CHAPTER 1

INTRODUCTION

In Thailand, there are about 163 species of snakes and 48 of them are dangerous venomous snakes belonging to two groups; Elapidae and Viperidae. In each year, there are approximately 7,000 snakebite patients. The most cases of snakes bite are from Malayan Pit Viper (*Calloselasma rhodostoma*), Green Pit Viper (*Trimeresurus* species) and Cobra (*Naja* species), respectively.[1] The snake venoms are used as an effective method of hunting and digesting prey. Snake venom is a complex mixture of proteins. The protein composition in venom varies widely from species to species[2]. As mentioned before, the snakebites in Thailand are still an important medical problem.

Green pit viper bites are common in Thailand. *Trimeresurus albolabris* and *Trimeresurus macrops* have been regarded as the most common snakes causing bites.[3] There have been several reports about biological activities of these two snake venoms.[3-4] Also, *Trimeresurus albolabris* venom has been studied and were reported proteins; Albolabrin[5] , Alboaggregins A, Alboaggregins B[6], and Alborhagin[7] protein in 1991, 1998, and 2001, respectively. Therefore, it is interesting to study on *Trimeresurus macrops* venom, which has been no previous report about the protein composition. The protein identification of this venom can give information and knowledge, which are important advantages for medical application and antivenom production.

Objective of this research

The aim of this research is to identify proteins from crude *Trimeresurus macrops* venom.

1.1 Snakes

1.1.1 General Background [8]

Snakes belong to the class *Reptilia*, subclass *Synaptosauria*, order Squamata and suborder *Serpentes*. There are 15 snake families and the venomous species, with only a few exceptions, belong to two of them: Elapidae and Viperidae. Cobras, mambas, kraits, coral snakes and sea snakes are elapids. They are all venomous snakes and can be found in warmer parts of the world, except Europe and Madagascar. The second family, Viperidae, includes vipers and pit vipers. These again are all venomous snakes that have long, hinged fangs. The difference between the true vipers and pit vipers is that the pit vipers have an additional sense organ located in front and just below the eyes. These are the so-called heat pits that allow pit vipers to hunt with remarkable accuracy, even in total darkness. There are three types of venomous snakes: opysthoglyph, proteroglyph and solenoglyph. The first type is mostly harmless or mildly venomous snakes. Their fangs are enlarged rear teeth with a groove that venom flows down while they are swallowing their prey. However, there are snakes of this type that are known to have killed humans before, for example the Boomslang (*Dispholidus typus*). Another good example of this type is the Mangrove snake (*D. dendrophila*). Proteroglyphs have small, fixed, non-movable front fangs. When they bite, they hang on and chew their prey to envenomate it. Cobras (*Naja*), mambas (*Dendroaspis*), kraits (*Bungarus*), taipans (*Oxyuranus*), coral snakes (*Micrurus*) and sea snakes are good examples of this type. They are some of the deadliest snakes in the world. Solenoglyphs have movable front fangs that fold back into the mouth until they are needed. These snakes are very dangerous for they can open their mouths almost 180 degrees with their fangs extended straight out. They can strike at any portion of the body and their attack is much unpredictable. Rattlesnakes (*Crotalus*), eyelash vipers (*Bothriechis*), gaboon vipers (*Bitis*), cottonmouths and copperheads (*Agkistrodon*) belong to this type. In Thailand, important poisonous snakes, which are most commonly found, include Malayan pit viper, Green pit viper and Cobra. Among these poisonous snakes, Green pit viper *Trimeresurus macrops* is one of the most common snakes causing bites.

Snake bite remains a public health problem in many countries, including Thailand, even though it is difficult to be precise about the actual number of cases. It is estimated that the true incidence of snake envenomation could exceed 5 million per

year. About 100,000 of these develop severe sequelae. The global disparity in the epidemiological data reflects variations in health reporting accuracy as well as the diversity of economic and ecological conditions. Snake venoms are used in both attack and defense. Therefore, they contain components designed not only to immobilize prey, but also to facilitate their digestion. Also, snake venoms can play an important in the production of diverse medication such as blood clots in stroke or heart attack victims.

1.1.2 Characteristics and Habitats of *Trimeresurus macrops* [1]

The common name of *Trimeresurus macrops* is Big-eyed Pit Viper. This snake is medium pit viper. The maximum lengths of body are 66 centimeter for male and 72 centimeter for female. The characteristics of *Trimeresurus macrops* are big triangle-shaped head, big yellow-rounded eyes and dark green skin (Figure 1.1). The habitats of *Trimeresurus macrops* are the center of Thailand, including Bangkok and the neighboring countries (Cambodia, Laos and Vietnam).

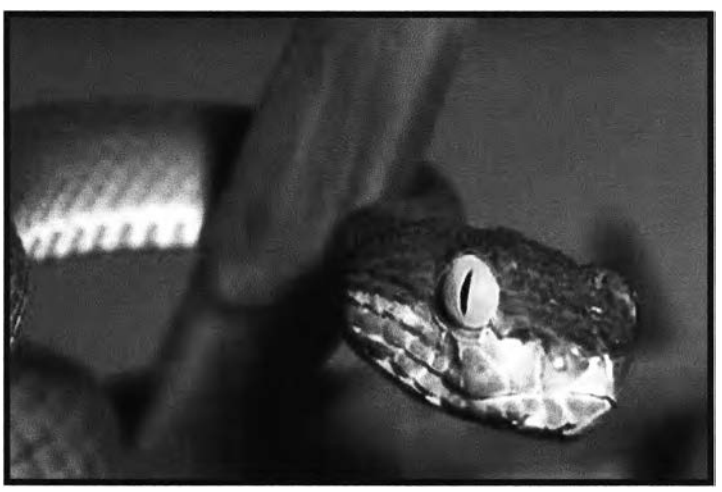

Figure 1.1 *Trimeresurus macrops* (Big-eyed Pit Viper).

1.2 Snake Venoms

Venoms are not composed of a single substance common to all poisonous snakes. Snake venoms are an aqueous solution of complex mixture. Over the 90% dry weight of venoms are proteins. These proteins include enzymes and toxins, each class being capable of modulating the physiological response of envenomed animals. Protein fractions are biologically more important than nonprotein ones as most of the biological activities reside in protein fractions.

1.2.1 Nonprotein Components of Snake Venoms [8]

The nonprotein components are divided into inorganic and organic constituents. Inorganic constituents are metal contents. Organic constituents are further classified into free amino acids and small peptides, nucleotides and related compounds, carbohydrates, lipids, and biogenic amines.

1) Inorganic Constituents

Metal Contents of snake venoms are calcium, zinc, magnesium, sodium, potassium, copper, manganese, iron and nickel. Calcium, zinc and magnesium were the most prevalent metals, copper was detected in some venoms. Since the major constituents of snake venoms are proteins which are charges macromolecules, it is quite natural that snake venoms contain various cations or anions to neutralize the charges. Some of the metals, especially monovalent cations, serve this purpose. Actually sodium is present in any venom in far greater quantity than other cations, but monovalent ions probably have relatively little significance in terms of biological and enzymatic activities. Some divalent metals are required as cofactors for many different enzymatic and biological activities.

2) Organic Constituents

In general, organic constituents of snake venoms are biologically less active. The level of nucleotides and related compounds, carbohydrates, lipids, and biogenic amines are low (1-2% of dry weight).

The amino acids and small peptides contents vary markedly from venom to venom and presumably results from exopeptidase activity in the venom. Venoms contain only a small amount of free amino acids: glycine, serine, cysteine, threonine, lysine, alanine, tyrosine, valine, phenylalanine, leucine, isoleucine, histidine, aspartic acid, glutamic acid, spermine, arginine, and proline. Two types of pyroglutamylpeptides which are pGlu-Asn-Trp and pglu-Glu-Trp, were isolated from the venom. A number of proline-rich peptides that potentiate bradykinin are present in

snake venoms. A tripeptide with unknown biological activity is also present in some snake venom.

Nucleotides and related compounds, study on the contents of venom nucleotides (and related compounds) has been very limited. Therefore, all these contents of snake venoms can be generalized. Derivatives of purine are one common finding in snake venoms. Also, adenosine and guanosine are natural constituents of venoms found in freshly milked venoms. Inosine and hypoxanthine could arise from adenosine by enzymatic action during the drying process. The total content of purine compounds amounts to 1.4 to 4.3% of venom dry weight. Thus the purine content is significant.

In snake venoms, carbohydrates are in form of glycoprotein rather than free sugars, and they are found in many types of venom. Most sugars are neutral sugars, amino sugars, or sialic acid (*N*-Acetylneuraminic acid). Neutral sugars include *D*-galatose, *D*-mannose, and *L*-fucose. Only *D*-glucosamine is present as an amino sugar.

The major components of lipids are phospholipid and phosphatidylcholine. All the fatty acids are present in the neutral and phospholipid fractions. Several biogenic amines in snake venoms are potent pain producers. They include such compounds as bradykinin, histamine, spermine, and serotonin. Also, acetylcholine like substance is present in some snake venoms.

1.2.2 Protein Components of Snake Venoms [9]

These protein components include enzyme, and polypeptide toxins.

1) Enzymes (Molecular Weight 150,000-13,000)

Many of enzymes in snake venom are hydrolased and possess a digestive role, for instance proteinases, exo-peptidases, endopeptidases, phosphodiesterases, and phospholipases. More than 20 enzymes have been detected in snake venoms, and 12 are found in all venoms (Table 1.1), although their levels differ markedly. Indeed, the enzyme levels of viperid and crotalid venoms fall on the range 80 to 95% of the total dry matter, whereas the corresponding range for elapid venoms is 25 to 70%. The enzyme content of hydrophid venoms is at the lower end of the elapid range.

Table 1.1 Enzymes Found in Various Snake Venoms.

Type of venom	Enzymes
All snake venoms	L-Amino acid oxidase, phosphodiesterases, 5′-nucleotidase, phosphomonoesterase, deoxyribonuclease, ribonuclease, adenosine triphosphatase, hyaluronidase, NAD-nucleosidase, peptidase, and phospholipase A_2*
Crotalid and viperid venoms	Endopeptidase, arginine ester hydrolase, kininogenase, thrombin-like enzyme, factor X activator, prothrombin activator
Elapid venoms	Acetylcholinesterase, phospholipase B, glycerophosphatase
Some venoms	Glutamic-pyruvic transaminase, catalase, amylase, β-glucosaminidase

*This enzyme is found in most venoms.

Hyaluronidase, which is present in all snake venoms, has a "spreading action", thereby facilitating the distribution of other venom components throughout the tissues of the prey. In addition, the viperid venoms contain enzymes involved with each of the three fundamental reactions associated with blood coagulation. In combination, these reverse the tendency for blood clotting and thereby further facilitate the distribution of venom throughout the prey. Several classes of enzymes induce direct toxic effects; indeed the majority of the toxic components of viperid and crotalid venoms are enzymes. Phospholipases, by cleaving membrane-bound phospholipids and converting them to lytic products cause cell lysis. Some phospholipases are specifically directed to presynaptic membranes while others are particularly active on muscle cells. *L*-Amino acid oxidase exerts its toxic influence on biological tissue by producing the powerful oxidizing agent hydrogen peroxide. Many venoms exhibit a yellow color. This is due to the presence of *L*-amino acid oxidase, which contains riboflavin as a prosthetic group. The more yellow the venom, the more riboflavin it contains. The hemorrhagic factors found in many viperid venoms possess molecular weight close to 50,000 and are probably hydrolytic enzymes. Some enzymes, for instance acetylcholinesterase and many of the arginine ester hydrolases, are capable of perturbing the normal physiological

response of the prey. The latter group possesses kinin-releasing activity, thereby triggering bradykinin production from endogenous precursors. Nerve growth factor (NGF) is also detected in a wide range of snake venoms including members of the elapid, viperid, and crotalid classes. Although NGF is not an enzyme, most forms have molecular weights in the range 20,000 to 35,000, and this component is frequently isolated in the enzyme-rich fractions of venoms. The role of NGF in the venoms is unknown, but it may potentiate the toxic effects of other venom components.

2) Polypeptide toxins (Molecular Weight 10,000-5,000)

In addition to enzymes, elapid venoms typically contain a range of polypeptide toxins. Postsynaptically acting neurotoxins that are capable of interacting with the nicotinic acetylcholine receptor, localized at skeletal muscle end plates are found in virtually all Elapidae and Hydrophidae venoms. Such toxins are also known as α-neurotoxins or curaremimetic toxins. Related neurotoxins (κ-neurotoxins) bind to neuronal nicotinic cholinoceptors. Cytotoxins (also called cardiotoxins) are present in cobra (*Naja*) venoms. Proteinase inhibitors of kunitz type are present in many viperid and elapid venoms, but mamba (*Dendroaspis*) venoms contain a homologous protein class, the dendrotoxins, which facilitate neurotransmitter release. Some mamba venoms also contain acetylcholinesterase inhibitors. In contrast to Elapidae venoms, those of the Viperidae and Crotalidae generally lack toxic polypeptides in this molecular weight range. However, some crotalid species do possess a low molecular weight (5kDa) myotoxin. All these polypeptide toxins are rich in both protein secondary structure and disulfide links. Consequently, they are generally robust molecules and relatively stable, once isolated.

1.2.3 Biological Effect of Snake Venom Toxins [1]

The snake venom toxins are divided by the effect on an organ system. The effects are seen in the cardiovascular, hematologic, respiratory and nervous systems. There are three types of toxin activities: hemotoxin, myotoxin and neurotoxins.

1) Hemotoxin

Hemotoxin affects blood circulatory system causing great pain, swelling, bleeding, hemorrhages (the damage of blood capillary tubes) and hemolysis (the rupture of erythrocyte membrane). Viperidae (pit vipers) snakes have hemotoxic venom.

2) Myotoxin

Myotoxin attacks the muscular system causing the destruction of muscle cells, muscular pain and paralysis. Only sea snakes in Elapidae produce this toxin.

3) Neurotoxins

Neurotoxin attacks the nervous system and causes death as a result of respiratory failure or heart failure. Cobras, kraits and mambas are examples of Elapidae that contain mainly neurotoxins.

1.3 Separation of Proteins [10]

A large number of isolation methods for proteins have been developed. Most of proteins purification involves with chromatography and electrophoresis, for examples, reversed-phase high-performance liquid chromatography (HPLC), ion-exchange chromatography (IEX), affinity chromatography (AC), hydrophobic interaction chromatography (HIC), gel filtration (GF), capillary electrophoresis (CE), and gel electrophoresis (GE). Each separation method is based on different principles. Proteins are purified using chromatographic techniques which separate according to differences in specific properties, as shown in Table 1.2. In many chromatographic methods, sample components compete with the eluant for sites on the adsorbent and the separation depends on different partitioning between mobile and stationary phase. The components pass with different velocities through the separation column. The high affinity components for the adsorbent are more retarded. Chromatograms are usually monitored by UV absorption at 280 nm. This wavelength is selected because the aromatic residues tyrosine and tryptophan posses strong absorptions in this region.

Table 1.2 Protein properties used during chromatographic purification.

Protein Properties	Purification Techniques
Charge	Ion-exchange chromatography (IEX)
Size	Gel filtration (GF)
Hydrophobicity	Reversed-phase high-performance liquid chromatography (HPLC), hydrophobic interaction chromatography (HIC)
Biorecognition (ligand specificity)	Affinity chromatography (AC)

In electrophoretic separation techniques, high separation efficiency can be achieved using a relatively limited amount of equipment. It is mainly applied for analytical rather than for preparative purposes. The electrophoretic separation is carried out under the influence of an electrical field, charged molecules and particles migrate in the direction of the electrode bearing the opposite charge. During this process, the substances are usually in an aqueous solution. Because of their varying ratios of charges to masses, different molecules and particles of a mixture will migrate at different velocities and will thus be separated into single zone. The electrophoretic mobility, which is a measure of the migration velocity, is a significant and characteristic parameter of a charged molecule or particle. It is dependent on the pK values of the charged groups and the size of the molecule of particle. Electrophoretic separations may be carried out in either free solution as in capillary (capillary electrophoresis) or free flow systems, or in stabilizing media such as films or gels (gel electrophoresis).

According to this research, the separations of proteins from *Trimeresurus macrops* venom utilise gel filtration chromatography and gel electrophoresis. The following details of basic principles will be focused on these two separation methods.

1.3.1 Gel Filtration Chromatography[9]

Gel filtration chromatography was introduced by Porath and Flodin in 1959.[11] Gel filtration chromatography separates proteins with differences in

molecular size. Large molecule can not enter the matrix. Intermediate size molecules can enter part of the matrix and small molecules freely enter the matrix. The function of the matrix is to provide continuous decrease in accessibility for the molecule of increasing size. The large molecules are eluted from the column first and the smallest are eluted last. The solvent in a gel filtration column is both in the space between the particles, *the void volume* V_0, and inside the particles, *the internal volume* V_i. A large molecule that is excluded from the interior has an elution volume $V_e = V_0$. A small molecule which all of V_i is accessible elutes with $V_e = V_0 + V_i$. A molecule of an intermediate size is excluded from the smaller pores, and the internal volume is only partially accessible. A distribution coefficient K_d is defined as the fraction of V_i accessible. The elution volume can be expressed as

$$V_e = V_0 + (K_d \times V_i)$$

and

$$K_d = (V_e - V_0)/ V_i.$$

$K_d = 0$ for molecules that are excluded from the interior of the particles, $K_d = 1$ for molecules to which the solvent in the both the void and the interior volume is accessible, and $K_d > 1$ indicates adsorption to the gel. The elution volume depends on both the shape and the molecular weight of the molecule. Most columns will fractionate within a particular molecular weight range, determined by the pore size of the bead. A good correction exists between molecular weight and elution volume for a set of molecules with similar gross structure, such as globular proteins. The exclusion limit gives the lower limit for molecules eluting in the void.

A great number of gel filtration supports are available, and they can be considered to two general classes: cross-linked natural or synthetic polymers and silica of different pore sized coated with a hydrophilic layer. The cross-linked polymers have a rather limited mechanical stability and restrictive using in conventional chromatography (low flow rate and low pressure column). In contrast to the polymeric supports, matrices suitable for size-exclusion HPLC must consist of small uniform particles that are rigid and can withstand high pressures. The two types of HPLC supports are silica based and organic based polymer. The examples of supports for gel filtration chromatography and typical fractionation ranges are presented in Table 1.3.

Table 1.3 Supports for Gel Filtration Chromatography.

	Fractionation ranges (kDa)
Low-pressure supports:	
Sephadex: Dextran, cross-linked with epichlorohydrin	≤0.7-600
Sepharose: Agar, porosity depending on amount of agar in matrix	10-40,000
Bio-Gel A: Agar	<10-150,000
Sepharose CL: Sepharose cross-linked with 2,3-dibromopropanol	10-40,000
Sephacryl: Allyl dextran cross-linked with *N,N'*-methylene bisacrylmide	5-80,000
Bio-Gel P: Polyacrylamide	0.1-400
HPLC supports:	
Silica-based: Porous silica with bonded hydrophilic groups, TSK supports of SW type	5-7000
Organic polymers with TSK supports of PW type: Hydrophilic hydroxylated ether	0.1-8000

The proteins of interest should elute between the extremes of K_d = 0 and 1. The fractionation range or the most useful working range of some common gels is given in Table 1.4.

Table 1.4 Protein Fractionation Range of Some Gels.

Sephadex	**kDa**	**HPLC supports**	**kDa**
G-25	1-5	Superose 12	3-100
G-50	1.5-30	Superose 6	5-5000
G-75	3-70	TSK G2000SW	5-100
G-100	4-100	TSK G3000SW	10-500
G-150	5-150	TSK SW	20-7000
G-200	5-250	TSK-30	1-20
Sephacryl S-300	10-1500	TSK-40	2-30

1.3.2 Gel Electrophoresis [12-13]

Gel electrophoresis provides a powerful separation for proteins in complex mixtures. Proteins are separated according to their ability to move under the influence of an electric field. Polyacrylamide gels were first used for electrophoresis by Raymond and Weintraub in 1959.[14] Cross-linked polyacrylmide gels are from the polymerization of acrylamide monomer in the presence of small amounts of *N,N'*-methylene-*bis*-acrylamide (crosslinking reagent) (Figure 1.2). The polymerization of acrylamide is initiated by the addition of ammonium persulfated and the base *N,N,N',N"*-tetramethylenediamine (TEMED). TEMED catalyzes the composition of persulfate ion to give a free radical.

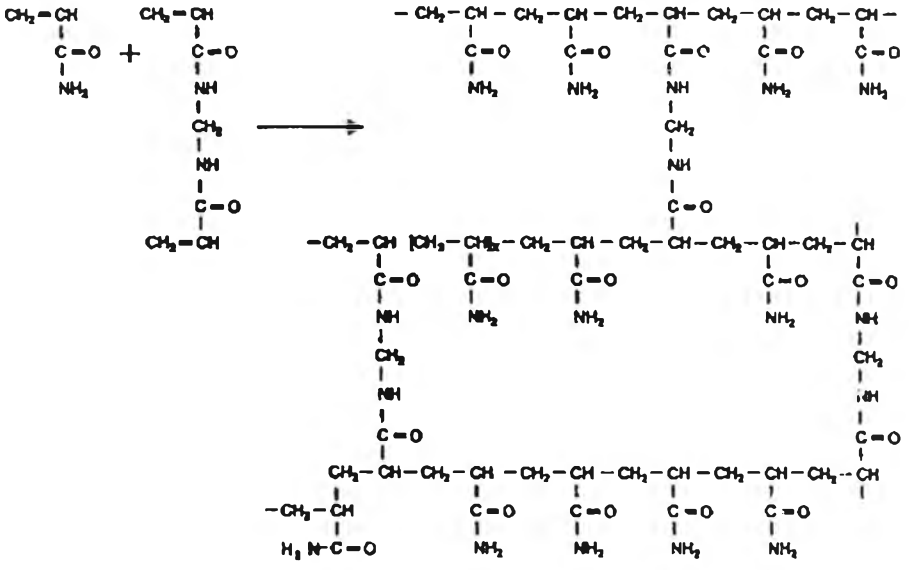

Figure 1.2 The polymerization reaction of acrylamide and methylenebisacrylamide.

The pore size restricts the movement of proteins. The pore size of the gel can be controlled by the total acrylamide concentration *T* and the degree of crosslinking *C*.

$$\%T = [(a + b) \times 100]/V, \quad \%C = (b \times 100)/(a+b)$$

a is the mass of acrylamide in g.

b is the mass of methylenebisacrylamide is g, and *V* is the volume in ml.

When C remains constant and T increases, the pore size decreases. The range of pore size varies from 4%T to 20 %T. Gel with higher %T has small pore sizes and more restrictive so it favors the movement of smaller proteins with little or no movement of large proteins.

1) Separations Based on Protein Molecular Weight

Sodium-dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE), reflecting the combination of SDS treatment of proteins with polyacrylamide gel (PAGE), is one-dimensional electrophoresis (1-D electrophoresis). All proteins are given a negative charge and move in the same direction towards the positive electrode. The charge of proteins can be produced by coating with the anionic detergent sodium dodecyl sulfate (SDS). The proteins are denatured and uncoiled by the effect of the SDS saturation, especially when using reducing reagents such as dithiothreitol to cleave disulfide bonds. Smaller proteins move more rapidly through the gel than larger proteins such that mixtures of proteins can be separated according to their molecular weight (M_r).

2) Separations Based on Protein Isoelectric Point

Isoelectric focusing (IEF) can be described as electrophoresis in a pH gradient set up between a cathode and anode with the cathode at a higher pH than the anode. In electric field, the proteins, which are amphoteric substance, move towards the anode or the cathode until they reach a position in the pH gradient where their net charges are zero. Proteins tend to be positively charged at pH values below their isoelectric pH and negatively charged above. The pH at which a protein has no net charge is called the "isoelectric point" or "pI" of the protein. The protein species migrate and focus (concentrate) at their isoelectric points. The focusing effect of the electrical force is counteracted by diffusion which is directly proportional to the protein concentration gradient in the zone. In an isoelectric focusing gel, the pH conditions are established in polyacrylamide gel by two techniques, carrier ampholytes (low molecular weight amphoteric species) or immobilines (acrylamide derivatives). The most use of isoelectric focusing experiments carried out in immobilized pH gradient (IPG) gel. IPG gel made from mixtures of immobilines with common gradient-mixing systems to establish the pH gradient. The advantages of

using IPG gel are allowing a long focusing time to ensure the focusing of the analyte proteins, loading relatively large amount of proteins, available to purchasing IPG in variety of pH ranges, and simplify the physical handling.

3) Two-Dimensional Electrophoresis [15]

Two-dimensional electrophoresis (2-D electrophoresis) is a powerful and high resolution method for the analysis of complex protein mixtures. Several thousand of proteins can be separated in one gel. This technique combines two steps of gel electrophoresis separation, and separates proteins according to two independent properties. The first-dimension step is isoelectric focusing, separates proteins according to their pI across the x-axis. Then, all proteins contained in isoelectric focusing gel are saturated with SDS and transferred to SDS-PAGE in the second-dimension step, separates proteins according to their M_r across y-axis. Each spot on the resulting 2-D gel corresponds to a single protein in the sample. 2-D electrophoresis is also an analytical method for measuring the M_r, pI, and relative amount of a protein in the mixture.

4) Protein Detection

The mostly applied methods in the detection of separated proteins from gel electrophoresis are Coomassie blue-staining and Silver-staining. Coomassie blue-staining colors the proteins fixed in a polyacrylamide gel with a dark blue dye. Generally, Coomassie blue-staining can detect as little as 0.5 pmol to 1 pmol of protein in a 1-D gel band, and 0.2 pmol to 0.5 pmol for 2-D gel. Silver-staining is an estimated 100-fold more sensitive than Coomassie blue-staining, giving limits of detection for 2-D gel bands in the 5 fmol range.

1.4 Mass Spectrometry [16]

Mass spectrometry (MS) measures the mass weight of molecules in term of the mass-to-charge ratio (m/z). Molecular weight measurements by mass spectrometry are based upon the production, separation, and detection of molecular ions. A typical components of mass spectrometer includes; ion source (Ionizes sample and generates gas phase ions), mass analyzer (Separates ions according to individual mass-to-charge ratios), detector (Detects and amplifies ions). The common two ionization methods for peptides and proteins; electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), which will be described in detail later. After the gas phase ions have been generated, they are expelled from the ion source and beamed in to mass analyzer, the region of ion separation. Many types of mass analyzer that commonly used for protein analysis applications are time of flight (Tof), quadrupole mass filter, ion trap, combinations of quadrupole Tof and Tof/Tof. Herein, Tof and quadrupole mass filter are mentioned here because these two types of mass analyzer were used in this research.

1.4.1 Matrix Assisted Laser Desorption Ionization/Time of flight Mass Spectrometry (MALDI/Tof MS) [16-18]

1) Matrix-Assisted Laser Desorption Ionization (MALDI)

Karas and Hillenkampm[19-20] introduced Matrix Assisted Laser Desorption/Ionization (MALDI) technique that could readily ionize biomolecules in a very sensitive manner. MALDI is a pulsed ionization technique which utilises the energy from a laser to desorp and ionize the analyte molecules in the presence of a light absorpting matrix (Figure 1.3). MALDI ions are created by mixing the analyte with the small organic molecule (the matrix) which absorbs at the wavelength of the laser. The analyte becomes incorporated into the crystal lattice of the matrix and is then irradiated with a laser. The laser causes the desorption and ionization of matrix and analyte, either by protonation (positively charged ions) or desorption (negatively charged ions). The ions are then accelerated into the MS analyzer. As MALDI is a pulsed ionization technique, which is ideally coupled with a Tof analyzer.

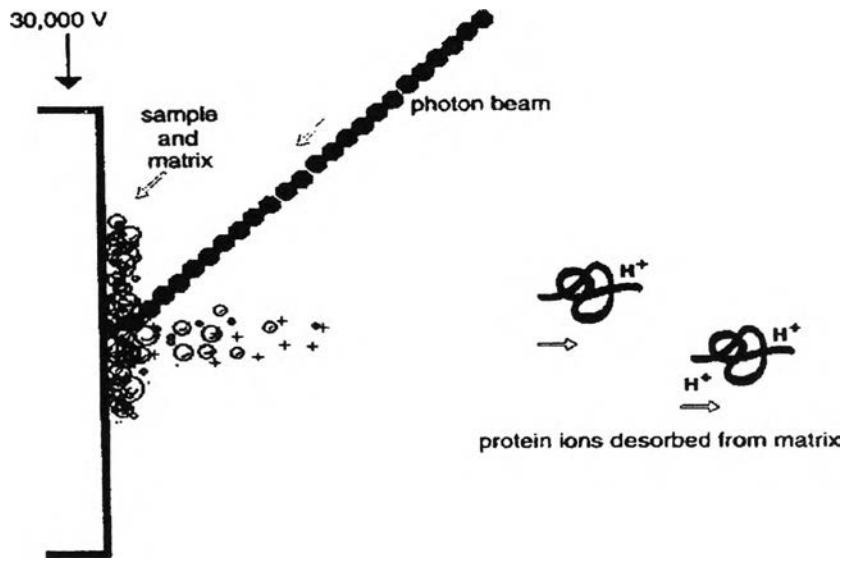

Figure 1.3 Matrix Assisted Laser Desorption/Ionization (MALDI) Source.

Among the chemical and physical ionization pathways suggested that MALDI are gas-phase photoionization, excited-state proton transfer, ion-molecule reactions, desorption of preformed ions, etc. The most widely accepted ion formation mechanism involves gas-phase proton transfer with photoionized matrix molecules (Figure 1.4). MALDI produces predominantly singly charged ions. Also, MALDI allows to the desorption and ionization of analytes with very high molecular mass in excess of 100,000 Da.

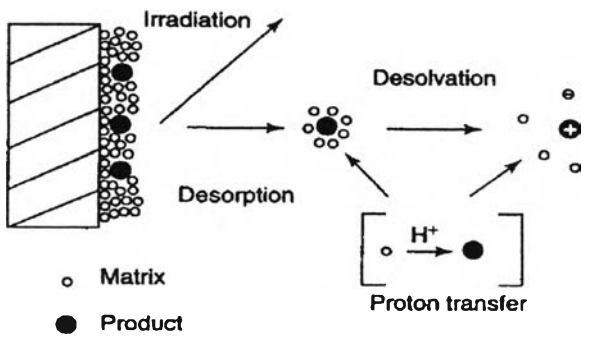

Figure 1.4 MALDI Ion Formation.

The typical wavelength of the UV lasers utilised is 337 nm. MALDI ions are generated under high vacuum ($5x10^{-6}$mbar). A wide range of matrices for biological mass spectrometry applications have been adopted for use with UV lasers (Table 1.5).

Table 1.5 Common MALDI matrices used in biological applications.

Matrix	Application
α-cyano-4-hydroxycinnamic acid	UV laser Peptides analysis & protein digests Analytes < 10 kDa
sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid)	Analysis of large polypeptides & proteins Analytes > 10 kDa
2,5-dihydroxybenzoic acid (2,5-DHB)	UV laser Protein digests & Proteins Oligosaccharides released from Glycoproteins
2,4,6-trihydroxyacetophenone (THAP)	UV laser Oligonucleotides < 3 kDa
3-hydroxy picolinic acid	UV laser Oligonucleotides > 3 kDa

The three widely used matrices for peptides and proteins are α-cyano-4-hydroxycinnamic acid (CCA), 2,5-dihydroxybenzoic acid (2,5-DHB), and sinapinic acid.

2) Time of Flight Mass Analyzer

Tof analyzer is one of the simplest mass analyzing devices and commonly used with MALDI ionization. Tof analysis is based on accelerating a set of ions to a detector with the same amount of kinetic energy. Because the ions have the same energy, yet a difference mass, the ions reach the detector at difference times. Mass-to-charge ratios are determined by recording the time that ions use to move in a field free region (a flight tube) from the source to the detector. The arrival time of an ion at the detector is dependent upon the mass, charge, and kinetic energy of the ion. A beam of ions is accelerated through a know potential V_s. When ion leaving the source, an ion with mass m and total charge $q = ze$ has a kinetic energy E_k

$$\frac{mv^2}{2} = qV_s = zeV_s = E_k$$

Replacing v by its value in the previous equation gives

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

The mass-to-charge ratio can be calculated from a measurement of t^2, the term in parentheses being constant. From the equation show that, all others being equal, the

lower the mass of ion, the faster it will reach the detector. The linear Tof mass spectrometer is the simplest example of Tof analyzer (Figure 1.5).

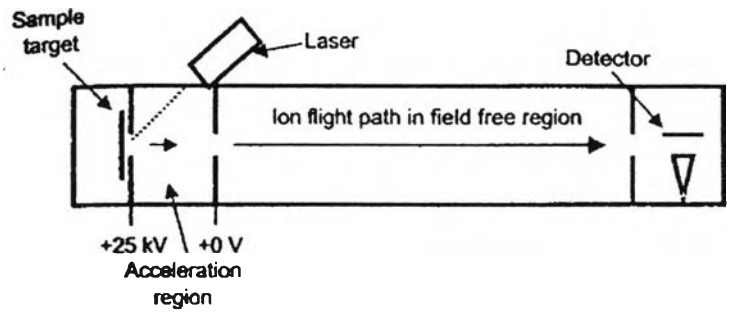

Figure 1.5 Linear Time-of-Fight Mass Spectrometer.

In principle, the upper mass range of a Tof instrument has no limit, which suitable for soft ionization techniques. The advantages of this instrument are high sensitivity and very fast scan speed. The drawback of the linear Tof analyzers is poor mass resolution. Mass resolution is affected by factors that create a distribution in flight times among ions with the same *m/z* ratio. Time-of-fight reflectron mass analyzer has been developed to improvement of mass resolution. The reflectron is an ion mirror, created by an electric field that reverses the flight path of ion (Figure 1.6). The most significant effect of reflectron is to focus ions with the same *m/z* but different velocities. Focusing is accomplished because these ions penetrate the reflectron to different degrees. The higher-velocity ions penetrate farther, and spend a longer time in the reflectron than the lower velocity ions. Another effect of a reflectron is to increase the effective flight tube. The longer Tof flight tube has better resolution.

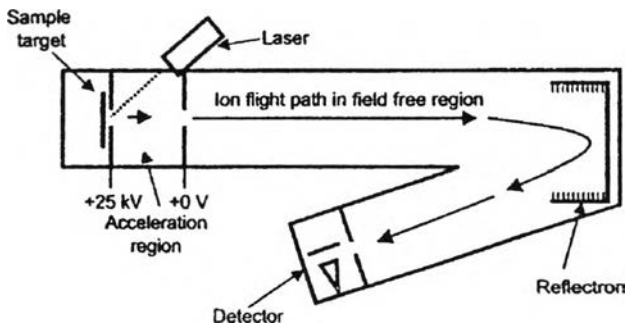

Figure 1.6 Reflectron Time of Fight Mass Spectrometer.

As mentioned above, the improving resolution and sensitivity of time of fight reflectron mass analyzer is an important contributor to the mass observed in

MALDI/Tof experiment. Also, it is an important consideration in the development of hybrid quadrupole-Tof instruments for tandem mass spectrometry described in detail later.

1.4.2 Electrospray Ionization Mass Spectrometry [16-18, 21]

1) Electrospray Ionization (ESI)

Fenn and co-workers described the use of electrospray for ionizing large biomolecule in 1989.[22-23] In electrospray ionization, analytes that acidic aqueous solution is sprayed through a small-diameter needle. Therefore, ESI ionizes is readily coupled to liquid-based (for example, chromatographic and electrophoretic). A high positive voltage is applied to the needle to produce a Taylor cone from which droplets of the solution are sputtered (Figure 1.7).

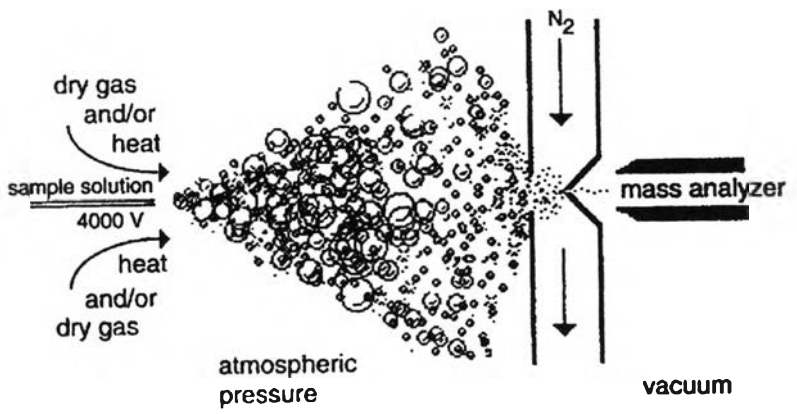

Figure 1.7 The Electrospray Ionization Source

Protons from the acidic conditions give the droplets a positive charge, causing them to move from the needle towards the negatively charged instrument. During this movement, evaporation, which processed by flow of gas (nitrogen) and heat, reduces the size of the droplets until the number and proximity of the positive charges spilt the droplet into a population of smaller, charged droplets. The evaporation and droplet-splitting cycle repeats until nanometer sized droplet are produced and can be directed into the mass spectrometer by appropriate electric field (Figure 1.8).

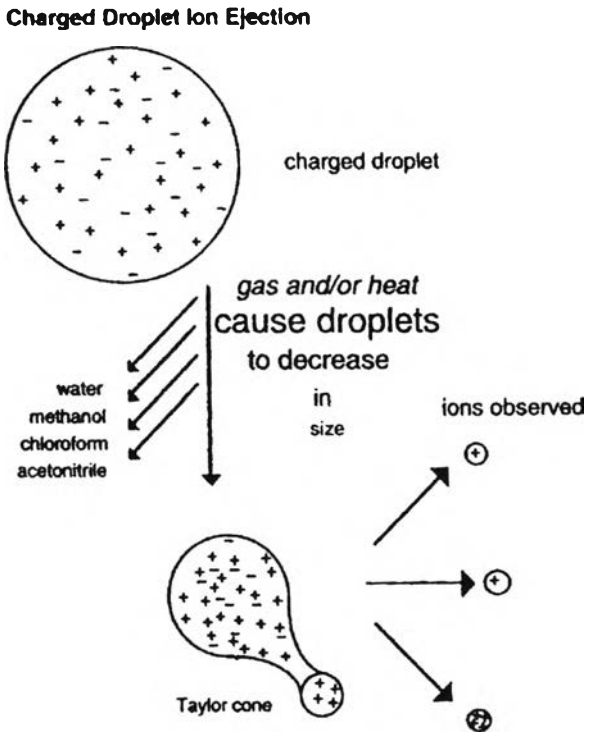

Figure 1.8 The Electrospray Ionization Formation.

The charges are statistically distributed over the analyte's potential charge sites, enabling the formation of multiply charged ions. Each multiply charged ion can be termed a charge state, and a distribution of charge states is characteristic of large macromolecules during ESI analysis. The high electric field in the electrospray source produces multiply-charged ions. For which

$$\frac{m}{z} = \frac{M + nH}{n}$$

where, M = molecular weight, n = number of charges and H = mass of proton

If a positive ion series is assumed to represent different protonation states, then the mass-to-charge ratio, A_1 and A_2, of adjacent ions in a multiply charged ion series appear at m/z of; $n_1 = n_2 + 1$, where n_1 is the number of charges on A_1 and n_2 is the number of charges on A_2. Then,

$$\frac{M + n_1H}{n_1} = A_1 \quad \text{and} \quad \frac{M + n_2H}{n_2} = A_2$$

Therefore,

$$n_2 = \frac{A_1 + H}{A_2 - A_1}$$

Thus, the estimation of mass and charge number can be calculated.

2) Quadrupole Mass Filter

The quadrupole mass filter is composed of four parallel hyperbolic rods, which the gas phase ions have to achieve a stable trajectory. The analyzer is operated by the application of a voltage (DC) and oscillating voltage (Radio Frequency, RF) to one pair of rods and DC voltage of opposite polarity and RF voltage of different phase to the opposite pair of rods (Figure 1.9). The alternating electric field helps to stabilize and destabilize the passing ions. The ions traverse through the space between the rods, according to equation noted in Figure 1.9, and only at specific voltages applied to the rods will certain m/z values be allowed to pass through the rods and reach the detector. The voltages are scanned to allow a wide mass range to be observed.

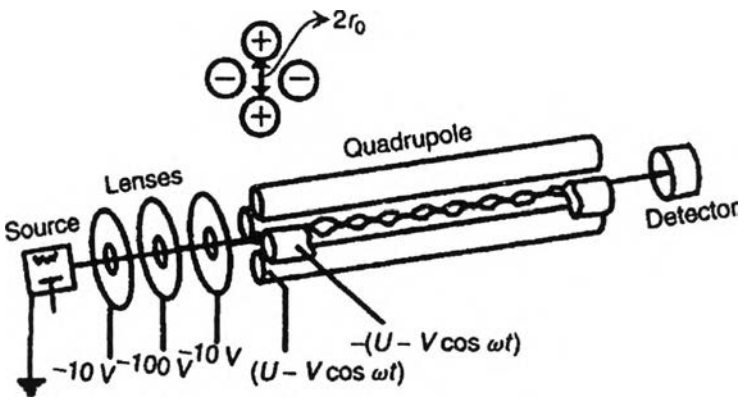

Figure 1.9 The Quadrupole Instrument.

The ion trajectory can be considered with Mathieu equation, leading to definition of two parameters.

$$a = \frac{8zeU}{mr_o^2\omega^2} \qquad q = \frac{4zeV}{mr_o^2\omega^2}$$

Where U = direct current (DC) potential, (V + cos ωt) = radio frequency potential,

ze = charge of ion and r_o = ½ of the distance between opposite rods

The parameter r_o and ω are maintained constants. U and V are the variables. The relation between a and q is

$$\frac{a}{q} = \frac{2U}{V} = \text{constant}$$

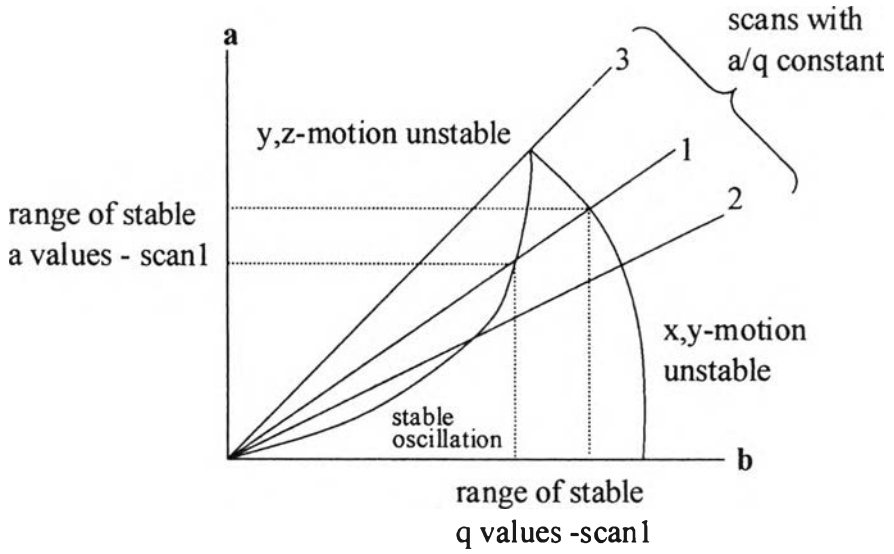

Figure 1.10 The Stability Region for Ion Trajectories in The Quadrupole Mass Filter.

For the graph in Figure 1.10, only the stable oscillation area of the graph contains values for both a and q that define stable ion motion along the z-axis, thereby allowing ions to pass along the axis of the quadrupole rods to the detector. For the other values of a and q ions diverge far enough from the z-axis that they collide with, or pass between, the rods. The lines marked 1, 2 and 3 in Figure 1.10 are called scan lines and correspond to variation of U and V such that U/V (a/q) remain constants. The quadrupole are low resolution instrument and thus function as a mass filter. Quadrupole mass analyzers have found commonly utility to interface with ESI and applied for using as instruments in tandem MS (Triple quadrupole and quadrupole Tof).

1.5 Tandem Mass Spectrometry [16, 18, 24]

Tandem mass spectrometry (MS/MS) is general method involving at least two stages of mass analysis. The first analyzer (MS1) is used to isolate a precursor ion, which then undergoes activation a fragmentation to yield product ions. The product ions are subsequently analyzed by a second mass analyzer (MS2). Tandem mass analysis is primarily used to obtain structural information. A tandem mass spectrometer can be classified into two types: tandem-in-space and tandem-in-time. The in-space mass spectrometers have two mass analyzers, and each mass analyzer performs separately to accomplish the difference stages of experiment. The classic example of tandem-in-space instrument is the tandem quadrupole instrument, commonly referred to as a "triple quadrupole" mass spectrometer. The combination of a quadrupole with time-of-flight instrument and time-of-flight with a reflectron are also used as tandem-in-space instruments. The tandem-in-time mass spectrometry performs as appropriate sequence of events in an ion storage device. The in-time instruments have only one mass analyzer. An ion trap mass spectrometer and ion cyclotron resonance instruments, also known as Fourier transform mass spectrometers, are the examples of tandem-in-time systems.

There are three modes of scanning in tandem MS: product ion scan, precursor ion scan, and neutral loss scan (Figure 1.11). These scan modes are distinguished by the relationship between the first and second stage of mass analysis. In standard mode of mass analysis, the first mass analyzer and the collision cell transmit all ions for mass analysis in the second mass analyzer. The product ion scan consists of selecting a precursor ion in the first mass analyzer and determining the product ions resulting from collision-induced fragmentation (CID) in second mass analyzer. The precursor ion scan consists of choosing a product ion and determining the precursor ions. The first mass analyzer is scanned to sequentially transmit the mass-analyzed ions into the collision cell for fragmentation. The second mass analyzer mass-selects the product ion of interest for transmission to the detector. All of precursor ions that produce ions with the selected mass through fragmentation thus are detected. The Neutral loss scan consists of selecting a neutral fragment and detecting all the fragmentations leading to the loss of that neutral fragment. Mass analysis in both mass analyzers is carried out by linking the scanning mass analyzers with a constant

mass difference. Fragmentation occurs in the collision cell. The detector signal is the result of precursor ions fragmenting to lose a specific neutral species, forming a product ion with a characteristic mass difference. In tandem instruments, only the tandem quadrupole instrument performs all of the experiments illustrated in Figure 1.11. The precursor ion scan and neutral ion scan mode cannot be preformed with time-based mass spectrometers. Because of the scan requires the focusing of the second spectrometer on a selected ion in the precursor ion scan mode and the scan requires that both mass spectrometers are scanned together in the neutral loss scan mode.

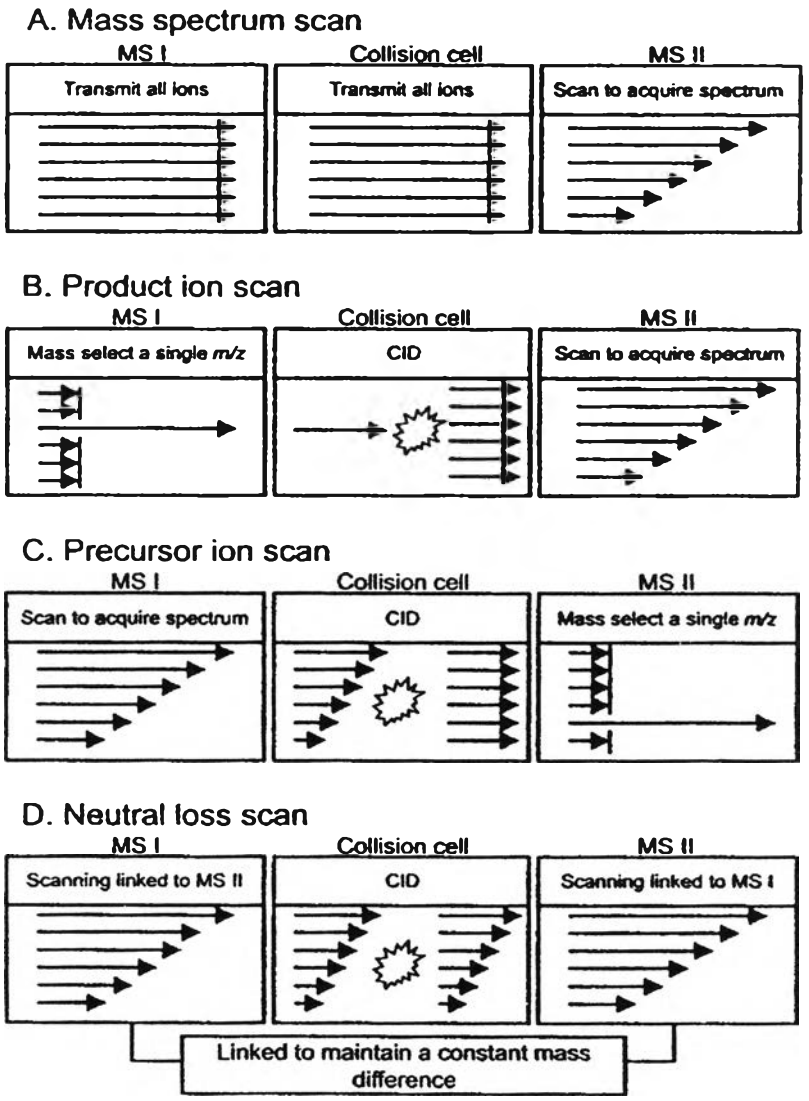

Figure 1.11 A Schematic Representation of The Tandem Mass Spectrometry Scan Modes.

1.5.1 Collisionally Activated Dissociation (CAD)

The fragmentation can be achieved by inducing ion-molecule collisions by a process know as collisionally activated dissociation (CAD) or collisionally induced dissociation (CID). The CAD process as 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. CAD 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 kinetic energy for internal energy transfers is controlled by the collisions of mobile species (the ion) and a static target (the collision gas). In practice, there are two groups of collision energy: high-energy collision, in the keV range, and low-energy collision, in the range of 1-100 eV. The high-energy collision is common for magnetic instruments and also uses helium as the common target gas. The low-energy collision occurs in quadrupole or ion trap instruments. The collision gas is more important than it is for the high-energy collisions. Heavier gases such as argon, xenon or krypton 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 CAD spectra give simpler, more clearcut fragmentation, whereas low-energy CAD spectra lead to more diverse fragmentation pathways, often including more rearrangements.

1.5.2 Tandem Mass Spectrometry for Peptide Sequencing

Tandem mass spectrometry obtains structural information on peptide and proteins by CAD, in which precursor ions are subjected to collisions and the structure of resulting ions is characterized. As result of the CID fragmentation the peptide precursor ion fragments predictably at each peptide amide bond along the peptide backbone yielding a distribution of product ions in complimentary ion series forming a ladder which is indicative of the peptide sequence. The nomenclature suggested by Roepstroff and Fohlman and used to describe the different product ions defines two set of ions that are named based on the peptide terminus retained in the

ion. The a-, b-, c-ions all contain the N-terminus of the peptide, while the x-, y-, z-ions all contain the C-terminus (Figure 1.12). There is a marked difference between the fragmentation observed at high and low energy. At high energy described in figure 1.13 can be generated. The high-energy tandem mass spectra present a greater number of fragment ions but also increasing the complexity and difficulty of interpretation. At low energy, the observed fragments are mostly b- and y-ion. These fragments then lose small molecules such as water or ammonia from functional on amino acid side chains.

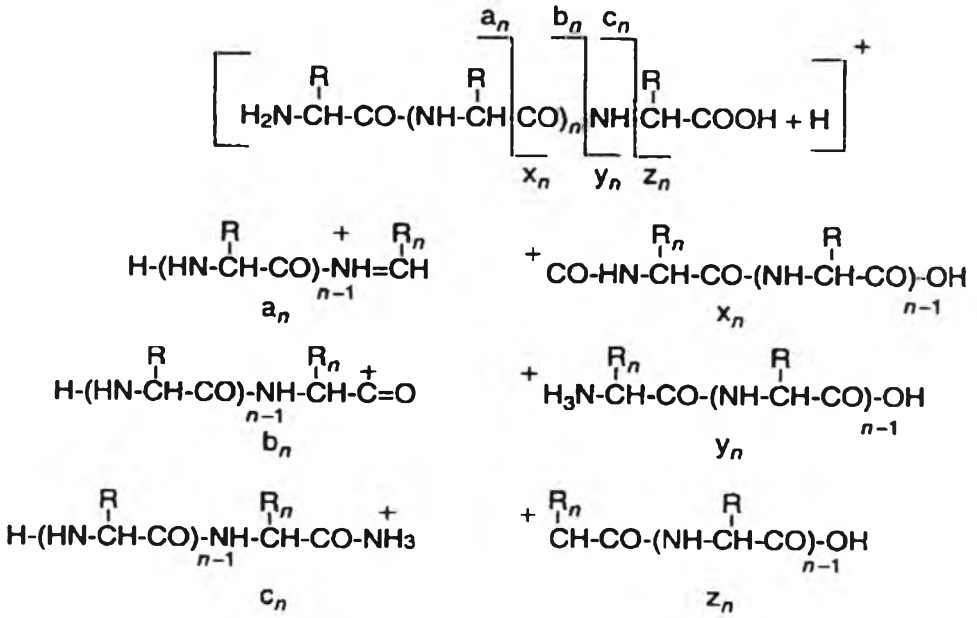

Figure 1.12 The Peptide Fragmentation.

In addition, there are two other types of fragments which appear among the low masses in the spectrum (Figure 1.13). The first type is called an internal fragment because these fragments have lost the initial N- and C-terminal sides. The second type of fragment is immonium ions of amino acids, labeled by a letter corresponding to the parent amino acid code. Even though these two fragments are rarely observed for all of peptide amino acids, those that appear yield information concerning the amino acid composition of the sample and also confirm the sequence.

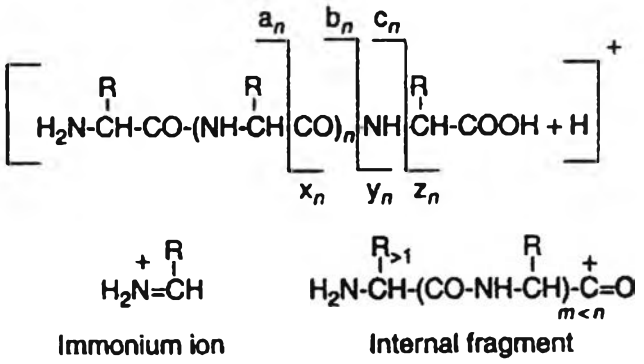

Figure 1.13 Imonium Ion and Internal Fragment.

The mass difference between consecutive ions within a series allows one to determine the identity of the amino acid (Table 1.6) and thus deduce the peptide sequence.

Table 1.6 The Masses of Common 20 Amino Acids.

Amino acid	Code (3 letters)	Code (1 letter)	Monoisotopic mass	Chemical mass
Glycine	Gly	G	57.02147	57.052
Alanine	Ala	A	71.03712	71.079
Serine	Ser	S	87.03203	87.078
Proline	Pro	P	97.05277	97.117
Valine	Val	V	99.06842	99.133
Threonine	Thr	T	101.04768	101.105
Cysteine	Cys	C	103.00919	103.144
Isoleucine	Ile	I	113.08407	113.160
Leucine	Leu	L	113.08407	113.160
Asparagine	Asn	N	114.04293	114.104
Aspartate	Asp	D	115.02695	115.089
Glutamine	Gln	Q	128.05858	128.131
Lysine	Lys	K	128.09497	128.174
Glutamate	Glu	E	129.04260	129.116
Methionine	Met	M	131.04049	131.198
Histidine	His	H	137.05891	137.142
Phenylalanine	Phe	F	147.06842	147.177
Arginine	Arg	R	156.10112	156.188
Tyrosine	Tyr	Y	163.06333	163.17
Tryptophan	Try	W	186.07932	186.213

1.6 Identification of Proteins

Proteins can also be viewed as being composed of smaller multi-amino acid subunit called peptides. It is often either necessary or advantageous to work with peptides rather than proteins. In instances, the protein is chemically or enzymatically digested by hydrolyzing selected amide bonds. Peptides have the same amino acid composition, N- and C-terminus as proteins. The amino acid sequence establishes the identity of protein and defines the primary structure of protein. The two commonly applied methods of generating amino acid sequence information are Edman degradation and mass spectrometry.

1.6.1 Edman Degradation [13, 18]

The oldest of the techniques used routinely for amino acid sequencing is Edman degradation. The chemical reaction used in the Edman degradation of proteins was first described in 1950s. Edman degradation uses the Edman reaction to systematically cleave amino acids from N-terminus of a protein or peptide, and then cleaved amino acid is identified by appropriate analytical methods. In the Edman reaction, phenylisothiocyanate reacts with the N-terminus of the protein to form a cyclic intermediate that facilitates hydrolysis of the adjacent amide bond. The effect of this reaction is to specifically remove the N-terminal amino acid from the protein and thereby generate a cleaved derivative of amino acid and a protein that is shorted by one amino acid and has new N-terminus (Figure 1.14). At each application or cycle of the Edman reaction, the cleaved amino acid can be recovered and identified by HPLC analysis. The current automated Edman sequenators are completely automated instrument system. At least 10 to 100 nanomole of the protein of interest was needed. There are several advantages of applying Edman degradation as protein sequencing method. For example, Edman degradation can be applied to intact proteins and also has good sensitivity. Moreover, the ease of operation of the instrument and the clarity of data add to the continued utility of the technique. However, Edman degradation is time consuming method. The time required to complete each cycle is about 45 minutes, which limit the analysis of on more two or three samples per day. Furthermore, Most native proteins have blocked N-terminally. There is because of post-translational modifications such as the addition of formyl or acetyl groups.

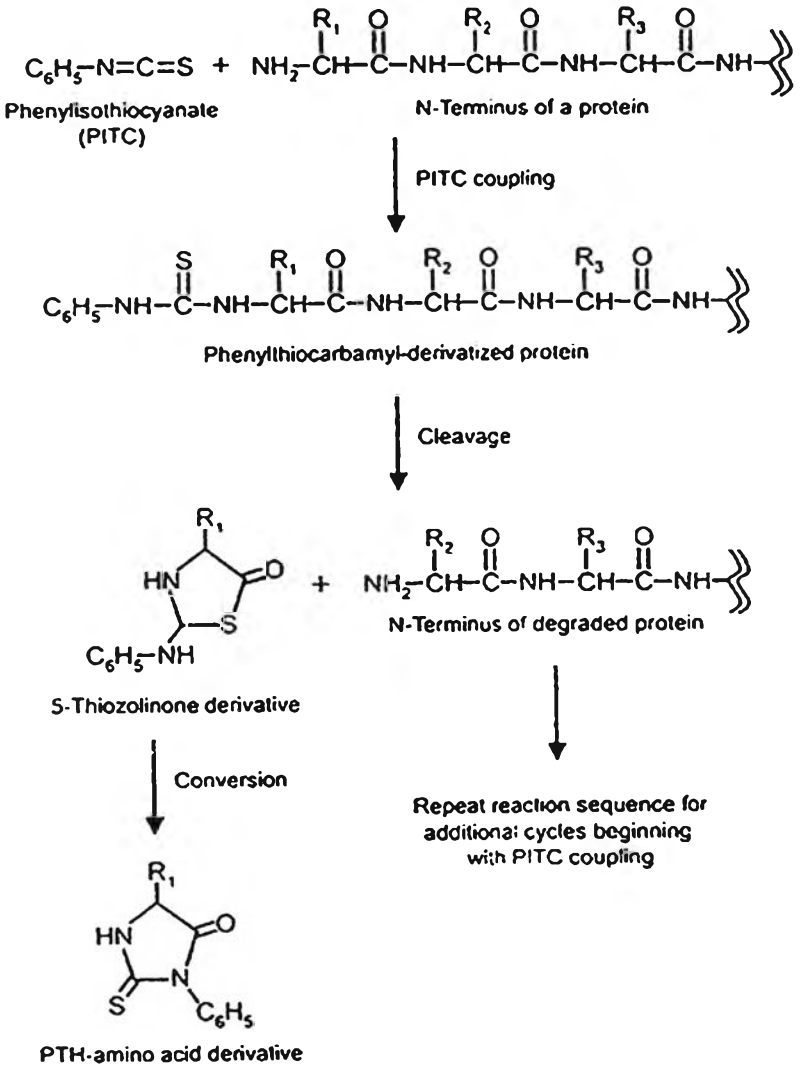

Figure 1.14 The Edman Reaction.

1.6.2 Mass Spectrometry [13, 16, 18]

Mass spectrometry is the most efficient way to identify proteins, especially the short time analysis, the high-sensitivity, and high information content in protein sequencing. The strategy for sequencing a protein using mass spectrometry starts with the precise determination of the molecular weight of that protein using MALDI or ESI MS (Figure 1.15). Accurate determination of molecular weight of a protein is useful for its identification and the determination of its purity.

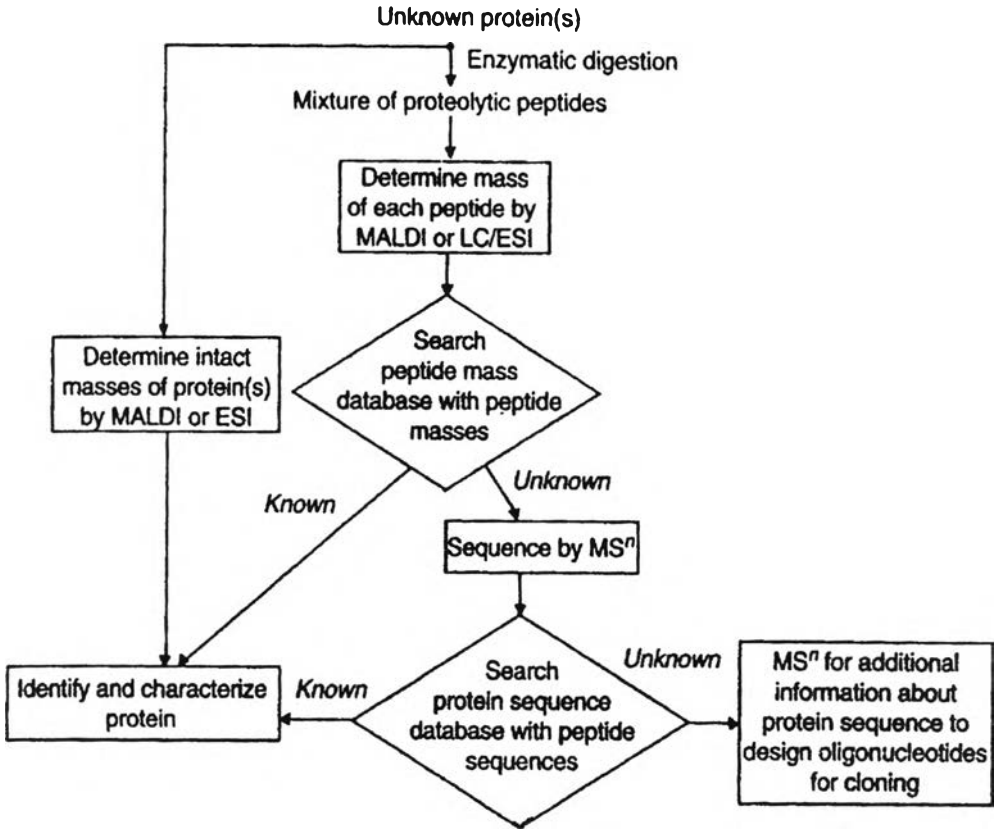

Figure 1.15 Strategy for The Identification of Proteins Based on Mass Spectrometry.

There are two approaches for protein identification using mass spectrometric data. The first approach is using mass spectrometry to detect mass of intact proteins and their digested peptides. These mass spectrometric data are compared with the data of known proteins in database. The second approach is using tandem mass spectrometry and CAD to obtain peptide amino acid sequence.

1) The Protein Sequence Databases

Protein identification is made by comparing the mass spectrometric data with the information that has been gathered into the sequence databases. The examples of the protein sequence databases are the GenBank database (www.ncbi.nlm.nih.gov/Genbank/index.html), the SWISS-PROT database (http://www.expasy.ch/sprot/sprot-top.htmL), TrEMBL (http://www.expasy.ch/srs5/), and dbEST (http://www.ncbi.nLm.nih.gov/dbEST/).

2) Protein Identification Using Peptide Mass Mapping

The peptide mass mapping usually performed using MALDI-MS. The method comprises protein digestion, MALDI/Tof analysis and sequence database search algorithms. The protein of interest can be enzymatically or chemically cleaved into its constituent peptides. There are many enzymes and chemical reagents available for protein cleavage (Table 1.7). The enzymatic digestion offers several advantages, including high specificity, minimal side reactions and good cleavage. The most common used enzyme is trypsin, which specifically cleaves protein on the C-terminal side of Lys and Arg, except those bonds are to proline (P) residues (K-P or R-P bonds). There are two types of digestion, in-gel digestion and in-solution digestion. The in-gel digestion methodology has become routine for proteins separated by 2D electrophoresis. Briefly, the in-gel digestion steps are protein spot picking, destaining, reduction and alkylation, tryptic digestion, and extraction tryptic peptides for analysis. For protein separated by other techniques (not gel electrophoresis), the digestion method is carried out by digest proteins in solution, so called "in-solution digestion".

Table 1.7 Specific Chemical and Enzymatic Cleavage of Protein.

(The cleavage is carboxyl side of X residue and amino acid side of Y residue)

Reagent	Cleavage site
Chemical cleavage	
Cyanogen bromide	Met-Y
Formic acid	Asp-Pro
Hydroxylamine	Asn-Gly
2-Nitro-5-thiocyanobenzoate	X-Cys
Phenyl isothiocyanate (Edman)	Terminal amino group of peptide
Enzymatic cleavage	
Trypsin	Arg-Y, Lys-Y
Chymotrypsin	Tyr-Y, Phe-Y, Trp-Y
Endoprotease V8	Glu-Y (Asp-Y)
Endoprotease Asp-N	X-Asp (X-Cys)

The resulting peptides from protein digestion are mass analyzed. The experimental peptides masses are compared with expected values of theoretical peptide masses from sequence database. The score is calculated and assigned. The score reflects the match between the theoretically and experimentally determined masses. Then, the protein can be identified as the most probability match. Several programs are

available to perform the peptide mass mapping search. There are MASCOT at www.matrixscience.com, profound at www.prowl.com, and MS-FIT at www.prospector.ucsf.edu/. The four important parameters for a peptide mass mapping search are peptide mass list, the cleavage agent, error tolerance (mass accuracy), and knowledge of peptide modifications. The peptide mass mapping is the easiest and fastest technique to identify proteins. However, not all the proteins using this technique can be identified. This can be caused by several reasons, for instance: insufficient of peptide information, post-translational modifications of the proteins, and query protein not exist in the protein database. Then, more specific information than molecular weight of peptides is required to identify protein, such as amino acid sequence using tandem MS and N-terminal-sequencing.

3) Protein Identification Using Tandem Mass Spectrometry

Tandem mass spectrometry can produce information specific to the amino acid sequence of peptide. The 'peptide sequence tag' is the approach using the peptide sequence information obtained by MS/MS. This approach allows a protein to be identified from a partial sequence and from mass differences between this sequence and the N-terminal and the C-terminal of the peptide resulting from cleavage of the protein. The other method that based on MS/MS but does not require interpretation in terms of the sequence of observed fragments is the method using the SEQUEST algorithm. An algorithm SEQUEST has been developed for the interpretation of fragmentation spectra from tandem MS. A correlation is searched between the fragmentation spectrum and peptide sequences contained in a database. First, the algorithm looks for all the peptides in the database that are the same mass as the precursor ions. Then, a measure of the similarity between the predicted fragments from the sequence obtained from the database and the fragments in the sample spectrum allows the most probable sequence to proposed. The sequence or even part of the sequence of a peptide is more specific than its molecular weight. There are many database search programs for use with mass spectrometric protein sequencing data (Table 1.8). The programs have been divided into three categories: programs that use amino acid sequences that must be produced by the interpreting the spectra, programs that use peptide molecular weight information, and programs that use the data from uninterpreted product ion spectra.

Table 1.8 Examples of the databases search programs and their Internet addresses that can be used for protein identification.

Program Name	Internet Address
Programs that use amino acid sequences for the search query	
FASTA	fasta.bioch.virginia.edu/fasta
BLAST	www.ncbi.nlm.nih.gov/blast/blast.cgi
MS-Edman	prospector.ucsf.edu/mshome3.2.html
Programs that use peptide molecular weights for the search query	
MS-Fit	prospector.ucsf.edu/mshome3.2.htm
MOWSE	srs.hgmp.mrc.ac.uk/cgi-bin/mowse
PeptideSearch	www.mann.embl-heidelberg.de/Services/PeptideSearch/PeptideSearchIntro.html
Programs that use the data from uninterpreted product ion spectra for the search query	
SEQUEST	thompson.mbt.washington.edu/sequest/
MS-Tag	prospector.ucsf.edu/mshome3.2.htm
PeptideSearch	www.mann.embl-heidelberg.de/Services/PeptideSearch/PeptideSearchIntro.html

1.7 Literature Reviews [5-7, 25-32]

There are many studies about protein purificaton and characterization of *Trimeresurus sp.* venoms. *Trimeresurus albolabris* venom contains a variety of platelet binding proteins. There are albolabrin[5], alborhagin[7], and alboaggregins A, B, and C[6, 25]. Albolabrin, which consists of 73 residues with six intramolecular disulfide bonds, was characterized by 1H-NMR technique.[26] It inhibits platelet aggregation. Alborhagin is metalloproteinase protein and induce platelet aggregation. Alboaggregins A, B, and C have the ability to stimulate platelet agglutination and aggregation. These alboaggregins were purified to homogeneity with ion exchange and hydrophobic HPLC. On SDS-PAGE under non-reducing conditions, the apparent molecular weights of alboaggregins A, B, and C were 52 kDa, 26 kDa, and 121 kDa, respectively.[25] In 1991, Weinstein et al. studied the characterization and amino acid sequence of 2 lethal peptides.[27-29] Two new lethal peptides, waglerin I and II, were purified from the venom of *Trimeresurus wagleri* by fast protein liquid chromatography and RP-HPLC, and sequenced using Edman degradation method.[27] These lethal toxins are neurotoxin and have molecular weight of 2504 and 2530 Da, respectively. In 1999, Zeng et al. studied an N-linked glycosylation in a novel C-lectin glycoprotein from the venom of *Trimeresurus stejnegeri*.[30] A C-lectin glycoprotein was observed by Edman degradation and liquid chromatography-electrospray mass spectrometry. The peptides obtained by trypsin cleavage were analyzed to confirm the amino acid sequence. The site of N-glycosylation which is the post-translation of C-lectin glycoprotein were identified by tandem MS. In 2002, Khow et al. studied a hemorrhagin as a metalloprotease in the venom of *Trimeresurus purpureomaculatus*.[31] A hemorrhagin was purified by gel filtration, ion exchange and affinity chromatography. The molecular weight determined by SDS-PAGE was 72 kDa. A hemorrhagin possesses hemorrhagic activity. In 2003, Nawarak et al. studied the protein components of snake venom in ten snake species from Elapidae and Viperidae families using multidimensional chromatographic methods such as RP-HPLC, SDS-PAGE, bioanalyzer lab-on-a-chip, and 2D-PAGE.[32] For the protein identification, proteins separated by 2D-PAGE were subsequently identified using peptide mass mapping method obtained from MALDI-MS and N-terminal sequences analyzed by Edman degradation.

In addition, several biological activities of the *Trimeresurus sp.* venoms were examined and compared.[3-4] Chanhome et al. studied the capacity of Thai green pit viper antivenom to neutralize the venoms of Thai *Trimeresurus* snakes and comparison of biological activities of these venoms.[4] The venoms from six species of *Trimeresurus sp.* in Thailand have been examined. They were *T. albolabris, T. macrops, T. popeiorum, T. hageni, T. purpureomaculatus*, and *T. kanburiensis.* The results shown that the *Trimeresurus sp.* venoms have lethal, hemorrhagic, proteolytic, phospholipase A, arginine ester, hydrolyze, and thrombin activities. The antivenom neutralized lethal and hemorrhagic activities of all these six snake venoms. *Trimeresurus purpureomaculatus* and *Trimeresurus popeiorum* obtained the strongest lethal toxicity and the strongest hemorrhagic activity, respectively. In contrast, the lethal toxicity and the hemorrhagic activity of *T. macrops* venom were lower than those of other six *Trimeresurus sp.* venoms. From database searching, there are 74 and 112 sequence proteins from *Trimeresurus sp.* venoms found in SWISS-PROT and TrEMBL database (www.expasy.org), respectively. However, there has been on report describing the purification and characterization of proteins in the venom of *Trimeresurus macrops.*