CHAPTER IV

RESULT

4.1 Expression of A Novel Snake Venom Serine Proteinase (SVSP) in *Pichia pastoris*

4.1.1 Amplification of GPV-PA using Polymerase Chain Reaction (PCR)

The structural gene of serine proteinse domain was amplified by PCR with a forward primer, GPVPAF, that has an *Eco*R I recognition site and six histidine residues for facilitating purification and a reverse primer, GPVPAR, that has an *Xba* I recognition site and UAA stop codon. After electrophoresis, the PCR product size was approximately 705 bp in length (Figure 2)

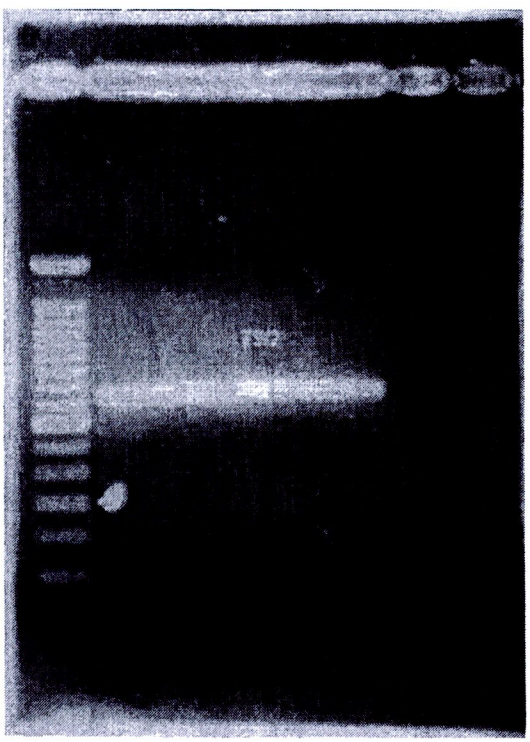

Figure 2 The GPV-PA coding sequence PCR product of approximately 705 bp in length electrophoresed on a 1.5% agarose gel.

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

The PCR product was extracted from 1.2% electrophoresis gel using the High Pure Plasmid Isolation kit, cloned into pGEM® T-vector and subsequently transformed to *E. coli*, JM 109. The positive plasmid clones were identified by the blue — white colony screening system. The white plasmid clones were purified and digested with *Eco*R I and *Xba* I to verify the presence of inserts and then sequenced using the T7 sequencing primer to confirm the correct and in-framed sequences of GPV-PA.

The selected clones were grown in LB broth with 100 μg/ml of ampicilin. The plasmid was purified again using High Pure PCR Product purification Kit (Gel Extraction Kit) to obtain a high yield of the purified insert.

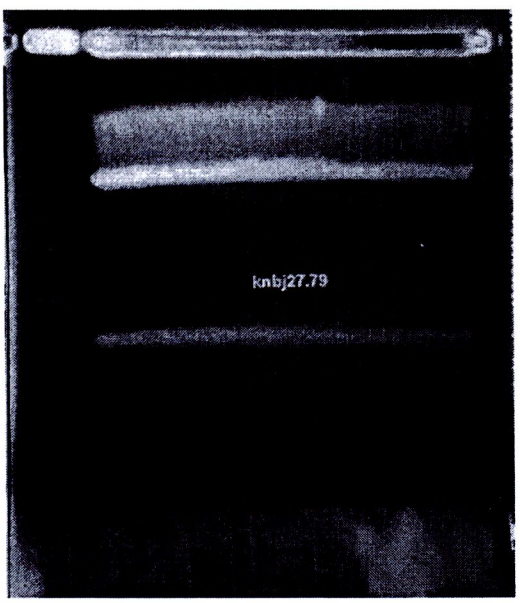

Figure 3 The restriction enzyme digested products of plasmid pGEM® T-vector containing the construct.

4.1.3 Ligation of GPV-PA into pPICZαA and Transformation into *E.* coli, JM 109

After the plasmid clone was confirmed by sequencing, the inserts in plasmid DNA were digested with *Eco*R I and *X*ba I. The digestion reactions were electrophoresed in 1.2% agarose gel (**Figure 3**). After gel extraction and purification, the digestion product was cloned into *Eco*R I and *X*ba I sites of the expression vector, pPICZαA. The recombinant plasmid was transformed into *E.* coli, JM 109, 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 tranformants. The recombinant plasmids were digested with *Eco*R I and *X*ba I as shown in **Figure 4**.

Figure 4 The restriction digested transformed pPICZαA vector. The picture shows inserts in the pPICZαA vector.

4.1.4 Sequence Alignment and Bioinformatic Analysis

The cDNA sequence from recombinant clone of GPV-PA was shown with redundant amino acid sequence after transalation (Figure 5)

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cgcccagctgcttaatttaatcaaataaagtgctgcttgatcaaaaagtctccgcttgggt
tatctgattaggttgatacggtacctcaagtttaagtaagggattggaatcttacaggca
aagagctttctgcgcagagttgaagctatggttctgatcagagtgctagcaaaccttctg
                                  M  V  L  I  R  V  L  A  N  L  L
atactacagctttcttacgcacaaaaatcttctgaactggtctttggaggtcgtccatgt
 I  L  Q  L                    S  S  E  L  V  F  G  G  R  P  C
aacataaatgaacatcgttcccttgttgtcttgtttaactccagcggttttctctgtggt
 N  I  N  E  H  R  S  L  V  V  L  F  N  S  S  G  F  L  C  G
gggactttgatcaatcaggattgggtggtcaccgctgcacactgtgacagtaataatttc
 G  T  L  I  N  Q  D  W  V  V  T  A  A  H  C  D  S  N  N  F
cagttgctgtttggtgtgcatagcaaaaagacactaaatgaggatgagcagacaagagac
 Q  L  L  F  G  V  H  S  K  K  T  L  N  E  D  E  Q  T  R  D
ccaaaggagaagttcttttgtcccaataggaaaaaggatgacgaagtggacaaggacatc
 P  K  E  K  F  F  C  P  N  R  K  K  D  D  E  V  D  K  D  I
atgttgatcaagctggacagttctgttaacaacagtgaacacatcgcgcctctcagcttg
 M  L  I  K  L  D  S  S  V  N  N  S  E  H  I  A  P  L  S  L
ccttccagccctcccagtgtgggctcagtttgccgtattatgggatggggcaaaaccata
 P  S  S  P  P  S  V  G  S  V  C  R  I  M  G  W  G  K  T  I
cctactaaagatatttatcccgatgtccctcattgtgctaacattaacatactcgatcat
 P  T  K  D  I  Y  P  D  V  P  H  C  A  N  I  N  I  L  D  H
gcggtgtgtcgaacagcttattcatggcggcaggtggcaaacacaacattgtgtgcaggt
 A  V  C  R  T  A  Y  S  W  R  Q  V  A  N  T  T  L  C  A  G
atcctgcaaggaggcaaagatacatgtcactttgactctgggggacccctcatctgtaat
 I  L  Q  G  G  K  D  T  C  H  F  D  S  G  G  P  L  I  C  N
gaacaattccatggcattgtatcttggggtgggcatccttgtggccaaccgcgggagcct
 E  Q  F  H  G  I  V  S  W  G  G  H  P  C  G  Q  P  R  E  P
ggcgtctacaccaatgtcttcgattatactgactggatccagagcattattgcaggaaat
 G  V  Y  T  N  V  F  D  Y  T  D  W  I  Q  S  I  I  A  G  N
Aaagatgcaacctgccccccgtgaaaactttgaaaaagttaagaggagaatatgtaaca
 K  D  A  T  C  P  P  –
tattagtacatctcttctatatccctaaccatatccgactacattggaatatattcccag
gcagaaaggtt
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Figure 5 The redundant amino acid sequence of GPV-PA was shown with highlight signal peptide prediction as SYAQK. The predicted N-glycosylation sites was underlined.

The inserted positive clones were sequenced, analyzed and compared with GENEBANK database using the BLAST N. The highest BLAST score of GPV-PA showed highest homology to *Viridovipera stejnegeri*, accession number AAC59686.1. An alignment showed 94 % of amino acids sequence identity (Figure 6).

Sequences producing significant alignments:	Score (bits)	E Value
gb|AAC59686.1| *Viridovipera stejnegeri* venom PA precursor	490	0.0
gb|AAG10789.1| *Trimeresurus jerdonii* serine proteinase2 precursor	485	0.0
gb|AAG10790.1| *Trimeresurus jerdonii* serine proteinase3precursor	450	0.0
gb|ABB76280.1| *Bothrops asper* thrombin-like enzyme	421	0.0
gb|ABD52886.1| *Lachesis muta* serine protease precursor	418	0.0
gb|AAN52350.1| *Viridovipera stejnegeri* venom serine protease 5	395	0.0
gb|AAQ02910.1| *Viridovipera stejnegeri* serine protease PA precursor	392	0.0
gb|AAF76378.1| *Deinagkistrodon acutus* thrombin-like protein DAV-PA..	390	0.0
gb|AAN52349.1| *Viridovipera stejnegeri* stejnefibrase 2 thrombin-like...	384	0.0

Figure 6 The homology search for cDNA sequence of GPV-PA of *C. albolabris*

The selected 4 venom serine proteinase were aligned using Geneous™ commercial software analysis. The consensus sequence indicates as green color with black highlight that indicate the high percentage of identity among these protein sequences. The pairwise statistic analysis of GPV-PA identity with other venom serine proteinase from alignment is 82.6 % (Figure 7).

In this analysis, the TSV-PA and GPV-PA are extensively identical protein with identity score as 94% (Figure 8). Furthermore, the phylogenetic tree analysis of four selected proteins were compared using the same computer program analysis that reveal distinct result of the intensive relation between TSV-PA and GPV-PA (Figure 8). In addition, the conserved cysteine residue alignment and signal peptide cleavage prediction analysisn were reported in GPV-PA (Figure 9 and 10).

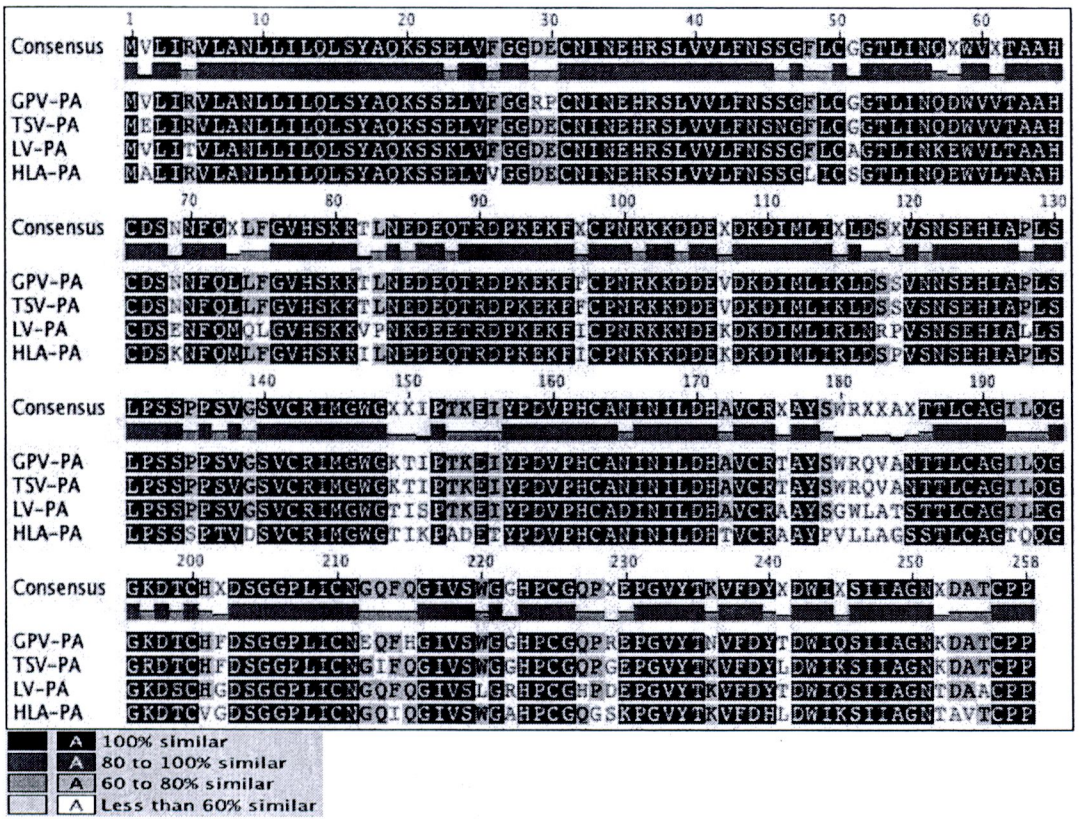

Figure 7 The protein sequence alignment of GPV-PA using Genious™ program.

```
GPV-PA    MVLIRVLANLLILQLSYAQKSSELVFGGRPCNINEHRSLVVLFNSSGFLCGGTLINQDWV
TSV-PA    MELIRVLANLLILQLSYAQKSSELVFGGDECNINEHRSLVVLFNSNGFLCGGTLINQDWV
          *:****************************::***************:**************


GPV-PA    VTAAHCDSNNFQLLFGVHSKKTLNEDEQTRDPKEKFFCPNRKKDDEVDKDIMLIKLDSSV
TSV-PA    VTAAHCDSNNFQLLFGVHSKKTLNEDEQTRDPKEKFFCPNRKKDDEVDKDIMLIKLDSSV
          ************************************************************


GPV-PA    NNSEHIAPLSLPSSPPSVGSVCRIMGWGKTIPTKDIYPDVPHCANINILDHAVCRTAYSW
TSV-PA    SNSEHIAPLSLPSSPPSVGSVCRIMGWGKTIPTKEIYPDVPHCANINILDHAVCRTAYSW
          :**********************************:************************


GPV-PA    RQVANTTLCAGILQGGKDTCHFDSGGPLICNEQFHGIVSWGGHPCGQPREPGVYTNVFDY
TSV-PA    RQVANTTLCAGILQGGRDTCHFDSGGPLICNGIFQGIVSWGGHPCGQPGEPGVYTKVFDY
          ****************:***************::*:*************:******:****


GPV-PA    TDWIQSIIAGNKDATCPP
TSV-PA    LDWIKSIIAGNKDATCPP
          :***:**************
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Figure 8 The protein sequence alignment of GPV-PA and *Viridovipera stejnegeri* (TSV-PA) using Genious™ program program. It reveals that they were 94 % identical.

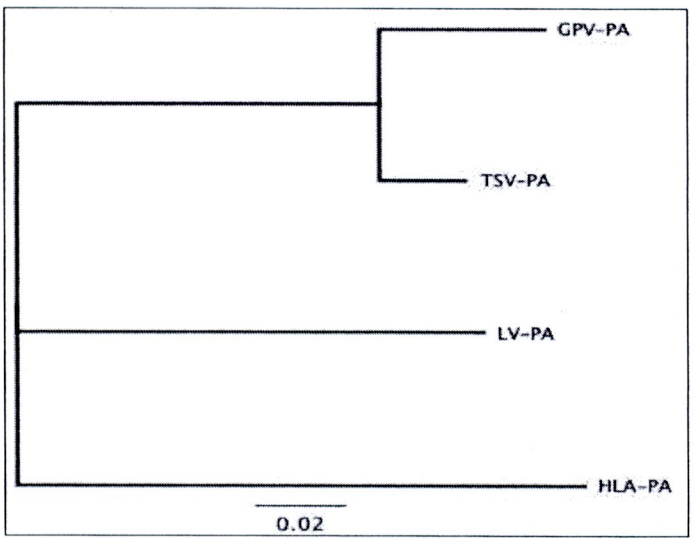

Figure 9 The phylogenetic tree prediction of GPV-PA and other snake venom proteins.

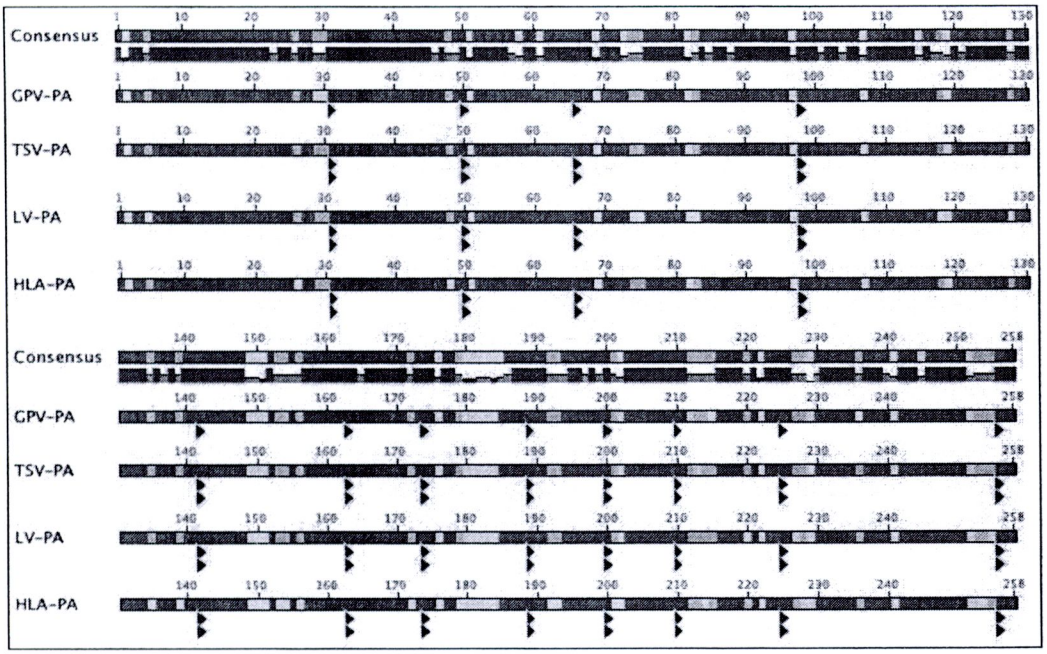

Figure 10 The conserved cysteine residues were represent as red arrow in the identical consensus motif of all protein comparing with GPV-PA.

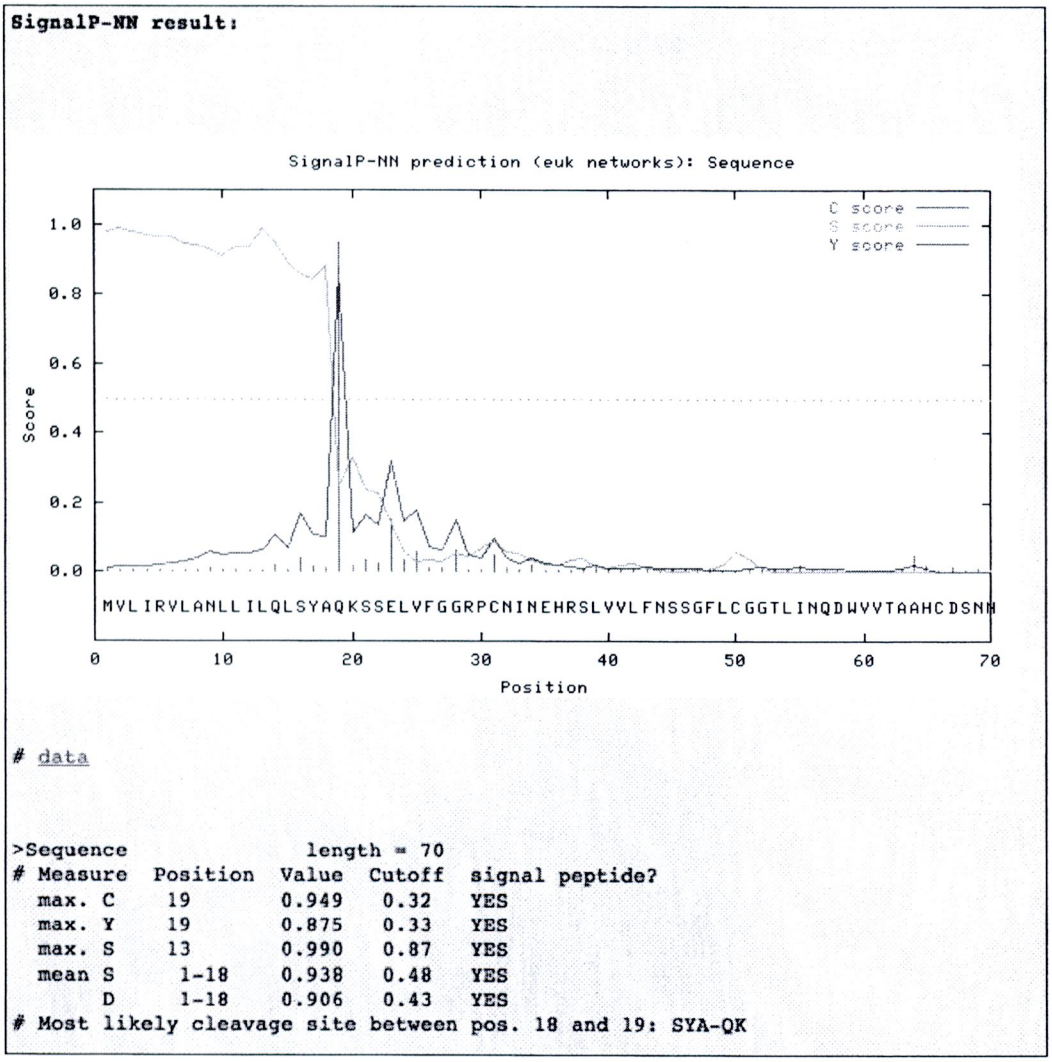

Figure 11 The signal peptide and its cleavage prediction in GPV-PA represent the cleavage site between amino acid residue 18 and 19 (SYA-QK). In this analysis latter sequence of its signal peptide revealed the conserve amino acid residue as KSSEL that determined the post translational protein modification of GPV-PA as secretory protein.

4.1.5 Tranformation of Recombinant pPICZαA into *Pichia pastroris*, X– 33

Prior to transformation into *Pichia pastroris*, recombinant pPICZαA was linearlized with *Sac* I (Figure 8). After that, the linearized recombinant pPICZαA was transformed into competent *Pichia pastroris* cells, X – 33. Approximately, 60 colonies of transformant were found after 4 days as shown in **Figure 13**.

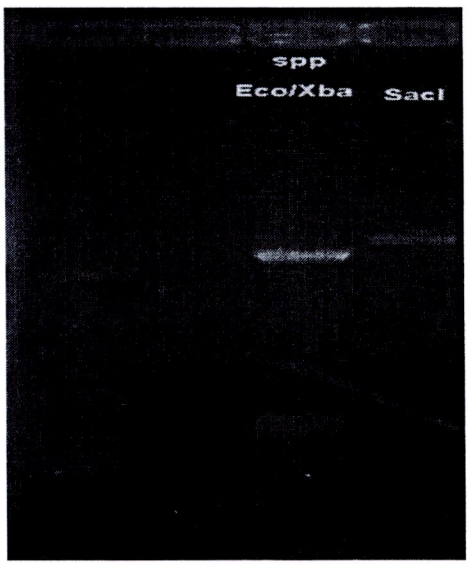

Figure 12 The *Sac* I restriction product with the size of approximately 3000 bp. Lane 2: pPICZαA vector with inserts digested by *Eco*R I and *X*ba I, Lane 3: pPICZαA vector linearized by *Sac* I.

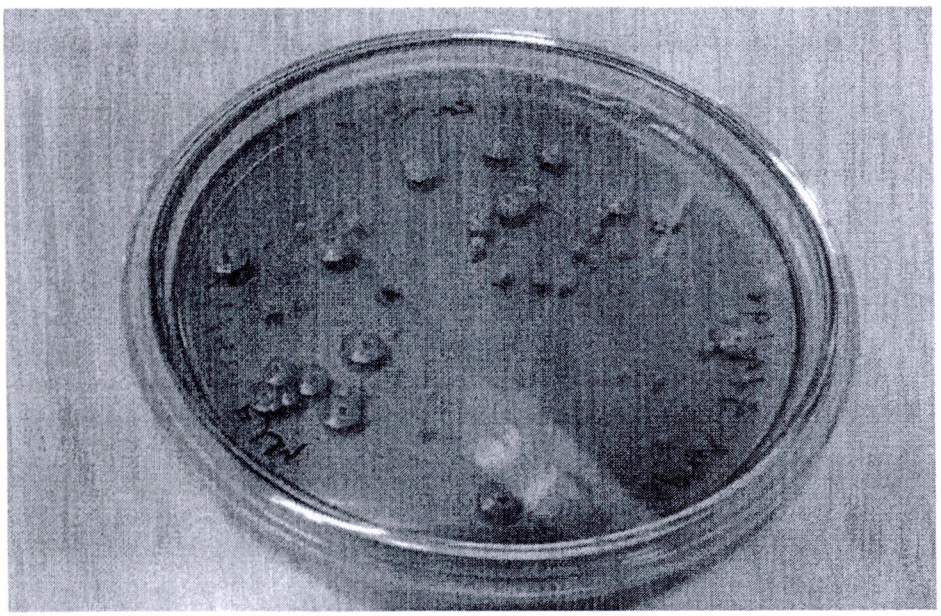

Figure 13 The colonies found on day 4 after transformation of a linearlized vector into *Pichia pastroris,* X-33 on a Zeocin-containing plate

4.1.6 Recombinant GPV-PA from *Pichia pastroris*

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 by using affinity chromatography. Subsequently, the recombinant protein was tested or its characteristics and functions. Moreover, increasing the concentration of methanol for induction as 1% could increase the efficacy of recombinant protein production as

4.2 Characteristics and functions of recombinant protein

4.2.1 Characteristics of GPVPA 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 chemi-luminescence on a photo film. They revealed that recombinant protein size of approximately 37 kDa. Then the concentration of the protein was measured by the micro BCA ™ Protein Assay using absorbance at 562 nm at 1:30 dilution of the protein sample. The calculated protein concentration was 5.45 µg/ml or 163.5 µg/ml for undiluted protein. Therefore, the yield of recombinant serine proteinase product in *Pichia pastroris* was 0.38 mg / Liter of culture medium. Moreover, increasing the concentration of methanol for induction as 1% could effect the efficacy

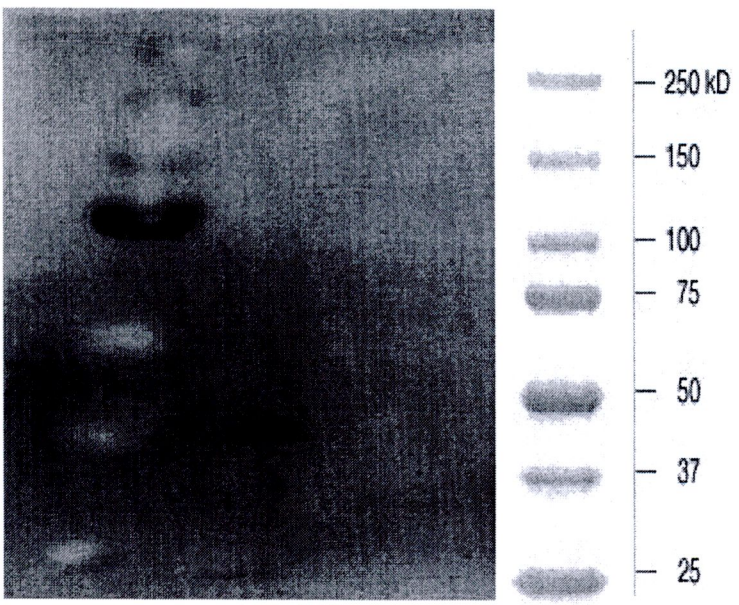

Figure 14 The affinity chromatography purified recombinant GPV-PA on Western blotting analysis of the purify protein visualized by chemi-luminescence.

4.2.2 Plasminogen activation assay

The average of plasminogen activator activity in GPV-PA compare to the activity of control plasminogen activator (U-PA) (Figure 12).

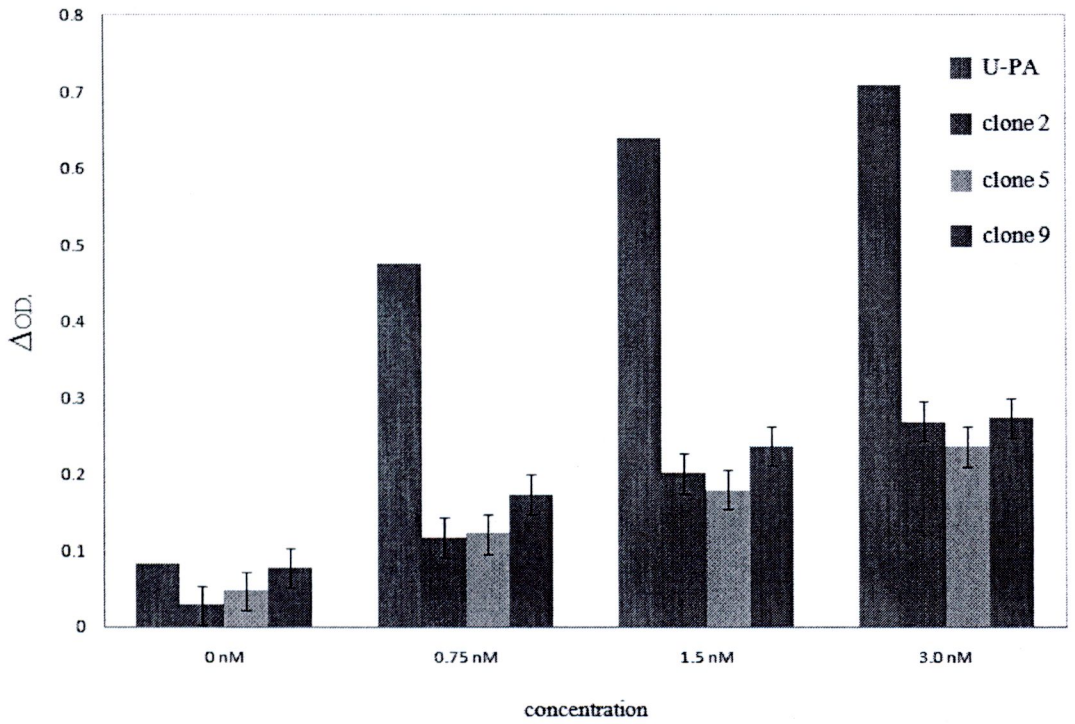

Figure 15. Plasminogen activation by GPV-PA. The delta OD represents absorbance changes GPV-PA added at the indicated concentrations time were measured for the absorbance (405 nm.) at 0 minute and 10 minutes. U-PA was used as standard and S2251 chromogenic substrate was used.

4.2.3 Platelet Aggregate assay

The purified recombinant protein GPV-PA was tested to investigate the function on platelet aggregation assay. The result showed that recombinant GPV-PA could not inhibit collagen-induced platelets aggregation in a dose dependent manner. At the concentration of 0, 5.0 and 10 μM, there was not aggregation.

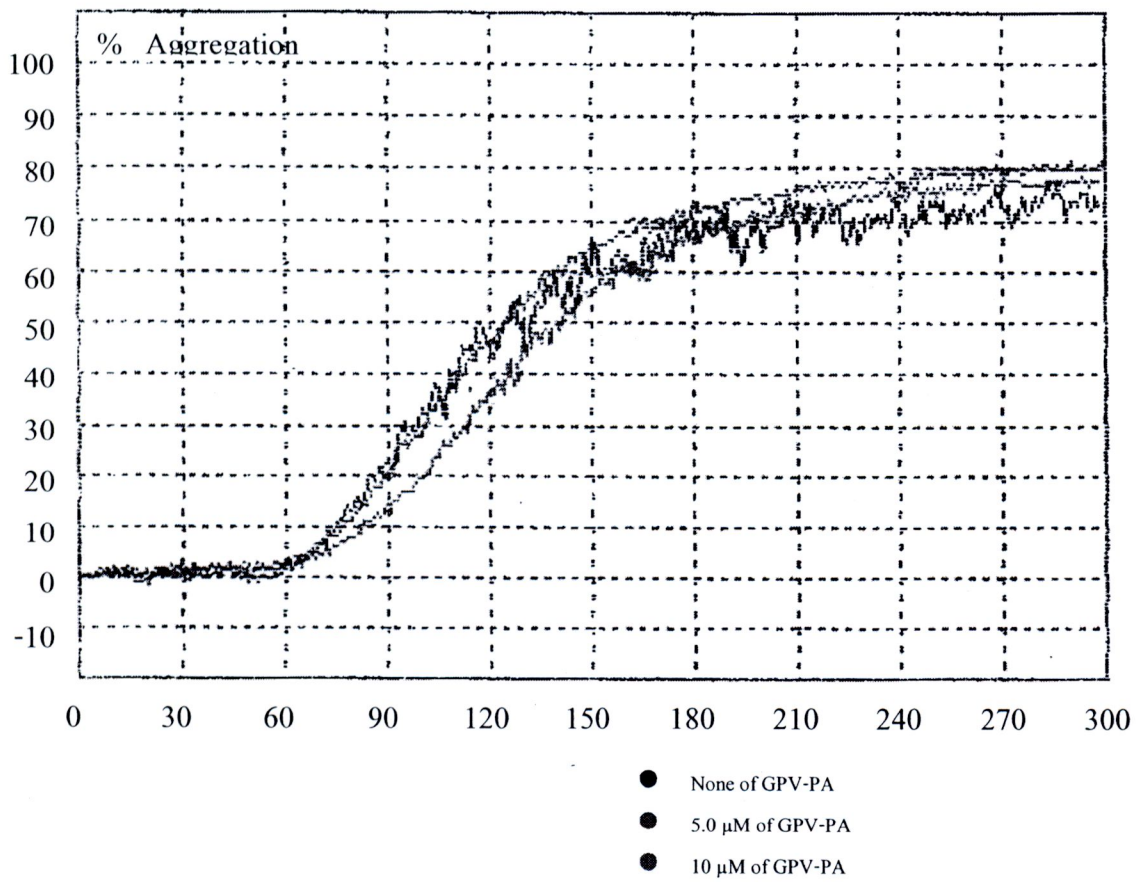

Figure 16. GPV-PA could not induce platelet aggregation by platelet aggregation assay.