III. MATERIALS AND METHODS

A. Experimental plan

The following approaches (**Figure 7**) were utilized to reach the goals of purification of expressed recombinant proteins and determination of the immunoreactivities of the GST-fusion proteins by serological test.

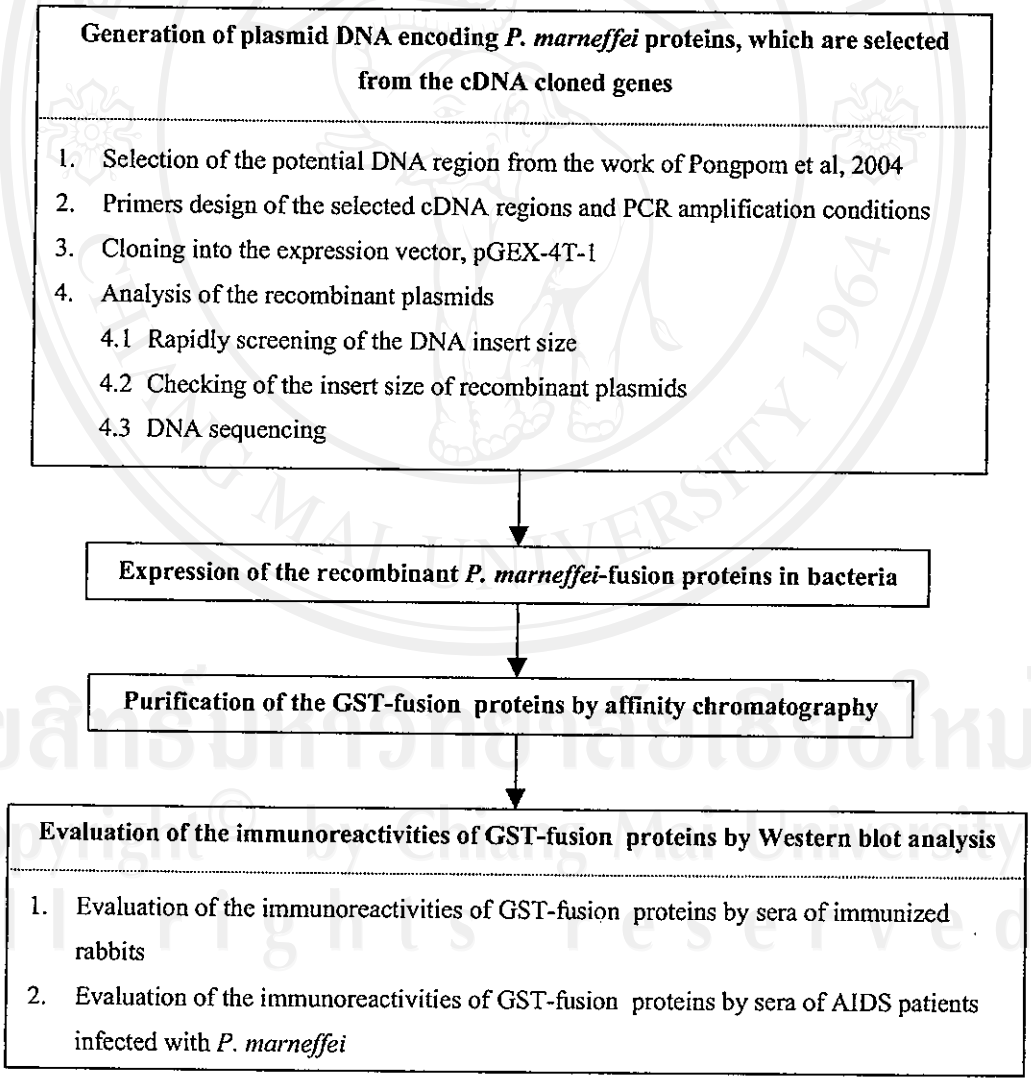

Figure 7. Diagram of the experimental plan

B. Selection of the potential DNA region for cloning

The nucleotide sequences of *P. marneffei* from the thesis research by Pongpom (2004) were analysed in order to select interesting DNA regions for subcloning into an expression plasmid. In this study, two potential genes were selected from 5 clones of cDNA library. These two coding regions were analyzed by using program nucleotide-nucleotide BLAST (www.ncbi.nlm.nih.gov) and predicted to encode putative HSP30, designated *P23*, and unknown protein, designated *P6* (Pongpom,2004). A hydropathy plot indicates potential transmembrane or surface regions in proteins (Kyte et al, 1982). The hydrophobicity of the amino acid determines where the amino acid will be located in the final structure of the protein. A Kyte-Doolittle Hydropathy Plots of *P6* and *P23* regions were predicted by the FASTA program (http://fasta.bioch.virginia.edu/o_fasta/grease.htm). Each amino acid is given a hydrophobicity score between –4.5 and 4.5. A score of 4.5 is the most hydrophobic and a score of –4.5 is the most hydrophilic. Hydrophilic groups which are typical polar, interacting with water by hydrogen bounding. All hydrophilic amino acids are pushed to the outside of the membrane. Hydrophobic groups, on the other hand, are nonpolar, unable to interact with water and locate inside of the bilipid membrane. In addition, Antigenicity Plots of *P6* and *P23* regions were predicted by program JaMBW Chapter 3.1.7 (http://hometown.aol.com/_ht_a/lucatoldo/myhomepage/JaMBW/3/1/7). A sequence of amino acids were submitted into the program, the antigenicity was computed and plotted along the polypeptide chain, as predicted by the algorithm of Hopp and Woods (1981). Primary structure analysis to predict the theoretical molecular weight (MW) for the protein sequence were computed by entering a protein sequence in a single letter code into the ExPASy program (http://us.expasy.org/tool/#primary).

C. Expression vector

The GST gene fusion system (Amersham Biosciences, Sweden) was used to generate recombinant plasmids and fusion proteins. The interesting gene fragment was inserted into the multiple cloning site (MCS) of the expression vector, pGEX-4T-1 vector. Expression is under the control of the *tac* promoter. The *tac* promoter was induced by the lactose analog IPTG. The pGEX-4T-1 vector was engineered with an

internal *lacI*^{q} gene. The *lacI*^{q} gene product, a repressor protein that binds to the operator region of the *tac* promoter, prevents the expression of the GST-fusion protein until induction by IPTG. The pGEX-4T-1 vector allowed mild elution conditions for release of the GST-fusion proteins from the affinity medium. Thus, effects on antigenicity and functional activity of the proteins were minimized. Functional tests can be performed using the intact fusion with GST. However, if the GST tag is necessary to remove, GST-fusion proteins containing a PreScission Protease, thrombin could be cleaved due to the pGEX-4T-1 vector has a thrombin cleavage recognition site. The expression vector, pGEX-4T-1 is shown in **Figure 8**.

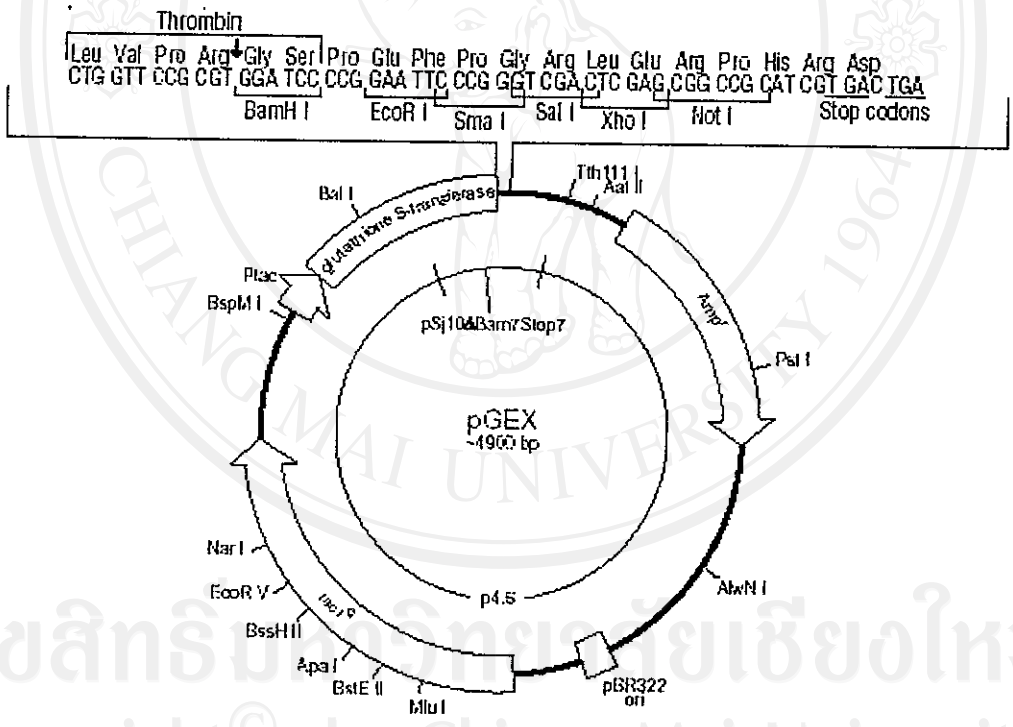

Figure 8. Map of the pGEX-4T-1 vector showing the reading frames and main features (This picture was taken from website:www.amersham.com)

D. Primers design of the selected cDNA regions and PCR amplification conditions

D.1 Primer design of the selected cDNA regions

The primers for amplification of the *P6* or *P23* used in these experiments have been designed according to the cDNA sequences of *P. marneffei* gene (Pongpom, 2004). The set of primers which were designed for cloning into the pGEX-4T-1 expression vector were covered the DNA region of *P6* at the nucleotide sequence 28 to 214 the whole nucleotide sequence of *P23*. The primers were extended with the oligonucleotides for recognition sites of the restriction enzymes, *Bam* HI and *Xho* I. The extended site were designed in order to amplify the DNA fragments which were in-framed with the GST gene in the expression vector, pGEX-4T-1.

D.2 PCR conditions

The 100 µl PCR reactions were performed. The reaction consisted of 50 ng of the plasmid, pZL1 containing the cDNA, *P6* or *P23*, 0.4 µM of each forward and reverse primer, 1X of PCR buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl pH 8.8 at 25 °C), 3 mM $MgSO_4$, 0.2 mM dNTP mixed and 1.25 units of Pfu® DNA polymerase (Fermentas Applied Biosystems, USA). The PCR amplification was performed using a GeneAmp® PCR system 2700. After the initial denaturing step at 95 °C for 5 min, 35 cycles were performed consisting of the denaturing step at 95 °C for 30 sec, the annealing step at 60 °C for 30 sec, and the extension step at 72 °C for 1 min. The final extension step was performed at 72 °C for 7 min. The PCR products were kept at 4 °C and analyzed by agarose gel electrophoresis.

E. Agarose gel electrophoresis

Agarose gel electrophoresis which is a highly effective method for identifying and purifying DNA fragments was used for determining the PCR products. The agarose gel electrophoresis protocol is as follow. The 1% (w/v) of SeaKem LE agarose (BioWhittaker Molecular Applications, USA) was prepared by melting with the 0.5X TAE buffer in a microwave oven. When the melted agarose was cooled to 55 °C in waterbath, it was poured into the i-Mupid minigel migration through (Cosmo Bio, Japan). The comb was then inserted in the gel that contained no bubbles on its

surface. After the gel had hardened, the gel comb was withdrawn and the gel casting platform containing the set gel in was placed to the electrophoresis tank. Then, 0.5X TAE buffer was added to electrophoresis tank to cover the gel to a depth just until the tops of the wells are submerged. Five µl of PCR products were prepared by mixing with 1 µl of 6X load buffer (0.09% bromphenol blue, 0.09% xylene cyanol FF, 60% glycerol and 60 mM EDTA). Six µl of samples and 100 bp Ladder DNA markers were loaded into the wells with micropipet. The voltage was set to 100 volts and the running time for 25 min. The gels were stained in 0.5 µg/ml of ethidium bromide in water by gently agitating for 10 min and destained by shaking in water for 30 min. The DNA were visualized by placing on a UV light source and photographed.

F. Cloning into the pGEX-4T-1 expression vector

F.1 Digestion of DNA with restriction endonucleases

The PCR products were purified with a NucleoSpin Extract columns (Macherey-Nagel, Germany) as described in the manufacturer's instructions. The purified PCR products were measured by using the Eppendorf BioPhotometer (Eppendorf-Netheler-Hinz GmbH, Germany). Three µg of PCR product were digested with 50 units of *Xho* I (New England BioLabs, England). The 30 µl of reactions consisted of 1X NEBuffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT pH7.9 at 25 °C). The reaction was incubated at 37 °C for 20 h and then digested with 50 units of *Bam* HI (New England BioLabs, England) at 37 °C for 4 h. The digested DNA was purified from the solution with a NucleoSpin Extract columns. The pGEX-4T-1 vectors were digested with *Xho* I and then digested with *Bam* HI. The digested DNA was purified from the solution with a NucleoSpin Extract columns.

F.2 Ligation of DNA

The purified digested PCR products and pGEX 4T-1 vector were incubated in the molar ratio of insert: vector is 5:1. The 10 µl of reaction consisted of 1 unit of T4 DNA ligase (Fermentas Applied Biosystems, USA), 1X DNA ligase reaction buffer (50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT and 5% (w/v)

polyethylene glycol-8000) and DEPC-treated water. The 10 µl of ligation control consisted of 0.5 µl of *Hind* III-digested λ DNA marker (Fermentas Applied Biosystems, USA), 1 unit of T4 DNA ligase, 1X DNA ligase reaction buffer and DEPC-treated water. The ligation mixture was incubated at 16 °C for 16 h and kept at 4 °C until used.

F.3 Preparation of the competent cells, *E.coli* Top10 and *E.coli* BL21

E. coli Top10 was used as a host for cloning the transformant plasmid, while *E.coli* BL21 was used as a host for expression of the GST fusion proteins. Preparation of the competent cells, one colony of *E. coli* Top10 or *E.coli* BL21 was inoculated into 5 ml of LB media in 100-ml flask with shaking 200 rpm at 37 °C overnight. The 1 ml of overnight culture was inoculated into 100 ml of fresh LB media in a 1-liter flask and incubated with shaking 200 rpm at 37 °C until the OD at 600 nm was approximately 0.5. The bacteria culture was transferred to 50 ml centrifuge bottle. The culture was centrifuged at 4,500 rpm at 4 °C for 10 min. The cell pellets in each centrifuge bottle were resuspended in 20 ml of ice-cold 0.1 M $CaCl_2$ and pooled together. The suspension was incubated on ice for 30 min and centrifuged at 4,500 rpm at 4 °C for 7 min. The cell pellets were collected and resuspended in 4 ml of ice-cold 0.1 M $CaCl_2$. The steriled glycerol was added to give a 20% final concentration and the 200 µl of competent cells were aliquoted immediately into pre-chilled 1.5-ml microtubes. They were freezed with ethanol in dry ice bath and kept at -70 °C until used for transformation.

F.4 Transformation of competent cells, *E.coli* Top10

Ten µl of ligation mixture were added to 100 µl of competent *E. coli* Top10 cells and incubated on ice for 30 min in transformation tube. The cells were heat-shocked at 42 °C for 45 sec and immediately incubated on ice for 2 min. The transformed cells were added to 900 µl of SOC media and incubated at 37 °C for 1 h. The culture was transferred to a clean 1.5-ml microtube, centrifuged at 14,000 rpm for 1 min. The supernatant (800 µl) was discarded and cell pellets were resuspended with 100 µl of the remaining media. The cell suspension was spread on LB agar plates

containing 100 μg/ml of ampicillin. The LB agar plates were incubated at 37 °C overnight.

F.5 Screening of the recombinant clones

F.5.1 Rapidly screening of the DNA insert size

Fourteen colonies were randomly selected for the screening of the DNA insert size. Plasmid DNA was isolated by the alkaline lysis method (Sambrook et al, 1989). Each colony was picked by using a steriled toothpick, incubated in 2 ml of LB media containing 100 μg/ml of ampicillin at 37 °C overnight with shaking 200 rpm. One ml of the overnight culture was pipetted into a microfuge tube and centrifuged at 14,000 rpm for 30 sec at 4 °C. The supernatant was removed. The bacterial pellet was left as dry as possible, then resuspended in 100 μl of ice-cold Solution I (25 mM Tris.HCl (pH 8.0), and 10 mM EDTA (pH 8.0)) by vortexing. Two hundred μl of freshly prepared of Solution II (0.2 N NaOH, and 1% SDS) was added. The contents was mixed by inverting the tube rapidly 6-8 times. One hundred and fifty μl of ice-cold Solution III (3 M potassium, and 5 M acetate) was added, then mixed by inverting the tube for 10 sec. The tube was stored on ice for 3-5 min, and then centrifuged at 14,000 rpm for 5 min at 4 °C. The supernatant was transferred to a fresh tube. An equal volume of phenol-chloroform was added and mixed by vortexing. After centrifugation at 14,000 rpm for 2 min at 4 °C, the supernatant was transferred to a fresh tube. The double-stranded DNA was precipitated with 2 volumes of ethanol, and mixed by vortexing. The mixture was allowed to stand for 2 min at room temperature (RT). After centrifugation at 14,000 rpm for 5 min at 4 °C, the supernatant was removed. Any drops of fluid adhering to the wall of the tube was removed. The pellet of double-stranded DNA was rinsed with 1 ml of 70% ethanol at 4 °C, then centrifuged at 14,000 rpm for 5 min at 4 °C. The supernatant was removed. The pellet of nucleic acid was air-dried for 10 min, then redissolved in 50 μl of TE (pH 8.0). Five μl of the DNA were loaded onto a 1% agarose gel. The gel was run at 50 volts for 60 min and observed under UV light after staining with ethidium bromide. Plasmid containing a DNA insert will show an increased band size

compared to a plasmid control without insert. The plasmid DNA was stored at -20 °C until used.

F.5.2 Checking the recombinant plasmids

One μg of recombinant plasmids were digested with 5 units of *Xho* I and *Bam* HI to determine the insert DNA. In the first step, 10 μl of the mixture consisted of 5 units of *Xho* I, 1X buffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT pH7.9 at 25 °C) and sterile distilled water, then incubated at 37 °C for 2 h. Five μl of the mixture was analysed by agarose gel electrophoresis. The second step, 5 units of *Bam* HI, 1X buffer *Bam* HI (150 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, solution pH 7.9 at 25 °C) and sterile distilled water were added into 5 μl of the remaining mixture. The mixture was incubated at 37 °C for 2 h, and analysed by agarose gel electrophoresis.

F.6 Transformation of the recombinant plasmid into the expression host, *E.coli* BL21

The purified pGEX-4T-1-*P6* clones no.1 and pGEX-4T-1-*P6* clones no.3 were re- transformed by adding 5 ng of each recombinant plasmid into 100 μl of competent *E. coli* BL21 cells and incubated on ice for 30 min in transformation tube. The cells were heat-shocked at 42 °C for 45 sec and immediately incubated on ice for 2 min. The transformed cells were added to 900 μl of SOC media and incubated at 37°C for 1 h. The culture was transferred to a clean 1.5-ml microtube, centrifuged at 14,000 rpm for 1 min. The 800 μl of supernatant was discarded and cell pellets were resuspended with 100 μl of the remaining media. The cell suspension was spread on LB agar plates containing 100 μg/ml of ampicillin. The LB agar plates were incubated at 37 °C overnight.

F.7 DNA sequencing

The recombinant plasmids, pGEX-4T-1-*P6* clones no.1 and pGEX-4T-1-*P23* clones no.3 were purified from *E. coli* BL21 by using NucleoSpin Plasmid Extraction kit (Macherey-Nagel, Germany) as described in the manufacturer's instructions. Fifty

ng of each purified recombinant plasmid was used as DNA template for amplification by using 5' sequencing primer and 3' sequencing primer. The 100 µl PCR reactions were performed. The reaction consisted of 50 ng of the recombinant plasmid, 0.4 µM of each forward and reverse primer, 1X of PCR buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl pH 8.8 at 25 °C), 3 mM $MgSO_4$, 0.2 mM dNTP mixed and 1.25 units of Pfu® DNA polymerase (Fermentas Applied Biosystems, USA). The PCR amplification was performed using a GeneAmp® PCR system 2700. After the initial denaturing step at 95 °C for 5 min, 35 cycles were performed consisting of the denaturing step at 95 °C for 30 sec, the annealing step at 60 °C for 30 sec, and the extension step at 72 °C for 1 min. The final extension step was performed at 72 °C for 7 min. Five µl of the PCR products were analyzed by 1% agarose gel electrophoresis. The remaining PCR products were purified by using NucleoSpin Extract columns (Macherey-Nagel, Germany) as described in the manufacturer's instructions. Twenty ng of the PCR products were used for DNA sequencing. Sequencing was performed in forward direction by using 5' sequencing primer. The 20 µl of sequencing reaction consisted of 20 ng of the PCR products, 4 µl of the BigDye® Terminator v3.1 cycle sequencing ready mixed (Applied Biosystems, USA), 2 µl of 5X sequencing buffer, and 3.2 pmol of the 5' sequencing primer. Twenty-five cycles of PCR for sequencing were performed using the following conditions; the denaturing step at 96 °C for 10 sec, the annealing step at 50 °C for 5 sec, and the extension step at 60 °C for 4 min. The PCR products were transferred to a new 1.5-ml microtube, then 2 µl of 3 M sodium acetate and 50 µl of 100% ethanol were added into 20 µl of sequencing reaction and mixed by inverting 4 times. The reaction was incubated on ice for 15 min, then centrifuged at 11,000 rpm at 4 °C for 30 min. The supernatant was removed, then washed 2 times with 70% ethanol by centrifugation at 11,000 rpm at 4 °C for 15 min. The DNA pellet was dried at RT for 20 min. The DNA sequences then were analysed by the ABI PRISM model 310 version 3.7 at the Bioservice Unit (Bangkok)

The sequences of the primers that were used for sequencing the recombinant plasmid:

5' pGEX sequencing primer : 5′-GGGCTGGCAAGCCACGTTTGGTG-3′

3' pGEX sequencing primer : 5′-CCGGGAGCTGCATGTGTCAGAGG-3′

G. Expression of recombinant proteins

The growth conditions for optimal expression of selected recombinant clones such as, media, growth temperature, culture density, induction conditions, and other variations were evaluated. In this study, one colony of each clone (pGEX-4T-1-*P6* clone no.1 or pGEX-4T-1-*P23* clone no. 3 in *E. coli* BL21) was inoculated into 5 ml of LB containing 100 μg/ml of ampicillin. The culture was incubated with shaking at 200 rpm at 25 °C for 12-18 h. The 12-18 h cultured was inoculated into 200 ml of fresh LB containing 100 μg/ml of ampicillin in a 1,000-ml flask. The culture was incubated with shaking at 200 rpm at 25 °C for 3-4 h or until the OD at 600 reached 1.0-1.5. The 1 M of IPTG was added into the culture to a final concentration of 0.1 mM and the culture was incubated for an additional 2 h. The culture was transferred to 50 ml centrifuge tubes and centrifuged at 4,500 rpm for 30 min at 4 °C to sediment the cells. The supernatant was discarded and the pellet was drained, then placed on ice. The prediction for MW of GST-fusion proteins were determined by MW prediction tool from www.expasy.com.

H. Purification of the recombinant proteins

H.1 Preparation of cells lysate

The cell pellet was resuspended in 1X PBS containing protease inhibitor (0.1 μ M iodoacetic acid (IAA), 0.1 mM phenylmethanosulfonyl fluoride (PMSF), and 1 mM ethylene diamine tetra acetic acid (EDTA)) to the final OD at 600 of 2.0. The cell suspension was lysed by using Branson Sonifier 250 (VWR Scientific, USA). Sonication was performed in short bursts on ice. The cells were sonicated on ice for 30 sec with duty cycle at 25%, and output control power no.2, and then soaked on ice for 30 sec. The sonication were repeated for 10 cycles. The supernatant containing soluble form of the recombinant protein was collected by centrifugation at 10,000 rpm at 4 °C for 10 min, and kept on ice for the purification by affinity chromatography.

H.2 Purification of the recombinant proteins by affinity chromatography

The soluble target protein was purified using GSTrap FF 1-ml column (Amersham Biosciences, Sweden). The GSTrap FF 1-ml column was equilibrated with ten column volumes of binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM

Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.3). The supernatant sample was applied to the column by the syringe, maintained a flow rate of 30 drops/min. The column was washed with ten column volumes of binding buffer. The recombinant protein was eluted from the column with ten column volumes of elution buffer (50 mM Tris-HCl, and 10 mM reduced glutathione, pH 8.0). The flow-through of elution buffer was collected in 1 ml fractions and performed on ice until the procedure has been successfully completed.

I. Detection of GST fusion proteins

The methods for detection of GST, GST-P6p and GST-P23p proteins were performed by SDS-PAGE analysis and Western blot analysis using horseradish peroxidase-conjugated monoclonal anti–GST antibody (HRP-anti GST) (Amersham Biosciences, Sweden). The GST, GST-P6p and GST-P23p proteins were separated by vertical SDS-PAGE as describe in J.2. The separated proteins from the electrophoresis gel were electrophoretically transferred to a 0.45 μm of pore size Hybond-C nitrocellulose membranes (Amersham Biosciences, Sweden) at 100 volts for 45 min at 4 °C (Towbin et al, 1979). Non-specific binding sites were blocked by immersing the membrane in 5% non-fat dried milk in PBS pH 7.2 containing 0.05% tween-20 (PBS-T) for 2 h at RT with gently shaking. The membrane was incubated in 1:5,000 dilution of the HRP-anti GST in PBS-T for 1 h at RT with gently shaking. The membrane was washed in 3 changes of PBS-T washing buffer for 5 min at RT. After washing, the blot was developed with the 4-chloro-1-naphthol substrate for 10-15 min at RT.

J. Determination of properties of the recombinant proteins

J.1 Quantitation of the recombinant proteins

Protein concentration was determined by the method of Bradford (Bradford, 1996) using the dry reagent concentration (Bio-Rad Laboratories, USA). The dry reagent concentration was diluted 1:5 in distilled water. A protein standard curve was determined at five concentrations of bovine serum albumin; 62.5, 125, 250, 500, and 1,000 μg/ml. The assay was started by adding 200 μl of diluted reagent to 10 μl of sample in a microtiter well plate. The mixture was incubated at RT for 5 min. The

absorbance at 595 nm was measured by Multiskan Readers (Labsystems, Finland) and the protein concentration was calculated from the standard curve by using Microsoft Excel program.

J.2 SDS-PAGE analysis

Vertical SDS-PAGE was performed by using the discontinuous buffer system (Laemmli, 1970). The stacking gel and separating gel for SDS-PAGE were prepared following the formula in **Table 1**. The protein sample was mixed with 2X SDS reducing sample buffer (625 mM Tris-HCL pH 6.8, 5% SDS, 10% glycerol, 5% 2-β-mercaptoethanol and 0.01% bromphenol blue) at a ratio 1:1, denaturated at 37°C for 30 min, and then loaded into a well. The electrophoresis was performed using 1X Tris-glycine buffer (25 mM Tris-HCl pH8.3, 192 mM glycine and 0.1% SDS) in the electrophoretic apparatus and run at a constant 100 volts for 1 h 45 min. The protein bands were stained in Coomassie staining solution (0.1% Coomassie brilliant blue R250, 40% methanol and 20% glacial acetic acid) at RT for 30 min and subsequently destained in destaining solution (40% methanol and 20% glacial acetic acid) until the background was clear. The gel was wrapped in a cellophane membrane and air-dried for the permanent record.

Table 1. Formula of SDS-PAGE stacking gel and separating gel (0.75 mm X 2 gels)

Solution	Separating gel (10%)	Stacking gel (4%)
Distilled water	4.0 ml	3.05 ml
1.5 M Tris-HCl pH 8.8	2.5 ml	-
0.5 M Tris-HCl pH 6.8	-	1.25 ml
Acrylamide solution	3.33 ml	0.65 ml
10% SDS	100 µl	50 µl
10% (w/v) Ammonium persulphate	50 µl	50 µl
TEMED	5 µl	5 µl
Total volume	10.0 ml	4.0 ml

K. Determination of the immunogenic activity of the recombinant proteins by specific antibodies

K.1 Immunogenic activity of the recombinant proteins by sera of immunized rabbits

To determine the immunogenic activity of the fusion proteins, rabbit polyclonal antibodies against crude proteins of *P. marneffei* F4, *C. neoformans* F109 or *H. capsulatum* H760 were generated. The crude proteins of *P. marneffei, C. neoformans* and *H. capsulatum* were prepared by the following methods. *P. marneffei, C. neoformans* or *H. capsulatum* were isolated from clinical specimens of AIDS patients who were admitted at Maharaj Nakorn Chiang Mai hospital during November 1993 to October 1994. The *P. marneffei* was isolated and confirmed for dimorphism. The pure culture was maintained in a mold phase at 25-28 °C on Sabauraud's dextrose agar. Yeast phase was obtained by culturing in the brain heart infusion broth medium with shaking incubation at 37 °C for 5 days. The fungal cells were pelleted by centrifugation at 4,500 rpm, 4 °C for 20 min. The cell pellet was resuspended in 1X PBS pH 7.3 containing protease inhibitors (50 mM IAA, 50 mM PMSF and 500 mM EDTA). The cell suspension was lysed using bead beater at 5,000 rpm for 30 sec for 6 cycles. The crude proteins in the supernatant were collected by centrifugation at 4,500 rpm, 4 °C for 30 min. These crude proteins were used for antibody production.

The 500 µg of the crude proteins prepared from *P. marneffei, C. neoformans* or *H. capsulatum* were mixed with an equal volume of complete Freund's adjuvant and injected intramuscularly into rabbits which were prebled. Two weeks after the primary immunization, the rabbit was boosted by subcutaneously injection with the same antigen emulsified in incomplete Freund's adjuvant. Two weeks after the first boost, immunization was repeated. Serum samples were taken 7-10 days after the third injection (Rosenberg, 1996) and subjected for Western blot analysis.

In Western blot assay, the recombinant protein samples and the crude concentrated culture filtrated *P. marneffei* antigens were run on SDS-PAGE and subsequently electroblotted onto a nitrocellulose membrane. The blot was incubated with a 1:100 dilution of the rabbit antiserum. After washing with PBS-T, the blot was incubated with 1:4,000 HRP-conjugated goat anti-rabbit IgG (Pierce Biotechnology, Perbio Science Erembodegen, Belgium), and washed again with PBS-T. The antigen-

antibody complex was detected with the 4-chloro-1-naphthol substrate for 10-15 min at RT (Sambrook et al, 1989).

K.2 Immunogenic activity of the recombinant proteins detected by sera of *P. marneffei*-infected patients

In Western blot assay, the recombinant protein samples and the crude concentrated culture filtrated *P. marneffei* antigens were run on SDS-PAGE and subsequently electroblotted onto a nitrocellulose membrane. The blot was incubated with a 1:50 dilution of the serum from each patient infected with *P. marneffei* at RT for 1 h. After washing with PBS-T, the blot was incubated with 1:2,000 HRP-conjugated goat anti-human IgG (Pierce Biotechnology, Belgium). The blot was washed with PBS-T. The antigen-antibody complex was detected with the 4-chloro-1-naphthol substrate for 10-15 min at RT (Sambrook et al, 1989).