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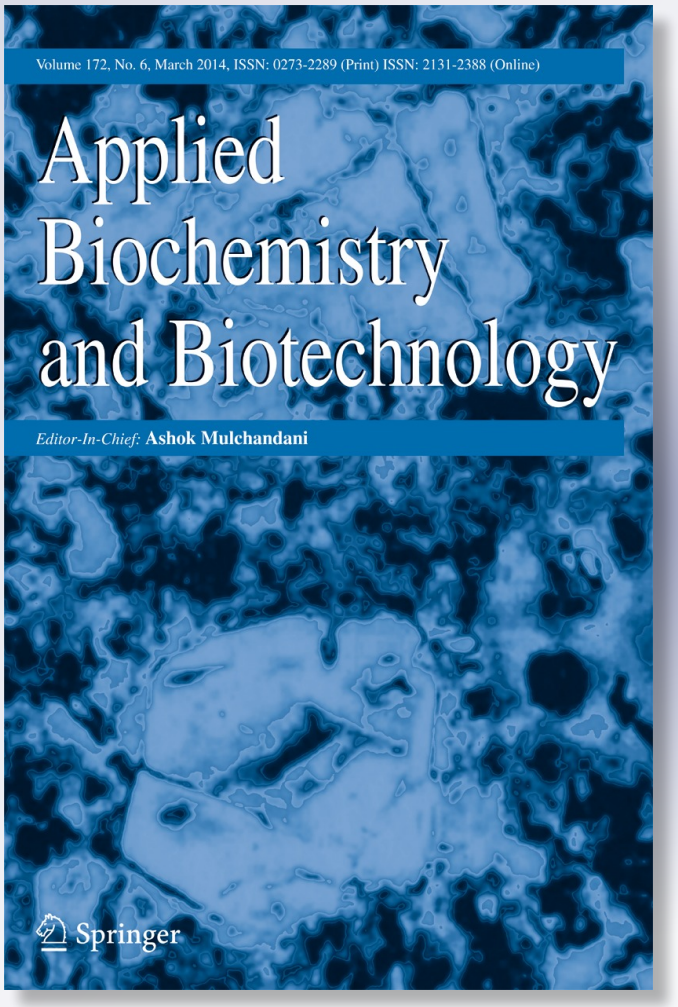

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Functional Mutations in and Characterization of VHH Against *Helicobacter pylori* Urease

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Abstract Manipulation of clinically significant antibodies can effectively improve the processes of diagnosis and treatment. Affinity maturation process has a significant role in improvement of antibodies efficiency. Error-prone PCR technique is one of the proposed methods for improvement of the affinity of antibodies. In the present research, a method was applied to camel heavy-chain antibody (VHH, nanobody) raised against UreC subunit of urease enzyme from *Helicobacter pylori*. This VHH was used as a starting molecule to construct a highly diversified phage displayed VHH library. The constructed library of nanobody mutants was subjected to several rounds of panning against UreC antigen. High-affinity mutant was selected. Our VHH (HMR23) showed 1.5-fold higher binding activity than the parental VHH. In addition, the mutant VHH presented a better performance in inhibition of urease activity at low concentrations retaining its specificity and thermal stability.

Keywords Affinity maturation · Error-prone PCR · *Helicobacter pylori* · Random library · Urease · VHH

Introduction

Current treatment to eradicate *Helicobacter pylori* as a significant public health challenge is antibiotic therapy. However, increasing occurrence of antibiotic resistance would further complicate the treatment of *H. pylori* infections [1, 2]. Consequently, it is important to seek alternative therapeutic strategies without drug resistance problems. A new approach to

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H. pylori treatment is the development of antibodies against specific *H. pylori* targets. Recently, antibody administration against urease enzyme for treatment of *H. pylori* infection has been studied as a new effective therapeutic strategy [3–8]. Urease is an important virulence factor for *H. pylori* and is critical for bacterial colonization in the gastric mucosa. Specific inhibition of urease activity has been proposed as a possible strategy to fight these bacteria [9]. Urease is a multimeric enzyme consisting of two subunits ureA (29.5 kDa) and ureB (66 kDa), at a ratio of 1:1 and is localized in cytoplasm and on the surface of *H. pylori* [10]. Because the enzyme active site is located in the large subunit (UreB, now known as UreC), and as UreC is present in all clinical isolates being highly conserved between strains, antibody against UreC would therefore be an excellent target therapy [10].

However, the antibodies' complex nature, large size, tissue accessibility, immunogenicity, interaction with patient immune system, and inadequate pharmacokinetics are important obstacles for their application [11–14]. Therefore, efforts made in the production of antibodies are aimed on reducing the size to be easily selected and manipulated [15, 16].

The variable domain of heavy-chain antibody in *Camelidae* (VHHs; also called nanobody) are the smallest fully functional antigen-binding fragments (~15 kDa) and can penetrate tissues more effectively than the conventional antibodies. They can also recognize uncommon or hidden epitopes. Nanobodies are featured with natural solubility in aqueous solution, no aggregation tendency, effective pharmacodynamics, proper expression level in bacteria or yeasts, higher thermo and chemical stability, capable of refolding, and also show high homology with the human VH (heavy chain variable domain) [17–19].

In our previous work, a nanobody against recombinant UreC subunit of urease enzyme of *H. pylori* was produced from immune phage library [20]. Although phage display is a very powerful technology for the isolation of antibodies, VHHs isolated from this library have low affinity and often require affinity optimization prior to clinical development [21]. In vitro and in vivo mutagenesis approaches have been employed to improve antibody affinity and stability [22].

Antibody engineering using techniques such as site-directed mutagenesis and random mutagenesis provides the potential to production of desirable antibodies. Among random mutagenesis methods, the error-prone PCR, based on the inaccurate amplification of genes, remains one of the most commonly approaches to generating libraries due to its simplicity, versatility, rapidness, and cheapness [22, 23]. This method consists of random mutagenesis and selected mutant library by panning. Phage display has become a powerful tool for selecting recombinant antibodies and antibody fragments with various specificities and affinities from antibody libraries [24].

In the present study, in an attempt to increase the affinity of anti-UreC-specific VHH isolated from an immune phage display library, we constructed a VHH mutant phage display library with higher affinity and better suppression of the urease enzymatic activity while retaining other essential functional properties, such as specificity and thermostability.

Materials and Methods

Recombinant UreC Antigen Preparation

UreC gene in pET-28a vector was obtained from our earlier work [25]. The recombinant antigen was expressed in *Escherichia coli* BL21 (DE3) and purified as described previously. Briefly, the UreC gene was amplified by PCR and cloned into pET-28a expression vector. Recombinant UreC was expressed in *E. coli* BL21 (DE3) and the protein was purified using

nickel nitrilotriacetic acid (Ni-NTA) affinity chromatography according to the manufacturer's instructions. UreC was also produced in pET-22 without His tag for affinity measurement in ELISA.

Error-prone PCR

For construction mutant library, UreC-specific VHH antibody previously constructed in our laboratory, raised from camelid phage library [20] was used as template.

A pair of primers F1 (5′-ACTGGCCCAGGCGGCCGAGGTGCAGC TGSWGSAKTCKG-3′) and R1 (5′-ACTGGCCGGCCTGGCCTGAGGAGACGGTGA CCWGGGTC-3′) were used for performing error-prone PCRs. F1 matched the framework 1 and F2 matched the framework 4 and amplified whole VHH gene. The underlined nucleotides are *Sfi*I restriction sites.

Two error-prone PCR reactions were carried out according Table 1. These Mutagenic reactions were done with the following conditions: initial denaturation at 94 °C for 5 min, then 35 cycles of 94 °C for 30 s, 66 °C for 30 s, and 72 °C for 90 s, followed by a 72 °C incubation period for 10 min.

Construction of Mutated VHH Library

Error-prone PCR products (400 bp) obtained by both PCRs were combined and purified from a low-melting 1 % agarose gel using agarose gel DNA extraction Kit. Purified mutant VHH fragments and pComb3x vector were digested overnight with *Sfi*I at 50 °C. DNA was purified again and ligated using T_4 DNA ligase. The ligation mixture was transformed into *E. coli* TG1 electrocompetent cells by electroporation. After incubation at 37 °C for 1 h in super optimal broth with catabolite repression (SOC) medium, transformation mixture (0.3 ml) was added to 200 ml Super broth (SB) medium containing 80 μg/ml ampicillin and incubated further (37 °C, with shaking) for approximately 24 h.

Colony PCR was performed to determine the presence of VHHs in the vector. The titer of transformants was determined by plating various amounts of the transformed cells on Luria Bertani (LB) ampicillin plates. Positive clones containing the VHH insert were identified in a standard PCR using the primers F1 and F2 and by restriction digestion of the vectors. The culture served as the UreC-specific VHH random mutagenesis library.

Table 1 Condition of error-prone PCR reactions

Test		Volume	Mutagenic materials						
			$MgCl_2$	dCTP	dGTP	dTTP	dATP	dITP	$MnCl_2$
Error-prone PCR 1	–	100 μl	7 mM	0.2 mM	0.2 mM	0.2 mM	0.2 mM	0.2 mM	0.3 mM
Error-prone PCR 2	Reaction 1	25 μl	1 mM	20 μM	0.2 mM	0.2 mM	0.2 mM	0.2 mM	–
	Reaction 2	25 μl	1 mM	0.2 mM	20 μM	0.2 mM	0.2 mM	0.2 mM	–
	Reaction 3	25 μl	1 mM	0.2 mM	0.2 mM	20 μM	0.2 mM	0.2 mM	–
	Reaction 4	25 μl	1 mM	0.2 mM	0.2 mM	0.2 mM	20 μM	0.2 mM	–

First reaction was performed 10 μL of 10× buffer, 5 ng/μl primers, 1 U Taq polymerase, 2 ng plasmid DNA with 7 mM MgCl2, 0.2 mM deoxyinosine triphosphate (dITP) and 0.3 mM $MnCl_2$ as mutagenic agents. The second PCR mixture contained was four 25-μl reactions performed with descending one of the dNTP (0.2 mM→ 20 μM), and adding 0.2 mM dITP

For displaying mutant library on phage particles, the transformed TG1 cells were infected with M13K07 helper phage. Recombinant phages were obtained by phage rescue by addition of polyethylene glycol (PEG) 6000/NaCl solution [26, 27] and were then collected for biopanning. Briefly, TG1 cells carrying pComb3x-mutant VHHs (library) were infected with 10^{11} plaque-forming units (pfu)/ml M13K07 helper phage. Infected bacteria were incubated for 30 min without shaking and then shaking for 30 min at 37 °C. A 50-μg/ml kanamycin was added to the culture. After overnight incubation at 37 °C, the recombinant phage particles were harvested, the phage particles in the supernatant were precipitated by addition 20 % (*w*/*v*) PEG 6000/2.5 M NaCl ice-cold solution. After centrifugation for 15 min at 15,000×*g*, the precipitated phage particles were collected and resuspended in 2–3 ml of sterile Tris-buffered saline (TBS) containing 1 % Bovine serum albumin (BSA) and phage-containing supernatant was stored at 4 °C, for biopanning. Library size was calculated by plating serial dilution aliquots on LB agar plates and incubated overnight at 37 °C.

Panning of Mutated Library

Biopanning for enriching the recombinant phages was performed on immobilized UreC antigen. A 96-well microtiter plate wells were coated with UreC (10 mg/ml) overnight at 4 °C and blocked for 2 h with BSA 3 %. Subsequently, 10^{11} pfu phages from the mutant library were added followed by 2 h incubation to allow phage binding. The wells were washed three times with Tris-buffered saline with 0.1 % Tween-20 (TBST). Bound phages were eluted with 100 mM glycine-HCl, pH 2.2, for 10 min and the elutions were neutralized with 35 μl of 1 M Tris-base, pH 9. The phage particles were amplified and purified as described in the "Construction of Mutated VHH Library." Eluted phages were amplified in *E. coli* TG1 and used for further rounds of selection.

Ascending washing times and amounts of Tween-20 along with different washing steps were used in subsequent panning cycles for selection of high affinity phages. Phage input and output titers were calculated for each panning round. After four rounds, enrichment of phage particles displaying VHH binding specifically to the UreC protein was monitored by phage ELISA. About 10^{11} freshly prepared phage of the initial library and each selection round were pipetted into wells with immobilized UreC protein (10 μg/ml) and incubated at 37 °C for 2 h. After three times washing of the wells with 200 μl of TBST, bound phage particles were detected with mouse anti-M13 antibody horseradish peroxidase (HRP) conjugate and 3.3′, 5.5′-tetramethyl benzidine (TMB) substrate, stopped with 3 N H_2SO_4, and absorbance was determined with ELISA microplate reader at 450 nm. BSA was used for negative control. After the final round of panning, 25 single clones were randomly picked from the third round of panning and grown in LB containing 80 mg/ml ampicillin and M13K07 helper phage. Cells were removed by centrifugation and phages in the supernatants were used in monoclonal phage ELISAs. Phages bound to microtiter plates coated with 10 μg/ml UreC were detected with HRP-conjugated anti-M13 antibody. Clones showing the highest affinity were subjected to DNA sequence analysis.

Production of Soluble Antibody Fragments

Antibody fragments from two positive clones, i.e., HMR13 & HMR23 with the highest absorbance in monoclonal phage ELISA, were selected for production of soluble mutant VHH antibody. For this purpose, HMR23 and HMR13 were electroporated into *E. coli* non-suppressor strain TOP10F′ and were plated on LB/ampicillin plates.

The selected VHH gene was cloned into the pET28a expression vector using primer set F2: 5′-ACTTCAGAATTCGAGGTGCAGCTGSWGSAKTCKG-3′ and R2: 5′-ACTACAAAGC

TTTTAGGAGACGGTGACCWGGGTC-3′ with *Eco*RI and *Hin*dIII restriction sites, respectively. The ligation product was transformed into *E. coli* BL21 (DE3), and the transformants were screened on LB agar containing 70 μg/ml kanamycin.

E. coli BL21 cells containing pET28a/VHH (OD_{600}=0.6) were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and incubated for 6 h at 37 °C. The cells were harvested and inclusion bodies were extracted by sonication in 8 M urea, pH 8. Protein expressions were detected on 15 % SDS-PAGE and confirmed by western blotting with HRP-conjugated mouse anti-His antibody (1:1,000, Abcam).

The recombinant protein was purified by a Ni-NTA affinity chromatography column. The proteins were eluted with 100 and 250 mM imidazole. Eluted proteins were dialyzed against PBS with descending concentrations of urea at pH 7.0. The recombinant proteins were analyzed by SDS-PAGE and western blotting.

Sequence Analysis of VHH Mutants

The amplified DNA from HMR13 and HMR23 and the parental colony were purified and subjected to DNA sequencing with F1 primer and the results were compared with the NCBI database. The DNA and protein sequences were analyzed with the ClustalW (version 2.1) multiple alignment program (http://www.ebi.ac.uk/clustalw). The parental VHH served as control.

Affinity Measurement

The affinity of the interaction between UreC expressed in pET-22 and parental and mutants VHHs were determined by ELISA. Affinity constant (K_{aff}) was determined using the method described by Beatty et al. [28]. Briefly, the wells of a 96-well plate were coated with UreC at 1.25, 2.5, 5, and 10 μg/ml concentrations in carbonate–bicarbonate buffer, pH 7.2 at 4 °C overnight. The plates were washed three times with PBS, 0.05 % Tween-20. One hundred microliters from each of 0, 1.25, 2.5, 5 and 10 μg/ml concentrations of purified VHHs in PBS buffer was added to UreC coated wells. After 2 h of incubation, the plates were washed and incubated for 1 h with 1:10,000 dilution of HRP/anti-His antibody conjugate. The plates were washed, developed with TMB substrate, and reaction was stopped with 3 N H_2SO_4. The absorbance was determined at 450 nm. K_{aff} was calculated according to Beatty's equation [28]:

$$n = [\mathrm{Ag}]/[\mathrm{Ag}']$$

$$K_{\mathrm{aff}} = (n-1)/2\left(n[\mathrm{Ab}']_t - [\mathrm{Ab}]_t\right)$$

Determination of Binding Specificity

The cross-reactivity of the same concentration of HMR23 and parental VHH was conducted by ELISA with various proteins such as UreC, recombinant C-terminal fragment of UreC protein, LPS from *Salmonella typhi* and *Vibrio cholera*, *Acinetobacter baumannii* biofilm associated protein (Bap), *Clostridium botulinum* neurotoxin type E, BSA, and live *H. pylori.* Various antigens were coated in microplate wells at 10 μg/ml concentrations and 10 μg/ml of the purified VHHs were added to each well. ELISA was performed.

Thermal Stability

Different batches of HMR23 and parental VHH were aliquoted and stored for 7 days at −20, 4, 25, and 37 °C. Each sample was analyzed on 15 % SDS-PAGE. Microtiter plates coated with 10 μg/ml concentrations of the recombinant UreC and 10 μg/ml of the nanobody incubated in different temperature were analyzed by ELISA.

Nanobody Inhibitory Effect on Urease Enzymatic Activity

H. pylori was cultured in brain heart infusion (BHI) for 48 h to reach 10^9 bacteria/ml. In microtiter plates, 10^9 *H. pylori* was incubated overnight with different concentrations of VHH anti-*H. pylori* urease (0–20 μg in PBS) at 4 °C. To each well, 100 μl of PBS buffer, pH 7.0, containing 500 mM urea and 0.02 % phenol red was added. The color development was monitored at 550 nm at 30-min intervals over 3 h at room temperature. BSA was used instead of VHH as negative control. Percent inhibition was determined by: % inhibition=[(activity without VHH−activity with VHH)/(activity without VHH)]×100 [6].

Molecular Modeling

Homology models for the high affinity mutant (HMR23) was constructed to identify the location of the residues changed by error-prone PCR and to see the effect of these mutations on the VHH properties.

The phyre2 program at http://www.sbg.bio.ic.ac.uk/phyre2 address was used to construct initial 3D structural models for parental and mutant VHH. This software aligns unknown protein sequence with known structure of homologous proteins and predicts the 3D structure of protein input.

Results

Library Construction and Screening

The gene encoding the anti-UreC VHH was mutated using error-prone PCR (Fig. 1a) and incorporated into an appropriate phagemid vector. The resulting library in transformed *E. coli* contained 4.0×10^7 unique members. PCR screening confirmed clones from the library to contain VHH with correct size (Fig. 1b). After four rounds of panning, 25 single clones from the third round showing the strongest enrichment (Fig. 2) were screened by phage ELISA. Approximately 80 % of VHH-phage clones tested with anti M13 antibody were positive, of which HMR13 and HMR23 exhibited better UreC recognition.

Expression of Nanobody in Soluble Form

Nanobody was expressed and purified using Ni-NTA affinity chromatography column. A 17-kDa nanobody eluted by 100 and 250 mM imidazole buffer as detected on 15 % SDS-PAGE (Fig. 3a) was confirmed by western blotting (Fig. 3b).

Nanobody Affinity Determination

Affinity analysis of the purified soluble VHH fragments and the immobilized UreC showed an affinity constant of 2.63×10^{-8} M^{-1} for HMR23 equivalent to 1.5-fold improvement as

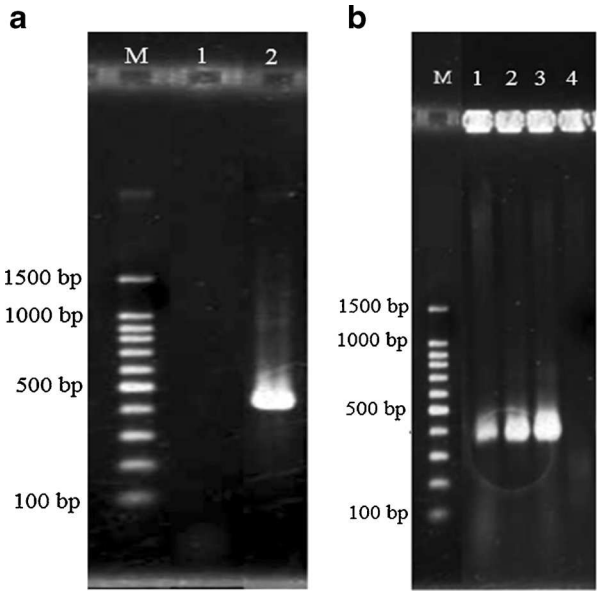

Fig. 1 **a** Agarose gel analysis of the error-prone PCR of anti-UreC VHH (400 bp). *Lane M*, DNA ladder; *Lane 1*, negative control (containing pET28a without gene as a template); *Lane 2*, error-prone PCR product. **b** Colony PCR from constructed mutant library. *Lane M*, DNA ladder; *Lanes 1*, *2*, *3*, PCR products from selected colonies; *lane 4*, negative control

compared to the parental VHH affinity. As HMR13 showed no enhanced affinity, the subsequent analyses were performed with HMR23.

Specificity Characterization

Tendency of VHH to bind UreC and *H. pylori* was higher than the other antigens (Fig. 4); therefore, mutations did not affect the specificity. Binding was detected using an anti-His tag antibody. The nanobody binding was specific to UreC, and no significant cross-reactivity was observed with other proteins.

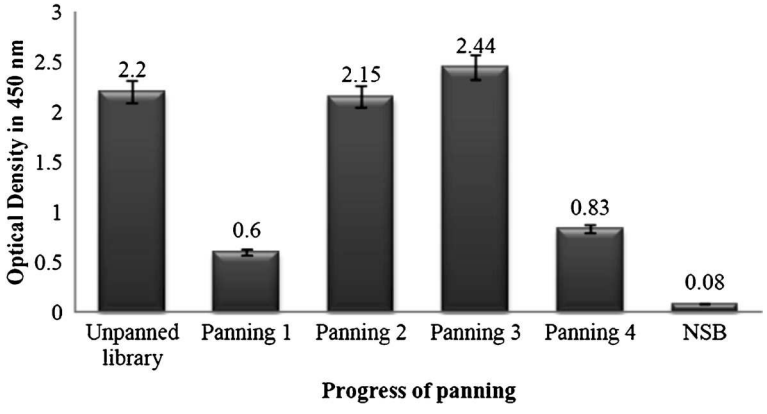

Fig. 2 Polyclonal phage ELISA to determine the UreC-specific enrichment of phages obtained after successive rounds of panning

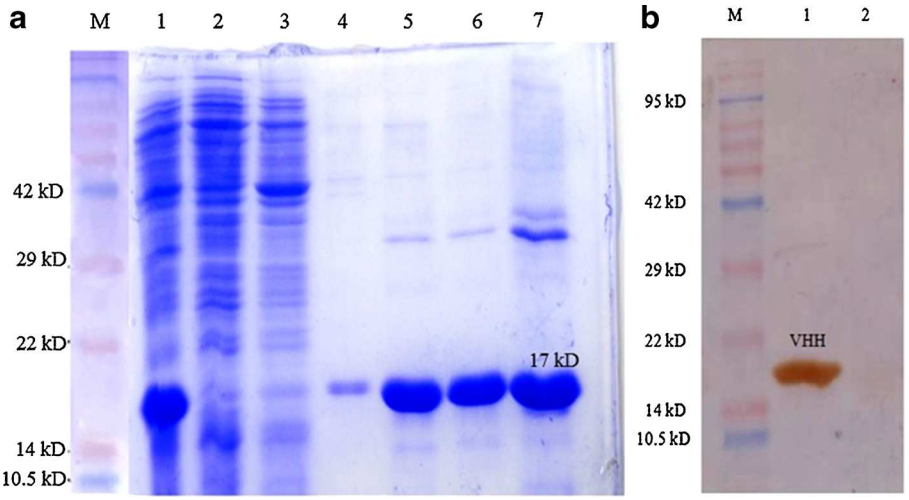

Fig. 3 SDS-PAGE and western blot analysis of HMR23. **a** Purification of HMR23 with Ni-NTA affinity column. *Lane M*, molecular weight marker; *lane 1*, the protein sample was loaded onto the column (total bacterial cell lysates induced with IPTG); *lane 2*: Column effluent from lane 1 before washing; *lanes 3* and *4*: output after washing with wash buffer containing 20 and 50 mM imidazole, respectively; *lanes 5* and *6*, after elution with buffer containing 100 mM imidazole; *lane 7*, elution after washing the column with buffer containing 250 mM imidazole. **b** Western blotting of HMR23 with anti-His tag antibody. *Lane M*, molecular weight marker; *lane 1*, nanobody after induction; *lane2*, negative control

Thermal Stability

Both parental and mutant nanobodies were stable at different temperatures, and thermostability of the HMR23 remained constant as compared with that of the parental VHH. Both parental and mutant nanobodies lost a small percentage of their binding activities. The mutation did not cause loss of thermal stability. About 80 % of the activity of both VHHs is retained at 37 °C (Fig. 5).

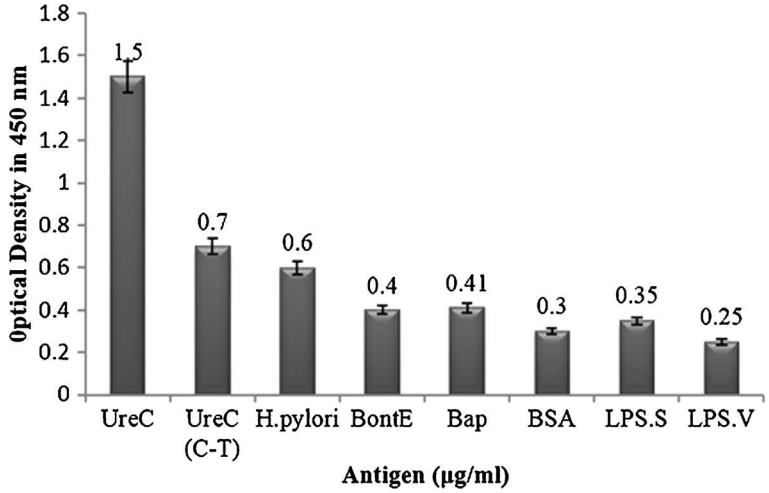

Fig. 4 Binding specificity of VHH antibody to UreC and other proteins

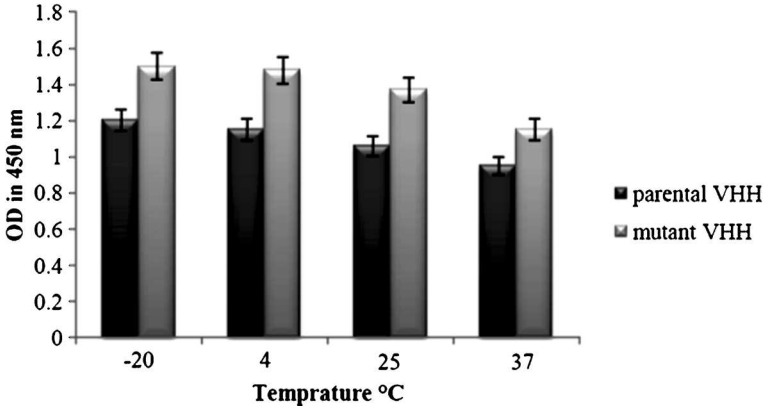

Fig. 5 ELISA analysis of VHHs incubated at different temperatures

Inhibitory Effects of VHHs on Urease Activity

H. pylori urease inhibition test was performed by preincubation of purified VHH with 9ydney strain 1 (SS1) strain. The inhibition percentages were calculated using data obtained after 30 min of the assay and comparing it with the control. Decrease in absorption at 550 nm indicated decreasing of Ph due to urease activity. The mutant and parental VHHs at 20 μg/ml concentration demonstrated the strongest inhibitory effect of approximately 68 and 60 %, respectively (Fig. 6). At higher concentrations of antibodies, inhibition test was plateau. The minimal amount of nanobody required for inhibition of urease was calculated to be 0.6 μg/ml. At a fixed UreC concentration, the inhibitory effect of VHHs was dose dependent.

Sequence Analysis of Mutant and Parental VHHs

DNA sequence of the HMR23 was translated into its protein complement and aligned with the parental VHH to identify the mutations (Fig. 7). Sequence analysis of the mutants showed a vast number of substitutions. Nucleotide deletion or insertion changing the open reading frame did not occur and length of proteins remained constant. Fifteen nucleotide substitutions and 13 amino acid

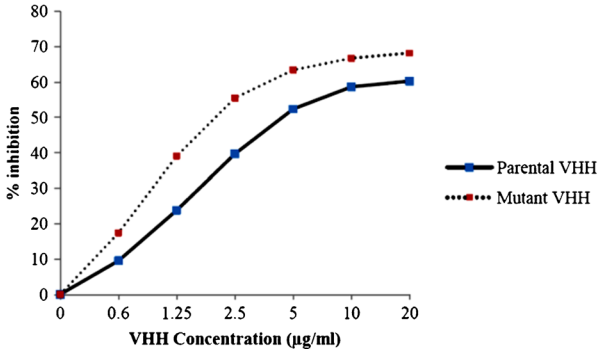

Fig. 6 Inhibition of UreC activity by mutant and parental VHHs

Fig. 7 Comparison of parental VHH antibody sequence with HMR23. Alignment was made by using ClustalW multiple sequence alignment. Identical amino acids in mutant and parental VHH are represented by a *dash*. Each CDR is shown in *bold*

changes occurred in HMR23 sequence. Two nucleotide substitutions occurred in third place which did not changed amino acid changes. The majority of the positions with the highest mutation frequencies occurred in framework regions (FRs). No change occurred in CDR1 and CDR3 except for three changes in CDR2. Figure 8 displays the status of the VHH structure changes.

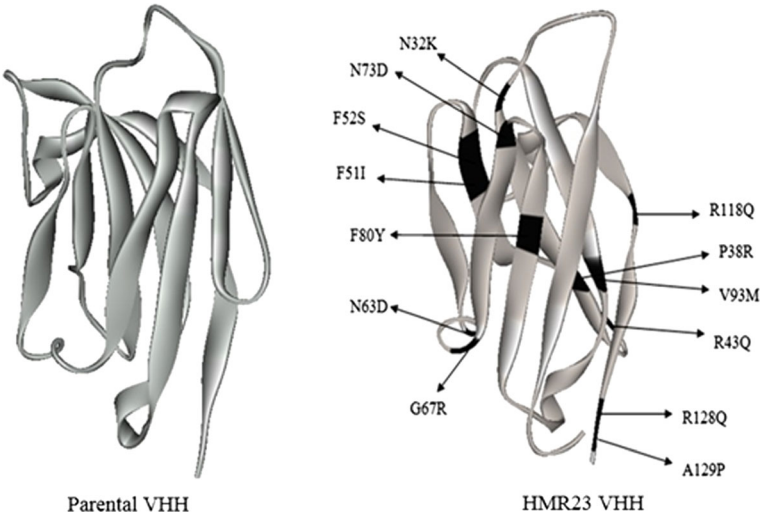

Fig. 8 Homology models of VHH against UreC, with emphasis on the modified residue (*marked black*) in error-prone PCR method

Structural Properties of Mutant VHH

Homology models constructed for the parental and HMR23 VHHs presented improved affinity and urease inhibitory for HMR23 VHH. Three dimensional models were constructed for each VHH which allowed the structural localization of the mutated residues (Fig. 8).

Discussion

With the possibility of protein improvement in the high mutation rate libraries, more unique functional mutants could be found in such libraries [29]. Combination of different mutagenesis techniques to enhance antibody affinity has been used. Random libraries was made by a mutator *E. coli* strain, error-prone PCR, and DNA shuffling yielded a scFv mutant with over 6,000-fold affinity improvement [30]. Antibodies generated by error-prone PCR, and selected by phage, bacterial, yeast, or ribosome display were reported to show 4–1,000-fold increased affinity [31]. In this study, error-prone PCR mutagenesis technique was employed to create a variety of nanobody genes against UreC.

In order to enhance errors brought about by Taq polymerase and consequent diversity of the library, several effective mutagenic factors such as different concentrations of $MgCl_2$, $MnCl_2$, dITP, and various dNTP ratios were used. The average number of 8 mutations in a gene length of 400 bp and 30 cycles of PCR have been reported [31]. In the present study, this number has increased to 13.

A urease-specific VHH with a 1.5-fold increased affinity compared to the parental VHH was isolated. This increased affinity could have been higher than 1.5-fold if some obstacles did not exists. First, the majority of mutations occurred in regions other than the CDR. In particular, CDR3 with having the greatest variation in length and sequence, has a prominent role in determining antibody specificity and affinity, was not changed whereas FRs showed many changes. Second, large affinity improvements by random mutagenesis have been reported only for scFvs, which are different from VHHs in terms of overall structure and antigen-binding mechanisms. Third, the results represent analysis of only a few clones. More clones must be analyzed to determine the effect of random mutations on VHH binding affinity.

Our results demonstrated that HMR23 recognizes UreC antigen without cross-reactivity with the tested proteins. Nagata (1992) showed that other bacteria and jack bean plant urease activity were not neutralized by monoclonal antibodies produced against *H. pylori* urease [6].

The results obtained from HMR23 thermal stability compared with De Genst reports (2006) [32] showed that 80 % VHH activity was retained after a week of storage at 37 °C [33].

At 20 μg/ml concentration of VHH, the inhibitory effects for parental and HMR23 were 60 and 68 %, respectively. In other studies, the inhibitory effect of monoclonal antibodies generated against the large subunit of urease was 80–100 % [5–7]. The above studies have been conducted with purified urease and the cell lysates, while we conducted the experiments with live bacteria. Urease activity of monoclonal antibodies has been found to be weaker than normal cells to inhibit the purified urease [6].

Mutation analysis indicates that changes in the error-prone PCR conditions were able to establish a balance between the types of mutations. A total of 13 amino acid changes observed together could be responsible for improved affinity HMR23. Homology models show that the mutations were not localized to any particular region of folded protein. Most of the changes (10 of 13) were replaced by residues with similar physicochemical properties. Nine of these mutations were more hydrophilic substitutions playing a role in enhancing polar interactions.

Previous studies have shown that positions 11, 14, 35, 37, 44, and 47 were involved in VHH solubility [34]. These positions were not altered in HMR23.

In conclusion, the VHH obtained in this study showed 1.5-fold higher affinity than the parental VHH and presented better inhibitory effect on urease activity while retaining its specificity and thermal stability.

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