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Quantitative Proteomics Analysis of Differentially Expressed Proteins in Aβ(17-42) Treated Synaptosomes

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QUANTITATIVE PROTEOMICS ANALYSIS OF DIFFERNTIALLY EXPRESSED PROTEINS IN Aβ(17-42) TREATED SYNAPTOSOMES

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DEDICATION

This thesis is dedicated to my parents Mohammed Yaqoob and Naseem Sultana, my brother Mohammed Ayub, my sisters Nazema Sultana, Kouser Sultana, Nayeem Sultana, Tahseen Sultana, and to all my friends who helped me and encouraged me to succeed. Thank you all for your unwavering support.

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ABSTRACT

Oxidative stress has been associated in the pathogenesis of numerous diseases such as neurodegenerative disorders, ischemia, and cancer. The brain is susceptible to oxidative stress due to its high content of peroxidizable unsaturated fatty acids, high consumption of oxygen per unit weight, high levels of free radicals, and comparatively low levels of antioxidant defense systems. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) can react with biomolecules such as proteins, lipids, carbohydrates, DNA, and RNA, which can lead to oxidative damage, cellular dysfunction, and can ultimately cause cell death. Down syndrome (DS) is the most common form of chromosomal abnormality found in live-born infants. DS patients have an extensive deposition of Aβ(17-42) peptide, which could contribute to their increased rate of developing Alzheimer's disease (AD). Since AD cannot be properly diagnosed until autopsy, development of a novel Down syndrome model using Aβ(17-42) could be beneficial in determining oxidative stress levels and their relationship to mild cognitive impairment (MCI), the earliest form of AD in order to possibly be used as a diagnostic tool for AD. We have found a significant difference between oxidative stress levels in Aβ(17-42) treated synaptosomes and control. By using proteomics, we have also identified several biomarkers including aldehyde dehydrogenase, aldolase, α-enolase, heat shock cognate 71, peptidyl-prolyl cis-trans isomerase, and ATP synthase α chain. Our present findings, suggest the role of Aβ(17-42) as one of the contributing factors in mediating oxidative stress in DS, and AD brain leading to neurodegeneration.

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LIST OF ABBREVIATIONS

Aβ	Amyloid beta
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
DS	Down syndrome
AD	Alzheimer's disease
MCI	Mild cognitive impairment
3-NT	3-nitrotyrosine
HNE	4-Hydroxy-2-nonenal
APP	Amyloid precursor protein
SP	Senile plaques
SOD	Superoxide dismutase
GPx	Glutathione peroxidase
Cat	Catalase
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
MS	Mass spectrometry
ESI	Electrospray ionization
MALDI	Matrix assisted laser desorption ionization
IEF	Isoelectric focusing

CHAPTER ONE
INTRODUCTION

The studies described in this thesis were performed to understand the role of amyloid beta peptide Aβ(17-42) induced oxidative stress and to develop of a possible Down syndrome model equivalent to that of mild cognitive impairment. Down syndrome (DS) is the most common form of chromosomal abnormality found in live-born infants and is the most common congenital cause of intellectual disability (Irving, Basu et al. 2008). It is caused by a genetic abnormality in which three copies of chromosome 21 are present instead of two (Nizetic 2001; Wiseman, Alford et al. 2009). DS patients have an extensive deposition of Aβ(17-42) peptide, which could contribute to their increased rate of developing Alzheimer's disease (AD). Alzheimer's disease is a neurodegenerative disorder that affects 5.5 million Americans and more than 35 million people worldwide (Querfurth and LaFerla 2010).

Alzheimer's disease is an age-related neurodegenerative disorder which is characterized by a progressive loss of memory and cognitive functions which ultimately results in severe dementia (Butterfield, Perluigi et al. 2006). AD is defined by the accumulation of two types of insoluble fibrous materials, extracellular amyloid-β peptide deposited in senile plaques and intracellular neurofibrillary tangles. Neurofibrillary tangles are principally composed of abnormal and hyperphosphorylated tau protein (Butterfield, Perluigi et al. 2006). AD is also pathologically further characterized by neuronal death and loss of synaptic connections within specific brain regions. Research has shown that mutations of the amyloid precursor protein (APP) genes, presenilin-1and presenilin-2, and cause inherited AD which leads to increased deposition of fibrillary β-amyloid in the brain (Butterfield, Perluigi et al. 2006). AD pathogenesis

has been proposed by several hypotheses including: amyloid beta cascade, oxidative stress, excitotoxicity, and inflammation. There is cumulative evidence suggesting a key role of oxidative stress in the pathophysiology of AD (Butterfield, Perluigi et al. 2006).

AD progresses through four stages: preclinical Alzheimer's disease (PCAD), mild cognitive impairment (MCI), early Alzheimer's disease (EAD), and late-stage Alzheimer's disease (late-stage AD). In PCAD, patients have a normal psychometric scores, but do meet the neuropathological criteria for AD (Aluise, Robinson et al. 2010). They also exhibit neuronal hypertrophy (Iacono, Markesbery et al. 2009) and have no significant hippocampal cell loss (West, Kawas et al. 2004). In MCI, symptoms include memory loss, poor judgment, confusion, disorientation, cognitive decline, and visuospatial cognition. As the disease progresses, the symptoms worsen over time, in late-stage AD, patients suffer from severe dementia and profound global cognitive deficits and in some cases immobility. In addition to loss of synaptic connections (Butterfield, Perluigi et al. 2006), it has been found that AD patients have a loss or shortening of dendritic spines (Mitsuyama, Futatsugi et al. 2009).

Oxidative stress is the imbalance between prooxidant and antioxidants in the body. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are highly reactive substances (Aruoma 1998; Stadtman and Berlett 1998; Stadtman 2006) that can react with biomolecules such as proteins, lipids, carbohydrates, DNA, and RNA (Table 1.1). ROS and RNS can cause oxidative damage to these biomolecules leading to cellular dysfunctions and ultimately may cause cell death (Butterfield, Reed et al. 2007).

Table 1.1 List of reactive oxygen and nitrogen species.

Primary radical species	Non radical species	Free radicals
OH•	H_2O_2	•C
NO•	HOCl	RS•
$O_2•^-$	CO	RSO•
$CO2•^-$	O_3, $^1O_3$, $^1O_2$	RSOO•
	ONO_2^-	R•
	$ONOCO_2^-$	ROO•
	N_2O_2, NO_2	$RSSR•^-$

DS is associated with various medical conditions including mental retardation, congenital heart disease, intestinal disorders, and defects of the endocrine and immune system. These patients also have an increased risk of developing leukemia and Alzheimer's disease (Zitnanova, Korytar et al. 2006). Other phenotypic features found in DS individuals include, abnormalities of neuromuscular tone, atlanto-axial instability (increased mobility at the articulation of the first and second cervical vertebrae of the atlantoaxial joint), characteristic skeletal and facial features, changes in the visual and audiovestibular functions. Among all of these features, virtually 100% of the individuals with DS have two features including mental retardation and neuropathological modifications (in individuals with over 35 years of age) similar to those observed in Alzheimer's disease brains (Nizetic 2001).

Research has shown that DS patients develop AD at a much earlier age, and therefore have a significantly higher risk of developing Alzheimer's disease (Zigman, Devenny et al. 2008). Individuals with Alzheimer's disease and Down syndrome develop neurofibrillary tangles, senile plaques and neuronal loss (Head, Lott et al. 2002). Extracellular deposition of

amyloid beta peptide Aβ(1-42) formed by the cleavage of amyloid precursor proteins by β and γ-secretase enzymes are found extensively in AD brain (Figure 1.1). Nonamyloidogenic amyloid beta fragments are formed by the sequential cleavage of Amyloid precursor protein (APP) by α- and γ-secretases that generates Aβ(17-42) peptide fragments (Figure 1.2). This is also known as the p3 fragment. Amyloid precursor protein gene is present on chromosome 21. Down syndrome individuals have high levels of Aβ deposition by the age of 40 and also develop early onset of dementia, however the average age of developing clinical dementia is 55 years and it widely varies (Schupf and Sergievsky 2002). The neuropathological manifestations of AD in DS is known to be because of triplication and overexpression of APP present on Chromosome 21(Schupf and Sergievsky 2002). Aβ(17-42) , which is also found in Down syndrome individuals, may serve as a model system to study early-onset AD (Jang, Arce et al. 2010).

H_2N-Asp^{1}-Ala^{2}-Glu^{3}-Phe^{4}-Arg^{5}-His^{6}-Asp^{7}-Ser^{8}-Gly^{9}-Tyr^{10}-Glu^{11}-Val^{12}-His^{13}-His^{14}-Gln^{15}-Lys^{16}-Leu^{17}-Val^{18}-Phe^{19}-Phe^{20}-Ala^{21}-Glu^{22}-Asp^{23}-Val^{24}-Gly^{25}-Ser^{26}-Asn^{27}-Lys^{28}-Gly^{29}-Ala^{30}-Ile^{31}-Ile^{32}-Gly^{33}-Leu^{34}-Met^{35}-Val^{36}-Gly^{37}-Gly^{38}-Val^{39}-Val^{40}-Ile^{41}-Ala^{42}-COOH

Figure 1.1 Amino acid sequence of Aβ(1-42) peptide.

H_2N-Leu^{17}-Val^{18}-Phe^{19}-Phe^{20}-Ala^{21}-Glu^{22}-Asp^{23}-Val^{24}-Gly^{25}-Ser^{26}-Asn^{27}-Lys^{28}-Gly^{29}-Ala^{30}-Ile^{31}-Ile^{32}-Gly^{33}-Leu^{34}-Met^{35}-Val^{36}-Gly^{37}-Gly^{38}-Val^{39}-Val^{40}-Ile^{41}-Ala^{42}-COOH

Figure 1.2 Amino acid sequence of Aβ(17-42) peptide.

Cleavage of APP by γ-secretase and BACE (β -amyloid precursor protein cleaving enzyme) between Tyr10 and Glu11 produces another nonamyloidogenic Aβ peptide, Aβ(11-40/42).Very little is known about this peptide fragment, but it is assumed to be nonpathogenic

(Jang, Arce et al. 2010). APP gene codes for a large, transmembrane protein expressed in both astrocytes and neurons (Capone 2001). The overall function of APP is not known precisely, however, various fragments are associated with stimulation of neurite outgrowth and synaptogenesis, promotion of cell survival, modulation of synaptic plasticity, regulation of cell adhesion, and neuroprotection against oxidative and excitototoxic insults (Capone 2001). Aβ(17-42) peptide fragments are the main component of the cerebellar preamyloid lesions in Down syndrome individuals and are speculated to induce neuronal toxicity (Jang, Arce et al. 2010), however their biophysical properties, pathological significance, and mechanism of toxicity in AD and DS remain unclear. Very little information is available about the structure and activity of Aβ(17-42). It could be possible that Aβ(17-42) could be one of the pathological agents that causes AD and DS (Jang, Arce et al. 2010).

Down syndrome individuals develop Alzheimer's disease pathology in a progressive age-dependent manner (Wisniewski, Wisniewski et al. 1985; Hof, Bouras et al. 1995) and are at a high risk for the progression of dementia (Lai and Williams 1989). The neuropathological manifestations of AD in DS individuals have been attributed due to triplication and overexpression of APP gene located on chromosome 21 (Schupf and Sergievsky 2002). Usually by the age of 40, almost all adult individuals with DS have significant neuropathology for the diagnosis of AD (Head and Lott 2004). As β-amyloid peptide is cleaved from APP, it might initially appear in soluble form in extracellular space or within neurons. It is found that plasma Aβ levels increase with increasing in age (Head and Lott 2004). DS individuals exhibit elevated levels of soluble Aβ peptide, which can interact with both the aging and developmental processes. It is speculated that the soluble species of Aβ may accumulate before extracellular Aβ and is more prone in causing neuronal dysfunction than extracellular Aβ in Down syndrome. It is

also believed that Aβ oligomers may be significantly important in causing neuronal dysfunctions before obvious neuronal loss in DS (Head and Lott 2004). Although DS individuals have a large amount of Aβ peptide that exists in a soluble form, it is found that insoluble deposits begin to form over time. Therefore intracellular accumulation of Aβ precedes extracellular Aβ deposition in DS. Intracellular accumulation of Aβ within neurons may be an important aspect for the age-associated extracellular Aβ deposition (Head and Lott 2004).

Reactive oxygen species and reactive nitrogen species cause oxidative damage when they interact with proteins, lipids, DNA, and RNA. Normal production of reactive oxygen species is typically counterbalanced by free radical scavenging enzymes including superoxide dismutase, glutathione peroxidase, and catalase.

Superoxide dismutase is an antioxidant enzyme found on chromosome 21 (Head and Lott 2004). This enzyme is found at higher levels in DS fetal brain when compared to control (Brooksbank and Balazs 1984). Superoxide dismutase enzyme converts superoxide radicals into hydrogen peroxide (Figure 1.3). Hydrogen peroxide, in turn, is converted to water by either catalase or glutathione peroxidase (Droge 2002). It is speculated that increased expression of superoxide dismutase or lack of sufficient levels of glutathione peroxidase (GSH) or catalase may lead to overproduction of hydrogen peroxide levels and oxidative damage in the DS brain (Head and Lott 2004).

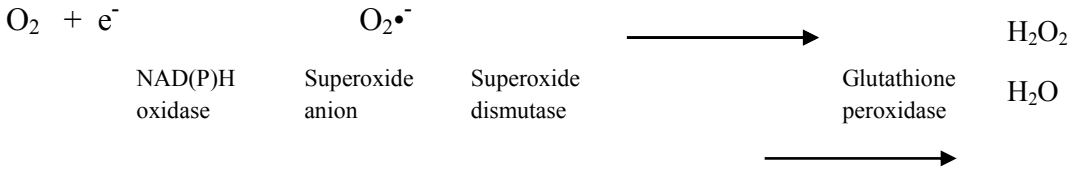


Figure 1.3 Formation of reactive oxygen species and clearance.

Oxidative damage may cause enhanced production of Aβ peptide and vice versa. Higher levels of Aβ may exacerbate ongoing oxidative damage as well. Abnormal APP expression may result from increase production of reactive oxygen species caused by mitochondrial dysfunction in DS (Head and Lott 2004). Soluble Aβ may also be detrimental to cholinergic and glutaminergic neurotransmitter systems, thereby effecting cognition in DS individuals. Not all adults having DS develop clinical signs of dementia in spite of having consistent AD neuropathology, although the risk of dementia increases with age in adult DS patients. Onset of clinical signs of dementia in DS individuals may be due to additional stimulating risk factors involving apolipoprotein E genotype, sex and estrogen. These factors may slow or accelerate the development of dementia in DS individuals (Head and Lott 2004).

Amyloid precursor proteins are present on Chromosome 21 in both AD and DS individuals. Aβ peptides are formed from APP by two proteolytic pathways. Two enzymes β and γ secretase cleave APP at certain specific ends to generate Aβ(1-42) peptides. These Aβ(1-42) peptide fragments accumulate in the brain and lead to the formation of senile plaques. Two enzymes α and γ secretase cleave APP at specific ends to generate Aβ(17-42) peptide in both DS and AD individuals. Persons with DS have extensive deposition of Aβ(17-42) peptide, and AD individuals have deposition of both Aβ(17-42) and Aβ(1-42) peptide. Extensive research has been done on Aβ(1-42) and it has been shown that this peptide is toxic and leads to the formation of senile plaque in the brain. Senile plaques are one of the neuropathological factors found along with neurofibrillary tangles, synapse loss, neuronal loss and neuronal dysfunctions in AD patients. Synaptosomes were used in the previous literature to study the effects of Aβ(1-42) and it has been concluded that it is a toxic peptide that induces oxidative stress to the AD brain. It has also been found in that several proteins are oxidiatively modified due to the incubation of Aβ(1-

42) in synaptosomes. As a consequence of oxidative stress induced by this peptide, oxidative damage to the cell may ultimately leads to cell death. As Aβ(17-42) peptide is found both in DS and AD, we used this peptide as our model to study its toxic effects. The extensive deposition of Aβ(17-42) peptide in Down Syndrome patients could contribute to their increased rate of developing AD. Investigating the toxic effect of this peptide would be useful and this may be helpful in future to possibly diagnose AD and prevent the progression of this devastating neurodegenerative disorder.

Since Alzheimer's disease cannot be properly diagnosed until autopsy, development of a novel Down syndrome model using Aβ(17-42) peptide could be beneficial in determining oxidative stress levels and their relationship to MCI, the earliest form of AD in order to possibly be used as a diagnostic tool for AD. This study demonstrated a significant difference between oxidative stress levels in Aβ(17-42) treated synaptosomes and control. By using proteomics, identification of potential biomarkers were assessed in Aβ(17-42) treated synaptosomes from Mongolian gerbils to provide insight into the proteins that are differentially expressed in the novel DS model and how they relate to proteins differentially expressed in MCI.

In this thesis, differentially expressed proteins were identified in Aβ(17-42) treated synaptosomes and their role in Down syndrome model will be compared in future with that of proteins of mild cognitive impairment. This could be helpful in the future as a possible diagnostic tool for the Alzheimer's disease.

CHAPTER TWO

BACKGROUND

2.1 Synapse

Human brain consists of approximately 10^{12} heterogeneous neurons that communicate with each other through the assistance of synapses. Approximately 10^{15} synapses are found in human brain (Pocklington, Armstrong et al. 2006). Synapses are either electrical or chemical contacts found between neurons. There are two different types of synapses located in the brain, electrical and chemical. Electrical synapses, also termed neuronal gap junctions, help in the propagation of electrical impulses from one cell to another and vice versa through direct, physical contact. They are often characterized by a relatively simple organization of membrane structure and associated organelles. Chemical synapses composed of a wide range of chemical neurotransmitters and neuropeptides that helps in the intercellular communication (Bai and Witzmann 2007).

Neuronal cell-cell communication occurs by chemical transmission, a complex protein – driven molecular mechanism, characterized by several events including synthesis, delivery, docking, fusion, neurotransmitter release, and reuptake (Bai and Witzmann 2007).

2.1.1. Structure

A synapse generally consists of three main components: a presynaptic component (presynaptic ending, axon terminal), a synaptic cleft, and a postsynaptic component (dendritic spine). A presynaptic ending is different from the post synaptic end by the presence of neurotransmitter-filled vesicles. The presynaptic axon terminal of the presynaptic component also consists of other organelles such as mitochondria, smooth endoplasmic reticulum,

microtubules, and neurofilaments (Figure 2.1). The synaptic cleft, composed of a dense plaque of intercellular material microfilament, is generally 20-30 nm wide and separates the pre- and postsynaptic membranes (Bai and Witzmann 2007).

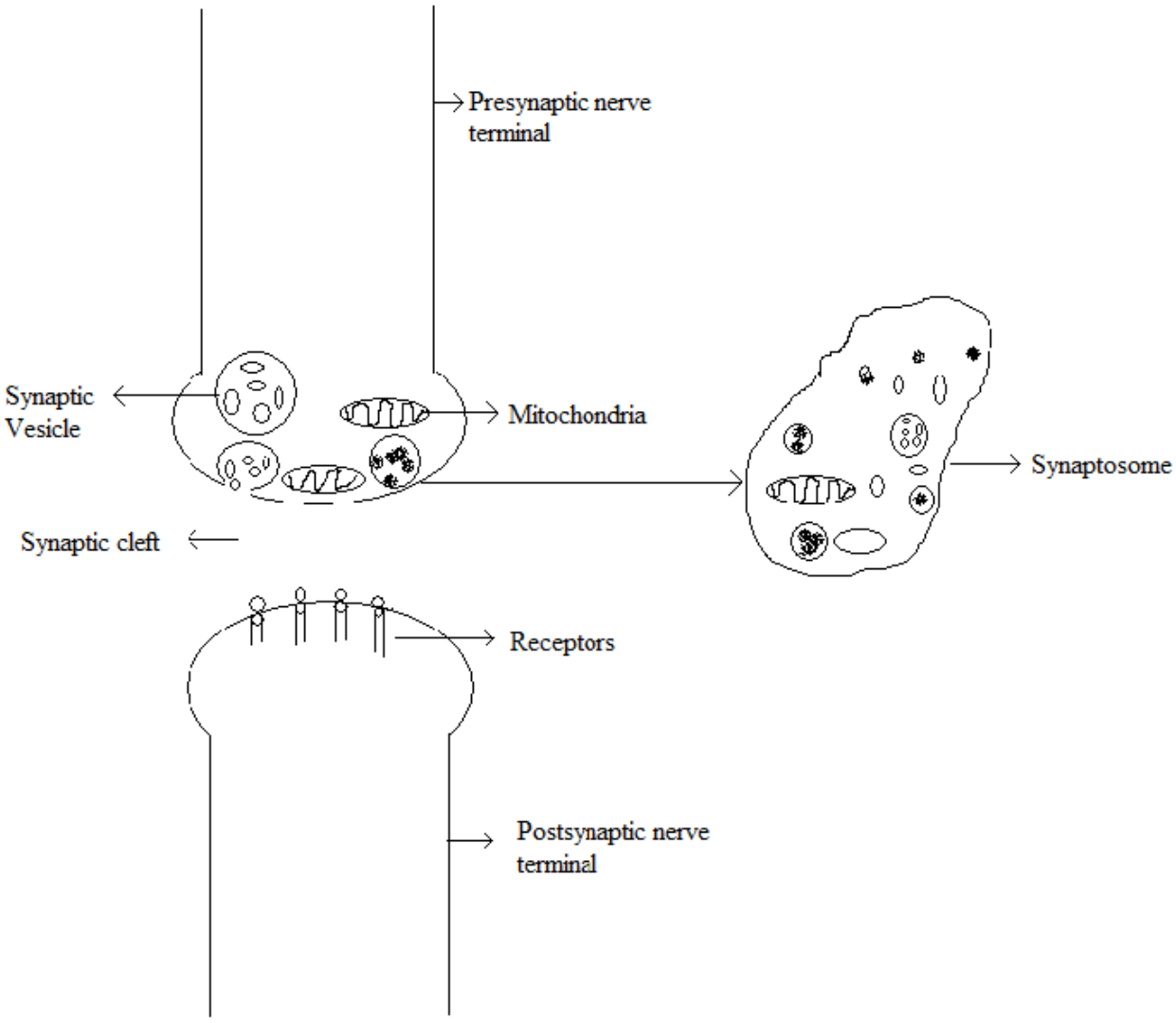


Figure 2.1 Illustration of a synapse.

2.1.2. Function

The synapse is considered to be the most important structure that helps in communication between cells. The signal transduction machinery and neurotransmitter receptors near the synapse respond to electrical activity and initiate biochemical changes in the nerve cell. During this process, they modify the brain in response to behavioral experience. The proteins associated with synapse correspond to several human disease genes and drug targets for therapeutics, and modulate cognitive illnesses (Pocklington, Armstrong et al. 2006). Synapse proteomic studies have helped in compiling a first draft of the protein composition of the synapse, revealing the high degree of molecular complexity (Pocklington, Armstrong et al. 2006).

2.2 Synaptosomes

Synaptosomes were first isolated by Hebb and Whittaker in 1958 (Hebb and Whittaker 1958).They are sealed presynaptic nerve terminals often having a portion of the target cell (Whittaker 1993). They are artificial, membranous sacs containing synaptic components. Synaptosomes contain mitochondria, and synaptic vesicles, beside postsynaptic membrane, and the postsynaptic density. These are prepared by sub-cellular fractionation of homogenized nerve tissue. They are often referred to as "pinched-off nerve endings," because during the homogenization of the nerve tissue, the lipid bilayers naturally reseal together after the axon terminals are torn off by the physical shearing forces during the homogenization process (Bai and Witzmann 2007). Synaptosomes are frequently used to study synaptic transmission because of the presence of molecular machinery for the release, uptake and storage of neurotransmitters (Bai and Witzmann 2007).

2.3 Oxidative stress

2.3.1 Overview

Oxidative stress may occur due to an imbalance between the prooxidant and antioxidant levels in the body. It has been implicated that oxidative stress play an important role in the pathogenesis of a number of diseases, including neurodegenerative disorders, and cancer. Brain is susceptible to oxidative damage because of its high utilization of oxygen, increased levels of polyunsaturated fatty acids, and addition to this the brain has relatively low antioxidants levels. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are highly reactive substances that can react with biomolecules such as proteins, lipids, carbohydrates, DNA, and RNA. ROS and RNS can cause oxidative damage to these biomolecules leading to cellular dysfunctions and can ultimately may cause cell death (Butterfield, Reed et al. 2007) The markers of oxidative stress commonly used in biological samples include protein carbonyls, 3-nitrotyrosine (3-NT) for protein oxidation and nitration. 4-hydroxy-2-trans-nonenal (HNE) is used as a marker for lipid peroxidation (Butterfield, Reed et al. 2007). Since proteins are more susceptible to the attack by ROS, they undergo oxidation which leads to loss of protein function as well as they get converted into more susceptible form that undergo degradation by proteasomes (Sultana and Butterfield 2009).

Oxidative stress can cause additional damage in brain via the overexpression of inducible nitric oxide synthase (iNOS) and neuronal NOS (nNOS) that increases the production of nitric oxide (NO•). Nitric oxide can react with superoxide anion ($O_2^{\bullet -}$) to generate peroxynitrite ($ONOO^-$). Peroxynitrite is very reactive; it can undergo a variety of chemical reactions. In the presence of CO_2, it can transform the available reactive targets forming modification like 3-NT

3-Nitrotyrosine is a covalent protein modification that can be used as a marker of nitrosative stress various disease conditions (Butterfield, Reed et al. 2007).

2.3.2 Oxidative stress and Down syndrome

Individuals with DS have unusually high oxidative stress levels. These persons have high vulnerability to oxidative stress caused by the overexpression of superoxide dismutase (SOD), an antioxidant enzyme coded on chromosome 21. The increased oxidative stress may cause oxidative damage to important macromolecules. The high levels of oxidative stress could be due to the excess activity of Cu-Zn superoxide dismutase (Cu, Zn-SOD) or may be due to an imbalance in other redox-related activities (Zitnanova, Korytar et al. 2006). A study found that DS patients have an elevated ratio of superoxide dismutase to catalase and glutathione peroxidase compared to controls, suggesting the role of redox imbalance causes accelerated aging (Zitnanova, Korytar et al. 2006).

2.3.3 Protein oxidation

Protein oxidation can lead to aggregation or dimerization of proteins, it may also lead to unfolding or conformational changes in the protein, by which the hydrophobic residues of proteins are exposed to an aqueous environment. As a result, proteins may lose their structural or functional activity. The accumulation of oxidatively modified proteins can cause harmful damage such as cellular dysfunction by alterations in protein expression, gene regulation, protein turnover, modulation of cell signaling, and apoptosis. Measuring the levels of protein oxidation, protein nitration, lipid peroxidation are some ways to determine protein modifications.

2.3.4 Indexing protein carbonylation

By measuring protein carbonylation, researchers can determine overall protein oxidation. Proteins become get oxidatively modified by interacting with ROS and RNS. Examples of these toxic species are listed in Table 1.1 (Please refer to Table 1.1, page 3). Free radicals are produced during the secondary reactions of these reactive oxygen species with proteins, lipids, and nucleic acids.

2.3.5 3-Nitrotyrosine (3-NT)

Nitration of tyrosine is a pathological event associated with several neurological diseases including Alzheimer's disease, amyotrophic lateral sclerosis, and Parkinson's disease (Castegna, Thongboonkerd et al. 2003). Protein nitration is a marker of protein oxidation, where a nitro group is generally attached to tyrosine residues at the third position to form 3-nitro tyrosine (3-NT). Nitrated proteins are susceptible to proteosomal degradation. Another toxic reactive nitrogen species, peroxynitrite ($ONOO^-$), is formed by the reaction of nitric oxide (NO•) with superoxide anion ($O_2•^-$). Peroxynitrite undergoes protonation and generates cytotoxic species that can oxidize and nitrate proteins (Figure 2.2) (Castegna, Thongboonkerd et al. 2003). Peroxynitrite can also react with carbon dioxide (CO_2) (Figure 2.3 a) and induce modifications such as protein nitration (Figure 2.3 b) (Butterfield, Reed et al. 2007).

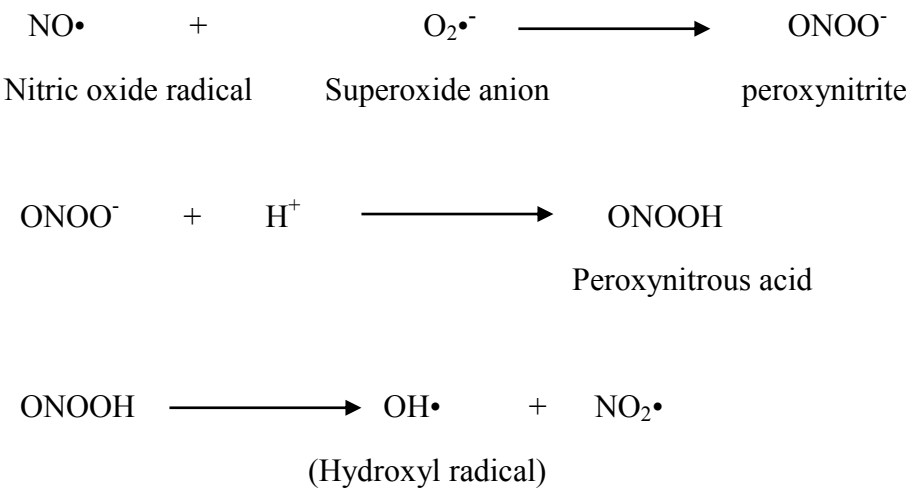


Figure 2.2 Reaction of nitric oxide to give peroxynitrite which can ultimately produce toxic hydroxyl radical (Contestabile 2001).

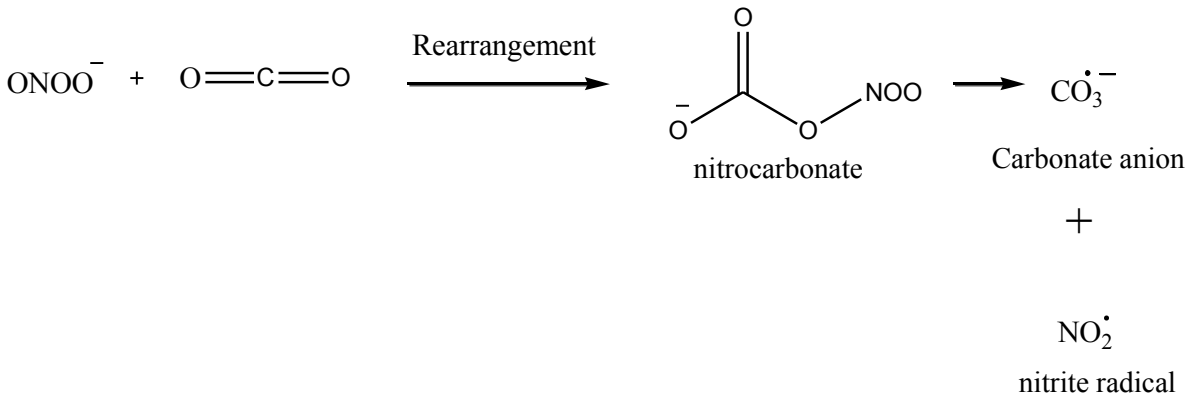


Figure 2.3 a) Nitration of tyrosine: Reaction of peroxynitrite and carbon dioxide (Butterfield, Reed et al. 2007).

Nitration of tyrosine occurs at the third position and sterically hinders phosphorylation, which may result in the structural change of proteins. Nitration may cause proteins to become dysfunctional and could eventually lead to cell death (Butterfield, Reed et al. 2007). Measuring levels of 3-nitrotyrosine can be used as a marker of nitrosative stress in a variety of disease conditions.

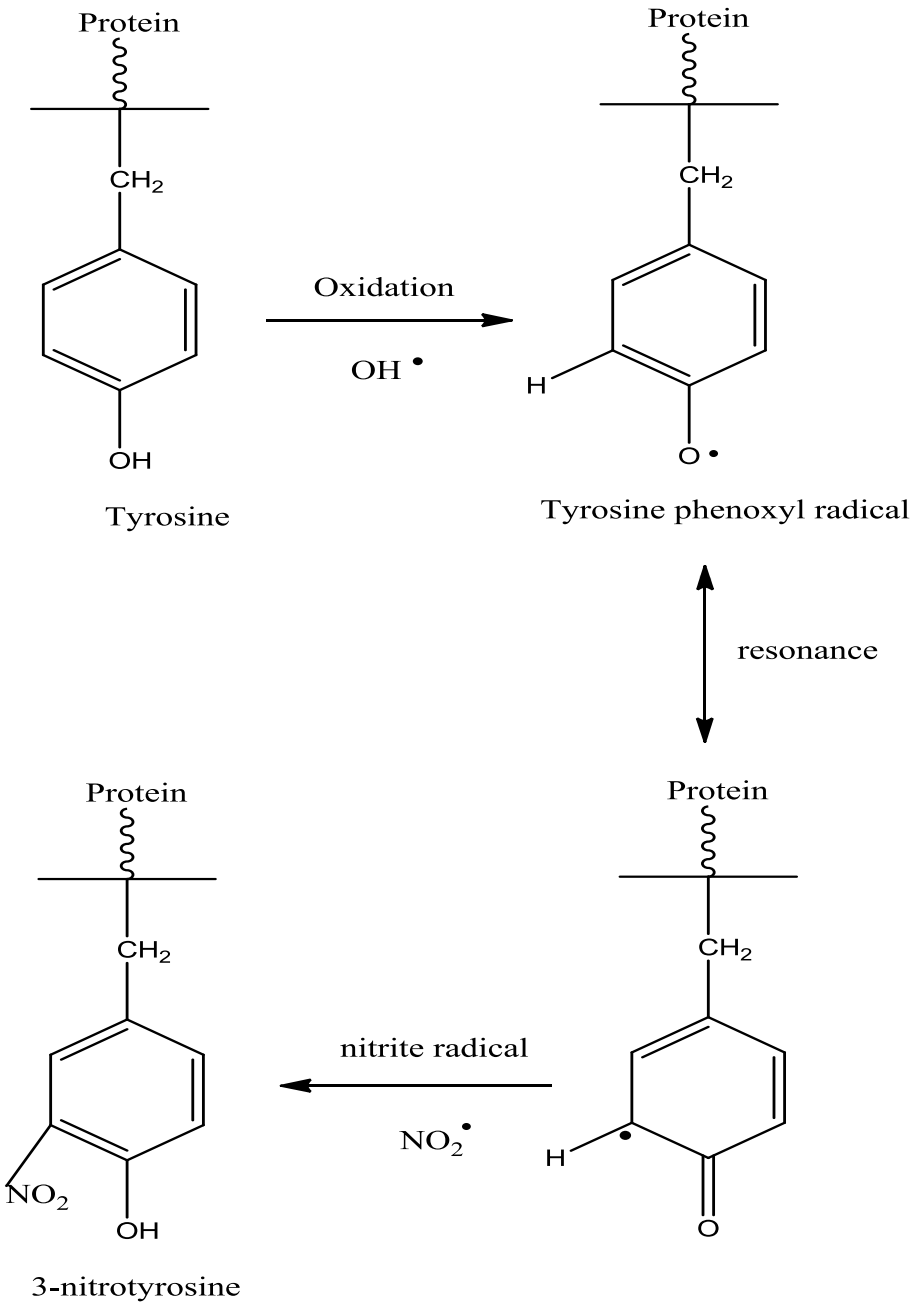


Figure 2.3 b) Nitration of tyrosine: Formation of 3-nitrotyrosine (Butterfield, Reed et al. 2007).

Nitric oxide is considered to be among the ten smallest molecules existing in nature (Contestabile 2001). The brain consists of neuronal nitric oxide synthase (nNOS), endothelial (eNOS), and the inducible (iNOS) isoforms in different cellular components. Each isoforms consumes L-arginine and produces equal amounts of L-citrulline and NO, and requires Ca^{2+}-calmodulin protein for activity (Ghafourifar and Cadenas 2005). NO exerts a wide range of function in several system, such as the cardiovascular system, peripheral nervous system (PNS), central nervous system (CNS) and immune system (Ghafourifar and Cadenas 2005). Oxidative stress could also stimulate damage to brain via the overexpression of inducible nitric oxide synthase (nNOS) and neuronal (nNOS), that increases the formation of nitric oxide (NO•) through catalytic conversion of L-arginine to citrulline (Butterfield, Reed et al. 2007). Nitric oxide can injure brain tissue through nitrosylation of proteins, DNA breaking, and other molecular interactions. At physiological pH, it can interact with the superoxide radical to produce peroxynitrite. Peroxynitrite is cytotoxic which can further accept a proton and decompose to produce the highly toxic hydroxyl radical (Contestabile 2001).

In addition to its harmful effects, nitric oxide has also been considered as an antioxidant and neuroprotective, due to its ability to scavenge toxic free radicals including superoxide, hydroxyl and peroxyl lipid radicals. It can also react with thiyl radical GS• which is formed from reduced glutathione (GSH) during oxidative stress, giving rise to a substance that further acts as an antioxidant, S-nitrosoglutathione (Contestabile 2001).

2.3.6 Lipid peroxidation

Lipid peroxidation has been extensively found in several neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and prion

disease (Sultana, Perluigi et al. 2006). Lipid peroxidation is considered as one of the major sources of free radical-mediated cell injury damaging membranes and generating a number of secondary products. The brain is vulnerable to lipid peroxidation because of its high utilization of oxygen, low levels of antioxidants, and high levels of polyunsaturated fatty acids which are the substrates for lipid peroxidation (Sultana, Perluigi et al. 2006). Lipid peroxidation is a complex process which involves the interaction of oxygen-derived free radicals with polyunsaturated fatty acids and resulting in production of different kinds of highly reactive electrophilic aldehydes (Figure 2.4). These reactive aldehydes are capable of easily attaching covalently with proteins and forming adduct with cysteine, lysine, or histidine residues. Malonaldehyde (MDA), 4-hydroxynonenal (HNE), and acrolein are among the aldehydes formed and represents the major lipid peroxidation products (Esterbauer, Schaur et al. 1991). Lipid peroxidation of membrane lipids can have dangerous effects including decreased activity of membrane-bound enzymes (i.e., sodium pumps), increased rigidity of the membranes, altered activity of receptors present on membranes, and membrane permeability. Radicals can attack membrane proteins and may induce lipid-lipid, protein-protein, and lipid-protein cross-linking that can have harmful effects on membrane functions (Sultana, Perluigi et al. 2006).

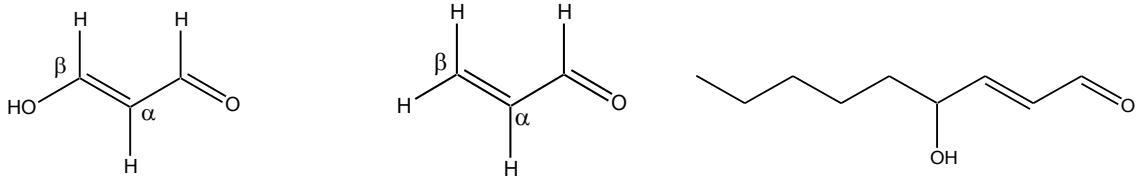


Figure 2.4 Products of lipid peroxidation (Sultana, Perluigi et al. 2006).

Oxidative degradation to lipid is known as lipid peroxidation. In this process, free radicals abstract a proton from the lipid in cell membrane, causing cell damage. It involves a free radical chain-reaction mechanism comprising of several steps (Figure 2.5) (Sultana, Perluigi et al. 2006).

Step 1: $LH + X\bullet \rightarrow L\bullet + XH$

Step 2: $L\bullet + O_2 \rightarrow LOO\bullet$

Step 3: $LOO\bullet + LH \rightarrow LOOH + L\bullet$

Step 4: $2\ LOO\bullet \rightarrow$ nonradical + O_2

Step 5: $LOO\bullet$ + antioxidants (TOH) $\rightarrow LOOH + TO\bullet$

Figure 2.5 Mechanism of lipid peroxidation by free radicals and inhibition by the antioxidant, tocopherol (Sultana, Perluigi et al. 2006).

Protein bound-HNE levels alter protein conformation and function (Butterfield, Reed et al. 2007). 4-Hydroxynonenal is an α,β unsaturated alkenal obtained by omega-6 polyunsaturated fatty acid peroxidation and is a cytotoxic endproduct of lipid peroxidation. HNE forms Michael adducts by covalently binding to cysteine, histidine, or lysine residues and can cause cell death by mediating oxidative stress (Sultana and Butterfield 2009). By measuring HNE levels, researchers can determine lipid peroxidation.

2.3.7 Source of free radicals

Free radicals may be defined as molecules or molecular fragments having one or more unpaired electrons. This unpaired electron(s) are usually responsible for the reactivity of the free

radicals. Mostly oxygen derived free radicals are considered to be the most important class produced in living systems (Valko, Leibfritz et al. 2007). Free radicals are produced during mitochondrial respiration, autooxidation of a variety of biological molecules and chemicals, irradiation damage, and are found as environmental pollutants. Free radicals are scavenged by enzymes like superoxide dismutase, glutathione peroxidase, and Vitamin C & E (Leibovitz and Siegel 1980). Superoxide radical ($O_2\bullet^-$) formation is the initial event in the production of free radicals. Superoxide dismutase reacts with superoxide radical and produces hydrogen peroxide (H_2O_2), which then can react with another superoxide radical to produce the hydroxyl radical, a more potent radical. Superoxide radical decomposes the hydrogen peroxide into hydroxyl radical (OH•), this reaction is catalyzed by ferric ion (Leibovitz and Siegel 1980). Free radicals can produce lipid peroxides. When free radicals attack polyunsaturated fatty acids it produces lipid peroxy radicals. Peroxy radicals can abstract a proton and electron and finally produces lipid peroxides. Lipid peroxides may decompose to produce aldehydes which can cross-link proteins, lipids, and nucleic acids (Leibovitz and Siegel 1980).

2.3.7.1 Reactive oxygen species

The body can be exposed to various reactive oxygen species that may damage proteins directly or indirectly. ROS can be produced by several different mechanisms such as irradiation (i.e. X-rays, γ-rays, and UV rays), inflammatory processes, metal-catalyzed oxidation (MCO) systems, electron-transport processes, glycation/glycoxidation, and environmental pollutants. These produce various types of oxygen free radicals, nonradical oxygen derivatives, 4-hydroxy-2-nonenal and others (please refer Table 1.1, page 3) (Stadtman and Berlett 1998).

The central nervous system is prone to get damaged when it is exposed to the attack by ROS. The brain is more prone to oxidative stress and stress related degeneration. Neural cells are enriched in mitochondria and therefore have a very high aerobic metabolism. In resting state brain consumes about 20% of the total oxygen (Contestabile 2001). As it is very active in oxidative metabolism, this results in large production of superoxide. In the electron transport chain, 2-5% of oxygen consumed, and it is converted into superoxide. Superoxide is converted into hydrogen peroxide and oxygen in the presence of superoxide dismutase (Smith, Kapoor et al. 1999). Hydrogen peroxide is converted into water by catalase or glutathione peroxidase (Figure 2.6). Under abnormal conditions, hydrogen peroxide in the presence of decompartmentalized transition metals (i.e. Cu and Fe) can be converted into highly toxic hydroxyl radical (Smith, Kapoor et al. 1999).

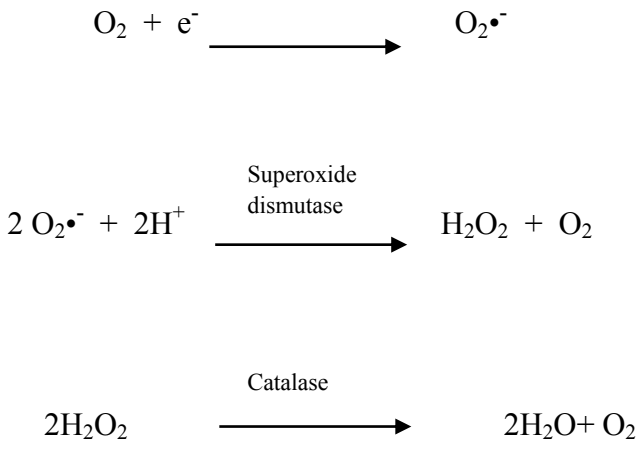


Figure 2.6 Formation of superoxide oxide, hydrogen peroxide and water molecules.

At very high concentrations, reactive oxygen species can cause damage to cell structures, nucleic acids, proteins and lipids. The hydroxyl radical is known to react with the DNA molecule, causing damage to purine and pyrimidine bases and also damaging deoxyribose sugar backbone (Valko, Leibfritz et al. 2007). As a result of these modifications to genetic material,

this triggers the initial step in mutagenesis, carcinogenesis, and aging. It is also known that metal can induce generation of ROS, they not only attack DNA but also effect cellular components involving polyunsaturated fatty acid residues of phospholipids. These residues are very sensitive to oxidation. HNE, a major product of lipid peroxidation, is weakly mutagenic but considered as a major toxic product of lipid peroxidation (Valko, Leibfritz et al. 2007).

The human body has a defense mechanism to protect against reactive oxygen species in the form of antioxidants. It mainly includes scavenger enzymes and some several vitamins including Vitamin C (ascorbic acid), Vitamin E (tocopherol), and Vitamin A (retinoic acid). Others include N-acetylcysteine and carotenoids that are derived exogenously and are precursors of endogenous antioxidants. Main antioxidant enzymes include superoxide dismutase (Cu, Zn SOD, Mn SOD, extracellular SOD), catalase and glutathione peroxidase. Under normal physiological conditions these defense mechanism prevent the damage cause by ROS. In certain abnormal conditions like aging, neurodegeneration, ROS production rises significantly, the defense mechanism of body is reduced that may endanger the structural and functional integrity of the cell. This condition may cause reversible or irreversible damage to the cell (Contestabile 2001).

2.3.7.2 Reactive nitrogen species

Proteins are highly sensitive to reactive nitrogen species (RNS) and reactive oxygen species (ROS) as they undergo oxidative modifications in the presence of them. Some of the reactive nitrogen species includes nitric oxide, peroxynitrite, and nitrogen dioxide.

2.3.7.3 Nitric oxide (NO•)

Nitric oxide (NO•) is a free radical that helps in various important oxidative biological signaling in a large variety of physiological processes, like neurotransmission, defense mechanisms, blood pressure regulation, smooth muscle relaxation, synaptic plasticity and immune regulation (Valko, Leibfritz et al. 2007). Nitrosative stress refers to overproduction of reactive nitrogen species. Nitrosative stress may occur when the balance between the production and neutralization of reactive nitrogen species is impaired in the body. Due to overproduction of reactive nitrogen species body cannot eliminate them and that leads to damage to proteins. Excess production of reactive nitrogen species can change the structure of proteins and may lead to inhibition of the normal protein function (Valko, Leibfritz et al. 2007).

During inflammatory processes, cells of the immune system produces nitric oxide (NO•) and superoxide anion ($O_2^{\bullet -}$). Nitric oxide and superoxide anion reacts with each other during this oxidative stress conditions and produce more oxidatively active peroxynitrite anion (ONOO-) molecule (Figure 2.7). This molecule can react with DNA causing DNA fragmentation and can also react with lipid to cause lipid oxidation (Valko, Leibfritz et al. 2007).

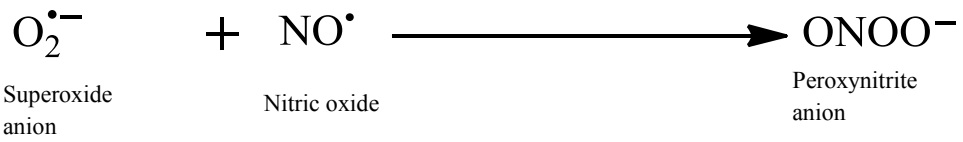


Figure 2.7 Formation of peroxynitrite.

2.4 Down syndrome and Alzheimer's disease

2.4.1 Overview

Alzheimer's disease is the most common cause of dementia. It is associated with distinct neuropathology by the deposition of extracellular amyloid beta plaques, intracellular neurofibrillary tangles, neuronal loss and dysfunction (Butterfield, Perluigi et al. 2006). Neurofibrillary tangles and amyloid plaques deposition in AD has been found extensively in the hippocampus, neocortex, and amygdala region of the brain (Nizetic 2001). There is cumulative evidence suggesting a key role of oxidative stress in the pathophysiology of AD. Free radicals produced during oxidative stress are believed to be critically important in AD and other neurodegenerative diseases. Oxidative stress is extensively elevated in the Alzheimer's disease brain (Butterfield, Boyd-Kimball et al. 2003). Oxidative stress in AD brain is manifested by: a) protein oxidation , as demonstrated by an increase levels of protein carbonyls and nitrated proteins, b) lipid peroxidation via increased levels of malonaldialdehyde, 4-hyroxy-2-nonenal, isoprostanes and altered composition of phospholipids, c) RNA and DNA damage, as indexed by increased levels of 8-hydroxyguanosine, and d) reactive oxygen and reactive nitrogen species (Butterfield, Perluigi et al. 2006).

Down syndrome is associated with immune deficiency, heart defects, and increased risk of leukemia and early development of Alzheimer's disease. The most common feature among all Down syndrome patients is the presence of mental retardation (Perez-Cremades, Hernandez et al. 2010). Down syndrome is linked with Alzheimer's disease in many different ways. Nearly all individuals with DS show similar distinctive neuropathological characteristics found in AD including β-amyloid (Aβ) plaques and neurofibrillary tangles (Lott, Head et al. 2006). DS

individuals develop Alzheimer's disease pathology with increase in age, and are at high risk of developing dementia. DS individuals almost develop AD neuropathology by the age of 40 (Head and Lott 2004). DS individuals have been found to have a reduced risk of developing hypertension (Wiseman, Alford et al. 2009).

2.4.2 Genetic factors

Research have shown that mutation of amyloid precursor protein genes, presenilin-1and presenilin-2, cause familial Alzheimer's disease that leads to increased deposition of fibrillary β-amyloid in the brain. AD has also been associated with the apolipoprotein E gene, allele 4, the endothelial nitric oxide synthase-3 gene, and the alpha-2-macroglobulin gene (Butterfield, Perluigi et al. 2006).

Down syndrome is most important genetic cause of intellectual disability (Irving, Basu et al. 2008). Down syndrome is caused by trisomy, a genetic abnormality in which three copies of chromosome 21(Moncaster, Pineda et al. 2010) is present instead of usual two. Abnormal cell division occurs during early fetal development. Down syndrome is the most common form of chromosomal abnormality found in live-born infants. This disorder occurs in 1 out of 800 live child births (Peterson 2006). Genetic risk factors associated with Down syndrome are Apolipoprotein E (ApoE), inflammation, oxidative stress and also early deposition of Aβ(17-42) peptide (Lott, Head et al. 2006).

Evidence shows that APP overexpression may lead to the development of AD in Down syndrome individuals. Many evidence shows that a lack of balance in the metabolism of free radicals may have a direct role in the development of neuropathology in DS. Along with APP,

chromosome 21 has other genes such as SOD1, Ets-2 and DSCR1 and they may lead to progressive oxidative damage (Lott, Head et al. 2006). Superoxide dismutase 1 (SOD1) overexpression has been considered as a lingering question in DS research. SOD1, located on chromosome 21, it is a cytosolic enzyme that catalyzes the conversion of superoxide to hydrogen peroxide and oxygen. In the next step, hydrogen peroxide is converted to water with the help of glutathione peroxidase or catalase. Alteration in the balance between hydrogen peroxide and antioxidant levels may produces excess of hydrogen peroxide, which then can undergo Fenton reaction to produce hydroxyl radical through its interaction with transition metals (Lott, Head et al. 2006). Under stress conditions, it has been found that an excess of superoxide releases "free iron" from iron-containing molecules. The released iron (Fe^{2+}) can take part in the Fenton reaction and produces hydroxyl radical (Figure 2.8) (Valko, Leibfritz et al. 2007).

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH\bullet + OH^-$$

Figure 2.8 Generation of hydroxyl radical (Valko, Leibfritz et al. 2007).

Ets-2, also found on chromosome 21, is a member of the Ets family of transcription factors, which is involved in various cellular processes like maturation, differentiation, and activation of signaling cascades. In DS individuals with AD and sporadic AD brain, Ets-2 expression is increased, which suggests a possible role of oxidative stress in both AD and DS (Lott, Head et al. 2006). DSCR is considered as stress-inducible factor that binds and inhibits calcineurin, a phosphatase responsible in multiple signaling pathways of the cell. It is interesting to note that DSCR1 expression stimulates gene expression of SOD1. Hence, altered expression of DSCR1 effect oxidative stress directly by increasing mitochondrial ROS production and indirectly by upregulation or overexpression of SOD1 (Lott, Head et al. 2006).

Trisomy 21 has a powerful downward effect on intelligence quotient (IQ) in Down syndrome individuals. Unlike normal children, they have a progressive decline in IQ during their first year of life. When they become adults, the IQ is usually lies between moderate to the severe retarded region (IQ = 25-55), while an average IQ is equivalent to 100 (Nizetic 2001). However, there are DS individuals who have a little to no defect in IQ. Trisomy 21 individuals are well-known to be at high risk of developing Alzheimer's disease during their middle age of life. Almost 100 % of the DS individuals show similar neuropathology to that of Alzheimer's disease by the age of 40 (Nizetic 2001). Down syndrome is therefore considered as an important but inadequate way to study the dose effects of specific chromosome 21 genes on the Alzheimer's disease pathogenesis (Nizetic 2001). There is a relationship between trisomy 21 and cancer in DS individuals; they have a 20-fold increase risk of developing childhood leukemia (Nizetic 2001).

2.4.3 Amyloid beta peptide

It has been shown that there is extensive accumulation of Aβ accumulation in brain of both Down syndrome and Alzheimer's disease (Wei, Norton et al. 2002). Aβ peptides are formed from amyloid precursor protein (APP) by two proteolytic pathways. One is formed by the sequential cleavage of APP by β and γ secretase that generate Aβ(1-42) peptide fragment (Figure 2.9 a), second is formed by the sequential cleavage of APP by α- and γ that produces Aβ(17-42) peptide fragments which is also known as p3 fragment (Figure 2.9 b). Aβ(1-42) peptide fragment has been found extensively in AD along with the deposition of Aβ(17-42) peptide fragment (Wei, Norton et al. 2002). DS patients have extensive deposition of Aβ(17-42) (Please refer to Figure 1.2, page 4) peptide, which could contribute to their increased rate of developing AD.

Aβ(17-42) is the major preamyloid fragment in DS while the major fragment in AD is Aβ(1-42). It is speculated that disruption of normal function of Amyloid precursor protein including altered processing or overexpression of APP is mostly responsible for amyloid plaque formation, neuronal loss, and dementia (Wei, Norton et al. 2002).

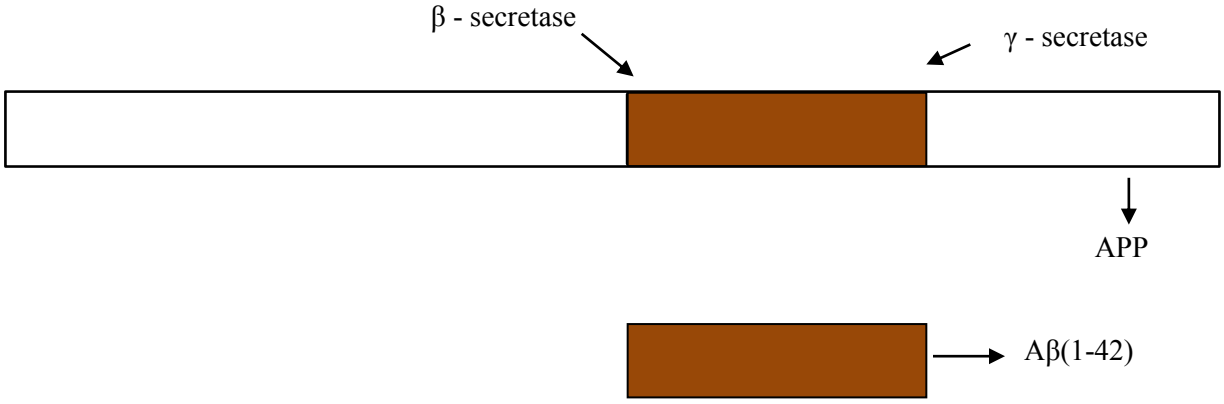


Figure 2.9 a) Formation of Aβ(1-42) by proteolytic cleavage of APP by β and γ secretase.

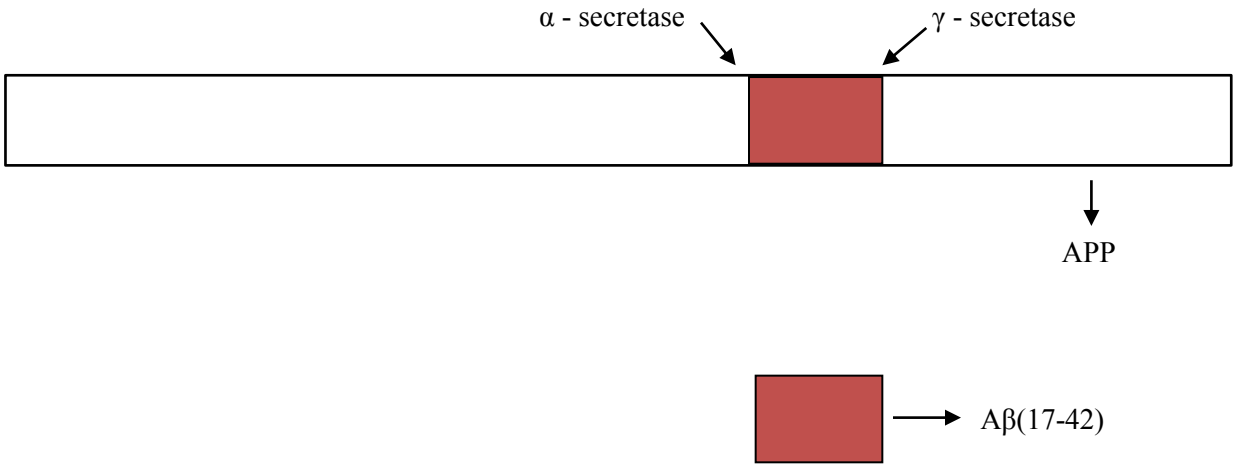


Figure 2.9 b) Formation of Aβ(17-42) by proteolytic cleavage of APP by α and γ secretase.

2.5 Proteomics

Proteomics deals with the large scale study of proteins, particularly of their structures and function (Butterfield, Perluigi et al. 2006). It involves the identification and quantification of proteins of interest pertaining to disease state (Figure 2.10). Proteomics also describes the information enclosed in genomic sequences in terms of the structure, function and control of biological processes comprehensively by the systematic analysis of the proteins expressed in a tissue or cell. The proteome is the protein complement expressed by a genome. It reflects the external conditions encountered or the cellular state of a cell. The analysis of proteome can be explored as a genome–wide assay to differentiate and to study cellular states. It is also viewed as to determine the molecular mechanisms that control them (Aebersold and Goodlett 2001). Mass spectrometry is one of the important tool that is being widely used in proteomics research (Nesvizhskii, Vitek et al. 2007). It has currently become the method of choice for identification, quantification and primary structure analysis of the protein components of complex sample mixtures in proteomic studies (Schmidt, Claassen et al. 2009). One of the key advancements in mass spectrometry (MS) based proteomics over the past few years is the huge increase in the quality of the data in terms of high accuracy and high resolution (Choudhary and Mann 2010). Mass spectrometry based proteomics have been used to generate several new biological and clinical insights such as the discovery of new proteins associated with organelles or signaling systems and the discovery of protein disease biomarkers (Aebersold 2009).

Two-dimensional gel electrophoresis is mostly used for separation of proteins in proteomic studies. Trypsin is a proteolytic enzyme that cleaves the protein at lysine and arginine residues of the carboxyl terminal of the polypeptide chain. Mass spectrometry is a widely used tool to

analyze the proteins of interest. MALDI-TOF is an instrument that has been used for determination of molecular weight of the resulting peptide. Database searching for the identification of proteins can be done by using search engine like MASCOT, Profound, MS-fit, and Peptident (Butterfield 2004). Other search engines include SEAQuest and Protein Prospector.

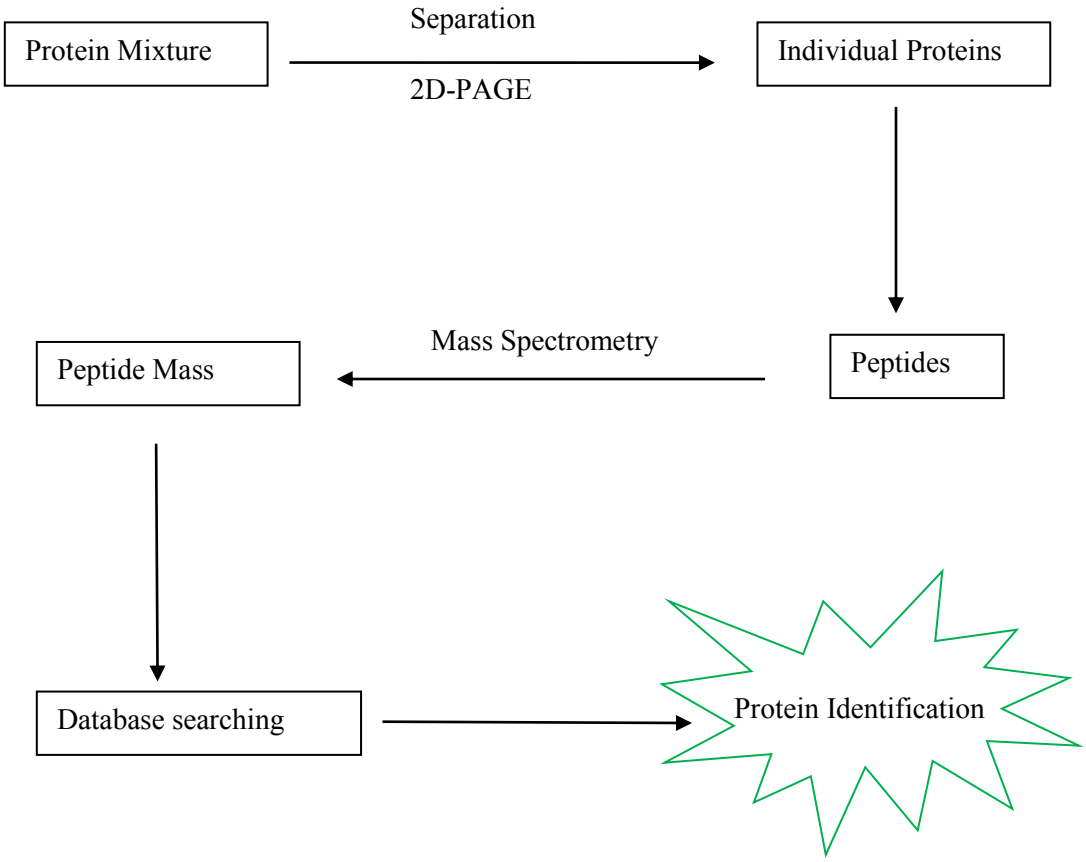


Figure 2.10 Basic scheme of proteomic analysis.

2.5.1 Background

Proteomics involves the systematic separation, identification, and quantification of many proteins from a sample (Sultana, Perluigi et al. 2006). Large scale increase in diversity of proteins is speculated to be because of alternative splicing and post-translational modification of proteins. Gene expression alone is not enough to fully characterized diversity of proteins; proteomics on the other hand is a very useful tool for characterizing cells and of interest. Proteomics helps to determine structure of proteins; it helps to determine modifications, localizations, and protein-protein interactions in addition to protein expression levels. Proteomics relies greatly on two-dimensional electrophoresis (2D-PAGE) for separation of proteins and mass spectrometry to identify the proteins of interest. 2D-gel electrophoresis uses isoelectric focusing and sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) in order to separate proteins from a sample (Sultana, Perluigi et al. 2006).

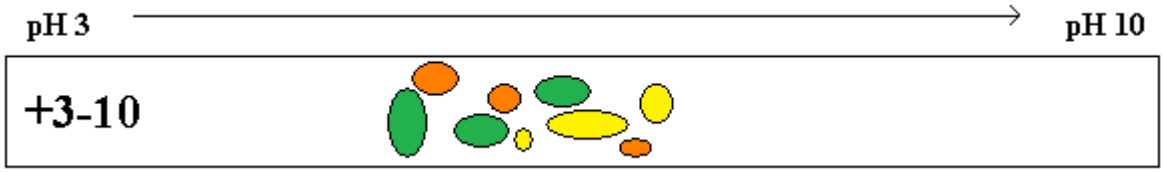
Proteins can also separated by using 2D-high performance liquid chromatography (2D-HPLC) that separate protein mixture by eluting the sample through series of columns and analyzed by coupling with mass spectrometer (Sultana, Perluigi et al. 2006). Alternatively, the expression profiles can also be done by using a technique known as isotopically coded affinity tags (ICAT). In this technique a mixture of samples are labeled with different isotopes that bind to specific amino acid –side chains. The isotopic labeling resulted is further analyzed by a mass spectrometer (Sultana, Perluigi et al. 2006). In proteomics studies, mass spectrometry is a key tool for analyzing the proteins of interest.

In 2D-gel electrophoresis, the proteins are first separated based on their isoelectric point during isoelectric focusing. Isoelectric point (pI) is the pH at which the protein has a net charge

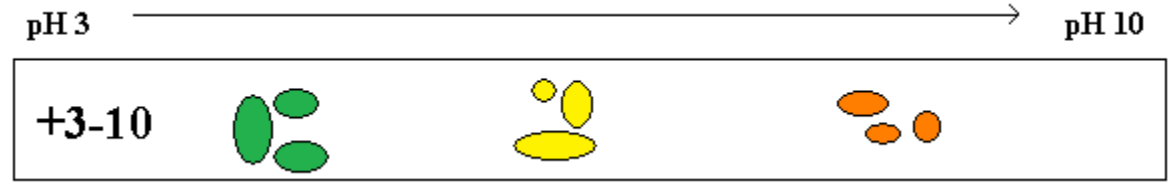
of zero and is subsequently immobilized. Proteins are mixed with rehydration buffer, which allows more dilute samples to be loaded and also allows large quantities of samples to be loaded and separated by preventing evaporation of proteins. The samples are place on an immobilized proteins gradient (IPG) strip that is composed of a polyacrylamide matrix. The rehydration buffer consists of urea (chaotropic agent that denatures proteins), thiourea (denatures proteins), CHAPS (chaotropic agent that denatures proteins), and dithiothreitol (reduces disulfide bonds), bromopheonol (tracking dye) and ampholyte (helps to move protein based on their isoelectric points). Thus the proteins are separated based on their isoelectric point in the isoelectric focusing (IEF) cells when electricity is passed.

In the second dimension, the proteins are separated based on their molecular mass. The IPG strips containing proteins that have been separated in the first dimension are treated with a buffer containing dithiothreitol (DTT). Then the strips are removed from the DTT solution and treated with iodoacetamide solution (IA). IA alkylates the thiol groups and prevents recombining. As IA is photosensitive, this reaction is done under dark. The strips are then loaded onto the gels. A marker is added for better comparison of migration rates of identified protein to those of known standards and voltage is applied. High molecular weight proteins travel slower and low molecular proteins travel faster and thus proteins get separated in the second dimension in the SDS-PAGE (Figure 2.11).

IPG Strip before application of current



IPG strip after application of current



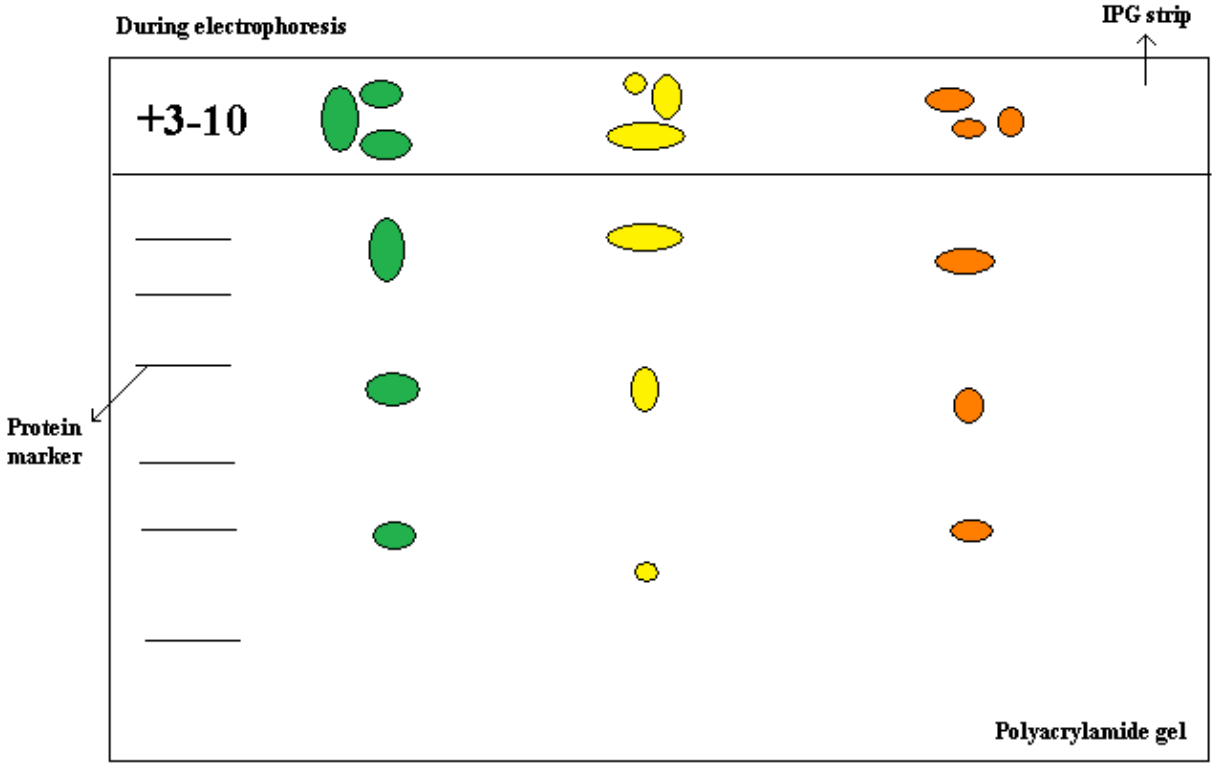


Figure 2.11 Overview of IEF and 2D gel electrophoresis.

2.5.2 2D gel electrophoresis

Two-dimensional polyacrylamide gel electrophoresis is a separation technique used to separate a mixture of proteins based on their isoelectric points and sizes. Isoelectric focusing combined with SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) commonly referred to as two dimensional (2D) electrophoresis.

IPG (immobilized pH gradient) strips were used for the separation of proteins based on their isoelectric points in an isoelectric focusing cell. SDS-PAGE is a process in which proteins are separated on the basis of their molecular weight. SDS stands for sodium dodecyl sulfate which is an anionic surfactant used to denature proteins to their primary structure and gives them a negative charge so that they can be attracted towards the positive terminal of polyacrylamide gel electrophoretic chamber.

Proteins present on the IPG strips were subjected to a voltage of 200V for 65 minutes in the 2D-gel electrophoresis apparatus (Butterfield 2004). The proteins were separated on the 8-16% Criterion polyacrylamide gels (Bio-Rad, Hercules, CA, USA). The gels were fixed with fixing solution in order to prevent movement of proteins in the 2D gel following electrophoresis. The gels were treated with SYPRO ruby stain (Bio-Rad, Hercules, CA, USA), a fluorescent stain that helps to visualize the proteins present on the gels. A UV transilluminator is used to help visualize the fluorescent gels while the Edovek photobox containing a built in camera, was used to take pictures of the gels. Images were analyzed using PDQuest software that gives the intensity of the proteins present on the gels. Significant differences in intensity were found and the excised proteins spots were send for mass spectrometric analyses.

2.5.3 Mass spectrometry

2.5.3.1 Overview

Mass spectrometry is a very important analytical tool with various applications in chemistry, biochemistry, pharmacy and medicine. It is used to examine various sequences of biomolecules, structural elucidation of unknown compounds including environmental and forensic analysis. It is also used in quality control of flavors, drugs, and polymers (Gross 2004). The most recent applications of mass spectrometry include the proteome and metabolome, high throughput in drug discovery and metabolism (Hoffmann and Stroobant 2007). Mass spectrometry is considered to be the most important tool in proteomics (Aebersold and Goodlett 2001).

A mass spectrometer consists of an ion source that generates ions, a mass analyzer that separates ions into mass-to-charge ratio (m/z) of the ionized samples, and a detector that detect the number of ions at each mass to charge value (Figure 2.12) (Aebersold and Mann 2003). Matrix assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) are the two techniques most widely used to volatilize and ionize the peptides and proteins for mass spectrometric analysis (Aebersold and Mann 2003). MALDI is usually associated with the time of flight (TOF) mass analyzer that measures the mass of the intact peptides (Aebersold and Mann 2003). MALDI-TOF has been used for the proteomics study in Alzheimer's disease (Castegna, Thongboonkerd et al. 2003).

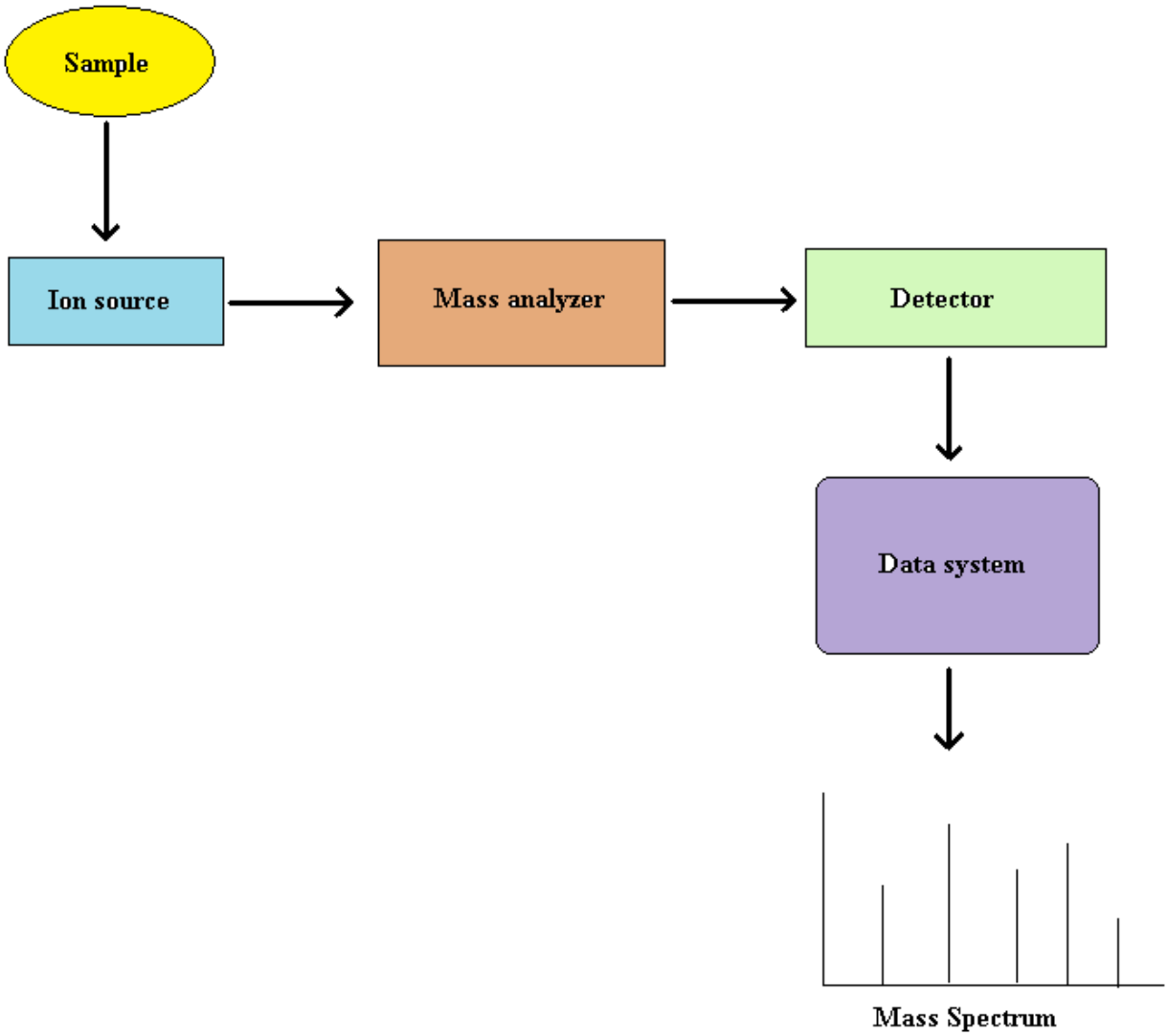


Figure 2.12 Block diagram of a mass spectrometer.

2.5.3.2 Electrospray Ionization (ESI)

Electrospray mass spectrometry (ESMS) was developed by Fenn for analyzing biological samples (Mann, Hendrickson et al. 2001). In this ionization technique, liquid is mixed with an analyte and pumped at low microliter-per-minute flow rates through a hypodermic

needle at high voltage to electrostatically disperse or electrospray. Small, micrometer-size droplets are formed, which them rapidly evaporate and transfer their charge onto analyte molecules. This ionization process takes place in atmospheric pressure and is therefore very tender, and little to no fragmentation of analyte ions are produced in the gas phase. The charged analyte molecules are then moved into the mass spectrometry for analysis with high efficiency. A nebulizer gas or some other device is frequently used to stabilize the spray (Mann, Hendrickson et al. 2001).

A wide range of compounds that are sufficiently polar which allows attachment of a charge on them can be analyzed by using ESMS. Proteins, sugars, oligonucleotides, and polar lipids can be easily analyzed. Since sugars have less sensitivity, sodium is used as a charging agent instead of hydrogen. There is no upper mass limit for this technique, large multiply charged ions of proteins, and peptides in positive and negative mode can be easily analyzed. During positive ion mode, protons are added to give the analyte a net positive charge, during negative mode, protons are abstracted to give the analyte a negative charge that can be analyzed by mass spectrometer based on their mass-to-charge (m/z) ratios. The charge distribution gives rise to the typical multiple charge envelopes. Deconvolution algorithm is used which sums up the signal intensity into a single peak at the molecular weight of the analyte. Very complex mixtures can be easily analyzed by ESMS, but as the molecular weight of the component increases, it is difficult to interpret the spectra (Mann, Hendrickson et al. 2001).

Electrospray is usually performed by infusion mode, the nanolectrospray format, or in combination with HPLC (High-performance liquid chromatography). During infusion mode, the sample or the analyte is introduced into a continuous liquid stream, a mixture of organic and

aqueous liquid (50:50 methonol:water), by means of an injection valves. Flow rates are usually adjusted between 0.5 and several microliters per minute. Samples must be free of salt and detergent for efficient analysis. Nanoelectrospray is another type of electrospray that operates in the absence of pumps and are operated at very low flow rates of nanoliters per minute. In nanoelectrospray analysis, a minute sample (upto 1µl) is sufficient for analysis. Complex sequencing experiments can be performed by nanoelctrospray as well. When combined with LC-MS (liquid chromatography and mass spectrometry), analysis of mixture of components of the sample takes place on–line as they come out from the chromatographic column. In this condition, sample cleanup, concentration, and separation all are achieved in a single step (Mann, Hendrickson et al. 2001).

2.5.3.3 MALDI

MALDI was principally introduced by Karas and Hillenkamp in 1988 (Hoffmann and Stroobant 2007). It is the best widespread used technique that produces intact gas-phase ions from a broad range of large, non-volatile and thermally labile compounds like proteins, oligonucleotides, synthetic polymers and large inorganic compounds. This method has easy sample preparation and has advantage of more tolerance to salts, buffers and detergents (Hoffmann and Stroobant 2007). MALDI is considered as a key technique in mass spectrometry-based proteomic studies. MALDI-MS is enormously sensitive, fairly tolerant to contaminants, and easy to apply available technique (Cramer 2009). It produces singly charged peptide ions. This technique suffers from disadvantages like low shot-to-shot reproducibility and strong reliance on the sample preparation method (Hoffmann and Stroobant 2007).

2.5.3.4 Advantages of MALDI

MALDI has several advantages including high-speed data acquisition, is a pulsed ionization technique, and has comparatively simple sample preparation. Other advantages of MALDI include high controllable sample consumption, sample archiving and recovery following analysis and also have high spatial resolution (down to 1μm) (Cramer 2009).

2.5.3.5 Principles of MALDI

The compound that is to be analyzed is dissolved in a matrix, a solvent containing small organic molecules. This mixture is allowed to dry and as a result a crystal is formed. The analyte molecules are distributed throughout the matrix. The matrix molecules should have a strong absorption at the laser wavelength (Hoffmann and Stroobant 2007). Two widely used matrices for the analyses of biomolecules are dihydrobenzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid (Mann, Hendrickson et al. 2001).The resulting crystal is then struck several times with a laser beam usually nitrogen lasers with a wavelength of 337nm is used (Mann, Hendrickson et al. 2001). Irradiation by the laser causes rapid heating of the crystals by the accumulation of a large amount of energy in the condensed phase due to the excitation of the matrix molecules. " The rapid heating causes localized sublimation of the matrix crystals, ablation of a portion of the crystal surface and expansion of the matrix into the gas phase, entraining intact analyte in the expanding matrix plume" (Hoffmann and Stroobant 2007).

Ion formation involves proton transfer from photoionized matrix to the analyte (desorption ionization). The ions in the gas phase are then accelerated by an electrostatic filed that takes the ions towards the analyzer (Hoffmann and Stroobant 2007) This technique is more

sensitive than other laser ionization techniques. It is used to image biological materials. It allows the desorption and ionization of analytes with very high molecular mass in excess of 100,000Da. Compounds can be cationized by adding a small quantity of alkali, copper or silver cations to the samples easily, if they are not easily protonated. Analysis of peptides, proteins and other biomolecules can be easily achieved by using this technique. The pulse ionization nature of the MALDI source is well suitable with the time-of flight mass analyzer (Hoffmann and Stroobant 2007).

2.5.3.6 MALDI Time-of-flight (TOF) Mass Spectrometer

Time of flight (TOF) analyzer has the capacity to analyze ions over a wide range of masses and therefore can analyze the high-mass ions produced by MALDI (Hoffmann and Stroobant 2007). The ions that are produced as a result of irradiation of laser light on the crystal (sample-matrix) are accelerated to a fixed amount of kinetic energy. The ions travel down in the flight tube of TOF mass spectrometer. Small ions have a higher velocity and can reach faster to the detector before the larger ions, producing a mass spectrum (Cramer 2009). Time of flight mass spectrometer in a reflectron mode allows all types of ions to reach the detector at the same time. Reflectron mode gives high resolution and high mass accuracy. MALDI ionization sources can also be combined with other mass analyzers like ion trap or Fourier transform mass spectrometers, this allows MS/MS analysis (Hoffmann and Stroobant 2007).

2.5.4 Expression proteomics in Down's syndrome

The protein expression analysis have been used as an aid in better understanding of the pathways involved in the pathogenesis of AD (Sultana, Perluigi et al. 2006). Significant

difference is found in terms of protein expression when we analyzed our protein spots by using PDQuest software. After determining the level of oxidative stress in Aβ(17-42) treated synaptosomes, the proteins were separated by using 2D-PAGE. The proteins were stained using Sypro Ruby stain and were visualized under UV light.

PDQuest software was used to identify expression profiles of the proteins. The software helps to calculate significant differences in the protein spots compared to control. If the fold value is less than one, this indicates that proteins are expressed less in oxidized samples vs. control. If the fold value is greater than one, it suggests that the proteins are overexpressed in the sample compared to control. Several protein spots were identified as being significantly differentially expressed. Those protein spots were excised and treated with trypsin. The resultant protein fragments were sent for mass spectrometric analysis to the University of Louisville's Core Mass Spectrometry Facility. By using proteomics, identification of potential biomarkers were assessed in Aβ(17-42) treated synaptosomes. Proteins that are differentially expressed in the novel Down syndrome model were compared to those of mild cognitive impairment, arguably the earliest stage of Alzheimer's disease.

CHAPTER THREE

METHODS

3.1 Sample preparation

Synaptosomes are isolated terminals of neurons, prepared by homogenization and fractionation of nerve tissue. First isolated by Hebb and Whittaker in 1958 (Hebb and Whittaker 1958), they were later identified by electron microscopy as detached synapses(Gray and Whittaker 1962). They contain all the components necessary to store, release, and retain neurotransmitters. Synaptosomes also contain mitochondria for ATP production and active energy metabolism (Breukel, Besselsen et al. 1997). For our study, synaptosomes were isolated from brain tissue of Mongolian gerbils. These animals were used because their synaptosomes have been extensively characterized and employed in neuroscience (Boyd-Kimball, Castegna et al. 2005). Synaptosomes were used to study consequences of protein oxidation by incubating the experimental set with amyloid beta Aβ(17-42) peptide (AnaSpec, San Jose, CA, USA). Amyloid beta peptide Aβ(17-42) was dissolved in phosphate-buffered saline (PBS), to a final concentration of 0.5mg/mL and preincubated at 37°C for 24 h prior to incubation with synaptosomes. This is done to aggregate the Aβ peptide and increase its solubility. As Aβ(17-42) is found in both Alzheimer's disease (AD) and Down syndrome (DS), the possible oxidative stress induced by this peptide was studied. The experimental set of synaptosomal preparations was incubated with Aβ(17-42) for 6 h at 37°C to possibly induce oxidative stress.

Following approval of animal protocols by the University of Kentucky Animal and Use Committee, the animals were fed with standard Purina rodent laboratory chow and housed in the University of Kentucky Central Animal Facility in a 12 hour light/dark phase. Synaptosomes

were isolated from three month old Mongolian gerbils at the University of Kentucky in their Division of Laboratory Animal Resources. Briefly, the animals were anaesthetized and sacrificed. Their brains were immediately isolated and dissected following sacrifice. The brain was homogenized with a Wheaton tissue homogenizer placed in ice containing 0.32M sucrose isolation buffer (4µg/ml leupeptin, 4µg/ml pepstatin, 5µg/ml aprotinin (ICN Biomedicals, Aurora, OH, USA), 2mM ethylene glycol-bistetraacetic acid (EGTA), 2mM ethylenediaminetetraacetic acid (EDTA), 20mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 20µg/ml trypsin inhibitor, and 0.2mM phenylmethanesulfonyl fluoride (PMSF), pH 7.4) (Boyd-Kimball, Castegna et al. 2005). The tissue was homogenized by 20 passes in the tissue homogenizer.

The homogenate obtained was centrifuged at 1500 *g* for 10 minutes. The supernatant was retained and the pellet was discarded. The supernatant was then centrifuged at 20,000 *g* for 10 minutes. This time, the pellet was retained and the supernatant was discarded. The pellet obtained was resuspended in 1 ml of 0.32M sucrose isolation buffer. Sucrose solutions (0.85M pH 8, 1.0M pH 8 and 1.18M pH 8.5) were prepared and layered in plastic centrifuge tubes by using 18 gauge syringe needles carefully to form a discontinuous sucrose gradient. The resuspended pellet was layered over the sucrose gradient (0.85M pH 8, 1.0M pH 8 and 1.18M pH 8.5 sucrose solutions each having 2mM EDTA, 10mM HEPES, and 2mM EGTA) and centrifuged by using a ultra centrifuge at 82,000 *g* for 60 minutes at 4^{o}C.

Synaptosomes were collected from the sucrose interface of 1.0M/1.18M layer and washed in Locke's buffer (154mM sodium bicarbonate ($NaHCO_3$), 5mM glucose, 5mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), pH 7.4) twice for 10 min at 32,000 *g*.

The synaptosomes obtained were assayed to determine protein concentration by using Pierce BCA method (Boyd-Kimball, Castegna et al. 2005).

3.2 Bicinchoninic acid (BCA) Protein Assay

Protein concentration was determined by the Bicinchoninic acid (BCA) method that involves protein mediated reduction of Cu^{2+} to Cu^{+} by the peptide bonds and amino acids. It involves the formation of a Cu^{2+} protein complex under alkaline conditions, followed by the reduction of the Cu^{2+} to Cu^{+} (Figure 3.1). BCA forms a complex with reduced copper (Cu^{+}) resulting in a soluble, purple solution that has a strong absorbance at 562nm. The intensity of color produced is directly proportional to the number of peptide bonds participating in the reaction. Bovine serum albumin (BSA) was used as a protein standard and the concentration of protein samples was determined by using Beer's law.

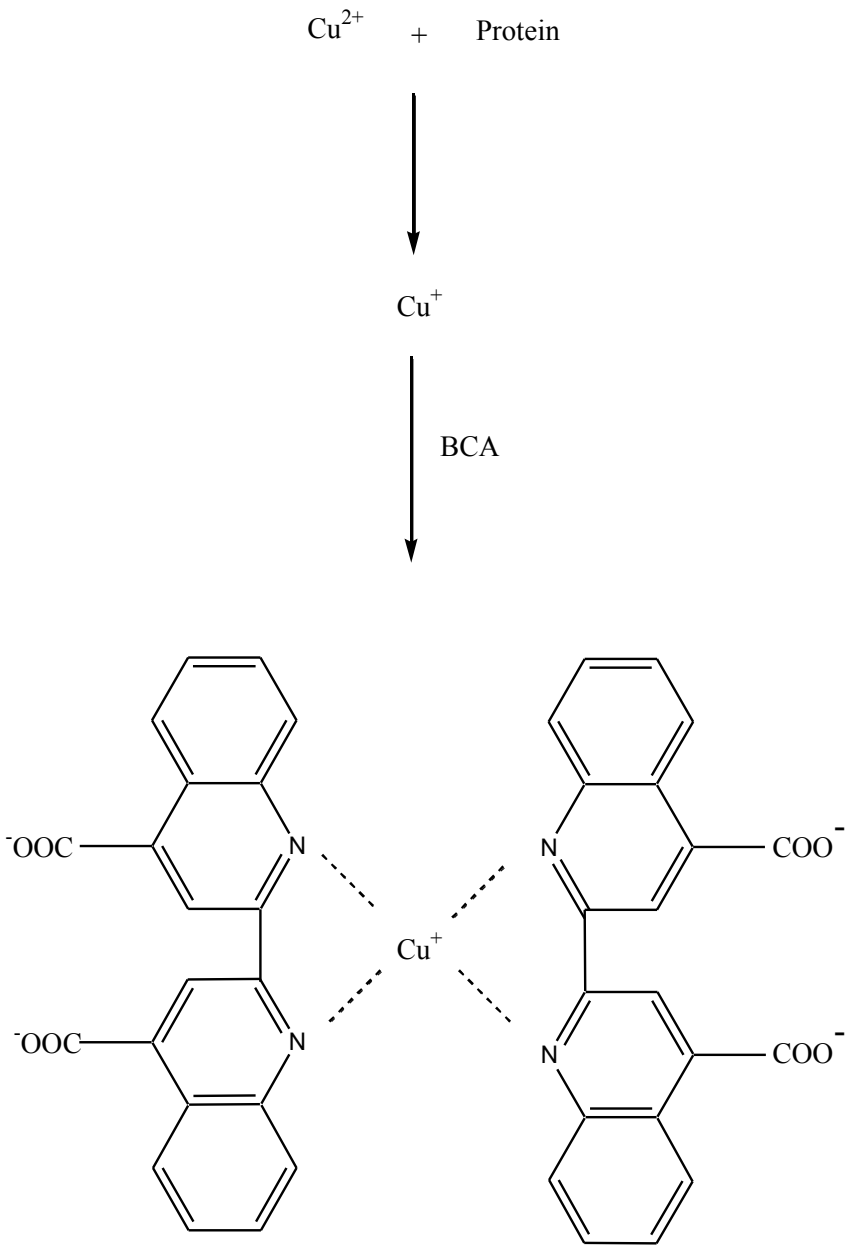


Figure 3.1 Reaction of BCA complex with Cu^{+} (Smith, Krohn et al. 1985).

3.3 Oxidative stress parameters

Oxidative stress parameters are used to study oxidative stress levels. Measuring protein oxidation by indexing protein carbonyls and nitration levels is a way to determine protein oxidation. Lipid peroxidation can be determined by the levels of 4-hydroxy-2-nonenal (HNE), malondialdehyde, isoprostanes and altered phospholipid composition. It has been found that lipid peroxidation can also be determined by elevated levels of thiobarbituric acid reactive substances (TBARS) (Markesbery 1997). Measuring the elevated levels of toxic carbonyls, 3-nitrotyrosine (3-NT), and HNE are consider to be among the earliest of these changes following an oxidative insult (Butterfield, Reed et al. 2007).

3.3.1 Protein carbonyls

Measuring protein carbonyl levels is one way of indexing protein oxidation. Protein carbonyls are stable and hence are widely used as markers to determine the extent of oxidation of proteins. Protein carbonyl levels can be experimentally determined by derivatization of the carbonyl groups with 2, 4-dinitrophenylhydrazine (DNPH) (Figure 3.2), followed by immunochemical detection of the resulting hydrazone product (Butterfield, Reed et al. 2007).

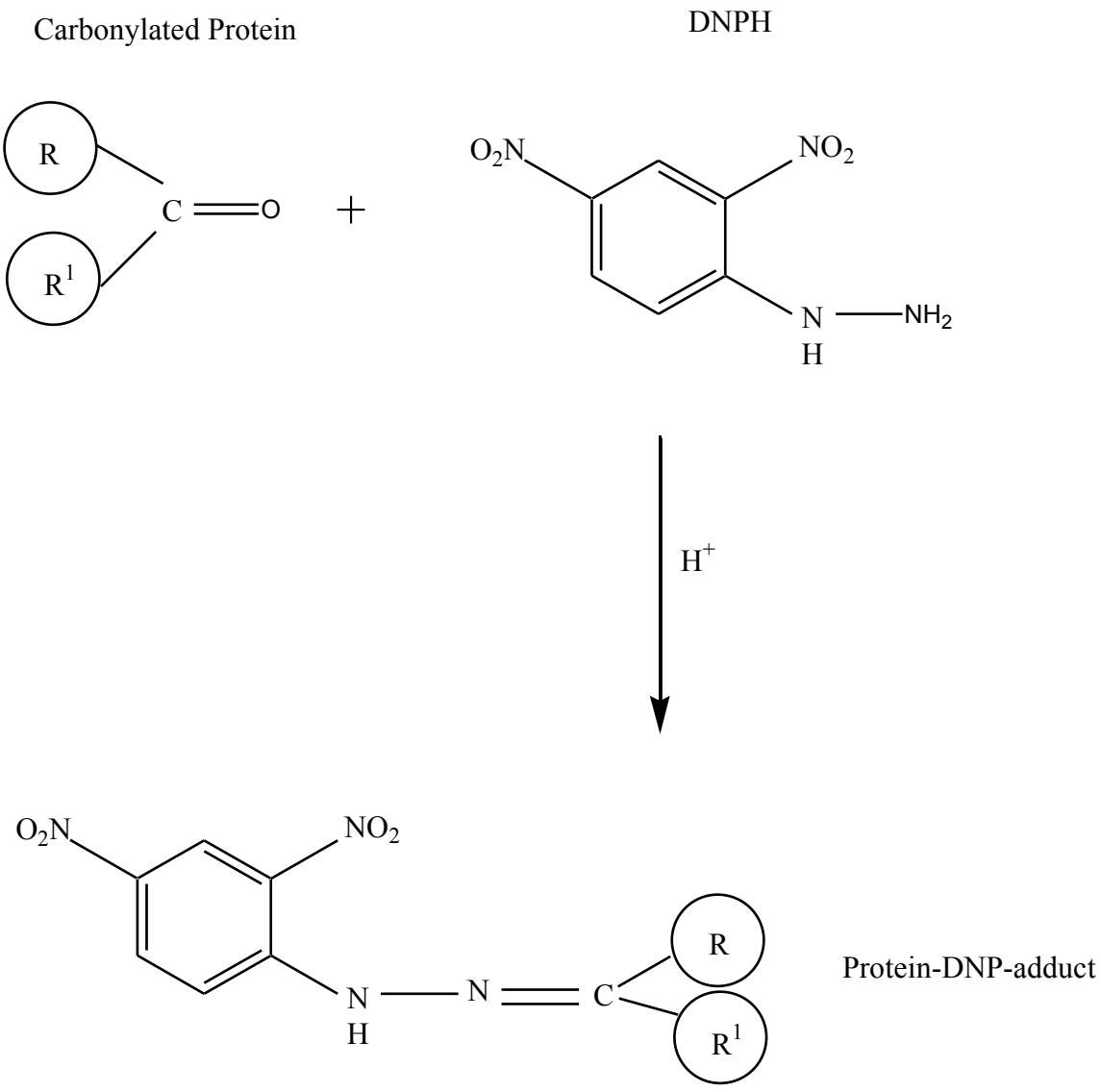


Figure 3.2 Reaction of protein carbonyls with 2,4-dinitrophenylhydrazine (DNPH) (Sultana and Butterfield 2009).

Protein carbonyl levels were measured by using the OxyBlot Protein Oxidation Kit (Chemicon International, Temecula, CA, USA) and Slot blot apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Briefly, 5µl of sample was incubated with 5µl of 12% sodium dodecyl sulfate (SDS) (Sigma, St. Louis, MO, USA) and 10µl of 10% 2,4-dinitrophenylhydrazine for 20

minutes at room temperature. 10% of DNPH was prepared by diluting DNPH with phosphate buffered saline (PBS), pH 8. After 20 minute incubation, the samples were neutralized by adding 7.5µl of neutralizing solution (2M Tris in 30% glycerol). After vortexing, 250ng of resulting sample solution was loaded per well in triplicates in the slot blot apparatus. Nitrocellulose membrane was placed on the top of the filter paper inside the slot blot apparatus. The membrane was precleaned with PBS and then sample solutions were loaded onto the nitrocellulose membrane under vacuum pressure. The membrane was dried overnight and blocked with 3% (w/v) bovine serum albumin prepared 50 ml of wash blot buffer (0.1% (w/v) NaCl, 0.04% (w/v) NaH_2PO_4, 0.24% (w/v) Na_2HPO_4, 0.02% (w/v) NaN_3, 0.04% (v/v) Tween 20), for 1 hour to prevent non-specific binding of the proteins. The membrane was incubated with a 1:100 dilution of anti-DNP polyclonal antibody (Millipore Corporation, Billerica, MA, USA) in wash blot buffer for two hours that acts as a primary antibody. After incubation with the primary antibody, the membrane was washed three times in wash blot buffer for 5 minutes each. Following three washes, a secondary antibody anti-rabbit IgG labeled with alkaline phosphatase (Sigma, St. Louis, MO, USA) added after diluting 1:6000 in wash blot buffer and incubated for 1 hour. The membrane was again washed with wash blot buffer three times for five minutes each. The membrane was then developed using SigmaFast BCIP/NBT substrate (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) (Sigma, St. Louis, MO, USA) (Figure 3.3). Blots were dried and scanned into TIFF files with Adobe Photoshop (San Jose, CA, USA) (Figure 3.4). Scanned images were quantified with Scion Image software.

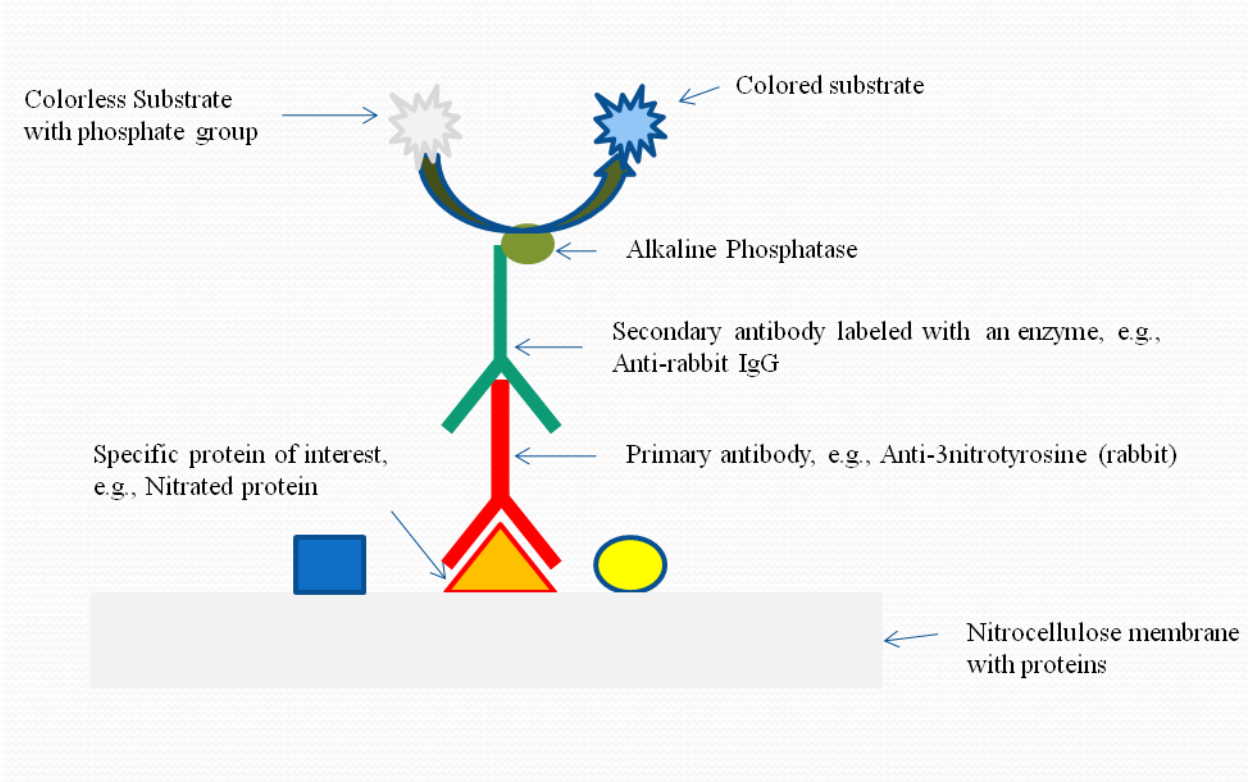


Figure 3.3 Illustration representing immunochemical detection of a nitrated protein.

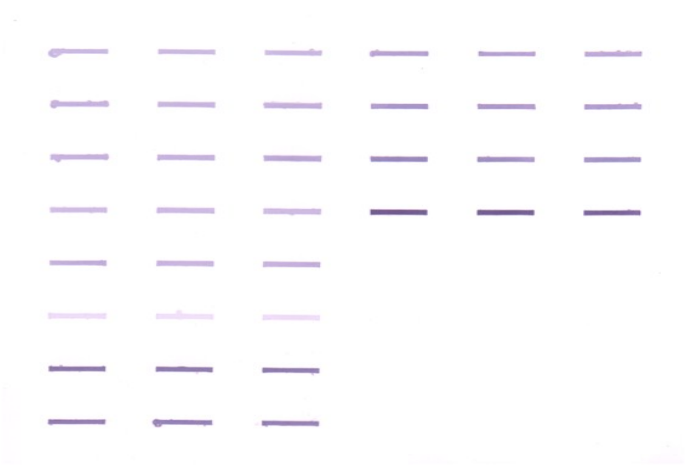


Figure 3.4 Representative slot blot.

3.3.2 4-Hydroxynonenal

Measuring protein bound lipid peroxidation product 4-hydroxynonenal (HNE) is a way of determining lipid peroxidation levels. Briefly, 5µl of sample was incubated with 5µl of Laemmli buffer (0.125 M Tris base pH 6.8, 20% (v/v) glycerol, and 4% (v/v) sodium dodecyl sulfate (SDS)) for 20 minutes at room temperature. After vortexing, 250ng of resulting sample solution was loaded per well in the slot blot apparatus (in triplicate). The sample solutions were loaded onto the nitrocellulose membrane under vacuum pressure. The membrane was dried overnight and blocked with 3% (w/v) bovine serum albumin (BSA) prepared in 50 ml of wash blot buffer for one hour to prevent nonspecific binding of proteins. The membrane was incubated with a 1:4000 dilution of anti-HNE polyclonal antibody (Alpha Diagnostic International Inc, San Antonio, Texas, USA) in wash blot buffer for 3 hours. After incubation with the primary antibody, the membrane was washed three times in wash blot for 5 minutes each.

Following three washes, a secondary antibody anti-rabbit IgG labeled with alkaline phosphatase (Sigma, St. Louis, MO, USA) added after diluting 1:6000 in wash blot buffer and incubated for 1 hour. The membrane was again washed with wash blot buffer three times for five minutes each. The membrane was then developed using SigmaFast BCIP/NBT substrate (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) (Sigma, St. Louis, MO, USA). Blots were dried and scanned into TIFF files with Adobe Photoshop (San Jose, CA, USA). Scanned images were quantified with Scion Image software.

3.3.3 3-nitrotyrosine

Measuring the 3-nitrotyrosine levels is a way of determining the levels of nitrated proteins. Nitrated protein levels were determined in a similar manner as 4-hydroxynonenal levels mentioned above. Briefly, 5µl of sample was incubates with 5µl of Laemmli buffer for 20 minutes at room temperature. 250ng of resulting sample solution was loaded per well in the slot blot apparatus (in triplicates) as mentioned above. The membrane was blocked with 3% bovine serum albumin (BSA); the membrane was incubated with a 1:3000 dilution of anti-3-nitrotyrosine polyclonal antibody (Sigma, St. Louis, MO, USA) in wash blot buffer for three hours. Following three washes the membrane was incubated with same secondary antibody as mentioned above in 1:6000 dilution for 1 hour. After three washes, the blots were developed as mentioned above; blots were dried, scanned, and quantified by scion image software.

3.4 Two dimensional gel electrophoresis

3.4.1 Overview

The proteins are first separated based on their isoelectric point (pI) in the first dimension in the isoelectric focusing (IEF) cells and are separated based on their sizes in the second dimension in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). High molecular weight proteins travel slower and low molecular weight proteins travel faster during the second dimension of electrophoresis. Thus, during two dimensional gel electrophoresis (2D-PAGE) of biological samples, the proteins are separated based on their isoelectric points (pI) and sizes. As a result of separation of proteins on the 2D gel, each individual spot on the gel represents a unique protein.

3.4.2 Sample preparation

Briefly, 150µg of the protein sample was precipitated by adding 100% cold trichloroacetic acid (TCA) to achieve a final concentration of 15% TCA and was placed on ice for 10 minutes. The precipitated proteins were centrifuged at 4000 g for 2 min at 4°C. The resulting protein pellets were washed three times each with 1ml of 1:1 (v/v) ethanol: ethyl acetate solution. The protein samples after the final wash were dissolved in 200µL of rehydration buffer containing 8M urea, 20mM dithiothreitol, 2M thiourea, 2.0% (w/v) CHAPS, 0.2% Biolytes and bromophenol blue.

3.4.3 Isoelectric focusing

The proteins are separated based on their isoelectric point during isoelectric focusing. This was performed by using IEF focusing cell and 110-mm pH 3-10 immobilized pH gradients (IPG) strips both obtained from Bio-Rad (Hercules, CA, USA). 200µL of sample solution were applied to a 110-mm IPG strips and electricity is applied for an hour and then 2mL of mineral oil was pipetted on the top of each strip to prevent the evaporation of proteins. The IPG strips were then actively rehydrated in an IEF cell for 16 hours at 50V. Isoelectric focusing of proteins was performed at a 20°C as follows: 300V for 2 h linearly, 500V for 2 h linearly, 1000V for 2 h linearly, 8000V for 8 h linearly, and 8000V for 10 h rapidly. The focused IPG strips were stored at -80°C until future use.

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

Two dimensional (2D) gel electrophoresis separates a mixture of proteins into single easily detectable protein spots based on isoelectric point (IEF) and molecular migration (M_r).

The 2D gel map helps to compare and match different sets of samples in order to identify isoforms, mutants, and posttranslationally modified proteins for statistical analysis. This technique gives high reproducibility and resolution. The proteins are first separated based on their isoelectric point (pI) in the first dimension in the isoelectric focusing (IEF) cells and are separated based on their sizes in the second dimension in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). High molecular weight proteins travel slower and low molecular weight proteins travel faster during the second dimension of electrophoresis. During two dimensional gel electrophoresis of biological samples, proteins are separated based on their isoelectric points and sizes. As a result of separation of proteins on the 2D gel, each individual spot on the gel represents a unique protein.

Proteins are separated based on their sizes in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For SDS-PAGE, the gel strips obtained after isoelectric focusing were allowed to thaw. The thawed strips were equilibrated for 10 minutes in equilibration buffer which consisted of 50mM Tris-HCl (pH 6.8) containing 6M urea, 0.5% dithiothreitol (DTT), 1% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) glycerol. After 10 minutes, they were re-equilibrated in the same buffer for 10 minutes this time DTT was replaced by 4.5% iodoacetamide (IA). For 2D gel electrophoresis, linear gradient (8-16%) precast Criterion Tris-HCl gels were used (Bio-Rad, Hercules, CA, USA).

Control and Aβ(17-42) strips were placed on the precast Criterion gels and this in turn placed in the 2D gel electrophoresis gel apparatus (Bio-Rad, Hercules, CA, USA). Electrophoresis was performed for 65 min at 200V.

3.4.5 Sypro Ruby staining

Following second dimension electrophoresis, the gels were incubated with fixing solution containing 10% (v/v) methanol, 7% (v/v) acetic acid for 20 minutes. The gels were then stained with 50mL of SYPRO Ruby gel stain (Bio-Rad, Hercules, CA, USA) for two hours on a slow continuous rocker. After staining, the gels were placed in 70mL distilled water overnight for destaining.

3.4.6 Image and statistical analysis

Following SYPRO ruby staining, each 2D gel was placed under a UV transilluminator (EDVOTEK, Bethesda, MD) to visualize different protein spots. The UV transilluminator (λ_{ex}=470nm, λ_{em}=618 nm) captures the fluorescent gel image with a built in digital camera. Gels were stored in distilled water at 4°C until spot excision for in-gel trypsin digestion. The images were analyzed for significant differences between the images in terms of protein spots by using PDQuest 2-D image analysis software (Bio-Rad, Hercules, CA, USA). This software matches and analyzes visualized protein spots among different gels and compares protein intensity between control and experimental gel images. It has powerful, automatching algorithms that identify and accurately match gel protein spots in terms of their intensity and determines if the spots are significantly different from each other. The principle of PDQuest software is similar to that of densitometric measurement. After the images were analyzed and matched through PDQuest software, the normalized intensity of each protein spot from individual gels was compared between groups for statistical analysis using student's t-test.

3.4.7 In gel trypsin digestion

For mass spectrometric analysis, the significant protein spots were excised from the gels by the method described by Thongboonkerd (Thongboonkerd, Luengpailin et al. 2002). Briefly, the significant protein spots from the 2D gels were excised by using a clean sharp razor blade and were transferred into new clean microcentrifuge tubes. The gel pieces were incubated with 0.1M ammonium bicarbonate (NH_4HCO_3) for 15 minutes at room temperature. To the same tube, acetonitrile was added to the gel pieces containing ammonium bicarbonate and incubated at room temperature for 15 minutes. Ammonium bicarbonate and acetonitrile solvents were removed after 15 minutes and gel pieces were allowed to dry in laminar flow hood for 30 minutes.

The gel pieces were rehydrated with 20µL of 20mM dithiothreitol (Bio-Rad, Hercules, CA, USA) prepared in 0.1M NH_4HCO_3 and incubated at 56°C for 45 minutes. The DTT solution was removed and 20µL of 55mM iodoacetamide (Bio-Rad, Hercules, CA, USA) prepared in 0.1M NH_4HCO_3 was added to the gel pieces and incubated for 30 minutes in the dark at room temperature. The iodoacetamide solution was removed and replaced with 200µL 50mM NH_4HCO_3 and incubated for 15 minutes at room temperature. To the same tube, 200µL of acetonitrile was added and incubated at room temperature for 15 minutes. The mixture of ammonium bicarbonate and acetonitrile solutions were removed from the tube and the gels were allowed to dry in a laminar flow hood for 30 minutes. Following drying in the laminar flow, the gel pieces were rehydrated with 20ng/µL of modified trypsin (Promega, Madison, WI, USA) in 50mM NH_4HCO_3 with the minimal volume to cover the gel pieces and allowed to incubate overnight (~18 hours) with shaking at 37°C. Following drying in the laminar flow, the gel pieces

were rehydrated with 20ng/µL of modified trypsin (Promega, Madison, WI, USA) in 50mM NH_4HCO_3 with the minimal volume to cover the gel pieces and allowed to incubate overnight (~18 hours) with shaking at 37°C.

3.4.8 Peptide Extraction

Four buffers: A (5% acetonitrile, 0.1% formic acid), B (95% acetonitrile, 0.1% formic acid), C (100 % acetonitrile), and D (50% acetonitrile, 0.1% formic acid) were prepared. The protein digest from the gel pieces was obtained after 18 hours of incubation following shaking. The digest was removed and placed in newly labeled microcentrifuge tubes. Sixty microliters of buffer A was added to the gel pieces until the gel piece were submerged in the buffer. The tubes were then sonicated in a waterbath for 15 minutes at room temperature. Ninety microliters of buffer B was added to the tubes and was allowed to sonicate for 15 minutes. The resulting supernatant obtained was mixed with the original peptide extract present in the already labeled microcentrifuge tubes. The tubes were centrifuged by using a high speed vacuum centrifuge until the peptide extract was concentrated to a volume of 10µL.

3.4.9 Protein clean up

A Supelco Zip tip consists of a 10µL pipette tip with a micro-volume bed of chromatography media fixed at its end. It is used for concentrating and purifying peptides or proteins in seconds for mass spectrometric analysis. Cleaning helps to bind the peptides to the column and attached peptides are transferred from the chromatographic column into new set of microcentrifuge tubes for mass spectrometric analysis. Ten microliters of buffer C was drawn up into the zip tip (Millipore Corporation, Billerica, MA, USA) and emptied to waste five times. The same zip tip was reequilibriated with 10µl of buffer A five times and discarded. The above

zip tip was used to draw up and gently expel the peptide extract present in the microcentrifuge tubes. This aspiration and release of sample was done for ten times in the microcentrifuge tubes.

The sample was washed in the zip tip with buffer A five times by drawing up 10µL of buffer A and aspirating it. Finally, 10µL of buffer D was drawn with the same zip tip and the resulting solution was transferred into a new labeled microcentrifuge tube. The eluent was drawn and gently expelled several times to remove the sample from the zip tip column completely. The tubes containing the peptides were sent to the University of Louisville's Core Mass Spectrometry Facility for mass spectrometric analysis and protein identification.

3.5 Mass spectrometry

A mass spectrometer consists of an ion source that generates ions, a mass analyzer that separates the ions into mass-to-charge ratio (m/z) of the ionized samples, and a detector that detects the number of ions at each mass to charge value (Aebersold and Mann 2003). Mass spectrometry is one of the important tool that is being widely used in proteomics research (Nesvizhskii, Vitek et al. 2007). Proteomics is one of the fast expanding filed used for systematic studies of protein structure, function, interactions and dynamics (Jensen 2006). It has currently become the method of choice for identification, quantification and primary structure analysis of the protein components of complex sample mixtures in proteomic studies (Schmidt, Claassen et al. 2009). One of the key advancements in mass spectrometry (MS) based proteomics over the past few years is the huge increase in the quality of the data in terms of high accuracy and high resolution (Choudhary and Mann 2010). Two ionization methods, matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) have been used to generate ions from large, nonvolatile analytes such as proteins and peptides without causing significant fragmentation of analyte.

These ionization techniques (MALDI and ESI) are also referred to as “soft” ionization methods because they do not generate fragments while ionizing the sample. ESI can easily be interfaced with chromatography and electrophoretic liquid-phase separation techniques and can quickly supplant fast ion bombardment as the ionization method of choice for proteins and peptide samples that are dissolved in liquid phase (Aebersold and Goodlett 2001). ESI is extensively used ionization technique in mass spectrometry (Oss, Kruve et al. 2010). ESI is capable of producing multiple charged species of analytes. It can be easily coupled with simple quadrupole or other mass analyzers with limited m/z range. MALDI, on the other hand, is more popular and is usually combined with a time-of flight (TOF) mass analyzer. MALDI ionization source generates predominantly singly charged ions that reduces the mass spectral complexity (Trimpin, Inutan et al. 2010). In this thesis all mass spectra recorded at the University of Louisville Core Mass Spectrometry Facility. The mass spectra were recorded on a LTQ orbitrap XL mass spectrometer connected with a nanospray nonomate ionization source. The LTQ-Orbitrap is considered as a very popular instrument in proteomics research because it combines high-resolution, high mass accuracy, and high sensitivity in a compact and robust instrument (Cox and Mann 2009).

3.5.1 Ion source

For mass spectrometric analysis, electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are the two ionization techniques most widely used to volatilize and ionize the proteins or peptides (Aebersold and Mann 2003).

3.5.1.1 Matrix assisted laser desorption ionization (MALDI)

Matrix assisted laser desorption ionization sublimates and ionizes the samples out of a dry, crystalline matrix by imparting laser pulses on the samples (Aebersold and Mann 2003). This method has easy sample preparation and has an advantage of being more tolerable to salts, buffers and detergents (Hoffmann and Stroobant 2007). MALDI is considered as a key technique in mass spectrometry-based proteomic studies. MALDI-MS is enormously sensitive, fairly tolerant to contaminants, and easy to apply available technique (Cramer 2009). MALDI has several advantages including high-speed data acquisition, is a pulsed ionization technique, and has comparatively simple sample preparation.

3.5.1.2 Electrospray Ionization (ESI)

In this process, liquid containing the analyte is pumped at low flow rates of microliter-per-minute at high voltage through a hypodermic needle. The analyte electrostatically becomes disperse, small, micrometer-sized droplets, which quickly evaporates and impart their charge onto the analyte molecule. As the ionization process takes place in atmospheric pressure, it is therefore very gentle and no fragmentation occurs in the gas phase. Furthermore, the molecules are moved into the mass spectrometer with high efficiency for analysis. The spray is stabilized by using a nebulizer gas or some other devices are often employed (Mann, Hendrickson et al. 2001).

Nanoelectrospray (nanoESI) technology offers the ability of increased sensitivity and lower sample consumption compare to that of conventional ESI. A nanoESI chip-based system, the NanoMate contains an array of 100 individual ESI nozzles on a single chip and a robotic arm which is capable of delivering the samples from a 96-well plate (Corkery, Pang et al. 2005). The

major advantages of this system include multiple-sample capability, low sample consumption, no sample carryover, and high sample throughput.

3.6 Database searching and Protein identification (Analysis of protein sequences)

Tryptic peptides were analyzed with an automated nanospray Nanomate Orbitrap XL MS/MS platform. The Orbitrap MS was operated by trained technicians in a data-dependent mode whereby the 8 most intense parent ions measured in the FT at 60,000 resolution were selected for ion trap fragmentation with the following conditions: injection time 50 ms, 35% collision energy, MS/MS spectra were measured in the FT at 7500 resolution, and dynamic exclusion was set for 120 seconds. Each sample was acquired for a total of ~2.5 minutes. MS/MS spectra were searched against the ipi_Rodent. Database using SEQUEST with the following criteria: Xcorr > 1.5, 2.0, 2.5, 3.0 for +1, +2, +3, and +4 charge states, respectively, and P-value (protein and peptide) < 0.01. IPI accession numbers were cross-correlated with SwissProt accession numbers for the final protein identification.

3.7 Enzyme assays

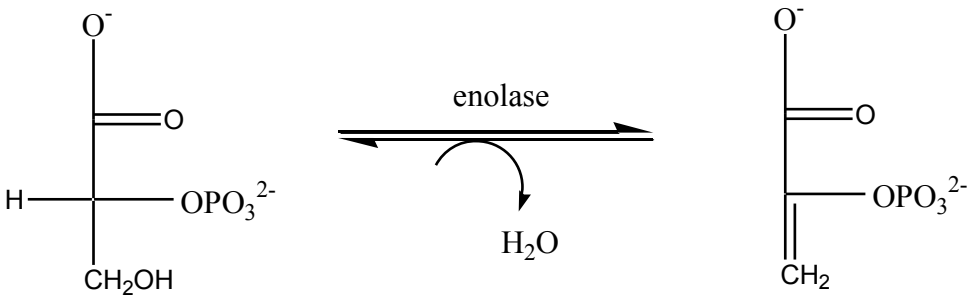
3.7.1 Overview

Reversible and/or irreversible modifications of sensitive proteins may occur due to oxidative stress; it may cause structural, functional and stability modulations of proteins (Butterfield, Perluigi et al. 2006). It has been found that in AD, oxidation of proteins may lead to conformational changes or unfolding in the protein, loss of structural and functional activity including aggregation and accumulation of oxidized proteins as cytoplasmic inclusions (Sultana, Perluigi et al. 2009). Oxidatively modified proteins may also have effects on normal physiological processes via disturbing cellular functions like variations in protein expression and

gene regulation, modulation of cell signaling, protein turnover, induction of necrosis and apoptosis (Sultana, Perluigi et al. 2009).

3.7.2 Alpha Enolase

The enzyme activity of alpha enolase was measured spectrophotometrically at 340nm. This enzyme is responsible for the conversion of 2-Phosphoglycerate to phosphoenolpyruvate in glycolyis, in this reaction a water molecule is liberated (Figure 3.5), The reaction mixture (0.2mL final volume) contained: 81mM triethanolamine (pH 7.4), 1.9mM 2-phosphoglycerate, 0.12mm β-nicotinamide adenine dinucleotide, reduced form, 25mM magnesium sulfate, 100mM potassium chloride, 1.3mM adenosine 5-diphosphate, 7 units pyruvate kinase, 10 units L-lactic dehydrogenase (Bergmeyer 1974) . 5µl of protein was used for this reaction and the assay was carried out at 25°C.



2-Phosphoglycerate Phosphoenolpyruvate

Figure 3.5 Conversion of 2-phosphoglycerate to phosphoenolpyruvate by enolase.

3.7.3 Aldehyde Dehydrogenase

The enzyme activity of aldehyde dehydrogenase was measured spectrophotometrically at 25 °C by following the rate of reduction of NAD^+ by observing the increase in A 340nm. The reaction mixture consists of 0.2mL final volume of 103mM Tris (pH 8)100mM, 0.67mM β-nicotinamide adenine dinucleotide (NAD^+), 100mM KCl, 10mM 2-mercaptoethanol, 2mM acetaldehyde, and 0.0007% (w/v) bovine serum albumin. 5µl of protein was used for this reaction and the assay was carried out at 25°C. Specific activity calculations was determined from protocols given by Sigma-Aldrich (St. Louis. MO, USA). Under these conditions a unit of enzyme activity is the amount of enzyme producing 1 pmol of NADH/min (Bostian and Betts 1978). An illustration of this reaction is given in Figure 3.6.

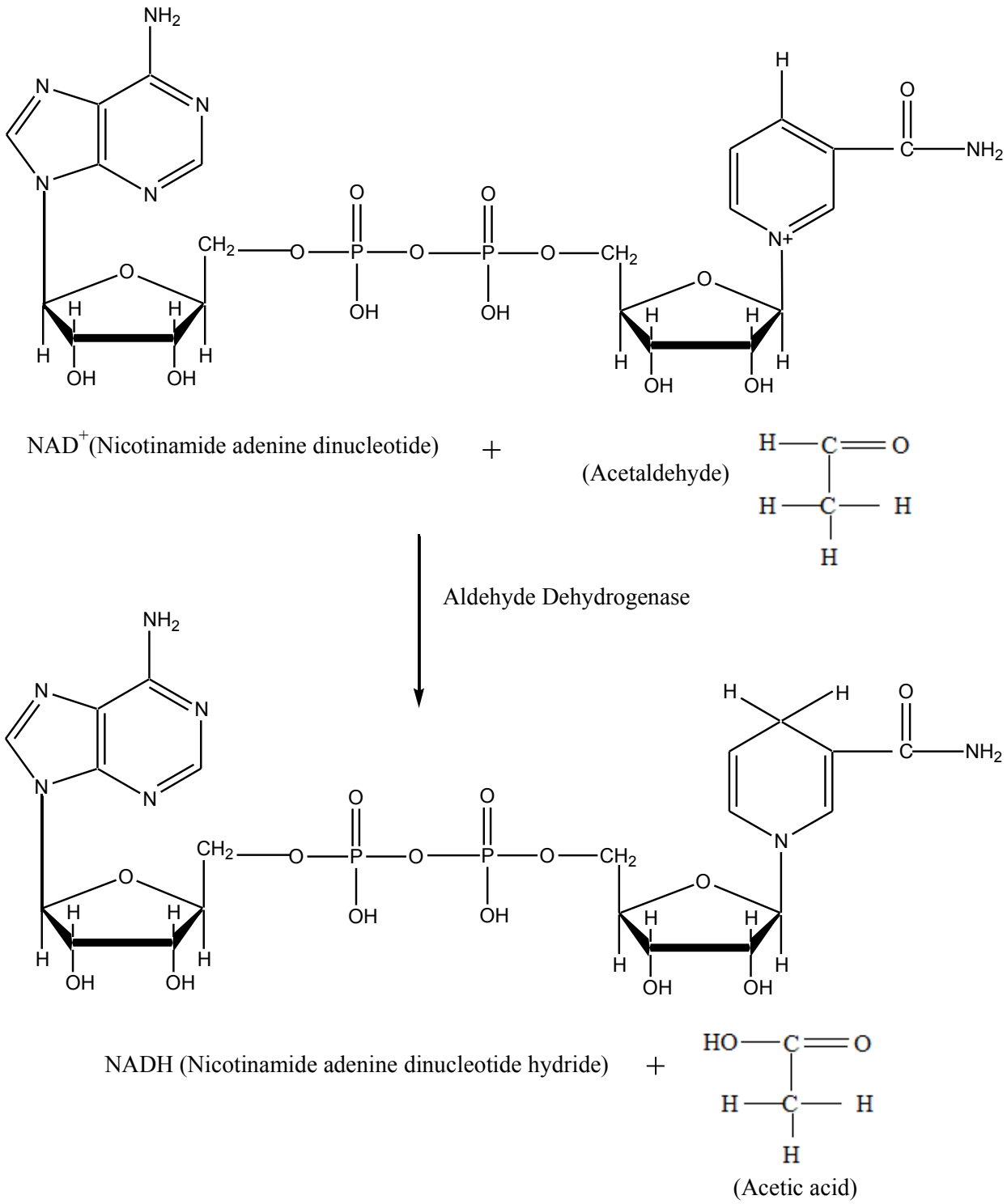


Figure 3.6 Enzymatic reaction of Aldehyde dehydrogenase.

3.7.4 Aldolase

The enzyme activity of aldolase enzyme was measured spectrophotometrically at 340nm. This enzyme is responsible for the breakage of Fructose 1,6-bisphosphate into Glyceraldehyde 3-phosphate and dihydroxyacetone phosphate in glycolyis (Figure 3.7). The reaction mixture (0.2mL final volume) contained: 90mM Tris (pH 7.4), 1.9mM fructose 1,6-bisphosphate solution, 0.13mM NADH (β-nicotinamide adenine dinucleotide hydride), and 5 units a-glycerophosphate dehydrogenase/triosephosphate isomerase (Bergmeyer 1974). 5µl of protein was used for this reaction and the assay was carried out at 25°C.

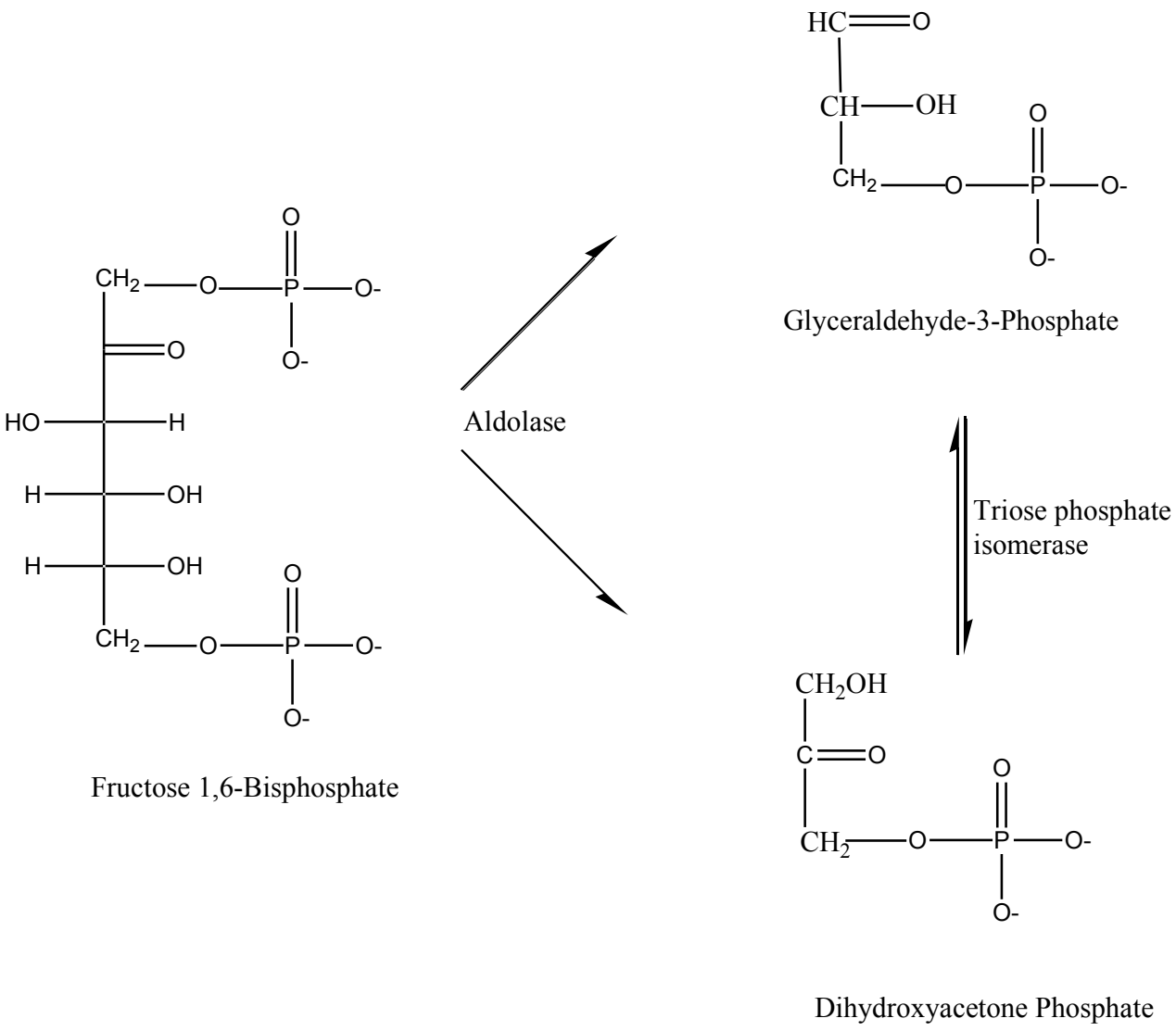


Figure 3.7 Enzymatic reaction of Aldolase and triose phosphate isomerase.

CHAPTER FOUR

Quantitative proteomics analysis of differentially expressed proteins in Aβ(17-42) treated synaptosomes

4.1 Overview

Oxidative stress has been associated in the pathogenesis of numerous diseases such as neurodegenerative disorders, ischemia, and cancer. Under oxidative stress conditions, the balance between the pro-oxidant and antioxidant levels is impaired. Certain environmental factors, stressors, or disease may cause an imbalance and as a result, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced. ROS and RNS have the ability to react with biomolecules including carbohydrates, proteins, lipids, DNA and RNA which leads to oxidative damage and which may ultimately lead to cellular dysfunction (Sultana, Perluigi et al. 2009). The brain is susceptible to oxidative stress due to its high content of peroxidizable unsaturated fatty acids, high consumption of oxygen per unit weight, high levels of free radicals, and comparatively low levels of antioxidant defense systems (Poon, Vaishnav et al. 2006). The typical markers of oxidative stress commonly studied to determine the oxidative stress levels include protein carbonyls, 3-nitrotyrosine (3-NT), free fatty acid release, 4-hydroxy-2-nonenal (HNE), acrolein, advanced glycation end products for carbohydrates, iso-and neuroprostane formation, 8-OH-2^{l}-deoxyguanosine, and altered DNA repair mechanisms (Sultana, Perluigi et al. 2009). Amyloid beta (Aβ), a 40-42 amino acid peptide is formed by the proteolytic cleavage of amyloid precursor protein (APP), by the action of two enzymes beta –and gamma-secretases. It has been discovered that mutations in APP, presenilin-1 or presenilin-2 lead to increased production of Aβ(1-42) and the early onset of Alzheimer's disease (AD) (Sultana, Perluigi et al. 2009). Protein expression analysis can potentially help in better understanding of the pathways

involved in pathogenesis of AD and therefore also help in depicting mechanism of progression of AD (Sultana, Perluigi et al. 2006). Protein oxidation can lead to loss of protein function, abnormal protein turnover, imbalance of cellular redox potential, interference with cell-cycle and eventually may lead to cell death which is observed in AD (Boyd-Kimball, Castegna et al. 2005). Oxidation of proteins that are involved in biosynthesis, cytoskeletal dynamics, energy production, and signal transduction may lead to their dysfunction (Poon, Vaishnav et al. 2006). Previous literature states that the proteins that are oxidized in AD brain affect various cellular functions including energy metabolism, proteosome function, glutamate uptake and excitotoxicity, neuritic connections, and neuronal communication (Boyd-Kimball, Castegna et al. 2005). One of the early events in the pathogenesis of AD is synaptic alterations. It has been speculated that synapse loss in the hippocampal dentate gyrus effects the connections between the hippocampus and the entorhinal cortex leading to memory deficits associated with AD (Boyd-Kimball, Castegna et al. 2005). Proteomics deals with the systematic study of proteins that helps to provide the complete view of the structure, function, and regulation of a given cell, tissue, or organism. Protein expression is found to be altered in disease conditions, hence proteomic studies can serve as a sensitive technique to expand insight into a host of biologic processes and phenotypes of both diseased and normal cells (Sultana, Perluigi et al. 2006).

4.2 Introduction

Alzheimer's disease is pathologically characterized by the presence of extracellular senile plaques (SP), intracellular neurofibrillay tangles, and loss of synapses (Sultana and Butterfield 2010). This disease is clinically characterized by progressive dementia. AD is a neurodegenerative disorder that affects 5.5 million Americans and more than 35 million people

worldwide (Querfurth and LaFerla 2010). Progressive memory impairment and intellectual ability are characteristics of Alzheimer's disease. Oxidative stress is believed to play an important role in the pathophysiology of this disease, manifested by protein oxidation, DNA oxidation, lipid peroxidation, advanced glycation end products, and formation of reactive oxygen species (ROS). Amyloid beta, redox metal ions, inflammation, microglia are some of the sources of free radicals important in AD brain (Sultana, Boyd-Kimball et al. 2006).

Down syndrome (DS), occurring in 1 out of 800 live child births, is the most common genetic cause of mild to moderate mental retardation that occurs in new born infants (Peterson 2006). DS is caused by trisomy 21(Jovanovic, Clements et al. 1998; Iannello, Crack et al. 1999; Capone 2001; Nizetic 2001; Lubec and Engidawork 2002; Zitnanova, Korytar et al. 2006; Zana, Janka et al. 2007; Zigman and Lott 2007; Ellis, Tan et al. 2008; Lockrow, Prakasam et al. 2009; Beacher, Daly et al. 2010), in which an extra chromosome is present instead of the usual two copies. Research was demonstrated that children born to women at the age of 35 or older are more prone develop DS (Peterson 2006).

Down syndrome individuals have unusually high oxidative stress. They age prematurely and develop Alzheimer's like brain changes during their 30s or 40s leading to dementia throughout their life. They also suffer from premature onset of cataracts and autoimmune diseases (Jovanovic, Clements et al. 1998). Sequential cleavage of APP by α- and γ secretases produces an Aβ(17-42) peptide fragment which is also known as p3 fragment. Aβ(1-42) peptide fragment has been found extensively in AD along with the deposition of Aβ(17-42) peptide fragment. Aβ(17-42) is the major preamyloid fragment in DS while the major fragment in AD is Aβ(17-42). DS patients have an extensive deposition of Aβ(17-42) peptide, which could contribute to their increased rate of developing AD (Wei, Norton et al. 2002).

There is ample evidence for the involvement of oxidative stress in the pathogenesis of Down syndrome and Alzheimer's disease (Zana, Janka et al. 2007). DS individuals have early mental decline and premature aging; they often suffer from diseases such as specific types of leukemias, growth retardation, thyroid disorders, celiac disease, and diabetes mellitus. Approximately 95% of the DS cases have a trisomy at chromosome 21 (Gulesserian, Kim et al. 2002; Zana, Janka et al. 2007). It is speculated that this genetic overexpression is responsible for dysmorphic features and the pathogenesis of the neurological, immunologic, endocrine, and biochemical alterations found in DS. By the age of 40, DS individuals develop senile plaques and neurofibrillary tangles similar to those seen in AD (Zana, Janka et al. 2007). Many of the DS individuals develop early onset of dementia by the age of 40, however, most develop clinical dementia by the age of 55 (Schupf 2002).

Individuals with DS have unusually high oxidative stress levels, this may occur due to excess activity of enzyme superoxide dismutase (SOD) which converts superoxide free radicals into hydrogen peroxide (H_2O_2) that gets converted into water by either glutathione peroxidase (GPx) or catalase (Cat). The ratio of SOD1 to GPx + Cat is altered in DS individuals. As a result of this, SOD1 ultimately produces more potentially damaging hydrogen peroxide than the antioxidant enzymes, Gpx and Cat, which can neutralize H_2O_2 to water thereby leading to severe oxidative imbalance (de Haan, Newman et al. 1992).

In order to study the oxidative stress levels induced by Aβ(17-42), we utilized immunochemical methods for detection of protein carbonyls, 3-nitrotyrosine , and 4-hydroxy-2-nonenal. 2D gel electrophoresis for separation of proteins and mass spectrometry for the identification of proteins from synaptosomes isolated from Mongolian gerbils was also implemented. It has been shown that Aβ(1-42), a core component found in the senile plaques,

induces protein oxidation in vitro and in vivo studies. Aβ(1-42) is a mediator of oxidative stress and it has been proposed that Aβ(1-42) induced oxidative stress plays an important role in the pathogenesis of AD (Boyd-Kimball, Castegna et al. 2005). Protein oxidation was observed when the Aβ(1-42) was incubated with isolated synaptosomes (Sultana, Perluigi et al. 2009). For our study, isolated synaptosomes were incubated with Aβ(17-42) to study the oxidative stress parameters. The proteins were separated by 2D gel electrophoresis and the oxidatively modified proteins were identified using mass spectrometry.

4.3 Experimental procedures

4.3.1 Chemicals

All chemicals were of the highest purity and obtained from Sigma Aldrich (St. Louis, MO, USA). Aβ(17-42) peptide was obtained from AnaSpec (San Jose, CA, USA) with HPLC and MS purity verification. Peptides were stored at -20°C until used. The OxyBlot protein oxidation detection kit was obtained from Chemicon International (Temecula, CA, USA). SYPRO Ruby stain was obtained from Bio-Rad (Hercules, CA, USA).

4.3.2 Sample preparation

Please refer to Chapter 3, Section 3.1, pages 44-46.

4.3.3 Synaptosomal incubation with amyloid beta peptide

Amyloid beta peptide Aβ(17-42) was dissolved in phosphate-buffered saline (PBS), to a final concentration of 0.5mg/1mL and preincubated at 37°C for 24 h prior to incubation with synaptosomes. The experimental set of synaptosomal preparations were incubated with Aβ(17-42) for 6 h at 37°C.

4.3.4 Two dimensional gel electrophoresis

Two dimensional (2D) gel electrophoresis separates a mixture of proteins into single easily detectable protein spots based on isoelectric point (IEF) and molecular migration (M_r), the two physiological properties. The 2D gel map helps in comparison and matching between different sets of samples to identify isoforms, mutants, and posttranslationally modified proteins for statistical analysis. This technique gives high reproducibility and resolution. Each protein spot in the 2D gel mostly represents a single protein in a sample (Sultana, Perluigi et al. 2006).

Please refer page Chapter 3, section 3.4.1 and 3.4.2, pages 53-54 for overview of the techniques employed for 2D gel electrophoresis.

4.3.4a Isoelectric focusing

Please refer page Chapter 3, section 3.4.3, page 54

4.3.4b Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE)

Please refer page Chapter 3, section 3.4.4, pages 54-55

4.3.5 Sypro Ruby staining

Please refer page Chapter 3, section 3.4.5, page 56

4.3.6 Image and statistical analysis

Please refer page Chapter 3, section 3.4.6, page 56

4.3.7 In gel trypsin digestion

Please refer page Chapter 3, section 3.4.7, pages 57-58

4.3.8 Peptide Extraction

Please refer page Chapter 3, section 3.4.8, page 58

4.3.9 Protein clean up

Please refer page Chapter 3, section 3.4.9, pages 58-59

4.3.10 Mass spectrometry

Please refer page Chapter 3, section 3.5, pages 59-62

4.3.11 Database searching and Protein identification (Analysis of protein sequences)

Please refer page Chapter 3, section 3.6, page 62

4.3.12 Statistical analysis

Statistical comparison of control and Aβ(17-42) treated gel images were performed using student's t-test. P values of <0.05 were considered to be significantly different.

4.4 Results

To assesses whether there were any changes in the proteomic profile in the control and Aβ(17-42) treated synaptosomes, differential protein expression was assayed by densitometric intensities on the gels. Five proteins showed increased expression, while one protein showed decreased expression. Mass spectrometry analysis allowed for the identification of differentially expressed proteins. The following enzymes were identified: aldehyde dehydrogenase, fructose 1,6-bisphosphate aldolase, ATP synthase, alpha enolase, dihydrolipoyllysine-acetyltransferase, peptidyl-propyl cis-transisomerase (PIN-1). Heat shock protein 71 was also identified along with

the enzymes. Slot blot analysis was performed for control and Aβ(17-42) treated synaptosomes, levels of protein carbonyls (Figure 4.1), levels of protein nitration (Figure 4.2), and HNE levels (Figure 4.3) were calculated, significant difference was found between the control and the experimental set, the p value of <0.005 was considered to be significantly different among control and experimental treated synaptosomes.

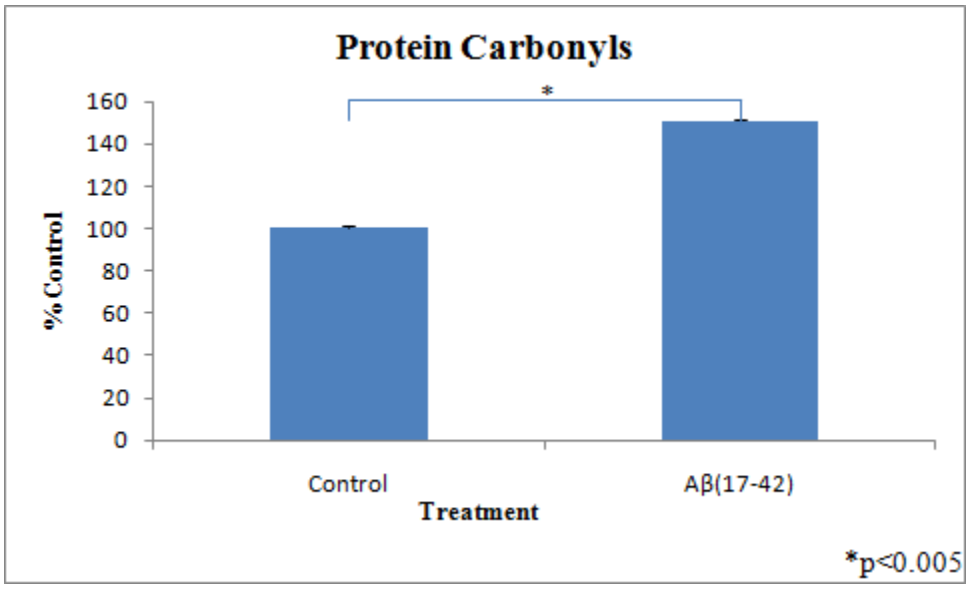


Figure 4.1 Protein carbonyl levels of control and Aβ(17-42) treated synaptosomes.

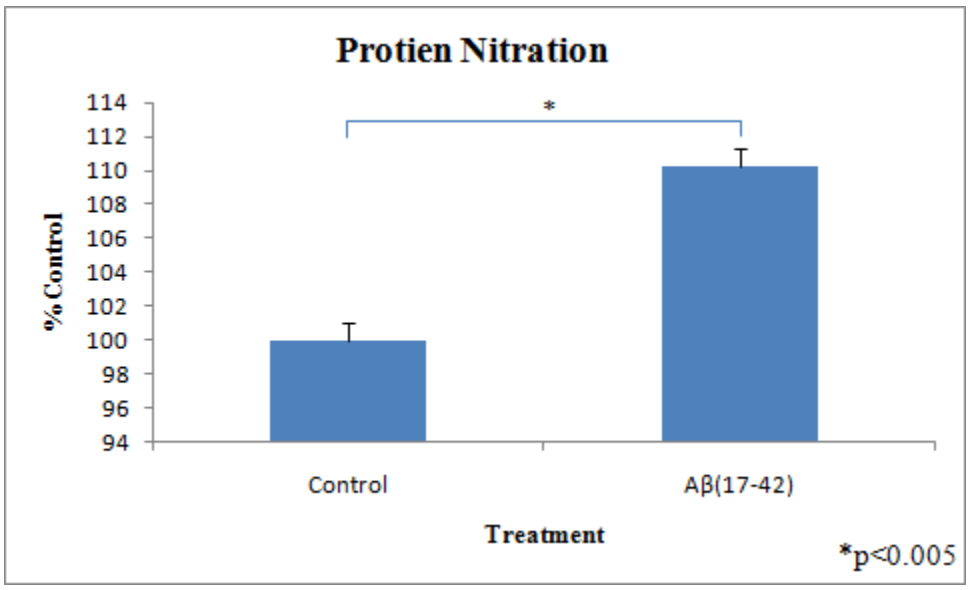


Figure 4.2 Protein nitration levels of control and Aβ(17-42) treated synaptosomes.

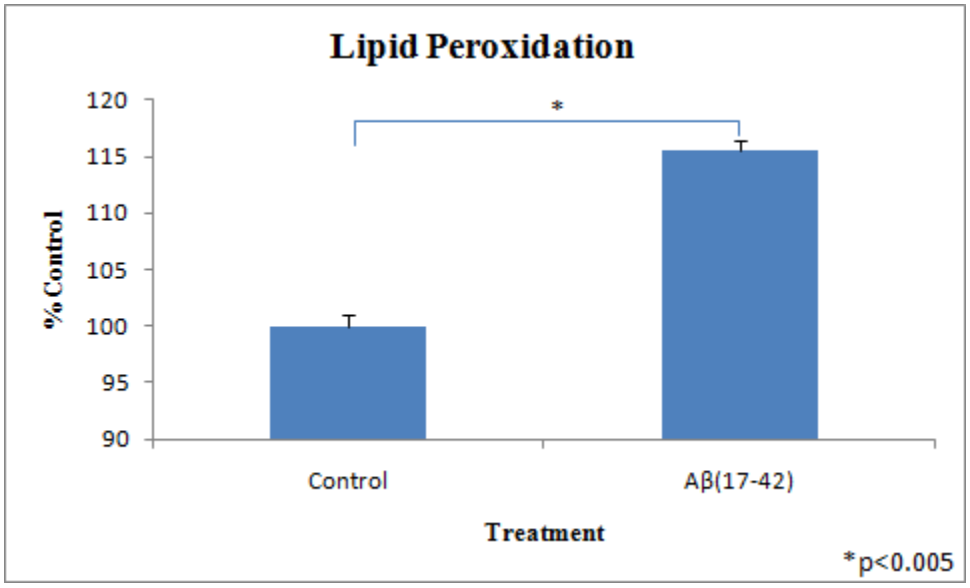


Figure 4.3 Protein bound HNE levels of control and Aβ(17-42) treated synaptosomes.

4.4.1 Proteins identified by mass spectrometry

After assessing the spots in PDQuest software, these spots were excised from the 2D gel and in-gel trypsin digestion was performed. Table 4.1 gives an overall summary of proteins identified by the mass spectrometry.

Table 4.1 Expression profile of significantly different proteins.

Protein identification	Peptide matches	pI, MW (Da)	Protein expression	P value
ATP synthase	3/4	9.22,59752	Downregulated	<0.002
Alpha enolase	3/5	6.37,47140	Upregulated	<0.0003
Aldehyde dehydrogenase	2/3	7.53,56537	Upregulated	<0.005
Heat shock cognate 71	10/17	5.37,70871	Upregulated	<0.003
Fructose 1,6-bisphosphate aldolase	2/2	8.30,39355	Upregulated	<0.005
Peptidyl-prolyl cis-trans isomerase	2/2	5.38,64697	Upregulated	<0.002

4.4.2 Identification of proteins found to be upregulated in the 2D gels

Protein expression refers to presence and abundance of proteins in the proteome. Proteins can be downregulated (less abundant or expressed less) or upregulated (more abundant or expressed more). Overexpression of protein may cause molecular crowding, which can result in changes in protein conformation. This may also lead to aggregation of proteins, inhibition of degradation of proteins, and production of protein formulations such as plaques, and fibrillary structures, which can promote pathological processes. Overexpression cannot be balanced by a

down regulation of other proteins in the cell; the cell may enter a disease state due to imbalance of homeostasis (Zabel, Sagi et al. 2006). It has been described that for diseases like Huntington's and Parkinson's disease, and other neurodegenerative disorders, mere overexpression of disease related protein causes disease (Zoghbi and Botas 2002). The proteins found to be upregulated are alpha enolase, aldehyde dehydrogenase, fructose 1,6-bisphosphate aldolase, peptidyl-prolyl cis-trans isomerase, and heat shock cognate 71 protein (Figure 4.4).

4.4.3 Identification of proteins found to be downregulated in the 2D gels

Differential protein expression is always context dependent. They occur within a specific context of a tissue, organ, environmental conditions, and individual fate (Schulenborg, Schmidt et al. 2006). Proteins that are expressed less or less abundant are considered to be downregulated. The protein found to be downregulated is ATP synthase using mass spectrometric analysis. (Figure 4.4).

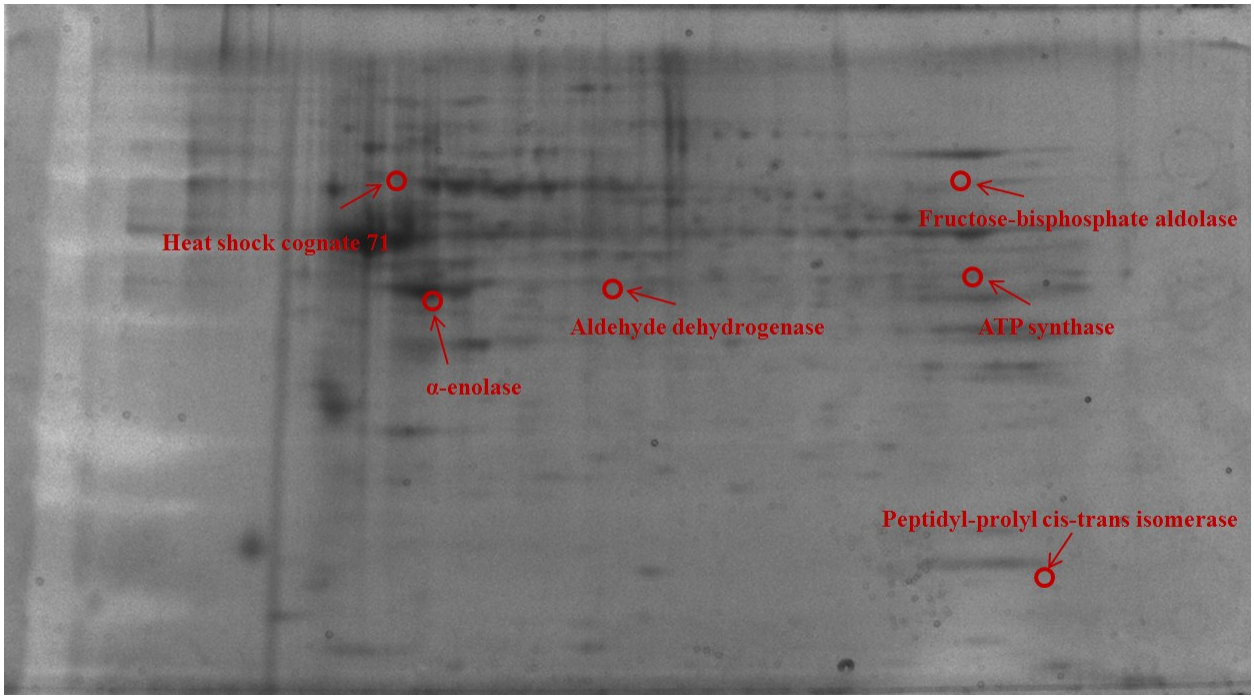


Figure 4.4 Identification of proteins found in 2D gels by mass spectrometry.

4.4.4 Enzyme assays

Enzyme assays were carried out for enolase, fructose 1,6-bisphosphate aldolase, aldehyde dehydrogenase, to check the enzymatic activity among the control and Aβ(17-42) treated synaptosomes. No significant difference ($p < 0.25$) was observed in the enzyme activity of enolase between the experimental set and control (Figure 4.5). No significant difference ($p < 0.1$) was observed between experimental and control samples in aldolase's enzymatic activity (Figure 4.6). The activity of aldehyde dehydrogenase was increased in experimental samples compared to control (data not shown).

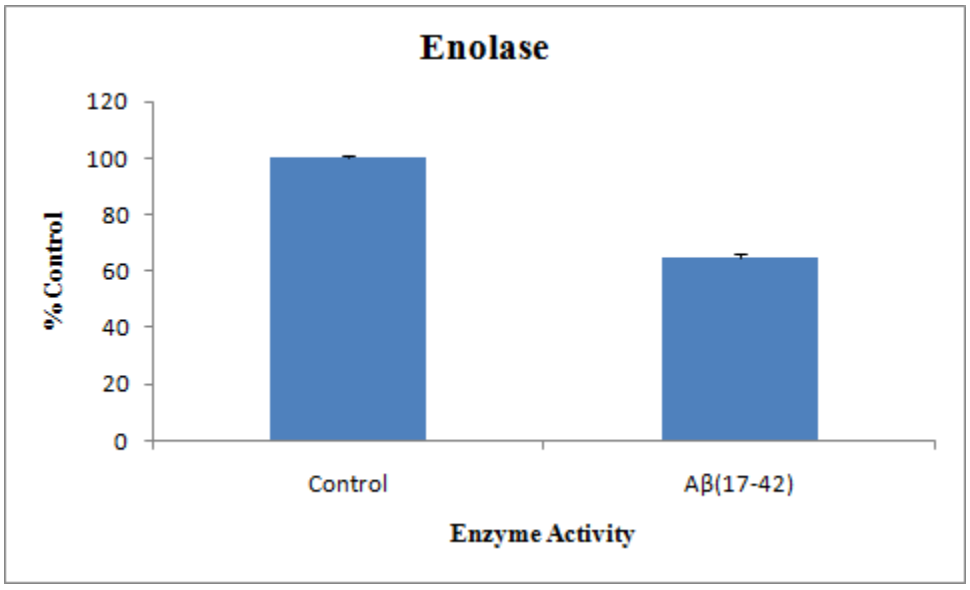


Figure 4.5 Enzyme activity of Enolase in control and Aβ(17-42) samples.

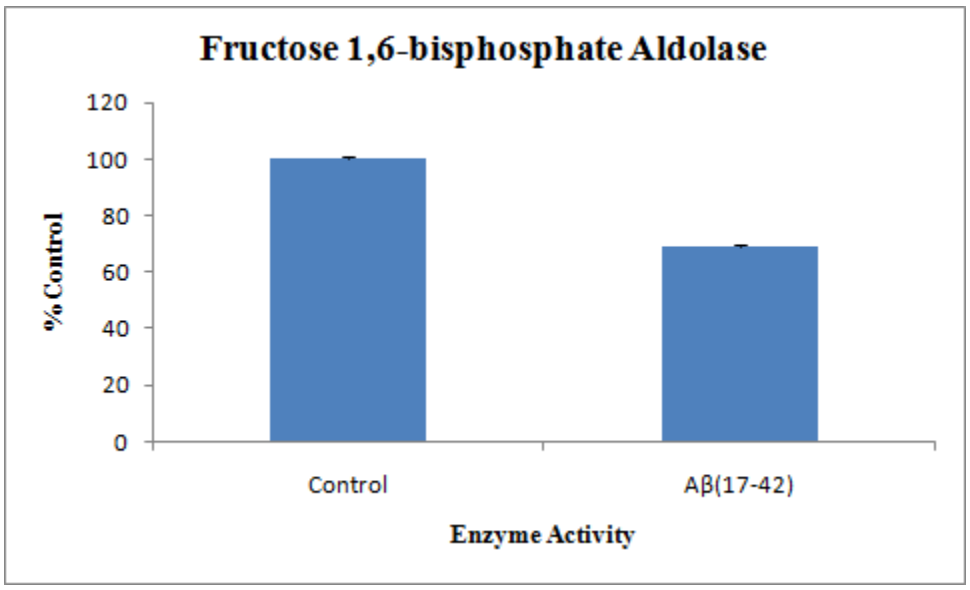


Figure 4.6 Enzyme activity of Fructose 1,6-bisphosphate aldolase in control and Aβ(17-42) samples.

4.5 Discussion

Alzheimer's disease is the most common cause of dementia among elderly people, and is characterized by extracellular deposition of neuritic plaques, amyloid beta (Aβ) and intracellular deposition of neurofibrillary tangles that are formed by the hyperphosphorylation of tau protein (Zigman and Lott 2007). As a result of dementia, the brain regions that control thought, memory, and language are affected and further causing serious decline to the patients preventing them to carry out normal daily activities. Overexpression of the amyloid precursor proteins (APP) is related to the deposition of amyloid in the brain of DS individuals and it appears to be critical to the development of AD in DS individuals (Zigman and Lott 2007).

There is increasing evidence that protein oxidation plays an important role in the development of AD. Additionally, there is increasing evidence that Aβ(1-42), may play a central role as a mediator of oxidative stress and in the pathogenesis of AD (Boyd-Kimball, Castegna et al. 2005). Synapse loss is speculated to be an earliest pathological event in AD, and a study

found that Aβ(1-42) incubation with synaptosomes causes oxidation. In this study, we examined the specific oxidative stress effects of Aβ(17-42) on synaptosomes. As Aβ(17-42) is found both in DS and AD, it could contribute to the development of AD at an earlier age in DS patients. The oxidative stress induced by Aβ(17-42) in our study suggests that it could be one of the contributing factor in the pathogenesis of AD. It has been shown that the Met35 residue Aβ(1-42), is a key amino acid residue involved in amyloid beta-peptide mediated toxicity, and consequently, the pathogenesis of AD (Boyd-Kimball, Sultana et al. 2005). Parallels can be drawn that Met35 found in the Aβ(17-42) also could contribute for the oxidative stress induced by this peptide.

Several proteins were differentially expressed in our Down syndrome model. We identified several proteins that were differentially expressed in 2D gels using mass spectrometry, these proteins are alpha enolase, fructose 1,6-bisphosphate aldolase, ATP synthase, peptidyl-prolyl cis-trans isomerase, aldehyde dehydrogenase, and heat shock cognate 71. Most of these proteins are directly or indirectly involved in the energy related metabolic processes. We found a reduction in enzyme activity of several enzymes suggesting Aβ(17-42) induced toxic effect to these enzymes leading to their decrease enzyme activity.

4.5.1 Alpha enolase

Enolase is a key glycolytic enzyme, which belongs to a new class of surface proteins that do not posses classical machinery for surface transport, but due are transported on the cell surface by unknown mechanism (Pancholi 2001). It is one of the most abundantly expressed cytosolic proteins found in many organisms. α- enolase is a metalloenzyme that catalyzes the dehydration of 2-phospho-D-glycerate to phopshoenolpyruvate in the catabolic direction in the glycolytic pathway (Pancholi 2001). Enolase has been shown to possess various different

regulatory function associated with hypoxia, ischemia, and Alzheimer's disease along with its usual role in glycolysis and gluconeogenisis. Enolase has been reported to be a neurotrophic factor (14-3-2), which is localized in the neuronal cells (Kolber, Goldstein et al. 1974), a hypoxic stress protein, and a strong plasminogen binding protein (Butterfield and Lange 2009). Enolase has been recently identified as one of the most frequently identified differentially expressed brain proteins in human and animal tissues (Petrak, Ivanek et al. 2008). It has been shown that α-enolase is one of the most consistently upregulated and oxidatively modified proteins in brain of early-onset AD, amnestic mild cognitive impairment, and late stage AD (Butterfield and Sultana 2007). Taken together, all of these findings suggest that enolase may possess one or more additional functions critical to brain cell survival along with its role in glucose metabolism. Furthermore, enolase could be integral to both normal and pathological brain function, and may possess other functions, normally necessary to preserve brain function. Up-regulation and loss of glycolytic enzyme activity of enolase is considered as a significant factor for the disease progression (Butterfield and Lange 2009).

Glucose is the main source of energy (ATP) production in the normal brain (Vannucci and Vannucci 2000), and decrease in the ATP production could cause disturbance in the ion homeostasis, cholesterol homeostasis, cholinergic defects, altered protein synthesis, transport, sorting and degradation of proteins and synaptic transmission, all of which could be detrimental to viability of the cell (Butterfield and Sultana 2007). These changes may expose phosphatidylserine to the outer membrane leaflet of the plasma membrane, which is usually present in the inner membrane; this loss of phospholipid asymmetry is an early signal of synaptosomal apoptosis (Mohmmad Abdul and Butterfield 2005). It has been shown previously that glycolytic enzymes are functionally are altered in AD (Butterfield and Sultana 2007) .

4.5.2 Fructose 1,6-bisphosphate aldolase (Aldolase)

Fructose 1,6-bisphosphate aldolase helps in breaking down fructose 1,6-bisphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, an important ATP generating step in the glycolytic pathway (Lorentzen, Siebers et al. 2004). Aldolase exists in three isoforms: A, B, and C. Aldolases A and C are preferentially involved in the glycolytic pathway and are mostly expressed in muscle and brain respectively. Aldolase B is typically expressed in liver and is also reportedly involved in gluconeogenesis (Sekar, Moon et al. 2010). As this is a glycolytic enzyme, aldolase is vitally important in the brain for the production of energy, as glucose is principle source of energy production. Identification of this enzyme, in addition to other glycolytic enzymes in the brain, suggests impaired energy metabolism pathway which could lead to a decrease in ATP production. As a consequence, it may lead to loss of synapses and synaptic function which may promote memory loss, since ATP is very crucial at nerve terminals for normal connections between neurons. Additionally, reduced ATP production may also alter glucose and glutamate transporters, cholinergic defects, altered protein synthesis, disturbances in cholesterol homeostasis, altered signal transduction, ultimately leading to cell death and consequently cognitive decline as observed in AD patients (Sultana, Perluigi et al. 2009).

4.5.3 Aldehyde dehydrogenase

Aldehyde dehydrogenases (ALDH) are a class of enzymes that detoxify and hence catalyze the removal of aldehydes present in the body (Chen, Sun et al. 2010). Aldehyde dehydrogenase catalyzes the oxidation of various aldehydes (i.e carbonyls) to carboxylic acids, and are known to play an important role in xenobiotic and endobiotic metabolism (Saini and Shoemaker 2010). Aldehyde dehydrogenases belong to a family of NADP-dependent enzyme

that catalyze the oxidation of a broad spectrum of aliphatic and aromatic aldehydes and have common structural and functional features. Each class is speculated to oxidize various substrates that may be derived from endogenous sources for examples amino acid, biogenic amine, or lipid metabolism, or from exogenous sources, such as aldehydes derived from xenobiotic metabolism (Lindahl 1992).Three classes of ALDH (for example ALDH1, ALDH2, and ALDH3) have been studied with respect to cytotoxic aldehyde metabolism. ALDH1 is present in cytosol, while ALDH2 is found in matrix of mitochondria and is responsible for acetaldehyde metabolism. ALDH3 is found extensively in lung and stomach (Hsu and Chang 1991). All three classes of ALDH enzymes metabolize HNE and utilize NAD^+ as a cofactor (Picklo, Montine et al. 2002). Acetaldehyde is considered neurotoxic product produced during the metabolic pathway of valine and threonine. It is also believed that accumulation of toxic acetaldehyde or aldehyde derivatives could affect the development of the pathogenesis of AD (Kamino, Nagasaka et al. 2000).

4.5.4 Heat shock proteins

Cells respond to sublethal heat stress by synthesis and accumulation of several members and compartmentally distinct families of heat shock proteins (Hsp). These proteins include Hsp70, Hsp90, Hsp60, and Hsp27. Some of these proteins have been found to be thermotolerant and resistant to other environmental stresses. Heat shock response has a cytoprotective role in a variety of metabolic disturbances and injuries, such as hypoxia, epilepsy, stroke, cell and tissue trauma, aging and neurodegenerative diseases (Calabrese, Colombrita et al. 2006).

The brain consumes a high rate of oxygen, as it has abundant lipid content, and has relatively low availability of antioxidant enzymes when compared with other body tissues. This makes the brain highly susceptible to oxidative stress. To overcome this vulnerability, the brain has developed networks that can combat these different kinds of stress. One such cellular stress

response is heat shock proteins, which protect cells from various forms of stress. Heat shock proteins serve as molecular chaperones, which exist in various types, among these Hsp32 (also called as HO1), Hsp60, and Hsp72 have been shown to play a protective role in the brain in regard to oxidative stress (Abdul, Calabrese et al. 2006). Heat shock cognate (Hsc71), is one of the isoforms of Hsp employed by the cell as a primary defense under unfavorable conditions. Hsc71 is specifically involved in the degradation of proteins with abnormal conformation; it can bind to a specific peptide region and label it for proteolysis and prevent protein aggregation (Castegna, Aksenov et al. 2002).

4.5.5 ATP synthase α

The α and β subunits of membrane bound ATP synthase complex bind ATP and ADP. It is speculated that α subunit is involved in the regulation of ATP synthase activity, while β subunit contributes to catalytic sites. The ATP synthase complex plays an important role in energy transduction in living cells (Walker, Saraste et al. 1982). ATP synthase is also known as complex V of the mitochondrial electron transport chain, helps in proton transport needed for the phosphorylation of ADP to produce ATP (Boyd-Kimball, Castegna et al. 2005). The α chain of ATP synthase is present in the inner membrane of mitochondria and is a part of Complex V of oxidative phosphorylation. It plays an important role in energy production. The β subunit is promotes catalytic sites. ATP synthase promotes ATP synthesis and release of ATP. It produces ATP by complex rotational movements of its subunits, and coupling the proton gradient generated by the respiratory chain (Sultana, Poon et al. 2006). ATP synthase complex is associated with proton transport necessary for the phosphorylation of ADP to produce ATP. The mitochondrial complex may get inactivated due to the oxidation of ATP synthase. Failure of

ATP synthase may decrease the activity of the entire electron transport chain and could contribute to impaired ATP production (Boyd-Kimball, Castegna et al. 2005).

4.5.6 Peptidyl-prolyl cis-trans isomerse

Peptidyl-prolyl cis-trans isomerase (PIN-1) and protein disulfide isomerase (PDI) are the two enzymes that help in the formation of correct disulfide bonds during folding of proteins. Protein disulfide isomerase are extensively found in the endoplasmic reticulum and it has been found that it helps in disulfide bond formation during the folding of nascent proteins in the endoplasmic reticulum, whereas PIN 1 are found in various cell compartments (Schonbrunner and Schmid 1992). PIN-1 catalyzes the isomerization of the peptide bond between pSer/Thr-Pro in proteins, thereby controlling their biological functions including protein assembly, folding, intracellular signaling, intracellular transport, transcription, cell cycle progression and apoptosis (Butterfield, Abdul et al. 2006). Normal function of PIN-1 includes phopshorylation of cytoskeletal proteins ex. Tau protein, response to DNA damage, and role in transcription. In most of the cells, including neurons, PIN-1 is mostly nuclear and its activity is required for the checkpoint of DNA replication. Several studies have shown that hyperphosphorylation of tau protein may occur due to improper activation of mitotic events in the cell cycle, thereby playing an important role in the progression of Alzheimer's disease (Butterfield, Abdul et al. 2006). PIN-1 is a chaperone enzyme that changes the peptide bond between a given amino acid and a proline, modifying it from the cis to the trans conformation and vice versa. This alteration caused by PIN-1 can cause remarkable structural modification, which can affect the properties of targeted proteins. PIN-1 also targets tau protein, a protein which is found to get hyper-phosphorylated in patients of Alzheimer's disease. Therefore, PIN-1 could be involved in the pathogenesis of AD (Sultana, Boyd-Kimball et al. 2006).

4.6 Conclusions

In this study, we identified 6 biomarkers that were differentially expressed in Aβ(17-42) treated synaptosomes. The proteins that were found to be upregulated include alpha enolase, aldehyde dehydrogenase, fructose 1,6-bisphosphate aldolase, peptidyl-prolyl cis-trans isomerase, and heat shock cognate 71. The protein found to be downregulated includes ATP synthase. It has been shown that protein oxidation may lead to protein conformational changes (Subramaniam, Roediger et al. 1997), and loss of protein function (Hensley, Hall et al. 1995; Kim, Vlkolinsky et al. 2000). Based on this concept, oxidation and successive loss of function of the proteins identified in our study would lead to the disruption of the synapse, neuronal communications, and impairment of energy metabolism. Taken together, the oxidative stress induced by Aβ(17-42) in synaptosomes in this current study is similar to the oxidative stress induced by Aβ(1-42) found in AD brain (Boyd-Kimball, Castegna et al. 2005).

Our present findings, suggest the role of Aβ(17-42) as one of the contributing factors in mediating oxidative stress in DS, and AD brain leading to neurodegeneration. Protein oxidation observed in our study suggests that oxidative stress may be an early event in the progression of neurodegenerative diseases. The loss of enzyme activity by oxidative modification of proteins may contribute to abnormal energy production found in many neurodegenerative disorders. Taken together these findings support the role of Aβ(17-42) as a mediator of oxidative stress and a causative agent in the pathogenesis of Down syndrome and Alzheimer's disease. Therapeutic strategies to modulate the protein oxidation during the early stages of the disease may prevent the progression of Alzheimer's disease.

CHAPTER FIVE

CONCLUSIONS AND FUTURE STUDIES

5.1 Conclusions

The studies presented in this thesis have investigated the role of Aβ(17-42) peptide induced oxidative stress with respect to the pathogenesis of Down syndrome (DS) and Alzheimer's disease (AD). The oxidative effects of Aβ(17-42) on synaptosomal preparation was examined. Oxidative stress occurs because of increased levels of reactive oxygen and nitrogen species that can react with biomolecules and may ultimately change the physiological function of the cells. We utilized the redox proteomics technique that combines two-dimensional gel electrophoresis, mass spectrometry, and protein databases to analyze several proteins from various samples.

Proteomic technique was used to identify the proteins that are differentially expressed in our novel Down syndrome model. We studied the oxidative stress parameters such as the levels of protein carbonyls, 3-nitrotyrosine, and 4-hydroxynonenal levels in the control and Aβ(17-42) synaptosomes. The proteins that were differentially expressed between control and Aβ(17-42) synaptosomes were identified by using mass spectrometry. 2D gel electrophoresis was used to separate proteins and PDquest software for used to determine the protein expression levels.

We have found a significant difference between oxidative stress levels in Aβ(17-42) treated synaptosomes and control. By using proteomics, we have also identified six biomarkers including aldehyde dehydrogenase, aldolase, α-enolase, heat shock cognate 71, peptidyl-prolyl cis-trans isomerase, and ATP synthase α chain. Enzyme assays were performed to determine enzymatic activity for α-enolase, aldehyde dehydrogenase, and aldolase. The activities of enolase

and aldolase were decreased, while the activity of aldehyde dehydrogenase was increased in experimental samples compared to control. Our findings show a decreased enzymatic activity in Aβ(17-42) treated synaptosomes compared to control samples. Decrease in enzyme activity suggests that the enzymes are susceptible to oxidative damage caused by oxidative stress. Our present findings, suggest the role of Aβ(17-42) as one of the contributing factors in mediating oxidative stress in DS, and AD brain leading to neurodegeneration. Protein oxidation observed in our study suggests that oxidative stress may be an early event in the progression of neurodegenerative diseases. The loss of enzyme activity by oxidative modification of proteins may contribute to abnormal energy production found in many neurodegenerative disorders. Therapeutic strategies to modulate the protein oxidation during the early stages of the disease may prevent the progression of Alzheimer's disease. Taken together these findings support the role of Aβ(17-42) as a mediator of oxidative stress and a causative agent in the pathogenesis of Down syndrome and Alzheimer's disease.

5.2 Future Studies

1. Based on the proteomic evaluation of the oxidative effects of Aβ(17-42) on synaptosomes, the proteins that are now differentially expressed in our novel Down syndrome model can be compared with that of proteins that are differentially expressed in the mild cognitive impairment, the earliest stage of Alzheimer's disease. If the same proteins are determined to have identical enzyme activities as found in our model, this may serve as a biomarker in future to possibly diagnose Alzheimer's disease. If the proteins differentially expressed in mild cognitive impairment are identical to our Down syndrome model, these proteins may serve as a biomarker in future to possibly diagnose

Alzheimer's disease. As the people with Down syndrome and mild cognitive impairment develops Alzheimer's disease with time, finding a biomarker would help in early diagnosis of Alzheimer's disease and may prevent the further progression of the early state to the late stage Alzheimer's disease in these patients.

2. Our complete study and experiments can be repeated and oxidatively modified proteins can be identified along with identification of differentially expressed proteins in our Down syndrome model. Proteomic analysis of proteins oxidized by Aβ(17-42) peptide may provide insight into the role of this peptide in the progression of Alzheimer's disease.
3. Currently the diagnosis of Alzheimer's disease is made based on symptoms, positron emission tomography (PET) scanning and psychomotor scores but proper diagnosis is not possible. Proper diagnosis of the AD can only be done at autopsy. Presence of extracellular deposition of amyloid beta plaques, and intracellular neurofibrillary tangles, and synapse loss confirms the Alzheimer's disease. As this histopathological identification is only possible at autopsy, development of a biomarker is highly desirable to diagnose Alzheimer's disease so that the progression of this disease can be prevented. Development of a new biomarker to possibly diagnose can save several lives and prevents the progression of this devastating neurodegenerative disorder. Further studies can be done to develop some therapeutic agents such as antioxidants, beta and gamma secretase inhibitor enzymes that can prevent the formation of the toxic Aβ(1-42) peptide from Amyloid precursor proteins, also developing some agents that can neutralize the already formed toxic oligomers of Aβ(1-42) so that amyloid plaque formation can be prevented, and thus may stop the progression of the Alzheimer's disease.

4. Superoxide dismutase is a cytosolic enzyme that catalyzes the conversion of superoxide to hydrogen peroxide and oxygen. In the next step, hydrogen peroxide is converted to water with the help of glutathione peroxidase or catalase. This two-step process causes the elimination of hydrogen peroxide and other noxious radicals. Alteration in the balance between hydrogen peroxide and antioxidant levels may produces excess of hydrogen peroxide, which then can undergo Fenton reaction to produce hydroxyl radical through its interaction with transition metals. Under stress conditions, it has been found that an excess of superoxide releases "free iron" from iron-containing molecules. The released iron (Fe^{2+}) can take part in the Fenton reaction and produces hydroxyl radical. Superoxide dismutase 1 (SOD1) overexpression is a lingering question in DS research. SOD1, located on chromosome 21along with amyloid precursor protein.

   Individuals with DS have unusually high oxidative stress levels. These persons are highly vulnerable to oxidative stress caused by the overexpression of SOD1. The increased oxidative stress may cause oxidative damage to important macromolecules. It has also been found that DS patients have an elevated ratio of superoxide dismutase to catalase and glutathione peroxidase. Overexpression of SOD1 may produce more hydrogen peroxide from the toxic superoxide anions because of its antioxidant properties. Due to overexpression of SOD1, more hydrogen peroxide is formed than it can be detoxified by other antioxidants glutathione peroxidase or catalase, as a result the production of excess hydrogen peroxide may cause various deleterious effect in the cell. It has been found that SOD1 is oxidatively modified in AD brain. As SOD1is found in both AD and DS, investigation of the enzyme activity of this protein would be very helpful in future to possibly diagnose AD.

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APPENDIX

DATA

C1	78	64	71
C2	74	69	79
C3	75	71	86
C4	62	67	68
C5	74	68	74
C6	38	42	39
E7	118	117	120
E8	113	117	116
E9	84	78	81
E10	95	86	90
E11	106	91	94

	% Control		
Control	100		Ttest
Aβ(17-42)	150.7256	p value	8.58E-08

Figure 4.1b Protein carbonyl levels of control and Aβ(17-42) treated synaptosomes (Supporting data)

C1	90	95	114
C2	119	132	141
C3	105	99	116
C4	110	112	128
C5	97	92	111
C6	106	116	121
C12	122	115	123
E7	129	131	134
E8	121	118	119
E9	119	121	115
E10	128	120	116
E11	135	126	130

	% control
Control	100
Aβ(17-42)	110.2707

	Ttest
p value	0.001976

Figure 4.2b Protein nitration levels of control and Aβ(17-42) treated synaptosomes (Supporting data)

C1	70	67	73
C2	72	81	82
C3	73	87	75
C4	71	79	84
C5	54	56	60
C6	76	73	64
C12	85	82	79
E7	73	71	88
E8	98	87	94
E9	91	111	87
E10	88	85	85
E11	70	74	71

	% Control		
Control	100		Ttest
Aβ(17-42)	115.5023	p value	0.0012

Figure 4.3b Protein bound HNE levels of control and Aβ(17-42) treated synaptosomes (Supporting data)

	Average of the initial concentration	Average of the final concentration	Difference of the final and initial concentration
C1	0.4685	0.4755	0.007
C2	0.4935	0.494	0.0005
C3	0.471	0.478	0.007
C4	0.4795	0.4835	0.004
C5	0.4925	0.493	0.0005
C6	0.4785	0.4835	0.005
E7	0.418	0.418	0
E8	0.409	0.4095	0.0005
E9	0.4195	0.425	0.0055
E10	0.432	0.441	0.009
E11	0.423	0.421	-0.002
C12	0.4205	0.4245	0.004

	% Control
Control	100
Aβ(17-42)	65

	Ttest
p value	0.257269

Figure 4.5b Enzyme activity of Enolase in control and Aβ(17-42) samples (Supporting data)

	Average of initial concentration	Average of final concentration	Difference of final and initial concentration
C1	0.824	0.7765	-0.0475
C2	0.827	0.791	-0.036
C3	0.819	0.7535	-0.0655
C4	0.719	0.701	-0.018
C5	0.738	0.704	-0.034
C6	0.795	0.774	-0.021
E7	0.757	0.7305	-0.0265
E8	0.805	0.7845	-0.0205
E9	0.726	0.704	-0.022
E10	0.7445	0.714	-0.0305
E11	0.6895	0.6675	-0.022
C12	0.6675	0.643	-0.0245

	% control
Control	100
Aβ(17-42)	69.00609

	Ttest
p value	0.094678

Figure 4.6b Enzyme activity of Fructose 1,6-bisphosphate aldolase in control and Aβ(17-42) samples (Supporting data)

VITA

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