CHAPTER IV

Methods

1. Identification of uridine phosphorylase gene

The bioinformatics underlying NCBI resources was used to study on enzyme uridine phosphorylase and model organisms. The selected model organisms were identified for their uridine phosphorylase gene using database of the National Center for Biotechnology Information (NCBI) available at *http://www.ncbi.nlm.nih.gov/Genbank/* (Figure 4-1).

All gene were then analyzed for sequence homologies using the BLAST program (Basic local alignment search tool) of NCBI available at *http://www.ncbi.nlm.nih.gov/BLAST/*, (Figure 4-2). The resulte homology sequence were then used to search for nucleotide sequence of a candidate gene of *P. falciparum* uridine phosphorylase.

The primer for amplifying the candidate DNA by PCR were designed from searching results.

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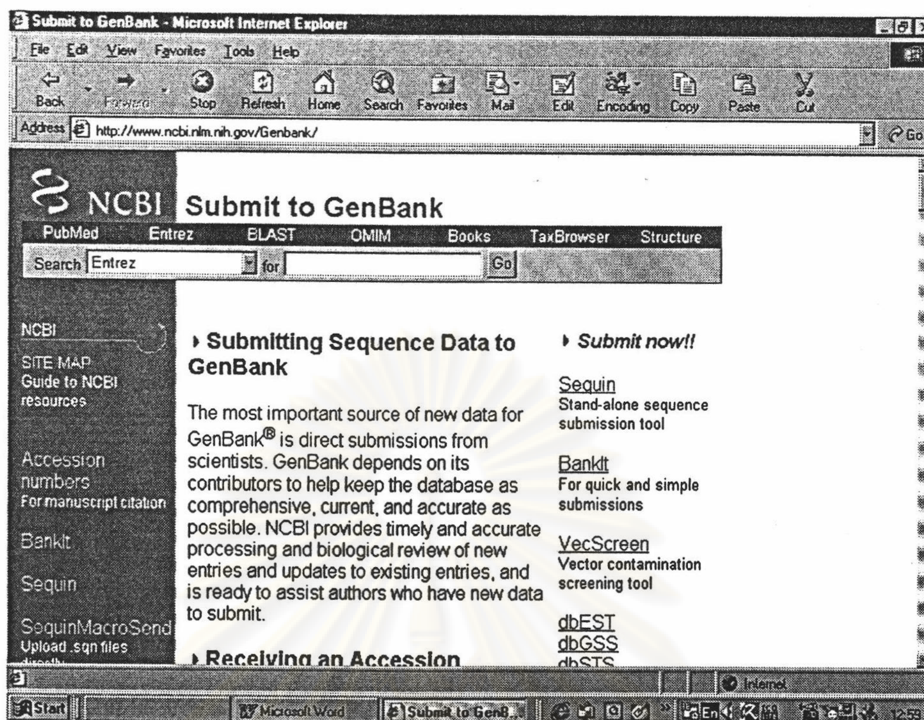

Figure 4-1 Web page of *http://www.ncbi.nlm.nih.gov/Genbank/*

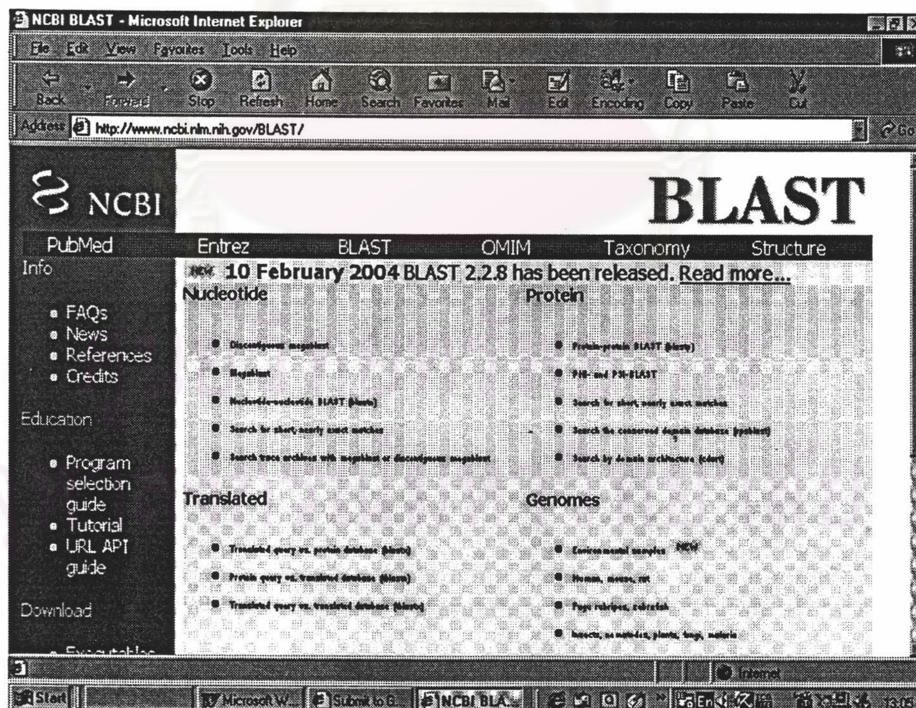

Figure 4-2 Web page of BLAST program.

2. Specimens

P. falciparum (T9 isolated) was cultivated by a modification of the candle jar method of Trager and Jensen (32), using a 5% hematocrit of human red cell type O suspended in RPMI 1640 medium supplemented with 25 mM Hepes, 32 mM $NaHCO_3$ and 10% fresh human serum type O. The cultures, started at low parasitemia (~1-2%), were changed with the medium twice daily until the cultures had ~30% parasitemia and then harvested for DNA preparation.

3. DNA extraction

A volume of 100 µl of intact *P. falciparum* was mixed with 1 ml of 10 mM Tris-HCL pH 7.5, 150 mM NaCl and 25 mM EDTA· SDS (10%). The mixture was then centrifuged at 13,000 rpm for 10 min at 4 °C and the supernatant was collected. Proteins were removed by extraction twice with an equal volume of phenol/chloroform/isoamyl alcohol(25:24:1,v/v) at 13,000 rpm for 10 min at 4 °C, then the upper aqueous phase was transfered to a fresh tube. DNA was precipitated by addition of ice-cold absolute ethanol and was then sedimented at 12,000 rpm for 5 min at 4 °C and washed twice with ice-cold absolute ethanol. The pellet was air dried and resuspended in an appropriate volume of TE buffer or sterile distilled water.

4. Calculation of DNA concentration

The DNA concentration was quantitated by measuring the absorbance (OD) at 260 nm . An OD_{260} of 1 corresponds to approximately

50 μg/ml for double-strand DNA. So that DNA concentration was calculated from the following equation ;

$$\text{DNA concentration } (\mu g/ml) = OD_{260} \times 50 \times \text{dilution factor}$$

5. Candidate gene amplification by polymerase chain reaction (PCR)

PCR was used to amplify DNA encoding *P. falciparum* uridine phosphorylase, the 5' ends of primer SK13 and primer SK14 (Appendix B) containing *Bam*HI and *Sac*I restriction sites, respectively, were used to amplify *P. falciparum* uridine phosphorylase DNA. The volume of 50 μl of PCR reaction, prepared in a 0.5 microfuge tube, consisted of 20.5 μl of sterile distilled water, 10 μl of *P. falciparum* gDNA, 5 μl of PCR buffer, 4 μl of dNTP, 5 μl of each primer and 2.5 U of *Taq* polymerase. The automated thermal cycler was used according to the time and temperature program as indicated in Table 4-1. Subsequently, 3 μl of PCR product was analysed using agarose gel electrophoresis.

Table 4-1 PCR parameters for candidate DNA amplification

Temp. / Stage	95 °C	48 °C	55 °C	68 °C	Cycle
1	3 min	1 min	-	2 min	1
2	1 min	-	1 min	3 min	30
3	1 min	-	1 min	10 min	1

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6. Agarose gel electrophoresis

The DNA fragments can be analysed by submarine agarose gel electrophoresis. The concentration of agarose gel depends on the size of DNA. In this thesis, 1% (w/v) agarose gel in 1X TAE was used. The agarose gel in 1X TAE was melted, poured into an electrophoretic tray and allowed to set at RT. The 1X TAE was used as an electrophoretic buffer.

The DNA sample was mixed with 1 μl of loading dye and loaded onto the prepared gel in 1X TAE running buffer at 0.80 v/cm and stoped when dye front reached the end of gel. The gel was stained with ethidium bromide (EtBr) solution for 5 min and then destained with water for 15-30 min. The pattern of DNA bands were observed under the UV light box.

7. Purification of PCR product using QIA quick PCR Purification Kit

A 5 volume of PB buffer was added to 1 volume of the PCR product and the tube was vortexed. The solution mixture was transfered to the microspin cup that seated in a receptacle tube, centrifuged at 13,000 rpm for 1 min and discard the PB buffer. Then, the microspin cup was added with 750 μl of PE washing buffer and centrifuged at 13,000 rpm for 1 min , discard the PE buffer and centrifuged again. The microspin cup was transfered to a fresh microcentrifuge tube and added with 30 μl of EB buffer directly onto the top of the fiber matrix at the bottom of the microspin cup. The tube was incubated at RT for 1 min and centrifuged at 13,000 rpm for 1 min, then discard the microspin cup. The purified PCR product was analyzed by submarine agarose gel electrophoresis.

8. Purification of DNA using QIAquick Gel Extraction Kit

The DNA fragment was excised from the agarose gel with clean, sharp scapel. Weight the gel slice in a tube and added with 3 volume of buffer QG to 1 volume of gel and the mixture was then incubated at 50 °C for 10 min or until gel melting, The mixture was added with 1 volume of isopropanol to 1 volume of gel and loaded into a Qai Quick spin column with the bottom outlet capped and centrifuged at 13,000 rpm for 1 min, then discarded the flow-through. The column was added with 0.5 ml of buffer QG through the bottom outlet capped of column and centrifuged at 13,000 rpm for 1 min, then discarded the flow-through and washed once with 0.75 ml of buffer QG. The DNA was eluted with 30 µl of buffer EB and analyzed by submarine agarose gel electrophoresis.

9. Cloning of the PCR product into the pDrive cloning vector

9.1 DNA ligation

The PCR product was ligated into the pDrive cloning vector by the ligation reaction that was performed in a total volume of 10 µl. The ligation reaction contained 1 µl of pDrive cloning vector, 1 µl of PCR product, 2 µl of sterile distilled water and 5 µl of 2x ligation master mix. The ligation mixture was spin briefly to mixed and incubated at 16 °C for 2 hrs, then proceed with transformation or stored at –20 °C until use.

9.2 *E. coli* transformation

The aliquot 50 µl of *E. coli* competent cells, strain *EZ*, and SOC medium were thaw on ice, then the ligation mixture was transfered to the

cells, gently mixed by hand and incubated on ice for 5 min. The tube was heated at 42 °C waterbath without shaking for 1 min and incubated on ice for 5 min. The mixture was then added with 250 µl of SOC medium, gently mixed and incubated at 37°C for 45 min. The mixture was centrifuged at 6,000 rpm for 1 min and removed the upper phase of mixture, then plate the transformation mixture onto the LB-ampicillin agar (LB agar with 100 µg/ml ampicillin which had spread with IPTG and x-gal) and incubated at RT until the transformation mixture had absorbed into agar. Invert the plate and incubated at 37 °C O/N.

10. <u>Plasmid extraction from *E. coli*</u>

10.1 Small scale plasmid preparation

A single white colony of the recombinant bacteria was grown at 37 °C O/N with shaking motion in 3 ml of LB broth containing 100 µg/ml ampicillin. The cells were harvested at 6,000 rpm for 5 min. The cell pellet was resuspended in 100 µl of ice-cold solutionI, then the cell suspension was added with 200 µl of freshy prepared solutionII and incubated on ice 5 min. The mixture was added with 150 µl of ice-cold solutionIII, gently mixed by invert and incubated on ice for 5 min; then bacterial chromosome and cells debris were removed by centrifugation at 10,000 rpm for 5 min. The supernatant was removed to a fresh tube and was then added with 0.7 volume of ice-cold isopropanol to 1 volume of the supernatant, gently mix and incubated at RT for 10 min. The tube was centrifuged at 10,000 rpm for 5 min, discard supernatant. The pellet was washed once with ice-cold 75 % ethanol, air dry and dissolved in 200 µl of sterile distilled water which added

with 0.5 μl of RNase, gently mix and incubate at 37 °C for 1 hr. The mixture was then added with an equal volume of phenol/chloroform (1:1,v/v), mix by vortex and incubated at RT for 5 min, then centrifuged at 10,000 rpm for 10 min. The aqueous phase was removed which carefully to a fresh tube and precipitated with 3M sodium acetate and ice-cold absolute ethanol at –20 °C O/N. The DNA pellet was collected at 12,000 rpm for 10 min and washed once with ice-cold 70 % ethanol. After drying, the pellet was resuspended in 30 μl of sterile distilled water and stored at –20 °C.

10.2 Medium scale plasmid preparation using Qaigen Plasmid Midi and Maxi Kit

A single white colony of the recombinant bacteria was grown at 37 °C O/N with shaking motion in 3 ml of LB broth containing 100 μg/ml ampicillin. The overnight culture was transfered to flask with 50 ml LB-ampicillin medium and incubated at 37 °C for 3 hrs with vigorous shaking. The cells were harvested at 6,000 rpm for 15 min and resuspended in 4 ml of P1 lysis buffer. The cells suspension was added with 4 ml of P2 buffer, gently mixed by invert and incubated at RT for 5 min. The mixture was added with 4 ml of ice-cold P3 buffer, gently mixed and incubated on ice for 15 min and was then centrifuged at 12,000 rpm for 45 min, at the same time, the tip was equilibrated with 4 ml of QBT buffer. The supernatant was transfered directly onto the top of the fiber matrix of the tip and was then washed twice with 10 ml of QC buffer. The tip was added with 5 ml of QF buffer to elute DNA. The plasmid DNA was precipitated with 3.5 ml of

isopropanol at 15,000 rpm for 30 min and washed once with 70 % ethanol, air dry and resuspended in sterile distilled water.

11. Restriction endonuclease digestion (33)

The recombinant plasmid DNA was analyzed by digestion with restriction enzymes. The digestion reaction was performed following the conditions of restriction enzymes, in this study, were consisted of *Bam*HI and *Sac*I . The digestion-reaction was performed in a total volume of 20 μl using 7.3 μl of sterile distilled water, 2 μl of reaction buffer, 0.2 μl of BSA, 10 μl of plasmid DNA, 0.5 μl of restriction enzyme (*Bam*HI) and incubated at 37 °C for 2.5 hrs. When the reaction was completed, precipitate with 3M sodium acetate and ice-cold absolute ethanol at –20 °C O/N. The DNA pellet was collected at 12,000 rpm for 10 min and washed once with ice-cold 70 % ethanol. After drying, the pellet was redissolved in 17.3 μl of sterile distilled water and was then digested with *Sac*I. The digestion product was analyzed by submarine agarose gel electrophoresis.

12. Automated DNA sequencing and analysis of DNA sequence

The candidate sequence (*P. falciparum* uridine phosphorylase sequence) was determined in both directions by the dideoxy chain termination method using an automated Applied Biosystems Procise sequencer. The sequence homologies were analysed using the BLAST program of the NCBI available at *http://www.ncbi.nlm.nlh.gov/BLAST/*

13. <u>Recombinant protein expression and purification of *P. falciparum* uridine phosphorylase from *E. coli*</u>

The construct plasmid, pDrive carrying PCR fragment of *P. falciparum* uridine phosphorylase gene, was subcloned into pQE30 expression vector. First, the construct plasmid was double digested with both of restriction enzymes, *Bam*HI and *Sac*I (Method 11). Second, *P. falciparum* uridine phosphorylase gene was purified from the digestion product using QIAquick Gel Extraction Kit (Method 8) and was then ligated into pQE30 expression vector, using 1 µl of pQE30 expression vector, 1µl of uridine phosphorylase DNA, 4 µl of sterile distilled water and 6 µl of 2x Ligation Master Mix. The ligation mixture was spin briefly to mix and incubated at 16 °C O/N and was then transformed into the DH-5α competent cells. The recombinant plasmid were analyed by restriction analysis of small-scale preparation method, then the recombinant plasmid from positive clone was transform into the SG13009 and M15 competent cells using LB-ampicillin-kanamycin media.

The expression of *P. falciparum* uridine phosphorylase gene in *E. coli* was induced by IPTG. First, the positive clones from SG13009 and M15 transformants were inoculated into 3 ml of LB-ampicillin-kanamycin medium and incubated at 37 °C O/N with vigorous shaking, the overnight culture was then diluted to ratio of 1:100 with LB-ampicillin-kanamycin medium and incubated at 37 °C O/N with vigorous shaking until OD_{600} was about 0.4-0.5. Second, the culture was added with IPTG to a final concentration of 1 mM and was then incubated at 18 °C O/N with vigorous

shaking. Finally, the cells were harvested at 6,000 rpm for 10 min and washed twice with ice-cold PBS and freeze until use.

All protein purification steps were performed on ice. Frozen cell pellets were resuspended in 1 ml of lysis buffer added with lysozyme to 1 mg/ml, genly mixed by hand and incubated on ice. The mixture was sonicated on ice for 6 times (30 sec burst /15 sec cooling), taking care to avoid foaming. Crude homogenate was centrifuged at 12,000 rpm for 20 min at 4 $^{\circ}C$, at the same time, the Ni-NTA spin column was equilibrated twice with lysis buffer with an open lid. The cleared lysate was loaded onto the Ni-NTA spin column and centrifuged at 2,000 rpm for 2 min, the Ni-NTA spin column was then washed twice with 600 μl washing buffer at 2,000 rpm for 2 min. The protein were eluted twice with 200 μl elution buffer at 2,000 rpm for 2 min. All samples, including lysate, flow-through, washing fraction and eluate were analyzed on 12% SDS-PAGE (Appendix A) and protein were visualized by staining with Coomassie blue.

Protein concentrations of all samples, including lysate, flow-through, washing fraction and eluate were determined by the method of Bio-Rad Protein Assay and using bovine serum albumin as a standard. Dilution sample with sample buffer may be necessary to reduce the concentration of protein in the assay. The standard protein was measured absorbance at 595 nm versus reagent blank and plot standard curve. The unknowns were read from standard curve.

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14. <u>Study on the enzyme uridine phosphorylase of *P. falciparum* recombinantly expressed in *E. coli*</u>

14.1 The enzyme assay method of uridine phosphorylase

The activity of uridine phosphorylase was assayed by following the phosphorylation of uridine. The reaction was monitored by the loss of uridine absorbance at 272 nm ($\in = 2900\ M^{-1}cm^{-1}$) on a Shimadzu spectrophotometer equiped with 37 °C thermostat waterbath. The assayed mixture contained 50 mM phosphate buffer pH 8.0 and 10 mM uridine. The reaction was initiated by addition of the enzyme and incubated at 37 °C for 1 min before measuring the absorbance.

The specific activity of uridine phosphorylase was calculated by the following equation ;

$$\text{Specific activity (nmol/min/mg)} = \frac{\text{Enzyme activity per fraction (nmol/min)}}{\text{Protein per fraction (mg)}}$$

14.2 Study on kinetic properties of uridine phosphorylase

Kinetic properties of uridine phosphorylase was determined by varying substrates (uridine) concentration and the amount of enzyme was fixed. After achieving the values of enzyme activity in each concentration of substrates, these values were plotted as Michealis-Menten kinetics and Lineweaver-Burk plot then were calculated the Michealis-Menten constant (K_m) and the turnover number (k_{cat}).

15. Enzyme molecular mass determination

The molecular mass of *P. falciparum* uridine phosphorylase was estimated by running SDS-PAGE compared with the standard protein marker.

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