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Proteomic Analysis of the Highly Detergent Resistant  
Radial component of Myelin

By

Vatsal M. Patel

Master of Science in Industrial Chemistry,

Sardar Patel University, 2007

Thesis

Submitted to the Department of Chemistry

In partial fulfillment of the requirements for

Master of Science in Chemistry

Wilfrid Laurier University

## Abstract

The wrapping of multiple layers of myelin membrane sheets around an axon plays an important role in normal neuronal function. In the central nervous system (CNS), oligodendrocytes are responsible for forming multiple myelin internodes which consist of lipids and proteins. Within these regions, there are various membrane microdomains including the radial component/tight junctions. The radial component is a junctional complex believed to stabilize the myelin membranes in the CNS myelin. In this study, the radial component from bovine and mouse myelin was isolated using differential detergent extraction. A highly detergent resistant fraction, containing previously identified proteins of the radial component (oligodendrocyte specific protein (OSP) and myelin oligodendrocyte basic protein (MOBP)), was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 2-D electrophoresis (with immobilized pH gradient (IPG) strip or liquid-phase isoelectric focusing (IEF) followed by SDS-PAGE), traditional Western blotting and multiplex fluorescent Western blotting, and mass spectrometry. The fraction was found to be significantly and consistently enriched in some high and low abundant proteins. The most abundant proteins identified are myelin basic protein (MBP), proteolipid protein, 2, 3-cyclic nucleotide 3 phosphodiesterase, cytoskeletal proteins (actin, tubulin, spectrin, neurofilament triplet M), glial fibrillary acidic protein, mitochondrial-type proteins (ATP synthase, ADP/ATP translocase) and ion transport sodium-potassium ATPase. A large-scale analysis of radial component myelin proteome was performed using a shot-gun approach. A total of 472 proteins were identified in bovine and 881 proteins in mouse. Furthermore, evidence implicates the presence of plasmalemmal voltage dependent anion channel (VDAC)

proteins in the myelin membrane. A select group of proteins (MBP, actin, tubulin, OSP, MOBP, VDAC, Na<sup>+</sup>/K<sup>+</sup> ATPase, and ATP synthase) identified were confirmed by Western blotting with protein specific antibodies. This study indicates that radial component of myelin can be isolated successfully and proteomic study reveals crucial information about high-abundance cytoskeletal and transport proteins and low-abundance signalling proteins. This information will aid in deciphering the roles of various proteins of the radial component in health and disease.

## **Acknowledgments**

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I would like to thank Dr. Masoud Jelokhani and Dr. Scott Smith for being on my committee and their advice.

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## **List of Abbreviations**

ADP: Adenosine diphosphate

ATP: Adenosine triphosphate

BCA: Bicinchoninic acid

BSA: Bovine serum albumin

CHAPS: (3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate)

CMC: Critical micelle concentration

CNP: 2, 3 -cyclic nucleotide 3 -phosphodiesterase

CNS: Central nervous system

DTT: Dithiothreitol

EDTA: Ethylene diamine tetraacetic acid

EGTA: Ethylene glycol tetraacetic acid

ELLS: Electron lucent linear structures

ESI-MS: Electrospray ionization-mass spectrometry

GFAP: Glial fibrillary acidic protein

GTP: Guanosine triphosphate

HCP: Hexachlorophene

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IEF: Isoelectric focusing

IPG: Immobilized pH gradients

LC-MS: Liquid chromatography - mass spectrometer

MAG: Myelin associated glycoprotein

MALDI-TOF: Matrix-assisted laser desorption ionization-time of flight

MBP: Myelin basic protein

MOBP: Myelin oligodendrocyte basic protein

MOG: Myelin oligodendrocyte glycoprotein

MS: Mass spectrometry

“MS”: Multiple sclerosis

MudPIT: Multidimensional protein identification technology

NADH: Nicotinamide adenine dinucleotide

NaCl: Sodium chloride

Na<sub>3</sub>VO<sub>4</sub>: Sodium vanadate

Na<sup>+</sup>/K<sup>+</sup>-ATPase: Sodium-potassium adenosine triphosphatase

ND: Not detected

OSP: Oligodendrocyte specific protein

PhosSTOP: Phosphatase inhibitor cocktail

pI: Isoelectric point

PIC: Protease inhibitor cocktail

PLP: Proteolipid protein

PMF: Peptide mass fingerprinting

PMSF: Phenylmethanesulfonylfluoride

PNS: Peripheral nervous system

PVDF: Polyvinylidene difluoride

RR“MS”: Relapsing-remitting multiple sclerosis

SDS: Sodium dodecyl sulfate

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SIRT2: Sirtuin-2

TBS: Tris buffer saline

TLC: Thin layer chromatography

TNE: Tris, sodium chloride, ethylene diamine tetraacetic acid

TTBS: Tris buffer saline with tween-20

TX-100: Triton-X 100

VDAC: Voltage dependent anion-selective channel

1-D: One-dimensional

2-D: Two dimensional

### **Sample ID**

A-24, E19 weeks

A-15, E27 weeks

A-26, E37 weeks

A-50, adult



# **Chapter 1 – Introduction**

## **1.1 Nervous System**

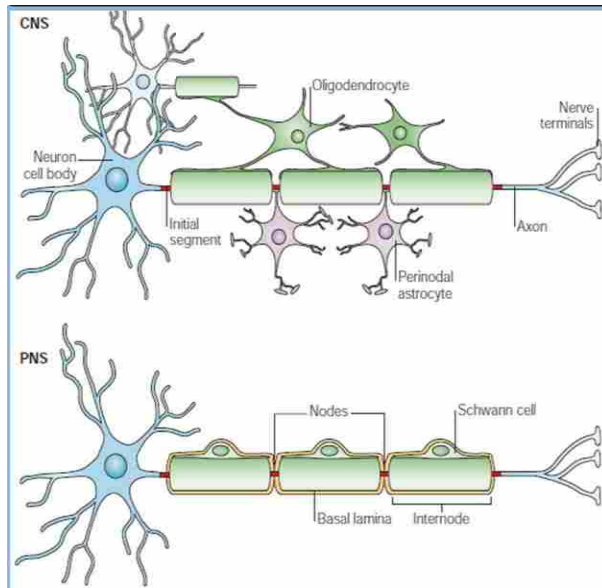
The nervous system is a network of specialized cells called neurons that coordinate the actions in our body and transmit signals between different parts of the body. The nervous system contains two main specialized types of cells, neurons and glial cells. Neurons send signals to other cells as electrochemical waves travelling along thin fibers called axons, which cause neurotransmitters to be released at junctions called synapses. Upon receiving synaptic signal, cells may be excited, inhibited or modulated. Sensory neurons are activated by physical stimuli and send signals which inform the central nervous system (CNS) of the state of the body and the external environment. Motor neurons situated either in the CNS or in peripheral ganglia, connect the nervous system to muscles or other organs. Central neurons make all of their input and output connections with other neurons. The important functions of glial cells are to support neurons and hold them in place, supply nutrients, insulate neurons electrically, destroy pathogens and remove dead neurons, and to provide guidance and directing the axons of neurons to their targets <sup>1</sup>.

The nervous system is basically responsible for controlling all the biological processes taking place in our body, as well as movement. Nervous system can be divided into two different categories, the CNS and peripheral nervous system (PNS). The CNS is made up of the brain and spinal cord, while the PNS consists of nerves in the body. In spite of high similarity between these two sub-systems, they differ greatly in their molecular organization and composition <sup>2</sup>. The CNS is known as the processing unit which receives and analyzes signals and consequently takes the required action. The PNS

is responsible for detecting and relaying signals from the nerve cells to the CNS. The nerve cells are responsive cells in the nervous system that process and transmit information by chemical signals within a neuron <sup>3</sup>.

## **1.2 Myelin Structure and Function**

Myelin is composed of a complex multilamellar sheath which is essential for the normal neuronal function. The formation of myelin occurs early in development and requires oligodendrocyte and Schwann cell proliferation and migration, followed by the wrapping and compaction of the plasma membrane around axons. The myelin membrane is responsible for the insulation and protection of the axon. Additionally, myelin plays a vital role in increasing the transfer efficiency of action potentials from one end of the axon to another. In axons, action potentials are transferred by saltatory movement, due to the myelin sheath <sup>4</sup>. The spaces between myelinated sections of the axon are known as nodes of Ranvier (Fig. 1.1), where the axon is exposed to the extracellular region and there are a large number of voltage-gated sodium channels that can open to allow the transfer of sodium ions into the cell due to the reduction in membrane potential. This reduction in potential depolarizes neighbouring nodes which allows the action potential to jump to the nearby node and thereby carry the electrochemical signal down the axon towards its final destination <sup>5</sup>.



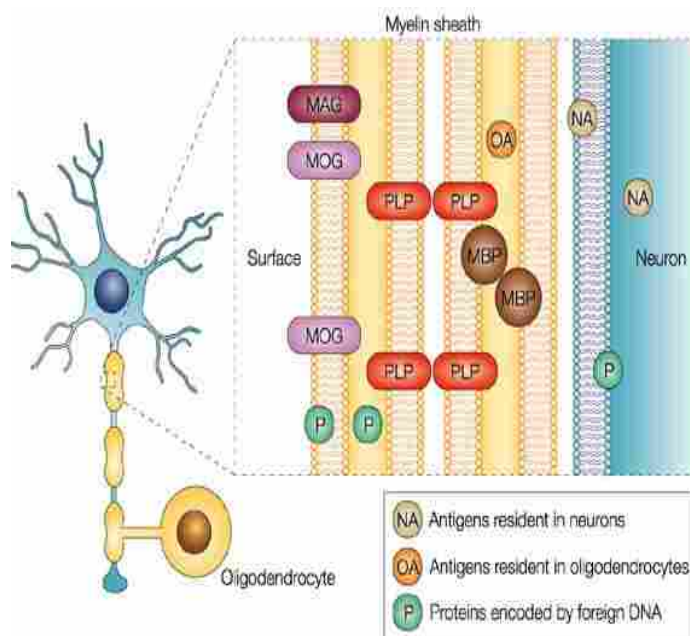
**Figure 1.1** - Myelinating glial cells, oligodendrocytes in the CNS or Schwann cells in the PNS, form the myelin sheath. Oligodendrocytes can myelinate different axons and several internodes per axon, whereas Schwann cells myelinate a single internode in a single axon<sup>5</sup>. Reprinted with the permission from Nature Publishing Group (Poliak, S., Peles, E. “The Local Differentiation of Myelinated Axons at Nodes of Ranvier” Nature Reviews Neuroscience **4** (2003): 968-980)

Glial cells are responsible for producing myelin sheath around axons of both the CNS and the PNS. Oligodendrocytes are responsible for producing myelin sheath in CNS; membrane sheets from single oligodendrocytes are capable of wrapping repeatedly around several separate axons. In the PNS, myelin is formed by Schwann cells which are capable of myelinating a single internode on a single axon (Fig. 1.1)<sup>4, 6</sup>. Within the myelin membrane, there are various types of membrane microdomains that may include caveolae, tetraspanin-enriched microdomains, lipid rafts, as well as the radial component/tight junction<sup>4</sup>.

### 1.2.1 Composition of Myelin

Myelin may be made by the two different cell types and varies in chemical composition and configuration, but performs the same insulating function. Myelin is composed of about 40% water; the dry mass of myelin is about 70-85% lipids and about 15-30% proteins. The major proteins present in myelin are the proteolipid protein (PLP) one of the most abundant proteins found in myelin and is thought to play a major role in

the differentiation of oligodendrocytes and the formation and maintenance of compact myelin membrane. Myelin basic protein (MBP) is the second most abundant protein found in the myelin sheath. MBP functions for proper myelin formation and compaction of myelin sheath. It exists as several different isoforms in myelin sheath<sup>7</sup>. These are the 21.5, 20.2, 18.5, 17.2, and 14kDa isoforms in the mouse, 21.5, 20.2, 18.5, 17.2, and 16kDa isoforms in the bovine and 21.5, 20.2, 18.5, and 17.2kDa isoforms in humans<sup>7,8</sup>. In human and bovine CNS, the 18.5kDa isoform of MBP is the major form, whereas the 14kDa isoform is the major isoform in adult mice and rat. Some of the other proteins present are the myelin oligodendrocyte glycoprotein (MOG), myelin associated glycoprotein (MAG), and 2, 3-cyclic nucleotide 3-phosphodiesterase (CNP).



**Figure 1.2** - Schematic diagram represents multilamellar myelin sheath with different proteins; associated myelin associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), and myelin basic protein (MBP)<sup>9</sup>. Reprinted with permission from Nature Publishing Group (Hemmer, B., Archelos, J., Hartung, H. “New concepts in the immunopathogenesis of multiple sclerosis” Nature Reviews Neuroscience **3** (2002): 291-301)

Protein organization and protein components of myelin can be seen in Fig. 1.2 and Table 1.1. Most myelin proteins are generally localized to a specific architectural subdomain of myelin; MBP and PLP are in the compact myelin, with MAG localized mainly in the innermost layers of myelin sheath, and MOG localized mainly on outermost

layers of myelin sheath. Although MAG is localized in innermost layers it is also found on outermost layers of myelin sheath. Table 1 compares the abundance of known myelin proteins determined using LC-MS/MS (ultra performance liquid chromatography separation coupled to detection with a quadrupole/time-of-flight mass spectrometer) with the literature values previously estimated from one-dimensional (1-D) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) <sup>10</sup>. The variation in the literature values for the composition of these proteins may be due to the different experimental approach. However, the sensitivity of LC-MS/MS allows for the detection of many low-abundant proteins.

**Table 1.1** - Comparison of composition of myelin proteins from previous estimations using SDS-PAGE and a recent LC-MS/MS analysis. Data compiled from reference 10.

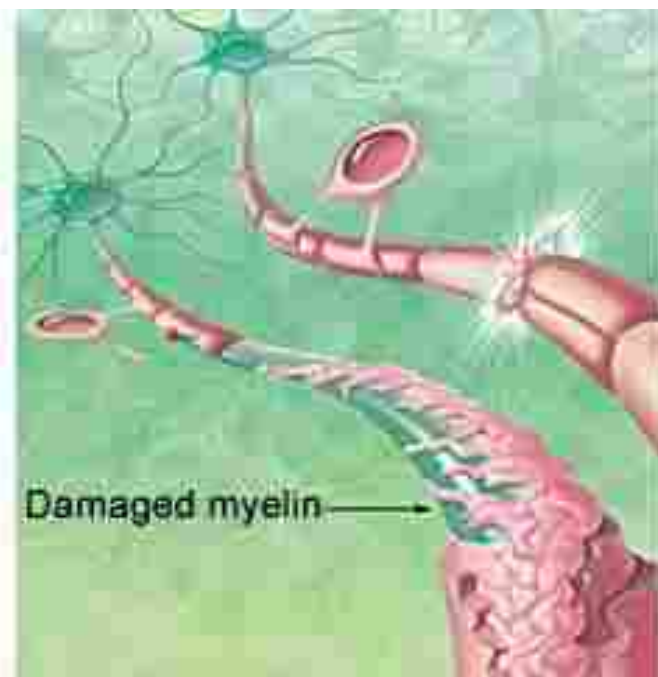
<b>Relative abundance of myelin proteins (%)</b>		
Protein	SDS-PAGE	LC-MS/MS
PLP	30-45	17
MBP	22-35	8
CNP	4-15	4
MOG	ND	1
MAG	1-4	1
SIRT2	ND	1
OSP	ND	1
Others	5-25	67

SIRT2-Sirtuin-2; OSP-Oligodendrocyte specific protein; ND-Not detected

### **1.3 Multiple Sclerosis**

Multiple sclerosis (“MS”) is the most common cause of non-traumatic disability in young adults, affecting approximately one million worldwide <sup>11</sup>. It is a chronic disease of the CNS, leading to major neurological disability in most patients over a course of several years or decades. “MS” is an inflammatory disease in which the fatty myelin sheaths around the axons of the brain and spinal cord are damaged, leading to demyelination and scarring as well as a broad spectrum of signs and symptoms such as

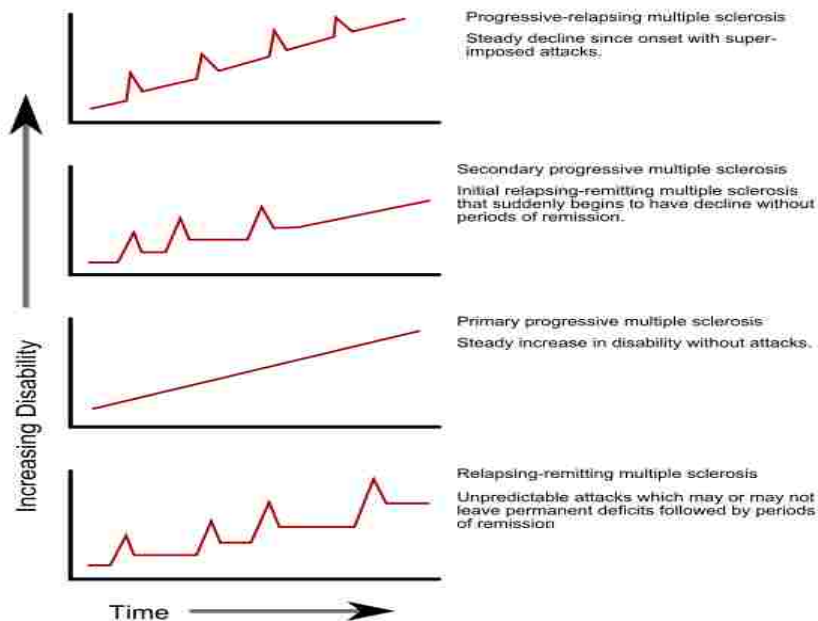
loss of sensitivity or numbness, muscle weakness, difficulties with coordination and balance, problems in speech or swallowing, and visual problems (Fig. 1.3) <sup>12</sup>. As discussed earlier, nerve cells communicate by sending electrical signals (called action potentials) down long fibers called axons, which are wrapped in an insulating substance called myelin. In “MS”, the body recognizes its own immune system as foreign body and starts attacking and damaging myelin. When myelin is lost, the electrical signal diminishes and the axons can no longer effectively conduct signals <sup>13</sup>.



**Figure1.3** - The effects of multiple sclerosis (“MS”) on myelin membrane. In normal nerves myelin is continuously wrapped forming a myelin sheath around axon. However, “MS” myelin is degraded and the axon gets exposed, reducing the efficiency of transmitting electrochemical signals, resulting in number of physiological symptoms. Figure adopted from <http://3.bp.blogspot.com/z5KjGM5bczU/Rjg0c7Jh3rI/AAAAAAAAAAdk/WDZgmHI-7QY/s320/myelin.jpg>

Although much is known about the steps involved in the disease process, the cause remains unknown. Progression of “MS” is divided into several subtypes using the past course of the disease in an attempt to predict the future course. They are important not only for prognosis but also for therapeutic decisions (Fig. 1.4). The four major subtypes are: 1) Relapsing-remitting “MS” (RR“MS”) is characterized by unpredictable relapses followed by periods of months to years of remission with no new signs of

disease activity; 2) Secondary progressive “MS”, accounts 65% of those with an initial RR“MS”, begin to have progressive neurologic decline between acute attacks without any definite periods of remission. Individuals will have an occasional relapses and minor remission; 3) Primary progressive subtype (accounts for 10-15% of individuals) is characterized by progression of disability without or only occasional and minor, remissions and improvements; 4) Progressive relapsing “MS” describes those who have a steady neurologic decline but also suffer clear superimposed attacks <sup>13</sup>.



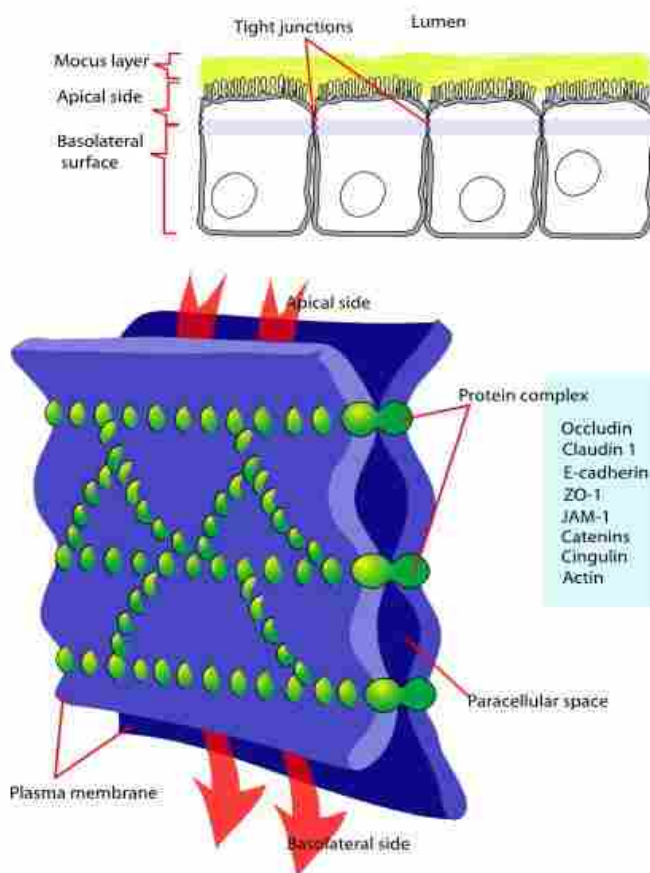
**Figure 1.4** - Clinical classification of multiple sclerosis. Figure adopted from [http://en.wikipedia.org/wiki/Multiple\\_sclerosis](http://en.wikipedia.org/wiki/Multiple_sclerosis)

## 1.4 Cellular Tight Junctions

In epithelial cells the junctional complex is located at the apical part of the membrane and consists of three components: tight junctions, adherens junctions, and desmosomes <sup>14</sup>. Under electron microscopy of an ultrathin section, a tight junction appears as fusions with the plasma membrane of the adjacent cells with no intercellular space, whereas the apposing membranes are 15-20nm apart in adherens junctions and

desmosomes. Immediately below the tight junction is the intermediate junction containing filamentous material with an intercellular space narrowed to 25-30nm.

The ability to form a seal between the cells is provided by epithelia which act as diffusion barriers between compartments with different compositions, good electrical and chemical gradients. Otherwise these gradients would disperse through intercellular space. This seal should be located between the lumen and the intercellular space so exchange of nutrients occurs between the adjacent cells. Later studies of both epithelia and endothelia revealed that the tight junction consists of a distinctive reticular (or meshwork of fibrils) embedded in the plane of the membrane (Fig. 1.5) <sup>15</sup>.



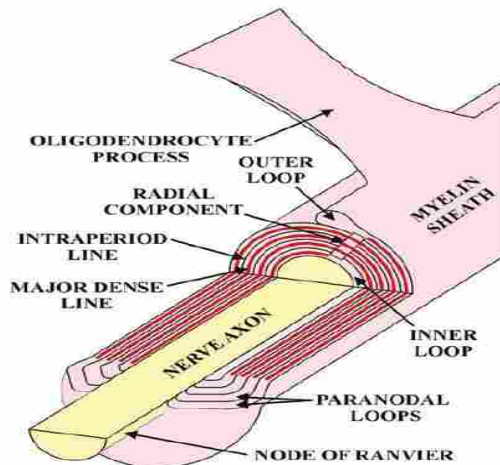
**Figure 1.5** – Schematic representing tight junction's array of proteins. Epithelial cells form tight junctions that prevent substance passing from lumen. Two adjacent cells form tight junctions. Figure adopted from [http://upload.wikimedia.org/wikipedia/commons/6/6d/Cellular\\_tight\\_junction\\_en.svg](http://upload.wikimedia.org/wikipedia/commons/6/6d/Cellular_tight_junction_en.svg)



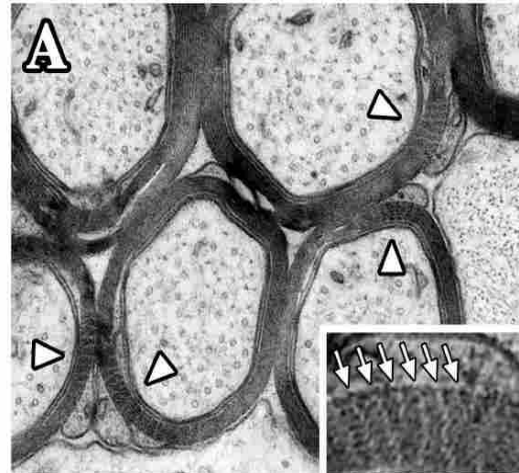
Tight junctions appear as flat meshwork filaments grouped in a narrow belt which surrounds the cell on the basolateral side at the outermost limit of the intercellular space. These junctions are adhesive intercellular structures that form a barrier to restrict paracellular transport of solutes and diffusion of membrane proteins. This structure of tight junctions suggests that each strand of tight junction behaves as resistive element. Moreover, the larger the number of strands, the higher is the electrical resistance<sup>15</sup>. Each strand is formed from a row of transmembrane proteins embedded in the plasma membrane (Fig. 1.5). The tight junctions consist of several integral membrane proteins; zonula occludens 1 (ZO-1), occludin, claudins, and junctional adhesion molecules<sup>16</sup>. Occludin has four transmembrane domains, two extracellular loops, and three cytoplasmic domains and binds directly to the ZO-1 and claudin<sup>16</sup>. Schwann cells form compacted and non-compacted multilamellar myelin sheath around the axon. In myelinating Schwann cells several types of junctional complexes such as tight, gap, and adherens junctions, can be found. These junctions are typically formed between epithelial cells but in myelinating Schwann cells they are formed between membrane lamellae of the same cell and are called autotypic junctions.

#### **1.4.1 Tight Junctions/Radial Component of CNS Myelin**

The first report of a radial structure was made by Peters in 1961<sup>2</sup>. In CNS myelin, tight junctions are structural units of the radial component. As seen in Fig. 1.6, transverse sections of internodal CNS myelin, the radial component consists of particular linear strands that run parallel to the axis of nerve fiber and radially through myelin sheath. Fig. 1.7 shows electron micrograph of optic nerve and the arrow head points shows multiple zigzag lines of radial component across compact myelin.



**Figure 1.6** - Transverse sections of internodal CNS myelin sheath <sup>4</sup>. Reprinted with permission from Springer (DeBruin, L., Harauz, G. “White Matter Rafting – Membrane Microdomains in Myelin” *Neurochem Res* **32** (2007): 213-228)



**Figure 1.7** - Electron micrograph of CNS myelin. Arrowheads point to the radial components, which appear as multiple white zigzag lines across the compact myelin. The inset shows radial component at a threefold higher magnification <sup>17</sup>.

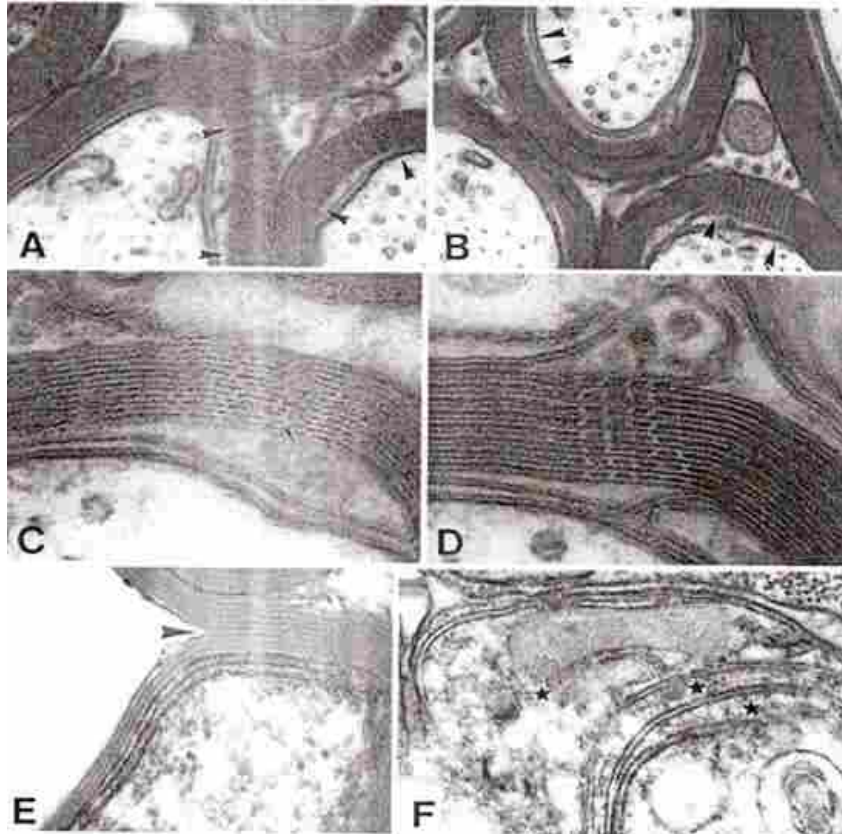
#### 1.4.2 Composition of Tight Junctions/Radial Component

Several integral membrane proteins such as occludins, junctional adhesion molecules, and claudins are present in tight junctions. The radial component can be isolated from CNS myelin with 0.5% triton-X 100 (TX-100) detergent treatment at room temperature <sup>20</sup>. The detergent insoluble fraction is found to be highly enriched in 21.5 and 17.22kDa isoforms of MBP, CNP, actin, and tubulin <sup>20</sup>. Later studies also identified OSP and myelin-associated oligodendrocyte basic protein (MOBP) as proteins that also are associated with radial component <sup>19</sup>. OSP is a member of the claudin family of tight junction-associated proteins, and is also termed as claudin-11. It is the third most abundant protein in CNS myelin contributing ~1-7% of total myelin <sup>2</sup>. OSP is involved in the membrane interaction at tight junctions and modulates the proliferation and migration of oligodendrocytes. It also acts as an auto-antigen in the development of autoimmune

demyelination. Occludin and claudin are the only two integral membrane proteins of tight junctions that increase cell-cell adhesion <sup>18</sup>.

### **1.5 Tight Junctions Complications During Diseases**

In past research, analysis has been done by comparing wild type mice and specific myelin protein-deficient mice. It has been shown how tight junctions are affected in the absence of MOBP. MOBP shares several similar characteristics with MBP, in terms of regional distribution and function; both MBP and MOBP are small basic proteins distributed throughout compact myelin. MOBP causes experimental allergic encephalomyelitis in experimental animals and is associated with multiple sclerosis <sup>19</sup>. Several isoforms of MOBP exist by differential splicing. Isoforms contain clusters of positively charged residues at the carboxy terminus that are unique to each isoform. These positively charged amino acids interact with phospholipids in the inner surface of the plasma membrane and become concentrated to the cytoplasmic faces of the myelin sheath which leads to the formation of major dense lines together with MBP. One isoform of MOBP is expressed in the embryo prior to myelination along with MBP and PLP. Thus to explore further the role of MOBP, MOBP-deficient mice were compared with wild type mice.



**Figure 1.8** - Electron micrographs of transverse sections of the optic nerve from wild type (A, C, E) and MOBP-deficient mice (B, D, F). The radial components are shown by arrowheads in A & B. At higher magnification C & D represents electron lucent linear structures. E & F are pictures where mice were exposed to hexachlorophene<sup>19</sup>. Reprinted with permission from Springer (Yoshikawa, H. "Myelin Associated oligodendrocytic

Basic Protein Modulates the Arrangement of Radial Growth of the Axon and the Radial Component of Myelin" *Med Electron Microsc* **34** (2001): 160-164)

Fig. 1.8 represents electron micrographs of transverse sections of optic nerve from wild type (A, C, E) and MOBP-deficient mice (B, D, F). The radial components in both wild type (A) and deficient mice (B) are indicated by arrowheads. In wild type mice, radial components are arrayed in an oblique and zigzag direction, whereas in deficient mice, radial components run straight and parallel to each other with narrower spaces between them. Also, electron lucent linear structures (ELLS) run obliquely across the major dense lines in the wild type mice while it is lacking in deficient mice. To examine the possible role of MOBP further, mice were subjected to the known dysmyelinating agent hexachlorophene (HCP). In wild type mice, on HCP exposure, myelin sheath splits at the intraperiod lines causing myelin vacuolation, whereas in MOBP deficient mice the

splitting of the myelin sheath is more frequent and there was widening of the major dense lines, which was not seen in wild type mice. Thus, MOBP is not only essential for compaction of myelin, but also plays an important role in limiting the extent of breakdown at the major dense lines in a certain pathological condition.

## **1.6 Rational and Objectives of the Research**

This research on radial component was inspired by previous research of Kirschner and colleagues<sup>20</sup> who isolated radial component by successive extractions using TX-100 detergent. It was found that radial component preserved its structure when treated with TX-100. The well-preserved radial component fraction was examined by SDS-PAGE, immunoblotting, and thin layer chromatography (TLC), and was found to be enriched in 21.5kDa and 17kDa isoforms of MBP, and later CNP, tubulin, actin, OSP, and MOBP were also identified<sup>20</sup>. All the components were not known and the functional role of radial component was not fully understood.

The main objective of this study was to further characterize the radial component as a distinct microdomain of the myelin membrane.

- The first objective of this study was to develop an effective isolation procedure for the radial component of CNS myelin.
- The second objective was to conduct an extensive proteomic analysis of the radial component.
- The third objective was to characterize/measure the expression level of key microdomain proteins in development of radial component.

Further, in this thesis Chapter 2 describes the methodology and bioanalytical techniques utilized. Chapter 3 describes about extensive proteomic analysis of the radial

component. In Chapter 4 some major and unique proteins are confirmed by Western blotting and multiplex fluorescent Western blotting and Chapter 5 provides an overall conclusion and examines future work.

### **1.7 Significance of the Research**

Myelin is not just the insulator of the axon. Myelin membrane microdomains are dynamic and have specific functional roles in myelinogenesis and in maintaining the integrity of the CNS white matter. Demyelination and dysmyelination have been implicated in a wide range of diseases and disorders. In this study, radial component from bovine and mouse myelin was extracted using differential detergent extraction. The fraction of radial component was examined by different proteomic techniques. This study indicates that radial component of myelin can be isolated successfully and proteomic studies reveal crucial information about high-abundant cytoskeletal and transport proteins and low-abundant signalling proteins. This information will aid in deciphering the roles of various proteins of the radial component in health and disease.

## **Chapter 2 – Methodology and Bioanalytical Techniques**

### **2.1 Isolation of Myelin**

Myelin is present throughout the nervous system, but it is concentrated in white matter of brain, spinal cord, and peripheral nerves. The extraction of lipid-rich myelin from tissue can be difficult; however, it is possible to isolate myelin in high yields and purity by conventional techniques of subcellular fractionation.

The isolation procedures fall into two groups, density gradient centrifugation and differential centrifugation. Upon homogenizing brain nerve tissue in an isotonic sucrose solution of low ionic strength, myelin peels off the axon and forms loose vesicles<sup>6</sup>. Because of their high lipid to protein ratio, myelin vesicles have the lowest buoyant density of any membrane fraction of the nervous system which can be separated by discontinuous gradient centrifugation containing sucrose solutions from 10.5- 30.5% (w/v). Myelin being less dense than 30% sucrose will form a band above that density during centrifugation. Larger cell particles such as nuclei, mitochondria, and synaptosomes will migrate through the dense sucrose layer to form a pellet while myelin will float on surface. Crude myelin is collected from the top of the layer, treated with water and centrifuged twice at low speeds to remove any contamination in supernatant. This method of isolation has proven to give pure myelin<sup>6,21</sup>.

### **2.2 Differential Detergent Solubilisation**

Differential extraction and precipitation of proteins is the first step in isolation protocols for membrane proteins. For membrane proteins which are usually insoluble in an aqueous buffer, detergents are generally added to the buffer for their extraction and purification. Detergents are compounds characterized by their amphiphilic structure (both

hydrophobic and hydrophilic). The tail of the detergent molecule is hydrophobic, usually consisting of a linear or branched hydrocarbon, whereas the hydrophilic head may have diverse chemical structures. Detergents are of three types: ionic, non-ionic, and zwitterionic. Ionic detergents contain head groups with either positive charges (cationic) or negative charges (anionic). However, ionic detergents denature proteins are not often included in the extraction since they can fully denature the proteins. Sodium dodecyl sulfate (SDS) is example of anionic detergent, and when in aqueous solution, forms globular micelles composed of 70-80 molecules with the dodecyl hydrocarbon in the core and the sulfate headgroups in the hydrophilic shell <sup>22</sup>.

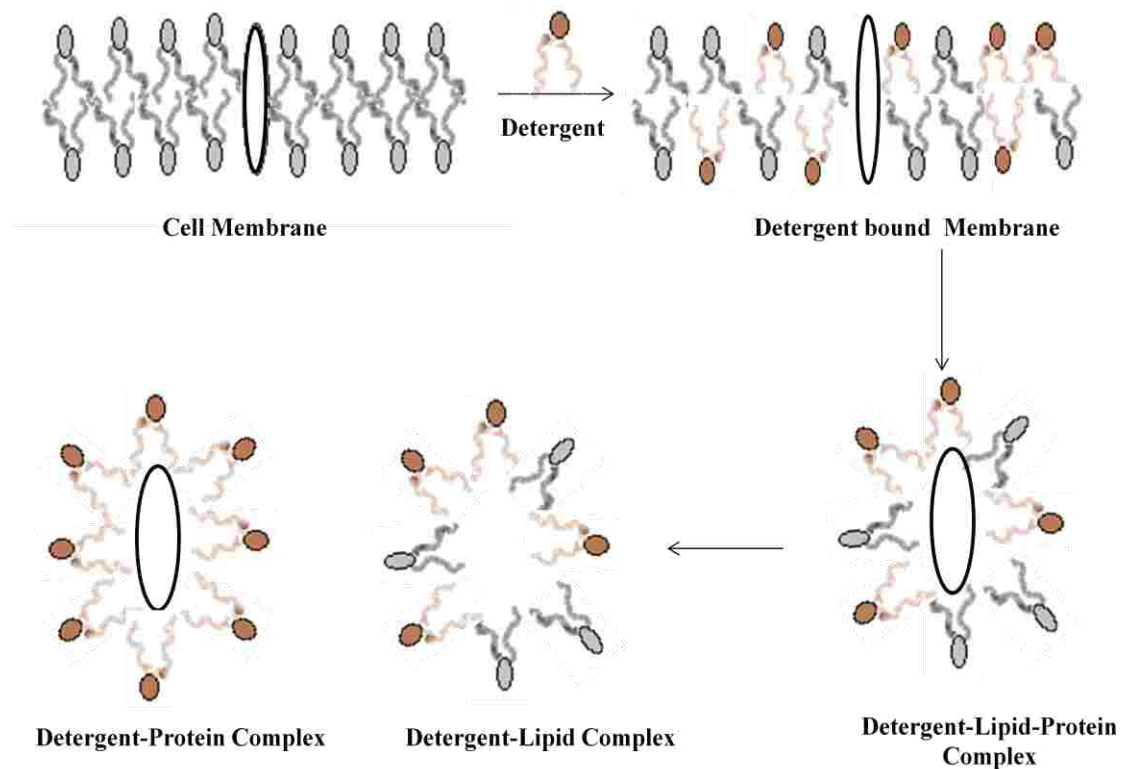
Non-ionic detergents such as TX-100 and Tween-20 have uncharged hydrophilic head groups and have a poly-oxyethylene polar region. Due to less denaturing properties than ionic detergents, non-ionic detergents are used to isolate functional proteins.

Zwitterionic detergents contain head groups with both positive and negative charges. These detergents are more effective than non-ionic detergents, as they disrupt lipid-lipid, protein-lipid interactions and prevent protein-protein interactions while causing less protein denaturation than ionic detergents. CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate) is the most commonly used zwitterionic detergent.

Detergent extraction of a membrane protein occurs as follows: 1) binding of detergent to the membrane and initiation of lysis; 2) solubilisation of the membrane in the form of detergent-lipid protein complex; and 3) further solubilisation of the detergent-lipid-protein complexes to give detergent-protein and detergent-lipid complexes (Fig. 2.1). The choice of detergent for the solubilisation of a membrane largely depends on the



physical properties of the membrane proteins. It is important to consider how detergent may affect native structures or activities of membrane proteins. Detergents that interfere with assays and are difficult to remove from the extract should not be considered.



**Figure 2.1** - Schematic representation of membrane protein solubilisation with detergents. Figure modified from Ahmed, H. Principles and Reactions of Protein Extraction, Purification, and Characterization. New York: CRC Press, 2005

Formation of micelles is the important property of detergents (Fig. 2.1). As membrane proteins become solubilised, they form mixed micelles with detergents, which prevents the hydrophobic domain of the protein from contacting the aqueous buffer. In order to choose an appropriate detergent, the most important consideration is the non-denaturing property. Therefore, non-ionic and zwitterionic detergents are often used to solubilise membrane proteins.

### **2.2.1 Lipid Raft Isolation**

Lipid rafts are microdomains of the plasma membrane that contain high concentrations of cholesterol and glycosphingolipids. They exist as distinct liquid-ordered regions of the membrane that are not solubilised by extraction with non-ionic detergents at 4°C. Lipid rafts have a low density and are insoluble in cold 1% TX-100<sup>23</sup>. Previously lipid rafts were isolated by removing cells into buffer containing 1% TX-100 followed by flotation in a 5% to 30% linear sucrose density gradient where they distribute in the top few fractions of the gradient because of low density<sup>23</sup>. Recently, a wide variety of detergents other than TX-100 have been used to isolate lipid rafts. DeBruin *et al.* found that lipid rafts can be isolated effectively by treating myelin with 1.5% CHAPS<sup>24</sup>.

### **2.3 Electrophoretic Separation**

Electrophoresis is an analytical tool that allows the examination of differential movement of charged molecules in an electric field. It is a method used to separate proteins by charge and/or size. Molecules are allowed to move through the gel made of polyacrylamide using an electric field. The sample to be analyzed is applied to the medium in a well in the gel and an electric current is applied by placing gel in an electrophoresis chamber. More complex mixtures are separated using two dimensional (2-D) gel electrophoresis.

#### **2.3.1 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis**

SDS-PAGE is a technique widely used in biochemistry, forensics, genetics and molecular biology to separate proteins according to their electrophoretic mobility (a function of length of polypeptide chain or molecular weight). The molecular weights of proteins may be estimated if they are subjected to electrophoresis in the presence of SDS

detergent, and a disulfide bond reducing agent, for example mercaptoethanol or dithiothreitol (DTT). This method is called denaturing electrophoresis. Nondenaturing (or native PAGE) is an electrophoretic technique in which protein analyzed still retains its biological activity or fold (conformation). The separation of proteins under these conditions where they maintain their native conformation is influenced by both charge and size.

When protein molecules are treated with SDS, the detergent disrupts the secondary, tertiary, and quaternary structure to produce linear polypeptide chains coated with negatively charged SDS molecules. The presence of mercaptoethanol assists in protein denaturation by reducing all disulfide bonds. Approximately one SDS molecule binds per two amino acids. The SDS molecules mask the surface charge of the proteins and create a net negative charge resulting from the sulfate groups on the SDS molecule. Therefore, polypeptide chains of a constant charge/size ratio is produced, and separation can be achieved only on the basis of size. Low molecular weight proteins travel faster in the gel, and proteins of high molecular weight move slower in the gel. Proteins molecular weights can be estimated by running appropriate standard proteins of known molecular weights on the same gel.

### **2.3.2 Solution-Phase Isoelectric Focusing**

Solution-phase isoelectric focusing (IEF) is an electrophoretic method in which amphoteric molecules are separated on the basis of their isoelectric point (pI). At physiological pH, lysine, arginine, and histidine residues in a protein are positively charged, while aspartic acid and glutamic acid carry a negative charge. Proteins carry either positive, negative, or zero net charge depending on the pH of their surroundings.

The pI is the pH at which protein has no overall net charge. There are various IEF systems available with different design. IEF fractionators supplied by Invitrogen™ consist of a series of sample chambers connected to each other with thin membranes called disks in between. The tube can be assembled with various combinations of disks and spacers to cover different pH range (standard pH range 3-10, extended pH range 3-12, or narrow pH range). The disk inserted between sample chambers creates chambers that will allow separation of proteins in specific pH range (inserting disks of pH 3 and pH 4.6 creates a chamber for fractionating proteins between pH 3-4.6). The protein sample is loaded into multiple sample chambers separated by disks and spacers. The one used for this study is MicroRotofor™ cell from BioRad laboratories. It consists of focusing chamber attached to the electrode assembly at the either end. The focusing chamber is designed to hold 2.5ml sample for fractionation. It is divided into 10 sections with nine evenly spaced monofilament polyester screens. Two sets of 10 holes are provided into opposite sides of the focusing chamber; one set is used for sample loading and the other for harvesting. Ion exchange membranes are inserted between the focusing chamber and electrode assemblies to separate the electrolytes from the sample. A pH gradient is established by loading acid at the anode and a base at the cathode. Establishment of a stable pH gradient is important in IEF which can be done by adding carrier ampholytes in resuspension buffer which partition themselves into smooth pH gradients and move from anode to the cathode. The protein sample that is solubilised in an IEF buffer with specific carrier ampholytes, is loaded into the focusing chamber. On applying electric current to the system, proteins start migrating based on their characteristic pI. Proteins carry a negative charge at pH's above their pI, and a positive charge at pH's below their pI.

Proteins will move toward the electrode with the opposite charge. A protein will either gain or lose protons during migration through a pH gradient. This decreases net charge and mobility of the protein, and when the pI is reached the protein will stop moving. The sample is harvested by positioning the focusing assembly in the harvesting chamber and applying a vacuum to the harvesting chamber.

### **2.3.3 Two Dimensional Gel Electrophoresis with Immobilized pH Gradients**

2-D gel electrophoresis is a powerful and widely used method for the analysis of complex protein mixtures extracted from cells, tissues, or other biological samples. This technique separates proteins according to two independent properties in two discrete steps: the first-dimension step IEF, works on the same principle as discussed in solution phase IEF, proteins are separated according to their pI; the second dimension step, SDS-PAGE, separates proteins according to their molecular weights. The 2-D process begins with sample preparation followed by immobilized pH gradient (IPG) strip (polyacrylamide gel on a plastic support film with varying pH ranges) rehydration. IPG strips are provided dry and must be rehydrated with the appropriate additives prior to IEF. The IPG strip is rehydrated overnight by placing in a strip holder containing sample resuspended in IEF rehydration buffer. Then IEF is performed. Next, for the second-dimension separation for the strip is equilibrated in two-step SDS-containing buffer. Following equilibration, the strip is placed horizontally on a denaturing SDS gel and electrophoresis is run for further separation of proteins. The final steps are visualization and analysis of the resultant two-dimensional spots. In brief, the experimental sequence for 2-D electrophoresis is: 1) sample preparation 2) IPG strip rehydration 3) IEF 4) IPG strip equilibration 5) SDS-PAGE 6) visualization 7) analysis.

## 2.4 Proteomic Analysis

Proteomics is the study of protein expression and function of multiprotein systems, in which focus is mostly on multiple, distinct proteins in their roles as part of a larger system. The readily available experimental tools for protein identification and characterization by mass spectrometry (MS) based methods have made a significant impact on proteomics. After running 1-D or 2-D SDS-PAGE, further steps are needed to obtain some useful information from the results. Commonly, this can be done by Western blotting where a specific antibody to particular proteins or to their modified forms can be used. More recently MS has been employed for rapid identification of proteins in sample mixtures by partial sequence analysis with the aid of database matching tools. There are different proteomic approaches to protein identification.

Bottom-up proteomics is an approach in which proteins are proteolytically digested into peptides prior to mass analysis, and the peptide masses and sequences are used to identify corresponding proteins<sup>25</sup>. Top-down proteomic uses masses of intact proteins and their fragments for successful identifications. Fragmentation reactions such as electron capture dissociation and electron transfer dissociation are also used because they yield more complete backbone sequencing and post translational modifications<sup>25</sup>. Shot-gun proteomics is the method of identifying proteins in complex mixtures by using multidimensional separation. Multidimensional protein identification technology (MudPIT) is a type of shot-gun proteomics in which the separation uses strong cation exchange and reversed phase chromatography directly coupled to tandem MS.

Protein identification by MS can be performed using sequence-specific peptide fragmentation or peptide mass fingerprinting (PMF). In PMF the unknown protein is first

cleaved into smaller peptides with proteases, mostly trypsin. For protein identification, the experimentally obtained masses are compared with the theoretical peptide masses of proteins stored in databases by means of mass search programs. The most commonly used technique is matrix-assisted laser desorption ionization-MS (MALDI-MS) <sup>26</sup>. The disadvantage of this technique is that the protein sequence has to be present in the database of interest. It fails to identify protein mixtures and to identify low molecular mass proteins and protein fragments due to the small number of detectable peptides. Complex mixtures of proteins require the additional use of tandem MS. The peptides are fragmented further with a collision in the quadrupole/time-of-flight or ion trap and the set of fragment masses gives the sequence for the peptide and can be used to search sequence databases for similar peptides. Complex protein mixtures can examine directly or the proteins or peptides can be separated by chromatographic or electrophoretic techniques including 2-D gel electrophoresis.

#### **2.4.1 Trypsin Digestion**

A commonly used approach for digestion of proteins separated by 1-D or 2-D SDS-PAGE is “In-Gel Digestion”. In order to obtain useful information such as specific protein identification after running and staining gels, the gel bands or protein spots of interest from 2-D gel electrophoresis are excised, destained, and treated with trypsin proteases. The gel pieces are destained before trypsin digestion as most of the stains, including coomassie brilliant blue and silver stain interfere in the digestion process. Staining protocols that use aldehyde fixatives or prolonged exposure to acids tend to fix the proteins in gels, thus making the proteins difficult to digest and elute from the gels. Another approach for protein digestion is “In-Solution Digestion”. The advantage of this

technique is that digestion is performed on whole proteomes without losing any protein but the disadvantage is down-stream analysis does not detect low-abundant proteins very well.

There are many enzyme proteases available such as chymotrypsin, endoproteinase Glu-C, Lys-C, Asp-N but trypsin is the most widely used protease in proteomic analysis. The proteins are digested with trypsin, which cleaves after lysine and arginine residues, unless either of these is followed by a proline residue. The spacing of lysine and arginine residues in many proteins is such that the length of many of the resulting peptides is compatible with MS analysis. In general, a 50kDa protein will give about 30 tryptic peptides. Due to the specificity of this protease, this results in basic charges at both the amino-terminal and carboxy-terminal ends of most peptides. Thus trypsin penetrates the gel matrix and digests proteins to peptides. Peptides are further extracted from gel pieces by sonicating, shrinking, and hydrating with acetonitrile and formic acid. Samples are completely dried under vacuum centrifuge, and prior to its analysis samples are resuspended in formic acid solution which helps in ionization of samples.

#### **2.4.2 Mass Spectrometric Analysis for Protein Identification**

Mass spectrometry is a standard tool in analysis of biological molecules and biological processes. It is an analytical technique that measures the mass-to-charge ratio of charged particles. Neutral molecules are ionized and their positively charged ion products are directed through an electric and/or magnetic field where they are separated on the basis of their mass-to-charge ratio. Measurement of the molecular mass of these species provides the molecular mass of the original molecule. Some molecular ions are unstable and disintegrate to produce fragment ions. These fragmentation processes are



useful in structural elucidation of smaller molecules. There are several programs available that allow the databases to be searched directly with the peptide fragment data. Chemical modifications such as phosphorylation of hydroxyl groups in serine and tyrosine residues, or addition of carbohydrate residues to the hydroxyl groups of serine, threonine, or to the amide nitrogen of asparagine residues can easily be detected by MS as the addition of phosphoryl group adds about 80Da, whereas addition of glucose residue adds about 180Da to the molecular mass of a protein.

Mass spectrometers have three essential parts: 1) source, which produces ions from the sample, 2) mass analyzer, which resolves ions based on their mass/charge ( $m/z$ ) ratio, and 3) detector, which detects the ions resolved by the mass analyzer. The mass spectrometry instruments that are used for most proteomics studies are matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) and electrospray ionization mass spectrometry (ESI-MS). In MALDI-TOF the sample is mixed with a chemical matrix, which contains organic molecules with a desirable chromophore that absorbs light at the specific wavelength. When the light is targeted from the source, the chemicals in the matrix absorb photons and become electronically excited. Excess energy is then transferred to the peptides in the sample, and they are ejected from the surface into the gas phase. The TOF analyzer measures the time it takes for the ions to fly from one end of the analyzer to the other and strike the detector.

ESI refers to the process by which ions are produced in the source of the instrument. ESI is commonly coupled to a quadrupole mass analyzer. The quadrupole mass analyzer consists of four circular metal rods arranged parallel to each other. It is responsible for filtering sample ions based on their  $m/z$  ratio. Direct current and

radiofrequency voltages are applied to the rods. Depending on the voltage applied to the rods, only ions of certain  $m/z$  ratio will reach the detector. To determine the amino acid sequence of a peptide, tandem mass spectrometry is employed. Often the peptides are separated by high performance or ultra performance liquid chromatography just prior to tandem mass spectrometric analysis (i.e., LC/MS/MS).

Hybrid mass spectrometers combining two different types of mass analyzers are widely used to achieve high performance. The combination of linear ion trap with the orbitrap analyzer has proven to be a popular instrument. The hybrid trap-trap instrument or orbitrap analyzer alone is named as exactive. Orbitrap consists of an outer barrel shape electrode and a coaxial inner spindle shape electrode that form an electrostatic field. Orbitrap is an ion trap where moving ions are trapped in an electrostatic field <sup>27</sup>. In an orbitrap, ions are injected around the central spindle shaped electrode where the electrostatic field forces them to move in complex spiral patterns. The linear trap collects the ion population and passes them to an intermediate C-trap for injection and analysis in the orbitrap analyzer at high resolution. The linear ion trap fragments the trapped ions, and records the signal of a mass dependent scan at low resolution. The C-trap accumulates and stores the ions where high energy collision fragmentations can be completed <sup>27</sup>. The last part is orbitrap where products are analysed at high resolution and mass accuracy. Depending on the requirements for the analysis, analyzers can be used independently or in series.

#### **2.4.2.1 Multidimensional Protein Identification Technology**

Another approach, direct analysis of protein complexes using mass spectrometry has been done with multidimensional protein identification technology (MudPIT). MudPIT eliminates the bias step of 1-D and 2D-PAGE and detects peptides of all isoelectric points, molecular weights, including those that are integral and low-abundance proteins. MudPIT allows the on-line separation of highly complex peptide mixtures directly coupled with mass spectrometry. A Single column is used in MudPIT that consists of fused silica packed with two orthogonal chromatographic resins, a strong cation exchange resin in the first dimension and reversed-phase resin in the second dimension. Trypsin digested samples are bound first to strong cation exchange resin followed by reversed-phase material from which they are eluted directly into the MS by using water and acetonitrile. This separation reduces the sample complexity entering the mass spectrometer.

## **Chapter 3 – Proteomic Analysis of the Radial Component, a Highly Detergent Resistant Fraction of Myelin**

Proteomics is the study of the proteome. It is defined as systematic analysis and documentation of the proteins in biological samples. Proteomics helps in finding the overall distribution of proteins in cells, in identifying and characterizing individual proteins of interest, and lastly in elucidating their relationships and functional roles. Myelin is a biologically active multilamellar membrane that is formed by oligodendrocytes in CNS and ensheathes axons. The loss or damage of the myelin sheath results in neurological diseases such as multiple sclerosis. As discussed in Chapter 1, myelin has various membrane microdomains. To prevent diseases associated with myelin damage or myelin, it is essential to understand the mechanism of its biogenesis and maintenance, which requires the identification of all myelin proteins. High-resolution separation technologies are required to achieve comprehensive proteome coverage and to increase the detection of low-abundance proteins. This chapter focuses on the proteomics analysis of radial component myelin from bovine and murine brain tissue. Radial component was isolated by differential detergent extractions followed by separation by various electrophoretic approaches and MS-based protein identification (see Fig. 3.1).

### **3.1 Reagents and Chemicals**

The water used throughout was obtained from a Milli-Q water purification system (Millipore).

#### **3.1.1 Isolation of Myelin**

Sucrose, sodium chloride (NaCl), ethylene diamine tetraacetic acid (EDTA), sodium vanadate ( $\text{Na}_3\text{VO}_4$ ) and phenylmethanesulfonyl fluoride (PMSF) were purchased

from Sigma-Aldrich, protease inhibitor cocktail (PIC) and phosphatase inhibitor cocktail (PhosSTOP) from Roche Canada, and tris from Bio Basic Inc. Frozen mouse brains were obtained from Rockland Immunochemicals. Protein concentration of isolated myelin was determined using the bicinchoninic acid (BCA) assay kit purchased from Pierce, Thermo Scientific.

### **3.1.2 Detergents**

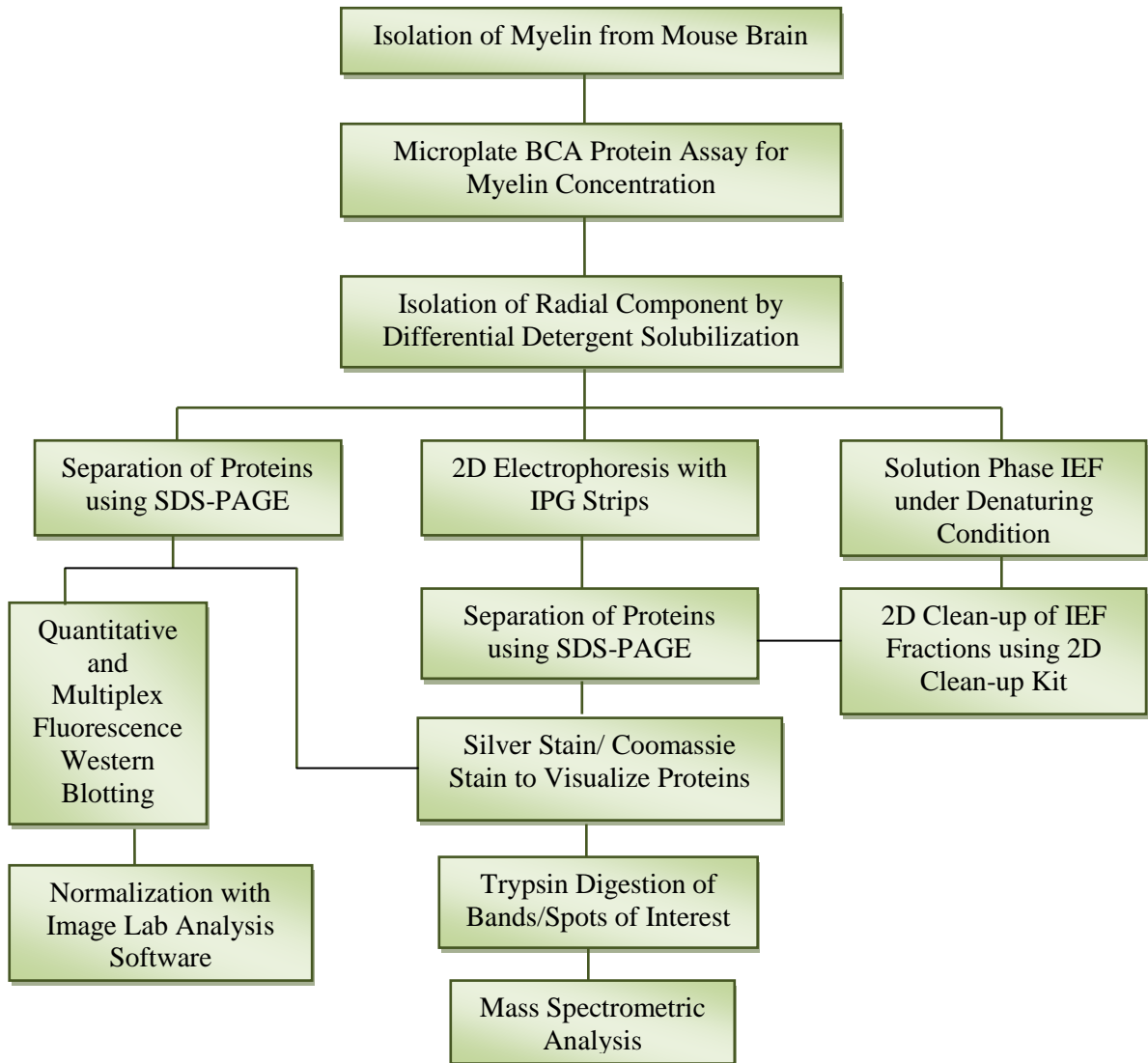
Detergents used in the extraction procedure were TX-100 purchased from Pierce, Thermo Scientific, CHAPS, and ASB-14 from Sigma-Aldrich, tween-20 from Bio-Rad and C<sub>12</sub>E<sub>9</sub> from Calbiochem.

### **3.1.3 SDS-PAGE and Staining Solutions**

Gels were cast with 30% acrylamide/ bis solution (37.5:1), and TEMED from Bio-Rad, ammonium persulfate from Sigma-Aldrich, and SDS from Bio Basic Inc. After running, SDS-PAGE gels were either silver stained or coomassie stained using sodium thiosulfate, silver nitrate, sodium carbonate, formaldehyde, acetic acid purchased from Sigma-Aldrich, methanol from Fisher Scientific, and Bio-Safe Coomassie G-250 stain from Bio-Rad.

### **3.1.4 Isoelectric Focusing**

IEF buffer was prepared with urea, thiourea, ASB-14, CHAPS, dithiothreitol (DTT) purchased from Sigma-Aldrich and TX-100 purchased from Pierce, Thermo Scientific, and carrier ampholytes of pH range (5-8, 7-9, and 9-11) purchased from Bio-Rad. IPG strips, 7cm length, pH 3-10 range were purchased from Bio-Rad.



**Figure 3.1** – Flow chart summarizing the isolation, separation technique, and characterization of radial component.

## 3.2 Experimental Procedure

### 3.2.1 Isolation of Myelin

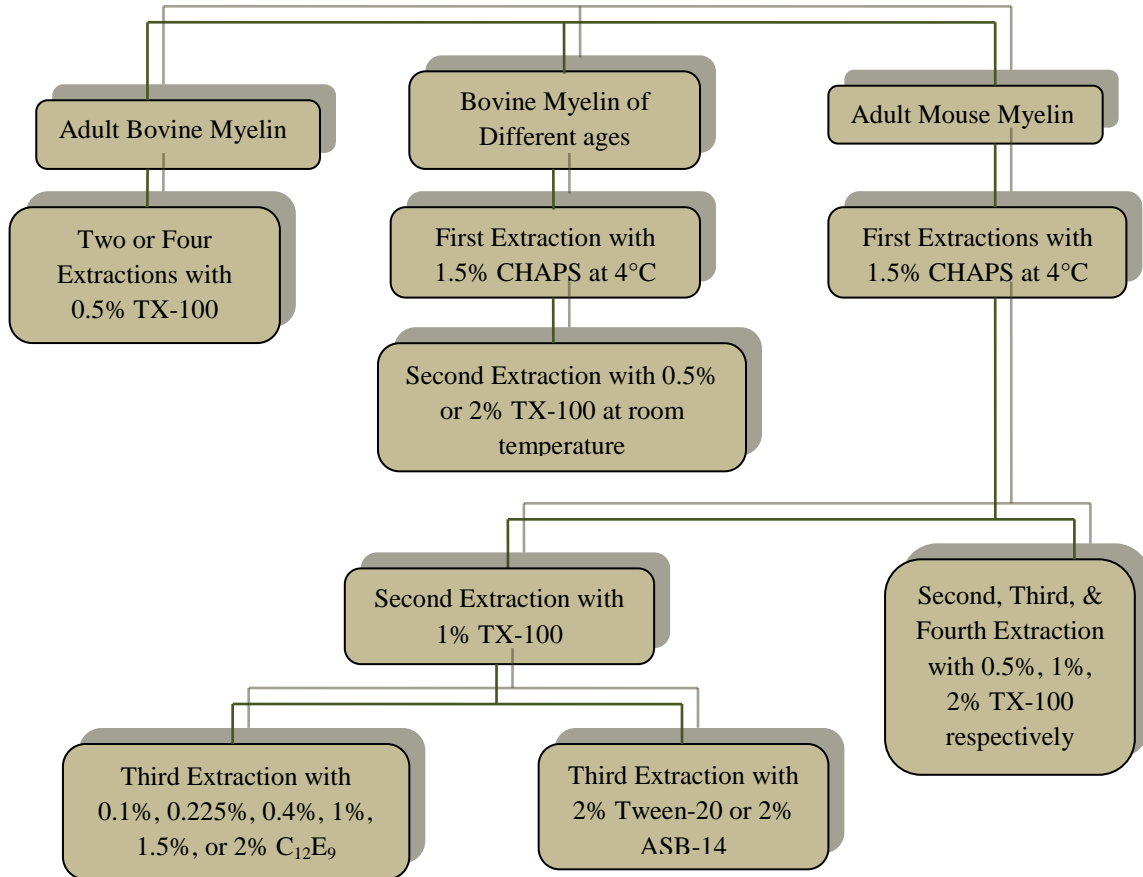
Myelin isolation is an important, first step without which experiments cannot proceed. Myelin was previously isolated from bovine brain tissue. Mouse myelin was isolated via sucrose cushions from tissue homogenates from whole frozen mouse brain samples using the method of Norton <sup>21</sup> (for complete procedure in detail, please see

Appendix 1). This method permits a high yield of myelin. In brief, the excised brain tissue was homogenized on ice in 10.5% sucrose in TNE (25mM tris buffer, pH 7.4; 150mM sodium chloride; 5mM EDTA) with a Dounce homogenizer and layered onto 30% sucrose in a 5ml ultracentrifuge tube. Myelin was separated from homogenate brain sample by repetitive steps of ultracentrifugation using a MLS-50 rotor at 35,000rpm in the Optima MAX Ultracentrifuge (Beckman Coulter). The isolated myelin vesicles were osmotically shocked with ice-cold water. The final myelin pellet was resuspended in myelin storage buffer containing protease and phosphatase inhibitors, aliquoted, and stored at -80°C until required. After each isolation, the protein concentration of myelin was determined using a microplate procedure for bicinchoninic acid (BCA) protein assay with the spectrophotometer set at wavelength 562nm (for complete procedure in detail, please see Appendix 2).

### **3.2.2 Myelin Treatment with Different Detergents**

Adult bovine myelin (sample ID; A-50) was subjected to two different detergent extraction protocols. The initial protocol involved two sequential extractions with 0.5% TX-100 as previously described by Kirschner<sup>20</sup>. The second extraction protocol included four sequential 0.5% TX-100 treatments. Control extractions were completed without the addition of TX-100. In brief, 500µg of myelin protein was treated for 30min at room temperature in 50mM phosphate buffer (pH 7.4) with or without 0.5% TX-100, along with protease inhibitors (100mM PMSF, 100mM Na<sub>3</sub>VO<sub>4</sub>, and 1X protease inhibitor cocktail). After addition, the mixture was mixed by vortexing at regular intervals over 30min at room temperature. The insoluble material was then pelleted by centrifuging at 14000×g for 15min (Eppendorf centrifuge 5415D) and the soluble fraction (supernatant)

was collected separately. Similarly second, third, or fourth extractions were performed as above (see Fig. 3.2).



**Figure 3.2** – Flow chart summarizing myelin extraction with different detergents.

For the most part, biochemical characterization of membrane microdomains depends on differential extraction methods. As discussed in Chapter 1, the myelin membrane is composed of several different types of functional microdomains. It is necessary to separate radial components from other types of microdomains prior to its analysis. Previously DeBruin *et al.* found that lipid rafts can be isolated by treating myelin with 1.5% CHAPS at 4°C.<sup>24</sup> This finding was incorporated into the extraction sequence in order to separate lipid rafts from the radial component. The extraction



protocols were performed on fetal and adult bovine myelin of different ages (sample ID A-24, E19 weeks; sample ID A-15, E27 weeks; sample ID A-26, E37 weeks; and sample ID A-50, adult). To microfuge tubes, 500 $\mu$ g of bovine myelin from different ages were added along with 1.5% CHAPS, 100mM PMSF, 100mM Na<sub>3</sub>VO<sub>4</sub>, and 1X protease inhibitor cocktail in TBS (tris buffered saline, pH 7.5) to a final volume of 1ml. After addition, the mixture was vortexed at regular intervals over 30min at 4°C. The insoluble material was then pelleted by centrifuging at 14000 $\times$ g for 15min at 4°C and the supernatants were collected separately. A second extraction was performed on the pellets with 0.5% or 2% TX-100 at room temperature.

To further characterize the radial component as a highly detergent resistant fraction, mouse myelin was extracted with a series of detergents. As summarized in Fig. 3.2, mouse myelin was extracted first with 1.5% CHAPS followed by second, third, and fourth extractions with different concentrations of TX-100, Tween-20, ASB-14, or C<sub>12</sub>E<sub>9</sub> (see Fig. 3.2). In all cases, final pellets were resuspended in 1ml resuspension buffer (1% SDS in 10mM tris buffer, pH 8.0). The pellets and the soluble fractions were subjected to electrophoretic separation and proteomic analyses as described below.

### **3.2.3 Electrophoretic Separations**

#### **3.2.3.1 SDS-PAGE**

The proteins in the insoluble and soluble fractions of bovine and mouse myelin were separated by SDS-PAGE. Prior to running one dimensional SDS gels, loading samples were prepared by taking 64 $\mu$ l of supernatant and pellet fractions and adding 16 $\mu$ l of 5X sample loading buffer (0.5M tris HCl, pH 6.8, glycerol, 10% w/v SDS, 0.5% w/v bromophenol blue). Then samples were centrifuged to remove any insoluble material. To

improve resolution of high and low molecular mass proteins, samples were separated on 12% and 14% resolving gels, respectively. Separation of IEF fractions (see section 3.2.3.2) were done on Criterion TGX precast gels 1mm thick, 4-20% (Bio-Rad). Criterion precast gels are wider and longer than traditional mini format gels and can accommodate more samples per gel. All other samples were separated on mini-gels with the Mini-Protean 3 cell (Bio-Rad) at constant 75V until the samples passed through the stacking gel, and then voltage was increased to 95V until run completes. Criterion TGX gels were run at constant voltage of 200V. Precision Plus Protein Standards (Bio-Rad) were used as protein ladders to estimate the molecular mass of protein bands on gel. After running SDS-PAGE, gels were either silver-stained (for complete procedure in detail, please see Appendix 3) or coomassie stained with Bio-Safe for protein visualization.

### **3.2.3.2 Solution-Phase IEF**

Solution-phase IEF was conducted in a MicroRotofor™ Liquid-Phase IEF Cell (Bio-Rad). The insoluble fraction pellet from adult bovine myelin, after a second extraction with 2% TX-100 (see section 3.2.2) was partially delipidated with 500µl ethyl ether and ethanol in a 2:1 ratio. The mixture was vortexed and kept on ice for 5min followed by centrifuged at 14000×g for 15min in cold room. The supernatant was discarded and pellets were air-dried to remove any traces of ethyl ether and ethanol. Pellets (in duplicate) were resuspended in 700µl of IEF buffer (7M urea, 2M thiourea, 50mM DTT, 2% ASB-14, 2% CHAPS, 2% TX-100, and 1% carrier ampholytes of pH range 5-8, pH 7-9, and pH 9-11). The samples were sonicated for 15-20min in ice water for complete solubilisation of pellets. The duplicates were then combined and 2.5ml sample was slowly loaded through the centremost port of the focusing chamber. As one

compartment fills, the sample slowly spreads and fills the adjacent compartments. The focusing assembly was placed into the focusing station with the anode assembly towards the left side and the cathode assembly towards the right side. The electrolytes utilized for the IEF gel run were 0.5M acetic acid (anode) and 0.1M sodium hydroxide (cathode). The IEF gel was run under denaturing conditions by setting the cooling switch to II (20°C) at a constant 2W (for complete procedure in detail, please see Bio-Rad MicroRotofor™ Liquid-Phase IEF Cell Instructional Manual). The voltage was recorded at regular intervals of 10min until a plateau was reached.

IEF fractions were harvested by applying an external vacuum in the harvesting chamber, and the pH was measured using a microelectrode. Each IEF fraction was precipitated using a 2-D Clean-up Kit (Bio-Rad) (for complete procedure in detail, please see Bio-Rad ReadyPrep 2-D Cleanup Kit Instructional Manual). The final pellets were resuspended in 16µl of resuspension buffer (1% SDS in 10mM tris buffer, pH 8.0) followed by addition of 4µl of 5X sample loading buffer (0.5M tris HCl, pH 6.8, glycerol, 10% w/v SDS, 0.5% w/v bromophenol blue). Samples were then subjected to SDS-PAGE.

### **3.2.3.3 2-D Gel Electrophoresis with IPG Strips**

Pellet fractions were resolubilised in 125µl of IEF rehydration buffer (7M urea, 2M thiourea, 50mM DTT, 2% w/v CHAPS, 2% w/v ASB-14, 0.018% w/v bromophenol blue, and 0.2% w/v carrier ampholytes of specific pH range). Prior to running first dimension separation, samples were centrifuged to remove any insoluble material. Each sample was added to the strip holder bases. The samples were loaded along the edge of the channel extending the whole length of the channel. IPG strips were placed into the

strip holder with the gel side facing down and rehydrated overnight at room temperature. Strips were overlaid with 1ml of mineral oil to prevent the evaporation of samples during the rehydration process. After rehydration was completed, excess mineral oil and loosely bound protein were removed by holding strips vertically and blotting on filter paper for a few minutes. Rehydrated IPG strips were placed in an IEF focusing tray; gel side down, with the anode side (marked “+” on IPG strips) against the anode (left) side in the focusing tray (for complete procedure in detail, please see Bio-Rad ReadyStrip IPG Strip Instructional Manual). IEF was started according to the programmed parameters in Table 3.1 below. Low voltage was applied initially for one and a half hours to improve entry of high molecular weight and loosely associated proteins into the IPG gel matrix.

**Table 3.1-** IEF Run conditions of first dimension gel electrophoresis with IPG strips.

<b>Step</b>	<b>Voltage</b>	<b>Time</b>	<b>Ramp</b>
1	150V	30min	Slow
2	750V	1hr	Slow
3	4000V	3hr	Linear
4	4000V	2hr	Linear

The final volt hours were typically set to 15000.

For second dimension SDS-PAGE, IPG strips were equilibrated in equilibration buffer I (0.375M tris HCl, pH 8.8, 6M urea, 20% glycerol, 2% SDS, and 2% DTT) followed by equilibration buffer II (0.375M tris HCl, pH 8.8, 6M urea, 20% glycerol, 2% SDS, and 2.5% iodoacetamide) for 10min each by placing on a rocker. The two equilibration steps ensure that cysteines are reduced and alkylated, which minimizes vertical streaking in the stained gel. Equilibration buffer I contains DTT which reduces sulfhydryl groups, while equilibration buffer II contains iodoacetamide which alkylates the reduced sulfhydryl groups. After completion second dimension separation was performed by placing strips horizontally on top of polymerized 12% resolving SDS gel.

### **3.2.4 Proteomic Analysis**

The technique used in this study of proteomics was trypsin-digestion of proteins followed by analysis of peptides with liquid chromatography coupled to tandem MS (LC/MS/MS). Samples were analyzed by a Thermo Fisher Q-Exactive quadrupole/orbitrap instrument. Proteome software Scaffold was used to validate peptide and protein identifications. Peptide sequence identification is most commonly used for proteomic analysis of complex samples where peptide masses may overlap even with a high resolution mass spectrometer.

#### **3.2.4.1 Trypsin Digestion**

In-gel trypsin digestion was performed on proteins that are separated by 1-D and 2-D electrophoresis (for complete procedure in detail, please see Appendix 4). The gel bands and protein spots of interest were excised and destained with 50mM ammonium bicarbonate. The gel pieces were dehydrated with 50% acetonitrile in 25mM ammonium bicarbonate, reduced with DTT (10mM) in ammonium bicarbonate for 10min, and then alkylated with iodoacetamide (100mM) in the dark for an additional 10min. The gel pieces were digested with trypsin (13ng/ $\mu$ l) (Promega) for 30-45min, and incubated at 37°C for 16-18hrs. Peptides were further extracted from gel pieces by sonicating, shrinking, and hydrating with acetonitrile and formic acid. Finally samples were completely dried with vacuum centrifuge, and prior to analysis, samples were resuspended in formic acid solution which helps in ionization of samples.

In-solution digestion was performed on the pellet fraction from adult bovine and mouse myelin using ProteaseMAX (Promega) (for complete procedure in detail, please see Appendix 5). ProteaseMAX is a surfactant that is designed to improve protein

digestion by providing a denaturing environment prior to protease addition <sup>28</sup>. It can improve protein identification of membrane proteins in the low nanogram level. The insoluble fraction pellets from detergent treatment of adult bovine and mouse myelin were washed with 10mM Tris buffer pH 7.8 to remove any residual salts and detergents. The pellets were then delipidated by washing with ice-cold acetone. ProteaseMAX (0.2%) and urea (8M) were added to the pellets and mixed until fully solubilised. The solubilised pellets were then incubated with 0.5M DTT at 56°C for 20min followed by incubation in the dark for an additional 20min with 0.55M iodoacetamide. The samples were digested with trypsin (1µg/µl) and ProteaseMAX (1%) for 3hrs at 37°C, and finally snap-frozen in dry ice and stored at -20°C until LC/MS/MS analysis.

#### **3.2.4.2 Mass Spectrometric Analysis**

All the samples after trypsin digestion were sent for mass spectrometric analysis at the Advanced Protein Technology Centre, The Hospital for Sick Children in Toronto. All the samples were analyzed on a Thermo Fisher Q-Exactive quadrupole/orbitrap LC/MS/MS instrument. Xcalibur processing and instrument control software.

**Database searching** - All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version Mascot) and X! Tandem (The GPM, thegpm.org; version 2007.01.01.1). X! Tandem was set up to search a subset of the NCBIInr\_20110515 database. Mascot was set up to search the NCBIInr\_20110515 database (selected for *Bos taurus*, unknown version, 38938 entries). Mascot and X! Tandem were searched with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 3.0 Da. Pyro-glu of glutamic acid and the N-terminus, s-carbamoylmethylcysteine cyclization of the N-terminus, pyro-glu of glutamine, deamidation of asparagine, oxidation of methionine and

acetylation of the N-terminus were specified in X! Tandem as variable modifications. Pyro-glu of the N-terminus, s-carbamoylmethylcysteine cyclization of the N-terminus, deamidation of asparagine and glutamine, oxidation of methionine and acetylation of the N-terminus were specified in Mascot as variable modifications.

**Criteria for protein identification** - Scaffold (version Scaffold\_3.3.3, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 80.0% probability as specified by the Peptide Prophet algorithm (Keller, A et al Anal. Chem. 2002;74(20):5383-92 as reference). Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, AI Anal Chem. 2003 Sep 1;75(17):4646-58 as reference).

Samples from solution-phase IEF were analyzed with similar conditions as described above, except only one search engine Mascot was used for analyses. Mascot was set up to search the NCBI nr\_20111113 database with 39115 entries. Protein identifications were accepted if they could be established at greater than 95% probability and contained at least 1 identified peptide.

Adult bovine and mouse myelin pellet samples digested with proteaseMAX surfactant using the shot-gun technique (MudPIT) were analyzed using Sequest (XCorr Only) (Thermo Fisher Scientific, San Jose, CA, USA; version 1.3.0.339) and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1)). Sequest (XCorr Only) was set up to search ipi.BBOVIN.v3.69.fasta (unknown version, 34641 entries) and ipi.MOUSE.v3.83.fasta (unknown version, 60010 entries). X! Tandem was set up to

search the ipi.BOVIN.v3.59 database (unknown version, 31571 entries) and ipi.MOUSE.v3.83 database (unknown version, 60010 entries). Sequest (XCurr Only) and X! Tandem were searched with a fragment ion mass tolerance of 0.0100 Da and a parent ion tolerance of 5.0 PPM. Iodoacetamide derivatives of cysteine was specified in Sequest (XCurr Only) and X! Tandem as a fixed modification. Deamidation of asparagine and glutamine and oxidation of methionine were specified in Sequest (XCurr Only) and X! Tandem as variable modifications. Criteria for protein identification was similar to the one described above with protein and peptide probability greater than 95% with at least 2 identified peptides.

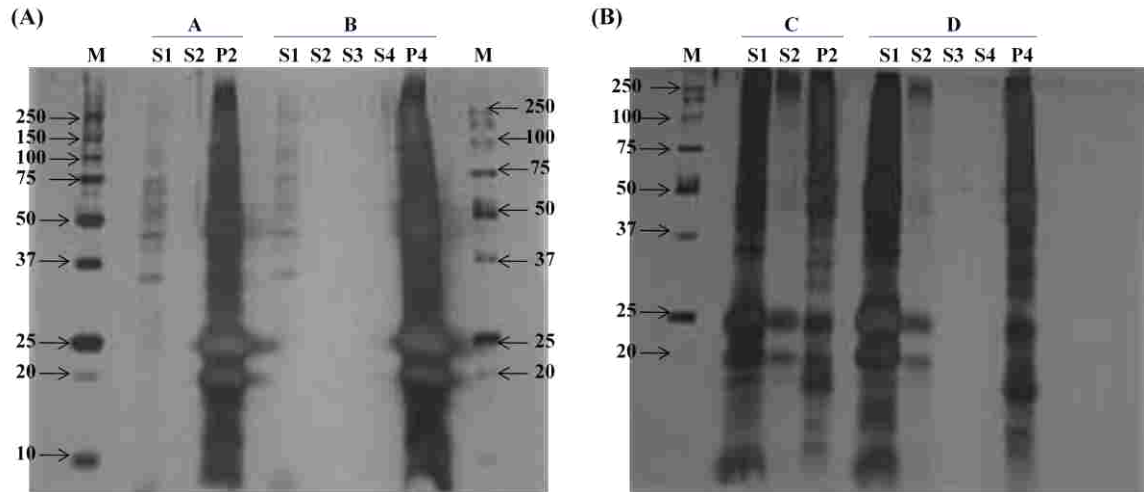
### **3.3 Results**

The purpose of this study was to further characterize the radial component in terms of its detergent extraction from myelin and its proteome. Previously, Kirschner and his colleagues found that the radial component is highly preserved with 0.5% TX-100 treatment<sup>20</sup>. It was noted that the radial component is present only within CNS myelin, and PNS sciatic nerve tissue is completely devoid of the radial component and its proteins<sup>20</sup>.

#### **3.3.1 Effects of Sequential Extractions with TX-100**

Myelin was extracted with different detergents and concentrations as outlined in section 3.2.2. Adult bovine myelin was extracted with two successive extractions with 0.5% TX-100 and with four successive extractions with 0.5% TX-100 (Fig. 3.3 (B)). Parallel extractions were performed with buffer only, no TX-100 (Fig. 3.3 (A)). Supernatant fractions for each extraction and the remaining pellets were subjected to SDS-PAGE analysis.





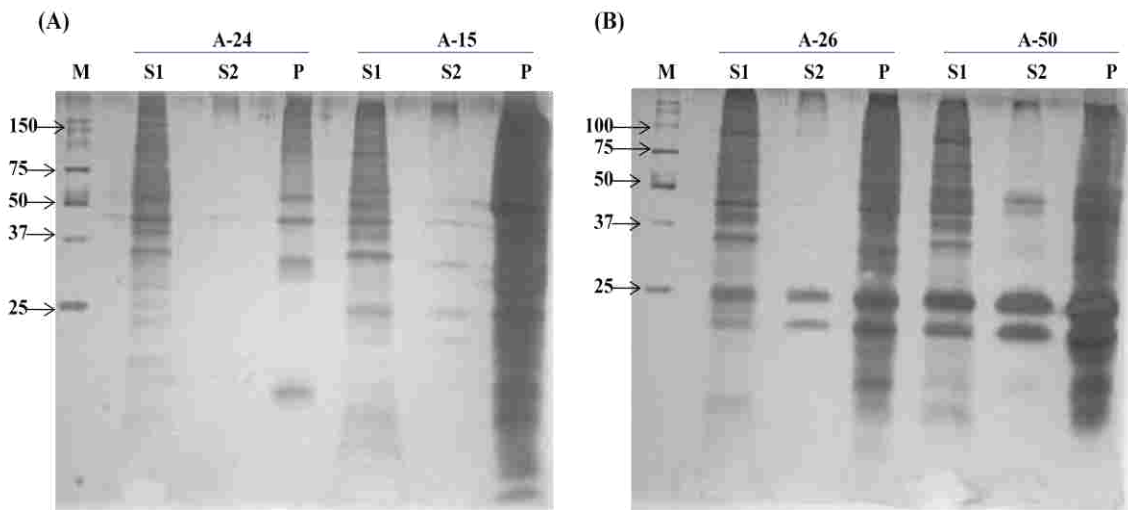
**Figure 3.3** – 1-D SDS-PAGE resolving adult bovine myelin fractions with and without TX-100 treatment. Equal volumes (20 $\mu$ l) of each fraction were loaded onto a 1mm 14% mini-gel. Proteins on the gel were visualized by silver staining. M-Unstained Precision Plus marker (Bio-Rad). A & B are supernatant and pellet of control (extraction without TX-100) samples, respectively. S1, S2, S3, and S4 refer to the supernatant from first, second, third, and fourth extraction respectively. P2 and P4 are pellets of control samples from two and four extractions respectively. (B) C & D are supernatant and pellet from extraction with 0.5% TX-100, respectively. S1, S2, S3, and S4 refer to the first, second, third, and fourth extraction with 0.5% TX-100 respectively. P2 and P4 are pellets obtained after two and four extractions respectively with 0.5% TX-100.

As seen in Fig. 3.3, the number of protein bands in supernatants of both the sets decreases as the number of extractions increases. In comparison to the detergent extractions, a minimal number of protein bands were observed in control supernatants with two and four extractions (Fig. 3 (A)). The proteins from the control extraction, detected in S1 fraction of A and B are proteins loosely associated with myelin membrane. There was no effect of successive extractions in control without detergent. In Fig. 3.3 (B), the supernatant S1-C and S1-D fraction which is the extract from the initial 0.5% TX-100 treatment, contained many protein bands from high to low molecular weight. In supernatants from the second extraction (S2-C and S2-D), major bands at 25kDa and 20kDa were observed as well as several bands above 37kDa. The detergent had negligible effect after first extraction and almost no effect after second extraction, and

thus a high amount of protein is still seen in the pellet. Proteins present in the pellet are highly resistant to successive detergent treatments with 0.5% TX-100.

### 3.3.2 Effects of Sequential Extractions with CHAPS and TX-100

The second extraction method employed the use of a zwitterionic detergent followed by a nonionic detergent. The myelin membrane has many different functional microdomains including lipid rafts that are enriched in cholesterol and glycosphingolipid. The treatment of myelin with 1.5% CHAPS effectively isolates lipid rafts from other microdomains<sup>24</sup>. Based on these findings, fetal and adult bovine myelin of different ages were extracted initially with 1.5% CHAPS to extract a fraction containing the lipid rafts, and then extracted with 0.5% TX-100.

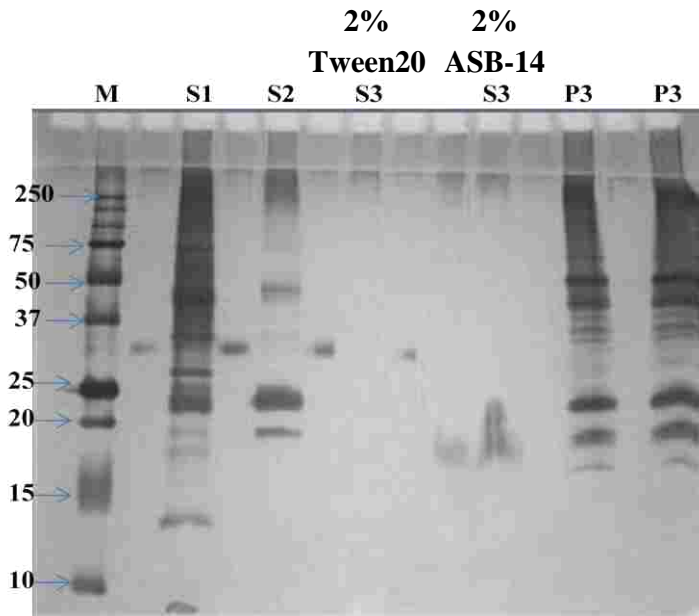


**Figure 3.4** – 1-D SDS-PAGE resolving soluble and insoluble fractions from fetal and adult bovine myelin of different age treated sequentially with CHAPS and TX-100. Samples were extracted with 1.5% CHAPS at 4°C followed by 0.5% TX-100 at room temperature. Equal volumes (20µl) of each fraction were loaded onto a 1mm 14% mini-gel. Proteins on the gel were visualized by silver staining. (A, B) M-Unstained Precision Plus marker. S1 & S2 are supernatant from first and second extraction with 1.5% CHAPS and 0.5% TX-100, respectively. P is final pellet obtained from extraction with 0.5% TX-100.

As seen in Fig. 3.4, the supernatant fraction (S1) from the extraction with 1.5% CHAPS which contains lipid rafts has a higher amount of proteins compared to supernatant fraction (S2) from the subsequent extraction with 0.5% TX-100. Supernatant S2 shows some high and low molecular weight protein bands and two prominent bands at 25 and 20kDa that are mostly MBP (as determined by mass spectrometric analysis and Western blotting). The number of protein bands increases with an increase in the development of the myelin.

### 3.3.3 Sequential Extractions with CHAPS, TX-100, Tween-20, and ASB-14

A third extraction method was employed where adult mouse myelin was sequentially extracted with 1.5% CHAPS followed by different sequence and concentrations of the detergent TX-100, Tween-20, ASB-14, or C<sub>12</sub>E<sub>9</sub> (see section 3.2.2).



**Figure 3.5** – 1-D SDS-PAGE resolving soluble and insoluble fractions from mouse myelin sequentially treated with CHAPS, TX-100, and Tween-20 or ASB-14. Myelin samples (0.5mg/ml) were extracted first with 1.5% CHAPS at 4°C followed by second extraction with 1% TX-100 and third extraction with 2% Tween-20 or 2% ASB-14 at room temperature. Equal volumes (20µl) of each fraction were loaded onto a 1mm 14% mini-gel. Proteins on the gel were visualized by silver staining. M-Unstained Precision Plus marker. S1 and S2 are supernatant from first and second extraction with 1.5% CHAPS and 1% TX-100 respectively. S3 are supernatants extracted with 2% Tween-20 or 2% ASB-14. P3 are the final pellets obtained from extraction with 2% Tween-20 or 2% ASB-14.

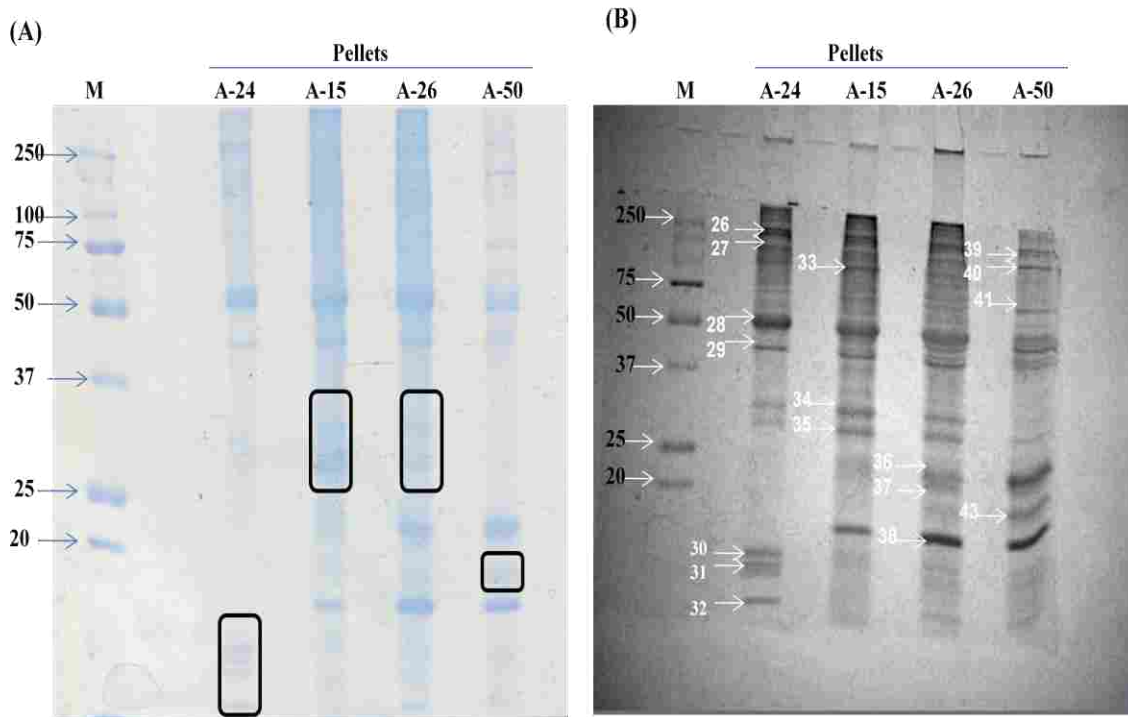
M-Unstained Precision Plus marker. S1 and S2 are supernatant from first and second extraction with 1.5% CHAPS and 1% TX-100 respectively. S3 are supernatants extracted with 2% Tween-20 or 2% ASB-14. P3 are the final pellets obtained from extraction with 2% Tween-20 or 2% ASB-14.

In the third method of extraction, adult mouse myelin samples were extracted with CHAPS, then with TX-100 followed by third extraction with Tween-20 or ASB-14. As seen in Fig. 3.5, only the supernatants from the CHAPS and subsequent TX-100 treatment contain a significant amount of protein. Further extraction with 2% Tween-20 or 2% ASB-14 does not solubilise any more protein. A third extraction was also performed with different concentrations of C<sub>12</sub>E<sub>9</sub> detergent. Even at 2%, C<sub>12</sub>E<sub>9</sub> detergent was not effective to further extract the proteins from the pellet. This suggests that insoluble fraction pellet which is the radial component has highly hydrophobic integral membrane proteins and is highly resistant to detergent. Among all the sequence of detergents used for extraction the best sequence found was CHAPS followed by TX-100 with the optimal concentration of 1.5% CHAPS and 2% TX-100. Similar profiles were found with 1% and 2% TX-100.

### **3.3.4 Proteomic Analysis of Pellet Fractions from Bovine Myelin**

Pellet fractions from bovine myelin of different ages were separated on SDS-PAGE using mini- and maxi- gel formats and major bands were excised, trypsin-digested, and analyzed by LC/MS/MS.

As seen in Fig. 3.6, A-24 pellet fraction has some lower molecular weight bands below 20kDa (black vertical box) which are very well defined but lack proteins between 37-20kDa. In A-15 and A-26 pellet fraction two major bands (black vertical box) were observed between 37kDa and 25kDa followed by A-50 with less intensity and almost absent in A-24. One unique band (black box) identified in A-50.



**Figure 3.6** – 1-D SDS-PAGE resolving the pellet fraction of bovine myelin of different ages treated with CHAPS then TX-100. A-24, A-15, A-26, and A-50 are final pellets extracted first with 1.5% CHAPS followed by 0.5% TX-100. Proteins on the gel were visualized by coomassie staining. M-Unstained Precision Plus marker. (A) Samples resolved on Protean cell-II (Bio-Rad). Equal volumes (25 $\mu$ l) of each fraction were loaded onto a 16cm, 1mm 14% gel. (B) Samples resolved on a mini-gel of 10 well, 14% resolving, 4% stacking with 1mm thickness containing the same samples and volumes as (A). White arrows indicate the protein bands which were excised and trypsin digested. (See text for box).

In order to identify specific proteins, 17 unique bands were analyzed. Mass spectrometry-identified proteins with probability greater than 99% and with two unique peptides sequenced are summarized. There are total 558 proteins identified from LC/MS/MS results of which 77 are high abundant with the unique peptides  $\geq 7$  and 481 are low abundant with unique peptides  $\geq 2$ . Only the highly abundant proteins are listed in Table 3.2.

**Table 3.2** – High abundant proteins identified in 1-D SDS-PAGE in bovine myelin of different ages. Protein band numbers refer to Fig. 3.6 (B). All the proteins with 100% probability with at least 7 unique peptides are listed.

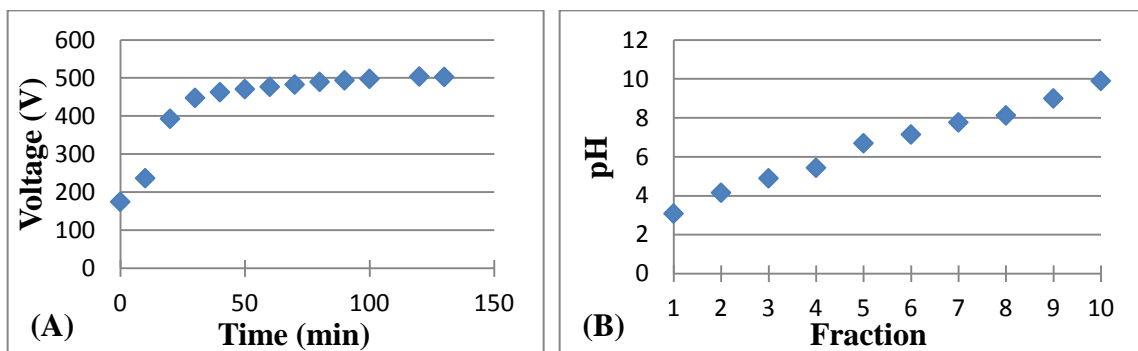
Band #	Protein	% Spectra	MW (kDa)	# Unique Peptides	%Coverage
<b>SAMPLE A-24</b>					
26	Spectrin $\alpha$	0.56%	285	101	48%
	Spectrin $\beta$	0.47%	274	74	36%
	Myosin-10	0.47%	229	74	44%
	Myosin-Va-like	0.33%	215	53	34%
	Tubulin $\beta$ -5	0.16%	50	18	52%
	Tubulin $\alpha$ -1A	0.14%	50	14	46%
	Centrosomal protein (170kDa)	0.11%	175	23	19%
27	p130Cas-associated protein	0.21%	131	32	38%
	Spectrin $\alpha$ chain	0.19%	285	39	19%
	Cytoplasmic linker associated protein 2	0.19%	165	31	28%
	Tubulin $\alpha$ -1A	0.13%	50	14	42%
	Neurofilament triplet M protein	0.12%	104	17	20%
	Tubulin $\beta$ -5	0.12%	50	14	37%
28	Tubulin $\alpha$ -1A	1.40%	50	25	67%
	Tubulin $\beta$ -5	0.94%	50	30	74%
	Tubulin $\beta$ -2	0.17%	50	9	75%
	Glial fibrillary acidic protein	0.13%	50	24	53%
	The Structure of F1-ATPase inhibited by resveratoral	0.12%	55	16	36%
	Tubulin $\beta$ -3	0.10%	50	11	68%
	Vimentin	0.10%	54	17	41%
29	Actin, $\beta$	0.61%	41.7	18	57%
	Doublecortin	0.11%	37.7	7	23%
30	Tubulin $\alpha$ -1A	0.32%	50	17	59%
	Tubulin $\beta$ -5	0.17%	50	14	52%
31	Hist1h2bj	0.52%	13.98	11	68%
	histone cluster 1, H2aj	0.32%	13.9	7	42%
	Hist1h4c	0.11%	11.3	6	52%
32	Histone H4	0.39%	11	15	61%
	Tubulin $\alpha$ -1A	0.12%	50	11	38%
<b>SAMPLE A-15</b>					
33	Hexokinase 1	1.10%	102.2	44	50%
	Na <sup>+</sup> /K <sup>+</sup> ATPase, $\alpha$ 3	0.38%	114	18	23%
	Nicotinamide nucleotide transhydrogenase	0.36%	108	24	26%
	Na <sup>+</sup> /K <sup>+</sup> ATPase $\alpha$ 1	0.12%	113	9	22%
34	VDAC 1	0.78%	31	22	75%

	VDAC2	0.34%	32	15	61%
	Ground State Structure Of F1- Atpase From Bovine Heart Mitochondria	0.33%	30	13	54%
	Prohibitin-2	0.25%	33	19	67%
	Sideroflexin-1	0.22%	36	16	62%
	Mitochondrial glutamate carrier 1	0.21%	35	15	61%
	Phosphate carrier protein, mitochondria	0.18%	40	15	43%
	Mitochondrial 2-oxoglutarate/malate carrier protein	0.18%	34	14	57%
	Mitochondrial carrier homolog 2	0.13%	33	10	42%
	ADP/ATP translocase 3	0.60%	33	27	68%
	ADP/ATP translocase 1	0.38%	33	15	73%
	Prohibitin	0.30%	30	17	73%
35	VDAC3	0.14%	31	9	39%
	Mitochondrial 2-oxoglutarate/malate carrier protein	0.12%	34	13	51%
	NADH dehydrogenase	0.12%	30	14	58%
<b>SAMPLE A-26</b>					
36	Myelin proteolipid protein	0.37%	30	13	35%
	ATP synthase subunit O	0.31%	23	8	51%
37	MBP	0.28%	18	9	50%
	PLP	0.18%	30	12	34%
38	MBP	0.46%	18	13	52%
<b>SAMPLE A-50</b>					
	Spectrin $\alpha$	0.67%	285	98	43%
	Spectrin $\beta$	0.62%	274	95	45%
	Glial fibrillary acidic protein	0.27%	50	27	60%
	Neurofilament, heavy polypeptide	0.22%	112	25	26%
	Myosin-10	0.19%	229	34	21%
39	Myosin heavy chain 14	0.17%	229	33	20%
	Tubulin $\alpha$ -1A	0.14%	50	14	41%
	Myosin-Va-like	0.14%	215	25	15%
	Neurofilament triplet M protein	0.12%	104	19	21%
	Calcium/calmodulin-dependent protein kinase II $\alpha$	0.12%	54	15	34%
	Tubulin $\beta$ -5	0.11%	50	15	39%
39	Contactin associated protein	0.11%	156	20	15%
	Ankyrin 2	0.10%	436	20	6%
40	Neurofilament triplet M protein	0.54%	104	45	40%
	Spectrin $\alpha$ chain	0.15%	285	30	14%
	Neurofilament, light polypeptide	0.86%	62	36	49%
	PLP	0.27%	30	7	27%
41	Heat shock cognate 71 kDa protein	0.23%	71.2	18	27%
	SLC25A12 a Calcium-binding mitochondrial carrier protein	0.21%	74.5	19	30%
	Glial fibrillary acidic protein	0.14%	50	12	29%
43	MBP	0.13%	21.3	8	41%

% Spectra- Protein percentage of total spectra; MW (kDa)- Molecular weight in kDa; # Unique peptides- Number of unique peptides identified in protein; % Coverage- Percentage of amino acids identified.

### 3.3.5 Solution Phase IEF

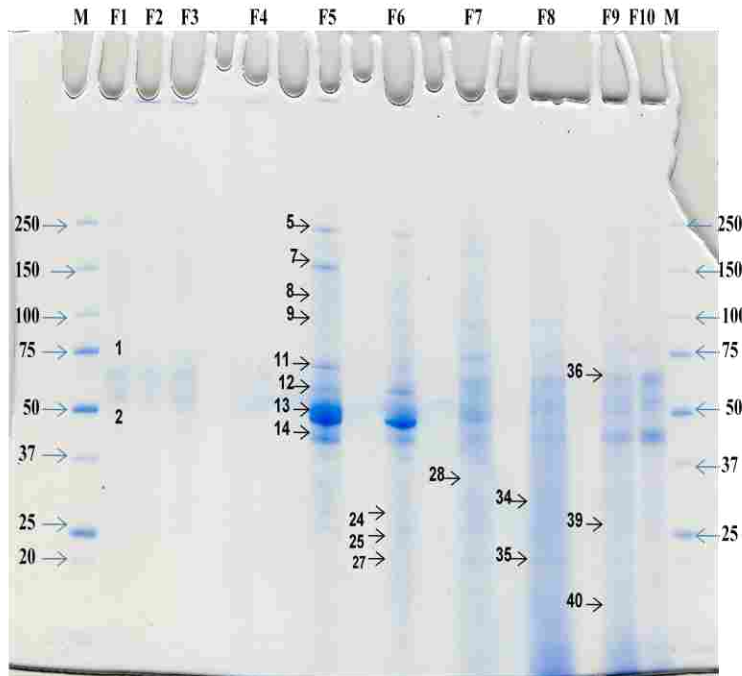
The effective study of low-abundance proteins often requires a fractionation step to reduce complexity of sample and to increase the concentration of low-abundance proteins compared to the original sample.



**Figure 3.7** – Solution-phase IEF of the pellet fraction of adult bovine myelin run under denaturing conditions at 20°C for 2hr 10min at 2W with electrolytes using 0.5M acetic acid and 0.1M NaOH. (A) Plateau of IEF voltage over time; (B) pH of fractions obtained from IEF run.

An adult bovine myelin sample was extracted and the pellet fraction was separated by solution-phase IEF as per process described in section 3.2.2. Fig. 3.7 (A) shows the change in voltage over the IEF run. The voltage increased throughout the run until the proteins reached their pI and then the voltage reached a plateau. The total run time was 2hr 10min. If the voltage does not plateau the run is incomplete, or if the voltage is allowed to increase after the plateau is reached, the run is over-focused. The proteins in either condition will not separate in correct fractions according to their pI. After run was completed the fractions 1 to 10 were harvested under vacuum and the pH was measured using a microelectrode. Fig. 3.7 (B) shows linear increase in pH. The fractions 1 to 5 are acidic in nature whereas fractions 6-10 are basic in nature.





**Figure 3.8** – 2-D separation of adult bovine myelin. Each IEF fraction (50 $\mu$ l) was initially precipitated using 2-D clean up kit (Bio-Rad), and then resolved on a 1mm thick, 4-20% Criterion TGX (Bio-Rad) precast gel followed by Coomassie staining. M-Unstained Precision Plus marker. The pH of each IEF fraction from F1 to F10 is as follows: 3.09, 4.16, 4.90, 5.44, 6.70, 7.15, 7.77, 8.13, 9.00, and 9.90. Bands which are marked were excised and trypsin digested for mass spectrometric analysis.

As seen in Fig. 3.8, fractions from F5-F8 with the pH range 6.7-8.13 contained the most proteins, followed by F9 and F10 with the pH 9.0 and 9.9, respectively. With fractions F1-F3, only very small amount of proteins were detectable between 75-50kDa. Thus, the majority of the proteins are in the basic pH range. A further separation of proteins in F5 and F6 was attempted on a lower pH range (5-8) IPG strips followed by SDS-PAGE. Unfortunately only a few higher molecular weight protein spots were observed upon staining.

For protein identification, 19 major bands from the various fractions were excised and trypsin digested. A total of 197 proteins were identified from mass spectrometric analysis. The major proteins present in each band are listed in Table 3.3.

**Table 3.3-** Major proteins identified from solution-phase IEF-SDS/PAGE separation. The band number corresponds to Fig 3.8. All the proteins with 100% probability, higher % spectra in particular band and with at least 2 unique peptides identified are listed.

Band #	Fraction pH	Protein	# Unique Peptides	% Coverage	MW (kDa)
1	3.09	Tubulin $\alpha$ -1D	5	16	50
		Tubulin $\beta$ -3	6	17	50
		GFAP	22	44	50
2	3.09	Vacuolar protein sorting associated protein-33B	2	4.1	71
		Tubulin $\beta$ -3	3	8.2	50
5	6.7	Spectrin $\alpha$	120	51	285
7		Neurofilament triplet M protein	41	47	104
8		GFAP	26	51	50
		Tubulin $\beta$ -3	12	28	50
9		GFAP	23	49	50
		Ubiquitin specific peptidase 5	13	22	93
11		Neurofilament light polypeptide	29	45	63
		Heat shock cognate protein	19	31	71
12		$\alpha$ -Internexin	26	53	55
		GFAP	20	43	50
13		Neurofilament light polypeptide	10	22	63
		Tubulin $\alpha$ -1D	16	44	50
14		Tubulin $\beta$ -3	18	54	50
		$\beta$ -Actin	18	55	42
24	7.15	Guanine nucleotide binding protein $\alpha$	11	32	40
25		Lactate dehydrogenase B chain	8	27	37
		VDAC 2	7	27	32
27	7.15	Prohibitin	13	53	30
		NADH dehydrogenase	10	45	30
		Heat shock cognate			
		VDAC2	2	9	32
28	7.77	Elongation factor Tu	18	43	49
		CNP	12	28	45
34	8.13	F1 ATPase	12	21	55
		Sirt2			
35	8.13	Lipophilin	3	13	27

		$\alpha$ -Crystallin B			
36	9	Calcium binding protein	15	25	75
39		VDAC1	8	30	31
		VDAC 2	7	27	32
		VDAC3	2	12	31
40		NADH dehydrogenase	3	23	21
	$\alpha$ -Crystallin B				

# Unique peptides- Number of unique peptides identified in protein; % Coverage- Percentage of amino acids identified; MW (kDa)- Molecular weight in kDa.

### 3.3.6 MudPIT Analysis of Adult Bovine and Mouse Myelin Detergent Resistant Fraction

Shot-gun proteomic analysis was performed on the detergent resistant pellet of adult bovine and mouse myelin in order to further characterize this proteome. In-solution digestion was performed using ProteaseMAX surfactant. As discussed in section 3.2.4.1 ProteaseMAX surfactant solubilises proteins including difficult membrane proteins and enhances protein digestion by providing a denaturing environment prior to protease addition. There are 472 high and low abundant proteins identified in bovine and 881 in mouse myelin. The major proteins found in the bovine and mouse samples are listed below in Table 3.4.

**Table 3.4** - The proteins identified by MudPIT analyses of pellet fraction from adult (A) bovine and (B) mouse myelin. Top 30 proteins with 100% probability and higher % spectra are listed.

(A)

Number	Protein	# Unique Peptides	% Coverage	MW (kDa)
1	GFAP	41	73	50
2	Tubulin $\alpha$ -1D	24	60	50
3	CNP	39	76	48
4	Tubulin $\beta$	32	72	50
5	MBP	15	39	36
6	Neurofilament light polypeptide	29	60	63
7	Spectrin $\alpha$	107	49	285
8	Spectrin $\beta$	82	41	274
9	PLP	9	26	30
10	531kDa protein	86	26	531
11	Dynein-1	77	23	532
12	Na <sup>+</sup> /K <sup>+</sup> -ATPase $\alpha$ -3	35	38	114
13	$\alpha$ -internexin	27	66	55
14	Clathrin heavy chain 1	39	32	192
15	Tubulin $\beta$ -4	7	72	50
16	ADP/ATP translocase 2	20	54	33
17	Syntaxin binding protein 1	25	49	68
18	ATP synthase $\alpha$	22	46	60
19	Microtubule associated protein-1B	35	22	270
20	$\alpha$ -crystallin B chain	14	80	20
21	Actin $\beta$	17	51	42
22	Neurofilament heavy polypeptide	25	28	112
23	N-ethylmaleimide-sensitive factor isoform 4	31	44	84
24	Dynamin 1	26	37	96
25	Calcium/calmodulin-dependent protein kinase II $\alpha$	18	38	54
26	478kDa protein	38	13	478
27	Heat shock cognate-12A	24	34	82
28	Tubulin $\beta$ -3	13	70	50
29	Hexokinase 1	28	37	102
30	Myosin-ID	26	35	116

# Unique peptides- Number of unique peptides identified in protein; % Coverage- Percentage of amino acids identified; MW (kDa)- Molecular weight in kDa.

**(B)**

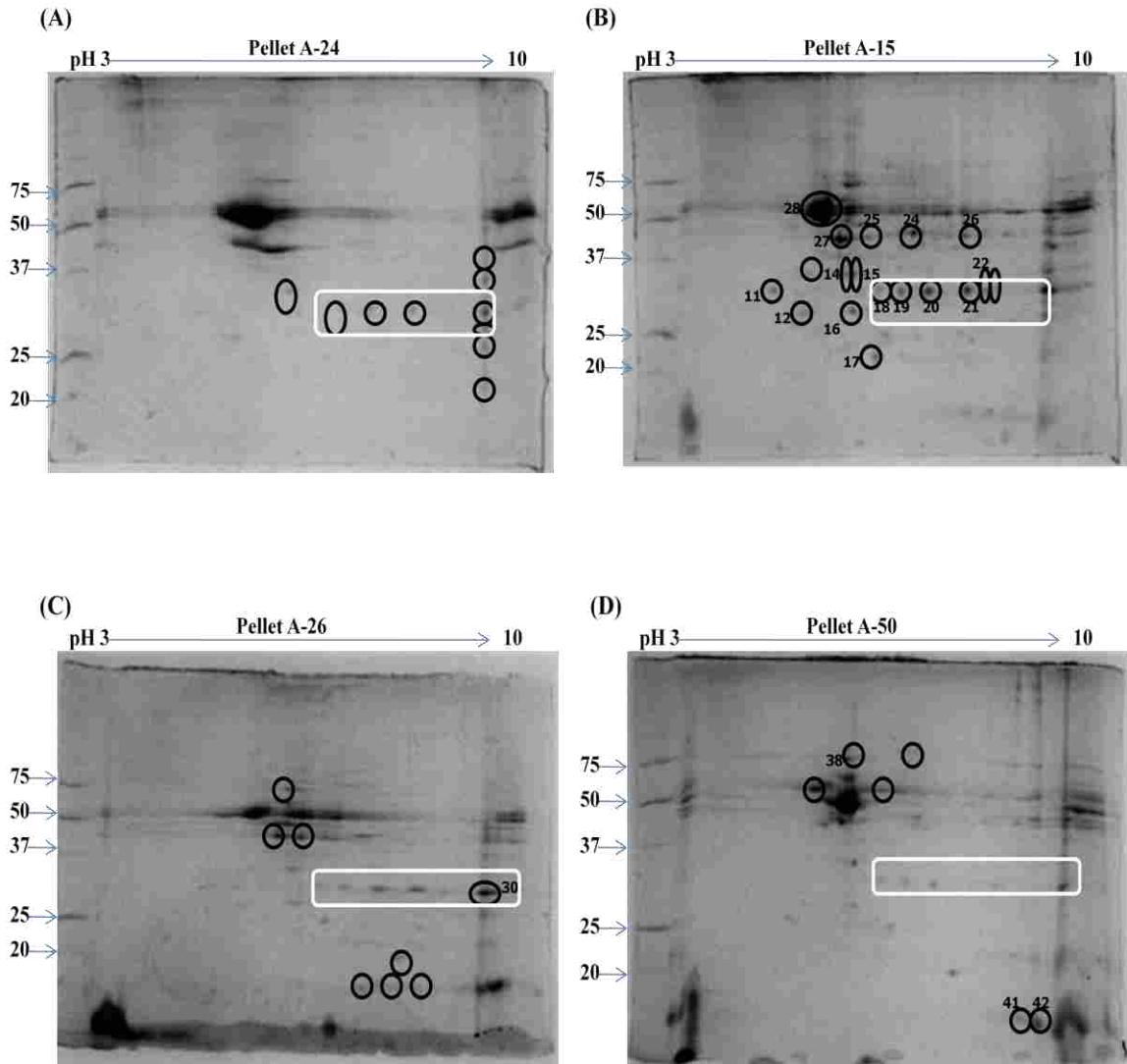
<b>Number</b>	<b>Protein</b>	<b># Unique Peptides</b>	<b>% Coverage</b>	<b>MW (kDa)</b>
1	Tubulin $\alpha$ -1A	31	74	50
2	CNP	32	74	45
3	Tubulin $\beta$ -2A	34	75	50
4	Na <sup>+</sup> /K <sup>+</sup> -ATPase $\alpha$ -3	43	52	112
5	Spectrin $\alpha$	113	56	285
6	Dynein 1 heavy chain 1	125	38	532
7	MBP	19	58	22
8	PLP	8	24	30
9	Neurofilament medium polypeptide	37	53	96
10	Clathrin heavy chain 1	70	55	192
11	Neurofilament light polypeptide	27	60	62
12	Spectrin $\beta$	83	45	274
13	ATP synthase $\alpha$	23	55	60
14	$\alpha$ -internexin	21	53	56
15	Plectin-1B2 $\alpha$	71	23	518
16	Microtubule-associated protein-1B	56	37	270
17	Dynamin-1	38	49	96
18	Dihydropyrimidinase-related protein 2	28	69	62
19	Myosin-1D	42	53	116
20	Isoform 4 of Inositol 1,4,5-trisphosphate receptor type 1	47	25	311
21	Glyceraldehyde-3-phosphate dehydrogenase	18	76	36
22	Microtubule associated protein-1A	53	28	300
23	Actin $\beta$	21	65	42
24	NAD-dependent deacetylase sirtuin-2	18	58	43
25	Tubulin $\beta$ -3	9	74	50
26	Syntaxin-binding protein 1	31	65	68
27	Cytokinesis protein 9	45	30	235
28	Fatty acid synthase	42	27	272
29	Adaptor protein-2 complex subunit $\beta$	32	40	105
30	ADP/ATP translocase 1	16	65	33

# Unique peptides- Number of unique peptides identified in protein; % Coverage- Percentage of amino acids identified; MW (kDa)- Molecular weight in kDa.

### **3.3.7 2-D Gel Electrophoresis Separation of Fetal and Adult Bovine Myelin.**

Since there are a number of proteins identified from the above 1-D and 2-D gel analysis, and whole myelin proteome analysis of adult bovine and mouse. Therefore, need arises to resolve further low abundant proteins. Another proteomic technique used for this study is 2-D gel electrophoresis using an IPG strip. This technique results in a more effective analysis, since there is a high degree of resolution within a specific pH range. IPG-IEF combined with SDS-PAGE is a powerful technique which separates complex mixtures.

A clear resolution of protein spots were observed on the second dimension SDS gel (Fig 3.9). Pellet fraction A-15 shows a high amount of protein spots present followed by A-26 and A-50 which are very close and then A-24. There are also some unique spots which are marked 18-22 in the white horizontal box around 30kDa, which were observed at all ages in bovine myelin sample, showing approximately the same molecular weight but with different pI values. These spots were identified as voltage-dependent anion-selective channel (VDAC) protein isoforms from mass spectrometric analysis. Vertical streaking was observed at pH 10 because proteins with pI values of 10 and higher run at the side of the gels. The protein spots which are circled were excised, trypsin digested and the spots, which are numbered, were analysed by LC/MS/MS for further protein identification. A total of 214 proteins were identified from 20 specific spots. The major proteins present in each spot are listed in Table 3.5.



**Figure 3.9** – 2-D gel electrophoresis of pellet fractions of fetal and mature bovine myelin of different ages. Samples were first extracted with 1.5% CHAPS followed by second extraction with 2% TX-100. First dimension (IPG-IEF) was performed using IPG strips, pH 3-10, run on Protean Isoelectric Focusing System (Bio-Rad). Second dimension SDS-PAGE was performed on 12% resolving mini-gel followed by Coomassie staining. (A), (B), (C), and (D) are the gel images with pellet samples A-24, A-15, A-26, and A-50 respectively. Spots which are marked were excised and trypsin digested for mass spectrometric analysis.

**Table 3.5-** The proteins in the detergent resistant fraction that were identified from 2-D gel electrophoresis with an IPG Strip. The spot number corresponds to Fig. 3.9. All the proteins with 100% probability, higher % spectra in particular spots and with at least 2 unique peptides identified are listed.

Spot #	Experimental pI	Protein	# Unique Peptides	% Coverage	MW (kDa)
12	5.1	14-3-3 protein, gamma	14	40	28
15	5.9	Guanine-nucleotide binding protein G(I)/G(S)/G(T) subunit beta-1	17	44	37
		Pyruvate dehydrogenase, E1 subunit beta	7	22	39
		Prohibitin	18	60	30
16					
17	6.4	ATP synthase subunit d	19	68	19
18	6.6	VDAC2	13	36	32
19	7	VDAC2	18	50	32
20	7.5	VDAC2	33	54	32
21	8.4	VDAC2	33	54	32
22	8.6	VDAC1	14	37	31
		VDAC2	8	30	32
24	7.2	CNP	7	16	45
		Elongation factor Tu	18	38	49
		Pyruvate dehydrogenase E1 subunit alpha	15	30	43
		Protein pelota homolog	10	25	44
25	6.4	Succinyl CoA ligase subunit beta	12	27	50
26	8.3	Citrate synthase	13	29	52
27	5.8	Actin, gamma	25	70	42
28	5.4	Tubulin beta-2A	33	75	50
		Tubulin alpha-1A	25	63	50
		ATP synthase subunit beta	22	62	52
30	10	VDAC1	20	61	31
38	6.2	Heat shock cognate 71kDa	35	44	71
41	9	Tubulin, beta-2A	4	9	50
		MBP	3	11	36
42	9.4	MBP	6	27	36

# Unique peptides- Number of unique peptides identified in protein; % Coverage- Percentage of amino acids identified; MW (kDa)- Molecular weight in kDa; pI- Approximate pI from the gel.



### 3.4 Discussion

Several detergent sequences and concentrations were applied to myelin to isolate the radial component and determine the proteome of this unique structural microdomain. The detergents chosen are currently used in membrane protein research, and have been applied to the solubilisation of myelin for 2-D gel electrophoresis <sup>29</sup>. As discussed in section 1.4.2, the structure of the radial component is well preserved in brain tissue treated with TX-100. Most researchers isolate lipid rafts from the plasma membrane with the cold nonionic detergent (typically TX-100), followed by separation on sucrose density gradient. In this study a differential detergent extraction methods were used to isolate a fraction containing lipid raft microdomains using CHAPS and then the remaining pellet was treated with 0.5%, 1%, or 2% TX-100 preserving the radial component. The radial component proteins remain intact with the CHAPS and TX-100 sequence of extraction. This fraction was found to be significantly and consistently enriched in 21.5, 18.5 and 17kDa MBP isoforms as well as myelin associated enzyme CNP, and the cytoskeletal proteins actin, and tubulin. OSP/claudin-11, a specific marker protein of this structure, was also identified from mass spectrometry results. The presence of these proteins was confirmed by Western blotting (see Chapter 5). Furthermore, the radial component proteome is comprised of many other structural, signaling, and enzymatic proteins.

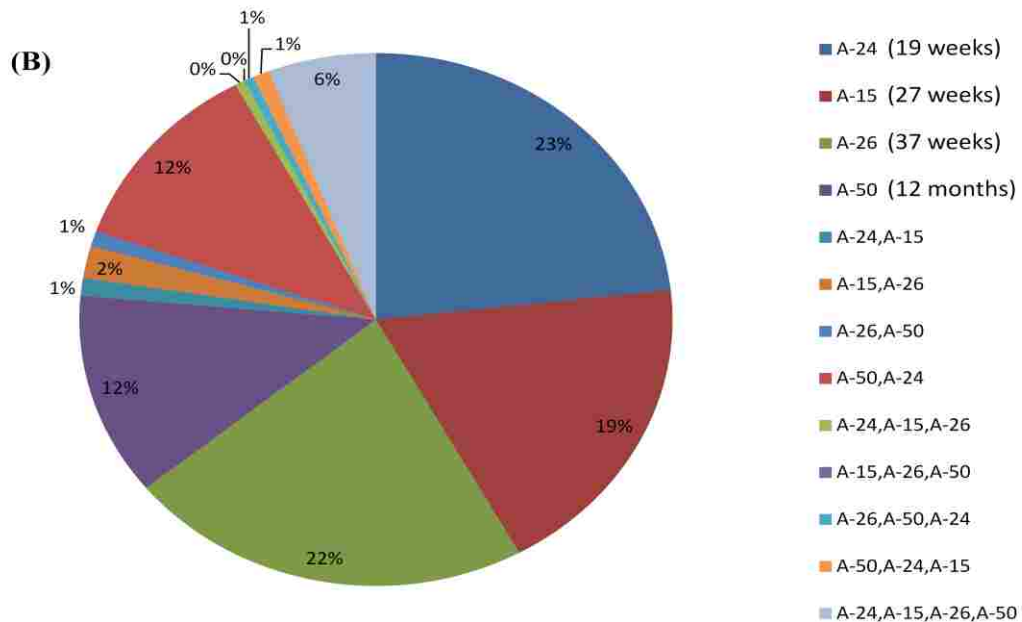
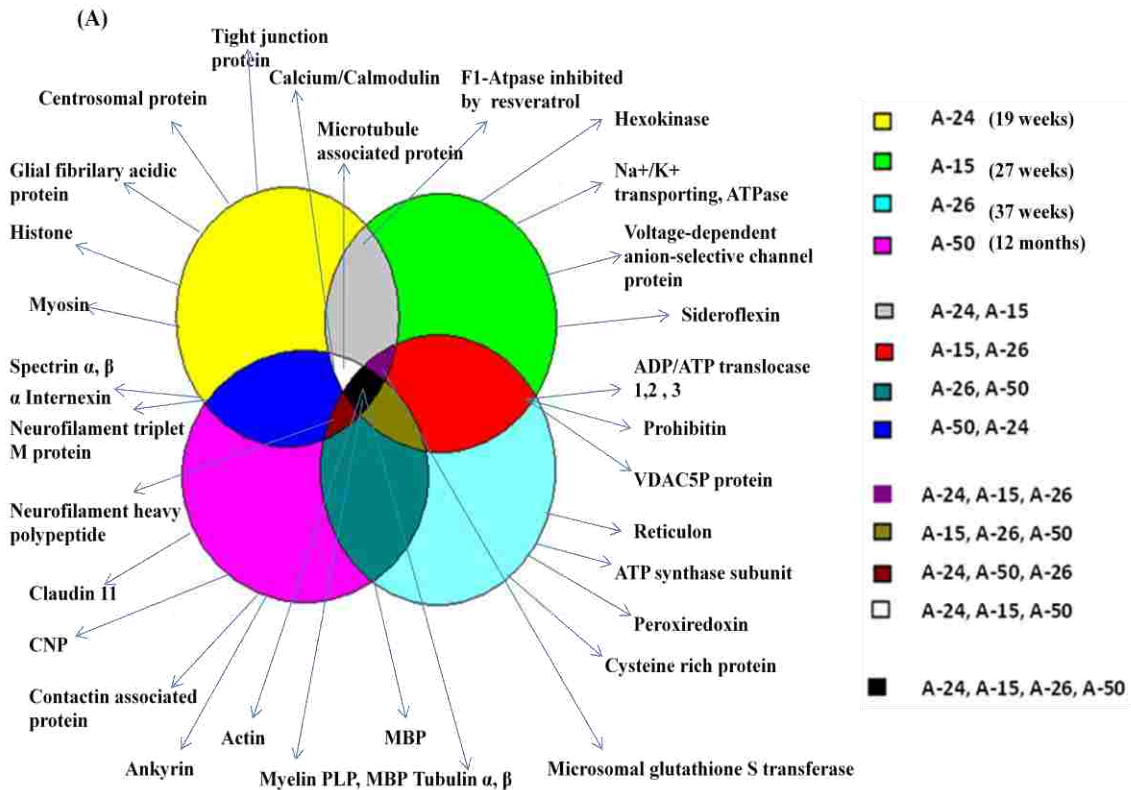
Several nonionic and zwitterionic detergents tested to solubilise the pellet remaining after CHAPS extraction. An important property of detergents is their formation of micelles. As discussed in section 2.2, nonionic and zwitterionic detergents are less denaturing and can be used to solubilise membrane proteins while retaining protein-

protein interactions. Critical micelle concentration (CMC) is the concentration above which surfactants form micelles and all additional surfactants added to the system will become micelles<sup>30</sup>. The CMC of the zwitterionic detergent, CHAPS, is 6-10mM with an average aggregation of 10 molecules per micelle is effective to solubilise more of lipids. CMC value of TX-100 is 0.2-0.9mM with an aggregation of 100-155 molecules per micelles whereas Tween-20, C<sub>12</sub>E<sub>9</sub>, and ASB-14 are 0.06mM, 0.08mM, and 8mM, respectively. The detergent concentrations in this study were above the CMC for each. The CHAPS supernatant and subsequent TX-100 pellet contained a significant amount of proteins. Further extractions with Tween-20, C<sub>12</sub>E<sub>9</sub>, or ASB-14 were not effective to solubilise any more protein from the radial component. The most effective detergent sequence for the isolation of radial component found was CHAPS followed by TX-100 with optimal concentrations of 1.5% CHAPS and 2% TX-100.

The detergent resistant fraction of myelin at all stages of myelination expressed several MBP isoforms. At the mid-myelination stage, MBP (21.5 and 18.5 and 17kDa isoforms) were found exclusively in the pellet fraction. As myelination continued, the 18.5kDa isoform was distributed into the supernatant fraction, whereas the 21.5kDa isoform remained in the pellet fraction. At the late myelination stage, some of the MBP isoforms 18.5, 17, and 14kDa were detected in the supernatant. The supernatant fraction contains the lipid rafts, as well as the completely detergent solubilised membrane proteins. The distribution of actin and CNP was similar to MBP, whereas tubulin and OSP only became associated with radial component during the maturation of myelin. Histone cluster proteins were detected at high levels during the early myelin developmental stage. Typically, these proteins are found in the nuclei of the cell that

package and order the deoxy ribonucleic acid (DNA) into structural units. Histones are components of chromatin that act as spools around which DNA winds, and play a role in gene regulation<sup>31, 32</sup>.

Performing large-scale proteomic analysis of the detergent resistant radial component from 1-D SDS-PAGE identified that this microdomain is highly enriched in proteins. There are a total 558 high and low abundant bovine proteins identified from LC/MS/MS results of which 77 are highly abundant with 100% probability and with at least 7 unique peptides identified (see Table 3.2). Proteins identified from 1-D SDS-PAGE can be grouped into several functional categories: 1) cytoskeletal proteins such as actin, tubulin, dynactin, glial fibrillary acidic protein (GFAP), microtubule associated protein, myosin, neurofilament polypeptides, and spectrin; 2) myelin specific proteins such as CNP, MAG, MOG, PLP, and MBP; 3) tight junction proteins such as OSP/ claudin11 and ZO-1; and 4) mitochondrial-type proteins such as ATP synthase, succinyl-CoA ligase, ADP/ATP translocase, calcium binding mitochondrial carrier protein, cytochrome c oxidase/reductase, NADH dehydrogenase, prohibitin, VDAC, and pyruvate dehydrogenase. These proteins are directly or indirectly importance to the growth and maintenance of myelin. Many of the proteins identified are cytoskeletal and associated with membrane trafficking including endocytosis and exocytosis.



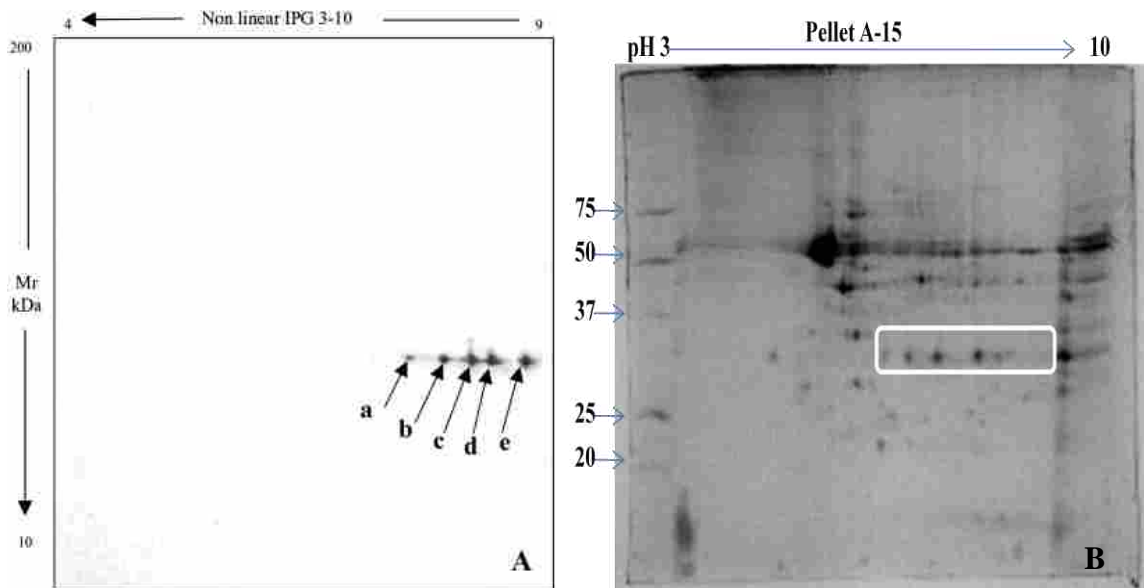
**Figure 3.10** – Proteins identified from fetal and mature bovine myelin of different ages by LC/MS/MS. (A) Venn diagram shows the high and low abundant proteins which are common between bovine myelin of different ages and which are present in each pellet fraction. (B) Pie-chart shows the percentage of proteins present in each pellet fraction and which are common between each other.

The Venn diagram shows the high and low abundant proteins identified by LC/MS/MS from 1-D SDS-PAGE in fetal and mature bovine myelin (Fig. 3.10 (A)). Pie-chart percentages of proteins which are unique to different ages show that 23% in A-24, 19% in A-15, 22% in A-26, and 12% protein in A-50. 12% of proteins were common between A-24 and A-50 and 6% of proteins were common among each pellet fraction (Fig. 3.10 (B)). Pooling several MS analyses, results indicated that all bovine samples have MBP, PLP, tubulin, actin, GFAP, and CNP as major proteins of radial component.

Various proteomic techniques have been applied towards the systematic protein composition analysis of the detergent resistant fraction. A number of proteins were determined from 1-D gel analyses. However, a need arises to resolve them further by utilizing solution-phase IEF because many could not be definitively identified by 1-D technique. As seen in Fig. 3.8, high amounts of proteins were found in fraction F5-F8, pI values between 6.7-8.1 followed by F9 and F10, pI values 9.0 and 9.9, respectively, which are basic in nature. Very few proteins are present in the acidic range with pI values between 3.09-5.44. In general most proteomes contain a majority of proteins in the pI 6-8 range. The wide range of pI values might be because proteins undergo post translational modification. Fraction (F5) band number 13 was highly abundant and was identified as  $\alpha$ - and  $\beta$ -tubulin from MS analysis. Proteomic analysis was surveyed the major bands from the gel. The resultant characterization by LC/MS/MS yielded 197 proteins. The major proteins present in each band are listed in Table 3.3 and includes tubulin, GFAP, spectrin, neurofilament light, medium and heavy polypeptide, actin, and VDAC.

A high degree of resolution is achievable with 2-D gel electrophoresis especially with cytosolic or soluble proteins. However, membrane and highly hydrophobic proteins

may be under-represented since these proteins tend to precipitate during IEF, which prevents the transfer of proteins to the second dimension SDS-PAGE analysis. To ensure the complete solubilisation of the pellet fraction, the IEF solution contained both CHAPS and ASB-14 as well as two chaotropic agents, urea and thiourea. This combination is effective in solubilising membrane proteins in brain tissue. The 2-D gel analysis of the pellet fractions from the different developmental bovine myelin samples indicated in each a series of 5-7 spots around 30kDa. Mass spectrometric analyses identified these protein spots as VDAC proteins. It exists in three different isoforms VDAC1, VDAC2, and VDAC3 and VDAC proteins can undergo post translational modification. Due to post translational modifications of these isoforms, the proteins with phosphorylation of Ser/Thr/Tyr and/or acetylation of lysine can appear over a range of pI values on the gel.



**Figure 3.11-** Comparison of 2-D gels from synaptosomes and myelin indicating VDAC isoforms. (A) 2-D immunoblotting of guinea pig brain synaptosomes with anti-VDAC antibody<sup>33</sup>. (B) 2-D gel electrophoresis of pellet fraction of fetal bovine myelin A-15 extracted with 1.5% CHAPS followed by 2% TX-100. 1-D (IPG-IEF) was performed using IPG strip, pH 3-10, run on Protean Isoelectric Focusing System (Bio-Rad). 2-D SDS-PAGE was performed on 12% resolving mini-SDS gel followed by Coomassie staining.

2-D electrophoresis is the most widely used method to define complex isoform patterns, including VDAC proteins. Liberatori and colleagues identified VDAC1 and VDAC2 isoforms in guinea pig brain synaptosomes by combining 2-D immunoblotting with anti-VDAC specific antibodies and MALDI-TOF MS (Fig. 3.11 (A))<sup>33</sup>. Synaptosomes were obtained from guinea pigs using discontinuous Ficoll gradient of 12% and 7%. Proteins were separated by 2-D electrophoresis using non-linear IPG strips (pH 3-10) and immunoblotted on nitrocellulose membrane and tested with commercially available anti-VDAC antibodies (Fig. 3.11 (A)). Five different spots of VDAC were reported with the same molecular weight but with different pI values (see Table 3.6). This can be compared directly with the myelin radial component. Fig. 3.11 (B) illustrates the 2-D gel electrophoresis of pellet fractions of fetal bovine myelin A-15 extracted with 1.5% CHAPS and 2% TX-100.

**Table 3.6** - Comparison of the VDAC isoforms identified in synaptosomes and the radial component from 2-D gel electrophoresis. Data for the synaptosomes were taken from reference (# 32). Data for the myelin radial component was compiled from Fig. 3.9.

Synaptosomes				Myelin radial component			
Spot	MW (kDa)	pI	ID	Spot	MW (kDa)	pI	ID
a	33	6.39	VDAC 2				
b	33	6.73	VDAC 2	18	32	6.6	VDAC2
c	33	7.04	VDAC 2	19	32	7.0	VDAC2
d	33	7.39	VDAC 1	20	32	7.5	VDAC2
e	33	8.26	VDAC 1	21	32	8.4	VDAC2
				22	31	8.6	VDAC1
				30	31	~10	VDAC1

MW (kDa)- Molecular weight in kDa; pI- Approximate pI from the gel; ID- Protein ID.

As discuss in section 3.3.7, and as seen in Fig. 3.11 (B), five unique spots in the horizontal white box around 30kDa were identified in all myelinating ages. The VDAC isoform identification obtained from LC/MS/MS and the approximate pI values listed in Table 3.6. Both guinea pig brain synaptosomes and bovine myelin radial component

contain VDAC1 and VDAC2. Since several spots indicate VDAC1 and VDAC2, these isoforms contain post translational modifications. Furthermore, there is some variation between guinea pig synaptosome and bovine myelin VDAC isoforms as different pI values are observed. From mass spectrometric analysis of the bovine radial component, several of the VDAC peptides from the 2-D gels were identified as acetylated; however, the acetylation could not be assigned to a specific lysine. Acetylation of a lysine residue would result in a VDAC isoform that is less basic.

Recently, Kerner *et al.* reviewed the different post translational modifications of VDAC in mitochondria<sup>34</sup>. More than one-third of the proteins are phosphorylated at one or multiple sites. Reversible post translational modifications affect enzymatic activity and have an impact on degradation of the proteins and translocation of the proteins from one compartment to another within the cell. Protein kinases catalyze phosphorylation of proteins most frequently at serine, threonine, and tyrosine residues. The reverse reaction is catalyzed by specific protein phosphatases. Another frequent post translation modification is acetylation. They are of two types; N-terminal acetylation which is irreversible and lysine side chain acetylation which is reversible. N-terminal acetylation is catalyzed by N-acetyltransferases and occurs on the N-terminal methionine. Reversible acetylation of lysine amino groups is catalyzed by protein acetyltransferases and deacetylases or histone deacetylases. They are grouped into class I and II histone deacetylases and class III acetylases are termed as sirtuins<sup>34</sup>. Sirt2 was consistently identified in the radial component proteome.

More than 50% of proteins identified in radial component had been previously identified in myelin. Some mitochondrial-type proteins were confirmed in this study by



Western blotting (see Chapter 5). Ishii *et al.* analyzed the human and mouse myelin proteomes using the shot gun approach of 1-D PAGE and LC/MS/MS<sup>35</sup>. The resultant characterization by LC/MS/MS on combining identified proteins from duplicate samples yielded a total of 678 proteins in human myelin fractions and 515 proteins from mouse myelin fractions of which 308 proteins overlapped between human and mouse myelin. This indicates that mice can be used as human models for myelin studies. These proteins were divided into various functional groups of classical myelin, cytoskeletal proteins, metabolism, glycolysis, endoplasmic reticulum, vesicular transport, cell adhesion, signaling, and mitochondrial proteins. The most abundant proteins found in human and mouse myelin fractions were classical myelin proteins, CNP, MAG, MOG, MBP, PLP, OSP/claudin11<sup>35</sup>.

In this thesis, a shot-gun proteomic approach on detergent resistant pellets of adult mouse and bovine myelin was performed using ProteaseMAX. The resultant characterization by MudPIT analysis yielded 472 proteins in bovine and 881 proteins in mouse myelin. The result obtained has high similarity with some differences. First 30 of the most abundant proteins with 100% probability and with higher % spectra are listed in Table 3.4 of which 22 proteins were overlapped between adult bovine and mouse myelin. GFAP is most abundant in bovine myelin and tubulin in mouse myelin. Proteins identified using MudPIT analysis include proteins previously been identified. Some of the most abundant include classical myelin proteins (CNP, MBP, PLP), cytoskeletal proteins (actin, tubulin, dynamin, and spectrin), ion transport  $\text{Na}^+/\text{K}^+$ -ATPase  $\alpha$ , and mitochondrial protein ADP/ATP translocase and ATP synthase. Significant levels of

hexokinase were found in bovine samples, whereas plectin,  $\alpha$ -internexin and cytokinases were abundant in mouse myelin radial component.

Dynamin is a microtubule-associated force-producing protein involved in producing microtubule bundles and is able to bind and hydrolyze guanosine triphosphate (GTP). Furthermore, dynamin is involved in the scission of newly formed vesicles from the membrane of one cellular compartment and targeting and fusion with another compartment.

Plectin acts as a link between the three main components of the cytoskeleton, actin microfilaments, microtubules, and intermediate filaments<sup>36</sup>. Besides forming a linker protein, plectin also is involved in connections between other cytoskeletally-related proteins. Desmosomes and hemidesmosomes, which link intermediate filament, have been shown to associate with plectin.

$\alpha$ -internexin is a class-IV neuronal intermediate filament protein that coexists with the neurofilament triplet proteins. In adult cells of the CNS,  $\alpha$ -internexin is found in the cytoplasm of neurons. It is involved in neuronal development and plays a role in axonal outgrowth<sup>37</sup>.

Hexokinase is an enzyme that phosphorylates hexoses, forming hexose phosphate. Mainly glucose phosphorylation where glucose is the most important substrate of hexokinases and glucose-6-phosphate is the most important product. Hexokinase isoforms I and II bind to mitochondrial outer membrane by interacting with the outer membrane VDAC<sup>38</sup>.

Monasterio-Schrader *et al.* provide meta-analysis of CNS myelin protein composition as identified by various proteomic studies. The integration of all myelin

proteome datasets yielded 1,280 proteins<sup>39</sup>. Decades ago it was believed that the number and variety of myelin proteins is very low because very few bands were visible upon 1-D gel separation and subsequent protein staining. These are mainly PLP, MBP, and CNP. The use of gradient-gels and/ or 2-D gels together with MS has turned out to be the most sensitive method to identify the protein constituents of myelin. Recently, the number of proteins identified per approach increased because of the advancements in proteomic methodology and MS instrumentation. Reproducibility of MS-based myelin protein analyses showed that more than half (52%) of the proteins were identified in at least two datasets which indicates its presence in CNS myelin<sup>39</sup>. Reproducibility was 100% in the earlier approaches, in which less than 150 proteins were identified and above 60% among the recent approaches with over 350 proteins identified. The proteins identified in all 10 different approaches are heat shock 70kDa protein, MBP, CNP, sirtuin 2, septin 8, and dihydropyrimidinase-like 2. Again, all these proteins were also identified in the radial component of myelin. GFAP which was identified as a major component in the bovine radial component, is a major intermediate filament of astrocytes; however, recent mRNA studies indicate GFAP is a myelin protein. Overall, the results obtained from radial component proteome are consistent with myelin proteomic analyses.

## **Chapter 4 – Confirmation of VDAC and other proteins in the myelin membrane and its radial component**

### **4.1 Introduction**

This chapter focuses on some of the important proteins which were identified from proteomic analysis of radial component. The proteins identified were further characterized and confirmed by Western blot. Percoll gradient separation of mouse myelin was also employed; fractions of myelin, synaptosomes, and mitochondria were analyzed to confirm whether VDAC, sodium-potassium adenosine triphosphatase ( $\text{Na}^+/\text{K}^+$ -ATPase), and adenosine triphosphatase (ATP) synthase are significant components of myelin.

#### **4.1.1 Voltage-Dependent Anion-Selective Channel Protein**

VDAC protein is a channel forming protein originally found in the outer mitochondrial membrane. VDAC is known to facilitate free permeability to low molecular-weight solutes across the outer mitochondrial membrane<sup>40</sup>. It is a small 30-35 kDa protein that exists in three different isoforms; VDAC1, VDAC2, and VDAC3. The structure of VDAC is known to be a  $\beta$  barrel shape composed of 19 antiparallel  $\beta$ -strands. Both VDAC1 and VDAC2 are able to form pores in lipid bilayers but there is no evidence of pore-forming for VDAC3<sup>41</sup>. Permeability of anions through the channel is greater than cations, thus providing a major pathway for ADP/ATP, succinate, and citrate which are exchanged between cytosol and mitochondria. In the mitochondrial membrane, the channel exhibits two different states (open or closed) depending on the transmembrane potential when reconstituted into a planar lipid bilayer. At zero or low membrane potential the channel remains in a high conductance state (open), whereas the

closed state is observed when the voltage exceeds to 20-30mV<sup>40, 41</sup>. VDAC has been originally identified as a mitochondrial protein; however, recent evidence showed that VDAC can also be present in the plasma membrane<sup>40, 42, 43</sup>. In the presence of nicotinamide adenine dinucleotide (NADH), VDAC in the plasma membrane is more sensitive to changes in applied voltage<sup>42</sup>. Baker *et al.* proposed that plasma membrane VDAC1 can function as a redox enzyme; it reduces extracellular ferricyanide in the presence of intracellular NADH<sup>42</sup>. Thus, VDAC1 has been characterized as a NADH: ferricyanide reductase. This can lead to transport of electrons across the plasma membrane.

#### **4.1.2 Sodium-Potassium Adenosine Triphosphatase**

Na<sup>+</sup>/K<sup>+</sup>-ATPase, also known as sodium-potassium pump, is an enzyme located in the plasma membrane that regulates the transport of sodium and potassium ions across the cell membranes by utilizing the energy released from the hydrolysis of ATP. Concentrations of both these ions across the membrane are interdependent as the same carrier transports both ions. The Na<sup>+</sup>/K<sup>+</sup>-ATPase helps in maintaining resting potential, exchange of substances between the cell and its surroundings, and the cell volume. The presence of this enzyme in epithelial cells allows the transport of salt and water across the cell and thereby maintains osmotic balance between inner and outer compartments<sup>44</sup>. Na<sup>+</sup>/K<sup>+</sup>-ATPase consists of two subunits,  $\alpha$  and  $\beta$  polypeptides, and aggregates of a large number of lipid molecules. The  $\alpha$  subunit contains an active catalytic site which undergoes phosphorylation. The  $\alpha$  subunit has three isoforms: isoform 1 is an ouabain-resistant and found in all cells, and isoforms 2 and 3 are ouabain-sensitive, with  $\alpha 2$  being distributed in skeletal muscle, heart, brain and  $\alpha 3$  in the neural tissue. The  $\beta$  subunit, a

glycosylated polypeptide, is important for the insertion of the  $\alpha$ - $\beta$  complex in the plasma membrane <sup>44</sup>.

Association of several enzymes with CNS myelin has been reported in the past. It is believed that ion movements via enzyme-dependent pathways may be an integral myelin function, as well. Early studies attributed  $\text{Na}^+/\text{K}^+$ -ATPase activity in myelin preparation to contamination by other membranes. However, Reiss *et al.* used myelin and microsomal fractions isolated from rat brains to analyze  $\text{Na}^+/\text{K}^+$ -ATPase activity associated with myelin <sup>45</sup>. The difference in ATPase activity in both these fractions was measured in the presence and absence of ouabain inhibitor. The  $\text{Na}^+/\text{K}^+$ -ATPase activity of myelin fractions prepared from rat brain showed threefold enrichment in activity compared to whole homogenate. This indicates that ATPase activity is due to myelin and not the microsomal contamination <sup>45</sup>.

#### **4.1.3 ATP synthase**

ATP synthase is located within the inner mitochondrial membrane. It is an important enzyme that provides energy for the cell through the synthesis of ATP. ATP synthase consists of two regions;  $F_0$  within the membrane and  $F_1$  above the mitochondrial membrane, inside the mitochondria.  $F_1F_0$  ATP synthase is responsible for the ATP synthesis in all living beings <sup>46</sup>. ATP synthase is composed of different unique subunits. The  $F_0$  domain uses the proton gradient to synthesised ATP from adenosine diphosphate (ADP) and inorganic phosphate. In the absence of the  $F_0$  domain, the ATP synthase enzyme behaves as an ATP hydrolase which is prevented by the  $F_1$  domain <sup>46</sup>. Recently, it has been reported that ATP synthase is present in the plasma membrane. On the surface of hepatocytes, ATP synthase “moonlights” as a high affinity receptor for apoA-1, the

major protein component in high density lipoproteins. Furthermore, ATP synthase subunits are found in the lipid rafts <sup>46</sup>.

## **4.2 Proteomic Techniques**

### **4.2.1 Western Blotting**

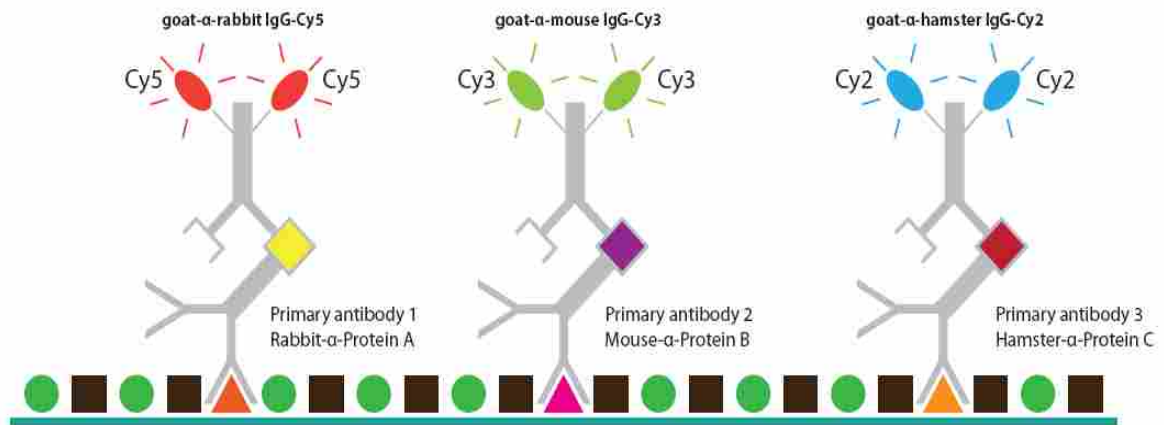
Western blot analysis is an extremely useful analytical technique which is used to detect specific proteins in a complex sample of tissue homogenate or extract. It uses gel electrophoresis to separate native or denatured proteins by the 3-D structure or by the length of the polypeptide. One of the important steps in the Western blot technique is the transfer of proteins from the gels onto fix solid support membranes made of nitrocellulose or polyvinylidene difluoride (PVDF) which make the proteins available to specific probes and allow quantitative detection. Proteins are transferred to the membrane by electrophoretic methods. There are mainly two types of electrophoretic transfer procedures, wet transfer and semi-dry transfer. In both these methods, the gel and membrane are sandwiched between filter papers wetted with buffer. In wet transfer, the gel sandwich cassette is submerged under transfer buffer in a tank, and in a semi-dry transfer, the gel sandwich cassette is in direct contact with the flat plate electrodes. An electric current pulls proteins from the gel onto a membrane. As a result, the proteins are exposed on a thin surface layer for detection. Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and proteins. During detection, the membrane is incubated in a primary antibody solution which binds to a specific protein, followed by incubation with a secondary antibody conjugated to an enzyme such as horseradish peroxidase or alkaline phosphatase which binds the primary antibody. Proteins on membranes are visualized using an appropriate chromogenic

substrate or chemiluminescence substrate to enhance the signal. For a variety of reasons, this traditionally takes place in a two-step process, although there are now one-step detection methods available for certain applications.

#### **4.2.2 Multiplex Fluorescent Western Blotting**

Multiplex fluorescent detection allows the detection and analysis of multiple proteins on a single Western blot. Chemiluminescence is a well characterized technique for sensitive detection of proteins <sup>47</sup>. However, this method can present limitations in applications where multiple proteins are of interest. For analysis of multiple targets, the blot must be stripped and reprobed for additional targets of interest <sup>47</sup>. Reprobing is time consuming, and some of the target protein on the blot is lost as a result of the stripping procedure. These limitations can be overcome by using direct fluorescent labeling of the secondary antibodies, combined with fluorescent light detection. The direct fluorescent detection of the antibodies allows for excellent sensitivity, linearity, and dynamic range <sup>48</sup>. Additionally, direct fluorescent detection allows for multiplex applications in which multiple proteins can be detected simultaneously on the same blot by the use of secondary antibodies labeled with different fluorescent dyes (Fig. 4.1). Each dye is selected to have unique spectral properties so that with proper selection of excitation and emission wavelengths, they can be independently excited and imaged.



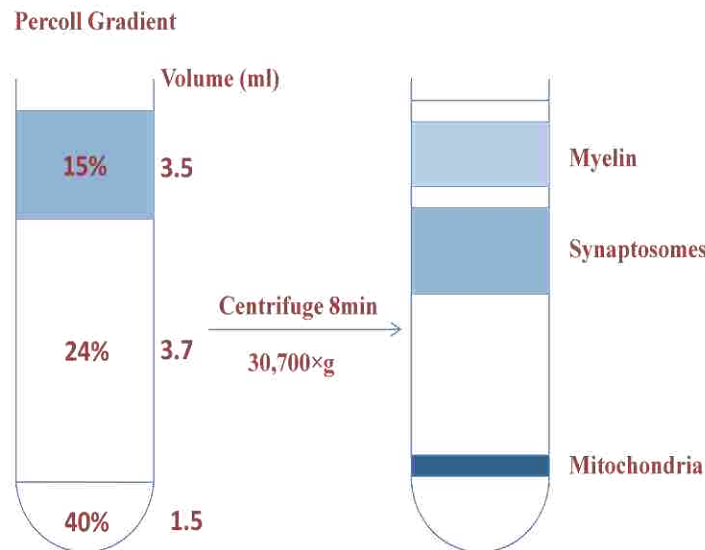


**Figure 4.1** – Fluorescent Western blot detection. Three primary antibodies bind to three independent antigens (triangles) on a membrane. Each primary antibody is bound by a secondary antibody that has been directly labeled with a Cyanine dye, Cy5, Cy3, or Cy2<sup>48</sup>.

#### 4.2.3 Isolation of Myelin via Percoll Density Gradient Centrifugation

For most tissues, myelin isolation procedures are based on two sucrose density gradient centrifugation steps each followed by hypoosmotic shock. This is able to generate an enriched myelin fraction that can be directly used for studies. This method has also been shown to produce myelin free of other cell structures<sup>6, 21, 49</sup>. However, previous proteomic research suggests that myelin may be contaminated with some mitochondrial proteins. Mass spectrometric analysis of insoluble fractions of radial component in chapter 3 shows the presence of some mitochondrial proteins, including VDAC. The need arises to obtain mitochondria-free myelin which can be done through Percoll density gradient centrifugation. Percoll is used as a tool for more efficient density separation. It is used for the isolation of cells, organelles, and viruses by density centrifugation. Percoll consists of colloidal silica particles of 15-30nm in diameter which have been coated with polyvinylpyrrolidone<sup>50</sup>. The method employed for isolation of myelin via Percoll gradient centrifugation was based on the method of Kristian (for

complete procedure in detail, please see Appendix 6) <sup>50</sup>. Here, Kristian describes a protocol for isolating brain mitochondria by using Percoll gradient centrifugation. Mitochondria were then purified further by resuspension in an isolation medium followed by centrifugation at high speed. Synaptosomes require further purification. Mitochondria can be separated from synaptosomes by resuspending the synaptosome fraction in 2ml isolation medium and disrupting synaptic membranes by applying high-pressure nitrogen decompression. The material from the nitrogen cavitation vessel is layered onto 24% Percoll followed by centrifugation. Further, the pellet containing free synaptic mitochondria is resuspended in 6ml isolation medium followed by centrifugation. The pellet is centrifuged again by resuspending in 0.5ml (10mg/ml) fatty-acid free bovine serum albumin (BSA) and 4.5ml isolation medium. Final synaptic mitochondria pellet is resuspended in isolation medium without ethylene glycol tetraacetic acid (EGTA).



**Figure 4.2** – Schematic representing the Percoll gradient and the distribution of brain homogenate fractions following centrifugation. Figure modified from Kristian <sup>50</sup>.

## **4.3 Experimental Procedure**

### **4.3.1 Western Blotting**

Mouse and bovine myelin fractions separated by SDS-PAGE were electrophoretically transferred onto nitrocellulose membrane (Bio-Rad) using tris-glycine buffer, pH 8.3 (for complete procedure in detail, please see Appendix 7). In this study, the wet transfer method was used, and electrotransfer was completed by using a constant current at 200mA for 2hr in cold temperature. Once proteins were transferred to membranes, the membranes were blocked in 5% skim milk solution for 2 hrs with gently shaking. Unoccupied binding sites on the membranes need to be blocked to prevent nonspecific binding of the antibodies which can lead to high background. Blocked membranes were incubated in primary antibody for 2-3hrs followed by incubation with a specific secondary antibody for 1hr (see Table 4.1). After each step, membranes were washed three times with 1X tris buffer saline with tween-20 (TTBS) to remove excess antibody and prevent non-specific binding. Detection of protein was by chemiluminescence using Super Signal West Femto solution (Fisher, Thermo Scientific). The prestained See Blue Plus 2 protein standard (Invitrogen) was used as a molecular weight marker. Proteins on membranes were imaged on VersaDoc 5000 (Bio-Rad) imaging system.

### **4.3.2 Multiplex Fluorescent Western Blotting**

The protocol followed for the multiplex fluorescent Western blotting was similar to the Western blot. The only difference was after incubating in primary antibody, membranes were incubated in specific fluorescently labeled secondary antibodies in the

dark (covered with aluminium foil) for 1hr. Membranes were then air dried and imaged using specific filters with the VersaDoc 4000 (Bio-Rad) imaging instrument.

**Table 4.1-** The primary and secondary antibody used with an appropriate dilution.

<b>Primary antibody</b>	<b>Dilution</b>	<b>Secondary antibody</b>	<b>Dilution</b>
MBP	1:500	Dylight 488 Anti-rat IgG	1:10,000
21.5kDa MBP	1:10,000	Dylight 649 Anti-mouse IgG	1:12,000
$\alpha$ -Tubulin	1:500	Dylight 649 Anti-mouse IgG	1:12,000
$\beta$ -Actin	1:2,000	Dylight 649 Anti-mouse IgG	1:12,000
Na <sup>+</sup> /K <sup>+</sup> -ATPase $\alpha$	1:2,000	Dylight 649 Anti-mouse IgG	1:12,000
ATP synthase/ATP5B	1:200	Dylight 649 Anti-goat IgG	1:12,000
MOBP	1:200	Dylight 649 Anti-goat IgG	1:12,000
Claudin11/OSP	1:200	Dylight 649 Anti-goat IgG	1:12,000
Claudin11/OSP	1:400	Anti-rabbit IgG-HRP	1:80,000
VDAC1	1:200	Anti-goat IgG-HRP or Dylight 649 Anti-goat IgG	1:2,000 or 1:12,000

#### **4.3.3 Isolation of Mouse Myelin via Percoll Density Gradient Centrifugation**

The isolation of myelin using the Percoll gradient method was based on a method described by Kristian with some modifications (for complete procedure in detail, please see Appendix 6). Briefly, two rounds of separation on gradients were performed with myelin samples, followed by water wash to ensure mitochondrial-free myelin. Sorbitol was used in an isolation medium instead of mannitol. On ice, two mouse brains (Rockland Inc.) were homogenized with an isolation medium (225mM sucrose, 75mM sorbitol, 1mM EGTA, 5mM HEPES) in a 2ml Dounce homogenizer with up and down strokes and rotating the Teflon pestle. The final volume of homogenate was adjusted to 10ml, divided into two polycarbonate centrifuge tubes, and centrifuged (1300×g, 3min, 4°C) (Beckman Coulter Allegra 64 R centrifuge, F1010 rotor). This resulted in a soluble fraction supernatant and an insoluble fraction pellet. The supernatant was collected into separate tubes and the pellet was resuspended in the isolation medium and centrifuged

again. Supernatant from both these steps were pooled and centrifuged at high speed (21,000×g, 10min, 4°C). The supernatant was discarded and the pellet was resuspended in 15% Percoll solution.

In two separate polycarbonate tubes, a step density gradient was prepared by adding 24% Percoll solution followed by introducing slowly a 40% Percoll solution to the bottom of the tube. The resuspended pellet in 15% Percoll solution was layered on the top, creating a sharp interface between the 24% and 15% Percoll solutions. The tubes were centrifuged at high speed (30,700×g, 8min, 4°C) using slow acceleration (6sec per 1000rpm) and deceleration (no brake). The purpose of slow acceleration and deceleration is to avoid disturbance of the Percoll gradient. This step redistributed the material into three major bands; myelin being very low in density accumulated at the top of the gradient; synaptosomes at the interface of the upper two layers; and mitochondria being very high in density separated at the interface between the 40% and 24% Percoll gradient (see Fig. 4.2). The myelin obtained was subjected to a second round Percoll gradient separation, followed by ice cold water washes. Mitochondria were concentrated and purified further by resuspending in 6ml isolation medium followed by centrifugation (16,700×g, 10min, 4°C). The loose mitochondria pellet was resuspended in 0.5ml (10mg/ml) BSA and 4.5ml isolation medium followed by centrifugation (6,900×g, 10min, 4°C). The final mitochondria pellet was resuspended in 0.1ml of the isolation medium with no EGTA. Synaptosomes were collected from the interface of the upper two layers, without further purification. The myelin, synaptosomes, and mitochondria were aliquoted and stored at -80°C.

#### **4.3.4 NADH: Ferricyanide Reductase Activity Assay**

Myelin fraction obtained from the above Percoll density gradient centrifugation was extracted with 1.5% CHAPS and 2% TX-100 as in chapter 3 section 3.2.2. The final pellet was resuspended in 5% TX-100 in 2X TBS. NADH: ferricyanide reductase activity was performed on soluble and insoluble fractions (for complete procedure in detail, please see Appendix 8). The assay was performed in a 96 well microtiter plate with optical black bottoms. Blanks consisted of reaction buffer (20mM sodium phosphate buffer, pH 7.5, 0.6% TX-100, 200mM NaCl) with 0.25mM NADH, and 0.25mM potassium ferricyanide. In triplicate, 50 $\mu$ l samples were analyzed. The reaction was initiated with the addition of 0.25mM potassium ferricyanide. The decrease in absorbance at 340nm was recorded as NADH oxidized to NAD<sup>+</sup> with the reduction of ferricyanide for 5min at every 10sec interval. Enzyme activity in samples was calculated by using the extinction coefficient of NADH at 340nm equal to 6220M<sup>-1</sup>cm<sup>-1</sup>.

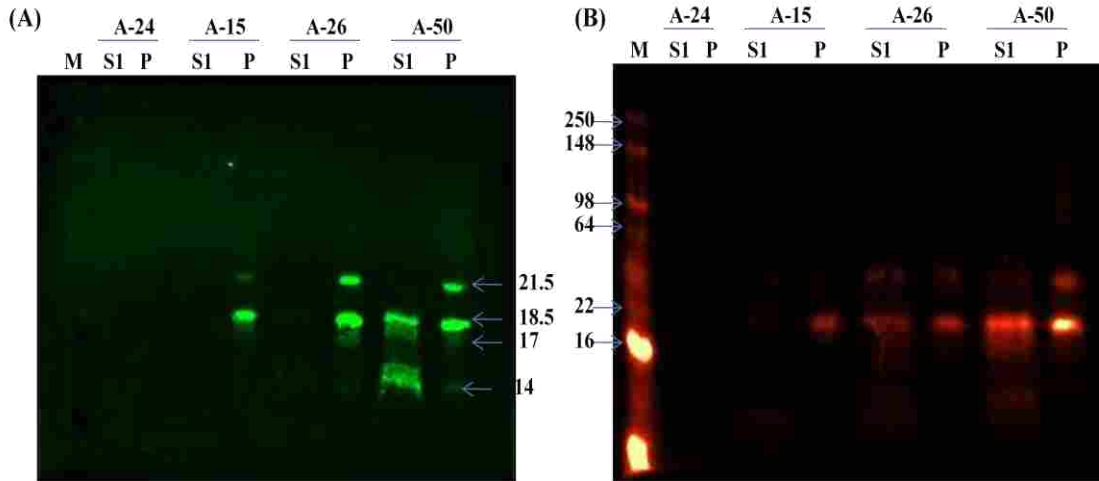
#### **4.4 Results**

Proteomic analysis in chapter 3 shows that there are more than 500 proteins in the radial component as identified by mass spectrometric analysis. Some of the proteins were further confirmed by Western blotting using specific antibodies.

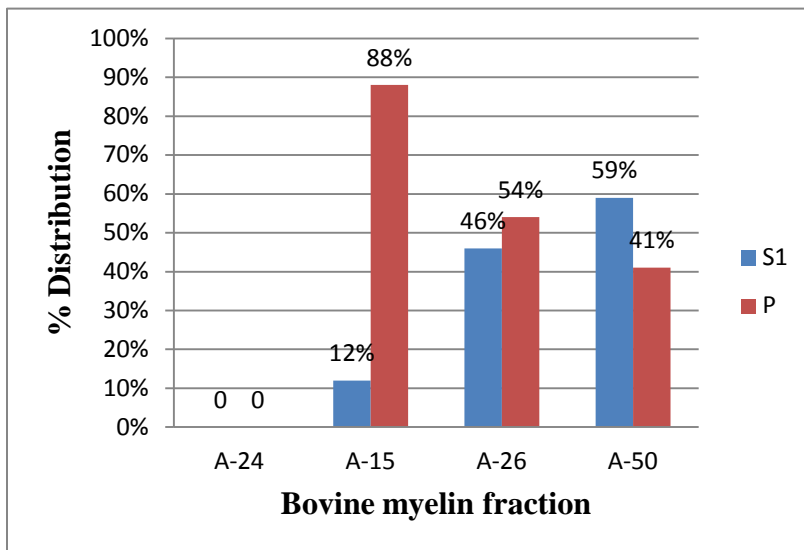
##### **4.4.1 Myelin Basic Protein in Bovine Myelin of Different Ages**

The soluble and insoluble fractions from fetal and adult bovine myelin extracted with 1.5% CHAPS and 0.5% TX-100 were separated on a 1mm thick, 14% resolving, 4% stacking denaturing SDS gel, resolved proteins from gels were transferred to nitrocellulose membrane and incubated with rat and mouse monoclonal MBP antibodies. Rat MBP antibody can detect different isoforms of MBP and mouse MBP antibody

detects specific 21.5kDa and 17.5kDa (exon II containing) isoforms. Membranes were then incubated in specific fluorescently labeled secondary antibodies, Dylight 488 anti-rat IgG and Dylight 649 anti-mouse IgG (Rockland Inc, PA, USA). The specific primary and secondary antibody dilutions are listed in Table 4.1.



**Figure 4.3** – Fluorescent Western blot detection of MBP in bovine myelin of different ages. Samples were separated on a 14% SDS-PAGE and transferred on a nitrocellulose membrane. Equal volumes (20 $\mu$ l) of each fraction were loaded into the well. M- SeeBlue Plus2 prestained marker. S1- Supernatant from the first extraction with 1.5% CHAPS at 4°C. P- Pellet from the second extraction with 0.5% TX-100 at room temperature. (A) Blot labeled with primary antibody rat MBP (Millipore) followed by specific secondary antibody Dylight 488 anti-rat IgG (Rockland Inc, PA, USA) (emission wavelength 518nm). Image was acquired on a VersaDoc 4000 system with a blue LED excitation source and a 530BP emission filter with an exposure time of 200sec. (B) Blot labeled with primary antibody mouse 21.5kDa MBP (abcam) followed by specific secondary antibody Dylight 649 anti-mouse IgG (Rockland Inc, PA, USA) (excitation/emission wavelength 646/674). Image was acquired on a VersaDoc 4000 system with a red LED excitation source and a 695BP emission filter with an exposure time of 400-500sec.



**Figure 4.4** – Percentage distribution of 21.5kDa MBP in fetal and mature bovine myelin fractions. The distribution was determined from the fluorescent Western blot (Fig. 4.3 (B)). Normalization was completed with Image Lab Analysis software.

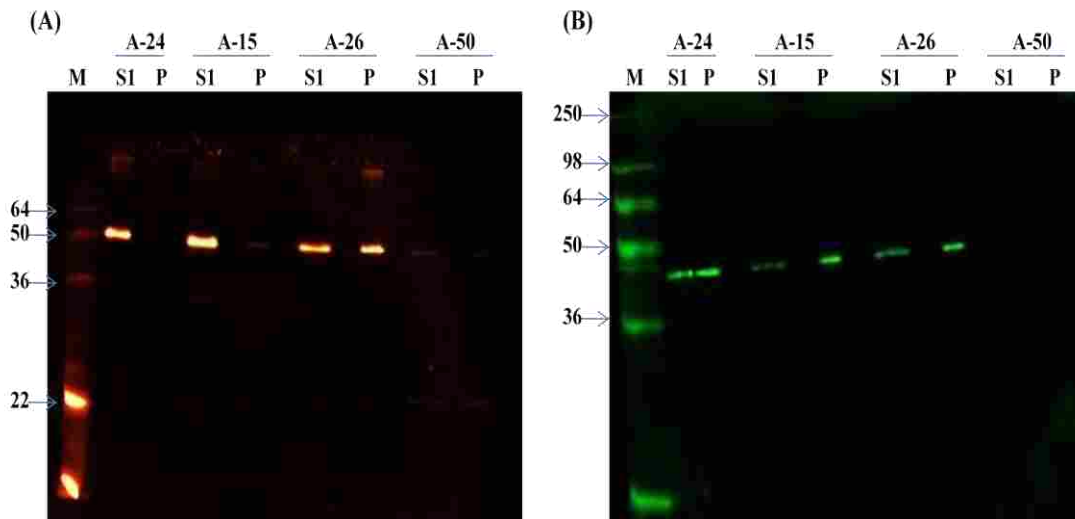
As seen in the Fig. 4.3 (A, B) and Fig. 4.4, MBP expression is very low during early development in fetal samples, (i.e. bovine myelin A-24 which is a 19 weeks old fetus, and A-15 which is 27 weeks old fetus) and expressed at high levels with the increase in age (A-26 which is 37 weeks old fetus and A-50 mature adult myelin). In Fig. 4.3 (A) supernatant and pellet fractions of A-24 does not show any sign of MBP, whereas in A-15, MBP is absent in supernatant originally extracted with 1.5% CHAPS, which is lipid rafts fractions but pellet shows presence of two isoforms of MBP 21.5 and 18.5kDa. In the A-26 supernatant, MBP is present but its intensity is low compared to the A-50 supernatant (Fig. 4.4). In the pellet A-15, only two isoforms of MBP are present, 21.5 and 18.5kDa, compared to A-26 and A-50, which has three isoforms 21.5, 18.5, and 17kDa and low amount of 14kDa. In the supernatants from A-26 and A-50, four isoforms can be seen; 20, 18.5, 17, and 14kDa with minimal resolution between 18.5 and 17kDa isoforms. In Fig. 4.4 (B) again it is confirmed that MBP is absent or present in very low intensity at lower ages and its level increases with the increase in age. It is interesting to see how



MBP becomes distributed in different fractions with age. As seen in Fig. 4.4, fetal bovine at an early stage completely lacks MBP. As myelin develops, the expression of MBP isoform 21.5kDa increases. At mid-myelination (A-15), 21.5kDa MBP is mostly distributed into the pellet fraction; however at the late-myelination stage (A-26), the isoform is distributed equally between the supernatant and pellet fraction. In mature myelin (A-50), a slightly higher amount is found in the supernatant.

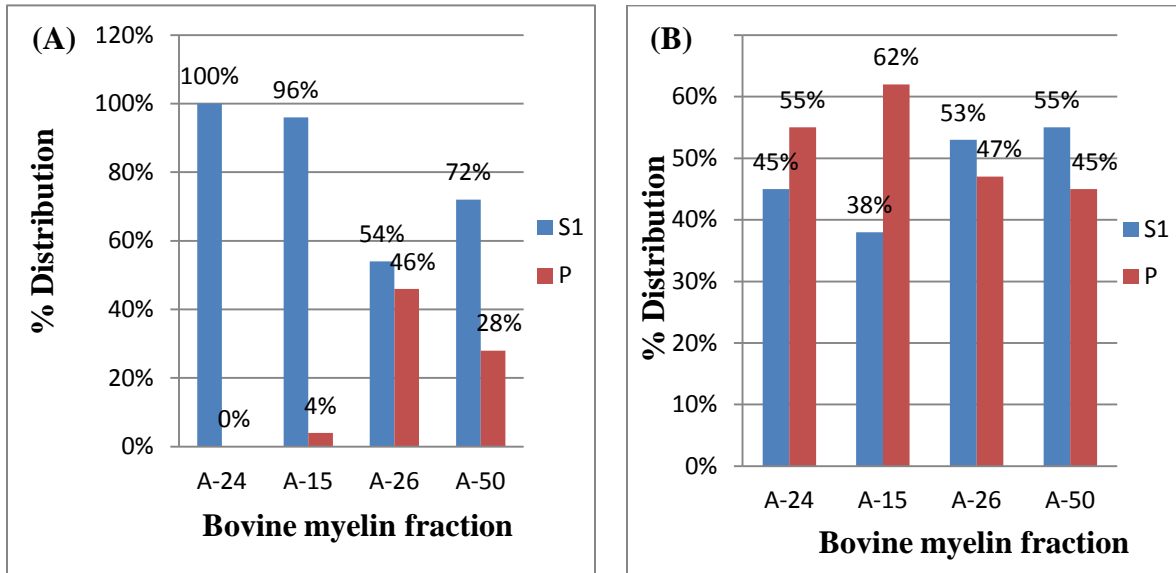
#### 4.4.2 Cytoskeletal Proteins in Bovine Myelin of Different Ages

Cytoskeletal proteins, actin and tubulin, were further confirmed in soluble and insoluble fractions from fetal and adult bovine myelin extracted with 1.5% CHAPS and 2% TX-100. Samples were separated either on 12% or 14% resolving SDS gels and transferred to nitrocellulose membrane.



**Figure 4.5** - Fluorescent Western blot detection of actin and tubulin in bovine myelin of different ages. Equal volumes (20 $\mu$ l) of each fraction were loaded into the well. (A) Samples were separated on a 14% resolving gel. (B) Samples were separated on a 12% resolving gel. Separated proteins were transferred to a nitrocellulose membrane. (A, B) M- SeeBlue Plus2 prestained marker. S1- Supernatant from the first extraction with 1.5% CHAPS at 4°C. P- Pellet from the second extraction with 2% TX-100 at room temperature. (A) Blot was incubated in primary antibody mouse  $\alpha$ -tubulin (1:500) (GenScript) (B) Blot incubated with primary antibody mouse  $\beta$ -actin (1:2000) (Sigma-Aldrich). Both the blots were then incubated in specific secondary antibody DyLight 649

anti-mouse IgG (1:12000). Images were acquired on a VersaDoc 4000 system with a red LED excitation source and a 695BP emission filter with an exposure time of 400-500sec.



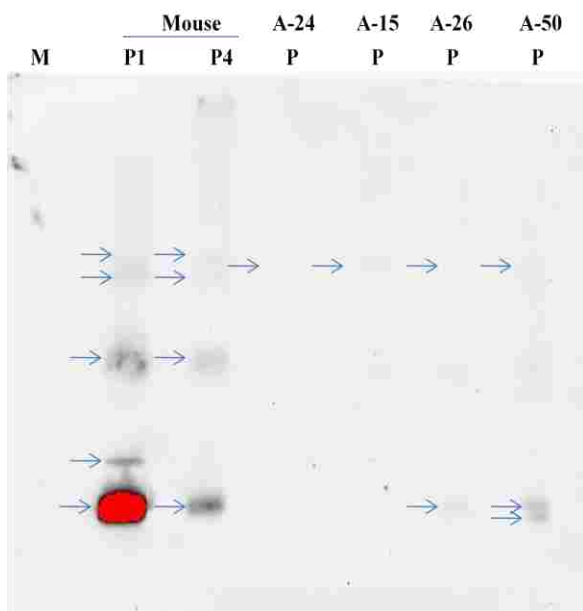
**Figure 4.6** – Percentage distribution of (A)  $\alpha$ -Tubulin and (B)  $\beta$ -Actin in fetal and mature bovine myelin fractions. The distribution was determined from the fluorescent Western blot (Fig. 4.5). Normalization was completed with Image Lab Analysis software.

As seen in Fig. 4.5 (A), tubulin is present in the supernatant and pellet fractions. The band is very intense and found in all ages of bovine myelin samples except in A-50, due to lower protein loading. During the early myelination stage, all or most of the tubulin was present in the supernatant fraction which contains the lipid rafts and absent in the pellet, which is the radial component fraction (Fig. 4.6 (A)). When myelin develops completely and becomes compacted, the amount of tubulin becomes equally distributed between supernatant and pellet. Fig. 4.5 (B) shows another cytoskeletal protein actin. Actin is also found in all stages of myelin development. Initially, the intensity of the band is higher in the pellet in lower age samples A-24 and A-15 compared to the supernatant. With an increase in age (A-26 and A-50), the actin band is more intense in the supernatant than the pellet. Percentage distribution of actin shows that initially actin is

higher in the radial component pellet in A-24 and A-15 compared to the supernatant (Fig. 4.6 (B)). On the contrary with the increase in age (A-26 and A-50), the actin becomes equal distributed in the supernatant and pellet fractions with a slight increase in the supernatant. Actin is also used as a reference protein. The presence of this protein in all fractions shows successful transfer of proteins. Overall, tubulin becomes associated with radial component only in mature myelin whereas actin is associated with radial component through all the stages of development.

#### 4.4.3 Oligodendrocyte Specific Protein in Radial Component

As discussed in chapter 1, the tight junction protein called OSP/claudin-11, 22kDa protein, is a member of the radial component and it has many important functional roles. In order to identify and confirm if the fractions extracted with CHAPS and TX-100 sequence preserved radial component, OSP/claudin-11 was used as a specific biomarker.



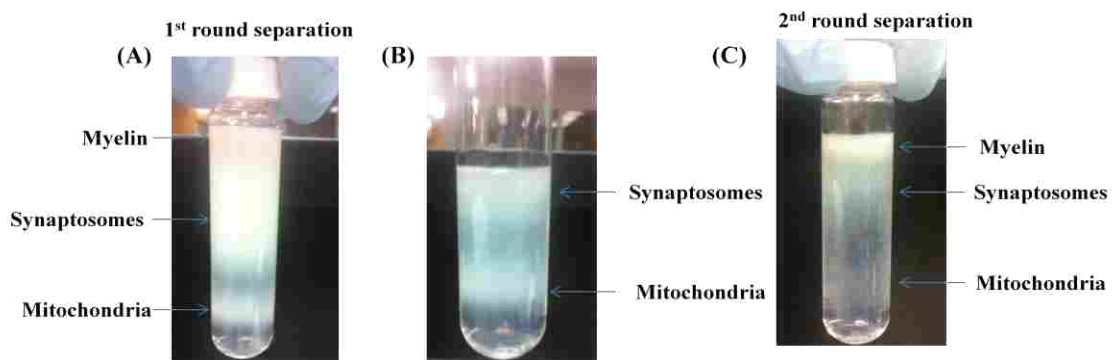
**Figure 4.7** – Western blot detection of OSP in mouse and bovine myelin extracted initially with 1.5% CHAPS followed by second, third, and fourth extractions with 0.5%, 1%, or 2% TX-100. Equal volumes (20 $\mu$ l) of each fraction were loaded into the well. Samples were separated on a 12% resolving gel and transferred to a nitrocellulose membrane. M- SeeBlue Plus2 prestained marker. P1- Pellet obtained from first extraction with 1.5% CHAPS from adult mouse myelin. P4 - Pellet obtained from the fourth extraction with 2% TX-100 from adult mouse myelin. P- Pellets obtained from second extraction with 2% TX-100 from fetal and adult bovine myelin A-24, A-15, A-26, and A-50. Blot incubated with primary antibody rabbit OSP (1:400) (abcam) followed by specific secondary antibody anti-rabbit IgG (1:80,000) (Sigma-Aldrich). Image acquired using electrochemiluminescence mixture with an exposure time of 100-150 sec.

fetal and adult bovine myelin A-24, A-15, A-26, and A-50. Blot incubated with primary antibody rabbit OSP (1:400) (abcam) followed by specific secondary antibody anti-rabbit IgG (1:80,000) (Sigma-Aldrich). Image acquired using electrochemiluminescence mixture with an exposure time of 100-150 sec.

As seen in Fig. 4.7, the OSP band is present in the pellet, the detergent resistant fraction. OSP is only found in the TX-100 resistant fraction and confirms successful isolation of radial component. The OSP band was also identified in fetal and mature bovine myelin fractions extracted with 1.5% CHAPS followed by 2% TX-100, but its intensity was low compared to adult mouse myelin. OSP is expressed at very low levels in the early myelin (i.e. A-24 and A-15) and increases as myelin develops and matures (i.e. A-26 and A-50).

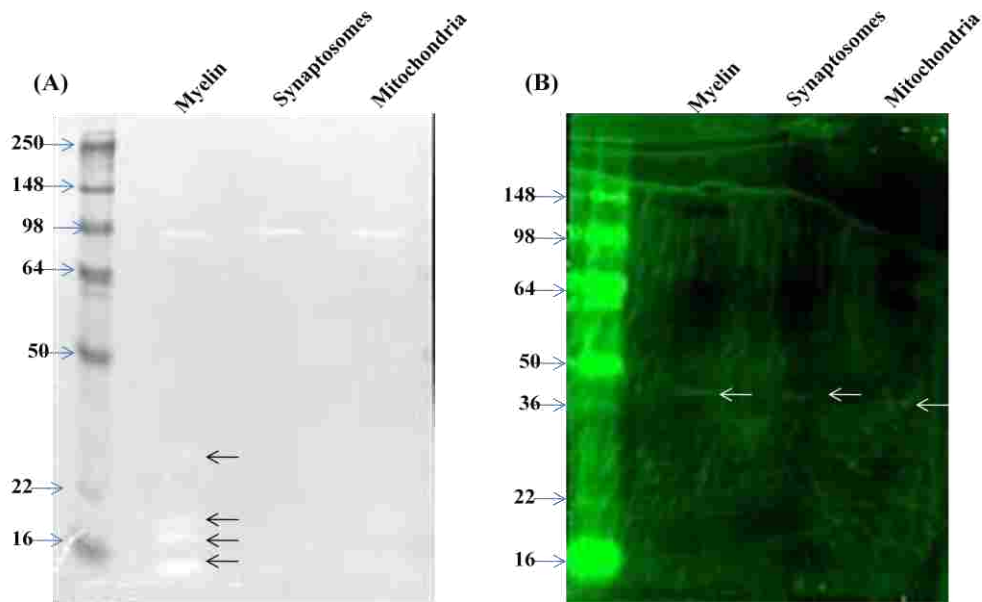
#### 4.4.4 Voltage Dependent Anion-Selective Channel and other Significant Proteins in Myelin, Synaptosomes, and Mitochondria

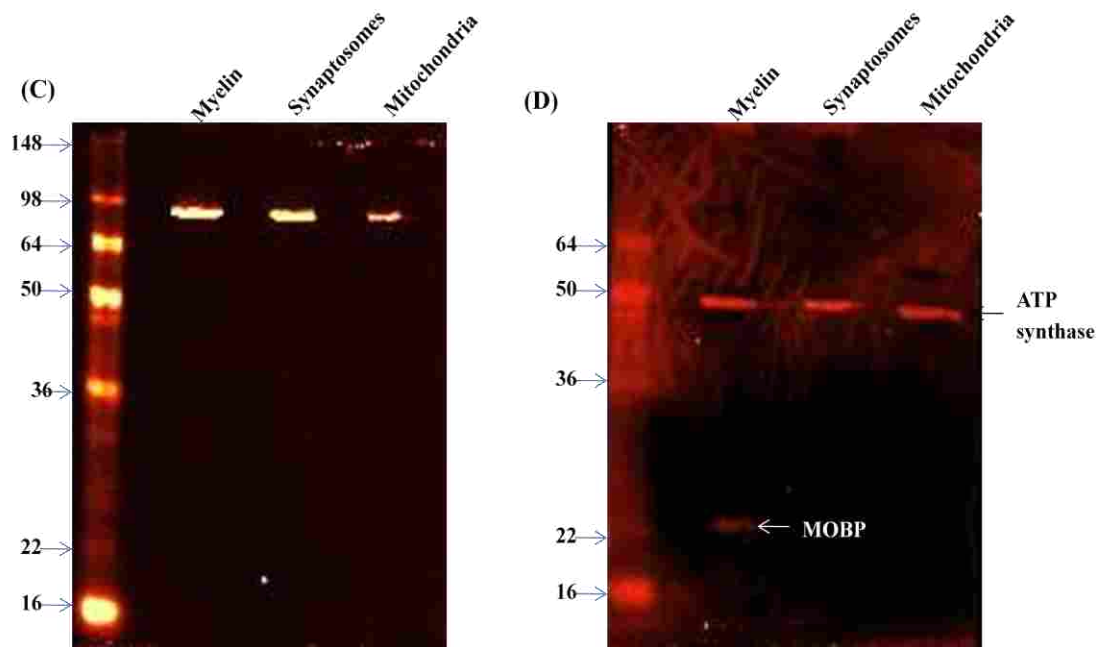
Homogenized adult mouse brain tissue was subjected to the Percoll density gradient centrifugation which distributed the material into three major bands. Fig. 4.8 shows the separation of three different layers distributing myelin, synaptosomes, and mitochondria from adult mouse myelin as described in section 4.3.3.



**Figure 4.8** - Isolation of myelin, synaptosomes, and mitochondria fractions via Percoll density gradient centrifugation. (A) First round separation of brain homogenate on Percoll gradient. (B) Synaptosomes and mitochondria fractions after removal of myelin from the top. (C) Second round separation of myelin on Percoll gradient separates into myelin, synaptosomes, and mitochondria

Myelin from a first round separation (Fig. 4.8 (A)) was collected from the top and was subjected to a second round of Percoll gradient separation (Fig. 4.8 (C)). Myelin obtained was further purified by two ice cold water washes. Synaptosomes were not further purified. Fractions were further subjected to multiplex fluorescent Western blot analysis of proteins specific to myelin as well as to the plasma membrane and the mitochondria.

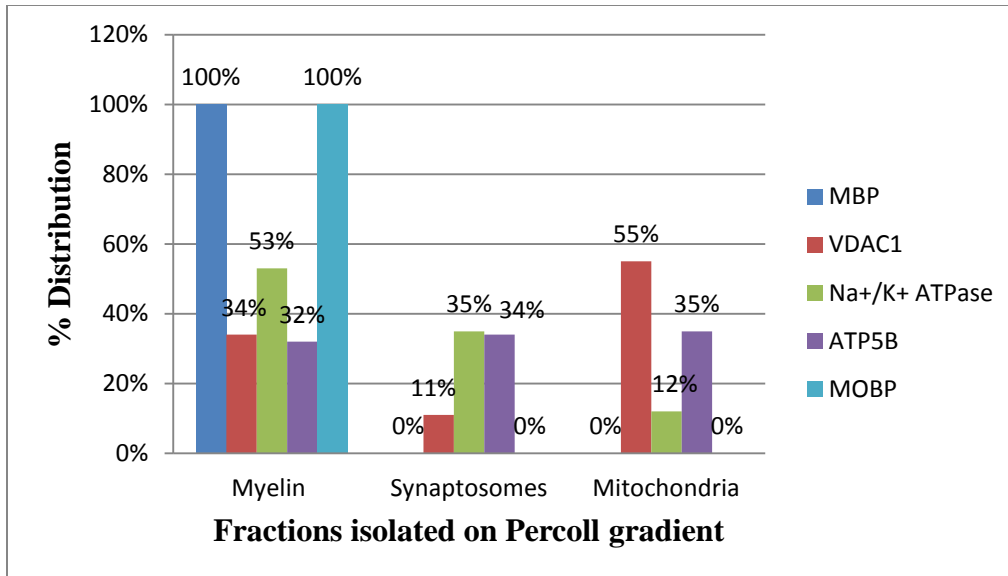




**Figure 4.9** – Fluorescent Western blot detection of MBP, VDAC1, Na<sup>+</sup>/K<sup>+</sup> ATPase  $\alpha$ , ATP5B, and MOBP in adult mouse myelin, synaptosomes, and mitochondria isolated by Percoll gradient centrifugation. Protein (10 $\mu$ g) was loaded from each fraction. Samples were separated on a 12% resolving gel and transferred to a nitrocellulose membrane. (A) Blot incubated with primary antibody rat MBP (1:500) (Millipore) followed by specific secondary antibody Dylight 488 anti-rat IgG (1:10000). Image was acquired on a VersaDoc 4000 system with a blue LED excitation source and a 530BP emission filter with an exposure time of 200sec. (B) Blot incubated with primary antibody goat VDAC1 (1:200) (Santa Cruz Biotechnology Inc.) followed by specific secondary antibody Dylight 649 anti-goat IgG (1:12000). (C) Blot incubated with primary antibody mouse Na<sup>+</sup>/K<sup>+</sup> ATPase  $\alpha$  (1:2000) (Santa Cruz Biotechnology Inc.) followed by specific secondary antibody Dylight 649 anti-mouse IgG (1:12000). (D) Blot incubated with two primary antibodies goat MOBP (1:200) (Santa Cruz Biotechnology Inc.) and ATP5B (1:200) (Santa Cruz Biotechnology Inc.) followed by specific secondary antibody Dylight 649 anti-goat IgG (1:12000). (B, C, D) Images were acquired on a VersaDoc 4000 system with a red LED excitation source and a 695BP emission filter with an exposure time of 400-500sec.

Equal amounts of protein (10 $\mu$ g) were loaded from each fraction. As seen in Fig. 4.9 (A), myelin shows the presence of three different MBP isoforms 21.5, 18.5, and 14kDa which are absent in the synaptosomes and mitochondria. Thus its percentage distribution (as shown in Fig. 4.10) is 100% in myelin fraction. In Fig. 4.9 (B), VDAC1 protein is present in all samples. VDAC, a mitochondrial protein, was expected in the

mitochondrial fraction, and a small amount of VDAC was observed in the synaptosomes since this fraction was not purified further. With an equal protein loading, the highest amount of VDAC1 (55%) was found in a mitochondria, a fair amount 34% in myelin (which is more than half of mitochondria) and less 11% in synaptosomes (Fig. 4.10). In Fig. 4.9 (C), Na<sup>+</sup>/K<sup>+</sup> ATPase  $\alpha$  which is 100-113kDa enzyme is present in all fractions. Synaptosome fraction shows the presence of Na<sup>+</sup>/K<sup>+</sup> ATPase  $\alpha$  band and a less intensity band is present in mitochondrial fraction. Percentage distribution of Na<sup>+</sup>/K<sup>+</sup> ATPase  $\alpha$  shows the highest amount (53%) was found in the myelin followed by 35% in the synaptosomes and 12% in the mitochondria (Fig. 4.10). Thus, it is possible that there is some synaptosomal presence in the mitochondrial fraction. However, it is highly unlikely that the myelin fraction is contaminated with mitochondria. In Fig. 4.9 (D), the Western blot was labeled with two different antibodies ATP5B (51kDa) and MOBP (25kDa), and it confirms the purity of the myelin fraction isolated by two rounds Percoll density gradient centrifugation. The ATP5B was present in all the three fractions, whereas MOBP myelin protein is present only within myelin. The ATP5B antibody detects the  $\beta$ -subunit of the F<sub>1</sub> complex of ATP synthase. ATP5B is present almost equally in all three fractions whereas MOBP is present only within myelin. Overall, presence of MBP and MOBP only within myelin fraction indicates that synaptosomes and mitochondria are not contaminated with myelin, and VDAC1, Na<sup>+</sup>/K<sup>+</sup> ATPase, and ATP synthase are myelin associated enzymes.



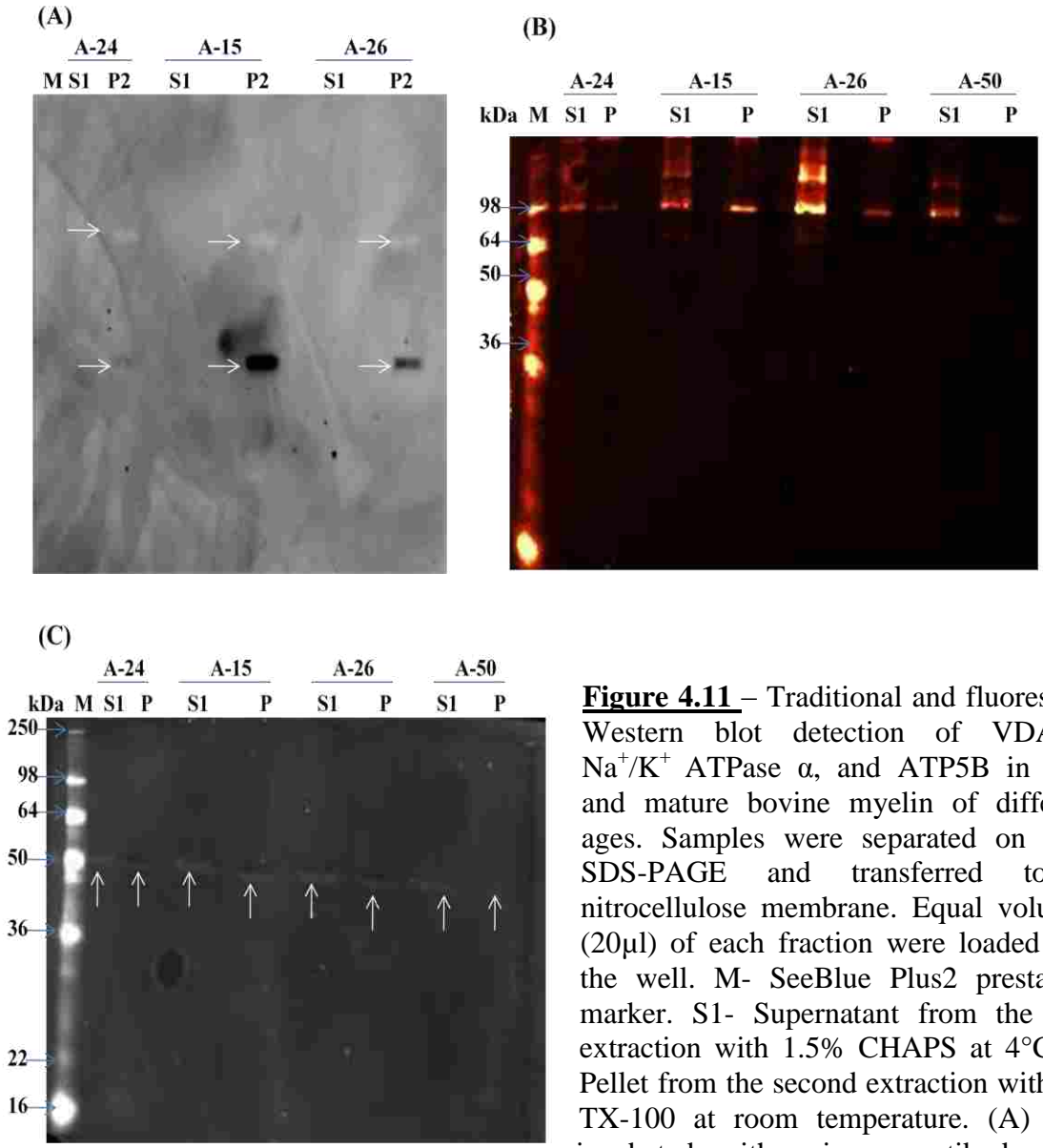
**Figure 4.10** – Percentage distribution of MBP, VDAC1, Na<sup>+</sup>/K<sup>+</sup> ATPase  $\alpha$ , ATP5B, and MOBP in adult mouse myelin, synaptosomes, and mitochondria isolated by Percoll gradient centrifugation. The distribution was determined from the fluorescent Western blot (Fig. 4.9). Normalization was completed with Image Lab Analysis software.

#### 4.4.5 Voltage Dependent Anion-Selective Channel and other Transport Proteins in

##### Radial component

The presence of VDAC, Na<sup>+</sup>/K<sup>+</sup> ATPase  $\alpha$ , and ATP5B were also identified in the pellet fraction from fetal and adult bovine myelin. Soluble and insoluble fractions extracted with 1.5% CHAPS and 2% TX-100 were separated on 12% resolving SDS gel and transferred to a nitrocellulose membrane.



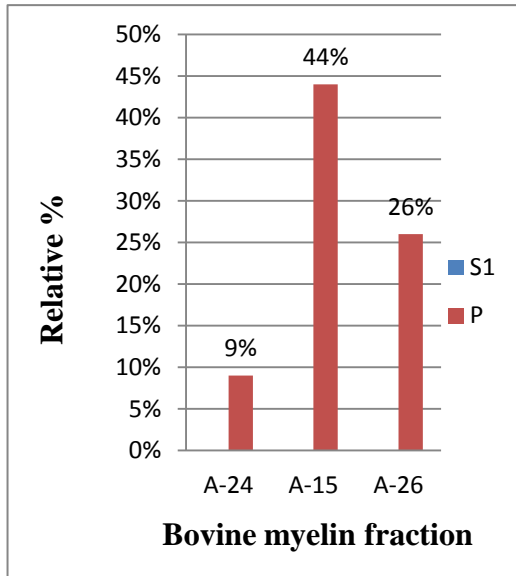


**Figure 4.11** – Traditional and fluorescent Western blot detection of VDAC1, Na<sup>+</sup>/K<sup>+</sup> ATPase α, and ATP5B in fetal and mature bovine myelin of different ages. Samples were separated on 12% SDS-PAGE and transferred to a nitrocellulose membrane. Equal volumes (20μl) of each fraction were loaded into the well. M- SeeBlue Plus2 prestained marker. S1- Supernatant from the first extraction with 1.5% CHAPS at 4°C. P- Pellet from the second extraction with 2% TX-100 at room temperature. (A) Blot incubated with primary antibody goat

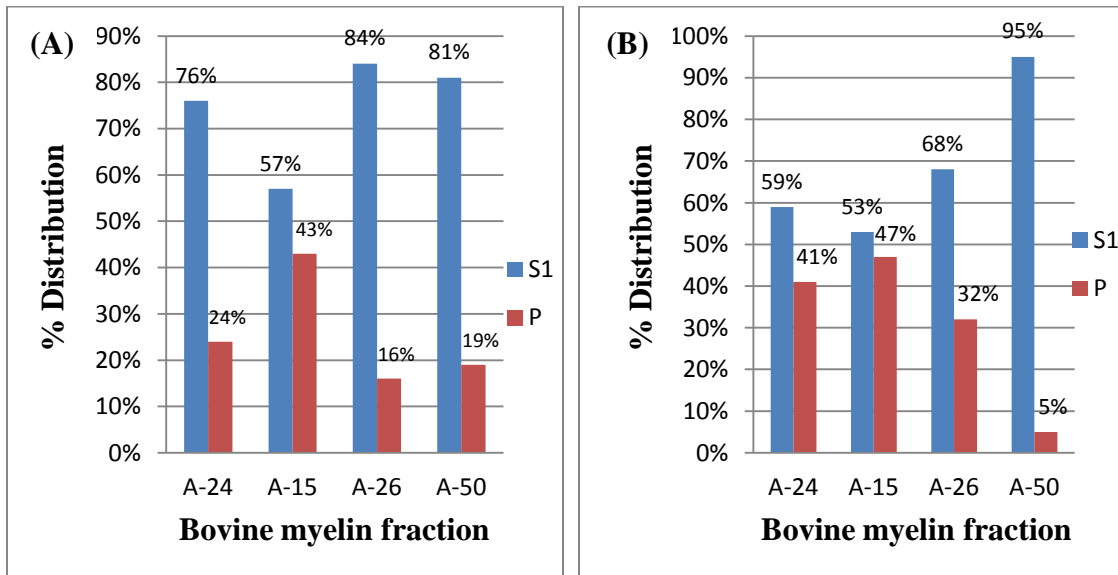
VDAC1 (1:200) followed by specific secondary antibody anti-goat IgG (1:2000). Image acquired with VersaDoc 4000 using enhanced chemiluminescence mixture with an exposure time of 100-150 sec. The proteins are marked with an arrow. (B) Blot incubated with primary antibody mouse Na<sup>+</sup>/K<sup>+</sup> ATPase α (1:2000) followed by specific secondary antibody Dylight 649 anti-mouse IgG-HRP (1:12000). (C) Blot incubated with primary antibody anti-goat ATP5B (1:200) followed by specific secondary antibody Dylight 649 anti-goat IgG (1:12000). Image was acquired on a VersaDoc 4000 system with a red LED excitation source and a 695BP emission filter with an exposure time of 400-500sec. The proteins are marked with an arrow.

As seen in Fig. 4.11 (A), VDAC1 is present in the pellet fraction, which represents the radial component of fetal bovine myelin A-24, A-15, and A-26. This

indicates that VDAC is associated with the radial component myelin. VDAC protein is absent in the supernatant which contains the lipid rafts. The band intensity is higher in the A-15 pellet, followed by A-26 and A-24. Adult bovine myelin A-50 did not show a VDAC band, due to low protein loading. Relative protein expression of VDAC1 is low in early myelin (A-24), high during mid-myelination (A-15) and then lower during late myelination (A-26) (Fig. 4.12). Fig.4.11 (B) shows the presence of  $\text{Na}^+/\text{K}^+$  ATPase  $\alpha$  in supernatant and pellet in all ages of bovine myelin samples. The percentage distribution of  $\text{Na}^+/\text{K}^+$  ATPase  $\alpha$  in Fig. 4.13 (A) shows  $\text{Na}^+/\text{K}^+$  ATPase  $\alpha$  is higher in the supernatant fraction than the pellet fraction. During early myelination  $\text{Na}^+/\text{K}^+$  ATPase  $\alpha$  is higher in the supernatant A-24 and as myelin develops it becomes equally distributed in the supernatant and the pellet. At the late myelination stage, again  $\text{Na}^+/\text{K}^+$  ATPase  $\alpha$  is higher in the supernatant and in a small amount in the pellet. Fig. 4.11 (C) shows ATP5B is present in the supernatant and the pellet in bovine myelin of all ages. In Fig. 4.13 (B), ATP5B is almost equally distributed in the supernatant and the pellet of early myelin and with an increase in age the distribution becomes greater in the supernatant compared to the pellet. This indicates that  $\text{Na}^+/\text{K}^+$  ATPase  $\alpha$  and the ATP synthase  $\beta$ -subunit are associated with both lipid rafts and radial component through all stages of development. MOBP was found only in the pellet A-24, A-15, and A-26 (figure not shown here) but with very low intensity. This indicates that MOBP is associated with the radial component.



**Figure 4.12** – Relative percentage of VDAC1 in fetal and mature bovine myelin of different ages. The relative % was determined from the Western blot (Fig. 4.11 A). Normalization was completed with Image Lab Analysis software. (Relative %-percentage of proteins in a lane)



**Figure 4.13** – Percentage distribution of (A)  $\text{Na}^+/\text{K}^+$  ATPase  $\alpha$ , and (B) ATP5B in fetal and mature bovine myelin of different ages. The distribution was determined from the fluorescent Western blot (Fig. 4.11 B and C). Normalization was completed with Image Lab Analysis software.

#### 4.4.6 NADH: Ferricyanide Reductase Activity and VDAC

Previous, investigations found that when VDAC is expressed in plasma membrane, it functions as a NADH-ferricyanide reductase by reducing extracellular ferricyanide in the presence of intracellular NADH<sup>42</sup>. For this assay, the myelin fraction, isolated by Percoll density gradient centrifugation, was extracted with 1.5% CHAPS followed by 2% TX-100. The final insoluble fraction pellet was resuspended in 5% TX-100 in 2X TBS buffer. The assay was performed as discussed in section 4.3.4. Reductase activity was measured on these soluble and insoluble fractions by monitoring the decrease in absorbance at 340nm, as NADH is oxidized to NAD<sup>+</sup> with the reduction of ferricyanide (graph shown in Appendix 9). Enzyme activity in samples was calculated by using the equation that follows:

Specific activity = U/mg protein

Where U is units of activity ( $\mu\text{mol}/\text{min}$ )

$$U = \Delta\text{Abs}/t \text{ (min)} \times 1/(\text{extinction coefficient of NADH (M}^{-1}\text{cm}^{-1}) \times \ell \text{ (cm)}) \times \text{volume of assay (L)} \times 10^6 \text{ (}\mu\text{mol/mol)}$$

Protein assayed for each fraction was 19 $\mu\text{g}$  (50 $\mu\text{l}$ ) for the supernatant (S1), 3.05 $\mu\text{g}$  (50 $\mu\text{l}$ ) for the supernatant (S2), and 2.35 $\mu\text{g}$  (50 $\mu\text{l}$ ) for the pellet. The pellet shows the highest specific activity present (see Table 4.2) which might be due to the presence of the VDAC protein. From Fig. 4.11 (A) VDAC is absent in supernatant; however, some specific activity which might be due to the presence of other enzymes such as cytochrome c reductase or NADH dehydrogenase.

**Table 4.2-** Specific activity of supernatant and pellet fractions from mouse myelin

Fractions from mouse myelin	Specific activity (U/mg)
Supernatant S1	0.112
Supernatant S2	0.137
Pellet P	0.120

#### 4.5 Discussion

MBP is the second most abundant protein after PLP in the CNS myelin. MBP is located at the cytoplasmic surfaces, and is responsible in multilayered compact myelin for adhesion of the cytosolic surface of plasma membrane, forming a major dense line, and extracellular surfaces adhere to each other and forms intraperiod line<sup>8</sup>. MBP binds to the cytosolic surfaces through electrostatic interactions with negatively charged lipids. Several isoforms of MBP exists by differential splicing. Isoform post-translational modifications, including deamidation, phosphorylation, deimination of MBP reduces its net positive charge. Phosphorylation occurs by the presence of enzymes in myelin such as protein kinase C, protein kinase A, glycogen synthase kinase, calmodulin-dependent kinase, and mitogen-activated protein kinase<sup>8</sup>. Once MBP is synthesized, its isoforms have been shown to segregate in different regions of the myelinating cell. It is interesting to see how myelin isoforms are distributed in different fractions with development. Our findings strongly suggest localization of 21.5, 18.5, and 17kDa isoforms in insoluble radial component fractions of compact myelin, and 18.5 and 14kDa isoforms of MBP in the soluble lipid rafts fraction which is consistent with previous findings<sup>24</sup>.

Actin and tubulin were identified as two cytoskeletal proteins present in compact myelin where they may be associated with MBP and the radial component<sup>20</sup>. MBP binds to these cytoskeletal proteins and forms bundles of microtubules by its polymerization.

MBP can bind actin filaments to the surface of negatively charge lipid vesicles, suggesting that it may be able to act as a membrane actin-binding protein <sup>8</sup>. Actin (42kDa) and tubulin (50kDa) were further identified in radial component myelin fractions. The distribution of these cytoskeletal proteins in soluble fraction lipid rafts and insoluble fraction radial component changes from early to late myelination stages. During early myelination, tubulin is found in the supernatant fraction, which contains lipid rafts. As myelination continued, the tubulin becomes equally distributed in the supernatant and the radial component. MBP isoform 21.5kDa is a major protein of radial component during early myelination, whereas tubulin becomes associated with radial component near the end of myelination. Actin is associated with the supernatant fraction and the radial component during early myelination. Kirschner and his colleagues found elevated levels of MBP isoforms with actin and tubulin in the radial component. Both these cytoskeletal proteins have been shown to have an affinity for MBP. MBP binding to these proteins might allow it to participate in transmission of extracellular signals to the cytoskeleton in oligodendrocytes and tight junctions in myelin <sup>8</sup>.

As discussed in sections 1.4 and 1.4.2, OSP is a member of the claudin family of tight junction-associated proteins, and is also termed claudin-11. OSP is the essential protein of this structure; no radial component is detected in OSP-deficient mice<sup>51</sup>. Kirschner *et al.* found that radial component preserved its structure in tissue treated with TX-100. Triton-resistant lipid and protein complexes from the CNS tissue were compared with triton-treated sciatic nerve from the PNS tissue. Sciatic nerve from PNS was completely devoid of radial component and protein found in triton-treated lipid and protein complexes recovered from CNS was absent in sciatic nerve <sup>20</sup>. Our findings

indicated that OSP is present only in the pellet fraction (radial component) of mature myelin and absent during early myelination. The presence of this protein in detergent resistant fractions confirms successful isolation of radial component. MOBP was identified in the pellet fraction of fetal and mature bovine myelin samples. This protein is associated with the radial component from early myelination stage. As discussed in section 1.5, MOBP plays an important role in normal arrangement of the radial component in CNS myelin. In MOBP- and MBP/MOBP-double deficient mice, the radial component is observed but of a condensed type <sup>19</sup>.

There is limited research indicating an association of Na<sup>+</sup>/K<sup>+</sup> ATPase and ATP synthase enzymes within CNS myelin. As discussed in section 4.1.2, Na<sup>+</sup>/K<sup>+</sup> ATPase is an enzyme located in the plasma membrane that maintains sodium and potassium gradients across the cell membranes by utilizing energy released from hydrolysis of ATP. Fluorescent Western blotting shows the presence of Na<sup>+</sup>/K<sup>+</sup> ATPase in supernatant and pellet fractions. The presence of this enzyme in myelin suggests that a sufficient amount of ATP is present within myelin sheath or produced within myelin sheath. F<sub>0</sub>F<sub>1</sub>- ATP synthase is responsible for most of the ATP synthesis in the cell. Recently, Ravera *et al.* investigated the presence of ATP synthase in myelin <sup>52</sup>. ATP hydrolysis assays, on isolated myelin vesicles indicated that a functional F<sub>0</sub>F<sub>1</sub>- ATP synthase was present which may be able to conduct an aerobic metabolism, to support the axonal energy demand <sup>52</sup>. As determined by fluorescent Western blotting Na<sup>+</sup>/K<sup>+</sup> ATPase and ATP synthase are expressed in the supernatant and the radial component during all stages of development.

Dyer *et al.* suggests that the radial component contains the molecular pumps which allow the movement of fluid molecules<sup>53</sup>. Junctional plaques (tight and adherens) in other cells play key roles in paracellular barrier function, which includes signal transduction, adhesion of cell membranes, and fluid movement between cells by aqueous pores and channels.

VDAC which is initially known to be a mitochondrial protein has been identified in the plasma membrane and functions to transport ions and maintain membrane potential. VDAC has been identified as a component of the radial component proteome from mass spectrometric analysis (see chapter 3). VDAC was further confirmed by using specific antibody that detects VDAC1 in fetal and adult bovine myelin fractions. However, it is associated only in the pellet fraction that represents the radial component. The relative percentage of VDAC1 varies from early to late myelination stages. Initially, during early myelination, it is present in very low amounts. As myelin develops and becomes compacted in late myelination, it is found in very high amounts. The possible role of the VDAC pore forming protein, in radial components is to transport ions and ADP/ATP molecules from an external source. Recent quantitative analyses strongly support that myelin supplies chemical energy to the neuron for its function<sup>54</sup>. This may be due to presence of specific enzymes for ATP production. The presence of VDAC in radial component was further confirmed by NADH: ferricyanide reductase activity in mouse myelin where VDAC functions as a redox enzyme. Reductase activity in the supernatant (S1) and the supernatant (S2) might be because of other enzymes such as cytochrome b<sub>5</sub> reductase or NADH dehydrogenase since no VDAC was found in these fractions as determined by Western blotting. The analysis of myelin, synaptosomes, and



mitochondria from adult mouse brain further confirm VDAC in myelin membrane. Synaptosomes and mitochondria were used as positive control. The presence of MBP and MOBP only within myelin fractions confirms that the Percoll gradient centrifugation is effective in isolating myelin from other cellular components of the brain tissue. Furthermore, VDAC in myelin membrane is not due to mitochondria contamination, since that relative abundance of VDAC in the myelin is approximately half of that found in the mitochondrial fraction (on an equal protein loading basis). If VDAC in the myelin fraction was due to mitochondrial contamination, over half of the protein in the myelin fraction would have to be from mitochondria. VDAC has been previously identified in synaptosomes; however, the expression of VDAC in synaptosomes seems to be half that found in the myelin. These results are the first to indicate that the myelin membrane possesses VDAC and this ion channel is localized to the radial component.

## Chapter 5 - Conclusions and Future Work

There were three main objectives to be achieved in this research; 1) to develop an effective isolation procedure for the radial component of CNS myelin, 2) to conduct an extensive proteomic analysis of the radial component, and 3) to characterize/measure the expression level of key microdomain proteins in development of radial component.

Our studies demonstrate that radial component of compact myelin was isolated successfully, as a highly detergent resistant fraction. Initial bovine and mouse myelin extractions were completed with different detergents and concentrations to further solubilise the proteins but the radial component was found to be highly resistant to detergents at a ratio of 4:1 (w/w) detergent-to-protein. The best sequence of detergents found was CHAPS which extracts lipid rafts followed by TX-100 which preserved radial component, with an optimal concentration of 1.5% CHAPS and 2% TX-100. Proteomic analysis of the detergent resistant radial component fractions reveals the presence of myelin-specific proteins, cytoskeletal, tight junction, and mitochondrial-type proteins. These include proteins previously found by Kirschner and colleagues<sup>20</sup>. The detergent resistant fraction of myelin at all stages of development has a high expression level of actin, tubulin, PLP, spectrin, MBP, MOG and CNP. Some additional proteins such as VDAC, Na<sup>+</sup>/K<sup>+</sup> ATPase, and ATP synthase were also prominent in this preparation.

The proteins identified by proteomics were also characterized and confirmed by Western blot. The MBP isoform 21.5kDa is a major protein of the radial component, especially during early myelination, whereas tubulin becomes associated with the radial component near the end of myelination. The distribution of actin was found similar to that of MBP. Fractions of myelin, synaptosomes, and mitochondria from aPercoll

gradient separation were analyzed to confirm the presence of VDAC, Na<sup>+</sup>/K<sup>+</sup> ATPase, and ATP synthase in myelin. The results show the presence of MBP and MOBP only in the myelin fraction isolated by Percoll density gradient centrifugation, indicating effective separation of myelin, synaptosomes and mitochondria. Western blotting indicated that VDAC, Na<sup>+</sup>/K<sup>+</sup> ATPase, and ATP synthase are associated with the radial component of myelin. NADH: ferricyanide reductase activity is functional in myelin and may be due to VDAC.

**Table 5.1** - List of previous and additional major proteins identified in radial component.

Previously identified proteins <sup>18, 19, 20</sup>	Additional major proteins identified in this study
MBP	VDAC 1, 2
CNP	Na <sup>+</sup> /K <sup>+</sup> ATPase
Tubulin	ATP synthase
Actin	GFAP
OSP/claudin 11	Spectrin
MAG	ADP/ATP translocase
MOG	Neurofilament light, medium, heavy polypeptide
PLP	Heat shock cognate protein
MOBP	Sirt2
	α-internexin
	Calcium/calmodulin-dependent protein
	NADH dehydrogenase
	Microtubule associated protein
	Dynamin

In the future, using the differential extraction method developed in this research, the difference between control and a spontaneously demyelinating mutant mouse should be compared. This may aid in understanding the structure and function of radial component in health and disease (including the destruction of the myelin sheath). Proteomic analysis shows that the radial component is highly enriched in many proteins; however, the functional role of all the proteins has not been studied yet. The role of

VDAC in the radial component of myelin needs to be further elucidated. The opening and closing of the channel of VDAC in plasmalamella can be performed by using electrophysiological techniques.

## Appendices

### Appendix 1. Protocol for Isolation of Myelin from Mouse Brain

Reference: Norton, W.T., Isolation of myelin from nerve tissue, Meth. Enzymol. 31, 435-444 (1974).

#### Reagents

##### **TNE, pH 7.4**

25 mM Tris	121.14 g/mol	3.03 g/L
150 mM NaCl	58.44 g/mol	8.77 g/L
5 mM EDTA	372.2 g/mol	1.86 g/L

add to 800 mL ddH<sub>2</sub>O, pH to 7.4 with 6 M HCl, then fill to 1 L  
store at 4°C for a maximum of 12 months

##### **30% sucrose in TNE**

33.81 g sucrose into 100 mL TNE  
Store at 4°C for a maximum of 3 months

##### **10.5% sucrose in TNE**

35 mL 30% sucrose diluted to 100 mL with TNE  
Store at 4°C for a maximum of 3 months

##### **2X TBS, pH 7.5**

50 mM Tris	121.14 g/mol	3.03 g
280 mM NaCl	58.44 g/mol	8.14 g
4 mM EDTA	372.2 g/mol	0.74 g

add to 400 mL ddH<sub>2</sub>O, pH to 7.5 with 6 M HCl, then fill to 500 mL  
store at 4°C for a maximum of 12 months

##### **100 mM PMSF**

- Dissolve 0.03484 g PMSF in 2.0 mL absolute EtOH
- Divide into 70 µl aliquots in green microfuge tubes
- Store at -20°C

##### **100 mM sodium vanadate**

- Dissolve 0.03678 g Na<sub>3</sub>VO<sub>4</sub> in 2.0 mL MilliQ H<sub>2</sub>O
- Divide into 70 µl aliquots in blue microfuge tubes
- Store at -20°C

##### **50X protease inhibitor cocktail (PIC)**

- Dissolve 1 tablet (PIC, Roche CAT# 04 693 116 001) in 1.0 mL MilliQ H<sub>2</sub>O
- Divide into 110 µl aliquots in pink microfuge tubes
- Store at -20°C

10X PhosSTOP (phosphatase inhibitor cocktail)

- Dissolve 1 tablet (Roche CAT# 04906845001) in 1.0 mL MilliQ H<sub>2</sub>O
- Divide into 70 µL aliquots in clear microfuge tubes, labelled P-STOP
- Store at -20°C

**Myelin Storage Buffer**

500 µL 2X TBS

40 µL PIC

20 µL 100 mM PMSF

20 µL 100 mM Na<sub>3</sub>VO<sub>4</sub>

20 µL PhosSTOP

400 µL H<sub>2</sub>O

**Procedure:**

1. To 10 mL of 10.5% sucrose in TNE, add 100 µL Na<sub>3</sub>VO<sub>4</sub>, 200 µL PIC, 100 µL PhosSTOP and 100 µL PMSF. Keep on ice. (Use this throughout, when it says 10.5 sucrose)
2. Homogenize whole mouse brain(s) (including meninges, grey matter, white matter) in ~1 mL of 10.5% sucrose in a Dounce homogenizer (on ice).
3. Bring final volume of homogenate to final volume of 2 mL using 10.5% sucrose.
4. Layer 2 mL of 30% sucrose in TNE in bottom of 5 mL ultracentrifuge tube (Beckman Cat. # 344057; Ultra-clear).
5. Overlay the 2 mL of mouse brain homogenate CAREFULLY onto the top of the 30% sucrose, being careful not to disturb the bottom layer (a 1 mL Eppendorf pipettor placed against the side of the tube, held almost perpendicular to the side of the tube seems to work best – let the brain homogenate trickle down and form a layer).
6. Balance tubes using analytical balance.
7. Load tubes into the buckets of the MLS-50 swinging bucket rotor.
8. Centrifuge for 61 minutes in the MLS-50 rotor at 35,000 rpm (in the Optima MAX ultracentrifuge).
9. Carefully remove the top sucrose layer (discard) and remove the floating white ‘myelin layer’ at the interface of the tube using a 1 mL Eppendorf pipettor or Pasteur pipette. Discard the rest of the tube contents. Be careful not to suck up too much of the sucrose and only the white layer. **\*\*Place the myelin into a 2 mL Eppendorf (centrifuge) tube.\*\***
10. Fill the tube to the 2 mL mark with ice-cold water. Vortex.
11. Spin in microfuge at 4°C at maximum speed for 10 minutes, discard supernatant.
12. Repeat steps 9 and 10, again.
13. Resuspend the washed pellet in 10.5% sucrose, to a **final** volume of 2 mL
14. Repeat ultracentrifugation steps 5-8 and wash steps 9-10. – only wash with cold water once
15. Resuspend in the pellet in a minimal volume (~100-500 µL—use 250µL, at first and if still viscous, add another 100µL) of Myelin Storage Buffer containing protease and phosphatase inhibitors (see above). Label tubes. Examples of the protease/phosphatase inhibitors are PMSF, sodium vanadate, Protease Inhibitor

- Cocktail (PIC) (Roche). If you're not concerned about post-translational modifications and possible protein degradation, leave them out.
16. Vortex well and aliquot into microfuge tubes. Aliquot 2 x 25  $\mu$ L of myelin for BCA protein assay.
  17. Flash-freeze the tubes in liquid N<sub>2</sub>. Store at -80 °C.

**Modification for SW50.1 rotor**

Centrifuge for 2 hrs in the SW50.1 rotor at 32,000 rpm for 60 minutes

**Modifications for SW 41 rotor**

Homogenize brains in ~3 mL 10% sucrose, transfer into 15 mL conical tube, and rinse homogenizer with 10% sucrose to bring the final volume to 5 mL.

In SW41 tube (hold ~10 mL), layer 5 mL 30% sucrose into bottom, then overlay sample (5 mL) using 1 mL pipette. Centrifuge at 26,800 rpm for 112 minutes.

## Appendix 2. Protocol for Micro-BCA protein Assay Kit (Pierce 23235)

### Microplate procedure

1. To the first 6 rows of the microplate, add 150  $\mu\text{L}$   $\text{H}_2\text{O}$ .
2. Prepare standards.
  - Working BSA standard solution, 0.08 mg/mL  
Dilute stock 2 mg/mL BSA standard (1:25)  
40  $\mu\text{L}$  stock + 960  $\mu\text{L}$   $\text{H}_2\text{O}$  in a microfuge tube
  - Add BSA standards to wells, in triplicate, as indicated in table.
  - Add  $\text{H}_2\text{O}$  to bring volume to 150  $\mu\text{L}$ .

$\mu\text{g}$ BSA	BSA (0.08 mg/mL)	$\mu\text{L}$ $\text{H}_2\text{O}$
0.8	10 $\mu\text{L}$	140
1.6	20 $\mu\text{L}$	120
2	25 $\mu\text{L}$	125
4	50 $\mu\text{L}$	100
6	75 $\mu\text{L}$	75

3. Add samples to wells, volumes from 1 to 150  $\mu\text{L}$  (in duplicate). If necessary, dilute samples to give 3-6  $\mu\text{g}$  protein/ well. Several dilutions may be necessary to target the correct concentration.

4. Add  $\text{H}_2\text{O}$  to sample wells to a final volume of 150  $\mu\text{L}$ .

5. Prepare the micro-BCA Working Reagent.

Reagent Mix		for 1 plate	for $\frac{1}{2}$ plate (<45 wells)
Reagent MA	25 parts	10.0 mL	3.75 mL
Reagent MB	24 parts	9.6 mL	3.60 mL
Reagent MC	1 part	0.4 mL	0.15 mL

6. Add 150  $\mu\text{L}$  Reagent Mix to each well.

7. Incubate plate at 37°C for 2 hours.

8. Measure at 562 nm.



### Appendix 3. Protocol for Silver Stain

Silver staining procedure requires several incubations and washes for various times which should be followed closely. All steps are performed at room temperature except for the silver nitrate incubations.

1. Transfer the gel section to a weigh boat lined with clean plastic wrap. Fix the gel with 100ml of 50% methanol, 10% acetic acid (**fixation solution**) for **30 minutes**. Occasionally agitate the weigh boat gently.
2. Carefully decant the solution into the silver staining waste container by the sink and then incubate the gel in 80-100ml of 5% methanol and 1% acetic acid for **15 minutes**.
3. **Decant the solution and quickly rinse** the gel in 50% methanol. Next wash the gel with double deionized water **three** times for five minutes.
4. Decant the water and incubate and incubate the gel in 80-100 ml of freshly prepared  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$  (sodium Thiosulfate, 0.2g/L or 0.02g/100ml) for **90 seconds** only.
5. Decant the sodium thiosulfate solution (**sensitization solution**) and wash the gel with double deionized water, three times for **30** seconds.
6. Incubate the gel in 80-100ml of chilled  $\text{AgNO}_3$  (Silver Nitrate, 0.2g/100ml) (**staining solution**) for **45-60 minutes** at 4 °C
7. Decant the silver nitrate solution into the waste container and wash the gel with double deionized water three times for **60 seconds** each.
8. Develop the gel in 80-100ml of  $\text{Na}_2\text{CO}_3$  (Sodium carbonate, 6g/100ml) HCOH (formaldehyde, 50  $\mu\text{l}$  of 37% formaldehyde/ 100ml),  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$  (sodium thiosulfate, 2ml from step 5 reagent/ 100ml) for **5 to 8 minutes**. Watch the gel carefully and when the protein band has stained to a light brown, stop the development using acetic acid. (**developing solution**)
9. Decant the above developer solution and incubate the gel in 80-100ml of 6% acetic acid (**stop solution**) for **10 minutes**. Then wash the gel 3-4 times with double deioined water.

## Appendix 4. Protocol for In-Gel Tryptic Digestion

References: Mass Spectrometry Facility, Hospital for Sick Children, Toronto  
Biological Mass Spectrometry Laboratory, UWO

To avoid keratin contamination:

- wear gloves (powder-free, non-latex) and a clean lab coat
- wipe down work surfaces with lint-free cloth moistened with MeOH/ H<sub>2</sub>O solution
- a hairnet and mask may be necessary

Prepare all solutions containing ammonium bicarbonate fresh daily.

Use gel-loading pipette tips when removing solutions from the gel pieces.

Use siliconized microfuge tubes to limit the binding of the proteins and peptides to the plastic tube. Rinse tubes with ethanol and let air dry (cover to keep dust off) before use.

1. Rinse gel with Milli-Q water twice for 5 minutes each time.
2. Excise protein spot/band on a clean glass surface (or in disposal Petri plate) using a scalpel. Cut the gel as close to the band as possible to reduce the volume of gel to be processed. Cut into 1-2 mm<sup>3</sup> pieces and place into a 1.5 mL siliconized microcentrifuge tube. Push gel pieces down with a pipette tip.
3. If storing the gel, add 40 µL of 1% acetic acid in Milli-Q water. Store overnight at 4°C until ready for digestion. (Alternatively, for long-term storage, add 50 µL 1:1 (v/v) MeOH: 50 mM NH<sub>4</sub>HCO<sub>3</sub>. Vortex, let stand 10 min. Remove supernatant. Add 50 µL acetonitrile. Let dehydrate for 15 min. Dry in vacuum centrifuge. Gel pieces can be store at 80°C for up to 3 months.)
4. Prepare the following solutions:

### **50mM ammonium bicarbonate (NH<sub>4</sub>HCO<sub>3</sub>)**

0.0396 g in 10.00 mL of Milli-Q water, pH should be ~8

### **10 mM dithiothreitol (DTT) in 50 mM ammonium bicarbonate**

0.0015 g DTT (electrophoresis grade) in 1.000 mL of 50 mM ammonium bicarbonate

### **100 mM iodoacetamide in 50 mM ammonium bicarbonate**

0.0185 g iodoacetamide in 1.000 mL of 50 mM ammonium bicarbonate

### **50% Acetonitrile/25 mM ammonium bicarbonate**

1 mL of acetonitrile (ACN)

1 mL of 50 mM ammonium bicarbonate

5. Remove the acetic acid from the tube, if present.

### **6. Destaining**

**Coomassie stained bands. This can also be used to wash Sypro-Ruby stained gels**

- Wash the gel with 50  $\mu$ L of 50 mM ammonium bicarbonate. Vortex mix and let stand for 5 minutes. Discard liquid into waste.
- Shrink the gel with 50  $\mu$ L of 50% acetonitrile/25 mM ammonium bicarbonate. Vortex mix and let stand for 10 minutes. Remove and discard the supernatant into waste. Gel pieces should have an opaque/ white appearance.

#### **Silver-stained bands**

- The silver-stained band/spot is incubated with a fresh mixture (v/v 1: 1) of 30 mM potassium ferricyanide (98 mg/10ml H<sub>2</sub>O) and 100mM sodium thiosulfate (248 mg/10ml H<sub>2</sub>O) for 15 minutes. Remove and discard the supernatant into waste.
  - Wash with 50  $\mu$ L deionized water. Vortex mix and let stand for 5 minutes. Discard liquid into waste.
  - Wash the gel with 50  $\mu$ L of 50 mM ammonium bicarbonate. Vortex mix and let stand for 5 minutes. Discard liquid into waste.
  - Shrink the gel with 50  $\mu$ L of 50% acetonitrile/25 mM ammonium bicarbonate. Vortex mix and let stand for 10 minutes. Remove and discard the supernatant into waste. Gel pieces should have an opaque/ white appearance.
7. Completely dry the gel pieces at ambient temperature in a vacuum centrifuge for 2-3 minutes.

#### **8. Reduction**

- Reduce with 30  $\mu$ L of 10 mM DTT for 30 minutes at 56°C (from 50 to 60°C is sufficient, room temperature is also adequate). Remove and discard the supernatant liquid into waste. (For cysteine-rich proteins, use 50 mM DTT.)

#### **9. Alkylation**

- Alkylate with 30  $\mu$ L of 100 mM iodoacetamide for 15 minutes in the dark at room temperature. (Wrap in foil.) Remove and discard the supernatant liquid into waste. (For cysteine-rich proteins, use 200 mM iodoacetamide.)

Peptides linked by disulfide bonds are first separated by reduction with DTT. To prevent the cysteine residues from recombining, they are alkylated with iodoacetamide to form a stable S-carboxymethyl derivative.

Steps 6 and 7 can be omitted if the gel spots come from a CBB-stained 2D gel where reduction and alkylation were completed between the 1<sup>st</sup> and 2<sup>nd</sup> dimension.

10. Shrink with 50  $\mu$ L of 50% acetonitrile/25 mM ammonium bicarbonate. Vortex mix and let stand for 15 minutes. Remove and discard the supernatant into waste.
11. Completely dry the gel pieces at ambient temperature in a vacuum centrifuge for 2-3 minutes.
12. The gel can be stored at 4°C overnight or at -20°C until digestion occurs.

#### **13. Digestion**

Prepare the trypsin solution:

**Trypsin Stock (0.2  $\mu$ g/ $\mu$ L)**: Add 500  $\mu$ L of 50 mM acetic acid to Promega Trypsin Gold 100  $\mu$ g (V5280). Aliquot into 10  $\mu$ l fractions. Store at -80°C. (50

mM acetic acid: 28.7  $\mu\text{L}$  glacial acetic acid into a final volume of 10.00 mL Milli-Q water. Store in glass vial.)

**13 ng/ $\mu\text{L}$  trypsin in 50 mM ammonium bicarbonate**

10  $\mu\text{L}$  of trypsin stock

140  $\mu\text{L}$  of 50 mM ammonium bicarbonate

- Cover the gel pieces with 13 ng/ $\mu\text{L}$  trypsin to the gel pieces (5  $\mu\text{L}$  for gel spot to 20  $\mu\text{L}$  for gel band). Let stand on ice for 30-45 min. to rehydrate the gel pieces. After 15-20 min., check samples, and add more trypsin solution if all the liquid has been absorbed.
- Before transferring to 37°C incubator, remove and discard any remaining solution. Add 5-20  $\mu\text{L}$  50 mM ammonium bicarbonate to cover the gel pieces.
- Place the digestion tube in a tube rack and wrap the rack plus tube completely with aluminum foil. Incubate at 37°C for 16-18 hours (overnight). The foil wrap helps minimize the amount of condensate that collects inside the reaction tube cap during the incubation and, thus, prevents the gel pieces from drying out overnight.

**14. Extraction of Peptides**

Prepare the following solutions:

**25 mM ammonium bicarbonate**

1 mL of 50mM ammonium bicarbonate

1 mL of Milli-Q water

**5% formic acid in water**

4.75 mL of Milli-Q water

250  $\mu\text{L}$  of formic acid

- a) Remove the tubes from the incubation. Sonicate for 5 min. in cool water (add some ice to keep water cool). Centrifuge briefly. From this point on the supernatant will be collected into a new 500  $\mu\text{L}$  siliconized microcentrifuge tube.
- b) Transfer the liquid to a new tube.
- c) Add 20  $\mu\text{L}$  of 25 mM ammonium bicarbonate to gel pieces. Vortex mix and let stand for 10 minutes. Sonicate for 2-3 min. in cool water. Add to the supernatant collected in step b).
- d) Add 20  $\mu\text{L}$  of 5% formic acid. Vortex mix and let stand for 10 minutes. Add to the supernatant collected in step b).
- e) Add 20  $\mu\text{L}$  of 100% acetonitrile. Vortex mix and let stand for 10 minutes. Add to the supernatant collected in step b).
- f) Add 20  $\mu\text{L}$  of 5% formic acid. Vortex mix and let stand for 10 minutes. Add to the supernatant collected in step b).

- g) Add 20  $\mu\text{L}$  of 100% acetonitrile. Vortex mix and let stand for 10 minutes. Add to the supernatant collected in step b).
- 15. Evaporate the solution to complete dryness in the vacuum centrifuge. Use only medium heat and it may take several hours.
- 16. Store at  $-20^{\circ}\text{C}$ .

## Appendix 5. Protocol for In-Solution Digestion of Membrane Proteins for MudPIT Analysis

### References:

ProteaseMAX surfactant, trypsin enhancer. Promega Technical Bulletin 1/10  
McClathy, D.B. and Yates, J.R. III, Comparison of Commercial Surfactants for MudPIT Analysis. Promega Corporation website. Updated 2010.

<http://www.promega.com/resources/articles/pubhub/comparison-of-commercial-surfactants-for-mudpit-analysis/> Accessed: February 21, 2012

### Use:

- Only MilliQ H<sub>2</sub>O
- Siliconized 1.5 mL microfuge tubes. Rinse tubes with USP-grade EtOH, followed by H<sub>2</sub>O, and let air-dry.
- Nitrile gloves

### Solutions (prepare fresh)

#### 50 mM Ammonium Bicarbonate (pH ~7.8)

Dissolve 98.8 mg NH<sub>4</sub>HCO<sub>3</sub> (MW=79.06) in 25.00 mL H<sub>2</sub>O.

#### 8 M Urea

Dissolve 480.5 mg of ultra-pure (MS grade) urea into a final volume of 1.00 mL H<sub>2</sub>O.

#### 0.5 M Dithiothreitol in 50 mM NH<sub>4</sub>HCO<sub>3</sub>

Dissolve 77.1 mg DTT (MW=154.25) in 1000 µL 50 mM NH<sub>4</sub>HCO<sub>3</sub>.

#### 0.55 M Iodoacetamide in 100 mM NH<sub>4</sub>HCO<sub>3</sub>

Dissolve 40.7 mg ICH<sub>2</sub>CONH<sub>2</sub> in 400 µL 50 mM NH<sub>4</sub>HCO<sub>3</sub>. Store in dark until use.

#### 1% ProteaseMax Solution

Add 100 µL 50mM (NH<sub>4</sub>)HCO<sub>3</sub> to vial (1mg; Promega V2071). Mix by swirling; not vortexing. Aliquot into 10-20 µL and store at -20°C.

#### 0.2% ProteaseMax Solution

Dilute 1% stock solution 1:5. Add 20 µL 1% stock to 80 µL 50mM (NH<sub>4</sub>)HCO<sub>3</sub>. Keep on ice.

#### 1µg/µL Trypsin Stock

Add 100 µL of 50 mM acetic acid to Promega Trypsin Gold 100 µg (V5280). Aliquot into 10 µL fractions. Store at -80°C. (50 mM acetic acid: 28.7 µL glacial acetic acid into a final volume of 10.00 mL Milli-Q water. Store in glass vial.)

Procedure:

For ~100-200 µg protein

1. Wash protein pellet with 250 µl 10mM Tris buffer pH 7.8 (to remove residual detergents and salts). Vortex. Centrifuge at 14,000xg at 4°C for 15 min.
2. Wash pellet with 300 µl ice-cold acetone (to remove lipids). Vortex. Centrifuge at 14,000 xg for 15 min. at room temperature. Let pellet air-dry but not completely.
3. Add 50 µl 0.2% ProteaseMax and 50 µl 8M urea. Vortex for 2 min. Continue mixing (on a shaker) until fully solubilised. May take up to 60 min.
4. Add 1 µl of 0.5 M DTT. Incubate at 56°C for 20 min.
5. Add 2.7 µl 0.55M iodoacetamide. Incubate at room temperature in the dark for 20 min.
6. Bring volume to 250 µl with 50mM (NH<sub>4</sub>)HCO<sub>3</sub>.
7. Add 4 µg trypsin (4 µl of 1 µg/µl Trypsin Stock) and 2.5 µl 1% ProteaseMax. Shake at 37°C for 3 hours
8. Snap-freeze in dry ice and store at -20°C until 2D LC MS/MS analysis.
9. Thaw, add formic acid to a final concentration of 5%. Centrifuge at 14,000xg for 30 min.
10. Analyze supernatant.

## Appendix 6. Protocol for the Isolation of Mitochondria from the CNS

Reference: Kristian, T. "Isolation of Mitochondria from the CNS" Current Protocols Neuroscience 52 (2010): 7.22.1-7.22.12

### Reagents

#### Bovine serum albumin (BSA)

10mg of fatty-acid free BSA in 1ml of water. Store at -20°C for maximum of 6 months

#### Isolation medium, pH 7.4

225mM Sucrose	342.3g/mol	7.70g
75mM Sorbitol	182.17g/mol	1.36g
1mM EGTA	380.4g/mol	0.038g
5mM HEPES	238.3g/mol	0.118g

Add to 100ml water, pH to 7.4 with Tris base. Store at 4°C for maximum of 1 month (Isolation medium with no EGTA has the same composition but with no EGTA)

#### Percoll solutions required for the isolation of two mouse brain

Final Percoll concentration (v/v)	100% Percoll (ml)	Isolation medium (ml)
24%	4.8	15.2
40%	4	6
15%	4	22.6

### Procedure:

1. Homogenize two mouse brains in 1ml of isolation medium in a Dounce homogenizer (on ice). Bring the final volume of homogenate to final volume of 10ml using isolation medium.
2. Transfer the homogenate equally into two 10ml polycarbonate tubes.
3. Centrifuge the homogenate 3min at 1300×g using F1010 fixed 35° angle rotor, 4°C in bench top ultracentrifuge (Beckman Coulter).
4. Collect the supernatant into new tubes and place it on ice. Avoid collecting fluffy loose material from top of the pellet.
5. Resuspend pellet in each tube in 5ml isolation medium followed by centrifuge 3min at 1300×g, 4°C. Collect the supernatant and combine it with the supernatant collected in previous step.
6. Centrifuge the pooled supernatant 10min at 21,000×g, 4°C. Discard the supernatant and resuspend the pellet in 3.5ml of 15% Percoll.
7. At this point prepare the Percoll gradient as described. Add 3.7ml of 24% Percoll into 10ml polycarbonate centrifuge tubes.
8. Using a pipet collect 1.5ml of 40% Percoll. Insert the pipet into the 24% Percoll, allow the tip touch to the bottom of the tubes and slowly introduce the 40% Percoll creating a discontinuous gradient. Prepare two tubes for two mouse brain.



9. Now slowly layer the pellet resuspended in 15% Percoll above the 24% Percoll.
10. Centrifuge 8min at  $30,700\times g$ ,  $4^{\circ}\text{C}$ , using slow acceleration (set the acceleration rate at 6sec per 1000rpm) and deceleration (no brakes).  
(At this centrifugation step material should redistribute into three major bands)
11. Very top layer above the gradient mostly contains myelin. Carefully remove the myelin layer from the top and resuspend in 3.5ml 15% Percoll for second round gradient separation.
12. Repeat steps 7-10. Carefully remove myelin layer and combine it from two tubes. Add 3-4ml ice cold water. Vortex and centrifuge 8-10min at  $30,700\times g$ ,  $4^{\circ}\text{C}$ . Myelin will be located at the bottom in a loose pellet. Discard the supernatant and collect the myelin from bottom, store at  $-80^{\circ}\text{C}$ .
13. Using pipet, collect the material banding near the interface of the upper two layers of the gradient. This contains mostly synaptosomes.
14. Collect the mitochondria accumulated at the interface between the bottom two layers 40% and 24% Percoll solutions in separate polycarbonate tubes.
15. Add 6ml of isolation medium to the mitochondria. Centrifuge fraction 10min at  $16,700\times g$ ,  $4^{\circ}\text{C}$ . Mitochondria will be at the bottom in the form of loose pellet.
16. Discard the supernatant and add 0.5ml of 10mg/ml fatty-acid free BSA, vortex, and then add 4.5ml of the isolation medium.
17. Centrifuge 10min at  $6900\times g$ ,  $4^{\circ}\text{C}$  will form firm pellet. Decant the supernatant and remove any remaining solution from wall of tube. Add 0.1ml of the isolation medium lacking EGTA to each tube and resuspend the pellet.

## **Appendix 7. Protocol for Western Blot**

### **10X Tris-Glycine Buffer, pH 8.3**

30.3g Tris base

144.0g glycine

Dissolve and bring total volume to 1000mL with MilliQ water. Do not adjust pH with acid or base. Store at 4 °C.

### **Transfer Buffer, pH 8.3**

25mM Tris, 192 mM Glycine, 20% methanol

To 1.0L graduate cylinder, add

100mL 10X Tris-Glycine buffer, pH 8.3

200mL methanol

Bring volume to 1000mL with MilliQ water. Chill on ice.

### **Electrotransfer procedure**

1. For each gel, 4 pieces of Whatmann 3MM filter paper (10.5 x 8.5 cm) and 1 piece of nitrocellulose (7.5 x 9.5 cm) is required.
2. Soak filter paper, nitrocellulose, and filter paper in a small tray containing the Transfer buffer.
3. Remove stacking gel and place gel in Transfer Buffer to remove the salts and detergents.
4. Assemble the blot. Make sure no air bubbles are trapped between each layer. Roll a glass rod over each layer, if needed. Assemble the gel sandwich on the black panel of the cassette in the following order:
  - a. Fiber pad
  - b. 2 pieces of filter paper
  - c. Gel
  - d. Nitrocellulose membrane
  - e. 2 pieces of filter paper
  - f. Fiber pad
5. Close the cassette and secure the latch.
6. Fill the tank completely with cold Transfer buffer. Add stir bar.
7. Place the cassette in the buffer tank with the black panel facing the black electrode.
8. Add the ice pack
9. Fill the buffer tank completely. Secure the lid. Turn on the stir plate and attach the electrodes to the HC power supply.
10. Set the current 200mA and electrotransfer for required time. If the required time is over 2 hours, the electrotransfer can be completed in the cold room.
11. Disassemble the cassette.
12. If Western blotting is not immediately completed, the nitrocellulose can be air dried on a filter paper and then wrapped in plastic and foil and store at 4 °C.

### **10X Tris Buffered saline with Tween-20 (TTBS)**

247 mM Tris, pH 7.6 mM KCl, 1.37 mM NaCl, 0.5% Tween-20

29.9 g Tris base  
2.0 g KCl  
80.1 g NaCl  
5.0mL 10% Tween – 20

Add to 600mL ddH<sub>2</sub>O, adjust pH to 7.6 and bring final volume to 1000mL. Store at 4°C.

### **5% milk solution**

2.0 g skim milk power  
Dissolve into 40mL 1X TTBS

### **2% gelatin solution**

0.8 g gelatin  
Dissolve into 40mL 1X TTBS. Gently heating maybe required to dissolve the gelatin.

Primary and secondary antibodies in 1X TTBS

### **General blotting procedure**

1. Wet dried nitrocellulose membrane in 1X TTBS
2. Block membrane in milk or gelatin solution for 2 hours with gently shaking. (Do not use milk for detection of phosphoproteins.)
3. Wash membrane with ~20mL 1X TTBS for 1 minute.
4. Wash 2 more times with 1X TTBS, each for 5 minutes.
5. Incubate membrane in primary antibody solution for 1 hour with gently shaking. (Longer incubation times may be needed. Overnight incubations can be completed at 4°C.)
6. Wash membrane in ~20mL 1X TTBS for 1 minute.
7. Wash 2 more times with 1X TTBS, each for 5 minutes.
8. Incubate membrane with secondary antibody solution for 1 hour with gently shaking.
9. Wash membrane in ~20mL 1X TTBS for 1 minute.
10. Wash 2 more times with 1X TTBS, each for 5 minutes.

### **Enhanced Chemiluminescence (ECL) detection**

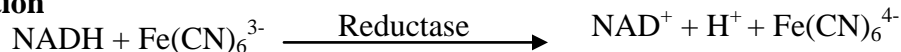
Using Amersham ECL Western Blotting Detection Reagents (GE HealthCare P/N 2106)

1. Prepare the ECL mixture; 1:1 volume of reagent A and reagent B. (In conical tube, mix 2mL reagent A and 2mL reagent B)
2. Place the drained membrane on a sheet of plastic wrap.
3. Carefully pipet the ECL mixture over the membrane. The surface tension will keep the solution on the membrane.
4. Incubate for 1.0 minute.
5. Drain the membrane and place on clean sheet of plastic wrap. Fold the plastic over the membrane, ensuring no bubbles form.
6. Immediately acquire the image with the CCD camera.

## Appendix 8. Protocol for NADH: Ferricyanide Reductase Activity Assay

*Reference: Langmuir 2007, 23, 3898-3905*

### Reaction



### Stock Solutions

20mM sodium phosphate buffer pH 7.5, 0.6% Triton-X100, 200mM NaCl

1. Dissolve 0.17g of  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  (MW 268.07g/mol), 0.18g of  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  (MW 137.99g/mol), and 1.169g of NaCl (MW 58.44g/mol) into 6mL of 10% Triton-X100
2. Dilute to 50mL with ultrapure water
3. Adjust pH to 7.5 with phosphoric acid, and bring up to final volume of 100mL with water.
4. Store at 4°C.

Cytochrome c reductase (Sigma C3381) Approximately 1unit/mg

Dissolve 25mg in 250 $\mu\text{L}$  sodium phosphate buffer. Approximately 1unit/10 $\mu\text{L}$

50mM  $\beta$ -NADH stock

Dissolve 0.035g of  $\beta$ -NADH (MW 709.41g/mol) in 0.5mL of 1X TBS buffer pH 7.2. Bring up to a final volume of 1mL with buffer. Aliquot into 60 $\mu\text{L}$  fractions and store at -80°C.

2.5mM  $\beta$ -NADH working solution (prepare fresh)

Dilute the 50mM stock solution to make a 1mL solution of 2.5mM  $\beta$ -NADH. Take 50 $\mu\text{L}$  of 50mM  $\beta$ -NADH stock and add 950 $\mu\text{L}$  1X TBS buffer pH 7.2.

2.5mM Potassium ferricyanide

Dissolve 0.0035g of potassium ferricyanide in 1mL of ultrapure water. Bring to final volume of 2mL with water.

\*All reagents are sufficient for 100 assays

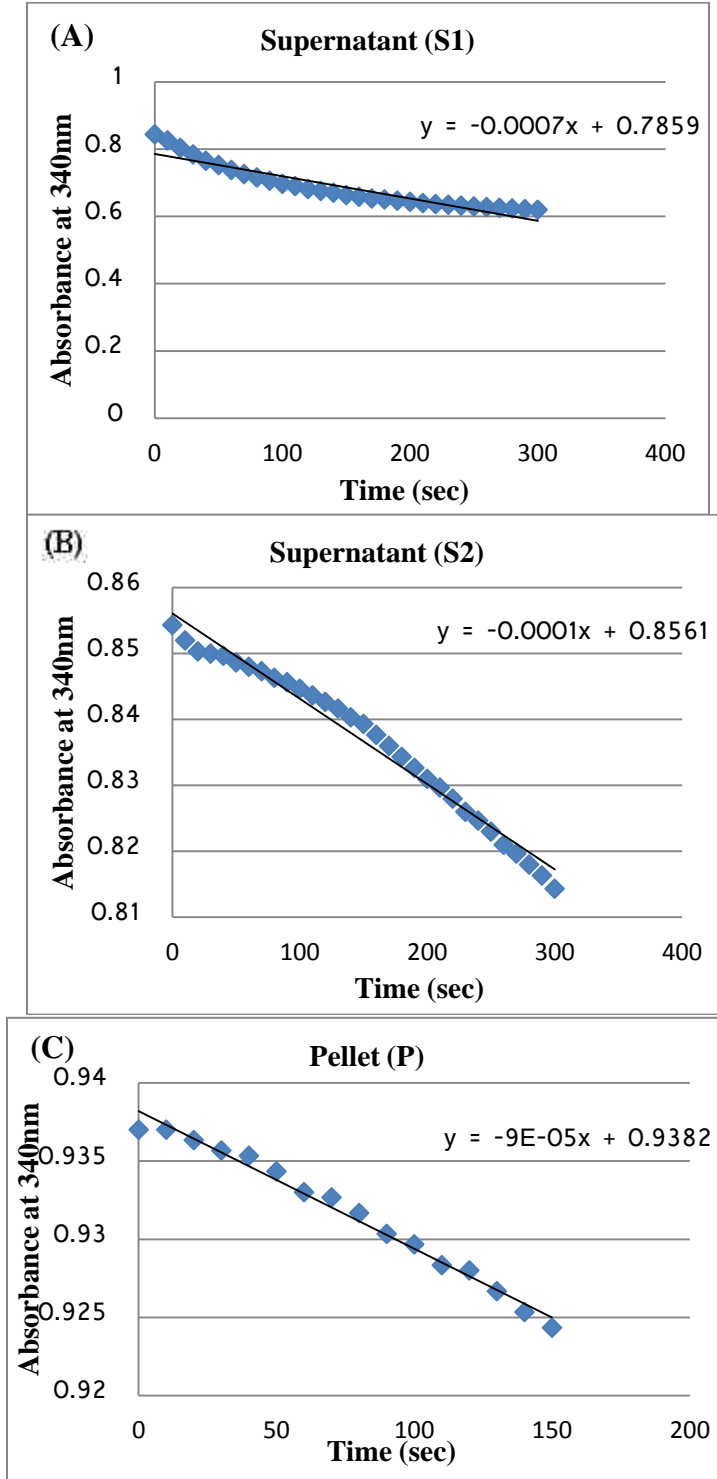
### Ferricyanide Assay Procedure:

1. Obtain a black 96 well microtiter plate with optical bottom (Nunc F96 plate Fisher 1256672)
2. Fill in the first 3 wells with blanks consisting of: 160 $\mu\text{L}$  buffer, 20 $\mu\text{L}$  NADH, and 20 $\mu\text{L}$  potassium ferricyanide
3. Load positive control into 1 well, consisting of 20 $\mu\text{L}$  of cytochrome c reductase, 20 $\mu\text{L}$  NADH and 140 $\mu\text{L}$  buffer.
4. Load 100 $\mu\text{L}$  buffer + 60 $\mu\text{L}$  sample + 20 $\mu\text{L}$  NADH, in triplicates.
5. To start the reaction, 20 $\mu\text{L}$  of potassium ferricyanide is added to all wells. This makes a total reaction volume of 200 $\mu\text{L}$ .

(Note: NADH and potassium ferricyanide quantity remains same as blank. Only buffer and sample volume changes in order to make total 200  $\mu\text{L}$ )

6. Measure fluorescence every 10 seconds for 5 minutes at the following conditions:  
Excitation wavelength: 340nm/ Emission wavelength: 460nm
7. OR: Measure absorbance at 340nm for 5 minutes at every 10 second interval
8. Plot data and calculate activity using the extinction coefficient of NADH,  
 $6220\text{M}^{-1}\text{cm}^{-1}$

## Appendix 9. Results of NADH: Ferricyanide Reductase Activity Assay



**Figure 5.1** – NADH: ferricyanide reductase activity assay on soluble and insoluble fraction from mouse myelin. (A) S1-supernatant extracted with 1.5% CHAPS at 4°C, 19µg (50µl) protein was used for analysis. (B) S2-supernatant extracted with 2% TX-100 at room temperature, 3.05µg (50µl) protein was used for analysis. (C) P-pellet extracted with 1.5% CHAPS at 4°C

followed by 2% TX-100 at room temperature, 2.35µg (50µl) protein was used for analysis. The reaction was initiated by adding 0.25mM (20µl) potassium ferricyanide in samples.

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