องค์ประกอบโปรตีนและฤทธิ์ทางชีวภาพของพิษงูจงอาง *Ophiophagus hannah*

นางสาวผกามาศ วงค์เตย์

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PROTEIN COMPOSITIONS AND BIOLOGICAL ACTIVITIES OF KING COBRA (*Ophiophagus hannah*) VENOM

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A Thesis Submitted in Partial Fulfillment of the Requirements
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อ. ที่ปรึกษา: รศ.ดร.พลกฤษณ์ แสงวณิช, 73 หน้า.

งานวิจัยนี้เกี่ยวข้องกับการเปรียบเทียบองค์ประกอบทางเคมีและฤทธิ์ทางชีวภาพของพิษงูจงอางที่มีอายุและถิ่นกำเนิดที่ต่างกัน การทดสอบฤทธิ์ทางชีวภาพได้เปรียบเทียบด้านความเป็นพิษต่อเซลล์มะเร็ง โดยพบว่าพิษงู KV2 มีค่าความเป็นพิษสูงกว่า พิษงู KV6 และ KV9 การเปรียบเทียบองค์ประกอบโปรตีนในพิษงูจงอางทั้ง 3 ตัวอย่าง ใช้อิเลคโตรฟอเรซิสทิศทางเดียว, อิเลคโตรฟอเรซิสแบบสองทิศทางและโครมาโทกราฟีของเหลวสมรรถนะสูงแบบรีเวอร์สเฟส พบว่า องค์ประกอบโปรตีนของพิษงูทั้ง 3 ตัวอย่างมีความคล้ายคลึงกัน แต่พิษงู KV2 มีความแตกต่างจากตัวอย่างอื่นอย่างเห็นได้ชัดโดยศึกษารูปแบบของโปรตีนด้วยวิธี โพลีอะคริลาไมด์เจลอิเล็คโตรฟอเรซิสแบบสภาพเสีย พบว่า KV2 มีแถบโปรตีนที่เข้มกว่า เช่นเดียวกับวิธีอิเลคโตรฟอเรซิสแบบสองทิศทาง พบว่า KV2 มีจุดโปรตีนที่มีมวลโมเลกุล ต่ำกว่า 14.4 กิโลดาลตัน มากกว่า KV6 และ KV9 และมีบางจุดโปรตีนที่ไม่พบในอีก 2 ตัวอย่าง คือ จุดโปรตีนที่ 2 และ 4 ซึ่งสามารถวิเคราะห์ชนิดของโปรตีนด้วยเทคนิคเปปไทด์แมสแมพพ์พบว่า เป็นโปรตีนในกลุ่มฟอสโฟไลเปส ส่วนในการศึกษารูปแบบโปรตีนด้วยเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูงแบบรีเวอร์สเฟส พบว่า ลำดับส่วนที่แยกได้จากพิษงู KV2 ใน acetronitrile ความเข้มข้นระหว่าง 31-50% มีความเข้มข้นโปรตีนสูงกว่าลำดับส่วนในช่วงเดียวกันของพิษงู KV6 และ KV9 จากผลทั้งหมดได้แสดงความแตกต่างทางด้านองค์ประกอบโปรตีนในพิษงูทั้ง 3 ตัวอย่าง ซึ่งเป็นสาเหตุความแตกต่างของความเป็นพิษของพิษงูแต่ละตัวอย่าง จากการแยกโปรตีนพิษงู KV2 ด้วยเทคนิคไอออนเอ็กเชนจ์และเจลฟิลเทรชันโครมาโทกราฟี ได้ ลำดับส่วน 4 ลำดับส่วน คือ 1.2.1, 1.2.2, 1.2.3 และ 1.2.4 จากการวิเคราะห์ลำดับกรดอะมิโนพบว่า ลำดับส่วน 1.2.2 มีลำดับกรดอะมิโนคล้ายคลึงกับ โปรตีนนาทริน (Natrin) จากพิษงูเห่า และ ลำดับส่วน 1.2.3 มีลำดับกรดอะมิโนคล้ายคลึงกับ โปรตีน โอฟานิน (ophanin) จากพิษงูจงอาง และนาทริน จากพิษงูเห่า

ภาควิชา.....เทคโนโลยีชีวภาพ..... ลายมือชื่อนิสิต.....ผกามาศ วงค์เตย์.....

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In this research involves comparison of protein composition and biological activities of different age and region of originate of three *Ophiophagus hannah* venoms. Biological activity was measured *via* cytotoxicity in cell line. KV2, crude venom showed higher toxicity than KV6 and KV9 venoms. Comparison of protein compositions of *O.hannah* venoms were performed using 1-D gel electrophoresis, 2D-gel electrophoresis and reverse-phase HPLC. Although protein compositions in all samples were similar, KV2 venom is very different from each other venom. The protein bands of KV2 in SDS-PAGE showed higher amount of all bands. 2DE gel of KV2 has many low molecular weight (14 kDa) spots. Spot No.2 and spot No.4 of KV2 are individual which are not found in KV6 and KV9. From peptides mass mapping by MALDI-TOF MS and amino acid sequence database searching, the amino acid sequence of protein spot 2 and spot 4 are similar to partial amino acid residue of phospholipase group from *O.hannah* venom. The RP-HPLC pattern of KV2 is different from other venoms. The eluted in range 31-50% ACN have high protein concentration than other venoms in same range. All of results indicated that variable protein composition were cause of difference in toxicity. The amino acid sequence of fraction 1.2.2 and 1.2.3, which were separated by ion exchange chromatography and gel filtration chromatography, were similar to partial amino acid residue of natrin and ophanin from *Naja atra* and *O.hannah* venom respectively.

Field of study:.....Biotechnology.....Student's signature:...Pakamas Wongtay.....
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LIST OF ABBREVIATIONS

µg	microgram
µl	microliter
µmol	micromolar
ACN	Acetonitrile
Bis	*N,N'*-methylenebisacrylamide
BSA	Bovine serum albumin
^{0}C	Degree Celsius
CCA	α-cyano-4-hydroxycinnamic acid
cm	centimeter
Da	Dalton
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
g	gram
h	hour
HPLC	High performance liquid chromatography
IAA	Iodoacetamide
IEF	Isoelectric focusing
kDA	Kilodalton
L	liter
M	Molar
mA	Milliampare
MALDI	Matrix Assisted Laser Desorption Ionisation
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation/Time of flight
MeOH	Methanol
min	Minute
mg	Milligram
mg/ml	Milligram per milliliter
ml	Milliliter
mm	Millimeter

mM	millimolar
MS	Mass spectrometry
M.w.	Molecular weight
m/z	Mass per charge
nm	Nanometer
PMF	Peptide mass fingerprint
rpm	Revolution per minute
rt	room temperature
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
T	Total acrylamide concentration (%)
TEMED	*N,N,N',N'*-tetramethylenebisacrylamide
TFA	Trifluoroacetic acid
TOF	Time of flight
Tris	Tris (hydroxymethyl)-aminoethane
V	Volt
v/v	Volume by volume
w/v	Weight by volume

CHAPTER I

INTRODUCTION

The snakebites are a serious health problem in many tropical and subtropical regions like Thailand, especially in agriculture and forestry has abundant snake. For instance approx. 3.3, 6.0 and 0.9 per 100,000 populations are estimated to die in Myanmar, Sri Lanka and Thailand respectively due to snake envenomation [1].

Ophiophagus hannah (King cobra) belongs to the Elapidae family, and is believed to be the world's largest poisonous snake. Elapidae snake venoms usually contain neurotoxins, phospholipaseA_2(PLA_2) enzymes and cardiotoxins (cytotoxins), especially neurotoxin with small molecules that can be absorbed very quickly into victims' blood circulation and cause death with short time. King Cobra venom has been chosen to study and analyze so as to provide information for further study and academic development.

From the report of the Queen Saovabha Memorial Institute shown different biological activity of crude venoms of *O.hannah* originating from different regions lead to the study of the composition of *O.hannah* venoms is focused on chemical substances of these venoms and comparison with the same species in different originated. The results will be used as basic information for venom analysis, medicinal production and medical treatment that have its own specific characteristics.

Objective of this research

In this work a comparative of biological activities and protein composition study was performed on the crude venom of *O.hannah* originating from different regions in Thailand.

CHAPTER II

THEORETICAL AND LITERATURE REVIEWS

2.1 Venomous snake [2]

Four families of snakes (*Atractaspididae*, *Colubridae*, *Elapidae*, and *Viperidae*) include species dangerous to humans, a total of roughly 450 species or about 19% of all snake species (Figure 2.1). In none of these families are all species lethal to humans, although all atractaspidine, elapid and viperid snakes are venomous. Generally speaking, the venoms most dangerous to human are those of snakes that specialize on warm-blooded prey. Because human physiology is similar to that of their prey, the venom reacts similarly in humans. But humans are also sensitive to snake venoms adapted to kill prey other than birds or mammals.

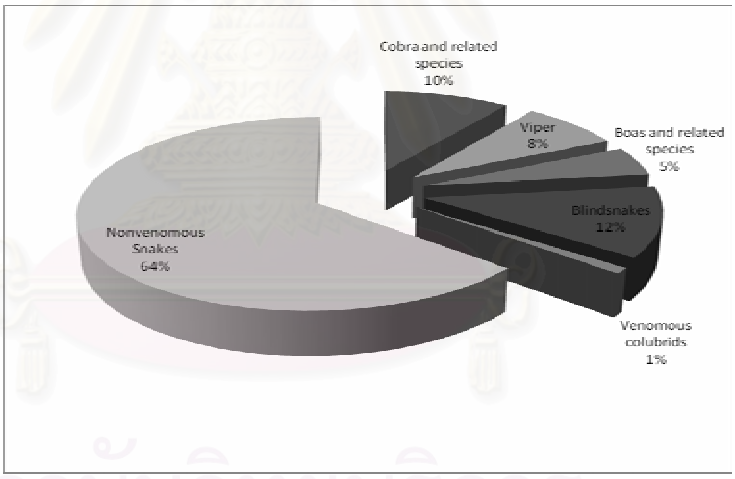


Figure 2.1 Proportions of venomous and nonvenomous snakes worldwide. Venomous snakes are here defined as those dangerous to humans.

Danger may vary with the volume of venom injected. Even mildly toxic venom is lethal if the snake injects enough of it. Conversely, a snake with highly toxic venom is not dangerous if it is small and incapable of breaking the skin, or if it does not bite in defense. Some species have venom delivery systems that do not permit them to deliver venom efficiently to large animals. Other species rarely come in contact with human.

In the large family Colubridae, about one-quarter of the species (over 600 species) have fangs, or at least enlarge and grooved maxillary teeth. But only four have cause human fatalities: the African boomslang (*Dispholidus typus*), Oriental tigersnake (*Rhabdophis tigrina*), African birdsnake (*Thelotornis kirtlandii*), and Peruvvian gray falseviper (*Tachymenis peruviana*). In the other three families, all species have fangs and should be considered potentially dangerous, though some are small or have venoms with weak effect on humans.

2.2 Snake Venom [2, 3]

In snakes, venom is an evolutionary adaptation to immobilize prey, secondarily used in defense. Venoms are highly toxic secretions produced in special oral glands. Because these oral glands are related to the salivary glands of other vertebrates, venom can be considered modified saliva. Venom immobilizes the prey when injected into its body, and in some cases initiates the digestive process by beginning the breakdown of the prey's tissues.

Each species has unique venom with different components and different amounts of toxic and nontoxic compounds. The more closely related two species of snakes, the more similar their venoms. It is probable that venoms and venom mechanisms evolved several times among snakes, increasing the diversity of venom chemistry and of the anatomy of the venom apparatus.

The chemistry of snake venoms is complicated. Venom are at least 90% protein (by dry weight), and most of the proteins in venoms are enzymes. About twenty-five different enzymes have been isolated from snake venoms, ten of which occur in the venoms of most snakes. Proteolytic enzyme, phospholipase, and hyaluronidases are the most common types. Proteolytic enzymes catalyze the breakdown of tissue proteins. Phospholipases, which occur in almost all snakes, vary from mildly toxic to highly destructive of musculature and nerves. The hyaluronidases dissolve intercellular materials and speed the spread of venom of vipers and pitvipers and promote the breakdown of a key structural component of connective tissue (the protein collagen); ribonucleases, deoxyribonucleases, nucleotidase, amino acid oxidases, lactate dehydrogenase, and acidic and basic phosphatases all disrupt normal cellular function, causing the collapse of cell metabolism, shock, and death.

Not all toxic chemical compounds in snake venom are enzymes. Polypeptide toxins, glycoprotein, and low-molecular-weight compounds are also present in mambas and colubrids. The roles of the other components of venom are largely unknown.

Every snake venom contains more than one toxin, and in combination the toxins have a more potent effect than the sum of their individual effects. In general, venoms are described as either neurotoxin (affecting the nervous system) or hemotoxin (affecting the circulartory system), although the venoms of many snakes contain both neurotoxin and hemotoxic components.

Venom components are broadly categorized by how they work to disrupt normal function.

Enzymes – found in all snake venoms – spur on physiologically disruptive or destructive processes.

Proteolysins – found mostly in viper and pit viper venom – dissolve cells and tissue at the bite site, causing local pain and swelling.

Cardiotoxins—associated mostly with elapids and vipers—have variable effect; some depolarize cardiac muscles and alter heart contraction, causing heart failure.

Hemorrhagins—occurring in the venom of vipers, pit vipers, and the king cobra—destroy capillary walls, causing hemorrhages near and distant from the bite.

Coagulation-retarding compounds—found in some elapids—prevent blood clotting.

Thrombosses—which some viper has—coagulate blood and foster clot formation throughout the circulatory system.

Hemolysins—which are in the venom of elapids, vipers and pit vipers—destroy red blood cells.

Cytolysins—components of viper and pit viper venom—destroy white blood cells.

Neurotoxins—found in elapids, viper, tropical rattlesnakes, and some North American Mojave rattlesnakes—block the transmission of nerve impulses to muscles, especially those associated with the diaphragm and breathing.

Venom composition can vary among individuals of the same species, and even in the same litter, but variation is greater among geographically different population. For example, Mojave rattlesnakes (*Crotalus scutulatus*) from eastern Arizona and

adjacent New Mexico have a special neurotoxin known as Majave toxin, but their venom lacks hemorrhagic and some proteolytic properties. Venom from Mojave rattlesnakes of central Arizona lacks the Mojave toxin but has strong hemorrhagic and proteolytic properties. Where the two populations overlap, individual rattlesnakes have venom with intermediate properties.

Venom toxicity may also vary over time in the same individual. Generally speaking, the venom of newborn and small juvenile snakes appears to be more potent than that of adult of same species. Also, a bite from a snake that has not fed recently, such as one that has just emerged from hibernation is more dangerous than that of one that has recently fed, because it has more venom to inject. Venom glands must replace venom lost with each strike-bite, and full replacement take time.

2.3 Mechanism of neurotoxin [4]

In general, neurotoxic venom affect to the neuromuscular junction which contains the membrane of the axon terminal or the motor neuron, also called presynaptic membrane, or presynaptic site; the motor end-plate of muscle cell, also called postsynaptic membrane, or postsynaptic site; and the space between them called synaptic cleft. Here the events of presynaptic membrane, synaptic cleft, and postsynaptic membrane is delineated:

2.3.1) Presynaptic membrane

The neurotransmitter, acetylcholine is synthesized in the cytosol of the axon terminal, and must be taken up by the synaptic vesicle. Acetylcholine release is triggered by the arrival of an action potential in the axon terminal. The depolarization of presynaptic membrane causes voltage-gated calcium channels in the active zones to open. The extracellular concentration of calcium is far higher than the intracellular concentration of calcium, so calcium ions will flood the axon terminal as long as the calcium channels are open. The resulting elevation in the internal calcium ion concentration is the signal that causes neurotransmitter to be released from synaptic vesicle. The vesicle releases their contents by a process called exocytosis. The membrane of the synaptic vesicle fuses to the presynaptic membrane at the active zone, allowing the contents of the vesicle to spill out the synaptic cleft.

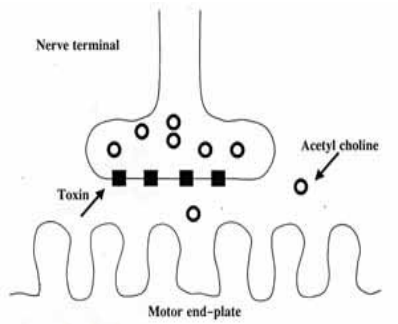


Figure 2.2 Pre-synaptic block

2.3.2) Postsynaptic membrane

Acetylcholine released in the synaptic cleft affect the muscle cell by binding to thousands of specific receptor proteins that are embedded in the motor endplate. The binding of neurotransmitter to the receptor is like inserting a key in a lock; this causes conformation changes in the protein. Receptor proteins also called Acetylcholine-gates sodium ion channels. They are membrane-spanning proteins consisting of five subunits that come together to form a pore between them. In the absence of neurotransmitter, the pore is closed. When neurotransmitter binds to a specific site on the extracellular region of the channel, the pore is open due to the conformation changes. The resulting elevation in the internal sodium ion concentration depolarizes the muscle cell from resting membrane potential, and eventually causes muscle to contract.

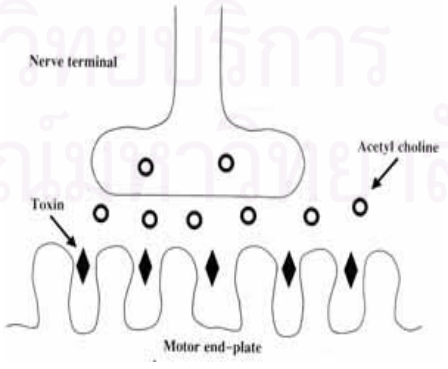


Figure 2.3 Post-synaptic block

2.4 King Cobra [2]

The king cobra or hamadryad (*Ophiophagus hannah*) is the world's largest venomous snake. These giant cobras are found throughout tropical Asia, from India eastward to Vietnam, southern China, and the Philippines, and southward through Malaysia and Indonesia. King cobras are not common anywhere, and are now considered rare in India. They are usually found in forests, often near streams, in mangrove swamps, and on tea and coffee plantations. They prefer habitats with heavy rainfull and dense undergrowth. King cobras readily ascend shrubs and low trees. Adults attain lengths of about 3 meters; only a few individuals grow larger. The largest hamadryads reside in the Malay Peninsula, and the maximum verified length is 5.58 meters.

Figure 2.4 King Cobra (*Ophiophagus hannah)*

The King cobra's generic name (*ophis*, snake; *phagein*, to eat) indicates its dietary preference: other snakes. Althrough it preys predominantly on nonvenomous species (including small pythons), king cobra also eat highly venomous snakes, such as cobra (*Naja*) and kraits (*Bungarus*). King cobras are cannibalistic and eat smaller individuals of their own species. They also prey on lizards, including large monitor lizards (*Varanus*). Active foragers, they hunt during the day.

King cobras mate in the late dry season, between January and March; the female lays a clutch of twenty to forty eggs in April, May, or June. *Ophiophagus hannah* is the only snake known to construct a nest in which to incubate its eggs. The nest consists of an untidy heap of leaves, twigs, and other vegetation, which the female scrapes together with her coils. The eggs are deposited and covered,

presumably benefiting from the heat generated by the decaying vegetation. Above them, the female hollows out a second chamber where she or her mate rests, guarding the eggs against predators. The other adult often remains nearby, but it is not known whether the male and female alternate guard duty. After sixty to ninety days, hatchlings 50 to 53 centimeters long emerge from the eggs. There is no evidence of any further parental care.

A favorite nest site for king cobras is the dead leaves that accumulate beneath clumps of bamboo. The nest looks no different from other heaps of dead bamboo leaves and is easily overlooked; the resident adult remains quietly coiled unless provoked. H. C. Smith recounts that “14 people accompanied by 7 dogs twice passed at different times within two yards of the nest, and yet the Hamadryad failed to show itself, and the nest remained undiscovered until! I prodded the heap of leaves with a small cane.”

Despite its massive size and fierce reputation, the king cobra is not an aggressive snake. Much more deliberate in its actions than the smaller, excitable cobra (*Naja*), it will quickly crawl away if given the opportunity. If cornered or attacked, however, it readily defends itself. Under attack, the king cobra coils, raises its forebody to a height approximating one-third of its body length—the world’s largest individual would have extended about 1.8 meters!—flattens its neck into a long narrow hood, and methodically strikes downward at its foe. Its venom is not as toxic as that of the common Indian cobra or krait, but because of its large venom glands it can inject a greater quantity of venom, enough to kill an Asian elephant. Fortunately, bites of humans are rare.

2.5 Separation Techniques

2.5.1 SDS-Polyacrylamide Gel Electrophoresis [5]

The separation of macromolecules in an electric field is called electrophoresis. The separation is according to charges of the molecules. The charge of a molecule can be either + or -, depending upon the pH of the buffer. If the electric filed is arranged in a way that the anions (-) are allowed to flow toward the anode (+), the system is known as an anionic system. Flow in the opposite direction, with cations (+) flowing to the cathode (-) is a cationic system.

Electrophoresis is probably the main technique for macromolecular separation in every biology laboratory. Most widely used electrophoresis technique for protein analysis is the polyacrylamide gel electrophoresis (PAGE). The PAGE can be performed under nondenaturing (native) or denaturing condition. The presence of polyacrylamide support provides sieving effect over the separation. Therefore, native PAGE separates the molecules by their sizes and charges. With sodium dodecyl sulfate included in the denaturing PAGE, the molecules are separated by their size only. The SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is therefore, a technique for the separation of polypeptides according to their sizes.

Sodium dodecyl sulfate (SDS, lauryl sulfate) is an anionic detergent. It binds to most soluble protein molecules in aqueous solutions over a wide pH range. All of the proteins become negatively charged by their attachment to the SDS anions. A constant amount of SDS is bound per unit mass of a typical polypeptide chain. The negative charges destroy (denature) most of the complex structure of proteins. In SDS-PAGE, the reducing agent is included for the reduction of intramolecular and intermolecular disulfide bonds. Under an electric field, the denatured polypeptides migrate through an acrylamide gel of known percentage towards the positive electrode (anode). The SDS-PAGE is thus an anionic system. Since the charge-to-mass ratio is nearly the same among SDS-denatured polypeptides, the final separation of proteins is dependent almost entirely on the differences in molecular weight (MW) of the polypeptides. In a gel of uniform density, the relative migration distance of a protein (R_f) is negatively proportional to the log of its MW. If the proteins of known MW are run simultaneously with the unknowns, the relationship between R_f and MW can be plotted, and the MWs of unknown proteins determined. The technique has become a standard means for molecular weight determination.

Polyacrylamide gels are formed from the polymerization of two compounds, acrylamide and *N*,*N*'-methylene-bisacrylamide (Bis). Bis is a cross-linking agent. The polymerization is initiated by the addition of ammonium persulfate along with *N*,*N*,*N*',*N*'-tetramethylethylenediamine (TEMED). The gels are neutral, hydrophilic, three-dimensional networks of long hydrocarbons cross-linked by methylene groups.

The separation of molecules within a gel is determined by the relative size of the pores formed within the gel. The pore size of a gel is determined by two factors, the total amount of acrylamide present (designated as %T) and the amount of cross-linker (%C). As the total amount of acrylamide increase or decrease in %C increase

the pore size. The percent gel to use depends on the molecular weight range of the protein to be separated. 5% gels are for proteins with MW ranging from 60,000 to 200,000 Daltons, 10% gels for a range of 16,000 to 70,000 Daltons and 15% gels for a range of 12,000 to 45,000 Daltons. Normally, the SDS-PAGE is used to determine the relative abundance of proteins in the samples and their approximate molecular weights. The purity of protein sample can be assessed.

2.5.2 Two-dimensional (2-D) electrophoresis [6]

Two-dimensional electrophoresis (2-D electrophoresis) is a powerful and widely used method for the analysis of complex protein mixtures extracted from cells, tissues, or other biological samples. This technique sorts proteins according to two independent properties in two discrete step: the first-dimension step, isoelectric focusing (IEF), separates proteins according to their isoelectric points (pI); the second-dimension step, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), separates protein according to their molecular weights (M_r,relative molecular weight). Each spot on the resulting two-dimensional array corresponds to a single protein species in the sample. Thousands of different proteins can thus be separated, and information such as the protein pI, the apparent molecular weight, and the amount of each protein is obtains.

2.5.3 Chromatography [7]

Chromatography is a process used to separate molecules on the basis of a chemical property such as molecular mass, charge, or solubility. The process uses a stationary phase and mobile phase, which may be either liquid or gas. The sample, which is often a complex mixture of molecules, is passed over or through the stationary phase by the flow of the mobile phase, which is called the eluting solvent or eluant. Molecules with different physical properties are partition differently between the stationary and mobile phases, resulting in a separation. Molecules that are strongly attracted to the stationary phase will be retarded or retained, relative to molecules that are not attracted strongly to the stationary phase. By choosing the appropriate stationary and mobile phases, it is possible to obtain effective separations of molecules that are only slightly different from each other.

2.5.3.1 Reversed-phase chromatography [8]

Reversed-phase high-performance liquid chromatography (RP-HPLC) involves the separation of molecules on the basis of hydrophobicity. The separation depends on the hydrophobic binding of the solute molecule from the mobile phase to the immobilized hydrophobic ligands attached to the stationary phase, i.e., the sorbent. A schematic diagram showing the binding of a peptide or a protein to a reversed-phase surface is shown in Fig. 2.5. The solute mixture is initially applied to the sorbent in the presence of aqueous buffer, and the solutes are eluted by the addition of organic solvent to the mobile phase. Elution can proceed either by isocratic conditions where the concentration of organic solvent is constant, or by gradient elution whereby the amount of organic solvent is increased over a period of time. The solutes are, therefore, eluted in order of increasing molecular hydrophobicity. RP-HPLC is a very powerful technique for the analysis of peptides and proteins because of a number of factors that include: (1) the excellent resolution that can be achieved under a wide range of chromatography conditions for very closely related molecules as well as structurally quite distinct molecules; (2) the experimental ease with which chromatographic selectivity can be manipulated through changes in mobile phase characteristics; (3) the generally high recoveries and, hence, high productivity; and (4) the excellent reproducibility of repetitive separations carried out over a long period of tome, which is caused partly by the stability of the sorbent materials under a wide rang of mobile phase condition. However, RP-HPLC can cause the irreversible denaturation of protein samples thereby reducing the potential recovery of the material in a biologically active form.

The RP-HPLC experimental system for the analysis of peptides and proteins usually consists of an *n*-alkylsilica-based sorbent from which the solutes are eluted with gradients of increasing concentrations of organic solvent such as acetonitrile containing an ionic modifier such as trifluoroacetic acid (TFA). Complex mixtures of peptides and proteins can be routinely separated and low picomolar- femtomolar amounts of material can be collected for further characterization. Separations can be easily manipulated by changing the gradient slope, the operating temperature, the ionic modifier, or the organic solvent composition.

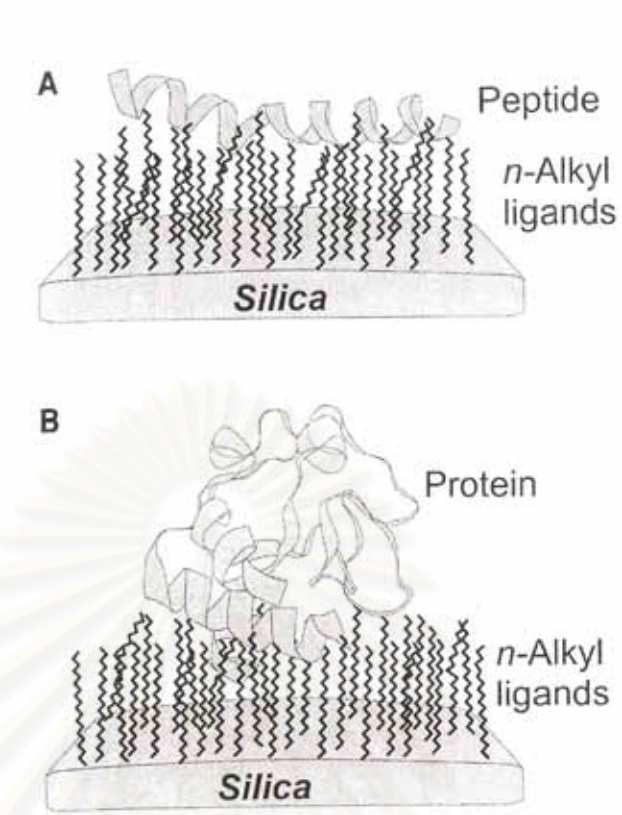


Figure 2.5 Schematic representation of the binding of (A) a peptide and (B) a protein, to an RP-HPLC silica-based sorbent.

The extensive use of RP-HPLC for the purification of peptides, small polypeptides with molecular weights up to 10,000, and related compounds of pharmaceutical interest has not been replicated to the same extent for larger polypeptides (molecular mass > 10 kDa) and globular proteins. The combination of the traditionally used acidic buffering systems and the hydrophobicity of the *n*-alkylsilica supports which can result in low mass yields or the loss of biological activity of larger polypeptides and proteins have often discouraged practitioner from using RP-HPLC methods for large-scale protein separations. The loss of enzymatic activity, the formation of multiple peaks for compositionally pure samples, and poor yields of protein can all be attributed to the denaturation of protein solutes during the separation process using RP-HPLC.

2.6 Identification Techniques

2.6.1 Mass Spectrometry [9]

Mass spectrometry is an analytic technique that measures the masses of individual molecules and atoms. As conceptualized in Figure2.6, the first essential step in mass spectrometry analysis is to convert the analyte molecules into gas-phase ionic species because one can experimentally manipulate the motion of ions, and to detect them (which are not possible with neutral species). The excess energy transferred to the molecule during the ionization event leads to fragmentation. Next, a mass analyzer separates these molecular ions and their charged fragments according to their *m/z* (mass/charge) ratio. The ions current due to these mass separated ions is detected by a suitable detector and displayed in the form of a mass spectrum. To enable the ions to move freely in space without colliding or interacting with other species, each of these steps is carried out under high vacuum (10^{-4} – 10^{-8} torr).

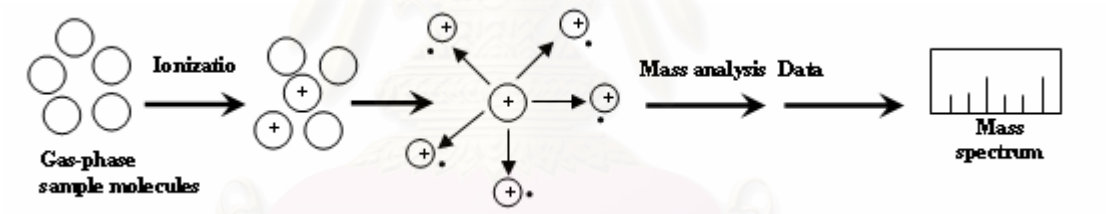


Figure 2.6 Basic concept of mass spectrometry analysis

Thus, a mass spectrometry consists of several essential functional units; they are depicted in Figure 2.7 in the form of a block diagram. These units are

- An inlet system to transfer a sample to the ion source
- A vacuum system to maintain a very low pressure in the mass spectrometer
- An ion source to convert the neutral sample molecule into gas-phase ions.
- A mass analyzer to separate and mass-analyze ionic species
- A detector to measure the relative abundance of the mass-resolved ions
- Electronics to control the operation of various units
- A data system to record, process, store, and display the data

The overall analytic capability of a mass spectrometry system depends on the combined performance of these individual units. Several ionization techniques have emerged, each with special purpose.

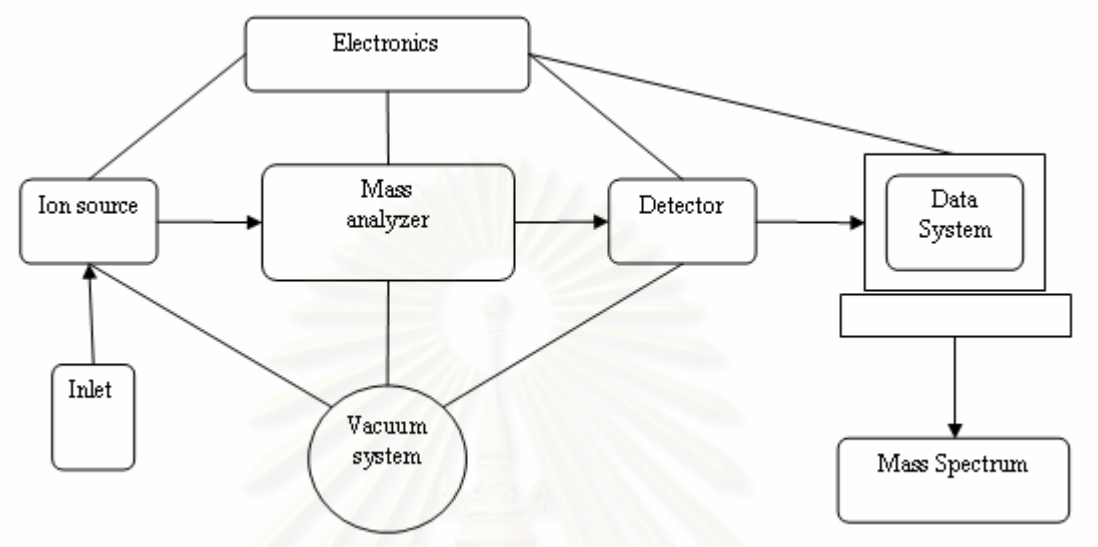


Figure 2.7 Basic components of a mass spectrometer

2.6.1.1 The ion source [10]

The ion source is the region of mass spectrometer where the gas phase ions are produced from sample molecule, the area of ion production. Various procedures are used to from gas phase ions from molecules, depending on the physical state of the analytes. Choices are available as to the types of ions produced (positively and negatively charged, radical cations and protonated molecules, etc.) and the degree, which these ions are internally excited. Internally excited molecular ions dissociate to produce fragment ions, which may reveal detail molecule structure. On the other hand, an intact molecular ion (such as the protonated molecule or radical anion) provides information on molecular weight.

The two ionization methods most commonly used to volatized and ionize the proteins or peptides for mass spectrometric analysis are:

1) Matrix-Assisted Laser Desorption/ionization (MALDI)
2) Electrospray ionization (ESI)

1.) Matrix-Assisted Laser Desorption/ionization (MALDI)

Matrix-assisted laser desorption/ionization is a technique that could redily ionize biomolecules in a very sensitive manner. MADLI is a pulse ionization technique which utilizes the energy from a laser to desorb and ionize the analyte molecules in the presence of a light absorbing matrix. As this is a plused ionization technique, generating packets of ion with each laser pulse, a pulsed analyzer is typically required for separation and resolution of the ions. Consequently, MALDI is routinely coupled with a time-of-flight analyzer (TOF)

Mechanism of matrix-assisted laser desorption ionization [11]

The matrix is believed to serve three major functions:

(1) Absorption of energy from the laser light. The matrix molecules absorb the energy from the laser light and transfer it into excitation energy of the solid system. Thereby an instantaneous phase transition of a small volume (some molecular layer) of the sample to gases species is induced. In this way the analyte molecules are desorbed together with matrix molecules, with limited internal excitation.

(2) Isolation of the biomolecules from each other. The biomolecules are incorporated in a large excess of matrix molecules, strong intermolecular forces are thereby reduced (matrix isolation). Incorporation of analyte into matrix crystal taking place upon evaporation of the solvents froms an essential prerequisite for successful MALDI analysis, it moreover provides an in-situ cleaning of the sample and is the reason for a high tolerance against contaminants.

(3) Ionization of the biomolecules. An active role of the matrix in the ionization of the analyte molecules by photoexcitation or photoionization of matrix molecules, followed by proton transfer to the analyte molecules is likely though not proven unequivocally to date.

A wide range of matrices for bio-mass spectrometry applications have been adopted for use with UV laser (the typical wavelength is 337 nm) such as α-cyano-4-hydroxycinnamic acid (CCA) for peptide analysis and protein digests.

2.) Electrospray ionization (ESI)

Electrospray ionization (ESI) is an atmospheric pressure ionization technique applicable to a wide range of compounds that are present in liquid matrices. The emergence of ESI represents a significant advance in the capabilities of mass spectrometry for the characterization of large biomolecules. It has also become the most widely used interface to combine HPLC with mass spectrometry. The wide popularity of ESI in these fields is due to its continous-flow operation, tolerance to different types of solvent, acceptance of wide solvent flow rates, and ability to generate intact multiple charged ions of fragile chemical and biochemical species. ESI uses a novel concept first introduced by Dole in 1968 for the generation of gas phase ions from electrically charged liquid droplet, which are produced by electrospraying the solution of an analyte at atmospheric pressure.

2.6.1.2 Mass Analyzer [9]

Ions can be separated on the basis of their mass-to-charge ratios using electric or magnetic fields arranged so as to spread them in time or space. The two mass analyzers most commonly used for mass spectrometric analysis are:

1) Time-of-flight (TOF)
2) Quadrupole (Q)

1) Time-of-flight (TOF)

A time-of-flight (TOF) mass spectrometer is one of simplest mass-analyzing devices. Since the 1990s, it has reestablished itself as a mainstream technique and is becommong increasingly useful in meeting the demands of contemporary research in biomedical sciences.

2) Quadrupole (Q)

The quadrupole mass spectrometer is the most widely used type of mass spectrometer. The mass separation in this instrument is accomplished solely by using electric fields. Quadrupoles are dynamic mass analyzer. Which means that ion

trajectories are controlled by a set of time-dependent forces that are generated by applying direct current (dc) and radiofrequency (rf) potentials to a set of electrodes.

2.6.1.3 Detector

After ions were separated by a mass analyzer, they reach at a detector for the detection of their mass and abundance. A detector measures the electric current in proportion with the number of ions striking it. Sensitivity, accuracy, resolution, and response time are the most important characteristics of any detector. These are major instruments for ion detection: Photomultiplier, Secondary electron multiplier and Post acceleration detector.

2.7 Protein identification using data from mass spectrometry [12]

Peptide mapping or peptide mass fingerprinting (PMF) refers to the identification of proteins using data from intact peptide masses (Figure 2.8). The principle of the technique is that each protein can be uniquely identified by the masses of its constituent peptides, this unique signature being known at the peptide mass fingerprint. Algorithms allowing database searching on the basis of peptide mass data were developed simultaneously by several groups in the early 1990s and have been implemented in a number of software packages, many of which are available over the internet (Table2.1). PMF involves the following steps:

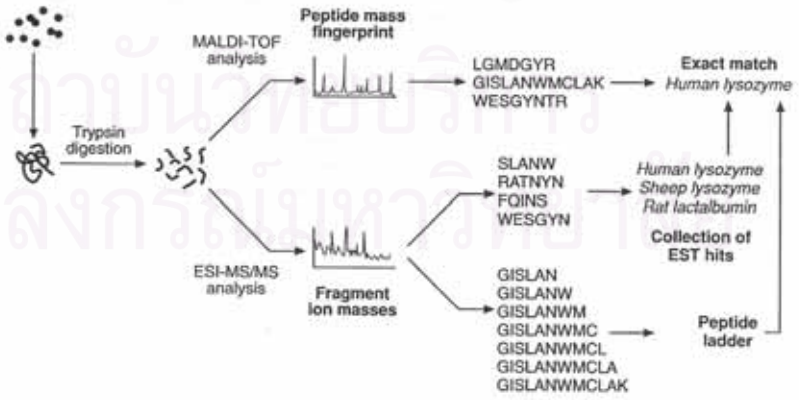


Figure 2.8 Protein identification by mass spectrometry. In a typical strategy, digested peptides are analyzed by MALDI-TOF MS in order to determine the masses of intact peptides.

1) The sample of interest should comprise a single protein or a simple mixture, e.g. an individual spot from a 2D-gel or a single LC fraction. The sample is digested with a specific cleavage reagent, usually trypsin.
2) The masses of the peptides are determined, e.g. by MALDI-TOF-MS
3) The experimenter chooses one or more protein sequence databases to be used for correlative searching. Examples include the SWISS-PROT and TrEMBL protein databases
4) The algorithm carries out a virtual digest of each protein in the sequence database using the same cleavage specificity as trypsin (or whichever other reagent has been used experimentally) and then calculates theoretical peptide masses for each protein.
5) The algorithm attempts to correlate the theoretical peptide masses with the experimentally determined ones.
6) Proteins in the database are ranked in order of best correlation, usually with a significance threshold based on a minimum number of peptides matched.

Table 2.1 Computer programs, most available over the internet that can be used to interpret or analyze MS data.

Program	Applications	URL
CIDentify	Search	http://www.immunex.com
Mascot	PMF, Tag, MS/MS	http://www.matrixscience.com
MassSearch	PMF	http://cbrg.inf.ethz.ch/Server/MassSearch.html
Mowse	PMF	http://www.hgmp.mrc.ac.uk/Bioinformatics/Webapp/mowse
MS BLAST	Search	http://dove.embl-heidelberg.de/Blast2/msblast.html
MS-FIT	PMF	http://prospector.ucsf.edu
MS-Seq	Tag	http://prospector.ucsf.edu
MS-Tag	MS/MS	http://prospector.ucsf.edu
PepFrag	MS/MS	http://prowl.rockefeller.edu/PROWL/prowl.html
PepMAPPER	PMF	http://wolf.bms.umist.ac.uk/mapper
PepSea	PMF, Tag	http://195.41.108.38/Pepsealntro.html
Peptldent	PMF	http://www.expasy.ch/tools/peptident.html
PeptideSearch	PMF, Tag	http://www.narrador.embl-heidelberg de/GroupPages/ PageLink/peptidesearchpage.html
ProFound	PMF	http://prowl.rockefeller.edu/PROWL/prowl.html
Sequest	MS/MS	http://fields.scripps.edu/sequest
Sonars MS/MS	MS/MS	http://www.proteometrics.com
Tagldent	Tag	http://www.expasy.ch/tools/tagident.html

2.8 Literature review

More than 72 proteins [13] have been purified and characterized from *O.hannah* venoms and their amino acid sequences have been determined. The majority of the venom components are neurotoxins, cardiotoxin and phospholipaseA_2.

The α-neurotoxins can bind much more specifically and tightly to nicotinic acetylcholine receptor (nAChR) at skeletal muscle neuromuscular junction, producing blockade of neuromuscular transmission at the postsynaptic membrane, leading animals to paralysis and quick death [14]. The α-neurotoxins are usually classified into short-chain neurotoxins (60-62 residues and four conversed disulfide bridges) and long-chain neurotoxins (65-72 residues and five conversed disulfide bridges) [15]. Several α-neurotoxins have been purified from *O.hannah* venom and well characterized, including nineteen long-chain neurotoxins, ten short-chain neurotoxins, five weak toxins and three muscarinic toxins (MTXs) which, belong to the neurotoxin group, members of which interfere in the transmission of nervous impulses by selectively binding to particular receptors in the nerve or muscular membrane. The major members of the cardiotoxin group exhibit quite different pharmacological properties, such as cytolysis, haemolysis and heart failure. Several cardiotoxins have been purified from *O.hannah* venom and well characterized, including six cardiotoxins

Snake venom phospholipaseA_2 (PLA_2) enzymes are low molecular weight (13-15 kDa) protein and exhibit a wide variety of pharmacological effect such as hemolysis, platelet aggregation inhibition, neurotoxicity and myonecrosis. PhospholipaseA_2 have been purified from *O.hannah* venom and well characterized, including three phospholipaseA_2.

Thioredoxin, ophanin, venom chymotrypsin inhibitor, L-amino acid oxidase and ohanin are also reported. (Table 2.2)

Venoms are exquisitely complex, composed of as many as a hundred different peptides, enzymes and toxins. Not only is the venom of every snake species sifferent, there are also subtle variations within each species. There are differences between venoms of juveniles and adults, and even among different geographic regions [34]. There differences may be due to different evolutionary pressures, like different ancestry, prey and environments. The variations between venom types and the number of venomous snakes worldwide create a rich molecular hunting ground for

researchers, such as seeking to design new drugs. The advantage of these venom derived toxins is that they seem to act only on certain types of cells. Chemotherapy and many other drug treatments do not distinguish between tumor cells and otherhealthy cells, causing debilitating side effects. But natural toxins have evolved to impact very specific targets. Such as a protein in venom gives valuable insight as to why a group of new heart medications calles integrin [35] antagonists that are designed to prevent blood clots from forming and causing heart attack during. In addition, Ancrod [36] was derived from the venom of a pit viper snake. That venom was utilized as an anticoagulant, helping blood flow more freely through vessels. Coumadin [37], also known by the generic name Warfarin, has been on the market for almost 60 years. It thins the blood, reducing the likelihood of blood clots that can block arteries and contribute to stroke or heart attack.

Table 2.2 Summary previous proteins found in *O.hannah* venom

Protein	**Amino acid/ Molecular weight**
Long-chain neurotoxin	
Neurotoxin 1 [16]	73/8,106
Neurotoxin b [16]	73/8,053
CM-9 [17]	73/7,957
Oh4 [18]	72/8,014
CM-11 [19]	93/10,210
Oh5 [20]	72/8,040
Oh-6A/6B [21]	91/9,857
Oh31 [22]	94/10,154
Oh34 [22]	90/9,867
Oh37 [22]	91/9,853
Oh55 [22]	93/10,210
Oh56 [22]	99/9,918
Oh57 [22]	91/10,058
LNTX1 [23]	94/10,536
LNTX8 [23]	94/10,509
LNTX22 [23]	93/10,381

Table 2.2 Summary previous proteins found in *O.hannah* venom (*continued*)

Protein	Amino acid/ Molecular weight
Long-chain neurotoxin	
LNTX28 [23]	91/9,885
LNTX37 [23]	94/10,563
LNTX2 [24]	93/10,244
Short-chain neurotoxin	
Oh9-1 [25]	57/6,516
Oh5 (precursor) [22]	78/8,984
Oh26 [22]	78/8,756
Oh32 [22]	77/8,601
Oh35 [22]	86/9,564
Oh46 [22]	78/8,893
SNTX6 [23]	78/8,849
SNTX11 [23]	78/8,931
SNTX14 [23]	78/8,909
SNTX26 [23]	78/8,895
Weak toxin	
DE-1 [26]	60/6,812
OH72 [22]	81/9,067
WNTX33 [23]	83/9,283
WNTX34 [23]	86/9,815
WTX DE-1 [24]	83/9,387
Muscarinic toxin	
MTX6 [23]	86/9,412
MTX38 [23]	86/9,684
Muscarinic toxin [24]	86/9,839
Cardiotoxin	
Oh27 [23]	84/9,311
CTX9 [24]	84/9,380
CTX14 [24]	84/9,256

Table 2.2 Summary previous proteins found in *O.hannah* venom (*continued*)

Protein	**Amino acid/ Molecular weight**
Cardiotoxin	
CTX15 [24]	84/9,352
CTX21 [24]	84/9,255
CTX23 [24]	84/9,296
PhospholipaseA_2	
PhospholipaseA_2, acidic 1 [27]	151/16,444
PhospholipaseA_2 [28]	146/15,901
PhospholipaseA_2, acidic 2 [29]	152/16,641
Other proteins	
Thioredoxin [28]	105/12,003
Ophanin [30]	239/26,869
Venom chymotrypsin inhibitor [31]	58/6,499
L-amino acid oxidase [32]	19/2,298
Ohanin [33]	190/21,174

CHAPTER III

EXPERIMENTAL

3.1 Snake venoms

Three Crude *Ophiophagus hannah* venoms (KV2, KV6 and KV9) were collected from different regions of Thailand and keep at 4°C (Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok Thailand). Three samples were prepared: KV2 collected from snake from Petchabun Province (3 year olds snake), KV6 collected from Southern of Thailand. (5 year olds snake) and KV9 collected from Southern of Thailand. (For this sample, there are no data on the age of snakes) Three *O.hannah* were cultured in Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok Thailand.

Fractions 1.2.1, 1.2.2, 1.2.3 and 1.2.4 were obtained from the Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok Thailand. The KV2 venom (100 mg) was fractionated on column Q-Sepharose column. The column was washed with 20 M Tris-HCl pH 7.4 and eluted with linear gradient of 0-0.5 M NaCl. The protein obsorption was monitored at 280 nm (Figure 1D of Appendix D) Fraction containing neurotoxicity (fraction 1) was fractioned on cellulofine and equilibrated with 25 mM Tris-HCl and 0.05 NaCl. The protein obsorption was monitored at 280 nm (Figure 2D of Appendix D). Fraction containing neurotoxicity (fraction 1.2) was fractioned on SP-Sepharose and eluted with linear gradient of NaCl (0-0.2 M NaCl in 50 mM Phosphate buffer pH 6.25). The protein obsorption was monitored at 280 nm (Figure 3D of Appendix D)

3.2 Chemicals and Reagents

Acetic acid: Merck Ag Darmstadt, Germany

Acetonitrile: Merck Ag Darmstadt, Germany

Acrylamide PAGE: Plusone pharmacia biotech, Sweden

Acrylamide: Amersham pharmacia biotech, Sweden

Ammonium persulfate: Plusone pharmacia biotech, Sweden

Bromophenol Blue: USB, U.S.A

Bovine serum albumin: Sigma, St. Louis, MO, U.S.A

Coomassie brilliant blue: USB, U.S.A

Ethanol: Merck Ag Darmstadt, Germany

Formic acid: Merck Ag Darmstadt, Germany

Glycine: USB, U.S.A

Methanol: Merck Ag Darmstadt, Germany

N, N'- methylenebisacrylamide; Plusone pharmacia biotech, Sweden

Phosphoric acid: J.T. Baker, U.S.A

SDS (Sodium Dodecyl Sulfate): USB, U.S.A

Trichloroacetic acid: BHD, VWR International Ltd., England

Trifluoroacetic acid: Fluka, Germany

Tris: USB, U.S.A

α-Cyano-4-hydroxycinnamic acid: Sigma Chemical, U.S.A

3.3 Apparatus and Instruments

Autopipette: Pipetman, Gilson, France

Freeze dryer: Labconco, U.S.A

Immobilized pH gradient strips (IPG strips) pH3-10 and PH 4-7: Amersham pharmacia biotech

Laboratory centrifuge; Biofuge pico Heraeus, Kendro, Germany

Matrix Assisted Laser Desorption Ionization-Time of Flight Mass spectrometer: MICROFLEX, Bruker Daltonics, Inc., USA

Microcentrifuge: Biofuge pico Heraeus, Kendro, Germany

Microcentrifuge tube: Axygen Scientific, U.S.A

Micropipettes adjustable from 2 to 1,000 µl: Gilson, France

Macrotrap desalting cartridge: Michrom Bioresource

Multiphor™ II Electrophoresis Unit: Amersham Pharmacia Biotech, Uppsala Sweden.

pH meter G-560E: Denver Instrument, U.S.A

Pipette tips: Bioline, U.S.A

Power Supply: EPS 301, Amersham pharmacia biotech, Sweden

Power Supply: EPS 3501 XL, Amersham pharmacia biotech, Sweden

SDS-PAGE: Hoefer^{TM} mini VE (minivertical), 8×9 cm gels, Amersham pharmacia biotech, Sweden

Spectrophotometer: TECAN, Austria

Speed vacuum centrifuge: Heto-Holten, Denmark

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

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

Water Bath Shaking: Memmert, Germany

3.4 Methods

3.4.1 Determination of protein by Bradford method

The crude venoms (1 mg) were dissolved with distilled water 1 ml. Protein solution were pipetted (maximum 10 µl) into 96 well platees, then added with the Bradford working solution (Appendix A) 100 µl to make a total volume of 110 µl and mixed thoroughly. The reaction mixture was read for optical density at 595 nm wavelength after 2 minutes but before 1 hour. For the quantitative of the protein in this research, micro assay method using a microplate reader has been used. Protein levels were evaluated against standard protein, bovine serum albumin.

3.4.2 Analysis of proteins in snake venom by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Polyaclyamide gel at 15% was used as separating gel and 3% of the gel was used as stacking gel. Tris glycine (25 mM Tris, 192 mM glycine) buffer pH 8.3 containing 0.25% w/v SDS was used as electrode buffer. Sample to be analyzed, was dissolved in tris buffer, containing 60 mM Tris, 2% w/v SDS, 25% v/v glycerol, 14.4 mM 2-mrcaptoethanol, and 0.1% w/v bromophenol blue, and boiled for 5 minutes prior to application to the gel. The electrophoresis was carried out at constant current of 15 mA, on a Mini-Protein (Hoefer mini VE) from cathode toward anode. When the electrophoresis was completed, the gel was stained with Coomassie blue R-250. The apparent molecular masses of protein were estimated using the low molecular weight marker kit which contain known protein standard, phosphorylase b, bovine serum albumin, egg albumin, carbonic anhydrase, soybean trypsin inhibitor, and

lactalbumin, with apparent molecular masses of 97.4, 66.2, 45.0, 31.0, 21.5 and 14.4 kDa, respectively.

3.4.3 Analysis of proteins in snake venom by Two-Dimensional Electrophoresis [6]

The 2-D electrophoresis separation was performed as described in the manual of 2-D Electrophoresis using immobilized pH gradients. For the first dimension IEF, the venom stock solution was mixed with the lysis buffer (8 M urea, 4% CHAPS, 2% IPG buffer pH 4-7). Then, this sample solution was mixed with rehydration solution (8 M urea, 2% CHAPS, , 2% IPG buffer pH 3-10 or 4-7, 0.2% DTT, 0.002% bromophenol blue). The rehydration solution with a sample solution was applied on pH 4-7 IPG strips (length 7 cm). The IPG strip was positoned on the Immobiline Drystrip Reswelling Tray and allowed to rehydrate overnight at room temperature. The rehydrated IPG strip was removed from the reswelling tray and transferred to the Immobiline Drystrip aligner. The IEF electrophoresis was carried out at 20 °C on the Multiphor II Electrophoresis Unit with Immobiline Drystrip Kit (Amersham Pharmacia Biotech). The running condition of IEF was follows: 200V for 0.001 kVh, 3500V for 2800 kVh and 3500V for 5200 kVh. After IEF, the IPG strip was equilibrated for 15 minutes in the equilibration solution (50mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 1% DTT, 0.002% bromophenol blue), then equilibrated for 15 minutes in the equilibration solution that replaced DTT with 2.5% iodoacetamide. Each IPG strip was embedded on top of the 2-D gel and covered with 0.5% agarose. Second dimentional SDS-PAGE was carried out on 20%T acrylamide gel. The running condition was as follow: 10 mA/gel for 15 minute and 20 mA/gel until bromophenol blue dye front reached the bottom of the gel. Lastly, the each gel was strained by coomassie brilliant blue G stained.

3.4.4 Coomassie Staining

The staining procedure consists of the five steps and all steps using the orbital shaker. After finishing the electrophoresis, the gel was removed and then placed in the staining plastic box. The first step was the gel fixing in 12% (w/v) TCA for 1 hour. The staining step was the second step took overnight with 160 ml of staining solution

(0.1% w/v Coomassie G-250 (Sigma, USA) in 2% H_3PO_4, 10% w/v ammonium sulfate) plus 40 ml of methanol (add during staining). The third step was the gel washing in 0.1 M Tris- H_3PO_4buffer at 6.5 for 3 min. Then, the gel was rinsed in 20 % v/v aqueous method for 1 min. Finally, the stabilizing step was the preserving gel in 20% aqueous ammonium sulfate.

3.4.5 In-gel digestion [38]

Protein bands or spots were manually excised from the polyacrylamide gels and transferred to a microcentrifuge tube. The gel pieces were washed three times with 100 µl of 100 mM ammonium bicarbonate/acetonitrile (1:1, vol/vol) and incubate with occasional vortexing for 30 min, depending on the staining intensity. After that add 500 µl of neat acetonitrile and incubate at room temperature with occasional vortexing, until gel pieces become white and shrink and then remove acetronitrile and dry gel pieces for saturate gel pieces process. When gel pieces were dried, add enough trypsin to cover the dry gel pieces and leave it in an ice bucket or a fridge. After 30 min, check if all solution was absorbed and add more trypsin, if necessary. Gel pieces should be completely covered with trypsin. Leave gel pieces for another 90 min to saturate them with trypsin and then add 10-20 µl of ammonium bicarbonate buffer to cover the gel pieces and keep them wet during enzymatic cleavage. In digestion process, place tubes with gel pieces into an air circulation thermostat and incubate samples overnight at 37 °C. The peptides were extracted with extraction buffer (1:2 (vol/vol) 5% formic acid/acetonitrile) 100 µl and incubate for 15 min at 37 °C in a shaker and then collected supernatant. For samples with much larger (or smaller) volume of gel matrix, add the extraction buffer such that the approximate ration of 1:2 between volumes of the digest and extraction is achieved. These solutions were dried and kept at -20°C for further analysis.

3.4.6 Analysis of proteins in snake venom by High performance Liquids Chromatography (HPLC)

Analysis of venoms was performed using a reverse-phase HPLC column (4.6 x 250 mm, Nucleosil7C18) attached to a L-4250, UV-VIS detector and L-7100 pump with a 20 µL sample injection loop. The separation was done by the gradient elution

of two solvent systems. The two solvents used were HPLC-grade acetonitrile (solution A) and 0.1% TFA DI-water (solution B). Both solution A and B were filtered through 0.45 µm pore size filter paper. Before sample injection, the column was equilibrated with solution B by flow the solution through the column at flow rate of 1 ml/min for at least 30 min. *O. hannah* venom (1 mg) was dissolved in 0.1 ml of distilled water. The solution was filtered through Millipore filter paper (0.45 µm pore size). The column was maintained at room temperature. A linear gradient of 0% ACN to 100% ACN (0-70% mobile phase B) in 60 min running times at a flow rate at 1 ml/min was used. Detection was done by UV detector set at 280 nm. The fractions collected were kept at 4 °C until need.

3.4.7 Protein Identification Methods

Mass spectra of protein and peptide were acquired using a MALDI/TOF mass spectrometer (MICROFLEX, Bruker, USA) operating in linear and reflecton modes. The dried samples were dissolved in 50% ACN/ 0.1% TFA and vortexed. The dried droplet method was used for MALDI-MS sample preparation. Cyano-4 hydroxycinnamic acid (CCA) was used as the matrix. For proteins, 1 µl of a sample solution was mixed with 1 µl of a matrix solution (saturated CCA in 50% ACN/ 0.1% TFA). For peptide mixtures, 1 µl of sample solution was mixed with 5 µl of matrix solution. Then, 1 µl of the mixture solution was spotted on MADLI target and allowed to dry at room temperature. The MALDI protein mass spectra were performed using a linear mode and calibrated using myoglobin (average mass 16,951 Da) and insulin (average mass 5,734.56 Da). MALDI peptide mass spectra were performed in reflectron mode and internally calibrated using Neurotensin(exact mass 1690.93 Da) and ACTH (exact mass 2,465.98Da). Peptide mass mapping obtained from each digested protein were searched against protein database via the MASCOT program (www.matrixscience.com). Search parameters allowed for carbamidomethylation of cysteine, one missed trypsin cleavages, and 100 ppm mass accuracy. Peptides in the mass range of 800-3,500 Da were selected for database search.

3.4.8 Antioxidant activity

Prepared DPPH solution at 200 µM in absolute ethanol (freshly prepare) and kept in dark by covered with aluminium foil until use. 1 mg/ml of Crude sample and vitamin E (positive control) was the stock. The appropriate concentration of sample were added in vary concentration such as 200, 100, 80, 60, 40, 20, 0 µg/ml. Added 800 µl of DPPH into tube. The mixture was shaken and incubated at 37°C for 30 min. The absorbance was measured at 517 nm.

3.4.9 Measurement of cytotoxicity

Cytotoxicity of *O.hannah* venoms were tested against cell line by MTT assay, analyzed at Antibody Production, The Institude of Biotechnology and Genetic Engineering, Chulalongkorn University.

3.4.10 Tandem MS

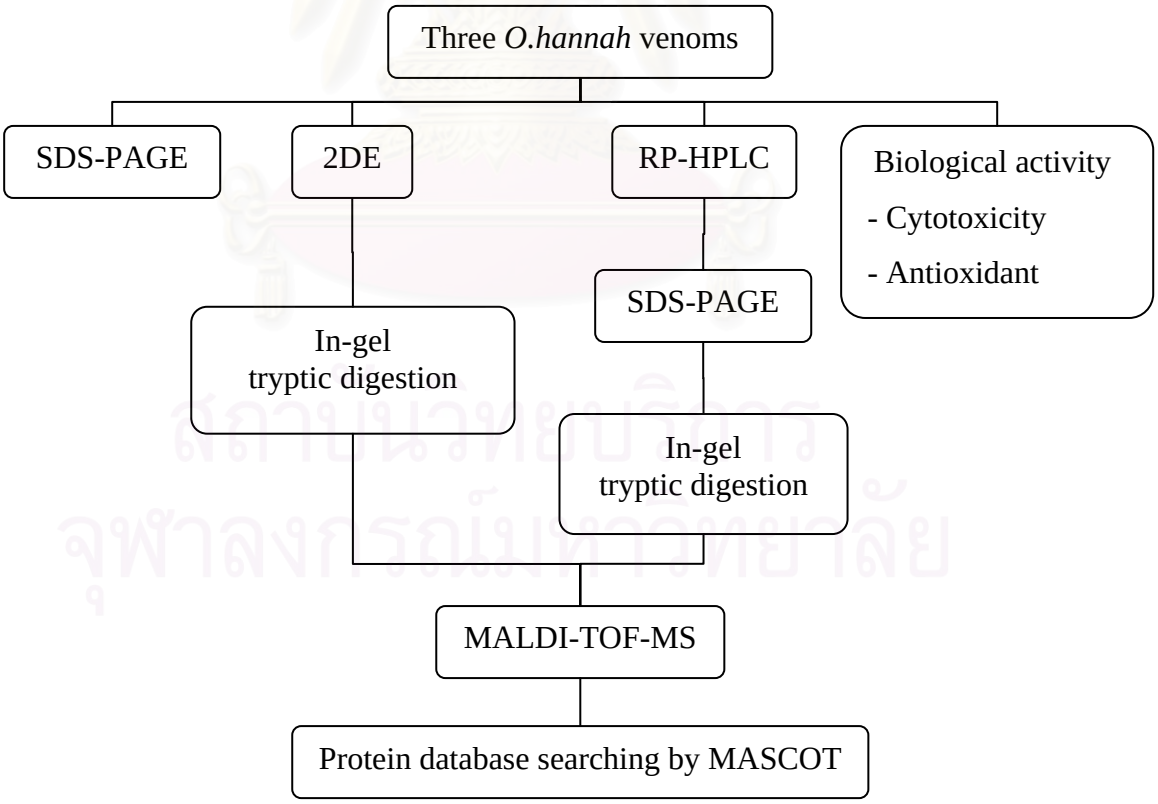
Tandem mass spectrometric analysis was performed on the ESI-Q-TOF mass spectrometer (Korea Basic Science Institute, Systems Biology Core Facility, Korea). The tandem MS spectra were searched against a protein sequence database using the MASCOT program (www.matrixscience.com).

CHAPTER IV

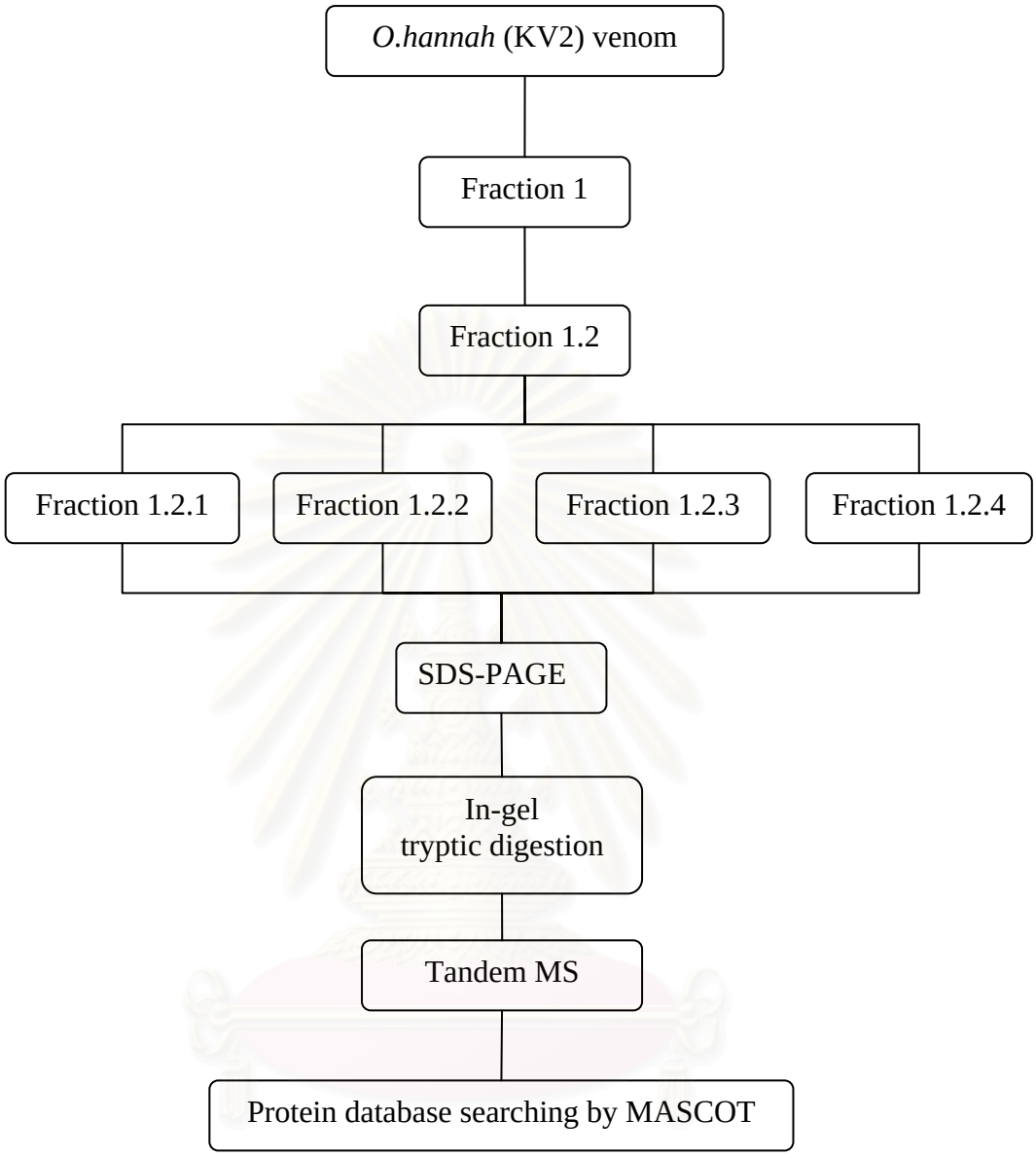
RESULTS AND DISCUSSION

4.1 Strategy to comparison biological activity and protein composition of *O.hannah* venom

The experimental procedure is depicted in Scheme 4.1 and 4.2. Scheme 4.1 shown strategies for comparison of biological activity and protein composition of three crude *O.hannah* venoms (KV2, KV6 and KV9) which were collected from different regions of Thailand. Strategy for analyzed protein fractions of KV2 venom were shown in Scheme 4.2. The report of the Queen Saovabha Memorial Institute showed that KV2 have toxicity higher than other venoms. They were interested proteins KV2 venom in with neuromuscular block activity for improving to new drug.



Scheme 4.1 Schematic of the combination strategy for comparison three venoms



Scheme 4.2 Schematic overview of strategy for analyzed protein of KV2 venom

4.2 Comparison of biochemical and biological activity of *O.hannah* venoms

The biochemical and biological activities of the three crude *O.hannah* venom samples are shown in Table 4.1. Protein contents of the three freezed-dried venom samples are all approximately 40% and the IC_{50} values of KV2 and KV6 samples are difference (In KV9, inhibition growth of cell line is lower 50%). That result indicated that venom from Petchabun Province (KV2) exhibited higher toxicity (IC_{50}, LD_{50}) than two venoms (KV6 and KV9) from same geographic origin. The cytotoxicity values are also the same with that reported earlier by Ahn [32] who reported values 3 µg/ml for human gastric carcinoma cell line (CRL 5971). The antioxidant values of three crude venoms have the EC_{50} lower than 50%, thus it cannot be calculate values of EC_{50}.

Table 4.1 The biochemical and biological activity of *O.hannah* venom

Biochemical and biological activity	**KV2**	**KV6**	**KV9**
Protein concentration (mg/ml)	0.39	0.36	0.38
(% (g/g) of crude venom protein)	(39)	(36)	(38)
Cytotoxicity * (IC_{50}) (µg/ml)	2.99	6.11	-
LD_{50}[†] (mg/kg) in mouse	1.65	4.41	3.89

[†] Data from Queen Saovabha Memorial Institute.

* Cytotoxicity on Hep-G2 cell line (MTT assay)

4.3 The analysis of venoms using SDS-PAGE

Protein pattern of the venom samples in SDS-PAGE is shown in Figure 4.1. The protein bands were distributed in the wide range molecular mass of between 97 kDa to 14.4 kDa. All venoms show a majority of protein bands with molecular masses higher than 45 kDa and lower than 20 kDa. The distribution patterns and the number of bands detected were similar for all samples, but differences were observed in intensity of several bands while amount of loading samples were same. Almost all of major bands in KV2 had higher intensity than in other samples except of the band

at 66 kDa which its intensity is lower than sample KV9. KV6 and KV9 exhibited two components clearly visible as separate bands in the area at 29 and 26 kDa whereas the samples KV2 exhibited only one band at 29 kDa. The varied intensity of protein bands in SDS-PAGE suggests the variations in protein composition of venoms. This variant might confer the differences in properties exhibited by the tree venoms.

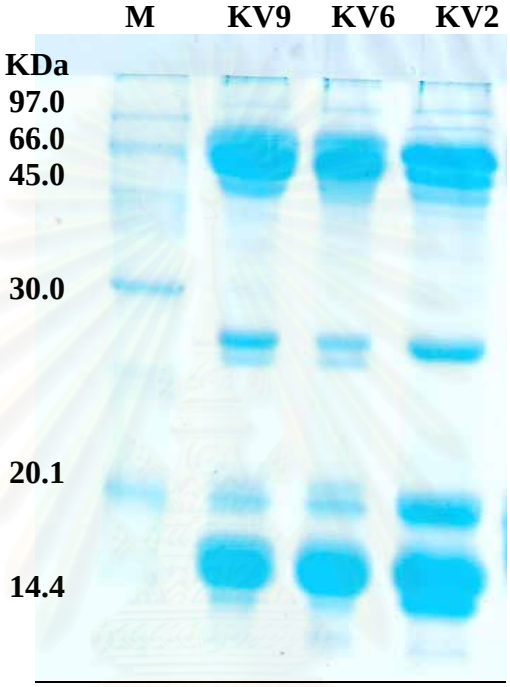


Figure 4.1 SDS-PAGE of three venoms. Lane1: KV9, Lane2: KV6 and Lane3: KV2.

4.4 Comparison of 2-DE gel patterns of venom samples from *O.hannah*

After that all sample were subjected to analyze by 2-DE gel. The results were shown in Figure 4.2 A-C. The IPG-strip at pH 3-10 has been used (Appendix E.), the result suggested that all the proteins are located in the acidic region. Consequently, the IPG-strip at pH 4-7 was selected for a further analysis. The 2-DE gels of three samples are shown in Figure 4.2- A-C.

The 2-DE gel images of three venoms are very similar. Almost of spots are located at molecular mass range between 97 kDa and 14.4 kDa. The protein spot profiles of three samples are abundant proteins with acidic pH and low molecular mass, in agreement with Nawarak reported [39]. Each spot in 2-DE gel represents individual protein that was summarized in Table 4.2

Table 4.2 Comparison number of spot protein from 2-DE gel of venom samples

Molecular mass range (kDa)	Number of spot protein		
	KV2	KV6	KV9
97-66	8	5	9
66-45	3	-	4
45-30	-	1	2
30-20	1	3	5
20-14	7	6	10
<14	7	1	1

Differences in pattern and number of spot from three venom samples are seen at molecular weight below 14.4 kDa, especially KV2 sample, which shown number protein spots more than other samples. KV2 sample have a lot of spots than KV6 and KV9 samples in all gel except a range of 45-30 kDa. This might be the reason why KV2 samples have a higher of the lethal toxicity and cyotoxicity than other samples that confirm with previous protein in *O.hannah* venom study [13], which suggested that the most of toxins in *O.hannah* venom have low molecular weight. This comparison of three venoms by 2-DE showed that the KV6 and KV9 have similarities in their compositions. The geographic origin variation might be one of reason for the protein composition variation.

Comparisons of different venom samples show the different distribution of protein spots. Many protein spots at the same location, presumed to be similar or same proteins, showed different intensities amoung the three samples. All of same protein in these sample are concluded in Figures 4.2 (Red circle in Figure 4.2), between KV6 and KV9 sample. (Green circle in Figure 4.2) Many protein spot occurred only in some gel. (Black circle in Figure 4.2)

4.5 Protein identification of protein spots

After the venoms were analyzed by two-dimensional gel electrophoresis and detected with Coomassie blue stained, some visible protein spots were excised and subjected to digestion with trypsin as described in section 3.4.5. The molecular weight of each tryptic fragment was analyzed by MALDI-TOF MS. The molecular mass results were used for performing of protein peptide mass mapping via MASCOT [40].

The search results are shown in Table 4.3, 4.4 and 4.5 even though many searched results were not reasonable, they might be new proteins or the data set is not enough for protein identification. Therefore, the sequencing techniques are required for further protein identification.

From the searching result, some of protein spots found in KV2 sample were higher score expect than the same protein which found in KV6 and KV9 sample (Figure 4.3) such as spot No.1 which appears in all of samples were match to ophanin [41], that are classified as CRISP family proteins, are secreted proteins found in the epididymis and granules of mammals, and they appear to have roles in sperm maturation and in the immune system. Function of this protein was weakly blocks contraction of smooth muscle elicited by high potassium-induced depolarization, but does not block caffein-stimulated contraction. May be target voltage-gated calcium channels on smooth muscle. Spot No.7 of KV2, spot No.10 of KV6 and spot No.13 of KV9 were match to Chain A of an acidic phospholipaseA2 (Figure 4.4), catalyzes the calcium-dependent hydrolysis of the 2-acyl groups in 3-sn-phosphoglycerides. This protein exhibits cardiotoxicity, myotoxicity, antiplatelet activity, and edema-inducing activity that show greater phospholipaseA2 activity and weaker cardiotoxic and myotoxic activity than a homologous acidic PLA2 from the same venom [42].

For the other spots that are same proteins in KV6 and KV9 can not be identified because the expected score and % protein coverage are too low to match any protein.

The gel of KV2 showed 11 individual protein spots. Some interested protein in this series as spot No.2 and spot No.4 which were match to protein in venom and expected score are significant. Spot No.2 and spot No.4 were match to phospholipaseA2 [28, 43] and phospholipaseA2 acidic 1 precursor [27] respectively, this protein is an acidic protein exhibiting cardiotoxicity, myotoxicity, and antiplatelet activity. The amino acid sequences are shown in Figure 4.5 and 4.6.

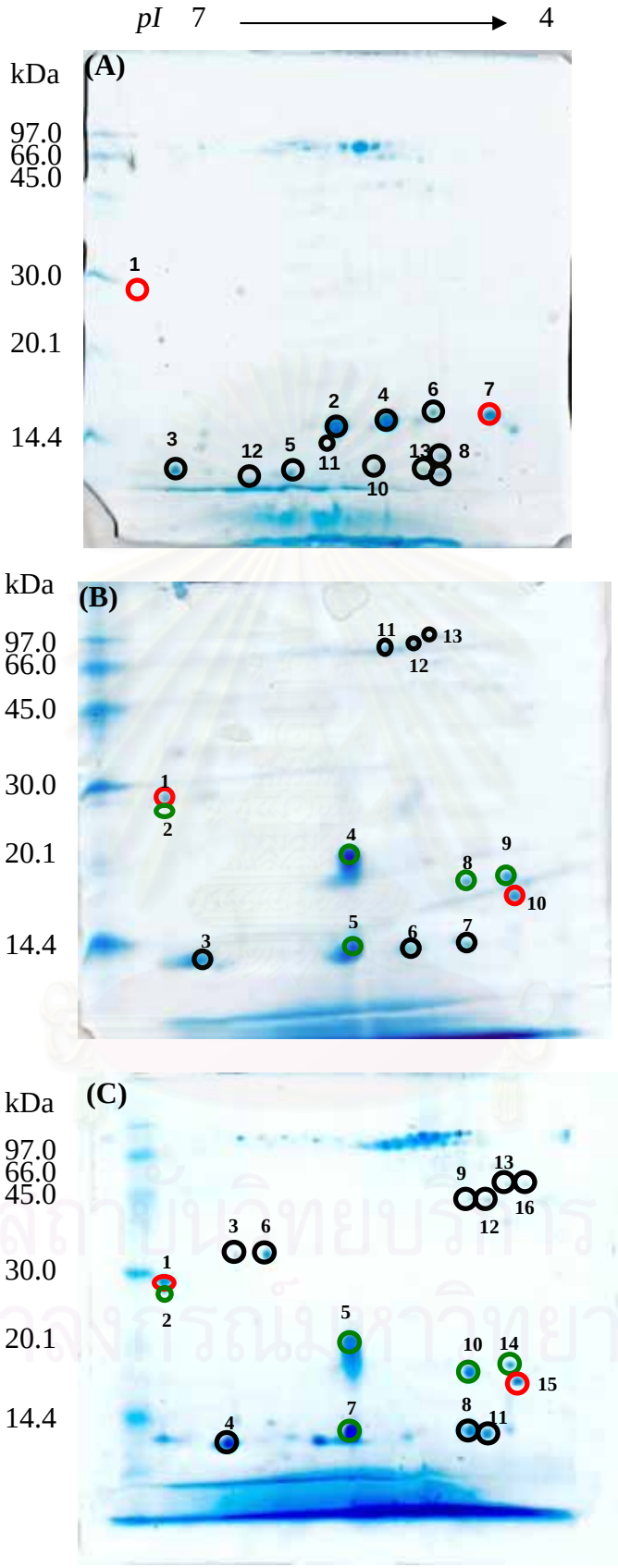


Figure 4.2 2-DE gel images of three venoms. Shown 2-DE for the same venom at pI 4-7. (A): KV2, (B): KV6 and (C): KV9

Table 4.3 Protein identification in *O. hannah* venoms (KV2)

Spot No.	App.mass/pI	Theor.mass/pI	No.of peptides matched	Sequence coverage (%)	score	Protein
1	31,945/6.9	26,851/6.90	5	21	60	Ophanin precursor (Opharin)
2	13,139/5.5	13,447/4.47	5	58	74	phospholipase A2
3	9,904/6.3	9,463/9.19	2	66	40	mCG147719
4	13,960/5.2	16,433/5.67	5	37	81	Phospholipase A2, acidic 1 precursor
5	9,904/5.7	7,072/5.83	2	45	40	ring finger protein 185
6	14,832/4.9	15,150/4.7	3	25	47	receptor activity modifying protein 3
7	14,832/4.5	13,182/4.41	5	43	85	Chain A, Structure of An Acidic Phospholipase A2
8	11,179/4.8	9,946/6.08	3	55	43	MHC class II beta chain
9	9,904/4.9	14,063/5.56	4	47	63	adenylate kinase 1
10	9,904/5.5	9,703/5.63	3	42	45	unnamed protein product
11	12,120/5.5	10,824/5.02	3	47	37	Bl-Beta II protein
12	9,904/6	19,064/7.85	3	30	48	Chain A, Structure Of The C2b Domain Of Rabphilin3a
13	10,312/5	15,728/5.13	2	27	30	Phospholipase A2 precursor

Table 4.4 Protein identification in *O. hannah* venoms (KV6)

Spot No.	App.mass/pI	Theor.mass/pI	No.of peptides matched	Sequence coverage (%)	score	Protein
1	36,925/6.9	26,851/6.90	4	20	39	ophanin precursor (Opharin)
2	35,562/6.9	42,768/9.67	7	32	52	hypothetical protein
3	16,764/6.7	14,279/6.81	3	55	42	hypothetical protein
5	17,406/5.6	13,730/4.32	3	69	52	MGC16385 protein
6	18,073/5.2	19,193/4.75	4	18	56	Translationally-controlled tumor protein
7	17,406/4.8	12,331/5.6	4	58	49	insulin 2
8	24,416/4.8	13,122/7.99	4	57	55	gonadotrophin alpha 1 subunit
9	24,416/4.5	27,219/6.52	3	16	46	rCG54731
10	21,812/4.5	13,182/4.41	3	31	46	Chain A, Structure Of An Acidic Phospholipase A2
11	69,976/5.3	60,132/5.87	6	12	45	titin
13	71.658/4.9	76,422/6.40	5	12	40	hypothetical protein

Table 4.5 Protein identification in *O. hannah* venoms (KV9)

Spot No.	App.mass/pI	Theor.mass/ pI	No.of peptides matched	Sequence coverage (%)	score	Protein
1	35,914/6.9	26,851/6.90	4	21	41	ophanin precursor (Opharin)
2	34,735/6.9	42,768/9.67	7	32	55	hypothetical protein
3	39,039/6.4	46,645/6.43	5	17	49	isocitrate dehydrogenase
4	14,585/6.5	13,560/4.8	2	47	37	EG545052 protein
6	39,039/6.2	33,729/6.16	5	38	67	cyclin-dependent kinase 4
7	15,080/5.6	13,730/4.32	2	53	34	MGC16385 protein
8	15,592/4.9	11,560/6.07	4	76	65	immunoglobulin heavy chain
9	51,845/4.8	43,344/6.64	6	27	54	leucine zipper
10	21,055/4.9	13,122/7.99	4	57	55	gonadotrophin alpha 1 subunit
11	15,592/4.8	12,305/8.62	3	73	55	immunoglobulin heavy chain
12	51,845/4.7	50,490/9.13	5	25	46	WDR40A protein
13	57,305/4.5	69,965/5.51	5	22	46	Prothrombin precursor
14	22,508/4.5	27,219/6.52	3	16	46	rCG54731
15	19,695/4.5	13,182/4.41	3	31	44	Chain A, Structure Of An Acidic Phospholipase A2
16	57,305/4.7	67,076/6.73	6	23	59	6-glucosidase

```
    1 MIAFTLLSLA VLQQSFGNVT DFNSESTRRQ KKQKEIVDLH NSLRRSVSPT
KV2_1 ---------- ---------- ---------- ----EIVDLH NSLRR-----
KV6_1 ---------- ---------- ---------- ----EIVDLH NSLR------
KV9_1 ---------- ---------- ---------- ----EIVDLH NSLR------

   51 ASNMLKMQWY PEAASNAERW ASNCNLGHSP DYSRVLEGIE CGENIYMSSN
KV2_1 ------MQWY PEAASNAERW ASNCNLGHSP DYSR------ ----------
KV6_1 ------MQWY PEAASNAERW ASNCNLGHSP DYSR------ ----------
KV9_1 ------MQWY PEAASNAERW ASNCNLGHSP DYSR------ ----------

  101 PRAWTEIIQL WHDEYKNFVY GVGANPPGSV TGHYTQIVWY KTYRIGCAVN
  151 YCPSSEYSYF YVCQYCPSGN MRGSTATPYK SGPTCGDCPS ACDNGLCTNP
  201 CTLYNEYTNC DSLVKQSSCQ DEWIKSKCPA SCFCHNKII
KV2_1 ---------- ---------- -----SKCPA   SCFCHNK
KV2_1 ---------- ---------- -----SKCPA   SCFCHNK
KV2_1 ---------- ---------- -----SKCPA   SCFCHNK
```

Figure 4.3 Comparison of amino acid sequence of protein spot KV2_1, KV6_1 and KV9_1 with the sequence of ophanin

```
     1 HLVQFNGMIR CTIPGSIPWW DYSDYGCYCG SGGSGTPVDE LDRCCQVHDN
 KV2_7 HLVQFNGMIR ---------- ---------- ---------- ----------
KV6_10 HLVQFNGMIR ---------- ---------- ---------- ----------
KV9_15 HLVQFNGMIR ---------- ---------- ---------- ----------

    51 CYTQAQQLTE CSPYSKRYSY DCSEGTLTCK ADNDECAAFV CDCDRVAAIC
 KV2_7 ---------- ------RYSY DCSEGTLTCK ADNDECAAFV CDCDRVAAIC
KV6_10 ---------- -------YSY DCSEGTLTCK ADNDECAAFV CDCDR-----
KV9_15 ---------- -------YSY DCSEGTLTCK ADNDECAAFV CDCDR-----

   101 FAGAPYNKEN INIDTTTRC
 KV2_7 FAGAPYNKEN INIDTTTRC
KV6_10 --------EN INIDTTTRC
KV6_15 --------EN INIDTTTRC
```

Figure 4.4 Comparison of amino acid sequence of protein spot KV2_1, KV6_1 and KV9_1 with the sequence of Chain A, Structure of An Acidic Phospholipase A2

```
    1 DLIQFGNMIQ CTVPGFLSWI KYADYGCYCG AGGSGTPVDK LDRCCQVHDN
KV2_2 ---------- ---------- -YADYGCYCG AGGSGTPVDK ---CCQVHDN
   51 CYTQAQKLPA CSSIMDSPYV KIYSYDESCR VAVTCKADND ECAAFICNCD
KV2_2 CYTQAQKLPA CSSIMDSPYV KIYSYDESCR ------ADND ECAAFICNCD
  101 RVAAYCFAAS PYNNNNYNID TX
KV2_2 R--------- ---------- --
```

Figure 4.5 Comparison of amino acid sequence of protein spot KV2_2 with the sequence of phospholipase A_2

```
    1 MNPAHLLVLS AVCVSLLGAS SIPPQPLHLI QFGNMIQCTV PGFLSWIKYA
KV2_2 ---------- ---------- ---------- ---------- --------YA
   51 DYGCYCGAGG SGTPVDKLDR CCQVHDNCYT QAQKLPACSS IMDSPYVKIY
KV2_2 DYGCYCGAGG SGTPVDKLDR ---------- ----LPACSS IMDSPYVK--
  101 SYDCSERTVT CKADNDECAA FICNCDRVAA HCFAASPYNN NNYNIDTTTR
KV2_2 -------TVT CKADNDECAA FICNCDR--- ---------- ----------
  151 C
KV2_2 -
```

Figure 4.6 Comparison of amino acid sequence of protein spot KV2_4 with the sequence of phospholipase A_2, acidic 1 precursor

4.6 The analysis of venoms using RP-HPLC

The RP-HPLC chromatogram is shown in Figure 4.7. The concentration of proteins of each fraction is shown in Table 4.6. RP-HPLC chromatograms from three venoms showed concentration of major protein peaks in range of 10 – 60 % ACN indicating that the venoms varied in hydrophobic properties. All of venoms showed two bands of peak, one hydrophilic and one moderately hydrophobic. KV6 and KV9 analyzed here have similarity pattern, the large protein peaks were eluted in the range 26-30 % ACN and small peaks were eluted in the range 31-50% ACN. The HPLC pattern of KV2 is different from other venom, the large protein peaks were eluted in the range 26-50 % ACN and small peaks were eluted at 10 and 18% ACN. The RP-HPLC results obtained also clearly confirmed that although the venoms had an overall similarity in their components, there were both qualitative and quantitative differences between the venoms from the three different regions. One of the most obvious differences between the venoms can be seen when the HPLC profiles for KV2 and other venom are compared in the region where the eluting gradient of acetonitrile has a concentration of 31–41%. It appears that the venom of KV2 contains higher concentration of the components which elute in this part of the gradient.

Differences were also visible in the HPLC profiles of the three samples of *O.hannah* venom obtained from snakes collected in different geographical regions, However it should be remembered that such differences may be also caused by factors other than geography. For example other workers have reported marked variations in quantitative and qualitative composition of snake venoms with respect to geographic origin, age, and sex of the spiders used

The eluted fractions were analyzed further by SDS-PAGE to check separation efficiency. Band proteins from SDS-PAGE were pooled for in-gel tryptic digestion. All digestion peptides were analyzed by MALDI-TOF MS.

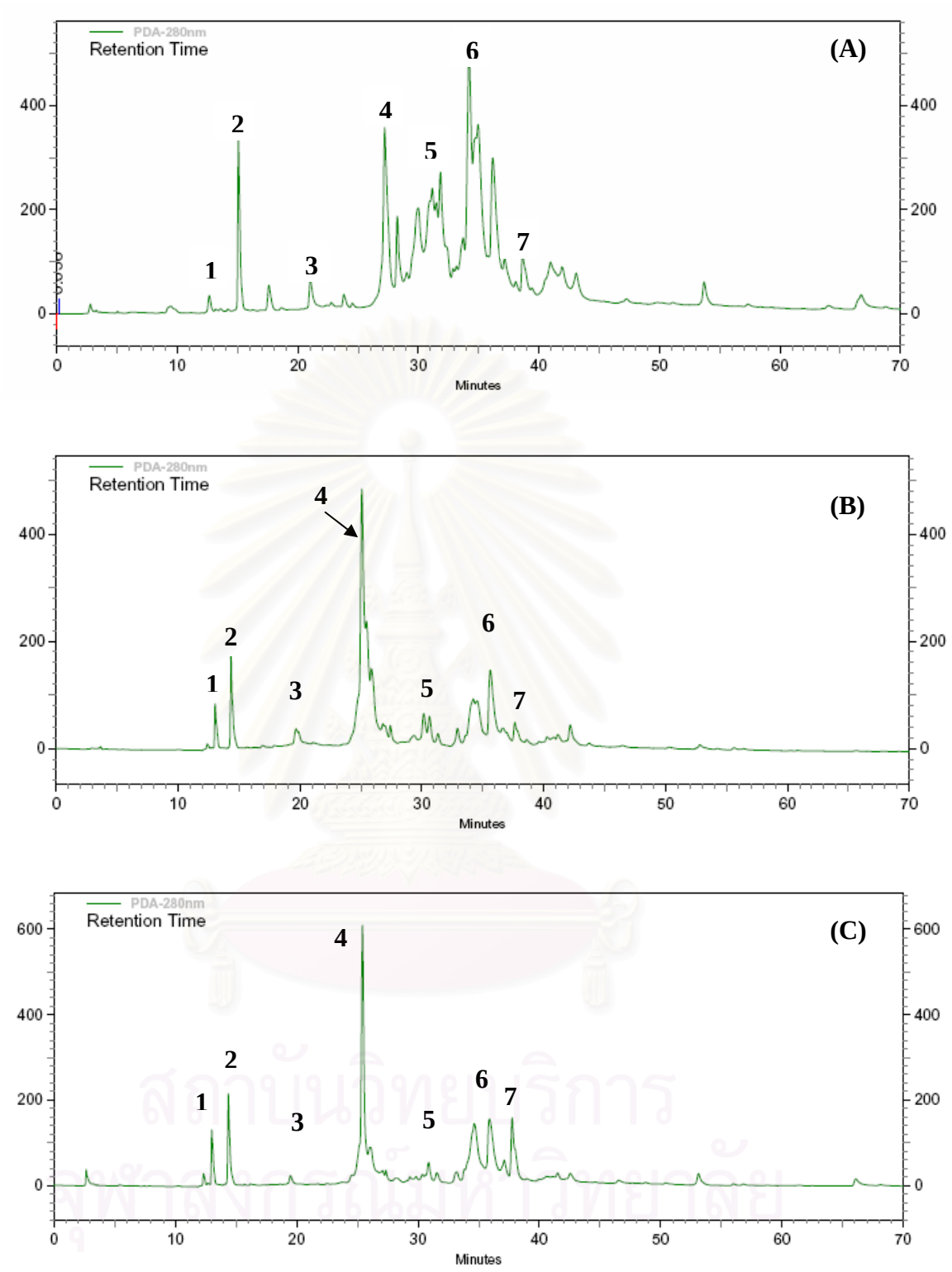


Figure 4.7 RP-HPLC separations for three *O. hannah* venoms. (A): KV2 venom, (B): KV6venom and (C): KV9venom

Table 4.6 Summarized %ACN to elute each of fractions and concentration of protein of each fraction

Fraction	% ACN	Concentration (mg/ml)		
		KV2	KV6	KV9
1	10	0.0450	0.0161	0.0588
2	12	0.0538	0.0089	0.0467
3	18	0.0250	0.0097	0.0153
4	26-30	0.4434	0.1965	0.2014
5	31-35	1.0322	0.2401	0.2642
6	37-41	1.7826	1.0931	1.1970
7	47-50	0.2332	0.1530	0.6364

4.7 Molecular weight of protein in each RP-HPLC fraction

The mass spectra of fraction 4 of all venoms are shown in Figure1-3F in Appendix F and the summarized in Table 4.7

Table 4.7 Mass per charge of each RP-HPLC fraction 4 of three venoms

Fraction 4	m/z
KV2	14034.59(A), 7016.45 (A^{2+}), 6472.79 (B), 3509.70 (B^{2+})
KV6	14069.35 (C), 7062.09 (C^{2+})
KV9	14019.22 (D), 7013.76 (D^{2+})

From Table 4.7, there are two proteins in KV2 venom fraction No.4. The m/z of 7016.45 and 3509.70 is doublet charged species of proteins which yield the molecular weight of 14033.59 and 6471.79, respectively. From fraction No.4 of KV6, the m/z of 7062.06 is singlet charge species and 14069.35 is doublet charge species of the protein so molecular weight is 14068. From fraction No.4 of KV9, the m/z of 7013.76 is singlet charge species and 14019.22 is doublet charge species of the protein so molecular weight is 14018. The fractions from KV2 were subjected to test for their cytotoxicity. The results suggested that the inhibition growth of cell line is lower 50%. RP-HPLC can cause the irreversible denaturation of protein samples

thereby reducing the potential recovery of the material in a biologically active form. After that, all fractions from RP-HPLC were performed further separation using by SDS-PAGE.

4.8 1-D gel electrophoresis of RP-HPLC fractions

The SDS-PAGE of all fractions from HPLC is shown in Figure 4.8. The results indicated that the distribution patterns and the number of bands detected were similar for all same fractions in three venoms.

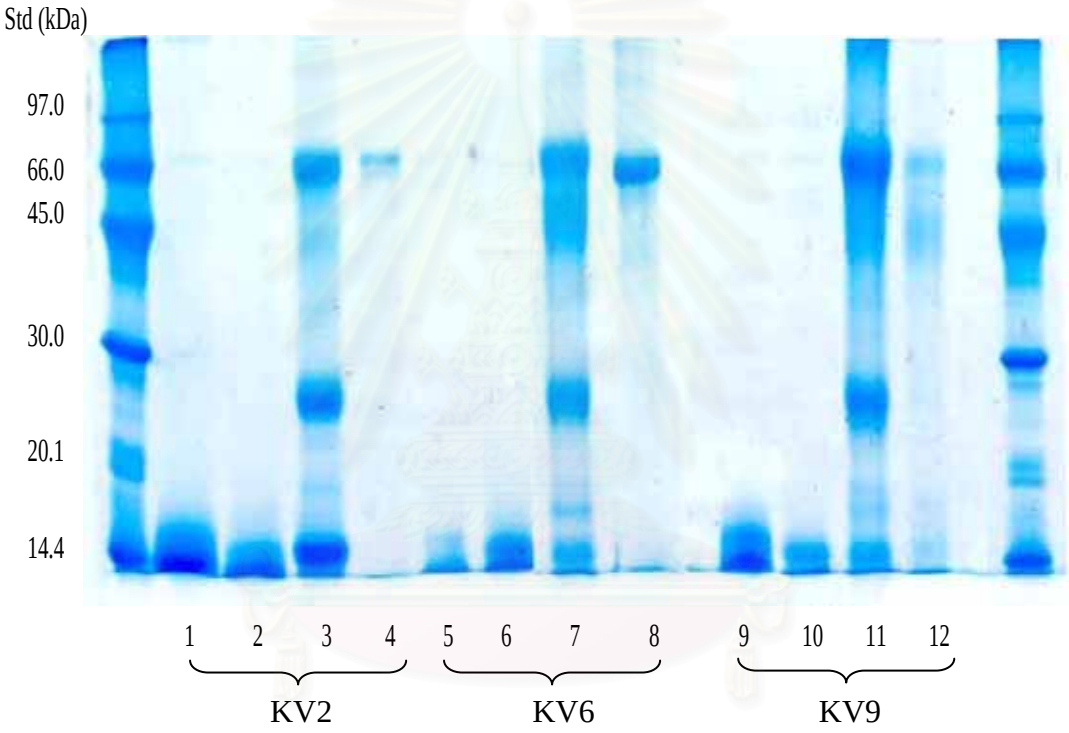


Figure 4.8 SDS-PAGE of HPLC fractions from three venoms: Lane 1: KV2_4, lane2: KV2_5, lane 3: KV2_6, lane 4: KV2_7, Lane 5: KV6_4, Lane 6: KV6_5, lane 7: KV6_6, Lane 8: KV6_7, Lane 9: KV9_4, lane 10: KV9_5, lane11: KV9_6 and lane 12: KV9_7

The bands were excised and subjected to trypsin digestion. The molecular weight of each tryptic peptides were analyzed by MALDI-TOF MS. The searched results were not significant. Sometimes more than one candidate for identity of the protein can be found, whereupon additional information obtained by sequencing techniques is generally required to constrain the search.

4.9 Protein Identification of fractions from ion exchange chromatography and gel filtration chromatography

The four fractions were collected from ion-exchange chromatography and gel filtration chromatography as shown in Appendix D. The purity of fractions was identified by SDS-PAGE. The result was shown in Figure 4.9 The SDS-PAGE gel shows the band at molecular weight rang 30-20.1 kDa both of Fraction 1.2.2 and 1.2.3 but Fraction 1.2.1 and 1.2.4 hasn't band so did not analyze any two fractions. Based upon results from SDS-PAGE bands were pooled for in-gel digestion. Digested peptides, obtained from the digestion of gel from SDS-PAGE, were analyzed by ESI-Q-TOF MS. (Korea Basic Science Institute, Systems Biology Core Facility, Korea).

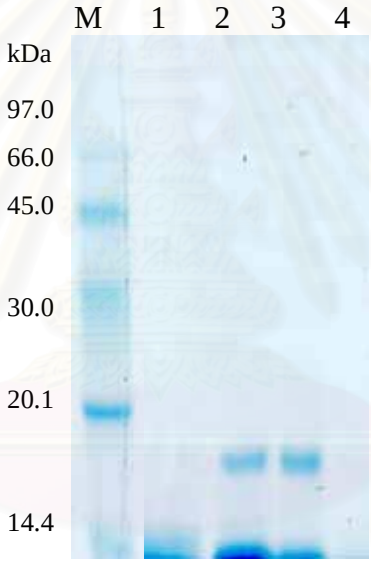


Figure 4.9 SDS-PAGE of the ion exchange and gel filtration fractions.
Lane1: Fraction 1.2.1 Lane2: Fraction 1.2.2 Lane3: Fraction 1.2.3
Lane4: Fraction 1.2.4 Lane M: molecular mass standards.

Peptide mass mapping and partial sequencing by ESI-Q-TOF MS of Fraction 1.2.2 and 1.2.3. From the result of database searching, partial amino acid sequence of fraction 1.2.2 and 1.2.3 were nearly coincided with partial amino acid sequence of ophanin and Natrin. A comparison of amino acid sequence between ophanin and Natrin and fraction 1.2.2 and 1.2.3 are shown below. The bold letters were shown similar amino acid sequence.

- Fraction 1.2.2 and Chain C, Crystal Structure of Natrin (*Naja Atra*) Score: 65
  Function: Inhibits carbachol-induced muscle contraction and weakly blocks muscle contraction evoked by potassium

```
Natrin:         N---------  RKKKQKEIVD[20] LHNSLRRRVS[30] PTASNMLKME[40]
Fraction1.2.2: ----------  ------EIVD   LHNSLR----  --------ME
Natrin:         WYPEAASNAE[50] RWANTCSLNH[60] ----------  I[121]
Fraction1.2.2: WYPEAASNAE   R
```

- Fraction 1.2.3 and ophanin (*Ophiophagus hannah*) Score: 73
  Function: Weakly blocks contraction of smooth muscle elicited by high potassium-induced depolarization, but does not block caffein-stimulated contraction.

```
Ophanin:         MIAFTLLSLA[10] ---------- ASNMLKMQWY[60]
Fraction 1.2.3: ----------  ---------- ------MQWY
Ophanin:         PEAASNAERW[70] ---------- SCFCHNKII[239]
Fraction 1.2.3: PEAASNAER
```

- Fraction 1.2.3 and Chain C, Crystal Structure of Natrin (*Naja Atra*) Score: 72

```
Natrin:         NVDFNSESTR[10] ---------- PASCFCRNKI I [121]
Fraction 1.2.3: NVDFNSESTR
```

CHAPTER V

CONCLUSION AND SUGGESTION

5.1 Conclusion

In this research, the proteomic approach was used to investigate protein profiles in three crude *Ophiophagus hannah* venoms (KV2, KV6 and KV9) which were collected from different regions of Thailand. Firstly, Protein content and cytotoxicity of crude venom were measured. Then they were separated by sodium dodecyl sulfate polyacrylamide gel electrophotesis, two-dimensional gel electrophoresis and the reverse-phase high performance liquids chromatography.

The variability in biochemical properties and biological activities in three venom samples were demonstrated. All samples have protein content around 40% of the dried venom weight. Cytotoxicity (IC_{50}) of KV2 and KV6 are 2.93 and 6.11 µg/ml respectively. From the result indicated that KV2 have the highest toxicity. The protein bands of three venoms in SDS-PAGE showed the similar pattern. There are slightly differed in the intensity of several bands especially KV2 which had higher intensity band than other samples.

2-DE gels of three venom samples indicated that the protein spots are also similar at acidic pH and low molecular mass. There are more spots in than KV6 and KV9 in all gel particularly in below low molecular mass protein. Protein identification of spots from 2-DE gel separation was performed by MALTI-TOF MS. From the search results indicated that spot No.2 of KV2 gel is phospholipaseA2. Spot No.4 of KV2 gel is phospholipaseA2, acidic 1 precursor. Spot No.7 of KV2 gel is chain A, structure of an acidic phospholipaseA2. The function of these proteins is exhibits cardiotoxicity, myotoxicity, antiplatelet activity. The expected score of other spots are not significant. This is more than one candidate for identity of the proteins has been found, whereupon additional information obtained by sequencing techniques is generally required to constrain the search.

Chromatograms from RP-HPLC of venom samples indicated that the venoms have an overall similarity in their components but differed in quantitative. Fraction 2, 5 and 6 of KV2 were higher concentration than KV6 and KV9.

The geographic origin variation was the factor of protein composition variation when were compared by SDS-PAGE 2-DE and RP-HPLC. KV6 and KV9 have similarities in protein compositions. Protein compositions of KV2 sample differed from other sample that cause of high toxicity of KV2.

Fraction 1.2.2 and 1.2.3 of KV2 was separated by ion exchange chromatography, gel filtration chromatography and SDS-PAGE. The amino acid sequence of fractions was analyzed by Tandem MS. The amino acid sequence of fraction 1.2.2 is similar to partial amino acid sequence of natrin from *Naja Atra* snake venom. The function of this protein is inhibits carbachol-induced muscle contraction and weakly blocks muscle contraction evoked by potassium. The amino acid sequence of fraction 1.2.3 is similar to partial amino acid sequence of ophanin from *O.hannah* and natrin from *Naja atra* snake venom. Function of ophanin is weakly blocks contraction of smooth muscle elicited by high potassium-induced depolarization, but does not block caffein-stimulated contraction.

5.2 Suggestion

The protein identifications of snake venoms by gel electrophoresis showed some difficulties such as the separation and identification of low molecular mass proteins. The alternative methods are required such as liquid chromatography. Moreover, for more precise protein identification, LC-MS/MS should be used for further confirmation of amino acid sequence.

Additionally, the different protein patterns and some different unique proteins are important and useful for further pharmaceutical applications and diagnostic.

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APPENDICES

Appendix A

Solutions in Bradford Assay

A rapid and reliable dye based assay for determining protein content in a solution. This assay is sometimes referred to as the Bio-Rad assay after the company which sells a widely used kit that is based on this method.

A. Bradford stock solution

	Final concentration	Amount
Ethanol	95% (v/v)	100 ml
Phosphoric acid	88% (v/v)	200 ml
Serva Blue G		350 mg

B. Bradford working buffer

	Final concentration	Amount
Ethanol	95% (v/v)	15 ml
Phosphoric acid	88% (v/v)	30 ml
Bradford stock solution		30 ml
Distilled water		to 500 ml

Filter through Whatman No. 1 paper, store at room temperature in brown glass bottle. Usable for several weeks, but may need to be refiltered.

Appendix B

Solutions in SDS-PAGE

The acrylamide, N, N'- methylenebisacrylamide, TEMED, ammonium persulfate and SDS in this experiments are extremely hazardous. Hazardous materials should be weighed in a fume hood while wearing a disposable dust mask and using double latex gloves for all protocols.

I. Stock Solutions

A. 2 M Tris-HCl (pH 8.8), 100 ml

	Final concentration	Amount
Tris-base	2 M	24.2 g
Distilled water		50 ml
HCl		adjust to pH 8.8
Distilled water		to 100 ml

Allow solution to cool to room temperature, pH will increase.

B. 1 M Tris-HCl (pH 6.8), 100 mL

	Final concentration	Amount
Tris-base	1 M	12.1 g
Distilled water		50 ml
HCl		adjust to pH 6.8
Distilled water		to 100 ml

Allow solution to cool to room temperature, pH will increase.

C. 10% SDS (w/v), 100 mL

	Final concentration	Amount
SDS	10% (w/v)	10 g
Distilled water		to 100 ml

Store at room temperature.

D. 50% glycerol (v/v), 100 ml

	Final concentration	Amount
100% glycerol	50% (v/v)	50 ml
Distilled water		to 50 ml

E. 1% bromophenol blue (w/v), 10 ml

	Final concentration	Amount
Bromophenol blue	1% (w/v)	100 mg
Distilled water		to 10 ml

Filtration will remove aggregated dye.

II. Working Solutions

A. Solution A (acrylamide stock solution)

(30% acrylamide, 0.8% N, N'- methylenebisacrylamide)

	Final concentration	Amount
Acrylamide	30% (w/v)	29.2 g
N, N'- methylenebisacrylamide	0.8% (w/v)	0.8 g
Distilled water		to 100 ml

Solution can be stored for months in the refrigerator.

B. Solution B (4x separating gel buffer)

(2 M Tris-HCl, pH 8.8, 10% SDS)

	Final concentration	Amount
2 M Tris-HCl (see stock solution A)	1.5 M	75 ml
10% SDS (see stock solution C)	0.4% (v/v)	4 ml
Distilled water		to 100 ml

Solution can be stored for months in the refrigerator.

C. Solution C (4x stacking gel buffer)

(1 M Tris-HCl, pH 6.8, 10% SDS)

	Final concentration	**Amount**
1 M Tris-HCl (see stock solution B)	0.5 M	50 ml
10% SDS (see stock solution C)	0.4% (v/v)	4 ml
Distilled water		to 100 ml

Solution can be stored for months in the refrigerator.

D. 10% Ammonium persulfate

	Final concentration	**Amount**
Ammonium persulfate	10%	0.5 g
Distilled water		to 5 ml

Stable for months in a capped tube in the refrigerator.

E. Electrophoresis buffer

	Final concentration	**Amount**
Tris-base	25 mM	3 g
Glycine	192 mM	14.4 g
SDS	0.1% (w/v)	1 g
Distilled water		to 1000 ml

pH should be approximately 8.3

F. 5x Sample buffer

(*1 M Tris-HCl, pH 6.8, 50% glycerol, 10% SDS, 2-mercaptoethanol)*

	Final concentration	**Amount**
1 M Tris-HCl (see stock solution B)	60 mM	0.6 ml
50% glycerol (see stock solution D)	25% (v/v)	5 ml
10% SDS (see stock solution C)	2% (v/v)	2 ml
2-mercaptoethanol	14.4 mM	0.5 ml
Distilled water		to 10 ml

Stable for weeks in the refrigerator or for months at -20^0 C.

III. Coomassie gel stain

	Amount
Coomassie blue R-250	1.0 g
Methanol	450 ml
Glacial acetic acid	100 ml
Distilled water	450 ml

IV. Coomassie gel destain

	Amount
Methanol	100 ml
Glacial acetic acid	100 ml
Distilled water	800 ml

V. Gel preparation

	Separating gel (10 ml)	Stacking gel (4 ml)
Solution A	5.0 ml	0.67 ml
Solution B	2.5 ml	-
Solution C	-	1.0 ml
10% Ammonium persulfate	50 µl	30 µl
TEMED	5.0 µl	5.0 µl
Distilled water	2.5 ml	2.3 ml

10% Ammonium persulfate and TEMED are added in last step because polymerization will be under way.

Appendix C

Solutions in Two dimensional gel electrophoresis

I. Stock Solutions

A. Lysis solution

(8M urea, 4% CHAPS, 2% Pharmalyte 3-10)

	Final concentration	Amount
Urea (FW 60.06)	8 M	19.2 g
CHAPS[†]	4% (w/v)	1.6 g
Pharmalyte 3-10	2%	800 µl
Double distilled H_2O		to 40 ml

[†] *Other detergent (Triton X-100, NP-40, and other non-ionic or zwitterionic detergents) can be used instead of CHAPS.*

B. Rehydration stock solution without IPG buffer[*]

(8M urea, 2% CHAPS, 0.002% bromophenol blue)

	Final concentration	Amount
Urea (FW 60.06)	8 M	12 g
CHAPS	2% (w/v)	0.5 g
bromophenol blue	0.002%	50 µl
Double distilled H_2O		to 25 ml

[*] *DTT and IPG Buffer or Pharmalyte are added just prior to use*

Store in 2.5 ml aliquots at -20 °C.

Bromophenol blue stock solution

	Final concentration	Amount
bromophenol blue	1%	100 mg
Tris-base	50 mM	60 mg
Double distilled H_2O		to 10 ml

C. Rehydration stock solution with IPG Buffer

(8M urea, 2% CHAPS, 0.5% or 2% IPG buffer, 0.002% bromophenol blue)

	Final concentration	Amount
Urea (FW 60.06)	8 M	12 g
CHAPS	2% (w/v)	0.5 g
IPG Buffer or Pharmalyte	0.5% (v/v) or 2% (v/v)	125 µl or 500 µl
bromophenol blue	0.002%	50 µl
Double distilled H_2O		to 25 ml

D. SDS equilibration buffer*

(50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, bromophenol blue)

	Final concentration	Amount
Tris-HCl, pH 8.8	50 mM	10.0 ml
Urea (FW 60.06)	6 M	72.07 g
Glycerol (87% v/v)	30% (v/v)	69 ml
SDS (FW 288.38)	2% (w/v)	4.0 g
bromophenol blue	0.002% (w/v)	400 µl of 1% solution
Double distilled H_2O		to 200 ml

** This is a stock solution. Prior to use DTT or iodoacetamide are added.*

E. 30% T, 2.6% C monomer stock solution

(*30% acrylamide, 0.8% N,N'-methylenebisacrylamide*)

	Final concentration	Amount
Acrylamide	50 mM	10.0 ml
N,N'-methylenebisacrylamide (FW 154.17)	6 M	72.07 g
Double distilled H_2O		to 200 ml

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

F. 4x resolving gel buffer

(*1.5 M Tris-HCl, pH 8.8*)

	Final concentration	Amount
Tris base (FW 121.1)	1.5 M	181.7 g
Double distilled H_2O		750 ml
HCl (FW 36.46)		adjust to pH 8.8
Double distilled H_2O		to 1 l

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

G. 10% SDS

	Final concentration	Amount
SDS (FW 288.38)	10% (w/v)	5.0 g
Double distilled H_2O		to 50 ml

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

H. 10% ammonium persulfate

	Final concentration	Amount
ammonium persulfate (FW 288.20)	10% (w/v)	0.1 g
Double distilled H_2O		to 1 ml

Prepare just prior to use.

I. Gel storage solution

(0.375 M Tris-HCl, pH 8.8, 0.1% SDS)

	Final concentration	Amount
4x Resolving gel buffer (see solution F)	1x	50 ml
10% SDS	0.1%	2 ml
Double distilled H_2O		to 200 ml

Store at 4 °C

J. SDS electrophoresis buffer

(*25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% SDS*)

	Final concentration	**Amount**
Tris-base (FW 121.1)	25 mM	30.3 g
Glycine (FW 75.07)	192 mM	144.0 g
SDS (FW 288.38)	0.1% (w/v)	10.0 g
Double distilled H_2O		to 10 l

Store at room temperature

K. Agarose sealing solution

	Final concentration	**Amount**
SDS electrophoresis buffer		100 ml
Agarose	0.5 %	0.5 g
Bromophenol blue	0.002% (w/v)	200 µl

II. Gel preparation

Final Gel Concentration	**15%**
Monomer solution (solution E)	50 ml
4x resolving gel buffer (solution F)	25 ml
10% SDS (Solution G)	1 ml
Double distilled water	23.5 ml
10% ammonium persulfate* (solution H)	500 µl
TEMED*	33 µl
Total volume	100 ml

* *Add after deaeration*

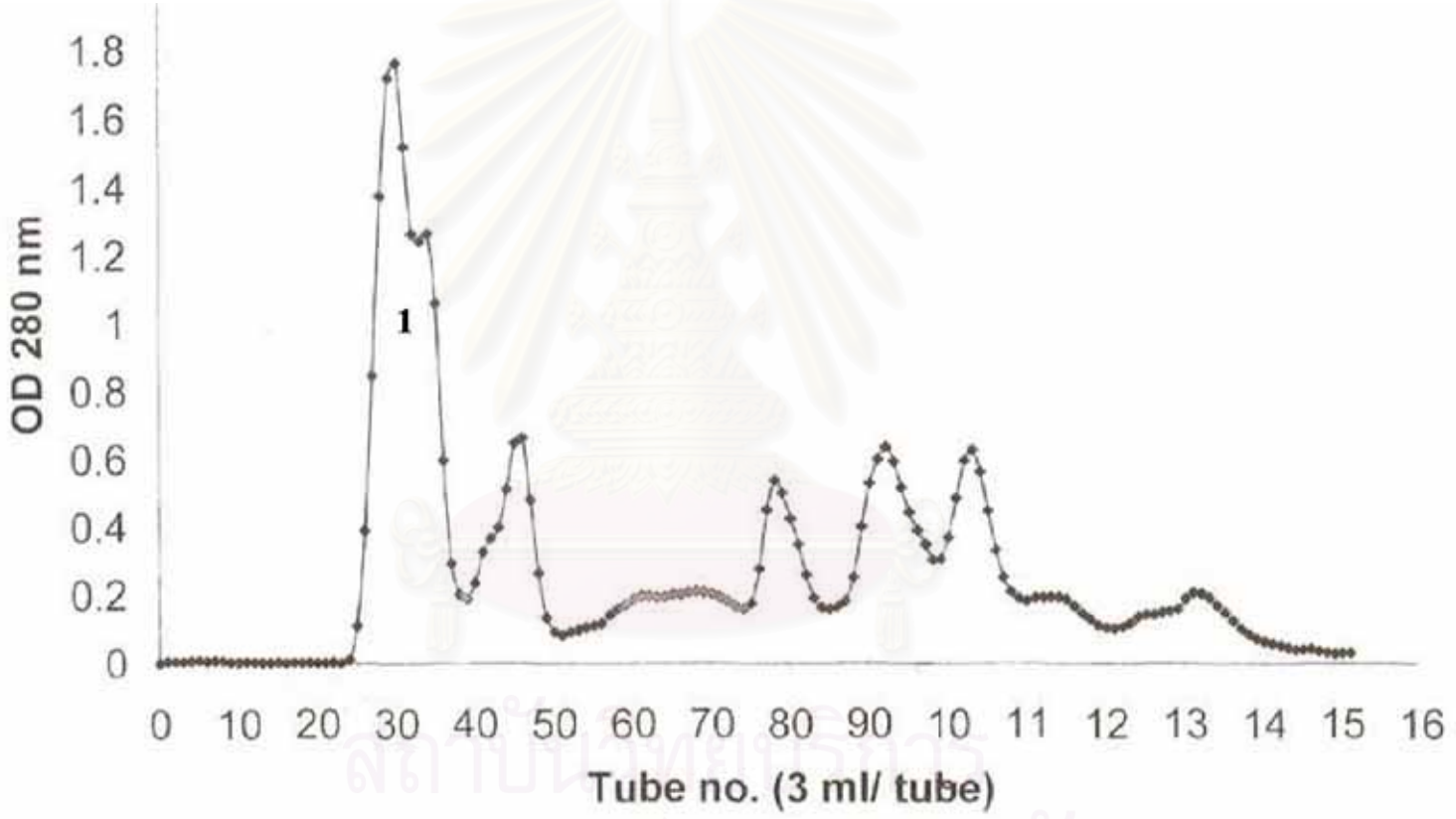

Figure 1D Ion exchange chromatogram of KV2 sample.

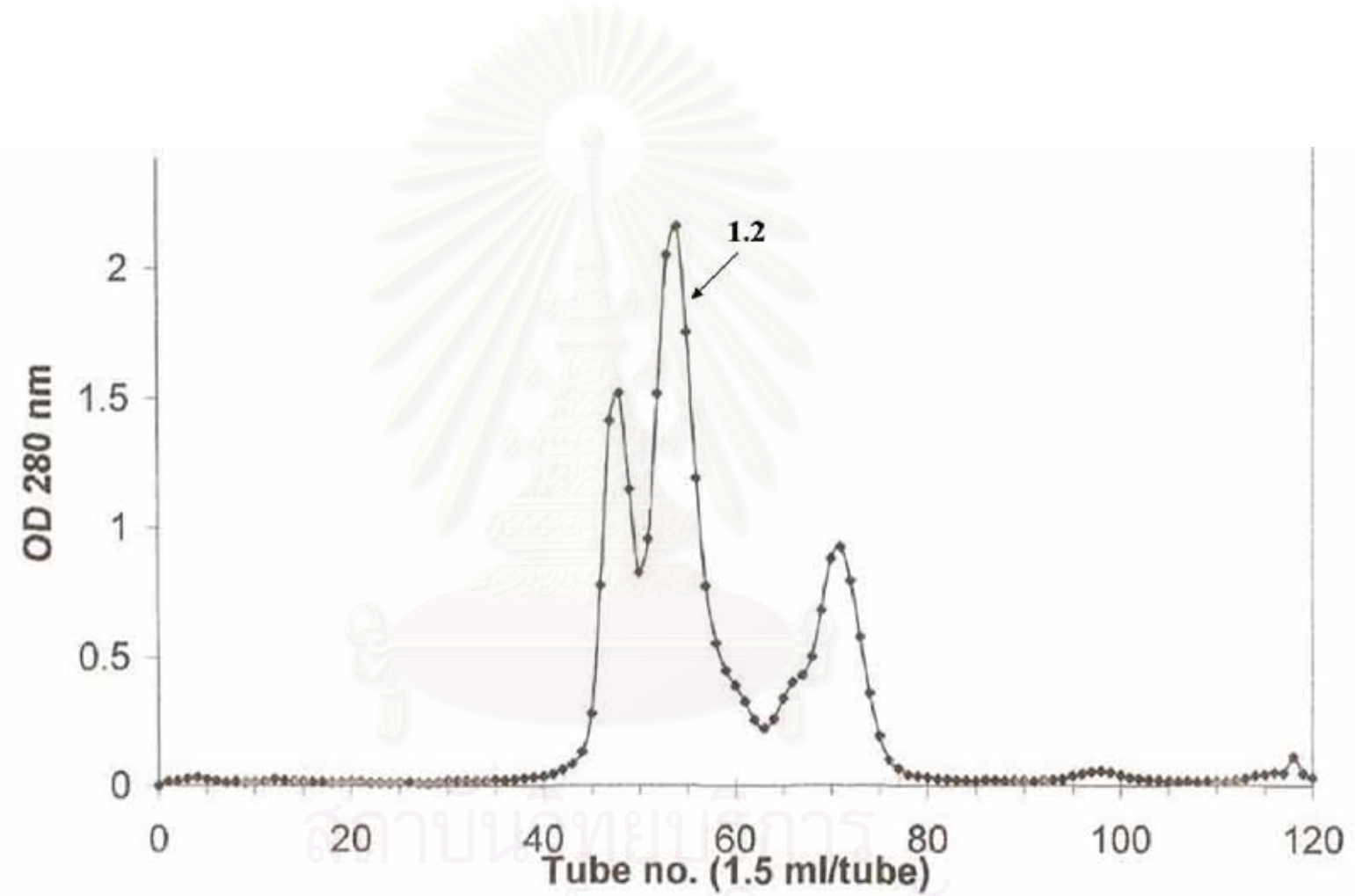

Figure 2D Gel filtration chromatogram of fraction 1 of ion exchange fraction

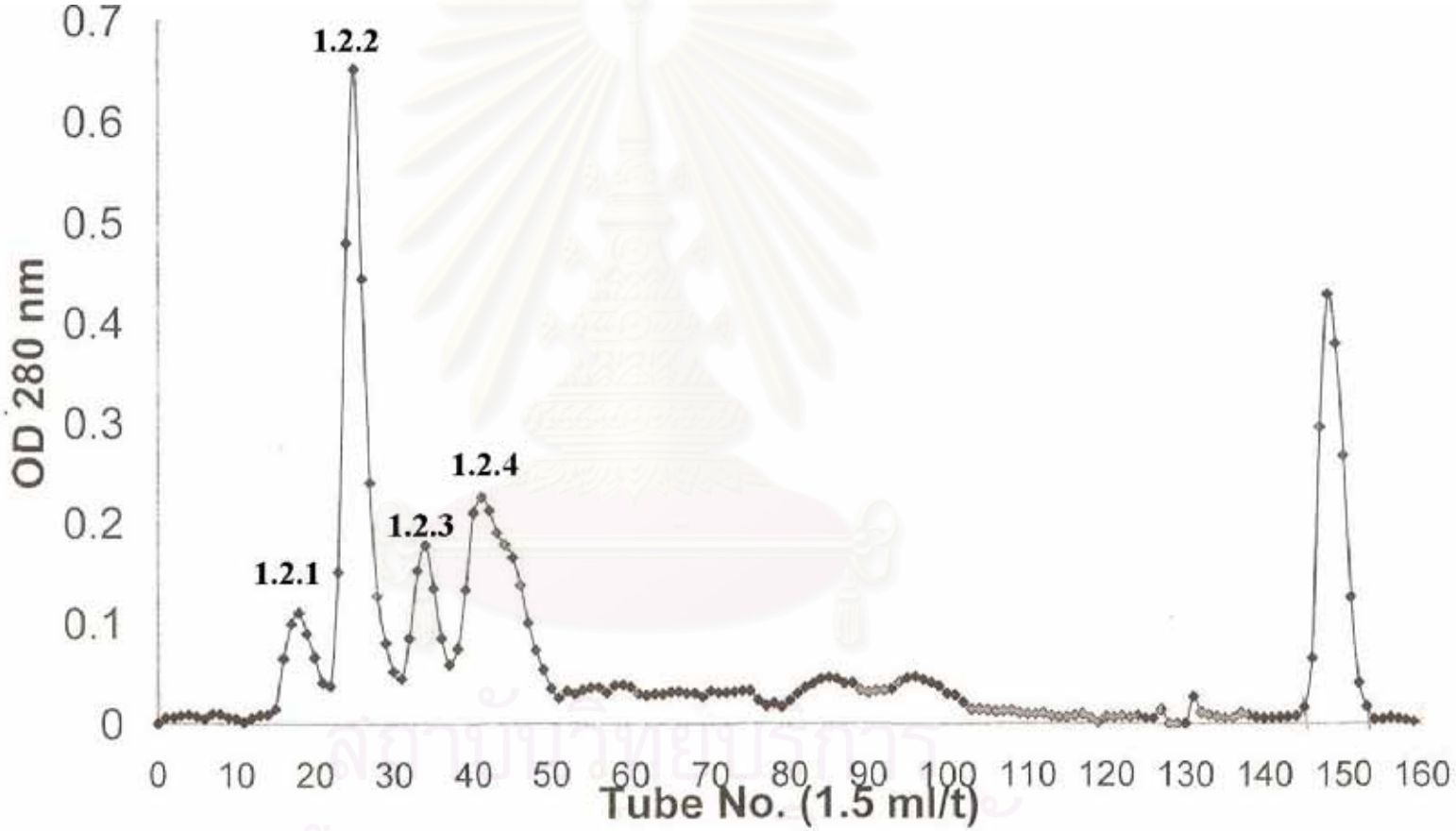

Figure 3D Ion exchange chromatogram of fraction 1.2 of gel filtration fraction.

Appendix E

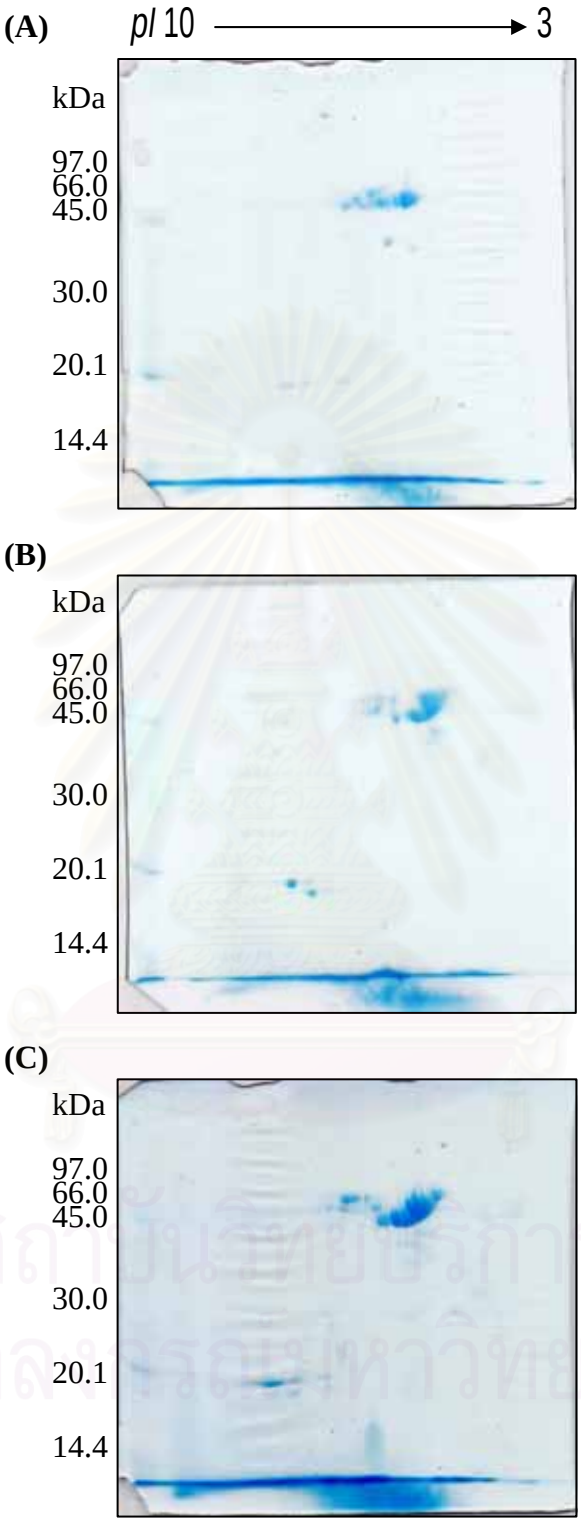

Figure1E. 2-DE gel images of three venoms shown 2-DE for pI 3-10

(A): KV2, (B): KV6, (C) : KV9

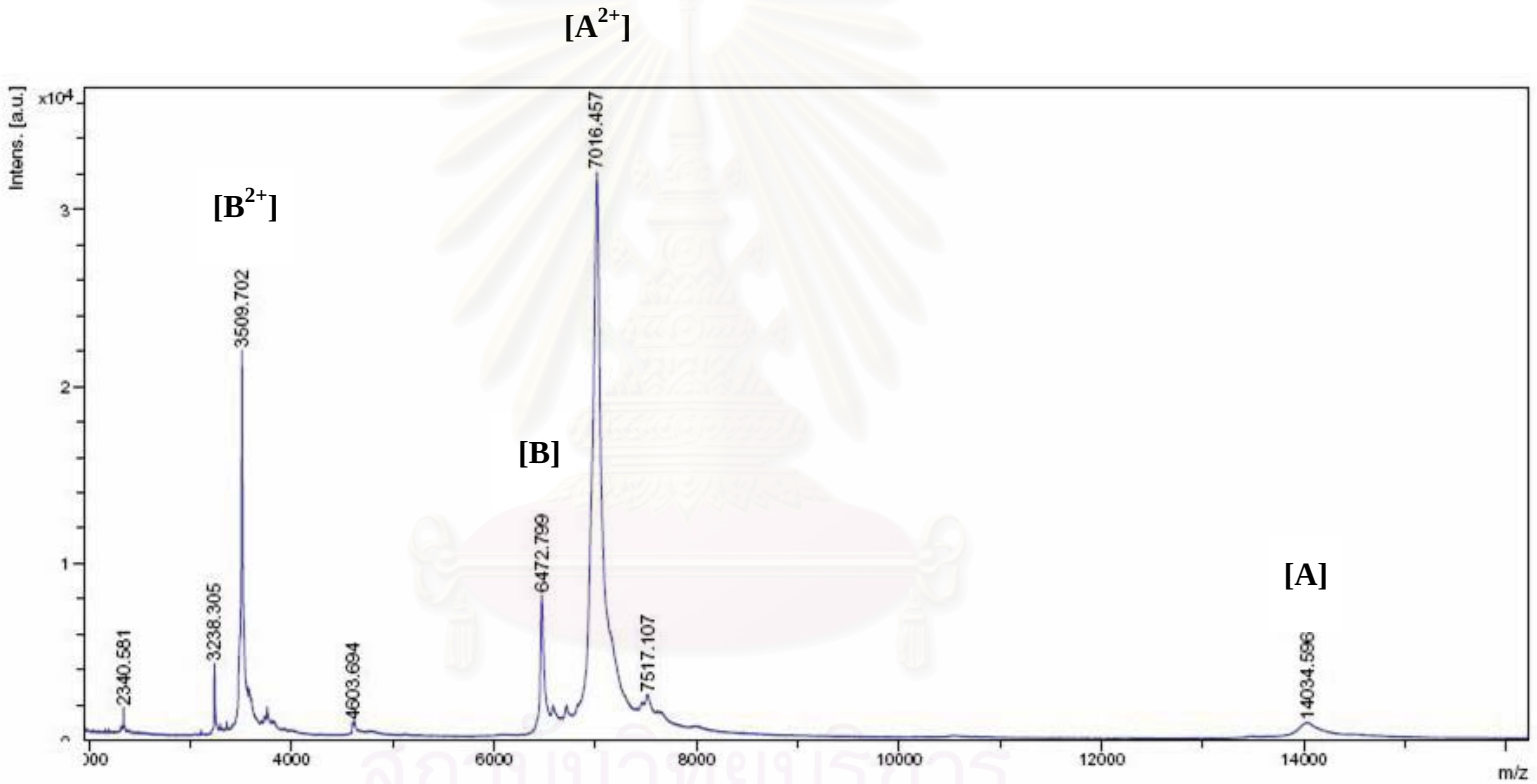

Figure 1F MALDI-TOF MS spectrum of fraction 4 of KV2

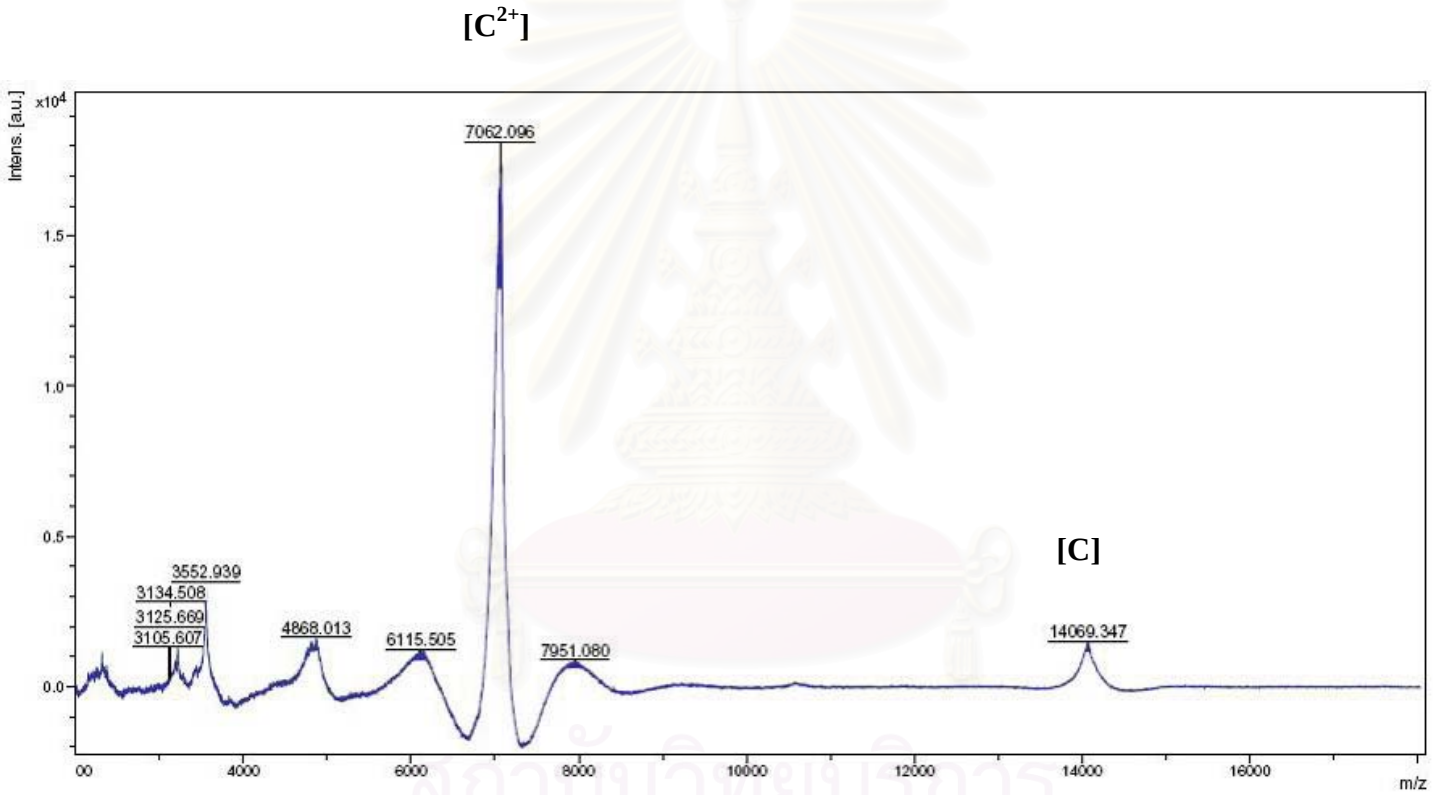

Figure 2F MALDI-TOF MS spectrum of fraction 4 of KV6

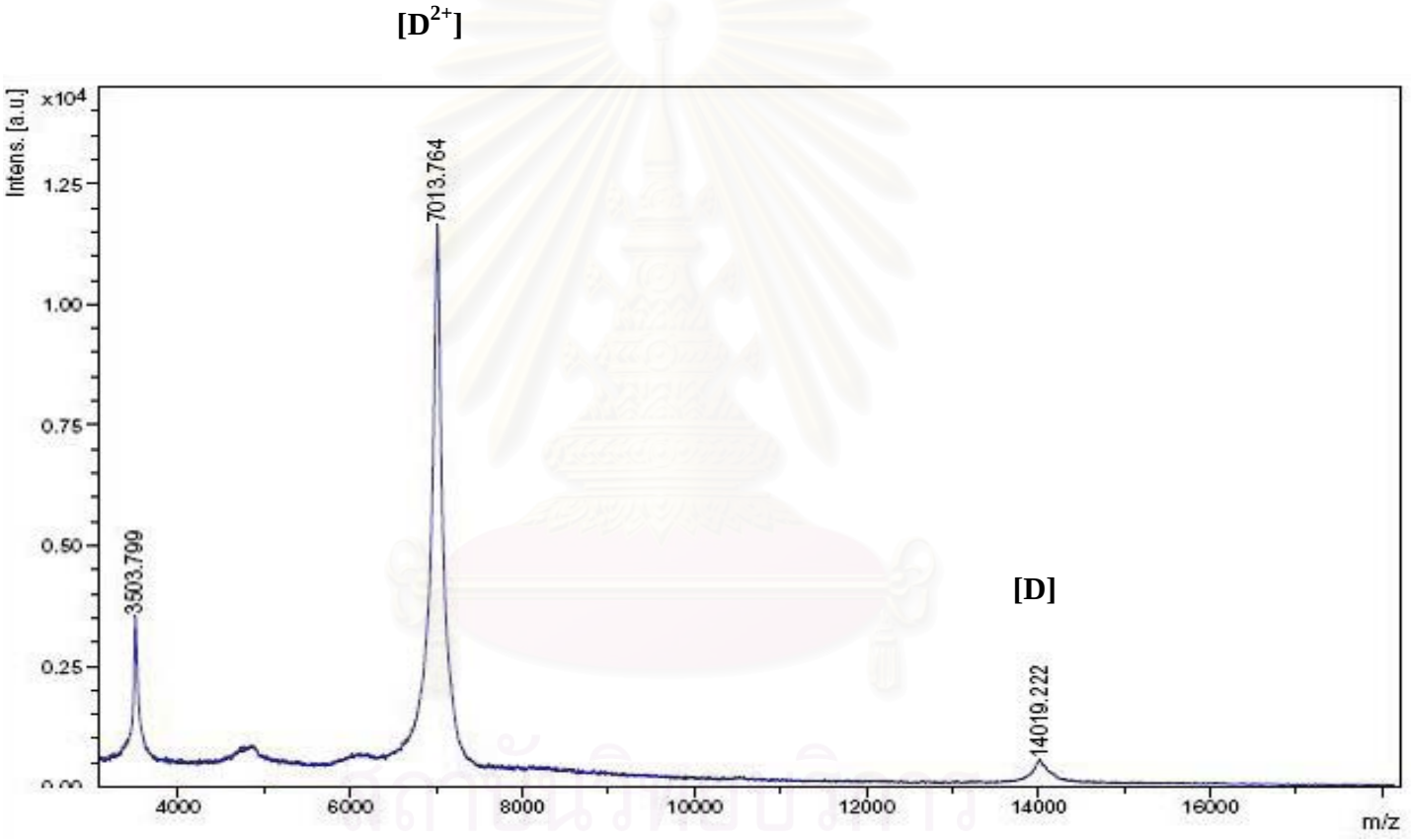

Figure 3F MALDI-TOF MS spectrum of fraction 4 of KV9.

VITA

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