ฟอสโฟโปรตีนจากเซลล์มะเร็งปอด A549

นางสาวอัจฉรา รับพร

PHOSPHOPROTEINS FROM LUNG CANCER CELL A549

Miss Atchara Rubporn

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Biotechnology

Faculty of Science

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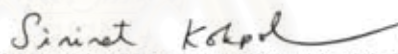Academic Year 2007

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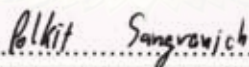
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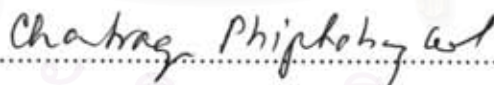
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อัจฉรา รับพร : ฟอสโฟโปรตีนจากเซลล์มะเร็งปอด A549. (PHOSPHOPROTEINS FROM LUNG CANCER CELL A549) อ. ที่ปรึกษา: รศ.ดร. พลกฤษณ์ แสงวณิช, อ. ที่ปรึกษาร่วม: ดร. จันทรกานต์ พิภพมงคล, 85 หน้า.

โรคมะเร็งปอดเป็นโรคที่ทำให้ผู้ป่วยมีอัตราการเสียชีวิตสูงเป็นอันดับสองรองจากมะเร็งตับ แต่มีแนวโน้มว่าในไม่ช้าโรคนี้จะกลายเป็นโรคที่ทำให้ผู้ป่วยมีอัตราการเสียชีวิตมากที่สุด เนื่องจากโรคนี้เป็นโรคที่ยากในการวินิจฉัยในระยะแรก อัตราการรอดชีวิตของผู้ป่วยโรคมะเร็งปอดจะขึ้นอยู่กับระยะของโรค นักวิจัยได้พยายามหาวิธีที่จะทำการตรวจโรคได้ตั้งแต่ในระยะเริ่มแรก หนึ่งในวิธีการเหล่านั้นได้แก่การใช้เทคนิคทางโปรตีโอมิค ดังนั้นวัตถุประสงค์ของงานวิจัยในครั้งนี้จึงทำการศึกษาเพื่อที่จะได้เข้าใจการแสดงออกของโปรตีนจากเซลล์มะเร็งปอด เปรียบเทียบกับเซลล์ปอดปกติ มุ่งเน้นเกี่ยวกับการศึกษาฟอสโฟโปรตีนโดยการใช้เทคนิคทางโปรตีโอมิค จากการศึกษาพบว่าเซลล์มะเร็งปอดมีการแสดงออกของโปรตีนหลายชนิดที่มีความแตกต่างจากโปรตีนของเซลล์ปอดปกติ นอกจากนี้ยังพบว่าโปรตีนบางชนิดที่พบในเซลล์ทั้งสองมีความแตกต่างกันในเชิงปริมาณ โปรตีนที่มีการแสดงออกมากขึ้นในเซลล์มะเร็งปอดส่วนใหญ่จะเป็นฟอสโฟโปรตีน ตัวอย่างเช่น Lamin AC 70 kDa Aldehyde dehydrogenase α-enolase Glyceraldehydes-3-phosphate dehydrogenase Pyruvate kinase Peroxiredoxin เป็นต้น ส่วนโปรตีนที่มีการแสดงออกเฉพาะในเซลล์มะเร็งปอด ได้แก่ Heterogenous ribonucleoprotein A1 Heterogenous ribonucleoprotein H1 Nuclear corepressor KAP1 Transketolase และ Cytokeratin18 นอกจากนี้ยังพบ Chaperonin ซึ่งเป็นฟอสโฟโปรตีน โดยโปรตีนชนิดนี้ทำหน้าที่เกี่ยวกับการเกิดปฏิกิริยาระหว่างกัน และการเปลี่ยนแปลงรูปร่างของโปรตีน การแสดงออกที่มากขึ้นของโปรตีนชนิดนี้อาจทำให้การเปลี่ยนแปลงรูปร่างของโปรตีนเกิดความผิดปกติและนำไปสู่การเกิดมะเร็งในระยะแรก ดังนั้นโปรตีนชนิดนี้อาจใช้เป็นตัวบ่งชี้ทางชีวภาพ (Biomarker) สำหรับตรวจหาและวินิจฉัยโรคมะเร็งได้ ซึ่งยังคงต้องทำการศึกษาต่อไป

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Lung cancer is the leading cause of death after liver cancer but it trends to be increased because it is hard to detect the symptoms of a disease in the first stage. Survival rate of the patient is up to the stage of the cancer. Researcher had tried to develop the methodologies for early detection of cancers. Proteomic analysis technique is selected. This research aimed to understand the expression of proteins from lung cancer cell line (A549) and compare with normal lung fibroblast cell line (MRC-5). The phosphoproteins were the major target of investigation by using proteomic analysis technique. The results were indicated that there were several proteins which present and absent in lung cancer cell line. Some proteins were difference in term of quantitation. The most of proteins which over-expressed in lung cancer cell line (A549) were phosphoproteins such as Lamin AC 70 kDa, Aldehyde dehydrogenase, α-enolase, Glyceraldehydes-3-phosphate dehydrogenase, Pyruvate kinase, Peroxiredoxin. Moreover some proteins were expressed only in lung cancer cell line (A549) such as Heterogenous ribonucleoprotein A1, Heterogenous ribonucleoprotein H1, Nuclear corepressor KAP1, Transketolase and Cytokeratin18. Furthermore, Chaperonin which is phosphoprotein was found. This protein functions on protein interaction and protein conformation. The over-expression of this protein in cells may cause abnormality of protein conformation and lead to early stage of cancer. Then this protein may be used as biomarker of lung cancer for early detection and clinical prognosis. Further studies on this topic are required.

Field of study.......Biotechnology.....　　Student's signature.....Atchara Rubporn..........

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CONTENTS

LIST OF FIGURE

LIST OF TABLES

LIST OF ABBREVIATIONS

2-DE	Two-dimensional gel electrophoresis
µg	microgram
µl	microliter
ACN	Acetronitrile
APS	Ammonium persulfate
Ar	Argon
Bis	*N,N'*-methylenebisacrylamide
°C	degree Celsius
C	Crosslinking factor [%]
CAD	Collision activated dissociation
CCA	α-Cyano-4-hydroxycinnamic acid
CHAPS	3-(3-cholamidopropyl)dimethylammonio-1-propane sulfonate
CID	Collision induced dissociation
Da	Dalton
DC	Direct current
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionization
ESI-Q/TOF	Electrospray ionization Quadrupole time-of-flight
eV	electron Volt
IEF	Isoelectric focusing
IPG	Immobilized pH gradients
He	Helium
HPLC	High-performance liquid chromatography
kDa	Kilodalton
kVh	kilovolt-hour
LC	Liquid chromatography
LMW	Low molecular weight
nM	Nanomolar
mA	Milliampere

MALDI	Matrix Assisted Laser Desorption Ionization
MALDI-TOF	Matrix Assisted Laser Desorption Ionization/Time of flight
mg	Milligram
mg/ml	Milligram per milliliter
ml	Milliliter
min	Minute
mm	Millimeter
mM	Millimolar
MS	Mass spectrometry
MS/MS	Tandem Mass spectrometry
m/z	Mass per charge
PAGE	polyacrylamide-gel electrophoresis
pI	Isoelectric point
PMM	Peptide mass mapping
ppm	parts per million
PSD	Post source decay
Q/TOF	Quadrupole Time of flight
RF	Radiofrequency
rpm	Revolutions per minute
SDS	Sodium-dodecyl sulfate
SDS-PAGE	Sodium-dodecyl sulfate polyacrylamide-gel electrophoresis
T	Total acrylamide concertration [%]
TCA	Trichloroacetic acid
TEMED	*N,N,,N',N'*-tetramethylethylenediamine
TFA	Trifluoroacetic acid
TOF	Time of flight
Tris	Tris(hydroxymethyl)-aminoethane
UV	Ultraviolet spectroscopy
V	Volt

CHAPTER I

INTRODUCTION

In Thailand, cancer is the third most leading cause of death after heart disease and accidents. Cancer is a disorder resulting from autonomous, uncontrolled cell growth and differentiation, invading and disrupting other tissues, and spreading to other areas of body, it effect on the body by destroying the surrounding adjacent tissues, it is replacing normal functioning cells in distant sites. Liver cancer is the most frequent malignancy in male but it trends to be that lung cancer may be the leading cause of death soon. Lung cancer is the malignant transformation and expansion of lung tissue, and is the most lethal of all cancers worldwide, responsible for 1.3 million deaths annually. It is caused mainly by cigarette smoking, and mostly affects men, being the leading cause of death of men between the ages of 40 and 65. With increased smoking among women, lung cancer is now occurring more frequently in women.

In resent years, "omics" analyses have been developed and promise to define "fingerprints" of patterns in malignant cells. These analyses derive their power from the simultaneous measurement of the expression level of multiple transcripts, protein products and/or protein modification (1). Proteome is the combining of two words, Protein and Genome. It was defined as all of proteins produced from all genes of the genome. The word "Proteomics" is the study of proteome, it is a powerful technique for comparing the total proteins from cells, tissues, or organisms. It is the large-scale study of proteins, particularly their structures and functions. Since proteins play a central role in the life of an organism, proteomics is instrumental in discovery of biomarkers, such as markers that indicate a particular disease. The scale of proteomic analysis varies according to its aim, such as determining the protein content of a whole organism, analyzing target proteins in a tissue or in cell. The direct evaluation of the proteins expressed in tumor cells offers information that can not be obtained by the study of DNA alterations or RNA expression pattern. The most important that genomic or genome analyses cannot detect is post-translational modifications (PTM) of proteins, such as proteolytic processing, phosphorylation, or glycosylation.

Phosphorylation is one of the most common and best characterized post-translational modifications of cellular proteins (2). It plays the central roll in many

biological and biomedical phenomena. It has increasingly become the focus of cell biology because it involved in gene expression, protein synthesis, signal transduction, which determine cell growth, cell division or differentiation and cancer. Phosphorylation acts as an on/off switch for many biochemical functions. It occurs by the reaction of enzyme, protein kinases. In eukaryotic, the typical acceptors for phosphorylation are the hydroxyamino acids serine, threonine and tyrosine. The identification of phosphoproteins is possible by many direct and indirect means, but the localization of the sites of phosphorylation remains a technical challenge because only few of phosphoproteins is known(3).

Objectives of this research

To study the alteration of protein expression in lung cancer cell line (A549) compare to normal lung fibroblast cell (MRC-5) focus on phosphoproteins by using proteomic technique such as two-dimension gel electrophoresis (2-DE) and identified proteins by mass spectrometry.

CHAPTER II

THEORETICAL AND LITERATURE REVIEW

Theoretical background

2.1 Cancer (4)

   Cancer is a class of diseases or disorders characterized by uncontrolled division of cells and the ability of these to spread, either by direct growth into adjacent tissue through invasion, or by implantation into distant sites by metastasis (where cancer cells are transported through the bloodstream or lymphatic system). Cancer may affect people at all ages, but risk tends to increase with age. It is one of the principal causes of death in developed countries. There are many types of cancer. Severity of symptoms depends on the site and character of the malignancy and whether there is metastasis. A definitive diagnosis usually requires the histological examination of tissue by a pathologist. This tissue is obtained by biopsy or surgery. Most cancers can be treated and some cured, depending on the specific type, location, and stage. Once diagnosed, cancer is usually treated with a combination of surgery, chemotherapy and radiotherapy. As research develops, treatments are becoming more specific for the type of cancer pathology. Drugs that target specific cancers already exist for several types of cancer. If untreated, cancers may eventually cause illness and death, though this is not always the case. The unregulated growth that characterizes cancer is caused by damage to DNA, resulting in mutations to genes that encode for proteins controlling cell division. Many mutation events may be required to transform a normal cell into a malignant cell. These mutations can be caused by radiation, chemicals or physical agents that cause cancer, which are called carcinogens, or by certain viruses that can insert their DNA into the human genome. Mutation Many forms of cancer are associated with exposure to environmental factors such as tobacco smoke, radiation, alcohol, and certain viruses. Some risk factors can be avoided or reduced. However, some carcinogens also appear to work through non-mutagenic pathways that affect the level of transcription of certain genes without causing genetic mutation. Cancer in Thailand is becoming a significant health problem. It is the leading cause of death in Thailand. Several cancers can be prevented by a nationwide campaign of health education

to prevent raw fish intake and an antismoking campaign. An appropriate cervical cancer and breast cancer screening program can improve the recent prevalence of both and lead to better results of treatment. Research related to the carcinogenesis mechanism of certain cancers can lead to greater understanding and a better plan of control.

2.2 Lung cancer (4)

Lung cancer is the malignant transformation and expansion of lung tissue, and is the most lethal of all cancers worldwide, responsible for 1.3 million deaths annually. It is caused mainly by cigarette smoking, and mostly affects men, being the leading cause of death of men between the ages of 40 and 65. With increased smoking among women, lung cancer is now occurring more frequently in women. While some people who have never smoked do still get lung cancer, this appears to be due to a combination of genetic factors and exposure to secondhand smoke. Air pollution may also contribute to the development of lung cancer. Current research indicates that the factor with the greatest impact on risk of lung cancer is long-term exposure to inhaled carcinogens. The most common means of such exposure is tobacco smoke. Treatment and prognosis depend upon the histological type of cancer, the stage (degree of spread), and the patient's performance status. Treatments include surgery, chemotherapy, and radiotherapy.

2.3 Proteomic tools

Proteomics is a powerful technique for comparing the total proteins from cells, tissues, or organisms. It is the large-scale study of proteins, particularly their structures and functions. Initially the strategy was to

1.   Separate protein components by two-dimensional gel electrophoresis (2-DE)

2.   Locate protein spots in the gel by staining

3.   Excise spots of interest

4.   Reduce, alkylate, and tryptic digestion each excised protein and

5.   Analyze the tryptic digests either by matrix-assisted laser desorption ionization (MALDI) or electrospray ionization (ESI) mass spectrometry

2.4 Protein extraction (5, 6)

The initial step of purification procedure involves recovery of the protein from its source. The complexity of this step depends on whether the interested protein is intracellular or extra cellular. If protein is an intracellular, collection of the cell or tissue is followed by their disruption. Most mammalian cells and tissues are easily disrupted. Most techniques rely on physical disruption of the cell membrane. A well known example is

homogenization. After homogenization step cellular debris can be removed by centrifugation of filtration. In this step the interest proteins are present in dilute solution, it become necessary to concentrate the large volume of dilute solution to make the extract volume for subsequence analysis and it is important to deactivate any enzyme such as trypsin by addition of enzyme inhibitor into lysis buffer to protect all of interest proteins.

2.5 Protein separation (5, 6)

Proteins are separated according to their difference physiochemical properties. The most commonly technique often used in this step is gel electrophoresis, one dimension and two dimension electrophoresis or chromatography technique such as gel filtration, ion exchange chromatography or used of affinity column for separate only target proteins or peptides.

2.6 Electrophoresis (7)

Electrophoresis separation techniques are at least as widely distributed as chromatographic methods. The main fields of application are biological and biochemical research, protein chemistry, as well as molecular biology. It is important to choose and carry out appropriate technique for specific separation. This technique is the process that applies an electric field across the mixture for moving charged molecules in solution. Molecules in electric field move with difference speed depend on their charge, shape and size. It is suitable for molecular separations, which carry out in free solution such as in capillary and free flow system, or stabling media, thin layer plates, films or gels.

2.6.1 Gel electrophoresis (7, 8)

Gel electrophoresis is the most common and standard technique for proteomic profiling analysis because it is a powerful separation, quantitation and characterization method for complex mixture of proteins, whole cell lysated or whole cell homogenates and tissues. Gel electrophoresis separation is carry out in gel, agarose or polyacrylamide gel. There are two types of system, continuous and discontinuous system. Continuous system used only one buffer for tanks and gel. Discontinuous system has a stacking gel, a non restrictive large pore gel and separating gel. Two gel layers used a difference buffer. In this system, the ions and proteins being migrating into the stacking gel when system started, this will cause the proteins concentrate in very thin zone. After that proteins were migrated

in stacking gel until they reach the separating gel. The resolution of this technique is excellent because peptides or proteins are concentrated in stacking gel before entering the separating gel (figure 2.1)
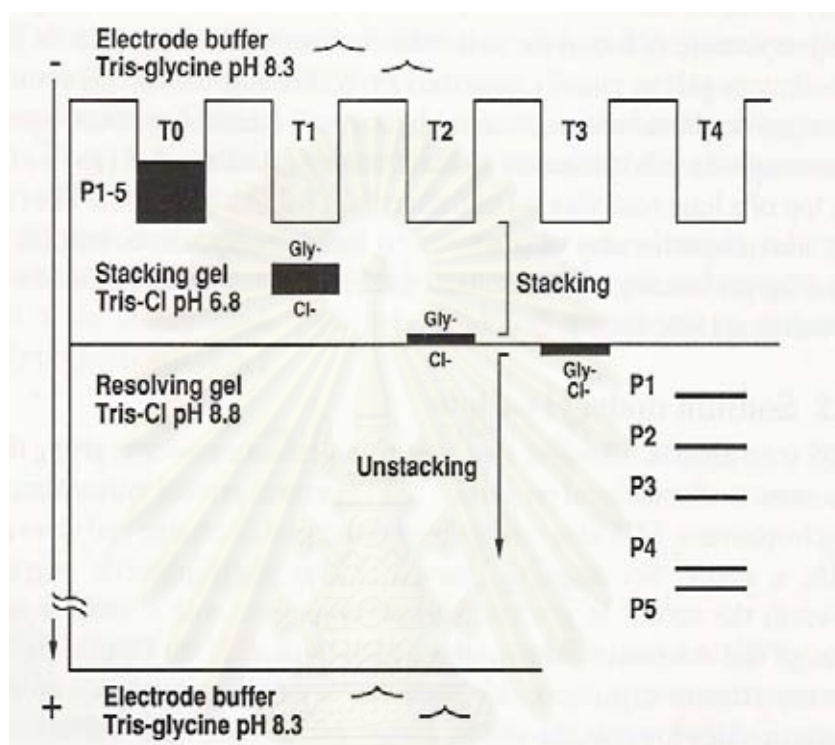
Figure 2.1   Gel electrophoresis: Explanation of the migration under multiple phase buffer condition, electrophoresis through the stacking (pH 6.8) and resolving (pH 8.8) gel of hypothetical sample containing 5 proteins (P1-5) is shown as function of time (T), T0: sample are loading to wells; T1: partial stacking of samples between loading; T2: complex stacking of samples; T3: unstacking take place with increased ionization of glycine in resolving gel; T4: resolution of samples

For protein separation, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is commonly used for separation and characterization the complex mixture of proteins for estimate the molecular weight. SDS-PAGE composes of two major components, sodium dodecyl sulfate and polyacryramide

Sodium Dodecyl Sulfate (SDS) (7)

Sodium dodecyl sulfate, an ionic detergent that used for denature proteins to give all of them in the same conformation properties and prevent protein interactions during the electrophoresis. It makes the charge of proteins themselves and formed anionic complexes to have net negative charge per unit mass.

Polyacrylamide gel (9)

Polyacrylamide gel (PEG) is chemically inert and stable by chemical co-polymerization of acrylamide monomer with cross link reagent, N, N'- methylene bis-acrylamide (figure 2.2) and free radical. The polymerization is catalyzed by ammonium persulfate and accelerator (N, N, N', N'-tetramethylenediamine, TEMED). Polymerization should take place under inert atmosphere because oxygen can act as a free radical trap, polymerization temperature should be maintained above 20°C to prevent an incomplete polymerization.
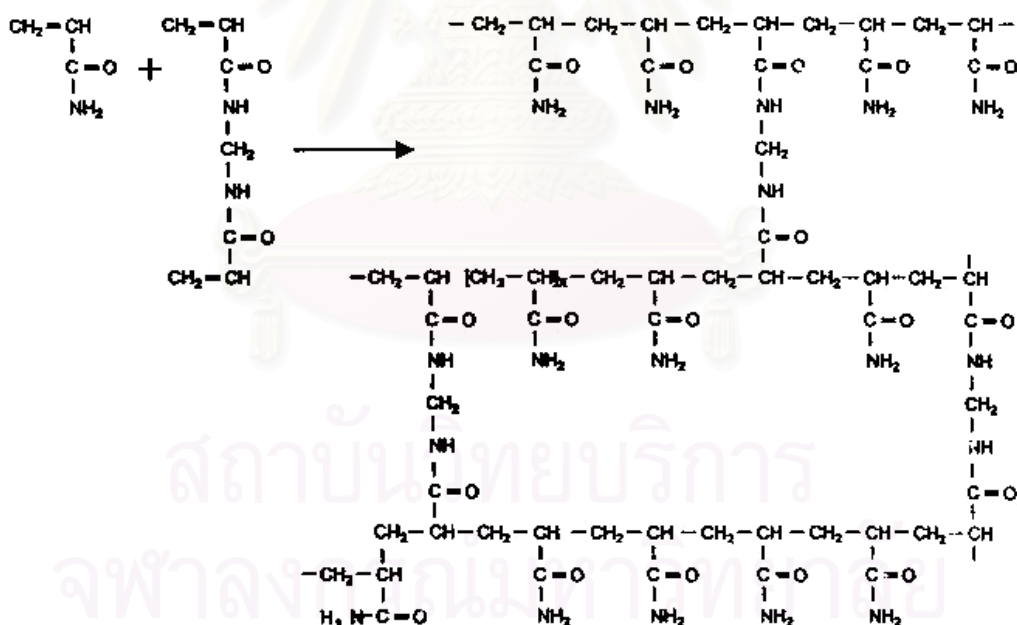
Figure 2.2　　The polymerization reaction of acrylamide and N, N'- methylene bis-acrylamide

　　　　The total acrylamide concentration (T) and the degree of cross-linking (C) can be exactly and reproducibly control the pore size

$$\%T = \frac{\text{acrylamide (g)} + \text{bis-acrylamide (g)}}{100 \text{ ml}} \times 100 \%$$

$$\%C = \frac{\text{bis-acrylamide (g)}}{\text{acrylamide (g)} + \text{bis-acrylamide (g)}} \times 100 \%$$

In general, best resolution is achieved when the pore size approximates the protein molecular size (14). The %T of the gel determines the range over which the protein M.W. is proportional to the rate of migration (Table 2.1). Gels may also be composed of a gradient of polyacrylamide (typically 5-20 %).

Table 2.1   Protein M.W. range resolved by SDS-PAGE

Polyacrylamide gel (%T)	M.W.range
6	30,000-200,000
8	20,000-175,000
10	15,000-150,000
12	10,000-100,000
15	6,000-50,000
5-20 gradient	6,000-250,000

2.6.2 Two-Dimensional Gel Electrophoresis (8, 10)

Two-dimensional gel electrophoresis (2-D electrophoresis) is nowadays frequently used in the laboratory. It is the most powerful protein separation technique and is applicable to soluble as well as membrane proteins. By combining isoelectric focusing with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), a high resolution and a high separation capacity is obtained. This technique sort's protein according to two independent properties in two discrete steps: the first dimension step, isoelectric focusing (IEF), separates proteins according to their isoelectric points (pI); the second dimension step, separates proteins according to their molecular weighs by SDS-

PAGE. These properties make two-dimensioned polyacrylamide gel electrophoresis (2D-PAGE) the methods of choice for the analysis of very complex protein mixtures. More than thousand, radioactively labeled proteins can be resolved and detected in a single two-dimension gel.

<div align="center">

1) First-dimension isoelectric focusing (7, 11)

</div>

Isoelectric focusing (IEF) is an electrophoretic method that separates proteins according to their isoelectric points (pI). Proteins, enzymes and peptides are such amphoteric molecules. 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 negative and positive charges of the amino acid side chains and amino- and carboxyl-terminal but the three-dimensional configuration of the protein also plays a role. The isoelectrtic point (pI) is the specific pH at which the net charges of the protein are zero. Proteins are positively charged at pH values below their pI and negatively charged at pH above their pI. If the net charge of a protein is plotted versus the pH of its environment (Figure 2.3), a continuous curve that intersects the x-axis at the isoelectric point pI will result.
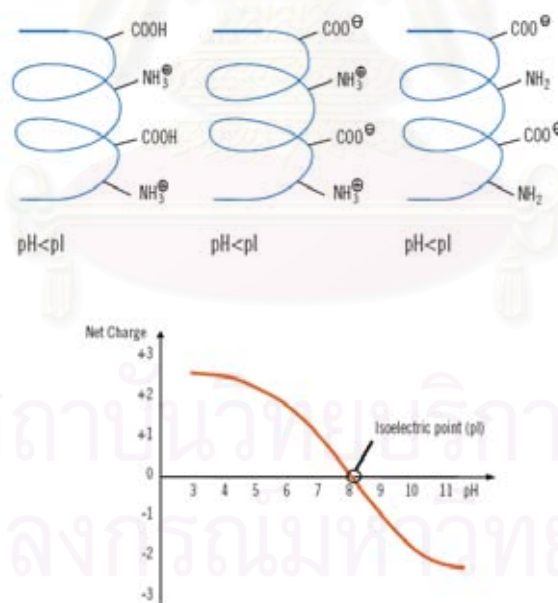
Figure 2.3   Plot of the net charge of a protein versus the pH of its environment. The point of intersection of the curve at the x-axis represents the isoelectric point of the protein

In a pH gradient and under the electric field, a protein will move towards the anode or the cathode until its reach a position on the pH gradient where its net charge is zero. If a protein should diffuse away from its pI, it immediately gains charge and migrates backs.

The focusing effect of IEF concentrates proteins at their pI and allows proteins to be separated based on very small charge differences. In an isoelectric focusing gel, the pH conditions are established in polyacrylamide gel by two techniques, carrier ampholytes (low molecular weight amphoretic species) and immobilines (acrylamide derivative). In the present, because of some limitations of the carrier ampholytes method, an alternative technique was developed: immobilized pH gradients or IPG. An immobilized pH gradient (IPG) is created with acrylamide derivatives with buffering groups (acidic or basic buffering group), the Immobilines, by co-polymerization of the acrylamide monomers in a polyacrylamide gel.

The general structure of immobiline reagent is: $CH_2=CH-CO-NH-R$,

where, R is weakly acidic or basic buffering groups.

To be able to buffer at a precise pH values, at least two different immobilines are necessary, an acid and base. Figure 2.4 is a graphic representation of the polyacrylamide matrix with attached buffering groups. The advantages of using IPG gel are allowing along focusing time to ensure the focusing of the analyst proteins, loading relatively large amount of proteins, available to purchasing IPG in variety of pH range and simplify the physical handing.
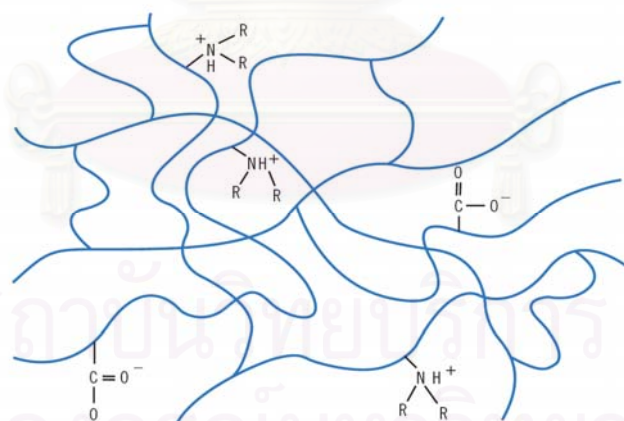
Figure 2.4 Immobilized pH gradient polyacrylamide gel matrix showing attached buffering group

2) Second-dimension SDS-PAGE (7)

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) is an electrophoretic method for separating proteins, enzymes and polypeptides according to their molecular weights (M.W.). This technique is performed in polyacrylamide gels

containing sodium dodecyl sulfate (SDS). The intrinsic electrical charge of the sample proteins is not a factor in the separation due to the presence of SDS in to the sample and the gel. SDS is an anionic detergent, when loading into the protein sample, the charge of the proteins is so well masked that anionic micelles with a constant net charge per mass unit result: 1.4 g SDS per g protein. For separation, proteins with small molecular weight move more rapidly through the gel than the proteins with high molecular weight (figure 2.5).
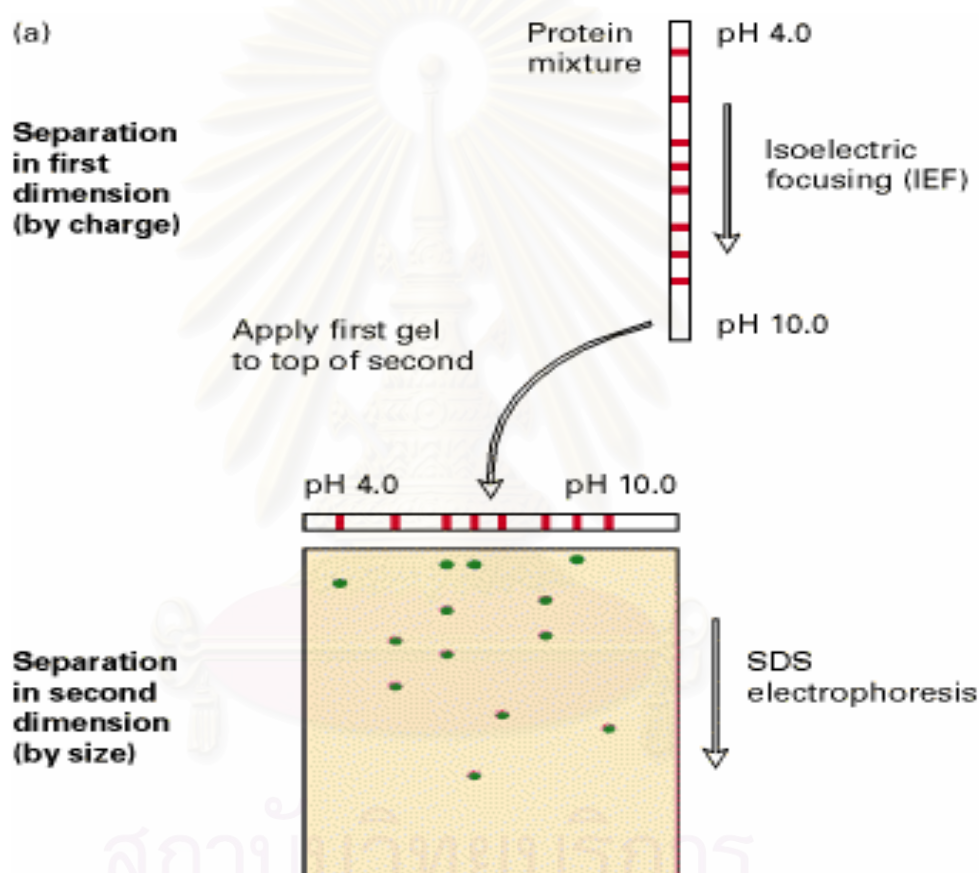
Figure 2.5   Two-dimensional Gel Electrophoresis

2.7 Protein Detection (12)

After the electrophoresis run is complete, the gel must be analyzed qualitatively or quantitatively to answer analytical or experimental questions.

Because most proteins and all nucleic acids are not directly visible, the gel must be processed to determine the location and amount of the separated molecules.

The mostly applied methods in the detection of separated proteins from gel electrophoresis are Coomassie blue staining and Silver-staining. Silver staining is the most sensitive non-radioactive method (below 1 ng). This technique is a complex, multi-step process utilizing numerous reagents for which quality is critical. Coomassie blue staining, although 50- to 100- fold less sensitive than silver staining, is simple method and more quantitative than silver staining. Coomassie blue staining is preferable when relative amounts of protein are to be determined by densitometry. For another methods used to detect proteins in general laboratory such as Autoradiography and Fluorography, Negative Zinc, Fluorescent staining etc.

2.8 Image Analysis (8, 13)

In 2D gels, the molecular weights of the proteins are determined based on their positions in the SDS-PAGE dimension are determined based on their position in the isoelectric focusing dimension and the relative amount of protein is determined based on the density of the staining. In SDS-PAGE, the molecular weight scale can be calibrated with standard run at the last lane of gel. The pI scale can be calibrated based on how the immobilized pH gradient strip was formed. There are two kinds of strips available in non-linear formats. The plots of pH in the strip versus position relative to the end of the strip in both formats are important. The amounts of protein can be determined by measuring the density of the gel bands using densitometer equipped with computer - controlled data recording system. This value is most accurately measured as a volume measurement that accounts for both the size of the band or spot and the density of staining. Scanning densitometers equipped with computer-controlled data recording system can make these measurements.

2.9 Mass Spectrometry (14, 15)

Mass spectrometry is a powerful technique for identifying unknown compounds, studying molecular structure and probing the fundamental principles of chemistry. In protein study, mass spectrometry is typically employed for protein primary structure analysis in the last stages of protein purification. Mass spectrometry not only allows the precise determination of the molecular weight of peptides and of proteins but also the determination of their sequences, especially when operates in tandem mode.

2.9.1 Principle of mass spectrometry (8, 14)

Mass spectrometer can be divided into three fundamental parts, namely the ion source, mass analyzer, and detector. The sample under investigation has to be introduced into the ion source of the instrument. Once inside the ion source the sample molecule are ionized to gas phase ions. These ions are extracted into the analyzer region of the mass spectrometer where they are separated according to their mass-to-charge ratios. The separated ions are detected and this signal sent to a data system where the m/z ratios are stored together with their relative abundance for presentation in the format of a mass spectrum.The analyzer and detector of the mass spectrometer, and often the ionization source too, are maintained under high vacuum (10^{-4}-10^{-6} torr) to give the ions a reasonable chance of traveling from on end of the instrument to the other without any hindrance from air molecule, reduce the collision and loss of ions (Figure 2.6)
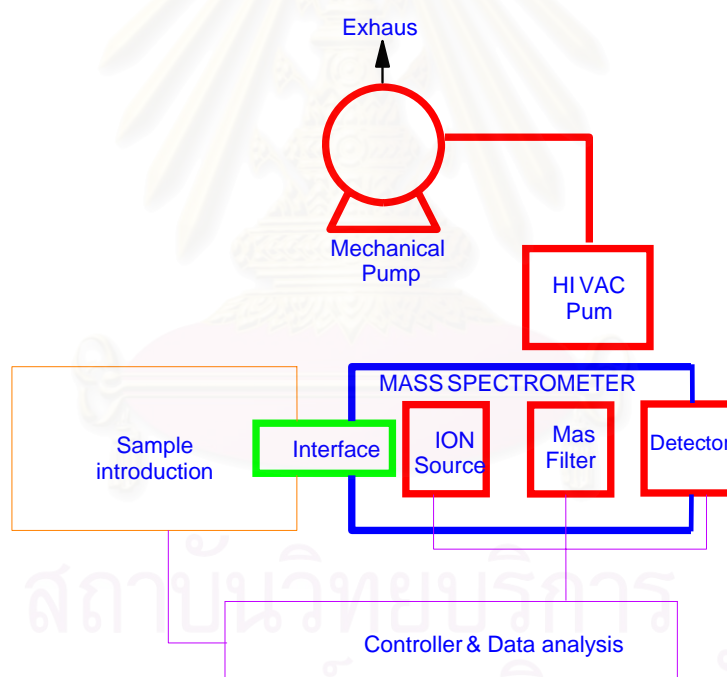
Figure 2.6 Mass spectrometer diagram

There are several ionization techniques which suitable for biological analysis. Only two techniques, matrix assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) are commonly used. The mass analyzers are time-of-flight (TOF) and quadrupole mass analyzer. Tandem mass spectrometry (MS/MS) is a combination of the

same or difference mass analyzers. One mass analyzer will feed ions to the other. For example, Q-TOF is the combination of quadrupole and Time-of-Flight analyzer.

2.9.2 Ionization technique

Electrospray Ionization (ESI)

In electrospray mass spectrometry, the analyte is presented in solution, such as for thermospray MS. A voltage of 2-3 kV is applied to the electrospray capillary, creating a strong electric field, e.g. for a 0.2 mm outer diameter capillary tip the field will be approximately 10^6 V/m. Ions in solution are repelled from the sides of the capillary and bud out to form a Taylor cone, shown in Figure 2.7(16). Small droplets are emitted from the tip of the cone. Lower flow rates and higher solution conductivities give smaller droplets; droplets of a few micrometer radii will be formed at a few microliter per minute flow rates and 10^{-4}-10^{-2} Sm^{-1} conductivity (from 10^{-5}-10^{-4} M electrolyte concentration).
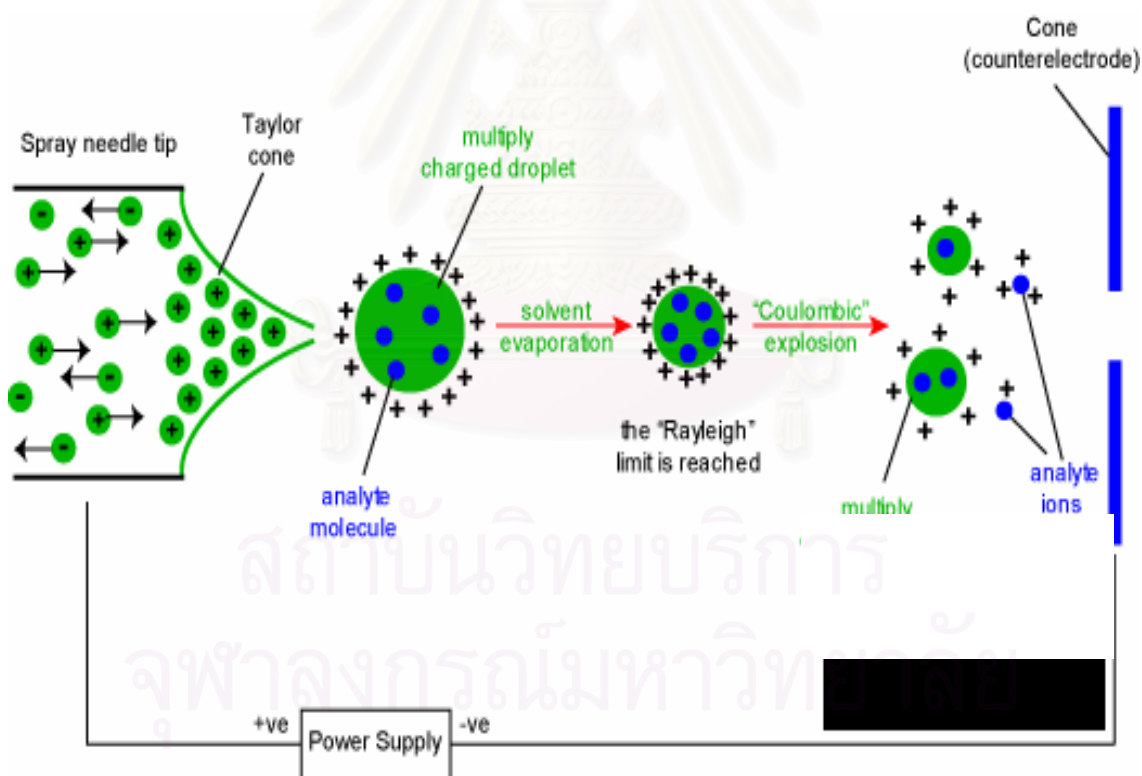
Figure 2.7   Schematic of Electrospray source: Positive ions are shown pushing out into a taylor cone before forming droplets

Droplets decrease in size due to evaporation of solvent. This has been observed experimentally: Taflin et al. trapped a droplet in a quadrupole ion trap and observed it using light-scattering techniques (17), while Gomez and Tang used 25 ms flash shadow graphs to observe droplets as they left the capillary, with Doppler anemometry to measure the diameter (18). As the droplet radius decreases, the electrostatic repulsion of the charges at the surface increases until coulombic fission occurs. This must occur at or before the Rayleigh stability limit, in fact it is assumed that it occurs at 0.8 q_{Ry}.

$$q_{Ry} = 8\pi(\varepsilon_0\gamma R^3)^{1/2}$$

The Rayleigh stability limit: q is charge; γ is surface tension; ε_0 is permittivity of vacuum

Fission is uneven, with jet emission of a string of small droplets, observed by Gomez and Tang. The offspring droplets observed were ~1/10th the radius of the parent droplet, with ~2% of the parent's mass and ~15% of the parent's charge. The mechanism for ion production from electrosprayed droplets of 1-10 nm radiuses is still contentious. Dole proposed a mechanism where successive coulombic fission events occurred until a single charged analyte remained, known as the charged residue model (19). Iribarne and Thomson suggested that analyte ions may be able to evaporate from the surface of a small droplet (<10 nm radius; ion evaporation model) before coulombic fission occurs (20). The two models are indistinguishable for droplets of radii below 1 nm. The ion evaporation model was developed to explicitly consider the surface-activity of analytes (21), which is known to be important, particularly if multiple analytes are 'competing' for ionization. The surface-activity effect can also be incorporated into the charged residue model, with surface ions more likely to form part of a daughter droplet. The ion evaporation method appears to be favoured for small ions with small solvation free energies and high surface activities; the charged residue model is favoured for macro-ions, such as proteins, where evaporation is unlikely to occur in the time available (~100-500 µsec to go from the capillary tip to the MS inlet). Many of the predictions made by the two models are similar and, given the timescales and small scales involved, it is hard to devise experiments to definitively distinguish between the processes.

2.9.3 Mass analyzer

1. Time-of-Flight Mass Analyzer (13, 14, 22)

Time-of-Flight (TOF) analyzers are one of the simplest MS analyzer in use today. Stephens developed TOF in the late 1940's, but until the 1990's its popularity was limited. Recent improvements in TOF technology, including orthogonal acceleration, ion mirrors (reflectrons), and high-speed electronics, have significantly improved TOF resolution. This improved resolution, combined with powerful and easy-to-use electrospray (ESI) and matrix-assisted laser desorption/ionization (MALDI) ion source, have made ToF MS a core technology for the analysis of both small and large molecules.

Principle

A time of flight mass spectrometer measures the mass-dependent time it takes ions of different masses to move from the ion source to the detector. This requires that the starting time (the time at which the ions leave the ion source) be well-defined. Therefore, ions are either formed by a pulsed ionization method (usually matrix-assisted laser desorption/ionization, or MALDI), or various kinds of rapid electric field switching are used as a 'gate' to release the ions from the ion source in a very short time.
Recall that the kinetic energy of an ion leaving the ion source is:

$$\frac{\mathrm{m}v^2}{z} = q\mathrm{V_s} = ze\mathrm{V_s} = \mathrm{E_k}$$

The ion velocity, v, is the length of the flight path, L, divided by the flight time, t:

Substituting this expression for v into the kinetic energy relation, we can derive the

$$t^2 = \frac{\mathrm{m}}{z}\left(\frac{\mathrm{d}^2}{2\mathrm{V}_s e}\right)$$

The mass-to-charge ratio can be calculated from a measurement of t^2, the terms in parentheses being constant. This equation also shoes that, all others factors being equal, the lower of an ion, the faster it will reach the detector. The simplest example is a linear TOF analyzer, where each ion is accelerated into the field-free region (the flight tube) and maintains the velocity it acquired by the acceleration until it hits the detector.

In principle, the upper mass range of a TOF instrument has no limit, which suitable for soft ionization techniques. For advantages of TOF mass analyzers are high sensitivity and very fast scan speed but it is poor mass resolution. Mass resolution is affected by a

distribution in flight times among ions with the same m/z ratio .Such as the ions leaving the ion source of a time-of-flight mass spectrometer have neither exactly the same starting times nor exactly the same kinetic energies (recall the "chromatic aberrations" discussed for magnetic sector mass spectrometers). Various time-of-flight mass spectrometer designs have been developed to compensate for these differences. A reflectron is an ion optic device in which ions in a time-of-flight mass spectrometer pass through a "mirror" or "reflectron" and their flight is reversed (Figure 2.8). The reflectron is a series of rings or grids that act as an ion mirror. This mirror compensates for the spread in kinetic energies of the ions as they enter the drift region and improves the resolution of the instrument. The output of an ion detector is displayed on an oscilloscope as a function of time to produce the mass spectrum
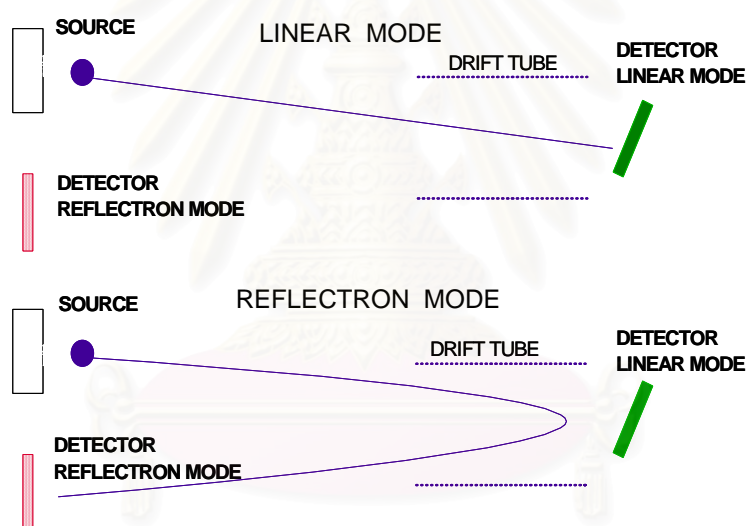
Figure 2.8   Linear and Reflectron Time-of-Flight Mass spectrometer

2. Quadrupoles

A quadrupole mass analyser is a low resolution mass filter (unit resolution for ions of up to a few thousand daltons) made up of four cylindrical metal rods (usually molybdenum), aligned in parallel along the z-axis. Ions pass through the quadrupole along the z-axis. A voltage, Φ, made up of a direct, U, and alternating, $V\cos \omega t$, potential is applied to each set of opposed rods, shown in Figure 2.9
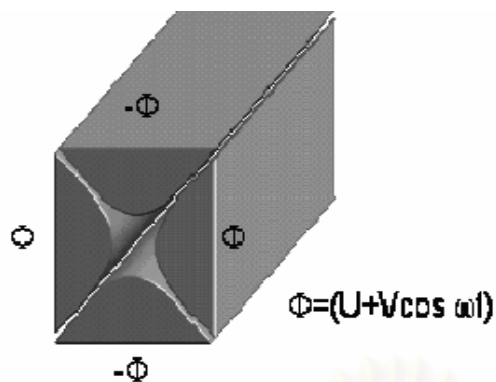
Figure 2.9   Schematic of quadrupole, with applied voltages. The rods shown are hyperbolic in shape, allowing the creation of symmetrical field

Ions passing through the quadrupole will experience an electric field resulting from the potentials applied to the two sets of rods. The alternating current leads to ion oscillation. The size of these oscillations is mass and charge dependent, giving the basis for mass selection. Ion motion in the x and y axes can be described by the Mathieu equation.

$$a = \frac{8e\text{U}}{mr_o^2\omega^2} \qquad q = \frac{4e\text{V}}{mr_o^2\omega^2}$$

e is charge of ion; U is the DC potential; V is RF potential; m is mass of ion; r_o is half distance between opposite rods and ω is the frequency

The parameters **a** and **q** define regions of stable ion trajectory, as shown by the stability diagram in Figure 2.10
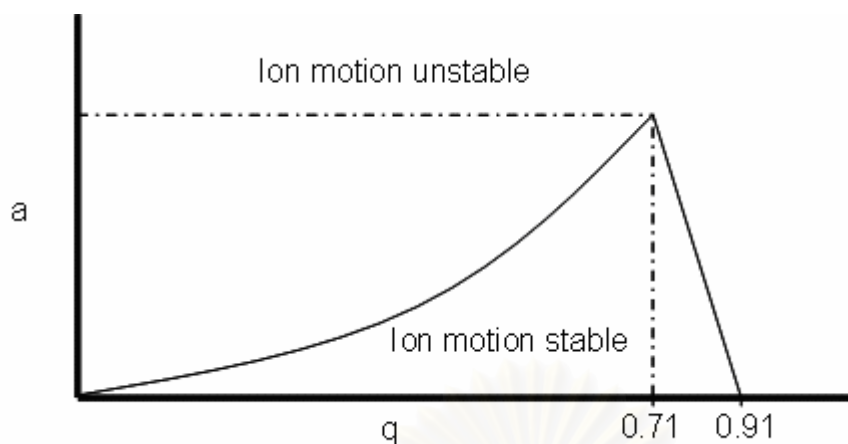
Figure 2.10 Stability diagram for an ion in quadrupole. The stability diagram illustrates that ions of m/z less than 78% (0.71/0.91* 100) of the optimal m/z will not be transmitted

In the quadrupole ω and r_o are constant. As U and V are varied ions of different mass to charge ratios will have stable trajectories through the quadrupole, i.e. will be transmitted. The resolution achieved is linked to the ratio of **a** to **q,** resulting in the different scan lines shown in Figure 2.11
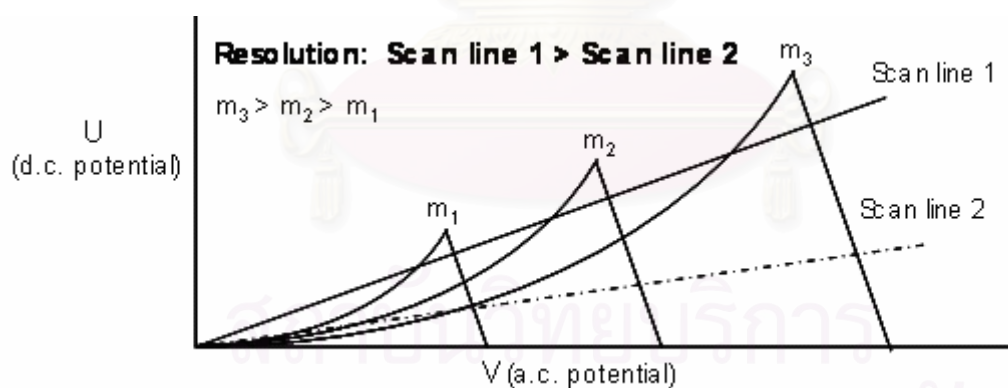
Figure 2.11 Stability diagram for three ions with difference mass to charge ratios. Both scan line 1 and 2 allow all three ions to pass through the quadrupole, scan line 1 has a steeper gradient and therefore gives higher resolution

2.10 Tandem Mass Spectrometry (MS/MS) (13, 22, 23)

Tandem mass spectrometry (MS/MS) was first used in the late 1960s. There is used to produce structural information about a compound by fragmenting specific sample ions inside the mass spectrometer and identifying the resulting fragment ions. This information

can then be pieced together to generate structural information regarding the intact molecule. Tandem mass spectrometry also enables specific compounds to be detected in complex mixtures because of their specific and characteristic fragmentation patterns. Tandem mass spectrometer is a mass spectrometer that has more than one analyser, in practice usually two. A collision cell into which an inert gas (e.g. argon, xenon) is admitted to collide with the selected sample ions and bring about their fragmentation separates the two analysers. The analysers can be of the same or of different types, the most common combinations being: Triple Quadrupole (QQQ), Quadrupole - Time-of-Flight (Q-TOF)

The four mains scan modes available using MS/MS are represented (Figure 2.12)
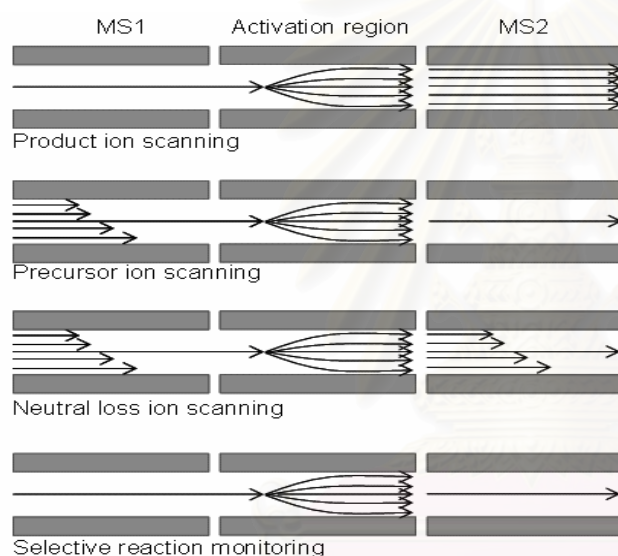
Figure 2.12 Common modes of tandem mass spectrometry

First mode is product or daughter ion scanning: the first analyser is used to select user-specified sample ions arising from a particular component; usually the molecular-related (i.e. $(M+H)^+$ or $(M-H)^-$) ions. These chosen ions pass into the collision cell, are bombarded by the gas molecules which cause fragment ions to be formed, and these fragment ions are analysed i.e. separated according to their mass to charge ratios, by the second analyser. All the fragment ions arise directly from the precursor ions specified in the experiment, and thus produce a fingerprint pattern specific to the compound under investigation. This type of experiment is particularly useful for providing structural information concerning small organic molecules and for generating peptide sequence information.

Precursor or parent ion scanning: the first analyser allows the transmission of all sample ions, whilst the second analyser is set to monitor specific fragment ions, which are generated by bombardment of the sample ions with the collision gas in the collision cell. Constant neutral loss scanning: this involves both analysers scanning, or collecting data, across the whole m/z range, but the two are offset so that the second analyser allows only those ions that differ by a certain number of mass units (equivalent to a neutral fragment) from the ions transmitted through the first analyzer. The last mode is selected/multiple reaction monitoring: both of the analysers are static in this case as user-selected specific ions are transmitted through the first analyser and user-selected specific fragments arising from these ions are measured by the second analyser. The compound under scrutiny must be known and have been well characterised previously before this type of experiment is undertaken.

2.10.1 Collision-induced Dissociation (CID) (13, 22)

If an ion collides with a neutral atom or molecule, some of the ion's kinetic energy can be converted into internal energy. This is called collisional activation. If there is enough excess internal energy to break chemical bonds, the ion will decompose. This is called collision-induced dissociation (CID) or collisionally activated dissociation (CAD). Both terms mean the same thing. The CID processes a sequence of two steps. The first step is very fast and corresponds to the collision between the ion and the target molecule when a fraction of the ion translational energy is converted into internal energy, bringing the ion into an excited state. The second step is the unimolecular decomposition of activated ion. CID is accomplished by selecting an ion of interest with a mass analyzer and introducing that ion into the collision cell, where the selected ion collides with the collision gas (Ar or He) atoms, resulting in fragmentation. For collision energy conversion to internal energy, the collisions of mobile species (the ion) and a static target (the collision gas) control the kinetic energy for internal energy transfers. In practice, there are two groups of collision energy: high-energy collisions, these refer to collisions where the precursor ion is accelerated to kinetic energies of approximately one kilovolt or higher. Low-energy collisions are referring to collisions where the precursor ions have kinetic energies in the range of a few eV to a few hundred eV. Fragmentation within triple quadrupole, qaudrupole ion trap and hybrid quadrupole ToF analyzers occur at low collisional energy, whilst fragmentations within a magnetic sector or ToF/ToF analyzer occur at high collisional energy. The collision gas is more important than it is for the high-energy

collisions. Heavier gases such as argon, xenon or kryton are preferred because they allow the transfer of more energy. In comparison of two-collision energy, the different fragmentation patterns are observed. The high-energy CID spectra give simpler, more clear-cut fragmentation, whereas low-energy CID spectra lead to more diverse fragmentation pathways, often including more rearrangements.

2.10.2 Electrospray Ionization Quadrupole/Time of Flight Mass spectrometer (ESI-Q/TOF MS) (8, 22)

ESI-Q/TOF mass spectrometry has electrospray ionization for ion source, a quadrupole mass filter for the mass analyzer and a time-of-flight mass analyzer for the second mass analyzer. ESI-Q-TOF MS used for molecular weight measurements, reaction monitoring, protein structural studies, peptide sequencing, nucleotide sequencing, macromolecule structure determination due to extended m/z range.

Quadrupole time-of-flight or hybrid analyzer is described in 1996 by Morris for oligosaccharide analysis and more recently Lobada et al., 2000; these instrument have rapidly become the instrument standard for MS/MS applications with in the theatre of proteomics. The user is able to acquire MS and most notably MS/MS data with high mass accuracy, resolution and sensitivity. The instrument is generally with interfaced with HPLC.
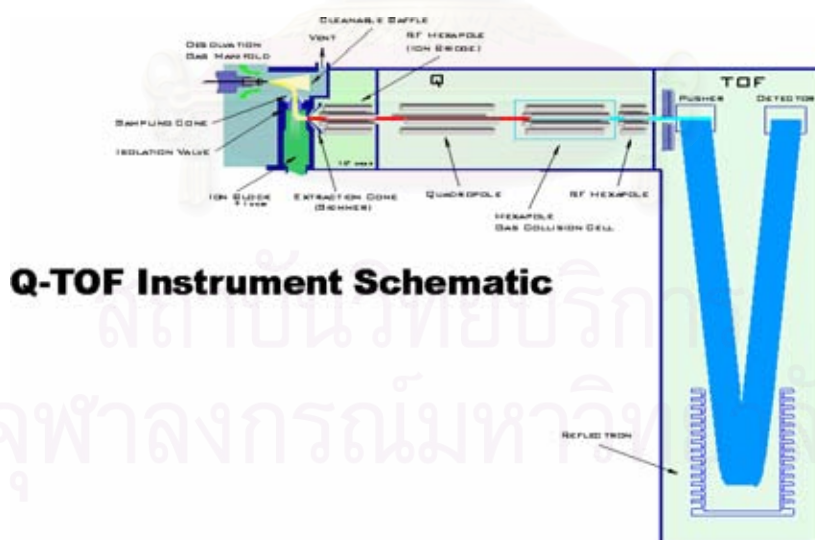
Figure 2.13   Schematic diagram of Electrospray Ionization Quadrupole/Time of Flight Mass spectrometer

2.11 Peptide Sequencing by Tandem Mass spectrometry (13, 22)

The most common usage of MS/MS in biochemical areas is the product or daughter ion scanning experiment, which is particularly successful for peptide and nucleotide sequencing. Roepstorff and Fohhlman, 1984, described this fragmentation nomenclature.

Peptides are three different types of bonds that can fragment along the amino acid backbone: the NH-CH, CH-CO, and CO-NH bonds. Each bond breakage gives rise to two species, one neutral and the other one charged, and only the charged species is monitored by the mass spectrometer. The charge can stay on either of the two fragments depending on the chemistry and relative proton affinity of the two species. Hence, there are six possible fragment ions for each amino acid residue and these are labelled as in the figure 2.13, with a, b, and c ions having the charge retained on the N-terminal fragment. The x, y", and z ions having the charge retained on the C-terminal fragment (Figure 2.14). The most common cleavage sites are at the CO-NH bonds which give rise to the b and/or the y" ions.
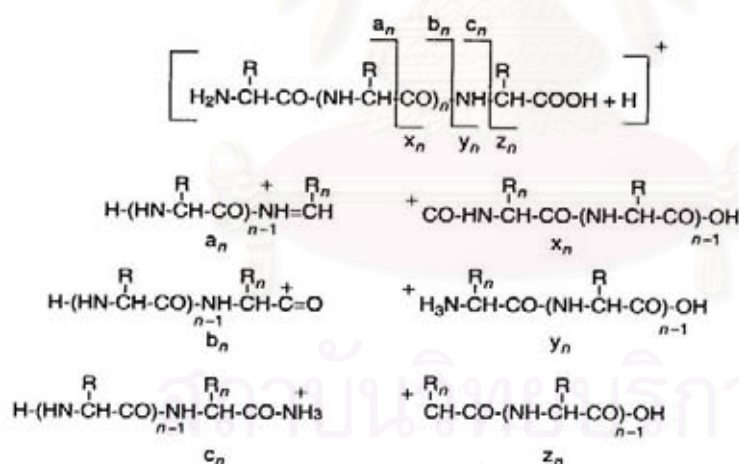
Figure 2.14 Structure of the peptide fragmentation

The extent of side-chain fragmentation detected depends on the type of analysers used in the mass spectrometer. A magnetic sector–magnetic sector instrument will give rise to high-energy collisions resulting in many different types of side-chain cleavages but also increasing the complexity and difficulty of interpretation. Quadrupole–quadrupole

and quadrupole–time-of-flight mass spectrometers generate low energy fragmentations with fewer types of side-chain fragmentations.

In addition, there are two other types of fragments, which appear among the low masses in the spectrum (Figure 2.15). The first type is called an internal fragment because these fragments have lost the initial N- and C- terminal side. The second type of fragment is immonium ions of amino acids; appear in the very low m/z range of the MS-MS spectrum. Each amino acid residue leads to a diagnostic immonium ion, with the exception of the two pair's leucine (L) and iso-leucine (I), and lysine (K) and glutamine (Q), which produce immonium ions with the same m/z ratio, i.e. m/z 86 for I and L, m/z 101 for K and Q. The immonium ions are useful for detecting and confirming many of the amino acid residues in a peptide, although no information regarding the position of these amino acid residues in the peptide sequence can be ascertained from the immonium ions.
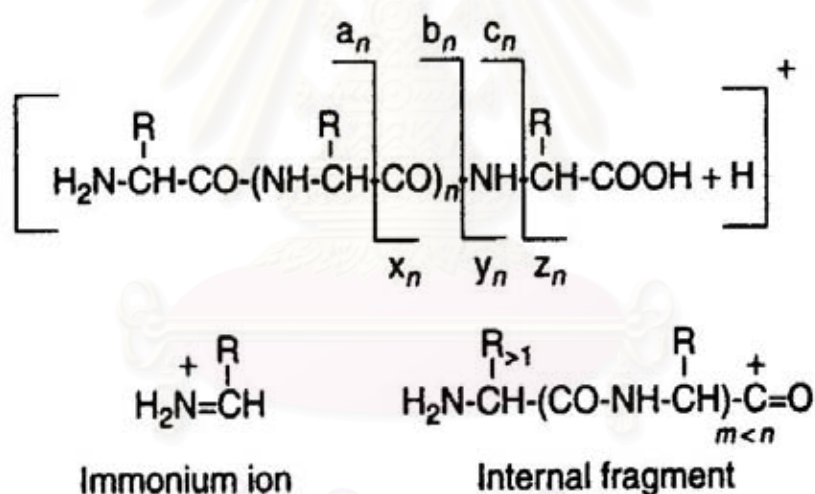
Figure 2.15 Immonium Ion and Internal Fragment

Protein and peptide structure differences relative to the residue masses in Table 2.2, which allow recognition of the N- and C- terminal of the peptide sequence.

Table 2.2 The residue masses of common 20 amino acids

Amino acid	Code (3 letters)	Code (1 letter)	Residue mass (Da)	Immonium ion (m/z)
Glycine	Gly	G	57.02	30
Alanine	Ala	A	71.04	44
Serine	Ser	S	87.03	60
Proline	Pro	P	97.05	70
Valine	Val	V	99.07	72
Threonine	Thr	T	101.05	74
Cysteine	Cys	C	103.01	76
Leucine	Leu	L	113.08	86
Isoleucine	Ile	I	113.08	86
Asparagine	Asn	A	114.04	87
Aspartate	Asp	D	115.03	88
Glutamine	Gln	Q	128.06	101
Lysine	Lys	K	128.09	101
Glutamate	Glu	E	129.04	102
Methionine	Met	M	131.04	104
Histidine	His	H	137.06	110
Phenylalanine	Phe	F	147.07	120
Arginine	Arg	R	156.10	129
Tyrosine	Tyr	Y	163.06	136
Tryptophan	Try	W	186.08	159

2.12 Methods for phosphoprotein analysis

Phosphorylation is of considerable interest due to its important role within cellular signal transduction (24). The generic analysis of phosphorylation is difficult, however, as the cell signaling proteins of interest are generally present at low abundance within the cell and the stoichiometry of phosphorylation (of a given protein) required for signal transduction may be low.

2.12.1 Mass spectrometric methods for selective detection of phosphopeptides

Mass spectrometric detection of phosphopeptides typically requires either prior enrichment of phosphopeptides or phosphopeptide-specific mass spectrometry (e.g. precursor ion scanning). The difficulty in detecting phosphopeptides may stem primarily from their low abundance relative to non-phosphorylated peptides (discussed in Ballif et al.(25)) however the negatively charged phosphate group is also likely to affect ionization efficiency. For electrospray mass spectrometry it has been suggested that reduced ionization efficiency may be a problem when the number of phosphate groups is greater than the number of basic groups (i.e. multiply phosphorylated tryptic peptides) (26).

The lability of the phosphate group under mass spectrometric conditions can be used to selectively identify phosphopeptides. The facile loss of the phosphate group (H_3PO_4) in mass spectrometry of serine and threonine phosphopeptides was noted in fast atom bombardment MS spectra (27) and the loss of H_3PO_4 or HPO_3 is a characteristic of mass spectrometric analysis of phosphopeptide, almost irrespective of the type of mass spectrometry used. In the positive mode, the tendency of phosphoserine and threonine residues to show a neutral loss of H_3PO_4 (98 Da) rather than HPO_3 (80 Da) can help to distinguish them from phosphotyrosine residues, which usually show a loss of 80 Da only (28).

In the following section the application of precursor ion and neutral loss scanning in phosphopeptide analysis is examined.

1) Precursor ion scanning

Precursor ion scanning, using a triple quadrupole mass spectrometer, can be used to detect phosphopeptides in the negative mode by identifying the precursors of the 79 Da (PO_3^-) fragment ion (29). Although in the negative mode phosphopeptide ionization should be favoured over that of less acidic non-phosphorylated peptides, negative mode peptide sequencing is not straightforward. Therefore a two stage experiment can be used, with negative ion mode precursor ion scanning to give a list of phosphopeptide masses, which are then sequenced in the positive mode (28, 30). If optimal pH sample buffers are to be used for the two modes, the sample must be split in two, or the buffer exchanged (e.g. via reverse-phase chromatography). If the peptide sample is reasonably complex, analysis can be online with RPLC separation, reducing the possibility of sequencing an unphosphorylated peptide of similar mass to the true precursor phosphopeptide. This LC-MS approach has recently been demonstrated with a hybrid triple quadrupole/linear ion

trap mass spectrometer capable of rapid switching from negative to positive mode (Q-Trap), allowing precursor ion scanning and phosphopeptide sequencing in a single experiment(8). A direct equivalent of -79 m/z precursor ion scanning in the positive mode is not possible, as the 80 Da HPO_3 fragment is not positively charged. To enable positive mode precursor ion scanning experiments for detection of phosphoserine and phosphothreonine containing peptides, peptide derivatisation is required. Phosphate β-elimination and Michael addition of 2-dimethylaminoethanethiol, followed by oxidation (thioether to sulfoxide) gave a peptide that released a sulfenic acid derivative fragment of 122 Da on low-energy CID (Figure 2.16) (31). This characteristic fragment could then be detected by precursor ion scanning. Furthermore, the addition of a basic tertiary amino group to the peptide should improve positive mode ionization efficiency. The disadvantages of this method are the inefficiency of β-elimination, especially for phosphothreonine residues, and the sample losses associated with the β-elimination and Michael addition.
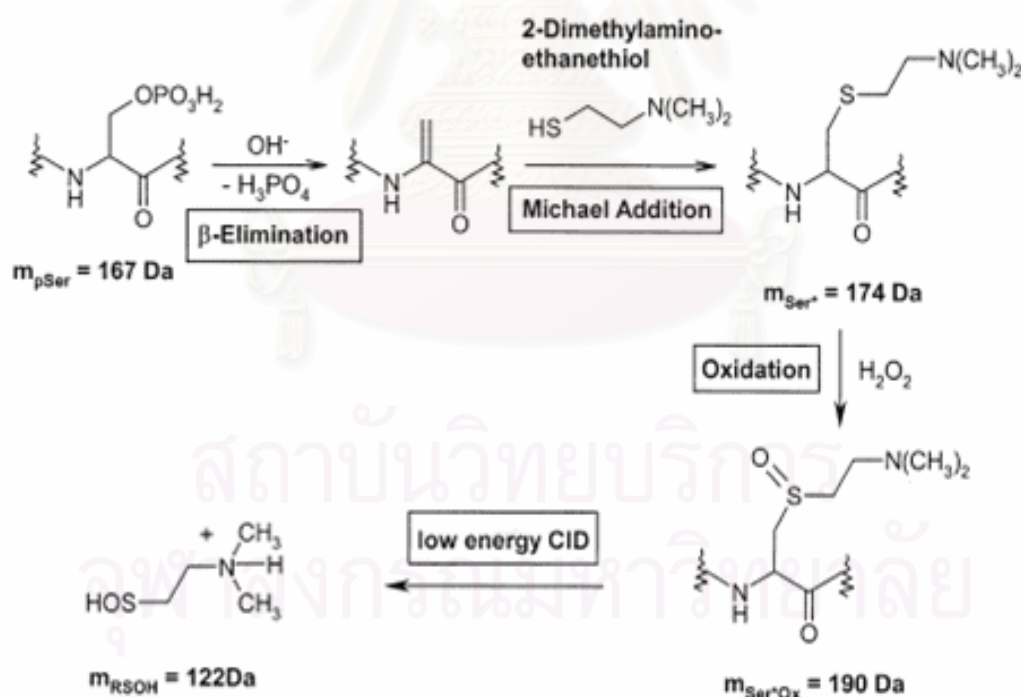
Figure 2.16   Phosphopeptide derivatization to allow detection by precursor ion scanning Collision induced dissociation of phosphotyrosine peptides yields a diagnostic immonium ion that can be used for positive mode precursor ion scanning, provided an instrument with high enough resolution to distinguish the 216.043 pTyr immonium ion from neighbouring

ions (e.g. AsnThr b_2 ion at 216.098) is used (32). This is possible with a quadrupole time-of-flight instrument, using a pseudo-precursor ion scanning experiment, as described below.

Although true precursor ion scanning requires a scanning instrument (e.g. a triple quadrupole instrument) a precursor ion scanning-like experiment can be performed on an quadrupole time-of-flight instrument (33). This experiment was designated 'precursor ion discovery' to distinguish it from a true precursor ion scanning experiment. The technique uses two survey modes: the first with low collision voltage, the second with high collision voltage. The latter mode gives fragmentation of all the ions present in the collision cell. If the product ion of interest (e.g. phosphotyrosine immonium ion) is detected in the resulting spectrum then the low collision energy spectrum is examined to give a list of possible precursor ions. This list is then interrogated by selection of each potential precursor for MS/MS. Obviously the fewer possible precursor ions the better, therefore good HPLC separation of the sample is essential.

2) Neutral loss scanning

In order to detect phosphopeptides in the positive mode, the neutral loss of 98 or 80 Da corresponding to H_3PO_4 or HPO_3 can be detected (34, 35). However this requires scanning for at least two separate losses (e.g. 49 m/z and 32.7 m/z for H_3PO_4) assuming the majority of phosphopeptides will be either doubly or triply charged. This neutral loss scan is less specific than the negative mode precursor ion scanning experiment as the collision energies for optimal neutral loss of the phosphate group from doubly charged peptides are high enough to allow backbone fragmentation of higher charge state peptides (35, 36).

The software described above for precursor ion scanning-like experiments on quadrupole ToF instruments can also be configured to search for a particular neutral loss (between the high and low collision energy survey modes) (33).

3) Hypothesis-driven mass spectrometry

In cases where the sample consists of a known protein, from an immunoprecipitation for example, masses of all the theoretically possible phosphopeptide can be calculated. This list can then be used to inform mass spectrometric analysis, after selecting those peptides that are within the analytical range of detection. The list of theoretical phosphopeptides can be filtered further according to kinase phosphorylation consensus motifs if required. This strategy has been implemented on a number of different instruments.

Chang et al. used a MALDI ion trap instrument, allowing MS^3 analysis. Putative phosphopeptide masses were selected and fragmented (37). Where a neutral loss of 98 m/z was observed, MS^3 was performed on the $[M+H-98]^+$ ion to confirm the sequence of the phosphopeptide.

Yi et al. also used a list of putative phosphopeptides (kinase consensus motif sites) but in an LC MS analysis, using an ion trap instrument (LCQ) (38). Analysis on-line with HPLC precludes the methodical selection of each putative phosphopeptide mass, instead a limited number of masses were targeted for fragmentation when detected in the survey scan- i.e. an 'include' list.

LC MS analysis was also used by Unwin et al. but in this case online with a hybrid triple quadrupole/linear ion trap instrument (QTrap) (39). The instrument was operated in triple quadrupole mode to interrogate a list of putative phosphopeptides by selective reaction monitoring, i.e. Q1 transmits a putative phosphopeptide mass, fragmentation occurs in the collision cell (Q2) and Q3 transmits only a particular fragment, e.g. the fragment resulting from neutral loss of phosphate. When the selective reaction monitoring indicates a phosphopeptide is present, the phosphopeptide is then sequenced using the linear ion trap mode. Again, the constraints of online analysis limit the number of reactions that can be monitored. In this case the authors limited the cycle time (including one product ion scan) to ~10 s, allowing from 65 to 107 transitions to be monitored (dwell times from 75 to 40 ms).

2.12.2 Phosphoprotein enrichment

Various phosphoprotein enrichment strategies exist and will be discussed in this section. However a general point is that phosphoprotein enrichment (e.g. by phospho-specific immunoprecipitation) followed by protein identification does not provide conclusive evidence that the protein is in fact phosphorylated unless a phosphopeptide is identified. This is due to the potential in each technique for false-positives (e.g. by non-specific binding of proteins during immunoprecipitation).

Immobilized metal affinity chromatography

Andersson and Porath established that phosphoserine had significantly higher affinity for Fe^{3+}-loaded sepharose iminodiacetic acid (IDA) than the other amino acids (40). They showed that phosphorylated and partially dephosphorylated ovalbumin could be retained and separated according to the degree of phosphorylation, using pH gradient elution. Since

then immobilized metal affinity chromatography (IMAC) has been used predominantly for phosphopeptide enrichment, but a number of recent papers have successfully employed IMAC at the protein level. Collins et al. tested two IMAC resins, sepharose IDA (tridentate) and agarose nitrilotriacetic acid (NTA; quadradentate; Figure 2.17) (41). The resin performance was assessed by comparing staining intensity of the eluate and the unbound fraction after SDS PAGE using a fluorescent stain with high affinity for phosphoproteins (Pro-Q diamond) (42). The tridentate IDA resin was found to retain significantly more phosphoprotein than the quadradentate NTA, with either iron or gallium as the chelated metal.
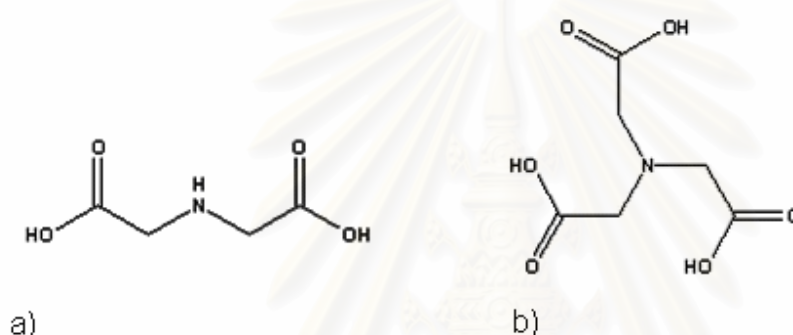
Figure 2.17   Metal chelators common used in IMAC

   a) imminodiacetic acid   b) nitrilotriacetic acid

Guerrera et al. used agarose NTA for protein IMAC and employed Western blotting (anti-phospho-p44/42 mitogen-activated protein kinase, anti-phosphotyrosine and anti-phosphoSer/Thr antibodies) to confirm enrichment of phosphoproteins (43). They found the choice of an acidic pH (~3.5) for sample loading was important to prevent non-specific binding of acidic proteins.

A commercial phosphoprotein affinity column is also available. While all information regarding the type of resin and buffer composition is proprietary, it is thought to use an IMAC-like enrichment and has been shown by fluorescent staining and phospho-specific Western blotting to effectively enrich phosphoproteins (44, 45 ,46, 47).

2.12.3 Phosphopeptide enrichment

Phosphopeptide enrichment and identification has the advantage that phosphorylation of the protein is by definition confirmed. However in large-scale phosphopeptide discovery approaches, a significant fraction of the identified proteins may be identified on the basis

of a single phosphopeptide from the particular protein. These so-called "one-hit wonders" require that the peptide identifications are of high confidence. Criteria for accepting protein identifications based on a single peptide are discussed in Rush *et* al. (48).

1) Immobilized metal ion affinity chromatography

After the initial demonstration of phosphoserine and phosphoprotein retention using Fe^{3+} IDA sepharose (40), the same material was shown to retain and concentrate casein phosphopeptides too (49). Acidic peptides, containing multiple glutamate residues, were also shown to be weakly retained by Fe^{3+}-IDA resin (50). The presence of tyrosine residues appeared to increase this retention. Figure 2.18 illustrates possible interactions between the iron and either phosphate or carboxyl groups. More than one carboxyl group is expected to be required for binding, consistent with the weaker affinity of acidic peptides than phosphopeptides for the IMAC resin.
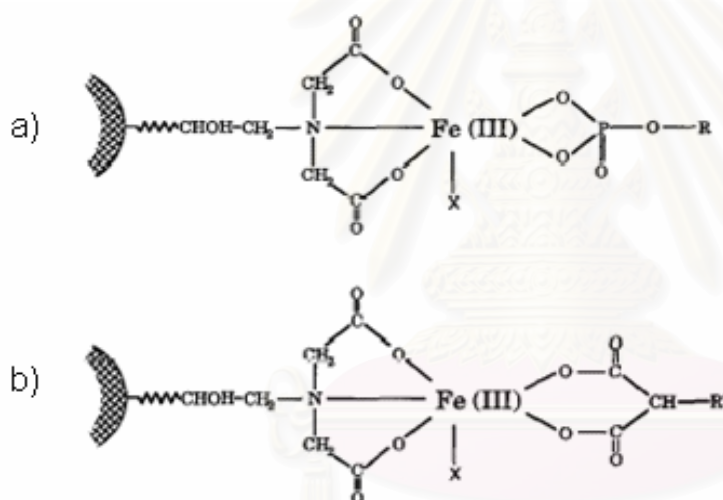
Figure 2.18   Proposed interactions between IDA-immobilized Fe^{3+} and
a) Phosphate   b) Carboxyl groups

On-line IMAC, i.e. electrospraying directly from an IMAC column, has been investigated. Nuwaysir et al. identified the β-casein phosphopeptides using on-line IMAC, despite the eluate being at ~pH 10 (non-optimal conditions for positive mode electrospray ionization) (51). More recently an IMAC column has been eluted onto a reverse phase C18 precolumn, which is then connected to an analytical column for LC MS analysis. This allows the solution sprayed into the mass spectrometer to be acidified and the sample is also desalted (removing phosphate buffer used for elution or any metal ions leaching from the column) (52). In subsequent studies this protocol was modified by the introduction of a methyl esterification step prior to IMAC enrichment (53, 54, 55). Methyl esterification of

aspartate, glutamate and C-termini carboxyl groups was shown to reduce binding of non-phosphorylated acidic peptides to the IMAC column, i.e. the specificity was increased. This method allowed the identification of over two hundred phosphopeptides, most of which were doubly phosphorylated, suggesting that competitive binding occurred with the conditions used (54).

The more commonly used Fe^{3+}-loaded IDA sepharose was compared to Fe^{3+}-loaded NTA sepharose for enrichment of phosphopeptides from a large protein (56). The same phosphorylation sites were identified in both cases, but IMAC using the NTA sepharose appeared to be more specific than the IDA Sepharose (with just five non-phosphorylated peptides in the eluate, compared to fifteen for the IDA).

A systematic study of different metals (Al, Fe, Ga, In, Ru, Sc, Y, Zr) showed that gallium (Ga^{3+}) was slightly more selective than iron (Fe^{3+}) for a phosphopeptide in a β-galactosidase tryptic digest. An additional advantage of Ga^{3+} was that elution of phosphopeptides occurred at a lower concentrations of ammonium hydroxide for Ga^{3+} IMAC than Fe^{3+} (57).

2) Phosphopeptide enrichment by ion-exchange chromatography

Separation of peptides by strong-cation exchange chromatography is a key part of 'shot-gun' multidimensional protein identification strategies. Recently ion-exchange chromatography has been used to enrich for phosphopeptides, taking advantage of the acidic phosphate moiety.

Nühse et al. employed strong anion exchange separation to fractionate peptides prior to IMAC phosphopeptide enrichment (58). They observed that the majority of non-phosphorylated peptide eluted in the first few fractions (0-150 mM sodium chloride) while the more acidic phosphopeptides eluted over a wider range (0-500 mM sodium chloride).

Beausoleil et al. used a strong cation exchange separation at a pH slightly more acidic than normal (pH 2.7)(59). This strategy was designed to take advantage of the lower pK_a of the phosphate group than the carboxyl groups to leave only the phosphate groups negatively charged. At pH 2.7 typical tryptic peptides would have a solution charge state of +2 while typical tryptic phosphopeptides would have a charge state of +1. Phosphopeptides were therefore enriched in the early-eluting fractions. Beausoleil et al. identified approximately two thousand sites of phosphorylation from a mammalian nuclear fraction using this approach. In a subsequent paper from the same group approximately five hundred sites of phosphorylation were identified using the same method (25). While these numbers are

impressive, the SCX separation provides only a partial enrichment of the phosphopeptides (60). Phosphopeptides within these SCX fractions can be identified either by exhaustive mass spectrometry or further enriched prior to mass spectrometric analysis. Gruhler et al. used a combination of SCX enrichment with subsequent IMAC enrichment to identify approximately seven hundred phosphopeptides.

Neutral species	pK_a	Reference
Phosphoric acid: H_3PO_4	1.8	(Saha et al., 1996)
Methyl phosphoric acid: $(CH_3)H_2PO_3$	1.1	(Saha et al., 1996)
Aspartic acid: $-(CH_2)CO_2H$	4	(Nielsen and Vriend, 2001)
Glutamic acid: $-(CH_2)_2CO_2H$	4.4	(Nielsen and Vriend, 2001)
C-terminal carboxyl: $-CO_2H$	3.8	(Nielsen and Vriend, 2001)

Table 2.3   Table of phosphate- and peptide-related pK_a values.
The exact pK_a value of these groups in peptides is expected to vary according to the surrounding amino acid sequence.

2.13 Protein Identification

Protein can be rapidly identified using mass spectrometry. It is especially the short time analysis, the high-sensitivity and high information content in protein sequencing. For general method to MS data can be used in four approaches for protein identification (61).

1) Peptide mass fingerprint (PMF) – MS mode. The mass measurement of each peptide derived from the enzyme digestion or chemical cleavage of the protein.

2) Peptide mass fingerprint and composition information. The molecular weights of each of the peptides derived from the enzyme digestion or chemical cleavage of the protein can be used alongside some composition information relating to one or more of the peptide.

3) Peptide mass fingerprint and sequence information. The molecular weight of each of the peptides derived from the enzyme digestion or chemical cleavage of the protein can be used alongside some direct sequence information realign to one or more the peptides.

4) Product ion MS/MS sequence data from one or more peptide-MS/MS mode.

As a rule, the mass spectrometric data use freely available databases containing amino acid sequences of protein. The example of the protein sequence databases were shown below (62):

- SWISS-PROT (www..expasy.ch/sprot-top.html) is a database of annotated protein sequence; it also contains addition information on function of the protein, its domain structure, posttranslational modification, etc.

- TrEMBL (www.expasy.ch/srs5/) is a supplement to SWISS-PROT, which contains all protein sequences, translated from nucleotide sequences of the EMBL database.

- NCBInr (www.ncbi.nLm.nih,gov/dbEST/) is a database containing sequences translated from DNA sequences of GenBank and also sequences from PDB, SWISS-PROT and PI database.

These databases are constantly updated and are usually characterized by the standardized data format.

For peptide, mass fingerprinting has several programs available to use. There is MASCOT at www.matrixscience.com, profound at www.prowl.com and MS-FIT at www.prospector.ucsf.edu/. The four important parameters for data search are peptides mass list, the cleavage agent, error tolerance (mass accuracy) and knowledge of peptide modification.

For protein identification by 2-DE, in gel digestion has important methodology to identify. There steps are excision of the relevant spots, in-gel proteolytic cleavage and extraction of the resulting peptides, the mass spectrometric (MALDI) analysis of this peptide mixture (peptide mass fingerprinting or PMF) can be performed. A subsequent protein database search can result in the identification of the organism is available. If this method does not result in unequivocal protein identification, individual peptides (parent) of the proteolytic map can be subject to further fragmentation analysis using MS/MS techniques (peptide fragmentation fingerprint or PFF). Hence, the identity of the original protein can be determined from the resulting fragment ion pattern (daughter ion spectrum) of a single proteolytic peptide by a further database search. This method even allows the identification of different components of an unseparated protein mixture. In addition to these database dependent techniques, ESI-MS/MS also allows direct sequencing of individual peptides from a proteolytic mixture without the availability of genetic information.

2.14   Protein detection by Western blot (Immunoblotting) (4)

Western blot (alternately, immunoblot) is a method to detect a specific protein in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native

or denatured proteins by the length of the polypeptide (denaturing conditions) (Figure 1) or by the 3-D structure of the protein (native/ non-denaturing conditions). The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are probed (detected) using antibodies specific to the target protein. There are now many reagent companies that specialize in providing antibodies (both monoclonal and polyclonal antibodies) against many thousands of different proteins. This has dramatically reduced the time to carry out a blot. Previously large animals (e.g. sheep, goat - lots of serum) had to be immunized with the target protein twice (secondary immune response generates high affinity antibodies). Then either serum could be purified and used (polyclonal antibodies) or B cells could be isolated from the animal and fused in vitro with mouse myeloma cells to generate hybridomas that then provided single-specificity antibodies (monoclonal antibodies). Commercial antibodies are expensive, though can be re-used (unbound antibody) between experiments. This method is used in the fields of molecular biology, biochemistry, immunogenetics and other molecular biology disciplines. (Figure 2.19).

The procedure can be broken down into a series of steps

1. Size separation of the proteins in the mixture by Polyacrylamide Gel Elecrophoresis (PAGE). The Proteins of the sample are separated using gel electrophoresis. Separation of proteins may be by isoelectric point (pI), molecular weight, electric charge, or a combination of these factors. Sampled proteins become covered in the negatively charged SDS and move to the positively charged electrode through the acrylamide mesh of gel. Smaller proteins migrate faster through this mesh and the protein are thus separated according to size (usually measured in kilo Daltons, kD). Proteins travel only in one dimension along the gel for most blots. Samples are loaded into wells in the gel. One lane is usually reserved for a marker, a commercially available mixture of proteins having defined molecular weights, typically stained so as to form visible, coloured band. When voltage is applied along the gel, proteins migrate into difference speeds. These difference rates of advancement separate into bands within each lane. It also possible to use a two-dimensional gel which spreads the proteins from a single sample out into two dimensions. Proteins are separated according to isoelectric point in the first dimension, and according to their molecular weight in the second dimension.
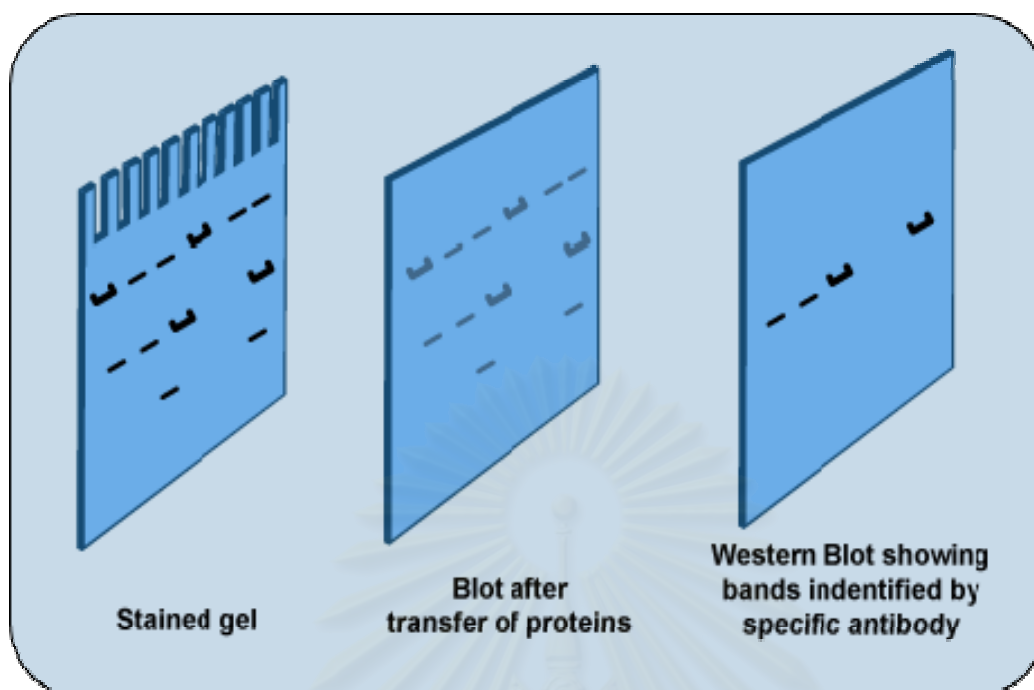
Figure 2.19   Concept of Western Blotting

2. Transfer of the separated proteins to a membrane while retaining their relative position. (Figure 2.20) In order to make the proteins accessible to antibody detection, they are moved from within the gel onto a membrane made of nitrocellulose or PVDF. The membrane is placed on top of the gel, and a stack of tissue papers placed on top of that. The entire stack is placed in a buffer solution which moves up the paper by capillary action, bringing the proteins with it. Another method for transferring the proteins is called electroblotting and uses an electric current to pull proteins from the gel into the PVDF or nitrocellulose membrane. The proteins have now moved from within the gel onto the membrane while maintaining the organization they had within the gel. As a result of this "blotting" process, the proteins are exposed on a thin surface layer for detection. Both varieties of membrane are chosen for their non-specific protein binding propert. Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein. Nitrocellulose membranes are cheaper than PVDF, but are far more fragile and do not stand up well to repeated probings.
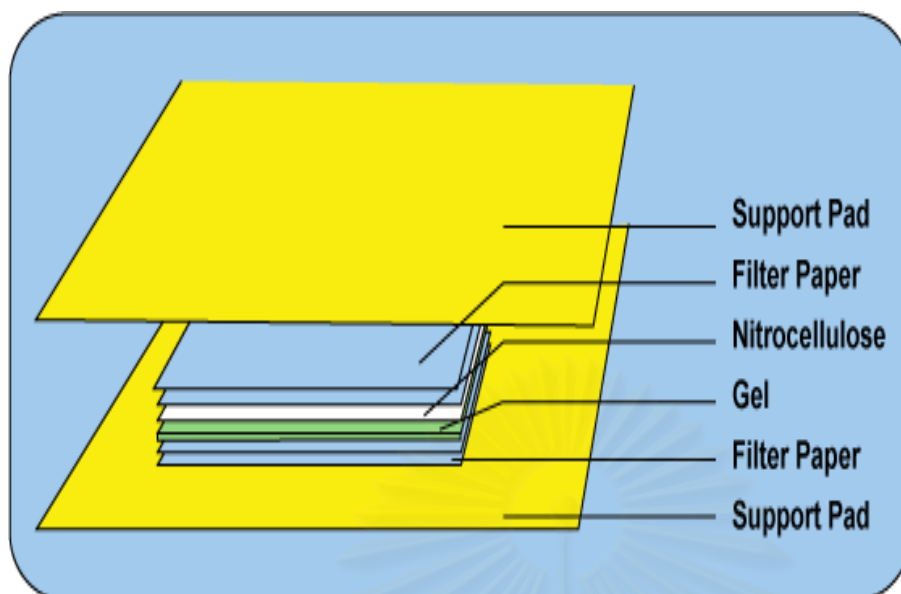
Figure 2.20 Arrangement of the gel, blot, filter paper and support pads for electrophoretic transfer of proteins for a Western Blot.

3. Detecting the protein under investigation by its specific reaction with an antibody and determination of its size relative to standard proteins of known size. During the detection process the membrane is "probed" for the protein of interest with a modified antibody which is linked to a reporter enzyme, which when exposed to an appropriate substrate drives a colorimetric reaction and produces a colour.

Primary antibody

Antibodies are generated when a host species or immune cell culture is exposed to the protein of interest (or a part thereof). Normally, this is part of the immune response, whereas here they are harvested and used as sensitive and specific detection tools that bind the protein directly.

After blocking, a dilute solution of primary antibody (generally between 0.5 and 5 micrograms/ml) is incubated with the membrane under gentle agitation. Typically, the solution is comprised of buffered saline solution with a small percentage of detergent, and sometimes with powdered milk or BSA. The antibody solution and the membrane can be sealed and incubated together for anywhere from 30 minutes to overnight. It can also be incubated at different temperatures, with warmer temperatures being associated with more binding, both specific (to the target protein, the "signal") and non-specific ("noise").

Secondary antibody

After rinsing the membrane to remove unbound primary antibody, the membrane is exposed to another antibody, directed at a species-specific portion of the primary antibody. This is known as a secondary antibody, and due to its targeting properties, tends to be referred to as "anti-mouse," "anti-goat," etc. Antibodies come from animal sources (or animal sourced hybridoma cultures); an anti-mouse secondary will bind to just about any mouse-sourced primary antibody. This allows some cost savings by allowing an entire lab to share a single source of mass-produced antibody, and provides for more consistent results. The secondary antibody is usually linked to biotin or to a reporter enzyme such as alkaline phosphatase or horseradish peroxidase. This means that several secondary antibodies will bind to one primary antibody and enhances the signal.

Most commonly, a horseradish peroxidase-linked secondary is used in conjunction with a chemiluminescent agent, and the reaction product produces luminescence in proportion to the amount of protein. A sensitive sheet of photographic film is placed against the membrane, and exposure to the light from the reaction creates an image of the antibodies bound to the blot.

CHAPTER III
EXPERIMENTAL

3.1 Materials and Instruments

3.1.1 Cancer and normal cell lines,

Lung cancer cell line (A549) and Lung fibroblast cell line (MRC-5) were received from Laboratory of Biochemistry, Chulabhorn Research Institute.

3.1.2 Instrument

Autopipette: Pipetman, Gilson, France

DUEL Gel Caster: Mighty Small SE245, Hoefer, U.S.A

ESI-Q-TOF Mass spectrometer: Micromass, UK

Hoefer TE series Transphor tank: Amersham Bioscience, U.S.A.

Homogenizer: IKA, Germany

IEF electrophoresis unit: Multiphor II, Amersham Pharmacia Biotech, U.S.A.

Microcentrifuge: Biofuge pico Heraeus, Kendro, Germany

Mini Trans Bloy cell: BioRad, U.S.A.

Orbital Shaker: Optima, Japan

Peptide Trap: Michrom BioResources, Germany

pH meter: Denver Instrument, U.S.A.

Power Supply: EPS 3500 XL, Pharmacia, England

Refrigarated centrifuge: Himac CR20B2, HITACHI, Japan

Sonicate: DHA-1000: Branson, U.S.A

Sonicator: BHA-1000, Branson, U.S.A

UV –Visible Spectrophotometer: Shimadzu, Japan

Labofuge 400R: Heraeus, Germany

Thermomixer comfort: Eppendorf, Germany

Speed vacuum centrifuge: Heto-Holten, Denmark

Vortex mixer: Vortex-Genie2, Scientific Industries, U.S.A

Water Bath Shaking: Memmert, Germany

3.1.3 Chemicals

Acetic acid: Merck Ag Darmstadt, Germany

Acetone: Merck Ag Darmstadt, Germany

Acrylamide PAGE: Plus-one Pharmacia Biotech, Sweden

Agaraose: Plus-one Pharmacia Biotech, Sweden

Anti-mouse IgG: Dako Cytomation, Denmark

Ammonium persulfate: Plus-one Pharmacia Biotech, Sweden

β-mercaptoethanol: Merck Ag Darmstadt, Germany

Bromophenol Blue: USB, U.S.A

CHAPS (3-[(3-cholamidopropyl)-dimthylammonio]-1-propane sulfonate): USB, USA

Coomassie Brilliant Blue R-250: Serva, U.S.A

Cocktail protease inhibitor: Sigma, U.S.A.

DTT (Dithiothrretiol): Plusone Pharmacia Biotech, Sweden

EDTA (Ethylenediaminetetraacetic acid): Merck Ag Darmstadt, Germany

Ethanol: Merck Ag Darmstadt, Germany

Glycerol: Plus-one Pharmacia Biotech, Sweden

Glycine: Plus-one Pharmacia Biotech, Sweden

Iodoacetamide: Sigma, U.S.A

Methanol: Merck Ag Darmstadt, Germany

Mouse anti cytokeratin 8 monoclonal antibody: Chemicon

Mouse anti cytokeratin 18 monoclonal antibody: Chemicon

N,N'-methylene-bis-acrylamide: Plus-one Pharmacia Biotech, Sweden

Nonidet NP-40: Amresco, U.S.A

SDS (Sodium Dodecyl Sulfate): Plus-one Pharmacia Biotech, Sweden

Tris: USB, U.S.A

TEMED: Invitrogen, U.S.A.

TFA: Merck, Germany

Trypsin: Promega, U.S.A.

Urea: Plusone Pharmacia Biotech, Sweden

3.1.4 Supplies

Hybond ECL Nitrocellulose Membrane: Amersham Bioscience, Germany

Hyperfilm ECL: GE Healthcare, UK

3.1.5 Kits

ECL plus Western blotting detection reagents: GE Healthcare, UK

Immobilized pH gradient strip pH 3-10NL, 7cm: Amersham pharmacia biotech, Sweden

Protein assay: BioRad, USA

Standard Low Molecular Weight Marker Protein: Amersham pharmacia biotech, Sweden

3.2 Methods

3.2.1 Cell collection

Both cells were gently washed with 0.25 M Sucrose 3 times, prior to scraping with a rubber policeman with 3 ml of 0.25M sucrose adding with cocktail inhibitor. The cell homogenate were centrifuge at 3,000 rpm, 4°C, 10 minute. The proteins concentration was determined by Bradford's method.

3.2.2 Protein Quantification

Protein content was determined by Bradford's method (63). Prepare five standards solution (1mL each) containing 0, 10, 20, 30, 40 and 50 µg/ml BSA were prepared. To a 1.4 ml plastic cuvett, 0.2 ml Protein assay solution (BioRad) was added followed by 0.8 ml of one of the protein standard solution or sample to be assayed (containing<100 µg of protein for <50 µg/ml standards). Gently mixed the solution for several times. After that recorded the absorbance spectrum of sample at 595 nm and repeat the steps above for each of the protein standards and for samples to be assayed. Then standard curve between the absorbance at 595 nm and protein concentration was plotted. To determine the protein concentration of sample from it absorbance, use the standard curve to find the concentration of standard that would have the absorbance as the sample.

3.2.3 Two-Dimensional Gel Electrophoresis

1) Isoelectric focusing and SDS-PAGE

The 2-D electrophoresis procedure was described in the manual of 2-D electrophoresis using immobilized pH gradients; Principle and Method (11). For the first dimension, proteins from cell were dissolved in lysis buffer (Appendix A). The total protein concentration was quantified by using Bradford's procedure (63). Then, added and mixed rehydration solution (see Appendix A) in the sample solution to total volume 125 µl contain an estimated 350 µg protein. Delivered the solution slowly at a central point in the slot of the Immobiline DryStrip Reswelling Tray and removed any large bubbles. Placed the pH 3-10 IPG strips (length 7 cm) on the solution and overlaid each IPG strip with 3 ml

of DryStrip Cover Fluid to minimize evaporation and urea crystallization. A minimum of 10 hours was required for rehydration at room temperature (Figure 3.1).
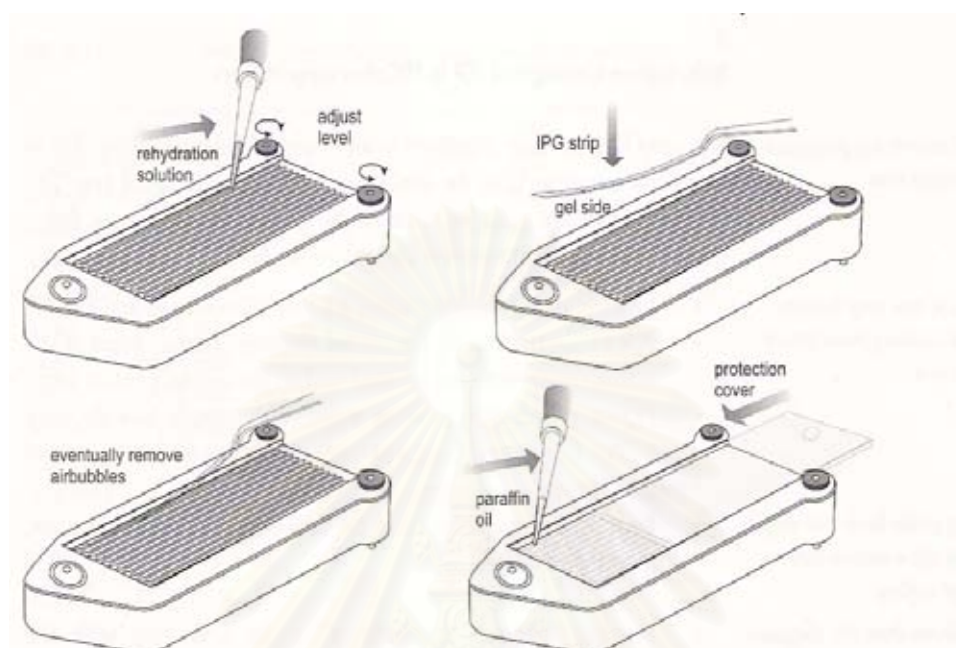
Figure 3.1 Rehydration of IPG strips in individual grooves in the reswelling tray

After rehydrated strip, removed a strip from the reswelling tray and transferred to the Immobiline Drystrip aligner. Placed the moistened electrode strips across the cathodic and anodic ends of the aligned IPG strips. The electrodes strips must be least partially contact the gel surface of each IPG strip. The IEF separation was carried out on the Multiphor II unit and connects the leads on the lid to the power supply (EPS 3500 XL). The separation conditions set the temperature at 20°C, current with 0.5 mA/strip and power with 5 W totals. The running condition according to the following gradient mode: 200 V for 1 min, 3,500 V for 1.5 h and 3,500 V for 1.5 h. Upon completion of IEF, each of the focused gel was put into a screw tube, the proteins were reduces by incubating with 1% w/v DTT for 10 min and alkylated with 2.5 % w/v iodoacetamide in 10 ml of equilibration buffer (30% v/v glycerol, 2% w/v SDS, 50 mM Tris-HCl pH 8.8, 6 M urea and 0.002% bromophenol blue) for 10 min. SDS-PAGE in the second dimension was carried out as described by Laemmli (1970) (15). The second dimensional, assemble glass plates for casting slab gel and label on plate with indelible marker at the resolving gel height. SDS

gel was 1.5 mm thick and consisted of a 6 cm separation gel of 12.5% w/v acrylamide overlaid with a 0.5 cm stacking gel of 4% w/v acrylamide. The resolving and stacking gel consisted of solutions in the Table 3.1

Table 3.1 Composition of the gel solutions for one gel

Stock solution*	12.5% *T* Resolving gel	4% *T* Stacking gel
Double distilled H_2O (ml)	7.23	1.525
Resolving buffer (ml)	5.5	-
Stacking gel buffer (ml)	-	0.625
30% T, 2.6 % C monomer (ml)	8.92	0.325
10% SDS (μl)	0.22	25.0
10% APS (μl)	110	12.5
TEMED (μl)	11	2.5
Total volume (ml)	22	2.515

* Preparation stock solution was described in Appendix A

The strips were then transferred to SDS-PAGE gel and sealed to SDS-PAGE gel with 0.5% agarose in SDS electrophoresis buffer. The SDS electrophoresis buffer contained 25 mM Tris-base, 192 mM glycine and 0.1% w/v SDS. For second dimension electrophoresis is used Hoefer miniVE vertical electrophoresis system and connects the leads on the lid to the power supply (EPS 3501XL). Electrophoresis was carried out at 12 mA/gel until the tracking dye had traversed the stacking gel and then at a 20 mA/gel until the dye had reached the bottom of the gel. The molecular weight markers were phosphorylase B (97,000 Da), bovine serum albumin (66,000 Da), ovalbumin (45,000 Da), carbonic anhydrase (31,000 Da), soybean trypsin inhibitor (21,000 Da) and lysozyme (14,400 Da).

2) Protein Detection by Coomassie blue Staining

After electrophoresis, proteins in the gel were stained by coomassie blue. The polyacrylamide gel was removed from between the glass plates and immersed in 40 mL of coomassie blue staining solution (filter through Whatman No. 1 filter paper before used). The staining gel was placed on a rotary shaker and gently shakes overnight. The staining

solution was removed and rinsed the gel extensively with H$_2$O. The gel in the destaining solution I was placed on a rotary shaker for 1-2 hr, followed by changed to destaining solution II (Appendix A) until background of the gel was cleared and spots appeared.

3) Phosphoprotein gel staining

Proteins from cell lysated were separated by 2-dimensional gel electrophoresis, the gel was fixed by immerse the gels in 100 ml of fix solution and incubate at room temperature with gently agitation for at least 30 minutes. The fixation step was repeated once more to ensure that all of SDS is washed out of the gel. The gel was washed with the ultrapure water 100 ml with gentle agitation for 10 minutes to remove methanol and acetic acid, and stained the gel by incubated in Pro-Q diamond phosphoprotein staining solution with gentle agitation in the dark for 90 minutes. The gel was destained with 20% acetonitrile, 50 mM sodium acetate, pH 4 for 30 minutes at room temperature, protected from light. After washed with ultrapure water 5 minute, 3 times, the stained gel was visualized on Typhoon^{TM} imager. The phosphoprotein stained gel were comparing with the coomassie blue stained gel.

4) 2-DE Data Analysis

The coomassie blue stained gels were scanned with at 300 dots per inch by Image densitometer (BIORAD, USA.). Spots were detected and quantified with Bio-2D software (64). Low molecular weight marker estimated molecular weights of protein spots were identified.

3.2.4 In-gel Digestion

In-gel digestion procedure has been modified from the method described in Chapter 6: the preparation of protein digests for mass spectrometric sequencing of the book of Protein Sequencing and Identification Using Tandem Mass Spectrometry (31). Rinsed the gel with water. Excised bands of interest with clean scalpel cutting as close to the edge of the spot or band as possible. It was important to reduce the volume of "background" gel. Choped the excised bands into cubes (*ca.* 1x1mm). Transferred gel particles into a microcentrifuge tube (0.5 ml or 1.5 ml eppendorf). Washed the gel particle with 100-500 µl of water (5 min, *ca.* 2-3 times). Spinned down and remove the liquid. Add acetonitrile/0.1M NH$_4$HCO$_3$ (1:1, *ca.* 3-4 times equal the volume of gel pieces) and wait for 10-15 min until the gel pieces shrunk-they become white and stick together. Spinned

the gel particles down and removed all liquid. Dried down the gel particle in a vacuum centrifuge.

Swelled the gel pieces in 10 mM dithiotreiol/0.1M NH$_4$HCO$_3$/1mM EDTA (added the liquid enough to cover gel) and incubated for 45 min at 60°C to reduce the protein. In-gel reduction was recommended even if proteins were reduced prior to an electrophoresis. Removed excess dithiotreitol solution and added 100 mM iodoacetamide/0.1 M NH$_4$HCO$_3$. Incubated for 30 min at room temperature in the dark. Removed iodoacetamide solution and washed the gel particles with 150-200 μl of 0.05 M Tris-HCl pH 8.5/50% acetonitrile (*ca.* 3-4 times). Rehydrated gel particles in the 180 μl digestion buffer (containing 100 μl of 0.1 M Tris-HCl, pH 8.5, 2 μl of 100 mM CaCl$_2$, 20 μl of ACN and 78μl of distilled water) and 20 μl of trypsin solution. Incubated for overnight at 37 °C. After overnight incubation kept 100 μl supernatant in a microcentrifuge tube (0.5 ml or 1.5 ml Eppendorf) and added 100 μl of 2% TFA, incubated for 30 min at 60 °C. After incubation, combined supernatant in before microcentrifuge tube and dried in a vacuum centrifuge. Added 30 μl of digestion buffer to the tube containing the gel pieces incubated for 10 min at 30 °C and sonicated for 5 min. After that added ACN 30 μl in extraction buffer, incubated for 10 min at 30 °C and sonicated 5 min. Removed and combined supernatant in before tube containing supernatant. Added 5% formic acid/ACN in gel, incubated for 10 min at 30 °C and sonicated for 5 min. Combined all supernatant in tube and dried in a vacuum centrifuge.

3.2.5 Protein Identification Methods

Tandem Mass spectrometry

A Tandem mass spectrometer analysis was performed on the ESI-Q-TOF mass spectrometer (Q-TOF-Micro, Micromass, Manchester, UK). Sample were injected into a CapLC (Waters, Milfold, MA, USA) System equipped with an auto sampler, gradient and auxiliary pump. Six μl was injected via "microliter pickup" mode and desalted on-line through a 300 μm × 5 mm C$_{18}$ Trapping cartridge (LC Packing, San Francisco, CA, USA). The sample was desalted at high flow (30μl/min) for 3 min. The peptides was separated on a 75 μm × 15 cm × 3 μm Å C18 100 A Symmetry column (Waters, Milfold, MA, USA) prior to introduction into the mass spectrometer. A typical reversed-phase was used from low to high organic over about 60 min. Mobile phase A was 3% acetronitrile, 0.1% formic

acid and B was 97% acetronitrile, 0.1% formic acid. The separation was performed using the following gradient: 0 min 7% B, 35 min 50% B, 45 min 80% B, 49 min 80% B, 50 min 7% B, and 60 min 7% B. The flow rate was 5 µl/min. The system utilized a split flow resulting in a column flow rate of approximately 200-300 nL/min.

MS/MS data was obtained using a Q-TOF MICRO (Micromass, Manchester, UK) filtered with Z-spray nanoflow electrospray ion source. The mass spectrometer was operated in positive ion mode with a potential of 3500 V applied to the nanoflow probe body. The collision energy was determined on the fly based on the mass and charge state of the peptides. Charge state recognition was used to switch only doubly and triply charged ions in MS-MS mode. Several trypsin autolysis ions were excluded. ProteinLynx Version (Micromass, Manchester, UK) to generate searchable peak lists processed the data. Initial protein identification was made by correlation of tandem mass spectra to entries in SWISS-PROT using Global Server (Version 2.0, Micromass). In addition, if clearly separated proteins were assigned to unreasonable such as different organism (animal, human, fungi) that could be traced to identical protein function an additional BLAST search (65) program of the sequence data against the clustered NCBI database (66).

3.2.6 Western blotting and Immunodetection

Cells were lysed in distilled water/cocktail inhibitor (990:10) and determined protein concentration by Bradford's method. Proteins were separated SDS-PAGE (12.5%T). After SDS-PAGE was finished, primary antibody (cytokeratin 8 and cytokeratin 18) were used at a 1:1000 dilution, the secondary (anti-mouse IgG) was used at 1:5000 dilution. The proteins were transferred onto a nitrocellulose membrane in Tris-Glycine, 20% methanol in a Hoefer TE series Transphor tank (Amersham Bioscience) at 400 mA for 1 hour (membrane should be pre-wetted in distilled water and equilibrate in transfer buffer for at lease 10 minutes before blotting). The membrane was washed 2 timed with 0.1% Tween in TBS (25 ml, 15 min; 25ml, 5 min) at room temperature. Non specific binding sites was blocked by immersing the membrane in 5% non fat dried milk, 0.1%(v/v) Tween 20 in TBS for 1 hour at room temperature on a orbital shaker. The primary antibody (diluted into 2.4 ml of the blocking buffer) was added to the membrane and incubated overnight at 4°C with gentle shaking. The membrane was washed as before, and incubated with the secondary antibody for 1 hour at room temperature with gentle shaking. The membrane was washed with 0.1% Tween in TBS (25 ml, 15 min; 25ml, 5 min x2). The detection

reagent was removed from storage at 2-8 °C and equilibrating was allowed to room temperature before opening was removed. Detection solution A and B were mixed in a ratio of 40:1 (for example 2 ml of solution A and 50 uL of solution B). The excess wash buffer was drained from the washed membrane and the protein was placed side up on a sheet of suitable clean surface. The mixed detection reagent was pipetted on to the membrane. Incubation was done for 5 minutes at room temperature. Chemiluminescent detection was processed by draining off the excess detection reagent, holding the membrane gently in forceps and touching the edge against a tissue paper. The blots protein was placed side down on to a fresh piece of saranwrap, wrapped up and gently smoothed out any air bubble. The wrapped blots were placed with protein side up in an x-ray film cassette. A sheet of autoradiography film was placed on the top of the membrane. The cassette was closed and exposed for 15 second (this stage should be carried out in the dark room using red safe light). A piece of film was developed immediately.

CHAPTER IV

RESULTS AND DISCUSSION

The proteins of MRC-5 lung fibroblast cell lineand A549 lung cancer cell line were analyzed as described in previous chapter. The results were shown and discussed in this chapter, respectively.

4.1   Comparison of proteins from normal lung fibroblast cell line (MRC-5) and lung cancer cell line (A549) by Two-dimensional gel electrophoresis

The analytical two-dimensional gel electrophoresis was established by using an Immobilized pH gradient, IPG strip pH 3-10NL, 7cm in first dimensional and 12.5% SDS-PAGE in the second dimension. Each of protein spot in gel represents individual proteins separate by their pI in the first dimension and by their molecular weight in the second dimension. The protein pattern of difference cell types were compared as shown in figure 4.1A and 4.1B. After analyzed the gel by using Bio-2D software the 2-DE result indicates that protein spots from MRC-5 (Figure 4.2 a) are more than from A549 (Figure 4.2 b). The identification of the differential proteins expression were further identified by mass spectrometry.

4.2   Phosphoproteins detection

The SDS-PAGE gels were fixed, washed with ultrapure water to remove methanol and acetic acid, incubated in Pro-Q Diamond phosphoprotein gel stain and protected from light for 90 minutes. After the gel were destained and washed with ultrapure water, they were visualized on a Typhoon Imager

Comparison of phosphoprotein stained gel with the coomassie blue are summarized in figure 4.3 and figure 4.4. The protein spots staining for phosphoprotein are indicated with blue circles. These proteins were further identified by mass spectrometry.

4.3   Identification of protein spots by Tandem mass spectrometry

    4.3.1 Protein expression level in both cell types

After the proteins were analyzed by two-dimensional gel electrophoresis, detected with Coomassie blue and Pro-Q Diamond phosphoprotein stain. The protein patterns from 2-DE gels of difference cell types were compared. The differences of protein expression from both cells were indicated by "D" series. Protein spots were manually excised (figure 4.5) and subjected to digestion with trypsin as described. ProteinLynx Version (Micromass, Manchester, UK) was used to generate searchable peak lists processed the data. Initial protein identification was made by correlation of uninterpreted tandem mass

spectra to entries in SWISS-PROT using Global server (Version 2.0, Micromass). In addition to, the amino acid sequence determination was obtained by Matrix science search, Mascot search engine (http://www.matrixscience.com) (67). Molecular weight, pI and search result of each spots were shown in Table 4.1

From the searching result, some of protein spots found in lung cancer cell line (A549) were higher expression than the same protein which found in normal lung fibroblast cell (MRC-5) such as spot D43 and D44 which had up regulation in A549 were matched to "pyruvate kinase", enzyme involved in glycolysis. It catalyzes the transfer of a phosphate group from phosphoenol pyruvate to ADP, yielding a pyruvate molecule and producing one ATP molecule. Spot D45 was matched to "α-enolase" or "enolase 1". It is multifunctional enzyme that as well as its role in glycolysis, plays a part in various processes such as growth control, hypoxia tolerance and allergic response, use as a diagnostic marker for many tumors. Spot D48 and D49 were matched to "peroxiredoxin"1 and 2 respectively. They are the antioxidant enzyme, induce peroxide levels, they mediate signal transduction and involved in redox regulation of the cells. They may play an important role in eliminating generated peroxides during metabolism. They might participate in the signaling cascades of growth factors and tumor necrosis factor by regulating the intracellular concentration of H_2O_2 (4, 68).

4.3.2 Identification for the difference proteins spots from each cell

After the proteins were analyzed by two-dimensional gel electrophoresis, detected with Coomassie blue stained and Pro-Q Diamond phosphoprotein stain. The protein patterns from 2-DE gels of difference cell types were compared. Some visible protein spots were manually excised and subjected to digestion with trypsin as described. ProteinLynx Version (Micromass, Manchester, UK) was used to generate searchable peak lists processed the data. Initial protein identification was made by correlation of uniterpreted tandem mass spectra to entries in SWISS-PROT using Global server (Version 2.0, Micromass). In addition to, the amino acid sequence determination was obtained by Matrix science search, Mascot search engine (http://www.matrixscience.com) (67). The gel of MRC-5 showed 16 individual protein spots. The spot in this gel was called "M" series (figure 4.6 a). Molecular weight, pI and search result of each spots were shown in Table 4.2. Some interested proteins in this series as spot M1, M2 and M8 which were matched with "collagens", the molecular weight of these proteins were quite high about 100 kDa up which in the same location of 2DE gel of A549 were not found any proteins in high

molecular weight area. Spot M7 was matched to "vinculin", the function of this protein was involved in cell adhesion and may be involved in the attachment of an actin based microfilaments to the plasma membrane and may also play important roles in cell morphology and locomotion. Spot M13 was matched to "stathmin" which is a highly conserved 17 kDa proteins. Its function as an important regulatory protein of microtubule dynamics has been characterized. There has the research reported that it is role in regulation of the cell cycle cause it to be an oncoprotein named oncoprotein 18 (op18), it can cause uncontrolled cell proliferation when mutated and not functioning properly. If stathmin is unable to bind to tubulin, it allows for constant microtubule assembly and therefore constant mitotic spindle assembly. With no regulation of the mitotic spindle, the cell cycle is capable of cycling uncontrollably resulting in unregulated cell growth characteristic of cancer cell. (4, 68)

The gel of A549 showed 12 individual protein spots. The spot in this gel was called "A" series (figure 4.6 b). Molecular weight, pI and search result of each spots were shown in Table 4.3. The interested proteins in this series were, spot A1 and A2 which matched to "chaperonin". Chaperonins are protein complexes that assist the folding of nascent, non native polypeptides into their native, functional state. These molecular machines use chemical energy, in the form of adenosine triphosphate (ATP), to promote protein folding in all cells. Chaperonins are the one in heat shock protein family; they are potential interest to cancer researchers, based on research that has shown that animals may respond to cancer vaccination. Tumor cells were attenuated and injected in small quantities into rodent, causing the rodent become immune to future full fledged tumor cell injection. While any relevance of animal research to humans has not been established, it is possible that the same may hold true for other species. Spot A7 was matched to "transketolase". Spot A8 and A10 were match to "cytokeratin 18" and "keratin 18". Cytokertins are intermediate filament keratins found in the intracytoplasmic cytoskeleton of epithelial tissue. There are 2 types of cytokeratins: the low molecular weight, acidic type I cytokeratins and the high molecular weight, basic or neutral type II cytokeratins. They are usually found in pairs comprising type I and II cytokeratin. Keratin 18 is a type I cytokeratin, it is together with its filament partner keratin 8, perhaps the most commonly found products of the intermediate filament gene family. Mutations in this gene have been linked to crypyogenic cirrhosis. The subset of cytokeratin which an epithelial cell express depend on the type of epithelium. The malignant counterparts of the epithelia (carcinogen), as the cytokeratin

profile trends to remain constant when epithelium undergoes malignant transformation, cytokeratins are wildly used for tumor diagnosis. The use of cytokeratin 8 and cytokeratin 18 as biomarker will be further study and discuss by immunoblotting technique.

(a)   **(b)**
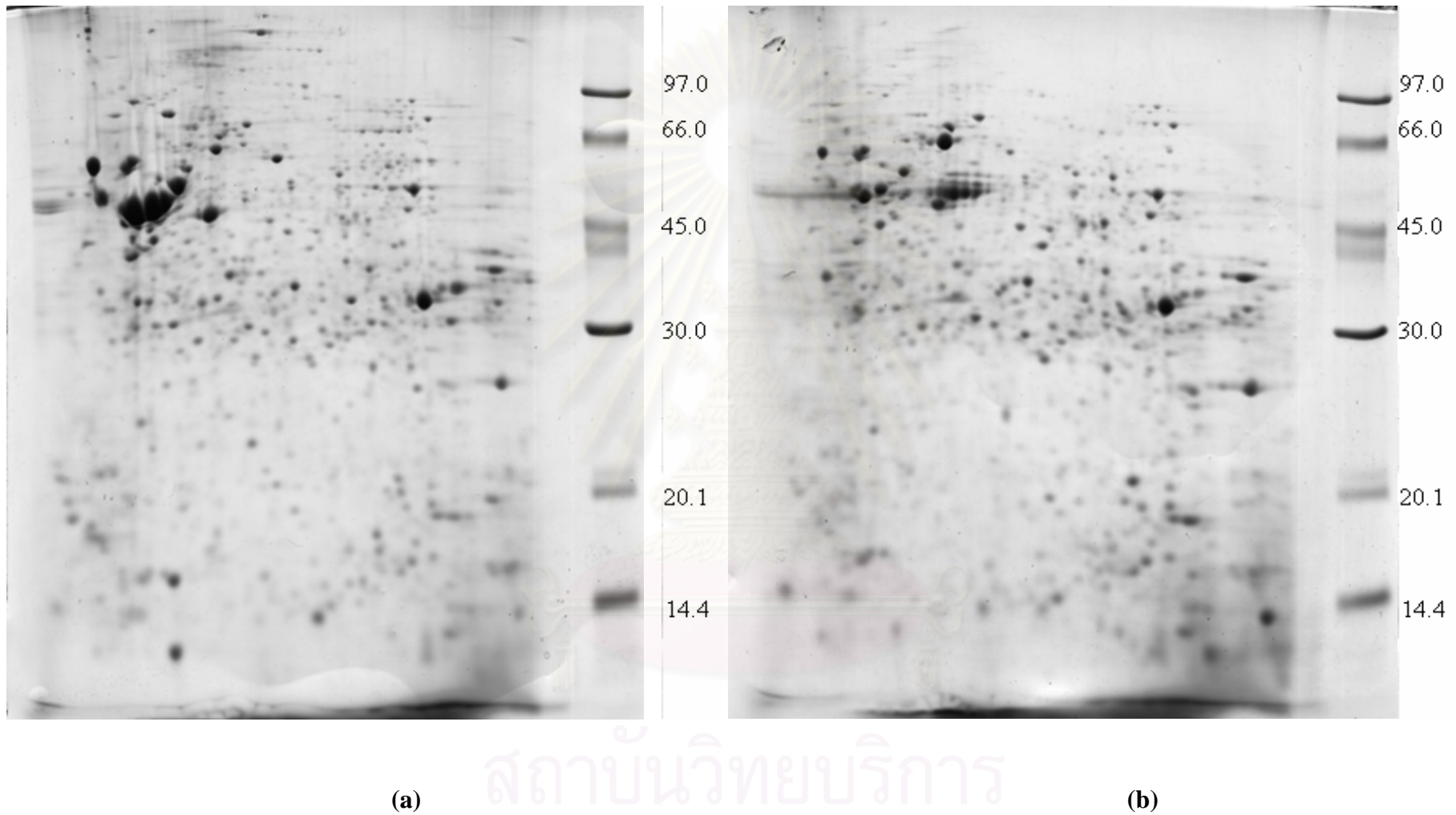
Figure 4.1   The 2DE comassie blue stain image compared between a) MRC-5 and b) A549

(**a**)   (**b**)
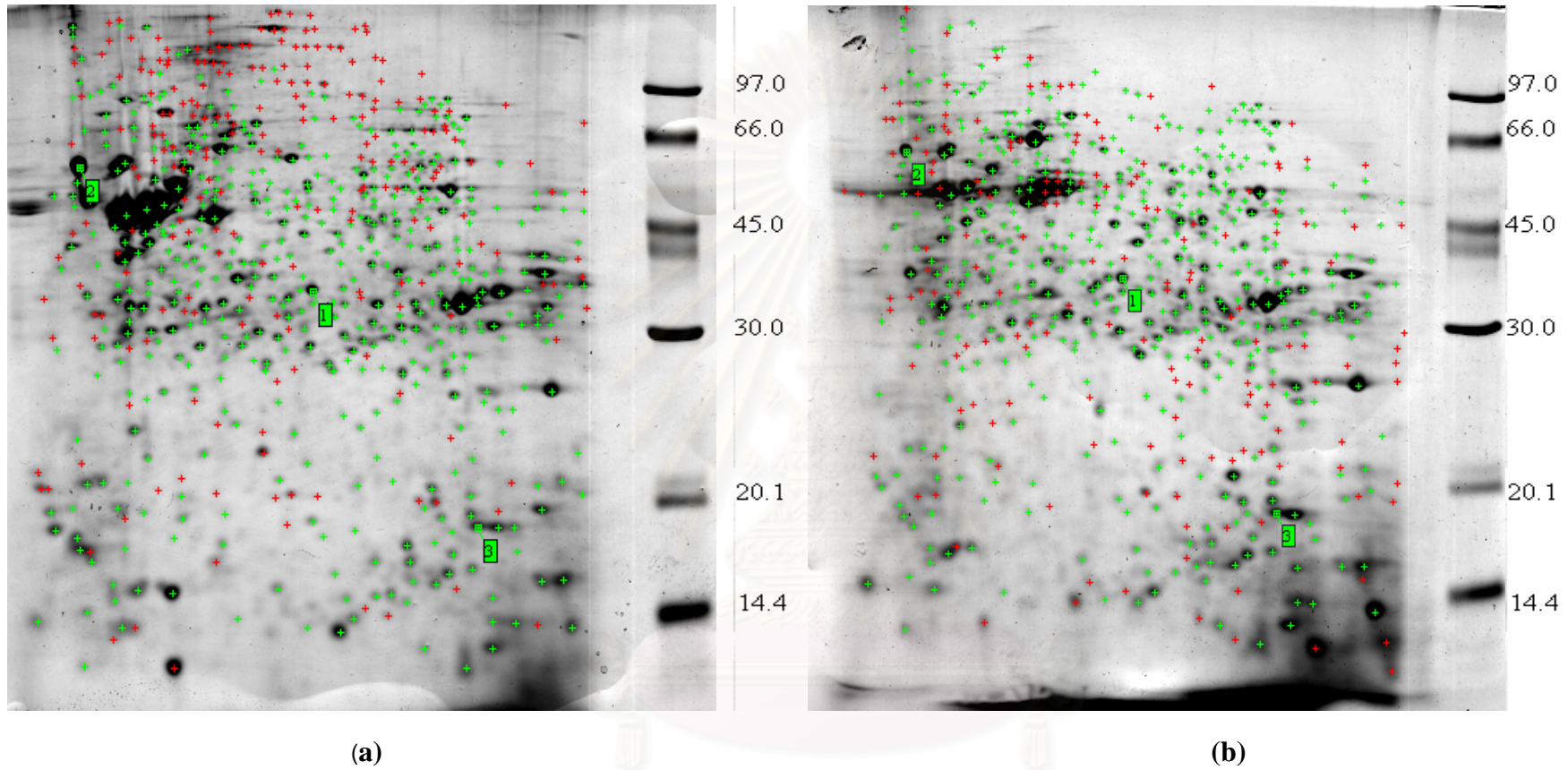
Figure 4.2   Gel match result from Bio-2D software between a) MRC-5 and b) A549

Red spots = Difference spots;   Green spots = Similar spots

Number 1-3 indicate for the marker spots.

(a)   (b)
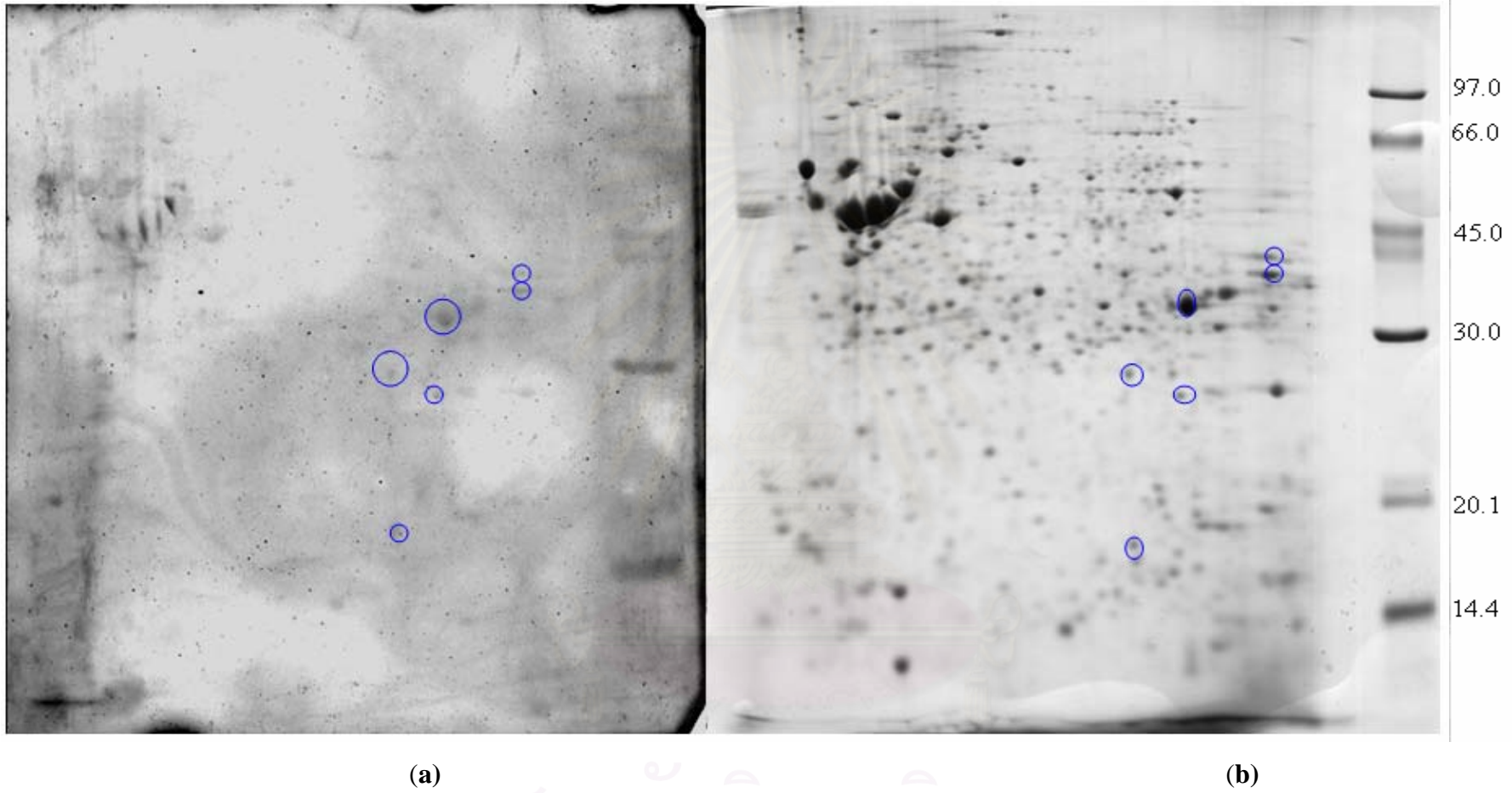
Figure 4.3   The Phosphostain gel (a) compared with Coomassie blue stain gel (b) of MRC-5
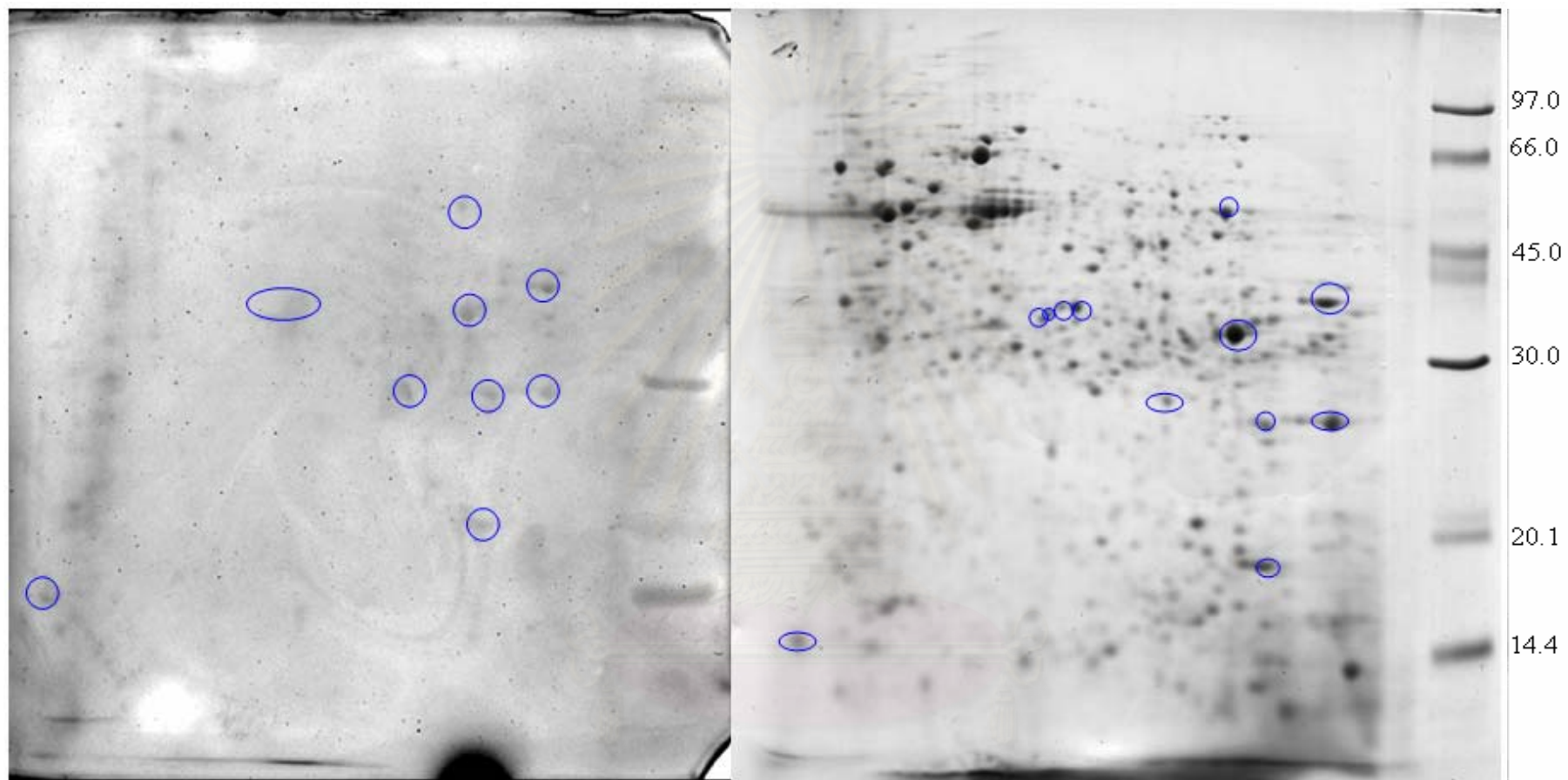
(a)   (b)

Figure 4.4   The Phosphostain gel (a) compared with Coomassie blue stain (b) of A549
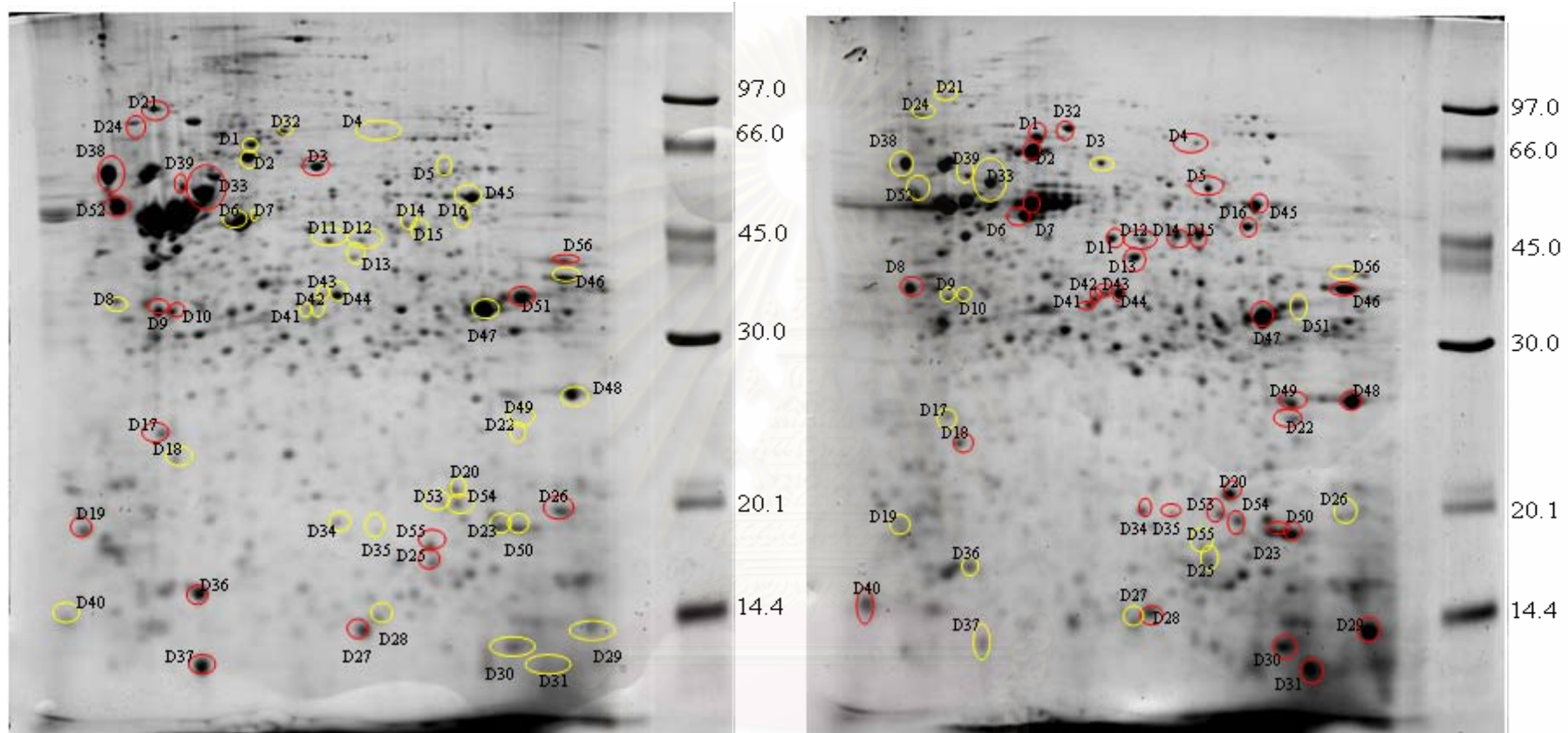
(a)   (b)

Figure 4.5   Differential expression of protein spots pattern a) A549, b) MRC-5

Red spots   = over expression spots;   Yellow spot   = under expression spots

Table 4.1   Identification and expression of protein spots from 2DE gel comparison between A549 and MRC-5

Spot	Description	MW	pI	Phospho-Stainning	Phospho-Protein	Phospho-peptide found	Protein Expression in A549	Protein Expression in MRC-5	Protein function
D1	Stress 70 protein mitochondrial	73,734	5.9	-	-	-	Up	Down	Chaperone / Stress response
D2	60kDa Heatshock protein mitochondrial precursor	61,016	5.6	-	√	√	Up	Down	Chaperone / Stress response
D3	Protein disulfide isomerase A3 precursor	56,747	5.9	-	-	-	Down	Up	Metabolism
D4	Lamin A C 70 kDa	74,095	6.6	-	√	-	Up	Down	Cytoskeleton / Mobility
D5	Aldehyde dehydrogenase 1A1	54,696	6.3	-	-	-	Up	Down	Metabolism
D6	Actin cytoplasmic 1 Beta actin	41,710	5.1	-	√	-	Up	Down	Cytoskeleton / Mobility
D7	Unidentified protein	-	-	-	-	-	Up	Down	Unknown
D8	Unidentified protein	-	-	-	-	-	Up	Down	Unknown

Table 4.1 Identification and expression of protein spots from 2DE gel comparison between A549 and MRC-5 (Cont.)

Spot	Description	MW	pI	Phospho-Stainning	Phospho-Protein	Phospho-peptide found	Protein Expression in A549	Protein Expression in MRC-5	Protein function
D9	Unidentified protein	-	-	-	-	-	Down	Up	Unknown
D10	Unidentified protein	-	-	-	-	-	Down	Up	Unknown
D11	2 Phosphopyruvate-hydratase, Alpha-enolase	47,008	7.2	-	√	√	Up	Down	Metabolism
D12	Unidentified protein	-	-	-	-	-	Up	Down	Unknown
D13	Hemoglobin beta chain	15,988	6.9	-	-	-	Up	Down	Binding / Folding
D14	Unidentified protein	-	-	-	-	-	Up	Down	Unknown
D15	Unidentified protein	-	-	-	-	-	Up	Down	Unknown
D16	2 Phosphopyruvate-hydratase, Alpha-enolase	47,008	7.2	-	√	√	Up	Down	Metabolism
D17	Unidentified protein	-	-	-	-	-	Down	Up	Unknown
D18	Unidentified protein	-	-	-	-	-	Up	Down	Unknown
D19	Unnamed protein product	59,492	5.2	-	-	-	Down	Up	Unknown

Table 4.1   Identification and expression of protein spots from 2DE gel comparison between A549 and MRC-5 (Cont.)

Spot	Description	MW	pI	Phospho-Stainning	Phospho-Protein	Phospho-peptide found	Protein Expression in A549	Protein Expression in MRC-5	Protein function
D20	Unnamed protein product	34,131	9.3	-	-	-	Up	Down	Unknown
D21	Vimentin	53,681	5.0	-	√	√	Down	Up	Cytoskeleton / Mobility
D22	Keratin type II, Cytokeratin 1	65,978	8.2	-	√	√	Up	Down	Cytoskeleton / Mobility
D23	Chain A, cyclophilin A complex with cyclosporin A	17,981	7.4	-	√	√	Up	Down	Binding / Folding
D24	Heat shock 70 kDa protein (GRP 78)	72,377	5.1	-	√	√	Down	Up	Chaperone / Stress response
D25	Unidentified protein	-	-	-	-	-	Down	Up	Unknown
D26	Keratin type II, Cytokeratin 1	65,978	8.2	-	√	√	Down	Up	Cytoskeleton / Mobility

Table 4.1   Identification and expression of protein spots from 2DE gel comparison between A549 and MRC-5 (Cont.)

Spot	Description	MW	pI	Phospho-Stainning	Phospho-Protein	Phospho-peptide found	Protein Expression in A549	Protein Expression in MRC-5	Protein function
D27	Unidentified protein	-	-	-	-	-	Down	Up	Unknown
D28	Glyceraldehyde 3 phosphate dehydrogenase	35,899	8.7	-	√	-	Up	Down	Metabolism
D29	Chaperonin 10	10,576	9.4	-	-	-	Up	Down	Chaperone / Stress response
D30	Annexin A2	38,552	7.6	-	-	-	Up	Down	Signal transduction / Transcription
D31	Unidentified protein	-	-	-	-	-	Up	Down	Unknown
D32	Unidentified protein	-	-	-	-	-	Up	Down	Unknown
D33	Vimentin	53,619	4.86	√	√	√	Down	Up	Cytoskeleton / Mobility
D34	Chlordecone reductase homolog	36,680	6.22	-	-	-	Up	Down	Other

Table 4.1   Identification and expression of protein spots from 2DE gel comparison between A549 and MRC-5 (Cont.)

Spot	Description	MW	pI	Phospho-Stainning	Phospho-Protein	Phospho-peptide found	Protein Expression in A549	Protein Expression in MRC-5	Protein function
D35	Aldo keto reductase	35,997	7.67	-	-	-	Up	Down	Protection / Detoxification
D36	hCG23783, isoform CRA_a	23,402	9.68	-	-	-	Down	Up	Unknown
D37	Chain A, crystal structure of Calcium free Human S100 A6	10,161	5.33	-	-	-	Down	Up	Other
D38	Calreticulin precursor CRP55 calregulin HACBP ERp60 grp60	48,111	4.09	√	-	-	Down	Up	Chaperone / Stress response
D39	Retinoblastoma binding protein7	47,790	4.89	√	-	-	Up	Down	Binding / Folding
D40	Histone H2A.2	13,899	10.2	√	√	-	Up	Down	Signal transduction / Transcription
D41	2 Phosphopyruvate-hydratase, Alpha-enolase	47,079	7.01	√	-	-	Up	Down	Metabolism

Table 4.1 Identification and expression of protein spots from 2DE gel comparison between A549 and MRC-5 (Cont.)

Spot	Description	MW	pI	Phospho-Stainning	Phospho-Protein	Phospho-peptide found	Protein Expression in A549	Protein Expression in MRC-5	Protein function
D42	Pyruvate kinase	57,841	7.58	√	√	√	Up	Down	Metabolism
D43	Pyruvate kinase	57,841	7.58	√	√	-	Up	Down	Metabolism
D44	Pyruvate kinase	57,841	7.58	√	√	√	Up	Down	Metabolism
D45	Enolase 1	47,139	7.01	√	√	√	Up	Down	Metabolism
D46	Heterogenous nuclear ribonucleoprotein A2/A1	35,984	8.67	√	-	-	Up	Down	Protein synthesis & Degradation
D47	Gleceraldehyde-3-phosphate dehydrogenase	36,031	8.26	√	√	-	Up	Down	Metabolism
D48	Peroxiredoxin 1	22,096	8.27	√	√	-	Up	Down	Protection / Detoxification
D49	Peroxiredoxin 2	22,096	8.27	√	√	-	Up	Down	Protection / Detoxification

Table 4.1 Identification and expression of protein spots from 2DE gel comparison between A549 and MRC-5 (Cont.)

Spot	Description	MW	pI	Phospho-Stainning	Phospho-Protein	Phospho-peptide found	Protein Expression in A549	Protein Expression in MRC-5	Protein function
D50	Cyclophilin A complexed with dipeptide Gly-Pro chain a	17,870	7.82	√	√	√	Up	Down	Binding / Folding
D51	Gleceraldehyde-3-phosphate dehydrogenase	36,031	8.26	√	√	-	Down	Up	Metabolism
D52	Calumenin	37,050	4.4	-	-	-	Down	Up	Other
D53	Chain A, Structure of Lamin AC Globular domain	13,360	8.9	-	-	-	Up	Down	Cytoskeleton / Mobility
D54	Transgelin	22,596	8.87	-	-	-	Up	Down	Binding / Folding
D55	R33729_1	11,326	7.03	√	-	-	Down	Up	Unknown
D56	Chain A, Aldolase A	39,264	8.39	√	√	√	Down	Up	Metabolism

(a)   (b)
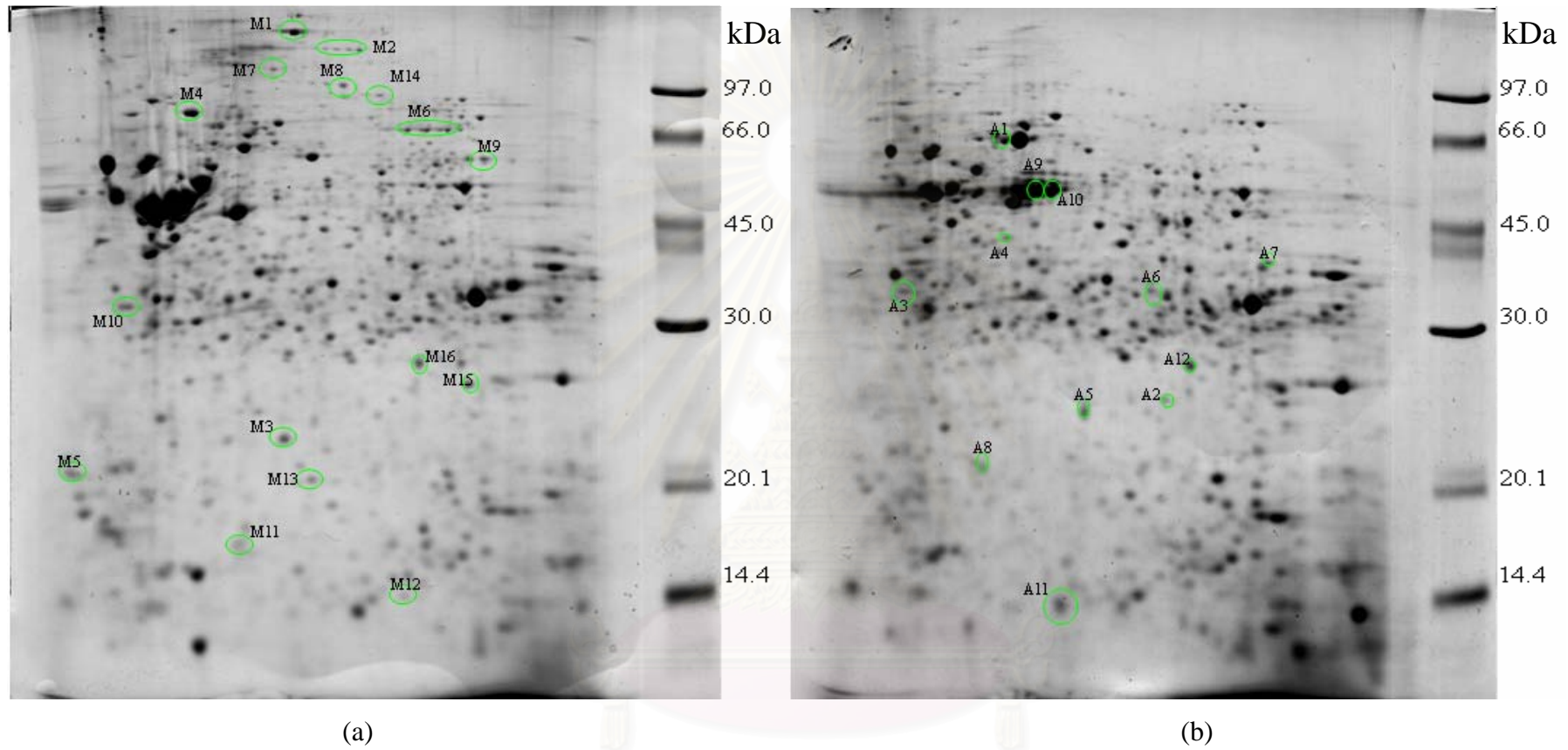
Figure 4.6   Difference type of proteins in each cell a) M series of MRC-5   b) A series of A549

Table 4.2   Identification of protein spots found only in lung fibroblast cell line MRC-5

Spot	Description	MW	pI	Peptide match	% cov.	Phospho-Stainning	Phospho-Protein	Phospho-peptide found	Protein Function
M1	Collagen, type I, alpha1	138,926	5.7	23	25	-	-	-	Chaperone / Stress response
M2	Collagen, type VI, alpha2	108,539	5.85	11	12	-	-	-	Chaperone / Stress response
M3	Chain A, Thioredoxin Peroxidase B	21,795	5.44	9	28	-	-	-	Metabolism
M4	BiP protein	70,888	5.23	26	41	-	-	-	Binding protein
M5	RAB 18, member RAS oncogene family	22,963	5.11	4	28	-	-	-	Apoptosis/ Cell cycle
M6	Lamin A/C isoform 2	65,096	6.4	21	46	√	√	√	Cytoskeleton / Mobility
M7	Chain A, crystal structure of Human full length Vinculin(residue1-1066)	115,928	5.77	20	22	-	√	√	Cell morphology
M8	Collagen, type VI, alpha2	108,539	5.85	11	11	-	-	-	Chaperone / Stress response

Table 4.2 Identification of protein spots found only in lung fibroblast cell line MRC-5 (Cont.)

Spot	Description	MW	pI	Peptide match	% cov.	Phospho-Stainning	Phospho-Protein	Phospho-peptide found	Protein Function
M9	Pyruvate kinase	57,841	7.58	16	34	-	√	√	Metabolism
M10	Laminin binding protein	31,774	4.84	2	10	√	-	-	Binding protein
M11	Unnamed protein product	59,492	5.17	4	9	-	-	-	Unknown
M12	Beta-2 microglobulin	12,791	5.77	2	18	-	-	-	Other
M13	Stathmin 1	17,292	5.76	6	40	-	√	√	Cytoskeleton / Mobility
M14	ESP-2	53,071	5.89	4	11	-	-	-	Apoptosis/ Cell cycle
M15	Manganese superoxide dismutase(MnSOD)	24,720	8.35	6	37	√	-	-	Metabolism
M16	Heterogeneous nuclear ribonucleoprotein H1	49,198	5.89	6	19	√	√	√	Protein synthesis & Degradation

Table 4.3   Identification of protein spots found only in lung cancer cell line A549

Spot	Description	MW	pI	Peptide match	% cov.	Phospho-Stainning	Phospho-Protein	Phospho-peptide found	Protein Function
A1	Chaperonin	60,986	5.7	22	48	-	√	√	Chaperone / Stress response
A2	Chaperonin	60,986	5.7	5	15	-	√	√	Chaperone / Stress response
A3	Heterogenous nuclear ribonucleoprotein A1 isoform a	34,175	9.51	2	9.7	-	√	-	Protein synthesis & Degradation
A4	nuclear corepressor KAP-1	88,479	5.52	2	2	-	√	-	Transcription
A5	Unnamed protein product	59,492	5.17	9	23	-	-	-	Unknown
A6	peroxisomal enoyl-coenzyme A hydratase-like protein	35,793	8.16	6	47	-	-	-	Metabolism
A7	Transketolase	67,751	7.9	11	25	-	√	-	Metabolism

Table 4.3   Identification of protein spots found only in lung cancer cell line A549 (Cont.)

| Spot	Description	MW	pI	Peptide match	% cov.	Phospho-Stainning	Phospho-Protein	Phospho-peptide found	Protein Function
A8	Cytokeratin 18(424AA)	47,305	5.27	22	45	-	-	-	Cytoskeleton / Mobility
A9	Keratin 18	48,003	5.39	17	41	-	-	-	Cytoskeleton / Mobility
A10	Cytokeratin 18(424AA)	47,305	5.27	26	58	-	-	-	Cytoskeleton / Mobility
A11	Unnamed protein product	59,492	5.17	9	17	-	-	-	Unknown
A12	Heterogenous nuclear ribonucleoprotein H1	49,198	5.89	5	17	√	√	√	Protein synthesis & Degradation
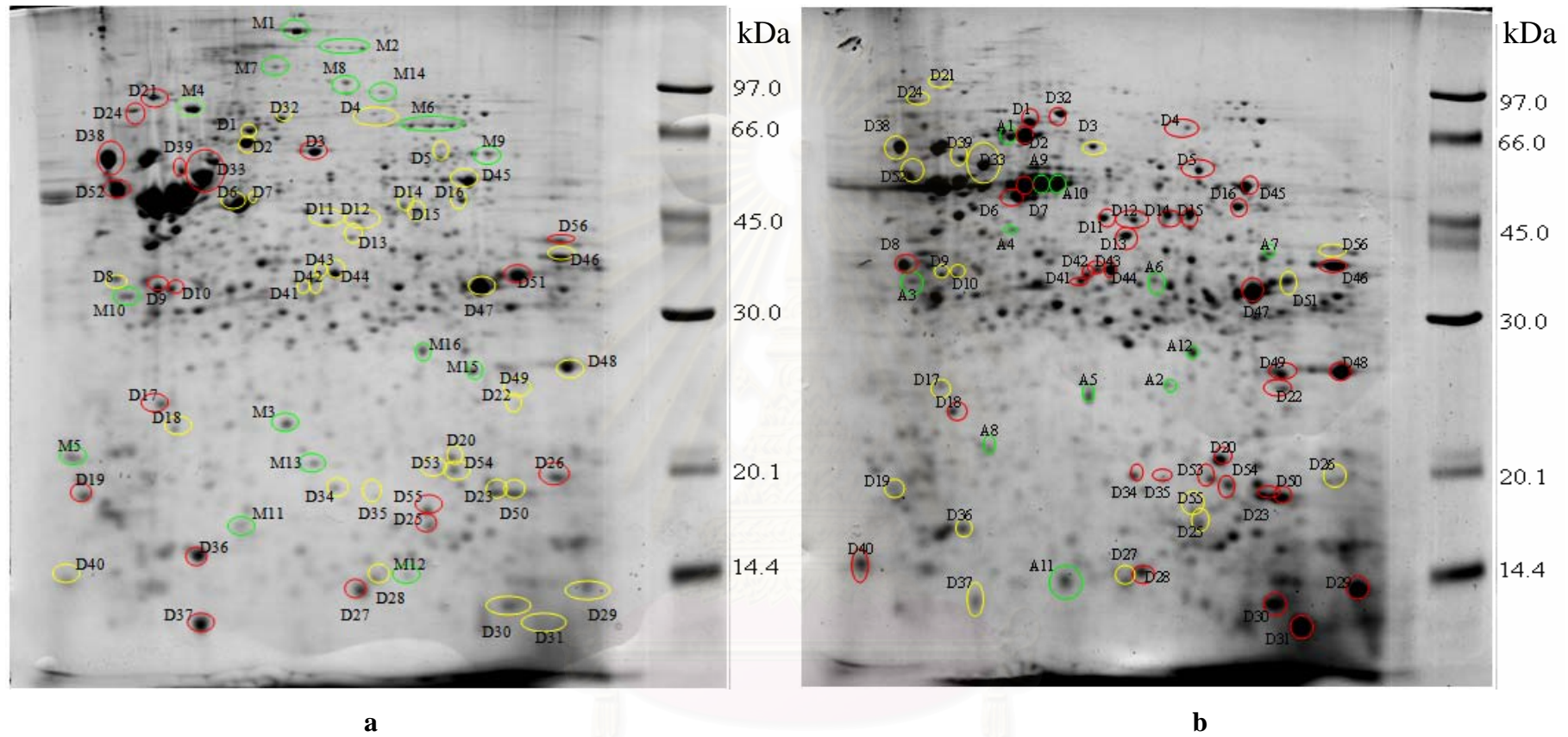
Figure 4.7 Summary of Protein spots pattern, (a) MRC-5 (b) A549

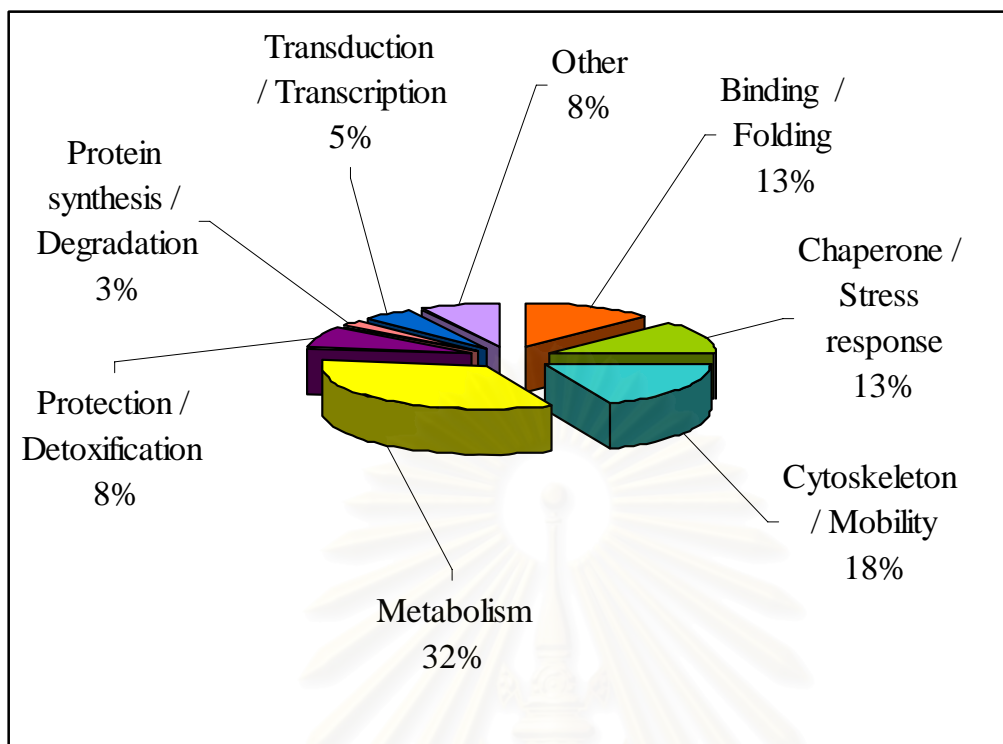Green spots = Different spots; Red spots = over expression spots; Yellow spots = under expression spots

Figure 4.8   Summary of protein function in D series spots
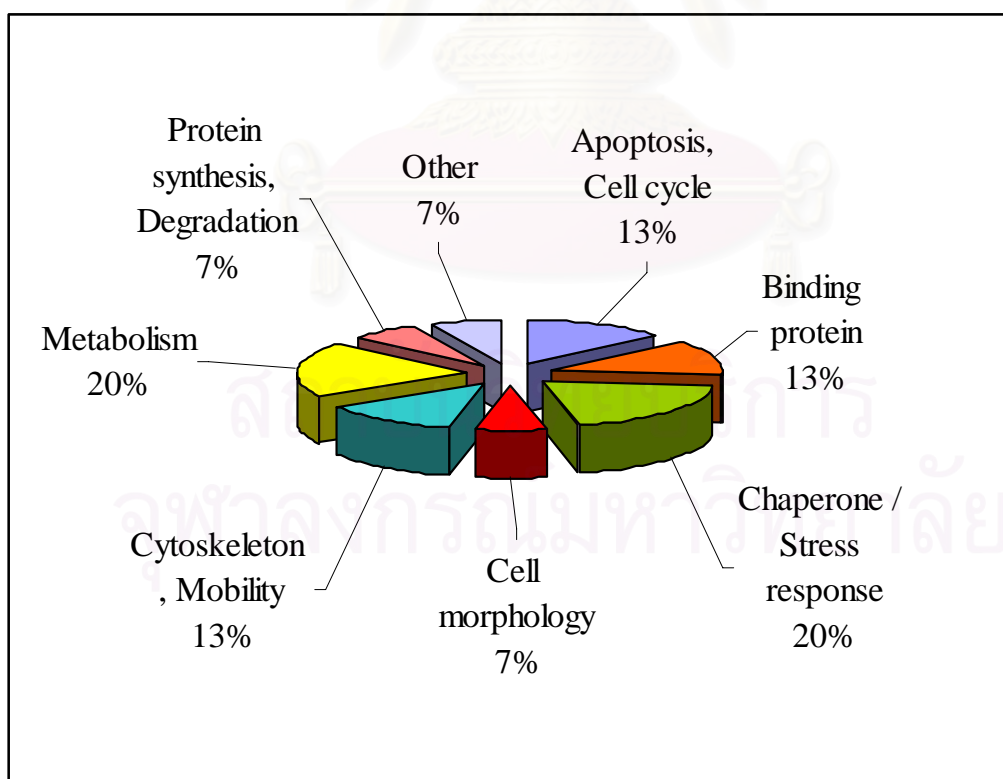
Figure 4.9   Summary of protein function in M series spots
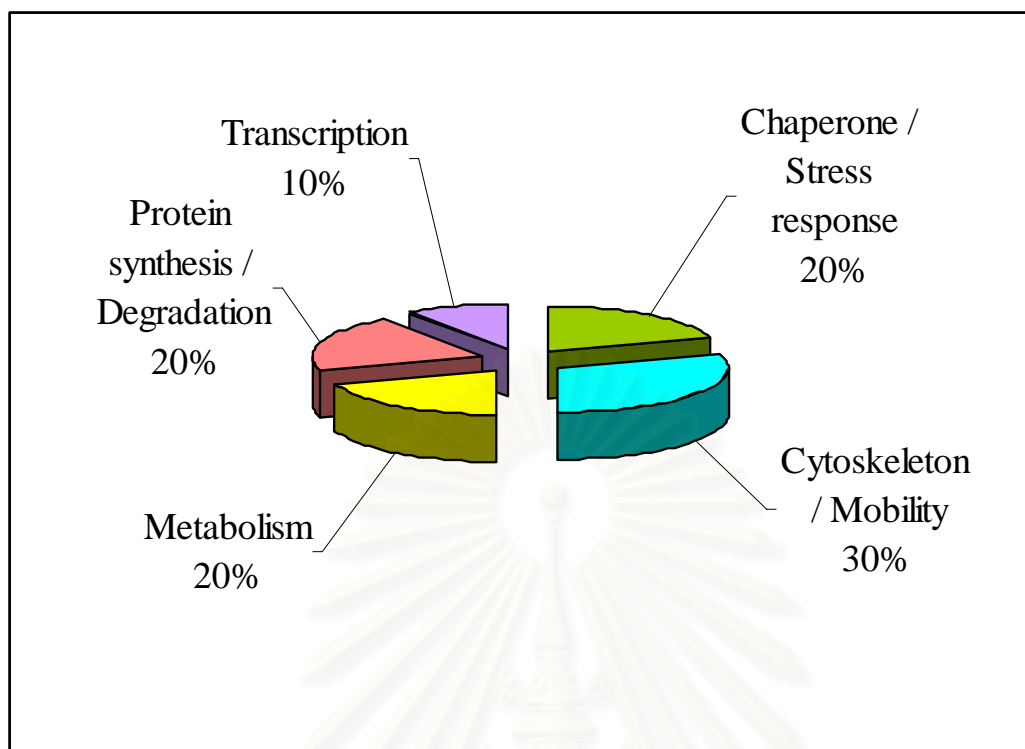
Figure 4.10   Summary of protein function in A series spots

4.3.3   Biomarker study by Western blotting and immunodetection

After separate proteins by 2DE technique and identified protein spots by mass spectrometry, cytokeratin8 (CK8) and cytokeratin18 (CK18) were found in difference expression from two cell type. The result shown that these proteins were found highly expression in A549 and seemed to be not expressed in MRC-5. To confirm this result, 1D immunodetection was used to confirm. The coomassie blue stain 12.5% gel of MRC-5 and A549 were shown in figure 4.6 (a) compare with anti cytokeratin8 stain gel in figure 4.6 (b) and anti cytokeratin18 stain gel in figure 4.6 (c).CK8 and CK18 were over expression in A549 cell line. This may be used as biomarker for lung cancer.

Figure 4.11 12.5% gel coomassie blue (a) compare with 1-DE immunodetection of cytokeratin8 (b) and cytokeratin18 (c)

lane1: Standard, lane2: MRC-5, lane3: A549

CHAPTER V
CONCLUSION

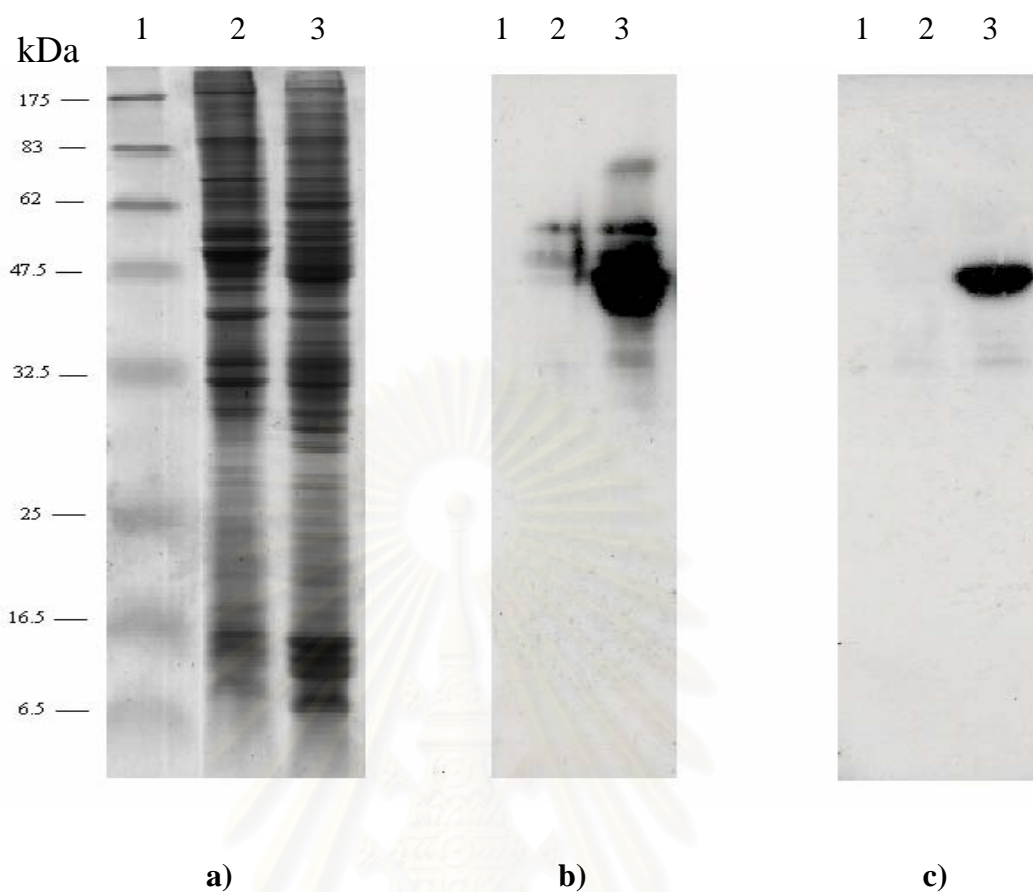In this research, the proteome techniques were used to identify the proteins in lung cancer cell (A549) compared with normal lung fibroblast cell (MRC-5). After extraction, whole cell proteins were analyzed by two-dimensional gel electrophoresis, stainned with coomassie blue and Pro-Q diamond phosphoprotein staining solution. The 2 DE gels of each cell type showed individual separate of protein spots. The comparison between 2-DE gels of two cell types showed the similar of the most of proteins from two cell types but the differences in the expression level of the proteins. Some proteins were over-expression and some were under-expression and the specific proteins were found. The over-expression of protein in A549 were the group of enzyme (23%) which were the phosphoproteins such as Pyruvate kinase (D42, D43 and D44), Enolase 1 (D45), Glyceraldehyde-3-phosphate dehydrogenase (D47), Peroxiredoxin 1 (D48), Peroxiredoxin 2 (D49). These groups of proteins may use as biomarkers because of the unusual expression of enzymes may effect on biological process that give an abnormality in cell division. Moreover some proteins were expressed only in lung cancer cell line (A549) such as Chaperonin (A1, A2), Heterogenous ribonucleoprotein A1 (A3), Nuclear corepressor KAP1 (A4), Peroxisomal enoyl- coenzyme A hydratase like protein (A6), Transketolase (A7) and Cytokeratin18 (A8-A10), Heterogenous ribonucleoprotein H1 (A12). The result found chaperonin (A1 and A2), phosphoprotein that has molecular weight 60986, pI 5.7 was highly expressed and seemed to be expressed only in A549, this protein may be used as biomarker because there are some groups reported that chaperonin performed functions on various intra-cellular process, play important role in protein interaction and protein conformation. The over-expression of chaperonin in cell may cause abnormality of proteins conformation and led to form the tumor.

Further more, 2DE spot indicated cytokeratin 18, keratin 18 (A8, A9 and A10) in A549 and was not express in MRC-5. Cytokeratins were usually found in pairs comprising type I and II, confirm this result by using 12.5%T 1-DE follow by anti- cytokeratin 8 and anti-cytokeratin 18 immunoblotting. The result indicated the appearance of highly band in the lane of A549, molecular weight of this protein is about 47.5 kDa which was equal to molecular weight of cytokeratin 8 and cytokeratin 18. These proteins may be used as biomarkers of lung cancer for early detection, diagnosis, monitoring disease and therapeutic treatment, to improve the long term survival of cancer patients. From this

research, some of protein spots can not be identified by this technique. It could be the concentration of proteins in gel spot is lower than the detection limit. However, the searching for cancer biomarkers using proteomic technology has a limitation because of cancer is a complex disease, it might require a panel of multiple biomarkers in order to achieve sufficient clinical prognosis.

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APPENDICES

Appendix A

Solution for 1D and 2D Electrophoresis

A. 0.25M sucrose

Sucrose	85.75 g
Distilled H_2O	to 1 L

B. 0.25M sucrose + Protease cocktail inhibitor

0.25M sucrose	20 ml
Cocktail inhibitor	40 µl

C. Lysis buffer

Urea	0.42 g
Thiourea	0.152 g
CHAPS	20 mg
DTT	20 mg
Ampholine	50 µl
Cocktail inhibitor	10 µl
Double distilled H_2O	to 1 ml

Prepare just prior to use. Store at room temperature.

D. Rehydrate buffer

Urea	240 mg
CHAPS	10 mg
IPG Buffer pH3-10	10 µl
DTT	1.4 mg
Double distilled H_2O	to 0.5 ml

Prepare just prior to use.

E. SDS equilibration buffer

	1st solution	2nd solution
1.5 M Tris-HCl, pH 8.8 (ml)	0.9	0.9
Urea (g)	3.24	3.24

Glycerol (ml)	2.7	2.7
SDS (mg)	90	90
Double distilled H$_2$O (ml)	3.015	3.015
DTT (mg)	90	-
Iodoacetamide (mg)	-	225

This solution prepared per 6 strips. Prepare just prior to use.

F. 30% T, 2.6 C monomer stock solution

Acrylamide	30 mg
N,N'-methylenebisacrylamide	0.8 g
Double distilled H$_2$O	to 100 ml

Filter solution through a 0.45 μm filter. Store at 4 °C in the dark

G. 10% SDS

SDS	5.0 g
Double distilled H$_2$O	to 50 ml

Filter solution through a 0.45 μm filter. Store at room temperature

H. 10% ammonium persulfate

Ammonium persulfate	0.1 g
Double distilled H$_2$O	1 ml

Prepare just prior to use.

I. SDS electrophoresis buffer

Tris-base	30.3 g
Glycine	144.0 g
SDS	10.0 g
Double distilled H$_2$O	to 10 l

Store at room temperature

J. Agarose sealing solution

SDS Electrophoresis buffer	1 ml
Agarose	5 mg

Bromophenol blue   1 μl

Add all ingredients into a 1.5 ml eppendorf. Swirl to disperse. Heat in a microwave oven low or heating until the agarose is completely dissolved. Do not allow the solution to boil over.

Fresh prepare and keep at 55°C until use

K. Coomassie Blue staining solution

Coomassie Brilliant Blue G-250	0.1 g
Methanol A.R. grade	40 ml
Acetic acid	10 ml
Double distilled H_2O	50 ml

Dissolved Coomassie Brilliant Blue G-250 in methanol until it is completely dissolved. Add Double distilled H_2O and acetic acid. Prepare just prior to use.

L. Destaining solution (coomassie blue)

	Destaining solution I	Destaining solution II
Methanol A.R. grade	40 ml	10 ml
Acetic acid	10 ml	5 ml
Distilled H_2O	to 100 ml	to 100 ml

Store at room temperature

M. Fixing solution for phosphostain

Methanol	100 ml
Acetic acid	20 ml
Distilled H_2O	to 100 ml

N. Destaining solution for phosphostain

1M sodium acetate pH4.0	50 ml
Acetonitrile	200 ml
Double distilled H_2O	750 ml

VITA

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