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Highly efficient production of VHH antibody fragments in *Brevibacillus choshinensis* expression system

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ABSTRACT

Anti-IZUMO1$_{PFF}$ VHH (variable domain of camelid heavy chain antibody) clones, N6 and N15, from immunized alpaca (*Lama pacos*) phage library were efficiently expressed and their VHH products were secreted into the culture medium of *Brevibacillus choshinensis* HPD31-SP3, e.g., at a level of 26–95 mg in 100 ml conventional flask culture. With a 3-L scale fed-batch culture for 65 h, the N15 VHH protein with C-terminal His-tag was produced at ~3 g/l culture medium. The N6 and N15 proteins were easily purified to apparent homogeneity by cation exchange and Ni-affinity chromatographies. Both proteins showed specific antigen-binding activity by ELISA and high antigen binding affinity, K_D = 6.0–8.6 nM, by surface plasmon resonance analysis. Size exclusion chromatography-multi-angle laser light scattering analysis revealed that N6 and N15 proteins purified were exclusively monomeric form in phosphate buffered saline. CD spectrum showed beta-sheet rich structure, consistent with a typical antibody structure and also suggested aromatic-aromatic interactions, as indicated by a positive peak at 232 nm. Thermal melting analysis of the N15 protein with C-terminal His-tag demonstrated a clear thermal transition with a Tm at 67 °C. The heat-denatured sample recovered antigen binding activity upon cooling, indicating a reversible denaturation.

Introduction

A host-vector system using genetically engineered *Brevibacillus choshinensis* HPD31-SP3 strain has been developed for secretory production of recombinant proteins [1]. This strain was engineered to remove the ability to form spores and the secretion of proteases. Furthermore, in this system, three promoters with low (P5), middle (P22) and high (P2) expression activity [2] were combined with four signal sequences, i.e., BbrPI [3], MWP [2], P22 [4] and R2L6 [5], to find an optimal combination for high expression of the target proteins. While the expression system proved promising, however, plasmid construction was rather complex and time-consuming. Recently, an easy plasmid construction method, termed "*Brevibacillus in vivo* cloning (BIC)[1]", has been developed, simplifying the overall expression technology drastically [4,6]. It would be great of interest to see how applicable this secretory expression technology is.

Antibodies are currently one of the most widely developed biopharmaceuticals [7–9]. While the whole antibody structures are essential in certain therapeutic applications that require Fc domains, there are some applications that require only antigen binding domains. Such smaller constructs are even preferred, for example, for antibody-drug conjugate (ADC) [10–12]. Small size makes penetration of ADC more efficient into solid tumors. Here, we have applied this expression technology to production of antigen binding domain of Camelid antibodies, VHH [13,14] or called

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[1] *Abbreviations used:* BIC, *Brevibacillus in vivo* cloning; ADC, antibody-drug conjugate; NDOM, N-terminal domain; HRP, horseradish peroxidase; TMB, tetramethylbenzidine; SPR, surface plasmon resonance; TCA, trichloroacetic acid.

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Nanobody [15]. Camelids, such as camel, llama and alpaca, produce uniquely structured antibodies composed only of heavy chains (heavy chain antibodies) as well as conventional tetrameric antibodies composed of two heavy and two light chains [11,13,14,16]. Here, we have observed that the *Brevibacillus* expression system was highly effective in the production of several VHH constructs.

Materials and methods

Bacterial strains and medium

B. choshinensis HPD31-SP3 (FERM BP-8479) was used as competent cells for transformation. The transformants were grown in TM and 2SY medium containing 50 microgram/ml neomycin (TMN and 2SYN). The composition of TM was as follows; 1% glucose, 1% Polypeptone (Nihon Pharmaceuticals, Tokyo, Japan), 0.5% meat extract (Kyokuto Pharmaceuticals, Ibaraki, Japan), 0.2% yeast extract (Nihon Pharmaceuticals, Tokyo, Japan), 0.001% $FeSO_4{\cdot}7H_2O$, 0.001% $MnSO_4{\cdot}4H_2O$ and 0.0001% $ZnSO_4{\cdot}7H_2O$, pH 7.0. The 2SY medium contained 2% glucose, 4% Phytone Peptone (Difco, Detroit, MI), 0.5% Bacto yeast extract (Difco, Detroit, MI), 0.001% $FeSO_4{\cdot}7H_2O$, 0.001% $MnSO_4{\cdot}4H_2O$, and 0.0001% $ZnSO_4{\cdot}7H_2O$, pH 7.2. Glucose was sterilized separately. Transformants were grown at 30 °C for 2–3 days with shaking in test tube or flask culture. Fed-batch culture was performed in 3-L-scale jar fermenter at 30 °C for 65 h.

Plasmid construction

The linearized *B. choshinensis* cloning and expression vectors, pBIC1, pBIC2, pBIC3 and pBIC4 [4], were used. These vectors were developed by us for the speedy application of BIC method for any target genes to be expressed. Three kinds of expression vectors harboring VHH gene with C-terminal His-tag, N-terminal His-tag and without His-tag, were constructed as follows. When the target gene is inserted into the vector between B and C positions in Fig. 1A, the expressed protein contains an amino (N)-terminal His-tag. When inserted into the vector between A and C positions, the expressed protein contains no His-tag. For the expression of target protein with carboxy (C)-terminal His-tag, a DNA fragment harboring target gene with C-terminal His-tag sequence will be readily inserted into the vector between A and C positions in Fig. 1A. These linearized pBIC1∼4 plasmid DNAs are available from TAKARA BIO, Japan, into which the target genes can be readily inserted. Namely, the target genes were made to contain ∼15 bp sequences at 5′- and 3′-ends that overlap with the 3′- and 5′-ends of the linearized vector. These two linearized DNA fragments undergo spontaneous *in vivo* recombination in *Brevibacillus* competent cells using vendor's protocol. PCR amplification of the target genes with such appropriate overlapping sequences were performed following manufacturer's instruction (primer sets shown in Table 1).

The Camelid antibody was generated against an N-terminal domain (NDOM) of $IZUMO1_{PFF}$ [17]. The NDOM of $IZUMO1_{PFF}$ was expressed in and purified from *Escherichia coli* BL21(DE3), and used to immunize alpaca (Miyazaki et al., submitted). The isolation of VHH clones, termed N6 and N15, was performed by biopanning against the NDOM antigen of VHH phage display library constructed from the peripheral blood monocytes of the immunized alpaca (Miyazaki et al., submitted). The N6 gene (DDBJ Accession Number: AB926002) was amplified by PCR and used to construct N6 with C-terminal His-tag (N6-C-His, Fig. 1B-1), N6 with N-terminal His-tag (N-His-N6, Fig. 1B-2), and N6 without His-tag (N6-nonHis, Fig. 1B-3). With regard to N15 (DDBJ Accession number: AB926006), only C- and N-terminal His-tag constructs, N15-C-His and N-His-N15 (Fig. 1B-1 and B-2), were prepared. The primer sets for amplification of N6 and N15 genes are summarized in Table 1.

Purification of VHH proteins expressed with or without His-tag

Culture supernatant, dialyzed against 25 mM Na-phosphate buffer (pH 6.0 for N6-nonHis and pH 6.7 for N6 and N15 constructs with His-tag) overnight, was applied to High-Trap SP column (GE

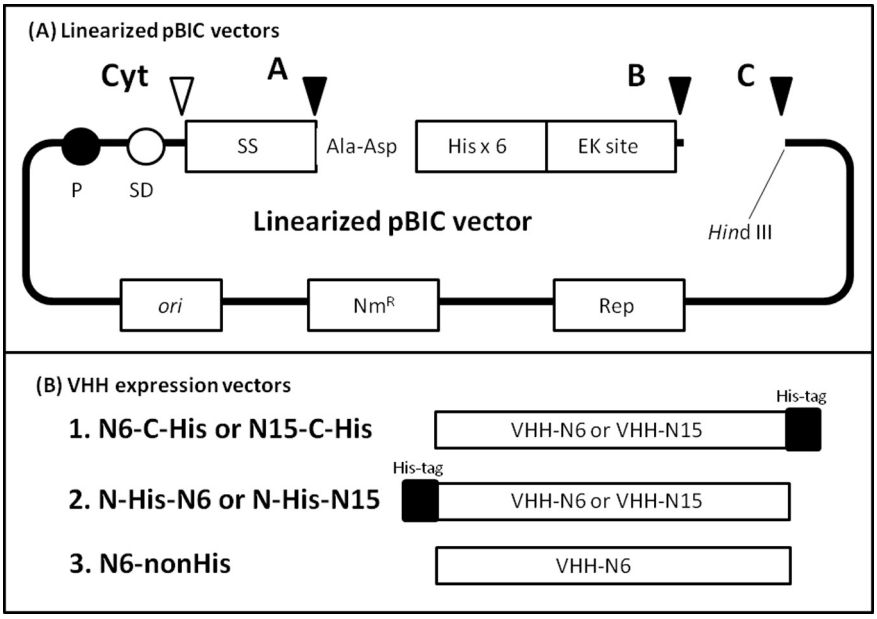

Fig. 1. Schematic illustration of pBIC expression vectors and VHH constructs. (A) structure of linearized pBIC vectors. P, P22 gene promoter; SD, Shine-Dalgarno sequence; SS, signal sequences. BbrPI, MWP, P22 and R2L6 signals for pBIC1-4, respectively; His x 6, His-tag; EK site, enterokinase cleavage site; *ori*, replication origin; Nm^R, neomycin resistant marker; Rep, gene for replication from pUB110. For secretory production, target gene can be inserted at (A)–(C) site for direct expression, and at (B) and (C) site for expression with N-terminal His-tag. For cytoplasmic expression, gene can be inserted at Cyt-C site. (B), schematic illustration of expression plasmids used in this study, N6 and N15 with C-terminal His-tag (1), N6 and N15 with N-terminal His-tag (2), and N6 without His-tag (3).