CHAPTER II

RESEARCH THEORETICAL

2.1 Antioxidant enzyme

Antioxidant enzyme is a group of antioxidant substance in nature which can reduce the energy from free radicals. Furthermore, they can give some of their electrons to free radicals to make those free radicals become stable molecules. Although free radicals are important for organisms in biological processes, the high level of free radicals can damage living cells and cause various diseases. Therefore antioxidant enzymes are important for maintaining cellular and systemic health and well-being. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) are well-known antioxidant enzymes in nature (Figure 2.1). SOD (EC 1.15.1.1) is the first enzyme in the pathway which catalys superoxide radical to hydrogen peroxide and molecular oxygen. Different SOD isozymes contain different metal-ions in their active site such as Cu plus Zn, Mn, Fe and Ni. Finally CAT (EC 1.11.1.6) and GPX (EC 1.11.1.19) contain a single selenocysteine (Sec) in their subunits continually catalyse hydrogen peroxide from the previous reaction to water and oxygen molecule.[29, 30]

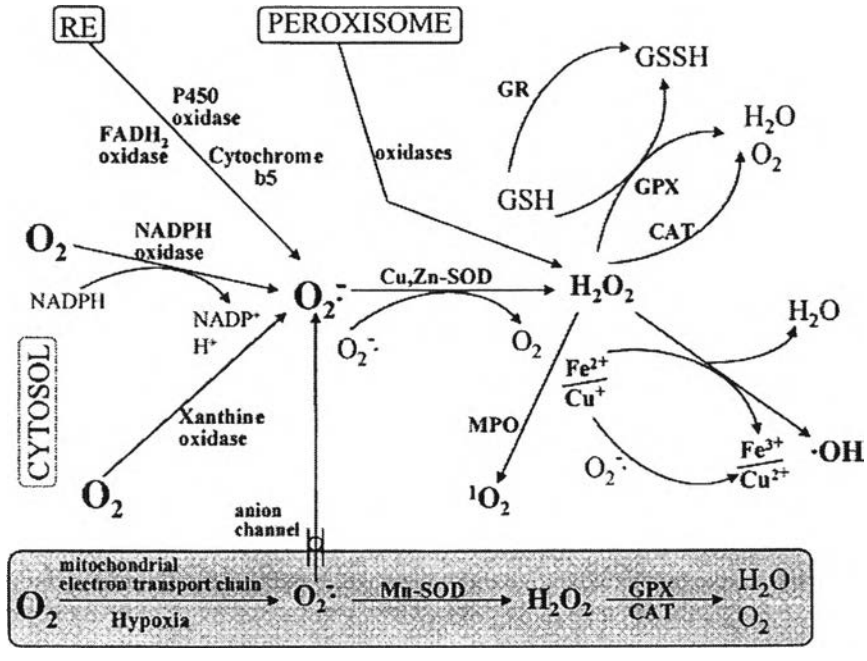

Figure 2. 1 Diagram of the reaction of antioxidant enzymes

2.2 Cyclic-peptide

Cyclic peptides are peptide with a head to tail cyclic backbone and comprise of 12-80 amino acid residues. The cyclic peptides containing one or more disulfide bond in their molecule are generally found in many organisms such as plants, bacteria, fungi and animals in (figure 2.2). Furthermore, these cyclic peptides are produced to be used in a host defense mechanism in organism according to their structure.[31] The cyclic peptides are varied in structure and size which can be divided into four group: (1) highly charged molecule (polyanion and polycation) mostly has function as a antimicrobial peptide (2) Non-polar cyclic peptide (3) cyclic peptide of mixed polarity and (4) cyclotide and cysteine knot with unusual topologies and a wide range of biological activities.[32] Since cyclic peptide structures are very stable, so

they have a potential bioactivity. Consequently, the cyclic peptides are currently applied as a scaffold in drug design and development.

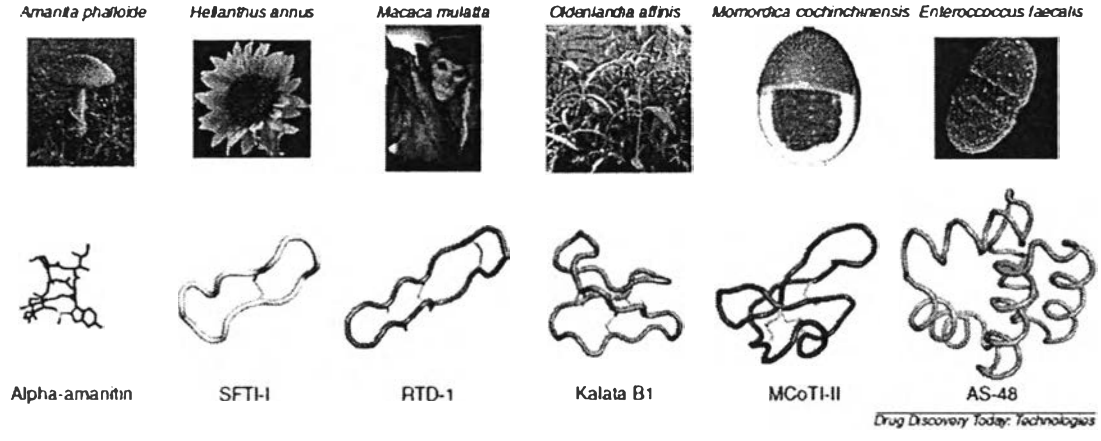

Figure 2. 2 Schematic of cyclic peptides found in many organisms[31]

2.3 Ion exchange chromatography

Ion chromatography is considered to be an indispensable tool in a modern analytical laboratory. Complex mixtures of anions or cations can usually be separated and quantitative amounts of the individual ions can be measured in a relatively short time. Higher concentrations of sample ions may require some dilution of the sample before introducing into the ion-chromatographic instrument. However, ion chromatography is also a superb way to determine ions the present of at concentrations at least the low part per billion (µg/l) ranges. Ion exchange chromatography separates compounds according to the nature and degree of their ionic charge. The net surface charge of samples varies according to the surrounding pH. When above its isoelectric point (pI), a sample will bind to an anion exchange. Contrastly, when below its pI a sample will bind to a cation exchanger. The column

to be used is selected according to its type and strength of charge. Ion exchange chromatography is divided into 2 types, anion exchange chromatography and cation exchange chromatography.

To release the samples in the order of binding tenacity, conditions are then altered so that bound substances are eluted differentially. This elution is usually performed by changes in pH or increases in salt concentration. At low salt concentration (low ionic strengths), all components will be tightly held on top of the column. When the ionic strength of the mobile phase is increased, the salt ions compete with the adsorbed sample ions for the bonded charges on the column. As a result, some of the sample components will be partially desorbed and start moving through the column. The higher concentration of salt, the larger number of desorbed the sample components. Moreover, the speed of the movement down the column increases. The samples come off the column matrix when the ionic strength of the buffer neutralizes their charge. The least charged molecules come off first while the most highly charged come off last.

Anion Exchange Chromatography (AEC)

The surface charge of the solutes which bind will be net negative, thus to get binding of a specific protein one should be above the pI of that protein. Commonly, used anion exchange resins are Q- resin, a Quaternary amine; and DEAE resin, DiEthylAminoEthane (Figure 2.3).

Q-anion exchanger DEAE-anion exchanger

Figure 2. 3 Q-resin and DEAE resin as anion exchange resin

AEC is often used as a primary chromatography step due to its high capacity. AEC is performed by using buffers at pH between 7 and 10 and running a gradient from a solution containing just this buffer to a solution containing this buffer with 1M NaCl. The salt in the solution competes for binding to the immobilized matrix and releases the protein from its bound state at a given concentration.

Cation Exchange Chromatography (CEC)

The surface charge of the solutes which bind will be net positive. Commonly used cation exchange resins are S-resin, sulfate derivatives; and CM resins, carboxylate derived ions (Figure 2.4).

S-cation exchanger **CM-cation exchanger**

Figure 2. 4 S-resin and CM-resin as cation exchange resin

CEC is less commonly used compared to AEC. Typically, CEC is performed using buffers at pH between 4 and 7 and running a gradient from a solution containing just this buffer to a solution containing this buffer with 1M NaCl. [33]

2.4 High performance liquid chromatography

High performance liquid chromatography (HPLC) in which the liquid mobile phase is mechanically pumped through a column that contains the stationary phase. An HPLC instrument, therefore, consists of an injector, a pump, a column, and a detector as show in figure 2.5. [34]

HPLC is primarily an analytical separation technique used to detect and quantitate analytes of interest in more or less complex mixtures and matrices. However, it is also used to isolate and purify compounds.[35]

The basic operating principle of HPLC is to force the analyte through a column of the stationary phase (usually a tube packed with small spherical particles with a certain surface chemistry) by pumping a liquid (mobile phase) at high pressure

through the column. The sample to be analyzed is introduced in small volume to the stream of mobile phase and is retarded by specific chemical or physical interactions with the stationary phase as it traverses the length of the column. The amount of retardation depends on the nature of the analyte, stationary phase and mobile phase composition. The time at which a specific analyte elutes (comes out of the end of the column) is called the retention time and is considered as a reasonably unique identifying characteristic of a given analyte. The use of pressure increases the linear velocity (speed) giving the components less time to diffuse within the column, leading to improve the resolution in the resulting chromatogram. Common solvents used include any miscible combinations of water or various organic liquids (the most common are methanol and acetonitrile). Water may contain buffers or salts to assist in the separation of the analyte components, or compounds such as trifluoroacetic acid which acts as an ion pairing agent.[36]

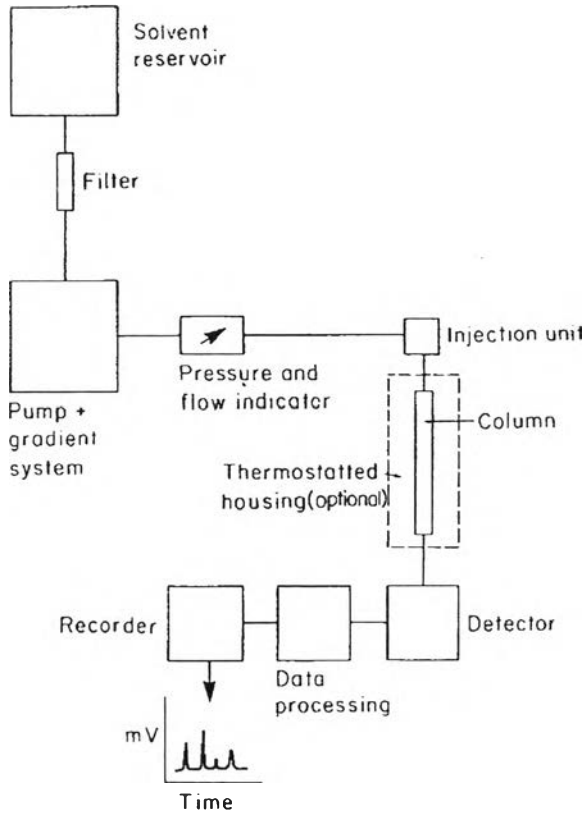

Figure 2. 5 The HPLC instrument consists of an injection, a pump, a column and a detector

2.5 Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis (2D-GE) is a well-known and potent method for the complex protein mixture separation in biological samples. The technique performs protein separation in two dimensions. The first dimension is isoelectric focusing (IEF) and then, the protein from the first dimension is continually separated based on their molecular weight in the second dimension (Figure 2.6). The detail of each dimension is described as the following. The first dimension step, called isoelectric focusing (IEF), is performed using acrylamide gel strip with immobilized pH gradient (IPG strips). Since proteins mixture can present in both

positively and negatively charge, when the electric current is applied, the proteins will move along the gel and will accumulate at the isoelectric point (pI) which the net charge on of protein is zero. The second dimension is performed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for separating protein according to molecular weight. The IPG strip from the first dimension is place at the top into SDS-PAGE before applying the electric current. SDS, a strong anionic detergent, will be disrupted the structure of protein by providing the net negatively charge to protein and leads to the formation of linear protein so the negative charge of protein will migrate from cathode (+) to anode (-) in SDS-PAGE based on molecular weight.[37, 38]

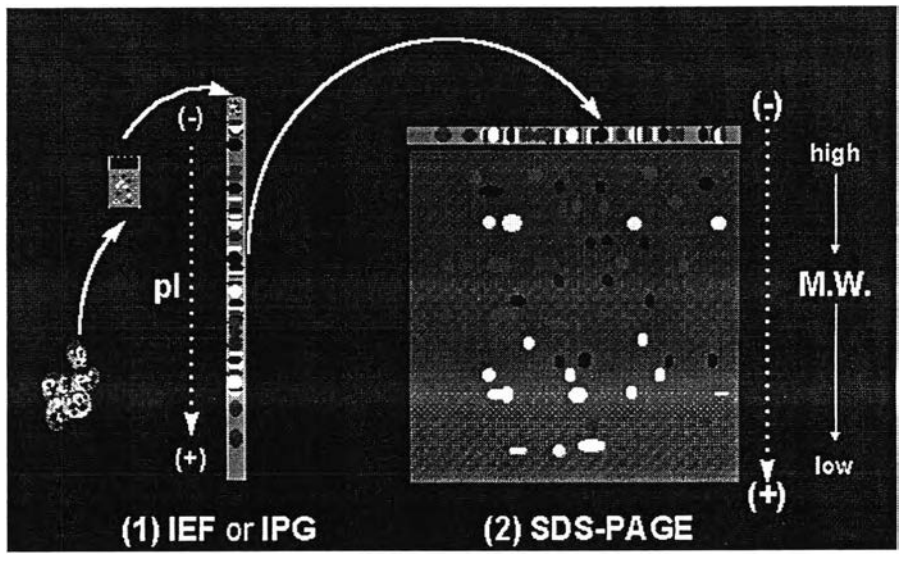

Figure 2. 6 Schematic of two-dimensional gel electrophoresis

2.6 Mass spectrometry for peptide sequencing

Mass spectrometry is a well-known method and widely used for peptide sequencing as a powerful tool. Diagram of mass spectrometry of peptide sequencing is represented in figure 2.7 which consists of ionization (ion-source), mass analyzer1 (MS1), collision cell, mass analyzer 2 (MS2) and detector. ESI and MALDI are the popular ion sources which are used to determine peptide sequencing because those tools are easy, robust and can convert whole protein or peptide to intact gas-phase ion. Furthermore, ESI also gives more advantages such as its ability to handle samples that have large masses and a soft-ionization. Therefore, it can analyze biological samples that are defined by non-covalent interactions. While the advantages of MALDI, for instance easy sample preparation, the method has tolerance to salts, detergents, buffer and so on as a sample contamination. The sample is ionized into positive charge ion before passing through mass analyzer (MS1). The mass anayzers are normally used to analyse parent ion or fragment ion such as ion-trap, quadrupole and time-of-flight (TOF). The parent ions are chosen in MS1 then continually fragmented at collision cell.

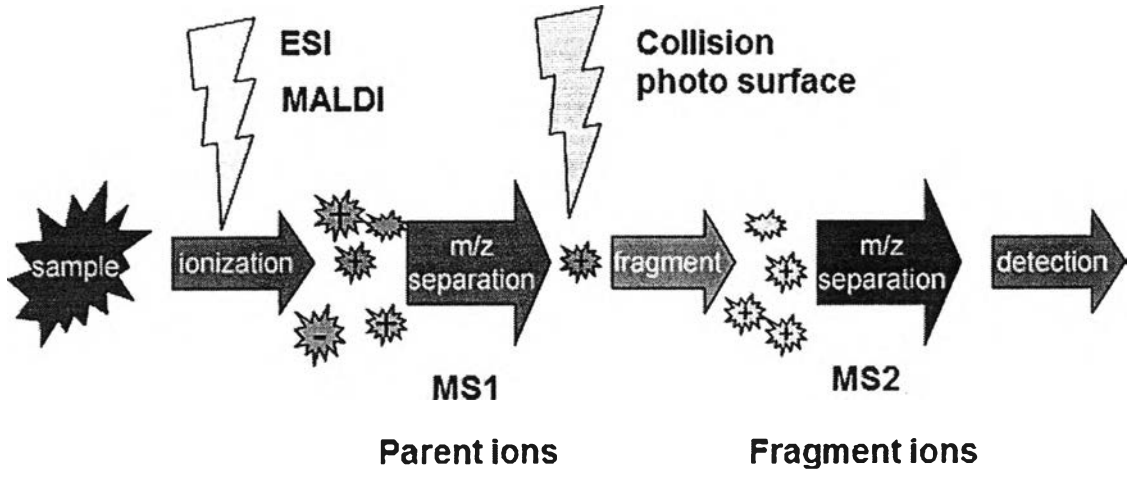

Figure 2. 7 Schematic of mass spectrometry for peptide sequencing

The pattern of peptide fragment ions in collision cell is shown in figure 2.8. Amide bond has the lowest bond energy so it will be cleaved and generates b-ion type contained N-terminus and the y-ion type contained C-terminus. All fragment ions are detected by MS2 and the data from fragment spectrum will be appeared in detector. The spectrum of b-ion and y-ion are useful for interpret for amino acid sequencing of peptide.[39]

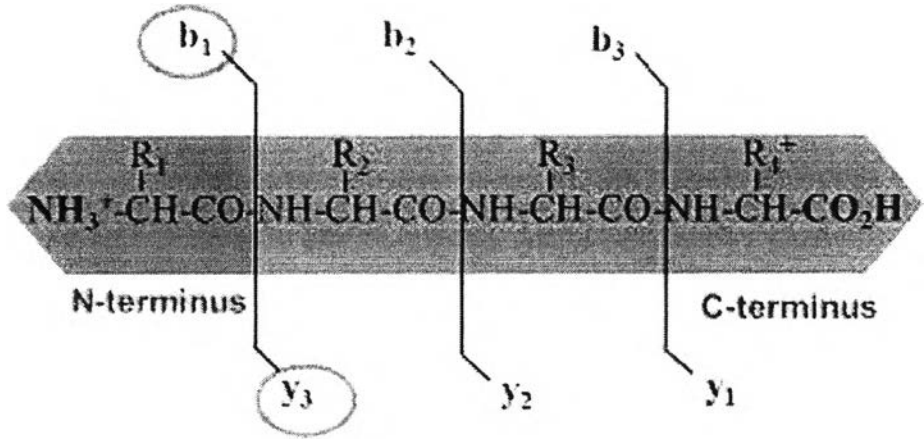

Figure 2. 8 The pattern of peptide fragmentation after pass collision cell

2.6.1 Electrospray quadrupole time of flight (ESI-QTOF)

ESI-QTOF is a mass spectrometry technique combined ion source (ESI) and two types of mass analyzers (quadrupole and time-of-flight) together for determining protein sequencing. ESI, also called soft-ionization, is an ion source to convert sample solution to positively charge under atmospheric pressure. The solvent from the charged parent droplet will be evaporated by inert gas such as N2 gas, giving charge progeny droplets (figure 2.9).[40]

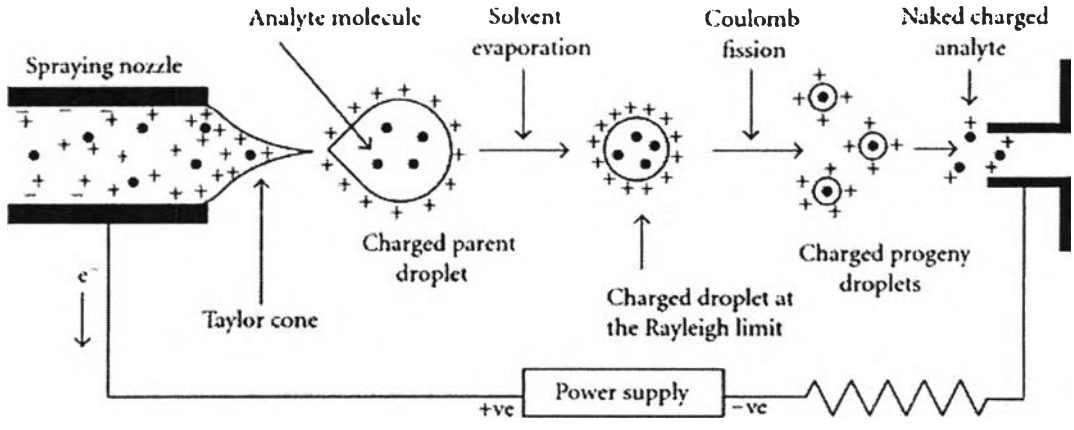

Figure 2. 9 Schematic of electrospray ionization

The ions are passed through quadrupole mass analyzer which is comprised of three main parts (Q0, Q1 and Q2). Q0 is used for focusing the ions which enter to the instrument. Q1 is a quardrupole tubes used for select the interesting parent ions. The interesting parent ions are fragmented in collision cell Q2 to fragment ions. Finally the fragment ions are moved to TOF mass analyzer to determine ion's mass to charge ratio via a time measurement. The mass to charge ratio of ion depends on the time of ion reaching to the detector, so heavier ions have lower velocity and take longer time to reach to the detector in figure 2.10.

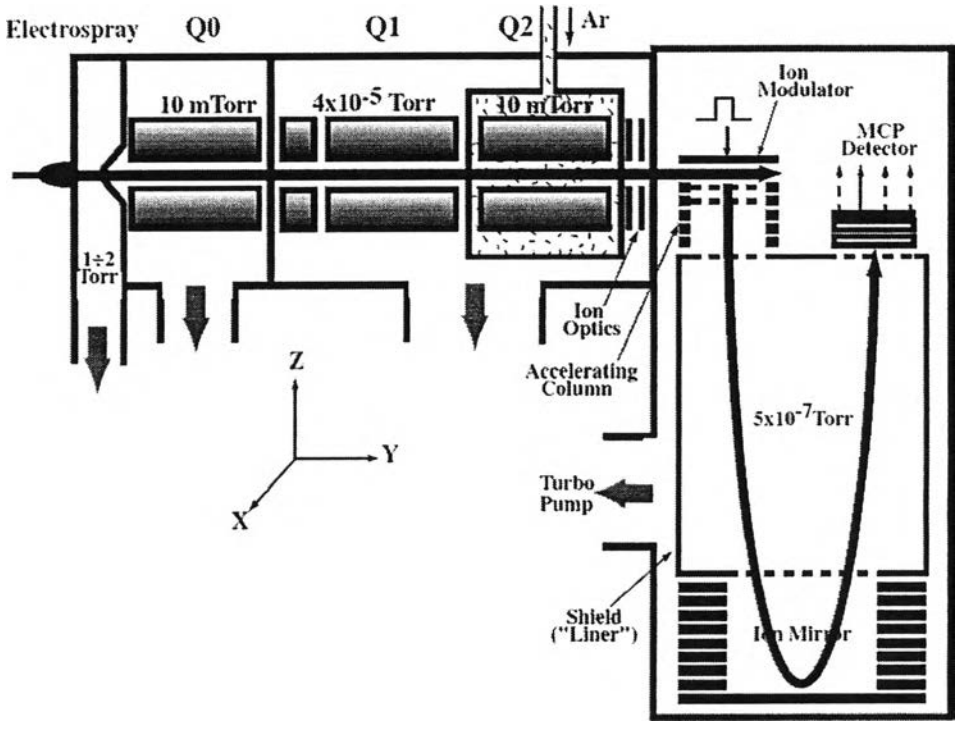

Figure 2. 10 Schematic of ESI-Q-TOF

2.6.2 Matrix-assisted laser desorption/ionization-time of flight/time of flight (MALDI –TOF/TOF)

MALDI-TOFTOF is mass spectrometry technique combined ion source (MALDI) and two mass analyzers (TOF) together for determining protein sequencing. MALDI technique starts by mixing the protein sample with small organic molecule, called matrix, then spoting on a MALDI target until the solution is dried and further formed crystal. The ionization reaction using MALDI occurs under vacuum condition. The crystal receiving rapid heating from radiation turns into a gas phase gives a positive charge ions, but the origin of ion produced from MALDI is still not clear (Figure 2.11).[41, 42] The ions are passed through TOF1 for selecting parent ions before being

fragmented at collision cell which are detected by TOF2 in figure 2.11. The principle of TOF analyzer is described in section 2.5.1.

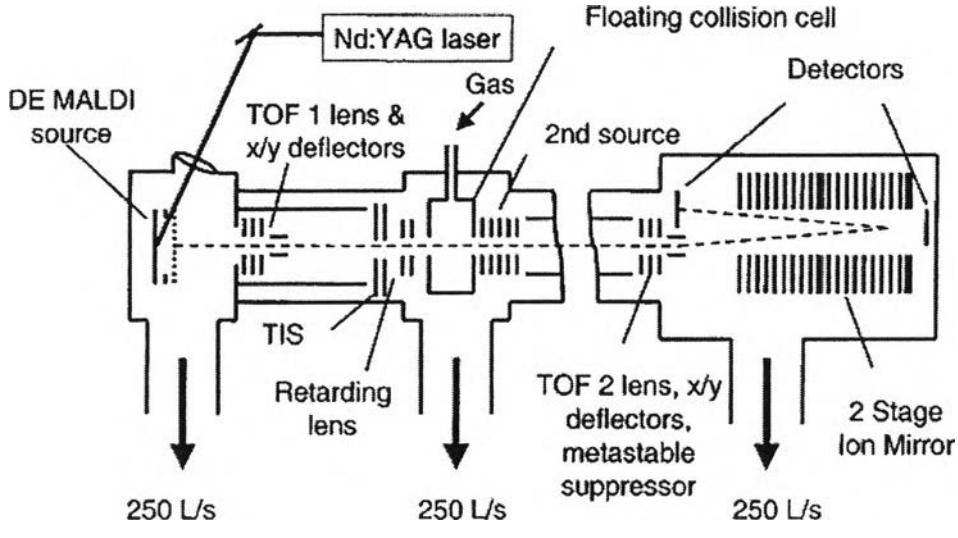

Figure 2. 11 Schematic of MALDI-TOF/TOF

2.7 Nuclear magnetic resonance (NMR)

Nuclear magnetic resonance (NMR) is a currently important technique for determining biological macromolecules such as protein and peptide. The principle of NMR technique is divided into four steps. Amino acid sequencing data is firstly required for determining protein structure by NMR technique. To study the protein structure by NMR, highly purify protein sample is required. Three different types of NMR experiment, one, two and three experiment (1D, 2D and 3D experiments), are used to analyze the protein structure respectively (Figure 2.12).

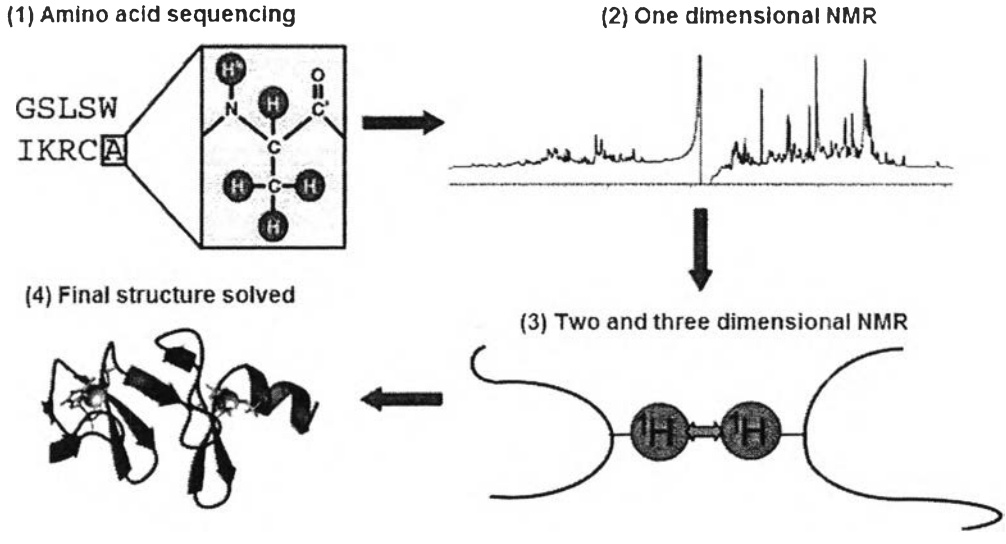

Figure 2. 12 Schematic of NMR experiments

1D experiment gives the 1D ^{1}H spectrum of protein which is used to indicate folded/unfolded of proteins. The properly folded protein peaks of 1D ^{1}H spectrum should be sharp and also show good chemical shift dispersion (Figure 2.13). TOCSY and NOESY spectrum are some types of 2D experiments. TOCSY spectrum can indicates an intra- amino acid residue correlation (within a residue) while NOESY spectrum can indicate an inter-residue correlation (between residues). Therefore, TOCSY peaks are used to identify the peaks belonging to each individual amino acid in the sequence and NOESY peaks are used to link the residues together giving the secondary structure of protein (beta-sheet or alpha-helix structures). Finally, all data from 1D and 2D experiments and the data from 3D experiment for example the peaks which are stacked on top in 2D spectrum can be applied to determine the structure of protein.[43, 44]

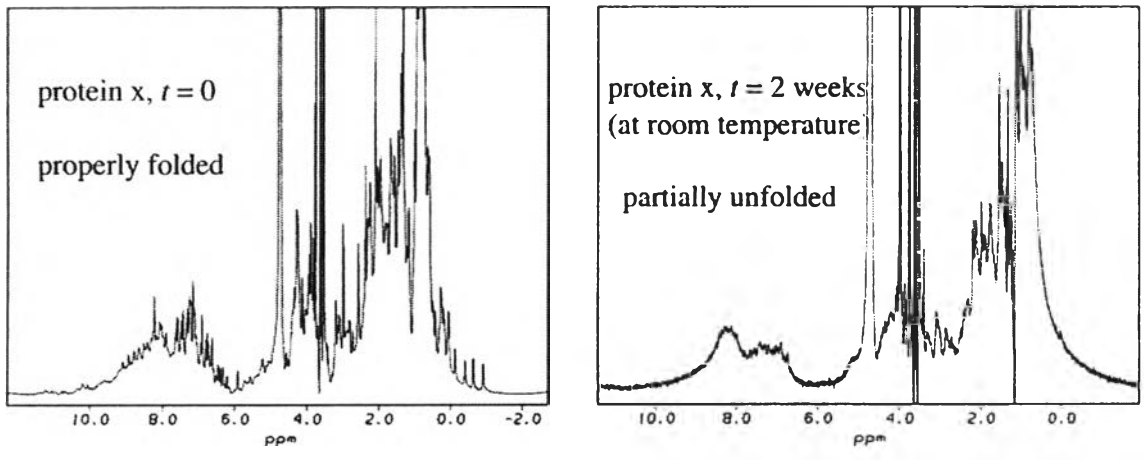

Figure 2. 13 1D ^{1}H spectrum of foled and unfoled of protein

2.8 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a powerful and scientific technique in molecular biology. The technique can use to amplify millions of copies of target DNA fragment. Consequently, the PCR technique is widely used in molecular biology because it is simple, quick and inexpensive, but doesn't require a very pure DNA fragment sources. The technique needs four basic factors (1) DNA polymerase and its buffer,(2) primers (3) a DNA template for constructing a new strand (4) deoxynucleotides substrate. The technique is divided into three steps as following. The first step is denaturation which the DNA template is denatured using high temperature to render it as single strand. The second step is annealing which primers are used to bind to DNA template strand to prime extension. The last step is primer extension using DNA polymerase and the most popular enzyme is *Taq* polymerase. All three steps are repeated from 28 to 35 times (Figure 2.14).[45]

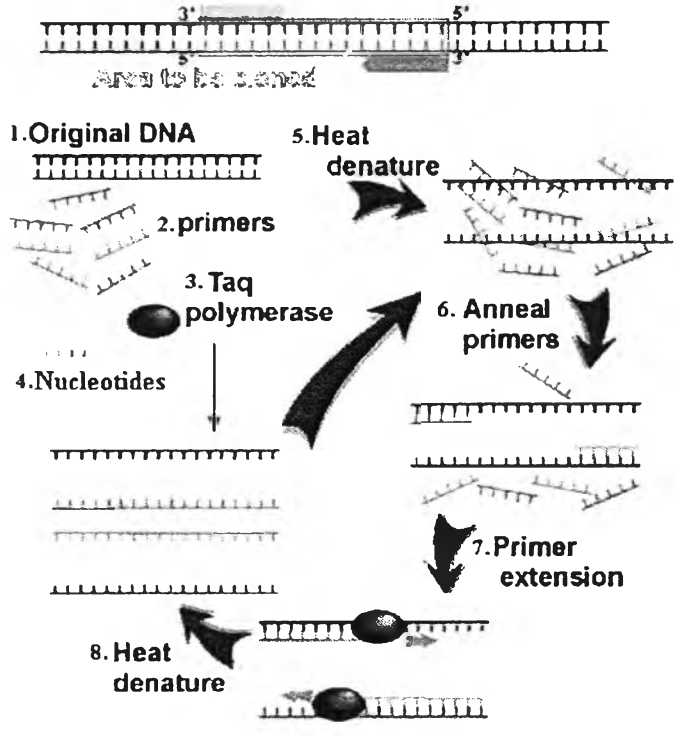

Figure 2. 14 Schematic of polymerase chain reaction (PCR)

2.8 MTT assay

MTT (3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, first described by Mosman in 1983, is the measurement of mitochondrial activity for calculate the number of cell that are dividing in the culture (cell proliferation). The tetrazolium rings of the yellow MTT are splited by mitochondrial reductase enzyme from viable cell and then they become purple formazan which is impermeable to cell membranes. A solubilization solution, usually using dimethylsulfoxide, is added to dissolve the purple formazan product into a colored solution. The number of surviving cells is calculated by measuring the absorbance of the colored solution by spectrophotometer (between 500-600 nm depending on the solubilization solution). The number of surviving cells is directly proportional to the level of the formazan product generated.[46]

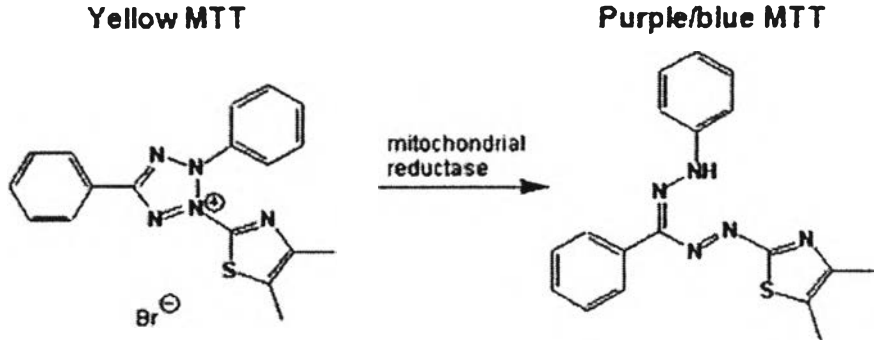

Figure 2. 15 The principle of MTT assay. The tetrazolium rings of the yellow MTT are splited by mitochondria reductase enzyme to be purple formazan