นิพนธ์ต้นฉบับ

การสร้างและผลิตโปรตีนเอนไซม์ Glyceraldehyde-3- phosphate dehydrogenese ของเชื้อ Plasmodium falciparum

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บทคัดย่อ

วัตถุประสงค์: Glyceraldehyde-3-phosphate dehydrogenase ของเชื้อ *Plasmodium falciparum* (pfGAPDH) เป็นเอนไซม์ใน glycolytic pathway มีความเหมือนกับเอนไซม์ GAPDH ของมนุษย์ 63.5% แต่ซีรัมที่ได้จากการฉีดกระตุ้นไม่ทำปฏิกิริยากับเม็ดเลือดแดงของมนุษย์ และพบว่ามีลำดับนิวคลีโอไทด์ไม่แตกต่างกันระหว่างเชื้อที่ทำการแยกได้ งานวิจัยนี้มีวัตถุประสงค์เพื่อผลิตรีคอมบิแนนท์โปรตีน pfGAPDH เพื่อใช้เป็นอิมมูโนเจนในการผลิตโมโนโคลนอลแอนติบดี

วิธีการ: ทำการสร้างรีคอมบิแนนท์พลาสมิด pfGAPDH-pET 15 b และนำเข้าสู่เซลเจ้าบ้าน คือ *E. coli* BL21(DE3) และตรวจสอบการสร้างโปรตีน โดย SDS-PAGE และ western blot analysis จากนั้นทำโปรตีนให้บริสุทธิ์โดยวิธี affinity chromatography

ผลการทดลอง: รีคอมบิแนนท์ plasmid ที่ได้ เมื่อนำเข้าสู่เซลเจ้าบ้านสามารถสร้าง รีคอมบิแนนท์โปรตีน pfGAPDH ได้ในปริมาณสูง และเมื่อผ่านการ purify จะได้โปรตีนมีความบริสุทธิ์สูง และโปรตีนดังกล่าวสามารถใช้เป็นอิมมูโนเจนในการฉีดกระตุ้นสัตว์ทดลองได้เป็นอย่างดี

สรุป: จากผลการทดลองพบว่า สามารถสร้างและผลิตโปรตีน pfGAPDH เพื่อใช้เป็นอิมมูโนเจน ในการฉีดกระตุ้นเพื่อผลิต monoclonal antibody ได้ โดยการศึกษาต่อไปจะเป็นการศึกษาคุณลักษณะของ monoclonal antibody ที่ได้ และนำไปประยุกต์ใช้ในการพัฒนาชุดตรวจวินิจฉัยมาลาเรียต่อไป **วารสารเทคนิคการแพทย์เชียงใหม่ 2549; 39: 27-35.**

คำหัส : *Plasmodium falciparum*, Glyceraldehyde- 3- phosphate dehydrogenase, *Escherichia coli* expression

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Abstract: Cloning and Expression of Glyceraldeyhe-3-Phosphate Dehydrogenase from *Plasmodium Falciparum.*

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Objective : Glyceraldehyde-3-phosphate dehydrogenase of *Plasmodium falciparum* (pfGAPDH) is the key enzyme in glycolytic pathway. Its gene has 63.5% identity to human GAPDH but the immune serum against this enzyme has no cross-reactivity with human erythrocyte and also conserved among various isolates. This study aims to clone the pfGAPDH gene for expression of pfGAPDH protein in *Escherichia coli* for using as immunogen to obtain monoclonal antibody.

Methods : Plasmid pfGAPDH-pET15b was constructed and transformed into *E. coli* BL21(DE3) for expression of pfGAPDH protein. The expressed His-pfGAPDH fusion protein was characterized by SDS-PAGE and western blot analysis. Then the purified protein was obtained by affinity chromatography.

Results : The pfGAPDH protein was expressed in *E. coli* at high level .By SDS-PAGE and western blot analysis, the protein band of 36.6 kDa was detected. Furthermore, the high purity protein was obtained and used as immunogen for immunization in BALB/c mice.

Conclusion : The pfGAPDH gene was cloned and expressed protein at high level in *E. coli.* The purified protein was successfully used as immunogen. Further study will carry on characterization of the obtained monoclonal antibody and applied for development of malarial diagnostic kit. **Bull Chiang Mai Assoc Med Sci 2006; 39: 27-35.**

Key words : *Plasmodium falciparum*, Glyceraldehyde- 3- phosphate dehydrogenase, *Escherichia coli* expression

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Introduction

The malaria situation is still endemic and minor epidemic especially in the border line of Thailand.[1] The prompt and accurate diagnosis is the key of effective disease management in malaria patients. The two diagnosis approaches are clinical diagnosis and microscopic diagnosis, however these do not allow satisfactory diagnosis of malaria.[2-3] Using microscopic examination for routine malaria diagnosis has many practical problems since it is labor-intensive and requires skilled operators.[4] Recently, rapid immunochromatography format is the

most prominent alternatives. However, its sensitivity still varies in the field study.[5-7] The other parasitic enzyme based antigen detection has been mentioned to supplement the available dipstick.

The glycolytic pathway is regarded as particularly important for the blood stage malaria parasite to grow and multiply. Utilization of glucose by parasitized red blood cells was reported to be increased 50 to 100 times more than that by non-parasitized ones.[8] Almost the increasing in utilization is the result of glycolytic enzymes expressed at high level by the parasite in anaerobic Embden-Meyerhoff-Parnas (EMP) pathway.[9]

Glyceraldehyde-3-phosphate dehydrogenase of *Plasmodium falciparum* (pfGAPDH) is a key enzyme in glycolysis and catalyzes the oxidative phosphorylation of glyceraldehydes 3 phosphate into 1,3 bisphosphoglycerate in the presence of NAD+ and inorganic phosphate. Its gene has 63.5% identity to human GAPDH but the immune serum against this enzyme has no cross-reactivity with human erythrocyte and also conserve among various isolates.[10] By the way, the detection of pfGAPDH for malarial diagnosis has not yet been conducted. Therefore, this study aims to clone pfGAPDH gene for expression of pfGAPDH protein from *Escherichia coli* for using as immunogen to obtain monoclonal antibody by hybridoma technology. Furthermore, it was demonstrated that the obtained monoclonal antibody could react with blood stage parasite by western blot analysis and immunofluorescence staining. The successful production of antigen and monoclonal antibody lead to the new developed test for malaria diagnosis.

Materials and methods

Parasites

Plasmodium falciparum strain K1 were maintained *in vitro* in RPMI 1640 medium to which was added human type O red blood cell and 10% heat-inactivated human serum. All cultures were placed in a humidified incubator at 37°C with gas controlled environment of 5% O_2, 5%CO_2, and 90% N_2 and fed according to the established procedures.[11]

Detection of parasites

Parasites was determined by light microscopy using Giemsa-stained thin smears and with fluorescent microscopy using immunostaining with monoclonal antibody.

Cloning of pGAPDH gene into plasmid *E. coli*

Total RNA was isolated from mixed stage saponin (0.1%) lysed *P. falciparum* (K1 strain) infected erythrocytes. The pfGAPDH gene was amplified from cDNA of *P. falciparum* that reverse transcriped from total RNA using specific primers of ORF of pfGAPDH containing *Nde* I and *Xho* I sites. The amplification was achieved by incubating the reaction mixture (50 µl) for 5 mins at 95 °C, 30 cycles of 1 min at 95 °C, 1 min at 55 °C, and 1 min at 72 °C and a final extension for 10 mins at 72 °C. The PCR products had a single deoxyadenosine (A) of the 3' end that were ligated with pCR 2.1 vector of TA Cloning Kit (Invitrogen) and transformed into *E. coli* (TOP 10 F'). The transformed colonies were selected by colony PCR. The plasmid of positive colony were extracted by QIAprep® spin miniprep kit (QIAGEN) and double digested with *Xho* I and *Nde* I, gel purification and cloned inframe into the bacterial expression vector pET 15b (Novagen). The transformants were pick up and selected by colony PCR.

Expression and Characterization of pGAPDH

The recombinant pfGAPDH was expressed in *E. coli* BL21 (DE3) with six histidine residues at the

N-terminal portion. The cells were cultured in 3 ml of LB broth containing 100 µg/ml of ampicillin at 30 °C, 2 hours. The expression was induced by the addition of 1 mM isopropyl thiogalactoside (IPTG) (Sigma) and cultured for additional 4 hours. The pellet was collected and lysed in SDS-PAGE sample buffer then lysate and debris were separated and applied to SDS-polyacrylamide gel elctrophoresis using 10% gels then stained with coomassie blue R-250, and immunoblotting using anti-His antibody.

Western blot analysis

Total cell lysates were run onto 10% gels. As molecular weight marker (Prestained SDS-PAGE standards, broad Range Bio-Rad) was used. Separated proteins were transfered electroelectically to nitrocellulose filter (Schleicher & Schuell). Blots were blocked and then incubated with anti-His antibody (1:500) (QIAGEN). After several washing steps, blots were incubate with 1:1000 dilution of a rabbit anti-mouse IgG Horseradish peroxidase conjugated IgG (DAKO) for 30 min, blots were developed using 3, 3-Diaminobenzidine (Sigma) to visualize bands.

Purification of pGAPDH from recombinant *E. coli*

Large scale culture of pfGAPDH expressed clones in *E.coli* BL21 (DE3) and induced with IPTG was prepared. Then crude extract was produced by sonication pellet of cultured *E.coli* in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl and 2.5 mM imidazole, pH 8.0). After centrifugation at 10,000xg, the supernate (containing soluble protein of interest) was loaded onto Ni-NTA resin (QIAGEN) and washed three times with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl and 20 mM imidazole, pH 8.0). The recombinant protein was recovered by using elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole, pH 8.0). After analysis by SDS-PAGE the purified protein was pooled and store at –20 °C. Protein concentration was determined using BSA standard.

Generation of monoclonal antibodies

Monoclonal antibodies against pfGAPDH was establish by Pongwit *et al.* Briefly, BALB/c mice were immunized and boosted with purified pfGAPDH protein. Then splenocytes were fused to the myeloma cells and selected in hypoxanthin aminopterin thymidine(HAT) media. Antibody from secreting cell line were screen for their activity by ELISA. Clones secreting anti-pfGAPDH mAb were expanded and cloned by limiting dilutions. Ascetic fluids with high antibody titers were then accumulated by injecting cloned hybrid cells intraperitoneally into mice treated with Pristane (Sigma) and purified according to published procedures.

Results

Cloning, Expression and Characterization of pfGAPDH

The ORF of pfGAPDH gene was amplify from cDNA of K1 strain. The PCR product of 1,013 bps was maintained in cloning vector pCR 2.1 and then subcloned into pET 15b expression vector to produce plasmid pfGAPDH-pET 15b. The plasmid was transformed into *E.coli* BL21 (DE3) host cell for expression. The positive clones were checked by PCR and double digestion (Fig. 1A and 1B). The recombinant pfGAPDH protein was expressed in *E. coli* BL21(DE3) with molecular weight of 36.6 kDa and no specific band was observed from lysates of *E. coli* alone and *E. coli* with plasmid pET-15b contained no insert (Fig. 2).

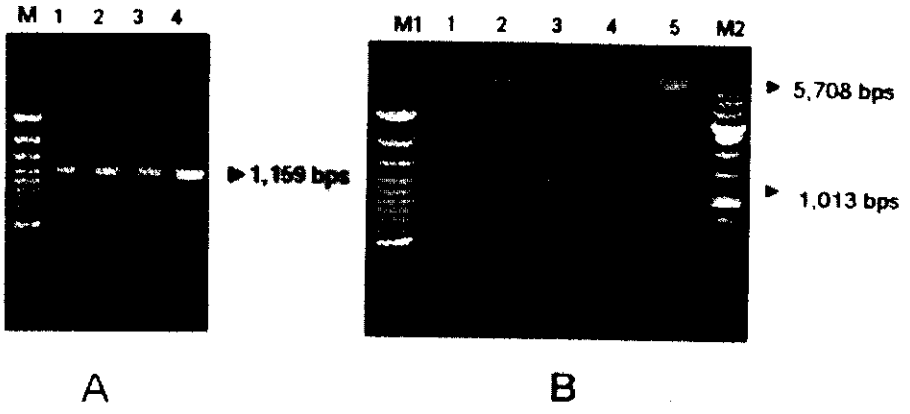

Figure 1. Agarose gel electrophoresis analysis of transformants that contained plasmid pfGAPDH –pET 15b. (A) By PCR (lane M: marker, lane 1-4 : positive clones). (B) The positives clones were confirmed by double digestion with restriction enzymes *NdeI* and *XhoI* (lane M1, M2 : marker, lane1-4 : positive clones, lane 5 : uncut).

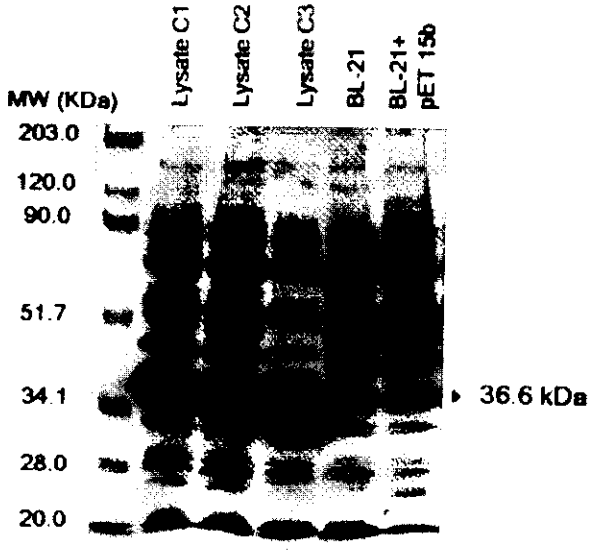

Figure 2. SDS-PAGE analysis of pfGAPDH expression. The lysate of *E. coli* BL21(DE3) transformed with plasmid pfGAPDH-pET15b clone 1-3(LysateC1-C3), lysate of *E. coli* BL21 (DE3) (BL-21) and lysate of *E. coli* BL21(DE3) transformed with pET-15b (BL-21+pET 15b) were applied to 10% SDS-PAGE then stained with coomassie blue R-250.

Purification of pfGAPDH protein

In order to use pfGAPDH protein as immunogen and screening for monoclones monoclones that secret monoclonal antibodies against pfGAPDH., large scale culture and purification of the protein was conducted. The 6xHis tagged at N-terminus of vector pET-15b was advantage for purification of fusion protein by affinity chromatography using Ni-NTA resin. As shown in Fig 3A. the obtained protein had high purity of about 90% and yield of 5 mg per one litre of culture medium, as judged by SDS-PAGE analysis. The purified protein was also analyzed by western blot analysis using anti-His antibody. Fig. 3B shows that the anti-His antibody recognized specific band of 36.6 kDa protein.

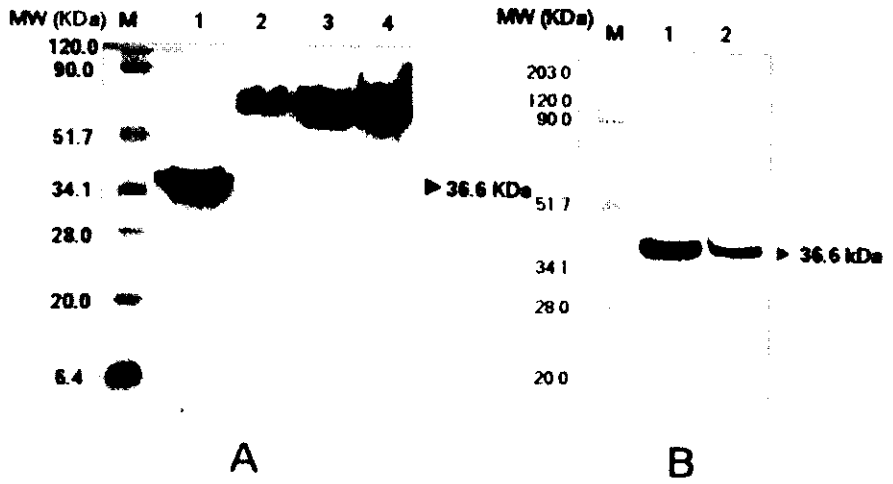

Figure 3. SDS-PAGE and western blot analysis of purified pfGAPDH protein. (A) SDS-PAGE to determine the purity and concentration of purified pfGAPDH protein compared with BSA standard protein. (M : protein marker, lane 1: purified pfGAPDH, lane 2-4 : BSA standard at concentration 5mg/ml, 10mg/ml and 15mg/ml respectively). (B) Western blot analysis of purified pfGAPDH protein eluted from Ni-NTA resin using anti-His antibody (lane M : protein marker, lane 1-2: purified protein from first elution and second elution, respectively).

Detection of pfGAPDH in parasites using anti-pfGAPDH mAb

In order to characterize the activity of the mAb against pfGAPDH (anti- pfGAPDH mAb), an immunoblotting and IFA were performed using the mAb. As shown in Fig. 4A, by western blot analysis, the anti- pfGAPDH mAb could recognize pfGAPDH protein in both the cultured parasites and recombinant protein from *E. coli* specifically. Furthermore, after purification of ascetic fluid, the mAb could recognize pfGAPDH of *P. falciparum* from clinical samples with no specific band was observed from human red blood cell (Fig. 4B). By IFA pfGAPDH was also detected at very high levels in cytosol of parasites in parasitized red blood cells as shown in Fig. 5A and 5B.

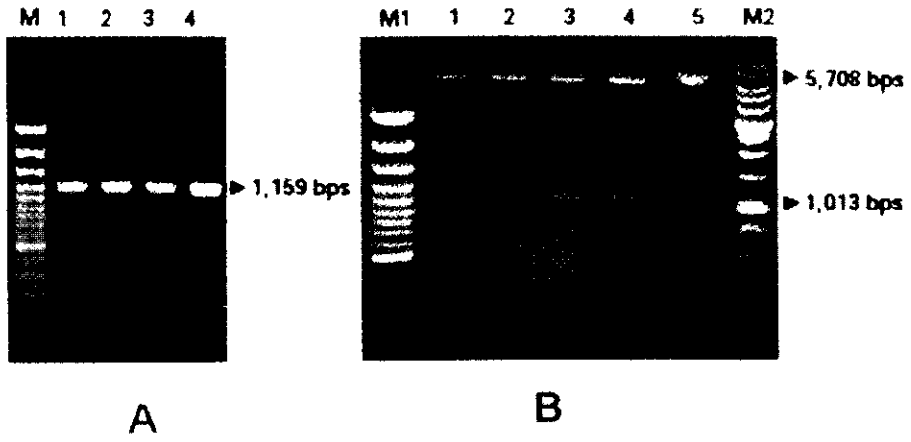

Figure 4. Determination of anti-pfGAPDH mAb activity. (A) Western blot analysis using ascetic fluid (1:5000) (M : protein marker, lane1-2: lysate of human red blood cells, lane 3-4: lysate of *P. falciparum* parasitized RBC in sample, lane 5-6: lysate of *P. falciparum* parasitized RBC in culture, lane 7-8: lysate of *E. coli* BL21(DE3), lane 9 : recombinant pfGAPDH protein). (B) Western blot analysis using purified mAb (1:5000)(lane 1 : lysate of *E. coli* BL21(DE3), lane2 : lysate of *P. falciparum* parasitized RBC in sample, lane 3 : lysate of human red blood cells, lane 4 : lysate of *P. falciparum* parasitized RBC in culture, lane 5 : recombinant pfGAPDH protein,). The arrows indicated protein bands of about 36.6 kDa that was recognized by anti-pfGAPDH mAb.

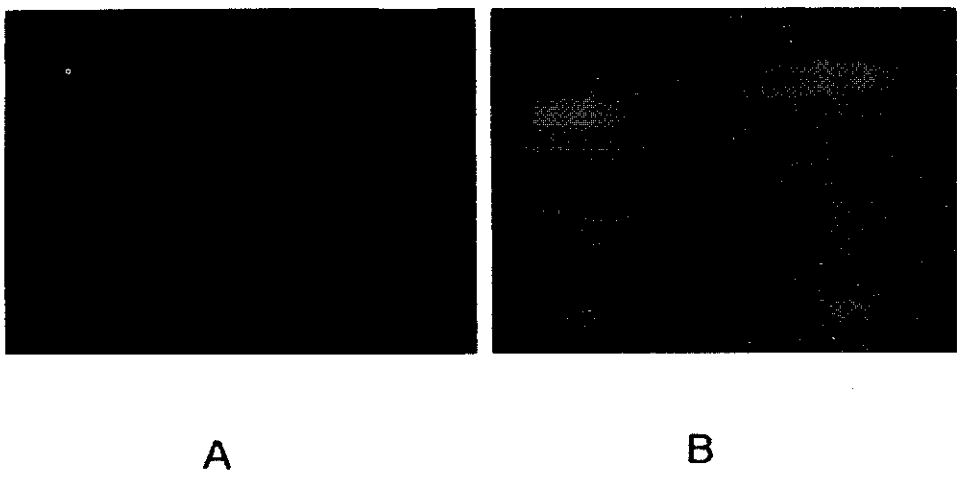

Figure 5. Detection of pfGAPDH in the parasite by immunofluorescent staining. (A) The thin film of blood stage *P. falciparum* was fixed and permeabilized and incubated in anti-pfGAPDH mAb followed by incubate in FITC-conjugated goat anti-mouse IgG and visualized by fluorescent microscopy. (B) The same area was visualized in bright field to positioned the parasites in red blood cells.

Discussion

Recently, rapid test or dipstick based on the detection of antigen(s) released from parasitized red blood cells for malarial diagnosis have been developed.[12-13] In the case of *P. falciparum*, rapid test based on detection of *P. falciparum* histidine-rich protein 2 (HRP-2)[14] or *Plasmodium*-specific lactate dehydrogenase (pLDH)[7,15] have been conducted and the sensitivity, specificity of these tests have been assessed compared with microscopy examination.[7,13,16] Although the specificity for detection are high (>90%), the sensitivity falls off at low levels of parasitemia. Therefore, further development appears necessary to improve the performance of the test.

The gene coding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) was first isolated from *Plasmodium falciparum* by Daubenberger *et al.* in 2000. Study in subcellular fractionation demonstrated that pfGAPDH was found in both the membrane-containing pellet and the supernatant fraction of parasite lysates.[17]The pfGAPDH could appropriately be antigen for detection in immunodiagnosis of malaria by establishment of monoclonal antibody for development of dipstick. Using protein from *in vitro* culture, purified pfGAPDH antigen was obtained very low yield and highly time consume for continuous culture parasites and process in purification. The large amount of pfGAPDH was also needed to screen the monoclones that secret monoclonal antibodies against pfGAPDH. More practical and easier way to obtain in amount and stable quality of pfGAPDH in each lot was producing recombinant pfGAPDH from *E. coli*. Our results demonstrated that the recombinant protein could be produced in *E. coli* employed pET 15b vector system, the expressed protein was recovered in the soluble fraction of the total extract then purified in native form and successfully used for immunization in BALB/c mice to produce monoclonal antibody. The obtained mAb could recognize parasite GAPDH protein at specific band of 36.6 kDa by western blot analysis. By IFA, the protein was stained specifically and localized in cytosol of parasites, note that this mAb has no cross-reactivity to human red blood cells that correlated well with previous report using rabbit polyclonal antibody.[10] This study suggested that it is a high potential for using this obtained anti-pfGAPDH mAb for further development of dipstick to improve capacity of detection.

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