METHODS DEVELOPMENT AND APPLICATION OF TWO-DIMENSIONAL GEL ELECTROPHORESIS AND MASS SPECTROMETRY IN PROTEOMICS

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ABSTRACT OF DISSERTATION

Meena Uma Rajagopal

The Graduate School
University of Kentucky
2006
METHODS DEVELOPMENT AND APPLICATION OF TWO-DIMENSIONAL GEL ELECTROPHORESIS AND MASS SPECTROMETRY IN PROTEOMICS

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Arts and Sciences at the University of Kentucky

By
Meena Uma Rajagopal
Lexington, KY
Director: Dr. Bert C. Lynn, Professor of Chemistry
Lexington, KY
2006
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ABSTRACT OF DISSERTATION

METHODS DEVELOPMENT AND APPLICATION OF TWO-DIMENSIONAL GEL ELECTROPHORESIS AND MASS SPECTROMETRY IN PROTEOMICS

The development of a highly sensitive ruthenium-based fluorescent staining solution is described in this dissertation. The in-house synthesized ruthenium complex (RuMS) containing both sulfonated and non-sulfonated ligand has detection limit of 1 ng of protein that is better than colloidal coomassie, silver and ruthenium complex containing all sulfonated ligands (RuBPS). RuMS stain has 100-fold dynamic range and does not interfere with subsequent mass spectral identification of proteins. The capability of in-house synthesis of the staining solution makes it a viable cost-effective alternative to the expensive commercially available fluorescent stain, Sypro Ruby. The low detection limit, broad linear dynamic range and compatibility with mass spectrometry, make the development of this stain a worthwhile pursuit. The staining solution was utilized in subsequent applications of two-dimensional gel electrophoresis (2-DE) technology.

Proteomics methodology utilizing 2-DE and mass spectrometry was applied to investigate the effect of malathion on the proteome of human neuroblastoma cells. Results indicated that out of 122 proteins that were identified from the neuroblastoma proteome, sixteen proteins were down-regulated while five proteins were significantly up-regulated after treatment with malathion. Significant down-regulation of calcium modulators like calmodulin and calgizarrin and other key chaperones makes the malathion-treated cells highly prone to oxidative stress. With increased awareness in pesticide related adverse effects, identification of altered proteins in malathion-treated human neuroblastoma cells is a critical finding.

Proteomics is a major area of research in the identification of biomarkers for diseases. A novel immunoprecipitation method developed in this work allowed for successful isolation and identification of albumin-interactome in cerebrospinal fluid (CSF) that is usually under-represented in standard CSF analysis using 2-DE. A key finding is the differential expression of various isoforms of proteins in CSF albumin-interactome from Alzheimer’s disease (AD) subjects. The data implicate the acidic isoform of prostaglandin D2 synthase (PGDS2) as a potential biomarker for AD. An understanding
of the differential expression of these protein isoforms in AD will provide insight into the etiology of the disease and this can have far-reaching implication on drug development leading to the cure or even prevention of the disease.

KEYWORDS: proteomics, 2-DE, mass spectrometry, OP toxicity and Alzheimer’s disease

Meena Uma Rajagopal

May 09, 2006
METHODS DEVELOPMENT AND APPLICATION OF TWO-DIMENSIONAL GEL ELECTROPHORESIS AND MASS SPECTROMETRY IN PROTEOMICS

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METHODS DEVELOPMENT AND APPLICATION OF TWO-DIMENSIONAL GEL ELECTROPHORESIS AND MASS SPECTROMETRY IN PROTEOMICS

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Arts and Sciences at the University of Kentucky

By
Meena Uma Rajagopal
Lexington, KY

Director: Dr. Bert C. Lynn, Professor of Chemistry
Lexington, KY
2006
Copyright © Meena Uma Rajagopal 2006
Dedicated to

my beloved mom Uma
sister Vidya

and
loving husband Rajagopal
ACKNOWLEDGEMENTS

I would like to sincerely thank Dr. Bert Lynn for accepting me in his group and for giving me this wonderful opportunity to learn new cutting-edge technologies. I am ever grateful for his guidance, support and patience. Every interaction with him has been a learning experience for me.

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TABLE OF CONTENTS

Acknowledgements .................................................................................................................. iii
List of Tables ........................................................................................................................... ix
List of figures .......................................................................................................................... x
List of files ................................................................................................................................ xii

CHAPTER ONE: INTRODUCTION

A FLUORESCENT RUTHERNIUM COMPLEX FOR STAINING PROTEINS
SEPARATED BY SDS-PAGE

1.1 Study goal.......................................................................................................................... 1
1.2 Study hypothesis............................................................................................................... 2
1.3 Study rational.................................................................................................................... 2
1.4 Specific aim....................................................................................................................... 3

PROTEOMIC INVESTIGATION OF MALATHION-INDUCED DIFFERENTIAL
PROTEIN EXPRESSION IN HUMAN NEUROBLASTOMA CELLS

2.1 Study goal.......................................................................................................................... 3
2.2 Study hypothesis............................................................................................................... 3
2.3 Study rational.................................................................................................................... 3
2.4 Specific aim....................................................................................................................... 4

COMPARISON OF ALBUMIN-INTERACTOME IN VENTRICULAR CSF
BETWEEN ALZHEIMER’S DISEASE AND NORMAL SUBJECTS

3.1 Study goal.......................................................................................................................... 4
3.2 Study hypothesis............................................................................................................... 4
3.3 Study rational.................................................................................................................... 4

v
CHAPTER TWO: BACKGROUND

2.1 Two-dimensional gel electrophoresis
   • Isoelectric focusing
   • Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

2.2 Mass spectrometry
   • Quadrupole ion trap mass spectrometer
   • Time-of-flight mass spectrometer

2.3 Ionization techniques
   • Electrospray
   • MALDI

2.4 Western blotting

2.5 Statistical treatment of data

CHAPTER THREE: A FLUORESCENT RUTHENIUM COMPLEX FOR STAINING PROTEINS SEPARATED BY SDS-PAGE

3.1 Introduction

3.2 Materials and Methods
   3.2a Chemicals
   3.2b Synthesis of RuBPS
   3.2c Synthesis of RuDS
   3.2d Synthesis of RuMS
   3.2e 1D gel electrophoresis
   3.2f Characterization of in-house synthesized ruthenium complexes
   3.2g Staining of mini gels
   3.2h Imaging of stained gels
   3.2i In-gel proteolysis
   3.2j In-gel proteolysis of proteins from silver-stained gels
3.2k MALDI-TOF mass spectrometry analysis.................................70
3.2l LC-MS/MS mass spectrometry analysis.................................70

3.3 Results
3.3a Characterization of ruthenium complexes............................71
3.3b Staining efficiency of various ruthenium complexes
    synthesized in-house.........................................................78
3.3c Optimization of in-house RuMS staining.............................78
3.3d Optimization of commercial RuMS staining..........................83
3.3e Sensitivity of various staining solutions.............................87
3.3f Linearity of staining response........................................87
3.3g Compatibility of stains with mass spectrometry....................93

3.4 Discussion
3.4a Characterization of in-house synthesized
    ruthenium complexes.......................................................104
3.4b Optimization of in-house RuMS staining............................106
3.4c Comparison of sensitivity of various staining solutions...........109
3.4d Linearity of staining response........................................110
3.4e Compatibility of stains with mass spectrometry....................111
3.4f Cost-effectiveness of RuMS stain......................................113

3.5 Conclusion...........................................................................116

CHAPTER FOUR: PROTEOMICS INVESTIGATION OF MALATHION-
INDUCED DIFFERENTIAL PROTEIN EXPRESSION IN HUMAN
NEUROBLASTOMA CELLS

4.1 Introduction...........................................................................119
4.2 Materials and Methods
4.2a Materials...........................................................................122
4.2b Cell culture.........................................................................123
4.2c Cell viability assay.............................................................123
4.2d Malathion treatment...............................................................124
4.2e 2-DE..............................................................................124
4.2f Staining of proteins.............................................................125
4.2g Image and statistical analyses............................................125
4.2h In-gel proteolysis of proteins.............................................125
4.2i MALDI-TOF mass spectrometry analysis..........................126
4.2j LC-MS/MS analysis.........................................................126
4.2k Database search..............................................................126
4.2l Western blotting...............................................................127

4.3 Results
4.3a Dose-dependent effects of malathion on cell viability..........128
4.3b Identification of proteins using mass spectrometry.............128
4.3c Analysis of SY5Y proteome after treatment with malathion...130
4.3d Validation of 2-DE results by western blotting...............146

4.4 Discussion
4.4a Cell viability.................................................................147
4.4b Alteration in levels of proteins after treatment with malathion...148

4.5 Conclusion........................................................................161

CHAPTER FIVE: COMPARISON OF ALBUMIN-INTERACTOME IN VENTRICULAR CSF BETWEEN ALZHEIMER’S DISEASE AND NORMAL SUBJECTS

5.1 Introduction........................................................................163

5.2 Materials and Methods
5.2a Materials......................................................................165
5.2b Human ventricular CSF samples......................................166
5.2c Efficiency of Microcon cut-off filters...............................166
5.2d Crude isolation of albumin-interactome
  using Microcon cut-off filters.............................................167
5.2e Crosslinking of human anti-albumin antibody
to Protein G beads……………………………………………………………167
5.2f Immunoprecipitation of albumin-interactome………………………….168
5.2g 1D gel electrophoresis……………………………………………………169
5.2h 2-DE……………………………………………………………………169
5.2i Image and statistical analyses……………………………………………169
5.2j Identification of proteins using mass spectrometry………………………170

5.3 Results
5.3a 2-DE of un-processed CSF………………………………………………170
5.3b Efficiency of Microcon cut-off filters……………………………………170
5.3c Crude isolation of albumin-interactome
   using Microcon cut-off filters………………………………………………174
5.3d Optimization of immunoprecipitation of
   albumin-interactome from CSF………………………………………………180

5.4 Discussion
5.4a Efficiency of Microcon cut-off filters……………………………………190
5.4b Crude isolation of albumin-interactome
   using cut-off filters……………………………………………………………191
5.4c Immunoprecipitation of albumin-interactome……………………………192

5.5 Conclusion……………………………………………………………………198

CHAPTER SIX: CONCLUSION
Conclusion……………………………………………………………………201

Appendix A……………………………………………………………………205
Appendix B……………………………………………………………………213
References……………………………………………………………………217
VITA……………………………………………………………………………236
LIST OF TABLES

Table 2.1 Types of different mass analyzers..........................................................19
Table 3.1 Peaks observed in MALDI-TOF mass spectrum of
different forms of ruthenium complexes synthesized in-house.......................73
Table 3.2 Summary of chromatographic data for various
in-house synthesized ruthenium complexes..................................................78
Table 3.3 Band volumes for different loads of
standard proteins in gels stained with different staining solution....................91
Table 3.4 Summary of MALDI-TOF data for different protein loads
of standard proteins from gels stained with various stains.............................95
Table 3.5 Summary of LC-MS/MS data for different protein loads
of standard proteins from gels stained with various stains...........................100
Table 3.6 Pros and cons of various fluorescent stains........................................115
Table 4.1 List of proteins altered due to treatment with malathion.................135
Table 5.1 Protein recovery using Microcon 30 kDa cut-off filter.......................173
Table 5.2 List of CSF proteins identified by LC-MS/MS ..............................177
Table 5.3 List of altered CSF proteins in CSF from AD subjects......................179
Table 5.4 List of proteins identified from 1D gel evaluating
three elution buffers by LC-MS/MS..............................................................182
Table 5.5 List of proteins identified from the 1D gel
after immunoprecipitation of albumin-interactome from CSF.....................189
LIST OF FIGURES

Figure 2.1 2-DE approach in bottom-up proteomics………………………………...8
Figure 2.2 Structures of common reagents used in 2-DE sample buffer………………11
Figure 2.3 Isoelectric focusing…………………………………………………………..13
Figure 2.4 General schematic of a mass spectrometer……………………………….18
Figure 2.5 Quadrupole ion trap mass analyzer………………………………………...22
Figure 2.6 Mathieu stability diagram for QIT…………………………………………..23
Figure 2.7 Mass selective instability scan………………………………………………27
Figure 2.8 Schematic of linear TOF……………………………………………………33
Figure 2.9 Effect of initial time, space and kinetic energy distributions in linear TOF………………………………………………………………………………..35
Figure 2.10 Schematic of ReTOF…………………………………………………………35
Figure 2.11 Different types of fragmentation that can occur in TOF…………………...37
Figure 2.12 Ion formation in electrospray………………………………………………40
Figure 2.13 Two theories for formation of charged analyte molecules from taylor cone………………………………………………………………………………42
Figure 2.14 Matrix assisted laser desorption ionization………………………………44
Figure 2.15 Structures of MALDI matrices…………………………………………46
Figure 2.16 Western Blotting…………………………………………………………….49
Figure 2.17 ECL detection…………………………………………………………………51
Figure 3.1 Structure of CCB R250………………………………………………………56
Figure 3.2 Structure of CCB G250………………………………………………………57
Figure 3.3 Structure of RuBPS………………………………………………………….61
Figure 3.4 Structure of RuDS…………………………………………………………….62
Figure 3.5 Structure of RuMS……………………………………………………………64
Figure 3.6 Excitation and Emission spectra for RuMS………………………………72
Figure 3.7 MALDI-TOF spectrum for RuNS…………………………………………74
Figure 3.8 MALDI-TOF spectrum for in-house RuMS………………………………75
Figure 3.9 MALDI-TOF spectrum for in-house RuDS………………………………76
Figure 3.10 MALDI-TOF spectrum for in-house RuBPS…………………………….77
Figure 3.11 Staining efficiency of various in-house Synthesized ruthenium complezes……………………………………………………………………………….79
Figure 3.12 Optimization of solvent for in-house RuMS staining…………………..80
Figure 3.13 Optimization of ethanol concentration for in-house RuMS staining…………………………………………………………………………………..81
Figure 3.14 Optimization of concentration of in-house RuMS……………………..82
Figure 3.15 Optimization of solvent for commercial RuMS…………………………83
Figure 3.16 Optimization of solvent for commercial RuMS…………………………84
Figure 3.17 Optimization of concentration of commercial RuMS……………………85
Figure 3.18 Comparison of staining efficiency between in-house And commercial RuMS……………………………………………………………………………….86
Figure 3.19 Representative gels stained with four different protein stains…………88
Figure 3.20 Linearity in staining standard proteins for CCB G250 and silver…………89
Figure 3.21 Linearity in staining standard proteins for RuMS and Sypro Ruby……...90
Figure 3.22 Histogram showing sensitivity of different protein stains………………92
Figure 3.23 MALDI-TOF spectra for 100 ng BSA from gels stained with four different stains

Figure 3.24 MS/MS fragmentation of BSA peptide from gels stained with four different stains

Figure 3.25 2-DE of 400 μg whole cell lysate stained with in-house RuMS

Figure 3.26 2-DE of 400 μg whole cell lysate from SY5Y cells treated with 50 μM malathion for 16 hours

Figure 3.27 2-DE of 400 μg whole cell lysate from SY5Y cells treated with 100 μM malathion for 24 hours

Figure 3.28 2-DE of 400 μg whole cell lysate from SY5Y cells treated with 100 μM malathion for 48 hours

Figure 3.29 Close-up of gel sections showing differential expression of UCH L1, rho GDI and GST proteins

Figure 3.30 Close-up of gel sections showing differential expression of calmodulin

Figure 4.1 Dose-dependent effect of malathion on cell viability

Figure 4.2 2-DE of 400 μg whole cell lysate protein from control SY5Y cells

Figure 4.3 2-DE of 400 μg whole cell lysate from SY5Y cells treated with 50 μM malathion for 16 hours

Figure 4.4 2-DE of 400 μg whole cell lysate from SY5Y cells treated with 100 μM malathion for 24 hours

Figure 4.5 2-DE of 400 μg whole cell lysate from SY5Y cells treated with 100 μM malathion for 48 hours

Figure 4.6 Close-up of gel sections showing differential expression of UCH L1, rho GDI and GST proteins

Figure 4.7 Close-up of gel sections showing differential expression of calmodulin

Figure 4.8 MS/MS spectrum for a tryptic peptide from UCH L1

Figure 4.9 MALDI-TOF mass spectrum for GRP 75

Figure 4.10 Western blotting analysis of UCH L1

Figure 4.11 Proposed model for malathion-induced cytotoxicity in human neuroblastoma cells

Figure 5.1 2-DE map of 150 μL of un-processed CSF

Figure 5.2 Efficacy of Microcon 30 kDa cut-off filter

Figure 5.3 Recovery of proteins using 30 kDa cut-off filter

Figure 5.4 Master gel containing all proteins spots present in two populations- AD and control

Figure 5.5 2-DE maps of proteins in albumin-interactome fraction isolated from CSF using cut-off filters

Figure 5.6 SDS-PAGE analysis of CSF proteins eluted from anti-albumin antibody attached Protein G beads

Figure 5.7 Schematic for immunoprecipitation of albumin-interactome from CSF

Figure 5.8 SDS-PAGE analysis of proteins isolated by immunoprecipitation from 10 μL of CSF

Figure 5.9 SDS-PAGE analysis of proteins isolated by immunoprecipitation from 100 μL of CSF

Figure 5.10 SDS-PAGE analysis of proteins isolated by immunoprecipitation from 250 μL of CSF

Figure 5.11 Optimized immunoprecipitation of albumin-interactome from CSF using anti-albumin antibody crosslinked Protein G beads
LIST OF FILES

MURajagopal-dissertation.pdf
CHAPTER ONE
INTRODUCTION

Successful completion of the Human Genome Project led to the surprising finding that there are far fewer genes in the human genome that actually code for proteins than there are proteins in the human proteome. This discrepancy implied that gene analysis alone is not sufficient to understand the complexity of life. The rapidly growing field of proteomics that involves the global analysis of proteins was initiated to enable better understanding of proteins- the functional entities of life. Marked improvements in various proteomics tools are essential for continued exploitation of this technology in various fields of research.

Proteomics is the subject of this dissertation. This work has been organized into three main data chapters. Two techniques that were employed in this work are two-dimensional gel electrophoresis and mass spectrometry. Development of a sensitive staining solution that is a key component of proteomics technology utilizing two-dimensional gel electrophoresis is discussed in this dissertation. The technology was applied in two different areas of research namely environmental toxicology and biomarker discovery.

A FLUORESCENT RUTHENIUM COMPLEX FOR STAINING PROTEINS SEPARATED BY SDS-PAGE

1.1 Study Goal

The goal of this project is to synthesize a ruthenium chelate, optimize the solvent composition so that the stain can be used to detect proteins separated by gel electrophoresis with better sensitivity than RuBPS and can be used as a cost-effective alternative to Sypro Ruby.
1.2 Study Hypothesis

The hypothesis is that ruthenium complex with both sulfonated and non-sulfonated ligands will provide better sensitivity than the RuBPS complex that contains all sulfonated ligands and the development of the stain in-house will prove to be cost-effective.

1.3 Study Rational

Commonly employed staining solutions to visualize proteins in polyacrylamide gels have inherent disadvantages associated with them like low linear dynamic response for silver stain and low detection limits for Coomassie blue. Improved techniques for the detection of proteins in 2-DE gels involve staining with fluorescent dyes, such as Sypro Ruby. Sypro Ruby is a ruthenium-based dye that has detection limit of approximately 1-2 ng of protein and about three orders of magnitude of linear dynamic range of quantification.

An alternative to Sypro Ruby staining based on ruthenium (II) tris (bathophenanthroline disulfonate) (RuBPS) has been developed by Rabilloud et al. We synthesized RuBPS in our lab following the published Rabilloud protocol to stain proteins. It was observed that RuBPS stain, an all-sulfonated ligand-containing complex had poor sensitivity compared to Sypro Ruby and could detect only about 5 ng of protein on the gel. This prompted us to explore the possibility of having both sulfonated and non-sulfonated ligands attached to the ruthenium complex. We predict that the presence of both sulfonated and non-sulfonated ligands attached to ruthenium chelate in an appropriate solvent may result in an additive, synergistic interaction of the different ligands of dye molecules with the proteins and in turn improve sensitivity. Since the dye can be synthesized in-house it provides for a cost-effective means to stain proteins.
1.4 Specific Aims

The specific aims are to synthesize and optimize the formulation of a ruthenium complex containing both sulfonated and non-sulfonated ligands, to achieve maximum sensitivity in detecting proteins in gels.

PROTEOMIC INVESTIGATION OF MALATHION-INDUCED DIFFERENTIAL PROTEIN EXPRESSION IN HUMAN NEUROBLASTOMA CELLS

2.1 Study Goal

The goal is to investigate the effect of malathion on the proteome of human neuroblastoma cells using 2-DE and mass spectrometry.

2.2 Study Hypothesis

The hypothesis is that treatment of human neuroblastoma cells with malathion, a safe organophosphate insecticide, does not cause significant alteration in levels of protein.

2.3 Study Rational

An increasing body of literature suggests that developmental exposure to the organophosphate chlorpyrifos results in biochemical and behavioral abnormalities in young animals. It was also reported that chlorpyrifos could cause developmental neurotoxicity in exposed young children. Malathion, on the other hand, is a more benign organophosphate and is being used in huge quantities to eradicate pests in both agriculture and the household. There is general lack of data in literature on the effects of pesticide exposure at the protein level. This study was initiated to conduct a proteomic investigation on the effect of pesticides and we predict that malathion, a safer organophosphate, compared to other potent organophosphates like chlorpyrifos, does not cause significant alteration in levels of protein.
2.4 Specific Aim

The specific aim is to study the effect of malathion, if any, on protein expression in human neuroblastoma using proteomic tools namely 2-DE and mass spectrometry.

COMPARISON OF ALBUMIN-INTERACTOME IN VENTRICULAR CEREBROSPINAL FLUID BETWEEN ALZHEIMER’S DISEASE SUBJECTS AND NORMAL SUBJECTS

3.1 Study Goal

The primary goal of this project is to identify biomarkers for Alzheimer’s disease by comparing the albumin-interactome in CSF between Alzheimer’s disease subjects and normal subjects.

3.2 Study Hypothesis

The hypothesis is that alterations in levels of proteins that constitute the CSF albumin-interactome will provide insight into Alzheimer’s disease pathology.

3.3 Study Rational

CSF is an ideal target to identify protein biomarkers for diseases like Alzheimer’s disease (AD) that affect the brain. Two-dimensional gel electrophoresis (2-DE) appears particularly well-suited for such analyses since it has the ability to resolve the complex protein mixture into individual protein spots in a polyacrylamide gel. However, a major challenge associated with the analysis of CSF by 2-DE is the very large dynamic range of CSF protein expression. The CSF proteome consists mostly of albumin, transferrin, immunoglobulins and these represent greater than 70% of the total protein in CSF. One way to overcome the dynamic range problem is to remove the most abundant proteins albumin and immunoglobulins using commercially available depletion kits. However,
there is increasing evidence that depletion of these most abundant proteins could lead to concomitant removal of other small proteins and peptides that might be interacting with the high abundant proteins. This would mean loss of valuable information about these potential biomarkers for the disease. The increased awareness that small proteins and peptides interact with the most abundant proteins like albumin led us to selectively isolate and identify the CSF albumin-interactome using immunoprecipitation technique.

3.4 Specific Aim

There are two specific aims in pursuit of this goal. The first specific aim is to isolate the albumin-interactome by immunoprecipitation and identify the protein components that constitute the albumin-interactome, using mass spectrometry.

The second specific aim is to compare the albumin-interactome in CSF between AD and control subjects to identify biomarkers for AD.
CHAPTER TWO

BACKGROUND

Main technologies utilized in the preparation of this work are 2-DE and mass spectrometry and this chapter provides background information and literature review on these two techniques. Statistical treatment of data is also briefly discussed here.

2.1 TWO-DIMENSIONAL GEL ELECTROPHORESIS (2-DE)

Two-dimensional gel electrophoresis (2-DE) is a powerful and widely used method for the analysis of complex mixture of proteins like biological fluids, cell extracts, tissue samples etc. This technique was first introduced by O’Farrell [1], Klose [2] and Scheele [3] in 1975 to map proteins from Escherichia coli, mouse and guinea pig respectively. 2-DE separates proteins by exploiting two properties of proteins in orthogonal directions. In the first dimension called the isoelectric focusing step, proteins are separated based on their isoelectric point (pI). In the second dimension step, sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) proteins are separated based on their molecular weight (Mr). Development of immobilized pH gradient (IPG) strips coupled with pre-cast polyacrylamide gels and introduction of new sensitive fluorescent stains have greatly improved the capacity, sensitivity and reproducibility of 2-DE.

In the original technique, carrier ampholytes were used to form a pH gradient under the influence of electric field and the first dimension isoelectric focusing was performed in carrier ampholyte containing polyacrylamide tube gels. In the current method developed by Gorg et al. [4, 5] the carrier ampholyte-generated pH gradient is replaced with IPG strips. This change overcomes limitations like reproducibility, handling and resolution of carrier ampholyte generated pH gradient gels. After 2-DE separation, proteins separated on a polyacrylamide gel are subjected to “in-gel” proteolytic digestion. The resulting peptides are analyzed by either matrix assisted laser desorption ionization (MALDI) or liquid chromatography tandem mass spectrometry (LC-MS/MS) and the mass spectrum is
searched against protein database for identification. The major steps involved in the proteomics approach utilizing 2-DE are sample preparation, protein separation by isoelectric focusing, equilibration with SDS buffer, separation by SDS-PAGE, protein detection using sensitive staining solution, analysis and quantification using image analysis software, spot excision and in-gel enzymatic digestion and finally identification using mass spectrometry (Figure 2.1). Each step in 2-DE will be discussed in detail in the following sections.

- **Sample preparation**

Appropriate sample preparation is absolutely essential for good 2-DE results. The method of sample preparation depends on the aim of the research. Various factors such as solubility, size, charge and isoelectric point (pI) of proteins of interest play critical role. Sample preparation/solubilization is important in reducing the complexity of a protein mixture. There is a wide range of solubilization cocktails available to researchers but it should be kept in mind that the sample to be loaded on a 2-DE must be of low ionic strength denaturing buffer that maintains the native charge of the protein and keeps them soluble.

An effective sample preparation procedure should be able to achieve the following:

1. **Solubilize the proteins including hydrophobic proteins**: denature the proteins to break the non-covalent interactions and break disulfide bonds.
2. **Prevent protein aggregation but keep the proteins in solution**.
3. **Prevent post-extraction modifications like degradation or chemical modifications of the proteins**.
4. **Remove IEF interfering species like nucleic acids and salts**. In 2-DE, proteins are the analyte and hence anything other than proteins are contaminants, some of which that can affect IEF must be completely removed.
5. **Provide proteins of interest at a detectable level** which might require removal of abundant proteins especially when handling biological fluids.
Figure 2.1 2-DE approach in bottom-up proteomics
• **Rationale for Solubilization**

Forces such as disulfide bonds and non-covalent interactions hold proteins together. The non-covalent forces are mainly ionic bonds, hydrogen bonds and hydrophobic interactions. The hydrophobic interaction is because of the presence of water and the side chains of apolar amino acids like leucine, valine, isoleucine, phenylalanine, tryptophan and tyrosine pack together and form “hydrophobic core” of the protein due to the thermodynamically unfavorable exposure to water molecules. Therefore, a good solubilization buffer should break the ionic bonds, hydrogen bonds, hydrophobic interactions and disulfide bonds under IEF-compatible conditions i.e. very low salts and other charged molecules. Rabilloud describes a detailed review on solubilization of proteins for electrophoresis [6].

Solubilization of proteins is usually achieved by the use of chaotropic agents, detergents, reducing agents, buffers and ampholytes. Structures of common reagents used are given in figure 2.2.

**Chaotropic agent**: Chaotropic compounds are chemicals that disrupt hydrogen bonding between and within proteins. They prevent protein aggregation and formation of secondary structure that can alter protein mobility. Neutral urea in concentration of 8M is usually used as a chaotrope in 2-DE that completely unfolds the protein to random coil and exposes the ionizable groups. However, care must be taken not to heat urea-containing solutions above 37°C as urea hydrolyses and carbamylation of protein occurs. Neither must the temperature of the solution go below 4°C since urea precipitates at this temperature. Thiourea is also sometimes used in addition to urea to increase the solubility of otherwise difficult-to-solubilize proteins like membrane proteins [7].

**Detergent**: In addition to the chaotropic agent, a detergent is also added to facilitate protein solubility. There are a number of cationic, anionic and zwitterionic compounds that can be used as detergents. For IEF, zwitterionic or non-ionic detergent is the best to use, as it is uncharged. The detergent aids in protein solubilization and prevents its
aggregation by disrupting the hydrophobic interactions. Originally the nonionic detergent Triton X-100 was used [1, 2] but subsequently it was shown that the zwitterionic detergent CHAPS was more effective. Detergents are usually used in concentrations up to 4% [8]. The anionic detergent SDS can be used as it is a very effective protein solubilizer but because of the charge and the ability to form associations with proteins it can affect IEF. The negative effect of SDS on IEF can be minimized by the addition of another detergent in combination with SDS and keeping the detergent to SDS ratio about 8:1 [9]. New detergents like SB 3-10 and ASB-14 that have great potential are being used in proteomics studies [10].

**Reducing agent:** Reducing agents are used in sample preparation to break the disulfide bond between cysteine residues between and within proteins. Compounds used as reducing agents in 2-DE are either sulfydryl or phosphine reducing agents. Examples of sulfydryl containing compounds are dithiothreitol (DTT) and β-mercaptoethanol (βME). Examples of phosphine reductants include tributylphosphine (TBP) and tris-carboxyethylphosphine (TCEP). The phosphine containing reductants are more effective than the sulfydryl compounds and can be used in lower concentrations. Following reduction of disulfide bonds, the free –SH groups are protected by alkylation using iodoacetamide. This prevents oxidative crosslinking of –SH groups.

**Carrier ampholytes and buffer:** Carrier ampholytes are small, amphoteric substances that have great buffering capacity at their pI. They are added in sample solubilization buffer to enhance protein solubility. They prevent protein aggregation due to charge-charge interaction. They are used typically in concentrations of <0.2% (w/v) in the sample buffer. Since they carry current, high concentrations of carrier ampholytes slow down IEF till they are focused at their pI and hence limit the voltage.
Figure 2.2 Structures of common reagents used in 2-DE sample buffer
**FIRST DIMENSION – ISOELECTRIC FOCUSING (IEF)**

The first dimension in 2-DE separation is the isoelectric focusing. IEF is an electrophoretic method that separates proteins according to their isoelectric point (pI). Proteins are amphoteric biomolecules carrying both negative and positive charges on the same molecule. The isoelectric point of a protein is the pH at which the protein carries no net charge. Proteins are positively charged at a pH below their isoelectric point and are negatively charged at a pH above their pI. For IEF separation, pH gradient is essential and mixture of proteins is separated in the pH gradient as they move under the influence of an electric field. A protein that is positively charged will move towards the cathode until it reaches a pH at which it carries a net zero charge (pH equal to its pI) and does not migrate further as it does not see the electric field at this point (Figure 2.3). If for some reason the protein diffuses from its pI point, it is immediately focused back to position. This is the focusing effect of IEF. The degree of resolution is determined by the pH gradient and voltage applied. Typically IEF is performed at high voltages. IEF carried out under denaturing conditions gives the best resolution, as the proteins are all completely denatured and exists in only one conformation.

The original method of IEF depended on pH gradient formed by carrier ampholytes in polyacrylamide tube gels [1, 2]. The carrier ampholytes comprise of small amphoteric molecules with pIs spanning a specific pH range. When voltage is applied, the carrier ampholyte with the most negative charge will move towards the anode, the molecule with the most positive charge will move towards the cathode and a pH gradient is established. However, the major disadvantage of a pH gradient formed by carrier ampholytes is the drift in the pH. These molecules are unstable and suffer batch-to-batch reproducibility. The polyacrylamide tube gels have low mechanical stability and also suffer from batch-to-batch differences.
Figure 2.3 Isoelectric focusing. A mixture of proteins resolved on a pH 3-10 IPG strip according to their pIs
Gorg et al. developed another method to form pH gradient polyacrylamide gels[4, 5, 11].
An immobilized pH gradient polyacrylamide gel strip was developed by covalently
incorporating a gradient of acidic and basic buffering groups into the polyacrylamide gel
at the time of its polymerization. The buffers are called acrylamido buffers that are well-defined molecules with a single acidic or basic buffering group linked to an acrylamide
monomer. The immobilized pH gradients are formed using two solutions, one containing
a relatively acidic mixture of acrylamido buffers and other containing basic mixture. The
concentrations of the various solutions define the pH range. During polymerization the
acrylamido portion of the buffer co-polymerizes with the acrylamide and bisacrylamide
monomers to form a polyacrylamide gel. The gel is cast onto a plastic backing for easy
handling and is usually dried which can be rehydrated with the sample.

Low voltage is applied sometimes to aid the sample rehydration, a process called active
rehydration that is carried out typically for 12 hours (overnight). IPG strips are a marked
improvement over tube gels in the following aspects:

- The first dimension separation is more reproducible because the covalently
  fixed gradient cannot drift
- The plastic backing provides easy handling
- With the IPG technology desired pH range can be developed
- The IPG strips have greater loading capacity for protein [11]
- The protein sample can be introduced into the IPG strip during rehydration
  [12]

Samples can be loaded onto an IPG strip either during rehydration or after rehydration of
the IPG strip via sample cups.
Sample loading during rehydration of the IPG strip is the most common method and has multitude of advantages such as:

- Large quantities of proteins can be loaded [12]
- Because the sample is not applied at one single place like when using sample cup, this method eliminates precipitation of proteins

The rehydration buffer also called sample buffer contains urea, DTT, CHAPS, IPG buffer and bromophenol blue (dye). The sample is dissolved in the rehydration buffer and added to the sample tray. The IPG strip is placed over the sample and is allowed to rehydrate for about 12 hours (or overnight). A low voltage is applied to facilitate the entry of protein into the gel during rehydration. A layer of mineral oil is added above the IPG strip mainly to minimize sample evaporation and urea crystallization. After the IPG strip is rehydrated with the sample, IEF can be performed. IEF is usually carried out at high voltages up to 4000 V. A typical IEF protocol generally proceeds through a series of voltage steps that begin at a low value. The initial low voltage is to separate the salt (which is collected by the paper wicks placed at either ends of the IPG strip) from the sample. The voltage is gradually increased to a final desired focusing voltage that is held for several hours. Many factors affect the time required for focusing. Optimization of the protocol is essential to achieve good separation. After focusing, the IPG strips are equilibrated with buffers containing DTT and iodoacetamide. The equilibration is to saturate the IPG strip with the SDS, reduce and alkylate any disulfide bonds.

The equilibration solution has several components in it. A solution of 50 mM tris, pH 8.8 maintains the IPG strip pH in a range appropriate for the second dimension separation and 6M urea and 30% glycerol reduces electroendosmosis by increasing the viscosity of the buffer and helps in the transfer of proteins from the first to the second dimension. DTT in the solution preserves reduced state of the denatured proteins while iodoacetamide alkylates the reduced disulfide bonds and residual DTT to prevent point streaking seen especially in silver stained gels.
SDS in the equilibration buffer denatures proteins and forms negatively charged protein-SDS complexes, which facilitates separation in the second dimension based on molecular weight. After equilibration of IPG strips, they are ready for the second dimension separation.

- **SECOND DIMENSION- SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)**

The separation of biomolecules in an electric field is called electrophoresis. The method of protein separation by electrophoresis employs polyacrylamide gel as a support medium and SDS as the denaturant and hence called SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS is an anionic detergent that denatures proteins by wrapping around their peptide backbone in a ratio of approximately 1.4 SDS to 1 protein. The bound SDS masks the charge of the protein itself giving it a net negative charge. The proteins are totally unfolded and carry a net negative charge and separation is based on molecular weight of the protein. The most commonly used buffer system is the tris-glycine system described by Laemmli [13].

*Polyacrylamide gel:* Polyacrylamide gel is the polymerization product of the monomer acrylamide and a crosslinking comonomer, most commonly bisacrylamide. The polymerization is started and propagated via a free radical reaction. The chemical N,N,N',N'-tetramethylethylenediamine (TEMED) causes ammonium per sulfate (APS) to produce sulfate radical. The radicals react with the acrylamide and bisacrylamide leading to polymerization. Single percentage acrylamide gels provide good resolution of proteins that fall within a narrow molecular weight range. However, gradient polyacrylamide gels give excellent resolution of wide range of molecular weight and the decrease in pore size allows for sharper protein spots. The percentage of total acrylamide monomer is referred to as %T and this value determines the pore size of the gel. The greater the %T, the smaller is the pore size of the gel. In gradient gels, percentage of acrylamide increases from top to bottom of the gel while the pore size decreases, allowing smaller proteins to travel more towards the bottom of the gel resulting in better separation.
2.2 MASS SPECTROMETRY

An essential tool in addition to the protein separation technology, for proteomics, is mass spectrometry (MS). MS is an ubiquitous analytical tool in a number of fields including chemistry, biochemistry, pharmacy and medicine. The history of MS dates back to 1897 when Sir J.J. Thomson of the Cavendish laboratory at the Cambridge University, while studying electrical discharges in gases discovered the electron. He then went on to build the first ever known mass spectrometer in the first decade of the 20th century for the measurement of mass to charge ratio \((m/z)\) of ions [14]. In this instrument, ions from discharge tubes were passed through both electrical and magnetic fields, by virtue of which they take parabolic trajectories. The ions were then detected on a fluorescent screen or photographic plate. Since then, MS has changed tremendously and is still undergoing improvements.

The basic principle of mass spectrometry is to generate ions of the analyte by suitable method, to separate the ions by their \(m/z\) values and to detect them quantitatively and qualitatively by their respective \(m/z\) value and abundance. A mass spectrometer consists of an ion source, a mass analyzer and a detector, which are operated under high vacuum (schematic shown in figure 2.4). Ions are separated in a mass analyzer on the basis of \(m/z\) and can be manipulated by electric or magnetic fields to direct ions to a detector in a \(m/z\) dependent manner. A mass spectrum is the two dimensional representation of the signal intensity versus the \(m/z\). The intensity of the peaks (signals) directly reflects the abundance of ionic species of that respective \(m/z\). The mass-to-charge ratio, \(m/z\), is dimensionless as it is a ratio between the mass number of the ion and the number of charge on it.

Instrumentation

Basic types of mass analyzers are given in Table 2.1. MS instrumentation has undergone tremendous change since its introduction in 1946 by Stephens [15]. Highly sensitive, robust instruments have been developed that can reliably analyze proteins. The two mass
analyzers that were used for the preparation of this dissertation namely, ion trap and time-of-flight are discussed below.

![Figure 2.4 General scheme of a mass spectrometer](image)

Figure 2.4 General scheme of a mass spectrometer
Table 2.1 Types of different mass analyzers

<table>
<thead>
<tr>
<th>TYPE</th>
<th>Acronym</th>
<th>Principle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time-of-flight</td>
<td>TOF</td>
<td>pulsed ion beam dispersed in time and separated by time of flight</td>
</tr>
<tr>
<td>Magnetic sector</td>
<td>B</td>
<td>Continuous ion beam is deflected in magnetic and separated based on the momentum</td>
</tr>
<tr>
<td>Quadrupole</td>
<td>Q</td>
<td>Continuous ion beam in Rf quadrupole field, separated on the basis of stable trajectories</td>
</tr>
<tr>
<td>Quadrupole ion trap</td>
<td>QIT</td>
<td>Ions trapped in the 3D trap under the influence of Rf field and separated based on stable trajectories</td>
</tr>
<tr>
<td>Fourier transform ion cyclotron</td>
<td>FT-ICR</td>
<td>Ions are trapped and separated by cyclotron frequency in a magnetic field</td>
</tr>
</tbody>
</table>
• QUADRUPOLE ION TRAP (QIT)

Introduction

The origin of ion trap goes back to the work of Paul and Steinwedel [16, 17] that was recognized by the presentation of shared 1989 Nobel Prize in Physics to Wolfgang Paul. QIT also known as 3D trap consists of an arrangement of three hyperbolic electrodes- a ring electrode and two end caps (Figure 2.5). In the normal mode of use, the end caps are connected to earth potential (grounded) while a radiofrequency (RF) fundamental potential (driving potential) typically 1 MHz is applied to the ring electrode. By virtue of the driving potential, ions are held stable within the ion trap. By gradually increasing the amplitude of the driving potential, ions become more energetic and their trajectories become unstable along the axis of symmetry (z axis) of the trap. As a result, the ions in order of increasing m/z value are ejected from the trap through holes in one of the end-cap and are detected by the channeltron electron multiplier. The resulting signal represents a mass spectrum. This mode of operation is called mass-selective ejection. The working principle of the 3D trap is based on creating stable trajectories for ions of a certain m/z or m/z range while removing unwanted ions by colliding them with the walls or by axial ejection from the trap due to their unstable trajectories.

Theory of the “perfect” Quadrupole ion trap

The theory of quadrupole ion trap is best explained by equations that govern both the electric field in the trap and the motion of ions inside. The potential \( \phi_{x,y,z} \) developed within the trap on applying both RF and DC (direct current) potential to the ring electrode is given by

\[
\Phi_{x,y,z} = \frac{(U - V \cos \Omega t)}{2} \left[ \frac{x^2 + y^2}{r_0^2} - 2z^2 \right] + \frac{(U - V \cos \Omega t)}{2}
\]

where,

- \( U \) is maximum DC potential and \( V \) the maximum RF potential
- \( \Omega \) is the angular frequency of the RF potential
r is the internal radius of the ring electrode
2z is the distance between the end-cap electrodes (for perfect quadrupole field \( r^2 = 2z^2 \) however a different relation was shown to work better) and
t is the time

The motion of ions in a quadrupole field is described mathematically by solutions to the Mathieu equation, which has the general form

\[
\frac{d^2 u}{d\xi^2} + (a_u - 2q_u \cos 2\xi) u = 0
\]

where,

\[ u = x, y \text{ and } z \text{ directions} \]
\[ \xi = \frac{\Omega t}{2} \]

The trapping parameters \( a \) and \( q \) which are fundamental for the operation of the ion trap is derived as

\[
q_z = \frac{4eV}{mr^2\Omega^2} \quad \text{and} \quad a_z = -\frac{8eU}{mr^2\Omega^2} \quad \text{Equation (1)}
\]

The form of an ion trajectory in the r-z plane has the general appearance of a Lissajous curve composed of two fundamental frequency components \( \omega_{r,0} \) and \( \omega_{z,0} \) of the secular motion with a super imposed micromotion of frequency \( \Omega/2\pi \) Hz. The graphical representation of stable solutions to the Mathieu equation is called the stability diagram (Figure 2.6). Ions can be stored in the trap provided they are stable in both r and z direction. The \( \beta_z = 1 \) line intersects the q\(_z\) axis at \( q = 0.908 \), this is the ejection limit of the ion from the trap. The parameter \( \beta \) appears in the solution to the Mathieu equation and defines the secular frequency of oscillation of the ion. When the values of U and V are held constant and the driving frequency \( \Omega \) is reduced, the values of both \( a_z \) and \( q_z \) will increase and at one point the \((a_z, q_z)\) coordinate of the ion will cross the boundary and the trajectory will become unstable. Keeping the frequency \( \Omega \) constant and increasing the V can achieve a similar effect.
Figure 2.5 Typical quadrupole iontrap comprising of the two endcap electrode and the ring electrode at the center
Figure 2.6 Mathieu stability diagram for quadrupole ion trap (QIT).
In most ion traps, end-caps are grounded and a RF potential is applied to the ring electrode. By gradually increasing the RF amplitude, the motion of ions become progressively more energetic and they eventually develop unstable trajectories in the order of increasing m/z. When \( q_z \) reaches the value of \( q_{eject} = 0.908 \) along the \( a_z = 0 \) line, the ion motion remains stable in the radial plane while the axial component of the trajectory becomes unstable so that ions are ejected from the trap. This method of ion ejection is referred to as mass selective axial instability [18]. Trapped ions of a given m/z oscillate at a frequency known as the secular frequency that is proportional to the angular frequency of the applied RF. Stafford et al. discovered that a helium damping gas of \( \sim 1 \) mTorr within the trap greatly improved both the sensitivity and the mass resolution of the instrument [19]. The light helium (He) atoms collide with heavier ions; this reduces their kinetic energy, and they migrate to the center of the trap. Consequently, when being ejected ions tend to start from essentially the same position along the axis of the trap hence improved resolution and with minimum radial dispersion forming a tightly focused ion beam that cleanly passes through holes in the end-cap electrode, resulting in greater sensitivity.

**Stretched geometry**

The performance of the quadrupole ion trap is affected by imperfections in the quadrupolar electric fields used to trap ions. For a perfect quadrupole field, the geometry of the ion trap should be such that \( r^2 = 2z^2 \). However, the commercial ion traps have a “stretched geometry” in which the shapes of the end-cap and the ring electrode are retained but the distance between them is extended by about 11%. This was shown to increase the signal intensity and improve resolution. The trapping parameters are now calculated using the actual \( r \) and \( z \) as follows:

\[
a_r = \frac{8eU}{m \left( r^2 + 2z^2 \right) \Omega^2}
\]

\[
q_z = -\frac{4eV}{m(r^2 + 2z^2)\Omega^2}
\]

and
Mass Range extension

By rearranging the equation for the trapping parameters, we get

\[
a_z = -\frac{16\ eU}{m\ (r^2 + 2z^2)\ \Omega^2}
\]

\[
q_r = \frac{8\ eV}{m\ (r^2 + 2z^2)\ \Omega^2}
\]

\[
\left(\frac{m}{z}\right)_{\text{max}} = \frac{4\ eV}{q_{ej} (r^2 + 2z^2)\ \Omega^2}
\]

where \(q_{ej}\) is 0.908 and \(V_{\text{max}}\) is the maximum value for the RF potential. In order to increase the \(m/z\) value for a given \(V_{\text{max}}\), we can either reduce \(r\) and/or \(\Omega\) or reduce the \(q\) value. The most efficient way to accomplish mass range extension is by resonant ion ejection. Resonant ejection requires cooling of ions towards the center of the trap with the help of Helium buffer gas. Resonance excitation is brought about by matching the frequency of the supplementary potential applied to the endcap electrodes to the secular frequency of the ion. The ion will absorb the energy from the applied field and the trajectory will increase linearly towards the endcap until the ion becomes unstable and is ejected.

Instrument and Its Operation

**LCQ Classic:**

The instrument used for the analysis/identification of proteins is the Thermo Electron Corporation LCQ Classic (San Jose, CA). The ions will be pre-formed by solution phase chemistry before the analyte reaches the probe. This is accomplished by adding a proton donor such as formic acid to the mobile phase. A potential of +4kV is applied at the heated capillary. By virtue of the potential, positive ions move towards the tip and a Taylor cone is formed. The Taylor cone emits very small charged droplets, which are desolvated by a combination of nitrogen gas (nebulizer) and heated capillary. The charged gas phase ion molecules are steered into the ion trap. There are two octapoles in
the LCQ Classic. The main purpose of these octapoles is to transmit ions from the exit of the skimmer to the ion trap and allow any remaining neutrals or opposite charge ions to be lost in vacuum. Ions are transmitted as a beam by the first octapole which then passes through the inter octapole lens and are refocused into the trap by the second octapole. The inter octapole lens has a tunable charge that is opposite to the ions of interest so that they are accelerated towards the second octapole. The second octapole has a even higher voltage so that the ions finally enter the ion trap. The RF frequency of the octapole is typically around 2400 Hz, which focuses ions into a beam. The octapoles act only as lens focusing ions and in no way take part in the mass selection. Once the ions enter the ion trap, it is here that they are selected and scanned-out to be detected.

There are four basic steps involved in an ion trap operation. These include collection, isolation, excitation and ejection of ions. During a full scan event which is the simplest, all ions regardless of mass are collected inside the trap and ejected resulting in a spectrum, that displays the ion count of each mass. RF is applied to the ring electrode to collect all ions. The endcap electrodes are held at ground. The RF frequency applied at the ring electrode in a LCQ Classic is 760 kHz. Due to this RF, the ions oscillate towards and away from the center of the trap along the radial axis. Due to the oscillating RF frequency, the trapped ions never hit the electrodes and thus are maintained inside the trap. The use of buffer gas greatly improved sensitivity and resolution of low mass ions. By cooling ions by collision with He gas, the kinetic energy of the ions entering the trap is reduced and their trajectories collapse to the center of the trap. With each collision some of the kinetic energy is converted to vibrational energy and heat, both of which quickly dissipate. The net result is loss of kinetic energy without much change in the vibrational energy to cause fragmentation. Thus ions cooled down in the center of the trap can be scanned out resulting in increased sensitivity and resolution.

The theory of ion trap has been discussed earlier in a previous section. In an LCQ, the above equations are even simpler. The r and z are dimensions of the ion trap which will remain constant for a trap, $\Omega$ the angular frequency of the drive potential is 760 kHz and the DC component U is zero, so rearranging the above q equation
The only variable that affects an ion’s stability is the q value, the ion’s m/e and the RF amplitude (V). All ions trapped inside an ion trap will have an a value of zero and a q value that extends from 0 to 0.908 (q=0.908 is called the edge of stability) depending upon their mass. At given RF amplitude, the q value is inversely proportional to the mass of the ion, meaning smaller ions will have larger q value than heavier ions. In LCQ, RF amplitude is ramped from a low voltage to high voltage and in this process the q value of the ions will increase. When an ion of particular mass reaches the qz value of 0.908, its trajectory will become unstable in the z direction (axial) and it is scanned out of the hole in the endcap into the detector (Figure 2.7).

Ramp RF amplitude and ions leave the trap in the order of increasing mass

Figure 2.7 Mass selective instability scan

When the q value is increased by increasing the RF voltage, the ion whose q value becomes 0.908 and that is close to the exit hole will eject out of the trap immediately while other ions of the same mass that continue to have stable trajectories and stay within the trap, will have chaotic motion from collisions with other ions and He atoms. This will affect the resolution. To prevent this, a RF potential is applied at the endcaps, which is exactly 180° out of phase from another. So, when the entrance endcap is of positive polarity, the positive ions are repelled from it and attracted towards the exit endcap as it
has negative polarity. However, in less than a second, the polarity of the endcaps interchange and the ions are again repelled from the exit endcap. In this way, the ions are oscillating in the trap back and forth in a group. Hence, when the q value of a particular mass of ion is reached, the same mass ions are ejected as a group and this increases resolution. This type of ejection is called resonance ejection. The frequency of RF applied to the endcap in the LCQ classic is about 320 kHz. This however reduces the q value to 0.83.

**Tandem Mass Spectrometry**

For a full scan, all ions that enter the ion trap are ejected and detected and a full MS scan spectrum is obtained. In the case of tandem mass spectrometry (MS/MS), the ions are all collected inside the trap and the ion of interested mass is specifically isolated from the other ions of different masses. Ejecting the other ions with different m/z values isolates ions of m/z of interest. This is accomplished by applying a resonant frequency equal to the secular frequency with which the ions oscillate and by doing so their trajectories are made unstable and they exit the trap. Now the isolated precursor ion has to fragment to give product ions for MS/MS. This is done by *excitation*. Excitation of precursor ion is accomplished by applying a RF voltage at the endcap. This voltage that is very small in amplitude causes the fragmentation of the precursor ion along with collision with He buffer gas. RF voltage applied to the endcap to bring about fragmentation is called tickle voltage. Before the precursor ion is excited it must be cooled to a lower q value, so that most of the fragment ions remain inside the trap (Remember, the q value is inversely proportional to the mass of the ion. If the precursor ion fragmented at a large q value then the fragment ions which are low in mass than the precursor ion will have greater q values and will be ejected out of the trap). So, in order to trap small fragment ions it is essential to cool down the precursor to a lower q value (not 0.83). The fragment ions will not resonate at the same frequency as the precursor ion (frequency is mass dependent) and hence will cool down due to collision with the He atoms down to the center of the trap.
The final step in both full scan and MS/MS is *ejection* in which ions are scanned out of the trap and to the detector in order of small mass to large mass. Ramping the ring electrode RF amplitude to increase the q value of all ions in the trap and scan them out from small to large mass – mass selective scanning performs ejection. At the same time, RF is applied to the endcap that makes the ions of same mass oscillate as a group and they scan out at the same time – resonance ejection.

Detection system comprises of the conversion dynode and electron multiplier. The conversion dynode is held at a very high voltage of about -15kV (for positive ions) and the ions exiting the trap are accelerated towards the conversion dynode because of the high negative potential. The ion strikes the dynode and secondary particles like electrons are emitted towards the electron multiplier. The electron multiplier increases the electron current for each ion. When a secondary particle strikes at the cathode, several electrons are given off, these electrons strike the inner walls of the cathode to give off more electrons, and thus signal is amplified. These electrons are detected by the anode and signal is digitized.

**Time-of-flight (TOF) mass analyzer**

**Introduction**

Stephens described the construction of a time-of-flight mass spectrometer in the year 1946 [15]. The principle of TOF is ions of different \( m/z \) are dispersed in time during their flight along the field-free drift path of known length. In other words, population of ions with different \( m/z \) but constant kinetic energy will have different velocities while moving in a field-free region of constant length. Provided the ions start at the same time or at least within a short time interval, the lighter ions will arrive earlier at the detector than the heavier ions. This however demands that ions emerge from a pulsed source. This separation-in-time technique is different from the separation-in-space of traditional magnetic sectors and quadrupole scanning instruments. The first commercial TOF
instrument was by Bendix in 1950. These were designed for gas-chromatography mass spectrometry (GC-MS). The main advantages of a TOF instrument are:

1. in theory, m/z is unlimited
2. from a single ionizing event, the mass spectrum is obtained within a very short period of time
3. highly sensitive
4. recent improvements in the TOF instrumentation allows for tandem mass experiments

**Principle of TOF Instruments**

1. **Velocity of ions**

The electric charge $q$ of an ion of mass $m$ is equal to the number of charge $z$ times the electron charge $e$

Thus,

$$q = ez$$

(Note: For singly charged ions $q = z$)

Force $F = Eq$

But $F = ma$ where $a$ is acceleration

Therefore,

$$Eq = ma$$

$$a = \frac{Eq}{m}$$

$$a = \frac{du}{dt}$$

$$u = \int \frac{Eq}{m} dt$$
\[ u = u_0 + \left( \frac{Eq}{m} \right) t \]

\[ t_a = \frac{u - u_0}{\frac{m}{q}} \]

We know that the potential energy of ions is converted to their kinetic energy,

\[ qE = \frac{1}{2} mv^2 \]

where \( E \) is electric field strength and \( v \) is drift velocity

Therefore,

\[ v = \left( \frac{2Eq}{m} \right)^{\frac{1}{2}} \]

Drift time \( t_D = \frac{D}{v} \) where \( D \) is length of the drift tube

\[ t_D = D \left( \frac{m}{2Eq} \right)^{\frac{1}{2}} \]  \text{ Equation (2) } \]

Observed time-of-flight \( \text{TOF} = t_0 + t_a + t_D + t_d \)

where,

\( t_0 \) is time after which the ions starts to accelerate

\( t_a \) time the ion actually accelerates

\( t_D \) time in the drift tube when it moves with constant velocity and

\( t_d \) is the response time of the detection system
Mass Resolution

In MS, mass resolution is usually given by $m/\Delta m$ where $\Delta m$ is the difference in mass. In TOFMS, since it is separation-in-time domain and from Equation 2,

$$m \propto t^2$$

Therefore,

$$\frac{dm}{dt} = 2At$$

Thus,

$$\frac{m}{\Delta m} = \frac{t}{\Delta t}$$

where,

$\Delta t$ is the full width at half maximum height of the peak (FWHM)

Linear Time-of-flight

A schematic of a linear TOF instrument is shown in figure 2.8. The analyte is in a sample holder or “target” on which a pulsed laser is focused. An acceleration voltage is applied between this target and an electrode that is grounded. Due to the potential applied, ions that are formed and desorbed during the laser pulse are extracted and accelerated into the flight tube. They drift down a field-free region and hit the detector at the end. This kind of instrument in which ions travel in a straight line from the point of formation/desorption to detection is called a linear TOF.
Figure 2.8 A schematic of a linear TOF instrument. Two ions of different mass are shown in the figure as blue and red filled circles. D is the length of the flight tube and E is the electric field applied to accelerate the ions.
The ions theoretically possess similar kinetic energy at the point of desorption and as they drift in the flight tube, they are separated by their masses. The lighter ions travel faster than the heavier ones. However in practice, the pulse is not felt by all ions to the same intensity and a kinetic energy distribution for each discrete $m/z$ exists. This lowers the resolution by creating a time-of-flight distribution for each $m/z$ [20]. Effects of initial time, space and kinetic energy distribution on mass resolution is shown in linear TOF are shown in figure 2.9.

**Solution to the energy distribution problem**

A technique called time-lag focusing or delayed extraction was introduced by Wiley and McLaren [21] to overcome the energy distribution. Ionization can be confined to a small distance and extraction of ions carried out with a delay in time. They devised an instrument with a pulsed two-grid ion source to compensate for the spatial distribution. In 1966, Mamyrin and coworkers proposed the use of “reflectron” (ReTOF), wherein the more energetic ions follow a longer trajectory to overcome the temporal spread due to difference in initial velocity [22]. The reflectron, essentially a mirror, consists of a decelerating and reflecting field (Figure 2.10). For ions of the same m/z entering such a field, those with higher kinetic energy (and velocity) will penetrate the decelerating field further than ions with lower kinetic energy. Therefore the faster ions will spend more time within the reflecting field, and ‘catch up’ with lower energy ions further down the flight path. By adjusting the reflectron voltages it is possible to achieve a time-focusing plane. In this ideal case, the resolution of the peaks in the mass spectrum will only be dependent on the time-width of ion formation. The angle of ion entry into the reflectron is set at slightly away from 90° and the ions take a different path after being reflected. This allows the detector to be placed in a path not along the axis of the ion entry from the source. The reflector increases the flight path and hence the dispersion in time-of-flight, the effect is negligible when compared to the capability of the reflectron to compensate for the initial energy difference. The other time-widening parameters that can affect resolution are temporal and spatial distribution (figure 2.9) and metastable ion formation.
Figure 2.9 Effect of initial time, space and kinetic energy distributions in linear TOF

Figure 2.10 Schematic of the ReTOF
**Temporal distribution:**

The differences in time of ion formation and limitations of the detection system contribute to the temporal distribution. The ions formed at different times with the same kinetic energy will travel the field-free region maintaining a constant difference in time.

The resolution is given by

\[
\frac{m}{\Delta m} = \frac{t}{\Delta t}
\]

As \(\Delta t\) is constant, the mass resolution can be improved by increasing the flight time by either decreasing the accelerating potential or by increasing the length of the flight tube. By applying the potential after a delay, all ions experience the accelerating voltage at about the same time and \(\Delta t\) is reduced.

**Spatial distribution**

When same mass ions are formed at the same time with the same kinetic energy but at different locations, the ions formed at the rear of the source will experience larger potential gradient than the ions formed close to the extraction grid. Ions at the rear will enter the flight tube later but will reach the detector faster than ions formed close to the grid because of the higher velocities. The extraction field can be adjusted such that the ions arrive at a space focus plane in the drift region at the same time. The space focus plane is independent of mass hence ions of different mass arrive at the plane at different times. Delayed extraction can also reduce the spatial distribution.

**Fragmentation and Mass Resolution**

Fragmentation occurs due to excess of internal energy in the ion during the ionization process. In MS, fragmentation can provide a wealth of information on the identity of the compound. If the compound is an organic molecule it provides information on the
functionality and if it is a protein the information can be used to deduce sequence information. The different types of fragmentation are shown in figure 2.11.

**Figure 2.11** Different types of fragmentation that can occur in TOF. Red filled circle is parent ion while black filled circle is fragment from the parent ion.
Prompt fragmentation: If the molecule fragments at the source due to the ionization impact, it is called prompt fragmentation. The fragment ions have same velocity but since they have different mass they arrive at the detector at different times.

Metastable fragmentation: If the fragmentation occurs in the acceleration region, then the ions reach the detector at times between the molecular ion and its fragment resulting in poor resolution. The ions cause tailing of the molecular ion peak and increased background noise.

Post-source fragmentation/decay: Any fragmentation is undesirable when the goal is to identify molecular ion. However, with MALDI the fragmentation can be used to get sequence information and is like a tandem MS/MS. Ions which fragment in the field-free region of the drift tube, retain essentially the same velocity as intact ions. This fragmentation is called post-source decay (PSD) and is a result of the laser irradiation and collision with other molecules (e.g. residual gas). The reflector will separate precursor and metastable decay ions by their difference in kinetic energy. PSD can be employed to obtain sequence information from peptides.

2.3 IONIZATION TECHNIQUES

Ionization techniques are critical for converting molecules into ions that can be analyzed by mass spectrometry. The challenge with biomolecules is to convert the zwitterionic molecules into gas-phase ions without fragmenting. However, there has been incredible progress in the ionization techniques that now successful biological mass spectrometry can be carried out. Two ionization techniques employed in the work towards this dissertation are discussed below.

Electrospray

Electrospray is essentially a transport process wherein ions in solution are transferred to gas phase. The application of ES as a source of gas phase ions for mass spectrometric
studies was first proposed by Dole [23] in 1968 but the work was very focused on polymers and was not explored any further. The groundbreaking application of ES was the ionization of biomolecules and their subsequent analysis by mass spectrometry, a work done by Yamashita and Fenn [24, 25] and John Fenn won the 2002 Nobel Prize in chemistry for his work on Electrospray-Mass Spectrometry (ESMS). The overall process of ES involves production of charged droplets at the capillary tip, shrinkage of the drop by solvent evaporation and subsequent disintegration to form a small drop with a charge on it and transfer into gas phase (Figure 2.12).

A solution of the analyte is passed through a capillary tube to which a potential of about 4 kV is applied. The counter electrode is a plate with an orifice leading to the mass analyzer. Since the capillary has a fine tip, the electric field is very high. The typical solution in the capillary is a polar solvent in which the analyte is soluble. When potential is applied, the positive and negative ions in solution will move under the influence of the electric field and in this case the positive ions will move towards the tip of the capillary i.e. towards the meniscus of the solution. Bombarding against the walls of the capillary neutralizes the negative ions. The positive ions at the very tip repel each other and this causes the meniscus to expand and a cone is formed called the Taylor cone[26] and if the potential is high enough, the cone expands and a fine jet of ions can be sprayed from the capillary tip. Drops of ions are drifted towards the counter electrode and in this process solvent evaporation occurs and the drop shrinks. As the drop reduces in size, it splits into smaller droplets and finally gas phase charged analyte molecules are formed which drift into the mass analyzer through the holes in the counter electrode.
Figure 2.12 Ion formation in Electrospray
There are two theories for the formation of charged analyte molecules from the Taylor cone – Charge residue model (CRM) and Ion evaporation model (IEM) (Figure 2.13). Each of these theories are discussed briefly below.

**Charge Residue Model**

This mechanism was proposed by Dole [23] depends on the formation of extremely small droplets which should contain only one ion. Solvent evaporation at atmospheric pressure from such a droplet will lead to a gas phase ion. As the drop shrinks, the Columbic energy exceeds the surface tension of the drop (Rayleigh limit) at which point fission occurs and the droplet is ripped apart. This fission process continues until small charged ion is formed.

**Ion Evaporation Model**

Iribane and Thomson proposed a new mechanism for the production of gas phase ions from charged droplets [27, 28]. Their theory describes that the radii of the droplets decrease to a given size (solvent evaporates) and direct ion emission takes place from the droplets due to Columbic repulsion. A detailed explanation on ESMS analysis of proteins will be discussed in the following section.
Figure 2.13 Diagram of two theories for the formation of charged analyte molecules from the Taylor cone.
Matrix assisted laser desorption ionization (MALDI)

Early 1960s was the advent of laser desorption ionization (LDI), which involves the irradiation of low-mass organic molecules with a high intensity laser to form ions that can be mass analyzed. Laser desorption, however, could not ionize large biomolecules. Matrix assisted laser desorption ionization (MALDI) was developed by Karas and Hillenkamp in the late 1980s [29] and is one of the two “soft ionization” techniques that was capable of ionizing proteins. The other soft ionization technique is the Electrospray (ES) that was discussed earlier. Koichi Tanaka received the Nobel Prize for Chemistry in 2002 [30], for his developments in the field of MALDI [31].

Principle:

MALDI involves the use of matrix to successfully ionize proteins. The analyte is mixed thoroughly with a small organic substance. The organic substance has strong absorbance at the laser wavelength that causes the co-crystallization of both matrix and analyte. The incorporation of analyte molecules in the lattice of the matrix crystal is the pre-condition for the laser desorption/ionization process. The crystallized surface of the sample is then exposed to laser (typically nitrogen laser 337 nm-UV wavelength) under high vacuum (Figure 2.14). This causes the evaporation of analyte molecules into gas phase. The target on which the sample is crystallized is floated at about 20 kV. Depending upon the polarity of ions, they are accelerated towards the mass analyzer, which is usually the TOF. The mechanism of ionization is still is topic of debate and is not very clear.
Figure 2.14 Matrix assisted laser desorption ionization (MALDI)
Lasers:

A number of laser wavelengths can be used ranging from ultraviolet (UV) to infrared (IR). UV lasers are emitting pulses of 3-10 ns duration while those of IR are in the range of 6-200 ns. UV lasers include Nd-YAG laser (yttrium-aluminum-garnet crystals doped with neodymium) at wavelengths of 335 nm (tripled frequency) or 266 (four-fold frequency) and nitrogen laser operating at a wavelength of 337 nm. IR lasers include Er-YAG laser (yttrium-aluminum-garnet doped with erbium) that work at wavelengths 294 μm.

Matrix:

The matrix is usually a small aromatic organic compound that can absorb energy at the laser wavelength. For the analysis of proteins, α-cyano-4-hydroxycinnamic acid (HCCA), dihydroxy benzoic acid (DHB) and sinapinic acid (SA) are the most used matrices. The structures of these matrices are shown in figure 2.15.

A good matrix must have the following properties:

- Have high extinction coefficient at the laser wavelength so that it can absorb the laser energy
- Be miscible with the analyte so that the analyte can co-crystallize with it
- Be stable in vacuum – should not sublime
- Have proper functional group to provide proton and hence act as a proton donor for the analyte
- Should be easily crystallized
Figure 2.15 Chemical structures of different matrices
There are a number of ways to “spot” the sample on a target (sample holder). These are all variations of the basic dried-droplet method which was introduced by Hillenkamp and Karas in 1988 [32] where the matrix is simply added to the sample on the target, mixed by pipetting up and down and allowed to air dry. The non-volatile substances may be washed away, but this method tolerates salts better than the other spotting techniques. The second common method is the Overlay method, which involves the use of a fast evaporation to form the first layer of crystals. Then a layer of mixture of analyte and matrix is applied and allowed to dry. The third method is the sandwich method; here the analyte is sandwiched between two layers of matrix.

\textit{Vacuum:}

Vacuum system is an integral part of any mass spectrometer. The reason for maintaining vacuum in a mass spectrometer is the “mean free path”. The mean free path is defined as the average distance between molecules before they collide with each other. It is given by the mathematical formula

\[ \text{Mean free path} = 5 \times 10^{-3} \text{ Torr cm} / P_{\text{torr}} \]

At atmospheric pressure (7.6 x 10^2 torr), the mean free path will be 2 x10^-6 inches, which is 500 nm. At this distance, there will be lot of collisions and the ions do not accelerate but travel with a constant velocity. The collisions could also bring about ion/ion reactions which are not desired. Hence the mean free path should be kept to a maximum and this can be achieved by decreasing the pressure in the system.

\textbf{2.4 WESTERN BLOTTING}

Blotting is the transfer of biomolecules separated on a gel onto a membrane like nitrocellulose or polyvinylidene difluoride (PVDF) (Figure 2.16). Blotting was first performed by Southern in 1975 to transfer DNA from agarose gels to nitrocellulose membranes [33]. Since then blotting has been applied to both RNA and proteins in both
agarose and polyacrylamide gels [34, 35]. Towbin et al. came up with the electrophoretic transfer of proteins from polyacrylamide gels [36]. Western blotting also known as protein blotting or immunoblotting consists of the following steps:

- Immobilization of proteins on a membrane by electrophoretic transfer from a gel (Figure 2.16)
- Unoccupied sites on the membrane are saturated (blocked) to prevent non-specific binding of the primary antibody – blocking is done with 5% non fat dry milk or bovine serum albumin (BSA)
- The blot is probed using primary antibody against the protein of interest
- Secondary antibody that is specific for the primary antibody is used for mainly amplification purposes. The secondary antibody can be radiolabeled or conjugated with an enzyme
- Incubation with detection reagents to generate signals that can be quantified
- Detection using autoradiography
- Analyze using image analysis software
**Figure 2.16** Gel-membrane sandwich in western blotting
A number of blotting membranes are available each with a particular advantage depending on the needs of the experiment. The most commonly used membranes are nitrocellulose (NC) and poly (vinylidene difluoride) (PVDF). Nitrocellulose was one of the first membranes used for western blotting. The membrane can be easily wetted and is compatible with a wide variety of detection systems. When using nitrocellulose, the transfer buffer should have methanol. PVDF is a hydrophobic support suitable for proteins. PVDF has a high binding capacity, is more mechanically, and chemically stable than nitrocellulose. PVDF membranes need to be pre-wetted with methanol but the transfer buffer need not contain methanol. Once the proteins are transferred to the membrane, blocked and incubated with the primary antibody for the specific protein of interest, the membrane is incubated with the secondary antibody that is specific for the primary antibody. The secondary antibody can be labeled with either enzyme or radioactive element. The labeling method involving enzyme conjugated secondary antibody specifically the horseradish peroxidase conjugated secondary antibody system (ECL) for detection will be discussed in detail.

Enhanced Chemiluminescence Labeling (ECL)

Luminescence is defined as the emission of light resulting from the dissipation of energy from a substance in an excited state. In chemiluminescence, the excitation is brought about by a chemical reaction. One of the most clearly understood system is the horseradish peroxidase/H$_2$O$_2$ catalyzed oxidation of luminol in alkaline conditions. The luminol is in an excited state following oxidation and then decays to the ground state by emitting the excess energy in the form of light. Enhanced chemiluminescence is achieved by carrying out the oxidation of luminol in the presence of enhancers like phenols [37] (Figure 2.17). This increases the light output approximately 1000 fold and also extends the time of light emission. The light produced by this enhanced chemiluminescence peaks after 5-20 min and the decays slowly. The maximum light emission is at 428 nm and this can be detected by exposure to X-ray film. The X-ray film is then developed and the bands analyzed using image analysis software.
**Figure 2.17** ECL detection
2.5 STATISTICAL TREATMENT OF DATA

Several statistical approaches were used to determine the significance of data obtained, including analysis of variance (ANOVA) with Dunnett’s post hoc test (ABSTAT) for cell viability data and 2-tailed student’s t test for the differential expression of proteins in 2-DE gels (PD Quest).

- Cell viability data

Cell viability studies were carried out in 96-well culture plates. The cell cultures were treated with various concentrations of malathion-25 µM, 50 µM, 100 µM, 250 µM, 500 µM, 1000 and 2000 µM of malathion. The cells in eight wells were treated with each concentration of malathion and the absorbance of the formazan crystals formed were recorded using a multi-well plate reader. The absorbance from malathion treated cells were calculated as percentage of absorbance from control cells (not treated with malathion). ANOVA with Dunnett’s post hoc test was performed to determine the significance of the data.

- Malathion study

2-DE was performed four times on whole cell lysate from control cell cultures (no malathion) and cell cultures treated with 100 µM malathion for 24 hours and 2 times each on 100 µM malathion for 48 hours, its corresponding control samples and 2 times each on 50 µM malathion for 48 hours and the corresponding control samples. The average number of spots detected from the fluorescence-stained gel images using the PD Quest software was about 400 spots in the treated and control gels. Spot quantity was measured using PD Quest software. Spot quantity is the total intensity of the spot and this corresponds to the amount of protein in the actual spot in the gel. PD Quest software uses Gaussian modeling to create “ideal” spots that can be easily identified and quantified. A Gaussian spot is a 3-D representation of the original scanned spot and this conversion of the scanned image to Gaussian image is carried out because the image of a spot conforms
to a Gaussian curve. The formula for calculating the quantity of Gaussian spots in PD Quest is:

\[ \text{spot height} \times \pi \times \sigma_x \times \sigma_y \]

where,

- spot height is the peak of the Gaussian representation of the spot that is measured in optical density (OD),
- \( \sigma_x \) is the standard deviation of the Gaussian distribution of the spot in the direction of the X axis and
- \( \sigma_y \) is the standard deviation of the Gaussian distribution of the spot in the direction of the Y axis.

Normalization of the spot quantity is carried out to avoid any non-expression related variations. The spot quantities were normalized to the total density of the gel image. In this normalization method, the raw quantity of each protein spot in the gel is divided by the total intensity of the all pixels in the image. Duplicate gels were run for 16 hour and 48 hour treated samples and their corresponding control samples (no treatment with malathion) while four gels each were run for 24 hour malathion treatment and its corresponding control sample. The PD Quest software was used to perform statistical analysis of the differential protein expression. The software allows the selection of various statistical tests that can be used to determine the significance of data. The normalized spot quantities from gels carried out for treatments and their corresponding control samples were subjected to student’s \( t \) test. Student’s \( t \) test determines the significance of difference in mean spot volumes between treated and control samples. The difference in spot volume was considered significant at \( p \) values <0.05 at confidence level of 95%.

- AD CSF study

A volume of 400 \( \mu \)L each of \textit{post mortem} human ventricular CSF from 6 AD subjects and 6 control subjects (normal) were individually subjected to crude isolation of albumin-
interactome using Microcon cut-off filters and 2-DE was carried out on each of these samples. The protein spot quantities were calculated using PD Quest software, as described above. Similar to the malathion study, spot quantities were normalized to total density of the gel image. Normalized spot quantities were compared between AD and control subjects and statistical significance of differences in spot quantities was determined by Student’s \( t \) test using the PD Quest software. The difference in spot volume was considered significant at \( p \) values <0.05 at confidence level of 95%.
CHAPTER THREE

A FLUORESCENT RUTHENIUM COMPLEX FOR STAINING PROTEINS SEPARATED BY SDS-PAGE

3.1 Introduction

Two-dimensional gel electrophoresis (2-DE) is a technique that involves the separation of complex mixtures of proteins based upon their isoelectric point and molecular weight [1]. 2-DE is a major player in proteomics, the success of which depends on the detection method used to visualize and quantify proteins in the gel. A myriad of staining and visualization protocols are available for proteins [38-43]. Radioactive labeling is by far the most sensitive method of detection [44] but because of inherent problems like hazards involved in handling radioactive materials, limited shelf-life, waste disposal and nonsuitability for subsequent mass spectrometry analysis, it has been replaced by other dyes.

Traditional protein stains that are commonly employed include Coomassie Brilliant Blue R-250 (CBB R-250), colloidal Coomassie blue G-250 (CBB G-250) and silver stains. Coomassie brilliant blue R-250 involves easy staining and de-staining of proteins in gels [45, 46]. However, the major disadvantage of this dye is its low sensitivity. CBB R-250 can detect μg to sub-μg levels of proteins [40, 47]. Therefore, either high loads of protein must be used or the analysis is limited to most abundant proteins. CBB R-250 is a nonpolar, sulfonated aromatic dye (Figure 3.1), a solution of which is made up in methanol and acetic acid, and the gel is incubated in this staining solution. Acetic acid enhances the ionic interaction of the dye with the basic amino acids like arginine and lysine as well as hydrophobic interaction with amino acids like tyrosine, tryptophan, histidine and phenylalanine in proteins [45]. The excess dye is removed by incubating the gel in a solution of methanol and acetic acid. Sensitivity is improved with the use of Coomassie G-250 (a dimethylated derivative of CBB R-250) (Figure 3.2, [48]). CBB G-250 interacts with the protein in a manner similar to CBB R-250 but it is a colloidal suspension and has
minimum background staining. CBB G-250 can detect 1 ng of protein; however, involves a number of repeated staining and de-staining steps and is laborious and time consuming.

Figure 3.1 Structure of Coomassie brilliant blue R-250 (CBB R-250)
Figure 3.2 Structure of Coomassie brilliant blue G-250 (CBB G-250)
Another protein stain that is commonly employed in gel electrophoresis is acidic silver nitrate (AgNO₃). Even though silver nitrate stain has better sensitivity (down to 0.5 ng of protein) than CBB R-250, it generally involves multiple steps that often require freshly prepared reagents and critical timing that result in constant hands-on activity. Typically, two kinds of silver staining are used for the detection of proteins in gels: acidic silver nitrate and alkaline silver diamine [42]. The acidic silver nitrate staining protocol relies on the impregnation of the gel with silver ions and its subsequent reduction to metallic silver at alkaline pH using formaldehyde [47, 49]. The alkaline silver diamine involves the formation of soluble silver diamine complexes with the proteins and subsequent reduction of the silver with formaldehyde in an acidified developer [47, 49]. Silver ions interact with amino acids like cysteine, lysine and histidine in proteins. Silver stain has poor dynamic range [50]. There is also evidence of peptide losses with silver stain and problems in identification by mass spectrometry [51].

An alternative to the use of the above mentioned colorimetric dyes is fluorescent protein labels. With recent developments in new imaging systems, fluorescent detection of proteins has re-surfaced. Fluorescent detection of proteins can be divided into two categories: covalent and non-covalent. In covalent labeling, proteins are derivatized with fluorescent compounds prior to separation by electrophoresis. A critical factor that should be kept in mind while using these compounds is the difference in migration of derivatized and un-derivatized proteins in the gel. Also, derivatization of proteins is not always 100% efficient, and in some cases could lead to other non-specific side reactions that can possibly affect interpretation of mass spectra. Non-covalent labeling, in contrast, is performed after electrophoresis. Here the fluorophore interacts directly with the protein or via SDS-protein complex non-covalently. Sypro Ruby is a ruthenium-based metal complex luminescent stain developed by Molecular Probes (Eugene, OR) [43]. Advantages of Sypro Ruby include high sensitivity and high linear dynamic range; however, it is highly expensive to buy commercially patented Sypro Ruby and hence not cost-effective. Rabilloud et al. reported the synthesis and use of Ruthenium [52] tris(bathophenanthroline disulfonate) [RuBPS] for detection of proteins in gels with minimal interference on subsequent analysis using mass spectrometry [53]. RuBPS is a
fluorescent ruthenium complex that can be synthesized in the lab and has been shown to
detect proteins separated by SDS-PAGE with high sensitivity and high linear range.
Since RuBPS can be prepared in the lab, it offers varied possibilities for modifications
and improvements in formulation. In addition, different staining and destaining protocols
have been reported for RuBPS [54-56]. Lamanda et al. have improved RuBPS staining
by mainly changing the reagent concentration, pH and solvent for staining and destaining
of the chelate.

We explored the possibility of using RuBPS staining solution in our lab and proceeded to
synthesize the ruthenium complex in-house using the Rabilloud protocol. Five hundred
milliliter of Sypro Ruby solution can be used to stain only 2 large format gels and it is not
advisable to re-use the stain. Considering the number of gels run in our lab on a daily
basis, we wanted to explore cost-effective options for fluorescent staining.

The goal of this project was to synthesize and optimize the formulation of a ruthenium
complex that could be used as a cost-effective fluorescent protein stain after gel
electrophoresis.

3.2 Materials and Methods

3.2a Chemicals

Low range marker proteins for SDS-PAGE (Bio-Rad Labs, CA) had equal amounts of
rabbit muscle phosphorylase B (97 kDa), bovine serum albumin (66 kDa), hen egg white
ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21
kDa), hen egg white lysozyme (14 kDa). The silver nitrate stain (ProteoSilver Plus) was
from Sigma (St. Louis, MO), Colloidal Coomassie Blue from Fisher Scientific (Fairlawn,
NJ) and Sypro Ruby was obtained from Molecular probes (Eugene, OR). Other
electrophoresis chemicals like SDS, ammonium persulfate, TEMED, acrylamide,
bisacrylamide, tris base and glycine were purchased from Sigma (St. Louis, MO).
Potassium pentachloro ruthenate was purchased from Alfa Aesar (Ward Hill, MA),
bathophenanthroline and bathophenanthroline disulfonate were purchased from GFS Chemicals (Columbus, OH) and the fluorescent RuMS was purchased from Biotium (Hayward, CA).

3.2b Synthesis of Ruthenium II tris (bathophenanthroline disulfonate) (RuBPS)

RuBPS (Figure 3.3) was synthesized using the protocol published by Rabilloud et al.[53]. Briefly, 0.089 g of potassium pentachloro aqua ruthenate (K₂Cl₅ Ru.H₂O) was dissolved in 10 mL of boiling water and kept under reflux. A reddish brown solution was obtained. A three molar equivalent of bathophenanthroline disulfonate, disodium salt (0.4 g) was added to the solution and refluxing was continued for about 20 min till the solution turned deep greenish brown. A reducing solution of 8 mM sodium ascorbate dissolved in 10 mL of water was prepared and added to the solution and refluxing was continued for another 20 min until the solution turned orange brown. After cooling, the pH was adjusted to 7 with sodium hydroxide and the volume was adjusted to 50 mL with absolute alcohol. The final concentration of the stock solution was approximately 500 μM.

3.2c Synthesis of Ruthenium II (bis (bathophenanthroline disulfonate) (bathophenanthroline)) (RuDS)

RuDS (Figure 3.4) was synthesized similar to RuBPS as described in the previous section. However, 2 molar equivalents of bathophenanthroline disulfonate disodium salt (0.2684 g) and one molar equivalent of bathophenanthroline (0.0833 g) were mixed with 0.089 g of ruthenium salt, refluxed and the synthesis continued as explained in the previous section. The concentration of RuDS solution is 500 μM.
Figure 3.3 Structure of Ruthenium II tris (bathophenanthroline disulfonate) (RuBPS)
Figure 3.4 Structure of Ruthenium II (bis (bathophenanthroline disulfonate) (bathophenanthroline)) (RuDS)
3.2d Synthesis of Ruthenium II (bis (bathophenanthroline) (bathophenanthroline disulfonate)) (RuMS)

The fluorescent ruthenium chelate RuMS (Figure 3.5) was synthesized as described previously [53] with slight modifications. A 0.089 g of potassium pentachloro aqua ruthenate (K$_2$Cl$_5$ Ru.H$_2$O) was dissolved in 10 mL of boiling water and kept under reflux. A reddish brown solution was obtained. One molar equivalent of bathophenanthroline disulfonate, disodium salt (0.1342 g) and 2 molar equivalents of bathophenanthroline (0.1666 g) were added and refluxing was continued for another 20 min until the solution turned deep greenish brown. A reducing solution of 8 mM sodium ascorbate dissolved in 10 mL of water was added to the refluxing solution and refluxing was continued for another 20 min until the solution turned orange brown. After cooling, the pH was adjusted to 7 with sodium hydroxide and volume adjusted to 50 mL with absolute alcohol. The final concentration of stock solution was approximately 500 μM.

3.2e 1D gel electrophoresis

Proteins that make up the low range molecular weight markers from Bio-Rad were separated by SDS-PAGE in the standard Laemmeli Tris-glycine system [13]. The molecular weight markers were diluted 100 and 2000 fold in SDS sample buffer to reach a concentration of 20 and 1 ng/μL respectively for each protein in the mixture. The required volumes were loaded on top of a 12% separating and 4% stacking mini polyacrylamide gel (7 cm) to give appropriate amounts of each protein band ranging from 100 ng to 0.5 ng. The running buffer contained 25 mM Tris, 192 mM glycine and 0.1% SDS, pH 8.3 and electrophoresis was performed at constant 150 V until the dye front reached the bottom of the gel.
Figure 3.5 Structure of Ruthenium II (bis (bathophenanthroline) (bathophenanthroline disulfonate)) (RuMS)
3.2f Characterization of in-house synthesized ruthenium complexes

- **Fluorescence spectroscopy**

Excitation and emission spectra of RuMS was obtained using Spex 1681 0.22 m spectrofluorometer. Emission wavelength was set to 610 nm and excitation spectrum was collected from 250 to 550 nm. For the emission spectrum, the excitation wavelength was set to 470 nm and the spectrum was collected from 500 to 750 nm.

- **MALDI-TOF mass spectrometry**

Both MALDI and laser desorption mass spectra were generated for the in-house synthesized ruthenium complexes using the Bruker Autoflex time-of-flight mass spectrometer (Billerica, MA). For MALDI spectra, a solution of 1 μL HCCA (10 mg/mL of α-cyano-4-hydroxycinnamic acid in 90% acetone and 2% TFA) was spotted on a sample target. A volume of 0.2 μL stock ruthenium complex solution was spotted on the matrix spot, allowed to air dry and spectra were acquired.

- **Paper chromatography**

Paper chromatography was carried out on a Whatmann filter paper with various percentages of solvents like tetrahydrofuran, water and ethanol as solvents. Two microliter of the in-house synthesized ruthenium complexes were spotted about 1 cm above the base of the paper and separation carried out in a closed chamber until the solvent front traveled about three fourths of the distance on the paper.

- **Crystallization**

Crystallization of in-house synthesized ruthenium complexes was carried out to obtain X-ray crystallography data on these different complexes. Various percentages of solvents like ethanol, isopropyl alcohol and THF were tried for this purpose.
3.2g Staining of mini gels

- **Colloidal Coomassie blue stain**

Colloidal Coomassie blue staining of SDS-PAGE gels were performed as described by Neuhoff et al. [48]. Briefly, gels were fixed with 50 mL of aqueous solution containing 1.3% (w/v) of ortho phosphoric acid and 20% (v/v) methanol for 1 hour. Staining solution was prepared by mixing 1 mL of freshly prepared staining solution B (containing aqueous 5% (w/v) Coomassie brilliant Blue G-250) and 40 mL of aqueous staining solution A (containing 2% w/v of ortho phosphoric acid, 10% w/v ammonium sulfate) with 10 mL of methanol. The gels were stained overnight in the staining solution. The next day, gels were transferred into a neutralization buffer containing aqueous 0.1 M Tris-base pH 6.5 (titrated with ortho phosphoric acid) for 2 min. The background was then washed with 25% methanol solution for less than 1 min and gels were transferred into an aqueous stabilizing solution containing 20% ammonium sulfate. The gels stay in the stabilization solution for a day and further staining was carried out as mentioned above without the fixing step. Staining was repeated three times which improved sensitivity and at the end of the third staining step about 0.5 ng of protein was detected. The volumes mentioned here are for one gel.

- **Silver stain**

Silver staining was performed using Pierce Proteosilver Plus kit (Rockford, IL). Staining was carried out as per manufacturer’s instruction. Briefly, the gels were fixed in 50% ethanol and 10% acetic acid solution overnight. Then the gels were washed with 30% ethanol for 10 min, decanted the ethanol solution and washed with ultra pure water for 10 min. The gels were incubated in the sensitizing solution (500 μL of Proteosilver Sensitizer in 50 mL ultra pure water) for about 10 min. Gels were washed twice with water for 10 min each and then incubated in silver solution (500 μL of Proteosilver Silver in 50 mL ultra pure water). After a brief wash in water, the gels were developed in the developer solution (2.5 mL of Proteosilver developer and 50 μL developer 2 in 50 mL of
ultrapure water) until desired staining is achieved and 2.5 mL of Proteosilver stop solution was added to this developer solution to stop the reaction and incubated the gel in this solution for about 5 min. Carbon dioxide formed was seen as bubbles in the solution. The solution was discarded and the gels were incubated in ultrapure water for about 15 min. The gels were stored in pure water. All solutions were prepared freshly before use. Silver nitrate binds to proteins (amino acids- cysteine, lysine and histidine) in the gel under weakly acidic pH. The silver ions bound to proteins are reduced by formaldehyde at alkaline pH to form metallic silver which gives the protein black to dark brown color the intensity of which depends on the concentration of protein present.

- **Sypro Ruby staining**

Gels were stained with Sypro Ruby per manufacturer’s instructions [43]. Briefly, gels were incubated in the Sypro Ruby solution overnight. The volume of staining solution used is about 50 mL per mini gel, which is roughly 10 times the volume of the gel. After staining, gels were rinsed in 10% methanol and 7% acetic acid for at least 30 min to remove residual dye and then stored in fresh pure water.

- **Ruthenium II tris (bathophenanthroline disulfonate) staining**

Staining and destaining for RuBPS was performed using the most recent modified protocol [56]. Briefly, proteins in the gel were fixed using 30% ethanol and 10% acetic acid solution overnight. The gels were washed with 20% ethanol three times 30 min each and then incubated in 150 nM (15 μL in 50 mL of 20% ethanol) staining solution for 6 hours. The gels were equilibrated with water for about 10 min and then background stain was removed by washing in destaining solution containing 40% ethanol and 10% acetic acid overnight. The gels were then washed in water for 10 min and scanned.
• **Ruthenium II (bis (bathophenanthroline disulfonate)) (bathophenanthroline) staining (RuDS)**

Staining of gels with the RuDS form of the ruthenium complex was performed similar to RuBPS staining protocol with some modifications. Gels were fixed overnight with 30% ethanol and 10% glacial acetic acid and washed with 20% ethanol solution three times 30 min each time. Gels were incubated in staining solution containing 150 nM of RuDS in 20% ethanol (15 μL of the stock in 50 mL of 20% ethanol) overnight, quickly rinsed with water and then destained with 10% ethanol and 5% acetic acid till desired intensity was obtained and the gel was scanned.

• **Ruthenium II (bis (bathophenanthroline) (bathophenanthroline disulfonate)) staining (RuMS)**

Various solvents were tested for optimal staining of proteins with RuMS. The protocol that gave best signal to noise ratio is described here. Briefly, RuBPS staining and destaining protocol [53] was used with slight modification. The gels were fixed overnight with 30% v/v ethanol and 10% v/v acetic acid. The gels were then rinsed with 20% ethanol three times 30 min each and incubated in staining solution overnight in 20% ethanol containing 150 nM of the ruthenium chelate (15 μL of stock solution in 50 mL 20% ethanol). Gels were washed for at least 15 min with water. Stained gels were stored in pure water.

• **Commercial RuMS staining**

A stock staining solution of 16 μM of commercial source of RuMS was prepared by dissolving 1 mg of the fluorescent compound in 50 mL of absolute alcohol. An aliquot of the stock staining solution (4 mL in 20% ethanol – 125nM) was used to stain proteins in gels.
3.2h Imaging of stained gels

Stained gels were scanned with a Versadoc 3000 scanner from Bio-Rad (Hercules, CA). Sypro Ruby and ruthenium chelate stained gels were visualized using 520 nm emission filter and UV light. CBB G-250 and silver stained gels were visualized on a white light background using a 610 nm emission filter. Exposure time for Sypro Ruby and ruthenium complex stained gels were 2.5 sec while for colloidal Coomassie and silver exposure time was 0.5 sec. Images were analyzed by Quantity One software. Band volumes were estimated after applying global background correction. The images were taken under essentially identical settings and with the same instrumentation to enable direct comparison.

3.2i In-gel proteolysis

Protein bands were excised from the gel using the Spot-cutter from Bio-Rad and in-gel proteolysis was carried out with trypsin. In brief, the gel pieces were washed 6 times alternating with 50 mM NH₄HCO₃ and 1:1 ACN: NH₄HCO₃ (50 mM) for 5 min each at 20°C and dried with ACN for 10 min at 20°C. A volume of 10 μL of trypsin (66.67 ng) in 25 mM NH₄HCO₃ was added and gel pieces allowed to swell for 30 min at 20°C and digested at 50°C for 4 hours. Peptides were extracted with 10 μL of 1% aq. formic acid at 20°C for 30 min.

3.2j In-gel trypsin digestion of proteins from silver stained gels

Trypsin digestion of proteins from silver stained gels was performed as described by Mann et al. [57] with slight modifications. In short, all pre-washing steps were omitted. The gels plugs were excised and shrunk with acetonitrile and dried in a vacuum centrifuge. A volume of 10 mM dithiothreitol (DTT) in 50 mM NH₄HCO₃ sufficient enough to cover the gels pieces was added and incubated at 56°C for 1 hour. After cooling the samples to room temperature, 55 mM iodoacetamide in 50 mM NH₄HCO₃ was added and incubated at room temperature for 45 min in the dark. The gel pieces were
then washed with 50 mM NH₄HCO₃ for 10 min, dehydrated with acetonitrile. Ten μL of trypsin (66.67 ng) in 25 mM NH₄HCO₃ was added and gel pieces allowed to swell for 30 min at 20°C and digested at 37°C overnight. Peptides were extracted with one change of 25 mM NH₄HCO₃ and three changes of 5% formic acid in 50% acetonitrile at room temperature for 20 min each time and dried down.

### 3.2k MALDI-TOF mass spectrometry

MALDI-TOF mass spectra were generated using Bruker Autoflex (Billerica, MA) in the reflectron mode. HCCA matrix solution was spotted on an Anchorchip target and 1.5 μL of peptide solution was spotted on top of the matrix and allowed to dry. On-target washing was carried out with 0.2% TFA. The spectra were externally calibrated against the monoisotopic mass of a standard solution containing 1 μg/μL bradykinin ([M+H]^+ 757.399), vasopressin ([M+H]^+ 1084.445), angiotension II ([M+H]^+ 1046.542), substance P ([M+H]^+ 1347.735) and oxidized form of substance P ([M+H]^+ 1363.731) and internally calibrated using the known trypsin autolysis peaks. Database search was performed using the Mascot software (www.matrixscience.com).

### 3.2l Liquid Chromatography-Tandem Mass spectrometry

The trypsin digested samples were analyzed using a Thermo Corporation’s LCQ Classic quadrupole ion trap mass spectrometer (San Jose, CA). The tryptic peptides were resolved on a fused silica capillary column. The column was fabricated in-house by packing 15 cm of Phenomenex Polymer X (3 μm particle size) into a 320 μm I.D fused silica capillary using a home-made stainless steel packing cell pressurized with helium to 1000 psi. The tryptic peptides were eluted using a typical gradient elution. Spectra were acquired with a probe spray voltage of 4 kV and a heated capillary temperature of 175°C.
3.3 Results

3.3a Characterization of ruthenium complexes

- **Fluorescence spectroscopy**

Excitation and emission spectra of the ruthenium complex RuMS synthesized in the lab were recorded using a Spex 1681 spectrofluorometer (Figure 3.6). There are two absorption peaks for the complex similar to Sypro Ruby, one at ~280 nm and a broad one at ~450 nm. The fluorescence emission spectrum has one broad peak at 610 nm.

- **MALDI-TOF mass spectrometry**

Mass spectrometry was employed to characterize the in-house synthesized ruthenium complexes. The base peak observed in all the spectra obtained for the various ruthenium complexes is an m/z of 766 (Figure 3.7-3.9). Table 3.1 gives the different mass peaks that were observed in the MALDI-TOF spectra for various in-house synthesized ruthenium complexes.

- **Paper chromatography**

The highly polar nature of sulfonic acid groups in the ruthenium complexes makes it difficult to separate the complexes using thin layer chromatography (TLC). Since the silanol groups in silica gel are also polar, the polar complex binds tightly to the solid phase and will not move much with the mobile phase. Under such circumstance, paper chromatography is a better option to resolve the polar complexes. Table 3.2 summarizes the chromatographic data for these compounds.
Figure 3.6 Excitation (A) and Emission (B) spectra of in-house RuMS
Table 3.1 Peaks observed in MALDI-TOF mass spectrum of different ruthenium complexes synthesized in-house. x – indicates ion not observed while ✓ - indicates observation of the ion in the mass spectrum

<table>
<thead>
<tr>
<th>Peaks (m/z)</th>
<th>Form of Complex</th>
<th>Probable ion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RuNS (all nonsulfonated ligand)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RuMS</td>
<td>RuDS</td>
</tr>
<tr>
<td>457.96</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>766.05</td>
<td>✓ (base peak)</td>
<td>✓ (base peak)</td>
</tr>
<tr>
<td>801.02</td>
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<td>✓</td>
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<tr>
<td>817.49</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>1098</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>1113</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>1194</td>
<td>x</td>
<td>✓</td>
</tr>
</tbody>
</table>

Found in sulfonated ligand mass spectrum

Ruthenium attached to two bathophenanthrolines and a Cl

Very small amount found in non sulfonated ligand mass spectrum

Ruthenium + three bathophenanthroline

Very small amount found in the non-sulfonated ligand

Ruthenium MS minus a SO₃
Figure 3.7 MALDI-TOF spectrum of ruthenium tris bathophenanthroline using HCCA matrix. Inset A is expanded mass range from 750-850 m/z and inset B is expanded mass range from 1080-1150 m/z.
Figure 3.8 MALDI-TOF spectra of in-house synthesized RuMS complexes using HCCA matrix. Inset A is expanded mass range from 750-850 m/z and inset B is expanded mass range from 1080-1150 m/z.
Figure 3.9 MALDI-TOF spectra of in-house synthesized RuDS complexes using HCCA matrix. Inset A is expanded mass range from 750-850 m/z and inset B is expanded mass range from 1080-1150 m/z.
Figure 3.10 MALDI-TOF spectra of in-house synthesized RuBPS complexes using HCCA matrix.
**Table 3.2.** Summary of chromatographic data for various in-house synthesized ruthenium complexes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R&lt;sub&gt;f&lt;/sub&gt; values (distance traveled by the component/distance traveled by the solvent)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
<td>50:50 water: THF</td>
</tr>
<tr>
<td>In-house RuMS</td>
<td>0.42, 0</td>
<td>0.16, 0.33</td>
</tr>
<tr>
<td>RuDS</td>
<td>0.42, 1</td>
<td>0.66, 0.83</td>
</tr>
<tr>
<td>RuBPS</td>
<td>0.42, 1</td>
<td>0.66, 0.83</td>
</tr>
<tr>
<td>Commercial RuMS</td>
<td>0.42</td>
<td>1.0</td>
</tr>
</tbody>
</table>

3.3b Staining efficiency of various ruthenium complexes synthesized in-house

A comparison of the effectiveness of three different in-house synthesized ruthenium complexes showed that RuMS form worked best by detecting 0.5-1 ng of protein (Figure 3.11). Sensitivity of the RuBPS was only about 5 ng of protein.

3.3c Optimization of in-house RuMS stain

A critical parameter for successful staining of proteins is the formulation of the stain in a suitable solvent. Various solvents like ethanol, methanol and isopropyl alcohol were used as solvent for the formulation of RuMS (Figure 3.12, 3.13). The concentration of RuMS in the working solution was also optimized (Figure 3.14). Desired staining of proteins with minimal background was obtained with 150 nM RuMS in 20% ethanol solution. When solvent other than ethanol was used or when greater concentration of ethanol was used in the formulation, there was increased background staining due to non-specific binding of the stain to the polyacrylamide matrix.
Figure 3.11 Serial dilutions of standard low range marker proteins stained with in-house synthesized ruthenium complexes. The amount of protein indicated is nanogram of each low-range marker protein.
Figure 3.12 Optimization of solvent for staining proteins with in-house synthesized RuMS. (A) 20% methanol, (B) 20% ethanol and (C) 20% isopropyl alcohol. The amount of protein indicated is nanogram of each low-range marker protein.
Figure 3.13 Optimization of ethanol composition for in-house RuMS staining solution. (A) 20% ethanol and (B) 50% ethanol. The amount of protein indicated is nanogram of each low-range marker protein.
Figure 3.14 Optimization of concentration of in-house RuMS in the staining solution. (A) 150 nM RuMS and (B) 200 nM RuMS. The amount of protein indicated is nanogram of each low-range marker protein.
3.3d Optimization of commercial RuMS stain

Commercial RuMS was purchased from Biotium (Hayward, CA) and used to stain proteins in gels. Figure 3.15 and 3.16 show the gel images for the optimization of solvent for commercial RuMS stain. Figure 3.17 is the gel image for the optimization of concentration of RuMS in the staining solution. The commercial RuMS staining was compared to the in-house synthesized RuMS stained gel (Figure 3.18).

**Figure 3.15** Optimization of solvent composition for commercial RuMS staining solution. Different solvents were used to stain the gels (A) 50% aq. ethanol, (B) 50% aq. isopropyl alcohol. The amount of protein indicated is nanogram of each low-range marker protein.
**Figure 3.16** Optimization of solvent composition for commercial RuMS staining solution. Different solvents were used to stain the gels (A) 20% aq. ethanol and (B) 20% aq. isopropyl alcohol. 20% aq. ethanol gave the maximum sensitivity with least background staining. The amount of protein indicated is nanogram of each low-range marker protein.
Figure 3.17 Optimization of concentration of commercial RuMS in the staining solution. (A) 62.5 nM (B) 125 nM and (C) 250 nM of commercial RuMS in staining solution. The amount of protein indicated is nanogram of each low-range marker protein.
Figure 3.18 Comparison in staining between in-house synthesized RuMS and commercial RuMS. The amount of protein indicated is nanogram of each low-range marker protein.
3.3e Sensitivity of various staining solutions

To assess the staining sensitivity of silver, colloidal coomassie, Sypro Ruby and in-house ruthenium II (bis (bathophenanthroline) (bathophenanthroline disulfonate)), serial dilutions of the low range molecular weight markers from 100 to 0.5 ng per lane was separated by SDS-PAGE. A batch of three gels was run for each stain and all 12 gels were run to the same pattern. Figure 3.19 gives the representative gels stained with colloidal Coomassie, silver, commercial Sypro Ruby and in-lab made ruthenium complex.

3.3f Linear dynamic range

To compare the linearity in staining of standard proteins between different stains, the volume of the protein band which represented the total pixel density of each band with the background density removed were measured using Quantity One imaging software and exported to Microsoft Excel (Table 3.3, Figure 3.20 and 3.21). The band volumes for 5 ng of standard proteins were normalized to values from Sypro ruby stained gel and the histogram plotted to compare sensitivity of the different stains (Figure 3.22).
Figure 3.19 Representative gels stained with colloidal CCB G250 (A), Silver nitrate (B), in-house synthesized RuMS (C) and Sypro Ruby (D). The amount of protein indicated is nanogram of each low-range marker protein.
Figure 3.20 Linearity of staining of standard proteins using colloidal Coomassie (A) and silver (B). Key for proteins (∗) phosphorylase B; (●) bovine serum albumin; (▲) ovalbumin; (×) carbonic anhydrase; (✽) trypsin inhibitor; (○) lysozyme
Figure 3.21 Linearity of staining of standard proteins using in-house RuMS (a) and Sypro Ruby (b). Key for proteins (•) phosphorylase B; (⋆) bovine serum albumin; (▲) ovalbumin; (×) carbonic anhydrase; (☆) trypsin inhibitor; (○) lysozyme
Table 3.3 Band volumes for different loads of standard proteins in gels stained with different staining solutions

<table>
<thead>
<tr>
<th>Amount of protein loaded (ng)</th>
<th>Phosphorylase</th>
<th>BSA</th>
<th>Ovalbumin</th>
<th>Carbonic anhydrase</th>
<th>Trypsin Inhibitor</th>
<th>Lysozyme</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Silver</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 1550 ± 8.9</td>
<td>1690 ± 9.3</td>
<td>1581 ± 9.8</td>
<td>1756 ± 8.1</td>
<td>2089 ± 18</td>
<td>1818 ± 12</td>
<td></td>
</tr>
<tr>
<td>50 1337 ± 10</td>
<td>1470 ± 7.5</td>
<td>1401 ± 9.7</td>
<td>1585 ± 9.0</td>
<td>1760 ± 13</td>
<td>1640 ± 16</td>
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<tr>
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<td>1132 ± 1.4</td>
<td>1108 ± 8.4</td>
<td>1260 ± 4.1</td>
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<td>1070 ± 5.6</td>
<td>1098 ± 4.3</td>
<td>1204 ± 2.1</td>
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<td>1114 ± 12</td>
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<tr>
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<td>1 201 ± 1.1</td>
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<td>202 ± 3.3</td>
<td>184 ± 2.8</td>
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</table>
**Figure 3.22** Histogram of normalized band volume for 5 ng of standard proteins (Phosphorylase A, BSA- bovine serum albumin, Oval- ovalbumin, CA- carbonic anhydrase, Tryp- trypsin inhibitor and Lyso- lysozyme) in gel stained with different stains.
3.3g Compatibility with mass spectrometry

Improved detection of protein alone is not sufficient for successful proteomic studies. The stain must be compatible with subsequent protein identification by mass spectrometry. To evaluate the compatibility of different stains with mass spectrometry, gels plugs were harvested and in-gel proteolysis was carried out with trypsin. The resulting tryptic peptides were subjected to both matrix assisted laser desorption ionization (MALDI-TOF) and liquid chromatography tandem mass spectrometry (LC-MS/MS).

- **Stain compatibility with MALDI-TOF**

Tryptic peptides were subjected to MALDI-MS as described earlier. A representative peptide mass fingerprint (PMF) analysis for all the 100 ng BSA protein is shown in figure 3.23. The summarized MALDI data from all the trypsin-digested proteins is provided in table 3.4

- **Stain compatibility with LC-MS/MS**

Data dependent MS/MS experiments were performed on a Thermo LCQ Classic quadrupole ion trap mass spectrometer. Mass spectra were searched against the human sub database from the Swiss-Prot database using Mascot algorithm. One missed trypsin cleavage was allowed and a variable modification of oxidation of methionine was set. Peptide tolerance and MS/MS tolerance were both set at ± 0.8 Da. Representative spectra are shown in figure 3.24. Table 3.5 summarizes the LC-MS/MS data for all the trypsin-digested samples.
Figure 3.23 MALDI-TOF spectra for 100 ng of bovine serum albumin from gels stained with different stains.
Table 3.4 Summary of MALDI-TOF data for different protein loads of standard proteins (BSA- bovine serum albumin and trypsin inhibitor) in gel stained with various stains- CCB G250- colloidal coomassie, silver nitrate, Sypro Ruby and in-house RuMS.

<table>
<thead>
<tr>
<th>Stain</th>
<th>Protein</th>
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Figure 3.24 MS/MS fragmentation of a BSA peptide (HLVDEPQNLIK) from gels stained with different stains as indicated. The ion intensity is given with parenthesis beside the peptide sequence in the figure.
Table 3.5 Summary of LC-MS/MS data for different protein loads of standard proteins (BSA: bovine serum albumin and Trypsin inhibitor) from gels stained with various stains

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103
3.4 Discussion

The primary aim of this work was to synthesize a fluorescent ruthenium complex to use as a cost-effective stain in visualizing proteins in gels after electrophoresis. A commercially available fluorescent protein stain is the Sypro Ruby, which has high sensitivity and linear dynamic range crucial for a successful proteomic study utilizing 2-DE. However, 500 mL of Sypro Ruby solution can be used to stain only two large format gels. Considering the huge number of gels run in a typical proteomic lab like ours, use of Sypro Ruby is not very cost-effective. Rabilloud et al. synthesized RuBPS in-lab to use as an alternative to the expensive Sypro Ruby [53]. This prompted us to test the usability of this RuBPS in our lab and we synthesized the chelate in-house using the protocol published by Rabilloud et al. As shown in figure 3.11, the sensitivity of the RuBPS stain synthesized in-house was relatively poor, with a detection limit of about 5 ng of protein. Since RuBPS is ruthenium attached to three bathophenanthroline disulfonate ligands, we explored the possibility of having both sulfonated and non-sulfonated bathophenanthroline moieties attached to the central ruthenium atom. Our rational was that different kinds of ligands may result in a synergistic effect in interacting with the protein and thereby enhance sensitivity. Analysis of the commercial Sypro Ruby by MALDI-TOF in our lab showed evidence for the presence of a non-sulfonated ligand in the solution (data not shown). This impelled us to synthesize ruthenium complex with both sulfonated and non-sulfonated ligands and hence two complexes RuMS and RuDS were synthesized in addition to RuBPS. Synthesis of RuMS and RuDS were carried out using modification of protocol published by Rabilloud [53].

3.4a Characterization of in-house synthesized ruthenium complexes

Various techniques like fluorescence spectroscopy, mass spectrometry and paper chromatography were carried out to characterize the in-house synthesized ruthenium complexes. Fluorescence spectroscopy of all the three ruthenium complexes synthesized in-house gave spectra similar to Sypro Ruby in overall appearance and in values for λ_ex and λ_em maxima. Figure 3.6 gives the excitation and emission spectra for RuMS, for
example. There are two absorption peaks, one at ~280 nm and a broad one at ~450 nm. The fluorescence emission spectrum has one broad peak at 610 nm. The similar spectra obtained for the ruthenium complexes indicate that they possess similar fluorescence properties.

RuNS (ruthenium attached to three bathophenanthroline ligands – no sulfonates) was bought from a commercial source and solution made up in-house. The various in-house synthesized ruthenium complexes and RuNS were subjected to MALDI-TOF mass spectrometry. Different matrices were employed like HCCA, SA and DCTB, but none enabled visualization of the expected m/z peak (RuNS (ruthenium tris bathophenanthroline) [M+H]⁺ = 1098, RuMS [M+H]⁺ = 1258, RuDS [M+H]⁺ = 1464 and RuBPS [M+H]⁺ = 1670). The base peak observed in all the spectra obtained was an m/z of 766. From spectra (Figures 3.7-3.10) obtained for the three complexes including RuNS in HCCA matrix, it is clear that each compound is breaking apart and some of the peaks observed match the m/z for fragments of the original complex. The peak with m/z = 766 could be the ruthenium ion attached to 2 bathophenanthrolines ligands. Table 3.1 gives different mass peaks that were observed in the MALDI-TOF spectra for the various complexes. The mass spectral characterization of in-house synthesized ruthenium complexes was not successful in clearly elucidating the structure.

Paper chromatography was performed to help us in the identification of these complexes. Table 3.2 summarizes the retention factors (Rf) obtained for ruthenium complexes in two different solvents. Two compounds were seen in each of the ruthenium complexes shown by the two Rf values. RuDS and RuBPS have similar retention factors indicating that the major constituents in both these preparations are the same compounds. RuMS, on the other hand, had different Rf values compared to RuDS and RuBPS. There is one spot in RuMS with Rf value of zero in water indicating that it is highly polar in nature. This could be the sulfonated ligand or some other polar ruthenium complex. All the three ruthenium complexes appeared to have one compound common to all. The commercial RuMS had an Rf value or 0.42 in water that is present in all the in-house synthesized ruthenium complexes. Comparison of Rf values between commercial and in-house RuMS
complex indicated that the in-house synthesized RuMS was indeed made up of RuMS complex and one other complex with Rf value of zero.

The three ruthenium complexes differed in terms of their staining abilities (Figure 3.11). In-house RuMS provided the greatest sensitivity with a limit of detection of 0.5 to 1 ng, versus 5-10 ng for RuBPS and 25-50 ng for RuDS. Since RuMS clearly had the best sensitivity among the three complexes, characterization of RuMS is important. Mass spectral data clearly indicates that the ruthenium complexes are highly unstable and fragment in the mass spectrometer. However, from the fragmentation pattern there is evidence for the presence of RuMS complex in the RuMS stock solution. Paper chromatography data indicates that the RuMS solution synthesized is a mixture of two complexes. The difficulty faced in obtaining crystals from the RuMS solution (in an attempt to characterize the complex by X-ray crystallography) further suggests that the solution is a mixture and not pure RuMS complex. Due to the paucity of this material, further structural elucidations were not pursued. However, the data suggests that the in-house synthesized RuMS solution contained RuMS and another minor component.

Since RuMS gave the most sensitivity in staining proteins, the following discussions will be mainly focused on RuMS. The next section is a discussion on the optimization protocols for RuMS staining that were carried out to achieve the maximum sensitivity.

3.4b Optimization of staining protocol using in-house RuMS

Solvent composition of the staining solution is highly critical for successful staining. Therefore, various solvents like ethanol, methanol and isopropyl alcohol were used as solvent for the formulation of RuMS stock solution. As seen in figures 3.12 and 3.13, the stain had little background and high sensitivity when ethanol was used as a solvent. The concentration of RuMS in the working solution was also optimized (Figure 3.14). Desired staining of proteins with minimal background staining was obtained with 150 nM RuMS in 20% ethanol solution. When solvents other than ethanol were used or when greater
concentrations of ethanol were used in the formulation, there was increased background staining due to non-specific binding of the stain to the polyacrylamide matrix.

The optimized staining protocol of the in-house synthesized RuMS detected proteins down to 0.5 ng in gels. As discussed earlier, greatest challenge was in characterizing the synthesized complex. Even though there was significant evidence for the presence of RuMS complex in the stock solution, there is lack of concrete data to support this observation. We looked at other ways to confirm the identification. In our search to do so, we came to know of a company Biotium (Hayward, CA) that was started during the course of this work, sells the fluorescent ruthenium complexes. The commercial RuMS was purchased in order to test its staining efficiency using standard marker proteins. The in-house synthesized RuMS stain was compared to this commercial RuMS in terms of sensitivity and specificity and to give further structural information on the synthetic stain by inference. Again for commercial RuMS, the formulation is very critical and hence various solvents like 20% v/v, and 50% v/v of both ethanol and isopropyl alcohol were used as solvents. As seen in figures 3.15 and 3.16, the stain had little background and high sensitivity when ethanol was used as the solvent. The background staining was high when isopropyl alcohol was used. Different percentage of ethanol also gave high background due to non-specific binding of the stain to the gel matrix. Therefore, the most suitable solvent for ruthenium complex to stain proteins with high sensitivity but with minimal background staining of the gel was obtained with 20% ethanol in the final working solution. Concentration of ruthenium complex in the staining solution was also optimized (Figure 3.17). Desired staining of the proteins with again minimal background staining was obtained with 100 nM of in-house RuMS and 125 nM commercial RuMS, both in 20% ethanol solution.

There exists subtle difference in staining efficiency of the in-house synthesized RuMS compared to the 99% pure commercial RuMS (Figure 3.18). In commercial RuMS stained gel, more non-specific binding occurs even at 10 ng of protein while there is negligible background with the in-house RuMS staining solution, leading us to speculate that the minor component present in the in-house synthesized RuMS solution enhanced
the staining ability of the ruthenium complex. From the paper chromatography data, we suspect this contaminant is most probably RuBPS, another form of ruthenium complex. RuMS and RuBPS have similar ligands attached to the central ruthenium atom. However, they differ in the number of sulfonate groups attached to the bathophenanthroline ligands. RuMS has two sulfonate groups attached to one bathophenanthroline ligand while RuBPS has six sulfonate groups. Thus RuMS is more hydrophobic than RuBPS complex. Goldstein et al. have reported the crystal structure of ruthenium tris bathophenanthroline complex (RuNS) in which they propose that the bathophenanthroline ligands of different molecules stack-up to form a cluster [58]. The features seen in the crystal and molecular structure of ruthenium tris bathophenanthroline can be used to construct models for the interaction between the different forms of ruthenium complexes in RuMS staining solution that can explain the improved sensitivity observed with the in-house RuMS.

In one plausible model, one of the hydrophobic bathophenanthroline groups of RuMS interact with hydrophobic residues in the protein. The other two ligands of RuMS, namely bathophenanthroline disulfonate and bathophenanthroline are aligned outside. The sulfonate group has a tetrahedral structure and since the group is not in the same plane as the aromatic ring, it destabilizes possible \( \pi \)-\( \pi \) stacking of its bathophenanthroline/phenyl groups with bathophenanthroline/phenyl groups of another ruthenium complex. However, the bathophenanthroline moiety with no sulfonate is accessible for \( \pi \)-\( \pi \) stacking and a second dye molecule can easily stack-up with this complex. In this way, the dye molecules stack-up with each other so as to allow for more dye molecules to bind to a protein, thereby significantly increasing the dye to protein ratio with an amplification in the signal generated. This stacking up of planar ring structures with extensive conjugation of \( \pi \) orbitals has been reported by Skoog et al. and Congdon et al. [59, 60]. The stacking of coomassie dye molecules with a similar structure as that of the ruthenium complex has been proposed for the improved sensitivity with the blue silver protocol [61]. If this same concept of \( \pi \)-stacking occurs in the case of our “in-house RuMS” staining protocol, this would very well explain the substantial increment in sensitivity. Due to the presence of two different forms of ruthenium complexes in the in-house synthesized RuMS staining solution, there are more numbers of dye molecules
binding to proteins. So even low levels of protein are detected since more dye molecules bind. On the other hand, with just the RuMS form in the staining solution, as in the case of commercial RuMS, there is only one form of ruthenium complex. Even though the stacking-up of the dye molecules is possible, RuMS being more hydrophobic than RuBPS, also non-specifically binds to the polyacrylamide gel matrix which considerably reduces sensitivity.

Hence by having both RuMS and RuBPS in a staining solution, we achieve the right balance in hydrophobicity and hydrophilicity of the final staining solution required to detect the proteins with improved sensitivity. The characterization of the in-house syntheized RuMS staining solution is necessary to validate the proposed model and future work should involve new experiments characterizing the material to ensure exploitation of the in-house synthesized RuMS stain’s potential to the fullest.

The optimized formulation of the in-house synthesized RuMS stain was compared with other stains for its sensitivity, linear dynamic range and compatibility with mass spectrometry.

3.4c Comparison of sensitivity of various stains

Figure 3.19 gives identically prepared gels stained with colloidal Coomassie, silver, commercial Sypro Ruby and in-lab synthesized RuMS. Clearly, colloidal Coomassie can detect up to 0.5 ng of protein, however only after repeated staining for three times. Silver can also detect down to 0.5 ng of protein but the background was darker at lower ng level of protein, and the gel showed inherent artifacts due to silver staining. Both Sypro ruby and in-lab synthesized fluorescent RuMS had comparable sensitivities and could detect 0.5 -1 ng of protein. Commercial RuMS had a sensitivity of about 1 ng. It is clear from the gels that there is some amount of difference in sensitivity from protein to protein which depends on the inherent amino acid composition of each protein and how the stains interact with them. Ovalbumin and lysozyme showed differential staining with colloidal Coomassie. This could be explained because of the heavy glycosylation on ovalbumin
that interferes with the binding of the dye to the protein as reported by Fountoulakis et al. [62]. As previously reported in colorimetric labeling of protein [63-65], lysozyme was more lightly stained with Coomassie down to about 10 ng. This could also be due to the glycosylation in lysozyme. In the case of silver staining, trypsin inhibitor and lysozyme proteins stained more intensely than the other protein markers and showed artifacts associated with this staining method. Sypro Ruby and in-lab synthesized RuMS showed much less protein to protein variability.

3.4d Linearity in staining response

To minimize protein-to-protein variability, stain and batch differences, the mean band volume from three gels run and stained with either of the dyes was plotted against the ng of protein loaded (Table 3.3, Figure 3.20 and 3.21). For each protein band selected, linear treadlines were added and $R^2$ values determined. The in-lab synthesized RuMS was found to be comparable to commercially available Sypro Ruby in terms of its linearity, but the staining intensity was greater than with Sypro Ruby and with greater signal to noise ratio. Both Sypro Ruby and in-house RuMS showed linear response from 0.5 to 100 ng protein. Silver staining, as reported earlier, is sensitive down to 1 ng and maybe even 0.5 ng of protein. But since the band volume at 0.5 ng protein load is very close to the band volume for 1 ng protein, it showed a linearity of dilution from 1 to 50 ng of protein. The linearity in response for colloidal Coomassie stained gels was 5 ng to 100 ng. There was considerable batch-to-batch difference in silver staining most likely owing to variability introduced due to critical timing. Relative standard deviation (%RSD) was less than ±5 for both Sypro Ruby and RuMS, which shows good batch-to-batch reproducibility.

Figures 3.20 and 3.21 give the graphs of the linear relationships for raw band volumes of proteins from gels after background subtraction. The band volumes for 5 ng of BSA from gels stained with the different staining solutions were normalized and histogram plotted (Figure 3.22). From the bar graph, we can see that there is no significant difference between the different stains – CCB G 250, silver nitrate, in-house RuMS and Sypro
Ruby- in terms of sensitivity. Though the stains had similar sensitivity, each stain had its own limitations. CCB G250 had sensitivity down in the ng levels of proteins only after repeated staining and destaining steps for at least three times. This is a very time consuming process that takes up to a week. Silver nitrate on the other hand, had very poor reproducibility due to a constant hands-on protocol that required routine preparation of fresh solutions. Sypro Ruby had good sensitivity but the major disadvantage was the cost involved in buying the stain. In-house synthesized RuMS had comparable sensitivity to Sypro Ruby and was very cost-effective.

3.4e Compatibility of stains with mass spectrometry

Once proteins are separated by 2-DE, they are visualized using a suitable staining solution and the protein spot profiles are analyzed using image analysis software. The protein spots of interest are then excised and subjected to enzymatic digestion. The resulting peptide fragments are then analyzed using mass spectrometry (MS). With fluorescent stains, the sensitivity of the protein stains has improved and the challenge is now to acquire good mass spectra from especially low abundance proteins. Therefore, it is imperative that the dye does not interfere with MS methods. Interfering dyes (like silver nitrate) can result in ion suppression resulting in fewer measurable peptides obtained and a reduction in signal intensity.

Two complementary MS methods used for proteomic studies are MALDI-TOF and ES-LC-MS/MS. To evaluate the compatibility of different stains with these two mass spectrometry methods, gel plugs were harvested and digested with trypsin. The resulting tryptic peptides were subjected to both matrix assisted laser desorption ionization (MALDI-TOF) and liquid chromatography tandem mass spectrometry (LC-MS/MS).

- **Stain compatibility with MALDI-TOF**

Tryptic peptides were subjected to MALDI-MS as described earlier. A representative peptide mass fingerprint (PMF) analysis for all the 100 ng BSA protein is shown in figure
3.23. The summarized MALDI data from all the trypsin digested proteins is provided in Table 3.4. The number of successfully identified peptides and the resultant sequence coverage were slightly better for the in-lab synthesized stain than for commercial Sypro Ruby. Although silver staining is a very sensitive visualization method, poor mass spectra were obtained. Out of 12 proteins that were subjected to MALDI-TOF after silver stain, peptides were detected for only 6 samples. No peptides from BSA were correctly identified after MALDI-TOF. Low peptide recovery and sequence coverage of silver stained proteins may be due to their modification by formaldehyde (alkylates α and ε amino groups of proteins) during the staining process. Unfortunately, formaldehyde cannot be omitted or substituted for in the staining protocol.

Colloidal Coomassie performed fairly well in the MS compatibility study, however it is very difficult to get rid of the stain completely, and this could pose difficulties with MS identification. Also the gel stained with colloidal Coomassie was stabilized in a saturated solution of ammonium sulfate which could interfere with PMF analysis. It should be noted from table 3.4 that different staining methods enabled different peptides to be identified. Our data indicate that both fluorescent stains namely; Sypro Ruby and in-house RuMS are fully compatible for protein identification by peptide mass fingerprinting using MALDI-TOF mass spectrometry.

- **Stain compatibility with ES-LC-MS/MS**

Data dependent MS/MS experiments were performed on a Thermo LCQ Classic quadrupole ion trap mass spectrometer. Mass spectra were searched against the human sub-database from the Swiss-Prot database using the Mascot algorithm. One missed trypsin cleavage was allowed and a variable modification of oxidation of methionine was set. Peptide tolerance and MS/MS tolerance were both set at ± 0.8 Da. Representative ES-LC-MS/MS spectra are shown in figure 3.24. Table 3.5 summarizes the LC-MS/MS data for all the trypsin digested samples. Silver staining yielded few matches when compared to other stains. Fewer peptides were seen for proteins from silver stained gels. Colloidal Coomassie seemed to perform fairly better than the silver staining method.
Sypro Ruby and in-lab synthesized stain gave the most number of peptides detected with highest Mowse score and sequence coverage.

In conclusion, all stains tested in this work demonstrate some degree of MS compatibility. Silver stain gave significantly better results with LC-MS/MS than MALDI-TOF and colloidal Coomassie gave slightly better results with LC-MS/MS than MALDI-TOF. The in-lab synthesized RuMS stain is slightly better than commercially available Sypro Ruby in terms of MS compatibility. More peptides were recovered and detected by both MALDI-TOF and LC-MS/MS for proteins stained with the in-lab stain and gave better Mascot search results with high Mowse score and sequence coverage.

- **Evaluation of protein sequence coverage**

The level of protein sequence coverage for the six marker proteins stained with different stains is shown in Tables 3.4 and 3.5. Coverage was determined from the Mascot search results for proteins that gave significant identification by searching against the protein database. For proteins that did not give a significant match but had peptides detected by the mass spectrometer, coverage was calculated manually. Sequence coverage for proteins extracted from in-lab RuMS stained gels was better than that obtained from Sypro Ruby stained gels. It is important to note here that higher protein coverage is not necessary for protein identification, especially with ES-MS/MS.

### 3.4f Cost-effectiveness of RuMS stain

The specific aim of this project was to synthesize a fluorescent ruthenium complex in-house to be able to use it as a cost-effective alternative to Sypro Ruby. The following section explains how RuMS synthesized in our lab proved to be a cost-effective protein stain.
In most proteomics labs, 2-DE is employed to analyze the proteome and hence staining solutions are used in huge quantities. The goal of this project is to synthesize a ruthenium complex that can be used as a cost-effective alternative to expensive Sypro Ruby. This section discusses the cost-effectiveness of the in-house synthesized RuMS staining solution in comparison to the commercial RuMS and Sypro Ruby. Sypro Ruby as mentioned earlier is highly sensitive and has linear response over a wide range of protein load but is highly expensive. An alternative to Sypro Ruby should be able to stain the proteins with similar sensitivity and give linear dynamic range but in a much more cost-effective manner. Commercial Sypro Ruby costs approximately $200 and it can be used to stain only two large format (20 x 20 cm) gels. A half liter solution of our in-house synthesized RuMS costs about $100 to buy the required chemicals and solvents and an aliquot of 1.5 mL is used to stain one gel. Hence 500 mL of the in-house staining solution can be used to stain ~ 330 gels. Chemicals worth $200 can be used to synthesize 1 L of staining solution which can be used to stain roughly about 600 gels. Commercial RuMS costs about $150 which can be used to make 250 mL of 16 nM solution. Forty mL of this staining solution can stain one large format gel. Hence one 250 mL solution can be used to stain 6 gels. A summary of pros and cons of the fluorescent stains evaluated is shown in the flow chart below (Table 3.6).
**Table 3.6** Pros and cons of various fluorescent stains.

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3.5 Conclusion

For a successful bottom-up proteomics involving 2-DE, an essential requirement is a protein staining technique that is sensitive, quantitative and compatible with MS. Using standard proteins on SDS-PAGE, we have showed that the ruthenium complex (RuMS) synthesized in our lab after optimization of the staining formulation is very similar in staining properties to commercially available fluorescent protein stain Sypro Ruby in terms of both sensitivity and linear dynamic range. Our results show that in-house RuMS has better compatibility with mass spectrometric identification than Sypro Ruby both in terms of the “goodness” of fit between the theoretical and observed peptide masses during the database search using the Mascot algorithm and in terms of better protein sequence coverage. The staining protocol is also simple and easy to follow with effectively no destaining required. Colloidal Coomassie and silver staining methods are commonly used but have pitfalls when used in proteomic studies, such as low linear dynamic range, batch-to-batch and protein-to-protein variations and incompatibility with further mass spectrometric identification of proteins. In-lab synthesized ruthenium complex stain is routinely used in our laboratory to stain 2-DE gels (example shown in Figure 3.25) and has given satisfactory results with respect to sensitivity and mass spectrometric identification by both MALDI-TOF and LC-MS/MS.

Even though the characterization of the RuMS solution was not successful, the presence of RuMS is evident from the mass spectral data. Other characterization attempts indicated the presence of an additional minor component present in the solution, which we speculate to be RuBPS form. Comparing the staining efficiency between the in-house synthesized RuMS and commercial RuMS, in-house RuMS had a better signal-to-noise ratio and this could be explained by the additional component in the RuMS solution. As explained earlier, presence of both RuMS and RuBPS enable a synergistic effect in the binding of the dye molecules to the protein and there is amplification of the signal. This allows for even low levels of proteins to be detected as number of dye molecules stack-up to give an amplified signal. The capability of synthesizing the fluorescent complex in-house proves to be very cost-effective. Sypro Ruby is highly expensive and 500 mL of
the solution can be used to stain only two large format gels; however for the same amount of money in terms of reagents (not including man power or quality control) 1 L of RuMS can be synthesized in-house which can be used to stain hundreds of gels. Thus, the optimized formulation of the in-house RuMS staining solution proved to be the most cost-effective alternative to the commercially available Sypro Ruby.
Figure 3.25 2-DE gel from 400 μg of SY5Y whole cell lysate stained with in-house synthesized RuMS staining solution. The gel image acquired using Versadoc was inverted so that the spots are darker than the background.
CHAPTER FOUR

PROTEOMIC INVESTIGATION OF MALATHION-INDUCED DIFFERENTIAL PROTEIN EXPRESSION IN HUMAN NEUROBLASTOMA CELLS

4.1 Introduction

Pesticides play a vital role in agricultural and industrial pest control. However, widespread use and overuse of these chemicals have increased awareness of the potential pesticide-related health hazards. An example is the class of organophosphates (OP) that are important chemical compounds used mainly as crop protective pesticides. Potent organophosphate such as chlorpyrifos is usable as an insecticide yet is also capable of causing learning disabilities especially in exposed young children [66]. Animal studies have reported that exposure to chlorpyrifos can cause developmental neurotoxicity by affecting basic processes of neuronal development like axonogenesis, neuritogenesis and synaptic function [66-69]. This led to the restriction in the use of chlorpyrifos in the United States [70, 71].

Malathion, another organophosphate (with a different leaving group) is used widely in both agriculture and domestic areas because it is thought to be a more benign OP. About 3.4 million pounds have been applied to non-agricultural areas such as golf courses, public and home gardens for the Mediterranean fruit fly quarantine and mosquito abatement to-date [72]. Close to 17 million pounds of malathion are sprayed annually in the U.S. to control boll weevils on cotton and other pests [73]. In 1999, malathion was also used in eradication of West Nile virus carrying mosquitoes.

On one end of the spectrum is the more potent OP chlorpyrifos shown to cause developmental neurotoxicity that has been banned from domestic use while on the other end there is the “less toxic” malathion that is used extensively for both agriculture and household purposes. The effect of the more potent OP, chlorpyrifos on levels of protein was studied in our lab using SY5Y as model system. Since it is equally important to
study the effect of a benign OP as a control that made up the other end of the spectrum of OP toxicity, the proteomic investigation on the effect of malathion was carried out. The initial hypothesis is that malathion does not cause any significant change in levels of protein. SY5Y cell cultures were used as model system to test the effect of malathion using proteomics techniques.

The primary mode of action of malathion and other OPs (including chlorpyrifos) in insects is inhibition of acetylcholinesterase (AChE) and thus interfere with normal functioning of the nervous system. In vivo AChE inhibition occurs when malaoxon, a metabolite of malathion, binds to acetylcholinesterase; thus, symptoms may be delayed after exposure owing to the need for metabolism. Covalent AChE binding results in accumulation of acetylcholine at the synapse causing repeated stimulation of neurons and a number of neurotoxic effects – especially muscarinic and nicotinic effects. Muscarinic effects include: Salivation, Lacrimation, Urination, Diarrhea, GI upset and Emesis (acronym: SLUDGE). Nicotinic effects include: tachycardia, muscle fasciculation and respiratory shutdown resulting from diaphragmatic failure, which ultimately causes death.

Previous studies have shown that malathion induces genotoxicity [74]. Using animal models, researches have shown that malathion greatly inhibits AChE in the cerebellum and hippocampus [75]. Experimental animal studies have revealed that malathion causes chromosomal aberrations and micronuclei formation [76-78]. Malathion is also now known to be an inhibitor of gap junction intercellular communication (GJIC) [79]. Akhgair et al. measured the levels of thiobarbituric acid reactive substances (TBARS) in the liver and blood of rats and showed that malathion inhibition of AChE activity was accompanied by oxidative stress [80]. In addition to its mutagenic, carcinogenic and teratogenic effects determined by in vitro and in vivo studies [81, 82], malathion reduces antibody titers to different antigens in a dose-dependent manner in experimental animals. This effect was most pronounced during the secondary immune response [83, 84]. Malathion was also shown to induce allergic reactions in children [85]. Rodgers et al. reported that malathion causes dysregulation of peritoneal mast cells [86].
There exists a number of possible ways humans get exposed to pesticides, which could be due to one or more of the following reasons: accidental poisoning, occupational exposure and off-target exposure from aerial spraying of the pesticide in crop fields. In view of the widespread use of pesticides, the potential is very high for repeated exposures to one or simultaneous exposure to various pesticides. Pesticide intoxication poses severe problems which can be exacerbated if not prevented. Most of the reports studying toxicity of malathion were focused on secondary effects after exposure, symptomology in particular. There is an overall lack of information on the protein targets and their levels that can be altered after exposure to pesticides. Significant biochemical pathways may be hindered by organophosphate insecticides exposed enzymes, receptors or structural proteins.

Proteomic investigation of the effect of malathion was performed using human neuroblastoma cell (SY5Y cells). The SY5Y neuroblastoma cell line was used as it exhibits neuronal phenotype which provides a convenient model system to study neurotoxicity of organophosphates [87]. SY5Y cells express muscarinic acetylcholine receptors [88], mostly subtype M3 [89] and exhibit higher activities of neurotoxic esterases (NTE) and acetylcholinesterase compared to other cell lines [90]. Barber et al. showed that organophosphate-treated human neuroblastoma cells gave similar responses to those in hens exposed to these compounds which are currently the Environmental Protection agency (EPA) prescribed animal model to test OP toxicity [91-93]. Barber et al. thereby validated SY5Y cells as a useful model and Ehrich et al. also validated the use of human neuroblastoma cells for the initial screening of OPs [89]. Criticism exists that neuroblastoma cells are not a part of “normal” tissue and the responses seen may be consequence of cancerous behavior. However, asking pointed questions and strategically designing the experiments with well-defined end-points can minimize this fear. As a minimum criterion, this involves carefully comparing the control (no malathion treatment) against the treated samples. Veronesi et al. agree that the in vitro system using human cell lines that house the target enzymes for the OPs are relevant to risk assessment [94]. Also cells of human origin provide good models for studying OP toxicity because of the direct correlation to humans as opposed to animal studies in which extrapolation of the results to humans is needed.
The initial hypothesis that malathion does not cause any significant change in levels of proteins was proved false. We present evidence that malathion does cause significant change in protein levels in SY5Y cells exposed to the organophosphate. With the aid of proteomic tools such as two-dimensional gel electrophoresis (2-DE) and mass spectrometry, we have identified the candidate SY5Y proteins whose levels were altered (up- or down-regulated) after treatment with malathion. Finally, western blot analysis was carried out to confirm alterations in levels of specific proteins seen in 2DE studies. We found that many chaperone proteins and metabolic enzymes were down-regulated coordinately, suggesting that malathion perturbs protein turnover. We hypothesized that the decrease in chaperone proteins may sensitize the cells to oxidative stress as shown by \textit{in vivo} and \textit{in vitro} studies.

4.2 Materials and Methods

4.2a Materials

Isoelectric pH gradient (IPG) strips (0.5 mm wide, 17 cm long, pH 3-10 linear), urea, Tris (electrophoresis grade), IPG rehydration/sample buffer containing 8M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte 3/10 ampholyte, trace bromophenol blue, readyprep overlay agarose and mineral oil were obtained from Bio-Rad (Hercules, CA). SDS, TEMED, ammonium persulfate, ammonium bicarbonate, acetonitrile, formic acid, acrylamide, bisacrylamlide and were purchased from Sigma (St.Louis, MO). For protein staining in gels, ruthenium complex prepared in our lab was employed. DTT, iodoacetamide, glacial acetic acid (ACS grade), glycine (tissue culture grade) and methanol (HPLC grade) were all purchased from Fisher Scientific (Fair Lawn, NJ). Lyophilized sequencing grade modified trypsin was purchased from Promega (Madison, WI). Fused silica capillary tubing 320 μm I.D. x 435 μm O.D. for liquid chromatography column was purchased from Polymicro Technologies (Phoenix, AZ) while the reverse phase packing material Polymer X (3 μm) was purchased from Phenomenex (Torrance, CA). All other LC accessories including zero dead volume (ZDV) unions, frits, ferrules were obtained from Upchurch Scientific (Oak Harbor, WA).
4.2b Cell Culture

SY5Y neuroblastoma (American Type Culture Collection CRL 2266) [87] cells were grown to confluence in 75 cm² flasks in Dulbecco’s minimum essential medium supplemented with 55 mM glucose, 26 mM NaHCO₃, 16 mM KCl, 1.36 mM pyruvic acid, 2 mM glutamine, 10 mg/mL gentamicin sulfate and 10%v/v clarified bovine serum (pH 7.4) in an environment of 94% air/6% CO₂ at 37°C. Cultures were split 1:3 every two to three days by removal of media and incubation in 0.25% trypsin/0.03% EDTA until cells detach. Five mL of media was then added and cells pelleted by centrifugation at 3,000 x g for 10min. For each malathion treatment, the cells were switched to Lock’s medium and treated with appropriate concentrations of malathion. Following treatment, cultures were washed extensively with phosphate buffer saline (pH 7.4) and cells were gently scraped into 3 mL PBS and pelleted by centrifugation at 800 x g for 10min. The pellet was then resuspended in 200 μL of distilled/deionized water and homogenized using a micro Dounce homogenizer. Cell viability test was checked by (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (MTT) assay and protein content determined using Pierce BCA method.

4.2c Cell Viability Assay

Confluent cultures were split from one flask into three 96-well culture plates. When confluent, cultures were switched to Lock’s medium consisting of 154 mM NaCl, 56 mM KCl, 2.3 mM CaCl₂, 1.0 mM MgCl₂, 3.6 mM NaHCO₃, 10 mM glucose, 5 mM HEPES (pH 7.2) with 10 mg/L gentamicin sulfate and treated with malathion (25 to 2000 μM) in 1% DMSO. Eight wells per concentration were treated with each concentration or with vehicle alone (control). After 24hours, cell viability is assayed by addition of 10 μL or 1 mg/mL MTT solution that is converted to formazan crystals by the cellular enzymes [95]. The level of formazan dye formed is directly correlated to metabolically active cells in culture. After addition of MTT, cultures were placed in the incubator for an additional 1 hour. The media was quickly aspirated and the formazan crystals dissolved in 100 uL DMSO. Formazan absorbance was then measured at 620 nm using a multi-well plate.
reader. Results were calculated for each well as percent of control absorbance and are expressed as the mean ± SD control absorbance. Cell viability was also assessed by adding 10 μL WST-1(4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate), which is converted to a water soluble formazan by reductase system and is a measure directly in the media. There was no significant difference in viability measured by the two assays. Using commercially available software, statistical analysis was carried out. ANOVA with Dunnett’s post hoc test was performed for differences.

4.2d Malathion Treatment

For proteomic analysis of changes in protein expression in SY5Y neuroblastoma cells, the cultures were treated with 100 μM malathion for 24 and 48 hours in the same way as the cell viability assay.

4.2e Two-dimensional gel electrophoresis

Pierce BCA method was used to determine protein content [96]. A protein concentration of 400 μg of whole cell lysate was dried in a vacuum centrifuge. A volume of 300 μL of rehydration buffer was added to the pellet and the solution was loaded on IPG strip and after 12 hours of active rehydration at 50 V, the proteins were focused in the Protean IEF cell from Bio-Rad using a 6 step program (100 V, 300 V, 500 V, 1000 V and 2500 V for 1 hour each and 5000 V for 16 hours). A limiting current (50 μA per strip) was maintained.

After IEF, the strips were equilibrated (30 min) with 6 M urea, 32 mM DTT, 0.375 M Tris-HCl pH6.8, 10%(w/v) SDS, 50% (v/v) glycerol and for 20 min in the dark with 63 mM iodoacetamide in the above equilibration buffer instead of urea. Each strip was loaded on an 8-16% gradient polyacrylamide gel and proteins were electrophoresed at 30 mA constant current using a Protean II XL (Bio-Rad) in a buffer that contained 25 mM Tris, 192 mM glycine and 0.1% SDS until the blue dye reached the bottom of the gel.
4.2f Staining of proteins

The 2-DE separated proteins were stained with a solution of Ruthenium II ((bis bathophenanthroline) (bathophenanthroline disulfonate)) that was synthesized in-lab using a modified protocol [53]. The gels were fixed in an aqueous solution containing 30% ethanol and 10% glacial acetic acid overnight. Gels were washed with aqueous solution of 20% ethanol three times and incubated overnight in solution containing the ruthenium complex. Thereafter, the gels were washed once with distilled water. Stained gels were stored in distilled water at 4°C.

4.2g Image and statistical analyses

Stained gels were scanned with a Versadoc 3000 scanner from Bio-Rad (Hercules, CA). Images were analyzed using the PD Quest software (Bio-Rad). Spots were detected and matched by both automated matching following the instructions in the software and also by manual matching. The spot volumes were normalized to the total density in the gel image to avoid any non-expression related variations between matched spots. The statistical significance of the data was determined by the Student’s $t$ test. A difference in spot volume was considered statistically significant at $p<0.05$ and confidence level of 95%.

4.2h In-gel proteolysis of proteins

Protein spots were picked and digested with trypsin using Bruker Daltonics prep/digest station. In brief, the gel pieces were washed 6 times alternating with 50 mM NH$_4$HCO$_3$ and 1:1 ACN: NH$_4$HCO$_3$ (50 mM) for 5 min each at 20°C and dried with ACN for 10 min at 20°C. A 10 μL volume of trypsin (66.67ng) solution in 25 mM NH$_4$HCO$_3$ was added and gel pieces were allowed to swell for 30 min at 20°C. Digestion was allowed to proceed for 4 hours at 50°C. Peptides were extracted after terminating the digestion with 1 μL of 1% aq. formic acid.
4.2i MALDI-TOF mass spectrometry analysis

MALDI-TOF mass spectra were generated using Bruker Autoflex (Billerica, MA) in the reflectron mode. The Bruker Daltonics prep/digest station was used to spot the matrix and the peptide solution on the anchorchip target. Briefly, matrix solution (10 mg/mL of α-cyano-4-hydroxycinnamic acid in 90% acetone and 2% TFA) was spotted and 1.5 μL of peptide solution was added to the matrix spot and allowed to air dry. On target washing was carried out with 0.2% TFA. Spectra were recorded and 100-300 laser shots were added to one spectrum. The spectra were externally calibrated against the monoisotopic mass of a standard solution containing 1 μg/μL Bradykinin ([M+H]+ 757.399), Vasopressin ([M+H]+ 1084.445), Angiotension II ([M+H]+ 1046.542), Substance P ([M+H]+ 1347.735) and oxidized form of Substance P ([M+H]+ 1363.731) and internally calibrated using known trypsin autolysis peaks.

4.2j LC-MS/MS analysis

The trypsin digested samples were also analyzed using a Thermo Electron Corporation’s LCQ Classic quadrupole ion trap mass spectrometer (San Jose, CA). The tryptic peptides were resolved on in-house constructed capillary column. The column was fabricated by packing 15 cm of Phenomenex Polymer X (3 μm particle size) into a 320 μm I.D fused silica capillary using a home-made stainless steel packing cell pressurized with helium to 1000 psi. Five μL of tryptic peptides were injected into the column and eluted at a flow rate of ~ 4 μL/min, using a binary gradient of 5-80% ACN (with 0.1% formic acid) within 55 min. Spectra were acquired with a probe spray voltage of 4 kV and a heated capillary temperature of 175°C.

4.2k Database search

- PMF

The MS data were searched against tryptic peptides sequences from the Swiss-Prot database using the Mascot algorithm. The search was restricted to trypsin generated
peptides allowing up to two missed cleavages. A variable modification for oxidation of methionine and carbamidomethylation of cysteines were set. The taxonomy was human and the peptide tolerance was ± 0.5Da.

- **LC-MS/MS**
  Mass spectra were searched against the human sub database from the Swiss-Prot database using Mascot algorithm. One missed trypsin cleavage was allowed and a variable modification of oxidation of methionine was set. Peptide tolerance and MS/MS tolerance were both set at ± 0.8 Da.

At the above settings for both PMF and LC-MS/MS, probability based Mowse scores greater than 33 indicated identity or extensive homology. Additional criteria like more than one peptide must be identified and the ion score of at least one peptide must be greater than 30 were used to identify proteins.

**4.2l Western blot analysis**

For western blotting after SDS-PAGE, a standard protocol after Towbin et al. was used [36]. Briefly, 25 μg of whole cell lysate from SY5Y treated with 100 μM malathion for 24 hours and the corresponding control samples were mixed with sample buffer containing 60 mM Tris-HCl pH 6.8, 10% glycerol, 10% SDS, 0.1% bromophenol blue and 5% β-mercaptoethanol, boiled for 5 min and on a 12% SDS-polyacrylamide gel. Electrophoresis was carried out at 100 V for about 2 hours. The proteins were blotted onto a nitrocellulose membrane at 100 V for 1 hour. Transfer of proteins to the nitrocellulose membrane is confirmed by Ponceau S staining. The membrane was blocked with blocking buffer (5% non fat milk in Tris buffer saline containing 0.05% Tween-20). After blocking for 1 hour 30 min, the membrane was incubated with the anti-human rabbit primary antibody in blocking buffer overnight at 4°C. Both UCH L1 and rhoGDI antibodies were used at 1:2000 dilutions. After washing, the membranes were incubated with the secondary peroxidase conjugated goat anti-rabbit antibody (1:2500 dilutions in wash buffer). Signals were developed with an enhanced chemiluminescence
(ECL) kit from GE Healthcare (Waukesha, Wisconsin). The developed ECL film was scanned and the tiff images were analyzed by Scion Image software. Excel was used to perform statistical analyses. The GAPDH protein was also probed for to check on the efficiency in loading the sample.

4.3 Results

4.3a Dose-dependent effects of malathion on cell viability

Malathion dose-dependently increased cell viability in SY5Y cells after exposure with concentrations up to 250 μM for 24 hours. The results were calculated for each well as percent of control absorbance (viability) and are expressed as the mean ± SD control absorbance (Figure 4.1). Cultures exposed to 1000 and 2000 μM malathion showed significantly decreased cell viability compared to control cultures. Surprisingly, lower concentrations of malathion (25 μM to 250 μM) significantly increased mitochondrial function.

4.3b Identification of proteins using mass spectrometry

Two-dimensional gel electrophoresis was carried out on 400 μg of whole cell lysate protein from SY5Y cells and 2-DE map of the whole cell lysate proteins were generated (Figure 4.2). Using PD Quest gel image analysis software around 400 proteins spots were detected on the 2-DE gel. A total of 122 proteins were identified using mass spectrometry after subjecting the proteins to in-gel trypsin proteolysis (Appendix A and B). Mass spectra were searched against the human sub database from the Swiss-Prot database using the Mascot algorithm [97]. The search parameters described in the methods section gave unambiguous protein identification with adequate number of peptides identified and reasonable sequence coverage for the proteins.
**Figure 4.1** Dose dependent effect of malathion on cell viability of SY5Y cultures. Cell viability was measured by MTT assay and the percentage control for each treatment is expressed as mean ± SD values.
4.3c Analysis of SY5Y proteome after treatment with malathion

2-DE was performed four times on whole cell lysate from control cell cultures (no malathion) and cell cultures treated with 100 μM malathion for 24 hours and 2 times each on 100 μM malathion for 48 hours and their corresponding control samples. The average number of spots detected from the fluorescence-stained gel images using the PD Quest software was about 400 spots in the treated and control gels. Figure 4.2 shows the typical 2-DE gel performed on the whole cell lysate from control sample (no malathion treatment). 2-DE maps of 400 μg of whole cell lysate from treated cell cultures are shown in figures 4.3 to 4.5. From the 400 spots, 123 spots were identified with both MALDI-TOF and LC-MS/MS combined (Appendix A and B). Twenty one proteins were differentially expressed in the malathion treated samples (p<0.05%) and the fold change relative to control is given in Table 4.1. Figure 4.6 shows the sections of gel for the differentially expressed proteins UCH L1, GST and for the protein rho GDI that was unchanged after treatment along with the histograms. Figure 4.7 gives the gel sections and the histogram for calmodulin that was down-regulated by about 2 fold when treated with 100 μM malathion for 48 hours. Sixteen proteins were down-regulated and 5 proteins were up-regulated in the malathion-treated SY5Y cultures. These differentially expressed proteins fall under different classes of proteins namely chaperone proteins, calcium binding proteins, cytoskeletal proteins and proteins involved in degradation pathways. A representative MS/MS spectrum along with the Mascot search results for protein UCH L1 is shown in figure 4.8. Figure 4.9 gives the MALDI-TOF mass spectrum for protein GRP 75.
Figure 4.2 A representative 2D gel image of 400 μg of control SY5Y samples (no Malathion) resolved first on a pH 3 to 10 IPG strip, separated on 8-16% gradient polyacrylamide gel and annotated with spot numbers. The protein spots were visualized by staining with in-lab synthesized fluorescent ruthenium complex. The numbers on the annotated gel indicate proteins that were identified by mass spectrometry and correspond to numbers assigned in Tables 1 and 2.
Figure 4.3 A representative 2D gel image of 400 μg of whole cell lysate from SY5Y cell cultures treated with 50 μM malathion for 16 hours and resolved first on a pH 3 to 10 IPG strip and separated on 8-16% gradient polyacrylamide gel. The spot numbers indicated in the gel are landmark proteins, the protein names of which are given in appendix A and B.
Figure 4.4 A representative 2D gel image of 400 μg of whole cell lysate from SY5Y cell cultures treated with 100 μM malathion for 24 hours and resolved first on a pH 3 to 10 IPG strip and separated on 8-16% gradient polyacrylamide gel. The spot numbers indicated in the gel are landmark proteins, the protein names of which are given in appendix A and B.
Figure 4.5 A representative 2D gel image of 400 µg of whole cell lysate from SY5Y cell cultures treated with 100 µM malathion for 48 hours and resolved first on a pH 3 to 10 IPG strip and separated on 8-16% gradient polyacrylamide gel. The spot numbers indicated in the gel are landmark proteins, the protein names of which are given in appendix A and B.
Table 4.1 List of proteins altered due to treatment with malathion. Seq.Cov. % denotes the percentage of protein sequence covered by the trypsin digested peptides

<table>
<thead>
<tr>
<th>Spot #</th>
<th>Protein</th>
<th>Swiss Prot ID</th>
<th>Theoretical Mr (kDa)</th>
<th>Theoretical pI</th>
<th>Seq. Cov. %</th>
<th>Mowse score</th>
<th>Peptides matched</th>
<th>Fold Changes</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Calmodulin</td>
<td>P62158</td>
<td>16.7</td>
<td>4.09</td>
<td>18</td>
<td>67</td>
<td>KMK, EAFR, DGDGTITTK DGNGYISAELR MADQLTEEQIAEFK EAFSLFDKGDGDTITTK, LTDESLSMiSSFCPR</td>
<td>0.92 0.84 0.49</td>
</tr>
<tr>
<td>6</td>
<td>Enhancer of rudimentary homolog</td>
<td>P84090</td>
<td>12.2</td>
<td>5.62</td>
<td>23</td>
<td>62</td>
<td>RQAQQAGK EKIYVLLR, MYEEHLKR</td>
<td>1.2 0.74 0.30</td>
</tr>
<tr>
<td>9</td>
<td>Calgizzarin</td>
<td>P31949</td>
<td>11.7</td>
<td>6.56</td>
<td>40</td>
<td>102</td>
<td>ISSPTETER TEFI$LSMINTELAATK NQKDGPVLD,DPGVLD, AVPSQKR</td>
<td>0.61 1.1 0.33</td>
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<tr>
<td>10</td>
<td>Ubiquitin</td>
<td>P62988</td>
<td>0.85</td>
<td>6.56</td>
<td>7</td>
<td>84</td>
<td>TLSDYNIQK TITLVEPSDTIENVK TITLVEPSDTIENVKAK</td>
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<td>12</td>
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<td>P09382</td>
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<td>5.34</td>
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<td>Spot #</td>
<td>Protein</td>
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<td>Theoretical Mr (kDa)</td>
<td>Theoretical pI</td>
<td>Seq. Cov.</td>
<td>Mowse score</td>
<td>Peptides matched</td>
<td>Fold Changes</td>
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<td>74</td>
<td>QAEILQESR LEAAYLDLQR KQAELQESR RLEAAYLDLQR</td>
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<td>7.82</td>
<td>10</td>
<td>184</td>
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<td>0.8 1.0 0.58</td>
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<td>Q04760</td>
<td>20.5</td>
<td>5.25</td>
<td>27</td>
<td>104</td>
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<td>25.7</td>
<td>7.89</td>
<td>20</td>
<td>71</td>
<td>MLLADQGQSWK PPHYTVIVYFPVR AFLASPEYNLPLINGNGKQ ALPGQLKLSSLQFQGGK</td>
<td>1.1 0.90 1.7</td>
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<td>Spot #</td>
<td>Protein</td>
<td>Swiss Prot ID</td>
<td>Theoretical Mr (kDa)</td>
<td>Theoretical pI</td>
<td>Seq. Cov. %</td>
<td>Mowse score</td>
<td>Peptides matched</td>
<td>Fold Changes</td>
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<td>94</td>
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<td>5.45</td>
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<td>AVTIFIR, NLIRDNR FSELTAEK, VGGRLEDTK MIIEEA,IADGYEQAAR QQISLATQMVR MLVIEQCKNRSR IDDIRKPGSESEE DVFELIKVEGK KQOQLATQMVR EKFEEMIQQIK</td>
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<td>Glucose regulated protein</td>
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<td>70.4</td>
<td>5.01</td>
<td>23</td>
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<td>Seq Cov %</td>
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Figure 4.6 Close-up of the gel sections showing differential expression of protein UCH L1(spot # 52), rho GDI (spot # 40) and GST (spot # 42) (A) and protein calmodulin (spot #1) (B) between control (CTRL), 16 hour (MAL 16), 24 hour (MAL 24) and 48 hour (MAL 48) malathion treated SY5Y cells. The protein spots are numbered corresponding to appendix A and B. The difference in expression is shown in the histogram at the bottom of the gel picture. The expression profile is presented as bar chart with error bars. Each bar represents intensity ± S.D. of blots from quadruplet runs for control (CTRL) and 24 hour malathion (MAL 24) treated samples while duplicate for 16 hour (MAL 16) and 48 hour malathion treatment (MAL 48).
**Figure 4.7** Close-up of the gel sections showing differential expression of protein calmodulin (spot #1) between control (CTRL), 16 hour (MAL 16), 24 hour (MAL 24) and 48 hour (MAL 48) malathion treated SY5Y cells. The protein spots are numbered corresponding to appendix A and B. The difference in expression is shown in the histogram at the bottom of the gel picture. The expression profile is presented as bar chart with error bars. Each bar represents intensity ± S.D. of blots from quadruplet runs for control (CTRL) and 24 hour malathion (MAL 24) treated samples while duplicate for 16 hour (MAL 16) and 48 hour malathion treatment (MAL 48).
Figure 4.8 Representative example of MS/MS spectrum for the peptide fragment MQLKPMEINPEMLNK along with the database search result for protein UCH L1
Figure 4.9 Representative MALDI-TOF spectrum for the for protein GRP 75 along with the matched peptides shown in bold black

Sequence Coverage: 30% Matched peptides shown in Bold Black

1 MISASRAAAA RLVGAASRG PTAARHQDSW NGLSHEAFR VL51 IKGAVVGIDL GTTNSCVAVM EGKOAKVLEN AEGARTTPSV VAFTADGERL 101 VGMPAKRQAV TNPNTFYAT KRLIGRRYDD PEVQKDKNV PFKVRASNG 151 DAWVEAHGKL YSPSQIGAFV LMKMKETAEN YLGHYAVN FLTPAYFNDS 201 QROATDKAGG ISGLNLVRVI NEPTAALAY GLDSDKSVI AYVDLGSSLGF 251 DISILEIQKG VFEV KSTDQ TFGLGGEDFDQ ALLRRHIVKEF KRETGVDTLK 301 DNMAALQVRE AAEKACELS SSVQTOINLP YLTMDSGPK HLNMKLTR 351 FEGIVTDLIR RTIAPCOKAM QDAEVKSDI GEILVGGMT RMPKVQQTVQ 401 DLGFGRSPSKA VNPDEAVALG AIQGGVLAG DVTDVLLLQV TPLSGLGETL 451 GGVFTKLINR NTTIPTK SQ VFSTAADGQT QVEIKVCQG REQADGMK 501 GOFTLIGG PAPRGVPO 551 SDAARKSTGQ REQVIIQSS 601 GGLSKDLD S 651 AYKKMASERE GSGGSGTGEQ KEDQKEEKGQ

MALDI-TOF mass spectrum of GRP 75
4.3d Validation of the 2-DE results by Western Blotting

To confirm the fidelity of results obtained by 2-DE, western blot analysis was carried out for two proteins: one protein that was significantly down-regulated namely UCH L1 and one protein rho GDI that was unchanged due to treatment with malathion. The western blot data correlate with the 2-DE result for the analyzed proteins (Figure 4.10).

![Western blot images]

**Figure 4.10** Western blot analysis of protein UCH L1 that was upregulated in malathion treated samples and rhoGDI that was changed due to treatment. The difference in expression is shown in the histogram. The expression profile is presented as grouped bar chart with error bars. Each bar represents relative intensity (% of control) ± S.D. of blots from duplicate runs.
Western blot bands were normalized to GAPDH bands and quantified in comparison to control cells. Data are given as percentages of the control value (mean ± SD; n = 2 experiments; p<0.05). Exposure to 100 μM malathion for 24 hours results in a significant decrease in UCH L 1 however, no significant change is seen in rho GDI protein.

4.4 Discussion

Large quantities of organophosphates are being used as insecticides to control pests in both agricultural and domestic areas. The widespread use of malathion has created awareness of related adverse effects on particularly non-target organisms. Most of the reports studying toxicity of malathion were focused on secondary effects after exposure and symptomology in particular. On the other hand, specific mechanisms involved in malathion-induced *in vitro* toxicity have not been extensively described. The study is the first proteomic investigation of the alteration in protein levels after treatment with malathion using SY5Y cells as model system. These studies enable us to better understand the underlying mechanisms by which malathion can potentially induce toxicity.

4.4a Cell viability

Cell viability measurements based on MTT assay assess the mitochondrial activity of healthy cells in a sample [95]. Treatment of SY5Y cells with 100 μM malathion for 24 hours lead to about 25% increase in the amount of MTT reduction compared to control cell cultures (not treated with malathion) (Figure 4.1). MTT is reduced to formazan by mitochondrial enzymes present exclusively in viable cells. Increased MTT reduction seen when cultures were treated with 25 to 250 μM malathion denotes enhancement of mitochondrial function in these cells. However, it does not provide any information on the actual number of viable cells. In other words there could actually be fewer viable cells present in the culture after treatment with malathion which show increased mitochondrial activity. Treatment with concentrations greater that 1000 μM malathion caused significant decrease in mitochondrial activity relative to control.
For our proteomic investigation, the SY5Y cell cultures may be treated with concentrations of malathion ranging from 25 to 250 μM (Figure 4.1). It was however, decided to treat the SY5Y cells with 100 μM malathion for two time periods namely: 24 hours and 48 hours and 50 μM malathion for 16 hours. This was done to enable comparison of malathion toxic effects with another study that involving another organophosphate insecticide, namely chlorpyrifos, on the SY5Y proteome. Chlorpyrifos was also being studied in the lab and it was important that similar responses to the cell viability assay for both chlorpyrifos and malathion were to be maintained to compare their toxic effects.

4.4b Alteration in levels of proteins after treatment with malathion

Investigation of the SY5Y proteome after treatment with malathion showed significant change in the levels of specific proteins when comparing results for control SY5Y samples (no treatment) and malathion-treated samples. SY5Y cultures treated with 50 μM malathion for 16 hours showed slight alteration in protein levels; these alterations became more conspicuous in cultures treated with 100 μM malathion for a period of 24 and 48 hours (Table 4.1). The PD Quest software used for detection of the protein spots was also used to estimate the protein spot volume that is directly proportional to the level of protein in the sample. Normalization of the spot volume to the total density in the gel image was carried out to avoid any non-expression related changes. A difference in spot volume (and hence the protein level) was considered statistically significant at p<0.05 and confidence level of 95%.

The proteins whose levels were altered significantly after treatment with malathion fall under five major classes of proteins: calcium binding proteins, chaperones, protein involved in degradation pathways, cytoskeletal proteins and other miscellaneous proteins. Each class of proteins is individually discussed in the following section.
• **Calcium binding proteins**

Calmodulin (spot #1) and calgizzarin (spot #9) are two calcium (Ca$^{2+}$) modulators whose levels were down-regulated by two and three fold respectively in SY5Y cell cultures after treatment with 100 μM malathion for a period of 48 hours. There exists numerous cellular processes within the eukaryotic cells that are Ca$^{2+}$ dependent and/or Ca$^{2+}$ regulated as reviewed by Clapham [98]. Calmodulin [99] is an ubiquitous protein that can bind up to 4 Ca$^{2+}$ ions and regulate various signaling pathways within the cell. Some of CaM targets include: cyclic nucleotide metabolism, phosphorylation pathways, dephosphorylation and calcium transport [100]. It has been reported that oxidative modification of CaM results in degradation of the protein by the proteosome and subsequently a decrease in the levels of functional CaM in rats [101]. Since CaM is the major Ca$^{2+}$ binding protein, reduction in calmodulin affects calcium levels within cells. Calgizzarin is another Ca$^{2+}$ binding protein that regulates various cellular events including protein phosphorylation, enzyme activation, interaction with cytoskeletal proteins and calcium homeostasis [102].

A balance in cytosolic calcium concentration is maintained not only by calcium binding proteins but also by various other intracellular calcium buffers like mitochondria [103-105]. But both calmodulin and calgizzarin that are primarily responsible for maintaining calcium homeostasis within the cell were significantly down-regulated in cell cultures treated with malathion compared to control cultures. The down-regulation of calmodulin and calgizzarin in malathion-treated SY5Y cell cultures may result in an imbalance in calcium concentration within the cell affecting various signaling pathways.

• **Chaperones**

Chaperones are proteins that assist in the proper folding of other proteins by preventing misfolding, unfolding misfolded proteins and maintaining unfolded conformations by providing molecular and physical environments for correct folding. Many chaperones are heat shock proteins (see more below) that are highly expressed during cellular stress to
facilitate proper protein folding [106]. Some chaperones act to repair the potential
damage caused by misfolding while others are involved in folding newly synthesized
proteins as they are extruded from the ribosome. Other types of chaperones are involved
in transport across membranes, for example in the mitochondria and endoplasmic
reticulum [107, 108]. Chaperone proteins that are involved in the proper folding of
nascent proteins into their native functional state are also called chaperonins.

a. Heat shock proteins

Heat shock proteins (Hsp) are present during normal conditions whose expression is
greatly induced during stress [109, 110]. A common aspect of stress is generation of
misfolded proteins; when the stress is intense heat shock proteins are highly expressed to
cope with the increase in misfolded proteins. For this reason Hsps are also referred to as
stress proteins. Hsps are also considered to play a general role in protection against
 cellular injury [111-113]. Hsps are highly conserved across species and since they play
vital role in protein translocation, folding and assembly are also known as molecular
chaperones [114, 115]. The Hsps are named according to their molecular weight and are
classified as small Hsps (between 10 – 30kDa) and large Hsps (greater than 30 kDa). A
number of heat shock proteins were identified in the whole cell lysate from SY5Y cells
(Appendix A and B and Table 4.1). The two heat shock proteins whose levels were
significantly altered after treatment with malathion are Hsp 27 and Hsp 60.

Hsp 27 belongs to a class of small heat shock proteins (sHsps) that can protect cells from
oxidative stress [116, 117]. sHsps act as molecular chaperones preventing unfolded
proteins from irreversible aggregation [118-120] and along with other factors such as
Hsp70 and adenosine tri-phosphate (ATP), they facilitate productive refolding of
unfolded proteins [121, 122]. Four protein spots #43, #48, #49 and #56 in figure 4.2 were
identified as Hsp 27 by mass spectrometry and the most acidic form [9] was down-
regulated significantly in SY5Y cells treated with malathion, yet without any significant
change in the total Hsp levels between control and treated samples. It is known that Hsp
27 has three phosphorylation sites (Ser15, Ser78 and Ser82) that can be potentially
phosphorylated by protein kinases [123, 124] and phosphorylation decreases the pI of a protein. The protein spots identified, as Hsp 27 may be the different phosphorylated forms. Both Lavoie et al. and Loktionova et al. report that phosphorylation of Hsp 27 changes the quaternary structure of Hsp 27 resulting in the formation of dimers and tetramers of the protein, which in turn is responsible for stabilization of actin microfilaments by acting as actin capping protein [125, 126]. Down-regulation of the most acidic isoform of Hsp 27 (phosphorylated) in malathion-treated cells suggests disruption of actin polymerization.

Hsp 60 is an essential mitochondrial protein that promotes the folding of several proteins imported into the mitochondrial matrix [127, 128]. Hsp 60 also refolds and prevents aggregation of denatured proteins [129]. Three protein spots #108, #112 and #113 were identified as Hsp 60 (Figure 4.2). Fratelli et al. report three isoforms of Hsp 60 in oxidatively stressed human T lymphocytes, a result of glutathionylation (post-translational modification) of the protein [130]. Our results indicated that the most acidic isoform was identified to be down-regulated in the malathion treated SY5Y cells. Taking into account that the main role of Hsp 60 is in the proper folding of proteins within the mitochondria, the down-regulation of the acidic form in the malathion treated SY5Y cell cultures could result in an accumulation of misfolded proteins within the mitochondria.

To reiterate, the acidic isoforms of both stress proteins Hsp 27 and Hsp 60 were significantly down-regulated in malathion-treated SY5Y cell cultures. The down-regulation of these proteins could also contribute to an increased susceptibility of the cells to oxidative stress since these chaperones are thought to attenuate the proteolysis of misfolded proteins (formed as a result of oxidative stress) by binding to them and assisting in their repair. Further characterization of the function of each protein isoform may reveal in-depth information about the relevance of these findings.
b. **Chaperonins**

A number of other chaperones were identified as significantly altered after treatment with malathion in SY5Y cells. These included tubulin-specific chaperone A, cyclophilin A, prohibitin, T complex 1 protein, glucose-regulated protein 78 (GRP 78) and glucose-regulated protein 75 (GRP 75). Tubulin specific chaperone A protein is essential for the formation of functional tubulin heteromers and binds to tubulin folding intermediate formed by the synthesis of tubulin polypeptide with chaperonin T complex protein [131]. T complex protein (TCP) in addition to its role in formation of tubulin, also aids in the folding of other proteins [132, 133]. It is thought to act in concert with various protein degradation and modification pathways and maintain cellular homeostasis. TCP was up-regulated 2.5 fold in the SY5Y cells treated with malathion. The up-regulation of TCP is an indication that SY5Y cells treated with malathion are stressed.

Spots #20, #21, #22 and #23 in figure 4.2 were all identified as peptidyl prolyl cis-trans isomerase (cyclophilin A). From the amino acid composition of the protein, the isoelectric point is expected to be 7.8; however, based on the computed course of the pH gradient and the positions of other identified proteins in the gel, the actual pI spans a range from 7 through 8. Three isoforms of cyclophilin A have been reported earlier with possibility of phosphorylation as PTM [134]. In our study, four different isoforms were identified. There was no significant change in total protein level, however, spot #22 was significantly down-regulated in malathion-treated samples. Cyclophilin A catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides and accelerates protein folding [135]. Cyclophilin A has been implicated in diverse functions such as the heat-shock response [136], in the nuclear import of some proteins [137] and in various signal transduction pathways [138], but its exact function is not fully understood. It has also been demonstrated that decreasing the level of cyclophilin A in neuronal B50 cells inhibits the activation of caspases that are involved in triggering apoptosis [139]. There is however, no information in the literature on the specific role of these different isoforms of the protein, but the significant change in the levels of one particular isoform (acidic isoform) after treatment with malathion is definitely worth exploring. It is possible that
malathion is triggering apoptosis in the SY5Y cells, but decreased cyclophilin A isoform (may be the functional isoform) renders the cells resistant to apoptosis.

Spot #67 was identified as prohibitin, a mitochondrial protein. Prohibitin is a membrane chaperonin that stabilizes mitochondrial proteins [140]. Prohibitin also plays a pivotal role in cell cycle regulation, aging, apoptosis and mitochondrial function [141, 142]. Coates et al. showed that prohibitin was up-regulated during metabolic stress due to imbalance in the synthesis of mitochondrial proteins [143]. Prohibitin was also up-regulated in malathion-treated SY5Y cells by a factor of 1.5 fold when compared to control cultures which could very well be due to malathion-induced mitochondrial stress.

Two glucose regulated proteins GRP 78 (spot #109) and GRP 75 (spot #110) were identified as significantly altered in SY5Y cells after treatment with malathion. GRP 78 has been implicated in unfolded protein response (UPR) during which it is up-regulated to correct the misfolded proteins [144]. GRP 78 transiently binds to a wide repertoire of proteins traversing the endoplasmic reticulum (ER) and facilitates their correct folding, glycosylation, assembly and turnover [145-147]. It is reported that GRP 78 induction protects cells from undergoing apoptosis and maintain cell viability under ER stress [148, 149]. Induction of the corresponding *grp78* gene can occur in response to different stimuli including glucose starvation, hypoxia and ER Ca$^{2+}$ pool depletion [150]. Interestingly, GRP 78 was also up-regulated in SY5Y cells treated with malathion. GRP 75 on the other hand was down-regulated significantly in malathion-treated SY5Y cells. GRP 75 is involved in the transport of proteins and their assembly inside the mitochondria [151]. Furthermore, GRP 75 may influence the pattern of proteins synthesized in the mitochondria, facilitate the assembly of mitochondrial proteins and then protect the proteins from proteolytic degradation [152, 153]. The alterations in levels of these ER and mitochondria specific glucose regulated proteins indicate malathion induced stress to these organelles in SY5Y cells.
• **Proteins involved in ubiquitin proteosome pathway**

Studies indicate that most intracellular protein degradation takes place *via* the ubiquitin proteosome pathway (UPP) [154]. The UPP maintains cellular homeostasis by removing misfolded dysfunctional proteins. Ubiquitin carboxy terminal hydrolase L1 (UCH L1) (spot # 52 and # 54) is an essential enzyme in the UPP that is specifically involved in the recycling of ubiquitin by releasing it from the polyubiquitin tail [154]. Choi *et al.* report the presence of three different isoforms of UCH L1 in the brain of Alzheimer’s disease (AD) patients [155]. It should be noted here that we have identified two isoforms that have similar molecular weight but different isoelectric points (pIs). The more acidic isoform was down-regulated about 2 fold when SY5Y cell cultures were treated with 100 μM malathion for a period of 48 hours. In addition to the hydrolase activity, it has been suggested that UCH L1 is also a regulator of apoptosis [156].

The UPP involves ubiquitination of damaged or misfolded proteins that is to signal the degradation of proteins; however, damaged proteins are not removed when ubiquitin is reduced or mutated [157]. There is evidence that UCH L1 associates with ubiquitin during the UPP [156, 157]. It has been reported that UCH L1 deletion in mice cause gracile axonal dystrophy (*gad*), a recessive neurodegenerative disease [158]. In our study both UCH L1 (an acidic isoform) and ubiquitin were identified to be down-regulated in malathion-treated SY5Y cell cultures. We know that under stress most proteins can become misfolded and non-functional. Down-regulation of these two critical proteins involved in the removal of dysfunctional proteins in the malathion treated SY5Y cells could render the cells more susceptible to the accumulation of toxic misfolded proteins. Further study is required to identify the PTM and elucidate the functional significance of the acidic isoform of UCH L1.

• **Cytoskeletal proteins**

Seven proteins spots (# 76, #77, # 78, #88, #89, #90, and #122 in figure 4.2) were identified as vimentin, the intermediate filament protein. The various isoforms had
different molecular weights and pIs. The isoforms differed in molecular weight may be result of fragmentation of the parent protein itself, while the isoforms that differed in pI resulted most likely from PTMs. Studies have reported that vimentin is essential for axonal initiation in differentiating neuroblastoma cells [159]. It is a major contributor to the mechanical strength of a cell. Significant down-regulation of one isoform of vimentin was observed in SY5Y cells exposed to malathion along with a down-regulation of total vimentin levels when compared to control cultures. Spot # 72 was identified as F actin capping protein; this is a cytoskeletal-associated protein that was significantly up-regulated by 5 fold in malathion-treated cultures. F actin capping protein is involved in capping the barbed ends of the actin filaments during polymerization and thus prevents the addition of actin units to the end [160]. The alterations in the levels of these structural proteins in malathion-treated SY5Y cells indicate disruption of cytoskeletal architecture.

- **Miscellaneous proteins**

Two isoforms of galectin-1 (spot # 12 and 13) were identified and both were down-regulated in SY5Y cell cultures treated with malathion. Galectin-1, a β-galactoside-binding lectin has been suggested to play a role in cell adhesion, proliferation and apoptosis [161]. Galectin-1 also acts as a negative growth regulator in neuroblastoma cells, and, in fact, galectin-1 is a probable effector in the sialidase-dependent growth control of neuroblastoma cells [162]. DJ-1 protein (spot # 44), a multifunctional protein involved in transcription and oxidative stress, is down-regulated after treatment with malathion. Deletion of the DJ-1 gene is associated with Parkinson’s disease which is thought to be due to the loss of the DJ-1 protein [163]. It has also been suggested that the loss of DJ-1 protein in neurons makes the cells more susceptible to oxidative stress and ER stress [164]. In the malathion-treated SY5Y cell cultures that show evidence for stress, down-regulation of DJ-1 protein can make these cells more vulnerable to ER stress.

Triose phosphate isomerase (spot # 62) catalyzes the isomerization of dihydroxyacetone phosphate to D-glyceraldehyde 3-phosphate [165]. The isomerization involves the
formation of the cytotoxic intermediate methylglyoxal, which is eliminated by glyoxalase 1 (spot #35) preventing the formation of advanced glycation end products [166]. Down-regulation of both these proteins in SY5Y cell cultures treated with malathion is intriguing but further investigation needs to be done to elucidate the significance.

Glutathione S transferase A4 (spot # 42) is a phase 2 detoxifying enzyme that conjugates glutathione to a number of toxic electrophiles [167]. Lechner et al. report that GST activity in rat liver homogenate was increased two fold after treatment with malathion [168]. The increased levels of this enzyme in malathion-treated SY5Y cells suggest that it may carry out a protective measure against the insult. It is clear that malathion has the potential to disrupt cellular antioxidant defenses in SY5Y cell cultures. Spot # 6 was identified as enhancer of rudimentary homolog and the protein is a transcriptional co-regulator that is highly conserved from plants to humans [169]. Little is known about its function, but its down-regulation observed in SY5Y cells treated with malathion can have consequences in the transcription machinery.

The alteration in protein levels in SY5Y cell cultures after treatment with malathion is an important finding in regard to the OP toxicity, since there has been limited literature in this area. The level of any protein in a cell at any given time is controlled by the rate of transcription of the gene, efficiency of translation of the mRNA into the protein and the rate of degradation of the protein in the cell. 2-DE offers a powerful way of taking a snapshot of the state cells are, at a given time. However it should be noted that whether the alteration in protein levels studied by 2-DE is due to imbalance in proteolysis and protein synthesis warrants further investigation. Based on the altered levels of major proteins identified in our study, we propose the following model for the effect of malathion (Figure 4.11).

Reports have indicated that organophosphates in addition to inhibiting AChE, can directly bind to muscarinic receptors (which are receptors for AChE) present in cells [170, 171]. Similarly, malathion can bind to muscarinic receptors present in the SY5Y cells. Binding of malathion to the receptor stimulates the phosphotidyl inositol pathway and activates the enzyme phospholipase C. Phospholipase C hydrolyzes phosphoinositide
bis-phosphate to inositol tri-phosphate (IP_3) and diacylglycerol (DAG), IP_3 acts as a second messenger and stimulates the release of calcium into the cytoplasm from intracellular stores like the ER. Increased cytoplasmic calcium has been reported as a definite event in the mechanism of many OP induced neuronal disorders [172, 173]. Elevation in intracellular calcium was also reported during an early phase of malathion induced apoptosis in the grass carp cell line ZC 7901 [174]. The increase in cytoplasmic calcium levels might be due to decreased activity of calcium expelling systems. Two calcium modulators: calmodulin and calgizzarin were significantly down-regulated in the malathion treated SY5Y cells. Ca^{2+} ATPase is another important calcium effluxing enzyme that extrudes calcium in order to maintain calcium homeostasis. Sharma et al. report the decrease in Ca^{2+} ATPase activity in hens after exposure to OP [175]. Though there is no direct indication of reduced Ca^{2+} ATPase in the malathion-treated SY5Y cells, the various other observations point out that malathion might increase cytoplasmic calcium levels. Since calcium is a known second messenger, increased levels of calcium within the malathion-treated SY5Y cells could lead to a cascade of intracellular events including impairment of mitochondrial oxidative phosphorylation and generation of free radicals [176].

Mitochondria start to accumulate calcium when the cytoplasmic calcium concentration is over a threshold value and this occurs via a uniporter that is driven by the mitochondrial membrane potential [177, 178]. Hansford [179] and McCormack [180] found three Ca^{2+}-stimulated mitochondrial dehydrogenases: pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and isocitrate dehydrogenase coupled to the electron-transport system. Activation of these dehydrogenases results in stimulation of both electron transport and ADP phosphorylation. The greater MTT reduction (mainly brought about by mitochondrial dehydrogenases) that was observed in the malathion treated SY5Y cell cultures can be explained by the stimulation of the above mentioned mitochondrial dehydrogenases by increased calcium concentration within the mitochondria. The uptake of calcium also activates mitochondrial phospholipases (like phospholipase A that catalyze the formation of arachidonic acid; reactive oxygen species generator) and seems
to trigger increased production of reactive oxygen species (ROS) [181-183] as well as release of mitochondrial proteins, some of which are proapoptogenic [184].

Cadenas et al. report that in normal cells up to 5% of the oxygen reduced by mitochondria is converted by complex I of the respiratory chain to superoxide [185]. While ROS are proposed to play important roles in coordinating and regulating a number of cellular signaling pathways (redox signaling), oxidative stress results when the formation of ROS exceeds the capacity of antioxidant defense systems [186]. Increased mitochondrial activity coupled to imbalance in Ca $^{2+}$ ion concentration within the mitochondria results in increased production of ROS. Oxidative stress has already been implicated in malathion-induced cytotoxicity [80]. The production of ROS damages the cell structures including the DNA, proteins and lipids [187].

Free radicals attack cell and organelle membranes, decrease the membrane fluidity by modifying lipids via a process termed lipid peroxidation and significantly alter membrane properties and possibly even disrupt the function of membrane-associated proteins [188]. Free radical oxidation causes proteins unfold/misfold [189-191]. Grune et al. demonstrated that oxidative modifications of proteins significantly accelerate protein aggregation and crosslinking [192]. To maintain proteins in a properly folded state, cells utilize a variety of chaperones that facilitate refolding, and if a protein is unable to be refolded it is degraded, often via the ubiquitin-proteasome pathway (UPP). However, in the malathion treated SY5Y cells, the chaperone proteins namely heat shock proteins, TCP, cyclophilin A and other proteins involved in UPP like ubiquitin and UCH L1 were significantly down-regulated. This means that in the SY5Y cells exposed to malathion, the damaged proteins may not be efficiently removed/degraded leading to their toxic accumulation.
Malathion binds to muscarinic acid receptor and stimulates the phospholipase pathway resulting in production of inositol tri phosphate (IP$_3$). IP$_3$ binds to its receptors present in endoplasmic reticulum (ER) and causes the release of Ca$^{2+}$ from the ER. Down-regulation of calcium modulators like calmodulin, calgizarrin and Ca-ATPase causes increase in cytosolic Ca$^{2+}$ and results in calcium influx into the mitochondria. Imbalance in Ca$^{2+}$ inside the mitochondria causes stimulation of various lipases, proteases and other enzymes. Activation of phospholipase A2 causes increased generation of reactive oxygen species (ROS). The electron transport chain (ETC) is also activated and this adds to the ROS produced. The increased ROS and down-regulation of antioxidant systems result in oxidative stress. The free radicals generated cause a number of undesirable effects that includes damage to the membrane and protein misfolding. The hydroxyl radical generated can cause ER stress that is seen in the up-regulation of GRP 78 protein. Both ER and mitochondria are stressed due to treatment with malathion that is seen by the up-regulation of the stress specific proteins. Down-regulation of UCH L1, ubiquitin and other chaperone proteins that are involved in the removal of misfolded proteins indicates potential accumulation within the cells that can lead to malathion-induced cytotoxicity.
Since malathion is thought to cause oxidative stress, one would predict that in response the levels of Hsps would be high in SY5Y cells exposed to malathion. Yet, 2-DE data find low levels of these proteins, thus the paradoxically low levels of Hsps accentuate the oxidative damage incurred by exposure to malathion leaving the cells vulnerable and more prone to such damage.

Another organelle that is susceptible to ROS damage is the ER. The free radicals generated within the mitochondria are converted to H₂O₂ by the enzyme superoxide dismutase [193]. The H₂O₂ so formed can react with intracellular Fe²⁺ ions possibly by Fenton reaction and result in the formation of hydroxyl radicals that are highly undesirable for ER and induce ER stress [194]. In addition to this oxidative stress due to ROS, ER is also susceptible to protein misfolding. Proteins unable to fold correctly cause ER stress and activate the unfolded protein response (UPR). As stated earlier, GRP 78 is a protein that is up-regulated when the ER is under stress due to misfolded proteins [148, 149]. In the SY5Y cells treated with malathion too the GRP 78 was significantly up-regulated indicating that malathion is capable of causing ER stress in these neuroblastoma cells. While increase in GRP 78 indicates ER stress, a decrease in DJ-1 protein levels also points out at an ER stressed environment. DJ-1 protein was down-regulated significantly when the SY5Y cells were treated with malathion.

In addition to ER, mitochondria being one of the major sites of ROS production in a cell are also highly sensitive to impairment in ROS levels. Prohibitin, a mitochondrial specific stress protein was significantly up-regulated in SY5Y cells treated with malathion. This indicates malathion-induced stress to the organelle. Chen et al. reported that malathion induced cytotoxicity in grass carp cells by directing damaging mitochondria due to intracellular Ca²⁺ elevation, generation of reactive oxygen species (ROS) and ATP depletion [174]. Taken together, our results suggest that malathion induces oxidative stress leading to possible misfolding of protein that can potentially accumulate and cause cytotoxicity in neuroblastoma cell cultures.
The 2-DE technique employed is a powerful tool to investigate differential protein expression in a complex system because it has the ability to resolve a complex mixture of proteins like that of the whole cell lysate. However, it should be realized that 2-DE can only provide a snapshot of the most abundant proteins that are expressed at a given time point. Although these results allow a glimpse of malathion toxicity at the proteome level, it is hard to know if such alteration is a cause or a consequence of the treatment. Future work should be focused on the sub-proteome of mitochondria and ER from SY5Y cells after treatment with malathion. This will provide insight into the biochemical pathways that are associated with stress caused by malathion.

Although neuroblastoma cells are the preferred model to test the in vitro toxicity caused by organophosphates, it should be realized that they are cancerous in. More precise information about the implication to human exposure can be deduced by employing primary cell lines (non-cancerous) or from animal studies. Overall, the current study provides a foundation for studies on cell culture, pesticide exposure and altered levels of proteins.

4.5 Conclusion

The hypothesis tested is exposure of SY5Y cells to malathion causes alteration in protein levels. The current study identified a number of stress response proteins, including calcium binding proteins, proteins involved in degradation pathways, cytoskeletal and tumor suppressor proteins to be significantly altered following treatment with malathion in the SY5Y cell cultures. The identification of differential protein expression in SY5Y cells after treatment with malathion is an important finding in regard to the OP toxicity, since there has been limited knowledge in this area.

In conclusion, the data obtained in this study represent the first proteome profiling of SY5Y cells response to malathion. It was observed that there was significant alteration in levels of proteins when the cells were treated with 100 μM malathion for a period of 24 hours that was exaggerated when the exposure was prolonged for 48 hours. This means
that the changes seen in protein levels could be an early indication of impending cellular damage. Cumulative evidence from previous experiments along with our data shows that oxidative stress is an important trigger for malathion toxicity. In our study, 122 proteins were identified from the SY5Y whole cell lysate out of which 21 proteins were significantly altered after treatment with malathion. Sixteen proteins were down-regulated while 5 proteins were up-regulated due to treatment with malathion. Our results suggest that exposure to malathion causes perturbation in calcium homeostasis within the mitochondria that leads to generation of free radicals, accumulation of misfolded proteins and cause stress to mitochondria and ER in the SY5Y cell cultures. With increased awareness in pesticide related adverse effects, this oxidative stress induced by malathion in human neuroblastoma cells becomes especially critical when it comes to non-target exposure of elderly individuals to malathion. Such individuals, who are already susceptible to the damaging effects of oxidative stress due to process of aging, are exposed to malathion there is all possibility that the undesirable effects be more exacerbated. However, implication of this finding has to be further investigated using either primary cell lines and/or animal models to be able to extrapolate the effect to humans as well.
CHAPTER FIVE

COMPARISON OF ALBUMIN-INTERACTOME IN VENTRICULAR CEREBROSPINAL FLUID BETWEEN ALZHEIMER’S DISEASE AND NORMAL SUBJECTS

5.1 Introduction

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder that is characterized by deterioration of cognitive functions and is the most common form of dementia [195]. AD symptoms form a continuum with normal aging-related memory loss and this makes diagnosis difficult. Currently the only way to confirm AD is during autopsy of the patient by examining the brain for the presence of senile plaques and neurofibrillary tangles that are hallmarks of AD [196]. Protein β amyloid 1-42 (Aβ42) is the main protein that constitutes the senile plaques in the brain [197, 198]. Cerebrospinal fluid (CSF) from AD patients has reduced levels of Aβ42 relative to normal individuals, but there is no clear explanation for this [199-202]. Neurofibrillary tangles in the brain of AD patients are a result of hyperphosphorylation of the tau protein (9). Aβ42, total tau (T-tau) and phosphorylated tau (P-tau) have been suggested as diagnostic markers for AD in clinical practice [203]. The changes in the levels of these proteins, however, overlap with other neurodegenerative disorders and hence are not specific to AD.

The development of valid and reliable biomarkers that signify the disease condition is essential for the clinical diagnosis of AD. Biological fluids like serum, urine and cerebrospinal fluid (CSF) are being tested for AD biomarkers. CSF is most suited for the identification of biomarkers for diseases affecting especially the brain. CSF produced by the choroids plexus is a complex mixture of proteins and a difference in levels of these proteins indicate a disease condition [204]. CSF is in direct contact with the extra-cellular space of the brain and there is constant exchange of substances between the CSF and the brain. Hence, any change in the brain due to the disease pathology must be reflected in the surrounding CSF.
The CSF proteome has been characterized by several researchers, some of them elucidating the proteome [75-89] and others aimed at discovering biomarkers for neurodegenerative diseases like AD [90-99]. A major challenge involved in the analysis of CSF is the complexity of the sample. There exists enormous dynamic range in the levels of CSF proteins ranging from μg to ng. Albumin and immunoglobulins are the most abundant proteins found in the CSF constituting on average about 50% and 25%, respectively [205]. The complexity of CSF makes fractionation an essential step in the analysis of the proteome. Different methods can be used to achieve this, which include acetone precipitation, ultrafiltration, HPLC, capillary isoelectric focusing, liquid IEF and traditional 2-DE. By a combination of albumin and immunoglobulin depletion and MUDPIT analysis, 249 proteins were identified in human ventricular CSF [206]. Normally, to facilitate the analysis of low abundance proteins in biological fluids like serum and CSF, the most abundant proteins- albumin and immunoglobulins- are depleted using commercially available depletion kits or by using specific antibodies [207-209]. Albumin is a carrier and transport protein that interacts with a number of other small proteins and peptides [210-212]. Depletion of albumin could lead to the concomitant removal of low molecular weight proteins and result in the loss of information from these potentially interesting proteins and peptides [213]. However, selective isolation of this “interactome” fraction (that consists of albumin interacting proteins) will enable identification of these low molecular weight proteins that are also potential biomarkers for the AD.

The primary goal of this project was to isolate and compare the albumin-interactome in CSF between AD and normal subjects. Changes in protein levels in this fraction of the CSF proteome can provide insights into the disease pathology and help understand the progression of the disease.

A crude isolation of the albumin-interactome was performed using membrane cut-off filters. A number of proteins along with their different isoforms as a result of post-translational modifications (PTM) were observed in the albumin-containing fraction that showed significant difference in expression levels between CSF from AD and normal
subjects. To pinpoint the proteins that make up the albumin-interactome, immunoprecipitation of albumin from CSF was carried out. Our results clearly indicated the successful isolation of the albumin-interactome by immunoprecipitation, which thereby enabled the identification of proteins that are otherwise difficult to identify by direct analysis of CSF. Analysis of the 2-DE gel from the cut-off filter study showed differences in expression of specific isoforms of proteins that constituted the albumin-interactome, emphasizing the importance of elucidation of PTMs along with studies related to levels of each isoform as opposed to examining the total protein content for biomarker discovery.

5.2 Materials and Methods

5.2a Materials

Cut-off filters were purchased from Millipore (Billerica, MA). Protein G sepharose beads were purchased from GE healthcare (Princeton, NJ) and the rabbit anti-human albumin antibody was a product of Research Diagnostics Inc. (Flanders, NJ). Spin-X centrifuge tube filters with cellulose acetate membranes having a pore size of 0.45 μm were acquired from Corning Incorporated Life Sciences (Acton, MA). Dimethyl pimelimidate (DMP), sodium borate, acrylamide, bisacrylamide, ethanolamine and ammonium persulfate were purchased from Sigma (St. Louis, MO). Sodium phosphate monobasic and dibasic, phosphoric acid, Tris-HCl, methanol, formic acid and acetonitrile were obtained from Fisher Scientific (Fair Lawn, NJ). Ammonium sulfate was obtained from EMD chemicals (Gibbstown, NJ). Protein staining reagent Coomassie G250, TEMED and the low-range marker proteins were purchased from Bio-Rad (Hercules, CA). Lyophilized sequencing grade modified trypsin was purchased from Promega (Madison, WI). Fused silica capillary tubing 320 μm I.D. x 435 μm O.D. for liquid chromatography column was purchased from Polymicro Technologies (Phoenix, AZ) while the reverse phase packing material Polymer X (3 μm) was purchased from Phenomenex (Torrance, CA). All other LC accessories including zero dead volume (ZDV) unions, frits, ferrules were obtained from Upchurch Scientific (Oak Harbor, WA).
5.2b Human ventricular CSF sample

Post mortem human CSF samples were obtained from Sanders-Brown Center on Aging (University of Kentucky, Lexington, KY). CSF samples were collected during autopsy from 6 AD and 6 normal control subjects. All AD subjects met NINCDS-ADRDA workgroup criteria for the clinical diagnosis of probable AD [214]. The subjects also met the criteria for histopathologic diagnosis of AD [215]. Control CSF samples were obtained from subjects who underwent annual neuropsychological testing as a part of normal volunteer, longitudinal aging studies at Sanders-Brown Center on Aging and were without a history of dementia or any other neurological disease. Evaluation of control brains revealed only age related alterations. CSF samples were collected as described previously by Lovell et al. [216]. Immediately after collection, 2 mL aliquots of CSF samples were stored at -80°C until analysis. The pilot study with Microcon filters used ventricular CSF samples from 6 AD and 6 normal age-matched individuals. For immunoprecipitation, 200 μL of CSF each from 4 AD samples were pooled together to form the pooled AD CSF sample and similarly 200 μL of CSF from 4 normal individuals were combined to form the pooled control CSF sample. The samples were pooled to minimize the individual differences in protein expression and normalize the amount of protein among the population.

5.2c Efficacy of Microcon cut-off filters

The efficacy of cut-off filters was tested using the standard low-range marker proteins. A 30 kDa cut-off filter was pre-rinsed with distilled water to get rid of any glycerol that might be present. A solution of 200 ng of each protein in the low-range marker proteins in 150 μL of 0.2% formic acid in 50% ACN was added to the pre-rinsed 30 kDa cut off filter and centrifugation was carried out at 4000 g for 15 min. A solution of 100 μL of 0.2% formic acid in 50% ACN was added to the filter and centrifugation was carried out at 1000 g for 1 min. The wash was repeated and the flow-through from the filter was collected. A volume of 100 μL of 0.2% formic acid in 50% ACN was added to the filter and the above 30 kDa fraction was collected after centrifugation at 1000 g for 1 min. The
flow-through and the fraction above 30 kDa (retentate) from the cut-off filter were dried in a vacuum centrifuge, 25 μL of sample loading buffer was added, boiled and the sample was loaded onto a 12% polyacrylamide gel. Electrophoresis was carried out at constant voltage of 200 V until the dye front reached the bottom of the gel.

5.2d Isolation of albumin-interactome using Microcon cut-off filters

A crude isolation of albumin-interactome was performed by subjecting a volume of 400 μL of post mortem human ventricular CSF from 6 AD and 6 age matched normal subjects (control) to fractionation based upon their molecular weight using Microcon 100 kDa and 50 kDa cut-off filters. The cut-off filters were pre-rinsed with distilled water to remove glycerol. CSF was added to the 100 kDa filter that is stacked on top of a 50 kDa cut-off filter and centrifugation was carried out at 2000 g for 30 min. The flow-through from the 50 kDa and the retentate from the above 100 kDa were collected. A solution of 200 μL of 1 mM DTT in 20% acetonitrile was added to the 50 kDa membrane and after incubation for 2 minutes, the between 100 and 50 kDa fraction was collected after centrifugation at 5000 g for 5 min. The wash was repeated two more times with 200 μL and 100 μL of DTT/ACN solution respectively. The washes from the 50 kDa filter were collected and added to another fresh pre-rinsed 50 kDa cut-off filter and centrifugation was carried out at 5000 g for 5 min to remove albumin (that has a molecular weight of ~ 60 kDa will remain above the filter). The flow through from the second 50 kDa cut-off filter that contains proteins interacting with albumin, was subjected to 2-DE.

5.2e Crosslinking of anti-human albumin antibody to Protein G beads

Immunoprecipitation of albumin from CSF was carried out after crosslinking to Protein G sepharose beads using a modified protocol published by Zhou et al. [213]. Briefly 300 μL of wet protein G sepharose beads were added to a Spin-X filter and centrifugation was carried out at 1500 g for 1.5 min. Five hundred micro liter of 0.1 M phosphate saline buffer (PBS) was added to the beads and centrifugation was carried out at 1500 g for 1.5 min. The PBS wash was repeated two more times after which the beads were incubated
with 100 μL of a 10 μg/μL stock solution of rabbit anti-human albumin antibody and 400 μL of PBS for 1 hour on a rocker at room temperature. The beads were subjected to centrifugation at 1500 g for 1.5 min to get rid of any unbound antibody. The beads were washed three times each with 500 μL of 0.2 M sodium borate buffer pH 9.0. The antibodies were crosslinked to Protein G using 500 μL of 25 mM DMP in 0.2 M sodium borate buffer pH 9.0. After incubation for 30 min at room temperature on a rocker, the beads were centrifuged at 1500 g for 1.5 min to remove the un-reacted DMP. The crosslinking of antibody to the beads was carried out two more times with 500 μL aliquots of DMP each time. The crosslinking reaction was stopped by a quick rinse with 500 μL of 0.1 M ethanolamine (pH 9.0) followed by incubation in 500 μL 0.1 M ethanolamine for 30 min at room temperature on a rocker. After incubation with ethanolamine, the beads were washed twice with 500 μL of PBS to remove any residual ethanolamine. The anti-albumin antibody conjugated protein G beads were re-suspended in PBS and stored at 4°C until use.

5.2f Immunoprecipitation of albumin-interactome

A volume of 300 μL of the rabbit anti-human albumin antibody conjugated Protein G beads was incubated with 250 μL of raw CSF at room temperature on a rocker for 1.5 hours. The depleted CSF was collected by centrifugation at 1500 g for 1.5 min and the beads were washed twice with 500 μL of PBS. Various solvents were tested for optimal elution of just the bound proteins. Fifty percent acetonitrile containing 0.2% formic acid was the ideal elution buffer under the given conditions and the proteins (after immunoprecipitation) were eluted using 200 μL of this solution after incubation at room temperature for 2 min. The beads were washed twice with 150 μL of elution buffer and the flow-through from the washes was collected in one eppendorf tube. The elute was dried in a vacuum centrifuge and re-suspended in 50 μL 1 X SDS loading buffer and loaded onto a 12% polyacrylamide gel and electrophoresis was carried out.
5.2g 1D gel electrophoresis

Proteins from the efficacy study were subjected to 1D gel electrophoresis. After drying the protein sample in the vacuum centrifuge, samples were re-suspended in 50 μL 1x loading buffer, boiled for about 5 min, loaded onto a 12% polyacrylamide gel and electrophoresis was carried out at constant voltage of 150 V. The gels were stained with a colloidal suspension of Coomassie G250.

5.2h 2-DE

The flow-through from the second 50 kDa cut-off filter was subjected to 2-DE. The sample was dried in a vacuum centrifuge, re-suspended in 125 μL of sample/rehydration buffer containing 8 M urea, 2% CHAPS, 50 mM DTT, ampholytes and bromophenol blue and loaded on IPG strip. After 12 hours of active rehydration period at 50 V, the proteins were focused in the Protean IEF cell from Bio-Rad using a 4-step program (300 V, 1000 V, 2500 V for 1 hour each and 4500 V for 4 hours). A limiting current (50 μA per strip) was maintained. After IEF, the strips were equilibrated (30 min) with 6 M urea, 32 mM DTT, 0.375 M Tris-HCl pH6.8, 10%(w/v) SDS, 50% (v/v) glycerol and for 20 min in the dark with 63 mM iodoacetamide in the above equilibration buffer instead of urea. Each strip was loaded on a 12% polyacrylamide gel and electrophoresis was carried out at 150 V using a Protean II mini (Bio-Rad) in a buffer that contained 25 mM Tris, 192 mM glycine and 0.1% SDS until the blue dye reached the bottom of the gel. The 2-DE separated proteins were stained with a solution of colloidal Coomassie G 250 [56].

5.2i Image and statistical analyses

Stained gels were scanned with a Versadoc 3000 scanner from Bio-Rad. Images were analyzed using the PD Quest software from Bio-Rad. Spots were detected and matched by both automated matching following the instructions in the software and also by manual matching. Normalization was carried out to avoid any non-expression related
variations between matched spots. A difference in spot volume was considered statistically significant at p<0.05 and confidence level of 95%.

5.2 Identification of proteins using mass spectrometry

Proteins of interest were excised from the gel and in-gel proteolysis was carried out with trypsin. In brief, the gel pieces were washed 6 times alternating with 50 mM NH₄HCO₃ and 1:1 ACN: NH₄HCO₃ (50 mM) for 5 min each at 20°C and dried with ACN for 10 min at 20°C. A volume of 10 μL of trypsin (66.67 ng) in 25 mM NH₄HCO₃ was added and gel pieces allowed to swell for 30 min at 20°C and digested at 50°C for 4 hours. Peptides were extracted with 10 μL of 1% aq. formic acid at 20°C for 30 min. The tryptic peptides were analyzed using a Thermo Electron Corporation’s LCQ Classic quadrupole ion trap mass spectrometer (San Jose, CA). Briefly, the tryptic peptides were resolved on a 15 cm in-house C18 packed capillary column and eluted using a typical gradient elution within 55 min. Spectra were acquired with a probe spray voltage of 4 kV and a heated capillary temperature of 175°C. The resulting mass spectra were searched against the human sub database from the Swiss-Prot database using Mascot algorithm and the protein was identified.

5.3 Results

5.3a 2-DE of un-processed CSF

A volume of 150 μL of CSF was subjected to 2-DE. It was observed that albumin, light and heavy chains of immunoglobulins were the major proteins that were present in the gel (Figure 5.1).

5.3b Efficacy of Microcon cut-off filters

The efficacy of Microcon cut-off filters was tested using low range molecular weight marker proteins. As seen from figures 5.2 and 5.3, greater than 80% of proteins less than
30 kDa were filtered through while only roughly about 20% remained above the filter. Seventy percent of the protein sample whose molecular weight same as that of the cut-off value is filtered through. The cut-off filters fractionated the proteins efficiently.

**Figure 5.1** A volume of 150 μL of un-processed CSF was subjected to 2-DE. A volume of 125 μL of sample buffer was added to the sample that was dried in vacuum centrifuge. 2-DE was carried out using a 7 cm IPG strip pH 3-10 and a mini (7 cm) 12% polyacrylamide separating gel. The gel was stained with colloidal Coomassie G250.
Figure 5.2 Efficacy of Microcon 30 kDa cut-off filter. A solution of 200 ng of each protein in the low-range standard proteins in 150 μL of 0.2% formic acid in 50% ACN was added to the pre-rinsed 30 kDa cut off filter and centrifugation was carried out at 4000 g for 15 min. The cut-off filter was washed with 2 x 100 μL of 0.2% formic acid in 50% CAN. The flow-through and the retentate were collected and loaded on a 12% polyacrylamide gel and electrophoresis carried out.
**Table 5.1** Protein recovery using Microcon cut-off filters

<table>
<thead>
<tr>
<th>Protein</th>
<th>Total protein (Band volume / %)</th>
<th>Flow through (Band volume / %)</th>
<th>Retentate (Band volume / %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbonic anhydrase</td>
<td>1534/100</td>
<td>923/60</td>
<td>496/32</td>
</tr>
<tr>
<td>Trypsin inhibitor</td>
<td>1344/100</td>
<td>1142/85</td>
<td>362/27</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>245/100</td>
<td>227/93</td>
<td>15/5</td>
</tr>
</tbody>
</table>

**Figure 5.3** Recovery of proteins using 30 kDa cut-off filters ( ), Retentate ( ), Flow through and ( ) whole protein.
5.3c Crude isolation of albumin-interactome using Microcon cut-off filters

Two molecular weight cut-off filters; 100 kDa and 50 kDa were employed to fractionate ventricular CSF and three fractions were obtained (above 100 kDa, between 100 and 50 kDa and below 50 kDa). The between 100 and 50 kDa fraction was denatured with DTT/ACN solution and filtered through another 50 kDa cut-off filter and the filtrate that contains only the small proteins interacting with albumin was analyzed by 2-DE. Figures 5.4 and 5.5 show the composite gel image containing proteins from both AD and control sample set and the representative images of CSF proteins from AD and control subjects generated by PD Quest software respectively.

Twenty one protein spots were identified and 9 proteins showed significant changes in expression levels between AD and control (p <0.05) (Table 5.2 and 5.3) subjects. In-gel proteolysis was carried out with trypsin, the tryptic peptides were separated in a reverse phase capillary column and the amino acid sequence was determined. Therefore, an accurate and unambiguous identification is achieved. Comparing the intensities of protein spots between six AD and six normal individuals, 9 proteins were significantly altered (p<0.05). These altered proteins are summarized in Table 5.2. Proteins that were significantly reduced in CSF of AD patients included one isoform (basic) of prostaglandin D2 synthase (PGDS2), one isoform (more basic) of transthyretin, and two isoforms of phosphotidyl ethanolamine binding protein. Proteins that were significantly up-regulated in AD included retinol binding protein and four isoforms of PGDS2 with one isoform being present exclusively in AD CSF.
Figure 5.4 Composite (master) gel from the image analysis software shows all protein spots present in both populations – AD and control. The spot numbers correlate to the protein names in Table 5.1.
Figure 5.5 Two-dimensional electrophoresis analysis of proteins in the albumin containing fraction after fractionation using cut-off filters. (A) AD and (B) control.
Table 5.2 List of proteins identified by LC-MS/MS in samples from the 2-DE gel using cut-off filters

<table>
<thead>
<tr>
<th>Spot #</th>
<th>Protein</th>
<th>Mr (kDa)</th>
<th>pI</th>
<th>Sequence coverage (%)</th>
<th>Mowse score</th>
<th>Peptides matched</th>
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<td>β 2 microglobulin</td>
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<td>80</td>
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<td>Transthyretin</td>
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<td>13</td>
<td>Carbonic anhydrase II</td>
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<td>6.86</td>
<td>16</td>
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<td>8</td>
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Table 5.3 Differential expression of proteins in AD and control (CTRL) ventricular CSF as determined by the image analysis software PD Quest.

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<th>spot #</th>
<th>Protein name</th>
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<th>Ratio AD:CTRL</th>
<th>CTRL (spot intensity)</th>
<th>Ratio CTRL:CTRL</th>
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<td>1.73</td>
<td>7408</td>
<td>1</td>
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<td>1</td>
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<td>10</td>
<td>PEBP</td>
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<td>0.45</td>
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5.3d Optimization of immunoprecipitation of albumin-interactome from CSF

Immunoprecipitation using antibodies is the most frequently employed technique to test whether two proteins are interacting in vivo. Albumin was used as the bait protein to fish out other small proteins that might be interacting with it in the CSF.

- Evaluation of elution buffer

Optimization of the elution condition was carried out and various solvents like 0.01% SDS, 20% ACN and 0.2% formic acid in 20% ACN (Figure 5.6) were evaluated for their ability to strip proteins bound to the bead without stripping the antibodies itself. This study evaluating different buffers in eluting proteins from beads, was carried out in which the rabbit anti-albumin antibodies used were not crosslinked to the beads.

Figure 5.7 is a schematic of immunoprecipitation of the albumin-interactome. The gel images shown in figures 5.8 to 5.10 are few attempts that were carried out to optimize the various parameters in the immunoprecipitation protocol. Figure 5.8 shows the gel image of one of the very first attempts to isolate the albumin-interactome from CSF. Ten microliter of Protein G bound sepharose beads were incubated with 10 μg of anti-albumin antibody at 4°C for 3 hours. The antibody was crosslinked to Protein G after incubation with DMP for 30 min. The crosslinking reaction was terminated by incubation with ethanolamine for 30 min. The antibody crosslinked beads were incubated with 10 μL of CSF for 4 hours at 4°C. Albumin interactome was eluted using 50 μL of 1X SDS-PAGE loading buffer. There was no protein observed in the elution fraction. The depleted CSF contained some amount of albumin but the bands were all skewed because of the presence of high amounts of PBS.

After a series of optimization experiments, it was decided to incubate the antibody with Protein G beads at room temperature for 1 hour for binding, incubate the antibody containing beads three times with freshly prepared DMP and finally incubate beads with CSF for 1 hour and 30 min at room temperature for sufficient binding of the albumin-
interactome with the antibody. In the next attempt, 200 μL of beads were incubated with 200 μg of anti-albumin antibody. Crosslinking was carried out as described in the earlier section with DMP and 100 μL of un-processed CSF was added and the sample was incubated at room temperature for 1 hour and 30 min. Proteins were stripped using 3 x 50 μL of 1X SDS loading buffer and SDS-PAGE was carried out (Figure 5.9).

**Figure 5.6** SDS-PAGE analysis of the CSF proteins eluted from the anti-albumin attached Protein G beads. 200 μL of CSF was applied to the beads and eluted with different elution buffers. Elute was dried and resuspended in loading buffer and electrophoresis carried out at 200 V and gel stained with colloidal Coomassie G 250. The proteins identified (numbered on the gel) are given in table 5.4
Table 5.4 Proteins identified from 1D gel evaluating three elution buffers, by LC-MS/MS

<table>
<thead>
<tr>
<th>Band number</th>
<th>Protein</th>
<th>Swiss-Prot</th>
<th>Molecular weight (KDa)</th>
<th>Sequence coverage (%)</th>
<th>Mowse score</th>
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<td>627</td>
</tr>
<tr>
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<td>P01870</td>
<td>50</td>
<td>26</td>
<td>203</td>
</tr>
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<td>3</td>
<td>Rabbit Ig Kappa b4 chain C</td>
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<td>25</td>
<td>36</td>
<td>140</td>
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<tr>
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<td>Human Serotransferrin</td>
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<td>P01840</td>
<td>25</td>
<td>23</td>
<td>63</td>
</tr>
<tr>
<td>6</td>
<td>Rabbit Ig gamma chain C</td>
<td>P01870</td>
<td>50</td>
<td>27</td>
<td>310</td>
</tr>
<tr>
<td>7</td>
<td>Human transthyretin</td>
<td>P02766</td>
<td>13.7</td>
<td>59</td>
<td>360</td>
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Figure 5.7 Schematic for the immunoprecipitation of the albumin-interactome from CSF using rabbit anti-albumin antibody (top box, immunoprecipitation; lower box, elution and analysis)
**Figure 5.8** SDS-PAGE analysis of proteins isolated by immunoprecipitation from 10 μL CSF using 10 μL of Protein G beads and 10 μg of anti-albumin antibody. Lane 1, 100 ng of low range molecular weight markers, Lane 2, 10 μL of un-processed CSF, Lane 3, elute fraction using 50 μL of 1X SDS loading buffer and Lane 4, depleted CSF fraction.
Figure 5.9 SDS-PAGE analysis of the CSF albumin-interactome using Protein G sepharose beads. The gel was stained with colloidal Coomassie G 250. Lane 1, 200 ng of low range marker proteins. Lane 2, 10 μg of standard HSA protein. Lane 3, 100 μg anti-albumin antibody. Lane 4, 100 μL of un-processed CSF. Lane 5, elute fraction using 50 μL 1X SDS-loading buffer.
To enable maximum enrichment of the albumin-interactome, larger volumes of Protein G beads, anti-albumin antibody and CSF were used. One milligram of anti-albumin antibody was added to 300 μL of wet Protein G beads to saturate all the binding sites with anti-albumin antibody. The antibody was covalently crosslinked to beads after incubation with DMP. Crosslinking reaction was terminated by the addition of ethanolamine and beads incubated with 250 μL of CSF (Figure 5.10). This protocol gave maximum recovery of albumin and also revealed the presence of other proteins (though faint) in addition to albumin. The presence of phosphate buffer saline (PBS) in the albumin depleted CSF fraction caused the lateral diffusion of proteins. This distortion was prevented by removal of PBS from the albumin depleted fraction using a 3 kDa cut-off filter. To verify if there is any interaction between the beads and the proteins in CSF, beads with no anti-albumin antibody were incubated with 250 μL of CSF and after washing off CSF, the beads were stripped and the elute loaded on the polyacrylamide gel (lane 6 Figure 5.10). There were some proteins in the CSF that non-specifically bound to the beads. Similarly, to test if the proteins in the elution fraction are a result of stripping of the Protein G and anti-albumin antibody itself, the beads were incubated with anti-albumin antibody, crosslinked and eluted using formic acid in ACN. Very faint high molecular weight protein bands were observed (lane 5 Figure 5.10). The proteins seen in lanes 5 and 6 of figure 5.10 were mostly high molecular weight proteins (> ~50 kDa) that are not a part of the fraction-of-interest (the fraction-of-interest being the low molecular weight proteins interacting with albumin).
Figure 5.10 Immunoprecipitation of albumin-interactome from CSF using anti-albumin crosslinked Protein G beads. Anti-albumin antibody was covalently crosslinked to Protein G using DMP and then beads were incubated with 250 μL CSF. Lane 1, 200 ng of standard proteins. Lane 2, 250 μL CSF. Lane 3, Elute fraction from beads using 0.2% formic acid in 50% ACN. Lane 4, albumin depleted CSF. Lane 5, Elute from beads incubated with anti-albumin antibody. Lane 6, Elute from beads incubated with CSF (no antibody)
Immunoprecipitation of the albumin-interactome fraction from pooled CSF samples from 6 AD and pooled CSF samples from 6 normal individuals was performed using anti-albumin antibody covalently crosslinked to Protein G beads. Candidate proteins bound to albumin were eluted using 0.2% formic acid in 50% ACN and analyzed by SDS-PAGE (Figure 5.11).

**Figure 5.11** Immunoprecipitation of albumin interactome from CSF using anti-albumin crosslinked Protein G beads. (A) Anti-albumin crosslinked Protein G beads were incubated with 250 μL pooled and control CSF from 6 individuals and albumin-interactome eluted using 0.2% formic acid in 50% ACN. Lane 1, standard marker proteins. Lane 2 and 3 are 250 μL of straight unprocessed AD and control CSF. Lane 4 and 5 are elutes from AD and control CSF samples respectively. (B) Aliquot of depleted CSF samples from AD (lane 2) and control (lane 3). The proteins with spot numbers labeled on the gel were identified by LC-MS/MS and are given in table 5.5.
**Table 5.5** List of proteins identified from the 1D gel after immunoprecipitation of albumin-interactome from CSF

<table>
<thead>
<tr>
<th>Band #</th>
<th>Protein</th>
<th>Mr (kDa)</th>
<th>Seq Covg (%)</th>
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</tr>
<tr>
<td>ii a</td>
<td>PGDS2</td>
<td>21</td>
<td>17</td>
<td>130</td>
<td>TMLLQPAGSLGSYSYR, AQGFTEDTIVFLPQTDK</td>
</tr>
<tr>
<td>ii b</td>
<td>CA II</td>
<td>29</td>
<td>32</td>
<td>262</td>
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</tr>
<tr>
<td>iii</td>
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</table>
5.4 Discussion

The CSF proteome holds great promise as a source of biomarkers for diseases like Alzheimer’s disease that affect the brain. Despite the potential, it is challenging to analyze such complex proteomes because of the dynamic range of protein concentrations that exists in the CSF. As seen in figure 5.1, albumin and immunoglobulins dominate the CSF proteome and this makes the detection of low abundant proteins very difficult. Thus, reduction of sample complexity is essential when analyzing CSF. One way to circumvent this dynamic range problem is to deplete the high abundant proteins using commercially available depletion kits based on affinity methods using specific antibodies against albumin and immunoglobulins. However, serum proteome studies indicate that such affinity depletion methods result in concomitant removal of small low molecular weight proteins along with albumin and immunoglobulins [213]. The hypothesis of this study is that the high abundance protein, albumin in CSF, acts as a “molecular sponge” and soaks up small low molecular weight proteins. Selective enrichment of these low molecular weight proteins interacting with the high abundance proteins using immunoprecipitation will enable un-biased analysis of this fraction of the proteome, and comparison of the albumin-interactome between AD and control subjects is likely to provide useful biomarkers for the disease.

5.4a Efficacy of Microcon cut-off filters

A crude isolation of the albumin-interactome was carried out using cut-off filters after fractionating the complex CSF. A key point is the efficacy of the Microcon filters that needs to be tested to confirm its ability to effectively separate a complex proteome such as the CSF. To test the efficacy of the filters, a volume equal to 200 ng of each standard marker proteins was added to a 30 kDa cut-off filter and centrifuged. The flow through (below 30 kDa) and the retentate (above 30 kDa) were collected and subjected to 1D gel electrophoresis (Figure 5.2). The efficiency of cut-off filters in filtering the proteins is dependent on the molecular weight of the proteins. Our data indicate that the cut-off filters separated the proteins based upon their molecular weight quite effectively.
The Microcon cut-off filters have been used earlier for sample preparation and trypsin digestion in proteomic analysis using standard proteins [217]. Our studies indicated that the Microcon filters can be used for fractionation of proteins based upon their molecular weight facilitating isolation of the albumin-interactome. Almost 80-90% of proteins less than the cut-off value were filtered through the cut-off filters but with increasing molecular weight the recovery was less.

5.4b Crude isolation of CSF albumin-interactome using Microcon cut-off filters

Cut-off filters were used to isolate albumin-interactome after fractionation of CSF proteome based upon molecular weight. Using two filters- 100 and 50 kDa- three fractions were obtained. The fraction between 100 and 50 kDa (containing albumin) was disrupted for non-specific interactions that can exist between proteins and filtered through a second 50 kDa filter. The filtrate was subjected to 2-DE. Nine unique proteins were identified along with their various isoforms as a result of post-translational modifications.

It should be noted here that the molecular weight of the proteins identified in this study were all less than 50 kDa. However, the proteins were initially from the fraction between 100 and 50 kDa and were denatured by the addition of DTT in ACN. Given that the cut-off filters passed approximately 90% of the proteins effectively (as seen from our efficacy study), the presence of these proteins in the between fraction indicated that they are either interacting with other proteins in the CSF or among themselves. Since the combined molecular weight is greater than their actual individual molecular weights they are hence retained in the fraction between 100 and 50 kDa. The addition of a solution of DTT in ACN disrupted this interaction and proteins smaller than 50 kDa were filtered through the second 50 kDa cut-off filter. To test our hypothesis that albumin acts a protein scaffold and interacts with other small proteins, immunoprecipitation using anti-albumin antibody coupled Protein G sepharose beads was carried out. Albumin along with other proteins that might be interacting with albumin was immunoprecipitated under non-denaturing condition.
This offers advantages:

(1) it is a rapid procedure to isolate/enrich the albumin interactome;
(2) the purity of proteins is high; and
(3) it allows for the identification of proteins interacting with albumin.

- Altered proteins in AD CSF

2-DE experiments on CSF fractions indicated the presence of eight proteins with molecular weight less than 50 kDa in the fraction between 100 and 50 kDa (after fractionation using cut-off filters). Out of these eight proteins, four unique proteins and their various isoforms were significantly altered in the CSF from AD subjects (Table 5.3). These proteins included PGDS2, transthyretin, PEBP and RBP. To test the hypothesis that the proteins might be interacting with albumin, immunoprecipitation of the albumin-interactome was carried out using anti-albumin antibody crosslinked Protein G beads.

5.4c Immunoprecipitation of albumin-interactome

A more precise isolation of the albumin-interactome was carried out using immunoprecipitation of albumin. A series of immunoprecipitation experiments were performed to optimize the various conditions for successful isolation of the albumin-interactome from the CSF. These experiments included optimization of the ratio of anti-albumin antibody and protein G beads for a given volume of CSF, determination and optimization of the necessary incubation times to ensure strong antigen-antibody interaction, the number and volume of wash steps to eliminate the CSF proteins non-specifically interacting with the beads itself, and optimization of the number of elution steps required to elute the bound protein fraction.
• **Evaluation of elution buffer**

In immunoprecipitation, elution conditions are intended to break the ionic, hydrophobic and hydrogen bonds that hold the antigen to the antibody. Ideally, elution should release only the protein bound to the antibody attached to Protein G beads and not the antibody itself. Three different elution buffers- 0.01% SDS, 20% ACN and 0.2% formic acid in 50% ACN were evaluated for their effectiveness in stripping the proteins from the Protein G beads. SDS and ACN both eluted about the same amount of proteins from the beads. Elution buffer containing 0.2% formic acid in 50% ACN gave more proteins than the other two solvents. All three buffers, however, stripped the anti-albumin antibody attached to the protein G beads (Figure 5.6, Table 5.4). In order to prevent the elution of the anti-albumin antibody itself, the antibody in further experiments was covalently crosslinked to Protein G beads using dimethylpimelimidate (DMP).

• **Optimization of immunoprecipitation of albumin-interactome**

The immunoprecipitation protocol involves a multitude of steps to isolate the target protein from a complex mixture of proteins like CSF. Various parameters were investigated to obtain an optimized protocol that can selectively isolate the albumin-interactome from CSF. From our elution buffer evaluation study, formic acid in ACN buffer stripped proteins from the beads better than SDS and 20% ACN; however it was also able to elute out anti-albumin antibodies (not crosslinked to Protein G in our elution buffer evaluation study). The elution of antibodies could, however, be prevented by covalently crosslinking the antibody to Protein G beads. Hence, for all further studies, the antibodies were chemically crosslinked to Protein G beads and 0.2% formic acid in 50% ACN was used as the elution buffer. The optimized immunoprecipitation protocol (given in experimental section) was repeated at least two times to verify the reproducibility and to establish standard operation procedures. A high reproducibility was achieved and the protein elution profiles were almost identical. Our immunoprecipitation results clearly supported our hypothesis that the proteins PGDS2, transthyretin and carbonic anhydrase
II were all interacting with albumin. The levels of these proteins were altered in CSF from AD patients.

Prostaglandin D synthase (PGDS2) was originally known as β trace and was discovered by Clausen in 1961 in human CSF [218]. PGDS2 is a sialic acid (SA) containing glycoprotein with a molecular weight of 27 kDa [219]. PGDS2 is produced by leptomeninges and the choroid plexus of the brain [220-222]. In addition to the enzymatic function in the synthesis of prostaglandin D, PGDS2 is also believed to be involved in the transport of various lipophilic moieties including thyroid hormone and retinoic acid in CSF [219, 223-225]. There is increasing evidence that isoforms of proteins are differentially altered in AD, hence it becomes imperative to study the differentially expressed levels of post-translational modifications in AD biomarker discovery to understand the neuropathology [226]. Several studies have investigated post-translational modifications (PTM) of PGDS2 in CSF of patients with neurological diseases. Hiraoka et al. have studied the microheterogeneity of PGDS2 in the CSF of patients with various neurological diseases like Amyotrophic lateral sclerosis (ALS), Alzheimer’s disease (AD) and Parkinson’s disease (PD) using capillary isoelectricfocusing [227]. Since PGDS2 has two oligosaccharide chains and each chain has two SA groups, the protein has in total four SA groups. This allows for the possibility of 8 different isoforms for the protein. Various studies have identified four isoforms of PGDS2 in human CSF [227, 228]. Studies investigating these different isoforms of PGDS2 will help to provide more insight about PGDS2 function in CSF. Currently, PGDS2 is used as a marker to detect the presence of CSF in nasal secretions from patients suffering from rhinorrhea [229]. PGDS2 was also reported as a marker for N-glycosylation defects in the brain [230].

Earlier work indicated that the acidic isoforms of PGDS2 were decreased in the CSF of patients suffering from neurological diseases [231, 232]. From our 2-DE study, 8 protein spots (with different pIs but almost the same molecular weight) were identified as PGDS2. The measurement of total PGDS2 in spot intensity in AD and control gels using PD Quest software indicated that the protein level was only slightly increased in AD (by 10%) when compared to levels in control samples. However, the three most acidic
isoforms were significantly up-regulated in AD, and one isoform at pI of approximately 7 was present exclusively in the AD sample. One basic isoform of PGDS2 was down-regulated significantly in AD. Since the various isoforms of PGDS2 have similar molecular weight but different pI, the most likely PTM of PGDS2 is glycosylation. Glycosylation of a protein changes its pI to be more acidic due to the addition of a carbohydrate moiety. Since the acidic isoforms of PGDS2 was found to be up-regulated in AD, our data suggests that there is increased glycosylation of PGDS2 in AD. Altered glycosylation of tau, cholinesterases and transferrin in AD have already been described, suggesting that aberrant glycosylation changes occur in AD [233-235]. Hashimoto et al. reported that sialylation of amyloid precursor protein (APP) increased the secretion of neurotoxic metabolites like amyloid beta peptide (Aβ) [236]. It was reported that hyperglycosylation of tau protein caused an increase in phosphorylation of tau resulting in hyperphosphorylated tau in the AD brain [233]. The differential expression levels of PGDS2 in CSF of AD patients further validated the impaired glycosylation in AD.

Since glycosylation plays a pivotal role in various cellular processes like signaling and protein folding, and is synergistic with phosphorylation events, it is most likely that alteration in glycosylation can induce signaling cascades that mediate neurodegeneration [237]. Watanabe et al. reported that serum albumin could bring about the synthesis of prostaglandin D2 from prostaglandin E (i.e. the function of PGDS2) in platelets [238]. This indicates a PGDS2-like activity of albumin in platelets. Hence, either, PGDS2 and albumin have a common substrate (PGE2) that might be responsible for the interaction of PGDS2 with albumin observed in the immunoprecipitation study and could explain the presence of PGDS2 in the albumin-interactome fraction or albumin is associated with PGDS2 and this combination can carry out prostaglandin E conversion to PGD2. PGDS2 was also reported to be present in human atherosclerotic plaques [239]. Since PGDS2 is a protein that is made up of mainly β sheets [240], it is possible that due to aberrant glycosylation of the protein, it aggregates in the brain of AD patients as well; however, more work is required to prove this speculation.
Two isoforms of transthyretin were identified in 2-DE gel and the more basic isoform was down-regulated three-fold in CSF from AD subjects. Transthyretin was also identified as one of the proteins present in the albumin-interactome. Transthyretin, formerly known as prealbumin, is a homotetrameric protein with a molecular weight of approximately 55 kDa. TTR is synthesized and released into the CSF by the choroid plexus [241]. A small amount of TTR synthesized in the liver also exists as a homotetramer in serum and constitutes about 0.5% of serum proteins [242]. Serum TTR cannot cross the blood brain barrier and hence TTR present in CSF is exclusively from the choroid plexus. TTR in CSF is predominantly in the monomeric form, but in the choroid plexus is a tetramer [231, 243].

Transthyretin was identified in the neurofibrillary tangles as well as in amyloid plaques in the brain of AD patients [244]. It was reported that the lower the levels of TTR in CSF, the more demented were the patients [52]. The monomeric form of TTR in the CSF was found to sequester amyloid β protein (Aβ), which is responsible for the formation of amyloid plaques in the brain of AD patients [245]. TTR forms soluble complexes with Aβ and inhibits its aggregation. However, TTR itself is capable of forming amyloid fibrils that are associated with diseases such as familial amyloid cardiomyopathy (FAC), senile systemic amyloidosis (SSA) and familial amyloiotic polyneuropathy (FAP) [246, 247].

Transthyretin is a β-sheet rich protein and any event triggering misconception can result in the stacking-up of these β sheets to form aggregates. Various PTMs of transthyretin have been detected in CSF and include sulfonation, cysteinylation and glutathione conjugation [248-250]. The isoforms that were detected in the 2-DE gels, in this study, were all of about the same molecular weight but had different isoelectric points. This suggested that the two isoforms were a result of either sulfonation or phosphorylation. The more basic isoform (with greater pl) was found to be down-regulated significantly by approximately three-fold in the CSF of AD patients. The down-regulation of TTR is in agreement with other published work [52]. The decrease in TTR
isoform could cause $\text{A} \beta$ to be more susceptible for aggregation. More investigations are required to identify the PTM in TTR.

Transthyretin was identified to be a component of the albumin-interactome through the described immunoprecipitation technique. One possible explanation for the presence of TTR in the albumin-interactome is the common substrates that both TTR and albumin share. TTR in CSF functions as a carrier protein mainly for thyroxin (T4) and vitamin A (retinol through the association with retinol binding protein-RBP) [251, 252]. The thyroid hormone and retinol are essential for normal development of brain [253, 254]. It was reported that TTR has high affinity for T4 while albumin has a low affinity but high capacity for T4 [255]. Albumin is the most abundant protein in CSF and being a known transport protein, it is quite possible that albumin is carrying with it other small proteins like TTR that have a common substrate such as T4 to lock onto. Our data show that retinol binding protein (RBP) was up-regulated by approximately 1.5 fold in the CSF of AD subjects. This is in agreement with results published earlier on the increased level of one isoform of RBP in the CSF from AD patients [226, 256, 257].

Two isoforms of phosphotidyl ethanolamine binding protein (PEBP) were identified in the 2-DE gel and both isoforms were significantly down-regulated by 2-fold in the CSF from AD subjects compared to control. In humans, PEBP is also known as neuropolypeptide h3 and contains the hippocampal cholinergic neurostimulating peptide (HCNP). Decreased levels of PEBP have been reported earlier in the hippocampus of brain from Tg2576 (mouse model of AD) [258]. It was also reported that PEBP immunoreactivities were found on amyloid plaques of both Tg2576 and AD brain sections [258]. The HCNP peptide is involved in the differentiation of neurons in the hippocampus where the protein also enhances the synthesis of choline acetyl transferase [259]. PEBP was not identified as a part of the albumin-interactome.

Other proteins like SOD1, cystatin C, $\beta$-2 microglobulin and carbonic anhydrase II (CA II) were identified in the cut-off filter study. However, only CA II was observed in the immunoprecipitated albumin-interactome fraction. This observation suggested that
proteins other than CA II are not interacting with albumin but might be interacting with other proteins including themselves and thus have molecular weights greater than their actual individual molecular weights and hence be retained in the fraction between 100 and 50 kDa. The other possibility is that the proteins are present in such low levels that they are not detected on the 1-D gel carried out after immunoprecipitation.

Most of the altered proteins detected in the CSF from AD subjects, in our study, are rich in β-sheets, and some have already been identified in the amyloid plaques and NFTs. Any event triggering misconformation could result in aggregation of these proteins. The proteins also exhibit different isoforms, and differential expression of the isoforms indicate the importance of elucidation of PTMs. Studies related to levels of each isoform are important for biomarker discovery instead of total protein content. The observation that levels of certain proteins are altered in CSF from AD subjects is of considerable interest but must be interpreted cautiously. For example, one interpretation is that protein deficiency triggers one or more cascades of molecular events that cause neurodegeneration. On the other hand, reduced levels of protein isoforms in a neurodegenerative disorder like AD may represent an expected secondary consequence of neuronal cell loss.

5.5 Conclusion

Measurements of changes in the expression of multiple proteins and their isoforms provide a powerful strategy for understanding pathophysiological processes of diseases. Owing to its proximity to brain, CSF is an ideal target to identify biomarkers for diseases like AD that mainly affect the brain. However, the challenge in analyzing the CSF proteome by 2-DE is the huge dynamic range of the proteins. The current trend in biomarker discovery is to deplete albumin from the CSF sample; however, it should be realized that this could lead to removal of small proteins and peptides that might be interacting with albumin. The present study involves analysis and identification of the albumin-interactome that constitute the albumin-interacting proteins and comparison of this albumin-interactome between AD and control subjects to help in identification of
biomarkers for the disease. To test our hypothesis that albumin acts as a protein scaffold and “soaks-up” other small proteins, a crude isolation of the albumin-interactome was carried out using cut-off filters. The presence of proteins whose molecular weight is less than 50 kDa, in the fraction between 100 and 50 kDa that predominantly contains albumin, indicates that the highly abundant carrier protein albumin can sequester low molecular weight proteins in CSF. To reaffirm the observation that these low molecular weight proteins in the fraction between 100 and 50 kDa were indeed interacting with the carrier protein albumin, immunoprecipitation of the albumin-interactome using albumin as the “bait” protein was carried out, and the albumin-interactome studied using 1-D gel electrophoresis.

Using this selective enrichment of the albumin-interactome, 21 protein spots were identified that comprised 9 unique proteins and their post-translational modifications. This is the first study to our knowledge that has successfully isolated albumin-interactome from the CSF and compared these fractions of CSF proteome between AD and control subjects for differences.

Various isoforms of prostaglandin D2 synthase, transthyretin, carbonic anhydrase II, β2 microglobulin, phosphatidyl ethanolamine binding protein, retinol binding protein, neuropolypeptide h3, SOD 1 and cystatin C were identified in the albumin containing fraction from cut-off filter study by 2-DE. Of these 9 proteins PGDS2, TTR and CA II were identified in the albumin-interactome after immunoprecipitation, using albumin as the bait protein. Our 2-DE results indicate alteration in the levels of various isoforms of proteins in the CSF from AD subjects. In particular, the acidic isoform of PGDS2 was up-regulated in the CSF from AD subjects. Other proteins that were altered in the CSF from AD subjects included the basic isoform of TTR, two isoforms of PEBP and one isoform of RBP. The observation that various isoforms of proteins were altered in the CSF from AD patients supports the glycosylation defects present in AD.

In conclusion, the data presented herein demonstrate that albumin indeed interacts with small low molecular weight proteins in CSF. The levels of these small proteins
constituting the albumin-interactome are significantly altered in the CSF from AD subjects. The ability to detect these proteins in the CSF was the key finding of this work. The alterations in levels of the isoforms of proteins like PGDS2 support the concept that glycosylation defects are implicated in Alzheimer’s disease. Furthermore, the data implicate the acidic isoform of PGDS2 as a potential biomarker of AD.
CHAPTER SIX

CONCLUSION

Proteomics is a rapidly expanding field of research that has carved a niche of its own in life sciences. At its core, proteomics is all about separation and identification—the process of taking a sample of interest, separating out all of the proteins within, and then identifying them. As one can imagine there exists an array of proteomics tools to help accomplish this formidable task of protein analysis. Specifically, two-dimensional gel electrophoresis and mass spectrometry—two milestone technologic breakthroughs facilitated the ability to simultaneously look at multitude of proteins. Tremendous improvements in both methodologies are providing improved strategies to speed biological discovery. Though many chromatographic techniques are being introduced, two-dimensional gel electrophoresis has been the method of choice for the analysis of proteomes for nearly 35 years mainly because of its enormous resolving power and ability to simultaneously separate thousands of proteins. A number of modifications to the 2-DE methodology have been introduced and improvements in key components of the technology such as use of immobilized pH gradient gels and fluorescent staining have greatly improved reproducibility and sensitivity.

The sensitivity of stains that are used to detect proteins after their separation by 2-DE, has been one factor influencing the amount of information that can be extracted from the 2-DE gels. The development of a fluorescent ruthenium complex (RuMS) for staining proteins separated by gel electrophoresis with high sensitivity, broad linear dynamic range and that is compatible with mass spectrometry for subsequent identification of proteins (as described in chapter three of this dissertation) is quite valuable in this context. With the low detection limit of 1 ng of protein and cost-effective potential available with this in-house synthesized RuMS staining solution, it is not difficult to see why the development of this staining solution is a very practical and worthwhile avenue to pursue. Knowledge of exact composition of the stain will prove to be very beneficial in being able to reproducibly synthesize the stain and exploit its potential to the fullest. One
can envision using different ratios of the commercially available different ruthenium complexes- RuMS, RuDS and RuBPS to probe and obtain the exact composition of the in-house RuMS staining solution that is a value added information.

Proteomics has diverse applications in various fields like medicine, toxicology, pharmacy and many others. The incredible potential of proteomics to discover new disease biomarkers and toxicity signatures were explored as described in this dissertation. The emerging field of toxicoproteomics integrates two disciplinary areas of toxicology and differential protein analysis and allows for better understanding of protein expression during toxicity and environmental stress for the advancement of public health. Proteomic techniques were applied to study the \textit{in vitro} effect of malathion, an organophosphate insecticide on protein expression using human neuroblastoma cells. Our studies indicated that out of 122 human neuroblastoma proteins identified, 21 proteins were significantly altered after treatment with malathion. Results suggest significant down-regulation of key calcium modulators and chaperones in the malathion-treated cells. A model was proposed based on the altered proteins that malathion induces oxidative stress leading to possible misfolding of protein that can potentially accumulate and cause cytotoxicity in neuroblastoma cell cultures. The identification of differential protein expression in neuroblastoma cells after treatment with malathion is an important finding in regard to the OP toxicity, since there has been limited knowledge in this area. Though neuroblastoma cells are the preferred model to study \textit{in vitro} OP toxicity, studies involving primary cell lines and animal models will provide more precise information on the implication of OPs on humans. One can also envision a similar kind of investigation performed on the liver proteome of OP exposed animals to identify protein targets of OP toxicity. The near-term payoffs of this type of work are two-fold. One is the understanding of mechanisms of toxicity and the other is the identification of biomarkers of exposure. While investigations presented here offered a basic foundation for understanding OP toxicity at the protein level, a closer examination involving analysis of sub-proteome will surely provide a greater appreciation of the underlying mechanisms of OP toxicity.
A major benefit of proteomic technology is its use in the development of biomarkers for diseases. Proteomic techniques can be applied to study innumerable human diseases ranging from cancer to infectious diseases. Alzheimer's disease (AD) affects more than 4.5 million Americans. There is no cure for Alzheimer's disease yet and the primary goal of biomarker research is to detect and diagnose the disease before symptoms surface. Proteomics is most suited for mining biomarkers and tools allow comparing protein profiles of normal and diseased body fluids such as blood, CSF and urine. A proteomic analysis of CSF albumin-interactome was undertaken to identify and compare the protein constituents of this fraction of the CSF proteome between AD and normal subjects. The novel immunoprecipitation-based proteomics approach to AD biomarker discovery proved successful in isolating and identifying the otherwise difficult-to-analyze albumin-interacting proteins and their isoforms. The immunoprecipitation method to analyze interactomes in CSF is both feasible and effective. With increased efforts to diagnose and treat AD by early intervention and even towards prevention, it is evident that biomarkers will gain significance as tools to aid research and ultimately clinical practice. Our results indicated presence of small proteins including prostaglandin D2 synthase (PGDS2) and transthyretin to be components of CSF albumin-interactome. A key finding is the alteration in levels of different isoforms of these proteins constituting the albumin-interactome in CSF from AD subjects and their potential to serve as biomarkers for AD. One can envision a careful evaluation of relative abundance of the different isoforms of the albumin-interactome proteins in CSF from AD, mild cognitive impairment and no cognitive impairment subjects be carried out to help generate a fingerprint model that can successfully delineate the various stages. A clear understanding of the post-translational modifications involved in these proteins will provide insight into the etiology of the disease. The methodology developed herein can also be applied to serum proteome that will enable non-invasive diagnosis of the disease. Though, this is a challenging endeavor, given the potential of various proteomics technologies, it should be possible to identify a sufficient number of biomarkers to revolutionize early detection.

Application of two vital proteomics methodologies namely 2-DE and mass spectrometry along with the in-house developed staining solution described in this dissertation clearly
demonstrates the power of the technology to analyze complex proteomes like whole cell lysate and cerebrospinal fluid. With the potential that proteomics carries with it, it will not be too far in the future that mass spectrometry-based proteomic technologies will play key role in clinical diagnosis of diseases. With development of miniature mass spectrometers, field application of the technology is a promising area of pursuit that will enable detection of for example, toxic chemicals during warfare. Questions like how the technology can be implemented and what costs are involved in doing so, to accomplish the above-said vision, must be considered. The versatility of various proteomics methodologies is endless and the alliance of various separation techniques with mass spectrometry has the potential to improve quality of life.
**Appendix A.** List of proteins identified on 2-DE gels by MALDI-TOF mass spectrometry

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<th>$M_r$ (kDa)</th>
<th>pI</th>
<th>% Sequence coverage</th>
<th># of peptides matched</th>
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REFERENCES

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VITA

Meena Uma Rajagopal was born in December 12 1976 in Madras, India.

Meena, after graduating with a Bachelors degree in Chemistry pursued her education in the field of Biochemistry and enrolled for Master’s degree at Meenakshi College, affiliated to University to Madras. She completed the Master’s degree in Biochemistry with honors and was awarded the Outstanding Achievement award by the University of Madras. Following graduation, Meena joined the PhD at the University of Kentucky. She joined Dr. Tae Ji’s lab and worked in the area of signal transduction in G protein coupled receptors and got her Master degree. Upon completion of Master’s degree, Meena worked for Dr. Haley for about a year, during which she learnt biochemical techniques like western blotting. Meena was fascinated by the research conducted in the neighboring lab of Dr. Lynn. She joined Dr. Lynn’s lab in the summer of 2003 and worked towards her PhD. She was involved in a number of proteomics projects that utilized mainly two-dimensional gel electrophoresis and mass spectrometry. She was the recipient of the Research Challenge Trust Fellowship from May 2000 to June 2005 and two travel awards to present her research at the annual American Society for Mass Spectrometry in 2004 and 2005. Meena is also a member of the American Society for Mass Spectrometry.

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May 09, 2006
Publications

1. Proteomic investigation of malathion induced differential protein expression in SY5Y cell cultures- manuscript submitted to EHP
2. Comparison of commonly used protein stains in terms of sensitivity, linear dynamic range and compatibility with mass spectrometry - manuscript in preparation
3. Comparison of albumin interactome in ventricular CSF between AD patients and normal individuals- manuscript in preparation

Presentations

1. Malathion induced differential protein expression in SY5Y cell culture models by Two Dimensional gel electrophoresis(2-DE) and LC-MS/MS- Meena Sundaramoorthy; Mark A. Lovell; Bert C. Lynn. 52nd ASMS conference on Mass Spectrometry- Nashville, TN
2. Proteomic analysis of human ventricular CSF albumin interactome and its relevance to Alzheimer’s disease - Meena Sundaramoorthy; Mark A. Lovell; Bert C. Lynn. 53rd ASMS conference on Mass Spectrometry- San Antonio, TX