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High-level expression of codon optimized foot-and-mouth disease virus complex epitopes and cholera toxin B subunit chimera in *Hansenula polymorpha*

Houhui Song,[a] Li Zhou,[a] Weihuan Fang,[b] Yong Li,[a] Xu Wang,[a] Hongbo Fang,[c] Xiangdong Li,[c] Mingyu Wu,[d] and Bingsheng Qiu[a,*]

[a] *Molecular Microbiological Centre, Institute of Microbiology, Chinese Academy of Sciences, 13# Zhongguancun Beiyitiao, Beijing 100080, PR China*
[b] *College of Animal Science, Zhejiang University, Hangzhou 310029, PR China*
[c] *College of Life Science, Beijing Normal University, Beijing 100875, PR China*
[d] *College of Life Science, Xi'an Jiaotong University, Xi'an 710049, PR China*

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Abstract

A codon optimized DNA sequence coding for foot-and-mouth disease virus (FMDV) capsid protein complex epitopes of VP1 amino acid residues 21–40, 135–160, and 200–213 was genetically fused to the N-terminal end of a 6× His-tagged cholera toxin B subunit (CTB) gene with the similar synonymous codons preferred by the methylotropic yeast *Hansenula polymorpha*. The fusion gene was synthesized based on a polymerase chain reaction (PCR) and subsequently overexpressed in *H. polymorpha*. The chimeric protein was successfully secreted into the culture medium (up to 100 mg/L) and retained the antigenicity associated with CTB and FMDV antibodies by Western blot analysis. The chimera after purification through Co^{2+}-charged resin column bound specifically to GM1 ganglioside receptor and thus retained the biological activity of CTB. This study has important implications in the construction of CTB chimera for mucosal vaccines against FMDV.

Keywords: Foot-and-mouth disease virus; Epitope; Cholera toxin B subunit; Synonymous codon; *Hansenula polymorpha*

Foot-and-mouth disease virus (FMDV) is the causative agent of an economically important disease that affects cattle and other cloven-hoofed animals. The precise location of B and T cell epitopes of the capsid protein VP1 has been established flanking amino acid residues 21–40, 135–160, and 200–213 [1–3]. These areas have been extensively used as synthetic peptides or as fusion proteins in the formulation of experimental immunogens for the induction of neutralizing antibodies and protection of natural and experimental hosts [4–6]. However, high-cost peptide vaccines have many problems such as short persistence and poor immunogenicity, or even integration into the host genome as in the cast of naked DNA vaccines [7]. For this reason, it is essential to consider the efficiency and practical use of vaccines based on T and B cell epitopes in order to induce an effective immunity.

One strategy is to develop mucosal vaccines employing bacterial enterotoxins such as cholera toxin B subunit (CTB) from *Vibrio cholera*. CTB is highly immunogenic when delivered mucosally and can act as a carrier to stimulate response to linked antigens, and as well as efficiently help present vaccines to the mucosal immune system [8,9]. Also, CTB adjuvants can regulate IL-12R expression and subsequent Th cell subset responses [10], which result in increased total IgG1, IgG2a, and IgA expression [11]. Furthermore, the conjugates to CTB can dramatically lower the threshold concentration of antigens required for immune cell activation and also permit low MHC II expressing antigen-presenting cells to prime for a specific immune response [12]. Therefore, the recombinant CTB provides

* Corresponding author. Fax: +86-10-6262-2101.
E-mail address: qiubs@sun.im.ac.cn (B. Qiu).

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a safe and potent mucosal adjuvant applied in the fields of immunology.

The methylotrophic yeast, *Hansenula polymorpha*, has been successfully used for large-scale production of heterologous proteins over the past 15 years because of its thermotolerance and capability of faster growth in simple defined media [13,14].

In this study, a codon optimized DNA sequence coding for FMDV capsid protein complex epitopes of VP1 amino acid residues 21–40, 135–160, and 200–213 was genetically fused to the N-terminal end of a 6× His-tagged CTB (hCTB) gene according to the codon usage preferred by *H. polymorpha*. The chimera was expressed in *H. polymorpha* on a scale of 3 L fermentations and examined by Western blot and ELISA analyses. To our knowledge, this paper constitutes the first report of the expression of VP1 complex epitopes and CTB chimera with high concentrations of purified protein in yeast systems, which could be directly applied in the development of mucosal vaccines.

Materials and methods

Organism and growth. *Hansenula polymorpha* wild-type *ATCC* (*JCM 3621*) was grown in YPD medium: 1% (w/v) difco yeast extract with 2% bacto-tryptone and 2% (w/v) dextrose. The selective medium was made by supplementing 100 μg zeocin/ml in the YPD medium. *Escherichia coli* DH5α was used for pGEM-T derived vector construction. It was grown in LB medium supplemented with 100 μg ampicillin/ml.

Gene modification and synthesis. Synonymous codon usage in genes from the yeast *H. polymorpha* was investigated based on a multivariate statistical analysis software (http://bioweb.pasteur.fr/seqanal/interfaces/codonw.html). The sequence coding for O-type-FMDV VP1 complex epitopes (amino acid residues 21-40, 135–160, and 200–213) was genetically fused to the N-terminal end of a 6× His-tagged CTB gene based on the synonymous codons preferred by *H. polymorpha* and named VP1-hCTB (Fig. 1). Three flexible linkers (amino acids: GGSGG) were added at the end of VP1 epitopes (Fig. 1).

The complete VP1-hCTB gene was assemblied by polymerase chain reaction (PCR) described elsewhere [15]. The purified PCR product was cloned into pGEMT-vector (Promega) and sequenced. The recombinant plasmid was named pGEMT-VP1-hCTB.

Plasmid construction. The plasmid pHFMDZ-alpha-VP1-hCTB was constructed by digestion of the pGEMT-VP1-hCTB vector with *Xho*I/*Xba*I and subsequent ligation of the fragment into the similarly digested pHFMDZ-alpha-A, we previously constructed [16].

Yeast transformation and screening. The competent cells of *E. coli* were transformed using one-step procedure [17]. The *H. polymorpha* transformation with the plasmid pHFMDZ-alpha-VP1-hCTB and the subsequent multiple copies screening were performed as described by Song et al. [16].

Fermentation conditions. Pre-culture from a single colony with multiple copies was grown for 12 h in a test tube (3 ml) with YPD medium supplemented with 100 μg/ml zeocin at 37 °C for 200 rpm. Then, the first pre-culture was inoculated into 300 ml pre-culture medium in shake flask and incubated for further 12 h until $OD_{600} = 8.0–10$ at the same conditions. The cells were harvested by centrifuging and resuspended in YPG medium (1% difco yeast extract with 2% bacto-tryptone and 2% glycerol). For the 3 L scale fermentations, a 5 L scale BIOFLO-IIc fermenter (New Brunswick Scientific, Edison, USA) containing 3 L YPG was inoculated with the second pre-culture cells. After complete consumption of initial glycerol in the medium, a pO_2-controlled feed was started to maintain glycerol concentrations between 0.05 and 0.4% (w/v) with YPG containing 50% glycerol. During the whole 80 h induction, the culture conditions were maintained as follows: pH 4.6, 37 °C, aeration rate 10 L/min, stirrer speed 600 rpm, and oxygen levels at a minimum value of 20% saturation.

Purification of recombinant VP1-hCTB. Secreted proteins in supernatant were harvested by centrifuging at 12,000 rpm for 30 min to discard the cell pellets. For 6× His-tagged VP1-hCTB, Co^{2+}-loaded resin (BD Biosciences Clontech) was used to purify the recombinant protein followed by elution using a gravity-flow column starting with extraction/wash buffer (50 mM sodium phosphate, 300 mM NaCl, and 10 mM imidazole, pH 7.0) to elute impurities and ending with the same buffer containing 150 mM imidazole to elute the targeted protein. Fractions were evaluated by 12% SDS–PAGE and stained with Coomassie brilliant blue R-250.

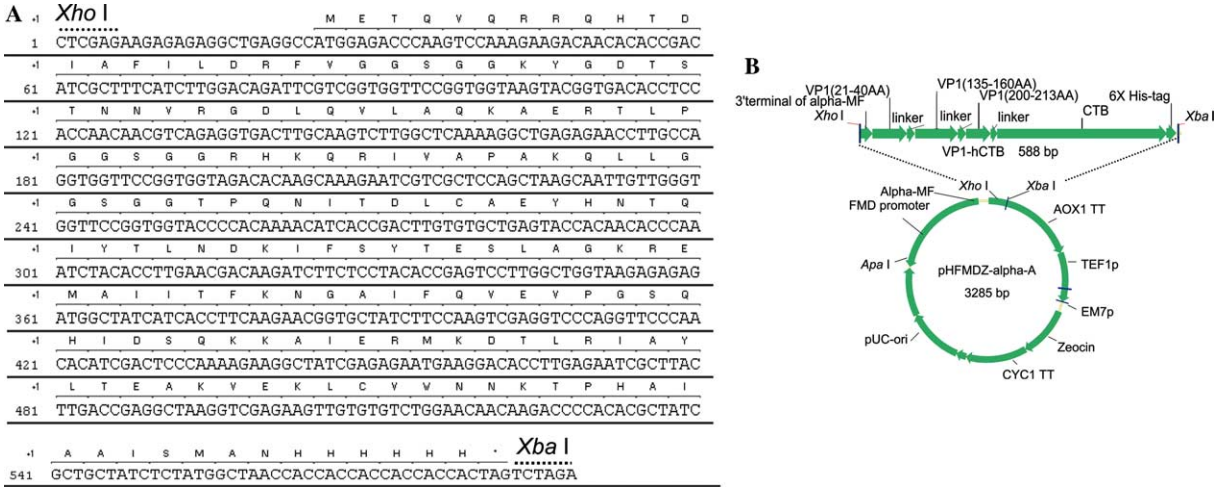

Fig. 1. Schematic representations of the gene design and expression vector. (A) The modified sequence of VP1-hCTB according to the optimized codon usage of *H. polymorpha*. The positions of restriction sites for *Xho*I and *Xba*I used to be cloned into pHFMDZ-alpha-A expression vector are underlined. The 6× His-tag coding sequence was added to the C-terminus of CTB sequence. The predicted molecular mass of the monomeric VP1-hCTB is about 20 kDa. (B) The physical map of VP1-hCTB and the construction of recombinant plasmid pHFMDZ-alpha-VP1-hCTB. *Xho*I/*Xba*I restriction sites and *Apa*I used to linearize the recombinant plasmid before transformations are indicated in the map.

Western blot analysis. The proteins were transferred to PVDF membranes after 12% SDS–PAGE. The membranes were blocked with 2.5% sodium caseinate in Tween 20/PBS (80 mM disodium hydrogen orthophosphate anhydrous, 20 mM sodium dihydrogen orthophosphate, and 100 mM sodium chloride) and further incubated with rabbit anti-CT antibodies (Sigma) and bovine anti-FMDV sera, respectively. Then, the membranes were incubated with HRP labeled goat anti-rabbit IgG (Sigma) and HRP labeled protein A/G (Pierce), respectively. The membranes were revealed with ECL Western blotting reagents (Amersham Biosciences).

GM1-ganglioside receptor ELISA binding assay. To evaluate the affinity of purified VP1-hCTB protein for the GM1-ganglioside receptor, GM1-ELISA was performed as described elsewhere [18] with minor modifications. The microtiter plate (NUNC) was coated with GM1-ganglioside receptor (Sigma), blocked with 2.5% sodium caseinate and followed by incubation with expressed samples, and blocked with sodium caseinate again. The plate was incubated with rabbit anti-CT antibodies and subsequently incubated with HRP labeled goat anti-rabbit IgG (Sigma). Color was developed using orthophenylenediamine (OPD) as substrate. The reaction was ended by addition of 2 M H_2SO_4 and read at 490 nm.

Results and discussion

Gene modification and synthesis

Gene fusion may be a practical alternative to chemical conjugation for making vaccines containing CTB. However, in bacterial and yeast systems, the level of gene expression is strictly related to specific codons [19–21]. Therefore, to improve the expression level of prokaryotic CTB and FMDV VP1 complex epitopes chimera in eukaryotic *H. polymorpha*, we directly designed and assembled the specific VP1 epitopes and CTB gene according to the codon usage preferred by *H. polymorpha* base on PCR.

Yeast transformation and screening

After transformation by electroporation, the multiple copy recombinant strains were selected by PCR as described by Song et al. [16]. A recombinant strain, containing multiple copies of VP1-hCTB, was used in the subsequent fermentations. Heterologous gene expression was controlled by the formate dehydrogenase promoter using glycerol as sole carbon source in the media.

Fermentation

The fermentation process was performed with glycerol as sole carbon source in the culture media. Complete consumption of the initial glycerol in the medium led to a rapid increase in the pO_2 value and indicated that feeding must be started with YPG containing 50% glycerol. It showed that the use of strictly carbon-limited conditions during the whole fermentation by pO_2 control strategy is essential and sufficient to ensure optimal production of VP1-hCTB.

Purification of recombinant VP1-hCTB

The recombinant protein was tagged with a 6× His-tag at its C-terminus. To obtain the best protein yield, the impurities were eluted using 10 mM imidazole followed by elution using 150 mM imidazole. The eluted fractions were analyzed by 12% SDS–PAGE (Fig. 2A). The optimum conditions were used for the expression of VP1-hCTB in larger volume.

Western blot analysis

To evaluate the antigenicity of the expressed fusion protein, the expressed samples were analyzed by 12% SDS–PAGE followed by transferring to PVDF membranes. The recombinant VP1-hCTB was confirmed by Western blot (Figs. 2B and C). The assay revealed that VP1-hCTB was strongly and specifically recognized by anti-CT antibodies and anti-FMDV sera, respectively. Immunological estimation showed a protein yield to be 100 mg/L.

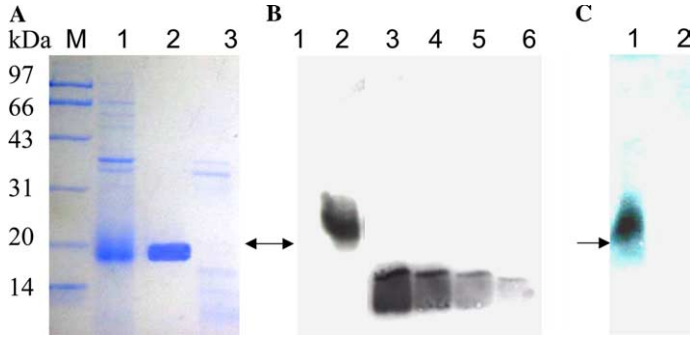

Fig. 2. Expression of the recombinant VP1-hCTB in *H. polymorpha*. (A) Recombinant proteins from supernatant (lane 1) of culture media and the purified VP1-hCTB (lane 2) using 150 mM imidazole were separated on a 12% SDS–PAGE by Coomassie blue staining (about 20 kDa, arrow indicated). Lane M, standard molecular weight marker; Lane 3, control supernatant of *H. polymorpha* transformed with pHFMDZ-alpha-A plasmid without any inserts. (B) The expressed VP1-hCTB (arrow indicated) was detected by anti-CT polyclonal antibodies (lane 2). Lane 1, control supernatant as shown in (A); lanes 3–6, commercial CTB standard for reference. The concentrations are 100, 50, 25, and 12.5 ng/lane, respectively. (C) The expressed VP1-hCTB (arrow indicated) was detected by anti-FMDV polyclonal antibodies (lane 1). Lane 2, control supernatant as shown in (A).

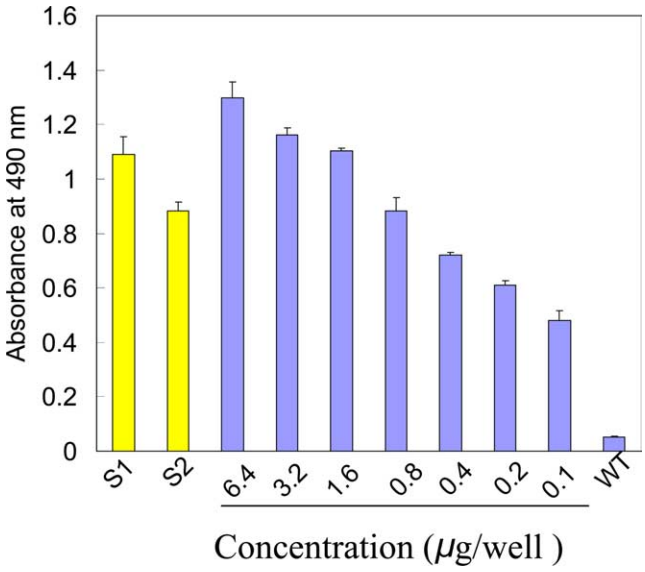

Fig. 3. GM1-ELISA analysis of expressed VP1-hCTB. The ELISA was performed by coating the microtiter plates with GM1-ganglioside. S1 and S2 represent the purified VP1-hCTB and supernatant of recombinant strain, respectively; WT represents control supernatant as shown in Fig. 2A. Commercial CTB standard was used for reference (from 6.4 μg to 0.1 μg/well). Standard deviation is marked at the top of each bar.

GM1-ganglioside receptor ELISA binding assay

GM1-ganglioside has been shown to be the specific receptor for CTB in vivo. However, for appropriate receptor binding a pentameric structure is required. Therefore, the purified VP1-hCTB was analyzed for its ability to bind GM1-ganglioside receptor using ELISA as described above. The presence of active pentameric form of VP1-hCTB was detected by its ability to bind with GM1-ganglioside (Fig. 3). The results confirmed that VP1-hCTB expressed in *H. polymorpha* is able to fold successfully in the form of pentamer with native conformation. We also quantitatively analyzed the expression level by GM1-ELISA, which is similar to Western blot analysis (Fig. 2B).

In conclusion, using *H. polymorpha* expression system, with secretory expression vector pHFMDZ-alpha-A constructed previously in our laboratory [16] and Co^{2+}-loaded resin, we were able to produce the purified VP1-hCTB chimera with antigenicity, which could be directly applied in mucosal vaccine against FMDV. It also confirmed that this novel expression system could be useful in the production of a wide variety of functional heterologous proteins, especially for mucosal vaccines.

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