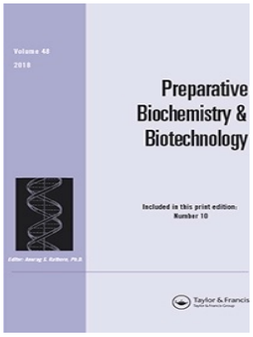

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Cytoplasmic and periplasmic expression of recombinant shark VNAR antibody in *Escherichia coli*

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ABSTRACT

Shark variable new antigen receptors (VNARs) are known to possess excellent heat-stability, and the long complementarity determining region 3 (CDR3) has permitted it to penetrate into the cleft region of antigens. The number of cysteine (Cys) residues contained within VNAR is greater than in conventional antibodies, entailing disulfide bond formation in both the inter- or intra-loop regions is required for interactions with the target protein antigens. Therefore, the selection of a suitable expression system is important to ensure the solubility and correct folding of functional VNAR protein production. Unlike higher organisms, the machinery for effecting posttranslational modifications of proteins in *Escherichia coli* (*E. coli*) are less sophisticated. To overcome this circumstance, a pDSB-28Y vector fusion with DsbA signal peptide was engineered for periplasmic H8VNAR production. Despite the periplasmic proteins showing a lower yield (62 μg/mL) than cytosolic proteins (468 μg/mL) that is obtained from pET-28a vector, it has demonstrated better performance than that of a cytosolic protein in terms of absorbance. However, these readings were still inferior to that of positive control mouse monoclonal antibody (mAb) C1-13 in this experiment. Therefore, further investigation is required to improve the binding affinity of selected recombinant VNAR towards malaria biomarkers.

KEYWORDS

Antibody; cytosolic; disulfide bonds; *E. coli* expression system; periplasmic; shark VNAR; single domain antibody

Introduction

The evolution of immunoglobulins from invertebrates began ~550 million years ago.[1] With the emergence of antibody surface display technology, interest has increased in new binders from less commonly used animals, including Variable domains of heavy chain-only (VHH) from camelids, and variable new antigen receptors (VNAR) from sharks. The unusual antibodies derived from these groups of animals have been reported to provide promising specificity and sensitivity for target antigens[2] As in all mammals, members of the camelid family produce immunoglobulins G that contains only a heavy chain domain. Owing to its peculiar structure, this antibody has been configured as "heavy chain only" antibodies (HCAbs).[3] HCAbs are slightly different from IgG, in that they also include both a constant (Fc) and a variable domain. The isolated variable domain region of camelids HCAbs is known as V_{HH} (variable domain of the heavy chain of HCAbs) or Nanobody® (Nb; Ablynx).[4]

In contrast to camelids, immunoglobulin new antigen receptor (IgNAR) is an intra-domain disulfide bond immunoglobulin superfamily protein that provided additional stability without adversely affecting antigen binding efficiency. This protein was naturally discovered from nurse shark (*Ginglymostoma cirratum*)[5] and wobbegong shark (*Orectolobus maculates*).[6] Unlike immunoglobulin (Ig) isotypes found in higher mammals, the variable domains of shark IgNAR (VNAR) is an unusual single domain antibody that contains only a single H-chain homodimer and lacks a light chain.[7] According to molecular analysis, the deletion of a large portion of framework region 2-complementarity determining region 2 (FR2-CDR2) has made VNAR the smallest variable domain with a size of ~12 kDa in the animal kingdom.[7] It may be due to VNARs have evolved from separate lineage from immunoglobulins and thus have never had a CDR2.[8,9]Moreover, VNAR domains possess an extraordinary CDR3 domain which is much longer than that of conventional antibodies. The inter- or intra-loop of CDR3 is typically influenced by the numbers of cysteine residues and the penetration capability into the cleft region of the target antigen.[10,11] Owing to peculiar structure, the excellent solubility and thermostability of these natural single domain fragments may be due to the substitutions of amino acid at heavy and

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light variable domains (VH-VL) interface, resulting to be more hydrophilicity rather than a hydrophobic interface as exhibited in conventional antibodies.[7,12]

Three IgNAR isotypes have been identified in IgNAR family, with Type 1 and Type 2 predominantly found in the shark immune system.[5,13] The number of Cys residues contained within shark VNAR domains is greater than in conventional antibodies, and entails disulfide bond formation in both the inter- or intra-loop regions that are required for interactions with the target protein antigens.[14] Therefore, the selection of a suitable expression system is important to ensure the solubility and correct folding required for expression of functional VNAR protein.[15,16]

The bacterial expression system is often the first choice in laboratories. It is amenable to produce recombinant proteins for a range of biological applications.[17] Rapid growth, high levels of protein production and, ability to multiplex both expression screening[18] and protein production,[19] explain why *Escherichia coli* is predominantly chosen as expression host in the molecular biology laboratory as well as in biotechnology industry. However, the machinery for effecting posttranslational modifications of proteins in *E. coli* is less sophisticated compared to higher organisms.[20] For example, the absence of eukaryote chaperones specialized in posttranslational modifications can lead to protein misfolding and aggregation.[21] In eukaryote cells, polypeptides expressed in the reducing cytoplasm compartment are generally directed to the endoplasmic reticulum (ER), where an oxidative environment facilitates correct folding including disulfide bond production. Similarly, the cytoplasm of prokaryote cells contains a reducing environment, but the absence of the specialized subcellular ER compartment can result in the failure to form disulfide bonds.[20] As a consequence, eukaryotic cell expression system remains an attractive production method for most recombinant human proteins required to resemble to naturally occurring *in vivo* form.[22]

An oxidizing environment is also present in the periplasm of *E. coli*. This is an important compartment for bacteria to perform enzymatic activities, and also functions as an export system for functional proteins.[23] Thus, the formation of disulfide bonds in recombinant proteins can be achieved by directing the protein to the bacterial periplasm using a signal sequence for translocation such as chaperones and foldases.[24–26] A range of leader peptides have been exploited as fusion partners to recombinant proteins to translocate unfolded precursors into the periplasm. These include SpA, PhoA, PelB, OmpA, OmpT, DsbA, TorT, and TolT.[27–33] In this manner, efficient expression of functional folding proteins can be achieved in *E. coli*.

The periplasmic disulfide oxidoreductase A protein (DsbA) belongs to the thioredoxin protein superfamily and is responsible for disulfide bond formation and rearrangement in *E. coli*.[34,35] DsbA, otherwise, in combination with other Dsb peptide family has been widely used to produce functional recombinant proteins in biomedical research. For instances, the performance and yield of functional scFv,[36] horseradish peroxidase,[37] human plasma retinol-binding protein,[38] nerve growth factor beta,[39] and brain-derived neurotrophic factor[40] have been improved using this fusion approach.

The recombinant VNAR proteins have been well expressed using proprietary expression vectors in a range of laboratories such as pIMS100,[16] pIMS147,[41] pEcan22,[42,43] pAK-300,[44] and pET-28a.[45] In addition, the PelB leader peptide is commonly used as a fusion protein partner for bacterial periplasmic targeting.[46,47] In this study, we attempted to produce recombinant H8VNAR which is specific to malaria *Plasmodium falciparum* histidine-rich protein 2 (PfHRP2) using a bacterial expression system. PfHRP2 has been widely used as a biomarker for detection of *Plasmodium falciparum* infection.[48–51] It is a water-soluble protein that is produced during the asexual stages and gametocytes of *P. falciparum*. PfHRP2 is abundantly expressed in the red cell, released during rupture of infected red cells and can remain in the blood for up to 28 days after the initiation of anti-malarial therapy thereby making it an excellent biomarker for diagnosis.[52–54]

To understand the binding ability, the proteins were expressed from different compartments of *E. coli*. Hence, a commercial pET-28a vector (Novagen, USA) was chosen for cytosolic recombinant H8VNAR protein expression. Meanwhile, an in-house constructed pDSB-28Y vector fused with DsbA signal peptide was used to synthesis periplasmic recombinant H8VNAR protein. The binding efficacy of these recombinant proteins towards malaria rPfHRP2 was further verified with positive control mouse mAb C1-13 (kindly provided by Dr. Martin Bubb, National Products Institute, South Africa).

Methods and materials

Plasmids and enzymes

pGEM-T (Promega, Madison, WI, USA) was used as the cloning vector. pET-28a (Novagen, Madison, WI, USA) was used to construct the expression vectors, also the backbone for vector pDSB-28Y construction. The restriction enzymes, *SpeI, NcoI* and *XhoI*, and T4 DNA ligase, were purchased from New England Biolabs Inc. (USA).

Chemicals and reagents

Luria–Bertani (LB) medium (w/v) containing 0.5% yeast extract, 1% tryptone, and 1% NaCl, was used for manipulation of cloning and recombinant VNAR expression. All antibiotics used for screening were obtained from Sigma Chemicals (USA).

Primers sequence

Table 1

Table 1. Oligonucleotide sequences of primers used in this study.

Primers Description	Nucleotide sequence (5′ → 3′)	Applications
DsbA_For	CATGCCATGGCGAAAAAGATTTGGCTGGCGCTGGCTGGT	*Nco*I-DsbA Forward
DsbA_Rev	GGACTAGTCGCCGCCGATGCGCTAAACGCT	*Spe*I-DsbA Reverse
H8VNAR_Dsb_For	GGACTAGTGCATGGGTGGACCAAACACCA	*Spe*I-H8VNAR domain Forward for pDSB-28Y
H8VNAR _For	CATGCCATGGCGGCATGGGTGGACCAAACACCA	*Nco*I-H8VNAR domain Forward
H8VNAR_Rev	CCGCTCGAGTTTCACGGTTAATGCGGTGCC	*Xho*I-H8VNAR domain Reverse
T7Seq_For	TAATACGACTCACTATAGGG	pET Vector Sequencing Forward
T7Seq_Rev	CTAGTTATTGCTCAGCGGTG	pET Vector Sequencing Reverse

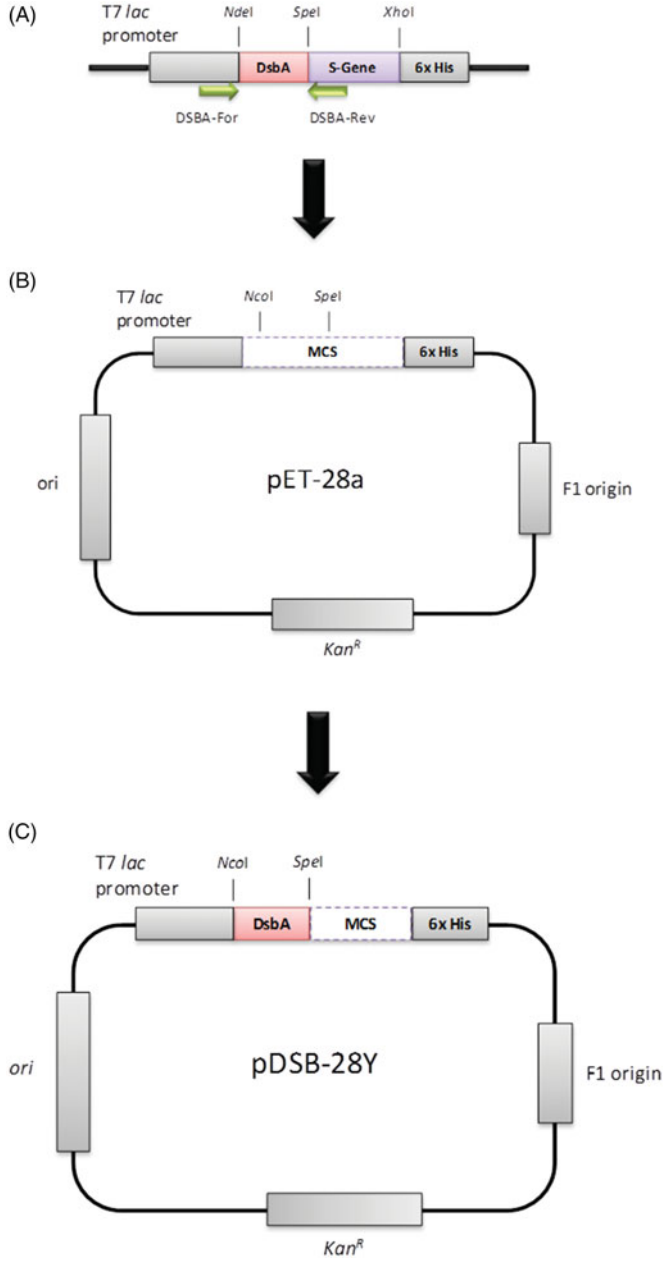

Figure 1. Schematic representation of pDSB-28Y expression vector construction. (A) PCR amplification of DsbA gene in pSSPET plasmic. (B) Cloning site of pET-28a. (C) Completion of pDSB-28Y plasmids construct.

Construction of recombinant plasmids pDSB-28Y for periplasmic expression

The template used for constructing DsbA signal peptides was derived from pSSPET vector provided by Dr. Mark Pearson, James Cook University, Australia. The DsbA sequence was PCR amplified using DsbA_primers as shown in (Table 1). The PCR reaction carried out was 95 °C for 15 min as a pre-cycle denaturing step; the 35 cycles of denaturing at 95 °C for 10 s, annealing at 55 °C for 1 min, and followed by extension at 72 °C for 2 min. The PCR products were then purified using Isolate II PCR Purification Kit™ (Bioline) prior to ligating into pET-28a vector which had been linearized with *Nco*I and *Xho*I restriction enzymes. The newly engineered expression plasmid designated as pDSB-28Y was used for periplasmic expression of VNAR protein in this study.

PCR amplification, sub-cloning, and transformation

The full length of H8VNAR was PCR amplified from a target clone using specially designed H8VNAR primers set as indicated in Table 1. The restriction site for expression of cytoplasmic and periplasmic recombinant H8VNAR proteins is shown in Figure 1. The PCR conditions employed for amplifying the VNAR gene fragments were similar to as mentioned before.

The purified PCR products were ligated into a pGEMT-Easy™ (Promega) plasmid vector using the T-A cloning procedure as outlined in the instruction manual. The next day, the ligated plasmid was transformed into fresh competent *E. coli* strain α-select™ (Bioline, USA) using the standard heat shock treatment of calcium-chloride method. The positive transformants were picked for colony PCR using gene-specific primers before analysis by gel electrophoresis. Transformants that contained the inserts with appropriate size were cultured in 10 mL LB broth with 100 µg/mL ampicillin and incubated at 37 °C with 250 rpm shaking overnight.

Plasmids extraction and restriction enzyme digestion

An overnight culture of transformed α-select cells (4 mL) was pelleted by centrifugation at 12,000 × *g* for 1 min. After supernatant was removed, plasmids extraction was performed using the High Pure Plasmid Extraction Kit™ (Roche Applied Science, USA) according to manufacturer instructions. The H8VNAR insert was excised from the pGEMT vector, and the pET-28a and pDSB-28Y vectors with a His-6 affinity tag at C-terminal were linearized with *Nco*I/*Xho*I or *Spe*I/*Xho*I double restriction enzyme digest, respectively. Reaction mixtures were made up with 26 µL of deionized water, 4 µL of bovine serum albumin (BSA), 4 µL of restriction enzyme buffer 4 (NEB, USA), 2 µL of *Spe*I (for pDSB-28Y) or *Nco*I (for pET-28a) restriction enzyme, 2 µL

of *Xho*I restriction enzyme and 2 µL purified plasmid. The mixture was incubated at 37 °C for 2 h. The mixture containing linearized vector was dephosphorylated by adding 5 µL of 10 × NEB buffer 3 and 3 µL of calf intestinal alkaline phosphatase (CIP) from NEB, followed by 1 h incubation at 37 °C.

Recombinant plasmids ligation and transformation

Agarose gel electrophoresis was undertaken to purify the band of linearized vectors pET-28a or pDSB-28Y and cut inserts (H8VNAR cDNA). Each of purified plasmids was ligated overnight in a mixing reaction consisting of purified H8VNAR insert at 4 °C. The recombinant plasmid was then transformed into *E. coli* BL21(DE3) competent expression cells and incubated at 37 °C overnight. The plasmid of the overnight culture was extracted and then subject to DNA sequence analysis.

Expression of His6-tagged H8VNAR protein

The expression of His-tagged H8VNAR protein was carried out by inoculating 100 µL glycerol stock of pET-28a/H8VNAR and pDSB-28Y/H8VNAR in BL21(DE3) cells into 20 mL LB/Kan (30 µg/mL) broth and incubating at 37 °C, with 200 rpm shaking overnight. The following day, 20 mL of overnight culture was then used to inoculate 1 L of fresh 2YT/Kan (30 µg/mL) broth. The 1 L culture was divided equally into four sterile 1 L flasks, with 250 mL culture in each. Flasks were then incubated at 37 °C in a shaking incubator at 250 rpm until OD_{600} reached ~0.5 (about 4 hours). IPTG was then added at a final concentration of 0.1 mM, followed by an additional incubation at 20 °C, with shaking at 200 rpm for 16 h.

Extraction of cytoplasmic protein fraction

Overnight cultures were centrifuged at 6000 × *g* at 4 °C in an Ultra Centrifuge for 40 min. The supernatants were discarded and the pellet resuspended in 30 mL of cold Tris-phosphate buffer (50 mM Tris-HCl, 20 mM sodium phosphate NaH_2PO_4, 500 mM $NaCl_2$, pH 8.0). After incubation at room temperature for 45 min with the addition of 100 µg/mL lysozyme, cells were lysed by passing through a French press twice with a pressure of 1000 psi. After incubating on ice for 5 min, the slurry was centrifuged at 20,000 rpm for 40 min at 4 °C. The supernatant (lysate) was collected and filtered through 0.45 µm filter twice. The supernatant was then dialyzed overnight at 4 °C against 6 L of protein binding buffer (50 mM Tris-HCl pH 8.0, 3 mM $MgCl_2$, 300 mM NaCl) prior to affinity purification.

Extraction of periplasmic protein fraction

After induction of protein expression, cell cultures were harvested at 6000 rpm at 4 °C using an Ultra Centrifuge (Beckman, USA) for 40 min. The supernatant was decanted and the periplasmic proteins extracted from the bacterial periplasm using 30 mL of ice-cold TES buffer (0.2 M Tris-HCL pH 8.0, 0.5 mM EDTA, 20% sucrose). The cell suspension was incubated on ice for 30 min. To lyse the cells by osmotic shock, 30 mL of 5 mM $MgSO_4$ was added, and the incubation continued for another 30 min on ice. The slurry was then centrifuged at 20,000 rpm for 40 min at 4 °C. The resulting supernatant was collected and twice filtered through 0.45 µm filter. The supernatant was then dialyzed overnight at 4 °C against 6 L of protein binding buffer (50 mM Tris-HCl pH 8.0, 3 mM $MgCl_2$, 300 mM NaCl) prior to affinity purification.

Protein purification

Recombinant His6-Tagged H8VNAR protein was purified using a Nickel-NTA Agarose Resin (Sigma-Aldrich, USA), according to the manufacturer instructions. Briefly, the Nickel-NTA Agarose Resin suspension was washed twice in 20 mL of VNAR equilibrium buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl). Approximately 1 mL of Nickel-NTA Agarose Resin was mixed into 50 mL of VNAR-containing *E. coli* total lysate or periplasmic extract in a 50 mL Falcon tubes (BD Bioscience, Oxford UK). The mixture was incubated at 4 °C overnight with head-over-head rotation. The following day, the resin was pelleted by centrifugation at 500 × *g* at 4 °C for 5 min. The supernatant was slowly decanted into a fresh 50 mL tube and labeled as the unbound protein sample. The resin was washed twice with VNAR equilibrium buffer followed by centrifugation at 500 × *g* at 4 °C for 5 min. The resin was then resuspended in a 10 mL of VNAR equilibrium buffer and packed into Poly Prep/flow columns (Bio-Rad Laboratories, USA). The packed column was then washed twice with 10 mL washing buffer 1 (50 mM Tris-HCl pH 8.0, 3 mM $MgCl_2$, 300 mM NaCl, 10 mM imidazole) and washing buffer 2 (50 mM Tris-HCl pH 8.0, 3 mM $MgCl_2$, 300 mM NaCl, 20 mM imidazole). The bound protein was then eluted with 2 mL of Elution buffers 1, 2, and 3 in order. Thus, the total 6 mL of eluate was collected in six fractions. Eluted fractions were analyzed by SDS-PAGE and Coomassie blue staining. Fractions containing the bulk of eluted VNAR were pooled and dialyzed against 1 L of PBS at 4 °C for 3 h using SnakeSkin™ Dialysis Tubing (Pierce Protein Biology Products, Thermo Fisher Scientific, Rockford, USA), with a molecular cutoff of 3.5 kDa. The PBS solution was changed and the sample dialyzed for another 3 h, before changing to a fresh 1 L PBS again at 4 °C overnight.

Protein analysis and concentration

After dialysis, the purified H8VNAR proteins were analyzed by SDS-PAGE (15%) and Coomassie blue staining. The Bradford method[55] was used to determine protein concentration using the Bio-Rad Protein Assay (Bio-Rad Laboratories) according to manufacturer instructions. Each protein sample was prepared in three dilutions and assayed in triplicate to obtain the mean reading.

rPfHRP2-specific VNAR protein sandwich ELISA assay

To determine the binding efficiency of recombinant antibodies, H8VNAR proteins purified from both cytoplasmic and periplasmic spaces were used as capture antibodies for the anti-rPfHRP2 assays in a sandwich format. A total of 100 µL/well recombinant H8VNAR proteins adjusted to 1 µg/mL in PBS was coated to 96 well microtiter plates prior to incubation overnight at 4 °C. Plates were washed 3 times with 0.1% PBST and blocked by 300 µL of 5% v/v Skim Milk in PBS (MPBS) at room temperature for 1 h with gentle shaking. 10-fold serial dilutions of recombinant PfHRP2 proteins, with starting concentration at 10 µg/mL were prepared in 96 wells culture plates. Protein dilutions (100 µL/well) were then transferred to the corresponding wells that had been coated with capture antibodies. After incubation at room temperature for 1 h, plates were washed 3 times with 0.1% PBST, excess solution was drained onto paper towels until completely dried. Bound antigens were then detected with 100 µl/well of mouse mAb C1-13 (10 µg/mL) (National Bioproducts Institute, South Africa). After incubation at room temperature for 1 h, plates were washed as described above. Detection was undertaken using 100 µL of goat anti-mouse antibody conjugated horseradish peroxidase (HRP) (Jackson, USA) diluted 1:5000 in 2% MPBS. Plates were incubated at room temperature for 1 h. After additional washing, 100 µL of freshly prepared *o*-Phenylenediamine dihydrochloride solution (Sigma, USA), prepared according to manufacturer recommendations, was added to each well and placed at room temperature in dark the until the color was developed. Absorbance was read at a wavelength of 405 nm using ELISA plate reader (Biotek Synergy HT, USA).

For positive control, an indirect ELISA format was undertaken. The capture mAb C1-13 was used to bind to immobilized recombinant PfHRP2 proteins. The detection and examination methods were described as above.

Results

Multiple commercial vectors (Novagen, USA) that matched BL21(DE3) host cells, including pET-22a, pET-28a, pET-32a, and pET-41a were investigated for soluble VNAR protein production. Of the results, pET-28a was demonstrated to possess better results with higher yield and solubility of cytosolic VNAR proteins

For periplasmic protein expression, a new expression vector based on pET28a backbone was constructed in this study. The target DsbA gene sequence amplified from pSSPET was cloned into the multiple cloning sites (MCS) of pET-28a. The *Nde*I restriction site in the N-terminal of DsbA was replaced by *Nco*I, whereas the *Spe*I site remains unchanged. This new vector was then termed as pDSB-28Y the following sections. The cloning strategy for constructing the recombinant expression vector of pDSB-28Y is illustrated in Figure 1.

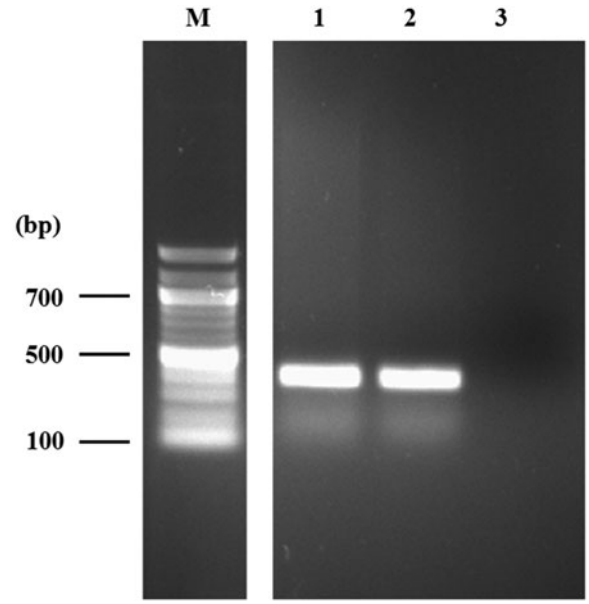

Figure 2. PCR amplification of H8VNAR genes showed a single band at 348 bp. Lane 1 represents H8VNAR insert PCR amplified for pET28a vector. Lane 2 represents H8VNAR gene PCR amplified for pDSB-28Y vector. Lane 3 represents PCR product with no cDNA template as negative control. Lane M represents 100 bp DNA ladder.

Gene cloning

The target H8VNAR gene isolated from shark immune VNAR phage library was amplified using appropriate primer sets for expression vectors of pET-28a and pDSB-28Y.[51] The PCR products of two H8VNAR genes for subcloning into the different expression vectors had the expected size of 348 bp (Figure 2). The PCR products were then cloned into pGEM-T easy vector using the T-A cloning protocol. After transformation to α-select *E. coli* cells, the positive H8VNAR/pGEM-T plasmid clone was confirmed by colony PCR (Figure 3). Following restriction enzyme digestion, excision of the appropriate fragment was confirmed by agarose gel electrophoresis. The fragment size of the H8VNAR gene was confirmed as 348 bp on the agarose gel, whereas the linearized pET-28a and pDSB-28Y expression vectors were about 1.2 kbp in size (Figure 4).

Cloning into expression vectors

For expression of recombinant H8VNAR proteins, the appropriate inserts were subcloned into the *Nco*I-*Xho*I cut sites of the pET-28a vector for cytoplasmic expression (Figure 5A), or *Spe*I-*Xho*I of the pDSB-28Y vector for periplasmic expression (Figure 5B). The ligated products were transformed into BL21(DE3) *E. coli* cells. To ensure each H8VNAR gene was in-frame, colony PCR and DNA sequencing were undertaken by randomly analyzing three clones from each of the two expression systems. The inserts were detected significantly different in both vectors with size of 348 bp for pET-28a and 411 bp for pDSB-28Y (Figure 6). It was due to an additional 63 bp nucleotides or 21 amino acid residues of DsbA signal peptides had been fused with H8VNAR in pDSB-28Y vector, resulting in longer length.

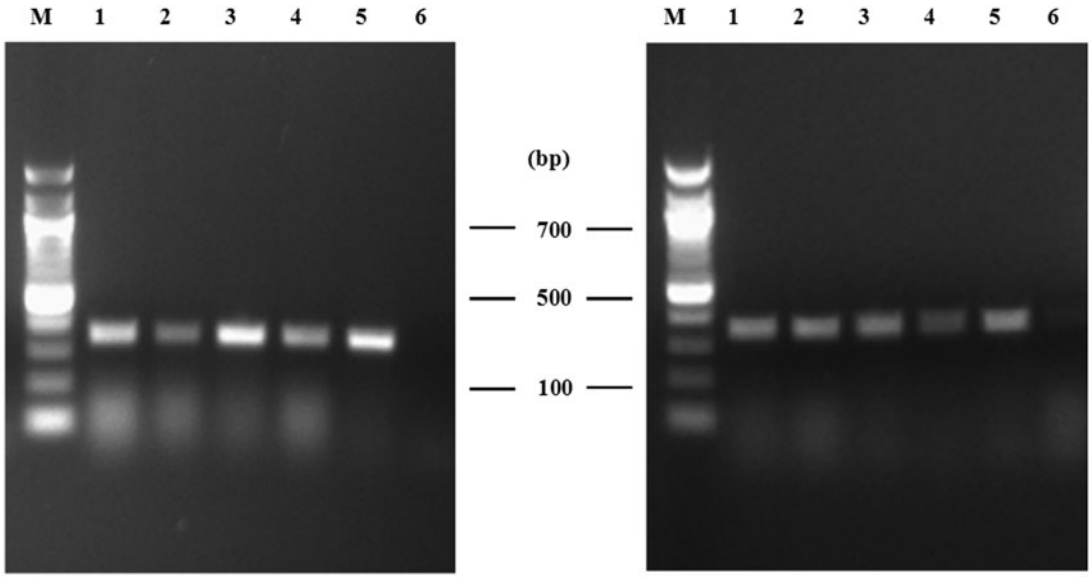

Figure 3. Colony PCR of pGEMT plasmids after cloning with H8VNAR gene. Gel A represents H8VNAR for pET28a cloning. Gel B represents H8VNAR for pDSB-28Y cloning. Lanes 1–5 show clones randomly picked for colony PCR; Lane 6 shows PCR product with no cDNA template as negative control; and Lane M shows 100 bp DNA ladder.

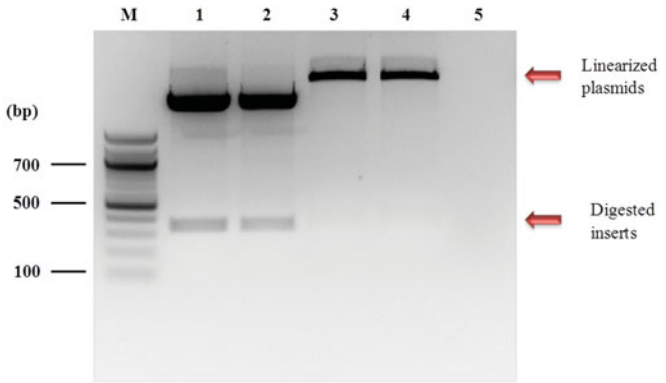

Figure 4. Restriction enzymes digestion of H8VNAR from pGEMT vectors and linearization of expression vectors. Lane 1 represents digested H8VNAR with *Nco*I/*Xho*I showed an insert at 348 bp; Lane 2 represents digested H8VNAR with *Spe*I/*Xho*I showed an insert at 348 bp; Lane 3 represents linearized pET-28a vector; Lane 4 represents linearized pDSB-28Y vector; Lane 5 represents PCR product with no cDNA template as negative control; and Lane M represents 100 bp DNA ladder.

DNA sequence analysis confirmed that both H8VNAR genes were in-frame with the start Met residue beginning the ORF of the H8VNAR gene (Figure 7). The H8VNAR gene was linked to a hexahistidine (6 × His) tag followed by a stop codon. For the recombinant gene designed for periplasmic expression, the required peptide sequence was confirmed to be fused to the H8VNAR gene sequence. The insertion of the *Spe*I restriction site could allow cloning of other VNAR genes at a later stage for recombinant protein expression in the periplasmic space.

Protein expression and purification

The expression and purification of H8VNAR proteins using both different expression vectors yielded a clearly visible band on SDS-PAGE stained with Coomassie Blue (Figure 8

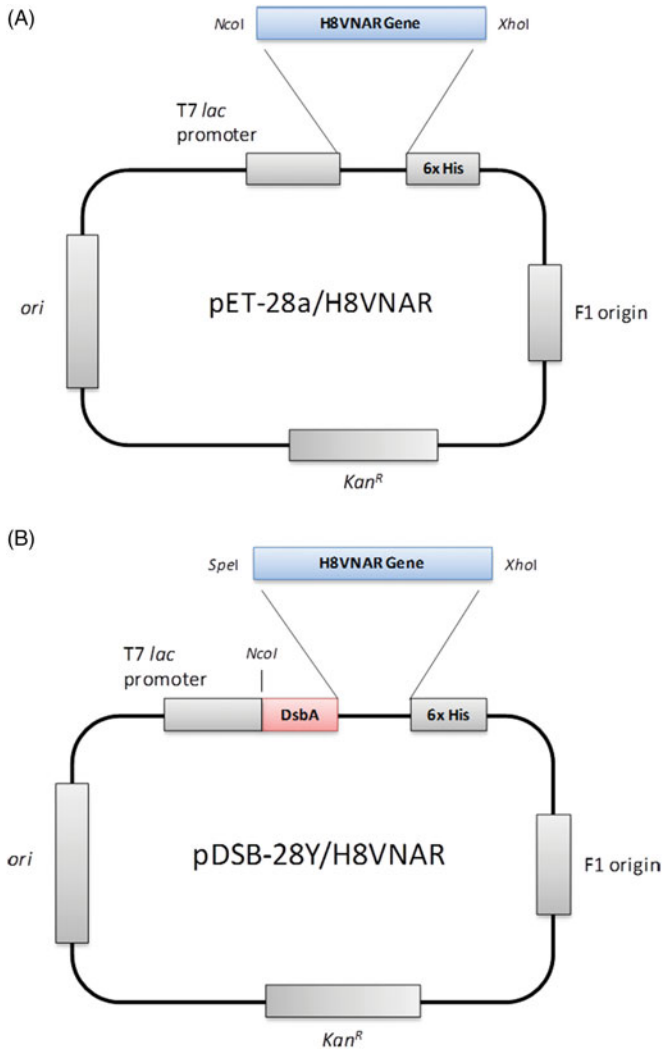

Figure 5. Cloning strategy of H8VNAR into different expression vectors. (A) represents cloning H8VNAR insert with appropriate restriction sites into pET-28a vector. (B) represents cloning H8VNAR insert with appropriate restriction sites into pDSB-28Y vector.

and 9). All H8VNAR proteins were present as well defined single bands at around 14 kDa in size, corresponding to the predicted molecular mass of H8VNAR including the 6 × His tag (13.57 kDa). In Western Blot analysis, a "split" band was detected in the IPTG-Induced total cell lysates of *E. coli* processed for periplasmic expression (Figure 9). However, this phenomenon was not observed in the cytoplasmic expression system (Figure 8). Nevertheless, only a single band was detected in both H8VNAR proteins after purification, suggesting the soluble recombinant VNAR proteins can be obtained using the bacterial expression system. The leakage of proteins from expression hosts was absence in the media. However, prolonged expression can lead to protein aggregation that eventually resulted in the formation of inclusion body in the *E. coli* expression host.

Protein concentration determination

The quantification of protein concentration of recombinant H8VNAR extracted in both cytoplasmic and periplasmic space of BL21(DE3) *E. coli* cells was determined using the standard Bradford method. The concentration of proteins was calculated and indicated in Table 2.

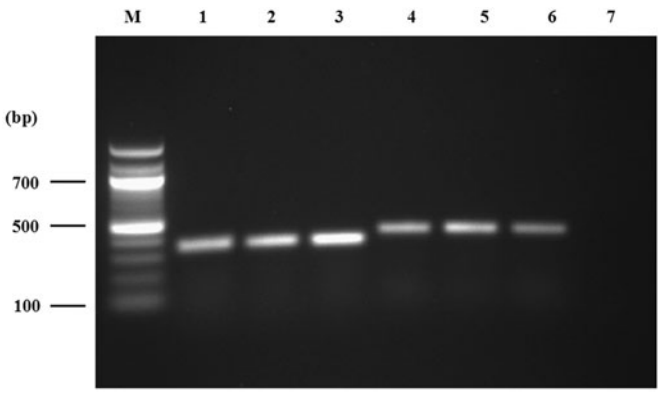

Figure 6. Colony PCR of H8VNAR transformants in BL21(DE3) *E. coli*. Lanes 1–3 represent three transformant clones randomly picked showing H8VNAR at 348 bp in pET-28a; Lanes 4–6 represent three transformant clones randomly picked showing fusion genes of H8VNAR and DsbA at 411 bp in the pDSB-28Y vector; Lane 7 represents PCR product with no cDNA template as negative control; and Lane M represents 100 bp DNA ladder.

Binding efficacy by sandwich ELISA

The binding ability of recombinant VNAR proteins was determined on ELISA plates in a sandwich format. The binding affinity was compared with commercial mAb C1-13 that performed in an indirect ELISA format. From the binding assay, both VNAR proteins gave positive responses when read on plate readers at OD 450 nm. The recombinant H8VNAR protein purified from periplasmic space showed better performance than that of cytosolic recombinant H8VNAR in terms of absorbance (Figure 10A). At a concentration of rPfHRP2 of 1 µg/mL, the OD reading of periplasmic VNAR protein (∼0.58) was about two-fold higher than that of protein produced by cytoplasmic expression (∼0.32). However, these readings were still inferior to that of mAb C1-13, the positive control in this experiment (OD of ∼1.4 at the same concentration of rPfHRP2) (Figure 10B). All OD readings demonstrated decreasing signal proportionally as the concentration of rHRP2 reduced. No binding was detected in both recombinant H8VNAR proteins and mAb C1-13 when the rPfHRP2 was replaced with 1% BSA as the negative control.

Discussion

The separate expression of variable heavy and light chain (VH and VL) domains using the microbial system in the initial experiment was not successful due to protein aggregation problem.[56,57] This drawback was not overcome until the synthetic single domain antibodies (sdAbs) were developed using antibody engineering approach. Although this technique can solve the problem of aggregation, the high cost and laborious process are disadvantages to certain applications, especially in the diagnostic platform.[58,59] Rather unexpectedly, the natural sdAbs have long been evolved in camelids[60] and shark[7] as part of their humoral immune system. Due to having several promising criteria to that of conventional mAbs, the isolation of antigen-specific heavy chain antibodies (VHH) of camelids, and V region of shark IgNAR (VNAR) have recently been focused to develop as new binders for targeting antigens from various diseases.[2]

Structural analysis revealed the disulfide bridges observed in VNAR domain are formed by the Cys residues that conserved within framework regions (FR) and complementary

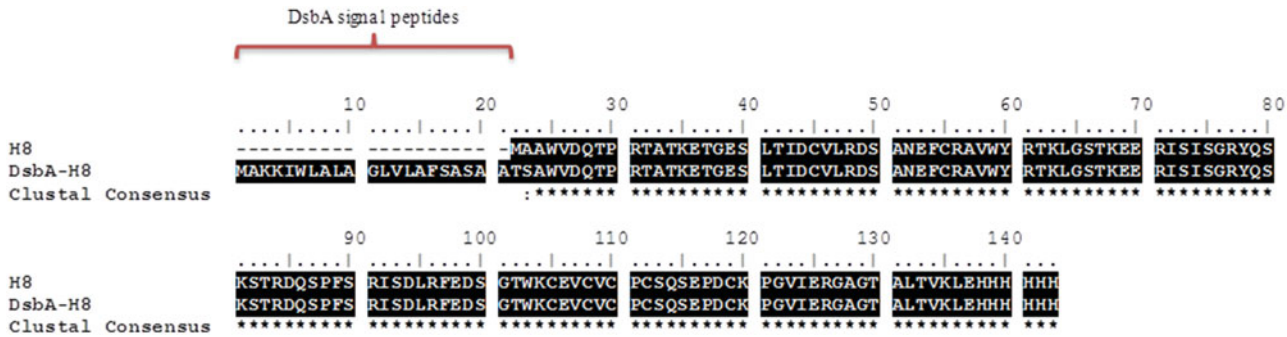

Figure 7. Deduced amino acids sequence alignment of H8VNAR and DsbA-H8VNAR fusion proteins. The 21 amino acid residues of DsbA signal peptides are highlighted at the C-terminal of DsbA-H8VNAR protein.

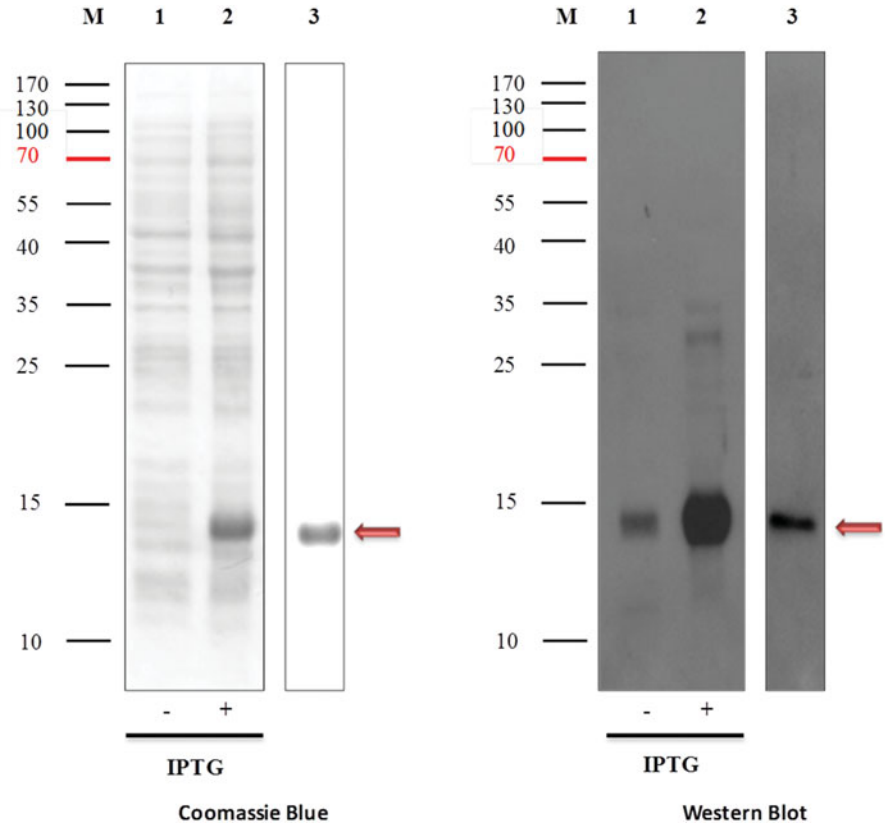

Figure 8. Expression and purification of recombinant cytosolic H8VNAR using pET-28a vector. Proteins were separated on 15% SDS-PAGE and stained with Coomassie Blue (left panel). H8VNAR was detected by Western Blot with anti-His tag antibody and a ~14 kDa band clearly apparent. Lane 1 represents total protein from non IPTG-induced bacteria cells; Lane 2 represents total protein from IPTG-induced bacteria cells; and Lane 3 represents purified cytosolic H8VNAR protein.

determining regions (CDRs).[61,62] To enhance the correct folding capability and stability, an appropriate signal peptide is required to fuze with VNAR proteins thereby the polypeptides can be secreted into the periplasmic space of *E. coli* for disulfide bond formation.[46] In contrast to cytoplasmic expression, translocation of protein into periplasm layer of *E. coli* has been verified to offer better performance in terms of simplifying the downstream processing, higher product solubility, and improved biological activity of the expressed proteins.[20,63] Furthermore, the proteins expressed in the periplasmic space can easily be extracted using a simple osmotic shock which is free from contamination with cytoplasmic proteins.[64]

Several types of signal peptides have been used for periplasmic expression of proteins such as DsbA, OmpA, PhoA, and PelB.[20] Of these peptides, DsbA is, however, one the most common fusion peptide used to produce antibody fragments, including scFv,[65] Fab,[66] and VHH.[67] In addition to antibody fragments, the recombinant human growth hormone was efficiently expressed into higher expression level and increased the solubility using DsbA signal peptides fusion method.[68]

H8VNAR was a clone isolated from a shark immune phage display library. This clone was exhibited to be positively reacting to recombinant PfHRP2 in the monoclonal assay. Based on the deduced amino acids results, H8VNAR contained four non-canonical Cys residues in the CDR3 region with a length of 20 amino acids in its CDR3.[51] So, it was predicted belonging to Type 1 NAR family and are proposed to form an interloop of disulfide bond.[62] Type 1 V_{NAR} was designated to have conserved cysteine residues in FR2 and FR4, with additional cysteine pairs (two or four) encoded within CDR3; Type 2 V_{NAR} has a single cysteine in each CDR1 and CDR3; Type 3 V_{NAR} has been detected predominantly in neonatal sharks.[13,69,70]

In this study, DsbA peptide was employed to express PfHRP2-specific H8VNAR protein using *E. coli* system. To compare the expression yield and binding activity, the gene encoding H8VNAR protein was simultaneously cloned into pET28a vector for expression cytoplasmic protein. The result shows the purified periplasmic H8VNAR with an expected band at ~14 kDa was separated in SDS-PAGE (Figure 9), suggesting the transportation of suggested DsbA signal sequence has efficiently directed VNAR protein from cytoplasmic into periplasmic space via co-translational SRP pathway.[71] It was speculated DsbA signal peptide can be an ideal fusion peptide that leads to produce soluble VNAR proteins with disulfide bond in *E. coli* expression system. Thus, crystallography is planned to determine the conformation of recombinant H8VNAR and verify the presence of disulfide bond in this protein molecule.

In terms of yield, H8VNAR protein extracted from periplasmic space has shown a lower yield compared with that of cytosolic proteins (Table 2). It may partly be affected by

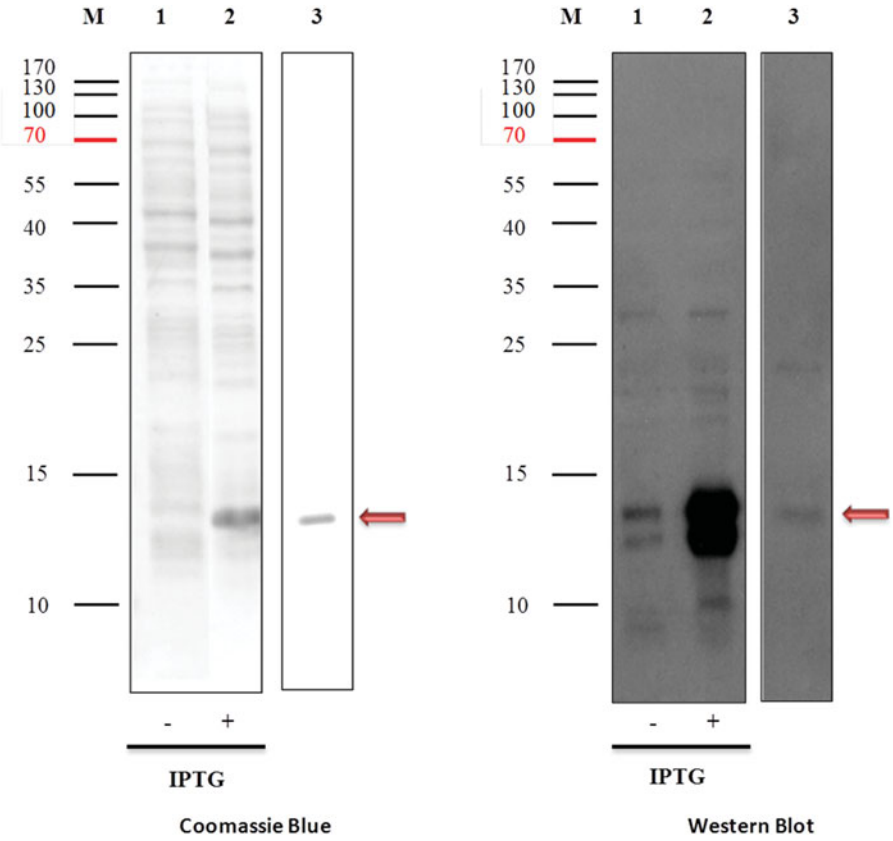

Figure 9. Expression and purification of recombinant periplasmic DsbA-H8VNAR using pDSB-28Y vector. Proteins were separated on 15% SDS-PAGE and stained with Coomassie Blue (left panel). DsbA-H8VNAR was detected by Western Blot with anti-His tag antibody and a ~14 kDa band clearly apparent. Lane 1 represents total protein from non IPTG-induced bacteria cells; Lane 2 represents total protein from IPTG-induced bacteria cells; and Lane 3 represents purified periplasmic DsbA-H8VNAR protein.

the maximum translocation proteins to periplasmic space which results in generally very low yields compared to proteins produced in cytoplasmic space.[72] Moreover, the limited exportation capacity of *E. coli* can lead to the accumulation of recombinant protein expressed in cytoplasmic space thereby turning the aggregated protein to inclusion body and reducing the binding ability of the protein to target its antigen.[73] Despite that, Aoki and coworkers demonstrated that the insoluble VNAR proteins in inclusion body can be redirected to soluble proteins by additional refolding procedure.[45]

One of the problems highlighted in retrospect is the cross-reactivity between anti-His antibody with His residues in this PfHRP2. The reason for this was an abundance of histidine residues in PfHRP2 (34% histidine).[49] To prevent this problem from occurring continuously, the ELISA assays in sandwich format was applied where mAb C1-13 was used as the detection antibody and detected by goat anti-mouse secondary antibody. The binding ability assays were evaluated and compared using ELISA assay. Nevertheless, an alternative to overcome this limitation is planned, including the production of anti-shark VNAR antibody, or modify the recombinant VNAR proteins by adding enzyme fusion conjugates such as horseradish peroxidase (HRP) or alkaline phosphatase (AP) to allow for signal development after binding.

Table 2. Solubility and concentration of purified recombinant cytosolic H8VNAR and periplasmic H8VNAR proteins.

Protein	Solubility	Concentration (μg/mL)
H8VNAR (Cytoplasmic)	Soluble	468
H8VNAR (Periplasmic)	Soluble	62

The low binding ability of recombinant PfHRP2-specific H8VNAR in these assays may partly be influenced by several factors in the prokaryote expression system, such as: (1) The expression of high-level proteins can often lead to low yield of producing active functional proteins in *E. coli*;[74,75] (2) Many commercially available of mAbs or polyclonal Abs today are still obtained from the ascites fluid, serum, or hybridomas.[76,77] In some cases, the production of therapeutic grade antibodies in mammalian cells expression system is preferable due to retaining the efficiency and functionality as to parental antibodies;[78,79] and (3) Although H8VNAR was identified as specific to malaria recombinant PfHRP2 protein, it might not be the highest affinity clone in this library.

To increase the binding specificity of VNAR towards their target antigens, protein engineering can be deployed to correct erroneous protein from eukaryote proteins in the bacterial expression system.[22] For instances, error-prone PCR and random mutagenesis of the amino acid residues in CDR codon have been used to improve the binding ability

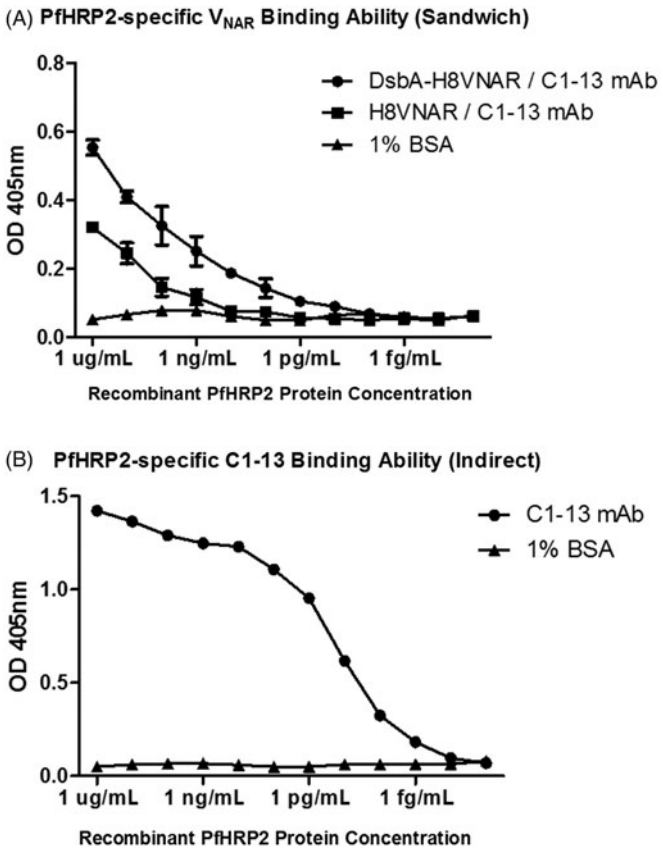

Figure 10. Comparison of binding curves of recombinant H8VNAR proteins with commercial mouse mAb C1-13 against 10-fold serial diluted HRP2 proteins by ELISA. (A) The binding affinity of purified cytosolic and periplasmic H8VNAR proteins was performed in a sandwich ELISA format. (B) The binding reactivity of positive control mAb C1-13 was performed in an indirect ELISA format. 1% BSA was used as negative antigen for rPfHRP2.

and protein functionality in several antibodies, including scFv[80] and camelids VHH.[81] Alternatively, optimization of gene expression system by using different promoters, signal peptides or co-expression of foldase are the other methods used to enhance the solubility and folding capability of the proteins strictly depending on correct disulfide bridges.[20,22] Since lacking appropriate anti-shark antibody the recombinant shark VNAR proteins have been tagged with human constant kappa (HuCκ)[16] or human Fc-region[82] at the C-terminal and expressed in *E. coli* or mammalian expression system, respectively. These fusion proteins can be detected by commercial available secondary antibody rather than raising anti-shark hybridomas.

In conclusion, the results presented in this study demonstrated the utilization of DsbA signal peptide potentially led to the expression of soluble recombinant shark VNAR proteins while enhancing folding capability was evidenced in the binding assays. Despite further optimization conditions under investigation, these findings and insights imply the expression of shark VNAR is possible using the system designated in this study. However, the readings shown by both H8VNARs were still inferior to mAb C1-13. Therefore, further investigation is required to improve the binding affinity of selected recombinant VNAR towards malaria biomarkers.

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