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การหาสภาวะที่เหมาะสมในการนำพลาสมิดเข้าสู่เซลล์เพื่อสร้างรีคอมบิแนนท์โมโนโคลนอลแอนติบอดีต่อเอนไซม์แลคเตทดีไฮโดรจีเนสของพลาสโมเดียมฟัลซิพารั่มในเซลล์สัตว์เลี้ยงลูกด้วยนม

ปนัดดา เทพอัคศร อภิชัย ประชาสุภาพ ดวงรัตน์ จุลอักษร อณิชา เลื่องชัยเชวง สกุลรัตน์ สุนทรฉัตราวัฒน์ และพรทิพย์ ไชยยะ

สถาบันชีววิทยาศาสตร์ทางการแพทย์ กรมวิทยาศาสตร์การแพทย์ ถนนติวานนท์ นนทบุรี 11000

บทคัดย่อ การศึกษาที่ผ่านมาคณะผู้วิจัยได้พัฒนาพลาสมิด pcDNA-pLDHAbHCγLCκ/C10 ที่บรรจุยีนอิมมูโนโกลบูลินที่จำเพาะต่อเอนไซม์แลคเตทดีไฮโดรจีเนสของ *Plasmodium falciparum* (pLDH) เพื่อใช้ในการสร้างรีคอมบิแนนท์โมโนโคลนอลแอนติบอดีต่อ pLDH การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาสภาวะที่เหมาะสมในการสร้างและผลิตรีคอมบิแนนท์แอนติบอดีต่อ pLDH ในเซลล์ 293F โดยศึกษาอัตราส่วนของพลาสมิดที่มีในท้องตลาด pEGFP-N1 ต่อปริมาตรน้ำยา transfection ที่นำพลาสมิดเข้าสู่เซลล์พบว่าอัตราส่วนระหว่างพลาสมิดดังกล่าวต่อน้ำยา transfection คือ 1 μg: 1.2 μl และเมื่อใช้อัตราส่วนดังกล่าวในการนำพลาสมิด pcDNA-pLDHAbHCγLCκ/C10 เข้าสู่เซลล์ 293F พบว่ามีการสร้างรีคอมบิแนนท์แอนติบอดีสูงสุดในวันที่ 4 หลังจากการ transfection เมื่อทดสอบโดยวิธี ELISA การศึกษาคุณสมบัติของรีคอมบิแนนท์โมโนโคลนอลแอนติบอดีที่ได้ โดย SDS-PAGE และ Western blotting พบว่า มีลักษณะไม่แตกต่างจากโมโนโคลนอลแอนติบอดีของหนูและพบว่ารีคอมบิแนนท์โมโนโคลนอลแอนติบอดีที่ได้มีความจำเพาะกับ pLDH ของเชื้อ *P. falciparum* ในเซลล์เม็ดเลือดแดงเมื่อทดสอบโดยวิธี IFA ดังนั้นรีคอมบิแนนท์โมโนโคลนอลแอนติบอดีที่สร้างได้นี้จึงมีความเป็นไปได้ที่จะนำไปพัฒนาสำหรับการตรวจวินิจฉัยโรคมาลาเรียที่เกิดจากเชื้อ *P. falciparum*

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Introduction

Antibody is a glycoprotein that is produced from B-lymphocytes in response to antigen, of which produced from a single clone of B-lymphocyte called monoclonal antibody (MAb). MAbs have been widely used in biomedicine especially for development of diagnostics and therapeutic agents.[1,2] Hybridoma technique, a method for development of hybridoma that produced MAb, was developed by Köhler and Milstein in 1975 by fusion of splenocytes which consist of various cell populations of lymphocytes including B lymphocytes from spleen of immunized mouse with immortal cancer cells.[3,4] The obtained hybridoma had both properties from mother cells: B lymphocytes and myeloma cells that could secrete antibodies and perform continuous growth, respectively.[5] The production of MAb from hybridoma cells can be performed by injection of hybridoma into the abdomen of a mouse called the ascites method, or culturing in flask or bioreactor as in vitro methods.[6] But concerning animal welfare, many countries in Europe and United States of America have banned or have laws and guidelines for limiting the ascites method of MAb production. Cultivation of hybridoma cells in flask or bioreactor was expensive and time-consuming.[7] Furthermore both methods were limited to no long term monoclonal antibody production because hybridoma cells were not stable.[8]

The recombinant DNA technology can be used to produce antibodies as recombinant monoclonal antibody especially in mammalian cells, by isolation of immunoglobulin gene from various sources of cells and cloning into expression vectors to express proteins in culture cells.[9,10,11,12,13] In previous study we have developed a recombinant plasmid containing immunoglobulin gene encoding monoclonal antibody against lactate dehydrogenase enzymes of *Plasmodium falciparum* (pLDH) for using in malarial diagnosis. In order to produce the recombinant monoclonal antibody by transient expression in mammalian cells, the optimal condition for protein expression should be determined. Therefore, this study was aimed to optimize primarily the transfection reagent used for transfection of the recombinant plasmid for expression of the MAb against pLDH in 293F cells modified from human embryonic kidney (HEK) 293 cell line that has been widely used for transient protein expression.[14,15] The obtained conditions was expected to use for production of recombinant monoclonal antibody against pLDH for further study in various malaria research and the experiments will be applied for optimization of expression of various proteins in mammalian cells.

Materials and Methods

1. Plasmids

1.1 pcDNA-pLDHAbHCγLCκ/C10

The plasmid pcDNA-pLDHAbHCγLCκ/C10 containing immunoglobulin heavy chain and light chain gene encoding monoclonal antibody against lactate dehydrogenase enzymes from *Plasmodium falciparum* (Medical Biotechnology Center, Medical Life Sciences Institute,

Department of Medical Sciences) was previously prepared, briefly, the variable region of heavy chain (V_H) and light chain (V_L) genes from scFv clone10 producing single chain variable fragment (scFv) antibody against pLDH were assembled together with mouse Cγ and Cκ for expression of recombinant monoclonal antibody against pLDH by blunt end cloning using restriction enzymes *HpaI* for V_H and *AfeI* for V_L, and then cloned into pcDNA3.4 containing Cγ and Cκ, respectively.

1.2 pEGFP-N1

pEGFP-N1 (Clonetech) encodes green fluorescence protein (GFP) with bright fluorescence and high expression in mammalian cells.[16, 17, 18]

2. Recombinant plasmodium lactate dehydrogenase enzymes antigen (pLDH)

The plasmid pET : LDH was previously prepared, briefly, the pLDH gene encoding for pLDH antigen of *P. falciparum* was amplified by PCR using specific primers to create restriction sites of *EcoRI* and *SalI*, at upsteam and downstream of the PCR product. After gel purification the PCR product was digested with *EcoRI* and *SalI* restriction enzymes and ligated with expression vector pET-21a (Novagen) which digested with the same restriction enzymes. The obtained recombinant plasmid was named pET : LDH.

Recombinant pLDH protein was prepared as previously described, briefly, the recombinant *E. coli* BL21(DE3) containing recombinant plasmids pET: pLDH were cultured in 100 ml LB medium and induced with 0.1 mM Isopropyl thiogalactoside (IPTG). Then cell pellets were harvested by centrifugation and sonicated in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl and 2.5 mM Immidazole, pH 8.0). After centrifugation the soluble protein pLDH in supernatant was purified using Ni-NTA resin (QIAGEN) according to manufacturer's protocol. The obtained purified protein was analyzed by SDS-PAGE and Western blotting and stored at −20°C in 50% glycerol.

3. Cell and cell culture media

The FreestyleTM 293F cells (Invitrogen™, Life Technologies) were propagated in disposable polycarbonate erlenmeyer flask with Freestyle™ 293 expression medium (Invitrogen™, Life Technologies). The viable cells were determined by Trypan blue exclusion method.[19] The cells were continuously cultured until the density of 1×10^6 viable cells/ml was reached, then the cells were subcultured to density of $1\text{-}2 \times 10^5$ viable cells/ml in 15 ml of fresh medium by incubating at 37°C and 8% CO_2 with orbital shaking at 140 rpm.

4. Parasites

The parasites, *Plasmodium falciparum strain* K1 were maintained by culture in erythrocyte (blood group O) at 1% hematocrit in 10 ml RPMI1640 medium with 25 mM HEPES, 2 mg/ml glucose, 5% sodium bicarbonate and 10% human serum (blood group AB). The culture was incubated in a humidified incubator at 37°C with gas controlled environment of 5% CO_2, 5% O_2 and 90% N_2 which was fed according to the established procedure.[20]

5. Optimization of pEGFP-N1: Transfection reagent ratio in 293 F cells

The 293F cells were cultured in 12 well plate at density of 1×10^6 cells/ml in 0.8 ml Freestyle™ 293 expression medium for 24 hours before transfection. Prior to transfection, the reagents were prepared as followings; 2 μg of pEGFP-N1 was diluted in Opti Pro SFM (Gibco, Life Technologies) to the total volume of 16 μl in 1.5 ml centrifuge tube for 6 tubes, and transfection reagent (Gen Carrier-1™, Epoch Life Science) was diluted individually in Opti Pro SFM at 1.2, 1.6, 2.0, 2.4, 2.8 and 3.2 μl to the total volume of 16 μl, respectively. After mixing both reagents together and incubating at room temperature for 20 minutes, the DNA-Transfection reagent complexes were added directly to each well, mixed gently and continued culturing with rotating at 140 rpm, 37°C and 8% CO_2 for 24 hours. The cell morphology and expression of GFP protein in each condition was observed under an inverted fluorescence microscope (EclipseTi-S, Nikon) at 24 hours post transfection.

6. Transfection of pcDNA-pLDHAbHCγLCκ/C10 into 293 F cells

293F cells were seeded into 30 ml of Freestyle™ 293 Expression medium with density of 6×10^5 - 7×10^5 cells/ml in 125 ml conical cell culture flask (Corning®, Sigma-Aldrich). The culture was incubated in a humidified incubator at 37°C and 8% CO_2 with orbital shaking at 140 rpm for 24 hours before transfection. On the day of transfection, the cells were counted to maintain the density of 1×10^6 cells/ml. To perform transfection using the obtained optimal condition of the plasmid DNA: Transfection reagent in 30 ml cell culture, 75 μg of recombinant plasmid pcDNA-pLDHAbHCγLCκ/C10 and 90 μl transfection reagent were each diluted in Opti Pro™ SFM to a total volume of 0.6 ml. Both reagents were mixed and incubated for 20 minutes at room temperature. 1.2 ml DNA-Transfection reagent complexes was added to the prepared cells dropwise and gently mixed the plate. Transfected cell culture was incubated at 37°C and 8% CO_2 with orbital shaking at 140 rpm for 7 days, using untransfected condition as negative control. The supernatant of transfected cell culture was collected daily at day 1-7 post transfection for ELISA assay. After that the culture supernatant were harvested for purification of recombinant monoclonal antibody.

7. Enzyme linked immunosorbent assay (ELISA)

Four hundred microliter of culture supernatant of day 1, 2, 3, 4, 5, 6 and 7 were collected from transfected cell culture and carried out to determine specific reactivity with recombinant pLDH protein by ELISA. Each 96 well plate was coated with 100 μl of carbonate bicarbonate buffer pH 9.6 containing 1 μg of recombinant pLDH protein at 4°C overnight. Then the plate was washed 3 times with 300 μl PBS and blocked with blocking buffer (PBS buffer containing 8% skim milk powder (Difco™, Becto Dickinson and Company) for 1 hour at room temperature. After washing, 100 μl of each sample was added to each well and incubated at room temperature for 1 hour. The plate was washed with PBST (PBS containing 0.05% Tween 20) for 3 times following with PBS 1 time then incubated with 100 μl of Rabbit anti-mouse immunoglobulins-HRP (Dako Cytomation) at dilution 1:2,000 for 1 hour. After washing with Phosphate Buffered Saline Tween-20 (PBST) for

4 times and 1 time with Phosphate Buffered Saline (PBS), 100 µl of TMB substrate solution (KPL, USA) was added to each well and incubated at room temperature for 30 minutes. After that 100 µl of 1 M HCL was added to stop the reaction and the plate was measured absorbance at 450 nm with reference absorbance at 630 nm.

8. Purification of recombinant protein

The recombinant monoclonal antibody was purified from the culture supernatant by Affinity chromatography using Protein A Sepharose™ Fast Flow(GE Healthcare Life sciences). The column was packed with 500 µl of Protein A Sepharose™ Fast Flow and equilibrated with 10 ml binding buffer (20 mM $NaHPO_4$ pH 7.0), then the culture supernatant containing recombinant antibody was applied through the column and washed the column with 10 ml of binding buffer. After that the bounded recombinant monoclonal antibody was eluted from the column by adding 1 ml of elution buffer (0.1 M Sodium citrate pH 3.0) following immediately with the addition of 100 µl neutralizing buffer(1M Tris-Cl pH 9.0). The purified recombinant monoclonal antibody was concentrated and changed the buffer to PBS pH 7.2 using an ultra-centrifugation filter of 10 kDa (Amicon, Merck Millipore). The purified protein was analyzed to determine the concentration using Nanodrop® spectrophotometer (ND-1000, Nano Drop Technologies, Inc.) and analyzed by SDS-PAGE and Western blotting against Rabbit anti-mouse IgG-HRP (Dako Cytomation), and then stored at -20°C until used.

9. Immunofluorescence assay (IFA)

Indirect immunofluorescence staining was carried out using purified recombinant monoclonal antibody against pLDH and mouse monoclonal antibody against pLDH (Arista BiologicalsInc, USA) as positive control. *P. falciparum* infected red blood cells from the cultures were harvested at 5% parasitemia, then the cells were smeared on microslide and fixed with cold 40% methanol in acetone at -80°C for 1 hour. Then 10 µl of 1mg/ml anti-pLDH mouse monoclonal antibody at dilution 1:100 or 1 mg/ml purified recombinant monoclonal antibody at the same dilution was added to the cells and incubated at room temperature for 45 minutes. The slide was washed 3 times with PBS and air dried. Ten microliter of Rabbit anti-mouse IgG (H + L) - FITC (Southern Biotech) at dilution 1:200 was added and the slide was incubated at room temperature for 30 minutes. The slide was then washed 3 times with PBS and air dried. After mounting with 10% glycerol in PBS and covering with a cover slip, the slide was observed under fluorescence microscope (EclipseNi, Nikon).

Results

1. Production of recombinant pLDH protein for ELISA

The recombinant plasmid pET: pLDH containing ORF of pLDH that was cloned inframe with 6 histidine residues (6 × His) was used for expression of pLDH-His fusion protein in *E. coli* BL21.

The obtained purified recombinant pLDH protein was shown the molecular weight of 33 kDa as analyzed by SDS-PAGE and Western blotting in (Figure 1).

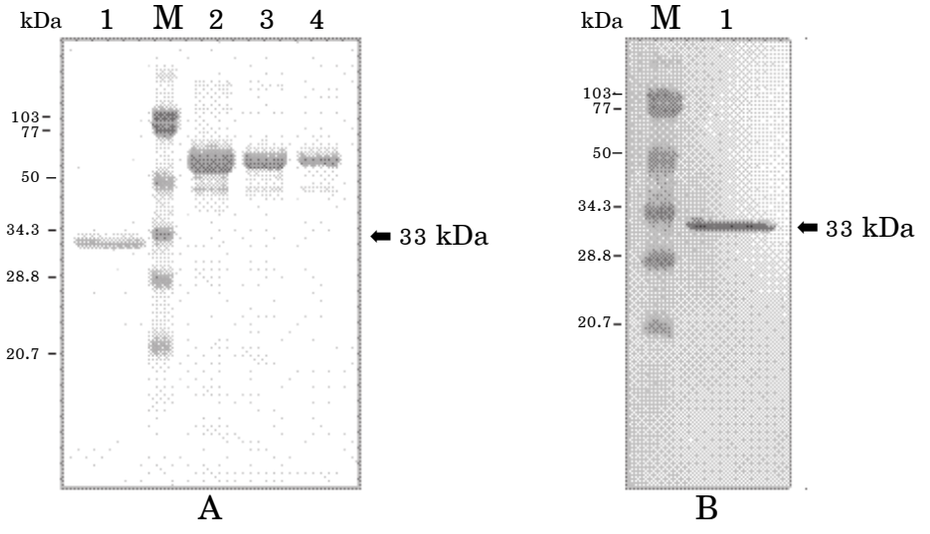

Figure 1 SDS-PAGE and Western blotting of purified recombinant pLDH protein
A: The affinity chromatography purified recombinant pLDH protein was analyzed by SDS-PAGE
B: Western blotting using anti-His monoclonal antibody
In A and B, lane 1 is purified recombinant pLDH protein and M is protein marker (Prestained SDS- PAGE standards Low Range, Bio-Rad) while lane 2-4 in A are bovine serum albumin standard at 20, 10 and 5 mg/ml, respectively.

2. Optimization of pEGFP-N1: Transfection reagent ratio for transfection into 293 F cells

To determine the optimal condition of the plasmid DNA to Transfection reagent ratio for transfection into 293F cells, various conditions of transfection were performed as shown in (Figure 2A) under inverted fluorescence microscope. The transfected cells in all conditions had normal morphology compared to non-transfected cells. The expression of GFP in transfected cells was observed under the same microscope and the fluorescence density was estimated by eye. As shown in (Figure 2B) the highest fluorescence density was visually detected at pEGFP-N1. Transfection reagentratio of 1μg: 1.2 μl. Noteworthy, the expression was not increased even the transfection reagent was increased up to 3.2μl as shown that the fluorescence density was not increased when using conditions of pEGFP-N1: Transfection reagent ratio 1 μg:1.4 μl. and 1μg : 1.6 μl.

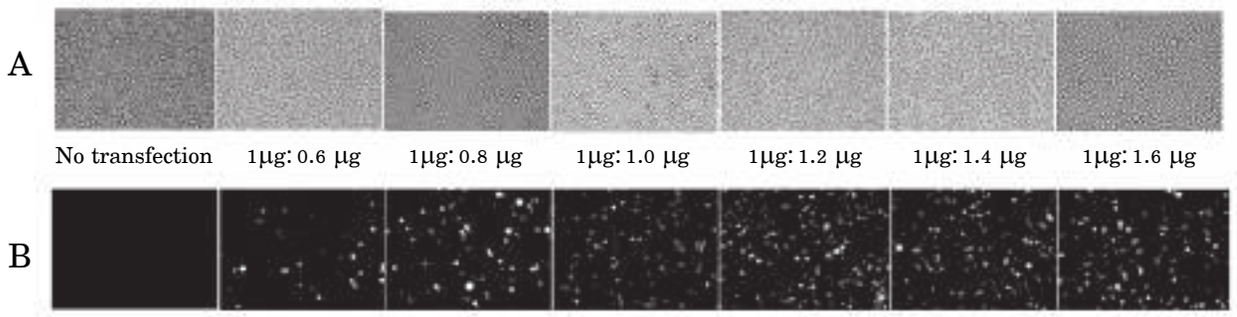

Figure 2 Optimization of pEGFP-N1: Transfection reagent ratio for transfection into 293F cells Various ratios of pEGFP-N1: Transfection reagent for transfection into 293F cells were determined. Cell morphology was observed under inverted fluorescence microscope at x20 magnification (A) and GFP expression was observed under the same microscope (B).

3. Determination of recombinant protein expression by ELISA

The reactivity of recombinant protein expressed by transfection of pcDNA-pLDHAbHCγLCκ/C10 into 293F cells ELISA was performed to determine the expression level of recombinant monoclonal antibody against pLDH in the supernatant of transfected cell cultures from day1-7 post transfection. The results showed that the expression of the recombinant monoclonal antibody was increased rapidly from day 0-4 and not increased more afterwards until day 7 of the study as shown in (Figure 3).

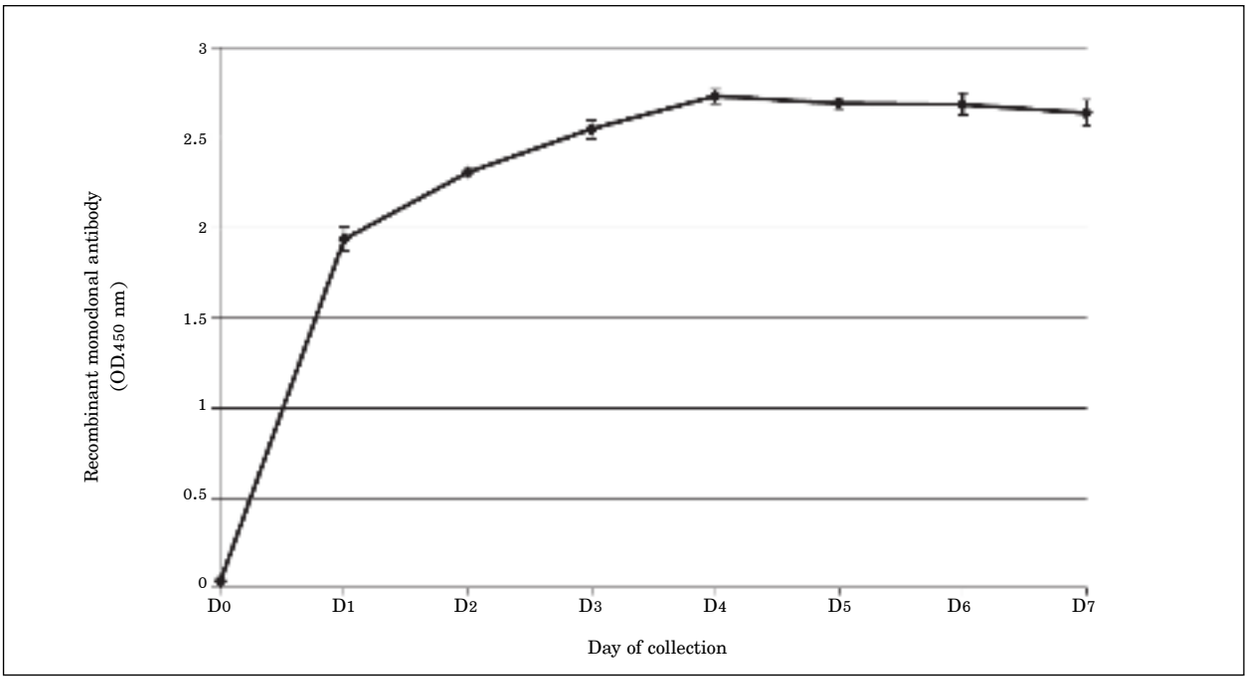

Figure 3 Determination of recombinant monoclonal antibody against *P. falciparum* LDH expression by ELISA

The supernatant of 293F transfected cell cultures transfected with pcDNA-pLDHAbHCγLCκ/C10: Transfection reagent ratio at 1 µg: 1.2 µl from day 1-7 post transfection were collected and determined the expressed recombinant monoclonal antibody against *P. falciparum* LDH by ELISA at absorbance 450 nm using culture medium before transfection (D0) as negative control.

4. Analysis of purified recombinant protein by SDS PAGE and Western-blot

The recombinant plasmid pcDNA-pLDHAbHCγLCκ/C10 containing immunoglobulin heavy chain and light chain genes encoding monoclonal antibody against pLDH was transfected into 293F cells to produce recombinant monoclonal antibody by using the obtained optimal condition. After 7 days of transfection the culture supernatant was collected and purified by Affiniy Chromatography. The obtained purified protein was analyzed by SDS-PAGE and Western blotting using mouse monoclonal antibody against pLDH as positive control. As shown in (Figure 4A) the recombinant monoclonal antibody and positive control had the same patterns with two major bands of 25 kDa for light chain and 50 kDa for heavy chain, respectively. The result of Western blotting in (Figure 4B) showed that the recombinant monoclonal antibody had specific reactivity to Rabbit anti- mouse IgG as same as the positive control.

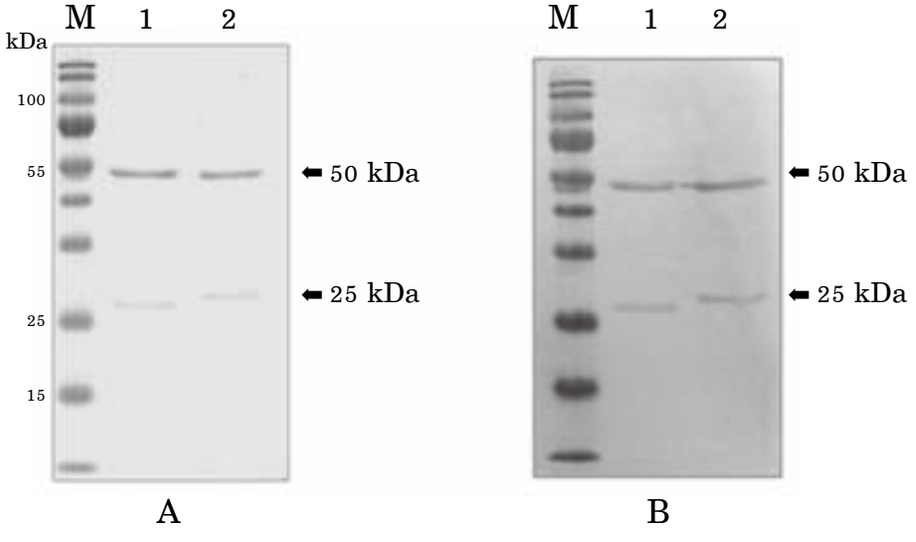

Figure 4 SDS-PAGE and Western blotting of purified recombinant protein against *P. falciparum* LDH

The purified recombinant protein was analyzed by SDS-PAGE (A) and by Western-blot (B). Lane 1 is mouse monoclonal antibody against pLDH (positive control), lane 2 is purified recombinant monoclonal antibody against pLDH, which M is protein marker (PageRuler™ prestained protein ladder, Thermo Scientific).

5. Determination of specific reactivity of recombinant protein by IFA

To determine recombinant protein antigen in *P. falciparum infected* cells, the IFA was performed while the mouse monoclonal antibody against pLDH was used as positive control. As shown in (Figure 5) when the infected cells were stained with purified recombinant monoclonal antibody against pLDH produced, the specific immunofluorescence was observed under fluorescence microscope (D) that had the same pattern as of that stained with positive control (B). Whereas, there was no specific signal observed in non-infected cells that were stained with positive control (A) and recombinant monoclonal antibody against pLDH (C), respectively.

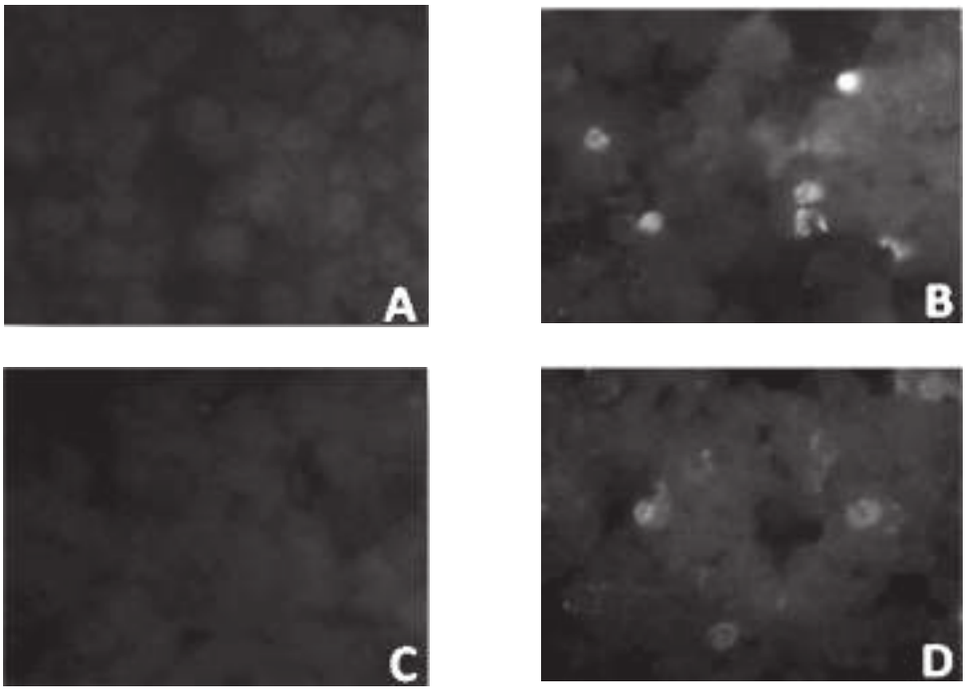

Figure 5 Determination of specific reactivity of recombinant monoclonal antibody produced against *P. falciparum* LDH to *P. falciparum* infected red blood cells by IFA

Non infected and *P. falciparu*m infected red blood cells were stained with mouse monoclonal antibody against pLDH (positive control) in A and B and stained with purified recombinant monoclonal antibody produced against pLDH in C and D, respectively. Specific immunofluorescene was observed under fluorescence microscope at 100x magnification.

A: Non infected red blood cells stained with mouse monoclonal antibody against pLDH

B: *P. falciparum* infected red blood cells stained with mouse monoclonal antibody against pLDH (positive control)

C: Non infected red blood cell stained with purified recombinant monoclonal antibody against *P. falciparu*m LDH

D: *P. falciparum* infected red blood cells stained with purified recombinant monoclonal antibody against *P. falciparum* LDH

Discussion

In this study the plasmid pcDNA-pLDHAbHCγLCκ/C10 containing immunoglobulin heavy chain and light chain genes was used for expression of recombinant monoclonal antibody against *Plasmodium falciparum* LDH (pLDH). The optimal protein expression was developed by optimizing transfection condition using pEGFP-N1 containing reporter gene expressed GFP protein of which fluorescence signal in living cells can be visually detected and had been reported as the advantage of this protein by Chalfie *et al.*[21]

The results showed that with various conditions of different volumes of the transfection reagent studied (1.2-3.2 µl/0.8 × 10^5 293F cells) the morphology of cells was normal as compared to non-transfected cells at 24 hours post transfection. Khodthong *et al* also studied in Hela cells and

found that toxicity of transfection reagent to the cells could be detected at 24 hours post transfection, whereas at 8 hours post transfection morphology of cells was normal.[22] These suggested that there was no toxicity to the cells due to these volumes.

In this study the obtained optimal condition for transfection into 293F cells was the ratio of pEGFP-N1 to Transfection reagent at 1 μg : 1.2 μl, whichwas used to scale up for expressing recombinant monoclonal antibody against pLDH using pcDNA-pLDHAbHCγLCκ/C10 in 293F cells and the optimal time was determined during day 1-7 post transfection. The recombinant monoclonal antibody in supernatant of transfected cell cultures from day 1-7 were determined for their specific reactivity to pLDH by ELISA. The recombinant monoclonal antibody could be detected from day 1 and increased rapidly to day 4 which showed the same pattern of recombinant monoclonal antibodies expression in HEK 293E cells using standard protocol transfection.[23] From day 5, the level of recombinant monoclonal antibody was not increased, it is assumed that plasmids were too diluted due to environmental factors and cell division that leaded to decrease of expression.[24]

As the purified monoclonal antibody was analyzed by SDS-PAGE and Western blotting which the results revealed the specific light chain and heavy chain approximately at 25 and 50 kDa[25], respectively, the molecular weight of light chain of recombinant monoclonal antibody was found slightly higher than those of mouse monoclonal antibody against pLDH, it might due to difference of amino acid sequence of both monoclonal antibodies. Furthermore, the recombinant monoclonal antibody could react specifically with pLDH antigen in *P. falciparum* infected cells by IFA suggested that it could detect pLDH antigen in native form. From the study, the recombinant monoclonal antibody against pLDH produced by the optimal condition developed, had potential to use further in development for *P. falciparum* malarial diagnosis.

Conclusion

In this study we obtained the optimal condition of the ratio of pcDNA-pLDHAbHCγLCκ/C10 to transfection reagent as 1 μg:1.2 μl for transfection into 293F cells to express monoclonal antibody against *Plasmodium falciparum* lactate dedyhrogenase and the highest amount was obtained by day 4 post transfection. The affinity chromatography purified recombinant monoclonal antibodyhad specific reactivity with pLDH antigen in *P. falciparum* infected cells by IFA.

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Transfection Optimization for Expression of the Recombinant Monoclonal Antibody against *Plasmodium falciparum* Lactate Dehydrogenase in Mammalian Cells

Panadda Dhepakson Apichai Prachasuphap Duangrat Jullaksorn Anicha Luengchaichaweng Sakulrat Soonthornchattrawat and Porntip Chaiya
Medical Life Sciences Institute, Department of Medical Sciences. Tiwanond Road, Nonthaburi 11000 Thailand.

ABSTRACT Recombinant plasmid pcDNA-pLDHAbHCγLCκ/C10 containing immunoglobulin gene encoding monoclonal antibody against lactate dehydrogenase enzyme of *Plasmodium falciparum* (pLDH) has been developed in our previous study. This study was aimed to optimize the condition of transfection reagent for expression of the recombinant monoclonal antibody against pLDH in 293F cells. By optimizing transfection condition for a commercial pEGFP-N1 plasmid to transfection reagent, the optimal ratiowas at 1 μg:1.2 μl in 293F cells. By this ratio, the plasmid pcDNA-pLDHAbHCγLCκ/C10 was also transfected into 293F cells to express the recombinant monoclonal antibody and maximal level was found in culture supernatant at day 4 as determined by ELISA. The recombinant monoclonal antibody protein pattern was similar to that of the mouse monoclonal antibody against pLDH as analyzed by SDS-PAGE and Western blotting. Furthermore, this monoclonal antibody could react specifically to pLDH antigen in *P. falciparum* infected cells by IFA. Thus, the recombinant monoclonal antibody had potential in development for *P. faciparum* malarial diagnosis.

Key words: Recombinant *Plasmodium falciparun* monoclonal antibody, 293F cells, transfection, *Plasmodium falciparum* lactate dehydrogenase