PROTEOMIC ANALYSIS OF BORON STRESS RESPONSE IN YEAST SACCHAROMYCES CEREVISIAE

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ABSTRACT

PROTEOMIC ANALYSIS OF BORON STRESS RESPONSE IN YEAST SACCHAROMYCES CEREVISIAE

Boron is a versatile element distributed in every part of the environment but most of its deposit reserves are localized in a few countries, Turkey being one of the most prominent. Boron is known to be an essential micronutrient for plants and some animals. Like any other essential element it has toxicity in high concentrations. Herein the mechanism of toxicity and the elements of the boron stress response were investigated in Saccharomyces cerevisiae with a proteomics approach. Boron is believed to have played a role in the evolution of life on earth. It has strongly electrophile organic compounds, the most important physiological form being boric acid. Boric acid has a capacity to bind cis-located hydroxl groups and some amino groups. Some of these groups are located at the active sites of some enzymes and at the carbohydrates with five-membered furanose rings. The riboses of some metabolically important molecules like S-adenosyl methionine, diadenosine phosphate family members and 3'end of RNAs are prone to be affected. The yeast cells subjected to boron in this study expressed higher amounts of carbohydrate metabolic enzymes, proteins involved in protein synthesis, protein folding and catabolism, redox homeostasis and nucleotide synthesis. All of these proteins are common to metal stress responses in yeasts. Some of them involve in other stress responses like peroxide, salt or herbicide stresses showing complex interplay between responses.

ÖZET

EKMEK MAYASI SACCHAROMYCES CEREVISIAE'DA BOR STRES YANITININ PROTEOMİK ANALİZİ

Bor her çevresel alanda bulunabilen çok yönlü bir elementtir, fakat maden rezervleri birkaç ülkeye dağılmıştır ki Türkiye burada öne çıkar. Bor bileşikleri en az bin yıldır bilinmektedir ve son zamanlarda teknolojide yeni kullanım alanları edinmektedir. Borun yeryüzündeki yaşamın evriminde rol oynadığına inanılır. Bor elementinin güçlü elektrofilik organik bileşikleri vardır ve bunların en önemli fizyolojik formu borik asittir. Borik asit aynı yönde yerleşmiş hidroksil ve bazı amino gruplarını bağlama kapasitesine sahiptir.. Bu gruplar bazı enzimlerin aktif bölgelerinde ve 5 karbonlu furanoz halkalı karbohidratlarda bulunur. S-adenozil metyonin, nikotinamid adenin dinükleotidler, adenozin fosfatlar, diadenozin fosfat ailesi üyeleri ve RNA'ların 3' uçları gibi metabolizmada önemli moleküllerin ribozları borik asitle etkileşmeye açıktır. Borun bitkiler ve bazı hayvanlar için esansiyel bir besin olduğu bilinmektedir. Diğer esansiyel elementler gibi bor da yüksek konsantrasyonlarda toksiktir. Toksiklik mekanizması ve bor stres yanıtının organizmalardaki elemanları henüz açığa kavuşmamıştır. Burada ekmek mayasında bor stres yanıtı proteomik yaklaşımla araştırıldı. Bora maruz kalan hücreler nispeten fazla miktarlarda karbohidrat metabolik enzimleri, protein sentezinde, protein katlanması ve yıkımında , redoks homeostazisinde ve nükleotid sentezinde yer alan proteinler eksprese ettiler. Tüm bu proteinler mayada metal stres yanıtında da yer alan proteinlerdi. Bazıları peroksit, tuz, veya herbisid stresleri gibi diğer streslerde de yer alan bu proteinler değişik streslere özgü yanıtların kendi aralarında iletişim halinde olduğunu göstermektedir.

TABLE OF CONTENTS

LIST OF FIGURES viii

LIST OF TABLES ix

CHAPTER 1. INTRODUCTION 1
1.1. The Chemistry of Boron 1
1.1.1. Unique Physicochemical Properties of Boron 2
1.1.2. Boron's Distribution in Nature 4
1.2. Biologically Relevant Boron 5
1.3. The Essentiality and Toxicity of Boron 7
1.4. The Molecular Basis for Boron Effect on Living Organisms 8

CHAPTER 2. MATERIALS AND METHODS 13
2.1. Yeast Growth and Medium 13
2.2. Protein Extraction from Yeast Cells 13
2.2.1. Chemical Lysis Procedure 13
2.2.2. Mechanical Disruption Procedure 14
2.3. Protein Quantification by Bradford Assay 14
2.4. Two Dimensional (2D) Gel Electrophoresis of Yeast Proteins 15
2.4.1. Isoelectric Focusing (IEF) 15
2.4.2. SDS Polyacrylamide Gel Electrophoresis 15
2.5. Mass Spectrometric Analysis of Protein Spots 16
2.5.1. MALDI TOF/TOF Analysis 16

CHAPTER 3. RESULTS AND DISCUSSION 18
3.1. Proteins Extracted by Two Procedures from Yeast Cells 18
3.2. Two-Dimensional Gel Electrophoresis of Yeast Cell Extracts 19
3.3. The Mass Spectrometric Analyses of Selected Spots 21

CHAPTER 4. CONCLUSION 26

REFERENCES 28

LIST OF FIGURES

Figure **Page**

Figure 1.1. The hydroboration reaction between a borane group and an alkene 6
Figure 1.2. The oxidation of an alkyl boron by hydroperoxide anion 6
Figure 1.3. The drawing of a monoester of boric acid 9
Figure 1.4. The drawing of a monoester of borate anion 9
Figure 1.5. The drawing of a borodiester 9
Figure 1.6. Boron complex formation in the active site of a serine protease 10
Figure 1.7. Ribose-containing nucleotide derivatives which make complexes with boron with varying affinities 11
Figure 3.1. Two dimensional gel appearances of proteins from A) Mechanically disrupted cell extract, B) Chemically lysed cell extract 20
Figure 3.2. The spots of proteins selected from the gel of the extract from boron-treated yeast cells 21
Figure 3.3. A representative MS/MS profile of a peptide with a 2160 Dalton parental ion belonging to the Fba 1 protein 23
Figure 3.4. Several adaptive response programs of yeast against variable stresses 25

LIST OF TABLES

Table **Page**

Table 1.1. Boron reserves and production in thousand metric tons estimated in years 2003 and 2004……..……...…5

Table 3.1. Protein concentrations of the yeast cell extracts in µg/ml……...…...18

Table 3.2. The list of gene products identified by the mass analysis of the spots……...22

Table 3.3. The proteins up-regulated in response to boron stress, their descriptions and their fragments recognized by MS/MS analyses…….…...24

CHAPTER 1

INTRODUCTION

1.1. The Chemistry of Boron

Boron is one of the most versatile elements in the periodic table as noted by M. Frederick Hawthorne who is the winner of 2009 Priestly medal bestowed by American Chemical Society. He is recognized by his work on clarifying the chemistry of boron. The accomplishment Hawthorne is most excited about is the creation of nontoxic carborane-containing liposomes that selectively target cancer cells for destruction by Boron Neutron Capture Therapy (BNCT).

In 'boron neutron capture therapy methods' he worked on, short-ranged (5-9 μm) alpha and lithium particles are released from the neutron capture reaction in minor stable isotope of boron: ^{10}B. This kind of therapy was incepted in as early as 1936 by Locher. Boron was incorporated into liposomes which were targeted to tissues to be treated (Hawthorne and Lee 2003). The main requirement for this therapy is selective targeting of especially aggressive tumor cells by sufficient quantities of ^{10}B atom (15-30μg/g or more) and their irradiation by low energy thermal neutrons (Ciofani, et al. 2009). Selective targeting is needed for sparing normal healthy tissues from ablative effects of ionizing radiation due to high linear energy transfer originating from particles created by neutron capture reaction of boron and for reducing the penetrating radiation of conventional radiotherapy (Schuller, et al. 2006). Special boron compounds are available to carry boron to its targets. One of several boron carriers recently tested was boron nitride nanotubes functionalized with a selective tumor targeting ligand, folate. Boron nitride nanotubes are structural analogs of carbon nanotubes with alternating boron and nitrogen atoms substituting for carbon atoms with almost no change in atomic spacing. Boron nanotubes composed of 50% boron atoms are taken into target cells by receptor-mediated endocytosis in this example. This special kind of treatment, although it is yet in trial phases for commercial usage, is a representative of future roles for boron containing therapeutic agents (Hawthorne 1998).

Boron nitride nanotubes can be constructed like carbon nanotubes. Boron can form stable covalently bonded molecular networks similar to carbon. These properties are exploited in making new boron compounds. To see the in-depth picture of boron's relations with organic compounds it is important to consider its unique physicochemical characteristics.

Among the most common naturally occurring or chemically produced boron compounds are sodium tetraborates (anhydrous, pentahydrate or decahydrate) , boric acid, boron oxide, boron sodium oxide tetrahydrate, boron tribromide, boron trifluoride, diborane, decaborane, pentaborane, borane, zinc borate, barium borate, boron phosphate etc. Carboranes are clusters composed of boron and carbon atoms (Plesek 1992). Some carboranes are extremely stable structures of molecular cages. Although boron compounds have been known almost a thousand years long, elemental boron was chemically identified only in 1824 by Jöns Jacob Berzelius after its isolation in 1808 by Humphry Davy, Louis Jacques Thênard and Joseph Louis Gay-Lussac . Pure boron could not be produced until 20.th century. Boron is an element with atomic number of 5 and atomic weight of 10.811 with isotopes ^{10}B and ^{11}B found in nature in 19.78% and 80.22% ratios respectively. Boron does not have a radioactive isotope with a half-life longer than 0.8 s. although it has 13 isotopes in total. Its electron configuration is [He] $2s^2 p^1$.

1.1.1. Unique Physicochemical Properties of Boron

Elemental boron never exists free in nature but is found as boron compounds. Elemental boron is a trivalent metalloid which located as the first element in group 13 (IIIA), period 2, neighboring carbon in the periodic table. Boron locates to the p-block , right hand side of the periodic table , with unfilled p orbitals, with the exception of noble elements, and therefore p orbitals are filled in the reactions.

Metalloids have properties of both metals and non-metals (Chedd 1969). Crystalline boron is a poor electrical conductor at standard temperatures but good conductor at high temperatures, whereas some of the metalloids are good semiconductors. Indeed magnesium diboride, a metal-boron compound, makes a layered material which is superconductor in relatively high temperatures, at 39 degrees

Kelvin (-234°C), one of the highest known transition temperatures (Tc) of any super-conductor.

Other than neutron capturing capacity of its enriched ^{10}B isotope used in BNCT and in the control of radiation in nuclear plants, boron's element and compounds have various significant properties exploited in a wide variety of uses. Depleted isotope ^{11}B confers heat resistance and radiation hardening and is used in borosilicate glasses and rad-hard electronics against the cosmic radiation. Lower lethal (LD50) dose against arthropods, fungi, bacteria and weeds makes them good pesticides or insecticides, antifungal, disinfectant, wood protector and weed killers (Quarles 2001). Borates clean and bleach by converting some water into hydrogen peroxide and by providing a basic (high pH) environment and buffering it. Borax-boric acid is used as flame retardant causing acid catalyzed dehydration reactions in wood to facilitate the formation of char and reduce the effective heat of combustion (LeVan and Tran 1990). In 2004, 4% of boron was consumed for this purpose in USA (Kelly and Survey 2005). Sixty four percent was consumed in the production of fiber glasses in the same year. Boric acid has major markets in textile fiber glass and in cellulose insulator as flame retardant. The boron added to the glass forms covalent bonds with oxygen atoms in the silica network. Borate additives lower melting and forming temperature of glass without increasing the electric conductivity, and gives resistance against thermal expansion.

Although boron is non-magnetic itself, its neodymium-iron-boron alloy is used as light-weight magnets in electronic devices. First commercial levitation magnetic train which makes super high speeds over magnetic field using boron magnets went into service in China in 2004. Boron easily makes alloys by melting at high temperatures with metals making mixtures with properties different from individual components.

Boron compounds function as light structural materials in forms of light-weight, high strength filaments. However crystalline boron, very hard material with a high melting point, has some forms that are somewhat analogous to diamond or carbon crystals. Boron exists in many different forms, known as allotropes which are modifications of an element within the same phase (i.e. different solid, liquid or gas forms). Amorphous boron contains atoms randomly bonded to each other without long range order. Crystals are α or β rhombohedral or tetragonal structures. Boron has several different possible structures because of the 3-bond structure of boron atoms which leads to asymetrical bonds in 3-D space. Metallic/crystalline form of boron transmits infrared light. The metallic form is hard, 9.3 on Mohs' scale, and a bad

conductor of electricity in room temperatures but a good conductor in high temperatures. On the other hand, boron's compounds can also have different forms, called polymorphism at this time. Boron nitride crystals are very hard, second in hardness only to diamond. Like diamond, it is an electrical insulator but excellent conductor of heat. Like graphite form of carbon, boron nitride has lubricating qualities as well, when it is composed of layers of fused hexagonal sheets analogous to graphite.

Nitrogen compensates for boron's deficiency of electron in boron nitrides. Chemically boron is an electrophile and boron compounds behave like Lewis acids. Lewis acids are electron-pair acceptors that react with Lewis bases and share their electron pairs to give Lewis adducts. For example trimethylboron reacts with ammonia to give the adduct Me_3BNH_3.

$$Me_3B + : NH_3 \longleftrightarrow Me_3B: NH_3 \qquad (1.1)$$

Electrophile substances readily bind to electron-rich substances to compensate for electron deficiency. This requirement for electron dominates the reactions of boron. Boron is the least electronegative nonmetal so it is usually oxidized in reactions (loses electrons). Sodium borohydride ($NaBH_4$) is a reducing agent used for reducing aldehydes and ketones into alcohols (Behr 2002). This boron compound is principally used in the production of sodium dithionite which is a bleaching agent for wood pulp. It is decomposed rapidly and exothermically in water especially in acidic pH. Decomposition releases toxic diborane gas and flammable explosive hydrogen gas. If it is handled safely and if hydrogen gas can be produced in controlled amounts, this is one of the candidate energy supplies in place of fossil fuels as 40 kg of this compound corresponds to 60 kg of fuel for the energy yield. The hydrogen can be generated catalytically in this reaction.

$$NaBH_4 + 2\ H_2O \rightarrow NaBO_2 + 4\ H_2\ (\Delta H < 0) \qquad (1.2)$$

1.1.2. Boron's Distribution in Nature

Boron is widespread in rocks, soil and water. However its concentration varies in a large range among different types of rocks. Its concentration is pretty high in ocean,

approximately 4.5mg/L (Woods 1994), especially as compared to other trace elements. Its entrance into the environment takes place by watering of rocks, mining and processing activities of boron ores, boric acid volatilization from seawater, geothermal releases and volcanic activity (Anderson, et al. 1994). Boron's concentrations in soils range from 10 to 300 mg/kg (average 30 mg/kg) depending on the physical and chemical features like pH, salinity, organic content, aluminium oxide, aluminium hydroxy and clay content and type of the soil (Sprague 1972, Biggar and Fireman 1960). The most common forms of boron found in nature are its sodium salts as in borax or boric acid (Woods 1994).

Boron is an essential micronutrient for plants and some vegetables, fruits and nuts contain significant amounts of boron. Dairy products, fish and meat contain much less boron (Nielsen 1991). Although boron is available in every part of the environment, its deposits are distributed among a few countries, making Turkey the most prominent one in reserves and the production all over the world.

Table 1.1. Boron reserves and production in thousand metric tons estimated in years 2003 and 2004. Reserves: Economical resources. Reserve base: All resources (Source: Kelly et al. 2005).

World Production, Reserves, and Reserve Base:	Production—all forms		Reserves	Reserve base
	2003	2004		
United States	1,150	1,130	40,000	80,000
Argentina	545	550	2,000	9,000
Bolivia	34	33	NA	NA
Chile	500	300	NA	NA
China	130	130	25,000	47,000
Iran	3	3	1,000	1,000
Kazakhstan	30	30	NA	NA
Peru	9	7	4,000	22,000
Russia	1,000	1,000	40,000	100,000
Turkey	1,400	1,400	60,000	150,000
World total (rounded)	4,800	4,600	170,000	410,000

1.2. Biologically Relevant Boron

Bioconjugates of boron have been synthesized essentially for targeting sufficient amounts of boron to each cancer cell for the BNCT (Mehta and Lu 1996). They are large, roughly spherical polymers bearing up to over 1000 atoms of boron. This field of

science expands as new stable and more specifically targeted carriers are explored as drug delivery systems.

Organoboron or organoborane compounds are organic derivatives of BH_3, also containing B-O and B-N bonds and they are important in enabling many chemical transformations, like hydroboration, in organic chemistry. A reaction of hydroboration between alkene (below left) and hydroborane (BH_3 or it may be represented by R-BH) (upper left) on the left is shown below.

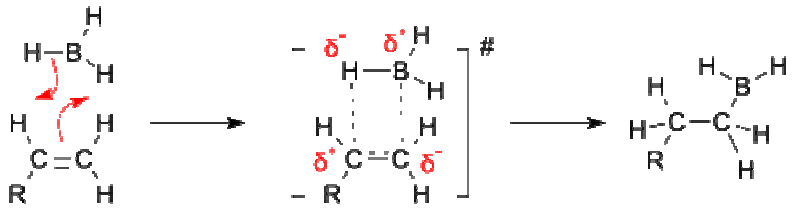

Figure 1.1. The hydroboration reaction between a borane group and an alkene.

The boron group, BH_2, continues adding to more alkenes. The C-B bond has low polarity, therefore alkyl boron compounds are in general stable, but easily oxidized

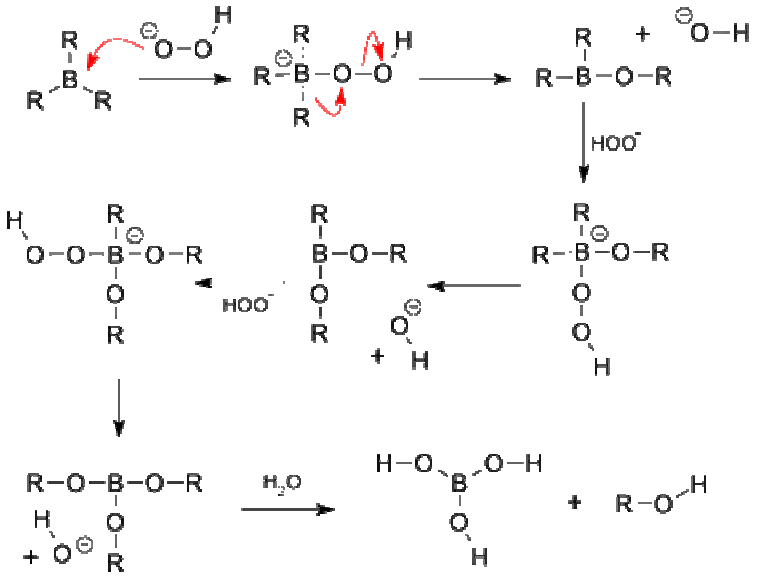

Figure 1.2. The oxidation of an alkyl boron by hydroperoxide anion.

(Noth 2004). In the oxidation step the nucleophilic hydroperoxide anion attacks the boron atom. The resultant molecule is orthoboric acid (common name is boric acid) and a neutral alcohol.

Organoboranes are classified as strong electrophiles. Electrophilic property depends on the molecular environment of the atom with unfilled outer orbitals. Vinyl or aryl groups, when bound to borons, donate electrons to boron and make it less electrophilic and the C-B bond gains a double bond character. Organoboranes with carbon replaced by oxygen are borinic esters, R_2BOR; boronic esters, $RB(OR)_2$; and borates $B(OR)_3$. Organoboron complexes occur in plants and are produced in vitro with biomolecules isolated from animal tissues. Typical physiological concentrations of boron in plants, animals and humans are 0.006 - 9.0 µmol/L. In physiological conditions, organoboron compounds are more often the result of interactions with hydroxyl or amine groups. The most common physiological form of boron compounds is boric acid (H_3BO_3) which is a very weak acid (pKa=9.25). It is the most probable form of dietary boron after ingestion (Hunt 2007). Boric acid accepts a hydroxyl ion (a Lewis acid) to form tetrahedral anion $B(OH)_4^-$ (Greenwood 1973).

$$B(OH)_3 + 2\ H_2O \leftrightarrow H_3O^+ + B(OH)_4^- \quad pKa=9.25\ (25°C) \qquad (1.3)$$

Undissociated (and uncharged) boric acid is very soluble in water. $B(OH)_3$ – saturate solution at 20°C equals 0.75 mol/L. Its permeability coefficient for transport across lipid bilayer is on the same order as urea and several orders of magnitude higher than that of ions. Boric acid and water molecules have similar molecular radii of 2.57 A° and 2.82 A° respectively and it was suggested that boric acid could replace water molecules that hydrate the polar head groups of lipid bilayers in biological membranes (Verstraeten, et al. 2005). It has the capacity of passing through these membranes with passive diffusion.

1.3. The Essentiality and Toxicity of Boron

Boron is believed to have served an essential function since the early evolution of life. It is satisfying the essentiality criteria for at least some members of every phylogenetic kingdom. These criteria involve: 1) Reacting with biological material or

forming chelates; 2) presence in healthy tissues of organisms at comparable concentrations; 3) toxicity occurring only at high intakes; 4) maintenance by homeostatic mechanisms of tissue concentrations during short term variations after intake; 5) prevention of growth and of completion of the life cycle by its depletion; 6) reduction of a physiologically important function resulting consistently from depletion; and; 7) when an integral part of an organic structure, reduction in performance of a vital function caused by depletion of the element.

Since 1923, boron is known to be an essential micronutrient for vascular plants (Warington 1923). Subsequent studies have revealed that boron is an ultra trace element for animals (Rowe and Eckhert 1999; Park, et al. 2004), and boron has also roles in microbial world (Anderson and Jordan 1961; Goldbach, et al. 2001). Boron plays a role in nucleic acid, carbohydrate and protein metabolism, sugar translocation, cell wall structure, membrane integrity and function, optimum growth and pollen germination in plants (Iwai, et al. 2002; O'Neill, et al. 2004; Miwa, et al. 2007). Other than vascular plants, boron is essential for diatoms, marine algal flagellates, heterocystous cyanobacteria, actinomycetes of the genus *Frankia* and bacillus boroniphilus which can tolerate more than 450 mM of boron (Ahmed, et al. 2007). Boron requirement for human is not clear but it was found to be essential for the embryonic development of zebra fish, rainbow trout and frogs (Rowe and Eckhert 1999). On the other hand, boron rich soils decrease crop production in different parts of the world. Different plant species demonstrate variable boron tolerance due to their differences in membrane permeability to boric acid (Hu and Brown 1997). Toxicity of borate has been documented for fish, mice, rats and dogs (Birge and Black 1977; Yazbeck, et al. 2005).

1.4. The Molecular Basis for Boron Effect on Living Organisms

The chemistry of boron favors formation of complexes with a lot of biomolecules. The most common ones are orthoborates, $B(OR)_3$, $(RO)B(OR')_2$, (RO)B(OR')(OR'') and orthoborates of polyhydric alcohols in addition to organoboron compounds including B-N bonds. B-N bond is isoelectronic to C-C bond. All these compounds are the results of borons interacting with OH (hydroxyl) or amine groups. Vast number of mono or polyhydroxy compounds with one or more OH groups in

suitable positions exists in cells to form boroesters. In structure shown below, boric acid forms a monoester with a dihydroxy compound by partial esterification (Hunt 2007).

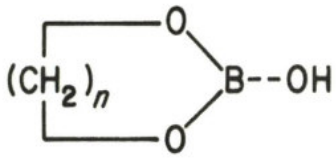

Figure 1.3. The drawing of a monoester of boric acid.

If a borate anion forms a monoester with a suitable dihydroxy compound, the structure shown below is formed again by partial esterification. A similar compound with same charge and monocyclic configuration can be formed by interaction of boric

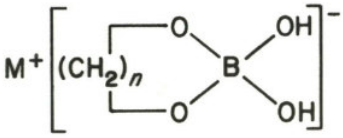

Figure 1.4. The drawing of a monoester of borate anion.

monoester with another OH group. These two types of boromonoesters can react with another dihydroxy compound and form borodiester by complete esterification which is a chelate complex with a tetrahedral configuration and a negative charge.

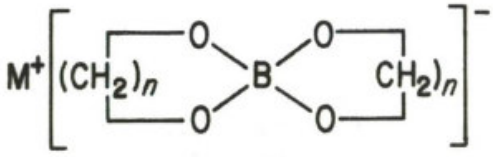

Figure 1.5. The drawing of a borodiester.

Two hydroxyl groups adjacent and on the same side (cis-diol) of the molecule are preferred in the boroester formation. Many of these interactions are rapidly reversible while some are extremely stable that take place in endurable structures like cell walls in plants. Apiose, a pectic polysaccharide with favorable hydroxyl positions makes complexes only with a boron atom to form boroester in dimeric structure of cross-linked rhamnogalacturonan II. Boron is esterified to 2 furanoid sugars (five - member cyclic structures) in spiro-cyclic borodiester structure like that shown above.

Compounds with cis-diols on furanoid rings make stronger bonds with boron than corresponding cis-diols in six-member rings of pyranoid sugar residues. This is suggested to have exerted evolutionary pressure on pyranoid sugars like glucose to be preferentially used in energy metabolism.

Boron can form reversible covalent bonds with the nitrogen atom of amine groups as well. This ability facilitates the formation of boron complexes with a large additional number of biological molecules other than polyols. Active sites of some enzymes affected by boron esterification would create important metabolic outcomes. Nanomolar concentrations of peptide boronic acids and milimolar concentrations of boric acid bind reversibly with the active sites of serine proteases as shown below (for example chymotrypsin). Boron-containing small molecules are utilized, based on the

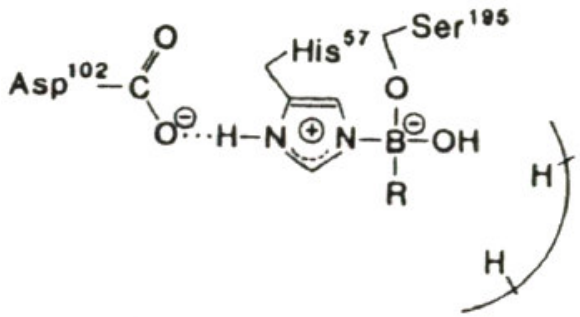

Figure 1.6. Boron complex formation in the active site of a serine protease.
(Source: Hunt C. D. 2007)

same principles (Pang and Martinis 2009). Ribose and its derivatives are in strongly-borate-complexing configuration. A number of ribose-containing nucleotides have been demonstrated to have boron complexes with different affinities. The highest known affinity for boron belongs to S-adenosylmethionine (SAM), a ribose-having methyl-group transfer co-substrate. Members in diadenosine phosphate (Ap_nA) family are next. They are signal nucleotides present in cells with active protein synthesis.

3' end of tRNA has a cis-diol group prone to bind borate which distinguishes it from aminoacyl-tRNA (McCutchan, et al. 1975). Borate-bound tRNA can not bind its aminoacid. Oligonucleotides of DNA show no complex formation with borate in physiological pH values (D'Acunto, et al. 2002). Only 3' ends of riboses from RNA molecules have cis-diol groups to complex with boron.

Oxidoreductase enzymes requiring pyridines of NAD and NADP (Nicotinamide adenine dinucleotide phosphate) or flavin nucleotide are competitively inhibited by borate or its derivatives. Diadenosine phosphate-derived boronated derivatives are used

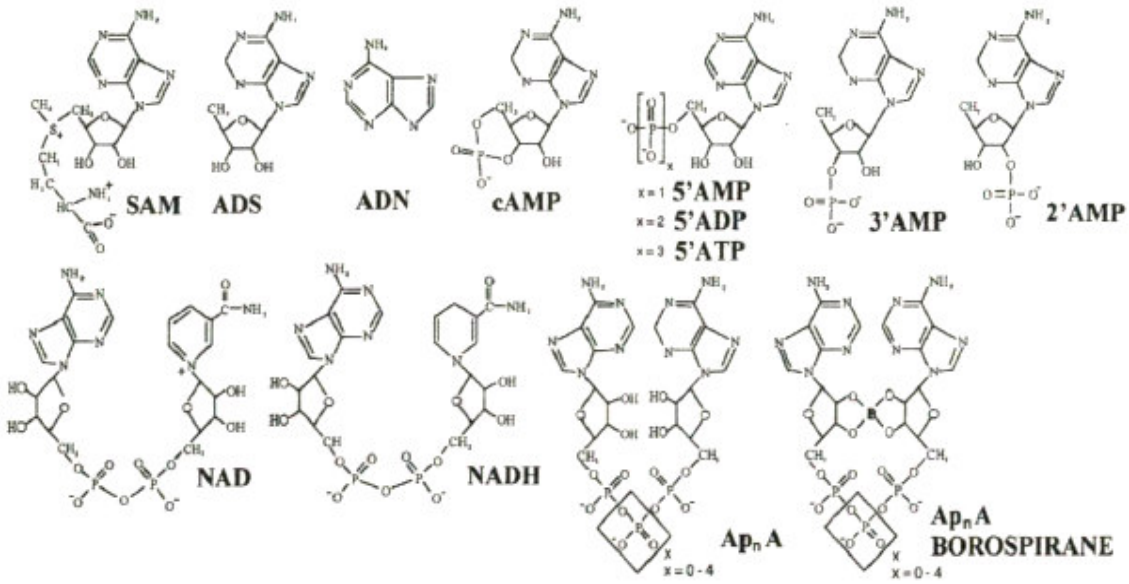

Figure 1.7. Ribose-containing nucleotide derivatives which make complexes with boron with varying affinities. SAM: S-adenosyl methionine. ADS: Adenosine. ADN: Adenine. AMP: Adenosine monophosphate. ADP: Adenosine diphosphate. ATP: Adenosine triphosphate. NAD: Nicotinamide adenine dinucleotide. NADH: Reduced form of NAD. (Source: Hunt C. D. 2007)

to inhibit nucleotide pyrophosphatase/phosphodiesterase enzymes (Eliahu, et al. 2010). Boron is certainly an unusual element which serves as a reversible enzymatic inhibitor, and it is not unreasonable to consider it as a stabilizer of ribose-containing signaling molecules with mirror or near-mirror halves.

There are homeostatic mechanisms that maintain short term variations and stability in tissue concentrations of boron. In humans, for example, milk boron concentration is under homeostatic control. Mean concentrations of milk boron did not change widely between different test populations (29-39 µg/L) and they were kept stable over the first four months of lactation in two reports by Hunt et al (Hunt, et al. 2004; Hunt, et al. 2005). Plasma boron concentrations are kept similarly in a narrow range even in case of excessive boron intake. Large increases in urinary boron despite small changes in plasma reveals an effective gradient-forming mechanism in plasma membranes of kidney. Cells can retain intracellular boron against a concentration gradient due to boron-specific active transporters in membranes or boron binding

molecules inside the cell. NaBC1 is a specific mammalian boron transporter discovered in epithelial cells. BTR1 is mammalian homolog of BOR1 (AtBOR1) boron transporter in Arabidopsis thaliana. Growth of Saccharomyces cerevisiae is induced by boron while keeping its intracellular concentrations of boron almost equivalent, no matter it is cultured in growth media with very low or high boron levels. A kind of homeostatic mechanism must be functioning to benefit from growth-inducing effects of boron while actively keeping its concentration low enough to avoid from deleterious effects. Hence this organism can be used as a model organism in the studies of boron essentiality or tolerance (Bennett, et al. 1999).

Apparent from molecular and in vitro studies that boron participates in both enzymatic and nonenzymatic processes (Schmidt, et al. 2010). Examples of boron-dependent reactions include plant hormone-sensitive NADH oxidase activity (Barr, et al. 1993), and the crosslinking of plant cell wall carbohydrates. Teratogenic effects of boron on embryos of mice might be caused by inhibition of histone deacetylase activity (Di Renzo, et al. 2007). In plants boron may affect metabolic pathways by binding apoplastic proteins to cis-hydroxyl groups of cell walls and membranes (Blevins and Lukaszewski 1998). In yeast, the cell wall integrity signalling triggered by the septation defect caused by irregular assembly of septin scaffold due to boron stimulates, like other threats to the integrity of the cell wall, protein kinase C (PKC) pathway leading to hyperphosphorylation of the PKC downstream effectors. These mechanisms of boron action provides us some insight to understand boron stress response and tolerance.

CHAPTER 2

MATERIALS AND METHODS

2.1. Yeast Growth and Medium

Saccharomyces cerevisiae WT strain, BY4741 (MATα his3 leu2 met15 ura3) was obtained from the yeast deletion library (Invitrogen). YPD rich liquid medium (2% glucose, 2% peptone, 1% yeast extract) was used for yeast cell growth. Cells were cultivated at 30°C in 500 ml Erlenmayer flasks and were harvested from 50ml of culture media containing the same batch of YPD with 50 mmol/L boric acid (in final concentration) or without any boric acid added. The addition of boric acid was approximately one hour before the completion of the growth, so the boron treatment lasted about one hour. Boric acid treated yeast cells were compared with no-treatment cells which were used as controls. Cells were grown up to Ods 1.2-1.5 at 600 nm in each flask corresponding to about 15-20 $x10^8$ cells in total. After harvesting, pellets were washed two times with sterile ultra pure water and once more with TE-PMSF buffer containing 1mM EDTA, 0.1 M Tris, 14 μM PMSF, pH 7,5 and a protease inhibitor cocktail (Sigma P8215) in concentrations recommended by the manufacturer. Afterwards pellets were re-suspended in 4 ml of the same buffer.

2.2. Protein Extraction from Yeast Cells

2.2.1. Chemical Lysis Procedure

One ml of this suspension, containing ca. 4-5$x10^8$ cells was added to 8 ml of the lysis solution, consisting of 0.1M NaOH, 0.05 M EDTA 2% SDS and 2% β-mercaptoethanol, in order to follow the protein extraction procedure described by Tobias von der Haar (Von Der Haar 2007), except that we performed the extraction in larger amounts. Briefly the suspension was incubated at 90^{o}C for 10 min and 170μL of 4M acetic acid was added prior to another round of incubation at the same conditions.

Lysate was cleared by centrifugation (10 minutes, 12000xg at 4°C). SDS-containing buffer was removed by adding 4ml methanol, 1ml chloroform and 3ml of water onto 1 ml of the extract one after another in this order and vortexed each time. It was spun for 5 min. Upper aqueous phase was removed carefully in order not to disrupt the layer of proteins located between the two phases. Three ml of methanol was added, vortexed and centrifuged for 5 min. to obtain the protein pellet. The pellet was dried under vacuum at ambient temperature (SpeedVac) and stored at -20°C.

2.2.2. Mechanical Disruption Procedure

Three ml of re-suspended cells in TE-PMSF buffer were disrupted in 2 ml. of glass beads (0.5 mm) by 4 rounds of vortexing for 30 sec. intervals interrupted with at least 30 sec. of chilling in ice. The cell extract was centrifuged at 12000xg, 4°C for 10 minutes and clear supernatant was used in subsequent steps. It was kept at -20°C till then.

2.3. Protein Quantification by Bradford Assay

Pellets were re-suspended in rehydration buffer before performing Bradford assay for protein quantification and 2D gel electrophoresis. Rehydration buffer was the same as the rehydration reagent described under IEF procedure below except for that it was lacking ampholytes and PMSF. Further purification of proteins was performed using chilled-acetone precipitation by adding 1 ml of cold (-20°C) acetone drop-wise onto 200 µl of sample, incubating at -20°C for 10 minutes and centrifuging at 10000xg for 5 minutes at -4°C. After discarding the supernatant and repeating the procedure once more, the precipitate was dried under vacuum.

Bradford reagent was prepared by dissolving 10 mg of Coomasie Brillant Blue (CBB) G-250 in 95% ethanol and adding 10 ml of 85 % ortho-phosphoric acid. This concentrated dye was diluted to 100 ml with distilled water. Standards of proteins were prepared with bovine serum albumin (BSA). Sample or standard to dye ratio was 1 to 20 and the color was read after 5-30 minutes at 595 nm in colorimeter.

2.4. Two Dimensional (2D) Gel Electrophoresis of Yeast Proteins

2.4.1. Isoelectric Focusing (IEF)

Rehydration reagent was consisting of 8M urea, 2M Thiourea, 1% (w/v) CHAPS, 20 mM DTT, 0.8% ampholyte 3-10, 100 mM Tris/HCl pH 7.5, 1 mM EDTA, and 14µM PMSF. The proteins in amount of 440 µg, determined by the Bradford reagent, were diluted in final volume of 330 µl of rehydration reagent, 300 µl of which was applied onto IPG strips (pH 3-10 non-linear, 17 cm). Rehydration procedure for transferring proteins into the strips was done actively at 50 volts for 15 hours in Biorad IEF system (Biorad Protean IEF Cell) with subsequent isoelectric focusing at 20^{o}C under mineral oil for 56 kVh (0 to 500V 2000Vh, 500V 2000Vh, 500 to 3500V 12kVh, 3500V 40kVh).

Strips were stored afterwards at -80^{o}C, wrapped in stretch film, till the second dimension no longer than 2 weeks.

Prior to SDS (sodium dodecyl sulphate) polyacrylamide gel electrophoresis, strips were equilibrated in equilibration buffers of 1: 6M urea, 0.375 M Tris-HCl pH 8.8, 2% SDS, 20% glycerol with 2% dithiothreitol (DTT) for sulphydryl group reduction for 15 minutes and then with 2.5% iodoacetamide for the alkylation of the reduced sulphydryl groups for another 15 minutes under gentle shaking. DTT and iodoacetamide were freshly added to the equilibration buffer just before the incubation. Strips were dipped into the running buffer for the removal of the excess of DTT and iodoacetamide.

2.4.2. SDS Polyacrylamide Gel Electrophoresis

For electrophoresis, 12% polyacrylamide gels were prepared in original Biorad's gel casting apparatus the day before the running day and stored at 4 ^{o}C overnight. Strips were placed over the gel over which 'overlay agarose' was poured to facilitate the transfer of the proteins into the gel with 16 mA of fixed current run in 'running buffer for electrophoresis' for one hour. Electrophoresis was carried out at 220 volts for about another 6 hours in vertical Biorad electrophoresis system (Biorad Protean II xi cell).

Gels were stained with colloidal Coomassie Brillant Blue. They were neutralized with Tris-phosphate buffer pH 6.5 for 3 minutes, destained in 2% methanol for 2 minutes and fixed in 20% ammonium sulfate no less than 24 hours on a shaker with gentle rotation. They were stored in 5% acetic acid afterwards.

2.5. Mass Spectrometric Analysis of Protein Spots

Spots on the gels that were under the detection limit (50ng) in no treatment gel but with apparent intensity in boron treated sample gels were identified by observing with naked eye. They were revealing the proteins upregulated under boron stress. They were then subjected to mass spectrometric analysis with ION TRAP or MALDI TOF/TOF system (Bruker autoflex III Smartbeam). Ion Trap mass spectrometric analysis was performed outside the institution and MALDI mass spectrometry was performed home.

2.5.1. MALDI TOF/TOF Analysis

Spots were excised in 2-4 mm diameter, divided into smaller pices and subjected to in-gel digestion by a three-day procedure with trypsin incubation lasting overnight (Shevchenko, et al. 1996). Gel pieces were rinsed overnight with the wash solution on the first day. On the second day gel pieces were dehydrated with acetonitrile, reduced and alkylated with DTT and iodoacetamide, dehydrated, rehydrated with ammonium bicarbonate and dehydrated once more followed by rehydration on ice with trypsin solution. Digestion was carried out at 37°C overnight. On the third day peptide digests are extracted with the extraction buffer. A background spot with apparently no protein content was handled in the same way with the protein spots for exclusion of the non-specific peaks arising mostly from autolytic fragments of trypsin.

Matrix was made up of α-cyano-4-cinnamic acid layered on golden laser target plate in two layers with second layer containing peptide fragments concentrated and desalted with ZipTip^{TM}. Spots of peptides layered with matrix onto the target were subjected to MS/MS analysis by TOF/TOF system with reflectron. After first MS analysis, specific peaks with higher intensity were selected and they were sequenced

after collision induced dissociation (CID) using Argon. Data were analyzed with Mascot™ search engine and the proteins were identified.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Proteins Extracted by two Procedures from Yeast Cells

In order to see the effects of boron treatment on proteomic changes in yeast, cells were treated with boric acid and total protein extracts were prepared from the samples. The protein concentrations of the extracts from control yeast cells obtained by chemical procedure were less than 30 % lower than that of mechanical one although approximately 3 times more cells were subjected to the mechanical disruption procedure (Table 3.1). The reason was the incomplete disruption of nearly half of the yeast cells by the mechanical forces exerted by glass beads under vortex mixing. This was mostly due to the endurance of yeasts' cell wall components. This endurance of control cells was apparently diminished in boron treated cells that is why the extract from these cells contained almost 20% more proteins. On the contrary, chemically disrupted boron treated cells contained almost 20% less proteins compared to that of controls.

Table 3.1. Protein concentrations of the yeast cell extracts in µg/ml.

Type of procedure	Boron treated yeast cells	Control yeast cells
Mechanical	2600	2200
Chemical	1250	1600

Boric acid reportedly disturbs cell wall synthesis in baker' yeast by stimulating cell wall integrity response leading to increased chitin synthase 3 activity (Schmidt et al. 2010). On the other hand, general inhibition of translation in response to toxic doses of boron was demonstrated to be mediated by Gcn2p (Uluışık, 2010). Gcn2p binds uncharged tRNAs in order to activate its kinase function normally in the scarcity of amino acids (Dong, et al. 2000). In high concentrations of intracellular boron, on the other hand, 3'end sugar moiety of a tRNA could be occupied by a boric acid or borate

molecule and probably could still hold its capacity to bind Gcn2p. While it was an adaptive response to the scarcity of nutrients carried out to reduce the ATP consumption, general inhibition of translation could be put into play by the unusual element of boron in an absolutely different condition. But this putative mechanism needs to be confirmed by further investigation. Nonetheless, inhibited levels of proteins can be seen in boron treated cells in lower line of table 3.1 when compared to approximately the same number of chemically-lysed control cells. The inhibition of translation has undoubtedly significant outcomes. One of these outcomes might be the insufficient amounts and/or activities of proteins important for the structure of a yeast cell wall. With the increased amount of chitin, impairment of a few enzyme activities which act on the glycosylation of wall structures has been shown to result in the aberrant composition and structure in cell wall like that in boron toxicity (Orlowski, et al. 2007).

3.2. Two-Dimensional Gel Electrophoresis of Yeast Cell Extracts

The yeast cells responded to the high concentrations of boron by stimulating the expression of some kind of proteins while they repressed their general expression affecting most of the proteins. The proteins relatively increased in amounts are quite significant in order to understand the patterns of boron stress response in yeasts and other eukaryotes. Herein the proteins from yeast cell extracts were separated by 2-dimensional gel electrophoresis (Figure 3.1). The first dimension was the separation of proteins according to their isoelectric points (pI), called isoelectric focusing (IEF). Each protein has a specific pI which is prone to change in some posttranslational modifications. Before this step sodium dodecyl sulphate (SDS), an anionic detergent used for the solubilization of hydrophobic proteins, must be removed because IEF is a 'SDS non-compatible' procedure. In addition, proteins from cell extracts obtained by two distinct processes were further purified with acetone to remove contaminating ions which cause horizontal and/or vertical streaks in the gel after staining. This process along with the removal of SDS may give rise to the loss of some proteins. It was evident by the comparison of the gels after second dimension of electrophoresis that the extracts from lysed cells by using NaOH and SDS reveal more spots of proteins (Figure 3.1). This was partly resulting from the use of a detergent to solubilize the membrane

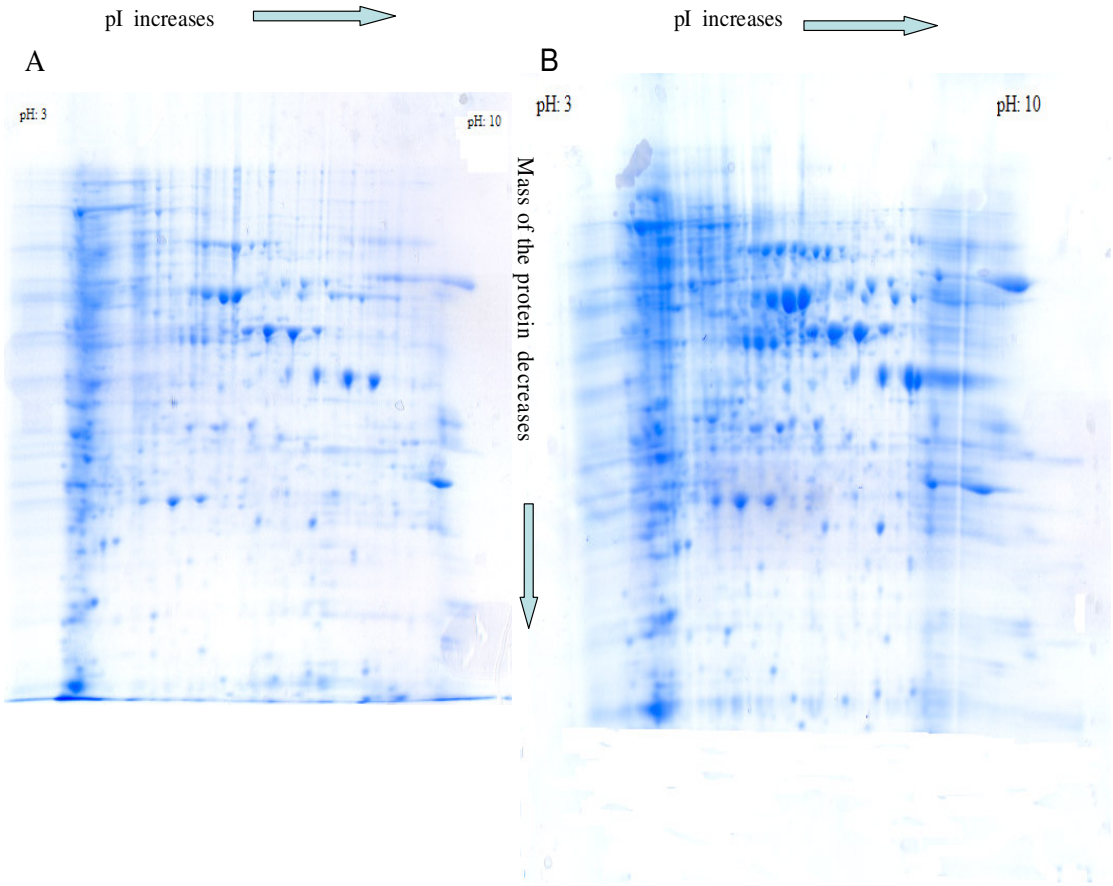

Figure 3.1. Two dimensional gel appearances of proteins from A) Mechanically disrupted cell extract, B) Chemically lysed cell extract.

proteins, although any particular detergent can not solubilize them all (Von Der Haar 2007). Again they may aggregate after the removal of the detergent. The detection limit of any spot stained with colloidal Coomasie Blue is 50 ng. If any spot is observed in only boron-treated cell extract, this does not mean that it doesn't exist in the control gel instead it may imply that it is expressed lower. Fifteen of such spots in the gel of boron-treated cells (Figure 3.2) were selected so as to determine the proteins expressed higher in these cells. The spots were subjected to mass spectrometric analyses after in-gel trypsin digestion. Trypsin cuts the carboxyl terminal side of the lysine or arginine residues unless next residue is proline.

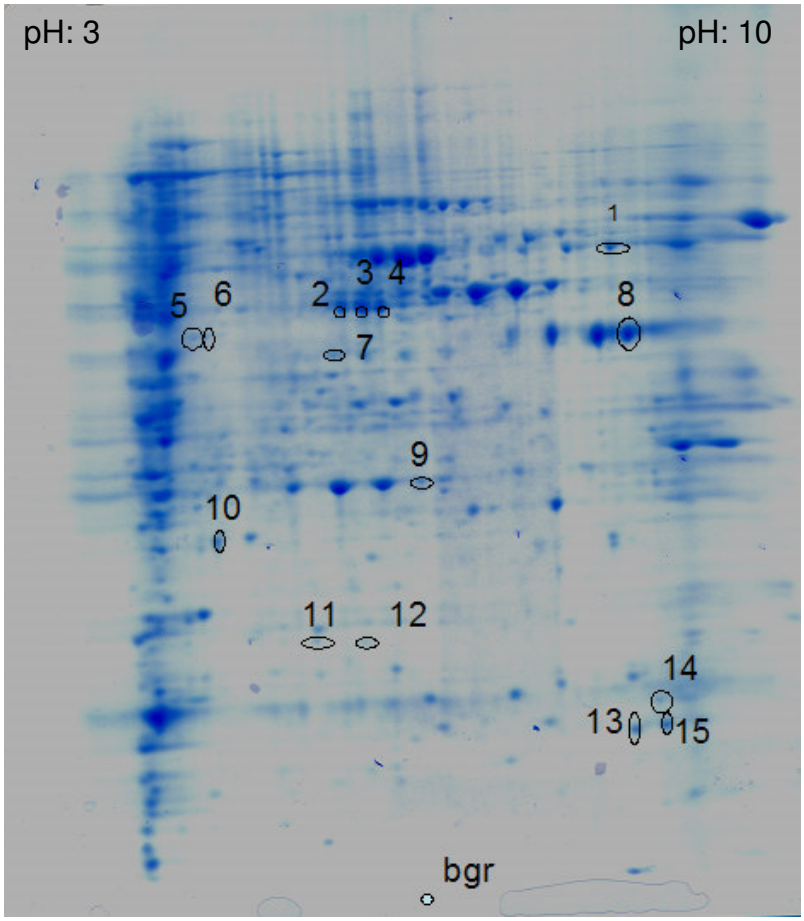

Figure 3.2. The spots of proteins selected from the gel of the extract from boron-treated yeast cells. Bgr indicates the spot selected for the background.

3.3. The Mass Spectrometric Analyses of Selected Spots

The proteins identified by mass spectrometric analyses are listed in table 3.2 and their matched peptide fragments are shown in table 3.3. Eleven out of 15 spots were recognized by the analyses while remaining 4 spots did not give typical dissociation profiles such as shown in figure 3.3 in MS/MS analysis. Nevertheless obtained data were sufficient to indicate that the significant part of the boron stress response involved energy metabolism enzymes and the components of the protein synthesis (table 3.3). Oxidative stress defense mechanisms are likely to be up-regulated too. Interestingly 3 out of 4 up-regulated glycolytic enzymes in our experiment (except for Pgk 1) were reported to be carbonylated under oxidative stress in Saccharomyces cerevisiae

Table 3.2. The list of gene products identified by the mass analysis of the spots.

GI NUMBER	GENE NAME	GENE DESCRIPTION	GROUP OF FUNCTION
6322790	FBA1/YKL060C	Fructose 1,6-bisphosphate aldolase	Energy metabolism
3724 or 6321631	TDH3/YGR192C	G-3 Phosphate dehydrogenase	Energy metabolism
4180	CDC19/YAL038w	Pyruvate kinase	Energy metabolism
1633520 or 10383781 or 13786936	PGK1/YCR012W	3-Phosphoglycerate kinase	Energy metabolism
6323613	TSA1/YML028W	Thioredoxin peroxidase	Redox homeostasis
6321574	PRE9/YGR135W	Alpha-3 subunit of the 20S proteasome	Protein catabolic process
119180 or 6323278	HEF3/YNL014W	Translational elongation factor EF-3	Protein synthesis
6325126	RPL5/YPL131W	Large subunit ribosomal protein L5e	Protein synthesis
6323391	ADE13/YLR359W	Adenylosuccinate lyase	Purine nucleotide synthesis
6320242	KRS1/YDR037W	Lysyl-tRNA synthetase	Protein synthesis
533365 or 6325151	SSE1/YPL106C	ATPase, component of Hsp 90	Protein folding

(Kim, et al. 2009). Carbonylation is an irreversible reaction that makes the proteins more prone to be degraded.

Another up-regulated mechanism seems to be the unfolded protein response. In addition, the induction of a purine and a tRNA synthesis enzyme against boron stress is also noteworthy. The involvement of carbohydrate metabolic enzymes in metal stress response is very common along with thioredoxin peroxidase in yeast (Yao 2003; Yin, et al. 2009). Cadmium stress up-regulates Krs1 and other amino acid metabolism enzymes like many other proteins common to boron stress in our example. Peroxide stress leads to similar protein folding, redox regulation and translational effects in Candida albicans but similarity is not evident in carbohydrate metabolism (Kusch, et al. 2007). On the other hand, Fba1p and a subunit of G-3 phosphate dehydrogenase enzyme were shown to bind boron in some plants (Wimmer, et al. 2009). It is not clear that boron-binding features of these proteins are common to other organisms or if they bind any other metals.

Yin et al. determined 6 core proteins responsive to all of cadmium, salt and peroxide stresses in C. albicans (Yin, et al. 2009). One of these was a translational elongation factor which may explain the divergence of transcriptomic results from those of proteomics in these experiments. This indicates that a significant part of control in

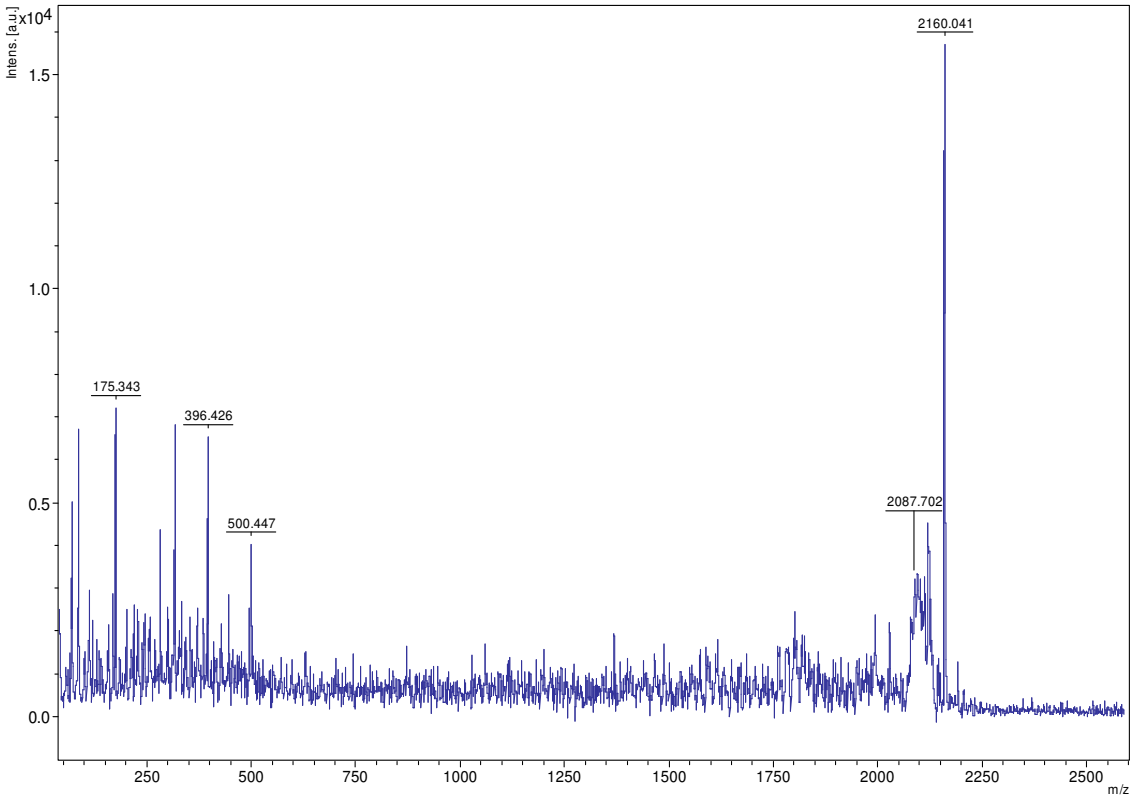

Figure 3.3. A representative MS/MS profile of a peptide with a 2160 Dalton parental ion belonging to the Fba 1 protein.

gene expression takes place on the level of translation in several stress conditions. In the case of an herbicide stress however, the yeast responds by stimulating the amino acid and nucleotide synthesizing enzymes without any sign of a translational inhibition (Teixeira, Santos et al. 2005). This response is likely to follow the depletion of the amino acid deposits in the vacuoles of these cells. In boron treated cells, tRNAs could be lacking their specific amino acids without any depletion of amino acid deposits. It is not clear whether this mode of action applies to metal stress, oxidative stress or even salt stress in yeast.

Table 3.3. The proteins up-regulated in response to boron stress, their descriptions and their fragments recognized by MS/MS analyses.

NAME OF THE PROTEIN	MATCHED PROTEIN SEQUENCES (all are not shown)	DESCRIPTIONS IN SGD
Fructose1,6-bisphosphate aldolase	**51**ARDSKSPIILQTSNGGAAYFAGKGIS NEGQNASIK**GAIAA AHYIR**SIAPA	Required for glycolysis and gluconeogenesis; catalyzes conversion of fructose 1,6 bisphosphate to glyceralde-hyde-3-P and dihydroxy-acetone-P; locates to mitochondrial outer surface upon oxidative stress
Glyceraldehyde-3Phosphate dehydrogenase	**301**LSPKFVK**LVSWYDNEYGYSTR**VVD	Involved in glycolysis and gluconeogenesis; tetramer that catalyzes the reaction of glyceraldehyde-3-phosphate to 1,3 bis-phosphoglycerate; detected in the cytoplasm and cell wall
Pyruvate kinase	I IYVDDGVLSFQVLEVVDDK EFGILK	Functions as a homotetramer in glycolysis to convert phosphoenol-pyruvate to pyruvate, the input for aerobic (TCA cycle) or anaerobic (glucose fermentation) respiration
3-Phosphoglycerate kinase	HELSSLADVYINDAFGTAHR AGAEIVPK	Catalyzes transfer of high-energy phosphoryl groups from the acyl phosphate of 1,3-bisphosphoglyce-rate to ADP to produce ATP; key enzyme in glycolysis and gluco-neogenesis
Thioredoxin peroxidase	**101**LADTNHSLSR**DYGVLIEEEGVALR**GL	Acts as both a ribosome-associated and free cytoplasmic antioxidant; self-associates to form a high-molecular weight chaperone complex under oxidative stress
Alpha-3 subunit of the 20S proteasome	KVTSTLLEQDTSTEK IHAQNYLK	The only nonessential 20S subunit; may be replaced by the alpha 4 subunit (Pre6p) under stress conditions to create a more active pro-teasomal isoform
Translational elongation factor EF-3	MPELIPVLSETMWDTK KLSSAELR	Stimulates EF-1 alpha-dependent binding of aminoacyl-tRNA by the ribosome
Large subunit ribosomal protein L5e	GYLADDIDADSLEDIYTSAHEAIR	Protein component of the large (60S) ribosomal subunit with similarity to E. coli L18 and rat L5 ribosomal proteins; binds 5S rRNA and is required for 60S subunit assembly

(cont. on next page)

Table 3.3 (cont.)

Adenylosuccinate lyase	HLGSLFSDAVQTASVQWFER	Catalyzes two steps in the 'de novo' purine nucleotide biosynthetic pathway; expression is represssed by adenine and activated by Bas1p and Pho2p
Lysyl-tRNA synthetase	AAAEGVANLHLDEATGEMVSK	The aminoacyl-tRNA synthetase specific for lysine
ATPase, component of Hsp 90	I IGLDYHHPDFEQESK LVAETEDR	ATPase that is a component of the heat shock protein Hsp90 chaperone complex; binds unfolded proteins; member of the heat shock protein 70 (HSP70) family

The yeast has several adaptive response programs against stress induced by variable factors (Teixeira, et al. 2011) reviewed in figure 3.4. Proteomic studies reveal the complexity arising from the intensive interplay between these distinctive programs.

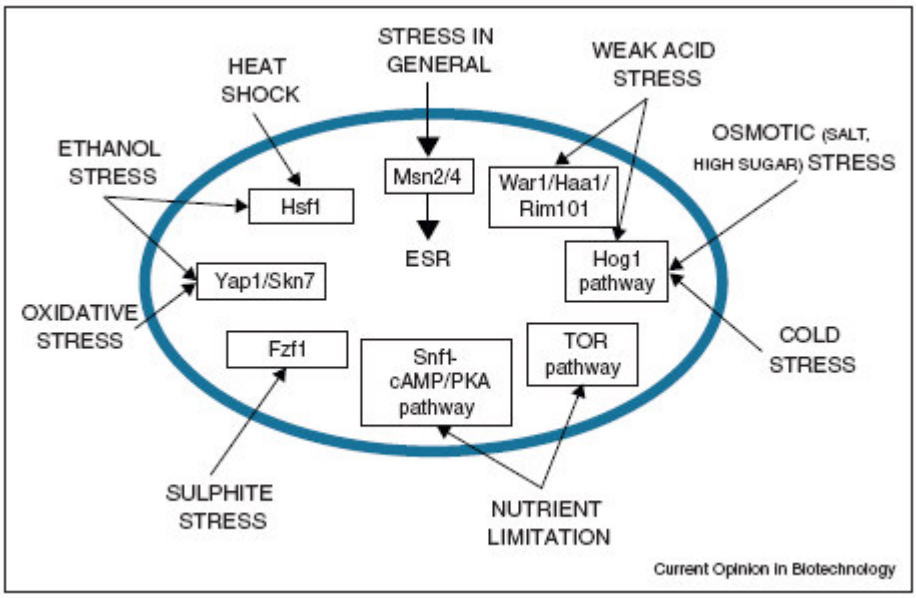

Figure 3.4. Several adaptive response programs of yeast against variable stresses. (Source: Teixeira, et al. 2011)

CHAPTER 4

CONCLUSION

The molecular interactions of boron compounds with metabolically important organic molecules are well documented. However it is still not clear why boron is essential for some plants and some other organisms. Although boron is required for the structure of apiose-containing cell walls, the importance of many other speculative enzymatic or non-enzymatic interactions remains to be demonstrated. Boron is under an efficient homeostatic control inside the cell. Its intracellular concentrations are mainly controlled by efflux pumps located in cell membranes. Boron may also be sequestered in limited locations where it is mostly needed or deposited like calcium in the cell. Recently its relevance to the signal transduction between outside and inside the cell is studied more intensively.

In the case of failure of the homeostatic mechanisms, when the concentration of boron in the environment of the cell exceeds some point, toxicity takes place. Up to that point cells respond the boron stress, as they do against other kinds of stresses, with distinctive sequence of events. In microarray data, yeast cells respond to the boron stress by inducing the transcription of mainly amino acid biosynthesis genes in a GCN4-dependent manner and by activating the transporter genes. ATR1 gene is the most prominent activated transporter gene which is known to be induced by transcription factors Yap1 and Gcn4. Yap1 plays a central role in activation of the oxidative stress response genes.

There are several proteome studies investigating oxidative and other stress-response mechanisms in yeasts. The proteome of the boron stress response is poorly investigated. Our study in this regard presents some information in addition to the transcriptomic data. Proteome studies have some challenges that can be overcome only by applying several different mass spectrometric approaches. One of these challenges is the wide dynamic range of protein concentrations, the other one is the solubility problems of especially membrane proteins. Nevertheless our data indicate that boron stress response in proteome resembles that of metal stress responses although it has some similarities with oxidative stress and herbicide stress responses. Another

indication is that protein synthesis is largely influenced which explains the discrepancy between the transcriptome and the proteome data obtained in cells under these kinds of stresses. Data from 2-D gel electrophoresis must be supported and enriched by LC/MS shotgun approaches in mass analyses.

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