# การสังเคราะห์และศึกษาผลของโปรตีนส่วนดิสอินทีกรินของเมตาโลโปรตีนเนส จากพิษงูเขียวหางไหม้ท้องเหลือง (ไตรเมอรีซูรัส อัลโบลาบรีส) ต่อเกร็ดเลือดของมนุษย์

นางสาวพอน สิงหามาตร



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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# EXPRESSION OF RECOMBINANT SNAKE VENOM METALLOPROTEINASE DISINTEGRIN DOMAIN FROM GREEN PIT VIPER, *TRIMERESURUS ALBOLABRIS*, AND CHARACTERIZATION OF ITS EFFECTS ON HUMAN PLATELETS

Miss Pon Singhamatr

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| Thesis Title | Expression of Recombinant Snake Venom Metalloproteinase Disintegrin Domain from Green Pit Viper, *Trimeresurus albolabris*, and Characterization of Its Effects on Human Platelets |
| By | Miss Pon Singhamatr |
| Field of Study | Medical Science |
| Thesis Advisor | Associate Professor Ponlapat Rojnuckarin, M.D., Ph.D. |

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พอน สิงหามาตร : การสังเคราะห์และศึกษาผลของโปรตีนส่วนดิสอินทีกรินของเมตาโลโปรตีนเนสจากพิษงูเขียวหางไหม้ท้องเหลือง (ไตรเมอรีซูรัส อัลโบลาบรีส) ต่อเกร็ดเลือดของมนุษย์ (EXPRESSION OF RECOMBINANT SNAKE VENOM METALLOPROTEINASE DISINTEGRIN DOMAIN FROM GREEN PIT VIPER, *TRIMERESURUS ALBOLABRIS*, AND CHARACTERIZATION OF ITS EFFECTS ON HUMAN PLATELETS) อ. ที่ปรึกษา รศ.นพ.พลภัทร โรจน์นครินทร์, จำนวน 80หน้า, ISBN 974-53-1374-2.

ดิสอินทีกรินเป็นกลุ่มโปรตีนชนิดหนึ่งที่มีผลในการยับยั้งการเกาะกลุ่มของเกร็ดเลือด ซึ่งมีลำดับกรดอะมิโนอาร์จีดีหรือเคจีดีในสายโปรตีน ลำดับกรดอะมิโนนี้สามารถยับยั้งกระบวนการการทำงานที่อาศัยอินทีกริน เช่น การปฏิสัมพันธ์ระหว่างเซลล์ การเกาะกลุ่มของเกร็ดเลือด เป็นต้น ดังนั้นดิสอินทีกรินจากพิษงูจึงอาจมีประโยชน์ในการรักษาโรคหลอดเลือดแดงอุดตัน ใช้ป้องกันการแพร่กระจายของมะเร็ง หรือในการสร้างแอนตีบอดีเพื่อใช้ในการผลิตชุดตรวจหาพิษงู วิทยานิพนธ์ฉบับนี้ศึกษาคุณสมบัติของโปรตีนส่วนดิสอินทีกรินของเมตาโลโปรตีนเนส (035 SVM) ต่อระบบเกร็ดเลือดของมนุษย์และศึกษาลำดับกรดนิวคลีอิกและลำดับกรดอะมิโนที่ครบถ้วนสมบูรณ์โดยเริ่มจากห้องสมุดซีดีเอ็นเอแรก (Primary cDNA library) ของต่อมพิษงูเขียวหางไหม้ท้องเหลือง

ผลการศึกษาลำดับกรดนิวคลีอิกและลำดับกรดอะมิโนของ 035 SVM จากห้องสมุดซีดีเอ็นเอจากต่อมพิษงูเขียวหางไหม้ท้องเหลืองโดยเทคนิค 5′-RACE พบว่า mRNA ของ 035 SVM มีจำนวนกรดนิวคลีอิกเท่ากับ 2,040 เบส และโปรตีนมีจำนวนกรดอะมิโน 484 ตัว ประกอบด้วยส่วน สัญญาณ โปรเป็ปไทด์ เมตาโลโปรตีนเนส โปรตีนคั่นกลาง และดิสอินทีกริน เมื่อพิจารณาจากลำดับกรดนิวคลีอิกพบว่า 035 SVM เป็นเมตาโลโปรตีนเนส/ดิสอินทีกรินชนิดที่สองและมีลำดับเหมือน Jerdonitin จาก *Trimeresurus jerdonii* ซึ่งมีลำดับกรดอะมิโนเหมือนกันเท่ากับ 84 เปอร์เซ็นต์ ส่วนดิสอินทีกรินของ 035 SVM ซึ่งมีจำนวนกรดอะมิโน 76 ตัว และมีลำดับกรดอะมิโนเคจีดีในสายเปปไทด์ได้ถูกผลิตในยีสต์ *Pichia pastoris* และแยกให้บริสุทธิ์ด้วยวิธีโครมาโตกราฟฟี่แบบจำเพาะ จากการเลี้ยงเซลล์พบว่าได้โปรตีนจำนวน 3.3 มิลลิกรัมต่อลิตร โปรตีนที่ได้จับกันเป็นคู่ (homodimer) มีขนาดประมาณ 11 และ 22 กิโลดาลตัน ในภาวะรีดิวส์และไม่รีดิวส์ ตามลำดับ และมีความสามารถในการยับยั้งการเกาะกลุ่มของเกร็ดเลือดมนุษย์เมื่อถูกกระตุ้นด้วยคอลลาเจน โดยมีค่า $IC_{50}$ เท่ากับ 976 นาโนโมล/ลิตร

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต _____

ปีการศึกษา 2547 ลายมือชื่ออาจารย์ที่ปรึกษา _____

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KEY WORDS: SNAKE VENOM METALLOPROTEINASE / DISINTEGRIN / *TRIMERESURUS ALBOLABRIS.*

PON SINGHAMATR : EXPRESSION OF RECOMBINANT SNAKE VENOM METALLOPROTEINASE DISINTEGRIN DOMAIN FROM GREEN PIT VIPER, *TRIMERESURUS ALBOLABRIS*, AND CHARACTERIZATION OF ITS EFFECTS ON HUMAN PLATELETS. THESIS ADVISOR : ASSOC. PROF. PONLAPAT ROJNUCKARIN, 80 pp. ISBN 974-53-1374-2.

Disintegrins are involved in inhibition of platelet aggregation. They contain RGD or KGD sequences that can inhibit integrin-mediated cell-cell interactions and platelet aggregation. Therefore, snake venom disintegrins are helpful in therapy of arterial thrombosis and tumor metastasis, and producing an antibody for development of a venom diagnostic test kit. The aim of this thesis is to analyze the full-length sequence of 035 SVM from the cDNA library of *Trimeresurus albolabris* venom gland and characterize functions of the disintegrin domain of 035 SVM on human platelets.

From the primary library, partial cDNA of 035 SVM was obtained. Using 5′-RACE method, the full-length sequence of 035 SVM was derived. Analysis of the nucleotide sequence found that the full-length sequence of 035 SVM mRNA was 2,040 bp in length. It was a type N-II snake venom metalloproteinase with 484 amino acid residues comprising signal region, pro-peptide, metalloproteinase domain, spacer region and disintegrin domain. The nucleotide sequence of 035 SVM showed 84 % amino acid sequence identical to *Trimeresurus jerdonii* jerdonitin. The disintegrin domain of 035 SVM, composed of 76 amino acids with a KGD sequence, was expressed in *Pichia pastoris* and purified using affinity chromatography. The yield of recombinant 035 disintegrin was 3.3 mg/liter of culture medium with molecular weight of approximately 11 kDa and 22 kDa on SDS-PAGE in reduced and non-reduced state, respectively. Recombinant 035 disintegrin was a homodimer that inhibited collagen-induced platelet aggregation with $IC_{50}$ of 976 nM.

Field of study Medical Science Student's signature _____

Academic year 2004 Advisor's signature _____

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| A° | Angstorm unit |
| ADAM | a disintegrin and metalloproteinase |
| ADP | adenosine diphosphate |
| Arg | arginine |
| Asp | aspartic acid |
| bp | base pair |
| BCA | bicinchoninic acid |
| °C | degree Celsius |
| cDNA | complementary deoxyribonucleic acid |
| cm | centimeter |
| Cys | cysteine |
| dNTPs | dATP, dTTP, dGTP and dCTP |
| DNA | deoxyribonucleic acid |
| *E. coli* | *Escherichia coli* |
| ECM | extracellular matrix |
| EDTA | ethylenediaminetetra acetic acid |
| FDA | food and drug administration |
| g | gram |
| Gly | glycine |
| GP | glycoprotein |
| HPLC | high performance liquid chromatography |
| $IC_{50}$ | concentration of inhibitor required to inhibit 50 % aggregation |
| IPTG | isopropy1-β-D-thiogalactopyranoside |
| kb | kilobase |

| | |
|---|---|
| kDa | kiloDalton |
| KGD | Lys-Gly-Asp |
| Ki | constant inhibition |
| L | Liter |
| LB | Luria-Bertani media |
| Lys | lysine |
| mg | milligram |
| ml | milliliter |
| mM | millimolar |
| M | molar |
| MLD | methionine leucine aspartic acid |
| ng | nanogram |
| nm | nanometer |
| N | normal |
| NMR | nuclear magnetic resonance |
| OD | optical density |
| ORF | open reading frame |
| pmol | picomole |
| PCR | polymerase chain reaction |
| $PLA_2$ | phospholipase $A_2$ |
| PNGaseF | peptide-N-glycosidase |
| RACE | rapid amplification of cDNA end |
| RGD | Arg-Gly-Asp |
| SDS | sodiumdodesylsulphate |
| SDS-PAGE | sodiumdodesylsulphate polyacrylamide gel electrophoresis |
| SVMPs | snake venom metalloproteinases |
| Thr | threonine |

| | |
|---|---|
| Tris-HCl | tris-(hydroxymethyl)-aminoethane |
| UTR | untranslated region |
| VGD | valine glycine aspartic acid |
| YPDS | yeast extract peptone dextrose medium |
| v/v | volume/volume |
| w/v | weight/volume |
| µg | microgram |
| µl | microliter |
| µm | micrometer |

# CHAPTER I

# INTRODUCTION

## 1.1 Background and Rationale

Snakebite is a common medical emergency. Its severity ranges from minimal local pain and swelling to severe pain, marked edema, tissue necrosis, hemorrhage, shock and death. Therefore, it must be promptly diagnosed and adequately treated to minimize local tissue destruction and systemic complications in order to prevent death. Snakes can be found throughout the world. Approximately 15 percent of the 3000 species of snakes worldwide are considered to be dangerous to humans (*1*). The incidence of venomous snakebite in Thailand is about 11,000 per year with the mortality rate around 0.02 per 100,000 (*2*, *3*). Most important species are Malayan pit viper (40%), Green pit viper (34%), Cobra (12%), and Russell's viper (10%). There are about 160 species of snake in Thailand and 47 of them are venomous. Two important families of the venomous snakes in Thailand are elapidae and viperidae that are neurotoxic and hematotoxic, respectively. Elapidae consists of Cobra, Spitting cobra, King cobra, Banded krait and Malayan krait. They are widely distributed throughout Thailand. Viperidae family can be classified into two subgroups: the true viper (Viperinae) and the pit viper (Crotalinae). The true viper subgroup consists of Russell's viper or *Daboia russelli*. They are most commonly found in the central and eastern parts of Thailand. The pit viper subgroup consists of Malayan pit viper and Green pit viper. Malayan pit vipers are found in the southern, eastern and northern parts of Thailand. Green pit vipers are the most common venomous snakes found most frequently in the central part of Thailand especially in Bangkok and the nearby areas (*4*).

### 1.1.1 Green pit viper

Green pit vipers are venomous snakes having long fangs used to transfer venom. Fangs are normally folded up against the upper jaw. Green pit vipers have a pair of heat-sensing pit organs located between nostrils and eyes on each side of the head. The pit organ is a thermo-receptor sensitive to very small changes in temperature. It is supplied with nerves and blood vessels and partially enclosed in a cavity in the side of the maxillary bone. Green pit vipers characteristically have a broad, lance-shaped head and vertical pupils. They are generally slender in shape and green in color with yellow markings. They are small snakes, usually attaining less than 1.5 meter in length. Green pit vipers are members of the genus *Trimeresurus*. The two most common species of *Trimeresurus* are *Trimeresurus albolabris* and *Trimeresurus macrops* (*4*) that are most prevalent in Bangkok and the nearby areas. *Trimeresurus albolabris* is a tree-dwelling snake with a red tail and a yellow belly.

### 1.1.2 Clinical signs and symptoms of green pit viper bites

Snake venom can be classified by their effects into three categories: neurotoxin, myotoxin and hematotoxin (*2*). Green pit viper venom affects hemostatic system. The clinical characteristic of green pit viper bites, similar to other venomous snakes, is the presence of one or more fang marks that look like puncture wounds with or without scratches. The toxic symptoms can be divided into two categories: local and systemic effects. The local effects start to appear within 30 to 60 minutes after pit viper envenomation. These effects include pain, edema, blister, hemorrhage and ecchymosis at the bite sites (*5*). The systemic effects include coagulopathy, hypofibrinogenemia and thrombocytopenia (*6*, *7*, *8*). Green pit viper bites may also result in hypotension, respiratory distress, or severe tachycardia.

### 1.1.3 Component of green pit viper venom

Approximately 90 percent of pit viper venom are proteins that can be categorized into serine proteinases, phospholipases $A_2$ (*9*), C-type lectins (*10*), metalloproteinases, and disintegrins (*11*, *12*, *13*).

Green pit viper serine proteinases have either fibrinogenolytic or fibrinolytic activities. However, one of the most prominent activity is called thrombin-like proteinase. Phospholipase $A_2$ ($PLA_2$) is the major component in the snake venom. $PLA_2$ is a non-glycosylated proteins that can induce a wide variety of pharmacological effects by interfering with several physiological functions, including endothelial injury, myotoxicity, thrombosis and hemostasis. Several isoforms of $PLA_2$ have been found in a single species and various isoforms are different among different species.

Snake venom C-type lectins contain amino acid sequence homology to the calcium regulatory domain of mammalian lectins. Proteins in this family typically comprise disulfide-linked αβ heterodimers. C-type lectins have been demonstrated to promote platelet aggregation by targetting von Willebrand factor, glycoprotein Ib-IX-V, glycoprotein VI, and possibly other platelet receptors.

Snake venom metalloproteinases (SVMPs) are multi-domain proteins that compose of a catalytic domain and one or several non-catalytic domains. These proteins have a molecular mass of 20 to 100 kDa comprising a signal peptide, a pro-sequence, a spacer region, a metalloproteinase domain, a disintegrin-like or disintegrin domain with or without a cysteine-rich carboxyl terminus. SVMPs are homologous to mammalian proteins in a disintegrin and metalloproteinase (ADAMs) family. However, ADAMs proteins have other domains besides those of SVMPs, e.g., an epidermal disintegrin-like domain, a transmembrane domain and a cytoplasmic domain. The metalloprotenase domain of SVMPs contains a zinc-

binding consensus sequence, HEXXHXXGXXH, which makes it belongs to the metzincins family of zinc-dependent metalloproteinase. Chelation of the $Zn^{2+}$ ion with EDTA or 1, 10-phenanthroline abolishes its proteolytic and hemorrhagic activities (*14*).

On the basis of their domain structure, SVMPs precursors can be classified into four main groups (Figure 1): 1) P-I, comprising only the metalloproteinase domain; 2) P-II, having a metalloproteinase domain followed by a disintegrin or disintegrin-like domain; 3) P-III, comprising metalloproteinase, disintegrin-like and cysteine-rich domains; and 4) P-IV, a group of enzymes that posseses, besides the three described domains, an additional lectin-like polypeptide linked by a disulfide bridge to the metalloproteinase-containing polypeptide chain. The disintegrin-like domain in class III and IV enzymes, as well as in some of class P-II metalloproteinases, has high sequence identity with venom disintegrins. However, the disintegrin-like domain does not contain the typical Arg-Gly-Asp (RGD) sequence that found in venom disintegrins.

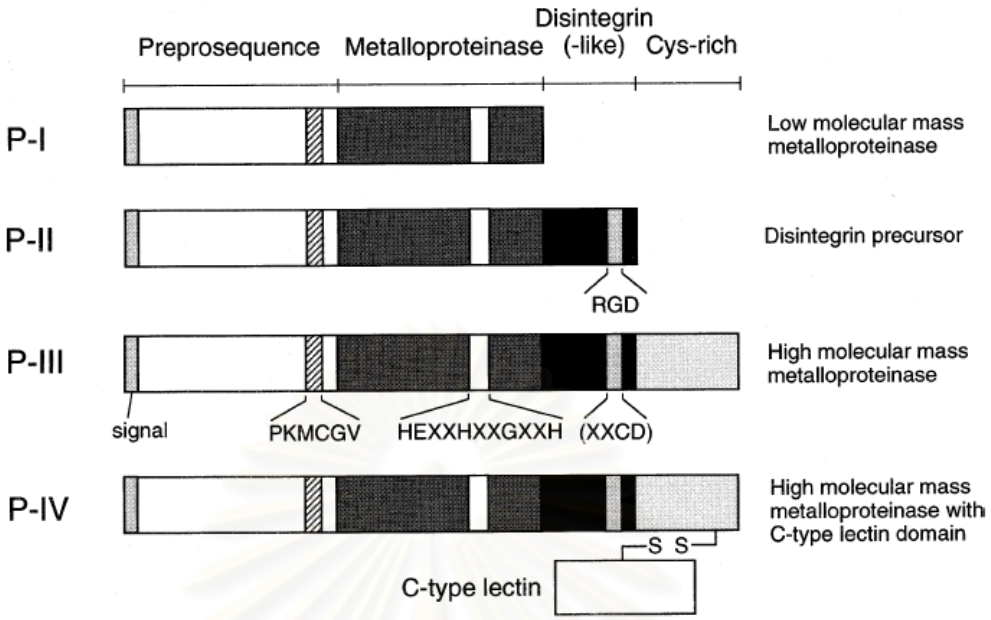



**Figure 1** Schematic structures of snake venom metalloproteinases.

The venom metalloproteinases generally show hemorrhagic activity. These protein families cause hemorrhage by digestion of components of extracellular matrix (ECM) proteins, e.g., collagen and laminin. In addition, these proteins can also digest some blood coagulation factors, including von Willebrand factor, enhancing its hemorrhagic effect.

Disintegrins, the main focus of this study, are small peptides ranging from 49 to 84 amino acid residues in size. They are usually derived from class P-II snake venom metalloproteinase precusors and concomitantly released with mature metalloproteinases. There is a report showing that the disintegrin domain is autoproteolysed from its metalloproteinase precusor in the absence of $Ca^{2+}$ at 37 $^{\circ}$C (*15*).

Disintegrins are classified into monomeric and dimeric disintegrins. Monomeric disintegrins can be divided, on the basis of their length and number of cysteine residue, to three groups: 1) short disintegrins, composed of 49 – 51 amino

acid residues including 8 cysteine residues; 2) medium-size disintegrins, composed of about 70 amino acid residues including 12 cysteine residues; 3) long disintegrins, composed of 84 amino acid residues including 14 cysteine residues (*16*). All cysteine residues in a disintegrin are involved in disulfide bond formation (*17*). However, the disulfide-bond patterns are slightly different among disintegrins.

The dimeric disintegrins are classified into homodimeric and heterodimeric groups. Both subunits of homodimeric disintegrin have identical polypeptide chains. An example of homodimeric disintegrin is contortrostatin that is isolated from *Agkistrodon contortrix contortrix*. Heterodimeric disintegrins have different lengths of mature proteins and nonidentical polypeptide chains. An example of heterodimeric disintegrin is EC3 that is isolated from *Echis carinatus*.

Disintegrin proteins contain either the Arg-Gly-Asp (RGD) or Lys-Gly-Asp (KGD) conserved sequences. The RGD sequence in these peptides is a potent inhibitor of integrins, such as $\alpha_{IIB}\beta_3$ (fibrinogen receptor on platelets surface). Disintegrin that possesses a KGD sequence, instead of RGD sequence, is also effective antagonist of the fibrinogen receptor. It has been found that sequences flanking the RGD or KGD site determined the specificity and affinity of the disintegrins to different integrins (*18*, *19*). In addition, the regulation of the binding affinity also involves the C-terminal region that is located near the RGD/KGD loop on 3-D structure exposing to the integrin receptor. Supporting this conclusion, there is a report showing that a change in amino acids adjacent to the C-terminus of the RGD alters its affinity to integrins (*20*, *21*).

### 1.1.4 Venom disintegrins acting on platelet aggregation (*9*, *10*, *22*, *23*)

Hemostasis is the process to arrest bleeding from an injuried blood vessel. This process involves clot formation and clot dissolution. These two opposite processes are in equilibrium. The hemostasis is achieved through an interaction of the vessel wall, platelet and coagulation .

Platelets are small discoid anucleate cells with 2 to 3 μm diameter. They remain in circulation for approximately 10 days. These cells take part in a series of complex reactions to prevent blood loss. To prevent damage of vessels at high shear rates, platelets adhere to von Willebrand factor and collagen in the subendothelium via their surface glycoprotein (GP) Ib-IX-V and collagen receptors, respectively (*24*, *25*). This process activates platelets, causing conformational changes of the fibrinogen receptor GP IIb-IIIa ($\alpha_{IIB}\beta_3$ integrin) inducing fibrinogen binding (Figure 2). In addition, binding of these ligands to their receptors enhances platelets activation and aggregation (*26*).

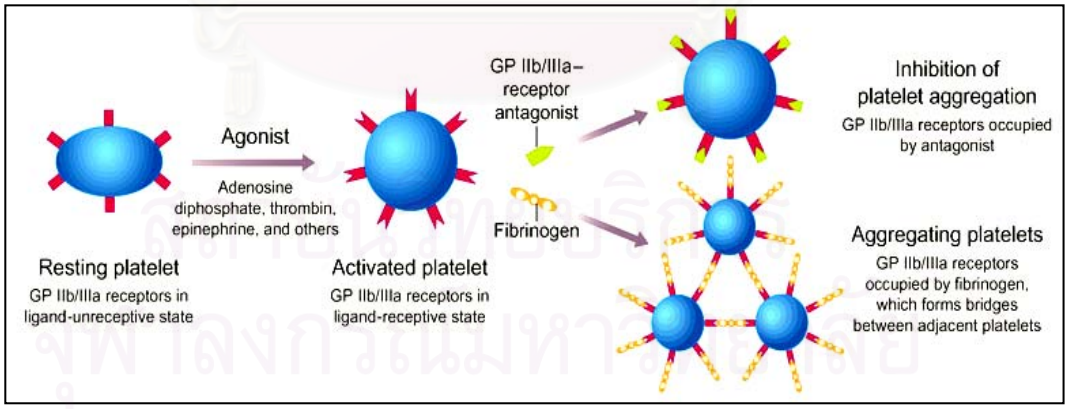



**Figure 2** Overview of the processes of platelet activation and aggregation and the inhibition of platelet aggregation by inhibitors of glycoprotein (GP) IIb/IIIa receptors.

Platelet aggregation is a key event in thrombus formation depending upon the binding of fibrinogen to glycoprotein (GP) IIb-IIIa receptor complex located on platelet surface, where the RGD sequence of fibrinogen is engaged. The RGD sequence is identified as the cell recognition site on fibronectin molecules and presented in a variety of ECM and plasma proteins, including fibrinogen, von Willebrand factor, collagen, vitronectin, thrombospondin as well as venom disintegrins.

Venom disintegrins are appeared to function as platelet aggregation inhibitors by binding to the platelet GP IIb-IIIa complex, blocking binding of adhesive proteins, such as fibrinogen and von Willebrand factor. In addition, disintegrins can block the adhesive function of other RGD-dependent integrins such as the vitronectin receptors ($\alpha_V\beta_3$ and $\alpha_V\beta_5$) and fibronectin receptor ($\alpha_V\beta_1$). These receptors involved in cell migration and invasion of tumor cells. Therefore, disintegrins show a therapuetic potential for the treatment of tumor metastasis, a process required cell-EMC interaction via integrins (*11*, *27*, *28*, *29*, *30*, *31*, *32*).

Although many reports have shown that disintegrins could be expressed in *Escherichia coli* (*E. Coli*) with full activity. *E. Coli* expression system is not appropriated for highly disulfide-bonded proteins because the cytoplasm of *E. Coli* possesses a reducing environment. Furthermore, there is also a problem in post-translational modification (*33*). Therefore, recombinant proteins from *E. coli* may be inactive. In contrast to *E. Coli*, *Pichia pastoris* is uniquely suited for foreign protein expression due to three key reasons: it can be easily manipulated at the molecular level, it can express proteins at high level, and it can perform post-translational modifications of proteins similar to higher eukaryotes (*34*).

This research studied the functions of a disintegrin domain from a snake venom metalloproteinase of *Trimeresurus albolabris* by expression of the disintegrin in *Pichia pastoris* system and also investigated the effects of the

disintegrin domain on human platelets. The results from this research is potentially useful for developing a novel antiplatelet agent. Furthermore, antibody to this protein may be used to develop a diagnostic kit or therapeutic antivenom for green pit viper bite patients.

### 1.2 Research Questions

Does the recombinant snake venom metalloproteinase disintegrin domain from *Trimeresurus albolabris* inhibit human platelet aggregation?

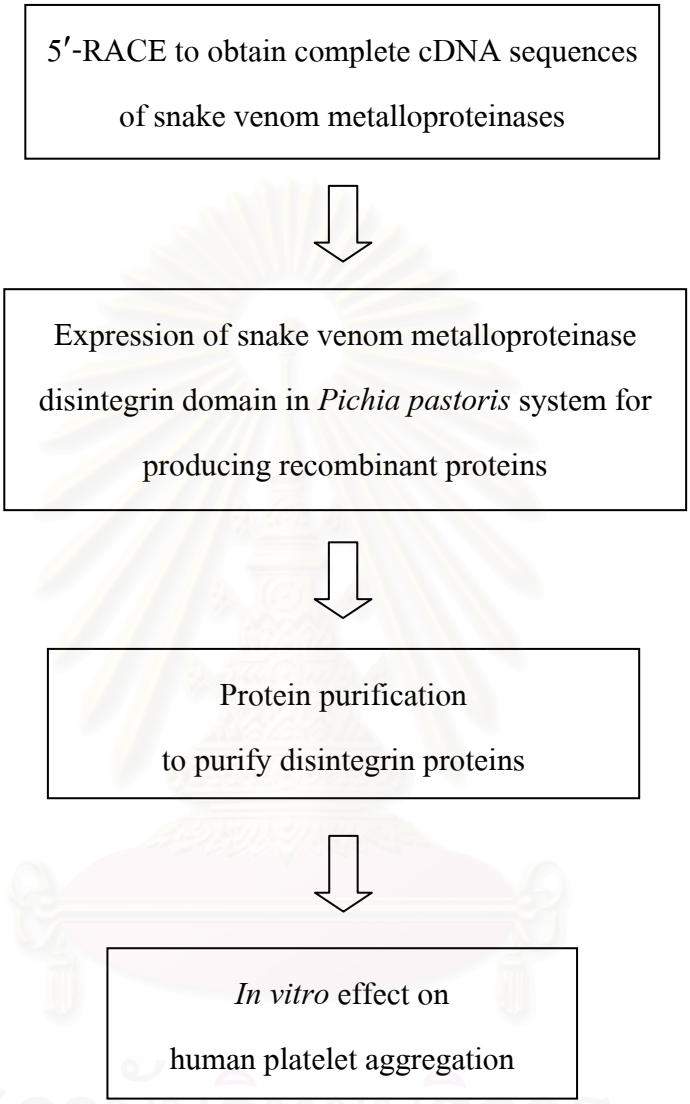
### 1.3 Objectives of the Study

1. To clone and analyze complete cDNA sequence of snake venom metalloproteinase from *Trimeresurus albolabris*.
2. To express and purify the snake venom metalloproteinase disintegrin domain in *Pichia pastoris* system.
3. To study the effects of snake venom metalloproteinase disintegrin domain on human platelets.

### 1.4 Keywords

Snake Venom Metalloproteinase, Disintegrin, *Trimeresurus albolabris*.

## 1.5 Conceptual Framework




## 1.6 Benefits and Applications

1. The study will give insights in the functions of snake venom metalloproteinase disintegrin domain on the mechanism of platelet aggregation.
2. These recombinant proteins are useful for making an antibody for developing a diagnostic test.
3. These disintegrins may be useful as antiplatelet agents in the future.

# CHAPTER II

# LITERATURE REVIEW

This thesis studied the expressions and effects of the recombinant venom disintegrin domain of snake venom metalloproteinase (SVMPs) from *Trimeresurus albolabris*. To the author's knowledge, this study has never been reported in the literature. In the following sections, a literature review related to the characteristics and the relationships of SVMPs and disintegrins, the effects of native and recombinant disintegrins on human platelets, and the production of recombinant disintegrins in *Pichia* expression system are presented.

## 2.1 SVMPs and Disintegrins: Characteristics and Relationships

SVMPs and disintegrins are important components of most viperid and crotalid venoms. SVMPs are varying in size from 20 to 100 kDa and composed of multidomain, e.g., a pro-peptide, a metalloproteinase, a disintegrin (-like), and a cysteine-rich domain. In contrast to SVMPs, disintegrins are cysteine-rich low molecular weight polypeptides, which contain tripeptide sequence recognized by integrin receptors. Notably, the amino acid sequence of disintegrin region of SVMPs shows a high degree of homology to the disintegrins protein (*11*). There is a study showing that the amino acid sequence of the metalloproteinase domain of flavostatin precursor is identical to that of HR2a which is a low molecular mass hemorrhagic factor of *Trimeresurus flavoviridis* venom composed only of a metalloproteinase domain (*38*). Therefore, it can be concluded that the flavostatin precursor belongs to metalloproteinase/disintegrin family. The metalloproteinase and disintegrin proteins in snake venom can be derived by proteolysis from a common metalloproteinase/disintegrin precursor.

The disintegrins comprise a large family of RGD-containing venom proteins. They can be divided into monomeric and dimeric groups (*17*). The striking features of disintegrins are the disulfide bonds, tripeptide sequence, amino acids adjacent to the tripeptide sequence, and C-terminal sequence.

The disulfide bond paring of trimestatin from *Trimeresurus flavoviridis* was studied by nuclear magnetic resonance (NMR) technique. The results showed, at 1.7 A° resolution, the rigid structure held together by 6 conserved disulfide bonds (*20*).

The tripeptide sequence is essential for binding to integrin receptor. Most monomeric disintegrins contain tripeptide sequence, RGD or KGD, whereas a minority of the dimeric disintegrins has VGD/MLD sequences, e.g., EC3 in its A and B subunits, respectively (*39*). The study of several short- and medium-sized disintegrins by NMR technique showed that the active tripeptide is located at the apex of a mobile loop protruding 14 – 17 A° from the protein core (*40*, *41*). The crystal structure of trimestatin shows that the RGD sequence is located at the tip of a long loop and there is irregular 15 residue hairpin loops from Thr43 to Cys57 (*20*).

The amino acid residues adjacent to the RGD sequence play an important role in ligand binding affinity. The Arg49 and Asp51 side-chains of the RGD sequence of trimestatin point to opposite directions allowing the unique RGD conformation without steric hindrance from neighboring side-chains. In addition, changing of the amino acids adjacent to the C-terminus of the RGD alters its affinity to integrins. The synthetic mutant of echistatin with an aspartic acid 27 in echistatin substituted with tryptophane showed increased inhibitory activity against platelet aggregation, increased potency to block fibrinogen binding to $\alpha_{IIB}\beta_3$, and decreased potency to block vitronectin binding to $\alpha_V\beta_3$ as compared to wild-type (*21*). Therefore, the

amino acids adjacent to the RGD sequence also affect the selectivity of disintegrins.

The affinity and selectiveness of snake venom disintegrins for integrin receptors also involve the C-terminal sequence. The amino acid residues in the C-terminal region of disintegrins are not highly conserved among the snake venom disintegrins. The NMR studies show that the C-terminal region locates near the RGD loop and exposes to the outside of the molecule. Moreover, deletion of the C-terminal amino acid of echistatin decreased the ability in binding to $\alpha_{IIB}\beta_3$ and, hence, decreased the inhibition of platelet aggregation (*21*).

**2.2 Effect of Native and Recombinant Disintegrins on Human Platelets**

Snake venom constitutes a rich source of biologically active proteins that interact with hemostatic system, either by promoting or inhibiting particular steps of platelet activation, coagulation cascade or fibrinolysis. Viper venom disintegrins, found in various snake venoms, are in a family of platelet aggregation inhibitors. Most of the venom disintegrins contain RGD or KGD sequence that is the structural motif recognized by the platelet fibrinogen receptor $\alpha_{IIB}\beta_3$. In addition, venom disintegrins also act as potent antagonists of several integrins including $\alpha_V\beta_3$ and $\alpha_5\beta_1$, that are expressed on vascular endothelial cells and some tumor cells (*42*).

Many articles have shown that the disintegrins can affect the platelet aggregation (*29*, *30*, *31*, *43*). Adinbitor, a novel disintegrin from Chinese snake (*Agkistrodon halys brevicaudus steineger*) cloned and characterized by Wang et al. (*44*), encodes a polypeptide, which composes of 73 amino acids, including 12 cysteines and an RGD motif. It was expressed in *E. Coli* with polyhistidine tag and purified using the His-Bind affinity chromatography. Wang et al. found that adinbitor could inhibit human platelet aggregation in a dose-dependent manner.

Human platelet aggregation is completely inhibited by recombinant adinbitor at concentration of 8.4 μM. The $IC_{50}$ value of recombinant adinbitor in inhibiting platelet aggregation is 6 μM. The rhodostomin, isolated from the venom of *Calloselasma rhodostoma*, consists of 68 amino acids, including 12 residues of cysteine and an RGD sequence. Recombinant rhodostomin expressed in *E. coli* and *Pichia pastoris* can inhibit platelet aggregation with a resulting constant inhibition (Ki) value of 263 nM and 70 nM, respectively, indicating that the recombinant protein from *Pichia pastoris* is more active than those from *E. coli* (*33*). The disintegrin domain of jararhagin, a P-III snake venom metalloproteinase, is isolated from *Bothrops jararaca* venom. The recombinant disintegrin is produced in *E. Coli* system after induction by 1 mM isopropyl-thio-β-D-galactopyranoside (IPTG). It inhibites collagen-induced platelet aggregation in a dose-dependent manner with $IC_{50}$ value of 8.5 μg/ml (*44*).

Like monomeric disintegrins, dimeric disintegrins have also been reported that they can serve as a potent inhibitor of human platelet aggregation. Contortrostatin is a homodimeric disintegrin isolated from *Agkistrodon contortrix contortrix* venom. Each chain of contortrostatin has 65 amino acid residues and an RGD sequence. Recombinant contortrostatin inhibit ADP-induced human platelet aggregation with $IC_{50}$ value of 250 nM. Acostatin, a heterodimeric disintegrin purified from *Agkistrodon contortrix contortrix* venom, contains 63 and 64 amino acids and designated acostatin 1 and acostatin 2, respectively. Each subunit of acostatin inhibit ADP-induced platelet aggregation in human PRP with $IC_{50}$ value of $65 \pm 19$ and $73 \pm 30$ nM, respectively.

In addition, it has been reported that the peptide that belongs to disintegrin family serves as a model for anti-platelet drug development (*45*, *46*). For example, abciximab, the drug for treatment of acute coronary syndromes, has received USA food and drug administration (FDA) approval with three other $\alpha_{IIB}\beta_3$ blockers.

Abciximab shares the same binding site as barbourin (*48*). Barbourin is a disintegrin protein found in the venom of the Southwestern pigmy rattle snake, *Sistrurus barbourii*. It composes of 73 amino acids and a KGD integrin binding site. Recombinant barbourin is produced in *Pichia pastoris* after induced by 0.5 % v/v methanol and purified by nickel column affinity chromatography using C-terminal hexahistidine tags. Recombinant barbourin is effective in inhibiting platelet aggregation either ADP-induced or thrombin-induced aggregation with its $IC_{50}$ value of 330 – 370 nM range (*49*, *50*). For the native disintegrin protein, there are many studies characterizing disintegrin proteins. Bothrasperin, a disintegrin isolated from Central American viperid snake, *Bothrops asper* venom, is fractionated by a combination of gel filtration, anion-exchange chromatography and reverse-phase high performance liquid chromatography. The purified protein has potent aggregation inhibitory activity on ADP-stimulated human platelet with an $IC_{50}$ value of 50 nM (*51*). Similar to bothrasperin, saxatilin is purified from fresh venom of *Gloydius Saxatilis*, a Korean snake, by a combination of gel filtration and reverse-phase HPLC. The purified saxtilin inhibited ADP-induced platelet aggregation with an $IC_{50}$ value of 127 nM (*30*).

### 2.3 Production of Recombinant Disintegrins in *Pichia* Expression System

*Pichia pastoris* is a methylotrophic yeast that has been developed to be a highly successful system for production of a variety of heterologous proteins for both basic research and industrial uses. For example, metalloproteinase domain of ADAM9 (*52*), barbourin (*50*), rhodostomin (*33*), laccase (*53*), β-mannanase (*54*), and bovine β-casein (*55*) have been successfully expressed in *Pichia pastoris* system in the correct active forms. *Pichia pastoris* is a suitable host strain for the production of proteins for several reasons. *Pichia pastoris* has a strong inducible promoter, *AOX1*, to induce high levels of transcription (*35*). Moreover, *Pichia*

*pastoris* has the potential to perform many post-translational modifications typically associated with higher eukaryotes, such as processing of folding, disulfide bridge formation, certain types of lipid addition as well as *O*- and *N*-linked glycosylation. In addition, *Pichia pastoris* does not secrete a lot of its own proteins into culture medium. The isolation of the interest protein, thus, can be facilitated.

# CHAPTER III

# MATERIALS AND METHODS

## 3.1 Materials

### 3.1.1 Obtaining Full Length cDNA

#### *3.1.1.1 Rapid Amplification of cDNA Ends (RACE)*

We use SMART™ RACE cDNA Amplification Kit purchased from CLONTECH Laboratories, Inc.

#### *3.1.1.2 Gene Specific Primers* (GSP)

Synthetic oligonucleotides were purchased from BGM

**Table 1** Oligonucleotides and their descriptions.

| Name | Sequence | Description |
|---|---|---|
| T7 | 5′- GTAATACGACTCACTATAGGGC -3′ | Sequencing primer from T7 promoter |
| SP6 | 5′-ACTCAAGCTATGCATCCAAC –3′ | Sequencing primer from SP6 promoter |
| SVM1 | 5′-GCGCTTCAGCAAATCTCTCTCTCTCCA-3′ | 1st5′-RACE PCR |
| SVMR | 5′-AAATCAAATCTGAGAGAAGCCAGAGGT TGAT-3′ | 2nd5′-RACE PCR |

**Table 1** Oligonucleotides and their descriptions. (Cont.)

| Name | Sequence | Description |
|---|---|---|
| SVMR 16F.2 | 5′- CGGAATTCCATCATCATCATCATCATG AGGTGGGAGAAGATTGTG-3′ | GSP for PCR of the N-terminal of disintegrin domain with 6xHis and *Eco*R I recognition site |
| SVMR 16R.2 | 5′- GCTCTAGATTAGCCATAGTAGCCAATC CAG-3′ | GSP for PCR of the C-terminal of disintegrin domain with stop codon and *Xba* I recognition site |
| 5′- *AOX1* | 5′- GACTGGTTCCAATTGACAAGC –3′ | *Pichia* sequencing primer |
| 3′- *AOX1* | 5′- GCAAATGGCATTCTGACATCC –3′ | *Pichia* sequencing primer |
| α- Factor | 5′- TACTATTGCCAGCATTGCTGC –3′ | *Pichia* sequencing primer |

#### *3.1.1.3 DNA Extraction and Purification from gel slice*

QIAquick® Gel Extraction Kit was purchased from QIAGEN Inc., U.S.A.

Wizard® SV Gel and PCR Clean-Up System was purchased from Promega, U.S.A.

#### *3.1.1.4 Cloning of RACE Products*

pGEM®-T Easy Vector System II was purchased from Promega, U.S.A. It contains *Eschericia coli*, JM 109 strain, pGEM®-T Easy Vector, T4 DNA Ligase and 2x Rapid Ligation Buffer.

Isopropyl-β-D-Thiogalactopyranoside (IPTG), Dioxane-Free, Formula weight 238.3 was purchased from Promega, U.S.A.

5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 100 mg was purchased from Promega, U.S.A.

#### *3.1.1.5 Enzymes*

| | |
|---|---|
| Tag DNA polymerase | (Invitrogen™ life technologies) |
| T4 DNA Ligase | (Promega) |
| *Eco*R I | (Sigma) |
| *Xba* I | (Promega) |
| *Sac* I | (harmacia Biotech) |



#### *3.1.1.6 DNA Sequencing*

We use ABI PRISM® BigDye® Terminator V.3.1 Cycle Sequencing Kit purchased from AB Applied Biosystems, U.S.A.

### 3.1.2 Expression of Disintegrins in *Pichia pastoris*

#### *3.1.2.1 Polymerase Chain Reaction*

Gene specific primers were purchased from Biogenomed.

#### *3.1.2.2 Pichia expression system*

EasySelect™ *Pichia* Expression Kit Version G, 122701, was purchased from Invitrogen™ life technologies.

#### *3.1.2.3 Proteins Detection*

*3.1.2.3.1 Sodiumdodesylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

Mini-Protein 3 Electrophoresis apparatus was purchased from Bio-Rad Laboratories, Ltd.

Prestained Protein Marker, Broad Range (Premixed Format) was purchased from New England BioLabs Inc.

Coomassie Brilliant Blue R-250 was purchased from USB, U.S.A.

*3.1.2.3.2 Western Blotting Hybridization*

Trans-Blot® SD semi-dry electrophoretic transfer cell was purchased from Bio-Rad Laboratories, Ltd.

Polyvinylidene difluoride (PVDF) membrane 0.45 μm was purchased from Bio-active Co., Ltd.

Mouse Anti-His antibody was purchased from Amersham Biosciences, Ltd.

Polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP was purchased from Dako Cytomation, Denmark.

3, 3′-Diaminobenzidine (DAB) tetrahydrochloride was purchased from PIERCE Biotechnology.

#### *3.1.2.4 Protein Purification*

Protein purification using Immobilized Metal Affinity Chromatography (IMAC). Talon Super-flow Metal Affinity Resin was purchased from BD Biosciences.

#### *3.1.2.5 Concentration of Protein*

Vivascience ultrafiltration was purchased from Vivascience Sartorius Group.

#### *3.1.2.6 Protein Quantitative Assay*

Micro BCA™ Protein Assay Reagent Kit was purchased from PIERCE Biotechnology.

### 3.1.3 Activity Assay

Collagen was purchased from Sigma, U.S.A.

## 3.2 Methods

### 3.2.1 Obtaining full length snake venom metalloproteinase/disintegrin cDNA

Green pit viper venom gland library has been previously prepared. Two partial sequence P-II SVMPs have been cloned from green pit viper, called clone 035 and 038 SVM.

#### *3.2.1.1 5′-RACE*

5′-RACE was used for generating full length cDNA. The templates of 5′-RACE-PCR are prepared by reverse transcriptase polymerase chain reaction (RT-PCR) using the joint action of the SMART IIA™ oligonucleotide and PowerScript™, a variant of MMLV reverse transcriptase, reverse transcription. For preparation of 5′-RACE-Ready cDNA, we synthesyzed the first strand cDNA using poly $A^+$ RNA in the reaction as followed. Firstly, 500 ng of poly $A^+$ RNA from venom gland of *Trimeresurus albolabris*, 1 µl of 5′-CDS primer, 1 µl of SMART IIA oligonucleotide and sterile $H_2O$ are combined to a final volume of 5 µl. It is, then, incubated at 70 °C for 2 minutes. When reach the given time, the reaction tube was kept on ice for 2 minutes. After that, the following reagents are added to the reaction; 2 µl of 5X first-strand buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 30 mM $MgCl_2$), 1 µl of DTT (20 mM), 1 µl of dNTP Mix (10 mM each), and 1 µl of PowerScript Reverse Transcriptase. The tube is then incubated at 42 °C for 1.5 hours in an air incubator. Finally, the first-strand reaction solution is diluted with Tris-EDTA buffer (10 mM Tris-KOH pH 8.5, 1 mM EDTA) and heated at 72 °C for 7 minutes.

At this point, we have 5′-RACE-Ready cDNA template. The SMART IIA™ oligonucleotide is incorporated in its 5′end. 5′-RACE is carried out by using the SMART RACE cDNA amplification Kit with gene specific primers based on nucleotide sequence derived from the primary library. The Tm should be between

60 – 70 °C. Firstly, PCR Master mix is prepared by combining 27 µl of PCR-Grade Water, 5 µl of 10X Advantage 2 PCR Buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 1 µl of dNTP Mix (10 mM each), and 1 µl of 50X Advantage 2 Polymerase. Secondly, 10 µl of 5′-RACE-Ready cDNA, 5 µl of 10X Universal Primer A Mix (UPM) that is complementary to SMART IIA oligonucleotide, and 10 µl of 10 pM Gene specific primer for clone 035 are then added to 34 µl of PCR Master mix as described. We used PE GeneAmp Systems 2400 thermal cycler for amplifying 5′-RACE fragments using 40 cycles with the temperature cycling parameters: 94 °C for 5 seconds of denaturation, 68 °C for 10 seconds of annealing and 72 °C for 3 minutes of extension.

#### *3.2.1.2 DNA Extraction and Purification from Gel Slice*

After amplification of 5′-RACE, the RACE products is electrophoresed on 1.2 % agarose gel. A band of DNA is excised from agarose gel with a sterile blade. The RACE products are purified by the NucleoTrap™ Gel Extraction Kit. Two volumes of NT 1 Buffer to one volume of gel are added and the tube is placed in 50 °C water bath incubator. After agarose gel is completely dissolved, the tube is centrifuged at 20,000 x g for 30 seconds and supernatant is discarded. Then, 500 µl the NT 2 Buffer is added, mixed, and centrifuged at 20,000 x g for 30 seconds. The supernatant is discarded, repeat this step once. 500 µl the NT 3 Buffer is then added and centrifuged at 20,000 x g for 30 seconds. The supernatant is discarded, this step is repeated once and the pellet is air-dried. Finally, EB buffer is added to elute DNA before centrifugation at 20,000 x g for 10 minutes. After that, DNA is precipitated using 0.3 M sodium acetate in 100% ethanol before centrifugation at 20,000 x g for 10 minutes. The supernatant is discarded. The pellet is washed with 1 ml of 70 % ethanol, and centrifuged at 20,000 x g for 10 minutes. The supernatant is discarded. The dry pellet is dissolved with TE buffer pH 8.0 (10 mM

Tris-HCl pH 8.0 and 1 mM EDTA pH 8.0) or distilled water, and stored at –20 °C until use.

#### *3.2.1.3 Cloning of RACE Products*

##### *3.2.1.3.1 Ligation of RACE Products into pGEM®-T easy Vector.*

After the 5′-RACE products are purified by the NucleoTrap Gel Extraction Kit, the 5′-RACE products are cloned into pGEM®-T easy Vector. The ligation procedure is carried out in a 10 µl. The ligation reaction mixture contains 5 µl of 2X Rapid Ligation Buffer (60 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP and 10% PEG), 50 ng of pGEM®-T easy Vector, 3 Weiss units of T4 DNA Ligase and an appropriate amount of A-tailing PCR products that optimized from the insert: vector ratio of 3:1 by using the following equation.

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \frac{3}{1} = \text{ng of insert}$$

Subsequently, deionized water is added to a final volume of 10 µl. Finally, the ligation reaction is mixed by pipetting and incubated at 4 °C for 16 – 18 hours.

##### *3.2.1.3.2 Transformation to E. Coli, JM 109*

10 µl ligation reaction is added to a sterile falcon tube Cat.#2059 on ice. JM 109 competent cells that is placed on ice until just thawed, are then mixed with DNA by gently flicking. Subsequently, 50 µl of competent cells are carefully transferred into falcon tube and gently mixed and placed on ice for 20 minutes. The reaction tube is then subjected to heat-shock for 40 – 50 seconds in a water bath at exactly 42 °C and immediately returned to ice for 2 minutes. The transformed cells are mixed with 450 µl of SOC medium and incubated at 37 °C for 1.5 hours with shaking at 150 rpm. Finally, 500 µl of the transformed cells are

plated on LB agar plate with 100 μg/ml amplicillin supplemented with 100 mM IPTG and 50 μg/ml of X-gal for blue/white screening. The plate is incubated at 37 °C for 16 – 24 hours.

*3.2.1.3.3 Preparation of plasmid DNA by Alkaline Lysis: Minipreparation*

Each colony of transformed bacteria is inoculated in 3 ml of LB broth containing 100 μg/ml of ampicillin. The culture is incubated overnight at 37 °C with shaking at 250 rpm. The culture cells are poured into 1.5 ml microcentrifuge tube and centrifuged at 20,000 x g for 10 minutes. An aliquot of the original culture is stored at –70 °C in 50 % glycerol. After centrifugation, supernatant is removed by aspiration and the pellet is resuspended in 500 μl of cold STE buffer (10 mM Tris-HCl pH 8.0, 0.1 M NaCl, 1 mM EDTA pH 8.0) and repeat centrifugation. The bacterial pellet is lysed in 100 μl of ice-cold Alkaline lysis Solution I (50 mM Glucose, 25mM Tris-HCL pH 8.0, and 10 mM EDTA pH 8.0) and vigorously vortexed. Subsequently, 200 μl of freshly prepared Alkaline lysis Solution II (0.2 N NaOH, 1% w/v SDS) was added to bacterial suspension. The tube is closed and mixed by gently inversion five times. The tube is stored on ice for 10 – 30 minutes. After that, 150 μl of ice-cold Alkaline lysis Solution III (5 M Potassium acetate, glacial acetic acid, and $H_2O$) is added and mixed. The tube is stored on ice for 3 – 5 minutes. The bacterial lysate tube is centrifuged at 20,000 x g for 10 minutes. The supernatant is transferred to a fresh tube. Then, an equal volume of phenol: chloroform is added. The tube is mixed by vortexing and then centrifuged at 20,000 x g for 10 minutes. The aqueous upper layer is transfered to a fresh tube. Finally, plasmid DNA is recovered by precipitation from the supernatant by adding 2 volumes of 100 % ethanol. The solution is mixed by vortexing and centrifuged at 20,000 x g for 10 minutes. The supernatant is removed by gentle aspiration. The tube is stood in an inverted position on a paper to allow all of the fluid to drain away. Then,

pellet is washed with 70 % ethanol and invert the tube several times. The tube is centrifuged at 20,000 x g for 10 minutes to recover the DNA. The supernatant is removed from the tube and open the tube at room temperature to allow ethanol evaporation. Finally, the pellet is dissolved with 50 µl of TE buffer pH 8.0. The DNA solution is mixed and stored at –20 °C.

*3.2.1.3.4 Restriction Endonuclease and Electrophoresis*

Approximately 500 ng of plasmid DNA is digested with 5 units of *Eco*R I according to manufacturer's protocol (Sigma), 1 µl of 10X Buffer (300 mM Tris-HCl pH 7.8, 100 mM $MgCl_2$, 100 mM DTT, and 10 mM ATP), and 0.1 mg/ml BSA. The digestion reaction is incubated overnight at 37 °C. After digestion, the reaction is electrophoresed on 1.5 % gel. Clones containing the insert of interest are selected for sequencing.

*3.2.1.3.5 DNA Sequencing*

The sequencing is performed using BigDye™ Terminator Cycle Sequencing Ready Reaction Kit. The PCR reaction is carried out in a 10 µl containing 4 µl of terminator ready reaction mix (AmpliTag DNA polymerase and FS with thermostable pyrophosphatase), 1 pM sequencing primer (T7 or SP6) and 1 µg DNA template. After incubation at 95 °C for 30 seconds, amplification is carried out for 25 cycles of the following thermal cycling parameters: 95 °C for 10 seconds of denaturation, 50 °C for 5 seconds of annealing, and 60 °C for 4 minutes of extension. The DNA is then precipitated by 65 % ethanol and 0.08 M sodium acetate pH 8.0. The solution is centrifuge at 25,000 x g for 20 minutes and the supernatant is removed by pipetting. The pellet is then washed with 1 ml of 70 % ethanol, and centrifuge tube at 25,000 x g for 8 minutes. Then the supernatant is removed. The pellet is dried in heated incubator at 95 °C for 2 minutes. Finally, the

DNA pellet is resuspended in 10 µl. Template Suppression Reagent (Perkin-Elmer) and loaded to the ABI PRISM sequencer.

*3.2.1.3.6 Alignment and Computational Searching Sequences Analysis*

The nucleotide sequences and their conceptual translation obtained from the clones of interest are compared against nucleotide or protein sequences in online databases by using BLAST N (Basic Local Alignment Search Tool) program via the World Wide Web. Alignments of sequence are made using CLUSTALW multiple sequence alignment program.

### 3.2.2 Expression of Disintegrin Domain in *Pichia pastoris*

#### *3.2.2.1 Amplification of Disintegrin by PCR*

PCR is used to amplify the cDNA fragment encoding the disintegrin domain. Two primers, SVMR16 F.2 and SVMR16 R.2, are used to amplified the disintegrin. The *Eco*RI and six histidine residues are incorporated into the forward primer for facilitating purification and detection. The *Xba*I recognition site and UAA stop codon are incorporated into the reverse primer. The PCR reaction is carried out in a 50 µl containing 10X PCR buffer (100mM Tris- HCl pH 8.3, 500 mM KCl, and 15 mM $MgCl_2$), 1.25 units of Tag DNA polymerase (Pharmacia), 10 pM of each primer, 25 mM $MgCl_2$, 25 mM of each dNTPs, and 200 ng DNA template. After incubation at 94 °C for 5 minutes, amplification is carried out for 30 cycles with the following temperature cycling parameters: 94 °C for 1 minute of denaturation, 53 °C for 1 minute of annealing, 72 °C for 1 minute of extension and a final extension at 72 °C for 5 minutes. The PCR products is electrophoresed in 1.2 % agarose gel. Then, gel is extracted and purified as described in Section 3.2.1.2. Subsequently, the DNA is subcloned into pGEM® -T vector and

transformed to *E. coli*, JM109 as described in Section 3.2.1.3.1 and 3.2.1.3.2, respectively. Plasmid clones are purified and restriction digested to verify the presence of inserts as described in Section 3.2.1.3.3 and 3.2.1.3.4, respectively.

#### *3.2.2.2 Digestion Plasmid DNA and Expression Vector*

After the plasmid clone is confirmed by sequencing the insert, plasmid DNA and expression vector, pPICZαA, are digested with *Eco*R I and *Xba* I, respectively. The digestion reaction is electrophoresed in 1.2 % agarose gel. Then, gel is extracted and purified as described in Section 3.2.1.2. After that, the DNA is precipitated by 0.3 M sodium actate in 90 % ethanol . Then, the solution is centrifuged at 25,000 x g for 20 minutes. The pellet is washed by 1 ml of 70 % ethanol, and centrifuged at 25,000 x g for 10 minutes. The pellet is then dried and dissolved in sterile distilled water.

#### *3.2.2.3 Ligation of Disintegrin into pPICZαA Vector*

Optimize an appropriate amount of plasmid DNA and pPICZαA vector as described before. The ligation reaction is carried out in a 10 µl. The ligation reaction mixture contains 3 µl of 2X Rapid Ligation Buffer (60 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP, and 10% polyethylene glycol), 1 µl of pPICZαA vector, 5 µl of digested disintegrin construct plasmid DNA, and 3 Weiss unit of T4 DNA Ligase. The ligation reaction is incubated at 4 °C overnight.

#### *3.2.2.4 Transformation of Ligated product into E. coli, JM109*

Transformation is performed by heat shock method. The procedure is described in Section 3.2.1.3.2. After transformation, plate 500 µl of the transformation is mixed onto Low Salt LB plate with 25 µg/ml Zeocin™ and

incubated at 37 °C, overnight. After that, transformants are isolated and analyzed for the presence and correct orientation of insert. Zeocin™-resistant colonies are picked, inoculated into 3 ml of Low Salt LB medium with 25 μg/ml Zeocin™ and inoculated overnight at 37 °C with shaking. The plasmid DNA is isolated by miniprep for restriction analysis and sequenced as procedure described in Section 3.2.1.3.3, 3.2.1.3.4 and 3.2.1.3.5, respectively.

#### *3.2.2.5 Linearization of the Plasmid DNA*

Prior to transformation into *Pichia pastoris*, we prepared 5 – 10 μg of plasmid DNA by minipreparation and linearized with the restriction enzyme, which cut one time in the 5′-*AOX 1* region of pPICZαA. 14 μl of plasmid DNA is mixed with 2 μl of 10X Buffer (300 mM Tris-HCl pH 7.8, 100 mM $MgCl_2$, 100 mM DTT, and 10 mM ATP), 0.1 mg/ml BSA and 1 unit of *Sac* I. The reaction is incubated at 37 °C for 16-18 hours. Aliquot of reaction is electrophoresed to verify complete linearization. The reaction is then inactivated using heat at 65 °C for 20 minutes. Then, plasmid DNA is precipitated by 2.5 volumes of 100 % ethanol and 1/10 volume of 3 M sodium acetate. Subsequently, the solution is centrifuged and pellet washed with 80 % ethanol, air-dried and resuspended in 5 μl sterile deionized water, and stored at –20 °C until use.

#### *3.2.2.6 Preparation of Pichia for Transformation*

Competent *Pichia* cells are prepared by chemical method as provided with the *Pichia* EasyComp™ Kit. *Pichia pastoris*, X-33 strain, is streaked on Yeast Extract Peptone (YEPD) plate to grow as single colonies. The YEPD plate is incubated at 30 °C for 2 days. After that, a single colony is inoculated in 10 ml of YEPD and incubated overnight at 30 °C in a shaking incubator (250 – 300 rpm). Yeast cells from overnight culture is then diluted to an $OD_{600}$ of 0.1 – 0.2 in 10 ml

of YEPD and allowed to grow at 30 °C in a shaking incubator until the $OD_{600}$ reaching 0.6 – 1.0. Subsequently, the cells are centrifuged at 500 x g for 5 minutes at room temperature. The cell pellet is washed in 10 ml of Solution I (ethylene glycol and DMSO) and centrifuged at 500 x g for 5 minutes at room temperature. Finally, the pellet is resuspended in 1 ml of Solution I containing ethylene glycol and DMSO. Competent cells are aliquoted in to 50 µl in 1.5 ml sterile microcentrifuge tubes and kept in -80 °C freezer.

***3.2.2.7 Transformation of the Linearized Plasmid DNA into Pichia pastoris, X-33***

The transformation is performed using the *Pichia* EasyComp™ Kit from Invitrogen. Solutions II and III are stored at room temperature before use. The 50 µl of competent cells are thawed at room temperature for each reaction. 3 µg of the linearized plasmid DNA is placed to the competent cells. Then, 1 ml of Solution II (PEG solution) is added to the DNA/cell mixture and mixed by vortexing or flicking the tube. After that, the transformation reaction is incubated at 30 °C for 1 hour in a water bath. The tube is vortexed every 15 minutes. Subsequently, the transformation reaction is subjected to heat shock at 42 °C for 10 minutes in water bath. The transformed cells are splited into 2 microcentrifuge tubes. Add 1 ml of YPD medium to each tube, incubate the transformed cells at 30 °C for 1 hour to allow expression of Zeocin™ resistance. After that, the transformed cells are centrifuged at 500 x g for 5 minutes at room temperature, resuspended in 500 µl of Solution III (Salt solution) and combined into one tube. The transformed cells are then centrifuged at 500 x g for 5 minutes at room temperature, and resuspended in 100 to 150 µl of Solution III. Finally, the transformed solution is plated on YPDS plate with 100 µg/ml Zeocin™ and incubated for 3 to 10 days at 30 °C.

### *3.2.2.8 PCR Analysis of Pichia Integrants*

Genomic DNA is isolated from 6 to 10 *Pichia* clones using the protocol from the EasySelect™ *Pichia* Expression Kit manual. Amplification of disintegrin gene is carried out with 5′ *AOX1* and 3′ *AOX1* primer including in the kit. PCR reaction is carried out in 50 µl reaction containing 10X PCR buffer (100mM Tris- HCl pH 8.3, 500 mM KCl, and 15 mM $MgCl_2$), 25 mM $MgCl_2$, 25 mM dNTPs, 0.2 pM of both 5′ *AOX1* and 3′ *AOX1* primers, and 200 ng genomic DNA. The PCR reaction is incubated at 95 °C for 5 minutes. Then, 1.25 units of Tag DNA polymerase is added to the reaction. After that, amplification is carried out for 35 cycles with the following temperature cycling parameters: 95 °C for 1 minute of denaturation, 53 °C for 45 seconds of annealing, 72 °C for 1 minute of extension and final extension at 72 °C for 7 minutes. Recombinant plasmids with and without insert are used as positive and negative controls, respectively. PCR products are analyzed on 1.5 % gel electrophoresis.

### *3.2.2.9 Small-Scale of Expression of Recombinant Pichia*

The purpose of this procedure is to determine the optimal method and conditions for expression of recombinant *Pichia* before starting large-scale expression. Firstly, a single colony is inoculated in 25 ml of BMGY in a 250 ml baffled flask and incubated at 30 °C in a shaking incubator until culture reaches an $OD_{600}$ equal 2 to 6. Then, cell pellets is harvested by centrifugation at 500 x g for 5 minutes at room temperature and resuspended to an $OD_{600}$ of 1.0 in BMMY. The culture is then placed in a 1 liter baffled flask, cover the flask with two layers of sterile gauze and return to incubator for continue growth. The methanol concentration is maintained at 0.5 % (v/v) every 24 hours for optimal induction during the entire expression period. At 24, 48, 72, and 96 hours, an aliquot of 10 ml of the expression culture is transferred to a 15 tube. The tubes are centrifuged at 25,000 x g for 10 minutes. The supernatant is transferred to a separate tube and

concentrated. Both supernatant and cell pellets are analyzed for protein expression by Coomassie stained SDS-PAGE .

#### *3.2.2.10 Large-Scale of Expression of Recombinant Pichia*

A single colony is inoculated in 10 ml of BMGY in a 250 ml baffled flask, and incubated at 30 °C in a shaking incubator for 16 – 18 hours or until culture reach an $OD_{600}$ equal 2 to 6. Subsequently, 10 ml of culture is inoculated in 100 ml of BMGY in a 500 ml baffled flask and grow at 30 °C with shaking until the culture reaches an $OD_{600}$ equal 2 to 6. After that, the cells are collected by centrifugation at 500 x g for 5 minutes at room temperature. To induce expression, supernatant is discarded and cell pellet is resuspended to an $OD_{600}$ of 1.0 in BMMY medium. Then, the culture is aliquoted into several 4 liters baffled flask, cover the flask with two layers of sterile gauze and continue to grow at 30 °C with shaking. The methanol concentration is maintained at 0.5 % (v/v) every 24 hours for induction expression until the time reach 96 hours. After that, the supernatant and cell pellets are separated by centrifuging at 25,000 x g for 10 minutes at room temperature. The cells are stored at –80 °C and the supernatant is concentrated by centrifuging concentrator.

#### *3.2.2.11 Concentration of Proteins*

The supernatant is separated by centrifugation and concentrated by ultrafiltration using Vivaspin concentrator that have MWCO of 5,000 Da. The supernatant is poured into the concentrator at maximum volume, and then the concentrator was placed in 50 ml centrifuge tube. Subsequently, the assembled concentrator was centrifuged at 25,000 x g for 40 minutes. The remaining sample from the bottom of the concentrated pocket is recovered with a pipette.

### 3.2.3 Purification of Recombinant Proteins

Recombinant disintegrin was purified according to protocol from BD TALON™ Metal Affinity Resins User Manual (BD Biosciences). All steps are carried out at 4 °C. The concentrated media is chromatographed on a cobalt-based immobilized metal affinity column (BioLogic™ LP System, Bio-Rad Laboratories, Ltd.). The column is equilibrated and washed with 50 mM sodium phosphate pH 8.0, 300 mM NaCl. The column is eluted with 50 mM sodium phosphate pH 5.0, 300 mM NaCl into 30 fractions. Absorbancy of each fractions are monitored at 280 nm. The appropriate fractions are analyzed by Coomassie-stained SDS-PAGE, pooled and concentrated using ultrafiltration, and diluted with 1X PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$ , and 2 mM $KH_4$) and stored at –20 °C.

### 3.2.4 Protein Detection

#### *3.2.4.1 Sodiumdodecylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Coomassie Brillient Blue Staining*

12 % of resolving gel and 5 % of stacking acrylamide gel containing 10% SDS are freshly prepared. After gel setting, the recombinant protein is mixed with ¼ volume of 2X sample buffer (100 mM Tris-HCl pH 6.8, 4% w/v SDS, 0.2% w/v bromophenol blue, 20% v/v glycerol, 200 mM with or without β-mercaptoethanol) and then denatured at 95 °C for 10 minutes and loaded into gel slots. Electrophoresis is performed at 125 volts for 90 minutes in 1X running buffer, pH 8.3 (0.25 M Tris-HCl, 1.92 M glycine, 1 % w/v SDS). After electrophoresis, the gel is soaked in Coomassie Brillient Blue Solution for 30 minutes with gentle agitation. After the staining solution is removed, the destaining solution (10% glacial acetic acid, 30% methanol) is added and incubated for 2 – 3 hours. The destaining solution is changed 3 to 4 times during incubation

#### *3.2.4.2 Western Blotting Hybridization*

After SDS-PAGE is used to separate the proteins by size, the proteins are transferred from polyacrylamide gel to PVDF membrane using electrophoresis. For transferring of proteins from 10 % or 13 % gels to PVDF membrane, semi-dry transfer is used. The polyacrylamide gel and PVDF membrane are soaked in transfer buffer for 20 minutes. Both of equilibrated gel and wetted membrane are sandwiched between sheets of transfer buffer-soaked thick filter papers, and then are place on Trans-Blot® SD cell. The proteins are transferred at 20 voltage for 40 minutes. When finished, the blotted membrane is immediately placed into the blocking solution (5 % v/v Non-fat Dry Milk in 1X PBS buffer, pH 7.4) for 1 hour at room temperature with gentle agitation and, then washed 3 times with 1X PBS buffer, pH 7.4, for 3 minutes each. The membrane is incubated with 1:3,000 dilution of Anti-His Antibody in blocking buffer for 1 hour at room temperature with gentle agitation. The membrane is, then, washed 3 times with 1X PBS buffer, pH 7.4, for 3 minutes each. After that, the membrane is incubated with 1:1,000 dilution of Horse radish peroxidase-conjugated rabbit Anti-Mouse IgG:HRP in blocking buffer for 2 hours at room temperature with gentle agitation. The membrane is, then, washed as described previously. For developing the blot, the membrane is soaked in the visualizing solution (1.66 mM 3, 3′-diaminobenzidine (DAB) tetrahydrochloride, 0.04 % $NiCl_2$ and 3 % $H_2O_2$). The reaction is allowed to occur in the dark for 5 minutes. Finally, the solution is removed and the reaction is stopped with $H_2O$ and let the membrane dry overnight.

### 3.2.5 Quantitative Assay for Recombinant Proteins

Protein concentration is determined using Micro BCA™ Protein Assay Reagent Kit (Pierce). The method utilizes bicinchoninic acid (BCA) as the detection reagent for $Cu^{+}$ that is formed when $Cu^{2+}$ is reduced by protein in an

alkaline environment. The bovine serum albumin standards (BSA) are diluted into 6 dilutions (0.025 – 0.1 mg/ml). Then fresh working reagent is prepared by mixing 25 parts of Micro BCA™ Reagent MA containing sodium carbonate, sodium bicarbonate and sodium tartrate in 0.2 N NaOH and 24 parts Reagent MB containing 4% bicinchoninic acid in water with 1 part of Reagent MC containing 4% cupric sulfate, pentahydrate in water. 150 µl of each standard or the sample solution replicate are pipetted into microplate wells and 150 µl of the working reagent is added to each well and mixed. The plate is covered and incubated at 37 °C for 2 hours. The reaction is then measured the absorbance at 570 nm on a plate reader.

### 3.2.6 Platelet Aggregation Assay

Platelet aggregation assay is performed using a Helenna Aggregometer. Venous blood (9 parts) from healthy donor who has not received any medication for at least 2 weeks is collected in 3.2 % sodium citrate (1 part). The whole blood is centrifuged at 1,000 x g for 10 minutes to obtain platelet-rich plasma (PRP) and platelet-poor plasma (PPP) is prepared from the remaining whole blood by centrifuging at 3,500 x g for 10 minutes. PRP is diluted to $250\times10^9$ platelets/L with PPP. Different amount of recombinant disintegrins are added to PRP and incubated at 37 °C for 10 minutes. Platelet aggregation is initiated by adding collagen (2 mg/ml). Light transmittance is recorded and the maximum aggregation response is obtained. The maximal aggregation in the absence of recombinant disintegrin is given a value of 100 % aggregation.

# CHAPTER IV

# RESULTS

## 4.1 Cloning of Full Length Metalloproteinase/Disintegrin from Green Pit Viper

### 4.1.1 5′-RACE

From the primary library of *Trimeresurus albolabris* venom gland, 2 clones of partial cDNA of metalloproteinase-disintegrin was obtained, designated 035 and 038 SVM (Figure 3). To obtain a complete cDNA of 035 SVM, 5′-RACE technique was used (Figure 4). The 5′-RACE products were subcloned into pGEM®-T easy vector and transformed to *Eschericia coli*, JM109. The positive clones were identified by blue-white color selection system. Plasmid clones were purified by alkaline lysis minipreparation method and digested with *Eco*R I to verify the presense of inserts. The digestion reaction were fractionated on gel electrophoresis to screen for clones that contained inserts (Figure 5). After electrophoresis, 10 of 35 clones show the presence of inserts and 3 of 10 of insert-positive clones were electrophoresed against undigest plasmids (Figure 6) confirming of their correct size. Then, the plasmids containing inserts were sequenced using ABI PRISM (Perkin-Elmer). The sequencing was performed using both T7 and SP6 sequencing primer.

**PARTIAL cDNA OF 035 SVM FROM THE PRIMARY LIBRARY**

TAATCAACCTCTGGCTTCTTCAGATTGATTTGAGATCCTCTTCCAGAAGG
TCACTTCCACAAGCCCAAAGAGACCCATCTGCATCCTACTAGAAATTATC
CTTAGCTTCCAGATGGCATCCAAATTTTGTAATATTTCTTCTCCACATTA
ATCTGTTTCCCTCTTGCTGAACAAAACCATTTTCCCACCACAAAAGCTCC
ATGGGCATGTACAACACCAAAGAGCTTATTTGCTGTCAAGAAAAAAAAAT
GGCCATTTTACCATTTGCCAGTTACAAAGCACATTTAATGCAACAAGTTC
TGCTTTTGAGCTGGTGATTCGAAGTCAATGCTTCCTCTCCCAAAATTTCA
TGTTGGCTTTCCAAGATGTAGCTGCTTCCATCAATAAACTAATTATTCTC
AAAAAAAAAAAAAAAAAA

**Figure 3** Partial cDNA of 035 SVM from the primary library.

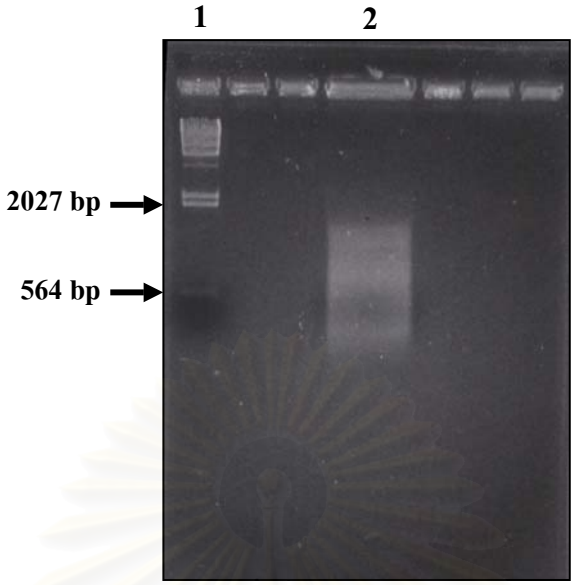



**Figure 4** 5′-RACE of 035 SVM. Lane 1: λ *Hin*d III molecular weight marker ; Lane 2: 5′-RACE of 035 SVM

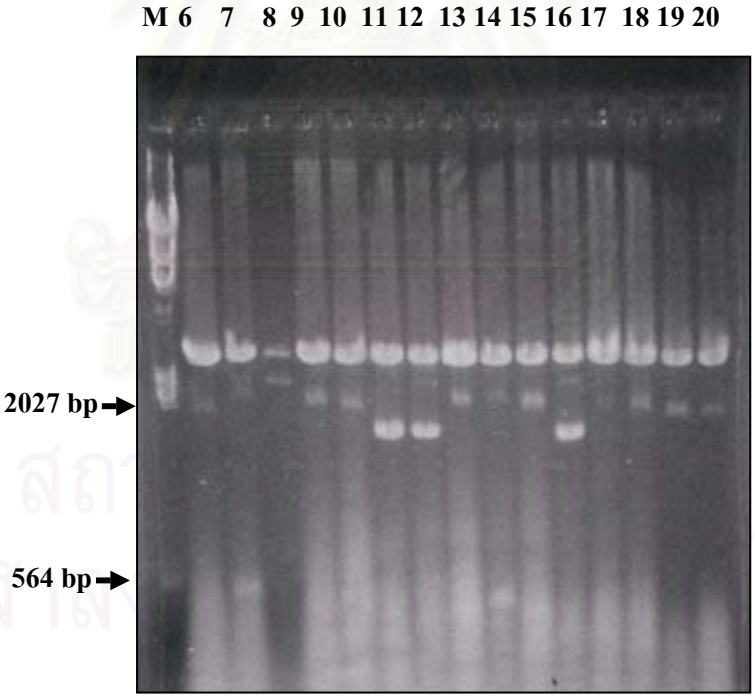



**Figure 5** Digestion of recombinant plasmid clone 1-35 of 035 SVM with *Eco*R I. The inserts were vary in size. Clone 7, 11, 12, 14 and 16 showed the presence of insert. Clones 1 to 5 and clones 21 to 35 are not shown. M = λ *Hin*d III molecular weight marker.

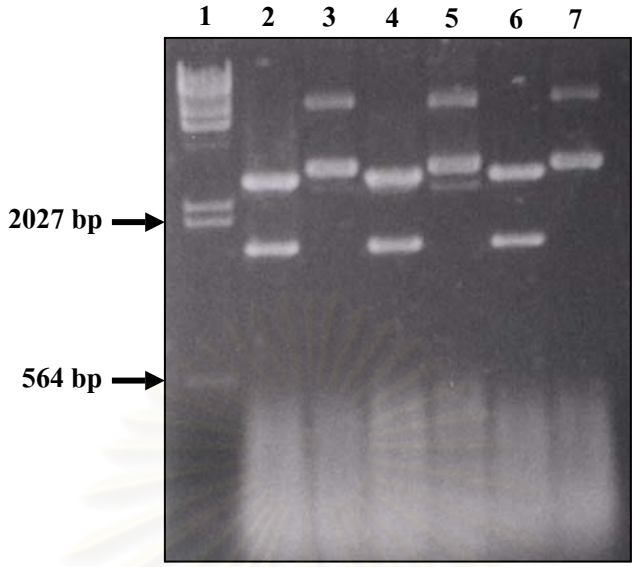



**Figure 6** Confirming of the inserts with the correct size. Clone 11, 12 and 16 were analyzed against undigested plasmids. Lane 1: λ *Hin*d III molecular weight marker ; Lane 2: digested Clone 12; Lane 3: undigested Clone 12; Lane 4: digested Clone 14; Lane 5: undigested Clone 14; Lane 6: digested Clone 16; and Lane 7: undigested Clone 16.

### 4.1.2 Sequence Alignment and Computational Searching Analysis

The insert-positive clones were sequenced and analyzed compared with GENBANK database using the BLAST N and CLUSTAL W program. The full nucleotide sequence of a metalloproteinase/disintegrin was designated as 035 SVM. The highest BLAST score of 035 SVM showed highest homology to *Trimeresurus jerdonii* jerdonitin, accession number AY 364231 (Figure 7). An alignment between amino acids sequence of 035 SVM and jerdonitin from *Trimeresurus jerdonii* venom showed 84 % amino acids sequence identity (Figure 8). The full length of 035 SVM was 2040 bp in length comprising 104 bp of 5′-UTR, 1452 bp of ORF, and 484 bp of 3′-UTR. A putative poly A signal (AATAAA) was detected at nucleotide positions 2004 – 2009 of the 3′-UTR. The ORF consists

of a 60 bp signal region containing the initial ATG codon, a 519 bp pro-peptide region containing a cysteine switch motif (PQMCQVT), a 597 bp metalloproteinase region containing $Zn^{2+}$-binding motif (HGLGHNLGIHH), a 48 bp spacer region, and a 228 bp disintegrin region (Figure 9). The 228 bp disintegrin region of 035 SVM encoded 76 amino acids containing KGD sequence and 13 cysteine residues. CLUSTAL W multiple sequence alignment results revealed that disintegrin region of 035 SVM was highly homologous to other disintegrins: 75 % homology with barbourin, 60 % homology with flavostatin, and 57 % homology with rhodostomin (Figure 10). Notably, 035 SVM disintegrin showed one additional cysteine residue compared with others.

| Sequences producing significant alignments: | Score (bits) | E Value |
|---|---|---|
| gi\|34329646\|gb\|AY364231.1\| Trimeresurus jerdonii jerdonitin... | 1822 | 0.0 |
| gi\|27461087\|gb\|AY071905.1\| Gloydius halys metalloproteinase... | 1013 | 0.0 |
| gi\|20530118\|dbj\|AB074143.1\| Trimeresurus flavoviridis mRNA ... | 938 | 0.0 |
| gi\|31742524\|gb\|AF149788.5\| Bothrops jararaca hemorrhagic me... | 916 | 0.0 |
| gi\|27922987\|dbj\|AB078904.1\| Agkistrodon contortrix contortr... | 900 | 0.0 |
| gi\|14595994\|gb\|AY037808.1\| Trimeresurus flavoviridis hemorr... | 900 | 0.0 |
| gi\|27922991\|dbj\|AB078906.1\| Agkistrodon piscivorus piscivor... | 894 | 0.0 |
| gi\|20530120\|dbj\|AB074144.1\| Trimeresurus flavoviridis mRNA ... | 894 | 0.0 |
| gi\|14325766\|dbj\|AB051849.1\| Trimeresurus flavoviridis mRNA ... | 894 | 0.0 |
| gi\|15991219\|dbj\|AB059571.1\| Trimeresurus elegans mRNA for e... | 894 | 0.0 |
| gi\|20069134\|gb\|AF490534.1\| Bothrops insularis cluster BITM0... | 890 | 0.0 |

**Figure 7** Homology searching of 035 SVM using BLAST N program.

```
CLUSTAL W (1.82) multiple sequence alignment

T. jerdonii  MIQVLLVTICLAVFPYQGSSIILESGNIDDYEVVYPRKVTALPKGAVQQKYEDTMQYEFK 60
035 SVM      MIQVLLVTICLAVFPYQGSSIILESGNVNDYEVVYPEKVTALPKGAVQQKYEDAMQYEFK 60
            ****************************::*******.***************:******

T. jerdonii  VNEEPVVLHLEKNKGLFSKDYSETHYSPDGREITTYPPVEDHCYYHGRIQNDADSTASIS 120
035 SVM      VNGEPVVLYLEKNKELFSENYSETHYSPDGREITTYPSVEDHCYYHGRIQNDADSTASIS 120
            ** *****:***** ***::*****************.**********************

T. jerdonii  ACNGLKGHFKLQGETYFIEPLKLPDSEAHAVFKYENVEKEDEAPKMCGVTETNWESDEPI 180
035 SVM      ACNGLKGHFKLRGKTYLIEPLKLPNSEAHAVFKYENVEKEDEAPQMCGVTETNWESDLPI 180
            ***********:*:**:*******:******************:*********** **

T. jerdonii  KKLSQIMIPPEQQRYIELVIVADHRMYTKYDGDKTEISSKIYETANNLNEIYRHLKIHVV 240
035 SVM      KKTSQLNLPLLEKRCIELVMVADHRMYTKYDGDKTEISSKIYEIANNLNVDYRPMKIRVA 240
            ** **: :*  ::* ****:********************** *****  ** :**:*.

T. jerdonii  LIGLEMWSSGELSKVTLSADETLDSFGEWRERDLLQRKRHDNAQLLTGMIFNEKIEGRAY 300
035 SVM      LIGTEIWSSGELSKVTLSADETLDSFGEWRERDLLKRKSHDNVQLLTGMIFNEKIEGRAY 300
            *** *:*****************************:** ***.****************

T. jerdonii  KESMCDPKRSVGIVRDHRTRPHLVANRMAHELGHNLGFHHDGDSCTCGANSCIMSATVSN 360
035 SVM      NKSMCDPKRSVGIVRDXRTRPHLVANRMAHGLGHNLGIHHDGDSCSCGANSCIMSATVSN 360
            ::************** ************ ******:*******:**************

T. jerdonii  EPSSRFSDCSLFQYSSDIIHNPFTSRCLYNEPSKTDIVSPSVCGNYYMEVGEDCDCGPPA 420
035 SVM      EPSSRFSDCSLNQYSNDIIYNPWTSYCLYNEPSKTDIVSPPVCGNYYLEVGEDCDCGPPA 420
            *********** ***.***:**:** **************.******:************

T. jerdonii  NCQNPCCDAATCRLTPGSQCADGLCCDQCRFMKKGTICRIARGDDLDDYCNGISAGCPRN 480
035 SVM      NCQNPCCDATTCKLTPGSQCAEGLCCAQCKFIEEGTVCRVAKGDWNDDHCTGQSGDCPWI 480
            *********:**:********:**** **:*:::**:**:*:**  **:*.* *..**

T. jerdonii  PFHA 484
035 SVM      GYYG 484
             ::.
```

**Figure 8** An alignment of deduced amino acid sequence of 035 SVM and *Trimeresurus jerdonii*. An alignment showed 84 % amino acids sequence identity. Asterisk represents the homology residues.

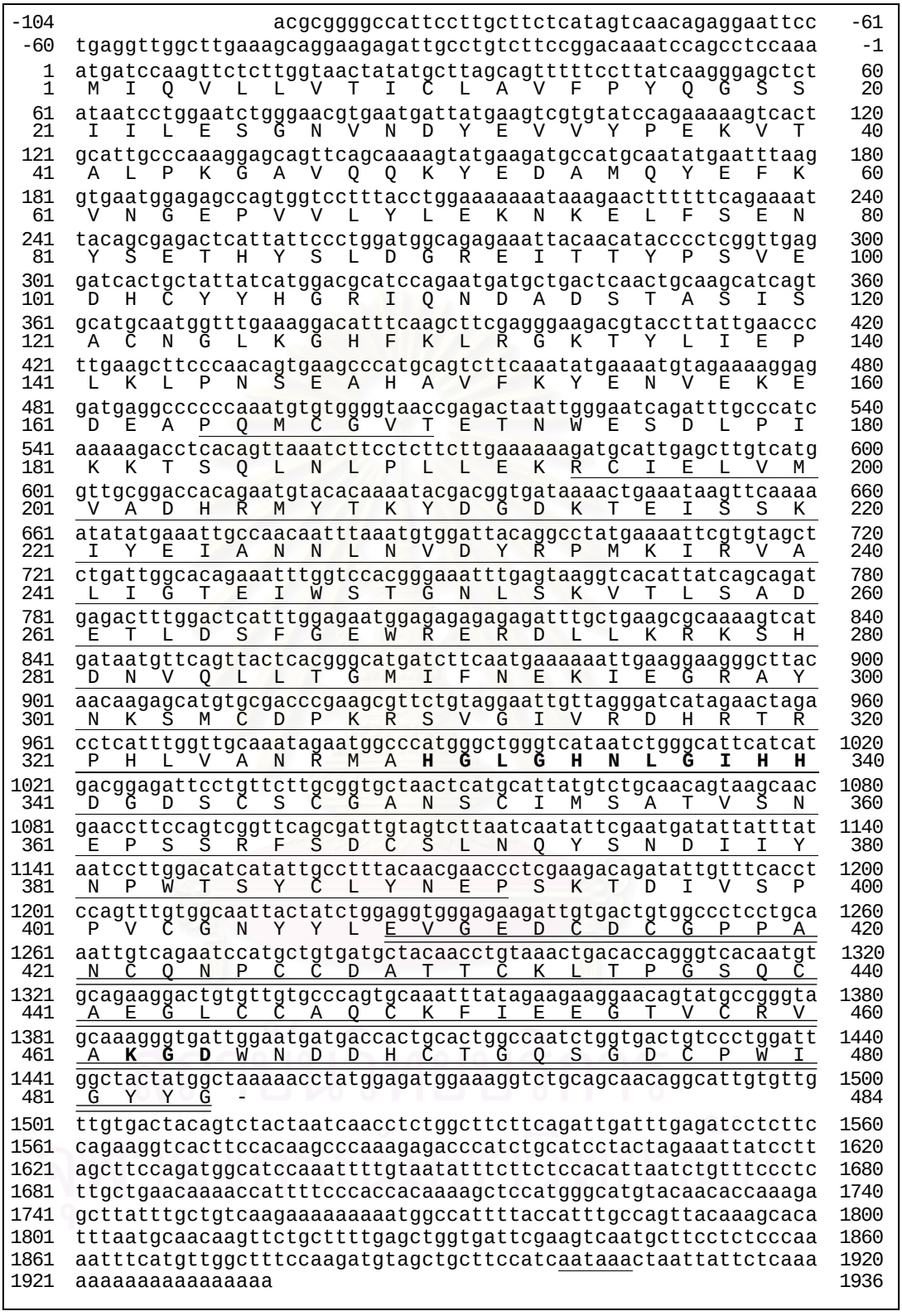



**Figure 9** Complete cDNA and deduced amino acid sequences of snake venom metalloproteinase/disintegrin (035 SVM) from *Trimeresurus albolabris*. The cysteine switch motif, regions corresponding to the metalloproteinase and poly A signal are indicated by single underline. The disintegrin region is indicated by double underline. The $Zn^{2+}$-binding motif and KGD sequences are indicated by bold fonts.

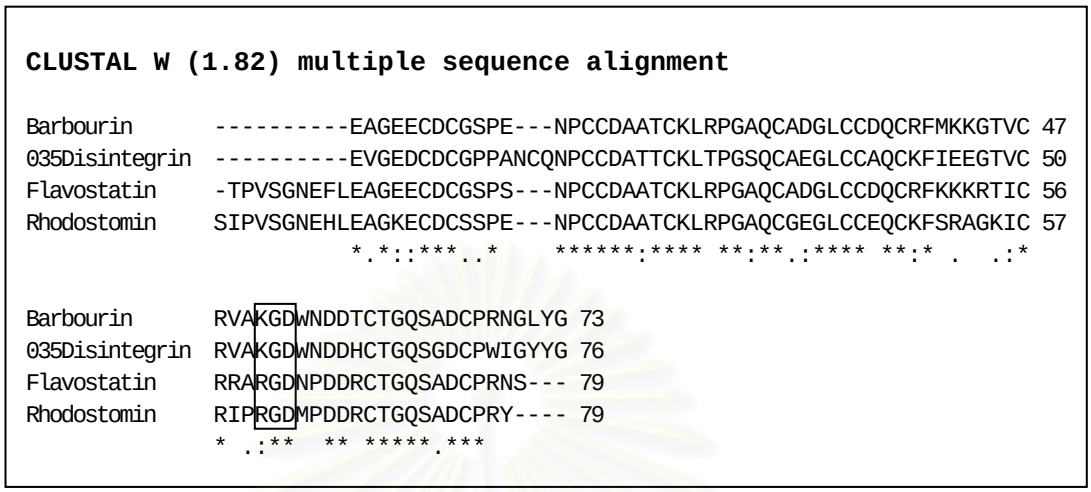



**Figure 10** Comparison of the deduced amino acid sequences of 035 disintegrin with those of other disintegrins. The tripeptide sequence is represented in the box line.

## 4.2 Expression of Disintegrin Domain in *Pichia pastoris*

### 4.2.1 Amplification of Disintegrin by PCR

The structural gene of disintegrin domain was amplified by PCR with forward primer, SVMR16F.2, that has an *Eco*R I recognition site and six histidine residues for facilitating purification and with reverse primer, SVMR16R.2, that has an *Xba* I recognition site and UAA stop codon. After electrophoresis, the PCR product size was approximately 230 bp in length (Figure 11).

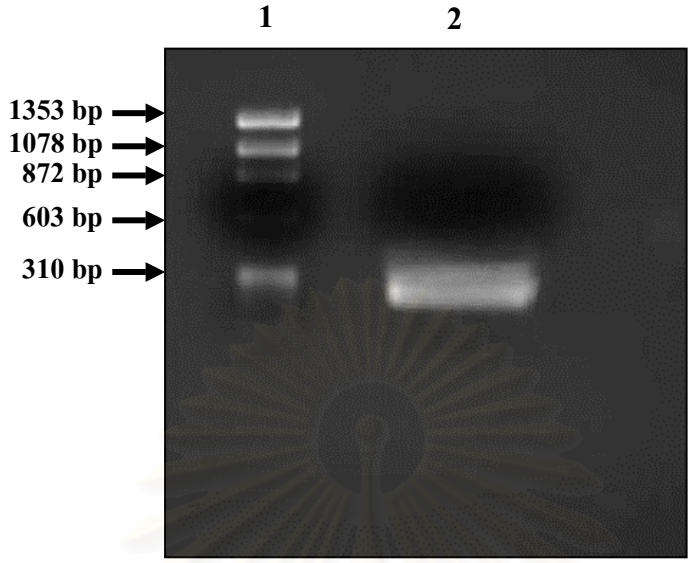



**Figure 11** PCR product of disintegrin domain. Lane 1, Molecular weight marker Phi X 174, Lane 2; PCR of disintegrin was approximately 230 bp in length.

**4.2.2 Ligation of Disintegrin into pGEM® T-vector and Transformation of *E. coli*, JM109**

The PCR product was extracted, purified and then cloned into pGEM® T-vector and transformed into *E. coli*, JM109. The positive clones were identified by blue-white color system (Figure 12). The white plasmid clones were purified and digested with *Eco*R I to verify the presence of inserts (Figure 13) and then sequenced with T7 sequencing primer.

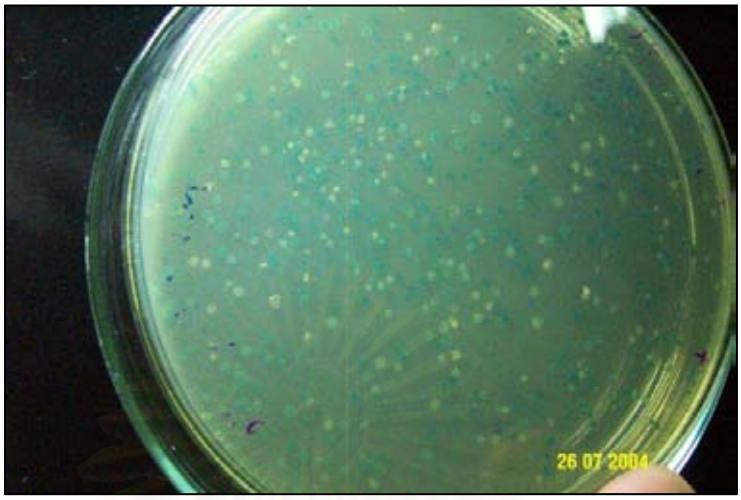



**Figure 12** Disintegrin gene transformed into *E. coli*, JM 109. The transformants were selected by a blue-white system.

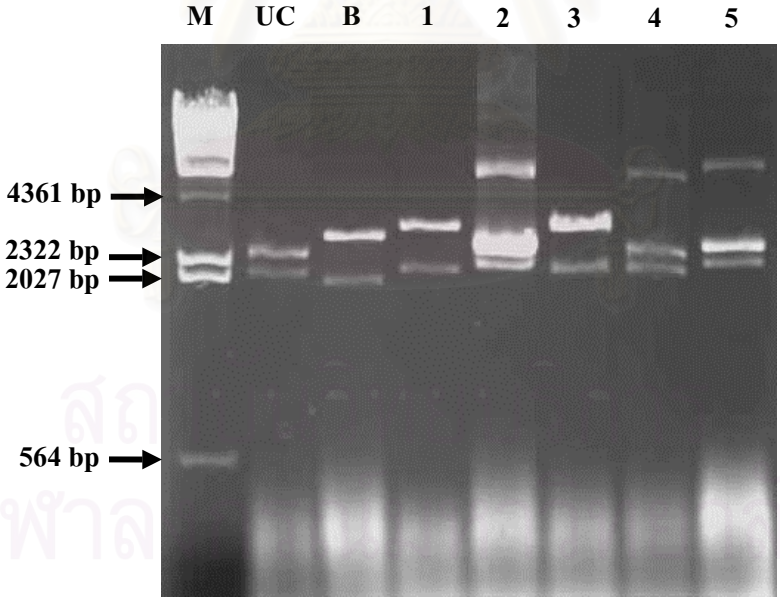



**Figure 13** The plasmids from white colonies that represented the presence of inserts showed shifted bands (1 and 3) when compared with a plasmid from blue colony (B) and undigested plasmid (UC). M = λ *Hin*d III molecular weight marker.

### 4.2.3 Ligation of Disintegrin into pPICZαA and Transformation of *E. coli*, JM109

After the plasmid clone was confirmed by sequencing the insert, plasmid DNA and expression vector, pPICZαA, were digested with *Eco*R I and *Xba* I. The digestion reaction were electrophoresed in 1.2 % agarose gel (Figure 14). After gel extraction and purification, the digestion product was cloned into the *Eco*R I and *Xba* I sites of expression vector, pPICZαA. The recombinant plasmid was transformed into *E. coli*, JM109, and the colony was selected by agar plate with low-salt LB and 25 μg/ml Zeocin™. As a result, there were about 200 Zeocin™-resistant transformants. The recombinant plasmids were digested with *Eco*R I as shown in Figure 15. The band of the correct clone was shifted after it was digested by *Eco*R I. The clones, which contained of insert, were about 3,900 bp in length. Finally, the correct clones were confirmed by sequencing.

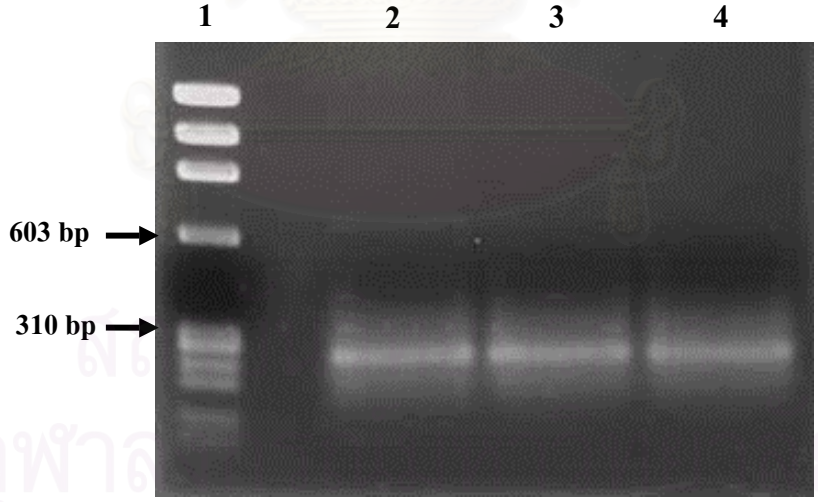



**Figure 14** Electrophoresis of plasmid-insert. Lane 1: molecular weight marker Phi X 174; Lane 2, 3 and 4: insert disintegrin digested from pGEMT®-vector.

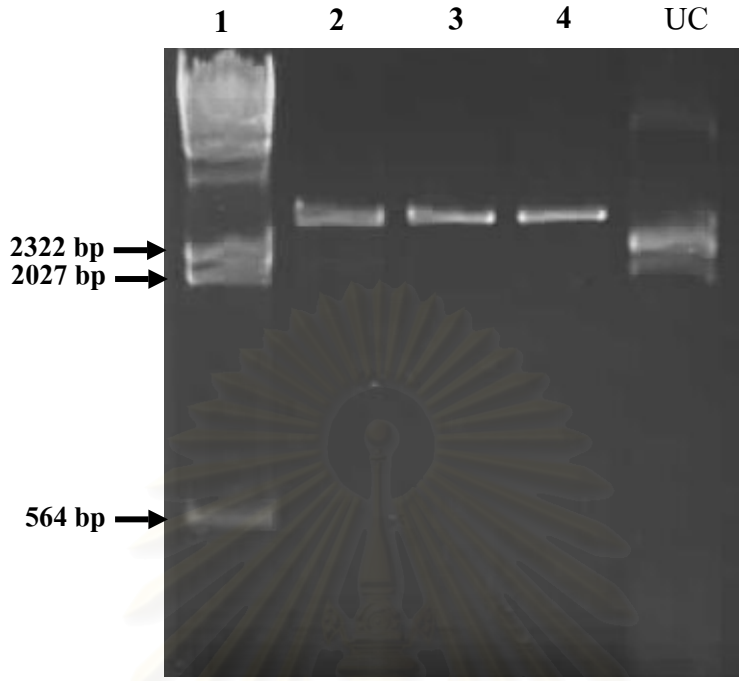



**Figure 15** Electrophoresis of recombinant pPICZαA. Lane 1; λ *Hin*d III molecular weight marker. Lanes 2-4; recombinant plasmid 1-3 were digested with *EcoR I*. UC = undigested recombinant plasmid.

### 4.2.4 Transformation of Recombinant pPICZαA into *Pichia pastoris*, X-33

Prior to transformation into *Pichia pastoris*, recombinant pPICZαA was linearized with *Sac* I as shown in Figure 16. After that, linearized recombinant pPICZαA was transformed into competent *Pichia* cells, X-33. Approximately 300 colony transformants were found in 4 days as shown in Figure 17. PCR results revealed that disintegrin gene had been integrated into *Pichia* genome (Figure 18) and the PCR product was 816 bp in length using 5′-*AOX1* primer.

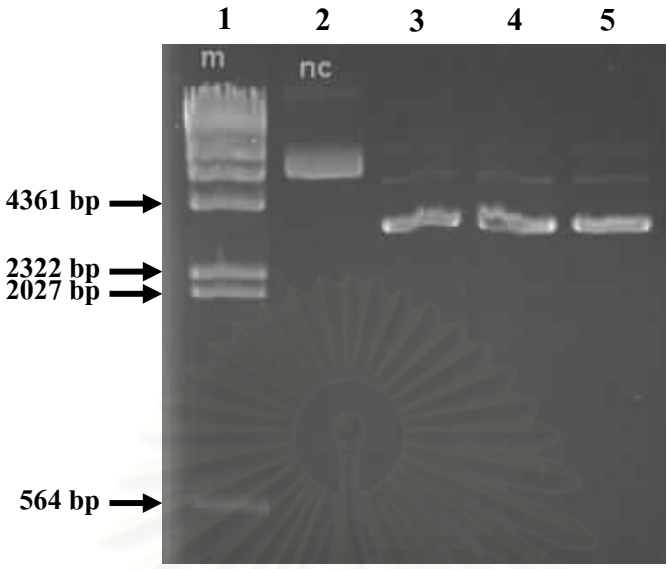



**Figure 16** Electrophoresis of linearized recombinant pPICZαA. Lane 1: λ *Hin*d III molecular weight marker; Lane 2: undigested recombinant pPICZαA; Lanes 3-5: recombinant pPICZαA digested with *Sac* I.


**Figure 17** Recombinant *Pichia pastoris*, X-33, on YPDS (100 μg/ml Zeocin®)

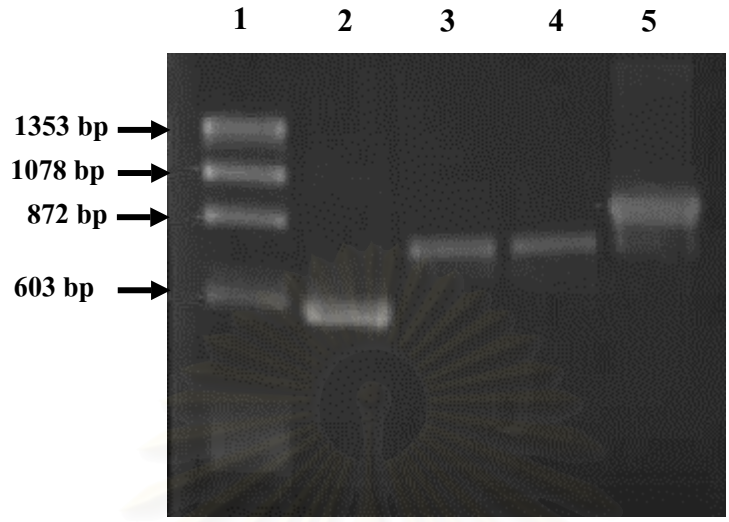



**Figure 18** PCR analysis of *Pichia* integrants. Lane 1: molecular weight marker phi X 174; Lane 2: pPICZαA vector; Lanes 3 and 4: recombinant *Pichia* with disintegrin construct clones 6 and 7; Lane 5: positive control: recombinant *Pichia* with another construct.

### 4.2.5 Expression of Recombinant disintegrin in *Pichia pastoris*, X-33 and Protein Purification

From the time-course experiments, the optimal incubation time for the maximum production of recombinant disintegrin was 96 hours (Figure 19). The apparent molecular mass of recombinant disintegrin was approximately 11 kDa in the reduced condition on SDS-PAGE. In addition, similar band was not found inside yeast cells (Figure 20). For large scale expression, recombinant *Pichia* was expressed in 1.0 liter of BMMY and induced by 0.5 % v/v methanol. The concentrated media was chromatographed on a cobalt-based immobilized metal affinity column. The elution fractions of recombinant disintegrin were monitored by its absorbance at 280 nm. and analyzed by Coomassie-stained SDS-PAGE as

shown in Figure 21 and pooled into 15 ml. For the detection and confirmation of recombinant disintegrin protein, the pooled fraction was determined by Western blotting probed with anti-His antibody (Figure 22) and coomassie-stained on SDS-PAGE in the reduced and non-reduced states (Figure 23). The recombinant 035 disintegrin showed a single band in both reduce- and non-reduced states on SDS-PAGE. The molecular mass of recombinant 035 disintegrin was approximately 11 kDa and 22 kDa in reduced and non-reduced condition, respectively.

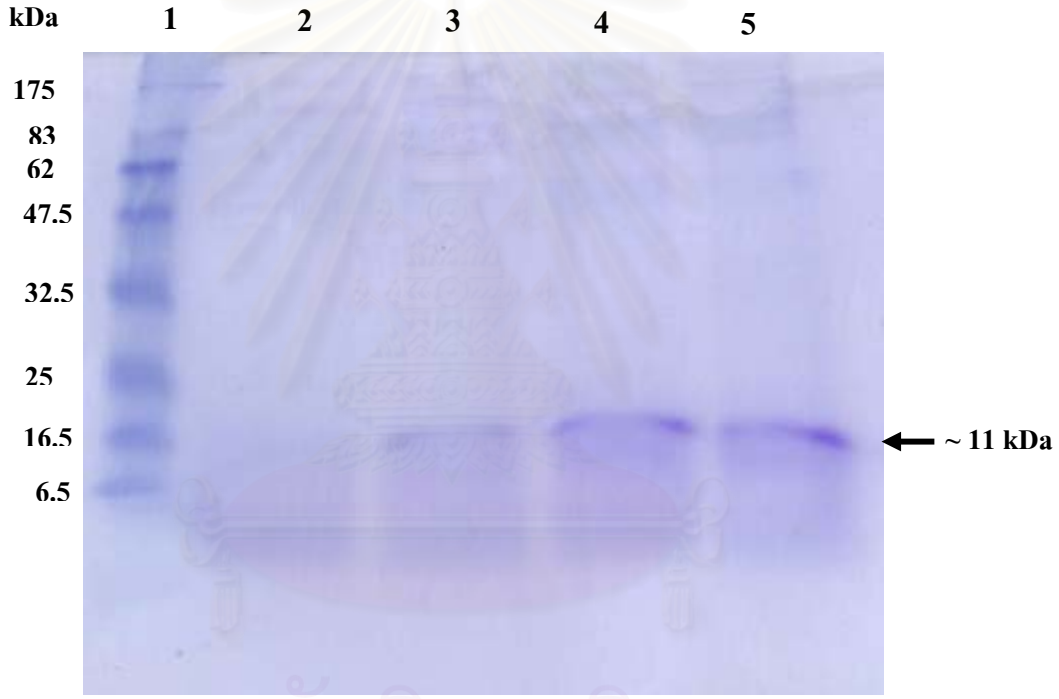



**Figure 19** Coomassie-stained SDS-PAGE showed time-course analysis of protein expression. Lane 1: prestained protein marker; Lanes 2 - 5: 035 disintegrin expression after methanol induction at 24, 48, 72 and 96 hours, respectively.

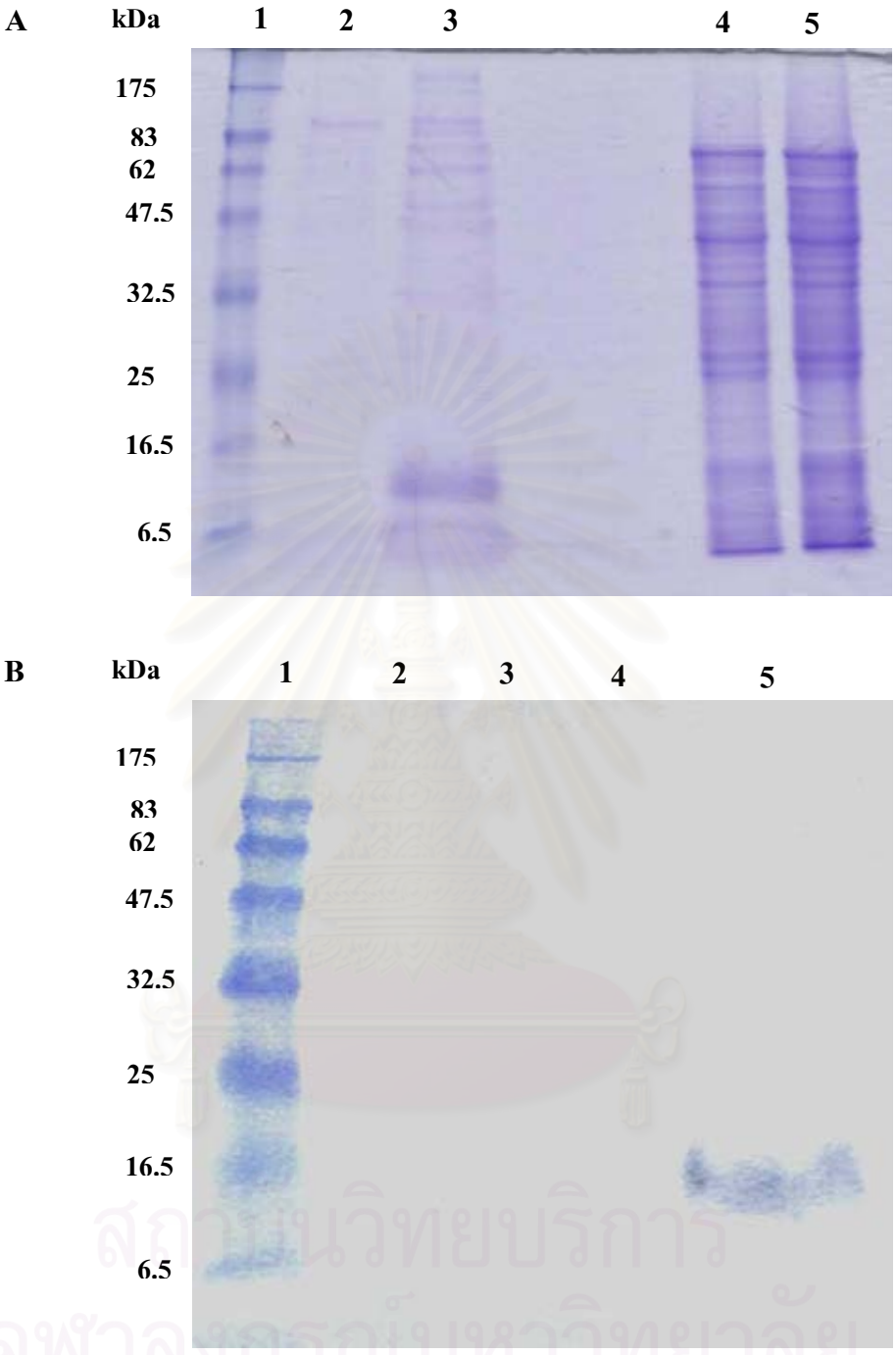



**Figure 20** A) Coomassie-stained SDS-PAGE of recombinant 035 disintegrin. Lane 1: prestained protein marker; Lane 2 and 4: supernatant and cell lysate from *Pichia* X-33 with pPICZαA; Lanes 3 and 5: supernatant and cell lysate from *Pichia* X-33 with recombinant disintegrin. B) Western blotting of 035 disintegrin. Lane 1: Prestained protein marker; Lanes 2 and 3: cell lysate and supernatant from *Pichia* X-33 pPICZαA; Lanes 4 and 5: cell lysate and supernatant from *Pichia* X-33 with recombinant disintegrin.

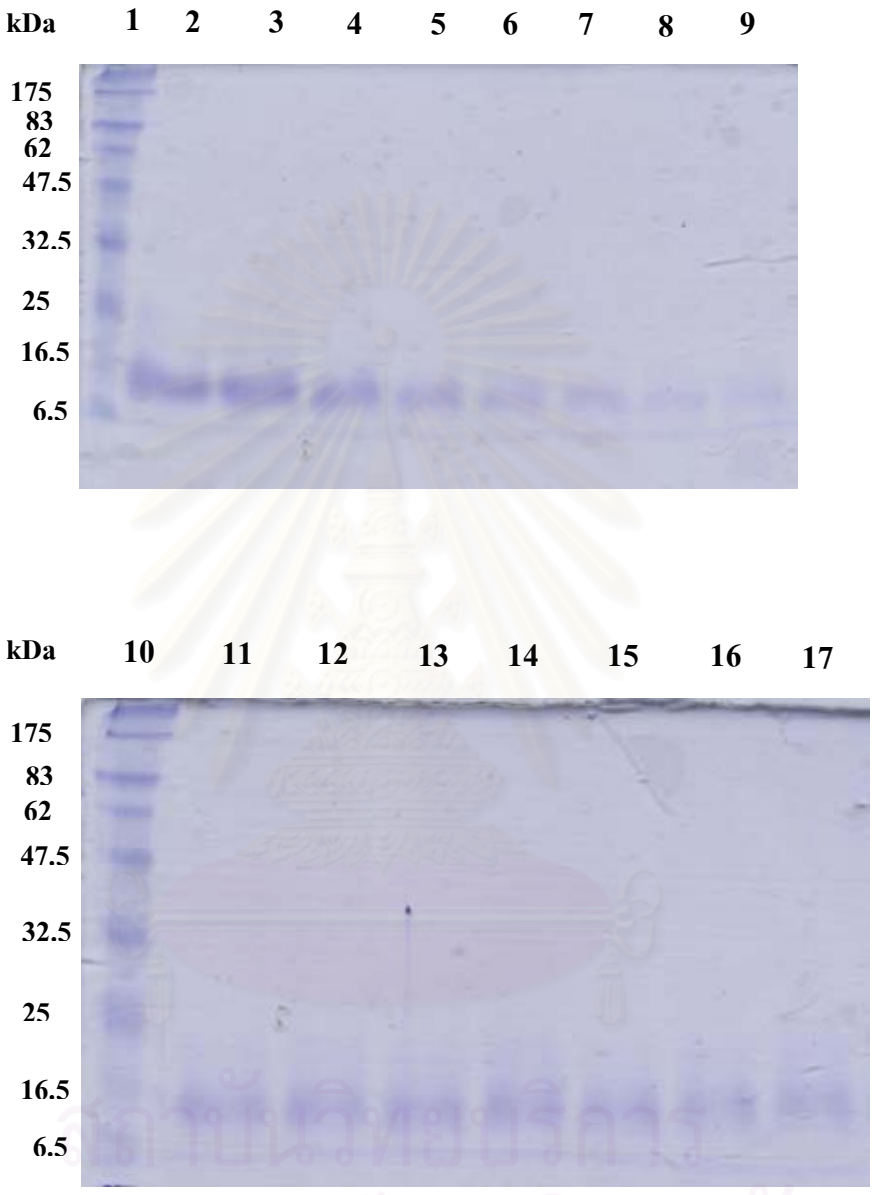



**Figure 21** Coomassie-stained SDS-PAGE of elution fractions. Lanes 1 and 10: prestained protein marker; Lanes 2 - 17: fractions 13 - 27.

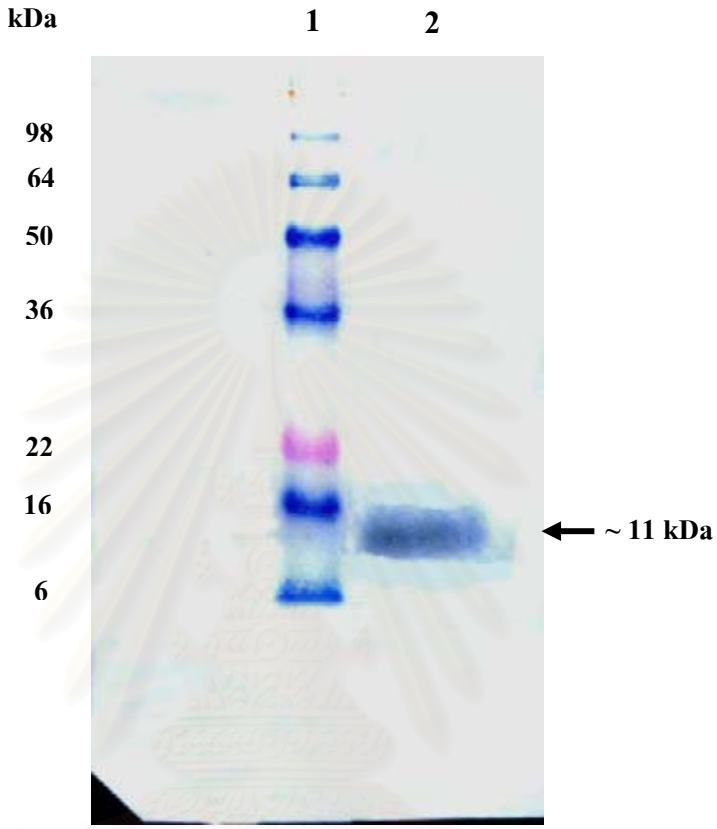



**Figure 22** Western blotting analysis of pooled fractions of recombinant 035 disintegrin. Lane 1: Prestained protein marker. Lane 2 : recombinant 035 disintegrin probed with anti-His antibody.

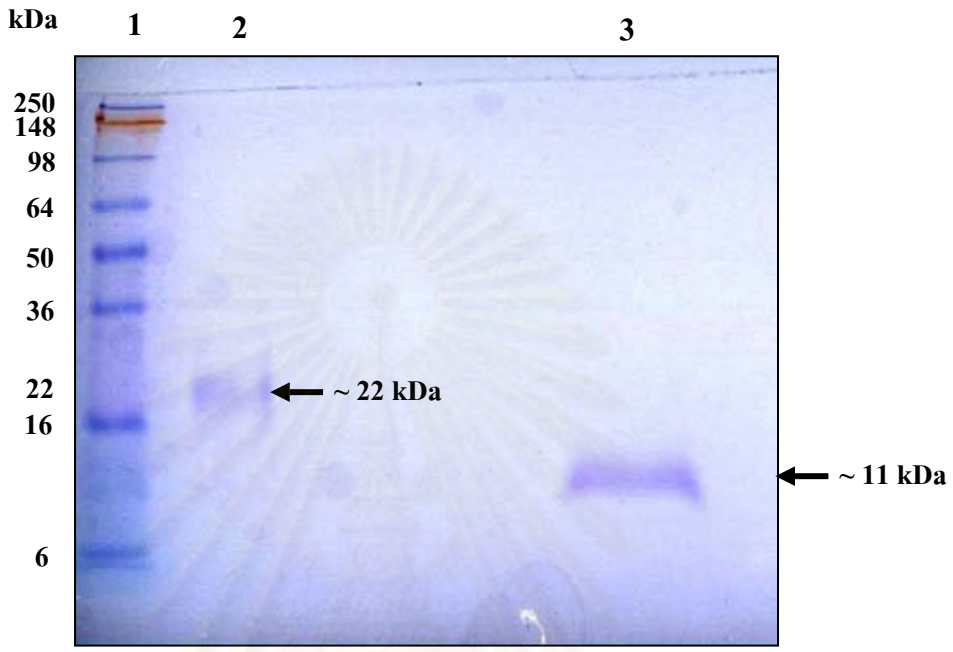



**Figure 23** SDS-PAGE of recombinant 035 disintegrin. Lane 1: prestrained protein marker; Lanes 2 and 3: recombinant 035 disintegrin under non-reducing and reducing conditions, respectively.

### 4.2.6 Quantitative Assay of Purified Recombinant Protein

To determine the production level of purified recombinant disintegrin, the Micro BCA™ Protein Assay was used for measuring the protein concentration. Absorbancy average of 0.603 at 570 nm at 1:4 dilution of protein sample was calculated protein concentration as 0.054 mg/ml (Figure 24). Thus, concentration of undiluted protein was 0.22 mg/ml. Therefore, the yield of recombinant disintegrin produced in *Pichia pastoris* was 3.3 mg/Liter of culture medium.

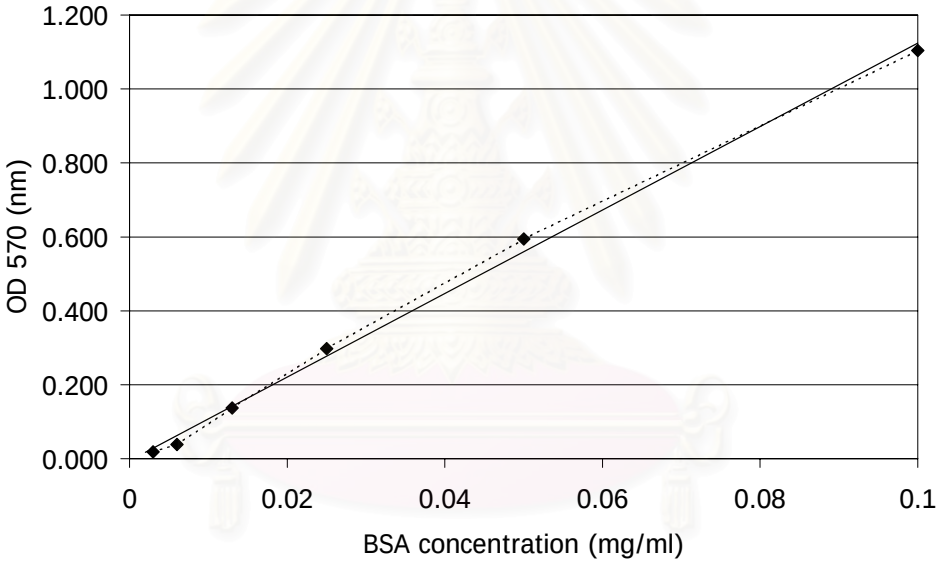



**Figure 24** Standard curve of protein concentrations fitted by linear regression.

### 4.2.7 Platelet Aggregation Inhibition of Recombinant Disintegrin

To examine the affect of recombinant 035 disintegrin protein on human platelets, *in vitro* collagen-induced platelet aggregation assay was carried out in human platelet-rich plasma. The maximum aggregation response obtained from addition of inducer was given a value of 100 % aggregation. The $IC_{50}$ value of recombinant 035 disintegrin was determined to be 976 nM using linear regression curve fitting algorithms (Figure 25).

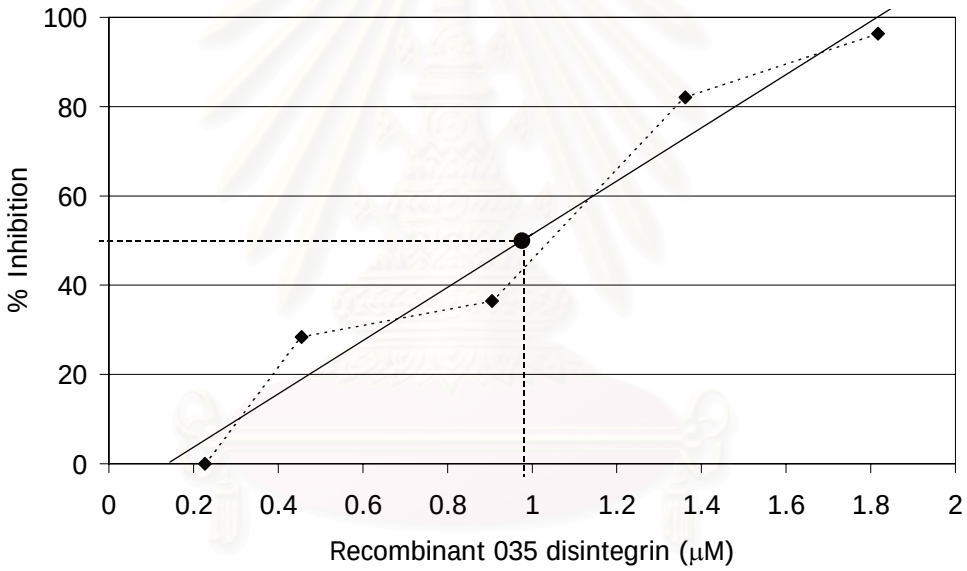



**Figure 25** Effect of recombinant 035 disintegrin on the aggregation of human platelets. $IC_{50}$ determination by curve fitting with linear regression.

# CHAPTER V

# DISCUSSION AND CONCLUSION

This is the first report of the complete nucleotide sequence of a cDNA encoding snake venom metalloproteinase/disintegrins from *Trimeresurus albolabris*. In addition, characterization and expression of its disintegrin domain in *Pichia Pastoris* were also studied in this research. The cDNA sequence of snake venom metalloproteinase/disintegrin is 2,040 bp in length. The deduced amino acid sequence composed of a signal region, a pro-peptide domain, a metalloproteinase domain, a spacer region and a disintegrin domain. Thus, the snake venom metalloproteinase/disintegrin of *Trimeresurus albolabris* belongs to the metalloproteinase/disintegrin family type N-II (Figure 26). Notably, the amino acid sequence of the conserved $Zn^{2+}$-binding motif in the metalloproteinase domain is different from other metalloproteinase/disintegrin families. Figure 27 indicated that metalloproteinase domain of 035 SVM displays an amino acid G, instead of amino acid E, in the conserved $Zn^{2+}$-binding motif sequence. Without $Zn^{2+}$ ion, the metalloprotein is inactive. From this observation, we predict that the metalloproteinase domain of 035 SVM do not have protease activity. The 035 SVM may serve as a precursor of a disintegrin.

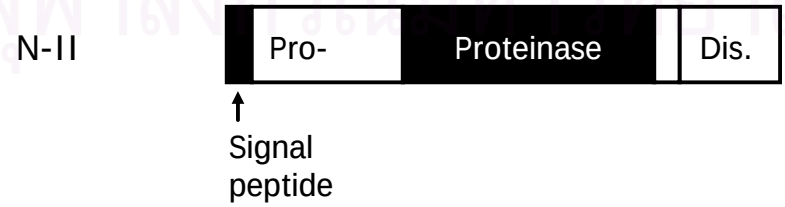



**Figure 26** Schematic representation of the type N-II SVMP nucleotide from *Trimeresurus albolabris*.

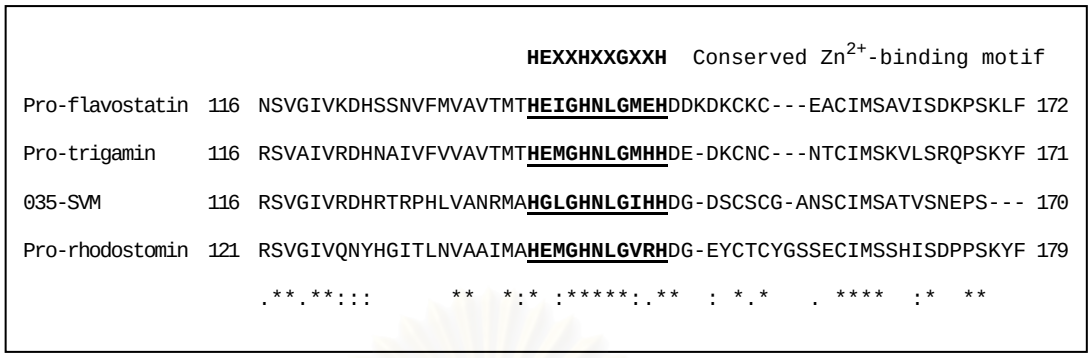



**Figure 27** Comparison of the $Zn^{2+}$-binding motif of metalloproeinase domain of 035 SVM with those of other metalloproteinase/disintegrin precursors.

From the analysis of cDNA encoding disintegrin of a snake venom metalloproteinase/disintegrin of *Trimeresurus albolabris*, it is found that the cDNA sequence of a disintegrin domain, 035 disintegrin, was 228 bp in length and composes of 76 amino acid residues including 13 cysteines. However, a molecular mass of 035 disintegrins on SDS-PAGE in the reduced and non-reduced condition were approximately 11 kDa and 22 kDa, respectively. Thus, 035 disintegrin of 035 SVM from *Trimeresurus albolabris* is a homodimeric disintegrin. It should be noted that 035 disintegrin has KGD sequence near the C-terminus, instead of the typical RGD sequence that is the binding motif of various cell surface integrins. From the deduced amino acid sequence of 035 disintegrin, there is an unpaired cysteine residue in sequence as shown in Figure 28. It has been reported that an unpaired cysteine has been found in bilitoxin-1. Bilitoxin-1 is a homodimeric disintegrin isolated from *Agkistrodon bilineatus* venom. It comprises disulfide-bonded polypeptides (*56*). Each chain of bilitoxin-1 contains 15 cysteine residues. In contrast to 035 disintegrin, bilitoxin-1 has MGD sequence, instead of typical RGD sequence, and it inhibits neither collagen- nor ADP-induced human platelet

aggregations. Therefore, the promoting the inhibitory mechanism of disintegrin depends on antagonist $\alpha_{IIB}\beta_3$ tripeptide sequence.

The conserved cysteine residues are involved in disulfide bond formation, which is important for maintaining the molecular structure and the biochemical function of a disintegrin (*32*) . From Figure 28 the positions of the cysteine residues of 035 disintegrin are similar to those found in the medium size disintegrins. If the cysteine residues of 035 disintegrin have identical disulfide bonds as found as in other medium size integrins, the cysteine residue at position 422 is perhaps unpaired or participating in an interchain disulfide bond with the unpaired cysteine residue of the other chain to form a dimeric disintegrin.


**Figure 28** Comparison of cysteine residues of 035 disintegrin with those of other medium-size disintegrins.

In this research, *Pichia pastoris* expression system was used to produce recombinant 035 disintegrin with methanol induction. There are many reports stated that recombinant proteins produced in *Pichia pastoris* result in high yields corrected folding and post translational modification (*33*, *57*). For example, Guo et al. found that rhodostomin produced in *Pichia pastoris* yieled 5 – 10 mg/Liter of culture medium and inhibited platelet aggregation with a *K*i value of 70 nM which is more potent than rhodostomin produced in *E. coli* (*K*i of 263 nM) system. In our

results, the yield of 035 disintegrin produced in *Pichia pastoris* was 3.3 mg/Liter culture medium that is less than rhodostomin. However, there was also reported in the literature that the expression level is strongly affected by induction temperature (*54*). Optimization of temperature may be helpful.

Bingze et al. reported that post-translational glycosylation of protein can be obtained when expressed in the *Pichia pastoris*. In addition, the study by Choi et al. stated that the molecular mass of Bovine β-casein, expressed in *Pichia pastoris*, was estimated to be 33.6 kDa on SDS-PAGE. They also found that when treated with peptide-N-glycosidase (PNGaseF), the molecular weight was migrated at 30 kDa on SDS-PAGE (*55*). They concluded that Bovine β-casein produced in *Pichia pastoris* was glycosylated. In our results, the molecular mass of 035 disintegrin calculated from amino acid sequence with the fusion of six histidine is 8.8 kDa, but it migrates at approximately 11 kDa and 22 kDa in the reduced and non-reduced condition on SDS-PAGE. We proposed that, similar to the conclusion stated by Choi et al., 035 disintegrin is glycosylated in *Pichia pastoris* expression system.

In this study, we characterized the platelet aggregation inhibitory activity of the recombinant 035 disintegrin in human PRP. Our results revealed that recombinant 035 disintegrin dose-dependently inhibited collagen-induced platelet aggregation with the $IC_{50}$ of 976 nM. There is a report by Marques et al. that recombinant barbourin, a KGD-containing monomeric disintegrin, can inhibit ADP-induced platelet aggregation with $IC_{50}$ value ranges from 330-370 nM (*50*). When compared with those of other RGD-containing monomeric disintegrins inhibiting collagen-induced platelet aggregation, e.g., jerdonin that has $IC_{50}$ value of 240 nM (*43*), it is found that recombinant 035 disintegrin is less active than native jerdonin. Contortrostatin is a homodimeric disintegrin that has RGD sequence in each chain. Recombinant contortrostatin inhibites ADP-induced platelet aggregation with $IC_{50}$ value of 250 nM (*58*). On the other hand, bilitoxin-1,

which is also a homodimeric disintegrin, does not have any inhibitory activity to both collagen- and ADP-induced human platelet aggregations. It can be seen that 035 disintegrin is less active than contortrostatin and more active than bilitoxin-1. In the future, the effects of 035 disintegrin on other integrins should be studied.

To induce platelet aggregation, the collagen directly binds to integrin $\alpha_2\beta_1$ receptors and leads to the conformational changes of integrin $\alpha_{IIB}\beta_3$ enhancing its affinity to fibrinogen containing RGD. The results from our study indicate that the recombinant 035 disintegrin of *Trimeresurus albolabris* can inhibit collagen-induced human platelet aggregation. It is not investigated in this study whether this process is occurred by blocking the binding of fibrinogen to integrin $\alpha_{IIB}\beta_3$ on platelet surface or by the binding of collagen to integrin $\alpha_2\beta_1$ on platelet surface. There is a report that KGD/RGD-containing disintegrin inhibit platelet aggregation by blocking the binding of fibrinogen to integrin $\alpha_{IIB}\beta_3$. However, further investigation on the molecular mechanism of 035 disintegrin is required for the understanding of its functional mechanism.

In summary, we characterized the full-length cDNA of snake venom metalloproteinase/disintegrin from the venom gland of green pit viper, *Trimeresurus albolabris* and expressed disintegrin domain of snake venom metalloproteinase/disintegrin in *Pichia pastoris* system. The structural domain of cDNA similar to the metalloproteinase/disintegrin class N-II of SVMPs, which consists of a signal peptide, a pro-peptide, an-inactive metalloproteinase domain, a spacer region, and a disintegrin domain. The disintegrin domain encoding 76 amino acids was expressed in *Pichia pastoris*. The yield of recombinant disintegrin was 3.3 mg/Liter culture medium. The recombinant disintegrin is a homodimeric disintegrin that has molecular mass approximately 11 kDa and 22 kDa on SDS-PAGE in the reduced and non-reduced states, respectively. Recombinant

disintegrin inhibited collagen-induced platelet aggregation with $IC_{50}$ value of 976 nM. This data give us deeper insight in the mechanism of platelet inhibition and bleeding caused by green pit viper venom. In addition, this novel disintegrin should be studied for its potentials to be a new antiplatelet agent in the future.

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# APPENDIX

## 1. Bacterial Media

### 1.1 LB Medium (per liter)

10g Bacto®-tryptone

5g Bacto®-yeast extract

5g NaCl

Adjust pH to 7.0 with NaOH.

### 1.2 LB Plates with Ampicillin

Add 15g agar to 1 liter of LB medium. Autoclave. Allow the medium to cool to 50 °C before adding ampicillin to a final concentration of 100 µg/ml. Pour 30-35 ml of medium into 85 mm petri dishes. Let the agar harden. Store at 4 °C for up to one month or at room temperature for up to one week.

### 1.3 LB Plates with Ampicillin/IPTG/X-Gal

Make the LB plates with ampicillin as above; then supplement with 0.5 mM IPTG and 80 µg/ml X-Gal and pour the plates. Alternatively, 100µl of 100 mM IPTG and 20 µl of 50 mg/ml X-Gal may be spread over the surface of an LB ampicillin plate and allowed to absorb for 30 minutes at 37 °C prior to use.

### 1.4 SOC Medium (100ml)

2.0 g Bacto®-tryptone

0.5 g Bacto®-yeast extract

1 ml 1M NaCl

0.25 ml 1M KCl

1 ml 2M $Mg^{2+}$ stock, filter sterilized

1 ml 2M glucose, filter sterilized

Add Bacto®-tryptone, Bacto®-yeast extract, NaCl and KCl to 97 ml distilled water. Stir to dissolve. Autoclave and cool to room temperature. Add 2 M $Mg^{2+}$ stock and 2 M glucose, each to a final concentration of 20 mM. Bring to 100 ml with sterile, distilled water. The final pH should be 7.0.

**2. *Pichia pastoris* Media**

**2.1 Low Salt LB (Luria-Bertani) Medium**

1 % Tryptone

0.5 % Yeast Extract

0.5 % NaCl

Adjutst to pH 7.0 with NaOH.

For 1 liter, dissolve 10 g tryphone, 5 g yeast extract and 5 g NaCl in 950 ml deionized water. Adjust the pH of the solution to 7.5 with NaOH and bring the volume up to 1 liter. Autoclave for 20 minutes at 15 lb/sq. in. Let cool to ~55 °C and add desired antibiotics at this point. Store at room temperature or at +4 °C.

**2.2 Yeast Extract Peptone Dextrose Medium - YPD or YEPD** (1 liter)

1 % Yeast Extract

2 % Peptone

2 % Dextrose (glucose)

Dissolve 10 g yeast extract and 20 g of peptone in 900 ml of water. Note: Add 20 g of agar if making YPD slants or plates. Autoclave for 20 minutes on liquid cycle. Add 100 ml of 10X D (20% Dextrose). The liquid medium is stored at room temperature. YPD slants or plates are stored at +4 °C. The shelf life is several months.

**2.3 Yeast Extract Peptone Dextrose Medium – YPDS + Zeocin™ Agar** (1 liter)

1 % Yeast Extract

2 % Peptone

2 % Dextrose (glucose)

1 M Sorbitol

2 % Agar

100 µg/ml Zeocin™

Dissolve 10 g yeast extract, 20 g peptone and 182.2 g sorbitol in 900 ml of water. Note: Add 20 g of agar and autoclave for 20 minutes on liquid cycle. Add 100 ml of 10X D (20% Dextrose). Cool solution to ~ 60 °C and add 1.0 ml of 100 mg/ml Zeocin™. Store YPDS or plates containing Zeocin™ at +4 °C in the dark. The shelf life is one to two weeks.

**2.4 Buffered Glycerol-Complex Medium and Buffered Methanol-Complex Medium – BMGY and BMMY** (1 liter)

1 % Yeast Extract

2 % Peptone

100 mM Potassium phosphate, pH 6.0

1.34 % YNB

$4 \times 10^{-5}$ % Biotin

1 % Glycerol or 0.5 % methanol

Dissolve 10 g yeast extract and 20 g peptone in 700 ml of water. Autoclave for 20 minutes on liquid cycle. Cool to room temperature, then add 100 ml 1 M potassium phosphate buffer (pH 6.0), 100 ml 10X YNB, 2 ml 500X B (0.02% Biotin), and 100 ml 10X GY (10% Glycerol) and mix well. For BMMY, add 100 ml 10X M (5% Methanol) instead of glycerol. Store media at +4 °C. The shelf life of this solution is approximately two months.

**3. Buffer**

**3.1 1X Equilibration/Wash Buffer** (pH 7.0)

| | |
|---|---|
| 50 mM | Sodium Phosphate pH 7.0 |
| 300 mM | NaCl |

**3.2 1X Equilibration Buffer** (pH 8.0)

| | |
|---|---|
| 50 mM | Sodium Phosphate pH 8.0 |
| 300 mM | NaCl |

**3.3 1X Elution Buffer** (pH 5.0)

| | |
|---|---|
| 50 mM | Sodium Phosphate pH 5.0 |
| 300 mM | NaCl |

**3.4 Alkaline Lysis Solution I**

| | |
|---|---|
| 50 mM | Glycose |
| 25 mM | Tris-Chloride, pH 8.0 |
| 10 mM | EDTA, pH 8.0 |

**3.5 Alkaline Lysis Solution II**

| | |
|---|---|
| 0.2 N | NaOH |
| 1 % (w/v) | SDS |

**3.6 Alkaline Lysis Solution III**

| | |
|---|---|
| 60 ml | 5 M Potassium Acetate |
| 11.5 ml | Glacial Acetic Acid |
| 28.5 ml | $dH_2O$ |

**3.7 STE BUFFER**

| | |
|---|---|
| 10 mM | Tris-Cl pH 8.0 |
| 0.1 M | NaCl |
| 1 mM | EDTA pH 8.0 |

**3.8 Tris-Glycine Buffer (1X)**

| | |
|---|---|
| 25 mM | Tris-Cl |
| 250 mM | Glycine |

**3.9 10X Tris EDTA (TE) pH 8.0**

| | |
|---|---|
| 100 mM | Tris-Cl, pH 8.0 |
| 10 mM | EDTA, pH 8.0 |

**3.10 1X Phosphate-Buffered Salive (PBS)**

| | |
|---|---|
| 137 mM | NaCl |
| 2.7 mM | KCl |
| 10 mM | $Na_2HPO_4$ |
| 2 mM | $KH_2PO_4$ |

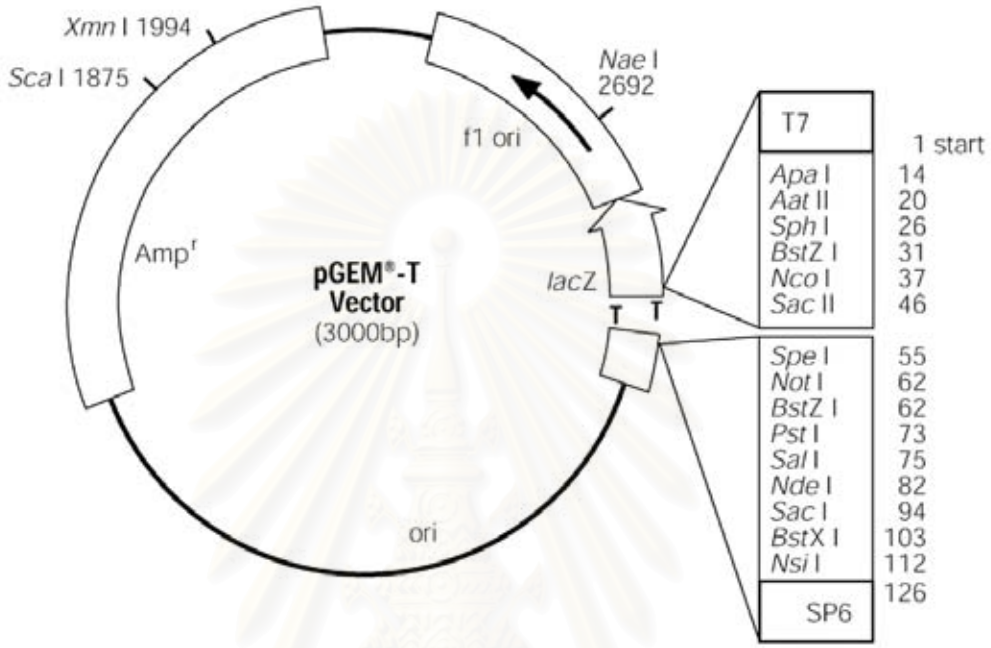
Dissolve 8 g of NaCl, 0.2 of KCl, 1.44 g of $Na_2HPO_4$ and 0.24 g of $KH_2PO_4$ in 800 ml of $dH_2O$. Adjust pH to 7.4 with HCl. Add $dH_2O$ to 1 liter and sterilize by autoclaving for 20 minutes at 15 psi (1.05 $kg/cm^2$) on liquid cycle. Store the buffer at room temperature.

**3.11 Blotting Transfer Buffer pH 8.3**

| | |
|---|---|
| 20 mM | Tris-Cl |
| 150 mM | Glycine |
| 20 % v/v | Methanol |

**4. Vector**

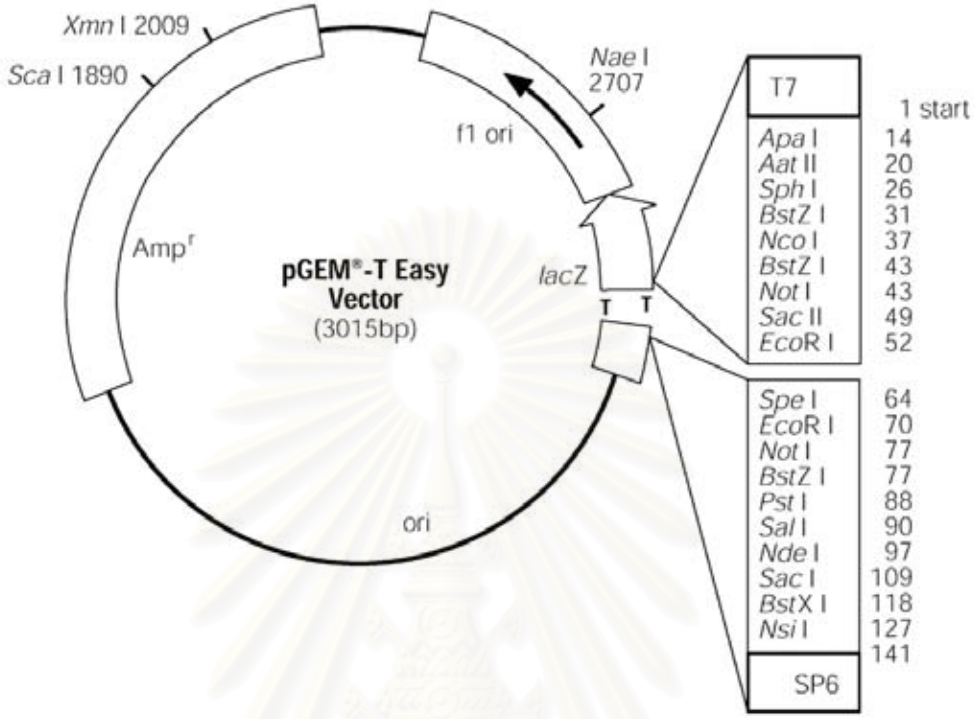
**4.1 pGEM®-T Vector Circle Map and Sequence Reference Points.**




pGEM® -T Vector sequence reference points:

| | |
|---|---|
| T7 RNA polymerase transcription initiation site | 1 |
| multiple cloning region | 10–113 |
| SP6 RNA polymerase promoter (–17 to +3) | 124–143 |
| SP6 RNA polymerase transcription initiation site | 126 |
| pUC/M13 Reverse Sequencing Primer binding site | 161–177 |
| lacZ start codon | 165 |
| lacoperator | 185–201 |
| β-lactamase coding region | 1322–2182 |
| phage f1 region | 2365–2820 |
| lacoperon sequences | 2821–2981, 151–380 |
| pUC/M13 Forward Sequencing Primer binding site | 2941–2957 |
| T7 RNA polymerase promoter (–17 to +3) | 2984–3 |

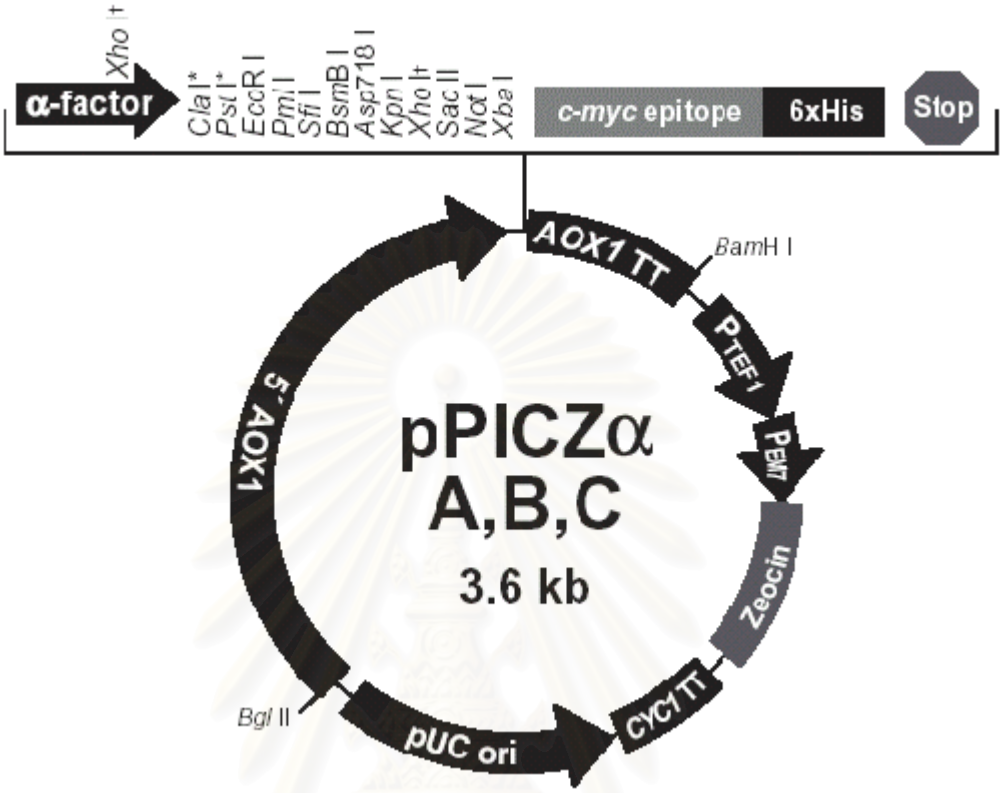
**4.2 pGEM®-T Easy Vector Circle Map and Sequence Reference Points.**




pGEM® -T Easy Vector sequence reference points:

| | |
|---|---|
| T7 RNA polymerase transcription initiation site | 1 |
| multiple cloning region | 10–128 |
| SP6 RNA polymerase promoter (–17 to +3) | 139–158 |
| SP6 RNA polymerase transcription initiation site | 141 |
| pUC/M13 Reverse Sequencing Primer binding site | 176–197 |
| lacZ start codon | 180 |
| lacoperator | 200–216 |
| β-lactamase coding region | 1337–2197 |
| phage f1 region | 2380–2835 |
| lacoperon sequences | 2836–2996, 166–395 |
| pUC/M13 Forward Sequencing Primer binding site | 2949–2972 |
| T7 RNA polymerase promoter (–17 to +3) | 2999–3 |

**4.3 Map of pPICZα A, B, C.**




**5. Others**

**5.1 DAB /$NiCl_2$ Visualization Solution**

| | |
|---|---|
| 5 ml | 100 mM Tris-C pH 7.5 |
| 120 µl | DAB stock (40 mg/ml in $H_2O$, stored in 100 µl aliquots at –20 °C |
| 25 µl | $NiCl_2$ stock (80 mg/ml in $H_2O$, stored in 100 µl aliquots at –20 °C |

Mix just before use.

**5.2 12 % Gel (5 ml) Resolving Gels for Tris-Glycine SDS-Polyacrylamide Gel Electrophoresis**

| | |
|---|---|
| 1.6 ml | $H_2O$ |
| 2.0 ml | 30 % acrylamide mix |
| 1.3 ml | 1.5 M Tris, pH 8.8 |
| 0.05 ml | 10 % SDS |
| 0.05 ml | 10 % ammonium persulfate |
| 0.002 ml | TEMED |

**5.3 5 % Stacking Gel (1 ml)**

| | |
|---|---|
| 0.068 ml | $H_2O$ |
| 0.17 ml | 30 % acrylamide mix |
| 0.13 ml | 1.0 M Tris, pH 6.8 |
| 0.01 ml | 10 % SDS |
| 0.01 ml | 10 % ammonium persulfate |
| 0.001 ml | TEMED |
| 0.1 % | SDS |

**5.4 2X SDS Gel-Loading Buffer**

| | |
|---|---|
| 100 mM | Tris-Cl, pH 8.8 |
| 4 % w/v | SDS |
| 0.2 % w/v | bromphenol blue |
| 20 % v/v | glycerol |
| 200 mM | dithiothreitol or β-mercaptoethanol |

# BIOGRAPHY

Miss Pon Singhamatr was born in 1975. She obtained the Bachelor's Degree from the Faculty of Allied Health Science, Chulalongkorn University in 1997. The Bachelor's study was supported by the scholarship from Chulalongkorn University. She has worked at Queen Sirikit National Institute of Child Health as a medical technologist since 1998. She continued her study for Master's Degree in Medical Science, Faculty of Medicine, Chulalongkorn University in 2002.