Expression, Purification and Affinity of Two Anti-FGF-2 Nanobodies

Ruibin Lu, Hui Zhao, Qiuling Xie and Sheng Xiong*

College of Life Science and Technology, Jinan University, Guangzhou 510632, China.

Abstract

Fibroblast growth factor 2 (FGF-2) is one of the most important cytokines in the process of tumor angiogenesis, so it can be neutralized by preparing anti-FGF-2 antibodies to inhibit tumor growth. A special single-domain antibody called nanobody, which comes from camel, with low immunogenicity, strong tissue penetration, and high expression level, is also an ideal molecule drug. Two anti-FGF-2 nanobodies that have been constructed were explored for the optimal concentration of IPTG to induce protein expression and were separated and purified by Ni-NTA affinity purification of his-tagged. The affinity constant and kinetic characteristics were showed by surface plasmon resonance (SPR) technology. When the concentration of IPTG was 1 mmol/L, the nanobodies had higher expression concentration of 20.6 mg/L and 18.5 mg/L, and their purity after purification by Ni-NTA affinity purification were about 93% and 92%. One of the affinity of nanobody measured by SPR technology was 10.9nM and the other was 46nM, but the kinetic characteristics were both slow binding and slow dissociation.

Keywords

FGF-2; Nanobody; SPR.

1. Introduction

FGF-2 is a multifunctional cytokine in human body, which is highly expressed in tumor tissues such as melanoma, breast cancer, lung cancer, and bladder cancer under pathological conditions [1]. When tumors begin to develop, metastasize [2], FGF-2 will play a major role in this process. On the one hand, FGF-2, as a mitogenic factor and chemokine, can directly promote their uncontrolled growth and proliferation of tumor cells in an autocrine or paracrine manner [3]. On the other hand, FGF-2 provides rich nutrition for the growth of tumor cells by promoting angiogenesis [4]. At present, there is no commercial therapeutic FGF-2 antibody, and the research of anti-FGF-2 drugs still has excellent prospects for development.

By cloning the variable region of the heavy-chain antibody (HCAb) in camel by genetic engineering, the smallest antibody fragment with complete antigen binding capacity can be obtained, which is called nanobody [5]. Because the nanobody is encoded by a single gene, it has a simple structure and low molecular mass which also leads to its expression in bacteriophages, E. coli, yeast and other microorganisms, and even large-scale production [6]. Secondly, it does not lead to complement-dependent cytotoxicity (CMC) reaction, so it has weak immunogenicity to humans and has excellent biocompatibility with humans [7]. Furthermore, he hydrophobic residues in the framework region are replaced by hydrophilic residues, which increase water solubility [8]. Therefore, current use of nanobody strategies to study new target therapeutic drugs is one of the new trends in antibody research and development.

SPR technology monitors the quality change of the sensor's metal surface through spectroscopy. When the analytical molecule is combined with the stationary phase on the sensor chip, the increase in surface quality will cause the refractive index to modify, which will cause the SPR angle to shift. By monitoring the angular deviation over time during the binding process, the machine can generate a real-time sensor map. Therefore, the machine monitors the angular deviation over time during this process, and can generate a real-time sensor map [9]. Therefore, the machine will record the angular deviation over time during this process, and can generate a real-time sensor map to obtain the dynamic process of antigen-antibody binding and dissociation.

In the previous study, alpaca was immunized with FGF-2, and a FGF-2 nanobody phage library with a storage volume of 1×10^{14} pfu/mL was constructed. Two similar specific targeting antigen FGF-2 nanobodies named nanobody1(Nb1) and nanobody2 (Nb2) were selected (18kDa), have been constructed on the PMECS plasmid in WK6 Escherichia coli. On this basis, this study carried out their soluble expression and purification, and detected the affinity constant and kinetic characteristics by SPR.

2. Method

2.1 Optimization of expression conditions

After inoculating 800 mL seed liquids (OD_{600}=1) in a 15 L bioreactor(10%v/v), the anti-FGF-2 nanobody was cultured and expressed at 37°C with a stirring speed of 300 rpm. Different concentrations of IPTG were added during the logarithmic period to induce expression. Under the conditions of a temperature of 28°C and a rotation speed of 500 rpm/h, the protein expression was induced to the plateau phase of the strain with feeding the material in a fixed flow rate mode. The value of OD600 each time point will be recorded. After the expressing bacteria were sonicated and centrifuged, expression content of the target protein was detected by SDS-PAGE to determine the optimal concentration of the inducer.

2.2 Anti-FGF-2 nanobody purification

The pellet was re-dissolved in PBS buffer after centrifuging the fermentation broth at 8000 rpm/min for 10 minutes. Centrifuge the sonicated Escherichia coli at 13500 rpm/min for 60 minutes. The supernatant was filtered through a 0.45 μm filter (Millipore, Billerica, MA), and then applied to a Ni-NTA affinity chromatography column, which was equilibrated with 20 mM imidazole at a flow rate of 4 mL/min for 25 minutes. The nanobody was eluted with 300 mM imidazole and then the protein concentration of the eluted fraction was measured using a NanodropTM 2000 spectrophotometer and SDS-PAGE.

2.3 Analysis of affinity and kinetics

The commercial FGF-2 was coupled to the surface of the CM-5 sensor chip through the amino coupling kit as the detection channel, the channel not coupled with FGF-2 was used as the reference channel, and the HBS-EP solution was used as the mobile phase. The chip was activated and blocked, and then different concentrations of anti-FGF-2 nanobody were injected under the conditions of 25°C and pH 7.4, and the binding dynamics of each concentration will be obtained. The 1:1 limgmuir binding mode was used to analyze the binding and dissociation constants, and the steady-state affinity constant KD was obtained.

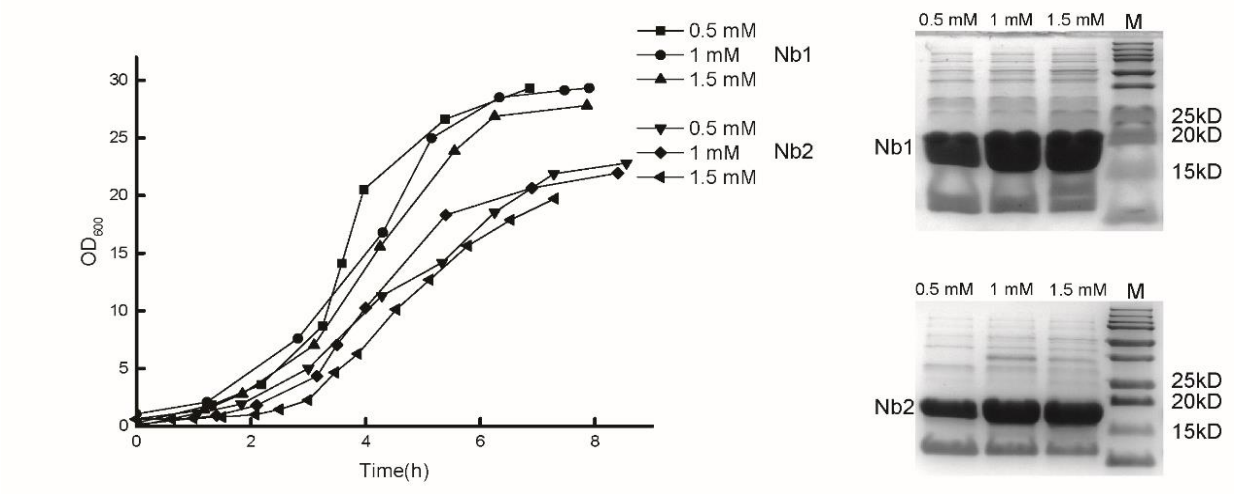

Fig. 1 Growth curve of WK6 Escherichia coli and concentration of expressed protein under different concentrations of IPTG

3. Results

3.1 The influence of IPTG on protein expression

Protein content per unit bacterial solution is displayed on SDS-PAGE as shown in Figure 1. At low concentrations, as the IPTG concentration increases, the expression of the target protein increases, and the expression reaches its peak when the final concentration is 1 mM, but at the same time, the growth of E. coli is also inhibited as the concentration increases, resulting in the unit protein expression decline. Therefore, 1mM IPTG was used as the induction concentration.

3.2 Separation and purification of nanobody

After the expressed bacteria were sonicated and centrifuged, the supernatant was purified by affinity chromatography on a nickel column, washed with low concentration 20 mM imidazole to remove impurities, and 300 mM imidazole was used to elute the target protein, as shown in Figure 2. SDS-PAGE is the result of concentration after elution. Protein purity of gray-scale scanning analysis was 93% and 92%, and the fermentation broth yield was at least 20.6 mg/L and 18.5 mg/L.

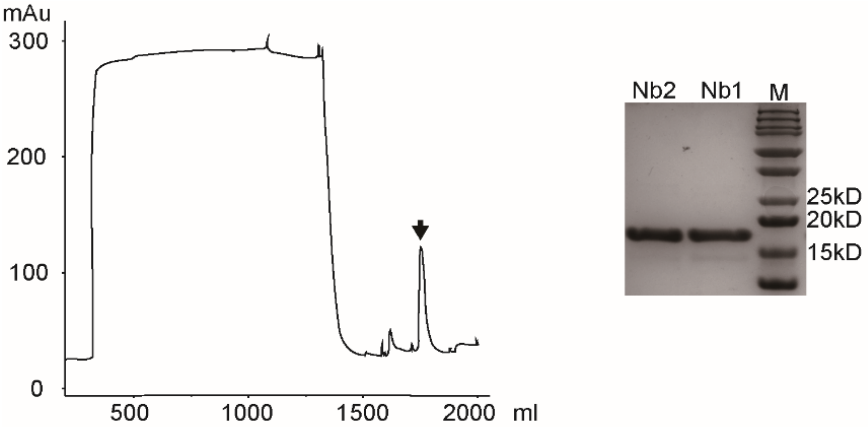

Fig. 2 Nanobody elution profile and SDS-PAGE purity identification

3.3 Analysis of affinity and kinetic characteristics

From the interaction map of the binding and dissociation of nanobodies and FGF-2, it was found that the binding reaction between the two antibodies and FGF-2 was slow binding and slow dissociation, or even non-dissociation. The limgmuir binding model was used to compare the experimental results. Data fitting was performed, and the affinity of the two antibodies was 10.9 nM and 46 nM, respectively.

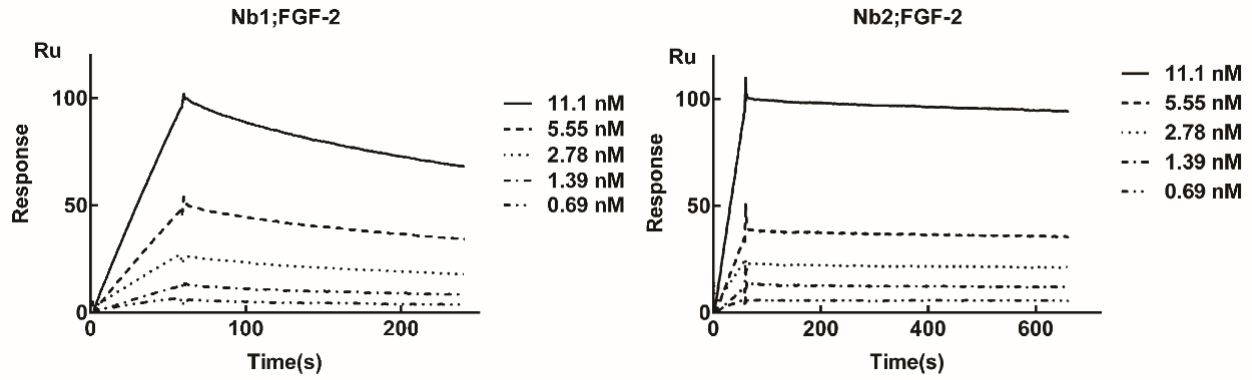

Fig. 3 The interaction map of FGF-2 after injecting different concentrations of nanobodies

4. Discussion

Traditional antibody production requires mammalian cells, which not only have high production costs, but also have a long production cycle, and is sensitive to high temperatures and difficult to store. This brings certain limitations to the application of antibodies. Although the amount of nanobody we obtained through prokaryotic system expression is not very high, it is also higher than the expression amount of cells, and it is greatly reduced compared with the time required for expression in mammalian cells and the production cost. In addition, other conditions can be changed to further increase protein expression.

SPR technology does not use fluorescent labels and isotope labels, thereby maintaining the natural activity of biomolecules, which allows real-time detection of the entire process of antigen-antibody interactions and rapid detection of the dynamic characteristics of antigen-antibody. Although the affinities of the two antibodies are similar, both have strong binding ability. But in fact, Nb2 dissociates very slowly, and the binding is still relatively strong within the longest dissociation time allowed by the machine, which also makes kinetic constant k_d is outside and constant k_a is approaching the limits that can be measured by the instrument. So the actual affinity is greater than this value. From the analysis of kinetic characteristics, Nb1 is more suitable as an anti-FGF-2 drug, and Nb2 is more suitable as a protective agent.

References

[1] HU M, HU Y, HE J, et al. Prognostic Value of Basic Fibroblast Growth Factor (bFGF) in Lung Cancer: A Systematic Review with Meta-Analysis [J]. PloS one, 2016, 11(1): e0147374.

[2] GUL N, VAN EGMOND M. Antibody-Dependent Phagocytosis of Tumor Cells by Macrophages: A Potent Effector Mechanism of Monoclonal Antibody Therapy of Cancer [J]. Cancer research, 2015, 75(23): 5008-13.

[3] TURNER N, GROSE R. Fibroblast growth factor signalling: from development to cancer [J]. Nature reviews Cancer, 2010, 10(2): 116-29.

[4] PRESTA M, DELL'ERA P, MITOLA S, et al. Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis [J]. Cytokine & growth factor reviews, 2005, 16(2): 159-78.

[5] MUYLDERMANS S, BARAL T N, RETAMOZZO V C, et al. Camelid immunoglobulins and nanobody technology [J]. Veterinary immunology and immunopathology,2009,128(1-3):178-83.

[6] BAGHBAN R, GARGARI S L, RAJABIBAZL M, et al. Camelid-derived heavy-chain nanobody against Clostridium botulinum neurotoxin E in Pichia pastoris [J]. Biotechnology and applied biochemistry, 2016, 63(2): 200-5.

[7] CORTEZ-RETAMOZO V, BACKMANN N, SENTER P D, et al. Efficient cancer therapy with a nanobody-based conjugate [J]. Cancer research, 2004, 64(8): 2853-7.

[8] JOVCEVSKA I, MUYLDERMANS S. The Therapeutic Potential of Nanobodies [J]. BioDrugs: clinical immunotherapeutics, biopharmaceuticals and gene therapy, 2020, 34(1): 11-26.

[9] MIURA K. An Overview of Current Methods to Confirm Protein-Protein Interactions [J]. Protein and peptide letters, 2018, 25(8): 728-33.