# IV. RESULTS

## A. Selection of the DNA regions of *P. marneffei* for subcloning

The amino acid positions 10 to 220 of *P6* gene showed high hydrophilic score indicating the surface regions, while the amino acid positions 220 to 360 showed hydrophobic score (**Figure 9A**). Therefore, the 187 amino acid residues of *P6* at amino acid positions 28 to 214 were selected for cloning into the pGEX-4T-1 vector. The hydropathy plot of *P23* showed the hydrophilic score in a whole amino acid sequence (**Figure 10A**). Thus, the region of 187 amino acid residues of *P23* was selected for cloning into the pGEX-4T-1 vector. The antigenicity along the polypeptide chain was predicted by the algorithm of Hopp and Woods (1981). The amino acid sequences of the selected region of *P6* and *P23* showed high antigenicity index, **Figure 9B** and **Figure 10B**, respectively. The predictions for N- and O-glycosylation sites of *P6* and *P23* proteins revealed that the *P6* contained one predicted N-glycosylation site at asparagines position 163 and a serine- and threonine-rich region for O-glycosylation, while the *P23* contained only serine- and threonine-rich region for O-glycosylation. For the predictions of primary structure analysis, theoretical MW of the *P6* and *P23* protein sequences are summarized in **Table 2**.

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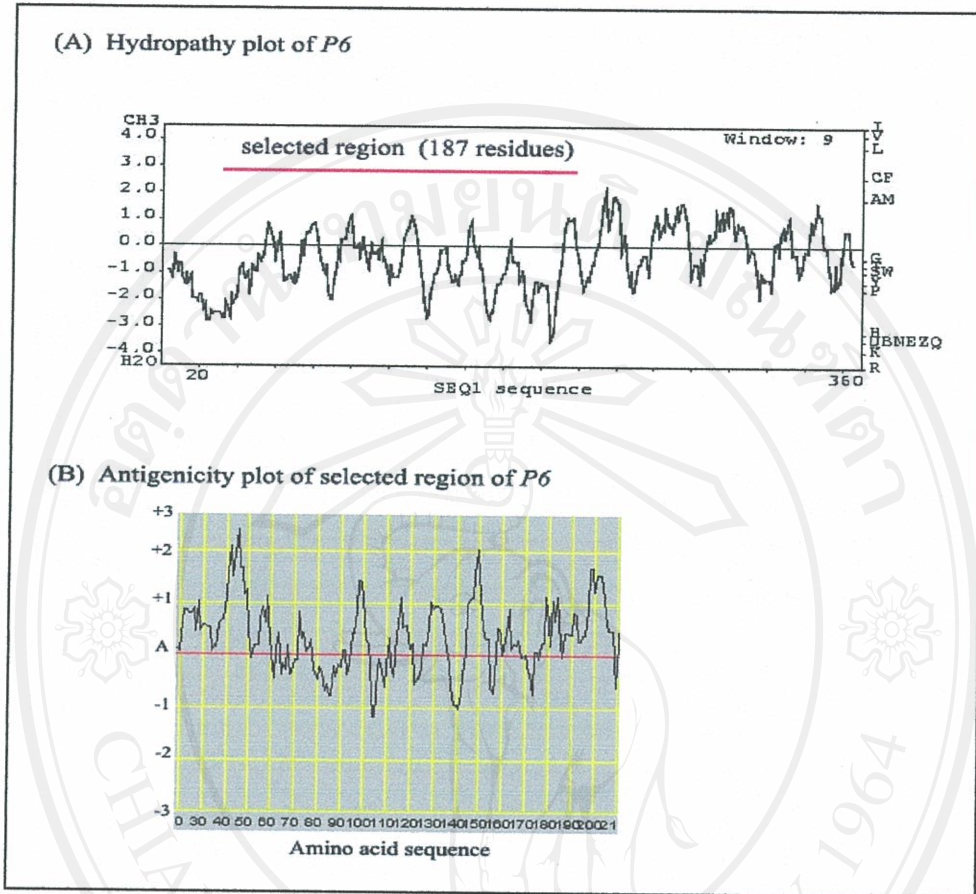


**Figure 9.** (A) Hydropathy plot of *P6* is predicted by using the FASTA program (http://fasta.bioch.virginia.edu/o_fasta/grease.htm with the Window size = 9). A red-line indicates the selected region containing 187 amino acid residues of *P6*. (B) Antigenicity plot of selected region of *P6* is predicted by using the program JaMBW Chapter 3.1.7 (http://hometown.aol.com/_ht_a/lucatoldo/myhomepage/JaMBW/3/1/7)

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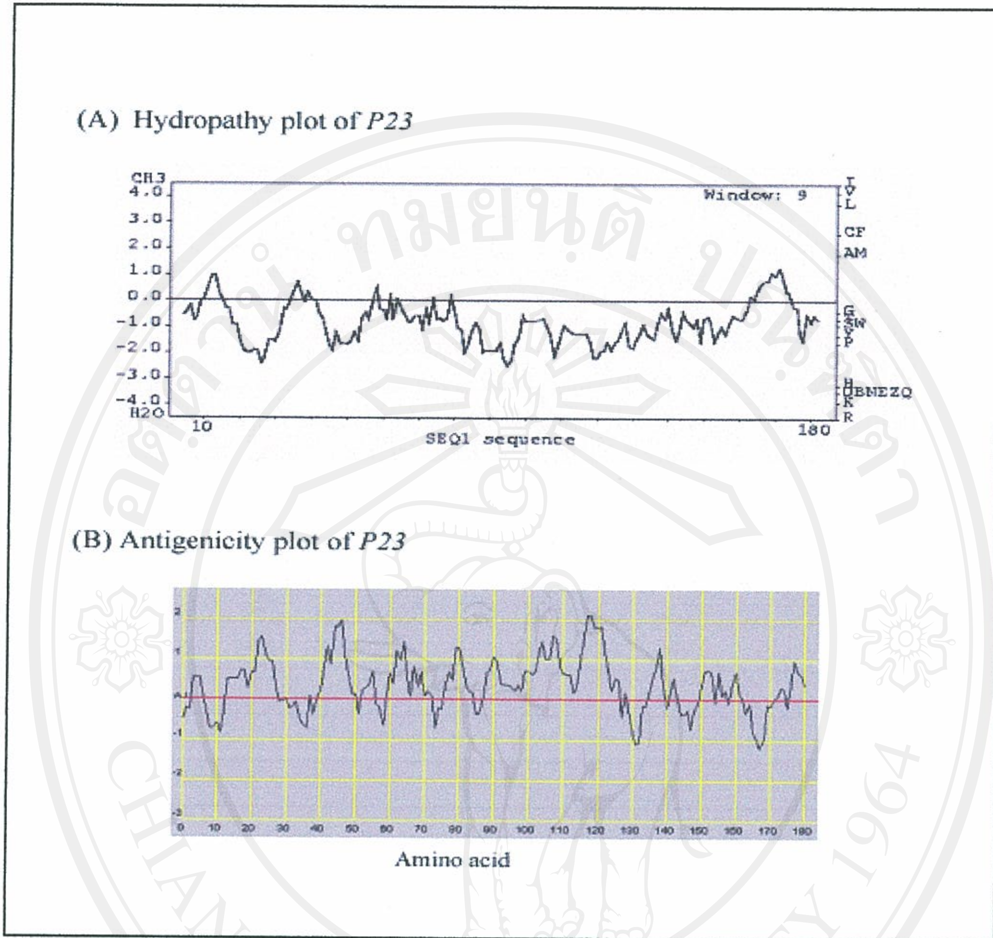


**Figure 10.** The selected region of *P23* containing 187 amino acid residues . (A) Hydropathy plot of *P23* is predicted by using the FASTA program (http://fasta.bioch.virginia.edu/o_fasta/grease.htm with the Window size = 9). (B) Antigenicity plot of selected region of *P23* is predicted by using the program JaMBW Chapter 3.1.7 (http://hometown.aol.com/_ht_a/lucatoldo/myhomepage/JaMBW/3/1/7)

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**B. Primers design of the selected cDNA regions**

The oligonucleotide primers were designed from the selected DNA sequence regions of clone *P6* and *P23*. The forward primers were extended with the recognition sites of the restriction enzymes, *Bam* HI. The reverse primers were extended with the recognition sites of the restriction enzymes, *Xho* I. The extended site were designed to amplify the DNA fragments which were in-framed in the vector. The nucleotide and translated amino acid sequence of *P6* are shown in **Figures 11** and **12**, respectively. The length of nucleotide sequence is 561 base pairs (bp) and translated amino acid sequence is 187 residues. The DNA fragment of these 561 bp of the *P6* or *P23* were cloned into an expression vector, pGEX-4T-1.

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*P6* **cDNA**

1 P F P S F L A T - K K M E T A N D A P S<br>
3 CCGTTCCCGTCTTTTCTTGCAACATGAAAGAAGATGGAGACTGCGAACGACGCACCCTCA

21 T T L E G Q H E A D A Q N S R T Q Q P Q<br>
63 ACTACACTGGAGGGACAACATGAAGCAGACGCTCAGAACAGCCGAACTCAACAACCCCAA

41 Q S P D S Q E D D A E E V R P P L P P R<br>
123 CAATCCCCCGACAGTCAAGAAGACGACGCAGAAGAAGTACGACCACCATTGCCACCACGT

61 P E T I D L L N E G I A F R T S T A R P<br>
183 CCTGAGACCATCGATCTGCTAAATGAAGGCATTGCCTTTCGTACCTCCACGGCGAGACCA

81 N L Q S H A T T A L S L T D I T G Q T N<br>
243 AACCTGCAGTCGCACGCGACAACGGCACTGTCATTGACAGACATTACCGGCCAAACGAAT

101 A D G R D G F V A G F G R T L L G R G L<br>
303 GCAGATGGAAGAGACGGTTTCGTTGCAGGCTTTGGGCGTACCTTGCTGGGGCGGGGACTA

121 R A K A S L S Q L N S A R G S E A G D T<br>
363 CGAGCAAAGGCTAGTTTGAGCCAACTGAATAGTGCTCGTGGTAGTGAAGCCGGGGACACC

141 A S V L S F A P N S E E G Q D E S L F G<br>
423 GCGAGCGTGTTAAGCTTTGCGCCGAATTCCGAGGAAGGCCAGGATGAGAGTCTGTTTGGG

161 E F A N E T N A Q D I S G N I E V L G Y<br>
483 GAGTTTGCAAATGAAACCAACGCGCAGGATATTTCTGGGAATATTGAAGTCCTGGGCTAT

181 D E Y P Q D G N E Y E F V E E F E P I G<br>
543 GATGAATATCCCCAGGACGGCAATGAGTACGAATTTGTAGAGGAATTCGAGCCGATAGGG

201 E L D E D G Q N E E S L L Q 187<br>
603 GAACTGGATGAGGATGGCCAAAAT**GAAGAATCACTGCTCCAG** 561

**Figure 11.** Nucleotide and translated amino acid sequences of *P6*. The length of nucleotide sequence is 561 base pairs and translated amino acid sequence is 187 residues. Underlined residues indicate the 5' primer binding site. Bold underlined residues indicate the 3' primer binding site.

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*P23* cDNA

1 M S L F H R S G D F A P L F R L L D D Y
1 ATGTCTCTCTTCCACCGCAGCGGCGACTTTGCTCCCCTCTTCCGTCTCCTCGACGACTAT

21 D L H R S G R D G Q T P A S S S I S S F
61 GACCTCCACCGCTCCGGACGCGACGGTCAGACTCCTGCCTCCAGTAGCATCTCAAGCTTC

41 A P R F D V R E S K D A Y H L D G E L P
121 GCGCCACGCTTCGACGTCCGCGAGTCCAAGGATGCTTACCATCTGGACGGCGAACTCCCC

61 G I A Q K D V E I E F S D P Q T L T I K
181 GGCATTGCTCAAAAGGACGTTGAAATCGAATTCTCCGACCCGCAGACATTGACCATCAAG

81 G R S V R E Y H T L P E N E N P H A P K
241 GGTCGCTCGGTTCGGGAATACCACACCCTTCCCGAGAACGAGAACCCTCATGCTCCTAAG

101 P A S V E D A P E S S D E T A V Q K S S
301 CCCGCTTCTGTCGAAGACGCACCCGAGTCCAGTGACGAGACAGCCGTCCAAAAGTCTTCC

121 D K K E V S K A Q G N G Y K Y W V S E R
361 GACAAAAAAGAGGTCTCGAAGGCTCAGGGTAACGGCTACAAGTACTGGGTCAGCGAGCGC

141 S V G E F H R S F N F P S R V D H N G V
421 TCAGTCGGCGAGTTCCATCGCTCATTCAACTTCCCTAGCCGCGTTGATCACAATGGCGTC

161 K A S L K N G V L T V T V P K A A P P T
481 AAGGCTAGTTTGAAGAATGGTGTTCTTACGGTGACGGTGCCCAAGGCTGCCCCTCCTACT

181 S R K I T I E - 187
541 A**GTCGCAAGATCACAATTGAG**TAA 561

**Figure 12.** Nucleotide and translated amino acid sequences of *P23*. The length of nucleotide sequence is 561 base pairs and translated amino acid sequence is 187 residues. Underlined residues indicate the 5' primer binding site. Bold underlined residues indicate the 3' primer binding site.

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The sequences of the primers that were used for amplification the interesting DNA region of the *P6* are shown in **Figure 13**. The forward primer, *Bam*-P6, was extended to the specific recognition site of the endonuclease restriction enzyme, *Bam* HI. The reverse primer, *Xho*-P6, was extended to the specific recognition site of the endonuclease restriction enzyme, *Xho* I

*Bam* HI
Forward primer:*Bam*-P6 5'-TCAGGATCCGAAGCAGACGCTCAGAAC-3'

*Xho* I
Reverse primer:*Xho*-P6 5'-CCGTAACTCGAGCTGGAGCAGTGATTCTTC-3'

**Figure 13.** The sequences of the primers used for amplification the interesting DNA region of the *P6*

The sequences of the primers that were used for amplification the interesting DNA region of the *P23* are shown in **Figure 14**. The forward primer, *Bam*-HSP30, was extended to the specific recognition site of the endonuclease restriction enzyme, *Bam* HI. The reverse primer, *Xho*-HSP30, was extended to the specific recognition site of the endonuclease restriction enzyme, *Xho* I

*Bam* HI
Forward primer:*Bam*-HSP30 5'-TCAGGATCCATGTCTCTCTTCCACCGCA-3'

*Xho* I
Reverse primer:*Xho*-HSP30 5'-CCGTAACTCGAGCTCAATTGTGATCTTGCGAC-3'

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**Figure 14.** The sequences of the primers used for amplification the interesting DNA region of the *P23*

## C. Generating the recombinant plasmids

C.1 Amplification of insert DNA, *P6* and *P23*

The plasmids, pZL1 containing the cDNA, *P6* or *P23* were selected and used as the templates in amplification reaction. The primer pairs, *Bam*-P6 and *Xho*-P6 as well as *Bam*-HSP30 and *Xho*-HSP30 were used to amplify *P6* and *P23* region, respectively. Electrophoresis of the amplified products was performed using 1% agarose gel at 100 volts for 20 min. The sizes of PCR products from both *P6* and *P23* were 561 bp as shown in **Figure 15**.

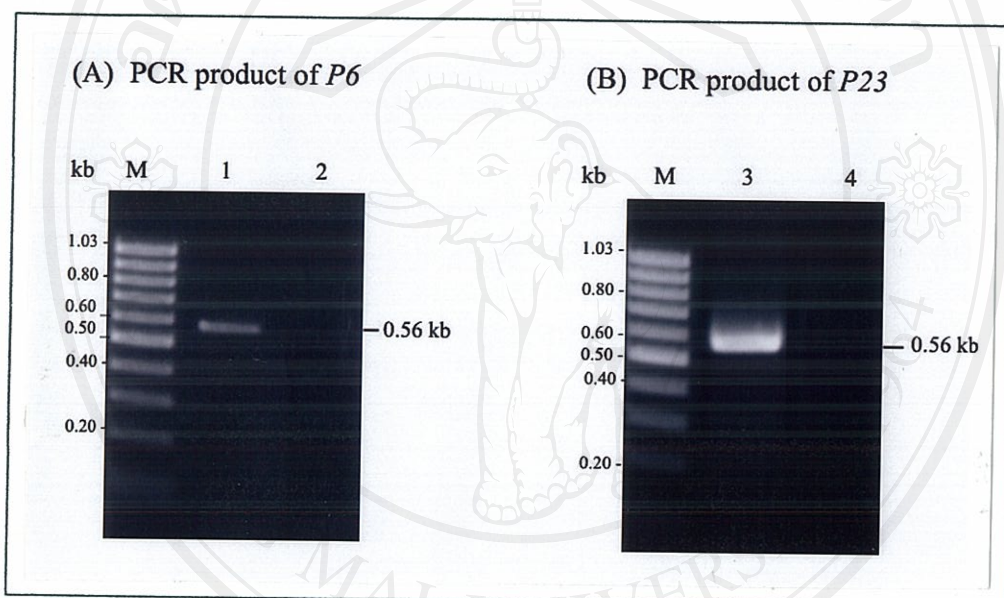


**Figure 15.** (A) Ethidium bromide staining of *P6* (A), and *P23* (B) PCR products on a 1% agarose gel.

Lane M, 100-bp ladder

Lane 1, PCR product of *P6* with expected size of 0.56 kb

Lanes 2 and 4, negative control that contained no template

Lane 3, PCR product of *P23* with expected size of 0.56 kb

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C.2 Purification and restriction endonuclease digestion of DNA

The pGEX-4T-1 vectors and the PCR products of *P6* and *P23* were purified from agarose gel with NucleoSpin Extract columns (Macherey-Nagel) as described in the manufacturer's instruction. The purified DNAs were digested with *Xho* I, and then with *Bam* HI. The digested DNA were purified from solution with a NucleoSpin Extract columns. The ethidium bromide staining digested DNA fragments are shown in **Figure 16**.

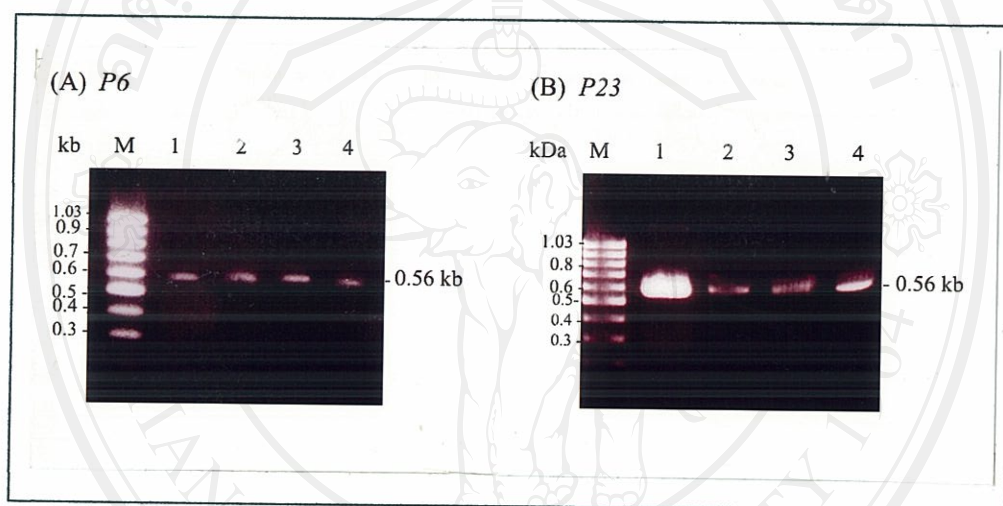


**Figure 16.** (A) Ethidium bromide staining of digested DNA fragments of *P6* PCR product (A) and *P23* PCR product (B) on a 1% agarose gel.

Lane M, 100-bp ladder

Lane 1, uncut PCR product

Lane 2, PCR products digested with *Xho* I

Lane 3, PCR products digested with *Xho* I and *Bam* HI

Lane 4, purified 0.56 kb of (A) *P6* fragment, (B) *P23* fragment digested with *Xho* I and *Bam* HI.

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C.3 Construction of recombinant plasmids

The PCR products and the pGEX-4T-1 were digested with *Xho* I and *Bam* HI to generate two cohesive ends as described in the Materials and Methods. The digested PCR products were purified using PCR purification kit. The digested pGEX-4T-1 plasmids were purified from the gel and then ligated to the digested PCR fragments to generate the recombinant plasmids. The diagram of the recombinant plasmid construction in pGEX-4T-1 is shown in **Figure 17**. The recombinant plasmids were transformed into the *E. colli* Top10 host. After incubation on LB agar plates containing 100 μg/ml of ampicillin at 37 °C overnight, 14 recombinant clones of pGEX-4T-1 containing DNA fragment of *P6* and pGEX-4T-1 containing DNA fragment of *P23* were randomly selected. Each isolated colony was streaked on a new plate and cultured in LB broth containing 100 μg/ml of ampicillin. The cultures were incubated at 25 °C overnight. Recombinant plasmids were isolated by alkaline lysis mini-preparation. The plasmids containing DNA inserts of *P6* or *P23* with an increased band size compared to that of no insert DNA on a 1% agarose gel are shown in **Figures 18** and **19**.

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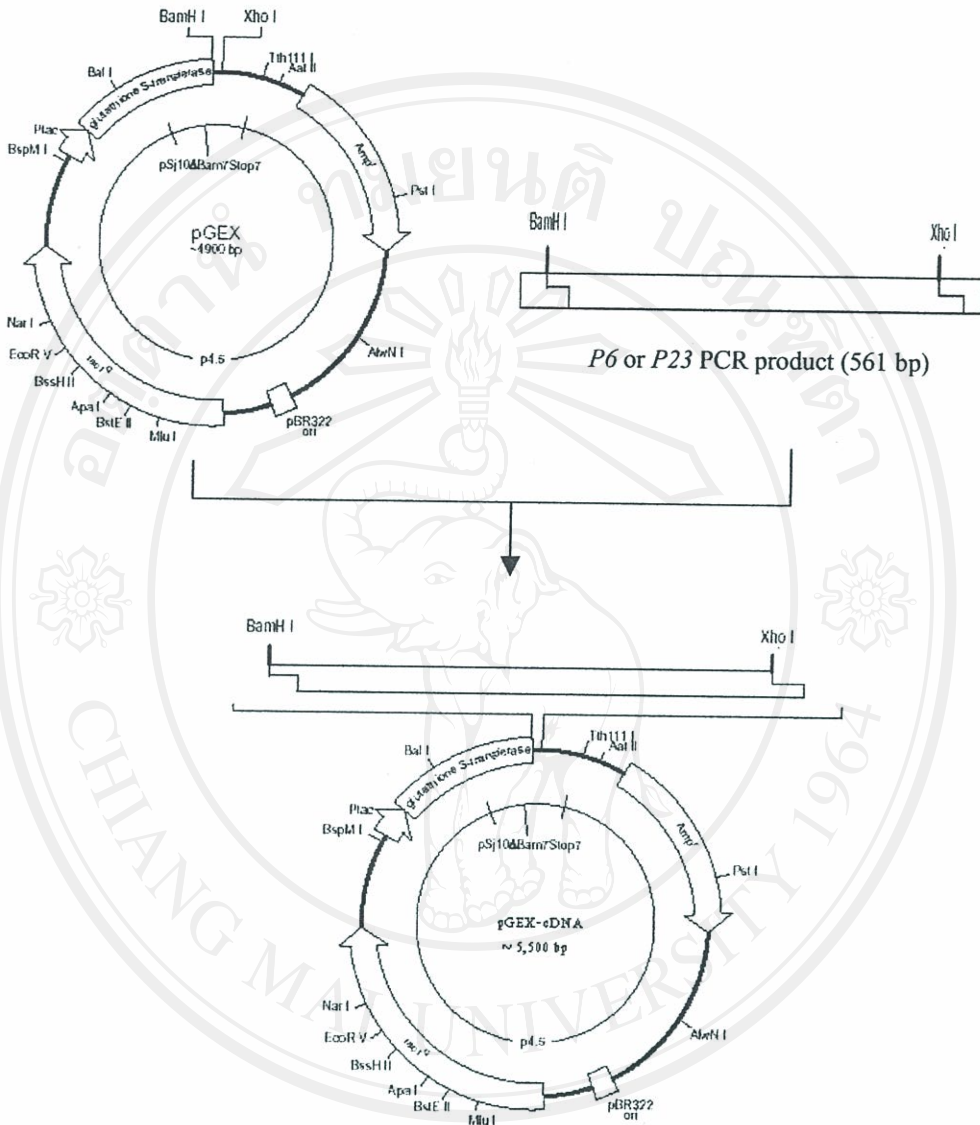


**Figure 17.** Schematic diagram of the recombinant plasmid construction in the pGEX-4T-1 vector. The figure illustrates construction of the recombinant plasmid containing either *P6* or *P23* gene cloned into *Bam* HI and *Xho* I sites of pGEX-4T-1 vector (This picture was taken from website:www.amersham.com).

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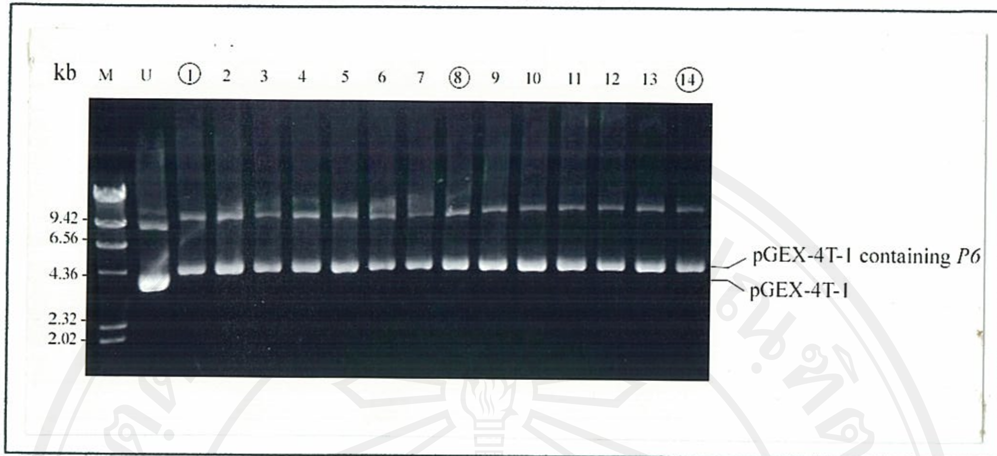


**Figure 18.** *P6* recombinant plasmid size screening. The pGEX- *P6* was purified by alkaline lysis mini-preparation and analyzed by 1% agarose gel electrophoresis at 50 volts for 1 h.

Lane M, *Hin*d III-digested λ DNA

Lane U, uncut pGEX-4T-1 vector

Lanes 1-14, uncut *P6* recombinant plasmids of clones number 1-14.

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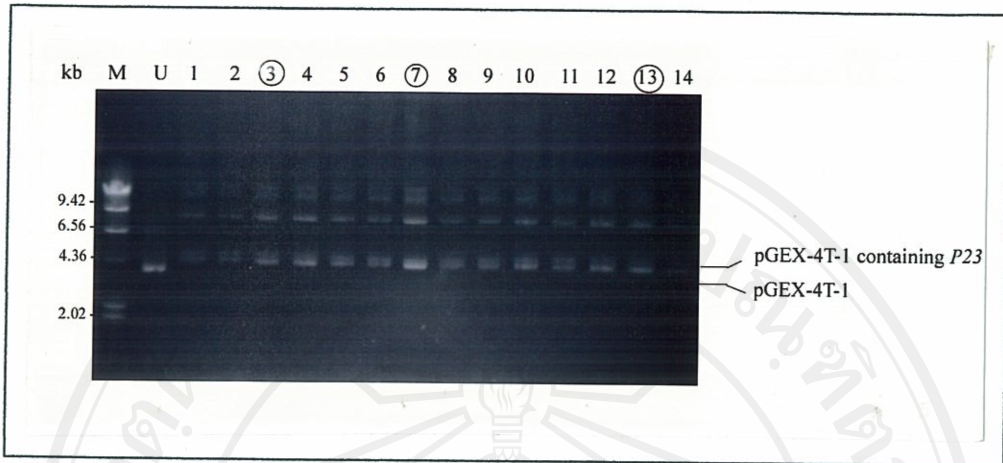


**Figure 19.** *P23* recombinant plasmid size screening. The pGEX- *P23* was purified by alkaline lysis mini-preparation and analyzed by 1% agarose gel electrophoresis at 50 volts for 1 h.

Lane M, *Hin*d III-digested λ DNA

Lane U, uncut pGEX-4T-1 vector

Lanes 1-14, uncut *P23* recombinant plasmids of clones number 1-14.

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C.4 Checking of the insert size of recombinant plasmids

Three clones of pGEX-*P6* (clones no.1, 8 and 14) and 3 clones of pGEX-*P23* (clones no.3, 7, and 13) were randomly selected for restriction analysis using *Xho* I and *Bam* HI are shown in **Figures 20** and **21**, respectively. All selected recombinant plasmids showed the expected restriction pattern, 0.56 kb and 4.96 kb. pGEX-*P6* clone no.1 and pGEX-*P23* clone no.3 were selected for sequencing and proteins expression.

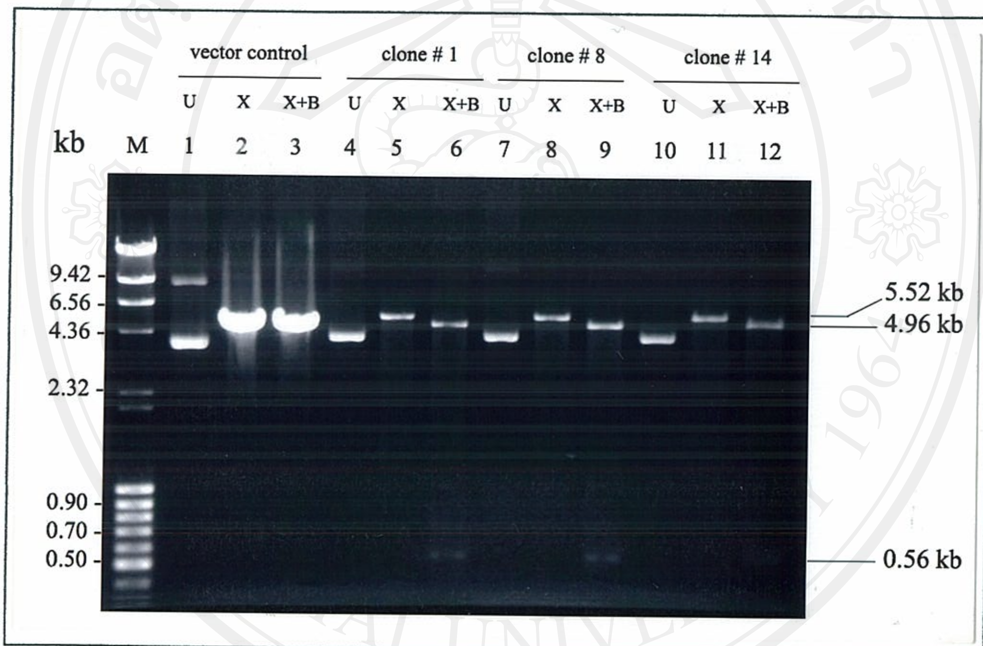


**Figure 20.** Restriction endonuclease analysis of the *P6* recombinant plasmids with *Xho* I and *Bam* HI. The uncut and digested plasmids were analyzed by 1% agarose gel electrophoresis at 50 volts for 40 min.

Lane M, *Hin*d III-digested λ DNA marker + 100-bp ladder

Lanes 1-3, pGEX-4T-1 vector

Lanes 4-6, pGEX-*P6* clone no.1

Lanes 7-9, pGEX-*P6* clone no.8

Lanes 10-12, pGEX-*P6* clone no.14

Lane U, uncut plasmid

Lane X, plasmid digested with *Xho* I

Lane X+B, plasmid digested with *Xho* I and *Bam* HI

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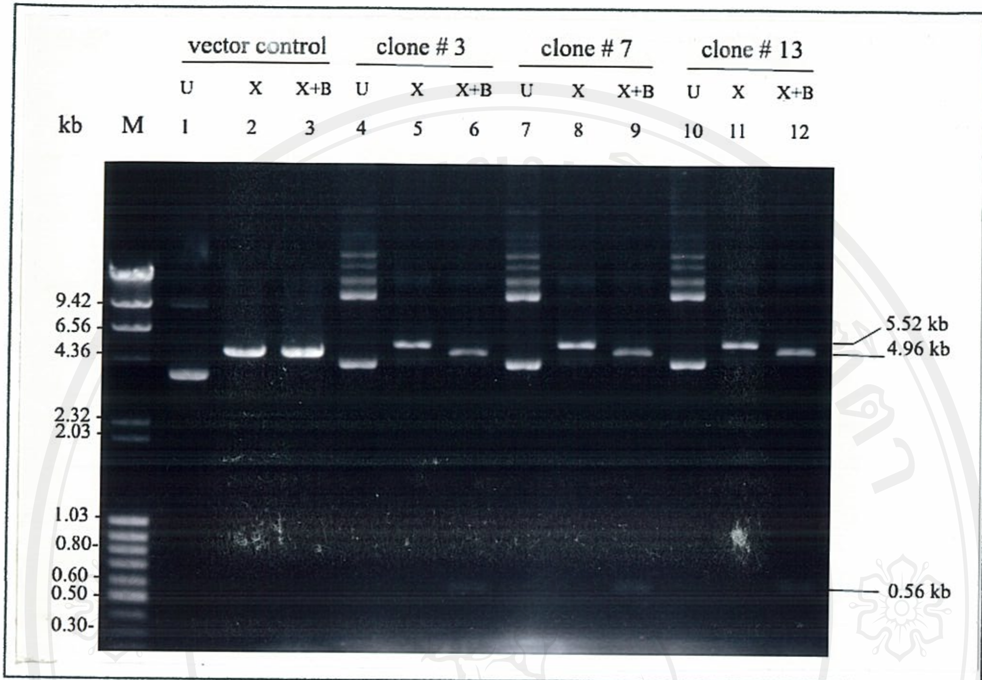


**Figure 21.** Restriction endonuclease analysis of the *P23* recombinant plasmids with *Xho* I and *Bam* HI. The uncut and digested plasmids were analyzed by 1% agarose gel electrophoresis at 50 volts for 40 min.

Lane M, *Hin*d III-digested λ DNA marker + 100-bp ladder

Lanes 1-3, pGEX-4T-1 vector

Lanes 4-6, pGEX-*P23* clone no.3

Lanes 7-9, pGEX-*P23* clone no.7

Lanes 10-12, pGEX-*P23* clone no.13

Lane U, uncut plasmid

Lane X, plasmid digested with *Xho* I

Lane X+B, plasmid digested with *Xho* I and *Bam* HI

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C.5 DNA sequencing

The recombinant plasmids, pGEX-*P6* clone no.1 or pGEX-*P23* clone no.3 was each re-transformed into *E.coli* BL21 and incubated in LB broth containing 100 μg/ml of ampicillin were purified from *E. coli* BL21, then were amplified by using 5' sequencing primer and 3' sequencing primer. The 716 bp of PCR products of both P6 and P23 were purified from the PCR reactions and used for DNA sequencing. Sequencing was performed using 5' sequencing primer. The nucleotide sequences of recombinant plasmids were sequenced by an automated DNA sequencer as described in the Materials and Methods. The nucleotide sequence of the recombinant plasmid revealed the correct orientation of 561 bp nucleotide sequences of *P6* or *P23* joined in frame with GST gene.

## D. Expression of GST-fusion proteins in *E. coli*

The recombinant proteins, GST-P6p and GST-P23p fusion proteins, were expressed by inducing with 100 μM of IPTG for 2 h at 25 $^{O}$C. The GST protein was expressed from the pGEX-4T-1 for using as protein control. The crude extracts of uninduced and induced samples were collected and analyzed on SDS-PAGE to determine expression levels of GST, GST-P6p and GST-P23p. The predicted MW of the GST-fusion proteins determining by MW prediction tool showed the predicted MW of GST-P6p was 47.2 kDa and predicted MW of GST-P23p was 47.7 kDa as shown in **Table 2**. The expression level of GST-P6p or GST-P23p after 2 h of IPTG induction was approximately 5-10 folds greater than uninduced sample. The GST-P6p fusion protein migrated at MW of 58 kDa (**Figure 22**), whereas the GST-P23p fusion protein migrated at MW of 53 kDa (**Figure 23**) on 10% SDS-polyacrylamide gel. After 2 h of 100 μM of IPTG induction, the 27-kDa of GST was expressed approximately 10 folds greater than uninduced sample as shown in **Figure 24**.

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**Table 2.** The prediction for molecular weight by MW prediction tool compared to molecular weight of the GST-P6p and GST-P23p proteins observed on 10% SDS-polyacrylamide gel.

| | **Molecular weight** (kDa) | | |
|---|---|---|---|
| | **GST** | **GST-P6p** | **GST-P23p** |
| Predicted MW | 27 | 47.2 | 47.7 |
| Observed MW | 27 | 58 | 53 |

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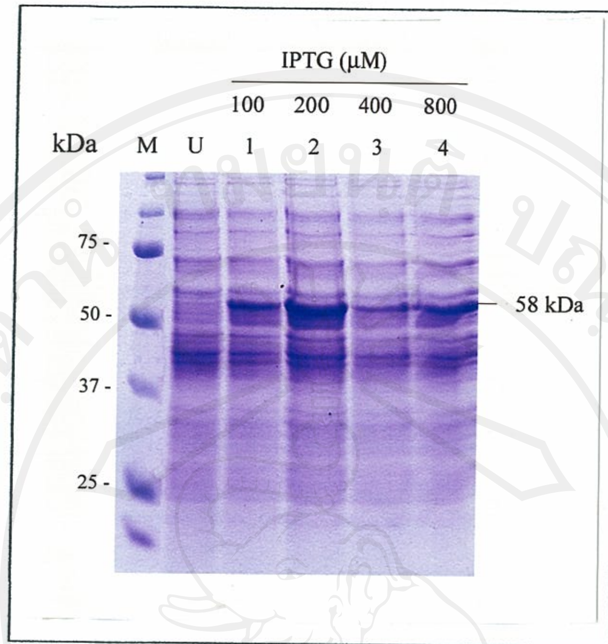


**Figure 22.** The SDS-PAGE analysis of uninduced and induced bacterial lysate containing GST-P6p fuison protein (58 kDa) with IPTG

Lane M, Prestained Precision Protein Standards marker

Lane U, uninduced culture.

Lane 1, induced culture with 100 µM of IPTG for 2 h

Lane 2, induced culture with 200 µM of IPTG for 2 h

Lane 3, induced culture with 400 µM of IPTG for 2 h

Lane 4, induced culture with 800 µM of IPTG for 2 h

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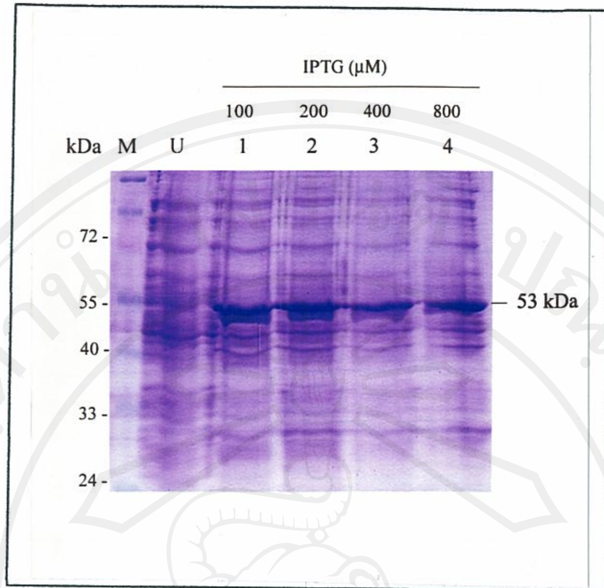


**Figure 23.** The SDS-PAGE analysis of uninduced and induced bacterial lysate containing GST-P23p fuison protein (53 kDa) with IPTG

Lane M, Prestained Precision Protein Standards marker

Lane U, uninduced culture.

Lane 1, induced culture with 100 μM of IPTG for 2 h

Lane 2, induced culture with 200 μM of IPTG for 2 h

Lane 3, induced culture with 400 μM of IPTG for 2 h

Lane 4, induced culture with 800 μM of IPTG for 2 h

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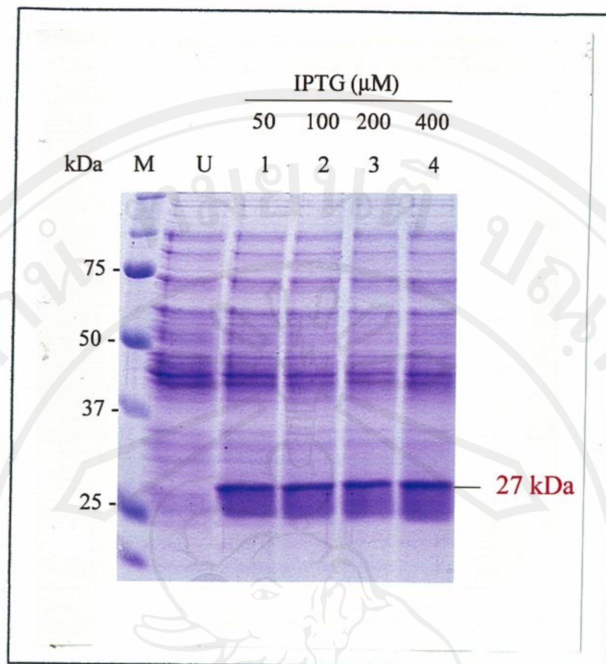


**Figure 24.** The SDS-PAGE analysis of uninduced and induced bacterial lysate containing GST protein (27 kDa) with IPTG

Lane M, Prestained Precision Protein Standards marker

Lane U, uninduced culture.

Lane 1, induced culture with 50 μM of IPTG for 2 h

Lane 2, induced culture with 100 μM of IPTG for 2 h

Lane 3, induced culture with 200 μM of IPTG for 2 h

Lane 4, induced culture with 400 μM of IPTG for 2 h

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**E. Purification of the recombinant proteins by affinity chromatography**

The proteins, GST-P6p, GST-P23p, and GST were purified from *E. coli* lysates by using GSTrap FF 1 ml column (Amersham Biosciences, Sweden). The recombinant proteins binded to the glutathione in the column. After the final step of purification, approximately 2 mg of proteins were recovered from the glutathione sepharose column. The purified GST-P6p fusion protein migrated at the MW of 58 kDa **(Figure 25)**. The purified GST-P23p migrated at the MW of 53 kDa **(Figure 26)**. The GST protein of 27 kDa was also seen as illustrated in **Figure 27**.

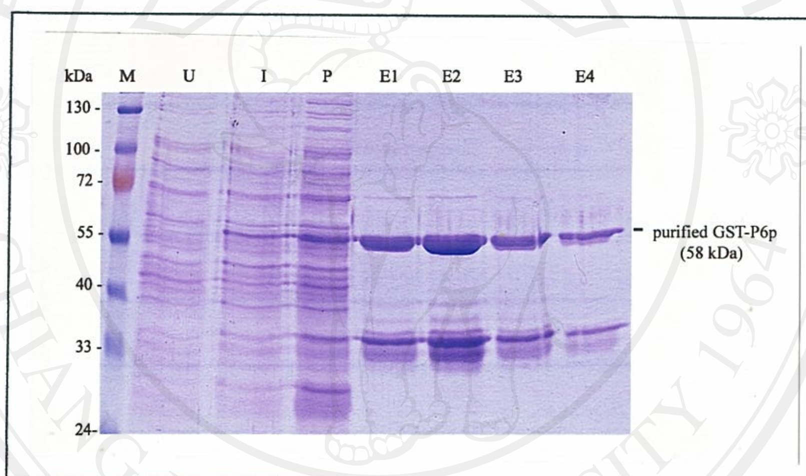


**Figure 25.** The SDS-PAGE analysis of unpurified and purified GST-P6p.
Lane M, 10-180 kDa Prestained Protein Ladder marker
Lane U, un-induced GST-P6p protein
Lane I, 0.1 OD of culture after 100 μM IPTG induction for 2 h
Lane P, unpurified GST-P6p
Lanes E1-E4, purified GST-P6p eluates no. 1, 2, 3, and 4, respectively

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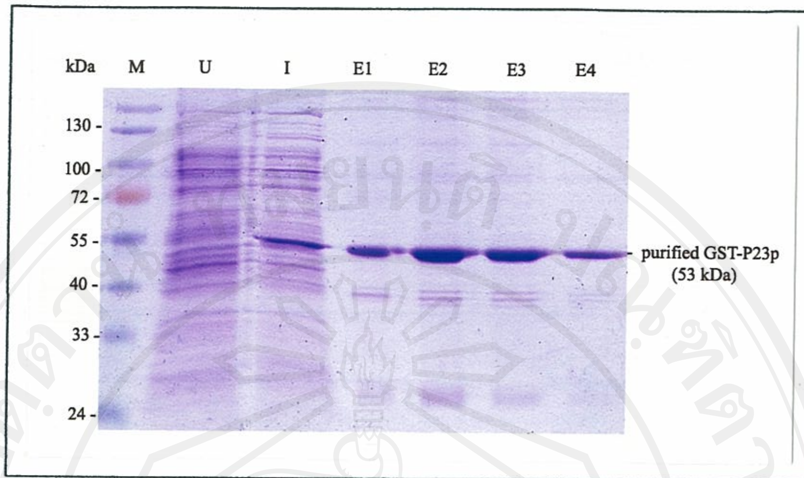


**Figure 26.** The SDS-PAGE analysis of unpurified and purified GST-P23p.
Lane M, 10-180 kDa Prestained Protein Ladder marker
Lane U, un-induced GST-P23p protein
Lane I, 0.1 OD of culture after 100 μM IPTG induction for 2 h
Lane P, unpurified GST-P23p
Lanes E1-E4, purified GST-P23p eluates no.1, 2, 3, and 4, respectively

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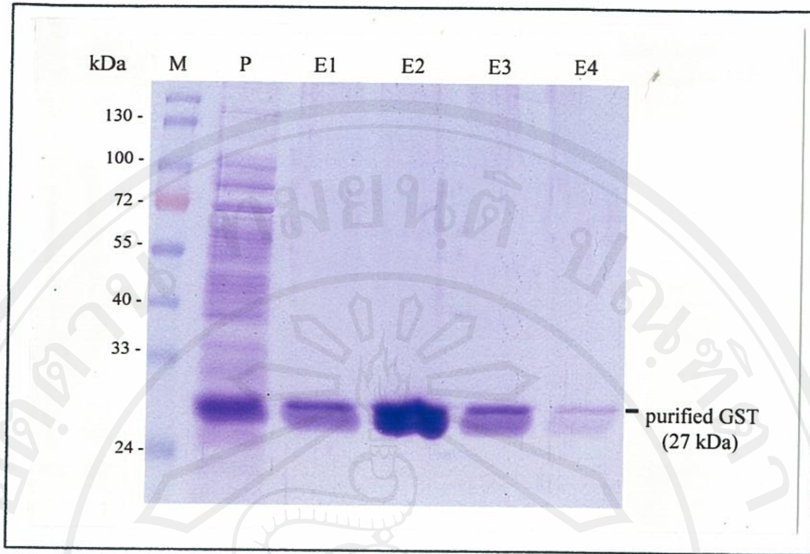


**Figure 27.** The SDS-PAGE analysis of unpurified and purified GST protein.
Lane M, 10-180 kDa Prestained Protein Ladder marker
Lane U, un-induced GST protein
Lane I, induced culture with 100 μM of IPTG for 2 h
Lane P, unpurified GST
Lanes E1-E4, purified GST eluates no.1, 2, 3, and 4, respectively

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Proteins concentration of each eluated fraction were measured by Bradford's method as shown in **Table 3**.

**Table 3.** The concentration of proteins eluted by GSTrap FF 1 ml.

| GST fusion protein | GST-P6p | | GST-P23p | |
|---|---|---|---|---|
| Eluate fraction no. | $OD_{595}$ | Protein conc. (μg/ml) | $OD_{595}$ | Protein conc. (μg/ml) |
| 1 | 0.479 | 420 | 0.359 | 250 |
| 2 | 0.856 | 900 | 0.572 | 550 |
| 3 | 0.430 | 350 | 0.432 | 410 |
| 4 | 0.337 | 220 | 0.341 | 225 |
| 5 | 0.299 | not detectable | 0.201 | not detectable |

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## F. Evaluation of the recombinant proteins by Western blot analysis

F.1 Detection of GST fusion proteins

Three µg of GST protein, GST-P6p, GST-P23p and *E.coli* BL21 lysates were separated on 10% SDS polyacrylamide gel and transferred to nitrocellulose membranes. The membrane was used for detection of GST fusion proteins by incubation with 1:5,000 dilution of HRP-anti GST antibody, and then detection with chromogenic substrate. The detection of GST fusion proteins is shown in **Figure 28**. The reactivity with purified GST protein shows single positive band of 27 kDa. The anti-GST antibody reacted to three proteins of purified GST-P6p at MW of 58-, 34- and 32-kDa. The reactivity with purified GST-P23p shows a positive band of 53 kDa.

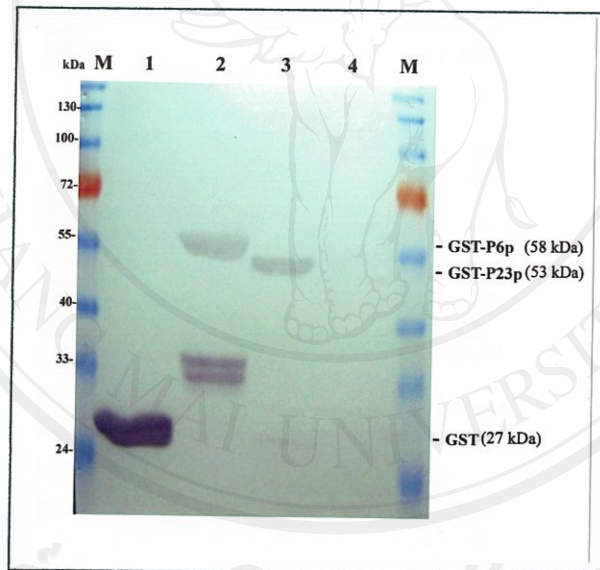


**Figure 28.** Western blot analysis of GST fusion protein using HRP-anti GST at dilution 1:5,000

Lane M, 10-180 kDa Prestained Protein Ladder marker

Lane 1, 3 µg of purified GST protein

Lane 2, 3 µg of purified GST-P6p

Lane 3, 3 µg of purified GST-P23p

Lane 4, 3 µg of *E. coli* BL21 lysates

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F.2 Immunogenic activity of the recombinant proteins detected by sera from crude antigen immunized rabbits

The protein component extracted from the yeast cells of *P. marneffei* F4, *C. neoformans* F109 and *H. capsulatum* H760 were separated on 10% SDS polyacrylamide gel. The antigenic proteins of *P. marneffei* F4, *C. neoformans* F109 and *H. capsulatum* H760 were analyzed by immunoblot assay employing rabbit antisera against concentrated crude antigens. At least ten proteins of MW in range from 200 to 25-kDa were identified. The results are shown in **Figure 29**. The rabbit pre-immuned serum could not react to the fungal proteins.

The purified GST protein, GST-P6p and GST-P23p were analyzed by immunoblot assay employing rabbit antisera against concentrated crude antigens as described above. The antibodies in a rabbit serum immunized with *P. marneffei* strain F4 could react with the purified GST-P6p **(Figure 30A, lane 2)**. Sera from one pre-immuned rabbit, two rabbits immunized with *P. marneffei* F4, one rabbit immunized with *P. marneffei* Y1, one rabbit immunized with *C. neoformans* F109 and one rabbit immunized with *H. capsulatum* H760 could not react with the purified GST protein, GST-P6p and GST-P23p **(Figure 31)**.

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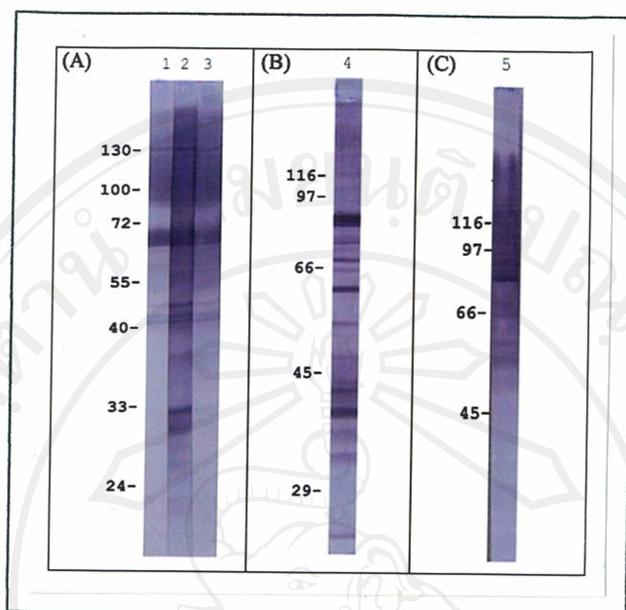


**Figure 29.** Immunogen profiles of concentrated crude antigens of *P. marneffei* F4 (A), *C. neoformans* F109 (B), and *H. capsulatum* H760 (C) detected with rabbit antisera at serum dilution 1:100. Three rabbits were immunized with concentrated crude antigens of *P. marneffei*, one rabbit immunized with that of *C. neoformans* and another rabbit immunized with that of *H. capsulatum*.

Lane 1, 2 µg of crude *P. marneffei* antigens reacted with rabbit antiserum immunized with secreted proteins of *P. marneffei* (Y1)

Lane 2, 2 µg of crude *P. marneffei* antigens reacted with rabbit antiserum immunized with cytoplasmic proteins of *P. marneffei* (no.1)

Lane 3, 2 µg of crude *P. marneffei* antigens reacted with rabbit antiserum immunized with cytoplasmic proteins of *P. marneffei* (no. 2)

Lane 4, 1 µg of crude *C. neoformans* antigens reacted with rabbit antiserum immunized with cytoplasmic proteins of *C. neoformans* (no.3)

Lane 5, 2 µg of crude *H. capsulatum* antigens reacted with rabbit antiserum immunized with cytoplasmic proteins of *H. capsulatum* (no.4)

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The Western blot analysis of GST-P6p, GST-P23p and GST protein with rabbit antisera that raised against concentrated crude antigens of *P. marneffei*, *C. neoformans* or *H. capsulatum* at serum dilution 1:100 are shown in **Figure 30**. The reactivity of recombinant proteins of GST-P6p with rabbit anti-*P. marneffei* antibody (rabbit no.1) gave positive band of 58 kDa **(Figure 30A, lane 2)**. The recombinant proteins of GST-P6p showed negative reactivity with rabbit antiserum against secreted antigens of *P. marneffei* (rabbit Y1) or with rabbit antiserum against cytoplasmic antigens of *P. marneffei* (rabbit no.2). Negative reactivity with rabbit antiserum against cytoplasmic antigens of *C. neformans* or *H. capsulatum* was observed (**Figure 30A**). The detection of GST-P23p and GST protein showed negative reactivity with rabbits antiserum against both cytoplasmic and secreted antigens of *P. marneffei* or with rabbits antiserum against cytoplasmic antigens of *C. neformans* or *H. capsulatum,* **Figure 30B** and **C**, respectively.

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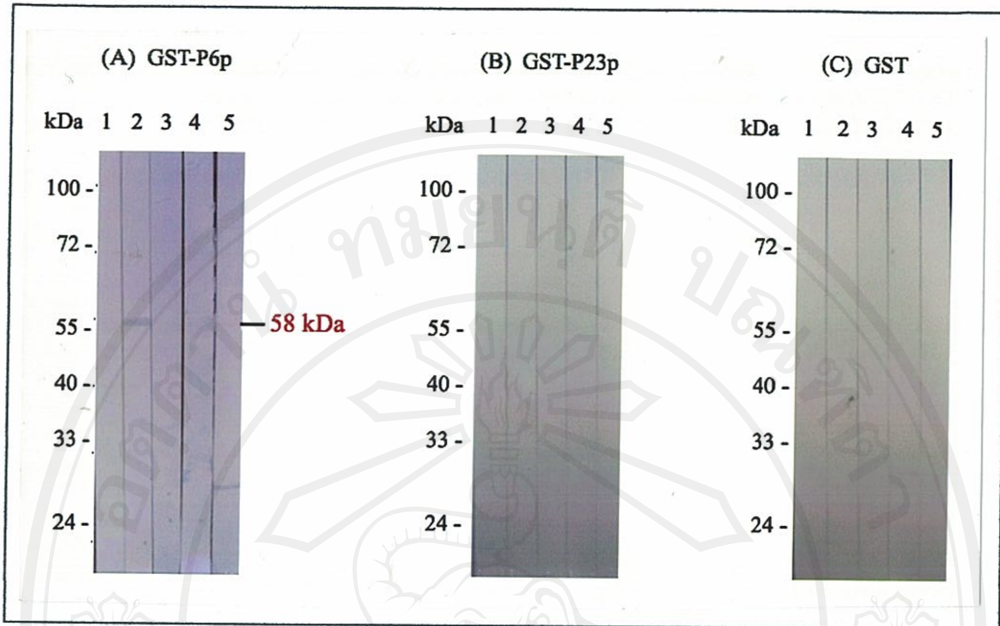


**Figure 30.** Western blot analysis of the, GST-P6p (A), GST-P23p (B), and GST protein (C) with rabbit antisera that raised against concentrated crude antigens of *P. marneffei*, *C. neoformans* or *H. capsulatum* at serum dilution 1:100.

Lane 1, rabbit antiserum against antiserum from rabbit injected with concentrated secreted antigens of *P. marneffei* (Rabbit Y1)

Lane 2, rabbit antiserum against antiserum from rabbit injected with concentrated cytoplasmic antigens of *P. marneffei* (Rabbit no.1)

Lane 3, rabbit antiserum against antiserum from rabbit injected with concentrated cytoplasmic antigens of *P. marneffei* (Rabbit no.2)

Lane 4, rabbit antiserum against antiserum from rabbit injected with concentrated cytoplasmic antigens of *C. neoformans* (Rabbit no.3)

Lane 5, rabbit antiserum against antiserum from rabbit injected with concentrated cytoplasmic antigens of *H. capsulatum* (Rabbit no.4)

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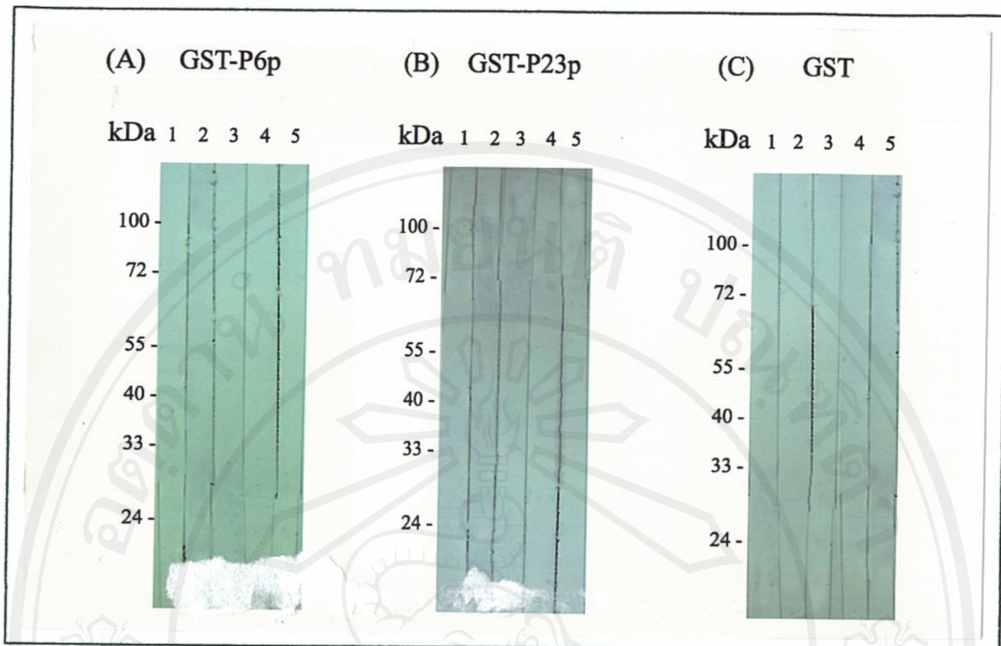


**Figure 31.** Western blot analysis of the, GST-P6p (A), GST-P23p (B), and GST proteins (C) with pre-immunized rabbit sera at serum dilution 1:100.

Lane 1, pre-immune rabbit serum (Rabbit Y1)

Lane 2, pre-immune rabbit serum (Rabbit no.1)

Lane 3, pre-immune rabbit serum (Rabbit no.2)

Lane 4, pre-immune rabbit serum (Rabbit no.3)

Lane 5, pre-immune rabbit serum (Rabbit no.4)

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F.3 Immunogenic activity of the recombinant proteins detected by sera from *P. marneffei*-infected patients.

Ten individual serum samples from AIDS patients with penicilliosis marneffei were used in this study. These samples had immunoreactivities with crude *P. marneffei* antigens by Western blot analysis (**Figure 32A**). Nine individual serum samples from laboratory personnels and pooled sera from normal healthy in non-endemic area (Sigma-Aldrich, USA) were used as normal serum controls. The immunoblots of normal sera with crude *P. marneffei* antigens are shown in **Figure 32B**.

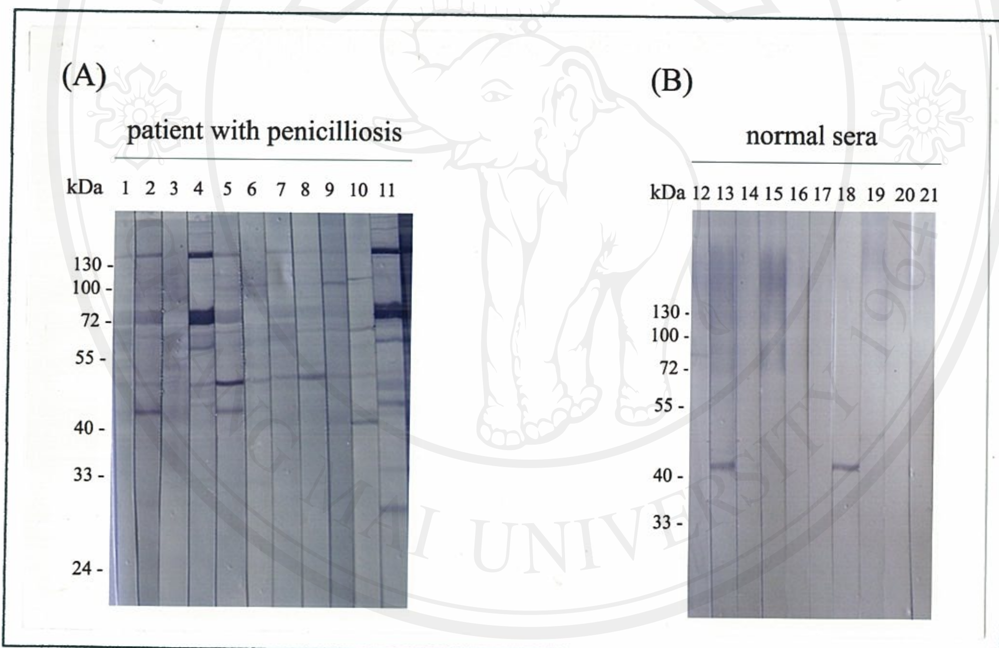


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**Figure 32.** Western blot analysis of cytoplasmic yeast antigens prepared from 5-day-old *P. marneffei* strain F4, recognized by human sera at serum dilution 1:50. (A) Ten individual serum from AIDS patients with penicilliosis marneffei reacted to cytoplasmic *P. marneffei* antigen (lanes 1-10) and pooled sera from 2 AIDS patients with penicilliosis marneffei which were used for screening *P6* and *P23* clones served as a positive control antiserum (lane 11). (B) Nine serum samples from laboratory personnels (lanes 12-20) and pooled normal healthy sera from non-endemic area (lane 21).

**Table 4.** Immunoreactivities of 10 individual serum samples (at serum dilution 1:50) from 10 AIDS patients with penicilliosis marneffei against cytoplasmic yeast phase antigens of *P. marneffei.*

| Patient No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Molecular weight (kDa) | | | | | | | | | | | |
| 27 | | | | | | | | | | | // |
| 31 | | | | | | | | | | | / |
| 40 | | | | | | | | | / | // | |
| 41 | | | / | | | | | | | / | / |
| 44 | / | // | / | | // | | | | | | // |
| 45 | | | | / | | | | | | | |
| 47 | | | | / | | | | | | | // |
| 50 | | | | / | / | / | / | / | | | // |
| 52 | / | / | | / | /// | / | / | // | / | / | / |
| 55 | / | | / | | | / | / | / | / | / | |
| 58 | / | | / | | | / | | | | / | |
| 62 | / | | | // | | | / | | | / | // |
| 67 | | / | / | / | / | / | / | / | / | // | / |
| 69 | / | | | / | | / | / | / | / | | / |
| 73-79 | / | // | | /// | // | | / | / | / | | /// |
| 88 | | | / | | | / | / | | / | / | |
| 94 | | / | / | | / | | / | / | // | / | |
| 97 | | / | | | | / | | | | / | / |
| 100 | | / | / | | / | | / | | | // | |
| >130 | / | // | | /// | // | | / | / | / | | /// |

/ = weakly positive, // = immediately positive, /// = strongly positive

* Pooled sera from 2 AIDS patients with penicilliosis marneffei which was used for screening *P6* and *P23* clones

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Serological responses of individual serum samples to GST-P6p are shown in **Figure 33**. Three μg of GST-P6p were electrophoresed on 10% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane. The protein-coated nitrocellulose membrane were cut into 20 of 4-mm strips. Each strip was incubated with 20 individual serum samples at serum dilution 1:50. One serum from an AIDS patient infected with *P. marneffei* (Patient no.5) showed strongly reactive bands of 58 and 34-32 kDa. Patient no.7 showed strongly reactive bands of 58 kDa, while 4 normal serum samples showed immediately reactive bands of 58, and 34-32 kDa. At the serum dilution of 1:100, only the serum samples from patients no. 5 and 7 had positive reactivities **(Figure 34A)**. Negative reaction was seen at the serum dilution of 1:200 (**Figure 34B**).

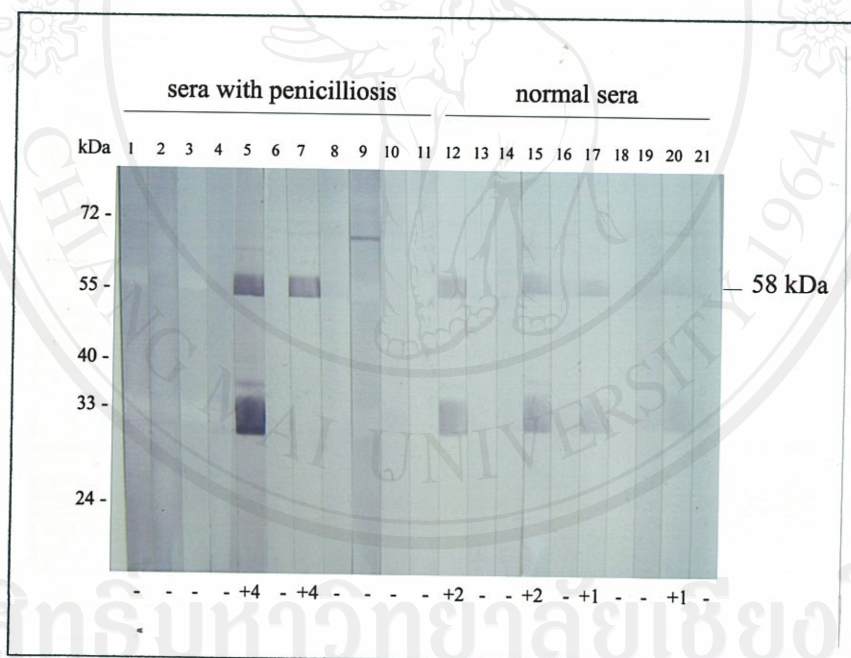


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**Figure 33.** Western blot analysis of the recombinant protein, GST-P6p, recognized by antibodies from human sera at serum dilution 1:50. Lanes 1-10, 10 individual serum samples of AIDS patients with penicilliosis marneffei, pooled sera from 2 AIDS patients with penicilliosis marneffei which was used for screening *P6* clones (lane 11), 9 serum samples from laboratory personnels (lanes 12-20) and pooled normal healthy sera from non-endemic area (lane 21).

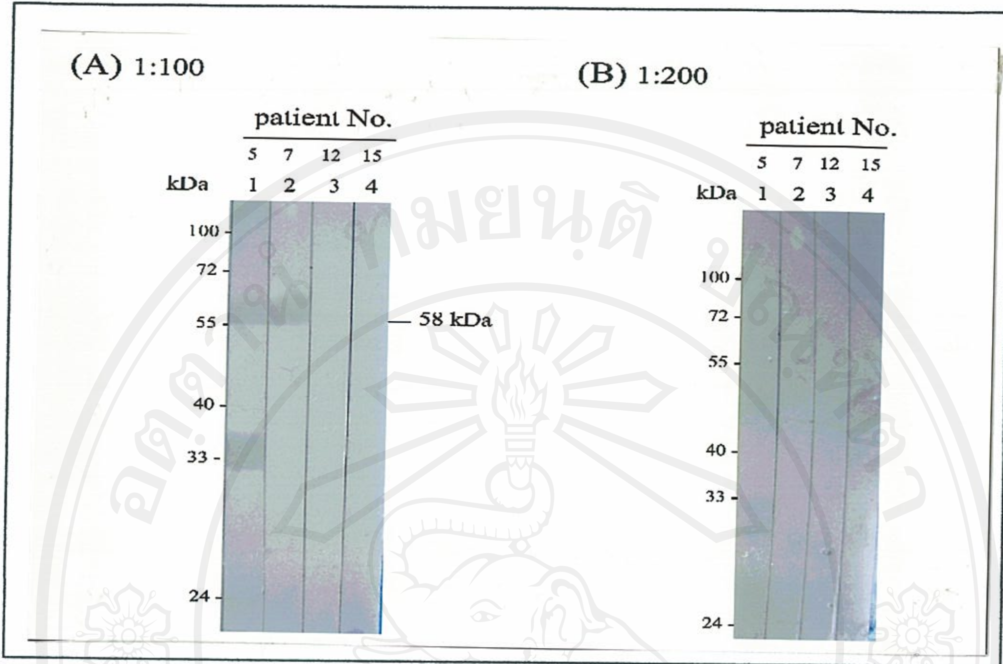


**Figure 34.** Western blot analysis of the recombinant protein, GST-P6p, recognized by antibodies from serum samples of AIDS patients with penicilliosis marneffei no. 5 and 7 (lanes 1-2), two individual normal serum samples from laboratory personnels no. 12 and 15 (lanes 3 and 4) at serum dilution 1:100 (A) and serum dilution 1:200 (B).

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Serological responses of all 20 serum samples to GST-P23p are shown in **Figure 35**. Three µg of GST-P23p were electrophoresed on 10% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane. The protein-coated nitrocellulose membrane were cut into 20 of 4-mm strips. Each strip was incubated with 20 individual serum samples at serum dilution 1:50. Two AIDS patients infected with *P. marneffei* (Patients no.8 and 10) showed strongly reactive bands of 53 kDa. Ten normal serum samples could not react to GST-P23p at serum dilution 1:50. All 20 serum samples could not react to GST protein at serum dilution 1:50 **(Figure 36)** .

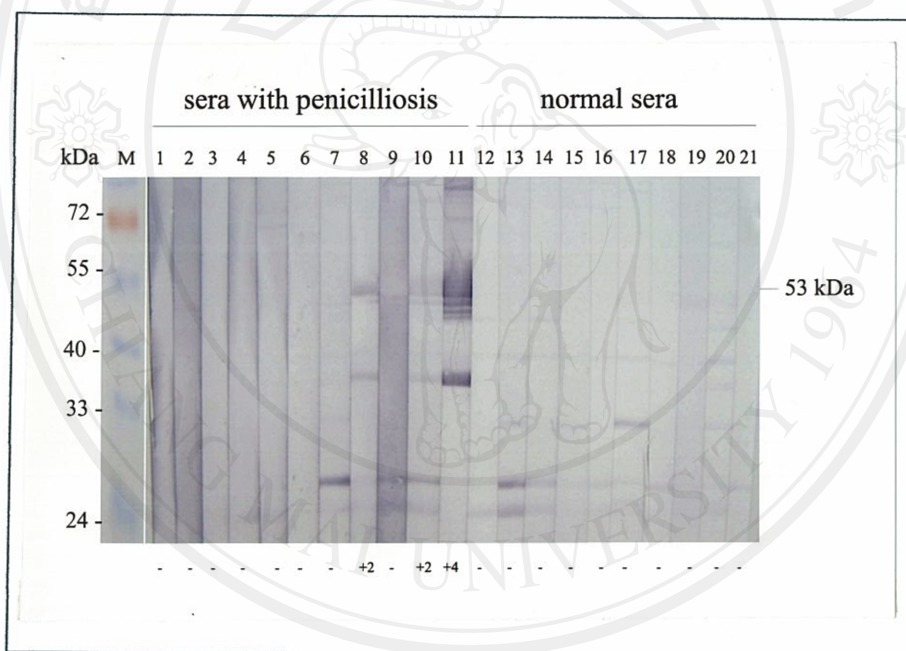


**Figure 35.** Western blot analysis of the recombinant protein, GST-P23p, recognized by antibodies from human sera at serum dilution 1:50. Lanes 1-10, 10 individual serum samples of AIDS patients with penicilliosis marneffei, pooled sera from 2 AIDS patients with penicilliosis marneffei which was used for screening *P6* clones (lane 11), 9 serum samples from laboratory personnels (lanes 12-20) and pooled normal healthy sera from non-endemic area (lane 21).

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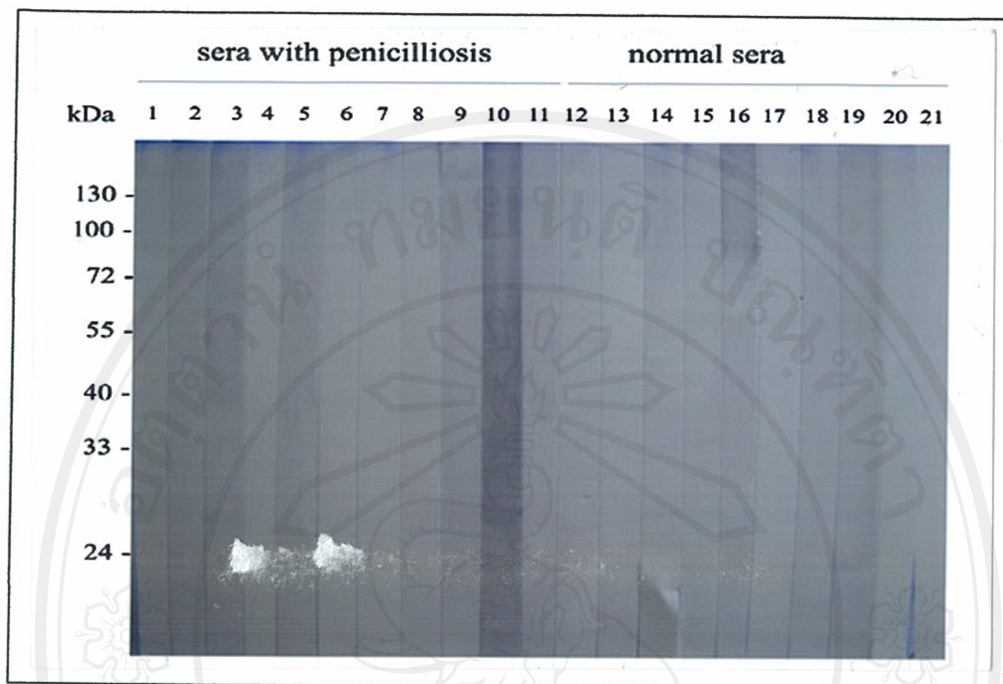


**Figure 36.** Western blot analysis of the recombinant protein, GST protein, recognized by antibodies from human sera at serum dilution 1:50. Lanes 1-10, 10 individual serum samples of AIDS patients with penicilliosis marneffei, pooled sera from 2 AIDS patients with penicilliosis marneffei which was used for screening *P6* clones (lane 11), 9 serum samples from laboratory personnels (lanes 12-20) and pooled normal healthy sera from non-endemic area (lane 21).

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