Journal of Bioscience and Bioengineering
VOL. xx No. xx, , 2018

www.elsevier.com/locate/jbiosc

Physicochemical improvement of rabbit derived single-domain antibodies by substitutions with amino acids conserved in camelid antibodies

Naoya Shinozaki,[1,2] Ryuji Hashimoto,[1] Masanori Noda,[2] and Susumu Uchiyama[2,3,*]

Modality Research Laboratories, R&D Division, Daiichi Sankyo Co., Ltd., Shinagawa R&D Center, 1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140-8710, Japan,[1] Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan,[2] and Okazaki Institute for Integrative Bioscience, National Institutes of Natural Sciences, 5-1 Higashiyama, Myodaiji, Okazaki, Aichi 444-8787, Japan[3]

Received 28 November 2017; accepted 9 January 2018
Available online xxx

Recently, we showed that immunized rabbit heavy chain variable regions (rVHs) can have strong antigen binding activity comparable to that of the camelid variable domain of the heavy chain of heavy chain antibody (VHH). These rVHs lack the light chain variable regions (rVLs), which exist in the authentic Fab format; thus, molecular surfaces at the interface region of rVHs are exposed to solvent. This physical feature may change physicochemical properties, such as causing reduced stability. By overcoming potential physicochemical issues through engineering the interface region, rVHs could become more useful as single-domain antibodies. In this study, we substituted amino acid residues conserved at the interface region of rVHs with those of VHHs. These substitutions included V37F, involving substitution of a residue in the hydrophobic core with a bulkier hydrophobic amino acid, and G44E/L45R, involving double substitutions of highly exposed residues with more hydrophilic ones. As expected, biophysical and structural characterizations showed that the V37F substitution markedly enhanced the thermal stability through increased hydrophobic packing, while G44E/L45R substitutions greatly reduced hydrophobicity of the interface. The quadruple substitutions of V37F/G44E/L45R/F91Y resulted in not only enhancements of thermal stability and reduction in hydrophobicity, both in an additive manner, but also synergistic improvement of purification yield. This quadruple mutant exhibited greatly reduced non-specific binding with improved colloidal stability owing to the reduced hydrophobicity. The approach used in this study should further enhance the utility of rVHs and promote research and development of single-domain antibodies.

[**Key words:** Single-domain antibody; Rabbit heavy chain variable region; Physicochemical improvement; Amino acid substitution; Thermal stability; Non-specific binding; Colloidal stability]

We have shown that various antigen-specific rabbit heavy chain variable regions (rVHs) can be obtained from immunized rabbit for producing single-domain antibodies (1). In contrast to the antigen-specific VHs of humans and mice (2–7), our study showed that some rVHs have very strong antigen binding activity with K_D values in the sub-nanomolar region. Since rabbits are very easy-to-handle animals for immunization compared with camelids and sharks, which have conventionally been used for single-domain antibody production, the use of rVHs is expected to promote the research and development of single-domain antibodies.

However, for the obtained rVHs, there is room for improvement, including the possibility of achieving better physicochemical properties (1). For example, structural stability was reduced due to the lack of partner light chain variable regions (rVLs) of the rVHs (8–10). Although rVHs could be structurally stabilized by the introduction of an additional disulfide bond, the improvement of purification yield was not clearly observed by disulfide bond introduction with the *Escherichia coli* expression system, which is a versatile protein production system (1). To employ rVHs for various applications, alternative methods should be established for stabilizing them. Additionally, rVHs showed non-specific binding, which should be reduced as much as possible, because non-specific binding to molecules other than the target may cause undesirable side effects upon use for therapeutic purposes. The non-specific binding of rVHs is considered to be due to exposure of the surface interacting with rVLs, which are composed mainly of hydrophobic amino acids (11). Exposure of this hydrophobic surface could also reduce colloidal stability (12). Decreases in structural stability and colloidal stability promote aggregate formation during production, purification, and/or storage (12,13), which is a potential cause of immunogenicity (13). In this context, there is clearly the possibility as well as the need to improve the physicochemical properties of rVHs, especially for their development as biopharmaceuticals.

Methods for improving the physicochemical properties of human VHs have been reported (14–17). One of the major ones is the substitution of amino acid residues with those conserved in camelid VHHs (camelization). For example, Davies et al. reported that a VH generated from human scFv, Ox13-VH, having a short transverse proton relaxation time in NMR spectroscopy, probably caused by aggregation through the exposed hydrophobic VL interaction surface, showed a normal value after the substitution of three residues

* Corresponding author at: Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel.: +81 6879 4216; fax: +81 6879 7442.
E-mail address: suchi@bio.eng.osaka-u.ac.jp (S. Uchiyama).

1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2018.01.006

located on the surface to mimic VHHs (18). In their other study, substitution of another two amino acids together with the introduction of an intradomain disulfide bond, which is frequently observed in VHHs, resulted in improved thermal stability of human VH (3). Such reports on human VH suggest that the introduction of conserved amino acids in VHH could be an attractive approach to improve the physicochemical properties of rVHs that also lack partner VLs.

In this study, we focused on the amino acid residues at the putative VL interaction interface of rVHs that differ from those at the homologous region in VHHs. Specifically, we strategically substituted such amino acids in rVHs with those in VHHs. First, we investigated the impacts of individual substitutions regarding the purification yield, antigen binding activity, thermal stability, and hydrophobicity of the molecular surface. Then, to improve the physicochemical properties further, we introduced multiple amino acid substitutions into rVHs. Our findings reveal that this is a rational and effective approach for improving the physicochemical properties of rVHs.

MATERIALS AND METHODS

Comparison of putative VL interaction interface of rVHs with corresponding region of VHHs To select the amino acid residues of rVHs for substitution with those of VHHs, we compared the amino acid residues of rVHs to VHHs, focusing on their region corresponding to the rVH-rVL interaction interface of rabbit Fv. In this study, all of the residues are numbered according to Kabat numbering (19) and the residues of rVH located on rVL interaction interface were determined as the residues of rVHs within 5 Å from all residues of rVLs in the available PDB structures of rabbit Fv (PDB IDs: 4HBC, 4HT1, 4JO1, 4JO4, and 4O4Y) using Discovery Studio Ver 4.0 (Accelrys, San Diego, CA, USA). Amino acid sequences of our previously obtained rVHs (1) and VHHs reported elsewhere (PDB IDs: 1HCV, 1BZQ, 1KXQ, 1OPQ, 1QD0, 1ZMY, and 1MEL) were then aligned with those of rVHs in rabbit Fvs, and the residues corresponding to "located on rVL interaction interface" were identified for our rVHs and reported VHHs. Identified residues of our rVHs were compared with those of VHHs to select the candidate residues for substitution.

To estimate the physicochemical change accompanying a substitution, modeled structures of rVHs were constructed and structural properties of candidate residues in rVHs and of corresponding residues in VHHs were investigated. The construction of modeled structures was carried out in accordance with our previous study (1), using the antibody structure prediction function of BioLuminate (Schrodinger, New York, NY, USA). For structural characterization, the accessible surface area (ASA) was measured for each candidate residue in the modeled structures of rVHs or the corresponding residue in the PDB structures of VHHs using Discovery studio. In addition, as described below, $\%ASA_{total}{}^{F}$ was calculated as the index of exposure and $\%ASA_{pol}$ as the index of hydrophilicity at the molecular surface.

$\%ASA_{total}^{F}$ was calculated using the following Eq. 1:

$$\%ASA_{total}{}^{F} = ASA_{total}{}^{F} \Big/ ASA_{total}{}^{UF} \times 100 \quad (1)$$

where $ASA_{total}{}^{F}$ and $ASA_{total}{}^{UF}$ represent the ASA of total component atoms of the residue of interest (ASA_{total}) in the folded and unfolded protein. $ASA_{total}{}^{F}$ was obtained as the ASA_{total} of the residue of interest in the modeled structures of rVHs or the PDB structures of VHHs. $ASA_{total}{}^{UF}$ was obtained as the ASA_{total} of the residue of interest maximally exposed to the solvent in the center of tripeptides, which was created with Discovery studio to mimic the unfolded state of protein. These tripeptides consisted of three consecutive residues in the amino acid sequence of rVH, having a candidate residue in the center and the defined rotation angle of peptide bond ($\varphi = -120°$, $\psi = 120°$).

$\%ASA_{pol}$ was calculated using the following Eq. 2:

$$\%ASA_{pol} = ASA_{pol} \Big/ ASA_{total}{}^{F} \times 100 \quad (2)$$

where ASA_{pol} is the ASA of polar components (N, carbonyl O, and OH) (20) and ASA_{pol} was obtained from the modeled structure of rVHs or the PDB structures of VHHs.

Preparation of rVHs The rVHs and those mutants in which amino acid residues in the region corresponding to the rVL interaction interface were substituted with those of VHHs were prepared using *E. coli*, as we performed in our previous study (1). In brief, *E. coli* BL21(DE3) strain (Merck Millipore, Darmstadt, Germany) was transformed with pFLAG-CTS vector (Sigma–Aldrich, St. Louis, MO, USA) containing genes encoding rVHs with FLAG and His tag. Transformants were grown in Luria–Bertani medium supplemented with 100 μg/mL ampicillin. When the optical density at 600 nm reached 1.0, rVH expression was induced by the addition of IPTG at a final concentration of 1 mM and cultured at 16°C overnight. Expanded *E. coli* and culture medium were separated by centrifugation and the cell pellets were collected and then subjected to osmotic shock. After the cell pellets had been suspended in 20 mM Tris-HCl (pH 8.0) supplemented with 0.5 M sucrose and 0.1 mM EDTA, five volumes of distilled water was added to break their outer membrane. The osmotic shock supernatant was mixed with the culture medium, and rVH was purified from this mixture using Ni Sepharose Excel (GE Healthcare UK Ltd., Little Chalfont, UK), followed by gel filtration using a Superdex 75 10/300 GL with the ÄKTA system (GE Healthcare UK Ltd.). The purified protein concentrations were determined from the absorbance at 280 nm with the extinction coefficients that were calculated from amino acid sequences using SEDNTERP ver. 1.09 (University of New Hampshire, Durham, NH, USA).

Evaluation of antigen-specific and non-specific binding The binding activity of rVHs to the antigen was evaluated by surface plasmon resonance (SPR) analysis using BIAcore T200 (GE Healthcare UK Ltd.). Measurement was carried out at 25°C using Series S Sensorchip CM 5 (GE Healthcare UK Ltd.), to which recombinant human ErbB-2/HER2 protein (ACROBiosystems, Newark, DE, USA) was immobilized by amine coupling. The kinetic parameters were determined by a 1:1 binding model of single cycle kinetics using BIAcore T200 Software (GE Healthcare UK Ltd.). Non-specific binding of rVHs was evaluated by ELISA. Purified rVHs were added at concentrations of 25, 50, and 100 nM to a Nunc MaxiSorp flat-bottomed 96-well plate (Thermo Fisher Scientific, Inc., Waltham, MA, USA), pre-coated with bovine serum albumin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). After washing with phosphate-buffered saline (PBS, pH 7.4) containing 0.05% Tween-20, non-specifically bound rVHs were detected with HRP-conjugated anti-FLAG M2 antibody (Sigma–Aldrich, St. Louis, MO, USA).

Evaluation of conformational stability For thermal stability evaluation and thermodynamic analysis of rVHs, differential scanning calorimetry (DSC) analysis was conducted, in accordance with the procedure described in a previous report (1). MicroCal VP-Capillary DSC (Malvern Instruments Ltd., Malvern, UK) was used for measurement at a heating rate of 60°C/h. To evaluate thermal stability, the T_{peak} value (the temperature at which heat capacity peaks) was determined with purified rVH at a concentration of 0.25 mg/mL in PBS (pH 7.4) using the software MicroCal Origin 7 (Malvern Instruments Ltd.). To obtain detailed thermodynamic parameters, DSC analysis was conducted at 1.0 mg/mL. The ΔH values were estimated by the integration of endothermic heat accompanying the unfolding of rVHs. The T_m values were determined as the temperature at which the integration of endothermic heat is equal to half the area of ΔH. In this analysis, we assumed that ΔC_P was constant and determined it as the difference between the baseline of folded and unfolded states in the DSC curve. The enthalpy change [$\Delta H(T)$], entropy change [$\Delta S(T)$], and free energy change [$\Delta G(T)$] were calculated as shown in Eqs. 3–5.

$$\Delta H(T) = \Delta H + \Delta C_P(T - T_m) \quad (3)$$

$$\Delta S(T) = \Delta S + \Delta C_P \ln(T/T_m) \quad (4)$$

$$\Delta G(T) = \Delta H(T) + T\Delta S(T) \quad (5)$$

Because ΔG becomes zero at T_m from Eqs. 3–5, ΔS can be calculated from $\Delta H/T_m$. To evaluate the hydrophobicity of the molecular surface, rVH was subjected to hydrophobic interaction chromatography (HIC) by a method similar to that described previously (21). The experiments were performed at 22°C using an Agilent 1100 HPLC System (Agilent, Santa Clara, CA, USA) with the TSKgel Butyl-NPR column (particle diameter 2.5 μm, inner diameter 4.6 mm, length 10 cm; Tosoh, Tokyo, Japan). Buffer A was 20 mM phosphate buffer (pH 6.9) supplemented with 2 M ammonium sulfate and buffer B was 20 mM phosphate buffer (pH 6.9). rVH was diluted with buffer A from 0.4 to 0.1 mg/mL, injected at a volume of 20 μL, and eluted with a linear gradient of increasing buffer B from 50% to 100% in 15 min at a flow rate of 1.0 mL/min.

Evaluation of colloidal stability The size distributions of rVHs were analyzed using sedimentation velocity analytical ultracentrifugation (SV-AUC). SV-AUC experiments were performed using a Proteomelab XL-I Analytical Ultracentrifuge (Beckman–Coulter, Fullerton, CA, USA). rVH samples of 0.1 and 1.0 mg/mL in PBS (pH 7.4) were measured. Runs were carried out at 60,000 rpm at a temperature of 20°C using aluminum double sector centerpieces and a four-hole An60 Ti analytical rotor equilibrated to 20°C. The sedimentation boundary was monitored with UV detection optics at 231 nm for 0.1 mg/mL and at 289 nm for 1.0 mg/mL. At least 150 scans were collected between 6.00 and 7.25 cm from the center of the rotation axis. All SV-AUC raw data were analyzed by the continuous $C(s)$ distribution model using the software program SEDFIT14.4 (22). The position of the meniscus and the frictional ratio (f/f_0) were varied as fitted parameters. Additional parameters for the analysis, which included partial specific volumes (H2-2-2 WT: 0.71 cm^3/g, H2-2-2 mutant with V37F/G44E/L45R/F91Y substitution: 0.71 cm^3/g), buffer density ($\rho = 1.00$ g/cm^3), and viscosity

Please cite this article in press as: Shinozaki, N., et al., Physicochemical improvement of rabbit derived single-domain antibodies by substitutions with amino acids conserved in camelid antibodies, J. Biosci. Bioeng., (2018), https://doi.org/10.1016/j.jbiosc.2018.01.006