Expression and Purification of bFGF Nanobody in Pichia Pastoris GS115

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Abstract

[Objective] To construct a Pichia expression system of bFGF Nanobody F3, and study the secretion and expression of bFGF Nanobody F3 protein in Pichia pastoris GS115, so as to obtain a method for large-scale expression of bFGF Nanobody. [Method] The F3 protein sequence and the eukaryotic expression vector pPIC9K and pPICzαA were analyzed, and the recombinant plasmids pPIC9K-F3 and pPICzαA-F3 were constructed. The plasmids were linearized by Sac I and then electroporated into Pichia pastoris GS15 and passed G418 and Zeocin antibodies. Sexual screening and colony PCR were used to obtain positive monoclonal strains. The positive monoclonal strain was induced for 96 hours under the conditions of 30°C, 220 rpm/min and 1% methanol. SDS-PAGE and Western Blotting were used to verify whether the F3 protein was expressed in the positive monoclonal strain. [Result] The recombinant plasmids pPIC9K-F3 and pPICzαA-F3 were successfully constructed, and F3 protein was successfully obtained from the induced expression supernatant, and its molecular weight was consistent with the theoretical value of 15kDa. [Conclusion] The Pichia secretory expression system of bFGF nanobody F3 was obtained through the successful induced secretion expression of F3 protein, which provided reference and laid a foundation for the subsequent large-scale expression of bFGF nanobody F3.

Keywords

bFGF; Nanobody; Pichia Pastoris.

1. Introduction

Fibroblast growth factor-2 (FGF-2) was originally isolated and purified from bovine brain tissue and bovine pituitary gland by researchers. It has mitogenic and proliferative effects on Balb/c 3T3 fibroblasts, and The isoelectric point is alkaline (pH=9.6), so it is also called basic fibroblast growth factor. Under normal circumstances, bFGF is mainly distributed in the pituitary gland, brain, nerve tissue, retina, adrenal gland, placenta and other parts of the body. It plays an important role in the process of angiogenesis, damaged tissue repair, wound healing, embryonic development and differentiation. Role [1]. Under pathological conditions, FGF-2 is highly expressed in tumor tissues such as melanoma, breast cancer, lung cancer, and bladder cancer. It is closely related to tumor occurrence and development, tumor metastasis, angiogenesis and prognosis [2- 5], and the current drugs targeting bFGF targets are mainly bFGF antibodies.

Since researchers prepared 4 bFGF antibodies for the first time in 1987 [6], bFGF antibodies have undergone more than 20 years of development of polyclonal antibodies, monoclonal antibodies, FGF-2 fragment antibodies, and humanized antibodies, and most of them The bFGF antibodies are limited to the research phase. Nanobodies are single domain antibodies (VHH) containing only the variable region of heavy chain antibodies, with a diameter in the nanometer range, and are the smallest antibody fragments that can bind to antigens found so far [7-10]. Nanobody F3 is a strain of anti-bFGF antibody screened by our laboratory, and its expression system is Escherichia coli expression system.

When nanobodies are expressed in E. coli, the expression cycle is short and the price is low, but they are easy to form inclusion bodies, lack post-translational modification functions, and remove endogenous proteins during the purification process [11-12]. The Pichia pastoris expression system has the function of post-translational modification. The target protein can be secreted to the outside of the cell through the signal peptide to facilitate purification. The expression level is usually higher than that of the E. coli expression system, which is easy to large-scale expression, fermentability and industrial production [13-16]. At present, the expression of Nanobody in Pichia pastoris has not been systematically and deeply studied. Therefore, in this study, the bFGF Nanobody F3 was used as the target protein, and recombinant plasmids pPIC9K-F3 and pPICzαA-F3 were constructed to explore the F3 protein in Biology. The secretion and expression in red yeast provides reference and lays a foundation for the industrialized large-scale production of Nanobodies.

2. Materials and Methods

2.1 Plasmids and strains

The eukaryotic expression vectors pPIC9K and pPICzαA, plasmid pMECS-F3 and Pichia pastoris are preserved in the author's laboratory, and Escherichia coli DH5α is provided by Shanghai Shenggong Biological Engineering Co., Ltd.

2.2 Reagents and instruments

T4 DNA ligase, restriction enzymes EcoR I, Not I and Sac I were purchased from TaKaRa; G418, Zeocin, ampicillin, methanol, TCA precipitation kit, mouse anti-6 × His monoclonal antibody, goat anti-mouse IgG-HRP; Medium MD, YPD, BMGY, BMMY preparation methods refer to Invitrogen Pichia pastoris operation manual (Invitrogen Corporation. A manual of methods for expression of recombinant proteins. USA). The PCR machine and the electrotransformer were produced by Eppendorf.

2.3 Construction of recombinant plasmids pPIC9K-F3 and pPICzαA-F3

The EcoR I restriction site was introduced at the 5'end of the F3 gene, and the His tag sequence and Not I restriction site were introduced at the 3'end. F3 gene, eukaryotic expression vector pPIC9K and pPICzαA were digested with EcoR I and Not I, respectively, and ligated with T4 DNA ligase at 37°C for 30 min. The enzyme-linked product was transformed into DH5α competent, and spread on Amp+ and Zeocin LB agar plates for constant temperature culture. After picking the positive monoclonal strains, the plasmids were extracted for double digestion with EcoR I and Not I, and sent to Shanghai Biosequence is used to verify whether the construction is successful.

2.4 Linearized recombinant plasmid and electrotransformed to Pichia pastoris GS115

The recombinant plasmids pPIC9K-F3 and pPICzαA-F3, which were successfully linearized and sequenced with the restriction endonuclease Sac I, were electro-transformed into GS115 competence. The electro-transformation parameters were 1500V, 5ms. At the same time, electrotransform the linearized plasmids pPIC9K and pPICzαA as blank controls. They were spread on YPD agar plates containing G418 and Zeocin, and incubated at 30°C for 3 days to observe bacterial growth. Pick several single colonies for colony PCR verification.

2.5 Expression, identification and comparison of recombinant pPIC9k-F3 and pPICzαA-F3 engineering bacteria GS115

Pick out the recombinant pPIC9k-F3 and pPICzαA-F3 engineering bacteria GS115 that are positive for colony PCR, and inoculate them in YPD liquid medium for culture activation. The activated strains are inoculated in BMGY medium and wait until the OD600 of the bacterial solution reaches 2-5. Centrifuge at 1500g for 5min, discard the supernatant to collect the bacteria, dilute the bacteria with BMMY medium to an OD600 of 1, induce culture at 220rpm/min and 30°C for 96h, supplement with 1% methanol every 24h, after the fermentation is over The cells and supernatant were collected separately, and the expression of the target protein was verified by SDS-PAGE and Western blotting.

2.6 Expression and purification of Nanobody F3

A recombinant engineered strain with high F3 protein expression was selected and cultured at 220rpm/min and 30℃ for 96h. The supernatant was collected by centrifugation. The supernatant was filtered with a 0.22μm filter and purified by Ni-NTA affinity chromatography column. . Equilibrate the column mixture with 1×PBS (pH 7.4). Then 100 mM imidazole was used to elute the impurity protein, and 300 mM imidazole was used to elute the target protein, and the results were detected by SDS-PAGE.

2.7 Statistical analysis

The data from this paper were evaluated using analysis of deviation and variance.

3. Results

3.1 Construction and identification of recombinant plasmids pPIC9K-F3 and pPICzαA-F3

The F3 gene sequence was cloned from the plasmid pMECS-F3 and verified and identified by 1% agarose electrophoresis. As shown in Figure 1A, an obvious band appeared near 500bp, which was consistent with the theoretical size of the F3 gene (400bp). The recombinant plasmid was transformed into E. coli DH5α competent. Amp+ and Zeocin were screened for positive monoclonal strains respectively. After the strains were expanded and cultured, the plasmids were extracted for double enzyme digestion, and identified by 1% agarose electrophoresis, as shown in Figure 1 B and C. Two bands clearly appeared after double digestion.

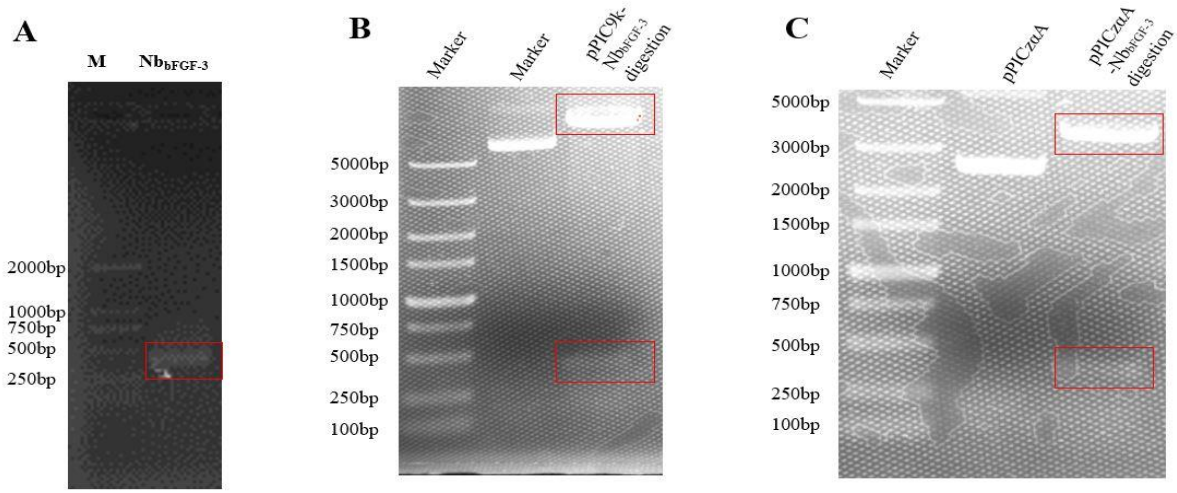

Figure 1. Construction and identification of recombinant plasmids pPIC9K-F3 and pPICzαA-F3

A: PCR amplification of F3 gene; B: Double restriction digestion of recombinant plasmid pPIC9K-F3; C: Double restriction digestion of recombinant plasmid pPICzαA-F3

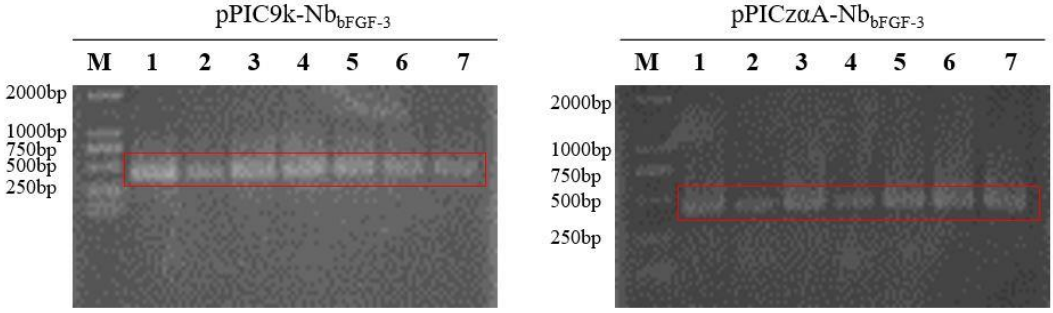

Figure 2. Colony PCR verification

A: Recombinant pPIC9k-F3 engineering bacteria GS115 colony PCR

B: Recombinant pPICzαA-F3 engineering bacteria GS115 colony PCR

3.2 Identification of Pichia pastoris positive transformants

Transform the plasmids pPIC9K-F3 and pPICzαA-F3 linearized by Sac I into Pichia pastoris GS115 by electrotransformation, and then culture them on YPD medium containing Amp+ and Zeocin until a single bacteria grows. Pick 7 Colony PCR was performed on a single colony. As shown in Figure 2, a specific band slightly larger than 250 bp was obtained. The result was in line with the theoretical value (400 bp), confirming that pPIC9K-F3 and pPICzαA-F3 have been successfully integrated into the yeast genome.

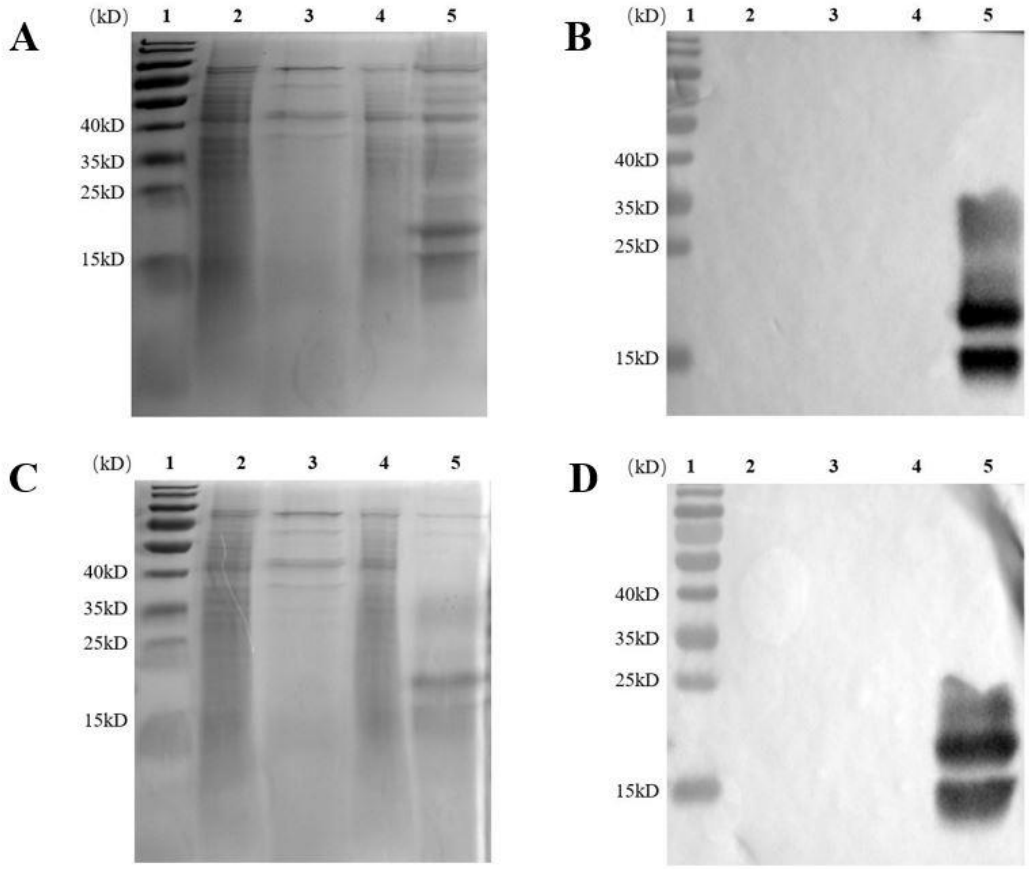

Figure 3. Recombinant pPIC9k-F3 and pPICzαA-F3 engineering strain GS115 induced expression

A:SDS-PAGE; B:Western blot; 1:Marker, 2:Culture precipitationof P. pastoris transformed with blank pPIC9k vector (negative control), 3:culture supernatant of P. pastoris transformed with blank pPIC9k vector (negative control), 4: Culture precipitationof P. pastoris transformed with the recombinant pPIC9k- F3, 4: culture supernatant of P. pastoris transformed with the recombinant pPIC9k- F3.

C:SDS-PAGE;D:Western blot; 1:Marker, 2:Culture precipitationof P. pastoris transformed with blank pPICzαA vector (negative control), 3:culture supernatant of P. pastoris transformed with blank pPICzαA vector (negative control), 4: Culture precipitationof P. pastoris transformed with the recombinant pPICzαA- F3, 4: culture supernatant of P. pastoris transformed with the recombinant pPICzα- F3.

3.3 Recombinant pPIC9k-F3 and pPICzαA-F3 engineering bacteria GS115 induced expression

The positive monoclonal bacteria that were verified by colony PCR were selected, and methanol induced expression for 96h. At the same time, electro-transformed linearized plasmids pPIC9K and GS115 of pPICzαA were used as blank controls. The cells and supernatant were collected by centrifugation, and the expression of the target protein was verified by SDS-PAGE gel electrophoresis

and Western blotting, as shown in Figure 3. It can be seen from the figure that compared with the blank control, it is around 15kDa Obvious bands appeared at the place, indicating that the target protein F3 was secreted in the culture supernatant.

However, the results of sample processing, SDS-PAGE and Western blotting showed that the target protein F3 expressed in the supernatant of the recombinant pPIC9k-F3 engineering bacteria GS115 needs to be concentrated by TCA precipitation to see obvious bands on SDS-PAGE However, the target protein F3 expressed in the supernatant of recombinant pPICzαA-F3 engineered bacteria GS115 can be seen in SDS-PAGE without TCA precipitation. Therefore, the recombinant pPICzαA-F3 engineered bacteria GS115 was selected for the expression of F3.

3.4 Expression and purification of F3 protein

The recombinant pPICzαA-F3 engineered strain GS115 frozen at -80 ℃ was activated and induced expression, the culture supernatant was collected by centrifugation, and the target protein F3 was purified by Ni-NTA affinity chromatography column. During the purification process, 1×PBS (pH7. 4) As a loading buffer and a balance solution, followed by 50 mM, 100 mM, 300 mM and 500 mM imidazole to elute the impurity protein and the target protein (Figure 4). It was found that the target protein was eluted in 300 mM imidazole.

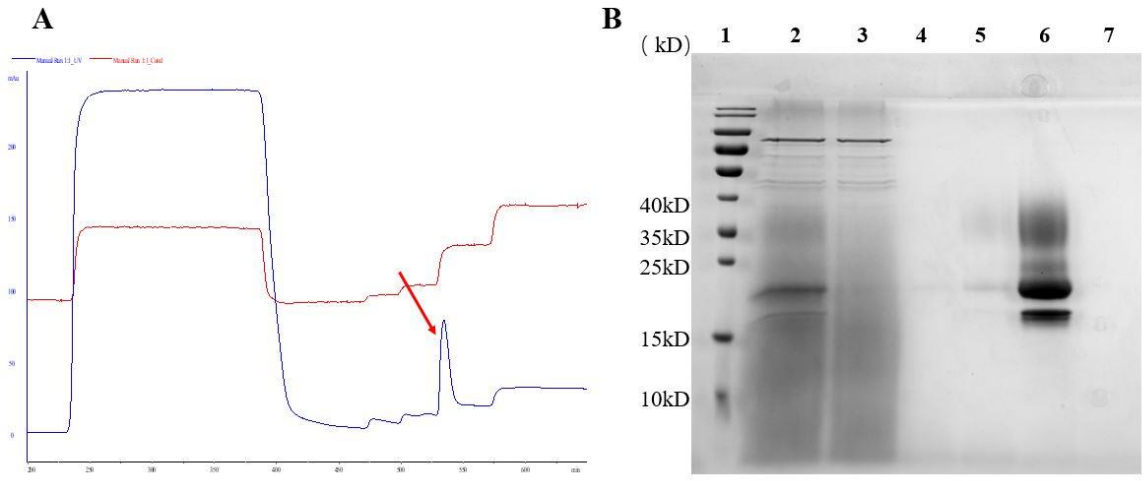

Figure 4. Expression and purification of F3 protein

A:F3 purification map; B:F3 purified SDS-PAGE; 1:Marker, 2:culture supernatant, 3:Flow-through, 4:Elution 1, 5:Elution 2, 6:Elution 3, 7:Elution 4.

4. Discussion

bFGF plays an important role in tumor growth, metastasis and angiogenesis, and is an important research object [17]. The most effective drug for the bFGF target is an antibody, but the current commercialized bFGF antibody in the world is limited to laboratory research and has not been used clinically, and it is a traditional IgG molecule. In addition, most of the current research on nanobody expression systems focuses on prokaryotic expression systems, such as E. coli expression systems; while eukaryotic expression systems are rarely used, such as Pichia pastoris expression systems, usually in Pichia pastoris expression systems. The target protein is secreted and expressed, and the expression level is higher than that in the E. coli expression system [18-19].

In this study, we first constructed the recombinant plasmids pPIC9K-F3 and pPICzαA-F3 and transformed them into Pichia pastoris GS115. We compared the expression level of the target protein F3 in pPIC9K-F3 and pPICzαA-F3 engineering bacteria GS115, and found The expression level is higher in pPICzαA-F3 engineering strain GS115, which is selected as the expression host of Nanobody F3. In the purification of the target protein F3 on the Ni-NTA affinity chromatography

column that induced the expression of the supernatant, it was found that it was eluted in 300nM imidazole solution, and the results were analyzed and verified by SDS-PAGE.

In order to meet industrialized large-scale production, the expression level of antibodies has always been a hot issue of concern to scientists, how to select an expression system, screen for high-expressing strains, and optimize the expression process. The pPICzαA-F3 engineered strain GS115 constructed in this research is a highly expressing strain, which can be used as a large-scale industrial seed for the target protein F3. The expressed and purified nanobody F3 can also be used as an antibody raw material for in vivo and in vitro diagnosis. Of course, in order to further meet the needs of industrialization, the expression process can be further optimized.

Acknowledgements

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