Parasitology International 72 (2019) 101938

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Sero-diagnostic potential of *Plasmodium falciparum* recombinant merozoite surface protein (MSP)-3 expressed in silkworm

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ARTICLE INFO

Keywords:
Merozoites surface protein 3
Plasmodium falciparum
Silkworm
Sero-diagnostic

ABSTRACT

Plasmodium falciparum is a blood protozoan parasite, transmitted by *Anopheles mosquitoes* vectors, that can cause morbidity and even leads to mortality in tropical countries. Strategies are directed to combat malaria including development of diagnostic tools, serological markers and vaccinations. A target under intensive studies is Merozoite Surface Protein (MSP)-3. The aim of this study is to express and purify recombinant MSP3 of *P. falciparum* (rPfMSP3) using silkworm expression system as a host for its large-scale production and to investigate its potential effectiveness for sero-diagnosis. The rPfMSP3 formed oligomers in a blue-native PAGE and its *N*-glycosylation was confirmed by periodic acid-Schiff staining and PNGase F treatment. The amyloid-like morphology of the rPfMSP3 oligomers was observed. Enzyme-linked immunosorbent assay showed that 60–70% of human samples from subjects living in malaria endemic areas in Indonesia detected the rPfMSP3. Western blot results showed that the rPfMSP3 was recognized by a malaria infected human serum but not by an uninfected human serum. The rPfMSP3 was successfully expressed in silkworm as a soluble protein and has the potential to be used in serological measurement for detecting PfMSP3-specific antibodies in sera from individuals living in endemic areas.

1. Introduction

Malaria is one of infectious diseases in tropical countries that leads to fatality. Malaria vaccines become important since antimalarial drugs in many parts of the world are faced to some resistance issues. Some vaccines are expected to generate specific antibodies that prevent *Plasmodium* parasite invasion of the red blood cells. Pre-erythrocytic and erythrocytic stage targeting malaria vaccines i.e. circumsporozoite protein (CSP), apical membrane antigen-1 (AMA-1), and merozoite surface protein 3 (MSP3) have been studied, and now are undergoing clinical trial [1]. To evaluate the antibody generated after MSP3 vaccination, availability of *P. falciparum* MSP3 recombinant protein is necessary. Besides, the PfMSP3 recombinant protein produced could be used as sero-diagnostic marker to assess malaria transmission in area that entering pre-elimination phase [2].

MSP3 is a soluble protein and expressed on surface of its merozoites by forming a protein complex with MSP1, which is linked to the surface of merozoites by a glycosylphosphatidylinositol anchor. MSP3 function is unclear, many hypothesize that it binds to receptors during invasions and has an ability to form extended oligomers. The MSP3 has three significant regions, alanine heptad repeated region, glutamic acid-rich region and leucine zipper region. The leucine zipper region contributes to the formation of its oligomers together with ^{192}YILGW^{196} sequence [3]. MSP3-based vaccines induce the protective immunity to *P. falciparum* [4,5]. MSP3-derived long synthetic peptide showed the significant protective efficacy in the Phase 1b trial [6]. In addition, MSP3 fused with glutamate-rich protein was also investigated, but its vaccine efficacy was low [7]. However, MSP3 was found as a vaccine candidate by wheat germ cell-free system-based immuno profiling even in low endemic area [8].

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https://doi.org/10.1016/j.parint.2019.101938
Received 5 April 2019; Received in revised form 31 May 2019; Accepted 11 June 2019
Available online 12 June 2019
1383-5769/

It was previously reported that MSP3 expressed in *Escherichia coli* formed amyloid-like fibrils in the same manner as native MSP3 on the surface of merozoites [3]. In addition, its recombinant MSP3 has heme-binding activity. Widely established *E. coli*-based expression approach provides high-yield heterologous protein production and at relatively low production cost. However, in this way, almost of proteins from eukaryotes are expressed insoluble form due to lack of proper post-translational modification. Insect-based systems including silkworm (*Bombyx mori*) offer a distinct advantage because of post-translational modification capability including glycosylation, phosphorylation and disulfide bond formation. In addition, protein expression levels in silkworm are relatively high. The recent development of a *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid [9], an *E. coli* and *B. mori* hybrid shuttle vector, enables to express proteins in silkworm pupae or larvae, reducing the time and eliminating the laborious steps of gene maniplulation [9,10].

Our study aims to express MSP3 of *P. falciparum* (PfMSP3) in silkworm larvae and purify the recombinant antigens from silkworm larval hemolymph for sero-diagnosis. The purified recombinant PfMSP3 (rPfMSP3) was characterized and its antigenicity was evaluated with enzyme-linked immunoassay (ELISA) and western blot approach using human sera from *P. falciparum*-infected and non-infected human to explore the possibility of the use of the rPfMSP3 as a serological marker of *P. falciparum*.

2. Materials and methods

2.1. Construction of recombinant bacmid

The MSP3 (25–354 aa from the ORF) cDNA was PCR amplified using polymerase KOD plus NEO (TOYOBO, Tokyo, Japan) with primers as shown in Table 1 from pGEM-pf3D7-XM001347593-MSP3 (Sino Biological, Beijing, China) as per standard protocol for the polymerase enzyme. The amplified cDNA was confirmed by agarose gel electrophoresis and purified by FastGene Gel/PCR Extraction kit (Nippon Genetics, Tokyo, Japan). The purified cDNA was cloned into pFastBac1 (Thermo Fisher Scientific K. K, Tokyo, Japan) at *Eco*R I and *Bam*H I restriction enzyme sites using DNA Ligation Kit Mighty Mix (Takara Bio Inc., Kusatsu, Shiga, Japan) as per kit protocol. Recombinant pFastBac1/rPfMSP3 was screened and used to make recombinant *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid as per standard protocol published earlier [9,11,12]. Subsequently the screened recombinant bacmid was sequenced (FASMAC, Atsugi, Japan).

2.2. Silkworm rearing and injection of bacmid

Fifth instars larvae (Ehime Sansyu Co. Ltd., Ehime, Japan) were reared on an artificial diet Silkmate S2 (Nihon Corp., Yokohama, Japan) and reared as reported previously [13]. Each silkworm injected with 50 µl recombinant bacmid DNA solutions containing 20 µg of BmNPV/rPfMSP3 bacmid DNA and 0.1% chitosan respectively in 2-(N-morpholino) ethanesulfonic acid buffer as per protocol published earlier [13]. At post injection 7th day, the silkworm larval hemolymph was harvested in tubes (Falcon, Lincoln Park, NJ, USA) containing 2 mM phenyl thiourea to inhibit the hemolymph melanization. These samples were then aliquoted and stored at −80 °C. Fat body collected from the above silkworm (approximately 0.1 g) was dissolved in 1 mL TBS buffer containing 0.1% (*v*/v) Triton X-100 and sonicated for 30 s using a pulse Sonicator (VC-130 PB, Sonics & Materials Inc., Newtown, CT, USA). The mixture was centrifuged at 10,000*g* at 4 °C for 10 min to separate supernatant and pellet respectively.

2.3. Purification of rPfMSP3

Hemolymph diluted with PBS (pH 7.4) by 2-fold was treated with 40% (saturation) ammonium sulfate (FUJIFILM Wako Pure Chem. Corp., Osaka, Japan) at 4 °C overnight. The hemolymph was centrifuged at 12,000 *g* and the supernatant applied to HiTrap Phenyl FF column chromatography (GE Healthcare Japan, Tokyo, Japan). The column was washed by PBS containing 8% (saturation) ammonium sulfate and proteins were eluted by PBS. Eluted rPfMSP3 was applied to DDDDK-tagged protein purification gel column chromatography (Medical & Biological Laboratories, Nagoya, Japan). The rPfMSP3 was eluted by glycine-HCl (pH 2.3) and immediately neutralized by 1 M Tris-HCl (pH 8.0).

2.4. Western blot and sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE)

Collected hemolymph and fat body were denatured and loaded on SDS-PAGE in 10% (*w*/*v*) acrylamide at 70 V. The SDS gels were subsequently trans-blotted on to Immobilon-P (Merck, Tokyo, Japan), blocked and probed with anti-DDDDK tag (Medical & Biological Laboratories, Japan) at 1:10,000 dilution in tris-buffered saline with tween-20 (TBS-T) for 1 h at room temperature as reported previously [13]. Subsequently washing was done and probed with secondary anti-mouse IgG-horseradish peroxidase (HRP) (GE Healthcare Japan). Then the membrane was washed and probed as reported previously [13].

2.5. Blue native poly-acrylamide gel electrophoresis (PAGE)

A 3–10% gradient polyacrylamide gel (ATTO, Tokyo, Japan) was prepared with reagents mentioned above without containing SDS. The cathode buffer (100 mM L-histidine, 0.02% Coomassie blue G-250, pH 8.0) and anode buffer (100 mM Tris-HCl, pH 8.0) was used to run the gel at 70 V at room temperature.

2.6. Glycan staining and PNGase F treatment

A 10% (*w*/*v*) SDS-PAGE gel was loaded with rPfMSP3 and electrophoresis performed as mentioned above. After electrophoresis the gel was treated as per protocol for Pierce glycoprotein staining (Thermo Fisher Scientific K.K.). In addition, denatured rPfMSP3 was treated as per protocol for PNGase F treatment kit (Takara Bio) according to the manufacturer's protocol. Treated proteins were applied to SDS-PAGE and detected by western blot.

2.7. Congo red assay and transmission electron microscopy (TEM)

Congo red solution (2.5 µM) was mixed with purified rPfMSP3 (200 µg/ml in PBS pH 7.5) in 1:1 ratio with a final volume of 200 µl and

Table 1
Primers used in this study.

Primer	Sequence
Forward primer	GGCGGATCCATGAAGATACTCCTTGCTATTGCATTAATGTTGTCAACAGTAATGTGGGTGTCAACACAAAAAGAAATTGTAAAAAAATATAATC
Reverse primer	GGCGAATTCTTACTTGTCGTCATCGTCTTTGTAGTCATGATTTTTAAAATATTTGGA

Forward primer underlined region is the bombyxin signal sequence followed by MSP3 sequence. Reverse primer underlined region is the DYKDDDDK affinity tag nucleotide sequence.

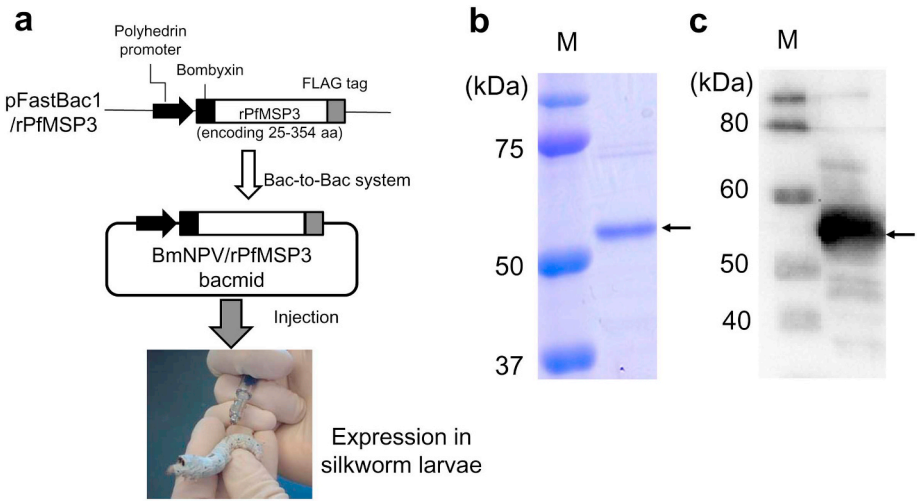

Fig. 1. Purification of rPfMSP3 from silkworm hemolymph. The rPfMSP3 from hemolymph was purified by ammonium sulfate precipitation and two-column chromatography. (a) Schematic representation of recombinant rPfMSP3. At N-terminal bombyxin signal (black shaded) for secretion of recombinant protein flagged by DYDDDDK tag (gray shaded) for affinity purification. (b) rPfMSP3 was detected by CBB staining in 10% (*w/v*) SDS-PAGE. (c) The western blot of purified rPfMSP3 using mouse anti-DDDDK tag antibody as a primary antibody. M in (b) and (c) denotes Magic XP (Thermo fisher scientific, Japan) protein marker. Black arrows indicate bands of interest rPfMSP3.

incubated for 5 min at room temperature. As a negative control Congo red solution only and with bovine serum albumin (BSA) was performed as mentioned above. The Congo red stained samples were scanned with 200 PRO NanoQuant (TECAN Japan, Kawasaki, Japan) in the range of 400–600 nm wavelength.

rPfMSP3 was loaded onto gilder grid and stained with 2% (*w/v*) phosphotungstic acid and observed under TEM (JEM 1400Plus, JEOL, Akishima, Japan) as per protocols described earlier [11].

2.8. Immunological assay

2.8.1. Evaluation of rPfMSP3 with ELISA

The ability of malaria exposed human sera to recognize rPfMSP3 was determined by ELISA method. For this purpose, we used available malaria sera kept in our laboratory. Sera were collected from people living in malaria endemic areas in Indonesia i.e. Banjarmasin (Kalimantan) and Sumba Tengah (Nusa Tenggara Timur) in 2013. Those areas had annual parasite incidences around 1.5°/₀₀ and 7°/₀₀, respectively, with *P. falciparum* and *P. vivax* as dominant species. Total 81 sera were collected from fever patients visiting a Primary Health Care in Banjarmasin or during Mass Blood Survey in Sumba Tengah. Fifty five sera were infected with *P. falciparum* and the rest were uninfected, or infected with other (*P. vivax* or *P. malariae*). Malaria infections were confirmed by microscopic examination. Sera from healthy people living in non-endemic area in Java were used to determine cut off point for ELISA reading.

ELISA was performed by coating rPfMSP3 (500 ng/well, 100 µl) into 96 well ELISA plates and incubating the plates overnight at room temperature. Following washing steps, plates were blocked with 1% BSA in PBS buffer (pH 7.0). Sera that were diluted 1:10 in buffer incubation solutions (100 µl) were added into duplicate wells and incubated at room temperature. After washing steps, 50 µl of solution containing alkaline phosphatase goat anti-human IgG diluted 1:1000 was added to each well. Enzymatic reaction was developed by addition of 1 mg/ml 4-nitrophenylphosphatase (Merck). Plates were read at 405 nm with an ELISA reader (Zenix, Zenix Diagnostic, Germany). Cut off points were set at 2 standard deviations above the mean absorbance at 405 nm from 28 individuals, unexposed to malaria from Jogjakarta city. Sensitivity and specificity were defined as follows,

$$Sensitivity = \frac{N_{tp}}{(N_{tp} + N_{fn})} Sensitivity = \frac{N_{tp}}{(N_{tp} + N_{fn})} \quad (1)$$

$$Specifity = \frac{N_{tn}}{(N_{tn} + N_{fp})} Specifity = \frac{N_{tn}}{(N_{tn} + N_{fp})} \quad (2)$$

where N_{tp} and N_{fn} of Eq. (1) denote number of true positive results and number of false-negative results, respectively; N_{tn} and N_{fp} of Eq. (2) number of true negative results and number of false positive results, respectively. True positive defines that the human sera collected from subject that had been confirmed as having *P. falciparum* infection is recognized by rPfMSP3. False negative rPfMSP3 indicates that the human sera collected from subject that had been confirmed as having *P. falciparum* infection has no PfMSP3 antibody.

2.8.2. Evaluation of recombinant rPfMSP3 in western blot

Western blots were performed using sera from malaria *P. falciparum*-infected and non-malaria (healthy donor and toxoplasmosis) patients. Briefly, 20 µl (0.5 µg) rPfMSP3 were denatured and loaded into SDS-PAGE 10% (*w/v*) acrylamide at 70 V. The SDS gels were subsequently trans-blotted on to Immobilon-P (Merck), blocked and probed with human sera at 1:10 dilution in TBS-T for 1 h at room temperature as reported previously [18]. Subsequently, washing was done and probed with the secondary anti-human IgG conjugated with alkaline phosphatase (1:1000). After washing step, proteins were detected by adding nitro blue tetrazolium/bromochloroindolyl phosphate (Thermo-Scientific) as a substrate.

3. Results

3.1. Purification of rPfMSP3

To express rPfMSP3 in silkworm larvae, its native signal sequence at its N-terminus (1–24 aa) was replaced with that of bombyxin from *B. mori*. In addition, FLAG tag was added to its C-terminus for the easy purification as shown in schematic representation (Fig. 1a). A recombinant BmNPV bacmid containing rPfMSP3 expression cassette was constructed and injected into silkworm larvae. The hemolymph was collected and rPfMSP3 purified by 40% (saturation) ammonium sulfate treatment, HiTrap Phenyl FF column chromatography and DDDDK-tagged protein purification gel column chromatography was confirmed by Coomassie Brilliant Blue (CBB) staining (Fig. 1b) and western blotting (Fig. 1c) at around 55 kDa. Approximately 234 µg of rPfMSP3 purified from 10 ml of the silkworm larval hemolymph.

3.2. Glycosylation analysis of rPfMSP3

In the rPfMSP3, 4 putative *N*-glycosylation sites (N18, 29, 69, 199) were identified using NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/). To analyze the *N*-glycosylation, CBB and glycoprotein staining respectively were performed (Fig. 2a–b). The rPfMSP3 was stained by periodic acid-Schiff method with HRP as the positive control and soybean trypsin inhibitor as the negative control. The

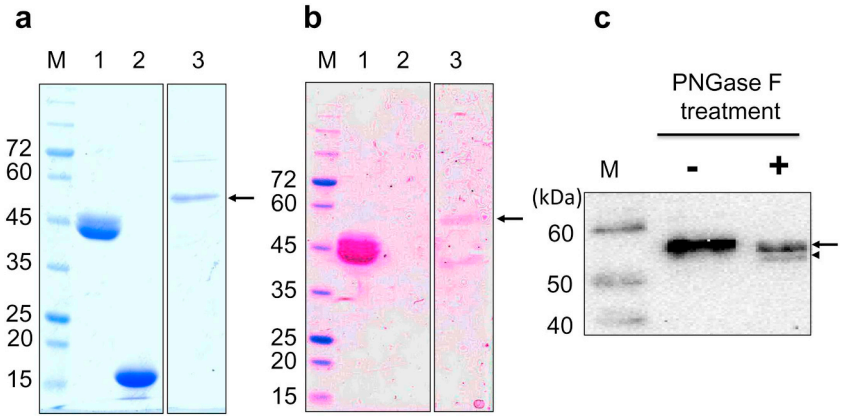

Fig. 2. Glycoprotein staining of rPfMSP3 purified from hemolymph by periodic acid-Schiff method. (a) Each protein was separated by SDS-PAGE. (b) the gel was stained by Glycoprotein staining kit (Thermo Fisher Scientific K.K.). M, 1, 2 and 3 in (a) and (b) denotes protein marker, HRP as positive control, soybean trypsin inhibitor as negative controls and rPfMSP3, respectively. (c) Deglycosylation of rPfMSP3 by PNGase F. rPfMSP3 treated with PNGase F (with and without) under the denature condition was separated by SDS-PAGE and proteins were detected by western blot. M denotes protein marker. Large and small arrows show the change in its molecular mass due to PNGase F treatment, respectively.

rPfMSP3 was clearly stained as well as the positive control, but the negative control was not. In addition, the treatment of rPfMSP3 with PNGase F produced the new band detected just beneath the rPfMSP3 by western blot (Fig. 2c). These results indicate that rPfMSP3 purified from silkworm hemolymph was *N*-glycosylated.

3.3. Oligomerization of rPfMSP3

To analyze this property of rPfMSP3, Congo red staining experiment was performed. Maximum absorbance in the presence of rPfMSP3 shifted to red (Fig. 3a). The red shift of maximum absorbance in the presence of rPfMSP3 expressed in silkworm was from 488 nm to 508 nm which shows the fibrillar assemble of MSP3 [3].

To analyze its amyloid formation or oligomerization, blue-native PAGE was carried out (Fig. 3b). A little smear band was observed above 146 kDa, suggesting that rPfMSP3 expressed in silkworm hemolymph may form trimers or tetramers. Morphology of the purified rPfMSP3 expressed in silkworm hemolymph was observed by TEM (Fig. 3c). Long fibril-like morphology were observed, which were similar one compared to the previous report [3]. The major content of these elongated fibrils are MSP3 which usually expressed during schizogony. These results indicate that the rPfMSP3 expressed in silkworm also formed amyloid-like fibrils.

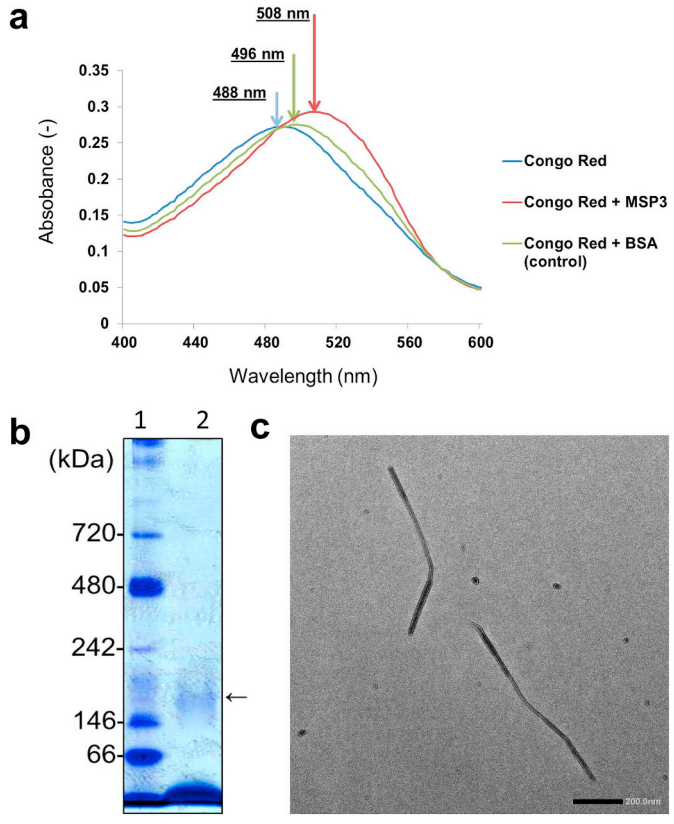

Fig. 3. Oligomerization of rPfMSP3 purified from silkworm hemolymph. (a) Spectra from Congo red staining of rPfMSP3. (b) Blue native-PAGE of purified rPfMSP3. Blue-native PAGE were carried out and the gel was destained, followed by staining again by CBB. Lanes 1 and 2 denote a protein marker and rPfMSP3, respectively. (c) TEM image of purified rPfMSP3. rPfMSP3 was loaded onto a TEM grid and stained by negative staining using phosphotungstic acid. Scale bar is of 200 nm.

3.4. ELISA analysis of rPfMSP3 with human sera

Using 50 sera collected from Banjarmasin, we found that 30 out of 41 *P. falciparum* malaria sera (73% sensitivity) was positive against the rPfMSP3 expressed from silkworm by ELISA; although it also recognized 4 out of 9 non-falciparum malaria samples, giving specificity 56% (5 out of 9 non-falciparum malaria samples were found to be true negative) (Supplementary material Tables S1 and S2, Table 2).

Using 31 sera collected from Sumba Tengah, the rPfMSP3 protein was reacted with 8 out of 14 sera from *P. falciparum* malaria patients giving sensitivity 57%, while specificity was 8 out of 17 (47%) (Supplementary material Tables S3 and S4, Table 2).

Table 2
Summary of ELISA results obtained from rPfMSP3 detected with human sera from endemic areas in Indonesia.

Endemic area	Number of samples	ELISA results	
		Positive	Negative
Banjarmasin samples			
Non Pf[a]	9	4	5
Pv[b]	8	4	4
Pm[c]	1	0	1
Pf[d]	41	30	11
Pf	24	19	5
Pf & Pv	17	11	6
Sumba Tengah samples			
Non Pf	17	9	8
Pv	11	5	6
Uninfected	6	4	2
Pf	14	8	6
Pf	11	6	5
Pf & Pv	3	2	1

These results are shown in Supporting information.
[a] Non *P. falciparum* malaria serum.
[b] *P. vivax* malaria serum.
[c] *P. malariae* malaria serum.
[d] *P. falciparum* malaria serum.

Table 3
Sensitivity and specificity of ELISA results using rPfMSP3 protein.

Group	Number of human sera	Positive	Negative
Non-*P. falciparum* malaria serum	26	13	13
P. falciparum malaria serum	55	38	17
Sensitivity	38/55 = 69%		
Specificity	13/26 = 50%		

ELISA results from those two different areas were summarized in Table 3. The rPfMSP3 reacted with 38 out of 55 *P. falciparum* malaria sera, giving it 69% sensitivity to *P. falciparum* patient sera and 50% specificity since it was also reacted with 13 out of 26 non-falciparum malaria samples (*P. vivax* malaria). Although the subjects were confirmed as infected by *P. vivax*, they also had circulating antibodies against *P. falciparum* including against PfMSP3 due to previous *P. falciparum* infection as they lived in endemic area with high *P. falciparum* exposures.

3.5. Western blot analysis of rPfMSP3 with human serum sample

Whether a *P. falciparum*-infected human serum recognizes rPfMSP3 purified from silkworm hemolymph was confirmed by western blot. For this analysis, rPfMSP3 was tested against three different infected sera groups: sera from *P. falciparum*-infected, healthy donor and *Toxoplasma*-infected human (Fig. 4). Non-malaria-infected human sera (healthy donor and toxoplasmosis sera) were used as a negative control. The rPfMSP3 was detected at around 55 kDa by the serum taken from *P. falciparum* patient, but not by the sera from non-malaria-infected human. Protein bands at around 75 kDa were detected by all sera including negative sera, but protein bands below 55 kDa were detected by only the serum from *P. falciparum*-infected human. The protein bands below 55 kDa might come from the degradation of rPfMSP3.

4. Discussion

The rPfMSP3 was successfully expressed in silkworm larvae as a secretory protein and purified from silkworm hemolymph. Estimated molecular mass of rPfMSP3 with FLAG tag sequence (not including the bombyxin signal sequence) is approximately 38 kDa, but that of rPfMSP3 expressed in silkworm larvae was 55 kDa. This result suggests that this discrepancy may come from post-translational modifications in silkworms. Therefore, the rPfMSP3 was treated by PNGase F to deglycosylate its *N*-glycans. However, deglycosylated rPfMSP3 observed at approximately 53–54 kDa, which does not correspond to that estimated from the amino acid sequence (38 kDa). When the PfMSP3 (21–354 aa) was expressed in *E. coli*, the molecular mass was observed at around 55 kDa [3,14]. MSP3 has a glutamic acid-rich region and this region may cause the discrepancy of its molecular mass in SDS-PAGE. Now this discrepancy has not been still solved. Additionally, in this study, the rPfMSP3 was observed above 146 kDa and PfMSP3 expressed in *E. coli* was also observed at almost the same molecular mass in the previous study [3]. rPfMSP3 expressed in silkworm larvae forms long fibril-like structures as PfMSP3 expressed in *E. coli* did (Fig. 3c). Moreover, Congo red analysis of rPfMSP3 expressed in silkworm larvae showed the shift of the maximum absorbance. These results suggest that some post-translational modifications in silkworms, except for its *N*-glycosylation, may contribute to the difference in properties between PfMSP3s.

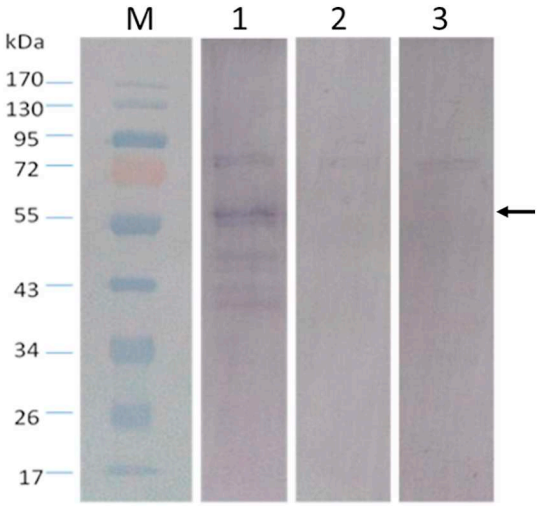

Fig. 4. Reactivity of the purified rPfMSP3 with patient sera infected by malaria falciparum. Each western blot strip of the purified rPfMSP3 was tested with *P. falciparum*-infected serum (lane 1), healthy donor (lane 2), and *Toxoplasma gondii*-infected serum (lane 3). M denotes protein marker (10–170 kDa Prestained protein marker, Crystalgen Inc). Arrow head indicate the *P. falciparum* sample recognized rMSP3 protein band at 55 kDa.

Our serological analysis shows the sensitivity and specificity of ELISA using the rPfMSP3 in this study were lower than those in previous studies using different serological markers of *P. falciparum* [15,16]. In addition, regarding other malaria, recombinant of *P. vivax* MSP1-42 expressed in *E. coli*, for example, when evaluated with sera from naturally infected individuals giving 87% sensitivity and 94% specificity [17]. In other studies, rMSP1 for *P. knowlesi* (expressed in *E. coli*) or rMSP1-33 gave sensitivity more than 90% and 100% specificity with other non-malaria although high cross-reaction with other malaria species was reported [18,19]. Limited number of human sera tested might also become limitation of our study as we could only use available sera in our laboratory.

Since PvMSP3a is not homologous to PfMSP3 and these MSP3s may be analogous [20], recombinant protein of PfMSP3 is become a necessity. Large amount of recombinant protein is preferable when it used to use as sero-diagnostic or surveillance marker for countries that entering pre- and pre-elimination phase such as Indonesia. Our recombinant PfMSP3 expressed in silkworm might help with the discrimination of the sera from human infected with falciparum malaria. However, in this study, it shows that 47% of sera from *P. vivax*-infected human is sero-positive by the ELISA using rPfMSP3 expressed from silkworm. Therefore, other serological markers could be used in combination with the rPfMSP3 to improve the sensitivity and specificity of its ELISA sero-diagnostic [21].

It also could not be ruled out that because the samples were taken from endemic area, it is possible that the antibodies detected here may come from the previous malaria infection due to the circulation of antibodies to *P. falciparum* in human blood for a period of 5 years or more [22]. Moreover, human sera tested need to be determined whether they are derived from early acute, or chronic patients revealed by its IgM positive-IgG negative, IgM positive-IgG positive and IgM negative-IgG positive respectively, as done by similar study while assessing sero-diagnostic evaluation because the induction of anti-*Plasmodium* antibodies by its first infection may take one to two weeks and these antibodies may be in the blood for only 3–6 months [2,23,24].

5. Conclusions

In this study, rPfMSP3 was expressed in silkworm larvae and secreted into its hemolymph by the addition of bombyxin signal sequence. rPfMSP3 was purified from hemolymph using ammonium sulfate precipitation and two-column chromatography. Approximately 234 μg of post-translationally modified rPfMSP3 was obtained from 10 ml of silkworm larval hemolymph. Purified rPfMSP3 had the similar properties to that expressed in *E. coli* and formed oligomers. In addition, sera from *P. falciparum*-infected humans showed the reactivity to the purified rPfMSP3. These results indicate that rPfMSP3 expressed in silkworms could be used in serology measurement for malaria infection.

Acknowledgements

The authors would like to express gratitude to, Puspawati (RS Ratu Zalecha, Banjarmasin), Fridolina Mau (Sumba Tengah), Arta Farmawati (Faculty of Medicine, Public Health and Nursing UGM), and Irena Agustiningtyas in providing sera used in this study. Thanks to Mr. Kenshin Takemura and Ms. Mikiko Hayashitani for their participation in MSP3 purification and transmission electron microscopy experiment.

Ethics approval and consent to participate

Human sera collection was ethically approved by Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine Public Health and Nursing, Universitas Gadjah Mada, Indonesia - DR. Sardjito general Hospital (Ref: KE/FK/0032/EC/2018).

Conflict of interest disclosure

The authors declare no commercial or financial conflict of interest.

Appendix A. Supplemental data

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