incubated with 100 µL of freshly prepared 1:100 dilution of goat anti-mouse IgG F(ab')$_2$-FITC (SouthernBiotech, Birmingham, Alabama, U.S.A.) for 60 minutes at RT in a humid chamber in the dark. After washing with 1X PBS as above, coverslips were mounted in Vectashield®, sealed, visualized and imaged digitally as previously described. Sulfatide O4 detection was performed as described above, using 5% fetal calf serum for blocking, 20 µg/mL mouse anti-cow O4 IgM antibody (Millipore Corporation) diluted in 1X PBS as primary antibody and 1:250 dilution of goat anti-mouse IgM-FITC (SouthernBiotech) as secondary antibody. Brief 30-60 second washes with 100 µL of fresh 1X PBS with gentle horizontal rotation were performed before and after secondary antibody incubation.

Lipid analysis

Lipids were separated from 10 mg of lyophilized BPNM modifying an established protocol [12], as described in the gravimetric analysis section. BPNM lipid extracts were evaporated to dryness under nitrogen stream at RT, and redissolved in 3-4 mL 2:1 $CHCl_3$: CH_3OH for analysis. Detection and quantification of the major myelin lipid subclasses was performed using colorimetric assays adapted from

previously described protocols [15]. All the reagents used were purchased from Sigma-Aldrich, St. Louis, Missouri, U.S.A. unless otherwise stated.

For phospholipid analysis, all glassware was washed with 98% concentrated sulfuric acid, rinsed in distilled water followed by methanol and chloroform and used immediately. A 1 mL aliquot of BPNM lipid was placed in a 50 mL glass beaker and dried completely under a nitrogen stream at 35°C on a VWR hotplate/stirrer in a fume hood. 2 mL of 70% perchloric acid (Alfar Aesar, Ward Hill, Massachusetts, U.S.A.) and soda lime glass beads (VWR Scientific) were added, and heated to an internal temperature of ~110°C to release inorganic phosphorus for at least 30 minutes (until digest was clear and colorless). The digest was diluted with distilled water (total volume 12.5 mL). 2.0 mL of freshly prepared and filtered 1% weight/volume 2, 4-diaminophenol dihydrochloride with 20% weight/volume sodium metabisulfite in distilled water was added and mixed, followed by 1.0 mL 8.4% weight/volume ammonium molybdate solution in distilled water. After 20 minutes, the solution was diluted to a final volume of 25 mL. 2 mL aliquots were placed in 24-well tissue culture treated polystyrene microplates (BD Labware, Franklin Lakes, New Jersey, U.S.A.). Absorbance was measured at 620 nm in quadruplicate using a BMG Labtech FluoStar Optima microplate reader relative to reagent blank wells. A standard curve using 0-100 µg phosphorus/ mL was

generated with KH_2PO_4, and read simultaneously with BPNM lipid phosphorus. The amount of phospholipid in the initial aliquot was calculated based on a molecular weight of 775 [13].

For galactolipid analysis, a dried 1 mL aliquot of BPNM lipid was resuspended in 2.0 mL of distilled water in a 50 mL glass beaker. 1.0 mL of 5% phenol solution in distilled water was added and mixed thoroughly, followed by 5.0 mL of concentrated sulfuric acid. The mixture was placed in a water bath at 80°C for 10 minutes. After cooling for 20 minutes at RT, 2 mL aliquots were placed in 24-well microplates and absorbance measured against reagent blanks at 490 nm in triplicate. A standard curve of 0-80 µg galactose/ mL was generated and read simultaneously with BPNM lipid. A molecular weight of 846 was used to determine the amount of galactolipid in the initial aliquot [13].

For total cholesterol analysis, a dried 1 mL aliquot of BPNM lipid was redissolved in 6.0 mL of glacial acetic acid in a 50 mL glass beaker. A stock solution of 2.5% weight/volume $FeCl_3.6H_2O$ in 85% H_3PO_4 was diluted in a 2:23 ratio with concentrated sulfuric acid, and 4 mL of this solution added to the glass beaker and mixed thoroughly. After cooling for 10 minutes at RT, 2 mL aliquots were placed in 24-well microplates and absorbance measured at 544 nm against reagent blanks in quadruplicate. A standard curve of 0-0.5 mg/mL cholesterol (>99% pure, Sigma-Aldrich) was generated and read at the
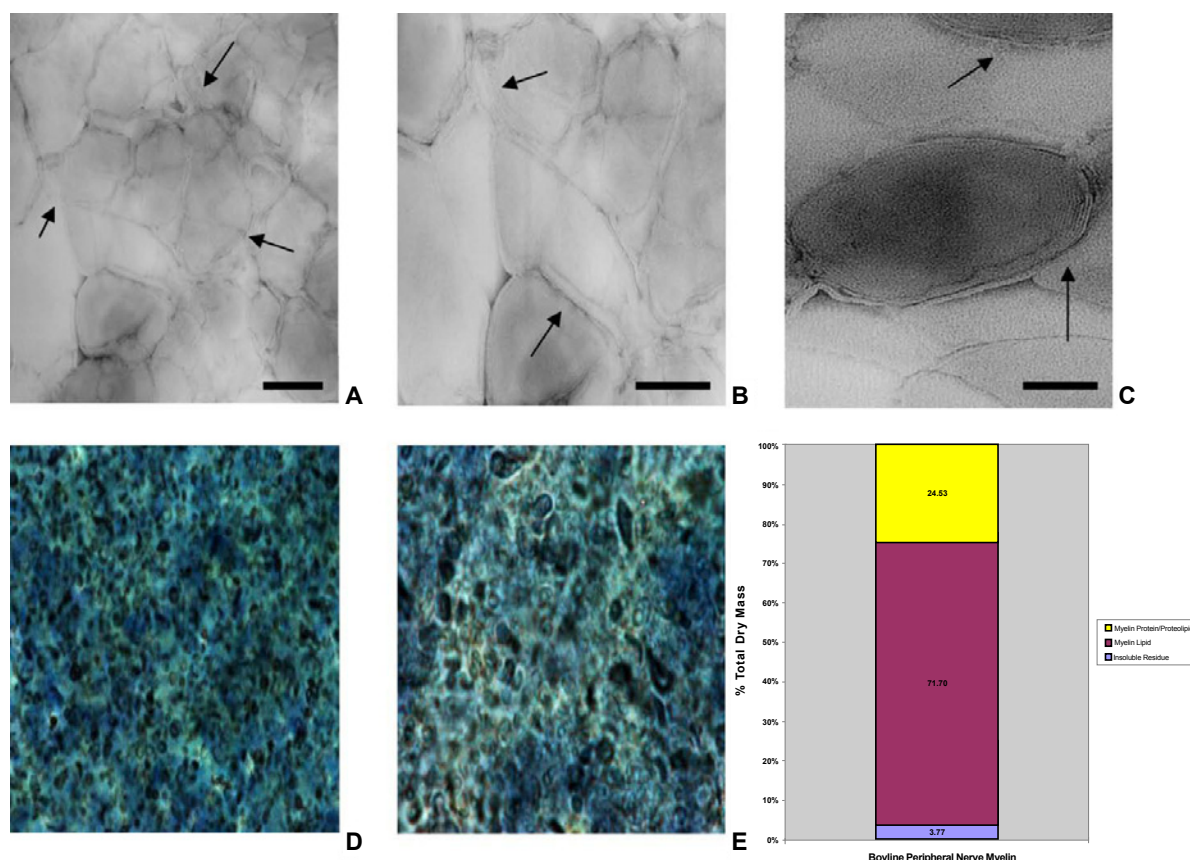
Figure 1: BPNM morphology and gravimetric analysis. Potassium phosphotungstate negatively-stained transmission electron microscopy, light microscopy of glutaraldehyde-fixed, osmium tetroxide post-fixed, 0.1% toluidine blue stained smears and gravimetric analysis were performed on purified lyophilized BPNM. Electron micrographs (A-C) demonstrate the repetitive nature of myelin lamellae (black arrows) and the absence of subcellular organelles. Scale bars represent 250 nm (A and B) and 100 nm (C). D and E show the brownish-black staining of myelin on light microscopy (original magnification 100X for D and 400X for E). Based on gravimetric analysis, BPNM was predominantly lipid, with approximately three times the amount of protein/proteolipid, as expected of peripheral nerve myelin (F). Numbers depict the relative percentages of each fraction to the total dry mass. Insoluble residue consists of predominantly large protein aggregates.
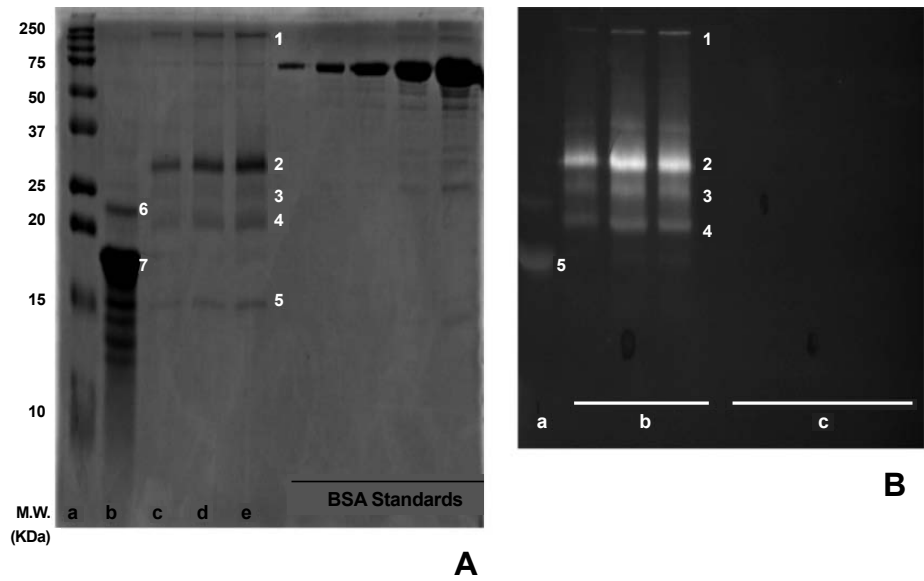
Figure 2: SDS-PAGE and fluorescent western blot of BPNM. Figure A demonstrates an electrophoretic pattern consistent with PNS myelin. Lane a consists of the molecular weight (M.W.) markers (in KDa) and lane b consists of 50% pure bovine CNS myelin basic protein (MBP). Lanes c-e consist of purified BPNM from 2 separate isolates (c and d/e). Based on M.W., 1 most likely represents periaxin, 2 represents P0, 3 represents PMP-22, 4 represents post-translationally modified MBP, while 5 represents P2. 6 represents proteolipid protein (not present in PNS myelin), while 7 represents the 17-18.5 kDa fractions of MBP. Figure B confirmed that the majority of the electrophoresed proteins were glycoproteins as expected of PNS myelin. Lane a consists of 50% pure bovine CNS MBP, lanes b consist of BPNM while lanes c consist of the BSA standards. The specificity of lens culinaris-FITC is demonstrated by the lack of staining of the BSA standards. P2, the fatty acid-binding basic protein is also not detected. 1 most likely represents periaxin, 2 represents P0, 3 represents PMP-22, 4 represents post-translationally modified MBP, while 5 represents partially glycosylated CNS MBP.

same time as BPNM lipid. The total amount of myelin lipid in each extract was calculated by adding the mean amounts of phospholipid, galactolipid and cholesterol, and multiplying by the total volume of 2:1 $CHCl_3:CH_3OH$ solvent used after initial lipid separation. The percentage of each lipid subfraction relative to total lipid dry weight was calculated for each extract.

Bovine peripheral nerve myelin-induced EAN

EAN was induced in 8-12 week old female Swedish Jim Lambert/Jackson (SJL/J) mice by subcutaneous injection of 2.5 mg BPNM in 1X PBS emulsified in a 1:1 ratio with Complete Freund adjuvant (Difco, Detroit, Michigan, U.S.A.), as previously published [3-6]. At expected maximal severity (day 30 post-induction), the sciatic nerves were harvested bilaterally and fixed in 3% phosphate buffered glutaraldehyde for plastic embedding and semi-thin sections, or placed in Tissue-Tek® optimum cutting temperature compound (Sakura Finetek, Torrance, California, U.S.A.) and immediately stored at -80°C for cryostat sectioning and indirect immunohistochemistry (for Schwann cells/myelin, axons, macrophages, T-lymphocytes and B-lymphocytes) as previously described [3-6].

Results

Structural characteristics of isolated BPNM

Cauda equina derived BPNM was verified by negatively stained TEM, a method designed to detect subcellular fractions of animal tissues [10]. The characteristic repetitive lamella pattern of myelin was seen. The lack of debris or cellular organelles was observed at several magnifications (Figures 1A-1C). Furthermore, light microscopy of glutaraldehyde-fixed, osmium tetroxide post-fixed BPNM smears counter-stained with 0.1% toluidine blue showed aggregates of myelin (Figures 1D and 1E). Gravimetric analyses demonstrated that lipids

accounted for ~72% of the dry mass of BPNM, while ~25% was protein/proteolipid and <4% insoluble residue (Figure 1F). These values are consistent with the known composition of myelin [1,13], verifying the isolation and purification process.

Protein composition of purified BPNM

SDS-PAGE demonstrated that BPNM was made up of 5 major protein fractions with molecular weights ~15 KDa, ~19 KDa, ~23 KDa, ~28 KDa and ~170 KDa. These are consistent with myelin proteins P2, MBP, peripheral myelin protein-22 (PMP-22), myelin protein zero (P0) and periaxin [1] as shown on Figure 2A. It is also possible that the 19 and 23 KDa bands represent or contain in part, *in situ* proteolytic fragments of P0 [16-18]. Fluorescent western blotting using LCA-FITC confirmed that BPNM consisted of predominantly glycoproteins [1], with partial staining of the MBP band indicating non-specific binding or contamination with a P0 proteolytic fragment. P2, a fatty acid binding-basic protein, was not detected, neither were the BSA standards, indicative of selective glycoprotein detection by LCA-FITC (Figure 2B). Based on quantitative band spot densitometry,

Protein	Sample A	Sample B1	Sample B2	Mean
P2	13.08	12.50	14.10	13.22
Myelin Basic Protein	18.08	25.50	26.28	23.29
Peripheral Myelin Protein-22	0.77	1.00	1.07	0.95
P0	64.62	57.50	54.70	58.94
Periaxin	3.46	3.50	3.85	3.60

Values represent percentages of proteins relative to total BPNM protein quantified on gels by spot densitometry (to 2 decimal points).

Table 1: Relative quantification of BPNM protein components based on SDS-PAGE.
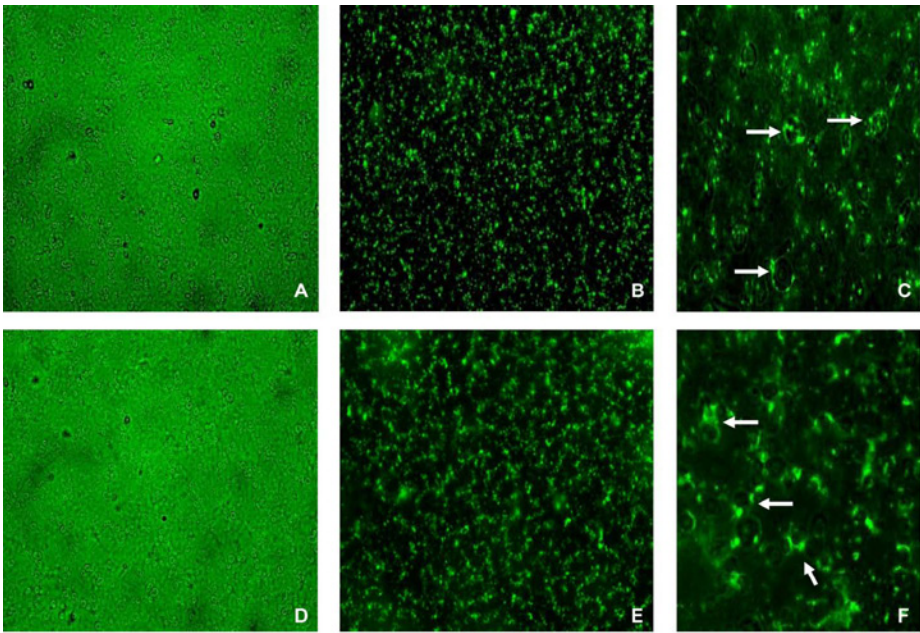
Figure 3: Phase contrast microscopy and indirect fluorescent immuno-histochemistry of BPNM. A and D are phase contrast micrographs of paraformaldehyde-fixed BPNM smears, demonstrating aggregates of purified myelin, while B and E demonstrate galactocerebroside and sulfatide immunoreactivity of these smears respectively (original magnification 100X). Partial phase contrast-fluorescent image composites confirm the localization of galactocerebroside (C) and sulfatide (E) to the myelin membrane (white arrows, original magnification 400X).

P0 accounted for ~59% of total bovine myelin protein, while MBP (~23%), P2 (~13%), periaxin (~4%) and PMP-22 (~1%) made up the other fractions detected (Table 1). These data verified the PNS origin of purified BPNM isolates.

Lipid detection and composition of purified BPNM

Indirect fluorescent immunohistochemistry of PFA-fixed BPNM smears demonstrated galactocerebroside (Figures 3A-C) and sulfatide (Figures 3D-F) expression. The corresponding phase contrast images (Figures 3A and 3D) clearly show aggregates of myelin that correspond to areas of immunoreactivity (Figures 3B and 3E). A partial phase contrast-fluorescent image composite confirmed the localization of galactocerebroside (Figure 3C) and sulfatide (Figure 3F) immunoreactivity on the myelin membranes. Phospholipids accounted for ~60% of the total dry weight of BPNM, while galactolipids and cholesterol accounted for ~24% and ~15% respectively (Table 2). These values are within the range expected for PNS myelin [1], with a reversal in percentages of galactolipids and cholesterol when compared to a previously described analysis of bovine cauda equina myelin [19]. These relatively straightforward colorimetric assays verified the PNS origin of the isolated myelin without need for chromatography.

Histopathology of BPNM-induced EAN in female SJL/J mice

Toluidine blue stained, basic fuchsin counterstained semi-thin plastic embedded sections demonstrated multi-focal demyelination with frankly demyelinated or thinly myelinated axons and reduced large myelinated axonal density associated with mononuclear cell infiltration at peak disease severity (Figure 4A). Indirect immunohistochemistry confirmed loss of the normal honeycomb myelin architecture due to loss or damage to Schwann cell membranes (Figure 4B) and reduced axonal density (Figure 4C) associated with intense mononuclear cell infiltrates. These infiltrates were predominantly macrophages (Figure 4D), followed by T-lymphocytes (Figure 4E) with scattered

B-lymphocytes (Figure 4F), consistent with a representative animal model of Guillain-Barré syndrome, as previously characterized and published [3-6].

Discussion

PNS myelin is still considered a valuable resource for studying inherited and acquired causes of peripheral nerve disease. The traditional methods of isolating myelin from mammalian tissues were initially described ~40-50 years ago, and utilize equipment and consumables that have been replaced or are no longer in use. Prior verification of myelin isolates required labor-intensive gravimetric analyses, SDS-PAGE and chromatography, often requiring the production of large quantities of myelin. The purpose of this study was to update these protocols and demonstrate several simplified methods of verifying the nature and purity of myelin isolates, utilizing widely available materials. This allows small scale rapid isolation, purification and verification of myelin isolates in the laboratory, prior to performing more labor-intensive and expensive analytical assays.

Negatively-stained TEM is the quickest way to accurately verify myelin and demonstrate lack of contamination. Light and phase contrast microscopy of BPNM smears can be used in situations where TEM is not readily available. An inability to detect galactocerebroside and sulfatide by indirect fluorescent immunohistochemistry could

Lipid Subclass	Sample A	Sample B	Mean
Phospholipid	60.7	59.8	60.3
Galactolipid	23.9	24.8	24.4
Cholesterol	15.3	15.4	15.4

Values represent percentages of lipid subclass relative to total BPNM lipid dry weight (to 1 decimal point).

Table 2: Relative quantification of BPNM lipid subclasses based on colorimetric assays.
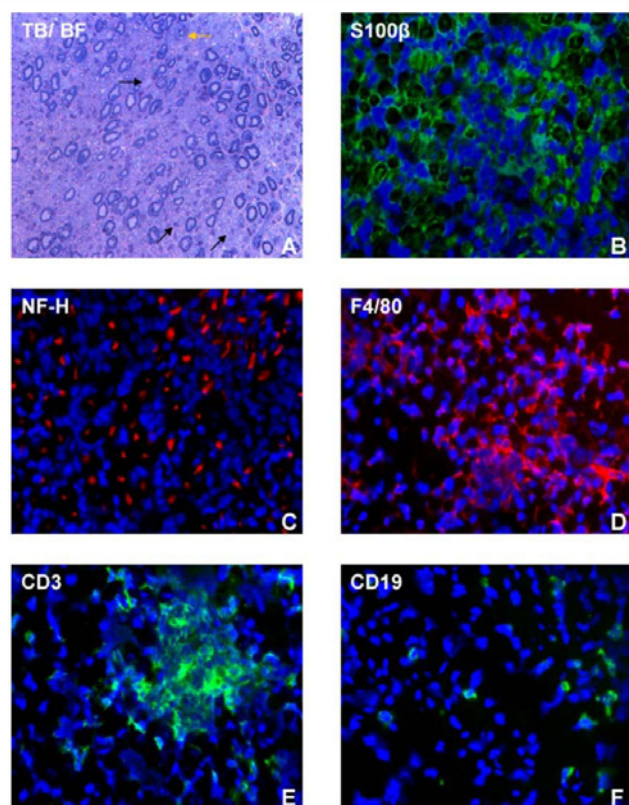
Figure 4: Histopathology of severe murine EAN. A photomicrograph of a representative toluidine-blue stained, basic fuchsin counterstained (TB/ BF) semi-thin plastic embedded axial section of a mouse sciatic nerve shows multifocal demyelination of axons (black arrows) associated with mononuclear cell infiltrates, with reduced density of large myelinated axons (A). An endoneurial microvessel is shown (yellow arrow). Indirect immunohistochemistry photomicrographs show loss of S100β immunoreactivity (green) associated with intense mononuclear cell infiltrate (blue), indicative of demyelination (B). There is loss of axonal density (red immunoreactivity for neurofilament-Heavy chain [NFH]) similarly associated with mononuclear cell infiltrate (blue), indicative of axonal loss in BPNM-induced murine EAN (C). Infiltrates are predominantly macrophages (F4/80+; red immunoreactivity) as shown in D. A focal collection of CD3+ T-lymphocytes and scattered CD19+ B-lymphocytes (green immunoreactivity) are shown in E and F respectively. Original magnification 400X.

imply galactolipid loss or isolation of another biological membrane. Gravimetric analytical procedures, although more labor-intensive, can provide more accurate determination of the lipid and protein components of isolated myelin. PNS myelin consists of 70-80% lipid and 20-30% protein by dry mass [1], so this method can rapidly verify that the extract is myelin prior to more detailed analyses.

We determined the protein components of BPNM based on their known molecular weights. Specific antibodies against bovine PNS and CNS myelin proteins are not commercially available or are difficult to generate. This limits the precise identification of protein components by western blot. The electrophoretic pattern and relative percentages of the protein components demonstrate that the myelin extracts were from the PNS, with P0 being the most abundant protein [1]. This difference is borne out using a commercially available 50% pure bovine brain MBP as a control in these experiments. Bovine brain MBP consists of several components that vary from 17-18.5 KDa in size. MBP may undergo post-translational modification resulting in slightly larger band sizes (19.0-20 KDa). Due to the lack of specific antibodies

against bovine myelin proteins, glycoprotein detection using LCA-FITC provides a rapid and inexpensive method to verify PNS myelin by fluorescent western blot. Glycoproteins are major constituents of PNS myelin and minor constituents of CNS myelin [1]. Where available, CNS myelin from the same animal source could be utilized as negative controls in these assays.

Colorimetric assays can be employed to estimate the relative proportions of the major myelin lipid subclasses. These values can be compared with data derived from previous studies or other species. In this study, the relative proportions of galactolipids and cholesterol in BPNM were reversed compared to a published report describing the isolation, purification and analysis of BPNM [19]. In that study, myelin was purified using a discontinuous sucrose gradient. We performed an additional continuous cesium chloride gradient to further purify myelin isolates, as large amounts of insoluble residues were reportedly obtained in some of their isolates [19].

We used rapid and relatively inexpensive colorimetric assays to quantify the major lipid subcomponents and indirect immunohistochemistry to detect galactolipids on intact myelin preparations; in contrast to chromatography employed in the older study [19]. Furthermore, that study did not analyze BPNM protein components. Thus, our study provides important information on BPNM isolates from the cauda equina utilizing protocols with readily available materials to the general laboratory scientist. Differences in myelin purification and lipid analysis methods could explain the observed variation in galactolipid and cholesterol. It is also possible that the age, strain, dietary changes and genetic modifications of cows from which the cauda equina were obtained are contributory factors.

BPNM extracted and verified using our protocols induced a severe inflammatory polyneuritis in female SJL/J mice that recapitulates essential features of the acute inflammatory demyelinating polyradiculoneuropathy variant of GBS. This model is reliable and reproducible with ~100% induction rates [3-6], providing a means to study molecular determinants and signaling pathways implicated in acute peripheral nerve inflammation with potential therapeutic implications in humans. Mouse models of peripheral nerve inflammation more closely resemble the human disorder than rat models due to similarities in neural-immune responses. In summary, we updated and developed adapted protocols for the isolation, purification and verification of BPNM, using tools readily available to most biomedical research laboratories in industrialized nations. These techniques can be readily applied by neuroscientists, immunologists, biochemists and molecular biologists interested in studying the structure-function relationships of myelin and other biological membranes, or animal models of peripheral neuroinflammation.

Acknowledgements

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