Research Article

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Cloning, expression, and functional assay of monoclonal antibody fab fragment against merozoite surface protein 1 (msp1) of *Plasmodium falciparum*.

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The cloning of antibody-encoding genes is a pivotal role for generation antibody variants with a higher affinity and a lower immunogenicity. This study was successfully cloned a fab fragment of monoclonal antibody against Merozoite Surface Protein 1 (MSP1) of *Plasmodium falciparum* from antibody-producing hybridoma and expressed in *E. coli* cell-free expression system. ELISA result showed that the generated antibody could bind with their antigen ($MSP1_{19}$) in specifically manner. Addition of DsbC, periplasmic disulfide bond isomerase, gave a higher ELISA signal, indicating that the addition of DsbC accelerated disulfide isomerization in the expression system resulted in proper folding of the generated antibody. The sequence analysis of the fab fragment genes consists of 663 nucleotide of Light Chain (LC) and 645 nucleotides of Heavy Chain (HC). The final products of this research are not only active antibodies but also the antibodies-encoding gene, which allows further engineering to produce the antibody variants with desired properties and applications.

Key words: ***Monoclonal antibody, Fab fragment, Merozoite Surface Protein 1 (MSP1), Plasmodium falciparum*.**

Malaria remains to be the most dangerous diseases in tropical and subtropical countries. World Health Organization (2008) reported that 3.3 billion people spread in 109 countries were infected and at risky condition due to the malarial disease, of which nearly 881,000 were reported death in 2008. Some papers revealed that *Plasmodium falciparum* was one of four distinct parasitic agents which cause malarial disease. The use of chemotherapy such as antimalarial drug to treat the parasite even leads to worse problem for instance creating antibiotic-resistance parasites (Ali *et al*., 2013). In this context, an alternative approach such as passive immunotherapy using specific antibodies is urgently needed.

Good *et al*., (1998) described that merozoite surface protein 1 (MSP1) was the most abundant protein on the surface of *P. falciparum.* Many studies also concluded that this protein played major role in blood cell invasion. Due to such important role, some researchers could derive antibodies from human sera which targeted the protein during blood cell infection (Good *et al*., 1998; deKoning-Ward *et al*., 2003; Egan *et al*., 1999; John *et al*., 2004; O'Donnell *et al*., 2001). Further study by John *et al*. (2004) concluded that $MSP1_{19}$ protein, one of MSP1 derivatives during parasitic invasion, has been associated with human resistance to the disease reinfection. The protective effects from $MSP1_{19}$ –targeting antibodies are postulated to occur by inhibiting the proteolytic cleavage of MSP1 needed to expose the 19kDa fragment and/or by blocking the $MSP1_{19}$kDa from binding to erythrocytes.

Immuno-epidemiologic studies suggest that $MSP1_{19}$ IgG responses are associated with reduced clinical symptoms, reduced parasitemia, and reduced risk of subsequent infection (Dodoo *et al*., 2008).

So far, MSP1-targeting antibodies have been generated using hybridoma technology. However, the technology has several drawbacks, especially contamination and mutation which could reduce the affinity and specificity of the antibody, cloning of the antibody-encoding gene is necessary (Ali *et al*., 2006). To avoid those weak spots, studies which cloned the specific antibody-encoding genes might be able to produce antibody variants with a lower immunogenicity (Coronella *et al*., 2000), a higher affinity (Rajval *et al*., 2005), an enhanced stability (Sturbe and Chen, 2004), or a modified utility (Maynard *et al*., 2002). Therefore, this study cloned Fab fragment of monoclonal antibody against MSP1 from anti-MSP1 monoclonal antibody-producing hybridoma. The obtained genes were subsequently used to generate cell-free expression cassettes, which then used for the antibody synthesis in cell-free expression system. Results of this experiment indicated that the antibody generated from the cassettes expression could recognize their antigen ($MSP1_{19}$) in specifically manner. Moreover, inclusion of DsbC gave a higher ELISA signal, indicating that the addition of the periplasmic disulfide bond isomerase accelerated disulfide isomerization in the expression system resulted in proper folding of the generated antibody. The results of this research are not only active antibodies but also the antibodies-encoding gene, which is valuable for further engineering to generate antibody variants with desired properties and applications.

MATERIALS AND METHODS

Synthesis of Fab Fragments

cDNA was synthesized from the MSP_1 monoclonal antibody-producing hybridoma kindly provided by Professor Hiroyuki Matsuoka (Jichi Medical School, Tochigi, Japan). The hybridoma cells were counted under a microscope and were diluted into one cell per well. Plates were sealed with Sealing Film (Takara Bio Inc., Japan) and immediately used for cDNA synthesis or stored at -80°C for later use. Reverse transcription was performed using SS III First-strand Synthesis System (Invitrogen, CA,USA) and subsequent PCR amplification of Light Chain (Lc) and Heavy Chain (Hc) were carried out using primers as described previously (Sabrina *et al*., 2010). To provide *in vitro* expression cassette, the T7 promoter and T7 terminator fragments were amplified from pRSET-B vector (Invitrogen, USA) and continued for overlapping PCR as described (Ali *et al*., 2005).

Generation of Monoclonal Antibody

The cell-free expression was carried out as described in previous research (Ali *et al*., 2005) with small modifications: 3 μl of each expression cassettes was transcribed and translated in 30 μl of mixture (56.4 mM Tris-acetate, pH 7.4, 1.22 mM ATP, 0.85 mM each of GTP, CTP and UTP, 50 mM creatine phosphate, 0.5 mM each of all 20 unlabeled amino acids, 4% polyethyl glycerol 6000, 34.6 μg/ml folinic acid, 0.17 mg/ml *E. coli* tRNAs, 35.9 mM ammonium acetate, 10 mM $Mg(OAc)_2$, 100 mM KOAc, 10 μg/ml rifampicin, 0.15 mg/ml creatine kinase, 7.6 μg/ml T7 RNA polymerase, 28.3% [v/v] S30 extract), 0,1 mM GSH, 1 mM GSSG , 36 μg/ml Protein disulfide-isomerase (PDI) or DsbC). At the same time, the reaction mixture without expression cassettes was used for control reactions. The cell free reaction mixture were incubated at 30°C for 60 minutes, and directly put on ice to stop the reaction. The activity of the Fab fragments was examined using ELISA against $MSP1_{19}$ antigen.

Analysis of Fab Sequences

The sequence of selected clone from this experiment was analyzed. The germline gene was determined using the IgBLAST database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/igblast).

Identification of complementary determining regions (CDRs) of the gene was performed by examining the sequence based on the Kabat definition (Martin *et al*., 2001).

RESULTS

Fab Fragments

In this experiment, the first strand cDNA from a hybridoma cell was synthesized using SS III First-Strand Synthesis System. The resulting cDNA were subsequently used as templates for first round PCR to obtain Lc and Hc gene separately using the established PCR condition mentioned in Material and Method.

The results of PCR amplification were shown in Figure 1.

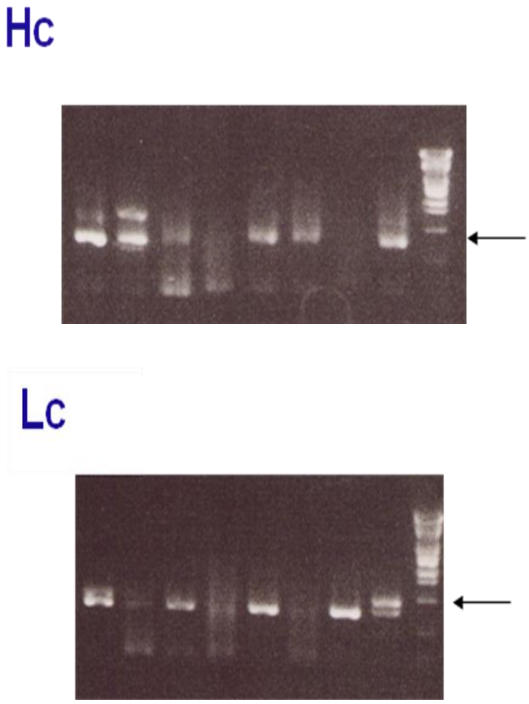


Figure 1. Agarose gel electrophoresis analysis of RT-PCR product originated from single hybridoma cell. The results of the amplification of the Lc (light chain) and Hc (heavy chain) genes are partly shown.

Anti-MSP1$_{19}$ Monoclonal Antibody

The amplified Lc or Hc was joined by overlapping PCR technique with the DNA cassettes for the T7 promoter (PT7) containing Shine Dalgarno sequence and T7 terminator (T7T). The genes construct (PT7-Lc-T7T and PT7-Hc-T7T) now ready for directly expressed in cell free protein synthesis. The overlapping PCR results of Lc and Hc (expression cassettes) were shown in Figure 2.

To produce naturally correct pairing of antibody genes, the overlapping product of pairing Lc and Hc were placed into the same reaction tube, transcribed and translated in a cell-free protein synthesis at 30° C for 1 hour. We added DsBc in cell free protein synthesis in order to improve folding of Fab fragment.

In this study indirect ELISA was performed using *in vitro* synthesized Fab fragments against the antigen to determine the Fab fragment affinity for antigen. ELISA against MSP1$_{19}$ protein was conducted and the result was shown in Figure 3. This result strongly suggests that the synthesized Fab was capable of binding the MSP1$_{19}$ protein.

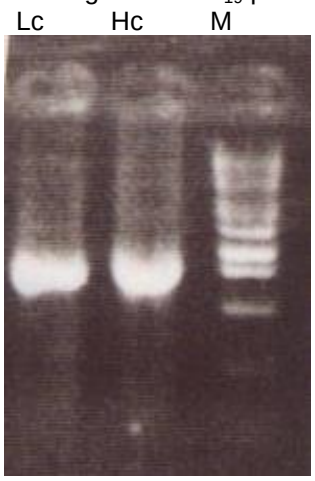


Figure 2. Agarose gel electrophoresis analysis of overlapping PCR products originated from single hybridoma cell, Lc = light chain, Hc = heavy chain, M = marker.

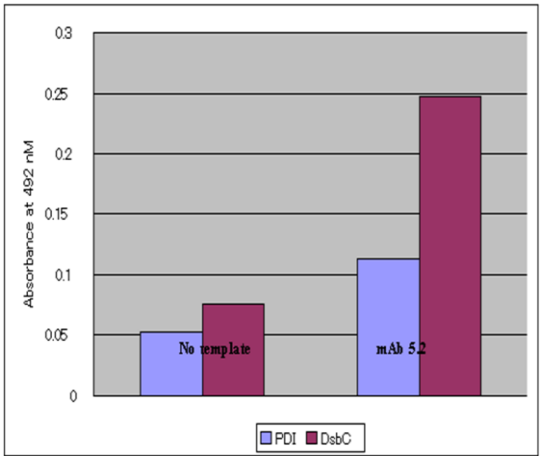


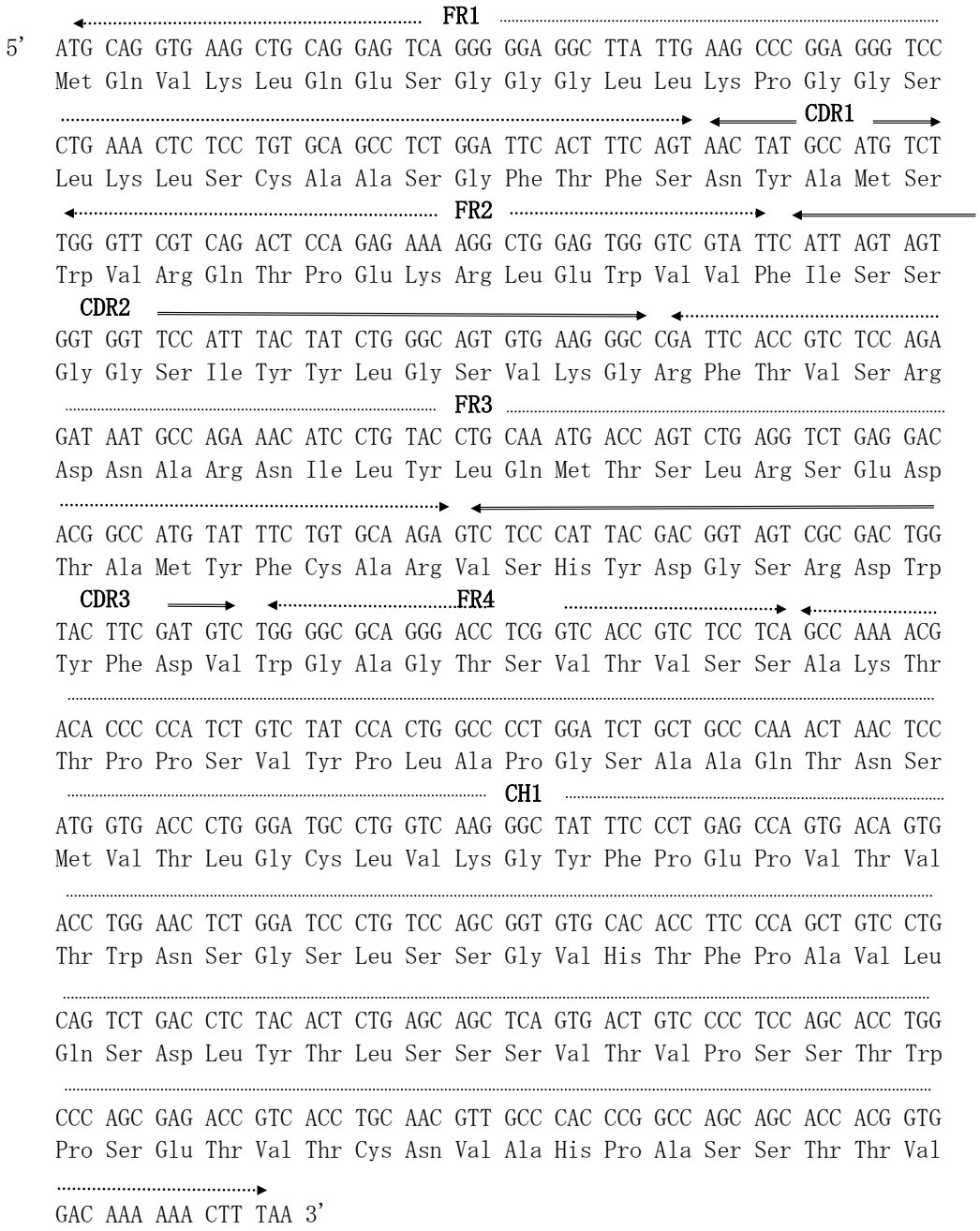
Figure 3. ELISA against MSP1 protein of Fab from single hybridoma cells and control with no template. The Fab was synthesized in an *E.* cell free protein synthesis system with addition of PDI or DsbC. Samples were taken at 60 min after the start of incubation.

Fab Sequences

The Lc and Hc gene were cloned into pGEMT vector and followed by sequencing. Results of sequencing were shown in Figure 4 and 5. The sequence analysis shows that the Lc sequence consists of 663 nucleotides sequence encoding 221 amino acid residues. The Hc sequence consists of 645 nucleotides sequence encoding 215 amino acid residues.

Figure 4. Sequences of Lc obtained from single hybridoma cells

Figure 5. Sequences of Hc obtained from single hybridoma cells



DISCUSSION

The specific amplification of Lc and Hc genes is important point in cloning of immunoglobulin genes from the cells. This is due to the fact that the immunoglobulin genes are highly diverse in their amino acid composition and nucleotide sequence (Coronella *et al*., 2000; Dohmen *et al*., 2005). Several important factors for successfulness of amplification are the initial amount of template, primer design,

and also the condition of RT-PCR.

Generation of Fab has several advantages over either the whole antibody or scFv format, for example, light chain (Lc) and heavy chain (Hc) of Fab are covalently linked by a disulphide bridge, which give native structure, proper folding, a higher binding affinity, and longer term storage. In comparison, scFv often forms unpredictable multimers, can be digested by proteases, and some scFvs show a reduced affinity of up to one order of magnitude compared to the corresponding Fab fragments. Moreover, construction of Fab in recombinant plasmid is simpler and faster than construction of scFv.

The framework 1 (FR1) regions from VL and VH were chosen as target sequences for forward primers in the amplification of variable regions. It is because the reverse primers anneal to the highly conserved C-terminal sequences of the constant region, minimizing the mismatch bias during PCR amplification (Coronella *et al*., 2000). The primers were also designed to have a homotag sequence at both ends as described (Ali *et al*., 2006). Using these primers, a PCR product with homo-priming sequences at both ends would be produced, and this would subsequently facilitate further amplification using a homotag-specific primer.

The use of SS III First-strand system for cDNAs synthesis has several advantages over the SS II, since the SS III provide higher specificity, higher yields of cDNA and also can generate longer cDNA. Since hybridoma was used as template in this experiment, the amplification was likely easier compare with using plasma or B cells as template. Because the concentration of mRNA in hybridoma is more higher than mRNA concentration in plasma or B cells

ELISA result shows that the Fab produced in cell free protein synthesis could bind with their antigen ($MSP1_{19}$ protein) in specifically manner as shown in Fig. 3. Furthermore by adding DsbC in cell free mixture the ELISA signal was higher compare without additional DsbC. Since DsbC is a periplasmic disulfide bond isomerase, the addition of DsbC will accelerate disulfide isomerization in cell-free protein synthesis system resulted in proper folding of the protein (Sturbe and Chen, 2004).

The sequence analysis of the Lc sequence consists of 663 nucleotides sequence encoding 221 amino acid residues containing four framework regions (FRs) and three complementarity determining regions (CDRs). The Lc sequence was found to be 98.3 % identical to the 8-21 germline gene (GenBank accession no. **Y15982**). The Hc sequence, which consists of 645 nucleotides sequence encoding 215 amino acid residues, containing four framework regions (FRs) and three complementarity determining regions (CDRs). The Hc sequence was found to be 92.7 % identical to VH7183.a37.59 germline gene (GenBank accession no. **AJ851868**). Results of CDR identification indicated that the CDR-L and CDR-H sequence had consistent characteristic with the CDRs sequence in the Kabat definition with a small difference (Maynard *et al*., 2002).

In conclusion, cloning and expression of gene coding for monoclonal antibody fragment against MSP1 of *P. falciparum* were successfully performed to produce not only the active antibodies but also the antibody-encoding gene. Furthermore, the obtained gene will allows further engineering to produce the antibody variants with higher affinity or desired properties and applications.

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