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Research article

Screening and identification of nanobody against inhibin α-subunit from a *Camelus bactrianus* phage display library

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ABSTRACT

Background: Inhibin is a member of the transforming growth factor family that influences reproduction in animals.
Objective: The purpose of this study was to obtain nanobodies from the phage antibody library constructed by us that can specifically bind to inhibin α-subunit.
Methods: In this study, camels were immunized with Kazakh sheep inhibin-α protein that expressed in BL21 *E. coli*, and the camel VHH nanobody phage display library was prepared using nested PCR. The nanobodies specifically binding to inhibin α-subunit in the library were screened by three rounds of immunoaffinity screening and phage enzyme-linked immunosorbent assay (phage ELISA). The functions of the selected nanobodies were identified using molecular simulation docking, ELISA affinity test, and sheep immunity test.
Results: A nanobody display library was successfully constructed with a capacity of 1.05×10^{12} CFU, and four inhibin-α-subunit-specific nanobodies with an overall similarity of 69.34 % were screened from the library, namely, Nb-4, Nb-15, Nb-26, and Nb-57. The results of molecular simulation docking revealed that four types of nanobodies were complexed with inhibin-α protein mainly through hydrophobic bonds. Immunity tests revealed that the nanobody Nb-4 could effectively inhibit sheep inhibin A/B and could significantly improve the FSH level in sheep.
Conclusion: Four inhibin α-subunit-specific nanobodies with biological functions were successfully screened. To the best of our knowledge, this is a new reproductive immunomodulatory pathway of inhibin α-subunit, which may change the secretion of FSH in the ovary, thus changing the estrous cycle of organisms.

1. Introduction

Inhibin is a water-soluble protein from testicular secretions and was first reported by McCullagh in 1932 [1]. It is secreted in the testis, passes through the blood, and regulates pituitary cells to inhibit the formation of castrated cells. Inhibin consists of two

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disulfide-linked subunits, designated as α and β. Among them, β subunits can be divided into 5 types: βA, βB, βC, βD, and βE. βA and βB are linked to the inhibin α-subunit to form inhibin A and inhibin B, respectively [2]. α, βA, and βB subunit precursors are encoded by *INHA*, *INHBA*, and *INHBB* genes, respectively. At present, inhibin has been proven to be the main negative feedback regulator of follicle stimulating hormone (FSH) secretion in mammals, which can inhibit the secretion of FSH in the pituitary gland [3–5]. Therefore, many studies have been focusing on improving the ovulation rate and birth rate of animals through active or passive immunization with inhibin. For example, researchers immunized female mice with rat inhibin monoclonal antibody and observed that after immunization, the number of healthy mice offspring, total production efficiency, and average litter size increased by 1.4 and 2.4 and from 15.3 to 21.2, respectively [6]. Immunizing 380-day-old partridge chickens with the recombinant plasmid pcISI containing inhibin could improve the follicular development and egg-laying performance of partridge chickens [7]. Immunizing Booroola sheep with inhibin-α antiserum increased the average ovulation rate of Booroola sheep from 2.7 to 4.6 [8]. Sows were passively immunized with inhibin antiserum; inhibin immunization combined with AIS injection could significantly improve ovulation rate and embryo recovery rate in sows [9]. However, due to the shortage of raw materials and high production costs of inhibin immunogen and inhibin antibody, popularization and use of inhibin immune technology is difficult. Therefore, it is necessary to explore a new type of inhibin immune preparation so that this technology can be used in practical production.

Nanobody is a stable and heritable single-domain antibody from camelidae and cartilaginous fishes. Because of low molecular weight, strong stability, good solubility, easy expression, and low immunogenicity, it has become a potential new antibody drug and has been widely used [10–12]. Researchers have constructed a transgenic rice expression system to express anti-rotavirus-specific nanobodies (MucoRice-ARP1). Mucorice-ARP1 is currently in clinical trials; it is expected to become an effective oral anti-rotavirus product and is suitable for large-scale production [13]. Virdi et al. replaced the antigen-binding fragment of secretory immunoglobulin A (SIgA; the main protective antibody on the surface of the gastrointestinal mucosa) with nanobodies and produced seed bioencapsulated SIgA analogues (VHH-SIgA) in *Arabidopsis thaliana*. They observed that seed extracts that produce VHH-SIgA antibodies inhibit the binding of bacteria to porcine intestinal villi cells in vitro [14,15]. In a previous study, a new starin Lactobacillus rhamnoides DSM 14870 was cretaed that can colonize the human vagina and express anti-HIV nanobodies in the form of soluble and surface covalent anchoring; trials are underway to vaccinate high-risk women infected with HIV with this strain [16]. Thus, nanobodies are widely used, have a potential use as therapeutics, and are likely to gradually replace conventional antibody drugs.

However, to date, no nanobodies of inhibin have been reported. In this study, the anti-inhibin α-nanobody library was constructed by phage display technology, and the inhibin α-specific nanobodies were screened from the library. The binding of the nanobody to inhibin α was studied using molecular simulation docking and indirect ELISA. Finally, the biological function of the nanobody was identified by passively immunizing Altai sheep. To the best of our knowledge, this is the first anti-inhibin α nanobody drug in its class and may help in further development of novel hormone immunization strategies.

2. Materials and methods

2.1. Experimental animals

Kazakh sheep were provided by the Xinjiang Shihezi cattle and sheep slaughterhouse, and the Xinjiang Shuangfeng camels were provided by the Shihezi Huaguoshan camel farm. Immunized sheep were provided by farms in Fuyun County, Xinjiang. Adult animals were used in this study.

2.2. Material

Escherichia coli BL21 (DE3) strain was purchased from Bao Bioengineering Co., Ltd. Previously isolated and stored *E. coli* TG1, M13KO7 helper phage, and pCANTAB 5E were used in this study.

RNA extraction kit, reverse transcription kit, plasmid extraction kit, and rubber recovery kit were purchased from Tiangen Biochemical Technology Co., Ltd. Camel peripheral blood lymphocyte extraction kit was purchased from Tianjin Yuyang Biological Products Technology Co., Ltd. Anti-His tag antibody, rabbit anti-camel IgG serum, and horseradish peroxidase-labeled mouse anti-rabbit IgG were purchased from Solaibao Biotechnology Co., Ltd. M13 phage monoclonal antibody, E-tag antibody, and all restriction enzymes were purchased from Beijing Yiqiao Shenzhou Technology Co., Ltd., Kingsray Biotechnology Co., Ltd., and NEB Corporation. Primer designing and sequencing were completed by Shanghai Shenggong Bioengineering Co., Ltd.

2.3. Amplification of the INHA gene

Adult Kazakh sheep ovaries were collected from the Shihezi cattle and sheep slaughterhouse in Xinjiang and stored at −80 °C. Two sets of specific primers were designed based on the full length of the *INHA* gene (GenBank, NM_001308579.1) sequence: *INHA-Eco*RI-F: 5′-*CCG GAA TTC ATG TGG CTT CAG CTG CTC CTC TTC*-3′, *INHA-Hind*III-R: 5′-*CCC AAG CTT GAT GCA AGC ACA GTG CTG GGT G*-3′. Total RNA was extracted from the ovarian tissue using the RNA extraction kit (TIANGEN) and reverse transcribed using the reverse transcription kit (TAKARA).

Using cDNA as a template and *INHA-Eco*RI-F and *INHA-Hind*III-R as primers, the *INHA* gene was cloned and agarose gel electrophoresis. The agarose gel recovery kit was used to recover the electrophoresis products. The recovered products were connected to the pMD19-T vector at 16 °C and used to transformed *E. coli* BL21-competent cells.

2.4. Construction and identification of prokaryotic expression vector

The laboratory-preserved pET32a strains were cultured, and plasmids were extracted. The extracted plasmids were digested with restriction enzymes *EcoR*I *and Hind*III. The *INHA* gene was ligated with pET32a at 16 °C and was used to transform *E. coli* BL21 (DE3) competent cells. The positive clones were identified using double restriction endonuclease digestion and sequencing.

2.5. Induced expression of recombinant plasmids

The positive clones were cultured in 50 mL LB liquid medium at 37 °C and 170 rpm until they reached the logarithmic growth phase. The culture medium was supplemented with 50 μL isopropylthio-D-galactoside (IPTG) (1 mM). At 0, 2, 4, 6, and 8 h, 1 mL sample medium was collected, mixed with protein loading buffer and heated at 100 °C for 10 min. The remaining bacteria were ultrasounded at 300 W, 10 s/10 s for 20 min and centrifuged for 20 min at 10,000 rpm. Further, some amount of supernatant and precipitate were mixed with the protein loading buffer, boiled, and used for polyacrylamide gel electrophoresis.

2.6. Inhibin α purification and Western blot analysis

The *INHA* gene in the prokaryotic expression vector was induced by IPTG at 37 °C for 6 h, and inhibin α was expressed in *E. coli*. The bacterial cells were collected by centrifugation, resuspended in phosphate buffer solution (PBS), and subjected to ultrasonication. Further, these samples were centrifuged, and the precipitate was resuspended in binding buffer [20 mM Tris-HCl (pH7.9), 5 mM imidazole, 0.5 M NaCl, and 8 M urea]. The inclusion body dissolution product was filtered and added to the nickel agarose gel column, and further, the inhibin α protein was eluted with the elution buffer [20 mM Tris-HCl (pH7.9), 5 mM imidazole, 0.5 M NaCl, and 8 M urea]. The eluted proteins were placed into dialysis bags, and 6, 4, 2, and 1 M urea and PBS were used for dialysis for 6–8 h to refold the inclusion body protein.

A little refolded protein was subjected to polyacrylamide gel electrophoresis. The gel block containing approximately 45 kD band was cut and transferred to a NC membrane at 2 W. The membrane was blocked with 5 % skimmed milk for 2 h at 37 °C. The NC membrane was washed three times with 1 × TBST buffer for 15 min each. Further, the membrane was incubated with 5 mL of mouse anti-His-tag antibody (1:1000) at 37 °C for 2 h, followed by washing with 1 × TBST buffer. Finally, the membrane was incubated with 5 mL of horseradish peroxidase-labeled rabbit anti-mouse IgG (1:5000) at 37 °C for 2 h, followed by washing with 1 × TBST buffer and detection of signals using 3,3′-diaminobenzidine (DAB).

2.7. Immunization of camels and determination of antibody titer

On the 1st, 14th, 21st, 28th, 35th, and 42nd days, adult male camels were subcutaneously immunized using the emulsification of recombinant inhibin α and an equivalent amount of adjuvant (the first immunization involved Freund's complete adjuvant, and the rest involved Freund's incomplete adjuvant) [17].

Bactrian camel blood was collected 5 days after the third and sixth immunizations, respectively, and serum was separated for the determination of antibody titer. Diluted inhibin α protein was used as an antigen to coat a 96-well plate. On the 2nd day, the enzyme standard plate was washed with PBST and blocked with 5 % skimmed milk at room temperature for 2 h. After washing, 1:2-diluted Bactrian camel serum in PBS was added to the plate and incubated at room temperature for 2 h. After washing the plate, the rabbit anti-camel polyclonal antibody labeled with HRP was added and incubated at room temperature for 2 h. After washing the plate, the TMB single component color developing solution was added and incubated for 10 min to develop the color. Further, the termination solution was added, and OD_{450nm} was measured.

2.8. Obtaining the first cDNA sequence of lymphocytes

From each camel, 50 mL blood was collected from the jugular vein before the immunization and 2 weeks after the sixth immunization. Using a camel peripheral blood lymphocyte extraction kit, 10^7 lymphocytes were extracted according to the manufacturer's instructions. The total RNA of lymphocytes was extracted using an RNA extraction kit, and the cDNA was prepared using a reverse transcription kit.

2.9. Amplification of VHH antibody sequences

The camel antibody heavy-chain variable region (VHH) was amplified using:

CALL001: 5′-*GTC CTG GCT GCT CTT CTA CAA AG*-3′,

CALL002: 5′-*GGT ACG TGC TGT TGA ACT GTT CC*-3′, VHH-Forward: 5′-*TCG CGG CCC AGC CGG CCC AGG TCC AAC TGC AGG AGT CTG GGG*-3′, and VHH-Reverse: 5′-*ATA AGA ATG CGG CCG CTG AGG AGA CGG TGA CCT GGG TCC CC*-3′ [18].

The first round of PCR was performed using cDNA as a template and Call001 and Call002 as upstream and downstream primers, respectively. The PCR products were subjected to 1 % agarose gel electrophoresis, and 700-bp products were recovered. Using the recovered product as a template and VHH-Forward and VHH-Reverse as upstream and downstream primers, respectively, the second round of PCR was conducted, and the PCR product was subjected to 1 % agarose gel electrophoresis.

2.10. Preparation of hybrid libraries

*Not*I and *Sfi*I enzymes were used to digest the pCANTAB 5E vector and recovered PCR product from the second round before and after immunization. Because the reaction temperatures of the two enzymes are different, *Not*I was first used to digest at 37 °C for 4 h, and the reaction was inactivated by heating at 65 °C for 20 min. *Sfi*I was used to digest at 50 °C for 4 h. The digested products were subjected to 1.0 % agarose gel electrophoresis. The digested fragments were recovered and purified, and the ligation reaction of pCANTAB 5E vector and VHH was performed using T4 ligase kit according to the manufacturer's instructions. Recombinant vector pCANTAB 5E-VHH was used to transform TG1 competent cells via electroporation. The transformed cells were cultured overnight on LB solid medium containing 100 μg/mL ampicillin. Overall, 34 positive clones were selected for bacterial liquid PCR. Overall, 100 μL of M13KO7 (1.5×10^{13}) helper phage was added to a nanobody library cultured with an OD_{600nm} of 0.6–0.8 and allowed to stand at 37 °C for 30 min for helper phage rescue. Further, 20 % PEG-NaCl was used to recover and purify the nanobody phage library.

2.11. Selection and identification of inhibin-α-specific nanobodies

In a 96-well plate, each well was coated with 0.4 μg inhibin α. The negative control involved coating with PBS. On the 2nd day, the plates were washed with PBST, and blocked with 5 % BSA at room temperature for 2 h. After washing, the diluted initial phage library was added into each well and incubated at 37 °C for 2 h. After washing the plates for 15 times, 0.3 M glycine (pH 2.4) was used to elute bacteriophage particles, and further, 20 μL of neutralization buffer was added. A 10 μL eluate was used to determine the bacteriophage titer. The remaining neutralized eluate was infected by *E. coli* TG1 ($OD_{600\ nm}$: 0.5–0.7), and the M13K07 helper phage was used to rescue the library. After expanding the library through the 2 YT medium, PEG-NaCl was used to collect a round of phage panning library and conduct the next round of panning, for a total of 3 rounds of panning.

After three rounds of panning, 96 monoclonal clones were randomly chosen and inoculated into 2 YT medium for culturing until the logarithmic growth period was reached. After the library rescue with the M13K07 helper phage, the precipitate was collected by centrifugation, and cultured overnight in 2 YT medium containing ampicillin and kanamycin. Further, the supernatant was collected after centrifugation at 10,000 *g* for 20 min, which is the VHH phage polyclonal supernatant. The 96-well plate was coated with inhibin protein. The negative control was PBS, and the positive control was the phage polyclonal supernatant. On the 2nd day, the 96 well plates were washed with PBST and blocked with 5 % BSA at room temperature for 2 h. After washing the enzyme-labeled plate, the prepared polyclonal supernatant was added to it and incubated at room temperature for 2 h. After washing the plates, HRP-labeled anti-E-tag polyclonal antibody (tag protein of pCANTAB5E carrier) was added and incubated at 37 °C for 1 h. TMB single component color developing solution was added to the 96-well plate after washing. After developing color at room temperature for 10 min, termination solution was added, and the OD_{450nm} value was measured. The 6 polyclonal supernatants with the highest OD_{450nm} value were selected for sequencing.

2.12. Molecular simulation docking

To verify the binding ability of nanobodies and inhibin α protein, the following procedures were performed on their gene sequences. First, the SWISS-MODEL online software (https://swissmodel.expasy.org/) was used to predict the three-dimensional structure of inhibin protein and nanobodies. Further, using the online ZDOCKServer software (https://zdock.umassmed.edu/), nanobodies and inhibin-protein docking of three-dimensional structures was simulated. Finally, the online Protein Interactions Calculator (http://pic.mbu.iisc.ernet.in/job.html) was used for interaction analysis. The results of protein docking were analyzed to determine the type of interaction between the two proteins. In addition, HEX software was used to predict the binding capacity of nanobodies to inhibin proteins.

2.13. Vector construction involving nanobody genes

The VHH-Forward and VHH-Reverse primers for nested PCR were changed, and a pair of new primers carrying *Eco*RI *and Hind*III was designed (VHH-*Eco*RI-F and VHH-*Hind*III). Among them, VHH-*Eco*RI-F carried the Flag tag sequence. The sequences were as follows: VHH-*Eco*RI-F: 5′-*CCG GAA TTC GAT TAC AAG GAT GAC GAC GAT AAG CAG GTC CAA CTG CAG GAG TCT-3*′ and VHH-*Hind*III-R: 5′-*CCC AAG CTT TGA GGA GAC GGT GAC CTG GGT-3*′ (Van Overbeke et al., 2015).

The pCANTAB-5E-VHH strains screened from the phage library were amplified and plasmids were extracted. The extracted plasmids were used as templates with VHH-*Eco*RI-F and VHH-*Hind*III-R as primers for PCR amplification, and the PCR products were analyzed using agarose gel electrophoresis and gel recovery. The further steps were the same as those for the construction of the prokaryotic expression vector for the *INHA* gene. First, the nanobody genes were inserted into the pMD19-T vector and used to transform DH5α competent cells. Further, double digestion of nanobody genes and the pET32a vector was performed, and the digested product was connected at 16 °C and used to transform *E. coli* BL21 (DE3) competent cells.

2.14. Induced expression and purification of nanobody proteins

The positive strains were cultured at 37 °C at 170 rpm and then induced with IPTG to express nanobody proteins. Overall, 1 mL culture medium was collected at 0, 2, 4, 6, and 8 h and subjected to polypropylene gel electrophoresis. The remaining bacterial culture medium was centrifuged and precipitated with a phosphate buffer (20 mL). After repeated freezing and thawing with liquid nitrogen

and ultrasonic fragmentation, the solution was centrifuged for 20 min at 4 °C and 10,000 rpm. The precipitate and supernatant were analyzed using polyacrylamide gel electrophoresis to determine the best induction time and expression form of recombinant protein for each nanobody.

The precipitate obtained after ultrasonic fragmentation was resuspended in binding buffer, dissolved at 4 °C for 16 h, and centrifuged for 20 min at 10,000 *g*. The collected supernatant was filtered through a 0.45-μm filter membrane and passed through a Ni-agarose gel column. The nanobody protein in the gel column was eluted using elution buffer. Finally, the protein was renatured with 6, 4, 2, and 1 M urea and 1 M PBS for 6–8 h, and the protein was identified using polyacrylamide gel electrophoresis.

2.15. Identification of the ability of nanobodies to combine with the inhibin α-subunit

The inhibin α protein was coated on a 96-well plate. After washing, the plate was blocked with 5 % skimmed milk at 37 °C for 2 h. After washing, the plate was incubated with nanobodies (serially 1:2-diluted, e.g., 250 μg/mL, 125 μg/mL, … … and 0.122 μg/mL) at 37 °C for 1 h and further with HRP-labeled mouse anti-Flag-tag polyclonal antibody (Beijing Solarbio Technology Co) at room temperature for 1 h. After 15 min of incubation with TMB single-component chromogenic solution at room temperature, the terminating solution was added, and the OD value was measured at 450 nm.

2.16. Animal immunity

Overall, 45 adult female sheep aged 3–5 years with similar times of estrus and diestrus were randomly divided into three groups: inhibin α recombinant protein immunization group (group A, n = 15), inhibin α-subunit-specific nanobody immunization group (group B, n = 15), and the normal saline group (group C, n = 15). All sheep were immunized twice (the initial and booster immunization). Before immunization, 10 mL sheep blood was collected from the jugular vein. In group A, inhibin α protein was mixed with Freund's adjuvant (1:1) before immunization (Freund's complete adjuvant was used for the first immunization and Freund's incomplete adjuvant was used for booster immunization). After emulsification, the sheep was subcutaneously injected in the neck. The first immune dose was 2 mL, and the protein concentration was 0.2 mg/mL. The dose of booster immunization was the same, and the concentration was halved. Group B was subcutaneously injected with inhibin α-subunit-specific nanobody. Initial and booster doses were 2 mL, and protein concentration was 0.2 mg/mL. Group C was subcutaneously injected with normal saline (both doses were 2 mL).

2.17. Sample collection, treatment, and analysis

On the 5th day after intensive immunization, all sheep blood samples were collected (10 mL from the jugular vein), kept on an ice

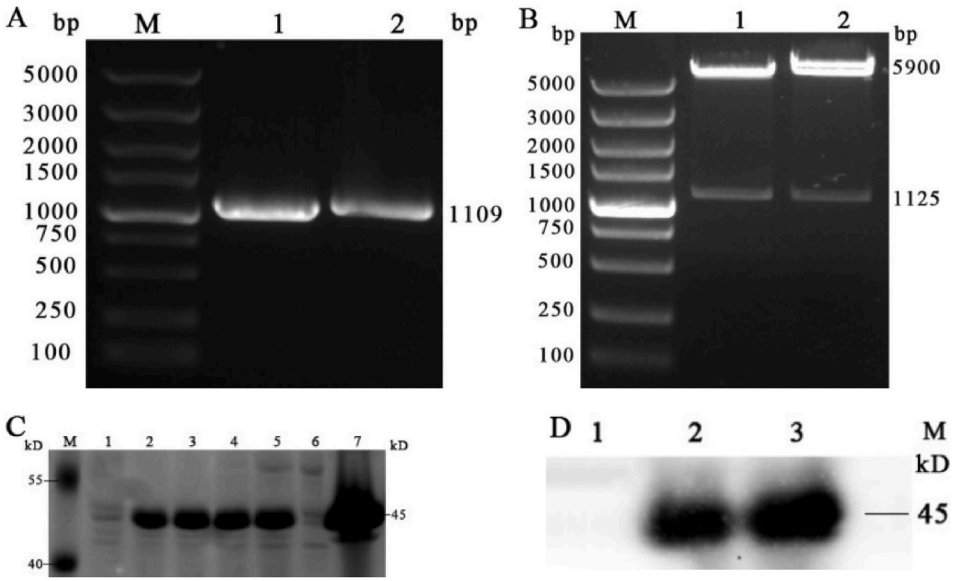

Fig. 1. (A) PCR product of *INHA* gene in Hazake sheep. Lane M: BM5000 DNA Marker. Lanes 1–2: The amplification product of *INHA* gene. (B) Enzyme digestion of recombinant plasmid pET32a(+)-*INHA*. Lane M. BM5000 DNA Marker. Lanes 1–2; pET32a(+)-*INHA* double enzyme digestion products. The prokaryotic expression vector pET32a(+)-*INHA* was verified using PCR and enzyme digestion. (C) Detection of prokaryotic expression protein of inhibin α-subunit using SDS gel electrophoresis. Lane M: Protein Marker (10–170 kD). Lanes 1–5: PAGE results of Rosetta (DE3) induced by 0.1 M IPTG after 0, 2, 4, 6, and 8 h. Lanes 6–7: Supernatant and precipitate after ultrasonic crushing. (D) Western blot results for inhibin α protein. Lane M: Protein marker. Lane 1: *E. coli* BL21 with empty pET32a (+) vector. Lanes 2–3: Purified inhibin α protein.

bag, and immediately brought to the laboratory. The samples were allowed to stand at room temperature for 4 h and centrifuged for 10 min at 25 °C and 4000 rpm under aseptic conditions. The obtained serum was stored in 5-mL EP tubes at −80 °C.

Serum reproductive hormones, including follicle-stimulating hormone (FSH), inhibin A (INHA), inhibin B (INHB), luteinizing hormone (LH), estrogen (E2), and progesterone (PROG), were detected using enzyme-linked immunosorbent assay (ELISA) using the manufacturer's instructions.

All data were analyzed using one-way ANOVA with GraphPad Prism 9.0 software.

2.18. Ethical statement

The experiments with animals were performed following the Ethical Principles in Animal Research adopted by the National Council for the Control of Animal Experimentation (CONCEA), and the protocol was approved by the Research and Ethical Committee of the Shihezi University (No. A2024251).

3. Result

3.1. Cloning of the sheep INHA gene and prokaryotic expression vector construction

Total RNA was extracted from ovarian tissue and reverse-transcribed to obtain cDNA. The PCR product was subjected to agarose gel electrophoresis, producing a specific band at 1109 bp, which was consistent with the expected size and was initially identified as a sheep *INHA* gene segment (Fig. 1-A). The constructed pET32a-*INHA* recombinant plasmid was double digested, and two specific bands were obtained at 5900 and 1125 bp (Fig. 1-B), which was consistent with the expected results. In addition, the sequencing results were consistent with the gene sequence in GenBank (XM004004955.1: the sheep *INHA* gene sequence), indicating that the prokaryotic expression vector of the *INHA* gene was successfully constructed.

3.2. Induced expression of inhibin α protein and identification using Western blot

The induced expression product of inhibin α protein was subjected to SDS-PAGE. As expected, the results revealed that the 45-kD protein was induced to express (Fig. 1-C). The optimal induction time of the inhibin α protein was 4 h. In addition, inhibin α protein was expressed as an inclusion body. It indicated that the inhibin α protein was successfully induced and expressed.

Western blot results for inhibin protein revealed that a single, clear band was obtained at 45-kD (Fig. 1-D). It indicated that the inhibin protein was successfully induced and expressed.

3.3. Construction of hybrid VHH libraries

After the Bactrian camels were immunized with inhibin α protein, non-anti-coagulated whole blood was collected after the third and sixth immunization, and the serum was collected to determine the antibody titer. The serum after the third immunization exhibited binding to the antigen inhibin α protein, according to indirect ELISA (Fig. 2-A), but the serum titer did not meet the requirements for blood collection this time. Indirect ELISA revealed that the serum after the sixth immunization exhibited binding to the antigen inhibin α-subunit at 1:10,24,000 (Fig. 2-B), and the serum titer met the requirement for blood collection this time.

Total RNA was extracted from the peripheral blood lymphocytes of camels before and after immunization, and the RNA was reverse-transcribed into cDNA. In the first round of PCR amplification in nested PCR, two bands were obtained, of which 700 bp was for the heavy chain antibody sequence (Fig. 3-A). In the second round of PCR amplification, a VHH gene sequence of approximately 400 bp (Fig. 3-B) was obtained. This indicated that the RNA extraction was successful, and the required *VHH* gene sequence was successfully cloned.

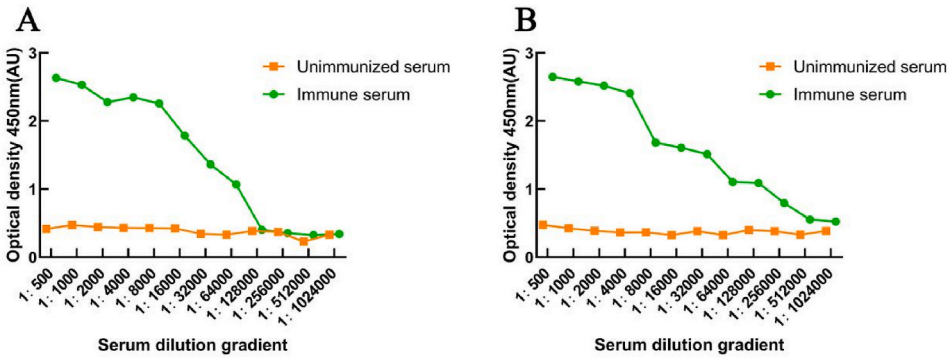

Fig. 2. Results of immune antibody titer of inhibin α protein in Bactrian camel. (A) Results of immune antibody titer of Bactrian camel for the third time. (B) Results of immune antibody titer of the 6th Bactrian camel.

In total, 34 clones were randomly selected from electroconversion-coated plates for bacterial liquid PCR. It was observed that all 34 clones were positive (Fig. 3-C), indicating that the positive rate after electroconversion reached 100 % and the electroconversion was successful.

3.4. Selection and sequencing of inhibin α-subunit-specific nanobodies

The nanobodies of inhibin α-subunits were panned using solid-phase biopanning technology. After the third round of panning, phage particles of inhibin α-subunit-specific nanobodies were abundantly enriched (Fig. 4-A). The degree of binding of the expression product to the inhibin α-subunit in 96 individual colonies was determined using ELISA (Fig. 4-B). From these clones, six positive clones with the highest degree of binding were selected for sequencing. Three of the six inhibin α-subunit-specific nanobodies (Nb-4) had the same sequence, whereas the other three nanobodies (Nb-15, Nb-26, and Nb-57) had different sequences (Fig. 4-C). The sequence alignment results revealed that the overall similarity of the four nanobodies was 68.75 %.

3.5. Molecular simulation docking

Because the binding site of the ovine inhibin α protein has not been determined yet, blind molecular docking was conducted. The results revealed that the inhibin α protein interacted strongly with the four nanobodies, and the binding sites were different (Fig. 5-ABCD). The main forces in the interaction between the inhibin α-subunit and nanobody complex were hydrophobic interaction and hydrogen bonding, according to the analysis. In addition, the four nanobodies have strong binding free energy from the inhibin α protein. Among the four types of nanobodies, the total free energy of nanobody Nb4 and the inhibin α-subunit was the highest (−576.28 kcal/mol).

3.6. Cloning of nanobody genes and construction of recombinant plasmid

After PCR amplification, agarose gel electrophoresis revealed that there were four specific target bands of 400 bp, which were preliminarily determined to be nanobody gene fragments (Supplementary Fig. 1A). These nanobody gene fragments recovered from the gel were ligated with a pET-32a (+) vector fragment and transformed into competent DH5α cells. Four single colonies were selected for propagation, and the four extracted plasmids were double digested. As expected, agarose gel electrophoresis revealed bands of

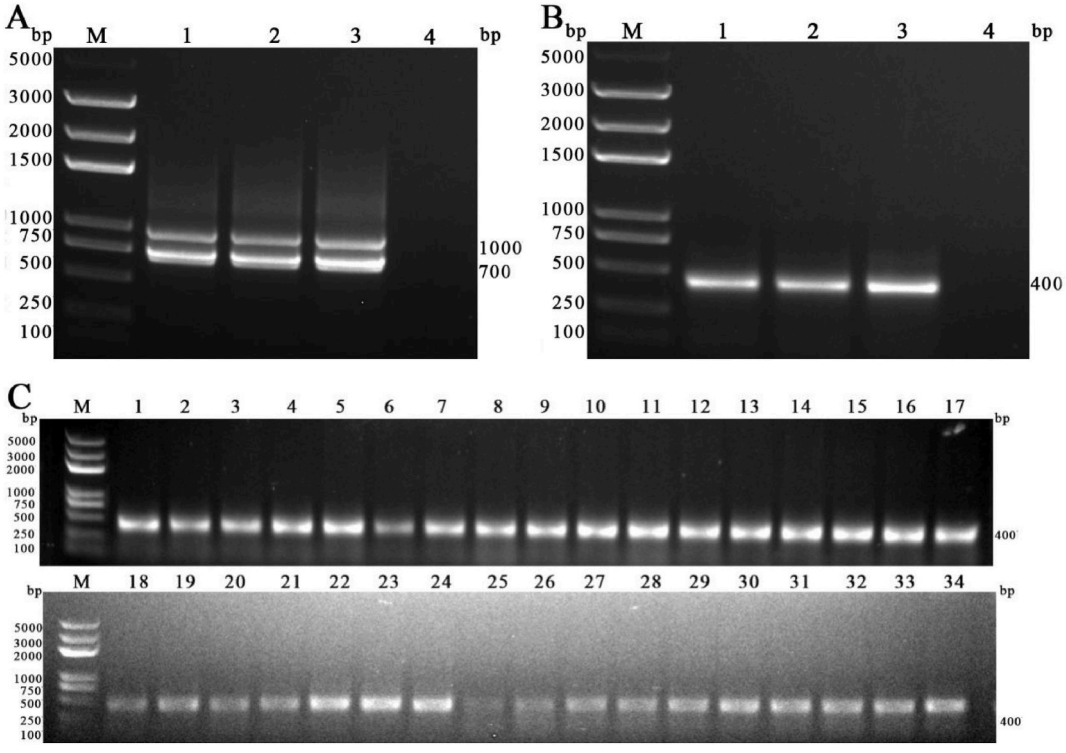

Fig. 3. (A) Electrophoresis of the first round of PCR products is given in lanes 1–3. Lane M: BM 5000 DNA Marker. Lane 4: Negative control (using water as the template). (B) Electrophoretic of the second round of PCR products is given in lanes 1–3. Lane M: BM5000 DNA Marker. Lane 4: Negative control (using water as the template). (C) Electrophoresis of PCR products of monoclonal bacteria in the original library are given in lanes 1–34. Lane M: BM5000 DNA Marker.

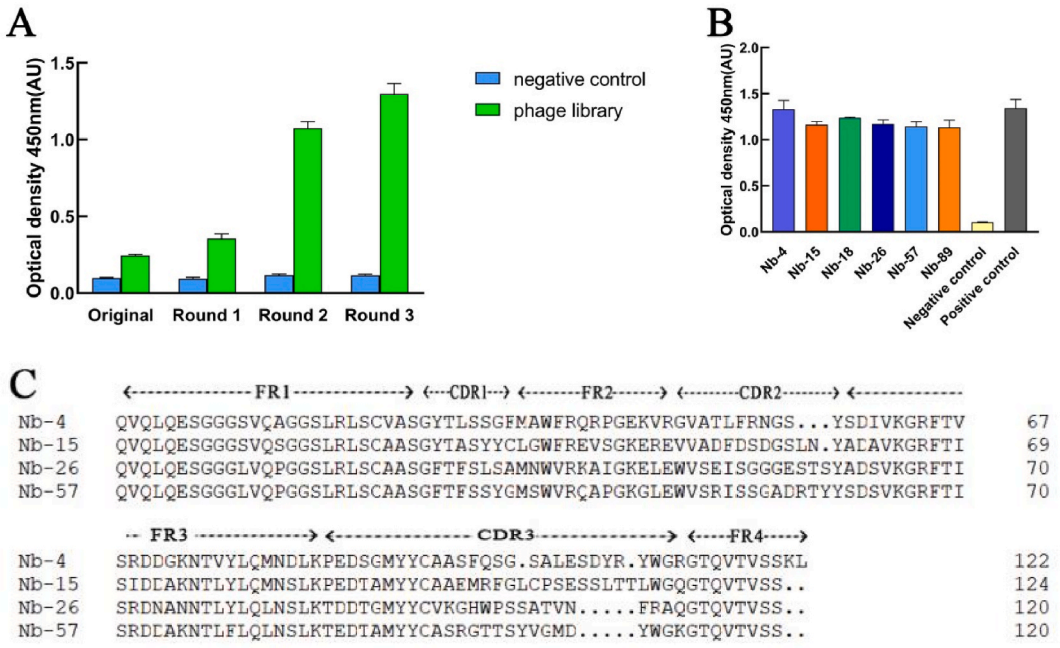

Fig. 4. (A) Polyclonal phage ELISA of inhibin α-subunit phage nanobodies from each round of biopanning. The coated antigen was inhibin α-subunit, and the blocking agent and negative controls were 5 % (w/v) skimmed milk. The OD_{450nm} values were expressed as the mean ± SD from the three measurements. (B) Analysis of the binding ability of recombinant nanobodies against inhibin α-subunit using indirect ELISA. Among the 96 positive clones, six exhibited the highest OD. The antigen of the test group, negative control, and positive control was inhibin α protein, PBS, and positive clones, respectively. (C) Sequence alignment results of four nanobody strains.

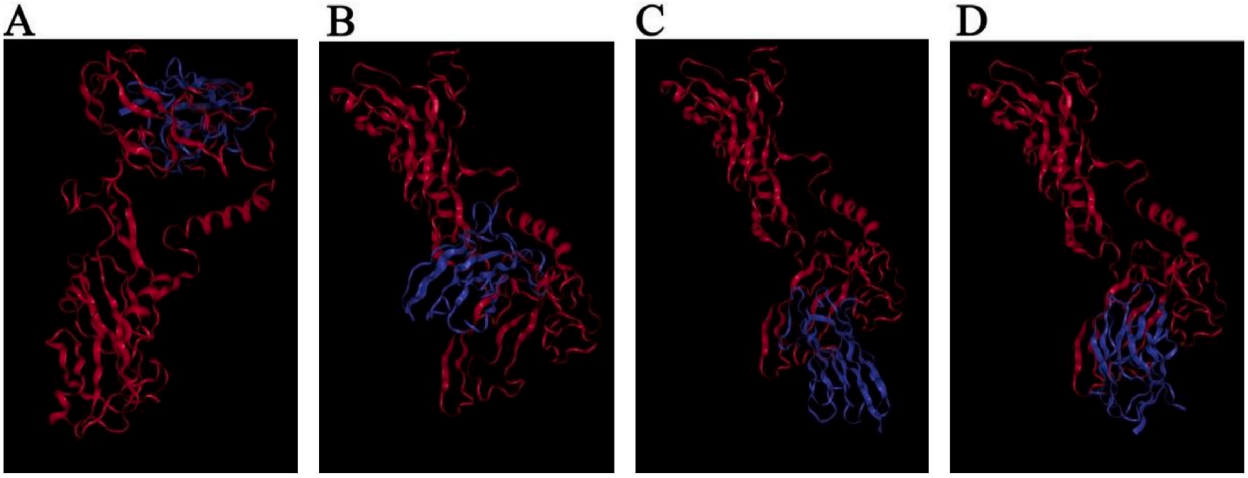

Fig. 5. Simulated docking of inhibin α with nanobodies. (A) Inhibin α-subunit and nanobody Nb-4. (B) Inhibin α-subunit and nanobody Nb-15. (C) Inhibin α-subunit and nanobody Nb-26. (D) Inhibin α-subunit and nanobody Nb-57. Inhibin α-subunit is colored in red and nanobodies in blue.

5900 and 400 bp (Supplementary Fig. 1B). These sequencing results were consistent with the sequence of nanobody genes, and there was no mutation. Therefore, it indicated that the recombinant plasmid pET32a-VHHs was successfully constructed.

3.7. Induced expression and purification of nanobody proteins

The size of nanobodies Nb-4, Nb-15, Nb-26, and Nb-57 was 15 kD (carrying His and Flag tags). The best induction time for each was 6, 6, 8, and 6 h, respectively, and the expression level was high, high, low, and low, which was expressed in the form of inclusion body (Supplementary Figs. 2A–D). The results revealed that the recombinant proteins of four nanobodies were induced and expressed successfully.

3.8. Affinity identification

The results of the ELISA revealed that all four nanobodies had high affinity for inhibin α-subunit at high concentration (Fig. 6-A). Among them, the nanobody Nb-4 could still bind to the inhibin α-subunit at 0.122 μg/mL, exhibiting the best affinity among the four nanobodies (Fig. 6-B).

3.9. Animal immunity

The hormone determination results revealed that no significant difference existed in the levels of six hormones (FSH, INHA, INHB, E2, PROG, and LH) before immunization between groups A (inhibin α protein), B (Nb-4), and C (normal saline), and the mean value was close. Thus, the experimental results compared with the control group could also explain the significant level of comparison between groups. The results revealed that the levels of INHA and INHB in groups A and B were significantly different from those in the control group after immunization and exhibited a downward trend after immunization (Fig. 7-A and B). This indicated that both inhibin α-subunit and Nb-4 immunization could reduce the levels of INHA and INHB. There was a significant difference in the level of FSH between group B and the control group after immunization, and an increasing trend was observed after immunization (Fig. 7-C). This indicated that Nb-4 immunization could increase the level of FSH. Although no significant difference existed in the level of FSH in group B, it also had the ability to improve the level of FSH. However, they had no significant effect on the levels of E2, P4, and LH (Fig. 7-D–F).

4. Discussion

Follicular development in mammals is a continuous and complex process regulated by various growth factors, pituitary gonadotropins, and steroid hormones in body [19,20]. Among them, inhibin plays an important role as a negative-feedback-regulating hormone on the hypothalamic-pituitary-gonadal axis. Inhibin combines with its own auxiliary receptor to form a complex, and competitively binds with activin to the activin type II receptor (AcvR II) to prevent the transcription of Fshb mRNA and the synthesis of FSH [21]. Therefore, blocking the binding of inhibin and AcvR II to promote the transcription of Fshb mRNA is the main goal of many studies on inhibin. Moreover, the idea of using inhibin immunity to increase animal fertility is emerging. Chen et al. revealed that active immunization of rhesus monkeys with an inhibin DNA vaccine increases their IgA and IgG antibody titers, which is an effective method to induce ovulation in primates and promote the reproduction of endangered rhesus monkeys [22]. Inhibin immunity can not only promote the development of follicles but also induce superovulation in animals. Many studies have reported that neutralization of endogenous inhibin using inhibin antiserum (IAS) is an effective method to mediate superovulation in mice. The combined use of IAS and equine chorionic gonad-otropin (eCG) was compared with eCG or IAS alone in conventional superovulation treatment. The combined group exhibited significantly increased number of ovulations in C57BL6 female mice [23]. Dan et al. revealed that the construction of a novel DNA vaccine p-TPA-SINH/TPASRFRP expressing inhibin α-subunit (1–32) and RFRP-3 genes has strong immunogenicity, and it increased the litter size of mice [24]. Huang et al. immunized Xinjiang Yemuller white sheep with a pEGFP-inhibin α-subunit recombinant vector. The results revealed that the recombinant vector had an important role in the secretion of FSH in Yemuller white sheep, indicating that it had an effect on ovarian function. It has been reported to have a regulating effect [25]. However, so far, no study has reported a specific blocker that targets the binding of inhibin to *AcvR*II. The four inhibin α-subunit nanobodies screened in this study can significantly bind to inhibin α-subunit.

Currently, it is not difficult to obtain active target protein by genetic recombination technology. However, in the actual application process, the acquisition of some special macromolecular proteins requires analysis of their specific structure and selection of appropriate vectors and bacteria to restore their correct expression form. The selection of a prokaryotic expression vector is most important for protein production. It can affect successful expression in the host, purification, and production of natural recombinant protein

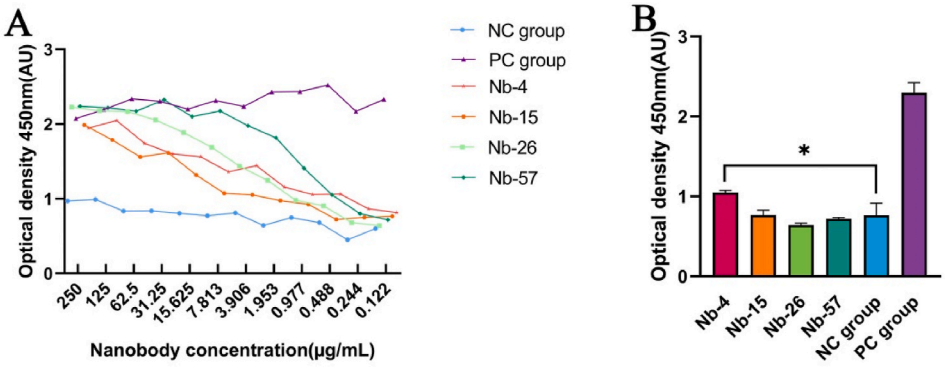

Fig. 6. (A) Affinity between inhibin α-subunit and four nanobodies at different concentrations. NC group is negative control group, and PBS group contained antigen-coated 96-well plate. PC group is positive control, and nanobody group contained antigen-coated 96-well plate. (B) Affinity between inhibin α-subunit and 0.122 μg/mL nanobodies.

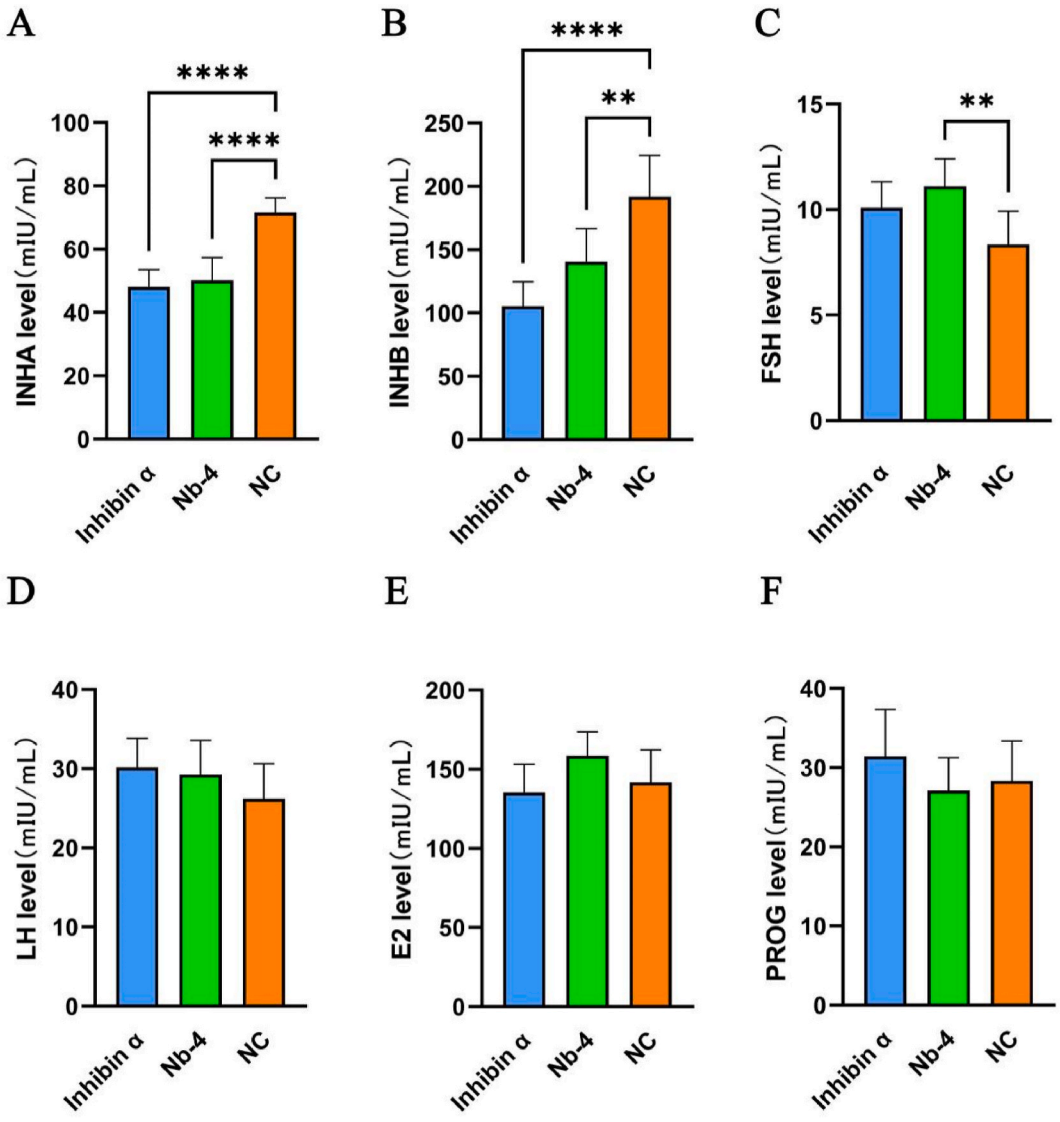

Fig. 7. Immunization results in sheep. NC is the negative control. (A) INHA, (B) INHB, (C) FSH, (D) LH, (E) E2, and (F) PROG levels in sheep after immunization.

activity. All the above qualitative and quantitative improvements can be obtained by inserting tags and protein fusions [26]. In the pET32a (+) prokaryotic expression vector used in this study, there is a trxA tag linked to the N-terminus of the insert, which has a redox function and improves the expression of inhibin α-subunit in *E. coli*. trxA in pET32a (+) leads to better expression of the recombinant protein [27].

After 6 h of IPTG induction, inhibin α protein levels stabilized. This may be because *E. coli* BL21 cells are similar to other cells in a way that their proliferation and growth proceeds through four phases, namely, the delay, log, stationary, and death phases [28]. We chose to add IPTG in the logarithmic phase; thus, it can be inferred that BL21 cells transformed with foreign genes entered the stationary phase after 6 h. However, the expression rate of foreign genes in BL21 cells may have slowed down due to conditions such as IPTG concentration or induction temperature.

Polyclonal and monoclonal antibodies currently in use are often less specific than desired, difficult to produce, and limited in number. A single-chain antibody in camelids has exhibited great potential [29]. On the other hand, inhibin immunity is difficult due to problems with inhibin immunogens. Tissue extraction of inhibin is difficult and expensive and is not suitable for market applications [30]. Chemically synthesized inhibin peptide fragment exhibits low immunogenicity because it cannot be correctly folded [31]. The effect of inhibin produced by genetic engineering technology is not very stable [32]. Therefore, in this study, we used the principle of antigen–antibody reaction to prepare nanobodies for inhibin α-subunit. The results indicated that after 11 weeks of immunization, the titer of inhibin α-subunit-specific antibodies in the blood of Bactrian camel was as high as 1:1,024,000. This indicated that the prepared inhibin α-subunit exhibits good immunogenicity. Successful enrichment from the library to specific phages is the key to obtain inhibin

α-subunit nanobodies. In this study, after 3 rounds of screening and enrichment, the number of bound phages increased by 1,200 times. This indicated that the third round of enrichment products was ready for high-throughput screening. In total, 96 positive monoclonal phage nanobodies (Nb-1 to Nb-96) were selected from the third round of enrichment of the library for phage ELISA. Six positive clones with the highest P/N were sequenced and identified. Surprisingly, the sequence of three nanobodies (Nb-4, Nb-18, and Nb-89) was the same. The fragments were all hydrophilic amino acids in the FR2 region. These hydrophilic amino acids increase the hydrophilicity of inhibin α-subunit-nanobody, which in turn increases the stability and solubility of nanobodies. By comparing other sequences in the hybrid library, the key complementarity determining regions (CDR) that bind to inhibin α-subunit are shown. However, the rules and mechanisms for binding the CDR to inhibin α-subunit remain to be studied.

To the best of our knowledge, this is the first study on the development of inhibin α-subunit nanobodies. Inhibin α-subunit is docked with inhibin βA/B in body to exert its biological function. Since the tertiary structure of the sheep inhibin α-subunit has not been resolved, and the INHA gene is conserved in the genomes of different animals, we first used the LOMETS method to predict its structure [33–35]. Based on its sequence, it has conservative and tertiary structural characteristics. Finally, we determined the inhibin α-subunit model. According to reports, the conservative mature inhibin α-subunit will have 2 N-glycosylation sites and a polyarginine cleavage site (Arg-XX Argor RXXR) [36], which is consistent with our sequence structure. The binding site of mature inhibin α-subunit and inhibin βA-subunit is in the C-terminal region of inhibin α-subunit. The selected inhibin α-subunit nanobody exhibited higher binding energy.

When inhibin α-subunit nanobodies have sufficient affinity with inhibin α-subunit, nanobodies can preferentially bind to inhibin α-subunit than inhibin β subunit. Thus, nanobodies change the structure of inhibin α-subunit, preventing it from binding to inhibin β subunit, thereby reducing inhibin levels in body. Therefore, the molecular docking method was used to evaluate the affinity of inhibin α-subunit nanobodies for inhibin α-subunit protein. The results indicated that inhibin α-subunit has a higher affinity (−576.28 kcal/mol) to Nb-4 than the reported predicted affinity of inhibin α-subunit to inhibin βA (−4.70 kcal/mol) [21]. On the other hand, although bioinformatic methods have some advantages such as short time and cost-effectiveness, due to some limitations such as limitations of docking algorithms, low accuracy, consideration of interactions under ideal conditions, and the inability to simulate complex internal environments [37], results often need to be confirmed through in vitro and in vivo assessments. Therefore, further experimental studies are needed to confirm the effectiveness of the antibody produced.

The results of inhibin α protein immunization and inhibin α-subunit-specific nanobody immunization revealed that both had certain effects on the content of reproductive hormones in sheep blood, especially on the level of inhibin A and inhibin B after immunization. Inhibin α-subunit is necessary for the synthesis of inhibin A/B, where the affinity of inhibin α-subunit with nanobody is much higher than that of inhibin α-subunit with β-subunit. Therefore, the nanobody preferentially binds to inhibin α-subunit and changes the conformation of inhibin α-subunit so that it cannot bind to β-subunit, thus reducing the synthesis of inhibin A and inhibin B. In addition, nanobody decreased the level of FSH in body, which may because of two aspects. First, the decrease in inhibin A/B weakens its ability to competitively bind to activin type II receptor (AcvR II), thus promoting the expression of Fshb (FSH β-subunit) mRNA in gonadotropin cells and strengthening the synthesis of FSH [38]. Second, inhibin A and inhibin B need to bind to the coreceptors TGFBR3 (Betaglycan) and TGFBR3L, respectively, to be able to competitively bind to AcvR II [39]. However, the existence of nanobodies may prevent inhibin A/B from binding to their respective coreceptors normally, thus preventing inhibin from performing normal physiological function and further increasing the synthesis and secretion of FSH.

According to the studies on inhibin, inhibin A/B forms a binary complex with TGFBR3/TGFBR3L and then competitively binds to AcvR II to inhibit the synthesis of Fshb mRNA [38]. Because FSH and LH share the same inhibin α-subunit but have different β-subunits, most researchers believe that inhibin A only inhibits FSH but has no effect on LH [40]. Therefore, no significant change in the LH level may be observed because inhibin content has no effect on the synthesis and secretion of LH.

Although the protein sequence and tertiary structure simulation of nanobodies we studied are consistent with the structure of the nanobody studied by Muyldermans [11], nanobodies may also have some unexpected physical properties including heat resistance, resistance to proteolysis at non-physiological pH (pH range 3.0–9.0), high pressure (500–750 MPa), and chemical denaturants (2–3 M guanidinium chloride, 6–8 M urea) [41]. These properties will hardly damage the binding capacity of inhibin α-subunit. Nanobodies have the advantages of small size, high affinity, and low purification cost [42]. However, due to their small size, their half-life in body is short, and their physiological effects cannot be fully exerted. At the same time, the molecular weight of the nanomolecules for the glomerular filtration effect is far below the renal threshold of 50–60 kD. Therefore, they are easy to get cleared through the kidneys, and the ideal binding effect cannot be achieved. However, inhibin mainly exists in the blood and ovarian tissues of the body [43]. Our future studies will focus on the optimization of nanobodies so that they exhibit the best effect.

In summary, this is the first study in which the prokaryotic expression system was used to express Kazakh sheep inhibin α-subunit, and we isolated four inhibin α-subunit-specific nanobodies from the Xinjiang Bactrian camel VHH library. Further, prokaryotic expression and affinity study were conducted on the selected nanobodies. The four nanobodies had a certain affinity with inhibin α-subunit. Finally, the sheep immune test was conducted with the nanobody Nb-4 with the best affinity. Nb-4 could normally function as an inhibin-synthesis blocker and promoted the synthesis and secretion of FSH. Our study provided a reference for the development of nanobodies in the field of hormone immunology.

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CRediT authorship contribution statement

Jifu Ma: Writing – original draft, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation. **Bakhet Bodai:** Writing – original draft, Software, Methodology. **Zhongmei Ma:** Writing – original draft, Software, Methodology. **Kezerbek Khalembek:** Resources, Investigation. **Jingang Xie:** Methodology, Conceptualization. **Rizabek Kadyken:** Resources, Formal analysis. **Mukhtar Baibatshanov:** Resources, Formal analysis. **Oralhazi Kazkhan:** Writing – review & editing, Project administration, Formal analysis, Conceptualization.

Declaration of competing interest

All authors declare that No conflict of interest exists.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e36180.

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