An approach to achieve highly soluble bioactive ScFv antibody against staphylococcal enterotoxin A in *E.coli* with pelB leader

Weifeng Chen1, Jinsong Wu1, Wei Chen1, Ping Hu1, Xiaohong Wang2,3*

1 School of Food and Bioengineering, Henan University of Animal Husbandry and Economy, 450046 Zhengzhou, China
2 Key Laboratory of Environment Correlative Dietology, Huazhong Agricultural University, Wuhan 430070, Hubei, People’s Republic of China
3 College of Food Science and Technology, Huazhong Agricultural University, Wuhan 430070, Hubei, People’s Republic of China
*Corresponding author: zhuimeng860209@163.com

Abstract. Staphylococcal enterotoxin A (SEA) is an extremely heat stable, highly toxic, and potent gastrointestinal toxin responsible for Staphylococcal intoxication in humans. Therefore, production of immunological reagents used for SEA detection is of prime importance. For this, two fusion expression vectors, one with pelB leader, the other without it were constructed and then transformed into *E. coli* BL21(DE3) to produce the single chain variable antibodies (ScFv) against SEA. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis, western blot and enzyme-linked immunosorbent assay were used for characterization of the ScFv. Results demonstrate that expression level of the soluble ScFv with pelB leader was 20 times higher than that without it and the binding activity of ScFv without pelB leader was far below as compared to the ScFv with it. This work provides an alternative method of fast and cheap production of soluble ScFv against SEA.

1.Introduction
Staphylococcal enterotoxins (SEs) is one of the most common food-borne diseases worldwide[1-3]. Among these SEs, serological type A is the most common enterotoxin, causing more than 75% of outbreaks, followed by SED, SEC and SEB[4-6]. Therefore, rapid, accurate, and reliable detection of SEs is necessary and important to protect public health from SFP[7, 8]. However, current detection methods for SEs are primarily antibody-dependent, so design and preparation of specific antibody with high affinity against them is crucial for routine observations of SEs. The antibodies used in these assays have been mainly polyclonal antibodies or monoclonal antibodies now. Monoclonal antibodies are having conspicuous privileges over polyclonal antibodies while being used in actual applications. However, the preparation time of hybridoma clones is relatively long, and over time, it is easy to lose the ability to secrete antibodies. In recent years, the rapid development in the field of biotechnology has introduced new methods of producing genetically engineered antibodies. Single-chain variable fragment antibodies (ScFv) have attracted widespread attention[9-11]. ScFv is a fragment of traditional antibody, which connects the variable domains of antibody heavy and light chains by connecting peptides[12]. However, single-chain variable antibody fragments, as heterologous
eukaryotic proteins, cannot be correctly modified and segregated into insoluble aggregates easily known as inclusion bodies when expressed using E. coli expression systems [13]. Though the form of inclusion bodies can prevent degradation of the target protein and the expression level is much higher than the soluble expression, but after denaturation and renaturation of the recombinant antibody, it tends to lose a lot of its activity [14, 15]. As an alternative strategy, some large tags such as MBP, GST seem to improve solubility, stability, translational efficiency [16, 17], but the fusion protein may affect the activity of the target protein by burying active binding site. So, it must be digested by the corresponding protease for further use and this step is relatively tedious and the yield is very low. Using a pelB gene which directs protein export and the relatively small fusion tag may avoid the above problems. The pelB leader is an amino acid sequence which directs the protein to the bacterial periplasm when attached to a protein and then the sequence is removed by a signal peptidase [18]. So, it can play an important role in protein purification and will not affect the activity of the protein [19].

In view of this, the aim of the present study was to construct the ScFv molecules for use in immunological detection of SEs. For this, two prokaryotic expression vector pET-28a-ScFv (+) and pET-28a/pelB-ScFv (+) were constructed and then transformed into Escherichia coli BL21 (DE3) cell to express anti-SEA ScFv in vitro. Afterwards the soluble expression levels and activity of the ScFv against SEA expressed with and without the pelB leader were compared. We thus developed an useful method for producing soluble anti-SEA ScFv using pelB signal peptides as directional signals, which laid the foundation for the future immuno-detection of SEA.

2. Materials and Methods

2.1 Materials and reagents

SEA was kindly provided by Dr. Jiang from Academy of Military Medical Sciences (Beijing, China). Plasmid pET-22b, pET-28a(+), DH5α and E. coli BL21 (DE3) strain were purchased from Novagen (Novagen, Germany). Ni-NTA spin columns were purchased from Roche (Indianapolis, IN). Restriction enzymes, dNTP, Pfu DNA polymerase, and T4 DNA ligase were obtained from Takara (Takara, Japan). PVDF membrane and Anti-His-Tag HRP conjugated antibody were purchased from Bio-Rad (Mississauga, Canada).

2.2 Construction of pET-28a-ScFv and pET-28a/pelB-ScFv plasmids

In our previous work, the ScFv gene was constructed (VH-linker-VL). It was then digested with Hind III and EcoR I, and then subcloned downstream of the His-Tag of pET-28a expression vector (Novagen) to generate pET-28a-scfv plasmid.

The introduction of the cytoplasmic secretion signal sequence into the pET-28a-ScFv plasmid requires two steps. In the first step, the ScFv gene was digested with EcoR I and Hind III and cloned into the E. coli expression vector pET-22b. pET-22b has a pelB secretion signal to direct the synthesis of foreign proteins through the plasma membrane. The vector pET-22b-ScFv was used as the basis of pET-28a/pelB-ScFv expression vector. In the second step, the pET-22b-ScFv plasmid obtained by digesting with Xba I and Xho I was cloned into the E. coli expression vector pET-28a vector. After the N-terminal His-Tag and TEV cleavage sites on the pET-28a vector were removed by enzyme digestion, and then replaced with the pelB secretion signal sequence, a pET-28a/pelB-scfv expression vector was generated.

2.3 Expression of anti-SEA ScFv antibodies and identifying by Western blotting

The two expression vectors constructed were transferred into E. coli BL21 (DE3), separately. The strain was cultured in 100 mL LB medium and grown overnight (37 °C, 200 rpm) up to OD600 0.6~1.0. Thn 1 mM IPTG was added and further grown at 37 °C, 28 °C and 16 °C, separately. The cells were collected and then insoluble, soluble, sonication and the total were separated by centrifugation of the cell lysates at 12, 000 rpm for 10 min at 4 °C and then analyzed by SDS-PAGE gel with 12 % acrylamide.
2.4 Purification of anti-SEA ScFv antibodies

In order to purify soluble ScFv antibodies, a pre-packed 5 mL Ni-NTA Spin Columns was washed with 40 mL binding buffer (50 mM KH$_2$PO$_4$, 300 mM NaCl, 25 mM imidazole, pH 8.0). 30mL of soluble ScFv was applied to the column and washed with 10 mL of buffer solution. The target proteins were then eluted with 250 mM imidazole in 50 mM KH$_2$PO$_4$, 300 mM NaCl, pH 8.0. The purification of the insoluble ScFv protein was similar to the procedure given by Guo and the detailed procedure was described below[20]. A pre-packed 5 mL Ni-NTA Spin Columns was washed with 40 mL binding buffer (8 M urea, 50 mM KH$_2$PO$_4$, 0.5 M NaCl, 40 mM imidazole, pH 8.0). 30 mL of the denatured ScFv antibodies and 15 mL of binding buffer were added to the column. Then a linear 8-0.5 M urea gradient refolding buffer (50 mM KH$_2$PO$_4$, 0.5 M NaCl, 20 mM imidazole, 1mM GSSH, 1 mM GSH, pH 8.0) was added. The refolded protein was then eluted by 40 mL refolding buffer to remove imidazole. The purified ScFv protein was then determined according to Bradford[21]. The anti SEA ScFv protein was boiled in 2x sample loading buffer for 5 min, and then verified by SDS-PAGE and westernblot assay.

2.5 Antigen binding assay of ScFv proteins by enzyme-linked immune-sorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) was used to verify the binding ability between SEA and ScFv. First, 100 µL 1 µg/mL SEA was coated on the ELISA plate wells and placed at 4 °C, overnight. The ELISA plate wells were washed with PBST three times. 5% skimmed milk was added for 1 h at 37 °C, then anti-SEA ScFv proteins with different dilution were incubated at 37 °C for 1 h. The anti-His-Tag HRP conjugated antibody was used to detect the content of ScFv antibody. The optical densities at 450 nm (OD450) was recorded with a MK3 microplate reader (Thermo, USA).

3. Results

3.1 Construction and expression of pET-28a-ScFv and pET-28a/pelB-ScFv plasmids

The ScFv gene was subcloned downstream of the His6-tag and T7-tag of the pET-28a expression vector to generate a pET-28a-ScFv plasmid. In order to increase the production of soluble ScFv, the pelB leader was inserted upstream of the start codon of the ScFv which had an effect on bioactivity and yield of soluble protein[19]. The coding regions of the two plasmids are shown in Fig 1.

E. coli BL21(DE3) cells transformed with the two plasmids described above were induced at 37 °C, 28 °C and 16 °C, separately. As shown in Fig 2A and 2B, SDS-PAGE analyses revealed that the target protein band appeared at the expected band position, and the size was about 27 kDa. The following western blot experiments further confirmed its expression, as shown in fig 2C. Temperature had little effect on the expression of ScFv protein. with the increase of temperature, the expression of ScFv protein in SDS-PAGE gel did not increase significantly.

Fig.1 Schematic diagram of pET-28a-ScFv fusion expression vector before and after modification.
Fig. 2 SDS-PAGE (A) and (B) profiles showed expression of anti-SEA ScFv at different temperatures and western blotting (C) further confirmed its expression. (A) M, molecular mass markers; Lane 1, empty plasmid (pET-28a) before induction; Lane 2, empty plasmid (pET-28a) after induction; lane 3, *E. coli BL21* (DE3) containing plasmid pET-28a-ScFv before induction; lanes 4–6, *E. coli BL21* (DE3) containing plasmid pET-28a-ScFv after induction at 16 °C, 28 °C, 37 °C, respectively; (B) Lane 1, empty plasmid (pET-28a) before induction; Lane 2, *E. coli BL21* (DE3) containing plasmid pET-28a/pelB-ScFv before induction; lanes 3–5, *E. coli BL21* (DE3) containing plasmid pET-28a-pelB-ScFv after induction at 16 °C, 28 °C, 37 °C, respectively. (C) M, molecular mass markers; Lane 1, empty plasmid (pET-28a) after induction; Lane 2, *E. coli BL21* (DE3) containing plasmid pET-28a-ScFv after induction; lanes 3, *E. coli BL21* (DE3) containing plasmid pET-28a/pelB-ScFv after induction.

### 3.2 Purification of anti-SEA ScFv with and without the pelB leader

With some optimizing the expression conditions of solubility such as IPTG concentration, temperature and time, the best soluble expression conditions for the target protein involved addition of IPTG to a final concentration of 1 mmol/L with shaking at 16 °C for 24 h. The soluble and insoluble fractions of the induced *E. coli* BL21(DE3) cells with and without the pelB leader were purified by Ni-NTA Spin Columns separately and subsequently quantified by Bradford assay. Results showed there was a correlation between the increased soluble expression of ScFv and the addition of the pelB signal peptide. For the pET-28a-ScFv construct, only a final yield of 400 μg L⁻¹ soluble target protein was obtained and the majority of the protein produced was insoluble, while the pET-28a/pelB-ScFv constructs had the greatest enhancement of soluble expression (over 10 mg L⁻¹) which was 20 times higher than that of the protein without leader as shown in Table 1. These results indicated that the ScFv expression with pelB signal peptide is a very powerful and versatile strategy to achieve the high-level production of soluble anti-SEA protein in *E. coli*.

| Table 1 Purification of anti-SEA ScFv from *E. coli BL21* (DE3) strain containing fusion expression vector |
|----------------------------------------------------------------------------------------------------------|
| Construct | a | b | c |
| pET-28a-ScFv | 3 | 0.380 | 60.06 |
| pET-28a/pelB-ScFv | 3 | 10.36 | 17.56 |

a Total protein (g), 3g of wet weight cells obtained from 1 L culture was utilized for purification of anti-SEA ScFv.

b Soluble ScFv (mg), after purification, the amount of total soluble ScFv was measured by Bradford assay.

c Insoluble ScFv (mg), after purification, the amount of total insoluble ScFv was measured by Bradford assay.

### 3.4 Binding activity assay of the ScFv by ELISA

In order to identify whether the addition of the signal peptide affects the activity of the ScFv protein, the total binding activity of all fractions were measured by ELISA after induction. Fig 3 showed that the two constructs can successfully express active protein after induction and the bioactivity values of the strain expressing ScFv with leader sequence was 2.3-fold higher than that without it. From the Fig
4. activity measurement of the periplasmic space expression of pET-28a/pelB-ScFv resulted in a clear affinity activity to SEA in a continuous cultivation which was much higher than that obtained from the plasmid pET-28a-ScFv lacking the respective leader sequence with time. For accurate determination of the biological activity of the ScFv protein, the soluble proteins with and without pelB leader were purified separately (Fig 5A) and the reactivity of the soluble ScFv proteins were tested by ELISA. As shown in Fig 5B, the binding activity of ScFv protein without pelB leader was far below as compared to ScFv protein with pelB leader. These datas above indicated that the N-terminal fusion of pelB leader to scFv could significantly influence the soluble protein expression level and the folding of the resulting proteins in periplasmic space. The reason may be that a pelB signal peptide could slow the aggregation process inadvertently and shift the target protein to a folding pathway to increase the amount and bioactivity of the ScFv protein. Thus we concluded that there could be a positive effect on the total yield and bioactivity of the target protein when using solubilizing pelB signal peptide.

Fig. 3 Biomass and biological activity of anti-SEA ScFv in E. coli BL21(DE3) using fusion expression vector pET-28a-ScFv and pET-28a/pelB-ScFv, respectively. 1 and 2 represented the E. coli BL21 (DE3) containing plasmid pET-28a-ScFv before and after induction, respectively; 3 and 4 represented the E. coli BL21 (DE3) containing plasmid pET-28a/pelB-ScFv before and after induction, respectively.

Fig. 4 The changes in biological activity of the anti-SEA ScFv in periplasmic space by both constructs over time.
Fig. 5 SDS-PAGE profiles (A) showed the purification of anti-SEA ScFv in soluble from two constructs, respectively and (B) ELISA showing binding of anti-SEA ScFv in soluble from the two constructs with SEA antigen. ELISA was performed with different dilutions of soluble anti-SEA ScFv antibody. The bound anti-SEA ScFv antibody was detected using anti-His-tag HRP-conjugated antibody.

5. Conclusion
In this study, we established two prokaryotic expression vector for increasing the production of soluble ScFv against SEA and the results demonstrated that the pelB signal peptide played an important role as a directional signal directing and boosting secretion of the target ScFv antibody to the periplasm; The present study provided a method of increased expression of a soluble recombinant target protein using a minimum-length pelB as signal sequence. We had thus developed useful methods for producing soluble anti-SEA ScFv using pelB signal peptides as directional signals.

Acknowledgements
The research was funded by the National Key R&D Program of China (2017YFC1600100), National Natural Science Foundation of China (31772054), the project of the department of education of Henan province (No. 18A550010), science and technology department of Henan province (No. 182102110303), doctor start-up fund (No. 53000153) and the funding for academic leaders (No. 84000141) of Henan University of Animal Husbandry and Economy.

References:
[1] N. Zeaki, R. Cao, P.N. Skandamis, P. R Dstr M, J. Schelin, Int J Food Microbiol, 44, 182-183 (2014).
[2] J.M. Hait, R.W. Bennett, S.R. Monday, J Aoac Int, 1(2019).
[3] B. Thomas, J.M. Njoroge, R.L. Jones, P. Maryann, J Aoac Int, 2(2019).
[4] Y.C.X.X. Huang, Anal Methods-UK, 6, 690(2014).
[5] L. Christiane, M. Tiphaine, M. Sabine, D. Sylviane, J Aoac Int, 5(2019).
[6] B.R. W, M. Foster, Collaborators, Journal of the Association of Official Analytical Chemists, 6(2020).
[7] P.K. Singh, R. Agrawal, D.V. Kamboj, G. Gupta, M. Boopathi, A.K. Goel, L. Singh, Appl Environ Microbiol, 76, 8184(2010).
[8] I.V. Pinchuk, E.J. Beswick, V.E. Reyes, Int J Food Microbiol, 2, 2177 (2010).
[9] H. Xiong, S. Li, Z. Yang, R.R. Burgess, W.S. Dynan, Pro Exp & Pur, 66, 180(2009).
[10] R. Bird, K. Hardman, J. Jacobson, S. Johnson, B. Kaufman, S. Lee, T. Lee, S. Pope, G. Riordan, M. Whitlow, Science, 242, 423(1998).
[11] Arne, Skerra, Andreas, Plückthun, Science, 240, 1038(1988).
[12] Z.X. Liu, G.H. Yi, Y.P. Qi, Y.L. Liu, J.P. Yan, J. Qian, E.Q. Du, W.F. Ling, Bio & Bio Res, 329, 444(2005).
[13] R.C. Stevens, Struture, 8, 177(2000).
[14] S. Jana, J.K. Deb, Appl Microbiol & Bio, 67, 289(2005).
[15] J.R. Swartz, Curr Opin Bio, 12, 195(2001).
[16] S. Agarwal, S. Jha, I. Sanyal, D.V. Amla, Jour Bio, 147, 64(2010).
[17] D. Esposito, D.K. Chatterjee, Curr Opin Bio, 17, 353(2006).
[18] D. Lundell, R. Greenberg, Y. Alroy, R. Condon, C. Shah, J I Microbiol, 5, 215(1990).
[19] B. Gao, D. Zhangsun, Y. Hu, Y. Wu, L. Sheng, L. Fang, X. Wu, J. Yu, S. Luo, Toxicon Official Journal of the International Society on Toxinology, 72, 81(2013).
[20] J.Q. Guo, S.Y. You, L. Li, Y.Z. Zhang, J.N. Huang, C.Y. Zhang, J Bio, 102, 177(2003).
[21] A.J. Dickson, Trends Bio Sci, 19, 558(1994).