INVESTIGATION OF HEAT STRESS-INDUCED PROTEINS OF COLD-ADAPTED *Pseudomonas marginalis* USING PROTEOMIC APPROACH

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ABSTRACT

INVESTIGATION OF HEAT STRESS-INDUCED PROTEINS OF COLD-ADAPTED *Pseudomonas marginalis* USING PROTEOMIC APPROACH

Temperature alteration is known as a common environmental stress condition which all living organisms encounter and response by producing evolutionary well-conserved specific proteins called heat stress or heat shock proteins in the cell in order to adapt and survive. In the current study, the induction of heat stress proteins in a cold-adapted bacterial strain of *Pseudomonas marginalis* cells grown under heat stress was investigated by proteomic approach. Five different temperatures, 5, 10, 15, 24, and 30˚C, were examined for the purpose of determining the optimum growth temperature for the bacterium. Consequently, 15˚C was observed as optimum temperature for growth while 30˚C was established as heat stress temperature. Total proteins from *Pseudomonas marginalis* cells in the late exponential phase of growth at these two temperatures were extracted and separated by two-dimensional polyacrylamide gel electrophoresis. Totally 1391 protein spots were visualized for 15˚C and 1384 protein spots for 30˚C. After comparing with 15˚C, 13 protein spots that were differentially expressed in the cells exposed to heat stress (30˚C) were cut from the gel and fragmented into their peptides by in-gel digestion method. Finally, these proteins were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and database searching. Among them, ribosome recycling factor, universal stress protein family and chaperonin GroEL were established as direct sensors of heat stress. As a result, the genes encoding these two heat stress proteins can be isolated and cloned into any other useful microorganism such as bacteria used for detoxification of industrial waste or used in bioremediation but not capable of surviving at high temperatures so that they can be efficient at those temperatures, too.

ÖZET

SOĞUĞA UYUM SAĞLAMIŞ *Pseudomonas marginalis*'in ISI ETKİSİYLE TETİKLENEN PROTEİNLERİNİN PROTEOMİK YAKLAŞIMLA ARAŞTIRILMASI

Sıcaklık değişimi tüm canlı organizmaların karşılaştığı ve uyum sağlayıp yaşamlarını sürdürmeleri için ısı stresi ya da ısı şoku proteinleri olarak adlandırılan, evrimsel süreçte iyi korunmuş özel proteinleri üreterek karşılık verdiği yaygın çevresel bir stres koşuludur. Bu çalışmada, soğuğa uyum sağlamış *Pseudomonas marginalis* bakteri ırkı hücrelerindeki ısı stresi proteinlerinin tetiklenmesi proteomik yaklaşımla araştırılmıştır. Bakteri için en elverişli büyüme sıcaklığının belirlenmesi amacıyla 5, 10, 15, 24 ve 30˚C olmak üzere beş farklı sıcaklık test edilmiştir. Bunun sonucunda, 15˚C en elverişli büyüme sıcaklığı olarak gözlemlenirken 30˚C ısı stresi sıcaklığı olarak saptanmıştır. Büyümenin geç logaritmik evresindeki *Pseudomonas marginalis* hücrelerindeki toplam protein izole edilmiş ve iki boyutlu poliakrilamit jel elektroforezi ile ayrıştırılmıştır. 15˚C için 1391, 30˚C için 1384 protein spotu görüntülenmiştir. Kontrol örneği (15˚C) ile karşılaştırıldığında, ısı stresine (30˚C) maruz bırakılmış hücrelerdeki 13 farklı protein spotu jelden kesilmiş ve jel içinde parçalama metodu ile peptitlerine parçalanmıştır. Son olarak, bu proteinler sıvı kromatografisi-tandem kütle spektrometresi (LC-MS/MS) ve veritabanı taraması ile tanımlanmıştır. Bunların arasından ribozom geridönüşüm faktörü, evrensel stres protein ailesi ve şaperonin GroEL, ısı stresinin doğrudan algılayıcıları olarak tespit edilmiştir. Sonuç olarak, bu iki ısı stresi proteinini kodlayan genler izole edilip, endüstriyel atıkların zehirli etkilerinin giderilmesinde ya da biyoremidasyonda kullanılan fakat yüksek sıcaklıklarda yaşayamayan bakteriler gibi yarar sağlayacak herhangi bir mikroorganizmaya klonlanarak bu sıcaklıklarda da etkin olmaları sağlanabilecektir.

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CHAPTER 1

INTRODUCTION TO HEAT STRESS

1.1. General Stress Response

All living microorganisms usually encounter quickly changing environmental conditions such as temperature alterations, nutrient and water availability, presence of toxic materials in nature. For the survival of all microorganisms, it is essential to sense and then adapt rapidly to these changes. The general response to stressful environmental conditions is changing the patterns of gene expression for the induction of specific proteins reducing damage in the cell so that the stress can be compensated. These adaptive responses occur very fast to enable the survival in extreme and rapidly changing environments.

Alterations in gene expression reasoned by environmental stress are frequently regulated at the level of transcription and translation or by post-translational modifications. Transcriptional level activation of genes is controlled by variations in the interactions between RNA polymerase and gene regulators. As a result of these associations, new gene products, stress proteins actually, are synthesized in order to overcome a certain physico-chemical stress. In other words, activation of these genes results in more resistive microorganisms to environmental stresses.

1.2. Heat Stress Response

Temperature change, a critical environmental factor, is known as the most common stress to which organisms are exposed in nature. To cope with critical conditions like protein unfolding caused by extreme temperatures, living organisms have developed a wide spectrum of complicated fundamental mechanisms which are known as heat shock responses. These are protective and homeostatic responses to prevent the cellular systems from potential stress-induced damage in proteins. In the course of stress period, cellular activities are reprogrammed quickly to secure survival. Consequence of these adaptive responses to heat stress is an enhanced expression of

heat shock genes, which in turn encode a set of specific proteins called heat shock proteins (Hsps).

1.2.1. Heat Shock Proteins (Hsps)

Hsps are a super family of evolutionary well conserved, ubiquitous proteins existing in a large variety of organisms (Lindquist and Craig 1988). These specific proteins have a significant role in the cell to control the stress response and protect organisms from destructive stress conditions. History of Hsps began with a study by Ritossa in 1962 that a short heat exposure of *Drosophila* larvae induced some new alterations in gene activity (Ritossa 1962). Subsequently, Tissières and his colleagues first reported the existence of Hsps in *Drosophila* (Tissières, et al. 1974). Unfortunately, it took many years to understand the main features of heat shock response for various organisms. In the following years, induction of these proteins was demonstrated in many cellular systems involving bacteria (O'Farrell 1975), yeast (Sanchez and Lindquist 1990), insects (Michaud, et al. 1997), worms (Rose and Rankin 2001), and mammals (Srivastava 2002).

In the early days of heat shock response studies, it was supposed that overexpression of Hsps is induced by elevated temperatures only. Later on, however, it was found that a wide variety of other environmental stress conditions such as exposure of cells to heavy metals, high salt, hypoxia, starvation, ethanol, and sodium arsenite can also trigger the synthesis of Hsps (Nover 1991). The common consequence of all these conditions is the accumulation of denatured, abnormally folded proteins. This unusual behaviour is detected by cellular systems which then induce the synthesis of Hsps to overcome the physical and chemical changes in the cell.

An interesting feature of Hsps is that some particular classes of these proteins have been highly conserved in all organisms throughout the evolution. For instance, there exists a 72% homology between 70 kDa-Hsps of yeast and *Drosophila* (Ingolia, et al. 1982) and 63% amino acid identity between their 84 kDa-HSPs (Hackett and Lis 1983, Farrelly and Finkelstein 1984). Also it was reported that 70 kDa-Hsp gene of *Drosophila* and human has 73% homology (Hunt and Morimoto 1985). This high level of conservation is said to prove the involvement of Hsps in a variety of fundamental cell processes, covering correct folding of newly synthesized proteins, protein translocation,

protein degradation, and cell signaling (Hartl 1996). In the view of these unique functions, heat shock response has become a determining factor for exploring gene regulation (Neidhardt, et al. 1984, Morimoto, et al. 1994). Therefore, Hsps have gained acceptance for being used as markers of cellular injury, and potential for diagnostic & therapeutic purposes. Moreover, they play important roles in human immune system (Christians, et al. 2002).

Heat shock proteins have been classified into five major groups based on their obvious molecular masses, which are Hsp100, Hsp90, Hsp70, Hsp60, and small Hsps (sHsps) (Table 1.1).

Table 1.1. Predominant Families of Heat Shock Proteins
(Source: Moseley 1997)

Class	Size range (kDa)	Intracellular Localization	Main Functions
Hsp100	100-110	Cytosol, nucleus	Protein folding, thermo tolerance
Hsp90	85-90	Cytosol, nucleus, endoplasmic reticulum	Protein translocation, receptor regulation
Hsp70	68-74	Cytosol, nucleus, endoplasmic reticulum, mitochondria	Protein folding and translocation, thermotolerance
Hsp60	60	Mitochondria	Protein assembly
Small Hsps	< 40	Cytosol, nucleus	Stabilization of microfilaments, cytokine signal transduction

1.2.2. Molecular Chaperones

Molecular chaperones are simply described as a specific group of protein family that assists the non-covalent folding/unfolding and assembly of other proteins under both optimal and stress growth conditions. The term molecular chaperone was first used by Laskey and co-workers in 1978 (Laskey, et al. 1978) to find the properties of an acidic nuclear protein, nucleoplasmin. These distinctive proteins play important roles in protein folding, translocation and degradation in ordinary cellular processes while they are also responsible for stabilization of proteins throughout assisting protein refolding under adverse conditions. They are located in almost every compartment of the cells. In addition, their highly conserved nature in evolution makes them essential for all life forms.

Aside from these main characteristics, molecular chaperone concept is proved to be directly related with heat stress response because it has been observed that the need for molecular chaperones increases if proteins are exposed to extreme temperatures. For the cell to survive stress, it is necessary to refold damaged proteins correctly or degrade them by proteolysis. At the same time, proteins with no damage need to be prevented from stress conditions leading to incorrect assemblies. In this respect, a number of molecular chaperones are defined as heat shock proteins which repair the potential damage of protein misfolding reasoned by heat stress. Therefore, these chaperones are named by the nomenclature of Hsps (i.e., Cpn60, Cpn70.). The heat shock proteins having chaperone function are shown in Table 1.2.

Table 1.2. Heat Shock Proteins and their Bacterial Homologues

Protein	Bacterial homolog	MW (kDa)	Theoretical pI	Reference
Hsp100	ClpB	96	5.37	Kitagawa, et al. 1991
Hsp40	DnaJ	39	7.98	Bardwell, et al. 1986
Hsp70	DnaK	69	4.83	Bardwell and Craig 1984
Hsp60	GroEL	57	4.85	Neidhardt, et al. 1981
Hsp10	GroES	10	5.15	Tilly, et al. 1983a
Hsp15	HsIR	15	9.94	Chuang and Blattner 1993
Hsp33	HsIO	33	4.65	Chuang and Blattner 1993
Hsp90	HtpG	71	5.09	Bardwell and Craig 1987
sHsps	IbpA	16	5.57	Allen, et al. 1992
sHsps	IbpB	16	5.19	Allen, et al. 1992

Although heat shock proteins comprehend the largest family of molecular chaperones, it must be noted that not all chaperones are heat shock proteins, and conversely, not all heat shock proteins act as chaperones.

1.2.3. Regulation of the Heat Shock Response

As indicated previously, the heat shock proteins and their functions are successfully well conserved in evolution. However, the control mechanisms of their expression significantly differ from an organism to another. Among the microorganisms, regulation of the heat shock response is best studied in Gram-negative bacteria *Escherichia coli* at which the control element responsible for the regulation of heat shock proteins is a heat shock sigma factor σ^{32}. This sigma factor is encoded by rpoH gene, and also it is the subunit of RNA polymerase (RNAP). Under stressful conditions the level of its synthesis and stability increases, which in turn induces the heat shock response (Arsène, et al. 2000). Production of DnaK-DnaJ-GrpE chaperone machinery activates the feedback system by binding to σ^{32} and inhibiting its activity (Tomoyasu, et al. 1998). Besides, binding of DnaK-DnaJ assists the degradation of σ^{32} by the ATP dependent metalloprotease, FtsH (Tatsuta, et al. 1998). Thus, DnaK-DnaJ machinery serves as a typical sensor of cellular stress and a regulator of heat shock transcription (Tomoyasu, et al. 1998).

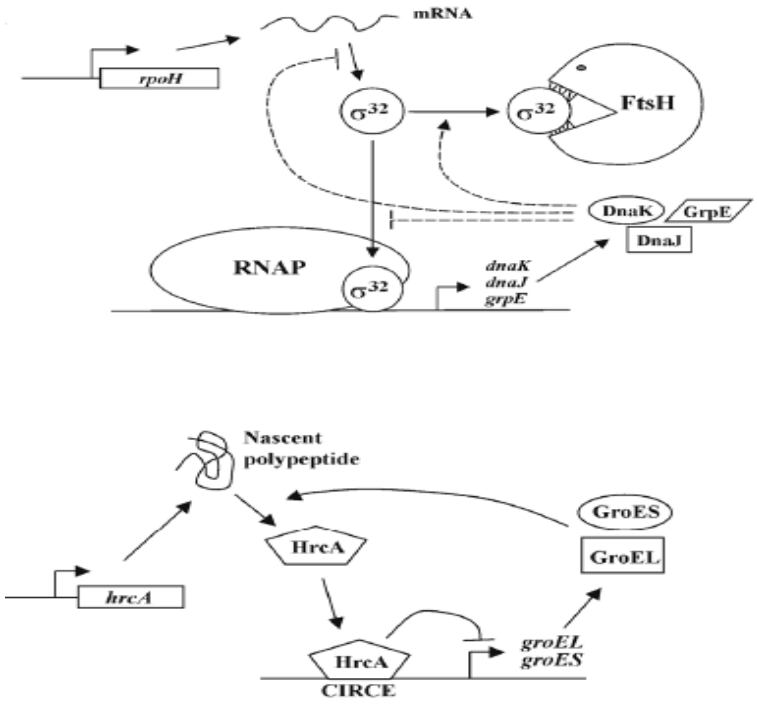


Figure 1.1. Regulatory Mechanisms of the Prokaryotic Heat Shock Response
(Source: Makarow and Braakman 2006)

Alternative to the sigma factor-induced regulation of the heat shock response, there exist another regulatory mechanisms that are controlled by transcriptional repressors. Of these repressors, HrcA is the most common one which regulates the heat shock response by binding to conserved inverted repeat DNA sequence (CIRCE). In this mechanism, feedback regulation is enabled by GroEL-GroES complex that promotes the folding of HrcA (Yura and Nagahigashi 1999).

CHAPTER 2

PROTEOMICS AND MASS SPECTROMETRY

2.1. Proteomics

Proteomics, an abbreviation of the words protein and genomics (Wilkins, et al. 1996), is an advancing branch of molecular biology that deals with the systematic, large-scale analysis of proteins. The principle underlying the proteomics is the notion of proteome, protein content of the genome, as a complete set of proteins produced by a given cell or organism under a defined set of conditions (Ashcroft 2003). Proteins play major roles in almost every biological function, so an extensive study of the proteins in the cell contributes a unique global perspective on how these molecules interact and cooperate to create and protect a working biological system. The cell corresponds to internal and external effects by adjusting the level and activity of its proteins, so changes in the proteome, either qualitative or quantitative, provide a snapshot of the cell in action. The proteome is a complex and dynamic entity that can be defined in terms of the sequence, structure, abundance, localization, modification, interaction and biological function of its components, providing a rich and varied source of data. An equally diverse range of technologies are required for the analysis of these various properties of the proteome (Twyman 2004).

The terms ‘’proteomics’’ and ‘’proteome’’ were first used by Marc Wilkins and colleagues in the early 1990s and reflect the terms ‘’genomics’’ and ‘’genome’’, which describe the entire collection of genes in an organism (Liebler 2002). These ‘’-omics’’ terms represent a redefinition of how to approach to biology and the workings of living systems (Figure 2.1).

Proteome analyses are accompanied by two-dimensional gel electrophoresis (2DE) for separation of proteins followed by protein identification by mass spectrometry (MS) and database searches. Due to the introduction of soft ionization methods to mass spectrometry like matrix assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) together with developments in sample preparation techniques and rapid increase of sequential information in databases, mass spectrometry has admitted of protein identification and characterization during the last decade

(Nyman 2001, Nägele, et al. 2003, Ashcroft 2003). On this basis, proteomics offers a very effective tool for analysis of hundreds of proteins expressed in a complex mixture at one time as different samples of bacteria cells (Antelmann, et al. 2004, Hecker and Volker 2004), yeast cells (Kolkman, et al. 2005), and plant cells (Bak-Jensen, et al. 2004). Contrary to plant, animal and yeast analyses, very few studies on filamentous fungi have been reported (Hofmann, et al. 2003, Nandakumar and Marten 2002.). Another important reason for protein analysis by MS is the rapid increase of sequential information in databases.

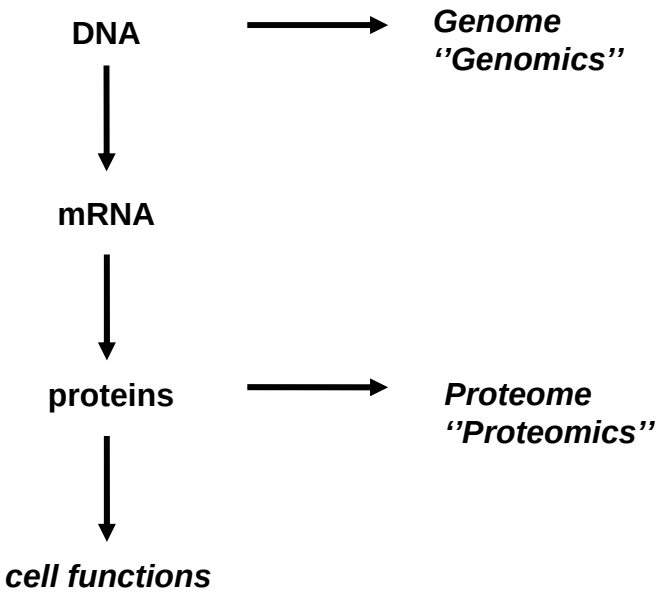


Figure 2.1. Biochemical Context of Genomics and Proteomics
(Source: Liebler 2002)

2.1.1. Need for Proteomics

Apart from classical understanding of proteomics, there is also more comprehensive approach that combines protein studies with genetic analysis such as mRNA analysis and genomics. In the view of this fact, many different areas of study are now grouped under the title of proteomics (Figure 2.2). These cover protein-protein interaction studies, protein modifications, protein function, and protein localization studies to name a few. Proteomics aims at not only the identification of all the proteins

in a cell but also creating a complete three-dimensional (3-D) map of the cell indicating the location of proteins which in turn requires the coordination of a large variety of disciplines like molecular biology, biochemistry, and bioinformatics (Graves and Haystead 2002).

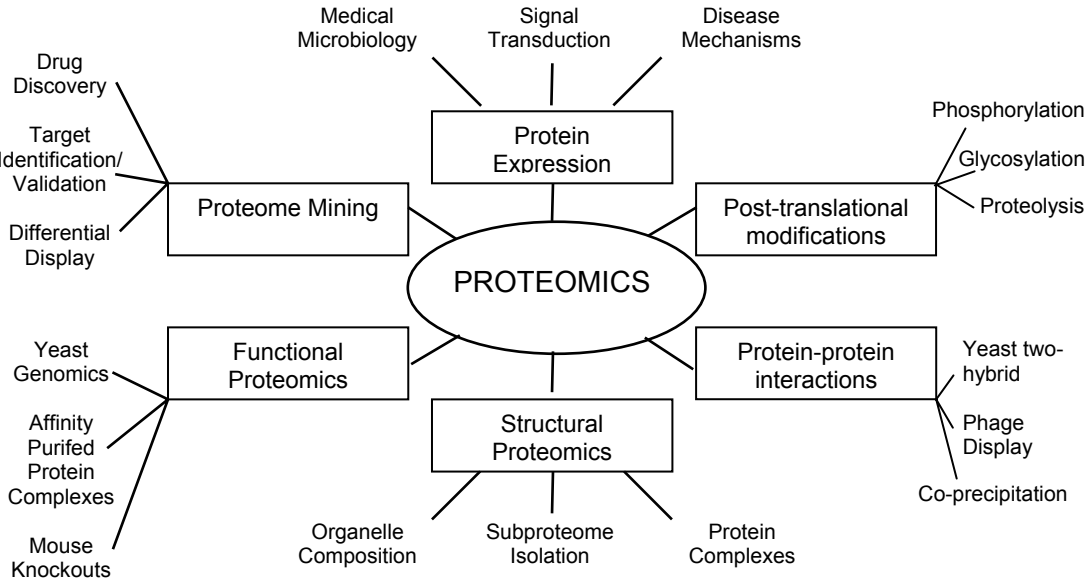


Figure 2.2. Types of Proteomics and their Application to Biology
(Source: Graves and Haystead 2002)

In comparison with transcriptome analysis, where the DNA-microarray technology is completely automated, proteome analysis demands manual work and bring about problems with 2DE. However, the basis of DNA-microarray technology is to measure steady-state mRNA levels whereas proteomics technology deals with proteins which are the active agents in cells. The direct measurement of protein levels and activity within the cell is the best determinant of overall cellular functions when it is compared to DNA and RNA (Seibert, et al. 2004, Nyman 2001). In addition, levels of mRNA do not define the current state of a living cell enough on account of the large variety of posttranslational modifications of proteins (PTMs) and mRNA splicing. The formation of mRNA is only the first step in a long sequence of events resulting in the synthesis of protein (Figure 2.3). Therefore, mRNA expression does not always correlate with corresponding protein level and is not necessarily a confidential marker of protein abundance (Nägele, et al. 2003, Wolff, et al. 2007, Kaczmarek, et al. 2002, Guina, et al. 2003).

Corresponding to the characterization of the proteome of a given cell or organism, it should be recognized that the proteome is dynamic as indicated in the previous section. The proteome of a cell will mirror the instant effect in the environment. Therefore, investigation of the proteome of a cell is like taking a ‘’snapshot’’ of the protein level at any given time (Graves and Haystead 2002).

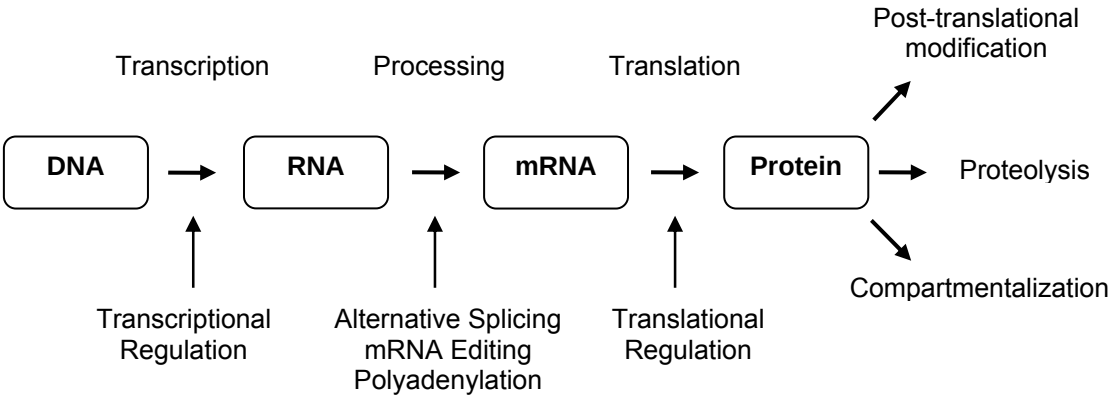


Figure 2.3. Mechanisms by which a Single Gene can give rise to Multiple Gene Products (Source: Graves and Haystead 2002)

2.1.2. Overview of Proteomics Methodology

Analytical protein identification is based on one essential fact: most peptide sequences of at least six or more amino acids are large enough to be successful in the proteome analysis. In other words, a typical six amino acid peptide maps to a single gene product. So if the sequence of the peptide can be obtained or its mass can accurately be measured, it will be possible to identify the protein using database of protein sequences (Liebler 2002).

Figure 2.4 describes the essential steps of the analytical proteomics approach. In the beginning, it is necessary to separate the protein mixture into proteins or digest it into peptide mixture in order to obtain peptides owing to the fact that peptides are sensitive to mass analysis compared to large molecular weight proteins. Then, the peptides are analyzed by either of two types of mass spectrometers; Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) instruments, used primarily

to measure the masses of peptides, and Electrospray Ionization (ESI)-tandem MS instruments which are used to obtain sequence data for peptides.

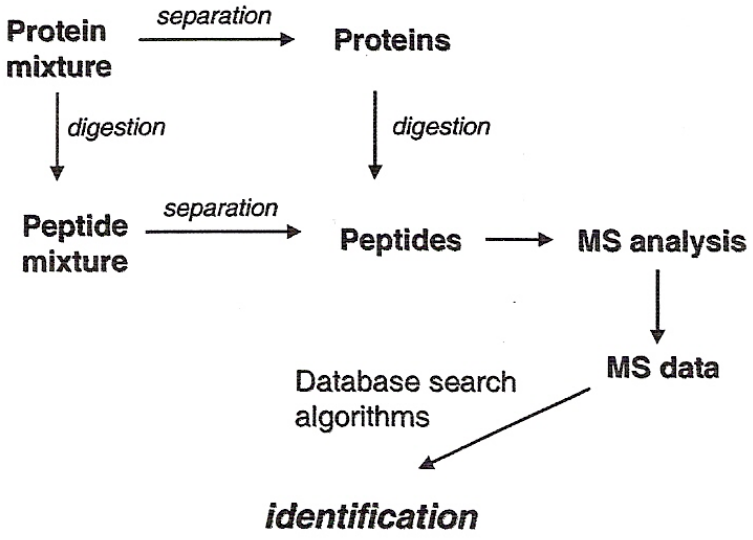


Figure 2.4. General Flow Scheme for Proteomic Analysis
(Source: Liebler 2002)

In the final part, the data collected with the mass spectrometers is used to identify sequences of peptides. Then, the corresponding proteins can be identified based on the sequence information of peptides using software-assisted database searching.

2.1.3. Types of Proteomics

Proteins are diverse molecules that can be studied in various different contexts, including sequence, structure, interactions, expression, localization and modification. Proteomics is divided into several major but overlapping branches, which embrace these different contexts and help to synthesize the information into a comprehensive understanding of biological systems (Twyman 2004).

2.1.3.1. Expression Proteomics

It is devoted to the quantitative analysis of protein abundance between samples differing by some variable. By this approach, protein expression of the entire proteome or of subproteomes between samples can be compared. Expression proteomics involves

the separation of complex protein mixtures, the identification of individual components and their systematic quantitative analysis. Information from this approach can be used to identify novel proteins in signal transduction or to identify disease-specific proteins by comparing alternative states, such as health and disease (Graves and Haystead 2002, Twyman 2004).

2.1.3.2. Structural Proteomics

Proteomics studies whose goal is to map out the structure of protein complexes or the proteins present in a specific cellular organelle are known as ‘’cell map’’ or structural proteomics (Blackstock and Weir 1999). Structural proteomics aims to identify all the proteins within a protein complex or organelle, determine where they are located, and characterize all protein-protein interactions (Graves and Haystead 2002).

2.1.3.3. Functional Proteomics

The most straightforward method to establish the function of a protein is to test that function directly. Functional proteomics is a relatively new development in which protein functions are examined directly but on a broad range (Twyman 2004). This approach allows a selected group of proteins to be studied and characterized and can provide essential information about protein signalling, disease mechanism or protein-drug interactions (Graves and Haystead 2002).

2.1.3.4. Interaction Proteomics

Interaction proteomics is concerned with the genetic and physical interactions amongst proteins as such in interactions between proteins and nuclear acids or small molecules. By protein interactions analysis describes how proteins function in pathways, networks, and complexes in addition to giving information about the function of individual proteins. It is a branch that relies on many different technology platforms to provide diverse information, and is closely related to functional proteomics and the large-scale analysis of protein localization. In a conceptive manner, the most ambitious prospect of interaction proteomics is the generation of proteome linkage maps based on

binary interactions between individual proteins and high-order interactions determined by the systematic analysis of protein complexes (Twyman 2004).

2.1.4. Bacterial Proteomics

Proteomics, especially 2DE, has been used from the outset to investigate the bacterial proteome under different growth conditions (Linn and Losick 1976, Neidhardt, et al. 1977, Agabian and Unger 1978) and various external stress factors (Young and Neidhardt 1978, Krueger and Walker 1984). Nevertheless, Fleischmann and his friends opened a new field for bacterial proteomics by completing first genome sequence of a bacterium, *Haemophilus influenzae* strain RD KW20 in 1995 (Fleischmann, et al. 1995). On the basis of a good-elucidated genomic sequence, introduction of a large-scale mass spectrometry (MS) techniques emerged to identify protein spots on a 2 dimensional gel. The complete genomic sequences of around 350 bacteria have been identified, and are available in public on web (J. Craig Venter Institute 2008) so that one can have the opportunity to choose between a diversity of bacteria for proteomic studies based on scientific interest.

In comparison to eukaryotic cells, bacteria offer a perfect organism model in terms of investigating regulatory networks, protein function, and cell differentiation due to their relatively small genomes and more simple adaptation processes. In parallel, some can be readily genetically manipulated which in turn makes them excellent organisms to study protein function. In addition, biotechnology and food industry are two areas in which bacteria use is very common. Optimization of product yields and quality can be achieved by comprehension of bacterial metabolism. Most importantly, human health is directly related to bacterial actions in points of immune-system maturation, nutrition digestion, and vitamin production. As a matter of fact, World Health Organization (WHO) reports 17 million deaths each year reasoned by microorganism infections (Brötz-Oesterhelt, et al. 2004). Not only do deaths occur in less-developed countries but also widespread in developed countries even in US (Armstrong, et al. 1999). The biggest cause of this event is resistance mechanisms developed by bacteria against antibiotics (Hiramatsu, et al. 2001). Under these circumstances, proteome studies are expected to provide good understanding and large vision into bacterial responses in order to discover new antimicrobial drug targets.

2.1.5. Applications of Proteomics

In general the most common type of application of proteomics is protein identification which includes the systematic quantitation of all the proteins found in a particular cell, tissue or organism. The main objective is to provide a complete quantitative breakdown of the proteome including all post-translational variants (Graves and Haystead 2002, Twyman 2004). In current practice, proteomics contains a number of principal applications:

- Mining: exercise of identifying all (or as many as) of the proteins in the sample.
- Protein-expression profiling: identification of proteins in a particular sample as a function of a particular state of the organism or as a function of exposure to drug, chemical, or physical stimulus.
- Protein-network mapping: proteomics approach to determine how proteins interact with each other in living systems.
- Mapping of protein modifications: task of identifying how and where proteins are modified (Liebler 2002).

The identification of proteomic profiles for diagnostics, prediction of therapeutic outcomes and drug target validation is gaining much attention. Other current and presumably growing targets for proteomics include protein engineering and biotechnological process development.

2.1.6. Challenges of Proteomics

In contrast to DNA studies, proteomics possesses a number of unique challenges. For one thing, there is no amplification technique, equivalent to the PCR, so the analysis of low-abundance proteins is one of the major drawbacks of proteomics. In addition, there are series of applications for high-throughput analysis of proteins and it is obvious that there is no technology form suitable for every application **(**Seibert, et al. 2004**,** Twyman 2004). Important difficulties must be overcome at every stage of analysis, from sample preparation through to database management (Figure 2.5).

Next challenge is the fact that study of proteins is more difficult than study of nucleic acids because of the secondary and tertiary structure of proteins, the possibility of their denaturation, or poor solubility of some proteins (Kaczmarek, et al. 2002).

Another problem particular to proteomics is that each protein gene product does not necessarily give rise to only one molecular entity in the cell since proteins are posttranslationally modified. Consequently, many proteins are present in multiple forms (Liebler 2002).

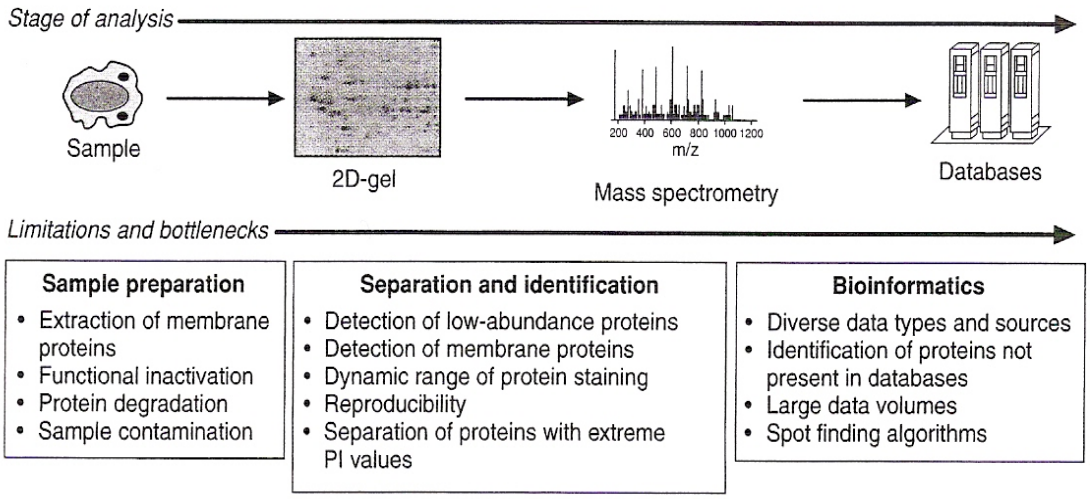


Figure 2.5. Challenges facing Proteomics with current technology
(Source: Twyman 2004)

Considering the tendency of life sciences to biological systems, analytical tools are required to identify the component parts of the system and find their responses to a varying environment. Thus, a great number of technologies on proteomics have been developed leading to many thousand proteomic studies. However, a small group of these works have tried to provide a comprehensive quantitative description of the biological system investigated. In spite of the major impact of mass spectrometry and peptide separation techniques on proteomics, the identification and quantification of all of the proteins in a biological system still remains an unmet technical challenge since data quality, in terms of information content, required for quantification by far exceeds that for protein identification (Figure 2.6).

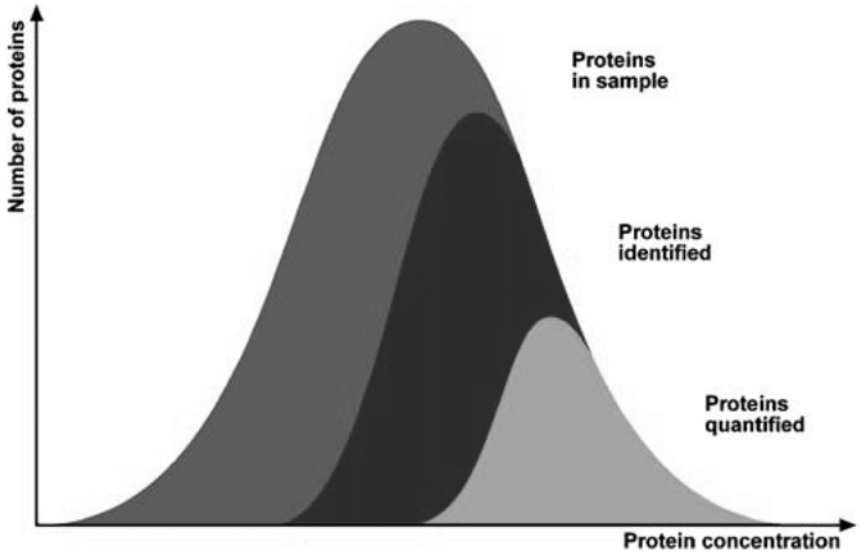


Figure 2.6. Schematic Representation of the Fraction of a Proteome that can be identified or quantified by Mass Spectrometry-Based Proteomics (Source: Bantscheff, et al. 2007)

Despite these limitations, when combined with other complementary technologies such as molecular biology, proteomics has outrageous potential to provide new insight into biology (Graves and Haystead 2002).

2.2. Separation and Quantitation Strategies for Proteins

The analysis of proteins, no matter on a small or large scale, necessitates techniques for the separation of protein mixtures into their individual components. For a eukaryotic cell, this may express 50,000 – 100,000 or more different types of protein when post-translational modifications are considered. Great chemical diversity is seen between these proteins; therefore, suitable separation methods representing all proteins equally are difficult to construct (Twyman 2004). These methods are required to resolve protein mixtures so that the proteins can be visualized, identified, and characterized.

The transcendent technique for protein separation and isolation is polyacrylamide gel electrophoresis, which is a central component of proteomic research since it provides an effective separation and quantitation technique for proteins in complex mixtures, including whole homogenates of cultured cells and tissues. Factors assisting this power are the union of high resolution to separate the mixture and high sensitivity to detect trace amounts of any protein. Considerable practical worries are that

gel electrophoresis is a reliable laboratory technique widely applicable to almost any protein in almost any type of sample (Kinter and Sherman 2000). Unfortunately, protein separation technology has been one of the branches of protein science that have shown little progress over the years. In spite of this, gel electrophoresis still exists as a most hardcore method to resolve complex mixtures of proteins, and it will stay a fundamental of proteomics until something replaces this technique (Graves and Haystead 2002).

2.2.1. General Principles of Gel Electrophoresis

Electrophoresis is known as a phenomenon in which any charged molecule in solution will migrate when an electric field is applied (Twyman 2004). The charge providing the movement of protein under the influence of an electric field can be produced either by uniform coating of the protein with the anionic detergent sodium dodecylsulfate (SDS) or by acid-base association-dissociation reactions of amine and carboxylic acid parts of the protein, depending on the type of electrophoresis being carried out. The migration rate or mobility varies according to the strength of electric field and the charge density of the molecule. In gel electrophoresis, this operation is carried out in a gel and the gel provides extra experimental parameters that alter the movement of protein in a manner to conclude specific types of separation. The two most common systems are gels that separate proteins according to their molecular weight and gels that separate proteins according to their isoelectric point (Kinter and Sherman 2000).

Since gels are formed by the polymerization of acrylamide, they are called ‘’polyacrylamide gels’’. Polyacrylamide gels are considerably easy to prepare with a number of operational parameters for the optimization of separation, namely total acrylamide content, the degree of cross-linking in the polyacrylamide, dimensions of the gel including both thickness and length (Kinter and Sherman 2000).

2.2.2. One-Dimensional Gel Electrophoresis

One-dimensional gel electrophoresis (1D-GE), which is the most commonly used analytical separation technique for all protein chemistry, is also substantially

beneficial for proteomic analysis. In 1D-GE, proteins are separated according to their molecular weights.

First, the protein is solubilized with a buffer system containing a thiol reductant (mercaptoethanol or DTT) and SDS. During mixing, SDS surrounds the protein homogeneously to form a micelle with a size directly proportional to molecular weight of the protein. So all the proteins gain a negative charge in constant proportion to molecular weight, which in turn means all movement is in the same direction, towards the positive electrode. Other effect of the SDS saturation is denaturation of proteins, especially when reagents such as dithiothreitol are included to cleave any disulfide bonds. Consequently, diversity coming from the complex tertiary structures for proteins is reduced to a simple rod-like shape within an SDS micelle. Then the gel is subjected to an electric field and protein-SDS complexes migrate through the cross-linked polyacrylamide gel at rates depending on their ability to penetrate the pore matrix of the gel. Smaller proteins migrate further through the gel than larger proteins so that the mixtures of proteins are resolved into bands in order of molecular weight. This type of electrophoresis is usually called as SDS-PAGE, representing the joining of SDS treatment of the proteins with polyacrylamide gel electrophoresis (PAGE) (Kinter and Sherman 2000, Liebler 2002).

2.2.3. Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis (2DE), first introduced for protein separation in the early 1970s by O'Farrell (O'Farrell 1975) , is a powerful and widely used electrophoretic technique that analyzes thousands of individual protein species extracted from cells, tissues, or other biological samples. Spot patterns are formed in 2DE analysis in which every single spot indicates an individual protein species to its specific coordinates. The intensity of an individual spot implies how much the cell has produced of that actual protein **(**Bendixen 2005**,** Shi, et al. 2004**).**

This method is actually a combination of two different and independent types of separations. The first dimension is isoelectric focusing (IEF), which separates proteins according to their isoelectric points (pI); the second dimension is sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins according to their molecular weights (MW).

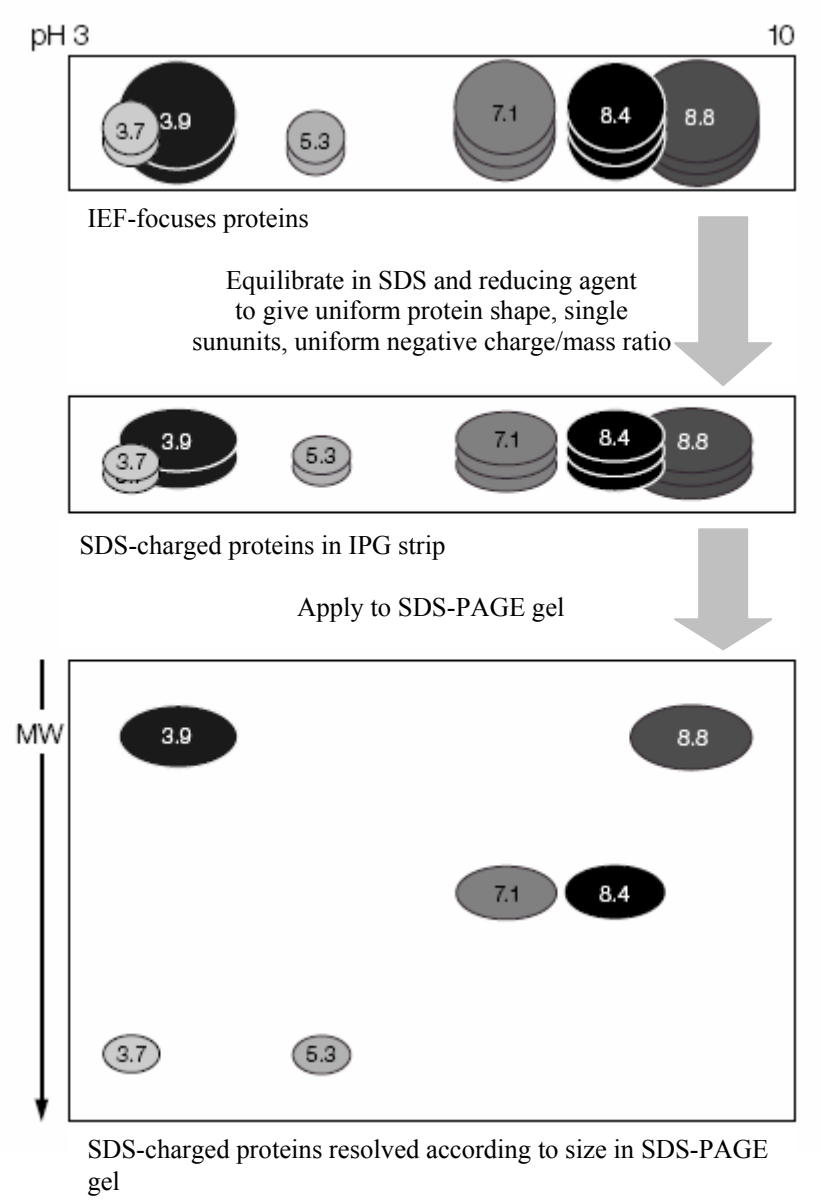


Figure 2.7. Schematic Diagram Showing Separation of Proteins by SDS-PAGE after Separation by IEF, Bio-Rad

2.2.3.1. First Dimension: Isoelectric Focusing

Isoelectric focusing (IEF) is an electrophoretic method that distinguishes proteins based on their isoelectric points (pI). Proteins are amphoteric molecules (zwitterions); they carry either positive, negative, or zero net charge, depending on the pH of their surroundings. The net charge of a protein is the sum of all the negative and positive charges of its amino acid side chains and amino- and carboxyl-termini. The pH at which a protein has no net charge is called the ‘’isoelectric point’’ or ‘’pI’’ of that

protein. Proteins are positively charged at pH values below their pI and negatively charged at pH values above their pI.

2.2.3.1.1. pH Gradients

The existence of a pH gradient is crucial to the IEF technique. When an electric field is applied, a protein will move to the position in the pH gradient where its net charge is zero. A protein carrying a positive net charge will move toward the cathode, becoming incrementally less positively charged as it moves through the pH gradient until it reaches its pI. A protein with a negative net charge will move toward the anode, becoming less negatively charged until it also reaches zero net charge. If a protein should diffuse away from its pI, it immediately gains charge and migrates back. This behaviour is called focusing effect of IEF, which concentrates proteins at their pIs and lets proteins be separated dependent upon very small charge differences. (Berkelman and Stenstedt 2002)

The pH gradient in an IEF gel can be set up by either of two techniques, carrier ampholytes and immobilines. The carrier ampholytes are collections of low molecular weight amphoteric molecules with pI values corresponding to a given pH range. They are designed to have high buffer capacities. In the beginning, no pH gradient exists in the gel due to even distribution of ampholytes. Under the electric field, however, the ampholytes themselves are exposed to electrophoresis. The most acidic ampholytes migrates towards the anode, the most basic ampholytes migrates towards the cathode and others find intermediate zones based on their pI values. By this way the system reaches an equilibrium precisely expressed by a continuous pH gradient. Slowly moving proteins, which can either be added to the gel before the electric field is applied or after prefocusing, then start to migrate towards their pI in the gel.

Numerous studies have used this approach and careful attention to detail can provide reproducible protein profiles (Lopez and Patton 1997). Nevertheless, use of carrier ampholytes brings about a number of drawbacks involving cathodic drift (Righetti 1983), low protein load capacity, pH gradient instability, and poor gel-to-gel reproducibility. Many of these problems are overcome by the introduction of immobilized pH gradient (IPG) gels in which the buffering groups are attached to the polyacrylamide matrix of the gel (Bjellqvist, et al. 1982, Görg, et al. 1985, Görg, et al.

1988). This methodology is now the standard approach in proteomics, where reproducibility is a keystone. The IPG gels are prepared using immobilines, a collection of nonamphoteric molecules containing a weak acid- or base- buffering group at one end, and acrylic double bond to assist the immobilization reaction at the other. By the help of casting a gradient gel using immobilines with the desired pH extremes, the pH gradient is established. The gel is run normally and remains stable yet pH gradient exists before the electric field is applied. When the sample is loaded, the proteins move towards their isoelectric points. Addition of carrier ampholytes to the IPG gel buffer is considered to increase protein solubility and block useless interactions between protein and the immobilized reagents.

Commercial immobilized pH gradient gels are cast, washed, dried, and cut into narrow (3.3 mm wide) IPG gel strips with different lengths and varying pH ranges. IPG gel strips offer the benefit of gradient stability over extended focussing runs (Bjellqvist, et al. 1982) although they are much more difficult to cast compared to the carrier ampholytes gels (Righetti 1983).

2.2.3.1.2. Protein Solubilization

Protein solubilization is a crucial step for successful 2D gel electrophoresis. Since proteins exist in their native form and are insoluble in that state, it is necessary to denature proteins to assist solubilization. Thus, proteins to be separated need to be well solubilized before and throughout the electrophoresis procedure. Solubilization of proteins includes the process of breaking all interactions involved in protein aggregation (Rabilloud 1996), which include disulfide bonds, hydrogen bonds, van der Waals forces, ionic interactions, and hydrophobic interactions. Unless these interactions survive proteins can aggregate or precipitate, which in turn causes artifacts or sample loss.

The sample rehydration buffer, also known as sample buffer, is required for both denaturation and solubilization of protein samples and rehydration of the IPG strips. The sample rehydration buffer characteristically contains a denaturing/chaotropic agent (urea or urea/thiourea), solubilizing agent (non-ionic or zwitterionic detergent and ampholytes), and reducing agent (DTT) (Table 2.1). The sample rehydration buffer must not influence the pI of the protein, must not increase the ionic strength of the

solution in order to apply high voltages during focusing without producing high currents. Final concentration of the components is needed to be optimized according to the solubility of proteins.

Table 2.1. Major Components of Rehydration Buffer, Invitrogen

Component	Function	Final Concentration
Urea Urea solutions are prepared fresh or stored frozen at -20°C. De-ionize urea solutions on a mixed-bed ion exchange resin using manufacturer's recommendations. Thiourea is used to increase solubility of some proteins (Rabilloud, 1998)	Denatures and solubilizes proteins	8 M urea or 9 M urea in some cases For urea/thiourea solution, use 5-8 M urea with 2 M thiourea
Detergent Use non-ionic or zwitterionic detergents such as CHAPS, NP-40, CHAPSO, and sulfobetaines (SB3-10) (Chevallet *et al.*, 1998). Ionic detergents such as SDS are not recommended.	Solubilizes the proteins and helps to maintain the proteins in solution during rehydration and IEF	Total detergent concentration range is 0.5-4%
Reducing Agent Use DTT or DTE (dithioerythritol). β-mercaptoethanol is not recommended for reduction (Righetti *et al.*, 1982).	Cleaves the disulfide bonds in the proteins	DTT or DTE at 20-100 mM
Ampholytes Use the appropriate carrier ampholytes based on the pH range of the IPG strip. May use ampholytes pH 3-10 for all pH ranges of IPG strips.	Helps in solubilizing proteins and aids in maintaining the pH gradient	0.2-2% **Note:** Higher ampholyte concentration requires longer focusing times.

2.2.3.1.3. Sample Application and Rehydration

Different protein estimation methods are being used to determine protein concentration for protein load on the IPG gel strip. Those are absorption methods, colorimetric methods, and fluorescent methods, listed below:

- Ultraviolet Absorption Methods
    - A280
    - A205
    - Extinction Coefficient at 280 nm
- Colorimetric Methods
    - Biuret Assay
    - Lowry Assay
    - BCA Protein Assay
    - Bradford Assay
    - Colloidal Gold Assay
    - Ninhydrin Assay
- Fluorescent Methods
    - Fluorescamine Protein Assay
    - o-Phtalaldehyde Protein Assay
    - CBQCA Protein Assay
    - NanoOrange Protein Assay

Loading equal amounts of proteins on the IPG gel strips is the basis of comparative proteomics. Different amounts of protein loaded on the IPG gel can cause poor resolution and less protein spot on the gel. Therefore, using an accurate and sensitive protein estimation method is very critical. The amount of protein loaded can be determined based on a number of variables including sensitivity of gel staining method, pH gradient and length of the IPG gel strip. The following table summarizes generally recommended protein loads for IPG strips based on strip length and pH range.

The IPG strips on which protein is loaded must be rehydrated in the rehydration buffer/sample buffer before the first dimension of 2D electrophoresis. IPG gel is allowed to swell to its original thickness (0.5 mm) and width (3 mm), and is usually performed for 11-16 hours. Solution used to rehydrate IPG strips prior to isoelectric focusing is the same as sample rehydration buffer described before.

Table 2.2. Approximate Protein Loads for ReadyStrip IPG Strips, Bio-Rad

pH Range	**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	10-100 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	20-100 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	

Rehydration of IPG strips combined with sample application can be performed using passive or active methods. In the case of passive manner, rehydration is carried out by placing the IPG gel side down in the channel of a focusing or rehydration tray which contains protein sample in a suitable rehydration solution. Smaller or larger rehydration buffer volumes can be used and the strips will swell to accommodate more liquid up to a point (Görg, et al. 2000). Large proteins cannot enter the gel until the pores are large enough to accept them, which only occur when the pores have swelled to their maximum size. Rehydration process can be observed if a trace amount of bromophenol blue is added to the sample solution. The IPG strips must be covered completely with mineral oil to prevent evaporation of buffer which will precipitate urea as it becomes more concentrated. On the other hand, in active rehydration IPG strips are rehydrated under a low voltage (50 V) to enhance protein absorption by the gel matrix.

Active rehydration is considered to allow large proteins to enter the strip by applying electrical ‘’pull’’. Due to applying voltage prior to absorption of rehydration buffer and protein by the gel, the pH of the protein’s environment will be equal to the pH of rehydration buffer, and the protein will migrate depending on its size. Hence, small proteins having higher mobility are more susceptible to be lost from the strip.

It is very crucial to incubate strips with sample for 11-16 hours before focusing for both passive and active rehydration. Because incubation provides a required time for

large proteins to enter the gel matrix following full gel hydration and attaining full pore size in the gel.

Considering the sample application methods, cup loading is another way to introduce protein sample. Application of the sample within a discrete zone to a previously rehydrated IPG strip is the basis of cup loading, and it is usually carried out at the anodic or cathodic end of an IPG strip. This method can be used at pH range between 6 to 11 in order to obtain better protein separations if the samples are contaminated with nucleic acids or glycoprotein's as well as for serum sample that has not been depleted of albumin. **(**Humphrey-Smith, et al. 1997, Görg, et al. 2000). However, this technique is disadvantageous in terms of ease of sample application, and also it can lead to sample precipitation (Rabilloud 1999).

2.2.3.1.4. Focusing Conditions

In the course of IEF run, focusing conditions will change with sample composition, sample complexity, and IPG pH range. Following the rehydration of IPG strips, isoelectric focusing is performed applying high voltages across the IPG strips. It is very important to have a power supply that is able operate at high voltage and low current. Applied current should not pass beyond 50 μA/IPG strip which in turn limits the voltage. At the beginning of IEF run, the current is high because of the movement of charged molecules. When the proteins begin to focus at their respective pIs, the current is observed to fall down slowly due to decrease in the charge on individual proteins and carrier ampholytes. After all the proteins reach their pIs, current reaches its minimal value, and at that point a high voltage is applied to focus the proteins into sharp, narrow zones. This high voltage is maintained for at least several thousand Volt-hours (Vh), the integral of the volts applied over the time. The number of volt-hours required to achieve a successful IEF run must be determined empirically.

The resolution of an IEF run is determined by the pH gradient, strip length, and applied electrical field. In agreement with theory and experiment **(**Andrews 1986**,** Garfin 2000**)**, the difference in pI between two resolved adjacent protein IEF bands (ΔpI) is directly proportional to the square root of the pH gradient and inversely proportional to the square root of the voltage gradient (field strength) at the position of

the bands. Hence, narrow pH ranges and high-applied voltages are expected to give high resolution (small ΔpI) in IEF (Garfin 2000).

In IEF cell system, temperature control is another important parameter. The currents that flow at the time of focusing may fall into the microampere range even though it may begin in the low milliampere range, which in turn cause the generation of little heat in the cell. Since isoelectric point of the proteins changes with temperature, isoelectric focusing requires effective cooling for close temperature control, which can be accomplished by a Peltier cooling plate or a horizontal ceramic cooling plate connected to a thermostatic circulator. The influence of temperature at which IEF with IPG is performed was studied and the optimum focusing temperature was found to be 20^{o}C (Görg, et al. 1991). At lower temperatures the presence of urea is not appropriate owing to the formation of urea crystals (Righetti 1983). Similarly higher temperatures result in protein carbamylation in the presence of urea. Furthermore, elevated temperatures enable more rapid focusing because of lower viscosity and increased mobility of proteins. However, temperature control is necessary in order to obtain purposeful comparison of 2D patterns.

2.2.3.1.5. IPG Strip Equilibration

Before running the second dimension it is strongly advised to equilibrate focused IPG strips in the presence of SDS in order to expedite efficient transfer of proteins from the first-dimension to the second dimension. This conditioning transitional step mainly aims at coating proteins with SDS for migration in the second dimension on the basis of molecular weight. So all molecules of the same protein will run out of the first-dimensional gel at the same time and proteins will be resolved as round spots in the second dimension.

Equilibration is usually performed in two steps. The first treatment includes reduction of proteins by equilibrating the isoelectric focusing gel in a solution, namely equilibration buffer one, containing 6 M urea, 50 mM Tris-HCl (pH 8.8), 2% (w/v) SDS, 30% (w/v) glycerol, and 1% (w/v) DTT. Urea together with glycerol reduces the effects of electro-endosmosis, movement of buffer within the IPG strip, by increasing the viscosity of the buffer (Görg, et al. 1985). After 10-15 minute treatment of equilibration buffer one, second equilibration is applied to the IPG gel strips for the

same time duration to alkylate sulfhydryl groups of the proteins. Equilibration buffer two is the same as first buffer, but contains 4% (w/v) iodoacetamide instead of DTT. DTT preserves the fully reduced state of denatured, unalkylated proteins by reducing sulfhydryl groups while iodoacetamide alkylates thiol groups on proteins, preventing their reoxidation during electrophoresis. (Görg, et al. 1987).

As indicated above, each equilibration step must be carried out for 10-15 minutes due to the fact that prolonged equilibration brings about protein loss, as high as 15 to 25%, particularly of low molecular weight species (Garrels 1989, Rickwood, et al. 1988). At the end of equilibration procedure, the IPG gel strips become ready for second dimension analysis.

2.2.3.2. Second Dimension: SDS-PAGE

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, is an electrophoretic technique that separates proteins according to their molecular weights irrespective of their charge. The main principle underlying this technique is the exposure of denatured proteins to the anionic detergent sodium dodecyl sulfate (SDS) within the polyacrylamide gel, which binds stoichiometrically to the polypeptide backbone and carries a large negative charge. The separation is not influenced by the intrinsic electrical charge of the proteins due to the presence of SDS in the sample and the gel. When in solution, SDS forms spherical micelles composed of 70-80 molecules with the dodecyl hydrocarbon in the core and hydrophilic sulfate groups in the head. When SDS binds to proteins, it creates complexes with a necklace-like structure composed of protein-decorated micelles connected by short flexible polypeptide segments (Ibel, et al. 1990). In the consequence of this necklace structure, great amounts of SDS are involved in the SDS-protein complex in a ratio roughly 1.4 g SDS/g protein. The existence of tens or hundreds of SDS molecules on each polypeptide eclipses any intrinsic charge of the proteins themselves so that anionic complexes formed have a constant net negative charge per unit mass. SDS molecules binds stoichiometrically to the proteins, that means the larger the protein, the higher binding capacity of SDS it has. This event brings two significant outcomes assuring molecular weight based-separation only, which are the facts that all SDS-protein

complexes have primarily the same charge density, and they sustain relative differences in molecular weights between proteins.

As mentioned in one dimensional gel electrophoresis, the gel enables the size-based separation by sieving the proteins as they migrate. This sieving effect is based on the pore size of the gel, which is then dependent on the gel concentration. Total acrylamide content of the gel is referred to as T%. For the polyacrylamide gels, the monomer is made up of the gelling agent acrylamide and also the cross-linking agent *bis*-acrylamide. Thus, the pore size becomes also dependent on *bis*-acrylamide content (C%) (Twyman 2004). Generally, pore size is controlled by varying the T% between 4 % and 20% while keeping C% constant. So gel conditions can be chosen according to molecular weights of the proteins to be separated and the desired degree of restriction. Gels having high T% (i.e.12%) have small pore sizes making them restrictive and favouring the movement of smaller proteins while little or no movement of larger proteins, and vice versa (Kinter and Sherman 2000). The molecular weights of the proteins in the sample can be predicted by including, in one of the lanes of the gel, a series of protein markers whose masses are already known.

The most frequently used buffer system to run second dimension SDS-PAGE is the tris-glycine electrophoresis buffer system which is described by Laemmli (Laemmli 1970). This buffer system separates proteins at high pH that brings the benefit of minimal protein aggregation and clean separation even at relatively high protein loads. On the other hand, the Laemmli buffer system has disadvantages in terms of a limited gel shelflife.

2.2.4. Protein Detection and Image Acquisition

The last step of 2D-PAGE is the detection of proteins that are separated and planned for in-gel digestion. Visualization of proteins can be commonly achieved by three well known techniques, namely Coomassie brilliant blue-staining, silver-staining, and fluorescent-staining. In general, amount of protein in the sample determines which type of staining method will be applied (Table 2.3).

Coomassie brilliant blue (CBB) is an organic dye used for colouring proteins in polyacrylamide gels. It is commercially available with different labels as G-250, R-250, and R-350 according to detection sensitivity. In spite of various staining procedures,

standard staining is usually performed mixing the dye with concentrated acid in ethanol or methanol. So, a colloidal suspension is generated, that develops intensely coloured complexes with proteins. Overnight immersion of the gel in this solution, saturates the gel and stains proteins. Coomassie brilliant blue is a commonly used protein detection technique due to its ease of use, high compability with mass spectrometry, and linear response for most proteins, it lacks the sensitivity for proteomic analysis with a detection limit below 10 ng. Moreover, interpretation of mass spectrometry data can be obstructed in situations where possible modifications of glutamic acid side chains occur by the dye. CBB also does not necessarily offer a successful staining for highly acidic, highly basic, or glycosylated proteins.

Table 2.3. Commonly used Stains for Visualization of Gel-Separated Proteins
(Source: Granvogl, et al. 2007)

Stain	**Time (h)**	**Detection Limit (ng per protein band)**	**Literature**
Coomassie colloidal (G-250)	15	1-16	Neuhoff,et al. 1988, Berggren, et al. 2000, Mackintosh, et al. 2003, Candiano, et al. 2004
Coomassie R-250	12-48	30-100	Patton 2002, Mackintosh, et al. 2003
Silver	0.25-3	0.5	Heukeshoven and Dernick 1985, Merril, et al. 1986
Fluorescent dyes: SyproRuby	5.5-12	0.5-5	Berggren, et al. 2000, Nishihara and Champion 2002, Lilley and Friedman 2004
Deep Purple	3.5	0.1	Mackintosh, et al. 2003
DIGE	0.75	0.025	Marouga, et al. 2005

Another commonly used method is silver staining which is based on reactions known from photography. The basic principle is diffusion of silver ions into the gel at acidic pH, their binding to sulfhydryl and carboxyl groups of amino acid side chains **(**Rabilloud 1990, Heukeshoven and Dernick 1985**,** Merril and Pratt 1986**)**, and reduction to metallic form at basic pH. Protein spots are black or brown coloured due to the mediation of silver crystallization by peptide backbone and functional groups of amino acids **(**Moritz and Meyer 2003**)**. Silver staining is about 100 times more sensitive than CBB staining **(**Switzer, et al. 1979) giving detection limits for 2D gel protein bands in the range of 0.5-1.0 ng. Despite this, silver staining protocols suffer from the

disadvantage of limited compatibility with downstream mass spectrometry since cysteine residues and alkylate-exposed amino groups can be modified by silver stains. It can also be troublesome due to the fact that precise timing is required between staining steps to get reproducible and coherent results. Furthermore, the intensity of silver stained protein spots does not remain constant over time which also explains poor reproducibility of the method. Including the negative aspects of silver staining, poor linear dynamic range that makes quantitative analysis unconvincing, and poor staining of certain proteins like glycoproteins can be considered.

Recently, a number of fluorescent dyes have been introduced into detection methods. These dyes consist of complexes of an organic compound and a heavy metal component (e.g. ruthenium; Sypro Ruby). Fluorescent staining combines the advantages of high sensitivity and compatibility with mass spectrometry **(**Berggren, et al. 2000**,** Mackintosh, et al. 2003**)**. Linear dynamic range spanning three orders of magnitude is another stunning trend of fluorescent dyes **(**Patton 2000**)**. However, these techniques require a high expense of hardware since fluorescent signal is not visible to human eye directly. Additionally, gels stained with fluorescent dyes can not be stored for long terms because they are temporarily light sensitive **(**Granvogl, et al. 2007**)**.

Following to the staining procedure, the abundance of different proteins on a 2D-gel is specified in terms of intensity, size and shape of the related spots. Hence, protein quantitation necessitates the conversion of an analogue gel image into digital data. In this way, objective comparisons of equivalent protein spots on different gels can be performed in order to observe up-regulated and/or down-regulated proteins as well as newly produced proteins. Since human eye is very subjective when judging protein spots, 2D-gels stained with CBB or silver are scanned by a charge-coupled device (CCD) camera or a densitometer. The image from a CCD camera is then analyzed by computer based software tools, such as DECODON Delta2D, Bio-Rad's PDQuest, for spot detection, matching and quantitation.

2.2.5. In-Gel Digestion

The in-gel digestion, a method introduced by Rosenfeld in 1992, is a critical step of sample preparation for the mass spectrometric analysis of proteins. After visualization of the gel, protein of interest requires to be divided into its peptides for

protein identification by mass spectrometry. Because generated peptides have molecular weights within the mass range of mass spectrometers. Mass spectrometry can still produce measurement errors whose magnitude increases as the length of the peptide chain gets longer. In addition, the fact that protein databases are constructed according to the peptide masses makes peptides preferable against intact proteins.

The term ‘’in-gel’’ represents that the protein is processed and digested while contained in the polyacrylamide gel piece. Although several modifications have contributed to increasing peptide yield and improving the quality of MS data, essential steps of the technique have remained unchanged. Basically, in-gel digestion includes cut of protein spots of interest from the gel, its destaining, reduction and alkylation of cysteines, and treatment of proteins with a specific protease. In this manner, the gel matrix is penetrated by the enzyme, which leads to digestion of the protein to peptides.

As noted earlier, the in-gel digestion of proteins with a specific enzyme generates a certain number of characteristic peptides with different molecular masses which are then used in mass spectrometry to identify the protein. For most proteome studies, trypsin is a universal choice due to its exclusive properties as a protease. Specifically, it cleaves an amino bond on the C-terminal side of lysine and arginine residues. The spacing of these two residues in many proteins provides the generation of the peptide masses that fits the range required for MS analysis **(**Olsen, et al. 2004**)**. Peptide fragments having 6-20 amino acids are optimum for MS analysis and database searches.

Table 2.4. Overview of Proteases used for In-Gel Digestion
(Source: Reinders, et al. 2004)

Endopeptidase	Type	Specificity	pH range	Inhibitors
Chymotrypsin	Serine	Y, F, W	1,5 – 8,5	Aprotinin, DFP, PMSF
Trypsin	Serine	R, K	7,5 – 9,0	TLCK, DFP, PMSF
Glu C	Serine	D, E	7,5 – 8,5	DFP
Lys C	Serine	K	7,5 – 8,5	DFGP, Aprotinin, Leupeptin
Arg C	Cysteine	R	7,5 – 8,5	EDTA, Citrate
Asp N	Metallo	D (N-terminal)	6,0 – 8,0	EDTA
Elastase	Serine	A, V, I, L, G	8,0 – 9,0	DFP, a1-Antitrypsin, PMSF
Pepsin	Acidic	F, M, L, W	2,0 – 4,0	Pepstatin
Subtilisin	Serine	nearly all	7,0 – 11,0	Phenole, DFP, PMSF
Thermolysine	Metallo	hydrophobic AA	7,0 – 9,0	EDTA
Papaine	Cysteine	R, K, G, H, Y	7,0 – 9,0	IAA, TLCK, TPCK
Proteinase K	Serine	hydrophobic AA	7,0	IAA
Thrombin	Serine	R	7,5	DFP, TLCK, PMSF
Factor X	Serine	I-E-G-R	8,3	DFP, PMSF

Beside trypsin, a number of proteases exist and are frequently used for in-gel digestion such as chymotrypsin, Asp-N, Lys-C, Glu-C and so on. However, these enzymes cut proteins at one defined amino acid **(**Michalski and Shiell 1999**)**; thus, they produce a less number of peptides with greater length. Table 2.4 shows commonly used proteases for in-gel digestion and their cleavage characteristics.

2.3. Mass Spectrometry

Mass spectrometry (MS) has been accepted as the most comprehensive and all-purpose analytical technique that measures mass-to-charge ratio (*m/z*) of molecules and atoms since the early 1900s. It has come into prominence in the fields of chemistry, physics, geology, archaeology, nuclear science, material science, environmental science, forensic science, and petroleum industry. MS majorly serves the purposes of molecular mass determination, structure elucidation, quantification at trace levels, and mixture analysis.

The history of MS began with cathode ray tube experiments of Sir J.J. Thomson in 1897 (Thomson 1897). He measured the mass-to-charge ratio of the negatively charged cathode ray particles by passing the collimated beam through crossed electric and magnetic fields. From the study of Thomson, MS has evolved over the 20^{th} century with tremendous technological improvements. High resolution mass spectrometry made exact mass measurements possible in 1950s while development of gas chromatography (GC) MS facilitated the analysis of complex mixtures in the following decade (Watson and Biemann 1964, Ryhage 1964). The 1980s and 1990s brought significant developments in instrumentation and ionization techniques.

Previously, the application of mass spectrometry to biology was ambiguous due to the absence of ionization methods applicable to biological compounds. Fortunately, introduction of soft ionization techniques have paved the way for mass analysis of large molecular mass biological compounds so that mass spectrometry has become an essential tool in biological research. More specifically, MS satisfies all basic requirements of proteomic studies, which are a broad dynamic detection range, high throughput and accurate protein identification, absolute protein quantification, the ability to cope with multiple proteins in a single spot, and characterization of post-translational modifications.

Major capabilities that render mass spectrometry so popular are listed below:

- Applicability to all elements
- Independence of sample type; volatile or nonvolatile, polar or nonpolar, and solid, liquid, or gaseous materials
- Excellent molecular specificity
- Hyper detection sensitivity in zeptomole levels, ability to detect a single molecule
- Unique versatility to determine the structures of most classes of unknown compounds
- Matchless ability to analyze complex samples when combined with high resolution separation devices

2.3.1. Basic Principle and Instrumentation

As mentioned previously, MS is an analytical technique that can measure the *m/z* of individual molecules and atoms. The first fundamental step in MS analysis is to convert the neutral analyte molecules into gas-phase ionic species to be able to control their motion. Fragmentation takes place if energies higher than ionization energies are used during ionization process. Then, ions are accelerated through a voltage difference towards a mass analyzer, which separates these molecular ions and their charged fragments based on *m/z* ratio. Finally, ion currents generated by these mass separated ions are recorded by a convenient detector and monitored in the form of a mass spectrum, a plot of *m/z* values versus abundance. A high vacuum (10^{-4} – 10^{-8} torr) is maintained throughout all components of the system to let ions move freely in space without colliding or interacting with other species.

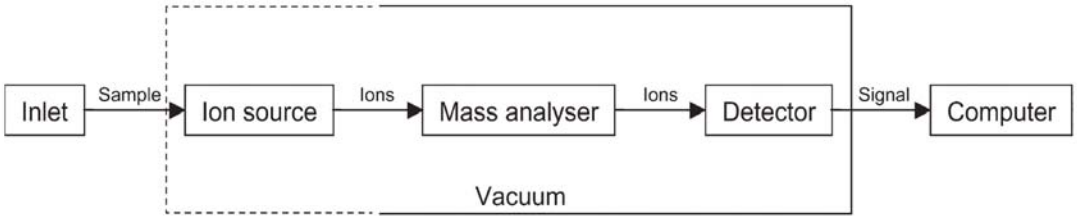


Figure 2.8. Basic Components of a Mass Spectrometer
(Source: Lane 2005)

2.3.2. Ionization Methods

Sample ionization is a keystone of mass spectrometry since a compound needs to be charged and ionized to be analyzed by a mass spectrometer. Ionization sources enable the conversion of neutral molecules into gas phase ionic species so that electric and magnetic fields can be used to exert forces on charged particles in a vacuum for mass analysis. The selection of ionization method is dependent on the nature of the sample investigated. In the case of biomolecules, soft ionization methods which provide mass spectra with less or no fragment ion content are being used. The most common of these methods include electrospray ionization (ESI), and matrix assisted laser desorption ionization (MALDI).

2.3.2.1. Electrospray Ionization (ESI)

Electrospray ionization (ESI) has become a popular mode of ionization technique for qualitative analysis of a broad variety of compounds in solution. The invention of ESI has given rise to a respectable progress in mass spectrometry. Dole laid the foundations of ESI in 1968 by producing gas phase ions from electrically charged liquid droplets (Dole, et al. 1968). The work of Dole then gave inspiration to Yamashita and Fenn for combining ESI with mass spectrometry (Yamashita and Fenn 1984). The gentile nature of this ionization explains the versatility of ESI-MS combination for the measurements of molecular masses of nonvolatile and thermally unstable compounds. Furthermore, it serves as an excellent interface to couple high performance liquid chromatography (HPLC) with MS for the analysis of complex samples.

In ESI process, solution of the sample is first mixed with a continuously flowing suitable solvent in a capillary tube that is a 1:1 mixture of water and an organic solvent (methanol, acetonitrile, or isopropanol). A potential difference of 3-4 kV applied between the capillary and the inlet to the mass spectrometer generates a fine spray of charged droplets forming a cone shape, known as a Taylor cone (Wilm and Mann 1994), while emerging from the tip of the capillary. A drying gas at the interface, usually nitrogen, helps the evaporation and removal of the solvent from the charged droplets. While the diameter of the charged droplet decreases, the charge density on its surface increases. When surface tension of the droplet and electrostatic repulsion on it

are equalized, charged droplet explodes to produce daughter droplets which are exposed to further evaporation. This cycle repeats itself until evaporation is completely finished. In the end, resulting desolvated, gas-phase ions are accelerated through mass analyzer and separated based on m/z ratio and all separated ions are detected with the detector.

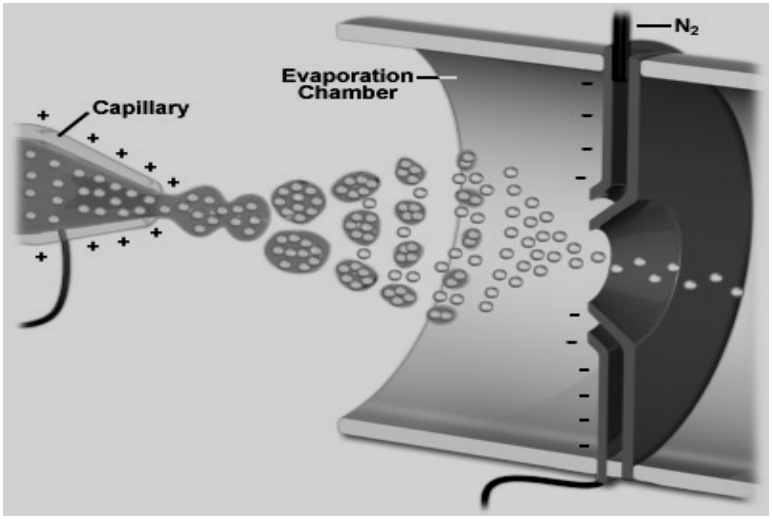


Figure 2.9. Formation of Gas-Phase Ions in ESI
(Source: Florida State University 2008)

2.3.2.2. Matrix-Assisted Laser Desorption/Ionization (MALDI)

Matrix-assisted laser desorption/ionization mass spectrometry is an established ionization technique, especially for macromolecules of biological importance. The invention of MALDI was first reported nearly at the same time by two groups, Karas and Hillenkamp (Karas and Hillenkamp 1988), and Tanaka and co-workers (Tanaka, et al. 1988). This approach has gained a wide acceptance for analysis of large biopolymers with masses up to 500 kDa. MALDI can also provide characteristics of speed, high sensitivity, ease of use while tolerating small amounts of contaminants like salts and surfactants.

An astonishing aspect elevating MALDI to a level where it has become an indispensible technique is mixing of the sample with a ‘’matrix’’, a low-molecular weight energy-absorbing organic acid. Matrix is the heart of MALDI process since it serves as laser energy absorbent and an energy transfer agent in order to prevent direct interaction of laser energy with the sample. Principally, the analyte is dispersed in a

large excess of matrix material (in the ratio of one-to-several thousands) which can strongly absorb energy at the wavelength of the laser radiation (typically a nitrogen laser at 337 nm). The analyte and matrix is then dissolved in an organic solvent, placed on a metallic probe or sample target, and allowed to dry. Evaporation of the solvent leaves matrix crystals surrounding analyte molecules. After the target is placed in the vacuum chamber of the mass spectrometer, a high energy laser beam is directed at the sample plate with about 10^6 W/cm^2 irradiance power. Most of the laser energy is efficiently absorbed by the matrix crystals causing evaporation of the matrix, and this energy is transferred to the analyte as heat in a controlled manner such that no fragmentation occurs. So analyte molecules are converted into gas-phase ions by gas-phase proton-transfer reactions. Once in the gas phase, analyte ions are then directed electrostatically into the mass analyzer.

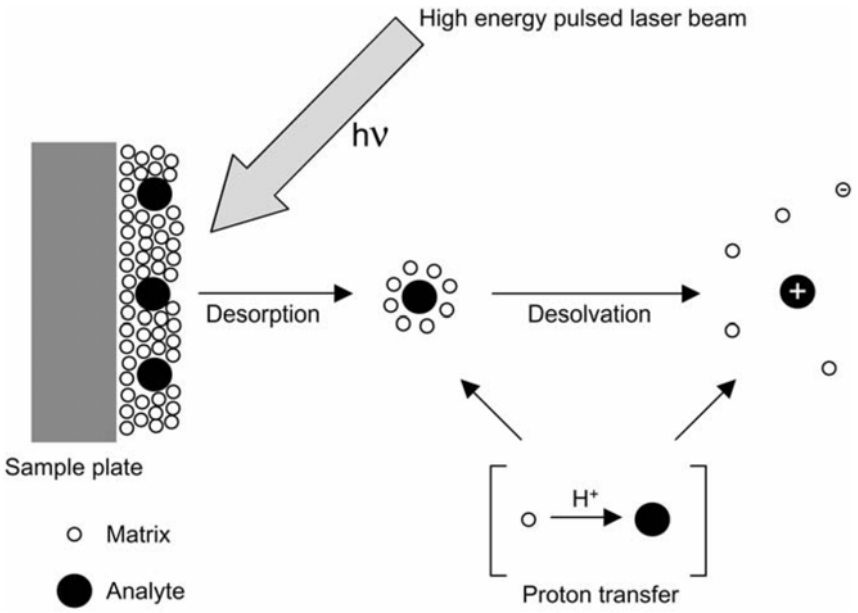


Figure 2.10. MALDI Process
(Source: Hoffmann and Stroobant 2003)

2.3.3. Mass Analyzers

At the end of ionization process, a mass analyzer is required to separate ions produced at the ion source according to their *m/z* ratios. Applying suitable electric and magnetic fields, ion motion can be controlled and manipulated in the mass analyzer in

order to detect the number of ions at each individual *m/z* value. The principle of distinguishing the motion of a charged particle relies on kinetic energy, momentum and velocity of the ion so that an analyzer can mass-resolve the ions. Mass analyzers maximize the transmission of all ions entering from ion source and help to focus all mass-resolved ions at a single focal point for the ease of detection. Fundamental instrumental parameters assigning the performance of mass analyzers include mass range, mass resolution, mass accuracy, detection sensitivity, and scan speed as much as design and associated ion optics.

For proteomics research, different types of mass analyzers can be used, such as time-of-flight (TOF), quadrupole ion trap, quadrupole linear, and Fourier transform ion cyclotron resonance (FT-ICR) analyzers. They are quite different in terms of experimental design and performance parameters like resolution, sensitivity, and mass accuracy. Intended for improved overall capability and making the use of different strengths, mass analyzers can be combined in tandem to build hybrid instruments, called tandem mass analyzers (MS/MS) such as quadrupole/quadrupole, magnetic sector/quadrupole, quadrupole/TOF, and ion trap/TOF geometries, etc. Tandem mass analyzers contain three main parts: a first mass analyzer can be used to isolate the ion of interest (precursor ion or parent ion) and second part can be used as a collision cell to fragment the parent (precursor) ions, a collision-induced dissociation device (CID) fragmenting the selected parent ions to form daughter (fragment) ions, and a third part can be used to separate daughter ions according to mass or energy. So an MS/MS spectrum from which structural assignments can be drawn is generated.

2.3.3.1. Time-of-Flight (TOF)

A time-of-flight (TOF) mass spectrometer, theoretically proposed in 1946 (Stephens 1946), is a simple type of mass analyzer. By the development of MALDI, TOF has been undergoing a renaissance in the field of biomedical sciences, and being used in coupling with MALDI to handle the pulse of ions. TOF takes the advantage of singly charged peptide ions produced by MALDI to relate the flight time with molecular mass.

TOF serves as a kind of velocity analyzer which separates ions based on their different velocities. A short pulse of ions exiting the source is given the same kinetic

energy to accelerate them towards the detector; hence, it is obvious that ions carrying the same charge with different mass will travel through a flight tube at different velocities; thus, it takes different amount of time to travel the same distance. The lighter ions travel down a field-free region faster and reach the detector earlier due to their greater velocity while it takes longer time for heavier ions because of their lower velocity. In this way, flight times of ions required to move through a field-free region between the source and the detector are measured, and packets of isomass ions are quantified. Conversion of time spectrum into a mass spectrum is managed by calibrating the analyzer through measuring the flight times of ions of known mass. So, mass spectrum is acquired.

In the past, TOF suffered from the limitation of poor resolution because of the ions' kinetic energy inhomogeneity, which results in recording different arrival times for ions of same mass. This kinetic energy (energy of ion formation) distribution is reduced by use of an energy-correcting device, named as ''reflectron''. A retarding field created by the reflectron enables larger pathways for more energetic ions to reach the detector at the same time with less energetic ions of the same mass (Figure 2.11). Mass resolution is, hence, improved. There exist also spatial (location of ion formation) and temporal (time of ion formation) distributions affecting mass resolution inversely. By a technique known as ''delayed extraction'', a time delay is set between ion formation and ion extraction from the source so that spatial and temporal distributions can be prevented.

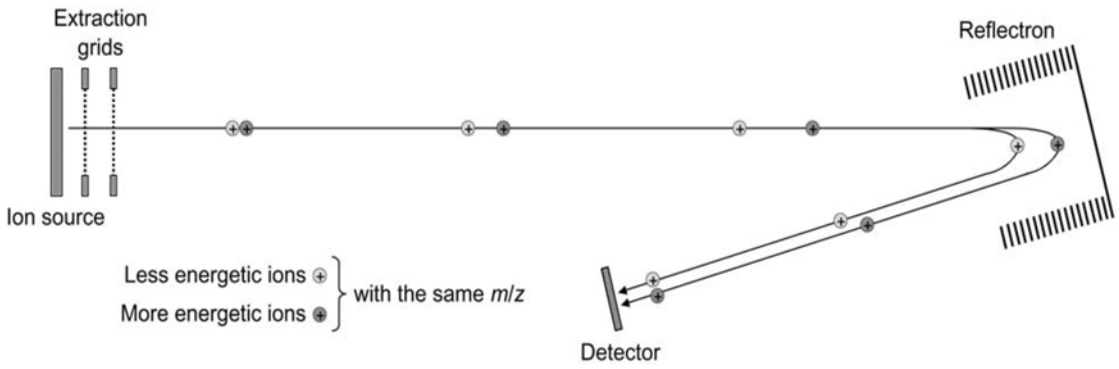


Figure 2.11. A Sketch of a Reflectron Time-of-Flight Mass Analyzer
(Source: Lane 2005)

To obtain more detailed structural information for the characterization of proteins, further analysis can be accomplished in reflectron mode MALDI-TOF MS by the production of fragment ions following the ionization, a method called ‘’post-source decay’’ (PSD). Applying different voltages, reflectrons can differentiate the fragment and precursor ions of same velocity based on their different kinetic energies. In the end, a very useful mass spectrum of fragment ions is obtained.

2.3.3.2. Quadrupole Ion-Trap

Quadrupole ion-traps are very compact, versatile and robust mass analyzers, first introduced in the early 1950s (Paul and Steinwedel 1953). The working principle is first trapping the ions and then detecting them according to their *m/z* ratios. The trap typically consists of three hyperbolic electrodes, a ring electrode and two end-cap electrodes. By applying DC and RF voltages to the electrodes, ions with a wide *m/z* range can be trapped within the space between the electrodes. So ions are confined by the RF field and they follow an eight-shaped oscillating trajectory related to their *m/z* ratio. Helium gas is introduced inside the trap to remove excess energy from the ions as the RF potential increases so that ions can remain closer to the centre of the electrodes. Increasing the DC and RF potentials makes ions of higher *m/z* unstabilized so that they are ejected from the trap along the axis of the end-caps. Oscillating frequencies are a function of ion masses; thus, ions with different *m/z* leave the trap at different voltages and times. A matchless virtue of an ion-trap is its ability to store fragment ions for further fragmentation analysis.

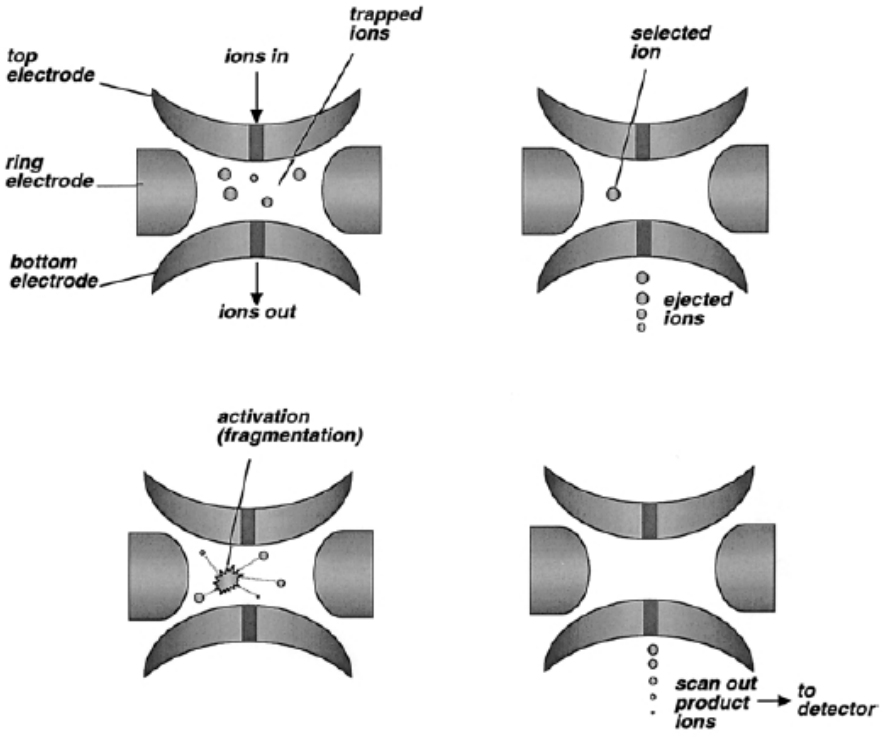


Figure 2.12. Schematic Diagram of an Ion-Trap
(Source: Liebler 2002)

2.3.4. Ion Detectors and Protein Identification

Following to the mass analysis, ions reach the ion detector for the detection of their mass and abundance. Ion current generated by the number of ions striking the detector is measured and amplified when necessary by electrometer tubes and vibrating reed electrometers. Electron multiplier tube is a common type of detectors used in MS. Faraday cup, photomultiplier conversion dynode, multichannel plate, charge detector are other detector types.

There exist two basic routes by which proteins are identified using MS. These are peptide mass fingerprinting (PMF) by MALDI-MS and peptide sequence tagging by ESI-MS. PMF is related to the identification of proteins using data from intact peptide masses. In this approach, proteins are identified by comparing the list of peptide masses obtained from proteolytic digestion of an unknown protein with a calculated list of all expected peptide masses for each entry in a protein database. If the theoretical peptide

masses are well-correlated with the experimental ones, protein is said to be identified. The major drawbacks of PMF include its incompatibility with protein mixtures and relatively pure sample requirement. On the other hand, peptide sequence tagging is well-suited with analysis of protein mixtures. This technique is based on fragmentation of peptides which produces a short stretch of amino acids. A partial amino acid sequence of a peptide is then obtained by the interpretation of the MS/MS spectrum. In both approaches, database searching benefits from algorithms that have been constructed simultaneously from the beginnings of 1990s.

2.4. Aim of the Study

The objective of this study was mainly to investigate the influence of heat stress to the protein levels of a cold-adapted *Pseudomonas marginalis* strain by proteomic analysis. After assigning a temperature of 15˚C as a control temperature while 30˚C as a heat stress temperature, bacterial cultures were grown until the late exponential phase at these temperatures. Following to the extraction of total protein content, a comparison of protein pattern expressions in these two different cultivations of *Pseudomonas marginalis* was carried out using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Differentially expressed protein spots were in-gel digested and identified by nanoscale capillary liquid chromatography coupled with electrospray ionization tandem mass spectrometry (nanoLC-ESI-MS/MS), and database searches (Mascot search engine and NCBInr protein database) in order to highlight the mechanism of heat stress response. The role of these heat stress-specific proteins in heat adaptation was also discussed.

CHAPTER 3

MATERIALS AND METHODS

3.1. Bacterial Strain and Growth Conditions

The cold-adapted mesophilic strain of *Pseudomonas marginalis* isolated from soil samples of Antarctica was used throughout this study. Bacterium was routinely propagated and maintained on Luria-Bertani (LB) agar at room temperature for 48 hours. The precultures of the strain were prepared by picking a single colony from *Pseudomonas marginalis* stock and inoculating into 20 ml of LB media in sterile 50 ml-falcon tubes and incubating at room temperature for 24 hours with shaking at 180 rpm. Experimental cultures were prepared by inoculation of these 24-hours-old precultures into sterile fresh LB media. Growth experiments were performed in 100 ml volumes of LB media, inoculated with 100 µl of the precultures, in 250 ml-Erlenmeyer flasks (1:1000 dilution). In order to apply heat stress, it was necessary to determine optimum growth temperature of the strain, thus; *Pseudomonas marginalis* cells were grown aerobically at five different steady-state temperatures, 5, 10, 15, 24, and 30˚C, for 72 hours with shaking at 180 rpm. Bacterial cell growth was monitored with a spectrophotometer by measuring the optical density at a wavelength of 600 nm (OD_{600}) every four hours during 3 days.

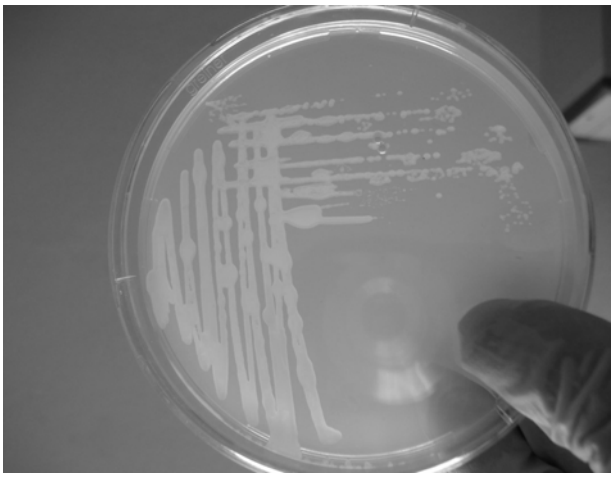


Figure 3.1. The Photograph of *Pseudomonas marginalis* Colonies on LB agar

- Preparation of LB media: 10 g of tryptone (AppliChem), 5 g of yeast extract (AppliChem), and 5 g of NaCl (AppliChem) were weighed and dissolved in 900 ml of distilled water, pH was adjusted to 7.00 with 1 M NaOH, and final solution was diluted to 1000 ml with distilled water. Finally, the prepared solution was autoclaved for sterilization. For the preparation of LB agar, 15 g of agar (AppliChem) was also added to prepared solution after adjusting pH to 7.00.

3.2. Heat Stress Treatment

After constructing growth curves for each temperature and calculating growth rates based on these growth curves, a temperature of 15˚C was observed as the optimum growth temperature for *Pseudomonas marginalis* strain being studied while 30˚C was chosen as the heat stress temperature. Consequently, cultures of *Pseudomonas marginalis* were grown at 15˚C and 30˚C as control unstressed and heat stressed samples, respectively until they reached late exponential phase which took nearly 36 hours (OD_{600} ~ 1.3) for both temperatures.

3.3. Protein Extraction from Bacterial Cells

The extraction of total protein content of *Pseudomonas marginalis* cells were performed by TRIzol method, which is a rapid way of extracting protein, DNA, and RNA from the cells. After 80 ml of samples had been taken from the cultures and transferred into 2 x 40 ml-centrifuge tubes, control (15˚C) and heat stress-exposed (30˚C) cells of *Pseudomonas marginalis* at the late exponential phase of growth were harvested by centrifugation at 15,000 x g for 10 minutes at 4^{o}C. Pellets were washed twice with 80 ml of ultrapure water (18 ohm) to facilitate the removal of any remaining salty material, and allowed to stand at -80˚C for 10 minutes. Then, frozen pellets were removed from -80˚C, put into the mortar, and liquid nitrogen was immediately poured into the mortar. Using a pestle and adding liquid nitrogen when evaporated, pellets were grinded well until they became homogenous powdered material weighing around 1 gram. After transferring into centrifuge tube, 10 ml of TRIzol reagent was added to the sample tube, vortexes well, and then 2 ml of chloroform (AppliChem) was added to the

final solution. The solution was allowed to stand at room temperature for 15 minutes following to shaking vigorously. Then, it was centrifuged at 13,000 x g for 17 minutes at 4˚C, and then three phases were observed; upper phase containing RNA, interphase with DNA, and lower phase containing whole protein. Protein phase (~10 ml) was transferred into another centrifuge tube using Pasteur pipet. Proteins were precipitated with using 15 ml of isopropyl alcohol (AppliChem) and overall solution was allowed to stand at room temperature for 10 minutes. Subsequently, the sample solution was centrifuged at 12,000 x g for 10 minutes at 4˚C, and supernatant was discarded. Protein pellet was washed three times with 20 ml of 0.3 M guanidine hydrochloride/95% ethanol solution. For each period of washing, the protein pellet was allowed to stand at room temperature for 20 minutes in wash solution, and centrifuged at 7,500 x g for 5 minutes at 4˚C. After three period of washing, protein pellet was solubilized in 1.5 ml of ethanol (AppliChem) and then transfer to the 2.0 ml-eppendorf tube to store at -80˚C for further analysis.

- Preparation of TRIzol reagent: 42 ml of phenol liquid (91%) (AppliChem), 9.4528 g of guanidine thiocyanate (AppliChem), 3.0448 g of ammonium thiocyanate (AppliChem), 0.8203 g of sodium acetate (Merck), and 5 ml of glycerol (AppliChem) were mixed. The volume was adjusted to 90 ml with distilled water and pH was adjusted to 5.00, and then the final volume was adjusted to 100 ml. The solution can be stored at 4˚C for several months. Final concentrations were: 38% phenol, 0.8 M guanidine thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate, and 5% glycerol.
- Preparation of guanidine hydrochloride/95% ethanol: 4.299 g of guanidine hydrochloride (AppliChem) was weighed and diluted to 150 ml with 95% ethanol (AppliChem).

3.4. Protein Sampling and Solubilization

Prior to protein sampling and solubilization, ethanol was removed using SpeedVac but not to full dryness. Then, the protein pellet was resuspended in 200 µl of rehydration buffer, and vortexes well for about 5 minutes in order to be solubilized

completely. Then, the mixture was centrifuged at 17,000 x g for 5 minutes at 4˚C, and supernatant was collected to clean ependorf for further analysis and the remaining solid residue was discarded.

- Preparation of rehydration buffer: 0.42 g of urea (AppliChem), 0.152 g of thiourea (AppliChem), and 0.04 g of CHAPS (AppliChem) were dissolved in 1 ml of ultrapure water. 0.01 g of DTT (AppliChem) and 25 µl of ampholyte pH: 3-10 (Fluka) were added immediately before use.

3.5. Determination of Protein Concentration

Before 2D-PAGE analysis, it was required to determine the concentration of proteins in the samples so that the same amounts of protein can be applied onto the gels for better comparison with control sample. Protein concentrations in the samples were determined based on the method of Bradford (Bradford 1976) with bovine serum albumin (BSA) as the standard. The principle is based on the event that the absorbance maximum for an acidic environment of the dye Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when the dye binds to the protein through Van der Waals forces and hydrophobic interactions and the result is the colour change from green to blue.

For Bradford assay, a standard curve of absorbance at 595 nm versus protein concentration is necessary to be constructed by using BSA protein as a standard. First, a stock solution of BSA was prepared. Then, standards, ranging from 1 µg/ml to 12 µg/ml were prepared from stock solution of BSA in 1 ml-plastic cuvettes according to the Table 3.1. After incubating at room temperature for 5 minutes, absorbance of the standards at 595 nm was measured by spectrophotometer, and the standard curve was constructed. In the end, a linear regression equation (y=mx+b) was obtained from which protein concentration could be calculated by substituting absorbance value for ‘’y’’ and solving the equation for ‘’x’’.

Table 3.1. Preparation Table for a Bradford Standard Assay using BSA

Sample	Sample Volume, µl	Distilled Water Volume, µl	Coomassie Reagent Volume, µl	Final Standard Concentration, µg/ml
Reference	0	800	200	0
BSA stock	50	750	200	2
BSA stock	150	650	200	6
BSA stock	200	600	200	8
BSA stock	250	550	200	10
BSA stock	300	500	200	12
Protein	2	798	200	?

- Preparation of Coomassie reagent: 10.0 mg of CBB G-250 (AppliChem) was dissolved in 5 ml of 95% ethanol. The solution was added on 85% phosphoric acid (AppliChem). The overall solution was filtered through Whatman No.1 filter paper. Filtered solution (~15 ml) was diluted to 100 ml with distilled water and stored in an amber bottle at 4˚C.
- Preparation of 40 µg/ml BSA stock solution: 40 mg of BSA (Fluka) was weighed and diluted to 1 ml with distilled water. 100 µl of this solution was diluted to 1 ml with distilled water. Again, 100 µl of the second solution was diluted to 1 ml with distilled water. Finally, 100 µl of the third solution was diluted to 1 ml with distilled water. By this way, 1:1000 dilutions were performed to get a concentration of 40 µg/ml.

Table 3.2. Absorbance Values of BSA Standards

Concentration of BSA Standard	Absorbance at 595 nm (OD_{595})
2 µg/ml	0.1105
6 µg/ml	0.2424
8 µg/ml	0.3129
10 µg/ml	0.3891
12 µg/ml	0.4199

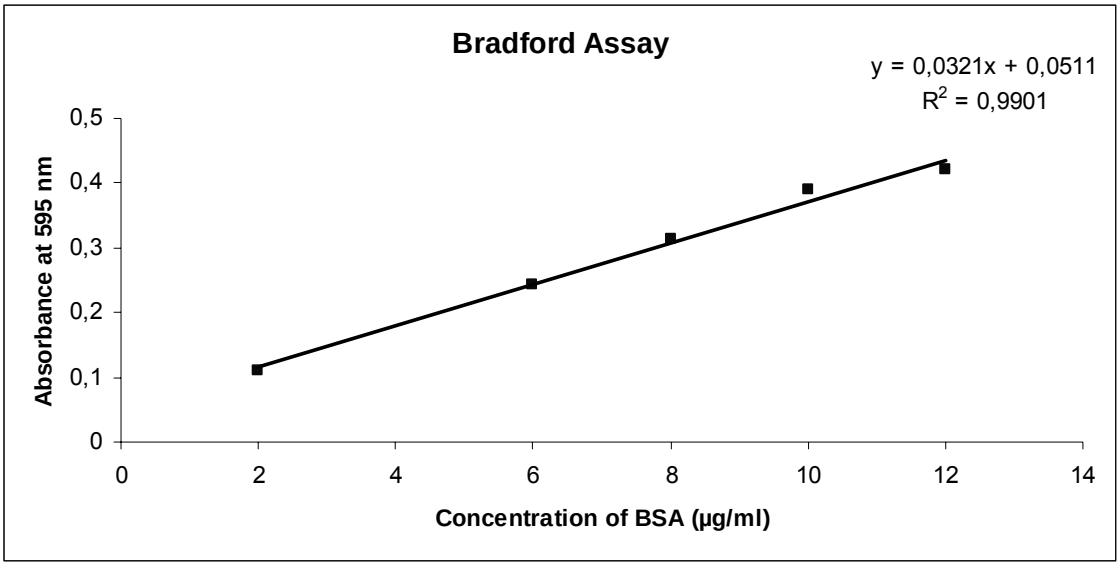


Figure 3.2. BSA Standard Curve

3.6. 2D-PAGE Analysis of *Pseudomonas marginalis* Proteins

3.6.1. Isoelectric Focusing

Isoelectric focusing was carried out on 17 cm-immobiline strips which provided a linear gradient from pH 3 to 10 (Bio-Rad) by a PROTEAN IEF Cell (Bio-Rad).

Firstly, the IEF focusing tray was cleaned and dried. Then, paper wicks were inserted on the wire electrodes to prevent direct contact with IPG strips, which could lead to undesired burns on strips. To enable electrical conductivity, paper wicks were wetted with 8 µl of ultrapure water. As it is mentioned, the same amount of total protein was required to be used for each electrophoretic run to perform objective comparisons of different protein patterns. For this purpose, protein samples of 15˚C and 30˚C were loaded into channels of the IEF focusing tray in such a way that totally 300 µg of protein was loaded for 350 µl of the mixture of protein sample and rehydration buffer (Table 3.3.). If formed, air bubbles were removed in case of uneven distribution of the sample in the IPG strip. Then, IPG strips were taken out from -20˚C and allowed to thaw for 10 minutes at room temperature. Afterwards, cover sheet was peeled from the IPG strip using forceps and the IPG strip was placed gently gel side down, onto the sample, in the IEF focusing tray so that the acidic end, marked with ‘’+’’, was at the

anode of the IEF cell. In order to assist in absorption of proteins by the strip, the IPG strip was lift and lowered twice or third times slowly. Subsequently, each strip was overlaid with 2 ml of mineral oil (Bio-Rad) to prevent evaporation and urea crystallization during the rehydration process. At last, the plastic lid of the tray was covered and waited for an hour at room temperature without voltage.

Table 3.3. Protein Concentrations determined by Bradford Assay and Amount of Protein Loads for IEF

Sample	**Protein Concentration**	**Protein Sample Volume (for 300 µg load)**	**Rehydration Buffer Volume**	**IPG Strip Total Load Volume**
15˚C	4.58 mg/ml	76 µl	274 µl	350 µl
30˚C	4.98 mg/ml	70 µl	280 µl	350 µl

After that, 0.5 ml of extra mineral oil was added on the strips again, and the PROTEAN IEF cell was programmed for active rehydration mode. The running condition of IEF cell at 20˚C was shown in Table 3.3. Total process was completed nearly in 20 hours. When isoelectric focusing was finished, IPG strips containing focused proteins based on their isoelectric points were removed from the focusing tray, and stored at -80˚C until required or prepared instantly for equilibration steps.

Table 3.4. PROTEAN IEF Cell Focusing Conditions

Step	**Voltage**	**Time, hours**	**Volt-Hours**	**Ramp**
Active Rehydration	50 V	12	---	---
Step 1	250 V	0.5	---	Linear
Step 2	10,000 V	2.5	---	Linear
Step 3	10,000 V	---	30,000	Rapid
Total	---	~ 20	~ 40,000	---

3.6.2. SDS-PAGE

SDS-PAGE experiments were performed in Bio-Rad PROTEAN II XL electrophoresis cell. Dimensions of the glass plates were 16 x 20 cm for inner plate and 18.3 x 20 cm for outer plate. Prior to analysis, the glass plates were assembled according to the instructions in the manufacturer's manual.

3.6.2.1. Pouring SDS-Polyacrylamide Gels

As it is stated above, SDS-polyacrylamide gel contains only the resolving gel but not the stacking gel. The resolving gel for SDS-PAGE should be prepared the day before SDS-PAGE analysis, and kept at 4˚C overnight. The composition of acrylamide for the resolving gel was chosen as 12% so that components of the gel could be prepared according to this value. Preparation of the resolving gel components were described below.

- Preparation of 30% acrylamide mix: 8.70 g of acrylamide (AppliChem) and 0.30 g of N, N´-methylenebisacrylamide (AppliChem) were taken into a falcon tube covered with aluminium foil, and dissolved in 30 ml of distilled water. The pH of the mixture was checked whether it was 7.0 or smaller, if not adjusted.
- Preparation of 1.5 M Tris-HCl pH 8.8: 3.6344 g of Tris-HCl (AppliChem) was dissolved in 20 ml of distilled water, and the pH was adjusted to 8.8 with 6 M HCl.
- Preparation of 10% SDS: 0.1 g of SDS (AppliChem) was dissolved in 1.0 ml of distilled water.
- Preparation of 10% ammonium persulfate: 0.1 g ammonium persulfate (Sigma) was dissolved in 1.0 ml of distilled water.

Two SDS-polyacrylamide gels were prepared for samples of 15˚C and 30˚C in a comparative manner. Since the volume of each gel mold was approximately 35 ml, necessary volume of each solution component was adjusted to prepare 70 ml of 12% SDS-polyacrylamide gel (Table 3.5).

Table 3.5. Required Volumes of 12% SDS-Polyacrylamide Gel Components for two gels

SDS-Polyacrylamide Gel Components	Volume, ml
dH_2O	23.1
30% acrylamide mix	28
1.5 M Tris-HCl pH 8.8	17.5
10% SDS	0.7
10% ammonium persulfate	0.7
TEMED	0.028

After each solution component had been prepared, they were mixed in a 100 ml-beaker in the order of distilled water, 30% acrylamide mix, 1.5 M Tris-HCl pH 8.8, and 10% SDS. Since polymerization will begin as soon as the TEMED (Sigma) has been added, TEMED and 10% ammonium persulfate were added to the mixture at the same time. The final mixture was then swirled rapidly and poured into the gap between the glass plates without delay. After polymerization had been complete (~30 minutes), upper side of the gels were covered with distilled water and they were kept at 4˚C overnight.

3.6.2.2. Equilibration of IPG Strips

IPG strips were taken out of -80˚C and allowed to thaw at room temperature for 10 minutes. They were placed gel side up in the equilibration tray, and then covered with 5 ml of equilibration buffer I. The tray covered with aluminium foil was placed on an orbital shaker and shaken gently for 10 minutes. After that, buffers were discarded and IPG strips were placed gel side up again in a new clean equilibration tray. Similarly, 5 ml of equilibration buffer II was added on and the tray was shaken gently for 10 minutes in the dark medium.

- Preparation of equilibration buffer I: 3.6036 g of urea, 2.5 ml of Tris-HCl pH 8.8, 0.2 g of SDS, 2 ml of glycerol, and 0.2 g of DTT were taken and diluted to 10 ml with distilled water. Final concentrations were 6 M urea, 0.375 M Tris-HCl, 2% SDS, 20% glycerol, and 2% DTT.

- Preparation of equilibration buffer II: 3.6036 g of urea, 2.5 ml of Tris-HCl pH 8.8, 0.2 g of SDS, 2 ml of glycerol, and 0.25 g of iodoacetamide (Sigma) were taken and diluted to 10 ml with distilled water. Final concentrations were 6 M urea, 0.375 M Tris-HCl, 2% SDS, 20% glycerol, and 2.5% iodoacetamide.

3.6.2.3. Running the Gel

After period of equilibration of IPG strip, a 100 ml-graduated cylinder was filled with 1X Tris/glycine/SDS buffer and any bubbles on the surface of the buffer was removed using a Pasteur pipette. Then, equilibrated IPG strips were dipped briefly into the graduated cylinder respectively to be rinsed in the buffer. After that, each strip was laid gel side up on to the longer (back) glass plate, and connected with the gel without any air bubble at the interface. The glass plates were then held vertically by placing them in the gel box, and one ml of preheated overlay agarose solution was pipetted into the IPG well of each gel. After allowing the overlay agarose solution to solidify for 5 minutes, the reservoir of the gel box and the gap between the gels were filled with 1X Tris/glycine/SDS running buffer. Water circulation for controlled cooling of electrophoresis was enabled and electrophoresis was started according to the conditions given in the Table 3.6. The migration of the Bromophenol Blue which is present in the overlay agarose solution, was used to monitor the progress of the electrophoresis. When it reached the bottom of the gel, electrophoresis was stopped.

Table 3.6. SDS-PAGE Running Conditions

Program	Power Condition	Approximate Run Time
Constant Current	16 mA	1 hour
Constant Voltage	180 V	6-7 hours

- Preparation of 1X Tris/glycine/SDS running buffer: 15.1 g of Tris-base (AppliChem) and 94 g of glycine (AppliChem) were dissolved in 900 ml of distilled water. To this mixture, 50 ml of 10% (w/v) SDS was added on and the final volume was adjusted to 1000 ml with distilled water.

200 ml of this solution was taken and diluted to 1000 ml with distilled water. It was stored at 4˚C.

- Preparation of overlay agarose solution: 3.03 g of Tris-base, 14.40 g of glycine, and 1.0 g of SDS were dissolved in 1000 ml of distilled water, and labelled as SDS electroporesis buffer. Then, 0.50 g of agarose (AppliChem) and 2.0 mg of Bromophenol Blue were added to the 100 ml of SDS electrophoresis buffer in a beaker closed with aluminium foil. The solution was swirled to disperse and heated until the agarose was completely dissolved. 2 ml of aliquots were dispensed in eppendorf tubes and stored at 4˚C.

3.6.3. Staining of the Gels

Colloidal Coomassie Blue (CBB) G-250 staining was preferred to visualize the proteins separated on the gels. The gels were separated from the glass plates carefully, and taken into plastic staining trays. Then, enough staining solution (~250 ml) was added to staining tray to completely cover each gel. The trays were placed on an orbital shaker with shaking at 25 rpm to stain the gels overnight. At the end of staining, the gels were washed twice with distilled water, and immersed in neutralization buffer for 3 minutes. After that, neutralization buffer was discarded, and the gels were washed with distilled water again. Next, the gels were destained with destaining solution for about 50 seconds, and immersed in fixation solution overnight after washing with distilled water. For long term storage, the gels were maintained in 5% acetic acid (AppliChem) solution at 4˚C.

- Preparation of staining solution: 40.0 g of ammonium sulfate (AppliChem), 8 ml of 85% phosphoric acid, and 0.5 g of CBB G-250 were diluted to 400 ml with distilled water and 100 ml of methanol (Merck) was added. It was stored at 4˚C.
- Preparation of neutralization buffer: 10.96 g of Tris-dihydrogen phosphate (AppliChem) was dissolved in 450 ml of distilled water, the pH was adjusted to 6.5 with NaOH, and the final volume was completed to 500 ml with distilled water. It was stored at 4˚C.

- Preparation of destaining solution (25% methanol): 125 ml of methanol was mixed with 375 ml of distilled water. It was stored at 4˚C.
- Preparation of fixation solution (20% ammonium sulfate): 100 g of ammonium sulfate was dissolved in distilled water to a volume of 500 ml. It was stored at 4˚C.

3.6.4. Image Acquisition and Data Analysis

The CBB stained gels were scanned and photographed by AlphaImager (Alpha Imatech) CCD camera. The image analysis, spot detection/matching/quantitation, and determination of different protein spots were then performed with the 3-day free licensed software package of DECODON Delta2D Version 3.6. The software autodetected protein spots and matched them automatically. For qualitative analysis, differences in spot position were eliminated thanks to warping strategy of the program which aligned gel images to visualize differences in expression levels.

3.7. In-Gel Digestion of Proteins

Spots of differentially expressed proteins were determined at the end of image analysis. The proteins were then in-gel digested using trypsin according to the protocol described below:

- The protein spot was cut from the gel and placed in a 1.5 ml-eppendorf tube.
- 200 µl of the washing solution was added to rinse the gel pieces overnight at room temperature.
- The washing solution was discarded and 200 µl of the fresh washing solution was added. The gel pieces were rinsed for 2-3 hours at room temperature and the washing solution was then removed from the sample.
- 200 µl of acetonitrile was added and the gel pieces were dehydrated for 5 minutes at room temperature. Acetonitrile was discarded.
- The gel pieces were dried 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. The solution was removed.

- 30 µl of 100 mM iodoacetamide was added and the protein was alkylated for 30 minutes at room temperature. The solution was removed.
- 200 µl of acetonitrile was added. The gel pieces were dehydrated for 5 minutes at room temperature. Acetonitrile was discarded.
- The gel pieces were dried in a vacuum centrifuge for 2-3 minutes.
- Trypsin reagent was prepared immediately. The solution was kept on ice.
- 30 µl of the trypsin solution was added to the sample which was then rehydrated on ice for 10 minutes with occasional vortex mixing.
- The gel pieces were centrifuged for 30 seconds and the excess trypsin solution was removed.
- 5 µl of 50 mM ammonium bicarbonate was added to the sample. The mixture was vortexed and centrifuged for 30 seconds. The digestion was carried out overnight at 37˚C.
- 30 µl of 50 mM ammonium bicarbonate was added to the digest. It was incubated for 10 minutes with occasional gentle vortex mixing. Supernatant was collected and transferred to a 0.5 ml-plastic micro centrifuge tube.
- 30 µl of the extraction buffer was added to the tube containing gel pieces. It was incubated for 10 minutes with occasional gentle vortex mixing. The supernatant was collected and transferred to a 0.5 ml-plastic micro centrifuge tube.
- Another 30 µl of the extraction buffer was added to the tube containing gel pieces. It was incubated again for 10 minutes with occasional gentle vortex mixing. The tube was centrifuged for 30 seconds. The supernatant was collected and transferred to a 0.5 ml-plastic micro centrifuge tube.
- The volume of the extract was reduced to < 20 µl by evaporation in a vacuum centrifuge.
- The volume of the digest was adjusted to ~20 µl with 1% acetic acid for mass spectrometric analysis.

    - Preparation of washing solution (25% methanol): 10 ml of methanol, 5 ml of distilled water, and 1 ml of acetic acid were mixed. Total volume was adjusted to 20 ml with distilled water.
    - Preparation of 100 mM ammonium bicarbonate: 0.2 g of ammonium bicarbonate was taken and diluted to 20 ml with distilled water.

- Preparation of 50 mM ammonium bicarbonate: 2 ml of 100 mM ammonium bicarbonate was mixed with 2 ml of distilled water.
- Preparation of 10 mM DTT: 0.0015 g of dithiothreitol was dissolved in 1.0 ml of 100 mM ammonium bicarbonate.
- Preparation of 100 mM iodoacetamide: 0.018 g of iodoacetamide was dissolved in 1.0 ml of 100 mM ammonium bicarbonate.
- Preparation of extraction buffer: 10 ml of acetonitrile, 5 ml of distilled water, and 1 ml of formic acid were mixed. The final volume was adjusted to 20 ml with distilled water.
- Preparation of trypsin reagent: 1 ml of ice-cold 50 mM ammonium bicarbonate was added to 20 µg of trypsin (final concentration: 20 ng/µl). The solution was kept on ice.

3.8. Mass Spectrometric Analysis and Protein Identification

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.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Effect of Temperature on Bacterial Growth

As described in Materials and Methods, *Pseudomonas marginalis* cultures were grown at five different temperatures, 5, 10, 15, 24, and 30˚C, for 72 hours in order to determine optimum growth temperature and apply heat stress. The effect of temperature on growth is shown in Figure 4.1. It is interesting to observe that lag phase of growth is getting longer as growth temperature decreases. This obviously indicates that bacterium needs much more time to prepare itself for growth at lower temperatures. It could also be argued that heat treatment induces bacterial growth.

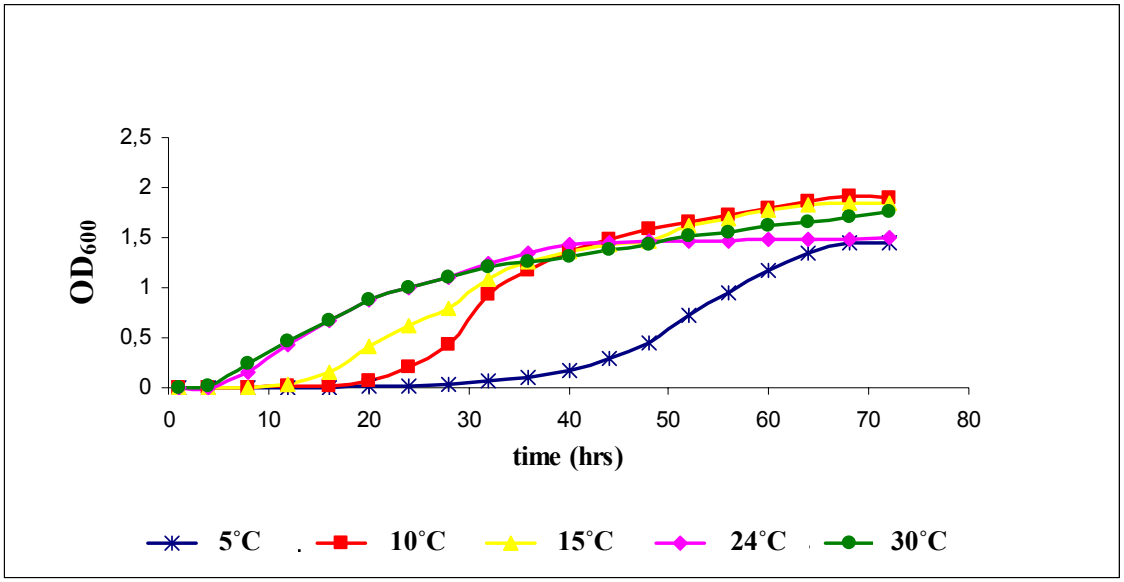


Figure 4.1. Growth Curves of *Pseudomonas marginalis* at 5 Different Temperatures

4.2. Kinetics of Cell Growth at Different Temperatures

After observing temperature dependence of bacterial growth, growth rates (μ) at each temperature were calculated using the formula $\mu = \frac{\ln I_1 - \ln I_0}{t_1 - t_0}$, where I stands for optical density at 600 nm (OD_{600}) at the beginning and the end of exponential phase in growth curve while t is the corresponding time in hours at these OD_{600} values.

By this way, the maximal growth was observed at 15˚C as 0.210 $hour^{-1}$, which in turn proved that this temperature was optimum growth temperature for *P. marginalis*. Other growth rates found at 5, 10, 24, and 30˚C were 0.075, 0.154, 0.189, and 0.162 $hour^{-1}$, respectively.

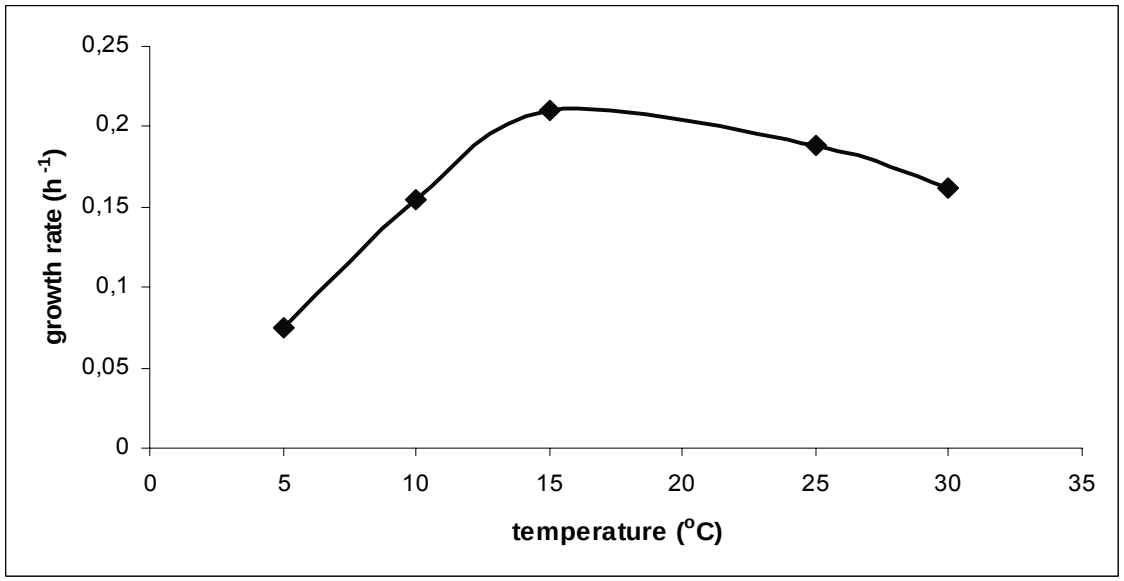


Figure 4.2. Calculated Growth Rates of *Pseudomonas marginalis* at 5 Different Temperatures

4.3. Heat Stress-Induced Proteins in *Pseudomonas marginalis*

For *Pseudomonas marginalis*, the synthesis of proteins in response to heat stress was evaluated by 2D-SDS-PAGE analysis. After protein samples from control unstressed (15˚C) and heat stressed (30˚C) bacterial cells grown to late exponential phase (OD_{600} =1.3) had been extracted, proteins were separated by two dimensional-

electrophoresis and the gels were stained with Coomassie colloidal blue as explained in previous chapter.

4.3.1. Differential Protein Expression to Heat Stress

At the end of 2DE analysis to gain an overview of heat stress-induced proteins, gel maps were obtained. Since proteome of an organism is not a stable entity, 2DE experiments were carried out three times and image analysis was performed according to the average gels constructed to show mutual proteins present in all three replicate gels for each condition. Considering the reproducibility of the protein maps, the overall pattern of protein synthesis was observed to be relatively stable except for minor differences. These inevitable differences in resolution might possibly be a result of small variations in the running and staining conditions. It was remarkable that some proteins were faintly stained or not stained by Coomassie blue. However, the effect of these qualitative and quantitative differences on the results might be relatively small and could be neglected.

In order to elucidate and compare protein synthesis patterns of *Pseudomonas marginalis* cultures to heat stress exposure, two individual gel images for control and stress conditions were imported into gel analysis software DECODON Delta2D Version 3.6. A total of 1391 protein spots were detected in representative protein profile of control cells while 1384 protein spots were detected in that of heat stressed-cells in the experimental window of molecular mass comprised between 10 to 200 kDa and with the pH ranging from 3 to 10. During the analysis, 667 spots were paired (matched) in both gels with a match quality of 0.6504144, and these pairs were used to compare protein expressions.

Comparative detailed analysis of the 2DE gels allowed us to distinguish alterations in the expression of several proteins and revealed that 13 new protein spots were differentially expressed in the heat stressed-cells (Figure 4.3 and 4.4). These spots were numbered and then cut out from the 30˚C gel for enzymatic digestion, mass spectrometry and database searches for identification. Magnified segments were used in Table 4.1 for the ease of observing differential expressions.

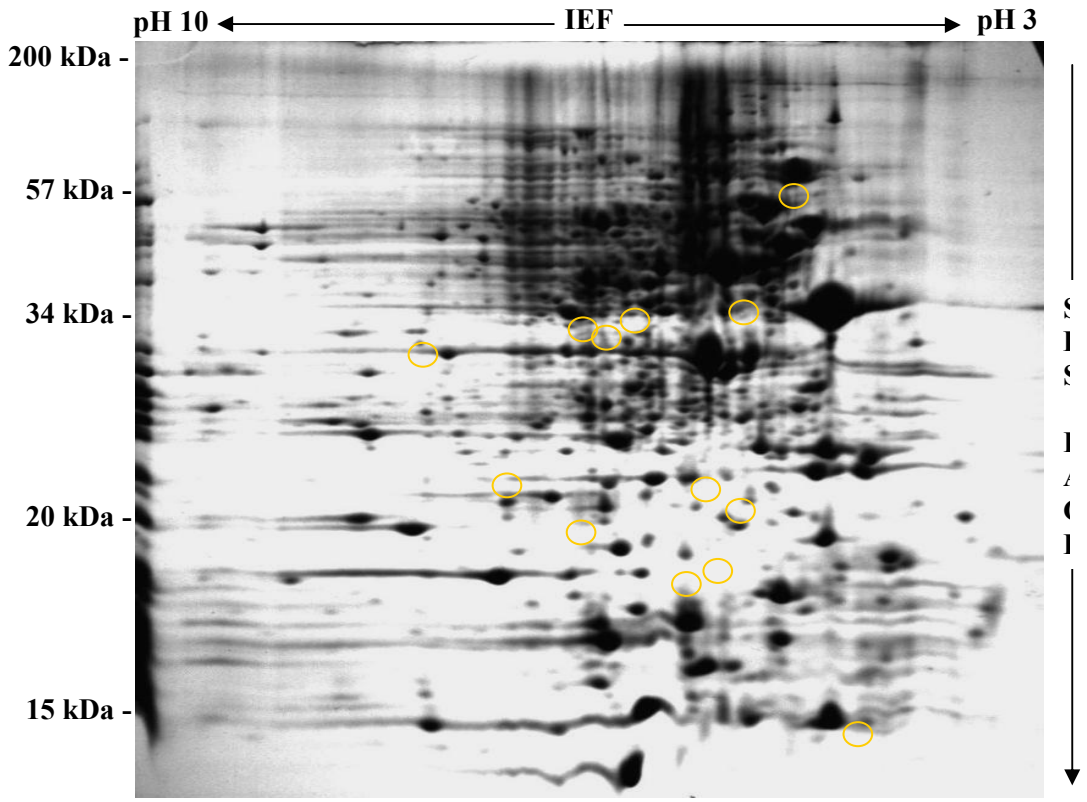


Figure 4.3. 2DE Protein Profile for Control Unstressed Cells of *Pseudomonas marginalis*

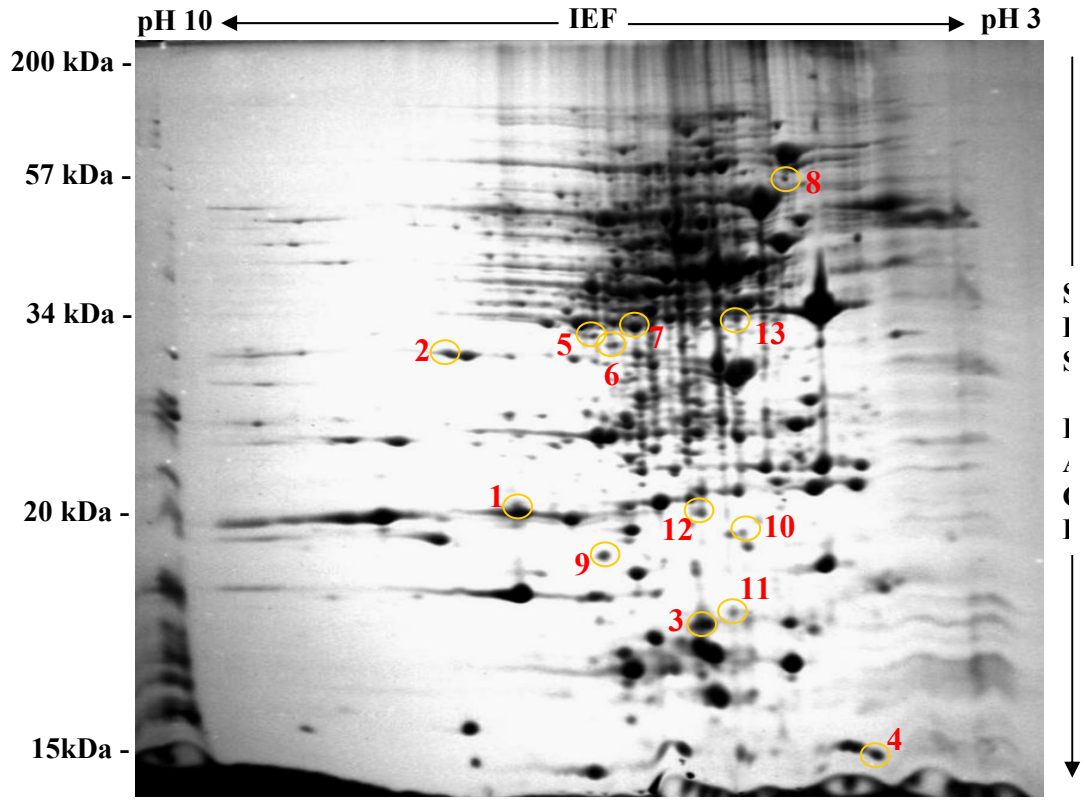
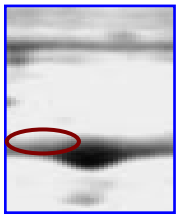
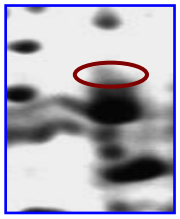
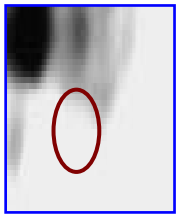
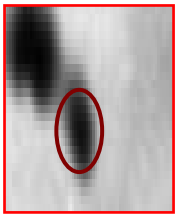
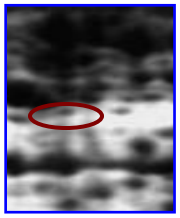
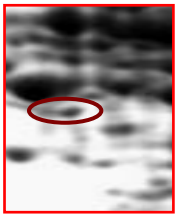
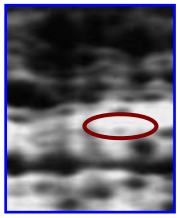
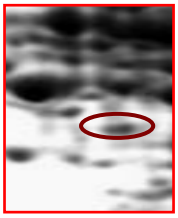
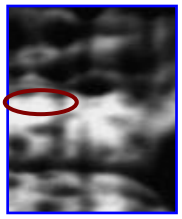
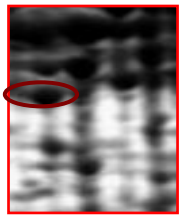


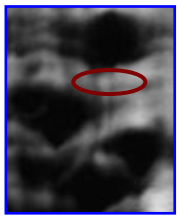
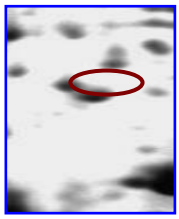
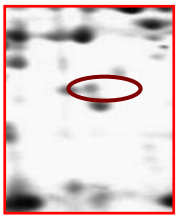
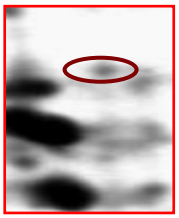
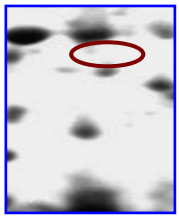
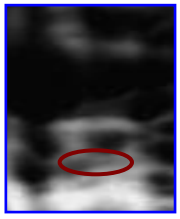
Figure 4.4. 2DE Protein Profile for Heat-Stressed Cells of *Pseudomonas marginalis*

Table 4.1. Segments of 2DE Gel Map

SPOT NO	CONTROL UNSTRESSED (15˚C) GEL FRAME	HEAT STRESSED (30˚C) GEL FRAME
1		
2		
3		
4		
5		
6		
7		

(cont. on next page)

Table 4.1. (cont.) Segments of 2DE Gel Map

8		
9		
10		
11		
12		
13		

4.3.2. Identification of Differentially Expressed Proteins

Following to the enzymatic digestion, proteins were characterized by nanoLC-ESI-MS/MS and mass spectrometric data was compared to the protein database for sequence matches. Reliable protein spot identification was performed thanks to

parameters such as sequence coverage, molecular mass and isoelectric point. Since there is no study on the nature of the 2DE protein patterns for *Pseudomonas marginalis*, database search resulted in protein matches of different organisms, most of which are *Pseudomonas fluorescens* origin. Table 4.2 summarizes the differential expression of 13 proteins that were characterized. The proteins were identified as follow: spot 1, ribosome recycling factor; spot 2, extracellular solute-binding protein/family 3; spot 3, MaoC-like dehydratase; spot 4, nucleoside diphosphate kinase; spot 5, FAD dependent oxidoreductase; spot 6, Zinc-containing alcohol dehydrogenase superfamily; spot 7, universal stress protein family; spot 8, chaperonin GroEL; spot 9, Ubiquinol-cytochrome c reductase/iron-sulfur subunit; spot 10, 50S ribosomal protein L5; spot 11, Glyoxalase/bleomycin resistance protein/dioxygenase; spot 12, lactoylglutathione lyase; and spot 13, recombination associated protein.

Of the proteins that have been identified, universal stress protein and chaperonin GroEL are of particular interest because they are direct sensors of heat stress treatment. Universal stress protein (UspA) is an autophosphorylating serine and threonine phosphoprotein (Freestone, et al. 1997). Figure 4.5 shows the dimer of UspA with an apparent molecular weight of 34 kDa. It was found to be unique in its almost universal responsiveness to a large variety of stress conditions, including stationary phase, starvation for carbon, nitrogen, phosphate, sulfate, and amino acids, and exposure to oxidants, metals, polymyxin, cycloserine, ethanol, antibiotics, and osmotic shock in addition to heat stress (Nyström and Neidhardt 1994, Gustavsson, et al. 2002, Kvint, et al. 2003).

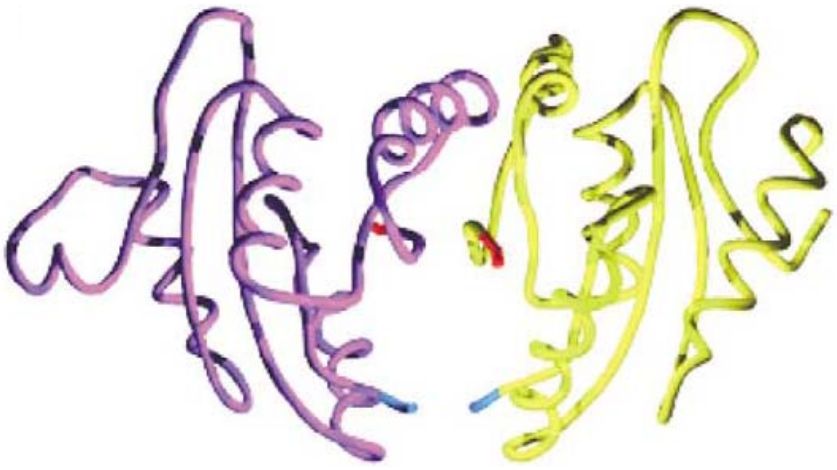


Figure 4.5. Diagram of the α Carbon Backbone Trace of UspA
(Source: Kvint, et al. 2003)

Table 4.2. List of Identified Heat Stress-Induced Proteins of *Pseudomonas marginalis*

Spot No.	Protein Identity	Accession No.(gi | NCBI)	Reference Organism	Matched Peptides*	Sequence Coverage (%)	Mascot Mowse Score	MW/pI (kDa)
1	Ribosome recycling factor	77457331	*Pseudomonas fluorescens PfO-1*	TLQVVAFER DANSSLKDLVK	10%	94	20.5/6.21
2	Extracellular solute-binding protein, family 3	77460756	*Pseudomonas fluorescens PfO-1*	IKESGVITLGHR YNLVTSQTRIPLVQNGTVDVECGSTTNNVER GKNVVTTAGTTSER AMNADKQMGMNVISAKDHGESFQMLESGRAVAFMMDDALLAGEAAK ATYASGEINK WFMQPIPPKGLNLNFPMSDELKALIANPTDK	47%	627	32.9/6.45
3	MaoC-like dehydratase	77461203	*Pseudomonas fluorescens PfO-1*	PYVPVAELK DYVGK SEWLTIDQERINLFAEATGDYQFIHVDPVKAAQTPFGSTIAHGFLSLSLIPK MVVNYGLDSVRFIQPVK KPGQWLLKATATLEIEGSDKPAYIAEPLSLCFV	76%	526	16.8/5.41
4	Nucleoside diphosphate kinase	28868637	*Pseudomonas syringae pv. tomato str. DC3000*	ADFADSIDANAVHGSDSEAAAAR	16%	92	14.9/ 5.41
5	FAD dependent oxidoreductase	77460953	*Pseudomonas fluorescens PfO-1*	RSDAGSLDMGAQYFTAR RFVTEVQR VEGAWLSGQEAAR	11%	275	35.8/6.09
6	Zinc-containing alcohol dehydrogenase superfamily	77459164	*Pseudomonas fluorescens PfO-1*	GILIDKDDSGYRANLQEISDEQLPDGDVTVRVAYSTLNFKDGLAITGSSPVVR LNGDWLIPLPK QLGASEIIDR GVTLAGINSVTQPK	26%	405	34.3/6.00
7	Universal stress protein family	156622675	*Pseudomonas sp. MIS38*	RIVAAVDPFHR VLASYADAYDIDVIVMGR	17%	128	34.1/5.67
8	Chaperonin GroEL	52424514	*Mannheimia succiniciproducens MBEL55E*	MLAGVNVLADAVK AVAAGMNPMDLK	4%	93	57.3/4.83
9	Ubiquinol-cytochrome c reductase, iron-sulfur subunit	77460913	*Pseudomonas fluorescens PfO-1*	IEPGQQMIAEWRGQPVFIVRRTEEILGNLKKIEGQLSDPTSK	21%	349	21.0/7.68
10	50S ribosomal protein L5	26987207	*Pseudomonas putida KT2440*	VIEHAVADLEK	6%	66	20.3/9.69

(cont. on next page)

Table 4.2. (cont.) List of Identified Heat Stress-Induced Proteins of *Pseudomonas marginalis*

11	Glyoxalase/bleomycin resistance protein/dioxygenase	77460142	*Pseudomonas fluorescens PfO-1*	AAEAIDFYKKAFGATEVMRLSMPDGGIGHAELR SGTLKDPYGHLWFLATRKEDLTQEQIEQR	40%	643	16.7/5.29
12	Lactoylglutathione lyase	70730757	*Pseudomonas fluorescens Pf-5*	FVFNHTMLR SLDFYTR FEALGCDFQK	15%	110	19.4/5.31
13	Recombination associated protein	70731752	*Pseudomonas fluorescens Pf-5*	QGLILVNSASPK EVLGTLPVRPLTVK VVTQLSLAWQDK	12%	148	34.3/4.93

***** Complete sequences of differentially expressed proteins were listed in Appendix A.

Concerning the regulation of UspA, differential expression of the *uspA* gene is elicited by elevated initiation of transcription from a σ^{70}-dependent promoter (Nyström and Neidhardt 1992). There are a number of regulators found to be responsible for the control of *uspA* expression. One regulatory pathway involves activation of the *uspA* promoter by alarmone guanosine tetraphosphate (ppGpp) via the b-subunit of RNA polymerase (Kvint, et al. 2000). There is also another mechanism proposed by Farewell and his colleagues in which the gene expression is controlled by fatty acid responsive transcription factor FadR (Farewell, et al. 1996). They suggested that the gene is a part of the FadR regulation so that UspA can take part in fatty acid/membrane lipid metabolism. Additionally, *uspA* is considered to be regulated by the FtsK protein according to the study of Diez and his co-workers (Diez, et al. 1997). In conclusion, increased levels of UspA synthesis during heat stress exposure are thought to improve the cell's survival capacity even though the exact biochemical function of Usp family proteins is still unknown.

Chaperonin GroEL, on the other hand, is a very common heat shock protein which is composed of two heptameric rings of identical 58 kDa subunits symmetrically stacked back to back to form a hollow cylinder of ~800 kDa (Martin, et al. 1994). Apart from heat stress, its synthesis is increased by a number of external factors like ultraviolet radiation, exposure of ethanol and heavy metals. The gene responsible for its production is *rpOH* which codes for a polypeptide with a molecular weight of 32 kDa, σ^{32}. This sigma factor acts as a regulator of the heat stress response by associating with RNA polymerase core (E) in order to form the $E\sigma^{32}$ holoenzyme (Grossman, et al. 1984, Landick, et al. 1984). The $E\sigma^{32}$ polymerase recognizes definite promoter sequences with two well conserved parts, -CCCTTGAA- and –CCCATTTA, which are accepted as the heat shock- (or σ^{32}-) promoter (Cowing, et al. 1985).

Chaperonins are commonly known as structures using the energy from ATP hydrolysis to assist protein folding in the cell (Bukau and Horwich 1998). Various electron microscopy studies have proved that GroEL binds many kinds of nonnative polypeptides and hydrophobic residues within its central cavity (Braig, et al. 1993, Fenton, et al. 1994, Buckle, et al. 1997). Although GroEL alone is enough to mediate the folding of some substrates, it usually needs other chaperonin known as GroES for protein folding (Schmidt, et al. 1994). GroES is a single heptameric ring of identical 10 kDa-subunits and acts as a fundamental component in protein folding. Because binding of GroES to the ends of the GroEL cylinder changes the conformation of GroEL by

doubling the volume of its central cavity (Chen, et al. 1994, Xu, et al. 1997). By this way, the formed GroEL-GroES complex provides a protected polar environment to initiate the folding of substrate polypeptide. Nevertheless, a different aspect of GroEL function was investigated by Zahn and his colleagues. According to their observations, the main function of GroEL is providing a hydrophobic surface which binds folding intermediates to prevent their aggregation but not a protected cavity (Zahn, et al. 1996).

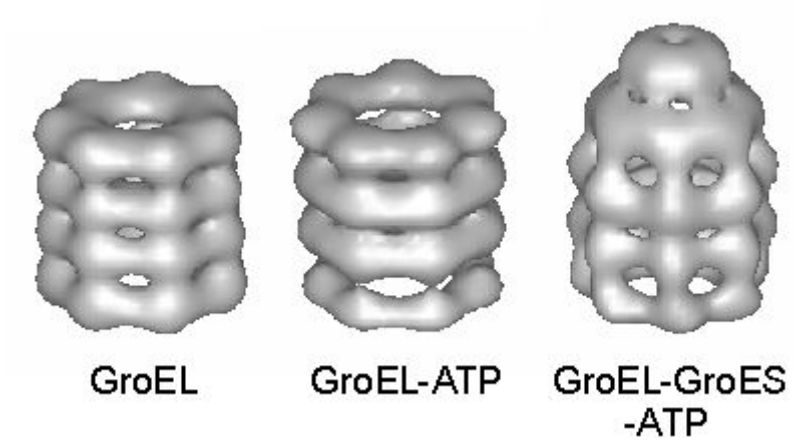


Figure 4.6. Surface-Rendered Views of 3D Reconstructions of GroEL, GroEL-ATP and GroEL-GroES-ATP from cryo-EM (Source: Chen, et al. 1994)

Regarding to the production of other proteins in response to heat stress, spot 1 protein ribosome recycling factor (RRF) attracts most attention. It is mainly responsible for dissociation of ribosomes from mRNA after termination of translation. By this way, RRF acts to recycle ribosomes so that they can be ready for another cycle of protein synthesis. The interesting aspect of RRF is the study of Van Bogelen and Neidhardt which revealed the fact that ribosomes or ribosome-associated factors take part in heat stress response (Van Bogelen and Neidhardt 1990). Therefore, it can easily be concluded that RRF is one of the critical proteins that play a certain role in heat stress adaptation.

On the other hand, ten proteins, probably involved in metabolic processes, were also found to be synthesized under heat stress conditions but their exact biochemical mechanism by which they protect cells from harmful effects of heat stress exposure could not been determined. Complementary studies should be performed to increase the understanding of their role in heat stress treatment.

CHAPTER 5

CONCLUSION

In the present study, the main objective was to use proteomic analysis for the investigation of heat stress response in cold-adapted strain of *Pseudomonas marginalis*. Two-dimensional SDS-PAGE analysis of cellular proteins of this bacterium exposed to heat stress at 30˚C allowed us to identify general and stress-specific proteins of *Pseudomonas marginalis*. Around 1400 proteins were detected with 2D analysis. Comparative analysis of 2D-SDS-PAGE gels for control and stress conditions revealed that 13 proteins of late exponential phase cells of *Pseudomonas marginalis* were differentially expressed in response to heat stress. Of these, universal stress protein and chaperonin GroEL were identified as direct indicators of heat stress treatment while ribosome recycling factor was also found to be involved in heat stress response. However, the mechanisms by which other ten proteins were synthesized in response to heat stress were unknown. They might presumably have various roles in cell physiology of temperature sensing. Further studies should be carried out to investigate their role in heat stress response. Based on these considerations, the heat stress response of *Pseudomonas marginalis* can be evaluated to be a little bit complex process.

When it comes to the aspects regarding the application of this research, because universal stress protein and chaperonin GroEL were found to be proteins of survival in heat stress environment, the genes responsible for these proteins can be isolated and cloned into any other useful microorganism such as bacteria used for detoxification of industrial waste or used in bioremediation but not capable of surviving at higher temperatures, maintaining their efficiency at those temperatures.

All things considered, to our knowledge, this study is of particular importance since it is the first study in which 2DE analysis, visualization of the general protein pattern, and identification of heat stress proteins were combined to explain mechanisms of heat stress response in *Pseudomonas marginalis*.

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APPENDIX A

SEQUENCES OF DIFFERENTIALLY EXPRESSED PROTEINS IN *Pseudomonas marginalis*

Sequences of matched peptides were highlighted in red.

1. Spot 1 - Ribosome recycling factor:

MINEIKKDAKERMTKSVESLAHNFGRIRTGQAHPSILEGVMVPYYGADTPIKQV
ANITVKDAR**TLQVVAFER**NMLGAVDKAIGSAGLNLNPTNLGELLLISMPALTE
ETRRGFTKQARDVAEDARVAVRNIRR**DANSSLKDLVK**EKEISEDEERRAAGEI
DDLTKKFVAEIDAKLAEKEKDLMAV

2. Spot 2 - Extracellular solute-binding protein, family 3:

MRIVPHILGAAIAAALISTPVFAAELTGTLKK**IKESGVITLGHR**DASIPFSYIADA
SGKPVGYSHDIQLAIVEAIKKDLDLPNLQVK**YNLVTSQTRIPLVQNGTVDVEC**
GSTTNNVERQQQVDFSVGIFEIGTRLLSKADSKYKDFDDLK**GKNVVTTAGTTS**
ERILK**AMNADKQMGMNVISAKDHGESFQMLESGRAVAFMMDDALLAGEA**
AKAKKASDWAVTGTPQSYEIYGCMMRKGDEPFKKAVDDAIK**ATYASGEINK**I
YEK**WFMQPIPPKGLNLNFPMSDELKALIANPTDK**AADDKKS

3. Spot 3 - MaoC-like dehydratase:

M**PYVPVAELKDYVGK**ELGR**SEWLTIDQERINLFAEATGDYQFIHVDPVKAA**
QTPFGSTIAHGFLSLSLIPKLMEDILILPQGVK**MVVNYGLDSVRFIQPVK**VNS
KVRLKVDLGEVTEK**KPGQWLLKATATLEIEGSDKPAYIAEPLSLCFV**

4. Spot 4 - Nucleoside diphosphate kinase:

MAVQRTFSIIKPDAVAKNVIGEITTRFEKAGLRVVASKLKQLSKAEAEGFYAEH
SARGFFGDLVAFMISGPVVVQVLEGENAIALNRELMGATNPKEAAAGTIR**ADF**
ADSIDANAVHGSDSEAAAAREISYFFAATEVTAR

5. Spot 5 - FAD dependent oxidoreductase:

MTVPIAIIGTGIAGLSAAQALTDAGHTVQLFDKSRGSGGRMSSK**RSDAGSLDM**
GAQYFTARDR**RFVTEVQR**WQSKGWVAEWAPQLYTFHGGQLNLSPDEQTRW
VGTPRMSAITRGLLDGLEVHFACRITEVYRGEEHWHLQDAEGFTHGPFSHVVIA
TPAPQATALLAAAPKLAGAAAGVKMDPTWAVALAFDTPLDTPIEGCFVQDSAL
DWLARNRSKPGRETTCDTWVLHATSAWSRQHIDLPKEAVIEQLHGAFAELLHS
AMPAPTFSLAHRWLYARPASSHEWGTLADADLGLYACGDWCLSGR**VEGAWL**
SGQEAARRLHEHLL

6. Spot 6 - Zinc-containing alcohol dehydrogenase superfamily:

MFK**GILIDKDDSGYRANLQEISDEQLPDGDVTVRVAYSTLNFKDGLAITGSS**
PVVRKFPMVPGIDLAGTVEVSSHPDYKVGDAVLLNGWGVGESHCGGLAQKAR
LNGDWLIPLPKAFTAAQAMAIGTAGYTAMLCVMALERNGVTPQQGEVLVTG
ANGGVGSFAIALLSKLGYRVVASTGRVSEHEYLK**QLGASEIIDR**ATLSEPGKPL
AKERWAGVIDSVGSHTLANACASTRAEGTVAACGLAQGMDFPASVAPFILR**G**
VTLAGINSVTQPKARRIEAWDRLTKDLDFALLPLISHEIGLGEALEAAPKLLAG
QLRGR VVVDVNR

7. Spot 7 - Universal stress protein family:

CPVPLHFVSHVQHVLPR**RIVAAVDPFHR**DGQYQGLNDRILYEAGKLASLCKAE
LDVIYAHDLSSISAAEFGFDHASAFFSSSAAKALFDAQGEAFRELAERNDIPAEN
QHMIMGDPAK**VLASYADAYDIDVIVMGR**VAHRGMGRLIGSTVEHLLYRMPCS
VWVVAPEKLE

8. Spot 8 - Chaperonin GroEL:

MAAKDVKFGNDARVK**MLAGVNVLADAVK**VTLGPKGRNVVLDKSFGAPTITK
DGVSVAREIELEDKFENMGAQMVKEVASKANDAAGDGTTTATVLAQAIVNEG
LK**AVAAGMNPMDLK**RGIDKAVAAVVTELKALSKPCETSKEIEQVGTISANSDS
IVGQLIAQAMEKVGKEGVITVEDGTGLEDELDVVEGMQFDRGYLSPYFINKPET
ATVELDSPFILLVDKKISNIRELLPVLEAVAKAGKPLLIIAEDVEGEALATLVVNT
MRGIVKVAAVKAPGFGDRRKAMLQDIAILTAGTVISEEIGMELEKATLEDLGQ
AKRVVINKDNTTIIDGIGDEAQIKGRVAQIRQQIEESTSDYDKEKLQERVAKLAG
GVAVIKVGAATEVEMKEKKDRVEDALHATRAAVEEGIVAGGGVALIRAASKA
AASLQGDNEEQNVGIKLALRAMESPLRQIVANAGEEASVVASAVKNGEGNFG

YNAGTEQYGDMIAMGILDPTKVTRSALQFAASIAGLMITTEAMVTELPKDDKL
DAAAAMGGMGGMGGMM

9. Spot 9 - Ubiquinol-cytochrome c reductase, iron-sulfur subunit:
MSNDGVNAGRRRFLVAATSVVGAAGAVGAAVPFVGSWFPSAKAKAAGAPVK
VNVSK**IEPGQQMIAEWRGQPVFIVRRTEEILGNLKKIEGQLSDPTSK**NSTQP
TYVDPEVRSIKPEILLLIGICTHLGCSPTFRPEVAPADLGKDWVGGYFCPCHGSH
YDLAGRVYKSQPAPLNLPVPPHSYETDDLIVIGVDTEKA

10. Spot 10 - 50S ribosomal protein L5:
MARLKEIYRNEIAPKLKEELKLSNVMEVPRVTKITLNMGLGEAIGDKK**VIEHAV**
ADLEKITGQKPVVTFARKSIAGFKVREGWPIGVKVTLRSDKMYEFLDRLLAISL
PRVRDFRGLNAKSFDGRGNYSMGVKEQIIFPEIDYDKIDALRGLDITLTTTARSD
DEGRALLRAFKFPFRN

11.Spot 11 - Glyoxalase/bleomycin resistance protein/dioxygenase:
MSVKPIPEGYHSTTPYLGIHK**AAEAIDFYKKAFGATEVMRLSMPDGGIGHAE**
LRIGDSAIMLGSPCDQGPLSNPDTAVSVGLHLYVTDVDKSFQRALEAGATSVSE
VKDQFYGDR**SGTLKDPYGHLWFLATRKEDLTQEQIEQR**AKEMFQQG

12. Spot 12 - Lactoylglutathione lyase:
MSLNELNTFPGVTAQPDAATAR**FVFNHTMLR**VKDITR**SLDFYTR**VLGFSLVEK
RDFPEAEFSLYFLALVDKAQIPADAGARTEWMKSIPGILELTHNHGTENDPAFA
YHNGNSDPRGFGHICISVPDIVAACER**FEALGCDFQK**RLNDGRMKSLAFIKDP
DGYWVEIIQPAPL

13. Spot 13 - Recombination associated protein:
MWFKNLLIYRLTQDLPFDAEALETALATKLARPCASQELTTYGFVAPFGKGED
APLVHVSQDFMLVAARKEERILPGSVVRDAVKEKVEEIEAEQMRKVYKKERD
QIKDEIIQAFLPRAFIRRSSTFAAIAPK**QGLILVNSASPK**RAEDLLSTLR**EVLGTL**
PVRPLTVKIAPTAIMTDWVKTQKAADDFFVLDECELRDTHEDGGIVRCKRQDL
TGEEIQLHLSTGK**VVTQLSLAWQDK**LSFMLDDKMVVKRLKFEDLLQDQAEQ
DGGDEALGQQDASFTLMMLTFGDFLPALFEALGGEEIPQGI