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Enhanced periplasmic expression of high affinity humanized scFv against Hepatitis B surface antigen by codon optimization

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ABSTRACT

Production of properly folded, functional recombinant antibodies in a prokaryotic system is governed by multiple factors like codon usage, plasmid copy number, upstream elements such as leader sequence, mRNA stability and presence of tightly controlled promoters. Here we present a strategy for enhanced production of the functional scFv in *Escherichia coli* by codon optimization. We have previously reported the generation of humanized scFv form of a potentially neutralizing mouse monoclonal antibody (5S) to the Hepatitis B surface antigen. However, the expression level of 5S-scFv in *E. coli* was fairly low which was possibly due to the presence of rare codons. In the native 5S-scFv gene, almost 58% of codons showed poor codon bias with varying degrees of rare occurrence in the *E. coli* genes. We therefore designed a synthetic gene encoding the 5S-scFv protein by using *E. coli* preferred codon usage. The codon-optimized scFv gene was further cloned into a T7 expression system with a C-terminus His-tag and expressed as a soluble protein mainly in the periplasm. The scFv was both purified by IMAC and detected on Western blot with this His-tag. Using the codon optimization strategy, we were able to achieve a more than 100-fold increased periplasmic expression of soluble scFv. Further, the purified scFv was stable and retained its antigen-binding affinity and epitope specificity. Interestingly, based on secondary structure prediction, we observed that the mRNA secondary structure, including that of the 5′-end, may not have a significant role in the increased expression of this optimized gene.

Introduction

In spite of universal vaccination and safe blood practices Hepatitis B is likely to continue as a major cause of morbidity and mortality. More than 350 million people worldwide are infected with Hepatitis B virus (HBV) [1,2] and a high proportion of these are likely to develop chronic infection which ultimately leads to chronic liver disease, cirrhosis and hepatocellular carcinoma [3]. Considerable evidence has accumulated that antibody targeting the Hepatitis B surface antigen (HBsAg) protects against infection [4]. Currently, HBIG (Hepatitis B Immunoglobulin) collected from the blood of human donors, is used for post-exposure prophylaxis in many cases such as accidental needle stick injuries, for prevention of vertical transfer of HBV infection from mother to child [5–7] and for passive immunotherapy of liver-transplant patients [8,9]. However, such blood-derived products are costly and can cause cross-contamination [10,11]. Therefore a recombinant antibody to HBsAg can be a suitable alternative to such a practice.

Although several recombinant antibodies against HBsAg have been reported in literature, none is available for clinical uses [12–14]. We have previously reported the humanization and characterization of a single chain variable fragment (scFv) form of a high affinity mouse monoclonal, 5S, against neutralizing epitope of HBsAg [15]. This antibody, which shows high affinity and stable binding in presence of various destabilizing agents, is therefore of therapeutic interest [16].

However, it shows a low level of soluble expression in an *Escherichia coli* host, thus limiting its use for further application. Expression of a properly folded protein is largely dependent on the intrinsic features of the gene encoding the scFv construct and the expression plasmid. Factors like codon usage, plasmid copy number [17,18], upstream elements such as leader sequence, stability of mRNA [19] and presence of tightly repressible promoters [20,21], all have an influence on expression yield.

When we analyzed the codon usage in our scFv gene, almost 58% of codons showed poor codon bias with varying degrees of rare occurrence in *E. coli* genes [22]. We interpreted that poor codon bias in the native scFv gene and use of phagemid vector may be a possible cause of inefficient translation and scFv production in *E. coli*. To circumvent this problem, we decided to optimize codon usage of

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scFv gene by replacing the poor codons with host-preferable codons that have higher corresponding tRNA concentrations [23–25].

In this work, we demonstrate a method of coding sequence engineering that has a positive effect on the level of scFv production. Taking advantage of the degeneracy of the genetic code and the proven successes of codon engineering in *E. coli*, we designed a synthetic gene encoding the 5S-scFv protein using the *E. coli* preferred codon usage. The codon optimized variant showed a significant increase in expression, and primarily expressed as a soluble protein in periplasmic space. The purified scFv fragment retained the high affinity binding and epitope specificity of the original 5S mouse monoclonal.

Materials

Expression vector pET-22b (Novagen, USA) was used for expressing the recombinant antibody fragment. *E. coli* strain BL21 (DE3) from Invitrogen was used as the gene expression host. The anti-His_6 monoclonal antibody IgG (BD Biosciences) was used to detect the expression of scFv-His fusion protein. Purified recombinant HBsAg expressed in a Pichia system was provided by Shanta Biotech, India and serum purified HBsAg was provided by Yashraj Biotech, India.

Methods

Generation and humanization of 5S-scFv

The strategy used for the generation of scFv is discussed in detail in our previous papers [15]. In brief, the variable regions of the heavy chain and light chain of 5S antibody were amplified by RT-PCR using RNA isolated from the mouse hybridoma cell line (5S), which produces an anti-HBsAg antibody (IgG1). The scFv of 5S was constructed by linking these amplified V_H and V_L with the help of an inert linker and cloned in a phagemid vector. The 5S-scFv was humanized by grafting its antigen-binding site onto the frameworks of the human consensus sequence showing highest sequence homology [15]. The structural significance of each mouse residue that differed from the consensus sequence was analyzed individually using the molecular model of 5S-scFv and only those residues were mutated, where the substitution was unlikely to result in any significant alteration in the structure and antigen binding properties of the mouse scFv.

Construction of native and codon-optimized 5S-scFv expression vectors

For native 5S-scFv expression vector construction, 5S-scFv gene was digested from pCANTAB-5E-5S-scFv plasmid using *Nco*I/*Not*I site and subcloned downstream of the pelB signal peptide sequence of pET-22b(+) vector with similar restriction sites to construct native 5S-scFv expression vector pET22b-5S-scFv. For codon-optimized 5S-scFv expression vector construction, the native 5S-scFv gene was optimized according to preferred codon usage in *E. coli* using proprietary algorithm 'Gene Designer software' (www.DNA20.com) that replaces rare codons, rare codon clusters and avoids sticky mRNA structures. We identified 57 rare codons [26], some rare codon clusters and 92 other less preferred codons in the native gene, which were substituted by the *E. coli* preferred codons in the synthetic gene (Fig. 1A and B). The optimized scFv gene was synthesized from 75–85 mer oligonucleotides and then inserted into the pET-22b expression vector. A schematic diagram of the PCR strategy and scFv gene cloning is shown in Fig. 2A and B, respectively). Briefly, eight oligonucleotide primers (Integrated DNA Technology, Germany) of 75–85 nucleotides in length with an overlap of 30 nucleotides were sequentially linked by overlap PCR to generate codon optimized V_H and V_L domain construct independently as previously described [27]. The nucleotide sequences of primers used in overlap extension are given in Table 1.

Three overlap PCR amplifications were required to generate each variable domain gene. Initially, the inner most pair of primers (1 and 2) was amplified with the PCR condition: for 20 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, followed by final extension of 10 min at 72 °C. The product of the initial assembly reaction was diluted 10 times and used as the template for further extension with the next set of adjacent external primers (3F and 3R). The third and final extension utilized primers 4F and 4R. The resulting PCR product containing V_H and V_L domain gene was gel purified and further joined by overlap PCR as 3′-end of V_H and 5′-end of V_L fragment are complementary to each other. Both the gene fragments were taken in equivalent molar ratio and linked by PCR (20 cycles, 60 °C annealing) without any primer. The resulting PCR product was diluted 10 times and amplified by pull through PCR (35 cycles, 50 °C annealing) using primer V_H1 and V_L4R. All the PCR based linking of oligonucleotides and final amplification of the codon-optimized scFv gene was accomplished by using high fidelity polymerase (Fusion). After digestion with *Nco*I and *Not*I, the assembled codon-optimized scFv gene was cloned into the pET-22b vector. (Fig. 2B).

Expression and purification of 5S-scFv

The recombinant plasmid, pET22b-5S-scFv was transformed into BL21 (DE3) competent cells. A single clone from transformed cells was selected and grown at 37 °C in 10 ml LB medium with 100 μg/ml ampicillin and 2% glucose. The seeded culture was inoculated into 100 ml fresh LB medium containing 2% glucose and shaken at 250 rpm for 1 h at 37 °C. The culture was harvested by centrifugation at 1500*g* at room temperature for 10 min. The bacterial pellet was re-suspended in 1 L fresh 2× YT medium (without glucose) in the presence of antibiotic and cultured at 27 °C. When the OD_{600} reached 0.8–1.0, 1 mM isopropyl-β-D-thiogalactosidase (IPTG) was added and incubation was continued at 27 °C for 6 h. The cells were then harvested by centrifugation at 6000*g* for 15 min at 4 °C and periplasmic extract were prepared by resuspending the subsequent pellet in 1/50 volume of ice-cold 1× TES buffer (0.2 M Tris–HCl, pH 8.0, 0.5 mM ethylenediaminetetra-aceticacid (EDTA), 0.5 M sucrose) and 1/40 volume of ice-cold 0.2× TES buffer and incubated on ice for 30 min. The re-suspended extract was centrifuged at 14,000 rpm to obtain the supernatant containing scFv which was stored at −20 °C. The periplasmic proteins were dialysed overnight against 3 L of PBS containing 10 mM imidazole. The recombinant humanized scFv was purified from the dialysed periplasmic extract by metal-affinity chromatography using HisLink™ protein purification resin (Promega) as per the manufacturer's protocol. In brief, the column containing HisLink™ resin (2 ml) was equilibrated with five column volumes of binding buffer (100 mM HEPES, 10 mM imidazole, pH 7.5). The periplasmic extract was allowed to pass through the column at a flow rate of 1 ml/min. The column was washed thoroughly by 10–20 volume of wash buffer (100 mM HEPES; 10 mM imidazole; pH 7.5) and the polyhistidine-tagged scFv was eluted out by three volume of elution buffer (100 mM HEPES; 250 mM imidazole; pH 7.5). The eluate was dialysed overnight against 3 L of 20 mM Tris–HCl pH 7.5, including 10% glycerol at 4 °C. Purified scFv was concentrated five times its original concentration by Centriprep-10 filter and the aliquots were stored at −70 °C. Protein concentrations were estimated by bicinchoninic acid (BCA) protein assays (Pierce, USA). Different dilutions of BSA were used to draw the standard curve, which was used to estimate the total protein content of a sample.