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Purification and functional characterization of a Camelid-like single-domain antimycotic antibody by engineering in affinity tag

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ABSTRACT

Single-domain single-chain variable fragment (scFv) antibody is sometimes critical for purification using affinity tagging strategy. We failed in our initial effort to purify a prematurely developed Camelid-like E-tagged short scFv-K2 antibody that contained a complete variable region of the heavy chain and partial region of the light chain by using an anti-E-tag affinity column. To expedite the purification of this altered but interesting antimycotic agent, we replaced a long and large E-tag by a short and hydrophilic 6×-Histidine (His_6) affinity tag by polymerase chain reaction. The short and compact His_6-tag was placed on the previously constructed expression vector pCANTAB 5 E that contained the large affinity E-tag sequence (13 amino acids) by PCR-based mutagenesis and was expressed in *Escherichia coli*. The recombinant protein can then be purified by immobilized metal affinity chromatography (IMAC) and be used for biochemical and other functional characterization. This His_6-tagged short scFv-K2 antibody (20 kDa) had strong cytocidal activity against *Saccharomyces* and *Candida* species with a IC_{50} value of 0.44×10^{-6} M and 1.10×10^{-6} M, respectively. Tag replacement facilitates the purification of a Camelid-like single-domain scFv antibody and after that meets its different functional characteristics. The present study reflects that the V_H domain of the scFv antibody is mainly responsible for its biological activity and single-domain scFv antibody may acts as a potent antimicrobial agent.

Introduction

The expression and subsequent purification of recombinant proteins or antibodies are widely employed in biochemical studies. Protein purification methods typically seek to recover the protein analyte free of contaminants, in high yield, without denaturing the analyte's biological activity. Affinity-based methods meet this requirement that depend on noncovalent bonding between a protein analyte and an immobilizing substrate [1]. Affinity tags are highly efficient and effective tools for purifying recombinant proteins from the crude extracts [2]. A powerful purification method involves the use of peptide affinity tags, which are fused to protein of interest and used to expedite protein purification via affinity chromatography [3,4]. The best fusion tags promote solubility, may function as purification handles and do not interfere with downstream application [5]. The tag sequence is incorporated into the recombinant protein by means of an expression vector positioned alongside the DNA sequence encoding the protein itself. Induction of the vector results in expression of the protein fused to the affinity tag, which can then be purified from the cell lysate. Affinity tagging has made possible the expression and purification of large numbers of proteins by a single purification scheme [6].

Six-histidine (His_6) tag is usually placed at either the N- or C-terminus of a recombinant protein, that allows the metal affinity resin charged with Ni^{2+} or some other transition metal ion (Co^{2+}, Cu^{2+}, Zn^{2+}) to selectively capture the target protein by metal–ligand coordination bonding [7,8]. This technique is known as immobilized metal affinity chromatography (IMAC). His_6-tag is a promising vehicle for rapid and inexpensive protein purification through IMAC. Absorption of the His_6-tagged protein is performed at neutral to slightly alkaline pH to prevent protonation and loss of binding capacity of the weakly basic histidine imidazole groups. Elution of the bound protein is caused by displacement with increasing the concentration of imidazole in the purification buffer or stripping the metals off the resins with strong metal chelators such as EDTA [7–12].

We employed on anti-idiotypic antibodies of a HM-1 killer toxin (HM-1) to generate mycotic drug. The purpose of this study was to investigate the feasibility of an alternative approach for vaccine production. Single-chain variable fragment (scFv) anti-idiotypic antibodies of HM-1 from the yeast *Williopsis saturnus* var. *mrakii*

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IFO 0895 have been produced by recombinant DNA technology from the splenocyte of a hyper immunized mouse by idiotypic vaccination with HM-1 killer toxin neutralizing monoclonal antibody (nmAb-KT) [13–18]. After successful phage-display panning, we isolated five different clones K1–K5. Among them two abundant group clones, K1 (48%; 19/40) and K2 (38%; 15/40) had two different lengths of cDNA insert (780 and 522 bp, respectively) and after clone expression produced two different types of soluble scFv antibody (30 and 20 kDa) [18]. The sequence data generated for scFv-K1 and scFv-K2 have been deposited to the GeneBank, under accession Nos.: GU319877 and GU319878, respectively. Antibody ELISA and Western blotting using horseradish peroxidase (HRP)-conjugated anti-E-tag antibody, both of these clones showed high binding affinity to the specific antigen nmAb-KT. So, we had a keen interest in these two clones for further functional characterization. Previously, we studied on scFv-K1 (a complete scFv antibody) and found that it was a good candidate against different fungal species growth [18]. On the other hand, Camelid-like incomplete scFv-K2 antibody, prematurely translated product had full V_H (variable region of the heavy chain) domain and partial V_L (variable region of the light chain) domain i.e., only initial 14 amino acids in V_L domain instead of 99 amino acids like the complete K1 clone but that had the E-tag sequence. Functional heavy-chain γ-immunoglobulin lacking of light chain exists naturally in Camelidae of which the single N-terminal domain is fully capable of antigen binding [19,20]. Herein, although the Camelid-like E-tagged short scFv-K2 antibody (20 kDa) would show high binding affinity during ELISA and Western blotting against the antigen nmAb-KT, affinity purification through anti-E-tag Sepharose column was not possible as was reported previously [18]. Probably there was no open arm or solvent accessible part of the E-tagged scFv-K2 antibody to bind with the E-tag specific monoclonal antibody resin of the anti-E-tag Sepharose column. The purification handle (E-tag sequence) might interact with another part of the V_H domain or linker region; hence the purification handle was concealed from binding with the anti-E-tag antibody resin of the column. Thus, we failed in our initial effort to purify *Escherichia coli* (*E. coli*) derived Camelid-like E-tagged short scFv-K2 antibody by using an anti-E-tag affinity column for further biochemical and functional analysis. We solved this problem by replacing the large-affinity E-tag with a small and strongly hydrophilic His_6-tag. For comprehensive functional analysis of scFv-K2, sufficient amount of purified antibody is required. Herein, we described the molecular sub-cloning, bacterial expression, purification of this His_6-tagged scFv-K2 antibody without CDR1–3 in V_L domain, and the functional properties of this antibody, which possesses an internal image of HM-1 killer toxin within its variable regions.

Materials and methods

Plasmids, strains, reagents, and enzymes

Anti-E-tag scFv-K2 antibody bearing plasmid pCANTAB 5 E constructions was described in our previous study [18]. The vector pCANTAB 5 H was derived from *E. coli* expression vector pCANTAB 5 E by replacing the large E-tag with a short and compact His_6-tag, and expression was performed using *E. coli* HB2151 non-suppressor strain (GE Healthcare, UK Ltd., UK). All synthesized oligos and PCR primers were purchased from Invitrogen, Japan. KOD-Plus mutagenesis kit, restriction enzyme Dpn I, and DNA ligation kit (Ligation High) were purchased from Toyobo Co. Ltd., Japan and another restriction enzyme NotI was from Takara Bio Inc., Japan. *Saccharomyces cerevisiae* A451 and *Candida albicans* ATCC 10231 were a kind gift from Nippon Roche Research Center. Bacterial expression vector pCANTAB 5 E, HRP-conjugated anti-E-tag antibody, 5 ml anti-E-tag Sepharose column and His-Trap Ni^{2+}–NTA (nickel–nitrilotriacetic acid) Sepharose columns (5 and 1 ml) were obtained from GE Healthcare UK Ltd., UK. Anti-6×-histidine rabbit antibody and anti-rabbit IgG (Fc)-alkaline phosphatase (AP) conjugate (goat) were purchased from Promega, USA. His_6-tag containing octapeptide (AHHHHHHA) was purchased from Sigma Genosys, Japan.

Plasmid reconstruction by DNA manipulation

Escherichia coli expression vector pCANTAB 5 E was reconstructed by replacing the large affinity E-tag via PCR-based mutagenesis using KOD-Plus mutagenesis kit. Isolated pCANTAB 5 E circular plasmid was digested with the restriction enzyme NotI to get linear DNA. Then, the DNA encoding the E-tag domain of scFv-K2 was excised at amino acid in between Ala-158 to Ala-172 and repaired to insert the DNA encoding His_6-tag by PCR amplification using the following two primers, forward 5′-CACCA TCACCATCACCATGCCGCATAGACTGTTGAAAGTTGT-3′ and reverse 5′-TGCGGTCATGGTGACCTTCTCCC-3′. The cycling program started with 94 °C for 2 min for denaturation and continued with 40 cycles of the following thermocycling conditions: 94 °C for 15 s, 51 °C for 30 s, and 68 °C for 5 min/cycle using KOD-Plus DNA polymerase (Toyobo Co., Ltd.). After purification by ethanol precipitation, the PCR products were treated with DpnI that digested methylated parental strands. Circular plasmids were made from the linear DNA using Ligation High and transformed into competent *E. coli* HB2151.

Sequencing of the sub-cloned mutant

Regions of the phage genome encoding the scFv insert of K2 mutant clones were amplified by PCR from bacterial cultures using the following two primers, forward 5′-CCATGATTACGCCAAGCT TTGGAGCC-3′ and reverse 5′-GTAAATGAATTTTCTGTA TGAGG-3′, and subjected to DNA sequence analysis. The amplified DNA portion of the scFv inserts were sequenced by using a CEQ 2000XL DNA analysis system with Dye Terminator Cycle Sequencing Quick Start kit (Beckman Coulter). The sequence alignment and manipulation were performed by using DNASIS program (Hitachi Software Engineering Co., Ltd.) and the Kabat Database system [21].

Sub-cloning and His_6-tagged scFv-K2 production

After successful tag replacement at the C-terminus of the recombinant antibody, the newly constructed pCANTAB 5 H plasmid was transformed into *E. coli* HB2151. Expression and periplasmic extraction of His_6-tagged scFv-K2 antibody was done as described by Yau et al. [22]. Briefly, transformants were cultured in 2× YT (10 g Bacto-yeast extract, 17 g Bacto-tryptone, 5 g NaCl per liter of water) containing 100 μg/ml ampicillin and 2% glucose at 30 °C. Production of the fusion protein was induced in *E. coli* cells in exponential growth phase by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) after which the cultures were grown for a further 12–16 h. Following growth and IPTG induction, bacterial cells were pelleted by centrifugation and resuspended in ice-cold TES buffer (0.2 M Tris–HCl (pH 8.0), 0.5 mM EDTA, 0.5 M sucrose) to collect soluble His_6-tagged scFv-K2 antibody in the periplasmic extract. Insoluble cell debris were removed by centrifugation and the collected periplasmic extract containing His_6-tagged scFv-K2 was dialyzed against phosphate buffered saline (PBS, pH 7.0) using a dialysis membrane (MWCO 15,000 Da) to remove some unwanted salts and non-specific small substances.