A Biochemical Analysis of the Factors Influencing P0 Oligomerization in Xenopus laevis Peripheral Nerve Myelin

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Spring 2004

Abstract

Protein zero (P0), the major structural protein of peripheral nerve myelin, is a ~30 kDa integral membrane glycoprotein consisting of an extracellular domain, a transmembrane domain, and a palmitoylated cytoplasmic domain. In native membranes of *Xenopus laevis* it exists primarily as a dimer. To determine the effects of glycosylation, acylation, and hydrophobic interactions on protein dimerization, I used SDS polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, and high-performance thin layer chromatography (HPTLC) to analyze the effects of deglycosylation, deacylation, and various detergent treatments on myelin isolated from *Xenopus laevis* sciatic nerve. These treatments showed no effect on P0 oligomerization, suggesting that glycosylation, acylation, and hydrophobic interactions disrupted by these detergents do not underlie P0 dimerization. The data points to the likelihood that covalent linkages contribute to P0 oligomerizaztion in Xenopus.

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Table of Abbreviations

Abbreviation	Full Name
βOG	N-octyl-β-D-glucopyranoside
CE	Cholesterol ester
CH	Hydroxy cerebrosides
CHAPSO	3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate
CN	Non-hydroxy cerebrosides
CNS	Central nervous system
CO	Cholesterol
DG	Diglyceride
DTT	Dithiothreitol
ECL	Enhanced chemiluminescent
EDTA	Ethylenediaminetetraacetic acid
FA	Fatty acid
GLC	Gas-liquid chromatography
HA	Hydroxylamine
HPTLC	High-performance thin layer chromatography
MG	Monoglyceride
P0	Protein zero
PAGE	Polyacrylamide gel electrophoresis
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PLP	Proteolipid protein
PNGase F	Peptide N-glycosidase
PNS	Peripheral nervous system
PS	Phosphatidylserine
PVDF	Polyvinylidene fluoride
SDS	Sodium dodecyl sulfate
SH	Hydroxy sulfatides
SM	Sphingomyelin
SN	Non-hydroxy sulfatides
TG	Triglyceride

Introduction

Myelin, which consists of a compact jelly-roll-like wrapping of lipid-rich membranes surrounding nerve axons, facilitates a much higher rate of nerve conduction between neurons than is possible in unmyelinated axons of the same diameter (Fig. 1). Myelin is produced by Schwann cells in the peripheral nervous system (PNS) and by oligodendroglial cells in the central nervous system (CNS);(Kirschner and Blaurock, 1992). The most abundant component of myelin is lipid; however, proper membrane packing has been shown to be dependent upon the protein content of the membrane (Inouye and Kirschner, 1990).

Protein zero (P0), a ~30kDa integral membrane glycoprotein, is the most abundant protein and is the primary structural factor in peripheral nerve myelin (Kirschner et. al., 1996). The protein consists of a palmitoylated cytoplasmic domain, a transmembrane domain, and a glycosylated extracellular domain (Fig. 2). Mutations in P0 lead to demyelination of peripheral nerves and underlie the clinical deficits in certain human peripheral neuropathies including Charcot-Marie-Tooth disease and Dejerine-Sottas syndrome (Keller and Chance, 1999). The sequence of the protein is highly conserved in human, mouse, frog, and other species (Fig. 3). Analogous proteins discovered in peripheral nerve myelin of teleosts, including zebrafish, are called IP1 and IP2.

P0 exists primarily as a dimer in native membrane from amphibian (Xenopus), but not from higher vertebrates, and the level of oligomerization is apparently unaffected by changes in pH or salt concentration (Thompson, et. al., 2002). It is possible that posttranslational modifications of the protein are responsible for P0 oligomerization. P0

contains an N-linked carbohydrate on Asn93 in the extracellular domain of the protein and a palmitoylated cysteine in the cytoplasmic domain that may play a role in dimerization. Deacylation of proteolipid protein (PLP), a protein analogous to P0 in the central nervous system, by hydroxylamine (HA) has been shown to reduce the adhesive properties of the protein and leads to decompaction of CNS myelin as shown by electron microscopy (Bizzozero et. al., 2001). It is possible that HA treatment could have a similar effect on P0 and peripheral nerve myelin.

It is also possible that hydrophobic interactions are responsible for P0 dimerization. X-ray diffraction studies of whole Xenopus sciatic nerves treated with the zwitterionic detergent CHAPSO show symmetrization of membrane spacing (Z. Haddadin et. al., unpublished data), suggesting that membrane packing, and possibly P0 oligomerization, has been disrupted. Palmitoylation of P0 could also contribute to hydrophobic interactions that stabilize P0 oligomers via a trans interaction of apposed P0 molecules.

Myelin lipids from PNS and CNS myelin have been analyzed in mice using high-performance thin layer chromatography (HPTLC) (Ganser et. al., 1988). The lipid content of Xenopus and zebrafish myelin is less understood, but can presumably be analyzed and compared to that of mouse myelin using the same methods. This method could also be used to verify the results of depalmitoylation of P0 with HA.

In this study, the factors influencing P0 oligomerization were examined using peripheral nerve myelin purified from *Xenopus laevis*. This organism was chosen for study because they are easily obtained and have large, well-developed sciatic nerves and a P0 sequence that is highly homologous to that of humans and mice. Also, the P0 in Xenopus peripheral nerve myelin exists primarily as a stable dimer that is easily

detectable in an SDS-PAGE gel and is absent in other species. Protein samples were separated and analyzed using SDS-PAGE and Western Blotting, and lipid samples were separated and analyzed using HPTLC, for the speed and specificity of analysis that these procedures allow. Using these procedures also allowed comparison with previously obtained data from Xenopus and mouse PNS myelin studies using similar methods. In many cases, samples of isolated PNS myelin from Xenopus were compared to samples of PNS and CNS myelin from mouse or zebrafish treated in parallel.

By subjecting the isolated myelin to several different biochemical treatments, we hoped to disrupt the Xenopus P0 dimer and elucidate what causes its unusual stability. The long-range goal of this research is to understand what underlies P0 protein-protein interactions in Xenopus peripheral nerve myelin, and thereby explain how PNS myelin protein stabilizes myelin structure.

Materials and Methods

Sample selection and preparation:

Sciatic nerves were isolated from adult female *Xenopus laevis* (Xenopus I, Inc., Dexter, MI) and sciatic nerves, brains, and optic nerves were isolated from adult female B6 mice (courtesy of Dr. T. Seyfried, Boston College) and homogenized in water with aprotinin (1μg/mL) to prevent proteolysis. Myelin was isolated using a discontinuous sucrose gradient (Norton and Poduslo, 1973). Lateral line and optic nerves were isolated from adult zebrafish and myelin was isolated using a modified discontinuous sucrose gradient protocol in which a lower volume of nerve homogenate and fewer washes maximized the yield.

Sample treatment:

Isolated myelin was treated with N-glycosidase F (PNGase F); (Sigma-Aldrich, Inc., St. Louis, MO), an enzyme that cleaves N-linked carbohydrates from denatured proteins, in order to remove the N-linked carbohydrate from P0. The protein was first denatured in .2%SDS and 1% β-mercaptoethanol at 100°C for 3 minutes, then sample buffer was added to a final concentration of 0.15% SDS, 0.75% β-mercaptoethanol, 100mM Na_2HPO_4, 25mM EDTA, and 1% Triton X-100. Enzyme was then added and the solution was incubated overnight (~16h) at 37°C. Enzyme activity was stopped by boiling in SDS-PAGE sample buffer. Isolated myelin was also treated with 0mM-667mM

hydroxylamine (HA) solution for 1 hr. at 30°C, and with 5,10, and 25mM CHAPSO (Sigma-Aldrich, Inc., St. Louis, MO) and N-octyl-β-D-glucopyranoside (βOG); (AG Scientific, Inc., San Diego, CA) for 1hr. at 37°C.

Protein visualization and analysis:

Treated and untreated myelin samples were separated using SDS-PAGE alongside broad-range molecular standards (BioRad, Inc., Hercules, CA). Samples were heated to 100°C in sample buffer (2% SDS, 10% glycerol, 0.1mM DTT, 0.01% bromophenol blue, 0.063M Tris-Cl) before loading. Gels were 8cm 4-20% Tris-HCl Novex precast polyacrylamide minigels (Invitrogen Inc., Carlsbad, CA), run at a constant voltage of 125V for approximately 90 minutes, until the dye front reached the end of the gel. Gels were stained using standard Coomassie blue protocol, stained using the Silver Stain Plus Kit (BioRad Inc., Hercules, CA), or blotted onto PVDF membrane (Millipore, Inc., Bedford, MA) using the Mini-trans Blot tank apparatus (BioRad Inc., Hercules, CA) at 35V for 2h. Western blotting was carried out with the ECL Western Blotting Analysis System (Amersham Biosciences, Inc., Piscataway, NJ). Primary P0 antibodies were monoclonal mouse IgG obtained from the laboratory of Dr. Richard Quarles. Glycoprotein detection was carried out after blotting on PVDF membrane using the ECL Glycoprotein Detection Module (Amersham Biosciences, Inc., Piscataway, NJ). Gels and film were scanned, and gels were dried in 5% glycerol solution between cellophane sheets.

Lipid analysis:

After treatment of isolated myelin with HA, its lipid content was analyzed using one-dimensional HPTLC. Each 50μL myelin sample contained either 1M or 0M HA solution, and was incubated for 45min at 30°C. The samples were then diluted with ddH_20 to 200μL and extracted with two washes of 200μL of pure chloroform. The chloroform was then evaporated under a steam of nitrogen gas and the contents resuspended in 100μL of chloroform. Aliquots of 15 μL of these samples were hand spotted onto a 10x10cm silica gel HPTLC plate (Whatman Inc., Clinton, NJ) along with a lipid standard mix (Nu-Chek Prep, Inc., Elysian, MN) and palmitic acid standard (Sigma-Aldrich, Inc., St. Louis, MO).

Neutral lipid standards include cholesterol ester (CE; cholesteryl oleate), triglyceride (TG; tristearin), diglyceride (DG; distearin), cholesterol (CO), free fatty acid (FA; stearic), and monoglyceride (MG; 1-monostearyl). Polar lipid standards include non-hydroxy and hydroxy cerebrosides (CN, CH), non-hydroxy and hydroxy sulfatides (SN, SH), ethanolamine phosphatides (PE), phosphatidic acid (PI), phosphatidylserine (PS), phosphatidylcholine (PC), and sphingomyelin (SM);(Nu-Chek Prep, Inc., Elysian, MN).

Polar lipids were separated with a mixture of methyl acetate/1-propanol/chloroform/methanol/.25% KCl/acetic acid (25:25:25:10/9/.3). Neutral lipids were separated with first with a mixture of chloroform/methanol/acetic acid (93.1:1.9:.1) to 1.5cm from the top of the plate, then redeveloped with a mixture of hexane/ethyl ether/acetic acid (89.3:5.7:.1) to the top of the plate. Color was developed by charring the plates at 140°C after immersing the plate in aqueous 10% $CuSO_4 \bullet 5H_2O$, 8% H_3PO_4 solution (Ganser et. al., 1988). Plates were then scanned and the digital chromatograms

were analyzed.

Results

Deacylation of P0 with HA

Treatment with HA showed no effect on the level of Xenopus P0 oligomerization in small concentrations (0-500mM) when compared to P0 from untreated myelin. In higher concentrations (>500mM), HA caused aggregation of the samples, preventing them from running properly on the gel (Fig. 4). After treatment of isolated myelin with 225mM HA, there was no difference in the oligomerization of P0 from either Xenopus or mouse (Fig. 5). HPTLC analysis of lipids from HA treated and untreated Xenopus myelin samples showed no significant increase in the level of free palmitic acid in the treated samples (Fig. 6). Samples of HA treated isolated myelin from mouse CNS and PNS did not show an increase in the level of free palmitic acid when compared to untreated samples (Fig. 7,8).

Deglycosylation of P0 with PNGase F

Deglycosylation of P0 from Xenopus and mouse showed no effect on P0 oligomerization. A band-shift representing the size of the removed carbohydrate moiety is observed, as well as an additional ~36kDa band which has been identified as the PNGase F enzyme (Fig. 9). Deglycosylation was confirmed by using the ECL glycoprotein detection kit on Western blots of treated and untreated myelin samples. Color developed in lanes with untreated samples of Xenopus and mouse myelin but did not develop in lanes with PNGase F treated samples (Fig. 10).

Detergent treatments:

Treatment of Xenopus myelin with 5-25mM CHAPSO and βOG also did not affect the level of P0 oligomerization (Fig. 11). Treatment of isolated myelin with 25 or 100mM HA as described above followed by treatment with 25mM CHAPSO showed no effect on the level of P0 oligomerization (Fig. 12)

Lipid analysis

The polar and neutral lipid content of Xenopus PNS myelin, both untreated and treated with HA, has been successfully visualized using HPTLC (Figs. 5,13). No significant increase in the level of free palmitic acid was visible in the HA treated sample when compared with the untreated sample. Lipids from mouse CNS and PNS myelin, untreated and treated with HA, have also been visualized using this method for comparison (Figs. 7,8,14,15). In these samples, there no significant increase in the level of free palmitic acid in the HA treated samples when compared with the untreated samples.

Discussion

Amphibian peripheral nerve myelin P0 forms stable dimers in native membrane that are unaffected by changes in pH and ionic strength (Thompson et. al., 2002). To test the possibility that hydrophobic forces, glycosylation, or palmitoylation underlie the stability of the P0 dimer, isolated PNS myelin from Xenopus was subjected to several biochemical treatments and the protein and lipid content was analyzed using SDS-PAGE and HPTLC.

Treatment of Xenopus myelin with HA and PNGase F reproducibly showed no change in the level of P0 oligomerization. These results suggest that palmitoylation and glycosylation of P0 do not play a role in oligomerization of the protein. The deglycosylation of P0 by this method was confirmed using glycoprotein specific detection, and the identities of the bands were confirmed by Western Blotting with anti-P0 antibody. Deacylation of P0 by treatment with HA was not confirmed by our HPTLC lipid analysis of isolated Xenopus myelin. Deacylation of myelin proteins in mouse isolated CNS and PNS myelin also was not apparent using this method. However, there is an apparent increase in the concentration of lipids from HA treated myelin samples. This may be due to more efficient extraction of lipids from HA treated myelin, although the exact cause is unclear. The lack of increase in free palmitic acid as visualized using HPTLC may be the result of a low relative amount of cleaved palmitic acid in the samples. The increase in free palmitic acid may not be significant in either mouse or Xenopus when compared to the high levels of other lipids in the sample. Deacylation of PLP from isolated mouse CNS myelin by HA under the same conditions was confirmed

by Bizzozero (2001) using gas-liquid chromatography (GLC) to monitor the level of covalently bound fatty acids on isolated PLP. This method is sensitive only to palmitic acid bound to protein, and could be used to show decreasing levels of bound fatty acid on isolated P0 as a more sensitive and selective means of confirming palmitic acid cleavage.

Treatment of isolated Xenopus myelin with CHAPSO or βOG did not affect the level of P0 oligomerization. Treatment with CHAPSO following HA treatment also appeared to have no effect. Although this data suggests that palmitoylation does not play a role in hydrophobic interactions that may stabilize the P0 dimer, it does not eliminate the possibility that hydrophobic interactions are responsible for P0 oligomerization. It is possible, although unlikely, that another detergent treatment or different incubation conditions would disrupt the P0 dimer.

Future plans for this project include combining the results of this research on isolated myelin with X-ray diffraction studies of whole Xenopus sciatic nerves to determine how protein-protein interactions in Xenopus PNS myelin, specifically the P0 dimer, are related to membrane packing. Also, as the forces that stabilize the P0 dimer have not yet been elucidated, research in this area should continue. Other detergent treatments or incubation conditions could be explored as a means to disrupting P0 oligomerization in isolated myelin from Xenopus to conclusively determine if this interaction is stabilized by hydrophobic interactions.

The possibility that another type of covalent linkage, possibly an Arg-Lys crosslink (formed by the sugar-catalyzed Maillard reaction);(Dyer 29), stabilizes the dimer should also be explored. The nature of the bond stabilizing the P0 dimer could be explored by isolation of the dimer from an SDS-PAGE gel followed by digestion of the protein with proteases. The fragments could then be separated on a preparative gel and

analyzed using mass spectrometry. By comparing these fragments with the results of an identical enzymatic treatment of known monomer, the protein fragment that contains the crosslink could be identified and the nature of the crosslink elucidated.

Acknowledgments

I would like to thank Dr. Dan Kirschner for his knowledge, guidance and encouragement, Dr. Deepak Sharma for his support and advice, the rest of my lab, especially Zaid Haddadin and Robin Avila, for their support, motivation and collaboration, and my family and friends.

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Figures

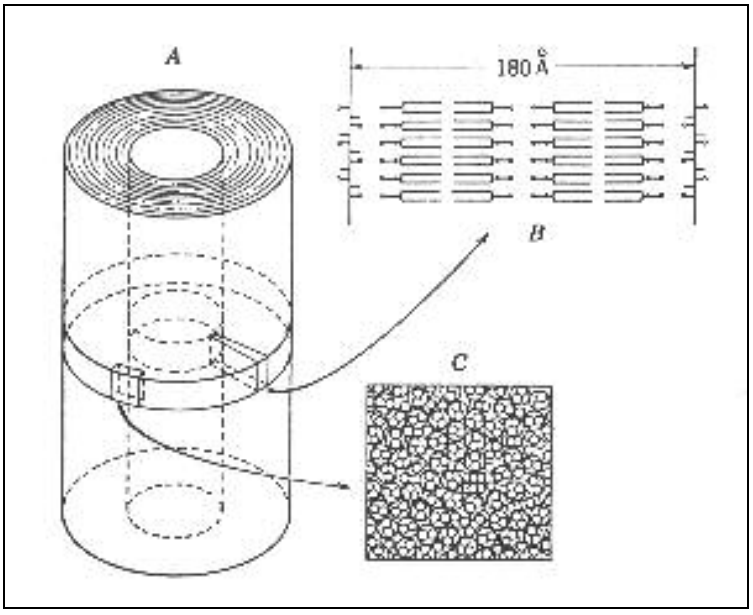

Figure 1. Organization of the myelin sheath surrounding an axon. **A.** Superstructure of the myelin sheath. **B.** Repeating lipid bilayers making up the myelin sheath. **C.** Cross-section through lipid membrane (Schmitt, 1950).

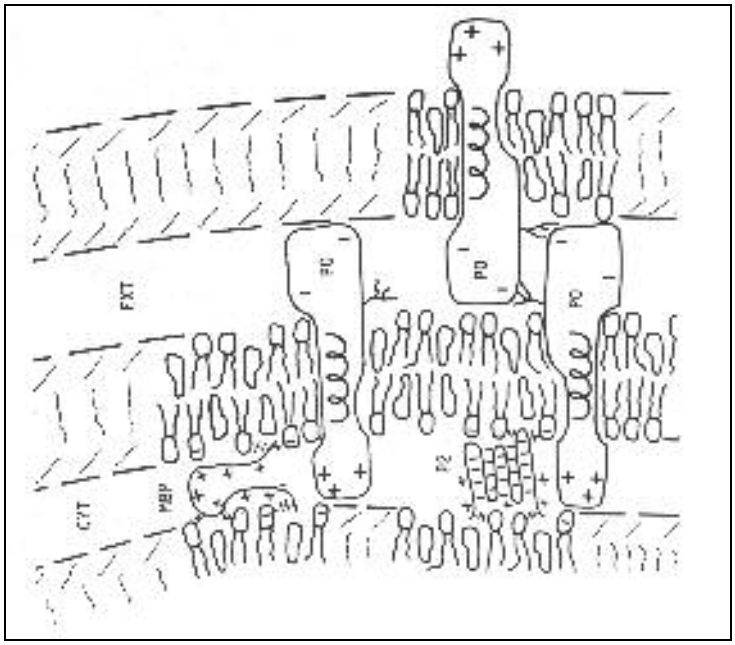

Figure 2. Schematic representation of PNS myelin protein orientation. Branches show carbohydrate chains. (Kirschner and Blaurock, 1992).

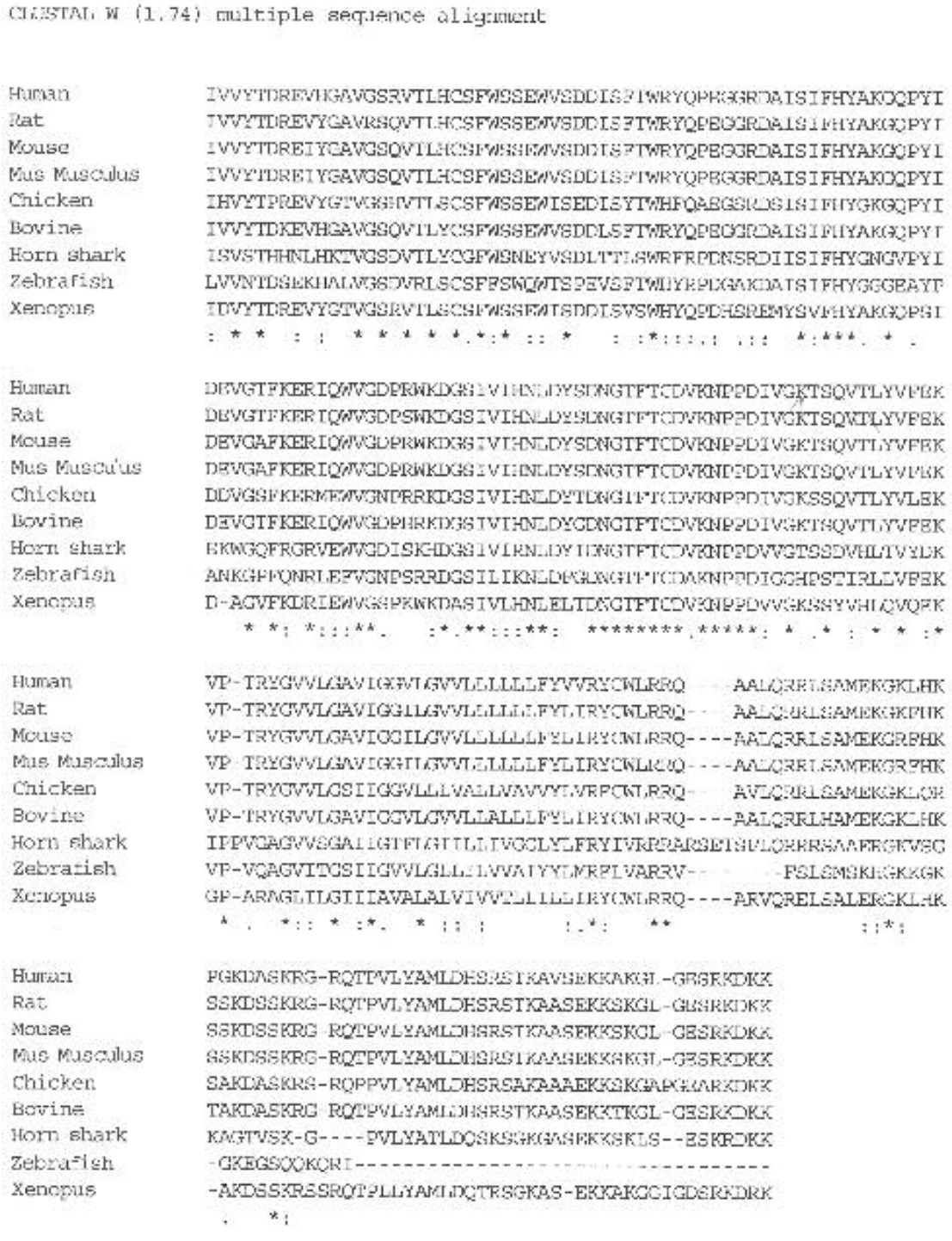

Figure 3. Sequence alignment of P0 protein from several species.

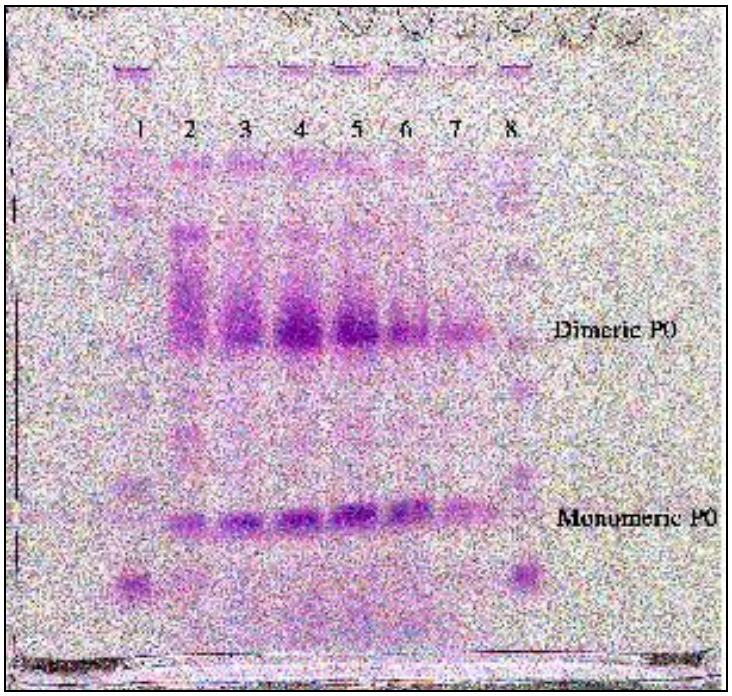

Figure 4. SDS-PAGE gel. Lanes 1,8 broad-range standard, lanes 2-7 isolated myelin from Xenopus treated with 0, 0.167, 0.5, 0.83, 1.33, and 1.67M HA.

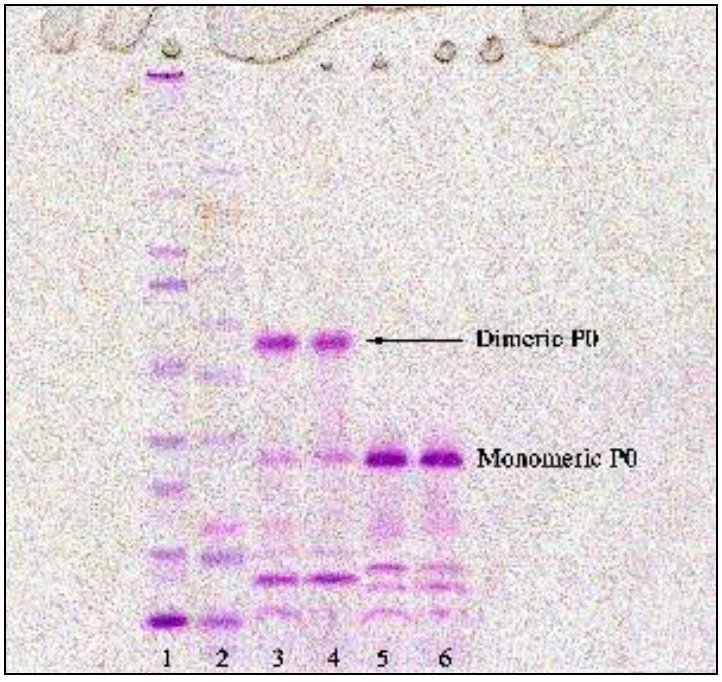

Figure 5. SDS-PAGE gel. Lane 1 broad-range standard, lanes 3,5 untreated myelin from Xenopus and mouse, lanes 4,6 myelin from Xenopus and mouse after 225mM HA treatment for 1hr. at 37°C.

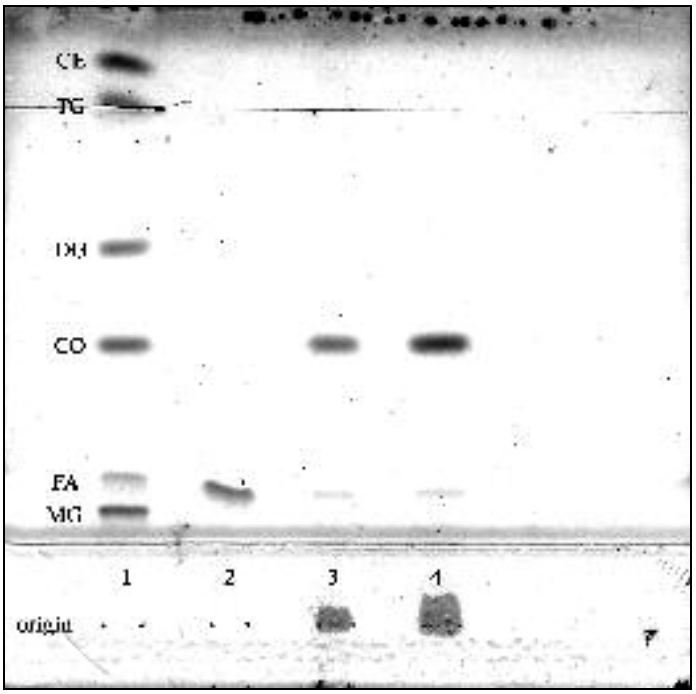

Figure 6. HPTLC plate, neutral lipids. Lane 1 neutral lipid standards, lane 2 palmitic acid, lanes 3,4 lipids isolated from untreated and HA treated isolated PNS myelin from Xenopus.

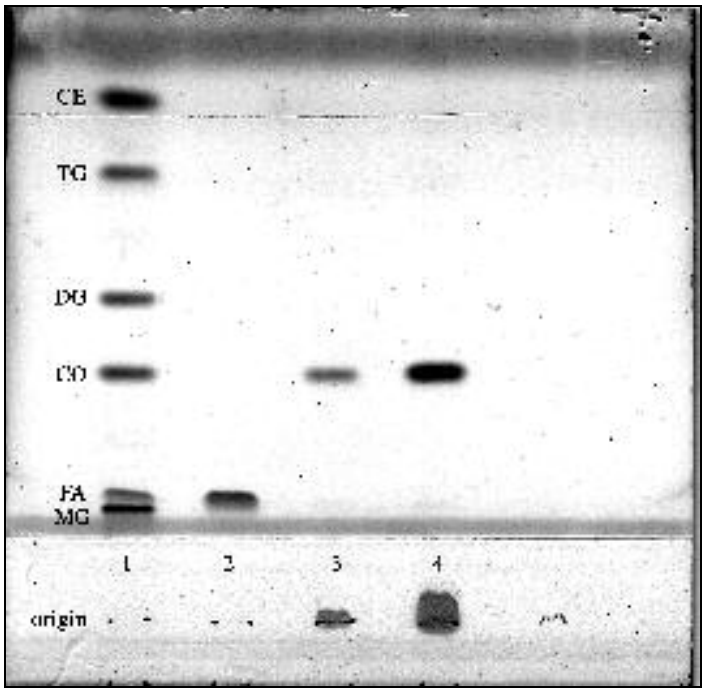

Figure 7. HPTLC plate, neutral lipids. Lane 1 neutral lipid standards, lane 2 palmitic acid, lanes 3,4 lipids isolated from untreated and HA treated isolated PNS myelin from mouse.

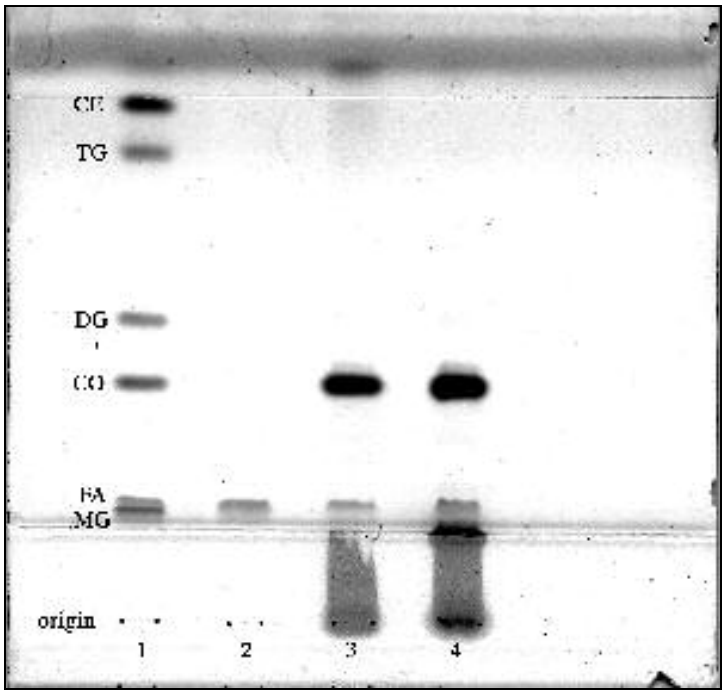

Figure 8. HPTLC plate, neutral lipids. Lane 1 neutral lipid standards, lane 2 palmitic acid, lanes 3,4 lipids isolated from untreated and HA treated isolated CNS myelin from mouse.

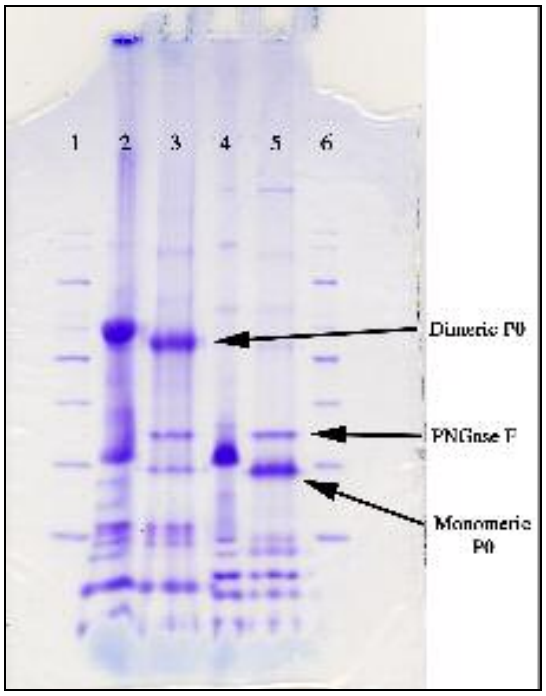

Figure 9. SDS-PAGE gel. Lanes 1,6 broad-range standard, lanes 2,3 PNS myelin isolated from Xenopus untreated and after PNGase F treatment, lanes 4,5 PNS myelin isolated from mouse untreated and after PNGaseF treatment.

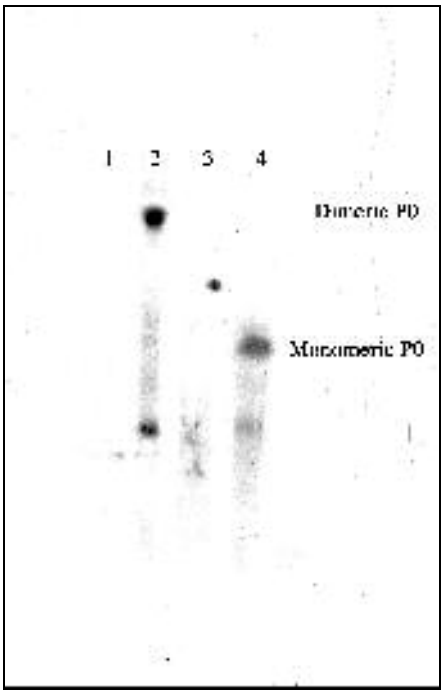

Figure 10. X-ray film from ECL glycoprotein detection. Lanes 2,4 isolated PNS myelin from Xenopus and mouse, lanes 1,3 isolated PNS myelin from Xenopus and mouse after PNGase F treatment (no color development).

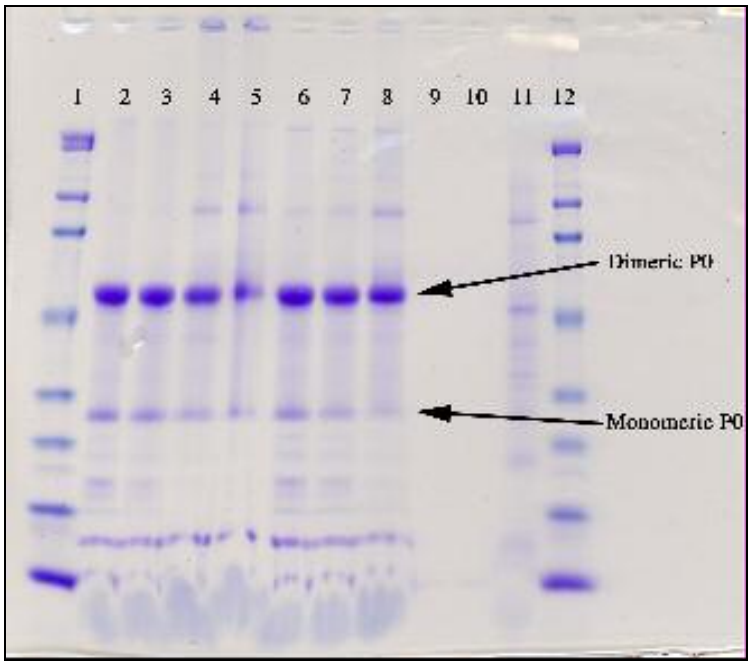

Figure 11. SDS-PAGE gel. Lanes 1,12 broad-range standard, lane 2 PNS myelin isolated from Xenopus lanes 3,4,5 PNS myelin isolated from Xenopus treated with 5,10, and 25mM CHAPSO, lanes 6,7,8 PNS myelin isolated from Xenopus treated with 5,10, and 25mM βOG.

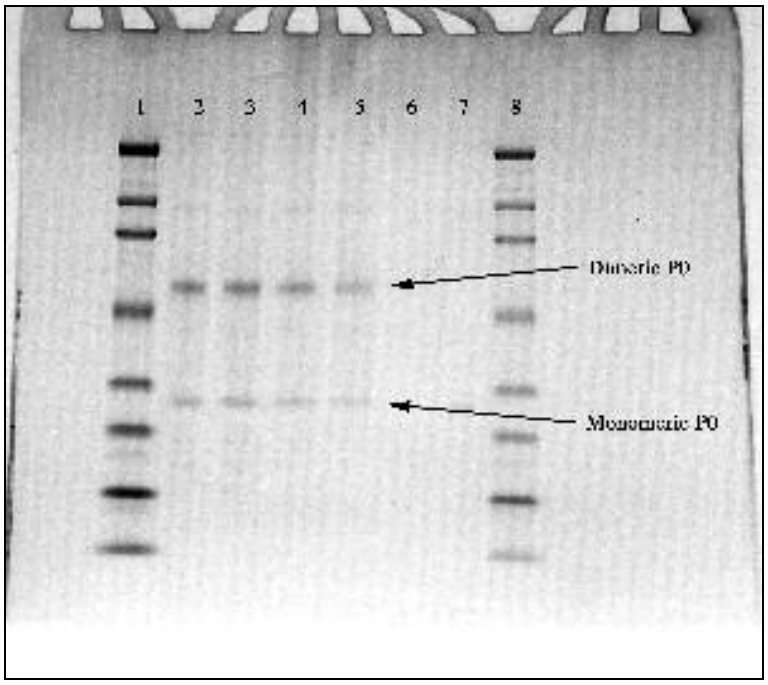

Figure 12. SDS-PAGE gel. Lanes 1,8 broad-range standard, lane 2 untreated PNS myelin isolated from Xenopus, lane 3 25mM HA treated PNS myelin from Xenpus, lane 4 10mM CHAPSO treated PNS myelin from Xenopus, lane 5 25mM HA and 10mM CHAPSO treated PNS myelin from Xenopus.

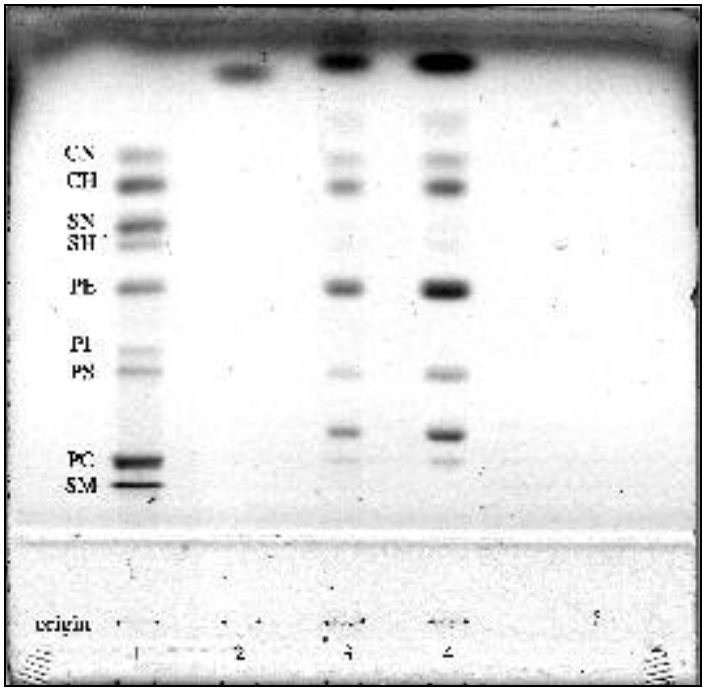

Figure 13. HPTLC plate, polar lipids. Lane 1 polar lipid standards, lane 2 palmitic acid, lanes 3,4 lipids isolated from untreated and HA treated isolated PNS myelin from Xenopus.

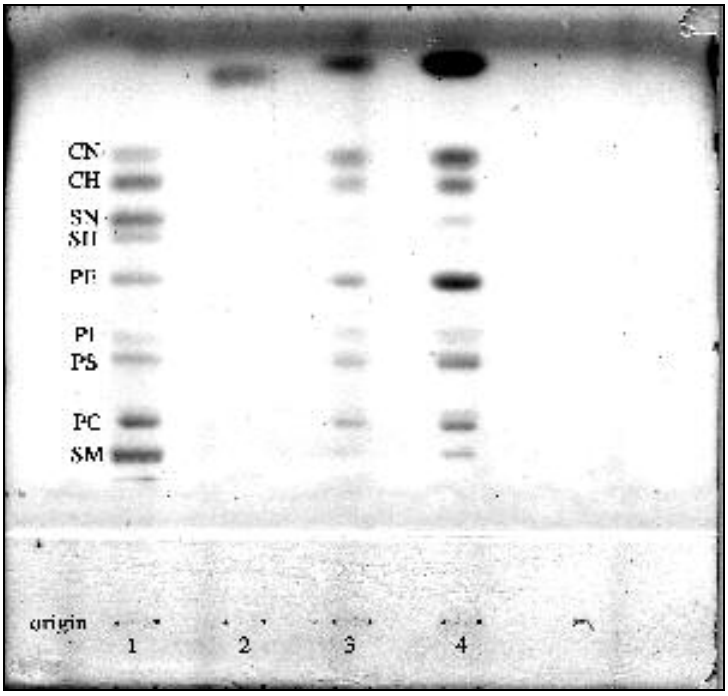

Figure 14. HPTLC plate, polar lipids. Lane 1 polar lipid standards, lane 2 palmitic acid, lanes 3,4 lipids isolated from untreated and HA treated isolated PNS myelin from mouse.

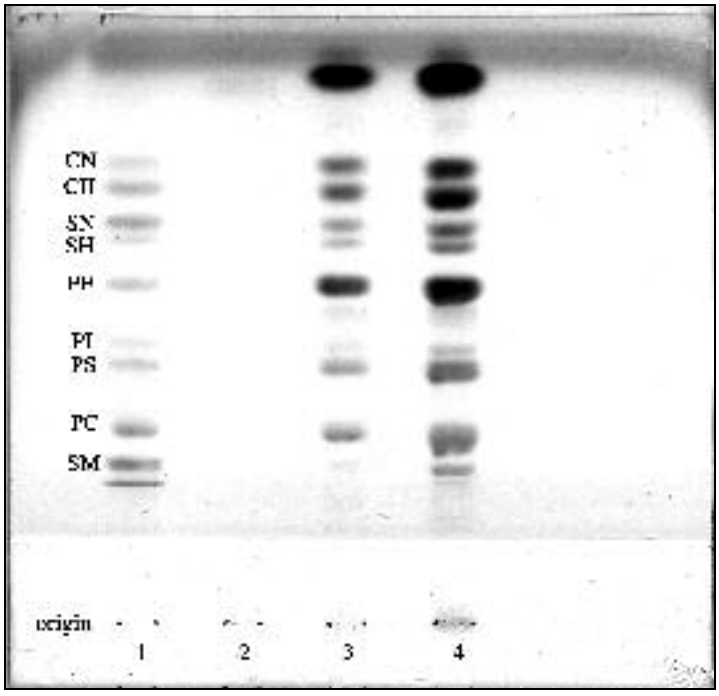

Figure 15. HPTLC plate, polar lipids. Lane 1 polar lipid standards, lane 2 palmitic acid, lanes 3,4 lipids isolated from untreated and HA treated isolated CNS myelin from mouse.