A PROTEOMIC APPROACH FOR IDENTIFYING BORON-STRESS TOLERANT PROTEINS IN BARLEY GENOTYPES

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Chemistry

by
Ahmet Emin ATİK

December 2007

İZMİR

We approve the thesis of **Ahmet Emin ATİK**

_____

Assoc. Prof. Dr. Talat YALÇIN
Supervisor

_____

Assist. Prof. Dr. H. Çağlar KARAKAYA
Co-Supervisor

_____

Prof. Dr. Bekir SALİH
Committee Member

_____

Prof. Dr. Figen ZİHNİOĞLU
Committee Member

_____

Assist. Prof. Dr. Gülşah ŞANLI
Committee Member

28 December 2007

Date

_____

Prof. Dr. Levent ARTOK
Head of the Chemistry Department

_____

Prof. Dr. Hasan BÖKE
Dean of the Graduate School of Engineering and Sciences

ACKNOWLEDGEMENTS

First of all, I would like to thank my supervisor Assoc. Prof. Dr. Talat YALÇIN, for his excellent guidance, understanding, endless support and help throughout this study. I believe, this thesis will not come into life without his great motivations and encouragement.

I would like to express my grateful thanks to my co-supervisor Assist. Prof. Dr. H. Çağlar KARAKAYA, for his generous help and valuable comments during thesis study.

Special thanks are extended to Assist. Prof. Dr. Ahmet KOÇ, for his valuable contributions and kind helps. I owe my thanks to Plant Molecular Genetics and Molecular Genetics Laboratory staff.

I am also grateful to my laboratory friends Çağdaş TAŞOĞLU, Alaattin KAYA, Ersin AKINCI and Ercan Selçuk ÜNLÜ for their sincere help and kindness during studies.

Also, I wish to acknowledge my colleagues in Chemistry Department in İYTE for their friendship for past three years and İYTE Scientific Research Project (2006 BAP 20) for funding.

Finally, but not the least, words have no meaning when it comes to express my gratitude to my family. Thanks for all their motivation, inspiration and cooperation throughout not only this study and also my hopeless times of all through my life. I extent my sincere appreciation to my brother İsa Doğan ATİK for his comments and suggestions throughout this study.

ABSTRACT

A PROTEOMIC APPROACH FOR IDENTIFYING BORON-STRESS TOLERANT PROTEINS IN BARLEY GENOTYPES

Boron is an essential micro-nutrient for plants. However, when boron is present at high concentrations in the soil or ground water, healthy plant growth and development can be affected by boron toxicity. Turkey constitutes about 72 % of the total boron reserves in the world. In Turkey, barley (*Hordeum vulgare*) is the second widely grown cereal after wheat. Boron toxicity is one of the major factors limiting the yield of barley in Central Anatolia of Turkey.

In the present study, proteomic approach was used to investigate the boron-stress tolerant proteins in Anadolu barley genotype (boron-tolerant). Eight-day-old barley plants were treated with 10 mM H_3BO_3 for seven days. Control plants received no boron treatment during this period. Total proteins of leaves were extracted and separated by two-dimensional polyacrylamide gel electrophoresis. Control and boron-treated Anadolu genotype's proteome maps were compared and the novel proteins were excised from gels which were newly expressed under boron stress. The proteins were fragmented into peptides using in-gel digestion technique. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis and database searching gave way to identify ten spots representing seven different proteins. Two spots were identified as the same protein and one protein could not identified. The identified seven proteins are namely, ribulose 1,5-bisphosphate carboxylase/oxygenase large chain (RuBisCo large chain), thaumatin-like protein TLP5, basic pathogenesis-related protein PR5, RNase S-like protein, vacuolar proton-translocating ATPase subunit E, PSI type III chlorophyll a/b-binding protein, and light-harvesting complex I; LHC I.

Among the identified seven proteins, vacuolar proton-translocating ATPase (V-ATPase) subunit E is the important one for boron tolerance in tolerant barley genotype. It is shown that the accumulation of excess boron in the vacuolar compartment of the plant cell by the help of V-ATPase subunit E protein. This is the known as internal tolerance mechanisms for Anadolu genotype of barley to survive under boron stress. It was proposed that, this might be the defense mechanism in boron-tolerant barley genotype under toxic boron concentrations.

ÖZET

ARPA GENOTİPLERİNDE BOR-STRESİNE TOLERANSLI PROTEİNLERİN PROTEOMİK YAKLAŞIM İLE TANIMLANMASI

Bor bitkiler için gerekli bir mikro-besindir. Topraktaki veya yer altı suyundaki fazla miktardaki bor, bor toksikliğine yol açarak bitkinin sağlıklı bir şekilde büyümesi ve gelişmesini etkiler. Türkiye'de bulunan bor kaynakları dünya toplam bor rezervinin yaklaşık olarak %72'sini oluşturmaktadır. Arpa (*Hordeum vulgare*) Türkiye'de buğdaydan sonra en çok üretilen tahıldır. Bor toksikliği Türkiye'nin Orta Anadolu'sunda yapılan arpa tarımında verimi sınırlayan bir faktördür.

Bu çalışmada, Anadolu (bora karşı dirençli) arpa genotipinde bor-stresine toleranslı proteinler proteomik yaklaşım kullanılarak incelenmiştir. Sekiz-günlük arpa bitkileri yedi gün boyunca 10 mM borik asite maruz bırakılmıştır. Kontrol bitkilerine bu süre içinde bor uygulanmamıştır. Yapraktaki toplam proteinler izole edilmiştir ve iki boyutlu poli akrilamit jel elektroforezi kullanılarak birbirlerinden ayrılmışlardır. Kontrol ve bora maruz bırakılmış Anadolu genotipinin proteom haritaları karşılaştırılmış ve bor stresi altında oluşan yeni proteinler jelden kesilip çıkartılmıştır. Jel içinde parçalama tekniği kullanılarak proteinler peptitlere parçalanmıştır. Sıvı kromatografisi-ikili kütle spektrometresi (LC-MS/MS) analizi ve veritabanı taraması yedisi farklı olan on tane proteinin tanımlanmasını sağlamıştır. İki tane protein aynı protein olarak tanımlanmıştır ve bir protein tanımlanamamıştır. Tanımlanan yedi tane proteinin isimleri şöyledir: ribuloz 1,5-bifosfat karboksilaz/oksijenaz büyük zincir (RuBisCo büyük zincir), thaumatin-benzer proteini TLP5, bazik patogenez-ilgili protein PR5, RNaz S-benzer protein, koful proton-translokasyon ATPaz alt birim E proteini, PSI tipi III klorofil a/b-bağlayıcı protein, ve ışık-toplayan komleks protein I; LHC I.

Tanımlanan yedi tane protein içinde, bora karşı dirençli arpa genotipinin bor toleransını sağladığı düşünülen koful proton-translokasyon ATPaz (V-ATPaz) alt birim E proteini en önemli olanıdır. V-ATPaz alt birim E protein sayesinde hücre içindeki fazla miktardaki bor hücrenin kofulunda biriktirildiği gösterilmiştir. Anadolu arpa genotipindeki dahili dayanıklılık mekanizması olarak bilinen bu mekanizma bor stresi altında bitkinin yaşamasını sağlamıştır ve bora karşı dirençli arpa genotipinin toksik bor konsantrasyonuna dayanıklılık mekanizması olarak önerilmiştir.

Dedicated to;

the memory of my father, grandmother and grandfather

and

my lovely mother for being with me in all my life...

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CHAPTER 1

INTRODUCTION

1.1. Boron

1.1.1. Physical and Chemical Properties

Boron is a metalloid element whose atomic number is 5 and the atomic mass is 10.81 g/mol with a chemical symbol B. The oxidation state of boron is 3+ and it is a member of group IIIA in the periodic table. Elemental boron exist as a solid at room temperature, either as a black monoclinic crystals or as a yellow-brown amorphous powder. It has two naturally occurring stable isotopes; ^{10}B and ^{11}B isotopes with relative abundances 19.78 % and 80.22 %, respectively.

The electron configuration of boron is $1s^2\ 2s^2\ 2p^1$ with three valence electrons. It is electron-deficient with vacant p-orbital; it does not form ionic bonds whereas forms stable covalent bonds. It makes bonds with electron-rich substances and always found to be bound to oxygen in nature. Boron containing compounds generally behave as a Lewis acid.

Boron compounds occur in the earth's crusts as a concentration of nearly 0.001%. It is not present in nature in elemental form, but it is found combined in borax ore or tincal ($Na_2B_4O_7.10H_2O$), boric acid (H_3BO_3), colemanite ($Ca_2B_6O_{11}.5H_2O$), kernite ($Na_2B_4O_7.4H_2O$), ulexite ($NaCaB_5O_9.8H_2O$), and borates (salts of boric acid).

The solubility of elemental boron is very low in water. In contrast most boron compounds are soluble in water. Borax is easily dissolved in water to form a mixture of boric acid and borates. Boric acid is a very weak acid having low dissociation constant ($5.81x\ 10^{-10}$ at 25 °C and pK_a=9.24). At low and neutral pHs, H_3BO_3 exists in unionized form while $B(OH)_4^-$ (meta-borate ion) form is predominates at high pHs (>9.3). It is shown that both species exist in equilibrium from pH 7.0 to 11.5; therefore pH is the main factor to determine which boron species is available at desired value (Darbouret, et al. 2000). The following equilibrium is established at stated pH range.

$$B(OH)_3 + H_2O \leftrightarrow B(OH)_4^- + H^+$$

1.1.2. Major Uses and Distribution in Nature

Boron compounds are mainly used in the manufacture of fiberglass insulation, borosilicate glass, ceramic glazes, porcelain enamel, laundry products, flame retardants, herbicides, insecticide, cosmetics, pharmaceuticals (as pH buffers), and as ingredients in mild antiseptic (Woods 1994). Besides these applications, it is also used in nuclear chemistry as a radiation shield, boron neutron-detecting instrumentation in cancer and brain tumor treatment and in agricultural plant fertilizers.

Boron enters the environment through natural processes and from anthropogenic sources. Natural processes for releasing boron can be classified as weathering of boron containing rocks, volcanic activity, and as a boric acid vapour from seawater. It also exits in nature by human activities such as using borates as a fertilizers in agriculture and using borates in home and industry, release from borate mining operations, glass and ceramic manufacturing, power generation using coal and sewage disposal of boron.

Turkey has nearly 72 % of total boron reserves of the world having 803 million tones (Kar, et al. 2006). The other known massive boron deposits are located in the USA, Argentina, Russia, Chile, China, and Peru. Turkey's total income from boron export was $192 million in 2002. Eti Mine Works Management possesses 31 % of total world production on a B_2O_3 basis where Turkey's main boron deposits are along the Eskişehir, Kütahya, and Balıkesir provinces.

Boron is widely distributed as undissociated boric acid with some borate ions in natural water. Through the several analysis of groundwater showed that boron concentration has a range is from 0.3 to 100 mg/L. However, boron concentration in fresh surface water should be in range from <0.001 to 2.0 mg/L with a mean value below 0.6 mg/L. It is also shown that seawater has average boron concentration around 4.5 mg/kg (WHO 1998). The average content of borate of all soils is 10-20 mg/kg where higher values (up to 100 mg/kg) in the Western USA and across the Mediterranean in Turkey are also indicated. These boron limit values strongly depend on local geology character of soil, especially in areas of boron mining and volcanic activity.

Boron forms several chemical compounds with oxygen, but water analysis reports boron concentration in terms of its elemental boron, without defining the actual compounds that are present.

1.2. Boron in Plant Structure and Function

Plants can be classified into three categories according to their boron requirement: having lowest demand for boron is called graminaceous plants, having intermediate demand-the remaining monocots and most dicots-, and the highest boron requirement is needed for latex-forming plants. It is easily seen that every plant species require different amount of boron; it can be optimum for one where toxic for other one (Blevins, et al. 1998).

Boron is known as an essential micronutrient for healthy plant growth and development for more than 80 years. It has a unique character among the essential elements by having narrow margin between deficiency and toxicity level. However effect of boron in plants is not fully clarified up to date but the most important functions that involved in plant physiology can be classified as a role in cell wall structure, membrane function, and specific metabolic activities (Bolaños, et al. 2004). Besides these fundamental aspects; scientists have speculated that boron is also needed for protein synthesis, sugar transport, cell division, fruit and seed development, hormone regulation, carbohydrate metabolism and nitrogen regulation, and root development.

Molecular biologists suggested that higher plants contain boron as water-soluble or water-insoluble forms. Nearly all water-soluble boron localized in the apoplastic region as boric acid. It is proved that boron crosslinks two pectic polysaccharide chains at the RG-II region through borate diester bonding. RG-II is a pectic polysaccharide with 12 different sugars and 20 different linkages and found in the walls of growing plant cell. For that reason, boron plays an important role for pectic network in cell walls (Matoh 1997) by effecting cell wall pore size. Loomis and his friends stated that nearly 90 % of the cellular boron is localized in the cell wall fraction (Loomis and Durst 1992). The formation of the dimer strongly depends on monomeric RG-II and the concentration of soluble boron in the medium. If the concentration of boron in the cell is in small amount, the dimer formation can not be achieved and results in larger cell wall pore size. In the same manner, if the concentration of soluble boron is above the required value, the amount of RG-II-B will decrease and the pore size will also be changed (Fleischer, et al. 1999). It can be concluded that any increase in soluble boron concentration in the cell effects the pore sizes which facilitate access of enzymes to the

apoplastic region and less protein could be bound to the cell wall due to a lower amount of binding sites (Wimmer, et al. 2003).

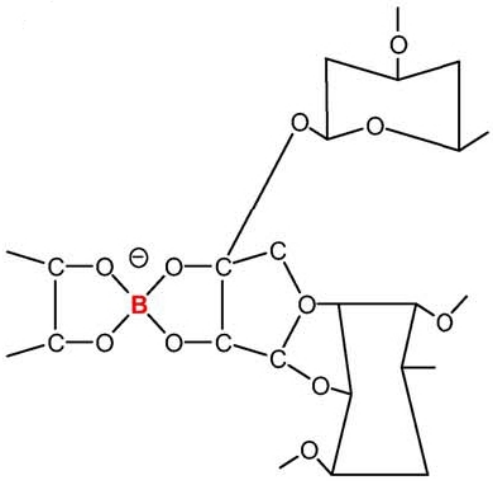

Figure 1.1. Cell Wall Boron-Rhamnogalacturonan II (RG-II-B) Complex
(Source: Bolaños, et al. 2004)

Referring to Hu and co-workers, pectin is showed to bind excess boron, thus protect the cell against to toxic effect (Hu, et al. 1996)

In addition, boron may affect metabolic activities by making complex with apoplast proteins and by interfering with manganese-dependent enzymatic reactions (Blevins, et al. 1998). Bolaños and his friends proposed that main function of boron in biological systems is stabilization of molecules with cis-diol groups (Bolaños, et al. 2004).

Boron is taken into plants in the form of boric acid. It is transported from roots to leaves through xylem vessels and accumulates in growing part of leaves and stems. During transportation some of the boron easily forms complexes with sugars and other compounds containing cis-hydroxyl groups. Excessive boron can be toxic with having more boron transport from roots to leaves. It is easily accumulated in the plant cell walls and extra boron may pass into the cytoplasm and effect cytoplasmic activities which are resulted as boron toxicity (Matoh 1997). It is important to define the chemistry of boron and uptake mechanism in plants for understanding the management of boron-toxicity and boron-deficiency for agriculture and environment.

According to the Brown and his colleagues, boron uptake is through passive permeation of plasma membrane (Brown, et al. 2002). The diffusion of boron is affected in the cell and in the apoplastic region because of complex formation.

1.3. Boron Toxicity

The mechanism of boron toxicity is still under debate. It is thought that soluble boron concentration plays an important role in the occurrence of boron toxicity in arid and semi-arid region of the world (Loomis and Durst 1992). Boron toxicity generally occurs in dry years with low rainfall regions. Boron can be easily absorbed by plant roots because of mobility of soluble boron in soils. Several approaches have been proposed about how boron caused toxicity in plants. Recently Wimmer and co-workers stated the influx of boron into the cell is easy when the concentration of boron is high in the medium (Wimmer, et al. 2003). Because of pH dependence of boron and its related compounds, boron is converted into the borates with complexing with putative ligands in the symplasm. Pyridine nucleotide coenzymes (NAD^+), ATP, RNA and several sugars are reported molecules that boron binds onto them and changes their function or changes enzyme activity in the cell (Ralston and Hunt 2000).

1.3.1. Sources of Boron

Boron is found in soils, rocks, surface, ground and ocean waters, and in the atmosphere. It is released mainly by natural processes all over the world. Large boron deposits, which are located in West and Central Anatolia of Turkey, may causes boron toxicity by having high concentration of boron in soils and groundwaters.

Boron enters to the environment mainly from irrigation waters, mining, and industrial applications.

Irrigation water, that contains high boron concentrations, is the most important contributor to boron toxicity in plants. The level of boron in irrigation waters is dependent on the geological conditions of soil. Boron level is well correlated with salinity where saline irrigation water contributes to an accumulation of boron in soils. Salinity increases boron toxicity symptoms in many plant species (Grieve and Poss 2000).

In Turkey, borate mines are located north of the town of Bigadiç and along the Simav River. Boron is present in groundwaters and rivers primarily as a result of leaching from rocks and soils containing boron compounds. The amount of boron in this river varies between 4 mgB/L to 7 mgB/L due to pollution from waste of boron mines whereas 0-0.5 mgB/L is the normal value for Simav River before reach to the mining area. Boron toxicity has also seen while using drainage water in agriculture as an irrigation that contains high level of boron. It is reported that if hot water extractable boron concentration exceeds 5.0 mg/kg in soil, it can be toxic to many plants (Nable, et al. 1997).

The main application of boron is using sodium perborate as laundry products. The waste of bleaching agents into the environment during production and end use causes boron accumulation in groundwater. These industrial effluents, irrigation waters, and surface mining have resulted on boron toxicity which can limit plant growth and decrease crop yield in soils of arid and semi-arid part of the world.

The form of boron in soil affects its availability to plants. Boron availability is totally dependent upon soil's physical and chemical properties such as clay, organic matter, pH, moisture and amount of leaching and rainfall.

1.3.2. Visible Symptoms of Boron Toxicity in Plants

Boron is known as immobile nutrient within plants which can not be translocated to new growth leaves. Therefore, boron toxicity symptoms location is in middle or older leaves. Avcı and his co-workers showed that there were no visible boron toxicity symptoms on barley plants even high (44.4 mg/L) plant boron concentration (Avcı, et al. 2005).

Visible boron toxicity symptoms can be classified as leaf burn, necrosis of the leaf tips, and necrotic and/or chlorotic patches along the leaf margins (Nable, et al. 1997). The severity of necrotic and chlorotic spots may vary if boron concentration in the medium changes. Moreover, leaf burn is not only the indicator for boron toxicity symptoms.

In contrast to leaves, roots do not show any visible symptoms against to boron toxicity. It is stated that boron concentration in the roots were low as compared to

leaves at high boron supplied soils, therefore it is concluded toxic concentration do not occur in the root of plants (Nable, et al. 1997).

1.3.3. Plant Tolerance to Boron Toxicity

The physiology of boron tolerance and boron toxicity are not well understood. However, exclusion mechanisms and internal tolerance mechanisms are thought to be the physiology of tolerance to boron toxicity in plants.

It is obvious that a level of boron above the optimum range causes significant changes in the activity of numerous enzymes and, consequently, the metabolism of higher plants (Shkolnik 1974). The possible boron toxicity tolerance mechanisms have been reported by many researchers in literature. The exclusion of boron from roots, reduced translocation to shoots and avoidance by means of shallow root systems are considered the main mechanisms involved in boron tolerance or resistance (Paull, et al. 1992, Nable 1988). In addition to these mechanisms, Nable also stated that boron tolerance of plant genotypes is related to their abilities to passively transport of boron as a result of differences in their membrane composition (Nable 1988).

Recently Hayes and Reid showed results of tolerant and sensitive barley genotypes to boron toxicity in terms of boron concentrations in their roots, leaves, and xylem. They demonstrated that tolerant variety able to maintain lower boron concentration in its structure against susceptible one due to actively boron efflux from tolerant roots which results lower concentration of boron in the xylem and finally less accumulation of boron in the shoot. As a conclusion, they proposed two models for boron efflux as the basis of boron tolerance in barley namely either by anion exchange or an anion channel. The lack of a capacity to efflux of borate anion in boron sensitive genotype is the main factor for boron toxicity in that plant species. They concluded that boron tolerance in barley is mediated by efflux of boron from the roots (Hayes and Reid 2004).

Every plant species have different boron necessity for normal growth. The following table indicates relative tolerance to boron of some crops.

Table 1.1. Relative Tolerance to Boron Toxicity of Some Crops
(Source: Keren and Bingham 1985)

Tolerant (2-4 mg B/L)	**Semi-tolerant (1-2 mg B/L)**	**Sensitive (up to 0.3 mg B/L)**
Carrot (*Daucus carota*)	Barley (*Hordeum vulgare*)	Bean (*Phaseolus vulgaris*)
Sugar Beet (*Beta vulgaris*)	Maize (*Zea mays*)	Apple (*Malus domestica*)
Alfalfa (*Medicago sativum*)	Potato (*Solanum tuberosum*)	Avocado (*Persea americana*)

1.4. Barley (*Hordeum vulgare* L.)

Barley (*Hordeum vulgare* L.) is a major staple and second widely grown crop in Central Anatolia of Turkey (Avcı, et al. 2005) after wheat and it is the fourth most important cereal after wheat, maize, and rice in the world. It is used for human consumption, animal feed, and in malt industry. Turkey has taken its role in world production in 6th place by producing approximately 7,600 out of 137,436 million tones by the year session of 2005/06.

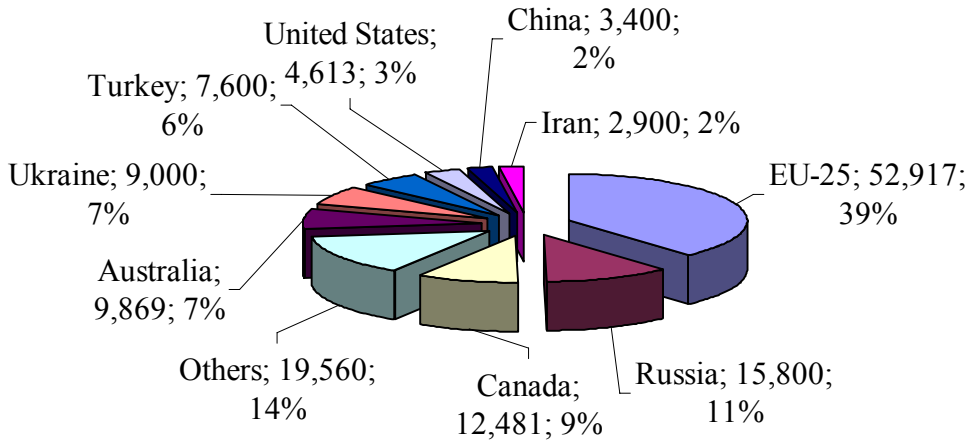

Figure 1.2. World Barley Producers by the Year of 2005/06
(Source: USDA 2007)

It is also stated that barley is also an indicator plant for boron toxicity (Krantz and Melsted 1964).

In Turkey, there are ten different barley genotypes namely Anadolu, Bülbül, Cumhuriyet, Erginel, Hamidiye, Obruk, Tarm-92, Tokak, Yea-1868, and Yesevi. There is large variation in tolerance to boron toxicity among these varieties. When the severity of toxicity symptoms on leaves, levels of shoot dry matter production, and grain yield taken into account, Hamidiye and Bülbül were identified as the most sensitive genotypes. On the other hand Obruk, Yea-1868, Cumhuriyet, and Erginel classified as moderately tolerant genotypes while Anadolu, Tarm-92, Yesevi, and Tokak are the most tolerant genotypes against boron toxicity (Torun, et al. 2003).

CHAPTER 2

PROTEOMICS AND MASS SPECTROMETRY

2.1. Origin of Proteome and Proteomics

The term proteome is used for total protein content expressed by genome in a cell, tissue or organism under a defined set of conditions (Wasinger, et al. 1995 and Wilkins, et al. 1996). Proteome was firstly proposed by Marc Wilkins, and co-workers in 1994 at the Conference on Genome and Protein Maps (Siena, Italy) as the "PROTEin complement expressed by a genOME". Unlike genome, total DNA content, proteome is highly dynamic- the cell responds to internal and external effects by changing the level and activity of its proteins, hence alteration in proteome content. It is clear that proteome is like a photographic snapshot of the proteins which are expressed at a given moment in a cell.

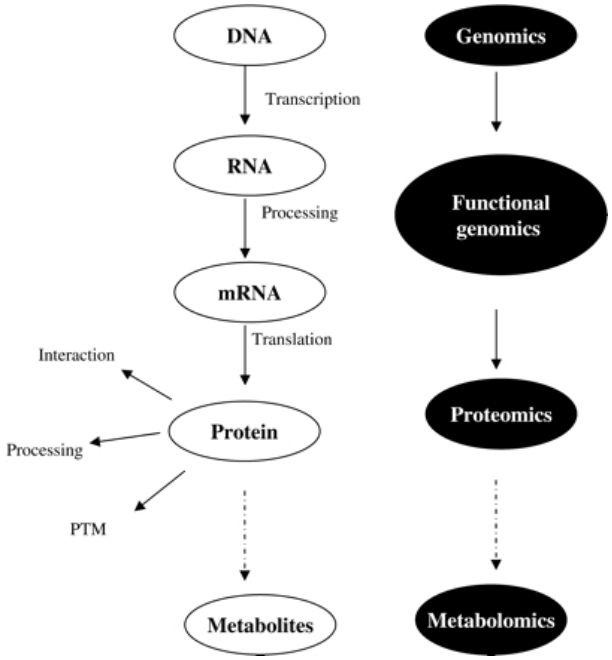

Figure 2.1. Overview of the Currently Available Disciplines for Large-Scale Analyses of Genes, Transcripts, Proteins and Metabolites (Source: Tilleman, et al. 2005)

Beyond the proteome, proteomics is the study of proteins with its expression levels, interactions, and their functions in a given cell, tissue or organism at any given time (Barbier-Brygoo and Joyard 2004). In deeper, proteomic analysis related with identification of amino acid sequence of proteins, determination of their relative amounts, their modification patterns, and their relations with other proteins.

2.1.1. Why Proteomics?

Proteomics is the identification and sequencing of proteins isolated from cell, tissue, and body fluids. Proteins have performed almost all cellular activities in cells; therefore it is very important to define the identity of the proteins individually. Proteins are made up with basic building blocks called amino acids and they are joined together by peptide bonds. It is well know that each protein is built from different arrangement of twenty natural amino acids and two modified derivatives of amino acids discovered until now.

Protein synthesis is two step process namely transcription and translation. Single strand of messenger RNA (mRNA) is produced in the nucleus by copying one strand DNA by the help of RNA polymerase enzyme. Then mRNA passes through to the cytoplasm for translation on the ribosome. Ribosome reads three nucleotides at a time by matching each three bases to its base pairing anticodon- site for amino acid attachment- which is located on tRNA. Afterwards tRNA transports the defined amino acids to the ribosome where each tRNA is specific for one amino acid. Finally amino acids are linked together by peptide bonds to forms proteins. In the past, it is believed that every gene is responsible for one protein, but this is not correct anymore. There is a considerable increase in complexity of proteins when gene expression is analyzed; in other words proteome is larger than the genome.

It is estimated that human genome consist of approximately 35,000 genes where expected encoded protein number may vary from 200,000 to 2 millions due to alternative splicing, post-translation modifications (PTMs) of proteins (phosphorylation, glycosylation, methylation, etc) and protein degradation (Rose, et al. 2004). The diversity of protein can not be explained only by gene expression analysis; hence proteomic analysis is required for identifying cell function in terms of its protein level.

Scientists believed that it can be easy to understand the aetiology of human diseases after completion of human genome sequence in 2002 but this is not come true in real life. Basicly, proteome of diseased cell will have differences at protein level over the normal cell proteome where genome remains constant over this period. Molecular characterization of human diseases can only be accomplished by proteomic based studies. In the past decade, proteomics is used for investigation infectious diseases and several cancer tumors (Adam, et al. 2003, Lehrer, et al. 2003, Rai, et al. 2002), Alzheimer's disease (Choi, et al. 2002, Mattila and Frey 1996) and Down syndrome (Greber, et al. 1999) by monitoring protein composition in certain body fluids such as serum, prostatic fluid, and urine.

2.1.2. Types of Proteomics

There are mainly three types of proteomics which are named as expression, structural, and functional proteomics.

Expression proteomics aims to identify the protein expression level of biological systems in terms of its response to the any physical and biochemical stresses. In other words, it deals with change in entire proteome in a cell, tissue, or organism at a certain time. Structural proteomics seeks to identify all the proteins within the cell or any organelle with their location. Its major role is mapping out the structure of protein in three dimensional space using X-ray crystallography and NMR spectroscopy methods. Functional proteomics is concerned with the identification of functions, activities, and interactions of all the proteins in proteome.

2.2. Plant Proteomics

Plant proteomics has entered the functional genomics era after the genome sequence of thale cress (*Arabidopsis thaliana*) (The Arabidopsis Genome Initiative 2000) and rice (*Oryza sativa*) (Goff, et al. 2002, Yu, et al. 2002) were completed. Although its infancy, it will be a very active field in biology with the increasing amounts of other plant genome and EST data.

Researchers have focused on protein isolation from cellular compartments of any cell or tissue instead of dealing with total protein complement. This is just because

resolution of protein spots on a 2D (two-dimensional) gel is limited, complete proteome of a cell divided into sub-proteomes in order to enhance the differentiation of spots easily. Therefore, in the last two decade researches have been carried out on different plant organs (Watson, et al. 2003), organelles and individual tissues such as seeds (Gallardo, et al. 2001), leaves (Donnelly, et al. 2005, Dani, et al. 2005), and roots (Yan, et al. 2005, Bona, et al. 2007). Proteomic studies in different subcellular compartments in plants can be classified as: cell wall (Chivasa, et al. 2002, Borderies, et al. 2003), chloroplast (Peltier, et al. 2000, Ferro, et al. 2003, Zolla, et al. 2002, 2003), chloroplast membrane (Peltier, et al. 2002, Zolla, et al. 2004), mitochondria (Kruft, et al. 2001, Millar, et al. 2001), nucleus (Bae, et al. 2003), and plasma membrane (Santoni, et al. 1998, Kawamura and Uemura 2003). Moreover, total protein profile of any part of cell can be altered due to environmental stresses. Several studies have been carried out with different plant species with abiotic and biotic stress conditions. Some of the abiotic stresses are classified as non-optimal temperatures (Amme, et al. 2006, Ferreira, et al. 2006), drought (Salekdeh, et al. 2002), salinity (Kav, et al. 2004), heavy metals (chemicals) (Ingle, et al. 2005) and soil nutrient problems (Kang, et al. 2004) whereas bacterial (Jorrin, et al. 2006), fungal (Campo, et al. 2004) and viral diseases (Ventelon-Debout, et al. 2003) are belong to the biotic stress category. It is well known that plants are immobile in soil, so they can easily change their leaf's and root's physiological and morphological structures to protect itself against biotic and abiotic stresses. For that reason, plants develop defense mechanisms to protect itself by changing its protein types and their expression levels when exposed to any given stress treatment.

Among the stresses, nutritional disorders have taken its role in the preliminary places. Dealing with nutrient(s) either deficiency or toxicity levels are classified as the most important problems for the soil management. Plant nutrients are categorized into two types: macro-nutrients and micro-nutrients. Plants require a right combination of macro- and micro-nutrients for healthy growth, development and reproduction, however, nutrient requirement changes among plants. Macro-nutrients are the elements which required relatively large amounts such as nitrogen, phosphorus, potassium, calcium, magnesium and sulfur whereas micro-nutrients are the elements that need by plants only small quantities such as iron, manganese, boron, zinc, copper, molybdenum and chlorine. On the other hand, carbon, hydrogen and oxygen are the nutrients absorbed in large amounts from air, water and soil. Up to date, there are 16 elements that have been found to be essential for healthy plant growth. These elements are

generally taken up in their ionic forms by roots of plants. Because of high cost and time consuming methods, clean-up procedures for excess amount of nutrients from soils have not been preferred. Similarly it is hard to define the deficiency problems for several elements in the soil. To manage these toxicity and deficiency problems, proteomic approach can be used to identify related proteins which are involved in defense and/or tolerance mechanisms in plants. Two-dimensional (2-DE) gel electrophoresis coupled with mass spectrometric analysis has been used after development of soft ionization methods (MALDI and ESI) for mass spectrometry.

2.3. Two-Dimensional Gel Electrophoresis

One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1-D SDS-PAGE or 1-DE) has been used for several decades to separate total protein extracts based on protein's molecular weight (size) difference. However 1-DE can not resolve more than 80-100 different protein components where cell proteomes are extremely complex having several thousand of proteins. O'Farrell firstly introduced high-resolution two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2-D SDS-PAGE or simply 2-DE) for separation complex protein mixture in 1975 (O'Farrell 1975). 2-DE is not only used for complete protein separation but also to analyze the protein alterations due to environmental stress conditions and to detect co- and post-translation modification which can not be determined from genome sequence. Mainly 2-DE is used as a component of proteomics and is the step used for separation of proteins for further characterization by mass spectrometry (MS).

2-DE separates protein mixture according to two distinct properties of proteins, isoelectric point (pI) in the first dimension and molecular mass (M_r) in the second dimension. More generally, isoelectric focusing (IEF) is coupling with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for total proteins separation. Today's modern 2-DE systems has a capacity to separate up to 10,000 protein spots on one gel theoretically by capability of approximately 100 protein separation in each dimension. Depending on the pore size in acrylamide gels and pH gradient used, 2-DE systems can resolve more than 5000 proteins simultaneously having nearly 2000 proteins routinely and able to detecting and quantifying protein amounts of nearly 1 ng per spot.

The resolution of proteins on gels and reproducibility of gels have been improved by the development of dry commercial IPG strips for the first dimension of 2-DE (Görg 1991). The availability of commercial IPG strips of different pH ranges and lengths, i.e. 3-10, 3-10 NL (nonlinear gradient), 4-7, 3-6, 5-8, 7-10, 3.9-5.1, 4.7-5.9, 5.5-6.7, 6.3-8.3 and 7, 11, 17, 18, 24 cm in length, respectively enables of broad view of proteome maps. The theory behind the IEF can be described as follows: protein sample is loaded to an IPG strips and an electric potential is applied across the strips. As all know, proteins carry either negative, positive, or zero net charge due to containig different side chains. Under the influence of electric field, proteins will move along the strip and will stop at the isolectric point of itself. Isoelectric point of a protein is the point where the net charge on a protein is zero. If the proteins have net negative charges they will be pulled to anode-the more positive end of the gel. In contrast, if the proteins have net positive charges they will be pulled towards the cathode-the more negative side of the strip.

The second dimension of 2-DE separates proteins according to their molecular weight, in other words their mobility in polyacrylamide porous gel. SDS-PAGE can be performed on horizontal or vertical systems (Görg, et al. 1995). Vertical systems are prefered when multiple runs in parallel are required. Pore size of the polyacryamide gel can be controlled by varying the total acrylamide content of the gel and cross-linker content of the total acrylamide. IPG strips having low polyacrylamide content can acts as a stacking gel due to concentrated, ready and nonrestictive protein zones within. Therefore there is no need to use stacking gel with vertical 2-DE systems.

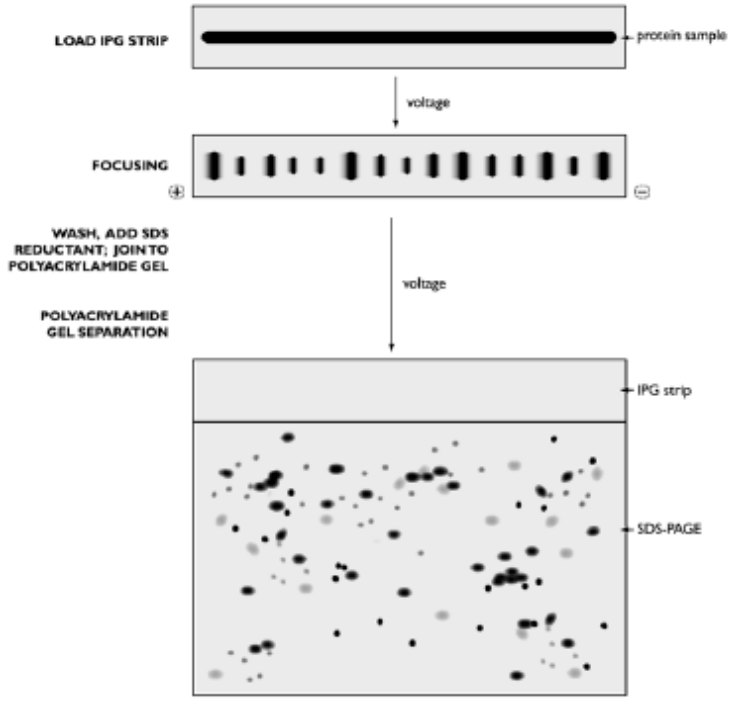

Figure 2.2. Schematic Representation of 2-DE SDS-PAGE
(Source: Liebler 2002)

However, 2-DE system has limitations, such as poor solubility of membrane and hydrophobic proteins (Adessi, et al. 1997), difficulties in resolving and identifying very acidic or basic proteins and low-abundance proteins and limited dynamic range. Although having limitations, 2-DE is still a powerful and versatile method and the most commonly used technique in proteome analysis.

2.3.1. First Dimension: Isoelectric Focusing

The first dimension in 2-DE system is the separation of proteins according to their isoelectric point. In the past, IEF was done with thin polyacrylamide gel rods in glass tubes with the aid of carrier ampholyte-generated pH gradients (O'Farrel 1975). Carrier ampholytes with the highest pI migrate towards to the anode and the carrier ampholytes with the lowest pI move to the cathode which provides a linear and continuous pH gradient. This method does not produce reproducible results. In the following years, an immobilized pH gradients was introduced to improve the reproducibility of 2-DE gels (Görg, et al. 2000). They are made with buffering

acrylamide derivatives which contain either a free carboxylic acid or tertiary amino group that is copolymerized with acrylamide and bis-acrylamide. These IPG strips are mechanically strong and their pH gradient can not drift. Additionally, IPGs can be in different pH ranges between pH 2.5 to pH 12 such as linear and nonlinear wide range (e.g. IPG 3-12), medium (e.g. IPG 4-7), narrow (e.g. IPG 4.5-5.5), and ultra-narrow (e.g. IPG 4.9-5.3) as well as with different lengths.

Table 2.1. The Properties of Commercial IPG Dry Strips

	Strip Length				
	7 cm	**11 cm**	**17 cm**	**18 cm**	**24 cm**
Strip dimensions					
Strip length	7.9 cm	11.8 cm	17.8 cm	19.0 cm	24.7 cm
Gel length	7.25 cm	11.0 cm	17.1 cm	18.0 cm	23.4 cm
Strip width	3.3 mm	3.3 mm	3.3 mm	3.3 mm	3.3 mm
Gel thickness	0.5 mm	0.5 mm	0.5 mm	0.5 mm	0.5 mm
Linear pH gradient range					
Broad range	3–10, 4–7, 3–10 NL*	3–10, 4–7, 3–10 NL	3–10, 4–7, 3–10 NL	3–10, 4–7, 3–10 NL	3–10, 4–7, 3–10 NL
Narrow range	3–6, 5–8, 7–10	3–6, 5–8, 7–10	3–6, 5–8, 7–10	3–6, 5–8, 7–10	3–6, 5–8
Micro range	3.9–5.1 4.7–5.9 5.5–6.7 6.3–8.3	3.9–5.1 4.7–5.9 5.5–6.7 6.3–8.3	3.9–5.1 4.7–5.9 5.5–6.7 6.3–8.3	3.9–5.1 4.7–5.9 5.5–6.7 6.3–8.3	
Gel composition	4%T/3%C	4%T/3%C	4%T/3%C	4%T/3%C	4%T/3%C
Storage	-20°C	-20°C	-20°C	-20°C	-20°C
Number of strips/package	12	12	12	12	12
Anode (acidic) end identified by:	+	+	+	+	+

*Nonlinear gradient

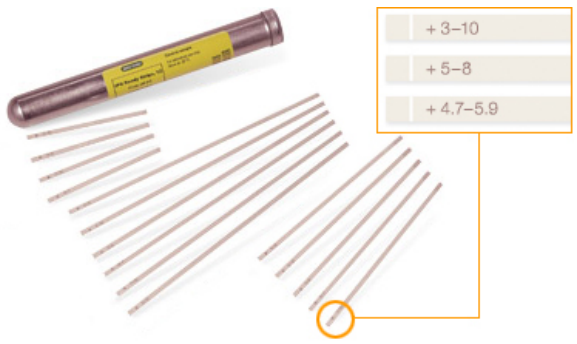

Figure 2.3. The Picture of IPG Strips with Different pH Ranges and Lengths

2.3.1.1. General Guidelines for IEF with IPGs

Before IEF, the IPG dry strip needs to be rehydrate (generally 12 hours) to return it to original thickness of 0.5 mm with a rehydration buffer containing chaotropes (e.g. urea and thiourea), non-ionic or zwitterionic detergents (e.g. CHAPS), reducing agents (e.g. DTT) and carrier ampholytes. Sample can be applied to the IPG dry strips either by including it in the rehydration solution or by applying it directly to the rehydrated IPG strip through cup-loading method. Cup-loading is commonly used approach to improve the resolution of basic protein's separation (Görg, et al. 2000). This sample loading method is preferred when the samples are contaminated with nucleic acids or other large non-proteinaceous molecules. There are several advantages of loading sample onto the IPG strip by including the sample in rehydration solution such as loading larger quantities of proteins and allows their proper separation, gives chance to load more dilute samples and minimizes protein precipitation.

The sample-loading/gel rehydration process (in-gel rehydration) can be accomplished in either a passive or active manner. In passive rehydration, the sample is put into the one well of focusing tray firstly, then IPG dry strip is placed gel-side down in the same well, that is, in contact with the protein sample. The strip is then covered with mineral oil to prevent evaporation and urea crystallization during rehydration and finally allowed to incubate overnight (12-16 hours). This method allows proteins loading naturally. In contrast, active rehydration is carried out in the presence of low voltage (typically 50 V) during rehydration period. It is believed that in-gel rehydration loading under low voltage procedure provides a more complete uptake of protein in the sample into the IPG strip and facilitate the entry of large proteins into the gel. The procedure steps (placing strips gel-side down and covering it with mineral oil) are the same with the passive rehydration. After overnight incubation time, with voltage or without any voltage, the IEF cell is programmed using appropriate protocol for focusing proteins according to their pI.

A common temperature for isoelectric focusing is 20 °C and this precise temperature control is maintained by peltier cooling within the IEF cell system. The temperature must be kept constant at specified value because any change in temperature may affect the pI of sample proteins and this causes spot position change in polyacrylamide gel. It is proved that 20 °C is the optimal temperature for IEF run and it

is required for enhancement of reproducibility (Görg, et al. 1991). Carbamylation of proteins may occur when the temperature is too much above 20 °C while much lower temperatures may cause precipitation of rehydration solution components such as urea.

Figure 2.4. PROTEAN IEF Cell System

Focusing conditions will vary with sample composition, sample complexity, and IPG pH range. The current should not be exceeded 50 µA/strip. The total voltage applied onto the strip is shown in volt-hour integrals (Vh) in IEF. If the applied Vh is not enough for focusing, the protein spots on a gel may undergo horizontal streaking. In the same way, when the proteins are focused too long, cysteines will become oxidized and the structure of protein will change. The modified proteins have different pI and start to migrate again with the horizontal streaks radiating from the spots. Hence, the best results are obtained with the shortest possible focusing steps at the highest possible voltage (Lopez 2007). The following tables show the parameters recommended for IEF program and the range of protein loads for IPG ReadyStrip by Bio-Rad company.

Table 2.2. Recommended PROTEAN IEF Cell Focusing Condition

ReadyStrip pH 3–10, 3–10 NL, 4–7, 5–8 Focusing Conditions

	Start Voltage	End Voltage	Volt-Hours	Ramp	Temperature
7 cm	0 V	8,000 V	8–10,000 V-hr	Rapid	20°C
11 cm	0 V	8,000 V	20–35,000 V-hr	Rapid	20°C
17 cm and 18 cm	0 V	10,000 V	40–60,000 V-hr	Rapid	20°C
24 cm	0 V	10,000 V	60–80,000 V-hr	Rapid	20°C

Table 2.3. Recommended Range of Protein Loads for ReadyStrip IPG Strips

	Strip Length			
ReadyStrip	**7 cm**	**11 cm**	**17 cm and 18 cm**	**24 cm**
3–10, 3–10 NL	5–100 µg	20–200 µg	50–300 µg	65–400 µg
4–7	10–150 µg	40–200 µg	80–300 µg	100–400 µg
3–6	10–150 µg	40–200 µg	80–300 µg	100–400 µg
5–8	10–150 µg	40–200 µg	80–300 µg	100–400 µg
7–10	20–200 µg	50–300 µg	100–300 µg	130–400 µg
3.9–5.1	10–125 µg	50–250 µg	100–400 µg	
4.7–5.9	10–80 µg	50–125 µg	100–250 µg	
5.5–6.7	10–80 µg	50–250 µg	100–400 µg	
6.3–8.3	10–125 µg	125–250 µg	200–500 µg	

Proteins are usually present in their native form in biological samples. They are not soluble at that state, therefore need to be denatured to help solubilization. Sample preparation for 2-DE requires the use of solubilization, desegregation, denaturation and reduction buffer which is called rehydration buffer. The components of buffer must have capability of converting all the proteins into a single conformation, preventing protein aggregates, deactivating proteases, providing cleavage of disulfide and hydrogen bonds, and getting hydrophobic proteins into solution. Sample solubilization solutions generally contain chaotropic agents, detergents, reducing agents, and ampholytes.

Chaotropic agents, such as urea and thiourea, disrupt hydrogen bonds and hydrophobic interactions both between and within proteins. Urea has ability to disrupt secondary protein structure when the total urea concentrations is 9 M. In addition, thiourea can improve proteins solubility (Rabilloud, et al. 1997) while solubilization of urea decreases if the buffer contains thiourea. Therefore urea concentration should not exceed 5-7 M when combined with 2 M thiourea. In other words, the total concentration of urea and thiourea should be in the range 7–9 M. Urea and thiourea can hydrolyze to cyanate and thiocyanate, respectively under heat treatment which can modify amino groups on proteins and give way for charge heterogeneity. Hence, samples containing chaotropes should not be heated above the 37 °C in the presence of protein. Guanidine hydrochloride is another chaotrope but it is not preferred because of its incompatibility with IEF in 2-DE system due to its charge.

Detergents are also used to disrupt hydrophobic interactions between and within proteins, provide effective protein extraction and solubilization. They are classified into three groups according to their hydrophilic group's ionic character: ionic (e.g. anionic-

SDS or cationic detergents), non-ionic (uncharged, e.g. octyl glucoside, dodecyl maltoside, Nonidet P-40 (NP-40) and Triton X-100), and zwitterionic (having both positively and negatively charged groups with a net charge of zero, e.g. CHAPS, its hydroxyl derivative-CHAPSO and ASB-14). SDS, effective solubilizing agent for hydrophobic and membrane proteins, interferes in isoelectric focusing step by horizontal streaking on gels and therefore cannot be the choice for IEF experiment. Therefore, non-ionic and zwitterionic detergents commonly used at a concentration range of 0.5-4% in isoelectric focusing. These detergents ensure protein solubilization and prevent aggregation. CHAPS is most commonly used one in proteomic studies in the last decade due to its high solubility and a relative lack of detergent-induced artifacts (Kulakowska, et al. 2007). Triton X-100 is preferred to recover hydrophobic proteins where CHAPS has failed to extract them. It can be concluded that chaotrope and detergent's concentration must be in agreement for total solubilization and disruption of hydrophobic interactions.

Reducing agents (reductant) are necessary for cleavage of intra- and intermolecular disulfide bonds, such as between cysteine residues, which promote unfolding of proteins. Dithothreitol (DTT), dithioerythritol (DTE) and β-mercaptoethanol belong to the class of sulfhydryl reductant while tributylphosphine (TBP) and tris-carboxyethylphosphine (TCEP) are the example of phosphine reductants class. In addition, these sulfhydryl or phosphine reductants are used for 2-D sample preparation step. DTT and DTE can be used at lower concentrations (e.g. ranging from 20 to 100 mM). However, DTT is charged and migrates to its pKa during IEF which can lead to a loss of solubility of some proteins. To overcome this problem, TBP and TCEP are used recently as remedies for the problems associated with the use of thiol reagents in concentration of 2mM. It is obvious that the use of non-charged phosphine reductants increase solubilization of proteins during IEF.

Both ionic strength and pH of the solution has a role on protein solubility. For this purpose, carrier ampholyte mixtures (sometimes referred to as IPG buffer) are a common added to sample preparation solutions because they increase both buffering power and ionic strength without causing any interference with IEF. It is used at a concentration range of 0.5-2% in isoelectric focusing for acting cyanate scavengers and also ensures uniform conductivity during IEF without altering the pH gradient of the IPG strip. Generally, sample extraction and solubilization can be done with the same solution used for rehydration prior to IEF. This is just because for simplifying the

experimental procedure and ensures that there is no IEF incompatible chemicals are introduced during sample preparation.

2.3.1.2. IPG Strip Equilibration

Prior to the second dimension separation, an equilibration step is necessary to saturate the IPG strip with SDS and for efficient transfer of the proteins into the SDS-PAGE gel. Strip equilibration is a two-step process to ensure the proteins are suitable for SDS-PAGE analysis. Equilibration buffer I, which contains 50 mM Tris-HCl (pH 8.8), 2% (w/v) SDS, 1% (w/v) DTT, 6 M urea and 30% (w/v) glycerol, is initially added onto focused IPG strips for 10-15 minutes (Görg, et al. 1988). This is followed by a further 10–15 minute equilibration in the same solution containing 4% (w/v) iodoacetamide instead of DTT which is called equilibration buffer II. Urea and glycerol is required to reduce electroendosmotic effects. Electroendosmosis is the movement of buffer within the IPG strip and is due to the fixed charge associated with the ampholytes contained within the strip. The usage of DTT in the first equilibrium buffer is cleavage of sulfhydryl bonds between cysteine residues within a protein. In contrast, iodoacetamide is alkylated any thiol groups in the protein preventing their reoxidation where reoxidation can result in streaking within the gels. Besides reoxidation, it also alkylates any remaining DTT to prevent point streaking.

The treatment of strips with these two equilibrium buffer is strongly recommended, since it considerably simplifies further protein identification with in-gel digestion procedure by mass spectrometry. After equilibration steps, the IPG strips are applied onto the surface of the second dimension vertical SDS-PAGE gels.

Table 2.4. The Minimum Required Equilibrium Buffers for One Strip

	Strip Length				
	7 cm	**11 cm**	**17 cm**	**18 cm**	**24 cm**
Equilibration buffer I	2.5 ml	4 ml	6 ml	6 ml	8 ml
Equilibration buffer II	2.5 ml	4 ml	6 ml	6 ml	8 ml

2.3.2. Second Dimension: SDS-PAGE

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, gives a rapid and relatively accurate way to determine the protein's molecular weight. The theory is simply based on electrophoretic mobility of proteins within polyacrylamide gel pores where smaller proteins move more rapidly through the gel than larger proteins such that mixtures of proteins are separated according to their molecular size.

Proteins are found into a variety of shapes such as compact or elongated in their native form. Therefore, protein mixture is firstly solubilized with a buffer solution containing SDS, an anionic detergent, which denatures secondary and tertiary protein structures. During mixing, the SDS binds to the protein in a ratio of 1.4 g SDS per gram of protein to form micelles. This creates a uniform mass-to-charge ratio between different proteins which allows separation on a polyacrylamide gel only by mass. Without SDS, proteins that are similar in molecular weights can move differently in polyacrylamide gels because of difference in folding pattern. SDS masks the charge of the proteins themselves by surrounding proteins with its negative charge. The magnitude of negative charge on a protein is proportional to the protein's molecular weight. The principle underlying SDS solubilization is the fact that all proteins are negatively charged for movement in the same direction, towards the positive electrode.

The most commonly used buffer for the second dimension is the discontinuous Tris–chloride/Tris–glycine buffer system of Läemmli (Läemmli 1970). In the classical SDS-PAGE, the gel divided into an upper "stacking" gel with low percentage and pH of 6.8 by having large pore size and a lower "resolving" gel with pH 8.8 with much smaller pores. However, development and usage of IPG strips have solved two stage gel preparation case, because proteins are already pre-separated by the IEF. Hence, today's 2-DE systems do not needed stacking gel.

Polyacrylamide gels are simple to prepare with sufficient number of parameters, which can be changed to optimize the specific separation, being considered. The parameters include the total acrylamide content, the degree of cross-linking in the polyacrylamide and the thickness and length of the gel. Total acrylamide content and the cross-linker content of the gel can be represented by % T and % C, respectively. At certain polyacrylamide percentage, there is a linear relationship between molecular weight and the relative migration distance of proteins in gels. The pore size can be

controlled by varying the % T from 4 % to 20 % T with constant % C at 2.7. The most appropriate acrylamide concentration is 12 % due to correct and linear separation of proteins from 7 kDa to 202 kDa. Gels with lower percentages have not good protein resolution in 2-DE maps and higher percentages makes extraction of proteins from gels more difficult for further protein identification, such as mass spectrometric analysis. In addition, the molecular weight of proteins can be roughly estimated by running standard of known molecular weights in the same gel.

Polyacrylamide gels are mainly composed of chains of polymerized acrylamide that cross-linked by a bifunctional agent that is called N, N′-methylenebisacrylamide, SDS, Tris-buffers, N, N, N′, N′ -tetramethylethylenediamine (TEMED) and ammonium persulfate (APS). The size of pores decreases as the bisacrylamide to acrylamide ratio increases. Additionally, APS provides the free radicals that drive polymerization of acrylamide and bisacrylamide while TEMED accelerates the polymerization of acrylamide and bisacrylamide by catalyzing the formation of these free radicals formed.

Overall, 2-DE is an analytical method for determining isoelectric point, molecular weight, and the relative amount of protein in mixture. The focused proteins on IPG strip is saturated with SDS and transferred to the molecular weight gel for the second dimension. The final gel is a kind of protein map that represent the separated proteins according to pI across the x-axis and according to molecular weight across the y-axis. Additionally, these gels are displayed with acidic side of the gel oriented on the left while basic side of the gel oriented on the right, and the protein's molecular weights decreasing from the top to the bottom of the gel.

2.4. Detection of Protein Spots and Image Analysis

The final component of 2-DE experiment is visualization of separated protein spots on gels either by universal or by specific staining methods. Universal staining methods for protein detection on two-dimensional gels include staining with Coomassie blue dye, silver staining, negative staining with metal cations (e.g. zinc imidazole), staining or labeling with organic or fluorescent dyes, detection by radioactive isotopes, and by immunological detection. Specific staining methods are used for detecting PTMs (e.g. phosphorylation, methylation, etc.) either employed directly on gel or after transferring onto an immobilizing membrane. Coomassie blue staining, silver staining

and fluorescence staining are the most preferred detection methods for proteomic researches. There are important properties required for ideal protein detection on two-dimensional gel. It should be sensitive (low detection limit), reproducible, compatible with mass spectrometry and it should also have linear and wide dynamic range. Unfortunately, there is no method currently that meets all these requirements together. Moreover, the gels are generally fixed overnight in ethanol/acetic acid/water mixture to eliminate any interfere compound which may come from detergents, carrier ampholytes, etc. before staining step.

Coomassie brilliant blue (CBB) staining is widely used because of its low cost and ease of use. It colors the proteins on a gel with dark blue. CBB R-250, CBB R-350 and CBB G-250 dyes are commercially available in either a standard or colloidal format.

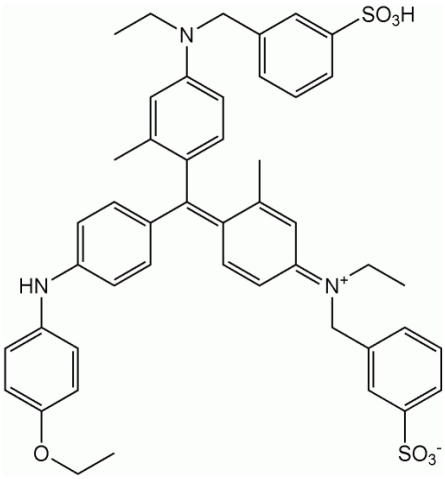

Figure 2.5. The Structure of Coomassie Blue Dye
(Source: Wikipedia 2007)

CBB stained gels have enough protein for mass spectrometry analysis. It is also compatible with mass spectrometry because of simple procedure for removal of dye from gel and protein. CBB staining has working detection range of approximately 50 ng to 1000 ng whereas colloidal Coomassie blue staining has working detection range 20 ng to 100 ng. The solution, used for colloidal Coomassie blue staining, contains ammonium sulfate which increases the interactions between the proteins and the dye. The procedure requires several steps and takes a very long time, but it gives nearly silver staining sensitivity. The classical method of CBB has a problem during destaining

due to spots are also partially destained. Quantification cannot be accurate and reproducible for this type of staining because steady state between dye and protein is not established totally.

Silver staining is another technique for detection proteins that have low abundance (less than 1.0 ng) while colloidal CBB staining detects approximately 50 ng of protein. Silver staining improves this protein detection value up to fifty-fold and it has a linear response over 0.02-0.8 ng/mm^2 (Merril 1990). It has a two-step process for color development. In the first step, silver is bound to the protein by soaking the gel in a dilute silver nitrate solution. In the second step, protein-bound silver is detected by reduction with formaldehyde. Some modifications are required to make silver staining compatible with mass spectrometry by eliminating glutaraldehyde and formalin from silver staining solutions. Glutaraldehyde and formalin fix the proteins in the gel, prevents both digestion and the recovery of any peptides formed. Therefore, the gels can not be fixed by with these chemicals. The most important advantage of silver staining is its sensitivity. Silver staining is estimated 50-fold more sensitive than Coomassie blue staining. Due to its high sensitivity, it is useful when dealing with qualitative variations such as presence or absence of protein spots. However, Coomassie blue staining is more preferable when the aim is to detect the quantitative changes on protein spots.

In the last years, fluorescent dyes were introduced and provide high detection sensitivity, dynamic range and reproducibility. Proteins can be stained with a fluorescent dye such as ruthenium II tris (bathophentroline disulfonate) (RuBPS) and SYPRO Ruby (Rabilloud, et al. 2001) after the electrophoretic separation. The detection limit is nearly 1-2 ng protein per spot, and it is compatible with mass spectrometry. However, their usage remains relatively limited due to their cost and technical difficulties.

After staining the gel, the gel images have to be converted into digital data using a scanner or camera and then analyzed with a computer program such as, PD Quest, Bio-Rad and Delta2D, Decodon. These programs have a capability for spot detection, spot filtering, spot editing, background correction, gel matching, normalization, quantification, etc.

Finally it can be concluded that the position of protein spots in polyacrylamide gel do not provide exact identification of it. For that reason, protein spots (i.e. newly expressed and up- or down-regulated) are excised from gel and digested (in-gel

digestion) into peptide fragments with specific enzyme (generally trypsin) and then identified using mass spectrometry and database searches.

2.5. In-Gel Digestion

In proteomic researches, in-gel digestion- digestion of the protein with proteolytic enzymes- is the most common method for producing peptides to identify protein by mass spectrometric analysis. This technique is used because the extraction of whole proteins from gels is inefficient and unavailable where extraction and recovery of peptides is easier one. It was developed by Shevchenko and co-workers (Shevchenko, et al. 1996, Wilm and Mann 1996) and is commonly applied to both one and two dimensional polyacrylamide gels (Rosenfeld, et al. 1992).

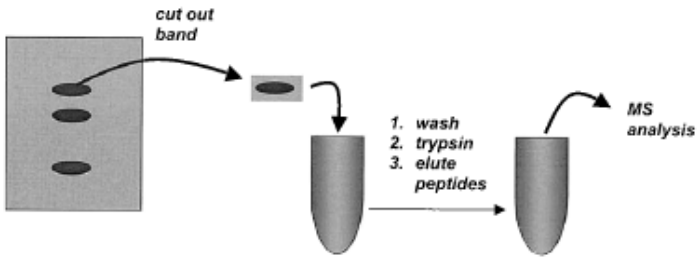

Figure 2.6. Schematic Representation of In-Gel Digestion
(Source: Liebler 2002)

Simply, the protein spot of interest is cut from the gel, destained, and the treated with a protease (most common trypsin). The enzyme penetrates the gel matrix and digests the protein into peptide fragments which then are eluted and recovered from the gel. Generally two days are required for completion of the analysis. On the first day, samples are prepared for overnight digestion and the following day samples are lyophilized and then reconstituted in a solution for MS analysis. The method involves reduction and alkylation of cysteine-containing peptides. These steps are useful when a higher coverage of the protein is required for protein sequencing.

The peptide mixture is needed to be purified to eliminate gel contaminants such as salts, buffers, and detergents that can interfere with MS (Yates 1998). The obtained peptides can be purified with ZipTips (Millipore) or Poros R2 (PerSeptive Biosystems,

Framingham, Mass.) (Wilm and Mann 1996) or by high-pressure liquid chromatography (HPLC).

Several other enzymes are also be used in in-gel digestion approach such as chymotrypsin, endoprotease Lys-C, endoprotease Arg-C, etc. The most commonly used protease is trypsin for sequencing experiments with tandem mass spectrometry (MS/MS). Because trypsin cleaves amide bonds in proteins at the C-terminal side of lysine (K) and arginine (R) residues, unless either of these is followed by a proline residue in the C-terminal direction. It also produces small peptides, generally in the mass range of 600-2500 Da.

The ideal protein digestion method would cleave proteins at certain specific amino acid residues to produce highest yield of peptides of optimal length for MS analysis. Peptides of between about 6-20 amino acid length are optimal for MS analysis and database comparisons. If the peptides are shorter than six amino acids, it may not produce unique sequence matches in database searches.

2.6. Mass Spectrometry

MS can be described as the study of gas-phase ions, and the main goal of mass spectrometric experiments is to characterize the structure of a molecules. MS became a valuable tool in the field of biochemistry by the development of fast atom bombardment (FAB) in 1981 (Barber, et al. 1981). Since early 1990's, MS has been living its golden age with the introduction of new soft ionization techniques, namely electrospray ionization by Fenn and co-workers (ESI) (Fenn, et al. 1989) and matrix-assisted laser desorption/ionization by Karas and Hillenkamp (MALDI) (Karas and Hillenkamp 1988). MS currently plays a central role in the identification and characterization of large biomolecules such as proteins and peptides. The most attractive properties of MS with these new ionization methods are high informational content of MS data, high sensitivity (low attomole levels), rapidity, versatility, and the accuracy of the method in the field of biological science.

2.6.1. An Overview of the Instrumentation

Mass spectrometers have mainly three essential parts, namely the ionization source, the mass analyzer, and the detector.

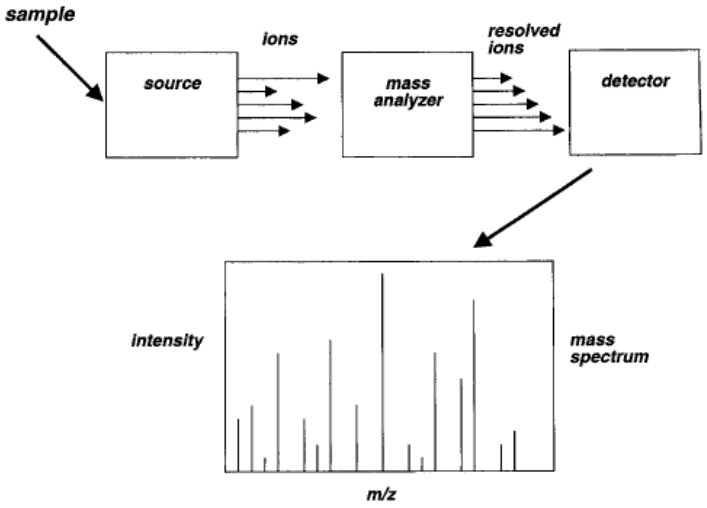

Figure 2.7. Schematic Representation of Mass Spectrometer
(Source: Liebler 2002)

The first part is the ionization source (e.g. ESI, MALDI) which produces ions from liquid and solid the sample. The second part is the mass analyzer (e.g. quadrupole, time-of-flight (TOF)) which resolves ions based on their mass-to-charge ratio (m/z). The third and last part is the detector (photomultiplier, microchannel plate, electron multiplier) which detects the ions resolved by the mass analyzer. In short, the basic process associated with a mass spectrometer is generation of gas-phase ions derived from an analyte and the measurement of those ions according to their mass-to-charge ratio. Each of these three parts of mass spectrometer is under vacuum-pump systems which is required for their function.

2.6.1.1. Sample Ionization Methods

The molecules have to be charged and dry for biological samples to be analyzed the by MS. As mentioned above, the two most common methods for converting sample molecules to desolvated ions are electrospray ionization and matrix-assisted laser

desorption/ionization. ESI and MALDI are called a “soft” ionization techniques, due to ionization without fragmentation which allow the formation of ions without significant loss of sample integrity and therefore provides molecular weight information. Ions are formed in both methods by the addition or loss of one or more protons.

2.6.1.1.a. Electrospray Ionization (ESI)

The design and working principle of electrospray ion sources used in today’s mass spectrometers is firstly described by Fenn and co-workers in 1985 (Whitehouse, et al. 1985). In the electrospray ionization of peptides, acidic aqueous solution is sprayed through a small needle. A positive and high voltage is applied to this needle to produce a Taylor cone from which droplets of the solution are sputtered. At that moment droplets take protons from acidic medium which causes to move from the needle towards the negatively charged instrument. During this movement, solvent evaporates and the sizes of the droplets decrease resulting in the formation of desolvated ions by the help of uncharged warm carrier gas such as nitrogen flowing around the outside of the capillary. As the solvent evaporates, the analyte molecules are forced closer together, repel each other and break up the droplets. The process continues until the analyte is free of solvent, and finally they can be directed into the mass analyzer of mass spectrometer by appropriate electric fields.

Nanospray and microspray are additional ESI ionization techniques used in the mass spectrometric analysis of peptides (Wilm and Mann 1996, Wilm, et al. 1996). In nanospray ionization, the microcapillary tube has a spraying orifice of 1 to 2 μm and flow rates nearly 5 to 10 nL/min. The advantage of low flow rates in nanospray ionization is reducing the amount of sample that consumed and increasing the time that required for analysis.

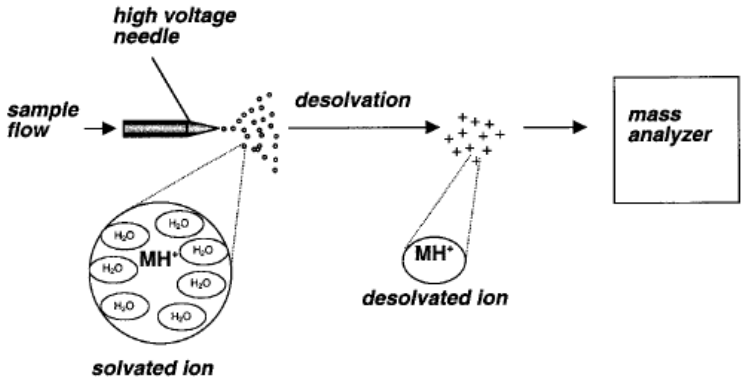

Figure 2.8. Schematic Representation of an ESI
(Source: Liebler 2002)

ESI and nanospray ionization are very sensitive techniques but the sensitivity is lost with the presence of non-volatile buffers and other additives, which should be avoided if possible. The ionization process occurs under atmospheric pressure in ESI, for that reason it may coupled to liquid chromatography systems and this property makes ESI useful equipment in proteomic studies.

2.6.1.1.b. Matrix-Assisted Laser Desorption/Ionization (MALDI)

Matrix-assisted laser desorption/ionization was first described by Karas and Hillenkamp in 1988 (Karas and Hillenkamp 1988). In MALDI, samples are usually mixed with the matrix solution in a ratio 1:1000, respectively. The matrix is a small organic molecule with a desirable chromophore that can absorbs the energy. The sample-matrix is then spotted on a target and allowed to dry and then it is transferred to vacuum system for the analysis. The co-crystallized peptides and matrix mixture are irradiated with UV-laser pulses. The laser pulses vaporize the matrix compound that also carries the protonated peptide into the gas phase. Finally the gas-phase ions are directed into the mass analyzer by appropriate electric fields.

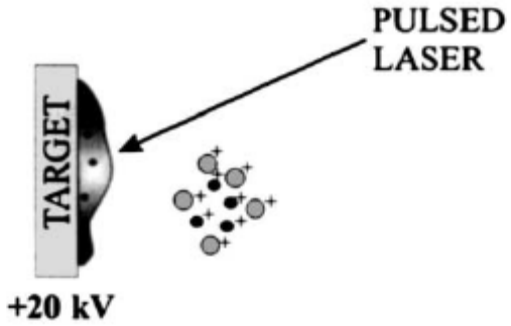

Figure 2.9 Formation of Ions in MALDI
(Source: Nyman 2001)

The most important points for successful MALDI analysis are the proper choice of matrix and sample deposition method, to achieve highest possible sensitivity and accuracy. Matrix purity should be of at least 99%, otherwise re-crystallization is recommended as impurities may negatively affect the formation of analyte/matrix crystals. The most popular matrices in proteomic studies are 2,5-dihydroxybenzoic acid (DHB) (Strupat, et al. 1991), 3,5-dimethoxy-4-hydroxycinnamic acid (SA) (sinapinic acid) (Beavis and Chait 1989), and α-cyano-4-hydroxycinnamic acid (CHCA) (Beavis, et al. 1992). Generally, CHCA is preferred for analysis of peptide maps, SA works best for larger proteins and DHB is usually used for hydrophobic, glyco- and phosphopeptides. In some cases, the mixtures of these matrixes are also found to be useful (Gonnet, et al 2003, Laugesen and Roepstorff 2003). Instead of multiple used stainless steel devices, disposable targets may be chosen to eliminate the risk of cross-contamination. There are different sample preparations protocols are also used for matrix and sample solution mixture. In dried droplet method, the matrix solution is mixed with the sample and the resulting mixture is deposited on the target plate (Karas and Hillenkamp 1988). On the other hand, a drop of analyte solution is deposited on a matrix-covered target surface in the thin layer method to improve accuracy and sensitivity (Vorm, et al. 1994).

MALDI can tolerate varying levels of some contaminants whereas ESI is easily effected by contaminating species.

2.6.1.2. Mass Analyzers

There are several mass analyzers for MS, but the most common and useful ones for biomolecules are quadrupole mass analyzer, time-of-flight (TOF), quadrupole ion traps, and Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry. The main function of the mass analyzer is to separate the ions formed in the ionization source of the mass spectrometer according to their mass-to-charge (m/z) ratios. Generally, TOF is connected to MALDI and triple quadrupole-TOF or ion traps coupled to ESI for analyzing large biomolecules. In most proteomic works, MALDI-TOF instruments and the ESI-tandem MS instruments are used where they completely operate in different ways and generate different but complementary information. MALDI-TOF MS enables for identification of proteins by peptide masses (peptide mass fingerprints; PMF) where ESI MS/MS describes peptide fragmentation.

In some cases, hybrid instruments are also used for structural and sequencing studies. There is more than one analyzer in this hybrid tandem mass spectrometry systems. More popular designs are quadrupole-quadrupole, magnetic sector-quadrupole, and quadrupole-time-of-flight geometries.

In proteomic approach, there are three types of tandem mass analyzers coupled to ESI source, namely triple quadrupole, quadrupole-ion trap, and quadrupole-TOF. The general theory behind them is generation of peptide ions in ESI source followed by analyzing in tandem MS analyzer which select a single m/z species. Meanwhile, the selected ion is subjected to collision-induced dissociation (CID) which produces fragment ions and neutral fragments. Then, the fragment ions are analyzed on the basis of their m/z to yield a product ion spectrum. Finally, the sequence of the peptide can be deduced from information which is collected in tandem mass spectrum.

2.6.1.2.a. Time-of-Flight Mass Analyzer (TOF)

A time-of-flight instrument is one of the simplest mass analyzers among them. Basically, the ions are introduced directly from the source of the instrument as a pulse. All the ions are given the same initial kinetic energy by the extraction pulse. Following acceleration, the ion enters a field-free region where it travels at a velocity that is inversely proportional to its m/z. In other words, ions with low m/z travel more rapidly

than ions with high m/z. This enables the instrument to record all ions as they arrive at the detector and so accounts for the techniques high sensitivity. To improve sensitivity, reflectron mode which is placed at the end of the drift zone can be used by refocusing of ions with the same m/z on the reflectron detector. In this way, the reflectron serves to increase the length of the flight tube. Ion manipulations are used to increase resolution (delayed extraction of ions from the source, two stage sources with complex voltage gradients, and reflectron technology). A commercial TOF instrument can typically achieve resolution of 10,000 or greater (separate m/z 1000.0 from m/z 1000.1) (Chernushevich, et al. 2001). Another technique which is called post-source decay (PSD) can also be used with a reflectron in TOF systems. In this technique, the voltage on the reflectron is modulated during analysis to allow the detection of fragments of peptide ions formed during ionization and acceleration down the flight tube. The big advantage of PSD spectra is the appearance of peptide immonium ions of general formula H_2N^+=CHR, where R is the amino acid side chain. It is obvious that immonium ions are indicators of the presence of specific amino acids in peptide fragments.

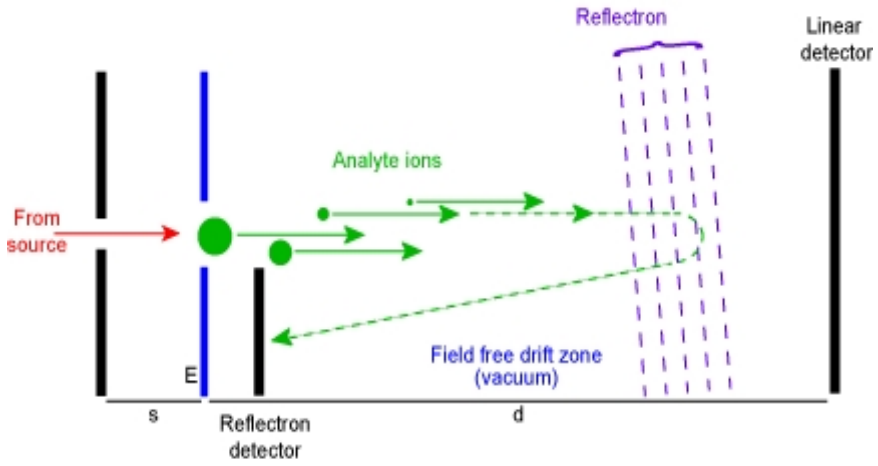

Figure 2.10. A Schematic of a Time-of-Flight Mass Spectrometer Operating in Reflectron Mode (Source: The University of Bristol 2007)

The MALDI-TOF system is a very sensitive method, which allows the detection of low (10^{-15} to 10^{-18} mole) quantities of sample with an accuracy of 0.1-0.01 %.

The MALDI Q-TOF MS gives both peptide mass fingerprints and amino acid sequence. One of the main advantage of this system is identifying a sample with amino

acid sequence when the peptide mass fingerprinting is failed without using a different mass spectrometer.

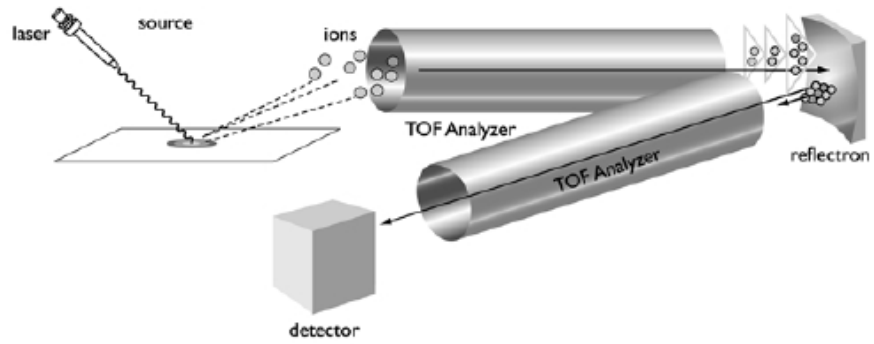

Figure 2.11. A MALDI-TOF Instrument Equipped with a Reflectron
(Source: Liebler 2002)

2.6.1.2.b. Quadrupole Ion Traps

Ion trap mass analyzers are used to trap molecules. The formed ions are directed into the ion trap, which consists of a top and bottom electrode and a ring electrode around the middle. Ions are trapped in that medium by the help of RF and DC voltages. At the same time a small amount of helium is used as a cooling gas which helps to control the distribution of energies of the ions.

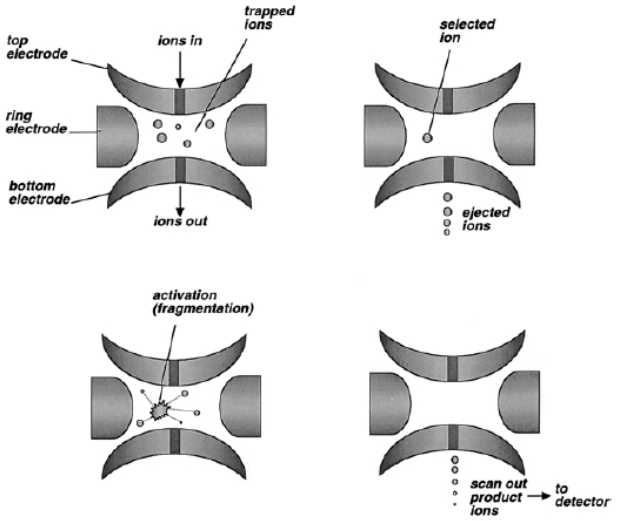

Figure 2.12. Schematic Representation of an Ion-Trap
(Source: Liebler 2002)

Mainly, quadrupole ion traps focus ions into a small volume with an oscillating electric field; ions are activated and ejected by electronic manipulation of this field. Ion traps are very sensitive, because they can concentrate ions in the trapping field for varying lengths of time.

The main advantage of an ion trap mass analyzer over quadrupole mass analyzer is the ability to allow ions to be "stored" and then selectively ejected from the ion trap, which increases sensitivity of the system (Yates 1998).

2.6.1.3. Detectors

The final component of mass spectrometer is detector. Simply, it records the charge induced or current produced when an ion passes by or hits the surface. Significant amplification is required to get a signal because the number of ions which leaves the mass analyzer is small at a given instant. Photomultiplier, microchannel plate, and electron multiplier are the examples of detector. Microchannel plate detectors are commonly used in modern commercial instruments (Dubois, et al. 1999).

Recently, there are two main approaches to mass spectrometric protein identification in proteomic researches, namely peptide mass fingerprinting (PMF) by MALDI and creating sequence-tags by ESI-MS/MS.

In PMF, the protein spot of interest is in-gel digested with an enzyme followed by recovering of the peptides from gel. The masses of peptides obtained from the proteolytic digestion of an unknown protein are then compared to the predicted masses of peptides form the theoretical digestion of proteins in a database search programs. If enough peptides from the real mass spectrum and the theoretical one overlap, it can be said that successful protein identification is achieved. However, PMF does not work well with protein mixtures. When a protein mixture is converted to a peptide mixture, it increases the complexity of the peptide mass fingerprint and limits the identification of protein.

In the second approach, peptides are fragmented into partial amino acid sequences in the mass spectrometer after in-gel digestion step. If the amino acid sequence of a peptide can be identified, it can be used to search databases to find the protein from which it was derived. A partial amino acid sequence is obtained by interpretation MS/MS spectrum (the sequence tag). Peptide mass tag searching is a more specific tool for protein identification than peptide mass fingerprinting. Additionally, one of the biggest advantages of utilizing MS/MS to obtain peptide amino acid sequence is that, unlike PMF, it is compatible with protein mixtures. The ability to identify proteins in mixtures is one of the great advantages of using MS as a protein identification tool.

2.7. Previous Studies

There are only limited studies which are related with barley and boron in literature. According to the Hayes and Reid, boron tolerance is mediated by efflux of boron from roots in barley. They carried out experiments with two Australian barley genotypes, namely Sahara (boron-tolerant genotype) and Schooner (boron-sensitive genotype). Sahara genotype was shown to be able to maintain root boron concentration up to 50 % lower than in the Schooner genotype. They stated that boron accumulation was rapid and reached a steady-state concentration in roots within 3 hours while this concentration was similar to the external medium in Sahara whereas the root concentration was maintained at a lower concentration in Schooner (Hayes and Reid 2004)

On the other hand, Torun and co-workers examined ten barley Turkish cultivars on soils containing normal and very high soluble boron concentration to study genotypic variation in tolerance to boron toxicity. They showed that among the barley genotypes examined Hamidiye and Bülbül were the most sensitive, and Anadolu and Tarm-92 the most tolerant ones. They found that boron tolerant and boron sensitive genotypes had very close tissue concentrations of boron, and even though Hamidiye contained the lowest boron concentration in flag leaves. As a conclusion, they showed that boron concentration in shoot and leaf are not reliable parameters in screening barley genotypes to boron tolerance whereas severity of boron toxicity leaf symptoms reflected much better results (Torun, et. al. 2003).

Recently, an Australian group worked for investigation of boron toxicity in barley using metabolomic approach. They explained the metabolite profiles of two genotypes, Clipper (boron-sensitive) and Sahara (boron-tolerant). The metabolic differences in roots were greater in Clipper than in Sahara at 200 and 1000 μM boron concentration. In contrast, leaf metabolites of both genotypes only responded at 1000 μM treatment. Additionally, metabolite levels were changed in the tips of leaves of the Clipper after growth in 1000 μM B compared to Sahara. This is in agreement with a gradual accumulation of boron from leaf base to tip in sensitive genotype (Roessner, et al. 2006).

According to the Mahboobi and colleagues cell wall uronic acid concentration, which is involved in binding of excess boron, were analyzed in leaf and root cell walls of Anadolu and Hamidiye genotypes. It is found that the cell wall uronic acid content has similar value under normal and excess supply of boron (Mahboobi, et al. 2001). Similarly, activity of nitrogen assimilating enzymes such as nitrate reductase and glutamate dehydrogenize did not correlate with boron toxicity susceptibility/tolerance of barley genotypes (Mahboobi, et al. 2002). In an other work, Karabal and co-workers were also studied antioxidant responses of tolerant and sensitive barley genotypes to boron toxicity. They suggested that antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR), do not have a role in boron toxicity tolerance mechanism (Karabal, et al. 2003).

The closest work to our study was done by Mahboobi and co-workers in 2000. They studied the changes in total protein profiles of barley genotypes, Anadolu (boron-tolerant) and Hamidiye (boron-sensitive) in response to toxic boron concentration. In root protein profile, one newly synthesized protein (molecular weight of nearly 35.0

kDa and pI value of 7.8) was detected in tolerant genotype while the same protein was not shown up in sensitive genotype. The amount of three proteins (Mr: 23.0, 45.0, and 45.0 kDa; and pI values of 6.1, 6.0, and 6.1 respectively) were increased as a boron treatment in both genotypes. In contrast, there were remarkable changes in total protein profile after boron treatment relative to control ones in leaf tissues. The amount of seven proteins (Mr: 14.0, 32.5, 33.0, 32.5, 34.2, 15.8, 15.8 kDa; and pI values of 5.0, 6.5, 6.7, 7.3, 7.7, 5.4, 8.0 respectively) were increased in tolerant genotype but the same proteins were unchanged in the susceptible one. Additionally there were two proteins (Mr: 22.5 and 29.0 kDa; and pI values of 4.5, and 4.9 respectively) increased in amount in tolerant and sensitive genotypes relative to control seedlings. They concluded that boron stress caused increases or decreases in a number of proteins in root and leaf tissues and these proteins may be involved in tolerance to boron toxicity (Mahboobi, et al. 2000).

2.8. The Aim of the Study

In this study, proteomic approach was used to identify boron-stress tolerant proteins in leaves of Anadolu (boron-tolerant) over Hamidiye (boron-sensitive) barley genotype and to explain the tolerance mechanism in boron-tolerant barley genotype. Eight-day-old seedlings of two barley genotypes were treated with 10 mM boric acid for seven days. Control plants received no boron treatment during this period. Total proteins of leaf tissues were extracted from control and treated plants and separated by two-dimensional gel electrophoresis (2-DE PAGE). Evaluation of two-dimensional gel patterns revealed several proteins to be differentially expressed as a result of boron stress in both genotypes. The protein spots of interest were excised from the gel and in-gel digested. The obtained peptides were analyzed by mass spectrometry and each protein was identified by the help of database search programs. Proteomic study provides an excellent opportunity to identify boron-stress responsive proteins and to explain the defense mechanism in boron-tolerant barley genotype (Anadolu) to boron toxicity. The understanding of plant stress physiology is well correlated with the changes in proteome content of cells.

In addition, newly synthesized, up-regulated, down-regulated, or totally disappeared proteins were compared in boron treated and control plants of an Australian boron-sensitive barley genotype, Sahara.

CHAPTER 3

EXPERIMENTAL

3.1. Plant Growth Conditions and Boron Treatment

In this study, three genotypes of barley (*Hordeum vulgare*; Anadolu, Hamidiye, and Sahara) were used. These three genotypes were designated as boron-tolerant (Anadolu and Sahara) or boron-sensitive (Hamidiye). Seeds were firstly washed with water, and then they were put into petri dishes that contain wet filter paper. They were allowed to germinate approximately for three days under sterile conditions without letting filter paper to dry. A plastic beaker containing half-strength Hoagland's solution which was covered with stretchable parafilm was prepared. After germination; approximately 15-20 seeds then were planted onto that beaker such that roots of the plants were in the solution whereas shoots and leaves were on the stretch film as it can be seen in figure 3.1. This solution contains all essential plant nutrients including macro- and micro-nutrients in balanced proportions. It consisted of 3.5 mM $Ca(NO_3)_2.4H_2O$, 2.5 mM KNO_3, 1 mM KH_2PO_4, 1 mM $MgSO_4.7H_2O$, 22 μM H_3BO_3, 4.5 μM $MnCl_2.4H_2O$, 0.35 μM $ZnSO_4.7H_2O$, 0.2 μM $CuSO_4.5H_2O$, 0.07 μM $NaMoO_4$, and a mixture of 15 μM EDTA.2Na, 14 μM $FeSO_4.7H_2O$ and 0.5 mM KOH whose pH was adjusted to 5.5. They were grown for 5 days under controlled environmental condition (23±2 ˚C with 16-h light/8-h dark photoperiod at a light intensity of 40 μmol m^{-2} s^{-1}). The nutrient solution was changed every five days and the water lost by evapotranspiration was compensated daily by addition of deonized water.

At the 8^{th} day of growth, boron was applied to a final concentration of 10 mM as boric acid. The control plants of each genotype continued to grow under normal Hoagland solution. Control plants (no boric acid treatment) and boron stressed plants were grown with the same physical parameters for additional 7 days. At the end of growing period, the leaf tissues of 15 days old seedlings were harvested and quickly wrapped with an aluminum foil, and immediately submerging them in liquid nitrogen to minimize proteolytic activity. The samples were taken from Anadolu, Hamidiye, and Sahara genotypes of both treated and control plants (Anadolu Control-AC, Anadolu Boron-AB, Hamidiye Control-HC, Hamidiye Boron-HB, Sahara Control-SC, and

Sahara Boron-SB). Sample were stored at -80 ˚C or they were freshly used for protein extraction.

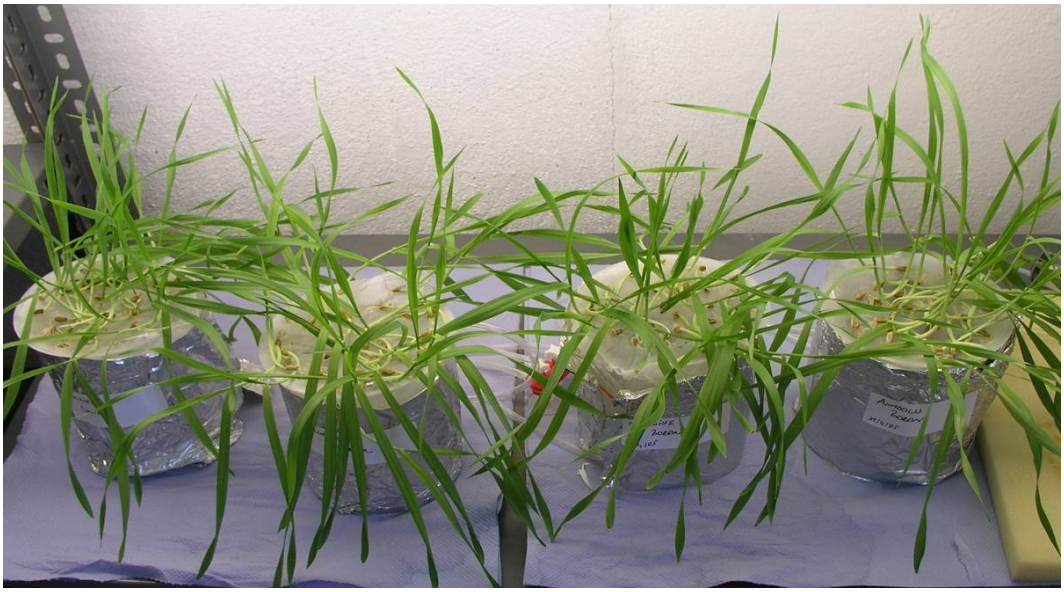

Figure 3.1. The Photograph of Control and Boron-treated Barley Genotypes, from Left to Right, Hamidiye Control (HC), Hamidiye Boron (HB), Anadolu Control (AC), and Anadolu Boron (AB), respectively

3.2. Protein Extraction from Leaves

All chemicals were in electrophoresis/biological grade and ultra pure water (18Ω) was used during study. All experiments were carried out at 4 ˚C unless other temperature is stated and unpowdered gloves were used throughout to avoid contamination.

Protein extraction was performed using TRIzol (phenol/guanidine isothio-cyanate) reagent. TRIzol is a quick and convenient reagent for use in the simultaneous isolation of RNA, DNA, and protein from a single sample. Preparation of 100 ml TRIzol was described in the following:

- 38 % phenol in saturated buffer: 38.0 ml phenol (Merck)
- 0.8 M guanidine thiocyanate: 11.816 g of guanidine thiocyanate (AppliChem)

- 0.4 M ammonium thiocyanate: 7.612 g of ammonium thiocyanate (AppliChem)
- 0.1 M sodium acetate: 0.8203 g of sodium acetate (Merck)
- 5 % Glycerol: 5.0 ml (AppliChem)
- dH_2O to 100 ml

After mixing of first five chemicals in a given amounts and homogenization, pH was adjusted to 5 for effective and complete protein extraction from tissues. And then the mixture was fulfilled to 100 ml with ultra pure water. It can be stored at 2-8 ˚C for several months.

Approximately 3 grams of frozen leaf samples of control and treated for each cultivars was ground by a mortar and pestle with liquid nitrogen to a powder. 30 ml TRIzol reagent was added to 3 grams of crushed leaf tissues for homogenization and the mixture was allowed to stand for 5 minutes at room temperature. Then 6.0 ml of chloroform (AppliChem) was added into homogenate, shaken vigorously for 15 seconds and allowed to stand for 2-15 minutes at room temperature. The resulting mixture was centrifuged at 13,000 x g for 17 minutes at 4 ˚C. By doing centrifugation, the mixture was separated into the 3 phases: a colorless upper aqueous phase containing RNA, an interphase containing DNA, and lower phase containing protein. Nearly 10 ml of aqueous protein phase were taken to a new and clean centrifuge tube and 40 ml of isopropanol (AppliChem) was added and allowed the samples to stand for at least 10 minutes at room temperature. Afterwards, the sample mixture was centrifuged again at 12,000 x g for 10 minutes at 4 ˚C. The supernatant was discarded and the pellet was washed 3 times with 40 ml of 0.3 M guanidine hydrochloride/95 % ethanol solution.

- Preparation of 0.3 M guanidine hydrochloride/95 % ethanol solution: 5.732 grams of guanidine hydrochloride (AppliChem) was taken and diluted to the 200 ml with 95 % of ethanol solution (AppliChem).

During each wash, samples were stored in wash solution for 20 minutes at room temperature and centrifuged at 7,500 x g for 5 minutes at 4 ˚C. At the end of the centrifugations, pellets were taken from centrifuge tube to the 1.5 ml eppendorf tube and covered with sufficient absolute ethanol (AppliChem). At this stage, pellets can be

stored -80 ˚C for further usage or immediately used in IEF, after solubilization of pellet with rehydration buffer.

3.3. Protein Solubilization with Rehydration Buffer

The excess ethanol above the pellets was taken carefully and the pellet was finally vacuum-dried in SpeedVac (Thermo Electron Corporation) not more than 5 minutes, until all ethanol was completely evaporated. Then the lyophilized pellet was dissolved in rehydration buffer that consist of urea, thiourea, CHAPS, DTT (AppliChem), and Ampholyte pH 3-10 (Fluka).

- The preparation of rehydration stock solution: 4.2 g of urea, 1.52 g of thiourea, and 0.4 g of CHAPS were weighed and dissolved in ultra pure water to a final solution volume of 10 ml. The solution can be gently heated to dissolve, but must not be heated above 30 ˚C to prevent urea breakdown. This stock solution can be stored at 4 ˚C up to one month.
- Just prior to use, 1 ml of stock solution was taken and followed by addition of 0.01 g of DTT and 25 µl of Ampholyte. DTT and ampholyte must be added fresh just before use.

The final concentration individual components are; 7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 65 mM DTT, and 2.5 % Ampholyte pH 3-10. The rehydration buffer was added to the lyophilized pellet in a sufficient amount for complete solubilization. Approximately, 600 µl of rehydration buffer was used per one leaf sample pellet throughout experiments. After addition enough buffer to pellet, it was thoroughly mixed and followed by vortexing for 10 minutes. Then the eppendorf tubes were centrifuged at 8,000 x g for 5 minutes at 4 ˚C and the supernatant was taken for IEF experiment. Before IEF, protein concentration was determined by Bradford method.

3.3.1. Bradford Protein Assay for Protein Determination

The Bradford protein assay is one of the spectroscopic analytical methods used to determine the total protein concentration of a sample (Bradford 1976). This method is

based on the absorption shift from 470 nm to 595 nm in the Coomassie brilliant blue G-250 dye when it binds to protein. Upon addition of sample, the dye will bind protein, resulting in a color change from green to blue. For that reason, the Bradford method is also known as colorimetric protein assay as the protein concentration increases; the color of the test sample becomes darker. The CBB G-250 dye is thought to bind to protein through an electrostatic attraction of the dye's sulfonic groups, theoretically to arginine, lysine, and histidine residues. Additionally, the dye also binds weakly to the tyrosine, tryptophan, and phenylalanine via van der Waals forces and hydrophobic interactions.

The Bradford assay has a linear dyanmic range, generally from 2µg/ml to 120 µg/ml, it is usually necessary to make dilutions of sample before analysis. As an analytical rule, a series of protein standards is needed to determine the protein concentration of a test sample. Thus, it is important to generate a standard curve using a protein of known concentration. Bovine serum albumin (BSA) was used as a standard protein to compare with sample protein concentration.

- Preparation of Coomassie Reagent: 10.0 mg of CBB G-250 (AppliChem) was dissolved in 5 ml of 95 % ethanol to which 10.0 ml of 85 % phosphoric acid (AppliChem) had been added and the whole was diluted to 100 ml with ultra pure water. The final solution was filtered through filter paper (Whatman No. 1) and was stored in an amber bottle at 4 ºC.
- Preparation of 200 µg/ml stock BSA standard: 0.0200 g of BSA was weighed and dissolved in water to a final volume of 1.0 ml.

Table 3.1. Preparation of BSA Standards from 0.2 mg/ml BSA and Test Sample for the Bradford Protein Assay

Test Sample	Sample Volume, µl	Water Volume, µl	Coomassie Reagent Volume, µl
Blank	0	800	200
BSA Standard – 1 µg/ml	5	795	200
BSA Standard – 2 µg/ml	10	790	200
BSA Standard – 4 µg/ml	20	780	200
BSA Standard – 6 µg/ml	30	770	200
BSA Standard – 8 µg/ml	40	760	200
Protein Sample	2	798	200

Blank, BSA standards, and protein samples were prepared according to Table 3.1 in disposable cuvettes and tested by using UV-visible spectrophotometer for absorbance measurement. The order of mixing these reagents is; water, either BSA or sample protein, and lastly Coomassie solution. Then mixture of these was allowed to incubate at room temperature for 5 minutes. Finally, absorbance of each sample was measured at 595 nm using a UV-visible spectrophotometer.

The standard curve was established by plotting the absorbance at 595 nm versus µg of protein in BSA standard samples. The best straight line was determined in the from of "y=mx + b" where y is absorbance reading at 595 nm and x is protein concentration. At the end, this equation was used to calculate the concentration of the protein sample based on the measured absorbance. It must be noted that, dilution may be required for samples for the resulting absorbance to fall within the linear range of the assay. Addition to this, the relationship between protein concentration and absorbance is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity. Table 3.2 shows the absorbance values for various BSA standards.

Table 3.2. Absorbance Values for BSA Standards

Concentration (µg/ml)	Absorbance at 595 nm
1	0.0900
2	0.1294
4	0.2712
6	0.3576
8	0.4374

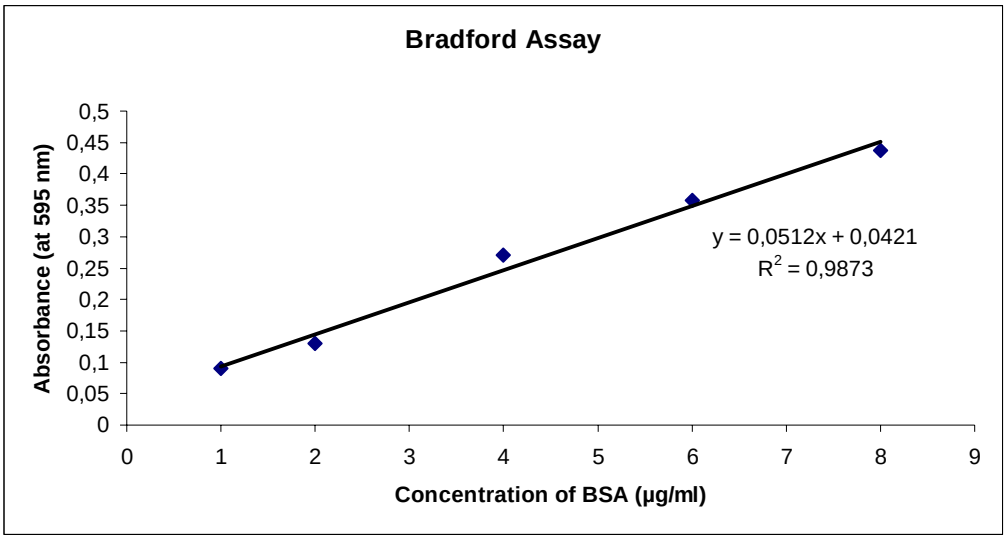

Figure 3.2 Standard Curve for BSA

3.4. 2-DE of total proteins from barley leaves

3.4.1. Isoelectric Focusing

The first dimension or simply isoelectric focusing (IEF) was carried out in IPG strips (pH 3-10, linear gradient, 17 cm, or pH 4-7, linear gradient, 17 cm, both from Bio-Rad) by using a Protean IEF Cell (Bio-Rad).

The focusing tray was selected corresponding to the IPG strip length chosen for the experiment and it must be clean and dry before IEF. Paper wicks were placed onto the electrodes and wetted with approximately 8 µl of nano-pure water which was supplied from Bio-Rad. Appropriate amount of protein sample and rehydration buffer, as described previous chapter, were placed into one well of the focusing tray. In our work, IEF experiments were carried out in parallel manner, i.e. Anadolu-Boron and Anadolu-Control at the same experimental conditions. Same amount of protein (350 µg) were loaded onto strips to compare up- and down-regulated proteins in treated and control plant's leaf protein samples. Rehydration buffer was also prepared as described in protein solubilization part. The mixture of buffer and protein sample, a rehydration buffer containing 350 µg protein/350 µL, was put slowly at a central point in the well and any bubbles, if formed, was removed which causes serious problems while focusing of proteins. Afterwards, strips were taken out from -20 ºC and let it to thaw for 5 minutes at room temperature. Then, the protective cover of the strip was peeled off and the strip was positioned with gel side down followed by laid onto the solution holding one end with forceps, and placed with the positive end of the strip on the positive electrode. In order to coat the entire strip, it was gently lift and lowered in the same well of focusing tray to ensure that the gel was completely contact with sample protein and rehydration buffer. The strip was then covered with 2 ml of mineral oil to minimize evaporation and urea crystallization. The cover of the focusing tray was placed and let it to stay one-hour on a bench.

After one-hour, IPG strips were actively rehydrated for 12 hours. Active rehydration was carried out in the presence of 50 V during rehydration period. After rehydration step, IEF voltage settings for 17-cm IPG strips was programmed as follows: phase 1, linear gradient up to 250 Volts in 15 minutes; phase 2, linear gradient up to 500 Volts in 30 minutes; phase 3, rapid gradient up to 1000 Volts in 1 hour; phase 4, linear gradient up to 5000 Volts in 3 hours; phase 5, linear gradient up to 10,000 Volts in 3 hours; and phase 6, linear gradient up 10,000 Volts at 70,000 Volts h^{-1}. Working temperature was set to 20 ºC in IEF. Total focusing time was almost 20 hours without rehydration step.

After the IEF run was complete, strips were removed from IEF focusing tray and were placed at -80 ºC or immediately equilibrated with equilibrium buffers for second dimension of the experiment.

3.4.2. Equilibration of Strips

The strips containing the focused proteins were equilibrated in equilibrium buffers which contain SDS for transforming the focused proteins into SDS-protein complexes. They had completely unfolded structure and carried only negative charges. Beside SDS, equilibrium buffer I had Tris-HCl pH 8.8, glycerol, urea, DTT, while equilibrium buffer II had iodoacetamide instead of DTT. DTT is necessary for cleavage of sulfhydryl bonds between cysteine residues within a protein, as a reductant. Iodoacetamide was used as an alkylation for reduced sulfhydryl groups in the protein and as a scavenger of the excess reductant.

- Preparation of equilibrium buffer I: 1.81 g of urea, 1.25 ml of Tris-HCl pH 8.8, 0.1 g of SDS, 1 ml of glycerol, and 0.1 g of DTT. The final volume was adjusted to 5 ml with water for one strip. The final concentration for equilibrium buffer I was 6 M urea, 0.375 M Tris-HCl, 2 % SDS, 20 % glycerol, and 2 % DTT.
- Preparation of equilibrium buffer II: 1.81 g of urea, 1.25 ml of Tris-HCl pH 8.8, 0.1 g of SDS, 1 ml of glycerol, and 0.125 g of iodoacetamide (AppliChem). The final volume was adjusted to 5 ml with water for one strip. The final concentration for equilibrium buffer II was 6 M urea, 0.375 M Tris-HCl, 2 % SDS, 20 % glycerol, and 2.5 % iodoacetamide.

After preparation of equilibrium buffers, strip was laid gel side up into the disposable equilibrium tray and treated with equilibrium buffer I for 15 minutes with gentle shaking. After 15 minutes, strip was taken from one well of tray and placed into another dry well and treated with equilibrium buffer II for 15 minutes with gentle shaking again.

3.4.3. SDS-PAGE

After removing strip from equilibrium buffers, strip needed to be rinsed with 1X Tris-Glycine-SDS (TGS) running buffer before attaching into the polyacrylamide gel.

- Preparation of 5X Tris-Glycine-SDS (TGS) running buffer: 15.1 g of Tris-base (AppliChem) and 94 g of glycine (AppliChem) were weighed and dissolved in 900 ml of water. Then, 50 ml of a 10 % (w/v) SDS solution was added and the final volume was adjusted to 1000 ml with water. It can be stored at 4 ºC.

To prepare 100 ml of 1X TGS buffer, 20 ml of 5X TGS buffer was diluted to a final volume of 100 ml with ultra pure water. The graduated cylinder was filled to a 100 ml with 1X TGS buffer and any bubbles on the surface of the buffer was removed by the help of plastic pasteur pipette. The equilibrated strip was rinsed with 1X TGS buffer for 15 seconds. Now, strip was ready for second dimension- separation of proteins according to their molecular weight differences.

Separation in the second dimension by SDS-PAGE was carried out in PROTEAN II xi Cell (Bio-Rad). 12 % polyacrylamide gel slabs containing 1.5 M Tris-HCl buffer, pH 8.8, 10 % SDS, 10 % ammonium per sulfate, TEMED were used and the running buffer being composed of 1X TGS buffer. Glass plate sizes are 16 cm cells with 16 x 20 cm for inner plate and 18.3 x 20 cm for outer plate. Solutions for preparing resolving gel for SDS-PAGE were described in the following.

- Preparation of 30 % acrylamide mixture: 29.0 g of acrylamide (AppliChem) and 1.0 g of N, N´-methylenebisacrylamide (AppliChem) were dissolved in a total volume of 60 ml of water and the solution was heated to 37 ºC to dissolve the chemicals. The total volume was fulfilled to 100 ml with water. This solution can be stored at 4 ºC in dark bottles not more than 1 month. During storage, acrylamide and bisacrylamide are slowly converted to acrylic acid and bisacrylic acid.
- Preparation of 1.5 M Tris-HCl, pH 8.8: 3.634 g of Tris-base was dissolved in 15 ml of water and pH was adjusted to 8.8 with concentrated (~6 M) HCl. Then the volume was fulfilled to 20 ml with water. It can be stored at 4 ºC.
- Preparation of 10 % SDS: 1.0 g of SDS was dissolved in water to a final volume of 1.0 ml.

- Preparation of 10 % ammonium per sulfate (APS): 1.0 g of APS was dissolved in water to a final volume of 1.0 ml. This solution should be prepared just before use.

12 % polyacrylamide gel was prepared by mixing 16.5 ml of water, 20.0 ml of 30 % acrylamide mixture, 12.5 ml of 1.5 M Tris-HCl (pH 8.8), 500 µl of 10 % SDS, 500 µl of 10 % ammonium per sulfate, 20 µl of TEMED were mixed to a final volume of 50 ml. Polymerization was started immediately after adding APS and TEMED. The mixture was then poured between two glass plates.

The casted SDS-polyacrylamide gel was kept for at least one day at 4 ºC prior to electrophoresis in order to reduce protein modifications caused by free acrylamide or reagents involved in the polymerization process.

Equilibrated IPG strip was placed onto the polyacrylamide slabs and sealed with 1 ml of 0.5 % (w/v) melted agarose (AppliChem) (0.5% agarose in 1X Tris-Glycine-SDS with traces of bromophenol blue). There must not be any air bubbles between the IPG strip and the resolving gel interface. Once the overlay has solidified, the gel was placed in a buffer tank and central cooling core was cooled with circulating water to hold the temperature near 10 ºC. The reservoirs were half filled with 1X TGS running buffer and electrophoresis was begun. Electrophoresis run was made at constant current at 16 mA/gel for 45 minutes and followed by constant voltage at 150 Volts for at least 7 hours until the blue dye front reached the bottom of the gel.

3.4.4. Staining and Destaining of Gel

After electrophoresis was finished, the gel was taken out from two glass plates carefully into a large tray for staining and destaining steps. Coomassie colloidal blue staining was used throughout the study.

- Preparation of Staining Solution: 40 g of ammonium sulfate (AppliChem) was dissolved in 280 ml of ultra pure water and mixed with 8 ml of 85 % phosphoric acid (AppliChem). Then 100 ml of methanol (Merck) was added and the final volume is adjusted to 500 ml with ultra

pure water. Finally, 0.5 g of CBB G-250 was added and mix thoroughly. It can be stored at 4 °C.

- Preparation of Neutralization Buffer: 0.1 M of Tris-phosphate, pH 6.5 was prepared by dissolving 10.96 g of Tris-dihydrogen phosphate (AppliChem) in water to a final volume of 500 ml. The pH was adjusted with NaOH to 6.5. It can be stored at 4 ºC.
- Preparation of Destaining Solution: 25 % methanol solution was prepared by mixing 125 ml methanol with 375 ml water. It can be stored at 4 ºC.
- Preparation of Fixation Solution: 20 % of ammonium sulfate (AppliChem) was prepared by dissolving 100 g of ammonium sulfate in water to a final volume of 500 ml. It can be stored at 4 ºC.

Firstly, gel was stained with sufficient staining solution at least 12 hours with gentle shaking. After overnight staining step, the solution was poured and the gel was washed with water. Then, gel was treated with neutralization buffer for three minutes with gentle shaking. The buffer solution was again poured and the gel was washed with water. The gel was mixed with destaining solution for less than one minute with gentle shaking again. After pouring the destaining solution gel was washed properly with water and treated with fixation solution for at least one day.

3.4.5. Image and Data Analysis of Gel

After staining and destaining steps, the photograph of the gel was taken by camera. PD Quest 8.0.1 version of image analysis software was downloaded from the Company's web site for one month free-trial use (Bio-Rad) for gel scanning and analysis. All available background subtraction and smoothing algorithms can be done with this program. It has a capability of the generation of statistical data concerning differentially expressed proteins while comparing sample's gels.

3.5. In-Gel Digestion

In gel digestion, excising protein spot from SDS-PAGE gel and cleavage of protein into peptides and sequencing of these fragments, is an central part of the proteomic studies. Shevchenko and co-workers described a protocol that is well adapted with further identification instruments (Shevchenko, et al. 1996). It is presented as a three-day procedure in which an initial washing of the protein spot and digestion reaction are carried out overnight. Recently, robotic instruments have been introduced, including spot detection, cutting from gel and with in-gel digestion steps in the same system. The preparation of in-gel digestion chemicals are described in the following:

- Preparation of wash solution: 10 ml of methanol (Merck) was added to 5 ml of water followed by addition of 1 ml of acetic acid and the total volume was adjusted to 20 ml with water. The final concentrations are 50 % (v/v) methanol and 5 % (v/v) acetic acid.
- Preparation of 100 mM ammonium bicarbonate: 0.2 g of ammonium bicarbonate (AppliChem) was dissolved in 20 ml of water.
- Preparation of 50 mM ammonium bicarbonate: 2 ml of 100 mM ammonium bicarbonate was mixed with 2 ml of water.
- Preparation of 10 mM DTT: 1.5 mg of dithiothreitol was placed in a 1.5 ml plastic centrifuge tube followed by addition of 1 ml of 100 mM ammonium bicarbonate for complete dissolving of DTT.
- Preparation of 100 mM iodoacetamide: 18 mg of iodoacetamide was placed in a 1.5 ml plastic centrifuge tube followed by addition of 1 ml of 100 mM ammonium bicarbonate for complete dissolving of iodoacetamide.
- Preparation of trypsin solution: 1 ml of ice cold 50 mM ammonium bicarbonate was added to 20 μg of sequencing-grade modified trypsin (V5111; Promega) and dissolved by drawing the solution into and out of the pipette. The trypsin solution was kept on ice until use. The final concentration is 20 ng/ml trypsin.
- Preparation of extraction buffer: 10 ml of acetonitrile (Merck) was added to 5 ml of water followed by addition of 1 ml of formic acid (Merck) and

the final volume was adjusted to 20 ml with water. The final concentrations were 50 % (v/v) acetonitrile and 5 % (v/v) formic acid.

The following digestion procedure was suitable for spots cut from 2D gel, on the order of 2mm to 4 mm diameter with a gel thickness of 1 mm, and have total volumes nearly ~20 µL.

Day One: Cutting Protein Spot and Washing the Gel Pieces

- Protein spot was cut from the gel as closely as possible with a sharp scalpel, and divided into smaller pieces. The gel pieces were placed in a 1.5 ml of plastic micro centrifuge tube. 200 µL of wash solution was added and the gel pieces were rinsed overnight at room temperature.

Day Two: Reduction, Alkylation, Washing out Reagents and Exchange of Buffers Followed by Digestion with Trypsin

- Wash solution was carefully removed from the sample and discarded.
- 200 µL of wash solution was added and the gel pieces were rinsed for additional 2-3 hours at room temperature. Then, wash solution was carefully removed from the sample and discarded.
- 200 µL of acetonitrile was added and the gel pieces were dehydrated for ~ 5 minutes at room temperature. When dehydrated, the gel pieces were an opaque white color and were smaller in size.
- Acetonitrile was carefully removed from the sample and discarded. The gel pieces were completely dried at ambient temperature in a vacuum centrifuge for 2-3 minutes.
- 30 µL of 10 mM DTT was added and the protein was reduced for 30 minutes at room temperature. Then, DTT was carefully removed from the sample and discarded.
- 30 µL of 100 mM iodoacetamide was added and the protein was alkylated for 30 minutes at room temperature. Then, iodoacetamide was carefully removed from the sample and discarded.

- 200 µL of acetonitrile was added and the gel pieces were dehydrated for ~5 minutes at room temperature. Acetonitrile was carefully removed from the sample and discarded.
- The gel pieces were rehydrated with 200 µL of 100 mM ammonium bicarbonate, incubating the samples for 10 minutes at room temperature. Then, ammonium bicarbonate was carefully removed from the sample and discarded.
- 200 µL of acetonitrile was added and the gel pieces were dehydrated for ~5 minutes at room temperature. Acetonitrile was carefully removed from the sample and discarded. The gel pieces were completely dried at ambient temperature in a vacuum centrifuge for 2-3 minutes.
- 30 µL of the trypsin solution was added to the sample and the gel pieces were allowed to rehydrate on ice for 10 minutes with occasional vortex mixing.
- The gel pieces were driven to the bottom of the tube by centrifuging the sample for 30 seconds. Excess trypsin solution was carefully removed from the sample and discarded.
- 5 µL of 50 mM ammonium bicarbonate was added to the sample and the mixture was vortexed. The sample was driven to the bottom of the tube by centrifuging the sample for 30 seconds. Digestion was carried out overnight at 37 ºC.

Day Three: Extraction of Peptides for Analysis

- 30 µL of 50 mM ammonium bicarbonate was added to the digest and the sample was incubated for 10 minutes with occasional gentle vortex mixing. The digest was driven to the bottom of the tube by centrifuging the sample for 30 seconds. The supernatant was carefully collected and the sample was transferred to a 0.5 ml plastic micro centrifuge tube.
- 30 µL of extraction buffer was added to the tube containing the gel pieces and incubated for 10 minutes with occasional gentle vortex mixing. The extract was driven to the bottom of the tube by centrifuging the sample for 30 seconds. The supernatant was carefully collected and combined the extract in the 0.5 ml plastic micro centrifuge tube.
- 30 µL of aliquot of the extraction buffer was added to the tube containing the gel pieces, and incubated for 10 minutes with occasional gentle vortex mixing. The extract was driven to the bottom of the tube by centrifuging the sample for 30

seconds followed by carefully collection of supernatant and combination of the extract in the 0.5 ml plastic micro centrifuge tube.

- The volume of the extract was reduced to less than 20 µL by evaporation in a vacuum centrifuge at ambient temperature. The extract must not be allowed to dry completely.
- The volume of the digest was adjusted to ~20 µL with acetic acid. Finally, the sample was ready for mass spectrometric analysis.

3.6. Protein Identification and Mass Spectrometric Analysis

Protein identification using nanoLC-ESI-MS/MS was performed by Proteome Factory (Proteome Factory AG, Berlin, Germany). The MS system consisted of an Agilent 1100 nanoLC system (Agilent, Boeblingen, Germany), PicoTip emitter (New Objective, Woburn, USA) and an Esquire 3000 plus ion trap MS (Bruker, Bremen, Germany). Protein spots were in-gel digested by trypsin (Promega, Mannheim, Germany) or thermolysine (Fluka, Seelze, Germany) and applied to nanoLC-ESI-MS/MS. After trapping and desalting the peptides on enrichment column (Zorbax SB C18, 0.3 x 5 mm, Agilent) using 1% acetonitrile/0.5% formic acid solution for five minutes, peptides were separated on Zorbax 300 SB C18, 75 µm x 150 mm column (Agilent) using an acetonitrile/0.1% formic acid gradient from 5% to 40% acetonitrile within 40 minutes. MS spectra were automatically taken by Esquire 3000 plus according to manufacturer's instrument settings for nanoLC-ESI-MS/MS analyses. Proteins were identified using MS/MS ion search of Mascot search engine (Matrix Science, London, England) and NCBInr protein database (National Center for Biotechnology Information, Bethesda, USA). Ion charge in search parameters for ions from ESI-MS/MS data acquisition were set to "1+, 2+ or 3+" according to the instrument's and method's common charge state distribution.

The following figure was summarized the overall procedure starting from plant growth to identification of proteins with mass spectrometry.

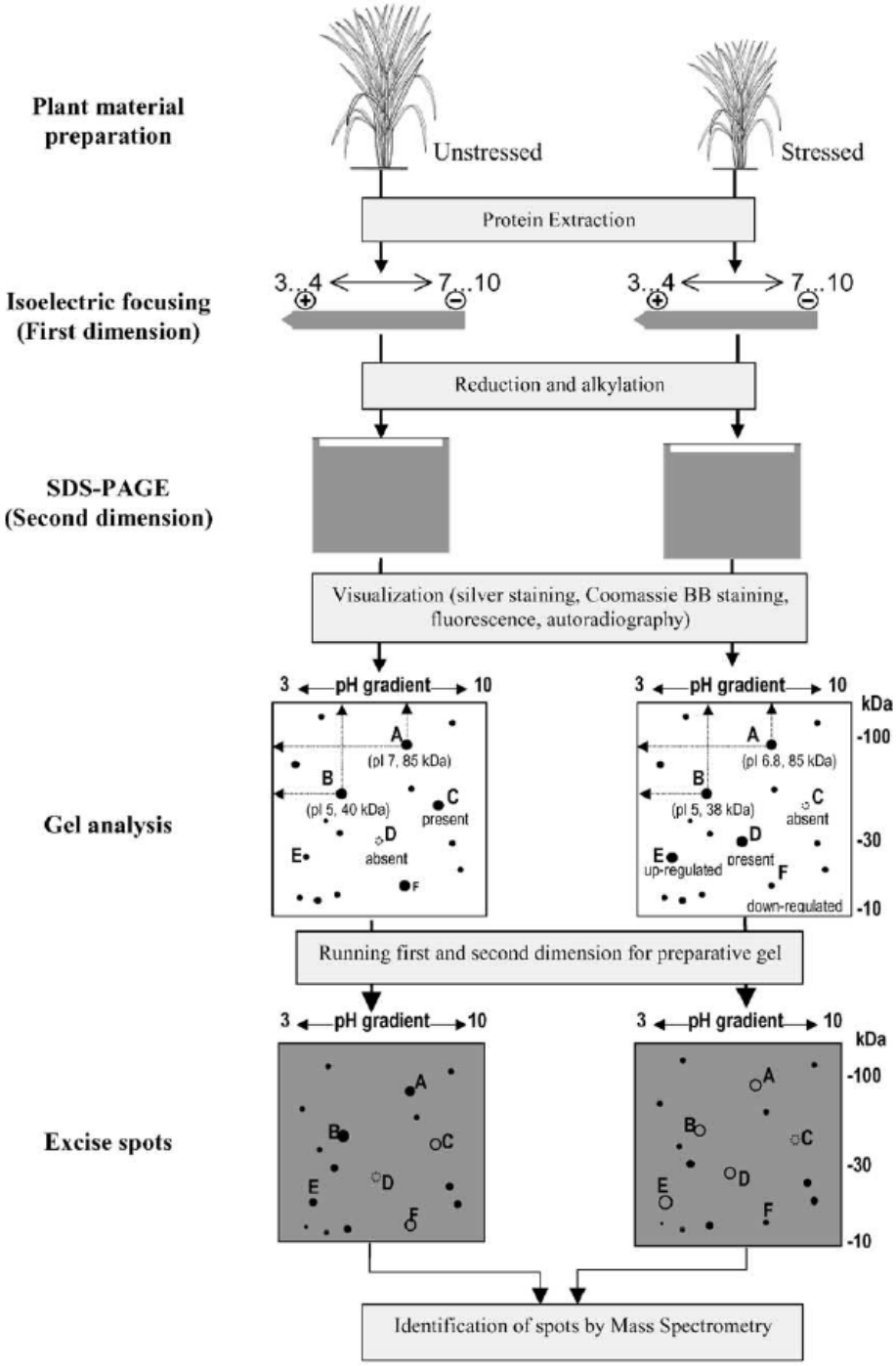

Figure 3.3. A Schematic Representation of Differential Display using 2D Gels (Source: Salekdeh, et al. 2002)

CHAPTER 4

RESULTS AND DISCUSSION

The study was divided into two parts; namely, identification of boron-stress tolerant proteins in Anadolu (boron-tolerant) over Hamidiye (boron-sensitive) barley genotype by nanoLC-ESI-MS/MS and comparison of boron-treated and control plants of Sahara (boron-tolerant) barley genotype in terms of its up- and down-regulated, totally disappeared, and newly formed proteins.

In literature, the usability of TRIzol-extracted proteins in proteomic applications, such as identification with mass spectrometry is still largely unknown. In our study, however, we were successful in identifying of proteins which were extracted by TRIzol.

4.1. Effects of Boron Toxicity on Growth

Plant response to boron in the soil varies widely among species, and as well as among genotypes within a species. After 10 mM boric acid treatment, boron tolerant genotypes were able to grow well in hydroponic solution media in which boron sensitive genotype was adversely affected by boron stress.

Boron toxicity manifests itself in a several ways in barley; including decreased shoot and root growth, reduced leaf chlorophyll, low photosynthetic rates due to loss of green leaf area as it can been in the figure 4.1. Leaf symptoms of toxicity in barley were characterized by chlorotic and/or necrotic patches, generally at the margins and tips of older leaves. This showed that the accumulation of boron at the end of transpiration stream. In addition, fewer leaves were developed and roots were browner compared to control plants. Plant growth was retarded and this leads to a reduction in total yield of crop when the soil has long-term exposure of high boron.

According to our observations, the barley genotypes Anadolu, Hamidiye and Sahara greatly differed in severity of leaf symptoms of boron toxicity. Approximately 80 % of Hamidiye genotype leaves showed reddish-brown neurotic spots and chlorotic spots whereas only 50 % of Anadolu genotype leaves showed similar symptoms under boron stress. On the other hand, Sahara genotype showed no neurotic spots along to its leaves, while the tip of older leaves only turned yellow.

Figure 4.1. Leaf Symptoms in Barley Leaves under Boron Stress. The First Photography was taken from Anadolu Genotype of Boron Stress and Control Leaves, whereas the Second Photograph was from Hamidiye Genotype under Boron Stress

4.2. Boron-Stress Responsive Proteins in Barley Leaves

Two barley genotypes (Anadolu and Hamidiye) differing in their sensitivity to boron were cultivated in the presence or absence of 10 mM boric acid for 7 days. Total proteins were extracted from leaves and the protein extracts from two control plants (Anadolu Control and Hamidiye Control) and two boron-stressed plants (Anadolu Boron and Hamidiye Boron) were analyzed by 2-DE. The proteome maps showed a broad distribution of spots in a pI range from 3.0 to 10.0 and a mass range nearly from 10 kDa to 120 kDa. There were clear differences between control and treated plants in the low and middle mass region (proteins less than 50 kDa).

Studies were carried out to see differences between total protein patterns of boron-treated and control plants and to identify novel proteins whose quantities were altered under boron stress. However, because of tolerance to boron toxicity, the boron-stress responsive proteins were examined on leaves of boron-tolerant barley, genotype Anadolu. The whole experiment, including plant culture and protein extraction and 2-DE experiments, was repeated three times in our laboratory and almost similar proteome patterns were obtained.

By using PD Quest 8.0.1 software program (Bio-Rad Laboratories), 152 proteins in Anadolu Boron, 107 proteins in Anadolu Control, 80 proteins in Hamidiye Boron, and 124 proteins in Hamidiye Control were detected based on the stated parameters such as selecting faint spot, small spot, and largest spot cluster on each gel.

The following gel images shows barley leaf proteins which was extracted from Anadolu and Hamidiye genotype. Proteins were separated in the first dimension on an IPG Strip pH 3.0-10.0 and in the second dimension on a 12 % acrylamide SDS-gel. The gels were stained with Coomassie colloidal blue staining. From left to right; Anadolu Control (A), Anadolu Boron (B), Hamidiye Control (C), and Hamidiye Boron (D).

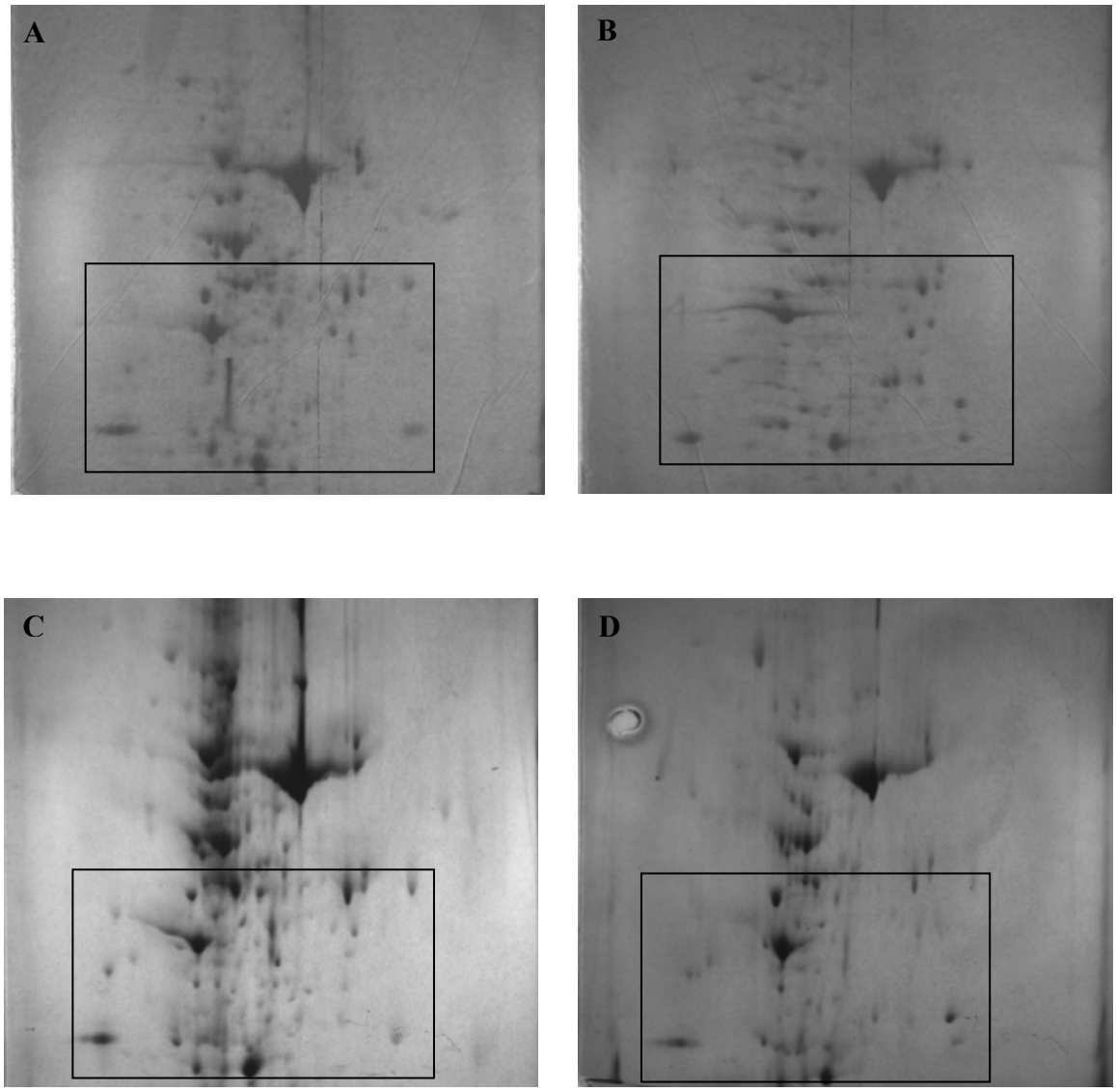

Figure 4.2. 2D-PAGE Gels of Anadolu and Hamidiye Leaf Proteins

As it can be seen in the above framed region, the main difference was between 15 kDa to 30 kDa mass regions. Comparative proteomic analysis was used to investigate the protein profiles under boron stress. The novel protein spots were numbered in the figure 4.3 in the Anadolu genotype of boron treated over control and excised for mass spectrometric analysis.

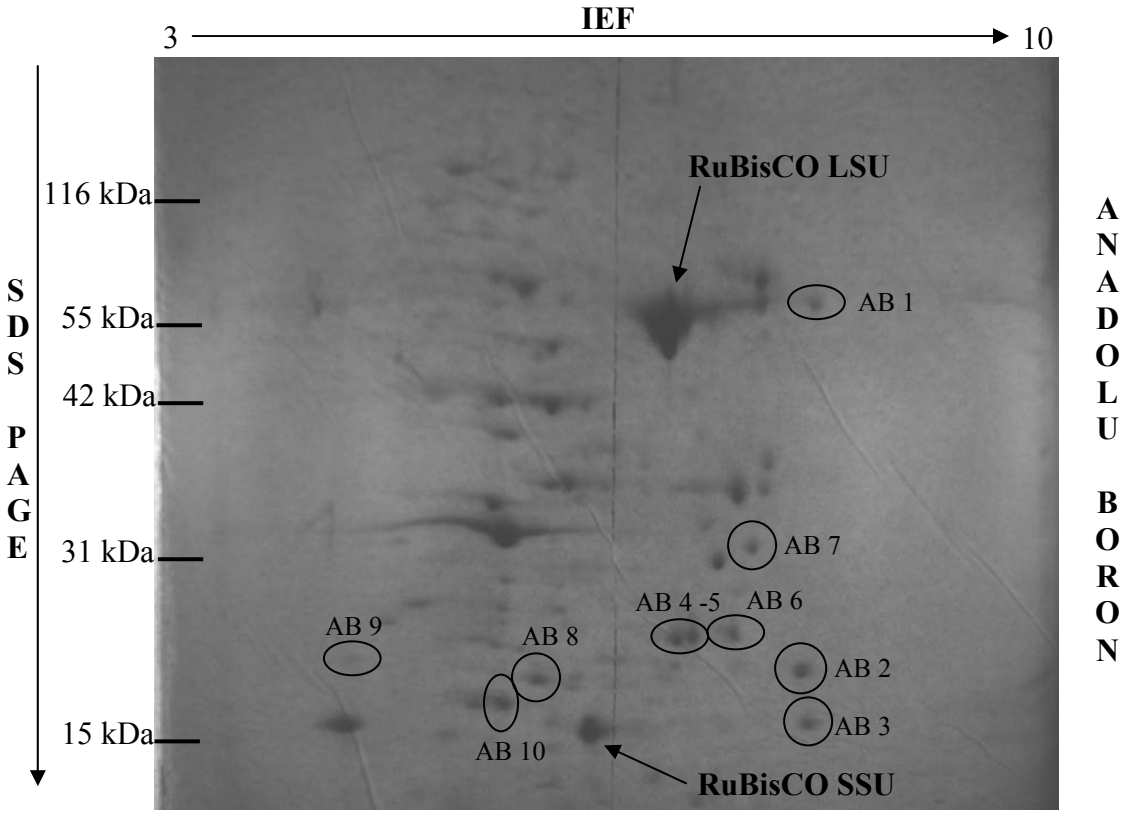

Figure 4.3. Representative 2-DE Map of Anadolu Boron Leaf Proteins

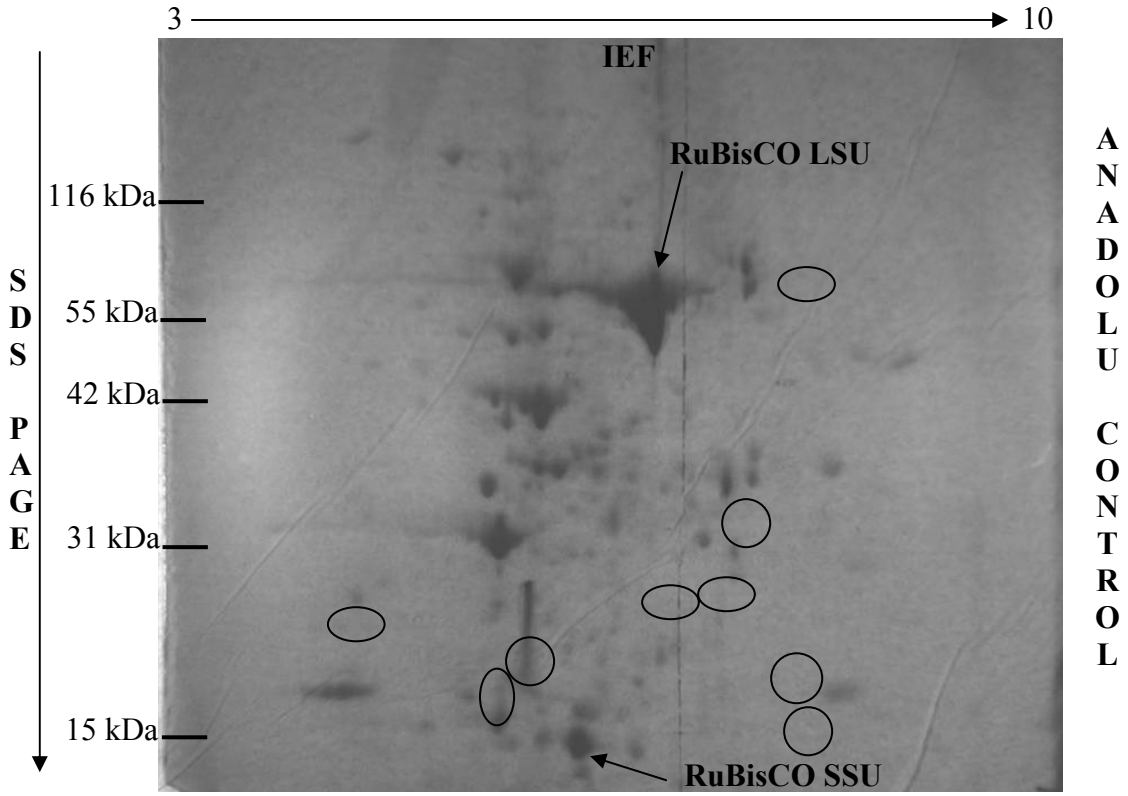

Figure 4.4. Representative 2-DE Map of Anadolu Control Leaf Proteins

Table 4.1 Boron Responsive Proteins, Identified by nanoLC-ESI-MS/MS (cont.)

Spot No	Protein Name	Sequence	AC Number (gi | NCBI) and Reference Organism	Sequence Covered (%)	Theoretical/ Observed Molecular Mass (kDa)	Theoretical/ Observed (pI)
AB 1	ribulose 1,5-bisphosphate carboxylase/oxygenase large chain (RuBisCo large chain)	MSPQTETK**AGVGFKAGVK**DYK**LTYYTPEYET KDTDILAAFR**VSPQPGVPPEEAGAAVAAESSTG TWTTVWTDGLTSLDRYKGRCYHIEPVAGEDSQ WICYVAYPLDLFEEGSVTNMFTSIVGVFGFKALR ALRLEDLRIPPTYSKTFQGPPHGIQVERDKLNKY GRPLLGCTIKPKLGLSAKNYGRACYECLRGGLDF TKDDENVNSQPFMRWRDRFVFCAEAIYKSQAET GEIKGHYLNATAGTCEEMIKRAVFARELGVPIV MHDYLTGGFTANTTLAHYCRDNGLLLHIHRAM HAVIDRQKNHGMHFRVLAKALRMSGGDHIHSG TVVGKLEGER**EMTLGFVDLLR**DDFIEKDRARGI FFTQDWVSMPGVIPVASGGIHVWHMPALTEIFG DDSVLQFGGGTLGHPWGNAPGAAANRVALEAC VQARNEGRDLAREGNEIIRAACKWSPELAAACE VWKAIK**FEFEPVDTIDK**	14017580 Triticum aestivum	10 %	52.8 / 55.0	6.22 / 7.20
AB 2	thaumatin-like protein TLP5	MAFSGVVHLIALVIAVAAAATDATTITVVNRCSY TIWPGALPGGGAR**LDPGQSWQLNMPAGTAGA RVWPRTGCTFDRSGRGRCITGDCAGALVCR**VSG **EQPATLAEYTLGQGGNR**DFFDLSVIDGFNVPM SFQPVGGAPCRAATCAVDITHECLPELQVPGGCA SACGKFGGDTYCCRGQFEHNCPPTYYSRFFKGK CPDAYSYAKDDQTSTFTCPAGTNYQIVLCPARN DLHMDQ	56682582 Hordeum vulgare	16 %	24.9 / 24.0	6.04 / 7.00

(cont. on next page)

Table 4.1 Boron Responsive Proteins, Identified by nanoLC-ESI-MS/MS (cont.)

AB 3	basic pathogenesis-related protein PR5	MASSRVVYLLAGLLLAALAATTDAATITVVNRC SYTVWPGALPGGGVRLDPGQSWALNMPAGTAG ARVWPRTGCTFDGSGRGRCITGDCNGVLACR**VS GQQPTTLAEYTLGQQGANK**DFFDLSVIDGFNVP MSFEPVGGCRAARCATDITKDCLKELQVPGGCA TCGKFGGDTYCCRGQFEHNCPPTNYSMFFKGKC PDAYSYAKDDQTSTFTCPAGTNYQIVLPLDQYQS ITWVNKE	2344818 Hordeum vulgare	8 %	25.2 / 20.0	6.54 / 7.10
AB 4	S-like RNase	MAQRTISLCLILGLLAAAAPANATSFDFYYLVLM WPGAYCVDSEYGCCVPKYGYPAEDFFVQSFTTF DLSLNKAIVRCNSDKPFDINKLEPIENNLNHYWS NIHCPRTDGTSTWKSEWRSYGVCSGLKEVDYFR AGLNLRK**NADVLGALAEQGINPDYR**LYSTEHIK WAVNQKLGVMPGVQCRDGPFGKKQLYQIYLCV DKNGETIIDCPKLPKLHCPEEVLFHPFHTWMLNA TSPAKIMLPTEA	20271131 Triticum aestivum	7 %	27.7 / 26.0	6.30 / 6.70
	RNase S-like protein	MAMRAISLCLILGLLAAAAPASATSFDFYYLILM WPGAYCADSDYGCCVPKYGYPAEDFFVEGFMT FDLSLNKAIVRCNSDKPFDVNKLEPIENNLNHYW SNIHCPRNDGTGTWKSEWRSYGVCSGLKLVDYF RAALNLRKKADVLGALAEQGINPDYR**LYNTEHI K**WAVNQKLGVMPGVQCRDGPFGKKQLYQIYLC VDKDGQIFIDCPKLPKLHCPEEVLFHPFHTWMLN ATSPAKITLPTEA	21954110 Hordeum vulgare	3 %	27.6 / 26.0	6.58 / 6.70
AB 5	RNase S-like protein	MAMRAISLCLILGLLAAAAPASATSFDFYYLILM WPGAYCADSDYGCCVPKYGYPAEDFFVEGFMT FDLSLNKAIVRCNSDKPFDVNKLEPIENNLNHYW SNIHCPRNDGTGTWKSEWRSYGVCSGLKLVDYF RAALNLRKKADVLGALAEQGINPDYR**LYNTEHI K**WAVNQKLGVMPGVQCRDGPFGKKQLYQIYLC VDKDGQIFIDCPKLPKLHCPEEVLFHPFHTWMLN ATSPAKITLPTEA	21954110 Hordeum vulgare	10 %	27.6 / 27.0	6.58 / 6.80

(cont. on next page)

Table 4.1 Boron Responsive Proteins, Identified by nanoLC-ESI-MS/MS (cont.)

AB 6	GTP-binding nuclear protein Ran1	MALPNQQTVDYPSFK**LVIVGDGGTGK**TTFVKR **HLTGEFEK**KYEPTIGVEVHPLDFFTNCGKIRFYC WDTAGQEKFGGLRDGYYIHGQCAIIMFDVTARL TYKNVPTWHRDLCRVCENIPIVLCGNKVDVKNR QVKAKQVTFHRKK**NLQYYEISAKSNYNFEKPF LYLAR**KLAGDGNLHFVESPALAPPEVHIDLAAQ ALHEEELQQAANQPLPDDDDEAFE	585777 Solanum lycopersicum	19 %	25.2 / 27.5	6.25 / 6.90
	putative small GTP-binding protein Ran	MSRAQALPDPAAVGYPSFKLILVGDGGTGKTTF VKR**HITGEFEK**RYEPTIGVEVRPLDFHTSRGKVR FCCWDTAGQEKFGGLRDGYYIHGHCAIIMFDVT SRLTYKNVPTWHKDICRVCDNIPIVLCGNKVDM KNRQVKAKMVTFHRKK**NLQYYEISAKSNYNFE KPFLYLAR**KLTGDMNLRFVEELALLPADVTIDLI AQQKIETEIAAAAAMPLPDEDEDGLM	50725032 Oryza sativa (japonica cultivar-group)	14 %	25.6 / 27.5	8.15 / 6.90
	GTPase Ran/TC4/GSP1 (nuclear protein transport pathway), small G protein superfamily (ISS)	MSAPPSNTPTFKLILVGDGGTGKTTFVKR**HLTGE FEK**KYLPTVGVSVHPLDFHTNCGPIRFDCWDTA GQEKFGGLRDGYYIHGQCAIIMFDVTSRTTYKN VPTWHRDITRVCEDIPIVLCGNKVDVRNRQVRA KSITFHRKK**NLQYYELSAKSNYNFEKPFLYLAR** KLSGNPQLQFTESPALAPPTVTVDLAEVAAYEKE LADAAAQPLPDEDDELLDA	116061061 Ostreococcus tauri	14 %	24.6 / 27.5	6.59 / 6.90
	RNase S-like protein	MAMRAISLCLILGLLAAAAPASATSFDFYYLILM WPGAYCADSDYGCCVPKYYPAEDFFVEGFMTF DLSLNKAIVRCNSDKPFDVNKLEPIENNLNHYW NIHCPRNDGTGTWKSEWRSYGVCSGLKLVDYFR AALNLRKK**ADVLGALAEQGINPDYRLYNTEHI K**WAVNQKLGVMPGVQCRDGPFGKKQLYQIYLC VDKDGQIFIDCPKLPKLHCPEEVLFHPFHTWMLN ATSPAKITLPTEA	21954110 Hordeum vulgare	10 %	27.6 / 27.5	6.58 / 6.90

(cont. on next page)

Table 4.1 Boron Responsive Proteins, Identified by nanoLC-ESI-MS/MS

AB 7	vacuolar proton-translocating ATPase (V-ATPase) Subunit E	MNDTDVSR**QIQQMVR**FIRQEAEEK**AGEISVSAE EEFNIEKLQLVEAEK**KKIRQEYERKEKQVDVRK **KIEYSMQLNASR**IK**VLQAQDDLVNK**MKEDAM KELLNISSNHHEYRNLLK**ELVVQGLLRLKEPAV LLR**CRKEDHHNVESVLHSAKNEYASKADVHEPE ILVDHSVYLPPSPSHDDKHGQICHGGVVLASRDG K**IVFENTVDAR**LEVVFRKKLPEIRK**LLVAA**	4099148 Hordeum vulgare	38 %	26.2 / 30.0	6.57 / 7.00
	Unknown protein	MNDADVAK**QIQQMVR**FIRQEADEKANEISVSAE EEFNIEK**LQLVEAEK**KKIRQEYERKEKQVEVRK **KIEYSMQLNASR**IK**VLQAQDDLVNK**MKDDAM KELLLVSHNHHEYKNLLK**DLIVQGLLR**LKEP	5565981 Zea mays	36 %	15.2 / 30.0	6.34 / 7.00
	Os01g0222500 protein	MMNDGDVARQLKQMTDFIRQEAVEKAAEIEAA AAEEFQIEK**LQLVEAEK**KRILEFERNEKQGDIKK KIEYSKQLNASRLEVLQAQDDLAMSMLEAAGKE LLYITRDHHVYKNLLRIFIVQSLLR**LKEPAVILR**C RKEDRELVESVLESAKNEYADKANIYPPEIMVDR NVYLPPAPSHYEAHGPSCSGGVVLASRDGKIVCE NTLDARLEVVFRKKLPEIRRSLLGQVAA	115435358 Oryza sativa (japonica cultivar-group)	7 %	26.4 / 30.0	6.18 / 7.00
AB 8	PSI type III chlorophyll a/b-binding protein	MAAQALVSSSLTSSVQTARQIFGSKPVASASQKK SSFVVKAAAAPPVKQGANRPLWVASSQSLSYLD GSLPGDYGFDPLGLSDPEGTGGFIEPRWLAYGEII NGRFAMLGAAGAIAPEILGKAGLIPAETALPWFQ TGVIPPAGTYTYWANYTLFVLEMALMGFAEHRR **LQDWYNPGSMGKQYFLGLEK**GLAGSGNPAYP GGPFFNPLGFGKDEKSLKELKLKEVKNGRLAML AILGYFIQGLVTGVGPYQNLLDHLADPVNNNVL TSLKFH	430947 Arabidopsis thaliana	7 %	29.1 / 23.0	8.61 / 6.20
AB 9	NOT IDENTIFIED	————————	————	————	————	————
AB 10	light-harvesting complex I; LHC I	PGRSGLPFCAYATTSGRVTMSAEWFPGQPRPAH LAGSSPRGLRVRPLGLATVPENFERFKESEIYHCR WAMLCVPGVLVPEALGLGNWVKAQEWAALPD GQATYLGNPVPWGNLPTILAIEFLAIAFAEQQRT MEKDPEKK**KYPGGAFDPLGFSK**DPAKFEELKL KEIKNGRLAMLAFVGFCVQQSAYPGTGPLENLA THLADPWHNNIGDIVIPR**NIYGP**	544700 Hordeum vulgare	8 %	24.2 / 21.0	8.11 / 6.00

In Table 4.1., the sequence of the each protein was shown with its other characteristic properties. The red colored amino acid sequence indicates the identified sequence by LC-MS/MS. This sequence was then entered into the database to identify and to name the protein.

Mass spectrometry analysis and database searching helped to identify 10 spots representing 7 different proteins. These seven proteins were consequence of boron stress. Two spots were identified as the same protein and one protein was not identified. The identified 7 proteins are namely, ribulose 1,5-bisphosphate carboxylase/oxygenase large chain (RuBisCo large chain), thaumatin-like protein TLP5, basic pathogenesis-related protein PR5, RNase S-like protein, vacuolar proton-translocating ATPase subunit E, PSI type III chlorophyll a/b-binding protein, and light-harvesting complex I; LHC I.

The identified three protein, thaumatin-like protein TLP5, basic pathogenesis-related protein PR5 and RNase S-like protein, are environmental stress related proteins that expressed in plant leaves under boron treatment. Other three protein, RuBisCo large chain, PSI type III chlorophyll a/b-binding protein and light-harvesting complex I, are photosynthesis related proteins. Toxic concentration of boron in plants causes reducing normal growth and development, leaf area loss due to inhibition of photosynthesis by decreasing of chlorophyll concentrations and CO_2 fixation. Vacuolar proton-translocating ATPase subunit E protein is the most important one which provides the living of plant under boron stress.

After boron stress, green leaf area was decreased due to neurotic and chlorotic patches in barley genotypes. To counteract boron stress, plants can change their gene expression and protein accumulation. In our study, it was obvious that barley leaves need to increase their photosynthesis activity to maintain their lives. This observation was confirmed by identification of three proteins which are related to photosynthetic mechanism. These proteins are ribulose 1,5-bisphosphate carboxylase/oxygenase large chain (RuBisCo large chain), PSI type III chlorophyll a/b-binding protein, and light-harvesting complex I; LHC I, spot 1, spot 8, spot 10, respectively.

In figure 4.3 and 4.4, RuBisCO LSU and RuBisCO SSU stand for large subunit of RuBisCO and small subunit of RuBisCO, respectively. These subunit's molecular weights are at approximately 55 kDa (for large chain) and 14 kDa (for small chain). In our study, spot 1 is identified as RuBisCO large subunit. To our knowledge, the expression of this protein is mainly due to demand of maintaining lives of barley leaves

under boron stress. Because of losing green leaf area, plants want to enhance its photosynthetic rate and this causes the expression of photosynthesis-related protein, RuBisCO.

RuBisCO, a critical enzyme in the photosynthesis, a short for ribulose 1,5-bisphosphate carboxylase/oxygenase. It catalyzes reaction between carbon dioxide (CO_2) and the five-carbon sugar ribulose 1, 5-bisphospate (RuBP), the first major step of carbon fixation in Calvin cycle. RuBisCO catalyzes the carboxylation or oxygenation of RuBP with carbon dioxide or oxygen, respectively. RuBisCO is probably the most abundant protein in leaves due to being 16 % of the protein content of the chloroplast, and it may be the most abundant on Earth. RuBisCO is slow compared to many enzymes; it is able to fix only a few carbon dioxide molecules per second whereas some enzymes can catalyze thousands of chemical reactions per second. In addition, RuBisCO is the primary rate-limiting enzyme of the Calvin cycle when the light is off. RuBisCO is only active during the day because RuBP is not being produced in the dark due to the regulation of several other enzymes in the Calvin cycle.

However, the dynamic range of protein accumulation is very large; this is a problem for leaf proteomic analysis because the preponderance of RuBisCO which masks the detection of proteins in small amount. There are some methods to get rid of RuBisCO while protein extraction was carried out from plant leaves.

Spot 2 is identified as thaumatin-like protein, TLP5. Thaumatin-like proteins (TLPs) are polypeptides of about 200 residues synthesized by plants in response to fungal infection. Their name is derived from the first member isolated from the seeds of the plant *Thaumatococcus danielli*. Some scientists have demonstrated that TLPs's action against pathogenic microorganism has been related to endo-β-1,3-glucanase activity and α-amylase inhibiting properties (Brandazza, et al. 2004). However, not all plant TLPs show glucanase activity. TLPs were assigned TLP1-TLP8 in order of increasing isoelectric points where TLP5 is thought to be a basic protein. These TLPs belong to the family of PR-5. N-terminal region of TLP5 closely resembles that a protein called avematin, a TLP isolated as an antifungal protein from oat seeds (*Avena sativa*) (Reiss, et al. 2001). TLP5 shows a C-terminal structure differing from that of the other TLPs by having additional nine residues on that side of the chain.

Spot 3 is found as basic pathogenesis-related protein, PR-5. Pathogenesis-related (PR) proteins are involved in plant responses to biotic and abiotic stress and they are a large group of low molecular mass proteins. PR proteins were originally identified in

tobacco plants infected with tobacco mosaic virus (TMV) (van Loon and van Kammen, 1970) and they have been known since then. Based on their primary structures, immunologic relationships, and enzymatic properties, PR proteins are currently grouped into seventeen families (PR-1 through 17) (Zhang 2006). PR proteins play an important role in protecting plants from pathogen infection and also has functions to alleviate the harmful effects to cells and organisms caused by natural stresses, such as cold, drought, osmotic stress, UV light, and metal toxicity. PR-5 proteins are a family of proteins that are induced by different phyto-pathogens in many plants. The family of PR-5 proteins includes proteins with several functions, such as roles in development, protection against osmotic stress and freezing tolerance in plants. There have been found three classes of PR-5 protein group in some plant, namely acid, basic and neutral isoforms.

The proteins of the PR-5 family are also called thaumatin-like proteins (TLPs) as they have a high degree of homology to thaumatin, a sweet-tasting protein. PR-5 proteins have been suggested to bind β-1,3-glucans, and hence they might have carbohydrate-binding properties that may result present in membrane-permeabilizing activity.

Spot 4-5-6 are identified as the same protein, RNase S-like protein. It is the member of plant's RNase T2 family. S-like RNases are homologous to S-RNases, other member of RNase T2 family, and it is not involved in self-incompatibility. The majority of S-like RNases in higher plants are induced during both senescence and phosphate starvation. They are believed to be components of a phosphate remobilization system that recycles phosphates during senescence or phosphate starvation to supplement the limited supply of phosphate available from soil. However, the main function of RNase-related proteins (RRPs) in plants remains largely unknown. The barley RRP gene was found specifically expressed in leaves and was induced by light, but its role has not yet been defined (Shimizu, et al. 2001, Gausing 2000).

Spot 8 and spot 10 were identified as PSI type III chlorophyll a/b-binding protein and light-harvesting complex I; LHC I, respectively. Photosystems are protein complexes involved in photosynthesis, simply uses light to reduce molecules. During oxygenic photosynthesis, photosystem I (PSI) and photosystem II (PSII) operate in series. They are located in distinct thylakoid membrane regions and have different light absorption properties. Under changing light conditions, the reversible phosphorylation of light harvesting chlorophyll a/b binding proteins (LHC II) represents a system for balancing the excitation energy between the two photosystems (Liu, et al. 2004). A

light-harvesting complex is one or more polypeptide chains containing photosynthetic pigments, which surrounds a photosystem and focuses light inward toward its core. Chlorophylls, with the carotenoids, are the most important in light-harvesting complexes in plants. Chlorophyll b is nearly identical to chlorophyll a except it formyl group instead of methyl group. This makes chlorophyll b absorb light at 400 and 500 nm wavelengths efficiently. Green plants have chloroplast, which is called a photosynthetic area, holds the pigment protein complexes, known as LHC, light-harvesting complexes, I and II in their thylakoid membrane.

Spot 7 is identified as vacuolar proton-translocating ATPase (H^+ V-ATPases or V-ATPase) subunit E. The plant V-ATPase is a primary-active proton pump located at the vacuolar membrane (tonoplast) and various other components of the endomembrane system of the plant cell (Li and Zhang 2004).

V-ATPases affect several cellular processes such as cell expansion, cytoplasmic pH and ion homeostasis and transient or permanent deposition of toxic ions, excess nutrients and metabolites in the vacuolar lumen. V-ATPase is known as a house-keeping enzyme by maintaining cytosolic ion homeostasis and cellular metabolism. On the other hand, it has functions as a stress response enzyme under environmental stress by changing expression levels of subunits.

V-ATPases consists of 14 different polypeptide subunits which are organized in two domains and they work together as a rotary machine (Inoue, et al. 2005). The V_1 domain is a membrane-peripheral complex of 650 kDa, which carries out ATP hydrolysis, is located on the cytoplasmic side of the membrane and it is composed of eight subunits (A, B, C, D, E, F, G and H). In addition to this, the V_0 domain is a membrane-integral complex of 260 kDa which is responsible for the translocation of protons from the cytoplasm to the vacuole and it contains six subunits (a, c, c', c", d and e). The following figure indicates the parts of the V-ATPase membrane protein.

The V_1 domain consists of 3 copies of subunits A and B that form a ring, 2 copies of E and G subunits, 1 or 2 copies of subunit of H and single copies of the remaining subunits. Three copies of subunit A (catalytic subunit) and subunit B (non-catalytic ATP binding) mediate the hydrolysis of ATP at three reaction sites (Golldack and Dietz 2001). The other remaining subunits are arranged between fixed peripheral stalk and central rotational stalk which connect the V_1 with V_0 domain. The V_0 domain composed of 4 or 5 copies of proteolipid subunits (c, c', and c") and single copies of the

remaining subunits which involved in proton translocation. These proteolipid parts are highly hydrophobic and they arranged to form a ring structure.

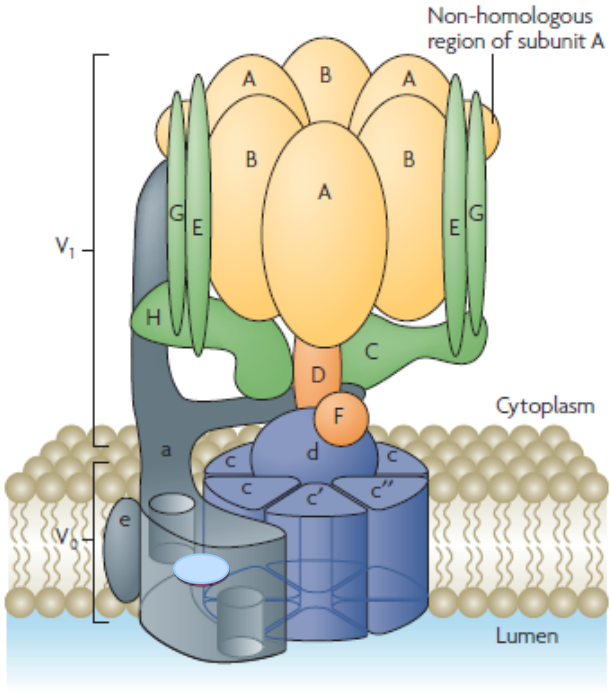

Figure 4.5. The Structure of V-ATPase
(Source: Forgac 2007)

V-ATPases operate by a rotary mechanism. The ATP hydrolysis provides required energy for rotation of catalytic sites of subunit A and B which are head groups of V_1 complex. This also facilitates rotation of D and F subunits that are connected to d and the proteolipid subunits of V_0. During rotation, protons are delivered from the cytoplasm into the vacuolar lumen through channels formed by subunit a. As a result of H^+ displacement, the V-ATPase generates an electrochemical gradient across the membrane which is the driving force for transport processes of ions and metabolites.

Isoforms of V-ATPase subunit E have been detected in several plant species (Kawamura, et al. 2000, Kluge, et al. 2003). It is also stated that accumulation of subunit E was slightly modified by salt stress in barley (Dietz, et al. 1995, Dietz and Arbinger, 1996).

We proposed that the excess boron in the cell is transported into the vacuole of the plant cell where they can be stored without causing any toxic effect. During proton translocation across the membrane of the vacuole, electrochemical gradient provides

excess boron to transfer from cytoplasm into the vacuole of the plant cell. The leaves of Anadolu barley genotype accumulated considerable amounts of boron, this is totally related to the accumulation of boron in the vacuolar compartment of the plant cell. This defense mechanism is called as internal tolerance mechanism for boron in boron-tolerant barley genotype. By doing so, we were able to show that Anadolu (boron-tolerant) barley genotype can survive under toxic boron concentration in our study.

4.3. Comparative Proteome Analysis of Sahara Leaves

In the previous work, mass spectrometric identification of proteins which were thought to be involved in tolerance to boron toxicity was done successfully.

In the second part of the experiment, comparative proteome analysis of Sahara (Australian barley genotype, boron tolerant) leaves from control and boron stress were studied. The protein profiles were compared in terms of its up-regulated, down-regulated, totally disappeared, and newly formed proteins. This experiment was done only once because there was not much seeds for triplicate analysis of Sahara genotype.

160 µg of each protein extract (Sahara Boron-SB, Sahara Control-SC) was loaded on an immobilized pH gradient strip (pH 4–7, linear) followed by a 12 % SDS-PAGE. Representative 2-DE gel images were visualized by Coomassie colloidal blue staining. Approximately 300 proteins were detected by using PD Quest 8.0.1 software program (Bio-Rad Laboratories). Up- and down-regulated proteins can be determined because same amount of protein was loaded onto IPG strips. Proteome maps of SC and SB showed a broad distribution of spots in a pI range from 4.0 to 7.0 and a mass range from 15 kDa to 140 kDa.

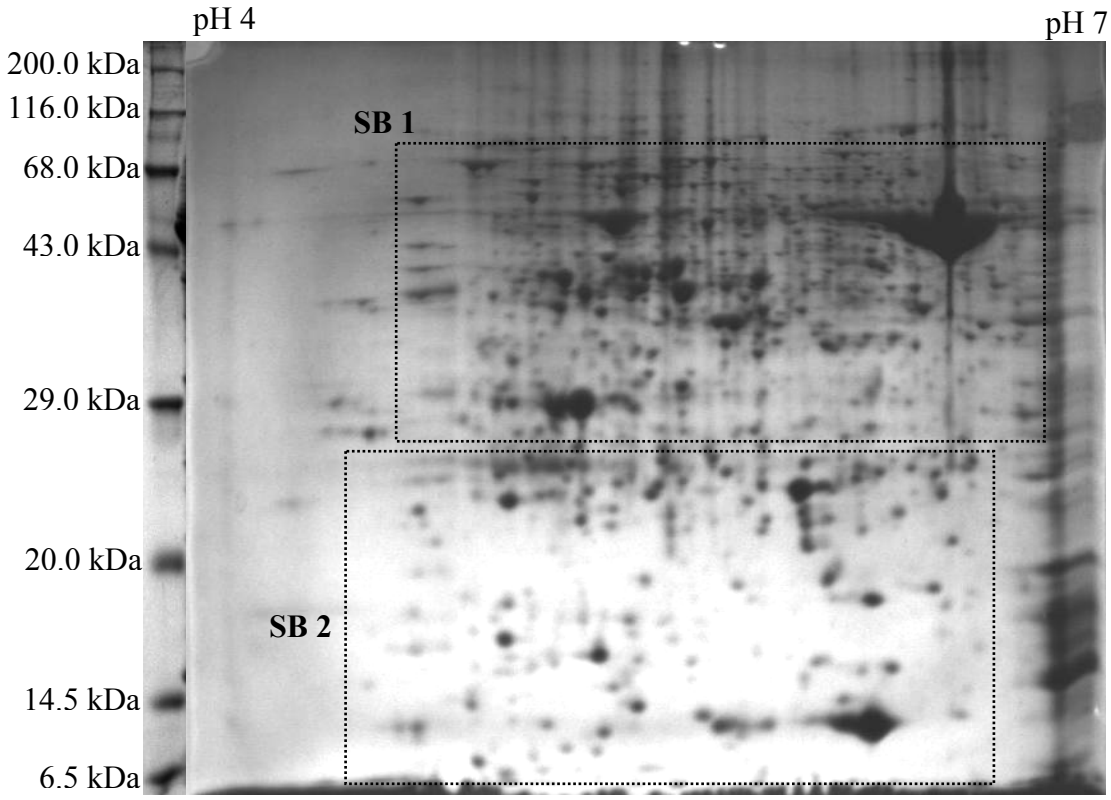

Figure 4.6. Representative 2-DE Map of Sahara Boron Leaf Proteins

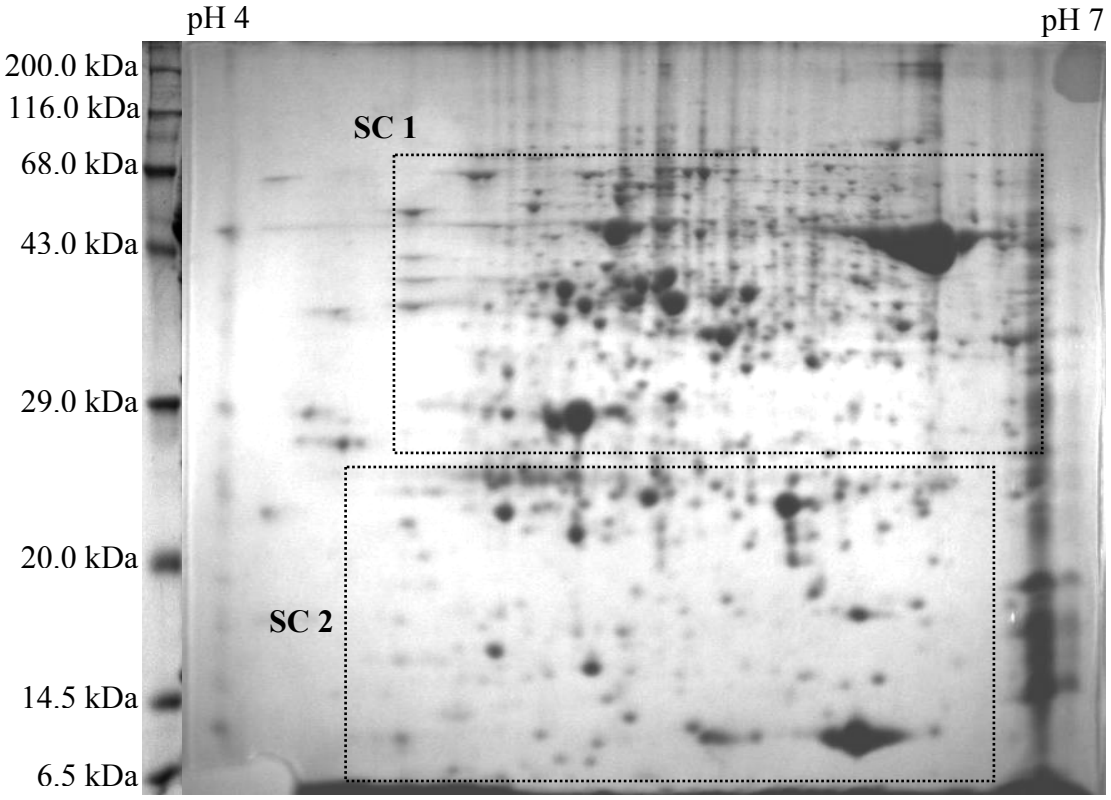

Figure 4.7. Representative 2-DE Map of Sahara Control Leaf Proteins

The framed regions were enlarged to indicate protein changes in control and treated Sahara leaves.

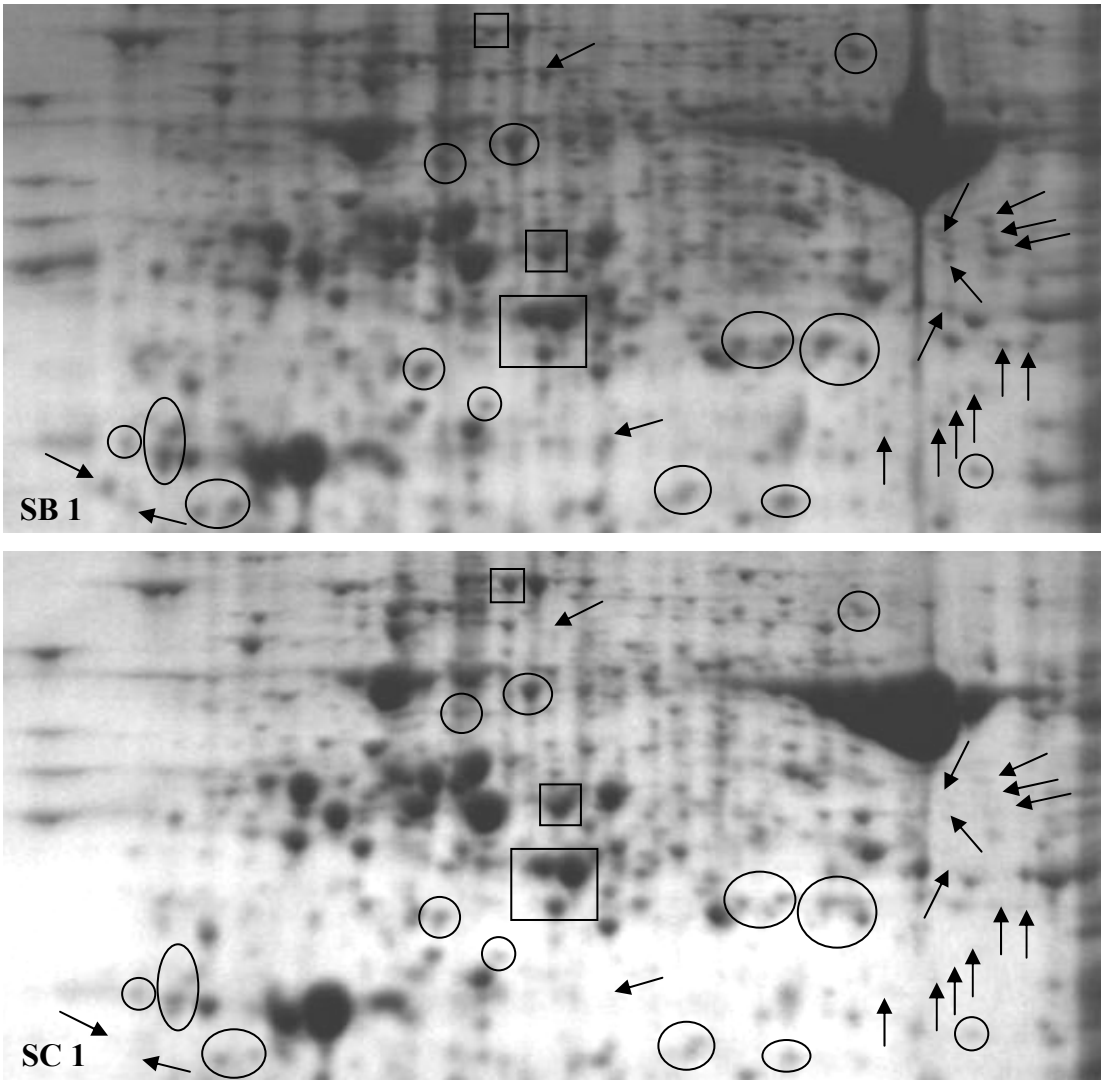

Figure 4.8. The Extended Gel Image of Sahara Boron and Sahara Control in High Mass Region

Arrows shows proteins exclusively present in the gels obtained from boron treated plants while circles indicates proteins significantly increased in the gels obtained from treated plants compared to those obtained from control plants. Additionally, boxes points out proteins decreased in the gels obtained from treated plants compared to those obtained from control plants.

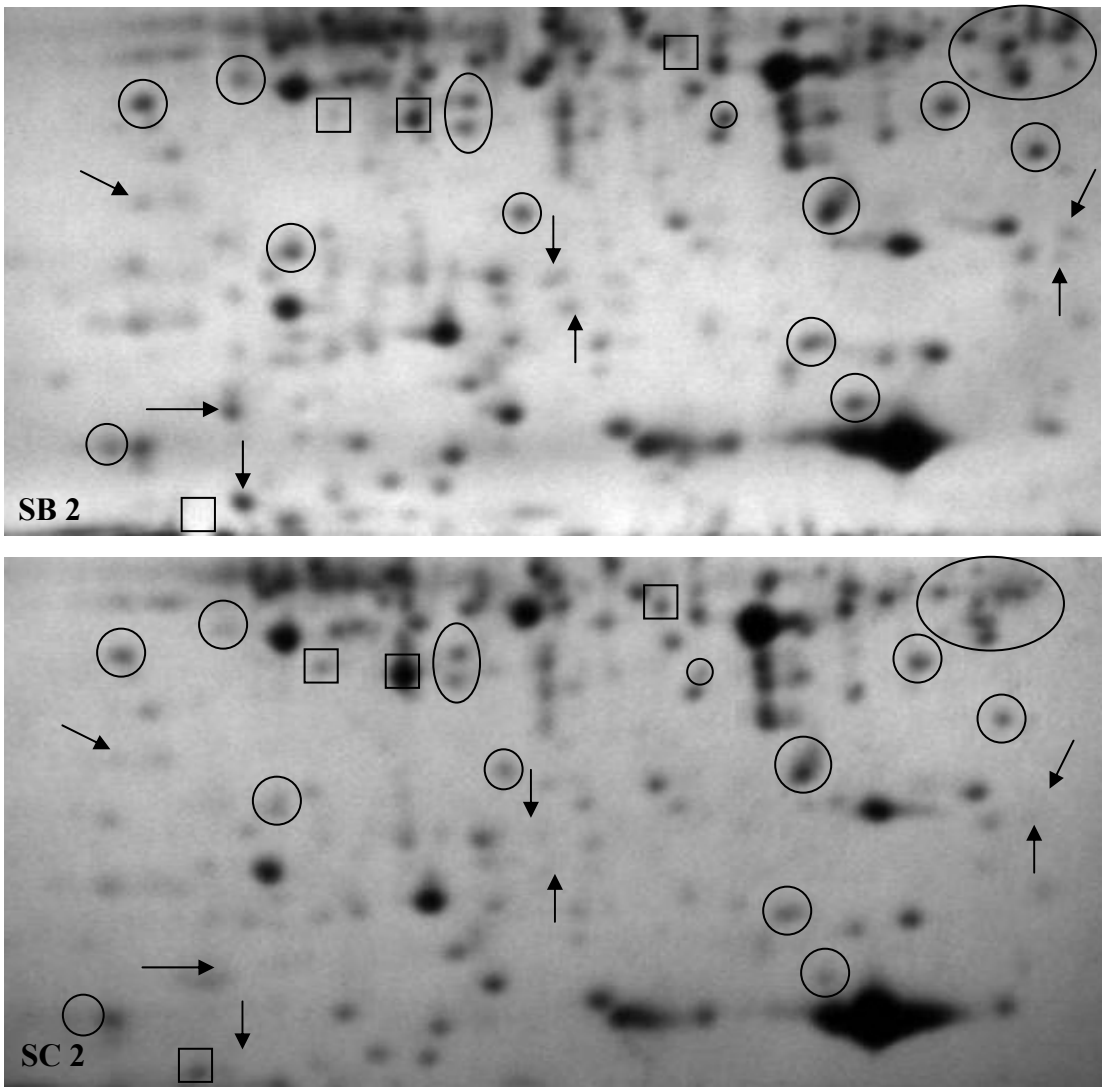

Figure 4.9. The Extended Gel Image of Sahara Boron and Sahara Control in Low Mass Region

In the same manner, arrows shows proteins exclusively present in the gels obtained from boron treated plants while circles indicates proteins significantly increased in the gels obtained from treated plants compared to those obtained from control plants. Additionally, boxes points out proteins decreased in the gels obtained from treated plants compared to those obtained from control plants.

It can be said that disappearance of the protein spots may be interpreted as the "turning off" of protein synthetic genetic machinery (genes) in response to boron treatments. Additionally, down-regulated proteins can also be explained with the same reason.

To our observations, the growth of three genotypes were reduced under boron treatments; more, however for the sensitive Hamidiye than for Anadolu and Sahara. Lastly, comparative proteome analysis of Sahara leaf proteins under control and stress conditions provided to detect up-regulated, down-regulated, newly expressed and completely disappeared proteins. For deeper analysis, mass spectrometric identification of these proteins could be done to explain defense mechanism to toxic boron concentrations.

The main theory is that every condition produces a unique set of proteins in the organisms. Proteins are the primary effector macromolecules of all living systems, and therefore virtually and adaptive response to environmental, physiological or pathological conditions will be reflected by alterations in protein activity, location and concentration. It is obvious that stress conditions have been shown to alter the protein composition of cells both qualitatively and quantitatively. In addition, higher plants synthesize new proteins which are involved in the stress tolerance mechanisms. Such proteins are thought to be potential markers for boron tolerance of barley genotypes in breeding program. For that reason, understanding the effect of boron on barley is an important issue for the improvement of the quality of these crops in boron rich soils.

CHAPTER 5

CONCLUSION

In this study, the main goal was to identify boron-stress tolerant proteins in boron-tolerant barley genotype by proteomic approach. One of the identified protein name as V-ATPase subunit E is the most important one for defense mechanism in boron-tolerant barley genotype. It is shown that excess boron ion in the cytoplasm of the cell is translocated into the vacuole of the plant cell by the help of V-ATPase subunit E. V-ATPases works by a rotary mechanisms. During rotation of head group of V-ATPase (subunits A and B), two-protons are transferred from the cytoplasm into the vacuolar lumen through channels. This generates an electrochemical gradient across the membrane which is driving force for transport boron ion into the vacuole of the plant cell. This is called internal tolerance mechanism without causing the plant cell to die under toxic boron concentrations. It is proposed that this might be the tolerance mechanism for boron in Anadolu genotype of barley.

In addition of V-ATPase protein, six more proteins were also identified by mass spectrometry. Three of them are photosynthetic mechanism related proteins due to the decreasing leaf green area which can be monitored as necrotic and chlorotic spots along the leaf margins and tips of leaves. In order to survive under these conditions plants needs to increase their photosynthesis mechanism. Photosynthesis can be effected by excess boron because it may causes activation or over expression of some genes in chloroplast genome. Other identified three proteins are generally expressed proteins under any environmental stresses.

Barley leaf proteins of Australian boron-tolerant genotype (Sahara) from control and 10 mM boric acid treatment stress group were also studied. It was shown that level of several proteins were changed after stress application such as up- and down-regulation and novel proteins were observed. Mass spectrometry must be conducted to this analysis for identifying boron-stress responsive proteins and to clarify the tolerance mechanism.

These two works indicates that certain proteins may be involved in tolerance to boron toxicity. Several proteins can be altered under boron treatment. The further step of this kind of studies is the identification of genes whose expression is induced or

enhanced under boron stress by identifying boron tolerant proteins firstly. These genes might be used in the breeding program for barley to improve crop tolerance to boron toxicity.

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