CHAPTER IV

RESULT

4.1 Expression of Snake Venom Metalloprotease Clone 038 (SVMP 038) in *Pichia pastoris*

4.1.1 Amplification of SVMP 038 using Polymerase chain reaction (PCR)

The coding sequence of P-II SVMP 038, comprising metalloprotenase and disitegrin domains, was amplified by PCR using the forward primer, which included 6-Histidine-tag residues for facilitating purification and *Eco*R I recognition site, and the reverse primer, SVMR038R2 that had an the *Xba* I recognition site and UAA stop codon. It was 859 bp in length (Figure2).

Figure 2 The SVMP 038 PCR product of approximately 859 bp in length electrophoresed on 1.5% agarose gel. Lane 1 is the 100 Bp Molecular weight ladder and lane 2 present the P-II SVMP from PCR product

4.1.2 Ligation of SVMP 038 into pGEM® T-vector and Transformation of *E. coli*, JM109

The PCR product was extracted from 1.2% electropheresis gel using the High Pure Plasmid Isolation kit, subsequently, cloned into pGEM® T-vector and transformed into *E. coli*, JM109. The positive plasmid clones were identified by blue-white color screening system. The white plasmid clones were purified and digested with *E*coR I and *Xba* I to verify the presence of inserts and then sequenced using T7 sequencing primer to confirm the correct and in-framed sequences of P-II SVM 038. The selected clones were grown in LB broth with 100 µg/ml of ampicilin. The plasmid was purified again using the High Pure PCR Product purification Kit (Gel Extraction Kit) to obtain high yield and purity of the insert.

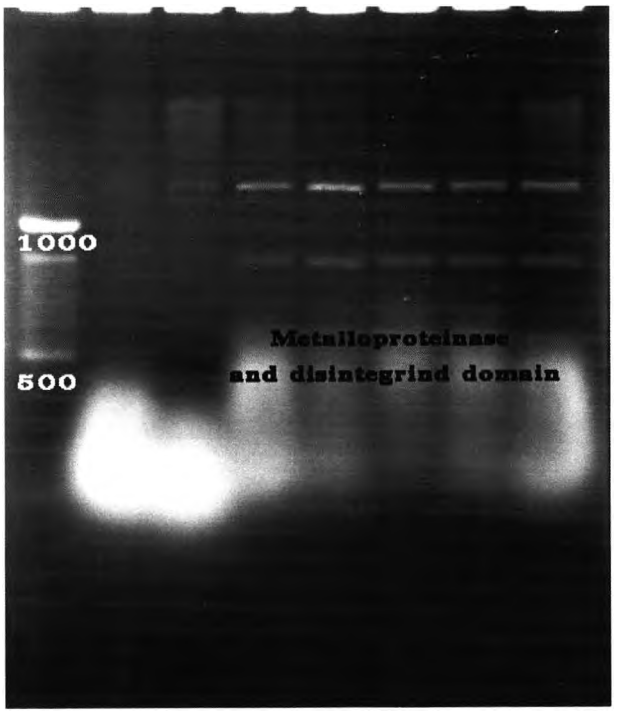

Figure 3 The restriction enzyme digested products of the plasmid pGEM® T containing the construct. Lane 1 is the 100 Bp molecular weight ladder. Lane 2 and 3 show undigested vector and lanes 4-8 show the digested vectors revealing the inserts.

4.1.3 Ligation of SVMP 038 into pPICZαA and Transformation into *E. coli*, JM109

After the plasmid clone was confirmed by sequencing, the inserts in plasmid DNA were digested with *Eco*R I and *Xba* I. The digestion reactions were electrophoresed in 1.2 % agarose gel (Figure 3). After gel extraction and purification, the digestion product was cloned into the *Eco*R I and *Xba* I sites of the expression vector, pPICZαA. The recombinant plasmid was transformed into *E. coli*, JM109, and the colony was selected on an agar plate of low-salt LB agar with 25 μg/ml Zeocin™. As a result, there were approximately 15 Zeocin™-resistance transformants. The recombinant plasmids were digested with *Eco*R I and *Xba* I as shown in Figure 4.

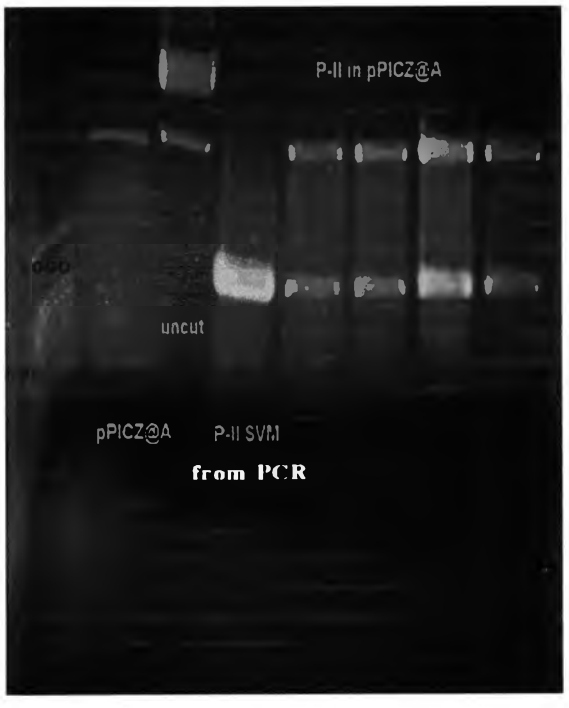

Figure 4 The restriction digested transformed pPICZαA vector. Lane 1 is 100 Bp marker ladder. Lane 2 and 3 show the undigested blank vectors. Lane 4 shows P-II SVM 038 from PCR as the marker and Lane 5 to lane 8 show inserts in the pPICZαA vector.

4.1.4 Sequence Alignment and Computational Searching Analysis

The insert-positive clones were sequenced and analyzed compared with GENBANK database using the BLAST N. The highest BLAST score of 038 SVM showed highest homology to *Trimeresurus jerdonii* jerdonitin, accession number AY 364231. An alignment showed 81 % amino acids sequence identity (Figure 5).

Sequences producing significant alignments:	Score (bits)	E Value
gb|AY364231.1| Trimeresurus jerdonii jerdonitin mRNA, complet...	830	0.0
gb|AY071905.1| Gloydius halys metalloproteinase mRNA, complet...	780	0.0
dbj|AB074143.1| Trimeresurus flavoviridis mRNA for hemorrhagi...	679	0.0
gb|AY204244.1| Gloydius saxatilis metalloproteinase/disintegr...	679	0.0
gb|AY835997.1| Macrovipera lebetina VLAIP-B mRNA, complete cds	673	0.0
gb|AF212305.1| Agkistrodon contortrix contortrix cont...	669	0.0
dbj|AB078906.1| Agkistrodon piscivorus piscivorus mRNA for pi...	665	0.0
gb|U86634.1| Agkistrodon contortrix laticinctus metal...	665	0.0
gb|DQ263750.1| Deinagkistrodon acutus agkihagin mRNA, complet...	664	0.0
dbj|AB078904.1| Agkistrodon contortrix contortrix mRNA for ac...	659	0.0
gb|M89784.1| Western diamondback rattlesnake preprom...	659	0.0
gb|AF051787.1| Gloydius halys metalloprotease (Mt-a) ...	659	0.0
gb|AY736107.1| Bothrops insularis insularinase and insularin ...	659	0.0

Figure 5 The homology search for the cDNA sequence of P-II SVMP clone 038 from *C. albolabris*.

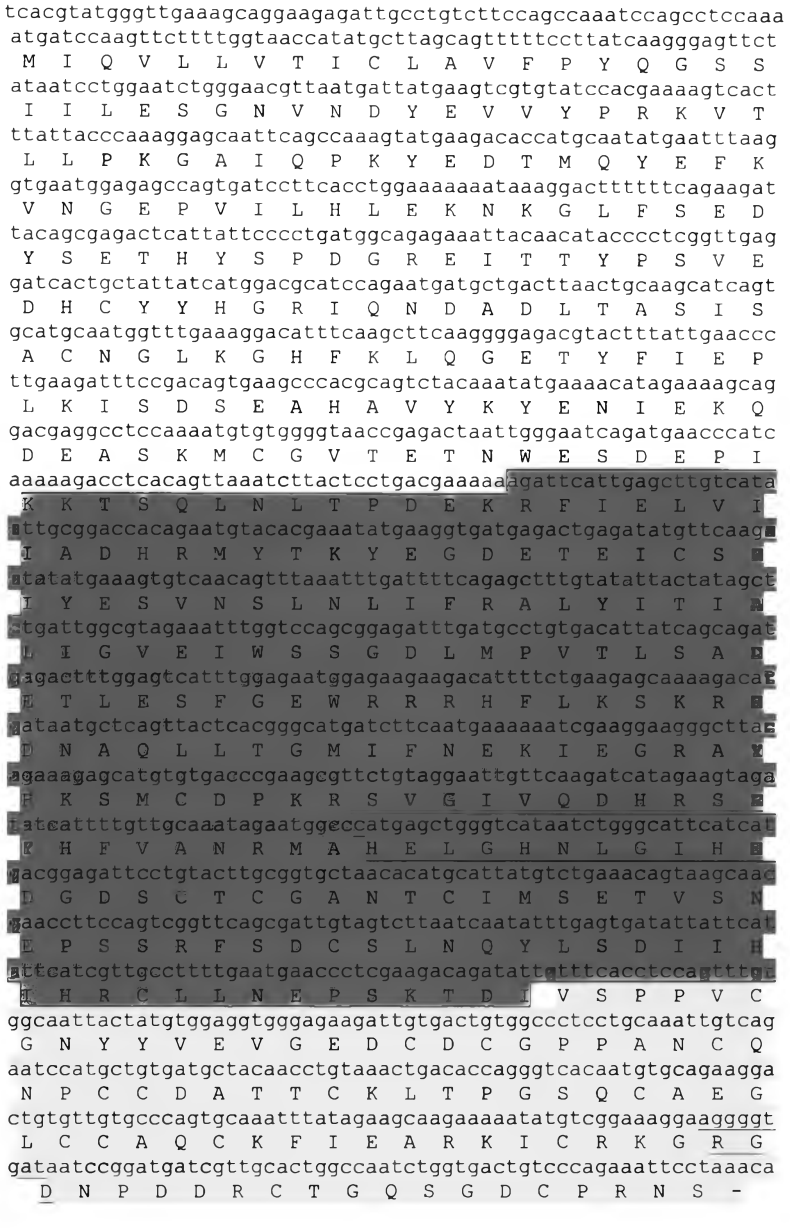

Figure 6 Show the cDNA sequence and conceptual translation of 038 SVMP: pre-prodomain was not highlight, shows the metalloproteinase domain with zinc binding motif in and shows the disintegrin domain. The RGD motif sequence was underlined.

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SVM_038         PRTEINSEQUENCEMIQVLLVTICLAVFPYQGSSIILESGNVNDYEVVYPRKVTLLPKGA
Jerdonitin      PRTEINSEQUENCEMIQVLLVTICLAVFPYQGSSIILESGNIDDYEVVYPRKVTALPKGA
                ****************************************:::********** *****

SVM_038         IQPKYEDTMQYEFKVNGEPVILHLEKNKGLFSEDYSETHYSPDGREITTYPSVEDHCYYH
Jerdonitin      VQQKYEDTMQYEFKVNEEPVVLHLEKNKGLFSKDYSETHYSPDGREITTYPPVEDHCYYH
                :* ************** ***:***********:******************.********

SVM_038         GRIQNDADLTASISACNGLKGHFKLQGETYFIEPLKISDSEAHAVYKYENIEKQDEASKM
Jerdonitin      GRIQNDADSTASISACNGLKGHFKLQGETYFIEPLKLPDSEAHAVFKYENVEKEDEAPKM
                ******** ****************************:.*******:****:**:***.**

SVM_038         CGVTETNWESDEPIKKTSQLNLTPDEKRFIELVIIADHRMYTKYEGDETEICSRIYESVN
Jerdonitin      CGVTETNWESDEPIKKLSQIMIPPEQQRYIELVIVADHRMYTKYDGDKTEISSKIYETAN
                ***************** **: :.*::::*:*****:*********:**:***.*:***:.*

SVM_038         SLNLIFRALYITIALIGVEIWSSGDLMPVTLSADETLESFGEWRRRHFLKSKRHDNAQLL
Jerdonitin      NLNEIYRHLKIHVVLIGLEMWSSGELSKVTLSADETLDSFGEWRERDLLQRKRHDNAQLL
                .** *:* * * :.***:*:****:*  *********:******.*.:*: *********

SVM_038         TGMIFNEKIEGRAYRKSMCDPKRSVGIVQDHRSRYHFVANRMAHELGHNLGIHHDGDSCT
Jerdonitin      TGMIFNEKIEGRAYKESMCDPKRSVGIVRDHRTRPHLVANRMAHELGHNLGFHHDGDSCT
                ***************::************:***:* *:***************:********

SVM_038         CGANTCIMSETVSNEPSSRFSDCSLNQYLSDIIH---IHRCLLNEPSKTDIVSPPVCGNY
Jerdonitin      CGANSCIMSATVSNEPSSRFSDCSLFQYSSDIIHNPFTSRCLYNEPSKTDIVSPSVCGNY
                ****:**** *************** ** *****     *** ***********.*****

SVM_038         YVEVGEDCDCGPPANCQNPCCDATTCKLTPGSQCAEGLCCAQCKFIEARKICRKGRGDNP
Jerdonitin      YMEVGEDCDCGPPANCQNPCCDAATCRLTPGSQCADGLCCDQCRFMKKGTICRIARGDDL
                *:**********************:**:********:**** **:*::  .*** .***:

SVM_038         DDRCTGQSGDCPRNS---
Jerdonitin      DDYCNGISAGCPRNPFHA
                ** *.* *..****.
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Figure 7 The protein sequence alignment of our SVMP_038 and Jerdonitin using CLUSTALW program. It reveals that they are 81% identical.

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

Prior to transformation into *Pichia pastoris*, recombinant pPICZαA was linearized with *Sac* I (Figure 8). After that, the linearized recombinant pPICZαA was transformed into competent *Pichia pastoris* cells, X-33. Approximately 60 colonies of transformants were found after 4 days as shown in Figure 9.

Figure 8 The *Sac* I restriction product with the size of approximately 3000bp Lane 1: 100 Base pair marker, Lane2: pPICZαA vector, Lane 3: pPICZαA vector with the insert, Lane 4-5: pPICZαA vector linearized by *Sac* I

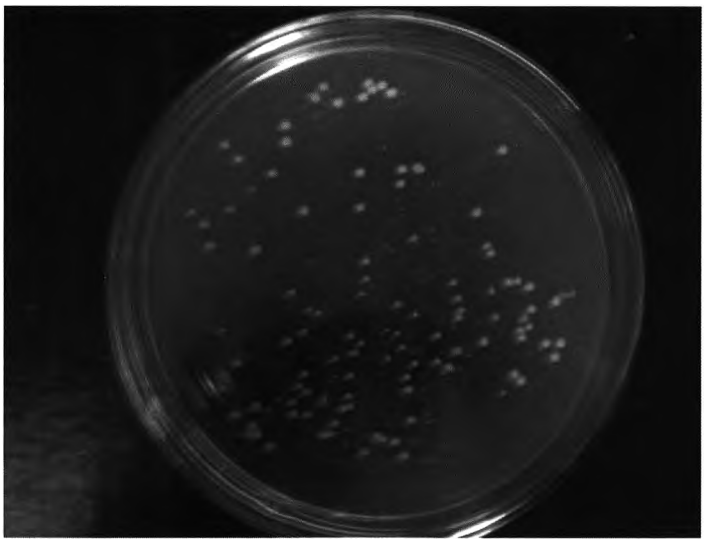

Figure 9 Colonies found on day 4 after transformation of a linearlized vector into *Pichia pastoris*, *X-33* on a Zeocin-containing plate

4.1.6 Recombinant SVM 038 from *Pichia pastoris*

After the positive colonies were identified, a clone was selected and cultured. We started to induce the expression of recombinant protein using 0.5% V/V methanol in BMMY media for 3 days. The media were precipitated and concentrated by VIVA spin at 6000 g centrifugation. The concentrated media were purified using affinity chromatography. The recombinant protein was, then, tested for its characteristics and functions.

4.2 Characteristics and functions of recombinant protein

4.2.1 Characteristics of SVMPs 038 Recombinant Protein

Purified protein was run on SDS PAGE (10% gel) and subjected to Western blot on PVDF membrane (Figure 10). After incubation with anti-histidine and anti-mouse HRP, the membrane was developed using ECL chemiluminassence on photo film. They revealed that recombinant protein size of approximately 32 kDa. Then, the concentration of the protein was measured by the microBCA ™Protein Assay using absorbance at 570nm at 1:30 dilution of the protein sample. The calculated protein concentration was 2.82 µg/ml or 84.5 µg/ml for undiluted protein. Therefore, the yield of recombinant disintegrin produce in *Pichia pastoris* was 8.5mg/Liter of culture medium.

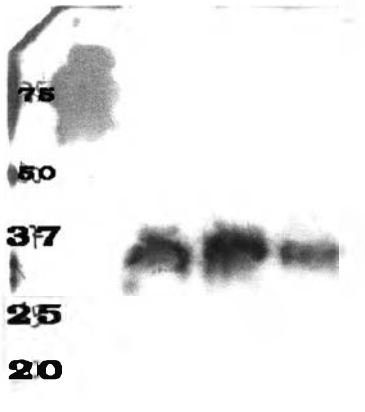

Figure 10 The affinity chromatography purified recombinant protein on Western blotting analysis of purify protein by chemiluminassence.

4.2.2 Activity of the SVMPs 038 Recombinant Protein on Platelet Aggregation

The purified recombinant protein SVM 038 was tested to investigate the function on platelet aggregation assay. The result showed that recombinant protein could inhibit collagen-induced platelets aggregation in a dose dependent manner. At the concentrations of 0, 4.25, 9.04 and 13.5 μM, there was 65%, 30% 10% and 0% aggregation, respectively (Figure 11). Using a curve fitting calculation, the IC_{50} of recombinant protein was 5.62 μM (Figure 11). However, there was no effect on ADP-induced platelet aggregation (the data was not shown).

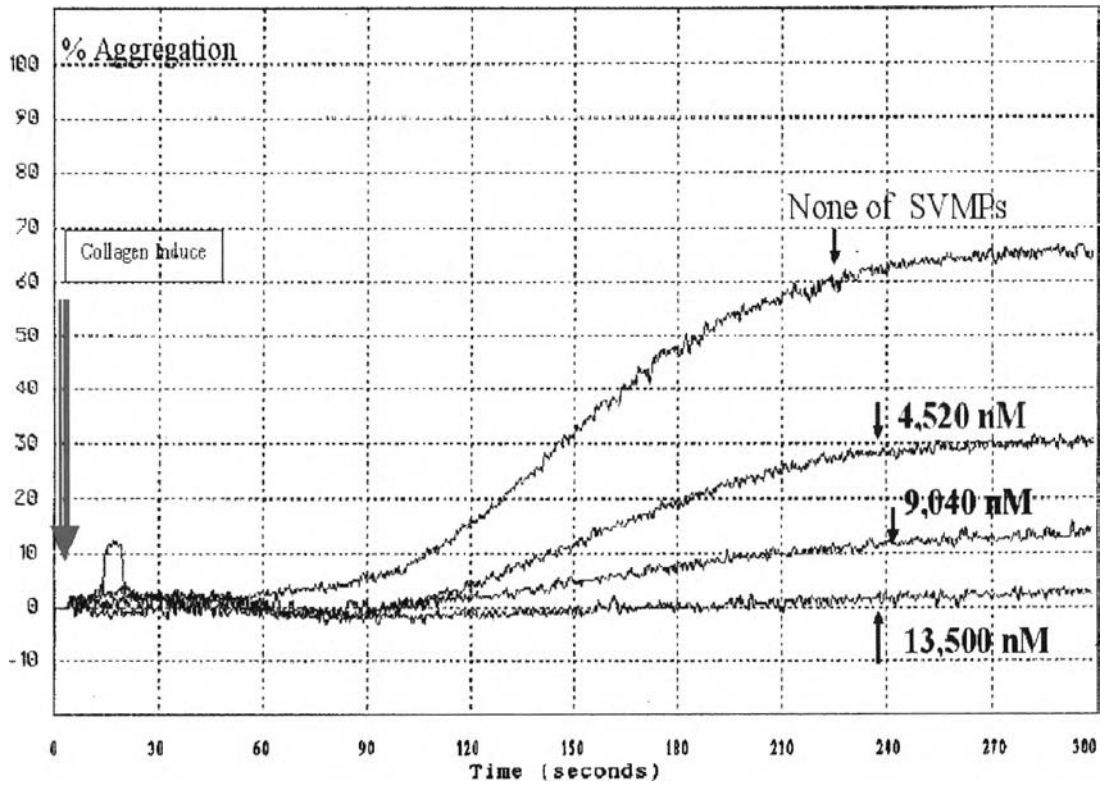

Figure 11 The inhibition of collagen-induced platelet aggregation by variable concentrations of recombinant SVM038.

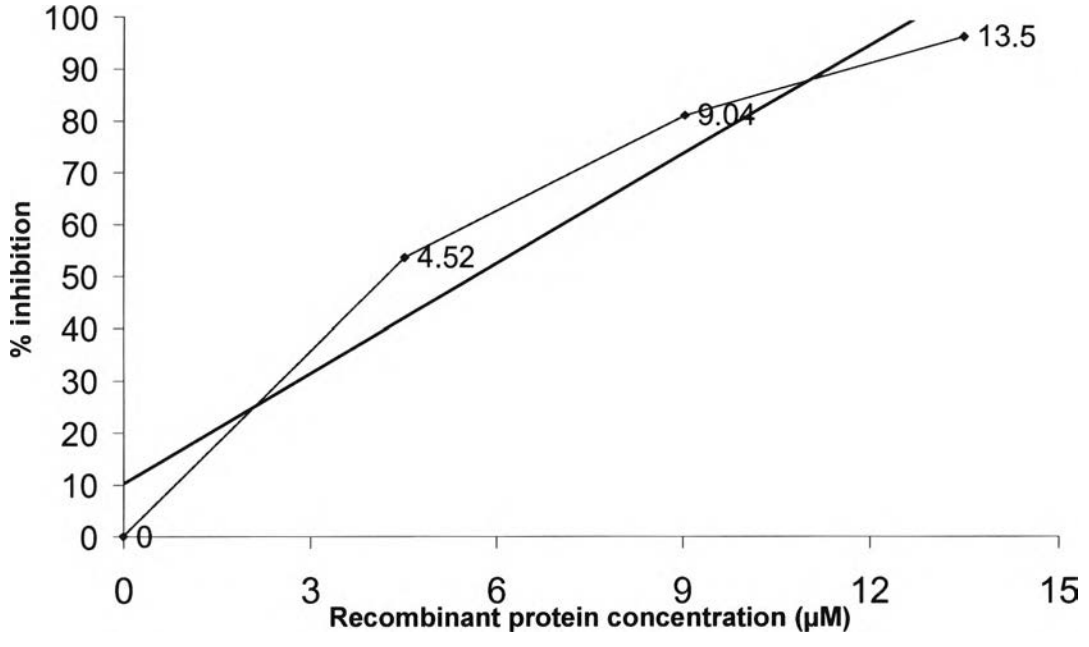

Figure 12 The effect of various concentrations of recombinant SVM038 on inhibition of collagen-induced human platelets aggregation. The IC_{50} determined from curve fitting is 5.62 µM